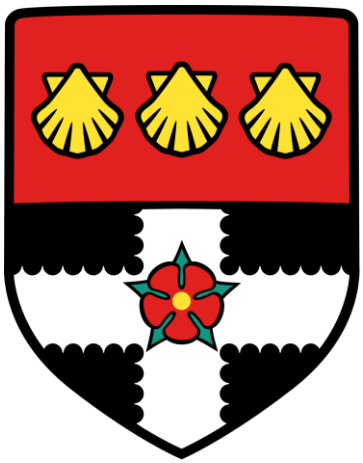
UNIVERSITY OF READING



Role of Connexin 62 (Cx62) in Platelets

A thesis submitted for the degree of Doctor of Philosophy

By

Khaled Ali Y Sahli

Institute for Cardiovascular and Metabolic Research
School of Biological Sciences

July 2018

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Signed: Khaled Ali Y Sahli

Date: July 2018

Abstract

Introduction: Connexins (Cxs), a large family of proteins, are expressed in numerous mammalian cells. Cxs oligomerise into hexameric hemichannels in the plasma membrane. The hemichannels on adjacent cells can dock together to form gap junctions (GJs), which regulate the direct trafficking of molecules (up to approximately 1 kDa) from one cell to another, and serve to allow coordinated responses between cells in tissues. Cx37 and Cx40 are expressed in human platelets and their selective inhibition in human platelets or deletion in transgenic mice attenuates platelet function. Notable levels of Cx62 transcripts have been reported in megakaryocytes, although nothing is known of the protein expression or function of this relatively recently discovered Cx in any tissue. The objective of this study was to determine the role of Cx62 in human platelets.

Results: The expression of Cx62 in human and mouse (Cx57, mouse homologue) platelets was confirmed by western blotting and immunocytochemistry. A novel mimetic peptide that targets the second extracellular loop of Cx62 was designed in this study. The newly designed Cx62 mimetic peptide, ^{62}Gap27, reduced hemichannel permeability and GJ-mediated intercellular communication. Several features of agonist-induced platelet activation, such as aggregation, degranulation, fibrinogen binding to integrin $\alpha_{IIb}\beta_3$, Ca^{2+} mobilisation and integrin $\alpha_{IIb}\beta_3$ outside-in signalling (which controls clot retraction and spreading), were inhibited by ^{62}Gap27. No effects were observed after treatment with the scrambled peptide control. The selectivity of ^{62}Gap27 for Cx62 was confirmed using Cx57 knockout mice. Additionally, Cx62 hemichannels were observed to function independently of Cx37 and Cx40 hemichannels. Thrombus formation (*in vitro* and *in vivo*) and tail bleeding were inhibited substantially by ^{62}Gap27, suggesting that Cx62 regulates thrombosis and haemostasis, respectively.

Experiments performed to explore the effects of Cx62 inhibition on platelet signalling revealed that treatment with ^{62}Gap27 can negatively-regulate tyrosine phosphorylation of multiple glycoprotein VI (GPVI) signalling mediators, including Syk, LAT phospholipase Cγ2; it also negatively regulates PKC substrate phosphorylation. In addition, treatment of resting and activated platelets with ^{62}Gap27 increased protein kinase A (PKA) activation, as observed via vasodilator-stimulated phosphoprotein VASP S157 phosphorylation. This activation was induced in a cyclic adenosine monophosphate (cAMP) -independent manner.

Conclusion: Cx62 and Cx57 are expressed in human and mouse platelets, respectively, where they play a fundamental role in platelet function, thrombus formation, and thereby haemostasis and thrombosis. Future work is required to determine the protein interactions of Cx62 and the mechanisms that allow platelets to control Cx function. Furthermore, the nature of the molecules that traffic through Cx62 hemichannels in isolated platelets or GJs within a thrombus also requires investigation.

Dedication

This thesis is dedicated to the memory of my brother, Ahmed Sahli. He died at the age of 34 in 2011 (RTA). His death is a great loss for his family members, friends and all who knew him. He was very kind, sympathetic, generous and supportive. He loved to help others and was always ready to do so. For me, he was not only a brother but a close friend and motivator. He motivated me to undertake my postgraduate studies. He used to tell me that I should have dreams, interests and goals for the future because life is meaningless without dreams. Moreover, he used to motivate me to aim for big goals in life. According to him, dreams do not have limits and if you dream, dream high. I am sad that I never told him how much I loved him. He will always occupy a special place in my heart. His motivation has played and will continue to play a great role in my life.

Acknowledgements

I owe a lot to my supervisor, Professor Jonathan Gibbins, who gave me the opportunity to conduct this research at the University of Reading. I am thankful not only for his support, encouragement and guidance throughout the research but also for his help with my health and family issues. I am also grateful to Ministry of Defense for the scholarship that helped me fulfil my dream.

I would like to thank Mrs. Helen Yeshayahoo. She has been a constant source of encouragement and support throughout my health and family issues. Many thanks to Gagan, he has not only proven to be a friend but also a helpful partner in the lab. He made working in the lab quite amusing. Thanks to Dr Bye, Dr Neline and Dr Amanda, as well, for their cooperation and suggestions. Special thanks to Sarah Alouda for her encouragement and support; she is like my sister. I would also like to thank Dr Paru, Dr Lisa and Tanya for their assistance with the experiments that involved mice. I would also like to thank the special one in the lab, Dr Stainer, for his continuous support. Thanks to Jo and Alex for their suggestions and advice. Previous lab members, including Mike Schenk, Marilena, Maria, and Sarah Ryan, and PhD students, including Mohammed, Sophie and Alex, supported me throughout the project. Special thanks to the individuals who donated blood for the project and to the members of the platelets group. It was really a wonderful experience working with the platelets group.

Last but not least, I would like to thank my family members and friends for their prayers, support and encouragement, without which I am unable to do anything. I am indebted to my wife not only for her support and care but for the patience she showed throughout the study. I have no words to express my gratitude to her. I am also grateful to my sons Ziyad and Tareq, who have always been a source of strength for me.

Publications

Manuscripts in preparation

- **Sahli KA**, Flora, G.D**,** Sasikumar P, Magrabi A, Holbrook L.M., Alouda S, Sage T, Stainer A.R, Adiyaman R, AboHassan, M, Crescente, M, Bye, A.P, McGuffin, L, Gibbins, J.M; *Connexin 62 hemichannels and gap junctions regulate platelet function and signalling through the activation of PKA in a cAMP-independent manner*.
- Flora GD**, Sahli KA**, Sasikumar P, Holbrook LM, Stainer AR, Unsworth AJ, Crescente M, Sage T, Gibbins JM; *The Pregnane X Receptor exhibits non-genomic effects to inhibit GPVI mediated platelet signalling*

Published

- Holbrook L, Sandhar GK, Sasikumar P, Schenk MP, Stainer AR, **Sahli K**, Flora GD, Bicknell AB, Gibbins JM; *A humanized monoclonal antibody that inhibits platelet-surface ERp72 reveals a role for ERp72 in thrombosis;* J Thromb Haemost. 2017; 16(2): 367–377

Presentations

1. **ORAL PRESENTATION: Regulation of platelet function by connexin62 hemichannels and gap junctions.** 9th UK Gap Junction meeting, Warwick, UK, December 2016
2. **POSTER: Role of Connexin 62 in Human Platelets.** UK platelet meeting, Leicester, UK, 2015
3. **POSTER: Connexin 62 Hemichannels and Gap junctions Regulate the Function of Platelets.** European Platelet Network conference (EUPLAN), Bäd Homburg (Germany), September 2016.
4. **POSTER: Connexin 62 Hemichannels and Gap junctions Regulate Platelet Function.** International Society on Thrombosis and Haemostasis (ISTH) Congress, Berlin, Germany, 2017.
5. **POSTER: Connexin 62 Hemichannels and Gap junctions Regulate Platelet Function via Activation of PKA in a cAMP Independent Manner.** International Gap Junction Conference 2017 (IGJC 2017), Glasgow, UK, 29th July – 2nd August 2017

List of Abbreviations

^{62}Gap27	Connexin 62 mimetic peptide
AC	Adenylyl cyclase
ACD	Acid citrate dextrose
AD	Autosomal dominant
ADP	Adenosine diphosphate
AKAP	A kinase anchoring protein
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C	Cangrelor
c-subunit	Catalytic subunit
C-terminal	Carboxyl-terminal
CalDAG-GEFI	Calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CLEC-2	C-type lectin-like receptor 2
COX1	Cyclooxygenase 1
CRAC	Calcium release-activated calcium channel
CRP-XL	Crosslinked collagen-related peptide
CTL	Cytotoxic T lymphocyte
Cx	Connexin
Cx37$^{-/-}$	Cx37-deficient
Cx40$^{-/-}$	Cx40-deficient
Cx57$^{-/-}$	Cx57-deficient
DAG	Diacylglycerol
DiOC6	3,3′-dihexyloxacarbocyanine iodide
DTS	Dense tubular system
E1	Connexin extracellular domain 1

E2	Connexin extracellular domain 2
ECM	Extracellular matrix
ECs	Endothelial cells
EC50	Half maximal effective concentration
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
Epac	Exchange proteins that are directly activated by cAMP
FcRγ	Fc receptor γ-chain
FII	Factor II
FITC	Fluorescein isothiocyanate
FV	Factor V
FVIII	Factor VIII
FIX	Factor IX
FRAP	Fluorescence recovery after photobleaching
FX	Factor X
FXI	Factor XI
FXII	Factor XII
GA	Glycyrrhetinic acid
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GJ	Gap junction
GJA	Gap junction protein alpha family
GJIC	Gap junction intercellular communication
GP	Glycoprotein
GPCR	G-protein-coupled receptor
GPO	G-Glycine, P-Proline, O-Hydroxyproline
GPRP	Peptide Gly-Pro-Arg-Pro
GTP	Guanosine triphosphate
HSC	Haematopoietic stem cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I	Indomethacin

Ig	Immunoglobulin
IP	Prostacyclin receptor
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T cells
M	MRS2179
M1	Connexin transmembrane domain 1
M2	Connexin transmembrane domain 2
M3	Connexin transmembrane domain 3
M4	Connexin transmembrane domain 4
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MK	Megakaryocyte
ms	millisecond
N-terminal	Amino-terminal
NO	Nitric oxide
NOS	Nitric oxide synthases
OCS	Open canalicular system
ODDD	Oculodentodigital dysplasia
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease-activated receptors
PBS	Phosphate-buffered saline
PECAM-1	Platelet–endothelial cell adhesion molecule 1
PGH2	Prostaglandin endoperoxide H2
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 3,4-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C

PM	Plasma membrane
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RabGDI	Rab GDP dissociation inhibitor
RhoGEFs	RhoGTPase nucleotide exchange factors
RIAM	Rap1b-interacting adaptor molecule
ROI	Region of interest
R-subunit	Regulatory subunit
Rp-8-CPT-cAMPS	Rp-adenosine 3′,5′-cyclic mono-phosphorothioate
SDS	Sodium dodecyl sulphate
SFK	Src family tyrosine kinase
sGC	Guanylyl cyclase
SH	Src homology
SH2	Src homology 2
SH3	Src homology 3
SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
SMC	Smooth muscle cell
SNARE	Soluble NSF attachment protein receptor
SOCE	Store-operated calcium entry
STIM1	Stromal interaction molecule 1
STORM	Stochastic optical reconstruction microscopy
TBST	Tris-buffered saline-TWEEN® 20
TMDs	Transmembrane domains
TP	Thromboxane A2 receptor
tSNARE	Target soluble NSF attachment protein receptor
TSP-1	Thrombospondin 1
TxA_2	Thromboxane A2
TxB_2	Thromboxane B2
TxS	Thromboxane synthase
VASP	Vasodilator-stimulated phosphoprotein
V_j	Transjunctional voltage
V_m	Transmembrane potential
vSNARE	vesicular soluble NSF attachment protein receptor

VWF	von Willebrand factor

List of Figures

List of Tables

Table of Contents

1 Introduction

1.1 Summary of the Purpose of the Project

Platelets perform an essential role through their ability to recognise and respond to tissue injury. This initiates a rapid response that culminates in singular platelets sticking together to form a thrombus or 'haemostatic plug'. Recent studies have demonstrated that contact-dependent signalling between platelets maintains thrombus growth and stability. Connexin (Cx) 37 and Cx40 play integral roles in contact-dependent interactions by forming hemichannels and gap junctions (GJs), which facilitate inter-cellular communication between platelets that serves to ensure full activation and thrombus stability (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). It was also reported that megakaryocytes (MK) express notable levels of Cx62 mRNA. Cx62 protein expression has not yet been reported in any cell type. This, and lack of reagents to block the function of Cx62 has hindered progress to establish the functions of this new connexin family member.

This study provides for the first time evidence for Cx62 and Cx57 (the mouse homologue) expression in human and mouse platelets respectively. We describe the design of a Cx62-selective peptide inhibitor (^{62}Gap27) that offers insights into the mechanism through which Cx62 hemichannels and GJs mediate their functions in platelets to facilitate haemostasis and thrombosis.

1.2 Platelets: A Historical Perspective

Platelets were originally known as ‘the dust of blood’ (Addison, 1841; Bizzozero, 1881; Bizzozero, 1882; Tocantins, 1948; Coller, 1984; Kuter, 1996). Several researchers claimed that they were the first to identify these blood components. In 1873, the disc-like structure of platelets was reported by Osler (Osler, 1873). Seven years later, Bizzozero carried out an anatomical study of platelets and reported their

involvement in haemostasis and experimental thrombosis (Bizzozero, 1881; Bizzozero, 1882; Coller, 1984). He also discovered that the formation of aggregates at an injury site mainly involves the adherence of platelets to the walls of blood vessels (Bizzozero, 1869; Bizzozero, 1882; Born, 1965; Coller, 1984). Researchers have been studying the mechanisms involved in the formation of platelets for more than a century (Born, 1967a; Born, 1967b). The generation of platelets by giant bone marrow megakaryocytes (MKs) was first demonstrated by James Homer Wright in 1906 (Wright, 1906; Kuter, 1996). Although Bizzozero discovered bone marrow MKs, he did not identify them as the precursors of platelets. Wright synthesised a polychrome staining solution (Wright's stain), with which he studied the similarities in the colours and shapes of MKs and the red-to-violet granules of platelets (Lee et al., 2002). Based on this demonstration, he is acclaimed as pioneer in the field of platelet formation studies. Since Bizzozero's ground-breaking research, the literature has expanded to include many studies, which have strengthened our knowledge of the structure and formation of platelets and their contribution to thrombosis and haemostasis (Born, 1965; Coller, 1984).

1.3 Platelet – Key Regulators of Haemostasis and Wound Repair

The processes of wound repair and blood clotting require platelets. Following vascular injury, the subendothelial extracellular matrix (ECM) is exposed. When platelets circulating in the blood encounter the ECM, they become activated; as a result, a haemostatic plug that prevents blood loss is formed. This process is critical for haemostasis and the prevention of excessive bleeding. Many physiological mechanisms are responsible for the regulation of platelet activity. These mechanisms prevent inappropriate, unwanted platelet activation. However, clots may be formed in the absence of vessel injury, a process known as thrombosis. Thrombosis is among the

most common causes of death globally (Gaziano et al., 2010). Thrombosis can occur through many mechanisms. For instance, rupture of an atherosclerotic plaque in an artery may lead to development of thrombus, which may cause its occlusion leading to myocardial infarction or stroke. Thrombosis is also a common pregnancy- or surgery-related complication (Elyamany et al., 2014; Otsuka et al., 2016; Khan et al., 2017). In 2014, 15% of all male deaths and 10% of all female deaths in the UK, a total of 69,000 mortalities, were associated with coronary heart disease. In the same year, 155,000 deaths were associated with cardiovascular disease and almost 2 million people suffered related episodes in UK hospitals; the number of cardiovascular disease-related prescriptions that year exceeded 313 million (Townsend et al., 2015). The effects of thrombosis on human health and the economy are undeniable. Hence, platelets have become the chief targets for existing and innovative antithrombotic drugs.

1.4 Platelet Formation

MKs are responsible for the production of platelets. They are bone marrow cells that arise from haematopoietic stem cells (HSCs) via a process known as megakaryopoiesis. MKs comprise 0.05%–0.1% of nucleated cells in the bone marrow; they may also be found in the pulmonary tract and blood (Levine et al., 1993; Huang et al., 2016). Every MK is capable of producing 5,000 to 10,000 platelets (Italiano and Shivdasani, 2003). The cytokine thrombopoietin, which is produced in the kidneys and the liver, regulates the formation of platelets. Thrombopoietin binds to the c-Mpl surface receptor on the megakaryocyte, which stimulates endomitosis a process in which MKs undergo cycles of DNA replication without the associated cell division, transforming megakaryocytes into giant cells with a large cytoplasm and thereby forming polyploid nucleus cells (de Sauvage et al., 1994; Kaushansky et al., 1994; Ravid et al., 2002; Italiano Jr and Hartwig, 2013). The process can generate a cell with a DNA content up to 128*n* within

one polylobulated nucleus (Gurney et al., 1994; Zimmet and Ravid, 2000; Sim et al., 2016). Endomitosis is responsible for generating an abundance of mRNA and protein, which is required for platelet synthesis and the development of the membrane system that forms proplatelet membranes (Zimmet and Ravid, 2000; Schulze et al., 2006; Machlus et al., 2014).

Changes in the cytoskeleton regulate the production of proplatelets from the elongated branching processes of the MKs. Thon et al. (2010) demonstrated how microtubule-based twisting forces affect the elongation of proplatelets and studied the role of actin in the process (Patel et al., 2005). The main role of microtubules is thought to be the transportation of organelles and granules into developing proplatelets (Richardson et al., 2005). The site of proplatelet formation is thought to be the bone marrow sinusoidal blood vessels. Blood flow helps to detach platelets from proplatelets via a biophysical process (Junt et al., 2007). The biomolecular mechanisms responsible for regulating this process have not been identified. Zhang et al. (2012) linked sphingosine 1-phosphate with the shedding of proplatelets in the sinusoidal blood vessels as mice lacking its receptor displayed thrombocytopenia.

The synthesis of platelets in the lung has been recognised for almost a century (Howell and Donahue, 1937). However, its importance was not completely understood until recently. Lefrançais et al. (2017) recently performed a study that showed the migration of bone marrow MKs into mouse lungs, where the extravascular spaces contain an abundance of mature and immature cells, including haematopoietic progenitor cells. Large quantities of platelets are produced by these lung MKs, accounting for half of all murine platelet production. In thrombocytopenic conditions, the progenitor cells in the lungs migrate back into the bone marrow, which helps to elevate low platelet counts

(Lefrançais et al., 2017). Therefore, the lungs are proposed to be one of the main platelet production sites.

1.5 The Regulation of Platelet Activation

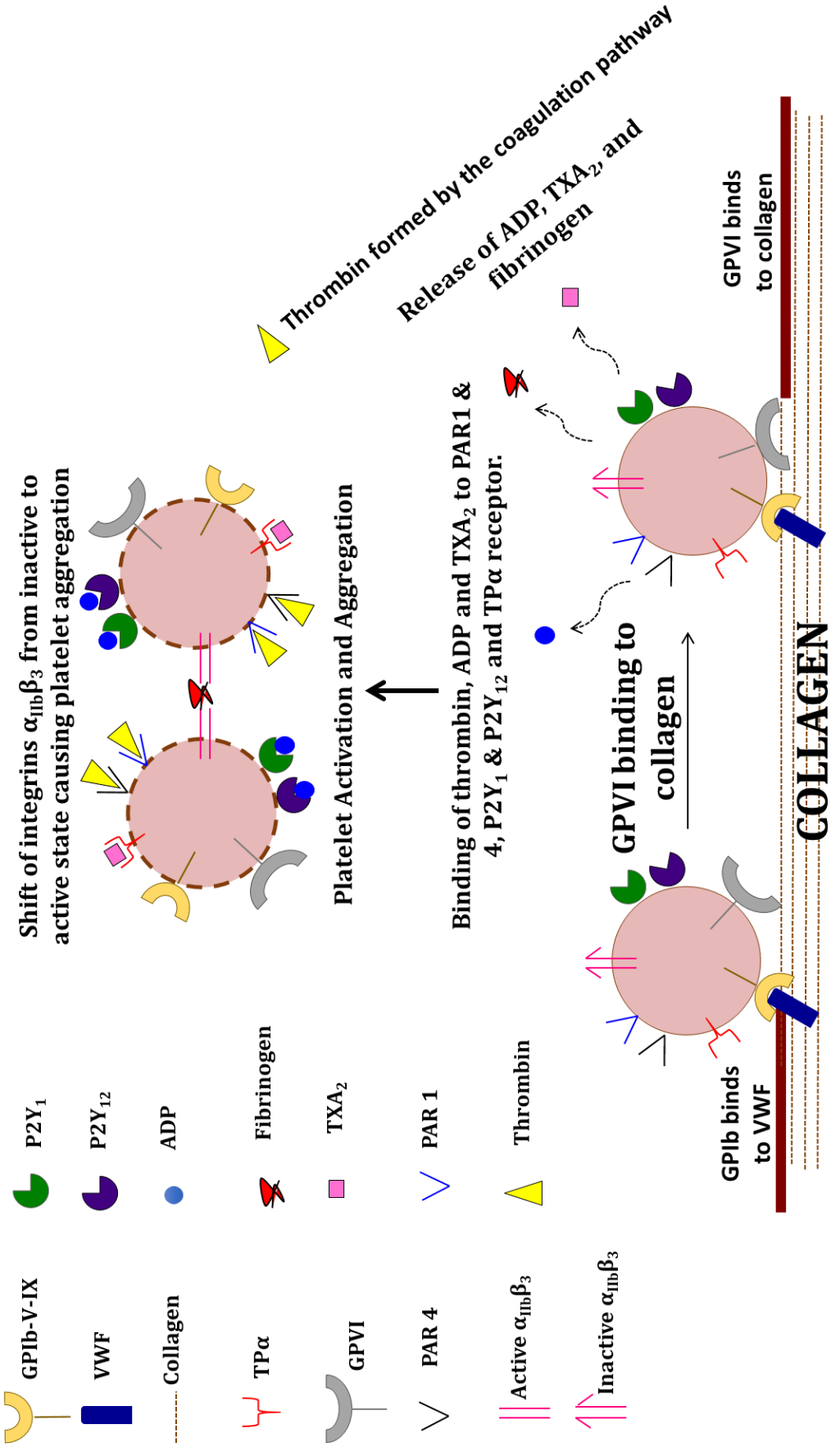
Platelets are responsible for the essential defence mechanism of haemostasis. Under ideal physiological circumstances, platelets are dormant. Immediately upon the detection of an injury, platelets bind to collagen, the extracellular matrix (ECM) protein in the vascular subendothelium. The ECM protein collagen is detected by glycoprotein (GP) receptors on the surfaces of the platelets. The interaction between collagen and GP receptors, which is part of a series of rapid reactions in the creation of a monolayer, that recruits more platelets which assists in healing to halt the loss of blood (Moroi et al., 1996; Polanowska-Grabowska et al., 1999; Nuyttens et al., 2011).

The reaction of platelet GPs with ECM proteins initiates tyrosine kinase signalling pathways that lead to their rapid activation. The downstream effects include intracellular Ca^{2+} mobilisation, the secretion of platelet granule contents and the activation of platelet integrins. Granule secretion and integrin activation result from the immediate mobilisation of Ca^{2+}. A positive feedback occurs in platelets due to the variety of activatory molecules released from the platelet granules; which prompts the platelets in close proximity to aggregate. Enhanced affinity for fibrinogen, amongst many other ligands, is achieved by the stimulation of a conformational change in integrin $\alpha_{IIb}\beta_3$. The stimulation of this integrin is seen in all platelet activation pathways. In addition to increasing the affinity of the integrin, this reaction catalyses fibrinogen binding, the creation of divalent crosslinks with fibrinogen and the accumulation of high numbers of platelets (Ma et al., 2007; Sánchez-Cortés and Mrksich, 2009).

The roles of platelets in clotting and the subsequent generation of a stable thrombus are as important as their roles in wound healing through activation and aggregation. Activated platelets translocate phosphatidylserine (PS) to the outer leaflet of the platelet surfaces, creating a platform for the assembly of protein complexes that control coagulation cascades. Also, procoagulant platelets have high levels of cytosolic Ca^{2+} and expose phosphatidylserine (PS) on their surfaces (Heemskerk et al., 2002; Keuren et al., 2005). Tenase and prothrombinase complexes are assembled by procoagulant platelets to help with the activation of coagulation factor X (FX) and prothrombin into FXa and thrombin (a soluble platelet agonist), respectively; binding sites for factor XI (FXI) and prothrombin are also provided by platelets (Heemskerk et al., 2002). Podoplelova et al. (2016) recently conducted a study to identify the procoagulant 'cap' regions in platelets. The coagulation factors are distributed randomly in procoagulant platelets, but there are high concentrations of the coagulation factors in the caps of the platelets. When activated, platelets release microparticles, which have strong procoagulant activity; the microparticles bind to factor VIII (FVIII) and FVa and assist in the formation of the prothrombinase complex (Sims et al., 1988; Gilbert et al., 1991). The procoagulant activity of these microparticles is 50- to 100-fold greater than that of the activated platelets (Sinauridze et al., 2007). The role played by platelets in the coagulation process is essential for the production of thrombin and the processing of fibrinogen into fibrin. Fibrin plays a crucial role in vessel injury healing; the developing clot is mainly composed of fibrin, which promotes the aggregation of blood cells to form a stable thrombus (Falati et al., 2002; Ariëns, 2013). Figure 1.1 shows a diagram of platelet activation and thrombus formation.

Figure 1.1:The different stages of platelet activation and thrombus formation.

Upon blood vessels injury, GPIbα, a component of the GPIb-IX-V complex, binds to collagen-bound von Willebrand factor (VWF), thereby initiating the adhesion of platelets. This is followed by direct binding between collagen and GPVI, resulting in the activation of platelets. This causes platelets to change shape, thereby activating integrins and stimulating the secretion of granules, resulting in conformational alterations in integrin $\alpha_{IIb}\beta_3$. This alteration improves the affinity of the integrin for VWF and fibrinogen. The activated form of integrin $\alpha_{IIb}\beta_3$ is essential for the aggregation of platelets and stabilization of the resultant thrombus.


GPIb-V-IX
VWF
Collagen
TPα
GPVI
PAR 4
Active $\alpha_{IIb}\beta_3$
Inactive $\alpha_{IIb}\beta_3$
$P2Y_1$
$P2Y_{12}$
ADP
Fibrinogen
TXA_2
PAR 1
Thrombin
Shift of integrins $\alpha_{IIb}\beta_3$ from inactive to active state causing platelet aggregation
Platelet Activation and Aggregation
Binding of thrombin, ADP and TXA_2 to PAR1 & 4, $P2Y_1$ & $P2Y_{12}$ and TPα receptor.
Thrombin formed by the coagulation pathway
Release of ADP, TXA_2, and fibrinogen
GPVI binding to collagen
GPVI binds to collagen
GPIb binds to VWF
COLLAGEN

1.6 Platelet Structure

Platelets are small cells with a diameter of 1–3.5 μm. They lack nuclei and range in volume from 7 to 10.5 fl. The circulating human platelet count ranges from 150 - 450 × 10^9 platelets/L (Giles, 1981; Martin et al., 1982). Each day humans produce approximately 1 × 10^{11} platelets, which have a lifespan of approximately 8–10 days before clearance after they enter the circulation (Stuart et al., 1975; Grozovsky et al., 2015). The clearance of platelets is not completely understood. Phagocytes in the liver and spleen eliminate old or defective platelets from the circulation (Italiano Jr and Hartwig, 2013). Some studies have suggested clearance via Ashwell-Morell receptor-mediated recognition and removal of senescent, desialylated platelets in the liver along with the clearance of apoptotic cells. The role of this receptor in stimulating TPO synthesis, which drives thrombopoiesis has also been suggested (Mason et al., 2007; Grewal et al., 2008; Grozovsky et al., 2015).

Various cell surface receptors are expressed on the platelet plasma membrane (PM); they are important for platelet activation and accumulation. Among these receptors are adhesion receptors, G-protein-coupled receptors (GPCRs) and integrins. They are discussed in greater detail in Section 1.7 and Section 1.8. A complicated cytoskeleton provides structural support to the platelet PM (Shin et al., 2017), as shown in Figure 1.2. This cytoskeleton is mainly composed of microtubules and actin filaments, along with spectrin networks, and is critical for the maintenance of the resting discoid shape of platelets. When the cytoskeletal network is activated, it is dynamically restructured by depolymerisation, reorganisation and polymerisation, which generates the shape alteration observed in activated platelets (Hartwig, 1992; Kaushansky et al., 2015; Sandmann and Koster, 2016).

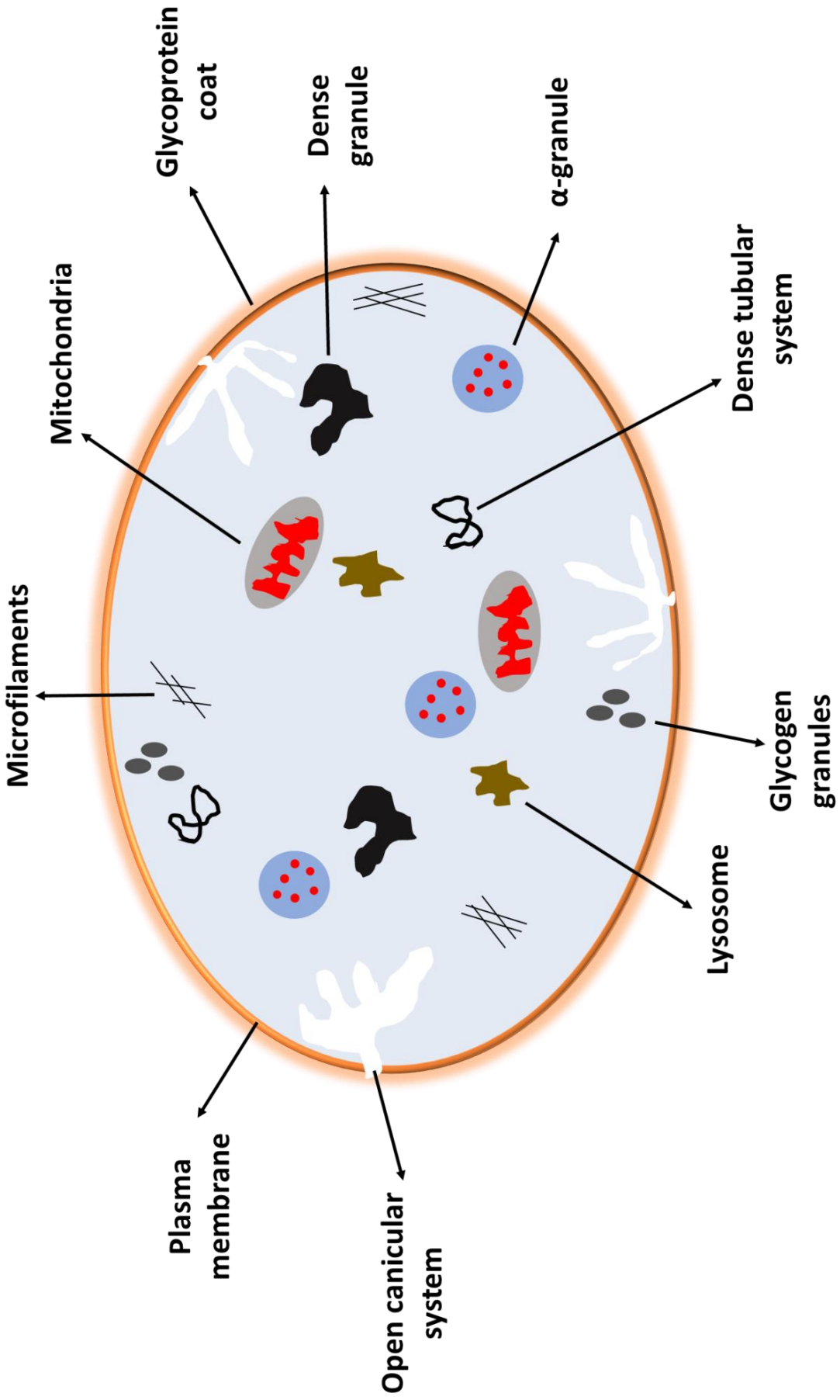
Behnke (1967) described the two membrane channel structures in platelets: the open canalicular system (OCS) and the dense tubular system (DTS) (Figure 1.2). The OCS is the site of granule release and substance entry via the platelet PM (Escolar and White, 1991; Fogelson and Wang, 1996; White, 2013). There are various channels in the DTS, which originate from the MK smooth endoplasmic reticulum, that are extensively distributed throughout the platelet and unlike OCS, are not contiguous with the external surface of the platelet (White, 1972; White, 2013). Ca^{2+} is actively sequestered in the DTS of quiescent platelets and quickly released after activation. Due to this movement of Ca^{2+}, there are rapid increases in the cytoplasmic Ca^{2+} levels of platelets that are crucial to their activation, as described in Section 1.9 (Gerrard et al., 1978; Ware et al., 1986; Ebbeling et al., 1992). ATPases in the DTS, which are important for the regulation of adenylyl cyclase (AC) activity, take part in managing GPCR downstream signalling (Cutler et al., 1978). Several secretory granules are present in platelets: α-granules, dense granules and lysosomes (Figure 1.2). α-granules are spherical granules with a diameter of approximately 200–500 nm. They are the main secretory granules in platelets (approximately 40–80 per platelet) (Italiano and Battinelli, 2009). They contain various molecules that are important for platelet adhesion and aggregation: proteins like von Willebrand Factor (VWF), fibronectin and fibrinogen; coagulation factors; growth factors like platelet-derived growth factor; and chemokines such as platelet factor 4 and transforming growth factor β (Stenberg et al., 1984; Harrison and Cramer, 1993; Maynard et al., 2007). In addition, they contain various transmembrane receptors, like integrin $\alpha_{IIb}\beta_3$, platelet–endothelial cell adhesion molecule 1 (PECAM-1) and GPIb-V-IX complex, the surface exposure of which is enhanced by platelet activation (Cramer et al., 1994; Berger et al., 1996; Jones et al., 2009; Clemetson and Clemetson, 2013). α-granules may be categorised by their types of cargo Italiano et al.

(2008), which include pro- and anti-angiogenic products that are released in distinct ways by platelets. Sehgal and Storrie (2007) provided support for this categorisation with their observation that fibrinogen and VWF are packaged in and secreted from α-granules in distinct manners. There are only 3–5 dense granules per platelet. They contain lower-molecular-weight components that are essential for regulating activation, such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), Mg^{2+}, Ca^{2+} and serotonin (Gear and Burke, 1982; Meyers et al., 1982; Jonnalagadda et al., 2012; Golebiewska and Poole, 2015).

After platelets are activated, α- and dense- granules release their contents across the OCS (where there is separate release of α- and dense granules) or across the non-OCS surface of the PM. The contents of the granules cause the further activation of platelets. The target platelet may be the same platelet (autocrine) or an adjacent platelet (paracrine); cumulatively, this process generates the positive feedback in platelets (Goggs et al., 2013; Golebiewska and Poole, 2015). Section 1.10 explains the granule secretion process. There have been considerably fewer studies on platelet lysosomes, which are defined by their acidic nature and their contents, like acid hydrolases. Lysosomes are also secreted in vivo following platelet activation (Ciferri et al., 2000; Polasek, 2005). Glycogen particles are also found in platelets where they function as an important source of energy, also, approximately 4–7 mitochondria are present in the granule zone of each platelet (Smyth et al., 2010a), and participate in prothrombotic redox signalling and apoptosis. Figure 1.2 shows a diagram of the ultrastructure of the platelet (Zharikov and Shiva, 2013; Rocha et al., 2014).

Figure 1.2: Diagrammatic representation of platelet ultrastructure.

An external glycoprotein coat surrounds the platelet plasma membrane. The sub-membrane area has abundant actin, microtubules and microfilaments that ensure that the resting platelets maintain their discoid shape and that modify the shape of activated platelets. There are various organelles in the cytosol, such as α- and dense granules, mitochondria, glycogen granules and lysosomes. There are two membrane systems in platelets: the open canalicular system (OCS), which provides for the transmission of molecules in and out of the platelets, and the dense tubular system (DTS), which functions as a store of Ca^{2+}.


Glycoprotein coat
Dense granule
α-granule
Mitochondria
Dense tubular system
Microfilaments
Glycogen granules
Lysosome
Plasma membrane
Open canicular system

1.7 Platelet Receptors

Platelet activation is a rapid, dynamic process. This complicated process is rigorously regulated by several proteins in a variety of pathways. When an agonist binds its surface receptor on a platelet, this serves as a stimulus that starts a signal transduction cascade through tyrosine kinase-dependent phosphorylation of several proteins, or stimulation of G-protein signalling which ultimately permits platelets to create aggregates. There are three broad classes of platelet receptors: (1) receptors that attach to immobilised matrix proteins like collagen, including the GPIb-V-IX complex, integrin $\alpha_2\beta_1$ and GPVI (Gibbins, 2004; Li et al., 2010); (2) GPCRs, the activation of which is brought about by soluble mediators in the plasma or secreted by platelets, including thrombin, ADP or thromboxane A2 (TxA_2) (Rivera et al., 2009); and (3) receptors that stabilise the thrombus, such as integrin $\alpha_{IIb}\beta_3$ and Eph receptors (Brass et al., 2004; Gibbins, 2004; Li et al., 2010). A detailed explanation of the signalling pathways downstream of these receptors is presented in Section 1.8.

1.8 Platelet Signalling

Platelets are involved in a wide range of intercellular interactions via complicated signalling mechanisms. Several signalling pathways are activated by the recruitment of signalling molecules to ligand-bound receptors, leading to conformational changes in platelets. Multiple factors may induce platelet signalling, including adhesion process or exposure to agonists, such as collagen, which triggers the secretion of granules resulting in an inside-out activation of integrin $\alpha_{IIb}\beta_3$, causing platelets to aggregate and form haemostatic plug (Badimon and Vilahur, 2008).

1.8.1 Platelet Collagen-Mediated Signalling

Almost 40% of all proteins that make up the ECM of the blood vessel wall are collagens. They are present as insoluble scaffolds with a triple helical structure made of three distinct polypeptide α-chains (Smethurst et al., 2007). The vessel wall receives mechanical and structural strength from the collagen, which also serves as a substrate for the attachment and activation of platelets (Farndale et al., 2004; Roberts et al., 2004). Twenty-five different varieties of collagen have been discovered, of which 10 are present in the human blood vessel; types I, II, III, IV, V, VI, VIII, XII, XIII and XIV. However, types I, III, V and VI (fibrillar collagens) and types IV and VIII (non-fibrillar collagen) are inherently thrombogenic (Barnes and Farndale, 1999; Ricard-Blum, 2011; Kauskot and Hoylaerts, 2012). Collagen-induced platelet activation commences with the interaction of the platelet GPIb–V–IX receptor complex with VWF bound to exposed collagen at the site of injury. This slows the movement of platelets, allowing a stable interaction of platelets with collagen via collagen receptors GPVI and integrin $\alpha_2\beta_1$, which leads to adhesion. The clustering of GPVI leads to the stimulation of intracellular tyrosine kinase signalling that leads to Ca^{2+} mobilisation, granule secretion, integrin $\alpha_{IIb}\beta_3$ activation and the creation of platelet aggregates (Jaffe et al., 1974; Alevriadou et al., 1993; Gibbins et al., 1997; Emsley et al., 1998; Knight et al., 1999; Cole et al., 2003; Inoue et al., 2003; Wu et al., 2003; Gibbins, 2004; Li et al., 2010; Nuyttens et al., 2011; Peyvandi et al., 2011).

1.8.1.1 VWF Receptor: the GPIb-IX-V Complex

GPIb-IX-V is a receptor complex with a unique structure that is exclusively found on MKs and platelets. A single platelet expresses up to 25,000 copies of the receptor (Berndt et al., 2001). Also, researchers proposed that the receptor comprised a single GPV subunit, two GPIX subunits, two GPIbβ subunits and two GPIbα subunits (Berndt

et al., 2001). More recently, however, researchers determined that GPIbα, GPIbβ, GPIX and GPV are present in the molar ratio of 2:4:2:1 (Luo et al., 2007a). Members of the leucine-rich repeat protein superfamily encode these subunits (Berndt et al., 2001).

Upon injury, initial tethering of platelets occurs via interaction between GPIb-IX-V complex (on platelet) and the plasma protein VWF (attached to collagen), which represents the first interaction between platelets and the damage area. The process requires the increased shear rates found in stenosed arteries, arterioles and small arteries (Savage et al., 1998). Tethering decreases the velocity of the platelets and facilitates their interaction with the damaged tissue via different receptors. GPIb-IX-V captures platelets at the site of damage. Integrin activation is crucial for stabilising platelet adhesion. Researchers had proposed that the on/off rate for GPIb-IX-V and VWF may be too transient to initiate signalling (Savage et al., 1996).

However, it was later demonstrated that GPIb-IX-V mediates weak intracellular signalling via PLCγ2 and SFK pathways, thereby leading to platelet activation and aggregation mediated by integrin $\alpha_{IIb}\beta_3$ (Yap et al., 2002; Mangin et al., 2003). Ozaki et al. (2005) proposed a hypothetical GPIb-IX-V signal transduction pathway, primarily located in lipid rafts. Mangin et al. (2004) and Mu et al. (2008) proposed the existence of constitutive binding between GPIb-IX-V and the p85 subunit of PI3K via 14-3-3ζ, which would be consistent with the Ozaki model. Moreover, GPIb-IX-V interacts with VWF to induce Src interaction with PI3K, thereby causing downstream signalling, such as PLCγ2 activation.

Individuals with dysfunctional GPIb-IX-V or who lack GPIb-IX-V develop Bernard-Soulier syndrome (Lopez et al., 1998), which is a congenital bleeding disorder characterised by giant platelets and mild thrombocytopenia. VWF is the primary ligand

for GPIb-V-IX and the lack of interaction between them majorly contributes towards the progression of Bernard-Soulier syndrome. Additionally, it has been reported that GPIb-V-IX interacts with various coagulation factors such as thrombin and factors VII, XI and XII. The absence of these interactions may also play a role in the bleeding defect manifested by Bernard-Soulier syndrome patients (Dumas et al., 2003; Lanza, 2006; Vanhoorelbeke et al., 2007).

1.8.1.2 Collagen Receptors: GPVI and $\alpha_2\beta_1$

The two main collagen receptors expressed by platelets are integrin $\alpha_2\beta_1$ and the immunoreceptor GPVI (Nieswandt and Watson, 2003; Pozgajova et al., 2006). Binding has been observed between $\alpha_2\beta_1$ and GFOGER, GPVI and GPO repeat sequences (collagen related peptide with GPO repeats where: G-Glycine, P-Proline, O-Hydroxyproline) and between $\alpha_2\beta_1$ or GPVI and non-specific regions of collagen fibrils. The receptors have distinct affinities for their cognate motifs. For instance, activated $\alpha_2\beta_1$ binds with high affinity, whereas GPVI binds with low affinity. GPVI first produces a rapid signal, after which activated $\alpha_2\beta_1$ mediates firm adhesion (Lanza et al., 1986; Nieswandt and Watson, 2003). These receptors work together, but must perform distinct roles to control platelet adhesion and thrombus development (Holtkotter et al., 2002; Massberg et al., 2003; Sarratt et al., 2005).

1.8.1.2.1 GPVI/FcRγ Complex

The physiological ligands of GPVI include laminin and collagen, which are present in the ECM surrounding damaged endothelial cells (ECs) (Inoue et al., 2006; Ruggeri and Mendolicchio, 2007). To study downstream signalling actions that are independent of integrin $\alpha_2\beta_1$ *in vitro*, synthetic ligands are used such as snake venom toxin convulxin, which binds GPIb-IX-V and GPVI (Jandrot-Perrus et al., 1997; Kanaji et al., 2003;

Canobbio et al., 2004; Wei et al., 2009), and collagen-related peptide, which binds only to GPVI (Asselin et al., 1997).

Human chromosome 19 carries the gene for GPVI, a 62-kDa type I transmembrane receptor (Clemetson et al., 1999; Wei et al., 2009) that is found only on platelets and MKs (Moroi and Jung, 2004; Jackson, 2011). It comprises an extracellular mucin-like stalk, a pair of immunoglobulin (Ig)-like domains, a transmembrane region, and a cytoplasmic tail of only 51 amino acids. In murine GPVI, the cytoplasmic tail comprises only 27 residues, indicating that the missing residues are not crucial for the function of GPVI (Moroi and Jung, 2004; Jackson, 2011).

As described by Horii et al. (2009), a fraction of GPVI is found on the surface of platelets as a dimer, which is believed to be associated with causing an increase in affinity for collagen. Signalling through Src family kinases (SFKs) is stimulated by ligand-mediated clustering of GPVI. The transmembrane region of GPVI is constitutively, non-covalently bound to FcRγ through a positively charged arginine residue (Nieswandt and Watson, 2003; Watson et al., 2005). The binding between GPVI and the γ-chain is crucial not only for signalling, but also for GPVI expression (Gibbins et al., 1996; Poole et al., 1997; Quek et al., 2000; Severin et al., 2012).

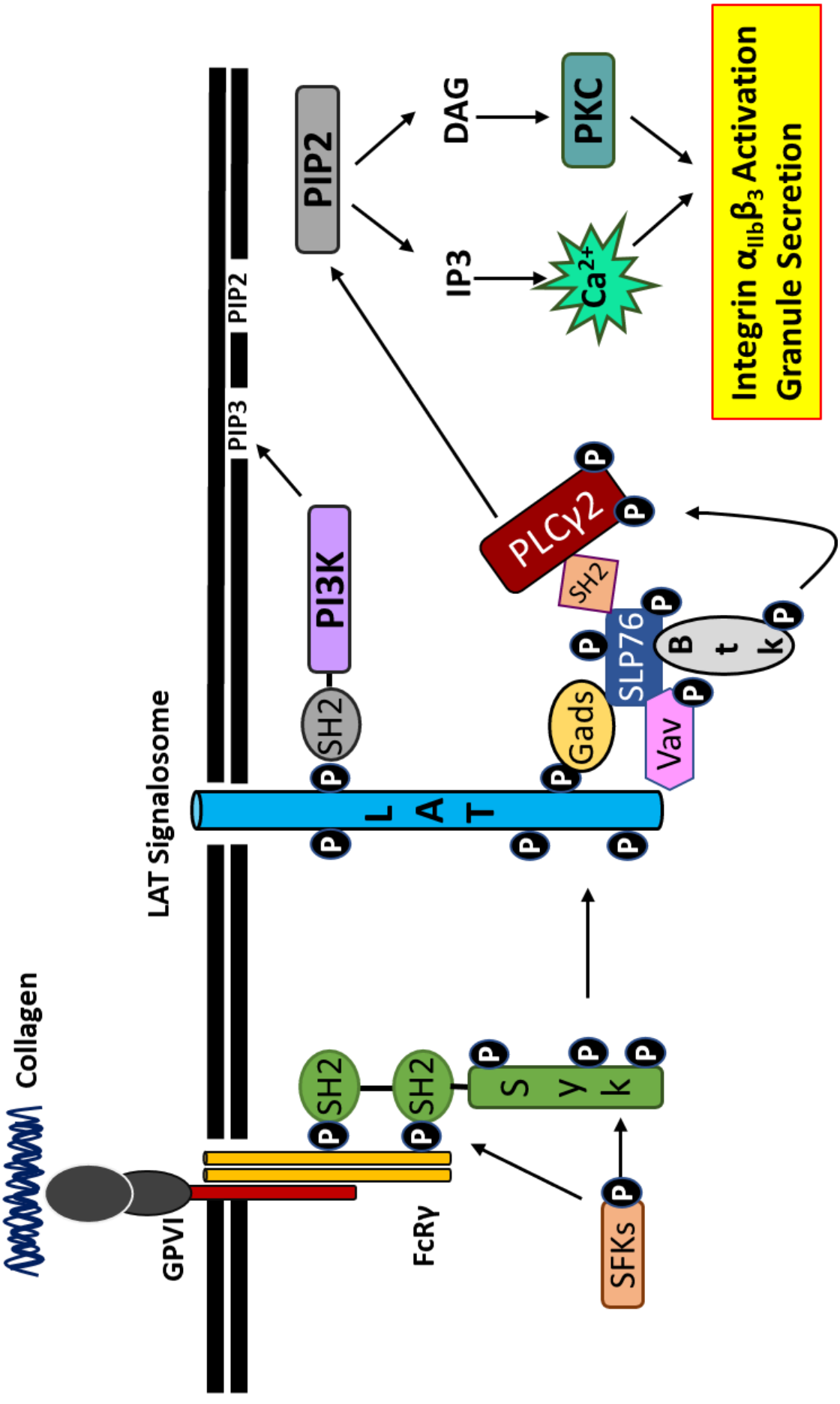
Severin et al. (2012) reported that SFKs are sequentially activated by GPVI. GPVI largely signals through Lyn and Fyn in addition to Src that minorly contributes in this process. Suzuki-Inoue et al. (2002) reported constitutive binding between the Src homology 3 (SH3) domain of Lyn and the cytoplasmic tail of GPVI, which is rich in proline residues. This binding causes phosphorylation of the $Yxx[L/I]x_{6\text{-}12}Yxx[L/I]$ motif in FcRγ. This conserved sequence is an immunoreceptor tyrosine-based activation motif (ITAM) (Gibbins et al., 1996; Poole et al., 1997). Binding of Syk, via

its Src homology 2 (SH2) domain, with the phosphorylated FcRγ ITAM causes autophosphorylation and additional SFK-mediated phosphorylation (Watson et al., 2005). The resultant activated protein promotes downstream signalling events in lipid rafts, including production of the linker for activation of T cells (LAT) signalosome, which comprises the adaptors LAT and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76), Tec family kinases, Gads/Grb2, effector proteins like phospholipase C (PLC) γ2 and phosphoinositide 3-kinase (PI3K) and the Vav family of guanine nucleotide exchange factors (Watson et al., 2005). This leads to formation of inositol 1,4,5-trisphosphate (IP_3) and the secondary messenger diacylglycerol (DAG). As shown in Figure 1.3, IP_3 and DAG stimulate Ca^{2+} release from intracellular spaces and protein kinase C (PKC) activation, respectively. These signalling events promote thrombus growth; they are reinforced by the activation of integrins, the discharge of α-granules and dense-granules and the release of the secondary mediators TxA_2 and ADP (Nieswandt and Watson, 2003; Offermanns, 2006).

Since redundant platelet receptors can mediate platelet activation, only rare cases of bleeding diathesis have been reported in individuals with qualitative or quantitative defects in GPVI. A recently reported case of bleeding diathesis was caused by a genetic defect in GPVI (Hermans et al., 2009; Matus et al., 2013). In addition, clinical cases of idiopathic thrombocytopenic purpura caused by low GPVI expression have been reported. The patients with idiopathic thrombocytopenic purpura produce autoantibodies against GPVI, which cause GPVI shedding and the removal of the GPVI/FcRγ complex from the surfaces of platelets (Boylan et al., 2004). Studies in GPVI-deficient mice have demonstrated the requirement of GPVI for collagen-induced platelet activation (Konishi et al., 2002; Lockyer et al., 2006; Matsumoto et al., 2007).

Figure 1.3: Glycoprotein VI signalling pathway.

Src family kinases (SFKs) Fyn and Lyn bind the glycoprotein (GP) VI receptor once GPVI binds to the collagen. Autophosphorylation of SFKs, which occurs due to collagen-induced GPVI clustering, leads to phosphorylation of the tyrosine residues in the Fc receptor γ-chain (FcRγ). The phosphorylated residues in FcRγ serve as docking sites for the protein-tyrosine kinase Syk, which contains a Src homology 2 domain. Syk phosphorylates the linker for activation of T cells (LAT) tyrosine residues, leading to the generation of the LAT signalosome. The signalosome comprises Gads, Vav and Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP-76); and it is responsible for recruiting phosphoinositide 3-kinase (PI3K), which triggers the conversion of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate. The interactions between Tec kinases and Btk aid the activation and phosphorylation of phospholipase C (PLC) γ2. Activated PLCγ2 produces diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C and IP_3 mobilises intracellular Ca^{2+}. These signalling events result in degranulation and enhance the affinity of integrin $\alpha_{IIb}\beta_3$. Ultimately, the signalling cascade promotes platelet aggregation.


Collagen
GPVI
FcRγ
SFKs
S
Y
k
SH2
SH2
LAT Signalosome
L
A
T
PI3K
SH2
PIP3
PIP2
PIP2
PLCγ2
SH2
SLP76
Gads
Vav
B
t
k
DAG
IP3
PKC
Ca2+
Integrin αIIbβ3 Activation
Granule Secretion

1.8.1.2.2 Integrin $\alpha_2\beta_1$

Integrin $\alpha_2\beta_1$, which is also called GPIa/IIa, is the most primitive known receptor expressed on platelets. It primarily acts as an adhesion receptor (He et al., 2003; Nieswandt and Watson, 2003). The expression of this receptor varies among individuals, from 2,000 to 4,000 copies per platelet (Best et al., 2003; Samaha et al., 2004), and preferentially binds to collagen types I–V (Holtkotter et al., 2002; Leitinger and Hogg, 2002). After platelet activation, its expression level remains unchanged. Resting platelets mostly express integrins in their low-affinity states; the integrins adopt their high affinity states after conformational changes instigated by inside-out signalling, which occurs upon platelet interaction with collagen (Jung and Moroi, 2000a; Jung and Moroi, 2000b).

In addition to its role in platelet adhesion, integrin $\alpha_2\beta_1$ binding improves the binding of collagen with GPVI. Therefore, it strengthens signalling via Ig receptors (Jung and Moroi, 2000a; Jung and Moroi, 2000b; Nieswandt and Watson, 2003). However, $\alpha_2\beta_1$ signalling is weaker than GPVI signalling and may make a relatively minor contribution to the formation of the thrombus, despite signalling through many of the GPVI signalling mediators, like PLCγ2, SLP-76, Syk and Src (Inoue et al., 2003). Pharmacological and genetic studies have revealed that $\alpha_2\beta_1$ makes only a small contribution to the formation of platelet aggregates and it inhibition/deletion is not against arterial thrombosis (Holtkotter et al., 2002; He et al., 2003; Farndale et al., 2004).

He et al. (2003) reported that mice deficient in integrin $\alpha_2\beta_1$ demonstrated reduced platelet attachment to collagen substrates. However, an intravital fluorescence microscopy study demonstrated that mice deficient in $\alpha_2\beta_1$ have equivalent platelet

adhesion and thrombus formation to wild type mice (Gruner et al., 2003). According to Nieswandt et al. (2001), β_1-null platelets are capable of attachment to collagen under high and low shear stress. Moreover, the size and number of normal and β_1-null platelet aggregates were comparable. However, platelet adhesion was not observed in the absence of GPVI expression.

1.8.1.3 CLEC-2 receptor

Human and mouse platelets express CLEC-2 on their surfaces (Suzuki-Inoue et al., 2006; Severin et al., 2011). Human chromosome 12 encodes the CLEC-2 gene. CLEC-2 is a type II membrane protein comprising a carboxyl-terminal (C-terminal) extracellular domain, an amino-terminal (N-terminal) cytoplasmic domain and a single transmembrane region. In addition to platelets, inflammatory dendritic cells express CLEC-2. Podoplanin, a type I transmembrane GP, is the endogenous ligand for the receptor (Chaipan et al., 2006; Suzuki-Inoue et al., 2007). Strong platelet aggregation is induced by podoplanin-expressing cancer cells (Tsuruo and Fujita, 2008). Moreover, podoplanin and CLEC-2 are important for lymphatic development, since mice lacking either demonstrate blood–lymphatic mixing (Uhrin et al., 2010; Herzog et al., 2013).

Rhodocytin, a toxin from snake venom, is used in vitro for CLEC-2-mediated platelet activation through Src and Syk family kinases, which leads to activation of PLCγ2. The toxin, obtained from *Calloselasma rhodostoma*, has also been used to affinity-purify CLEC-2 (Severin et al., 2011; Suzuki-Inoue et al., 2011). CLEC-2 induced protein tyrosine phosphorylation and platelet activation are blocked by the Syk inhibitors PRT-060318 and R406 (Spalton et al., 2009; Hughes et al., 2013) and SFK inhibitors like PP2 (Severin et al., 2011).

CLEC-2 signals through a mechanism distinct from that of GPVI. It signals via a hemITAM sequence (YxxL) in its tail region, which is phosphorylated by Syk (Severin et al., 2011; Hughes et al., 2013). CLEC-2 signalling involves the two SH2 domains of Syk and, accordingly, its signalling is inhibited by point mutations in these domains (Fuller et al., 2007). Syk activation results in the assembly of the LAT signalosome and downstream regulation is thereby similar to that stimulated by GPVI (Suzuki-Inoue et al., 2006; Fuller et al., 2007).

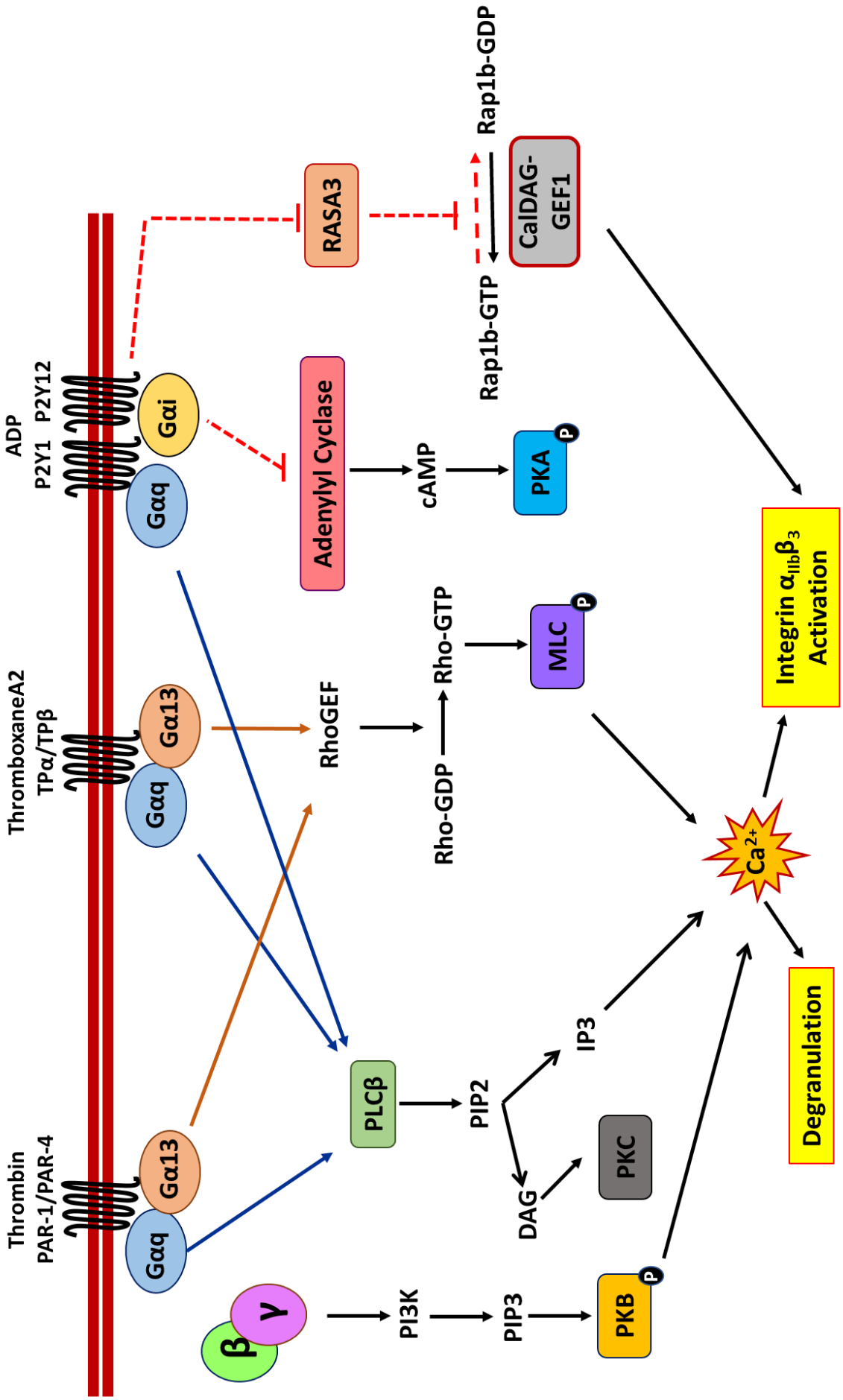
1.8.2 Platelet Activation through Activation of GPCRs

GPCRs constitute a large group of membrane receptors; the human genome contains more than 800 GPCRs (Fredriksson and Schioth, 2005). Structurally, they consist of an intracellular C-terminus, seven transmembrane domains and an extracellular N-terminus. Although the transmembrane and extracellular domains serve as binding pockets for certain ligands, the N-terminus is the primary ligand-binding site (Dohlman et al., 1987; Lerner et al., 1996; Verrall et al., 1997). Important receptors for the activation of platelets include TP receptor, which is activated by TxA_2, the ADP receptors $P2Y_1$ and $P2Y_{12}$ and the thrombin protease-activated receptors PAR-1 and PAR-4 on human platelets (PAR-3 and PAR-4 on mouse platelets). Immediately upon agonist binding with GPCRs, the GPCR intracellular and transmembrane subunits undergo conformational changes leading to G-protein interaction and activation. The heterotrimeric protein structure of G-proteins comprises G_α, G_β and G_γ subunits. The α-subunit is bound to guanosine diphosphate (GDP) in an inactive resting state. Activation of G-proteins involves the replacement of GDP with guanosine triphosphate (GTP), followed by dissociation of the α-subunit from the $G\beta\gamma$ dimer (Sondek et al., 1996; Oldham and Hamm, 2008).

Classification of G-proteins is done on the basis of the sequence and function of their Gα subunits, which includes four families; $G_{\alpha q}$, $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha 12/13}$. $G_{\alpha q}$ family signals via the activation of activation of Phospholipase Cβ (PLCβ). $G_{\alpha s}$ is coupled with adenylyl cyclase, whereas, $G_{\alpha i}$ function by down-regulating adenylyl cyclase activation and thus modulating cyclic adenosine monophosphate (cAMP) levels within the cell. $G\alpha_{12/13}$ involves signalling via activation of RhoGTPase nucleotide exchange factors (RhoGEFs) (Liebmann and Bohmer, 2000; Ellis, 2004; Siehler, 2009; Harden et al., 2011). The Gβγ dimer also stimulates intracellular signals that activate platelets through various pathways. When the GTP bound to the α-subunit is hydrolysed to GDP, the G_{α} subunit again binds the $G_{\beta\gamma}$ dimer and the G-protein trimer links with the GPCR (Kleuss et al., 1994). A schematic diagram of the GPCR signalling that causes platelet activation is shown in Figure 1.4.

Figure 1.4: Major G-protein-coupled receptor-mediated signalling pathways in platelets.

Upon activation, platelets release numerous soluble agonists that trigger activatory signalling through G-protein-coupled receptors. The adenosine diphosphate (ADP) receptor $P2Y_1$, the protease-activated receptor (PAR)-1 and PAR-4 receptors for thrombin and the thromboxane A2 receptor (TP) are associated with $G_{\alpha q}$, which coordinates with phospholipase C (PLC) β to cause phosphatidylinositol 3,4-bisphosphate (PIP2) cleavage, thereby forming inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These events stimulate the secretion of granules and increases in cytosolic Ca^{2+} levels, which enhance the affinity of $\alpha_{IIb}\beta_3$ for fibrinogen. PAR-1, PAR-4 and TP also signal through $G_{\alpha 13}$. The myosin light chain (MLC) is phosphorylated by Rho kinase, which causes degranulation, cytoskeletal rearrangement and, ultimately, conformational changes in platelets. The ADP receptor $P2Y_{12}$, which is associated with $G_{\alpha i}$, decreases cyclic adenosine monophosphate (cAMP) formation triggered by adenylyl cyclase. These events suppress the activation of protein kinase A (PKA) and enhance the activation of platelets. Also, RASA3 activity is inhibited by $P2Y_{12}$ sensitisation upon platelets activation which promote Rap1b activation. The Gβ/γ dimer also signals through phosphoinositide 3-kinase (PI3K) activation, which causes protein kinase B (PKB) activation.


ADP
P2Y1 P2Y12
Gαi
Gαq
RASA3
CalDAG-GEF1
Rap1b-GDP
Rap1b-GTP
Adenylyl Cyclase
cAMP
PKA
P
ThromboxaneA2
TPα/TPβ
Gα13
Gαq
RhoGEF
Rho-GTP
Rho-GDP
MLC
P
Integrin $\alpha_{IIb}\beta_3$ Activation
Thrombin
PAR-1/PAR-4
Gα13
Gαq
PLCβ
PIP2
IP3
DAG
PKC
Ca^{2+}
Degranulation
β
γ
PI3K
PIP3
PKB
P

1.8.2.1 Thrombin Receptors: PARs

The coagulation cascade involves multiple enzymatic reactions which take place at sites of injury, leading to the formation of the soluble plasma serine protease thrombin, which is also known as factor IIa (Hoffman and Monroe, 2001). The inactive zymogen prothrombin is converted into active thrombin, which generates insoluble fibrin polymers from soluble fibrinogen via its serine protease activity. Thrombin also serves as a major agonist for platelets (Coughlin, 2000). Thrombin activates platelets through cleavage of protease activated receptors (PARs) on the cell surface (Coughlin, 2005). There are two PAR receptors, PAR-1 and PAR-4, expressed on human platelets (Kahn et al., 1999). PAR-4 is found on human and mouse platelets, whereas PAR-1, the chief thrombin receptor on human platelets, is absent from mouse platelets where PAR-3 replaces PAR-1 (Kahn et al., 1998). Human platelets express approximately 1,000–2,000 copies of PAR-1 (Brass et al., 1992; Ramstrom et al., 2008).

Coughlin (2000) reported that PAR-1 and PAR-4 couple with the heterotrimeric G-proteins $G_{\alpha 12/13}$ and G_q. PAR-1 and PAR-4 trigger PLCβ to generate DAG and IP_3, leading to the discharge of Ca^{2+} and PKC activation. These two receptors bind the α-subunit of the G_q proteins. In addition, these receptors activate the $G_{\alpha 12/13}$ pathway. This, in turn, causes Rho kinase activation and cytoskeletal rearrangements. Like PAR-4, PAR-3 is expressed on mouse platelets. However, PAR-3 is not involved in transmembrane signalling. In contrast to the other members of its family, PAR-3 serves as a cofactor that positions thrombin in the proximity of PAR-4 on platelets in mice(Coughlin, 2000).

1.8.2.2 ADP Receptors: $P2Y_1$ and $P2Y_{12}$

Damaged endothelial cells (ECs), red blood cells and activated platelets release ADP in the region of a vascular injury. ADP supports the activation of platelets via positive feedback. It is also crucial for recruiting platelets to the developing thrombus (Offermanns, 2006). Platelets express two ADP receptors: $P2Y_1$ and $P2Y_{12}$ (Gachet et al., 2006). Human platelets express 10 times fewer $P2Y_1$ molecules (approximately 150) than $P2Y_{12}$ molecules. The coactivation of these two ADP receptors is important for ADP-induced platelet activation, as the experimental deletion or inhibition of one of the receptors results in reduced platelet aggregation (Gachet, 2006; Gachet et al., 2006; Murugappa and Kunapuli, 2006). This finding implies that these receptors work in coordination to rapidly, stably activate platelets.

$P2Y_1$ associates with the G_q and $P2Y_{12}$ associates with the G_i heterotrimeric G-protein α-subunit. In platelets, $P2Y_1$ is responsible for the mobilisation of Ca^{2+}, conformational changes and transient aggregation. On the other hand, $P2Y_{12}$ inhibits adenylyl cyclase (AC)-dependent increases in the concentration of cyclic adenosine monophosphate (cAMP) and activates Rap1b and PI3K which are required for integrin activation (Lova et al., 2002; Dorsam and Kunapuli, 2004; Hardy et al., 2004; Gachet et al., 2006).

It has been demonstrated that Ras GTPase-activating protein 3 (RASA3) antagonises the activation of Rap1b through CalDAG-GEFI in order to balance the activation and inhibition of integrins on platelets (Stefanini et al., 2015; Stefanini and Bergmeier, 2016). The levels of expression of CalDAG-GEFI and RASA3 are comparable, as revealed by plateletomic studies (Rowley et al., 2011; Simon et al., 2014). Based on these reports, researchers have highlighted possible role of RASA3 to play in platelet activation. Researchers validated this function of RASA3 by demonstrating RASA3-

mediated inhibition of Rab1-dependent platelet activation (Stefanini et al., 2015; Stefanini and Bergmeier, 2016).

Moreover, a potential model for the involvement of RASA3 in Rab1 signalling was proposed by Stefanini et al. (2015) based on these findings. They proposed that a highly active form of RASA3 is maintained on the platelet plasma membrane, which impedes the activation of Rab1 through CalDAG-GEFI and sustains the quiescent state of the platelets. On the other hand, activated platelets cause $P2Y_{12}$ sensitisation and consequential PI3K activation. RASA3 activity is inhibited by activated kinases, which depresses the fast exchange of nucleotides on Rap1 and integrin activation (Stefanini et al., 2015; Stefanini and Bergmeier, 2016). Furthermore, the activation of $P2Y_{12}$ leads to constant aggregation of platelets and amplification of signalling induced by integrin $\alpha_{IIb}\beta_3$, TxA_2 and collagen (Lova et al., 2002; Dorsam and Kunapuli, 2004; Hardy et al., 2004; Gachet et al., 2006).

1.8.2.3 TxA_2 Receptor: TP

TxA_2 is a short-lived lipid mediator. It is released by activated platelets to improve and stabilise activation signals and to recruit more platelets to the developing thrombus (Hamberg et al., 1975). Its role as a strong vasoconstrictor has also been established (Francois et al., 2004). Activated platelets generate TxA_2 from prostaglandin H2 via thromboxane A synthase (TxS) (Huang et al., 2004; Nakahata, 2008). Since TxA_2 is lipid soluble, it can cross the plasma membrane to bind TP expressed by the same or a nearby platelet (Shen and Tai, 1998; Suzuki-Inoue et al., 2002; Davi and Patrono, 2007). Like the ADP receptor $P2Y_1$ and the thrombin receptors PAR-1 and PAR-4, activated TP associates with the $G_{12/13}$ and G_q α-subunits (Djellas et al., 1999). Individuals with abnormally low levels of TxA_2 develop a minor bleeding disease,

suggesting that TxA_2 is an important positive-feedback agonist for platelets (Mumford et al., 2010; Dawood et al., 2012). In agreement with this, TP-deficient mice experience prolonged bleeding (Thomas et al., 1998).

1.8.3 Integrin $\alpha_{IIb}\beta_3$ Signalling

Integrin $\alpha_{IIb}\beta_3$ is a GP that is expressed abundantly on the surface of platelets. A single resting human platelet may express 60,000 to 80,000 copies of this GP, which is also known as GPIIb/IIIa. The GPIIa and GPIIb genes reside on human chromosome 17 (Shattil et al., 1998). Platelet α-granules also contain $\alpha_{IIb}\beta_3$, which is transported to the platelet surface upon activation, thereby causing a 30%–50 % increase in the surface exposure of this integrin (Shattil et al., 1998; Shattil and Newman, 2004). Shattil and Newman (2004) reported that α_{IIb} subunit is found in only platelets and MKs, although the β_3 subunit is expressed by various cells. Integrin $\alpha_{IIb}\beta_3$ facilitates platelet binding with VWF, fibronectin and fibrinogen. Resting platelets contain this GP in its low-affinity state. The stimulation of platelet-activation pathways, as through P_2Y receptors, PARs and GPVI, usually results in the activation of integrin $\alpha_{IIb}\beta_3$ through a phenomenon referred to as inside-out signalling (Ma et al., 2007). This process results in dramatic conformational changes in integrin $\alpha_{IIb}\beta_3$ (Offermanns, 2006).

The interaction between the membrane-proximal cytosolic regions of the α- and β-subunits of integrin $\alpha_{IIb}\beta_3$ maintains the integrin in its inactive conformation by forcing the extracellular domains to remain in the bent position (Luo et al., 2007b). Platelet activation results in DAG production and an increase in the Ca^{2+} concentration. Consequently, calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) is activated and subsequently activates Rap1b (Crittenden et al., 2004; Stefanini et al., 2009; Joo, 2012). There is a strict preservation of the fine

balance between the GTP loading and unloading of Rab1 in platelets. This balance is important to avoid needless platelet activation and subsequent thrombosis. Rab1 can switch between GTP-bound active and GDP-bound inactive states with the help of a pair of regulatory proteins. The guanine nucleotide exchange factor CalDAG-GEFI exchanges GDP for GTP, which leads to Rab1 activation, and GTPase activating proteins (GAPs) are responsible for GTP hydrolysis, which inhibits Rab1 activation (Bos et al., 2007).

The GAP RASA3 is crucial for restraining Rab1 activation by CalDAG-GEFI; RASA3 in platelets is maintained in an extremely active state, thereby down regulating Rab1 activation and keeping platelets in a non-adhesive, quiescent state. However, platelet activation terminates RASA3 activity, which permits GTP loading and activation of Rab1, leading to integrin activation (Iwashita et al., 2007; Stefanini et al., 2015; Stefanini and Bergmeier, 2016). The $P2Y_{12}$ receptor is targeted by the antithrombotic drug clopidogrel bisulphate, which mainly works by prevention of RASA3 inactivation, thereby limiting Rab1 signalling and integrin activation (Stefanini et al., 2015; Stefanini and Bergmeier, 2016).

Activated Rap1b is required for integrin activation via the development of the integrin activation complex (Crittenden et al., 2004; Stefanini et al., 2009; Joo, 2012), which until recently was believed to comprise talin, Rap1b and Rap1b-interacting adaptor molecule (RIAM). The attachment of talin to the β-subunit breaks the bond of the β-subunit with the α-subunit of the integrin, leading to a conformational change. This change occurs with a switchblade-like movement and activates the integrin so that it can bind to its substrate molecules (Han et al., 2006; Luo et al., 2007b; Banno and Ginsberg, 2008; Li et al., 2010).

RIAM-null mice have recently been developed (Stritt et al., 2015), and are viable, fertile and healthy. In RIAM-null mice, platelets surprisingly demonstrate intact β_3 and β_1 integrin activation, normal aggregation and adhesion under flow conditions and in static environments. Likewise, arterial thrombus development and haemostasis are indistinguishable in RIAM-null and wild type mice. This implies that the activation and normal functioning of integrins do not essentially require RIAM and that there may be alternative pathways of talin recruitment (Stritt et al., 2015). Kindlin-3 is also involved in the activation of integrin $\alpha_{IIb}\beta_3$. The dysfunction or absence of this protein in humans or mice, respectively, causes impaired integrin activation (Moser et al., 2008; Malinin et al., 2009).

Ligand-mediated $\alpha_{IIb}\beta_3$ clustering activates outside-in signalling, formation of filopodia and lamellipodia, secretion and clot retraction (Shattil et al., 1998). The outside-in signalling is required for platelet adhesion and aggregation at the site of damage (Shattil and Newman, 2004). GPVI signalling and $\alpha_{IIb}\beta_3$-mediated outside-in signalling events share similarities (Watson et al., 2005; Kasirer-Friede et al., 2007). Moreover, $\alpha_{IIb}\beta_3$ clustering induced by fibrinogen binding results in the sequential activation of SFKs and Syk, the recruitment of SLP-76 and other adaptor proteins and the downstream activation of enzymes such as Vav, PI3K and PLCγ2 (Watson et al., 2005; Kasirer-Friede et al., 2007).

As in signalling downstream of GPVI activation, after integrin clustering, Fyn and Src bind the β_3 subunit and interact with Syk (Woodside et al., 2001). However, there are also important differences between GPVI and integrin $\alpha_{IIb}\beta_3$ signalling. It is not known if Syk interacts independently with the β_3 subunit or if an ITAM-expressing protein supports this interaction (Woodside et al., 2001; Mocsai et al., 2006; Hughes et al.,

2015). Finally, the transmembrane adaptor protein LAT is not involved in $\alpha_{IIb}\beta_3$ signalling, but it is important for GPVI signalling (Wonerow et al., 2002). There are also differences between integrin $\alpha_{IIb}\beta_3$ signalling in the platelets of mice and humans. For instance, FcγRIIa is involved in $\alpha_{IIb}\beta_3$ signalling in humans, but not in mice (Boylan et al., 2008).

Perturbation of $\alpha_{IIb}\beta_3$ function or the absence of $\alpha_{IIb}\beta_3$ causes a serious bleeding disease called Glanzmann thrombasthenia. The disease is characterised by defective platelet adhesion and aggregation (Nurden, 2006). It is phenocopied in mice by the removal of the α_{IIb} or β_3 subunit (Hodivala-Dilke et al., 1999). Mice lacking the α_{IIb} and β_3 subunits have been used to explore haematopoiesis and the role of platelets (Tropel et al., 1997; Emambokus and Frampton, 2003). Mice deficient in the β_3 subunit experience increased tail bleeding times and spontaneous haemorrhage, and intravital microscopy has revealed that such mice are incapable of forming thrombi at damaged sites (Ni et al., 2000).

Owing to its crucial role in platelet adhesion and accumulation, $\alpha_{IIb}\beta_3$ serves as a pharmacological target for preventing ischaemic cardiovascular events. The function of $\alpha_{IIb}\beta_3$ can be inhibited with humanised antibodies (e.g. abciximab), analogues of an RGD-containing peptide and peptides based on those (e.g. eptifibatide) derived from snake venom (Coller and Shattil, 2008). However, these inhibitors are used infrequently due to the associated risk of excessive bleeding (Quinn et al., 2003).

1.9 Calcium Signalling in Platelets

Intracellular Ca^{2+} is an important second messenger for the regulation of numerous cellular mechanisms. Cardiomyocyte function, for example, is controlled by calcium-induced release of Ca^{2+} (Berridge et al., 2003). Elevation of the intracellular Ca^{2+}

concentration is as an important signalling step for most of the main mechanisms involved in platelet activation, including PKC activation, integrin inside-out signalling, calmodulin (CaM) binding, Ca^{2+}-dependent protease activity and cytoskeletal rearrangements (Hathaway and Adelstein, 1979; Varga-Szabo et al., 2009; Li et al., 2010).

Ca^{2+} can enter the cytosol of a platelet from the extracellular environment via ion channels or by release from the endoplasmic reticulum within the cell. Upon depletion of calcium from DTS, there is a rapid influx of calcium across the plasma membrane (Liou et al., 2005; Grosse et al., 2007; Tolhurst et al., 2008). This process is regarded as store-operated calcium entry (SOCE). Stromal interaction molecule 1 (STIM1) is a calcium sensor located on the membrane of DTS in a bound state with calcium through its EF-hand domain. (Zhang et al., 2005; Stathopulos et al., 2008). Depletion of calcium from the DTS is followed by dissociation of calcium-EF domain complex (Liou et al., 2005; Grosse et al., 2007). Zhang et al. (2005) demonstrated that this bond is broken in response to IP_3-triggered release of Ca^{2+} from the DTS. After detachment, STIM1 is transported to the cell membrane, and its subsequent interaction with Orai1, allowing calcium influx (Vig et al., 2006; Tolhurst et al., 2008). Hou et al. (2012) et al. (2012) reported that Orai1 proteins contain 4 transmembrane domains (TMDs). Moreover, crystal structure studies have revealed that the fourth TMD interacts with STIM1.

SOCE-independent pathways also mediate Ca^{2+} influx into platelets via transient receptor potential cation channel subfamily C member 6 (TRPC6), which serves as a store-independent, non-selective cation entry channel. Ca^{2+} enters via this channel after it is activated by agonist (Hassock et al., 2002; Mahaut-Smith, 2012; Vemana et al., 2015). Ca^{2+} may also use direct, receptor-operated pathways to enter platelets. The

$P2X_1$ purinoceptor, for instance, binds ATP and acts as a homomeric, non-selective cation channel (Vial et al., 1997; Sage et al., 2000; Mahaut-Smith et al., 2004; Mahaut-Smith et al., 2011; Mahaut-Smith, 2012). The abovementioned means for Ca^{2+} entry work to elevate the cytosolic concentration of calcium after activation of platelet.

Calcium- and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) is an intracellular signalling molecule expressed in MKs and platelets. Ca^{2+} binding to their respective motifs in CalDAG-GEFI results in TxA_2 generation, granule secretion and integrin activation (Crittenden et al., 2004; Bergmeier and Stefanini, 2009). Persistently elevated Ca^{2+} concentrations trigger scramblase enzyme activity, leading to the expression of PS on the plasma membrane, which reinforces the activation and aggregation of platelets (Ramstrom et al., 2003; Freyssinet and Toti, 2010).

1.10 Platelet Degranulation

Dense and α-granules in platelets contain various activatory molecules (Section 1.6). After platelet activation, these granules combine with the membrane of the OCS or the PM and release their contents into the extracellular environment where the contents function in an autocrine or paracrine way to further stimulate platelet activation (Golebiewska and Poole, 2015). Soluble NSF attachment protein receptors (SNAREs) are at the centre of the molecular systems that form the basis of platelet granule secretion (Sollner et al., 1993; Blair and Flaumenhaft, 2009). These membrane-associated proteins are related to platelet granules (vesicular SNAREs or vSNAREs) or the OCS/PM (target SNAREs or tSNAREs) (Duman and Forte, 2003; Sudhof and Rothman, 2009). The interaction between the coiled-coil domains in these two groups of SNAREs leads to the secretion of granules. The interaction causes the granules to come into contact with the OCS or PM, thereby producing energy, causing membrane

fusion, creating pores, and ultimately leading to the secretion of the contents of α- and dense granules (Harbury, 1998; Antonin et al., 2002).

SNAREs are related to various chaperone proteins that participate in granule secretion. The actin cytoskeleton also plays an extensive role, particularly through actin polymerisation and association with SNARE proteins (Polgar and Reed, 1999; Woronowicz et al., 2010). There is a high degree of similarity between the mechanisms through which α- and dense granule are secreted, although distinct SNARES are involved ((Flaumenhaft et al., 1999; Golebiewska et al., 2015). There is also an association between the regulatory proteins Sec1/Munc and SNARES, suggesting the existence of a mechanism that prevents incorrect granule secretion (Shirakawa et al., 2004).

1.11 Platelet Regulation by the Endothelium

Although platelets are essential for haemostasis, their inappropriate activation can cause thrombotic conditions such as myocardial infarction. Hence, platelet activity is constitutively blocked so that circulating platelets are maintained in a quiescent state in normal conditions. In the setting of tissue injury and thrombus development, the size of the developing thrombus is controlled by these negative regulatory pathways. Platelets are negatively regulated by prostacyclin (PGI_2) and nitric oxide (NO) generated by the healthy vascular endothelium (Figure 1.5) (Marcus and Safier, 1993; Clemetson, 1999).

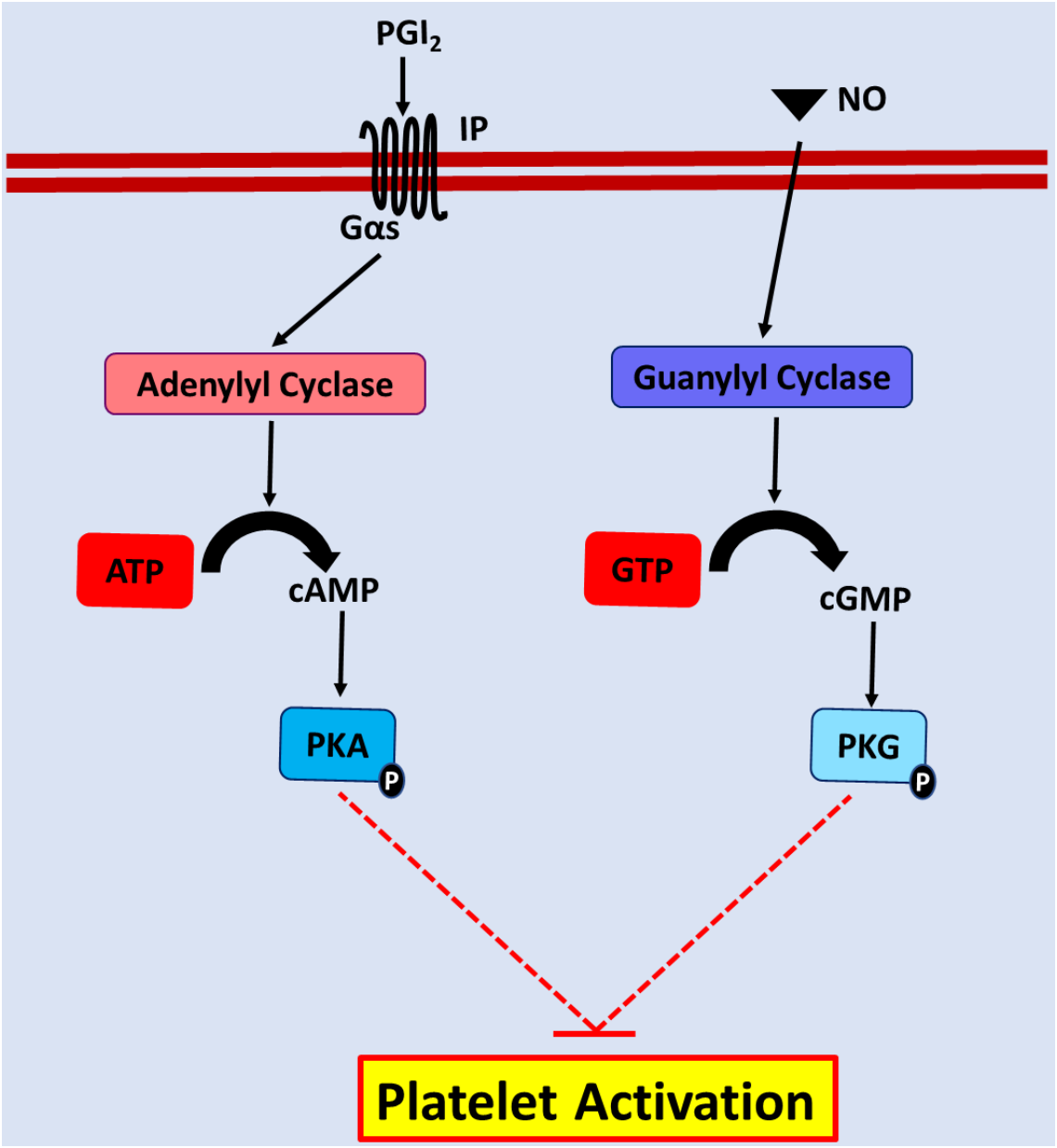


Figure 1.5: Inhibitory signalling pathways mediated by GPCRs.

Binding of prostacyclin (PGI_2) with prostacyclin receptors (IP) coupled to $G_{\alpha s}$ induces inhibitory signalling in platelets. The receptor triggers adenylyl cyclase (AC) activity to produce cyclic adenosine monophosphate (cAMP), which causes protein kinase A (PKA) phosphorylation. Consequently, Ca^{2+} mobilisation, Rap1b activation and platelet adhesion are suppressed, thereby preventing the activation of platelets. Nitric oxide (NO) also binds the guanylyl cyclase (sGC), which inhibits platelet activation via cyclic guanosine monophosphate (cGMP) generation and protein kinase G (PKG) phosphorylation. ATP, adenosine triphosphate; GTP, guanosine triphosphate

1.11.1 Platelet Regulation by NO

Normal ECs generate NO, which is biosynthesised by NO synthases (NOS) (Furchgott and Zawadzki, 1980). L-arginine is oxidised by NOS, which leads to the generation of NO and L-citrulline (Radomski et al., 1990). NOS exists as three isoforms with differential tissue expression: NOS1 (neuronal), NOS2 (inducible) and NOS3 (endothelial, known as eNOS) (Hanafy et al., 2001). NO can pass across the cell membrane, enabling it to bind its intracellular receptor guanylyl cyclase (sGC) (Bellamy and Garthwaite, 2002). The binding of NO causes a conformational change in sGC, which improves its catalytic activity. Consequently, GTP hydrolysis is initiated to form cyclic guanosine monophosphate (cGMP), thereby increasing the concentrations of cGMP in platelets (Figure 1.5). The chief effector target of cGMP is protein kinase G (PKG) (Naseem and Roberts, 2011).

PKG is a member of the AGC kinase family, comprises three functional domains: the catalytic, regulatory and N-terminal domains. Two cGMP-binding sites constitute the regulatory domain, which is responsible for the conformational change that leads to enzyme activation (Hofmann, 2005). PKG exists in two isoforms, PKGI and PKGII; the former is more abundant in platelets. The isoforms have different N-terminal domains, which confer different substrate specificities (Hofmann, 2005). Activated PKG phosphorylates the serine/threonine residues of different substrates, leading to the inhibition of platelet activation (Bult et al., 1988). Massberg et al. (1999) reported an increase prothrombotic phenotype in mice deficient in PKG. Conversely, Zhang et al. (2011) reported defective platelet aggregation in platelet-specific sGC-knockout mice suggesting stimulatory role of sGS.

1.11.2 Platelet Regulation by PGI_2

PGI_2 is a physiological inhibitor of platelet activation and a strong vasodilator with a half-life of only 3 minutes (Vane and Botting, 1995; Parente and Perretti, 2003). The membrane phospholipids of platelets are hydrolysed by calcium-dependent cytosolic Phospholipases A2 (PLA2) at their sn-2 position, resulting in the generation of lyso-lipids and arachidonic acid (Irvine, 1982). Platelet cyclooxygenase 1 (COX1) oxidises arachidonic acid to generate prostaglandin G2, which can be converted by the hydroperoxide activity of COX1 into the unstable prostaglandin endoperoxide H2 (PGH2). Subsequently, various synthases may act on PGH2 to form TxA_2, PGI_2, prostaglandin E2, prostaglandin D2 and prostaglandin F2α. Although platelets are deficient in prostaglandin D2, they express TxS, which forms TxA_2 from PGH2 (Needleman et al., 1976). PGI_2 synthase, which is abundantly expressed in ECs, generates PGI_2 from PGH2. PGI_2 is then discharged into the bloodstream (Vane and Botting, 1995). When PGI_2 binds the IP receptor, AC is activated, leading to the activation of the cAMP signalling pathway and elevated protein kinase A (PKA) expression causing inhibition of various aspects of platelet activation such as Ca^{2+} mobilisation, integrin activation and cytoskeleton rearrangement (Figure 1.5) (Yan et al., 2009).

PGI_2 is one of the strongest physiological inhibitors of platelets (Moncada et al., 1976; Weksler et al., 1977; Moncada, 1982). Increased PGI_2 generation has been reported during platelet aggregation in atherosclerotic mice (FitzGerald et al., 1984). Deficiency of the gene for PGI_2 synthase, results in platelet hypersensitivity and increased vulnerability to vascular diseases in mice (Yokoyama et al., 2002). Reduced concentrations of PGI_2 have been linked with atherosclerosis, stroke (Narumiya et al., 1999; Gawaz, 2004) and cardiovascular conditions, such as myocardial infarction

(Stein et al., 1985; Akopov et al., 1993). Rubin (1995) also reported a link between the decreased generation of PGI_2 and the pathogenesis of pulmonary hypertension. Patients with vascular disorders demonstrate reduced PGI_2 sensitivity (Sinzinger et al., 1981; Fitscha et al., 1985). These effects have been proposed to exist due to damage to PGI_2-mediated regulatory pathways (Murata et al., 1997; Yang et al., 2002).

1.12 Cellular Communication

All biological systems communicate with their environments via various mechanisms. The cells of higher organisms have developed a wide range of communication mechanisms due to the structural and functional complexity of these organisms. Cell–cell communication usually involves the exchange of spatial and temporal information, which is vital for the organisation of tissues and the preservation of the living system.

Cell signalling has been extensively studied due to its involvement in the pathogenesis of human diseases. Soluble chemical messengers are produced by cells during cell–cell communication. Target cells receive these signals via receptors, then second messengers spread the signal within the receiving cell. Signalling occurs not only between the cells of a single organism, but also between the cells of different living systems. For instance, cell signalling occurs between the uterus and the embryo (Mohamed et al., 2005). In addition, *Saccharomyces cerevisiae* releases a peptide signal into the environment during mating. This signal, mating-factor pheromone, binds its receptor on other cells, thereby rendering the receptive cell ready for mating (Lin et al., 2004). Similarly, bacterial cells exchange signals with other bacterial cells, immune cells and epithelial cells within the human gastrointestinal tract (Clarke and Sperandio, 2005).

Cell signalling is categorised by the target cell and its distance from the signalling cell. Endocrine signals, such as hormones, are carried by the blood to distant target cells. Paracrine signals, like neurotransmitters, target nearby cells. Juxtacrine signals can target the signalling cell or immediately adjacent cells. They pass through membrane lipids or proteins. Autocrine signals are produced by a cell, secreted, then influence the same cell via receptor binding. Finally, intracrine signals remain inside the signalling cell (Clarke and Sperandio, 2005).

Cell-to-cell contact is required for some methods of cellular communication. In other cases, gap junctions (GJs) are formed that link the cytoplasms of communicating cells. In cardiac muscle, GJs facilitate the transmission of an action potential from the site of a cardiac pacemaker to surrounding cells, thereby mediating heart contraction (Rohr, 2004). The immune system uses all these mechanisms of cell–cell communication.

1.12.1 Direct Intercellular Communication

Cellular communication can be intercellular or intracellular. Intracellular signaling is triggered by extracellular signals that generate responses within a target cell equipped with appropriate receptors to respond to external stimuli. Whereas, direct intercellular communication occurs between cells that are physically connected with each other and can therefore transmit signals/molecules directly from the cytosol of one cell to another through channel-like structures (See section 1.13) (Mittelbrunn and Sanchez-Madrid, 2012; Torday and Rehan, 2012).

More than 50 years ago, Furshpan and Potter (1959) identified low-resistance electrical transmission between the axons of crayfish. Later, researchers found that very small cellular junctions, which allow the passage of minute entities, are formed between non-

neuronal cells (Revel and Karnovsky, 1967). These structures became known as GJs which are involved in intercellular communication (Goodenough and Revel, 1970).

1.13 Connexins and GJs

1.13.1 Structure and Location of Connexins

Connexins (Cxs) are a large group of proteins with similar basic structures: C- and N-terminal domains present near the cytoplasmic side of the cell membrane, one intracellular loop, two extracellular loops and four transmembrane α-helices (Figure 1.6) (Harris, 2001). Mice have 20 Cx family proteins, whereas humans have 21. The proteins are differentiated by their molecular masses (Table1.1) (Sohl and Willecke, 2003; Martin and Evans, 2004). The isoforms have different molecular masses because of variations in the amino acid sequences of their C-terminal domains. Researchers have also detected variations in their cytoplasmic loops (van Veen et al., 2001). Several tyrosine, threonine and serine residues are present in the C-terminus; these residues can be phosphorylated and have been implicated in the regulation of Cxs (Kemp and Pearson, 1990; Bevans and Harris, 1999).

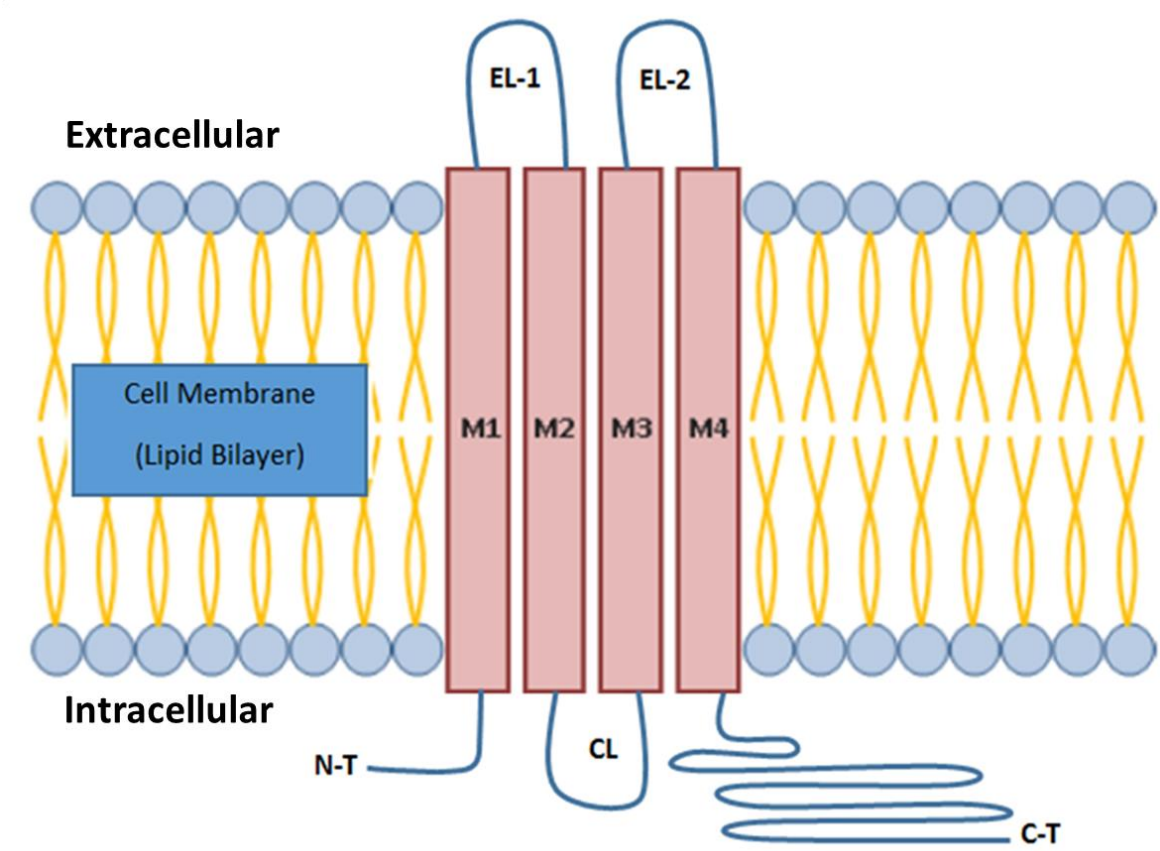


Figure 1.6: The basic structure of connexins.

Connexins are proteins that contain an amino-terminus (N-terminus, or N) and carboxyl-terminus (C-terminus, or C) in the cytoplasm, one intracellular loop, two extracellular loops (E1 and E2) and four transmembrane domains (M1–M4). The amino acid sequences of the four transmembrane domains, which are highly conserved between family members, are crucial for docking in the cellular membrane. However, the amino acid sequences and lengths of the cytoplasmic domains vary.

1.13.1.1 Transmembrane Domains

Topological experiments revealed that approximately 20 amino acid residues constitute each transmembrane domains (connexin four transmembrane domains). Detailed analysis led to the hypothesis that the third transmembrane domain (M3) comprises a chain of well-organised polar amino acids that form an amphipathic α-helix. Accordingly, the M3 domain of a Cx is believed to form the innermost lining of the transmembrane pore (Milks et al., 1988).

1.13.1.2 Cytoplasmic Domains

The cytoplasmic domains of Cxs, in contrast to the extracellular and transmembrane domains, contain varied amino acids sequences. The disparities between the different Cxs arise from structural disparities in their cytosolic regions (Vinken et al., 2006). Acidification and X-ray diffraction experiments revealed that the cytoplasmic domains of all Cxs are structurally different. Moreover, they demonstrate great flexibility in the intercellular channel where they participate in the opening and closing of the pore (Makowski et al., 1977). According to the particle–receptor hypothesis, the cytoplasmic carboxyl group, particularly in Cx43, serves as an independent binding site which identifies and binds non-covalently with a distinct peptide sequence near the pore, thereby opening and closing the pore of the channel (Morley et al., 1996). Post-translational modifications, like phosphorylation, primarily affect the cytoplasmic carboxyl tails of Cxs that control their arrangement into GJs (Warn-Cramer et al., 1998).

1.13.1.3 Extracellular Domains

The highest degree of sequence conservation has been observed in the extracellular loops E1 and E2. These loops characteristically feature three cysteine residues in the

conserved motifs CX_6CX_3C in E1 and CX_4CX_5C in E2 (Hoh et al., 1991; Elfgang et al., 1995), where X signifies any amino acid and the numeral indicates the number of residues. The cysteine residues in the extracellular loops have been proposed to be involved in the formation of disulphide bridges within molecules. Although there are no disulphide bonds between opposing connexons, they do exist between pairs of extracellular loops (Dupont et al., 1989; John and Revel, 1991; Rahman and Evans, 1991; Foote et al., 1998). The function of GJs is perturbed by the disruption of these motifs. The secondary and tertiary structures arising from disulphide bonding are functionally important (Dahl et al., 1992).

1.13.2 GJ Intercellular Channels

GJs are membrane-bound protein channels comprising of tightly packed, clustered molecules that link the cytoplasm of two cells and facilitate bidirectional transmission of entities like metabolites, ions, electrical currents, peptides (up to 1 kDa) and second messengers (Goodenough et al., 1996; Neijssen et al., 2005). Transmission of a wide variety of entities through these structures is crucial for phenomena like cell death, inflammation and development (Montecino-Rodriguez et al., 2000; Matsue et al., 2006).

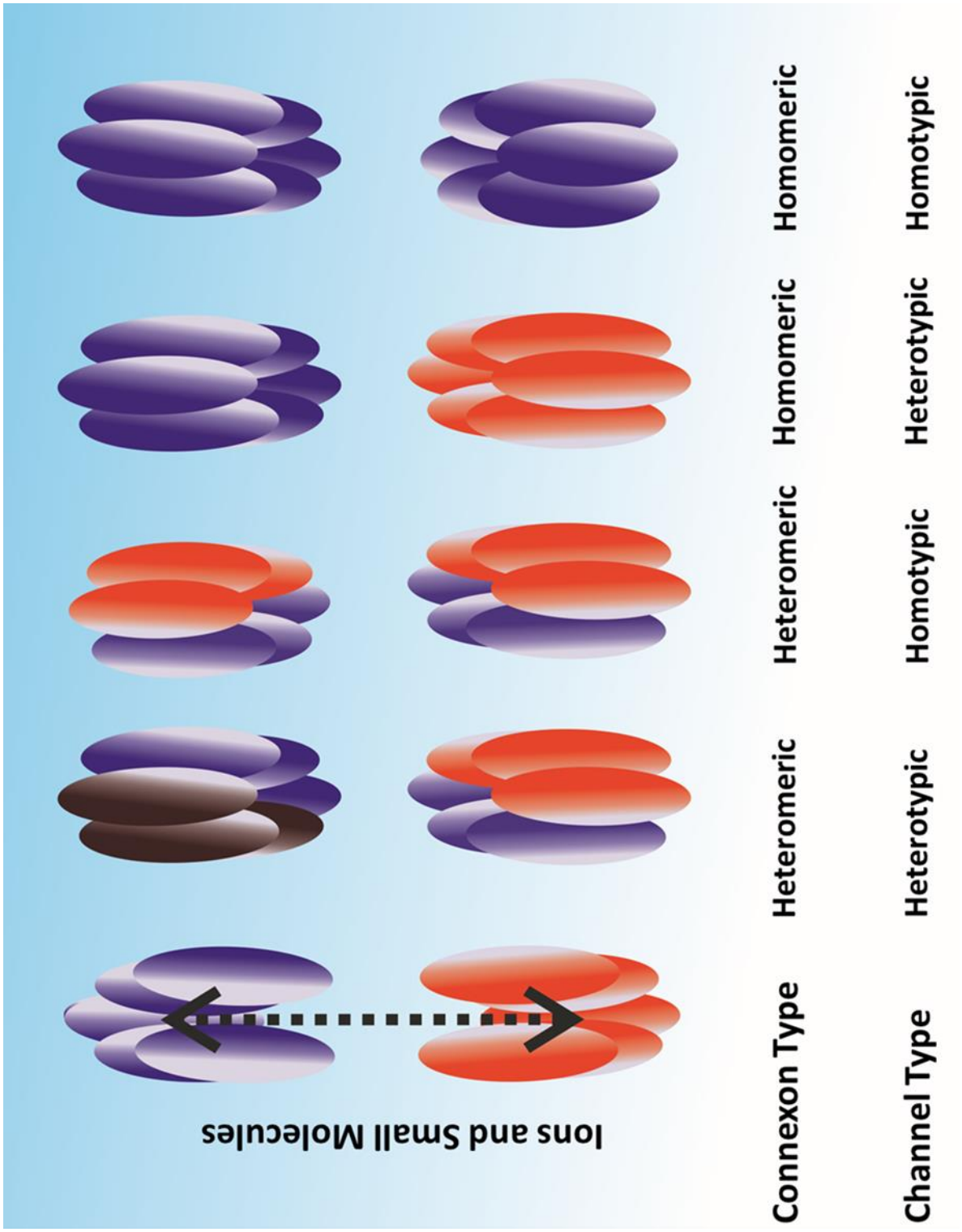
1.13.2.1 Structure of GJ Channels

GJs are composed of building blocks known as hemichannels Figure 1.7. Each hemichannel contains six Cx subunits organised to form a central pore; the hexameric transmembrane structure is known as a connexon. Connexons can dock with the connexons of nearby cells or may function as hemichannels. Hemichannels were previously proposed to be formed only temporarily during GJ formation (Unger et al., 1999; Hemler, 2005; Saez and Leybaert, 2014), but may also work as free channels.

Connexons can be heteromeric or homomeric. Homomeric connexons comprise six identical Cxs, whereas heteromeric connexons contain multiple Cx isoforms (He et al., 1999).

Figure 1.7: Gap junctions develop between opposing membranes of adjacent cells.

Connexons on all cell surfaces dock with those on a nearby cell to create conductive channels. A gap junction plaque is formed by a large grouping of these channels. Every connexon comprises six connexin protein subunits, which are positioned at right angles to the cell membrane to form a central pore. The pore functions as a conduit for ions and molecules with a low molecular mass (up to 1 kDa). Homomeric connexons comprise only one kind of connexin subunit, whereas heteromeric connexons have a mixture of different kinds of connexins. Gap junction channels may comprise homotypic channels or heterotypic channels; adapted from (Bloomfield and Volgyi, 2009).


Ions and Small Molecules
Connexon Type
Heteromeric
Heteromeric
Homomeric
Channel Type
Heterotypic
Homotypic
Heterotypic
Homotypic

1.13.3 Assembly of GJ Intercellular Channels

During the GJ intercellular channel formation stage, the Cx genes are transcribed and translated. Then, six Cx monomers are non-covalently oligomerised into connexons and transferred to the cell membrane. Finally, they dock with adjacent connexons in the membrane and aggregate to form GJ plaques. GJ channels are removed by internalisation as whole plaques or parts of a GJ. Connexons and their constituent Cx proteins are then degraded (Figure 1.8) (Evans et al., 2006; Koval, 2006; Laird, 2006).

1.13.3.1 Half-life of Plasma Membrane GJ Plaques

In pulse–chase analyses, labelled Cxs in tissue-cultured organs and cells have a half-life of only 1.5–3.5 hours (Traub et al., 1989; Darrow et al., 1995; Beardslee et al., 1998; Musil et al., 2000; Thomas et al., 2005). Cell membrane proteins usually have a turnover rate >24 hours, whereas nuclear and cytosolic proteins involved in signal transduction are degraded rapidly. A short turnover rate is of crucial importance for a regulatory factor. Since GJ organisation seems to be a coordinated self-assembly phenomenon, slower degradation of Cx would result in increased formation of GJs and increases intercellular exchange. Reducing the turnover rate of Cxs quickly leads to organisation of cell surface Cx43 into functional, persistent GJs (Musil et al., 2000; VanSlyke and Musil, 2002; Laird, 2006).

1.13.3.2 Biosynthesis and Assembly of Cxs into GJ Intercellular Channels

Once synthesised, Cxs are sent to the endoplasmic reticulum where they exist as four-transmembrane domain proteins (Falk et al., 1994; Zhang et al., 1996; Falk and Gilula, 1998; Ahmad et al., 1999). Although the exact site of oligomerisation is still unclear, it has been reported to occur in endoplasmic reticulum–Golgi apparatus intermediates. It has also been proposed to occur in the trans-Golgi network, from which the connexons

are transported to the cell membrane (Zhang et al., 1996; Ahmad and Evans, 2002). Vanslyke et al. (2009) found that the levels of expression of Cx43 and Cx32 affect connexon organisation during transportation to the trans-Golgi network. This finding implies that the folding, oligomerisation and transport of connexons takes place gradually.

The usual secretory mechanism transports connexons to the cell surface. Connexons are sent from the endoplasmic reticulum to the cis-medial and trans-Golgi cisternae. Then, they enter the trans-Golgi network prior to transfer to the cell membrane (Musil and Goodenough, 1993; Laird et al., 1995; Koval et al., 1997; VanSlyke and Musil, 2000; Lauf et al., 2002; Thomas et al., 2003).

In tissue culture, fluorescent Cxs utilise several transport intermediates of various shapes and sizes before reaching the cell surface (Jordan et al., 1999; Lauf et al., 2002; Thomas et al., 2005). Microtubules may participate in the transportation of Cxs by increasing the efficiency of the transport process, as exposure to nocodazole, which causes microtubular disruption, perturbs the localisation of Cxs (George et al., 1999; Martin et al., 2001; Johnson et al., 2002; Lauf et al., 2002; Thomas et al., 2005). However, brefeldin A, which disrupts the Golgi apparatus, does not alter Cx transportation, indicating that the process can use an alternative mechanisms that does not require the Golgi network (George et al., 1998; George et al., 1999; Martin et al., 2001). After reaching the cell membrane, Cxs undergo lateral diffusion and dock with adjacent connexons, thereby forming GJs. This regulated process involves N- and E-cadherin (Musil et al., 1990; Jongen et al., 1991; Meyer et al., 1992; Wei et al., 2005). Fluorescent recovery after photobleaching and pulse–chase experiments indicated that

newly synthesised GJ channels reside at the edge of GJ plaques and that older channels accumulate in the centres of the plaques (Gaietta et al., 2002; Lauf et al., 2002).

Under normal physiological conditions, hemichannels or undocked connexons in the cell membrane remain closed (Saez et al., 2005; Evans et al., 2006). They open in conditions with low extracellular Mg^{2+} and Ca^{2+} concentrations (Valiunas and Weingart, 2000; Valiunas et al., 2002). The phosphorylation status of connexons and the surrounding physiological conditions determine if hemichannels dock or remain independent (Saez et al., 2005).

1.13.3.3 GJ Degradation

The turnover of GJs requires cooperation between cells. Annular junctions are formed to internalise intact GJ plaques into the cytosol of one of the junction-forming cells (Jordan et al., 2001). The factors that determine which cell internalises the vesicle remain to be determined.

Electron microscopic imaging revealed that the old channels in the centre of a plaque are internalised. This finding implies that large parts of membrane can be endocytosed. Moreover, connexons can be degraded without the destruction of the whole GJ (Gaietta et al., 2002; Falk et al., 2009). The number of GJs can be controlled by endocytosis by either internalisation of whole GJ plaques or the internalisation and degradation of small vesicles from the centre of the GJ, which renews the channels in a plaque (Falk et al., 2009).

Cxs are degraded by either lysosomes or ubiquitin-regulated proteasomes. Electron microscopy studies utilising lysosomal enzyme inhibitors, like ammonium chloride and leupeptin, revealed that Cx-enriched portions of membrane fuse with lysosomes after

internalisation (Qin et al., 2003; Berthoud et al., 2004; Leithe and Rivedal, 2004a). Similarly, studies with specific inhibitors of proteasomal degradation revealed that Cx43 serves as a ubiquitin substrate (Laing and Beyer, 1995; Musil et al., 2000; Leithe and Rivedal, 2004b). Some researchers propose that lysosomes are responsible for the degradation of Cxs from the cell membrane and that proteasomes are responsible for the degradation that occurs in the endoplasmic reticulum as a quality control mechanism for protein folding and arrangement (Laird, 2006; Salameh, 2006).

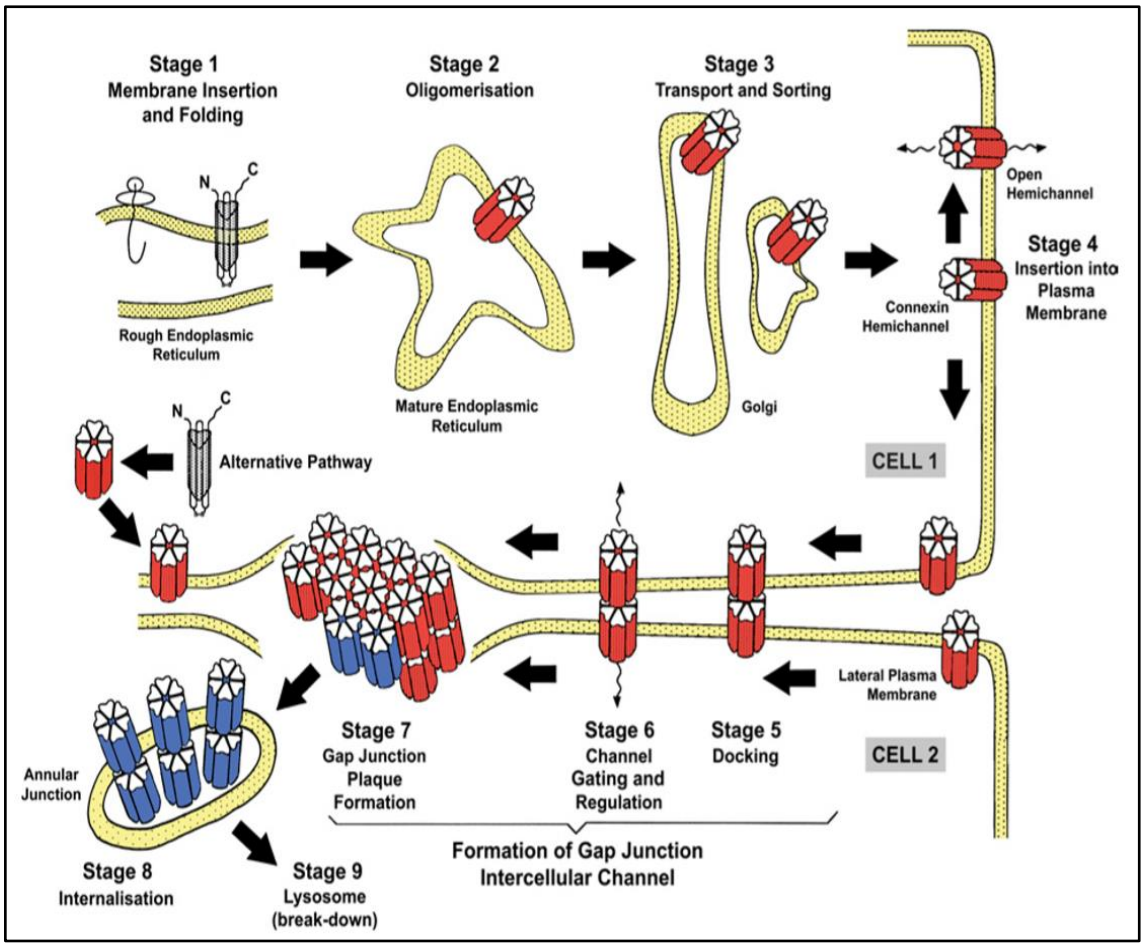


Figure 1.8: Formation and dissociation of gap junctions, and the generation and transport of connexin hemichannels.

Gap junctions are formed and regulated via multiple stages. (1) Connexin hemichannels (red/new hemichannel) are formed in the initial stages of the secretory pathway. (2 and 3) Connexin hemichannels are translocated through the secretory pathway, involving areas of the Golgi apparatus and the endoplasmic reticulum. (4) A poorly studied insertion mechanism that involves connexin 26 and potentially other connexins has been reported. It does not include direct involvement of the Golgi apparatus. Connexin hemichannels are inserted into the plasma membrane. (5) The inserted connexin hemichannels dock with their binding partners. (6) The connexin binding partners on the attached cell open the channels. (7) The processes of channel-gating and regulation may occur while gap junction channels aggregate into large plaques. (8) The gap junctions are internalized (blue) into one of the attached cells and (9) Lysosomal hydrolysis degrades the gap junctions (Evans et al., 2006). C, carboxyl-terminus; N, amino-terminus

1.13.4 GJ Modulators

GJ intercellular communication (GJIC) occurs in all multicellular living systems. Few reagents are known to block the process. GJ channels are protected from the extracellular space and are, thus, not subject to direct modulation.

1.13.4.1 Chemical Inhibitors

Chemicals capable of blocking communication through GJs are called GJ blockers. Researchers have performed *in vitro* studies with these blockers to explore the function of electrical synapses. Communication through GJs can be assessed as the transfer of dye between cells and the electrical coupling of nearby cells (Guan et al., 1997; Cruikshank et al., 2004). There is limited information on the influence of these blockers on the physiology of the brain and the behaviour of living systems. However, chemicals that have been established as good GJ blockers have been used to treat disorders like cardiac arrhythmia, ulcer and malaria (Wenckebach, 1923; Pinder et al., 1976; Achan et al., 2011; Noubiap, 2014). Unfortunately, these chemicals have well-established side effects in humans and many studies have reported the toxicity of these chemicals in experimental animal models (Manjarrez-Marmolejo and Franco-Perez, 2016).

1.13.4.2 Glycyrrhetinic acid

Glycyrrhetinic acid (GA) is a strong, non-selective blocker of hemichannels (IC_{50} for Cx46 and Cx50 are 250 μM and 100 μM, respectively) (Eskandari et al., 2002) and GJ channels (IC_{50} of 18α-GA and 18β-GA are 1.5 μM and 2 μM, respectively) (Davidson et al., 1986; Davidson and Baumgarten, 1988). GA also inhibits 11β-hydroxysteroid dehydrogenase (IC_{50} = 300 nM) (Su et al., 2007). Like carbenoxolone, 18β-GA inhibits voltage-sensitive calcium currents (Matchkov et al., 2004). Prolonged exposure to mild concentrations and short exposure to high concentrations of GA cause water and

sodium retention, elevated potassium excretion, increases in body weight, suppression of the renin–angiotensin–aldosterone system, alkalosis, muscle paralysis and hypertension (Omar et al., 2012).

GA is a component of liquorice which is employed as a flavouring compound in drugs, foods and beverages (Asl and Hosseinzadeh, 2008). Heavy intake of liquorice may result in headache and hyper-mineralcorticoidism, leading to oedema, loss of potassium, sodium retention, paralysis, hypokalaemia and renin–angiotensin–aldosterone (Dobbins and Saul, 2000; van den Bosch et al., 2005; Asl and Hosseinzadeh, 2008). Researchers have used GA in vitro to explore the roles of GJs in neuronal oscillations, neurotoxicity induced by glutamate and epileptic discharges (de Curtis et al., 1998; Hughes et al., 2004; Leznik and Llinas, 2005; Proulx et al., 2006).

1.13.4.3 Carbenoxolone

The glycyrrhetinic acid derivative carbenoxolone is a GJ blocker. It is structurally similar to steroids and molecules in the root of the liquorice plant. It is used to treat inflammation and oral, oesophageal and peptic ulceration. Upon systematic administration, it may cause electrolyte imbalance (Spray et al., 2002).

Although it does not demonstrate specificity for a specific Cx, carbenoxolone blocks GJs in mutant human fibroblast cell lines (half maximal inhibitory concentration [IC_{50}] = 3 μM) (Davidson et al., 1986; Davidson and Baumgarten, 1988). It also blocks voltage-gated Ca^{2+} channels (IC_{50} = 48 μM) (Vessey et al., 2004). In addition, it inhibits 11β-hydroxysteroid dehydrogenase, thereby improving the efficiency of endogenous glucocorticoid hormones (Bujalska et al., 1997).

1.13.4.4 Cx Mimetic Peptides

Researchers have developed short synthetic peptides, called Cx mimetic peptides, which are comparable to selected sequences in the extracellular loops of Cxs. To develop these peptides, researchers screened different peptides from external loop 1 and 2 of Cx32 for their efficiencies in slowing the synchronised contraction caused by the clustering of myocytes isolated from the hearts of chicks (Warner et al., 1995). These myocytes express an unknown Cx with sequence similarity to the well-studied mammalian Cx32 (Barker and Gourdie, 2002). Likewise, Bao et al. (2004) used short synthetic Cx peptides to analyse the docking of connexons on paired *Xenopus* embryos. Inhibitor peptides synthesised from the extracellular sequences adjacent to the 2nd and 4th transmembrane domains of Cx43, called GAP26 (VCYDKSFPISHVR) and GAP27 (SRPTEKTIFII and SRPTEKNVFIV), respectively, have proven to be experimentally useful (Dahl et al., 1994; Chaytor et al., 1997; Oviedo-Orta et al., 2000; Oviedo-Orta et al., 2001). The SRPTEK sequence, which is present in the majority of Cxs, is mimicked by GAP27. Moreover, the efficiency of this GJ-inhibitory peptide is considerably improved by adding the amino acids found in the 4th transmembrane domain (Chaytor et al., 1999).

Researchers have reported the usefulness of these reversible GJIC inhibitors for experimentally manipulating GJs. The inhibitors can block electrical communication and the transmission of calcium waves across sets of confluent cells (Boitano and Evans, 2000; Isakson et al., 2001). They also block the transfer of fluorescent dyes in different cells culture (Oviedo-Orta et al., 2000; Oviedo-Orta et al., 2001). Owing to technical issues, it is difficult to demonstrate the influence of mimetic peptides on GJIC. However, the specificities of these peptides for GJs make them suitable for exploring the functions of organs and tissues.

1.13.5 Permeability of GJ Channels

Many factors influence GJ-mediated intercellular coupling, including the probability of a single channel being open, the permeability or conductance of a single channel and the number of channels available for conductance (van Veen et al., 2001). Up to 1 kDa molecules can pass through GJs (Harris, 2001). The pore diameter, usually in the range of 6.5 to 15 Å, is determined by the type of connexon (Veenstra et al., 1994; Beblo and Veenstra, 1997; Harris, 2001). This pore size is sufficient to permit the transport of water, anions and cations (e.g. Ca^{2+}, K^{+}, Na^{+} and Cl^{-}) and the majority of second messengers (e.g. IP_3, ATP, adenosine, ADP, cGMP, cAMP, glutamate, small interfering RNAs and polypeptides) (Bevans et al., 1998; Harris, 2001). Junctional channels are non-selectively permeable to smaller molecules. However, the selectivity of the channel becomes evident for larger molecules (Harris, 2001).

The permeability of a GJ is not solely determined by molecular size; the net charge of the permeant is also influential. Fluorescent probes of the same size but with opposite charges have varying permeabilities through GJs comprising different connexon isoforms (Kanaporis et al., 2011). GJs comprising Cx43 or Cx32 demonstrate variations in selectivity. The former demonstrates greater cation selectivity than the latter. However, GJ channels comprising Cx43 exhibit a maximal unitary conductance approximately twice that of GJs comprising Cx32 (Harris, 2002). In addition, the permeabilities of those GJs to AMP, ADP, ATP and adenosine are different (Goldberg et al., 2002).

There are at least two distinct mechanisms of GJ gating (Bukauskas and Peracchia, 1997; Bukauskas and Peracchia, 2000; Skerrett et al., 2000; Harris, 2001; Moreno et al., 2002). These mechanisms are voltage gating and chemical or loop gating. The voltage

gating pathway is faster; it is voltage-driven and can change the conformation of the channel from completely open to almost closed within milliseconds (ms). Chemical gating is a slower mechanism (up to 30 ms) that completely closes a channel in response to chemical changes (chemical gating) or voltage alterations (loop gating). (Bukauskas and Peracchia, 1997; Harris, 2001; Bukauskas et al., 2002).

The number of open channels (often termed the 'open probability') and the unitary conductivity of open junctional channels influence the macroscopic GJ conductivity. Regulatory pathways can have contrasting consequences for the probability of the open state and the average conductivity of open channels or the size of the channel opening. This implies that a single stimulus can decrease the permeability of the channel for large-sized molecules while enhancing the passage of small ions and improving electrical coupling (Kwak et al., 1995; Dhein, 1998). This phenomenon has been demonstrated for Cx43 in numerous cell lines. For Cx43, the residual state demonstrates greater anion selectivity and permits the maintenance of cell-to-cell communication while discouraging metabolic coupling (Bukauskas et al., 2002). Thus, it is difficult to define the effects of various conditions on GJs as 'increases' or 'decreases' in GJ-mediated intercellular coupling. It is more accurate to state how the permeability, selectivity and electrical coupling are affected.

1.13.6 Cx Regulation

Different factors are involved in the strict regulation of the synthesis of Cxs, the assembly and breakdown of GJs and the conductance and permeability of junctional channels. These factors include the transmembrane potential (V_m), transjunctional voltage (V_j), phosphorylation status, CaM binding, intracellular Ca^{2+} concentration and pH (Figure 1.9) (Rabionet et al., 2002).

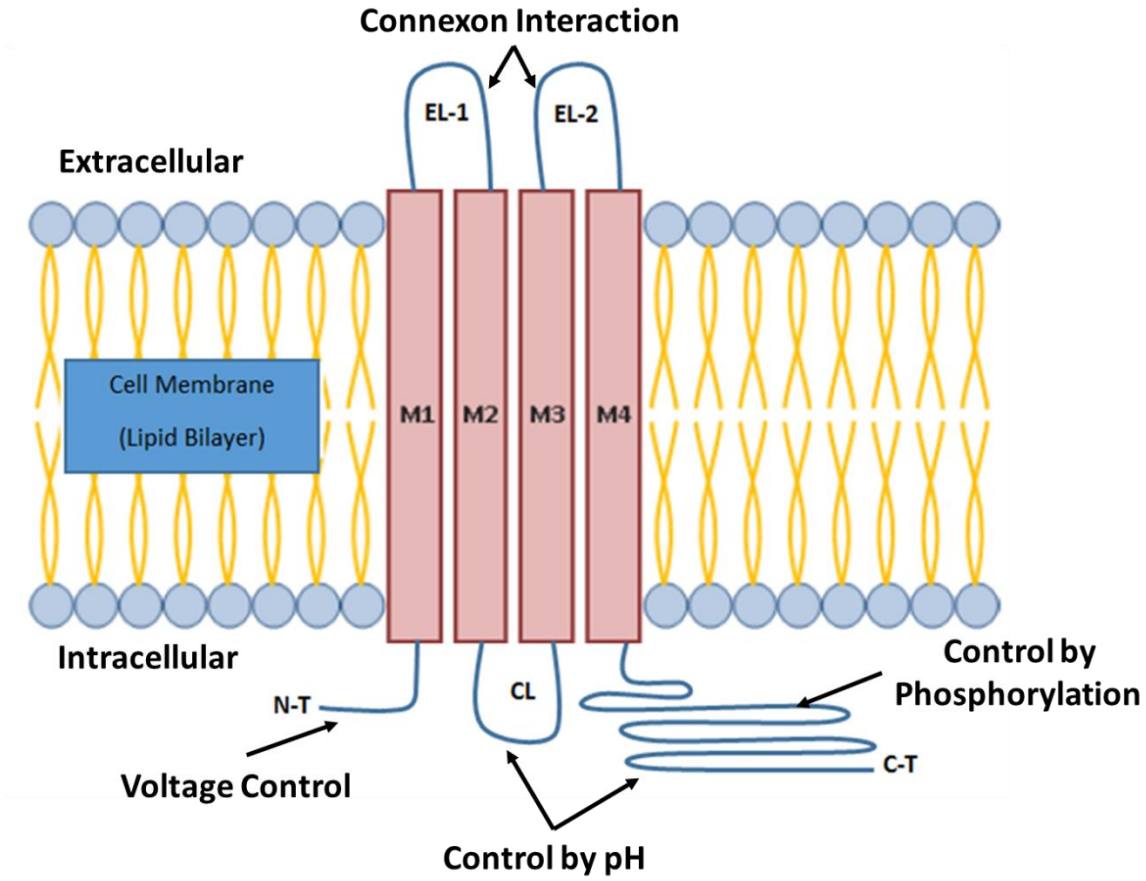


Figure 1.9: Connexin regulation by environmental and intracellular factors.

Different factors regulate connexins, such as voltage, phosphorylation status and pH adapted from (Rabionet et al., 2002). EL-1, extracellular domain 1; EL-2, extracellular domain 2; M1, transmembrane domain 1; M2, transmembrane domain 2; M3, transmembrane domain 3; M4, transmembrane domain 4; N-T, NH_2 terminus; C-T, COOH terminus

1.13.6.1 Transmembrane Potential (V_m) and Transjunctional Voltage (V_j)

Cx channels in the membrane are subjected two kinds of voltage: V_j, the voltage between the cytoplasm of two connected cells, and V_m, the voltage between the cytoplasm and the extracellular space (Segal, 2000; Bargiello and Brink, 2009; Bavamian et al., 2009).

Voltage changes have different effects on the GJ channels, depending on their Cx isoforms. Some channels are affected only by V_j, whereas others are affected by V_j and V_m. Our understanding of the mechanisms underlying voltage gating is still rudimentary. However, researchers have proposed that each connexon is equipped with two different gates, a fast gate regulated by V_j and a slow gate regulated by V_j plus V_m, at different structural locations in the Cxs, based on the observation that mutations in different parts of the proteins cause changes in these gates (Gonzalez et al., 2007).

At either of the two voltage polarities, the fast V_j-gating transforms the channel from its completely open state to a residual state. Based on the isoform of the Cx, hemichannels close under positive or negative potentials at the cytoplasmic surface. Cxs 31, 32, 37, 40, 43, 45 and 57 close in response to negative potential and Cxs 26, 30 and 50 close in response to positive potential. Intriguingly, Cx46 closes in response to both via different gating mechanisms (Rackauskas et al., 2010). Earlier research studies indicated that charged residues behave as voltage sensors. They are present at the N-terminal domain and regulate the voltage polarity of the fast V_j-gating mechanism (Bukauskas et al., 2002). Thus, although the N-terminal domain contributes to gating, the most acknowledged mechanism for fast V_j-gating is the chain-and-ball model, which involves binding of the flexible C-terminal domain with the cytoplasmic loop, leading to partial closure of the pore (Gonzalez et al., 2007).

Negative cell potentials always result in the closure of the slow V_j gate, which mediates changes between the residual and the completely closed state. Chemical gating is also linked with this mechanism because reductions in pH and exposure to lipophiles and Ca^{2+} completely close the residual-state, slow, V_j-sensitive gates (Bukauskas and Peracchia, 1997; Peracchia, 2004). Conversely, a completely closed channel results from V_m depolarisation, which increases the rate of the slow transitions (Bukauskas and Weingart, 1994). Revilla et al. (2000) investigated mutations in Cx43 and found that its V_m sensor is in a distinct area of the C-terminal domain rather than in the area that participates in fast V_j-gating. Nevertheless, interactions between the two gating sensors remain worthy of consideration.

1.13.6.2 Intracellular pH

As described by Stergiopoulos et al. (1999), increased acidification of the cytoplasm adversely affects GJ-mediated intercellular communication. However, different channels demonstrate varying sensitivities to pH based on their Cx isoforms. Cx43 channels partially close in response to minor decreases in intracellular pH. On the other hand, a similar degree of closure of Cx32 GJ channels requires a greater reduction in pH (Morley et al., 1996). Researchers have studied the pH sensitivities of different Cx isoforms in oocyte pairs and found that the relative pH sensitivities of Cxs are 50 > 46 > 45 > 26 > 37 > 43 > 40 > 32 (Stergiopoulos et al., 1999). According to Liu et al. (1993), channel pH sensitivity may depend on the C-terminal portion of the protein, since removal of that portion abrogates the pH regulation of Cx43. Similarly, Stergiopoulos et al. (1999) reported similar findings for Cxs 37, 40 and 50, but not 45. Intriguingly, these investigations also demonstrated that channels comprising truncated Cx43 or Cx40 can be regulated by the C-terminal domain of the other molecule.

The residual state of GJ channels can be changed by removing the C-terminal domain; this alteration can be reversed through the co-expression of the carboxyl group as a distinct protein (Anumonwo et al., 2001; Moreno et al., 2002). Based on these observations, some researchers proposed that a ball-and-chain mechanism may regulate pH and chemical gating, as for fast V_j-gating (Delmar et al., 2004). Nevertheless, doubts remain that pH gating can be engaged through direct protonation of certain histidine residues in the carboxyl domain (Trexler et al., 1999) or through indirect protonation of intracellular amino sulfonates, such as taurine (Harris, 2001).

1.13.6.3 Intracellular Ca^{2+} Concentrations and CaM

Numerous researchers have studied the role of Ca^{2+} in the regulation of GJ channels (Peracchia, 2004; Lurtz and Louis, 2007). They initially found that electrical uncoupling is triggered by intracellular administration of Ca^{2+} (De Mello, 1975; Rose and Loewenstein, 1975). The pathways that induce uncoupling in conditions such as ischaemia are associated with several pathophysiological phenomena, such as the accumulation of amphipathic lipid metabolites and gradual and persistent increases in the intracellular levels of H^+ and Ca^{2+} (Wu et al., 1993; Dekker et al., 1996). Uncoupling also plays roles in arrhythmias and conduction abnormalities downstream of other inducers (Beardslee et al., 2000).

The cell type also determines the sensitivity of GJ channels to intracellular levels of Ca^{2+}. However, it remains to be determined if Ca^{2+} influences gating directly or through intracellular messengers. Several studies have reported that Ca^{2+} may cause the closure of GJs by activating CaM, which can directly act as a gating regulator (Peracchia et al., 2000; Peracchia, 2004). According to Sotkis et al. (2001), the acid-soluble protein CaM interacts with Ca^{2+} and the resulting complex binds basic amphiphilic α-helix domains.

CaM has been found to colocalise with Cxs and interacts with Cx isoforms 38, 32, 37, 43, 44 and 50. Rackauskas et al. (2010) have reported that GJ closure by intracellular Ca^{2+} during ischaemia may contribute to protecting healthy cells from membrane depolarisation and the release of metabolites through the GJ by isolating these cells from damaged cells in a process called 'healing over'.

1.13.6.4 Phosphorylation

Phosphorylation regulates Cxs by altering the molecular structures of their channels, their mean open time, open probability, intracellular trafficking and their assembly into GJ plaques. However, this mechanism must be more extensively characterised. The impact of phosphorylation may vary based on the biochemical environment, the site of phosphorylation and the Cx isoform (van Veen et al., 2001). Numerous protein kinases may be able to phosphorylate tyrosine, threonine and serine residues that are abundantly present in the C-terminal domain. The cytoplasmic loops of Cx36 and Cx56 may undergo phosphorylation (Rackauskas et al., 2010).

Some of the Cx43 serine residues that undergo phosphorylation include 255, 279, 282, 364 and 368. Mitogen-activated protein kinases (MAPKs) can phosphorylate serines 255, 279 and 282, PKC can phosphorylate 368, and PKA phosphorylates 364. The net charge of the protein can be affected by phosphorylation, which may cause changes in pH or voltage sensitivity. Extensive study of the regulation of Cx43 through phosphorylation has indicated that PKA phosphorylates Cx43, resulting in elevated channel permeability and cell-to-cell communication (Lampe and Lau, 2000). Conversely, Cx43 phosphorylation by MAPK and PKG decreases GJ intercellular communication in cardiomyocytes. The impact of Cx43 phosphorylation by the α and ε isoforms of PKC is even more complicated. It increases electrical conductance between

paired cardiomyocytes, but also reduces cell-to-cell permeability and channel conductance (Kwak and Jongsma, 1996; Schulz and Heusch, 2004).

1.14 GJs in Health and Disease

Hemichannels and GJIC are involved in the regulation of the signalling and physiology of various organs and organ systems, including cardiac myocytes, arterial ECs, bone marrow stromal cells and oocytes, and the cells of the skin, bone, breast, ovary, testis, kidney, ear, retina, lung, liver, lens, heart and central nervous system as summarised in Table 1.1. Connexons also act as channels for molecular exchange between the extracellular space and the cytoplasm (Dorshkind et al., 1993; Kidder and Mhawi, 2002; Goodenough and Paul, 2003; John et al., 2003; Rohr, 2004; Pointis and Segretain, 2005; Bruzzone and Dermietzel, 2006; McLachlan et al., 2007; Gershon et al., 2008; Figueroa and Duling, 2009; Goodenough and Paul, 2009; Hanner et al., 2010; Batra et al., 2012).

A wide range of human disorders involve mutations in the Cx genes. A mutation in Cx26 accounts for over 50% of hereditary skin problems and deafness (Denoyelle et al., 1997; Kelsell et al., 1997). Cx32 mutations result in X-linked Charcot–Marie–Tooth syndrome, a widespread peripheral demyelinating condition (Bergoffen et al., 1993). Similarly, Paznekas et al. (2003) reported that a mutation in Cx43 causes oculodentodigital dysplasia (ODDD). A mutation in Cx47 causes Pelizaeus–Merzbacher-like disease, a central demyelinating condition (Uhlenberg et al., 2004). Mutations in Cx46 or Cx50 are seen in cataract patients (Gong et al., 2007; Jiang, 2010). As shown in Table 1.1, some mutations cause total channel function loss and other mutations cause changes in the properties of the channels compared to their wild type counterparts (Schalper et al., 2009).

Table 1.1: Connexin (Cx) proteins, genes, and their sites of expression and related disorders

(Oyamada et al., 2005; Pfenniger et al., 2011; Morel, 2014)

Cx (Human)	Cx (Mouse)	EXPRESSION	FUNCTION	ASSOCIATED DISEASES
Cx26 (GJB2)	**Cx26 (GJB2)**	Cochlea, skin, liver, placenta, breast, lung, and brain	Involved in hearing. Growth, maturation & stability of the epidermis.	Bart-Pumphrey syndrome Vohwinkel syndrome
Cx30 (GJB6)	**Cx30 (GJB6)**	Brain, inner ear, hair follicles and skin	Transport of ions and nutrients between adjacent cells.	Clouston syndrome Nonsyndromic deafness
Cx31 (GJB3)	**Cx31 (GJB3)**	Skin, cochlea, placenta, kidney, testes, eye, and PNS	Auditory functions	Erythrokeratodermia variabilis
Cx32 (GJB1)	**Cx32 (GJB1)**	Liver, pancreas, kidney, nervous system (Schwann cells) and Platelets	Communication across the outer myelin layers and the interior of the Schwann cell.	Charcot Marie Tooth syndrome, Demyelination neuropathy
Cx37 (GJA4)	**Cx37 (GJA4)**	Brain, heart, ovary uterus and endothelial cells of blood vessels	Intercellular communication between platelet	Erythrokeratodermia variabilis
Cx40 (GJA5)	**Cx40 (GJA5)**	Vascular cells (Blood and lymphatic vessels) and Platelets	Regulation of vasomotor tone (nervous stimulation inside the muscles of blood vessels that maintain contraction)	Cardiac arrhythmia Hypertension
Cx 43 (GJA1)	**Cx 43 (GJA1)**	Ubiquitously present including skin, heart, eye, and brain	Development of cardiac muscles and ensures synchronous contraction of muscles	Heart abnormalities Oculodentodigital dysplasia
Cx 45 (GJC1)	**Cx 45 (GJC1)**	Heart	Development and function of the embryonic heart along with maintenance of cardiac rhythmicity.	Cardiac arrhythmia
Cx 47 (GJC2)	**Cx 47 (GJC2)**	Oligodendrocytes	Synthesis of multi-lamellar myelin membranes surrounding axons.	Hypomyelinating leukodystrophy Lymphedema
Cx50 (GJA8)	**Cx50 (GJA8)**	Lens	Development and maturation of lens	Cataract
Cx46 (GJA3)	**Cx46 (GJA3)**	Lens	Lens hemostasis	Cataract Hypoxia
Cx62 (GJA10)	**Cx57 (GJA10)**	Cx57 Retinal cell mouse. **Cx62 unknown**	**Unknown**	**Unknown**

1.15 GJs in Circulating Cells

1.15.1 Lymphocyte Gap Junctional Communication and Antigen-mediated Responses

Specialised mechanisms enable the immune system to recognise, analyse and respond to foreign and self-antigens. These mechanisms depend on molecular structures in immune cells, which include antigen-presenting cells (APCs), B cells and T cells. Cx43 is the chief GJ protein in the immune system. The immunological synapse also uses Cx43 (Mendoza-Naranjo et al., 2011). A growing number of reports indicates a role for Cx43 in the intercellular and intracellular signalling that activates lymphocytes (Oviedo-Orta et al., 2010; Machtaler et al., 2011). Several studies have demonstrated the roles of Cx43 in antigen-specific effector T cell responses, such as the destruction of tumour cells by cytotoxic T lymphocytes (CTLs) (Benlalam et al., 2009), antigen presentation (Neijssen et al., 2005) and phagocytosis (Anand et al., 2008). Although the function of GJ channels in the development of lymphocytes has been studied by numerous researchers, the investigations are hindered by the short lifespans of primary cells and the inability of the lymphocytes to adhere with the culture supports. However, research on the involvement of Cx43 in the peripheral and central immune responses in inflammatory and normal conditions has benefited from the utilisation of animal models, especially tissue-targeted knockout mice (Nguyen and Taffet, 2009).

It has been recently demonstrated that GJs composed of Cx43 might contribute to antitumor responses in individuals infected with bacteria, as the GJs aid in the transfer of antigenic peptides to dendritic cells, where they are processed and presented to CTLs to promote the destruction of cancer cells (Benlalam et al., 2009). This research strengthens the hypothesis that GJs promote antigen-specific antitumor killing using

bacteria or components of bacterial cells (Terman et al., 2006; Fuchs and Bachran, 2009).

1.15.2 Communication of Platelets via GJs

Platelets express a broad range of signalling molecules and receptors that are responsible for regulating the development of firm contacts between platelets and may stimulate adherence-dependent signalling through homotypic interactions (Brass et al., 2004). GJ proteins, particularly Cx37 and Cx40, have recently been detected in platelets, where they are proposed to have essential functions (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

Our research group have demonstrated that platelets strongly express two members of the Cx family, Cx37 and Cx40, which are involved in hemichannel formation. Inhibitor (human) and knockout (mice) experiments have provided evidence that these two Cxs play roles in clot retraction, a coordinated phenomenon that is dependent on platelets (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Researchers have demonstrated that the inhibition of Cx37 and Cx40 with the Cx-mimetic peptides ^{43,37}Gap27 and ^{40}Gap27, respectively, decreases outside-in signalling via integrin $\alpha_{IIb}\beta_3$, which is responsible for clot retraction. The same two Cxs function in other activation stages, such as platelet aggregation, integrin activation, α-granule secretion and Ca^{2+} signalling (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

Subsequent studies with Cx37 inhibitors in Cx40-deficient platelets and Cx40 inhibitors in Cx37-deficient platelets showed that these Cxs perform their roles independently of each other. These findings are consistent with the observation that these Cxs were unable to exhibit heterotypic interactions during co-immunoprecipitation experiments. The physiological significance of Cx40 for sustained haemostasis is highlighted by the

inhibitory effect of ^{40}Gap27 on haemostasis, studied in the phenotype of Cx40$^{-/-}$ mice (Vaiyapuri et al., 2013). Taken together, the available studies indicate that GJ blockers inhibit thrombosis and thrombus formation in vitro in human blood under arterial flow conditions. However, additional studies are required to investigate the exact mechanisms by which platelet Cxs regulate these processes.

In contrast with our group findings, the deletion of Cx37 (Cx37$^{-/-}$) results in enhanced platelet aggregation in response to collagen, thrombin or ADP stimulation in comparison to wild type mice (Cx37$^{+/+}$). Furthermore, treatment with ^{37,43}Gap27 enhances platelet aggregation above the level observed in scrambled control treated samples (Angelillo-Scherrer et al., 2011). It was also observed that Cx37$^{-/-}$ knockout mice show increased thrombus propensity compared to wild type mice (Cx37$^{+/+}$) which indicated that thrombus propensity is limited by communication via Cx37 GJ channels (Angelillo-Scherrer et al., 2011). Angelillo-Scherrer et al. (2011) concluded that earlier studies provided evidence that GJ intercellular communication between Cx37-expressing platelets can control thrombus propensity.

1.16 Expression of Cxs during Atherosclerosis

The vasculature normally expresses four Cxs. Cx37 and Cx40 are chiefly found on ECs in healthy arteries. They form complexes with eNOS, which implies that they may be involved in NO-mediated, endothelium-dependent vasodilation (Kwak et al., 2002; Alonso et al., 2010; Pfenniger et al., 2010). Moreover, Wolfle et al. (2007) demonstrated that endothelium-dependent vasodilation of small arteries, such as arterioles, in mice requires Cx40. Gabriels and Paul (1998) reported higher expression of Cx43 by some ECs at the branching points of arteries. These areas are subjected to increased turbulent flow. Similarly, Kwak et al. (2002) reported the expression of Cx43

and Cx45 on SMCs in the arterial media. Intriguingly, research in an atherosclerosis model in LDL-receptor-deficient mice revealed alterations in the expression of Cxs in large arteries, such as the aorta, during atherosclerosis (Figure 1.10) (Meens et al., 2012).

Unique Cx expression is the distinguishing feature of plaques at different stages, such as advanced atheromas or initial fatty streaks (Kwak et al., 2002). Cx37 and Cx40 are expressed by ECs in early atheroma development. Cx37 is also expressed by macrophage-derived foam cells. Similarly, intimal SMCs upregulate Cx43 compared to SMCs that are not associated with plaques. Further alterations in Cx expression are seen in advanced atheromas (Burnier et al., 2009; Meens et al., 2012). No Cxs are expressed on the ECs coating an advanced atheroma, except for Cx43-expressing ECs found in the shoulder region. Cx37 and Cx43 are expressed by foam cells near the necrotic centre, and intimal SMCs downregulate Cx43 expression compared to that in non-plaque-associated SMCs. Within the medial layer of the artery in an advanced atheroma, SMCs begin to express Cx37. Alterations in the patterns of Cx expression can cause differential regulation of the interactions between different atheroma-associated cell types (Burnier et al., 2009; Meens et al., 2012).

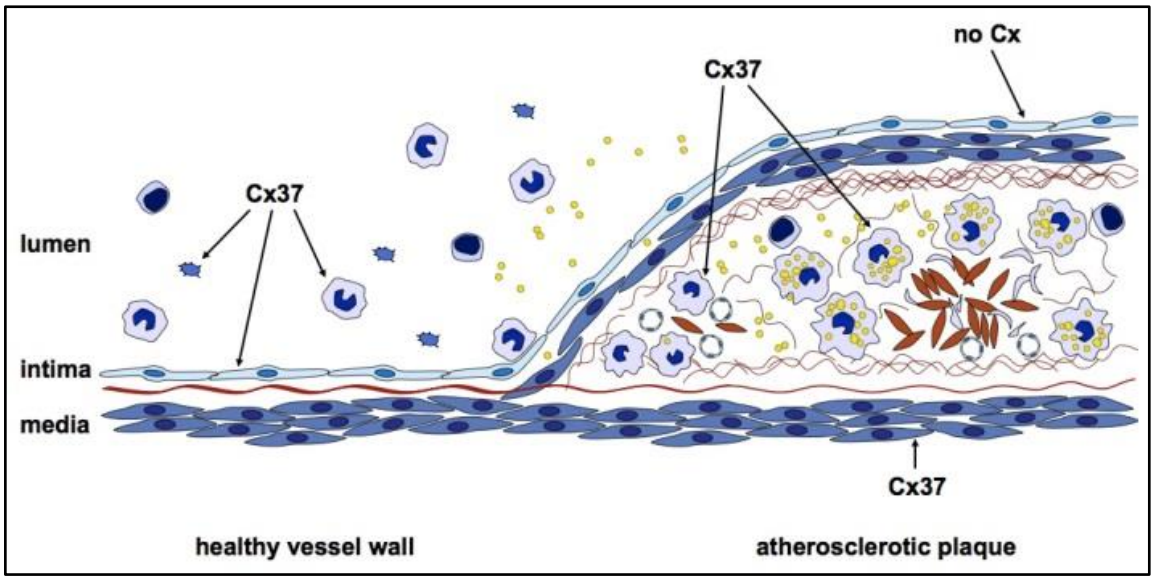


Figure 1.10: Differential expression of connexins during the development of atherosclerotic plaques.

Connexin (Cx) 37 is expressed by monocytes and endothelial cells in the healthy vessel wall (left). On the other hand, macrophages and smooth muscle cells express Cx37 in the atherosclerotic plaque (right). Cx37 is not expressed by the endothelial cells that cover the lesion (Meens et al., 2012).

1.17 Aims

Under normal physiological conditions, platelets are independently circulating cells; however, upon activation, they come into close contact with each other to form a thrombus. This contact endures for an extended period, during which the platelets collaborate. The stability of a thrombus is regulated by persistent internal signalling. The consequential clot retraction is also regulated by this signalling and is essential for repair of the damaged artery tissue (Calderwood, 2004). GJs and connexons have been studied in various cell types, where they mediate stable cell interactions. However, recent reports indicate that Cxs are also capable of regulating the activities of circulating cells, such as T cells and monocytes. This enables the transfer of small molecules between the cytosol of adjacent cells, facilitating contact-dependent inter-cellular communication that enables coordinated cellular responses (Wong et al., 2006; Bermudez-Fajardo et al., 2007; Derouette et al., 2009a; Derouette et al., 2009b; Oviedo-Orta et al., 2010; Kar et al., 2012; Koval et al., 2014).

Communication via hemichannels alone in isolated cells has also been observed. The functions of Cxs (mainly Cx37, Cx40 and Cx43) have been well characterised in the cardiovascular system, including the vasculature (Brisset et al., 2009; Elgueta et al., 2009; Jansen et al., 2010). However, the expression of Cxs in circulating blood cells, such as platelets, macrophages, lymphocytes, neutrophils and dendritic cells, is a relatively recent finding that suggests more widespread roles for these proteins and intercellular communication than previously recognised (Anand et al., 2008; Sarieddine et al., 2009; Machtaler et al., 2011; Glass et al., 2015; Vaiyapuri et al., 2015a; Molica et al., 2017).

The expression of Cx37 and Cx40 in platelets has recently been reported. Treatment with selective mimetic peptides that target Cx37 and Cx40 (^{37,43}Gap27 or ^{40}Gap27, respectively) interfered with hemichannel and GJ function and inhibited platelet fibrinogen binding, aggregation and degranulation (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Both Cxs positively regulate platelet function as their selective deletion in mice reduces platelet function. A fluorescence recovery after photobleaching (FRAP) study facilitated the detection of intercellular communication between the platelets in a thrombus mediated by Cx37 GJs; this communication was prevented by treatment with ^{37,43}Gap27 and non-selective GJ blockers (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012). Clot retraction, a process that consolidates and stabilises thrombus formation, was also attenuated in ^{37,43}Gap27- or ^{40}Gap27-treated samples (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). These findings indicate a possible role for GJs in the regulation of thrombus stability in a contact-dependent manner.

In addition to Cx37 and Cx40, notable levels of Cx62 mRNA transcripts in MKs have been detected (Vaiyapuri et al., 2012). The levels observed were comparable to or higher than those in other circulating cells, such as B cells, T cells and monocytes. Cx62 is a newly identified member of the Cx family and its expression at the protein level has yet not been reported in any cell types. However, mouse Cx57, which is homologous to human Cx62, has been detected in retina cells (Sohl and Willecke, 2003; Oyamada et al., 2005; Sohl et al., 2010; Morel, 2014). In contrast to other extensively studied Cx family members, such as Cx37, Cx40 and Cx43, little is known about the role of Cx62. The lack of reagents to block the function of Cx62 has hindered research progress.

The hypothesis of this project is that the physiological functions of platelets are controlled by cellular communication mediated through Cx62 (hemichannels and GJs), and that this communication plays a role in the development of thrombotic disease.

The main goals of the research were to:

- determine if Cx62 and Cx57 are expressed in human and mouse platelets, respectively
- formulate a selective inhibitor that targets the second external loop of Cx62
- identify the functions of platelet Cxs in haemostasis and thrombotic disease
- analyse the mechanism of Cx-mediated signalling in the regulation of platelets

The findings of the study may reveal new mechanisms and molecules to target therapeutically.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents

Professor Richard Farndale (University of Cambridge, Cambridge, UK) supplied the crosslinked collagen-related peptide (CRP-XL). Protease-free bovine serum albumin (BSA) was obtained from First Link (Wolverhampton, UK). Cx62 mimetic peptide (^{62}Gap27), scrambled peptide, Gly-Pro-Arg-Pro (GPRP), cangrelor, indomethacin, rabbit anti-GJA10 antibody, bovine thrombin, digitonin, phosphate-buffered saline (PBS) tablets, EGTA (Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), fibrinogen from human plasma, ADP, 3,3′-dihexyloxacarbocyanine iodide (DiOC6; 98%), H89, GF109203X, LY29400 and the AKT inhibitor IV were purchased from Sigma–Aldrich (Poole, UK). Vena8 Fluoro+™ Biochips were purchased from Cellix OEM Microfluidic Solutions (Dublin, Ireland). MRS2179 was obtained from Abcam (Cambridge, UK). Poly-L-lysine-coated 12-mm coverslips were obtained from VWR (Leicestershire, UK). ProLong™ Gold Antifade Mountant was purchased from Life Technologies (Carlsbad, CA, USA). Microscopic slides (glass) and Alexa Fluor® 488-conjugated phalloidin were purchased from Thermo Fisher Scientific (Loughborough, UK). Whatman 3-mm chromatography paper, Fura-2 AM, calcein AM and Tris were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Formaldehyde (16%) was purchased from Agar Scientific (Essex, UK). The Chrono-Log CHRONO-LUME® kit was obtained from Chrono-Log (Havertown, PA, USA). Mini-PROTEAN® TGX™ precast polyacrylamide gels (4%–20% and 10% gradient), dual-stained molecular weight markers and polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad (Hemel Hempstead, UK). U46619 (a TxA_2 analogue), SQ 22536, PKI 14-22 and Rp-adenosine 3′,5′-cyclic mono-phosphorothioate

(Rp-8-CPT-cAMPS) were obtained from Tocris Biosciences (Bristol, UK). Black and clear 96-well flat bottom plates were purchased from Greiner Bio-One (Frickenhausen, Germany). The thromboxane B_2 (TxB_2) enzyme-linked immunosorbent assay (ELISA) kit was obtained from Cayman Chemical (Cambridge, UK). A cAMP ELISA kit was purchased from Cell Signaling Technology (Hitchin, UK). All other reagents were of analytical grade and purchased from Sigma–Aldrich or Thermo Fisher Scientific.

2.1.2 Animals

Wild type ($Cx57^{+/+}$) and Cx57-deficient ($Cx57^{-/-}$) mice were provided by International Mouse Phenotyping Consortium. C57BL/6 mice were purchased from Envigo (Huntingdon, UK).

2.1.3 Antibodies

All details regarding the primary and secondary antibodies used in this project, including their applications and dilutions, are listed in Table 2.1 and Table 2.2, respectively.

Table 2.1: Primary antibodies that were used for this project

Antibody	Host	Application	Dilution	Source and catalogue no.
Polyclonal anti-Cx62	Rabbit	Western blotting Immunofluorescence STORM microscopy	1:1,000 1:100 1:50	Sigma–Aldrich (Poole, UK) SAB2105481
Monoclonal anti-GPIb	Mouse	Immunofluorescence	1:100	Thermo Fisher Scientific (Loughborough; UK) PM6/248
Monoclonal anti-Integrin β3	Mouse	STORM microscopy	1:50	Santa Cruz Biotechnology (Calne, UK) sc-52685
Polyclonal anti-human fibrinogen FITC conjugated	Rabbit	Flow cytometry	1:50	Dako (Glostrup, Denmark) F0111
Anti-human CD62P monoclonal antibody–PE-Cy™5	Mouse	Flow cytometry	1:50	BD Biosciences (Franklin Lakes, New Jersey, USA) 551142
Monoclonal anti-integrin α2 chain (GPIa)	Rat	Flow cytometry	1:50	emfret ANALYTICS (Würzburg, Germany) M070-1
Monoclonal anti-GPVI	Rat	Flow cytometry	1:50	emfret ANALYTICS M011-1
Monoclonal anti-Integrin $\alpha_{IIb}\beta_3$	Rat	Flow cytometry	1:50	emfret ANALYTICS M025-2
Monoclonal anti-GPIba	Rat	Flow cytometry	1:50	emfret ANALYTICS M040-2
Anti-phospho-VASP (Ser157)	Rabbit	Western blotting	1:1,000	Cell Signaling Technology (Hitchin, UK) 3111

Table 2.1: Primary antibodies that were used for this project

Antibody	Host	Application	Dilution	Source and catalogue no.
Phospho-VASP (Ser239)	Rabbit	Western blotting	1:1,000	Cell Signaling Technology (Hitchin, UK) 3114
Anti-phospho-tyrosine 4G10	Mouse	Western blotting	1:1,000	Merck Millipore (Watford, UK) 05-321
Anti-phospho-tyrosine PKC	Rabbit	Western blotting	1:1,000	Cell Signaling Technology #2261
Anti-phospho-Syk (Y525/526)	Rabbit	Western blotting	1:1,000	Abcam (Cambridge, UK) ab58575
Anti-phospho-LAT (Y200)	Rabbit	Western blotting	1:1,000	Abcam ab68139
Anti-phospho-PLCγ2 (Y1217)	Rabbit	Western blotting	1:1,000	Cell Signaling Technology 3871
Monoclonal anti-14-3-3 ζ	Mouse	Western blotting	1:1,000	Santa Cruz Biotechnology (Calne, UK) sc-293415
Polyclonal anti-actin	Goat	Western blotting	1:1,000	Santa Cruz Biotechnology sc-1615
DyLight® 649 anti-GPIbα	Rat	*In vivo* thrombosis assay	0.2 µg/gm weight of mice	emfret ANALYTICS M040-3

Table 2.2: Secondary antibodies that were used for this project

Antibody	Host	Application	Dilution	Source and catalogue no.
Alexa Fluor® 488-conjugated anti-mouse IgG	Donkey	Immunofluorescence Western blotting	1:500 1:1,000	Life Technologies (Paisley, UK) A-21202
Alexa Fluor® 488-conjugated anti-goat IgG	Donkey	Western blotting	1:1,000	Life Technologies A-11055
Alexa Fluor® 555-conjugated anti-mouse IgG	Donkey	STORM microscopy	1:50	Life Technologies A-31570
Alexa Fluor® 647-conjugated anti-rabbit IgG	Donkey	Immunofluorescence STORM microscopy Western blotting	1:500 1:50 1:1,000	Life Technologies A-31573
Cy5-conjugated anti-rabbit IgG	Goat	Western blotting	1:1,000	Life Technologies A-10523

2.2 Methods

2.2.1 Cell Preparation

2.2.1.1 Preparation of Washed Human Platelets

On the day of experimentation, healthy volunteers (drug-free) donated their blood after providing informed consent according to procedures approved by the University of Reading Research Ethics Committee. Platelets were prepared by differential centrifugation. Blood (50 mL) was drawn into a syringe containing the anticoagulant 3.8% (w/v) sodium citrate (5 mL). Acid citrate dextrose (ACD; 7.5 mL; 85 mM sodium citrate, 71 mM citric acid and 110 mM glucose) was added to the blood samples, which were then centrifuged at 102 *g* for 20 minutes to obtain platelet-rich plasma (PRP) that was free of erythrocytes and leukocytes. The platelets were then separated by subjecting the PRP to centrifugation at 1,413 *g* for 10 minutes following the addition of PGI_2 (10 μL; prepared at 125 μg/mL in ethanol).

Platelets were resuspended and washed in modified Tyrode's-HEPES buffer (25 mL; 134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$ and 5 mM glucose; pH 7.3) with ACD (3 mL) and PGI_2 (10 μL; 125 μg/mL). Platelet counts were determined using a model Z2™ COULTER COUNTER® (Beckman Coulter, High Wycombe, UK). The platelets were then pelleted by centrifugation at 1,413 *g* for 10 minutes, resuspended in modified Tyrode's-HEPES buffer (1 mL) and adjusted to the desired concentration with modified Tyrode's-HEPES buffer. Prior to the use of washed platelets, they were incubated at 30 °C for 30 minutes to ensure the recovery of functional platelet responses.

2.2.1.2 ADP-sensitive platelets

To prepare ADP-sensitive washed platelets, blood was collected into 3.8% (w/v) sodium citrate (without ACD). The blood was subjected to centrifugation for 20 minutes at 102 *g* and 20 °C to generate PRP. PRP was collected and subjected to centrifugation for 20 minutes at 350 *g* to isolate platelets. During preparation, the discharge of ADP from platelets was minimised by carrying out centrifugation at low speeds to minimise the desensitisation of ADP receptors that can be caused by high-speed centrifugation. The supernatant was discarded and the pellet was resuspended in modified Tyrode's-HEPES buffer (4×10^8 cells/mL).

2.2.1.3 Preparation of PRP

Whole blood was collected in vacutainers containing 3.2% sodium citrate to prepare PRP. The vacutainers were subjected to centrifugation at 102 *g* for 20 minutes. The resulting PRP was then separated and allowed to rest at 30 °C for 30 minutes in a water bath to assure the recovery of functional platelet responses prior to experimentation.

2.2.1.4 Preparation of Mouse Platelets

On the day of the experiment, mouse blood was collected through cardiac puncture after termination by rising CO_2 concentration and cervical dislocation as per Schedule 1 of the Animals (Scientific Procedures) Act 1986. After the mice were euthanised, their blood was drawn through cardiac puncture into a syringe containing 4% sodium citrate (1 part sodium citrate to 9 parts blood). Red blood cells and leukocytes were eliminated by reducing the concentration of blood with Tyrode's-HEPES buffer (1 mL) followed by centrifugation at 203 *g* for 8 minutes. The upper layer comprising PRP was gently aspirated with a pipette. After the addition of PGI_2 (final concentration, 12.5 ng/mL) to the PRP, the platelets were subjected to centrifugation at 1,028 *g* for 5 minutes. The

resulting platelet pellet was resuspended in modified Tyrode's-HEPES buffer (4×10^8 cells/mL) and left to rest at 30 °C for 30 minutes.

2.2.2 Western Blot Analysis

2.2.2.1 Sample Preparation

Mouse or human platelets prepared by differential centrifugation (as described in Sections 2.2.1.1 and 2.2.1.4) were lysed in 6× Laemmli sample treatment buffer comprising 4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol, 0.5 M Tris, 0.001% (w/v) Brilliant Blue R and 10% (v/v) 2-mercaptoethanol. The samples were heated for 5 minutes at 95 °C.

2.2.2.2 Platelet Signalling Studies

Washed human platelets (4×10^8 cells/mL), used to study platelet signalling, were incubated with reagents to block secondary signalling (20 μM indomethacin, 1 μM cangrelor, 100 μM MRS2179 and 1 mM EGTA), which diminish the propensity of platelets to aggregate. Platelets were exposed to appropriate control or Cx inhibitor or cell signalling inhibitors for 5 minutes, followed by stimulation with thrombin or CRP-XL under stirring conditions in an aggregometer (1,200 rpm at 37 °C). Samples were lysed with 6× Laemmli reducing buffer. Prior to storage at −20 °C, the samples were heated for 5 minutes at 95 °C.

2.2.2.3 SDS-Polyacrylamide Gel Electrophoresis

The proteins in the extracts of the platelet lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) after heating to 95 °C for 10 minutes in 6× Laemmli reducing buffer. The samples and molecular weight standards were loaded onto 4%–20% acrylamide gradient gels (Bio-Rad precast gels; Bio-Rad, Watford, UK).

The gels were run at a constant voltage (100 V) for 90 minutes in a Mini-PROTEAN® II apparatus (Bio-Rad, Watford, UK) with Tris-glycine buffer (2 mM Tris, 192 nM glycine and 0.1% [w/v] SDS; pH 8.3) in the running reservoir.

The separated proteins were transferred to PVDF membranes (Bio-Rad, Watford, UK) by semi-dry transfer. PVDF membranes, soaked in methanol, were placed under the resolving gels. Four sheets of 3-mm filter paper soaked in anode buffer (300 mM Tris base and 20% [v/v] methanol; pH 10.4) were placed below the membranes and 4 sheets of 3-mm filter paper soaked in cathode buffer (25 mM Tris base and 40 mM 6-amino-n-hexanoic acid; pH 9.4) were placed above the resolving gels. The proteins were transferred from the gels to the membranes by applying a constant voltage (15 V) for 2 hours.

2.2.2.4 Immunoblotting

The PVDF membranes were blocked with 5% (w/v) BSA dissolved in 1% (v/v) Tris-buffered saline–TWEEN® 20 (TBST; 20 mM Tris, 137 mM NaCl and 0.1% [v/v] TWEEN® 20; pH 7.6) for 1 hour at room temperature. The membranes were incubated with the primary antibodies, which were diluted in 1% (v/v) TBST with 2% (w/v) BSA, overnight at 4 °C.

After overnight incubation, the membranes were washed with TBST (3 × 5 minutes) to remove unbound antibodies. Fluorescently labelled secondary antibodies diluted in 1% (v/v) TBST containing 2% (w/v) BSA were then applied to the membranes, which were incubated for 1 hour at room temperature in the dark. The membranes were then washed with TBST (3 × 5 minutes) and their fluorescence visualised using a Typhoon FLA 9500 fluroimager (Amersham Biosciences, Buckinghamshire, UK). Image Quant

software version 8.1 (GE healthcare) was used to quantify the fluorescence intensities of the individual bands.

2.2.3 Immunofluorescence Microscopy of Human Platelets

Human PRP was prepared from whole blood as described in Section 2.2.1.3. The PRP was fixed with 4% (v/v) formaldehyde for 15 minutes, then subjected to centrifugation at 950 *g* for 11 minutes. The pellet was resuspended twice in Tyrode's-HEPES buffer (1 mL), followed by final centrifugation at 950 *g* for 10 minutes.

The final pellet was resuspended in Tyrode's-HEPES buffer (500 µL) containing 1% (w/v) protease-free BSA. Poly-L-lysine-coated 1.5-mm glass coverslips were placed in 6×6-well culture plates accompanied by a wet tissue in the empty spaces to maintain humidity. Platelets (90 µL) were added to each coverslip using a cut pipette tip, then incubated at 37 °C for 90 minutes.

The non-adhered platelets were removed by washing with PBS (3 × 2 minutes) and the coverslips were blocked with 0.2% (v/v) Triton™ X-100, 2% (v/v) donkey serum and 1% (w/v) BSA for 60 minutes at room temperature. Following permeabilization, fixed platelets were stained with primary antibodies diluted 1:100 in 0.2% (v/v) Triton™ X-100, 2% (v/v) donkey serum and 1% (w/v) BSA. The unbound antibodies were removed by washing with PBS (3 × 2 minutes).

The coverslips were further incubated with secondary antibodies diluted 1:200 in 0.2% Triton™ X-100, 2% donkey serum and 1% BSA for 60 minutes at room temperature in the dark. The unbound antibodies were removed by washing with PBS (3 × 2 minutes). The platelets were fixed in 4% formaldehyde for 5 minutes. The coverslips were washed with PBS (3 × 2 minutes) and mounted with mounting medium (Life

Technologies, Carlsbad, CA, USA). The platelets were imaged with a 100× oil immersion lens on a Nikon A1R confocal microscope. Two filters were used: one to visualise Alexa Fluor® 488 (excitation at 490 nm and emission at 525 nm) and the other to visualise Alexa Fluor® 647 (excitation at 650 nm and emission at 665 nm).

2.2.4 Super Resolution Stochastic Optical Reconstruction Microscopy

PRP was obtained as described (section 2.2.1.3). Tyrode's-HEPES buffer was used to dilute the PRP (1:20). The polymerisation of fibrin was prevented by treatment with GPRP (0.5 mg/mL). The samples were activated with thrombin (1 U/mL) for 5 minutes, then the unstimulated and stimulated samples were fixed with 2% (v/v) formyl saline and subjected to centrifugation for 15 minutes at 500 *g*. After removal of the supernatants, the pellets containing the platelets were resuspended in Perm Buffer III (100 µL; BD Biosciences, Oxford, UK) and incubated on ice for 30 minutes. Then, the platelets were washed with Tyrode's-HEPES buffer (2 × 20 minutes) and subjected to centrifugation at 500 *g*. The supernatant was discarded and the resultant pellet was resuspended in Tyrode's-HEPES buffer (50 µL). The samples were incubated with the primary antibodies (diluted 1:50; mouse monoclonal IgG against integrin β_3 and rabbit polyclonal IgG against Cx62) at 4 °C overnight. The following day, platelets were washed twice with Tyrode's-HEPES buffer (2 mL) followed by centrifugation for 20 minutes at 550 *g*. The samples were incubated with the secondary antibodies (diluted 1:50 in Tyrode's-HEPES buffer; Alexa Fluor® 647-labelled donkey anti-rabbit to detect Cx62 and Alexa Fluor® 555-labelled goat anti-mouse to detect β_3 integrin) at 37 °C for 30 minutes. Then, the platelets were washed with Tyrode's-HEPES buffer (2 mL) and subjected to centrifugation for 20 minutes at 550 *g*. The resulting pellet was suspended in Tyrode's-HEPES buffer (100 µL). Finally, platelets (100 µL) were applied to ibidi® slides coated with poly-L-lysine. The slides were incubated at 4 °C

overnight to allow the platelets to adhere. The next day, the unbound platelets were removed and blinking buffer was added (Stock A: 0.90 g/mL catalase [Sigma–Aldrich], 0.182 mM Tris [2-carboxyelthyl] phosphine hydrochloride [Sigma–Aldrich], 2.27% [v/v] glycerine, 1.14 mM KCl, 0.91 mM Tris-HCl [pH 7.5], 0.045 mg/mL glucose oxidase [Sigma–Aldrich] and 5 mL diH_2O; stock B: 36 mg/mL glucose, 3.6% [v/v] glycerine and 36 mL H_2O; and stock C: 0.09 M mercaptoethylamine-HCl [Sigma–Aldrich] and 1 mL diH_2O). For 3D stochastic optical reconstruction microscopy (STORM) imaging of the platelets, the 100× oil immersion lens of the microscope was used.

2.2.5 Platelet Subcellular Fractionation Using a Sucrose Density Gradient

Prior to the day of experiment, 60%–55%, 50%–45% and 40%–35% sucrose solution gradients in PBS were prepared by layering 2 mL of each concentration of the sucrose in the designated tubes (60% at the bottom and 35% on the top). The tubes were incubated at 4 °C overnight to form linear sucrose gradients. On the day of the experiment, drug-free, healthy individuals donated blood (100 mL) after providing full consent. The blood was collected in two syringes containing 3.8% (w/v) sodium citrate (5 mL). The blood in both syringes was treated with ACD (7.5 mL; 85 mM sodium citrate, 71 mM citric acid and 110 mM glucose) then subjected to centrifugation at 102 *g* for 20 minutes to pellet the red and white blood cells. PRP was collected and treated with PGI_2 (20 μL, 125 μg/mL in ethanol). The PRP was subjected to centrifugation at 1,413 *g* for 10 minutes to pellet the platelets from the plasma. Thereafter, the platelet-poor plasma was removed and the pelleted platelets were resuspended in Tris-citrate buffer (15 mL; 63 mM Tris, 95 mM NaCl, 5 mM KCl and 12 mM citric acid; pH 6.5/HCl) with PGI_2 (10–15 μL), then subjected to centrifugation for 10 minutes at 1,413 *g*. The platelet pellet was resuspended in Tris-citrate buffer (2.5 mL) and

maintained in a water bath (37 °C) for 30 minutes. Non-aggregating conditions were maintained by treating the platelets with the calcium chelator EGTA (1 mM), and indomethacin (10 μM) or apyrase (10 U/mL) to minimise the release of TxA_2 or metabolism of ADP, respectively. The platelets were homogenised by nitrogen cavitation (4639 Cell Disruption Vessel, Parr Instrument Company). A pressure of 1200 psi N_2 was applied to the platelet suspension, then quickly released after 15 minutes a total of three times. The platelet homogenates were subjected to centrifugation at 500 *g* for 10 minutes at 4 °C to eliminate partially disrupted cells and other cellular debris. Then, the supernatants were placed over a linear sucrose gradient (35%–60%) and subjected to ultracentrifugation at 200,000 *g* for 2 hours at 10 °C. Thirteen fractions (1 mL each) were carefully obtained from the tops of the gradients and preserved at −20 °C for future testing.

2.2.6 Calcein Dye Efflux

Before preparing washed platelet suspensions (section 2.2.1.1), PRP was loaded with calcein AM (0.5 μM; Thermo Fisher Scientific, Waltham, MA, USA) for 30 minutes at 37 °C. The platelets were then treated with scrambled peptide or ^{62}Gap27 for 5 minutes. Next, the platelets were stimulated with thrombin (0.1 U/mL). In order to prevent fibrin polymerisation, the thrombin-treated samples were also treated with GPRP (25 μg/mL). Stimulation was carried out with gentle mixing for different time periods over 5 minutes. Finally, the reaction was stopped with 0.2% (v/v) formyl saline. Flow cytometry (488 nm excitation, 530 ± 30 nm emission) was performed with a BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK). For each sample, 10,000 events, gated on platelets by forward scatter (1,520–16,000,000) and side scatter (152–1,600,000), were collected. The data were analysed with the built-in BD Accuri™ C6 Plus software (version 1.0.264.21).

2.2.7 Fluorescence Recovery after Photobleaching (FRAP)

The 100× objective of a Nikon AIR confocal microscope was employed for FRAP. Each of the eight wells of each ibidi® slide was coated with fibrinogen (100 μg/mL) and collagen (10 μg/mL) in modified PBS for 1 hour. Then, 1% (w/v) BSA was added to the wells followed by a 1-hour incubation to prevent the binding of platelets to the glass. The wells were washed three times with PBS. Calcein-loaded PRP was added to the coated coverslips and incubated for 45 minutes. Unbound platelets were washed from the wells with PBS (three washes). The samples were then treated with the scrambled peptide or ^{62}Gap27 (100 μg/mL) for 5 minutes.

A high-intensity laser (488 nm) was trained on the central circular area (8-μm-diameter region of interest [ROI]) of the thrombus for 300 milliseconds, resulting in an 85% loss of fluorescence. Then, a 488-nm wavelength laser was used to excite the samples and the fluorescence emission was detected at 500–520 nm. Finally, fluorescence recovery was recorded for 500 seconds. The 100× oil immersion objective of an A1R confocal microscope was used to capture images of single sections every second for 500 seconds. Five thrombi from each of seven donor samples treated with scrambled peptide or ^{62}Gap27 were analysed. NIS-Elements software (Nikon, Tokyo, Japan) was used to compute the mean fluorescence intensities. For each time point, the average fluorescence intensities were computed for the background, non-bleached (reference) and bleached areas. The correction of the fluorescence signals for background was performed and the recovery rate was calculated as follows:

$$\text{Normalised Bx} = \frac{\left(\frac{Bx - Bg}{Rx - Bg}\right) - \left(\frac{Bb - Bg}{Rb - Bg}\right)}{\left(\frac{Bi - Bg}{Ri - Bg}\right) - \left(\frac{Bb - Bg}{Rb - Bg}\right)}$$

where $\mathbf{B}_x$ refers to the fluorescence intensity of the bleached ROI recorded at *x* seconds, $\mathbf{R}_x$ refers to the fluorescence intensity of the reference ROI (non-bleached region) recorded at *x* seconds, $\mathbf{B}_b$ refers to the fluorescence intensity of the bleached ROI immediately after photobleaching, $\mathbf{R}_b$ refers to the fluorescence intensity of the reference ROI immediately after the photobleaching, $\mathbf{B}_i$ refers to the initial fluorescence intensity of the bleached ROI, $\mathbf{R}_i$ refers to the initial fluorescence intensity of the reference ROI and $\mathbf{B}_g$ refers to the average fluorescence intensity of the background ROI.

2.2.8 Platelet Aggregation Assay

Aggregation assays on washed platelets were performed by light transmission aggregometry in an optical platelet aggregometer (Chrono-Log, Havertown, PA, USA). Platelets (225 µL; 4×10^8 cells/mL) were incubated with an inhibitor of Cx function or an appropriate control for 5 minutes. The platelets were stimulated with CRP-XL or thrombin (12.5 µL) while being stirred at 1,200 rpm at 37 °C. The aggregation traces were recorded for up to 3 minutes.

2.2.9 α-granule Secretion and Fibrinogen Binding

P-selectin exposure and fibrinogen binding to the platelet surface were detected by flow cytometry as measures of the secretion of α-granules and integrin $\alpha_{IIb}\beta_3$ activation, respectively. Fluorescein isothiocyanate (FITC)-labelled rabbit anti-human fibrinogen antibody (Dako, Ely, UK) was used to measure fibrinogen binding. PE-Cy™5-labelled

mouse anti-human CD62P antibody (BD Biosciences, Oxford, UK) was used to measure the exposure of P-selectin. The assay volume (50 µL), comprising human or mouse PRP (5 µL), an inhibitor of Cx function or appropriate control (5 µL) and each of the antibodies (1 µL) in modified Tyrode's-HEPES buffer (39 µL), was incubated for 5 minutes at room temperature in the dark.

The platelet agonists thrombin (in the presence of 25 µg/mL GPRP to prevent fibrin polymerisation) or CRP-XL (5 µL) were added and incubated for an additional 20 minutes. The reaction was stopped by the addition of 0.2% (v/v) formyl saline. Suitable isotype controls, EGTA (final concentration, 1 mM; for fibrinogen binding) or isotype controls for P-selectin exposure were used as negative controls for the antibody responses. A BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK) and BD Accuri™ C6 software were used for the acquisition of the flow cytometry data. The median fluorescence intensity was calculated for 10,000 gated events. Fluorescence in FL1-A and FL3-A channels were used to analyse fibrinogen binding and P-selectin exposure, respectively.

2.2.10 Platelet Receptor Expression

To quantify the expression of receptors in $Cx57^{+/+}$ and $Cx57^{-/-}$ platelets, mouse PRP ($Cx57^{+/+}$ or $Cx57^{-/-}$) was incubated with antibodies against integrin $\alpha_2\beta_1$ (FITC-labelled), integrin $\alpha_2\beta_3$ (FITC-labelled), GPVI (Cy5-labelled) and GPIb (Cy5-labelled) for 10 minutes. The samples were left unstimulated or stimulated with CRP-XL (0.5 µg/mL), then fixed with 0.2% (v/v) formyl saline (250 µL). The samples were analysed (10,000 events each) on a BD Accuri™ C6 flow cytometer (BD Biosciences, Oxford, UK). The events were gated on platelets using forward scatter (1,520–16,000,000) and side scatter (152–1,600,000). BD Accuri™ C6 Plus software (version 1.0.264.21) was used for data analysis.

2.2.11 Dense Granule Secretion

Dense granule secretion was determined by measuring changes in the extracellular ATP concentration. These changes were observed concurrently with aggregation in a Model 700 Whole Blood/Optical Lumi-Aggregometer with the use of a luciferase kit (Chrono-Log, Havertown, PA, USA). ATP release from dense granules was monitored with a bioluminescence system comprising D-luciferin, firefly luciferase and magnesium. ATP interactions with these reagents produce light, in direct proportion to the ATP concentration, which is observed and quantified using a lumi-aggregometer.

Platelets (4×10^8 cells/mL; 200 µL) were pre-treated with an inhibitor of Cx function or appropriate control (12.5 µL) at 37 °C for 5 minutes. Luciferase (25 µL) was added under stirring conditions during the last 2 minutes of the incubation. The platelets were stimulated with the indicated concentrations of thrombin or CRP-XL (12.5 µL) under stirring conditions (1,200 rpm at 37 °C). ATP release and aggregation at 37 °C were recorded for 3 minutes.

2.2.12 TxB_2 ELISA

The TxB_2 measurements were performed with a TxB_2 immunoassay kit based on a competitive ELISA (Cayman Chemical, Cambridge, UK), according to the manufacturer's instructions. Washed platelets (4×10^8 cell/mL; 225 µL) were treated with an inhibitor of Cx function or appropriate control (12.5 µL) in glass cuvettes and incubated for 5 minutes. The samples were then activated with CRP-XL or thrombin (12.5 µL). After 5 minutes, stop solution (1 mM EGTA and 10 µM indomethacin) was added to terminate the reaction. The samples were then immediately subjected to centrifugation for 2 minutes at 12,000 rpm and the supernatants were frozen at −80 °C. Later, the supernatants were thawed and diluted 1:40 in ELISA buffer (0.01% [w/v] sodium azide, 1 mM EDTA, 400 mM NaCl, 0.1% [w/v] BSA and 100 mM

phosphate). The dilutions (50 µL) were plated in wells coated with polyclonal goat anti-mouse IgG antibodies. To determine the relationship between the TxB_2 concentration and absorbance, TxB_2 standards (serial dilutions, 50 µL) were prepared. TxB_2-acetylcholinesterase (50 µL) and anti-TxB_2 monoclonal antibody (50 µL) were added to each well, then the plate was incubated at room temperature for 2 hours. After incubation, the plate was washed 4 times with washing buffer. Next, Ellman's reagent (200 µL) was added to each well and the plate was incubated in the dark. A NOVOstar plate reader (BMG Labtech, Aylesbury, UK) was used to determine the absorbances of the wells at 405 nm. A standard curve was plotted using the absorbance readings for the TxB_2 standards. The inverse function was used to compute the TxB_2 concentrations from the test sample readings.

2.2.13 Analysis of Intracellular Ca^{2+} Mobilisation

The mobilisation of Ca^{2+} from intracellular stores into the platelet cytosol was measured in a fluorescence-based 96-well plate assay. As described in Section 2.2.1.1, whole human blood was used to prepare PRP. PRP was incubated with 2 µM Fura-2 AM for 60 minutes at 30 °C. The PRP was washed and subjected to centrifugation at 350 *g* for 20 minutes, then resuspended in modified Tyrode's-HEPES buffer at (4×10^8 cell/mL).

Fura-2-loaded platelets were incubated with an inhibitor of Cx function or appropriate control for 5 minutes at 37 °C, then stimulated with the agonists thrombin and CRP-XL. A NOVOstar plate reader (BMG Labtech, Aylesbury, UK) was used to measure the fluorescence (excitation at 340 and 380 nm and emission at 510 nm).

The ratio of the excitation signals at 340 and 380 nm was used to estimate the concentration of Ca^{2+}. The cells were lysed with digitonin (5 µM) to release the Fura-2

into the assay buffer (which contained 1 mM $CaCl_2$) and facilitate the measurement of the maximum fluorescence ratio. The minimum fluorescence ratio was measured by chelating Ca^{2+} ions with EGTA (10 mM) and Tris base (10 mM; added to ensure that the pH remained alkaline for optimum Ca^{2+} buffering by EGTA). Non-Fura-2-loaded cells at the same final density were used to measure the autofluorescence levels (Ohlmann et al., 2004). Using the calibration values from above, experimental $[Ca^{2+}]_i$ concentrations were calculated using the following equation:

$$[Ca^{2+}]_i = K_d \times \frac{S_f}{S_b} \times \frac{R - R_{min}}{R_{max} - R}$$

where $\mathbf{K_d}$ is the dissociation constant of Fura-2 (224 nm); $\mathbf{S_f}$ and $\mathbf{S_b}$ are the values of the fluorescence after excitation at 380 nm, corrected for autofluorescence, with zero or saturating levels of Ca^{2+}, respectively; **R** is the ratio of 340/380 nm fluorescence, corrected for background fluorescence; $\mathbf{R_{min}}$ and $\mathbf{R_{max}}$ are the ratio limits at zero or saturating levels of Ca^{2+}, respectively.

2.2.14 Clot Retraction Assay

As described in Section 2.2.1.3, human PRP was prepared and rested at 30 °C for 30 minutes. Red blood cells (5 µL) and an inhibitor of Cx function or appropriate control were mixed with the PRP (200 µL). The mixture was adjusted to a final volume of 1 mL with modified Tyrode's-HEPES buffer and incubated for 5 minutes at room temperature. Thrombin (50 µL; final concentration, 1 U/mL) was added to initiate clot generation. A surface for the formation and contraction of the clot was established by placing a sealed glass capillary in the middle of a glass test tube. Clots were observed at

30-minute intervals for 90 minutes and recorded by photography. At the end of the study, the clots were weighed with a microbalance (lower weight means greater contraction).

2.2.15 Platelet Spreading

To study platelet spreading, glass coverslips coated with fibrinogen (100 μg/mL in modified PBS) were placed in 6-well plates. After coating for 1 hour, 1% (w/v) BSA was added to the coverslips followed by a 1-hour incubation to prevent platelets from binding the glass. The coverslips were then washed three times with PBS. The washed platelet suspensions (2×10^7 cells/mL) that had been incubated for 5 minutes with an inhibitor of Cx function or appropriate control were then added to the coverslips and incubated at 37 °C for 45 minutes. Unbound platelets were removed and the coverslips were washed three times with PBS. Then, the coverslips were fixed in 0.2% (v/v) formyl saline for 10 minutes. The coverslips were again washed three times with PBS. Next, the platelets were permeabilised with 0.2% (v/v) Triton™ X-100 for 5 minutes, then washed three times with PBS. The coverslips were incubated with Alexa Fluor® 488-conjugated phalloidin for 1 hour in the dark to label filamentous actin. The supernatants were removed, the coverslips were washed with PBS and placed on glass slides and fluorescence was preserved by adding ProLong™ Gold Antifade Mountant. The 100× oil immersion lens of the Nikon A1R confocal microscope (Nikon, Tokyo, Japan) was used to image samples (excitation at 488 nm from an argon laser, emission between 500 and 520 nm). Images were taken in a single focal plane. In order to determine platelet adhesion, the numbers of platelets in five random images of each coverslips were counted. Platelets were categorised as spread fully (lamellipodia formed), partially spread (defined as filopodia) or adhered (not spreading). Finally, the relative frequencies of these groups were computed.

2.2.16 cAMP ELISA

A cAMP immunoassay kit based on a competitive ELISA (Cell Signaling Technology, Hitchin, UK) was used to assess cAMP levels, according to the protocol provided by the manufacturer. Washed platelets (4×10^8 cell/mL; 225 µL) were added to a glass cuvette. An inhibitor of Cx function or appropriate control (12.5 µL) was added to the cuvettes, which were then incubated for 5 minutes. Then, thrombin or CRP-XL (12.5 µL) was added for activation. After 5 minutes of stimulation, lysis buffer (Triton™ X-100: 1% polyethylene glycol octylphenol ether) was added to the samples, which were immediately frozen at −20 °C. The samples (50 µL) were later thawed and added to microwells coated with cAMP XP® rabbit monoclonal antibody. The association between cAMP concentration and absorbance was determined using cAMP standards (serial dilutions, 50 µL). The assay plate was covered and incubated on a horizontal orbital plate shaker for 3 hours at room temperature. After incubation, the contents of the wells were removed and the wells were washed with 1× washing buffer three times. Then, 3,3',5,5'-tetramethylbenzidine substrate (100 µL) was added to the wells and the plate was incubated for 30 minutes. Stop solution was added to terminate the reaction. The absorbance at 450 nm was periodically determined with a NOVOstar plate reader (BMG Labtech, Aylesbury, UK). A standard curve was plotted from the absorbance readings of the cAMP standards. cAMP concentrations were computed for the test sample readings via the inverse function.

2.2.17 *In Vitro* Thrombus Formation under Flow

Whole human blood was incubated with the lipophilic dye DiOC6 (5 µM) at 30 °C for 1 hour. Vena8 BioChip microfluidic channels were coated with type I collagen (100 µg/mL) for 1 hour. Channels were washed with modified Tyrode's-HEPES buffer to remove excess collagen. Whole blood was incubated with an inhibitor of Cx function or

an appropriate control for 5 minutes. Then, the blood samples were perfused through the collagen-coated channels at an arteriolar shear rate of 20 dyn/cm^2. An argon laser was used to excite fluorescence (488 nm) and emission was recorded at 500–520 nm. Thrombus formation on the microfluidic chip was observed through the 20× objective of the Nikon A1R confocal microscope. Images of single sections were obtained every second for 600 seconds. Finally, NIS-Elements software (Nikon, Tokyo, Japan) was used to compute the mean thrombus fluorescence intensity.

2.2.18 *In Vivo* Thrombus Formation

The protocol described by Falati et al. (2002) was used to study *in vivo* thrombus formation. C57BL/6 mice were anaesthetised with intraperitoneally administered atropine (0.25 mg/kg), xylazine (12.5 mg/kg) and ketamine (125 mg/kg). When needed, pentobarbital (5 mg/kg) was used to sustain anaesthesia. After exteriorisation of the cremaster muscle and removal of the connective tissue, an incision was made in the muscle, resulting in its adherence as a single layer to the glass slide. A buffer (135 mM NaCl, 4.7 mM KCl, 2.7 mM $CaCl_2$ and 18 mM $NaHCO_3$; pH 7.4) was used to hydrate the muscle.

Before the injury (made with a MicroPoint Ablation Laser Unit; Andor Technology, Belfast, UK), an inhibitor of Cx function or appropriate control and DyLight® 649-conjugated anti-GPIbα antibody (to label platelets; 0.2 μg/g mouse weight), were introduced into the circulation through a cannula in the carotid artery. After 5 minutes of administration of an appropriate control or inhibitor of Cx function, the formation of thrombi was observed with an Olympus BX61W1 microscope (Olympus, Tokyo, Japan). A Hamamatsu digital camera (C9300; Hamamatsu Photonics, Welwyn Garden City, UK) with charge-coupled device camera in 640×480 format was used to obtain

images before and after injury. The images were analysed with SlideBook 6 software (Intelligent Imaging Innovations, Denver, CO, USA).

The protocol from the Home Office licence was followed for the sacrifice of the mice. The protocol was also approved by the Animal Welfare and Ethics Research Board and the University of Reading local ethics review panel. Since the procedure calls for expertise in microsurgery, it was performed with the assistance of Dr Parvathy Sasikumar.

2.2.19 Tail Bleeding Assay

C57BL/6 mice were anaesthetised by intraperitoneal administration of xylazine (12.5 mg/kg) and ketamine (125 mg/kg). Moreover, an inhibitor of Cx function or appropriate control was administered through injection via the femoral vein. After 5 minutes of infusion, the tips of the tails (0.3 cm) were cut with a scalpel and immediately placed in tubes with saline in a manner that prevented the cut ends of the tails from touching the walls of the tubes. The bleeding time was recorded until blood flow stopped or for up to 20 minutes.

The mice were sacrificed according to the protocol that was approved by the University of Reading local ethics review panel, the Animal Welfare and Ethics Research Board and the Home Office. These assays were performed with the assistance of Dr Lisa Holbrook.

2.2.20 Statistical Analyses

The statistical significance of the findings for more than two groups was determined by one-way analysis of variance (ANOVA) with post-hoc Dunnett's multiple comparisons test. Student's *t*-test was used for comparisons between two groups. The *in vitro* thrombus formation assay was analysed by two-way ANOVA with Sidak's multiple

comparisons test. The tail bleeding and *in vivo* thrombosis assay data, which were not normally distributed, were analysed by non-parametric Mann–Whitney U-test. Data for which the *P*-value for the mean ± standard error of the mean was ≤0.05 were considered statistically significant. The statistical analyses were performed with Prism software (version 7.00, GraphPad, San Diego, CA).

3 Expression of Cx62 in Platelets and Design of a Cx62 Mimetic Peptide ^{62}Gap27

3.1 Introduction

Cxs comprise 4 helical transmembrane domains, a pair of extracellular loops, an intracellular loop, a C-terminus and an N-terminus. Transmembrane domains 1–4 and extracellular loops 1 and 2 are highly conserved in all members of the Cx family. Conversely, the intracellular loop, C-terminus and N-terminus in the cytoplasm are of variable compositions and lengths; these components are distinctive for each kind of Cx. After synthesis in the endoplasmic reticulum, Cxs undergo oligomerisation in the endoplasmic reticulum and Golgi apparatus or the trans-Golgi network, thereby forming hexameric connexons (Laird, 2006; Thevenin et al., 2013). This is followed by transportation of the connexons to the cell membrane. After reaching the cell membrane, connexons from one cell may dock with corresponding connexons on an adjacent cell, thereby forming a GJ channel which allows the transportation of entities up to ~1 kDa. Based on the Cx isoforms that form the channels, they are categorised as homomeric or heteromeric (Koval et al., 2014).

The importance of Cxs for tissue homeostasis is illustrated by the polymorphisms and mutations in Cx genes that are linked with pathological conditions (Pfenniger et al., 2011; Kelly et al., 2015). In recent years, researchers have studied the formation and roles of Cx37 and Cx40 in platelets (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Interestingly, MKs express higher levels of Cx62 transcripts than other blood cells, such as monocytes, T cells and B cells (Vaiyapuri et al., 2012).

Cx62 protein expression has not been reported in any cells. Accordingly, the aim of this chapter was to determine if Cx62 and Cx57 are expressed in human and mouse platelets, respectively. Cx57 in mouse is reported to be homologous to human Cx62

(Sohl and Willecke, 2003; Morel, 2014). In addition, a mimetic peptide targeting the second external loop of Cx62 (^{62}Gap27) was developed to explore the functions of Cx62 hemichannels and GJs in platelets.

3.2 Expression of Cx62 in Platelets

mRNA transcriptomic profiling established the expression of notable levels of Cx37, Cx40 and Cx62 in MKs (Vaiyapuri et al., 2012). The expression of Cx37 and Cx40 proteins have been confirmed in human platelets through immunoblot analysis (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). The aim of this initial section of work was to determine if Cx62 (human) and Cx57 (mouse) are expressed in platelets.

Immunoblotting was performed with polyclonal rabbit anti-human Cx62 antibodies that target the N-terminus of Cx62. Given the >98% sequence similarity of the N-terminus of human Cx62 and mouse Cx57, it was anticipated that the antibody binds Cx62 proteins on human cells and Cx57 protein on mouse cells.

The proteins from human and mouse platelet lysates (4×10^8 cells/mL) were separated by SDS-PAGE, then immunodetected with rabbit polyclonal anti-Cx62. The PVDF membranes were further incubated with a fluorescently labelled secondary antibody prior to visualisation with a Typhoon™ FLA 9500 fluorimager. Bands of sizes ranging between 50 and 75 kDa were detected in extracts from the human (lanes 1, 2, 3 and 4) and mouse (lanes 5 and 6) platelets from different donors (Figure 3.1). A protein of approximately 62 kDa was also detected in MEG-01 cells (lane 7). The HeLa cells (lane 8) were not found to express Cx62, which is in agreement with previous studies reporting the absence of any form of Cx in HeLa cells (Saez et al., 2005).

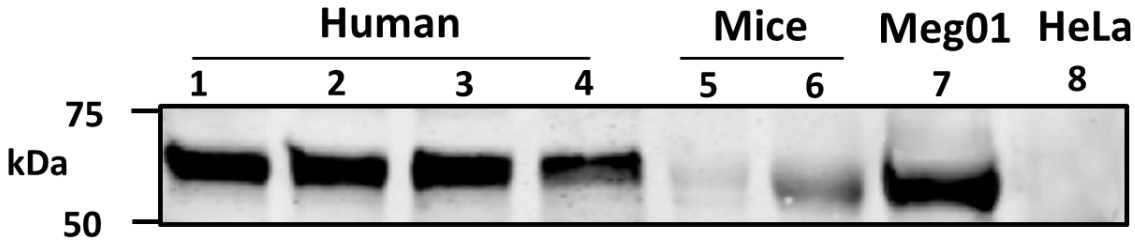


Figure 3.1: Expression of Cx62/57 in mouse and human platelet lysates and a MEG-01 lysate.

Immunoblot analysis verified the presence of Cx62 in human (lanes 1–4, each representing a different donor) and Cx57 in mouse (lane 5 and 6) platelets and in the MEG-01 cell lysate (lane 7). MEG-01 cells and human or mouse platelets (4×10^8 cells/mL) were collected through differential centrifugation and lysed using reducing Laemmli buffer. A HeLa cell lysate (lane 8) served as negative control. Loading control showed that there are no differences between samples. A Typhoon™ FLA 9500 fluorimager was used for membrane visualisation (GE Healthcare, UK). Data are representative of 4 separate experiments.

3.3 Subcellular Localisation of Cx62 in Platelets

Although the expression of Cx62 and Cx57 was verified in human and mouse platelets, respectively, the subcellular locations of the Cxs within the platelets remained unknown. The large numbers of Cxs could reside in the membranes of platelets and form GJs with the connexons of other platelets upon stimulation. In order to test this hypothesis, permeabilised resting and activated platelets were immunostained for Cx62 and visualised by confocal microscopy.

Human PRP from the blood was incubated for 30 minutes to allow platelets to rest. Then, platelets were activated with U46619 (non-aggregating condition) in the presence of integrilin (4μM). U46619 was used as an agonist because it stimulates gentle activation of platelets with minimal shape change, which is helpful for the study of the distribution of Cxs. Resting and activated platelets were then fixed in 4% (v/v) formaldehyde for 15 minutes. Next, the platelets were permeabilised with 0.2% (v/v) Triton™ X-100, then incubated with or without the rabbit polyclonal anti-Cx62 antibody. The GPIb surface receptor was detected with mouse monoclonal anti-GPIb antibody (in order to stain the exterior surface of the plasma membrane (PM). Finally, the samples were incubated with Alexa Fluor®647- and Alexa Fluor®488-conjugated secondary antibodies for the detection of Cx62 and GPIb, which are shown in red and green, respectively (Figure **3.2**). Human platelets without primary antibody treatment were used as a negative control.

The expression of Cx62 in platelets was detected by confocal imaging. Cx62 was found to be organised in a punctate fashion in platelets with low levels of the molecules detected close to the plasma membrane of unstimulated platelets (The GPIb staining marked the cell boundary) (Figure **3.2**a). In contrast, following stimulation of platelets

with U46619, the majority of Cx62 appeared to mobilise toward the platelet plasma membrane where it was localised in a punctate appearance (Figure **3.2**b).

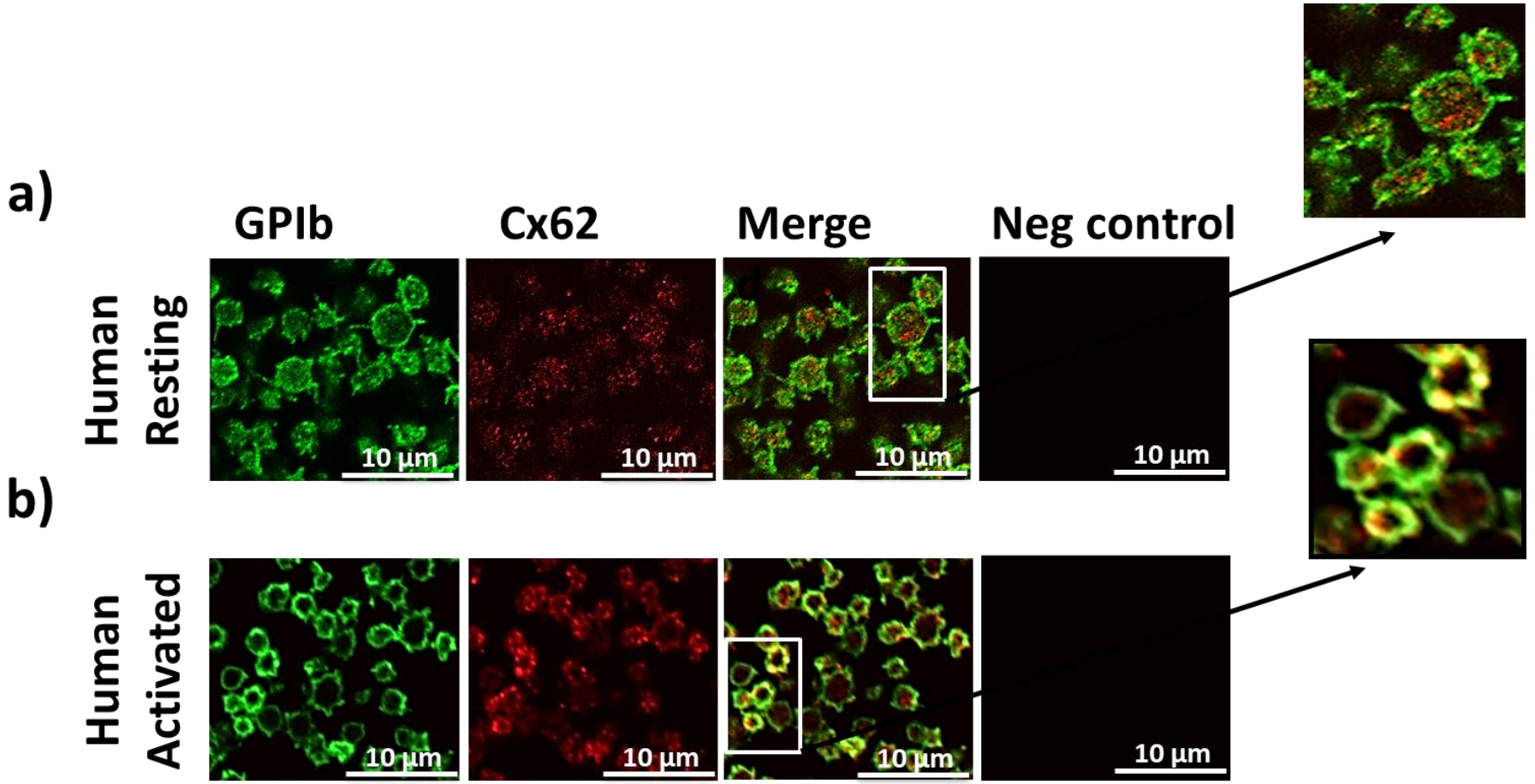


Figure 3.2: Detection of the localisation of Cx62 in platelets by immunocytochemistry.

(a) Resting and **(b)** activated (with 5 µM U46619 in the presence of integrelin) platelets were fixed in 4% (v/v) formaldehyde, then permeabilised with 1% BSA, 2% donkey serum and 0.2% Triton™ X-100. After washing, the platelets were subjected to blocking followed by incubation with 1 µg/mL anti-Cx62 and anti-GPIb. Secondary antibodies conjugated with Alexa Fluor® 488 (GPIb; green) and Alexa Fluor® 647 (Cx62; red) were used to detect the binding of the primary antibodies. A Nikon A1R confocal microscope and 100× oil immersion lens were used for visualisation.

3.4 Subcellular Distribution of Cx62 Studied using Super-Resolution Microscopy (STORM)

Considering the punctate distribution of Cx62 and its mobilisation toward the plasma membrane after platelet activation, it was hypothesised that platelets may have multiple storage pools of Cx62. However, it is not possible to verify this possibility by conventional confocal microscopy, owing to the small size of platelets (2–5 μm in diameter), the limited spatial resolution of the technique and the limitations of the diffraction resolution of the system (~250 nm) (White et al., 1987; Jonkman and Brown, 2015; Westmoreland et al., 2016). These limitations can be overcome by Stochastic Optical Reconstruction Microscopy (STORM), which provides the highest resolution of the existing light microscopy systems. This enables the observation of the trafficking and localisation of proteins in small cells like in platelets within a nanostructure scale (20 to 25 nm resolution) (Olivier et al., 2013). Thus, form of super resolution microscopy enables substantially greater resolution than confocal microscopy. Therefore, STORM was used to visualise the localisation of Cx62 in permeabilised resting and activated human platelets.

Human PRP was rested for 30 minutes at 37 °C and diluted in HEPES buffer. Platelets, either resting or thrombin-activated (1 U/mL, as optimised in our lab for this assay; in the presence of 25 μg/mL GPRP), were fixed in 2% [v/v] formyl saline, then subjected to centrifugation for 15 minutes at 500 *g*. The pellet was resuspended in permeabilisation buffer (BD Phosflow Perm Buffer III). The platelets were washed twice in HEPES buffer, then incubated with rabbit polyclonal anti-Cx62 and mouse monoclonal anti-β_3 integrin antibodies. Secondary antibodies conjugated to Alexa Fluor® 555 (green) and Alexa Fluor® 647 (red) were used to detect β3- and Cx62-probed platelets. Human platelets without primary antibody treatment were used as a

negative control. The prepared slides were observed using a Nikon N-STORM using a 100× oil immersion lens. Data acquisition was performed using NIS-Elements software.

It was observed that Cx62 molecules were distributed distinctly inside the cytosol of resting platelets with few molecules on the surface (Figure 3.3a). A large proportion of Cx62 seemed to be transported to platelet membrane upon activation, and were observed arranged in a clustered appearance which may represent the formation of hemichannels (Figure 3.3b). These data are in alignment with the previous observations obtained by confocal microscopy (Figure 3.2).

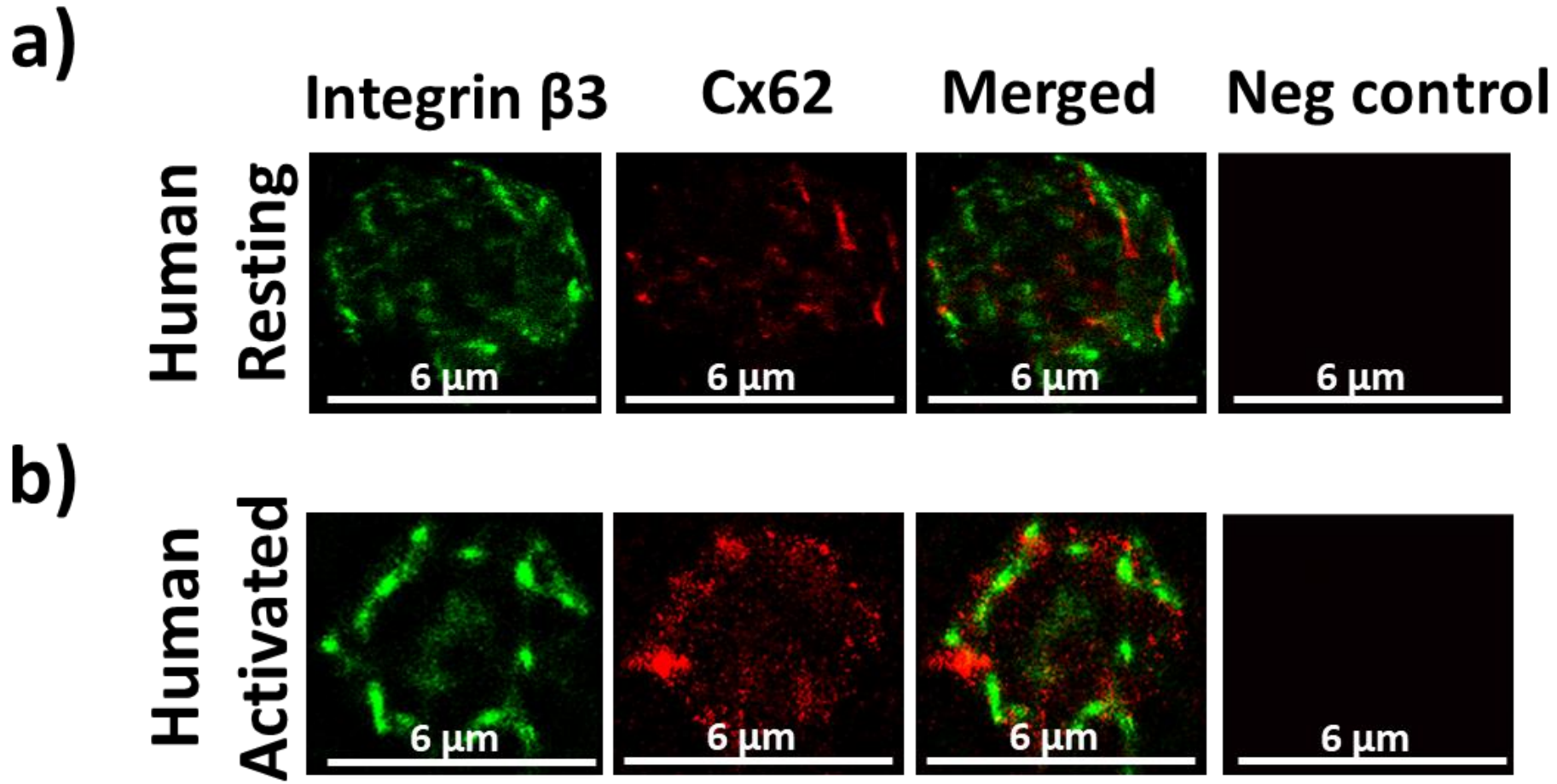


Figure 3.3: Distribution of Cx62 in resting and activated platelets as assessed by STORM microscopy.

Resting **(a)** and activated **(b)** platelets visualised by STORM. Resting and thrombin (1 U/mL)-activated platelets were fixed in 2% (v/v) formyl saline and permeabilised with BD Phosflow™ Perm Buffer III. Secondary antibodies conjugated to Alexa Fluor® 555 (green) and Alexa Fluor® 647 (red) were used to detect the β_3- and Cx62-probed platelets, respectively. A Nikon N-STORM, NIS-Elements and a 100× oil immersion lens were used for visualisation. Data are representative of >3 separate experiments.

3.5 Distribution of Cx62 in Platelet Subcellular Fractions

After determining the subcellular localisation of Cx62 and observing that it is transported to the membrane after activation, the potential link between the cytoplasmic pool of Cx62 in resting platelets and the various subcellular fractions was explored. Nitrogen cavitation was used to obtain platelet lysates that were then fractionated via sucrose density gradient ultracentrifugation. This resulted in the separation of the organelles and granular compartments of the platelets from the membranes and cytosol (Niessen et al., 2007; Crescente et al., 2016).

Washed human platelets (12×10^8 cells/mL) were subjected to homogenisation by nitrogen cavitation in a cell disruption vessel (Section 2.2.5). Homogenised platelets were then introduced to the top of the sucrose density gradient (35%–60%) followed by centrifugation for 2 hours at 200,000 g at 10 °C. Thirteen subcellular fractions (1 mL each), ranging from low to high density, were obtained from the platelets. The fractions were subjected to SDS-PAGE, then immunoblotted for the detection of Cx62. The low-density fractions (1–7) are known to contain surface proteins and other membrane structures, like cytosol-derived proteins and components of the DTS. Conversely, high-density fractions (8–13) are known to contain heavy cellular compartments such as granules and intact organelles.

The distribution of these molecules was verified based on the localisation of marker proteins in the fractionation blots. β_3 integrin, a surface protein, was principally found in the low-density fractions. However, it was also detected in the high-density fractions, consistent with the presence of integrin β_3-bearing α-granules. The cytosolic marker Rab GDP dissociation inhibitor (RabGDI) and the DTS-resident protein calreticulin were restricted to the low-density fractions (1, 2 and 3). Thrombospondin -1 (TSP-1),

which marks α-granules, was mostly found in the high-density fractions. Surprisingly, it was also found in the low-density fractions, likely because mechanical stresses exerted during nitrogen cavitation resulted in the dissociation of the granules and consequent discharge of their contents into the low-density fractions, which has been reported by Crescente et al. (2016) (Figure 3.4).

Cx62 was isolated in the low-density fractions along with membrane proteins, in accordance with the immunofluorescence findings. Intriguingly, the localisation of Cx62 was similar to that of the DTS marker calreticulin. Moreover, the high-density fractions that contain granules did not contain Cx62, indicating that the punctate organisation of Cx62 observed by immunofluorescence imaging is unlikely to be associated with granules (Figure 3.4).

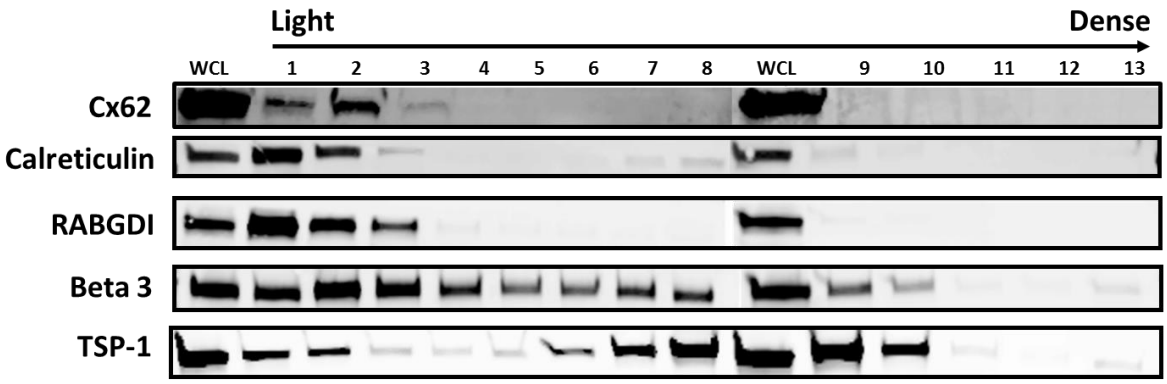


Figure 3.4: Cx62 is distributed in low-density subcellular platelet fractions.

Ultracentrifugation was utilised to separate platelet homogenates on a sucrose density gradient. The fractions were separated by SDS-PAGE and immunoblotted for Cx62, β_3 integrin, calreticulin, RabGDIb and TSP-1. The lower-density platelet fractions (lanes 1–6) are identified by the surface marker integrin β_3, the DTS protein calreticulin and the cytosolic marker RabGDIb. The α-granule protein TSP-1 was used to identify the heavier fractions. A Typhoon™ FLA 9500 fluorimager was utilised to examine the immunoblots (GE Healthcare, UK). The results are representative of 3 individual experiments.

3.6 Deletion of Cx37 or Cx40 Does Not Alter the Expression of Cx57 in Platelets

The deletion of a gene from any cell may result in altered expression of other protein in that cell. This phenomenon has been noted for some Cx proteins (Nelles et al., 1996; Nagy et al., 2003; Simon and McWhorter, 2003). It has been reported that the deletion Cx37 or Cx40 does not affect the expression of Cx40 or Cx37, respectively, in platelets (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). In this study, the potential influence of the ablation of Cx57 on the expression Cx37 and Cx40 was investigated. This is important because compensatory effects would limit the understanding gained from experimental work with $Cx57^{-/-}$ mice and its relevance to Cx62 in human platelets. Furthermore, the effect of the deletion of Cx37 or Cx40 on the expression of Cx57 was evaluated.

In addition, it is possible that Cx57 could play a previously unrecognised role in Cx37-deficient ($Cx37^{-/-}$) and Cx40-deficient ($Cx40^{-/-}$) platelet functions. To address these issues, the expression levels of Cx57, Cx37 and Cx40 were evaluated in the platelets of wild type, $Cx57^{-/-}$, $Cx37^{-/-}$ and $Cx40^{-/-}$ mice by immunoblotting (Figure 3.5).

After SDS-PAGE to separate proteins from the murine platelet lysates (wild type, $Cx57^{-/-}$, $Cx37^{-/-}$ and $Cx40^{-/-}$ samples), the membranes were incubated with antibodies against Cx57, Cx37 and Cx40. Fluorescently labelled secondary antibodies were used to detect specific staining and the membranes were visualised with a Typhoon™ FLA 9500 fluorimager. As shown in Figure 3.5a and b, Cx57 was not detected in the $Cx57^{-/-}$ platelet lysates. This is also confirming the specificity of the Cx62/57 antibody. Similarly, Cx37 (Figure 3.5c) and Cx40 (Figure 3.5d) were not detected in their respective gene-deficient mouse platelets. However, the levels of Cx57 expression were comparable in wild type, $Cx37^{-/-}$ (Figure 3.5c) and $Cx40^{-/-}$ (Figure 3.5d) platelets. In

addition, similar levels of Cx37 (Figure 3.5a) and Cx40 (Figure 3.5b) were observed in platelets from wild type and $Cx57^{-/-}$ mice. Thus, the ablation of Cx57 does not affect the expression of Cx37 or Cx40 in platelets, indicating an independent mode of Cx expression (Figure 3.5).

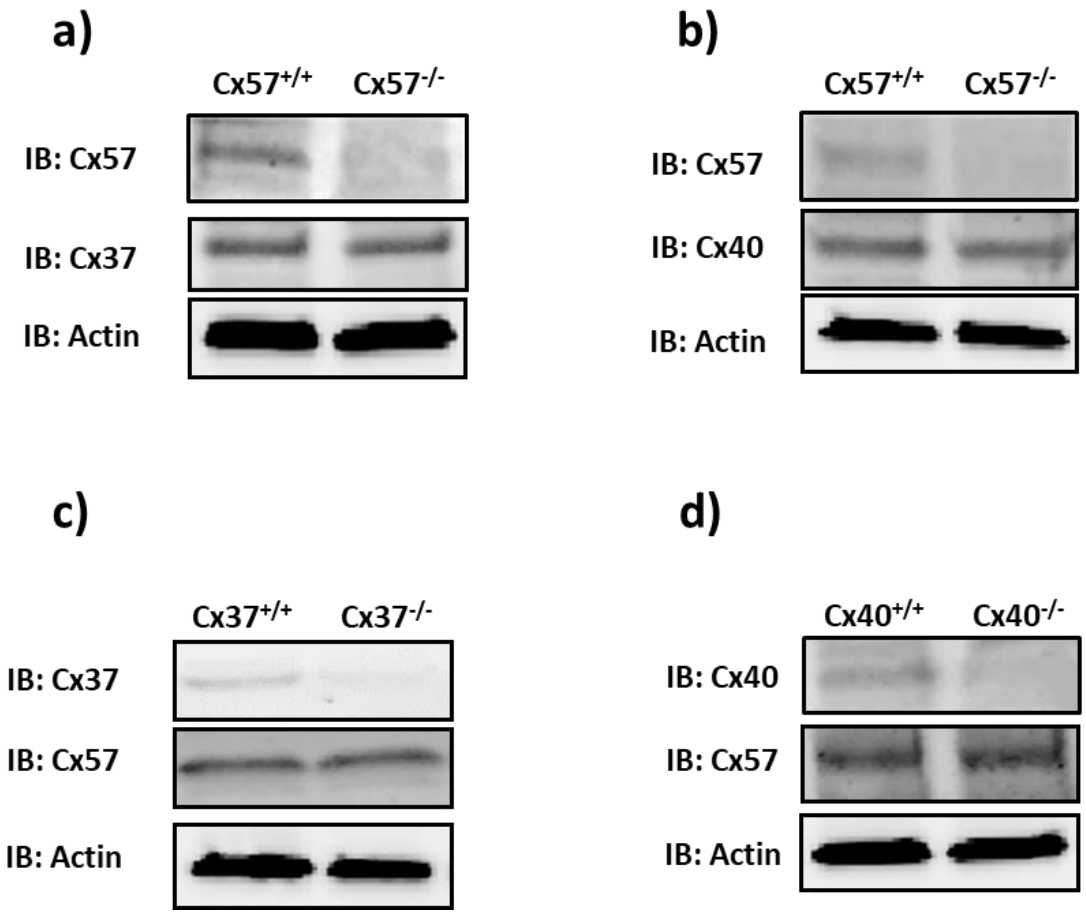


Figure 3.5: Characterisation of Cx57$^{-/-}$, Cx37$^{-/-}$and Cx40$^{-/-}$ platelets confirmed the independent expression of the Cxs in platelets.

Wild type (Cx57$^{+/+}$) and connexin 57-deficient (Cx57$^{-/-}$) platelets were used to analyse the expression of **(a)** Cx37 and **(b)** Cx40 by immunoblotting. The Cx57 expression level was assessed in **(c)** wild type (Cx37$^{+/+}$) and Cx37-deficient (Cx37$^{-/-}$) and **(d)** wild type (Cx40$^{+/+}$) Cx40-deficient (Cx40$^{-/-}$ platelets. Actin was detected by immunoblotting as a loading control. A Typhoon™ FLA 9500 fluorimager was used to image the immunoblots (GE Healthcare, UK). The results are representative of 3 individual experiments.

3.7 Design of the Cx62 Mimetic Peptide: ^{62}Gap27

There are few chemical inhibitors available for the study of Cx-mediated intercellular communications (Salameh and Dhein, 2005; Bodendiek and Raman, 2010; Verselis and Srinivas, 2013). For instance, carbenoxolone is a potent Cx function blocker (Spray et al., 2002). However, beside targeting Cxs, it blocks several other channels, such as pannexins, which limits its use substantially (Vaiyapuri et al., 2012; Taylor et al., 2014). Therefore, the inhibition of Cx-mediated intercellular communication is generally accomplished through short, 'designer' Cx mimetic peptides that rapidly and reversibly prevent Cx channel function. For example, Gap26 and Gap27 target the first and second extracellular loops of Cxs, respectively, and have been used to study the roles of Cx37, Cx43 and Cx40. Initially, mimetic peptides (Gap26 and Gap27) were used to inhibit gap junctional coupling in various mammalian cells and tissues (Warner et al., 1995; Kwak and Jongsma, 1999; Ujiie et al., 2003; Martin et al., 2005; Young et al., 2008; Billaud et al., 2011). The mimetic peptides bind to Cx hemichannels, thereby lowering their electrical conductivity and potently hindering the entry of small dyes and the discharge of signalling molecules.

Having established the presence of Cx62 in human platelets, we further explored its function in human platelets. Due to the lack of an existing Cx62 targeting peptide, a mimetic peptide (^{62}Gap27) that targets the second external loop of Cx62 was designed. The second external loop of most of the Cxs have been targeted extensively while designing Gap27 mimetic peptides and this concept was utilised for the development of ^{62}Gap27 (Evans et al., 2012). For example, the mimetic peptides ^{40}Gap27 and ^{37,43}Gap27 target the second external loops of Cx40 and Cx37, respectively, in platelets; they are associated with down-regulation of a range of platelet functions (Vaiyapuri et

al., 2012; Vaiyapuri et al., 2013). Their specificities have been confirmed using knockout mouse platelets (Vaiyapuri et al., 2013).

The Cx sequences were accessed in the PubMed database. Thereafter, UniProt (http://www.uniprot.org/uniprot/Q969M2) was used to examine the structure of Cx62, including the positions and lengths of all topological and transmembrane domains, which aided in the identification of the second external loop. ClustalW (www.ebi.ac.uk/tools/clustalw) was used for multiple sequence alignments of the human Cx sequences (Appendix 8.1). To avoid false positive data, a negative control scrambled peptide was designed using Mimotopes (http://www.mimotopes.com/peptideLibraryScreening.asp), a web-based tool.

Basic Local Alignment Search Tool (BLAST) was used to ensure that the sequences are not present in proteins other than Cx62. Similarly, this test was applied to the scrambled peptide. As the sequence of human Cx62 is homologous to Cx57 in mouse (Morel, 2014), the sequence of the Cx62 mimetic peptide targets the second external loop of Cx62 and Cx57 (Appendix 8.2). The sequences of the Cx62 mimetic peptide and the scrambled peptide are shown in Figure 3.6.

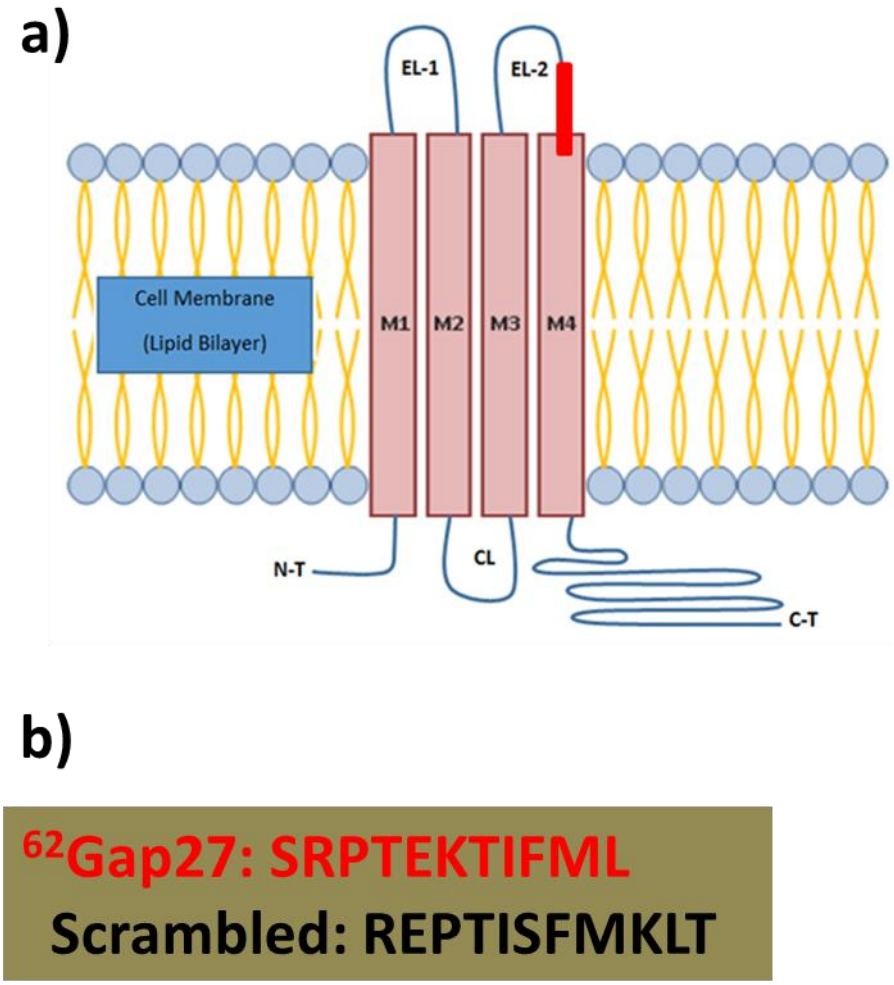


Figure 3.6: The design of ^{62}Gap27 and the targeted location within Cx62.

(a) Cxs are proteins with their N-termini (NT) and C-termini (CT) in the cytoplasm; they also contain one intracellular loop, two extracellular loops (EL-1 and EL-2) and four transmembrane domains (M1-M4). **(b)** The location of ^{62}Gap27, which targets the second external loop of Cx62 (in red), along with the sequence of the scrambled control peptide (in black).

3.8 The actions of ^{62}Gap27 on Platelets

^{62}Gap27 was investigated for its potential mode of actions in platelets by evaluating its effects on the permeability of hemichannels and GJs that contribute to intracellular and intercellular communication in platelets.

3.8.1 ^{62}Gap27 Inhibits the Permeability of Hemichannels

The varied tasks of many tissues and cells are achieved through coordinated cell interactions. Cell-to-cell interactions via GJs organise cell functions, including intercellular propagation of calcium waves and the spread of electric potentials. Currently, researchers speculate that undocked hemichannels may permit the diffusional exchange of ions and small molecules between intra- and extracellular spaces (Patel et al., 2014). This concept is supported by the fact that hemichannel opening permits the release of small signalling molecules (e.g. cyclic nucleotides, ATP, nicotinamide adenine dinucleotide [NAD^+], glutamate and adenosine) and the influx of metabolically relevant molecules (e.g. glucose) (Patel et al., 2014; Saez and Leybaert, 2014). This role of hemichannels is evident in the homeostatic disparities in various diseases; cell death in numerous pathological conditions is modulated by enhanced hemichannel opening. Cellular damage in a number of animal models of human diseases can be reduced by hemichannel blockade using Gap27 (Retamal and Saez, 2014; Saez and Leybaert, 2014). The effect of the ^{62}Gap27 inhibitor peptide was tested on hemichannel permeability.

Flow cytometry was performed to investigate the efflux of calcein (anionic, 0.62-kDa fluorescent dye) from calcein-loaded platelet cytosol. Calcein-loaded PRP was treated with scrambled control peptide (100 µg/mL) or ^{62}Gap27 (100 µg/mL) for 5 minutes prior to stimulation with thrombin. The reaction was stopped with 0.2% (v/v) formyl

saline and the samples were analysed using flow cytometry. The median fluorescence intensities of the platelet samples were recorded and compared with the unstimulated sample. Upon stimulation with thrombin (0.1 U/mL), calcein-associated fluorescence was approximately 50% lower in the scrambled peptide-treated cells in comparison to the non-stimulated cells, indicating release of dye. However, treatment with ^{62}Gap27 prevented this stimulation-induced loss of fluorescence (Figure 3.7). This finding suggests that Cx62 hemichannels regulate platelet permeability and the trafficking of molecules across the plasma membrane (PM).

At the same concentration of thrombin (0.1 U/mL), ^{62}Gap27 was unable to inhibit P-selectin exposure on the platelet surface (a marker of α-granule secretion and platelet function). This suggests that the effects of ^{62}Gap27 observed on the permeability of hemichannels were not simply due to a reduction in the activity of platelets.

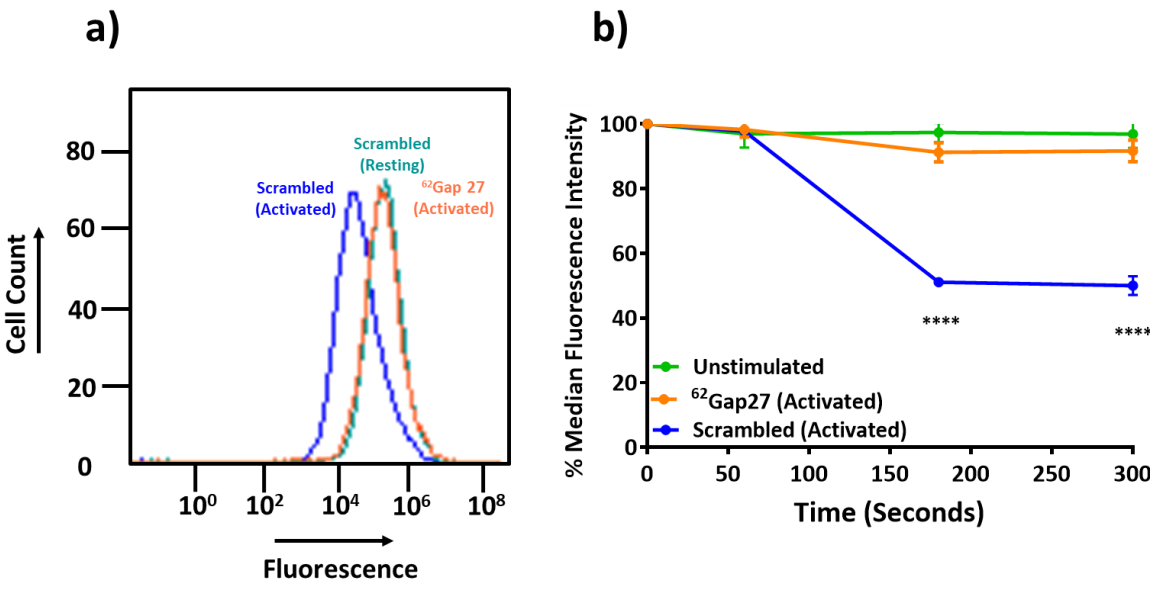


Figure 3.7: Thrombin-induced efflux of the anionic dye calcein through hemichannels is blocked by ^{62}Gap27.

The efflux of calcein was calculated using flow cytometric analysis. Calcein-loaded platelets incubated with ^{62}Gap27 or scrambled peptide (100 µg/mL) were stimulated with thrombin (0.1 U/mL). **(a)** Histograms of calcein fluorescence for unstimulated platelets (green) and thrombin-stimulated platelets in the presence of scrambled peptide (blue) or ^{62}Gap27 (100 µg/mL) (orange). **(b)** Calcein efflux following thrombin stimulation for varying time periods as indicated. Median fluorescence intensity was analysed for unstimulated and stimulated samples treated with scrambled peptide or ^{62}Gap27. Data represent Mean ± SEM (n = 6). ****$P \leq 0.0001$ was calculated by two-way ANOVA.

3.8.2 ^{62}Gap27 Blocks Intercellular Communication through platelet GJs

Cell–cell communication is a major aspect of developmental biology, which may be possible to harness for the engineering or repair of tissues. Intercellular chemical and mechanical signals comprise cell-to-cell communication, which are important for tissue homeostasis. GJs permit the exchange of small metabolites, ions and second messengers. GJIC, or direct signalling, occurs through GJ channels and is particularly important for coordinating the activities of cells. Cx-mediated GJIC plays essential, varied roles in diverse tissues and cells, such as cell growth, differentiation and apoptosis. In cardiac muscle, GJs facilitate the transmission of an action potential from the site of a cardiac pacemaker to surrounding cells, thereby mediating heart contraction (Rohr, 2004). Alterations to GJ channels by Gap27 change their properties; they lower their electrical conductivity and block the entry of small reporter dyes and the discharge of small molecules, like ATP (Evans and Boitano, 2001; Evans et al., 2006; Yeager and Harris, 2007; Dobrowolski and Willecke, 2009). Therefore, the ability of ^{62}Gap27 to modulate GJIC between platelets was investigated.

Fluorescence recovery after photobleaching (FRAP) experiment was performed to examine direct intercellular communication between platelets in calcein-loaded platelets that were allowed to spread on fibrinogen and collagen. Prior to photobleaching, platelets were permitted to spread for 45 minutes, then incubated with scrambled control peptide (100 µg/mL) or ^{62}Gap27 (100 µg/mL) for 500 seconds.

The level of fluorescence intensity was examined in the region of interest (ROI) (diameter, 8 µm) as described in Chapter 2 (Section 2.2.7). It was observed that the fluorescence intensity of scrambled peptide-treated platelet aggregates recovered by 17% (in 500 seconds) in comparison to the pre-bleached value (Figure 3.8a) However,

the recovery was restricted to only 8% in the ^{62}Gap27-treated samples (Figure 3.8b). These results suggest the involvement of Cx62 GJ-mediated intercellular communication between the platelets in a thrombus.

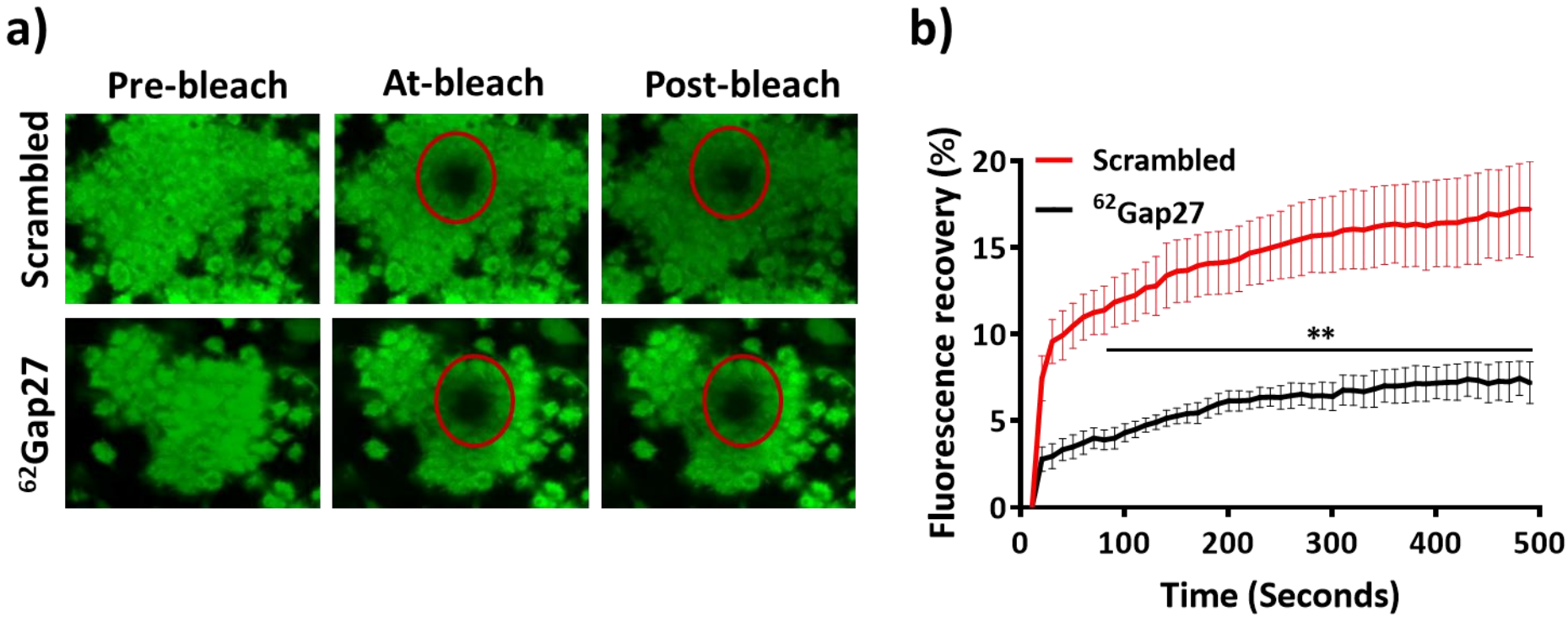


Figure 3.8: ^{62}Gap27 inhibits intercellular communication through GJs.

Calcein-loaded platelets were treated with scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes prior to their stimulation on fibrinogen- and collagen-coated coverslips for 45 minutes and FRAP analysis was performed. The samples were excited at 488 nm with an argon laser and emission was detected at 500–520 nm. A Nikon A1R confocal microscope (100× objective) was used to analyse the fluorescence recovery. **(a)** Representative images show fluorescence recovery (Pre-bleach, At-bleach and Post-bleach) in samples treated with scrambled or ^{62}Gap27. **(b)** Quantified data shows mean fluorescence recovery intensity of scrambled and ^{62}Gap27-treated samples, which were normalised to the level of fluorescence at bleach point (shown in red circle). Data represent Mean ± SEM (n = 7). $^{**}P \leq 0.01$ was calculated by two-way ANOVA.

3.9 Discussion

Platelets circulate as independent discoid bodies in the intact vasculature. Using integrin $\alpha_{IIb}\beta_3$ and the adhesion proteins fibrinogen and VWF (Gibbins, 2004), platelets aggregate after injury to form a thrombus. As suggested by Prevost et al. (2005), sustained signalling between the aggregated platelets stabilises the thrombus. Once thrombus formation is complete, the synchronised process of clot contraction occurs, including thrombus retraction via platelet integrin $\alpha_{IIb}\beta_3$ and the binding of the ends of the wound (Calderwood, 2004).

Cxs comprise a family of channel-forming proteins that are distributed in several cell types. A hemichannel is formed by oligomerisation of six Cx monomers in the endoplasmic reticulum, which is later conveyed to the PM. Another observation revealed that communication can occur by hemichannels alone in isolated cells (Retamal and Saez, 2014). A GJ is formed by the docking of hemichannels on neighbouring attached cells (size of the pore, ~2–3 nm); which allows the direct flow of signalling molecules (up to 1 kDa). The intercellular communication mediated by GJs facilitates the synchronisation of cellular responses. The roles of various Cxs, especially Cx37, Cx40 and Cx43, are well characterised in the vasculature. More recently, researchers have demonstrated the expression of Cxs in circulating blood cells, such as platelets, neutrophils, monocytes and lymphocytes (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Pfenniger et al., 2013; Vaiyapuri et al., 2013; Glass et al., 2015). This suggests a wider-ranging role for these proteins and intercellular communication than previously appreciated.

3.9.1 Cx62 is Expressed by Platelets and MKs

An ultrastructural analysis of the bone marrow along with bone marrow cultures showed that Cxs are expressed in and form GJs between reticular cells and haematopoietic cells, suggesting that leukocytes and platelets could express Cxs (Campbell, 1980; Allen and Dexter, 1982). The possibility that Cxs play important roles in platelet aggregation was supported by the finding that Cxs mediate important intercellular communication and adhesion functions in inflammatory and epithelial cells (Kameritsch et al., 2012; Naus and Giaume, 2016). The expression of Cxs in platelets was confirmed by transcriptomic analysis of MKs, which revealed the expression of mRNAs for 16 Cxs, amongst which Cx37, Cx40 and Cx62 were the most highly expressed; Cx37 and Cx40 protein were also found to be in human platelets (Vaiyapuri et al., 2012). However, the lack of a specific Cx62 detection antibody prevented the analysis of its expression in platelets. The expression of Cx62 and Cx57 in human and murine platelets and MKs was confirmed, which supports a megakaryocytic origin for human Cx62.

3.9.2 Subcellular Distribution of Cx62 in Human Platelets

The formation of GJs between aggregated platelets has been reported in a number of studies. It is important to note that a number of methods and techniques have been employed to track the passage of various fluorescent dyes through aggregated platelets (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012). The role of Cxs in platelet function is supported by the observation that inhibition or deletion of Cx37 hinders dye diffusion within a platelet thrombus (Angelillo-Scherrer et al., 2011).

Cx37 and Cx40 were the only Cxs known to be expressed and function in platelets. This study shed light on the previously unknown role of Cx62 in platelets and

compared its function to those of Cx37 and Cx40 by analysing Cx62 and Cx57 in human and murine platelets, respectively. The scattered, punctate subcellular localisation of Cx62 in platelets was revealed by confocal microscopy. In resting platelets, minimal immunofluorescent Cx62 staining was observed at the platelet surface and the majority of Cx62 appeared to be distributed throughout the cytoplasm in a punctate arrangement. However, platelet activation tended to mobilise a large proportion of Cx62 toward the platelet surface, suggesting the creation of functional GJs and a possible contribution to platelet activation.

To better interpret the pattern of Cx62 staining, super-resolution microscopy was used in resting and stimulated platelets. Undocked hemichannels have been reported on the cell surface (Laird, 2006; Patel et al., 2014; Saez and Leybaert, 2014), which is consistent with the minimal surface distribution of Cxs in resting platelets and may reflect a minor pool of Cx62 hemichannels that facilitate the release or uptake of various mediators at the platelet surface. On the other hand, the reports of GJs in platelet aggregates explain the peripheral mobilisation of Cx62 following platelet stimulation and suggest that Cx62 GJs participate in platelet activation.

3.9.3 Cx62 Co-fractionates with Low-density Fractions of Platelet Proteins

Synthesis of Cxs takes place in the endoplasmic reticulum, as for standard transmembrane proteins (Falk et al., 1994). The structures of the proteins change so that they are similar to their native transmembrane oligomerised arrangement (Falk and Gilula, 1998) before they are conveyed to the PM by the Golgi apparatus (Musil and Goodenough, 1993; Laird et al., 1995; Koval et al., 1997; Thomas et al., 2004). In fact, anucleated platelets do not have all distinct organelles present in nucleated cells, but they include the membranous dense tubular system (DTS), which is considered the

remnant of the smooth endoplasmic reticulum (White, 1972). Our analysis of the subcellular fractions demonstrated that Cx62 was present exclusively where the DTS and several other membrane systems are present in the lighter fractions, but not in the high-density fractions that contain granules. Cx62 has an intriguingly similar distribution pattern to calreticulin, which is used to label the DTS compartment amongst the platelet fractions (van Nispen Tot Pannerden et al., 2009). Comparable localisation of Cxs to the endoplasmic reticulum in nucleated cells is also supported by this observation (Falk et al., 1994) and emphasises that the punctate staining for Cx62 in the platelet may be due to its DTS localisation. Furthermore, it has been suggested that Cxs are chaperoned by protein disulphide-isomerases, as the extracellular loops of Cxs that are exposed in the endoplasmic reticulum form disulphide bonds (John and Revel, 1991). Protein disulphide-isomerases are also thought to localise to the platelet DTS and mobilised in activated platelets, which was dependent on actin polymerisation and independent of membrane fusion (Crescente et al., 2016). Taken together, these findings suggest that localisation of Cx62 to the DTS, rather than the platelet cytosol, accounts for its distribution in the low-density fractions and the surface-linked pools.

3.9.4 Ablation of Cx57 has no Effect on the Expression of Cx37 and Cx40 in Platelets

At the site of vessel damage, where platelets adhere, crosslink and aggregate to form a thrombus, the platelets come into close proximity with each other. A number of receptors and adhesion proteins on the platelet surface and several soluble mediators support and facilitate platelet function (Angelillo-Scherrer et al., 2011). Different possibilities exist that might explain the potential role or functions of connexons and GJs in platelet function, based on the nature of GJ-facilitated intercellular communication in different cells and tissues, such as oocytes (Kidder and Mhawi,

2002), nerve cells (Bruzzone and Dermietzel, 2006), bone marrow stromal cells (Dorshkind et al., 1993) and cardiac muscle (Rohr, 2004).

Several studies confirmed the expression of Cx40 and Cx37 in MKs and platelets and investigated their possible roles in controlling platelet functions by utilising Cx deletion and inhibition (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Similarly, the priority in this study was to analyse the effects of Cx62 inhibition and deletion on platelets.

The ablation of the expression of a target Cx can alter the expression of other Cxs, suggesting a possible interdependency amongst the isoforms of Cx proteins. Atrial myocytes in which Cx43 has been ablated have lower immunofluorescence signals for Cx45 and Cx40 than non-ablated controls (Desplantez et al., 2012a). Moreover, in the liver, inhibition of Cx32 was accompanied by lower expression of Cx26 (Nelles et al., 1996). Cx ablation commonly results in lower expression of non-ablated Cxs (Simon and McWhorter, 2003). However, overexpression is also possible; Cx32-deficient mouse brain tissue has higher Cx43 and Cx47 expression than wild type brain tissue (Nagy et al., 2003). No compensatory or ablative effects on the expression of the abundantly expressed Cx37 or Cx40 were observed in $Cx57^{-/-}$ platelets. The results of this study are consistent with those in $Cx40^{-/-}$ platelets, in which no compensatory effects on the expression of Cx37 were observed (Vaiyapuri et al., 2013). Similarly, no compensation in the level of Cx40 was reported by the same group following the ablation of $Cx37^{-/-}$ (Vaiyapuri et al., 2012).

3.9.5 Design of ^{62}Gap27 Mimetic Peptide

Following the confirmation of the expression of Cx62 in platelets, a mimetic peptide that targets the second external loop of Cx62 was designed using ClustalW

(www.ebi.ac.uk/tools/clustalw). This mimetic peptide also targets the second external loop of Cx57 in mice. In addition, a scrambled peptide was developed as a control which help to avoid false positive results. Both of the peptides are absent from other protein sequences and the specificity of ^{62}Gap27 for Cx62 (human) and Cx57 (mouse) was confirmed using BLAST.

3.9.6 ^{62}Gap27 Modulates Hemichannel and GJ Functions

Gap27 peptides designed against different Cxs have been shown bind to unopposed hemichannels and alter their functions, such that molecules like glutamate (e.g. Cx43 in astroglia) or ATP (e.g. Cx43 in leukocytes) are not released (Eltzschig et al., 2006; Orellana et al., 2011; Retamal and Saez, 2014; Saez and Leybaert, 2014). Upon longer incubation times, the peptides inhibit gap junctional communication, possibly by interfering with the docking of apposed hemichannels to create functional GJ channels (Evans and Boitano, 2001; Leybaert et al., 2003). Broadly speaking, hemichannel activity, GJ formation and gap junctional conductance in cells or tissues can be influenced by mimetic peptides; it has been suggested that the peptide Gap27 may have specific effects on Cx43 as opposed to Gap26, which may affect an array of Cxs (Wright et al., 2009).

The findings suggest that single hemichannels that have not formed GJs or GJs formed to release molecules are reduced by ^{62}Gap27 as observed in Figure 3.7 and Figure 3.8. The release of molecules can be limited by ^{62}Gap27, which binds to the external domains of the channels, resulting in closure. Moreover, intercellular coupling communication is impaired by the formation of Cx62 hemichannel–^{62}Gap27 peptide complexes which move crosswise in the PM and cause the close of GJs. Closure can also be mediated by ^{62}Gap27 peptides that diffuse into intercellular spaces and bind

Cx62 hemichannels and GJs. In the sub-plasma membrane, Cx channel gating may be controlled indirectly by changes in Ca^{2+}. ^{62}Gap27 may also affect Ca^{2+} entry into cells, thereby causing hemichannel and GJ closing. In addition, researchers have speculated that Gap27 and Gap26 may diffuse into pre-existing GJ plaques and change their channel conductances without disrupting their structures (Martin et al., 2005).

The findings presented in this chapter demonstrate the expression and localization of Cx62 and Cx57 in human and mouse platelets, respectively. Cx62 traffics towards the plasma membrane upon stimulation, suggesting that it plays a role in inter-cellular communication through the formation of hemichannels and GJs. Moreover, expression of Cx62 is not altered by deletion of Cx37 and Cx40 genes in platelets. A novel mimetic peptide targeting the second external loop of Cx62/57 (^{62}Gap27) was successfully designed. It regulated the permeability of the Cx62 hemichannel and inter-cellular communication via GJs. The next chapter will investigate the effects of ^{62}Gap27 on platelet function and the selectivity of ^{62}Gap27.

4 ^{62}Gap27 Negatively Regulates Platelet Function and Acts Selectively Via Cx62

4.1 Introduction

Connexins (Cxs) can be formed on the surface of platelets where they participate in cell–cell adhesion and support the activity of adhesive receptors such as ephrins and Eph kinases. However, the functions of gap junctions (GJs) in the regulation of platelet adhesion requires detailed research (Brass et al., 2004; Vaiyapuri et al., 2015a; Vaiyapuri et al., 2015b)

In the 1980s, research on the formation of GJs between stromal cells and haematopoietic cells in the bone marrow and cell cultures suggested that Cxs may be expressed on the surfaces of leukocytes and platelets (Campbell, 1980; Allen and Dexter, 1982; Campbell, 1986). In more recent studies by our laboratory, the mRNA transcripts of 16 Cxs were detected in MKs, amongst which Cx37, Cx40 and Cx62 were the most highly expressed (Vaiyapuri et al., 2012). The expression of two Cx proteins, notably Cx37 and Cx40, has been confirmed in platelet (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Moreover, GJ formation within thrombi has been demonstrated using techniques that permit the tracking of the transfer of fluorescent dyes (neurobiotin and calcein) between platelets. Remarkably, the diffusion of these dyes is inhibited upon blocking or deletion of Cx37 (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012). Although Cx32 and Cx43 mRNA transcripts have also been detected in platelets, only studies on the functions of Cx37 and Cx40 on platelet aggregation and thrombus formation have been performed.

Our lab identified the roles of Cx37 and Cx40 hemichannels and GJs in platelets. The deletion of Cx37 was associated with reduced fibrinogen binding and granule secretion by platelets (Vaiyapuri et al., 2012). This suggests the involvement of hemichannels in the regulation of platelet functions. Similarly, blocking or deletion of Cx40 in platelets

inhibits the expression of P-selectin, a marker of α-granules, on the surface of activated platelets (Vaiyapuri et al., 2013). Haemostasis and thrombus formation *in vitro* and *in vivo* were also suppressed in samples treated with ^{37,43}Gap27 or ^{40}Gap27 (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

Blocking Cx37 with ^{37,43}Gap27 in Cx40$^{-/-}$ murine platelets reduced P-selectin exposure and fibrinogen binding in platelets stimulated with CRP-XL. Similarly, blocking Cx40 with ^{40}Gap27 in Cx37$^{-/-}$ platelets inhibited P-selectin exposure and fibrinogen binding in CRP-XL-stimulated platelets. Taken together, these data suggest that these two Cx hemichannels operate independently (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Importantly, ^{37,43}Gap27 and ^{40}Gap27 have been confirmed to selectively and specifically inhibit Cx37 and Cx40, respectively, in experiments using Cx37$^{-/-}$ and Cx40$^{-/-}$ mouse platelets (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

In contrast to these findings, Angelillo-Scherrer et al. (2011) reported that the deletion of Cx37 results in enhanced platelet aggregation in response to collagen, thrombin or ADP stimulation. This discrepancy may be due to:

(i) the concentration of the agonist used to study the function Cx37 in platelets is either too high or too low (e.g. 2μM ADP is generally not sufficient to cause platelet aggregation in many donors)

(ii) the failure of the Cx blocker such as carbenoxolone, 18β-GA and ^{37,43}Gap27 to cause platelet activation or increase platelet sensitivity (Vaiyapuri et al., 2012).

Cx62 is a comparatively recently identified member of the Cx family; its expression has not been reported in any cell types. However, Cx57, the mouse homologue to Cx62, has been identified in retina (Sohl et al., 2010). Little information is available on the role of

Cx62, in contrast to that available on the roles of the members of the Cx family (e.g. Cx37, Cx40 and Cx43) that have been investigated extensively in different cell types. Cx62 mRNA transcripts have been detected in megakaryocytes (MKs). However, an investigation of its function in platelets has been impeded by the lack of reagents to study its function. In this study, the expression of Cx62 in platelets was confirmed and the mimetic peptide ^{62}Gap27 was developed to study functions of Cx62 in platelets. The major objective of this chapter was to determine the roles of Cx62 hemichannels and GJs in platelet function.

4.2 Role of Cx62 in the Regulation of Platelet Aggregation

Platelet aggregation in the presence of ^{62}Gap27 was investigated to determine whether modulating the function of Cx62 had an impact on platelet activation. The effects of ^{62}Gap27 were evaluated on the responses induced by a range of platelet agonists that target different pathways of platelet activation, including crosslinked collagen-related peptide (CRP-XL, a selective GPVI agonist) (Knight et al., 1999); thrombin; ADP and U46619, a TxA_2 receptor agonist.

4.2.1 ^{62}Gap27 Inhibits CRP-XL-induced Platelet Aggregation

It has been previously observed that mimetic peptides targeting Cx37 and Cx40 were able to inhibit platelet aggregation stimulated by CRP-XL. To investigate the effects of ^{62}Gap27 on CRP-XL-mediated platelet aggregation, washed human platelets (4×10^8 cells/mL) were incubated with a range of concentrations of ^{62}Gap27 (25, 50 and 100 μg/mL) or scrambled peptide (100 μg/mL) for 5 minutes. These concentrations were selected based on previous studies using other Gap27 peptides (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). To maintain consistency amongst the donors, the concentration of CRP-XL used to stimulate platelets was optimized for each donor to attain 50% of the maximum level of aggregation (EC_{50}) in 3 minutes (concentrations ranged from 0.25 to 0.30 μg/mL). Aggregation responses (changes in light transmission) were recorded using an optical aggregometer with constant stirring (1,200 rpm) for 3 minutes at 37 °C.

The effects of the treatment of platelets with ^{62}Gap27 or scrambled peptide for 5 minutes prior to a 3-minute stimulation with CRP-XL are shown in Figure 4.1a. A substantial, concentration-dependent reduction in platelet aggregation was observed in 3 minutes (~42% and ~65% inhibition in samples treated with 50 and 100 μg/mL

^{62}Gap27, respectively, in comparison to scrambled peptide-treated samples; Figure 4.1b). No differences in platelet aggregation were observed between the scrambled peptide-treated and untreated samples (data not shown). These findings indicate that ^{62}Gap27 inhibits platelet aggregation downstream of GPVI stimulation.

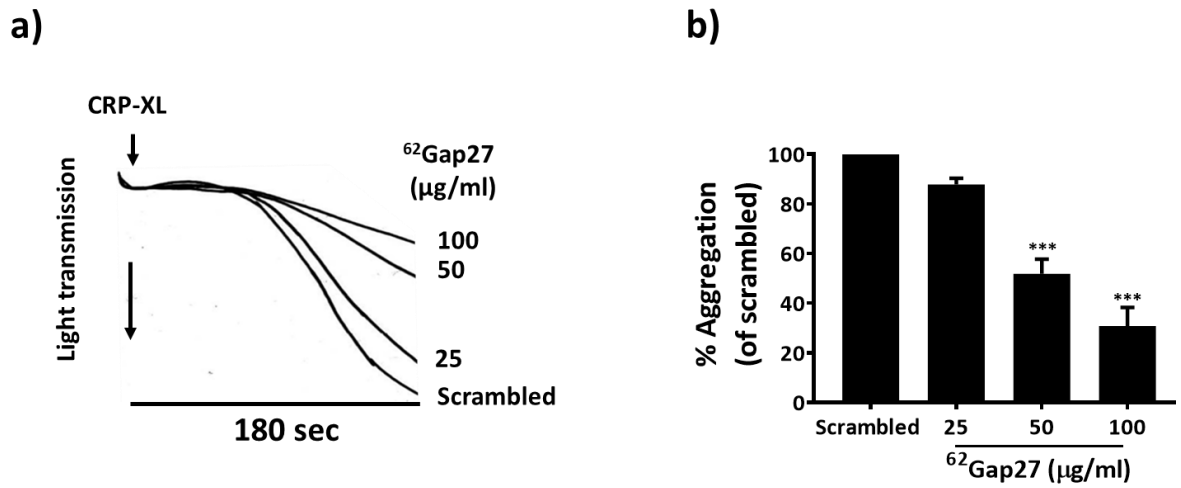


Figure 4.1: ^{62}Gap27 inhibits CRP-XL-induced platelet aggregation.

Washed human platelets (4×10^8 cells/mL) were pre-incubated for 5 minutes with ^{62}Gap27 (25, 50 and 100 μg/mL) or scrambled peptide (100 μg/mL), then stimulated with CRP-XL (EC_{50}: 0.25–0.3 μg/mL). Changes in light transmission were monitored and recorded for 180 seconds. **(a)** Representative aggregation traces. **(b)** Cumulative data from multiple donors. The percentage of aggregation was measured at 180 seconds with the aggregation of the scrambled peptide group set as 100%. Data represent the mean ± SEM ($n \geq 5$) analysed by one-way ANOVA. ***$P \leq 0.001$

4.2.2 ^{62}Gap27 Inhibits Thrombin-induced Platelet Aggregation

Thrombin is a highly potent platelet agonist that belongs to the serine protease family. PAR-1 and PAR-4 are thrombin receptors that are expressed on the surface of human platelets and belong to the class of G-protein-coupled receptors (GPCRs). In mice, PAR-1 receptor is replaced by PAR-3. PAR-1 can respond to low concentrations of thrombin, whereas PAR-4 becomes activated only at higher concentrations (Angiolillo et al., 2010; Li et al., 2010) and collectively stimulate powerful activation of platelet function. Given its inhibition of platelet aggregation downstream of GPVI stimulation, the effects of ^{62}Gap27 were investigated on thrombin stimulation to determine whether its actions are restricted to GPVI pathway or it functions through a general mechanism.

Washed human platelets (4×10^8 cells/mL) were pre-incubated with ^{62}Gap27 (25, 50 and 100 μg/mL) or scrambled peptide (100 μg/mL) for 5 minutes and stimulated with thrombin for 3 minutes. As for CRP-XL, we determined the EC_{50} concentration of thrombin over 3 minutes (concentration ranged from 0.05 to 0.07 U/mL).

Changes in light transmission were assessed for 180 seconds as a measure of aggregation (Figure 4.2a). Incubation with 50 and 100 μg/mL ^{62}Gap27 reduced significantly thrombin-stimulated platelet aggregation by approximately 35% and 60%, respectively, in comparison to treatment with the scrambled peptide (Figure 4.2b). No differences in thrombin-stimulated platelet aggregation were observed between the scrambled peptide-treated and untreated samples (data not shown). Thus, ^{62}Gap27 can influence platelet aggregation induced by the GPVI receptor agonist CRP-XL and the GPCR agonist thrombin.

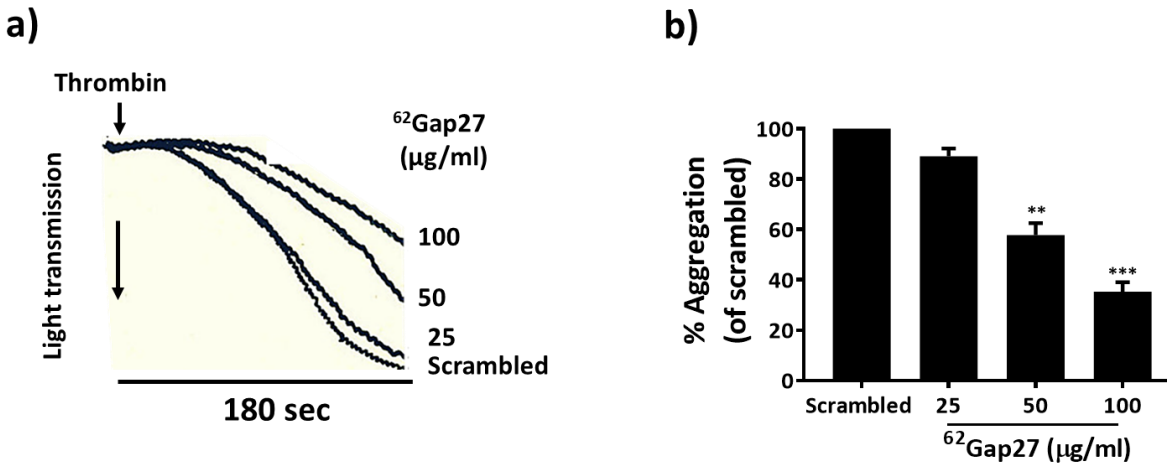


Figure 4.2: ^{62}Gap27 inhibits thrombin-induced platelet aggregation.

Washed human platelets (4×10^8 cells/mL) were pre-treated with the ^{62}Gap27 (25, 50 and 100 µg/mL) or scrambled peptide (100 µg/mL) for 5 minutes. Thrombin (EC_{50}: 0.05–0.07 U/mL) was then used to stimulate the platelets. Changes in light transmission were measured for 180 seconds to assess aggregation. **(a)** Representative aggregation traces. **(b)** Cumulative data from different donors. The percentage of aggregation obtained with the scrambled peptide treatment was set as 100%. Data represent the mean ± SEM ($n \geq 4$) analysed by one-way ANOVA. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$

4.2.3 ^{62}Gap27 Attenuates ADP- or U46619-stimulated Aggregation

Sustained platelet activation downstream of CRP-XL or thrombin relies, to an extent, on the release of prothrombotic secondary agonists (Nieswandt and Watson, 2003). Amongst the critical factors responsible for the propagation of the growing thrombus is GPCR signalling driven by the binding of the soluble ligands ADP and TxA_2 (Woulfe, 2005; Offermanns, 2006). ADP binds to its receptors $P2Y_1$ and $P2Y_{12}$, which are coupled to G_q and G_i, respectively (Hechler et al., 1998; Jin et al., 1998; Hollopeter et al., 2001). TxA_2 binds to the TP receptor, which is coupled to the G proteins G_q and $G_{\alpha 13}$ (Knezevic et al., 1993; Djellas et al., 1999; Paul et al., 1999). Given the ^{62}Gap27-mediated inhibition of thrombin stimulation, the effect of ^{62}Gap27 was examined on platelet aggregation induced by other GPCR agonists, including U46619 (TP receptor agonist) and ADP.

^{62}Gap27 impaired U46619-mediated (EC_{50} range: 0.25–0.4 μM, identified for each donor) platelet aggregation by ~50% and ~65% at concentrations of 50 and 100 μg/mL, respectively, in comparison with treatment with the scrambled peptide (Figure 4.3ai, aii). Similarly, ^{62}Gap27 negatively regulated the platelet aggregation induced by ADP (EC_{50} range: 5–10 μM, identified for each donor) by ~45% and ~70% at concentrations of 50 and 100 μg/mL, respectively (Figure 4.3bi, bii). Moreover, treatment with ^{62}Gap27 resulted in partially reversible platelet aggregation, a feature which is frequently observed when granule secretion is inhibited (Born, 1962). No differences in ADP- or U46619-stimulated platelet aggregation were observed between the scrambled peptide-treated and untreated samples (data not shown).

These findings suggest that ^{62}Gap27 negatively regulates platelet aggregation induced by a range of agonists that stimulate cell signalling through different receptors. These

findings are similar to those observed after inhibition with the mimetic peptide that targets Cx37 and Cx40, suggesting a potential common mechanism of Cx action.

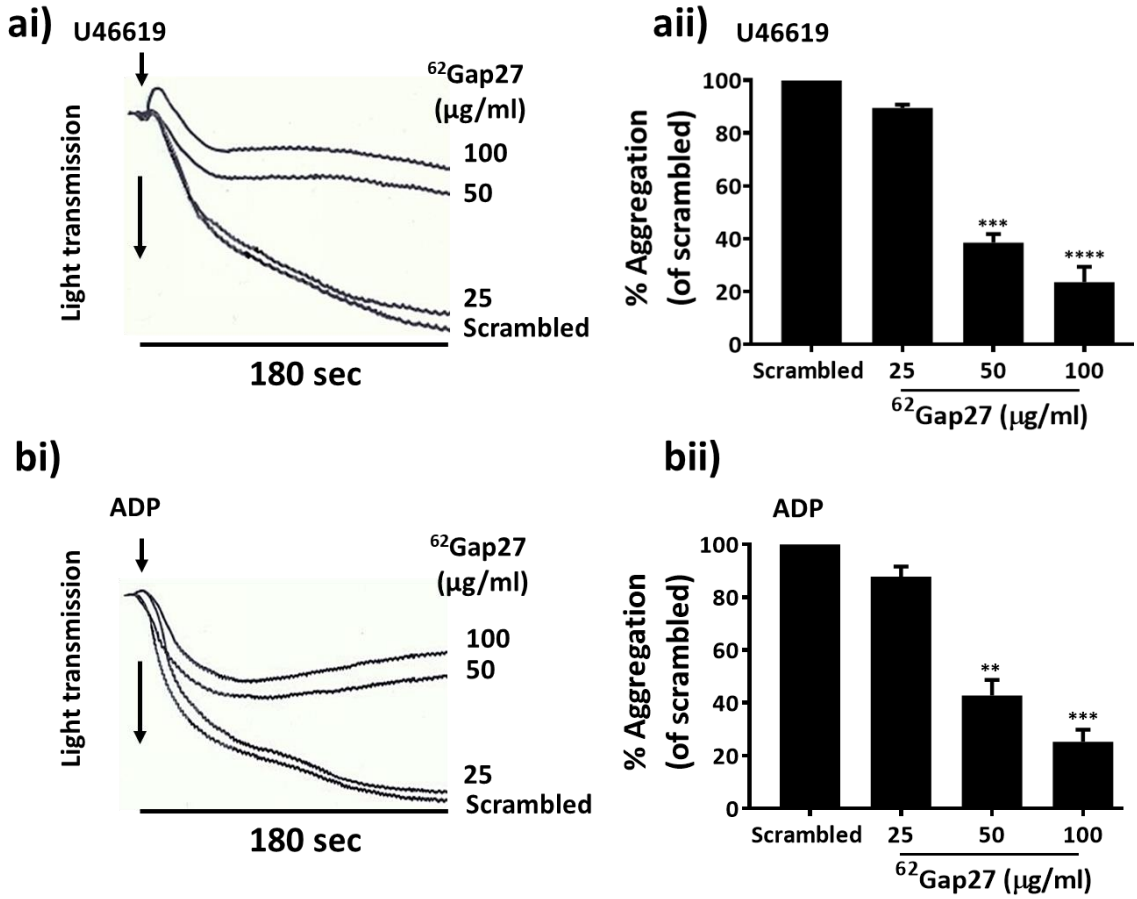


Figure 4.3: ^{62}Gap27 inhibits U46619- and ADP-mediated platelet aggregation.

Washed human platelets (4×10^8 cells/mL) were treated with scrambled peptide or ^{62}Gap27 (25, 50 and 100 µg/mL) followed by stimulation with **(a)** U46619 (EC_{50}: 0.25–0.4 µM) or **(b)** ADP (EC_{50}: 5–10 µM). The changes in light transmission were recorded for 180 seconds at 37 °C with stirring (1,200 rpm). (i) Representative traces of platelet aggregation induced by U46619 or ADP after treatment with ^{62}Gap27 are shown. (ii) The percentage of aggregation in scrambled peptide-treated samples was set as 100%. The statistical differences in the mean ± SEM ($n \geq 3$) were assessed by one-way ANOVA. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$

4.3 Integrin $\alpha_{IIb}\beta_3$ Affinity is Inhibited by ^{62}Gap27

Integrin $\alpha_{IIb}\beta_3$ plays an essential role in platelet aggregation and is necessary for thrombus formation. All platelet agonists, which act via different platelet receptors, transform integrin $\alpha_{IIb}\beta_3$ from its low-affinity state to a high-affinity state in activated platelets; this process is called inside-out signalling. This feature is vital for the formation of platelet aggregates (Shattil and Newman, 2004; Ma et al., 2007; Li et al., 2010). Since, ^{62}Gap27 negatively regulated platelet aggregation stimulated by a range of agonists, its effects on integrin $\alpha_{IIb}\beta_3$ activation were examined, an event that precedes platelet aggregation.

Fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ was studied by flow cytometry (on individual platelet populations) as a measure of the extent of integrin $\alpha_{IIb}\beta_3$ activation. Human PRP was mixed with HEPES buffer containing FITC-labelled rabbit anti-human fibrinogen antibody, which fluoresces at 519 nm following excitation at 495 nm. The samples were incubated with ^{62}Gap27 (25, 50 and 100 µg/mL) or scrambled peptide for 5 minutes. The platelets were then stimulated for 20 minutes (with occasional gentle mixing) with CRP-XL (0.25 µg/mL) or thrombin (0.05 U/mL) in the presence of the peptide Gly-Pro-Arg-Pro (GPRP 25 µg/mL) to prevent fibrin polymerisation. Samples were fixed with 0.2% (v/v) formyl saline and median fluorescence of 10,000 platelet-gated events was measured using a flow cytometer.

Treatment with 50 and 100 µg/mL ^{62}Gap27 inhibited fibrinogen binding by 35% and 55%, respectively, after stimulation with CRP-XL (0.25 µg/mL) in comparison with treatment with the scrambled peptide control (Figure 4.4a). Moreover, approximately 30% and 60% reductions in binding were caused by 50 and 100 µg/mL ^{62}Gap27, respectively, in thrombin-stimulated (0.05 U/mL) samples in comparison with the

binding in the scrambled peptide-treated controls (Figure 4.4b). No differences in CRP-XL- or thrombin-mediated fibrinogen binding were observed between the scrambled peptide-treated and untreated samples (data not shown). This inhibition of fibrinogen binding is consistent with the ability of ^{62}Gap27 to negatively regulate platelet aggregation after CRP-XL (Figure 4.1) or thrombin (Figure 4.2) stimulation.

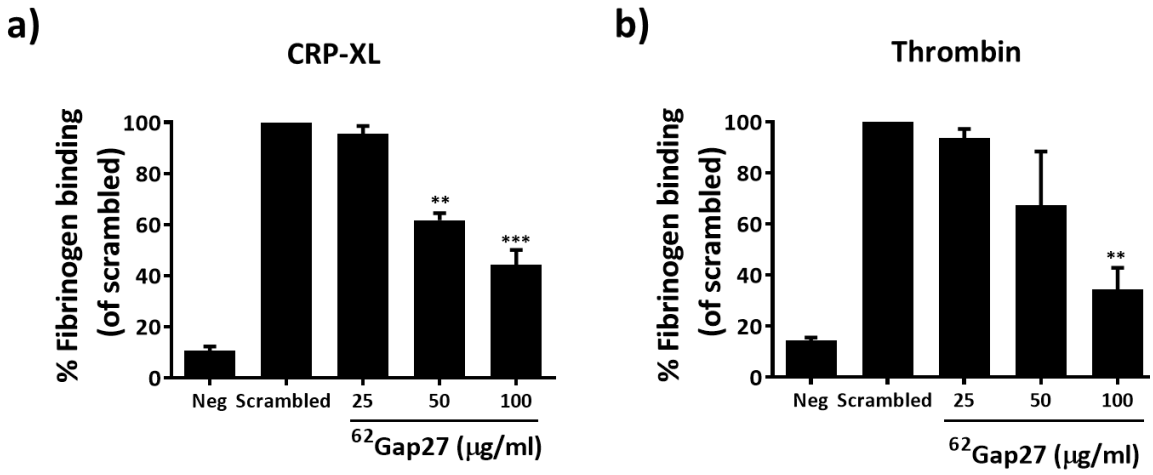


Figure 4.4: ^{62}Gap27 inhibits CRP-XL- and thrombin-mediated fibrinogen binding.

Human PRP was incubated with ^{62}Gap27 (25, 50 and 100 μg/mL) or scrambled peptide for 5 minutes, then stimulated with **(a)** CRP-XL (0.25 μg/mL) or **(b)** thrombin (0.05 U/mL) for 20 minutes and analysed by flow cytometry. Integrin $\alpha_{IIb}\beta_3$ activation was measured by the binding of anti-fibrinogen antibody to the platelets. Unstimulated platelets treated with FITC-conjugated anti-human fibrinogen antibody served as negative control (Neg). The degree of fibrinogen binding in the presence of scrambled peptide was set as 100%. Data represent the mean ± SEM ($n \geq 6$) of the median fluorescence values. Data were analysed with one-way ANOVA. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$

4.4 ^{62}Gap27 Inhibits α-Granule Secretion by Platelets

α-granules release a range of platelet-activating factors, coagulation proteins, protease inhibitors, soluble adhesion proteins and growth factors upon platelet activation. Platelet interactions with leukocytes within thrombi are supported by the exposure of P-selectin, which is released from α-granules, on the platelet surface (Koedam et al., 1992). The exposure of P-selectin, a classical marker of platelet α-granules, on the surface of platelets was measured to assess the effects of ^{62}Gap27 on α-granule secretion.

P-selectin exposure on platelet surface was measured by flow cytometry following incubation with a PE-Cy™5-labelled mouse anti-human CD62P (P-selectin) antibody, which fluoresces at 670 nm following excitation at 488 nm. Median fluorescence values of platelet samples treated with the control and inhibitory peptides were obtained to determine the potential contribution of Cx62 to the extent of P-selectin exposure.

Human PRP was incubated in HEPES buffer containing PE-Cy™5-labelled mouse anti-human CD62P antibody for 5 minutes at room temperature in the presence of the ^{62}Gap27 mimetic peptide (25, 50 and 100 μg/mL) or the scrambled peptide. Prior to analysis by flow cytometry, the platelets were stimulated for 20 minutes with CRP-XL (0.25 μg/mL) or thrombin (0.05 U/mL) in the presence of GPRP (25 μg/mL). Reactions were terminated by fixing samples with 0.2% (v/v) formyl saline and median fluorescence intensities for 10,000 platelet-gated events were recorded by a flow cytometer.

As shown in Figure 4.5a, a concentration-dependent inhibition of P-selectin exposure was observed in samples incubated with ^{62}Gap27 (~30% and ~50% inhibition by 50 and 100 μg/mL, respectively, compared to the exposure in scrambled peptide-treated

samples) following stimulation with CRP-XL. On the other hand, 50 µg/mL and 100 µg/mL ^{62}Gap27 facilitated reduction of ~25% and ~55%, respectively, in samples treated with thrombin (Figure 4.5b). No differences in CRP-XL- or thrombin-mediated P-selectin exposure were observed between the scrambled peptide-treated and untreated samples (data not shown). These outcomes were consistent with and comparable to the reduction in fibrinogen binding following platelet stimulation with CRP-XL or thrombin in the presence of ^{62}Gap27 (Figure 4.4a, b).

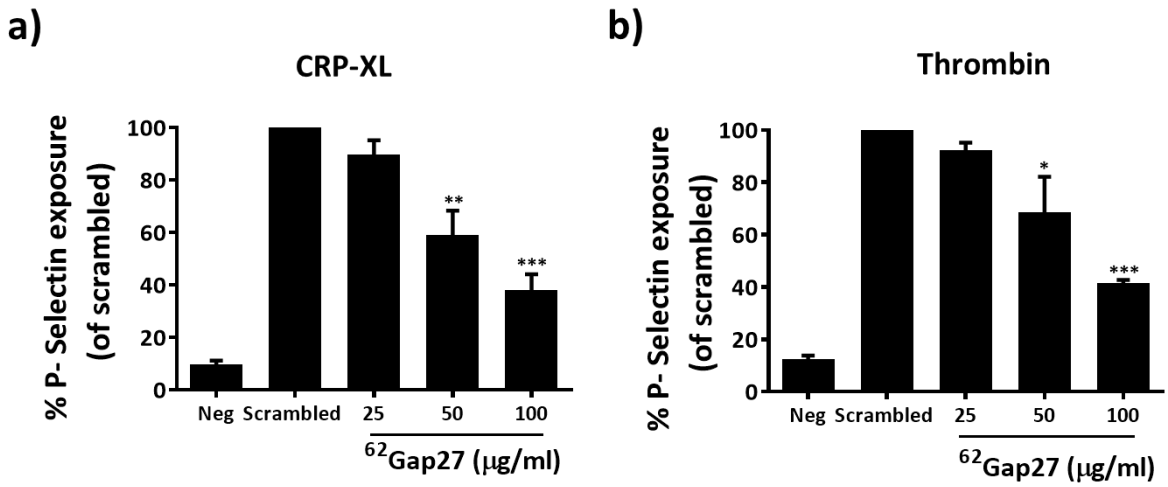


Figure 4.5: P-selectin exposure mediated by CRP-XL or thrombin is inhibited by ^{62}Gap27.

Human PRP was treated with ^{62}Gap27 (25, 50 and 100 μg/mL) or scrambled peptide, followed by stimulation with **(a)** CRP-XL (0.25 μg/mL) or **(b)** thrombin (0.05 U/mL) for 20 minutes. The α-granule secretion was then measured by flow cytometry as the level of P-selectin exposure on the platelet surface. Unstimulated platelets incubated with PE-Cy™5-labelled mouse anti-human CD62P (P-selectin) antibody served as negative control (Neg). The level of P-selectin exposure in the presence of the scrambled peptide was set as 100%. Data represent the mean ± SEM of the median fluorescence intensities ($n \geq 6$). Data were analysed by one-way ANOVA. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$

4.5 Cx62 Hemichannels Function Independently of Cx37 and Cx40 in Platelets

As Cx amino acid sequences, including the Gap27 sequence, are very similar (80%–95% identical), studying the specificity of a mimetic peptide is important. The specificities of the actions of the selective inhibitors of Cx40 (^{40}Gap27) and Cx37 (^{37,43}Gap27) have been tested previously to confirm the absence of inhibitory influence on Cx40$^{-/-}$ and Cx37$^{-/-}$ mouse platelets, respectively (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Hetero-oligomeric GJs (isoforms of two different hemichannels) can function on various cell types (He et al., 1999; Bloomfield and Volgyi, 2009). The heteromeric or homomeric associations between Cx37 and Cx40 in murine platelets have also been explored using ^{40}Gap27 or ^{37,43}Gap27 on Cx37$^{-/-}$ and Cx40$^{-/-}$ platelets. Cx37 and Cx40 were unable to form hetero-oligomeric hemichannels and regulated platelet function independently of each other (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). A similar approach was used in this study to clarify the specificity of ^{62}Gap27 by assessing its effects on Cx40$^{-/-}$ and Cx37$^{-/-}$ knockout mouse platelets.

The independence of Cx57 hemichannels from Cx37 and Cx40 was assessed by measuring CRP-XL-stimulated fibrinogen binding in Cx37$^{-/-}$, Cx37$^{+/+}$, Cx40$^{-/-}$ and Cx40$^{+/+}$ mouse PRP that had been treated with ^{62}Gap27. CRP-XL-stimulated (0.5 µg/mL) fibrinogen binding in Cx37$^{-/-}$ mouse platelets was hindered by ^{62}Gap27 (100 µg/mL) to the same extent as that in Cx37$^{+/+}$ platelets (approximately 30%; Figure 4.6a, b). Similarly, a comparable level of inhibition (approximately 30%) of fibrinogen binding was exhibited by ^{62}Gap27 (100 µg/mL) in Cx40$^{-/-}$ and Cx40$^{+/+}$ mouse platelets (Figure 4.7a, b).

Taken together, these findings indicate that Cx62 hemichannel contributes to platelet regulation independently of Cx37 and Cx40 and supports the selectivity of ^{62}Gap27.

However, the selectivity of ^{62}Gap27 would need to be conclusively established in Cx57$^{-/-}$ mouse or Cx62 gene knockdown experiments.

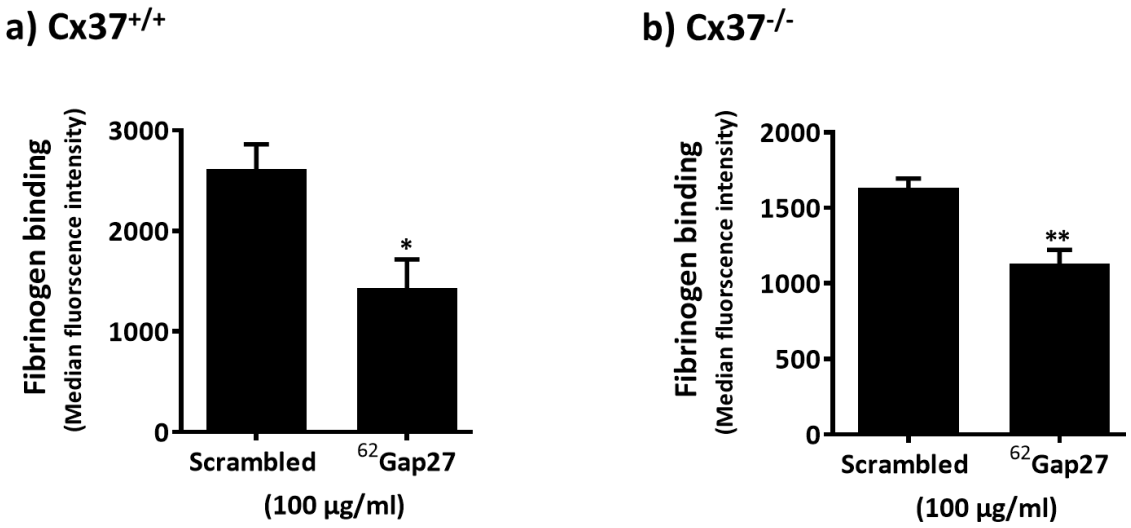


Figure 4.6: The regulation of platelet function by Cx62 hemichannel is not dependent on Cx37 hemichannel.

Mouse PRP was incubated with ^{62}Gap27 (100 µg/mL) or scrambled peptide for 5 minutes in the presence of a FITC-labelled anti-fibrinogen antibody. The fibrinogen binding levels of **(a)** wild type Cx37$^{+/+}$ and **(b)** Cx37$^{-/-}$ knockout mouse platelets were recorded by flow cytometry after CRP-XL (0.5 µg/mL) stimulation. The figure shows the median fluorescence intensity of fibrinogen binding in the presence of ^{62}Gap27 compared with binding in the presence of the scrambled peptide. Data are shown as mean ± SEM (n = 3), analysed by Student's *t*-test. *$P \leq 0.05$ and **$P \leq 0.01$

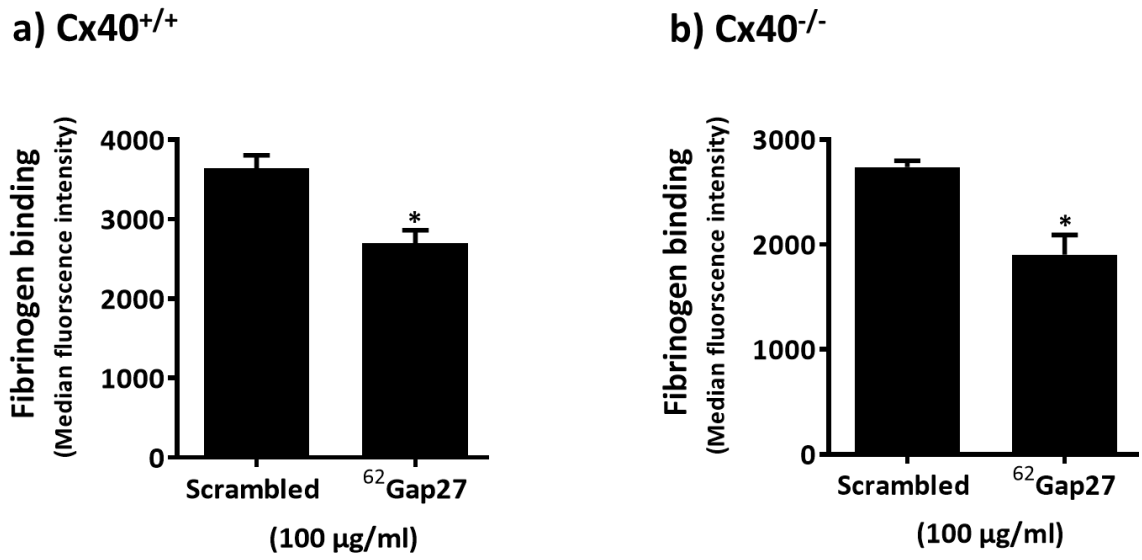


Figure 4.7: The regulation of platelet function by Cx62 hemichannel is independent of Cx40 hemichannel.

Mouse PRP was incubated with ^{62}Gap27 (100 µg/mL) or scrambled peptide (100 µg/mL) in the presence of FITC-labelled anti-fibrinogen antibody. The degree of fibrinogen binding in **(a)** wild type Cx40$^{+/+}$and **(b)** Cx40$^{-/-}$ knockout mouse platelets was recorded by flow cytometry after CRP-XL (0.5 µg/mL) stimulation. The figure shows the median fluorescence intensity of fibrinogen binding in the presence of ^{62}Gap27 compared with that in the presence of the scrambled peptide. Data represent the mean ± SEM (n = 4), analysed by Student's *t*-test. *$P \leq 0.05$

4.6 ^{62}Gap27 Selectively Inhibits the Function of Cx62

Cx62 is structurally similar to Cx57 in mouse and ^{62}Gap27 targets identical sequences in the second external loops of human Cx62 and mouse Cx57 (Appendix 8.2). Moreover, other Gap27 mimetic peptides target multiple Cxs that share specific sequences. ^{37,43}Gap27 targets Cx37, Cx43 and Cx46, which share its target sequence. The effects of ^{62}Gap27 were assessed in Cx57$^{-/-}$ mice (homologous to human Cx62) (obtained from the International Mouse Phenotyping Consortium) to determine the selectivity of ^{62}Gap27 for Cx62.

In order to determine that the effects of ^{62}Gap27 are indeed mediated via Cx57, the specificity of ^{62}Gap27 was examined by comparing the CRP-XL-stimulated fibrinogen binding in control (Cx57$^{+/+}$) and Cx57$^{-/-}$ mouse platelets in the presence of ^{62}Gap27.

Prior to the investigation of ^{62}Gap27 selectivity, the expression levels of GPVI (Figure 4.8a), integrin $\alpha_2\beta_1$ (Figure 4.8b), integrin $\alpha_{IIb}\beta_3$ (Figure 4.8c) and GPIb (Figure 4.8d) were evaluated in control and Cx57$^{-/-}$ mice to assess if the deletion of Cx57 influenced the expression of these receptors in platelets. The expression of these receptors on the wild type and Cx57$^{-/-}$ platelets were noted to be similar under resting and activated (CRP-XL, 0.5 µg/mL) conditions.

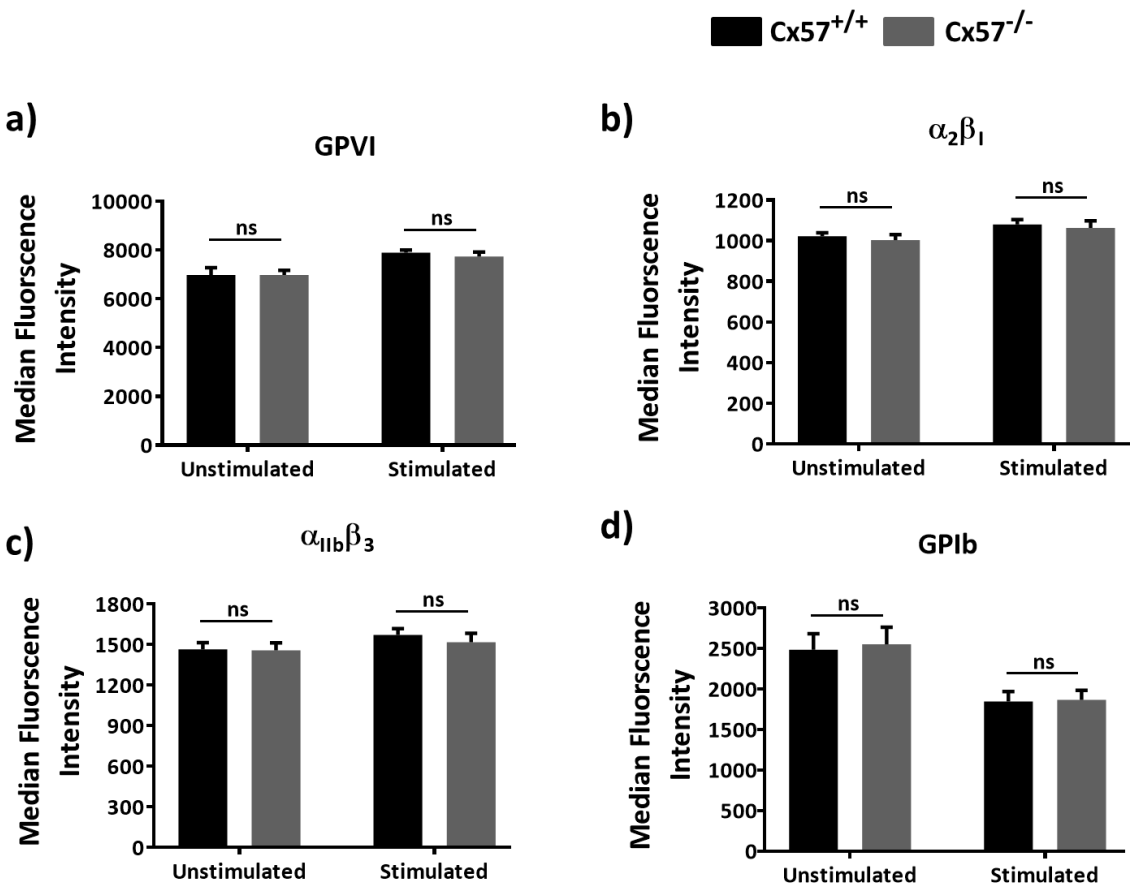


Figure 4.8: Cx57 ablation does not affect receptor levels on mouse platelets.

The expression levels of platelet receptors on resting and activated (CRP-XL, 0.5 µg/mL) platelets from control ($Cx57^{+/+}$) and $Cx57^{-/-}$ mice were analysed by flow cytometry: **(a)** GPVI, **(b)** integrin $\alpha_2\beta_1$, **(c)** integrin $\alpha_{IIb}\beta_3$ and **(d)** GPIb. The mean ± SEM (n = 6) of the median fluorescence intensities are shown.

To assess the selectivity of ^{62}Gap27, mouse PRP (obtained from both Cx57$^{-/-}$ and Cx57$^{+/+}$) was incubated with FITC-conjugated anti-human fibrinogen antibody, then with ^{62}Gap27 (100 μg/mL) or scrambled control (100 μg/mL) for 5 minutes. The PRP was further stimulated at room temperature with CRP-XL (0.5 μg/mL) for 20 minutes with occasional gentle mixing. Samples were then fixed in 0.2% (v/v) formyl saline and analysed by flow cytometry (10,000 platelet-gated events).

^{62}Gap27 inhibited CRP-XL-mediated fibrinogen binding in wild type mouse platelets by 50% compared with the scrambled control (Figure 4.9a). However, it had no impact on CRP-XL-stimulated Cx57$^{-/-}$ mouse platelets (Figure 4.9b). These data confirm that the inhibitory actions of ^{62}Gap27 on platelets are mediated through Cx57/62.

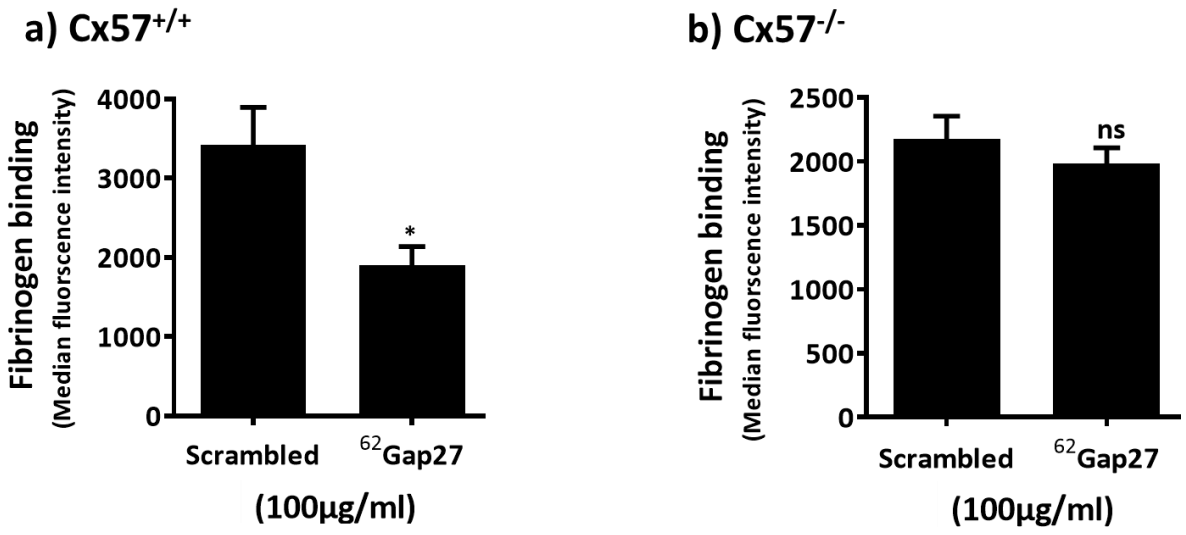


Figure 4.9: Cx62 is selectively inhibited by ^{62}Gap27.

PRP from **(a)** wild type Cx57$^{+/+}$ or **(b)** Cx57$^{-/-}$ knockout mice was incubated with FITC-labelled rabbit anti-fibrinogen antibody prior to treatment with scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes. Then, the samples were stimulated with CRP-XL (0.5 µg/mL) for 20 minutes. The cells were fixed in 0.2% formyl saline and the samples were examined on a flow cytometer. The data were analysed with Student's *t*-test and displayed as the mean ± SEM (n = 6). *$P \leq 0.05$

4.7 The Role of Cx62 in the Regulation of Dense Granule Secretion upon Platelet Activation

Human platelets contain 3–8 dense granules each. The granules are rich in nucleotides (ADP and ATP), serotonin, calcium and polyphosphates (Morrissey, 2012). Platelet aggregation is associated with the secretion of dense granules, which contribute to agonist-induced platelet exocytosis and platelet activation via the autocrine and paracrine actions of the released factors. The reinforcement of the initial activation events and propagation of the activation signal require the release of these mediators from dense granules. The release of ATP from dense granules as a secondary event during platelet aggregation has been identified as a fundamental step in the formation of platelet aggregates and is vital for the development of stable aggregates (Hechler and Gachet, 2011). Therefore, the effects of ^{62}Gap27 on ATP release were investigated as a marker of dense granule secretion.

This assay involves monitoring ATP release from dense granules using a bioluminescent system comprising D-luciferin, firefly luciferase and magnesium. The interactions of these reagents with ATP produce light, in direct proportion to the ATP concentration, which is detected and quantified using a lumi-aggregometer.

Platelets were pre-incubated with ^{62}Gap27 (25, 50 and 100 µg/mL) or scrambled peptide (100 µg/mL) for 5 minutes and then stimulated with CRP-XL (0.5 µg/mL) or thrombin (0.05 U/mL) for 3 minutes before the levels of ATP release were assessed.

As shown in Figure 4.10ai, incubation of platelets with ^{62}Gap27 (50 and 100 µg/mL) inhibited CRP-XL-stimulated ATP release by approximately 45% and 65%, respectively, in comparison to treatment with scrambled peptide control (Figure 4.10aii). Similarly, ATP release in thrombin-stimulated platelets was inhibited by approximately

35% and 50% in the presence of ^{62}Gap27 (50 and 100 µg/mL, respectively) (Figure 4.10bi, bii). The scrambled peptide had no effect on the level of ATP release when compared with non-peptide treated samples (data not shown).

These data indicate that ^{62}Gap27 influences the secretion of dense granules in platelets. Furthermore, such findings may explain the mimetic peptide-induced reduction in platelet aggregation, as dense granules are rich in ADP, ATP, and serotonin, which act as secondary platelet agonists.

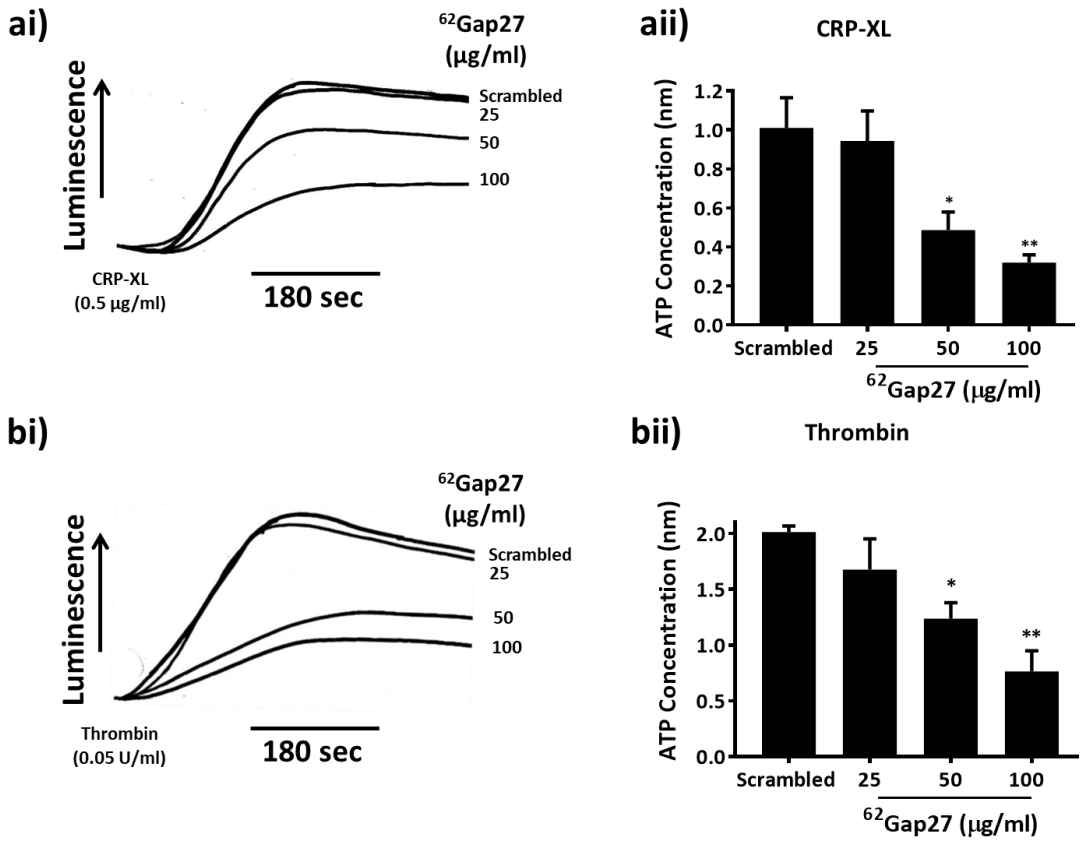


Figure 4.10: ATP secretion by CRP-XL- and thrombin-stimulated platelets is inhibited by ^{62}Gap27.

ATP release was measured from platelets pre-incubated with ^{62}Gap27 (25, 50 and 100 μg/mL) or scrambled peptide (100 μg/mL) for 3 minutes, then stimulated with **(a)** CRP-XL (0.5 μg/mL) or **(b)** thrombin (0.05 U/mL). Changes in ATP release were observed concurrently with aggregation in an optical lumi-aggregometer with a luciferase detection system. (i) Representative ATP release over 3 minutes. (ii) ATP secretion quantified data. The traces are representative of 5 individual experiments. Data represent the mean ± SEM (n = 5) as analysed by one-way ANOVA. *$P \leq 0.05$ and **$P \leq 0.01$

4.8 ^{62}Gap27 Decreases Thromboxane B_2 (TxB_2) Production

TxA_2 is a platelet agonist and potent vasoconstrictor that is produced by platelets (Gryglewski et al., 1978). The release of Ca^{2+} from intracellular stores is associated with the activation of platelet phospholipase A2, which leads to the hydrolysis of membrane phospholipids and the release of arachidonic acid (Balsinde et al., 2002). The aforementioned conversion occurs owing to the actions of the aspirin-sensitive cyclo-oxygenase (COX-1) and thromboxane synthase (TxS) and leads to the production of TxA_2 (Tazawa et al., 1996). TxA_2 disseminates across the plasma membrane (PM) and acts in an autocrine or paracrine fashion, due to its short half-life, to promote positive-feedback activation and aggregation (FitzGerald, 1991; Schrör, 2016). Unstable TxA_2 is rapidly converted to the inactive but stable lipid TxB_2 (Hamberg et al., 1975; FitzGerald, 1991). Since ^{62}Gap27 negatively regulated platelet aggregation and secretion from both α- and dense granules, its effects on TxA_2 production were studied. Given the short half-life (approximately 30 seconds) of TxA_2 and its non-enzymatic hydrolysis into its inactive form TxB_2, the levels of TxB_2 were measured as an indirect approximation of the concentration of TxA_2 (Seidel et al., 2011).

Washed platelets were treated with ^{62}Gap27 (50 and 100 μg/mL) or scrambled peptide for 5 minutes. Then, aggregation was induced by CRP-XL (1 μg/mL) or thrombin (0.1 U/mL) for 5 minutes. To ensure the maximum production and release of TxB_2 from activated platelets, high concentration of agonists was used. Non-stimulated samples served as negative controls. After stimulation with CRP-XL or thrombin in an aggregometer, the platelets that had been treated with scrambled peptide or ^{62}Gap27 were separated from the supernatant by centrifugation at 12,000 rpm for 2 minutes. Levels of TxB_2 in the plasma samples were evaluated by ELISA. A standard curve,

obtained by running a set of standards, was used to determine the TxB_2 levels in the ^{62}Gap27- and scrambled peptide-treated samples and the unstimulated samples.

The unstimulated samples did not contain detectable levels of TxB_2, whereas the scrambled treated samples stimulated with CRP-XL (Figure 4.11a) or thrombin (Figure 4.11b) contained approximately 1,800 pg/mL to 2,000 pg/mL, respectively. TxB_2 levels were diminished by approximately 50% upon incubation with ^{62}Gap27 (100 μg/mL) compared with the levels following scrambled peptide treatment (Figure 4.11a, b).

In conclusion, ^{62}Gap27 negatively regulates the secretion of positive-feedback mediators from platelets, which further suppresses platelet aggregation and, possibly, thrombus formation.

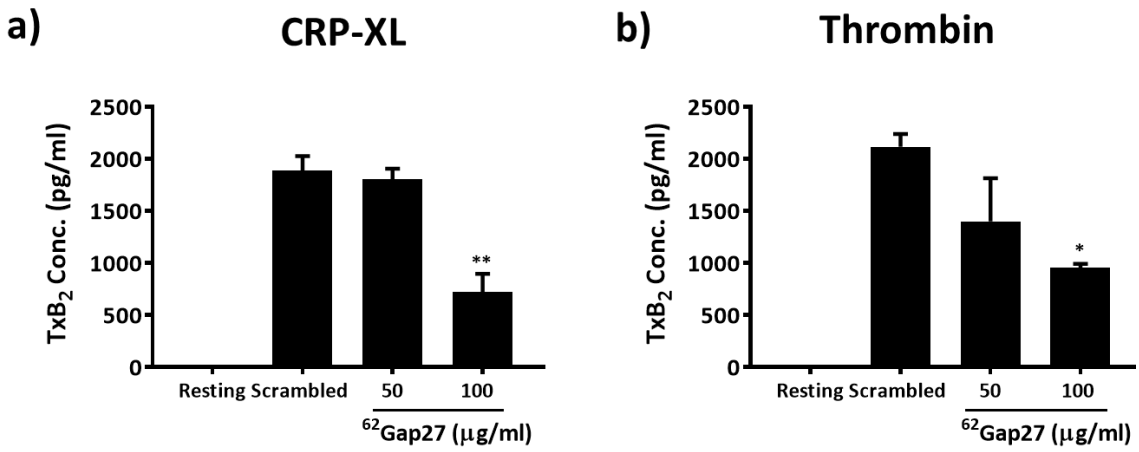


Figure 4.11: ^{62}Gap27 inhibits TxB_2 production.

Washed human platelets (4×10^8 cells/mL) were pre-incubated with ^{62}Gap27 or scrambled peptide for 5 minutes, then activated with **(a)** CRP-XL (1 μg/mL) or **(b)** thrombin (0.05 U/mL) in an aggregometer for 5 minutes at 37 °C. At the end of the reactions, 1 mM EGTA with 20 μM indomethacin was added, then the samples were subjected to centrifugation at 12,000 rpm for 2 minutes at room temperature to separate the supernatants, which were immediately frozen at −80 °C. A TxB_2 ELISA kit was used to measure the concentration of TxB_2, the stable metabolite of TxA_2, in accordance with the manufacturer's standards. The figures show the TxB_2 levels (pg/mL) following incubation of the platelets with ^{62}Gap27 or scrambled peptide and activation with **(a)** CRP-XL or **(b)** thrombin. Data, presented as the mean ± SEM (n = 5), were analysed by one-way ANOVA. *$P \leq 0.05$ and **$P \leq 0.01$

4.9 The Inhibitory Effects of ^{62}Gap27 on CRP-XL- or Thrombin-induced Activation are Independent of their Signalling through TP or ADP Receptors

Full platelet activation requires the synergistic actions of prothrombotic factors, such as ADP or TxA_2, that are released by activated platelets and signal through platelet surface receptors to augment platelet activation (Hirata et al., 1991; Daniel et al., 1998). Having observed the ability of ^{62}Gap27 to inhibit platelet aggregation stimulated by ADP and U46619 (TxA_2 mimetic peptide), experiments were performed to determine if the inhibitory effects of ^{62}Gap27 on CRP-XL or thrombin-stimulated platelet aggregation are solely due to their ability to inhibit the actions of TxA2 and ADP secreted after stimulation, or whether this may be attributed to the direct actions of other aspects of platelet activation.

CRP-XL- or thrombin-mediated platelet aggregation assays were performed on washed platelets (4×10^8 cells/mL) incubated with the cyclooxygenase (COX-1) inhibitor indomethacin and the ADP receptor antagonists MRS2179 (blocks $P2Y_1$ receptor) and cangrelor (blocks $P2Y_{12}$ receptor) for 5 minutes to block secondary mediator effects. The saturating concentration of each of these inhibitors was identified by treating platelets with a range of concentrations. The chosen concentrations [20 μM indomethacin, 1 μM cangrelor and 100 μM MRS2179 for CRP-XL-stimulated aggregation (Figure 4.12a, b) and 30 μM indomethacin, 1 μM cangrelor and 100 μM MRS2179 for thrombin-stimulated aggregation (Figure 4.13a, b)] were sufficient to completely abolish secondary mediator signalling. Higher concentrations of CRP-XL (1 μg/mL) or thrombin (0.1 U/mL) were used than in the earlier experiments to ensure 50% aggregation was still achieved within 5 minutes despite the inhibition of secondary mediator signalling.

Inhibition of COX-1 by the saturating concentration of indomethacin inhibited platelet aggregation by 40% upon CRP-XL stimulation (Figure 4.12a) and by 20% upon thrombin stimulation (Figure 4.13a). Reductions in platelet aggregation of 30% in response to CRP-XL- (Figure 4.12b) and 35% in response to thrombin (Figure 4.13b) were observed upon treatment with saturating concentrations of the ADP receptor antagonists cangrelor and MRS2179. A combination of saturating concentrations of all three inhibitors inhibited platelet aggregation by 55% after CRP-XL (Figure 4.12c) or 60% after thrombin (Figure 4.13c) stimulation. Having established the effects of the inhibitors that target the secondary mediators, further experiments were performed in the presence of ^{62}Gap27. In the presence of the high concentrations of CRP-XL or thrombin, ^{62}Gap27 had no effect. However, in the presence of cangrelor, MRS2179 and indomethacin, the addition of ^{62}Gap27 caused a 20% greater inhibition of platelet aggregation following stimulation with CRP-XL (Figure 4.12c, d) or thrombin (Figure 4.13c, d).

These findings suggest that defects in the secretion of ADP or synthesis of TxA_2 were not the only factors associated with the inhibitory effects of ^{62}Gap27 on platelet functions.

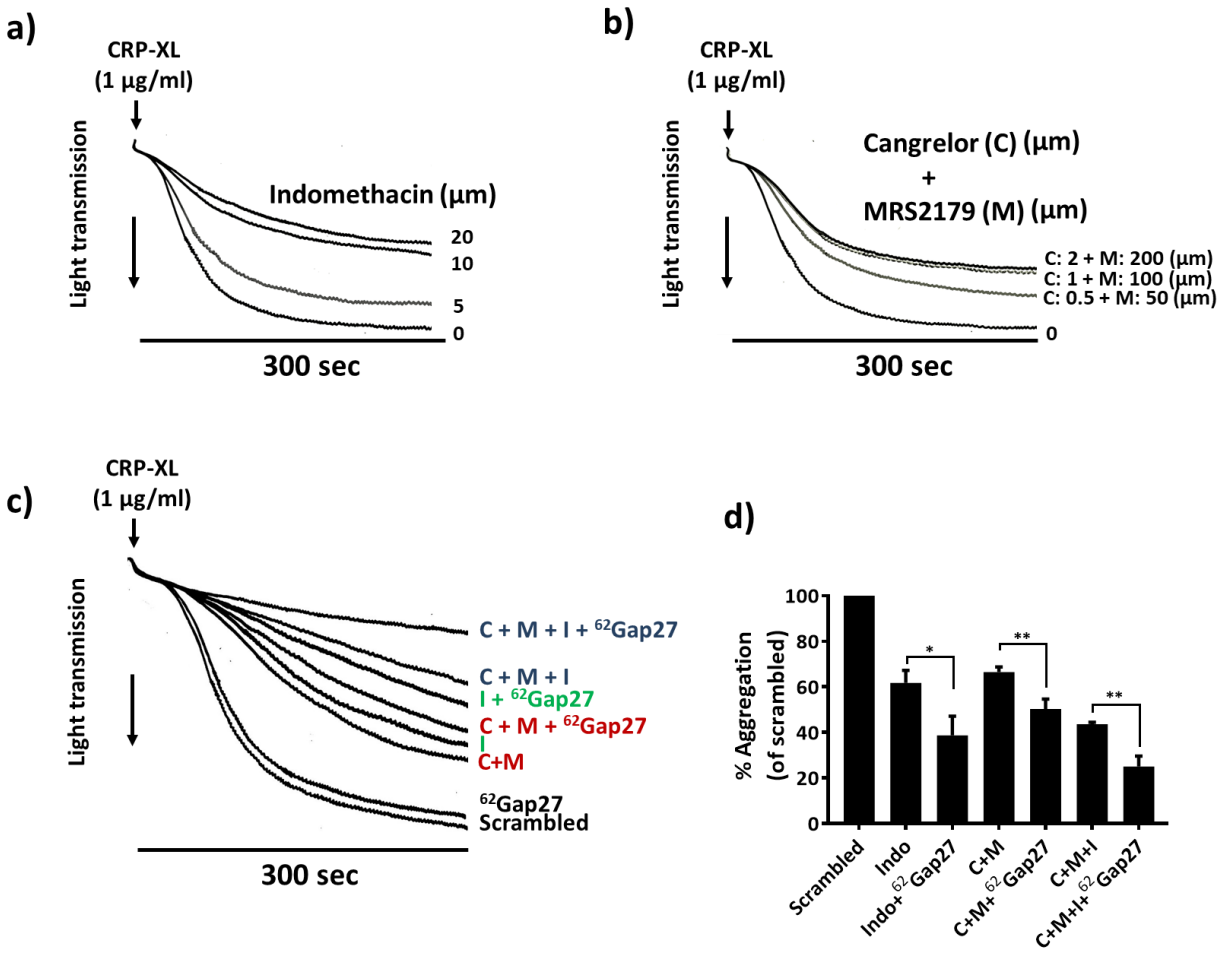


Figure 4.12:The effects of ^{62}Gap27 on CRP-XL-mediated platelet aggregation are not restricted to the inhibition of granule secretion.

Washed platelets (4×10^8) were incubated with different concentrations of **(a)** indomethacin (I; 5, 10 and 20 µM) or **(b)** the ADP receptor antagonists cangrelor (C; 0.5, 1 and 2 µM) and MRS2179 (M; 50, 100 and 200 µM). Platelet aggregation was induced with CRP-XL (1 µg/mL). Aggregation was observed for 5 minutes to determine the saturating concentrations. **(c)** CRP-XL-stimulated platelet aggregation in the presence and absence of ^{62}Gap27 (100 µg/mL) along with indomethacin (I; 20 µM) or cangrelor (C; 1 µM) and MRS2179 (M; 100 µM) or the combination of all inhibitors. The figure shows representative aggregation traces. **(d)** The platelet aggregation data for platelets stimulated with CRP-XL in the presence or absence of ^{62}Gap27 with indomethacin (I+^{62}Gap27) or cangrelor and MRS2179 (C+M+^{62}Gap27) or the triple combination (I+C+M+^{62}Gap27). Aggregation of scrambled peptide-treated platelets in the absence of secondary mediator inhibitors was set as 100% platelet aggregation. Data represent the mean ± SEM (n = 5), analysed by Student's *t*-test. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$

Abbreviations: I – Indomethacin, C – Cangrelor, M – MRS2179, S – Scrambled Peptide and ^{62}Gap27 – connexin 62 mimetic peptide

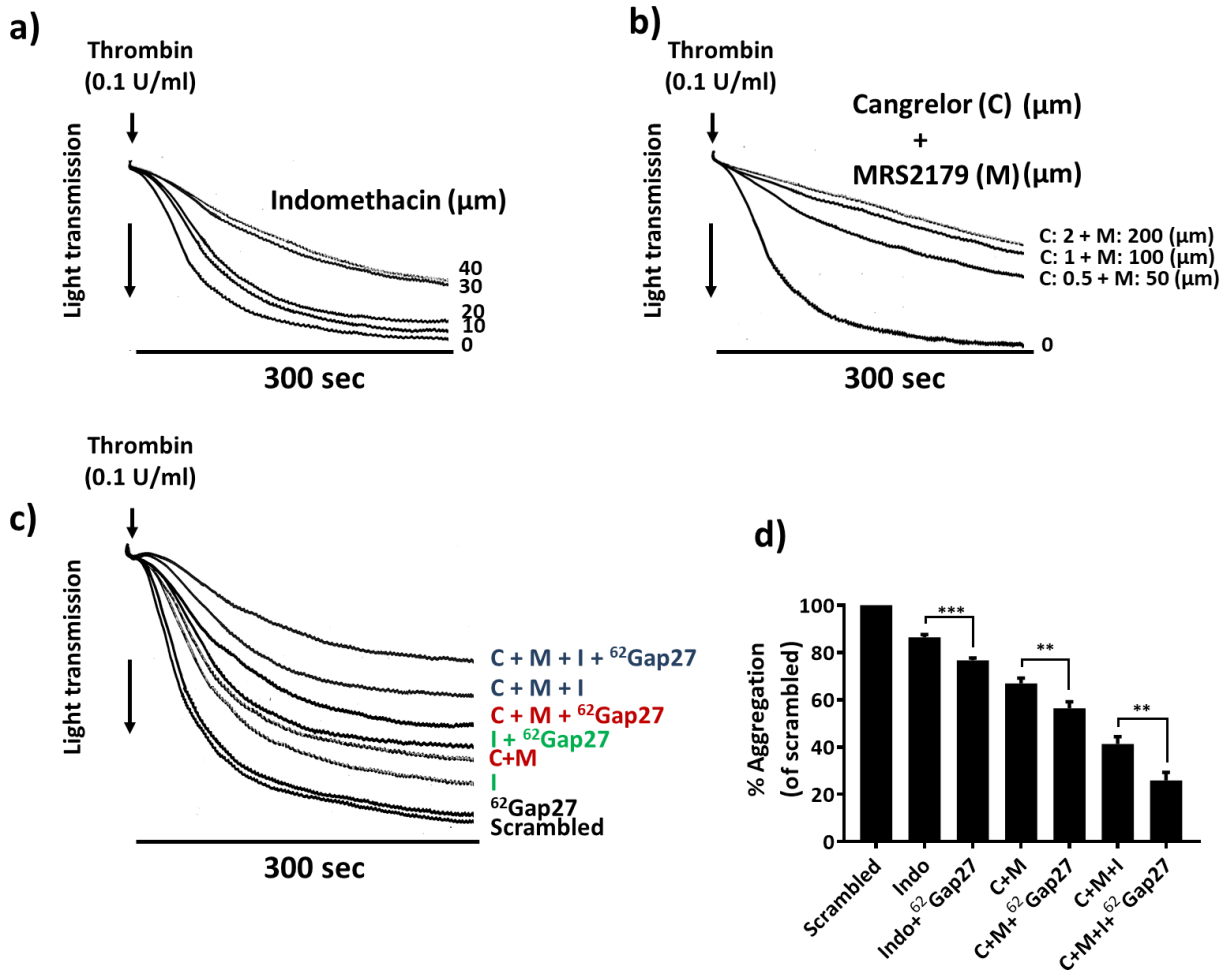


Figure 4.13:The inhibitory effects of ^{62}Gap27 on thrombin-induced platelet aggregation are not solely dependent on defects in ADP release or TxA_2 synthesis.

Washed platelets (4×10^8) were incubated with different concentrations of **(a)** indomethacin (I; 5, 10 and 20 µM) or **(b)** the ADP receptor antagonists cangrelor (C; 0.5, 1 and 2 µM) and MRS2179 (M; 50, 100 and 200 µM). Platelet aggregation was induced with thrombin (0.1 U/mL). Aggregation was observed for 5 minutes to determine the saturating concentrations. **(c)** Thrombin-stimulated platelet aggregation in the presence and absence of ^{62}Gap27 (100 µg/mL) along with indomethacin (I; 20 µM) or cangrelor (C; 1 µM) and MRS2179 (M; 100 µM) or the combination of all inhibitors. The figure shows representative aggregation traces. **(d)** The platelet aggregation data for platelets stimulated with thrombin in the presence or absence of ^{62}Gap27 with indomethacin (I+^{62}Gap27) or cangrelor and MRS2179 (C+M+^{62}Gap27) or the triple combination (I+C+M+^{62}Gap27). Aggregation of the scrambled peptide-treated platelets in the absence of secondary mediator inhibitors was set as 100% platelet aggregation. Data represent the mean ± SEM (n = 5), analysed by Student's *t*-test. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$

Abbreviations: I – Indomethacin, C – Cangrelor, M – MRS2179, S – Scrambled Peptide and ^{62}Gap27 – connexin 62 mimetic peptide

4.10 Role of Cx62 in CRP-XL- and Thrombin-stimulated Ca^{2+} Mobilisation

Platelet activation in haemostasis and thrombosis requires agonist-induced amplification of cytosolic Ca^{2+} concentrations. Calcium plays a central role in multiple steps of platelet activation, such as the reorganisation of the actin cytoskeleton during conformational changes (Hathaway and Adelstein, 1979), degranulation and inside-out activation of integrin $\alpha_{IIb}\beta_3$ (Shattil and Brass, 1987). Thus, platelet activation is accompanied by an elevation of the cytosolic Ca^{2+} concentration. The main sources of intracellular Ca^{2+} are the agonist-induced release of Ca^{2+} that had been sequestered in the DTS and subsequent Ca^{2+} influx through the plasma membrane (PM). Several ion channels, which are store- or receptor-operated, facilitate the entrance of Ca^{2+} into the cell from the extracellular environment (Varga-Szabo et al., 2009; Li et al., 2010; Mahaut-Smith et al., 2011; Mahaut-Smith, 2012). The entrance of Ca^{2+} into platelets mainly occurs through store-operated Orail channels (via SOCE) and purinergic $P2X_1$ channels (Hoth and Penner, 1992; Berridge et al., 2003; Mahaut-Smith et al., 2004; Fung et al., 2007; Tolhurst et al., 2008; Bergmeier and Stefanini, 2009; Smyth et al., 2010b). It has also been established by Hassock et al. (2002) that thrombin activates the transient receptor potential channel 6 (TRPC6) via the production of DAG. Intriguingly, researchers have found that Ca^{2+} entrance via TRPC6 channels usually occurs in the presence of greater levels of thrombin. Therefore, it can be postulated that this mechanism affects platelet physiology depending on the extent of platelet stimulation (Harper and Poole, 2011; Mahaut-Smith, 2012). As the release of Ca^{2+} from platelets plays important roles in granule secretion and inside-out platelet activation signalling, the effects of ^{62}Gap27 on intracellular Ca^{2+} mobilisation and the influx of Ca^{2+} following CRP-XL or thrombin stimulation were studied.

The ratiometric dye Fura-2AM was used to monitor the levels of calcium. This dye binds to free intracellular Ca^{2+}; it is excited by wavelengths between 340 and 380 nm and emits at 510 nm. The emission values directly relate to intracellular Ca^{2+} levels. Ca^{2+} mobilisation was measured in the presence or absence of EGTA. Since EGTA chelates extracellular Ca^{2+}, thereby enabling the measurement of only Ca^{2+} that is released from the internal stores. Fura-2AM-loaded platelets were incubated with ^{62}Gap27 (50–100 μg/mL) or scrambled peptide (100 μg/mL) for 5 minutes, then stimulated with CRP-XL (0.25 μg/mL) or thrombin (0.05 U/mL) for 3 minutes. Intracellular Ca^{2+} mobilisation was measured by spectrofluorimetry.

Treatment with 50 and 100 μg/mL ^{62}Gap27 following CRP-XL stimulation inhibited peak Ca^{2+} mobilisation by approximately 30% and 45%, respectively, compared with the treatment with the scrambled peptide (Figure 4.14ai, aii). Likewise, treatment with 50 and 100 μg/mL ^{62}Gap27 inhibited peak Ca^{2+} levels by 30% and 55%, respectively, in thrombin-stimulated platelets (Figure 4.14bi, bii).

Ca^{2+} mobilisation was also measured in the presence of EGTA. Treatment with saturating EGTA levels (2 mM) in the presence of 100 μg/mL scrambled peptide inhibited peak Ca^{2+} mobilisation by 50% in comparison to treatment with scrambled peptide control alone. Following stimulation with CRP-XL or thrombin, treatment with ^{62}Gap27 (100 μg/mL) in the presence of EGTA inhibited peak Ca^{2+} mobilisation by 45% compared with treatment with the scrambled peptide in the presence of EGTA (Figure 4.15a, b). The scrambled peptide had no effect on the level of Ca^{2+} mobilisation when compared with non-peptide treated samples (data not shown).

These data provide evidence that the negative regulation of Ca^{2+} mobilisation by ^{62}Gap27 can be at least partially explained by its inhibitory effect on Ca^{2+} release from intracellular stores. However, an influence on Ca^{2+} influx cannot be ruled out.

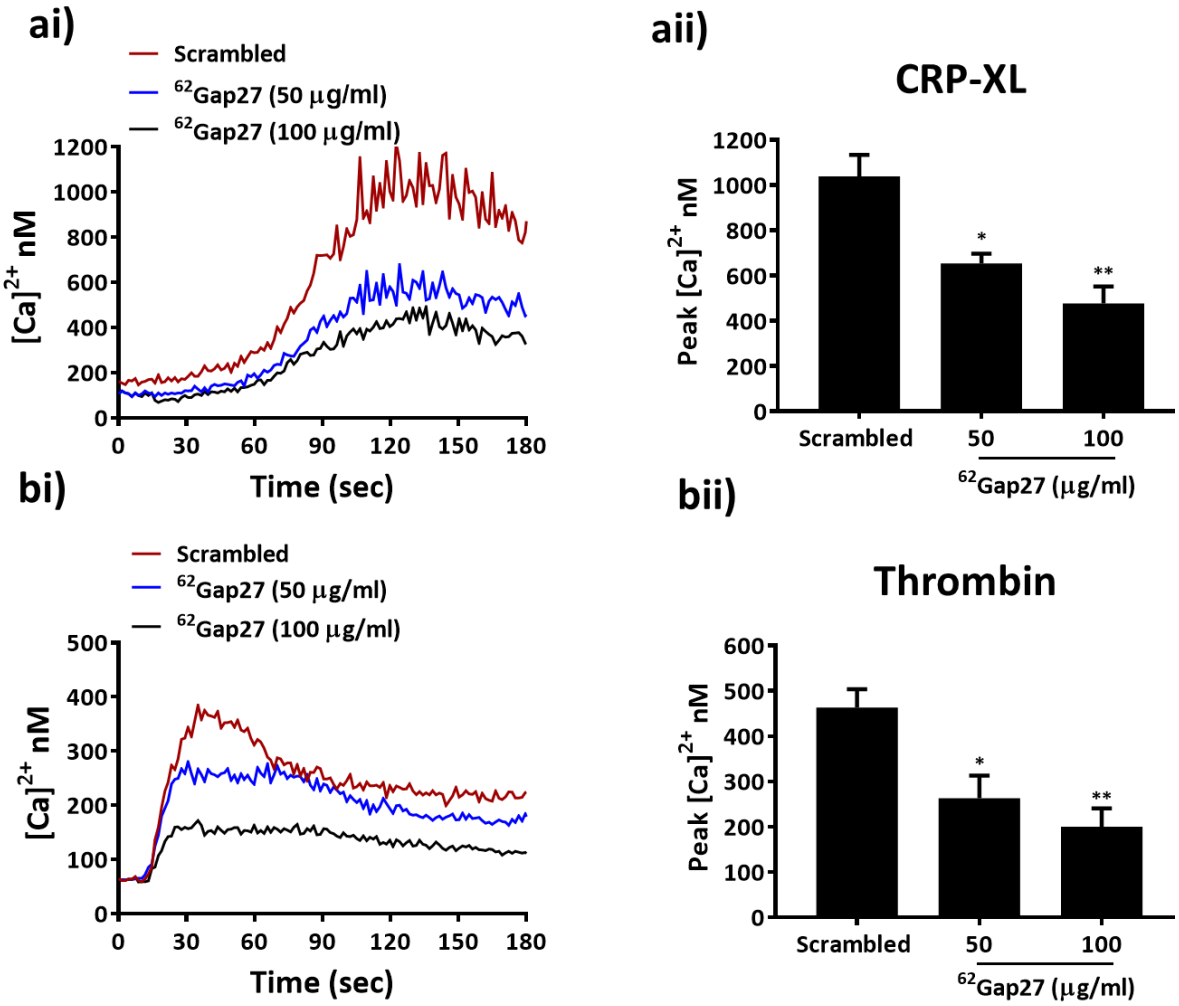


Figure 4.14: Cytosolic Ca^{2+} mobilisation is inhibited by ^{62}Gap27.

Fura-2AM-loaded platelets were incubated with ^{62}Gap27 (50 or 100 µg/mL) or scrambled peptide (100 µg/mL) for 5 minutes, then stimulated with **(ai)** CRP-XL (0.25 µg/mL) or **(bi)** thrombin (0.05 U/mL) for 3 minutes. Spectrofluorimetry was used to measure the intracellular mobilisation of Ca^{2+}. **(aii and bii)** The inhibition of the peak cytoplasmic Ca^{2+} concentration after treatment with ^{62}Gap27 compared with treatment with the scrambled peptide; traces are representative of 7 individual experiments. The data represent the mean ± SEM (n = 7), as analysed by one-way ANOVA. *$P \leq 0.05$ and **$P \leq 0.01$

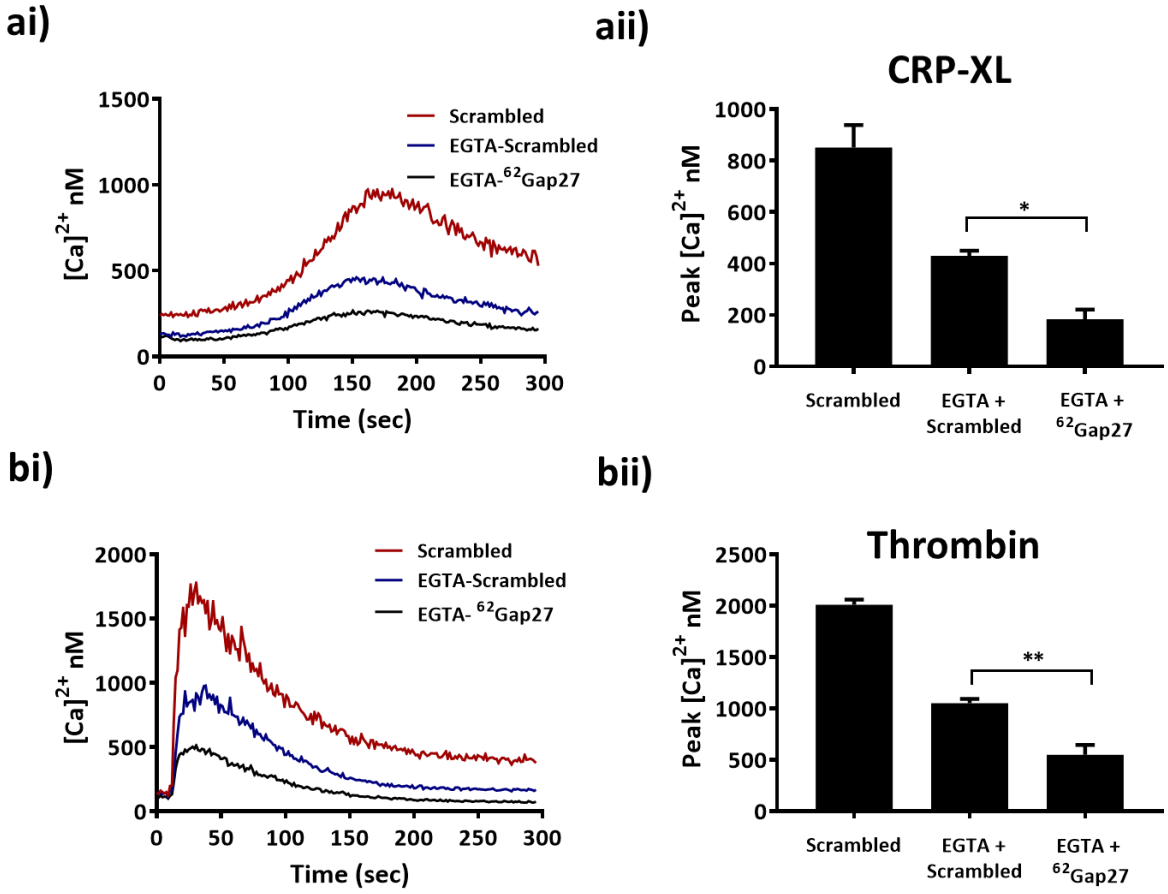


Figure 4.15: ^{62}Gap27 inhibits cytosolic Ca^{2+} mobilisation in the presence of EGTA.

Fura-2AM-loaded platelets were incubated with ^{62}Gap27 (50 or 100 µg/mL) or scrambled peptide (100 µg/mL) for 5 minutes in the presence of EGTA, then stimulated with **(ai)** CRP-XL (0.5 µg/mL) or **(bi)** thrombin (0.05 U/mL) for 5 minutes. Spectrofluorimetry was used to measure intracellular mobilisation of Ca^{2+}. **(aii and bii)** The inhibition of the peak cytoplasmic Ca^{2+} concentration after treatment with ^{62}Gap27 in contrast to treatment with scrambled peptide; traces are representative of 5 individual experiments. The mean ± SEM (n = 5) are depicted. Data were analysed by one-way ANOVA. *$P \leq 0.05$ and **$P \leq 0.01$

4.11 ^{62}Gap27 Attenuates Platelet Adhesion and Spreading on Fibrinogen

Signals can be transferred bidirectionally via integrin $\alpha_{IIb}\beta_3$ across the plasma membrane. 'Inside-out signalling' is initiated in platelets by the binding of talin and kindlin to the cytoplasmic domains of integrin β subunits, which leads to increased affinity of integrin $\alpha_{IIb}\beta_3$ for fibrinogen (Tadokoro et al., 2003; Moser et al., 2008). The high-affinity integrin binds fibrinogen, which creates fibrinogen bridges between platelets and leads to the development of stable platelet aggregates (Bonnefoy et al., 2001). In addition to the physical interaction that this facilitates, the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ activates intracellular signalling known as 'outside-in' signalling. Following integrin binding to fibrinogen, $G_{\alpha 13}$ interacts with the cytoplasmic tail of β_3, which stimulates SFKs, particularly c-Src, to bind β_3 (Obergfell et al., 2002; Gong et al., 2010). This leads to tyrosine phosphorylation of the cytoplasmic tail of β_3, leading to improved interactions between β_3 and cytoskeletal proteins, such as myosin heavy chain. The improved interactions stimulate cytoskeletal reconfiguration and clot retraction (Jenkins et al., 1998). c-Src also causes transient inactivation of RhoA, which encourages platelet spreading (Arthur et al., 2000). The impact of ^{62}Gap27 on outside-in signalling can be assessed by investigating platelet distribution on fibrinogen.

Glass coverslips were coated with fibrinogen (100 μg/mL) for 1 hour, then blocked with bovine serum albumin (BSA) for 1 hour to prevent binding of the platelets to the glass. The washed platelets (2×10^7 cells/mL) were incubated with ^{62}Gap27 or scrambled peptide for 5 minutes, then plated on the coated coverslips. Unbound platelets were removed after 45 minutes at 37 °C by washing with phosphate-buffered saline (PBS). Next, the samples were fixed in 0.2% (v/v) formyl saline, washed with PBS, and permeabilised with 0.2% Triton™ X-100. After washing, the samples were

incubated with Alexa Fluor® 488-conjugated phalloidin to stain the actin in the adhered platelets for visualisation with a confocal microscope. For each sample, five images were obtained from random locations on each slide. Images were analysed blind and platelets categorised into 3 groups: adhered (not spread), filopodia (partially spread) or lamellipodia (fully spread). The percentage of platelets in each group was computed for the distinct experimental treatments.

Representative images of platelet adhesion and spreading on fibrinogen after treatment with scrambled peptide or ^{62}Gap27 are presented in Figure 4.16a. Incubation with ^{62}Gap27 (50 and 100 μg/mL) inhibited the adhesion of platelets to fibrinogen-coated coverslips by approximately 70% compared to that observed after incubation with scrambled peptide (Figure 4.16b).

Platelets treated with ^{62}Gap27 (50 and 100 μg/mL) and allowed to adhere to fibrinogen were prevented from completely spreading as compared to the scrambled peptide-treated controls (Figure 4.16c). Approximately 80% of the platelets were completely spread in scrambled peptide-treated samples, but the percentage decreased to 45% and 35% for those treated with 50 and 100 μg/mL ^{62}Gap27, respectively. After treatment with 50 and 100 μg/mL ^{62}Gap27, 35% and 45% of platelets, respectively, had filopodial extensions as compared to 18% in the scrambled control group. Finally, a higher percentage of samples treated with ^{62}Gap27 (20% for 50 μg/mL and 25% for 100 μg/mL) adhered to fibrinogen, but did not spread, than scrambled control-treated platelets (2%; Figure 4.16c).

Taken together, these data indicate that integrin $\alpha_{IIb}\beta_3$ is incapable of attaining a high-affinity state after treatment with ^{62}Gap27, which decreases its ability to attach to fibrinogen and reduces platelet spreading. These findings are in agreement with the

^{62}Gap27-induced decrease in fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in CRP-XL- or thrombin-activated samples.

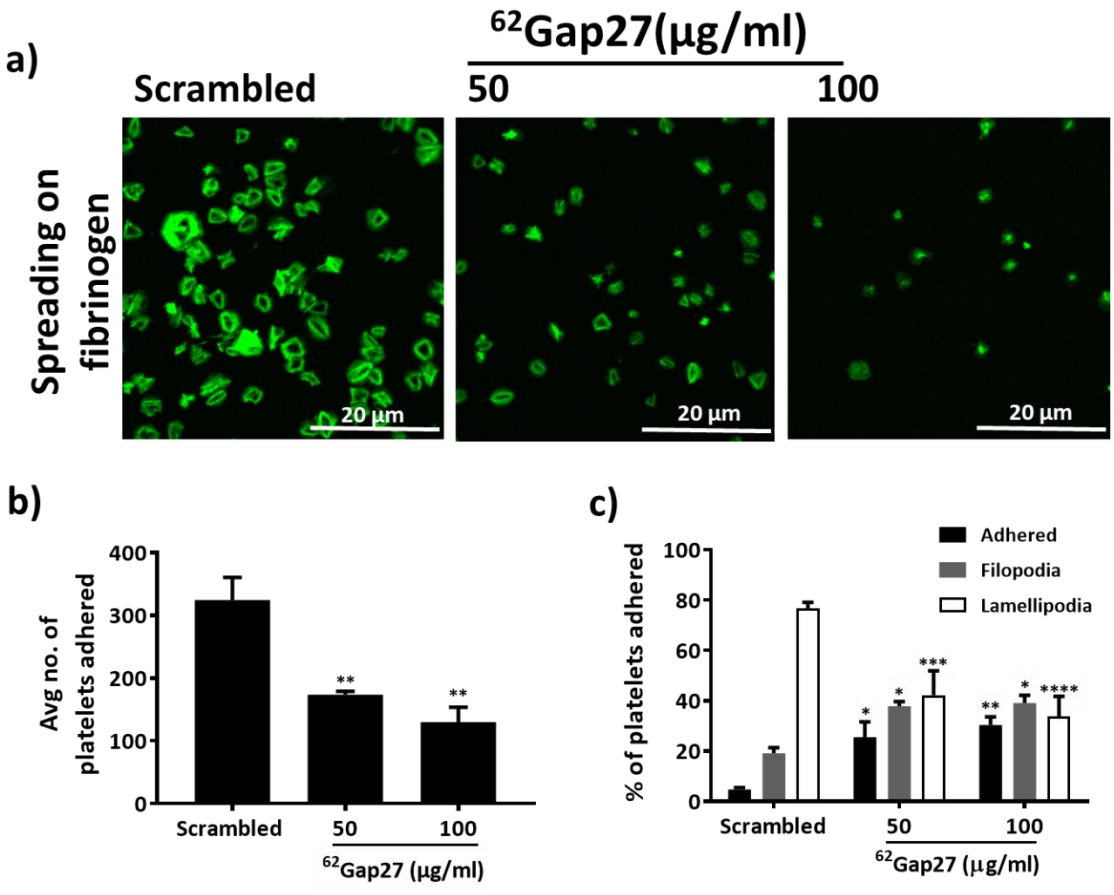


Figure 4.16: ^{62}Gap27 inhibits platelet adhesion to and spreading on fibrinogen.

Washed human platelets (2×10^7) were treated with scrambled peptide or ^{62}Gap27 for 5 minutes, then transferred to fibrinogen-coated (100 µg/mL) coverslips for 45 minutes at 37 °C. The samples were fixed in 0.2% (v/v) formyl saline and permeabilised with 0.2% Triton™ X-100. The coverslips were incubated with Alexa Fluor® 488-conjugated phalloidin for 1 hour, then mounted to slides using ProLong™ Gold Antifade Mountant. The samples were visualised with a Nikon A1R confocal microscope and a 100× oil immersion lens. **(a)** For every sample, five images were obtained at different locations on the slide. Representative images of platelet adhesion and spreading on fibrinogen are shown. **(b)** The numbers of platelets (average) adhered in each different condition are shown. **(c)** Spreading platelets were grouped into three categories: adhered (not spread), filopodia (partially spread) and lamellipodia (fully spread). The findings are presented as the percentage of the total platelets adhered. The data represent the mean ± SEM (n = 5) and were analysed by one-way ANOVA. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$

4.12 Effect of ^{62}Gap27 on Clot Retraction

Fibrinogen binding results in platelet clustering, which propagates outside-in signalling to reinforce platelet activation and render aggregation irreversible. The purpose of the cascade is to assist in platelet conformational changes, spreading and clot retraction to balance the aggregates and subsequent thrombus (Shattil et al., 1998; Shattil and Newman, 2004; Li et al., 2010). After development of the clot, contractile forces are exerted by the platelets through the actin–myosin cytoskeleton. The cytoskeleton is attached to the internal domain of integrin $\alpha_{IIb}\beta_3$ and the fibrin network in the clot is bound to the external domain; the contractile forces reduce the size of the clot (Shattil et al., 1998; Shattil and Newman, 2004; Li et al., 2010). This process is known as clot retraction; it is believed to be important for wound repair and stable thrombus formation (Law et al., 1999a; Tucker et al., 2012; Cines et al., 2014). It has been reported previously that Cx37 and Cx40 play an important role in regulating contact-dependent signalling in platelets, which is initiated following the formation of thrombus (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Therefore, the impact of ^{62}Gap27 on intra-clot signalling was studied in a clot retraction assay.

Human PRP (200 µL) was pre-incubated with ^{62}Gap27 (25, 50 and 100 µg/mL) or scrambled peptide (100 µg/mL) at room temperature for 5 minutes. Red blood cells (5 µL) were added to aid visualisation. Thrombin (50 µL; final concentration 1 U/mL) was added to the samples to initiate clot formation. A sealed glass capillary was situated in the centre of the glass test tube to establish an area for the formation and retraction of the clot. Pictures of clot formation were taken every 30 minutes and the clots were weighed (indicative of clot retraction) after 90 minutes, at which point the scrambled peptide-treated sample appeared to retract completely.

The clot weight in the ^{62}Gap27-treated sample (100 µg/mL) was three-fold higher than that in the scrambled peptide sample (Figure 4.17). The clot weight was influenced by the ^{62}Gap27 mimetic peptide, which confirms that Cx62 plays an important role in the modulation of integrin $\alpha_{IIb}\beta_3$-mediated outside-in signalling.

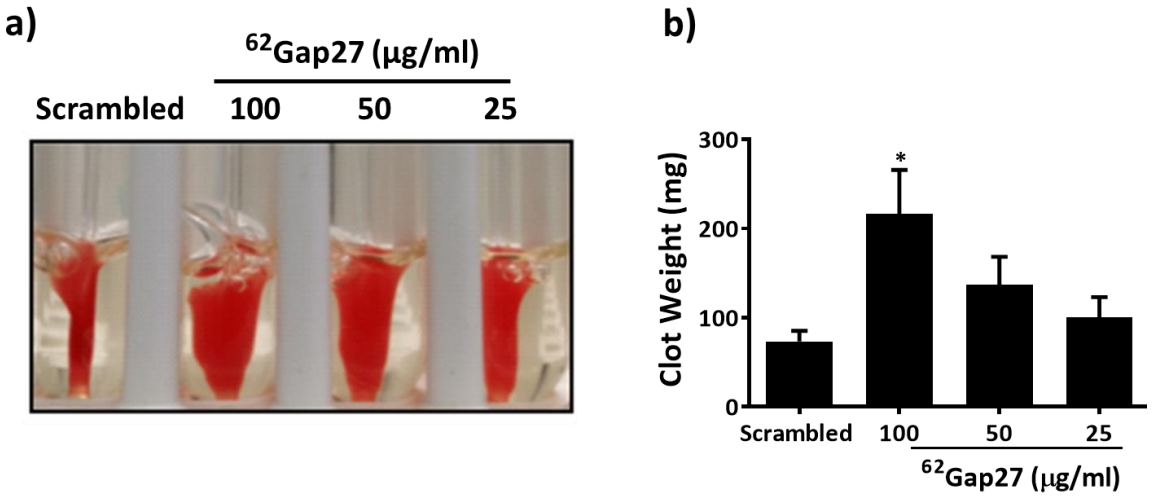


Figure 4.17: ^{62}Gap27 inhibits clot retraction.

Human PRP and red blood cells (5 µL) were incubated with scrambled peptide (100 µg/mL) or ^{62}Gap27 (25, 50 and 100 µg/mL) at room temperature for 5 minutes. Thrombin (1 U/mL) was used to stimulate clot generation. A surface was provided for clot formation by situating a glass capillary in the centre of a glass tube. **(a)** Clots were monitored over a period of 90 minutes. **(b)** Clot retraction was recorded after 90 minutes by calculating clot weight (mg). The cumulative data show the mean ± SEM of the clot weight (n = 5) as analysed by one-way ANOVA. *$P \leq 0.05$

4.13 Discussion

Within the intact vasculature, platelets circulate as independent discoid bodies. However, following injury, they bind amongst themselves to create a thrombus, using integrin $\alpha_{IIb}\beta_3$ as well as the adhesion proteins fibrinogen and VWF (Gibbins, 2004). According to Prevost et al. (2005), the thrombus is stabilised by sustained signalling between the platelets within the thrombus. However, not much information is available about the regulation of this process. Thrombus formation is followed by clot contraction, which is a synchronised response stimulated by platelet integrin $\alpha_{IIb}\beta_3$ that leads to thrombus retraction, further stabilisation of the clot and binding of the wound ends (Calderwood, 2004). Since platelets are regulated both (i) in isolation in the plasma and (ii) in a contact-dependent manner in a thrombus, researchers have studied their expression of Cx hemichannels and GJs in platelets and the roles they play in the various stages of platelet function (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

Previous studies confirmed the expression of Cx37 and Cx40 in platelets (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Platelet functions, including aggregation, fibrinogen binding and granule secretion, are inhibited by specific peptide inhibitors of Cx37 and Cx40, indicating regulatory roles for Cx40 and Cx37 in platelet function. Cx37 and Cx40 mimetic peptides also inhibit thrombus formation *in vitro* and in mouse models (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Research on mouse platelets that lack Cx37 or Cx40 has confirmed that these two Cxs appear to function independently of each other (Vaiyapuri et al., 2013).

4.13.1 ^{62}Gap27 Affects a Range of Platelet Functions

The aggregation of platelets upon stimulation with different platelet agonists was analysed in the presence of the selective mimetic peptide ^{62}Gap27, which targets the

second external loop of Cx62. Platelet responses were suppressed in the presence of ^{62}Gap27 following stimulation with a range of agonists, suggesting that GPVI and GPCR receptor function may be controlled by Cx62.

Platelet aggregation was inhibited by ^{62}Gap27 at a stage prior to the development of platelet aggregates, indicating ^{62}Gap27-mediated regulation of GJs. Typically, primary signalling events are amplified by the secretion of key molecules, which are stored in intracellular granules, that trigger platelet aggregation. Reduced binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ hinders the stabilisation of platelet aggregates. The level of fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ receptor in CRP-XL- or thrombin-stimulated platelets was studied to explore the role of Cx62 on the affinity activation of integrin $\alpha_{IIb}\beta_3$. ^{62}Gap27 inhibited the enhancement of the affinity of integrin $\alpha_{IIb}\beta_3$ for and binding to fibrinogen. These data were consistent with the ability of ^{62}Gap27 to inhibit platelet aggregation responses to CRP-XL and thrombin stimulation.

Incubation with ^{62}Gap27 blocked the secretion of platelet α-granules and dense granules, as it dampened P-selectin exposure on the platelet surface and ATP release, respectively. Also, fibrinogen binding was reduced upon treatment with ^{62}Gap27.Flow cytometric analysis facilitates the evaluation of the activity of a single platelet. Thus, the fibrinogen binding and P-selectin exposure data indicate that ^{62}Gap27 regulates hemichannels in platelets (early stage of platelet activation). Thus, Cx62, like Cx37 and Cx40 (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013), may play an important role in both the initiation and stabilisation of thrombi. In addition, the inhibitory effects of ^{62}Gap27 in the presence of cangrelor, MRS2179 and indomethacin suggest that the blockade of ADP receptors and TxA_2 synthesis are not the only mechanisms responsible for the inhibition of platelet aggregation.

4.13.2 ^{62}Gap27 Specifically Acts through Cx62 and does not Cross-react with Cx37 and Cx40

Gap27 peptides are widely used to study the functions of Cxs in different cell types. These mimetic peptides, which target the second external loops of Cxs, work by closing hemichannels and preventing the formation of GJs (Evans et al., 2012). Previous studies have shown that Gap27 peptides can inhibit a wide range of platelet functions (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). Similarly, ^{62}Gap27 impaired many platelet functions in this study. The selectivity of a Gap27 mimetic peptide is crucial due to the high degree of similarity between Cx sequences. The selectivity studies for ^{62}Gap27, which were performed in Cx57$^{-/-}$ mice, revealed that ^{62}Gap27 acts specifically via mouse Cx57 (homologous to human Cx62). In addition, Cx62 hemichannels contribute to platelet regulation independently of Cx37 and Cx40, as demonstrated by the effects of ^{62}Gap27 in Cx37$^{-/-}$ or Cx40$^{-/-}$ mouse platelets. Therefore, ^{62}Gap27 is not cross-reactive with Cx37 and Cx40.

4.13.3 ^{62}Gap27 Inhibits Ca^{2+} Mobilisation in Platelets

The activation of GPVI and thrombin receptors initiates the recruitment of Ca^{2+} from internal stores. This Ca^{2+} mobilisation influences granule secretion and the conformational changes that enhance the affinity of integrin $\alpha_{IIb}\beta_3$ for fibrinogen, thereby leading to platelet aggregation and thrombus formation. ^{62}Gap27 inhibited Ca^{2+} mobilisation in platelets stimulated with CRP-XL or thrombin. This decrease accounts for the reductions in α- and dense granule release, integrin $\alpha_{IIb}\beta_3$ affinity modulation and subsequent platelet aggregation. In addition, in the presence of EGTA, which blocks Ca^{2+} influx, the overall levels of cytosolic Ca^{2+} were lower than those in the absence of EGTA. ^{62}Gap27 also inhibited the cytosolic Ca^{2+} elevation stimulated through CRP-XL or thrombin in the presence of EGTA. This observation indicates that

Cx62 modulates cytosolic Ca^{2+} levels both following release from intracellular stores and via calcium influx.

The effects of ^{62}Gap27 on Ca^{2+} mobilisation may be due to its influence on ion channel functionality. For example, it may act in cooperation with Orai1 (Braun et al., 2009), TRPC6 (Hassock et al., 2002) or $P2X_1$ (Fung et al., 2007), which are important Ca^{2+} channels in platelets. Our results indicate that Cxs have an important impact on the discharge of Ca^{2+} from the intracellular stores in platelets. They may also regulate Ca^{2+} influx through the plasma membrane. Notably, hemichannel opening in other cell types is controlled by extracellular Ca^{2+} levels (Goodenough and Paul, 2003), although the precise mechanism is unclear. Thus, intercellular signalling by diffusion of messengers, such as IP_3 and Ca^{2+}, amongst platelets is stimulated by Cxs upon contact or improvements in the interactions that encourage outside-in signalling.

4.13.4 ^{62}Gap27 Suppresses Clot Retraction and Spreading

The effects of ^{62}Gap27 on outside-in signalling in clot retraction were also studied. This enabled an understanding of the effects of ^{62}Gap27 on platelets in a more physiological environment that includes the coagulation system, plasma proteins and other blood cells (Shattil et al., 1998; Osdoit and Rosa, 2001; Shattil and Newman, 2004; Li et al., 2010). Previous studies have shown that Cx37 and Cx40 mimetic peptides can block clot retraction (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). In agreement with the findings of those studies, clot weight increased three-fold following treatment with ^{62}Gap27 compared with the weight of the scrambled peptide-treated sample, which indicates that ^{62}Gap27 influences outside-in signalling via integrin $\alpha_{IIb}\beta_3$.

In addition, treatment with ^{62}Gap27 inhibited platelet spreading on fibrinogen, which suggests that it suppressed outside-in signalling. Inadequate adhesion to fibrinogen

suggests that integrin $\alpha_{IIb}\beta_3$ did not adopt an open conformation after treatment with ^{62}Gap27. This may partially explain the decreases in spreading and clot retraction, as the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ is required to initiate outside-in signalling, which is regulated by $G_{\alpha 13}$ and Rho GTPase (Arthur et al., 2000; Flevaris et al., 2007).

Despite our limited knowledge about the mechanisms through which Cx62 modulates platelet function, a wide range of platelet functions were inhibited by ^{62}Gap27. These include Ca^{2+} mobilisation and dense granule secretion. In conjunction with results from previous studies, our data suggest that many important functions are controlled by Cx62 via currently unknown hemichannels. Previous work showed that GJs form amongst platelets in a thrombus, which suggests that GJs may play a role in constant contact-dependent signalling to control thrombus stability and clot retraction (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

The possibility remains that the development of aggregates after platelet stimulation strictly requires the function of hemichannels and GJs between neighbouring platelets. A better understanding of the nature of the GJ- and hemichannel-dependent signalling events that modulate platelet activation and clot retraction is necessary. In addition, the roles of the Cxs expressed on platelets in the interactions of platelets with the cells of the vasculature must be identified. It is critical to determine the type of signalling molecules that are transmitted by the platelet hemichannels or GJs.

In summary, the results presented in this chapter demonstrate an inhibitory effect of ^{62}Gap27 on human and mouse platelet function. Platelet aggregation stimulated by a range of agonists was inhibited by ^{62}Gap27 treatment whereas treatment with a scrambled peptide did not affect aggregation. ^{62}Gap27 inhibited CRP-XL- or thrombin-stimulated fibrinogen binding, P-selectin exposure, ATP release and TxB_2 synthesis,

indicating a fundamental role for Cx62 hemichannels in platelet activation, granule secretion and the inside-out signalling that leads to affinity upregulation of integrin $\alpha_{IIb}\beta_3$. Consistent with this, treatment of platelets with ^{62}Gap27 also reduced peak Ca^{2+} mobilisation, platelet spreading and clot retraction. Taken together, these findings suggest that Cx62 regulates inside-out and outside-in signalling. The effect of ^{62}Gap27 was selective for Cx62, as observed using $Cx57^{-/-}$ mice, and Cx62/57 hemichannels function independently of Cx37 and Cx40. The negative regulation of numerous platelet functions via ^{62}Gap27 may be due to its influence on different signalling pathways (GPVI and GPCR) in platelets. The next chapter will discuss the inhibitory effects of ^{62}Gap27 on thrombus formation (*in vitro* and *in vivo*) and on haemostasis. It also focuses on the mechanisms through which Cx62 function is controlled in platelets.

5 ^{62}Gap27 Regulates GPVI and Cyclic Nucleotide Signalling in Platelets to Reduce Thrombosis

5.1 Introduction

Following vascular injury, circulating platelets tether to the injured site, leading to platelet adhesion, aggregation and, ultimately, the formation of a haemostatic plug. The aggregation stage involves the secretion of signalling molecules by the platelets that act via autocrine and paracrine signalling systems to ensure the stimulation and accumulation of other platelets at the injury site. ADP, which binds to $P2Y_1$ and $P2Y_{12}$ receptors, is released from the dense granules of activated platelets (Jantzen et al., 1999). Similarly, TxA_2 is a signalling molecule that is rapidly secreted by activated platelets; it binds to the TP receptor (Li et al., 2003).

Complicated intracellular signalling pathways in platelets control their responses and activation statuses based on external signals. For example, PKC and PLC are vital signals for the recruitment, stimulation and accumulation of platelets at the site of injury. Upon PKC and PLC signalling, the levels of Ca^{2+} and DAG are increased, leading to inside-out signalling (Balendran et al., 2000).

In contrast, cGMP and cAMP are vital signals that negatively regulate platelets ensuring that in the undamaged circulation they remain in a quiescent state and prevent unnecessary platelet activation. The activation of sGC and AC elevates the levels of cGMP and cAMP respectively. This is followed by cGMP and cAMP mediated activation of PKG and PKA respectively, which down-regulates thrombus growth. These pathways mainly function to negatively regulate platelet activation via different pathways (Massberg et al., 1999; Offermanns, 2006).

^{62}Gap27 can inhibit various platelet activities, such as aggregation, degranulation, outside-in signalling and Ca^{2+} mobilisation, that are essential processes for platelet activation and thrombus formation and are directly regulated by different signalling

pathways in platelets. In this chapter, the effects of ^{62}Gap27 on thrombosis and haemostasis were investigated. Additionally, this study was extended to determine if the ^{62}Gap27-mediated effects on platelets are due to its ability to regulate platelet signalling mechanisms.

5.2 ^{62}Gap27 Inhibits Thrombus Formation *In Vitro*

Given the ability of ^{62}Gap27 to restrict platelet activation downstream of various platelet activators, such as GPVI (CRP-XL) and GPCR (thrombin, ADP and U46619) agonists, further experiments were performed to investigate its effects on the regulation of thrombus formation *in vitro* under arterial flow conditions. It is critical to examine the impact of ^{62}Gap27 in a flow assay that approximates physiological conditions. The assessment of thrombus formation in whole blood using a flow assay facilitates the exploration of the impact of ^{62}Gap27 on platelets under condition more akin to those encountered *in vivo*. Traditional *in vitro* assays, like the aggregation assay, are performed with isolated platelets that are stirred in the presence of an agonist. Therefore, in those assays, various factors that affect thrombus formation are not considered. For example, platelets are briefly exposed *in vivo* to subendothelial collagens, which facilitates the formation of a platelet monolayer. Then, prothrombotic molecules secreted from platelets are mainly responsible for the recruitment of additional platelets to augment thrombus formation. In addition, under physiological conditions, platelets are consistently affected by other blood cells, plasma proteins and shear rates, which regulate their function and the stability of the thrombus.

Vena8 Biochips were coated with type I collagen (100 μg/mL) and citrated human blood (collected in a vacuum-sealed tube) was incubated with the lipophilic dye 3,3′-dihexyloxacarbocyanine iodide (DiOC6; 5 μM) for 1 hour at 30 °C. Excess collagen was washed from the coated channels of the microfluidic chips with modified Tyrodes-HEPES buffer. The DiOC6-loaded blood was incubated with the scrambled peptide or ^{62}Gap27 (100 μg/mL) for 5 minutes at 30 °C prior to perfusion across the collagen-coated microfluidic channels under arterial flow conditions (20 dyn/cm^2). An argon laser was used to excite fluorescence at 488 nm and emission was observed at 500–520

nm. Images of thrombus formation, focused on a single section, were obtained with a Nikon A1R confocal microscope (20× objective lens) every second for 600 seconds. NIS-Elements software was used to determine mean thrombus fluorescence intensity, which was normalised to the level of fluorescence near the end of the experiment in the sample incubated with the scrambled peptide.

Figure 5.1 shows representative images from near the end of the experiment. The scrambled peptide-treated samples created large, bright, stable thrombi that continued to grow in fluorescence intensity over 600 seconds. The thrombi in the ^{62}Gap27-treated samples were smaller and unstable dissociating under the flow conditions and therefore they did not reach to the level of thrombus size developed in the absence of ^{62}Gap27. Overall, ^{62}Gap27 decreased the fluorescence intensities of thrombi by approximately 65% after 600 seconds (Figure 5.1a, b). This observation suggests that ^{62}Gap27 inhibits platelet stimulation, even when plasma proteins and other blood cells are present, and has the potential to impact substantially on thrombus formation.

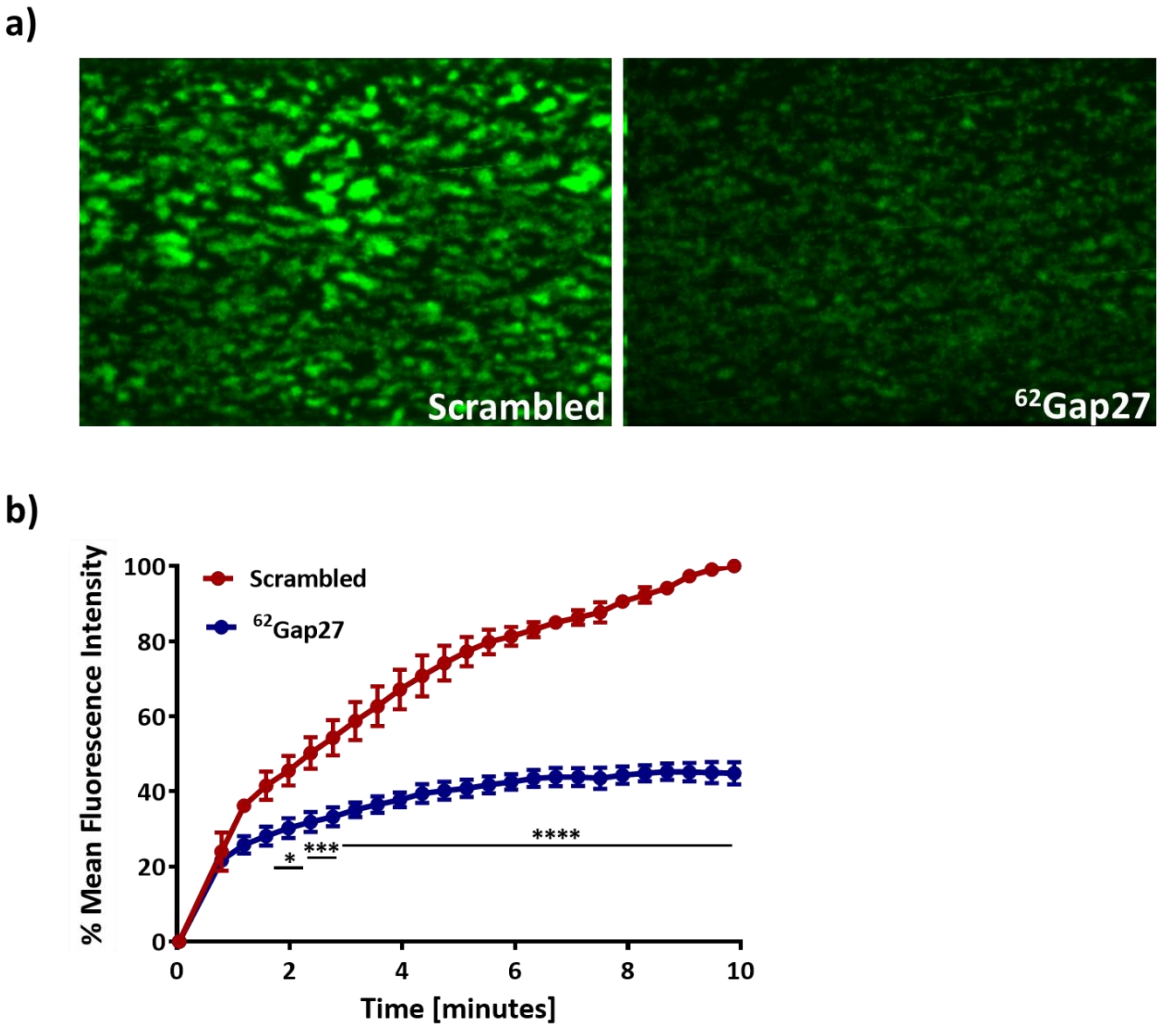


Figure 5.1: ^{62}Gap27 attenuates thrombus formation *in vitro*.

Citrated human blood was incubated with DiOC6 (5 µM) for 1 hour at 30 °C, then treated with scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes. The loaded blood was then perfused across Vena8 Biochips (coated with 100 µg/mL collagen) under arterial flow conditions (20 dyn/cm^2). **(a)** Images are representative of thrombus formation (endpoint) in samples treated with scrambled peptide or ^{62}Gap27. An argon laser was used to excite the samples and emission was observed at 500–520 nm. A Nikon A1R confocal microscope (20× objective) was used to observe thrombus formation; images were saved every second for 600 seconds. **(b)** The mean thrombus fluorescence intensities for the scrambled peptide- and ^{62}Gap27-treated samples are shown. The values were obtained using NIS-Elements software and normalised to the fluorescence level of the scrambled peptide-treated sample at the end of the experiment. Data represent the mean ± SEM (n = 5), analysed by two-way ANOVA. *$P \leq 0.05$, ***$P \leq 0.001$ and ****$P \leq 0.0001$

5.3 ^{62}Gap27 Does Not Alter Platelet Adhesion to Collagen

A thrombus mainly comprises platelets working together, but the base of the thrombus includes platelets that interact directly with collagen. Hence, to study if ^{62}Gap27 decreased thrombus size by preventing preliminary platelet–collagen interactions, *in vitro* thrombus formation experiments were performed in the presence of the integrin $\alpha_{IIb}\beta_3$ antagonist integrilin to block platelet–platelet interactions. The lack of interaction between platelets would prevent thrombus formation, allowing the study of the effects of ^{62}Gap27 specifically on platelet adhesion.

Whole human blood loaded with $DiOC_6$ and pre-treated with integrilin (4 μM) to prevent aggregation was perfused through Vena8 Biochips coated with type I collagen (100 μg/mL) along with scrambled peptide or ^{62}Gap27 under arterial flow conditions (20 dyn/cm^2). The samples were excited with an argon laser and emission was observed at 500–520 nm. Thrombus formation was observed with a Nikon A1R confocal microscope (20× objective) and images were obtained every second for 300 seconds.

^{62}Gap27 did not alter platelet adhesion to collagen (Figure 5.2a, b). Thus, ^{62}Gap27 appears to regulate platelet activity within thrombi thereby influencing the propagation and growth of thrombi, but has no effect on the initial attachment of platelets to collagen.

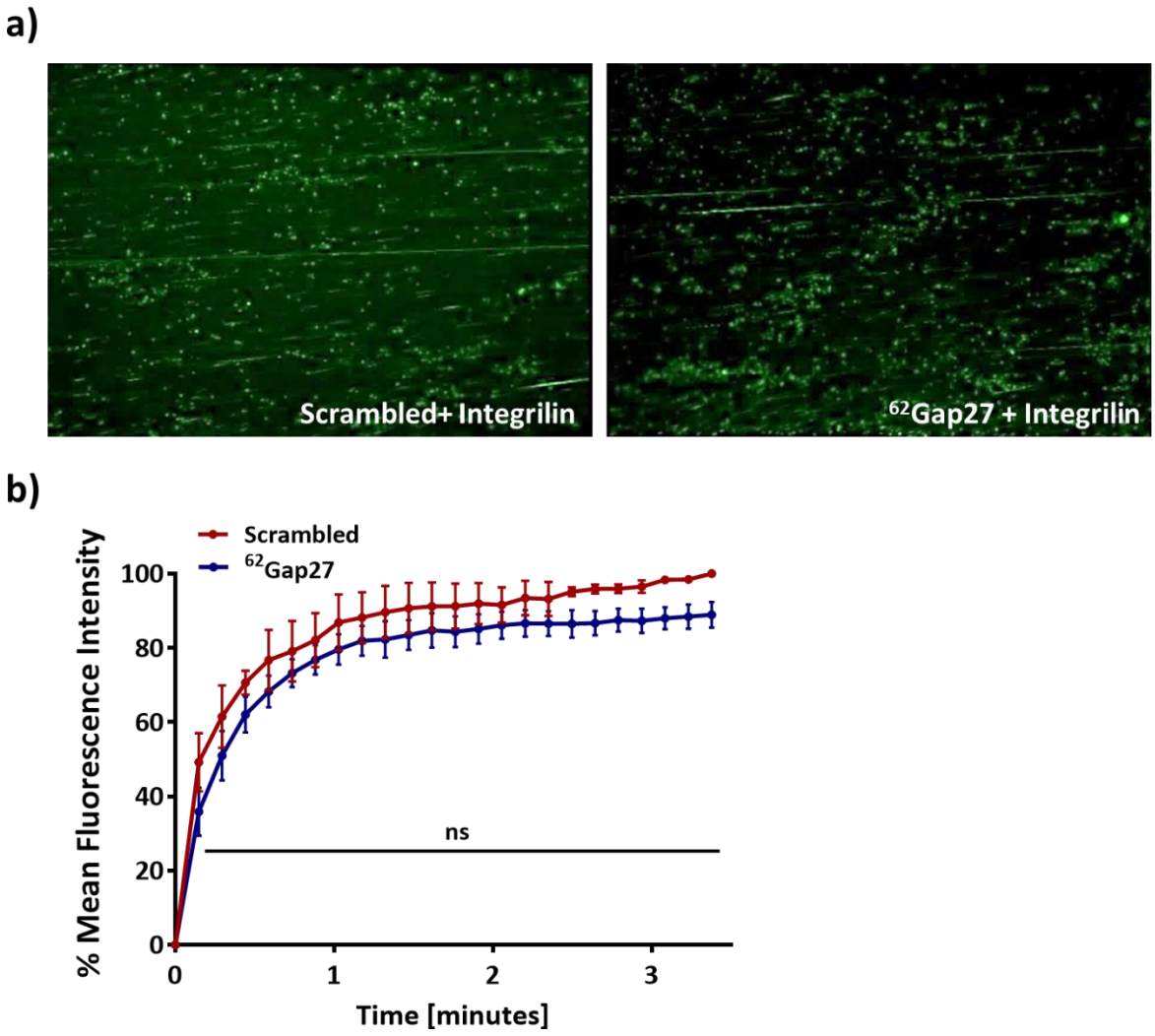


Figure 5.2: ^{62}Gap27 does not decrease platelet adhesion to collagen during thrombus formation *in vitro*.

Citrated human blood was incubated with DiOC6 (5 µM) for 1 hour at 30 °C, then treated with the scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes in the presence of integrilin (4 mM). The loaded blood was perfused across Vena8 Biochips (coated with 100 µg/mL collagen) under arterial flow conditions (20 dyn/cm^2). **(a)** The images are representative of thrombus formation (endpoint) in the samples treated with the scrambled peptide or ^{62}Gap27. An argon laser was used to excite the samples and emission was observed at 500–520 nm. A Nikon A1R confocal microscope (20× objective) was used to observe thrombus formation; images were saved every second for 300 seconds. **(b)** The mean thrombus fluorescence intensities for the scrambled peptide- and ^{62}Gap27-treated samples are shown. The values were obtained using NIS-Elements software and normalised to the fluorescence level of the scrambled peptide-treated sample at the end of the experiment. Data represent the mean ± SEM (n = 5).

5.4 ^{62}Gap27 Inhibits Thrombosis and Haemostasis in Mice

Given that ^{62}Gap27 negatively regulates thrombus formation *in vitro*, its influence on thrombosis and haemostasis was analysed *in vivo.* The *in vivo* thrombus formation assay allows examination of platelet function in a more physiological environment than the *in vitro* assay due to presence of factors such as endothelial derived factors, blood pressure, blood flow and metabolism.

This *in vivo* assay measures the potential of fluorescently labelled platelets to create thrombi after laser-induced injury to an arteriole in the mouse cremaster muscle. The cremaster muscles of the testicles of anaesthetised C57BL/6 mice were exteriorised, the connective tissue was removed, and an incision was created. The muscle was placed on a glass slide as a single sheet and completely hydrated with buffer. The platelets were labelled with DyLight® 649-conjugated anti-GPIbα antibody. Scrambled control peptide (100 µg/mL) or ^{62}Gap27 (100 µg/mL) were injected intravenously. The cremaster arteriole wall was injured with a MicroPoint ablation laser unit. Previous work from our lab has established that this injury causes damage to endothelial cells (ECs) and exposure of collagen but does not rupture the vessel (Sasikumar et al., 2018). Thrombus formation was monitored for 5 minutes. Images were obtained with a digital camera prior to and following injury, then analysed with SlideBook (version 6) software. Procedures on these mice require micro-surgery expertise and were therefore performed in collaboration with Dr P Sasikumar.

Large, stable thrombi were formed in the scrambled peptide-treated mice (100 µg/mL) (Figure 5.3ai), which is typical of non-treated mice (Unsworth et al., 2017a). ^{62}Gap27 treatment (100 µg/mL) had an adverse effect on the production and stability of thrombi, as indicated by constant thrombus mobilisation and reconstruction. The peak

fluorescence intensity was used to determine the maximum size of the thrombus. The intensity of the peak fluorescence was almost 80% lower in mice that had undergone treatment with ^{62}Gap27 than in mice treated with the scrambled control peptide (Figure 5.3aii, aiii). This finding suggests that fewer platelets accumulate to create a thrombus following ^{62}Gap27 treatment compared to treatment with scrambled peptide.

A tail bleeding assay was performed on C57BL/6 mice to determine the effects of ^{62}Gap27 on haemostasis. The volume of ^{62}Gap27 (stock: 2 mg/ml) or scrambled peptide (stock: 2 mg/ml) injected into their femoral veins was expected to give a concentration of 100 μg/mL in the blood, assuming a total blood volume of 2 mL in a 25-g mouse. After 5 minutes, the tips of the tails (≤0.3 cm) were removed with a sharp razor blade and placed in sterile saline (37 °C). The time until the bleeding stopped (seconds) was measured.

The scrambled peptide-treated mice bled for approximately 450 seconds, whereas the mean bleeding time for the mice treated with ^{62}Gap27 extended to almost 983 seconds (Figure 5.3b). Prolonged bleeding time in ^{62}Gap27-treated mice reflects the potential involvement of Cx62/57 in the regulation of haemostasis *in vivo*.

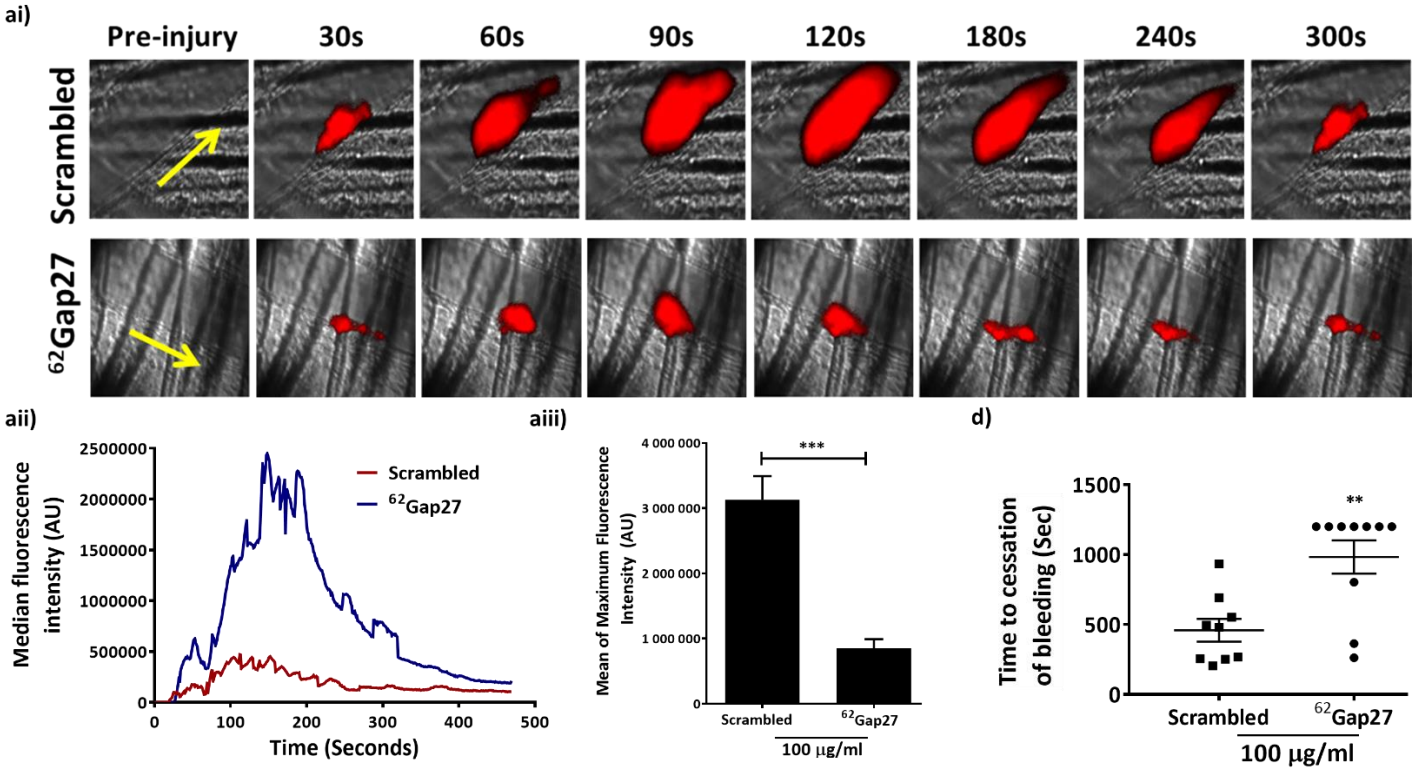


Figure 5.3: ^{62}Gap27 restricts thrombus formation and increases bleeding time in C57BL/6 mice.

(a) Thrombus formation in C57BL/6 mice was assayed by intravital microscopy in a laser-induced injury model. DyLight® 649-conjugated anti-GPIbα antibody was used to fluorescently label the platelets. The scrambled peptide or ^{62}Gap27 was injected intravenously into C57BL/6 mice. After 5 minutes, a laser injury was inflicted. **(ai)** The representative images of thrombi were captured at the indicated intervals. The direction of blood flow is shown by an arrow. **(aii)** The median fluorescence intensities of 21 thrombi from 5 mice each in the scrambled and ^{62}Gap27 groups and **(aiii)** the means of the peak fluorescence intensities of the thrombi were calculated. **(b)** C57BL/6 mice that had been treated with the scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes (n = 10) were tail-bled to determine the bleeding time following the cutting of the tail tip. Data represent the mean ± SEM. *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ were calculated with the non-parametric Mann–Whitney U test.

5.5 ^{62}Gap27 Suppresses GPVI-mediated Signalling

^{62}Gap27 significantly inhibited several CRP-XL-regulated platelet functions and thrombus formation *in vivo*. These findings indicate that the inhibitory effects of ^{62}Gap27 may result from the modulation of signalling downstream of GPVI. Therefore, the impact of ^{62}Gap27 on the GPVI signal transduction pathway was examined. Binding of GPVI to collagen leads to GPVI clustering and phosphorylation of the FcR γ-chain on the immunoreceptor tyrosine activation motif (ITAM) by Src family kinases (SFKs), including Lyn and Fyn, (Gibbins et al., 1996; Briddon and Watson, 1999). Following the phosphorylation of FcR γ-chain, Syk binds to this and is stimulated (Shiue et al., 1995) and in turn activated form of Syk phosphorylates the adaptor protein LAT (Zhang et al., 1998).

LAT serves as nucleus for the assembly of a signalling complex of proteins (signalosome) incorporating key effectors for platelet activation (Gibbins et al., 1998; Gross et al., 1999). The signalosome results in the recruitment of phosphoinositide 3-kinase (PI3K) and the subsequent conversion of PIP2 into PIP3, followed by PIP3-induced activation and phosphorylation of PLCγ2 at the platelet membrane (Barry and Gibbins, 2002). Activated PLCγ2 catalyses the production of IP_3 and DAG, which are essential messengers (Quek et al., 1998; Oda et al., 2000). IP_3 stimulates the release of Ca^{2+} from intracellular storage sites (Varga-Szabo et al., 2009) and DAG activates some PKC isoforms. These events are indispensable for the modification of platelet shape, integrin activation and the release of granule contents into the circulation (Hathaway and Adelstein, 1979; Shattil and Brass, 1987; Watson et al., 2005).

The impact of ^{62}Gap27 on GPVI signalling was examined by first incubating washed platelets (4×10^8 cell/mL) with indomethacin (20 μM), cangrelor (1 μM), MRS2179

(100 µM) and EGTA (1 mM) to prevent platelet aggregation (stimulated by secondary mediators such as TxA_2 and ADP), then treated with scrambled peptide (100 µg/mL) or ^{62}Gap27 (50 and 100 µg/mL) for 5 minutes before activation with CRP-XL (1 µg/mL) for 90 seconds. Finally, the tyrosine phosphorylation of GPVI signalling proteins was observed by immunoblotting.

Consistent with previous studies, treatment with CRP-XL caused a substantial increase in the tyrosine phosphorylation of several proteins (Unsworth et al., 2017a). Pre-incubation with ^{62}Gap27 treated samples had a significantly lower total tyrosine phosphorylation level compared to scrambled peptide-treated sample. Approximately 32% lower phosphorylation was observed after treatment with 50 µg/mL ^{62}Gap27, with further reduction to 41% in samples treated with 100 µg/mL ^{62}Gap27 (Figure 5.4a, b).

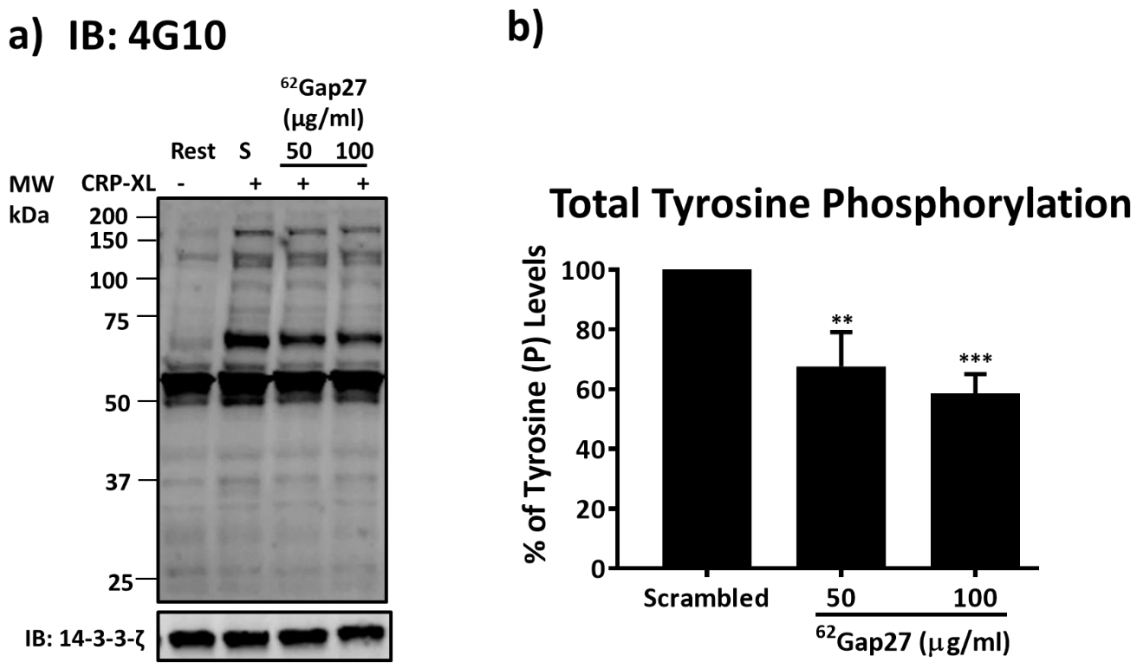


Figure 5.4: ^{62}Gap27 inhibits tyrosine phosphorylation in CRP-XL-activated platelets.

Platelets (4×10^8 cells/mL) were pre-treated with scrambled control peptide or ^{62}Gap27 (50 and 100 µg/mL) for 5 minutes, then activated with CRP-XL (1 µg/mL) for 90 seconds in the presence of indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA (1 mM). Laemmli sample buffer was used to lyse the samples and whole cell lysates were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with an anti-phospho-tyrosine antibody (4G10) to detect tyrosine phosphorylation. **(a)** A representative immunoblot is presented. **(b)** The quantified tyrosine phosphorylation level after treatment with ^{62}Gap27 is shown as the proportion of the phosphorylation in the scrambled peptide-treated samples. 14-3-3-ζ was detected by immunoblotting as the loading control. The results are displayed as the mean ± SEM ($n \geq 3$). $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ were calculated by one-way ANOVA.

Abbreviations: S – Scrambled Peptide

Since total CRP-XL-mediated tyrosine phosphorylation was suppressed by ^{62}Gap27, the effect of ^{62}Gap27 was further analysed on the preliminary GPVI signalling events, with a specific focus on Syk, LAT and PLCγ2. In alignment with previous studies, treatment with scrambled peptide resulted in CRP-XL-mediated increases in phosphorylation of these signalling components, which were similar to those observed in untreated samples (Unsworth et al., 2017a). ^{62}Gap27 inhibited the phosphorylation of Syk at its autophosphorylation site Y525/526 (Sada et al., 2001). Pre-treatment with 50 and 100 µg/mL ^{62}Gap27 for 5 minutes inhibited CRP-XL-stimulated (90 seconds) tyrosine phosphorylation of Syk by 20% and 45%, respectively (Figure 5.5ai, ii).

Activated Syk phosphorylates the transmembrane protein LAT, thereby triggering a signalosome complex to form that organises downstream signalling. Scrambled peptide-treated samples demonstrated a profound CRP-XL-stimulated enhancement in the phosphorylation of LAT at Y200 (the equivalent of Y171; phosphorylated by SFKs and Syk) (Jiang and Cheng, 2007). Incubation with ^{62}Gap27 suppressed this tyrosine phosphorylation by 29% and 50% at the concentrations of 50 and 100 µg/mL, respectively (Figure 5.5bi, ii).

The LAT signalosome participates in the recruitment and phosphorylation of PLCγ2. Consistent with the suppression of the phosphorylation of the initial GPVI signalling proteins Syk and LAT, lower levels of PLCγ2 phosphorylation at Y1217 (a Bruton's tyrosine kinase (Btk) phosphorylation site) (Watanabe et al., 2001) were observed in the presence of ^{62}Gap27. PLCγ2 phosphorylation was decreased by nearly 25% and 52% upon treatment with 50 and 100 µg/mL ^{62}Gap27, respectively (Figure 5.5ci, ii).

The negative impact of ^{62}Gap27 may not be limited to the modulation of early GPVI signalling. It may affect downstream entities, as both PLCγ2 activation and CRP-XL-

triggered Ca^{2+} mobilisation are also inhibited by ^{62}Gap27. In particular, IP_3-stimulated Ca^{2+} mobilisation and serine/threonine PKC family activation through DAG, which are events that take place after the phosphorylation of PLCγ2 (Yacoub et al., 2006; Varga-Szabo et al., 2009; Bye et al., 2016).

Hence, the effect of ^{62}Gap27 on PKC activity was investigated with an antibody raised against phosphorylated PKC substrate recognition sequence. Upon stimulation with CRP-XL, scrambled peptide-treated samples exhibited an increase in PKC substrate phosphorylation, which was reduced by approximately 40% and 50% in the presence of 50 and 100 μg/mL ^{62}Gap27, respectively (Figure 5.6a, b).

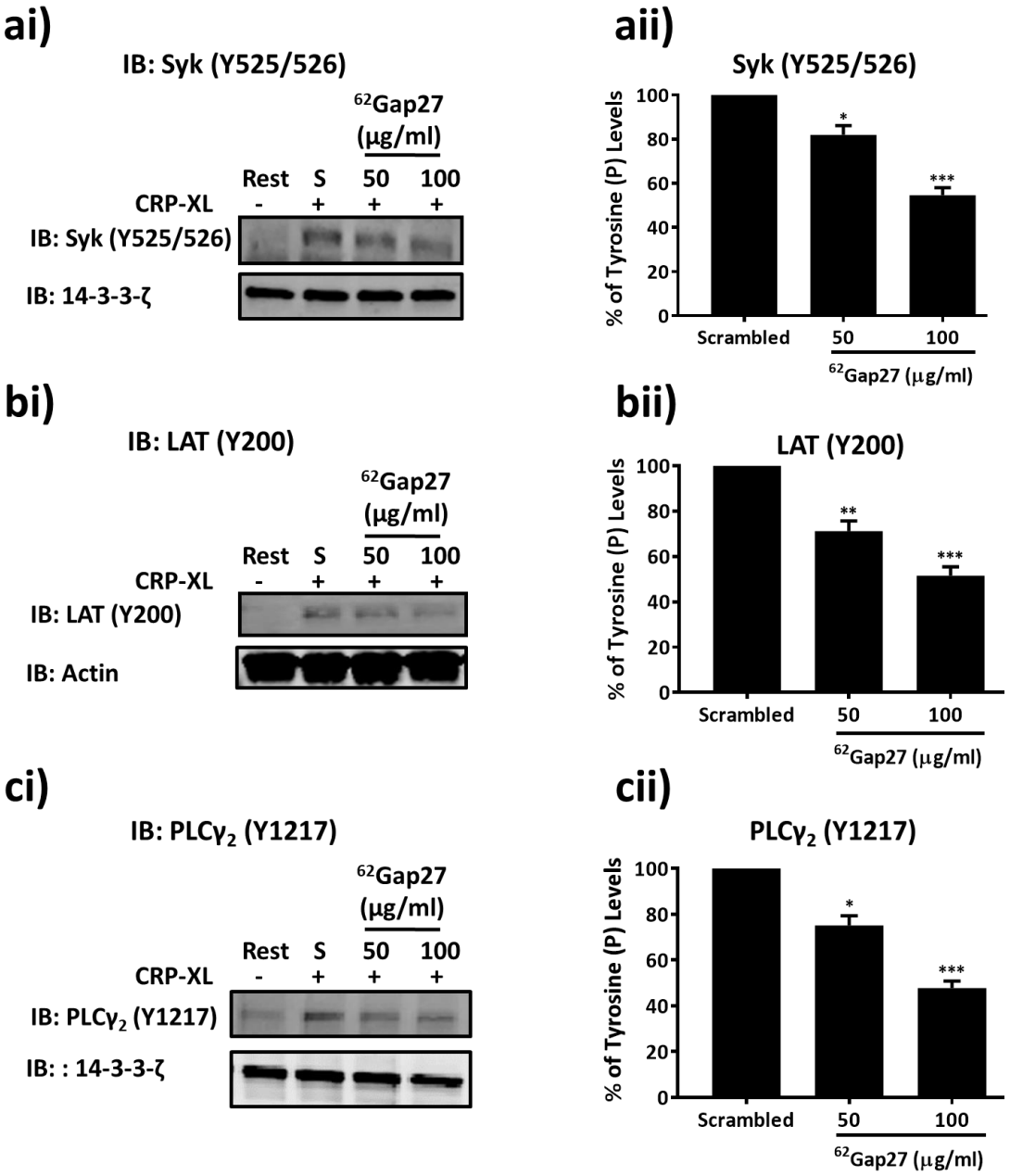


Figure 5.5: ^{62}Gap27 inhibits CRP-XL-stimulated Syk, LAT and PLCγ2 tyrosine phosphorylation.

Washed platelets (4×10^8 cells/mL) were treated with the scrambled peptide or ^{62}Gap27 (50 and 100 μg/mL) for 5 minutes, then activated with CRP-XL (1 μg/mL) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA acid (1 mM). Laemmli sample buffer was used to lyse the samples, which were separated by SDS-PAGE and transferred to PVDF membranes. Site-specific phospho-antibodies for Syk (Y525/526), LAT (Y200) and PLCγ2 (Y1217) were used to determine their phosphorylation levels. Representative immunoblots are shown for the phosphorylation levels of **(a)** Syk, **(b)** LAT and **(c)** PLCγ2 following treatment with scrambled peptide or ^{62}Gap27. The quantifications of the phosphorylation levels of **(a)** Syk, **(b)** LAT and **(c)** PLCγ2 in samples treated with ^{62}Gap27 are presented as the proportions of the levels in the scrambled peptide-treated samples. Actin or 14-3-3-ζ was detected by immunoblotting as the loading control. The results represent the mean ± SEM ($n \geq 3$). *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ were calculated by one-way ANOVA.

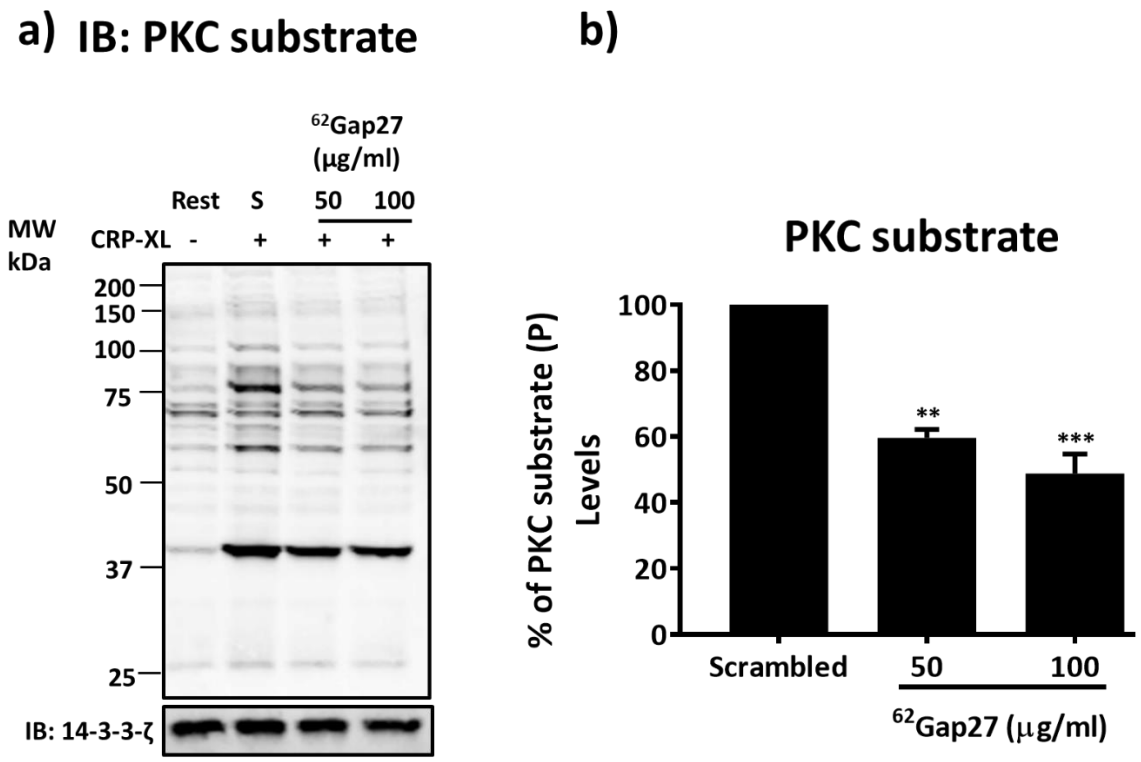


Figure 5.6: ^{62}Gap27 inhibits CRP-XL-stimulated PKC substrate recognition sequence phosphorylation.

The washed platelets (4×10^8 cells/mL) were incubated with the scrambled peptide or ^{62}Gap27 (50 and 100 μg/mL) for 5 minutes, then activated with CRP-XL (1 μg/mL) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). Laemmli sample buffer was used to lyse the samples, which were separated by SDS-PAGE and transferred to PVDF membranes. An antibody against the phosphorylated PKC substrate recognition sequence was used to determine its level of phosphorylation in the blot. **(a)** A representative immunoblot for ^{62}Gap27-treated samples is shown. **(b)** The quantified data for the levels of PKC substrate phosphorylation in the presence of ^{62}Gap27 were calculated as the percentage of that in the scrambled peptide-treated controls. 14-3-3-ζ was detected by immunoblotting as the loading control. The results represent the mean ± SEM ($n \geq 3$). $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ were calculated by one-way ANOVA.

Abbreviations: S – Scrambled Peptide

5.6 ^{62}Gap27 Does Not Affect Initial GPCR Signalling in Platelets

Given the established negative regulation of ^{62}Gap27 on the GPCR agonist thrombin-mediated platelet aggregation, degranulation and Ca^{2+} mobilisation, an investigation of thrombin-induced platelet signalling in presence of ^{62}Gap27 was carried out. Thrombin is a platelet agonist that signals through $G_{\alpha q}$. The thrombin receptors PAR-1 and PAR-4 are coupled to $G_{\alpha q}$ proteins (Brass, 2003). The phosphorylation of PLCβ following receptor activation drives the cleavage of PIP2 to generate IP_3 and DAG. As a result, DAG and IP_3 increase the intracellular Ca^{2+} levels, activate PKC, promote degranulation and upregulate integrin $\alpha_{IIb}\beta_3$ (Offermanns, 2006).

Washed platelets (4×10^8 cells/mL) were incubated in non-aggregating conditions with indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA (1 mM), followed by incubation with the scrambled peptide or ^{62}Gap27 (50 and 100 µg/mL) for 5 minutes. Finally, the platelets were activated with thrombin (0.1 U/mL) and tyrosine phosphorylation was assessed via immunoblotting.

Treatment with scrambled peptide resulted in a considerable rise in the level of thrombin-induced total tyrosine phosphorylation. No differences in total tyrosine phosphorylation levels were observed between the platelets treated with ^{62}Gap27 (50 and 100 µg/mL) and those treated with the scrambled peptide for 5 minutes after activation with thrombin (0.1 U/mL) (Figure 5.7a). Given the decrease in thrombin-stimulated Ca^{2+} mobilisation, which is related to the PKC activator DAG, in the presence of ^{62}Gap27, the effect of the mimetic peptide on the degree of serine/threonine PKC substrate phosphorylation was assessed. Upon stimulation with thrombin, scrambled peptide-treated samples exhibited increased PKC substrate phosphorylation.

Similarly, platelets pre-incubated with ^{62}Gap27 had comparable thrombin-activated phosphorylation of PKC to scrambled peptide-treated platelets (Figure 5.7b).

Although ^{62}Gap27 has inhibitory effects on various platelet functions and Ca^{2+} mobilisation downstream of GPCR agonists (Chapter 4, section 4.10), no evidence was found that it affects total tyrosine and PKC phosphorylation. This may be because the impact of ^{62}Gap27 on platelet activity and Ca^{2+} mobilisation was observed only at low GPCR agonist concentrations (determined mainly using EC_{50}). As it was not possible to detect phosphorylation levels at such low agonist concentrations, the impact of ^{62}Gap27 was examined in the presence of a high agonist concentration, which may explain the lack of effect on GPCR signalling. Furthermore, the impact of ^{62}Gap27 on GPVI signalling and its ability to restrict platelet responses to various agonists were modest. This indicates that there may be another mechanism that accounts for the ability of ^{62}Gap27 to restrict platelet functions activated by various agonists acting on distinct signalling pathways.

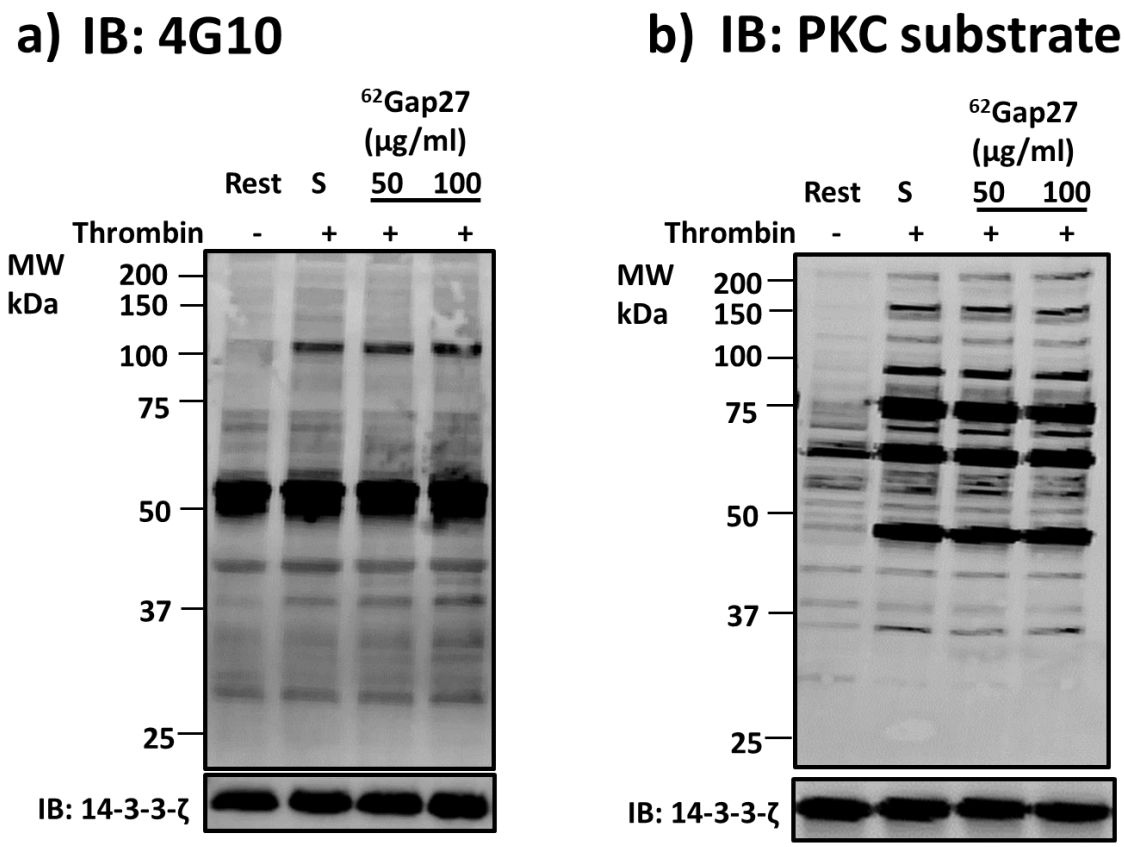


Figure 5.7: Thrombin-induced total tyrosine and PKC phosphorylation levels are not altered in the presence of ^{62}Gap27.

Washed human platelets (4×10^8 cells/mL) incubated in the presence of indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA (1 mM) were treated with the scrambled peptide or ^{62}Gap27 (50 and 100 µg/mL) for 5 minutes, followed by thrombin stimulation (0.1 U/mL). Laemmli sample buffer was used to lyse the samples and whole cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes. Antibodies to detect (**a)** total tyrosine phosphorylation and (**b)** PKC phosphorylation were used for immunoblotting. 14-3-3-ζ was detected by immunoblotting as a loading control. Representative blots from 5 distinct experiments are presented.

Abbreviations: S – Scrambled Peptide

5.7 ^{62}Gap27 Upregulates Inhibitory Pathway-mediated Signalling

^{62}Gap27 did not affect GPCR-regulated signalling in platelets (4G10 and PKC). However, ^{62}Gap27 inhibited various platelet functions, including thrombus production (*in vitro* and *in vivo*) and haemostasis, downstream of different agonists across several signalling pathways. This may suggest another mechanism that explains the ability of ^{62}Gap27 to inhibit platelet functions activated by various agonists acting on the GPVI and GPCR signalling pathways. Hence, the study was expanded to assess if the aforementioned effects of ^{62}Gap27 on platelets are mediated by platelet signalling pathways distinct from GPVI signalling.

The principal endothelium-borne inhibitors of platelet stimulation are PGI_2 and NO. These inhibitors raise the levels of the cyclic nucleotides cAMP and cGMP. AC is activated by PGI_2 through the IP receptor, a G_s-coupled receptor (Offermanns, 2006). Subsequently, PKA is activated, triggering the phosphorylation of various signalling molecules, including Rap1b (Grunberg et al., 1995), which regulates integrin activation. However, the details of the mechanism are still unclear. The elevation of PKA mediated protein phosphorylation is associated with the downregulation of various aspects of platelet activation, such as aggregation, Ca^{2+} mobilisation and spreading (Yan et al., 2009).

In contrast, NO directly activates sGC. NO diffuses passively across the platelet membrane, where it attaches to and activates sGC. This causes the synthesis of cGMP, which, in turn, stimulates PKG activity (Radomski et al., 1987a; Radomski et al., 1987b). The activated form of PKG suppresses integrin activation, platelet adhesion and aggregation (Roberts et al., 2004). Therefore, the effects of ^{62}Gap27 on the inhibitory systems in platelets were examined.

The effects of ^{62}Gap27 on the levels of phosphorylation of VASP-S157 and VASP-S239 (PKA- and PKG-selective phosphorylation sites, respectively) were investigated in resting human platelets.

To establish that the assay was capable of detecting PKA- and PKG-dependent phosphorylation of VASP, platelets were stimulated with PGI_2 and PAPA-Nonoate (a NO donor), which stimulated high levels of VASP-S157 and -S239 phosphorylation, respectively (Figure 5.8a, b). Following treatment of platelets with ^{62}Gap27 (50 and 100 μg/mL) for 5 minutes, the phosphorylation of VASP-S157 was increased by approximately 7 and 12 folds, respectively (Figure 5.8ai, aii), but not VASP-S239 phosphorylation (Figure 5.8bi, bii). The scrambled peptide did not affect the phosphorylation of either site. Taken together, these findings indicate that the inhibitory influence of ^{62}Gap27 on platelet activation is also regulated by the upregulation of PKA activity and cyclic nucleotide signalling.

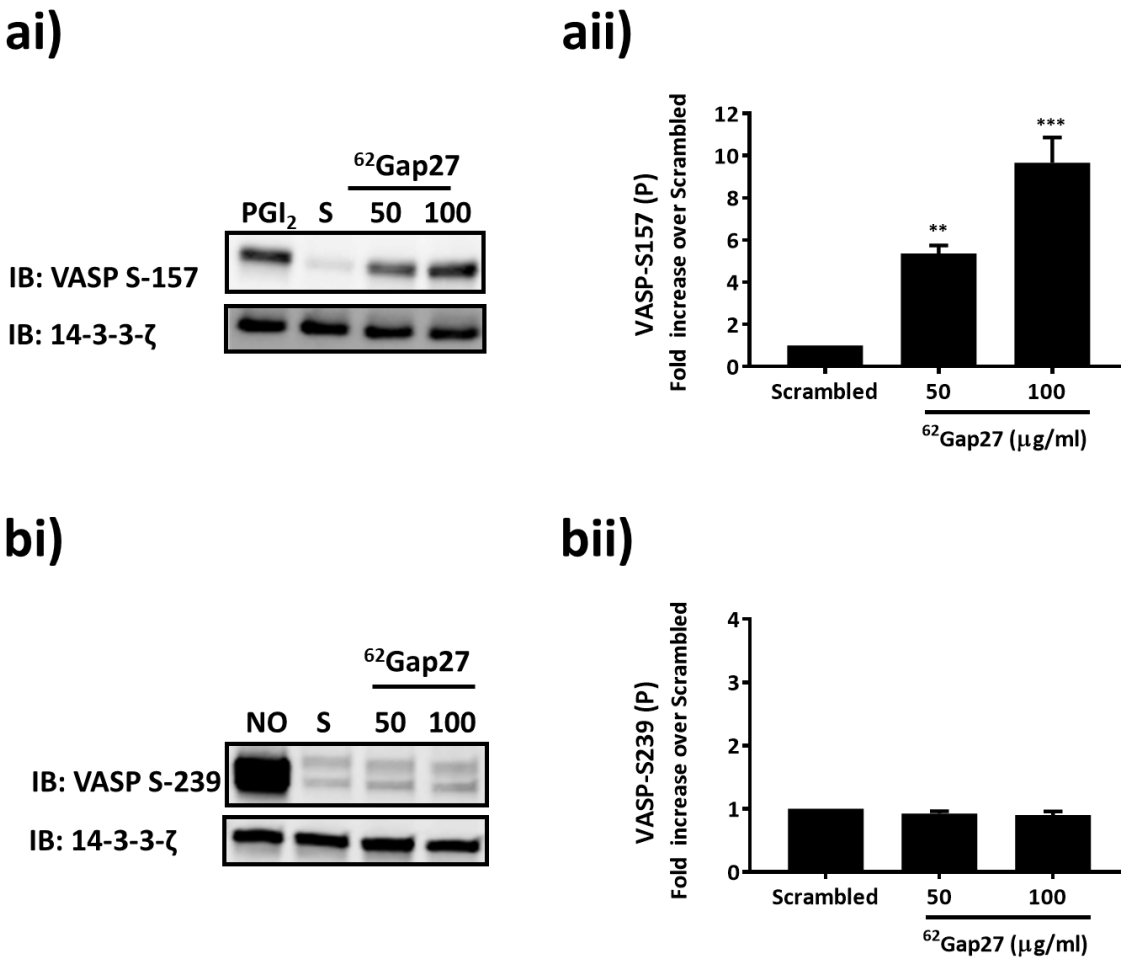


Figure 5.8: ^{62}Gap27 upregulates PKA but not PKG activity in resting platelets.

Resting washed human platelets (4×10^8 cells/mL) were treated with the scrambled peptide (100 μg/mL) or ^{62}Gap27 (50 and 100 μg/mL) for 5 minutes. Platelets were treated with PGI_2 (1 μg/mL) or PAPA-Nonoate (100 μM) for the stimulation of PKA- and PKG-mediated phosphorylation, respectively. Laemmli sample buffer was used to lyse the samples before separation by SDS-PAGE and transfer to PVDF membranes. The samples were then tested for VASP-S157 and VASP-S239 phosphorylation (the PKA- and PKG-selective phosphorylation sites, respectively). 14-3-3-ζ was detected by immunoblotting as a loading control. The results represent the mean ± SEM ($n \geq 5$). $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ were calculated by one-way ANOVA.

Abbreviations: S – Scrambled Peptide and NO – PAPA-Nonoate

5.8 ^{62}Gap27 Does Not Modulate cAMP Levels

cAMP is an important second messenger in cells. It participates in signal transduction in platelets to ensure that they remain inactive and do not form blood clots (Armstrong et al., 1989). When PGI_2 from the endothelium binds its $G_{\alpha s}$-coupled receptors on platelets, it increases cAMP concentrations by activating AC, which converts ATP to cAMP (Baragli et al., 2008). When the levels of cAMP are increased, PKA is activated, which inhibits platelets through the phosphorylation of VASP-S157 (Aszodi et al., 1999). Given the increased phosphorylation of VASP-S157 in the presence of ^{62}Gap27, the dependence of the increase on cAMP was investigated.

Unstimulated or stimulated (CRP-XL: 1 μg/mL or thrombin: 0.1 U/mL) washed platelets were pre-incubated for 5 minutes with scrambled peptide (100 μg/mL) or ^{62}Gap27 (50 and 100 μg/mL), then stirred in an aggregometer for 5 minutes. Lysis buffer was used to stop the reaction. Treatment with PGI_2 served as a positive control. The concentrations of cAMP in the samples were examined with a cAMP ELISA kit (Cell Signaling Technology, Hitchin, UK). A standard curve was produced using cAMP provided in the assay kit that was used to determine cAMP concentrations in the samples.

Interestingly, cAMP levels were not elevated in the presence of 50 and 100 μg/mL ^{62}Gap27 or scrambled peptide. Elevated cAMP levels were, however, detected in PGI_2-treated samples (Figure 5.9a, b, c). These findings suggest that ^{62}Gap27-induced phosphorylation of VASP-S157 is mediated through a mechanism that does not involve the elevation of cAMP levels. It was, therefore, important to determine whether the phosphorylation of VASP-S157 was indeed mediated by PKA.

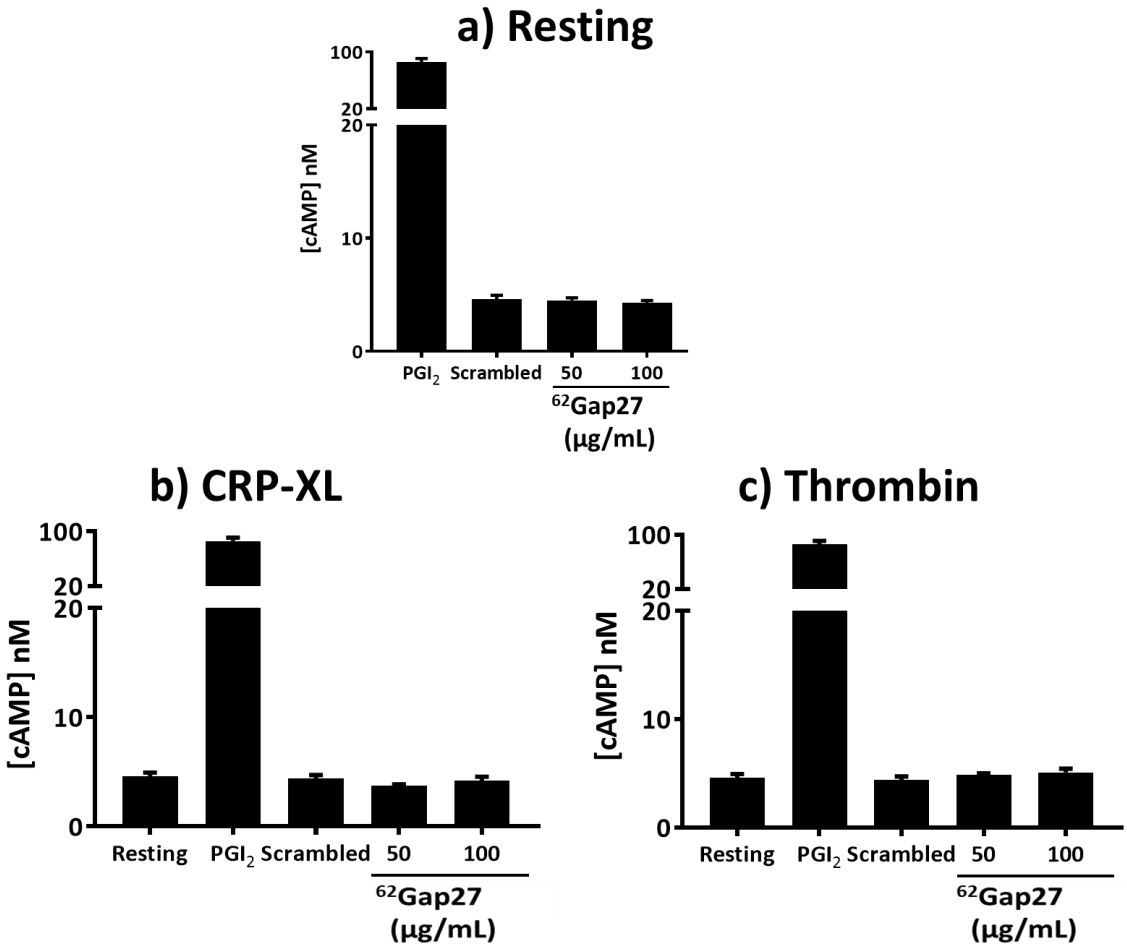


Figure 5.9: ^{62}Gap27 does not augment cAMP levels in platelets.

Washed human platelets (4×10^8 cells/mL) were incubated with the scrambled peptide (100 µg/mL) or ^{62}Gap27 (50 or 100 µg/mL), then left unstimulated **(a)** or stimulated with **(b)** CRP-XL (1 µg/mL) or **(c)** thrombin (0.1 U/mL) for 5 minutes at 37 °C in an aggregometer. Treatment with PGI_2 (1 µg/mL) was used as a positive control. Lysis buffer was used to stop the reactions and the samples were frozen. A cAMP ELISA kit was used according to the manufacturer's instructions to determine the concentrations of cAMP. The concentrations (pg/mL) produced by platelets incubated with ^{62}Gap27, scrambled peptide or PGI_2 in **(a)** unstimulated conditions or after stimulation with **(b)** CRP-XL or **(c)** thrombin are presented. The data represent the mean ± SEM (n = 12).

5.9 ^{62}Gap27 Activates PKA Independently of cAMP

PKA is a heterotetramer comprised of a dimer of regulatory subunits (R-subunits) that are attached to each other via two catalytic submits (c-subunits). Four molecules of cAMP attach cooperatively at the cAMP-binding sites on the R-subunits, thereby releasing the active c-subunits to phosphorylate the serine and threonine residues of PKA substrates (Skalhegg and Tasken, 2000). The PKA c-subunit structure includes an N-terminal loop, a small linker sequence and a larger helical C-terminal loop. The c-subunit N-terminal region participates in Mg-ATP binding and the C-terminal region includes the substrate binding sequence and initiates the catalytic reaction (Taylor et al., 2008).

Various compounds exploit the structural hallmarks of PKA to decrease its function. For example, H89 is a competitive antagonist that binds the ATP-binding region on the PKA c-subunit (Chijiwa et al., 1990). When the ATP-binding site is blocked on the c-subunit, it is unable to phosphorylate the corresponding serine and threonine residues, thereby inhibiting its function. PKA can also be inhibited by compounds like Rp-adenosine 3′,5′-cyclic mono-phosphorothioate (Rp-8-CPT-cAMPS), which compete for the cAMP-binding site on the R-subunit of PKA and prevent enzyme activation (de Wit et al., 1984). Furthermore, SQ 22536, (also known as 9-(tetrahydro-2-furanyl)-9H-purin-6-amine) inhibits AC activity and prevents cAMP production (Unsworth et al., 2017a; Unsworth et al., 2017b). ^{62}Gap27 has the ability to increase VASP-S157 phosphorylation without influencing cAMP levels. Hence, it is important to identify the underlying mechanisms through which ^{62}Gap27 controls VASP-S157 phosphorylation in the presence of these compounds (AC, cAMP and PKA blockers).

Further studies on the impact of ^{62}Gap27 on VASP-S157 phosphorylation were performed in resting human platelets. Resting platelets were incubated with H89 (10 µM), SQ 22536 (100 µM) or Rp-8-CPT-cAMPS (200 µM) for 5 minutes, then treated with 100 µg/mL scrambled peptide or ^{62}Gap27 for 5 minutes. The concentrations of these inhibitors were based on previously reported concentrations optimised in our lab for these assays (Unsworth et al., 2017a; Unsworth et al., 2017b).

The washed platelet samples treated with the positive control PGI_2 showed high levels of VASP-S157 phosphorylation. Following treatment with H89 (10 µM) (Figure 5.10a), SQ22536 (100 µM) (Figure 5.10b) and Rp-8-CPT-cAMPS (200 µM) (Figure 5.10c), VASP phosphorylation was reversed and no elevation was seen in the presence of PGI_2. These findings demonstrate that PGI_2-mediated VASP-S157 phosphorylation is mediated by PKA in cAMP dependent manner.

In contrast, after treatment with the PKA inhibitor H89 (10 µM), ^{62}Gap27-stimulated VASP-S157 phosphorylation was reversed (Figure 5.10a). This finding confirms that the effects of ^{62}Gap27 on VASP-S157 are mediated via the upregulation of PKA activity. ^{62}Gap27-stimulated PKA phosphorylation was not inhibited after treatment with SQ 22536 (100 µM) (Figure 5.10b), the inhibitor of AC function, or by Rp-8-CPT-cAMPS (200 µM) (Figure 5.10c), the competitive inhibitor of cAMP binding in PKA. This suggests that ^{62}Gap27 utilises a PKA activation mechanism that is induced independent of cAMP.

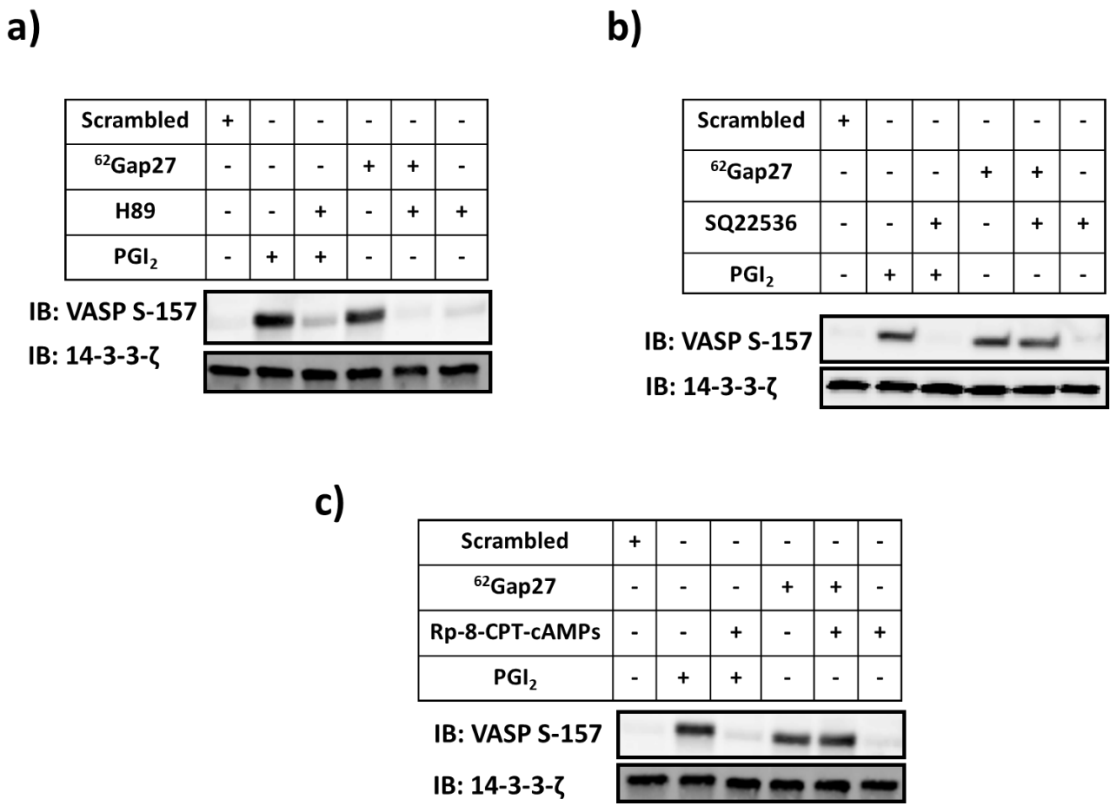


Figure 5.10: ^{62}Gap27 modulates PKA independently of cAMP.

Resting washed human platelets (4×10^8 cells/mL) were treated with **(a)** H89 (10 µM), **(b)** Rp-8-CPT-cAMPS (200 µM) or **(c)** SQ 22536 (100 µM) for 5 minutes before treatment with the scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes. Then, the samples were assayed for VASP-S157 phosphorylation. Treatment with PGI_2 (1 µg/mL), which activates PKA by stimulating AC, was used as a positive control. Lysis of the samples was carried out using Laemmli sample buffer prior to separation by SDS-PAGE, then the samples were transferred to PVDF membranes. 14-3-3-ζ was detected by immunoblotting as a loading control. The impact of ^{62}Gap27 on VASP-S157 phosphorylation is shown in representative blots from 4 distinct experiments.

To exclude the impact of other serine/threonine kinases that may have a role in VASP-S157 phosphorylation, the effects of additional kinase inhibitors were examined. VASP-S157 may also be directly phosphorylated *in vitro* by PKC (Chitaley et al., 2004). In thrombin-stimulated platelets, VASP-S157 is phosphorylated by PKC-dependent and -independent methods (Pula et al., 2006; Wentworth et al., 2006). Various proteins, including GSK3α (Sakamoto et al., 2006) and 14-3-3-ζ (Powell et al., 2002), are considered common substrates of PKB/AKT and PKA. PI3K, upstream of PKB, stimulates VASP-S157 and the inhibition of PI3K has been reported to partially restrict thrombin-stimulated VASP-S157 phosphorylation (Gambaryan et al., 2010). Therefore, the phosphorylation of VASP-S157 in ^{62}Gap27-treated platelets downstream of PKC, PKB/AKT and PI3K was examined.

Prior to these investigations, the effects of ^{62}Gap27 were examined on the baseline tyrosine (Figure 5.11a) and PKC substrate phosphorylation (Figure 5.11b) levels in unstimulated samples and compared with samples treated with scrambled peptide. The phosphorylation levels were found to be similar to each other (Figure 5.11a, b).

The experiments were performed in resting human platelets with PKC, PKB/AKT or PI3K inhibitors. Resting platelets were incubated with pan-PKC inhibitor GF109203X (10 µM), PI3K inhibitor LY29400 (100 µM) or AKT inhibitor IV (5 µM) for 5 minutes, then treated with scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes. The concentrations of these inhibitors were based on previously reported concentrations optimised in our lab for these assays (Unsworth et al., 2017a; Unsworth et al., 2017b).

^{62}Gap27-induced VASP phosphorylation in platelets was not inhibited after treatment with pan-PKC inhibitor GF109203X (10 µM) (Figure 5.12a), PI3K inhibitor LY29400 (100 µM) (Figure 5.12b) or AKT inhibitor IV (5 µM) (Figure 5.12c). In alignment with

experiments done to evaluate baseline phosphorylation levels, these findings reflect a direct effect of ^{62}Gap27 on PKA activation. The established restricted platelet activation induced by ^{62}Gap27 may be attributable to its regulation of GPVI signalling and PKA activity independently of cAMP.

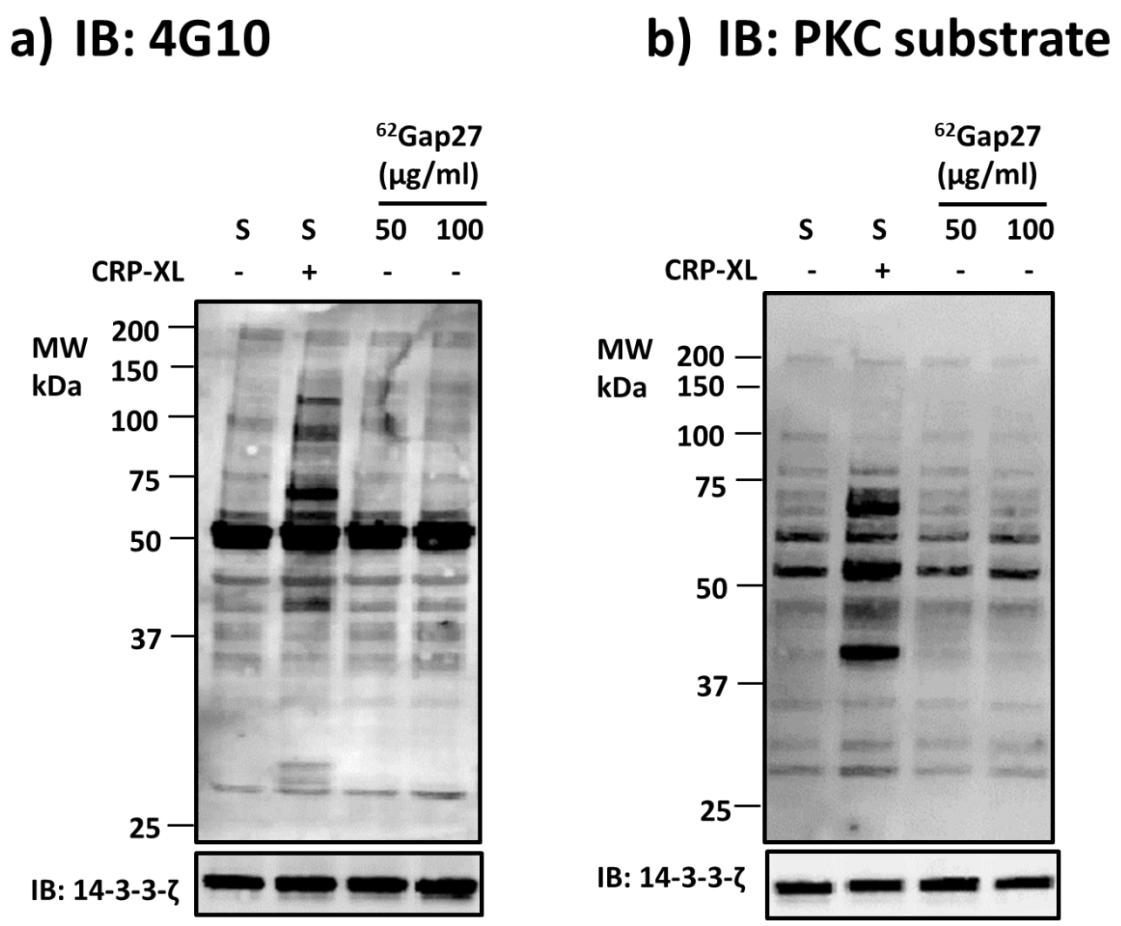


Figure 5.11: ^{62}Gap27 does not enhance total tyrosine phosphorylation and PKC phosphorylation.

Resting washed human platelets (4×10^8 cells/mL) were treated with the scrambled peptide or ^{62}Gap27 (50 and 100 µg/mL) for 5 minutes. Scrambled peptide-treated samples stimulated with CRP-XL (1 µg/mL), which induces total tyrosine phosphorylation and PKC phosphorylation, was used as positive controls. Laemmli sample buffer was used to lyse the samples and whole cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes. Antibodies to detect total tyrosine phosphorylation **(a)** and PKC phosphorylation **(b)** were used for immunoblotting. 14-3-3-ζ was detected by immunoblotting as a gel loading control. Representative blots from 5 distinct experiments are presented.

Abbreviations: S – Scrambled Peptide

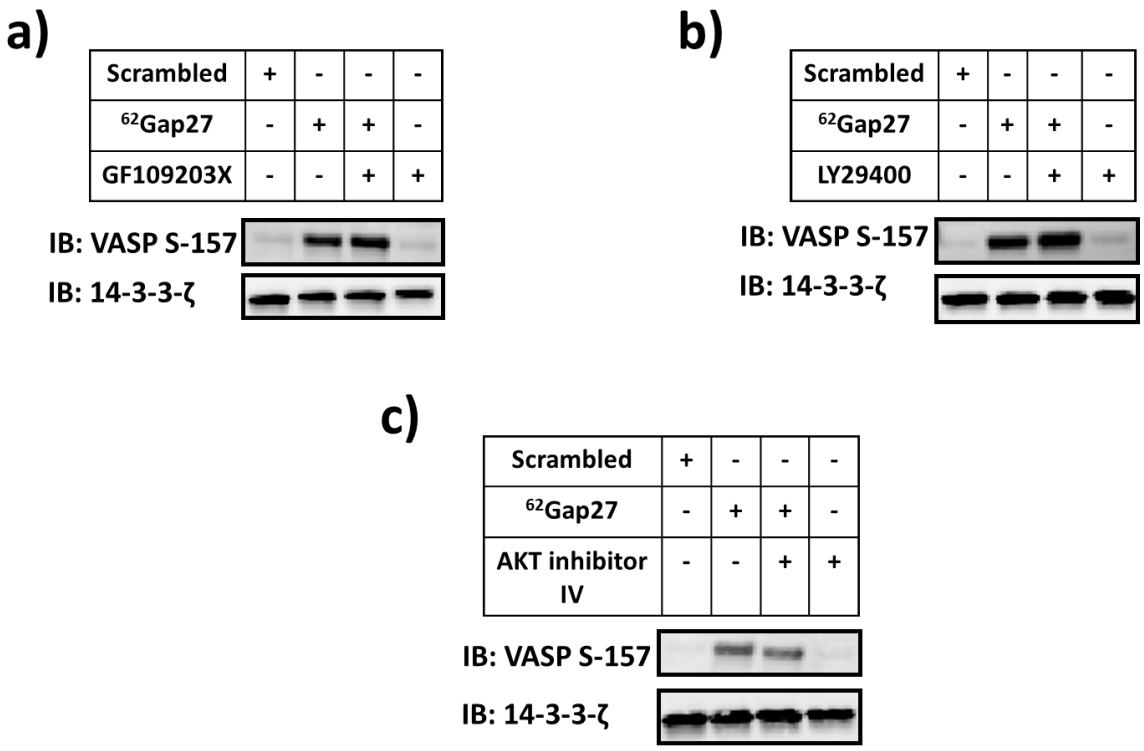


Figure 5.12: ^{62}Gap27 modulates PKA independently of PKB, PKC and PI3K.

Washed resting human platelets (4×10^8 cells/mL) were treated with **(a)** GF109203X (10 µM), **(b)** LY29400 (100 µM), or **(c)** AKT inhibitor IV (5 µM) for 5 minutes, then with scrambled peptide or ^{62}Gap27 (100 µg/mL) for 5 minutes. Then, the phosphorylation of VASP-S157 was assessed. Laemmli sample buffer was used to lyse the samples before separation by SDS-PAGE and transfer to PVDF membranes. 14-3-3-ζ was detected by immunoblotting as a loading control. The impact of ^{62}Gap27 on VASP-S157 phosphorylation is depicted in representative blots from 5 distinct experiments.

5.10 Discussion

A multitude of inhibitory and activatory signalling pathways regulate platelet function, haemostasis and thrombosis. Platelet activation is suppressed by inhibitory signals produced by the healthy vasculature. Platelet activation and thrombus formation are initiated when activatory signals are produced at an injured site. The growth of the thrombus is counteracted by negative signalling regulators that suppress the activatory signals. The positive activation mechanisms occur rapidly and if they occur when not needed, severe issues like stroke and myocardial infarction may occur. As demonstrated in the previous chapter, ^{62}Gap27 blocks platelet activation and Cx62 activity. These effects were observed for PRP and washed platelets. In this chapter the effects of ^{62}Gap27 on thrombus formation (*in vivo* and *in vitro*) and haemostasis were studied along with the mechanism through which ^{62}Gap27 regulates platelet activation.

5.10.1 Inhibition of Thrombus Formation and Haemostasis by ^{62}Gap27

Since ^{62}Gap27 inhibited several pathways linked with the activation of platelets, it appears that Cx62 is important for the regulation of haemostasis and thrombus formation. It suppressed thrombus formation and stability *in vitro*. In addition, ^{62}Gap27 suppressed integrin $\alpha_{IIb}\beta_3$-mediated fibrinogen binding. Compared with the scrambled peptide-treated controls, the ^{62}Gap27-treated samples displayed attenuated platelet–platelet interactions, leading to persistent thrombus dissociation and reduced thrombus growth.

Given the observed inhibition of thrombus formation by ^{62}Gap27 *in vitro*, the influence of ^{62}Gap27 on haemostasis and thrombosis was studied. ^{62}Gap27 treatment caused a considerable reduction in arterial thrombosis upon laser-mediated vessel injury. Moreover, ^{62}Gap27 treatment considerably prolonged tail bleeding time. This indicates

that blocking Cx62 perturbs the regulation of the haemostatic response and that Cx62 serves as a crucial factor for the activation of platelets after injury.

5.10.2 Negative Regulation of the GPVI-mediated Signalling Pathway in Platelets By ^{62}Gap27

Given the inhibitory effect of ^{62}Gap27 on platelet function, haemostasis and thrombosis, its effects on the different pathways involved in platelet signalling were examined. Ca^{2+} mobilisation from intracellular reserves and CRP-XL-stimulated total tyrosine phosphorylation were inhibited by ^{62}Gap27. These phenomena play crucial roles in ensuring persistent platelet activation. Their inhibition by ^{62}Gap27 led us to investigate its influence on specific molecules in the GPVI signalling pathway.

^{62}Gap27 inhibited Syk phosphorylation. Syk is essential for the activation of other effectors that cause $\alpha_{IIb}\beta_3$ activation (Suzuki-Inoue et al., 2004; Hughes et al., 2010). Accordingly, Syk inhibition after GPVI stimulation inhibits all downstream functional responses (Zhang et al., 1998; Law et al., 1999b; Andre et al., 2011). In agreement with earlier reports, considerable inhibition of LAT phosphorylation downstream of Syk inhibition was observed. LAT is an adaptor molecule that acts as a Syk substrate (Zhang et al., 1998). ^{62}Gap27 also inhibited PLCγ2 phosphorylation. PLCγ2 induces the dissociation of PIP2 to form DAG and IP_3. The discharge of Ca^{2+} from the DTS of platelets is stimulated by IP_3. Thus, the inhibition of PLCγ2 phosphorylation may account for the decreased mobilisation of Ca^{2+} in ^{62}Gap27-treated platelets (Watson et al., 2005).

In addition to affecting the initial steps of GPVI-stimulated signalling, ^{62}Gap27 decreased considerably PKC phosphorylation. According to Harper and Poole (2010), PKC plays a supportive role in the regulation of thrombus formation, platelet

aggregation, integrin activation, TxA_2 synthesis and the secretion of granules. Other researchers have reported negative regulatory functions of PKC on the discharge of Ca^{2+} and receptor desensitisation (Mundell et al., 2006; Strehl et al., 2007; Harper and Poole, 2010; Unsworth et al., 2011). As PKC is the chief protein involved in GPVI signalling pathways, it appears that ^{62}Gap27 affects the initial and late steps in GPVI receptor signalling.

PLCγ2 and Syk phosphorylation participate in both inside-out and outside-in signalling. Given the ^{62}Gap27-mediated inhibition noted in their phosphorylation levels to regulate GPVI signalling, it is plausible that the inhibition of these proteins also regulates outside-in signalling via integrin $\alpha_{IIb}\beta_3$, as observed in the inhibition of platelet spreading and clot retraction. Once bound to VWF or fibrinogen, the integrin $\alpha_{IIb}\beta_3$ stimulates tyrosine phosphorylation-dependent signalling within the platelet, thereby stabilising the developing thrombus (Arias-Salgado et al., 2003) together with PLCγ2 (Wonerow et al., 2003) and Syk (Woodside et al., 2001). In agreement with these findings, ^{62}Gap27 reduced thrombus formation.

5.10.3 PKA Activation by ^{62}Gap27 is Independent of cAMP

Although ^{62}Gap27 has substantial inhibitory activity for various platelet functions induced by different agonists, it does not influence thrombin-stimulated, GPCR-mediated platelet signalling. This finding implies that platelet functions triggered by different agonists may be inhibited by ^{62}Gap27 through different mechanisms. Hence, the effects of ^{62}Gap27 on various inhibitory pathways affecting platelets were investigated.

Resting platelets were used in this study to examine the influence of ^{62}Gap27 on the degree of phosphorylation of VASP-S239 and VASP-S157 (PKG- and PKA-specific

phosphorylation sites, respectively). When resting platelets were treated with ^{62}Gap27, VASP phosphorylation was upregulated considerably compared to phosphorylation levels in samples treated with scrambled peptide. Conversely, VASP-S239 phosphorylation was unaffected.

Treatment with the PKA inhibitor H89 (10 μM), which inhibits binding between PKA and ATP, reversed the effect of ^{62}Gap27 on VASP phosphorylation. PKA phosphorylation commonly occurs through increased cAMP generation induced by AC. Despite phosphorylation of VASP at S157, no increase in the cAMP concentration in platelets treated with ^{62}Gap27 was observed. Accordingly, exposure to another PKA inhibitor (Rp-8-CPT-cAMPs; 200 μM) that competes for the cAMP-binding site in PKA did not control the PKA phosphorylation triggered by ^{62}Gap27. This finding implies that PKA is regulated independently of cAMP. Researchers have previously reported increases in PKA activity irrespective of cAMP levels (Wentworth et al., 2006; Gambaryan et al., 2010).

PKB/AKT, PI3K and PKC reportedly participate in VASP phosphorylation (Unsworth et al., 2017a; Unsworth et al., 2017b). However, treatment with AKT inhibitor IV (5 μM), PI3K inhibitor LY29400 (100 μM) or pan-PKC inhibitor GF109203X (10 μM) did not prevent the ^{62}Gap27-induced phosphorylation of VASP, indicating that PKA activation is regulated by Cx62 through ^{62}Gap27.

VASP plays an essential role in the regulation of platelet activation. It belongs to the Ena/VASP family and is found in focal adhesions, cell–cell contacts and stress fibres (Reinhard et al., 2001). According to Fox (2001), VASP regulates the polymerisation of actin and the bundling of actin filaments through profilin binding, thereby mediating the recruitment of actin monomers to sites containing VASP. Harbeck et al. (2000)

reported that binding between VASP and actin filaments is reduced by VASP phosphorylation by PKA, which inhibits the supportive role of VASP for actin polymerisation. Jensen et al. (2004) also reported the involvement of VASP in the inhibition of platelet shape changes mediated by cAMP/PKA. This finding is in agreement with the observation that VASP-S157 phosphorylation by PKA is strongly linked with the inhibition of platelet conformational changes.

VASP participates in the activation of $\alpha_{IIb}\beta_3$. In the absence of VASP, the inhibitory effect of cAMP on collagen-mediated platelet activation is considerably reduced (Aszodi et al., 1999). Moreover, there is a correlation between the inhibition of $\alpha_{IIb}\beta_3$ activity and VASP-S157 phosphorylation mediated by increased cAMP/PKA activity (Horstrup et al., 1994). These findings suggest that the phosphorylation of VASP is important for the inhibition of integrin $\alpha_{IIb}\beta_3$ activation mediated by cAMP/PKA.

IP_3 binds to and activates the IP_3 receptor (IP_3R)/Ca^{2+} channel. This IP_3R (channel) is a substrate of PKA (Ferris et al., 1991). PKA-mediated phosphorylation interferes with IP_3 production/binding, thereby inhibiting platelet function (Cavallini et al., 1996).

These findings indicate the presence of another mechanism, besides GPVI signalling, through which ^{62}Gap27-mediated inhibition of platelet activation occurs via cAMP-independent PKA upregulation. The increase in PKA phosphorylation appears to be the chief effect of ^{62}Gap27 treatment, since it is observed before platelet stimulation. This finding implies that the effects of ^{62}Gap27 were caused by increased PKA activity, irrespective of cAMP, which negatively regulates platelet functions.

6 General Discussion

6.1 Introduction

Vascular injury is normally followed by the formation of a blood clot by platelets and coagulation cascade to stop bleeding (Gibbins, 2004). In diseased blood vessels, unnecessary platelet activation may occur, for example by events like the rupture of atherosclerotic lesions, leading to the development of blood clots in vessels or thrombosis. Development of thrombosis in veins (venous thrombosis) may lead to localised blockage of the blood flow, whereas arterial thrombosis may hinder the flow of blood to other organs, like the heart or brain, leading to myocardial infarction or ischaemic stroke, respectively (Gibbins, 2004; Davi and Patrono, 2007). Antithrombotic treatment is capable of lowering the risk of thrombosis, yet, the anti-platelet treatments available at present are not refined and they lack efficacy in many patients and can cause serious side effects, particularly bleeding (Barrett et al., 2008). Hence, there is a clinical need to develop effective and safe antithrombotic drugs. This calls for detailed research into new drug targets to facilitate the development of novel drugs targeting new mechanisms of action.

Circulating platelets aggregate into a multicellular structure called a thrombus upon activation, a highly reactive process controlled by complex signalling (Gibbins, 2004). The process of platelet activation culminates with integrin $\alpha_{IIb}\beta_3$ present on the platelet surface binding with fibrinogen, which supports platelet–platelet adhesion and thereby brings the cells close to one another to facilitate signalling dependent on cell–cell contact (Gibbins, 2004; Davi and Patrono, 2007; Barrett et al., 2008; Li et al., 2010). In addition to intracellular signalling, intercellular communication also occurs upon platelet aggregation. Studies indicate that GJs regulate contact-dependent signalling

events in platelets within a thrombus and contribute to the stability and growth of the thrombus (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013).

In contrast to intracellular signalling, intercellular communication occurs in tissues via GJs, which are formed by the docking of hemichannels from two cells in close proximity to each other. Hemichannels are hexameric structures formed from monomers of Cxs. When two hemichannels dock with each other, a pore is formed with a diameter of approximately 2–3 nm. Molecules of up to 1 kDa can be transported through this pore in either direction (Goodenough and Paul, 2009). Hemichannels are also known to act as conduits for the transport of molecules (Goodenough and Paul, 2003; John et al., 2003). Researchers have discovered more than 20 Cxs in mammals, which are capable of constructing heteromeric or homomeric hemichannels that form diversely regulated GJs with differing conductances (Falk et al., 1997; Segretain and Falk, 2004). The physiology of hemichannels and GJs has been researched extensively in various cells with persistent contact. However, it has recently been discovered that Cxs can regulate the function of certain circulating cells, like T cells and monocytes (Oviedo-Orta et al., 2010). Although platelets are anucleated circulating cells, they come close to each other in a thrombus where persistent signalling between platelets is required for thrombus stability (Prevost et al., 2003).

Researchers have recently discovered that Cx37 and Cx40 are expressed in human platelets where they are involved in the construction of hemichannels and GJs. Targeting these Cxs with their selective inhibitors (^{37,43}Gap27 for Cx37 and ^{40}Gap27 for Cx40) or deletion of Cx genes (Cx37 or Cx40) in mice was associated with significant inhibition of different platelet functions, such as reduced fibrinogen binding, α-granule secretion, Ca^{2+} mobilisation and platelet aggregation. In addition, clot retraction was substantially decreased by Cx37 and Cx40 inhibition, which indicates decreased

outside-in signalling via integrin $\alpha_{IIb}\beta_3$ (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). The same researchers detected considerable expression of mRNA transcripts for Cx37, Cx40 and Cx62 in MKs (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013). However, the functions of Cx62 remained unknown until this study.

The hypothesis explored in this study was that platelets may express Cx62, which could be involved in GJ formation to permit cell–cell interactions and the signalling required for thrombus endurance.

6.2 Cx62 in Platelets

Cx37, Cx40, Cx43 and Cx45 are the main, abundantly expressed cardiovascular Cxs. Cx37 is encoded by the GJA4 gene on human chromosome 1 (Sohl and Willecke, 2003), Cx40 by the GJA5 gene on chromosome 17, Cx43 by the GJA1 gene on chromosome 6 and Cx45 by the GJA7 gene on chromosome 17. Base on gene structure, the length of the cytoplasmic loop and the sequences of the conserved domains, Cxs are categorised into five groups, which are signified by Greek letters (Cruciani and Mikalsen, 2006; Abascal and Zardoya, 2013). Cx37, Cx40 and Cx43 are members of the α (GJA) group, Cx32 belongs to the β group and Cx45 belongs to the γ group. The regional expression of these Cxs differs in the heart and vascular system. Cxs are also found on the surfaces of blood cells. Although red blood cells do not express Cxs, they do express Pannexin 1 on their surfaces (Begandt et al., 2017). Cx37 and Cx40 are expressed on platelets at notable levels, whereas Cx32 and Cx43 are expressed at low levels (Vaiyapuri et al., 2015a; Molica et al., 2017). Monocytes express Cx37 and Cx43. Cx43 is also found on T cells, B cells and neutrophils (Pfenniger et al., 2013; Glass et al., 2015).

Notable levels of the mRNA transcripts of different Cxs are expressed in MKs as they bud into vessels in bone marrow. MKs also expresses a variety of Cx proteins, including substantial levels of Cx37, Cx40 and Cx62 (Vaiyapuri et al., 2012). Cx37 and Cx40 have been studied extensively in different types of cells, whereas Cx62 is a newly discovered Cx that needs to be characterised. It belongs to the α group and is encoded by the GJA10 gene found on human chromosome 6. Mouse Cx57 is homologous to human Cx62 and is also encoded by GJA10 (Sohl and Willecke, 2003).

The expression of Cx62 on human and mouse platelets was confirmed in this study. The internal structures of platelets contain notable levels of Cx62 (in the resting state) and a large proportion of these proteins is transported to the cell membrane upon activation. This observation suggests that, upon activation, this Cx forms hemichannels in the cell membrane followed by the formation of GJs through docking with hemichannels on nearby platelets, thereby permitting communication amongst platelets.

The mechanism involved in the transport of Cx62 to the cell membrane upon platelet activation still needs to be determined. Cxs are synthesised in the endoplasmic reticulum, then sent to the cell membrane through forward trafficking. This transport involves the secretory pathway and the Golgi apparatus. The majority of Cx proteins are known to be transported in this way. However, Cx26, which is expressed in the liver, Schwann cells, cochlea and oligodendrocytes, is transported through Golgi-independent (Martin et al., 2001) and Golgi-dependent (Thomas et al., 2005) mechanisms. The oligomerisation of Cxs to form hemichannels is initiated in the endoplasmic reticulum. The oligomerisation continues in the Golgi apparatus and is stabilised in the trans-Golgi network (Laird, 2006). Certain Cxs, like the eye lens-specific Cx46 and the cardiovascular-specific Cx43, remain monomeric in the endoplasmic reticulum and Golgi apparatus, only oligomerising in the trans-Golgi

network. Vesicles carrying hemichannels, particularly the ones formed of Cx43, are sent to the cell membrane via microtubules and actin filaments. This transport process is promoted by the tethering of microtubule plus ends at adherens junction proteins (Shaw et al., 2007).

These compartments are not present in nucleus-lacking platelets, but platelets possess a specialised, membranous DTS, which can be regarded as a primitive endoplasmic reticulum (White, 1972). During subcellular fractionation, Cx62 was only present in the lower-density cells fractions, which are anticipated to carry the DTS and other membrane structures (but not granules). It was intriguing to observe the similar distribution patterns of Cx62 and calreticulin. The protein calreticulin marks the DTS (van Nispen Tot Pannerden et al., 2009). This similarity in distribution suggests that in nucleated cells, Cx62 is likely localise to the endoplasmic reticulum (Falk et al., 1994).

6.3 Design of ^{62}Gap27

After the expression of Cx62 was detected in human platelets in this study, its role in platelet physiology was investigated. As no Cx62 mimetic peptides had previously been developed, the mimetic peptide ^{62}Gap27 was developed for this study. The second external loop of Cx62 is targeted by this peptide. The second external loops of the majority of Cxs have been targeted for the development of mimetic peptides. However, a high level of sequence similarity exists between Gap27 peptides that target different Cx isoforms. Consequently, careful consideration is required while designing Gap27 inhibitors to minimise cross-reactivity. ^{37,43}Gap27, for instance, targets Cx37, 43 and 46. Considering that Gap27 has been used to target other Cx isoforms, the selectivity of ^{62}Gap27 had to be tested to reduce the chances of false positive results. In this study, selectivity was determined by the gold standard of testing the effect of the peptide in

mice that are deficient in Cx57. ^{62}Gap27 was found to be selective and active against only Cx62 (Cx57 in mice) since treatment of Cx57$^{-/-}$ platelets with ^{62}Gap27 did not affect CRP-XL-stimulated fibrinogen binding.

Cx62 hemichannel permeability and GJIC were inhibited by ^{62}Gap27. However, the full mechanism of the inhibitory activity of the peptide requires elucidation. Although this mechanism is unknown for other Cxs and their mimetic peptides, numerous hypotheses have been put forward by researchers (Evans and Boitano, 2001). One hypothesis suggests that the peptides may inhibit the docking of hemichannels, which is required to give rise to a GJ, by binding the second external loop of hemichannels (Leybaert et al., 2003). Consequently, fewer hemichannels would be available for the formation of GJs. Since the half-life of a Cx is 1–5 hours, the inhibition of GJs is anticipated to take place in a comparable time period. Interestingly, using electrophysiological analysis, the kinetics of Gap26/Gap27 inhibition of Cx43 GJs has been revealed as a few minutes (Desplantez et al., 2012b). Interestingly, Cxs are detected in platelets where no protein synthesis occurs. This might be due to two possibilities: (i) Cxs last longer in platelets than in other cell types or (ii) their expression is detected predominantly in the newest platelets within the population.

Another hypothesis is that ^{62}Gap27 may interact directly with GJs, thereby causing the dissociation of hemichannels from GJs or negatively affecting the permeability of GJs. The dissociation of hemichannels from GJs is possible and, if persistent, would cause GJs to be unzipped, internalised and broken down. Such internalisation could be prompted via different physiological stimuli, such as wound healing and ischaemia (Solan and Lampe, 2014). Researchers have proposed that particular phosphorylation patterns of Cx43 determine if the GJs are unzipped and completely internalised (Solan and Lampe, 2014). Although it is plausible that mimetic peptides could reach the

intercellular space between hemichannels through the edge of the junctional plaque, there is scant evidence for the unzipping action in the available literature.

The interaction of the Gap27 peptide with the GJ may negatively affect the permeability of the GJ, as has been reported with P180–195, a peptide that targets the second extracellular loop of Cx43. This peptide comprises amino acids S180–Q195 and has been shown to reduce dwelling in sub-conductance whereas the main conductance state remains unaffected (Kwak and Jongsma, 1999). However, in that study, the cells were exposed to the peptide for a long period (overnight). Considering the scarcity of evidence for the unzipping of GJs and for the interactions between mimetic peptides and GJs affecting permeability, these mechanisms require study in greater depth.

The available literature contains evidence for interactions between Gap27 peptides and single hemichannels, rather than hemichannels in GJs (Leybaert et al., 2003). Desplantez et al. (2012b) reported that the inhibition of hemichannels occurs more quickly than that of GJs. Using macroscopic current measurements, researchers have observed the inhibition of hemichannels within 5 minutes. Conversely, the inhibition of GJs occurs within 10 minutes to hours (Desplantez et al., 2012b). These findings suggest that hemichannels are inhibited initially, which eventually results in GJ inhibition. Although hemichannel inhibition occurs within a few minutes, this is not considered a quick process given channel dynamics. The process may be slow because the interaction site on the extracellular loop is initially unavailable and access requires conformational changes, like unfolding in order for peptide to access this. It could also be due to the fact that other associations with Cx domains occur deep within the pore of the channel and the inhibition can only occur in the open state of the channel. This second possibility requires further investigation.

6.4 The Role of Cx62 in Platelet Function

After verifying the expression of Cx62/57 on human and mouse platelets, the role of this Cx in platelet function was studied with the novel inhibitor ^{62}Gap27. Treatment with 100 μg/mL ^{62}Gap27 caused an inhibition of almost 50% of the aggregation induced by various agonists, including U46619, ADP, thrombin and CRP-XL. This finding implies that Cx62 can regulate platelet physiology via various pathways. Accordingly, decreased agonist-stimulated fibrinogen binding (50%) was observed in mice lacking Cx57 compared to that in wild type mice.

The importance of Cx62 for the maintenance of haemostasis was revealed by examination of bleeding time in mice. Mice treated with ^{62}Gap27 bled for 983 seconds, whereas mice treated with the scrambled peptide bled for 450 seconds. Similarly, the volume, size and number of thrombi were smaller in the samples treated with the inhibitor than in those treated with the scrambled peptide in the *in vitro* thrombus formation assay. Thrombus growth was 70% lower in the *in vivo* thrombus formation assay in the mice treated with the mimetic peptide. Clot retraction was also suppressed by the inhibitor, indicating the vital role of gap junctional intercellular communication (GJIC in the reinforcement of thrombi. These findings were in agreement with the earlier findings following the inhibition of Cx37 by ^{37,43}Gap27 and Cx40 by ^{40}Gap27 (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013; Vaiyapuri et al., 2015a; Molica et al., 2017).

6.5 The Functions of Cx62 Hemichannels in Initial Platelet Activation

Several functional studies performed on individual platelets revealed that hemichannels containing Cx37 and Cx40 are essential for initial platelet activation. The involvement of hemichannels in the trafficking of different signalling entities between the

extracellular space and the cytoplasm has been studied in different cell types, including leukocytes (Vaiyapuri et al., 2012; Pfenniger et al., 2013; Vaiyapuri et al., 2013; Glass et al., 2015; Vaiyapuri et al., 2015a). In this study, the roles of Cx62 hemichannels in early platelet activation, the binding of fibrinogen and secretion of granules were studied through flow cytometry on individual platelet populations. Similarly, isolated platelets from mice deficient in Cx57 revealed the potential role of Cx57 hemichannels in fibrinogen binding. Thus, hemichannels comprising Cx62 are involved in early platelet activation and recruitment to the developing thrombus. However, the signalling entities transported through these channels remain unknown.

6.6 The Functions of Cx62 GJs in Thrombus Formation and the Maintenance of Stability

GJs are responsible for permitting the transport of signalling entities between cells; they also participate in cell–cell adhesion to ensure persistent contact. Inside the thrombus, platelets strongly adhere to each other. Although the importance of fibrinogen binding via integrin $\alpha_{IIb}\beta_3$ for the maintenance of contact between platelets is well established, studies are on-going to identify other important receptors, cell adhesion proteins (like Eph kinases) and their respective ligands that are involved in fibrinogen binding. The role of ephrins in controlling contact-based cell communication is also well established (Brass et al., 2004; Vaiyapuri et al., 2015b).

Our lab have recently investigated the roles of Cx37 and Cx40 in the regulation of thrombus stability and various platelet functions by treating platelets with specific inhibitors (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013; Vaiyapuri et al., 2015a). In contrast to our lab findings Angelillo-Scherrer et al. (2011), reported that the deletion of Cx37 results in enhanced platelet aggregation in response to different agonists. In this study, when Cx62/57 was inhibited with ^{62}Gap27 in human or mouse platelets,

thrombus formation and stability were lower than in the scrambled peptide-treated controls. Moreover, clot retraction was also suppressed by treatment with ^{62}Gap27. These findings indicate that GJs comprising Cx62 are required for platelet adherence and communication between platelets within a thrombus, which are required for clot retraction. Nevertheless, the signalling entities exchanged via these channels must be identified.

6.7 Attenuation of Ca^{2+} Mobilisation by ^{62}Gap27

In this study, Ca^{2+} mobilisation was negatively regulated by ^{62}Gap27. This inhibition was identified to be partly an outcome of reduced Ca^{2+} release from the internal store in platelets treated with ^{62}Gap27. However, its effect on the influx of Ca^{2+} cannot be denied. Platelets contain numerous channels for Ca^{2+}, like $P2X_1$, TRPC6, STIM1 and Orai1. These channels work in coordination to control intracellular Ca^{2+} exchange (Varga-Szabo et al., 2009; Li et al., 2010; Procter et al., 2016). Tolhurst et al. (2008) identified the association between Orai1 and STIM1 for the control of Ca^{2+} mobilisation. Noe et al. (2010) found that Cx43-based GJs can link with $P2X_1$ in cardiomyocytes. Hence, it is possible that Cx62 associates with another calcium channel to influence Ca^{2+} mobilisation. ^{62}Gap27 can negatively influence the permeability of Cx62-based hemichannels. The passage of IP_3 through the cell membrane may be affected by this reduced permeability, thereby negatively influencing Ca^{2+} mobilisation.

6.8 ^{62}Gap27 Downregulates Platelet Function Through the Activation of PKA

Phosphorylation is the chief regulatory step in many cellular phenomena. The increased phosphorylation observed in cancers (Radivojac et al., 2008) and genetic disorders

(Cohen, 2001) indicates the importance of reversible phosphorylation. In cells other than platelets, the downstream effects of cAMP are mediated by exchange proteins that are directly activated by cAMP (Epac) (de Rooij et al., 1998), PKA (Hayes and Mayer, 1981) and cyclic nucleotide-gated ion channels (Nakamura and Gold, 1987). Conversely, in platelets, cAMP signalling is mediated by only PKA due to the scarcity of cAMP-activated Epac. In addition, cyclic nucleotide-gated ion channels are not expressed in platelets (Schwarz et al., 2001; Schmidt et al., 2013).

The available literature contains substantial evidence of the involvement of PKG and PKA in the regulation of the phosphorylation of different Cxs, including Cx32, C35/36, Cx40, Cx43 and Cx50, in various cells (van Rijen et al., 2000; Shah et al., 2002; Lampe and Lau, 2004; Ouyang et al., 2005; Liu et al., 2011; Pidoux and Tasken, 2015). In this study, ^{62}Gap27 increased the phosphorylation of PKA but not the cAMP level. Indeed, other studies have also reported that the phosphorylation of PKA can be stimulated by collagen or thrombin in a cAMP-independent manner (Wentworth et al., 2006; Gambaryan et al., 2010). Therefore, PKA phosphorylation inhibits platelet activation by affecting the secretion of granules and cytoskeletal rearrangements and by increasing Ca^{2+} levels (Schwarz et al., 2001).

6.8.1 PKA Regulates Platelet Rearrangement and Secretion

The role of integrin $\alpha_{IIb}\beta_3$ in platelet activation is well established. Platelet activation also involves cytoskeletal proteins that drive the polymerisation of actin filaments and cytoskeletal rearrangements downstream of signalling. Actin polymerisation is regulated by VASP, a well-known membrane-bound protein that acts as a substrate for PKA (Harbeck et al., 2000). When VASP is phosphorylated by PKA at S157, the binding of VASP with filamentous actin is suppressed, which suppresses actin

polymerisation. Researchers have also found an association between the downregulation of the fibrinogen receptor $\alpha_{IIb}\beta_3$ and VASP-S157 phosphorylation (Horstrup et al., 1994). In addition, when VASP is knocked out in mice, platelet aggregation is perturbed (Aszodi et al., 1999), which indicates the importance of VASP in the regulation of platelet function.

In addition to its effects on cytoskeletal proteins, PKA also acts on IP_3R, an intracellular channel for the transportation of Ca^{2+} (Ferris et al., 1991). IP_3, produced in response to agonist-stimulated platelet activation, binds to its receptor, thereby opening the channel and causing an increase in the intracellular concentration of Ca^{2+}. This process is impeded by PKA-mediated phosphorylation, which causes platelet inhibition (Cavallini et al., 1996).

6.8.2 The Role of PKA in TP Desensitisation

In this study, ^{62}Gap27 inhibited the synthesis of TxA_2. In platelets, $G_{\alpha13}$ is phosphorylated at T203 by PKA (Manganello et al., 1999; Manganello et al., 2003). This phosphorylation of $G_{\alpha13}$ inhibits platelet Rho activation, leading to the inhibition of the Rho/Rho-kinase pathway via destabilisation of the association between $G_{\alpha13}$ and its $\beta\gamma$ subunit and stabilisation of the association between $G_{\alpha13}$ and TxA_2 receptors (Manganello et al., 2003). Moreover, TP is a PKA substrate in HEK293 cells (Habib et al., 1997). Researchers have found that TP undergoes desensitisation mediated by PGI_2 or the prostaglandin D2 receptor. This desensitisation takes place via phosphorylation of TPβ isoform of the human TXA_2 receptor at S329 by PKA. Even though the phosphorylation of TxA_2 receptor in platelets has not been verified yet; desensitisation of this receptor by PGI_2 or the prostaglandin D2 receptor has been detected in human platelets. Moreover, the PKA inhibitor H89 inhibits this desensitisation (Walsh et al.,

2000; Foley et al., 2001). Taken together, these findings indicate that TP is desensitised by PKA through the phosphorylation of relevant G-proteins or TP, leading to the inhibition of the Rho pathways or PLC activation.

6.8.3 Possible Mechanisms of the Upregulation of PKA Phosphorylation by ^{62}Gap27

In this study, ^{62}Gap27 induced PKA phosphorylation; however, the exact mechanism involved remains unclear. A number of possible hypotheses can be tested to determine the mechanism. Firstly, when platelets are activated, Cx62 hemichannels are transported to the PM where they give rise to GJs to permit intercellular communication between platelets within the thrombus. It is possible that PKA remains inactive as a component of the regulatory mechanism for Cx62. Treatment with ^{62}Gap27 may activate PKA. This hypothesis can be tested by immunoprecipitation or co-immunoprecipitation experiments. Due to limited resources, this hypothesis was not tested during this study.

Secondly, Cxs are subjected to several post-translational modifications and are regulated through phosphorylation. The translocation of Cx43 towards plasma membrane involves two kinases: PKA and PKB (Paulson et al., 2000; Shah et al., 2002). This could explain the increase in PKA activity in platelets treated with ^{62}Gap27. The blockade of Cx62 could lead to interference in GJIC in the thrombus.

Finally, it is also possible that Cx62 associates with a kinase anchoring protein (AKAP) to regulate signalling through GJs via PKA. The regulation of subcellular microdomains is mediated by PKA bound to an AKAP, which ensures the maintenance of the specificity of cAMP–PKA signalling in response to extracellular stimuli. GJs are channels made up of Cxs that allow the transport of small molecules between cells.

Although PKA signalling is required for the promotion of human trophoblast fusion, signalling through Cx43 GJs is also required for this phenomenon. Ezrin, a well-known AKAP, has recently been shown to regulate trophoblast fusion. Furthermore, the dissociation of the ezrin–Cx43 bond inhibits Cx43 phosphorylation by PKA, thereby inhibiting GJ signalling and, eventually, cell fusion (Pidoux and Tasken, 2015). Researchers have hypothesised that the opening of the Cx43 GJ is regulated by the PKA–ezrin–Cx43 macromolecular complex in response to nucleotide signalling in various cell types (Pidoux and Tasken, 2015). It is, therefore, possible that Cx62 uses the same mechanism to interact with PKA. These hypotheses should be investigated in detail.

6.9 Conclusions and Future Research

The available literature provides evidence of the importance of hemichannels and GJs for the control of haemostasis and thrombosis (Vaiyapuri et al., 2012; Vaiyapuri et al., 2013; Vaiyapuri et al., 2015a). The early stages of platelet activation require hemichannels; GJs are required in the later stages of thrombus development and stabilisation. Together, hemichannels and GJs permit the transport of signalling entities between platelets, which may include cyclic nucleotides, IP_3 and Ca^{2+}. In particular, hemichannels mediate the trafficking of molecules between the extracellular space and platelets, whereas GJs are responsible for the regulation of the signalling amongst platelets in the thrombus and clot retraction. However, more work is required to establish this. GJs accumulate on the cell surface to form GJ plaques, thereby sustaining cell–cell adhesion and allowing effective coordination between cells (Goodenough and Paul, 2009). These plaques can be formed on the surfaces of platelets where they participate in cell–cell adhesion and support the activity of adhesive receptors like ephrins, Eph kinases and integrin $\alpha_{IIb}\beta_3$ (Prevost et al., 2003; Brass et al., 2004; Prevost

et al., 2005; Vaiyapuri et al., 2015b). However, the functions of GJs for the regulation of platelet adhesion require further detailed research. It remains unclear if Cxs on the surfaces of platelets mediate functional interactions between cells circulating in vessels. Another important area of research is the determination of the different signalling entities that are transported through hemichannels and GJs. Similarly, an understanding of the molecular mechanisms involved in controlling the conductance of these channels will prove to be immensely helpful in exploring the therapeutic, particularly antithrombotic, potential of these structures.

6.9.1 Identification of the Signalling Molecules Transported Through Cx62 GJs

Functional studies must be performed to identify the intercellular signalling that occurs through the Cx62 GJs. This can be achieved with a ligand microarray that enables the spatial organisation of the platelets associated with fibrinogen. The exact locations of the platelets can be determined through micro-collection of fibrinogen-coated spots. By spreading on the fibrinogen, platelets can adhere with other nearby platelets, resulting in the formation of a network that can be used to investigate intercellular signalling, channel regulation and the entities being transported.

The exchange of signals between platelets can be studied *in vitro* by ligand array of thrombi developed from human or mouse blood. In this study, intercellular communication between calcein-loaded platelets was studied through FRAP. In the future, the respective contribution of every Cx should be identified using selective inhibitors for the each of the Cxs. The available literature lacks thorough information about the entities transported through gated channels. However, ADP, ATP, Ca^{2+}, cyclic nucleotides and IP_3 are known to be transported through these structures (Evans

and Boitano, 2001; Evans et al., 2006; Yeager and Harris, 2007; Dobrowolski and Willecke, 2009; Leybaert et al., 2017).

6.9.2 Cx62 Protein Phosphorylation

Researchers have identified tyrosine, threonine and serine phosphorylation sites in Cx43. Mutagenesis experiments revealed that these sites are involved in transport through Cxs, membrane insertion, GJ formation, internalisation and permeability regulation (Solan and Lampe, 2009; Johnstone et al., 2012; Chen et al., 2013). They may also work in coordination; their combined action remains unclear, although they are likely to be involved in the main steps of regulating signalling through GJs. Kinases like casein kinase, PKA, PKC, Src, and MAPK and Ca^{2+}/CaM-dependent mechanisms have been reported to be involved in the regulation of Cxs (Saez et al., 1990; Richards et al., 2004; Solan and Lampe, 2009; Johnstone et al., 2012), but the importance of Cx62 phosphorylation remains unclear.

6.9.3 Cx62 GJ–Protein Interactions

Cx62 GJIC may be regulated by Cx-associated proteins like protein kinases (PKG, PKA and c-Src) and regulatory protein phosphatases. In addition, structural proteins like microtubules and zona occludens have recently been detected in the GJs formed by Cx32, Cx36, Cx43 and Cx45. Researchers have also proposed that Cxs can be involved in processes other than the trafficking of molecules, like regulation of the cytoskeleton and transcription (Leybaert et al., 2017). Hence, it will be important to identify other proteins that interact with Cx62 GJs to understand their coordinated actions.

6.9.4 Concluding Remarks:

- Cx62/57 is expressed in human and mouse platelets and transported towards the cell membrane upon platelet activation

- The new mimetic peptide ^{62}Gap27 selectively targets the second external loop of Cx62/57
- Treatment with ^{62}Gap27 inhibits a wide range of platelet functions
- Cx62 hemichannel functions independently of Cx37 and Cx40 in platelets
- Thrombus formation (*in vitro and in vivo*) and haemostasis were inhibited by ^{62}Gap27
- ^{62}Gap27 stimulates PKA phosphorylation independently of cAMP

7 References

Abascal, F. & Zardoya, R. (2013). Evolutionary analyses of gap junction protein families. *Biochim Biophys Acta,* **1828**, 4-14.

Achan, J., Talisuna, A. O., Erhart, A., Yeka, A., Tibenderana, J. K., Baliraine, F. N., Rosenthal, P. J. & D'Alessandro, U. (2011). Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malar J,* **10**, 144.

Addison, W. (1841). On the colourless corpuscles and onthe molecules and cytoblasts in the blood. *London Med Gaz,* **30**, 144-152.

Ahmad, S., Diez, J. A., George, C. H. & Evans, W. H. (1999). Synthesis and assembly of connexins in vitro into homomeric and heteromeric functional gap junction hemichannels. *Biochem J,* **339 (Pt 2)**, 247-53.

Ahmad, S. & Evans, W. H. (2002). Post-translational integration and oligomerization of connexin 26 in plasma membranes and evidence of formation of membrane pores: implications for the assembly of gap junctions. *Biochem J,* **365**, 693-9.

Akopov, S., Darbinian, V., Grigorian, G., Kosyan, A. & Gabrielian, E. (1993). Elevated velocity of prostacyclin degradation in blood as a possible risk factor in patients with cerebrovascular disorders. *Eur Neurol,* **33**, 252-5.

Alevriadou, B. R., Moake, J. L., Turner, N. A., Ruggeri, Z. M., Folie, B. J., Phillips, M. D., Schreiber, A. B., Hrinda, M. E. & McIntire, L. V. (1993). Real-time analysis of shear-dependent thrombus formation and its blockade by inhibitors of von Willebrand factor binding to platelets. *Blood,* **81**, 1263-76.

Allen, T. D. & Dexter, T. M. (1982). Ultrastructural aspects of erythropoietic differentiation in long-term bone marrow culture. *Differentiation,* **21**, 86-94.

Alonso, F., Boittin, F. X., Beny, J. L. & Haefliger, J. A. (2010). Loss of connexin40 is associated with decreased endothelium-dependent relaxations and eNOS levels in the mouse aorta. *Am J Physiol Heart Circ Physiol,* **299**, H1365-73.

Anand, R. J., Dai, S., Gribar, S. C., Richardson, W., Kohler, J. W., Hoffman, R. A., Branca, M. F., Li, J., Shi, X. H., Sodhi, C. P. & Hackam, D. J. (2008). A role for connexin43 in macrophage phagocytosis and host survival after bacterial peritoneal infection. *J Immunol,* **181**, 8534-8543.

Andre, P., Morooka, T., Sim, D., Abe, K., Lowell, C., Nanda, N., Delaney, S., Siu, G., Yan, Y., Hollenbach, S., Pandey, A., Gao, H., Wang, Y., Nakajima, K., Parikh, S. A., Shi, C., Phillips, D., Owen, W., Sinha, U. & Simon, D. I. (2011). Critical role for Syk in responses to vascular injury. *Blood,* **118**, 5000-10.

Angelillo-Scherrer, A., Fontana, P., Burnier, L., Roth, I., Sugamele, R., Brisset, A., Morel, S., Nolli, S., Sutter, E., Chassot, A., Capron, C., Borgel, D., Saller, F., Chanson, M. & Kwak, B. R. (2011). Connexin 37 limits thrombus propensity by downregulating platelet reactivity. *Circulation,* **124**, 930-9.

Angiolillo, D. J., Ueno, M. & Goto, S. (2010). Basic principles of platelet biology and clinical implications. *Circ J,* **74**, 597-607.

Antonin, W., Fasshauer, D., Becker, S., Jahn, R. & Schneider, T. R. (2002). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nature Structural & Molecular Biology,* **9**, 107.

Anumonwo, J. M., Taffet, S. M., Gu, H., Chanson, M., Moreno, A. P. & Delmar, M. (2001). The carboxyl terminal domain regulates the unitary conductance and voltage dependence of connexin40 gap junction channels. *Circ Res,* **88**, 666-73.

Arias-Salgado, E. G., Lizano, S., Sarkar, S., Brugge, J. S., Ginsberg, M. H. & Shattil, S. J. (2003). Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A,* **100**, 13298-302.

Ariëns, R. (2013). Fibrin (ogen) and thrombotic disease. *Journal of thrombosis and haemostasis: JTH,* **11**, 294.

Armstrong, R. A., Lawrence, R. A., Jones, R. L., Wilson, N. H. & Collier, A. (1989). Functional and ligand binding studies suggest heterogeneity of platelet prostacyclin receptors. *Br J Pharmacol,* **97**, 657-68.

Arthur, W. T., Petch, L. A. & Burridge, K. (2000). Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. *Curr Biol,* **10**, 719-22.

Asl, M. N. & Hosseinzadeh, H. (2008). Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. *Phytother Res,* **22**, 709-24.

Asselin, J., Gibbins, J. M., Achison, M., Lee, Y. H., Morton, L. F., Farndale, R. W., Barnes, M. J. & Watson, S. P. (1997). A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. *Blood,* **89**, 1235-42.

Aszodi, A., Pfeifer, A., Ahmad, M., Glauner, M., Zhou, X. H., Ny, L., Andersson, K. E., Kehrel, B., Offermanns, S. & Fassler, R. (1999). The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *Embo j,* **18**, 37-48.

Badimon, L. & Vilahur, G. (2008). Coronary atherothrombotic disease: progress in antiplatelet therapy. *Rev Esp Cardiol,* **61**, 501-13.

Balendran, A., Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M. & Alessi, D. R. (2000). A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Czeta (PKCzeta) and PKC-related kinase 2 by PDK1. *J Biol Chem,* **275**, 20806-13.

Balsinde, J., Winstead, M. V. & Dennis, E. A. (2002). Phospholipase A(2) regulation of arachidonic acid mobilization. *FEBS Lett,* **531**, 2-6.

Banno, A. & Ginsberg, M. H. (2008). Integrin activation. *Biochem Soc Trans,* **36**, 229-34.

Bao, X., Chen, Y., Reuss, L. & Altenberg, G. A. (2004). Functional expression in Xenopus oocytes of gap-junctional hemichannels formed by a cysteine-less connexin 43. *J Biol Chem,* **279**, 9689-92.

Baragli, A., Grieco, M. L., Trieu, P., Villeneuve, L. R. & Hebert, T. E. (2008). Heterodimers of adenylyl cyclases 2 and 5 show enhanced functional responses in the presence of Galpha s. *Cell Signal,* **20**, 480-92.

Bargiello, T. & Brink, P. (2009). Voltage-gating Mechanisms of Connexin Channels. *In:* Harris, A. L. & Locke, D. (eds.) *Connexins: A guide.* Springer Humana Press.

Barker, R. J. & Gourdie, R. G. (2002). JNK bond regulation: why do mammalian hearts invest in connexin43? *Circ Res,* **91**, 556-8.

Barnes, M. J. & Farndale, R. W. (1999). Collagens and atherosclerosis. *Experimental Gerontology,* **34**, 513-525.

Barrett, N. E., Holbrook, L., Jones, S., Kaiser, W. J., Moraes, L. A., Rana, R., Sage, T., Stanley, R. G., Tucker, K. L., Wright, B. & Gibbins, J. M. (2008). Future innovations in anti-platelet therapies. *Br J Pharmacol,* **154**, 918-39.

Barry, F. A. & Gibbins, J. M. (2002). Protein kinase B is regulated in platelets by the collagen receptor glycoprotein VI. *J Biol Chem,* **277**, 12874-8.

Batra, N., Kar, R. & Jiang, J. X. (2012). Gap junctions and hemichannels in signal transmission, function and development of bone. *Biochim Biophys Acta,* **1818**, 1909-18.

Bavamian, S., Klee, P., Allagnat, F., Haefliger, J.-A. & Meda, P. (2009). Connexins and Secretion. *In:* Harris, A. L. & Locke, D. (eds.) *Connexins: A guide.* Springer Humana Press.

Beardslee, M. A., Laing, J. G., Beyer, E. C. & Saffitz, J. E. (1998). Rapid turnover of connexin43 in the adult rat heart. *Circ Res,* **83**, 629-35.

Beardslee, M. A., Lerner, D. L., Tadros, P. N., Laing, J. G., Beyer, E. C., Yamada, K. A., Kleber, A. G., Schuessler, R. B. & Saffitz, J. E. (2000). Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. *Circ Res,* **87**, 656-62.

Beblo, D. A. & Veenstra, R. D. (1997). Monovalent cation permeation through the connexin40 gap junction channel. Cs, Rb, K, Na, Li, TEA, TMA, TBA, and effects of anions Br, Cl, F, acetate, aspartate, glutamate, and NO3. *J Gen Physiol,* **109**, 509-22.

Begandt, D., Good, M. E., Keller, A. S., DeLalio, L. J., Rowley, C., Isakson, B. E. & Figueroa, X. F. (2017). Pannexin channel and connexin hemichannel expression in vascular function and inflammation. *BMC Cell Biol,* **18**, 2.

Bellamy, T. C. & Garthwaite, J. (2002). Pharmacology of the nitric oxide receptor, soluble guanylyl cyclase, in cerebellar cells. *Br J Pharmacol,* **136**, 95-103.

Benlalam, H., Jalil, A., Hasmim, M., Pang, B., Tamouza, R., Mitterrand, M., Godet, Y., Lamerant, N., Robert, C., Avril, M. F., Neefjes, J., Tursz, T., Mami-Chouaib, F., Kieda, C. & Chouaib, S. (2009). Gap junction communication between autologous endothelial and tumor cells induce cross-recognition and elimination by specific CTL. *J Immunol,* **182**, 2654-64.

Berger, G., Masse, J. M. & Cramer, E. M. (1996). Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood,* **87**, 1385-95.

Bergmeier, W. & Stefanini, L. (2009). Novel molecules in calcium signaling in platelets. *J Thromb Haemost,* **7 Suppl 1**, 187-90.

Bergoffen, J., Scherer, S. S., Wang, S., Scott, M. O., Bone, L. J., Paul, D. L., Chen, K., Lensch, M. W., Chance, P. F. & Fischbeck, K. H. (1993). Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science,* **262**, 2039-42.

Bermudez-Fajardo, A., Yliharsila, M., Evans, W. H., Newby, A. C. & Oviedo-Orta, E. (2007). CD4+ T lymphocyte subsets express connexin 43 and establish gap junction channel communication with macrophages in vitro. *J Leukoc Biol,* **82**, 608-12.

Berndt, M. C., Shen, Y., Dopheide, S. M., Gardiner, E. E. & Andrews, R. K. (2001). The vascular biology of the glycoprotein Ib-IX-V complex. *Thromb Haemost,* **86**, 178-88.

Berridge, M. J., Bootman, M. D. & Roderick, H. L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol,* **4**, 517-29.

Berthoud, V. M., Minogue, P. J., Laing, J. G. & Beyer, E. C. (2004). Pathways for degradation of connexins and gap junctions. *Cardiovasc Res,* **62**, 256-67.

Best, D., Senis, Y. A., Jarvis, G. E., Eagleton, H. J., Roberts, D. J., Saito, T., Jung, S. M., Moroi, M., Harrison, P., Green, F. R. & Watson, S. P. (2003). GPVI levels in platelets: relationship to platelet function at high shear. *Blood,* **102**, 2811-8.

Bevans, C. G. & Harris, A. L. (1999). Direct high affinity modulation of connexin channel activity by cyclic nucleotides. *J Biol Chem,* **274**, 3720-5.

Bevans, C. G., Kordel, M., Rhee, S. K. & Harris, A. L. (1998). Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. *J Biol Chem,* **273**, 2808-16.

Billaud, M., Dahan, D., Marthan, R., Savineau, J. P. & Guibert, C. (2011). Role of the gap junctions in the contractile response to agonists in pulmonary artery from two rat models of pulmonary hypertension. *Respir Res,* **12**, 30.

Bizzozero, G. (1869). Sul midollo delle ossa. *Il Morgagni*.

Bizzozero, G. (1881). Su di un nuovo elemento morfologico del sangue dei mammiferi e della sua importanza nella trombosi e nella coagulazione. *L'Osservatore,* **17**, 785–7.

Bizzozero, J. (1882). Ueber einen neuen Fornnbestandteil des Blutes und dessen Rolle bei der Thrombose und Blutgerinnung. *Archiv fur pathologische Anatomie und Physiologie und fur klinische Medecin,* **90**, 261-332.

Blair, P. & Flaumenhaft, R. (2009). Platelet α–granules: Basic biology and clinical correlates. *Blood reviews,* **23**, 177-189.

Bloomfield, S. A. & Volgyi, B. (2009). The diverse functional roles and regulation of neuronal gap junctions in the retina. *Nat Rev Neurosci,* **10**, 495-506.

Bodendiek, S. B. & Raman, G. (2010). Connexin modulators and their potential targets under the magnifying glass. *Curr Med Chem,* **17**, 4191-230.

Boitano, S. & Evans, W. H. (2000). Connexin mimetic peptides reversibly inhibit Ca(2+) signaling through gap junctions in airway cells. *Am J Physiol Lung Cell Mol Physiol,* **279**, L623-30.

Bonnefoy, A., Hantgan, R., Legrand, C. & Frojmovic, M. M. (2001). A model of platelet aggregation involving multiple interactions of thrombospondin-1, fibrinogen, and GPIIbIIIa receptor. *J Biol Chem,* **276**, 5605-12.

Born, G. V. (1965). PLATELETS IN THROMBOGENESIS: MECHANISM AND INHIBITION OF PLATELET AGGREGATION. *Ann R Coll Surg Engl,* **36**, 200-6.

Born, G. V. (1967a). Mechanism of platelet aggregation and of its inhibition by adenosine derivatives. *Fed Proc,* **26**, 115-7.

Born, G. V. (1967b). Possible mechanisms of platelet aggregation by ADP and of its inhibition. *Thromb Diath Haemorrh Suppl,* **26**, 173-4.

Born, G. V. R. (1962). Aggregation of Blood Platelets by Adenosine Diphosphate and its Reversal. *Nature,* **194**, 927-929.

Bos, J. L., Rehmann, H. & Wittinghofer, A. (2007). GEFs and GAPs: critical elements in the control of small G proteins. *Cell,* **129**, 865-77.

Boylan, B., Chen, H., Rathore, V., Paddock, C., Salacz, M., Friedman, K. D., Curtis, B. R., Stapleton, M., Newman, D. K., Kahn, M. L. & Newman, P. J. (2004). Anti-GPVI-associated ITP: an acquired platelet disorder caused by autoantibody-mediated clearance of the GPVI/FcRgamma-chain complex from the human platelet surface. *Blood,* **104**, 1350-5.

Boylan, B., Gao, C., Rathore, V., Gill, J. C., Newman, D. K. & Newman, P. J. (2008). Identification of FcgammaRIIa as the ITAM-bearing receptor mediating alphaIIbbeta3 outside-in integrin signaling in human platelets. *Blood,* **112**, 2780-6.

Brass, L. F. (2003). Thrombin and platelet activation. *Chest,* **124**, 18s-25s.

Brass, L. F., Stalker, T. J., Zhu, L., Lu, B., Woulfe, D. S. & Prevost, N. (2004). Boundary events: contact-dependent and contact-facilitated signaling between platelets. *Semin Thromb Hemost,* **30**, 399-410.

Brass, L. F., Vassallo, R. R., Jr., Belmonte, E., Ahuja, M., Cichowski, K. & Hoxie, J. A. (1992). Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J Biol Chem,* **267**, 13795-8.

Braun, A., Varga-Szabo, D., Kleinschnitz, C., Pleines, I., Bender, M., Austinat, M., Bosl, M., Stoll, G. & Nieswandt, B. (2009). Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation. *Blood,* **113**, 2056-63.

Briddon, S. J. & Watson, S. P. (1999). Evidence for the involvement of p59fyn and p53/56lyn in collagen receptor signalling in human platelets. *Biochem J,* **338 (Pt 1)**, 203-9.

Brisset, A. C., Isakson, B. E. & Kwak, B. R. (2009). Connexins in Vascular Physiology and Pathology. *Antioxidants & Redox Signaling,* **11**, 267-282.

Bruzzone, R. & Dermietzel, R. (2006). Structure and function of gap junctions in the developing brain. *Cell Tissue Res,* **326**, 239-48.

Bujalska, I., Shimojo, M., Howie, A. & Stewart, P. M. (1997). Human 11 beta-hydroxysteroid dehydrogenase: studies on the stably transfected isoforms and localization of the type 2 isozyme within renal tissue. *Steroids,* **62**, 77-82.

Bukauskas, F. F., Bukauskiene, A. & Verselis, V. K. (2002). Conductance and permeability of the residual state of connexin43 gap junction channels. *J Gen Physiol,* **119**, 171-85.

Bukauskas, F. F. & Peracchia, C. (1997). Two distinct gating mechanisms in gap junction channels: CO2-sensitive and voltage-sensitive. *Biophys J,* **72**, 2137-42.

Bukauskas, F. F. & Peracchia, C. (2000). Distinct Behaviors of Chemical and Voltage Sensitive Gates of Gap Junction Channel. *In:* Peracchia, C. (ed.) *Gap junctions. Molecular basis of cell communication in health and disease.* Academic Press.

Bukauskas, F. F. & Weingart, R. (1994). Voltage-dependent gating of single gap junction channels in an insect cell line. *Biophys J,* **67**, 613-25.

Bult, H., Fret, H. R., Van den Bossche, R. M. & Herman, A. G. (1988). Platelet inhibition by endothelium-derived relaxing factor from the rabbit perfused aorta. *Br J Pharmacol,* **95**, 1308-14.

Burnier, L., Fontana, P., Angelillo-Scherrer, A. & Kwak, B. R. (2009). Intercellular communication in atherosclerosis. *Physiology (Bethesda),* **24**, 36-44.

Bye, A. P., Unsworth, A. J. & Gibbins, J. M. (2016). Platelet signaling: a complex interplay between inhibitory and activatory networks. *Journal of Thrombosis and Haemostasis,* **14**, 918-930.

Calderwood, D. A. (2004). Integrin activation. *J Cell Sci,* **117**, 657-66.

Campbell, F. R. (1980). Gap junctions between cells of bone marrow: an ultrastructural study using tannic acid. *Anat Rec,* **196**, 101-7.

Campbell, F. R. (1986). Ultrastructural studies of intercellular contacts (junctions) in bone marrow. A review. *Scan Electron Microsc,* 621-9.

Canobbio, I., Balduini, C. & Torti, M. (2004). Signalling through the platelet glycoprotein Ib-V-IX complex. *Cell Signal,* **16**, 1329-44.

Cavallini, L., Coassin, M., Borean, A. & Alexandre, A. (1996). Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. *J Biol Chem,* **271**, 5545-51.

Chaipan, C., Soilleux, E. J., Simpson, P., Hofmann, H., Gramberg, T., Marzi, A., Geier, M., Stewart, E. A., Eisemann, J., Steinkasserer, A., Suzuki-Inoue, K., Fuller, G. L., Pearce, A. C., Watson, S. P., Hoxie, J. A., Baribaud, F. & Pohlmann, S.

(2006). DC-SIGN and CLEC-2 mediate human immunodeficiency virus type 1 capture by platelets. *J Virol,* **80**, 8951-60.

Chaytor, A. T., Evans, W. H. & Griffith, T. M. (1997). Peptides homologous to extracellular loop motifs of connexin 43 reversibly abolish rhythmic contractile activity in rabbit arteries. *J Physiol,* **503 (Pt 1)**, 99-110.

Chaytor, A. T., Martin, P. E., Evans, W. H., Randall, M. D. & Griffith, T. M. (1999). The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication. *J Physiol,* **520 Pt 2**, 539-50.

Chen, V. C., Gouw, J. W., Naus, C. C. & Foster, L. J. (2013). Connexin multi-site phosphorylation: mass spectrometry-based proteomics fills the gap. *Biochim Biophys Acta,* **1828**, 23-34.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. & Hidaka, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem,* **265**, 5267-72.

Chitaley, K., Chen, L., Galler, A., Walter, U., Daum, G. & Clowes, A. W. (2004). Vasodilator-stimulated phosphoprotein is a substrate for protein kinase C. *FEBS Lett,* **556**, 211-5.

Ciferri, S., Emiliani, C., Guglielmini, G., Orlacchio, A., Nenci, G. G. & Gresele, P. (2000). Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost,* **83**, 157-64.

Cines, D. B., Lebedeva, T., Nagaswami, C., Hayes, V., Massefski, W., Litvinov, R. I., Rauova, L., Lowery, T. J. & Weisel, J. W. (2014). Clot contraction: compression of erythrocytes into tightly packed polyhedra and redistribution of platelets and fibrin. *Blood,* **123**, 1596-603.

Clarke, M. B. & Sperandio, V. (2005). Events at the host-microbial interface of the gastrointestinal tract III. Cell-to-cell signaling among microbial flora, host, and pathogens: there is a whole lot of talking going on. *Am J Physiol Gastrointest Liver Physiol,* **288**, G1105-9.

Clemetson, J. M., Polgar, J., Magnenat, E., Wells, T. N. & Clemetson, K. J. (1999). The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. *J Biol Chem,* **274**, 29019-24.

Clemetson, K. J. (1999). Primary haemostasis: sticky fingers cement the relationship. *Curr Biol,* **9**, R110-2.

Clemetson, K. J. & Clemetson, J. M. (2013). Platelet Receptors. *In:* Michelson, A. D. (ed.) *Platelets.* 3 ed.: Academic Press.

Cohen, P. (2001). The role of protein phosphorylation in human health and disease. The Sir Hans Krebs Medal Lecture. *Eur J Biochem,* **268**, 5001-10.

Cole, V., Staton, J., Eikelboom, J., Hankey, G., Yi, Q., Shen, Y., Berndt, M. & Baker, R. (2003). Collagen platelet receptor polymorphisms integrin α2β1 C807T and GPVI Q317L and risk of ischemic stroke. *Journal of Thrombosis and Haemostasis,* **1**, 963-970.

Coller, B. S. (1984). Bizzozero and the discovery of the blood platelet. *Lancet,* **1**, 804.

Coller, B. S. & Shattil, S. J. (2008). The GPIIb/IIIa (integrin alphaIIbbeta3) odyssey: a technology-driven saga of a receptor with twists, turns, and even a bend. *Blood,* **112**, 3011-25.

Coughlin, S. R. (2000). Thrombin signalling and protease-activated receptors. *Nature,* **407**, 258-64.

Coughlin, S. R. (2005). Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost,* **3**, 1800-14.

Cramer, E. M., Berger, G. & Berndt, M. C. (1994). Platelet alpha-granule and plasma membrane share two new components: CD9 and PECAM-1. *Blood,* **84**, 1722-30.

Crescente, M., Pluthero, F. G., Li, L., Lo, R. W., Walsh, T. G., Louriero, S., Ali, M. S., Vaiyapuri, S., Schenk, M. P., Holbrook, L. M., Falet, H., Jones, I. M., Poole, A. W., Kahr, W. H. & Gibbins, J. M. (2016). Intracellular Trafficking, Localization, and Mobilization of Platelet-Borne Thiol Isomerases. *Arterioscler Thromb Vasc Biol*.

Crittenden, J. R., Bergmeier, W., Zhang, Y., Piffath, C. L., Liang, Y., Wagner, D. D., Housman, D. E. & Graybiel, A. M. (2004). CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. *Nat Med,* **10**, 982-6.

Cruciani, V. & Mikalsen, S. O. (2006). The vertebrate connexin family. *Cell Mol Life Sci,* **63**, 1125-40.

Cruikshank, S. J., Hopperstad, M., Younger, M., Connors, B. W., Spray, D. C. & Srinivas, M. (2004). Potent block of Cx36 and Cx50 gap junction channels by mefloquine. *Proc Natl Acad Sci U S A,* **101**, 12364-9.

Cutler, L., Rodan, G. & Feinstein, M. B. (1978). Cytochemical localization of adenylate cyclase and of calcium ion, magnesium ion-activated ATPases in the dense tubular system of human blood platelets. *Biochim Biophys Acta,* **542**, 357-71.

Dahl, G., Nonner, W. & Werner, R. (1994). Attempts to define functional domains of gap junction proteins with synthetic peptides. *Biophys J,* **67**, 1816-22.

Dahl, G., Werner, R., Levine, E. & Rabadan-Diehl, C. (1992). Mutational analysis of gap junction formation. *Biophys J,* **62**, 172-80; discussion 180-2.

Daniel, J. L., Dangelmaier, C., Jin, J., Ashby, B., Smith, J. B. & Kunapuli, S. P. (1998). Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem,* **273**, 2024-9.

Darrow, B. J., Laing, J. G., Lampe, P. D., Saffitz, J. E. & Beyer, E. C. (1995). Expression of multiple connexins in cultured neonatal rat ventricular myocytes. *Circ Res,* **76**, 381-7.

Davi, G. & Patrono, C. (2007). Platelet activation and atherothrombosis. *N Engl J Med,* **357**, 2482-94.

Davidson, J. S. & Baumgarten, I. M. (1988). Glycyrrhetinic acid derivatives: a novel class of inhibitors of gap-junctional intercellular communication. Structure-activity relationships. *J Pharmacol Exp Ther,* **246**, 1104-7.

Davidson, J. S., Baumgarten, I. M. & Harley, E. H. (1986). Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. *Biochem Biophys Res Commun,* **134**, 29-36.

Dawood, B. B., Lowe, G. C., Lordkipanidze, M., Bem, D., Daly, M. E., Makris, M., Mumford, A., Wilde, J. T. & Watson, S. P. (2012). Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. *Blood,* **120**, 5041-9.

de Curtis, M., Manfridi, A. & Biella, G. (1998). Activity-dependent pH shifts and periodic recurrence of spontaneous interictal spikes in a model of focal epileptogenesis. *J Neurosci,* **18**, 7543-51.

De Mello, W. C. (1975). Effect of intracellular injection of calcium and strontium on cell communication in heart. *J Physiol,* **250**, 231-45.

de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A. & Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature,* **396**, 474-7.

de Sauvage, F. J., Hass, P. E., Spencer, S. D., Malloy, B. E., Gurney, A. L., Spencer, S. A., Darbonne, W. C., Henzel, W. J., Wong, S. C., Kuang, W. J. & et al. (1994). Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature,* **369**, 533-8.

de Wit, R. J., Hekstra, D., Jastorff, B., Stec, W. J., Baraniak, J., Van Driel, R. & Van Haastert, P. J. (1984). Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases. *Eur J Biochem,* **142**, 255-60.

Dekker, L. R., Fiolet, J. W., VanBavel, E., Coronel, R., Opthof, T., Spaan, J. A. & Janse, M. J. (1996). Intracellular Ca2+, intercellular electrical coupling, and mechanical activity in ischemic rabbit papillary muscle. Effects of preconditioning and metabolic blockade. *Circ Res,* **79**, 237-46.

Delmar, M., Coombs, W., Sorgen, P., Duffy, H. S. & Taffet, S. M. (2004). Structural bases for the chemical regulation of Connexin43 channels. *Cardiovasc Res,* **62**, 268-75.

Denoyelle, F., Weil, D., Maw, M. A., Wilcox, S. A., Lench, N. J., Allen-Powell, D. R., Osborn, A. H., Dahl, H. H., Middleton, A., Houseman, M. J., Dode, C., Marlin, S., Boulila-ElGaied, A., Grati, M., Ayadi, H., BenArab, S., Bitoun, P., Lina-Granade, G., Godet, J., Mustapha, M., Loiselet, J., El-Zir, E., Aubois, A., Joannard, A., Levilliers, J., Garabedian, E. N., Mueller, R. F., Gardner, R. J., Petit, C. & et al. (1997). Prelingual deafness: high prevalence of a 30delG mutation in the connexin 26 gene. *Hum Mol Genet,* **6**, 2173-7.

Derouette, J. P., Desplantez, T., Wong, C. W., Roth, I., Kwak, B. R. & Weingart, R. (2009a). Functional differences between human Cx37 polymorphic hemichannels. *J Mol Cell Cardiol,* **46**, 499-507.

Derouette, J. P., Wong, C., Burnier, L., Morel, S., Sutter, E., Galan, K., Brisset, A. C., Roth, I., Chadjichristos, C. E. & Kwak, B. R. (2009b). Molecular role of Cx37 in advanced atherosclerosis: a micro-array study. *Atherosclerosis,* **206**, 69-76.

Desplantez, T., McCain, M. L., Beauchamp, P., Rigoli, G., Rothen-Rutishauser, B., Parker, K. K. & Kleber, A. G. (2012a). Connexin43 ablation in foetal atrial myocytes decreases electrical coupling, partner connexins, and sodium current. *Cardiovascular Research,* **94**, 58-65.

Desplantez, T., Verma, V., Leybaert, L., Evans, W. H. & Weingart, R. (2012b). Gap26, a connexin mimetic peptide, inhibits currents carried by connexin43 hemichannels and gap junction channels. *Pharmacol Res,* **65**, 546-52.

Dhein, S. (1998). Gap junction channels in the cardiovascular system: pharmacological and physiological modulation. *Trends Pharmacol Sci,* **19**, 229-41.

Djellas, Y., Manganello, J. M., Antonakis, K. & Le Breton, G. C. (1999). Identification of Galpha13 as one of the G-proteins that couple to human platelet thromboxane A2 receptors. *J Biol Chem,* **274**, 14325-30.

Dobbins, K. R. & Saul, R. F. (2000). Transient visual loss after licorice ingestion. *J Neuroophthalmol,* **20**, 38-41.

Dobrowolski, R. & Willecke, K. (2009). Connexin-caused genetic diseases and corresponding mouse models. *Antioxid Redox Signal,* **11**, 283-95.

Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. (1987). A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry,* **26**, 2657-64.

Dorsam, R. T. & Kunapuli, S. P. (2004). Central role of the P2Y12 receptor in platelet activation. *J Clin Invest,* **113**, 340-5.

Dorshkind, K., Green, L., Godwin, A. & Fletcher, W. H. (1993). Connexin-43-type gap junctions mediate communication between bone marrow stromal cells. *Blood,* **82**, 38-45.

Duman, J. G. & Forte, J. G. (2003). What is the role of SNARE proteins in membrane fusion? *American Journal of Physiology - Cell Physiology,* **285**, C237.

Dumas, J. J., Kumar, R., Seehra, J., Somers, W. S. & Mosyak, L. (2003). Crystal structure of the GpIbalpha-thrombin complex essential for platelet aggregation. *Science,* **301**, 222-6.

Dupont, E., el Aoumari, A., Briand, J. P., Fromaget, C. & Gros, D. (1989). Cross-linking of cardiac gap junction connexons by thiol/disulfide exchanges. *J Membr Biol,* **108**, 247-52.

Ebbeling, L., Robertson, C., McNicol, A. & Gerrard, J. M. (1992). Rapid ultrastructural changes in the dense tubular system following platelet activation. *Blood,* **80**, 718-23.

Elfgang, C., Eckert, R., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F. & Willecke, K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J Cell Biol,* **129**, 805-17.

Elgueta, R., Tobar, J. A., Shoji, K. F., De Calisto, J., Kalergis, A. M., Bono, M. R., Rosemblatt, M. & Saez, J. C. (2009). Gap junctions at the dendritic cell-T cell interface are key elements for antigen-dependent T cell activation. *J Immunol,* **183**, 277-84.

Ellis, C. (2004). The state of GPCR research in 2004. *Nature Reviews Drug Discovery,* **3**.

Eltzschig, H. K., Eckle, T., Mager, A., Kuper, N., Karcher, C., Weissmuller, T., Boengler, K., Schulz, R., Robson, S. C. & Colgan, S. P. (2006). ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. *Circ Res,* **99**, 1100-8.

Elyamany, G., Alzahrani, A. M. & Bukhary, E. (2014). Cancer-Associated Thrombosis: An Overview. *Clinical Medicine Insights. Oncology,* **8**, 129-137.

Emambokus, N. R. & Frampton, J. (2003). The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity,* **19**, 33-45.

Emsley, J., Cruz, M., Handin, R. & Liddington, R. (1998). Crystal structure of the von Willebrand Factor A1 domain and implications for the binding of platelet glycoprotein Ib. *J Biol Chem,* **273**, 10396-401.

Escolar, G. & White, J. G. (1991). The platelet open canalicular system: a final common pathway. *Blood Cells,* **17**, 467-85; discussion 486-95.

Eskandari, S., Zampighi, G. A., Leung, D. W., Wright, E. M. & Loo, D. D. (2002). Inhibition of gap junction hemichannels by chloride channel blockers. *J Membr Biol,* **185**, 93-102.

Evans, W. H. & Boitano, S. (2001). Connexin mimetic peptides: specific inhibitors of gap-junctional intercellular communication. *Biochem Soc Trans,* **29**, 606-12.

Evans, W. H., Bultynck, G. & Leybaert, L. (2012). Manipulating connexin communication channels: use of peptidomimetics and the translational outputs. *J Membr Biol,* **245**, 437-49.

Evans, W. H., De Vuyst, E. & Leybaert, L. (2006). The gap junction cellular internet: connexin hemichannels enter the signalling limelight. *Biochem J,* **397**, 1-14.

Falati, S., Gross, P., Merrill-Skoloff, G., Furie, B. C. & Furie, B. (2002). Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med,* **8**, 1175-81.

Falk, M. M., Baker, S. M., Gumpert, A. M., Segretain, D. & Buckheit, R. W., 3rd (2009). Gap junction turnover is achieved by the internalization of small endocytic double-membrane vesicles. *Mol Biol Cell,* **20**, 3342-52.

Falk, M. M., Buehler, L. K., Kumar, N. M. & Gilula, N. B. (1997). Cell-free synthesis and assembly of connexins into functional gap junction membrane channels. *Embo j,* **16**, 2703-16.

Falk, M. M. & Gilula, N. B. (1998). Connexin membrane protein biosynthesis is influenced by polypeptide positioning within the translocon and signal peptidase access. *J Biol Chem,* **273**, 7856-64.

Falk, M. M., Kumar, N. M. & Gilula, N. B. (1994). Membrane insertion of gap junction connexins: polytopic channel forming membrane proteins. *J Cell Biol,* **127**, 343-55.

Farndale, R. W., Sixma, J. J., Barnes, M. J. & de Groot, P. G. (2004). The role of collagen in thrombosis and hemostasis. *J Thromb Haemost,* **2**, 561-73.

Ferris, C. D., Cameron, A. M., Bredt, D. S., Huganir, R. L. & Snyder, S. H. (1991). Inositol 1,4,5-trisphosphate receptor is phosphorylated by cyclic AMP-dependent protein kinase at serines 1755 and 1589. *Biochem Biophys Res Commun,* **175**, 192-8.

Figueroa, X. F. & Duling, B. R. (2009). Gap junctions in the control of vascular function. *Antioxid Redox Signal,* **11**, 251-66.

Fitscha, P., Kaliman, J. & Sinzinger, H. (1985). Platelet sensitivity to antiaggregatory prostaglandins (PGE1,D2,I2) in patients with peripheral vascular disease. *Am J Hematol,* **19**, 13-9.

FitzGerald, G. A. (1991). Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. *Am J Cardiol,* **68**, 11b-15b.

FitzGerald, G. A., Smith, B., Pedersen, A. K. & Brash, A. R. (1984). Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation. *N Engl J Med,* **310**, 1065-8.

Flaumenhaft, R., Croce, K., Chen, E., Furie, B. & Furie, B. C. (1999). Proteins of the exocytotic core complex mediate platelet alpha-granule secretion. Roles of vesicle-associated membrane protein, SNAP-23, and syntaxin 4. *J Biol Chem,* **274**, 2492-501.

Flevaris, P., Stojanovic, A., Gong, H., Chishti, A., Welch, E. & Du, X. (2007). A molecular switch that controls cell spreading and retraction. *J Cell Biol,* **179**, 553-65.

Fogelson, A. L. & Wang, N. T. (1996). Platelet dense-granule centralization and the persistence of ADP secretion. *Am J Physiol,* **270**, H1131-40.

Foley, J. F., Kelley, L. P. & Kinsella, B. T. (2001). Prostaglandin D(2) receptor-mediated desensitization of the alpha isoform of the human thromboxane A(2) receptor. *Biochem Pharmacol,* **62**, 229-39.

Foote, C. I., Zhou, L., Zhu, X. & Nicholson, B. J. (1998). The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions. *J Cell Biol,* **140**, 1187-97.

Francois, H., Athirakul, K., Mao, L., Rockman, H. & Coffman, T. M. (2004). Role for thromboxane receptors in angiotensin-II-induced hypertension. *Hypertension,* **43**, 364-9.

Fredriksson, R. & Schioth, H. B. (2005). The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Mol Pharmacol,* **67**, 1414-25.

Freyssinet, J. M. & Toti, F. (2010). Formation of procoagulant microparticles and properties. *Thromb Res,* **125 Suppl 1**, S46-8.

Fuchs, H. & Bachran, C. (2009). Targeted tumor therapies at a glance. *Curr Drug Targets,* **10**, 89-93.

Fuller, G. L., Williams, J. A., Tomlinson, M. G., Eble, J. A., Hanna, S. L., Pohlmann, S., Suzuki-Inoue, K., Ozaki, Y., Watson, S. P. & Pearce, A. C. (2007). The C-type lectin receptors CLEC-2 and Dectin-1, but not DC-SIGN, signal via a novel YXXL-dependent signaling cascade. *J Biol Chem,* **282**, 12397-409.

Fung, C. Y., Cendana, C., Farndale, R. W. & Mahaut-Smith, M. P. (2007). Primary and secondary agonists can use P2X(1) receptors as a major pathway to increase intracellular Ca(2+) in the human platelet. *J Thromb Haemost,* **5**, 910-7.

Furchgott, R. F. & Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288**, 373-6.

Furshpan, E. J. & Potter, D. D. (1959). Slow post-synaptic potentials recorded from the giant motor fibre of the crayfish. *J Physiol,* **145**, 326-35.

Gabriels, J. E. & Paul, D. L. (1998). Connexin43 is highly localized to sites of disturbed flow in rat aortic endothelium but connexin37 and connexin40 are more uniformly distributed. *Circ Res,* **83**, 636-43.

Gachet, C. (2006). Regulation of platelet functions by P2 receptors. *Annu Rev Pharmacol Toxicol,* **46**, 277-300.

Gachet, C., Leon, C. & Hechler, B. (2006). The platelet P2 receptors in arterial thrombosis. *Blood Cells Mol Dis,* **36**, 223-7.

Gaietta, G., Deerinck, T. J., Adams, S. R., Bouwer, J., Tour, O., Laird, D. W., Sosinsky, G. E., Tsien, R. Y. & Ellisman, M. H. (2002). Multicolor and electron microscopic imaging of connexin trafficking. *Science,* **296**, 503-7.

Gambaryan, S., Kobsar, A., Rukoyatkina, N., Herterich, S., Geiger, J., Smolenski, A., Lohmann, S. M. & Walter, U. (2010). Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. *J Biol Chem,* **285**, 18352-63.

Gawaz, M. (2004). Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium. *Cardiovasc Res,* **61**, 498-511.

Gaziano, T. A., Bitton, A., Anand, S., Abrahams-Gessel, S. & Murphy, A. (2010). Growing Epidemic of Coronary Heart Disease in Low- and Middle-Income Countries. *Current problems in cardiology,* **35**, 72-115.

Gear, A. R. & Burke, D. (1982). Thrombin-induced secretion of serotonin from platelets can occur in seconds. *Blood,* **60**, 1231-4.

George, C. H., Kendall, J. M., Campbell, A. K. & Evans, W. H. (1998). Connexin-aequorin chimerae report cytoplasmic calcium environments along trafficking pathways leading to gap junction biogenesis in living COS-7 cells. *J Biol Chem,* **273**, 29822-9.

George, C. H., Kendall, J. M. & Evans, W. H. (1999). Intracellular trafficking pathways in the assembly of connexins into gap junctions. *J Biol Chem,* **274**, 8678-85.

Gerrard, J. M., White, J. G. & Peterson, D. A. (1978). The platelet dense tubular system: its relationship to prostaglandin synthesis and calcium flux. *Thromb Haemost,* **40**, 224-31.

Gershon, E., Plaks, V. & Dekel, N. (2008). Gap junctions in the ovary: expression, localization and function. *Mol Cell Endocrinol,* **282**, 18-25.

Gibbins, J., Asselin, J., Farndale, R., Barnes, M., Law, C. L. & Watson, S. P. (1996). Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. *J Biol Chem,* **271**, 18095-9.

Gibbins, J. M. (2004). Platelet adhesion signalling and the regulation of thrombus formation. *J Cell Sci,* **117**, 3415-25.

Gibbins, J. M., Briddon, S., Shutes, A., van Vugt, M. J., van de Winkel, J. G., Saito, T. & Watson, S. P. (1998). The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. *J Biol Chem,* **273**, 34437-43.

Gibbins, J. M., Okuma, M., Farndale, R., Barnes, M. & Watson, S. P. (1997). Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor γ-chain. *FEBS Letters,* **413**, 255-259.

Gilbert, G. E., Sims, P. J., Wiedmer, T., Furie, B., Furie, B. C. & Shattil, S. J. (1991). Platelet-derived microparticles express high affinity receptors for factor VIII. *J Biol Chem,* **266**, 17261-8.

Giles, C. (1981). The platelet count and mean platelet volume. *Br J Haematol,* **48**, 31-7.

Glass, A. M., Snyder, E. G. & Taffet, S. M. (2015). Connexins and pannexins in the immune system and lymphatic organs. *Cell Mol Life Sci,* **72**, 2899-910.

Goggs, R., Harper, M. T., Pope, R. J., Savage, J. S., Williams, C. M., Mundell, S. J., Heesom, K. J., Bass, M., Mellor, H. & Poole, A. W. (2013). RhoG protein regulates platelet granule secretion and thrombus formation in mice. *J Biol Chem,* **288**, 34217-29.

Goldberg, G. S., Moreno, A. P. & Lampe, P. D. (2002). Gap junctions between cells expressing connexin 43 or 32 show inverse permselectivity to adenosine and ATP. *J Biol Chem,* **277**, 36725-30.

Golebiewska, E. M., Harper, M. T., Williams, C. M., Savage, J. S., Goggs, R., Fischer von Mollard, G. & Poole, A. W. (2015). Syntaxin 8 regulates platelet dense granule secretion, aggregation, and thrombus stability. *J Biol Chem,* **290**, 1536-45.

Golebiewska, E. M. & Poole, A. W. (2015). Platelet secretion: From haemostasis to wound healing and beyond. *Blood reviews,* **29**, 153-162.

Gong, H., Shen, B., Flevaris, P., Chow, C., Lam, S. C., Voyno-Yasenetskaya, T. A., Kozasa, T. & Du, X. (2010). G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. *Science,* **327**, 340-3.

Gong, X., Cheng, C. & Xia, C. H. (2007). Connexins in lens development and cataractogenesis. *J Membr Biol,* **218**, 9-12.

Gonzalez, D., Gomez-Hernandez, J. M. & Barrio, L. C. (2007). Molecular basis of voltage dependence of connexin channels: an integrative appraisal. *Prog Biophys Mol Biol,* **94**, 66-106.

Goodenough, D. A., Goliger, J. A. & Paul, D. L. (1996). Connexins, connexons, and intercellular communication. *Annu Rev Biochem,* **65**, 475-502.

Goodenough, D. A. & Paul, D. L. (2003). Beyond the gap: functions of unpaired connexon channels. *Nat Rev Mol Cell Biol,* **4**, 285-94.

Goodenough, D. A. & Paul, D. L. (2009). Gap junctions. *Cold Spring Harb Perspect Biol,* **1**, a002576.

Goodenough, D. A. & Revel, J. P. (1970). A fine structural analysis of intercellular junctions in the mouse liver. *J Cell Biol,* **45**, 272-90.

Grewal, P. K., Uchiyama, S., Ditto, D., Varki, N., Le, D. T., Nizet, V. & Marth, J. D. (2008). The Ashwell receptor mitigates the lethal coagulopathy of sepsis. *Nat Med*, **14**, 648-55.

Gross, B. S., Melford, S. K. & Watson, S. P. (1999). Evidence that phospholipase C-gamma2 interacts with SLP-76, Syk, Lyn, LAT and the Fc receptor gamma-chain after stimulation of the collagen receptor glycoprotein VI in human platelets. *Eur J Biochem*, **263**, 612-23.

Grosse, J., Braun, A., Varga-Szabo, D., Beyersdorf, N., Schneider, B., Zeitlmann, L., Hanke, P., Schropp, P., Muhlstedt, S., Zorn, C., Huber, M., Schmittwolf, C., Jagla, W., Yu, P., Kerkau, T., Schulze, H., Nehls, M. & Nieswandt, B. (2007). An EF hand mutation in Stim1 causes premature platelet activation and bleeding in mice. *J Clin Invest*, **117**, 3540-50.

Grozovsky, R., Giannini, S., Falet, H. & Hoffmeister, K. M. (2015). Novel mechanisms of platelet clearance and thrombopoietin regulation. *Current opinion in hematology*, **22**, 445-451.

Grunberg, B., Kruse, H. J., Negrescu, E. V. & Siess, W. (1995). Platelet rap1B phosphorylation is a sensitive marker for the action of cyclic AMP- and cyclic GMP-increasing platelet inhibitors and vasodilators. *J Cardiovasc Pharmacol*, **25**, 545-51.

Gruner, S., Prostredna, M., Schulte, V., Krieg, T., Eckes, B., Brakebusch, C. & Nieswandt, B. (2003). Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo. *Blood*, **102**, 4021-7.

Gryglewski, R. J., Dembinska-Kiec, A. & Korbut, R. (1978). A possible role of thromboxane A2 (TXA2) and prostacyclin (PGI2) in circulation. *Acta Biol Med Ger*, **37**, 715-23.

Guan, X., Cravatt, B. F., Ehring, G. R., Hall, J. E., Boger, D. L., Lerner, R. A. & Gilula, N. B. (1997). The sleep-inducing lipid oleamide deconvolutes gap junction communication and calcium wave transmission in glial cells. *J Cell Biol*, **139**, 1785-92.

Gurney, A. L., Carver-Moore, K., de Sauvage, F. J. & Moore, M. W. (1994). Thrombocytopenia in c-mpl-deficient mice. *Science*, **265**, 1445-1448.

Habib, A., Vezza, R., Creminon, C., Maclouf, J. & FitzGerald, G. A. (1997). Rapid, agonist-dependent phosphorylation in vivo of human thromboxane receptor isoforms. Minimal involvement of protein kinase C. *J Biol Chem*, **272**, 7191-200.

Hamberg, M., Svensson, J. & Samuelsson, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A*, **72**, 2994-8.

Han, J., Lim, C. J., Watanabe, N., Soriani, A., Ratnikov, B., Calderwood, D. A., Puzon-McLaughlin, W., Lafuente, E. M., Boussiotis, V. A., Shattil, S. J. & Ginsberg, M. H. (2006). Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Curr Biol*, **16**, 1796-806.

Hanafy, K. A., Krumenacker, J. S. & Murad, F. (2001). NO, nitrotyrosine, and cyclic GMP in signal transduction. *Med Sci Monit*, **7**, 801-19.

Hanner, F., Sorensen, C. M., Holstein-Rathlou, N. H. & Peti-Peterdi, J. (2010). Connexins and the kidney. *Am J Physiol Regul Integr Comp Physiol*, **298**, R1143-55.

Harbeck, B., Huttelmaier, S., Schluter, K., Jockusch, B. M. & Illenberger, S. (2000). Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. *J Biol Chem*, **275**, 30817-25.

Harbury, P. A. B. (1998). Springs and zippers: coiled coils in SNARE-mediated membrane fusion. *Structure,* **6**, 1487-1491.

Harden, T. K., Waldo, G. L., Hicks, S. N. & Sondek, J. (2011). Mechanism of Activation and Inactivation of Gq/Phospholipase C-β Signaling Nodes. *Chemical reviews,* **111**, 6120-6129.

Hardy, A. R., Jones, M. L., Mundell, S. J. & Poole, A. W. (2004). Reciprocal cross-talk between P2Y1 and P2Y12 receptors at the level of calcium signaling in human platelets. *Blood,* **104**, 1745-52.

Harper, M. T. & Poole, A. W. (2010). Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation. *J Thromb Haemost,* **8**, 454-62.

Harper, M. T. & Poole, A. W. (2011). Store-operated calcium entry and non-capacitative calcium entry have distinct roles in thrombin-induced calcium signalling in human platelets. *Cell Calcium,* **50**, 351-8.

Harris, A. L. (2001). Emerging issues of connexin channels: biophysics fills the gap. *Q Rev Biophys,* **34**, 325-472.

Harris, A. L. (2002). Voltage-sensing and substate rectification: moving parts of connexin channels. *J Gen Physiol,* **119**, 165-9.

Harrison, P. & Cramer, E. M. (1993). Platelet alpha-granules. *Blood Rev,* **7**, 52-62.

Hartwig, J. H. (1992). Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol,* **118**, 1421-42.

Hassock, S. R., Zhu, M. X., Trost, C., Flockerzi, V. & Authi, K. S. (2002). Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood,* **100**, 2801-11.

Hathaway, D. R. & Adelstein, R. S. (1979). Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity. *Proc Natl Acad Sci U S A,* **76**, 1653-7.

Hayes, J. S. & Mayer, S. E. (1981). Regulation of guinea pig heart phosphorylase kinase by cAMP, protein kinase, and calcium. *Am J Physiol,* **240**, E340-9.

He, D. S., Jiang, J. X., Taffet, S. M. & Burt, J. M. (1999). Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells. *Proc Natl Acad Sci U S A,* **96**, 6495-500.

He, L., Pappan, L. K., Grenache, D. G., Li, Z., Tollefsen, D. M., Santoro, S. A. & Zutter, M. M. (2003). The contributions of the alpha 2 beta 1 integrin to vascular thrombosis in vivo. *Blood,* **102**, 3652-7.

Hechler, B. & Gachet, C. (2011). P2 receptors and platelet function. *Purinergic Signal,* **7**, 293-303.

Hechler, B., Leon, C., Vial, C., Vigne, P., Frelin, C., Cazenave, J. P. & Gachet, C. (1998). The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood,* **92**, 152-9.

Heemskerk, J. W., Bevers, E. M. & Lindhout, T. (2002). Platelet activation and blood coagulation. *Thromb Haemost,* **88**, 186-93.

Hemler, M. E. (2005). Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol,* **6**, 801-11.

Hermans, C., Wittevrongel, C., Thys, C., Smethurst, P. A., Van Geet, C. & Freson, K. (2009). A compound heterozygous mutation in glycoprotein VI in a patient with a bleeding disorder. *J Thromb Haemost,* **7**, 1356-63.

Herzog, B. H., Fu, J., Wilson, S. J., Hess, P. R., Sen, A., McDaniel, J. M., Pan, Y., Sheng, M., Yago, T., Silasi-Mansat, R., McGee, S., May, F., Nieswandt, B., Morris, A. J., Lupu, F., Coughlin, S. R., McEver, R. P., Chen, H., Kahn, M. L.

& Xia, L. (2013). Podoplanin maintains high endothelial venule integrity by interacting with platelet CLEC-2. *Nature,* **502**, 105-9.

Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S. & Narumiya, S. (1991). Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature,* **349**, 617-20.

Hodivala-Dilke, K. M., McHugh, K. P., Tsakiris, D. A., Rayburn, H., Crowley, D., Ullman-Cullere, M., Ross, F. P., Coller, B. S., Teitelbaum, S. & Hynes, R. O. (1999). Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest,* **103**, 229-38.

Hoffman, M. & Monroe, D. M., 3rd (2001). A cell-based model of hemostasis. *Thromb Haemost,* **85**, 958-65.

Hofmann, F. (2005). The biology of cyclic GMP-dependent protein kinases. *J Biol Chem,* **280**, 1-4.

Hoh, J. H., Lal, R., John, S. A., Revel, J. P. & Arnsdorf, M. F. (1991). Atomic force microscopy and dissection of gap junctions. *Science,* **253**, 1405-8.

Hollopeter, G., Jantzen, H. M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R. B., Nurden, P., Nurden, A., Julius, D. & Conley, P. B. (2001). Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature,* **409**, 202-7.

Holtkotter, O., Nieswandt, B., Smyth, N., Muller, W., Hafner, M., Schulte, V., Krieg, T. & Eckes, B. (2002). Integrin alpha 2-deficient mice develop normally, are fertile, but display partially defective platelet interaction with collagen. *J Biol Chem,* **277**, 10789-94.

Horii, K., Brooks, M. T. & Herr, A. B. (2009). Convulxin forms a dimer in solution and can bind eight copies of glycoprotein VI: implications for platelet activation. *Biochemistry,* **48**, 2907-14.

Horstrup, K., Jablonka, B., Honig-Liedl, P., Just, M., Kochsiek, K. & Walter, U. (1994). Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. *Eur J Biochem,* **225**, 21-7.

Hoth, M. & Penner, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature,* **355**, 353-6.

Hou, X., Pedi, L., Diver, M. M. & Long, S. B. (2012). Crystal structure of the calcium release-activated calcium channel Orai. *Science,* **338**, 1308-13.

Howell, W. H. & Donahue, D. D. (1937). The production of blood platelets in the lungs. *The Journal of Experimental Medicine,* **65**, 177-203.

Huang, J. S., Ramamurthy, S. K., Lin, X. & Le Breton, G. C. (2004). Cell signalling through thromboxane A2 receptors. *Cell Signal,* **16**, 521-33.

Huang, N., Lou, M., Liu, H., Avila, C. & Ma, Y. (2016). Identification of a potent small molecule capable of regulating polyploidization, megakaryocyte maturation, and platelet production. *Journal of Hematology & Oncology,* **9**, 136.

Hughes, C. E., Finney, B. A., Koentgen, F., Lowe, K. L. & Watson, S. P. (2015). The N-terminal SH2 domain of Syk is required for (hem)ITAM, but not integrin, signaling in mouse platelets. *Blood,* **125**, 144-54.

Hughes, C. E., Pollitt, A. Y., Mori, J., Eble, J. A., Tomlinson, M. G., Hartwig, J. H., O'Callaghan, C. A., Futterer, K. & Watson, S. P. (2010). CLEC-2 activates Syk through dimerization. *Blood,* **115**, 2947-55.

Hughes, C. E., Sinha, U., Pandey, A., Eble, J. A., O'Callaghan, C. A. & Watson, S. P. (2013). Critical Role for an acidic amino acid region in platelet signaling by the

HemITAM (hemi-immunoreceptor tyrosine-based activation motif) containing receptor CLEC-2 (C-type lectin receptor-2). *J Biol Chem,* **288**, 5127-35.

Hughes, S. W., Lorincz, M., Cope, D. W., Blethyn, K. L., Kekesi, K. A., Parri, H. R., Juhasz, G. & Crunelli, V. (2004). Synchronized oscillations at alpha and theta frequencies in the lateral geniculate nucleus. *Neuron,* **42**, 253-68.

Inoue, O., Suzuki-Inoue, K., Dean, W. L., Frampton, J. & Watson, S. P. (2003). Integrin α2β1 mediates outside-in regulation of platelet spreading on collagen through activation of Src kinases and PLCγ2. *The Journal of Cell Biology,* **160**, 769.

Inoue, O., Suzuki-Inoue, K., McCarty, O. J., Moroi, M., Ruggeri, Z. M., Kunicki, T. J., Ozaki, Y. & Watson, S. P. (2006). Laminin stimulates spreading of platelets through integrin alpha6beta1-dependent activation of GPVI. *Blood,* **107**, 1405-12.

Irvine, R. F. (1982). How is the level of free arachidonic acid controlled in mammalian cells? *Biochem J,* **204**, 3-16.

Isakson, B. E., Evans, W. H. & Boitano, S. (2001). Intercellular Ca2+ signaling in alveolar epithelial cells through gap junctions and by extracellular ATP. *Am J Physiol Lung Cell Mol Physiol,* **280**, L221-8.

Italiano, J. E. & Battinelli, E. M. (2009). Selective sorting of alpha-granule proteins. *Journal of thrombosis and haemostasis : JTH,* **7**, 173-176.

Italiano, J. E., Jr., Richardson, J. L., Patel-Hett, S., Battinelli, E., Zaslavsky, A., Short, S., Ryeom, S., Folkman, J. & Klement, G. L. (2008). Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood,* **111**, 1227-33.

Italiano, J. E., Jr. & Shivdasani, R. A. (2003). Megakaryocytes and beyond: the birth of platelets. *J Thromb Haemost,* **1**, 1174-82.

Italiano Jr, J. E. & Hartwig, J. H. (2013). Megakaryocyte Development and Platelet Formation. *In:* Michelson, A. D. (ed.) *Platelets.* 3 ed.: Academic Press.

Iwashita, S., Kobayashi, M., Kubo, Y., Hinohara, Y., Sezaki, M., Nakamura, K., Suzuki-Migishima, R., Yokoyama, M., Sato, S., Fukuda, M., Ohba, M., Kato, C., Adachi, E. & Song, S. Y. (2007). Versatile roles of R-Ras GAP in neurite formation of PC12 cells and embryonic vascular development. *J Biol Chem,* **282**, 3413-7.

Jackson, S. P. (2011). Arterial thrombosis--insidious, unpredictable and deadly. *Nat Med,* **17**, 1423-36.

Jaffe, E. A., Hoyer, L. W. & Nachman, R. L. (1974). Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc Natl Acad Sci U S A,* **71**, 1906-9.

Jandrot-Perrus, M., Lagrue, A. H., Okuma, M. & Bon, C. (1997). Adhesion and activation of human platelets induced by convulxin involve glycoprotein VI and integrin alpha2beta1. *J Biol Chem,* **272**, 27035-41.

Jansen, J. A., van Veen, T. A., de Bakker, J. M. & van Rijen, H. V. (2010). Cardiac connexins and impulse propagation. *J Mol Cell Cardiol,* **48**, 76-82.

Jantzen, H. M., Gousset, L., Bhaskar, V., Vincent, D., Tai, A., Reynolds, E. E. & Conley, P. B. (1999). Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost,* **81**, 111-7.

Jenkins, A. L., Nannizzi-Alaimo, L., Silver, D., Sellers, J. R., Ginsberg, M. H., Law, D. A. & Phillips, D. R. (1998). Tyrosine phosphorylation of the beta3 cytoplasmic domain mediates integrin-cytoskeletal interactions. *J Biol Chem,* **273**, 13878-85.

Jensen, B. O., Selheim, F., Doskeland, S. O., Gear, A. R. & Holmsen, H. (2004). Protein kinase A mediates inhibition of the thrombin-induced platelet shape change by nitric oxide. *Blood,* **104**, 2775-82.

Jiang, J. X. (2010). Gap junctions or hemichannel-dependent and independent roles of connexins in cataractogenesis and lens development. *Curr Mol Med,* **10**, 851-63.

Jiang, Y. & Cheng, H. (2007). Evidence of LAT as a dual substrate for Lck and Syk in T lymphocytes. *Leuk Res,* **31**, 541-5.

Jin, J., Daniel, J. L. & Kunapuli, S. P. (1998). Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem,* **273**, 2030-4.

John, S., Cesario, D. & Weiss, J. N. (2003). Gap junctional hemichannels in the heart. *Acta Physiol Scand,* **179**, 23-31.

John, S. A. & Revel, J. P. (1991). Connexon integrity is maintained by non-covalent bonds: intramolecular disulfide bonds link the extracellular domains in rat connexin-43. *Biochem Biophys Res Commun,* **178**, 1312-8.

Johnson, R. G., Meyer, R. A., Li, X. R., Preus, D. M., Tan, L., Grunenwald, H., Paulson, A. F., Laird, D. W. & Sheridan, J. D. (2002). Gap junctions assemble in the presence of cytoskeletal inhibitors, but enhanced assembly requires microtubules. *Exp Cell Res,* **275**, 67-80.

Johnstone, S. R., Billaud, M., Lohman, A. W., Taddeo, E. P. & Isakson, B. E. (2012). Posttranslational modifications in connexins and pannexins. *J Membr Biol,* **245**, 319-32.

Jones, C. I., Garner, S. F., Moraes, L. A., Kaiser, W. J., Rankin, A., Ouwehand, W. H., Goodall, A. H. & Gibbins, J. M. (2009). PECAM-1 expression and activity negatively regulate multiple platelet signaling pathways. *FEBS Lett,* **583**, 3618-24.

Jongen, W. M., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M. & Yamasaki, H. (1991). Regulation of connexin 43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin. *J Cell Biol,* **114**, 545-55.

Jonkman, J. & Brown, C. M. (2015). Any Way You Slice It-A Comparison of Confocal Microscopy Techniques. *J Biomol Tech,* **26**, 54-65.

Jonnalagadda, D., Izu, L. T. & Whiteheart, S. W. (2012). Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. *Blood,* **120**, 5209-16.

Joo, S.-J. (2012). Mechanisms of Platelet Activation and Integrin αIIβ3. *Korean Circulation Journal,* **42**, 295-301.

Jordan, K., Chodock, R., Hand, A. R. & Laird, D. W. (2001). The origin of annular junctions: a mechanism of gap junction internalization. *J Cell Sci,* **114**, 763-73.

Jordan, K., Solan, J. L., Dominguez, M., Sia, M., Hand, A., Lampe, P. D. & Laird, D. W. (1999). Trafficking, assembly, and function of a connexin43-green fluorescent protein chimera in live mammalian cells. *Mol Biol Cell,* **10**, 2033-50.

Jung, S. M. & Moroi, M. (2000a). Activation of the platelet collagen receptor integrin alpha(2)beta(1): its mechanism and participation in the physiological functions of platelets. *Trends Cardiovasc Med,* **10**, 285-92.

Jung, S. M. & Moroi, M. (2000b). Signal-transducing mechanisms involved in activation of the platelet collagen receptor integrin alpha(2)beta(1). *J Biol Chem,* **275**, 8016-26.

Junt, T., Schulze, H., Chen, Z., Massberg, S., Goerge, T., Krueger, A., Wagner, D. D., Graf, T., Italiano, J. E., Jr., Shivdasani, R. A. & von Andrian, U. H. (2007). Dynamic visualization of thrombopoiesis within bone marrow. *Science,* **317**, 1767-70.

Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H. & Coughlin, S. R. (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest,* **103**, 879-87.

Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jr., Tam, C. & Coughlin, S. R. (1998). A dual thrombin receptor system for platelet activation. *Nature,* **394**, 690-4.

Kameritsch, P., Pogoda, K. & Pohl, U. (2012). Channel-independent influence of connexin 43 on cell migration. *Biochim Biophys Acta,* **1818**, 1993-2001.

Kanaji, S., Kanaji, T., Furihata, K., Kato, K., Ware, J. L. & Kunicki, T. J. (2003). Convulxin binds to native, human glycoprotein Ib alpha. *J Biol Chem,* **278**, 39452-60.

Kanaporis, G., Brink, P. R. & Valiunas, V. (2011). Gap junction permeability: selectivity for anionic and cationic probes. *Am J Physiol Cell Physiol,* **300**, C600-9.

Kar, R., Batra, N., Riquelme, M. A. & Jiang, J. X. (2012). Biological role of connexin intercellular channels and hemichannels. *Arch Biochem Biophys,* **524**, 2-15.

Kasirer-Friede, A., Kahn, M. L. & Shattil, S. J. (2007). Platelet integrins and immunoreceptors. *Immunol Rev,* **218**, 247-64.

Kaushansky, K., Lichtman, M. A., Prchal, J., Levi, M. M., Press, O. W., Burns, L. J. & Caligiuri, M. (2015). *Williams Hematology.* New York, USA: McGraw-Hill Education.

Kaushansky, K., Lok, S., Holly, R. D., Broudy, V. C., Lin, N., Bailey, M. C., Forstrom, J. W., Buddle, M. M., Oort, P. J. & Hagen, F. S. (1994). Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature,* **369**, 568-571.

Kauskot, A. & Hoylaerts, M. F. (2012). Platelet receptors. *Handb Exp Pharmacol,* 23-57.

Kelly, J. J., Simek, J. & Laird, D. W. (2015). Mechanisms linking connexin mutations to human diseases. *Cell Tissue Res,* **360**, 701-21.

Kelsell, D. P., Dunlop, J., Stevens, H. P., Lench, N. J., Liang, J. N., Parry, G., Mueller, R. F. & Leigh, I. M. (1997). Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. *Nature,* **387**, 80-3.

Kemp, B. E. & Pearson, R. B. (1990). Protein kinase recognition sequence motifs. *Trends Biochem Sci,* **15**, 342-6.

Keuren, J. F. W., Wielders, S. J. H., Ulrichts, H., Hackeng, T., Heemskerk, J. W. M., Deckmyn, H., Bevers, E. M. & Lindhout, T. (2005). Synergistic Effect of Thrombin on Collagen-Induced Platelet Procoagulant Activity Is Mediated Through Protease-Activated Receptor-1. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **25**, 1499.

Khan, F., Vaillancourt, C. & Bourjeily, G. (2017). Diagnosis and management of deep vein thrombosis in pregnancy. *BMJ,* **357**.

Kidder, G. M. & Mhawi, A. A. (2002). Gap junctions and ovarian folliculogenesis. *Reproduction,* **123**, 613-20.

Kleuss, C., Raw, A. S., Lee, E., Sprang, S. R. & Gilman, A. G. (1994). Mechanism of GTP hydrolysis by G-protein alpha subunits. *Proc Natl Acad Sci U S A,* **91**, 9828-31.

Knezevic, I., Borg, C. & Le Breton, G. C. (1993). Identification of Gq as one of the G-proteins which copurify with human platelet thromboxane A2/prostaglandin H2 receptors. *J Biol Chem*, **268**, 26011-7.

Knight, C. G., Morton, L. F., Onley, D. J., Peachey, A. R., Ichinohe, T., Okuma, M., Farndale, R. W. & Barnes, M. J. (1999). Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc Res*, **41**, 450-7.

Koedam, J. A., Cramer, E. M., Briend, E., Furie, B., Furie, B. C. & Wagner, D. D. (1992). P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. *J Cell Biol*, **116**, 617-25.

Konishi, H., Katoh, Y., Takaya, N., Kashiwakura, Y., Itoh, S., Ra, C. & Daida, H. (2002). Platelets activated by collagen through immunoreceptor tyrosine-based activation motif play pivotal role in initiation and generation of neointimal hyperplasia after vascular injury. *Circulation*, **105**, 912-6.

Koval, M. (2006). Pathways and control of connexin oligomerization. *Trends Cell Biol*, **16**, 159-66.

Koval, M., Harley, J. E., Hick, E. & Steinberg, T. H. (1997). Connexin46 is retained as monomers in a trans-Golgi compartment of osteoblastic cells. *J Cell Biol*, **137**, 847-57.

Koval, M., Molina, S. A. & Burt, J. M. (2014). Mix and match: investigating heteromeric and heterotypic gap junction channels in model systems and native tissues. *FEBS Lett*, **588**, 1193-204.

Kuter, D. J. (1996). The physiology of platelet production. *Stem Cells*, **14 Suppl 1**, 88-101.

Kwak, B. R. & Jongsma, H. J. (1996). Regulation of cardiac gap junction channel permeability and conductance by several phosphorylating conditions. *Mol Cell Biochem*, **157**, 93-9.

Kwak, B. R. & Jongsma, H. J. (1999). Selective inhibition of gap junction channel activity by synthetic peptides. *J Physiol*, **516 (Pt 3)**, 679-85.

Kwak, B. R., Mulhaupt, F., Veillard, N., Gros, D. B. & Mach, F. (2002). Altered pattern of vascular connexin expression in atherosclerotic plaques. *Arterioscler Thromb Vasc Biol*, **22**, 225-30.

Kwak, B. R., van Veen, T. A., Analbers, L. J. & Jongsma, H. J. (1995). TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. *Exp Cell Res*, **220**, 456-63.

Laing, J. G. & Beyer, E. C. (1995). The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J Biol Chem*, **270**, 26399-403.

Laird, D. W. (2006). Life cycle of connexins in health and disease. *Biochem J*, **394**, 527-43.

Laird, D. W., Castillo, M. & Kasprzak, L. (1995). Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. *J Cell Biol*, **131**, 1193-203.

Lampe, P. D. & Lau, A. F. (2000). Regulation of gap junctions by phosphorylation of connexins. *Arch Biochem Biophys*, **384**, 205-15.

Lampe, P. D. & Lau, A. F. (2004). The effects of connexin phosphorylation on gap junctional communication. *Int J Biochem Cell Biol*, **36**, 1171-86.

Lanza, F. (2006). Bernard-Soulier syndrome (hemorrhagiparous thrombocytic dystrophy). *Orphanet J Rare Dis*, **1**, 46.

Lanza, F., Cazenave, J. P., Beretz, A., Sutter-Bay, A., Kretz, J. G. & Kieny, R. (1986). Potentiation by adrenaline of human platelet activation and the inhibition by the alpha-adrenergic antagonist nicergoline of platelet adhesion, secretion and aggregation. *Agents Actions,* **18**, 586-95.

Lauf, U., Giepmans, B. N., Lopez, P., Braconnot, S., Chen, S. C. & Falk, M. M. (2002). Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. *Proc Natl Acad Sci U S A,* **99**, 10446-51.

Law, D. A., DeGuzman, F. R., Heiser, P., Ministri-Madrid, K., Killeen, N. & Phillips, D. R. (1999a). Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3 signalling and platelet function. *Nature,* **401**, 808-11.

Law, D. A., Nannizzi-Alaimo, L., Ministri, K., Hughes, P. E., Forsyth, J., Turner, M., Shattil, S. J., Ginsberg, M. H., Tybulewicz, V. L. & Phillips, D. R. (1999b). Genetic and pharmacological analyses of Syk function in alphaIIbbeta3 signaling in platelets. *Blood,* **93**, 2645-52.

Lee, R. E., Young, R. H. & Castleman, B. (2002). James Homer Wright: a biography of the enigmatic creator of the Wright stain on the occasion of its centennial. *Am J Surg Pathol,* **26**, 88-96.

Lefrançais, E., Ortiz-Muñoz, G., Caudrillier, A., Mallavia, B., Liu, F., Sayah, D. M., Thornton, E. E., Headley, M. B., David, T., Coughlin, S. R., Krummel, M. F., Leavitt, A. D., Passegué, E. & Looney, M. R. (2017). The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. *Nature,* **544**, 105-109.

Leithe, E. & Rivedal, E. (2004a). Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43. *J Cell Sci,* **117**, 1211-20.

Leithe, E. & Rivedal, E. (2004b). Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment. *J Biol Chem,* **279**, 50089-96.

Leitinger, B. & Hogg, N. (2002). The involvement of lipid rafts in the regulation of integrin function. *J Cell Sci,* **115**, 963-72.

Lerner, D. J., Chen, M., Tram, T. & Coughlin, S. R. (1996). Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *J Biol Chem,* **271**, 13943-7.

Levine, R. F., Eldor, A., Shoff, P. K., Kirwin, S., Tenza, D. & Cramer, E. M. (1993). Circulating megakaryocytes: delivery of large numbers of intact, mature megakaryocytes to the lungs. *Eur J Haematol,* **51**, 233-46.

Leybaert, L., Braet, K., Vandamme, W., Cabooter, L., Martin, P. E. & Evans, W. H. (2003). Connexin channels, connexin mimetic peptides and ATP release. *Cell Commun Adhes,* **10**, 251-7.

Leybaert, L., Lampe, P. D., Dhein, S., Kwak, B. R., Ferdinandy, P., Beyer, E. C., Laird, D. W., Naus, C. C., Green, C. R. & Schulz, R. (2017). Connexins in Cardiovascular and Neurovascular Health and Disease: Pharmacological Implications. *Pharmacol Rev,* **69**, 396-478.

Leznik, E. & Llinas, R. (2005). Role of gap junctions in synchronized neuronal oscillations in the inferior olive. *J Neurophysiol,* **94**, 2447-56.

Li, Z., Delaney, M. K., O'Brien, K. A. & Du, X. (2010). Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol,* **30**, 2341-9.

Li, Z., Zhang, G., Le Breton, G. C., Gao, X., Malik, A. B. & Du, X. (2003). Two waves of platelet secretion induced by thromboxane A2 receptor and a critical role for phosphoinositide 3-kinases. *J Biol Chem,* **278**, 30725-31.

Liebmann, C. & Bohmer, F. D. (2000). Signal transduction pathways of G protein-coupled receptors and their cross-talk with receptor tyrosine kinases: lessons from bradykinin signaling. *Curr Med Chem,* **7**, 911-43.

Lin, J. C., Duell, K. & Konopka, J. B. (2004). A microdomain formed by the extracellular ends of the transmembrane domains promotes activation of the G protein-coupled alpha-factor receptor. *Mol Cell Biol,* **24**, 2041-51.

Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., Jr. & Meyer, T. (2005). STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. *Curr Biol,* **15**, 1235-41.

Liu, J., Ek Vitorin, J. F., Weintraub, S. T., Gu, S., Shi, Q., Burt, J. M. & Jiang, J. X. (2011). Phosphorylation of connexin 50 by protein kinase A enhances gap junction and hemichannel function. *J Biol Chem,* **286**, 16914-28.

Liu, S., Taffet, S., Stoner, L., Delmar, M., Vallano, M. L. & Jalife, J. (1993). A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: the carboxyl tail length. *Biophys J,* **64**, 1422-33.

Lockyer, S., Okuyama, K., Begum, S., Le, S., Sun, B., Watanabe, T., Matsumoto, Y., Yoshitake, M., Kambayashi, J. & Tandon, N. N. (2006). GPVI-deficient mice lack collagen responses and are protected against experimentally induced pulmonary thromboembolism. *Thromb Res,* **118**, 371-80.

Lopez, J. A., Andrews, R. K., Afshar-Kharghan, V. & Berndt, M. C. (1998). Bernard-Soulier syndrome. *Blood,* **91**, 4397-418.

Lova, P., Paganini, S., Sinigaglia, F., Balduini, C. & Torti, M. (2002). A Gi-dependent pathway is required for activation of the small GTPase Rap1B in human platelets. *J Biol Chem,* **277**, 12009-15.

Luo, B. H., Carman, C. V. & Springer, T. A. (2007b). Structural basis of integrin regulation and signaling. *Annu Rev Immunol,* **25**, 619-47.

Luo, S. Z., Mo, X., Afshar-Kharghan, V., Srinivasan, S., Lopez, J. A. & Li, R. (2007a). Glycoprotein Ibalpha forms disulfide bonds with 2 glycoprotein Ibbeta subunits in the resting platelet. *Blood,* **109**, 603-9.

Lurtz, M. M. & Louis, C. F. (2007). Intracellular calcium regulation of connexin43. *Am J Physiol Cell Physiol,* **293**, C1806-13.

Ma, Y. Q., Qin, J. & Plow, E. F. (2007). Platelet integrin alpha(IIb)beta(3): activation mechanisms. *J Thromb Haemost,* **5**, 1345-52.

Machlus, K. R., Thon, J. N. & Italiano, J. E., Jr. (2014). Interpreting the developmental dance of the megakaryocyte: a review of the cellular and molecular processes mediating platelet formation. *Br J Haematol,* **165**, 227-36.

Machtaler, S., Dang-Lawson, M., Choi, K., Jang, C., Naus, C. C. & Matsuuchi, L. (2011). The gap junction protein Cx43 regulates B-lymphocyte spreading and adhesion. *J Cell Sci,* **124**, 2611-21.

Mahaut-Smith, M. P. (2012). The unique contribution of ion channels to platelet and megakaryocyte function. *J Thromb Haemost,* **10**, 1722-32.

Mahaut-Smith, M. P., Jones, S. & Evans, R. J. (2011). The P2X1 receptor and platelet function. *Purinergic Signal,* **7**, 341-56.

Mahaut-Smith, M. P., Tolhurst, G. & Evans, R. J. (2004). Emerging roles for P2X1 receptors in platelet activation. *Platelets,* **15**, 131-44.

Makowski, L., Caspar, D. L., Phillips, W. C. & Goodenough, D. A. (1977). Gap junction structures. II. Analysis of the x-ray diffraction data. *J Cell Biol,* **74**, 629-45.

Malinin, N. L., Zhang, L., Choi, J., Ciocea, A., Razorenova, O., Ma, Y. Q., Podrez, E. A., Tosi, M., Lennon, D. P., Caplan, A. I., Shurin, S. B., Plow, E. F. & Byzova, T. V. (2009). A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. *Nat Med,* **15**, 313-8.

Manganello, J. M., Djellas, Y., Borg, C., Antonakis, K. & Le Breton, G. C. (1999). Cyclic AMP-dependent phosphorylation of thromboxane A(2) receptor-associated Galpha(13). *J Biol Chem*, **274**, 28003-10.

Manganello, J. M., Huang, J. S., Kozasa, T., Voyno-Yasenetskaya, T. A. & Le Breton, G. C. (2003). Protein kinase A-mediated phosphorylation of the Galpha13 switch I region alters the Galphabetagamma13-G protein-coupled receptor complex and inhibits Rho activation. *J Biol Chem,* **278**, 124-30.

Mangin, P., David, T., Lavaud, V., Cranmer, S. L., Pikovski, I., Jackson, S. P., Berndt, M. C., Cazenave, J. P., Gachet, C. & Lanza, F. (2004). Identification of a novel 14-3-3zeta binding site within the cytoplasmic tail of platelet glycoprotein Ibalpha. *Blood,* **104**, 420-7.

Mangin, P., Yuan, Y., Goncalves, I., Eckly, A., Freund, M., Cazenave, J. P., Gachet, C., Jackson, S. P. & Lanza, F. (2003). Signaling role for phospholipase C gamma 2 in platelet glycoprotein Ib alpha calcium flux and cytoskeletal reorganization. Involvement of a pathway distinct from FcR gamma chain and Fc gamma RIIA. *J Biol Chem,* **278**, 32880-91.

Manjarrez-Marmolejo, J. & Franco-Perez, J. (2016). Gap Junction Blockers: An Overview of their Effects on Induced Seizures in Animal Models. *Curr Neuropharmacol*, **14**, 759-71.

Marcus, A. J. & Safier, L. B. (1993). Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. *Faseb j,* **7**, 516-22.

Martin, J. F., Trowbridge, E. A., Salmon, G. L. & Slater, D. N. (1982). The relationship between platelet and megakaryocyte volumes. *Thromb Res,* **28**, 447-59.

Martin, P. E., Blundell, G., Ahmad, S., Errington, R. J. & Evans, W. H. (2001). Multiple pathways in the trafficking and assembly of connexin 26, 32 and 43 into gap junction intercellular communication channels. *J Cell Sci,* **114**, 3845-55.

Martin, P. E. & Evans, W. H. (2004). Incorporation of connexins into plasma membranes and gap junctions. *Cardiovasc Res,* **62**, 378-87.

Martin, P. E., Wall, C. & Griffith, T. M. (2005). Effects of connexin-mimetic peptides on gap junction functionality and connexin expression in cultured vascular cells. *Br J Pharmacol,* **144**, 617-27.

Mason, K. D., Carpinelli, M. R., Fletcher, J. I., Collinge, J. E., Hilton, A. A., Ellis, S., Kelly, P. N., Ekert, P. G., Metcalf, D., Roberts, A. W., Huang, D. C. & Kile, B. T. (2007). Programmed anuclear cell death delimits platelet life span. *Cell,* **128**, 1173-86.

Massberg, S., Gawaz, M., Gruner, S., Schulte, V., Konrad, I., Zohlnhofer, D., Heinzmann, U. & Nieswandt, B. (2003). A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med,* **197**, 41-9.

Massberg, S., Sausbier, M., Klatt, P., Bauer, M., Pfeifer, A., Siess, W., Fassler, R., Ruth, P., Krombach, F. & Hofmann, F. (1999). Increased adhesion and aggregation of platelets lacking cyclic guanosine 3',5'-monophosphate kinase I. *J Exp Med,* **189**, 1255-64.

Matchkov, V. V., Rahman, A., Peng, H., Nilsson, H. & Aalkjaer, C. (2004). Junctional and nonjunctional effects of heptanol and glycyrrhetinic acid derivates in rat mesenteric small arteries. *Br J Pharmacol,* **142**, 961-72.

Matsue, H., Yao, J., Matsue, K., Nagasaka, A., Sugiyama, H., Aoki, R., Kitamura, M. & Shimada, S. (2006). Gap junction-mediated intercellular communication between dendritic cells (DCs) is required for effective activation of DCs. *J Immunol,* **176**, 181-90.

Matsumoto, Y., Takizawa, H., Gong, X., Le, S., Lockyer, S., Okuyama, K., Tanaka, M., Yoshitake, M., Tandon, N. N. & Kambayashi, J. (2007). Highly potent anti-human GPVI monoclonal antibodies derived from GPVI knockout mouse immunization. *Thromb Res,* **119**, 319-29.

Matus, V., Valenzuela, G., Saez, C. G., Hidalgo, P., Lagos, M., Aranda, E., Panes, O., Pereira, J., Pillois, X., Nurden, A. T. & Mezzano, D. (2013). An adenine insertion in exon 6 of human GP6 generates a truncated protein associated with a bleeding disorder in four Chilean families. *J Thromb Haemost,* **11**, 1751-9.

Maynard, D. M., Heijnen, H. F., Horne, M. K., White, J. G. & Gahl, W. A. (2007). Proteomic analysis of platelet alpha-granules using mass spectrometry. *J Thromb Haemost,* **5**, 1945-55.

McLachlan, E., Shao, Q. & Laird, D. W. (2007). Connexins and gap junctions in mammary gland development and breast cancer progression. *J Membr Biol,* **218**, 107-21.

Meens, M. J., Pfenniger, A. & Kwak, B. R. (2012). Risky communication in atherosclerosis and thrombus formation. *Swiss Med Wkly,* **142**, w13553.

Mendoza-Naranjo, A., Bouma, G., Pereda, C., Ramirez, M., Webb, K. F., Tittarelli, A., Lopez, M. N., Kalergis, A. M., Thrasher, A. J., Becker, D. L. & Salazar-Onfray, F. (2011). Functional gap junctions accumulate at the immunological synapse and contribute to T cell activation. *J Immunol,* **187**, 3121-32.

Meyer, R. A., Laird, D. W., Revel, J. P. & Johnson, R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J Cell Biol,* **119**, 179-89.

Meyers, K. M., Holmsen, H. & Seachord, C. L. (1982). Comparative study of platelet dense granule constituents. *Am J Physiol,* **243**, R454-61.

Milks, L. C., Kumar, N. M., Houghten, R., Unwin, N. & Gilula, N. B. (1988). Topology of the 32-kd liver gap junction protein determined by site-directed antibody localizations. *Embo j,* **7**, 2967-75.

Mittelbrunn, M. & Sanchez-Madrid, F. (2012). Intercellular communication: diverse structures for exchange of genetic information. *Nat Rev Mol Cell Biol,* **13**, 328-35.

Mocsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L. & Lowell, C. A. (2006). Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat Immunol,* **7**, 1326-33.

Mohamed, O. A., Jonnaert, M., Labelle-Dumais, C., Kuroda, K., Clarke, H. J. & Dufort, D. (2005). Uterine Wnt/beta-catenin signaling is required for implantation. *Proc Natl Acad Sci U S A,* **102**, 8579-84.

Molica, F., Stierlin, F. B., Fontana, P. & Kwak, B. R. (2017). Pannexin- and Connexin-Mediated Intercellular Communication in Platelet Function. *Int J Mol Sci,* **18**.

Moncada, S. (1982). Prostacyclin and arterial wall biology. *Arteriosclerosis,* **2**, 193-207.

Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature,* **263**, 663-5.

Montecino-Rodriguez, E., Leathers, H. & Dorshkind, K. (2000). Expression of connexin 43 (Cx43) is critical for normal hematopoiesis. *Blood,* **96**, 917-24.

Morel, S. (2014). Multiple roles of connexins in atherosclerosis- and restenosis-induced vascular remodelling. *J Vasc Res,* **51**, 149-61.

Moreno, A. P., Chanson, M., Elenes, S., Anumonwo, J., Scerri, I., Gu, H., Taffet, S. M. & Delmar, M. (2002). Role of the carboxyl terminal of connexin43 in transjunctional fast voltage gating. *Circ Res,* **90**, 450-7.

Morley, G. E., Taffet, S. M. & Delmar, M. (1996). Intramolecular interactions mediate pH regulation of connexin43 channels. *Biophys J,* **70**, 1294-302.

Moroi, M. & Jung, S. M. (2004). Platelet glycoprotein VI: its structure and function. *Thromb Res,* **114**, 221-33.

Moroi, M., Jung, S. M., Shinmyozu, K., Tomiyama, Y., Ordinas, A. & Diaz-Ricart, M. (1996). Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. *Blood,* **88**, 2081-92.

Morrissey, J. H. (2012). Polyphosphate: a link between platelets, coagulation and inflammation. *Int J Hematol,* **95**, 346-52.

Moser, M., Nieswandt, B., Ussar, S., Pozgajova, M. & Fassler, R. (2008). Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med,* **14**, 325-30.

Mu, F. T., Andrews, R. K., Arthur, J. F., Munday, A. D., Cranmer, S. L., Jackson, S. P., Stomski, F. C., Lopez, A. F. & Berndt, M. C. (2008). A functional 14-3-3zeta-independent association of PI3-kinase with glycoprotein Ib alpha, the major ligand-binding subunit of the platelet glycoprotein Ib-IX-V complex. *Blood,* **111**, 4580-7.

Mumford, A. D., Dawood, B. B., Daly, M. E., Murden, S. L., Williams, M. D., Protty, M. B., Spalton, J. C., Wheatley, M., Mundell, S. J. & Watson, S. P. (2010). A novel thromboxane A2 receptor D304N variant that abrogates ligand binding in a patient with a bleeding diathesis. *Blood,* **115**, 363-9.

Mundell, S. J., Jones, M. L., Hardy, A. R., Barton, J. F., Beaucourt, S. M., Conley, P. B. & Poole, A. W. (2006). Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. *Mol Pharmacol,* **70**, 1132-42.

Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S. & Narumiya, S. (1997). Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature,* **388**, 678-82.

Murugappa, S. & Kunapuli, S. P. (2006). The role of ADP receptors in platelet function. *Front Biosci,* **11**, 1977-86.

Musil, L. S., Cunningham, B. A., Edelman, G. M. & Goodenough, D. A. (1990). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J Cell Biol,* **111**, 2077-88.

Musil, L. S. & Goodenough, D. A. (1993). Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell,* **74**, 1065-77.

Musil, L. S., Le, A. C., VanSlyke, J. K. & Roberts, L. M. (2000). Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J Biol Chem,* **275**, 25207-15.

Nagy, J. I., Ionescu, A. V., Lynn, B. D. & Rash, J. E. (2003). Coupling of astrocyte connexins Cx26, Cx30, Cx43 to oligodendrocyte Cx29, Cx32, Cx47: Implications from normal and connexin32 knockout mice. *Glia,* **44**, 205-18.

Nakahata, N. (2008). Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacol Ther,* **118**, 18-35.

Nakamura, T. & Gold, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature,* **325**, 442-4.

Narumiya, S., Sugimoto, Y. & Ushikubi, F. (1999). Prostanoid receptors: structures, properties, and functions. *Physiol Rev,* **79**, 1193-226.

Naseem, K. M. & Roberts, W. (2011). Nitric oxide at a glance. *Platelets,* **22**, 148-52.

Naus, C. C. & Giaume, C. (2016). Bridging the gap to therapeutic strategies based on connexin/pannexin biology. *Journal of Translational Medicine,* **14**, 330.

Neijssen, J., Herberts, C., Drijfhout, J. W., Reits, E., Janssen, L. & Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. *Nature,* **434**, 83-8.

Nelles, E., Butzler, C., Jung, D., Temme, A., Gabriel, H. D., Dahl, U., Traub, O., Stumpel, F., Jungermann, K., Zielasek, J., Toyka, K. V., Dermietzel, R. & Willecke, K. (1996). Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. *Proc Natl Acad Sci U S A,* **93**, 9565-70.

Nguyen, T. D. & Taffet, S. M. (2009). A model system to study Connexin 43 in the immune system. *Mol Immunol,* **46**, 2938-46.

Ni, H., Denis, C. V., Subbarao, S., Degen, J. L., Sato, T. N., Hynes, R. O. & Wagner, D. D. (2000). Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen. *J Clin Invest,* **106**, 385-92.

Niessen, J., Jedlitschky, G., Grube, M., Bien, S., Strobel, U., Ritter, C. A., Greinacher, A. & Kroemer, H. K. (2007). Subfractionation and purification of intracellular granule-structures of human platelets: an improved method based on magnetic sorting. *J Immunol Methods,* **328**, 89-96.

Nieswandt, B., Brakebusch, C., Bergmeier, W., Schulte, V., Bouvard, D., Mokhtari-Nejad, R., Lindhout, T., Heemskerk, J. W., Zirngibl, H. & Fassler, R. (2001). Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *Embo j,* **20**, 2120-30.

Nieswandt, B. & Watson, S. P. (2003). Platelet-collagen interaction: is GPVI the central receptor? *Blood,* **102**, 449-61.

Noe, L., Peeters, K., Izzi, B., Van Geet, C. & Freson, K. (2010). Regulators of platelet cAMP levels: clinical and therapeutic implications. *Curr Med Chem,* **17**, 2897-905.

Noubiap, J. J. (2014). Shifting from quinine to artesunate as first-line treatment of severe malaria in children and adults: saving more lives. *J Infect Public Health,* **7**, 407-12.

Nurden, A. T. (2006). Glanzmann thrombasthenia. *Orphanet J Rare Dis,* **1**, 10.

Nuyttens, B. P., Thijs, T., Deckmyn, H. & Broos, K. (2011). Platelet adhesion to collagen. *Thromb Res,* **127 Suppl 2**, S26-9.

Obergfell, A., Eto, K., Mocsai, A., Buensuceso, C., Moores, S. L., Brugge, J. S., Lowell, C. A. & Shattil, S. J. (2002). Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. *J Cell Biol,* **157**, 265-75.

Oda, A., Ikeda, Y., Ochs, H. D., Druker, B. J., Ozaki, K., Handa, M., Ariga, T., Sakiyama, Y., Witte, O. N. & Wahl, M. I. (2000). Rapid tyrosine

phosphorylation and activation of Bruton's tyrosine/Tec kinases in platelets induced by collagen binding or CD32 cross-linking. *Blood*, **95**, 1663-70.

Offermanns, S. (2006). Activation of platelet function through G protein-coupled receptors. *Circ Res,* **99**, 1293-304.

Ohlmann, P., Hechler, B., Cazenave, J. P. & Gachet, C. (2004). Measurement and manipulation of [Ca2+]i in suspensions of platelets and cell cultures. *Methods Mol Biol,* **273**, 229-50.

Oldham, W. M. & Hamm, H. E. (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol,* **9**, 60-71.

Olivier, N., Keller, D., Gonczy, P. & Manley, S. (2013). Resolution doubling in 3D-STORM imaging through improved buffers. *PLoS One,* **8**, e69004.

Omar, H. R., Komarova, I., El-Ghonemi, M., Fathy, A., Rashad, R., Abdelmalak, H. D., Yerramadha, M. R., Ali, Y., Helal, E. & Camporesi, E. M. (2012). Licorice abuse: time to send a warning message. *Ther Adv Endocrinol Metab,* **3**, 125-38.

Orellana, J. A., Froger, N., Ezan, P., Jiang, J. X., Bennett, M. V., Naus, C. C., Giaume, C. & Saez, J. C. (2011). ATP and glutamate released via astroglial connexin 43 hemichannels mediate neuronal death through activation of pannexin 1 hemichannels. *J Neurochem,* **118**, 826-40.

Osdoit, S. & Rosa, J.-P. (2001). Fibrin Clot Retraction by Human Platelets Correlates with αIIbβ3Integrin-dependent Protein Tyrosine Dephosphorylation. *Journal of Biological Chemistry,* **276**, 6703-6710.

Osler, W. (1873). An account of certain organisms occurring in the liquor sanguinis. *Proceedings of the Royal Society of London,* **22**, 391-398.

Otsuka, F., Yasuda, S., Noguchi, T. & Ishibashi-Ueda, H. (2016). Pathology of coronary atherosclerosis and thrombosis. *Cardiovascular Diagnosis and Therapy,* **6**, 396-408.

Ouyang, X., Winbow, V. M., Patel, L. S., Burr, G. S., Mitchell, C. K. & O'Brien, J. (2005). Protein kinase A mediates regulation of gap junctions containing connexin35 through a complex pathway. *Brain Res Mol Brain Res,* **135**, 1-11.

Oviedo-Orta, E., Gasque, P. & Evans, W. H. (2001). Immunoglobulin and cytokine expression in mixed lymphocyte cultures is reduced by disruption of gap junction intercellular communication. *Faseb j,* **15**, 768-74.

Oviedo-Orta, E., Hoy, T. & Evans, W. H. (2000). Intercellular communication in the immune system: differential expression of connexin40 and 43, and perturbation of gap junction channel functions in peripheral blood and tonsil human lymphocyte subpopulations. *Immunology,* **99**, 578-90.

Oviedo-Orta, E., Perreau, M., Evans, W. H. & Potolicchio, I. (2010). Control of the proliferation of activated CD4+ T cells by connexins. *J Leukoc Biol,* **88**, 79-86.

Oyamada, M., Oyamada, Y. & Takamatsu, T. (2005). Regulation of connexin expression. *Biochim Biophys Acta,* **1719**, 6-23.

Ozaki, Y., Asazuma, N., Suzuki-Inoue, K. & Berndt, M. C. (2005). Platelet GPIb-IX-V-dependent signaling. *J Thromb Haemost,* **3**, 1745-51.

Parente, L. & Perretti, M. (2003). Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. *Biochem Pharmacol,* **65**, 153-9.

Patel, D., Zhang, X. & Veenstra, R. D. (2014). Connexin hemichannel and pannexin channel electrophysiology: how do they differ? *FEBS Lett,* **588**, 1372-8.

Patel, S. R., Hartwig, J. H. & Italiano, J. E. (2005). The biogenesis of platelets from megakaryocyte proplatelets. *Journal of Clinical Investigation,* **115**, 3348-3354.

Paul, B. Z., Jin, J. & Kunapuli, S. P. (1999). Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem,* **274**, 29108-14.

Paulson, A. F., Lampe, P. D., Meyer, R. A., TenBroek, E., Atkinson, M. M., Walseth, T. F. & Johnson, R. G. (2000). Cyclic AMP and LDL trigger a rapid enhancement in gap junction assembly through a stimulation of connexin trafficking. *J Cell Sci,* **113 (Pt 17)**, 3037-49.

Paznekas, W. A., Boyadjiev, S. A., Shapiro, R. E., Daniels, O., Wollnik, B., Keegan, C. E., Innis, J. W., Dinulos, M. B., Christian, C., Hannibal, M. C. & Jabs, E. W. (2003). Connexin 43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. *Am J Hum Genet,* **72**, 408-18.

Peracchia, C. (2004). Chemical gating of gap junction channels; roles of calcium, pH and calmodulin. *Biochim Biophys Acta,* **1662**, 61-80.

Peracchia, C., Sotkis, A., Wang, X. G., Peracchia, L. L. & Persechini, A. (2000). Calmodulin directly gates gap junction channels. *J Biol Chem,* **275**, 26220-4.

Peyvandi, F., Garagiola, I. & Baronciani, L. (2011). Role of von Willebrand factor in the haemostasis. *Blood Transfusion,* **9**, s3-s8.

Pfenniger, A., Chanson, M. & Kwak, B. R. (2013). Connexins in atherosclerosis. *Biochim Biophys Acta,* **1828**, 157-66.

Pfenniger, A., Derouette, J. P., Verma, V., Lin, X., Foglia, B., Coombs, W., Roth, I., Satta, N., Dunoyer-Geindre, S., Sorgen, P., Taffet, S., Kwak, B. R. & Delmar, M. (2010). Gap junction protein Cx37 interacts with endothelial nitric oxide synthase in endothelial cells. *Arterioscler Thromb Vasc Biol,* **30**, 827-34.

Pfenniger, A., Wohlwend, A. & Kwak, B. R. (2011). Mutations in connexin genes and disease. *Eur J Clin Invest,* **41**, 103-16.

Pidoux, G. & Tasken, K. (2015). Anchored PKA as a gatekeeper for gap junctions. *Commun Integr Biol,* **8**, e1057361.

Pinder, R. M., Brogden, R. N., Sawyer, P. R., Speight, T. M., Spencer, R. & Avery, G. S. (1976). Carbenoxolone: a review of its pharmacological properties and therapeutic efficacy in peptic ulcer disease. *Drugs,* **11**, 245-307.

Podoplelova, N. A., Sveshnikova, A. N., Kotova, Y. N., Eckly, A., Receveur, N., Nechipurenko, D. Y., Obydennyi, S. I., Kireev, II, Gachet, C., Ataullakhanov, F. I., Mangin, P. H. & Panteleev, M. A. (2016). Coagulation factors bound to procoagulant platelets concentrate in cap structures to promote clotting. *Blood,* **128**, 1745-55.

Pointis, G. & Segretain, D. (2005). Role of connexin-based gap junction channels in testis. *Trends Endocrinol Metab,* **16**, 300-6.

Polanowska-Grabowska, R., Simon, C. G., Jr. & Gear, A. R. (1999). Platelet adhesion to collagen type I, collagen type IV, von Willebrand factor, fibronectin, laminin and fibrinogen: rapid kinetics under shear. *Thromb Haemost,* **81**, 118-23.

Polasek, J. (2005). Platelet secretory granules or secretory lysosomes? *Platelets,* **16**, 500-501.

Polgar, J. & Reed, G. L. (1999). A critical role for N-ethylmaleimide-sensitive fusion protein (NSF) in platelet granule secretion. *Blood,* **94**, 1313-8.

Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G., Saito, T., Tybulewicz, V. L. & Watson, S. P. (1997). The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *Embo j,* **16**, 2333-41.

Powell, D. W., Rane, M. J., Chen, Q., Singh, S. & McLeish, K. R. (2002). Identification of 14-3-3zeta as a protein kinase B/Akt substrate. *J Biol Chem,* **277**, 21639-42.

Pozgajova, M., Sachs, U. J., Hein, L. & Nieswandt, B. (2006). Reduced thrombus stability in mice lacking the alpha2A-adrenergic receptor. *Blood,* **108**, 510-4.

Prevost, N., Woulfe, D., Tognolini, M. & Brass, L. F. (2003). Contact-dependent signaling during the late events of platelet activation. *J Thromb Haemost,* **1**, 1613-27.

Prevost, N., Woulfe, D. S., Jiang, H., Stalker, T. J., Marchese, P., Ruggeri, Z. M. & Brass, L. F. (2005). Eph kinases and ephrins support thrombus growth and stability by regulating integrin outside-in signaling in platelets. *Proc Natl Acad Sci U S A,* **102**, 9820-5.

Procter, N. E., Hurst, N. L., Nooney, V. B., Imam, H., De Caterina, R., Chirkov, Y. Y. & Horowitz, J. D. (2016). New Developments in Platelet Cyclic Nucleotide Signalling: Therapeutic Implications. *Cardiovasc Drugs Ther,* **30**, 505-513.

Proulx, E., Leshchenko, Y., Kokarovtseva, L., Khokhotva, V., El-Beheiry, M., Snead, O. C., 3rd & Perez Velazquez, J. L. (2006). Functional contribution of specific brain areas to absence seizures: role of thalamic gap-junctional coupling. *Eur J Neurosci,* **23**, 489-96.

Pula, G., Schuh, K., Nakayama, K., Nakayama, K. I., Walter, U. & Poole, A. W. (2006). PKCdelta regulates collagen-induced platelet aggregation through inhibition of VASP-mediated filopodia formation. *Blood,* **108**, 4035-44.

Qin, H., Shao, Q., Igdoura, S. A., Alaoui-Jamali, M. A. & Laird, D. W. (2003). Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. *J Biol Chem,* **278**, 30005-14.

Quek, L. S., Bolen, J. & Watson, S. P. (1998). A role for Bruton's tyrosine kinase (Btk) in platelet activation by collagen. *Curr Biol,* **8**, 1137-40.

Quek, L. S., Pasquet, J. M., Hers, I., Cornall, R., Knight, G., Barnes, M., Hibbs, M. L., Dunn, A. R., Lowell, C. A. & Watson, S. P. (2000). Fyn and Lyn phosphorylate the Fc receptor gamma chain downstream of glycoprotein VI in murine platelets, and Lyn regulates a novel feedback pathway. *Blood,* **96**, 4246-53.

Quinn, M. J., Byzova, T. V., Qin, J., Topol, E. J. & Plow, E. F. (2003). Integrin alphaIIbbeta3 and its antagonism. *Arterioscler Thromb Vasc Biol,* **23**, 945-52.

Rabionet, R., Lopez-Bigas, N., Arbones, M. L. & Estivill, X. (2002). Connexin mutations in hearing loss, dermatological and neurological disorders. *Trends Mol Med,* **8**, 205-12.

Rackauskas, M., Neverauskas, V. & Skeberdis, V. A. (2010). Diversity and properties of connexin gap junction channels. *Medicina (Kaunas),* **46**, 1-12.

Radivojac, P., Baenziger, P. H., Kann, M. G., Mort, M. E., Hahn, M. W. & Mooney, S. D. (2008). Gain and loss of phosphorylation sites in human cancer. *Bioinformatics,* **24**, i241-7.

Radomski, M. W., Palmer, R. M. & Moncada, S. (1987a). Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. *Br J Pharmacol,* **92**, 181-7.

Radomski, M. W., Palmer, R. M. & Moncada, S. (1987b). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem Biophys Res Commun,* **148**, 1482-9.

Radomski, M. W., Palmer, R. M. & Moncada, S. (1990). Characterization of the L-arginine:nitric oxide pathway in human platelets. *Br J Pharmacol,* **101**, 325-8.

Rahman, S. & Evans, W. H. (1991). Topography of connexin32 in rat liver gap junctions. Evidence for an intramolecular disulphide linkage connecting the two extracellular peptide loops. *J Cell Sci,* **100 (Pt 3)**, 567-78.

Ramstrom, S., Oberg, K. V., Akerstrom, F., Enstrom, C. & Lindahl, T. L. (2008). Platelet PAR1 receptor density--correlation to platelet activation response and changes in exposure after platelet activation. *Thromb Res,* **121**, 681-8.

Ramstrom, S., Ranby, M. & Lindahl, T. L. (2003). Platelet phosphatidylserine exposure and procoagulant activity in clotting whole blood--different effects of collagen, TRAP and calcium ionophore A23187. *Thromb Haemost,* **89**, 132-41.

Ravid, K., Lu, J., Zimmet, J. M. & Jones, M. R. (2002). Roads to polyploidy: the megakaryocyte example. *J Cell Physiol,* **190**, 7-20.

Reinhard, M., Jarchau, T. & Walter, U. (2001). Actin-based motility: stop and go with Ena/VASP proteins. *Trends Biochem Sci,* **26**, 243-9.

Retamal, M. A. & Saez, J. C. (2014). Hemichannels; from the molecule to the function. *Front Physiol,* **5**, 411.

Revel, J. P. & Karnovsky, M. J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J Cell Biol,* **33**, C7-c12.

Revilla, A., Bennett, M. V. & Barrio, L. C. (2000). Molecular determinants of membrane potential dependence in vertebrate gap junction channels. *Proc Natl Acad Sci U S A,* **97**, 14760-5.

Ricard-Blum, S. (2011). The Collagen Family. *Cold Spring Harbor Perspectives in Biology,* **3**, a004978.

Richards, T. S., Dunn, C. A., Carter, W. G., Usui, M. L., Olerud, J. E. & Lampe, P. D. (2004). Protein kinase C spatially and temporally regulates gap junctional communication during human wound repair via phosphorylation of connexin43 on serine368. *J Cell Biol,* **167**, 555-62.

Richardson, J. L., Shivdasani, R. A., Boers, C., Hartwig, J. H. & Italiano, J. E. (2005). Mechanisms of organelle transport and capture along proplatelets during platelet production. *Blood,* **106**, 4066-4075.

Rivera, J., Lozano, M. L., Navarro-Nunez, L. & Vicente, V. (2009). Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica,* **94**, 700-11.

Roberts, D. E., McNicol, A. & Bose, R. (2004). Mechanism of collagen activation in human platelets. *J Biol Chem,* **279**, 19421-30.

Rocha, D. S., de Souza, S. K., Onsten, T. G. H., da Silva, R. S. M. & Frizzo, M. E. (2014). A simple method to quantify glycogen from human platelets. *Journal of Cytology & Histology,* **5**, 1.

Rohr, S. (2004). Role of gap junctions in the propagation of the cardiac action potential. *Cardiovasc Res,* **62**, 309-22.

Rose, B. & Loewenstein, W. R. (1975). Permeability of cell junction depends on local cytoplasmic calcium activity. *Nature,* **254**, 250-2.

Rowley, J. W., Oler, A. J., Tolley, N. D., Hunter, B. N., Low, E. N., Nix, D. A., Yost, C. C., Zimmerman, G. A. & Weyrich, A. S. (2011). Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood,* **118**, e101-11.

Rubin, L. J. (1995). Pathology and pathophysiology of primary pulmonary hypertension. *Am J Cardiol,* **75**, 51a-54a.

Ruggeri, Z. M. & Mendolicchio, G. L. (2007). Adhesion mechanisms in platelet function. *Circ Res,* **100**, 1673-85.

Sada, K., Takano, T., Yanagi, S. & Yamamura, H. (2001). Structure and function of Syk protein-tyrosine kinase. *J Biochem,* **130**, 177-86.

Saez, J. C. & Leybaert, L. (2014). Hunting for connexin hemichannels. *FEBS Lett,* **588**, 1205-11.

Saez, J. C., Nairn, A. C., Czernik, A. J., Spray, D. C., Hertzberg, E. L., Greengard, P. & Bennett, M. V. (1990). Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca2+/calmodulin-dependent protein kinase II. *Eur J Biochem,* **192**, 263-73.

Saez, J. C., Retamal, M. A., Basilio, D., Bukauskas, F. F. & Bennett, M. V. (2005). Connexin-based gap junction hemichannels: gating mechanisms. *Biochim Biophys Acta,* **1711**, 215-24.

Sage, S. O., Yamoah, E. H. & Heemskerk, J. W. (2000). The roles of P(2X1)and P(2T AC)receptors in ADP-evoked calcium signalling in human platelets. *Cell Calcium,* **28**, 119-26.

Sakamoto, K., Arnolds, D. E., Fujii, N., Kramer, H. F., Hirshman, M. F. & Goodyear, L. J. (2006). Role of Akt2 in contraction-stimulated cell signaling and glucose uptake in skeletal muscle. *Am J Physiol Endocrinol Metab,* **291**, E1031-7.

Salameh, A. (2006). Life cycle of connexins: regulation of connexin synthesis and degradation. *Adv Cardiol,* **42**, 57-70.

Salameh, A. & Dhein, S. (2005). Pharmacology of gap junctions. New pharmacological targets for treatment of arrhythmia, seizure and cancer? *Biochim Biophys Acta,* **1719**, 36-58.

Samaha, F. F., Hibbard, C., Sacks, J., Chen, H., Varello, M. A., George, T. & Kahn, M. L. (2004). Measurement of platelet collagen receptor density in human subjects. *Arterioscler Thromb Vasc Biol,* **24**, e181-2.

Sánchez-Cortés, J. & Mrksich, M. (2009). The Platelet Integrin αIIbβ3 Binds to the RGD and AGD Motifs in Fibrinogen. *Chemistry & biology,* **16**, 990-1000.

Sandmann, R. & Koster, S. (2016). Topographic Cues Reveal Two Distinct Spreading Mechanisms in Blood Platelets. *Sci Rep,* **6**, 22357.

Sarieddine, M. Z., Scheckenbach, K. E., Foglia, B., Maass, K., Garcia, I., Kwak, B. R. & Chanson, M. (2009). Connexin43 modulates neutrophil recruitment to the lung. *J Cell Mol Med,* **13**, 4560-70.

Sarratt, K. L., Chen, H., Zutter, M. M., Santoro, S. A., Hammer, D. A. & Kahn, M. L. (2005). GPVI and alpha2beta1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow. *Blood,* **106**, 1268-77.

Sasikumar, P., AlOuda, K. S., Kaiser, W. J., Holbrook, L. M., Kriek, N., Unsworth, A. J., Bye, A. P., Sage, T., Ushioda, R., Nagata, K., Farndale, R. W. & Gibbins, J. M. (2018). The chaperone protein HSP47: a platelet collagen binding protein that contributes to thrombosis and hemostasis. *J Thromb Haemost,* **16**, 946-959.

Savage, B., Almus-Jacobs, F. & Ruggeri, Z. M. (1998). Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell,* **94**, 657-66.

Savage, B., Saldivar, E. & Ruggeri, Z. M. (1996). Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell,* **84**, 289-97.

Schalper, K. A., Orellana, J. A., Berthoud, V. M. & Saez, J. C. (2009). Dysfunctions of the diffusional membrane pathways mediated by hemichannels in inherited and acquired human diseases. *Curr Vasc Pharmacol,* **7**, 486-505.

Schmidt, M., Dekker, F. J. & Maarsingh, H. (2013). Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. *Pharmacol Rev,* **65**, 670-709.

Schrör, K. (2016). *Acetylsalicylic Acid.* New Jersey, USA: John Wiley & Sons.

Schulz, R. & Heusch, G. (2004). Connexin 43 and ischemic preconditioning. *Cardiovascular Research,* **62**, 335-344.

Schulze, H., Korpal, M., Hurov, J., Kim, S.-W., Zhang, J., Cantley, L. C., Graf, T. & Shivdasani, R. A. (2006). Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis. *Blood,* **107**, 3868-3875.

Schwarz, U. R., Walter, U. & Eigenthaler, M. (2001). Taming platelets with cyclic nucleotides. *Biochem Pharmacol,* **62**, 1153-61.

Segal, S. S. (2000). Integration of blood flow control to skeletal muscle: key role of feed arteries. *Acta Physiol Scand,* **168**, 511-8.

Segretain, D. & Falk, M. M. (2004). Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. *Biochim Biophys Acta,* **1662**, 3-21.

Seidel, H., Rahman, M. M. & Scharf, R. E. (2011). Monitoring of antiplatelet therapy. Current limitations, challenges, and perspectives. *Hamostaseologie,* **31**, 41-51.

Severin, S., Nash, C. A., Mori, J., Zhao, Y., Abram, C., Lowell, C. A., Senis, Y. A. & Watson, S. P. (2012). Distinct and overlapping functional roles of Src family kinases in mouse platelets. *J Thromb Haemost,* **10**, 1631-45.

Severin, S., Pollitt, A. Y., Navarro-Nunez, L., Nash, C. A., Mourao-Sa, D., Eble, J. A., Senis, Y. A. & Watson, S. P. (2011). Syk-dependent phosphorylation of CLEC-2: a novel mechanism of hem-immunoreceptor tyrosine-based activation motif signaling. *J Biol Chem,* **286**, 4107-16.

Shah, M. M., Martinez, A. M. & Fletcher, W. H. (2002). The connexin43 gap junction protein is phosphorylated by protein kinase A and protein kinase C: in vivo and in vitro studies. *Mol Cell Biochem,* **238**, 57-68.

Shattil, S. J. & Brass, L. F. (1987). Induction of the fibrinogen receptor on human platelets by intracellular mediators. *J Biol Chem,* **262**, 992-1000.

Shattil, S. J., Kashiwagi, H. & Pampori, N. (1998). Integrin signaling: the platelet paradigm. *Blood,* **91**, 2645-57.

Shattil, S. J. & Newman, P. J. (2004). Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood,* **104**, 1606-15.

Shaw, R. M., Fay, A. J., Puthenveedu, M. A., von Zastrow, M., Jan, Y. N. & Jan, L. Y. (2007). Microtubule plus-end-tracking proteins target gap junctions directly from the cell interior to adherens junctions. *Cell,* **128**, 547-60.

Shen, R. F. & Tai, H. H. (1998). Thromboxanes: synthase and receptors. *J Biomed Sci,* **5**, 153-72.

Shin, E.-K., Park, H., Noh, J.-Y., Lim, K.-M. & Chung, J.-H. (2017). Platelet Shape Changes and Cytoskeleton Dynamics as Novel Therapeutic Targets for Anti-Thrombotic Drugs. *Biomolecules & Therapeutics,* **25**, 223-230.

Shirakawa, R., Higashi, T., Tabuchi, A., Yoshioka, A., Nishioka, H., Fukuda, M., Kita, T. & Horiuchi, H. (2004). Munc13-4 is a GTP-Rab27-binding protein regulating dense core granule secretion in platelets. *J Biol Chem,* **279**, 10730-7.

Shiue, L., Green, J., Green, O. M., Karas, J. L., Morgenstern, J. P., Ram, M. K., Taylor, M. K., Zoller, M. J., Zydowsky, L. D., Bolen, J. B. & et al. (1995). Interaction of p72syk with the gamma and beta subunits of the high-affinity receptor for immunoglobulin E, Fc epsilon RI. *Mol Cell Biol,* **15**, 272-81.

Siehler, S. (2009). Regulation of RhoGEF proteins by G(12/13)-coupled receptors. *British Journal of Pharmacology,* **158**, 41-49.

Sim, X., Poncz, M., Gadue, P. & French, D. L. (2016). Understanding platelet generation from megakaryocytes: implications for in vitro–derived platelets. *Blood,* **127**, 1227-1233.

Simon, A. M. & McWhorter, A. R. (2003). Decreased intercellular dye-transfer and downregulation of non-ablated connexins in aortic endothelium deficient in connexin37 or connexin40. *J Cell Sci,* **116**, 2223-36.

Simon, L. M., Edelstein, L. C., Nagalla, S., Woodley, A. B., Chen, E. S., Kong, X., Ma, L., Fortina, P., Kunapuli, S., Holinstat, M., McKenzie, S. E., Dong, J. F., Shaw, C. A. & Bray, P. F. (2014). Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. *Blood,* **123**, e37-45.

Sims, P. J., Faioni, E. M., Wiedmer, T. & Shattil, S. J. (1988). Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem,* **263**, 18205-12.

Sinauridze, E. I., Kireev, D. A., Popenko, N. Y., Pichugin, A. V., Panteleev, M. A., Krymskaya, O. V. & Ataullakhanov, F. I. (2007). Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost,* **97**, 425-34.

Sinzinger, H., Silberbauer, K., Horsch, A. K. & Gall, A. (1981). Decreased sensitivity of human platelets to PGI2 during long-term intraarterial prostacyclin infusion in patients with peripheral vascular disease--a rebound phenomenon? *Prostaglandins,* **21**, 49-51.

Skalhegg, B. S. & Tasken, K. (2000). Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA. *Front Biosci,* **5**, D678-93.

Skerrett, I. M., Smith, J. F. & Nicholson, B. J. (2000). Mechanistic Differences Between Chemical and Electrical Gating of Gap Junctions. *In:* Peracchia, C. (ed.) *Gap junctions. Molecular basis of cell communication in health and disease.* Academic Press.

Smethurst, P. A., Onley, D. J., Jarvis, G. E., O'Connor, M. N., Knight, C. G., Herr, A. B., Ouwehand, W. H. & Farndale, R. W. (2007). Structural Basis for the Platelet-Collagen Interaction. *Journal of Biological Chemistry,* **282**, 1296-1304.

Smyth, J. T., Hwang, S. Y., Tomita, T., DeHaven, W. I., Mercer, J. C. & Putney, J. W. (2010b). Activation and regulation of store-operated calcium entry. *J Cell Mol Med,* **14**, 2337-49.

Smyth, S. S., Whiteheart, S., Jr., J. E. I. & Coller, B. S. (2010a). Platelet Morphology, Biochemistry, and Function. *In:* Kaushansky, K., Seligsohn, U., Lichtman, M., Beutler, E., Kipps, T. & Prchal, J. (eds.) *Williams hematology.* 8 ed.: McGraw-Hill.

Sohl, G., Joussen, A., Kociok, N. & Willecke, K. (2010). Expression of connexin genes in the human retina. *BMC Ophthalmol,* **10**, 27.

Sohl, G. & Willecke, K. (2003). An update on connexin genes and their nomenclature in mouse and man. *Cell Commun Adhes,* **10**, 173-80.

Solan, J. L. & Lampe, P. D. (2009). Connexin43 phosphorylation: structural changes and biological effects. *Biochem J,* **419**, 261-72.

Solan, J. L. & Lampe, P. D. (2014). Specific Cx43 phosphorylation events regulate gap junction turnover in vivo. *FEBS Lett,* **588**, 1423-9.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. & Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature,* **362**, 318-24.

Sondek, J., Bohm, A., Lambright, D. G., Hamm, H. E. & Sigler, P. B. (1996). Crystal structure of a G-protein beta gamma dimer at 2.1A resolution. *Nature*, **379**, 369-74.

Sotkis, A., Wang, X. G., Yasumura, T., Peracchia, L. L., Persechini, A., Rash, J. E. & Peracchia, C. (2001). Calmodulin colocalizes with connexins and plays a direct role in gap junction channel gating. *Cell Commun Adhes*, **8**, 277-81.

Spalton, J. C., Mori, J., Pollitt, A. Y., Hughes, C. E., Eble, J. A. & Watson, S. P. (2009). The novel Syk inhibitor R406 reveals mechanistic differences in the initiation of GPVI and CLEC-2 signaling in platelets. *J Thromb Haemost*, **7**, 1192-9.

Spray, D. C., Rozental, R. & Srinivas, M. (2002). Prospects for rational development of pharmacological gap junction channel blockers. *Curr Drug Targets*, **3**, 455-64.

Stathopulos, P. B., Zheng, L., Li, G. Y., Plevin, M. J. & Ikura, M. (2008). Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. *Cell*, **135**, 110-22.

Stefanini, L. & Bergmeier, W. (2016). RAP1-GTPase signaling and platelet function. *J Mol Med (Berl)*, **94**, 13-9.

Stefanini, L., Paul, D. S., Robledo, R. F., Chan, E. R., Getz, T. M., Campbell, R. A., Kechele, D. O., Casari, C., Piatt, R., Caron, K. M., Mackman, N., Weyrich, A. S., Parrott, M. C., Boulaftali, Y., Adams, M. D., Peters, L. L. & Bergmeier, W. (2015). RASA3 is a critical inhibitor of RAP1-dependent platelet activation. *J Clin Invest*, **125**, 1419-32.

Stefanini, L., Roden, R. C. & Bergmeier, W. (2009). CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood*, **114**, 2506-2514.

Stein, R. W., Papp, A. C., Weiner, W. J. & Wu, K. K. (1985). Reduction of serum prostacyclin stability in ischemic stroke. *Stroke*, **16**, 16-8.

Stenberg, P. E., Shuman, M. A., Levine, S. P. & Bainton, D. F. (1984). Redistribution of alpha-granules and their contents in thrombin-stimulated platelets. *The Journal of Cell Biology*, **98**, 748-760.

Stergiopoulos, K., Alvarado, J. L., Mastroianni, M., Ek-Vitorin, J. F., Taffet, S. M. & Delmar, M. (1999). Hetero-domain interactions as a mechanism for the regulation of connexin channels. *Circ Res*, **84**, 1144-55.

Strehl, A., Munnix, I. C., Kuijpers, M. J., van der Meijden, P. E., Cosemans, J. M., Feijge, M. A., Nieswandt, B. & Heemskerk, J. W. (2007). Dual role of platelet protein kinase C in thrombus formation: stimulation of pro-aggregatory and suppression of procoagulant activity in platelets. *J Biol Chem*, **282**, 7046-55.

Stritt, S., Wolf, K., Lorenz, V., Vogtle, T., Gupta, S., Bosl, M. R. & Nieswandt, B. (2015). Rap1-GTP-interacting adaptor molecule (RIAM) is dispensable for platelet integrin activation and function in mice. *Blood*, **125**, 219-22.

Stuart, M. J., Murphy, S. & Oski, F. A. (1975). A Simple Nonradioisotope Technic for the Determination of Platelet Life-Span. *New England Journal of Medicine*, **292**, 1310-1313.

Su, X., Vicker, N., Lawrence, H., Smith, A., Purohit, A., Reed, M. J. & Potter, B. V. (2007). Inhibition of human and rat 11beta-hydroxysteroid dehydrogenase type 1 by 18beta-glycyrrhetinic acid derivatives. *J Steroid Biochem Mol Biol*, **104**, 312-20.

Sudhof, T. C. & Rothman, J. E. (2009). Membrane fusion: grappling with SNARE and SM proteins. *Science*, **323**, 474-7.

Suzuki-Inoue, K., Fuller, G. L., Garcia, A., Eble, J. A., Pohlmann, S., Inoue, O., Gartner, T. K., Hughan, S. C., Pearce, A. C., Laing, G. D., Theakston, R. D.,

Schweighoffer, E., Zitzmann, N., Morita, T., Tybulewicz, V. L., Ozaki, Y. & Watson, S. P. (2006). A novel Syk-dependent mechanism of platelet activation by the C-type lectin receptor CLEC-2. *Blood*, **107**, 542-9.

Suzuki-Inoue, K., Inoue, O. & Ozaki, Y. (2011). Novel platelet activation receptor CLEC-2: from discovery to prospects. *J Thromb Haemost*, **9 Suppl 1**, 44-55.

Suzuki-Inoue, K., Kato, Y., Inoue, O., Kaneko, M. K., Mishima, K., Yatomi, Y., Yamazaki, Y., Narimatsu, H. & Ozaki, Y. (2007). Involvement of the snake toxin receptor CLEC-2, in podoplanin-mediated platelet activation, by cancer cells. *J Biol Chem*, **282**, 25993-6001.

Suzuki-Inoue, K., Tulasne, D., Shen, Y., Bori-Sanz, T., Inoue, O., Jung, S. M., Moroi, M., Andrews, R. K., Berndt, M. C. & Watson, S. P. (2002). Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. *J Biol Chem*, **277**, 21561-6.

Suzuki-Inoue, K., Wilde, J. I., Andrews, R. K., Auger, J. M., Siraganian, R. P., Sekiya, F., Rhee, S. G. & Watson, S. P. (2004). Glycoproteins VI and Ib-IX-V stimulate tyrosine phosphorylation of tyrosine kinase Syk and phospholipase Cgamma2 at distinct sites. *Biochem J*, **378**, 1023-9.

Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H. & Calderwood, D. A. (2003). Talin binding to integrin beta tails: a final common step in integrin activation. *Science*, **302**, 103-6.

Taylor, K. A., Wright, J. R., Vial, C., Evans, R. J. & Mahaut-Smith, M. P. (2014). Amplification of human platelet activation by surface pannexin-1 channels. *J Thromb Haemost*, **12**, 987-98.

Taylor, S. S., Kim, C., Cheng, C. Y., Brown, S. H., Wu, J. & Kannan, N. (2008). Signaling through cAMP and cAMP-dependent protein kinase: diverse strategies for drug design. *Biochim Biophys Acta*, **1784**, 16-26.

Tazawa, R., Green, E. D., Ohashi, K., Wu, K. K. & Wang, L. H. (1996). Characterization of the complete genomic structure of human thromboxane synthase gene and functional analysis of its promoter. *Arch Biochem Biophys*, **334**, 349-56.

Terman, D. S., Bohach, G., Vandenesch, F., Etienne, J., Lina, G. & Sahn, S. A. (2006). Staphylococcal superantigens of the enterotoxin gene cluster (egc) for treatment of stage IIIb non-small cell lung cancer with pleural effusion. *Clin Chest Med*, **27**, 321-34.

Thevenin, A. F., Kowal, T. J., Fong, J. T., Kells, R. M., Fisher, C. G. & Falk, M. M. (2013). Proteins and mechanisms regulating gap-junction assembly, internalization, and degradation. *Physiology (Bethesda)*, **28**, 93-116.

Thomas, D. W., Mannon, R. B., Mannon, P. J., Latour, A., Oliver, J. A., Hoffman, M., Smithies, O., Koller, B. H. & Coffman, T. M. (1998). Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *J Clin Invest*, **102**, 1994-2001.

Thomas, M. A., Zosso, N., Scerri, I., Demaurex, N., Chanson, M. & Staub, O. (2003). A tyrosine-based sorting signal is involved in connexin43 stability and gap junction turnover. *J Cell Sci*, **116**, 2213-22.

Thomas, T., Jordan, K., Simek, J., Shao, Q., Jedeszko, C., Walton, P. & Laird, D. W. (2005). Mechanisms of Cx43 and Cx26 transport to the plasma membrane and gap junction regeneration. *J Cell Sci*, **118**, 4451-62.

Thomas, T., Telford, D. & Laird, D. W. (2004). Functional domain mapping and selective trans-dominant effects exhibited by Cx26 disease-causing mutations. *J Biol Chem*, **279**, 19157-68.

Thon, J. N., Montalvo, A., Patel-Hett, S., Devine, M. T., Richardson, J. L., Ehrlicher, A., Larson, M. K., Hoffmeister, K., Hartwig, J. H. & Italiano, J. E. (2010). Cytoskeletal mechanics of proplatelet maturation and platelet release. *The Journal of Cell Biology,* **191**, 861-874.

Tocantins, L. M. (1948). Historical notes on blood platelets. *Blood,* **3**, 1073-82.

Tolhurst, G., Carter, R. N., Amisten, S., Holdich, J. P., Erlinge, D. & Mahaut-Smith, M. P. (2008). Expression profiling and electrophysiological studies suggest a major role for Orai1 in the store-operated Ca2+ influx pathway of platelets and megakaryocytes. *Platelets,* **19**, 308-13.

Torday, J. & Rehan, V. (2012). *Evolutionary Biology, Cell–Cell Communication, and Complex Disease.* Hoboken, New Jersey: John Wiley & Sons.

Townsend, N., Bhatnagar, P., Wilkins, E. & Wickramasinghe, K. (2015). Cardiovascular disease statistics 2015. *In:* Dicks, E. (ed.) *Cardiovascular disease statistics.* London: British Heart Foundation.

Traub, O., Look, J., Dermietzel, R., Brummer, F., Hulser, D. & Willecke, K. (1989). Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. *J Cell Biol,* **108**, 1039-51.

Trexler, E. B., Bukauskas, F. F., Bennett, M. V., Bargiello, T. A. & Verselis, V. K. (1999). Rapid and direct effects of pH on connexins revealed by the connexin46 hemichannel preparation. *J Gen Physiol,* **113**, 721-42.

Tropel, P., Roullot, V., Vernet, M., Poujol, C., Pointu, H., Nurden, P., Marguerie, G. & Tronik-Le Roux, D. (1997). A 2.7-kb portion of the 5' flanking region of the murine glycoprotein alphaIIb gene is transcriptionally active in primitive hematopoietic progenitor cells. *Blood,* **90**, 2995-3004.

Tsuruo, T. & Fujita, N. (2008). Platelet aggregation in the formation of tumor metastasis. *Proc Jpn Acad Ser B Phys Biol Sci,* **84**, 189-98.

Tucker, K. L., Sage, T. & Gibbins, J. M. (2012). Clot retraction. *Methods Mol Biol,* **788**, 101-7.

Uhlenberg, B., Schuelke, M., Ruschendorf, F., Ruf, N., Kaindl, A. M., Henneke, M., Thiele, H., Stoltenburg-Didinger, G., Aksu, F., Topaloglu, H., Nurnberg, P., Hubner, C., Weschke, B. & Gartner, J. (2004). Mutations in the gene encoding gap junction protein alpha 12 (connexin 46.6) cause Pelizaeus-Merzbacher-like disease. *Am J Hum Genet,* **75**, 251-60.

Uhrin, P., Zaujec, J., Breuss, J. M., Olcaydu, D., Chrenek, P., Stockinger, H., Fuertbauer, E., Moser, M., Haiko, P., Fassler, R., Alitalo, K., Binder, B. R. & Kerjaschki, D. (2010). Novel function for blood platelets and podoplanin in developmental separation of blood and lymphatic circulation. *Blood,* **115**, 3997-4005.

Ujiie, H., Chaytor, A. T., Bakker, L. M. & Griffith, T. M. (2003). Essential role of Gap junctions in NO- and prostanoid-independent relaxations evoked by acetylcholine in rabbit intracerebral arteries. *Stroke,* **34**, 544-50.

Unger, V. M., Kumar, N. M., Gilula, N. B. & Yeager, M. (1999). Three-dimensional structure of a recombinant gap junction membrane channel. *Science,* **283**, 1176-80.

Unsworth, A. J., Flora, G. D., Sasikumar, P., Bye, A. P., Sage, T., Kriek, N., Crescente, M. & Gibbins, J. M. (2017a). RXR Ligands Negatively Regulate Thrombosis and Hemostasis. *Arterioscler Thromb Vasc Biol,* **37**, 812-822.

Unsworth, A. J., Kriek, N., Bye, A. P., Naran, K., Sage, T., Flora, G. D. & Gibbins, J. M. (2017b). PPARgamma agonists negatively regulate alphaIIbbeta3 integrin

outside-in signaling and platelet function through up-regulation of protein kinase A activity. *J Thromb Haemost,* **15**, 356-369.

Unsworth, A. J., Smith, H., Gissen, P., Watson, S. P. & Pears, C. J. (2011). Submaximal inhibition of protein kinase C restores ADP-induced dense granule secretion in platelets in the presence of Ca2+. *J Biol Chem,* **286**, 21073-82.

Vaiyapuri, S., Flora, G. D. & Gibbins, J. M. (2015a). Gap junctions and connexin hemichannels in the regulation of haemostasis and thrombosis. *Biochem Soc Trans,* **43**, 489-94.

Vaiyapuri, S., Jones, C. I., Sasikumar, P., Moraes, L. A., Munger, S. J., Wright, J. R., Ali, M. S., Sage, T., Kaiser, W. J., Tucker, K. L., Stain, C. J., Bye, A. P., Jones, S., Oviedo-Orta, E., Simon, A. M., Mahaut-Smith, M. P. & Gibbins, J. M. (2012). Gap junctions and connexin hemichannels underpin hemostasis and thrombosis. *Circulation,* **125**, 2479-91.

Vaiyapuri, S., Moraes, L. A., Sage, T., Ali, M. S., Lewis, K. R., Mahaut-Smith, M. P., Oviedo-Orta, E., Simon, A. M. & Gibbins, J. M. (2013). Connexin40 regulates platelet function. *Nat Commun,* **4**, 2564.

Vaiyapuri, S., Sage, T., Rana, R. H., Schenk, M. P., Ali, M. S., Unsworth, A. J., Jones, C. I., Stainer, A. R., Kriek, N., Moraes, L. A. & Gibbins, J. M. (2015b). EphB2 regulates contact-dependent and contact-independent signaling to control platelet function. *Blood,* **125**, 720-30.

Valiunas, V., Beyer, E. C. & Brink, P. R. (2002). Cardiac gap junction channels show quantitative differences in selectivity. *Circ Res,* **91**, 104-11.

Valiunas, V. & Weingart, R. (2000). Electrical properties of gap junction hemichannels identified in transfected HeLa cells. *Pflugers Arch,* **440**, 366-79.

van den Bosch, A. E., van der Klooster, J. M., Zuidgeest, D. M., Ouwendijk, R. J. & Dees, A. (2005). Severe hypokalaemic paralysis and rhabdomyolysis due to ingestion of liquorice. *Neth J Med,* **63**, 146-8.

van Nispen Tot Pannerden, H. E., van Dijk, S. M., Du, V. & Heijnen, H. F. (2009). Platelet protein disulfide isomerase is localized in the dense tubular system and does not become surface expressed after activation. *Blood,* **114**, 4738-40.

van Rijen, H. V., van Veen, T. A., Hermans, M. M. & Jongsma, H. J. (2000). Human connexin40 gap junction channels are modulated by cAMP. *Cardiovasc Res,* **45**, 941-51.

van Veen, A. A., van Rijen, H. V. & Opthof, T. (2001). Cardiac gap junction channels: modulation of expression and channel properties. *Cardiovasc Res,* **51**, 217-29.

Vane, J. R. & Botting, R. M. (1995). Pharmacodynamic profile of prostacyclin. *Am J Cardiol,* **75**, 3a-10a.

Vanhoorelbeke, K., Ulrichts, H., Van de Walle, G., Fontayne, A. & Deckmyn, H. (2007). Inhibition of platelet glycoprotein Ib and its antithrombotic potential. *Curr Pharm Des,* **13**, 2684-97.

VanSlyke, J. K. & Musil, L. S. (2000). Analysis of connexin intracellular transport and assembly. *Methods,* **20**, 156-64.

VanSlyke, J. K. & Musil, L. S. (2002). Dislocation and degradation from the ER are regulated by cytosolic stress. *J Cell Biol,* **157**, 381-94.

Vanslyke, J. K., Naus, C. C. & Musil, L. S. (2009). Conformational maturation and post-ER multisubunit assembly of gap junction proteins. *Mol Biol Cell,* **20**, 2451-63.

Varga-Szabo, D., Braun, A. & Nieswandt, B. (2009). Calcium signaling in platelets. *J Thromb Haemost,* **7**, 1057-66.

Veenstra, R. D., Wang, H. Z., Beyer, E. C. & Brink, P. R. (1994). Selective dye and ionic permeability of gap junction channels formed by connexin45. *Circ Res,* **75**, 483-90.

Vemana, H. P., Karim, Z. A., Conlon, C. & Khasawneh, F. T. (2015). A critical role for the transient receptor potential channel type 6 in human platelet activation. *PLoS One,* **10**, e0125764.

Verrall, S., Ishii, M., Chen, M., Wang, L., Tram, T. & Coughlin, S. R. (1997). The thrombin receptor second cytoplasmic loop confers coupling to Gq-like G proteins in chimeric receptors. Additional evidence for a common transmembrane signaling and G protein coupling mechanism in G protein-coupled receptors. *J Biol Chem,* **272**, 6898-902.

Verselis, V. K. & Srinivas, M. (2013). Connexin channel modulators and their mechanisms of action. *Neuropharmacology,* **75**, 517-24.

Vessey, J. P., Lalonde, M. R., Mizan, H. A., Welch, N. C., Kelly, M. E. & Barnes, S. (2004). Carbenoxolone inhibition of voltage-gated Ca channels and synaptic transmission in the retina. *J Neurophysiol,* **92**, 1252-6.

Vial, C., Hechler, B., Leon, C., Cazenave, J. P. & Gachet, C. (1997). Presence of P2X1 purinoceptors in human platelets and megakaryoblastic cell lines. *Thromb Haemost,* **78**, 1500-4.

Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R. & Kinet, J. P. (2006). CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. *Science,* **312**, 1220-3.

Vinken, M., Vanhaecke, T., Papeleu, P., Snykers, S., Henkens, T. & Rogiers, V. (2006). Connexins and their channels in cell growth and cell death. *Cell Signal,* **18**, 592-600.

Walsh, M. T., Foley, J. F. & Kinsella, B. T. (2000). The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for prostacyclin-mediated desensitization. *J Biol Chem,* **275**, 20412-23.

Ware, J. A., Johnson, P. C., Smith, M. & Salzman, E. W. (1986). Effect of common agonists on cytoplasmic ionized calcium concentration in platelets. Measurement with 2-methyl-6-methoxy 8-nitroquinoline (quin2) and aequorin. *J Clin Invest,* **77**, 878-86.

Warn-Cramer, B. J., Cottrell, G. T., Burt, J. M. & Lau, A. F. (1998). Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem,* **273**, 9188-96.

Warner, A., Clements, D. K., Parikh, S., Evans, W. H. & DeHaan, R. L. (1995). Specific motifs in the external loops of connexin proteins can determine gap junction formation between chick heart myocytes. *J Physiol,* **488 (Pt 3)**, 721-8.

Watanabe, D., Hashimoto, S., Ishiai, M., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T. & Tsukada, S. (2001). Four tyrosine residues in phospholipase C-gamma 2, identified as Btk-dependent phosphorylation sites, are required for B cell antigen receptor-coupled calcium signaling. *J Biol Chem,* **276**, 38595-601.

Watson, S. P., Auger, J. M., McCarty, O. J. & Pearce, A. C. (2005). GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost,* **3**, 1752-62.

Wei, A. H., Schoenwaelder, S. M., Andrews, R. K. & Jackson, S. P. (2009). New insights into the haemostatic function of platelets. *Br J Haematol,* **147**, 415-30.

Wei, C. J., Francis, R., Xu, X. & Lo, C. W. (2005). Connexin43 associated with an N-cadherin-containing multiprotein complex is required for gap junction formation in NIH3T3 cells. *J Biol Chem,* **280**, 19925-36.

Weksler, B. B., Marcus, A. J. & Jaffe, E. A. (1977). Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. *Proc Natl Acad Sci U S A,* **74**, 3922-6.

Wenckebach, K. F. (1923). Cinchona derivatives in the treatment of heart disorders. *Journal of the American Medical Association,* **81**, 472-474.

Wentworth, J. K., Pula, G. & Poole, A. W. (2006). Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated on Ser157 by protein kinase C-dependent and -independent mechanisms in thrombin-stimulated human platelets. *Biochem J,* **393**, 555-64.

Westmoreland, D., Shaw, M., Grimes, W., Metcalf, D. J., Burden, J. J., Gomez, K., Knight, A. E. & Cutler, D. F. (2016). Super-resolution microscopy as a potential approach to diagnosis of platelet granule disorders. *J Thromb Haemost,* **14**, 839-49.

White, J. G. (1972). Interaction of membrane systems in blood platelets. *Am J Pathol,* **66**, 295-312.

White, J. G. (2013). Platelet Structure. *In:* Michelson, A. D. (ed.) *Platelet.* 3 ed.: Academic Press.

White, J. G., Amos, W. B. & Fordham, M. (1987). An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J Cell Biol,* **105**, 41-8.

Wolfle, S. E., Schmidt, V. J., Hoepfl, B., Gebert, A., Alcolea, S., Gros, D. & de Wit, C. (2007). Connexin45 cannot replace the function of connexin40 in conducting endothelium-dependent dilations along arterioles. *Circ Res,* **101**, 1292-9.

Wonerow, P., Obergfell, A., Wilde, J. I., Bobe, R., Asazuma, N., Brdicka, T., Leo, A., Schraven, B., Horejsi, V., Shattil, S. J. & Watson, S. P. (2002). Differential role of glycolipid-enriched membrane domains in glycoprotein VI- and integrin-mediated phospholipase Cgamma2 regulation in platelets. *Biochem J,* **364**, 755-65.

Wonerow, P., Pearce, A. C., Vaux, D. J. & Watson, S. P. (2003). A critical role for phospholipase Cgamma2 in alphaIIbbeta3-mediated platelet spreading. *J Biol Chem,* **278**, 37520-9.

Wong, C. W., Christen, T., Roth, I., Chadjichristos, C. E., Derouette, J. P., Foglia, B. F., Chanson, M., Goodenough, D. A. & Kwak, B. R. (2006). Connexin37 protects against atherosclerosis by regulating monocyte adhesion. *Nat Med,* **12**, 950-4.

Woodside, D. G., Obergfell, A., Leng, L., Wilsbacher, J. L., Miranti, C. K., Brugge, J. S., Shattil, S. J. & Ginsberg, M. H. (2001). Activation of Syk protein tyrosine kinase through interaction with integrin beta cytoplasmic domains. *Curr Biol,* **11**, 1799-804.

Woronowicz, K., Dilks, J. R., Rozenvayn, N., Dowal, L., Blair, P. S., Peters, C. G., Woronowicz, L. & Flaumenhaft, R. (2010). The Platelet Actin Cytoskeleton Associates with SNAREs and Participates in α-Granule Secretion. *Biochemistry,* **49**, 4533-4542.

Woulfe, D. S. (2005). Platelet G protein-coupled receptors in hemostasis and thrombosis. *J Thromb Haemost,* **3**, 2193-200.

Wright, C. S., van Steensel, M. A., Hodgins, M. B. & Martin, P. E. (2009). Connexin mimetic peptides improve cell migration rates of human epidermal keratinocytes and dermal fibroblasts in vitro. *Wound Repair Regen,* **17**, 240-9.

Wright, J. (1906). The origin and nature of blood plates. *Boston Med Surg J,* **154**, 643–5.

Wu, J., McHowat, J., Saffitz, J. E., Yamada, K. A. & Corr, P. B. (1993). Inhibition of gap junctional conductance by long-chain acylcarnitines and their preferential accumulation in junctional sarcolemma during hypoxia. *Circ Res,* **72**, 879-89.

Wu, Y., Asazuma, N., Satoh, K., Yatomi, Y., Takafuta, T., Berndt, M. C. & Ozaki, Y. (2003). Interaction between von Willebrand factor and glycoprotein Ib activates Src kinase in human platelets: role of phosphoinositide 3-kinase. *Blood,* **101**, 3469-76.

Yacoub, D., Theoret, J. F., Villeneuve, L., Abou-Saleh, H., Mourad, W., Allen, B. G. & Merhi, Y. (2006). Essential role of protein kinase C delta in platelet signaling, alpha IIb beta 3 activation, and thromboxane A2 release. *J Biol Chem,* **281**, 30024-35.

Yan, R., Li, S. & Dai, K. (2009). The critical roles of cyclic AMP/cyclic AMP-dependent protein kinase in platelet physiology. *Frontiers of Biology in China,* **4**, 7-14.

Yang, J., Wu, J., Jiang, H., Mortensen, R., Austin, S., Manning, D. R., Woulfe, D. & Brass, L. F. (2002). Signaling through Gi family members in platelets. Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *J Biol Chem,* **277**, 46035-42.

Yap, C. L., Anderson, K. E., Hughan, S. C., Dopheide, S. M., Salem, H. H. & Jackson, S. P. (2002). Essential role for phosphoinositide 3-kinase in shear-dependent signaling between platelet glycoprotein Ib/V/IX and integrin alpha(IIb)beta(3). *Blood,* **99**, 151-8.

Yeager, M. & Harris, A. L. (2007). Gap junction channel structure in the early 21st century: facts and fantasies. *Curr Opin Cell Biol,* **19**, 521-8.

Yokoyama, C., Yabuki, T., Shimonishi, M., Wada, M., Hatae, T., Ohkawara, S., Takeda, J., Kinoshita, T., Okabe, M. & Tanabe, T. (2002). Prostacyclin-deficient mice develop ischemic renal disorders, including nephrosclerosis and renal infarction. *Circulation,* **106**, 2397-403.

Young, E. J., Hill, M. A., Wiehler, W. B., Triggle, C. R. & Reid, J. J. (2008). Reduced EDHF responses and connexin activity in mesenteric arteries from the insulin-resistant obese Zucker rat. *Diabetologia,* **51**, 872-81.

Zhang, G., Xiang, B., Dong, A., Skoda, R. C., Daugherty, A., Smyth, S. S., Du, X. & Li, Z. (2011). Biphasic roles for soluble guanylyl cyclase (sGC) in platelet activation. *Blood,* **118**, 3670-9.

Zhang, J. T., Chen, M., Foote, C. I. & Nicholson, B. J. (1996). Membrane integration of in vitro-translated gap junctional proteins: co- and post-translational mechanisms. *Mol Biol Cell,* **7**, 471-82.

Zhang, L., Orban, M., Lorenz, M., Barocke, V., Braun, D., Urtz, N., Schulz, C., von Bruhl, M. L., Tirniceriu, A., Gaertner, F., Proia, R. L., Graf, T., Bolz, S. S., Montanez, E., Prinz, M., Muller, A., von Baumgarten, L., Billich, A., Sixt, M., Fassler, R., von Andrian, U. H., Junt, T. & Massberg, S. (2012). A novel role of sphingosine 1-phosphate receptor S1pr1 in mouse thrombopoiesis. *J Exp Med,* **209**, 2165-81.

Zhang, S. L., Yu, Y., Roos, J., Kozak, J. A., Deerinck, T. J., Ellisman, M. H., Stauderman, K. A. & Cahalan, M. D. (2005). STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. *Nature,* **437**, 902-5.

Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P. & Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell,* **92**, 83-92.

Zharikov, S. & Shiva, S. (2013). Platelet mitochondrial function: from regulation of thrombosis to biomarker of disease. *Biochem Soc Trans*, **41**, 118-23.

Zimmet, J. & Ravid, K. (2000). Polyploidy: occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system. *Exp Hematol*, **28**, 3-16.

8 Appendix

8.1 Appendix 1: Multiple alignments of connexins protein obtained from ClustalW (www.ebi.ac.uk/tools/clustalw).

The green and red colours represent ^{62}Gap26 and ^{62}Gap27 sites, respectively.

```
Full=Connexin-31.1      ---MNWSIFEGLLSG-VNKYSTAFGRIWLSLVFIFRVLVYLVTAERVWSDDHKDFDCNTR 56
Full=Connexin-30.3      ---MNWAFLQGLLSG-VNKYSTVLSRIWLSVVFIFRVLVYVVAAEEVWDDEQKDFVCNTK 56
Full=Connexin-31        ---MDWKTLQALLSG-VNKYSTAFGRIWLSVVFVFRVLVYVVAAERVWGDEQKDFDCNTK 56
Full=Connexin-25        ---MSWMFLRDLLSG-VNKYSTGTGWIWLAVVFVFRLLVYMVAAEHVWKDEQKEFECNSR 56
Full=Connexin-26        ---MDWGTLQTILGG-VNKHSTSIGKIWLTVLFIFRIMILVVAAKEVWGDEQADFVCNTL 56
Full=Connexin-30        ---MDWGTLHTFIGG-VNKHSTSIGKVWITVIFIFRVMILVVAAQEVWGDEQEDFVCNTL 56
Full=Connexin-32        ---MNWTGLYTLLSG-VNRHSTAIGRVWLSVIFIFRIMVLVVAAESVWGDEKSSFICNTL 56
Full=Connexin-59        --MGDWNLLGDTLEE-VHIHSTMIGKIWLTILFIFRMLVLGVAAEDVWNDEQSGFICNTE 57
Full=Connexin-37        --MGDWGFLEKLLDQ-VQEHSTVVGKIWLTVLFIFRILILGLAGESVWGDEQSDFECNTA 57
Full=Connexin-43        --MGDWSALGKLLDK-VQAYSTAGGKVWLSVLFIFRILLLGTAVESAWGDEQSAFRCNTQ 57
Full=Connexin-46        --MGDWSFLGRLLEN-AQEHSTVIGKVWLTVLFIFRILVLGAAAEDVWGDEQSDFTCNTQ 57
Full=Connexin-40        --MGDWSFLGNFLEE-VHKHSTVVGKVWLTVLFIFRMLVLGTAAESSWGDEQADFRCDTI 57
Full=Connexin-50        --MGDWSFLGNILEE-VNEHSTVIGRVWLTVLFIFRILILGTAAEFVWGDEQSDFVCNTQ 57
Full=Connexin-62        --MGDWNLLGGILEE-VHSHSTIVGKIWLTILFIFRMLVLRVAAEDVWDDEQSAFACNTR 57
Full=Connexin-31.9      --MGEWAFLGSLLDA-VQLQSPLVGRLWLVVMLIFRILVLATVGGAVFEDEQEEFVCNTL 57
Full=Connexin-40.1      --MEGVDLLGFLIIT-LNCNVTMVGKLWFVLTMLLRMLVIVLAGRPVYQDEQERFVCNTL 57
Full=Connexin-36        --MGEWTILERLLEAAVQQHSTMIGRILLTVVVIFRILIVAIVGETVYDDEQTMFVCNTL 58
Full=Connexin-45        ---MSWSFLTRLLEE-IHNHSTFVGKIWLTVLIVFRIVLTAVGGESIYYDEQSKFVCNTE 56
Full=Connexin-47        MTNMSWSFLTRLLEE-IHNHSTFVGKVWLTVLVVFRIVLTAVGGEAIYSDEQAKFTCNTR 59
Full=Connexin-31.3      ---MCGRFLRRLLAE-ESRRSTPVGRLLLPVLLGFRLVLLAASGPGVYGDEQSEFVCHTQ 56
Full=Connexin-23        ---MSLNYIKNFYEG-CVKPPTVIGQFHTLFFGSIRIFFLGVLGFAVYGNEALHFICDPD 56
                                :           .  . .   .   :*:..       : ::   * *..

Full=Connexin-31.1      QPGCSNVCFDEFFPVS-HVRLWALQLILVTCPSLLVVMHVAYREVQEKRHREAHGENS-- 113
Full=Connexin-30.3      QPGCPNVCYDEFFPVS-HVRLWALQLILVTCPSLLVVMHVAYREERERKHHLKHGPNA-- 113
Full=Connexin-31        QPGCTNVCYDNYFPIS-NIRLWALQLIFVTCPSLLVILHVAYREERERRHRQKHGDQC-- 113
Full=Connexin-25        QPGCKNVCFDDFFPIS-QVRLWALQLIMVSTPSLLVVLHVAYHEGREKRHRKK------- 108
Full=Connexin-26        QPGCKNVCYDHYFPIS-HIRLWALQLIFVSTPALLVAMHVAYRRHEKKRKFIKGEIKS-- 113
Full=Connexin-30        QPGCKNVCYDHFFPVS-HIRLWALQLIFVSTPALLVAMHVAYYRHETTRKFRRGEKRN-- 113
Full=Connexin-32        QPGCNSVCYDQFFPIS-HVRLWSLQLILVSTPALLVAMHVAHQQH-IEKKMLRLEGHG-- 112
Full=Connexin-59        QPGCRNVCYDQAFPIS-LIRYWVLQVIFVSSPSLVYMGHALYRLRVLEEERQRMKAQLRV 116
Full=Connexin-37        QPGCTN[VCYDQAFPIS-HIR]YWVLQFLFVSTPTLVYLGHVIYLSRREERLRQKEGELRAL 116
Full=Connexin-43        QPGCENVCYDKSFPIS-HVRFWVLQIIFVSVPTLLYLAHVFYVMRKEEKLNKKEEELKVA 116
Full=Connexin-46        QPGCENVCYDRAFPIS-HIRFWALQIIFVSTPTLIYLGHVLHIVRMEEKKKEREEEEQLK 116
Full=Connexin-40        QPGCQN[VCYDQAFPIS-HIR]YWVLQIIFVSTPSLVYMGHAMHTVRMQEKRKLREAERAKE 116
Full=Connexin-50        QPGCENGCYDEAFPIS-HIRLWVLQIIFVSTPSLMYVGHAVHYVRMEEKRKSREAEELGQ 116
Full=Connexin-62        QPGCNN[ICYDDAFPIS-LIR]FWVLQIIFVSSPSLVYMGHALYRLRAFEKDRQRKKSHLRA 116
Full=Connexin-31.9      QPGCRQTCYDRAFPVS-HYRFWLFHILLLSAPPVLFVVYSMHR-------AGKEAGGAEA 109
Full=Connexin-40.1      QPGCANVCYDVFSPVS-HLRFWLIQGVCVLLPSAVFSVYVLHRGATLAALGPRRCPDPRE 116
Full=Connexin-36        QPGCNQACYDRAFPIS-HIRYWVFQIIMVCTPSLCFITYSVHQSAKQRERRYSTVFLALD 117
Full=Connexin-45        QPGCENVCYDAFAPLS-HVRFWVFQIILVATPSVMYLGYAIHKIAKMEHGEAD-----KK 110
Full=Connexin-47        QPGCDNVCYDAFAPLS-HVRFWVFQIVVISTPSVMYLGYAVHRLARASEQERRRALRRRP 118
Full=Connexin-31.3      QPGCKAACFDAFHPLS-PLRFWVFQVILVAVPSALYMGFTLYHVIWH------------- 102
Full=Connexin-23        KREVNLFCYNQFRPITPQVSFSALQLVIVLVPGALFHLYAACKSINQECILQKP------ 110
                        :      *::   *::       :: : :  *      .

Full=Connexin-31.1      ------------------------------------------------------------
Full=Connexin-30.3      ------------------------------------------------------------
Full=Connexin-31        ------------------------------------------------------------
Full=Connexin-25        ------------------------------------------------------------
Full=Connexin-26        ------------------------------------------------------------
Full=Connexin-30        ------------------------------------------------------------
Full=Connexin-32        ------------------------------------------------------------
Full=Connexin-59        ELEEVEFEMPRDRRR---------------------------------------------- 131
Full=Connexin-37        PAKDPQVERALAAI----------------------------------------------- 130
Full=Connexin-43        QTDGVNVDMHLKQI----------------------------------------------- 130
Full=Connexin-46        RESPSPKE----------------------------------------------------- 124
Full=Connexin-40        VRGSGSYEYPVAE------------------------------------------------ 129
Full=Connexin-50        QAGTN--GGPDQG------------------------------------------------ 127
Full=Connexin-62        QMENPDLDLEEQQRI---------------------------------------------- 131
Full=Connexin-31.9      AAQC--------------------------------------------------------- 113
```

```
Full=Connexin-40.1      PASG------------------------------------------------------------ 120
Full=Connexin-36        RDPPESIGGPGGTGGGGSGGGKREDKKLQNAIVNGVLQNTENTSKETEPDCLEV------ 171
Full=Connexin-45        AARSKPYAMRWKQHRALEETEEDNEEDPMMYPEMELESDK-------------------- 150
Full=Connexin-47        GPRRAPRAHLPPPHAGWPEPADLGEEEPMLGLGEEEEEEETGAAEGAGEEAEEAGAEEAC 178
Full=Connexin-31.3      ----------------WELSGKGKEEETLIQ------------------------------ 117
Full=Connexin-23        ------------------------------------------------------------

Full=Connexin-31.1      ------------GRLYLNPGKKR-----GGLWWTYVCSLVFKASVDIAFLYVFHSFYPKY 156
Full=Connexin-30.3      ------------PSLYDNLSKKR-----GGLWWTYLLSLIFKAAVDAGFLYIFHRLYKDY 156
Full=Connexin-31        ------------AKLYDNAGKKH-----GGLWWTYLFSLIFKLIIEFLFLYLLHTLWHGF 156
Full=Connexin-25        --------------LYVSPGTMD-----GGLWYAYLISLIVKTGFEIGFLVLFYKLYDGF 149
Full=Connexin-26        ------------EFKDIEEIKTQKVRIEGSLWWTYTSSIFFRVIFEAAFMYVFYVMYDGF 161
Full=Connexin-30        ------------DFKDIEDIKKQKVRIEGSLWWTYTSSIFFRIIFEAAFMYVFYFLYNGY 161
Full=Connexin-32        ------------DPLHLEEVKRHKVHISGTLWWTYVISVVFRLLFEAVFMYVFYLLYPGY 160
Full=Connexin-59        ---------LEQELCQLEKRKLNKAPLRGTLLCTYVIHIFTRSVVEVGFMIGQYLLY-GF 181
Full=Connexin-37        ----------ERQMAKISVAEDGRLRIRGALMGTYVASVLCKSVLEAGFLYGQWRLY-GW 179
Full=Connexin-43        ----------EIKKFKYGIEEHGKVKMRGGLLRTYIISILFKSIFEVAFLLIQWYIY-GF 179
Full=Connexin-46        ----------PPQDNPSSRDDRGRVRMAGALLRTYVFNIIFKTLFEVGFIAGQYFLY-GF 173
Full=Connexin-40        -----------KAELSCWEEGNGRIALQGTLLNTYVCSILIRTTMEVGFIVGQYFIY-GI 177
Full=Connexin-50        -----------SVKKSSGSKGTKKFRLEGTLLRTYICHIIFKTLFEVGFIVGHYFLY-GF 175
Full=Connexin-62        ---------DRELRRLEEQKRIHKVPLKGCLLRTYVLHILTRSVLEVGFMIGQYILY-GF 181
Full=Connexin-31.9      ------------APGLPEAQCAPCALRARRARRCYLLSVALRLLAELTFLGGQALLY-GF 160
Full=Connexin-40.1      ------------QRRCPRPFGERGGLQVPDFSAGYIIHLLLRTLLEAAFGALHYFLF-GF 167
Full=Connexin-36        -------KELTPHPSGLRTASKSKLRRQEGISRFYIIQVVFRNALEIGFLVGQYFLY-GF 223
Full=Connexin-45        ------ENKEQSQPKPK--HDGRRRIREDGLMKIYVLQLLARTVFEVGFLIGQYFLY-GF 201
Full=Connexin-47        TKAVGADGKAAGTPGPTGQHDGRRRIQREGLMRVYVAQLVARAAFEVAFLVGQYLLY-GF 237
Full=Connexin-31.3      -------GREGNTDVPG---AGSLR-----LLWAYVAQLGARLVLEGAALGLQYHLY-GF 161
Full=Connexin-23        ------------------------------IYTIIYILSVLLRISLAAIAFWLQIYLF-GF 140
                                                       *   :  :            ::

Full=Connexin-31.1      ILPPVVKCH--ADPCPNIVDCFISKPSEKNIFTLFMVATAAICILLNLVELIYLVSKRCH 214
Full=Connexin-30.3      DMPRVVACS--VEPCPHTVDCYISRPTEKKVFTYFMVTTAAICILLNLSEVFYLVGKRCM 214
Full=Connexin-31        NMPRLVQCAN-VAPCPNIVDCYIARPTEKKIFTYFMVGASAVCIVLTICELCYLICHRVL 215
Full=Connexin-25        SVPYLIKCD--LKPCPNTVDCFISKPTEKTIFILFLVITSCLCIVLNFIELSFLVLKCFI 207
Full=Connexin-26        SMQRLVKCN--AWPCPNTVDCFVSRPTEKTVFTVFMIAVSGICILLNVTELCYLLIRYCS 219
Full=Connexin-30        HLPWVLKCG--IDPCPNLVDCFISRPTEKTVFTIFMISASVICMLLNVAELCYLLLKVCF 219
Full=Connexin-32        AMVRLVKCD--VYPCPNTVDCFVSRPTEKTVFTVFMLAASGICIILNVAEVVYLIIRACA 218
Full=Connexin-59        HLEPLFKCH--GHPCPNIIDCFVSRPTEKTIFLLFMQSIATISLFLNILEIFHLGFKKIK 239
Full=Connexin-37        TMEPVFVCQ--RAPCPYLVDCFV[SRPTEKTIFII]FMLVVGLISLVLNLLELVHLLCRCLS 237
Full=Connexin-43        SLSAVYTCK--RDPCPHQVDCFLSRPTEKTIFIIFMLVVSLVSLALNIIELFYVFFKGVK 237
Full=Connexin-46        ELKPLYRCD--RWPCPNTVDCFISRPTEKTIFIIFMLAVACASLLLNMLEIYHLGWKKLK 231
Full=Connexin-40        FLTTLHVCR--RSPCPHPVNCYV[SRPTEKNVFIV]FMLAVAALSLLLSLAELYHLGWKKIR 235
Full=Connexin-50        RILPLYRCS--RWPCPNVVDCFVSRPTEKTIFILFMLSVASVSLFLNVMELGHLGLKGIR 233
[Full=Connexin-62]      QMHPLYKCT--QPPCPNAVDCFV[SRPTEKTIFML]FMHSIAAISLLLNILEIFHLGIRKIM 239
Full=Connexin-31.9      RVAPHFACA--GPPCPHTVDCFVSRPTEKTVFVLFYFAVGLLSALLSVAELGHLLWKGRP 218
Full=Connexin-40.1      LAPKKFPCT--RPPCTGVVDCYVSRPTEKSLLMLFLWAVSALSFLLGLADLVCSLRRRMR 225
Full=Connexin-36        SVPGLYECN--RYPCIKEVECYVSRPTEKTVFLVFMFAVSGICVVLNLAELNHLGWRKIK 281
Full=Connexin-45        QVHPFYVCS--RLPCPHKIDCFISRPTEKTIFLLIMYGVTGLCLLLNIWEMLHLGFGTIR 259
Full=Connexin-47        EVRPFFPCS--RQPCPHVVDCFVSRPTEKTVFLLVMYVVSCLCLLLNLCEMAHLGLGSAQ 295
Full=Connexin-31.3      QMPSSFACR--REPCLGSITCNLSRPSEKTIFLKTMFGVSGFCLLFTFLELVLLGLG--- 216
Full=Connexin-23        QVKSLYLCDARSLGENMIIRCMVPEHFEKTIFLIAINTFTTITILLFVAEIFEIIFRRLY 200
                               *          : * :..  **.::             : . ::

Full=Connexin-31.1      ECLA-ARKAQAMCTGH------HPHGTTSSCKQD--DLLSGDLIFLGSDSHPPLLPDRPR 265
Full=Connexin-30.3      EIFG-PRHRRPRCREC------LPDTCPPYVLSQGGHPEDGNSVLMKAGSAPVDAGGYP- 266
Full=Connexin-31        RGLH-KDKPRGGCSPS------SSASRASTCRCHH-KLVEAGEVDPDPGNNKLQASAPNL 267
Full=Connexin-25        KCCL-QKYLK-----------------KPQVLSV--------------------------- 223
Full=Connexin-26        GKSK-KPV------------------------------------------------------ 226
Full=Connexin-30        RRSK-RAQTQKNHPNH------AL-----KESKQNEMNELISD---SGQNAITGFPS--- 261
Full=Connexin-32        RRAQ-RRSNPPSRKGS------GFGHRLSPEYKQNEINKLLSEQDGSLKDILRRSPGTGA 271
Full=Connexin-59        RGLWGKYKLKKEHNEF------HANKAKQNVAKYQSTSANSLKRLPSAPDYNLLVEKQTH 293
Full=Connexin-37        RGMRARQGQDAPPT-------------QGTS-SDPYTD-----QVFFYLP------VGQG 272
Full=Connexin-43        DRVKGKSDPYHATS-------------GALSPAKDCGS-----QKYAYFNGCSSPTAPLS 279
Full=Connexin-46        QGVTSRLGPDASEAPL------GTADPPPLPPSSRPPA-----VAIGFPPYYAHTAAPLG 280
Full=Connexin-40        QRFVKPRQH-----------------MAKCQLSGPS-------------------VGIV 258
Full=Connexin-50        SALKRPVEQPLGEIPE------KSLHSIAVSSIQKAKGYQLLEEEKIVSHYFPLTEVGMV 287
Full=Connexin-62        RTLYKKSSSEGIEDETGPPFHLKKYSVAQQCMICSSLPERISPLQANNQQQVIRVNVPKS 299
Full=Connexin-31.9      R--------------------------AGERDNRCNRAHEEAQKLLPPPPPPP------- 245
Full=Connexin-40.1      RRPGPPTSPSIRKQS------------GASGHAEGRRTDEEGGREEEGAPAPPGARAGGE 273
Full=Connexin-36        LAVR-----------------------GAQAKRKSIYEIRNKDLPRVSVPNFGRTQSSDS 318
Full=Connexin-45        DSLNSKR--------------------RELEDPGAYNYPFTWNTPSAPPGYNIAVKPDQ 298
Full=Connexin-47        DAVRGRRGPPASAP--------------APAPRPPPCAFPAAAAGLACPPDYSLVVRAAE 341
Full=Connexin-31.3      ---RWWR-------------------------------------TWKHKSSSSKYFLTSESTR 239
Full=Connexin-23        FPFRQ--------------------------------------------------------- 205
```

```
Full=Connexin-31.1      DHVKKTIL------------------------------------------------------ 273
Full=Connexin-30.3      ------------------------------------------------------------
Full=Connexin-31        TPI----------------------------------------------------------- 270
Full=Connexin-25        ------------------------------------------------------------
Full=Connexin-26        ------------------------------------------------------------
Full=Connexin-30        ------------------------------------------------------------
Full=Connexin-32        GLAEKSDRCSAC-------------------------------------------------- 283
Full=Connexin-59        TAVYPSLNSSSVFQPNPDNHSVNDEKCILDEQETVLSNEISTLSTSCSHFQHISSNNNKD 353
Full=Connexin-37        PSSPP--------------------------CPTYNG---LSSS---------------- 287
Full=Connexin-43        PMSPPGYK----------------LVTGDRNNSSCRNYN----KQAS---------------- 306
Full=Connexin-46        QARAVGYPGAPP--PAADFKLLALTEARGKGQSAKLYNGHHHLLMT---------------- 324
Full=Connexin-40        QSCTPPPD------------FNQCLE---NGPGGKFFNPFSNNMAS---------------- 289
Full=Connexin-50        ETSPLPAKP-----------FNQFEEKISTGPLGDLSRGYQETLPSYAQVGAQEVEGEGP 336
Full=Connexin-62        KTMWQIPQPRQLEVDPSNGKKDWSEKDQHSGQLHVHSPCPWAGSAGNQHLGQQSDHSSFG 359
Full=Connexin-31.9      ----------------------------------PPPALPSRRPGPEPCAP-----PAY 265
Full=Connexin-40.1      GAGSPRRTSRVSGHTKIPDEDESEVTSSASEKLGRQPRGRPHREAAQDPRGSGSEEQPSA 333
Full=Connexin-36        AYV----------------------------------------------------------- 321
Full=Connexin-45        ---IQYTELSNAKIAYKQNKANTAQEQQYGSHEENLPADLEALQREIRMAQERLDLAVQA 355
Full=Connexin-47        RARAHDQNLANLALQALRDGAAAGDRDRDSSPCVGLPAASRGPPRAG-APASRTGSATSA 400
Full=Connexin-31.3      --------------------------RHKKATDSLPVVET---------KEQFQEAVPG 263
Full=Connexin-23        ------------------------------------------------------------


Full=Connexin-31.1      ------------------------------------------------------------
Full=Connexin-30.3      ------------------------------------------------------------
Full=Connexin-31        ------------------------------------------------------------
Full=Connexin-25        ------------------------------------------------------------
Full=Connexin-26        ------------------------------------------------------------
Full=Connexin-30        ------------------------------------------------------------
Full=Connexin-32        ------------------------------------------------------------
Full=Connexin-59        THKIFGKELNGNQLMEKRETEGKDSKRNYYSRGHRSIPGVAIDGENNMRQSPQTVFSLPA 413
Full=Connexin-37        --EQNWANLTTEER--------LASSRPPLF------------------------------ 308
Full=Connexin-43        --EQNWANYSAEQNRMGQAGSTISNSHAQPFDF---------------------------- 337
Full=Connexin-46        --EQNWANQAAERQPPALKAYPAASTPAAPSPVGSSSPPLAHEAEAGAAPLLLDGSGSSL 382
Full=Connexin-40        --QQNTDNLVTEQVRGQE--QTPGEGFIQVRYGQKPEVP---------------------- 324
Full=Connexin-50        PAEEGAEPEVGEKKEEAERLTTEEQEKVAVPEGEKVETP---------------------- 375
Full=Connexin-62        LQNTMSQSWLGTTTAPRNCPSFAVGTWEQSQDPEPSGEPLTDLHSHCRDSEGSMRESGVW 419
Full=Connexin-31.9      AHPAPASLRECGSGRGKASPATGRRDLAI-------------------------------- 294
Full=Connexin-40.1      APSRLAAPPSCSSLQPPDPPASSSGAPHLRARKSEWV------------------------ 370
Full=Connexin-36        ------------------------------------------------------------
Full=Connexin-45        YSHQNNPHGPREKKAKVGSKAGSNKSTASSKSGDGKTSVWI-------------------- 396
Full=Connexin-47        GTVGEQGRPGTHERPGAKPRAGSEKGSASSR--DGKTTVWI-------------------- 439
Full=Connexin-31.3      RSLAQ------EKQRPVGPRDA---------------------------------------- 279
Full=Connexin-23        ------------------------------------------------------------


Full=Connexin-31.1      ------------------------------------------------------------
Full=Connexin-30.3      ------------------------------------------------------------
Full=Connexin-31        ------------------------------------------------------------
Full=Connexin-25        ------------------------------------------------------------
Full=Connexin-26        ------------------------------------------------------------
Full=Connexin-30        ------------------------------------------------------------
Full=Connexin-32        ------------------------------------------------------------
Full=Connexin-59        NCDWKPRWLRATWGSSTEHENRGSPPKGNLKGQFRKGTVRTLPPSQGDSQSLDIPNTADS 473
Full=Connexin-37        ----------------------LDPPPQNGQKPPS-------------RPSSSASKKQY 332
Full=Connexin-43        --------PDDNQNSKKLAAGHELQPLAIVDQRPSS-------------RASSRASSRPR 376
Full=Connexin-46        EGSALAGTPEEEEQAVTTAAQMHQPPLPLGDPGRAS-------------KASRASSGRAR 429
Full=Connexin-40        --------NGVSPGHR---------LPHGYHSDKR---------------RLSKASSKAR 352
Full=Connexin-50        --------GVDKEGEKEEPQSEKVSKQGLPAEKTPSLCPELTTDDARPLSRLSKASSRAR 427
Full=Connexin-62        IDRSRPGSRKASFLSRLLSEKRHLHSDSGSSGSRNSSCLDFPHWENSPSPLPSVTGHRTS 479
Full=Connexin-31.9      ------------------------------------------------------------
Full=Connexin-40.1      ------------------------------------------------------------
Full=Connexin-36        ------------------------------------------------------------
Full=Connexin-45        ------------------------------------------------------------
Full=Connexin-47        ------------------------------------------------------------
Full=Connexin-31.3      ------------------------------------------------------------
Full=Connexin-23        ------------------------------------------------------------
```

```
Full=Connexin-31.1      ------------------------------------------------------------
Full=Connexin-30.3      ------------------------------------------------------------
Full=Connexin-31        ------------------------------------------------------------
Full=Connexin-25        ------------------------------------------------------------
Full=Connexin-26        ------------------------------------------------------------
Full=Connexin-30        ------------------------------------------------------------
Full=Connexin-32        ------------------------------------------------------------
Full=Connexin-59        LGGLSFEPGLVRTCNNPVCPPNHIVSLTNNLIGRRVPTDLQI------------------ 515
Full=Connexin-37        V----------------------------------------------------------- 333
Full=Connexin-43        PDDLEI------------------------------------------------------ 382
Full=Connexin-46        PEDLAI----------------------------------------------------- 435
Full=Connexin-40        SDDLSV----------------------------------------------------- 358
Full=Connexin-50        SDDLTV----------------------------------------------------- 433
Full=Connexin-62        MVRQAALPIMELSQELFHSGCFLFPFFLPGVCMYVCVDREADGGGDYLWRDKIIHSIHSV 539
Full=Connexin-31.9      ------------------------------------------------------------
Full=Connexin-40.1      ------------------------------------------------------------
Full=Connexin-36        ------------------------------------------------------------
Full=Connexin-45        ------------------------------------------------------------
Full=Connexin-47        ------------------------------------------------------------
Full=Connexin-31.3      ------------------------------------------------------------
Full=Connexin-23        ------------------------------------------------------------


Full=Connexin-31.1      ----
Full=Connexin-30.3      ----
Full=Connexin-31        ----
Full=Connexin-25        ----
Full=Connexin-26        ----
Full=Connexin-30        ----
Full=Connexin-32        ----
Full=Connexin-59        ----
Full=Connexin-37        ----
Full=Connexin-43        ----
Full=Connexin-46        ----
Full=Connexin-40        ----
Full=Connexin-50        ----
Full=Connexin-62        KFNS 543
Full=Connexin-31.9      ----
Full=Connexin-40.1      ----
Full=Connexin-36        ----
Full=Connexin-45        ----
Full=Connexin-47        ----
Full=Connexin-31.3      ----
Full=Connexin-23        ----
```

8.2 Appendix 2: Multiple alignments of connexin 62 in human and connexin 57 in mouse obtained from ClustalW (www.ebi.ac.uk/tools/clustalw). The red highlighting represents Gap27 sites.

```
Connexin62              MGDWNLLGGILEEVHSHSTIVGKIWLTILFIFRMLVLRVAAEDVWDDEQSAFACNTRQPG 60
Connexin57              MGDWNLLGGILEEVHSHSTIVGKIWLTILFIFRMLVLGVAAEDVWDDEQSAFACNTQQPG 60
                        ************************************ ******************:***

Connexin62              CNNICYDDAFPISLIRFWVLQIIFVSSPSLVYMGHALYRLRAFEKDRQRKKSHLRAQMEN 120
Connexin57              CNNICYDDAFPISLIRFWVLQIIFVSSPSLVYMGHALYRLRDFEKQRQKKKLYLRAQMEN 120
                        ****************************************** ***:**:** :*******

Connexin62              PDLDLEEQQRIDRELRRLEEQKRIHKVPLKGCLLRTYVLHILTRSVLEVGFMIGQYILYG 180
Connexin57              PELDLEEQQRVDKELRRLEEQKKIHKVPLKGCLLRTYVLHILTRSVLEVGFMIGQYILYG 180
                        *:********:*:*********:************************************

Connexin62              FQMHPLYKCTQPPCPNAVDCFVSRPTEKTIFMLFMHSIAAISLLLNILEIFHLGIRKIMR 240
Connexin57              FQMHPIYKCTQAPCPNSVDCFVSRPTEKTIFMLFMHSIAAISLLLNILEIFHLGIRKIMR 240
                        *****:*****.****:****************************************

Connexin62              TLYKKSSSEGIEDETGPPFHLKKYSVAQQCMICSSLPERISPLQANNQQQVIRVNVPKSK 300
Connexin57              ALDGKSSSGNTENETGPPFHSTNYSGTQQCMVCSSLPERISLLQANNKQQVIRVNIPRSK 300
                        :*  **** . *:******* .:** :****:********* *****:*******:*:**

Connexin62              TMWQIPQPRQLEVDPSNGKKDWSEKDQHSGQLHVHSPCPWAGSAGNQHLGQQSDHSSFGL 360
Connexin57              SMWQIPHPRQLEVDVSCGKRDWAEKIESCTQLHVHSPCPHDRSARIQHPGQQPCHSVFGP 360
                        :*****:******* * **:**:** : . *********   **  ** ***. ** **

Connexin62              QNTMSQSWLGTTTAPRNCPSFAVGTWEQSQDPEPSGEPLTDLHSHCRDSEGSMRESGVWI 420
Connexin57              KNAMSQSWFGTMTASQHRPSSALETWERSQGPEASGRSLTDRQSHFQGSDGSARESGVWT 420
                        :*:*****:** **.:: ** *: ***:**.**.**..*** :** :.*:** ******

Connexin62              DRSRPGSRKASFLSRLLSEKRHLHSDSGSSGSRNSSCLDFPHWENSPSPLPSVTGHRTSM 480
Connexin57              DRLGPGSRKASFLSRLMSEKGQRHSDSGSSRSLNSSCLDFSHGENSPSPLPSATGHRASM 480
                        **  ************:*** : ******* * *******.* *********.****:**

Connexin62              VRQAALPIMELSQELFHSGCFLFPFFLPGVCMYVCVDREADGGGDYLWRDKIIHSIHSVK 540
Connexin57              VSKSSH-----VDSPPHS-----SFIIHETYVYVY------------------------ 505
                        * :::       :.  **     .*::  . :**

Connexin62              FNS 543
Connexin57              ---
```