Activation of Nkx2.5 transcriptional program is required for adult myocardial repair

Carmen de Sena-Tomás1, Angelika G. Aleman2, Caitlin Ford3, Akriti Varshney4,5, Di Yao1, Jamie K. Harrington1, Leonor Saúde6, Mirana Ramialison5,7 & Kimara L. Targoff1,8

The cardiac developmental network has been associated with myocardial regenerative potential. However, the embryonic signals triggered following injury have yet to be fully elucidated. Nkx2.5 is a key causative transcription factor associated with human congenital heart disease and one of the earliest markers of cardiac progenitors, thus it serves as a promising candidate. Here, we show that cardiac-specific RNA-sequencing studies reveal a disrupted embryonic transcriptional profile in the adult Nkx2.5 loss-of-function myocardium. nkre−/− fish exhibit an impaired ability to recover following ventricular apex amputation with diminished dedifferentiation and proliferation. Complex network analyses illuminate that Nkx2.5 is required to provoke proteolytic pathways necessary for sarcomere disassembly and to mount a proliferative response for cardiomyocyte renewal. Moreover, Nkx2.5 targets embedded in these distinct gene regulatory modules coordinate appropriate, multi-faceted injury responses. Altogether, our findings support a previously unrecognized, Nkx2.5-dependent regenerative circuit that invokes myocardial cell cycle re-entry, proteolysis, and mitochondrial metabolism to ensure effective regeneration in the teleost heart.

1 Division of Cardiology, Department of Pediatrics, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA. 2 Department of Physiology & Cellular Biophysics, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA. 3 Department of Genetics & Development, College of Physicians & Surgeons, Columbia University, New York, NY 10032, USA. 4 Monash Biomedicine Discovery Institute, Monash University, Clayton, VIC 3800, Australia. 5 Australian Regenerative Medicine Institute & Systems Biology Institute Australia, Monash University, Clayton, VIC 3800, Australia. 6 Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, 1649-028 Lisboa, Portugal. 7 Murdoch Children’s Research Institute & Department of Paediatrics, The University of Melbourne, Parkville, VIC 3052, Australia. 8 Columbia Stem Cell Initiative, Columbia University, New York, NY 10032, USA. ✉ email: kl284@columbia.edu
Due to the limited responsiveness of endogenous cellular and molecular repair mechanisms in injured myocardium, cardiac dysfunction remains a key cause of morbidity and mortality in patients. Coronary artery disease leads to myocardial infarction which results in fibrotic scarring, adverse remodeling, and heart failure. Furthermore, in patients with congenital heart disease, cardiac ischemia yields cardiomyocyte (CM) loss and the induction of inappropriate healing responses. Despite these pathogenic mechanisms that ensue following injury, recent reports demonstrate that adult CMs preserve the capacity to replicate in both mice and humans. Therefore, identifying key factors that enhance CM proliferation offers opportunities to stimulate myocardial repair in patients suffering from cardiac dysfunction or hypoplasia.

Interestingly, in contrast to adult mice and humans, neonatal mice and adult zebrafish retain the ability to undergo CM dedifferentiation and proliferation, optimizing cardiac regenerative potential. Specifically, new CMs emerge following division of pre-existing CMs to repopulate the injury site. Furthermore, despite the propensity of most mammalian CMs to undergo polypliodization, a positive correlation between the maintenance of a mononuclear, diploid state, and retention of cardiac regeneration capacity in both mice and zebrafish has been established. Taken together, exploiting the pro-regenerative mechanisms innate in zebrafish and applying them to diseased human hearts would provide an arsenal of tools to awaken constructive healing in both adult and pediatric patients.

Parallel transcriptional networks have been shown to be essential for both development and regeneration of several tissues including the heart. For example, reactivation of genes essential during cardiogenesis is critical to stimulate CM production in damaged myocardium. Thus, we took advantage of our prior investigation of the Nkx2.5 transcriptional regulatory pathways in the zebrafish embryo to provide insights into the developmental mechanisms that are harnessed for activation of cardiac regenerative potential. The homeodomain transcription factor Nkx2-5, is one of the most commonly mutated genes associated with human congenital heart disease (CHD) and is a master regulator of cardiac development. Studies in model organisms highlight crucial roles of vertebrate and invertebrate homologs of Nkx2-5 in cardiac specification and morphogenesis. While the functions of Nkx genes in early development have been examined, it remains unclear whether Nkx2-5 is necessary to mount an efficient response for CM renewal. In this study, we inspect the distinct cellular and molecular processes regulated by Nkx2-5 in injured myocardium to elucidate effective therapeutic strategies for myocardial regeneration.

Our previous work illuminates that temporally controlled expression of nkh2.5 is required to preserve chamber-specific identity maintenance in both the first and second heart fields during discrete developmental time windows. Moreover, in the context of otherwise embryonic lethal nkh2.5−/− embryos, early overexpression (21 somites) of nkh2.5 is sufficient to sustain cardiac function and ensure embryonic viability into adulthood. These results regarding the temporal regulation of nkh2.5 broaden our appreciation of the essential functions of early cardiac transcriptional regulation in establishing long-standing myocardial health. Thus, we exploited this unique model to study the role of nkh2.5 in the adult heart following injury. Here, we show that rescued nkh2.5−/− fish demonstrate a diminished regenerative response following amputation of the ventricular apex. Compromised sarcomere dedifferentiation and decreased proliferation dampen the requisite regrowth of new CMs from pre-existing CMs in the nkh2.5−/− fish. Moreover, the epicardium fails to penetrate the regenerate to establish the tissue environment necessary to induce a cascade of signals required for healing. Altogether, our data illustrate the critical function of Nkx2.5 in the stressed adult myocardium to invoke regenerative cues for CM dedifferentiation, renewal, and effective patterning of a supportive microenvironment. Understanding the mechanisms guiding Nkx2.5-dependent cardiac regeneration will have an impact on the management of patients with CHD or cardiomyopathies associated with Nkh2.5 mutations.

Results
Embryonic rescue generates nkh2.5 loss-of-function fish. Given the embryonic lethality of mutations in nkh2.5, we pioneered an inducible, overexpression reagent to study the role of nkh2.5 in the adult myocardium. In nkh2.5−/− embryos, heat shock of Tg(hsp70l:nkh2.5-EGFP) during heart tube formation (21 somites) rescues the cardiac chamber proportion and identity defects normally observed during embryogenesis (Fig. 1A). These results led us to investigate the gross morphology and chamber-specific characteristics in age- and size-matched, heat-shocked, non-transgenic wild-type, Tg(hsp70l:nkh2.5-EGFP), and nkh2.5−/−;Tg(hsp70l:nkh2.5-EGFP) (hereafter referred to as nkh2.5−/−) fish (Fig. 1). Remarkably, the myocardial architecture appears normal in adult nkh2.5−/− fish (MT0) as compared to non-transgenic (WT0) and transgenic (TG0) wild-type fish following heat shock at 21 somites (Fig. 1B–D). Employing Acid Fuchsin-Orange G (AFOG), there is no evidence of tissue damage or collagen deposition in non-transgenic wild-type, transgenic wild-type, and nkh2.5−/− fish (Fig. 1E–G). Tropomyosin immunofluorescence delineates clearly ordered and uniformly spaced z-disks in all three genotypes (Fig. 1N–P). Furthermore, ventricular myosin heavy chain (vmhc) is expressed uniformly throughout the ventricles of the non-transgenic wild-type, transgenic wild-type, and nkh2.5−/− fish (Fig. 1H–J). However, while atrial myosin heavy chain (amhc) expression is visualized appropriately in the atrium in all genotypes, it is uniquely expressed in the ventricle of the nkh2.5−/− fish (Fig. 1M), a finding that is reminiscent of ectopic atrial (S46+) CMs in nkh2.5−/− embryos. Although these results reinforce the temporal requirement for Nkx2.5 in ventricular identity maintenance, we observed no evidence of gross histological or structural malformations of the ventricle or atrium in the nkh2.5−/− fish.

To substantiate our findings with quantitative strategies, we applied previously established morphometrics to assess for normal postembryonic cardiac growth. Precisely, we observed no statistically significant difference in the percentage of trabeculation between non-transgenic, transgenic wild-type, and nkh2.5−/− hearts (Fig. 1Q). However, ventricular volume is slightly increased in the nkh2.5−/− when compared to non-transgenic and transgenic wild-type fish, suggesting mild ventricular expansion (Fig. 1R). To assess the significance of this subtle variability in chamber size, we tested for possible signs of cardiac failure by subjecting the fish to an exercise routine. In a swim tunnel, we measured endurance, the average time during which the fish maintained swimming activity. We found that the nkh2.5−/− hearts were significantly more prone to cardiac failure compared to non-transgenic and transgenic wild-type hearts (Fig. 1S). Together, we conclude that, although future investigation may help to unravel these slight differences in ventricular size, Nkx2.5 is not necessary following embryogenesis to ensure long-term viability and fitness into adulthood.

Molecular signatures of adult nkh2.5−/− hearts are perturbed. Despite intact cardiac formation in the nkh2.5−/− compared to wild-type fish, we posited that there may be alterations in the underlying molecular signatures given the absence of this critical cardiac transcription factor. Thus, we performed RNA sequencing (RNA-seq) analysis of ventricular apices extracted from non-transgenic wild-type, transgenic wild-type, and nkh2.5−/− hearts.
We investigated the fundamental molecular similarities and differences of these three genotypes by retrieving the subsets of statistically significant, differentially expressed genes (DEGs). To isolate the set of DEGs that are misregulated in the \(nkx2.5^{-/-}\) fish (MT0) independent of the control sample used (WT0 or TG0), we established a subgroup of 1073 genes (hereafter referred to as group C or the ‘overlap’ group, Fig. S1A). This intersection category reflects the critical genes that are differentially expressed in the \(nkx2.5^{-/-}\) heart when compared to both wild-type conditions while excluding those genes that are misregulated between the transgenic and non-transgenic wild-type samples.

We then explored the Gene Ontology (GO) categories most highly enriched in each of the comparisons. Although several GO terms were similarly enriched in the MT0 vs WT0 (group A) and
Fig. 1 Adult nkx2.5−/− fish have structurally normal hearts. A Schematic of the experimental design and genotype identification. B-D Representative images of hearts stained for Troponymosin (green) and DAPI (blue) indicate cardiac muscle and nuclei, respectively, in wild-type (WT0) (n = 11) (B), Tg(hsp70:nkx2.5-EGFP) (TGO) (n = 6) (C), and nkx2.5−/− (MT0) (n = 7) (D) adult hearts. Scale bar, 100 µm. E-G The same sections in the top panels are stained with Acid Fuchsin-Orange G (AFOG) to indicate fibrin (red), myocardium (orange), and collagen (blue) in non-transgenic wild-type (WT0) (n = 11) (E), transgenic wild-type (TGO) (n = 6) (F), and nkx2.5−/− (MT0) (n = 7) (G) adult hearts. Scale bar, 100 µm. H-M In situ hybridization for vmhc (H-J) and amhc (K-M) in non-transgenic wild-type (WT0) (n = 11) (H, K), transgenic wild-type (TGO) (n = 6) (I, L), and nkx2.5−/− (MT0) (n = 7) (J, M) adult hearts. In nkx2.5−/− hearts, amhc is expressed ectopically in the ventricle (arrows) (n = 7/7). N-P Tropomyosin immunostaining elucidates well-organized z-disks in non-transgenic wild-type (WT0) (n = 11) (N), transgenic wild-type (TGO) (n = 6) (O), and nkx2.5−/− (MT0) (n = 7) (P) ventricular myocardium. Scale bar, 10 µm. Q-R Morphometric evaluation of trabecular myocardium in non-transgenic wild-type (WT0) (n = 5), transgenic wild-type (TGO) (n = 5), and nkx2.5−/− (MT0) (n = 5) adult hearts and ventricular volume in non-transgenic wild-type (WT0) (n = 8), transgenic wild-type (TGO) (n = 8), and nkx2.5−/− (MT0) (n = 7) adult hearts. Mean and standard error of each data set are shown. Unpaired, two-tailed t-test demonstrates no statistically significant differences between WT0 and TGO (p = 0.1428) and WT0 and MT0 (p = 0.8124) in Q. Unpaired, two-tailed t-test illustrates no statistically significant difference between WT0 and TGO (p = 0.2770) and statistically significant difference between WT0 and MT0 (p = 0.0490) in R. S Swim endurance assessed in non-transgenic wild-type (WT0) (n = 6), transgenic wild-type (TGO) (n = 6), and nkx2.5−/− (MT0) (n = 6) fish at maximal water speed. Mean and standard error of each data set are shown. Unpaired, two-tailed t-test shows no statistically significant differences between WT0 and TGO (p = 0.3866) and WT0 and MT0 (p = 0.3409). Source data are provided as a Source Data file.

While this methodological strategy of defining the ‘overlap’ category captures variations in the molecular profile unique to the loss of nkx2.5 gene function, we questioned whether hsp701 promoter leakiness could explain the observed differences between TGO and WT0. Our prior data illustrates absence of Tg(hsp70:nkx2.5-EGFP) expression during embryogenesis. We thus inspected the potential for overexpression in the adult heart. Interestingly, qPCR analysis of nkx2.5 expression illuminates no statistically significant difference between levels detected in TGO and WT0 hearts (Fig. S1B). Given that the presence of Tg(hsp70:nkx2.5-EGFP) does not explain the differences observed between TGO and WT0 datasets, we dissected the most highly enriched GO terms in group D (Fig. 2A) and found autophagy, mitochondrion organization, and ribosome biogenesis. Thus, we conclude that aberrations in these ‘housekeeping’ functions constitute the primary differences in this comparison and are distinct from the molecular changes observed in the MT0 comparisons. Indeed, while ‘housekeeping’ functions represent >75% of the DEGs in the TGO vs WT0 comparison, these GO terms compose only ~5% of the DEGs in the MT0 comparisons, reinforcing the benefits of utilizing the ‘overlap’ dataset to isolate genetic differences attributed to the loss of nkx2.5 gene function in the adult heart.

In this ‘overlap’ set, we investigated the genetic interaction network pertaining to the heart development GO category. Our studies identify bmp4, a vital signal in cardiac development and regeneration, as a core node (Fig. 2B). bmp4 is upregulated in the nkx2.5−/− fish when compared to both non-transgenic and transgenic wild-type fish. Our results are closely aligned with previous murine studies in which a negative feedback loop is identified between Nkx2.5 and Bmp2, the functional equivalent of zebrafish Bmp4. Given that bmp4 serves as a central connector in our RNA-seq dataset, we propose that modulation of myriad Nkx2.5 effectors, both direct and indirect, function to disrupt the cardiac development regulatory program in the nkx2.5−/− fish. As verification of the biological significance of this gene regulatory network (GRN), we detected statistically significant upregulation of versican (vcav1) in nkx2.5−/− compared to the wild-type myocardium employing RNAscope strategy (Fig. 2C–E). Taken together, our results illuminate that overexpression of Nkx2.5 at 21 somites rescues nkx2.5−/−–associated embryonic defects but results in enduring perturbation of the essential developmental pathways throughout adulthood.

Activation of nkx2.5 occurs following myocardial injury. Given that the Nkx2.5 downstream network is modified despite normal myocardial infrastructure in nkx2.5−/− hearts, we hypothesized that the integrity of the Nkx2.5 transcriptional profile is robust enough to obfuscate gross structural abnormalities. We speculated that stress or injury would expose a more dramatic disruption in the molecular program transcriptionally regulated by Nkx2.5 in the adult heart. Yet, the specific nature of nkx2.5 expression in the mature wild-type zebrafish heart upon injury has yet to be fully elucidated. Previous studies have reported expression of nkx2.5 along the resection plane13,23, while others have been unable to detect nkx2.5 transcripts through in situ hybridization (ISH)12. Thus, we performed ventricular sections in wild-type fish, as previously described. Using RNAscope, our data reveal expression of nkx2.5 throughout the ventricular myocardium in uninjured and at 7 and 14 days post amputation (dpa) (Fig. 3A–C). Moreover, upon examination of the injured area, it is evident that a few cells are also expressing nkx2.5 inside the regenerate (Fig. 3B, C). Intriguingly, at 7 dpa, minimal nkx2.5 expression is observed in the wound area. However, the abundance of transcripts increases progressively over time, a pattern that reflects the process of redifferentiation of newly generated CMs. We repeated the injury in wild-type fish carrying Tg(nkx2.5:ZsYellow) and detected both endogenous ZsYellow and Tropomyosin through immunostaining. Our RNAscope findings were reinforced by co-expression of ZsYellow and Tropomyosin in both uninjured and injured hearts (Fig. 3D–F). Together, these data substantiate our conclusion that Nkx2.5 is expressed in the adult zebrafish myocardium and in the regenerate following ventricular amputation.

Our next goal was to clarify the lineage-specific expression patterns of nkx2.5 in the adult heart. To delineate endothelial and epicardial cells from CMs, we performed immunostaining with Tg(nkx2.5:ZsYellow) in conjunction with Raldh2 antibody. At
7 dpa, ZsYellow+ cells do not co-localize with Raldh2+ cells suggesting absence of nkx2.5 expression in the endothelial and epicardial lineages (Fig. 3G–G‴). Furthermore, we used vimentin to label fibroblasts in Tg(nkx2.5:ZsYellow)fish and identified no evidence of overlapping expression between endogenous ZsYellow+ signal in CMs and Vimentin+ fibroblasts (Fig. 3H–H‴)40. From these data, we confirm that expression of Nkx2.5 is limited to the CM population in the adult heart.

Nkx2.5 is required for myocardial regeneration. Confirmation of nkx2.5 expression in the amputation plane of the adult zebrafish myocardium provided further ammunition to our hypothesis that loss of Nkx2.5 transcriptional regulation in the context of injury would intensify the disruption in the downstream molecular program. To address this question, we performed ventricular resections in genotyped ntx2.5−/−fish. We selected this injury tactic to circumvent inadvertent activation of the hsp70l transgene given previous studies documenting transgenic expression for as long as six days following cryoinjury41. To verify that the physical trauma of amputation does not yield a similar effect, we measured the magnitude and duration of nkx2.5 transcriptional activation in Tg(hsp70l:nkx2.5-EGFP)fish following ventricular resection, but in the absence of heat shock. Executing qPCR, we observed peak expression 1 hour post amputation and normalization to endogenous levels by 2 dpa (Fig. S2F). Moreover, employing an anti-GFP antibody, we implemented immunostaining to detect the spatiotemporal expression of Nkx2.5-EGFP following ventricular amputation. Our results uncover parallel, albeit slightly delayed in comparison to qPCR evaluation of nkx2.5-EGFP transcripts (Fig. S2F), expression of Nkx2.5-EGFP with diminution of the protein by 2 dpa (Fig. S2A–E). Nkx2.5-EGFP is primarily evident within ventricular myocardium and not specifically at the injury border. Moreover, Nkx2.5-EGFP expression is cytoplasmic initially...
Although the ventricular myocardium in \( \text{Tg(nkx2.5:ZsYellow)} \) fi compared to the non-transgenic and transgenic wild-type hearts (Fig. S5A, B). Our comparison, we focused on 14 genes derived from the over-expression (Fig. 1M, S5C), the healing response in both control systems, TG7 vs TG0 and WT7 vs WT0. From this approach, we identified 882 and 408 DEGs that are commonly upregulated and downregulated, respectively (Fig. S6A, B, green). Applying GO enrichment for these shared DEGs, we affirmed that the normal cardiac regenerative transcriptional profiling is recruited as illustrated by highly enriched categories such as extracellular matrix organization, actin cytoskeleton organization, and mitotic sister chromatid segregation (Fig. S6C). Furthermore, we validated the RNA-seq expression profile of essential genes known to be imperative for repair mechanisms using qPCR. For the WT7 vs WT0 comparison, we focused on 14 genes derived from the overlapping dataset representing the shared DEGs in the RNA-seq

![Fig. 3 Adult zebrafish cardiomyocytes activate nkx2.5 expression following injury. A-C Representative images of RNAscope in situ hybridization on sections of uninjured \((n = 3)\) (A) and injured wild-type hearts at 7 dpa \((n = 3)\) (B), and 14 dpa \((n = 3)\) (C) highlight nkx2.5 expression in the entire ventricle, expanding into the regenerate. Dashed lines represent amputation planes. Scale bar, 50 μm. D-F Co-localization of Tg(nkx2.5:ZsYellow) and Tropomyosin delineates nkx2.5 expression in the myocardium in both uninjured \((n = 4)\) (D) and injured wild-type hearts at 7 dpa \((n = 5)\) (E) and 14 dpa \((n = 6)\) (F). Higher magnification panels (D′-F′) indicate the areas outlined in the rectangular boxes. Scale bar, 100 μm. G-H′ Anti-Raldh2 \((n = 4)\) (G-G′) and anti-Vimentin \((n = 5)\) (H-H′) immunostainings in Tg(nkx2.5:ZsYellow) fish highlight the myocardial-specific expression of nkx2.5. Higher magnification panels (G″-H″) indicate the areas outlined in the rectangular boxes. Scale bar, 50 μm.](Image 452x434 to 538x499)
control systems (Fig. S6A, B, green). While only two genes reached statistical significance globally, we ascertained that 11 of the 14 transcripts were similarly up or downregulated by qPCR, corroborating the accuracy of our wild-type sequencing data (Fig. S6D, E). Finally, PCA comparing all RNA-seq samples shows that duplicates for each condition cluster together, indicating that the DEGs are not due to noise (Fig. S6F). Second, the first dimension tends to separate injured versus uninjured samples, while the second dimension tends to separate samples by genotype (WT, TG, and MT). In summary, these data indicate robust, global transcriptional differences in MT versus WT or TG samples. However, we observed a larger separation between the WT0 and TG0 samples, which we have resolved to be secondary to differences in 'housekeeping' functions (Fig. S1C), and account for this variation in our analysis by removing the contribution of these DEGs to the WT0 and TG0 comparison by generating the 'overlap' set (Fig. 2A, group C). Together, we show that the intersection of the regenerative responses in both control systems reflects previously identified cellular repair processes and that our RNA-seq transcriptional profiles echo in vivo alterations in transcriptional regulation.

Nkx2.5 deficiency disrupts proliferation and proteolysis. Following validation of the endogenous reparative mechanisms in our control fish after amputation (Fig. S6), we aimed to probe the underlying causes of the diminished regenerative potential in the nkk2.5−/− fish at 7 dpa. To accomplish this goal, we generated an UpSet plot to extract the specific gene sets that authentically

---

**Fig. 4 Nkx2.5 is required for myocardial regeneration.** A Schematic of the experimental design. B–D Impaired healing is evident in the nkk2.5−/− hearts (n = 6) (D) when compared with the non-transgenic wild-type (n = 6) (B) and transgenic wild-type (n = 6) (C) AFOG-stained adult hearts at 30 days post-amputation (dpa). Scale bar, 100 μm. B′–D′ Higher magnification images of the rectangular boxes in B–D. Scale bar, 100 μm. E, F Quantification of the percentage of scar tissue per ventricle (E) and the degree of scar severity (F) in non-transgenic wild-type (n = 6), transgenic wild-type (n = 6), and nkk2.5−/− (n = 6) fish illustrate the dramatically compromised injury response in the absence of nkk2.5 gene function. Mean and standard error of each data set are shown. Unpaired, two-tailed t-test demonstrates no statistically significant difference between WT30 and TG30 (p = 0.6065), but statistically significant difference between WT30 and MT30 (p = 0.0251) in E. Chi-squared test shows no statistically significant difference between WT30 and TG30 (p = 0.5582) and statistically significant difference between WT30 and MT30 (p = 0.0357). Source data are provided as a Source Data file.
Fig. 5 Network analysis underscores proliferative failure and proteolysis dampening in Nkx2.5 loss-of-function model. A UpSet plot representing DEGs (FC > 0.5; FDR < 0.05) in nine relevant comparisons. Only selected interactions are illustrated: set A (turquoise) – only non-transgenic and transgenic wild-type-specific DEGs following injury, set B (magenta) – only nfkx2.5−/− specific DEGs following injury, and set C (black) – DEGs common in non-transgenic wild-type, transgenic wild-type, and nfkx2.5−/− fish following injury. B GO analysis of sets A, B, and C. All significantly enriched GO terms from each set are included for analysis by DAVID. Color and size of circles correspond to log (q-value) and enrichment, respectively. C-E Network analyses of genes associated with significantly enriched GO terms from set A (C), set B (D), and set C (E). Color represents the fold change in injured compared to uninjured wild-type CMs (WT7 vs WT0) (C, E) and injured compared to uninjured nfkx2.5−/− CMs (MT7 vs MT0) (D). Red is upregulated and blue is downregulated, as shown in the key.

represent the injury response and are not confounded by differences in genotypes or controls (Fig. 5A). In this analysis strategy, the rows represent all appropriate two-way comparisons between the six individual samples (WT0, TG0, MT0, WT7, TG7, and MT7), summarizing the dataset size and delineating which comparisons are incorporated into the column sets. After contemplating appropriate inclusion and exclusion criteria, we narrowed in on three essential columns to scrutinize the reparative processes that are and are not initiated in the nfkx2.5−/− fish independent of GRN aberrations from the loss of nfkx2.5 function. We identified set A (Fig. 5A, turquoise column) as those genes that are deployed in wild-type hearts following amputation but fail to be recruited in the absence of nfkx2.5 gene function. Set B (Fig. 5A, magenta column) denotes DEGs that are utilized in the nfkx2.5 loss-of-function model but are not activated in control scenarios. Finally, set C (Fig. 5A, black column) embodies the DEGs that are appropriately enlisted in the nfkx2.5−/− fish, consistent with wild-type regenerative mechanisms.

The aforementioned set A encompasses 277 genes that fail to be activated in the nfkx2.5−/− fish during cardiac regeneration (Fig. 5A, set A, turquoise column). This group is functionally enriched for several cellular processes including cell cycle, translation, integrin-mediated signaling pathway, and ATP synthesis coupled proton transport (Fig. 5B, set A). Further network analysis employing set A illuminates upregulation of the cell cycle and integrin-mediated signaling pathways, pinpointing critical, regenerative mechanisms that are boosted in the wild-type but not in the nfkx2.5−/− fish following amputation (Figs. 5C and S7). Specifically, while wild-type fish mount a proliferative response and mediate extracellular matrix (ECM) production following amputation19,40,42,43, nfkx2.5 loss-of-function model fails to mirror these cellular reactions. Moreover, downregulation of the tricarboxylic acid cycle and the ATP synthesis coupled proton transport modules and similar dampening of the mitochondrial translation pathways in injured wild-type (but not nfkx2.5-deficient) myocardium corroborate previous results emphasizing the necessary depression in metabolism during myocardial repair13,44-46. Further aligned with our findings that nfkx2.5−/− fish demonstrate abnormal metabolism (Fig. 5C), recent murine studies describe an adult Nkx2-2 mutant model with significant abnormalities in mitochondrial respiratory pathways47. Altogether, analysis of set A accentuates that failure of the appropriate injury response in two essential cellular processes, CM proliferation and mitochondrial OXPHOS activity, is due to the loss of Nkx2.5 function.

Next, we queried the molecular pathways that are inappropriately regulated in the nfkx2.5−/− fish and are not altered in the wild-type conditions (Fig. 5A, set B, magenta column). Set B encompasses 165 genes that are functionally enriched in proteasome-mediated ubiquitin-dependent protein and proteolysis involved in cellular protein catabolic processes (Fig. 5B, set B). Applying these GO terms, we produced a network depicting downregulation of several, closely linked members of the psmb gene family, proteosome 26S subunit, non-ATPase (Figs. 5D and S8). Proteasome-mediated ubiquitin-dependent protein catabolic processes degrade proteins in a precise and fastidious fashion employing the 19S regulatory subunit of the 26S proteasome48. More specifically, in the context of zebrafish cardiac regeneration, expression of psmb1 and psmb3 have been discovered in wound-edge CMs49,50, evolving a functional role in sarcomere disassembly12,49,50. Thus, failure to stimulate expression of these proteolytic drivers may impair the ability of nfkx2.5-deficient hearts to undergo dedifferentiation12,13, an indispensable step in CM replenishment.

Finally, we investigated those genes which are differentially expressed in the same direction in non-transgenic wild-type,
NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-022-30468-4
ARTICLE

Nkx2.5 targets identify nodes that link regenerative GRNs. To determine the key effectors mediating these specific injury responses, we harnessed data from our previous studies to uncover a consolidated set of Nkx2.5 direct targets identified in mouse HL-1 cells by DamID. We then intersected our RNA-seq results with these DamID targets to identify the key GRNs nodes that function immediately downstream of Nkx2.5 to promote regeneration. To perform this overlap, we ascertained the zebrafish orthologues of the Ensembl IDs of the mouse DamID targets using BioMart (release 100). Overlapping these datasets with the entire gene lists in sets A and B from our RNA-seq results (Fig. 5A; independent of GO annotation) highlights interconnecting nodes that are under the control of Nkx2.5 and link the enriched modules for cell cycle, mitochondrial metabolism, and proteolysis (Fig. 6). When comparing uninjured and injured non-transgenic wild-type hearts (Fig. 6A), our analysis supports prior data by exhibiting appropriately diminished ATP-dependent and translational pathways and stable protein catabolic processes to support CM renewal. Moreover, proliferative genes are triggered following injury to simulate repair (Fig. 6A). Importantly, Nkx2.5 effectors coordinate these GRNs through critical hubs, such as ect2, p53b3, and p5md7, which assemble a consolidated response to injury. Alternatively, when evaluating the uninjured and injured Nkx2.5−/− fish (Fig. 6B), these crucial modules either fail to be inhibited, in the case of mitochondrial translation, or fail to be activated, in the case of proliferation. Further, inappropriate downregulation of the genes required for sarcomere disassembly exacerbates the collapse in reparative mechanisms (Fig. 6B). Altogether, integration of our new RNA-seq dataset and our existing DamID target list uncovers strategic nodes by which Nkx2.5 synchronizes the adult myocardial injury response.

To inspect the biological significance of these GRNs, we implemented in vivo analyses to detect expression of crucial nodes in non-transgenic wild-type and Nkx2.5−/− fish at 7 dpa. ect2, a putative direct target of Nkx2.5 from our DamID study and confirmed to be bound by Nkx2-5 in human pluripotent cell-derived cardiomyocytes, is well positioned to promote cell cycle re-entry and sarcomere disassembly during cardiac regeneration given the communication that it establishes between these two modules (Fig. 6A, B). Previous studies have elucidated the function of ect2 in contractile ring assembly and initiation of cytokinesis; Ect2 activity converts RhoA-GDP (guanosine diphosphate) to RhoA-GTP (guanosine triphosphate) at the spindle. Employing transient dominant negative expression in zebrafish, recent reports have illuminated the role of ect2 loss-of-function in inhibiting cytokinesis, inducing polyploidization, and ultimately, in yielding a loss of cardiac regenerative potential. Applying RNAscope technology, we documented upregulation of ect2 expression in the injury zone and wound edge of injured, non-transgenic wild-type apices (Fig. 7A), validating the importance of Ect2 function in CM cytokinesis. Moreover, ect2 expression is significantly depleted in injured Nkx2.5−/− hearts which supports our hypothesis that Nkx2.5 is required upstream of ect2 in promoting cell cycle reentry and proliferation in regenerating myocardium (Fig. 7B). Finally, our conclusion is further reinforced by evidence that Nkx2.5 and ect2 transcripts co-localize in individual nuclei at the injury border (Fig. 7C, D), suggesting cell autonomous interactions.

Two additional Nkx2.5 effectors, p53b3 and p5md7, sit high in the Nkx2.5 transcriptional hierarchy linking the mitochondrial translation and the proteolysis modules (Fig. 6A, B). Given data depicting p53b3 and p5md1 transcripts in the wound edge following cardiac injury in wild-type zebrafish, downregulation of these proteasome gene family members has the potential to prevent proteolytic mechanisms necessary to disassemble mature CM architecture. We postulate that Nkx2.5 regulates p53b3 and p5md7 expression following ventricular injury and, in the Nkx2.5 loss-of-function model, depleted p53b3 and p5md7 expression restrains dedifferentiation of pre-existing CMs. Employing ISH, we document robust expression of p53b3 (Fig. 7E) and p5md7...
**Fig. 7 Nkx2.5 regulates targets, ect2, psmb3, and psmd7, to orchestrate regenerative mechanisms.** A, B Representative images of RNAscope analysis on sections of injured hearts at 7 dpa display decreased ect2 expression in the regenerating myocardium of the nkx2.5−/− (n = 3) (A) fish. Dashed lines represent amputation planes. Scale bar, 50 μm. C, D Additional RNAscope analysis employing both nkx2.5 and ect2 probes simultaneously highlight co-localization of transcripts in individual nuclei at the injury border zone in non-transgenic wild-type fish (n = 3) (C). Higher magnification panel (D) indicates the area outlined in the rectangular box. Scale bar, 50 μm. E-H In situ hybridization for psmb3 (E, F) and psmd7 (G, H) in non-transgenic wild-type (n = 5) (E, G) and nkx2.5−/− (n = 5) (F, H) adult hearts. In nkx2.5−/− hearts, both psmb3 and psmd7 are downregulated in the injured area. Scale bar, 30 μm. I, J Quantification of psmb3 (I) and psmd7 (J) signal, detected by in situ hybridization, reveals statistically significant diminution of both genes in the nkx2.5−/− (n = 5) compared with the wild-type (n = 5) injured myocardium. Mean and standard error of each data set are shown. Unpaired, two-tailed t-test illustrate statistically significant differences in (I) (p = 0.0297) and (J) (p = 0.0468). Source data are provided as a Source Data file.

(Fig. 7G) in the regenerate of non-transgenic wild-type hearts. Strikingly, there is diminished expression of these proteasome genes in the wound of nkx2.5−/− fish (Fig. 7F, H). Moreover, pixel intensity quantification corroborates these histological findings; both psmb3 and psmd7 exhibit a statistically significant decrease in expression in the wound (Fig. 7I, J). Altogether, we conclude that Nkx2.5 operates via key effectors, ect2, psmb3, and psmd7, to orchestrate regenerative mechanisms involving mitochondrial metabolism, dedifferentiation, and cytokinesis to repopulate CMs following injury.

**Nkx2.5 promotes proliferation and dedifferentiation.** Considering this defective transcriptional response in nkx2.5−/− fish, we examined the source of the impaired healing in vivo by evaluating the CM proliferation indices at 7 dpa and 14 dpa in wild-type and nkx2.5−/− fish. Given CM-specific nkx2.5 expression (Fig. 3), we hypothesized that Nkx2.5 is required cell autonomously to induce dedifferentiation, cell division, and redifferentiation of new CMs. Our data reveal a statistically significant decrease in the CM proliferation index in animals with reduced nkx2.5 transcriptional activity versus animals with intact nkx2.5 function at 7 dpa (Fig. 8A, B, E). In contrast, there is no statistically significant discrepancy at 14 dpa in the proliferative rates between wild-type and nkx2.5−/− fish (Fig. 8C–E). These findings illuminate an essential role for Nkx2.5 in promoting CM proliferation at 7 dpa when an escalation in cellular renewal is required for myocardial regeneration, validating the GRNs created from the RNA-seq data (Figs. 5C and 6).

We next examined the injured nkx2.5−/− fish for evidence of impaired sarcomere disassembly given downregulation of the proteolysis module of the Nkx2.5-dependent GRN (Figs. 5D and 6). Prior work has detected reactivation of embryonic cardiac sarcomeric proteins at the wound edge, defining embryonic cardiac myosin heavy chain (embCMHC) as a marker of undifferentiated CMs. To assess whether nkx2.5 regulates dedifferentiation and, therefore, re-expression of immature myosins, we performed immunostaining with embCMHC and MF20 at 7 dpa (Fig. 8F, G). While wild-type hearts demonstrate co-localization of embCMHC and MF20 along the border of the regenerate (Fig. 8F), nkx2.5−/− myocardium shows minimal expression of embCMHC at the site of injury (Fig. 8G). These findings are further corroborated with immunostaining directed against activated leukocyte adhesion molecule a (Alcam), another marker of dedifferentiation, demonstrating diminished Alcam expression in the nkx2.5−/− compared to the wild-type regenerates (Fig. 8I). From these data, we conclude that adult CMs fail to dedifferentiate in the absence of nkx2.5 gene function. Taken together, our results validate GRNs underscoring that Nkx2.5 is required cell autonomously to instruct proteolytic pathways for dedifferentiation of mature CMs to an immature myocardial fate (Figs. 5D and 6).

**Nkx2.5 non-cell autonomously limits epicardial integration.** Although our data establish that CM-specific nkx2.5 expression directly regulates CM proliferation and dedifferentiation via Nkx2.5-dependent cell cycle and proteolysis modules, we sought to evaluate whether Nkx2.5 also mediates non-cell autonomous regenerative functions. Both the cardiac endocardial and epicardial lineages exhibit sentinel roles in heart regeneration and interaction among these specific cell types allows for orchestration of the complex healing process. Coronaries arteries are required to regenerate in order to perfuse new myocardium and revascularization has been shown to occur within hours of cryoinjury to stimulate CM proliferation. Thus, using nkx2.5−/− fish carrying Tg(kdrl:EGFP) tag16, we investigated the process of angiogenesis in wild-type and nkx2.5 loss-of-function animals. Interestingly, we observed normal EGFP+ endothelial tubes in the wound area.
embCMHC stain is depicted in wild-type (A, n = 6 in C) and nkkx2.5−/− (n = 8 in B, n = 6 in D) hearts indicate proliferating CMs (arrows). Scale bar, 100 μm. E Quantification of PCNA+/Mef2+ cells in wild-type and nkkx2.5−/− hearts. n = 8 at 7 dpa and n = 6 at 14 dpa for both genotypes. Mean and standard error of each data set is shown. Unpaired, two-tailed t-tests show statistical differences between WT7 and MT7 (p = 0.0033), WT7 and WT14 (p = 0.0143), and MT7 and MT14 (p = 0.0369). F, G Representative images of embCMHC/MF20 immunostaining illustrating diminished expression of immature myosin in the nkkx2.5−/− (n = 6) compared to the wild-type (n = 6) wound border. Scale bar, 100 μm. H Quantification of integrated signal density of the embCMHC stain is depicted in wild-type (n = 6) and nkkx2.5−/− (n = 6) injury borders. Mean and standard error of each data set are shown. Unpaired, two-tailed t-test indicates a statistically significant difference (p = 0.0248). Source data are provided as a Source Data file.

Fig. 8 Nkx2.5 is required for cardiomyocyte proliferation and dedifferentiation in the regenerating heart. A–D Representative images of PCNA/Mef2 immunostaining in wild-type (n = 8 in A, n = 6 in C) and nkkx2.5−/− (n = 8 in B, n = 6 in D) hearts indicate proliferating CMs (arrows). Scale bar, 100 μm. E Quantiﬁcation of PCNA+/Mef2+ cells in wild-type and nkkx2.5−/− hearts. n = 8 at 7 dpa and n = 6 at 14 dpa for both genotypes. Mean and standard error of each data set is shown. Unpaired, two-tailed t-tests show statistical differences between WT7 and MT7 (p = 0.0033), WT7 and WT14 (p = 0.0143), and MT7 and MT14 (p = 0.0369). F, G Representative images of embCMHC/MF20 immunostaining illustrating diminished expression of immature myosin in the nkkx2.5−/− (n = 6) compared to the wild-type (n = 6) wound border. Scale bar, 100 μm. H Quantiﬁcation of integrated signal density of the embCMHC stain is depicted in wild-type (n = 6) and nkkx2.5−/− (n = 6) injury borders. Mean and standard error of each data set are shown. Unpaired, two-tailed t-test indicates a statistically signiﬁcant difference (p = 0.0248). Source data are provided as a Source Data file.

establishing that nkkx2.5 expression is not responsible for coronary angiogenesis during cardiac regeneration (Fig. S11).

Next, we compared the regenerative response in the epicardium, a thin mesothelial cell layer overlying the atrial and ventricular chambers, between wild-type and nkkx2.5−/− fish. Previous studies have elucidated the essential roles of epicardial activation, proliferation, and colonization of the regenerate39,60. Specifically, epicardial cells undergo an epithelial-to-mesenchymal (EMT) transition to yield epicardial derived cells (EPDCs). These EPDCs invade the injury site between 7 dpa and 30 dpa to provide a cellular scaffold and produce perivascular cells and myofibroblasts, ultimately in support of CM proliferation and angiogenesis39,60. As the wound heals, the epicardium undergoes EMT and establishes a thickened epicardial layer covering the injured myocardium60–64. Employing immunostaining with Tg(tcf21:DsRed2) to label the epicardium in wild-type and nkkx2.5−/− fish, we discerned expression at the injury site in wild-type and nkkx2.5−/− fish at 14 dpa (Fig. 9A–D). Yet, impaired integration of tcf21+ cells in the nkkx2.5−/− compared to wild-type fish is evident; tcf21+ epicardial cells fail to penetrate the regenerate and remain exterior to the CM layer (Fig. 9B, D). Quantification of EPDC migration reveals a statistically signiﬁcant decrement in the distance measured from the apex of the heart to the maximal interior position of the Tcf21:DsRed2+ cells when comparing wild-type and nkkx2.5−/− hearts (Fig. 9E). Taken together, these data indicate that, while the epicardium mounts a suitable response to tissue damage in the loss-of-function nkkx2.5 model, compromised epicardial penetration into the wound weakens the healing potential in the nkkx2.5−/− compared to wild-type fish.

We sought to clarify the underlying cellular mechanism responsible for this deﬁcient epicardial inﬁltration into the regenerate. Speciﬁcally, we tested our hypothesis that Nkx2.5 mediates non-cell autonomous functions in addition to the CM-intrinsic roles of dedifferentiation and proliferation. Thus, we performed ex vivo assays using non-transgenic wild-type and nkkx2.5−/− fish carrying Tg(tcf21:dsRed), as previously described65. At 3 dpa, we resected the ventricular apices, cultured them in ﬁbrin-coated plates, and quantiﬁed the outgrowth of epicardial cells after four days. There were no signiﬁcant differences identiﬁed following quantiﬁcation of epicardial cell expansion between the wild-type and the nkkx2.5−/− cultured apices (Fig. 9F–H). Moreover, employing EdU incorporation, we established that the proliferation rate in the wild-type epicardial cultured cells is the same as that in the nkkx2.5−/− epicardium (Fig. 9I–K). These ﬁndings support our conclusion that the defect in epicardial inﬁltration of the regenerate is due to mechanisms extrinsic to the epicardial cells. This conclusion aligns elegantly with our network analyses which invoke a small, integrin-mediated signaling pathway module (itgb2, itba6a, and itgb1l) that fails to be stimulated in the nkkx2.5 loss-of-function model (Fig. 5C). Given the importance of ECM remodeling in cardiac regeneration43,45,66, we expect that future investigation of targets of Nkx2.5 will elucidate novel ECM regulatory networks...
exemplifying non-cell autonomous functions of this cardiac-specific transcription factor.

Discussion

These data offer a unique perspective on mechanisms regulated by Nkx2.5 that are responsible for building new muscle following injury in the mature zebrafish heart. In particular, we show that CM-specific expression of nskx2.5 is required for cardiac regeneration following amputation of the ventricular apex in an innovative, adult Nkx2.5 loss-of-function zebrafish model. Using this genetic tool, we harnessed both new RNA-seq and our prior DamID data to reveal that the developmental molecular profile regulated by Nkx2.5 is perturbed in the adult nskx2.5-deficient myocardium. Moreover, our multi-variate network analysis underlines Nkx2.5 as a vital regulator of CM renewal, functioning to promote cell cycle re-entry and to maintain proteolysis genetic modules. Direct targets, to promote cell cycle re-entry and to maintain proteolysis genetic modules. Direct targets, to promote cell cycle re-entry and to maintain proteolysis genetic modules. Direct targets, to promote cell cycle re-entry and to maintain proteolysis genetic modules.

| $Tg(tcf21:dsRed)$ | $n$skx2.5$^{-/}$; $Tg(tcf21:dsRed)$ | $Tg(tcf21:dsRed)$ | $n$skx2.5$^{-/}$; $Tg(tcf21:dsRed)$ |
|-------------------|-----------------------------------|-------------------|-----------------------------------|
| $A$               |                                    | $B$               |                                    |
|                   |                                    | $C$               |                                    |
|                   |                                    | $D$               |                                    |
|                   |                                    | $E$               |                                    |
|                   |                                    | $F$               |                                    |
|                   |                                    | $G$               |                                    |
|                   |                                    | $H$               |                                    |
|                   |                                    | $I$               |                                    |
|                   |                                    | $J$               |                                    |
|                   |                                    | $K$               |                                    |
|                   |                                    | $L$               |                                    |
|                   |                                    | $M$               |                                    |
|                   |                                    | $N$               |                                    |
|                   |                                    | $O$               |                                    |
|                   |                                    | $P$               |                                    |
|                   |                                    | $Q$               |                                    |
|                   |                                    | $R$               |                                    |
|                   |                                    | $S$               |                                    |
|                   |                                    | $T$               |                                    |
|                   |                                    | $U$               |                                    |
|                   |                                    | $V$               |                                    |
|                   |                                    | $W$               |                                    |
|                   |                                    | $X$               |                                    |
|                   |                                    | $Y$               |                                    |
|                   |                                    | $Z$               |                                    |

**Fig. 9 Impaired epicardial migration in the nskx2.5 loss-of-function model.** A-D Myosin Heavy Chain (MHC) immunostaining in injured $Tg(tcf21:dsRed)$ ($n=6$) (A, C) and nskx2.5$^{-/}$; $Tg(hsp70;nskx2.5-EGFP);Tg(tcf21:dsRed)$ ($n=5$) (B, D) at 14 dpa exhibits aborted epicardial penetration in the nskx2.5$^{-/}$ heart. Scale bar, 50 μm. E EPDC migration quantification strategy measures the distance from the apex of the heart to the maximal interior position of the Tcf21:DsRed$^+$ cells in injured wild-type ($n=6$) and nskx2.5$^{-/}$ ($n=5$) hearts (A-D). Mean and standard error of each data set are shown. Unpaired, two-tailed t-test demonstrates a statistically significant difference between WT14 and MT14 ($p=0.0035$). F, G Ex vivo epicardial migration assay in Tg(tcf21:dsRed) ($n=14$) (G) and nskx2.5$^{-/}$;Tg(hsp70;nskx2.5-EGFP);Tg(tcf21:dsRed) ($n=14$) (H) explants. H, I Quantification of the epicardial migration showing no statistical difference between the wild-type ($n=14$) and the nskx2.5$^{-/}$ ($n=14$) cultured apices (F, G). Additional quantification of Edu$^+$ tcf21:dsRed$^+$ cells indicates normal proliferative potential in cultured nskx2.5$^{-/}$ ($n=14$) versus wild-type epicardium ($n=12$) (J, K). Mean and standard error of each data set are shown with no statistically significant differences detected by unpaired, two-tailed t-tests between WT and MT ($p=0.8794$ in (H) and $p=0.6165$ in (D). J, K Edu proliferation assay in wild-type ($n=12$) (K) and nskx2.5$^{-/}$ ($n=14$) (L) epicardial tcf21:dsRed$^+$ cells. Scale bar, 50 μm. Source data are provided as a Source Data file.

While it is clear that evolutionarily conserved developmental genetic signatures are critical to facilitate regenerative responses from cuttlefish to mouse, the toolkits required to investigate the underlying essential mechanisms remain underdeveloped. There is a scarcity of genetic lines with loxP sites flanking genes of interest that limits our ability to institute conditional deletions efficiently. Furthermore, while some pivotal cardiac transcription factors have been examined functionally, many members of the cardiac developmental transcriptional hierarchy have yet to be investigated. Although murine studies have benefitted from conditional deletion of Nkx2.5 to examine its roles in the adult...
cardiac conduction system and trabecular myocardium, the Nkx2-5 requirement in myocardial repair was not addressed due to the limited regenerative potential in these models. Here, we describe the application of an innovative technique expediting rescue of otherwise lethal nkx2.5−/− embryos using heat shock overexpression without confounding evidence of non-specific defects. We have taken advantage of our nkx2.5-deficient fish to dissect the cell type-specific functional requisites, uncovering inappropriate responses in proliferation, sarcomere disassembly, and mitochondrial metabolic pathways in the CMs and cell extrinsic abnormalities in ECM deposition of the regenerative niche. This unique model provides a substrate to study the distinct molecular profiles that are altered in the Nkx2-5 loss-of-function myocardium, mimicking late-stage challenges faced by adult patients with Nkx2-5-associated CHD.

Our data also illustrate insights generated from a comprehensive assessment of the injured Nkx2-5 loss-of-function adult model and multi-variate network interpretation of transcriptional profile alterations in various genetic and temporal conditions. By probing each comparison, we identified distinct responses – both appropriate and inappropriate – that are regulated by Nkx2.5. These findings suggest that therapeutic measures need to be tailored to the individual clinical scenarios in order to ameliorate pathological mechanisms and redirect the interlocked chain of events that ensue following myocardial damage. Specifically, in the context of adult CHD patients carrying NKK2-5 mutations, we envisage that discrete and directed remedies, such as stimulation of cell cycle re-entry and suppression of mitochondrial translation, would augment successful regenerative outcomes. Moreover, the proteasome-mediated ubiquitin-dependent protein catabolic processes necessitate preservation to ensure proper dedifferentiation of Nkx2.5-deficient CMs. Our data suggest that modulation of the rapid inflammatory response following injury occurs aptly and, therefore, does not need remediation to support effective repair. Taken together, careful delineation of the molecular and cellular processes that go awry following injury in both wild-type and genetically altered myocardium will enhance our understanding of regenerative strategies and permit recruitment of explicit endogenous mechanisms deficient in each patient-specific context.

While evidence of CM-specific expression of nkx2.5 in the adult zebrafish heart, diminished proliferation, and arrested dedifferentiation illuminate cell intrinsic functions of Nkx2.5 following injury, impaired epicardial migration in vivo points to the contribution of CM extrinsic roles to the decreased reparative capability in nkx2.5−/− fish. In light of our ex vivo studies depicting normal epicardial migration and proliferation and our network analyses illustrating failure of nkx2.5−/− fish to upregulate an integrin-mediated ECM module, we speculate that Nkx2.5 directly or indirectly regulates crucial constituents of the cardiac matrix. Further investigation is required to uncover the key players acting downstream of Nkx factors. Yet, recent reports indicate that distinct embryonic ECM cargo can augment cytoskeletal in postnatal rat CMs in vitro and in vivo, providing a glimpse of therapeutic potential. Moreover, zebrafish studies have advanced our appreciation of the mechanisms responsible for the cardiac valve regeneration, further emphasizing the importance of studying ECM regulation in adult Nkx2.5 loss-of-function models given the incidence of valvular defects in these patients. To address questions on the impact of Nkx2.5-regulated ECM on cardiac and valvular repair, future inquires of the regenerative proteome with emerging techniques is necessary to understand global protein-protein interaction dynamics.

Altogether, our studies underscore that activation of the embryonic Nkx2.5 transcriptional network is critical in triggering productive intrinsic and extrinsic cellular responses to myocardial damage in the adult zebrafish heart. Exploiting insights into the specific mechanistic processes that are hampered in the nkx2.5−/− fish such as CM proliferation and dedifferentiation, our work open doors for the cultivation of targeted therapies in Nkx2-5-deficient myocardium. Studies demonstrating opportunities for myriad factors to instruct CM regeneration indicate that therapeutic intervention is feasible as long as the coordination of systematic approaches is realized. The identification of previously unrecognized Nkx2.5 targets, cc12, psmb3, and psmd7, as critical nodes in orchestrating cell cycle re-entry, proteolysis, and mitochondrial metabolism in response to injured myocardium offers initial steps to achieve this mission.

**Methods**

**Zebrafish lines and ventricular resections.** We used adult zebrafish carrying the following previously described mutation and transgenes: nkx2.5−/−f70171, Tg(lsp7/00:nkx2.5-EGFP)cko229, Tg(nkx2.5:ZsYellow)677, Tg(fli1:DsRed2)pd43709, and Tg(kdr:EGFP)f1668. Adult nkx2.5−/−:Tg(lsp7/00:nkx2.5-EGFP) fish were generated as previously described and experiments were implemented with one transgenic parent per cross. Ventricular apices were resected in adult fish (3- to 18-months-old), employing a previously described technique. All the zebrafish experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Columbia University.

**Heat shock conditions.** Embryos from outcrosses of fish carrying Tg(lsp7/00:nkx2.5-EGFP) were maintained at 28.5 °C and exposed to heat shock at 37 °C for 21 somites. To implement heat shock, 50 embryos were placed in 2.5 mL of filtered embryo medium in a 24-well Petri dish on top of a covered heat block for one hour. Three hours following initiation of heat shock, transgenic embryos were identified by visualization of ubiquitous EGFP expression. Non-transgenic sibling embryos exposed to heat shock served as controls.

**Histological analysis.** Zebrafish were anesthetized with tricaine and then fixed in 4% paraformaldehyde overnight at 4 °C. Subsequently, the dissected hearts were washed several times with PBS and cryopreserved with 30% sucrose before immersion in O.C.T. Subsequently, the hearts were sectioned at 10 μm with a cryostat (Leica CM3050 S) and collected for Acid Fuchsia Orange G (AFOG), RNA in situ hybridization (ISH), RNA-scope, and immunostaining.

AFOG staining was performed as previously described on 6–10 animals per time point. Briefly, slides were dried for one hour at room temperature and re-fixed with 4% PFA for five minutes. Subsequently, they were incubated in a pre-heated Bouin’s solution for two hours at 60 °C followed by one hour at room temperature. After extensive washes with H2O2, the slides were rinsed in 1% phosphoric acid for five minutes. Following a five-minute wash with H2O2, they were incubated for 10 min in AFOG staining solution (0.5% Aniline Blue, 1% Orange G, 1.5% Acid Fuchsin, pH 1.09). Finally, the slides were rinsed in H2O2, dehydrated, cleared in xylene, and mounted in Cytoseal 60 (Thermo Fisher Scientific).

ISH was performed as previously described with minor modifications. Briefly, slides were brought to room temperature for one hour before performing post-fixation in 4% PFA for 20 min. Subsequently, they were washed with PBS, treated with proteasine K (10 μg/ml) for 10 min at 37 °C, washed again with PBS prior to another 4% PFA fixation for 5 min, washed again with PBS, and treated with HCl 0.07 N for 15 min of shaking. Then, the sections were washed with PBS and a pre-hybridization buffer (0.2 M NaCl, 5 mM EDTA, 12 mM Tris HCl pH 7.5, 14 mM Na3HPO4, 7H2O, 6 mM NaH2PO4, 50% formamide, 10% dextran sulfate, 10 mg/ml yeast RNA, 1x Denhardt’s) at 65 °C for two hours. Hybridization of the probe was performed overnight at 65 °C, covered with a coverslip in a humidified chamber. The following day, the sections were washed three times with PBS for 5 minutes. Next, the sections were washed with PBS and a pre-hybridization buffer (50% formamide, 5x SSC, 0.1% Tween-20) for 30 min at 65 °C and three more times with solution II (50% formamide, 2x SSC, 0.1% Tween-20) for another 30 min, each at 65 °C. Subsequently, the sections were washed three times with maleic acid buffer (MAB) with 0.1% Tween-20 (MAB) for ten minutes at room temperature. The sections were then incubated in a blocking solution (70% MAB, 20% Normal Goat Serum, and 10% blocking reagent) for two hours at room temperature. Next, the anti-DIG-AP antibody (11093274910, Roche; 1:2000) was applied in blocking solution overnight at 4 °C with a coverslip in a humidified chamber. On the following day, the sections were washed with MAB for ten minutes, again for three one-hour-long washes, and finally three washes with an alkaline-phosphate buffer (100 mM NaCl, 50 mM MgCl2, 100 mM Tris pH 9.5, 0.1% Tween-20). The sections were developed in BM-purple (Roche) at 37 °C until signal was detected. The following probes were used: amhc (myh6; ZDB-GENE-031112-1)80, vmhc (myh7; ZDB-GENE-991123-5)81, psmb3 (ZDB-GENE-040426-2)82, and psmd7 (ZDB-GENE-031181-5)83.

Immunostaining was performed as previously described. Briefly, the hearts were collected in cold PBS and brought to room temperature. Following washes...
with PBS plus 0.1% Tween20 (PB1), sections were permeabilized with methanol for one hour at room temperature, blocked in blocking solution (PBS containing 2% sheep serum, 0.2% Triton X-100, 1% DMSO) for 30 min at room temperature, and incubated with primary antibodies in blocking solution at 4 °C overnight. Sections were then incubated with secondary antibodies between one and three hours at room temperature. Finally, the slides were mounted with Vectashield medium (Vector Laboratories) and DAPI prior to imaging.

MetZ/PCNA staining was performed as previously described93. Briefly, the slides were equilibrated to room temperature prior to performing antigen retrieval in 10 mM citrate buffer at 98 °C for 20 min. Then, they were cooled for 20 min, rinsed in H2O twice, and immersed in pre-cooled acetone (−20 °C) for 10 min. Subsequently, acetone was allowed to evaporate for 20 min at room temperature. The slides were then washed once in H2O, washed twice in PIB, and incubated in blocking buffer (5% normal goat serum, 0.1% Tween20) for 30 min at room temperature. Incubation with primary antibody was performed in antibody dilution buffer (1% BSA, 0.3% Triton X-100 in PIB) overnight at 4 °C, and secondary antibody for two hours at room temperature.

Primary antibodies used were anti-PCNA (WIB00511M2, Sigma, 1:200), anti-Mef2 (sc-313, Santa Cruz Biotechnology 1:50), anti-tropomyosin (CH1, Developmental Studies Hybridoma Bank, 1:100), anti-myosin heavy chain (E59, Developmental Studies Hybridoma Bank, 1:25), anti-GFP (A-11122, Invitrogen, 1:200), and anti-Ctnmb1 (G24969, Clone 20D1, 1:200), anti-nid2b (GTX124302, GeneTex, 1:500), anti-vimentin (4D-C, Developmental Studies Hybridoma Bank, 1:35), anti-embCMBHC (N2.261, Developmental Studies Hybridoma Bank, 1:50), anti-myosin heavy chain (MF20, Developmental Studies Hybridoma Bank, 1:200), and anti-AIcm (ZN-8, Developmental Studies Hybridoma Bank, 1:10). The following secondary antibodies were used (1:500): Alexa Fluor 488 Goat anti-Mouse IgG (A-11001, Invitrogen), Alexa Fluor 594 Goat anti-Mouse IgG (A-11941, Invitrogen), Alexa Fluor 680 Goat anti-Mouse IgG (A-11942, Invitrogen), Alexa Fluor 568 Goat anti-Mouse IgG (A-11212, Invitrogen), Invitrogen, Alexa Fluor 488 Goat anti-Mouse IgG (A-11941, Invitrogen), Alexa Fluor 680 Goat anti-Mouse IgG (A-12043, Invitrogen), and Alexa Fluor 488/680 anti-Rabbit IgG (A-21070, Invitrogen) with absolute log2 fold-change (FC) > 0.5 and false discovery rate (FDR) < 0.2 in R (version 3.6.0).

Venn overlaps of DEGs were generated with the VennDiagram package (version 1.71)96. Hierarchical clustering was performed using the gplots:heatmap.2 function which employs Euclidean distance measures with the average linkage method (version 3.1.3)97. The UpSet plot was generated with the UpSetR package (version 1.4.0)101. Functional enrichment was performed using Metascape 3.5102 and validated by orthogonal approaches using DAVID (version 6.8)103 and Panther (version 14.1)104, employing default parameters. Gene Ontology (GO) plots were created using the ggplot2 package (version 3.3.2).

Overlap with DamID targets of Nkx2-5 in mouse E1L-1 cells (GSE44902) was performed by first retrieving the zebrafish orthologues of the mouse DamID targets using BioMart (release 100)33. Then, the intersection of the Ensembl IDs for the zebrafish DamID targets and zebrafish DEGs were obtained with BioVenn (version 1.1.3)105. Network reconstructions were performed by retrieving protein-protein interacting relationships using STRING (version 11.0, default parameters, https://string-db.org) and visualized with Cytoscape (version 3.8.0)106. For networks associated with GO terms, interactions only between genes associated with specified GO terms were examined. However, the networks highlighting DamID candidates as hubs (Fig. 6) were reconstructed based on an overlap of all targets and sets A and B (Fig. 5). The first neighbors of the DamID targets and modules with more than seven nodes were included for analysis.

**Quantitative and statistical analysis.** All quantifications were performed blinded. Unless otherwise indicated, experiments were implemented with at least four biological replicates and at least three samples per replicate. For quantification strategies, three non-consecutive sections with the largest area were measured to assign an index for each heart. To calculate the proportion of scar tissue, the section with the largest injury site was selected and normalized to the ventricular area. Furthermore, the severity of the scar was classified as previously described94. Ventricular volume was calculated on 10 µm sections by quantifying the ventricular area in each section and multiplying by the distance between each section12. The percentage of trabeculation was calculated as the percentage of muscular tissue within the ventricular area using the threshold tool and manually excluding the internal cavity area32. For quantitative analyses, two-tailed Student’s t-test was requested from the corresponding author.

References
1. Benjamin, E. J. et al. Heart disease and stroke statistics-2018 update: a report from the American Heart Association. *Circulation* 137, e67–e492 (2018).
2. Sieweke, J. T. et al. Prediction of heart failure and death in an adult population of Fontan patients. *Cardiol. Young* 29, 602–609 (2019).
3. Bersell, K. et al. Neuregulin1/ErbB4 signaling induces cardiomyocyte dedifferentiation and proliferation. *Nat. Cell Biol.* 17, 627–638 (2015).

**Data availability**
Previously published datasets employed in this manuscript include the zebrafish reference genome GRCz10-91 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000002035.5/) and Nkx2-5 DamID targets under accession number GSE44902. The RNA sequencing data newly reported in this paper is available under accession number GSE184966. Source data are provided with this paper. All other relevant data may be requested from the corresponding author.

Received: 9 December 2020; Accepted: 3 May 2022; Published online: 27 May 2022
5. Hsieh, P. C. et al. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. *Nature Med.* 13, 970–974 (2007).

6. Polizzotti, B. D. et al. Neuregulin stimulation of cardiomyocyte regeneration in mice and human myocardium reveals a therapeutic window. *Sci. Transl. Med.* 7, 281ra245 (2015).

7. Senyo, S. E. et al. Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* 493, 433–437 (2013).

8. Xin, M. et al. Hippo pathway effector Yap promotes cardiac regeneration. *Proc. Natl Acad. Sci. USA* 110, 13839–13844 (2013).

9. Bergmann, O. et al. Evidence for cardiomyocyte renewal in humans. *Science* 324, 98–102 (2009).

10. Molls, M. et al. Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc. Natl Acad. Sci. USA* 110, 1446–1451 (2013).

11. Beltrami, A. P. et al. Evidence that human cardiac myocytes divide after myocardial infarction. *New Engl. J. Med.* 344, 1750–1757 (2001).

12. Jopling, C. et al. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* 464, 606–609 (2010).

13. Kikuchi, K. et al. Primary contribution to zebrafish heart regeneration by gata4(+)/cardiomyocytes. *Nature* 464, 601–605 (2010).

14. Porrello, R. E. et al. Transient regenerative potential of the neonatal mouse heart. *Science* 331, 1078–1080 (2011).

15. Marques, I. J., Lupi, E. & Mercader, N. Model systems for regeneration: zebrafish. *Development* 146, dev167692 (2019).

16. Mestas, G., Turka, C. S. & Denervier, M. A. Cardiomyocyte proliferation in zebrafish and mammals: lessons for human disease. *Cell. Mol. Life Sci.* 74, 1367–1378 (2017).

17. Gonzalez-Rosa, J. M. et al. Myocardial polyolipolydism creates a barrier to heart regeneration in zebrafish. *Dev. Cell* 44, 433–446.e437 (2018).

18. Patterson, M. et al. Frequency of mononuclear diploid cardiomyocytes underlies natural variation in heart regeneration. *Nat. Genet.* 49, 1346–1353 (2017).

19. Pronobis, M. I. & Poss, K. D. Signals for cardiomyocyte proliferation during zebrafish heart regeneration. *Curr. Opin. Physiol.* 14, 78–85 (2020).

20. Tzahor, E. & Poss, K. D. Cardiac regeneration strategies: staying young at heart. *Science* 356, 1035–1039 (2017).

21. Schindler, Y. L. et al. Hand2 elevates cardiomyocyte production during zebrafish heart development and regeneration. *Development* 141, 3112–3122 (2014).

22. Gupta, V. et al. An injury-responsive gata4 program shapes the zebrafish cardiac ventricle. *Curr. Biol.* 23, 1221–1227 (2013).

23. Lepilliaux, A. et al. A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* 127, 607–619 (2006).

24. Brunee, B. G. The developmental genetics of congenital heart disease. *Nature* 451, 943–948 (2008).

25. Prall, O. W. et al. An Nkx2.5/Bmp2/Smad1 negative feedback loop controls heart progenitor specification and proliferation. *Cell* 128, 947–959 (2007).

26. Targoff, K. L., Schell, T. & Yelon, D. Nkx genes regulate heart tube extension and exert differential effects on ventricular and atrial cell number. *Dev. Biol.* 320, 321–342 (2008).

27. Lyons, I. et al. Myogenic and morphogenetic defects in the heart tubes of mutants of the tinman-related genes, XNkx2-3 and XNkx2-5. *Dev. Biol.* 288, 435–446 (2006).

28. Grow, M. W. & Krieg, P. A. Tinman function is essential for vertebrate heart field development. *Development* 372, 371–379 (2013).

29. Green, R. A., Paluch, E. & Oegema, K. Cytokinesis in animal cells. *Annu. Rev. Cell Dev. Biol.* 28, 29–58 (2012).

30. Salmin, P. et al. Dual epimorphic and compensatory mode of heart regeneration in zebrafish. *Dev. Biol.* 399, 27–40 (2015).

31. Ogawa, M. et al. Kruppel-like factor 1 is a core cardiacomyogenic trigger in the mouse. *Science* 272, 201–205 (1996).

32. Monroe, T. O. et al. YAP partially reprograms chromatin accessibility to directly induce adult cardiogenesis in vivo. *Dev. Cell* 48, 765–779.e767 (2019).

33. Gonzalez-Rosa, J. M., Peralta, M. & Mercader, N. Pan-epicardial lineage tracing reveals that epicardium-derived cells give rise to myocardioblasts and perivascular cells during zebrafish heart regeneration. *Dev. Biol.* 370, 173–186 (2012).

34. Kim, J. et al. PDGF signaling is required for epicardial function and blood vessel formation in regenerating zebrafish hearts. *Proc. Natl Acad. Sci. USA* 107, 17206–17210 (2010).

35. Zhou, B. et al. Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J. Clin. Invest.* 121, 1894–1904 (2011).

36. Gonzalez-Rosa, J. M. et al. Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. *Development* 138, 1663–1674 (2011).

37. Smart, N. et al. Thymosin beta4 facilitates epicardial neovascularization of the injured adult heart. *Ann. N. Y. Acad. Sci.* 1194, 97–104 (2010).

38. Kim, J. et al. In vitro culture of epicardial cells from adult zebrafish heart on a fibrin matrix. *Nat. Protoc.* 7, 247–255 (2012).

39. Maro, J. et al. Collagen XII contributes to epicardial and connective tissues in the zebrafish heart during outgrowth and regeneration. *PLoS ONE* 11, e0165497 (2016).

40. Tarkoda, R. et al. Ablation of Nkx2-5 at mid-embryonic stage results in premature lethality and cardiac malformation. *Cardiovasc. Res.* 91, 289–299 (2011).
68. Kasahara, H. et al. Nkx2.5 homeoprotein regulates expression of gap junction protein connixin 43 and sarcomere organization in postnatal cardiomyocytes. J. Mol. Cell. Cardiol. 35, 243–256 (2003).

69. Callaghan, N. I. et al. Reversion to developmental pathways underlies rapid arm regeneration in juvenile European cuttlefish, Sepia officinalis (Linnaeus 1758). J. Exp. Zool. B Mol. Dev. Evol. 332, 113–120 (2019).

70. Witman, N. et al. Recapitulation of developmental cardiogenesis governs the morphological and functional regeneration of adult newt hearts following injury. Dev. Biol. 354, 67–76 (2011).

71. Conway, S. J. & Molkentin, J. D. Periostin as a heterofunctional regulator of cardiac development and disease. Curr. Genomics 9, 548–555 (2008).

72. Grego-Bessa, J. et al. Notch signaling is essential for ventricular chamber development. Dev. Cell 12, 415–429 (2007).

73. de Wit, L. et al. Cellular and molecular mechanism of cardiac regeneration: a comparison of newts, zebrafish, and mammals. Biomolecules 10, 1204 (2020).

74. Grajeskova, V. et al. Analysis of a conditional gene trap reveals that tbx5a is required for heart regeneration in zebrafish. PLoS ONE 13, e0197293 (2018).

75. Pashmforoush, M. et al. Nkx2-5 pathways and congenital heart disease; loss of ventricular myocyte lineage specification leads to progressive cardiomyopathy and complete heart block. Cell 117, 373–386 (2004).

76. Choquet, C. et al. Deletion of Nkx2-5 in trabecular myocardium reveals the developmental origins of pathological heterogeneity associated with ventricular non-compaction cardiomyopathy. PLoS Genet. 14, e1007502 (2018).

77. Wu, C. et al. Modulation of mammalian cardiomyocyte cytokinesis by the extracellular matrix protein thrombospondin-1. J. Cell Sci. 127, 896–907 (2020).

78. Bensimon-Brito, A. et al. TGF-beta signaling promotes tissue formation during cardiac valve regeneration in adult zebrafish. J. Mol. Biol. 521, 709–725 (2021).

79. Kefalos, P. et al. Reactivation of Notch signaling is required for cardiac valve regeneration in adult zebrafish. Dev. Cell 44, 15–25 (2019).

80. Bensimon-Brito, A. et al. TGF-beta signaling promotes tissue formation during cardiac valve regeneration in adult zebrafish. J. Mol. Biol. 521, 709–725 (2021).

81. Joziasse, I. C. et al. Genes in congenital heart disease: atrioventricular valve defects. Cardiovasc. Res. 117, 328–337 (2018).

82. de Wit, L. et al. Cellular and molecular mechanism of cardiac regeneration: a comparison of newts, zebrafish, and mammals. Biomolecules 10, 1204 (2020).

83. Lam, S. S. et al. Directed evolution of APEX2 for electron microscopy and proximity labeling. Nat. Methods 12, 51–54 (2015).

84. Mohamed, T. M. A. et al. Regulation of cell cycle to stimulate adult cardiomyocyte proliferation and cardiac regeneration. Cell 173, 104–116.e12 (2018).

85. Leach, J. P. et al. Hippo pathway deficiency reverses systolic heart failure after infarction. Nature 550, 260–264 (2017).

86. Basat, E. et al. The extracellular matrix protein agrin promotes heart regeneration in mice. Nature 547, 179–184 (2017).

87. Zhou, Y. et al. Latent TGF-beta binding protein 3 identifies a second heart field in zebrafish. Nature 474, 645–648 (2011).

88. Choi, J. et al. FoxH1 negatively modulates Ifk1 gene expression and vascular formation in zebrafish. Dev. Biol. 304, 735–744 (2007).

89. Sanchez-Iranzo, H. et al. Tbx5a lineage tracing shows cardiomyocyte plasticity during zebrafish heart regeneration. Nat. Commun. 9, 428 (2018).

90. Berdougo, E. et al. Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. Development 130, 6121–6129 (2003).

91. Yelon, D., Horne, S. A. & Stainier, D. Y. Restricted expression of cardiac myosin heavy chain genes reveals regulated aspects of heart tube assembly in zebrafish. Dev. Biol. 214, 33–37 (1999).

92. Misinato, M. A. et al. Extracellular component hyaluronic acid and its receptor Hmmr are required for epicardial EMT during heart regeneration. Cardiovasc. Res. 107, 487–498 (2015).

93. Zhao, L. et al. Notch signaling regulates cardiomyocyte proliferation during zebrafish heart regeneration. Proc. Natl Acad. Sci. USA 111, 1403–1408 (2014).

94. Cao, J. et al. Single epicardial cell transcriptome sequencing identifies Caveolin 1 as an essential component in zebrafish heart regeneration. Development 143, 232–243 (2016).

95. Dobrzensky, T. et al. An optimised pipeline for parallel image-based quantitative classification of gene expression and genotyping after in situ hybridisation. Biol. Open 7, bio031096 (2018).

96. Kikuchi, K. et al. Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. Dev. Cell 20, 397–404 (2011).

97. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21 (2012).

98. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930 (2014).

99. Chen, H. & Boutros, P. C. VennDiagram: a package for the generation of highly-collapsible Venn and Euler diagrams in R. BMC Bioinform. 12, 35 (2011).

100. Warnes, G. et al. gplots: various R programming tools for plotting data. R package version 3.0. https://CRAN.R-project.org/package=ggplot2 (2020).

101. Conway, J. R., Lex, A. & Gehrlehnger, N. UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinformatics 33, 2335–2340 (2017).

102. Zhou, Y. et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. 10, 1523 (2019).

103. Huang da, W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57 (2009).

104. Mi, H. et al. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. Nucleic Acids Res. 47, D419–D426 (2019).

105. Hulsen, T., de Vlieg, J., & Alkema, W. BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. BMC Genomics 9, 488 (2008).

106. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504 (2003).

107. Cao, J. & Poss, K. D. Explant culture of adult zebrafish hearts for epicardial regeneration studies. Nat. Protoc. 11, 872–881 (2016).

Acknowledgements

We thank Kenneth D. Poss, Caroline E. Burns, C. Geoffrey Burns, and Nadia Mercader for sharing protocols and Ian C. Scott and Enzo R. Porello for their critical reading of the manuscript. We appreciate the support of Traude Beilharz for the bioinformatic data analysis involved in this project and the expert advice of Jingli Cao regarding techniques for the study of zebrafish cardiac regeneration. We are grateful to the Targoff Laboratory for constructive feedback and to Joshua Barber for his expert zebrafish care. We also thank the Conifocal and Specialized Microscopy Shared Resource of the Herzberg Integrative Cancer Center at Columbia University Irving Medical Center, the Stem Cell Core Facility of the Columbia Stem Cell Initiative, and the Comparative Pathology Unit, the Bioimaging Unit, and the Zebrafish Unit at the Instituto de Medicina Molecular João Lobo Antunes (IMM-ILA). This work was supported by grants to K.L.T. from the National Institutes of Health (R01 HL131438-01A1), Cds-T. from Fundación para la Ciencia e Tecnología (FCT) (PTDC/BIA-BID/28572/2017), and M.R. from the State Government of Victoria and the Australian Government for the Australian Regenerative Medicine Institute and by Novo Nordisk Foundation (NNF21CC0073729).

Author contributions

C.d.S.-T. and K.L.T. conceived of the project and designed the experiments. C.d.S.-T., A.G.A., C.F, D.Y., and J.K.H. optimized the rescue experiments necessary to create the nkleZ/fish, generated data, and produced figures. A.V. and M.R. performed bioinformatic analysis. K.L.T. provided funding for the project. C.d.S.-T., A.V., L.S., M.R., and K.L.T. wrote, reviewed, and edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-30468-4.

Correspondence and requests for materials should be addressed to Kimara L. Targoff.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.