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Abstract

Aims. Protein kinases are potential therapeutic targets for heart failure, but most studies of cardiac protein kinases derive from other systems, an approach that fails to account for specific kinases expressed in the heart and the contractile cardiomyocytes. We aimed to define the cardiomyocyte kinome (i.e. the protein kinases expressed in cardiomyocytes) and identify kinases with altered expression in human failing hearts. **Methods and Results.** Expression profiling (Affymetrix microarrays) detected >400 protein kinase mRNAs in rat neonatal ventricular myocytes (NVMs) and/or adult ventricular myocytes (AVMs), 32 and 93 of which were significantly upregulated or downregulated (>2-fold), respectively, in AVMs. Data for AGC family members were validated by qPCR. Proteomics analysis identified >180 cardiomyocyte protein kinases, with high relative expression of mitogen-activated protein kinase cascades and other known cardiomyocyte kinases (e.g. CAMKs, cAMP-dependent protein kinase). Other kinases are poorly-investigated (e.g. Slk, Stk24, Oxsr1). Expression of Akt1/2/3, BRaf, ERK1/2, Map2k1, Map3k8, Map4k4, MST1/3, p38-MAPK, PKCδ, Pkn2, Ripk1/2, Tnni3k and Zak was confirmed by immunoblotting. Relative to total protein, Map3k8 and Tnni3k were upregulated in AVMs *vs* NVMs. Microarray data for human hearts demonstrated variation in kinome expression that may influence responses to kinase inhibitor therapies. Furthermore, some kinases were upregulated (e.g. NRK, JAK2, STK38L) or downregulated (e.g. MAP2K1, IRAK1, STK40) in human failing hearts. **Conclusions.** This characterization of the spectrum of kinases expressed in cardiomyocytes and the heart (cardiomyocyte and cardiac kinomes) identified novel kinases, some of which are differentially expressed in failing human hearts and could serve as potential therapeutic targets.

Key words: protein kinases, heart, cardiac myocytes, postnatal development, human heart failure

Abbreviations: AMPK, AMP-activated protein kinase; AVMs, adult ventricular myocytes; BSA, bovine serum albumin; CAMK, calcium/calmodulin-dependent protein kinase; DCM, dialted cardiomyopathy; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HF, heart failure; IHF, ischaemic heart failure; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MS, mass spectrometry; NVMs, neonatal ventricular myocytes; PKC, protein kinase C.

1. Introduction

Protein kinases regulate many aspects of cell function and represent one of the largest supergene families. The kinome concept was crystallised in 2002 with the report of the human kinome (the full protein kinase complement of the genome),[1] followed by the mouse kinome.[2] There are >500 potential protein kinases in the mammalian genome classified into 8 superfamilies (AGC, Atypical, CAMK, CK1, CMGC, STE, TK, TKL) according to homology within their catalytic domains. Additional kinases (classified as "other") have greater variability. Some kinome members are pseudokinases lacking one or more critical features required for a fully active enzyme, but these are increasingly recognised as important regulators of protein kinase signalling.[3]

Protein kinases are potential therapeutic targets for heart failure (HF).[4-8] However, protein kinase regulation and function are usually studied in proliferating cells in relation to cancer for which they are attractive therapeutic targets.[9] This is delivering an increasing range of protein kinase inhibitors as cancer therapies, but kinases that promote cancer are often required for cardiac function and at least some of these kinase inhibitors have cardiotoxic effects in a significant percentage of patients.[10-12] The emphasis on protein kinases in non-cardiac, cancerous cells also overlooks selective expression and specific roles of protein kinases in the heart, particularly in the highly-specialised contractile cardiomyocytes.

Mammalian cardiomyocytes withdraw from the cell cycle in the perinatal period. Postnatally, as the animal grows, terminally-differentiated cardiomyocytes enlarge and increase their contractile apparatus, a process associated with changes in gene expression.[13] Adult cardiomyocytes hypertrophy (increase in size without cell division) in response to an increased workload. This may be beneficial and reversible (as in pregnancy or endurance exercise) but, in pathological conditions (e.g. hypertension or following myocardial infarction in which surviving cardiomyocytes hypertrophy to maintain cardiac output in the face of cardiomyocyte loss) it may become deleterious and lead to HF. Protein kinase signalling plays a significant role in regulating these events,[5-7] but the relative importance of individual kinases is not clear and many highly-expressed cardiomyocyte kinases remain unstudied in this context. Here, we addressed the question of which protein kinases are expressed in cardiomyocytes. We assessed the changes during postnatal development, comparing profiles in rat neonatal ventricular cardiomyocytes (NVMs) with adult ventricular cardiomyocytes (AVMs). We also examined RNA expression profiles of human myocardial biopsies, identifying changes in kinase mRNA expression associated with HF.

2. Methods

2.1 Cardiomyocyte and neonatal non-cardiomyocyte preparation

Sprague-Dawley female rats with 2 day litters (Harlan SeraLab Ltd. UK) were housed in the Imperial College Central Biomedical Services or the University of Reading facility with water and food *ad libitum*. The facilities are UK registered with Home Office certificates of designation. All procedures in these facilities were performed in accordance with UK regulations. Sprague-Dawley male rats (300-350 g) were from Harlan SeraLab Ltd. UK or Charles River Laboratories Canada Inc. and were housed in the University of Reading or Montreal Heart Institute facility with water and food *ad libitum*. Work with adult male rats was undertaken in accordance with local institutional animal care committee procedures and either the U.K. Animals (Scientific Procedures) Act 1986 or the Canadian Council on Animal Care. Rat NVMs and non-myocytes were prepared and cultured from Sprague-Dawley rats as previously described[14] and detailed in the Supplemental Methods. Rat AVMs were prepared from male Sprague Dawley rats (300-350 g) as previously described.[15,16] AVMs were collected under gravity to give negligible contamination with non-myocytes, then

washed in warmed PBS (37°C, 3 × 5 ml) with collection by centrifugation (5 min, 60×g, 20°C). Further details are in the Supplemental Methods.

2.2 RNA preparation, microarray analysis and qPCR

Total RNA was prepared from NVMs and AVMs as described in the Supplemental Methods. cRNA was prepared as previously described.[14] Fragmentation of antisense cRNA and hybridization to Affymetrix rat genome 230 2.0 arrays was performed at the CSC/IC Microarray Centre (Imperial College London) according to the manufacturer's instructions. Data were exported to ArrayExpress (ArrayExpress ID: E-MTAB-2832). qPCR was performed as described in the Supplemental Methods with specific primers (Supplemental Table 1). Values for selected mRNAs were normalized to Gapdh expression. Samples for microarray analysis were prepared from 3 independent cardiomyocyte preparations. Samples for qPCR were from at least 3 independent cardiomyocyte preparations and were not those used for microarray analysis.

Microarray data (.CEL files) were analysed using GeneSpring (Agilent Technologies) 12.6.1 (cardiomyocytes) or 13 (human microarray data), using the PLIER16 algorithm with normalisation per gene to the gene median. To identify changes in kinase mRNA expression in human failing hearts, datasets were downloaded from ArrayExpress (E-GEOD-57338,[17] E-GEOD-29819,[18] E-GEOD-26887,[19] E-GEOD-21610,[20] E-GEOD 1145, E-GEOD-5406[21]). The numbers of patients studied are provided in Supplemental Table 2. Full details of microarray data analysis are provided in the Supplemental Methods.

2.3 Analysis of the kinase proteome

NVMs and AVMs were prepared as described in the Supplemental Methods. Two independent cardiomyocyte samples (NVM samples were prepared from 15 rat hearts for each preparation; AVM samples were from a single heart each) were shipped to KiNative™ for kinase profiling as described in [22,23] and described in full in the Supplemental Methods.

2.4 Western blotting

Recombinant human MKK1 (MAP2K1) and p38-MAPKα (MAPK14) were expressed as glutathione S-transferase (GST) fusion proteins and prepared as previously described.[24-26] Other recombinant human GST-fusion proteins were obtained commercially (AKT1, R & D Systems, 1775-KS-010; RIPK1, Abnova, H00008737-P01; PKN2 Life Technologies Ltd., PV3879). Concentrations of recombinant proteins were determined relative to BSA standards on Coomassie Brilliant Blue-stained gels. Cardiomyocyte samples were prepared and immunoblotting performed as previously described,[14] with additional details in the Supplemental Methods. Details of antibodies are in Supplemental Table 3. Samples for immunoblotting were from at least 3 independent cardiomyocyte preparations and were not those used for proteomics.

2.5 Immunostaining

NVMs were immunostained for troponin T as described in [14] and myofilamentous actin was counterstained with Texas Red®-X phalloidin as described in [27]. Full details are provided in the Supplemental Methods. Coverslips were mounted using fluorescence mounting medium (Dako) and viewed with a Zeiss Axioskop fluorescence microscope using a 40× objective. Digital images captured using a Canon PowerShot G3 camera were reduced in size and superimposed using Adobe Photoshop 7.0.

3. Results

3.1 mRNA expression profiling of protein kinases and pseudokinases in neonatal and adult rat cardiomyocytes

mRNA expression profiles for Sprague-Dawley rat NVMs (2-4 d, as cardiomyocytes exit the cell cycle[28]) and AVMs were compared using Affymetrix rat genome 230 2.0 microarrays.

Hierarchical clustering of samples segregated AVMs from NVMs (Supplemental Figure 1A) with differential expression (>2-fold, false discovery rate p<0.05) of 4720 mRNAs (Supplemental Spreadsheet 1). As expected,[28] expression of cell cycle genes declined in AVMs relative to NVMs, together with Orc2-6 and Mcm2-7, genes critical for DNA replication (Supplemental Figure 1, B and C). We also detected the expected isoform switching of mRNAs for α- and β-myosin heavy chains and thin filament proteins, with downregulation of atrial natriuretic factor (Supplemental Figure 1D).[29,30] NVM cultures inevitably contain some non-myocytes. By immunostaining cardiomyocytes for troponin T and counterstaining all cells with phalloidin, we estimated the number of non-myocytes as ~5% (Supplemental Figure 2, A-F). Orc1-6 mRNAs were significantly lower in NVMs compared with neonatal cardiac non-myocytes, whilst non-myocytes had negligible expression of myocyte-specific genes (Supplemental Figure 2, G and H). Because RNA was prepared from freshly isolated AVMs collected under gravity, these cells have negligible non-cardiomyocyte content. Thus, the microarray gene expression profiles are essentially those of cardiomyocytes.

We identified microarray probesets for 438 protein kinases, 408 of which were detected in NVMs and/or AVMs (Figure 1A; Supplemental Spreadsheet 2). Thirty-two and 93 were upregulated or downregulated (>2-fold; p<0.05), respectively, in AVMs relative to NVMs, and these were distributed between protein kinase families (Figure 1, B and C). Some changes (Figure 2, A and B) were expected (e.g. upregulation of Pdk1/2/4[31] and downregulation of Cdk1/4[28] in AVMs) or predicted (e.g. upregulation of Ttn in AVMs). However, other kinases substantially upregulated in AVMs have not been well-studied in cardiomyocytes (e.g. Adck3, Hipk2). We selected 20 AGC kinases for validation by qPCR. mRNA expression ratios for AVMs:NVMs for the two methods were within 2-fold for 15 kinases (Figure 2C). Both methods confirmed downregulation in AVMs *vs* NVMs for protein kinase C δ (PKCδ; Prkcd) and Cdc42bpb, although qPCR revealed a greater degree of downregulation. The data for PKCδ are consistent with previous work showing that the protein is downregulated in AVMs.[32] Mast3 was the only anomaly with an AVM:NVM ratio of 1.0 using microarrays and 0.25 with qPCR. This may reflect the 5% analysis error, or result from expression of alternatively-spliced transcripts, since many kinases (including MAST3 in humans) are alternatively spliced.[33] To confirm that the detection threshold was appropriate, we compared Ct values. Consistent with microarray data, Ct values for Sgk1, Mast2, Mast3 and Mast4 (21.8-25.0) were substantially lower than Sgk2 and Mast1 (33.0; considered undetectable) (Figure 2D).

3.2 Protein expression of cardiomyocyte protein kinases

There are several global proteomics studies of rodent and human hearts from which data for expressed protein kinases can be mined (e.g. [34-38]). However, cardiomyocytes constitute ~70% of heart volume, but only ~30% the cell number.[39] To identify highly expressed cardiomyocyte kinases, we used ActivX ATP probes for affinity purification of protein kinases in cardiomyocytes prior to identification/quantification by mass-spectrometry (MS) using KiNativ™.[40] This affinity purification approach concentrates ATP-binding proteins, simplifying the MS spectra for analysis and facilitating protein kinase identification. Nevertheless, the spectra remain complex and 321 protein and lipid kinases were targeted for identification (Supplemental Spreadsheet 3; Supplemental Tables 4-7). We detected over 180 protein kinases [some isoforms (e.g. JNK1/2/3) could not be distinguished because discriminating peptide sequences were not obtained] and 12 lipid kinases in cardiomyocytes (Supplemental Spreadsheet 3).

The most highly represented pathway was the extracellular signal-regulated kinase (ERK) 1/2 cascade (Figure 3; Supplemental Spreadsheet 3). c-Jun N-terminal kinase (JNK) and p38-MAPK pathways were also highly-expressed and other established cardiomyocyte protein kinases were detected [e.g. calcium/calmodulin-dependent protein kinases (CAMKs),[41] AMP-activated protein kinase (AMPK)[42], cAMP-dependent protein kinase (PKA)[43]]. Downstream components of the Akt pathway[44] were detected, but not Akt isoforms themselves. Akt1 was screened for (Supplemental Table 4), but the peptide selected may

be phosphorylated,[45,46] potentially causing complications. Several cardiomyocyte protein kinases identified are poorly-investigated in the heart including STE20 kinases [Slk, Stk24 (MST3), Oxsr1], MLK kinases (Zak, Ilk), and others [e.g. BRaf, Stk38/38l (NDR1/2), Cdk5]. We previously studied MST3 and NDR1/2 proteins in NVMs,[47,48] and it is reasonable to expect that other kinases should be detectable at the protein level. Indeed, Zak and Map4k4 were detected in cardiomyocytes by immunoblotting, with Zak being expressed predominantly as the smaller isoform (52 kDa cf. 92 kDa)[49] and multiple isoforms of Map4k4[50] (Supplemental Figure 3, A and B). Many kinases detected with microarrays were not detected by proteomics. Some (e.g. PKCα/ε[32]) are expressed in cardiomyocytes and, for these, expression levels are clearly below the level of detection by proteomics. For kinases not detected by proteomics, Supplemental Tables 4 and 5 provide references for proteins detected in cardiomyocyte or heart extracts, Supplemental Table 6 provides references for mRNAs detected in heart and Supplemental Table 7 lists kinases that remain to be studied in cardiomyocytes.

Proteomics data were validated by immunoblotting. Quantitative immunoblotting [with glutathione S-transferase (GST) fusion proteins as standards] was used for MKK1/2 (a highly represented kinase detected by proteomics), p38-MAPKs (less abundant) and Pkn2 (low relative levels), in addition to Akt1/2/3 (not detected by proteomics) and Ripk1 (not studied by proteomics) (Supplemental Figure 4). It should be noted that GST increases the relative molecular mass and, because smaller proteins are transferred more efficiently, concentrations of endogenous proteins may be overestimated. Furthermore, the antibodies used cannot distinguish between MKK1/MKK2, p38-MAPKs and Akt1/Akt2/Akt3. MKK1/2 were detected at 9.8- and 6.3-fold higher levels than p38-MAPKs in NVMs and AVMs, respectively (~7.0 and ~7.8-fold higher in the proteomics study), with substantially lower levels of Pkn2 (Figure 4, A and B). Akt1/2/3 were detected at similar levels to p38-MAPKs, whilst Ripk1 was more highly expressed. For all, expression was lower in AVMs than NVMs, but with a smaller relative decrease for Ripk1 (~2.1-fold).

To determine if there is differential expression in cardiomyocytes relative to cardiac non-myocytes, we compared selected protein kinases in neonatal or adult rat hearts with NVMs or AVMs, respectively. In neonates, MKK1/2, Akt1/2/3, ERK1/2, MST1 and Pkn2 expression was similar in hearts or NVMs, whilst p38-MAPKs and MST3 were more highly expressed in hearts, and Ripk1 and Pkn2 were enriched in NVMs (Figure 4C, Supplemental Figure 5). In adults, Ripk1 was similarly expressed in AVMs and adult hearts, and Pkn2 remained enriched in AVMs, but other kinases were expressed at higher levels in whole hearts (Figure 4D, Supplemental Figure 5). Thus, relative levels of expression of different protein kinases in cardiomyocytes and non-myocytes vary during postnatal development with some protein kinases (e.g. Pkn2) remaining more highly enriched in cardiomyocytes.

The proteomics data cannot compare levels of expression in NVMs with AVMs on a per cell basis because of the increase in size during postnatal development (membrane capacitance, an index of cell size, increases from 13 pF in 1-2 d NVMs to 156 pF in AVMs[51,52]) with increased expression of, for example, contractile proteins. Nevertheless, two kinases, Map3k8 and Tnni3k, were considerably more highly expressed in AVMs than NVMs (Supplemental Figure 3C) and such kinases are likely to play a particularly important role in the adult state. Most protein kinases were more highly expressed in NVMs (Akt1/2/3, BRaf, ERK1/2, MAP4K4, MKK1/2, MST1, MST3, p38-MAPK, PKCδ, Pkn2, Ripk1, Ripk2, Zak; Supplemental Figure 3D). However, because of the increase in cell size, at least some are probably expressed at similar or higher levels in AVMs on a per cell basis (as for PKCα and PKCε[32]).

3.3 Protein kinase mRNAs in human failing hearts

To explore variability of protein kinase expression in human hearts and determine whether expression profiles change in heart failure (HF), we mined Affymetrix microarray data from the ArrayExpress database (Supplemental Table 2). Initially, we used E-GEOD-57338 with data for samples from non-failing (NF) male (n=63) and female (n=73) left ventricles,[17] and detected 402 protein kinase mRNAs (Supplemental Spreadsheet 4). Only ADCK3 was

differentially expressed in male *vs* female hearts (>1.2-fold; p<0.05). There was variation in expression between patients, although some protein kinase mRNAs showed much greater variation than others (Figure 5). We compared data for male NF samples with samples from male patients with dilated cardiomyopathy (DCM; n=63) or ischaemic heart failure (IHF; n=81), and female NF samples with samples from female patients with DCM (n=19) or IHF (n=14) (i.e. 4 groups), identifying kinases that were significantly different in the failing hearts (>1.25-fold, p<0.05). Candidate HF kinases were selected if significantly changed in 3 (15 kinases) or 4 (16 kinases) of these groups (Supplemental Spreadsheet 5). We then interrogated other, smaller datasets for expression of these kinases (Supplemental Spreadsheets 6-10). Of the candidate HF kinases, mRNAs for NRK (but not the related kinase MAP4K4), JAK2 (but not JAK1), EPHA3, STK38L and KIT were significantly upregulated in HF samples relative to NF controls in all datasets studied, regardless of aetiology, with upregulation of NTRK2, ADRBK2 and MAPK10 in all but one of the datasets (Figure 6). Furthermore, mRNAs for MAP2K1 and IRAK1 (but not related kinases MAP2K2 or IRAK4) were downregulated in all HF samples relative to NF controls, with downregulation of MAP2K3, MAP3K3, TESK1, PIM1 and STK40 in at least 3 of the additional 5 datasets studied (Figure 7). It remains to be established whether the changes contribute to or are a consequence of the HF phenotype.

4. Discussion

As enzymes with substrate binding sites and active sites for catalysis, protein kinases are ideal targets for small molecule therapies, features being exploited in cancer therapeutics. Protein kinases play an important role in the development of heart failure, and many key protein kinases in heart and in cardiomyocytes have been studied, with much emphasis on their potential as therapeutic targets.[4-8] However, there has not been a systematic analysis of protein kinases that regulate cardiomyocyte and cardiac function. To start to address this, we present here the first global analysis of the rat cardiomyocyte kinome and human cardiac kinome.

Our first approach used microarrays for mRNA expression profiling of rat cardiomyocytes. We identified 408 protein kinases with detectable expression in NVMs and/or AVMs, most of which did not change substantially during postnatal development (Figure 1; Supplemental Spreadsheet 2). This is a higher proportion of the total kinome than might be anticipated but, given the general importance of protein kinases in cellular functions, perhaps not entirely unexpected. The relative levels of expression of the kinases is important and, whilst we can gauge whether or not a kinase is likely to be expressed at high or low levels from microarray data (e.g. Ttn, Pink1 and Mylk3, with raw fluorescence values >1000 in AVMs, are probably highly expressed compared with Akt3 and Cdk1, with raw fluorescence values ~100), protein expression levels are more relevant than transcript levels.

mRNA expression profiling remains far more sensitive than global proteomics profiling approaches. One reason is that global proteomics systems favour detection of abundant proteins, and protein kinases (as regulatory enzymes rather than functional components of, for example, the cytoskeleton or mitochondria) are not necessarily abundant. Thus, even when the system is simplified by analysis of subcellular fractions or individual organelles (as in [37,38]), previous studies of heart samples reported only limited numbers of protein kinases (see, for example, [34-38]). There are fewer proteomics studies of protein kinases in cardiomyocytes and these are usually highly focused. For example, proteomics has been used to study the PKCε interactome, identifying 12 kinases.[53] With >400 kinases detected at the mRNA level, we needed a different approach to confirm protein expression of as many kinases as possible. To increase the profiling capability for protein kinases at the protein level, we used ActivX ATP probes for affinity purification of protein kinases in AVMs and NVMs prior to identification and quantification by MS. As with any technique, this system is not perfect and, although >400 protein and lipid kinases can be identified, not all

are detected in the screen including some (Ttn and Pink1) that are highly expressed at the mRNA level in cardiomyocytes (Figure 2A; Supplemental Spreadsheet 2). Additionally, because of the complexity of the MS data, it was also still necessary to limit the screen to 321 kinases. Nevertheless, we detected over 180 protein kinases in NVMs and/or AVMs, some of which have not been studied previously in relation to cardiac disorders.

For the kinases detected by proteomics, we could clearly establish that they are expressed as proteins and gain some insight into the relative levels of expression. We validated our proteomics data by immunoblotting and extended the data to some kinases that were not studied using proteomics (Akt, Ripk1). Immunoblotting also has limitations, most particularly in the availability of specific and sensitive antibodies. For this study, we screened a number of different antibodies to different kinases, many of which could not be used because of a lack of specificity and/or insufficient sensitivity. However, subject to antibody availability, immunoblotting is undoubtedly very powerful for studies of individual protein kinases, allowing comparison in different cells (e.g. AVMs *vs* NVMs), tissues (e.g. cardiomyocytes vs whole hearts) and, though not undertaken here, subcellular compartmentalisation. Further information can be gained relating to isoform expression as with Zak and Map4k4 (Supplemental Figure 3), although sometimes antibodies cannot distinguish between isoforms (e.g. Akt1/2/3). For the remaining kinases that were studied but not detected by proteomics, this could be because expression was below the level of detection. For some, this is clearly the case given that they have been detected and studied in cardiomyocytes previously (Supplemental Table 4). Some have been detected in heart extracts (Supplemental Table 5), whilst others remain to be investigated at the protein level (Supplemental Tables 6 and 7). For the remaining kinases not studied by proteomics, further studies are clearly required although many have already been shown to be expressed in cardiomyocytes and/or heart and have been actively investigated (Supplemental Tables 4 and 5).

Our cardiomyocyte kinome data highlight protein kinases that are expressed and may be therapeutic targets for cardiac disorders and other diseases. From the cancer therapies already in clinical use, there is clear variation in the cardiac responsiveness to protein kinase inhibitors with, for example, cardiac dysfunction in up to 9.4% of patients treated with Herceptin[10,11] and ~7% of patients treated with the MKK1/2 inhibitor trametinib.[12] Our data demonstrate differential expression of the cardiac kinome between patients (Figure 5; Supplemental Spreadsheet 4) that may influence the degree to which they respond to therapeutic administration of kinase inhibitors. The human cardiac kinome data also provide an indication of potential changes in protein kinase expression in failing hearts (Figures 6 and 7; Supplemental Spreadsheets 5-10). The approach we used was facilitated by publication of microarray data from a large cohort of 313 patients (E-GEOD-57338[17]) giving us the opportunity to compare male and female hearts, in addition to NF and failing hearts. None of the other datasets available had sufficient numbers, particularly of NF samples, to initiate the study. Furthermore, in several cases, NF samples were biased towards females whilst the HF samples were biased towards males. With E-GEOD-57338, we could identify changes common to male *vs* female DCM and IHF. E-GEOD-57338 also allowed us to establish that there was little difference in protein kinase mRNA expression between males and females, allowing us to interrogate the other datasets. The consistency of many of the changes between 5 different datasets from different investigators using patient cohorts with different aetiologies was highly notable, strongly suggesting that the changes we identified are common features of HF. Clearly, the changes in mRNA expression remain to be validated at the protein level, whether the changes are cause or consequence remains to be determined and the effects of the changes remains to be established.

In summary, we present the first study of the rat cardiomyocyte and human cardiac kinomes. The data highlight the importance of many well-characterised protein kinase pathways in the heart, and establish the potential importance of novel kinases for further study. The latter represent potential novel, therapeutic targets for HF. Understanding their input into the cardiomyocyte signalling network and their role in cardiomyocyte function will also be essential for "fine-tuning" current therapeutic approaches for HF.

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FIGURE LEGENDS

Figure 1. Expression profiling of protein kinase mRNAs in rat adult ventricular myocytes (AVMs) and neonatal ventricular myocytes (NVMs). mRNA expression profiling for AVMs or NVMs (n=3, duplicate hybridisations) used Affymetrix microarrays. A, Heatmap for relative expression of all protein kinases/pseudokinases expressed in AVMs and/or NVMs. Data were normalised to the gene median and are mean values on a $\log_2$ scale. B, Numbers of each protein kinase superfamily detected in AVMs and/or NVMs that were similarly expressed (within 2-fold; black), more highly expressed in AVMs (blue) or more highly expressed in NVMs (green). C, Differentially expressed protein kinase mRNAs grouped according to protein kinase family. Data were normalised to the gene median and are mean values for AVMs (cyan) or NVMs (yellow).

Figure 2. A and B, Protein kinase mRNAs subject to postnatal developmental regulation in cardiomyocytes. Differentially expressed protein kinase transcripts were identified from the microarray data. Expression ratios are shown for the 30 protein kinases with the highest relative level of expression in AVMs (A) or NVMs (B). C and D, Validation of microarray data (solid bars, mean values) using qPCR (open bars, means ± SEM, n=3). Expression ratios (AVMs/NVMs) (C) or Ct values (D) are shown.

Figure 3. Protein expression of cardiomyocyte protein kinases. Protein kinases in NVMs (A) or AVMs (B) were affinity purified, then identified and quantified by mass-spectrometry (MS). Expression of each kinase was calculated as the percentage of the MS signal for that kinase relative to the total MS signal for all kinases detected. The area of each circle in the diagram is proportional to the percentage value of the kinase relative to the total pool of kinases detected. Data are shown for the principal MAPK cascades, other important cardiomyocyte kinases, components of the Akt pathway (pink, although p70S6k and Gsk3 are also phosphorylated by ERK1/2 and RSKs, respectively) and other kinases within the 30 most highly represented kinases. Relatively under-investigated kinases in cardiomyocytes are highlighted in dark blue. Common names are in capitals; otherwise gene symbols are used.

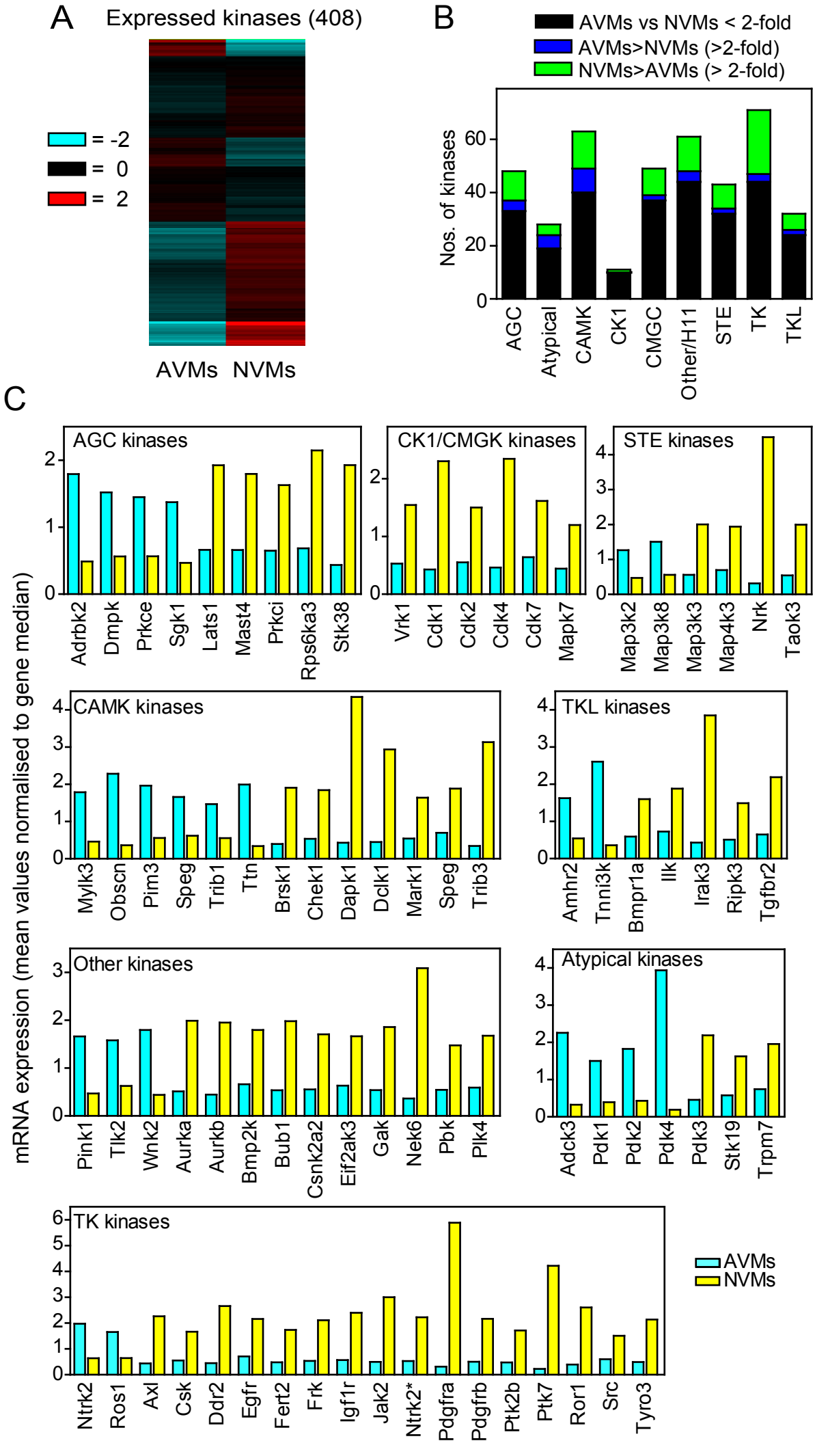
Figure 4. Analysis of protein kinase expression by immunoblotting. A, Quantitative immunoblotting of MKK1/2, p38-MAPK, Pkn2, Akt1/2/3 and Ripk1 in NVMs (solid bars) and AVMs (open bars) relative to GST fusion protein standards (means ± SEM, n=3). Primary data are in Supplemental Figure 4. Concentrations are in fmol/µg protein. B, Proteomics data for MKK1/2, p38-MAPK and Pkn2 (mean values, n=2). C and D, Immunoblot analysis comparing expression of selected kinases in neonatal (C) or adult (D) cardiomyocyte and whole heart extracts (means ± SEM, n=3/4). Primary data are in Supplemental Figure 5.

Figure 5. Protein kinase mRNA expression in non-failing human heart. Affymetrix Rat 1.1 ST Gene microarray data (E-GEOD-57338) from male (n=73) or female (n=63) hearts were mined for protein kinases. A, Heatmap showing hierarchical clustering of all detected protein kinases and patients. The bar above indicates male (blue) and female (red) samples. B-D, Detail of the variation of specific kinases. Data were normalised to the gene median and are mean values on a $\log_2$ scale. Heatmaps are from -2 (cyan) through 0 (black) to +2 (red).

Figure 6. Protein kinase mRNAs upregulated in human heart failure. Analysis of female and male left ventricular samples from E-GEOD-57338 (57338F and 57338M) comparing microarray data for non-failing (black bars) with dilated cardiomyopathy (DCM, grey bars) or ischaemic heart failure (IHF, white bars) identified candidate protein kinase markers of heart failure, with upregulation of NRK (A) not MAP4K4 (B), JAK2 (C) not JAK1 (D), and upregulation of EPHA3, (E) NTRK2 (F), STK38L (G), ADRBK2 (H), KIT (I) and MAPK10 (J). Other datasets were interrogated for expression of these kinases: E-GEOD-26887 [non-

diabetic heart failure (grey), diabetic heart failure (white)]; E-GEOD-29819 [DCM (grey), arrhythmogenic right ventricular hypertrophy (white)]; E-GEOD-21610, E-GEOD-1145 and E-GEOD-5406 [DCM (grey), IHF (white)]. NRK and EPHA3 were not represented on the microarrays used in E-GEOD-5406.

Figure 7. Identification of protein kinase mRNAs downregulated in human heart failure. Analysis of female and male left ventricular samples from E-GEOD-57338 (57338F and 57338M) comparing microarray data for non-failing (black bars) with dilated cardiomyopathy (DCM, grey bars) or ischaemic heart failure (IHF, white bars) identified candidate protein kinase markers of heart failure, with downregulation of MAP2K1 (A) not MAP2K2 (B), IRAK1 (C) not IRAK4 (D), and downregulation of MAP2K3, (E) RPS6KA2 (F), MAP3K6 (G), TESK1 (H), PIM1 (I) and STK40 (J). Other datasets were interrogated for expression of these kinases: E-GEOD-26887 [non-diabetic heart failure (grey), diabetic heart failure (white)]; E-GEOD-29819 [DCM (grey), arrhythmogenic right ventricular hypertrophy (white)]; E-GEOD-21610, E-GEOD-1145 and E-GEOD-5406 [DCM (grey), IHF (white)]. STK40 was not represented on the microarrays used in E-GEOD-5406.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Expressed kinases (408)
= -2
= 0
= 2
AVMs
NVMs
B
AVMs vs NVMs < 2-fold
AVMs>NVMs (>2-fold)
NVMs>AVMs (> 2-fold)
Nos. of kinases
AGC
Atypical
CAMK
CK1
CMGC
Other/H11
STE
TK
TKL
C
mRNA expression (mean values normalised to gene median)
AGC kinases
Adrbk2
Dmpk
Prkce
Sgk1
Lats1
Mast4
Prkci
Rps6ka3
Stk38
CK1/CMGK kinases
Vrk1
Cdk1
Cdk2
Cdk4
Cdk7
Mapk7
STE kinases
Map3k2
Map3k8
Map3k3
Map4k3
Nrk
Taok3
CAMK kinases
Mylk3
Obscn
Pim3
Speg
Trib1
Ttn
Brsk1
Chek1
Dapk1
Dclk1
Mark1
Speg
Trib3
TKL kinases
Amhr2
Tnni3k
Bmpr1a
Ilk
Irak3
Ripk3
Tgfbr2
Other kinases
Pink1
Tlk2
Wnk2
Aurka
Aurkb
Bmp2k
Bub1
Csnk2a2
Eif2ak3
Gak
Nek6
Pbk
Plk4
Atypical kinases
Adck3
Pdk1
Pdk2
Pdk4
Pdk3
Stk19
Trpm7
TK kinases
Ntrk2
Ros1
Axl
Csk
Ddr2
Egfr
Fert2
Frk
Igf1r
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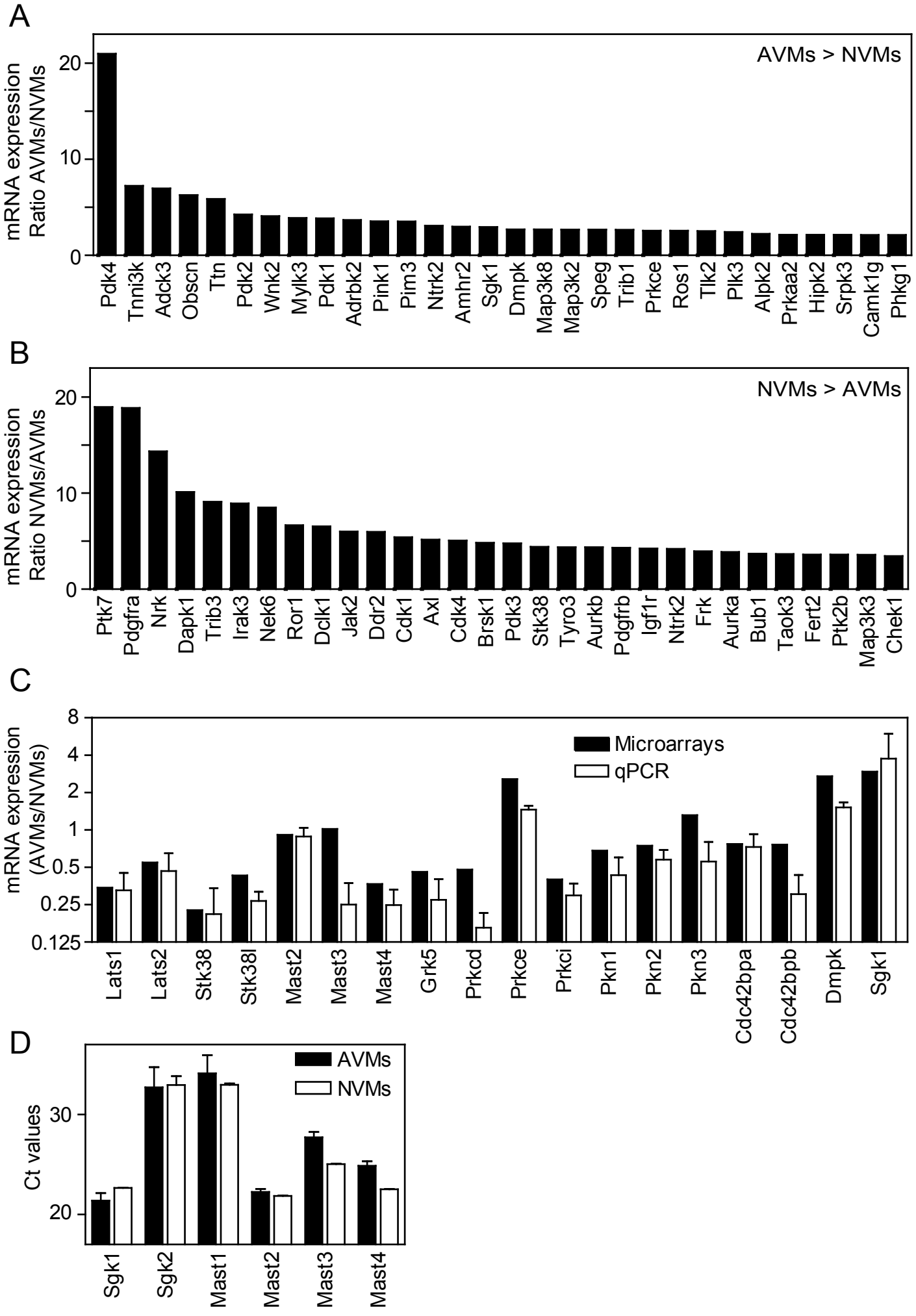
Ror1
Src
Tyro3
AVMs
NVMs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AVMs > NVMs
mRNA expression
Ratio AVMs/NVMs
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10
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Pdk4
Tnni3k
Adck3
Obscn
Ttn
Pdk2
Wnk2
Mylk3
Pdk1
Adrbk2
Pink1
Pim3
Ntrk2
Amhr2
Sgk1
Dmpk
Map3k8
Map3k2
Speg
Trib1
Prkce
Ros1
Tlk2
Plk3
Alpk2
Prkaa2
Hipk2
Srpk3
Camk1g
Phkg1
B
NVMs > AVMs
mRNA expression
Ratio NVMs/AVMs
20
10
0
Ptk7
Pdgfra
Nrk
Dapk1
Trib3
Irak3
Nek6
Ror1
Dclk1
Jak2
Ddr2
Cdk1
Axl
Cdk4
Brsk1
Pdk3
Stk38
Tyro3
Aurkb
Pdgfrb
Igf1r
Ntrk2
Frk
Aurka
Bub1
Taok3
Fert2
Ptk2b
Map3k3
Chek1
C
mRNA expression
(AVMs/NVMs)
8
4
2
1
0.5
0.25
0.125
Microarrays
qPCR
Lats1
Lats2
Stk38
Stk38l
Mast2
Mast3
Mast4
Grk5
Prkcd
Prkce
Prkci
Pkn1
Pkn2
Pkn3
Cdc42bpa
Cdc42bpb
Dmpk
Sgk1
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20
AVMs
NVMs
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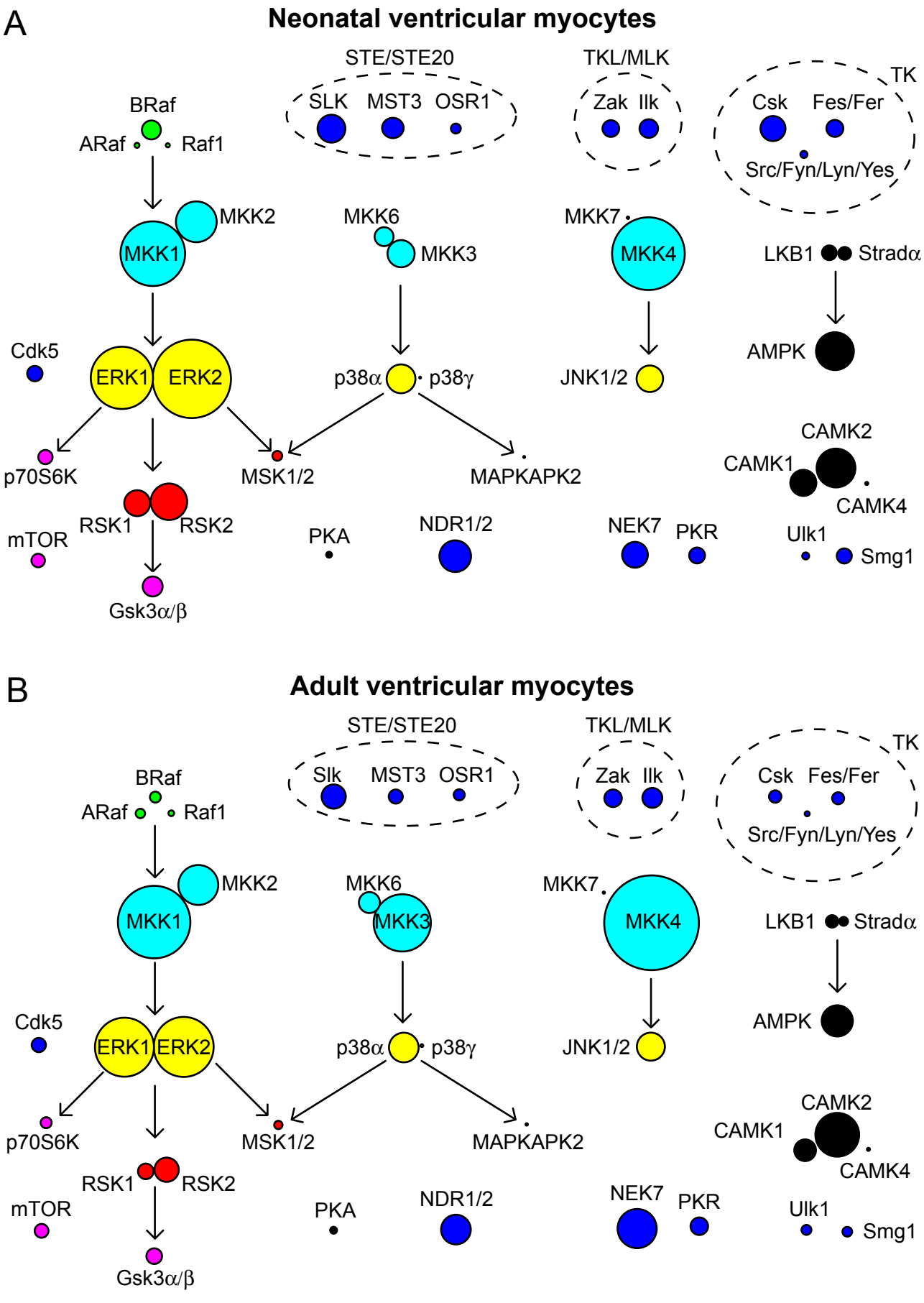
Sgk2
Mast1
Mast2
Mast3
Mast4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STE/STE20
SLK MST3 OSR1
TKL/MLK
Zak Ilk
TK
Csk Fes/Fer
Src/Fyn/Lyn/Yes
BRaf
ARaf Raf1
MKK2
MKK1
MKK6
MKK3
MKK7
MKK4
LKB1 Stradα
AMPK
Cdk5
ERK1 ERK2
p38α p38γ
JNK1/2
p70S6K
MSK1/2
MAPKAPK2
CAMK2
CAMK1
CAMK4
RSK1 RSK2
mTOR
PKA
NDR1/2
NEK7
PKR
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Smg1
Gsk3α/β
B
Adult ventricular myocytes
STE/STE20
Slk MST3 OSR1
TKL/MLK
Zak Ilk
TK
Csk Fes/Fer
Src/Fyn/Lyn/Yes
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ARaf Raf1
MKK2
MKK1
MKK6
MKK3
MKK7
MKK4
LKB1 Stradα
AMPK
Cdk5
ERK1 ERK2
p38α p38γ
JNK1/2
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MSK1/2
MAPKAPK2
CAMK2
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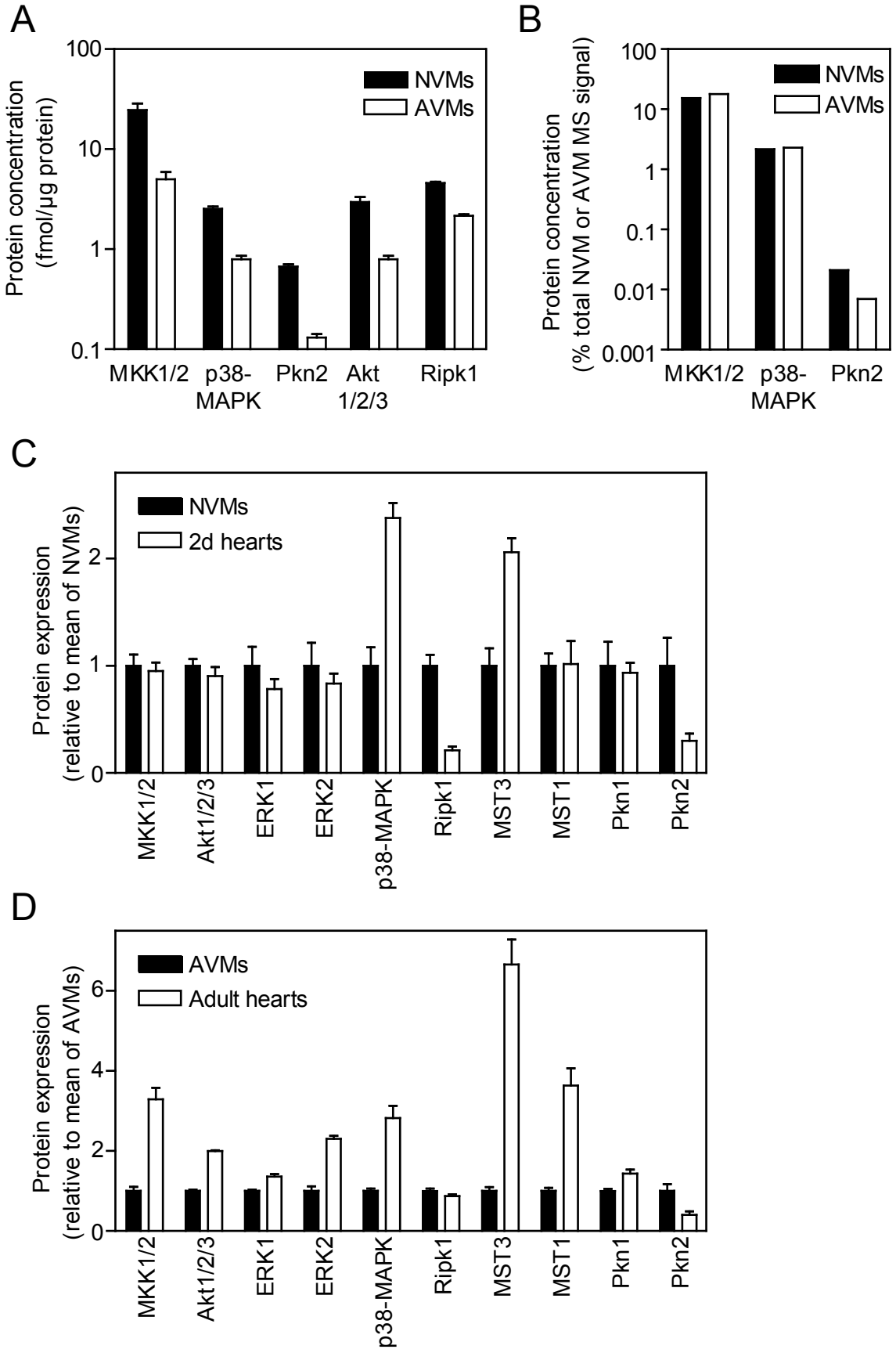
Gsk3α/β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NVMs
AVMs
MKK1/2
p38-MAPK
Pkn2
Akt 1/2/3
Ripk1
B
Protein concentration (% total NVM or AVM MS signal)
100
10
1
0.1
0.01
0.001
NVMs
AVMs
MKK1/2
p38-MAPK
Pkn2
C
Protein expression (relative to mean of NVMs)
NVMs
2d hearts
2
1
0
MKK1/2
Akt1/2/3
ERK1
ERK2
p38-MAPK
Ripk1
MST3
MST1
Pkn1
Pkn2
D
Protein expression (relative to mean of AVMs)
AVMs
Adult hearts
6
4
2
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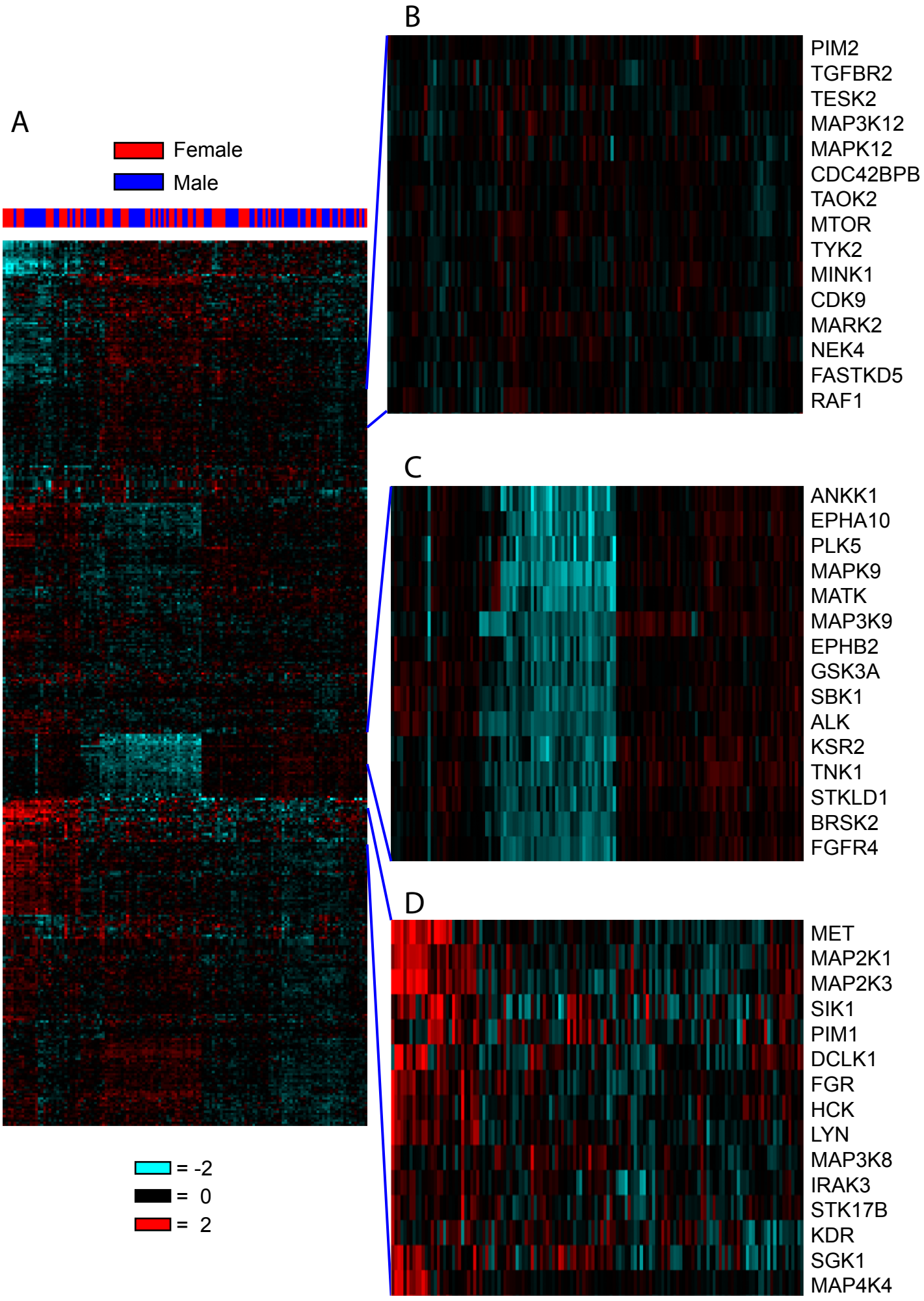
Pkn2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MATK
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ALK
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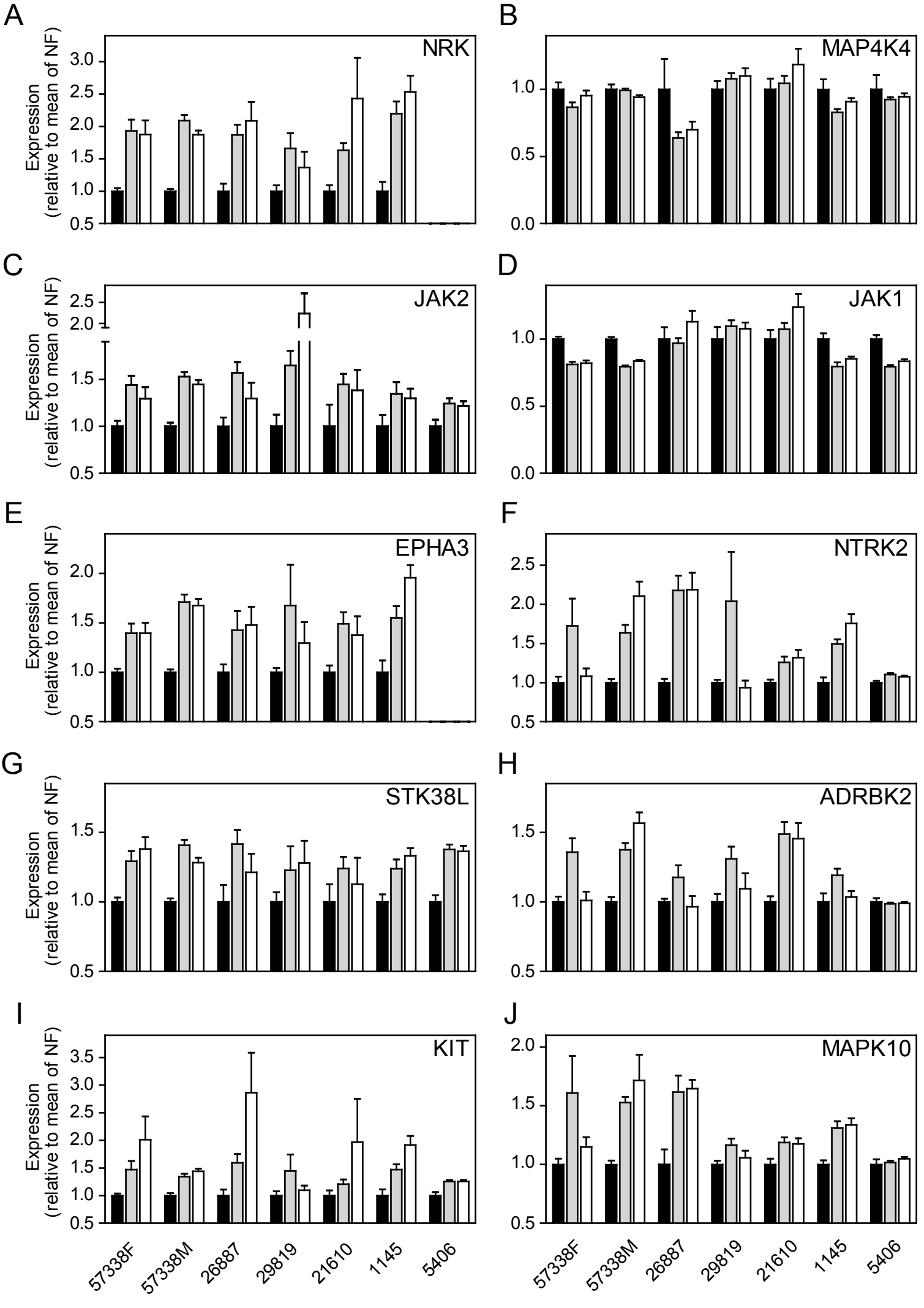
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H
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J
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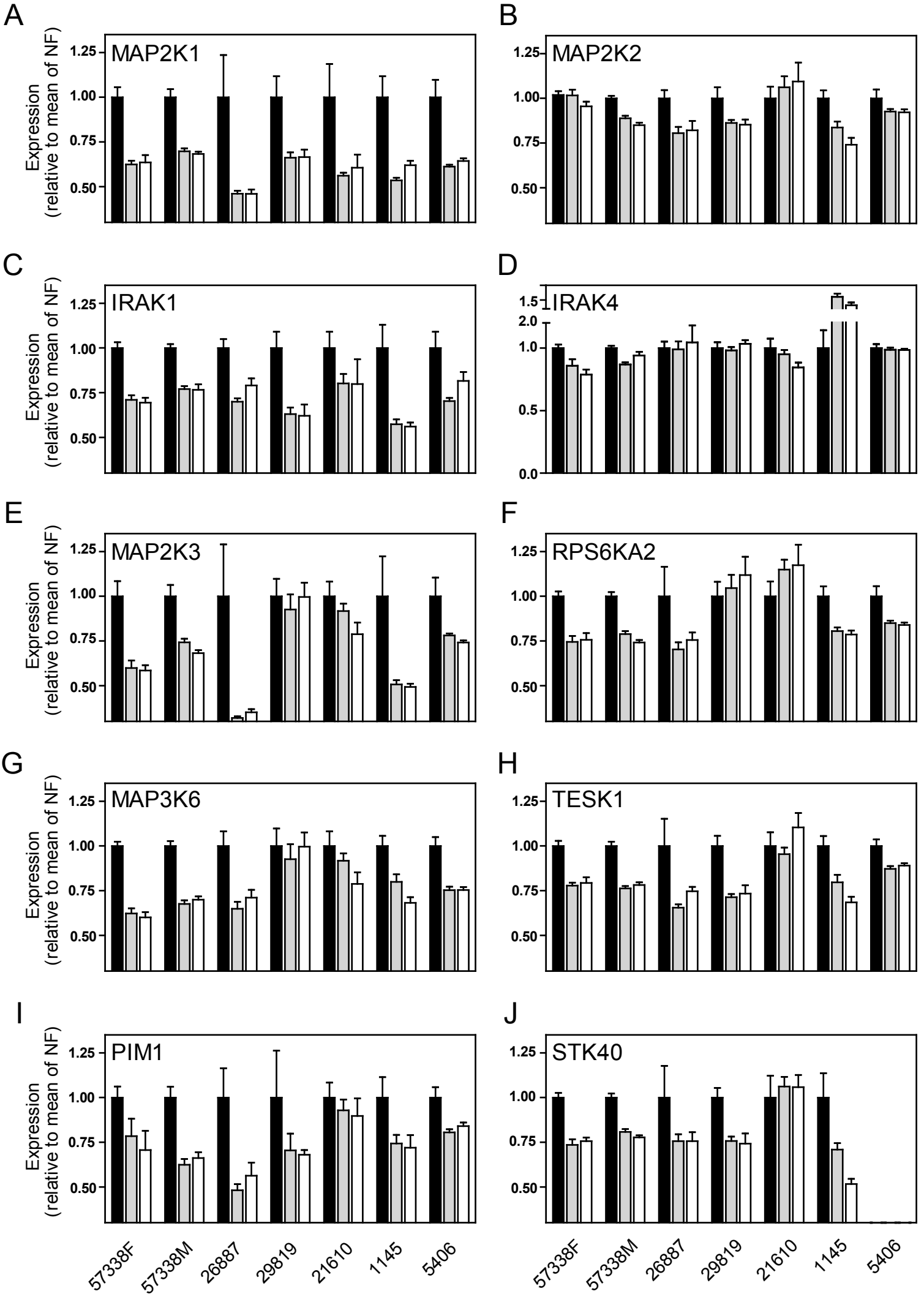
Expression (relative to mean of NF)
57338F
57338M
26887
29819
21610
1145
5406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H
TESK1
I
PIM1
J
STK40
Expression (relative to mean of NF)
57338F
57338M
26887
29819
21610
1145
5406

Supplemental Methods

Cardiomyocyte preparation and culture of neonatal non-cardiomyocytes

Sprague-Dawley female rats with 2 day litters were purchased from Harlan SeraLab Ltd. UK and were housed in the Imperial College Central Biomedical Services or the University of Reading facility with water and food *ad libitum*. All facilities are UK registered with Home Office certificates of designation. All procedures in these facilities were performed in accordance with UK regulations. Neonatal rats were culled by schedule 1 (cervical dislocation) for which additional approval and licences are not required according to UK regulations. Adult male Sprague-Dawley rats were purchased from Harlan SeraLab Ltd. UK or Charles River Laboratories Canada Inc. and were housed in the University of Reading or Montreal Heart Institute facility with water and food *ad libitum*. Work with adult male rats was undertaken in accordance with local institutional animal care committee procedures and either the U.K. Animals (Scientific Procedures) Act 1986 or the Canadian Council on Animal Care.

Rat neonatal ventricular myocytes (NVMs) were prepared and cultured from Sprague-Dawley rats as previously described.[1] Ventricles were dissected from neonatal (1–2 d) Sprague-Dawley rat hearts and dissociated by serial digestion with 0.4 mg/ml collagenase and 0.6 mg/ml pancreatin sterile digestion buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM Na_2HPO_4, 5.6 mM glucose, 5.4 mM KCl and 0.8 mM $MgSO_4$, pH 7.35). The first digestion supernatant (5 min, 37°C, 160 cycles/min in a shaking waterbath) was removed and discarded. Cell suspensions from subsequent digestions (4×25 min, 37°C 136 cycles/min shaking) were recovered by centrifugation (5 min, 60×g) and the cell pellet resuspended in plating medium (Dulbecco's modified Eagle's medium (DMEM)/medium 199 [4:1 (v/v)], 15% (v/v) foetal calf serum (FCS), 100 units/ml penicillin and streptomycin). Cells were pre-plated on plastic tissue culture dishes (30 min) to remove non-cardiomyocytes. The cells remaining on the pre-plates were cultured in Dulbecco's modified Eagle's medium (DMEM)/medium 199 [4:1 (v/v)], 5% (v/v) foetal calf serum (FCS), 100 units/ml penicillin and streptomycin until confluent. They were then trypsinized and divided between two dishes. After 24 h, cells were harvested for RNA extraction.

For biochemistry and molecular biology experiments, non-adherent viable cardiomyocytes were plated at a density of 4×10^6 cells/dish on 60 mm Primaria dishes pre-coated with sterile 1% (w/v) gelatin (Sigma-Aldrich UK). After 18 h myocytes were beating spontaneously. For immunostaining experiments, cardiomyocytes were plated at 1.5×10^6 cells/dish on 35 mm Primaria dishes containing glass coverslips pre-coated with sterile 1% (w/v) gelatin followed by laminin (20 µg/ml in PBS; Sigma-Aldrich UK). The plating medium was withdrawn and cells were incubated in serum-free maintenance medium (DMEM/medium [4:1 (v/v)], 100 units/ml penicillin and streptomycin) for a further 24 h. Immunostaining studies indicated that NVM cultures for immunostaining contained up to ~5% non-myocytes (Supplemental Figure 2).

Rat adult ventricular myocytes (AVMs) were prepared from male Sprague Dawley rats. In the UK, all animal experiments were approved by the Imperial College London or University of Reading ethics committee and performed according to the the U.K. Animals (Scientific Procedures) Act 1986. Rats (200-250g) were anaesthetised with a lethal intraperitoneal dose of Euthatal (pentobarbital sodium, 60 mg/kg). Once the plane of anaesthesia was such that they no longer responded to noxious stimuli (toe pinch), 100 units of heparin (1000 units/ml) was administered via the femoral vein. The chest cavity was opened and the heart and lungs were removed into modified ice-cold KHBBS (25 mM $NaHCO_3$, 119 mM NaCl, 35 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 equilibrated with 95% O_2/5% CO_2) whilst the heart was still beating. The surrounding tissues were removed from the heart before aortic cannulation. In Canada, all animal experiments were approved by the Montreal Heart Institute ethics committee and performed according to the guidelines of the Canadian Council on Animal Care. Rats (150-180g) were injected intraperitoneally with 500 U of heparin and anesthetised with pentobarbital (60 mg/kg). Once

the plane of anaesthesia was such that they no longer responded to noxious stimuli (toe pinch), rats were decapitated with a with a small animal guillotine (Harvard Apparatus) and the hearts rapidly removed, cannulated via the aorta, and subjected to retrograde perfusion. Cardiac myocytes were isolated as described previously as previously described.[2,3] AVMs were collected and initially washed under gravity such that there was negligible contamination with non-myocytes, then washed in warmed PBS (37°C, 3 × 5 ml) with collection by centrifugation (5 min, 60×g, 20°C).

RNA preparation, microarray analysis and qPCR

Total RNA was prepared from NVMs using RNA Bee (AMS Biotechnology Ltd) according to the manufacturer's instructions. For microarray studies of AVM RNA expression profiles, cardiomyocytes were homogenized in Tri Reagent® (Sigma Aldrich). Chloroform (0.2 ml) was added and samples vortexed, then centrifuged and the upper aqueous layer was collected. An equal volume of 70% (v/v) ethanol was added and the RNA was purified using Qiagen RNeasy Mini Kits according to the manufacturer's instructions. RNA was eluted in RNase-free water. For qPCR analysis of AVM mRNAs, cardiomyocytes were pelleted and resuspended in RNA Bee and samples processed as for NVMs. RNA purity was assessed from the A_{260}/A_{280} (values of 1.9–2.1 were considered acceptable). RNA concentrations were determined from the A_{260}.

For microarrays, two separate samples were prepared from each of three preparations for hybridisation (i.e. 6 samples were hybridised for each of NVMs and AVMs). For NVMs, equal amounts of RNA from three individual preparations were pooled to generate a single sample. For AVMs, RNA was prepared from cardiomyocytes from a single heart. cRNA was prepared as previously described.[1] Fragmentation of antisense cRNA and hybridization to Affymetrix rat genome 230 2.0 arrays was performed at the CSC/IC Microarray Centre (Imperial College London) according to the manufacturer's instructions. Data were exported to ArrayExpress (ArrayExpress ID: E-MTAB-2832).

qPCR was performed as previously described.[1] cDNAs were synthesized using High Capacity cDNA Reverse Transcription Kits with random primers (Applied Biosystems) according to the manufacturer's instructions. Primers were from Eurofins (glyceraldehyde 3-phosphate dehydrogenase, Gapdh) or PrimerDesign (Supplemental Table 1). qPCR was performed using an ABI Real-Time PCR 7500 system (Applied Biosystems). Optical 96-well reaction plates were used containing (in each well) 12.5 µl iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc.), 5 µl primers and 7.5 µl (1 µg) cDNA template. qPCR was performed using absolute quantification with standard curve protocol at 50°C (2 min), 95°C (10 min) and then 40 cycles of 95°C (15 s) and 60°C (1 min). Dissociation curve analysis was performed to confirm the absence of aberrant amplification products. Values for selected RNAs were normalized to Gapdh expression.

Microarray data analysis: rat cardiomyocytes

Microarray data (.CEL files) were imported into GeneSpring 12.6.1 (Agilent Technologies) using the PLIER16 algorithm with normalisation per gene to the gene median. For cardiomyocytes, probesets were filtered by expression removing those below the lowest 20th percentile in all samples for either NVMs or AVMs. Probesets within the array that detected protein kinases were identified. Gene identities were confirmed by BLAST search of probeset target sequences using the Entrez nucleotide database (www.ncbi.nlm.nih.gov/BLAST). Expressed kinases (i.e. those above the lowest 20th percentile of the full dataset) were selected. Differentially expressed kinases in NVMs *vs* AVMs were identified (>2-fold difference; moderated T test with a Benjamini and Hochberg false discovery rate correction, $p<0.05$).

Microarray data analysis: human myocardial samples

To identify changes in kinase mRNA expression in human failing hearts, we mined existing datasets publicly available from ArrayExpress or GEO databases (E-GEOD-57338,[4] E-GEOD-29819,[5] E-GEOD-26887,[6] E-GEOD-21610,[7] E-GEOD-1145 and E-GEOD-5406[8]).

Information on the patients from each of these studies is provided in Supplemental Table 2. No additional human myocardial samples were taken. The data were downloaded as .CEL files (E-GEOD-57338, E-GEOD-29819, E-GEOD-26887, E-GEOD-21610) or normalised data (E-GEOD-1145, E-GEOD-5406)

For E-GEOD-57338, the following groups were analysed separately: females, non-failing (NF) *vs* dilated cardiomyopathy (DCM); females, NF *vs* ischaemic heart failure (IHF); males, NF *vs* DCM; males, NF *vs* IHF. Data were imported and analysed using GeneSpring 13.0 using the PLIER16 algorithm with normalisation per gene to the gene median. Probesets within the array that detected protein kinases were identified and gene identities were confirmed by BLAST search of probeset target sequences using the Entrez nucleotide database (www.ncbi.nlm.nih.gov/BLAST). Expressed kinases (i.e. those above the lowest 20th percentile) were selected. Differentially expressed kinases in each of the four groups were identified (>1.25-fold difference; $p<0.05$, moderated T test with a Benjamini and Hochberg false discovery rate correction). The same approach was used for the other datasets but, because sample numbers were much less, all data in each dataset were imported and analysed together with no distinction between males and females. Kinase mRNAs that were significantly changed in E-GEOD-57338 were clustered according to the number of groups in which they were identified and those that changed in 3 or 4 of the groups were selected. The data for these kinases were mined from the other datasets for independent analysis.

Analysis of the kinase proteome

NVMs were dissociated from ventricles from 2-3 d rats as previously described.[1] Cells were pre-plated (to remove non-cardiomyocytes) on plastic tissue culture dishes (45 min, 37°C) in Dulbecco's modified Eagle's medium (DMEM)/medium 199 [4:1 (v/v)] containing 15% (v/v) FCS and 100 units/ml penicillin and streptomycin. Non-adherent cells were collected and recovered by centrifugation (5 min, 60×g, 20°C). AVMs were prepared as previously described[15] and collected and initially washed under gravity. Cell pellets were then washed in warmed PBS (37°C, 3 × 5 ml) with collection by centrifugation (5 min, 60×g, 20°C). Pellets were frozen and stored at -80°C. Two independent cardiomyocyte samples (NVM samples were prepared from 15 rat hearts for each preparation; AVM samples were from a single heart each) were shipped to ActivX Biosciences for in situ kinase profiling using the KiNative™ platform as described in [9,10]. ATP and ADP acyl-nucleotide probes were synthesized as described previously.[10] Cell pellets were lysed by sonication in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, phosphatase inhibitors [Cocktail II AG Scientific #P-1518]). After lysis, samples were cleared by centrifugation and supernatants collected for probe-labeling. Desthiobiotin-adenosine triphosphate-acylphosphate probe (ATP probe; 50 µl of 10× aqueous solution) was added to each sample to give a final concentration of 5 µM, and incubated with the samples for 10 minutes. Samples were prepared for MS analysis as described previously.[10,11] Briefly, probe-labeled lysates were denatured and reduced in [6 M urea, 10 mM dithiothreitol (DTT); 65°C, 15 min], alkylated (40 mM iodoacetamide, 37°C, 30 min), and gel filtered (BioRad 10DG) into 10 mM ammonium bicarbonate, 2 M urea, 5 mM methionine. The desalted protein mixture was digested with trypsin (0.015 mg/ml; 1 h, 37°C), and desthiobiotinylated peptides captured using high-capacity streptavidin resin (12.5 µl; Thermo Scientific). Captured peptides were washed extensively (150 µl per wash) with three different wash buffers: (A) 10 times with 1% (v/v) Triton X100, 0.5% tergitol, 1 mM ethylene diamine tetra-acetic acid (EDTA) in phosphate buffered saline (PBS); (B) 60 times with PBS; (C) 15 times with HPLC grade water. Peptides were eluted from the streptavidin beads using two 35 µl washes of a 50% CH_3CN/water mixture containing 0.1% trifluoroacetate (TFA) at room temperature.

Samples were analyzed by LC-MS/MS as described previously.[10] Samples were analyzed on Thermo LTQ ion trap mass spectrometers coupled with Agilent 1100 series micro-HPLC systems with autosamplers, essentially as described,[11] using a custom target list comprising 321 unique rat kinase peptides that had been previously identified during the characterization of various samples in data dependent mode.[9] For signal

extraction/quantitation, typically up to four ions were selected for based on their presence, intensity, and correlation to the reference MS/MS spectrum. The resulting chromatographic peaks from each run were then integrated. Each sample was analysed in duplicate. The means of the integrated peak values were calculated for each peptide in each sample (NVM1, NVM2, AVM1, AVM2). When multiple peptides were derived from a single kinase, MS values were added. For peptides that were not unique, the values were allocated according to the proportion of signals for unique peptides where possible. The means of the replicates were used to give an average estimate for NVMs and AVMs. The MS value for each kinase was expressed as the percentage of the total MS integration values for NVMs or AVMs.

Western blotting

Recombinant human MKK1 (gene symbol MAP2K1) and p38-MAPKα (MAPK14) were expressed as glutathione S-transferase (GST) fusion proteins and were prepared as previously described.[12-14] Other recombinant human GST-fusion proteins were obtained commercially (AKT1, R & D Systems, 1775-KS-010; RIPK1, Abnova, H00008737-P01; PKN2 Life Technologies Ltd., PV3879). Concentrations of recombinant proteins were determined relative to bovine serum albumin (BSA) standards on Coomassie Brilliant Blue-stained gels. Cardiomyocyte samples were prepared for immunoblotting as previously described.[1] Protein concentrations were determined by Bio-Rad Bradford assay using BSA standards. Proteins (cardiomyocytes and recombinant protein standards) were separated by SDS-polyacrylamide gel electrophoresis on 10% or 8% (w/v) polyacrylamide gels and transferred electrophoretically to nitrocellulose. Proteins were detected as previously described[1] using primary antibodies as indicated in Supplemental Table 3. Bands were detected by enhanced chemiluminescence using ECL Prime Western Blotting detection reagents with visualisation using an ImageQuant LAS4000 system (GE Healthcare). ImageQuant 7.0 software (GE Healthcare) was used for densitometric analysis of the bands. Data analysis used GraphPad Prism version 4.0.

Immunostaining

NVMs were plated at 1.5×10^6 cells/dish on 35 mm Primaria dishes containing glass coverslips pre-coated with sterile 1% (w/v) gelatin followed by laminin (20 µg/ml in PBS; Sigma-Aldrich UK) in DMEM/medium [4:1 (v/v)] containing 100 units/ml penicillin and streptomycin and 15% (v/v) foetal calf serum. The plating medium was withdrawn and cells were incubated in serum-free maintenance medium (DMEM/medium [4:1 (v/v)], 100 units/ml penicillin and streptomycin) for a further 24 h. Cells were washed with ice-cold PBS and fixed in 3.7% (v/v) formaldehyde in PBS (10 min, room temperature). Cardiomyocytes were permeabilised with 0.1% (v/v) Triton X-100 (10 min, room temperature) in PBS and non-specific binding blocked with 1% (w/v) fatty acid free BSA in PBS containing 0.1% (v/v) Triton X-100 (10 min, room temperature). All incubations were at 37°C in a humidified chamber, and coverslips were washed three times in PBS after each stage of the immunostaining procedure. Cardiomyocytes were stained with mouse monoclonal primary antibodies to troponin T (1/40, 60 min; Stratech Scientific, Cat. no. MS-295-P1) with anti-mouse immunoglobulin secondary antibodies coupled to Alexa-Fluor 488 (1/200, 60 min; Invitrogen). Myofilamentous actin was counterstained with Texas Red®-X phalloidin (5 U/ml, 20 min; Life Technologies Inc.). Coverslips were mounted using fluorescence mounting medium (Dako) and viewed with a Zeiss Axioskop fluorescence microscope using a 40× objective. Digital images captured using a Canon PowerShot G3 camera were reduced in size and superimposed using Adobe Photoshop 7.0.

Supplemental methods: References

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Supplemental Table 1. Primers used for qPCR.

Gene symbol	Accession no.	Product length (bp)	Position	Sense primer	Antisense primer
Cdc42bpa	NM_053657	129	24-152	TCCCTCCCCCTCCATACATT	GCAACATAATCCTGAAACGAATCC
Cdc42bpb	NM_053620	104	2282-2385	GAGGTCCTGATGCTGAAAGATAAG	CTCTCCCGTTCGTACTTGTCT
Dmpk	XM_006223105	119	1731-1849	TACAGGAGCGGATGGAGATG	TGCCCCAGGGTTCATACAC
Gapdh	NM_017008.3	93	552-644	CCAAGGTCATCCATGACAACTT	AGGGGCCATCCACAGTCTT
Grk5	NM_030829	91	230-320	GGGAAGGGGGTGGAGGAA	CGGAGGTCTTCACACTGGTT
Lats1	NM_001134543	116	5150-5265	GGAGGAATTGTGGAGTCATAAC	ATGTTAATAAGCCTTACGAAAATGAA
Lats2	NM_001107267	95	1742-1836	ACAAAAAGCAGATCCAGACCTC	ATATGGGGAGTAACTCTTGATTCG
Map2k3	NM_001100674	85	1292-1376	TTCTGGTCCTGGGCATTCG	AGGTCTGATTCTTTGGCACTTG
Map2k6	NM_053703	94	299-392	CGGGGTGGTGGAGAAGATG	CCGTTTCTGCTCCTGGCTAT
Mapk12	NM_021746	118	1393-1510	GTGCTTTTATCCCAAGTCATCCA	TGTTCTGCCAGGGTCATCTC
Mapk14	NM_031020	112	1308-1419	CGAATGGAAGAGCCTGACCTA	TGAAGTGGGATGGACAGAACA
Mapkapk2	NM_178102	95	1734-1828	GTCCTTTTCCCCACTCCTCAT	CATCCCTATAACAACCTCCACAAT
Mast1	NM_181089	116	220-335	GCCCATTTCTCGTTTGCTTCT	CAGGAGGATGAGACGGTTGAG
Mast2	NM_001108005	98	719-816	CAGTCTCTTCATCGTGTTCCTC	TGTGCTAAAATGCTTCGTCAGA
Mast3	NM_001134796	94	2181-2274	AGGGTGACGAGACGAATGAC	TCGGAACTGCTGTAGACCTTG
Mast4	XM_006231870	114	8176-8289	GAGGGCACACAGGGACTTA	CTTCTTGGACTCACAGCGTAA
Myh6	NM_017239	93	1865-1957	TACCAGAAGTCCTCCCTCAAAC	CTTGCCTCCTTTGCCTTTCC
Myh7	NM_017240	124	4186-4309	TGGCTCAGAGGCTTCAGGA	CGCTCCACATCCACCATCA
Orc1	NM_177931	105	1085-1189	CTTCTCTTCGTGCCCGTAGA	ACTCTTCCTCTTCTTGGTCACTT
Orc2	NM_001012003	96	678-773	CGAAAAAGAGTCAAGGTCAGAATAG	CCAAGTCACCTGCTTTGTCTC
Orc3	NM_001025282	147	751-897	ATCGCCACATCTCCTGTTATTATC	AAAGGGAAACTGAGGGGTAAGAA
Orc4	NM_199092	113	517-629	CCTTTCGTTTCTTCTGGAAGC	GGAGTGTTTGGTTTTTCTGATGA
Orc5	NM_001014186	121	904-1024	CGCAGTGGGAAACATTACAGAA	GCAAGGTACGCAGCAATAAGAA
Orc6	NM_001033690	89	930-1018	AACAACCAGCAAAAGACATAGAAG	TTTCCATTCCTCATAATCCTGTGT
Pkn1	NM_017175	85	1891-1975	CTGCCCTCCACCTCATGTAG	GGGTCTCCTGGGTCTCTGAA
Pkn2	NM_001105755	105	1923-2027	GCTATTCCCACAGTAAATCATTCTG	GGCTGGAGGTGCGAGTTC
Pkn3	NM_001047861	125	2122-2246	CACCCGTTCTTGCTCTCTCT	TGGGGCTCAGGAAAGACATC
Prkcd	NM_133307	105	2133-2237	GTAACAGGAAACATCAGGCTTCA	AGGGGATTTCACTTTGGGCTTA
Prkce	NM_017171	91	1582-1672	GCGGAAACACCCTTATCTAACC	GTCTCCACCGTTTACATATTCCAT
Prkci	NM_032059	86	1252-1337	GAGCGAGGGATAATTTATAGAGATTTG	ATGCCGTAGTCAGTGAGTTTG
Sgk1	NM_001193568	76	338-413	CAGGAGCCCGAACTTATGAAC	AGGATGGACCCAGGTTGATTT
Sgk2	NM_134463	75	1052-1126	CGTGGTACTGACAGATTTCGG	GTGCCGCAGAAGGTGGAT
Stk38	NM_001015025	121	105-225	TTCCCCTGCCTCCCACTG	ATCAAGTCCTAATCACAACGCATAA
Stk38l	NM_001083336	130	784-913	CGGGACATCAAGCCAGACA	GTGGGTTGTGTGTGAGGTTTC
Tnni1	NM_017184	104	223-326	CGCCCTTCAGGACTTATGC	TGATCTCTCTCGTGTTGTGGA
Tnni3	NM_017144	110	204-313	GCCACATGCCAAGAAAAAGTC	GTCGCTCCTCTGCCTCAC

Supplemental Table 2. Affymetrix microarray datasets used for analysis of kinase mRNA expression in human heart failure. NF, non-failing. DCM, dilated cardiomyopathy. IHF, ischaemic heart failure. NDHF, non-diabetic heart failure. DHF, diabetic heart failure. ARVC, arrhythmogenic right ventricular cardiomyopathy. * left ventricular samples studied (i.e. right ventricular samples not included in analysis). ** analysis performed only on samples before ventricular assist device support. # Hannenhalli et al. report that 86 IHF samples and 108 DCM samples were studied, but the annotations indicate 108 IHF and 86 DCM. For this study, the dataset annotations were used.

ArrayExpress/GEO accession no.	Patients studied (n)	Technology	Reference
E-GEOD-57338 GSE57338	Female NF (63) Female DCM (19) Female IHF (14) Male NF (73) Male DCM (63) Male IHF (81)	Affymetrix Human Gene 1.1 ST Array	Liu et al, 2015
E-GEOD-26887 GSE26887	NF (5) NDHF (12) DHF (7)	Affymetrix Human Gene 1.0 ST Array	Greco et al, 2012
E-GEOD-29819 * GSE29819	NF (6) DCM (7) ARVC (6)	Affymetrix Human Genome U133 Plus 2.0 Array	Gaertner et al, 2012
E-GEOD-21610 ** GSE21610	NF (8) DCM (21) IHF (9)	Affymetrix Human Genome U133 Plus 2.0 Array	Schwientek et al, 2010
E-GEOD-1145 GSE1145	NF (11) DCM (27) IHF (31)	Affymetrix Human Genome U133 Plus 2.0 Array	Not available
E-GEOD-5406 # GSE5406	NF (16) DCM (86) IHF (108)	Affymetrix Human Genome U133A Array	Hannenhalli et al, 2006

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Supplemental Table 3. Antibodies used for immunoblotting.

Target	Source	Catalogue no.	Dilution
AKT1/2/3	Cell Signaling Technology	4691	1/1000
BRaf (H145)	Santa Cruz Biotechnology Inc.	sc-9002	1/750
ERK1/2	Cell Signaling Technology	4695	1/1000
MAP3K8 (M20)	Santa Cruz Biotechnology Inc.	sc-720	1/1000
MAP4K4 (HGK)	Cell Signaling Technology	3485	1/750
MKK1/2	Cell Signaling Technology	8727	1/1000
MST1	Cell Signaling Technology	3682	1/1000
MST3	BD Transduction Laboratories	611056	1/1000
p38-MAPK	Cell Signaling Technology	9212	1/1000
PKCδ	BD Transduction Laboratories	610397	1/500
PKN1	BD Transduction Laboratories	610686	1/1000
PKN2	Cell Signaling Technology	2612	1/750
RIPK1	Cell Signaling Technology	4926	1/1000
RIPK2	Cell Signaling Technology	4142	1/1000
TNNI3K	Sigma	SAB4502101	1/500
ZAK	Thermo Scientific	PA5-29317	1/1000
HRP-anti-mouse immunoglobulins	Dako	P0260	1/5000
HRP-anti-rabbit immunoglobulins	Dako	P0448	1/5000

Supplemental Table 4. Protein kinases not detected in ActivX proteomics experiment but detected by microarrays: kinases with protein expression in cardiomyocytes confirmed in previous studies. Where possible, references to initial reports are given. Reviews are cited for some kinases that are well-studied in cardiomyocytes. We apologise to the many other investigators whose work is not mentioned.

* Not detected in proteomics experiment; other kinases were not searched for.
# Identified in embryonic development.

Gene symbol	Gene name	Microarrays (raw fluorescence values)		Reference
		AVMs	NVMs	
Adrbk2*	Adrenergic, beta, receptor kinase 2	95	26	Vinge et al. [1]
Akt1*	v-Akt murine thymoma viral oncogene homolog 1	1022, 1868	686, 1709	DeBosch et al. [2] Liu et al. [3]
Akt2	v-Akt murine thymoma viral oncogene homolog 2	378, 473, 600	268, 327, 510	DeBosch et al. [4]
Alpk3 #	Alpha-kinase 3	959	505	Hosoda et al. [5]
Bmpr1a	Bone morphogenetic protein receptor, type IA	121, 262, 42	338, 519, 155	Sui et al. [6]
Bmpr2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	237, 253, 146	156, 223, 260	Sui et al. [6]
Camk2b	Calcium/calmodulin-dependent protein kinase II beta	61	96	Singh et al. [7]
Camk2d	Calcium/calmodulin-dependent protein kinase II delta	112, 1354, 341	135, 707, 217	Xu et al. [8]
Cdk8	Cyclin-dependent kinase 8	118	276	Kim et al. [9]
Csf1r*	Colony stimulating factor 1 receptor	63	89	Postiglione et al. [10]
Dapk3	Death-associated protein kinase 3	383	759	Chang et al. [11]
Ddr2	Discoidin domain receptor tyrosine kinase 2	50, 28, 86	238, 203, 543	Grigore et al. [12]
Dmpk	Dystrophia myotonica-protein kinase	3206	1186	Mussini et al. [13]
Dyrk2	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2	251, 217, 372	245, 249, 360	Weiss et al. [14]
Epha3	Eph receptor A3	41, 64, 92	61, 113, 160	Li et al. [15]
Erbb3 #	v-Erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	54, 75	57, 59	Hao et al. [16]
Fgfr1	Fibroblast growth factor receptor 1	333, 279, 95	746, 490, 229	Liu et al. [3]
Fgfr2	Fibroblast growth factor receptor 2	66, 48, 100, 48	99, 187, 121, 56	Liu et al. [3]
Fgfr3	Fibroblast growth factor receptor 3	37, 57	55, 86	Liu et al. [3]
Flt1*	FMS-related tyrosine kinase 1	144, 124, 128, 258, 174	117, 73, 79, 107, 92	Takahashi et al. [17]
Grk4*	G protein-coupled receptor kinase 4	148	83	Dzimiri et al. [18]
Grk6*	G protein-coupled receptor kinase 6	123, 48, 125	148, 76, 312	Dzimiri et al. [18] Yi et al. [19]
Hspb8	Heat shock protein B8	1509, 1863	839, 1367	Sui et al. [6]
Igf1r	Insulin-like growth factor 1 receptor	80, 48, 156, 77	220, 95, 383, 1312	Leri et al. [20]
Ikbkb*	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	107	164	Dhingra et al. [21]
Insr	Insulin receptor	99, 172, 74	85, 146, 89	van Echten et al. [22]
Jak2*	Janus kinase 2	58, 78	402, 407	McWhinney et al. [23]
Kdr*	Kinase insert domain receptor	676	446	Takahashi et al. [17]
Kit #	v-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	51	84	Torella et al. [24] Leri et al. [25]
Lck*	Lymphocyte-specific protein tyrosine kinase	93	95	Ping et al. [26]
Map3k8	Mitogen-activated protein kinase kinase	193	72	Kim et al. [27]

	kinase 8			
Mapk11*	Mitogen-activated protein kinase 11	60	62	Kim et al. [28]
Met #	Met proto-oncogene	49	44	Rappolee et al. [29]
Mknk1	MAP kinase-interacting serine/threonine kinase 1	175	142	Tuxworth et al. [30]
Mknk2	MAP kinase-interacting serine/threonine kinase 2	1018	815	Tuxworth et al. [30]
Mylk3	Myosin light chain kinase 3	3341, 1876	829, 501	Ai et al. [31]
Ntrk2	Neurotrophic tyrosine kinase, receptor, type 2	17, 58	101, 166	Okada et al. [32]
Ntrk3	Neurotrophic tyrosine kinase, receptor, type 3	69	64	Kawaguchi-Manabe et al. [33]
Obscn	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	516	82	Borisov et al. [34]
Pak1	p21 protein (Cdc42/Rac)-activated kinase 1	105	128	Clerk et al. [35]
Pak2*	p21 protein (Cdc42/Rac)-activated kinase 2	99, 421, 96, 156	188, 701, 104, 450	Kim et al. [27]
Pdgfrb	Platelet derived growth factor receptor, beta polypeptide	113, 49, 35	991, 78, 185	Chintalgattu et al. [36]
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1	1466, 1021, 1437	414, 325, 283	Puthanveetil et al. [37]
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2	2083, 841	546, 179	Puthanveetil et al. [37]
Pdk4	Pyruvate dehydrogenase kinase, isozyme 4	1003, 2329	57, 89	Puthanveetil et al. [37]
Pdpk1	3-phosphoinositide dependent protein kinase 1	305	395	Rubio et al. [38]
Pim1	Pim-1 oncogene	196	161	Muraski et al. [39]
Pim3	Pim-3 oncogene	1137	323	Liu et al. [40]
Pink1	PTEN induced putative kinase 1	1110, 3869	332, 1025	Billia et al. [41]
Plk1	Polo-like kinase 1	106	122	Coxon et al. [42]
Prkca*	Protein kinase C, alpha	112, 141	130, 250	Clerk et al. [43]
Prkcb*	Protein kinase C, beta	54	85	Bowling et al. [44]
Prkcd	Protein kinase C, delta	271	568	Clerk et al. [43]
Prkce*	Protein kinase C, epsilon	364, 664	170, 215	Clerk et al. [43]
Prkg1	Protein kinase, cGMP-dependent, type I	217	233	Wollert et al. [45]
Ptk2b*	PTK2B protein tyrosine kinase 2 beta	38	137	Bayer et al. [46]
Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	156, 75	164, 98	This study
Ripk2	Receptor-interacting serine-threonine kinase 2	256	189	This study
Rps6ka2	Ribosomal protein S6 kinase polypeptide 2	175	147	Li et al. [47]
Sgk1	Serum/glucocorticoid regulated kinase 1	1335	453	Aoyama et al. [48]
Speg	SPEG complex locus	518	193	Liu et al. [49]
Taf1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor	82	130	Servant et al. [50]
Tec*	Tec protein tyrosine kinase	96	114	Bony et al. [51]
Tgfbr1*	Transforming growth factor, beta receptor 1	69, 90	83, 256	Devaux et al. [52]
Tnni3k	TNNI3 interacting kinase	1304	180	Vagnozzi et al. [53]
Ttn	Titin	4538	773	Hidalgo et al. [54]

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Supplemental Table 5. Protein kinases not detected in ActivX proteomics experiment but detected by microarrays: kinases with protein expression confirmed in hearts in previous studies. We apologise to any other investigators whose work is not mentioned.

* Not detected in proteomics experiment; other kinases were not searched for.
# Identified in global proteomics studies only

Gene symbol	Gene name	Microarrays (raw fluorescence values)		References
		AVMs	NVMs	
Aak1 *#	AP2 associated kinase 1	204, 97, 76, 32, 81	272, 83, 129, 50, 59	Aye et al. [1] Fernandez-Sanz et al. [2] Lundby et al. [3] Su et al. [4]
Abl2 #	v-Abl Abelson murine leukemia viral oncogene 2 (arg, Abelson-related gene)	78	142	Fernandez-Sanz et al. [2] Lundby et al. [3]
Acvrl 1#	Activin A receptor type II-like 1	68	108	Lundby et al. [3]
Adck1 #	Aarf domain containing kinase 1	109	127	Fernandez-Sanz et al. [2] Lundby et al. [3]
Adck3 #	Aarf domain containing kinase 3	1963	282	Lundby et al. [3]
Adck5 #	Aarf domain containing kinase 5	99	136	Fernandez-Sanz et al. [2]
Akt3	v-Akt murine thymoma viral oncogene homolog 3	61, 31, 39	102, 113, 75	Taniyama et al. [5]
Alpk2 #	Alpha-kinase 2	899	402	Su et al. [4]
Axl *	Axl receptor tyrosine kinase	134	690	Batlle et al. [6]
Blk *	B lymphoid tyrosine kinase	70, 332	50, 159	Ping et al. [7]
Bmp2k #	BMP2 inducible kinase	87	235	Lundby et al. [3]
Bub1 #	Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	32	118	Fernandez-Sanz et al. [2]
Camk1g #	Calcium/calmodulin-dependent protein kinase IG	121	57	Fernandez-Sanz et al. [2]
Camk2a *	Calcium/calmodulin-dependent protein kinase II alpha	151	76	Aye et al. [1] Lundby et al. [3] Bayer et al. [8]
Cdc42bpa #	CDC42 binding protein kinase alpha	126	163	Fernandez-Sanz et al. [2] Lundby et al. [3]
Cdk12 #	Cyclin-dependent kinase 12	106, 39	160, 80	Fernandez-Sanz et al. [2] Lundby et al. [3]
Cdk14 #	Cyclin-dependent kinase 14	341, 43	481, 100	Fernandez-Sanz et al. [2]
Cdk18 #	Cyclin-dependent kinase 18	143, 106	89, 103	Fernandez-Sanz et al. [2]
Epha5 #	Eph receptor A5	85	44	Lundby et al. [3]
Epha6	Eph receptor A6	53	49	DuSablon et al. [9]
Fastk #	Fas-activated serine/threonine kinase	528	376	Fernandez-Sanz et al. [2]
Fastkd2 #	FAST kinase domains 2	201	165	Fernandez-Sanz et al. [2]
Fastkd5 #	FAST kinase domains 5	98	120	Fernandez-Sanz et al. [2]
Fgr *	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	63	79	Ping et al. [7]
Gak *#	Cyclin G associated kinase	151	520	Lundby et al. [3]
Hipk1 #	Homeodomain interacting protein kinase 1	381	471	Fernandez-Sanz et al. [2]
Hipk3 #	Homeodomain interacting protein kinase 3	231, 88	232, 47	Lundby et al. [3]
Hunk #	Hormonally upregulated Neu-associated kinase	80	91	Fernandez-Sanz et al. [2]
Irak3 #	Interleukin-1 receptor-associated kinase 3	37	334	Fernandez-Sanz et al. [2]
Jak3 *#	Janus kinase 3	105	166	Lundby et al. [3]
Kalrn #	Kalirin, RhoGEF kinase	187, 206	124, 240	Lundby et al. [3]

Lrrk1 #	Leucine-rich repeat kinase 1	78	108	Fernandez-Sanz et al. [2]
Map2k5	Mitogen activated protein kinase kinase 5	137	250	Nicol et al. [10] Lundby et al. [3]
Map3k14 #	Mitogen-activated protein kinase kinase kinase 14	102	145	Lundby et al. [3]
Mapk4	Mitogen-activated protein kinase 4	55	86	Dingar et al. [11]
Mapk6	Mitogen-activated protein kinase 6	461	722	Dingar et al. [11]
Mapkapk 3 *	Mitogen-activated protein kinase-activated protein kinase 3	155	139	Aye et al. [1] Moise et al. [12]
Mapkapk 5	Mitogen-activated protein kinase-activated protein kinase 5	148	174	Dingar et al. [11]
Melk #	Maternal embryonic leucine zipper kinase	37	86	Lundby et al. [3]
Mylk2 *#	Myosin light chain kinase 2	376	295	Lundby et al. [3]
Mylk4	Myosin light chain kinase family, member 4	104	109	Herrer et al. [13]
Nek3 #	NIMA-related kinase 3	82	126	Lundby et al. [3]
Pak3	p21 protein (Cdc42/Rac)-activated kinase 3	49	64	Buscemi et al. [14]
Pak4	p21 protein (Cdc42/Rac)-activated kinase 4	134	205	Nekrasova et al. [15]
Pak6 #	p21 protein (Cdc42/Rac)-activated kinase 6	251	161	Lundby et al. [3]
Pdk3 #	Pyruvate dehydrogenase kinase, isozyme 3	56, 46	283, 206	Fernandez-Sanz et al. [2] Lundby et al. [3]
Peak1 #	NKF3 kinase family member	102	175	Fernandez-Sanz et al. [2] Lundby et al. [3]
Pragmin #	Pragma of Rnd2	39	68	Lundby et al. [3]
Prkcg *	Protein kinase C, gamma	69	54	Liu et al. [16]
Prkch	Protein kinase C, eta	79, 154	49, 74	Lundby et al. [3] Ping et al. [17]
Prkx	Protein kinase, X-linked	129, 101	214, 133	Li et al. [18]
Riok1 #	RIO kinase 1 (yeast)	102	165	Lundby et al. [3]
Ripk4 *#	Receptor-interacting serine-threonine kinase 4	98	98	Lundby et al. [3]
Ror1 #	Receptor tyrosine kinase-like orphan Receptor 1	20	130	Fernandez-Sanz et al. [2]
Srpk2 #	SRSF protein kinase 2	206	372	Fernandez-Sanz et al. [2] Lundby et al. [3]
Srpk3 #	SRSF protein kinase 3	218	102	Lundby et al. [3]
Tek #	TEK tyrosine kinase, endothelial	327	203	Lundby et al. [3]
Tie1 *	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1	512	252	Fernandez-Sanz et al. [2] Shyu [19]
Trib3	Tribbles homolog 3 (Drosophila)	19, 20, 53	191, 332, 295	Ti et al. [20]
Trpm7 #	Transient receptor potential cation channel, subfamily M, member 7	97, 99	234, 278	Lundby et al. [3]
Wnk3 #	WNK lysine deficient protein kinase 3	39	55	Fernandez-Sanz et al. [2]

Supplemental Table 5. References

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Supplemental Table 6. Protein kinases not detected in ActivX proteomics experiment but detected by microarrays: kinases with mRNA expression only confirmed in hearts in previous studies. We apologise to any other investigators whose work is not mentioned.

* Not detected in proteomics experiment; other kinases were not searched for.

Gene symbol	Gene name	Microarrays (raw fluorescence values)		mRNA expression	Reference
		AVMs	NVMs		
Clk1	CDC-like kinase 1	832	465	+++	Xie et al. [1]
Clk4	CDC-like kinase 4	173	211	+++	Schultz et al. [2]
Dapk2	Death-associated kinase 2	110	71	+++	Kawai et al. [3]
Dclk2*	Doublecortin-like kinase 2	142	145	+	Ohmae et al. [4]
Ddr1	Discoidin domain receptor tyrosine kinase 1	134, 253	284, 532	+	Di Marco et al. [5]
Dstyk	Dual serine/threonine and tyrosine protein kinase	55, 64, 45	118, 111, 95	++	Peng et al. [6]
Epha4	Eph receptor A4	94, 39, 48, 46	153, 51, 56, 88	+	Dries et al. [7]
Epha7*	Eph receptor A7	106, 34, 226	120, 56, 149	+	Dries et al. [7]
Flt4	Fms-related tyrosine kinase 4	91	67	+	Aprelikova et al. [8]
Hipk4	Homeodomain interacting protein kinase 4	106	76	++	Arai et al. [9]
Ick	Intestinal cell kinase	154	303	++	Abe et al. [10]
Mok	MOK protein kinase	99	105	+	Miyata et al. [11]
Pask	PAS domain containing serine/threonine kinase	91	90	+	Miao et al. [12]
Pbk	PDZ binding kinase	39	106	++	Gaudet et al. [13]
Prkg2	Protein kinase, cGMP-dependent, type II	30	68	+	Uhler et al. [16]
Pxk	PX domain containing serine/threonine kinase	84, 134	198, 293	++	Mao et al. [17]
Ros1	c-Ros oncogene 1 , receptor tyrosine kinase	103	40	+++	Matsushime et al. [18]
Ryk	Receptor-like tyrosine kinase	260, 334	624, 710	+	Wang et al. [19]
Stk17b	Serine/threonine kinase 17b	73, 66, 170	246, 182, 281	+++	Sanjo et al. [20]
Stk35	Serine/threonine kinase 35	173	170	++	Vallenius et al. [21]
Stk36	Serine/threonine kinase 36	150, 413	99, 247	+	Osterlund et al. [22]
Trib1	Tribbles homolog 1	312, 95, 621	105, 45, 209	++	Okamoto et al. [23]
Trib2	Tribbles homolog 2	129, 306	214, 565	+++	Okamoto et al. [23]
Trio	Triple functional domain (PTPRF interacting)	41, 89, 183, 210	109, 208, 380, 472	+++	Debant [24]
Tssk2	Testis-specific serine kinase 2	61	39	+	Hao et al. [25]
Uhmk1	U2AF homology motif (UHM) kinase 1	225, 94	413, 121	+	Maucuer et al. [26]
Ulk2	Unc-51 like kinase 2	160	161	+++	Yan et al.[27]
Vrk1	Vaccinia related kinase 1	46	134	+	Nezu et al. [28]

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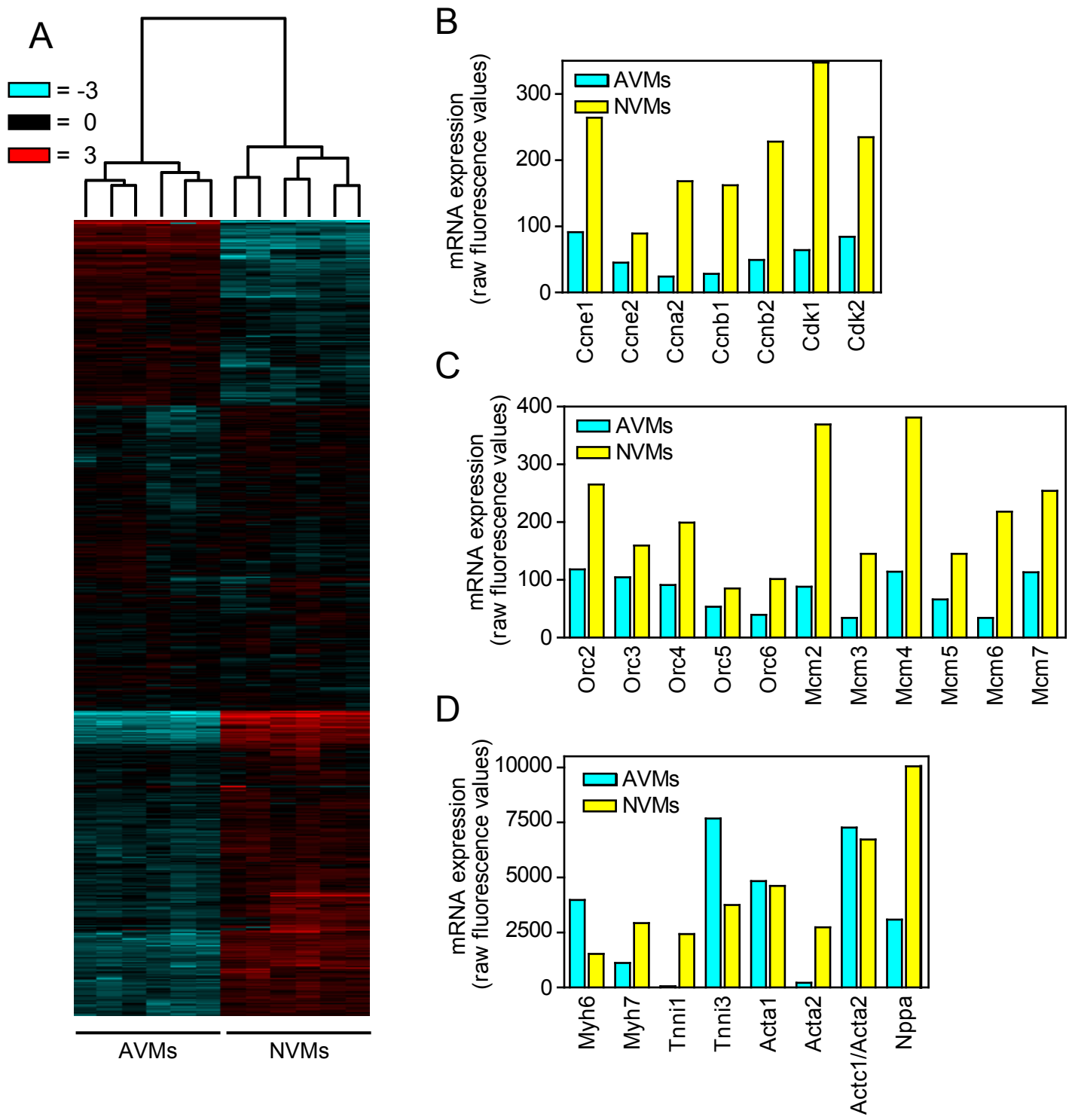
Supplemental Table 7. Protein kinases not detected in ActivX proteomics experiment but detected by microarrays: kinases for which there are no additional publications on expression in heart.

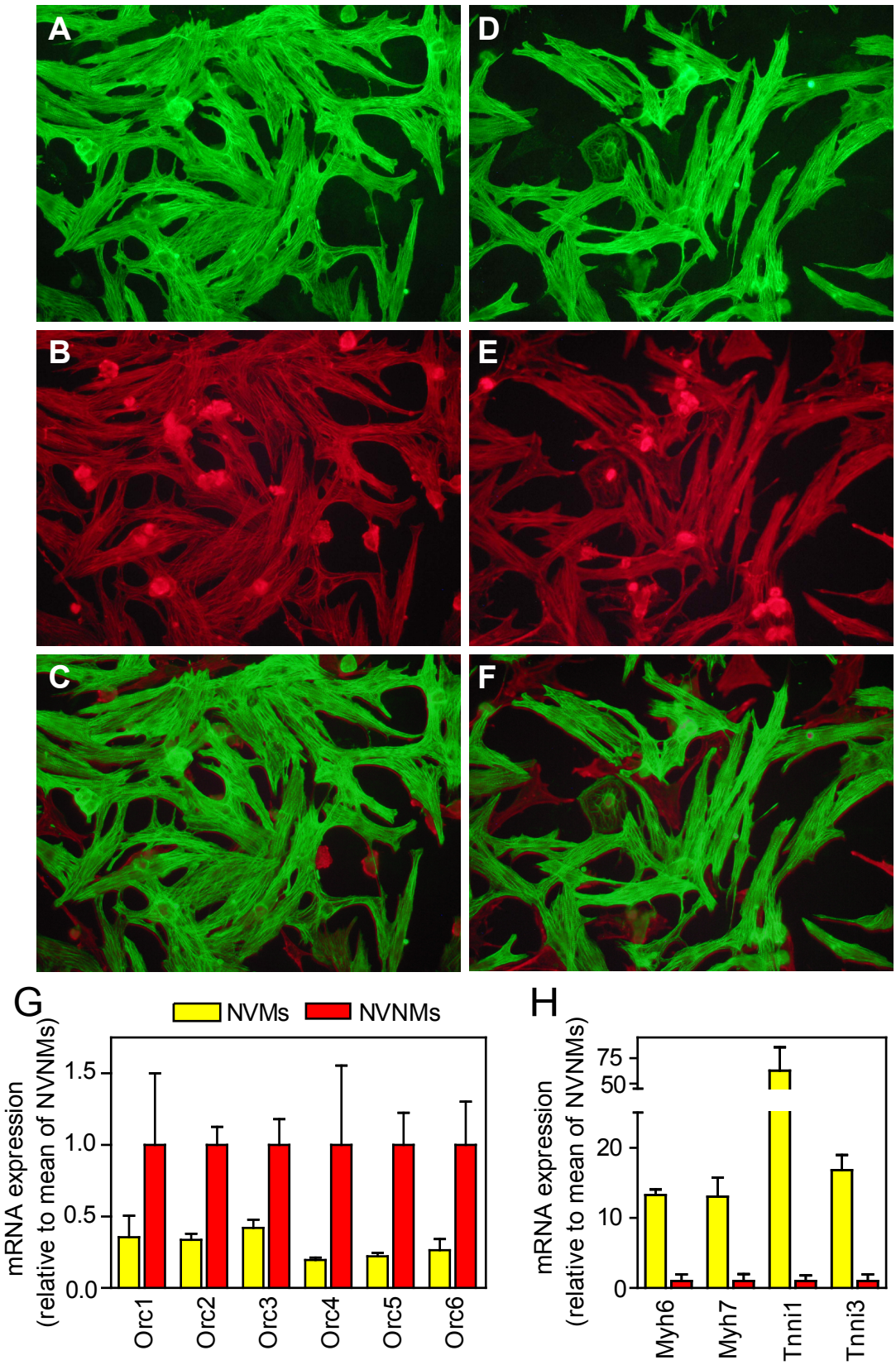
* Not detected in proteomics experiment; other kinases were not searched for.

Gene symbol	Gene name	Microarrays (raw fluorescence values)	
		AVMs	NVMs
Acvr1	Activin A receptor, type I	248, 469, 96	393, 265, 163
Acvr2a	Activin A receptor, type IIA	103, 127	190, 287
Acvr2b	Activin A receptor, type IIB	123	143
Adck2	Aarf domain containing kinase 2	103	105
Adck4	Aarf domain containing kinase 4	147	285
Amhr2	Anti-Mullerian hormone receptor, type II	153	51
Aurkc	Aurora kinase C	133	169
Bckdk	Branched chain ketoacid dehydrogenase kinase	755	514
Bcr	Breakpoint cluster region	322, 274	298, 299
Brsk1	BR serine/threonine kinase 1	37	179
Brsk2	BR serine/threonine kinase 2	147	224
Bub1b	Budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	33	65
Camkk1	Calcium/calmodulin-dependent protein kinase kinase 1, alpha	87	122
Camkv	CaM kinase-like vesicle-associated	45, 112	54, 113
Cdk19	Cyclin-dependent kinase 19	162	173
Cdkl3	Cyclin-dependent kinase-like 3	45	50
Dapk1	Death associated protein kinase 1	60, 24	516, 289
Epha10	EPH receptor A10	30	53
Epha8	Eph receptor A8	153	92
Ephb6	Eph receptor B6	73	75
Fastkd3	FAST kinase domains 3	73	114
Gak*	Cyclin G associated kinase	151	520
Grk1	G protein-coupled receptor kinase 1	75	62
Hck*	Hemopoietic cell kinase	60	111
Hipk2	Homeodomain interacting protein kinase 2	1190, 109, 310, 814	509, 83, 129, 268
Insrr	Insulin receptor-related receptor	141	200
Jak3*	Janus kinase 3	105	166
Limk1*	LIM domain kinase 1	88	105
Limk2	LIM domain kinase 2	88	105
Map3k12*	Mitogen activated protein kinase kinase kinase 12	353	465
Map3k9*	Mitogen-activated protein kinase kinase kinase 9	70	86
Mapk15	Mitogen-activated protein kinase 15	83	67
Matk	Megakaryocyte-associated tyrosine kinase	75	68
Mertk*	c-Mer proto-oncogene tyrosine kinase	65	101
Mylk2*	Myosin light chain kinase 2	376	295
Nrbp2	Nuclear receptor binding protein 2	318	379
Nrk	Nik related kinase	19	269
Pdgfra	Platelet derived growth factor receptor, alpha polypeptide	66	1244
Pik3r4	Phosphoinositide-3-kinase, regulatory subunit 4	144	277
Pkdcc	Protein kinase domain containing, cytoplasmic homolog (mouse)	344	310
Pkn3	Protein kinase N3	258	196
Plk3	Polo-like kinase 3	126	52
Plk4	Polo-like kinase 4	27	76
Plk5	Polo-like kinase 5	89	69
Pskh1	Protein serine kinase H1	259	213

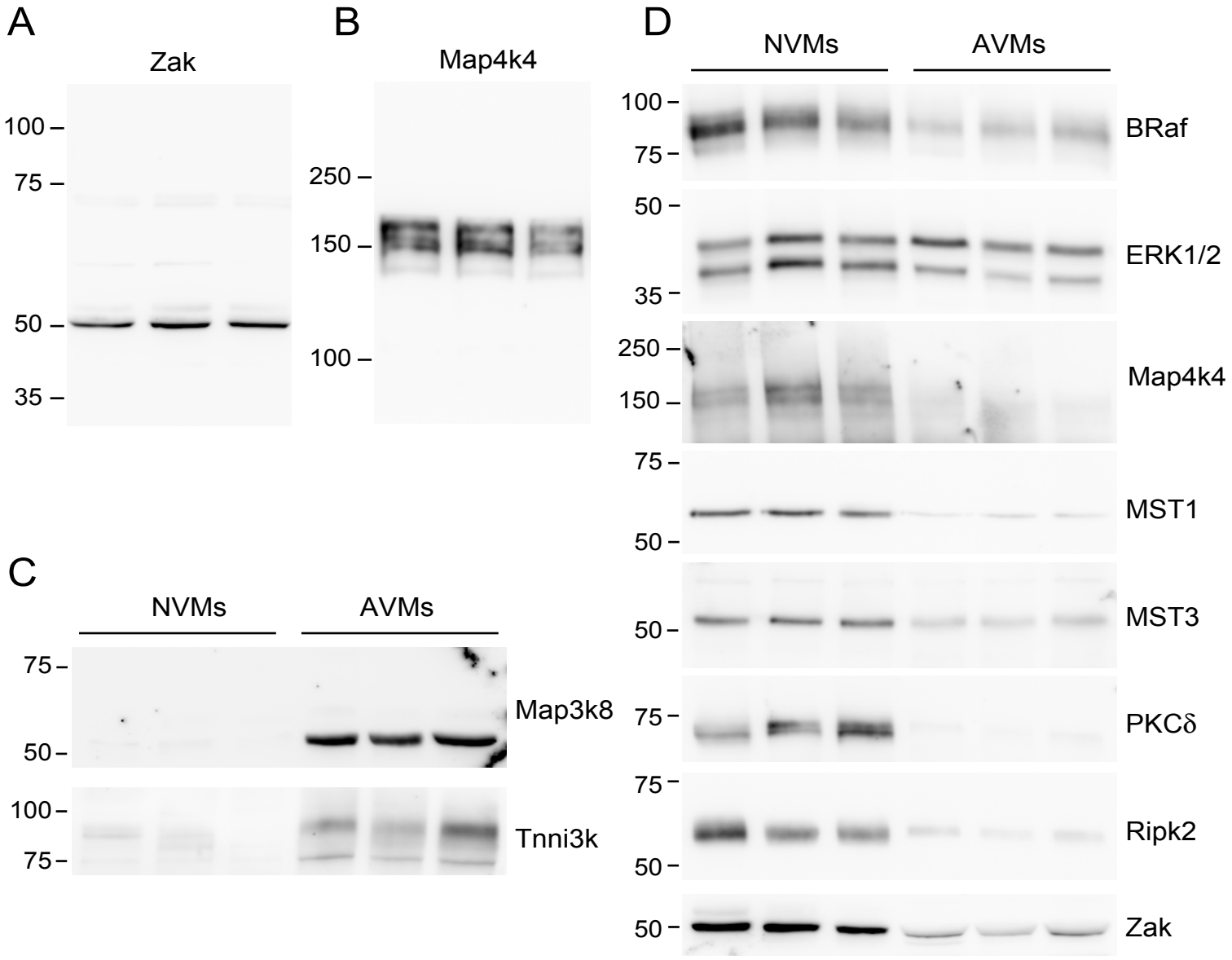
Ptk7	PTK7 protein tyrosine kinase 7	41	779
Ret	Ret proto-oncogene	51, 64, 77	76, 35, 162
Riok2	RIO kinase 2 (yeast)	79	120
Riok3	RIO kinase 3 (yeast)	283, 132, 163	728, 259, 353
Sbk1	SH3-binding domain kinase 1	137, 238	75, 108
Scyl1	SCY1-like 1 (S. cerevisiae)	152, 325	204, 432
Scyl2	SCY1-like 2 (S. cerevisiae)	139	248
Scyl3	SCY1-like 3 (S. cerevisiae)	61	69
Sgk196	Protein kinase-like Protein SgK196	113, 53, 102	197, 62, 253
Sik2*	Salt-inducible kinase 2	54	76
Stk19	Serine/threonine kinase 19	38	107
Stk32c	Serine/threonine kinase 32C	53	38
Stk40	Serine/threonine kinase 40	272	207
Stradb	STE20-related kinase adaptor beta	418	263
Tbrg4	Transforming growth factor beta regulator 4	496, 198	401, 171
Tesk2	Testis-specific kinase 2	50	69
Tex14	Testis expressed 14	69, 90	83, 256
Tp53rk	TP53 regulating kinase	59, 54, 98	72, 108, 118
Ttbk2*	Tau tubulin kinase 2	83	90
Ttk	Ttk protein kinase	38	73
Tyro3*	TYRO3 protein tyrosine kinase	29	127
Vrk3	Vaccinia related kinase 3	371	316
Zap70*	Zeta-chain (TCR) associated protein kinase 70	209, 81	123, 55



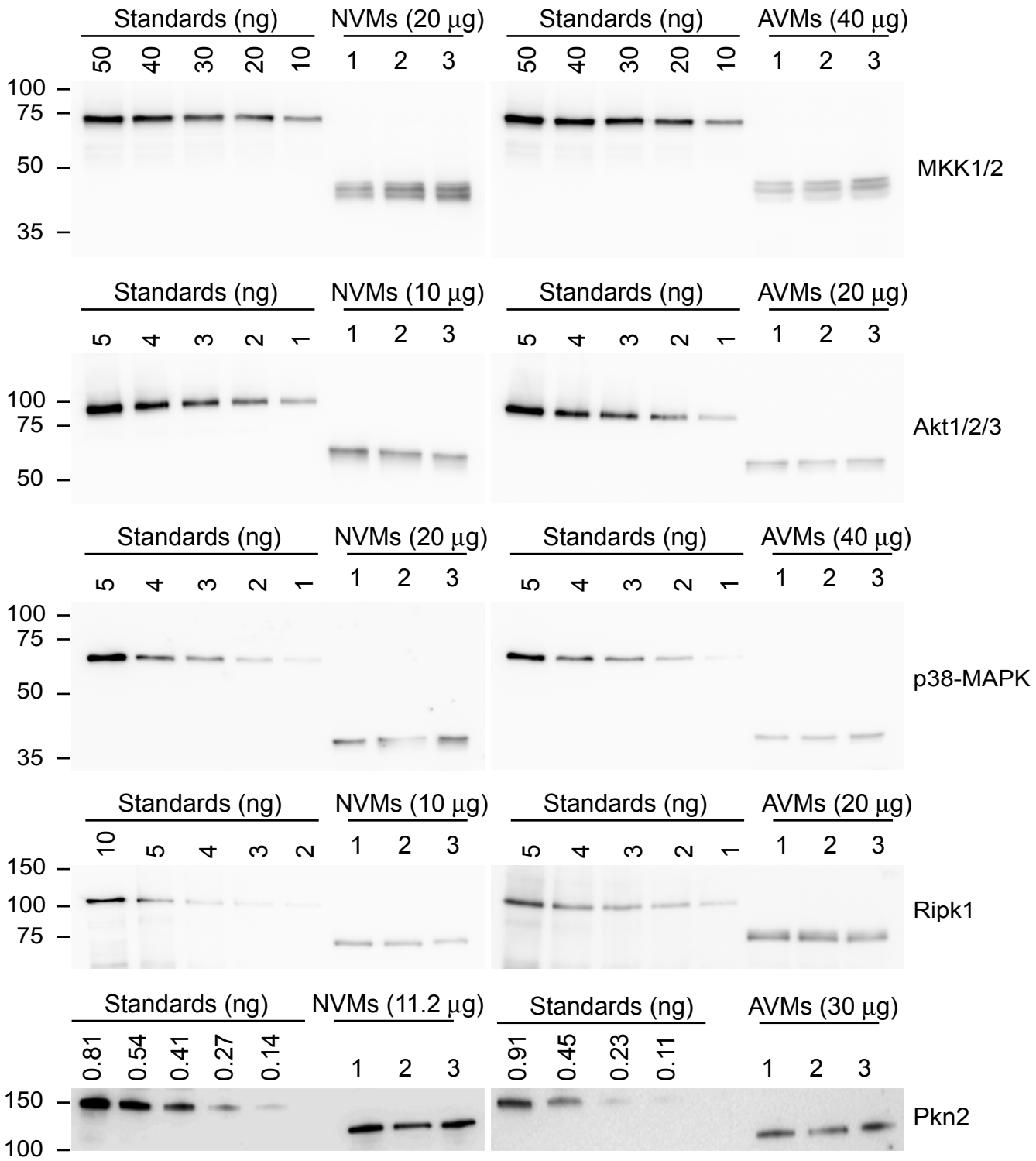
Supplemental Figure 1. mRNA expression profiling in rat adult ventricular myocytes (AVMs) and neonatal ventricular myocytes (NVMs). Affymetrix microarrays were used for mRNA expression profiling of AVMs or NVMs (n=3, duplicate hybridisations) with GeneSpring for data analysis. A, Heatmap for relative expression of all expressed mRNAs detected in AVMs and/or NVMs. Data were normalised to the gene median and are individual values on a log2 scale. Hierarchical clustering used a Euclidean similarity measure with Ward's linkage rule. B-D, Expression data for cell cycle-dependent genes (B), genes required for DNA replication (C) and genes established to be regulated during postnatal cardiomyocyte development (D). Results are mean raw fluorescence values for NVMs (yellow) and AVMs (cyan).



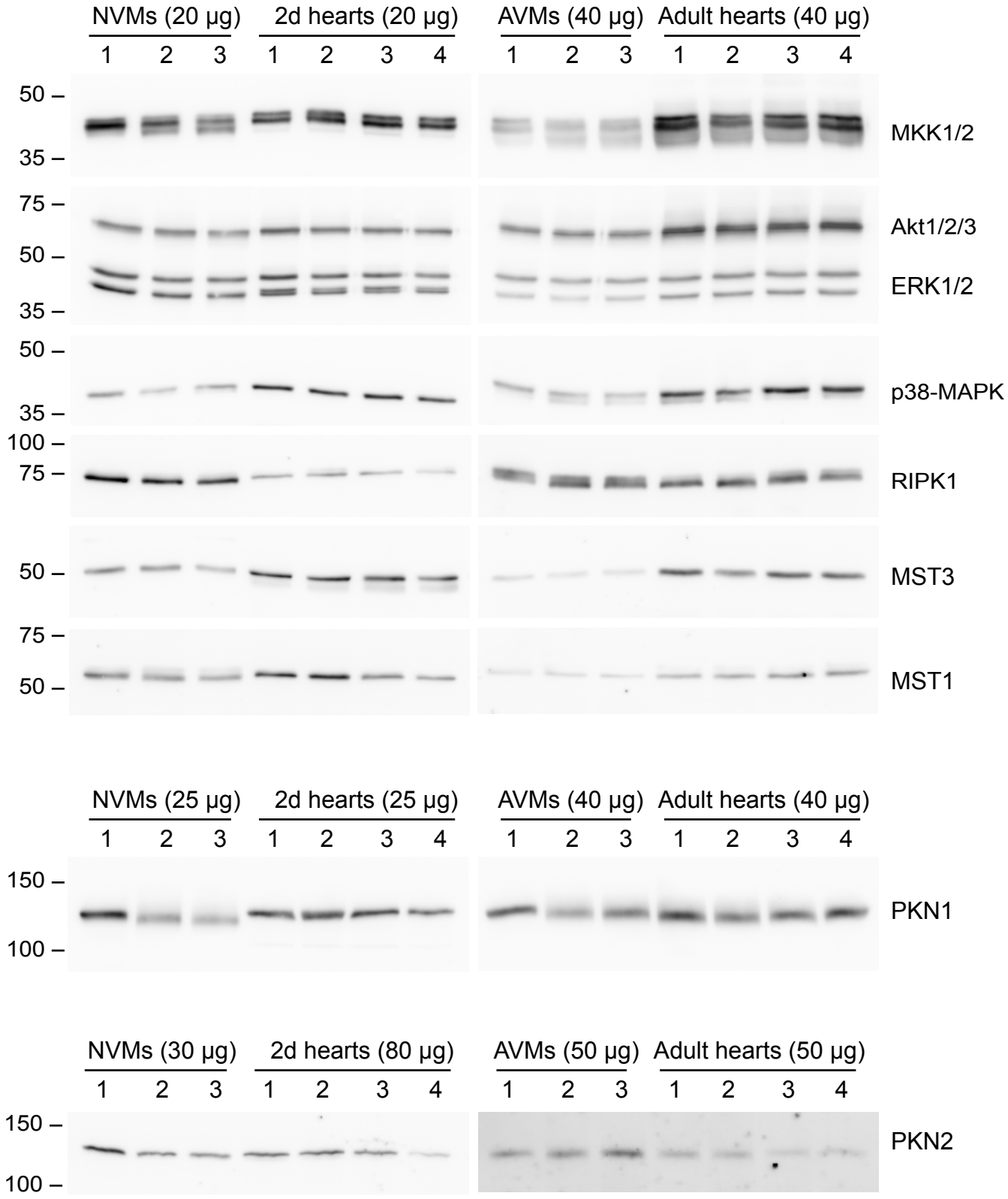
Supplemental Figure 2. Comparison of neonatal cardiomyocytes and cardiac non-myocytes. Cells in the NVM cultures were immunostained with antibodies to troponin T to identify cardiomyocytes (green, A and D) and counterstained with phalloidin to identify all cardiac cells (red, B and E). Images from A and D were overlaid onto B and E, respectively, in panels C and F. Results are representative of multiple experiments. G and H, qPCR analysis of Orc1-6 (G) and myocyte-specific gene expression (H, yellow bars) in NVMs and neonatal ventricular non-myocytes (NVNMs, red bars). Results (relative to Gapdh and normalised to the mean of values in NVNMs) are means ± SEM (n=3).



Supplemental Figure 3. Protein expression of selected protein kinases in rat adult ventricular myocytes (AVMs) and neonatal ventricular myocytes (NVMs). NVM and AVM extracts (20 µg and 40 µg, respectively) were immunoblotted with antibodies to the indicated protein kinases. A, Zak was detected in NVMs as the smaller isoform previously referred to as MLK7 (~52 kDa) rather than the longer isoform (~92 kDa). B, Map4k4 was detected as multiple isoforms in NVMs. C, Map3k8 and Tnni3k were more abundant in AVMs than NVMs. D, BRaf, ERK1/2, Map4k4, MST1, MST3, PKCδ, Ripk2 and Zak were more highly expressed in NVMs than AVMs. Each lane represents an independent cardiomyocyte preparation.



Supplemental Figure 4. Quantitative immunoblotting of selected protein kinases in rat adult ventricular myocytes (AVMs) and neonatal ventricular myocytes (NVMs). Proteins from individual cardiomyocyte preparations were immunoblotted for MKK1/2, Akt1/2/3, p38-MAPK, Ripk1 and Pkn2 alongside GST fusion protein standards of known concentration.



Supplemental Figure 5. Protein expression of selected protein kinases in cardiomyocytes compared with whole hearts. Proteins from individual cardiomyocyte or cardiac preparations were immunoblotted for MKK1/2, Akt1/2/3, ERK1/2, p38-MAPK, Ripk1 MST1, MST2, Pkn1 and Pkn2.