#### Cardiac Mitogen Signaling During Zebrafish Heart Regeneration

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

#### **ABSTRACT**

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#### Abstract

Adult zebrafish demonstrate a remarkable capacity to regenerate heart tissue following injury, and thus have served as a valuable model for developing our understanding of cardiac repair and regeneration. Recent work has identified and characterized multiple cardiac mitogens all of which can drive cardiomyocyte (CM) division in the absence of injury. Despite these impressive responses, little is known regarding the shared specific molecular mechanisms of CM proliferation that lie downstream of these unique ligand-receptor interactions. Here, we found that the tumor suppressor p53 was significantly suppressed during regeneration which correlated with increases in the transcription of p53's primary negative regulator Mdm2. Using established and newly generated transgenic lines we demonstrated that experimentally altering cellular p53 levels affects CM proliferation. Inducible overexpression of the cardiac mitogens Nrg1 and Vegfaa demonstrated similar findings with increased mdm2 transcription and p53 suppression during regeneration along with augmented CM proliferation with loss of p53. Furthermore, we observed significant overlap between gata4, a critical cardiomyocyte transcription factor, and mdm2 gene expression domains during development, following heart injury, and with mitogen stimulation suggesting potential interactions between these two genes. Our findings indicate a novel injury and mitogen-induced function of Mdm2 to repress p53 during zebrafish heart regeneration. Here we also investigated the presence of additional cardiac mitogens, specifically HB-EGF, an ErbB ligand. We found that both HB-EGF paralogs are present in the zebrafish heart and are both transcriptionally upregulated near the site of injury. A newly generated set of novel HB-EGF transgenic reporters, knock-outs, and overexpression lines will further investigate the importance of these early findings and HB-EGF signaling which will add to our understanding of heart regeneration.

iv

### Contents

Lists of figuresvi
Acknowledgementsviii
1. Introduction1
1.1 Heart disease1
1.2 Proposed regeneration strategies2
1.3 Heart regeneration models4
1.4 The source of new cardiomyocytes
1.5 Discovery of cardiac mitogens10
1.6 Reactive oxygen species and cardiac regeneration14
1.7 p53 and heart regeneration15
2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>
<ul> <li>2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish</li></ul>

3.1 Summary	43
3.2 Results and discussion	44
3.2.1 HB-EGF transcription is activated following cardiac injury	44
3.2.2 HB-EGF transcription is activated following zebrafish spinal cord injury	48
3.2.3 HB-EGF double knockout zebrafish are viable	50
3.2.4 HB-EGF overexpression lines	52
3.3 Discussion	53
3.4 Experimental procedures	,,,56
4. Discussion	59
4.1 p53 signaling and cardiomyocyte regeneration	60
4.1.1: How do p53 and mdm2 drive dedifferentiation	60
4.1.2: MDM2 overexpression	61
4.1.3: Evaluate the effects of p53 knock-out in mice	62
4.1.4: Viral mediated delivery of <i>mdm2</i> to cardiomyocytes	63
4.1.5: Effects of Gata4 KO and overexpression on p53	64
4.1.6: Evaluate efficiency of cell cycle entry in p53 KO	64
4.1.7: Evaluate Mdm2 and p53 in other regeneration contexts	65
4.1.8: HB-EGF overexpression	66
5. Conclusion	67
References	69
Biography	83

## List of figures

Figure 1: Tp53 and <i>mdm</i> 2 are dynamically expressed after heart injury	20
Figure 2: Tp53 regulation of downstream transcriptional regulators	22
Figure 3: <i>mdm</i> 2 expression is induced after heart injury	23
Figure 4: Generation and characterization of an <i>mdm2:EGFP</i> reporter line	24
Figure 5: Tp53 or Mdm2 modulation alters CM proliferation during regeneration	26
Figure 6: $tp53^{+/+}$ and $tp53^{-/-}$ transcriptomic data	27
Figure 7: mdm2 deletion mutations are lethal in zebrafish embryos	28
Figure 8: Effects of a dominant-negative Mdm2 construct in zebrafish embryos	28
Figure 9: Modulation of Tp53 and <i>mdm</i> 2 in Nrg1-overexpressing hearts	31
Figure 10: Mitogens activate <i>mdm</i> 2 regulatory sequences and have increased pote in <i>tp53<sup>-/-</sup></i> mutants	ency 32
Figure 11: <i>mdm2</i> induction is associated with highly cardiogenic areas during heart development and regeneration	34
Figure 12: Expression of <i>mdm</i> 2-directed EGFP and embryonic myosin heavy chair during heart regeneration	า 35
Figure 13: <i>hbegfa</i> and <i>hbegfb</i> are induced following cardiac amputation injury.	45
Figure 14: Generation and characterization of an <i>hbegfa:EGFP</i> reporter line	46
Figure 15: Generation and characterization of an <i>hbegfb:</i> EGFP reporter line	47
Figure 16: <i>hbegfb<sup>GFP</sup></i> expression is induced in border zone CMs and cells present clot	within 48
Figure 17: <i>hbegfa</i> is strongly induced following zebrafish spinal cord transection	50
Figure 18: Generation of HB-EGFa and HB-EGFb knockout lines	51
Figure 19: Heart amputations of HB-EGF double knock-out mutants	52

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#### **1. Introduction**

#### 1.1 Heart disease

Currently nearly 5 million individuals are affected by heart disease in the United States alone. This contributes to an estimated 300,000 yearly deaths (Go et al., 2013b). Myocardial infarction (MI), resulting from the occlusion of one or more coronary arteries and resultant ischemia, is an overwhelming cause of morbidity and mortality causing nearly 1 out of every 6 deaths in the United States in 2009 (Go et al., 2013a). The mainstays of medical and surgical treatment of MI has focused on expedient myocardial reperfusion strategies either by percutaneous means or with coronary artery bypass grafting. Both of these techniques have revolutionized the field of cardiology and have resulted in significant declines in mortality, however these interventions are unable to stimulate meaningful regeneration of lost cardiac tissue. Despite these advances, the myocardial injury in MI survivors commonly represents the loss of 1 billion cardiomyocytes (CMs). CM necrosis initiates a local inflammatory response leading to fibroblast recruitment and cardiac scarring that is thought to be irreversible. The resultant clinical sequelae include cardiac aneurysm formation, additional MIs and heart failure. While heart transplantation currently offers the only definitive treatment for heart failure, graft availability remains a limiting factor and immunosuppression carries its own significant morbidity. Given the estimated annual health care expenditure of 18 billion dollars on heart failure, the development of therapies to enable the survival or replacement of lost myocardium would represent enormous socioeconomic gain (Go et al., 2013b). For these reasons both scientists and clinicians believe that these patients represent the greatest potential beneficiaries of the developing field of regenerative medicine. Thus, the field of cardiac regeneration is a multidisciplinary research area

enlisting specialists in a multitude of fields as diverse as basic science and biomedical engineering to medicine whose collective goal is to prevent or reverse heart failure following MI.

#### **1.2 Proposed regeneration strategies**

In an effort to augment the regenerative capacity of mammalian cardiomyocytes three broad strategies have been and continue to be investigated; stem cell-based therapies, cellular reprograming, and the stimulation of endogenous repair mechanisms (Tzahor and Poss, 2017). Stem cell-based therapies represent a large, heterogeneous collection of approaches that are predicated on the hypothesis that the delivery of a variety of multipotent cells (embryonic stem cells, induced pluripotent stem cells, skeletal myoblast, bone marrow mononuclear cells, etc....) to the diseased animal or organ will result in improved cardiac function and/or regeneration (Laflamme and Murry, 2011; Martin-Puig et al., 2008). Numerous mechanisms by which these cells confer their regenerative potential have been proposed, including, but not limited to, the transdifferentiation of transplanted cells into cardiac tissue, paracrine mechanisms, cell fusion, & participation in angiogenesis (Laflamme and Murry, 2011; Martin-Puig et al., 2008). This stem-cell based approach is highlighted by work performed in the lab of Charles Murry at the University of Washington whose lab sought to evaluate the therapeutic potential of human embryonic-stem-cell-derived cardiomyocyte (hESC-CMs) transplantation into the infarcted heart of a non-human primate model. Although the delivery of 1x10<sup>9</sup> hESC-CMs resulted in remuscularization of the infarct and partial electrical coupling, severe arrhythmias were noted in all treated animals and the study failed to demonstrate that hESC-CMs improved the function of the post-infract heart (Chong et al., 2014; Liu et al., 2018; Shiba et al., 2016). To date no stem-cell based

therapy has demonstrated significant improvement in cardiac function however these studies continue to generate profound interest and financial support (Eschenhagen et al., 2017).

Cellular reprograming, the second proposed cardiac regeneration strategy, is built upon pioneering work performed by Takahashi and Yamanka, in which adult mouse fibroblasts, transduced with a collection of specific transcription factors, demonstrated cellular transformation into stem-cell like cells called inducible pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Further work has uncovered the ability to direct these cells into specific fates, such as hematopoietic stem cells and neurons (Szabo et al., 2010; Vierbuchen et al., 2010). With approximately 50% of cardiac tissue being composed of fibroblasts the ability to reprogram these resident fibroblasts into cardiomyocytes would represent an attractive therapeutic option. Work investigating this concept has demonstrated both in vitro and in vivo that the transduction of cardiac fibroblasts with a set of three transcription factors, Gata4, Mef2c, and Tbx5 is sufficient to reprogram these cells into cardiomyocytes (leda et al., 2010; Qian et al., 2012). Although the efficiency of successful reprograming following local delivery of transcription factors is only 10-15%, in vivo reprogramming performed in mice following coronary ligation has shown significant improvement across multiple measures of cardiac function, specifically ejection fraction, stroke volume, and cardiac output when compared to control animals (Qian et al., 2012). Numerous studies have refined this initial work with the addition of the transcription factor Hand2 along with coadministration of Thymosin  $\beta$ 4 both improving reprogramming efficiency (Song et al., 2012; Srivastava et al., 2012). While murine work has steadily advanced, the efficient reprogramming of human fibroblast has been elusive. Additional transcription factors combined with microRNAs has resulted in both low efficiency over extended culture

periods combined with incomplete reprogramming resulting in a failure to achieve spontaneous contractility (Fu et al., 2013; Nam et al., 2013; Wada et al., 2013). Recently, an alternative reprogramming approach, using a screen of 89 small molecules known to facilitate reprogramming, identified 9 compounds that efficiently reprogrammed human fibroblasts *in vitro* into contractile cardiomyocytes, however, future work will be required to investigate these compounds *in vivo* (Cao et al., 2016).

While the first two proposed cardiac regeneration strategies represent exotic and cutting edge science, the third strategy-- to utilize the endogenous repair mechanisms of the host's native tissue to boost cardiac function remains equally elusive despite its seemingly more pedestrian nature. Work over the past decade has challenged the long-held belief that cardiomyocytes represent a terminally differentiated, post-mitotic state that are incapable of further cell division. Elegant studies on human cardiac tissue using atmospheric Carbon-14, a byproduct of Cold War nuclear testing, have demonstrated that cardiomyocytes are replaced at a low but detectable level throughout life (Bergmann et al., 2009; Bergmann et al., 2015). Recent work in mice has demonstrated remarkable CM proliferation and improved post-infarct cardiac function with the delivery of activated forms of Erbb2 and Yap (D'Uva et al., 2015; Morikawa et al., 2017). The benefits of such an approach are numerous. Unlike stem-cell based therapies no immunogenic material is required thus obviating the need for, and the associated risks of immunosuppressant therapy. Additionally, the lack of a pluripotent cell-state eliminates the potential for teratoma formation—a potential devastating complication of stem cell therapies.

#### 1.3 Heart regeneration models

The first successful complete cardiomyocyte regeneration to occur in nature was described in adult zebrafish *(Danio rerio)* in 2002 (Poss et al., 2002). The subsequent

two decades have established the zebrafish as a key tool in the study of cardiac regeneration as they have the highest demonstrated capacity for heart regeneration in adult animals amongst laboratory model systems (Gonzalez-Rosa et al., 2011). Anatomically the zebrafish heart contains a single atrium, a single ventricle and an outflow tract, or bulbus arteriosus. Microscopically, similar to their mammalian counterparts, the hearts are composed of an endocardial layer which is in contact with the intraluminal blood flow. A myocardium, composed of cardiomyocytes, is responsible for cardiac contraction, and finally an outer endocardial layer (Poss et al., 2002). Currently several methods are employed to induce cardiac injury in the zebrafish model. These include surgical resection of the cardiac apex, cryoinjury, and inducible genetic CM ablation (Chablais et al., 2011; Gonzalez-Rosa et al., 2011; Kikuchi et al., 2011a; Schnabel et al., 2011; Wang et al., 2011). Each of these injury models possess unique benefits along with restrictions. Specifically, apical resection removes the tissue entirely and can be difficult to learn and reproduce (Chablais et al., 2011; Poss et al., 2002). Additionally, a large amputation often results in death of the organism. Cryoinjury was developed to mitigate these issues, creating a larger, more severe, defect along with the retention of proinflammatory devitalized tissue (Chablais et al., 2011). However, regeneration can take an extended period of time (~120 days) verses 30 to 60 days in the amputation model. Both of these techniques allow for the ability to visualize the spatial-temporal patterns associated with regeneration which is not true with inducible CM ablation, as ablation results in the loss of CMs throughout the myocardium in a random distribution. Additionally, the endocardium and epicardium remain uninjured in this injury model. Nevertheless, the ablation model serves as an excellent screening tool for gene expression and allows for the reproducible injury of a large number of fish throughout the myocardium which is useful in the creation of next generation sequencing

datasets such as RNAseq and ATACseq (Goldman et al., 2017; Kang et al., 2016; Wang et al., 2011).

As a model organism the zebrafish has revealed multiple critical components of cardiac regeneration. First, that the proliferation of existing CMs is the primary cellular source of muscle without a major contribution by stem cells (Choi et al., 2013; Gonzalez-Rosa et al., 2012; Joplin et al., 2010; Kikuchi et al., 2011a; Kikuchi et al., 2010). Second, that the environment aided by non-muscle epicardial and endocardial cells stimulates regeneration from this CM source by promoting neovascularization and releasing CM mitogens (Kikuchi et al., 2011c; Kim et al., 2010; Lepilina et al., 2006; Serluca, 2008). The insights gained from these investigations of zebrafish heart development and regeneration have influenced and guided the way in which cardiac regeneration is viewed in mammals. Mirroring important zebrafish findings, recent studies have revealed the stimulation of CM division in adult mammals following injury (Bergmann et al., 2009; Bergmann et al., 2015; Bersell et al., 2009; Senyo et al., 2013). This low, but measurable, cellular turnover demonstrates that the mammalian heart maintains analogous regenerative machinery found in zebrafish, however, its reduced activation following injury results in negligible CM regeneration.

Since these initial observations in zebrafish, naturally-occurring cardiomyocyte regenerative ability following heart cauterization has been documented in other teleost such as the giant danio (*Devario aequipinnatus*), and goldfish (*Carassius auratus*) (*Grivas et al., 2014; Lafontant et al., 2012*). Other teleosts such as medaka (*Oryzias latipes*) lack cardiac regenerative capacity, an observation recently exploited to contrast transcriptional changes between injured medaka and zebrafish revealing significant differences in immune responses between the two teleosts (Ito et al., 2014; Lai et al., 2017). Amphibians such as the newt (*Notophthalmus viridescens*) and AxolotIs

(*Ambystoma mexicanum*) have also demonstrated cardiac regenerative capacity following injury (Flink, 2002; Mercer et al., 2012; Mercer et al., 2013; Witman et al., 2011). Nevertheless, despite these examples of successful heart regeneration, the advanced genetics along with well-characterized and validated techniques the zebrafish remains the dominant non-mammalian model for heart regeneration research.

There remain significant differences between the zebrafish and mammalian hearts that are thought to contribute to the zebrafish's pro-regenerative nature. Specifically, throughout the life of the zebrafish, cardiomyocytes remain both mononuclear and diploid while mammalian cardiomyocytes become largely binucleate and polypoid during development (Patterson et al., 2017). Interestingly the presence of an increased number of mammalian mononuclear diploid cardiomyocytes in mice has been associated with increased cardiomyocyte proliferation after injury, while experimentally induced binucleated polypoid cardiomyocytes in zebrafish blocks their regenerative potential (Gonzalez-Rosa et al., 2018; Patterson et al., 2017). Additionally, the reduced systolic blood pressure of zebrafish compared to mammals (5 mmHg vs 120 mmHg) and relative hypoxemia have been suggested as major contributors to the zebrafish regenerative capacity (Elhelaly et al., 2016; Nakada et al., 2017). Indeed, reductions in either mammalian cardiac strain or oxygen tension have both been associated with increased cardiomyocyte proliferation (Canseco et al., 2015; Nakada et al., 2017). For these and other not yet understood reasons the use of mammalian heart regeneration models have been increasingly used to understand heart regeneration.

Work in the past decade has highlighted that the neonatal mouse is capable of heart regeneration after ventricle apical resection, cryoinjury, genetic ablation of cardiomyocytes, and left anterior descending artery (LAD) ligation during the first 7 postnatal days, after which this regenerative capacity is lost (Darehzereshki et al., 2015; Lavine et al., 2014; Mahmoud et al., 2014). Understanding what drives the loss in regenerative potential has been an area of significant investigation. Interesting, recent data suggest a relationship between regenerative capacity, thyroid hormone signaling, and metabolic changes in the developing mouse (Hirose et al., 2019; Naqvi et al., 2014). While the innate ability of the injured adult mouse heart to regenerate is insignificant, an increasing body of work has demonstrated meaningful regeneration with the overexpression of genes such as YAP and ErbB2 creating an incredibly useful model to investigate acquired regenerative ability with molecular and genetic manipulations (D'Uva et al., 2015; Monroe et al., 2019).

#### 1.4 The source of new cardiomyocytes

Despite the demonstration that mammalian cardiomyocytes do not represent a terminally differentiated and non-proliferative cell (Bergmann et al., 2009; Bergmann et al., 2015) the cellular source of new cardiomyocytes during regeneration remained elusive, with potential sources including cardiac stem-cell populations (Beltrami et al., 2003), proliferation of existing cardiomyocytes (Lechene et al., 2006), and bone marrow derived stem cells (Orlic et al., 2001). Early work suggested that the delivery of bone-marrow-derived c-kit<sup>+</sup> hematopoietic stem cells were capable of regenerating damaged cardiac tissue (Balsam et al., 2004; Orlic et al., 2001), however others were unable to reproduce these findings (Murry et al., 2004) leading the field to hypothesize that c-kit<sup>+</sup> cardiac progenitor cells resided within the cardiac tissue and were responsible for the production of new cardiomyocytes. Initial studies proved promising with reports that isolated c-kit<sup>+</sup> cells from rat myocardium could differentiate into multiple lineages including cardiomyocytes, and that once isolated and injected into diseased myocardium were capable of regenerating this tissue (Beltrami et al., 2003; Hosoda et al., 2009).

While additionally studies built upon these initial findings with increasingly profound cardiomyocyte regenerative potential of c-kit<sup>+</sup> cells (Ellison et al., 2013; Jesty et al., 2012), an increasing number of reports suggested that *in vivo* c-kit<sup>+</sup> cardiac progenitor cells lacked or contained very little ability to produce new cardiomyocytes (van Berlo et al., 2014; Zaruba et al., 2010). To address this fundamental question scientist developed an elegant lineage trace analysis in mice by transgenically inserting a Cre recombinase into the endogenous Kit locus and subsequently crossing this mouse with a Rosa26-CAG-*loxP*-STOP-*loxP*-eGFP reporter line. This double transgenic thus allowing for the irreversible labeling of cells that currently express, or have ever expressed, c-kit. From this double transgenic, it was observed that 6 months post tamoxifen-induced recombination and labeling, only 0.0055% of adult cardiomyocytes were GFP<sup>+</sup>. To further evaluate whether injury and the resultant increase in cell turnover would increase the number of labeled cardiomyocytes, left anterior descending artery ligation or chemical injury was performed revealing an increase in labeled cardiomyocytes to 0.016% and 0.007% with respect to the injury (van Berlo et al., 2014). These findings thus strongly suggest that the c-kit <sup>+</sup> population contributes a particularly small amount of new cardiomyocytes during development, cardiac homeostasis, and following injury and have been independently reproduced (Sultana et al., 2015). A 2017 consensus statement on cardiomyocyte regeneration published in the journal, *Circulation*, indeed recognizes that the contribution of resident stem/progenitor cells contribute to fewer than 0.01% of new cardiomyocytes per year (Eschenhagen et al., 2017).

As the debate over the contribution of cardiac progenitor cells to cardiomyocyte renewal continued, others investigated alternative sources of new cardiomyocytes. In zebrafish, it was observed that *gata4*, a well-known transcription factor critical for proper cardiac development, was transcriptionally upregulated in border zone CMs following

ventricular amputation. With this observation, a double transgenic fish containing a tamoxifen-inducible Cre driven by the regulatory sequences of either *gata4* or *cardiac myosin light chain 2 (cmlc2)* in addition to an inducible reporter demonstrated that pre-existing CM's that express *gata4* following injury regenerate the amputated ventricular apex (Kikuchi et al., 2010). Subsequent lineage trace investigations in the mouse recapitulated these findings (Senyo et al., 2013), thus providing strong cross-species evidence that following injury, new cardiomyocytes are the result of induced division of pre-existing CMs.

#### 1.5 Discovery of cardiac mitogens

With an increasing amount of work demonstrating that cardiomyocytes are capable of low, but measurable proliferative capacity during homeostasis and following cardiac injury, attention has focused on attempts to harness and augment this endogenous proliferative capacity. An unexpected clinical observation emerged following the development and initial use of trastuzumab, a monoclonal antibody used in the treatment of Erbb2<sup>+</sup> breast cancer. While this novel therapy had excellent oncologic outcomes in many recipients, it was nevertheless discovered that approximately 3% of patients receiving treatment developed overt congestive heart failure, with up to 30% developing a less pronounced, subclinical disease (Cote et al., 2012). These observations have led to an increasing amount of work focused on the Neuregulin1-Erbb2/4 signaling axis and the demonstration that cardiomyocyte-specific deletions of Erbb2/4 result in cardiomyopathies and poor stress response (Crone and Lee, 2002; Garcia-Rivello et al., 2005; Ozcelik et al., 2002). Similar results were found with Nrg1-heterozygous knockout mice, in which these mice demonstrate poor stress tolerance (Liu et al., 2005). Within the context of these findings investigators have hypothesized that administration of Nrg1,

a known Erbb2/4 ligand and agonist might therefore have effects opposite that which has been seen with trastuzumab. To investigate this hypothesis, a transgenic zebrafish was engineered containing  $\beta$ -actin2:loxP-mTagBFP-STOP-loxP-Nrg1 that when combined with *cmlc2:CreER* allows for cardiomyocyte-specific, tamoxifen-inducible expression of Nrg1. Following adult recombination an 84% increase in the cardiomyocyte proliferation index was observed by 7 days post cardiac injury. Additionally, in the absence of injury, Nrg1 overexpression resulted in marked cardiac proliferation with nearly 460% increase in cortical wall thickness by 30 days post tamoxifen treatment (Gemberling et al., 2015). Similar to these Nrg1 overexpression data in zebrafish, the establishment of a transgenic mouse, allowing for cardiomyocytespecific overexpression of a constitutively active Erbb2, resulted in mammalian cardiomyocyte hypertrophy, dedifferentiation, and proliferation (D'Uva et al., 2015). Interestingly, the authors also noted that decreasing Erbb2 levels as measured by transcript and total and active, phosphorylated, protein levels corresponded with the decreased regenerative capacity of mice from P1 to P7 (D'Uva et al., 2015). These data support a hypothesis that decreasing amounts of Erbb2 within mammalian cardiomyocytes is a prominent driver of decreased proliferative capacity in mammalian systems. Indeed, numerous clinical studies enrolling patients following acute myocardial infarction or chronic congestive heart failure have failed to demonstrate significant improvement in cardiac function with Nrg1 delivery despite seemingly favorable alterations in remodeling and angiogenesis (Gao et al., 2010; Jabbour et al., 2011). Nevertheless, Nrg1-Erbb2/4 signaling remains an important area of ongoing research.

Like Erbb2, additional known oncogenes are being increasingly evaluated for their ability to promote mammalian cardiomyocyte proliferation. Recently, a large amount of attention has been focused on the Hippo-Yap signaling pathway. The Hippo signaling

pathway contains a myriad of components. Specifically, Mst1/2 kinases complex with a scaffold protein, Salvador, resulting in the phosphorylation of the tumor suppressor Lats. Activated Lats thereby phosphorylates the transcriptional co-activators Yap and Taz resulting in their nuclear exclusion and inactivity. In mice, *Salv*- deficient hearts develop severe cardiomegaly with further studies identifying and focusing on Yap as the critical Hippo-pathway effector molecule regulating cardiomyocyte development (von Gise et al., 2012; Xin et al., 2011). To evaluate the Hippo pathway in adult mice, conditional knockout lines for *Salv*, *Lats1* and *Lats2* were generated and crossed with a *Myh6<sup>creERT2</sup>* transgene allowing for cardiomyocyte specific deletion. Excitingly, following cardiomyocyte-specific deletion of these Hippo pathway genes, cardiomyocytes were observed re-entering the cell cycle and proliferating (Tao et al., 2016). Further studies revealed that loss of cardiomyocyte Hippo pathway signaling or the expression of a constitutively active Yap protein are sufficient for increased cardiomyocyte regeneration by both histologic imaging and functional studies (Monroe et al., 2019; Morikawa et al., 2017).

While not technically a direct cardiomyocyte mitogen the pro-angiogenic growth factor Vegf has been demonstrated in zebrafish to promote cardiomyocyte proliferation in the absence of injury and significantly augment cardiomyocyte proliferation following ventricular resection injury (Karra et al., 2018). The ability of Vegf to act as an indirect cardiac mitogen is likely due to its ability to hypervascularize the heart as a transgenic zebrafish containing a  $\beta$ -actin2:loxP-mTagBFP-STOP-loxP-Vegfaa, allowing for the inducible overexpression of Vegfaa in cardiomyocytes, results in a significant increase in cardiac angiogenesis which seems to precede the increase in cardiomyocyte proliferation (Karra et al., 2018).

Recent work utilizing a chemical screen identified the vitamin D analogue, alfacalcidol as a potent cardiac mitogen capable of increasing cardiomyocyte proliferation in the absence of injury and by augmenting cardiomyocyte proliferation following ventricular resection (Han et al., 2019). This serves to highlight the broad range of cardiac mitogens that have been discovered; from cell signaling receptors and extracellular matrix components, to hormones, growth factors, and oncogenes our understanding of the mechanisms that allow these unique signals to converge and thus drive cardiomyocyte proliferation will undoubtedly provide insights that help steer the future of heart regeneration (Bassat et al., 2017; D'Uva et al., 2015; Gemberling et al., 2015; Han et al., 2019; Karra et al., 2018; Monroe et al., 2019). Nevertheless, a significant amount of work remains on identifying additional cardiac mitogens. In particular, in addition to Nrg1 numerous other Erbb ligands exist such as Nrg2-Nrg4, EGF, HB-EGF, amphiregulin, betacellulin, epiregulin, and TGF- $\alpha$  all of which have unique binding affinities for the four Erbb receptors (Fuller et al., 2008). Zebrafish deficient in Nrg2 demonstrate deficiencies in trabeculation and unlike Nrg1, the overexpression of Nrg2 failed to induced significant changes in cardiomyocyte proliferation (Rasouli and Stainier, 2017). Among these Erbb ligands, Heparin Binding-EGF-like Growth Factor (HB-EGF) is a particularly attractive candidate for a multitude of reasons. It has well-known roles in mammalian cardiac development with genetic deletion resulting in dilated cardiomyopathy, depressed cardiac function, and enlarged cardiac valves (Iwamoto et al., 2003; Jackson et al., 2003). HB-EGF, which preferentially binds to Erbb1 and Erbb4, is initially translated containing a transmembrane domain. This relatively inactive proHB-EGF remains bound to the cell membrane. Ectodomain shedding occurs via cleavage by ADAM-family metalloproteases at the juxtamembrane domain to release the activated, soluble form of HB-EGF (sHB-EGF) (Higashiyama et

al., 1991; Higashiyama et al., 2008). sHB-EGF has been demonstrated to have significant mitogenic activity towards hepatocytes, smooth muscle cells and fibroblasts (Ito et al., 1994; Iwamoto and Mekada, 2000; Kiso et al., 2003; Prenzel et al., 1999). Interestingly, the inhibition of ectodomain shedding *in vivo* has been demonstrated to significantly abrogate pressure overload-induced cardiac hypertrophy in mice suggesting that the release of sHB-EGF has pathologic consequences (Asakura et al., 2002). Additionally, the overexpression of proHB-EGF in infarcted rabbit hearts increased remodeling with negative consequences on post-MI cardiac function (Ushikoshi et al., 2005). In contrast, *in vitro* work has suggested potential mitogenic functions of HB-EGF on cardiomyocytes (Ieda et al., 2009). While these studies provide seemingly contrasting views of HB-EGF its role in cardiac regeneration has never been rigorously investigated in a regenerative context such as zebrafish heart regeneration.

#### 1.6 Reactive oxygen species and cardiac regeneration

Interestingly, investigating transcription factors that were upregulated in Hippodeficient hearts uncovered increased protein levels of Pitx2 and RNAseq confirmed postnatal downregulation of *Pitx2* transcripts. Apical resection of *Pitx2* cardiomyocyte conditional knockout mice at P1 were unable to fully regenerate compared to their wild type controls as demonstrated by scarring and decreased cardiac function (Tao et al., 2016). Conversely, the creation of a cardiomyocyte-specific *Pitx2* gain of function resulted in decreased scar size when compared to controls. Co-immunoprecipitation experiments demonstrated that Pitx2 and Yap interact *in vivo* and this interaction is required for the activation of genes necessary for control of reactive oxygen species (Tao et al., 2016). Numerous studies have investigated the role of reactive oxygen species on cardiomyocyte regeneration in more detail and it has been demonstrated that

as postnatal cardiomyocyte metabolism transitions from glycolytic to oxidative metabolism, ROS is increased resulting in decreased regenerative capacity (Puente et al., 2014). By exposing neonatal mice to a hypoxic environment  $(15\% O_2)$  compared to hyperoxic (100%  $O_2$ ) and measuring cardiomyocyte proliferation, investigators were able to conclude that oxygen levels can indeed affect regenerative capacity (Puente et al., 2014). Expanding upon these studies, scientist moved these observations into adult mice. Adult mice were exposed to a chronic hypoxic environment for 2 weeks, after which their hearts were assessed and determined to have a significant increase in cardiomyocyte proliferation by measure of the mitosis marker phosphorylated histone H3 Ser10 (pH3S10) (Nakada et al., 2017). In a similar manner, adult mice were subjected to chronic hypoxic conditions and LAD ligation was performed to evaluate the effect of hypoxia on the regenerative response. Multiple metrics were improved in the mice subjected to hypoxic conditions including reduced scar size, improved systolic function, and increased ejection fraction (Nakada et al., 2017). In addition to these improved cardiac performance values, the hypoxia group showed increased markers of cardiomyocyte proliferation as evidenced by a 20-fold increase in BrdU-positive cardiomyocytes, increase in cardiomyocytes positive for pH3S10, and aurora B (Nakada et al., 2017). Finally, using a lineage trace analysis, it was demonstrated that the source of these new proliferating cardiomyocytes was indeed pre-existing cardiomyocytes as opposed to a progenitor/stem cell source (Nakada et al., 2017).

#### 1.7 p53 and heart regeneration

The tumor suppressor gene *TP53*, discovered 40 years ago, is the most widely mutated gene in human cancers (Lane and Crawford, 1979; Mantovani et al., 2019). Tp53 is a highly conserved transcription factor that has critical roles in a variety of cell

processes including cell cycle regulation, DNA repair, apoptosis, and senescence across multiple species (Belyi et al., 2010; Berghmans et al., 2005; Fridman and Lowe, 2003; Hafner et al., 2019; Kruiswijk et al., 2015; Vousden and Lu, 2002). In cardiomyocytes, Tp53 has been implicated as a key regulator of the cardiac transcriptome, and increased Tp53 levels have been shown to be associated with late cardiac hypertrophy and remodeling (Mak et al., 2017; Nomura et al., 2018). Tp53 levels are typically kept low under homeostatic conditions, primarily through the activity of an E3 ubiquitin ligase, Mdm2. Mdm2 contains a C-terminal ligase function that, when the protein is bound to Tp53 via its N-terminal Tp53 binding domain, ubiquitinates Tp53 and targets it for proteosomal degradation (Chua et al., 2015; Michael and Oren, 2003; Nomura et al., 2017). These interactions between Tp53 and Mdm2 have been demonstrated to play a critical role in maintaining CM homeostasis in mice (Stanley-Hasnain et al., 2017). The direct binding of Mdm2 to Tp53 has also been shown to physically block and thus inhibit the transactivational domain of Tp53 (Oliner et al., 1993). Following a cellular insult like ionizing radiation exposure, Mdm2 is prevented from binding to Tp53, allowing for its accumulation and transcriptional activation of target genes (Guo et al., 2013; Wade et al., 2013). As molecular damage resolves, Tp53 acts as its own negative regulator by participating in a complex that directly increases *mdm2* transcription (Berghmans et al., 2005; Chua et al., 2015; Pant et al., 2013). Additional Tp53-independent roles for Mdm2 have been elucidated. (Bohlman and Manfredi, 2014; Gu et al., 2009).

# 2. Tp53 suppression by cardiomyocyte mitogens promotes heart regeneration in zebrafish

This chapter has been published as Shoffner et.al, Cell Reports 2020. Line creation and validation were all performed by myself. I performed the experiments involving the dnMdm2 and p53<sup>-/-</sup> lines. Valentina Cigliola was responsible for western blots, imaging, and ingenuity pathway analysis. Nutisha Lee performed heart surgeries and imaging. Jianhong Ou performed all biostatistics. The paper was written by myself and Valentina Cigliola with extensive revisions performed by myself, Valentina Cigliola and Ken Poss.

#### 2.1 Summary

Recently, a number of factors have been identified and shown to boost adult zebrafish CM proliferation if experimentally augmented during regeneration (Gemberling et al., 2015; Han et al., 2019; Karra et al., 2018; Missinato et al., 2018; Wu et al., 2016). A subset of these factors, including Neuregulin1 (Nrg1), Vascular endothelial growth factor a (Vegfa), and analogues of vitamin D like alfacalcidol (Gemberling et al., 2015; Han et al., 2019; Karra et al., 2018), have mitogenic effects even in the absence of injury. How these factors in particular exert their effects may be enlightening for the field, given that they possess the capacity as single entities to jumpstart a complex process of cardiogenesis, angiogenesis, and epicardial tissue growth, all seemingly without injurydependent influences like cell death, changes in tissue tension, and inflammation. Here, a new potential role of Tp53 during innate heart regeneration in zebrafish arose unexpectedly, as bioinformatic assessment of transcriptomes indicated that the network of Tp53-regulated genes is among the most suppressed during heart regeneration and with mitogen stimulation. We find that this suppression is likely due to regulated induction of *mdm2*, which we observe in CMs during regeneration as well as other cardiogenic events. Genetic reduction of Tp53 levels increased CM proliferation, either during heart regeneration or upon direct mitogen stimulation, whereas myocardial inhibition of Mdm2 decreased CM proliferation. Our experiments indicate that zebrafish heart regeneration is enabled by a mechanism in which mitogens alone, or after injury, suppress Tp53, enhancing CM proliferation.

#### 2.2 Results and discussion

#### 2.2.1 Tp53 protein levels transiently decrease after heart injury

To uncover transcription factors that regulate gene expression during heart regeneration, we performed additional bioinformatic analyses of published RNA-seq datasets using Ingenuity Pathway Analysis (IPA) software, a curated database of literature-derived information on biological interactions that integrates omics data to suggest regulatory mechanisms. Comparisons were made between samples from hearts of uninjured zebrafish and those from hearts of zebrafish receiving a genetic ablation injury that destroys ~50% of all CMs (Kang et al., 2016; Kramer et al., 2014; Wang et al., 2011) (Fig. 1A, B; Fig. 2). The most significant activated upstream regulators included factors involved in inflammation, such as TNF and IL6. ErbB2 and Vegf signaling, known to impact CM proliferation (D'Uva et al., 2015; Gemberling et al., 2015; Lai et al., 2017); (Bersell et al., 2009; Karra et al., 2018), were also found among the list of upstream regulators. Several transcription factors were identified as possible suppressed upstream regulators (i.e. molecules whose suppression results in the observed gene expression changes), including KLF15 and RB1. Surprisingly, among the most suppressed upstream regulators at 7 days post injury was the transcription factor Tp53 and its downstream transcriptional network (Fig. 1B; Fig. 2). By 14 dpi, this was no

longer the case. To our knowledge, no studies to date have evaluated the role of Tp53 in the context of innate heart regeneration.

To assess changes in Tp53 levels during heart regeneration, we performed Western blotting on proteins isolated from whole hearts at different time points after CM ablation. We found that Tp53 levels were dynamic after injury, decreasing to ~40% of uninjured levels at 7 dpi, and then recovering to ~14-fold those of uninjured fish at 14 dpi (Fig. 1C), changes that were consistent with the analyses of our transcriptome data.

Mdm2 is the primary regulator of Tp53. Based on our previously published transcriptome datasets, *mdm*2 transcripts are increased at 7 and 14 dpi at 1.7 and 2.6 fold those of uninjured levels, respectively (Fig. 3A and (Goldman et al., 2017; Kang et al., 2016)). To visualize these changes, we performed *in situ* hybridization using injured and regenerating hearts. *mdm*2 was rarely detectable in uninjured hearts. By contrast, increased *mdm*2 expression was detected in the cortical muscle layer near the injury site at 7 days following resection of the ventricular apex (dpa), though variable and sometimes difficult to detect (Fig. 1D). *mdm*2 expression remained detectable at 14 dpa but no longer by 30 dpa (Fig. 3C). Additionally, induced genetic ablation of CMs led to *mdm*2 expression throughout the ventricular wall and trabecular compartment by 7 dpi (Fig. 3B).

To more clearly define the spatiotemporal pattern of *mdm2* expression during heart regeneration, we generated a BAC transgenic reporter line containing long sequence stretches upstream (80 kb) and downstream (122 kb) of the *mdm2* start codon and flanking an EGFP cassette (*TgBAC(mdm2:EGFP<sup>pd312</sup>)*, hereafter referred to as *mdm2:EGFP* (Fig. 4A). As expected, based on the known requirement for Mdm2 during development (Chua et al., 2015), *mdm2*:EGFP fluorescence was evident throughout the entire bodies of developing larvae by 3 days post fertilization (dpf) (Fig. 4B). Adult

*mdm2:EGFP* fish hearts displayed low EGFP expression in the absence of injury, but EGFP fluorescence was sharply increased in both compact and trabecular muscle near the injury site upon ventricular resection at 7 and 14 dpa (Fig. 1G, Fig. 4C). Thus, Tp53 levels, and ostensibly the expression of its direct and indirect target genes, are transiently suppressed during heart regeneration, concomitant with transcriptional induction of the Mdm2 negative regulator.



#### Figure 1: Tp53 and *mdm*2 are dynamically expressed after heart injury.

(A) Experimental design and bioinformatic data analysis. (B) Selected top transcription factors acting as upstream regulators of differentially expressed genes in adult hearts 7 days after CM ablation (7 dpi). The p-value measures the statistical significance of the overlap between differentially expressed genes and the genes under predicted control of a regulator, and the activation score infers the activation state of a regulator, either activating (positive) or inhibiting (negative). (C) Western blot on protein extract of control ( $\beta$ -actin2:loxp-mCherry-STOP-loxp-DTA<sup>pd36</sup>), 7 dpi and 14 dpi (*cmlc2:CreER;*  $\beta$ -actin2:loxp-mCherry-STOP-loxp-DTA<sup>pd36</sup>) ventricles and quantification showing injury-induced regulation of Tp53 protein. Tamoxifen-induced ablation adjusted to ensure ablation of approximately 50-60% of CMs. Error bars indicate SEM (\*\*\*P < 0.001 unpaired t-test). (D) In situ hybridization for *mdm*2 expression (violet) in sections of uninjured and 7-day-post resection (7 dpa) ventricles. Arrows identify sites of increased *mdm*2 expression following ventricle amputation. (E) Sections images of uninjured and 7 dpa *mdm*2:EGFP hearts. Tnnt marks CMs. Boxes correspond to the region magnified in the right panels. Scale bars: 100 µm.



#### Figure 2: TP53 regulation of downstream transcriptional regulators.

Analysis of transcriptome data of 7 dpi regenerating zebrafish published by Kang et al., 2016. Solid arrows indicate direct and dashed arrow indicates indirect interactions. Predictions on the direction and intensity of activation and inhibition by TP53 were made by IPA knowledgebase based on published literature.



#### Figure 3: *mdm*2 expression is induced after heart injury.

(A) Genome browser tracks indicating expression of  $mdm^2$  7 days and 14 days after induced CM ablation (dpi), versus uninjured ventricles. (B) In situ hybridization for  $mdm^2$  (violet) in sections of uninjured and 7 dpi ventricles. VEH, vehicle; TAM, tamoxifen. (C) In situ hybridization for  $mdm^2$  in ventricular sections at different time points after resection of the ventricular apex (dpa). Dashed lines approximate the resection plane. Scale bars: 100 µm.



#### Figure 4: Generation and characterization of an *mdm2:EGFP* reporter line.

(A) Cartoon showing the *mdm2:EGFP* BAC transgene. (B) Larval *mdm2*-directed fluorescence, shown at 3 days post fertilization (dpf). (C) Images showing *mdm2*-directed EGFP at different time points after resection of the ventricular apex. Tnnt marks CMs. Dashed lines approximate resection plane. Scale bars: 100 µm.

## 2.2.2 Genetic modulation of Tp53 controls CM proliferation during heart regeneration

To investigate the function of Tp53 during regeneration, we resected the ventricular apices of zebrafish with null mutations in *tp53* (*tp53*<sup>M214K</sup>) and quantified CM proliferation at 7 dpa (Berghmans et al., 2005). *tp53*<sup>M214K</sup> mutants showed a more than doubling (123%) increase in their average CM proliferation index (n = 8, 9; Fig. 5A-C). Transcriptome sequencing of uninjured and regenerating *tp53*<sup>M214K</sup> hearts revealed significant expression changes from wild-types in a relatively small number of genes, 42 and 82, respectively, which represent a variety of cellular processes as characterized by gene ontology terms and IPA (Fig. 6). These data suggest a function for Tp53 in restricting CM proliferation during regeneration.

To increase levels/activity of Tp53 in heart muscle, we generated a new transgenic line to express an Mdm2 isoform lacking its C-terminal ubiquitin ligase and shown to have dominant-negative activity in previous studies (Chua et al., 2015; Jones et al., 1995). We placed this *m2DN* cassette downstream of a constitutive promoter and a *loxP*-flanked fluorescent reporter gene and stop signal (*Tg*( $\beta$ -actin2:loxP-BFP-STOP-loxP-dnMdm2-2A-mCherry)<sup>pd3t3</sup>), hereafter referred to as  $\beta$ -act2:BS-m2DN). Published homozygous *mdm2* mutants are embryonic lethal (Chua et al., 2015), as were new *mdm2<sup>pd314</sup>* mutants that we generated by CRISPR-based deletion, with no larvae surviving past 24 hours due to unregulated Tp53 activity (Figures 7A, B and 8A-D). To evaluate the effects of inhibiting Mdm2 function on CM proliferation, we crossed  $\beta$ -act2:BS-m2DN with *cmlc2:CreER* fish, for 4-Hydroxytamoxifen (4-HT)-inducible, Cremediated release of m2DN expression in CMs (Figures 5D-F and Figure 8). Adult *cmlc2:CreER*;  $\beta$ -act2:BS-m2DN and  $\beta$ -act2:BS-m2DN control clutch mates were treated with 4-HT 3 days before ventricular resection. *cmlc2:CreER*;  $\beta$ -act2:BS-m2DN animals

demonstrated a nearly 50% reduction in CM proliferation at 7 dpa when compared to controls (n = 12, 15; Figures 5D-F). Together, these data indicate that the suppression of Tp53-mediated gene regulation, at least in part by programmatic induction of its negative regulator Mdm2, provides a significant boost to CM proliferation upon cardiac injury in zebrafish.



Figure 5: Tp53 or Mdm2 modulation alters CM proliferation during regeneration. (A, B) Images of  $tp53^{+/+}$  and  $tp53^{-/-}$  hearts at 7 days after ventricular amputation (dpa). Arrows indicate Mef2<sup>+</sup>/PCNA<sup>+</sup> nuclei. (*C*) Quantification of CM proliferation at 7 dpa indicating an increased proliferation index in  $tp53^{-/-}$  mutants. (D and E) Images of  $\beta$ act2:BS-m2DN and cmIc2:CreER;  $\beta$ -act2:BS-m2DN hearts at 7 dpa and 10 days post tamoxifen administration (dpt). Arrows indicate Mef2<sup>+</sup>/PCNA<sup>+</sup> nuclei. (*F*) Quantification of CM proliferation showing a reduction in the proliferation index animals expressing a dominant-negative Mdm2. Scale bars: 100 µm. Error bars indicate SD (\*\*\**P* < 0.001, Mann–Whitney *U* test).



#### Figure 6: $tp53^{+/+}$ and $tp53^{-/-}$ transcriptomic data.

(A) Heatmap showing 42 genes differentially modulated in uninjured ventricles (Table S5). Black bars,  $tp53^{+/-}$ . Grey bars,  $tp53^{+/+}$ . (B, C) Barplot showing different biological processes assessed by GO analysis (B), and a list of selected differentially modulated canonical pathways assessed by Ingenuity Pathway Analysis (C; Table S7). (D) Top gene network composed by genes differentially modulated between uninjured  $tp53^{+/+}$  ventricles. (E) Heatmap showing 82 genes differentially modulated between  $tp53^{+/+}$  ventricles at 7 days after genetic CM ablation (Table S6). (F, G) Barplot showing different biological processes assessed by GO analysis (F), and a list of selected differentially modulated canonical pathways assessed by Ingenuity Pathway Analysis (G; Table S8). (H) Top gene network composed by genes differentially modulated in regenerating  $tp53^{+/-}$  and  $tp53^{+/+}$  ventricles.



Figure 7: *mdm*<sup>2</sup> deletion mutations are lethal in zebrafish embryos.

(A, B) Images showing  $mdm2^{+/+}$ ,  $mdm2^{+/-}$ , and  $mdm2^{-/-}$  embryos from an  $mdm2^{+/-}$  cross at 24 hours post fertilization (hpf).



#### Figure 8: Effects of a dominant-negative Mdm2 construct in zebrafish embryos.

Mutations within or loss of this mdm2 critical domain have been demonstrated both to phenocopy and to fail to rescue Mdm2 knock-out mice and zebrafish. Moreover, the injection of *m2DN* mRNA into single-cell zebrafish embryos recapitulated the *mdm2* mutant phenotype in wild-type embryos, but had limited effects in *tp53* mutant embryos.
(A) Cartoon schematic of  $\beta$ -act2:BSm2DN transgene. (B) Percentage of larvae recapitulating the mdm2<sup>-/-</sup> mutant phenotype upon injection of m2DN mRNA into single-cell embryos. (C, D) Images showing m2DN-injected tp53<sup>-/-</sup> and tp53<sup>+/+</sup> larvae 24 hours post fertilization (24 hpf). Asterisks indicate embryos with gross morphological defects.

#### 2.2.3 CM mitogens trigger *mdm2* induction

Cardiac damage is a massive biologic insult, resulting in a constellation of cellular events including and not limited to inflammation, fibrinogenesis, angiogenesis, muscle dedifferentiation, CM proliferation, and muscle patterning (Gonzalez-Rosa et al., 2017; Sadek and Olson, 2020; Tzahor and Poss, 2017; Vujic et al., 2019). Previously, we reported that the extracellular factor Nrg1 causes overt CM proliferation when ectopically expressed in the adult heart (Gemberling et al., 2015). In this way, adult cardiogenesis is uncoupled from injury, enabling assessment of the impact of the mitogenic signal itself. To determine effects of Nrg1 overexpression on expression of *mdm2*, we first performed ISH on *cmlc2:CreER;*  $\beta$ -act2:BSNrg1 fish 14 days after induction of the *nrg1* transgene. Whereas *mdm2* was undetectable in control hearts as described above, *nrg1* overexpression for 14 days led to strong and consistent *mdm2* expression in CMs within the cortical wall by ISH (Fig. 9, top panels). Similarly, when the *mdm2:EGFP* transgene was crossed into the *cmlc2:CreER;*  $\beta$ -act2:BSNrg1 background, 14 days of *nrg1* overexpression strongly induced EGFP throughout the cortical layer (Fig. 10A).

*Tp53* and *Mdm*<sup>2</sup> are known to be part of an autoregulatory *feedback loop*, conserved in zebrafish, with Tp53 binding to an upstream regulatory element of *mdm*<sup>2</sup> to activate transcription (Berghmans et al., 2005; Pant et al., 2013). To test whether *mdm*<sup>2</sup> induction with mitogenic stimulation reflects this mechanism of regulation, we first performed western blotting on proteins isolated from *cmlc2:CreER;*  $\beta$ -act2:BSNrg1 hearts and matching controls and found that Tp53 protein levels were reduced upon 14 days of ectopic Nrg1 expression (Fig. 9B,C). We then performed *mdm*<sup>2</sup> ISH using *cmlc2:CreER; βact2:BSNrg1; tp53*<sup>M214K</sup> mutant zebrafish. As in wild-type zebrafish, ectopic Nrg1 expression increased *mdm2* transcript levels throughout the outer cortical muscle layer in the presence or absence of functional Tp53 (Fig. 9A) (Berghmans et al., 2005). Taken together, these results suggest that mitogen stimulation induces *mdm2* in a Tp53-independent fashion.

To examine effects of a second CM mitogen, we tested the effects of induced myocardial Vegfa overexpression, which hypervascularizes the adult zebrafish heart in the absence of injury but also has mitogenic effects on CMs (Karra et al., 2018). Visualization of EGFP in *cmlc2:CreER;*  $\beta$ -act2:BSVegfa; mdm2:EGFP hearts revealed analogous findings as with *nrg1* overexpression, with *mdm*2:EGFP fluorescence activated in areas of proliferating CMs (Fig. 10B). Thus, two independent mitogens, having unique receptors and presumed mechanisms, can directly activate myocardial transcription of *mdm*2 in the absence of injury.

To determine whether the presence of Tp53 functionally impacts mitogen-stimulated CM proliferation, we crossed *cmlc2:CreER;*  $\beta$ -act2:BSNrg1 transgenes into the *tp53*<sup>M214K</sup> background. We found that, whereas 14 days of induced *nrg1* overexpression sharply increased the cortical CM proliferation index in wild-type animals from baseline levels to ~24%, the index in *tp53*<sup>M214K</sup> animals was ~37%, representing a ~54% higher level (n = 7, 12; Fig. 10C-G). Together, these findings indicate that mitogen presence, either experimentally expressed or injury-induced, induces *mdm2* and suppresses the Tp53 signaling network, and that this suppression of Tp53 has the effect of boosting CM proliferation.



#### Figure 9: Modulation of Tp53 and *mdm2* in Nrg1-overexpressing hearts.

(A) In situ hybridization for *mdm2* on ventricular sections with (right) or without (left) Nrg1 overexpression in  $tp53^{+/+}$  and  $tp53^{-/-}$  animals. *mdm2* is similarly induced in both tp53genotypes, at the peripheral edge of cortical muscle. Scale bars: 100 µm. (B) Western blot to detect Tp53, from control cardiac proteins and proteins extracted 14 days after Nrg1 induction. (C) Quantification of western blot protein bands. Error bars indicate SEM (\*\*\**P* < 0.001 unpaired t-test).





(A, B) *mdm2:EGFP* expression is activated in the ventricular wall after induced *nrg1* (A) or *vegfaa* (B) overexpression (dpt, days post tamoxifen treatment). High-mag views only shown in (B). (C) Quantification of CM EdU incorporation indices in ventricular walls of control (CreER-) or *nrg1*-overexpressing hearts at 14 dpt in  $tp53^{+/+}$  and  $tp53^{-/-}$  backgrounds. (D-G) Section images of ventricular walls from groups in (C), indicating greater Nrg1-induced EdU incorporation in Mef2<sup>+</sup> cells in  $tp53^{-/-}$  ventricles. Boxes correspond to the region magnified in adjacent panels. Arrows indicate Mef2<sup>+</sup>EdU<sup>+</sup> cells CMs. Scale bars 100 µm. Error bars indicate SD (\*\*\**P* < 0.001, Mann–Whitney *U* test).

#### 2.2.4 Tp53 regulation occurs in multiple cardiogenic contexts

Visual markers for dedifferentiated muscle in zebrafish include activation of regulatory sequences for the cardiogenic transcription factor gene gata4, as well as expression of the form of myosin heavy chain that is prominent in embryonic heart muscle but diminished in adults (Kikuchi et al., 2010; Wu et al., 2016). Importantly, gata4 activation also marks an emergent population of juvenile CMs from which the cortical muscle of the adult ventricular wall is derived (Gupta et al., 2013a; Gupta and Poss, 2012), as well as CMs stimulated to proliferate upon transgenic Nrg1 stimulation (Gemberling et al., 2015). To determine if *mdm*2 induction is a general feature of highly cardiogenic tissue in zebrafish, we assessed co-localization between mdm2- and gata4directed fluorescence in several contexts. We found that mdm2-directed EGFP fluorescence closely co-localized with gata4-directed dsRed fluorescence after ventricular resection injury (Figure 11A). Moreover, gata4-directed fluorescence was consistently present in a small domain at the base of the 4 wpf ventricle that partially overlaps with mdm2-directed fluorescence. By 7 wpf mdm2-directed EGFP fluorescence was evident in emerging and expanding cortical gata4<sup>+</sup> CMs (Figures 11B, C). In addition, mdm2- and gata4-directed fluorescence were tightly co-localized in adult cmlc2:CreER; β-act2:BSNrg1; mdm2:EGFP; gata4:dsRed fish after 14 days of nrg1 overexpression (Figure 11D). Interestingly, *mdm*2:EGFP and embryonic myosin heavy chain were expressed in the same region in each context, but they were expressed in adjacent CMs and did not colocalize to the same expressing cells (Fig. 12). These findings suggest a broad role for Tp53 suppression and *mdm2* induction during particularly active cardiogenesis.



## Figure 11: *mdm*2 induction is associated with highly cardiogenic areas during heart development and regeneration.

(A) Section confocal images of 7 dpa regenerating ventricles indicating co-localization of *gata4*- and *mdm2*-directed fluorescent reporter proteins. Boxes correspond to the region magnified in the panels. (B) Whole mount images of juvenile hearts at 4 and 7 weeks post fertilization (wpf) showing *gata4*- and *mdm2*-directed fluorescence at the ventricular base at 4 wpf, and expanding on the surface muscle by 7 wpf. (C) Cartoon showing co-localization of *mdm2* and *gata4* domains in emerging and proliferating juvenile CMs of the cortical wall. (D) *mdm2:EGFP* and *gata4:DsRed* fluorescence are co-activated in the ventricular walls of control (left) and *cmlc2:CreER;*  $\beta$ -act2:BSNrg1 (right) ventricles by 14 days of tamoxifen-induced overexpression. Dashed lines delineate cortical from trabecular muscle. Scale bars 100 µm.

#### mdm2:EGFP



## Figure 12: Expression of *mdm2*-directed EGFP and embryonic myosin heavy chain during heart regeneration.

Immunofluorescence staining for EGFP and embMHC at 7 dpa indicates co-expression but not co-localization of the two markers. Dashed line indicates approximate injury plane. Right panels are high-mag views of box. Scale bars: 50 µm.

## 2.3 Discussion

Tp53 has been widely studied in cultured cells and live model systems, where it regulates fundamental biological processes like apoptosis, DNA repair, senescence and cell proliferation in myriad contexts. Here we report transient Tp53 suppression as a regulatory event that underlies and contributes to the strong proliferative response of adult zebrafish CMs in response to injury. Our findings indicate a mechanism in which mitogens that are present in cardiac injury sites activate myocardial expression of the negative Tp53 regulator Mdm2, which transiently reduces Tp53 levels and facilitates cell cycle entry (Figure 5).

Tp53 has been implicated in multiple settings relevant to heart regeneration, most notably the regeneration of amputated limbs in salamanders (Yun et al., 2013). During limb regeneration, Tp53 protein levels are transiently suppressed early as the regeneration blastema forms, before recovering as new tissue is patterned. Moreover, pharmacological modulation of Tp53 activity reduced the ability of limb cells to form a blastema and differentiate properly, with the authors ultimately proposing that Tp53 suppression is key for post-mitotic limb cells to re-enter the cell cycle and contribute to blastema formation (Yun et al., 2013). A direct relationship between Tp53 levels and cellular differentiation was also reported more than a decade ago in the context of cellular reprogramming, when *Tp53* mutant mouse fibroblasts were found to be enhanced in their capacity to undergo conversion to pluripotent cells by reprogramming factors (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009; Utikal et al., 2009). Furthermore, human somatic cells have been shown to more readily undergo reprogramming upon acquisition of mutations in *TP53* (Merkle et al., 2017). Our findings on Tp53 involvement in zebrafish heart regeneration parallel these findings, as Tp53 suppression marked by *mdm2* expression closely associates with markers of cardiomyocyte dedifferentiation and division after injury. Thus, evidence is building toward a model in which Tp53 suppression is a broad mechanism to promote dedifferentiation across many species and contexts.

There are many potential regulators of Tp53 expression or activity present in cardiac injury sites, including macromolecular damage, inflammation, and hypoxia. Each of these stimuli would be predicted to increase Tp53 levels and promote cell cycle arrest or apoptosis based on the literature (Helton and Chen, 2007; Munoz-Fontela et al., 2016; Williams and Schumacher, 2016). We speculate that an innate ability to counter this effect and reduce Tp53 levels, for instance by enhancing Mdm2 presence in response to injury, can facilitate division of source cells to an extent that it outcompetes scarring. Importantly, however, while injury itself might constitute a regulatory influence on Tp53 levels, we also show that mitogens themselves induce *mdm2* expression and repress the Tp53 network. In fact, assessment of multiple cardiogenic contexts during heart

development and regeneration revealed *mdm*<sup>2</sup> expression spatiotemporally localized with the areas of most active cardiogenesis. Mdm<sup>2</sup> has pro-oncogenic effects that include Tp53 regulation but also other mechanisms (Marine and Lozano, 2010), and it is possible that induction of *mdm*<sup>2</sup> expression and the suppression of its targets that facilitate dedifferentiation during heart or limb regeneration is a vestige of the regulation that supports mammalian tumorigenic dedifferentiation and cell division. Identification of the transcription factors and regulatory elements that place *mdm*<sup>2</sup> expression under the control of mitogens can help illuminate how defining events in regeneration that involve dedifferentiation occur naturally without the threat of tumorigenesis.

Our findings together with those of others suggest that Tp53 occupies an important position in the transition of cell differentiation states, where it can play a critical role during innate regeneration. As suppression of Tp53 can augment CM proliferation following injury or growth factor stimulation, we postulate that applications to safely and transiently reduce Tp53 in CMs of MI patients might help boost CM proliferation and provoke clinically significant regeneration.

## 2.4 Experimental procedures

**Zebrafish.** Wild-type or transgenic zebrafish of the EK/AB strain were used for all experiments. *β-actin2:loxp-mCherry-STOP-loxp-DTA<sup>pd36</sup>* (Wang et al., 2011), *β-actin2:loxp-mTagBFP-STOP-loxp-nrg1<sup>pd107</sup>* (Gemberling et al., 2015), *β-actin2:loxp-mTagBFP-STOP-loxp-vegfaa<sup>pd262</sup>* (Karra et al., 2018), *cmlc2:CreER<sup>pd10</sup>* (Kikuchi et al., 2010), *gata4:EGFP* (Heicklen-Klein and Evans, 2004), *gata4:dsRed2<sup>pd28</sup>* (Karra et al., 2015), *tp53<sup>M214K</sup>* (Berghmans et al., 2005) transgenic mutant or fish have been previously described. Resection of ~20% of the cardiac ventricular apex was performed as described previously (Poss et al., 2002). To induce expression of *nrg1* or *vegfaa* in

CMs, adult *cmlc2:CreER;*  $\beta$ -act2:BS-nrg1 or  $\beta$ -actin2:loxp-mTagBFP-STOP-loxp-vegfaa zebrafish were treated for 24 hours with 5  $\mu$ M tamoxifen. To induce recombination in adult *cmlc2:CreER*;  $\beta$ -act2:BS-m2DN fish, animals were bathed 3 sequential days in 5  $\mu$ M 4-HT (Sigma) for 10-12 hours. Heart injuries were performed 3 days after the final 4-HT treatment. To induce ablation of CMs, adult *ZCAT* animals were treated for 24 hours with 0.5 - 1.0  $\mu$ M tamoxifen titrated to ablation of ~50% CMs (Wang et al., 2011). For EdU-incorporation experiments 10 mM EdU (Sigma) was injected intraperitoneally once daily for 3 days before collection. Procedures involving animals were approved by the Institutional Animal Care and Use Committee at Duke University.

**Ingenuity pathway and gene ontology analyses.** Pathway analysis of was performed using Ingenuity Pathway Analysis (IPA) software on the following published datasets: ventricle 7 dpi vs sham GSE75894 (Kang et al., 2016), ventricle 14 dpi vs sham GSE81865 (Goldman et al., 2017). Mouse orthologues of differentially expressed genes with p < 0.01 and -1 <log2FC <1, retrieved via www.ensembl.org/biomart/martservice, were analyzed to find upstream regulators and pathways showing differential modulation upon injury. IPA analyses were performed with the following settings: Expression Value Type (Exp Log Ration), Reference set (Ingenuity Knowledge Base), Relationships to consider (Direct and Indirect Relationships), Interaction networks (70 molecules/network; 25 networks/analysis or 30 molecules/network; 25 or 10 network/analysis), Data Source (all), Confidence (Experimentally Observed), Species (Human, Mouse, Rat), Tissue & Cell Lines (all), Mutations (all). Gene Ontology analyses were performed with The PANTHER database (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org).

**RNA extraction and sequencing.** Zebrafish ventricles were collected and placed in cold PBS while still pumping to help decrease intra-ventricular blood. Atrium and outflow tract were removed and ventricles (5 per sample) were homogenized in Trizol using a Tissue Lyser II (QIAGEN). RNA was extracted using the standard Trizol protocol, genomic DNA removed using RNA clean and Concentrator Kit (Zymo Research) and processed for preparation of libraries. Single-end 50-bp sequencing, with 30 million reads/sample was performed at BGI. RNA-Seq reads were trimmed by Trim Galore (0.6.4, with -q 15) and then mapped with TopHat (v 2.1.1, with parameters --b2-very-sensitive --no-coverage-search and supplying the UCSC danRer10 refSeq gene annotation). Gene-level read counts were obtained using the htseq-count (v1.6.1) by the reads with MAPQ greater than 30. DESeq2 (v 1.26.0) was employed for differential expression analysis. The RNA-Seq data generated in this study have been deposited in the NCBI GEO database under accession number GSE146859.

**Western blotting.** Zebrafish ventricles were collected and placed in cold PBS while still pumping to help decrease intra-ventricular blood. Atrium and outflow tract were removed and ventricles (8-10 per sample) were homogenized in RIPA buffer containing Proteinase and Phosphatase inhibitor (Thermo Fisher#78442) and Phenylmethanesulfonyl fluoride solution (Sigma #93482). Samples were denatured at 95°C for 5 min, quantified and tissue lysates were analyzed on Mini-Protein tetra cell (Bio-Rad) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in Tris/glycine/SDS buffer. After electrophoresis proteins were were transferred to a PVDF membrane using the Mini-Protein tetra cell in Tris/glycine buffer (v/v). Membranes were blocked for 1 h at room temperature using 5% BSA in Tris-buffered saline and Tween-20 (TBST), then were incubated with primary anti-Tp53 antibody (GTX128135, 1:500) and anti-GAPDH (Proteintech 60004-1-IG, 1:500) overnight at 4°C. Membranes

were incubated with appropriate HRP-conjugated secondary antibodies (Thermo Fisher Scientific), washed in TBST and developed with Pierce ECL western blotting substrate. WB were quantified as explained in (H, 2017).

*βact2:BS-dnMdm2-2A-mCherry* zebrafish line generation. A 2A-mCherry fragment was amplified from a *g4DN* template described previously (Gupta et al., 2013a), generated using the primer sequences 2A-mCherry forward: CCGGCGCGCCT GCTACGAACTTCTCTCTGTTAAAGCAAGCAGGGGACGTGGAAGAAAACCCTGGTCC TATGGTGAGCAAGGGCGAGGAGGACAAC; 2A-mCherry reverse: GATATCGCGGCCGCTTACTTGTACAGCTCGTCCATGCC. The PCR product was ligated into the Ascl/Notl site of the *β-act2:loxP-TagBFP-STOP-lox-P*vector (Gupta et al., 2013a). *Mdm2* cDNA lacking the C-terminal RING finger domain was amplified using the primer sequences *mdm2* forward: ATGGCAACAGAGAGTTGTTTAAGCAG; *mdm2* dominant negative reverse: GCACGTAGCGGGAAGGC. The PCR product was ligated into the Agel/Ascl site of the *β-act2:loxP-TagBFP-STOP-lox-P2A-mCherry*. The full name of this transgenic line is *Tg(β-actin2:loxP-mTagBFP-STOP-loxP-loxP-dnmdm2)<sup>pd313</sup>*.

*mdm2:EGFP* zebrafish line generation. The translational start codon of *mdm2* in the BAC clone CH211-209G11 (BACPAC Resources Center) was replaced with the EGFP sequence by Red/ET recombineering technology (GeneBridges) as described in reference (Kikuchi et al., 2011b). The entire construct was flanked with I-Scel sites allowing meganuclease mediated transgenesis as previously described (Thermes et al., 2002). After purifying the final BAC with nucleobond BAC 100 kit (Clontech) the BAC was co-injected along with I-Scel into one-cell embryos. A single founder was isolated and propogated. The full name of this transgenic line is *TgBAC(mdm2:EGFP)*<sup>pd312</sup>.

*mdm2*<sup>*pd314*</sup> mutants were generated using injection of CRISPR/Cas9 and gRNAs at the one-cell stage as previously described (Tornini et al., 2017). Exon 3 was targeted using oligo 5'-

gcgTAATACGACTCACTATA<u>GG</u>GCAATTGAAAAGCCTGTTAGAGGGTTTTAGAGCTA GAAATAGC-3' (target sequence underlined). Intron 3 was targeted using oligo 5'gcgTAATACGACTCACTATA<u>GG</u>GGGTGGGGTTTTACAACAACAGGGTTTTAGAGCTA GAAATAGC-3' (target sequence underlined) resulting in a 1.7 kb deletion. Deletion lines were genotyped using primers: mdm2\_KO\_for 5'- GCAGTTCTCAGATCAGCAAGGTTG-3'; mdm2\_prom\_rev 5'- GCTGAAAAGTGACCTTCGCCATC-3'; and mdm2\_geno\_rev 5'-GGCAAATATCCAGATAGTGCCACC-3'. Wild-type embryos yield an expected amplicon of 455 bp, with homozygous mutants containing a single 180 bp amplicon. Heterozygotes produce 455 bp and 180 bp amplicons.

Histological analysis and imaging. Primary and secondary antibody staining for immunofluorescence was performed as described in (Kikuchi et al., 2011b). Antibodies used in this study were anti-troponin T (mouse; Neomarkers, MS-295-PABX), anti-Mef2 (rabbit; Abcam) at 1:100, anti-EGFP (rabbit; Abcam) at 1:200, Alexa Fluor 488 (rabbit; Life Technologies) at 1:200, Alexa Fluor 594 (mouse and rabbit; Life Technologies) at 1:200. Confocal imaging was performed using a Zeiss LSM 700 or a Zeiss LSM 880 microscope. Tissue for CM proliferation were placed immediately into ice-cold 30% sucrose in PBS before being transferred to cold TFM (VWR). Hearts were flash frozen in dry ice ethanol bath. After sectioning hearts were fixed for 15 min with 3.7% formaldehyde (Sigma). Sections were stained for EdU, washed with PBS + 0.2% Triton (Sigma).Staining for Mef2, imaging, and counting were performed as previously described (Kikuchi et al., 2011b). Hearts for whole mount imaging were collected and fixed overnight in 4% paraformaldehyde at 4°C and imaged as previously described

(Gupta et al., 2013a).

*mdm2* mRNA injections. Dominant negative *mdm2* cDNA was transcribed and subcloned into the multicloning site of a pCS2<sup>+</sup> vector containing an SP6 promotor and poly-A tail using ClaI and EcoRIrestriction sites. mRNA was generated using the SP6 mMachine mMessage kit(Ambion) and 200 ng/uL was injected into single-cell embryos as described (Rosen et al., 2009).

# 3. HB-EGF, an Erbb ligand, is dynamically expressed in multiple regeneration contexts

## 3.1 Summary

Multiple studies investigating Erbb2 and its agonist, Nrg1, have shown farranging effects on cardiac development, CM survival, metabolism, angiogenesis, and myofiber structure in addition to CM proliferation (D'Uva et al., 2015; Parodi and Kuhn, 2014). Despite the understandable focus on Nrg1-Erbb2 signaling, additional Erbb2 agonists such as Heparin-Binding Epidermal Growth Factor (HB-EGF) are present in both zebrafish and mammalian hearts (Iwamoto et al., 2003). Mammalian studies investigating HB-EGF signaling in cardiac development have demonstrated its requirement, specifically in valve formation (Iwamoto et al., 2010; Iwamoto et al., 2003; Nanba et al., 2006). Additional *in vitro* work has implicated HB-EGF as possessing potential cardiomyocyte mitogenic properties (Ieda et al., 2009). Despite these intriguing studies, critical gaps exist in our understanding of HB-EGF signaling in adult animals that prevent the exploitation of this information to maximally affect cardiac regeneration.

In this study we, for the first time, demonstrate transcriptional activation of both paralogues of HB-EGF present in zebrafish following ventricular injury. Generation of HB-EGF double knock-out zebrafish reveal developmental viability along with unaffected growth, maturation, and fertility. Early studies investigating cardiac regenerative potential in the absence of HB-EGF suggest a potential critical role for HB-EGF signaling during zebrafish heart regeneration. Finally, evaluating HB-EGF transcriptional activation during spinal cord regeneration demonstrates significant HB-EGF activation within multiple cell types. These data suggest that HB-EGF signaling participates during zebrafish organ regeneration across multiple contexts.

## 3.2 Results and discussion

#### 3.2.1 HB-EGF transcription is activated following cardiac injury

Transcriptome sequencing indicates low *hbegfa/b* mRNA levels in uninjured zebrafish hearts (Kang et al., 2016). Interestingly, a significant increase in both paralogues of HB-EGF (hbegfa and hbegfb) transcripts are noted at 7 days following genetic ablation of ~ 50% of CMs correlating with the time of greatest CM proliferation (Wang et al., 2011). hbegfa mRNA levels are elevated relative to hbegfb in the uninjured heart, however, like *hbegfb*, are increased following CM ablation. In situ hybridization performed with anti-sense RNA probe against Hbegfa/b confirm that both isoforms are expressed in the heart and are dynamic following cardiac injury (Figure 13). To localize the cellular sources of *hbegf*, we generated an *hbegfb<sup>GFP</sup>* knock-in reporter line (Figure 15). Imaging of the *hbegfb<sup>GFP</sup>* reporter demonstrates a lack of significant expression in the absence of injury. Following injury there is a significant increase in expression in cardiomyocytes near the injury by 7 dpa. Interestingly, GFP expression is noted within the clot in what remains an unidentified cell type. The GFP expression notably resolves over the regenerative course and is decreased considerably by 14 dpa (Figure 16). In addition to this reporter, we generated an hbegfa BAC transgenic construct with EGFP replacing the first exon of HB-EGF to better visualize *hbegfa* expression dynamics. This BAC contains approximately 67kb of genetic information upstream of the start codon in addition to nearly 30kb of downstream sequence and therefore should accurately reflect Hbegfa transcriptional activity (Figure 14). Experiments investigating the expression of hbegfa using the novel hbegfa:EGFP BAC reporter are currently in progress.



Figure 13: *hbegfa* and *hbegfb* are induced following cardiac amputation injury. In situ hybridization for *hbegfa* (A) expression (violet) and *hbegfb* (B) in sections of uninjured and 7-day-post resection (7 dpa) ventricles.



**Figure 14: Generation and characterization of an** *hbegfa:***EGFP reporter line.** (**A**) Cartoon showing the *hbegfa:*EGFP BAC transgene. (**B**) Larval *hbegfa-*directed fluorescence, shown at 3 days post fertilization (dpf).









## Figure 16: *hbegfb<sup>GFP</sup>* expression is induced in border zone CMs and cells present within clot.

(A) Images showing *hbegfb*-directed EGFP at different time points after resection of the ventricular apex. Tnnt marks CMs. Dashed lines approximate resection plane.

## **3.2.2 HB-EGF transcription is activated following zebrafish spinal cord injury**

In addition to work done on HB-EGF in cardiac development and regeneration,

multiple studies have evaluated its role in nervous system development and

regeneration. HB-EGF has long been known to be expressed throughout the nervous

system where, among other functions, contributes to neuronal survival along with glial

cell proliferation (Ayuso-Sacido et al., 2010; Hayase et al., 1998; Kornblum et al., 1999).

Previous work in zebrafish identified HB-EGFa as necessary for muller glia dedifferentiation during retinal regeneration (Wan et al., 2012). Like the zebrafish heart, the adult zebrafish is capable of meaningful spinal cord regeneration following complete spinal cord transection (Mokalled et al., 2016). With these known functions of HB-EGF in mind we sought to investigate the dynamics of HB-EGF expression during adult zebrafish spinal cord regeneration. For both *hbegfa* and *hbegfb* there was no evidence of expression in the absence of injury. However, by 1 week after spinal cord transection there was a dramatic increase in *hbegfa* expression at the site of injury. Co-staining with the neuronal marker GFAP demonstrated significant overlap indicating a neuronal source of the expressed *hbegfa* (Figure 17A). Despite the increase in *hbegfa*, *hbegfb* expression remained largely unchanged with no significant increase following spinal cord injury (Figure 17B). Further work characterizing the expression pattern of *hbegfa* during spinal cord regeneration is ongoing.





## 3.2.3 HB-EGF double knockout mutants are viable

Α

The known importance of HB-EGF during development and in a variety of

biologic contexts combined with our demonstration of its dynamic expression during

zebrafish heart and spinal cord regeneration suggest that further investigation is

warranted to characterize its functions and requirements during regeneration. Therefore,

using CRISPR/Cas9 gene editing we generated mutants for both HB-EGFa and HB-

EGFb. These deletions were engineered to remove significant portions of the coding

sequence of each in addition to removing the well-conserved EGFR binding domains

located near the C-terminus in both paralogs (Figure 18). Individually, HB-EGFa<sup>-/-</sup> and HB-EGFb<sup>-/-</sup> mutants were found to develop normally and display intact fertility. We therefore crossed fish heterozygous for both mutations with one another. Mutants with deletions of both paralogs, HB-EGFa<sup>-/-</sup>; HB-EGFb<sup>-/-</sup> along with their wild-type clutch mates, HB-EGFa<sup>+/+;</sup> HB-EGFb<sup>+/+</sup> were selected and found to develop into adulthood without obvious abnormalities.

We next chose to perform heart resections on adult zebrafish comparing the 30 dpa regenerative capacity of the HB-EGF double mutants to their wild-type clutch mates. Interestingly, all wild-type and HB-EGF double mutants showed minor regeneration defects with small amounts of residual fibrin and collagen by 30 dpa (n = 12, 10; Fig. 19). However, 3/10 double mutants failed to regenerate with formation of an unusually large aneurysmal scar (Figure 19). This finding was notably not seen in any of the wild type controls and we are continuing to explore this finding with ongoing experiments.



#### Figure 18: Generation of HB-EGFa and HB-EGFb knockout lines.

(A) CRISPR sgRNAs were directed towards the 5' UTR and Exon 4 to delete nearly all of the coding sequence of HB-EGFa. (B) CRISPR sgRNAs directed towards Exon 3 and Intron 4 result in significant deletion of HB-EGFb coding region including conserved Erbb binding domain located in Exon 4.



#### Figure 19: Heart amputations of HB-EGF double knock-out mutants.

(A) 10x magnification demonstrates uniquely large aneurysmal scar present at 30 dpa on 3/10 dKO mutants. (B) 20x magnification of image (A) upper panel compared to non-regenerating WT heart at 30 dpa highlights size difference of scar between dKO and WT. (C) AFOG staining of panel (B) demonstrates presence of massive collagen-poor scar in dKO (upper panel) compared to smaller collagen-rich scar in WT (lower panel).

## 3.2.4 HB-EGF overexpression lines

Our work using a Nrg1 overexpression line of zebrafish demonstrated that Nrg1 alone, when expressed at high levels in cardiomyocytes, was capable of promoting significant cardiomyocyte proliferation (Gemberling et al., 2015). We therefore felt that investigations into the effect of HB-EGF overexpression were critical to develop our understanding of HB-EGF. To further investigate this, lines allowing for the inducible overexpression of proHB-EGF and sHB-EGF were generated in a manner similar to the Nrg1 overexpression. The cDNA for either proteins were placed under the control of a beta –actin promoter and a stop codon flanked by LoxP sites. This, when combined with a cardiomyocyte-specific *Cre-recombinase* (*cmlc2:CreER*) and treated with tamoxifen allows for the overexpression of the downstream transcript. Evaluation of both proHB-EGF and sHB-EGF overexpression will be particularly interesting. Work has previously

demonstrated critical biologic differences between the two molecules and their independent overexpression may help to provide interesting insights helping to guide future investigations. Work would be aimed at evaluating cardiomyocyte proliferation, but we would also investigate changes in the proliferation of the endocardium and epicardium in addition to vasculature density, recruitment of inflammatory cells and fibroblasts, along with increases and decreases in fibrosis. Initial work has demonstrated no obvious changes in cell proliferation with the overexpression of proHB-EGF however, marked increases in proliferation is observed with the overexpression of sHB-EGF. These results are preliminary and further work is required to identify the identity of the proliferation cells and the effects that overexpression has during regeneration.

## 3.3 Discussion

HB-EGF has been intermittently studied for its roles in cardiac development and post-myocardial infarction. These investigations have largely provided contradictory evidence positioning HB-EGF as a cardiac mitogen, proliferation inhibitory signal, and pro-fibrosis molecule (Asakura et al., 2002; Ayuso-Sacido et al., 2010; Higashiyama et al., 1991; Ito et al., 1994; Iwamoto and Mekada, 2000; Iwamoto et al., 2003). These likely context-dependent functions of HB-EGF provided an intriguing foundation and basis for our investigations into HB-EGF and its function during adult zebrafish heart and spinal cord regeneration.

Our studies using *in situ hybridization* along with novel HB-EGF reporter lines suggest that *hbegf* is strongly induced following both heart and spinal cord injury. Moreover, its peak expression, approximately 7 dpa, is associated with the time at which maximum cardiac regeneration is observed. These observations combined with the known importance of HB-EGF in developmental contexts provide an exciting basis for further investigation with genetic deletion to determine the function and necessity of HB-EGF during these regenerative contexts.

Genetic deletion and generation of HB-EGF mutants revealed no evidence of developmental lethality or the developmental phenotypes that have been demonstrated in mammalian hearts. Specifically, there was no observed incidence of dilated cardiomyopathy or valve defects in adult HB-EGF mutants. Unlike its mammalian homologues, the zebrafish is known to have undergone a genetic duplication event resulting in two paralogues of HB-EGF. To eliminate the potential that these paralogs effectively compensate for each thus eliminating the appearance of the above phenotypes we generated double knockouts with mutants in both *hbegfa* and *hbegfb*. Like their single mutant counterparts, these double knockout mutants demonstrated normal development. While it is possible that compensation from additional Erbb ligands such as Nrg1 provide the signaling required for normal development, it is more likely that HB-EGF is dispensable, despite being expressed, during development (Figures 14 and 15). During adult zebrafish cardiac regeneration it is unknown if the observation of the large aneurysmal collagen-poor scar represents a true regeneration defect and phenotype or if it is simply an artifact as it was only present in 30% of the observed hearts. Nevertheless, its presence is unusual and was absent in all of the WT clutch mates in a well-controlled blinded experiment. It is possible that its presence in a relatively small number of the observed hearts is related to the injury size with larger injuries resulting in a predisposition towards the formation of this phenotype, and smaller injuries being protected. Indeed, this phenotype has been observed previously in cryoablated zebrafish hearts treated with a TGF $\beta$ /Activin pathway inhibitor (Chablais and Jazwinska, 2012). As cryoablation is known to generate a significantly larger injury than amputation this may explain the phenotypic difference. Additionally, the similarities

between these two phenotypes provides an interesting potential connection between HB-EGF and TGFβ/Activin signaling during zebrafish heart regeneration. Further work will be required to investigate these potential relationships including investigating the regeneration phenotype of the HB-EGF double mutants in the setting of cryoablation.

Like the injury-induced expression of *hbegfa* in heart regeneration the expression of *hbegfa* after spinal cord injury is an intriguing finding. Current work utilizing the HB-EGF double mutant is investigating the function of HB-EGF during spinal cord regeneration by investigating histologic evidence of spinal cord regeneration in addition to functional regeneration capacity as measured by swim endurance as previously described (Mokalled et al., 2016).

Further work is required to adequately characterize HB-EGF and its contribution to heart and spinal cord regeneration. Similar to our work on Nrg1, lines allowing for the overexpression of proHB-EGF and sHB-EGF in cardiomyocytes are currently under development. These will allow for novel observations and provide further insights into HB-EGF and regenerative biology. The net output of all of these novel lines will, regardless of the results, contribute an important piece to our understanding of heart and spinal cord regeneration.

## 3.4 Experimental procedures

**Zebrafish.** Wild-type or transgenic zebrafish of the EK/AB strain were used for all experiments. Resection of ~20% of the cardiac ventricular apex was performed as described previously (Poss et al., 2002). Spinal cord injuries were performed as previously described (Mokalled et al., 2016). Procedures involving animals were approved by the Institutional Animal Care and Use Committee at Duke University.

Histological analysis and imaging. Primary and secondary antibody staining for

immunofluorescence was performed as described in (Kikuchi et al., 2011b). Antibodies used in this study were anti-Myosin heavy chain (F59, mouse; Developmental Studies Hybridoma Bank) at 1:50, anti-troponin T (mouse; Thermo) at 1:100, anti-EGFP (rabbit; Abcam) at 1:200, Alexa Fluor 488 (rabbit; Life Technologies) at 1:200, Alexa Fluor 594 (mouse and rabbit; Life Technologies) at 1:200, and anti-GFAP (Sigma, G9269,1:1000). Acid Fuschsin-Orange G staining and immunofluorescence was performed on 10 mm cyrosections as described (Poss et al., 2002). Confocal imaging was performed using a Zeiss LSM 700 or a Zeiss LSM 880 microscope. Hearts for whole mount imaging were collected and fixed overnight in 4% paraformaldehyde at 4°C and imaged as previously described (Gupta et al., 2013a). Spinal cords were collected, fixed, and imaged as previously described (Mokalled et al., 2016).

*hbegfa:EGFP* zebrafish line creation. The translational start codon of *hbegfa* in the BAC clone CH73-269I13 (BACPAC Resources Center) was replaced with the EGFP sequence by Red/ET recombineering technology (GeneBridges) as described in reference (Kikuchi et al., 2011b). The entire construct was flanked with I-Scel sites allowing meganuclease mediated transgenesis as previously described (Thermes et al., 2002). After purifying the final BAC with nucleobond BAC 100 kit (Clontech) the BAC was co-injected along with I-Scel into one-cell embryos. A single founder was isolated and propogated. The full name of this transgenic line is *TgBAC(hbegfa:EGFP*).

*hbegfb*<sup>GFP</sup> line creation. This novel line of zebrafish was kindly shared by Yanchao Han, PhD. These were generated using TALEN-directed knockin and PhiC31 mediated recombination. Briefly, a pair of TALEN mRNAs were transcribed in vitro and injected to zebrafish embryos to generate double strand breaks at the ATG region of hbegfb, together with a single strand oligo composed of attP sequences and flanking

homologous sequences of the TALEN cut site. Stable attP knocking-in F1 zebrafish were inter-crossed and F2 embryos were injected with PhiC310 mRNA, flpase mRNA as well as a donor plasmid containing attB, GFP-SV40 polyA, and two FRT sites flanking the vector sequences. Finally, stable *hbegfb*<sup>GFP</sup> zebrafish were isolated based on GFP expression and sequenced to ensure correct integration.

*hbegfa* line generation. mutants were generated using injection of CRISPR/Cas9 and gRNAs at the one-cell stage as previously described (Tornini et al., 2017). The 5'-UTR was targeted using oligo 5'-

gcgTAATACGACTCACTATA<u>GG</u>TGGCCACGTTCATATTTAAGCGG GTTTTAGAGCTAGAAATAGC-3' (target sequence underlined). Exon 4 was targeted using oligo 5'-gcgTAATACGACTCACTATA<u>GG</u>AGCCCTTGCTGTGGTAGCTGTGG GTTTTAGAGCTAGAAATAGC-3' (target sequence underlined) resulting in a 1.5 kb deletion. Deletion lines were genotyped using primers: hbegf\_KO\_for2 5'-GCAGGTAACCATACCAGGGATAAAAGG-3'; hbegf\_KOgeno\_rev1 5'-GGTAAAGACGAAAAGACGCAAGACTG-3'; and hbegf\_KO\_rev1 5'-CAGGAGGAGGCCAATGATGG-3'. Wild-type embryos yield an expected amplicon of 322 bp, with homozygous mutants containing a single 130 bp amplicon. Heterozygotes

*hbegfb* line generation. mutants were generated using injection of CRISPR/Cas9 and gRNAs at the one-cell stage as previously described (Tornini et al., 2017). Exon 3 was targeted using oligo 5'-gcgTAATACGACTCACTATA<u>GG</u>ACGGATGTTTGGGTTTGG GTTTTAGAGCTAGAAATAGC -3' (target sequence underlined). Intron 4 was targeted using oligo 5'-gcgTAATACGACTCACTATA<u>GG</u>CTATGACCGCCAGCGCTG GTTTTAGAGCTAGAAATAGC-3' (target sequence underlined) resulting in a 2.4 kb

produce 322 bp and 130 bp amplicons.

deletion. Deletion lines were genotyped using primers: hbegfb\_KO\_for1 5'-GCACTGACATCACTCTTGCTCAAC-3'; hbegfb\_KO\_SeqRev2 5'-CCATGAATGCAGAAATCTTTGTATTCCTCC-3'; and hbegfb\_KO\_rev1 5'-GCCTCGAGTTTGACCTTTTCTTCG-3'. Wild-type embryos yield an expected amplicon of 171 bp, with homozygous mutants containing a single 270 bp amplicon. Heterozygotes produce 171 bp and 270 bp amplicons.

*β-act2:BS-proHBEGF-2A-mCherry* line generation. A 2A-mCherry fragment was amplified from a *g4DN* template described previously (Gupta et al., 2013a), generated using the primer sequences 2A-mCherry forward: CCGGCGCGCCT

GCTACGAACTTCTCTCTGTTAAAGCAAGCAGGGGACGTGGAAGAAAACCCTGGTCC TATGGTGAGCAAGGGCGAGGAGGACAAC; 2A-mCherry reverse:

GATATCGCGGCCGCTTACTTGTACAGCTCGTCCATGCC. The PCR product was ligated into the Ascl/Notl site of the  $\beta$ -act2:loxP-TagBFP-STOP-lox-Pvector (Gupta et al., 2013a). Full length *hbegf* cDNA was amplified using the primer sequences proHBEGF forward: ATGTCTTGGACTATCCCAAAGAAAAGG; proHBEGF reverse:

CTGTTTTACTGACGTTGCCTCGAG. The PCR product was ligated into the Agel/Ascl site of the  $\beta$ -act2:loxP-TagBFP-STOP-lox-P2A-mCherry. The full name of this transgenic line is  $Tg(\beta$ -actin2:loxP-mTagBFP-STOP- loxP-proHBEGF-P2A-mCherry).

*β-act2:BS-sHBEGF* line generation. *hbegf* cDNA truncated at the transmembrane domain was amplified using the primer sequences sHBEGF forward:

ATGAACTTTTTAACAGTCTTGCGTCTTTTCG; sHBEGF reverse:

GGGGCGGCCGCTCA CAGAGAGAAATCGTGACATCGCTC. The PCR product was ligated into the Agel/Notl site of the  $\beta$ -act2:loxP-TagBFP-STOP-lox- Nrg1. The full name of this transgenic line is  $Tg(\beta$ -actin2:loxP-mTagBFP-STOP- loxP-sHBEGF).

## 4. Discussion

Meaningful cardiac regeneration in human hearts remains an elusive goal despite an increasing body of work that demonstrates that a variety of ligands, receptors, intracellular signaling molecules, and extracellular matrix components all possess the capacity to boost cardiomyocyte proliferation in mammals such as mice following cardiac injury (Bassat et al., 2017; D'Uva et al., 2015; Gemberling et al., 2015; Karra et al., 2018; Monroe et al., 2019; Morikawa et al., 2017). The work in this thesis works to broaden this expanding knowledge of the potential ligands and mitogens involved in heart regeneration while attempting to focus the collective efforts spanning the field of cardiomyocyte regeneration through the effects of downstream signaling via mdm2/p53. Here we demonstrate that multiple mitogens, signaling through unique receptors, increase mdm2 expression with concomitant decrease in p53 as cardiomyocytes enter proliferation. Moreover, natural and mitogen-stimulated proliferation of cardiomyocytes is significantly augmented by the loss of p53 in zebrafish. The further co-localization of mdm2 and gata4 expression in dividing cardiomyocytes suggests that these two molecules are linked and together play important roles in the differentiation

Our work on HB-EGF in the setting of both heart and spinal cord regeneration demonstrates dynamic *hbegf* expression in both contexts. HB-EGF's potential to affect either of these processes is compelling particularly when combined with our preliminary heart regeneration data using the novel HB-EGF double knockout mutants. These reporters along with their corresponding mutants position these reagents to uncover new and potentially exciting insights into signaling that underlies successful heart and spinal cord regeneration.

As I move forward with this work a multitude of questions remain that deserve investigation. For example, how are p53 and mdm2 related to cardiomyocyte cell

differentiation and what other molecules do they interact with? Are these processes conserved in mammalian systems and can p53 suppression be used to increase cardiomyocyte proliferation after cardiac ischemia? What are the effects of HB-EGF overexpression on cardiomyocyte proliferation and does the overexpression of sHB-EGF compared to proHB-EGF result in different outcomes?

## 4.1 p53 signaling and cardiomyocyte regeneration

## 4.1.1 How do p53 and mdm2 drive dedifferentiation

Perhaps the most intriguing finding in our work on mdm2 and p53 is the close association between these and the marker of less differentiated cardiomyocytes, Gata4. This association was extremely tight not only after injury, but also during the corticalization phase of heart development and with Nrg1 stimulation suggesting that these signaling processes are somehow linked. Unfortunately, we currently know little more than this. Do p53 and mdm2 drive differentiation, and if so, how? Does mdm2 have binding partners in addition to p53 that allow for a direct role in cell differentiation and what is controlling mdm2 during this process? These are all intriguing questions and the technology is currently available to begin rigorous *in vivo* investigations. Previous work in the Poss lab has demonstrated the feasibility of using streptavidin-based enrichment to isolate macromolecules that are associated with a specific protein in a cardiomyocytespecific manner (Goldman et al., 2017). For our purposes we would engineer a zebrafish that expresses both an Mdm2 or p53 protein that has been tagged with a biotin-ligase recognition protein (BLRP) and FLAG tag along with a cardiomyocyte-specific biotin ligase (BirA), cmlc2:BirA. This would allow for us to enrich proteins from cardiomyocytes that are bound to Mdm2 and p53 during periods of cardiomyocyte proliferation: development, regeneration, and mitogen stimulation thus providing a rich database of

potential critical mediators of cardiomyocyte cell differentiation. To identify transcriptional targets of p53 during regeneration CHiP analysis could be performed. This work has a high likelihood of yielding novel insights and it is the most important next step in progressing our understanding of the roles of mdm2 and p53 in establishing cardiomyocyte cell differentiation.

#### 4.1.2 MDM2 overexpression

Our work suggests that regulation of p53 by its primary negative regulator mdm2 is a critical component of cardiomyocyte proliferation. Work in tumor biology has demonstrated that although the majorities of cancers contain a p53 mutation resulting in loss of p53 activity the overexpression of mdm2 is often found in cancers with intact p53 (Oliner et al., 1992; Oliner et al., 2016). It would therefore be interesting to evaluate if the overexpression of MDM2 in zebrafish cardiomyocytes would allow for increased cardiomyocyte proliferation. These experiments could be undertaken in multiple ways, specifically with either a constitutively expressed mdm2 driven by the cardiomyocytespecific *cmlc2*-promoter or with the tamoxifen-inducible overexpression of *mdm2* in a manner similar to previous overexpression lines that have already been created in this and other studies (Gemberling et al., 2015; Gupta et al., 2013b; Karra et al., 2018; Karra et al., 2015). While the former option lacks the elegance of the latter and exposes the cardiomyocytes to the effects of increased *mdm2* overexpression throughout the lifespan it does mitigate the complexity and pitfalls of developing functional reagents such as gene silencing, leakiness of cre recombinase, and poor recombination. As the p53 knock out line used in our work had normal rates of cardiomyocyte proliferation in the uninjured zebrafish I would expect little change in proliferation in the absence of injury. Should the overexpression of *mdm2* result in decreased levels of p53, as measured by western

blots using antibody specific for p53, there should be an increased rate of cardiomyocyte proliferation following cardiac injury. This would an additional insight and tool for understanding these signaling interactions during heart regeneration and suggest directions for therapeutic interventions in mammalian systems.

### 4.1.3 Evaluate the effects of p53 knock-out in mice

As we describe a significant increase in the rate of cardiomyocyte proliferation in zebrafish lacking functional p53 it would be important to evaluate whether this is true in other less naturally regenerative organisms. p53 has been incredibly well-studied in mice and therefore a multitude of genetically engineered mice are readily available for investigations (Ambs et al., 1998; Marino et al., 2000). The most straight forward approach to this question would be to use a p53 null mouse and perform left anterior descending artery (LAD) ligation on the adult mouse. Subsequent work would evaluate whether the p53 null has a greater rate of cardiomyocyte proliferation following LAD ligation in addition to any changes in death or scar size when compared to its wild-type clutch mates. Using the pro-regenerative neonatal mouse could also be employed. Here, following amputation of the ventricular apex, measurement of cardiomyocyte proliferation could be performed. Additionally, as neonatal mice retain natural regenerative capacity for 7 days we could examine the possibility that p53 loss could extend this regenerative capacity (Porrello et al., 2011). Additionally, as conditional p53<sup>loxP/loxP</sup> null mice have been generated these could be used in both experiments described above to evaluate cardiomyocyte proliferation with loss of p53 effecting only cardiomyocytes by using a cardiomyocyte-specific cre-recombinase. This could be of particular importance as recent work has highlighted defects in neonatal mouse cardiac regeneration with

conditional loss of p53 signaling in fibroblasts, thus the ability to target cardiomyocyte p53 could be crucial to our understanding (Sarig et al., 2019).

#### 4.1.4 Viral mediated delivery of *mdm*<sup>2</sup> to cardiomyocytes

Although the overexpression of *mdm*<sup>2</sup> or knockout of p53 might yield interesting results both of these represent significant genetic alterations to the organism and thus would not represent a feasible method for potential therapeutic use. With this is mind it would be interesting to utilize AAV-directed delivery of a construct overexpressing mdm2. This approach has been previously used successfully and allows the systemic delivery of virus with heart specific transfection and over of the construct containing the gene of interest (Prasad et al., 2011). While the delivery and overexpression of unaltered MDM2 may result in significant downregulation of p53, MDM2 is a highly regulated protein and it is possible that at multiple levels the effects of MDM2 overexpression would be mitigated by the endogenous regulatory components (Fahraeus and Olivares-Illana, 2014). We would therefore plan to genetically alter the MDM2 gene at multiple sites to reflect various post-translational modifications that have been experimentally determined in vivo to have critical effects on MDM2 and its downregulation (Carr et al., 2016; Gannon et al., 2012; Zhou et al., 2001). This is similar to genetic changes that have been used with both Erbb2 and YAP in mice to augment their pro-regenerative abilities (D'Uva et al., 2015; Monroe et al., 2019). With the systemic delivery of a virus containing a constitutively active MDM2 construct we would evaluate the ability for this novel MDM2 to suppress p53 protein and signaling within cardiomyocytes and determine whether it is able to recapitulate the augmented proliferation findings of p53 knock out we observed in zebrafish.

#### 4.1.5 Effects of Gata4 KO and overexpression on p53

Our data along with mouse data from p53 null mice suggest a relationship between p53 and *gata4* expression (Mak et al., 2017). Unfortunately, it is unknown how these two critical proteins are related to each other. Several potential relationships exist and these could be further evaluated using currently available reagents. First, it is possible that in order for cardiomyocytes to proliferate the expression of the transcription factor gata4 is induced which drives an increase mdm2 and subsequent suppression of p53. This relationship has been described previously but has not been further characterized by other work (Han et al., 2017). Using the gata4 over expression and gata4 dominant negative lines that have been generated by the Poss lab we could evaluate both *mdm2* expression and p53 protein levels with each of these scenarios (Gupta et al., 2013b; Karra et al., 2015). Additionally, using the p53 null zebrafish combined with the *gata4:eGFP* line we could evaluate if there is an increase in *gata4* expression at baseline and during regeneration. These experiments would allow insights into the relationships between these critical molecules and develop a model for further studies.

## 4.1.6 Evaluate efficiency of cell cycle entry in p53 KO

In order to further investigate Mdm2 and p53's relationship to cell differentiation and proliferation it would be interesting to evaluate the dynamics by which cells with alterations in these genes de-differentiate and begin to proliferate. These experiments would be performed by combining the p53 KO line with the *gata4:eGFP* line. Using both wild-type and p53 null zebrafish we would perform heart amputations and evaluate the expression of *gata4* as an indicator of the status of cardiomyocyte differentiation with higher levels of *gata4* expression indicating less differentiated cardiomyocytes. We
would evaluate these injured fish at an increased number of time points in an effort to capture expedited transition to a less differentiated state. Decreased cardiomyocyte differentiation could also be examined by using antibodies specific for embryonic myosin heavy chain. Emerging technology could allow for novel approaches to evaluate cardiomyocyte differentiation. In particular, single-cell RNAseq, has significant potential. Comparing p53 null and wild-type zebrafish, cells clustered based on expression of known markers such as gata4 could be evaluated in terms of the co-expression of additional known or unknown genes involved in cellular differentiation. Differences in cell percentages of cell types, timing, and differentiation status could be thoroughly evaluated between the two lines. As p53 acts primarily as a transcription factor these data could be combined with single-cell ATACseq to evaluate for alterations in chromatin accessibility. Additionally, we would compare the dynamics of EdU incorporation to examine if p53 KO hearts, while capable of increased cardiomyocyte proliferation at 7 dpa, also demonstrate evidence of earlier proliferation. If the p53 null zebrafish do in fact display a shortened time frame between injury and cardiomyocyte dedifferentiation and proliferation when compared to their wild-type clutch mates it would strongly suggest that p53 acts as a regeneration and cellular differentiation 'block' and that its presence, as seen in our work, is a barrier to

### 4.1.7 Evaluate Mdm2 and p53 in other regeneration contexts

regeneration.

Previous work has evaluated the role of p53 in salamander limb regeneration which has informed our work in zebrafish heart regeneration (Yun et al., 2013). It would be important to utilize the regenerative capacity of the zebrafish to investigate whether the relationship between p53 suppression and regeneration is maintained in additional regeneration contexts. With the *mdm2:eGFP* BAC reporter line we would plan to investigate *mdm2* expression dynamics following fin and spinal cord injuries. This would provide initial insights into mdm2/p53 and guide further inquiries. Additional work would utilize the p53 null mutants in order to understand how loss of p53 would affect these natural regeneration processes.

#### 4.1.8 HB-EGF and heart regeneration

Our initial experiments investigating the ability of the HB-EGF double knock out were inconclusive resulting in observed regenerative differences seen in only 3/10 HB-EGF double knockouts. Nevertheless, the observation encourages additional experiments. Initially, it will be important to repeat the HB-EGF double knockout injury experiment using cryablation. One potential explanation regarding the low observance of the phenotype is the size of the injury. Cryoinjury is capable of inducing a significantly larger injury than what is survivable with amputation. Therefore, a larger injury may have the effect of generating a more consistent phenotype across observed hearts. Additionally, should the HB-EGF double knock out recapitulate the observed phenotype it will be important to understand where HB-EGF signaling resides in relationship to known regeneration signaling. This could be evaluated by examining whole transcriptome RNAseq in HB-EGF dKO fish following injury compared to uninjured dKO along with appropriate WT controls.

66

### 5. Conclusion

The years that I spent as a graduate student have proven to be exciting and fruitful years in the heart regeneration field. We have seen robust evidence of the ability of mammalian cardiomyocytes to proliferate with the targeted overexpression of a range of molecules including cell cycle regulators, mitogens, and extracellular matrix proteins. Furthermore, important work has concluded, with rigorous and reproducible experiments, that cardiac progenitor cells do not contribute meaningfully to cardiomyocyte regeneration. Nevertheless, the ability to translate these developments into treatment strategies for patients with myocardial damage remains unrealized. This is undoubtedly the result of a myriad of factors. Differences between mouse and human cardiomyocytes are certainly a source. The ErbB2 receptor, the target of Nrg1, has been shown to be downregulated postnatally in the mouse helping to explain why Nrg1 has little effect in adult mice or humans. In vivo work with cardiomyocyte reprogramming has proven to be a promising area of research, however the successful reprogramming of mouse fibroblasts has proven difficult to recapitulate when using human cells. The focus of my work has therefore been an attempt to understand the most fundamental and critical aspects of the molecular signaling that are necessary to drive cardiomyocyte proliferation and regeneration. This would theoretically define novel targets that could selectively, and powerfully, drive regeneration. Whether p53 and mdm2 will ultimately fulfill these roles will remain to be determined with future studies.

Many of the demonstrations of induced cardiomyocyte proliferation have involved genetic overexpression of a gene. Additionally, the overexpressed gene is often highly modified to enhance its function. While this functions well in order to cause cardiomyocyte proliferation, this strategy lacks an 'off switch' which will be a fundamental hurdle necessary to develop a therapeutic. Work in our lab has attempted to address this by placing the genes under the control of injury responsive enhancers. This strategy would allow for the overexpression of the targeted gene to be driven by injury and inflammation, the resolution of which would result in cessation of gene overexpression.

While this may ultimately prove to be the highest hurdle, our ability to get genetic information to the cardiomyocytes remains a formidable challenge. Work in mice has demonstrated successful use of AAV-mediated viral delivery. This and similar methods of delivery will require increased investigations in humans prior to the start of clinical studies. In particular, not only will the efficiency of viral delivery be important, but the importance of minimizing off-target effects will be critical if the delivered material consist of activated genes known to be involved in oncogenesis.

These are all challenges that will be met. I am confident that with further research our abilities to deliver the molecular information necessary to drive heart regeneration in a controllable manner will develop. I hope that this work will contribute in some way to that future.

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# **Biography**

Adam Shoffner attended the University of Kansas where he graduated with highest honors with a Bachelor's of Science in Microbiology. During Adam's years at the University of Kansas he worked in the laboratory of Dr. Matthew Buechner studying *C. elegans* as a model for nephron formation in patients with polycystic kidney disease. After graduation Adam spent two years at the National Institutes of Health in the laboratory of Dr. Steven Holland investigating a newly discovered pathogenic bacterium, *Granulibacter bethesdensis.* Adam then enrolled in medical school at Yale School of Medicine. He graduated in 2012 and began his residency in General Surgery at Duke University School of Medicine shortly thereafter. In the Spring of 2020 Adam was accepted into the Cardiothoracic Surgery Fellowship at Duke.

Adam will graduate this fall with his doctorate in Cell Biology. His graduate research on cardiac mitogens and their shared signaling pathways was conducted in the laboratory of Kenneth Poss. He received an F32 from the National Institutes of Health to support his research.