Stimulation of glycolysis promotes cardiomyocyte proliferation after injury in adult zebrafish

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**Abstract**

Cardiac metabolism plays a crucial role in producing sufficient energy to sustain cardiac function. However, the role of metabolism in different aspects of cardiomyocyte regeneration remains unclear. Working with the adult zebrafish heart regeneration model, we first find an increase in the levels of mRNAs encoding enzymes regulating glucose and pyruvate metabolism, including pyruvate kinase M1/2 (Pkm) and pyruvate dehydrogenase kinases (Pdk1s), especially in tissues bordering the damaged area. We further find that impaired glycolysis decreases the number of proliferating cardiomyocytes following injury. These observations are supported by analyses using loss-of-function models for the metabolic regulators Pkma2 and peroxisome proliferator-activated receptor gamma coactivator 1 alpha. Cardiomyocyte-specific loss- and gain-of-function manipulations of pyruvate metabolism using Pdk3 as well as a catalytic subunit of the pyruvate dehydrogenase complex (PDC) reveal its importance in cardiomyocyte dedifferentiation and proliferation after injury. Furthermore, we find that PDK activity can modulate cell cycle progression and protrusive activity in mammalian cardiomyocytes in culture. Our findings reveal new roles for cardiac metabolism and the PDK-PDC axis in cardiomyocyte behavior following cardiac injury.

**Keywords** cardiomyocyte regeneration; cardiomyocyte proliferation; glycolysis; metabolism; zebrafish

**Subject Categories** Development; Metabolism

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**Introduction**

Cardiac metabolism is essential to produce adenosine triphosphate (ATP), which is required for cardiac homeostasis and function (Neely & Morgan, 1974; Kolwicz et al., 2013). During development, embryonic cardiomyocytes (CMs) mainly utilize glucose metabolism for ATP production (Piquereau & Ventura-Clapier, 2018). As the heart grows, CMs exhibit metabolic changes and rely mostly on fatty acid oxidation (Cho et al., 2006; Chung et al., 2007, 2010; Kreipke et al., 2016). It has also been shown that under conditions of cardiac disease, such as ischemia and heart failure, CMs revert to glucose as an energy source (Das et al., 1987; Doenst et al., 2013).

Unlike adult mammals, adult zebrafish regenerate their heart effectively after injury (Poss et al., 2002). During cardiac regeneration, CMS re-express hand2 (Schindler et al., 2014), a transcription factor gene required for cardiac development, as well as an embryonic myosin (Sallin et al., 2015), and they also exhibit sarcomere disassembly (Jopling et al., 2010; Ben-Yair et al., 2019; Beisaw et al., 2020). These and other studies (Kikuchi et al., 2010; Wu et al., 2016; Grajevskaja et al., 2018) suggest that in zebrafish, CMs undergo dedifferentiation to supply new CMs during cardiac regeneration. Thus, CMs may utilize similar regulatory mechanisms during development and regeneration, yet the role of cardiac metabolism in these processes remains unclear.

Pyruvate dehydrogenase kinase plays an essential role in pyruvate metabolism (Gudi et al., 1995; Takubo et al., 2013; Zhang et al., 2014). Glucose is initially converted to pyruvate through several glycolytic intermediates, and then, pyruvate is converted to lactate via anaerobic fermentation (Lehninger et al., 2005), or to acetyl-coenzyme A (acetyl-CoA) by the PDC (Behal et al., 1993). By inhibiting the conversion of pyruvate to acetyl-CoA, PDK induces the conversion of pyruvate to lactate, via anaerobic fermentation (Korotchkina & Patel, 2001; Koukourakis et al., 2005). These studies indicate that PDK regulates the shift of pyruvate metabolism between glycolysis and OXPHOS (Korotchkina & Patel, 2001; Koukourakis et al., 2005; Takubo et al., 2013; Park et al., 2018). Higher expression of PDKs in cancer and stem cells, which rely mainly on glycolysis, has been associated with their enhanced proliferation and migration (Takubo et al., 2013; Zhang et al., 2014; Park et al., 2018).
Here, we first show that glycolytic enzymes are upregulated at the transcriptional level during cardiac regeneration. Pharmacological and genetic manipulations as well as CM-specific modulation of glycolytic activity reveal its importance in CM proliferation after injury. We also found that modulation of pyruvate metabolism can affect mammalian CM cell cycle progression and cell behavior. Transcriptomic analyses show that PDK3 overexpression (OE) leads to higher levels of genes encoding cell cycle regulators in CMs. These data indicate that metabolism plays an important role in CM proliferation following cardiac injury.

Results and Discussion

Glycolysis and pyruvate metabolism modulate cardiomyocyte dedifferentiation and proliferation following cardiac injury

Adult mammalian CMs rely mainly on fatty acid oxidation as a source of energy (Cho et al., 2006; Chung et al., 2007, 2010; Kreipke et al., 2016). We started by performing proteomic analysis and found higher levels of fatty acid oxidation enzymes in adult zebrafish ventricles compared to those in juveniles (Fig 1A and B). These findings are consistent with mammalian models, in which adult CMs express higher levels of fatty acid oxidation enzymes (Puente et al., 2014; Fukushima et al., 2016).

It has been suggested that zebrafish CMs utilize similar regulatory mechanisms during cardiac development and regeneration (Poss et al., 2002; Jopling et al., 2010; Kikuchi et al., 2010; Wu et al., 2016). We have recently shown that glycolysis plays an important role during zebrafish cardiac development (Fukuda et al., 2019). Thus, we wanted to examine whether the expression levels of glycolytic enzyme genes were changed in zebrafish ventricles after cryoinjury. We found significant upregulation of many of them in the tissues bordering the damaged area when compared to remote areas (Fig 1C). This finding is supported by a single-cell RNA-sequencing analysis in regenerating zebrafish hearts (Honkoop et al., 2019). Notably, we found high upregulation of pdk2b, pdk3b, and pdk4 (Fig 1C), which encode key enzymes that regulate pyruvate metabolism and promote glycolysis in a number of cell types including CMs (Zhao et al., 2008) and cancer cells (Koukourakis et al., 2005; Leclerc et al., 2017; Peng et al., 2018). We also measured lactate levels, which are indicative of glycolytic activity, and found higher levels in regenerating cardiac tissues (Fig EV1A). These and other observations (Honkoop et al., 2019) suggest that adult zebrafish CMs switch from fatty acid oxidation to glycolytic metabolism in response to cardiac injury. In order to examine the role of glycolysis and Pdk function following cardiac injury, we treated zebrafish with 2-deoxy-D-glucose (2-DG) and dichloroacetate (DCA), which decrease glycolysis and Pdk function, respectively. Notably, these treatments led to a decrease in CM proliferation in the border zone following injury (Figs 1D and EV1B). We also found that the re-expression of embryonic myosin, which has been reported to indicate CM dedifferentiation during cardiac regeneration (Sallin et al., 2015), was reduced when glycolysis or Pdk function was decreased (Fig 1E). These data suggest that glycolysis and pyruvate metabolism as regulated by Pdk play a role in border zone CMs following cardiac injury.

Figure 1. Glycolysis and pyruvate metabolism play an important role following cardiac injury.

- **A** 30 and 150 days post fertilization (dpf) zebrafish ventricles were isolated and relative protein levels determined (n = 3 biological replicates).
- **B** KEGG over-representation analysis for selected categories of upregulated proteins in 150 dpf hearts compared to 30 dpf hearts.
- **C** qPCR analysis of mRNA levels of glycolytic enzyme genes in 5 dpf remote and wound border cardiac tissue (n = 2–3 technical replicates using pooled cDNA from 10 ventricles for each condition (n = 2 for pkm2 and pdk4, and n = 3 for the other genes)).
- **D, E** Immunostaining of heart sections for PCNA and MEF2D or N261.261 and MEF2E (E) in 5 dpf animals treated with PBS, 2-DG, or DCA; magnified view of area in white boxes shown below; white dashed lines outline the wound area; arrowheads point to PCNA+ or N261.261+ CMs; percentage of PCNA+ or N261.261+ CMs in the border zone shown on the right (n = 4–5 ventricles).

Data information: Error bars, s.e.m.; P values were calculated by Bayesian-moderated t-test (limma); (A, B), two-tailed unpaired t-test (C), or ordinary one-way ANOVA with Dunnett’s multiple comparison test (D, E). Scale bars, 50 µm.

Genetic manipulation of metabolic enzymes affects cardiomyocyte dedifferentiation and proliferation following cardiac injury

Next, we assessed whether the loss of specific metabolic enzymes affected CM behavior after cardiac injury. We first examined the role of Pkm2. Mammalian Pkm gives rise to two splice isoforms, Pkm1 and Pkm2; Pkm2 promotes glucose metabolism toward lactate, while Pkm1 promotes glucose metabolism toward acetyl-CoA (Lehninger et al., 2005; Christofk et al., 2008). Knockdown of Pkm2 has been reported to lead to decreased glycolytic activity in human umbilical vein endothelial cells (Stone et al., 2018) and intestinal stem cells (Kim et al., 2019), as well as upregulation of Pkm1 (Lunt et al., 2015; Zheng et al., 2016). Similarly, Pkm2 OE in mouse CMs leads to enhanced glycolysis (Magadum et al., 2020).

The zebrafish genome contains two pkm genes (pkma and pkmb), with pkma encoding both an orthologue of mammalian Pkm1 (pkma1) and an orthologue of mammalian Pkm2 (pkma2), while pkmb encodes another orthologue of mammalian Pkm2 (Stone et al., 2018). Expression analysis in adult ventricles indicates that pkma is upregulated in tissues bordering the injured area, while pkmb is not (Fig 1C), suggesting a role for pkma following cardiac injury. In pkma2 mutant zebrafish, the pkma1 isoform, which promotes glucose metabolism toward acetyl-CoA, remains intact, suggesting that the metabolism of pyruvate to lactate is impaired while pyruvate oxidation is increased. These data indicate that a zebrafish pkma2 mutant constitutes a potential model of impaired glycolysis, as pyruvate is expected to be converted preferentially to acetyl-CoA but not lactate. Thus, we examined pkma2−/−; pkmb+−/− animals, which do not display obvious cardiac defects compared to WT (Fig EV2A–C), and found that they exhibited a reduction in the number of proliferating CMs after cardiac injury (Figs 2A and EV2D). Consistently, the expression of embryonic myosin was also decreased in pkma2−/−; pkmb+−/− animals (Fig 2B). We also
Figure 1.
Figure 2. Loss of pkma2 and ppargc1a affects cardiomyocyte dedifferentiation and proliferation following cardiac injury.

A–D Immunostaining of heart sections for PCNA and MEF2 (A, C) or N2.261 and MEF2 (B, D) in 5 dpci pkma2+/−; pkmb+/−; pkma2−/−; pkmb−/− (A, B) or ppargc1a+/+ and ppargc1a−/− (C, D) animals; magnified view of area in white boxes shown below; arrowheads point to PCNA+/− (A, C) or N2.261+ (B, D) CMs; white dashed lines outline the wound area; percentage of PCNA+/− (A, C) or N2.261+ (B, D) CMs in the border zone shown on the right (n = 4–6 ventricles of each genotype).

E, F AFOG staining of heart sections from 60 dpci pkma2+/−; pkmb+/− and pkma2−/−; pkmb−/− (E) or 45 dpci ppargc1a+/+ and ppargc1a−/− (F) animals; black dashed lines outline the scar area; scar area measured on the right (n = 3–5 ventricles of each genotype).

Data information: Error bars, s.e.m.; P values were calculated by two-tailed unpaired t-test. Scale bars, 50 μm.
examined pkma2−/− animals and found that they exhibit a similar decrease in PCNA+ and N2.261+ CMs following cardiac injury (Figs EV2E and F). Together, these results suggest that reducing glycolysis by affecting Pkm2 function leads to an attenuation in CM dedifferentiation and proliferation during regeneration.

Conversely, in order to determine whether enhanced glycolytic activity can promote cardiac regeneration, we examined the role of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (pparc1a), which encodes a key regulator of mitochondrial oxidative metabolism (Lin et al., 2005). Forced Pparc1a expression in CMs enhances oxygen consumption and mitochondrial respiration in vitro and in vivo (Lehman et al., 2000), while Pparc1a-deficient mice exhibit decreased oxygen consumption in their left ventricular tissues (Lehman et al., 2008). Zebrafish pparc1a mutant hearts exhibit reduced mitochondrial oxidative metabolism and increased glycolytic activity (Marin-Juez et al., 2019) without obvious cardiac defects (Fig EV2A–C). Altogether, these data suggest that in pparc1a mutant hearts, pyruvate oxidation is reduced while metabolism of pyruvate to lactate is increased, and thus, pparc1a mutants constitute a potential model of increased glycolysis. First, we examined the mRNA expression levels of pparc1a and its targets atp5pf and ndufb5 and found that these genes were downregulated in the cardiac tissues bordering the damaged area at 5 dpci (Fig EV2H). Notably, pparc1a mutants exhibited an increase in proliferating CMs (Figs 2C and EV2C) as well as in the expression of embryonic myosin (Fig 2D) following cardiac injury, suggesting that promoting glycolysis in CMs can increase their dedifferentiation and proliferative potential. Furthermore, we examined pkma2−/−; pkmb+/− animals at 60 dpci. We performed Acid Fuchsin Orange G (AFOG) staining to examine scar size. Notably, pkma2−/−; pkmb+/− animals exhibited a significantly larger scar area compared to pkma2+/−; pkmb+−/− animals (Fig 2E). However, pparc1a mutants did not exhibit a significant increase in scar area (Fig 2F). Together, these results provide further evidence that key regulators of pyruvate metabolism and glycolysis play an important role in CM dedifferentiation and proliferation in the early stages of cardiac regeneration.

Cardiomyocyte-specific modulation of pyruvate metabolism regulates cardiomyocyte behavior following cardiac injury

Expression analysis showed that multiple pdk genes were highly upregulated in tissues bordering the damaged area following cardiac injury (Fig 1C). Pdk promotes the conversion of pyruvate to lactate by inactivating the PDC through phosphorylation of pyruvate dehydrogenase E1 alpha 1 subunit (Pdha1), a catalytic subunit of the PDC (Korotchkina & Patel, 2001; Rardin et al., 2009; Park et al., 2018). To impair glycolysis specifically in CMs, we used a form of Pdha1a (Pdha1aSTA), which cannot be phosphorylated by Pdk, leading to enhanced pyruvate conversion to acetyl-CoA (Hitosugi et al., 2011; Fan et al., 2014; Fukuda et al., 2019). Using the HOTcre system (Hesselson et al., 2009), we generated a Tg(hsp70:lOXP-STOP-lOXP-pdha1aSTA-T2A-mCherry) line, which, when combined with the Tg(myl7:Cre-ERT2) line and following tamoxifen and heat-shock treatments, expresses pdha1aSTA specifically in CMs to impair glycolysis (Fig EV3A). Consistent with our previous results with DCA treatments, which inhibit Pdk function, the number of proliferating CMs following cardiac injury was decreased in Tg(myl7:Cre-ERT2); Tg(hsp70l:lOXP-STOP-lOXP-pdha1aSTA-T2A-mCherry) animals following heat-shock treatments (Figs 3A and B, and EV3C). Conversely, in order to stimulate glycolysis in CMs, we generated a Tg(hsp70l:lOXP-STOP-lOXP-pdk3b-T2A-mCherry) line and found that when pdk3b was overexpressed in Tg(myl7:Cre-ERT2); Tg(hsp70l:lOXP-STOP-lOXP-pdk3b-T2A-mCherry) animals (Fig EV3B), the number of proliferating CMs following cardiac injury was increased (Figs 3C and EV3D). We also examined the effects of pdk3b OE in non-injured hearts and saw no significant increase in the number of proliferating CMs (Fig EV3E). In line with their effects on CM proliferation, pdha1aSTA OE in CMs led to a decrease in the number of CMs exhibiting embryonic myosin expression, while pdk3b OE led to an increase (Fig 3D and E). Altogether, these results suggest that pyruvate metabolism as regulated by the PDK-PDC axis plays an important role in CM dedifferentiation and proliferation following cardiac injury. We next examined whether metabolic modulation in CMs affects scar size during cardiac regeneration, and found that pdha1aSTA OE led to the retention of a larger scar area at 60 dpci (Fig 3F), while pdk3b OE did not affect scar area (Fig 3G). Together with the data using metabolic enzyme mutant models, these results suggest that in the early stages of cardiac regeneration, glycolysis promotes CM dedifferentiation and proliferation and that genetic manipulations to enhance glycolytic activity do not facilitate scar resolution at later stages.

Modulation of pyruvate metabolism regulates primary rat neonatal cardiomyocyte cell cycle progression and behavior

We also tested the role of pyruvate metabolism in primary rat neonatal cardiomyocytes (RNCMs). To examine the role of pyruvate metabolism modulation in CM cell cycle progression, Ki67 expression was analyzed in RNCMs overexpressing PDHA1STA or PDK3 in growth culture medium containing 10% bovine serum as well as...
Figure 3.

A. Schematic representation of the experimental design.

B. Immunofluorescence images showing the percentage of PCNA+ CMs in control and pdha1aSTA OE mice.

C. Immunofluorescence images showing the percentage of PCNA+ CMs in control and pdk3b OE mice.

D. Immunofluorescence images showing the percentage of N2.261+ CMs in control and pdha1aSTA OE mice.

E. Immunofluorescence images showing the percentage of N2.261+ CMs in control and pdk3b OE mice.

F. Immunohistochemical analysis of muscle, fibrin, and collagen at 60 dpci in control and pdha1aSTA OE mice.

G. Immunohistochemical analysis of muscle, fibrin, and collagen at 60 dpci in control and pdk3b OE mice.
Figure 4.
in non-growth culture medium. We found that PDHA1STA OE significantly decreased the number of Ki67+ RNCMs in growth conditions (Fig 4A), while PDK3 OE increased the number of Ki67+ RNCMs (Fig 4B) in non-growth conditions. PDK3 OE also led to an increase in the number of pH3+ RNCMs (Fig EV4C). We then determined cell numbers and found that PDHA1STA OE significantly decreased the number of RNCMs (Fig EV4D), while PDK3 OE did not lead to obvious changes (Fig EV4E). Next, we investigated the role of pyruvate metabolism in an in vitro scratch assay which is used to examine the ability of cells to fill a wound-like gap in a confluent cell monolayer (Cormier et al., 2015). We first examined CM cell cycle progression in the scratch assay and found no significant differences in the number of Ki67+ CMs in the area close to the scratch border compared to the remote area (Fig EV4A and B). We then examined CM behavior and found that RNCMs close to the scratch border exhibited membrane protrusions (Fig 4C). Similar observations have been reported when analyzing zebrafish and mouse regeneration models, where CMs replenish to the injured area (Itou et al., 2012; Morikawa et al., 2015; Tahara et al., 2016; Marin-Juez et al., 2019; preprint: Aharonov et al., 2020). We analyzed the effects of pyruvate metabolism modulation on CM behavior in the scratch assay and found that PDHA1STA OE led to an increase in the number of CM membrane protrusions (Figs 4C and EV4C). To gain insight into the signaling pathways and effectors that are regulated by PDK3 OE in RNCMs, we performed transcriptomic analysis. Notably, the resulting data indicate that PDK3 OE led to increased levels of genes encoding factors which promote DNA replication and cell cycle (Figs 4D–F and EV4F and G). Altogether, these data indicate that pyruvate metabolism regulated by PDK also promotes mammalian CM cell cycle progression and protractive behavior.

Glycolysis plays an important role in the proliferation of cancer cells (Liberti & Locasale, 2016), endothelial cells (De Bock et al., 2013; Wilhelm et al., 2016), and neural progenitor cells (Zheng et al., 2019), as well as during cardiac (Wang et al., 2018; Honkoop et al., 2019; Magadum et al., 2020) and skeletal muscle (Wagner et al., 1976; Magadum & Engel, 2018) regeneration. Here, we showed that CM-specific modulation of glycolysis also regulates CM dedifferentiation and proliferation during regeneration. In addition, our transcriptomic data show that PDK3 OE, which enhances glycolysis, leads to higher levels of cell cycle regulator gene expression in RNCMs. It will of course be important to investigate further how glycolysis regulates these processes.

Our data indicate that increased glycolysis by Pdk3 OE promotes CM dedifferentiation following cardiac injury. It was reported that in regenerating CMs, mitochondria exhibit an immature structure and that the levels of mitochondrial gene expression are reduced (Honkoop et al., 2019), indicating lower mitochondrial activity. In addition, mitochondrial function increases during CM maturation in the developing heart (Menendez-Montes et al., 2016). Thus, reduced mitochondrial activity, and thereby reduced OXPHOS, may be important for CM dedifferentiation and proliferation during regeneration.

It has also been shown that the levels of glycolytic enzymes increase following cardiac ischemia and in failing hearts (Das et al., 1987; Doenst et al., 2013). However, adult mice do not exhibit robust cardiac regeneration. Recent data show that the upregulation of PKM2 levels following cardiac injury is limited and thus possibly insufficient to promote CM proliferation in an ischemic adult mouse model (Magadum et al., 2020), while Pkm2 OE induced CM proliferation after cardiac injury (Magadum et al., 2020). Together, these data suggest that the levels of glycolytic gene expression in mice following cardiac injury might be a limiting factor in terms of promoting CM proliferation. It will be interesting to further investigate the differences in metabolic changes between zebrafish and mouse cardiac injury models.

During cardiac regeneration, it is assumed that newly divided CMs have to undergo maturation to generate fully functional cardiac tissue. During development, the metabolic shift from glycolysis to OXPHOS plays an important role in CM differentiation and maturation (Cho et al., 2006; Chung et al., 2007). Our data in zebrafish indicate that while enhanced glycolysis by Pdk3 OE promotes CM proliferation during the early stages of cardiac regeneration, scar resolution at later stages does not appear to be significantly affected. However, it remains unclear whether, in conditions of Pdk3 OE, the newly formed CMs have undergone maturation and also whether CM maturation promotes scar resolution. Clearly, many questions remain to be addressed to fully understand the role of metabolism during cardiac regeneration.

Together, our data indicate that cardiac metabolism regulated by the PDK-PDC axis promotes CM proliferation following cardiac injury in zebrafish as well as cell cycle progression of mammalian CMs in culture.

Materials and Methods

Zebrafish

All zebrafish husbandry was performed under standard conditions in accordance with FELASA guidelines (Alestrom et al., 2019) and institutional (MPG) and national ethical and animal welfare guidelines. The following transgenic lines and mutants were used: Tg(cryaa:DsRed,-5.1myl7:CreERT2)st10 (Kikuchi et al,
Cryoinjury and quantification

Cryoinjury was performed as previously described (González-Rosa et al., 2011). Confocal images were processed using the ZEN software (ZEISS), and PCNA−, N2.261+, or pH3+ CMs in the 50-µm region adjacent to the injured area at 5 dpci were counted. The percentage of PCNA−, N2.261+, or pH3+ CMs in the border zone was calculated by dividing the number of PCNA−, N2.261+, or pH3+ CMs by the total number of CMs.

Pharmacological treatments

Adult zebrafish were treated with PBS, 2-DG (1 nmol/kg body weight; Sigma-Aldrich), or DCA (0.5 nmol/kg body weight; Sigma-Aldrich) by intraperitoneal (IP) injections (Kinkel et al., 2010) from 1 to 4 dpci and analyzed at 5 dpci.

Tamoxifen and heat-shock treatments

To induce Cre-ERT2-mediated recombination, animals were treated with 10 µM 4-hydroxytamoxifen (4-HT; Sigma-Aldrich) by IP injection. Cryoinjury was performed 24 h after 4-HT injection. Heat-shock treatments were performed in a 37 °C water bath for 1 h per day from 1 to 4 dpci, and animals were analyzed at 5 dpci. To examine 60 dpci hearts in transgenic animals (Fig 3F and G), heat-shock treatments were performed five to seven times a week from 1 to 59 dpci.

Staining

Zebrafish heart sections and RNCMs were fixed in 4% paraformaldehyde. Anti-MEF2 1:50 (sc-313, Santa Cruz Biotechnology), anti-PCNA 1:500 (PC10, Santa Cruz Biotechnology), anti-N2.261 1:100 (N2.261, DSHB), anti-DesRed 1:500 (632496, Takara), anti-MHC 1:500 (MF20, DSHB), anti-phospho-histone H3 1:200 (06-570, EMD Millipore), and anti-cardiac troponin I (CTNI) 1:500 (ab56357, Abcam) were used. After washing with PBS, samples were stained with Alexa 568, Alexa 488, or Alexa 647 secondary antibodies 1:500 (Life Technologies), followed by 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) 1:2,000 (Merck) staining to visualize DNA. To count PCNA−, N2.261+, or pH3+ CMs in regenerating ventricles, we analyzed more than three sections from each heart. Acid Fuchsin Orange G (AFOG) staining was performed using the A.F.O.G. Kit (BIOCOST).}

Cell culture

Rat neonatal (P1–P3) CMs were isolated as previously described (Fukuda et al., 2017), and CMs were further separated by density gradient centrifugation (Golden et al., 2012). Cells were plated onto 0.1% gelatin-coated (Sigma) plates and cultured in DMEM/F12 (Gibco) supplemented with 5% horse serum, l-glutamine, Na pyruvate, penicillin, and streptomycin at 37 °C and 5% CO2. 10% FBS (HyClone) was added to the medium for the growth conditions. CMs were transfected with adenovirus vectors and cultured for 72 h in growth medium or non-growth medium. Samples were then fixed with 4% PFA and stained with anti-CTNI and DAPI. Five different 1,000 µm regions per sample were analyzed for CM numbers, and average values were used.

Lactate measurements

Lactate levels were measured using a PicoProbe Lactate Fluorometric Assay Kit (BioVision). For each condition, the cryoinjured area and adjacent tissue from 10 ventricles were collected in PBS and homogenized. Samples were centrifuged, and the supernatant was used to measure lactate levels according to manufacturer’s protocol. Fluorescence was detected with a FLUOSTar Omega instrument (BMG).

Adenovirus vectors

Adenovirus vectors for transfection into CMs were generated using the AdEasy system (Agilent Technologies). The adenovirus vector encoding Pdk3 was previously described (Fukuda et al., 2019). To amplify Pdha1 (NM_0088810), mouse tissues, the following primers were used: Pdha1 (forward 5′-TAGAGATCTCGAGTTGAGTTAGAGAGATGC-3′ and reverse 5′-GGATATTCTTGGGCTCGAGAACAGAGTCG-3′) and P3 (NM_001080688) and pdha1a (NM_213393) were isolated by RT–PCR and cloned into a vector containing a promoter of heat-shock cognate 70-kd protein, like (hs70), and two I-SceI restriction sites. The following primers were used to amplify the cDNA: pdk3b (forward 5′-TTCACAGTCGCCCTCGCCCTCGCCTCGCTGTTTACCTCTG-3′ and reverse 5′-GCCCTCTCCACCGGCCCTCATGAAACAGAGATGC-3′) and reverse 5′-GCCCTCTCCACCGGCCCTCATGAAACAGAGATGC-3′). Plasmids were then injected into one-cell-stage embryos with I-SceI (NEB).

Scratch assay

CMs were cultured in growth medium until the plates were confluent and then transfected with adenovirus vectors encoding the gene of interest. After 24 h of culture, a sterilized plastic ruler was laid on the plate, and a sterile pipette tip was used to make a scratch in the CM layer as previously described (Cormier et al., 2015). After removing the dead cells, the plates were incubated for 3 days and then analyzed. To quantify CM membrane protrusions in the scratch assay, the number of CM membrane protrusions in an area of 1,000 µm height × 200 µm width was determined. The average value from two different regions per
sample was used. Values were normalized to the average value of control.

**Imaging**

A Zeiss spinning disk confocal microscope (CSU-X1, Yokogawa) and ORCA-Flash4.0 sCMOS camera (Hamamatsu) were used to acquire images. Time-lapse imaging for the scratch assay was performed using an SP8 microscope (Leica).

**RT–qPCR**

The miRNeasy Mini Kit (Qiagen) was used for total RNA extraction, and cDNA was synthesized using the SuperScript Second Strand Kit (Life Technologies). The CFX Connect Real-Time System (Bio-Rad) and Dynamo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific) were used. Technical replicates were analyzed for each sample. Primer sequences are shown in Table EV1 and Ct values in Table EV2.

**Statistical analysis**

Benjamini–Hochberg-corrected P values as well as standard errors for the fold changes represented by error bars were calculated using limma for proteomic analysis (Fig 1A and B). Comparative statistics between two sample groups was performed using two-tailed unpaired t-test and between more than two sample groups using ordinary one-way ANOVA with Dunnett’s multiple comparison test for parametric data. Benjamini–Hochberg correction was used for RNA-seq data analysis.

**Mass spectrometry**

Zebrafish ventricles were homogenized with a grinder in SDS lysis buffer (4% SDS in 0.1 M Tris–HCl pH 7.6) and heated at 70°C for 10 min. For DNA shearing, samples were sonicated prior to sedimentation of cell debris by centrifugation at 16,000 g for 10 min. Cleared supernatants were subjected to estimation of protein concentration (DC protein assay, Bio-Rad). Protein samples were separated according to their molecular weight by SDS–PAGE (NuPAGE 4–12% Bis-Tris Gel, Thermo Scientific), stained by InstantBlue Coomassie staining (Expe- deon), and subjected to in-gel digestion (Shevchenko et al, 2006). Finally, samples were desalted and stored on STOP And Go Extraction (STAGE) tips (Rappsilber et al, 2003) prior to analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Capillary LC was performed in line to a Q Exactive HF mass spectrometer (Thermo Scientific) via an electro-spray ionization (ESI) source using a nano-UHPLC System (EASY-nLC 1000, Thermo Scientific), as well as an in-house packed 70 μm ID, 15-cm reverse-phase column emitter (ReproSil-Pur 120 C18-AQ, 1.9 μm, Dr. Maisch GmbH) with a buffer system comprising solvent A (5% acetonitrile and 1% formic acid) and solvent B (80% acetonitrile and 1% formic acid). Relevant instrumentation parameters were extracted using MARMoSET (Kiweler et al, 2019) and are included in the Appendix information. Raw data processing was done using the MaxQuant suite of algorithms (v. 1.6.8.0) (Cox & Mann, 2008) against the Danio rerio UniProtKB database (canonical and isoforms; downloaded on 2019/08/19; 62,099 entries) with parameters documented in the supplementary material and employing label-free quantitation (Cox et al, 2014). Downstream bioinformatics analysis was performed using a limma-based R pipeline (Ritchie et al, 2015) (https://github.com/bhagwataditya/autonomics), on logarithmized intensities provided by MaxQuant and including an over-representation analysis by Fisher’s exact test, testing for enrichment of KEGG annotation terms in protein groups significantly ($P \leq 0.05$) upregulated in 150 dpf vs. 30 dpf hearts as compared to the detectome, the total of all detected protein groups.

**RNA-seq analysis**

Total RNA was isolated from RNCMs using the miRNeasy Mini Kit (Qiagen). RNA and library preparation integrity were verified with LabChip GX Touch 24 (Perkin Elmer). 1 μg of total RNA was used as input for VAHTS Stranded mRNA-seq Library Preparation following manufacturer’s protocol (Vazyme). Sequencing was performed on a NextSeq 500 instrument (Illumina) using v2 chemistry, resulting in an average of 37 M reads per library with 1 × 75 bp single-end setup. The resulting raw reads were assessed for quality, adapter content, and duplication rates with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Trimmomatic version 0.39 was employed to trim reads after a quality drop below a mean of Q20 in a window of 5 nucleotides (Bolger et al, 2014). Only reads between 30 and 150 nucleotides were cleared for further analyses. Trimmed and filtered reads were aligned vs. the Ensembl Rat genome version rn6 (version 94) using STAR 2.6.1d with the parameter “–outFilterMismatchNoverLmax 0.1” to increase the maximum ratio of mismatches to mapped length to 10% (STAR: ultrafast universal RNA-seq aligner) (Dobin et al, 2013). The number of reads aligning to genes was counted with featureCounts 1.6.5 tool from the Subread package (Liao et al, 2014). Only reads mapping at least partially inside exons were admitted and aggregated per gene. Reads overlapping multiple genes or aligning to multiple regions were excluded. Differentially expressed genes were identified using DESeq2 version 1.26.0 (Love et al, 2014). Only genes with a minimum fold change of ±2 (log2 $\geq 1$), a maximum Benjamini–Hochberg-corrected P-value of 0.05, and a minimum combined mean of 5 reads were deemed to be significantly differentially expressed. The Ensembl annotation was enriched with UniProt data (release 17.12.2018) based on Ensembl gene identifiers (Activities at the Universal Protein Resource (UniProt)). Gene Ontology analyses were performed using KEGG (https://www.genome.jp/kegg/kegg.html), Reactome (https://reactome.org/), and KOBAS 2.0 (Xie et al, 2011). Two separate tests were performed per contrast using only either up- or down-regulated genes for analysis. The results were combined keeping only gene sets that showed significant over-representation at FDR < 0.2 in only one input list (i.e., that were clearly enriched for up- or down-regulated genes, but not both). The Top10 gene sets considering enrichment FDR were selected per direction of regulation. The dashed line represents an FDR of 0.05, while the values in brackets denote the number of regulated genes vs. total genes in the respective pathway (Figs 4D and EV4F and G).
Data availability

The proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD016235 (http://www.ebi.ac.uk/pride/archive/projects/PXD016235). The transcriptomics data have been deposited to the GEO database (accession number GSE147073; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147073).

Expanded View for this article is available online.

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Author contributions

RF designed and conducted most experiments; RF and DYRS analyzed the data and wrote the paper; RM-J and HES designed and performed the experiments; AB and RM-J helped with the writing; RR helped with live imaging; CK and SG conducted transcriptome analysis; and AK, AMB, and JG conducted proteome analysis. All authors commented on the manuscript.

Conflict of interest

The authors declare no competing financial interests.

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