

Myocardial NF- κ B activation is essential for zebrafish heart regeneration

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Heart regeneration offers a novel therapeutic strategy for heart failure. Unlike mammals, lower vertebrates such as zebrafish mount a strong regenerative response following cardiac injury. Heart regeneration in zebrafish occurs by cardiomyocyte proliferation and reactivation of a cardiac developmental program, as evidenced by induction of *gata4* regulatory sequences in regenerating cardiomyocytes. Although many of the cellular determinants of heart regeneration have been elucidated, how injury triggers a regenerative program through dedifferentiation and epicardial activation is a critical outstanding question. Here, we show that NF- κ B signaling is induced in cardiomyocytes following injury. Myocardial inhibition of NF- κ B activity blocks heart regeneration with pleiotropic effects, decreasing both cardiomyocyte proliferation and epicardial responses. Activation of *gata4* regulatory sequences is also prevented by NF- κ B signaling antagonism, suggesting an underlying defect in cardiomyocyte dedifferentiation. Our results implicate NF- κ B signaling as a key node between cardiac injury and tissue regeneration.

heart regeneration | cardiomyocyte | zebrafish | NF- κ B | epicardium

Heat failure is an epidemic, with 870,000 new cases diagnosed annually in the United States (1). Failing hearts are characterized by progressive myocyte loss and replacement fibrosis, ultimately leading to susceptibility to fatal arrhythmia and pump dysfunction. Although major strides have been made in the treatment of heart failure, mortality remains high and additional therapies are needed. Recently, low-grade cardiomyocyte turnover has been reported in the mammalian heart (2, 3). Therapeutically augmenting this turnover toward regeneration of lost cardiac tissue is an attractive strategy for the treatment of heart failure. However, a better understanding of the mechanisms for heart regeneration is needed before regenerative therapies can be developed for patients.

In contrast to humans, zebrafish are capable of impressive regeneration following cardiac injury (4). Heart regeneration in zebrafish proceeds by reexpression of developmental factors along with cardiomyocyte proliferation and cytokinesis. Notably, *gata4* regulatory sequences are induced at the site of injury, and it is these cardiomyocytes that primarily contribute to the regenerate (5). The induction of a developmental program is seemingly required for regeneration, because functional inhibition of Gata4 impairs both cardiomyocyte proliferation and heart regeneration (6). Although most attention has been paid recently to cardiomyocytes, heart regeneration is a complex process that also involves the activation of embryonic programs in epicardial and endocardial cells (7–9). Remarkably, mechanisms for heart regeneration appear to be conserved across species; thus, studies in zebrafish have the potential to inform strategies for mammalian heart regeneration (10).

Regeneration is broadly defined as the replacement of a lost or damaged body part following injury. As such, tissue growth during regeneration is inextricably linked to the injury response. Earlier studies have identified response pathways such as retinoic acid signaling, JAK/Stat3 signaling, H₂O₂ signaling, and HIF1 signaling that are required for heart regeneration (7, 11–13). More recently, the inflammatory response itself has been implicated in guiding regeneration. Inflammatory responses are sufficient to stimulate neurogenesis through a regenerative program in the adult zebrafish

brain (14). Conversely, loss of macrophages impairs appendage regeneration in zebrafish and salamanders, and ablation of macrophages disrupts heart regeneration in neonatal mice (15–19). However, molecular links between the injury response and the induction of a developmental program in regenerating tissues await discovery.

NF- κ B factors were first identified nearly 30 years ago as a family of transcription factors capable of binding κ light chain enhancers in lymphocytes (20). Since then, NF- κ B signaling has been shown to have broad effects in a variety of tissues, influencing cell survival, tissue growth and proliferation, and chromatin structure (21). In the heart, NF- κ B signaling has been implicated as a hypertrophic influence and has been linked to expression of cardiac response genes like ANF and β -MHC (22–24). Classically, NF- κ B factors are sequestered in the cytoplasm through interaction with I κ B. Following stimulation, I κ B is targeted for proteasomal degradation, and NF- κ B factors are released for activation. Not surprisingly, NF- κ B signaling is the prototype inducible transcription factor and an important component of the early injury response in a variety of tissues (21). Here, we show that NF- κ B activity is induced following cardiac injury and required for heart regeneration. Importantly, NF- κ B has broad effects on the regenerative response, with evident contributions to cardiomyocyte dedifferentiation, cardiomyocyte proliferation, and epicardial injury responses. These studies offer a link between the injury response and the induction of a regenerative program.

Results

NF- κ B Activity Is Induced During Zebrafish Heart Regeneration. We previously established a model for genetic cardiomyocyte ablation that results in widespread regenerative responses, inducing

Significance

Heart failure occurs when the heart cannot pump enough blood to meet the body's demands. The heart most often weakens through the loss of muscle cells, called cardiomyocytes. Heart failure is a devastating disease, and one possible cure is to replace the lost cardiomyocytes through regeneration. Unlike humans, zebrafish can efficiently regenerate their hearts after injury. Interestingly, zebrafish heart cells are similar to human heart cells at the molecular level. Understanding how zebrafish can regenerate cardiac tissue can help identify regenerative therapies for humans. Here, we find that NF- κ B signaling is a link between the injury response and the regenerative program in zebrafish.

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proliferation in a high proportion of spared cardiomyocytes (25). To identify pathways related to regeneration, we used RNA sequencing to compare transcriptional profiles of ventricles at 7 days after genetic cardiomyocyte ablation (dpi) to profiles of uninjured ventricles. We found large changes in global gene expression during regeneration, with enrichment for targets of NF- κ B signaling. To confirm increased NF- κ B activity during regeneration, we performed quantitative PCR (qPCR) for NF- κ B target genes by using RNA from regenerating ventricles at 7 dpi and uninjured ventricles. Regenerating hearts were significantly enriched for the NF- κ B target genes *nfk1*, *nfk2*, *nfkbia*, *nfkbiab*, and *traf2b*, further suggesting increased NF- κ B activity during regeneration (Fig. 1A). We confirmed induction of increased NF- κ B activity following injury by in situ hybridization for the NF- κ B target genes *nfkbia* and *nfkbiab* in regenerating hearts (Fig. 1B–E). As in mammals, zebrafish have five members in the family of NF- κ B transcription factors. To identify predominant NF- κ B transcription factors during regeneration, we performed in situ hybridization for the annotated NF- κ B members on sections from regenerating hearts following cardiomyocyte ablation (Fig. S1). We found NF- κ B1 to be induced in the compact muscle of regenerating zebrafish hearts. As additional evidence for the presence of NF- κ B1 in cardiomyocytes, we assayed for the presence of NF- κ B1 in sections of mouse hearts. We found the murine NF- κ B1 colocalizes with cardiomyocyte nuclei, further supportive of NF- κ B1 activity in cardiomyocytes (Fig. S1). Of note, *NFKB1* polymorphisms have been linked to human cardiomyopathy, and *Nfk1^{-/-}* mice develop more fulminant heart failure following coronary ligation compared with wild-type controls (26–28). To more clearly identify which cell types displayed increased NF- κ B activity during zebrafish heart regeneration, we used a recently described transgenic reporter strain that reports NF- κ B activity with eGFP fluorescence (29). In the uninjured heart, we found eGFP fluorescence throughout the compact muscle, indicative of increased NF- κ B activity. As early as 1 d after resection injury (dpa), NF- κ B activity could be detected by eGFP fluorescence adjacent to the injury, and this activity increased during regeneration (Fig. 1F–I). Colocalization studies with cardiac troponin revealed strong NF- κ B activity, most notably in regenerating muscle.

NF- κ B Activity Is Required for Zebrafish Heart Regeneration. The strong spatiotemporal association of NF- κ B activity with regenerating muscle suggests a functional role for NF- κ B signaling

during heart regeneration. To test this idea, we established a new transgenic zebrafish line for conditional expression of a super-repressor I κ B (I κ BSR) to inhibit NF- κ B signaling [Tg(*factin2:loxP-mCherry-STOP-loxP-mTagBFP-2A-I κ BSR^{pl14}*); hereafter referred to as *fact2:RS-I κ BSR*] (Fig. 2A). Because I κ BSR is resistant to phosphorylation, NF- κ B transcription factors remain in the cytoplasm and NF- κ B activity is diminished (30). This mutant I κ BSR has been well described in mammalian systems and has also been shown to inhibit NF- κ B signaling in zebrafish embryos (29, 31). To determine the effect of diminished NF- κ B signaling in cardiomyocytes, we crossed *fact2:RS-I κ BSR* with *cmlc2:CreER* fish (5), enabling Cre-mediated recombination and expression of *BFP* and *I κ BSR* specifically in cardiomyocytes upon 4-HT treatment. First, we generated *cmlc2:CreER*; *fact2:RS-I κ BSR*; *NF- κ B:eGFP* transgenic fish to confirm inhibition of NF- κ B activity in cardiomyocytes. As expected, induced transgenic *I κ BSR* suppressed NF- κ B reporter activity in adult cardiomyocytes (Fig. 2B and C). Because we noted marked NF- κ B activity in the compact muscle of adult hearts, we assessed whether long-term NF- κ B inhibition has adverse effects on cardiac homeostasis. We did not observe any alteration in fish survival or cardiac structure, even at 60 d after recombination (Fig. 2B and C).

To determine the requirement for myocardial NF- κ B signaling during heart regeneration, we treated *cmlc2:CreER*; *fact2:RS-I κ BSR* fish with 4-HT and resected ventricular apices. We noted that animals with impaired NF- κ B signaling had defective regeneration at 30 dpa, evidenced by large gaps in muscle (Fig. 2E). By contrast, control animals consistently regenerated a contiguous wall of heart muscle (Fig. 2D and F). With a scoring system to assess regeneration, our analysis indicated that animals with a block in NF- κ B signaling had significant regenerative defects at 30 dpa compared with control animals ($P < 0.001$, Kruskal–Wallis test, $n = 25$). Similarly, fibrin/collagen staining revealed scar tissue at the amputation site in I κ BSR-expressing animals that was typically not present in control animals (Fig. 2F and G). These experiments reveal a requirement of NF- κ B signaling in zebrafish heart regeneration, but not for homeostatic cardiac maintenance.

NF- κ B Activity Is Required for the Cardiomyocyte Regenerative Program. During regeneration, new cardiomyocytes emerge through the division of preexisting cardiomyocytes (5, 32). Based on the localization of NF- κ B activity to regenerating cardiomyocytes and the effects on overall muscle regeneration, we tested whether

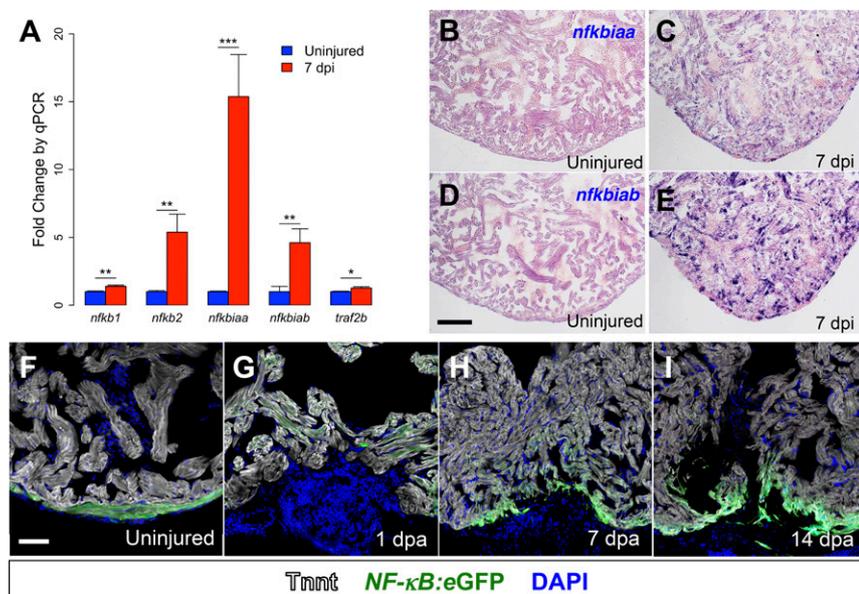


Fig. 1. NF- κ B activity is induced by injury and during regeneration. (A) qPCR for NF- κ B target genes in regenerating ventricles at 7 dpi in *cmlc2:CreER*; *fact2:RS-DTA* animals treated with tamoxifen ($n = 6$) compared with ventricles from *cmlc2:CreER*; *fact2:RS-DTA* animals treated with vehicle ($n = 6$). Error bars indicate SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test, two-tailed. Each replicate consists of a pool of 2–3 ventricles. (B–E) In situ hybridization for *nfk1a* or *nfk1b* on sections from ventricles of *cmlc2:CreER*; *fact2:RS-DTA* animals treated with vehicle (B and D) or tamoxifen (C and E). Violet staining indicates expression. (F–I) Time course of NF- κ B:eGFP induction during regeneration in sections from an uninjured ventricle, a 1 dpa ventricle, a 7 dpa ventricle, and a 14 dpa ventricle. Ventricles are counterstained with an antibody against Tnnt (gray) to delineate cardiomyocytes. (Scale bars: 100 μ m.)

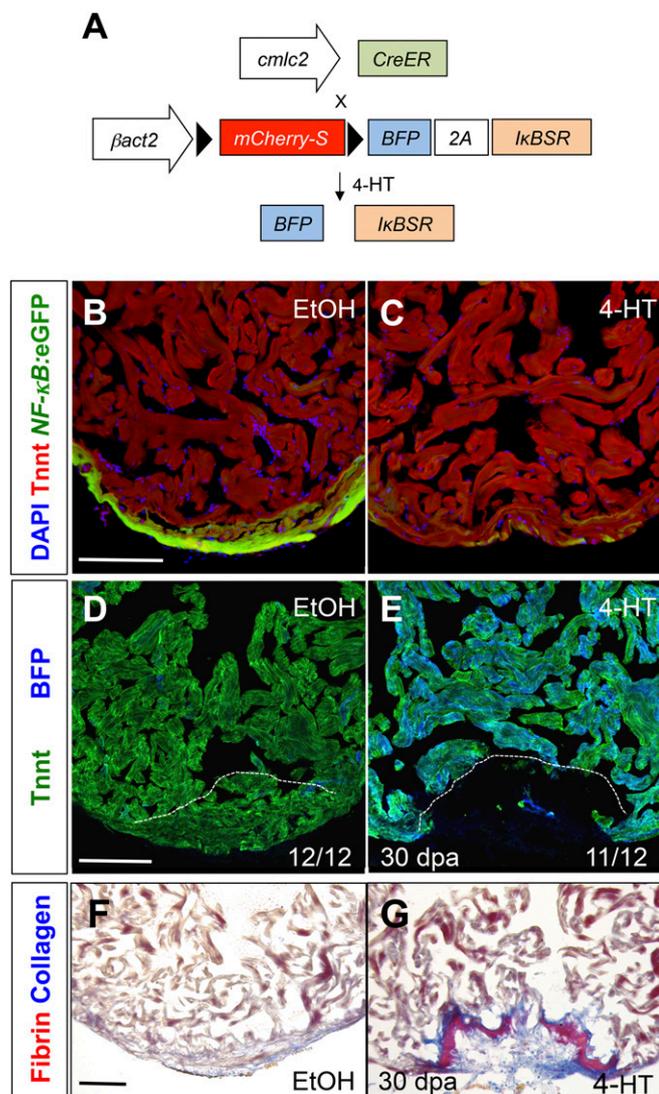


Fig. 2. NF- κ B is required for heart regeneration. (A) Schematic of $\beta act2:RS-I\kappa BSR$ transgene. (B and C) Section images of $cmlc2:CreER; \beta act2:RS-I\kappa BSR; NF-\kappa B:eGFP$ hearts 60 d after treatment (dpt) with vehicle ($n = 12$) or 4-HT ($n = 12$). (D and E) Section images of $cmlc2:CreER; \beta act2:RS-I\kappa BSR$ ventricles at 30 d after amputation after treatment with vehicle or 4-HT. Images are stained for troponin to denote cardiac muscle. Dashed line indicates approximate resection plane. (F and G). Section images of $cmlc2:CreER; \beta act2:RS-I\kappa BSR$ ventricles at 30 d after amputation after treatment with vehicle or 4-HT. Images are stained with acid fuchsin orange G (AFOG) to identify scar (blue) and fibrin (red). (Scale bars: 100 μ m.)

altering NF- κ B activity affects cardiomyocyte proliferation. We treated $cmlc2:CreER; \beta act2:RS-I\kappa BSR$ zebrafish with 4-HT or vehicle and assayed ventricles at 7 dpa, a stage of peak cardiomyocyte proliferation. We observed a nearly 50% decrease in cardiomyocyte proliferation in animals with impaired NF- κ B activity (9.1%) versus animals with intact NF- κ B (17.3%) ($P = 0.005$, Mann-Whitney) (Fig. 3 A–C). Because NF- κ B is also known to affect cell survival, we also examined the effect of NF- κ B inhibition on cardiomyocyte apoptosis following injury. TUNEL staining of hearts with and without intact NF- κ B signaling revealed no gross differences in cardiomyocyte apoptosis at 7 dpa (Fig. S2). Together, these data reveal a cell-autonomous role for injury-induced NF- κ B activity to promote or enable cardiomyocyte proliferation during heart regeneration.

The pattern of NF- κ B activation in cardiomyocytes was similar to the induction of *GATA binding protein 4* (*gata4*) regulatory

sequences, previously described in compact muscle and cardiomyocytes adjacent to the injury site (5). Moreover, the inhibition of cardiomyocyte proliferation by blockade of NF- κ B was similar to the decrease in proliferation that we have observed in transgenic animals expressing a dominant negative version of Gata4 in cardiomyocytes (6). To test for an association of NF- κ B with *gata4*, we crossed *NF- κ B:eGFP* animals with a new *gata4:DsRed2* reporter strain that we generated for this study. We noted that *gata4* regulatory sequences were activated in cardiomyocytes with NF- κ B activity (Fig. 3 D–J), suggesting a functional relationship. Although NF- κ B has been linked to a fetal gene program during hypertrophy, we were unaware of a direct interaction of NF- κ B with *gata4* regulatory sequences. To test the requirement of NF- κ B signaling in injury-induced activation of *gata4* regulatory sequences, we crossed $cmlc2:CreER; \beta act2:RS-I\kappa BSR$ with *gata4:eGFP* transgenic fish. In these experiments, control ventricles displayed typical induction of *gata4* regulatory sequences throughout the compact layer and adjacent to the injury site. However, fish with diminished NF- κ B activity did not activate detectable reporter fluorescence from *gata4* regulatory sequences (Fig. 3 J–Q). This requirement of NF- κ B activity seems specific to induction of *gata4* regulatory sequences, because suppression of NF- κ B was not sufficient to silence *gata5* regulatory sequences during regeneration (Fig. S3).

To further test direct interaction of NF- κ B with *gata4* regulatory sequences, we performed chromatin immunoprecipitation with an antibody against NF- κ B1 on extracts from hearts with and without intact cardiomyocyte NF- κ B signaling. We performed a bioinformatics analysis of the *gata4* promoter by using the JASPAR database and noted an increased density of NF- κ B1 binding sites ~12 kb upstream of the *gata4* transcriptional start site (Fig. 3R) (33). Interestingly, this site is just proximal to an enhancer required to direct *gata4* expression in the cardiac ventricle of zebrafish embryos (34). Using primers specific to this region of the *gata4* promoter, we observed evidence for direct interaction of NF- κ B1 with the *gata4* promoter in animals with intact NF- κ B signaling. By contrast, animals without intact NF- κ B signaling displayed less binding of NF- κ B1 with the *gata4* promoter (Fig. 3S). These data suggest that NF- κ B directly interacts with *gata4* regulatory sequences and is required for their induction during regeneration.

The induction of *gata4* regulatory sequences during regeneration has been suggested as evidence for cardiomyocyte dedifferentiation along with sarcomere dissociation (5, 32). We noted that cardiomyocytes with intact sarcomeres, such as in uninjured ventricles, display intense immunofluorescence for cardiac Troponin T (Tnnt). By contrast, regenerating ventricles show reduced Tnnt staining intensity in cardiomyocytes at the injury site by 7 dpa. Interestingly, we found that cardiomyocytes with diminished NF- κ B signaling stained more brightly for Tnnt during regeneration than controls, suggestive of a defect in sarcomere disassembly ($P = 0.028$, $n = 13$ ventricles; Fig. S3). Together, alterations in sarcomere disassembly and in the induction of *gata4* regulatory sequences suggest that NF- κ B signaling contributes to cardiomyocyte dedifferentiation.

Blocking NF- κ B Activity in Cardiomyocytes Has a Pleiotropic Effect on Regeneration. The colocalization of NF- κ B activity with activated *gata4* regulatory sequences and the requirement for NF- κ B to activate these sequences suggest that NF- κ B acts upstream of *gata4* during regeneration. We hypothesized that restoration of *gata4* to cardiomyocytes might be able to rescue the defect in regeneration we observed with NF- κ B blockade. To test our hypothesis, we established a new transgenic line Tg($\beta actin2:loxP-mTag-STOP-loxP-gata4$)^{pd115} (hereafter referred to as $\beta act2:BS-gata4$) to overexpress *gata4* in cardiomyocytes (Fig. S4). As we noted earlier, blockade of NF- κ B in cardiomyocytes resulted in impaired regeneration by 30 dpa (Fig. 4B). By contrast, we found

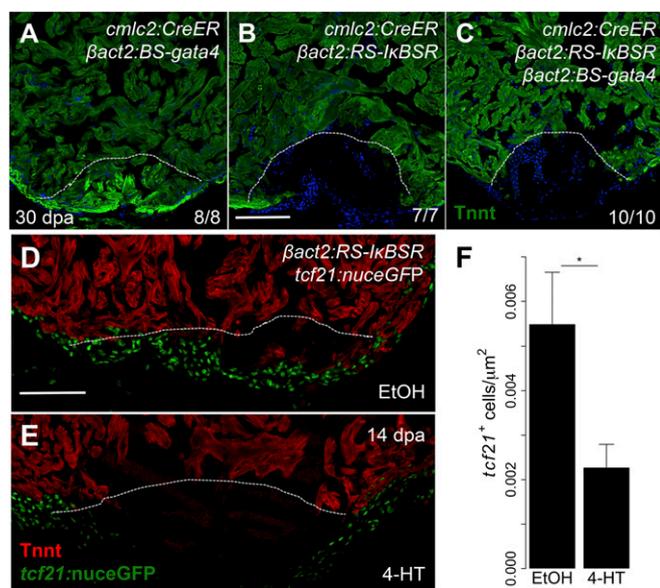


Fig. 4. NF- κ B has pleiotropic inhibitory effects on heart regeneration. (A–C) Section images of *cmlc2:CreER; βact2:BS-gata4*, *cmlc2:CreER; βact2:RS-lkBSR*, and *cmlc2:CreER; βact2:BS-gata4; βact2:RS-lkBSR* ventricles at 30 dpa after treatment with 4-HT. Images are stained for Tnnt to demarcate cardiac muscle. Dashed line indicates approximate resection plane. (D and E) Tilescan images of *cmlc2:CreER; βact2:RS-lkBSR; tcf21:nucGFP* ventricles at 14 dpa after treatment with vehicle or 4-HT. Images are stained for Tnnt to denote cardiac muscle. (F) Quantification of *tcf21*⁺ cell responses in *cmlc2:CreER; βact2:RS-lkBSR* hearts treated with vehicle ($n = 10$) or 4-HT ($n = 12$) at 14 dpa. Error bars indicate SE. * $P < 0.05$, Student's t test, two-tailed. (Scale bars: 100 μ m.)

to a stem cell-like state and increased tumorigenesis (44). One mechanism by which NF- κ B signaling may broadly work is to induce lineage-restricted developmental programs by activation of lineage-restricted enhancers. In macrophages, for instance, NF- κ B signaling coordinates rapid activation of tissue-restricted enhancers in response to inflammatory stimuli (45). Here, we add to this paradigm by showing a link between NF- κ B and regeneration through cardiomyocyte proliferation and dedifferentiation. Further, our observed defects in epicardial responses during regeneration by suppression of NF- κ B signaling in cardiomyocytes suggests that NF- κ B contributes to a cross-talk of cardiomyocytes and epicardial cells that is required to coordinate regeneration.

Although NF- κ B is required for heart regeneration, NF- κ B activity is also induced in response to injury in poorly regenerative tissues such as the adult mammalian heart. Interestingly, the mammalian heart up-regulates a fetal gene expression program with stress, but unlike zebrafish, does not undergo widespread cardiomyocyte proliferation or regeneration (46). One possibility to reconcile this discrepancy is that the accessible targets to NF- κ B signaling are different in fish and mammals. Another possibility is that the level and timing of signaling may be important to facilitate efficient regeneration. For example, early inhibition of Stat3 can promote satellite muscle cell expansion, but prolonged inhibition impairs regeneration by limiting progenitor cell differentiation (47). Similarly, prolonged NF- κ B activity may be maladaptive for the mammalian heart by promoting cardiomyocyte atrophy and fetal reprogramming of cardiomyocytes (22). Ultimately, the different physiologic states of zebrafish and mammalian hearts may contribute to differential NF- κ B signaling with injury. Future investigations into the relative amount of NF- κ B activity and sites of NF- κ B occupancy in cardiomyocytes in zebrafish and mammals may prove important in reconciling regenerative capacity among species.

Materials and Methods

Zebrafish. Wild-type or transgenic zebrafish of the EK/AB strain were used for all experiments. *βactin2:loxP-mCherry-STOP-loxP-DTA*, *cmlc2:CreER*, *NF- κ B:eGFP*, *gata4:eGFP*, *gata5:eGFP* and *tcf21:nucGFP* transgenic fish have been described (5, 7, 25, 29, 34, 48). Procedures involving animals were approved by the Institutional Animal Care and Use Committee at Duke University.

Generation of Transgenic Zebrafish. To generate *gata4:DsRed2* zebrafish, *DsRed2* cDNA was cloned downstream of the 14.8-kb *gata4* promoter (34). The entire construct was flanked by I-SceI sites for transgenesis. To generate *βact2:RS-lkBSR*, a bicistronic construct encoding mTag-BFP (Evrogen) and lkBSR (Addgene, plasmid 12329) separated by a viral 2A peptide was ligated into the *βactin2:loxP-DsRed-STOP-loxP-eGFP* plasmid after excision of eGFP with AgeI/NotI. To generate *βact2:BS-gata4* zebrafish, *gata4* was amplified from 7 dpf zebrafish embryo cDNA and cloned in frame to pFA6a-6xGLY-FLAG (Addgene; plasmid 20751) resulting in a C-terminal FLAG tagged protein. The resulting *gata4-FLAG* construct was then digested with XmaI/PspOMI and cloned into the *βactin2:loxP-mTagBFP-STOP-loxP-eGFP* plasmid after excision of eGFP with AgeI/NotI. Each construct was coinjected into one cell-stage wild-type embryos with I-SceI. One founder was isolated and propagated. The full names for these transgenic lines are *Tg(gata4:DsRed2)^{pd28}*, *Tg(βactin2:loxP-mCherry-STOP-loxP-mTagBFP-2A-lkBSR)^{pd114}*, and *Tg(βactin2:loxP-mTagBFP-STOP-loxP-gata4)^{pd115}*. To induce recombination in adult fish, *cmlc2:CreER; βact2:RS-lkBSR* fish and *cmlc2:CreER; βact2:BS-gata4* fish were bathed in 4 μ M 4-HT (Sigma) for 24 h.

Histological Analysis and Imaging. Probes for *nfkbiaa*, *nfkbiab*, *nfkbi1*, *crel*, *relb*, and *p65* were generated from 6 dpf zebrafish cDNA by using the primer sequences in Table S1. In situ hybridization probes were prepared and hybridizations were performed with the aid of an InSituPro robot (Intavis) as described (25). Primary and secondary antibody staining for immunofluorescence was performed as described in ref. 7. Primary antibodies used in this study were anti-PCNA (mouse; Sigma) at 1:200, anti-Mef2 (rabbit; Santa Cruz Biotechnology) at 1:75, anti-troponinT (mouse; Thermo) at 1:150, anti-NF- κ B1 (rabbit; Abcam) at 1:50, and anti-FLAG (mouse; Sigma) at 1:100. Confocal imaging was performed using a Zeiss LSM 700 or Leica SP8 upright confocal microscope. AFOG staining was performed as described in ref. 4. Mef2/PCNA imaging to assess cardiomyocyte proliferation was performed and quantified as described in ref. 7. Regenerates were scored on a scale from 1 to 3 after staining for Tnnt as described in ref. 50.

To quantify sarcomeric integrity, hearts were stained with anti-Tnnt and imaged by using identical imaging parameters. Images were processed by using the EBImage package of R/Bioconductor (51). To quantitatively assess sarcomere integrity, we imaged each heart and ascertained the sum intensity for Tnnt staining and divided by the calculated muscle area. For each heart, three different sections were used. A Student's t test was used to determine a significant difference between groups.

Epicardial density was performed on 10 μ M cryosections by taking confocal images across 2-tiles and z-stacks at a 2 μ m step at 20 \times magnification. For each heart, three maximal intensity projection images corresponding to the largest injury were imaged. Images were then coded by using a random number generator. For each image, a blinded reader traced and measured wound area using ImageJ. The number of *tcf21:nucGFP* nuclei within the wound was then counted and recorded. For each heart, the average number of nuclei per square μ m was determined and used for calculations. A Student's t test was used to determine a significant difference between groups.

Quantitative PCR. RNA was extracted by using whole ventricles from ZCAT fish and quantitative PCR was performed by using Roche Master Mix, Roche upl probes, and Roche LightCycler 480 system as described (49). Primer efficiency was tested, and only primers with an efficiency between 1.95 and 2.05 were used. Primer sequences and their corresponding upl are provided in Table S2.

Chromatin Immunoprecipitation. Hearts were extracted and whole ventricles were dissected in PBS with heparin (100 U/ml). Hearts were rinsed in PBS containing protease inhibitors (Roche), cross-linked in 1% formaldehyde for 10 min, and rinsed again in PBS. After homogenization in lysis buffer, chromatin was sonicated with a Bioruptor (Diagenode). ChIP was then performed by using an antibody against NF- κ B1 (Rabbit; Abcam) or Rabbit-IgG (Invitrogen) at 1 μ g of antibody/5 μ g of chromatin by using the Magnify ChIP System (Invitrogen). ChIP was performed per manufacturer protocol with the exception that incubation with primary antibody was performed overnight. To detect binding to the *gata4* promoter, 5' CCCCTGCGTGATATGTCT 3' and 5' TACGTTTTTCGGCGGTAAC 3' primers were used for PCR.

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