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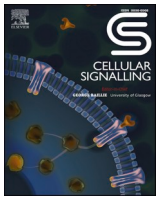

Mitogen-activated protein kinase signalling in rat hearts during postnatal development: MAPKs, MAP3Ks, MAP4Ks and DUSPs

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ABSTRACT

Mammalian cardiomyocytes become terminally-differentiated during the perinatal period. In rodents, cytokinesis ceases after a final division cycle immediately after birth. Nuclear division continues and most cardiomyocytes become binucleated by ~11 days. Subsequent growth results from an increase in cardiomyocyte size. The mechanisms involved remain under investigation. Mitogen-activated protein kinases (MAPKs) regulate cell growth/death: extracellular signal-regulated kinases 1/2 (ERK1/2) promote proliferation, whilst c-Jun N-terminal kinases (JNKs) and p38-MAPKs respond to cellular stresses. We assessed their regulation in rat hearts during postnatal development (2, 7, 14, and 28 days, 12 weeks) during which time there was rapid, substantial downregulation of mitosis/cytokinesis genes (*Cenpa/e/f, Aurkb*, *Anln*, *Cdca8*, *Orc6*) with lesser downregulation of DNA replication genes (*Orcs1–5*, *Mcms2–7*). MAPK activation was assessed by immunoblotting for total and phosphorylated (activated) kinases. Total ERK1/2 was downregulated, but not JNKs or p38-MAPKs, whilst phosphorylation of all MAPKs increased relative to total protein albeit transiently for JNKs. These profiles differed from activation of Akt (also involved in cardiomyocyte growth). Dual-specificity phosphatases, upstream MAPK kinase kinases (MAP3Ks), and MAP3K kinases (MAP4Ks) identified in neonatal rat cardiomyocytes by RNASeq were differentially regulated during postnatal cardiac development. The MAP3Ks that we could assess by immunoblotting (RAF kinases and Map3k3) showed greater downregulation of the protein than mRNA. MAP3K2/MAP3K3/MAP4K5 were upregulated in human failing heart samples and may be part of the "foetal gene programme" of re-expressed genes in disease. Thus, MAPKs, along with kinases and phosphatases that regulate them, potentially play a significant role in postnatal remodelling of the heart.

1. Introduction

Mammalian cardiomyocytes become terminally-differentiated soon after birth [1–3]. The current consensus is that cardiomyocyte proliferation in the adult mammalian heart is <1 % per annum and, whilst this may suffice for general maintenance, it is not physiologically meaningful for cardiac repair in disease. Induction of cardiomyocyte cell cycle re-entry is an attractive therapeutic concept for cardiac regeneration, but greater understanding of the molecular basis of cell cycle withdrawal and prevention of re-entry is required.

Cardiomyocyte terminal differentiation occurs in stages, and nuclear division (endomitosis) can continue after cessation of cytokinesis, producing binucleated cells [2]. In rats and mice, cardiomyocyte cell division ceases by ~4 days postnatally, nuclear division is completed by ~11–14 days and 90–95 % of adult cardiomyocytes are binucleated. The principle holds for other species and, for example, binucleation occurs in 40–60 % of human cardiomyocytes [2,4]. After birth, maturational growth of the heart results from an increase in size of individual myocytes (i.e. hypertrophy or eutrophy) [5]. Adult cardiomyocytes hypertrophy to accommodate increases in workload as occurs during

Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; DMEM, Dulbecco's modified Eagles Medium; DUSP, dual-specificity phosphatase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; fpkm, Fragments Per Kilobase per Million mapped fragments; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAP3K, MKK kinase; MAP4K, MAP3K kinase; MCM, minichromosome maintenance; MEKK, MAPK/ERK kinase kinase; MKK, MAPK kinase; NGF, nerve growth factor; ORC, origin recognition complex; RNASeq, RNA sequencing; SDS, sodium dodecyl sulphate; TBS, Tris-buffered saline; TBST, TBS containing 0.1 % Tween 20.

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pregnancy or exercise (physiological hypertrophy), or with pathophysiological stresses (e.g. hypertension, myocardial infarction) [5,6]. This is associated with increases in cardiomyocyte size and myofibrillar content. In addition, there are changes in gene expression in pathological hypertrophy including re-expression of genes normally expressed early in development (the "foetal gene programme") [1,5].

In proliferating cells, cell cycle entry is promoted by the extracellular signal-regulated kinases 1/2 (ERK1/2), the prototypic mitogen-activated protein kinases (MAPKs), and ERK1/2 play an important role throughout the cell cycle [7,8]. Progression through the cell cycle and mitosis is regulated by cyclin-dependent kinases (CDKs) activated by cyclins [9,10]. Checkpoints ensure that each stage of the process is properly completed: cell growth and DNA replication are completed before mitosis commences, and completion of mitosis is generally linked to nuclear division and cytokinesis. Mechanisms operate to prevent re-replication of DNA without mitosis, and further nuclear division without cytokinesis. Thus, DNA replication is "licensed" with origin recognition proteins (ORCs) acting at initiation start sites for DNA replication [11], and DNA replication is controlled by systems including the minichromosome maintenance (MCM) replicative DNA helicase complex [12]. Mitosis requires, for example, expression of centromere proteins to attach chromosomes to the spindle (e.g. Cenpa, Cenpe, Cenpf [13–15]), whilst cytokinesis requires induction of aurora kinases (e.g. Aurkb), borealin (Cdca8) and anilin (Anln) [16–18]. Thus, the process of cell division is generally well understood, but how this relates to terminal differentiation remains to be established.

Initial studies demonstrated that cyclins/CDKs are downregulated and CDK inhibitors (CDKIs) are upregulated in cardiomyocytes in the early postnatal period [19,20]. Many studies have since focused on specific transcription factors and signalling pathways [21], and recent "omics" approaches report global changes in cardiomyocyte gene expression during postnatal development (e.g. [22–27]). However, phenotypic changes are influenced by post-translational modifications (e.g. protein phosphorylation/dephosphorylation) regulated by intracellular signalling networks. The relatively little emphasis on MAPKs in cardiomyocyte terminal-differentiation is, therefore, surprising. Studies of ERK1/2 in the heart focus on their role in promoting cardiomyocyte hypertrophy and cardioprotection [28–31], but the degree/duration of ERK1/2 signalling influences proliferation vs differentiation in other cells [8] and they may play a role in cardiomyocyte terminal differentiation. Other MAPKs, c-Jun N-terminal kinases (JNKs) and p38-MAPKs, respond to cellular stresses and their role(s) in cardiac hypertrophy and cell death are being established [28,32,33]. With the stresses on the cardiovascular system following birth, these kinases may also be expected to be activated and affect cardiomyocyte differentiation.

MAPKs are activated by dual-specificity MAPK kinases (MKKs) that phosphorylate Thr and Tyr residues in a T-X-Y motif (where X is any amino acid) [7]. These MKKs are well-established: ERK1/2 are activated by MKK1/2, JNKs are activated by MKK4/7, and p38-MAPKs are activated by MKK3/6 [28]. MKKs are phosphorylated and activated by MKK kinases (MAP3Ks). RAF family kinases are MAP3Ks for the ERK1/2 cascade [34], but various MAP3Ks operate for JNKs and p38-MAPKs, potentially being activated by different types of stress [35]. MAP3Ks may be regulated by MAP3K kinases (MAP4Ks) but their regulation and activities are not well-understood, and activation of MAPK signalling by MAP4Ks may result from increased cellular stress [36]. MAPK inactivation is equally as important for regulatory signalling, and the dual-specificity phosphatase family (DUSPs) includes MAPK phosphatases that dephosphorylate both phospho-Thr and phospho-Tyr residues required for activity [37,38].

Here, we investigated the regulation and activation of ERK1/2, JNKs and p38-MAPKs in postnatal development of rat hearts, placing their activation in the context of changes in expression of genes required for DNA replication and cytokinesis. We compared MAPK profiles with activation of Akt (also known as protein kinase B), a key protein kinase implicated in cardiac growth and cytoprotection [39]. These kinases were all activated during postnatal development but with different temporal profiles. We determined the expression profiles of DUSPs and MAP3Ks/MAP4Ks that may influence MAPK activation during rat postnatal cardiac development, also assessing which MAP3Ks/MAP4Ks are regulated in human heart failure. We conclude that MAPKs and the kinases/phosphatases that regulate them, are likely play a significant role in postnatal remodelling of the heart.

2. Materials and methods

2.1. Ethics statement and tissue samples

2.1.1. Human heart samples

Human heart samples were obtained from the University of Pittsburgh, U.S.A. and were previously reported in [40]. Left ventricular samples from failing human hearts were from patients who consented to a protocol approved by the University of Pittsburgh Institutional Review Board and collected under IRB 0404033. Non-failing heart samples were collected under University of Pittsburgh CORID #451 (Committee for Oversight of Research and Clinical Training Involving Decedents), with consent being obtained by the local Organ Procurement Organization (OPO), CORE (Center for Organ Recovery and Education). Samples were transferred to University of Reading via a Biological Material Transfer Agreement (University of Pittsburgh MTA00009000) for the study of MAP3Ks and MAP4Ks and the proteins with which they interact. Samples were stored at University of Reading as frozen, de-identified acellular non-viable samples for which further approvals are not required. Patient information is provided in Supplementary Table S1.

2.1.2. Postnatal rat heart development

Rats were brought into the BioResource Unit at University of Reading (UK registered with a Home Office certificate of designation; establishment licence no. XF75DBBCB). Procedures were performed in accordance with UK regulations and the European Parliament Directive 2010/63/EU for animal experiments. All work was undertaken in accordance with local institutional animal care committee procedures at the University of Reading and the U.K. Animals (Scientific Procedures) Act 1986. Rats were culled by a schedule 1 procedure (cervical dislocation followed by removal of the heart) for which additional Home Office approvals are not required.

Sprague-Dawley CD® IGS rats were purchased from Charles River (Crl:CD(SD)). Female rats were purchased for delivery with 2 d neonates (n = 6 litters; 10 pups per litter). Male and female pups were used equally for the 2, 7 and 14 d samples and each sample used for analysis of a specific time point was from a different litter. For the 2 d samples, pups were sacrificed immediately (4 hearts per sample). Remaining pups were housed with the females and sacrificed at 7 and 14 d (3 hearts per sample). Male rats were used for the 28 d and 12 week time points and these were sacrificed immediately on delivery. Females with litters were housed in open top NKP cages (total area 1632 cm^2) supplied with aspen sawdust bedding, sizzle nesting, cardboard tunnels and housing plus additional enrichment (e.g. chew sticks and millet). Animals were provided with water and food (SDS RM3 expanded pelleted food) ad libitum, with a 12:12 light/dark cycle and room temperature of 21 °C. For tissue harvest, the hearts were cut open, briefly rinsed with ice-cold PBS, blotted dry and snap frozen in liquid N_2. Hearts were ground to powder under liquid nitrogen and samples taken for preparation of RNA and proteins.

2.2. Rat neonatal ventricular cardiomyocytes

Female Sprague-Dawley CD® IGS rats with 2–3 day litters were delivered and housed overnight in the BioResource Unit as described in Section 2.1. Neonates (males and females from at least 2 different litters per preparation) were culled at 3–4 days. Rat neonatal ventricular cardiomyocytes were prepared as described in [41]. In brief, hearts were

harvested, the atria were removed and ventricles were washed in ice-cold buffer. Heart tissue was subjected to sequential digestion with collagenase and pancreatin, and the cells were collected into plating medium [Dulbecco's Modified Eagles Medium (DMEM) and M199 at a ratio of 4:1] containing 15 % (*v*/v) foetal calf serum with penicillin and streptomycin. To remove cardiac non-myocytes, cells were preplated onto Primaria tissue culture dishes (30 min, 37 °C) at a density equivalent to 10 hearts per 60 mm dish. Non-adherent cardiomyocytes were plated in plating medium onto 60 mm Primaria tissue culture plates precoated with 1 % (*w*/*v*) gelatin (Sigma-Aldrich). Cells were seeded at a density of 4×10^6 cells per dish to ensure that the final cultures were confluent and beating. Serum was withdrawn after 16 h, and the cells were cultured for a further 20 h prior to harvest. This approach minimises the growth of cardiac non-myocytes (estimated at ~5 % of the total cell population [41]). We routinely use cardiomyocytes from 3 to 4 day neonates because they are the most mature cardiomyocytes we can culture routinely for intracellular signalling studies. They are sufficiently robust with enough plasticity for quiescence to be imposed with complete removal of serum and growth factors, whilst maintaining survival. Cardiomyocytes from older animals require addition of growth factors (e.g. insulin) that affect protein kinase activation and gene expression.

2.3. RNA preparation from heart tissue and qPCR

Rat heart powders or human left ventricular samples (10–20 mg) were weighed into Eppendorf Safe-Lock tubes on dry ice. RNA Bee (1 ml; AMS Biotechnology Ltd) was added as the tube was brought to 4 °C and powders were homogenised in the tubes on ice using a plastic pestle. The RNA was prepared according to the manufacturer's instructions and as described in [41]. RNA was dissolved in nuclease-free water and the purity assessed from the A_{260}/A_{280} measured using an Implen NanoPhotometer (values were 1.8–2.0). Concentrations were determined from the A_{260} and RNA was stored at −80 °C. Total RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription Kits with random primers (Applied Biosystems) in a final volume of 20 μl according to the manufacturer's instructions. cDNAs were diluted 1:1 with nuclease-free water and stored at −20 °C. qPCR was performed with a StepOnePlus Real-Time PCR system (ThermoFisher Scientific) using 1 μl of the cDNA. Optical 96-well reaction plates were used with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories Inc.) according to the manufacturer's instructions (see Supplementary Table S2 for primer sequences). Results were normalised to *Gapdh*, and relative quantification was obtained using the ΔCt (threshold cycle) method; relative expression was calculated as $2^{-\Delta\Delta Ct}$ [42] and normalised to the mean of the 2 d values (rat hearts) or the mean of the control hearts (patients).

2.4. RNA preparation from cardiomyocytes and RNASeq

Cardiomyocytes were prepared from 3 to 4 d neonates as described in Section 2.2. RNA was prepared using 1 ml RNA Bee per dish according to the manufacturer's instructions. RNA was provided to Novogene for RNA sequencing using their Illumina platform. Processed data for all samples ($n = 4$) are provided in Supplementary Spreadsheet S1 as original counts and fpkm (Fragments Per Kilobase per Million mapped fragments). Fpkm are defined as the number of fragments of a gene divided by the total sequencing depth, and the ratio is then divided by the gene length (i.e. the total length of exons from one gene).

2.5. Western blotting

Rat heart powders (15–20 mg) were homogenised in 6 vol extraction buffer [20 mM Tris pH 7.5, 1 mM ethylene diamine tetra-acetic acid, 10 % (*v*/v) glycerol, 1 % (v/v) Triton X-100, 100 mM KCl, 5 mM NaF, 0.2 mM Na_3VO_4, 5 mM $MgCl_2$, 0.05 % (v/v) 2-mercaptoethanol, 10 mM benzamidine, 0.2 mM leupeptin, 0.01 mM trans-epoxy succinyl-L-leucylamido-(4-guanidino)butane, 0.3 mM phenylmethylsulphonyl fluoride, 4 μM microcystin]. Samples were vortexed intermittently whilst being incubated on ice for 10 min. Samples were centrifuged (10,000*g*, 10 min, 4 °C), the supernatants were collected, a sample was taken for protein assay and the remainder was boiled with 1/3 vol sample buffer [300 mM Tris-HCl pH 6.8, 10 % (*w*/*v*) sodium dodecyl sulphate (SDS), 13 % (*v*/v) glycerol, 130 mM dithiothreitol, 0.2 % (w/v) bromophenol blue]. Protein concentrations were determined by BioRad Bradford assay using a 1/5 dilution (v/v) in H_2O of the dye reagent concentrate and bovine serum albumin (BSA) standards (1–10 μg). Patient samples were those prepared in [40] with homogenisation in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Cat. Nos. 11,836,170,001 and 04906837001). Following centrifugation (3000*g*, 15 min, 4 °C), the supernatants were collected. Protein quantification used a Pierce BCA Protein Assay kit and samples were boiled with 1/3 vol sample buffer.

Proteins were separated by SDS-polyacrylamide gel electrophoresis using 8 % (for RAF kinases in rat hearts), 12 % (for Gapdh in rat hearts) or 10 % (all other proteins) (*v*/v) polyacrylamide resolving gels with 6 % (v/v) stacking gels until the dye front reached the bottom of the gel (200 V; ~50 min). Prestained BioRad Dual Color molecular weight markers were loaded in the left lane (3 μl; Cat. No. 1610374). Proteins were transferred to nitrocellulose (0.45 μm, Cytiva) using a BioRad semi-dry transfer cell (10 V, 60 min). Non-specific binding sites were blocked (15 min) with 5 % (*w*/*v*) non-fat milk powder in Tris-buffered saline (20 mM Tris-HCl pH 7.5, 137 mM NaCl) containing 0.1 % (*v*/v) Tween 20 (TBST). Blots were incubated with primary antibodies in TBST containing 5 % (w/v) BSA (overnight, 4 °C). Details of antibodies are provided in Supplementary Table S3. Antibodies for Braf and Raf1 (total or phosphorylated proteins), phospho-Akt(T308) and phospho-ERK1/2, total Akt and total ERK1/2 were used on the same blots simultaneously. Blots were washed with TBST (3 × 5 min, room temperature), incubated with horseradish peroxidase-conjugated secondary antibodies in TBST containing 1 % (*w*/*v*) non-fat milk powder (60 min, room temperature) and then washed again in TBST (3 × 5 min, room temperature). Primary antibodies were used at 1/1000 dilutions. Horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulins (catalogue no. P0448) and anti-mouse immunoglobulins (catalogue no. P0477) were from Dako (supplied by Agilent) and used at 1/5000 dilution.

Bands were detected using ECL Prime enhanced chemiluminescence reagents and visualised with an ImageQuant LAS4000 system (reagents and equipment from GE Healthcare, now Cytiva). Image capture was by accumulating increments and all images were documented as .gel (proprietary) or .tif files. A white light image was captured to detect the molecular weight markers. Specific bands were identified on the basis of relative molecular mass. Densitometric analysis was performed with ImageQuant TL 8.1 software (GE Healthcare, now Cytiva) using short incremental exposures to avoid saturation of any bands. Raw values were normalised to Gapdh; values for phosphorylated kinases were also normalised to those of the total kinase as indicated. Values were normalised to the mean of 2 d samples (rat hearts) or the non-failing control heart samples (patients). Blots were cropped for presentation in the Figures. Full original blots are in Supplementary Figs. S1–3.

3. Results

3.1. DNA replication, mitosis and cytokinesis markers during postnatal cardiac development in rats

Cytokinesis in rodent cardiomyocytes ceases shortly after birth, but most nuclei divide again at approximately 4–7 days [2,4]. To assess how genes/proteins are regulated during postnatal development, rat hearts were harvested at the approximate time of the final cell division (2 days), when cells become binucleated (7 days), when binucleation is

complete and rapid growth commences (14 days), as the growth rate starts to reduce post-weaning (28 d), and in the young, mature adult (12 weeks). To confirm that events associated with cell cycle withdrawal could be detected, we selected genes associated with DNA replication and cytokinesis and confirmed expression in an RNASeq dataset for 3–4 day rat ventricular myocytes (Supplementary Spreadsheet S1). mRNAs for centromere proteins required for spindle attachment (*Cenpa*, *Cenpe*, *Cenpf* [13–15]), proteins required for cytokinesis (*Aurkb*, *Anln* and *Cdca8*) [16–18], and proteins required for DNA licensing (origin of recognition proteins, *Orc1-6* [11]) or unwinding (the MCM DNA helicase

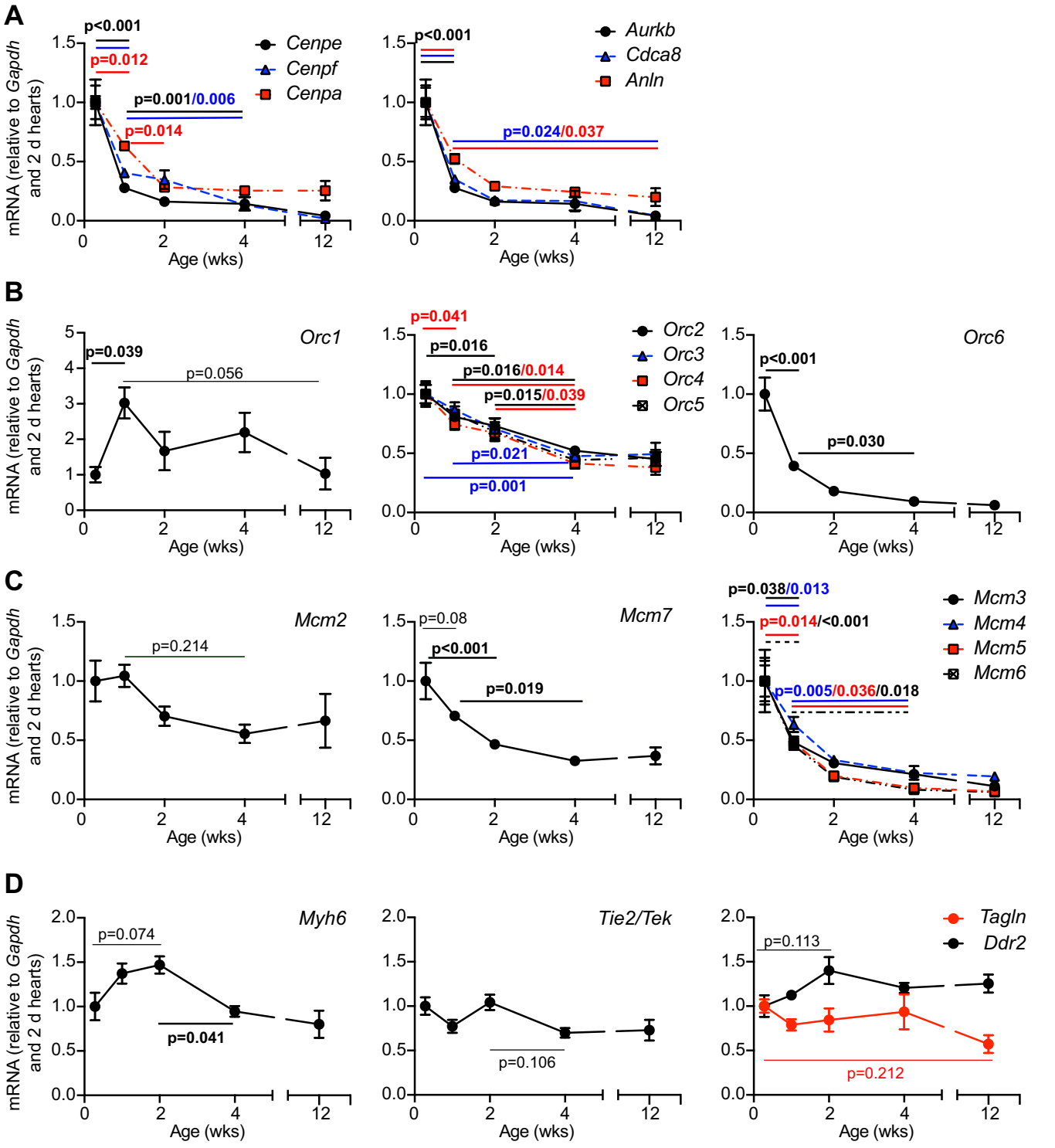

Fig. 1. Regulation of expression of mRNAs for selected genes associated with cytokinesis and DNA replication during postnatal development of rat hearts. RNA was isolated from rat hearts collected 2 d, and 1, 2, 4 and 12 weeks after birth ($n = 6$). mRNA expression for the indicated genes was assessed by qPCR, normalised to *Gapdh* and then expressed relative to the means of the 2 d samples. A, Expression of genes associated with mitosis and cytokinesis. B, Expression of mRNAs for origin recognition proteins. C, Expression of Mcm mRNAs of the DNA helicase complex. D, Markers for cardiomyocytes (*Myh6*), endothelial cells (*Tie2/Tek)*, smooth muscle cells (*Tagln*) and fibroblasts (*Ddr2*). Results are means ± SEM. Statistical analysis used one-way ANOVA with Holm-Sidak's post-test. Significant differences are in bold type.

complex, *Mcms 2-7* [12]) were all expressed in these cells at readily detectable levels (Supplementary Table S4).

Expression of the selected genes was assessed in rat hearts by qPCR. Ct values for *Gapdh* remained approximately constant across the developmental stages studied (Supplementary Fig. S1A) and this was used as the housekeeping gene. *Cenpa*, *Cenpe*, *Cenpf*, *Aurkb*, *Anln*, and *Cdca8* mRNAs were rapidly and substantially downregulated between 2 and 7 days, with a further decrease such that by 12 weeks, these genes

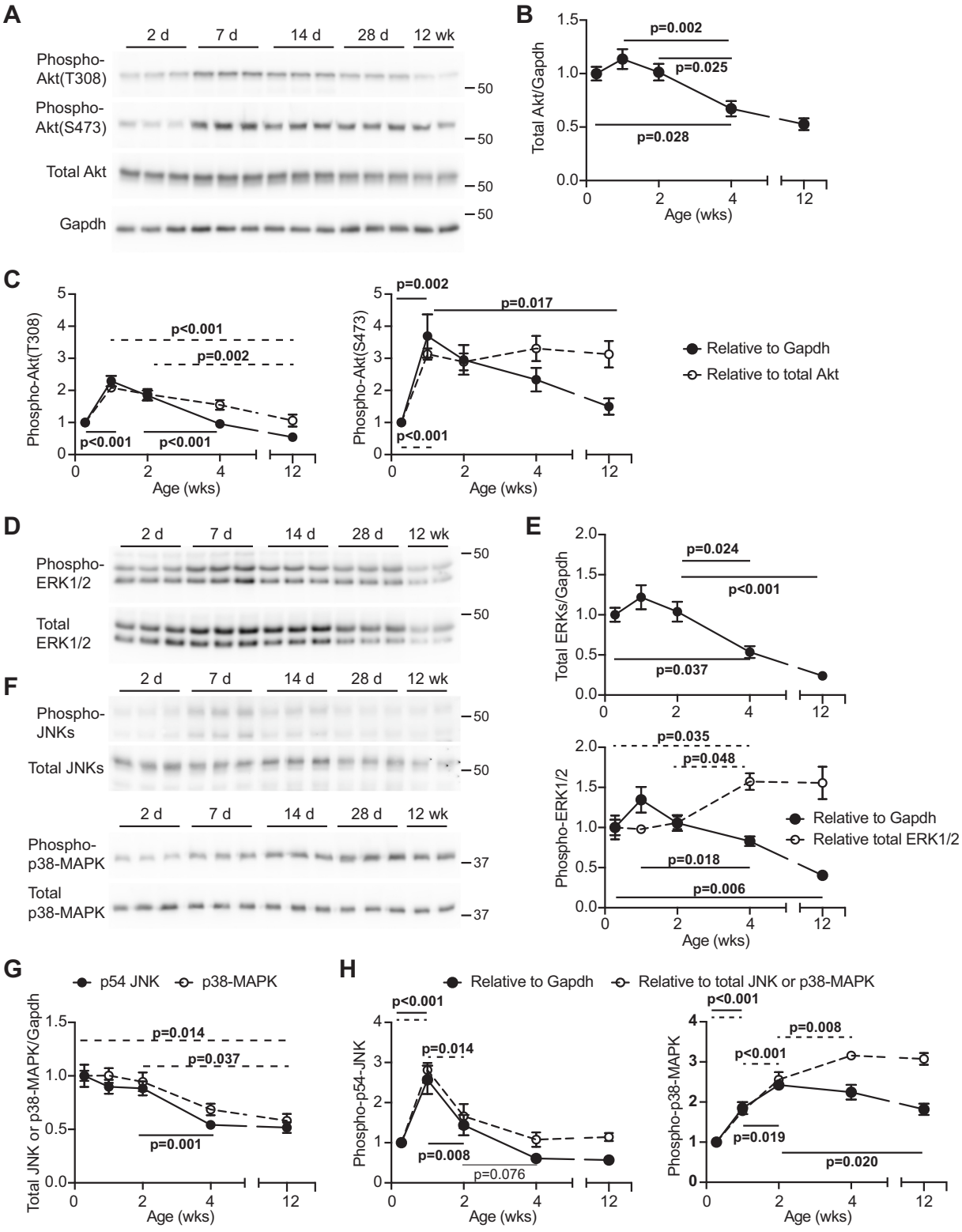

Fig. 2. Regulation of Akt and MAPK signalling during postnatal development of rat hearts. Protein samples (35 μg) from rat hearts collected 2 d, and 1, 2, 4 and 12 weeks after birth (n = 6) were immunoblotted for phosphorylated or total proteins as indicated. The samples were also immunoblotted for Gapdh (25 μg protein). Samples were immunoblotted across 2 blots (14 samples per blot). Representative blots from the same set of samples are shown in A, D, and F, with densitometric analysis in the other panels. Results are means ± SEM. Statistical analysis used one-way ANOVA with Holm-Sidak's post-test. Significant differences are in bold type.

were < 5 % of the 2 d expression levels (Fig. 1A). The data are consistent with termination of cytokinesis in cardiomyocytes soon after birth. mRNAs for core ORC proteins (*Orcs 2–5*) were downregulated from 2 d to 28 d, after which expression was stable at ~38–49 % of the original level (Fig. 1B). In contrast, expression of *Orc1* mRNA (associated with regulation of the complex [11]) initially increased before subsiding to the original levels. Orc6 is linked to cytokinesis [11] and *Orc6* mRNA declined substantially between 2 and 7 d with a further decline to 28 d, a profile similar to the cytokinesis and spindle markers (Fig. 1A). *Mcms 3-6* were substantially downregulated between 2 and 7 days with a further decline through to 12 weeks, but downregulation of *Mcm7* was more limited and *Mcm2* expression was largely sustained (Fig. 1C). Thus, there is downregulation of genes for DNA replication, but the potential for DNA replication is retained. *Myh6* mRNA expression increased to some degree over the first 2 weeks after birth but declined to the 2 d level by 4 weeks (Fig. 1D). mRNA markers for other proliferative cardiac cell types (*Tie2/Tek* for endothelial cells [43], *Tagln* (Sm22a) for smooth muscle cells [44], *Ddr2* for fibroblasts [45]) were not significantly changed during postnatal cardiac development (Fig. 1D). The data indicate that relative proportions of different cardiac cell types do not change significantly during maturational growth of the heart whether or not they retain proliferative potential.

3.2. MAPK and Akt signalling during postnatal cardiac development in rats

We next assessed expression and activation of ERK1/2, JNKs, p38-MAPKs, and Akt in rat hearts by immunoblotting for total and phosphorylated (i.e. activated) proteins. We did not detect any significant change in Gapdh protein expression in rat hearts during postnatal cardiac development and this was used for normalisation (Supplementary Fig. S1B). Expression of total Akt decreased ~30 % between 14 and 28 d and, by 12 weeks, expression was 52.6 ± 0.05 % that at 2 d (mean ± SEM; $n = 6$) (Fig. 2, A-B). Samples were immunoblotted for phosphorylation of the two sites required for full activity of Akt (Thr308 and Ser473). Both significantly increased between 2 and 7 d (Fig. 2, A and C) indicating that Akt signalling is activated during maturational growth of the heart. Akt(Ser473) phosphorylation relative to total Akt was sustained through to adulthood, although Akt(Thr308) declined to some degree.

Total ERK1/2 protein expression in rat hearts was substantially downregulated between 14 and 28 d, with a further decline to 12 weeks (24.0 ± 0.02 % relative to 2 days; Fig. 2D, lower blot; 2E, upper panel). Relative to Gapdh, phosphorylated (i.e. activated) ERK1/2 also declined, but the ratio of phosphorylated/total ERK1/2 significantly increased between 14 and 28 d (Fig. 2D, upper blot; Fig. 2E, lower panel). JNKs are expressed as multiple isoforms of ~46 and 54 KDa but the lower band detected by the antibody we used appeared too small for the 46 kDa isoforms, so we focused only on the upper band of ~54 kDa (Supplementary Fig. S2, centre blots). Total levels of expression of p54-JNKs and p38-MAPKs declined between 14 and 28 d, but to a lesser degree than ERK1/2, expression at 12 weeks relative to 2 d being 51.6 ± 0.05 % and 58.1 ± 0.06 %, respectively (Fig. 2F-G). Phosphorylation of JNKs increased significantly between 2 and 7 d, but this was transient and phosphorylation declined to basal levels by 28 d (Fig. 2H, left panel). Phosphorylation of p38-MAPKs also increased between 2 and 14 d, and remained above the 2 d level at 12 weeks (Fig. 2H, right panel). Thus, whilst cardiac ERK1/2 expression declined substantially during postnatal development, with some decrease in JNKs and p38-MAPKs, there was increased phosphorylation and activation of all the MAPKs relative to total protein during this period.

MAPKs are dephosphorylated and inactivated by DUSPs [37,38], of which some are upregulated in response to growth stimuli or cellular stresses (Dusp1, Dusp2, Dusp4, Dusp5 and Dusp10). Some have greater selectivity for ERK1/2 (Dusp4, Dusp5, Dusp6, Dusp7, Dusp9) whilst others favour JNKs or p38-MAPKs (Dusp1, Dusp2, Dusp8, Dusp10, Dusp16). These were all were readily detected at the mRNA level in 3–4 d rat neonatal ventricular cardiomyocytes by RNASeq except for *Dusp9* (Supplementary Table S5). Of the DUSPs favouring ERK1/2 (Fig. 3A), *Dusp5* mRNA was significantly upregulated between 2 and 7 d and declined thereafter, and *Dusp4* mRNA showed a similar profile although the change was not statistically significant. *Dusp7* was largely unchanged and *Dusp6* expression declined. Surprisingly, *Dusp9* was induced at 7–14 d, returning to the basal minimal level by 28 d. Of the DUSPs favouring JNKs and p38-MAPKs (Fig. 3B), *Dusp1* mRNA was significantly upregulated between 2 and 14 d. *Dusp2* and *Dusp8* mRNAs had a trend towards upregulation, but the increase was not statistically significant. *Dusp10* expression was significantly downregulated with an indication that *Dusp16* mRNA may also be downregulated. The data highlight the potential for DUSPs to regulate MAPK signalling in postnatal rat heart development.

3.3. MAP3Ks and MAP4Ks in rat heart development

MAP3Ks form a signalling node, feeding signals into the MAPK cascades, whilst MAP4Ks may regulate the MAP3Ks [35,36]. We mined RNASeq data from rat neonatal ventricular cardiomyocytes prepared from 3 to 4 d hearts for expression of MAP3Ks and MAP4Ks (Supplementary Table S5), and used qPCR for mRNA profiling of kinases with fpkm values >1 (Supplementary Table S6). Of the MAP3Ks for the ERK1/2 cascade, *Araf* and *Braf* did not show any significant change in mRNA expression during postnatal development, whilst there was a significant, though relatively small decrease in *Raf1* (Fig. 4A). Of the potential MAP3Ks for the JNK and p38-MAPK cascades, *Map3k1*, *Map3k10*, *Map3k11* and *Taok3* were not significantly changed (Supplementary Table S6). Most of the rest were modestly downregulated to ~50 % including *Map3k2*, *Map3k3* and *Map3k4* (Fig. 4B; Supplementary Fig. S4). *Map3k8* and *Map3k12* were significantly increased, albeit at different times (Supplementary Table S6). Of the potential MAP4Ks, *Map4k4* and *Map4k5* were significantly downregulated (Supplementary Table S6; Supplementary Fig. S4).

To compare mRNA expression with protein levels, we focused first on Raf kinases for which antibodies are available for immunoblotting. Despite the limited change in mRNA expression, all three RAF kinases were significantly downregulated in rat hearts during postnatal development between 2 and 4 weeks, although Araf was downregulated to a lesser degree than Braf or Raf1 [42.8 ± 0.06 % (Araf) vs 23.5 ± 0.02 % (Braf) and 23.9 ± 0.03 % (Raf1) at 12 weeks relative to 2 d] (Fig. 4, C–D). Antibodies are available for the activating Ser phosphorylation site for Braf (Ser445) and Raf1 (Ser338) [40]. Because of the decline in total protein, these phosphorylations declined relative to Gapdh, but phosphorylation of Braf in particular was preserved relative to total Braf protein (Fig. 4, C and E). Antibodies for most other MAP3Ks were insufficiently sensitive to compare expression in neonatal and adult rat hearts. However, Map3k3 protein declined significantly between 2 and 14 d, then further between 14 and 28 d (Fig. 4F). Thus, during postnatal heart development, expression of MAP3Ks relative to total protein does not necessarily match expression at the mRNA level, with a more pronounced decline in protein.

3.4. MAP3Ks and MAP4Ks in human failing hearts

Various genes expressed in early heart development may be re-expressed in cardiomyocytes and the heart as a response to pathophysiological stresses (e.g. *Myh7*, *Nppa* [1,5]). Since several MAP3Ks/MAP4Ks were downregulated during postnatal development, we assessed mRNA expression in left ventricular samples from heart failure patients compared with control samples (Supplementary Table S7). We have previously reported that RAF kinases are upregulated in human heart failure [40]. Of the remaining MAP3Ks, *MAP3K2* and *MAP3K3* mRNAs were significantly upregulated in failing hearts (1.44 ± 0.11 and 1.69 ± 0.21 fold change, respectively) (Fig. 5A, upper panels).

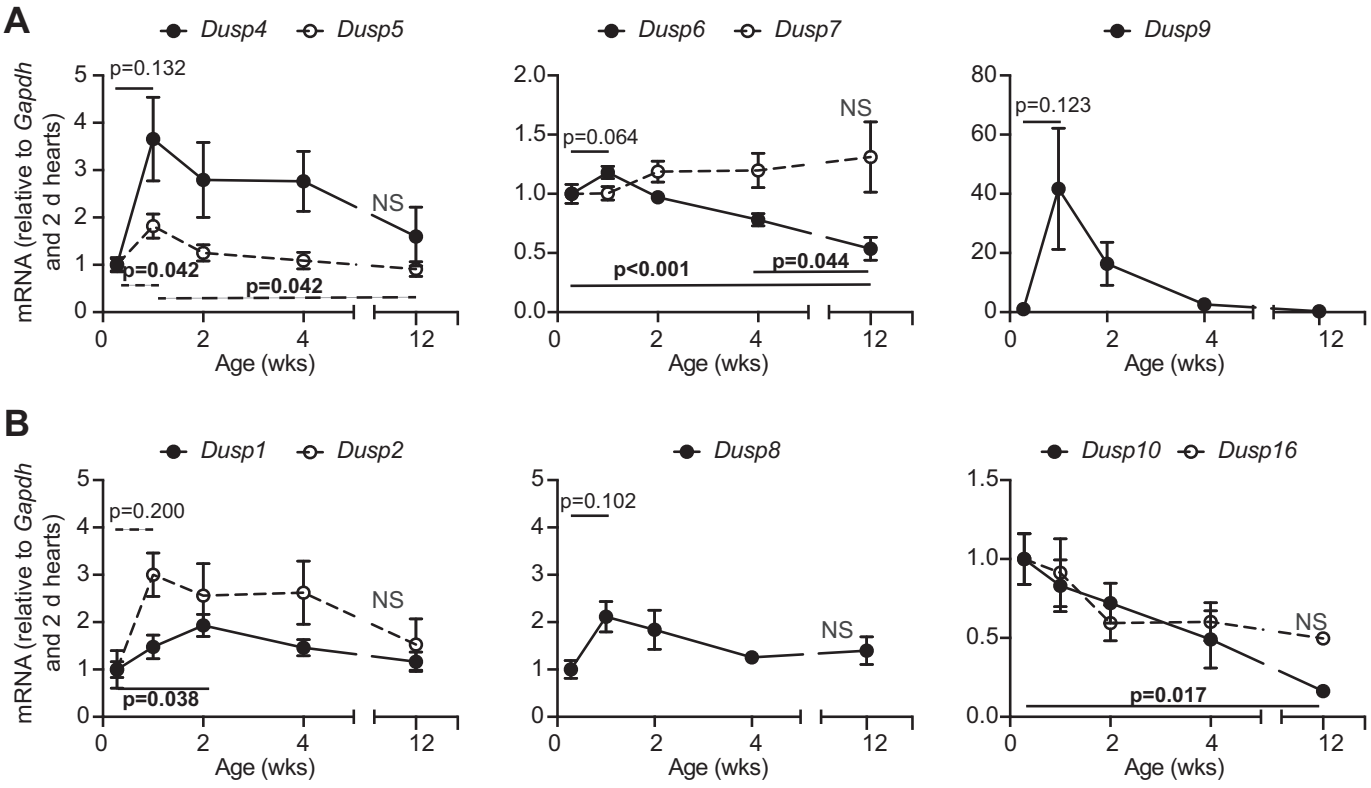

Fig. 3. Regulation of expression of mRNAs for DUSPs with potential to dephosphorylate MAPKs during postnatal development of rat hearts. RNA was isolated from rat hearts collected 2 d, and 1, 2, 4 and 12 weeks after birth (n = 6). mRNA expression for the indicated genes was assessed by qPCR, normalised to *Gapdh* and then expressed relative to the means of the 2 d samples. A, Expression of DUSPs with selectivity for ERK1/2 over JNKs and/or p38-MAPKs. B, Expression of DUSPs with selectivity for JNKs and/or p38-MAPKs over ERK1/2. Results are means ± SEM. Statistical analysis used one-way ANOVA with Holm-Sidak's post-test. Significant differences are in bold type. NS, no statistical significance.

Immunoblotting confirmed the increase in expression of MAP3K3 (Fig. 5B). Of the MAP4Ks, *MAP4K5* was significantly upregulated but to a limited degree (1.21 ± 0.06 fold change) whilst *MAP4K1* was perhaps downregulated (0.66 ± 0.06 relative to non-failing hearts), but this was not statistically significant (Fig. 5A, lower panels).

4. Discussion

The mechanism of mammalian cardiomyocyte terminal-differentiation is an important biological conundrum with major implications for human health. Without meaningful cardiomyocyte cell division, the heart has virtually no potential for regeneration and relies on cardioprotective systems to survive pathological insults such as myocardial infarction. Eventually, the heart becomes unable to deliver blood to the body and fails. Heart failure already affects millions of people worldwide [46], and investigators continue to explore various ways of supporting a failing heart, from pharmacotherapy, through mechanical support to implantation of stem cells. Arguably, the most effective solution currently is still a heart transplant, and the question remains of whether the heart could repair itself. For this to be possible, understanding how and why cardiomyocytes become terminally-differentiated becomes vitally important to develop new ways of reversing the process. Here, we focused on the MAPKs, well-known and well-established signalling pathways that regulate cell fate and which respond to growth stimuli or pathophysiological stresses. We recorded how these pathways are regulated in the heart as cardiomyocytes withdraw from the cell cycle and become binucleated, switching from hyperplastic to hypertrophic growth. The data demonstrate differential regulation of the MAPKs, their upstream MAP3K/MAP4K activators and the inhibitory DUSPs, highlighting the potential importance of the pathways in cardiomyocyte terminal-differentiation. There remain many questions, each of which could merit lengthy debate, so we have focused on some key points for consideration.

The triggers of cardiomyocyte differentiation potentially relate to adaptive responses of cardiomyocytes and the heart to the profound stresses associated with newborn life. With a sudden need for independent oxygenation and thermogenesis, the circulatory system is remodelled, and systemic vascular resistance and arterial blood pressure increase [47]. This is a huge workload, and the immature cardiomyocytes respond with an increase in myofibrillar and mitochondrial content and organisation, along with a switch in energy production from anaerobic glycolysis to oxidative phosphorylation [48,49]. There are additional increases in adrenergic drive, insulin-like growth factors and other systemic hormones (e.g. cortisol, thyroid hormones) [50,51]. These events potentially activate the intracellular signalling pathways highlighted in this study, but the pathways themselves may also drive the changes. Dissecting cause from consequence is difficult and observational correlations offer a way to eliminate some options whilst supporting others. Akt was activated between 2 and 7 d (Fig. 2A–C), a time of rapid cardiomyocyte growth and metabolic switching to oxidative phosphorylation, and the established role of Akt in physiological growth and metabolism [39] suggests it is a driver of these changes. JNKs and p38-MAPKs were also activated at 2–7 d (Fig. 2G–H). The transient activation of JNKs suggests this may be a response to pathophysiological stresses, such as the increase in oxidative stress as mitochondrial flux increases or mechanical strain resulting from the elevated workload. The sustained response of p38-MAPK suggests it may be involved in maintaining differentiation. Previous studies indicate that the α isoform is activated selectively in the right ventricle leading to apoptosis and a smaller size than the left ventricle [33]. We did not determine if there was selective activation in either ventricle, nor did we consider isoform specificity with respect to p38-MAPK activation, but sustained

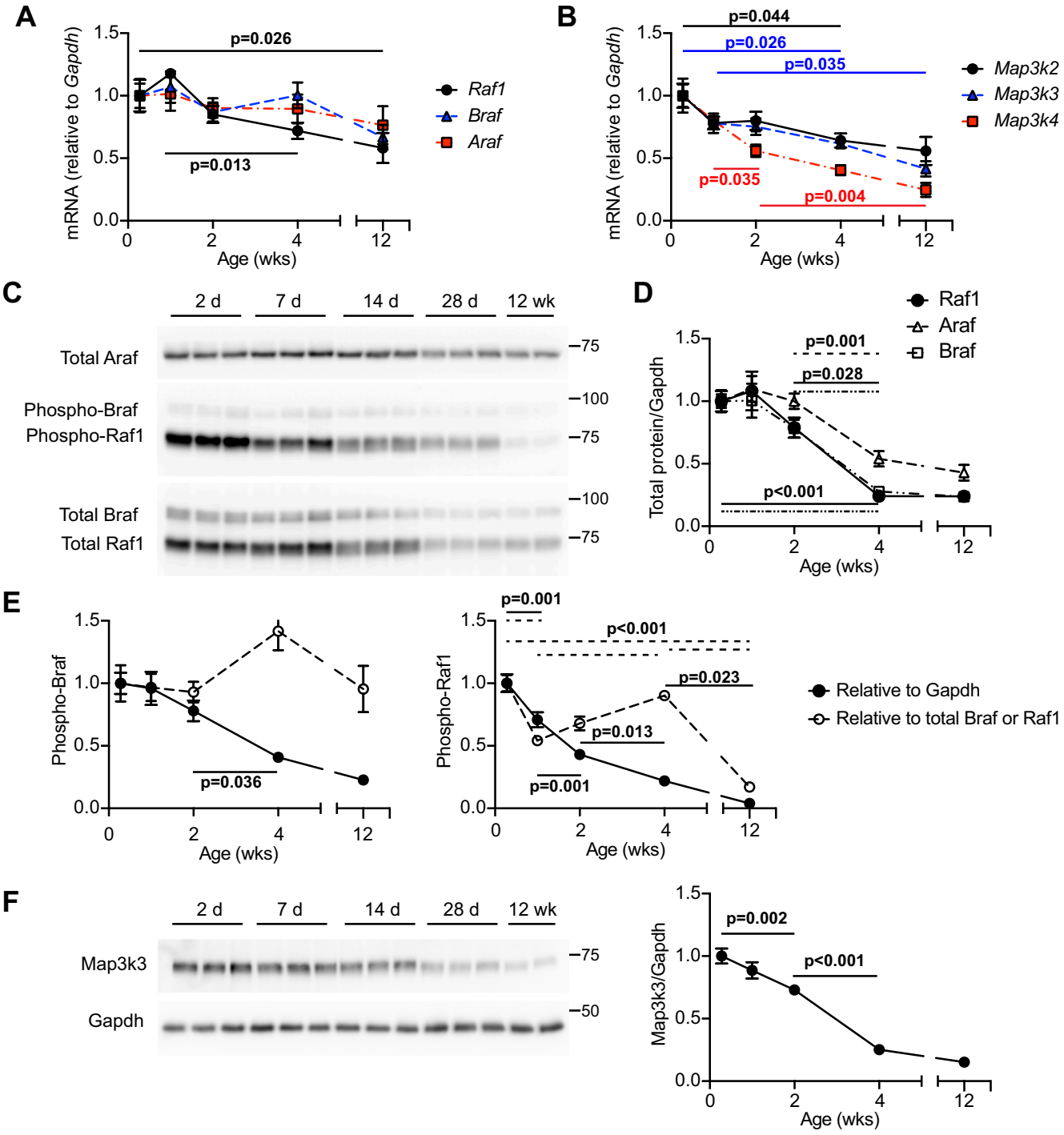

Fig. 4. Regulation of MAP3Ks during postnatal development of rat hearts. RNA or protein was prepared from rat hearts collected 2 d, and 1, 2, 4 and 12 weeks after birth (n = 6). A-B, mRNA expression for the indicated genes was assessed by qPCR, normalised to *Gapdh* and then expressed relative to the means of the 2 d samples. C-F, Protein samples were immunoblotted for phosphorylated or total proteins as indicated. Representative blots for RAF kinases (35 μg per lane) are in C, with densitometric analysis in D-E. These blots are of the same samples as presented in Fig. 2 and the data were normalised to the Gapdh blot in Fig. 2A. A representative blot for Map3k3 is in panel F (35 μg per lane; left panel) with its corresponding blot for Gapdh (25 μg per lane) and densitometric analysis shown on the right. These were from a second set of samples. Results are means ± SEM. Statistical analysis used one-way ANOVA with Holm-Sidak's post-test. Significant differences are in bold type.

activation would not be compatible with continued apoptosis in either ventricle so it seems likely that p38-MAPK has additional roles in the heart through the postnatal period.

The ERK1/2 cascade has great potential as a driving force for terminal differentiation but has not been fully investigated in this context. ERK1/2 signalling clearly promotes cell proliferation and the pathway plays a key role in cancer but, depending on the stimulus, it can instead promote differentiation [8]. For example, in the PC12 neuronal cell line, epidermal growth factor (EGF) induces cell division whereas nerve growth factor (NGF) promotes differentiation potentially due to differences in the intensity and duration of the ERK1/2 signal (EGF induces transient activation; NGF promotes sustained activation) [52–54].

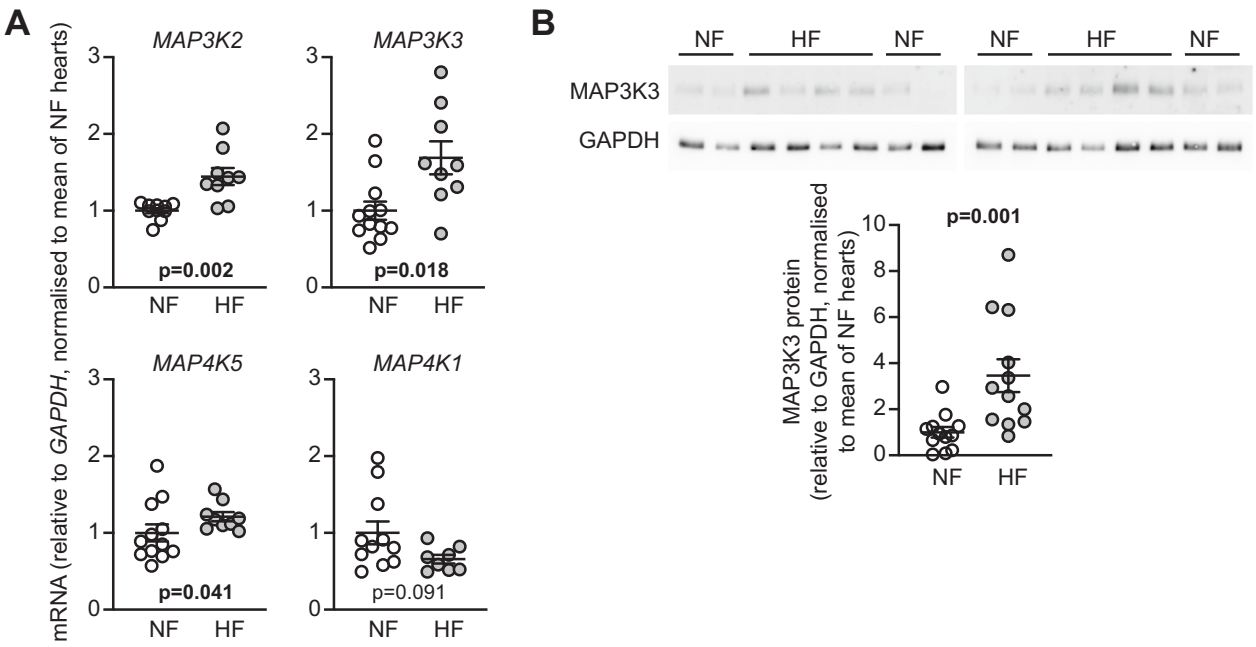

Fig. 5. Expression of MAP3K2 and MAP3K3 in failing human hearts. mRNA or protein were prepared from left ventricular samples from patients with heart failure (HF) or from non-failing explanted hearts (NF). A, *MAP3K2*, *MAP3K3*, *MAP4K5*, and *MAP4K1* mRNA expression was assessed by qPCR, normalised to *Gapdh* and expressed relative to the means of the NF samples. B, Protein samples (100 μg) were immunoblotted for MAP3K3 and the blots reprobed for GAPDH. Representative blots are in the upper panels with densitometric analysis below. Results are means ± SEM with individual points shown. Statistical analysis used Mann-Whitney tests.

Consistent with this, overexpression of EGF receptors or repetitive activation of ERK1/2 by re-application of EGF to induce a sustained response promotes differentiation rather than proliferation [55,56]. In rat hearts, activation of ERK1/2 was sustained to 28 d, despite a decline in total protein after 14 d (Fig. 2D–E), consistent with a role for ERK1/2 in driving differentiation. However, it is still not known how sustained ERK1/2 activation leads to differentiation in PC12 cells, so further work is needed to understand the mechanisms involved. In proliferating cells, ERK1/2 translocate to the nucleus where they phosphorylate transcription factors and modulate gene expression to elicit phenotypic changes [57]. Activated ERK1/2 is increased in the nucleus of neonatal cardiomyocytes on stimulation with endothelin-1 or the α_1-adrenergic agonist phenylephrine, although there is limited evidence of any net accumulation of total ERK1/2 [58]. ERK1/2 clearly play a significant role in regulating changes in cardiomyocyte gene expression [41,58,59], so it is probable that effects to promote differentiation are similarly elicited via effects in the nucleus.

For all regulatory systems, it is essential to consider methods of inactivation in addition to activation, which is why we assessed expression of the MAPK-directed DUSPs and MAP3Ks/MAP4Ks in relation to MAPK activation. DUSPs appear to be regulated largely at the level of expression and at least some are induced by the MAPKs themselves [37,38]. Dusps 1, 2, 4, 5, 8 and 9 mRNAs were generally upregulated at 7–14 days, a time at which the MAPKs were activated, whilst Dusps 6, 10 and 16 were downregulated (Fig. 3). Dusps 1, 2, 4 and 5 are nuclear-localised [37,38], suggesting particular control of MAPK activities in the nucleus, presumably to control gene expression. Dusp6 potentially influences the basal level of phosphorylation of cytoplasmic ERK1/2 [60], so the decline in expression may account for the increase in relative phosphorylation of ERK1/2 overall. The transient expression of Dusp9 (Fig. 3A, right panel) is intriguing since this coincides with a likely increase in oxidative stress in the heart. Moreover, Dusp9 also dephosphorylates ASK1, an oxidative-stress responsive MAP3K, and inhibits pathological hypertrophy associated with hypertension [61].

In contrast to the DUSPs, more of the MAP3Ks/MAP4Ks were downregulated in the heart, with the greatest changes occurring after 14 d (Fig. 4; Supplementary Table S6). It was challenging to identify antibodies suitable for immunoblotting, but for those we could study (RAF kinases and Map3k3), there was a far greater decrease in protein expression than in the mRNA. This highlights the importance of assessing protein expression to allow for rates of translation and protein turnover, but another consideration is the cellular environment. The increase in cardiomyocyte size between the neonate and adult is estimated as ~12-fold according to membrane capacitance [62,63], with a large increase in proteins required for contractile function and energy production. Consequently, expression of signalling proteins such as those involved in MAPK signalling is largely sustained relative to cell number. The increase in contractile apparatus and mitochondria with improved intracellular organisation in an adult cardiomyocyte [48,49] establishes additional physical barriers to signal transduction, and questions of how signals are transmitted across the cell (e.g. transmission of the ERK1/2 signal to the nucleus) become more perplexing. Signal compartmentalisation has been studied in adult cardiomyocytes in relation to the cAMP pathway. Here, there are clear constraints that dictate regional production of cAMP from β-adrenergic receptors expressed in specific subdomains of the membrane, in addition to the phosphodiesterases that contain the signal [64]. Similar studies for the MAPKs have yet to be done. Thus, although MAP3K/MAP4K mRNAs may be relatively unchanged or downregulated, with (potentially) greater downregulation at the protein level, subcellular compartmentalisation of individual MAP3Ks/MAP4Ks that respond to specific stimuli may result in high signal potential in specific sites within the cell.

The physical barriers imposed by the contractile apparatus and the organisation within an adult cardiomyocyte could not only impede intracellular signal transmission, but could also constitute a physical block for cytokinesis. However, the contractile apparatus in adult cardiomyocytes in primary culture can be disassembled as they "de-differentiate" [65], and this presumably occurs in the <1 % of cardiomyocytes that divide. Moreover, cessation of cytokinesis occurs early in postnatal development of the mammalian heart when cardiomyocytes still have an immature phenotype [48]. The block in cytokinesis, therefore, seems unlikely to be simply due to the physical barriers imposed by contractile machinery and (since nuclei continue to divide to produce bi- or multi-nucleated cardiomyocytes) seems more likely to be

a first stage of commitment to the terminal differentiation process. Consistent with this, there was very rapid downregulation of genes required for cytokinesis (Fig. 1A). Genes required for mitosis (*Cenpe*, *Cenpf*) were downregulated with a similar profile but, since the final nuclear division occurs at 4–7 days and we only studied 2 and 7 days, a more detailed time course is necessary to distinguish between cessation of cytokinesis and the final round of mitosis. Our study assessed expression in whole hearts, rather than individual cells. At 2 days, potentially 30 % of the cells are cardiomyocytes with a single nucleus, but by 14 days, most cardiomyocytes are binucleated, so ~46 % of nuclei derive from cardiomyocytes. The profound downregulation of cytokinesis genes suggests that almost all of the cells in a mature heart are not undergoing significant replication. Consistent with this, the proportions of different cardiac cell types appeared similar during the postnatal developmental period (Fig. 1D). This might be expected for a stable, mature heart, but it remains to be established if the mechanisms are the same in cardiomyocytes and proliferating cells and if there is a more permanent block in cardiomyocytes.

In an era of "omics" technologies, global approaches have been used to identify changes in gene/protein expression during postnatal development of rodent hearts. These include genetic/epigenetic assessment of hypermethylated genes and regions of heterochromatin, RNASeq profiling of mRNA expression, proteomics and metabolomics (e.g. [22–27]). The nucleic acid technologies are particularly powerful, allowing assessment of the full genome/transcriptome and, with single-cell transcriptomics, subpopulations of cardiomyocytes have been identified according to the transcriptome of individual nuclei. These studies generate vast amounts of data, but are difficult to interpret. "Omics" approaches favour abundance whereas, for cell regulation, the genes/proteins that matter are not necessarily expressed at the same high level as the genes/proteins required for cell "operations" (e.g. for the contractile machinery in a cardiomyocyte) and the total amount of protein may be less important than functional activity regulated by post-translational modifications (e.g. phosphorylation). To understand the signalling mechanisms associated with cardiomyocyte terminal differentiation, we argue that greater insight can be gained with more focused approaches, particularly when informed by "omics" data. For our study, we used RNASeq data from rat neonatal cardiomyocytes to identify the MAP3Ks/MAP4Ks and DUSPs expressed in these cells and tracked these genes specifically in the postnatal developmental period (Supplementary Tables S4-S5; Supplementary Spreadsheet S1). mRNAs for MAP3Ks, MAP4Ks and DUSPs were not highly expressed at the mRNA level with values <100 fpkm (mRNAs for mitochondrial, contractile or ribosomal proteins were > 1000 fpkm). Expression of mRNAs for ERK1/2 and Akt were of the same order (93 and 55 fpkm for ERK1/2; 146, 74, and 12 fpkm for the 3 Akt isoforms) and these clearly play an important role in regulating cardiomyocyte responses, so it is not unreasonable to expect that the levels of expression of MAP3Ks/MAP4Ks/DUSPs are meaningful with respect to cardiomyocyte signalling. Our data are comparable to other studies. For example, Talman et al. [66] used RNASeq transcriptomics for rat hearts at 2, 4, 9 and 23 days. The relative expression of the genes we studied in 3–4 d cardiomyocytes was comparable with the 4 d hearts and our qPCR profiles between 2 and 14 d were similar to the changes seen between 2 and 9 days (other differences between ages are difficult to correlate) (Supplementary Tables S5, S6 and S8).

Many of the MAP3Ks that we report were identified many years ago as upstream kinases for one of more of the stress-regulated MAPK cascades and generally showed little specificity for either JNKs or p38-MAPKs that were activated in tandem in most conditions. The best evidence for distinct regulation of the pathways is with ischaemia/reperfusion: p38-MAPKs are activated by ischaemia whilst JNKs are activated on reperfusion [67]. In ischaemia, the upstream MAP3K is most likely to be ASK1 (MAP3K5) which is selective for p38-MAPK activation in cardiomyocytes [68,69]. It is not clear if other MAP3Ks are selective for JNKs or p38-MAPKs or how they are activated by different cellular stresses, and many MAP3Ks have yet to be properly investigated in cardiomyocytes. For example, Map3k8 (Tpl2/Cot) has potential to activate ERK1/2 or p38-MAPK [70] and was upregulated during postnatal rat heart development (Supplementary Table S6). Moreover, Map3k12 (DLK, [71]) was also transiently upregulated and is amongst the more highly expressed mRNAs in cardiomyocytes, but there are few studies of this kinase.

With 20 MAP3Ks potentially signalling to JNKs and p38-MAPKs, there is much to discuss but we will focus on Map3k3 (also known as Mekk3 or MAPK/ERK kinase kinase 3) since it was the only MAP3K for JNKs and p38-MAPKs we could assess at the protein level in our development study. Map3k3 was downregulated during postnatal development of rat hearts, but MAP3K3 expression was increased in left ventricular samples from failing hearts compared with non-failing hearts (Figs. 4B, F and 5). This suggests it could be amongst the genes expressed early in cardiac development that are re-expressed in disease (the "foetal gene programme") [1,5]. However, although Map3k3 is required for embryonic development and embryos from Map3k3 knockout mice die at E11, this is due to a failure in angiogenesis [72] and subsequent studies focused on its role in endothelial cells and cerebral cavernous malformations [73]. Map3k3 is closely related to Map3k2 (also known as Mekk2) [74]. Strikingly, *Map3k2* mRNA expression was almost identical to that of *Map3k3* in rat hearts and patient samples (Figs. 4B and 5). In non-cardiomyocytes, there is evidence that the Map3k3/Map3k2 dimer signals to p38-MAPK or an alternative member of the ERK family, ERK5, but can also activate other signalling pathways including NFκB and the Hippo signalling pathway [74,75]. Hippo signalling is particularly implicated in determination of organ size (including the heart) and may play a substantial role in balancing cardiomyocyte proliferation and differentiation [76,77]. Thus, Map3k3/Map3k2 are in a pivotal position to orchestrate several signalling pathways simultaneously to influence cardiomyocyte growth during the important postnatal period of development.

5. Conclusion

Many investigators over many years have considered how and why cardiomyocytes become terminally-differentiated: it is interesting biologically and important for the increasing population with impaired quality of life as a result of cardiac diseases. Such is the barrier to cell cycle progression that studies of cardiomyocyte signalling are impeded by the inability of cardiomyocytes to divide, with no satisfactory cell lines available and limited potential for experimental use of primary cells. As large scale "omics" technologies have developed, more data are available to inform future investigations, but these alone cannot provide the answers. Studies of individual proteins via gene knockout or overexpression also have their place, but again cannot address how intracellular signalling networks operate to elicit and maintain a fully differentiated state in cardiomyocytes. A systems biology approach is needed for which fundamental observational data such as those presented here are essential. We demonstrated how signalling through the MAPKs changes in the postnatal heart and illustrated how expression of potential pathway components may modulate the response. But we have yet to address expression and activation in different cardiac cell types or cells in different regions of the heart, and further studies are needed to consider signal compartmentalisation. Integration of the MAPK signals with other signalling networks that operate is another facet for future exploration. This interplay between various signalling pathways is likely to be an important feature in determining when and how cardiomyocytes switch from hyperplastic to hypertrophic growth.

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CRediT authorship contribution statement

Hajed O. Alharbi: Writing – review & editing, Investigation, Funding acquisition, Formal analysis. **Peter H. Sugden:** Writing – original draft. **Angela Clerk:** Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2024.111397.

References

[1] M. Cui, Z. Wang, R. Bassel-Duby, E.N. Olson, Genetic and epigenetic regulation of cardiomyocytes in development, regeneration and disease, Development 145 (2018), https://doi.org/10.1242/dev.171983.

[2] N. Velayutham, E.J. Agnew, K.E. Yutzey, Postnatal cardiac development and regenerative potential in large mammals, Pediatr. Cardiol. 40 (2019) 1345–1358, https://doi.org/10.1007/s00246-019-02163-7.

[3] T.R. Heallen, Z.A. Kadow, J. Wang, J.F. Martin, Determinants of cardiac growth and size, Cold Spring Harb. Perspect. Biol. 12 (2020), https://doi.org/10.1101/cshperspect.a037150.

[4] C.G. Dimasi, J.R.T. Darby, J.L. Morrison, A change of heart: understanding the mechanisms regulating cardiac proliferation and metabolism before and after birth, J. Physiol. 601 (2023) 1319–1341, https://doi.org/10.1113/JP284137.

[5] G.W. Dorn II, J. Robbins, P.H. Sugden, Phenotyping hypertrophy: eschew obfuscation, Circ. Res. 92 (2003) 1171–1175, https://doi.org/10.1161/01.RES.0000077012.11088.BC.

[6] M. Maillet, J.H. van Berlo, J.D. Molkentin, Molecular basis of physiological heart growth: fundamental concepts and new players, Nat. Rev. Mol. Cell Biol. 14 (2013) 38–48, https://doi.org/10.1038/nrm3495.

[7] A. Martin-Vega, M.H. Cobb, Navigating the ERK1/2 MAPK cascade, Biomolecules 13 (2023) 1555, https://doi.org/10.3390/biom13101555.

[8] R. Nussinov, W. Zhang, Y. Liu, H. Jang, Mitogen signaling strength and duration can control cell cycle decisions, Sci. Adv. 10 (2024) eadm9211, https://doi.org/10.1126/sciadv.adm9211.

[9] M.G. Koliopoulos, C. Alfieri, Cell cycle regulation by complex nanomachines, FEBS J. 289 (2022) 5100–5120, https://doi.org/10.1111/febs.16082.

[10] M. Malumbres, M. Barbacid, Cell cycle, CDKs and cancer: a changing paradigm, Nat. Rev. Cancer 9 (2009) 153–166, https://doi.org/10.1038/nrc2602.

[11] B.P. Duncker, I.N. Chesnokov, B.J. McConkey, The origin recognition complex protein family, Genome Biol. 10 (2009) 214, https://doi.org/10.1186/gb-2009-10-3-214.

[12] Y. Hatoyama, M.T. Kanemaki, The assembly of the MCM2-7 hetero-hexamer and its significance in DNA replication, Biochem. Soc. Trans. 51 (2023) 1289–1295, https://doi.org/10.1042/BST20221465.

[13] H. Nagpal, B. Fierz, The elusive structure of centro-chromatin: molecular order or dynamic heterogenetity? J. Mol. Biol. 433 (2021) 166676 https://doi.org/10.1016/j.jmb.2020.10.010.

[14] B. Craske, J.P.I. Welburn, Leaving no-one behind: how CENP-E facilitates chromosome alignment, Essays Biochem. 64 (2020) 313–324, https://doi.org/10.1042/EBC20190073.

[15] A. Varis, A.L. Salmela, M.J. Kallio, Cenp-F (mitosin) is more than a mitotic marker, Chromosoma 115 (2006) 288–295, https://doi.org/10.1007/s00412-005-0046-0.

[16] O. Afonso, A.C. Figueiredo, H. Maiato, Late mitotic functions of Aurora kinases, Chromosoma 126 (2017) 93–103, https://doi.org/10.1007/s00412-016-0594-5.

[17] N.M. Tuan, C.H. Lee, Role of anillin in tumour: from a prognostic biomarker to a novel target, Cancers (Basel) 12 (2020), https://doi.org/10.3390/cancers12061600.

[18] M.S. van der Waal, R.C. Hengeveld, A. van der Horst, S.M. Lens, Cell division control by the chromosomal passenger complex, Exp. Cell Res. 318 (2012) 1407–1420, https://doi.org/10.1016/j.yexcr.2012.03.015.

[19] R.A. Poolman, G. Brooks, Expressions and activities of cell cycle regulatory molecules during the transition from myocyte hyperplasia to hypertrophy, J. Mol. Cell. Cardiol. 30 (1998) 2121–2135, https://doi.org/10.1006/jmcc.1998.0808.

[20] R.A. Poolman, R. Gilchrist, G. Brooks, Cell cycle profiles and expressions of p21CIP1 and p27KIP1 during myocyte development, Int. J. Cardiol. 67 (1998) 133–142, https://doi.org/10.1016/s0167-5273(98)00320-9.

[21] D. Mahiny-Shahmohammady, L. Hauck, F. Billia, Defining the molecular underpinnings controlling cardiomyocyte proliferation, Clin. Sci. (Lond.) 136 (2022) 911–934, https://doi.org/10.1042/CS20211180.

[22] P. Hu, J. Liu, J. Zhao, B.J. Wilkins, K. Lupino, H. Wu, L. Pei, Single-nucleus transcriptomic survey of cell diversity and functional maturation in postnatal mammalian hearts, Genes Dev. 32 (2018) 1344–1357, https://doi.org/10.1101/gad.316802.118.

[23] R. Gilsbach, M. Schwaderer, S. Preissl, B.A. Gruning, D. Kranzhofer, P. Schneider, T.G. Nuhrenberg, S. Mulero-Navarro, D. Weichenhan, C. Braun, M. Dressen, A. R. Jacobs, H. Lahm, T. Doenst, R. Backofen, M. Krane, B.D. Gelb, L. Hein, Distinct epigenetic programs regulate cardiac myocyte development and disease in the human heart in vivo, Nat. Commun. 9 (2018) 391, https://doi.org/10.1038/s41467-017-02762-z.

[24] R. Windmueller, J.P. Leach, A. Babu, S. Zhou, M.P. Morley, A. Wakabayashi, N. B. Petrenko, P. Viatour, E.E. Morrisey, Direct comparison of mononucleated and binucleated cardiomyocytes reveals molecular mechanisms underlying distinct proliferative competencies, Cell Rep. 30 (2020) 3105–3116 e3104, https://doi.org/10.1016/j.celrep.2020.02.034.

[25] Z. Li, F. Yao, P. Yu, D. Li, M. Zhang, L. Mao, X. Shen, Z. Ren, L. Wang, B. Zhou, Postnatal state transition of cardiomyocyte as a primary step in heart maturation, protein, Cell 13 (2022) 842–862, https://doi.org/10.1007/s13238-022-00908-4.

[26] B.I. Garay, S. Givens, P. Abreu, M. Liu, D. Yucel, J. Baik, N. Stanis, T.M. Rothermel, A. Magli, J.E. Abrahante, N.A. Goloviznina, H.A.N. Soliman, N.R. Dhoke, M. Kyba, P.W. Alford, S.C. Dudley Jr., J.H. van Berlo, B. Ogle, R.R.C. Perlingeiro, Dual inhibition of MAPK and PI3K/AKT pathways enhances maturation of human iPSC-derived cardiomyocytes, Stem Cell Rep. 17 (2022) 2005–2022, https://doi.org/10.1016/j.stemcr.2022.07.003.

[27] J. Bai, Y. Lin, J. Zhang, Z. Chen, Y. Wang, M. Li, J. Li, Profiling of chromatin accessibility in pigs across multiple tissues and developmental stages, Int. J. Mol. Sci. 24 (2023), https://doi.org/10.3390/ijms241311076.

[28] B.A. Rose, T. Force, Y. Wang, Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale, Physiol. Rev. 90 (2010) 1507–1546, https://doi.org/10.1152/physrev.00054.2009.

[29] I. Kehat, J.D. Molkentin, Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling in cardiac hypertrophy, Ann. N. Y. Acad. Sci. 1188 (2010) 96–102, https://doi.org/10.1111/j.1749-6632.2009.05088.x.

[30] I. Kehat, J. Davis, M. Tiburcy, F. Accornero, M.K. Saba-El-Leil, M. Maillet, A. J. York, J.N. Lorenz, W.H. Zimmermann, S. Meloche, J.D. Molkentin, Extracellular signal-regulated kinases 1 and 2 regulate the balance between eccentric and concentric cardiac growth, Circ. Res. 108 (2011) 176–183, https://doi.org/10.1161/CIRCRESAHA.110.231514.

[31] A. Clerk, S.U. Amadi, S.J. Smith, P.H. Sugden, Inhibiting the extracellular signal-regulated kinase 1/2 (ERK1/2) cascade in cancer and the heart: for better or worse, in sickness and health? IJDDP 3 (2024) 100006 https://doi.org/10.53941/ijddp.2024.100006.

[32] R. Romero-Becerra, A.M. Santamans, C. Folgueira, G. Sabio, p38 MAPK pathway in the heart: new insights in health and disease, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21197412.

[33] T. Yokota, J. Li, J. Huang, Z. Xiong, Q. Zhang, T. Chan, Y. Ding, C. Rau, K. Sung, S. Ren, R. Kulkarni, T. Hsiai, X. Xiao, M. Touma, S. Minamisawa, Y. Wang, p38 mitogen-activated protein kinase regulates chamber-specific perinatal growth in heart, J. Clin. Invest. 130 (2020) 5287–5301, https://doi.org/10.1172/JCI135859.

[34] D.E. Durrant, D.K. Morrison, Targeting the Raf kinases in human cancer: the Raf dimer dilemma, Br. J. Cancer 118 (2018) 3–8, https://doi.org/10.1038/bjc.2017.399.

[35] K. Mordente, L. Ryder, S. Bekker-Jensen, Mechanisms underlying sensing of cellular stress signals by mammalian MAP3 kinases, Mol. Cell 84 (2024) 142–155, https://doi.org/10.1016/j.molcel.2023.11.028.

[36] H.C. Chuang, X. Wang, T.H. Tan, MAP4K family kinases in immunity and inflammation, Adv. Immunol. 129 (2016) 277–314, https://doi.org/10.1016/bs.ai.2015.09.006.

[37] K.I. Patterson, T. Brummer, P.M. O'Brien, R.J. Daly, Dual-specificity phosphatases: critical regulators with diverse cellular targets, Biochem. J. 418 (2009) 475–489, https://doi.org/10.1042/bj20082234.

[38] R. Liu, J.D. Molkentin, Regulation of cardiac hypertrophy and remodeling through the dual-specificity MAPK phosphatases (DUSPs), J. Mol. Cell. Cardiol. 101 (2016) 44–49, https://doi.org/10.1016/j.yjmcc.2016.08.018.

[39] A. Clerk, P.H. Sugden, The insulin receptor family in the heart: new light on old insights, Biosci. Rep. 42 (2022), https://doi.org/10.1042/BSR20221212.

[40] A. Clerk, D.N. Meijles, M.A. Hardyman, S.J. Fuller, S.P. Chothani, J.J. Cull, S.T. E. Cooper, H.O. Alharbi, K. Vanezis, L.E. Felkin, T. Markou, S.J. Leonard, S. W. Shaw, O.J.L. Rackham, S.A. Cook, P.E. Glennon, M.N. Sheppard, J.C. Sembrat, M. Rojas, C.F. McTiernan, P.J. Barton, P.H. Sugden, Cardiomyocyte BRAF and type 1 RAF inhibitors promote cardiomyocyte and cardiac hypertrophy in mice in vivo, Biochem. J. 479 (2022) 401–424, https://doi.org/10.1042/BCJ20210615.

[41] A.K. Marshall, O.P.T. Barrett, T.E. Cullingford, A. Shanmugasundram, P.H. Sugden, A. Clerk, ERK1/2 signaling dominates over RhoA signaling in regulating early changes in RNA expression induced by endothelin-1 in neonatal rat cardiomyocytes, PLoS One 5 (2010) e10027, https://doi.org/10.1371/journal.pone.0010027.

[42] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[43] V. Martin, D. Liu, J. Fueyo, C. Gomez-Manzano, Tie2: a journey from normal angiogenesis to cancer and beyond, Histol. Histopathol. 23 (2008) 773–780, https://doi.org/10.14670/HH-23.773.

[44] M. Mericskay, Z. Li, D. Paulin, Transcriptional regulation of the desmin and SM22 genes in vascular smooth muscle cells, Curr. Top. Pathol. 93 (1999) 7–17, https://doi.org/10.1007/978-3-642-58456-5_2.
[45] A. Vijayakumar, M. Wang, S. Kailasam, The senescent heart-"age doth wither its infinite variety", Int. J. Mol. Sci. 25 (2024) https://doi.org/10.3390/ijms25073581.
[46] G. Savarese, P.M. Becher, L.H. Lund, P. Seferovic, G.M.C. Rosano, A. Coats, Global burden of heart failure: a comprehensive and updated review of epidemiology, Cardiovasc. Res. 118 (2023) 3272–3287, https://doi.org/10.1093/cvr/cvac013.
[47] C.M.J. Tan, A.J. Lewandowski, The transitional heart: from early embryonic and fetal development to neonatal life, Fetal Diagn. Ther. 47 (2020) 373–386, https://doi.org/10.1159/000501906.
[48] S. Salameh, V. Ogueri, N.G. Posnack, Adapting to a new environment: postnatal maturation of the human cardiomyocyte, J. Physiol. 601 (2023) 2593–2619, https://doi.org/10.1113/JP283792.
[49] T. Sakamoto, D.P. Kelly, Cardiac maturation, J. Mol. Cell. Cardiol. 187 (2024) 38–50, https://doi.org/10.1016/j.yjmcc.2023.12.008.
[50] M. Li, S.E. Iismaa, N. Naqvi, A. Nicks, A. Husain, R.M. Graham, Thyroid hormone action in postnatal heart development, Stem Cell Res. 13 (2014) 582–591, https://doi.org/10.1016/j.scr.2014.07.001.
[51] S.S. Jonker, S. Louey, Endocrine and other physiologic modulators of perinatal cardiomyocyte endowment, J. Endocrinol. 228 (2016) R1–18, https://doi.org/10.1530/JOE-15-0309.
[52] S. Traverse, N. Gomez, H. Paterson, C. Marshall, P. Cohen, Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor, Biochem. J. 288 (1992) 351–355, https://doi.org/10.1042/bj2880351.
[53] T.T. Nguyen, J.C. Scimeca, C. Filloux, P. Peraldi, J.L. Carpentier, E. van Obberghen, Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. Distinct effects of the neurotrophic factor, nerve growth factor, and the mitogenic factor, epidermal growth factor, J. Biol. Chem. 268 (1993) 9803–9810, https://doi.org/10.1016/S0021-9258(18)98418-8.
[54] C.J. Marshall, Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation, Cell 80 (1995) 179–185, https://doi.org/10.1016/0092-8674(95)90401-8.
[55] S. Traverse, K. Seedorf, H. Paterson, C.J. Marshall, P. Cohen, A. Ullrich, EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor, Curr. Biol. 4 (1994) 694–701, https://doi.org/10.1016/s0960-9822(00)00154-8.
[56] H. Ryu, M. Chung, M. Dobrzynski, D. Fey, Y. Blum, S.S. Lee, M. Peter, B. N. Kholodenko, N.L. Jeon, O. Pertz, Frequency modulation of ERK activation dynamics rewires cell fate, Mol. Syst. Biol. 11 (2015) 838, https://doi.org/10.15252/msb.20156458.
[57] K. Flores, S.S. Yadav, A.A. Katz, R. Seger, The nuclear translocation of mitogen-activated protein kinases: molecular mechanisms and use as novel therapeutic target, Neuroendocrinology 108 (2019) 121–131, https://doi.org/10.1159/000494085.
[58] E. Amirak, S.J. Fuller, P.H. Sugden, A. Clerk, p90 ribosomal S6 kinases play a significant role in early gene regulation in the cardiomyocyte response to Gq protein-coupled receptor stimuli, endothelin-1 and alpha1-adrenergic receptor agonists, Biochem. J. 450 (2013) 351–363, https://doi.org/10.1042/BJ20121371.
[59] T.E. Cullingford, T. Markou, S.J. Fuller, A. Giraldo, S. Pikkarainen, G. Zoumpoulidou, A. Alsafi, C. Ekere, T.J. Kemp, J.L. Dennis, L. Game, P. H. Sugden, A. Clerk, Temporal regulation of expression of immediate early and second phase transcripts by endothelin-1 in cardiomyocytes, Genome Biol. 9 (2008) R32, https://doi.org/10.1186/gb-2008-9-2-r32.
[60] M. Maillet, N.H. Purcell, M.A. Sargent, A.J. York, O.F. Bueno, J.D. Molkentin, DUSP6 (MKP3) null mice show enhanced ERK1/2 phosphorylation at baseline and increased myocyte proliferation in the heart affecting disease susceptibility, J. Biol. Chem. 283 (2008) 31246–31255, https://doi.org/10.1074/jbc.M806085200.
[61] L. Jiang, L. Ren, X. Guo, J. Zhao, H. Zhang, S. Chen, S. Le, H. Liu, P. Ye, M. Chen, J. Xia, Dual-specificity phosphatase 9 protects against cardiac hypertrophy by targeting ASK1, Int. J. Biol. Sci. 17 (2021) 2193–2204, https://doi.org/10.7150/ijbs.57130.
[62] E. Cerbai, R. Pino, L. Sartiani, A. Mugelli, Influence of postnatal-development on I (f) occurrence and properties in neonatal rat ventricular myocytes, Cardiovasc. Res. 42 (1999) 416–423, https://doi.org/10.1016/s0008-6363(99)00037-1.
[63] T. Banyasz, I. Lozinskiy, C.E. Payne, S. Edelmann, B. Norton, B. Chen, Y. Chen-Izu, L.T. Izu, C.W. Balke, Transformation of adult rat cardiac myocytes in primary culture, Exp. Physiol. 93 (2008) 370–382, https://doi.org/10.1113/expphysiol.2007.040659.
[64] S.R. Agarwal, R.T. Sherpa, K.S. Moshal, R.D. Harvey, Compartmentalized cAMP signaling in cardiac ventricular myocytes, Cell. Signal. 89 (2022) 110172, https://doi.org/10.1016/j.cellsig.2021.110172.
[65] L.B. Bugaisky, R. Zak, Differentiation of adult rat cardiac myocytes in cell culture, Circ. Res. 64 (1989) 493–500, https://doi.org/10.1161/01.res.64.3.493.
[66] V. Talman, J. Teppo, P. Poho, P. Movahedi, A. Vaikkinen, S.T. Karhu, K. Trost, T. Suvitaival, J. Heikkonen, T. Pahikkala, T. Kotiaho, R. Kostiainen, M. Varjosalo, H. Ruskoaho, Molecular atlas of postnatal mouse heart development, J. Am. Heart Assoc. 7 (2018) e010378, https://doi.org/10.1161/JAHA.118.010378.
[67] M.A. Bogoyevitch, J. Gillespie-Brown, A.J. Ketterman, S.J. Fuller, R. Ben-Levy, A. Ashworth, C.J. Marshall, P.H. Sugden, Stimulation of the stress-activated mitogen-activated protein kinases subfamilies in perfused heart. p38/RK mitogen-activated kinases and c-Jun N-terminal kinases are activated by ischemia-reperfusion, Circ. Res. 79 (1996) 162–173, https://doi.org/10.1161/01.res.79.2.162.
[68] S.J. Harding, G.J. Browne, B.W. Miller, S.A. Prigent, M. Dickens, Activation of ASK1, downstream MAPKK and MAPK isoforms during cardiac ischaemia, Biochim. Biophys. Acta 2010 (1802) 733–740, https://doi.org/10.1016/j.bbadis.2010.06.005.
[69] D.N. Meijles, J.J. Cull, T. Markou, S.T.E. Cooper, Z.H.R. Haines, S.J. Fuller, P. O'Gara, M.N. Sheppard, S.E. Harding, P.H. Sugden, A. Clerk, Redox regulation of cardiac ASK1 (apoptosis signal-regulating kinase 1) controls p38-MAPK (mitogen-activated protein kinase) and orchestrates cardiac remodeling to hypertension, Hypertension 76 (2020) 1208–1218, https://doi.org/10.1161/HYPERTENSIONAHA.119.14556.
[70] D. Xu, M.L. Matsumoto, B.S. McKenzie, A.A. Zarrin, TPL2 kinase action and control of inflammation, Pharmacol. Res. 129 (2018) 188–193, https://doi.org/10.1016/j.phrs.2017.11.031.
[71] K.A. Koster, M. Dethlefs, J. Duque Escobar, E. Oetjen, Regulation of the activity of the dual leucine zipper kinase by distinct mechanisms, Cells 13 (2024) 333, https://doi.org/10.3390/cells13040333.
[72] J. Yang, M. Boerm, M. McCarty, C. Bucana, I.J. Fidler, Y. Zhuang, B. Su, Mekk3 is essential for early embryonic cardiovascular development, Nat. Genet. 24 (2000) 309–313, https://doi.org/10.1038/73550.
[73] C. Qi, R.S. Bujaroski, J. Baell, X. Zheng, Kinases in cerebral cavernous malformations: pathogenesis and therapeutic targets, Biochim. Biophys. Acta, Mol. Cell Res. 1870 (2023) 119488, https://doi.org/10.1016/j.bbamcr.2023.119488.
[74] J. Moscat, M.T. Diaz-Meco, A. Albert, S. Campuzano, Cell signaling and function organized by PB1 domain interactions, Mol. Cell 23 (2006) 631–640, https://doi.org/10.1016/j.molcel.2006.08.002.
[75] J. Lu, Z. Hu, Y. Deng, Q. Wu, M. Wu, H. Song, MEKK2 and MEKK3 orchestrate multiple signals to regulate hippo pathway, J. Biol. Chem. 296 (2021) 100400, https://doi.org/10.1016/j.jbc.2021.100400.
[76] F. Meng, B. Xie, J.F. Martin, Targeting the hippo pathway in heart repair, Cardiovasc. Res. 118 (2022) 2402–2414, https://doi.org/10.1093/cvr/cvab291.
[77] K.I. Watt, K.F. Harvey, P. Gregorevic, Regulation of tissue growth by the mammalian hippo signaling pathway, Front. Physiol. 8 (2017) 942, https://doi.org/10.3389/fphys.2017.00942.