

Kv 1.3-induced hyperpolarization is required for efficient Kaposi's sarcoma-associated herpesvirus lytic replication

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Accepted Version

Carden, H., Harper, K. L., Mottram, T. J., Manners, O., Allott, K. L., Dallas, M. L. ORCID: <https://orcid.org/0000-0002-5190-0522>, Hughes, D. J., Lippiat, J. D., Mankouri, J. and Whitehouse, A. (2024) Kv 1.3-induced hyperpolarization is required for efficient Kaposi's sarcoma-associated herpesvirus lytic replication. *Science Signaling*, 17 (845). eadg4124. ISSN 1937-9145 doi: <https://doi.org/10.1126/scisignal.adg4124> Available at <https://centaur.reading.ac.uk/117368/>

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To link to this article DOI: <http://dx.doi.org/10.1126/scisignal.adg4124>

Publisher: AAAS

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1 **K_v1.3 induced hyperpolarisation is required for efficient Kaposi's sarcoma-associated herpesvirus**
2 **lytic replication**

3

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17

18 **Running Title: K_v1.3 is required for KSHV reactivation**

19

20

21 **Keywords:**

22 KSHV; antiviral; ion channel; K_v1.3; NFAT

23

24 Report: 4111 words (Main text: Summary, Introduction, Results, Discussion, Figure Legends)

25 Figures: 4

26 Supplementary Figures: 8

27 Supplementary Table: 1

28

29 **Summary**

30

31 Host factors critical for virus replication can identify new targets for therapeutic intervention.
32 Pharmacological and genetic silencing approaches reveal that Kaposi's sarcoma-associated
33 herpesvirus (KSHV) requires a B cell expressed voltage-gated K⁺ channel, K_v1.3, to enhance lytic
34 replication. The KSHV replication and transcription activator protein upregulates K_v1.3 expression,
35 leading to enhanced K⁺ channel activity and hyperpolarisation of the B cell membrane. Enhanced K_v1.3
36 activity promotes intracellular Ca²⁺ influx, leading to Ca²⁺ driven nuclear localisation of NFAT and
37 subsequent NFAT1-responsive gene expression. Importantly, KSHV lytic replication and infectious
38 virion production were inhibited by both K_v1.3 blockers and K_v1.3 silencing. These findings provide
39 new mechanistic insight into the essential role of host ion channels during KSHV infection and highlight
40 K_v1.3 as a potential druggable host factor.

41 Introduction

42

43 Ion channels are multi-subunit, pore-forming membrane proteins that control rapid and selective
44 passage of ions across the plasma membrane and membranes of subcellular organelles ¹. As such, ion
45 channels have roles in controlling the ion homeostasis of the cell and its organelles, action potential
46 firing, membrane potential and cell volume. Given these various functions and their ubiquitous nature,
47 ion channel dysregulation is implicated in multiple diseases, known as channelopathies ² and may also
48 enhance cell proliferation and invasion of tumour cells. Interestingly, several stages of virus
49 replication, including virion entry, virus egress and the maintenance of an environment conducive to
50 virus replication have been in-part, suggested to be dependent on virus-mediated manipulation of ion
51 channel activity ^{2,3}. This is reinforced by observations that pharmacological modulation of ion channels
52 can impede virus replication, highlighting ion channels as promising candidates for targeted anti-viral
53 therapeutics. Importantly, some ion-channel blocking drugs are in widespread human use for ion
54 channel-related diseases, highlighting potential for drug repurposing.

55

56 Kaposi's sarcoma-associated herpesvirus (KSHV) is a gamma 2-herpesvirus linked to the development
57 of Kaposi's sarcoma (KS), a highly vascular tumour of endothelial lymphatic origin, and several other
58 AIDS-associated malignancies ⁴⁻⁷. KSHV exhibits a biphasic life cycle consisting of latent persistence or
59 lytic replication. In contrast to other oncogenic herpesviruses in which latent gene expression drives
60 tumorigenesis, both the latent and lytic replication phases are essential for KSHV-mediated
61 tumorigenicity ⁸. Latency is established in B cells and the tumour setting, where viral gene expression
62 is limited to the latency-associated nuclear antigen (LANA), viral FLICE inhibitory protein, viral cyclin,
63 kaposins and several virally-encoded miRNAs ⁹⁻¹¹. Upon reactivation, KSHV initiates lytic replication
64 leading to the orchestrated expression of >80 viral proteins, sufficient for the production of infectious
65 virions ^{12,13}. In KS lesions, most infected cells harbour the virus in a latent state. However, a small
66 proportion of cells undergo lytic replication leading to the secretion of angiogenic, inflammatory and
67 proliferative factors that act in a paracrine manner on latently-infected cells to enhance tumorigenesis
68 ¹⁴. Lytic replication also enhances genomic instability ¹⁵ and sustains KSHV episomes in latently-
69 infected cells that would otherwise be lost during cell division ¹⁶. The ability to inhibit lytic replication
70 therefore represents a therapeutic intervention strategy for KSHV-associated diseases ^{17,18}.

71

72 The transition from latent infection to lytic replication is controlled by host and viral factors ^{19,20}. They
73 converge on the regulation of the latency associated nuclear antigen (LANA) and the master regulator
74 of the latent-lytic switch, KSHV replication and transcription activator (RTA) protein ²¹. Notably, agents

75 that mobilize intracellular calcium (Ca^{2+}) induce KSHV-RTA expression and enhance lytic replication ²²,
76 however this activity can be blocked with calcineurin-dependent signal transduction inhibitors ²³.
77 Cytoplasmic Ca^{2+} concentrations are regulated by a network of ion channels and transporters ²⁴. To
78 date, a specific role for host cell ion channels during KSHV lytic replication or any herpesvirus have yet
79 to be fully defined. B lymphocytes, the primary site of KSHV latency, are regulated by a network of
80 transporters and ion channels that control the cytoplasmic concentrations of Ca^{2+} , magnesium (Mg^{2+})
81 and zinc (Zn^{2+}), which act as important second messengers to regulate critical B cell effector functions
82 ²⁵. The repertoire of ion channels in B cells include potassium (K^+) channels, Ca^{2+} channels, P2X
83 receptors and transient receptor potential (TRP) channels, in addition to Mg^{2+} and Zn^{2+} transporters.
84 To-date, a role for these channels during KSHV infection has not been described.

85

86 Combining electrophysiological and biochemical approaches, we show that KSHV activates a voltage-
87 gated K^+ channel $\text{K}_v1.3$, the pharmacological and genetic silencing of which inhibits KSHV lytic
88 replication. We further define the mechanism for this dependence by showing that $\text{K}_v1.3$ activation
89 leads to hyperpolarisation-induced Ca^{2+} influx, enhancing the nuclear localisation of NFAT1, which is
90 required to drive virus replication. We therefore reveal the essential role of $\text{K}_v1.3$ in KSHV lytic
91 replication.

92 Results

93

94 K⁺ channels are required for efficient KSHV reactivation

95

96 K⁺ channels represent the largest family of ion channels with >70 genes identified in the human
97 genome ²⁶. To determine if their activity is required for efficient KSHV lytic replication, virus
98 reactivation assays were performed in the presence of potassium chloride (KCl) to collapse cellular K⁺
99 channel gradients, or broad spectrum K⁺ channel blockers, tetraethylammonium (TEA) and quinidine
100 (Qn). All inhibitors were used at non-toxic concentrations measured by MTS and annexin V-based flow
101 cytometry assays during both latent and lytic phases (**Figure S1**). KSHV reactivation was assessed in
102 TReX BCBL1-RTA cells, a latently infected KSHV B-lymphocyte cell line expressing a Myc-tagged viral
103 RTA under the control of a doxycycline-inducible promoter. TReX BCBL1-RTA cells reactivated for 24 h
104 in the presence of each K⁺ channel inhibitor showed a drastic reduction in the expression of early
105 ORF57, delayed early ORF59 and the late minor capsid ORF65 proteins (**Figure 1A, Figure S2**). No such
106 reduction was observed in the expression of Myc-RTA or GAPDH, highlighting specific effects on lytic
107 replication as opposed to dox-induced induction. This indicates a requirement for K⁺ channel function
108 during KSHV lytic replication.

109

110 K⁺ channel subfamilies include voltage-gated K⁺ channels (K_v), calcium-activated K⁺ channels (K_{Ca}),
111 inwardly rectifying K⁺ channels (K_{ir}) and two-pore domain K⁺ channels (K_{2P}) channels. To identify the
112 specific K⁺ channel(s) required for KSHV lytic replication a more specific drug regime was utilised. 4-
113 aminopyridine (4-AP), a non-selective K_v blocker, led to a concentration-dependent reduction in lytic
114 replication (**Figure 1B, Figure S2**), suggestive of a role for K_v channels. Electrophysiological studies have
115 identified an array of K_v channels expressed within B lymphocytes, with a member of the *Shaker*
116 related family, K_v1.3, most extensively characterised ²⁷. Specific K_v1.3 blockers margatoxin (MgTX) and
117 ShK-Dap²² showed a concentration-dependent reduction of ORF57 protein production, implicating a
118 role for K_v1.3 during KSHV lytic replication (**Figure 1C-D, Figure S2**). Similar inhibition of KSHV lytic
119 replication was observed in KSHV infected BCBL-1 and iSLK cells in the presence of ShK-Dap²² (**Figure**
120 **S3**). In contrast, TRAM-34, a blocker of B lymphocyte K_{Ca}3.1 channels, showed no effect in TReX BCBL1-
121 RTA cells (**Figure 1E, Figure S2**). ShK-Dap²²-mediated K_v1.3 inhibition also lead to a dramatic reduction
122 in a range of KSHV lytically expressed genes across the complete temporal cascade (**Figure 1F, Figure**
123 **S4**).

124

125 To confirm a role for K_v1.3, TReX BCBL1-RTA cells were stably transduced with lentivirus-based shRNAs
126 depleting K_v1.3 by >85% (**Figure 1G and 1H, Figure S2**). Reactivation assays showed that K_v1.3 silencing
127 led to a significant reduction in ORF57 mRNA levels (**Figure 1I**) and lytic proteins compared to control
128 (**Figure 1J and 1K, Figure S2**). K_v1.3 depletion also reduced infectious virus production by ~85%,
129 measured by reinfection of naive cells with supernatants from K_v1.3 depleted cells and qRT-PCR of
130 viral mRNA (**Figure 1L**) and LANA-immunostaining (**Figure 1M**). To ensure our K_v1.3 depletion studies
131 were not due to off-target effects, complementation assays were performed using a lentivirus
132 expressing a K_v1.3 shRNA-resistant expression construct. Results showed this rescued KSHV lytic
133 replication and infectious virion production in the K_v1.3 depleted cell line, measured by ORF57 protein
134 production (**Figure 1K, Figure S2**) and LANA immunostaining of reinfected supernatants (**Figure 1M**).
135 Together, results show K_v1.3 channels are required for efficient lytic replication.

136

137 **KSHV enhances K_v1.3 expression and activity**

138

139 We next assessed whether KSHV modulated K_v1.3 activity. qRT-PCR and immunoblotting showed K_v1.3
140 expression increased in reactivated TReX BCBL1-RTA cells compared to latent cells (**Figure 2A, Figure**
141 **S2**). To elucidate whether the increased K_v1.3 expression led to enhanced K⁺ efflux during lytic
142 replication, whole-cell patch clamp analysis was performed. Electrophysiological recordings revealed
143 a voltage-gated outward K⁺ current present in latent TReX BCBL1-RTA cells that was significantly
144 enhanced in reactivated cells (**Figure 2B**). To conclusively determine that K_v1.3 channels were
145 responsible, recordings were repeated in the presence of ShK-Dap²², which led to a dramatic inhibition
146 of the K⁺ current (**Figure 2B**). A similar reduction was observed in K_v1.3 depleted cells, compared to
147 controls (**Figure 2C**). We also observed that reactivated TReX BCBL1-RTA cells exhibited a significantly
148 more hyperpolarised membrane compared to latent cells (**Figure 2D**), which was reversed upon K_v1.3
149 depletion (**Figure 2E**). Membrane hyperpolarisation was confirmed using a membrane potential-
150 sensitive dye, bis (1,3-dibutylbarbituric acid) trimethine oxonol; DiBAC₄(3). Results showed a time-
151 dependent decrease in fluorescence intensity in control lytic cells, consistent with enhanced
152 membrane hyperpolarization, whereas no reduction was observed in K_v1.3 depleted cells (**Figure 2F**).
153 Conversely, addition of the calcium ionophore A23187, which induces depolarisation, enhanced
154 DiBAC₄(3) fluorescence (**Figure 2F**). Together results demonstrate that KSHV lytic replication increases
155 K_v1.3 expression, resulting in enhanced K_v1.3 currents and hyperpolarisation.

156

157 **KSHV RTA mediates the upregulation of K_v1.3 during lytic replication**

158

159 Given that membrane hyperpolarisation was observed as early as 4 h post-reactivation, we examined
160 whether any KSHV early proteins induced K_v1.3 expression. A549 and U87 cells were transiently
161 transfected with control GFP, RTA-GFP or ORF57-GFP expression constructs and K_v1.3 transcript levels
162 were assessed by qRT-PCR at 24 h post-transfection. RTA-GFP alone was sufficient to induce K_v1.3
163 expression in a dose-dependent manner (**Figure S5A-B**), confirming KSHV RTA as the direct inducer of
164 K_v1.3 expression. Specificity Protein (Sp) 1 functions as a co-adaptor for RTA-mediated transactivation
165 and is known to regulate K_v1.3 expression²⁸. We therefore examined a potential cooperative role for
166 Sp1 during the upregulation of K_v1.3 during lytic replication. RTA-GFP transfections were performed
167 in the presence of Mithramycin A, a selective Sp1 inhibitor that displaces Sp1 binding from its target
168 promoter²⁹. Mithramycin A suppressed RTA-mediated increase in K_v1.3 expression (**Figure S5C**), but
169 had little effect on the upregulation of the IL-6 promoter, suggesting an in-direct mechanism whereby
170 Sp1 recruits RTA to the K_v1.3 promoter. ChIP assays confirmed an association of both RTA and Sp1
171 with the K_v1.3 promoter, which significantly increased during lytic replication (**Figure S5D**). This reveals
172 RTA as the driver of K_v1.3 expression during KSHV lytic replication.

173

174 **K_v1.3 induced membrane hyperpolarisation drives Ca²⁺ influx required for KSHV reactivation**

175

176 In B lymphocytes, K_v1.3 maintains a hyperpolarised membrane potential necessary to sustain the
177 driving force for Ca²⁺ entry, which indirectly modulates an array of Ca²⁺-dependent cellular processes.
178 We therefore assayed Ca²⁺ influx into TReX BCBL1-RTA cells during KSHV lytic replication using the
179 ratiometric Ca²⁺ dye Fura-Red. We observed an increase in cytoplasmic Ca²⁺ over a 24 h period of lytic
180 reactivation, that was absent in K_v1.3-depleted TReX BCBL1-RTA cells (**Figure 3A, Figure S6A**). To
181 determine whether Ca²⁺ influx defines the requirement of K_v1.3 for efficient lytic replication, we
182 assessed what effect Ca²⁺ depletion, by EGTA chelation, had on lytic replication. Ca²⁺ depletion led to
183 the cytoplasmic accumulation of KSHV RTA and a corresponding reduction in lytic gene expression
184 across the temporal cascade, indicated by a reduction in ORF65 protein levels (**Figure 3B**) and KSHV-
185 encoded transcripts (**Figure S6B**). Conversely mimicking Ca²⁺ influx, by reactivating TReX BCBL1-RTA
186 cells in the presence of the Ca²⁺ ionophore A23187, slightly enhanced ORF57 protein levels compared
187 to control cells (**Figure 3C, Figure S2**), aligning with previous findings²². Notably, A23187 rescued KSHV
188 lytic replication in K_v1.3 depleted cells, suggesting A23187 overrides the dependence of KSHV on K_v1.3
189 (**Figure 3D, Figure S2**).

190

191 To confirm an extracellular source of Ca²⁺ is required for KSHV lytic replication, we compared KSHV
192 replication in TReX BCBL1-RTA cells cultured over 24 hours in normal growth media or calcium-free

193 media. Results showed that the lack of extracellular calcium dramatically reduced the levels of KSHV
194 lytic replication. Importantly, lytic replication was rescued upon returning the cells to normal growth
195 media (**Figure 3E, Figure S2**). Moreover, metallic divalent ions can inhibit Ca^{2+} permeation via voltage-
196 dependent Ca^{2+} channels³⁰. Therefore we assessed what affect the presence of Ni^{2+} had upon KSHV
197 lytic replication. Results showed a significant reduction in virus replication, confirming extracellular
198 Ca^{2+} is required (**Figure 3F, Figure S2**). In contrast Thapsigargin, which inhibits ER calcium pumps
199 leading to depletion of ER Ca^{2+} stores³¹, had little effect on KSHV lytic replication (**Figure 3G, Figure**
200 **S2**). These data suggest extracellular Ca^{2+} influx is essential for KSHV lytic replication and is induced by
201 $\text{K}_v1.3$ -mediated hyperpolarisation.

202

203 To identify candidate Ca^{2+} channel(s) required for KSHV-mediated Ca^{2+} influx, lytic replication was
204 assessed in presence of various Ca^{2+} channel modulating drugs at non-cytotoxic concentrations (**Figure**
205 **S1**). Incubation with either 2-APB, an inhibitor of intracellular calcium levels and transient receptor
206 potential (TRP) channels, BTP2 a blocker of store-operated Ca^{2+} entry and Nifedipine, an L-type
207 voltage-gated Ca^{2+} channel inhibitor, all showed little effect on KSHV lytic replication. In contrast,
208 Mibefradil a specific inhibitor of T-type voltage-gated Ca^{2+} channels dramatically reduced the levels of
209 KSHV lytic replication (**Figure S7**). This suggests that T-type voltage-gated Ca^{2+} channels may have a
210 role in $\text{K}_v1.3$ -mediated hyperpolarisation-induced calcium influx, although the exact channel is yet to
211 be determined.

212

213 **KSHV-mediated Ca^{2+} influx initiates NFAT1-mediated gene expression**

214

215 Ca^{2+} influx initiates multiple signalling pathways, including the serine/threonine phosphatase
216 calcineurin and its target transcription factor NFAT (nuclear factor of activated T cells)²⁴.
217 Dephosphorylation of cytoplasmic NFAT proteins by calcineurin unmask their nuclear localization
218 sequences, leading to nuclear translocation and NFAT-responsive gene expression. We therefore
219 determined whether the calcineurin-mediated nuclear import of NFAT1 was important for KSHV lytic
220 replication. In the presence of calcineurin/NFAT1 inhibitors, cyclosporin A (CsA) and VIVIT, a dose-
221 dependent reduction in ORF57 protein production (**Figure 4A, Figure S2**) and a reduction in KSHV lytic
222 genes was observed (**Figure S8**). To investigate whether KSHV-mediated hyperpolarisation and Ca^{2+}
223 influx promoted the nuclear translocation of NFAT, the nuclear/cytoplasmic distribution was
224 compared in latent versus lytic TREx BCBL1-RTA cells using immunofluorescence analysis. Results
225 showed that NFAT1 translocates to the nucleus in lytic cells, but remains cytoplasmic during latency
226 (**Figure 4B**). The nuclear localisation of NFAT1 was dependent on $\text{K}_v1.3$ -mediated hyperpolarisation

227 and calcineurin activity, has it was prevented by ShK-Dap²² and CsA, respectively (**Figure 4B**).
228 Consistent with enhanced NFAT nuclear localisation, we observed an increase in NFAT-responsive
229 gene expression during KSHV lytic replication, which was reduced in the presence of ShK-Dap²² (**Figure**
230 **4C**), and upon K_v1.3 depletion (**Figure 4D**). Interestingly, a number of NFAT-responsive genes, such as
231 COX-2 and ANGPT2 are upregulated during KSHV infection and implicated in KSHV-mediated
232 pathogenesis³²⁻³⁴. Together, data suggest that KSHV-induced hyperpolarisation, mediated by K_v1.3,
233 and the subsequent Ca²⁺ influx enhances NFAT1 nuclear localisation and NFAT-driven gene expression.

234 **Discussion**

235

236 Discovery of cellular determinants that control KSHV lytic induction can inform new therapeutic
237 targets for anti-KSHV drug development. Ion channels control a range of cellular processes that are
238 co-opted by viruses ^{2,3}. Accordingly, ion channels have emerged as druggable host targets to prevent
239 both RNA and DNA virus replication. Given the known dependence of KSHV lytic replication on Ca²⁺
240 signalling ²³, coupled to previous studies demonstrating VZV and HSV-1 activating Na⁺ and Ca²⁺ family
241 members ^{35,36}, we investigated the role of B-cell expressed ion channels during KSHV lytic reactivation.
242 We reveal that KSHV requires a B-cell expressed voltage-gated K⁺ channel, K_v1.3, to enhance lytic
243 replication and KSHV RTA protein upregulates K_v1.3 expression via indirect Sp1-mediated
244 transactivation. Enhanced K_v1.3 expression and activity led to hyperpolarisation of B-cell membrane
245 potential, initiating Ca²⁺ influx. At present the channel which drives the extracellular Ca²⁺ influx is
246 unknown, however studies suggest a potential T-type channel. This Ca²⁺ elevation enhances the
247 nuclear localisation of NFAT1 and KSHV RTA, which are both essential to drive KSHV lytic replication.
248 At present the exact role of Ca²⁺ in RTA-mediated nuclear import is yet to be elucidated however
249 potential mechanisms may involve enhanced recruitment of nuclear import proteins, or the
250 unmasking of nuclear localisation signals ³⁷. Together, this reveals that K_v1.3-mediated
251 hyperpolarisation and Ca²⁺ influx are direct contributors to KSHV lytic replication in B cells.

252

253 A striking feature of KSHV is the homology of its numerous ORFs to cellular genes ¹³. These virus-
254 encoded proteins contribute to KSHV-associated pathogenesis by subverting cell signalling pathways.
255 Many viruses encode viroporins ³⁸ that modulate the ionic milieu of intracellular organelles controlling
256 virus protein stability and trafficking. However, no known viroporin exists amongst KSHV ORFs and it
257 is likely that evolution has tailor-made KSHV proteins to regulate the expression of host ion channels
258 to regulate Ca²⁺ signalling during infection. Tumorigenesis represents a by-product of this regulation,
259 since enhanced K_v1.3 expression correlates with the grade of tumour malignancy in various cancers
260 ³⁹. Notably, features of KS tumours mirror the phenotypic effects of K_v1.3 overexpression, including
261 the enhanced expression of inflammatory and angiogenic cytokines and uncontrolled cell cycle
262 progression. This suggests that KSHV-driven activation of K_v1.3 is similar to channelopathies, diseases
263 characterised by altered function of ion channel proteins or their regulatory subunits.

264

265 Several ion channel inhibitors target K_v1.3, comprising small organic molecules such as quinine and
266 4AP or peptides purified from venom ^{28,40}. Venom-derived peptides are highly stable and resist
267 denaturation due to the disulphide bridges formed within the molecules ⁴⁰. Like margatoxin, most are

268 derived from scorpion venom, such as agitoxins, kaliotoxin, maurotoxin and noxiustoxin, whereas ShK,
269 is a peptide isolated from the sea anemone *Stichodactyla helianthus*⁴¹. Given the abundance of
270 natural sources for K_v1.3-inhibition a safe, effective therapeutic based on these compounds is a
271 promising target for prevention. Additionally, it is interesting to note that the CD20 monoclonal
272 antibody rituximab, a known K_v1.3 inhibitor, substantially improves KSHV patients outcome⁴².

273

274 Finally, K_v channels have been previously identified as a restriction factor to the entry of both Hepatitis
275 C virus⁴³ and Merkel cell polyomavirus⁴⁴, through their abilities to inhibit endosome acidification-
276 mediated viral membrane fusion. Whilst the inhibition of endosomal acidification reduces the entry
277 and trafficking of KSHV virions, our electrophysiological analysis revealed enhanced cell surface K_v1.3
278 activity during lytic replication that directly contributed to the hyperpolarised membrane potential of
279 cells required for efficient KSHV replication. Thus, whilst additional roles of K_v1.3 in endosomes cannot
280 be excluded, our data suggest a divergent role of K_v1.3 during infection that may be cell-type and/or
281 virus specific.

282

283 **Limitations of the study.** Although we reveal the requirement of K_v1.3 for KSHV reactivation it is
284 important to note that compounds targeting K_v1.3 are only in preclinical development⁴⁵. Moreover,
285 although Ca²⁺ influx is essential for KSHV lytic replication and results suggest the involvement of a T-
286 type voltage-gated Ca²⁺ channel, the exact channel is yet to be identified. Another key limitation of
287 this study is that results are based on *in vitro* studies and further studies targeting these channels
288 using appropriate *in vivo* models are now warranted.

289

290 **Acknowledgements**

291 This work was supported by Rosetrees Trust PhD studentship, M662 (Whitehouse) and Royal Society
292 University Research Fellowship, G:480764 (Mankouri). We thank Professor Jae Jung, University of
293 Southern California School of Medicine, Los Angeles, for the TReX BCBL1-RTA cells. KSHV ORF59 and
294 ORF65 antibodies were kind gifts from Professors Britt Glaunsinger (University of California, Berkeley)
295 and SJ Gao (University of Pittsburgh).

296

297 **Author Contributions**

298 Conceptualization (JM, AW); Data curation (HC, KLH, TJM, MLD, DJH, KLA, OM); Formal Analysis (HC,
299 KLH, TJM, MLD, DJH, KLA, OM, JDL, JM, AW); Funding acquisition (AW, JM); Investigation (HC, KLH,
300 MLD, DJH); Writing—original draft (HC, KLH, JM, AW); Writing—review & editing (All authors).

301

302 **Competing interests:** There are no financial and non-financial competing interests.

303 **Figure Legends**

304

305 **Figure 1. K_v1.3 channels are required for efficient KSHV lytic replication.**

306 (A-E) TREx BCBL1-RTA cells remained unreactivated or pre-treated with non-cytotoxic concentrations
307 of (A) general K⁺ inhibitors, 25 mM KCl, 100 μM Qn and 10 mM TEA for 45 minutes prior to reactivation
308 with doxycycline hyclate for 48 hr, or increasing amounts of (B) 4AP (C) MgTX, (D) ShK-Dap²² and (E)
309 TRAM34. Cell lysates were probed with ORF57-, ORF59- or ORF65-specific antibodies. GAPDH was
310 used as a measure of equal loading.

311 (F) TREx BCBL1-RTA cells remained unreactivated or pre-treated with 100 pM ShK-Dap²² for 45
312 minutes prior to reactivation with doxycycline hyclate, samples were harvested at 0, 8, 16 and 24
313 hours post-reactivation, total RNA was extracted and relative ORF57 transcript levels were analysed
314 by qRT-PCR using GAPDH as a reference. Fold change was determined by $\Delta\Delta C_t$ and statistical
315 significance analysed using a non-paired t-test, ** = p<0.01.

316 (G-J) Scramble and K_v1.3-depleted cells lines were reactivated with doxycycline hyclate for 24 hr. Total
317 RNA was extracted and relative transcript levels of (G) K_v1.3 or (I) ORF57 were analysed by qRT-PCR
318 using GAPDH as a reference. Fold change was determined by $\Delta\Delta C_t$ and statistical significance analysed
319 using a non-paired t-test, *** = p<0.001. Cell lysates were probed with (H) K_v1.3- or (J) ORF57-, ORF59-
320 or ORF65-specific antibodies and GAPDH used as a measure of equal loading.

321 (K) Scramble, K_v1.3-depleted or K_v1.3-rescued cell lines were reactivated for 24 h, cell lysates were
322 probed with ORF57 and K_v1.3-specific antibodies and GAPDH used as a measure of equal loading.

323 (L) Scramble and K_v1.3-depleted cell lines were reactivated for 72 hr, prior to the culture medium being
324 incubated with HEK-293T cells. Total RNA was extracted and relative ORF57 transcript levels were
325 analysed by qRT-PCR using GAPDH as a reference, results show the mean of three biological replicates
326 with error bar as standard deviation, *** = p<0.001.

327 (M) Scramble, K_v1.3-depleted or K_v1.3-rescued cell lines were reactivated for 72 hr, prior to the culture
328 medium being incubated with HEK-293T cells. Cells were then probed with a LANA-specific antibody
329 and DAPI-stained before imaging on a Zeiss LSM880 confocal microscope.

330

331 **Figure 2. Increased K⁺ currents during KSHV lytic replication is dependent on K_v1.3 expression.**

332 (A) TREx BCBL1-RTA cells remained unreactivated or were reactivated with doxycycline hyclate for 24
333 hr. (i) Total RNA was extracted and relative K_v1.3 transcript levels were analysed by qRT-PCR using
334 GAPDH as a reference. Fold change was determined by $\Delta\Delta C_t$ and statistical significance analysed using
335 a non-paired t-test, **** = p<0.0001. (ii) Cell lysates were probed with K_v1.3 and ORF57-specific
336 antibodies and GAPDH used for equal loading.

337 (B-C) Mean current density voltage relationships for K^+ currents (n=5 for all populations, statistical
338 significance *** = $p < 0.001$, **** = $p < 0.0001$) from (B) unreactivated and reactivated TREx BCBL1-RTA
339 at 16 hr; cells were pre-treated for 24 hours with DMSO control or 100 pM ShK-Dap²² and (C) Scramble
340 and $K_v1.3$ -depleted cells lines remained unreactivated or were reactivated with doxycycline hyclate
341 for 24 hr.

342 (D-E) Pooled data highlighting resting membrane potentials in (D) latent and lytic TREx BCBL1-RTA cells
343 or (E) Scramble and $K_v1.3$ -depleted cells lines (statistical significance ** = $p < 0.01$).

344 (F) Membrane polarisation of TREx BCBL1-RTA cells was measured by Flow cytometry after a 5 min
345 incubation with DiBAC4(3) in unreactivated and reactivated control and $K_v1.3$ -depleted TREx BCBL1-
346 RTA cells (n=3, statistical significance * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

347

348 **Figure 3. Ca^{2+} influx is essential during KSHV lytic replication and sufficient to override the effect of**
349 **$K_v1.3$ knockdown.**

350 (A) Fura Red staining of calcium ratios were measured in unreactivated and reactivated control and
351 $K_v1.3$ -depleted TREx BCBL1-RTA cells by Flow cytometry, the calcium ionophore A23187 was used as
352 a positive control (n=3, statistical significance * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

353 (B) TREx BCBL1-RTA cells were pretreated with 1mM EGTA 45 minutes prior to reactivation (i) cells
354 were probed with RTA-specific antibodies and DAPI-stained before imaging on a Zeiss LSM880
355 confocal microscope, (ii) TREx BCBL1-RTA cells remained unreactivated or pre-treated with non-
356 cytotoxic concentrations of 1mM EGTA for 45 minutes prior to reactivation for 24 hr. Cell lysates were
357 probed with ORF65-specific antibodies. GAPDH was used for equal loading.

358 (C-D) Unreactivated and reactivated (C) Control or (D) $K_v1.3$ -depleted cells lines were assessed for
359 levels of lytic replication in the presence of the calcium ionophore A23187, added to cells
360 simultaneously to Dox. Cell lysates were probed with ORF57-specific antibodies and GAPDH used for
361 equal loading.

362 (E) TREx BCBL1-RTA cells were incubated in normal or calcium free media for 24 hours prior to
363 reactivation. Cells were also rescued from calcium free media for 12 hours prior to reactivation. Cell
364 lysates were probed with ORF57-specific antibodies and GAPDH used for equal loading.

365 (F-G) TREx BCBL1-RTA cells remained unreactivated or pre-treated with non-cytotoxic concentrations
366 of (F) Nickel chloride or (G) Thapsigargin for 45 minutes prior to reactivation for 24 hr. Cell lysates
367 were probed with ORF57-specific antibodies. GAPDH was used for equal loading.

368

369 **Figure 4. KSHV-mediated calcium influx initiates NFAT1 nuclear localisation and NFAT1-mediated**
370 **gene expression.**

371 (A) TREx BCBL1-RTA cells remained unreactivated or were pre-treated with non-cytotoxic dose-
372 dependent concentrations of inhibitors (i) CsA and (ii) VIVIT for 45 minutes prior to reactivation with
373 doxycycline hyclate for 24 hr and probed with ORF57-specific antibody, GAPDH was used for equal
374 loading.

375 (B) TREx BCBL1-RTA cells remained unreactivated or were pre-treated with inhibitors and (i) probed
376 with endogenous NFAT1 or ORF57-specific antibodies and DAPI-stained before imaging on a Zeiss
377 LSM880 confocal microscope (ii) Quantification of NFAT1 immunofluorescence using Image J.

378 (C-D) Total RNA was extracted from unreactivated and reactivated (C) TREx BCBL1-RTA cells at 24 hr,
379 or cells pre-treated with ShK-Dap²² and (D) Scrambled control and K_v1.3-depleted cells lines. Relative
380 NFAT-responsive transcript levels were analysed by qRT-PCR using GAPDH as a reference. Fold change
381 was determined by $\Delta\Delta C_t$ and statistical significance analysed using a non-paired t-test, * = p<0.05, **
382 = p<0.01, *** = p<0.001.

383

384 STAR METHODS

385

386 Key Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
GAPDH (60004-1)	Proteintech	60004-1-Ig
ORF57 (207.6)	Santa Cruz	sc-135746
ORF59	Britt Glaunsinger; University of California, Berkeley (gift)	
c-Myc (9E10)	Sigma-Aldrich	M4439-100UL
Kv1.3	Sigma-Aldrich	MFCD02097210
Sp1 (EPR22648-50)	Abcam	ab231778
ORF65	SJ Gao; University of Pittsburgh (gift)	
LANA	Sigma-Aldrich	MABE1109
NFAT1	Abcam	ab244310
Goat anti-mouse HRP	Agilent Technologies	P044701-2
Goat anti-rabbit HRP	Agilent Technologies	P044801-2
Alexa Fluor 488 anti-rabbit	ThermoFisher	A-11008
Alexa Fluor 568 anti-mouse	ThermoFisher	A-11004
Bacterial and virus strains		
BL21(DE3)	Thermo Scientific	C600003
Chemicals, peptides, and recombinant proteins		
2-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma-Aldrich	P1585
4-Aminopyridine (4AP)	Sigma-Aldrich	275875
Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBAC4(3))	ThermoFisher	B438
2-APD	Tocris	1224
BTP-2	Abcam	223499-30-7
Calcium ionophore A23187 (A23187)	Sigma-Aldrich	C7522
Cyclosporin A (CsA)	Generon	AOB5973
DiBAC ₄ (3)	ThermoFisher	B438
Doxycycline hyclate (Dox)	Sigma-Aldrich	D9891-1G
Fura Red	ThermoFisher	F3020
LunaScript RT SuperMix (5X)	New England Biolabs	M3010
Margatoxin (MgTX)	Sigma-Aldrich	M8437
Mithramycin A	Insight Biotechnology	sc-200909
Mibefradil (Mib)	Cambridge Bioscience	15037-5mg-CAY
Nickel Chloride	Sigma-Aldrich	654507
Nifedipine (Nif)	Sigma-Aldrich	N7634
Quinine hydrochloride dihydrate (Qn)	Sigma-Aldrich	Q1125
Puromycin	ThermoFisher	A1113803
SensiMix SYBR green master mix	Bioline	QT650-05
ShK-Dap ²² (ShK)	Bio-Techne	3220/100U
Sodium butyrate	Sigma-Aldrich	B5887
Thapsigargin	ThermoFisher	T7458

Tetraethylammonium chloride (TEA)	Sigma-Aldrich	T2265
TRAM-34 (TRAM)	Sigma-Aldrich	T6700
Vivit	Tocris	3930
Critical commercial assays		
EZ-ChIP	Merck Millipore	17-371
Pierce chromatin prep module	Thermo Scientific	26158
CellTiter 96 AQueous One Solution Cell Proliferation Assay	Promega	
Monarch® Total RNA Miniprep Kit	New England Biolabs	T2010S
Experimental models: Cell lines		
Human: A549	ATCC	CCL-185
Human: BCBL1	ATCC	RRID:CVCL_0165
Human: HEK-293T	ATCC	CRL-3216
Human: iSLK-BAC16	Prof. J. Jung; University of Southern California (gift)	
Human: TReX BCBL1-RTA	Prof. J. Jung; University of Southern California (gift)	
Human: U-87 MG	Prof. J. Ladbury; University of Leeds (gift)	HTB-14
Oligonucleotides		
See Supplementary Table 1 for Oligonucleotides	Integrated DNA Technologies	
Recombinant DNA		
Human Foetal Brain cDNA library	Invitrogen	D883001
pEGFP-N1	Clontech	CB2370178
pLENTI-CMV-Kv1.3-Zeo	This Paper	
pORF57-GFP	Detailed in Ref 49	
pRTA-EGFP	Detailed in Ref 49	
pVSV.G	Dr. E. Chen; University of Westminster (gift)	
psPAX2	Dr. E. Chen; University of Westminster (gift)	
pLKO.1 with KCNA3-targeting hairpin	Dharmacon	TRCN0000044121
Scramble shRNA	Addgene	Addgene plasmid # 1864
Software and algorithms		
ImageJ	https://imagej.nih.gov/ij/	https://imagej.nih.gov/ij/
Graphpad prism	Dotmatics	https://www.graphpad.com/scientific-software/prism/
Rotor-Gene Q	Qiagen	https://www.qiagen.com/gb/resources/
CytExpert Software for the CytoFLEX Platform	Beckman Coulter	https://www.beckman.com/flow-cytometry/research-flow-cytometers/cytoflex/software

Patchmaster	Heka	https://www.heka.com/downloads/downloads_main.html#down_patchmaster
Zen Microscopy software (Black and Blue)	Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html

387

388 **Resource availability**

389 **Lead contact**

390 Further information and requests for resources and reagents should be directed to and will be fulfilled
 391 by the lead contact, Adrian Whitehouse (a.whitehouse@leeds.ac.uk).

392

393 **Materials availability**

394 Plasmids and all unique reagents generated in this study are available from the lead contact with a
 395 completed Materials Transfer Agreement.

396

397 **Experimental model and subject details**

398

399 **Cells**

400 TReX-BCBL-1-RTA cells (kindly provided by Prof. Jae Jung, University of Southern California) are a BCBL-
 401 1-based primary effusion lymphoma (PEL) B cell line engineered to express exogenous Myc-tagged
 402 RTA upon addition of doxycycline, triggering reactivation of the KSHV lytic cycle. BCBL1, A549 and HEK-
 403 293T cell lines were purchased from the American Type Culture Collection (ATCC). U-87 MG cells
 404 (kindly provided by Prof. J. Ladbury, University of Leeds) are a human brain glioblastoma astrocytoma
 405 cell line. iSLK-BAC16 cells (also provided by Prof. Jae Jung, University of Southern California) are a
 406 Caki1-derived renal carcinoma cell line, latently infected with bacterial artificial chromosome 16
 407 (BAC16)-derived KSHV. A549, iSLK, U87 and HEK-293T cells were grown in DMEM (Life Technologies)
 408 supplemented with 10% foetal calf serum (FCS) (Life Technologies) and 1% penicillin/streptomycin
 409 (P/S). TReX BCBL1-RTA and BCBL1 cells were grown in RPMI 1640 medium (Life Technologies)
 410 supplemented with 10% FCS and 1% P/S or calcium free RPMI (Genaxxon Bioscience), TReX BCBL1-RTA
 411 were maintained under hygromycin B (Life Technologies) selection (100 µg/ml). Reactivation into the
 412 lytic cycle was induced using 2 µg/ml doxycycline hyclate, (Sigma) for TReX BCBL1-RTA or with 2 mM
 413 sodium butyrate and 20 ng/ml 2-O-tetradecanoylphorbol-13-acetate (TPA) (both Sigma). All cells were
 414 maintained at 37°C in a humidified incubator with 5% CO₂.

415

416 **Method details**

417

418 **Lentivirus-based shRNA Knockdown and Rescue**

419 Lentiviruses were generated by transfection of HEK-293T cells seeded in 12-well plates using a three-
420 plasmid system⁴⁶. Per 6-well, 4 µl of lipofectamine 2000 (Thermo Scientific) were used together with
421 1 µg of pLKO.1 plasmid expressing shRNA against the protein of interest (Dharmacon), 0.65 µg of
422 pVSV.G, and 0.65 µg psPAX2. pVSV.G and psPAX2 were a gift from Dr. Edwin Chen (University of
423 Westminster, London). Eight hours post-transfection, media was changed with 2 mL of DMEM
424 supplemented with 10% (v/v) FCS. 500,000 TReX BCBL1-RTA cells in 6 well plates were infected by spin
425 inoculation with the filtered viral supernatant for 60 min at 800 x g at room temperature, in the
426 presence of 8 µg/mL of polybrene (Merck Millipore). Virus supernatants were removed 7 h post-spin
427 inoculation and cells were maintained in fresh growth medium for 48 h prior to selection in 3 µg/mL
428 puromycin (Sigma-Aldrich). Stable cell lines were generated after 8 days of selection. All shRNA
429 plasmids were purchased from Dharmacon. Scramble shRNA was a gift from Professor David Sabatini
430 (Addgene #1864). K_v1.3 codon exchange plasmids were generated via inverse PCR mutagenesis
431 utilising a pLENTI-CMV-K_v1.3-ZEO plasmid generated via Gibson Assembly. The mutagenesis process
432 involved exchanging the wobble base of each codon of the 20bp targeted by the shRNA constitutively
433 expressed within the cells. Thus, the resulting K_v1.3 RNA transcripts show resistance to shRNA activity,
434 restoring expression in transfected cells. The plasmids were transfected in to the ΔKv1.3 TReX-BCBL1-
435 RTA cell line following the three-plasmid system described above, with the shRNA-resistant pLENTI-
436 CMV-K_v1.3-ZEO plasmid replacing the pLKO.1 plasmid, and zeomycin used for selection at 250 µg/ml.

437

438 **Transient Transfections**

439 Plasmid transfections were performed using Lipofectamine 2000 (Life Technologies), at a ratio of 2 ug
440 plasmid to 1 ul Lipofectamine in 100 ul opti-MEM. Transfection media was incubated at room
441 temperature for 15 minutes before 1x 10⁶ cells were treated, dropwise. Cells were harvested after 24
442 hours.

443

444 **Immunofluorescence**

445 Cells were cultured overnight on poly-L-lysine (Life Technologies) coated glass coverslips in 24-well
446 plates. Cells were fixed with 4% paraformaldehyde (Calbiochem) for 10 min and permeabilised with
447 0.1% Triton X-100 for 20 min⁴⁷. Cells were blocked in PBS containing 1% BSA for 1 h at 37°C and
448 labelled with primary antibodies for 1 h at 37°C. Cells were washed five times with PBS and labelled
449 with appropriate secondary antibodies for 1 h at 37°C. Cells were washed five times with PBS and

450 mounted in VECTASHIELD containing DAPI (Vector Labs). Images were obtained using a Zeiss LSM880
451 confocal microscope and processed using ZEN 2009 imaging software (Carl Zeiss)⁴⁸.

452

453 **Electrophysiology**

454 TREx BCBL1-RTA cells seeded onto poly-L-lysine (Life Technologies) coated glass coverslips and were
455 transferred to a recording chamber, containing 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 10 mM HEPES-
456 NaOH, pH 7.2, 2 mM CaCl₂, 10 mM glucose, and mounted on the stage of a Nikon Eclipse inverted
457 microscope. Patch pipettes (5–8 MΩ) were filled with a solution consisting of: 140 mM KCl, 5 mM
458 EGTA, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES KOH, pH 7.2, 10 mM glucose. Voltage-clamp recordings
459 were performed using a HEKA EPC-10 integrated patch clamp amplifier controlled by Patchmaster
460 software (HEKA). Series resistance was monitored after breaking into the whole cell configuration. To
461 examine K⁺ currents, a series of depolarizing steps were performed from –100 to +60 mV in 10 mV
462 increments for 100 ms. Resting membrane potential was measured using the current clamp mode of
463 the amplifier. Results are shown as the mean ± SEM of n number of individual cells. Statistical analysis
464 was performed using an unpaired Student's T test. p<0.05 was considered statistically significant.

465

466 **Flow Cytometry**

467 Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBAC₄(3)) and Fura Red (both ThermoFisher) were
468 added to cells at a final concentration of 1 μM in RPMI-media. Cells were incubated at 37°C with Fura
469 Red for 30 min or DiBAC₄(3) for 5 min and washed in PBS. Cells were analysed on a CytoFLEX Flow
470 Cytometer (Beckman). Data were quantified using CytExpert software (Beckman).

471

472 **Proliferation (MTS) assays**

473 Cellular viability was determined using non-radioactive CellTiter 96 AQueous One Solution Cell
474 Proliferation Assay (MTS) reagent (Promega), according to the manufacturer's recommendations^{48,49}.
475 TREx BCBL1-RTA cells (~20,000) were seeded in triplicate in a flat 96-well tissue culture plates
476 (Corning) and treated with the indicated inhibitors for 24 h. CellTiter 96 AQueous One Solution
477 Reagent was added to the cells for 1 h at 5% CO₂, 37°C. Absorbances were measured at 490 nm using
478 an Infinite plate reader (Tecan).

479

480 **Two-step quantitative reverse transcription PCR (qRT-PCR)**

481 Total RNA was extracted using the Monarch[®] Total RNA Miniprep Kit (New England Biolabs) as per the
482 manufacturer's protocol. RNA (1 μg) was diluted in a total volume of 16 μl nuclease-free water, and 4
483 μl LunaScript RT SuperMix (5X) (New England Biolabs) was added to each sample. Reverse

484 transcription was performed using the protocol provided by the manufacturer. cDNA was stored at -
485 20°C, RNA was stored at -80°C. Quantitative PCR (qPCR) reactions (20 µl) included 1X SensiMix SYBR
486 green master mix (Bioline), 0.5 µM of each primer and 5 µl template cDNA (used at 1:200 dilution in
487 RNase-free water). Cycling was performed in a RotorGene Q instrument (Qiagen) ⁴⁸. The cycling
488 programme was a 10 min initial preincubation at 95°C, followed by 40 cycles of 95°C for 15 sec, 60°C
489 for 30 sec and 72°C for 20 sec. After qPCR, a melting curve analysis was performed between 65°C and
490 95°C (with 0.2°C increments) to confirm amplification of a single product. To assess primer
491 amplification efficiency (AE), for each gene of interest a standard curve was constructed using a pool
492 of cDNA derived from unreactivated and reactivated cells. At least four different dilutions of pool
493 cDNA were quantified to generate a standard curve. The slope of the standard curve was used to
494 calculate the AE of the primers using the formula: $AE = (10^{-1/\text{slope}})$. For gene expression analysis all
495 genes of interest were normalised against the housekeeping gene GAPDH (ΔCT) ⁵⁰. A summary of all
496 the primers used in this study is provided in Supplementary Table 1.

497

498 **Chromatin immunoprecipitation (ChIP)**

499 Formaldehyde-crosslinked chromatin was prepared using the Pierce Chromatin Prep Module (Thermo
500 Scientific) following the manufacturer's protocol. Cells (2×10^6) were digested with six units of
501 micrococcal nuclease (MNase) per 100 µl of MNase Digestion buffer in a 37°C water bath for 15 min.
502 These conditions resulted in optimal sheared chromatin with most fragments ranging from 150–300
503 base pairs in size. Immunoprecipitations were performed using EZ-ChIP kit (Millipore) kits overnight
504 at 4°C and contained 50 µl of digested chromatin (2×10^6 cells), 450 µl of ChIP dilution buffer and 1.5
505 µg of RNAPII antibody (clone CTD4H8) (Millipore) or isotype antibody, normal mouse IgG (Millipore).
506 qPCR reactions were performed using either 2 µl of immunoprecipitated DNA or 2 µl of input DNA as
507 template ⁵¹.

508

509 **Immunoblotting**

510 Protein samples were separated on SDS-PAGE gels and transferred to nitrocellulose membranes
511 (Amersham) via semi-dry transfer using a Trans-Blot® Turbo™ blotter (BioRad). Membranes were
512 blocked in TBS + 0.1% Tween 20 and 5% dried skimmed milk powder and probed with relevant primary
513 antibodies followed by horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse and
514 polyclonal goat anti-rabbit secondary antibodies (Dako). Membranes were treated with EZ-ECL
515 (Geneflow) and imaged using a G-Box (Syngene).

516

517 **Quantification and statistical analysis**

518 Statistical analysis as specified in figure legends were performed with Prism 9 (GraphPad software Inc.,
519 San Diego, California, United States). Graphical data shown represent mean \pm standard deviation of
520 mean (SD) using three or more biologically independent experiments. Differences between means
521 was analysed by unpaired Student's t test, or distribution with two sample Kolmogorov-Smirnov test
522 as detailed in the figure legends. Statistics was considered significant at $p < 0.05$, with * $P < 0.05$,
523 ** $P < 0.01$, *** $P < 0.001$.

524

525 **Data and code availability**

526 All data reported in this paper will be shared by the lead contact upon request. This paper does not
527 report original code. Any additional information required to reanalyze the data reported in this paper
528 is available from the lead contact upon request.

529

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