ruvbl2 is a negative regulator of zebrafish cardiomyocyte proliferation

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ABSTRACT

In contrast to mammals, which are unable to regenerate their hearts, the zebrafish is capable of heart regeneration through robust proliferation of pre-existing cardiomyocytes. The mechanisms that regulate the regenerative discrepancy between zebrafish and mammals remain largely unknown. In this thesis, we explore the genetic programs that govern cardiomyocyte proliferation in zebrafish during embryogenesis and regeneration, as well as the genetic programs that influence their cardiomyocyte DNA content. First, we find that fosf2, while a critical regulator of second heart field myocardial accretion and ventricular chamber formation, is not essential for regeneration. Additionally, we demonstrate that the evolutionarily conserved innate immune receptor, c5ar1, is required for cardiomyocytes to proliferate and regenerate after injury. Furthermore, we find that experimental induction of polyploidization in zebrafish cardiomyocytes is sufficient to block regeneration after injury. Finally, we demonstrate that ruvbl2 is a negative regulator of cardiomyocyte proliferation in the zebrafish embryo. Interestingly, we find that myocardial or endocardial overexpression of ruvbl2 is sufficient to suppress cardiomyocyte proliferation, demonstrating both the autonomous and non-autonomous role of ruvbl2 in regulating cardiomyocyte proliferation during cardiac growth. After cardiac injury during adulthood, we find that cardiomyocyte-specific overexpression of ruvbl2 is sufficient to dampen the cardiomyocyte proliferative response and leads to scar formation. Taken together, these studies provide a
robust framework with which to dissect the molecular mechanisms that regulate cardiomyocyte proliferation during embryogenesis and adulthood, as well as a means to understand the role of cardiomyocyte ploidy during regeneration after injury. Collectively, these data bolster our understanding of cardiac regeneration, allowing for the development of novel therapeutic strategies to enhance proliferation of cardiomyocytes in patients after myocardial infarction.
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Cardiovascular Disease and Myocardial Infarctions: The Root of the Problem

To date, cardiovascular disease is the leading cause of deaths worldwide. Ischemic heart disease accounts for 17% of global deaths each year, and roughly one in three Americans will die annually as a result of cardiovascular disease (World Health Organization Statistics, 2018). Forty percent of deaths caused by cardiovascular disease result directly from myocardial infarctions, more commonly known as heart attacks (World Health Organization Statistics, 2018; Benjamin et al., 2019). While a myocardial infarction on its own is not always fatal, a heart attack can be deadly when it leads to heart failure, a condition that disrupts the heart’s ability to pump blood and function effectively. Roughly one-third of all myocardial infarctions lead to heart failure, and studies show that heart failure is the primary mechanism behind the morbidity and mortality rates associated with myocardial infarction (Hellermann et al., 2002; Yancy et al., 2013; McKee et al., 2009; Cahill T.J., and Khardanda, R.K. 2017).

Heart failure as a result of a myocardial infarction both structurally and functionally impairs the contractile capacity of cardiomyocytes within the heart. The damage to cardiomyocytes from a heart attack is often compounded, as individuals who suffer one myocardial infarction are subsequently at an increased risk for recurrent infarctions (Benjamin et al., 2019); World Health Organization Statistics, 2018). As heart failure and cardiomyocyte damage contribute to global deaths on such a profound scale, discovering ways to help the heart recover from myocardial infarctions has been the core focus of research endeavors within cardiovascular biology.
Clinically, a myocardial infarction is defined as the death of cardiac muscle due to prolonged ischemia, or oxygen deprivation, in the heart. When plaque within the coronary arteries ruptures, a blood clot, or thrombus, often results. This blood clot then occludes the vessels which supply blood to the heart, leading to a sustained, limited flow of oxygen supplied to cardiomyocytes (Figure 1.1). In the region of the occluded artery (the infarct zone), cardiomyocytes begin to die by necrosis and apoptosis. As many as one billion cardiomyocytes die in a single ischemic event (Laflamme and Murry, 2005). As the cardiomyocytes die, signals released at the infarct zone recruit immune cells adjacent to the occluded artery. These immune cells infiltrate the site of injury to phagocytose the dead tissue (Burke and Virmani, 2007; Goldstein et al., 2000; Saraste et al., 1997).

The body’s response to a myocardial infarction helps to explain the likelihood of heart failure and subsequent mortality rates observed in heart attack patients. In mammals, the cardiomyocytes lost due to injury are never replaced by new cardiac muscle. Instead, fibroblasts display a rapid proliferative response to injury and invade the infarct zone, over time forming a non-contractile fibrotic scar to replace the lost cardiac tissue (Figure 1.2). This scarring of the heart leads to the progressive loss of cardiac contractility, which ultimately compromises heart function and leads to heart failure (Laflamme and Murry, 2005). The inability of mammalian hearts to replace cardiomyocytes with new cardiac muscle is thus linked to the poor prognosis of cardiovascular patients with reduced cardiac contractility.

In an attempt to compensate for the cardiomyocytes lost to injury, the heart will undergo various forms of cardiac remodelling after a myocardial infarction. In many cases, these remodelling efforts do not succeed in restoring the heart to its full
Figure 1.1. Myocardial infarction results in the death of cardiomyocytes. A. Rupture of plaques (yellow) within the coronary vessels results in the formation of a thrombus (clot) that B. occludes the vessels supplying oxygen to the heart. Sustained loss of oxygen and subsequent death of cardiomyocytes is defined as a myocardial infarction.
Figure 1.2. **Mammals are unable to regenerate their hearts after injury.** After cardiac injury such as a myocardial infarction, cardiomyocytes die by apoptosis and necrosis. Additionally, fibroblasts invade the wound area and form a permanent, non-contractile, fibrotic scar after injury. Lastly, an insufficient number of cardiomyocytes is capable of proliferating after injury to re-populate the cardiomyocytes that die during a myocardial infarction.
functioning capacity, and contribute to the incidence of heart failure and negative outcomes in cardiovascular patients (Laflamme and Murry, 2005; Sutton and Sharpe, 2000; Yancy et al., 2013). Early remodelling events, such as the thinning of the ventricular wall and dilation of the ventricle, take place in the myocardium surrounding the fibrotic scar. These events progressively increase the mechanical stress on the ventricular wall, and lead to further contractile dysfunction (Laflamme and Murry, 2005).

Later cardiac remodelling events take place in an effort to remedy this dysfunction. Such later events involve the thickening (hypertrophy) of the ventricle, which is intended to ameliorate the progressive dilation of the ventricle observed in early remodelling events. Ventricular hypertrophy in the later stages is also meant to stabilize the overall contractile functioning of the heart (Pfeffer and Braunwald, 1990; Sutton and Sharpe, 2000). However, the later remodelling events often cannot overcome damage caused by early remodelling and the even earlier loss of cardiomyocytes due to myocardial infarction. Ultimately, the reduced number of cardiomyocytes that survive after injury must sustain the full energetic demands of the body. The strain involved in compensating for the lost cardiomyocytes is another factor that leads to heart failure and death.

Taken together, the lack of newly-formed cardiac muscle in the wake of a myocardial infarction, and the insufficiency of the heart’s remedial response after injury can explain the likelihood of heart failure and death in cardiovascular patients. Current research in the realm of regenerative biology is aimed at investigating the possibility of heart regeneration in mammals. If cardiomyocytes are found to be capable of proliferation, this process could alleviate many of the cardiac issues that
develop post-heart attack, decreasing the likelihood that a myocardial infarction will lead to heart failure, and reducing fatalities worldwide.

An Historical View of the Heart as a Post-Mitotic Organ: Early Evidence

For the past 150 years, the pervading view in the field has held that the human heart is a post-mitotic organ incapable of regeneration (Carvalho and de Carvalho, 2010; Karsner et al., 1925; Tam et al., 1995). This was not always the case; initially, clinicians in the early 19th century believed that cardiac hypertrophy was due exclusively to cardiac hyperplasia (cardiomyocyte proliferation), and that heart regeneration might be possible. However, this theory was ultimately challenged by early macroscopic studies of the heart which declared no evidence of mitotic cardiomyocytes in hypertrophic hearts (Karsner et al., 1925). This research laid the foundation for what was to become dogma in the field of cardiovascular research for almost a century: that the heart was incapable of proliferation and, therefore, regeneration.

Over time, despite scientists’ belief that the heart was incapable of regeneration, evidence for mitotic cells in the heart has increased. Early evidence emerged which documented the presence of mitotic cardiomyocytes in children with hypertrophied hearts, hinting at the possibility that there is a time window, however restricted, for cardiomyocyte proliferation in humans (Macmahon, 1937). By the 1960s and early 1970s, mitotic cardiomyocytes were observed in rats up to four weeks of age, and by the late 1970s, work by (Astorri et al., 1977) described cardiac proliferation in diseased hearts (Sasaki et al., 1968; Zak, 1974). These
findings set the stage for recent studies which have provided more definitive
evidence of cardiomyocyte proliferation in the human heart, and encouraged further
exploration of the possibility of heart regeneration in mammals.

Evidence For Limited Cardiomyocyte Renewal in Mammals: Recent Findings

There is now substantial experimental evidence that provides support for
cardiomyocyte proliferation in the human heart. Studies that measured radioactive
carbon-14 ($^{14}$C) from nuclear testing during the Cold War era provided early
evidence for cardiomyocyte turnover in humans (Bergmann, 2019; Bergmann et al.,
2009; 2015a). After the bomb blasts, atmospheric $^{14}$C was incorporated by plants,
and then by the humans that consumed those plants. This designated a “birthdate”
of $^{14}$C in the human cardiomyocytes, as scientists could clearly identify the initial time
frame in which $^{14}$C had been absorbed. By radiolabeling and tracking levels of $^{14}$C in
the cardiomyocytes of humans over time, scientists were able to measure the rate of
cardiomyocyte proliferation. As the levels of $^{14}$C increased in the total number of
cardiomyocytes’ genomic DNA, this provided evidence that cardiomyocytes
underwent division, and gave a sense of the rate at which this process took place.

By measuring the levels of $^{14}$C in cardiomyocyte nuclei years after the nuclear
blasts, it was determined that the rate of cardiomyocyte turnover in the average 25-
year-old human heart is around 1% (Bergmann et al., 2009). Importantly, the rate of
cardiomyocyte turnover was also found to decline exponentially over one’s life,
reaching a mere 0.45% by the age of 75 (Bergmann et al., 2009; 2015a; Mollova et
al., 2013) reviewed in (Bergmann, 2019). Additionally, researchers found that most
cardiomyocytes in the human heart are generated by age 10, and that the total
number of cardiomyocytes in the heart remains relatively constant as we age, supporting the finding that the rate of proliferation is rather low (Bergmann et al., 2015b). However, these data demonstrate that, despite low rates of proliferation, cardiomyocytes are indeed capable of dividing over time. These findings thus combat the pervasive view that proliferation is not possible, and point to the potential of manipulating cardiomyocyte proliferation to enhance repair after injury (Mohamed et al., 2018) (Figure 1.3).

The results of the $^{14}$C studies on humans are congruous with findings from formal experiments performed in the mouse, confirming the relevance of these discoveries beyond humans and extending it to mammals more broadly. In the mouse, cardiomyocyte production also occurs within a restricted time window. Myocytes have been found to proliferate between post-natal day 1 (P1) and post-natal day 7 (P7), at which point cardiomyocytes undergo a final round of replication without cytokinesis and become binucleated (Alkass et al., 2015; Soonpaa et al., 1996).

Additionally, while it is widely accepted that the adult mouse heart is unable to mount a robust proliferative response to injury, seminal work has identified that a low level of post-injury cardiomyocyte production in the mouse is derived from pre-existing cardiomyocytes (Senyo et al., 2013). This mirrors the current standing of scientific research on human cardiomyocyte proliferation, in that there is general consensus that the adult human heart is unable to regenerate, although there exists evidence for low levels of cardiomyocyte proliferation.

In the case of both human and mouse hearts, it is now understood that mammals are unable to regenerate their hearts due to the cardiomyocyte’s inability to divide after
Figure 1.3. Mammalian hearts exhibit limited cardiomyocyte renewal. Atomic blasts released radiolabeled carbon-14 ($^{14}\text{C}$) during the Cold war era. $^{14}\text{C}$ was then incorporated into cardiomyocytes during the synthesis phase of cell cycle. These studies on human hearts revealed that the heart proliferates into adulthood, albeit at low levels. These data highlight the possibility of therapeutic intervention strategies for boosting proliferation of cardiomyocytes after injury.
injury (Laflamme and Murry, 2005). At the same time, recent discoveries have provided evidence of limited cardiomyocyte proliferation in these species. Looking to other animal models whose cardiomyocytes demonstrate a more robust ability to proliferate after injury provides an opportunity to examine this cardiac proliferative response in a different context, and to determine whether boosting the limited levels of proliferation in mammals may ever amount to a truly regenerative capacity.

**Zebrafish Cardiomyocytes Mount a Robust Proliferative Response to Cardiac Injury**

Using a variety of injury models, scientists have demonstrated that the adult zebrafish heart, in contrast to the adult mammalian heart, is able to mount a robust proliferative response to injury reviewed by (Gemberling et al., 2013; González-Rosa et al., 2011; Poss, 2002; Wang et al., 2011) reviewed by (González-Rosa et al., 2017). Following a series of critical regenerative events after injury, pre-existing zebrafish cardiomyocytes adjacent to the plane of injury (wound-edge cardiomyocytes) de-differentiate by breaking down their sarcomeres, and then re-enter the cell cycle. These cardiomyocytes then divide to replace the cardiomyocytes damaged from the injury (Jopling C et al. 2010)(Poss, 2002). These pre-existing cardiomyocytes also activate a gata4 regulatory sequence to regenerate cardiac muscle that was damaged by injury (Gupta et al., 2013; Kikuchi et al., 2010) (Figure 1.4).
Figure 1.4. The zebrafish heart mounts a robust proliferative response to cardiac injury. In response to a cardiac injury, the cardiomyocytes that comprise the zebrafish heart mount a robust proliferative response to injury. Specifically, a population of pre-existing cardiomyocytes that are responsive to a gata4 regulatory sequence (green), de-differentiate and breakdown their sarcomeres to re-enter the cell cycle and replace lost cardiac tissue after injury.
Because the cardiomyocytes in the zebrafish heart have a significant proliferative response and regenerate after injury, the zebrafish remains a highly attractive model by which to elucidate the mechanisms critical for the regulation of cardiomyocyte proliferation.

Past research regarding cardiomyocyte proliferation in the zebrafish has focused on the many factors that regulate proliferation in an attempt to understand how this process is made possible. Numerous studies have determined a variety of events and factors that are essential to regeneration in zebrafish. Some such events take place in the early hours after injury, whereas others take days after the injury to develop. Other factors contribute to the process of proliferation even in the absence of cardiac injury, and will induce proliferation after constitutive expression in the heart.

**Early Events after Injury Discovered to Contribute to Cardiomyocyte Proliferation in the Zebrafish**

Across injury models, zebrafish demonstrate an early inflammatory response and recruitment of phagocytes to the infarct zone as early as three hours after injury (de Preux Charles et al., 2016; Huang et al., 2013a). This inflammatory response is critical for cardiomyocyte proliferation to occur. Studies have demonstrated that early upregulation of pro-inflammatory cytokines is required for efficient vascularization to the wound area. Efficient vascularization is necessary for the subsequent proliferation of cardiomyocytes, which in turn is needed to regenerate the damaged cardiac muscle (Huang et al., 2013a). Just as the inflammatory response from the zebrafish immune system is a critical factor in launching the proliferative response,
recent evidence points to an increasing requirement for the immune system to initiate the pro-regenerative cascade of events after injury. Accordingly, the role of the immune system in modulating regeneration after injury remains an important area of scientific inquiry.

In addition to the immune system, a variety of other factors have been discovered to play an important role in cardiomyocyte proliferation in zebrafish. The estrogen hormone, for example, has been shown to positively regulate the expression of these early inflammatory cytokines critical for regeneration after injury (Xu et al., 2019).

Additionally, regulatory T cells seem to enhance the zebrafish’s regenerative capacity. It has been observed that a subset of regulatory T cells invades the wound area beginning around three days post-injury, and serves to induce the secretion of cardiomyocyte mitogenic factors such as neuregulin 1 (Nrg1) and Igf1. Complete regeneration was impaired without the invasion of these regulatory T cells into the regenerating heart after injury, demonstrating their critical role in the regenerative process (Hui et al., 2017).

Macrophages have also been identified as critical early regulators of the regenerative response that promotes cardiomyocyte proliferation. Through comparative transcriptomic analyses, scientists studied the evolutionarily divergent medaka teleost species, which cannot regenerate, alongside the zebrafish. Their results demonstrated that the early immune response to injury is the critical difference between the non-regenerative medaka and the highly regenerative zebrafish (Lai et al., 2017). Specifically, early recruitment of macrophages to the wound area was evident in the zebrafish and absent in the medaka. Furthermore,
preventing early recruitment of macrophages after injury resulted in the retention of a fibrotic scar, and was sufficient to block vascularization and proliferation of cardiomyocytes (Lai et al., 2017). More recently, work from Sanz-Morejon A et al. (2019) described that the wt1b macrophage subtype is essential for regulating macrophage migration and retention in the wound area. Furthermore, wt1b mutant zebrafish exhibit defects in macrophage migratory patterns and demonstrate subsequent defects in cardiomyocyte proliferation after injury (Sanz-Morejón et al., 2019). Taken together, these results indicate that defects in macrophage recruitment can lead to a reduced capacity for proliferation, and demonstrate the importance of macrophages in regulating regeneration.

Recent studies have also pointed to the importance of early vascularization of vessels in the regenerating heart as a requisite step for cardiomyocyte proliferation after injury (Marín-Juez et al., 2016). In the zebrafish, new coronary vessels were observed sprouting into the wound area by 15 hours post-injury. In one study, the expression of a dominant negative form of the pro-angiogenic factor vegfaa blocked angiogenesis of the coronary vessels into the wound area, thereby dampening cardiomyocyte proliferation and preventing regeneration from taking place (Marín-Juez et al., 2016). This outcome supports the notion that vascularization is necessary for cardiomyocyte proliferation. This work was further bolstered by research on chemokine receptors and their role in vascularization. Endothelial cells that form the coronary vessels express the chemokine receptor cxcr4a, which both migrates to and vascularizes the wound area. Importantly, cxcr4a mutants were found unable to accomplish this migration and vascularization. As a result, cxcr4a mutants fail to form the vascular network that is essential for removal of the fibrotic
scar after cryoinjury, and effectively prevent a proliferative response (Harrison et al., 2015). That the absence chemokine receptors prevent vascularization was also found to prevent proliferation further highlights the critical connection between early vascularization and regeneration.

In addition to the demonstrated importance of an early post-injury response from the immune system, macrophages and vascular sprouting, there is also evidence that later organ-wide transcriptional changes can affect cardiomyocyte proliferation in the zebrafish. For example, genetic ablation of tc21+ epicardial cells is sufficient to suppress cardiomyocyte proliferation and block regeneration after cardiac injury (Wang et al., 2015).

Researchers have also studied the activation of the endocardium as it pertains to the promotion of cardiomyocyte proliferation after injury. Upon injury, the retinoic acid synthesizing enzyme raldh2 is expressed broadly by the endocardium and epicardium. Eventually, this expression becomes restricted to the injury area to provide instructive cues that drive cardiomyocyte proliferation (Kikuchi et al., 2011). In addition to expressing raldh2, the epicardium also expresses early embryonic genes such as tbx18 and wt1b which, post-injury, similarly provide important cues for regeneration (González-Rosa et al., 2011; Lepilina et al., 2006; Schnabel et al., 2011). These studies demonstrate the role of organ-wide cellular responses in affecting the heart’s regenerative process.

Research on the Jak/Stat pathway shows a similar tendency for an organ-wide response to include upregulation broadly in the heart at first, and then in a more restricted manner to govern cardiomyocyte proliferation. The Jak/Stat pathway has demonstrated upregulation in response to injury throughout the entire heart as early
as one day after injury, and then becomes restricted to cardiomyocytes by seven
days post-injury (Fang et al., 2013). It has been demonstrated that il11a and lifra
ligands secreted by myeloid and endocardial cells activate the jak/stat pathway in
cardiomyocytes to promote their proliferation, further reinforcing the connection
between organ-wide responses to injury and regeneration (Fang et al., 2013).

It is important to note that these early events precede cardiomyocyte
proliferation and are critical mainly in priming and instructing a proliferative response
(Lepilina et al., 2006; Münch et al., 2017). Upon injury, both the endocardium and
epicardium are rapidly activated and broadly express genes that later become
restricted towards the wound edge to transmit signals which permit cardiomyocyte
proliferation. Taken together, these data demonstrate that early activation of the
epicardium and endocardium, in addition to an early immune response within the
zebrafish heart, are required to prime the pro-regenerative response after injury.

**Later events after injury discovered to contribute to cardiomyocyte
proliferation in the zebrafish**

As previously discussed, a variety of early signals to cardiomyocytes come
from immune, epicardial, and endocardial cells in the hours after injury. Additional
signals from cholinergic neurons have been observed during the days following
injury, and also seem to be required to stimulate proliferation. Studies which
chemically inhibited cholinergic signalling and overexpressed the neuronal
chemorepellent semaphorin3aa showed that neurons are potent regulators of
cardiomyocyte proliferation. In these instances, myocyte proliferation was
suppressed when the neural signals were disrupted (Mahmoud et al., 2015).
Additionally, NF-κB has been demonstrated to be expressed in cardiomyocytes after injury (Karra et al., 2015). In the presence of cardiomyocyte-specific suppression of NF-κB after injury, the de-differentiation of cardiomyocytes is blocked, and the proliferation required for regeneration is impaired (Karra et al., 2015).

**Mitogenic factors sufficient to induce cardiomyocyte proliferation in the Zebrafish heart**

In addition to the above-mentioned events and signals which are responsive to a cardiac injury, mitogenic signals have been discovered to induce cardiomyocyte proliferation in the zebrafish even in the absence of an injury to the heart.

Though we have a very limited understanding of the regulators of *de novo* cardiomyocyte proliferation, some molecular programs have been documented as sufficient to induce cardiomyocyte proliferation. The paracrine growth factor neureglin (*nrg1*) is one of the most well-studied examples of a mitogenic factor that stimulates cardiomyocyte proliferation. *Nrg1* is secreted from endocardial and perivascular cells in the ventricular wall, with secretion increasing after injury to promote myocyte proliferation (Gemberling et al., 2015). However, it has also been found that transgenic overexpression of *nrg1* in cardiomyocytes in the absence of injury is sufficient to induce cardiac hyperplasia and expansion of the ventricular wall, resulting in cardiomegaly even without cardiac damage (Gemberling et al., 2015).

More recently, it has been shown that vitamin D is also a potent mitogen that regulates cardiomyocyte cell cycling and acts through the neureglin/ErBB2 signaling pathway. A transgene constitutively expressing an active form of vitamin D in
cardiomyocytes was found sufficient to cause cardiomegaly, which was associated with a concomitant increase in cardiomyocyte proliferation. These data demonstrate that vitamin D signalling through the ErBB2 pathway is sufficient to induce cell cycle re-entry in cardiac myocytes (Han et al., 2019). Conversely, chemical inhibition of vitamin D signalling was found to mitigate cell cycle entry both in the embryo during growth and after injury (Han et al., 2019). This provides another example of a factor that allows proliferation to take place even in the absence of injury to the heart.

Insulin signalling has also been identified as a potent, required mitogenic pathway for inducing cardiomyocyte proliferation (Huang et al., 2013b). Specifically, igf2b secreted from the endocardium and epicardium binds to its cognate receptor igfr on the surface of cardiomyocytes to drive cardiomyocyte proliferation. Also on the surface of myocytes, overexpression of a dominant negative form of the insulin growth factor 2b receptor, dnigf1ra, was sufficient to dampen proliferation in cardiomyocytes (Huang et al., 2013b). Similarly, the p38α MAP kinase was found to be a negative regulator of cardiomyocyte proliferation, and active p38α prevented myocytes from entering the cell cycle (Engel, 2005a). These data demonstrate the potent role of mitogens in stimulating myocyte proliferation even independently of a cardiac injury, and identify the critical negative regulators that are essential for the prevention of aberrant proliferation of cardiac myocytes.

Despite prior research into the many factors which contribute to cardiomyocyte proliferation in the zebrafish, we still have a very limited understanding of the agents required for regulating the cell cycle within cardiomyocytes both throughout development and after injury. Therefore, studying the factors that regulate proliferation in cardiomyocytes after injury is critical to
understand how we can enhance cell cycle re-entry and boost the heart’s endogenous capacity to regenerate after injury.

**Using the Zebrafish Embryo to Study the Factors that Contribute to Cardiomyocyte Proliferation**

In order to cultivate a better understanding of the aforementioned factors which contribute to and regulate cardiomyocyte proliferation, some scientists have elected to examine the developing zebrafish embryo. Widespread proliferation takes place in the heart as the embryo develops, and this process provides an opportunity to further investigate the mechanisms that govern cardiomyocyte proliferation.

Cardiogenesis occurs in the developing zebrafish embryo as a result of two distinct phases. The first phase consists primarily of the specification of cardiac progenitors, beginning early in development in the anterior lateral plate mesoderm. By 24 hours post-fertilization (hpf), these progenitors accrete at the arterial and venous poles of the developing heart tube, where they are continuously specified until around 48 hpf (Chen and Fishman, 1996; Yelon et al., 2000). At this point, the second phase of cardiogenesis begins as the embryonic zebrafish heart begins to grow predominantly through cardiomyocyte proliferation (de Pater et al., 2009) (Choi et al., 2013; Qu et al., 2008; Ribeiro et al., 2007; Sedletcaia and Evans, 2011). From 48 hpf onwards, therefore, defects in cardiomyocyte proliferation can be measured and modelled in the embryo (**Figure 1.5**).
Figure 1.5. Cardiomyocytes in the embryonic zebrafish heart begin to grow predominantly by proliferation beginning at 48 hpf. During embryogenesis, the zebrafish heart is largely specified until 48 hpf. Beginning around 48 hpf, the heart begins to grow predominately by proliferation of cardiomyocytes. V= ventricle, AT= atrium, CM= cardiomyocyte
Tools with which to measure cardiomyocyte proliferation

A wide variety of tools have been developed for use in understanding and dissecting the molecular programs that regulate cardiomyocyte proliferation. These tools, used to analyse proliferation, have allowed for a better understanding of both the factors that are sufficient to drive cardiomyocyte proliferation, and those that are required for the process to occur.

Markers of proliferating cells

One of the most common methods to assay for proliferation is through the use of nucleotide analogs, such as BrdU, EdU, or tritiated thymidine. These analogs are incorporated into genomic DNA during the S phase of DNA synthesis, and allow for short- or long-term assessment of the proliferation of cells and their daughters (Jahangiri et al., 2016; O'Meara et al., 2015).

Analogs such as BrdU do not measure the bona fide cell division of cytokinesis or M-phase, but instead are reliable markers in indicating cell cycle progression (Iatropoulos and Williams, 1996). One example of such a marker is histone H3 phosphorylation, a post-translational modification to the histone H3 during the G2 and M phases of the cell cycle reviewed by (Hans and Dimitrov, 2001).

Another widely used metric for cell proliferation is Proliferating Cell Nuclear Antigen (PCNA), which is highly expressed in the G1-S phase of cell cycle, as well as the G2-M phase of the cell cycle (Connolly and Bogdanffy, 1993; Kurki et al., 1988). Ki67, one of the most commonly used markers of proliferation across disciplines, is used to label cells in all parts of the cell cycle without labelling non-cycling cells. However, Ki67 bears certain disadvantages, as it has been demonstrated to degrade
slowly and is still present in cells that have already exited the cell cycle (Miller et al., 2018).

Importantly, both nucleotide analogs as well as PH3 modifications are not cardiomyocyte-specific markers. As such, they require cell-specific cardiomyocyte co-labels in order to determine the extent of cardiomyocyte-specific proliferation. There are several cardiomyocyte-specific markers commonly used to directly quantify myocyte proliferation. Aurora B, for example, localizes to the centromeric ring formed during mitosis (Engel, 2005b). Anillin, an F actin binding protein, localizes to the cytokinetic bridge that forms just prior to cytokinesis during cell division (Engel et al., 2006).

**Cardiomyocyte counts**

Cell count experiments are a bona fide metric used to measure cell proliferation by tracking expanding cell numbers. The expansion of cell populations can be directly quantified *in vitro* or *in vivo* when coupled with cell type specific markers. (Jahangiri et al., 2016; Romar et al., 2016).

**Cardiomyocyte de-differentiation**

An important structural component of the cardiomyocyte is the sarcomere – the contractile apparatus of the cardiomyocyte (Fenix et al., 2018). It has long been observed that the disassembly of the sarcomeric apparatus is a step consistently associated with the proliferation of mature cardiomyocytes (Ahuja, 2004; Jopling et al., 2010; Kaneko et al., 1984; Uribe et al., 2018). Though it has yet to be formally tested whether or not sarcomere disassembly is both necessary and sufficient to
induce myocyte proliferation, cardiomyocytes re-entering the cell cycle seem to be less differentiated and possess more disassembled sarcomeres. It has been observed in zebrafish that upon injury to the ventricle, cardiomyocytes break down their sarcomeres, de-differentiate to express more embryonic genes such as embryonic myosins, re-enter the cell cycle, and re-differentiate to regenerate damaged cardiac muscle (Fan et al., 2015; Jopling et al., 2010; Sallin et al., 2015). Observing cardiomyocyte de-differentiation could be an additional means by which to track proliferation rates.

Clonal Analysis

Genetic tools can be used to permanently label cardiomyocyte populations for lineage tracing. It is possible to follow the expansion and, therefore, proliferation of cell populations using recombination technology such as Cre/loxP (Gupta et al., 2013), FLP/FRT (Sereti et al., 2018), Rox/Dre (Li et al., 2018), bipartite systems such as Gal4/UAS (Kawakami et al., 2016), or photoconvertible systems such as Kaede (Felker et al., 2018).

Investigating the Factors that Regulate Cardiomyocyte Proliferation in Zebrafish: Thesis Goal

The central theme of my thesis has been to understand the series of factors that regulate cardiomyocyte proliferation both in the context of cardiogenesis in the embryo and in the context of regeneration after injury. Our work has implicated a variety of novel critical regulators of cardiomyocyte proliferation after injury and has furthered our knowledge of important barriers to regeneration. Our hope is that
broadening our understanding of the factors important for enhancing proliferation after injury will bring us one step closer to generating novel therapeutic applications for patients after myocardial infarction.

**ruvbl1 (pontin) and ruvbl2 (reptin) as regulators of cardiomyocyte proliferation**

*Initial discovery of ruvbl1 and ruvbl2 and their role in DNA repair and cell cycle*

The ruv proteins have been defined as ATPases associated with diverse cellular activities (AAA+). These proteins, namely ruvA (TIP49, TIP49a, ruvbl1, pontin) and ruvB (TIP48, TIP49b, ruvbl2, reptin), were initially discovered in bacteria, and are highly conserved across species including bacteria, flies, worms, plants and humans (Bauer et al., 2000; Schořová et al., 2019). Both ruvbl1 and ruvbl2 were found to be essential for growth and viability in eukaryotes such as yeast and drosophila (Bauer et al., 2000; Schořová et al., 2019). Similarly, ruvbl1 and ruvbl2 knockouts were associated with early embryonic lethality in the mouse (Arnold et al., 2012; Bereshchenko et al., 2012).

It has now been over 20 years since ruvbl2 was initially discovered to be an important regulator of DNA repair, recombination and cellular proliferation (Lloyd et al., 1984; Otsuji et al., 1974; Sargentini and Smith, 1989; Sharples et al., 1990; Stacey and Lloyd, 1976). Research on ruvbl2 has spanned several decades and over time has contributed to our understanding of its function in relation to DNA repair and the cell cycle.
In early studies, mutations in ruvA (ruvbl1) and ruvB (ruvbl2) were found within the bacterial operon ruv due to their increased sensitivity to Y- and UV irradiation (Otsuji et al., 1974). Additionally, it was observed that E.coli carrying these ruv mutations also demonstrated septation/cytokinesis defects during the cell cycle, implying that ruv is necessary for proliferation (Otsuji et al., 1974; Sargentini and Smith, 1989). Later studies showed that DNA damage was sufficient to induce ruv expression, suggesting that ruv proteins may be important for DNA repair (Shurvinton and Lloyd, 1982). Studies characterizing ruv mutants in E. coli demonstrated defects in DNA recombination after DNA damage, further underscoring the connection between ruv proteins and DNA repair (Shurvinton and Lloyd, 1982).

Several years later, studies showed that bacterial ruvB (ruvbl2) could bind ATP, dATP and ADP. This evidence provided the first characterization of bacterial ruvB’s ATPase activity, activity which is critical to ruvB’s ability to function effectively and to carry out DNA repair and proliferation. Furthermore, it was also determined that ruvA could bind both single and double stranded DNA (Iwasaki et al., 1989; Shiba et al., 1991; Shinagawa et al., 1988). Importantly, both ruvA (ruvbl1) and ruvB (ruvbl2) were found to complex together with ATP to bind DNA and undergo efficient branch migration during homologous recombination. These studies demonstrated that the ATPase activity and DNA binding was critical to repair damaged DNA sequences or to facilitate DNA replication (Shiba et al., 1991).

Furthermore, both ruvbl1 and ruvbl2 proteins were found to contain highly conserved walker A and walker B domains, which are essential for their function in hydrolyzing ATP. Mutations specifically within these protein domains render the protein unable to hydrolyze ATP and bind DNA, resulting in defects in DNA repair.
and cellular proliferation (Gentili et al., 2015; Grigoletto et al., 2013; Hishida et al., 1999; 2004). The results of these studies serve to highlight the relationship between ruvbl1 and ruvbl2 proteins and the process of DNA replication and repair.

ruvbl2 and ruvbl1 subcellular localization: location confers function

Extensive research has demonstrated that the subcellular localization of both ruvbl1 and ruvbl2 determines their function. By analysing where ruvbl1 and ruvbl2 are found in the cell, we infer the role they might play during cell cycle. Both ruvbl1 and ruvbl2 are found within the nucleus as part of the INO80, SWR and TIP60 chromatin remodelling reviewed by (Gallant, 2007; Hota and Bruneau, 2016; Jin et al., 2005; Jónsson et al., 2001a; Mizuguchi et al., 2004). The chromatin remodelling complexes ability to function is an ATP dependent process that requires ruvbl1 and ruvbl2. Specifically, as part of these chromatin remodeling complexes, ruvbl1 and ruvbl2 are necessary to catalyse the exchange of the histone H2A with the histone H2A.Z, a marker of open chromatin and active transcription. ruvbl1 and ruvbl2 are also needed to acetylate histones for the activation of transcription (Redon et al., 2002; Jha and Dutta, 2009). Additionally, it has been observed that ruvbl1 and ruvbl2 are critical for the assembly of these chromatin remodelling complexes, such as INO80, where ruvbl2 is required to recruit the actin related protein Arp5 to the INO80 complex. The assembly of arp5 into INIO80 by ruvbl2 is essential for IN080 activity (Jónsson et al., 2001b; Shen et al., 2003). These ATP-dependent chromatin remodelling complexes have diverse roles from stem cell fate and differentiation to regeneration. The important role of ruvbl1 and ruvbl2 in allowing the complexes to function implicates these proteins in a diversity of biological processes (reviewed by Hota and Bruneau, 2016).
ruvbl1 and ruvbl2 also act within the nucleus to modulate the transcriptional activities of proto-oncogenes such as c-myc and β-catenin. Early in vitro studies identified that c-myc interacts with the N-terminal of ruvbl1 and ruvbl2 to regulate proliferation (Etard et al., 2005; Wood et al., 2000). This interaction was also confirmed in vivo where the overexpression of either ruvbl1 or ruvbl2 was sufficient to significantly increase mitosis in the Xenopus embryo through the c-myc/miz-1 pathway. Furthermore, it was demonstrated that the knockdown of either ruvbl1 or ruvbl2 resulted in early embryonic lethality, which could be rescued by the overexpression of c-myc (Etard et al., 2005). The genetic interaction among ruvbl1, ruvbl2 and c-myc is also conserved in Drosophila, where ruvbl1 or ruvbl2 mutant embryos exhibit embryonic growth arrest and are outcompeted by cell-competition during embryogenesis. Additionally, there is a marked reduction in the viability of offspring with ruvbl1 and ruvbl2 mutants in a myc hypomorphic background (Bellosta et al., 2005). Interestingly, in the fly, it seems that there is a stronger interaction between pontin and c-myc. Taken together, these data demonstrate a genetic interaction among c-myc, ruvbl1, and ruvbl2 in regulating cellular proliferation, providing us with further insight as to how ruvb1 and ruvbl2 are involved in this process.

In addition to these interactions with c-myc, ruvbl1 and ruvbl2 have also been shown to interact antagonistically with β-catenin in regulating proliferation. Past research identified ruvbl2 as a transcriptional repressor of canonical Wnt-signalng, and identified ruvbl1 as a transcriptional activator of β-catenin both in vitro and in vivo (Bauer et al., 2000; Kim et al., 2005). These data were confirmed in zebrafish,
where *ruvbl1* and *ruvbl2* were found to exert antagonistic roles in heart growth during embryogenesis through β-catenin mediated interactions (Rottbauer et al., 2002).

There is mounting evidence that *ruvbl1* and *ruvbl2* expression in the cytoplasm are associated with tumorigenesis. While there is little evidence for the role of *ruvbl1* and *ruvbl2* in regulating EMT under physiological conditions, EMT in tumorigenic conditions has been strongly associated with the cytoplasmic localization of *ruvbl1* and *ruvbl2* (Baron et al., 2016; Lauscher et al., 2007; Makino et al., 1998; Ni et al., 2009; Ren et al., 2013; Taniuchi et al., 2014; Xie et al., 2009). Specifically, *ruvbl1* and *ruvbl2* expression in the cytoplasm have been found to help regulate the epithelial to mesenchymal transition (EMT) in cells during cancer metastasis.

Confirming these observations, it has also been demonstrated that siRNA mediated downregulation of *ruvbl1* and *ruvbl2* is associated with less E-cadherin and vimentin signalling, respectively, which is indicative of EMT. Additionally, there is further evidence that *ruvbl2* regulates its downstream target, meprin α, a matrix metalloprotease involved in EGFR signalling and EMT further indicating the potential of *ruvbl2* in the process of EMT (Breig et al., 2017; Broder and Becker-Pauly, 2013). It has yet to be determined whether the cytoplasmic localization of reptin or pontin is sufficient to induce EMT during tumorigenesis, but it is clear that these ruv proteins play a role in EMT (Figure 1.6).

*ruvbl2 and cell cycle regulation*

Both *ruvbl1* and *ruvbl2* are described as critical regulators of cell cycle. Seminal work from Gentili et al. (2015) demonstrated that *ruvbl1* and *ruvbl2* are dynamically expressed during mitosis (Gentili et al., 2015). Despite existing in a
Figure 1.6. *ruvbl1/ruvbl2* are ATPases associated with diverse cellular activities. Canonical Walker A and B motifs are highly conserved across species and are essential for ATPase activity of *ruvbl1* and *ruvbl2*. In the nucleus, *ruvbl1/ruvbl2* interact with c-myc and β-catenin as well as chromatin remodeling complexes to regulate cellular proliferation. Cytoplasmic expression of *ruvbl1/ruvbl2* is associated with EMT during cancer metastasis and overall poor clinical prognosis.
complex together, *ruvbl1* and *ruvbl2* seem to have discrete roles during cell division as they exhibit distinct localization patterns during cytokinesis. While both *ruvbl1* and *ruvbl2* are found to be diffusely expressed during interphase, during late mitosis *ruvbl2* localizes to the mitotic spindle apparatus and midbody, and *ruvbl1* localizes to the centrosomes and mitotic spindle (Skop et al., 2004; Fielding et al., 2008; Gartner et al., 2003; Sigala et al., 2005; 2004). Taken together, these observations suggest that *ruvbl1* and *ruvbl2* play a role in regulating mitosis. Additionally, the distinct localization patterns of *ruvbl1* and *ruvbl2* during cytokinesis suggest discrete biological roles for each protein in regulating mitosis.

In order to test the functional role of *ruvbl1* and *ruvbl2* in regulating mitosis, a series of knockdown experiments were performed to assess their necessity during mitotic progression. *ruvbl1* knockdown *in vitro* exhibited a delayed cell cycle transition from pro-metaphase to anaphase, associated with an increase in the number of lagging chromosomes. These findings are indicative of improper microtubule attachment and defective mitotic progression as a result of *ruvbl1* knockdown (Gentili et al., 2015; Thompson and Compton, 2011). siRNA mediated knockdown of the *ruvbl1* ATPase domains was also shown to block proliferation of epithelial cells *in vitro*, demonstrating that *ruvbl1* is required during mitotic progression and growth (Gentili et al., 2015). Furthermore, *ruvbl2* knockdown was associated with failed cell cycle progression throughout mitosis. When combined, *ruvbl1* and *ruvbl2* knockdown result in defective microtubule formation and cell cycle progression (Ducat et al., 2008). Studies which focused on the depletion of either *ruvbl1*, *ruvbl2*, or ATPase mutants of either *ruvbl1* or *ruvbl2* have shown that these
proteins are required for the chromosomal condensation that is associated with the completion of mitosis (Magalska et al., 2014).

Additional evidence points to reptin and pontin as critical regulators of cell cycle. The mammalian cell cycle is tightly regulating by a number of proteins, including E2Fs (Fan and Bertino, 1997). Both ruvbl1 and ruvbl2 are recruited by e2f1, a critical transcription factor and regulator of G1-S transition phase of cell cycle, to open chromatin regions and carry out transcription of e2f target genes. Additionally, ruvbl2 knockdown results in a decreased expression of H2A.Z, open chromatin regions at e2f targets, and a concomitant decreased in cellular proliferation in vitro (Tarangelo et al., 2015). As essential components of the of the telomerase ribonucleoprotein complex, ruvbl1 and ruvbl2 are also required for telomerase biogenesis during S phase and for efficient cell cycle progression (Venteicher et al., 2008). Taken together, these data implicate ruvbl1 and ruvbl2 as critical regulators of cell cycle and mitotic progression.

ruvbl2 and cancer

As previously mentioned, the cytoplasmic expression of ruvbl1 and ruvbl2, while not formally tested, is commonly associated with tumorigenesis (Grigoletto et al., 2011). These observations have prompted further analysis of various human cancers and their interactions with ruv proteins, leading to the discovery that ruvbl1 and ruvbl2 seem to be important regulators of aberrant cell cycling and genomic stability in cancer. Namely, ruvbl1 and ruvbl2 have been shown to be overexpressed in hepatocellular carcinoma and non-cell lung carcinoma (Ocak et al., 2014; Rousseau et al., 2007). ruvbl1 is also found to be overexpressed in colon cancer
Table 1.1. *ruvbl1/ruvbl2* are overexpressed in a wide variety of cancers List of documented cancer types in which *ruvbl1* and *ruvbl2* are shown to be over-expressed. (adapted from Mao YQ and Houry WA, 2017).

<table>
<thead>
<tr>
<th>TISSUE AFFECTED</th>
<th>CANCER TYPE</th>
<th>RUVBL1/PONTIN</th>
<th>RUVBL2/REPTIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>Esophageal squamous cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>Gastric Cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>Pancreatic ductal adenocarcinoma</td>
<td></td>
<td></td>
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<tr>
<td>Liver</td>
<td>Hepatocellular carcinoma</td>
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<td></td>
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<tr>
<td>Colon, Rectum</td>
<td>Colorectal cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Renal cell carcinoma</td>
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<tr>
<td>Bladder</td>
<td>Micropapillary carcinoma</td>
<td></td>
<td></td>
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<tr>
<td>Lung</td>
<td>Non-small cell lung cancer</td>
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<tr>
<td>Lung</td>
<td>Small cell lung cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>Early-stage breast cancer and ductal carcinoma</td>
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<tr>
<td>Ovary</td>
<td>Ovarian</td>
<td></td>
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<tr>
<td>White Blood Cell</td>
<td>Acute Myeloid Leukemia</td>
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<tr>
<td>White Blood Cell</td>
<td>Lymphoma</td>
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</tr>
</tbody>
</table>
Both *ruvbl1* and *ruvbl2* are shown to be overexpressed in greater than 75% of hepatocellular carcinoma and colon cancers, respectively, and are generally associated with poor patient prognoses.

Both *ruvbl1* and *ruvbl2* interact with p53, a potent tumor suppressor and regulator of the cell cycle (Brown et al., 2009; Maslon et al., 2010; Ocak et al., 2014; Zhao et al., 2015). Specifically, *ruvbl1* binds to a mutated form of p53 that confers gain of function activity and aberrant proliferation of tumor cells (Zhao et al., 2015). In contrast, *ruvbl2* binds to suppress the wild-type form of p53, which has been found to promote cancer cell proliferation (Mao and Houry, 2017; Maslon et al., 2010). Given the expansive role that *ruvbl1* and *ruvbl2* play in cell cycle regulation and tumorigenesis, designing therapeutics to target *ruvbl1* and or *ruvbl2* to regulate aberrant or insufficient cell cycling is an attractive next step.

Recent research in therapeutics approaches designed to target the ruv proteins has shown promising results. Studies of mouse xenografts of hepatocellular carcinoma demonstrate that *ruvbl2* knockdown results in tumor regression (Ménard et al., 2010). Additionally, studies have shown that *ruvbl1* knockdown induces cell death in *in vitro* models of leukemia (Breig et al., 2014). Finally, molecular dynamic simulations have lead to the discovery of small molecule inhibitors of pontin sufficient to inhibit cell proliferation *in vitro* (Elkaim et al., 2012). Small molecules for reptin have been developed but are yet to be tested in their ability to reduce tumor growth (Javary J et al. 2018).
ruvbl2 (reptin) in regulating heart size during zebrafish embryogenesis (Rottbauer et al., 2002)

In addition to their critical role in regulating cell cycle, both ruvbl1 and ruvbl2 are also required to regulate proper growth of the developing zebrafish heart. An ENU chemical screen performed in zebrafish embryos identified a mutation within the endogenous ruvbl2 locus which resulted in embryos exhibiting a curved body phenotype as well as a cardiac edema by 72 hpf (Rottbauer et al., 2002). This mutation is characterized by a single point mutation resulting in an in-frame insertion of 9 base pairs in an intergenic region between exons 7 and 8 and a novel splice acceptor site. The resulting mutation was termed liebeskummer (lik), German for ‘lover’s grief,’ as zebrafish embryos with this mutation developed hearts that overproduce ventricular cardiomyocytes (Figure 1.7).

By analyzing these mutants, the Rottbauer study provided critical insights into how ruvbl1 and ruvbl2 regulate growth of the developing zebrafish heart. This study was the first to describe the ruvbl2lik mutation as a gain of function mutation, due to the increase of ATPase activity in ruvbl2lik mutants. They also performed a series of cell transplantation experiments in which ruvbl2lik cardiomyocytes were transplanted into a wild-type recipient ventricle. The resulting expansion of ruvbl2lik mutant cardiomyocytes in the wild-type recipient ventricle suggested that ruvbl2lik acts cell autonomously within cardiomyocytes to regulate myocyte expansion. Additionally, knockdown of ruvbl1 resulted in an increase in the total number of ventricular myocytes in zebrafish embryos and suggested that ruvbl1 was acting as a suppressor of cardiac growth. Lastly, the authors concluded that ruvbl1 and ruvbl2
control heart growth during embryogenesis through antagonistic regulation of β-catenin (Figure 1.8).

Ultimately, the data of the Rottbauer study provided compelling evidence that \textit{ruvbl1} and \textit{ruvbl2} are critical regulators of heart growth. However, it remained to be formally tested whether \textit{ruvbl1} and \textit{ruvbl2} were essential regulators of proliferation that might account for the increased number of ventricular myocytes in \textit{ruvbl2}\textsuperscript{ulk} mutants or \textit{ruvbl1} morphants. Further study is also required to determine whether \textit{ruvbl2} and \textit{ruvbl1} function early in development to increase the specification of cardiac progenitors, which might account for an increase in ventricular numbers later in development. This thesis is a detailed characterization of \textit{ruvbl2} during zebrafish embryogenesis and regeneration.
Figure 1.7. *ruvbl2*<sup>lik</sup> mutants exhibit increased ventricular cardiomyocyte numbers. An ENU chemical screen identified a single point mutation within the endogenous *ruvbl2* locus that results in an increase in the total number of ventricular cardiomyocytes. *ruvbl2<sup>lik</sup>* mutants also exhibit a curved body phenotype and die by 4 days post fertilization.

V= ventricle, AT= atrium, CM= cardiomyocyte (adapted from Rottbauer et al. 2002)
Figure 1.8. *ruvbl1/ruvbl2* antagonistically regulate heart growth during embryogenesis.

*Ruvbl2*<sup>lik</sup> was described as a gain of function mutation that resulted in embryos with an increase in the total number of ventricular myocytes. *Ruvbl1* knockdown resulted in an increase in ventricular myocytes. The proposed model was that *ruvbl1/ruvbl2* exert antagonistic roles in regulating heart growth, where *ruvbl1* suppresses myocyte growth and *ruvbl2* supports myocyte growth (adapted from Rottbauer et al. 2002).
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RUVEBL2 is a negative regulator of cardiomyocyte proliferation in zebrafish
ATTRIBUTIONS

Contributions to the work described in this chapter were made by Michka Sharpe, Juan Manuel González, Spencer Jeffrey, Katherine Copenhaver Caroline E. Burns and C. Geoffrey Burns.

CEB, CGB, MS and JMGR conceived of and designed the study. MS performed immunofluorescence staining, genotyping, BrdU proliferation assays, sectioning of adult ventricles, AFOG staining of adult ventricles, design and injection of constructs, confocal microscopy in adults and embryos and figure preparation. JMGR generated \textit{ruvbl2}^{Δ/Δ} mutants, designed and injected constructs and performed cryoinjuries for regeneration studies. SJ and KC performed genotyping of \textit{ruvbl2} mutants.
ABSTRACT
The limited proliferation of pre-existing cardiomyocytes in the mammalian heart remains a barrier to cardiac regeneration. In contrast, cardiomyocytes that comprise the zebrafish heart mount a robust proliferative response after injury, making the zebrafish an attractive model for understanding cardiac regeneration after injury. In order to identify critical regulators of cardiomyocyte proliferation, we used an embryonic model of proliferation in zebrafish and identified ruvbl2 as a critical regulator of cardiomyocyte proliferation. We generated whole locus deletion mutants of ruvbl2 in the zebrafish embryo and discovered that ruvbl2 is a negative regulator of cardiomyocyte proliferation. Furthermore, we found that ruvbl2 mutant ventricular cardiomyocytes enter cell cycle significantly more than their wild-type counterparts. Additionally, overexpression of ruvbl2 during regeneration is sufficient to dampen cardiomyocyte proliferation and impair regeneration after injury. Collectively, our study has identified a novel regulator of cardiomyocyte proliferation that may offer novel therapeutic avenues for promoting proliferation after cardiac injury.
INTRODUCTION

Myocardial infarction results in a significant loss of cardiomyocytes that compromises heart function. Proliferation of cardiomyocytes is required to regenerate lost tissue (Laflamme and Murry, 2005). However, limited proliferation of pre-existing cardiomyocytes in the adult mammalian heart remains a major barrier to cardiac regeneration (Bergmann et al., 2015; Senyo et al., 2013). In contrast, zebrafish cardiomyocytes are able to mount a robust proliferative response after cardiac injury to regenerate damaged myocardium (Foglia and Poss, 2016; lez-Rosa and Mercader, 2012; Poss et al., 2002). Dissecting the signals that support cardiomyocyte proliferation is critical to elucidating the mechanisms governing cardiac regeneration. However, we still have a limited understanding of the signals sufficient that regulate cardiomyocyte proliferation (Bersell et al., 2009; Gemberling et al., 2013; González-Rosa et al., 2017a; leda et al., 2009; Jackson et al., 1990; Pasumarthi, 2004). Therefore, studying factors regulating cardiomyocyte cell division will be critically important for achieving cardiac regeneration.

ruvbl2 (reptin) belongs to the family of AAA+ proteins associated with broad cellular activities including cell proliferation and viability (reviewed by (Gallant, 2007; Jha and Dutta, 2009)). Initially, ruvbl2 mutants were discovered in bacteria that exhibited defects in DNA repair and cell cycle (Otsuji et al., 1974; Sargentini and Smith, 1989). Across many species, ruvbl2 is a critical regulator of growth and viability (Carrie N et al. 2012; 1998) (Bereshchenko et al., 2012; 1998). Additionally, ruvbl2 is expressed dynamically during mitosis, where expression is diffuse during interphase and is then restricted to the mitotic spindle and midbody during late cell cycle (Gentili et al., 2015). Further evidence for ruvbl2 as an essential regulator of
cardiomyocyte proliferation has been demonstrated in the nucleus where \textit{ruvbl2} acts as a transcriptional regulator of the proto-oncogenes β-catenin and c-myc to drive cell proliferation (Bauer et al., 2000; Grigoletto et al., 2011; Wood et al., 2000). Overexpression of \textit{ruvbl2} increased cell proliferation though interactions with c-myc during Xenopus embryogenesis whereas knockdown of \textit{ruvbl2} resulted in a significant reduction of mitotic cells and early embryonic lethality (Wood M et al. 1999;) (Etard et al., 2005; Wood et al., 2000). Additionally, \textit{ruvbl2} overexpression is associated with oncogenesis, most notably in hepatocellular carcinoma and is often associated with overall poor prognosis in patients (Grigoletto et al., 2013; Raymond et al., 2015; Rousseau et al., 2007). Knockdown of \textit{ruvbl2} in mouse xenografts results in decreased tumour proliferation and overall size (Ménard et al., 2010). While there is substantial evidence for \textit{ruvbl2} in broadly regulating proliferation, very little is known about the tissue specific role of \textit{ruvbl2} in regulating proliferation.

A previous study identified a putative gain of function mutation within the \textit{ruvbl2} locus, \textit{ruvbl2\textsuperscript{ik}}, which resulted in an increased total number of ventricular cardiomyocytes in zebrafish embryos. Furthermore, cell transplantation experiments of \textit{ruvbl2\textsuperscript{ik}} mutant cardiomyocytes into wild-type recipient ventricles resulted in an expansion \textit{ruvbl2\textsuperscript{ik}} mutant cardiomyocytes, suggesting the possibility that \textit{ruvbl2} acts cell autonomously within cardiomyocytes to increase total cardiomyocyte numbers. These data also hinted at the possibility of \textit{ruvbl2} as a regulator of zebrafish cardiomyocyte proliferation. However, the role of \textit{ruvbl2} in regulating zebrafish cardiomyocyte proliferation remained previously unknown (Rottbauer et al., 2002). Here, we show that \textit{ruvbl2} is a negative regulator of zebrafish cardiomyocyte
proliferation. Ultimately, we provide evidence for a novel therapeutic target to induce cardiomyocyte proliferation after cardiac injury.
RESULTS

cuvbl2 regulates zebrafish cardiomyocyte cell numbers and proliferation rates

Previously, it was reported that a single point mutation within the endogenous cuvbl2 locus, cuvbl2lik, resulted in increased total ventricular myocytes (Rottbauer et al., 2002). However, the mechanism by which these cuvbl2 mutants increased cardiomyocyte cell numbers remained unknown. Cardiomyocytes in the developing heart go through two distinct phases of growth- differentiation and proliferation. In the developing zebrafish heart, differentiation of cardiomyocytes occurs until around 48 hpf (de Pater et al., 2009). After 48 hpf, the zebrafish heart begins to grow predominantly through cardiomyocyte proliferation (de Pater et al., 2009). To assess the cardiac phenotype of cuvbl2lik/lik mutants, we performed an in-cross of cuvbl2lik/+ adults carrying the Tg(cmlc2:nucGFP) transgene that labels all cardiomyocyte nuclei. cuvbl2lik/lik mutants exhibit a characteristic curved body phenotype and are found in Mendelian ratios (Figure 2.1.A-B). To learn if there were defects in cardiac specification of cuvbl2lik/lik mutants, we quantified GFP+ cardiomyocyte nuclei of cuvbl2lik/lik mutants and wild-type siblings at 48hpf (Figure 2.5.). Compared to wild-type siblings, cuvbl2lik/lik mutants exhibited no difference in the total number of ventricular cardiomyocytes at 48hpf (Figure 2.5.). Next, we quantified total ventricular cardiomyocyte numbers at 72 hpf, the time at which the heart is beginning to grow through cardiomyocyte proliferation, and found that cuvbl2lik/lik mutants had significantly more ventricular myocytes compared to controls (Figure 2.1.C-E). In order to determine if increased ventricular cardiomyocyte numbers of cuvbl2lik/lik mutants was due to increased proliferation rather than increased specification of cardiac progenitors, we performed a BrdU pulse from 48-72 hpf and found that
*ruvbl2*<sup>lik/lik</sup> mutant cardiomyocytes cycle 1.5 fold more than their wild-type siblings by 72hpf (Figure 2.1.F-H). These data demonstrate that the increased ventricular cardiomyocyte numbers in *ruvbl2*<sup>lik/lik</sup> mutants is due to increased proliferation rather than increased specification of cardiac precursors.
**Figure 2.1.** *ruvbl2* is cardiomyocytes exhibit increased cardiomyocyte proliferation. (A) wild-type and (B) *ruvbl2* embryos at 72 hpf respectively. (C-D) Confocal single plane projection images of control (C) and (D) and *ruvbl2* hearts at 72 hpf. (E) Quantification of total ventricular cardiomyocytes in WT and *ruvbl2* hearts; means represented as fold change mean±s.d, n=5 embryos per group; ***P<0.005 by Student’s T test. Single plane confocal projection images of (F) control and (G) *ruvbl2* hearts at 72 hpf. White boxes highlight regions of interest (F’,G’) and yellow arrows point to proliferating cardiomyocytes in (F’) control and (G’) *ruvbl2* hearts respectively. (H) Quantification of ventricular cardiomyocyte proliferation in control and *ruvbl2* hearts; means represented as fold change mean±s.d, n=6 embryos per group; *P<0.05 by Student’s T test. V, ventricle; AT, atrium; scale bars: 25 µm for confocal images and 100 µm for embryo images.
Figure 2.2. ruvbl2 is a negative regulator of cardiomyocyte proliferation. (A) Schematic of ruvbl2 locus deletion targeting strategy. sgRNAs (blue) were designed to target regions outside of the 5’UTR and 3’UTR of the endogenous ruvbl2 locus to generate a ruvbl2 locus deletion (ruvbl2ΔΔ). PCR primers (orange and purple) were designed to amplify a genomic region flanking the 5’UTR (orange) and 3’UTR (purple) that would otherwise be too large to amplify in an unedited WT allele. (B) Representative DNA gel of ruvbl2ΔΔ using genotyping primers. WT primers and ruvbl2ΔΔ genotyping primers are able to distinguish between WT allele and the ruvbl2ΔΔ allele. Brightfield images of (C) wild-type and (D) ruvbl2ΔΔ embryos at 72 hpf respectively. (E-F) Confocal projections of wild-type (E) and (F) ruvbl2ΔΔ hearts at 72 hpf. (G) Quantification of total ventricular cardiomyocytes in WT and ruvbl2ΔΔ hearts; means represented as fold change mean± s.d, n=6 embryos per group; ***P<0.005 by Student’s T test. Single plane confocal projection images of (H) control and (I) ruvbl2ΔΔ hearts at
Figure 2.2. continued. 72 hpf. White boxes highlight regions of interest (H’,I’) and yellow arrows point to proliferating Figure cardiomyocytes in control (H’) and ruvbl2Δ/Δ (I’) hearts respectively. (J) Quantification of cardiomyocyte proliferation in wild-type siblings compared with ruvbl2Δ/Δ mutants. (Data are represented as fold change mean ± s.d, n=6 embryos per group; *P<0.05 by Student’s T test. (K) Genetic complementation assay strategy for determining whether ruvbl2ΔΔ/ΔΔ is a loss of function mutation. Heterozygous adults ruvbl2ΔΔ/+ and ruvbl2ΔΔ/+ were in-crossed and embryos were analysed. Brightfield images of (L) wild-type and (M) ruvbl2ΔΔ/ΔΔ embryos at 72 hpf. Confocal projection images of (N) wild-type and (O) ruvbl2ΔΔ/ΔΔ embryos at 72 hpf. (P) Quantification of total ventricular cardiomyocytes in WT and ruvbl2ΔΔ/ΔΔ hearts; means represented as fold change mean ± s.d, n=6 embryos per group; ***P<0.005 by Student’s T test. Single plane projection confocal images of (Q) control and (R) ruvbl2ΔΔ/ΔΔ hearts at 72 hpf. White boxes highlight regions of interest (Q’,R’) and yellow arrows point to proliferating cardiomyocytes in (Q’) control and (R’) ruvbl2ΔΔ/ΔΔ hearts respectively. (S) Quantification of ventricular cardiomyocyte proliferation in control and ruvbl2ΔΔ/ΔΔ hearts; means represented as fold change mean ± s.d, n=6 embryos per group; *P<0.05 by Student’s T test. V, ventricle; AT, atrium; Scale bars: 25 µm for confocal images and 100 µm for embryo images.
Next, in order to determine if \textit{ruvbl2} was required for cardiomyocyte proliferation, we used CRISPR/Cas9 to create a deletion of the endogenous \textit{ruvbl2} locus (Figure 2.2.A). These locus deletion mutants, \textit{ruvbl2}^{Δ/Δ}, can be identified in Mendelian ratios by PCR amplification of genomic regions flanking the \textit{ruvbl2} locus (Figure 2.2.B). Additionally, \textit{ruvbl2}^{Δ/Δ} mutants survive until 4dpf and exhibit the curved body phenotype characteristic of \textit{ruvbl2}^{lik/lik} mutants (Figure 2.2.C-D). We performed an in-cross of \textit{ruvbl2}^{Δ/+} adults carrying the \textit{Tg(cmlc2:nucGFP)} transgene to visualize cardiomyocyte nuclei. At 48 hpf, we observed no difference in ventricular myocyte numbers between \textit{ruvbl2}^{Δ/Δ} mutants and their wild-type siblings (data not shown). However, differences between \textit{ruvbl2}^{Δ/Δ} mutants and their wild-type siblings arose at 72 hpf where \textit{ruvbl2}^{Δ/Δ} mutants exhibited 1.5 fold more ventricular cardiomyocytes compared to wild-type siblings (Figure 2.2.E-G). A BrdU pulse from 48 to 72 hpf revealed BrdU incorporation in \textit{ruvbl2}^{Δ/Δ} mutant cardiomyocytes 1.5 fold more than wild-type siblings (Figure 2.2.H-J). These data demonstrate that \textit{ruvbl2} acts as a negative regulator of cardiomyocyte proliferation in zebrafish.

Because the \textit{ruvbl2}^{Δ/Δ} mutation phenocopied the \textit{ruvbl2}^{lik/lik} mutation in the gross curved body morphology, cardiomyocyte proliferation rates and overall myocyte numbers, we sought to test the hypothesis that \textit{ruvbl2}^{lik} was a bona-fide loss of function mutation. Next, we performed a genetic non-complementation assay by in-crossing \textit{ruvbl2}^{+/lik} and \textit{ruvbl2}^{+/Δ} adults. We reasoned that if \textit{ruvbl2}^{lik/lik} was a gain-of-function mutation as had been previously described (Rottbauer et al., 2002), then the \textit{lik} allele would be sufficient to complement the \textit{ruvbl2} locus deletion allele and result in 100% of phenotypically wild-type animals. We therefore hypothesized that \textit{ruvbl2}^{lik/Δ} zebrafish embryos would look phenotypically wild-type and exhibit
normal rates of myocyte proliferation (Figure 2.2.K). After in-crossing \textit{ruvbl2}^{lik/+} and \textit{ruvbl2}^{Δ/+} adults, we observed that 25% embryos displayed a curved body phenotype (Figure 2.2.L-M). Additionally, we quantified the total ventricular cardiomyocytes and found a 1.3 fold increase in \textit{ruvbl2}^{lik/Δ} cardiomyocytes compared with wild-type siblings (Figure 2.2.N-P). To determine if these differences in ventricular cardiomyocyte numbers could be attributed to increased proliferation to increased proliferation, embryos were BrdU pulsed from 48 hpf to 72 hpf and we observed a 1.4 fold increase in BrdU incorporation of \textit{ruvbl2}^{lik/Δ} cardiomyocytes (Figure 2.2.Q-S). These data demonstrate that the \textit{ruvbl2}^{lik} is a loss-of-function mutation.

\textit{ruvbl2} acts in the myocardium and endocardium to suppress cardiomyocyte proliferation

To determine where in the developing heart \textit{ruvbl2} was expressed, we performed RNA scope \textit{in situ} hybridization in whole mount in wild-type zebrafish embryos at 72 hpf and probed for \textit{ruvbl2} transcripts as previously described (Figure 2.3.)(Gross-Thebing et al., 2014). In order to validate our technique, we tested our \textit{ruvbl2} probe in embryos over-expressing \textit{ruvbl2} specifically in cardiomyocytes as well as in \textit{ruvbl2}^{Δ/Δ} mutants (Figure 2.3.A-B). We observed \textit{ruvbl2} expression primarily in the developing endocardium (Figure 2.3.C’) and myocardium (Figure 2.3.C’’) at 72 hpf.

Based on our observation that \textit{ruvbl2} is expressed broadly in in the myocardium and endocardium, we sought to determine the tissue in which \textit{ruvbl2} regulates myocyte proliferation.
Given the cardiomyocyte phenotype in \textit{ruvbl2}^{\Delta\Delta} mutants described by Rottbauer et al., we first set out to test whether \textit{ruvbl2} was acting in cardiomyocytes to regulate proliferation. We generated and isolated transgenic lines in which \textit{ruvbl2} was overexpressed specifically in the myocardium Tg(cmlc2:nucGFP-P2A-ruvbl2) (\textbf{Figure 2.3.D}). In this transgenic line, \textit{ruvbl2} is expressed together with GFP in a bicistronic construct labels cardiomyocyte nuclei for performing quantifications of ventricular myocyte numbers and proliferation rates. Additionally, we generated a second transgene that overexpresses \textit{ruvbl2} in the endocardium of the developing zebrafish heart, Tg(fli1a:nucGFP-P2A-ruvbl2) (\textbf{Figure 2.3.D}). In this transgenic line, \textit{ruvbl2} is expressed together with GFP in a bicistronic construct that labels endocardial nuclei for analysing the effects of endocardial overexpression of \textit{ruvbl2} on myocardial proliferation. Embryos were then given a BrdU pulse from 48 hpf to 72 hpf. We found that overexpression of \textit{ruvbl2} in myocardium and endocardium was sufficient to rescue the hyperproliferation defect of \textit{ruvbl2}^{\Delta\Delta} mutants (\textbf{Figure 2.3.E-I}). These data demonstrate that \textit{ruvbl2} regulates the acts in both the myocardium and endocardium to regulate cardiomyocyte proliferation.
Figure 2.3. *ruvbl2* acts in the endocardium and myocardium to regulate cardiomyocyte proliferation. (A-C’’) Single plane confocal projection images using RNA scope *in situ* hybridization to detect *ruvbl2* transcripts. Projection confocal images of (A-A’) Tg+ myo *ruvbl2*Δ/Δ, (B-B’) *ruvbl2*Δ/Δ and (C-C’’) WT control hearts 72 hpf. White arrows highlight *ruvbl2* transcripts in embryos overexpressing *ruvbl2* in the myocardium (A-A’). Orange arrows point to *ruvbl2*+ cardiomyocytes in wild-type embryos and yellow arrows indicate an *ruvbl2* transcripts in the endocardium of wild-type embryos; n=3-4 embryos per group. (D) Schematic of transgenes constructed to overexpress *ruvbl2* in the myocardium and endocardium, respectively. (E-H) Single plane confocal projections of BrdU pulsed (E) wild-type, (F) *ruvbl2*Δ/Δ (G) cardiomyocyte specific *ruvbl2* overexpression: myo-*ruvbl2*Δ/Δ (H) endocardial specific *ruvbl2* overexpression: endo-*ruvbl2*Δ/Δ embryos at 72 hpf. (I) Quantification of ventricular cardiomyocyte proliferation comparing control, *ruvbl2*Δ/Δ, myo-*ruvbl2*Δ/Δ and endo-*ruvbl2*Δ/Δ hearts at 72 hpf; means represented as fold change mean±s.d, n=5-6 embryos per group; *P*<0.05 by Student’s T test. V, ventricle; AT, atrium; Scale bars: 25 µm for confocal images.
**ruvbl2 suppress zebrafish cardiomyocyte proliferation during regeneration**

To determine whether *ruvbl2* functions to suppress myocyte proliferation in the context of adult cardiac regeneration, we asked whether tissue-specific overexpression of *ruvbl2* in the myocardium would be sufficient to suppress cardiomyocyte proliferation during regeneration. We performed cryoinjury to animals carrying the Tg(cmlc2:ruvbl2) and Tg(fli1a:ruvbl2) transgenes and assessed proliferation and regeneration and 7dpi and 60dpi respectively (Figure 2.4.A). We observed a reduction in cardiomyocyte proliferation in animals overexpressing *ruvbl2* both in the myocardium and endocardium (Figure 2.4.B-E). Additionally, we observed a retention of the fibrotic scar when *ruvbl2* is expressed in either the endocardium or myocardium by 60 dpi (Figure 2.4.F-I). These data demonstrate that *ruvbl2* is acting as a negative regulator of proliferation in the regenerating myocardium and endocardium. Because the *cmlc2* and *fli1a* promoters are active throughout development, we next sought to determine whether broad overexpression of *ruvbl2* in the heart after development was completed would be sufficient to block regeneration after injury.

We generated and isolated a transgenic line in which *ruvbl2* is expressed under the temporal control of the heat shock promoter Tg(hsp70l: *ruvbl2*) (Figure 2.4.J). An increase in temperature from 28.5°C to 39°C activates the heat shock promoter which then drives the expression of *ruvbl2* and GFP. Zebrafish carrying the Tg(hsp70l: *ruvbl2*) transgene were cryoinjured and given a single heat shock once daily. At 7 dpi and 60 dpi, animals were analysed for cardiomyocyte proliferation and scarring respectively (Figure 2.4.J). Compared to wild-type, broad overexpression of *ruvbl2* was sufficient to dampen proliferation by 7dpi (Figure 2.4.K-M). Acid Fuchsin
orange G staining revealed defects in regeneration of Tg(hsp70l: ruvbl2) hearts. While wild-type hearts exhibit a thickened ventricular wall and minimal collagen deposition at the wound-site (highlighted by white asterisk), Tg(hsp70l: ruvbl2) hearts exhibited significantly more collagen deposition and have a disrupted and incompletely closed myocardial wall (highlighted by black asterisk) (Figure 2.4.N-P).
Figure 2.4. *ruvbl2* acts in the myocardium, endocardium and broadly throughout the adult heart to suppress cardiomyocyte proliferation during zebrafish cardiac regeneration. (A) Schematic of experimental design for regeneration experiments. Adult zebrafish carrying the Tg(cmlc2:ruvbl2) or Tg(fli1a:ruvbl2) to overexpress *ruvbl2* in the myocardium and endocardium respectively. Experimental endpoints were at 7dpi and 60dpi to assay for defects in cardiomyocyte proliferation and regeneration respectively. (B-D) Sections of WT, Tg(cmlc2:ruvbl2) and Tg(fli1a:ruvbl2) hearts immunostained for Mef2c and PCNA at 7dpi. Yellow arrows highlight proliferation cardiomyocytes. (E) Quantification of % cardiomyocyte proliferation at 7dpi. Means
Figure 2.4 continued. represented as fold change mean±s.d, n= hearts per group; *P<0.05 by one-way ANOVA. (F-H) Sections of (F) WT, (H) Tg(cmlc2:ruvbl2) and Tg(fli1a:ruvbl2) hearts stained with acid fuchsin orange G (AFOG) at 60dpi. (F'-H') Boxed regions highlight magnified regions in AFOG stained hearts. White asterisks highlight regenerated wound area. Black asterisks highlight fibrotic scar tissue. (I) Quantification of scar size in the ventricles of WT, Tg(cmlc2:ruvbl2) and Tg(fli1a:ruvbl2) hearts at 60 dpi. Means represented as fold change mean±s.d, n= 6 hearts per group; *P<0.05 by one-way ANOVA. (I) Schematic of experimental design for regeneration experiments. Adult zebrafish carrying the Tg(hsp70l:ruvbl2) transgene were given a single heat shock per day following cryoinjury to the ventricle. Experimental endpoints were at 7dpi and 60dpi to assay for defects in cardiomyocyte proliferation and regeneration respectively. (J-K) Sections of WT and Tg(hsp70l:ruvbl2) hearts immunostained for Mef2c and PCNA at 7dpi. Yellow arrows highlight proliferation cardiomyocytes (L) Quantification of % cardiomyocyte proliferation at 7dpi; means represented as fold change mean±s.d, n= 4-5 hearts per group; *P<0.05 by Student’s T (M-N) Sections of (M) WT and (N) Tg(hsp70l:ruvbl2) hearts stained with acid fuchsin orange G (AFOG) at 60dpi. (M'-N') Boxed regions highlight magnified regions in AFOG stained hearts. White asterisks highlight regenerated wound area. Black asterisks highlight fibrotic scar tissue. *P<0.05 by Student’s T test. V, ventricle; AT, atrium.
DISCUSSION

Substantial efforts have been given to the study of the critical regulators of cardiomyocyte proliferation in the hopes of improving patient outcomes after myocardial infarction. However, we still have a limited understanding of the intrinsic and extrinsic factors that regulate cardiomyocyte proliferation (Foglia and Poss, 2016; González-Rosa et al., 2017b). While most studies performed thus far have isolated critical positive regulators of zebrafish cardiomyocyte proliferation few studies have identified potent negative regulators of cardiomyocyte proliferation (Engel, 2005; Evans-Anderson et al., 2008; Mohamed et al., 2018).

We took advantage of the ruvbl2lik mutant, the first loss of function mutation characterized to date that results in an overproduction of cardiomyocytes. By establishing that ruvbl2 is a negative regulator of CM proliferation, we have added to the repertoire of critical regulators of cardiomyocyte proliferation. Building on early observations from Rottbauer et al. 2002 of reptin lik mutants, we sought to we examine the role of ruvbl2 in zebrafish cardiomyocyte proliferation. Here, we demonstrate that ruvbl2 is a negative regulator of cardiomyocyte proliferation in zebrafish. Moreover, we demonstrate that myocardial or endocardial ruvbl2 overexpression is sufficient to dampen cardiomyocyte proliferation and rescue the cardiomyocyte proliferation defect in ruvbl2Δ/Δ mutants and during cardiac regeneration. Our results implicate ruvbl2 as a critical regulator for supporting zebrafish cardiomyocyte proliferation.

Further studies are required to determine the transcriptional targets of ruvbl2 that might be acting in the endocardium or myocardium to regulate cell cycle in cardiomyocytes. ruvbl2 has been widely described to be a required component of a
number of protein complexes such as the INO80 chromatin remodelling complex reviewed by (Gallant, 2007; Hota and Bruneau, 2016; Jin et al., 2005; Jónsson et al., 2001; Mizuguchi et al., 2004). It will also be important to determine whether or not ruvbl2 functions as part as one of these chromatin remodelling complexes in either the endocardium or myocardium for regulating proliferation. Ultimately, our study offers a novel therapeutic avenue for modulating ruvbl2 to promote cardiomyocyte proliferation after cardiac injury.
MATERIALS AND METHODS

Zebrafish husbandry
Zebrafish were grown and maintained according to animal protocols approved by the Massachusetts General Hospital and Boston Children’s Hospital Institutional Animal Care and Use Committee. The following zebrafish strains were utilized:

Tg(cmlc2:nls-GFP)fb18 (González-Rosa et al., 2018), ruvbl2fk (Rottbauer et al., 2002) and wild-type (TuAB). Adult densities were maintained at around 3-4 fish per liter. For all experiments, zebrafish water temperature was maintained at 28°C unless raised to 39°C for heat shock treatments.

Genotyping of Mutant Lines
Zebrafish fins were clipped and DNA was extracted through alkaline lysis. Fins were boiled in a lysis solution (2N NaOH, 0.05M EDTA and ddH2O) for 20 minutes at 95°C, neutralized in 1M Tris·HCl (pH 5.0) and stored at -20°C. PCR amplification was performed to identify animals carrying mutant alleles. For each genotype, an in-cross of heterozygous adult animals was performed to generate homozygous mutant embryos which could be identified by gross curved-body morphology, a 25% Mendelian distribution and PCR based amplification.

Identifying and ruvbl2Δ/Δ mutant embryos
The genotyping strategy for distinguishing homozygous ruvbl2Δ/Δ mutants from heterozygous and wild-type siblings was performed as described (Figure 1I). An in-
cross of previously identified \textit{ruvbl2}^{+/\Delta} heterozygous animals was performed to generate homozygous \textit{ruvbl2}^{\Delta/\Delta} mutants embryos. Two genomic primers (\textit{ruvbl2} genomic-1 and genomic -2) that amplified regions outside of the sgRNA cut sites were generated (Figure 1I). Because the endogenous \textit{ruvbl2} locus is 12kb, primers are unable to amplify a large enough genomic region. Therefore, amplification using our genomic primers only occurs if there is a complete locus deletion. The following primers were used:

\begin{verbatim}
ruvbl2 genomic-1: TCCAGAACTCATGTAGACGGT
ruvbl2 genomic 2: GTGGCTTGAGGGTCATGAGA
\end{verbatim}

The PCR program used for distinguishing homozygous \textit{ruvbl2}^{\Delta/\Delta} mutants from heterozygous and wild-type siblings was the following: 1) denaturation: 94°C for 3 minutes, 2) annealing: 94°C for 30 seconds, 3) and elongation at 72°C for 40 seconds. A total of 34 cycles were used. A 266bp product is generated in \textit{ruvbl2}^{\Delta/\Delta} heterozygous animals and no band is generated in wild-type siblings.

\textit{Genotyping of ruvbl2}^{ijk} \textit{heterozygous adult fish}

\textit{ruvbl2}^{ijk} mutants were generated by an \textit{N-ethyl_N-nitrosourea} (ENU) chemical screen. The \textit{ruvbl2}^{ijk} mutant is a point mutation that results in a frame shift insertion of 9bp and formation of a novel splice acceptor site (Rottbauer et al. 2002) For distinguishing \textit{ruvbl2}^{ijk} heterozygous from wild-type siblings, the following primers were used:

\begin{verbatim}
ruvbl2^{ijk} FOR
5’GCCAAACCTCATATTCCAGGCTTTTCATGTGCTTAAATGTTAAATGACCTCATAATGTCATATTTCAG-3’
\end{verbatim}
ruvbl2\textsuperscript{lik} REV

5’ GAAGTTCCCCCTCTGGACACTGCACAAACTGCG-3’

The PCR program used for distinguishing homozygous ruvbl2\textsuperscript{lik} heterozygous animals from wild-type siblings was the following: 1) denaturation: 95°C for 5 minutes, 2) annealing: 95°C for 30 seconds, 3) and elongation at 65°C for 30 seconds. A total of 34 cycles were used. A 309bp product is generated in ruvbl2\textsuperscript{lik} heterozygous animals and no band is generated in wild-type siblings. To obtain homozygous ruvbl2\textsuperscript{lik} homozygous mutants, an in-cross of previously identified ruvbl2\textsuperscript{lik} heterozygous adults was performed. Homozygous ruvbl2\textsuperscript{lik} mutants were identified by characteristic curved body phenotype as previously demonstrated (Rottbauer et al. 2002).

Generation of Transgenic Lines

(1) Generation of Tg(cmlc2:nlsEGFP-P2A-ruvbl2) line

To generate the Tg(cmlc2:EGFP-P2A-ruvbl2) line, Gibson assembly was performed to generate the following construct: (1) 0.9 kb of the cmlc2 promoter to drive expression of (2) a nuclear localized enhanced green fluorescent protein (nlsEGFP); in a bicistronic cassette encoding (3) the zebrafish ruvbl2 coding sequence followed by (4) a polyadenylation sequence in cardiomyocytes. Tol2 sites flanked the entire construct. Line complete name according to zfin archives.

(2) Generation of Tg(fli1a:nlsEGFP-P2A-ruvbl2) line

To generate the Tg(fli1a:EGFP-P2A-ruvbl2) line, Gibson assembly was performed to generate the following construct: (1) \(\approx\) 7 kb the fli1a promoter (Lawson et al., 2001) to
drive expression of (2) a nuclear localized enhanced green fluorescent protein (nlsEGFP); in a bicistronic cassette encoding (3) the zebrafish ruvbl2 coding sequence followed by (4) a polyadenylation sequence in endocardial cells. Tol2 sites flanked the entire construct. Line complete name according to zfin archives.

(3) Generation of Tg(hsp70l:3XHA-ruvbl2) line

To generate the Tg(hsp70l:3XHA-ruvbl2) line, Gibson Assembly was performed to generate the following construct: (1) ≈1.5 kb of the hsp70l promoter (Kwan et al., 2007) was cloned/subcloned to drive expression of (2) three copies of the HA tag fused to the N-terminus of zebrafish ruvbl2 (3XHA-ruvbl2) followed by a (3) Xenopus β-globin 3’ UTR, a (4) bGH polyadenylation signal and (5) A transgensis control in which GFP is expressed under the control of the Elf1α promoter in the opposite orientation of the heat-shock cassette. Tol2 sites flanked the entire construct. Line complete name according to zfin archives. Minimums of three independent founders were isolated to validate consistent expression patterns and phenotypes.

Heat shock treatments

Heterozygous ruvbl2+/Δ animals hemizygous for the Tg(hsp70l:3XHA-ruvbl2) allele were given a single heat shock per following injury. Each day, animals were heat-shocked for 1 hour at 39°C to activate the heat shock transgene and then returned to 28.5°C as previously described (Lee et al., 2005). At 60 dpi, animals were sacrificed and fixed in 4% PFA in preparation for histology.
**BrdU Proliferation Assay**

Zebrafish embryos were pulsed with 5-Bromo-2′deoxyuridine (BrdU) (5 mg/mL in 1% DMSO, Sigma, USA) from 48 to 72 hpf. BrdU staining was performed as previously described (Jahangiri et al., 2016; Tkatchenko, 2006). To generate a proliferation index, total MF20+ cardiomyocyte nuclei co-labeled with BrdU were divided by the total number of MF20+ cardiomyocyte nuclei in zebrafish embryonic hearts at 72 hpf.

**Whole mount immunofluorescence**

Whole-mount immunostaining was performed as previously described (Jahangiri et al., 2016). Primary antibodies used were anti-DsRed (1:500, Clontech, USA), anti-Tropomyosin (clone CH1, 1:100, Developmental Studies Hybridoma Bank, USA), rat anti-BrdU (1:100, Abcam, USA), mouse anti-BrdU (1:50, Sigma, USA), mouse anti-GFP (1:50, Santa Cruz, USA), rabbit anti-GFP (anti-Ruvbl2 (1:200, Abcam, USA), anti-myosin heavy chain (1:50 clone MF20, Developmental Studies Hybridoma Bank, USA). Alexa (405, 488, 555, 647)-conjugated antibodies (Invitrogen, USA) were used to reveal primary antibody signal. Nuclei were stained with DAPI (1:1000, Life Technologies, USA).

**Whole Mount RNA Scope in situ hybridization**

RNA Scope ® (ACDbio) in situ hybridization was performed as previously described by (Gross-Thebing et al., 2014) for zebrafish embryos. Probes for *ruvbl2* were purchased from ACDbio.
Regeneration studies

All regeneration studies and immunofluorescence of adult tissues were performed as previously described (González-Rosa et al., 2011). Acid fuchsin-orange G (AFOG) staining was performed as previously described (Zhao et al., 2014) in adult hearts. Muscle, fibrin and collagen were stained brown-orange, red and blue respectively. Scar size was quantified as previously described (González-Rosa et al. 2018).

Imaging

Zebrafish embryos were embedded in 0.9% low-melt agarose (Lonza, USA) and imaged on a Nikon Eclipse Ti confocal microscope with a 40x Nikon Plan Apo water objective. Data were analyzed using Fiji software (Schindelin et al., 2012).

Statistical Analysis

All experiments were analyzed using the mean ± standard deviation (SD). Significance was calculated using an unpaired Student’s T-test for comparing the mean between two groups. A one-way analysis of variance (ANOVA) was performed to calculate the mean of three or more groups. For analysis of the phenotypic and genotypic distribution frequencies of ruvbl2 embryos, a Chi-square goodness of fit test was performed. Data were analyzed using GraphPad Prism 6 software. All experimental values with p<0.05 were considered to be significant.
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CHAPTER 3

The AP-1 transcription factor component fosl2 potentiates the rate of myocardial differentiation from the zebrafish second heart field.
ATTRIBUTIONS

Contributions to the work described in this chapter were made by Leila Jahangiri, Michka Sharpe, Natasha Novikov, Juan Manuel González, Asya Borikova, Kathleen Nevis, Noelle Paffett-Lugassy, Long Zhao, Meghan Adams, Burcu Guner-Ataman, Caroline Burns and Geoffrey Burns.

CEB, CGB and LJ conceived of and designed the study. LJ generated fosl2 mutants and performed immunofluorescence on zebrafish embryos. MS performed immunofluorescence staining, genotyping, EdU proliferation assays, confocal microscopy, ventricular resection injuries of fosl2 mutants, sectioning and AFOG staining of fosl2 mutant ventricles. JMGR performed TUNEL staining, confocal microscopy and prepared figures. NPL, NN, BGA, MA and KN performed RNA in situ hybridizations. AB performed Western Blot analyses. LZ performed qPCR analyses. CEB and CGB prepared figures. MS contributions culminated in second authorship.
ABSTRACT

In vertebrates, the muscle layer of the embryonic heart forms through two successive phases of cardiomyocyte differentiation. Initially, cardiomyocytes derived from first heart field (FHF) progenitors assemble the linear heart tube. Thereafter, second heart field (SHF) progenitors differentiate into cardiomyocytes that elongate the heart tube through accretion to the poles over a well-defined developmental window. Although deficiencies in heart tube extension can lead to life-threatening congenital heart defects, the variables controlling the initiation, rate, and duration of SHF-mediated myocardial accretion remain obscure. Here, we demonstrate that a component of the dimeric AP-1 transcription factor, Fos-like antigen 2 (Fosl2), potentiates the rate of myocardial accretion from the zebrafish SHF. Fosl2 null embryos initiate myocardial accretion appropriately, but the production of SHF-derived ventricular cardiomyocytes is sluggish, resulting in a ventricular myocardial deficit coupled with an accumulation of undifferentiated progenitors. These findings demonstrate that Fosl2 promotes the progenitor to cardiomyocyte transition. Surprisingly, mutant embryos eventually correct the myocardial deficit by extending the differentiation window. Lastly, overexpression of Fosl2 also compromises the production of SHF derived ventricular cardiomyocytes, a phenotype consistent with precocious depletion of the progenitor population. Taken together, our data implicate the AP-1 transcription factor complex in maximizing the rate of cardiomyocyte production from the SHF and uncover the existence of regulatory mechanisms to ensure that a precise number of SHF-derived cardiomyocytes are produced irrespective of embryonic stage.
INTRODUCTION

During vertebrate embryogenesis, cardiac muscle derives from progenitor cells in two successive waves [reviewed in (Abu-Issa and Kirby, 2007; Vincent and Buckingham, 2010)]. The earliest cardiomyocytes differentiate bilaterally in lateral plate mesoderm (LPM) from first heart field (FHF) progenitors, migrate to the midline, and assemble the linear heart tube. By contrast, second heart field (SHF) progenitors remain undifferentiated in LPM and come to reside in pharyngeal mesoderm where the poles of the linear heart tube attach to the embryo proper. Over a well-defined developmental window, SHF progenitors differentiate into nascent cardiomyocytes that are added progressively to the poles of the heart tube (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Termed myocardial accretion, this process significantly elongates the heart tube through the de novo production of new myocardial segments. At the arterial pole, SHF progenitors produce myocardium for the right ventricle and outflow tract (OFT) (Kelly et al., 2001; Rana et al., 2007; Verzi et al., 2005). They also produce a collar of smooth muscle at the base of the aortico-pulmonary trunk (Harmon and Nakano, 2013; Waldo et al., 2005). To balance cellular egress from the SHF resulting from cardiomyocyte differentiation, SHF cells proliferate to maintain the progenitor pool (Cai et al., 2003; Hutson et al., 2010; Tirosh-Finkel et al., 2010; van den Berg et al., 2009).

Genetic or environmental insults that compromise myocardial accretion at the arterial pole impede formation of the RV and OFT and lead to embryonic lethality (Both et al., 2004; Cai et al., 2003; Prall et al., 2007). Milder defects leave the embryonic OFT shortened and susceptible to misalignment with the ventricles (Ward et al., 2005; Yelbuz et al., 2002). OFT misalignment can result in rightward shifting of
the aorta at birth, a defining feature of the congenital heart defects Tetralogy of Fallot and Double Outlet Right Ventricle (Nakajima, 2010). Therefore, subtle disturbances in SHF-mediated myocardial accretion are sufficient to cause serious congenital heart defects.

Successive phases of cardiomyocyte differentiation also generate the embryonic myocardium in zebrafish. Within the first 24 hours post-fertilization (hpf), FHF-derived cardiomyocytes form the linear heart tube comprising both atrial and ventricular cardiomyocytes (de Pater et al., 2009; Lazic and Scott, 2011; Yelon et al., 1999). Between 24 and 48 hpf, SHF progenitors differentiate into cardiomyocytes that are progressively accreted to the heart tube’s arterial pole (de Pater et al., 2009; Hami et al., 2011; Lazic and Scott, 2011; Zhou et al., 2011). SHF progenitors also proliferate during this developmental window (Nevis et al., 2013; Zeng and Yelon, 2014; Zhou et al., 2011). When accretion is complete, the FHF and SHF-derived cardiomyocytes inhabit roughly the proximal and distal halves of the ventricular myocardium, respectively. As in higher vertebrates, SHF progenitors in zebrafish also give rise to myocardium and smooth muscle in the OFT (Hami et al., 2011; Zeng and Yelon, 2014; Zhou et al., 2011). After SHF-mediated accretion is complete, the zebrafish ventricle grows through cardiomyocyte proliferation (Choi et al., 2013; Liu et al., 2010).

As a point of convergence for many signaling pathways, the dimeric transcription factor AP-1 has been implicated in myriad molecular, cellular, developmental and pathologic processes [reviewed in (Eferl and Wagner, 2003)]. It comprises members of the c-Fos and c-Jun protein families and functions as a transcriptional activator or repressor depending on biological context (Suzuki et al.,
1991). Prior work has implicated AP-1 in SHF-mediated OFT morphogenesis because c-jun null mice are born with rightward shifting of an unseptated OFT (Eferl et al., 1999). Conditional deletion of c-jun within Isl1-expressing cells, including SHF progenitors also causes rightward shifting of the aorta (Zhang et al., 2013b). Therefore, despite genetic evidence that AP-1 functions within SHF progenitors to ensure correct alignment of the embryonic OFT, the cellular basis of OFT misalignment in c-jun null mice remains incompletely understood. Moreover, because c-fos-family member knockout mice have not been reported to exhibit defects in cardiac morphogenesis, (Eferl and Wagner, 2003; Karreth et al., 2004), a potential role for Fos proteins in AP-1-dependent OFT alignment also remains unclear. Lastly, although an in vitro study implicated the Fos family member Fos-like (or related) antigen 2 (Fosl2 or Fra-2) and AP-1 complexes in the regulation of cardiomyocyte differentiation, this function has yet to be corroborated in vivo. To learn if fosl2 regulates heart development in zebrafish, we created and characterized fosl2 null and overexpressing zebrafish embryos. Our work implicates Fosl2-containing AP-1 complexes in potentiating the rate of cardiomyocyte differentiation specifically from SHF progenitors.
RESULTS

Isolation of fosl2 null alleles

Fosl2 is a highly conserved protein across vertebrate species. To understand the function of Fosl2 during zebrafish development, we analyzed embryos injected with an anti-sense morpholino targeting a splice site in the fosl2 pre-mRNA. When compared to control animals, 72 hpf morphants displayed pericardial edema and smaller ventricles (Fig. 3.1.A-D), suggesting preliminarily that Fosl2 supports ventricular morphogenesis and/or growth. However, because multiple studies have reported discrepancies between morphant and genetic loss-of-function phenotypes (Broeder et al., 2009; Hinits et al., 2012; Kok et al., 2015; Rossi et al., 2015; Wright et al., 2004), we opted to isolate null alleles of fosl2 using TALEN-mediated genome editing before implicating Fosl2 in ventricular development.

The first mutant allele we isolated, fosl2b15, carries an 18bp deletion that removes six amino acids near the N-terminus (Fig. 3.1.E). Homozygous fosl2b15 mutants express significantly lower levels of fosl2 transcript (Fig. 3.1.F) demonstrating that the deletion undermines transcript production and/or stability. Consistent with this finding, fosl2b15 mutant embryos have severely reduced Fosl2 as revealed through western blotting analysis (Fig. 3.1.G) using an antibody we raised against the C-terminus of the zebrafish protein (Fig. 3.1.E). The second mutant allele we isolated, fosl2b16, contains a 16bp deletion that shifts the open reading frame after codon 16 of 341 total. As a result, 16 additional divergent amino acids are encoded prior to a premature stop codon (Fig. 3.1.E). As expected, we did not detect Fosl2 protein in fosl2b16 mutant embryos (Fig. 3.1.G) because the antibody’s epitopes are truncated by the deletion (Fig. 3.1.E). Together, the severe
reductions in mRNA and protein observed in fosl2\textsuperscript{b15} homozygous embryos, the confirmed magnitude of the Fosl2\textsuperscript{b16} truncation, and the observation that both classes of mutants display indistinguishable phenotypes (see below) suggests strongly that both deletions create null alleles.

\textit{Fosl2 null embryos exhibit small ventricles comprising fewer cardiomyocytes}

When we analyzed cardiac chamber morphology, we learned that mutant ventricles appeared grossly smaller than those of siblings (Fig. 3.1.H,I), a finding consistent with the morphant phenotype (Fig. 3.1.C,D). Quantification of cardiomyocytes at 48 hpf revealed that both alleles reduced ventricular cell number by \textasciitilde18\% (Fig. 3.2.A-D) while gross atrial size (Fig. 3.1.C,D) and cell number (Fig. 3.1.A-D) were unaffected. Because ventricular and OFT morphogenesis are interrelated developmental processes (Hami et al., 2011; Zhou et al., 2011), we analyzed mutants for the production of Elastin2 (Eln2) by OFT smooth muscle cells (Grimes et al., 2006; Miao et al., 2007). At 52 hpf, mutant OFTs were devoid of Eln2 when control animals were positive (Fig. 3.2. E,F). Taken together, our data demonstrate that inactivation of zebrafish Fosl2 causes cardiomyocyte and smooth muscle marker deficiencies in the ventricle and OFT, respectively.
Figure 3.1. – *fosl2* null embryos exhibit defects in cardiogenesis. (A,B) Bright field images of control (CTRL; A; n=44) and *fosl2* morphant (MO*^{fosl2}; B; n=35) embryos at 72 hours post fertilization (hpf). Morphant embryos exhibit pericardial edema (black arrow). (C,D) Confocal images of GFP+ hearts in 72 hpf control (C; n=6) and *fosl2* morphant (D; n=9) *Tg(cmlc2:GFP)* embryos. (E) Schematic diagrams of wild-type (WT) zebrafish Fosl2 with its basic leucine zipper domain (bZip) and the predicted protein products of two *fosl2* mutant alleles, *fosl2^{fb15}* and *fosl2^{fb16}* generated through TALEN-mediated genome editing. The black line highlights the C-terminal region of the protein used to raise polyclonal antiserum. (F) Bar graph showing the relative levels of *fosl2* mRNA in control and *fosl2^{fb15}-/-* embryos at 48 hpf as measured by quantitative PCR (n=6 biological replicates per group). Error bars represent one standard deviation. ****p<0.0001. (G) Western blots of 30 hpf whole-embryo lysates from control and mutant animals probed with Fosl2 (zFosl2) or α-Tubulin (a-Tub) antiserum. (H,I) Confocal images of GFP+ hearts in 42 hpf control (H; n=10) and mutant (I; n=4) *Tg(cmlc2:GFP)* embryos. V, ventricle. A, atrium. Scale bars: 50mm.
To narrow the developmental window during which the ventricular deficit emerges, we counted cardiomyocytes at 24 hpf when differentiation of the FHF-derived linear heart tube is largely complete (de Pater et al., 2009; Lazic and Scott, 2011). From this analysis, we learned that mutant heart tubes are indistinguishable from those in control animals (Fig. 3.2.G-J). This observation supports three conclusions: 1) the ventricular deficiency in null animals becomes evident between 24 hpf and 48 hpf, 2) inactivation of fosl2 does not negatively effect the rate of FHF progenitor differentiation, and 3) fosl2 null embryos do not suffer from generalized developmental delay, a finding confirmed independently by assaying the rate of hepatobiliary morphogenesis.

**Impaired cardiomyocyte differentiation from SHF progenitors in fosl2 null embryos**

Next, we sought to decipher the cellular mechanism(s) underlying the ventricular deficiency that emerges in mutant animals between 24 and 48 hpf. In theory, a decrease in cardiomyocyte proliferation might explain fewer ventricular cells. However, previous work has demonstrated that cardiomyocyte proliferation is virtually non-existent in zebrafish embryos during this time frame (de Pater et al., 2009). We also ruled out increased cardiomyocyte apoptosis as a potential cause of the cellular deficiency.
Figure 3.2. – A ventricular cardiomyocyte deficit emerges in fosl2 mutant embryos subsequent to unperturbed linear heart tube morphogenesis. (A-C) Confocal images of fluorescent cardiomyocyte nuclei in hearts of 48 hour post fertilization (hpf) control sibling (CTRL; A; n=11), fosl2fb15-/- (B; n=5), and fosl2fb16-/- (C; n=5) Tg(cmlc2:dsRed2-nuc) embryos. (D) Bar graph showing average total, atrial, and ventricular cardiomyocyte numbers in each experimental group. Error bars represent one standard deviation. **p<0.01. n.s., not significant. ****p<0.0001. (E,F) Confocal images of ventricular and outflow tract (OFT) regions of 52 hpf control (E; n=10) and null mutant (F, n=4) embryos co-stained with antibodies recognizing striated muscle (MF20, red) or OFT smooth muscle (a-Elastin2, ELN2, green). (G-I) Confocal images of fluorescent cardiomyocyte nuclei in linear heart tubes of 24 hpf control (G; n=12), fosl2fb15-/- (H; n=5), and fosl2fb16-/- (I; n=5) Tg(cmlc2:dsRed2-nuc) embryos. (J) Bar graph showing average cardiomyocyte numbers in each experimental group at 24 hpf. Error bars represent one standard deviation. n.s., not significant. V, ventricle. A, atrium. Scale bars: 50mm.
Because the ventricular cardiomyocyte deficit emerges during the SHF-mediated myocardial accretion window, we next assayed SHF function directly in fosl2 mutants. To that end, we utilized a cardiomyocyte photoconversion assay (de Pater et al., 2009; Lazic and Scott, 2011) to quantify FHF- and SHF-derived ventricular cardiomyocytes at 48 hpf. The assay employs a transgenic strain, Tg(myl7:nlsKikGR) that continuously expresses the photoconvertible Kikume Green-Red (KikGR) protein in cardiomyocyte nuclei (Lazic and Scott, 2011). At 24 hpf, FHF-derived cardiomyocyte nuclei are labeled with photoconverted red protein by exposure to UV light. At 48 hpf, FHF-derived cardiomyocytes retain the red label while simultaneously expressing newly synthesized green KikGR. On the contrary, SHF-derived cardiomyocytes express green KikGR exclusively because they differentiate after photoconversion. Ultimately, counting the numbers of red and green cardiomyocyte nuclei in the proximal ventricle and green-only cardiomyocyte nuclei in the distal ventricle reveals the numbers of FHF- and SHF-derived cells, respectively.

Consistent with a previous report (Lazic and Scott, 2011), we found that ~40% of cardiomyocytes in wild-type ventricles are derived from the SHF at 48 hpf (Fig. 3.3.A-C,G). In fosl2 mutants, this percentage decreased to 28%, revealing an almost 30% reduction in SHF-derived cardiomyocytes (Fig. 3.3.D-G). To evaluate the status of SHF progenitors themselves, we performed in situ hybridization for the SHF marker ltbp3 (Zhou et al., 2011). Mutant embryos harbored qualitative increases in ltbp3 transcripts (Fig. 3.3.H,I) that we quantified as 2-fold higher with qPCR (Fig. 3.3.J). Mutant embryos also expressed elevated levels of mef2cb (Fig. 3.3.J), an additional marker of SHF progenitors (Lazic and Scott, 2011). We quantified SHF
progenitor numbers by counting extra-cardiac nkh2.5+ cell nuclei adjacent to the arterial pole (Paffett-Lugassy et al., 2013; Zhou et al., 2011) and learned that the elevation in SHF markers reflected a 38% increase in SHF progenitor cells. This accumulation cannot be explained by an increase in SHF progenitor proliferation. Taken together, our data demonstrate that mutant embryos accumulate SHF progenitors at the expense of their differentiated progeny. These data support a model in which Fosl2 directly or indirectly facilitates the SHF progenitor to cardiomyocyte transition at the arterial pole.

Consistent with a direct role, we learned that 24 hpf zebrafish embryos express fosl2 transcripts on both the ventricular and extra-cardiac sides of the arterial pole. At later time points, fosl2 expression is less evident in the heart proper but remains strongly expressed on the extra-cardiac side where SHF progenitors reside. Fosl2 transcripts were also distributed broadly throughout the head but sparsely in the trunk and tail. Transcripts encoding c-Jun, a potential AP-1 binding partner for Fosl2 (Eferl and Wagner, 2003) were also observed on both sides of the linear heart tube’s arterial pole and later in the extra-cardiac region inhabited by SHF progenitors. The similarities between fosl2 and c-jun expression are consistent with the possibility that Fosl2 pairs with C-Jun to form the AP-1 complex that potentiates the rate of cardiomyocyte differentiation from the zebrafish SHF.
Figure 3.3. – Fosl2 potentiates the progenitor to cardiomyocyte transition during SHF-mediated ventricular growth. (A-G) Cardiomyocyte photoconversion assay. Control sibling (CTRL; A-C; n=23) and fosl2^{fb16/-} (D-F; n=6) Tg(myl7:nlsKikGR) embryos were photoconverted at 24 hours post fertilization (hpf) and imaged by confocal microscopy at 48 hpf in the red (A,D) and green (B,E) channels. Merged images are shown in (C, F). Arrowheads highlight SHF-derived green-only cardiomyocytes. (G) Bar graph showing the average numbers of total ventricular cardiomyocytes, green and red positive cardiomyocytes, and green only cardiomyocytes. Error bars represent one standard deviation. ****p<0.0001. n.s., not significant. (H,I) Ventral images of 48 hpf control (H; n=16) and fosl2 null (I; n=5) embryos stained with a riboprobe for the SHF marker ltbp3. Arrowheads highlight extra-cardiac SHF progenitors. (J) Bar graph showing relative levels of the SHF markers ltbp3 (n=9 biological replicates per group) and mef2cb (n=3 biological replicates per group) in control and mutant embryos at 48 hpf as measured by quantitative PCR. Error bars represent one standard deviation. ***p<0.001. *p<0.05. (K,L) Confocal images of the arterial poles in control (K; n=12) and fosl2 mutant (L; n=4) embryos carrying the Tg(nkx2.5:nZsYellow) and Tg(cmlc2:GFP) transgenes co-stained with antibodies recognizing ZsYellow (red) or GFP (green). (M) Bar graph showing the average numbers of extra-cardiac SHF progenitor cells (red nuclei without yellow cytoplasm) in both
Figure 3.3. continued. Experimental groups. Error bars represent one standard deviation. ***p<0.001. (N-R) Double in situ hybridization analysis of fosl2 (blue) and cmic2 (red) in whole mounted (N-Q) and sagitally cryosectioned (R) 24 hpf (N,O) and 36 hpf (P-R) embryos. Anterior to the left in (N-P, R). Boxed region in (N) is magnified in (O). (Q) shows an anterior and dorsal view of the embryo following removal of the head. Open arrowheads in (O), (Q), and (R) highlight fosl2+ cells on either side of the arterial pole (AP; closed arrow heads). Greater than 20 embryos per group were evaluated. VP, venous pole. Scale bars: 50mm.

Fosl2 null embryos overcome sluggish accretion by extending the SHF differentiation window

To understand the natural course of the fosl2−/− cardiac phenotype, we evaluated mutant embryos at 72 hpf for ventricular cardiomyocyte numbers and OFT Eln2 expression. Unexpectedly, mutant embryos were indistinguishable from control siblings (Fig. 3.4.A-E), demonstrating that phenotypic resolution had occurred between 48 and 72 hpf. Consistent with a full phenotypic recovery, mutant animals reach adulthood in the expected Mendelian ratios and are fertile. Adult animals are indistinguishable from siblings with regard to animal size, heart size, heart morphology, and cardiac regenerative capacity. By contrast, the ventricular cardiomyocyte deficiency observed in fosl2 morphants at 48 hpf persists at 72 hpf when severe OFT deficiencies also become evident (Fig 3.1.A-C, ). The discordance between mutant and morphant phenotypes at 72 hpf suggests that morpholino toxicity and/or off-target effects contribute to the morphant phenotype or that genetic mutants are uniquely equipped to deploy compensatory mechanisms (Rossi et al., 2015). To test the former, we injected fosl2−/− embryos with the fosl2 morpholino and evaluated the compound mutant-morphant embryos for OFT deficiencies at 72 hpf.
Figure 3.4. – *fosl2* mutants recover from their ventricular deficit. (A,B) Confocal images of hearts from 72 hours post fertilization (hpf) control sibling (CTRL; A; n=12) and *fosl2* (B; n=5) mutant Tg(cmlc2:dsRed2-nuc) embryos. (C) Bar graph showing the average numbers of total, atrial, and ventricular cardiomyocytes in control, *fosl2*<sup>b15</sup>-/-, and *fosl2*<sup>b16</sup>-/- (n=3) embryos. Error bars represent one standard deviation. n.s., not significant. (D,E) Confocal images of OFT regions in control (D; n=15) and *fosl2* mutant (E; n=4) embryos co-stained with antibodies recognizing striated muscle (MF20, red) or OFT smooth muscle (anti-Elastin2, ELN2, green). Scale bars: 50mm.
The OFT phenotype of compound mutant-morphants was indistinguishable from pure morphants demonstrating that the OFT deficiencies observed in morphants arise from morpholino toxicity and/or off-target effects independent of knocking down fosl2 protein expression (Fig.S6).

Next, we sought to determine why fosl2 embryos exhibit a deficit in SHF derived ventricular cardiomyocytes at 48 hpf. First, we tested the hypothesis that mutant embryos exhibit a delay in initiating the accretion process. To that end, we photoconverted and analyzed control and mutant hearts immediately prior to (23 hpf) and shortly after (32 hpf) accretion commences, respectively. During this short time frame, control and mutant embryos produced equivalent numbers of SHF-derived cells (Fig. 3.5.A-G), indicating that Fosl2 mutants initiate accretion in a timely fashion. Taking into account that mutant animals produce fewer cardiomyocytes between 24 and 48 hpf (Fig. 3.2.A-G), we deduced that the ventricular deficit at 48 hpf results from sluggish SHF differentiation between 32 and 48 hpf. To test this conclusion explicitly, we quantified the number of SHF-derived cardiomyocytes produced between 36 and 48 hpf and found that mutant animals accreted significantly fewer during this developmental window. Taken together, these data demonstrate that the ventricular deficiency present at 48 hpf results from sluggish accretion between 36hpf and 48 hpf.

Next, we ascertained whether the phenotypic recovery results from differentiation of the accumulated SHF progenitors (Fig. 3.3. H-M) beyond 48 hpf, the developmental stage when accretion is largely complete in wild-type embryos. To that end we photoconverted cardiomyocytes at 48 hpf and performed image analysis.
at 60 hpf. Whereas control embryos produced two SHF-derived cardiomyocytes during this 12-hour window (Fig. 3.5.H-J,N) null animals produced five times more (Fig. 3.3.K-M,N). Because the number of cardiomyocytes accreted in mutant embryos after 48 hpf is roughly equivalent to the deficit observed (Fig. 2D, it is unlikely that reprogramming of atrial cardiomyocytes (Zhang et al., 2013a) contributes to the phenotypic recovery. Furthermore, an increase in ventricular cardiomyocyte proliferation between 48 hpf and 72 hpf does not explain the phenotypic recovery. Ultimately, these data demonstrate that mutant animals extend their SHF accretion window to offset the ventricular deficiency caused by sluggish differentiation.
Figure 3.5. The ventricular deficit resolves in fosl2 mutants through extension of the SHF-mediated cardiomyocyte accretion window. Cardiomyocyte photoconversion assay. Control sibling (CTRL; A-C; n=11) and fosl2 null (D-F; n=7) Tg(myl7:nlsKiKGR) embryos were photoconverted at 23 hours post-fertilization (hpf) and imaged by confocal microscopy at 32 hpf in the red (A,D) and green (B,E) channels. Merged images are shown in (C and F). (G) Bar graph showing the average numbers of total ventricular cardiomyocytes, green and red positive cardiomyocytes, and green-only cardiomyocytes. Error bars represent one standard deviation. n.s., not significant. Control (H-J; n=19) and fosl2 mutant (K-M; n=5) Tg(myl7:nlsKiKGR) embryos were photoconverted at 48 hpf and imaged by confocal microscopy at 60 hpf in the red (H,K) and green (I,J) channels. Merged images are shown in (J,M). (N) Bar graph showing the average numbers of total ventricular cardiomyocytes, green and red positive cardiomyocytes, and green-only cardiomyocytes. Error bars represent one standard deviation. n.s., not significant, ****p<0.0001. Scale bars: 50mm.
Lastly, to determine if another *fos* family member might facilitate the phenotypic recovery by compensating for the loss of *fosl2*, we analyzed wild-type and *fosl2/-* embryos for the expression of *c-fos* at the arterial pole. In both wild-type and *fosl2/-* embryos, *c-fos* expression was not observed in or near the heart despite the existence of strong staining in the brain. These data suggest that upregulation of *c-fos* in the mutant does not account for the phenotypic recovery.

**Overexpression of *fosl2* perturbs SHF mediated cardiomyocyte accretion**

Lastly, we sought to characterize the phenotype of embryos overexpressing Fosl2. To that end, we injected one cell stage zebrafish embryos with full-length *fosl2* mRNA and quantified the numbers of atrial and ventricular cardiomyocytes at 48 hpf. Whereas atrial number was unaffected, ventricular cardiomyocytes were reduced by 31% ([Fig. 3.6.A-C](#)). Using the cardiomyocyte photoconversion assay, we learned that Fosl2 overexpression specifically reduced the number of SHF-derived ventricular cardiomyocytes by 86% ([Fig. 3.6.D-J](#)). The cardiomyocyte deficit was accompanied by qualitative ([Fig. 3.6.K, L](#)) and quantitative decreases in *ltbp3* expression (>50%, [Fig. 6M](#)) and SHF progenitor cell number (>65%; [Fig. 6N-P](#)). The co-occurrence of reduced SHF progenitors and their myocardial progeny is consistent with a model wherein Fosl2 overexpression increases the propensity of SHF progenitors to differentiate, rather than proliferate, resulting in precocious depletion of the progenitor pool. This would significantly reduce the number of SHF progenitors capable of producing the full complement of ventricular cardiomyocytes. Although this model is consistent with the reported sufficiency of Fosl2 to increase the differentiation of osteoblasts ([Bozec et al., 2010](#)) and drive precocious
differentiation of keratinocytes (Wurm et al., 2015), we cannot rule out other cellular mechanisms that might explain the overexpression phenotype.
Figure 3.6. – Overexpression of Fosl2 compromises SHF-mediated ventricular growth. (A,B) Confocal images of hearts in 48 hours post-fertilization (hpf) control sibling (CTRL; A; n=11) and Fosl2 overexpressing (B; n=5) Tg(cmlc2:nlsdsRedexpress) embryos. (C) Bar graph showing the average numbers of total, atrial, and ventricular cardiomyocytes in both experimental groups. n.s., not significant. ***p<0.001. (D-I) Cardiomyocyte conversion assay. Control (n=7) and Fosl2-overexpressing (n=5) Tg(myl7:nlsKiKGR) embryos were photoconverted at 24 hpf and imaged by confocal microscopy at 48 hpf in the red (D, G) and green (E, H) channels. Merged images are shown in (F, I). (J) Bar graph showing the average numbers of total ventricular cardiomyocytes, green and red positive cardiomyocytes, and green only cardiomyocytes. Error bars represent one standard deviation. ****p<0.0001. n.s., not significant. (K,L) Ventral images of control (n=30) and Fosl2 (n=35) overexpressing 48 hpf embryos stained with a riboprobe for the SHF marker ltbp3. (M) Bar graph showing the relative levels of ltbp3 mRNA at 48 hpf in both experimental groups as measured by quantitative PCR (n=3 biological replicates per group). **p<0.01. (N-O) Confocal images of the arterial poles in control (N; n=7) and Fosl2-overexpressing (N; n=6) embryos carrying the Tg(nkx2.5:nZsYellow) and Tg(cmlc2:AmCyan) transgenes co-stained with antibodies recognizing ZsYellow (red) or AmCyan (green). (M) Bar graph showing the average numbers of extra cardiac SHF progenitor cells (red nuclei without yellow cytoplasm) in both experimental groups at 48 hpf. Error bars represent one standard deviation. ****p<0.0001. Scale bars: 50mm.
DISCUSSION

Our study implicates Fosl2, a component of the dimeric AP-1 transcription factor, in potentiating the rate of cardiomyocyte differentiation from SHF progenitors in zebrafish. In Fosl2-null animals, the production of SHF-derived cardiomyocytes initiates appropriately, but sluggish differentiation results in a ventricular cardiomyocyte deficit and progenitor cell abundance. Null animals eventually resolve this phenotype by extending the accretion window until appropriate numbers of ventricular cells are achieved.

Our data provide in vivo relevance to a study demonstrating that Fosl2-containing AP-1 activity is required for cardiomyocyte differentiation from P19 embryonal carcinoma cells in vitro (Eriksson and Leppä, 2002). Furthermore, they are consistent with a report that conditional deletion of c-Jun in Isl1+ cells, including SHF progenitors, causes double outlet right ventricle (Zhang et al., 2013b), a manifestation of compromised SHF biology (Ward et al., 2005). Our data bring up the possibility sluggish cardiomyocyte accretion from the mouse SHF might account for the rightward shifting aorta observed in c-jun null mice. Although overt congenital heart defects have not been reported in Fosl2 knockout mice (Karreth et al., 2004), genetic redundancy with other Fos family members might compensate completely or facilitate a phenotypic recovery from sluggish differentiation. If genetic redundancy exists in the mouse or zebrafish, then introducing additional fos-family null alleles into the backgrounds of fosl2-/- animals would be predicted to reveal or exacerbate cardiac phenotypes, respectively. In that regard, our data bring up the possibility that Fosl2 mutations might cooperate with other genetic lesions that compromise SHF biology to cause congenital heart disease in the human population.
An important insight from our study is that the completion of myocardial accretion is not linked to developmental stage. At 48 hpf, when accretion has virtually ceased in WT embryos, *fosl2*-/− SHF progenitors continue producing cardiomyocytes to overcome the deficit. The cellular mechanism(s) ensuring that sufficient ventricular cardiomyocytes are produced, even in the face of sluggish differentiation, remain unknown. Perhaps SHF progenitors are capable of sensing ventricular size by monitoring contractility, shear stress, and/or oxygenation. Under this scenario, if one or more of these variables were below threshold, then the SHF responds by continuing to produce cardiomyocytes until that threshold is crossed. Alternatively, perhaps SHF progenitors are pre-programmed with limited quantities of an intrinsic determinant that controls asymmetric cell divisions. If true, the appropriate number of cardiomyocyte progenitors would be produced independent of the any delays to differentiation. Lastly, the mechanism(s) that extends the accretion window in *fosl2*-/− embryos also appears to delay the onset of smooth muscle production perhaps through activation of a checkpoint.

The transcriptional targets of Fosl2 that positively regulate cardiomyocyte differentiation remain elusive. Although several transcriptional targets have been identified for Fosl2 (Bozec et al., 2010; Bozec et al., 2008; Luther et al., 2014) and critical regulators of SHF differentiation have been described (Rochais et al., 2009), we are not aware of any genes that fit into both categories. With regard to cellular differentiation, Fosl2 transcriptionally activates osteocalcin (Bozec et al., 2010) and members of the epidermal differentiation complex (Wurm et al., 2015) to positively regulate osteoclast and epidermal differentiation, respectively. However, due to the lineage restricted and non-cardiac nature of these particular targets, they are unlikely
to mediate Fosl2’s role in heart development. We attempted to identify transcriptional targets of Fosl2 using two unbiased approaches, ChIP-sequencing and RNA-sequencing of mutant embryos, but neither approach produced a convincing list of candidate targets presumably because our antibody is not suitable for immunoprecipitation and the transcriptional changes underlying accumulation of SHF progenitors do not stand out in the context of whole embryos, respectively.

Together, the external development of zebrafish embryos combined with longitudinal assessments of cardiomyocyte production provide a highly-sensitive method for uncovering SHF phenotypes that might otherwise go unnoticed in higher vertebrates. Despite the SHF phenotype, zebrafish fosl2 mutants appear grossly normal during embryogenesis. Therefore, specific assays for SHF function are required before ruling out SHF phenotypes in zebrafish mutants. The observation that Fosl2 overexpression might cause precocious differentiation of SHF progenitors in vivo provides rationale for testing the hypothesis that AP-1 activity would improve the efficiency and/or reduce the time required to differentiate SHF progenitors down the cardiomyocyte lineage in vitro for myocardial replacement therapies.
METHODS

Zebralish strains

Zebralish were grown and maintained according to animal protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee. The following zebralish strains were utilized: Tg(cmlc2:GFP)$^f_1$ (Burns et al., 2005), Tg(cmlc2:dsRed2-nuc)$^f_2$ (Mably et al., 2003), Tg(myl7:nlsKikGR)$^{hsc6}$ (Lazic and Scott, 2011), Tg(nkx2.5nZsYellow)$^{fb17}$ (Paffett-Lugassy et al., 2013), Tg(myl7:nlsDsRedExpress)$^{hsc4}$ (Takeuchi et al., 2011) and Tg(nkx2.5:ZsYellow)$^{fb7}$ (Zhou et al., 2011).

Morpholino injections

One-cell stage wild-type, Tg(cmlc2:GFP), or fosl2-/- embryos were injected with ~1 nanoliter of a morpholino (5’-GCGCTGAGACACATCTGTGCATACC-3’; 1-3 ng/ml; Gene Tools, Philomath, Oregon, USA) targeting the first splice donor site in the fosl2 pre-mRNA. Sibling embryos were injected with a Standard Control oligo at the same dose (Gene Tools, Philomath, Oregon, USA).

Isolation of fosl2 null alleles

DNA constructs encoding TALENs designed to cut between two binding sites (5’ - TACGAC ACATCCTCCCGC - 3’ and 5’ - TGGTGTCGGGTTGCGCCG - 3’) just downstream of the fosl2 ATG were obtained from the Genetic Perturbation Platform of the Broad Institute. Standard methods were utilized to generate and identify fosl2 alleles carrying insertions or deletions (Hwang et al., 2014). The fosl2$^{fb15}$ allele harbors an 18 bp deletion (D 5’ - CTCCGCGGAGAGCCG - 3’) and the fosl2$^{fb16}$
allele carries a 16 bp deletion (D 5’- GCAGCAGCTACCGGC – 3’). Sibling embryos were used as controls in all experiments. \( Fosl2^{fb15/-} \) and \( fosl2^{fb16/-} \) embryos are indistinguishable with respect to cardiomyocyte number at all stages analyzed. If not otherwise specified, \( fosl2^{fb15/-} \) embryos are shown.

**Genotyping**

Mutant genotypes were identified by capillary electrophoresis of carboxyfluorescein (6-FAM)-labeled amplicons generated with the following primers:

Forward, 5’ – AAAAGGCAACATAAATTGGGAGTGC – 3’ and Reverse, 5’ - GGCGCTGAGACACATCTGTGCATA – 3’. We chose to attach a 6-FAM moiety on the Forward primer. When compared to the wild-type amplicon (337 bp), mutant amplicons are smaller by 18 (\( fosl2^{fb15} \)) or 16 (\( fosl2^{fb16} \)) base pairs. For western blotting and qPCR analyses, embryonic tail tips were collected prior to lysing animals individually in wells of a 96-well plate. The lysates were frozen until genotyping was complete.

**Quantitative Polymerase Chain Reaction (qPCR)**

The trunks and tails of embryos were excluded from the analysis as described (Zhou et al., 2011) to eliminate any potential influence of notochord (\( ltbp3 \)) or skeletal muscle (\( mef2cb \)) expression. Animals were lysed individually in TRIzol Reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). After genotyping (see above), the lysates of at least three groups of 10 embryos from each cohort (control, \( fosl2/- \) embryos, or \( fosl2 \) mRNA-injected) were pooled prior to total RNA purification using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA).
and first strand synthesis using the Superscript III First-Strand Synthesis Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR analysis was performed using Fast SYBR Green Master Mix (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. The 2^{-DDCT} method (Livak and Schmittgen, 2001) was used to measure differential expression levels after normalization to 18S ribosomal RNA (McCurley and Callard, 2008).

qPCR primer sequences:

ltbp3-F, 5’ – CGCCCAAAACAGGCTTGTAGTAGT – 3’
ltpb3-R, 5’ – CACTCTTCGGTAAACCGG – 3’
mef2cb-F, 5’ – CTCTCACTTATGTCAAGGTCAAT – 3’
mef2cb-R, 5’ – GAGACTATCAGCGGTCAGC – 3’
fosl2-F1, 5’ – AAGGAATCCCTTTGCTTGGA – 3’
fosl2-R1, 5’ – GGTAACTGGAGCCGAGGGATG – 3’
18S-F, 5’ – TCGCTAGTTGGCATCGTTATG – 3’
18S-R, 5’ – CGGAGGTTCGAAGACGATCA – 3’

Generation of custom polyclonal anti-serum recognizing zebrafish Fosl2

Using Gateway Cloning technology (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA), we generated a DNA construct for inducible expression of a 6xHis-tagged fragment of Fosl2 (a.a. 151 to 317) in bacteria. The bacterially-produced Fosl2 protein was affinity-purified using the HisPur Cobalt Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), separated further from other proteins.
by SDS-polyacrylamide gel electrophoresis, and electroeluted from a Fosl2-containing gel slice prior to injection into rabbits (Rockland Immunochemicals, Limerick, PA, USA). The IgG fraction was purified from rabbit serum internally designated “rabbit #1, bleed 2” using the Protein A IgG Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Western blotting

Animals were lysed individually in 4ml of lysis buffer [10mM Tris-HCl (pH=7.5), 10mM NaCl and 0.5% NP-40]. After genotyping (see above), 10 wild-type or mutant whole embryo lysates were pooled and microfuged on high for 2 minutes at room temperature. The supernatant was diluted with 2X sample buffer and boiled for 5 minutes before being separated by SDS-PAGE. The protein was transferred to PVDF paper and blocked overnight in 5% milk. Fosl2 anti-serum and anti-α-tubulin antibody (DM1A; EMD Millipore, Billerica, MA, USA) were used at dilutions of 1:500 and 1:4000 in block solution, respectively. HRP-conjugated anti-rabbit (Cell Signaling, Danvers, MA, USA, catalog number 7074) and anti-mouse (Cell Signaling, Danvers, MA, USA, catalog number 7076) secondary antibodies were used at dilutions of 1:10,000. Blots were developed using Pierce ECL Western Blotting Substrate (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

Immunofluorescence, cell counting and photoconversion

Cardiomyocyte nuclei were counted in embryos carrying the myl7:nlsDsRedExpress or cmlc2:dsRed2-nuc transgene following immunostaining as described (Nevis et al., 2013; Zhou et al., 2011). Cardiomyocyte photoconversion and analysis were
performed as described (Lazic and Scott, 2011; Paffett-Lugassy et al., 2013). MF20 and Eln2 immunostaining was performed as described (Zhou et al., 2011). SHF progenitor nuclei were visualized and counted in Tg(nkx2.5:nZsYellow), Tg(cmlc2:GFP) double transgenic animals after immunostaining as described (Nevis et al., 2013; Zhou et al., 2011). Embryos were imaged as described (Zhou et al., 2011).

**In situ hybridization**

A plasmid containing full-length zebrafish fosl2 (MGC:158347) was obtained from a commercial source (Open Biosystems, GE Dharmaco, Lafayette, CO). A cDNA encoding amino acids 31-339 of Fosl2 was PCR amplified and TOPO-cloned (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) into pCRBTII to generate pCRBTII-fosl2par(tial). From this plasmid, an anti-sense riboprobe for detecting fosl2 was transcribed using T7 polymerase after linearization with BamHI.

To generate a c-jun anti-sense riboprobe, a plasmid containing the zebrafish c-jun cDNA (MGC:77457) was obtained from a commercial source (Open Biosystems, GE Dharmaco, Lafayette, CO), linearized with EcoRI, and transcribed with T7 polymerase. To generate a c-fos anti-sense riboprobe, a plasmid containing zebrafish c-fos (MGC:77885) was obtained from a commercial source (Open Biosystems, GE Dharmaco, Lafayette, CO). The plasmid was used to PCR amplify a 647 bp fragment of c-fos that was cloned into pCS2+. The resulting plasmid was linearized with BamHI and transcribed with T3 polymerase. Anti-sense riboprobes for detecting ltbp3 (Zhou et al., 2011), cmlc2 (Yelon et al., 1999), and nkx2.5 (Paffett-Lugassy et al., 2013) were produced as described. Fosl2, ltbp3, nkx2.5, c-jun, and c-
fos probes were synthesized using the SP6/T7 DIG RNA labeling kit (Roche Diagnostics Corporation, Indianapolis, IN, USA) substituting T3 polymerase as needed. Cmlc2 probe was generated using the same kit but with Fluorescein RNA labeling mix (SP6/T7; Roche Diagnostics Corporation, Indianapolis, IN, USA). Whole mount in situ hybridizations were performed essentially as described (Thisse and Thisse, 2008). DIG and Fluorescein based in situ hybridizations were developed in NBT/BCIP (Roche Diagnostics Corporation, Indianapolis, IN, USA) and INT/BCIP (Roche Diagnostics Corporation, Indianapolis, IN, USA), respectively. Embryos were cryosectioned as described (Zhao et al., 2014). In photographs of stained embryos, the nkd2.5+ hepatobiliary system was circumscribed with the Lasso Tool in ImageJ (Schneider et al., 2012). The area of the circumscribed region was calculated using pixel number and size, the latter being determined empirically with a photograph of a stage micrometer taken under identical microscope settings.

**Fosl2 overexpression**

A full-length fosl2 cDNA was cloned into Clal-digested pCS3+MT to generate pCS3+fosl2MT. One-cell stage embryos were injected in the yolk with ~1 nanoliter (150pg) of full-length fosl2 mRNA transcribed from pCS3+fosl2MT using the mMESSAGE mMACHINE SP6 Transcription Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) after linearization with HindIII.
Detection of apoptotic cell death

TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling) was performed on 36 hpf Tg(nkx2.5:ZsYellow) embryos as described previously (Espín et al., 2013).

Analysis of SHF cell proliferation

EdU labeling and staining was performed as previously described (Mahler et al., 2010; Nevis et al., 2013) using the Click-iT EdU imaging kit (Invitrogen, Carlsbad, CA). Briefly, 36 hpf Tg(nkx2.5:ZsYellow) embryos were incubated on ice for 30 min in 10 mM EdU, rinsed 3 times in E3 medium and chased until 48 hpf when embryos were processed for antibody staining with anti-rCFP and Click-iT-Alexa-647 antibodies. Embryos were counterstained with DAPI and their distal ventricle and extra-cardiac ZsYellow+ regions were imaged by confocal microscopy. A SHF proliferation index (number of Edu+, ZsYellow+ cells/DAPI+, ZsYellow+ cells) was calculated for each embryo and averaged.

Adult morphology and regeneration assessments

Adult hearts were dissected, fixed and processed as described (González-Rosa and Mercader, 2012). Apex amputations and AFOG staining were performed as described (Poss et al., 2002, Zhao et al., 2014).

Analysis of cardiomyocyte cell proliferation

BrdU labeling and staining was performed as previously described (Tkatchenko, 2006) with several modifications. Briefly, Tg(nkx2.5:nZsYellow) embryos were
incubated in 5 mg/ml BrdU, 1% DMSO in E3 medium from 48 hpf to 72 hpf at 28 °C, rinsed 3 times in E3 medium and fixed overnight in 4% PFA. Fixed embryos were rinsed in PBST, bleached in the dark for 20 min (using 0.8% KOH, 0.9% H₂O₂ and 1% Tween-20 in distilled water), permeabilized using 1% Triton-X100 in PBS for 2 h and equilibrated in DNAse I buffer (40 mM Tris-HCl pH 8.0, 10 mM MgSO₄, 1 mM CaCl₂) for 30 min at 37 °C. Equilibrated embryos were treated with DNase I (1:50 in equilibration buffer) for 2 h at 37 °C, rinsed 3 times in PBSTw (PBS + 0.1% Tween20) and subjected to immunofluorescent staining with anti-rCFP and anti-BrdU antibodies. The ventricles of stained embryos were imaged and analyzed. A ventricular proliferation index (number of BrdU+, nZsYellow+ cells, nZsYellow+ cells) was calculated for each embryo and averaged.

Protein sequence analyses

ClustalW2 (Larkin et al., 2007) was used to align Fosl2 proteins from several species. The bZIP domain was identified by the conserved domain feature (Marchler-Bauer et al., 2015) associated with NCBI’s Standard Protein Blast.

Statistical Analysis

Unpaired, two-tailed t-tests assuming equal standard deviations were used to calculate p-values in Prism6 software (GraphPad Software, Inc., La Jolla, CA).
REFERENCES


CHAPTER 4

Complement receptor c5ar1 plays an evolutionarily conserved role in successful cardiac regeneration
ATTRIBUTIONS

Contributions to the work described in this chapter were part of a scientific collaboration between the Lee, Whited, Macrae and Burns laboratories. Individual contributions to the work described were made by Niranjana Natarajan, Yamen Abbas, Donald M Bryant, Juan Manuel González-Rosa, Michka Sharpe, Aysu Uygur, Lucas H Cocco-Delgado, Nhi Ngoc Ho, Norma Gerard, Craig Gerard, Calum Macrae, Caroline E Burns, C Geoffrey Burns, Jessica Whited and Richard T Lee.

RTL and JW conceived of and designed the study. Contributions from the Burns lab were as follows: MS and JMGR designed zebrafish experiments as part of the collaborative project. MS performed ventricular resections of adult zebrafish hearts, administered c5ar1 antagonists and sectioned adult hearts. JMGR sectioned adult hearts, performed immunofluorescence of adult sections, analyzed proliferation in injured hearts and prepared figures. MS contributions culminated in fifth authorship.
ABSTRACT
Defining conserved molecular pathways in animal models of successful cardiac regeneration could yield insight into why adult mammals have inadequate cardiac regeneration after injury. Insight into the transcriptomic landscape of early cardiac regeneration from model organisms will shed light on evolutionarily conserved pathways in successful cardiac regeneration. Here we describe a cross-species transcriptomic screen in three model organisms for cardiac regeneration – axolotl, neonatal mice and zebrafish. Apical resection to remove ~10 – 20% of ventricular mass was carried out in these model organisms. RNA-seq analysis was performed on the hearts harvested at three time points – 12, 24 and 48 hours post-resection. Sham surgery was used as internal control. Genes associated with inflammatory processes were found to be upregulated in a conserved manner. Complement receptors (activated by complement components, part of the innate immune system) were found to be highly upregulated in all three species. This approach revealed induction of gene expression for Complement 5a receptor1 (C5aR1) in the regenerating hearts of zebrafish, axolotls and mice. Inhibition of C5aR1 significantly attenuated the cardiomyocyte proliferative response to heart injury in all three species. Furthermore, following left ventricular apical resection, the cardiomyocyte proliferative response was abolished in mice with genetic deletion of C5aR1. These data reveal that activation of C5aR1 mediates an evolutionarily conserved response that promotes cardiomyocyte proliferation following cardiac injury and identify complement pathway activation as a common pathway of successful heart regeneration.
INTRODUCTION

While some organisms like zebrafish, axolotl and newt exhibit cardiac regenerative ability throughout life\textsuperscript{1-5}, mammalian hearts have limited natural regenerative potential with the exception of a narrow regenerative window demonstrated in neonatal mice\textsuperscript{6}. Adult mammals, including humans, fail to regenerate significant myocardium following injury. Instead, the injured muscle is replaced with scar tissue, compromising the contractility of the remaining myocardium when the extent of injury is severe\textsuperscript{2, 7, 8}. This inability of the adult mammalian heart to regenerate significant amounts of tissue can lead to subsequent heart failure, a leading cause of mortality\textsuperscript{7, 9}.

Upon injury in animals that can regenerate myocardium, some cardiomyocytes undergo de-differentiation and proliferation to regenerate the lost tissue\textsuperscript{4}. Lineage mapping of newly-derived cardiomyocytes in regenerating hearts has shown that new cardiomyocytes are primarily produced via cardiomyocyte cell division in zebrafish\textsuperscript{1, 10, 11} and neonatal mice\textsuperscript{6}. Furthermore, it has also been shown that cardiomyocyte renewal in adult uninjured mouse hearts occurs through cardiomyocyte division\textsuperscript{12}. The evidence that a significant majority of new cardiomyocytes are derived from pre-existing cardiomyocytes has focused attention on cardiomyocyte division as a key step in successful cardiac regeneration\textsuperscript{13}.

In this study, we leveraged three different animal models of successful cardiac regeneration to obtain insights into early events that may activate the cardiomyocyte cell cycle after cardiac injury. We aimed to define a conserved molecular pathway that plays a critical role early in the heart regenerative response using a cross-species comparative genomic approach. We performed an RNA-sequencing screen in zebrafish (\textit{Danio rerio}), axolotl (\textit{Ambystoma mexicanum}) and neonatal mouse (\textit{Mus musculus}), 12, 24 and 48 hours after cardiac apical resection. With the availability of recent tools to conduct such large-scale and multi-species studies, gene expression profiling in multiple species with enhanced capacity for cardiac regeneration is a powerful method to identify genes and pathways that play critical roles in cardiac regeneration. In addition to the upregulation of cell cycle and inflammatory genes across all three species, components of complement signaling...
were also upregulated in the regenerating hearts. Complement 5a receptor, C5aR1, was one of the most significantly upregulated genes early in the regenerating hearts of all species analyzed.

C5aR1 is a G-protein coupled receptor (GPCR) activated by complement 5a, a peptide anaphylotoxin generated by the cleavage of full length complement 5 protein\textsuperscript{14}. The complement pathway is a component of the innate immune system that serves to augment the ability of the immune system to identify and initiate the clearance of foreign material\textsuperscript{15}. Synthesized by the liver as zymogens, sequential proteolytic cleavage of complement proteins upon activation by external cues results in complement peptides that activate complement receptor GPCRs\textsuperscript{15, 16}. Expression of complement components has previously been noted in other regenerating tissues such as the axolotl and newt limb, mammalian liver, and chick embryonic retina\textsuperscript{17-20}. C5a has been shown to play a crucial role in murine liver regeneration\textsuperscript{21}, and several studies implicate C5a and C5aR in ischemic injury and cardiac dysfunction after sepsis\textsuperscript{22-24}. Here we show that complement signaling is required to initiate cardiomyocyte proliferation during heart regeneration.
MATERIALS AND METHODS

The data and pipelines from our RNA-seq experiments have been deposited with GEO, the material will be publicly released on 1.3.18. Additional data and methods pertinent to the findings of this study are available from the corresponding author (Richard T Lee) upon reasonable request.

Animals:
All experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by the Harvard University Institutional Animal Care and Use Committee.

Wild-type (WT) CD-1 and C57Bl/6 mice were obtained from Charles River Laboratories. C5ar1 knock-out mice were obtained from the laboratory of Dr. Craig Gerard, Boston Children’s Hospital.

For RNASeq experiments, zebrafish of strain Wild-type AB and Wild-type TuAB were produced, maintained, and housed in the laboratory of Dr. Calum MacRae, Massachusetts General Hospital. Ventricular apical resection was performed on 9-month-old zebrafish. For inhibition of C5aR1 transgenic zebrafish Tg(cmIc2:nlsDsRed-Express)hsc4 were used25. Adult zebrafish used for experiments were grown and maintained in compliance with the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

Wild-type black axolotl were maintained and housed in the laboratory of Dr. Jessica Whited, Brigham Regenerative Medicine Center. Sub-adult axolotls were used for ventricular resection, leucistic axolotls were used for C5aR1 inhibition studies.

Ventricular apical resection:
Zebrafish: Ventricular apical resection was performed on zebrafish between 4-6 months of age as previously described1.
Axolotl: Axolotl resection surgeries were performed on 8-12-months old axolotls (as previously described by Cano-Martinez et al., 2010)\textsuperscript{26}. Animals were anesthetized in tricaine mesylate (MS-222) for 20 minutes. Lateral thoracotomy was then performed to expose the heart ventrally. Microsurgery scissors were used to resect approximately 10 - 20\% of the ventricle. The control—or sham—animal group underwent the thoracotomy but not apical resection. After a blood clot was formed, a 6-0 prolene suture was then used to suture the incision in the thoracic cavity.

Neonatal mice: Neonatal mouse resection procedures were performed on 1-day old pups as described previously\textsuperscript{6, 27}. 1-day-old mice were anesthetized on ice for 4 minutes. All mice underwent a lateral thoracotomy. Resected animals received a $\sim$10\% resection of the ventricle to expose the left ventricular chamber and the control surgery animals (“sham”) only received a thoracotomy.

**RNA isolation and sequencing:**

Total RNA from the lower half of the ventricle of resected and sham hearts was isolated using Trizol at three different time points: 12, 24, and 48 hours post-surgery. Three biological replicates were used for each time point and condition in each species. For neonatal mice and axolotls, each replicate represents myocardium from a single animal. Due to the smaller size of the zebrafish, each biological replicate represents three pooled tissue samples. Poly-adenylated mRNA was isolated using the Wafergen #400047 protocol, and RNA-sequencing libraries were prepared with the IntegenX Directional PrepX mRNA kit as previously described\textsuperscript{28}. Paired-end (50 base pairs) sequencing was performed using an Illumina HiSeq 2500. Poly-adenylated mRNA isolation, library preparation, and sequencing were performed by the Biopolymers Facility at Harvard Medical School.
RNAseq data analysis:
Tophat 2 was used to align paired-end sequence reads to the GRCm30 genome for mouse and GRCz10 genome for zebrafish\(^{29}\). Cufflinks was used to estimate gene expression levels in aligned reads and the resulting data was processed with Cuffdiff to identify differentially expressed genes between resected and sham groups at each time point and species\(^{30}\). In contrast to zebrafish and the mouse, the full genome of the axolotl has not been sequenced. Thus, we took an approach were we first mapped the axolotl RNA-sequencing data to a previously published transcriptome that was constructed using the Trinity program\(^{31-33}\). RSEM was used to quantify gene expression\(^{34}\), and differential gene expression analyses were performed using EBseq\(^{35}\). For all species, a given gene had to be identified as differentially expressed (False Discovery Rate (FDR) adjusted p-value <0.05) by the programs described above and have a fold change of >1.2 in order to be considered in our final analyses. After identifying differentially expressed genes using the criteria described above, we constructed two datasets for each species that consisted of either genes that were 1) upregulated or 2) downregulated at 12, 24, or 48 hours post-surgery. We performed this step in order to account for species-specific time differences during early heart regeneration.

Orthology and Gene Ontology (GO) Analyses
Ensembl BioMart was used to identify the mouse orthologs of differentially expressed zebrafish genes\(^{36}\). Axolotl genes were annotated using Trinotate as previously described\(^{33}\). The Web-based Gene Set Analysis Toolkit (WebGestalt) was used to perform Gene Ontology analyses on the set of genes that were either upregulated during heart regeneration in both neonatal mice and zebrafish or downregulated during heart regeneration in both neonatal mice and zebrafish at 12, 24, or 48 hours post-surgery\(^{37,38}\). \textit{M. musculus} was used as the organism of interest, Overrepresentation Enrichment Analysis (ORA) was used as the method of interest, and gene ontology was used for the functional database. Ensembl gene I.D.s were used as inputs, and the genome was used as the reference set for enrichment analysis. GO terms with an FDR-adjusted p-value less than 0.05 were considered to be statistically significant.
Inhibition of C5aR1 using PMX205 following apical resection:
Axolotl: 0.05 mg PMX205\textsuperscript{39, 40} (Tocris, \#5196) was delivered via i.p. injection for 14 days post-resection.

Zebrafish: PMX205 (R&D, 5196, 2.5 mg kg\textsuperscript{-1}) or vehicle control were injected intraperitoneally into adult zebrafish. Injections were initiated 1 hour prior to ventricular resection and then daily for 10 days after ventricular amputation.

Neonatal mouse: On the day of surgery, 0.025 mg PMX205 was administered by a subcutaneous injection 30 minutes before resection and 0.025 mg was delivered post resection. For the next three days, 0.05 mg PMX205\textsuperscript{39, 40} (Tocris, \#5196) was administered.

Histology, Immunofluorescence and western:
Zebrafish: Zebrafish hearts were extracted and fixed as described\textsuperscript{41}. Hearts were then processed for histological and immunofluorescence analysis as described\textsuperscript{42}. Primary antibodies used were anti-PCNA (sc-56, 1:50, Santa Cruz Biotechnologies, USA), anti-DsRed (1:200, Clontech, USA) and anti-Tropomyosin (clone CH1, 1:100, Developmental Studies Hybridoma Bank, USA). Alexa (488, 568, 633)-conjugated antibodies (Invitrogen, USA) were used to detect primary antibody signal. Nuclei were stained with DAPI and slides were mounted in FluorSave (Millipore, USA). We tried two antibodies for C5aR1 localization – SantaCruz sc-56 and Thermo Fisher MA1-81761. We observed best signal with SantaCruz anti-C5aR1 in our immunostaining experiments.

Axolotl and Mouse: Hearts were fixed with 4% paraformaldehyde (PFA) overnight followed by cryoprotection through a sucrose gradient of 10\%, 15\%, 20\%, and 30\% sucrose in PBS. Samples were left in each sucrose solution till the tissue sank to the bottom of the vial, and then left in 30\% sucrose overnight at 4\°C. The following day samples were embedded in OCT and sectioned longitudinally (12\textmu M).
Primary antibodies

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Secondary antibodies

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<td>Alexa 488, 568, 647</td>
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Western blot: Mouse anti C5a (Abcam) was used at a 1:1000 dilution to detect C5a levels in resected and sham hearts, 48 hours post-resection. 50ug tissue lysate was loaded per well in the western blot. Cell signaling rabbit anti GAPDH was used at 1:2000 for loading control.

EdU incorporation assay: EdU was purchased from Carbosynth, Inc. Primary cardiomyocytes were isolated from wild-type mice and cultured in fibronectin – coated dishes at a density of 300,000 cells per well. 72 hours post – plating, cells were treated with varying concentrations of C5a (saline for control) and incubated with medium containing 20 µM EdU for 24 hours. Following treatment, cells were fixed and stained with PCM-1 (Abcam), EdU (Sulfo-Cy3 azide) and DAPI and imaged using CellDiscoverer (Zeiss).

**Imaging:**

Zebrafish: A total of 3 sections per heart were quantified for each group. DsRed+/PCNA+ cardiomyocyte nuclei were divided by the total DsRed+ cardiomyocytes to generate a proliferation index.

Axolotl and mouse: Fluorescently stained sections were imaged with Zeiss LSM 700 (mouse) and Zeiss Axiozoom (Axolotl), (HCBI, Harvard University). A total of 5 sections per heart were quantified for each group in a blinded manner.
Statistical analysis:
All data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using Prism 7 software (Graph Pad). After normality testing using the Wilk-Shapiro test; two-tailed Student’s t-test was used to compare data from individual experimental groups. A value of p<0.05 was considered significant.
RESULTS

Transcriptomic analyses indicate a conserved role for the inflammatory response and metabolic regulation during early neonatal mouse and zebrafish heart regeneration.

We utilized three model organisms capable of successful cardiac regeneration: neonatal mice, zebrafish, and axolotls. We resected ~10 – 20% of the ventricular myocardium in all three species, following which RNA-sequencing was carried out on the regenerating hearts of each species at 12, 24, and 48 hours post-resection. We undertook this approach to assess differential gene expression at three different time-points as studies have indicated that cardiac regeneration occurs at different rates across the three species; neonatal mice successfully accomplish regeneration in as few as 21 days while zebrafish and axolotl heart regeneration take 60 days or more to replace the lost myocardial tissue$^{6,43}$. RNA-sequencing on sham-operated hearts at each time point was used as control for data analysis. These internal controls were performed to account for potential systemic effects due to surgical opening of the thoracic cavity and for the rapid growth of the neonatal mouse during the first few days after birth. The zebrafish and the mouse genomes are well annotated and enabled feasibility of an RNA sequencing experiment with multiple samples. Even though the axolotl genome is incomplete, a high number of transcriptomes and various annotations exist to assist in analysis$^{33}$.

Following acquisition of the RNA-sequencing data, we found that 1433 genes were differentially expressed in neonatal mice, 4502 genes were differentially expressed in zebrafish, and 4639 gene contigs were differentially expressed in axolotl heart regeneration across all time points. We undertook a systematic computational approach to refine the dataset in order to identify genes that are functionally important for heart regeneration across all three species (Figure 4.1.A). First, we identified orthologous mouse and zebrafish genes that were consistent in terms of their differential expression (i.e. both upregulated or both downregulated) during early regeneration (Figure 4.1.A). We found that 233 genes were commonly upregulated during the first two days of heart regeneration (Figure 4.1.B). These
genes represented 23.5% of upregulated mouse genes (233/990 genes) and 11.1% of mouse genes (233/2103) with an orthologous relationship to upregulated zebrafish genes. Furthermore, we identified 64 genes common to zebrafish and mice that were downregulated during this early time period (Figure 4.1.C). These genes constitute 12.1% of mouse genes that were downregulated within the first two days of heart regeneration (64/527) and 4.1% (64/1572) of genes corresponding to the mouse orthologs of downregulated zebrafish genes.

Next, we attempted to identify conserved gene expression modules by performing overrepresentation analyses on the common differentially expressed genes (Figure 1A). Using WebGestalt\textsuperscript{38}, we found that the set of genes commonly upregulated during mouse and zebrafish heart regeneration were significantly enriched for Gene Ontology (GO) terms such as “Inflammatory Response”, “Regulation of Immune System Process”, and “Immune System Process” (FDR <0.05; Figure 4.1.D). These data suggest that regulation of the inflammatory response during early heart regeneration is a conserved process across species and is consistent with previous studies demonstrating the functional importance of the early immune response to regeneration\textsuperscript{44-46}. Similarly, we also observed significant enrichment (FDR <0.05) for “Cell proliferation” (Figure 4.1.D), which is consistent with cardiomyocyte division as well as possibly non-myocytes during heart regeneration\textsuperscript{6, 7, 10, 11}. Biological processes such as “Fatty Acid Oxidation”, “Lipid Oxidation”, and “Fatty Acid Metabolic Process” had the highest ratio of enrichment among significant GO terms (FDR <0.05) in the set of genes commonly downregulated in neonatal mouse and zebrafish heart regeneration (Figure 1E). These data suggest that fundamental changes in metabolic processes during early heart regeneration are evolutionarily conserved across species.
Figure 4.1. RNA-seq analysis pipeline to identify evolutionarily conserved genes involved in early heart regeneration. Ventricular myocardium was resected, and RNA-sequencing was performed at 12, 24, and 48 hours post-injury in neonatal mice, axolotl, and zebrafish. Each time point was internally controlled with sham surgery. (A) Schematic of transcriptomic approach for identifying genes whose expression was conserved during early heart regeneration. Black dots represent genes, and gray squares represent computational filtration steps. (B) Intersection of genes that were upregulated at 12, 24, or 48 hours relative to sham controls in neonatal mice and zebrafish. (C) Intersection of genes that are downregulated at 12, 24, or 48 hours relative to sham controls in neonatal mice and zebrafish. (D) Gene ontology (GO) analysis of commonly upregulated neonatal mouse and zebrafish genes. (E) Gene ontology (GO) analysis of commonly downregulated neonatal mouse and zebrafish genes.
Cross-species gene expression profiling indicates a conserved role for complement components during early heart regeneration.

Motivated by our ability to uncover broader biological processes that are known to be essential for regeneration, we focused our attention on genes belonging to the “Inflammatory Response” classification that are commonly upregulated during mammalian and zebrafish heart regeneration (Figure 4.1.D). This particular category comprised 38 genes and had the highest enrichment ratio relative to other GO terms in the upregulated gene set (Figure 4.1.D). We then identified 15 inflammatory response genes that were also upregulated during early axolotl heart regeneration (Figure 4.2.A); these comprised the highest confidence set of upregulated genes during early regeneration across the three species (Figure 4.2.B). The 15 inflammatory genes identified to be upregulated in early cardiac regeneration is listed in Figure 2B. It is important to note that the top two inflammatory genes found to be upregulated in a conserved manner are both complement receptors, C5aR1 and C3aR1.

The 15 genes included complement component 5a receptor (C5aR1) and complement component 3a receptor (C3aR1), which serve as the receptors for complement components C5a and C3a, respectively, and have been shown to mediate complement signaling in macrophages47 (Figure 4.2.A). The complement cascade is an ancient component of the innate immune system. Recent studies have shown that complement may also mediate cross-talk between the innate and adaptive immune response48. Upon binding to their corresponding receptors, complement peptides C5a and C3a act as potent inflammatory mediators that can trigger the degranulation of endothelial cells, mast cells, and phagocytes, and they function as chemoattractants for neutrophils, monocytes, and macrophages49. Complement proteins have been noted in several regenerating tissues such as newt lens, axolotl limb and mouse liver19, 20. Interestingly, the expression of C5 and C3 was observed in regenerating limb tissues such as blastema and wound epidermis, suggesting that the complement system could play functional roles in other regenerative contexts beyond heart regeneration20. Although most of the evidence supporting the expression and role for the complement system in regeneration is
mainly observational, Strey et.al. showed mechanistic evidence supporting the crucial role of C5a signaling in murine liver regeneration after partial hepatectomy\textsuperscript{21}.

The evolutionarily conserved upregulation of complement components observed during early heart regeneration in neonatal mouse, zebrafish, and axolotl, along with studies implicating the complement system in regenerative processes outside of the heart, suggest a role of complement receptors in cardiac regeneration. Of the two receptors identified, C5aR1 was upregulated during regeneration (Figure 4.2.A). When normalized to expression levels in sham-operated animals, C5aR1 expression in all three species was upregulated significantly, peaking at slightly different time points within the first 48 hours (Figure 4.2.B). Specifically, we observed the highest fold-change in C5aR1 expression at 24 hours post-injury in mice and 48 hours post-injury in zebrafish and axolotls. We speculate that the time differences in expression may be attributed to species-specific differences in the heart regeneration response and could possibly reflect the different rates at which heart regeneration occurs in these species. Taken together, our analyses suggested that conserved activation of C5aR1 during early heart regeneration could play a role in cardiac regeneration.
Figure 4.2. Schematic of analysis pipeline used to identify upregulated inflammatory genes in mouse, zebrafish an axolotl (A). Inflammatory response genes including complement components and associated proteins that are upregulated in early cardiac regeneration in an evolutionarily conserved manner. The list is ordered according to fold change; genes with higher fold change in mouse are first, followed by genes in decreasing order. Note that the top two genes are receptors for activated complement components, C5aR1 and C3aR1. (B). Complement 5a receptor 1 (C5aR1) is upregulated in early cardiac regeneration in zebrafish, axolotl and mouse at the timepoints shown, in hours (C), expression of C5aR1 is normalized to sham levels (dotted line).
Inhibition of C5aR1 severely attenuates cardiomyocyte proliferation following apical resection:

Based on our finding that upregulation of complement signaling is an evolutionarily conserved mechanism in early cardiac regeneration, we sought to test the hypothesis that C5aR1 signaling is required for successful cardiac regeneration after apical resection in zebrafish, axolotl and mice. We utilized a peptide C5aR1 antagonist, PMX205\textsuperscript{39, 40} to inhibit C5aR1 activity across the three species. PMX205 is a highly selective C5aR1 antagonist with an IC\textsubscript{50} of 31nM\textsuperscript{50}. Although studies have focused on the immunomodulatory role of C5aR1, it is known to be expressed in other tissues including liver, kidney and heart\textsuperscript{14}. We tested the hypothesis that C5aR1 receptor function is required for effective cardiac regeneration by administering PMX205\textsuperscript{39, 40} after apical resection in zebrafish, axolotl and neonatal mice. PMX205 was administered to the animals following cardiac apical resection (vehicle treatment of resected animals and sham surgery with vehicle / PMX205 administration for control). Cardiomyocyte proliferation was quantified by immunostaining for proliferation markers phospho-Histone 3 (pH3, mouse and axolotl) and PCNA (zebrafish). The total number of proliferating cardiomyocytes per field of view was quantified after imaging of immunostained heart sections.

Due to differences in the rate at which cardiac regeneration occurs in the three species, different lengths of treatment with PMX205 were employed for each species studied. Apical resection was performed on zebrafish (sham for control), following which PMX205 was administered for 10 days at 0.05mg / zebrafish by i.p. injection. Zebrafish hearts were harvested 14 days post-resection and stained with proliferation marker PCNA. A significant reduction in the number of proliferating cardiomyocytes per unit area was observed in resected zebrafish hearts that were treated with PMX205 (Figure 3A,B). Quantification of proliferating cardiomyocytes from the experimental groups showed that PMX205 effected ~ 2-fold reduction in cardiomyocyte proliferative response in zebrafish following apical resection (Figure 4.3.C).
Figure 4.3. Inhibition of C5aR1 decreases proliferating cardiomyocytes after apical resection in zebrafish. (A) resected zebrafish treated with vehicle, representative image, (B) resected zebrafish treated with PMX205, representative image, cardiomyocyte marker tropomyosin (red), PCNA (green), cardiomyocyte nuclei (MEF2, blue); scale bar - 50 µm (C) quantification of proliferating cardiomyocytes in vehicle and PMX205 treated hearts of zebrafish, * p<0.01, n=5 (vehicle), 8 (PMX205).
Axolotls received 14 days of PMX205 administration (0.05mg/kg) following apical resection. Axolotl hearts were harvested following PMX205 treatment and stained for pH3 and the cardiomyocyte marker MF20 to quantify cardiomyocyte proliferation. Inhibition of C5aR1 significantly decreased cardiomyocyte cell cycle activity after apical resection in axolotl (Figure 4.4.A-C), revealed by a reduction of pH3 positive cardiomyocytes per field of view in resected axolotl hearts treated with PMX205. Quantification of the number of proliferating cells per field of view showed a ~ 2-fold reduction in the number of pH3 positive cardiomyocytes in axolotl hearts treated with PMX205 (quantified blindly in Figure 4.4.D).

As cardiac regeneration in neonatal mice is faster than in axolotl and zebrafish, we assessed the effect of PMX205 treatment for 4 days after apical resection on neonatal mice (P1). Hearts from experimental mice were harvested 7 days post-resection and proliferation was assessed by staining for pH3. We observed a significant reduction of pH3 positive cardiomyocytes in the PMX205 treated animals compared to vehicle treatment, consistent with the reduction of cardiomyocyte proliferation we observe in axolotl and zebrafish (Figure 4.4.E-H). As the mouse model is the most relevant for mammalian heart regeneration among the three model organisms used, the effect of C5aR1 inhibition was then assessed at the histological level by quantifying scarring of the murine heart in the different experimental groups, 21 days post injury by Masson Trichrome staining. An increase in scarring of the ventricular apex was observed in the group of animals treated with PMX205, further demonstrating that C5aR1 signaling is crucial for the initiation of effective cardiac regeneration (Figure 4.4.I-L). While minimal scarring was observed in resected hearts treated with vehicle (Figure 4.4.J, L), we observed small but consistent scars in the ventricular apex of mice treated with PMX205 after resection (Figure 4.4.K,L). To further investigate the role of C5aR1 on cardiomyocyte proliferation, we quantified proliferation in primary cardiomyocytes treated with C5aR1 agonist C5a for a period of 24 hours by measuring EdU incorporation. We did not observe a significant increase in cardiomyocyte proliferation upon C5a treatment (Figure S1). This indicates that the proliferative effect elicited by C5a – C5aR1 signaling is potentially invoked after cardiac injury. Overall, our results across the
three species support the hypothesis that C5aR1 plays an important role in heart regeneration and is essential for the initiation of a cardiomyocyte proliferative response required for successful heart regeneration. Our findings also indicate that one possible function of the C5aR1 pathway is to inhibit collagen deposition and scarring in mammalian heart regeneration.
Figure 4.4. Inhibition of C5aR1 in axolotl and mouse. Inhibition of C5aR1 results in a reduction of proliferating cardiomyocytes in axolotl hearts. Cardiomyocyte marker MF20 is shown in red, phospho Histone 3 (pH3) is shown in green and nuclei are in blue. (A) Sham surgery of axolotl hearts, (B) resected – vehicle treatment and (C) resected – PMX205 treatment; scale bar - 100µm. Quantification of proliferating cardiomyocytes is summarized in (D), * p<0.05, n=4.

Effect of C5aR1 inhibition on cardiomyocyte proliferation in mouse (E-H). Cardiac Troponin T (cTnT, cardiomyocyte marker) is shown in red, phosphoHistone 3 staining is in green and nuclei are in blue. (E) sham surgery of mouse hearts, (F) resected – vehicle treatment and (G) resected – PMX205 treatment, scale bar - 20µm. Quantification of cardiomyocyte proliferation is summarized in (H), * p<0.05, n=4.

Effect of C5aR1 inhibition on scar formation after apical resection (I-L). Masson trichrome images of hearts 21 days post-resection show an increase in scar formation in PMX205 treatment after apical resection. Representative images of sham surgery (I), resected – vehicle treatment, resected – PMX205 treatment. Scar infiltration is quantified in (L), * p<0.05, n≥6, scale bar 100 µm.
C5aR1 is primarily expressed in cardiomyocytes following apical resection:

The observed upregulation of C5aR1 expression in the early regenerating myocardium could be due to immune infiltration of the heart following injury, since C5aR1 is expressed in myeloid cells\(^5\). Macrophages play an essential role in cardiac regeneration, and depletion of macrophages in neonatal mice results in incomplete regeneration\(^4\). However, despite extensive scarring and inhibition of microvasculature formation in the regenerating heart, macrophage depletion does not appear to cause a decrease in cellular proliferation following cardiac injury\(^4\). This suggests that decreased cardiomyocyte proliferation upon C5aR1 inhibition may not be due to the contribution of C5aR1 from macrophages. C5aR1 expression has been noted in cardiomyocytes\(^2\), \(^5\), \(^3\) as well as endothelial cells, where the C5a component of complement has been shown to activate the endothelium\(^5\)\(^4\)\(^-\)^\(^5\)\(^6\). C5aR1 expression has been shown to be upregulated in ischemic cardiomyocytes in adult mice following ischemia/reperfusion injury\(^2\). To identify the cell types expressing C5aR1 in the neonatal mouse heart during regeneration, we conducted extensive immunostaining analyses in the neonatal mouse heart following apical resection (sham for control). We found that C5aR1 was primarily expressed in cardiomyocytes (Figure 4.5.A, seen by co-localization with cardiac Troponin T), 48 hours post-resection in neonatal mice. Hearts from littermate sham controls did not have expression of C5aR1 (Figure 4.5.B). We validated the antibody by staining sections from hearts from C5aR1 neonates, 48 hours post-resection. We do not observe any signal from the antibody in C5aR1 knock-out sham and resected hearts (Figure S2). Furthermore, we also observed co-localization of C5aR1 with the endothelial marker CD31 in the injured hearts (Figure 4.5.C). However, we did not observe localization of C5aR1 staining with the macrophage marker CD68 in injured neonatal hearts (Figure 5D). In-depth immunostaining and imaging analyses of C5aR1 expression pattern in the injured neonatal heart show expression of C5aR1 in the injured zone at the apex (site of resection, Figure S3A), while, no significant expression was observed in the border and the remote zones (Figure S3 B,C respectively). Expression of C5aR1 in cardiomyocytes following cardiac injury circumstantially supports the concept that C5aR1 plays an important role in cardiomyocyte proliferation after injury and suggests that either cardiomyocytes or cardiac
endothelial cells—or both—are cells likely to be receiving the C5a signal during heart regeneration.

A majority of the complement components are secreted proteins, synthesized mainly by the liver as zymogens. Complement component 5, upon activation by complement 5 convertase, is cleaved to form C5a and C5b in target tissues. Therefore, we carried out western analysis of neonatal murine hearts 48 hours post-injury to detect activation of C5 and cleavage to C5a. (sham for control). We observed a significant increase in C5a production in the injured heart 48 hours post resection in mice, in comparison with sham hearts of littermate animals (Figure S4), n=3 (all groups); *p<0.05. This increase of C5a levels upon cardiac resection accompanied the upregulation of C5aR1 observed in our RNA-seq screen, supporting a role for C5a-C5aR1 signaling in early cardiac regeneration. This data also gives further confidence in the approach of pursuing the consequences of genetic ablation of the C5aR1 receptor in the process of heart regeneration.
Figure 4.5. Localization of C5aR1 to cardiomyocytes and endothelial cells after apical resection in neonatal mice. Localization of C5aR1 to cardiomyocytes and endothelial cells after apical resection in neonatal mice. C5aR1 (red) localizes with cardiomyocyte marker Troponin T (green) in neonatal mouse hearts, 48 hours after apical resection (nuclei are in blue), scale bar 20 µm (A). C5aR1 upregulation is absent in hearts from littermate sham controls (B). C5aR1 (red) expression localizes with endothelial marker CD-31 (green) (C), scale bar - 20 µm. C5aR1 (red) does not localize with macrophage marker CD68 (green) in resected hearts (D), scale bar - 20 µm.
Genetic deletion of C5aR1 abolishes cardiomyocyte proliferation following apical resection

To further evaluate the role of C5aR1 in cardiac regeneration, a murine global genetic deletion model of C5aR1 was used. Cardiomyocyte proliferation was assessed in C5aR1 knock-out mice and wild-type mice after apical resection. Our hypothesis was that the cardiomyocyte proliferative response 7 days after apical resection would be reduced in C5aR1 KO mice in comparison to C5aR1 wild-type (WT) mice. Consistent with the regenerative response observed by Porrello et al. in neonatal mice, the littermate WT mice had an increase in the fraction of proliferating cardiomyocytes 7 days post resection (Figure 4.6 A-E), while in the C5aR1 KO mice, no significant induction of cardiomyocyte proliferation is observed. This is consistent with the results of the receptor inhibition experiments, showing an essential role of C5aR1 in cardiomyocyte proliferation during regeneration.

Furthermore, we analyzed scarring in the hearts of C5aR1 KO and littermate WT mice 21 days post-resection to investigate the effect of C5aR1 in cardiac regeneration at a longer time-point. We observed moderate scarring of the apex 21 days post-resection in C5aR1 KO mice, while no significant differences in scarring were observed after resection in matched wild-type control mice (Figure 4.6 F-J). Although we did not observe a complete inhibition of apical regeneration of the hearts of C5aR1 knock-out mice, an increase in fibrosis observed indicates that the overall regenerative process is partially hampered upon loss of C5aR1 (Figure 4.6 F-J). This increase of fibrosis was not observed in littermate wild-type hearts after resection, further supporting a role for C5aR1 in effective cardiac regeneration and minimization of fibrosis following an injury.

Expression of C5aR1 has been reported mainly in myeloid cells and macrophages; therefore, we quantified macrophage infiltration after apical resection in C5aR1 wild-type and knock-out hearts, 48 hours post-resection, by immunostaining. While we saw a significant increase in the macrophage infiltration of the injured zone in C5aR1 wild-type mice 48 hours post-resection; we did not see any significant difference in macrophage infiltration between C5aR1 knock-out sham
and resected hearts (Figure S5). Since we observed localization of C5aR1 with the endothelial marker CD31, we analyzed the number of blood vessels in the apex, 7 days post-resection, in C5aR1 knock-out and wild-type mice. The total number of blood vessels per field of view was quantified. We did not see a significant difference in the number of blood vessels between sham and resected hearts of both C5aR1 knock-out and wild-type mice (Figure S6). Therefore, further downstream experiments need to be carried out with endothelial cell-specific deletion for estimating the effect of C5aR1 on angiogenesis after cardiac injury.
Figure 4.6. Mice lacking C5aR1 mice have a reduction in cardiomyocyte proliferation after apical resection compared to wild-type littermate controls. Representative images from C5aR1 wild-type sham surgery (A), C5aR1 wild-type resected (B), C5aR1 knock-out sham surgery (C) and C5aR1 knock-out resected (D). Cardiac troponin T (green), phosphoHistone 3 (red) and nuclei (blue), scale bar - 20 \( \mu m \). Quantification of proliferating cardiomyocytes shows the absence of proliferative response upon resection in C5aR1 knock-out hearts (E). *\( p < 0.05 \), \( n \geq 4 \). Measurement of fibrosis 21 days after apical resection (F-J). Representative Masson Trichrome stained images from C5aR1 wild-type sham surgery (F), C5aR1 wild-type resected (G), C5aR1 knock-out sham surgery (H) and C5aR1 knock-out resected (I), scale bars, 100 \( \mu m \). Quantification of fibrosis in cardiac apex after resection (J), *\( p < 0.05 \), \( n=8 \).
DISCUSSION

Achieving cardiac regeneration in adult mammals is an important goal for cardiovascular research. Although it is now known that adult mammalian cardiomyocytes can undergo cell proliferation to generate new cardiomyocytes, the rate of proliferation is insufficient to replace lost cardiomyocytes and restore function after a cardiac injury\(^5\), \(^6\). Previous work has established multiple models of successful heart regeneration, including neonatal mice, zebrafish, and axolotl\(^1\)-\(^4\). Over the past decade, studies of cardiac regeneration in different experimental models has shed light on several shared mechanisms across the species including stimulation of an essential immune response, a role for nerves and a critical role of cardiomyocyte division\(^4\), \(^28\), \(^46\). However, we still do not understand the barriers to heart regeneration that lead to extensive scarring and eventual heart failure in humans who have major cardiac injuries. This study leveraged similarities among model organisms that have substantial heart regenerative capacity to define a regenerative molecular pathway in the heart across species. The use of three unique model systems to identify the transcriptomic landscape of early cardiac regeneration provided insight into an evolutionarily conserved pathway underlying regeneration.

Our observation that C5aR1 is important for effective cardiac regeneration in an evolutionarily-conserved manner fits with the existing body of literature supporting a role for the complement system in mediating regenerative responses\(^19\). Although the role of macrophages in cardiac regeneration has been previously examined\(^46\), the role played by the immune system in initiating the cardiac regenerative response is not clearly understood. C5a and its receptor C5aR1 have been previously studied in the adult murine heart after septic shock, ischemia-reperfusion and in hypertension\(^24\), \(^52\), \(^53\), \(^59\), \(^60\). Activation of C5aR1 due to excessive C5a generation in sepsis has been shown to contribute to cardiac dysfunction as excessive C5a results in increased ROS and impaired Ca\(^{2+}\) handling in the cardiomyocytes\(^52\), \(^53\). However, it has also been shown that C5a - C5aR1 signaling decreases hypertension-induced fibrosis in an angiotensin II – induced hypertension model\(^61\). Our finding that C5aR1 knock-out mice have increased scarring of the heart following apical resection agrees with the observations made by Weiss et al., who found that loss of C5aR1
results in increased cardiac fibrosis upon stress or cardiac injury. Furthermore, C5aR1 has been shown to be upregulated in the adult mouse heart; following ischemia / reperfusion, C5aR1 upregulation was evident in ischemic cardiomyocytes. However, the role of C5aR1 and complement signaling in initiating cardiomyocyte proliferation and cardiac regeneration has not been investigated.

Following apical resection, we observe an upregulation of C5aR1 expression in cardiomyocytes and endothelial cells in the neonatal murine heart (Figure 5). This suggests a role for non-myeloid C5aR1 in heart regeneration. Although we did not observe localization of C5aR1 with the macrophage marker, differences in macrophage infiltration of the injured zone between C5aR1 knock-out and wild-type mice necessitate further downstream studies into the interplay of immune cells and complement components in early cardiac regeneration. It should be noted that differences in macrophage infiltration were not quantified by flow cytometry. Cell-specific conditional C5aR1 deletion models in cardiomyocytes, endothelial cells and myeloid cells need to be examined in future experiments to define the cell-type(s) expressing C5aR1 that contributes to cardiomyocyte proliferative response following injury. Furthermore, the intracellular signaling mechanisms by which cardiomyocyte division is initiated after an injury by C5a signaling in the adult heart may provide new insights. Although we did not observe a significant increase in cardiomyocyte proliferation upon treatment with C5a in vitro, it is possible that the effect of C5a – C5aR1 on cardiomyocyte proliferation could be mediated by paracrine signals from non-myocytes.

A pilot experiment was conducted to analyze the therapeutic potential of C5aR1 activation in adult murine cardiac regeneration, following an ischemia – reperfusion (I/R) injury. A pilot dose of C5a was administered for four days after I/R. We observed a marginal, non-significant increase in cardiomyocyte proliferation in the adult mouse heart after I/R and treatment with C5a. Future analyses of the mechanistic processes underlying the proliferative effect seen by C5aR1 activation need to be conducted. Additionally, elucidation of the mechanisms that lead to the transcriptional upregulation of the receptor gene, C5aR1, following injury should
provide important details for how the system is regulated. Finally, we have not yet defined the dynamics of the entire complement cascade in these models in comparison to myocardium that fails to regenerate, such as in adult mammals, and these studies might reveal that complement activation is different or identical in these settings.

Currently, treatment for myocardial infarction focuses on reperfusion and limiting ventricular remodeling; therapy targeted at replenishing lost cardiomyocytes has not entered the clinical arena. Our findings uncover a molecular pathway involving the interplay of the complement system and cardiac regeneration. We suggest that defining the molecular events of successful heart regeneration could eventually allow us to understand what events are absent in myocardium that cannot regenerate.
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CHAPTER 5

Myocardial polyploidization creates a barrier to heart regeneration in zebrafish
ATTRIBUTIONS

Contributions to the work described in this chapter were made by Juan Manuel González Rosa, Michka Sharpe, Dorothy Field, Mark H Soonpaa, Loren J Field, Caroline E Burns, C. Geoffrey Burns.

JMGR conceived of the study. JMGR designed generated transgenic lines, performed ventricular resections, cardiomyocyte dissociations, RNA Scope in situ hybridization, immunofluorescence of adult sections, BrdU analysis of adult sections, confocal microscopy of adult hearts, tamoxifen induced recombination experiments, scar analysis and figure preparation. MS performed immunofluorescence of ect2 mutants, confocal microscopy of ect2 mutant embryos, generated Kaplan-Meyer survival curves, performed tamoxifen induced recombination experiments, sectioned hearts and performed AFOG staining to assess for regeneration. DF, MHS and LJF performed mouse experiments. MS contributions culminated in second authorship.
ABSTRACT
Correlative evidence suggests that polyploidization of heart muscle, which occurs naturally in post-natal mammals, creates a barrier to heart regeneration. Here, we move beyond a correlation by demonstrating that experimental polyploidization of zebrafish cardiomyocytes is sufficient to suppress their proliferative potential during regeneration. Initially, we determined that zebrafish myocardium becomes susceptible to polyploidization upon transient cytokinesis inhibition mediated by dominant negative Ect2. Using a novel transgenic strategy, we generated adult animals containing mosaic hearts composed of differentially labeled diploid and polyploid-enriched cardiomyocyte populations. Diploid cardiomyocytes outcompeted their polyploid neighbors in producing regenerated heart muscle. Moreover, hearts composed of equivalent proportions of diploid and polyploid cardiomyocytes failed to regenerate altogether, demonstrating that a critical percentage of diploid cardiomyocytes is required to achieve heart regeneration. Our data identify cardiomyocyte polyploidization as a barrier to heart regeneration and suggest that mobilizing rare diploid cardiomyocytes in the human heart will improve its regenerative capacity.
INTRODUCTION

Cells containing two sets of homologous chromosomes are diploid, while those with greater than two complete sets, either enclosed within a single nucleus or separated into multiple nuclei, are termed polyploid (Davoli and de Lange, 2011). Polyploid cells arise naturally during the maturation of certain lineages when diploid cells fuse or complete DNA replication without mitosis and/or cytokinesis (Orr-Weaver, 2015). Although polyploidization correlates with terminal differentiation in some lineages, cell cycle exit is not a universal feature of polyploid cells. For instance, polyploid hepatocytes contribute robustly to regenerated liver segments through proliferation with or without ploidy reversal (Duncan et al., 2010). Therefore, the consequences of polyploidization are context specific.

Adult mammalian hearts lack any appreciable capacity to regenerate. By contrast, injured hearts of neonatal mice (Porrello et al., 2011) and some non-mammalian vertebrates, including salamanders (Becker et al., 1974; Nakamura et al., 2016; Oberpriller and Oberpriller, 1974) and zebrafish (González-Rosa and Mercader, 2012; Poss et al., 2002), mount an impressive regenerative response driven by myocardial proliferation (Foglia and Poss, 2016). Despite their clinical importance, the variables that promote or restrict myocardial regeneration remain obscure. One often-noted difference between non-regenerative and highly regenerative hearts is the DNA content of their cardiomyocytes (Bersell et al., 2009; Kikuchi, 2014; Vivien et al., 2016). Specifically, non-regenerative hearts contain a majority of polyploid cardiomyocytes (Brodsky et al., 1994) that form early in life when their diploid predecessors complete one round of DNA replication without mitosis and/or cytokinesis (Alkass et al., 2015; Brodsky et al., 1980; Li et al., 1996;
Soonpaa et al., 1996). By contrast, highly regenerative hearts contain a majority of cardiomyocytes that are diploid (Oberpriller et al., 1988), or assumed to be diploid based on nucleation studies (Wills et al., 2008). Although these observations reveal an inverse correlation between ploidy and regeneration in the myocardium, a causal relationship has yet to be explored.

In rodents, the transition from diploid (1 x 2c) to polyploid myocardium occurs within the first post-natal week and results in myocardium composed of >90% polyploid cardiomyocytes that are largely binucleated (2 x 2c) (Li et al., 1996; Soonpaa et al., 1996). In humans, myocardial polyploidization occurs within the first two decades, which causes a similarly high percentage of cardiomyocytes to become polyploid (Mollova et al., 2013; Sandritter and Adler, 1976). However, the adult human heart contains 30% binucleated (2x2c) and ~60% mononucleated cardiomyocytes with DNA contents ranging from 4c to 16c (1x4c, 1x8c, 1x16c). Therefore, polyploid myocardium is a common feature of the non-regenerative hearts of adult mammals despite species-specific differences in DNA content and nuclear number.

In neonatal mice, the timing of myocardial polyploidization coincides with the disappearance of cardiac regenerative potential (Porrello et al., 2011), which has led to speculation of causality (Vivien et al., 2016). However, the post-natal maturation of the heart is accompanied by numerous transitions within cardiomyocytes, resident non-cardiomyocyte populations, extra-cardiac cells, and the extra-cellular matrix (Vivien et al., 2016). Despite the notion that one or more of these transitions are responsible for suppressing regenerative capacity, the causative factors remain largely unidentified.
In an effort to move beyond a correlation, we set out to design an experimental strategy for testing the hypothesis that myocardial polyploidization in zebrafish is sufficient to dampen heart regeneration. Important features of this system would include: 1) a mechanism for creating polyploid cardiomyocytes through inducible cytokinesis inhibition during hyperplastic growth of the heart, 2) a counter mechanism for re-enabling cytokinesis during adulthood both prior to cardiac injury and during regeneration, and 3) indelible reporter labeling of induced polyploid cardiomyocyte populations to compare their regenerative capabilities side by side with unlabeled diploid cardiomyocytes in vivo.

Our studies indicate that induced myocardial polyploidization is sufficient to dampen cardiomyocyte proliferation in a highly regenerative setting. Moreover, they uncover a requirement for a critical percentage (>50%) of diploid cardiomyocytes to achieve heart regeneration. Ultimately, these conclusions provide rationale for identifying therapeutic approaches to amplify the rare population of diploid cardiomyocytes in the human heart as a means to induce natural heart regeneration in disease states.
RESULTS

Zebrafish cardiomyocytes are mononucleated, diploid, and upregulate Ect2 during heart regeneration.

Zebrafish cardiomyocytes were assumed to be diploid based on their predominantly mononuclear nature (Wills et al., 2008). However, because mononuclear cells can be either diploid or polyploid, we set out to make direct measurements of cardiomyocyte ploidy in adult animals. To that end, we developed an imaging and analysis platform to survey cardiomyocyte nucleation and DNA content in single-cell dissociations of adult ventricles. Cardiomyocytes containing a single 2c nucleus (1 x 2c) were categorized as diploid (Figure 5.1.A), while those containing two ≥2c nuclei (2 x ≥2c) or a single ≥4c nucleus (1 x ≥4c) were classified as polyploid. To unequivocally identify cardiomyocytes and distinguish multinucleated cells from mononucleated aggregates, we analyzed dissociations from Tg(cmlc2:nucGFP), Tg(cmlc2:CAAXmKate) zebrafish in which cardiomyocyte nuclei and plasma membranes were labeled with GFP and mKate, respectively (Figures 5.1.B-1D and Figure S1A-S1H). This approach virtually eliminated the possibility of falsely counting cell aggregates as polyploid cardiomyocytes, which is a drawback of flow cytometric analysis. Nucleation analysis alone revealed that ~99% of cardiomyocytes in homeostatic hearts are mononucleated (Figure 5.1.E), consistent with a previous report (Wills et al., 2008). DAPI-fluorescence intensity was used to quantify the DNA contents of cardiomyocyte nuclei relative to a reference population of non-myocardial diploid cells (Figure S1I-S1Y). When plotted on a histogram, the distribution of cardiomyocyte ploidy, taking into account both nuclear number and DNA content (Figures 5.1.F-1H), clustered into a single major peak that
aligned with the diploid peak (Figure 1I). Altogether, this analysis revealed that ~99% of zebrafish cardiomyocytes are diploid (1x2c), whereas ~1% are polyploid with one (1x4c) or two (2x2c) nuclei (Figure 5.1.K). Thus, in contrast to the non-regenerative polyploid hearts of adult mammals, the highly regenerative zebrafish myocardium is diploid, which further bolsters the inverse correlation observed between myocardial ploidy and regenerative capacity.

In adult mammals, a small number of cardiomyocytes surrounding infarcted myocardium re-enter the cell cycle, undergo DNA replication, but largely fail to complete cytokinesis, which increases ploidy further (Ebert and Pfitzer, 1977; Senyo et al., 2013). To determine if cardiac injury induces myocardial polyploidization in zebrafish, we surveyed cardiomyocyte nucleation and ploidy both during (7 days post-resection; dpr) and after (60 dpr) heart regeneration. While the percentage of binucleated cardiomyocytes remained constant at both time points (Figure 5.1.E), the proportion of mononucleated 4c cells increased at 7 dpr (Figures 5.1.J-1K), which we attribute to diploid cardiomyocytes cycling through G2 (Poss et al., 2002). At 60 dpr, the percentage of mononucleated 4c cells remained elevated (Figure 5.1.K), despite lowered cardiomyocyte proliferation at this stage (Poss et al., 2002). These data suggest that a small proportion of bona fide polyploid cardiomyocytes are created during regeneration. Nonetheless, they constitute only ~1% of the total population, demonstrating that successful cytokinesis, rather than polyploidization, is the predominant outcome of zebrafish cardiomyocytes entering the cell cycle during regeneration.

We set out to test the hypothesis that doubling the DNA content of zebrafish cardiomyocytes would be sufficient to suppress their proliferative potential following
injury. To that end, we sought an experimental tool to achieve transient inhibition of cardiomyocyte cytokinesis during cardiac growth, which occurs primarily by hyperplasia in zebrafish (Wills et al., 2008). We searched the literature to identify dominant-negative proteins that were previously demonstrated to promote cytokinesis failure. We learned that dominant-negative forms of Racgap1, Kif23, or Ect2 are potent inhibitors of cytokinesis in cultured mammalian cells (Hirose et al., 2001; Liu et al., 2004; Tatsumoto et al., 1999). Racgap1 and Kif23 perform essential functions in central spindle formation. At the spindle, Ect2 catalyzes the conversion of RhoA-GDP to RhoA-GTP, which is required for contractile ring assembly and cytokinesis initiation (Green et al., 2012). Previous studies, using gene knockdown and knockout approaches, have demonstrated that Ect2 is essential for cytokinesis both in cultured cells and in several model organisms including zebrafish (Green et al., 2012; Hoijman et al., 2015).

To determine whether racgap1, kif23, and ect2 are induced in proliferating cardiomyocytes, we performed qPCR on uninjured and regenerating hearts. We learned that all three genes are significantly upregulated at 7 dpr, with ect2 showing the strongest induction (Figure 5.1.L), which persuaded us to evaluate its localization during regeneration. Using RNAscope in situ hybridization, we documented ect2 expression in cardiomyocytes along the wound edge (Figure 5.1.M-1N) where proliferation is the highest (Sallin et al., 2015). These data are consistent with the notion that cardiomyocyte cytokinesis relies on Ect2 function in zebrafish.

Interestingly, by mining a publically available RNA-sequencing dataset (O’Meara et al., 2015), we discovered that Ect2 expression declines in mouse
neonatal cardiomyocytes (Figure S2) as they become polyploid (Alkass et al., 2015; Soonpaa et al., 1996) and the regenerative window closes (Porrello et al., 2011). This observation suggests that Ect2 downregulation might be a natural mechanism to induce cardiomyocyte polyploidization in mammals.
Figure 5.1. Zebrafish cardiomyocytes are mononucleated, diploid, and upregulate Ect2 during heart regeneration. (A) Schematic depicting cardiomyocyte nucleation and ploidy. (B) Cardiomyocytes (white arrows) from dissociated Tg(cmlc2:nucGFP) hearts. (C,D) Mononucleated and binucleated Tg(cmlc2:nucGFP) cardiomyocytes. (E) Average percentages of mononucleated (MonoN) and binucleated (BiN) cardiomyocytes in uninjured (UI), 7 dpr and 60 dpr ventricles (mean±s.d., n=9309, 9269, 9578 total cells respectively from 4 biological replicates per group; 2 pooled ventricles per replicate; n.s., not significant by one-way ANOVA test). (F,H) DAPI-stained mononucleated diploid, binucleated tetraploid, and mononucleated tetraploid cardiomyocytes isolated from Tg(cmlc2:nucGFP) hearts. Insets show DAPI signal. (I) Distributions of non-cardiomyocyte (gray) and cardiomyocyte (green) DNA content in homeostatic ventricles (n=779 and 552 cells, respectively, from 3 biological replicates; 1 ventricle per replicate). (J) Distributions of cardiomyocyte DNA content in 7 dpr and 60 dpr ventricles (blue and orange bars, respectively; overlap appears in brown). Insets are magnifications to show low frequency events (black arrows, tetraploid cardiomyocytes). (K) Quantification of indicated cardiomyocyte populations from UI, 7 dpr and 60 dpr ventricles (mean±s.d., n=3250, 2699 and 4306 total cells from 7, 3 and 4 biological replicates per group, respectively; 3 ventricles per replicate; ****P<0.0001; **P<0.01 by one-way ANOVA followed by Tukey’s multiple comparisons test). (L) qPCR analysis showing relative expression of three genes
**Ect2 is required for zebrafish cardiomyocyte cytokinesis and for maintaining their diploid state**

To learn if Ect2 is required for cardiomyocyte cytokinesis in zebrafish, we performed nucleation and ploidy analyses on cardiomyocytes from *ect2* null-mutant embryos (Amsterdam et al., 2004) carrying the *cmlc2:nucDsRed-Express* transgene (Takeuchi et al., 2011), which labels cardiomyocyte nuclei with red fluorescence. Using brightfield microscopy, we identified *ect2* mutant embryos by their pronounced body curvatures, small heads and necrotic brains (Figure S3). To measure cardiomyocyte ploidy in these animals, we first determined nuclear number for individual cardiomyocytes in confocal Z-stacks using nuclear red fluorescence and a plasma membrane marker. We then quantified the ploidy of each nucleus based on DAPI fluorescence intensity. Compared to control siblings, mutant hearts at 30 hours post fertilization (hpf) contained a 2-fold increase in polyploid cardiomyocytes (Figures S4A-S4C). This difference grew to 9-fold at 72 hpf (Figures 5.2.A, 2B and 2D), which is soon after hyperplastic growth of the heart commences (Choi et al., 2013). These data demonstrate that proliferating zebrafish cardiomyocytes become polyploid in the absence of Ect2 activity.
Next, we tested whether a dominant negative Ect2 protein (dnEct2) would inhibit cytokinesis and induce polyploidization in cardiomyocytes during cardiac growth. Previous reports demonstrated that overexpression of a C-terminally truncated mouse ECT2 protein, which lacks the GEF catalytic domain but retains RhoA-GDP binding activity, behaves as a dominant negative protein (dnECT2) by interfering with the catalytic conversion of RhoA-GDP to RhoA-GTP by wild-type ECT2 (Oceguera-Yanez, 2005; Sakata et al., 2000; Tatsumoto et al., 1999). We isolated and analyzed transgenic animals [Tg(cmlc2:GFP-P2A-dnEct2), abbreviated cmlc2:GdnEct2] continuously expressing GFP and an analogously-truncated zebrafish Ect2 protein (dnEct2) in all cardiomyocytes from embryonic stages. At 72 hpf, transgenic embryos displayed a 6-fold increase in polyploid cardiomyocytes (Figures 5.2.C and 5.2.D), a phenotype similar to that observed in ect2 mutants. To rule out perturbations in cell cycle entry caused by dnEct2 expression, we analyzed cardiomyocyte DNA synthesis and found no significant differences between cmlc2:GdnEct2 and sibling embryos (Figures S4D-S4F). During larval stages, cmlc2:GdnEct2 zebrafish were morphologically indistinguishable from siblings until 25 days post fertilization (dpf) (12.5 mm standardized standard length, SSL, Parichy et al., 2009) when their growth slowed, signs of heart failure emerged (Figure 5.2.E), and survival plummeted (Figure 5.2.F). An analysis of transgenic hearts at 30 dpf (14.5 mm SSL) revealed that ventricles of relatively normal size were composed of drastically fewer cardiomyocyte nuclei, 75% of which were polyploid with DNA contents ranging from 4c-16c (Figures 5.2.G-2L). Cardiomyocyte diameter measurements revealed significant cellular hypertrophy (Figure S4G-S4I). These data demonstrate that continuous expression of dnEct2 expression in zebrafish
cardiomyocytes impairs cytokinesis, induces polyploidization, and causes cells to grow by hypertrophy instead of hyperplasia. While tolerated initially, hypertrophic growth eventually becomes pathologic and heart failure ensures. We created these animals simply as a proof of concept that dnEct2 expression inhibits cardiomyocyte cytokinesis in zebrafish. They were not used for regeneration studies because ongoing experimental inhibition of cardiomyocyte cytokinesis after injury would mask any inherent proliferative potential polyploid cardiomyocytes might possess.
Figure 5.2. Loss of Ect2 function causes polyploidization of zebrafish cardiomyocytes. (A-C) Confocal projections of 72 hpf embryonic hearts from control (CTRL), ect2-/-, and Tg(cmlc2:GdnEct2) animals carrying the Tg(cmlc2:nucDsRed) transgene. Single confocal planes of boxed regions are shown at higher magnification with Alcama immunostaining to highlight plasma membranes. White and yellow arrows indicate diploid and polyploid cardiomyocytes, respectively. (D) Quantification of indicated cardiomyocyte populations in the indicated cohorts at 72 hpf (mean±s.d, n=4, 4 and 4 embryos for ect2+/+, ect2-/-, non-Tg, and Tg(cmlc2:dnEct2), respectively. ****P<0.0001; ***P<0.001; **P<0.01; *P<0.05 by one-way ANOVA followed by Tukey’s multiple comparisons test. (E) 45 dpf (e19.5 mm SSL) Tg(cmlc2:GdnEct2) and non-Tg sibling animals with arrowheads highlighting scale bristling (green), blood pooling (white), and pericardial edema (purple) in Tg(cmlc2:dnEct2) zebrafish. 68/77 Tg(cmlc2:GdnEct2) and 0/88 non-Tg siblings developed these phenotypes. (F) Representative Kaplan-Meier plot for Tg(cmlc2:GdnEct2) and non-Tg cluchmates from one of three independent
Design of a new transgenic system to create polyploid cardiomyocytes and track their contributions to regeneration

Therefore, we designed a double transgenic system, with two variations, to create adult zebrafish whose hearts contained cardiomyocyte populations that were: 1) significantly enriched in polyploid cells created by transient dnEct2 expression during cardiac growth and 2) permanently labeled with GFP. The first variation caused only a subset of the myocardium to become GFP+ and polyploid enriched, which permitted a side-by-side comparison of the regenerative capacities of diploid and polyploid cardiomyocyte populations in the same hearts. The second caused the entire myocardium to become GFP+ and polyploid enriched, which significantly boosted the overall polyploid proportion and allowed us to evaluate the consequences for cardiac regenerative fitness.

For the first variation, we generated the cmlc2:(iF-S)lox-mG transgene (Figure 5.3.A), which contains a cardiomyocyte-specific promoter upstream of a “floxed” cassette for co-expression of mOrange fluorescent protein and 4-HT-inducible
Flipase. This cassette also contains a polyadenylation signal to prevent downstream transcription of a second cassette for Cre-dependent expression of membrane GFP (mG or GFP) in cardiomyocytes. Next, we generated the $hsp:(P^*-S)^{FRT}$-$Cre$-$dnEct2$ transgene (Figure 3B), which contains the zebrafish heat shock promoter upstream of a “blank” expression cassette flanked by FRT recombination sites. This cassette also contains a polyadenylation signal to prevent downstream transcription of a second expression cassette for Flipase-dependent heat shock-mediated expression of Cre recombinase and dnEct2.

To create adult animals whose hearts were composed of GFP- diploid and GFP+ polyploid enriched cardiomyocyte populations, we treated 24 hpf double transgenic embryos with 4-HT to induce Flipase-mediated excision of $(P^*-S)$ from the $hsp:(P^*-S)^{FRT}$-$Cre$-$dnEct2$ transgene in a random subset of cardiomyocytes (Figures 5.3.C and S5). Thereafter, this myocardial subset carried the recombined $hsp$:Cre-$dnEct2$ transgene, which conferred heat shock dependent co-expression of Cre recombinase and dnEct2. It also became permanently labeled with GFP following a single heat shock at 48 hpf, which induced Cre-mediated excision of $(iF-S)^{lox}$ from $cmlc2:(iF-S)^{lox}$-$mG$ to create $cmlc2:mG$. Animals were grown for 3 weeks (up to ~10 mm SSL) at normal temperature to allow equivalent expansion of the GFP- diploid and GFP+ polyploid-susceptible cardiomyocyte populations through hyperplasia. Thereafter, animals were heat-shocked daily for 3 months to activate dnEct2 expression, inhibit cardiomyocyte cytokinesis, and induce polyploidization specifically within the GFP+ subset. Next, we injured adult hearts by ventricular apex amputation and analyzed regeneration in the absence of heat shocking and experimental cytokinesis inhibition (Figure 5.3.C).
Before injury, these animals and their hearts were grossly indistinguishable from controls (Figures 5.3.D and 5.3.E). Prominent subsets of GFP+ myocardium were present, in both the trabecular and compact layers (Figure 5.3.E), indicating that transient expression of dnEct2 during cardiac growth did not undermine cardiomyocyte viability. On average, approximately half of the myocardium was GFP+ in the mosaic hearts (50.28 ± 13.27 %, mean ± SD; Figure 3F, n=19).

Next, we assessed the efficiency of our experimental strategy for inducing cardiomyocyte polyploidization within the GFP+ subset of cardiomyocytes. We learned that approximately half of the GFP+ cardiomyocytes were polyploid with 4c DNA content, which represents an ~50-fold enrichment over the native population (Figures 3G and 3H). The majority of the polyploid cardiomyocyte population was mononucleated (Figure 3H), similar to that in human hearts (Mollova et al., 2013; Sandritter and Adler, 1976). Given that ~50% of the myocardium was GFP+ (Figure 5.3.F), and that ~50% of the GFP+ cardiomyocytes became polyploid (Figure 5.3.G-3H), ~25% of the myocardium in mosaic hearts was polyploid. Importantly, GFP+ cardiomyocytes contained sarcomeres that were indistinguishable from neighboring cells (Figures 5.3.I), ruling out a defect in cardiomyocyte differentiation. Polyploid cardiomyocytes in mosaic hearts were two times larger on average than their diploid counterparts (Figure 5.3.I and 5.3.J).

The second variation of our transgenic system conferred polyploid susceptibility to the entire myocardium during cardiac growth. To that end, we replaced inducible Flipase with constitutive Flipase to generate cmhc2:(F-S)lox-mG (Figure 5.4.A). After heat shocking double transgenic embryos once during embryogenesis (Figure 5.4.B), the entire myocardium turned GFP+ (data not
shown), indicative of homogeneous excision of \((P^*-S)\) from \(hsp:(P^*-S)^{FRT}\)-\(Cre\)-\(dnEct2\) to create \(hsp:Cre\)-\(dnEct2\). After three weeks of growth without heat shock treatments followed by three months of daily heat shocking (Figure 5.4.B), these animals and their GFP+ hearts were grossly indistinguishable from sibling animals not exposed to heat shocking during growth (Figures 5.4.C and 5.4.D). No evidence of DNA damage or apoptosis resulting from \(dnEct2\) expression was detectable (Figures S6A-S6C and S6E-S6F). Control hearts were ~99% diploid (Figures 5.4.E and 5.4.F), indicating that polyploidization does not occur in the absence of heat shocking from transgene leakiness. Similar to the GFP+ population in partially enriched hearts (Figure 5.3.F), approximately half of the homogenous GFP+ myocardium in heat-shocked animals was polyploid with 4c DNA content, with similar proportions containing one (1 x 4c) or two (2 x 2c) nuclei (Figures 5.4.E and 5.4.F).
Figure 5.3. Experimental strategy to generate mosaic hearts containing permanently labeled polyploid cardiomyocytes through transient cytokinesis inhibition. (A-C) Transgenes and experimental strategy used to create adult zebrafish with mosaic hearts composed of diploid (GFP-) and polyploid-enriched (GFP+) cardiomyocyte populations. Detailed description of the experimental strategy is provided in Figure S7. (D) External appearance of a double-transgenic adult zebrafish with a mosaic heart comprised of GFP- diploid and GFP+ polyploid-enriched cardiomyocytes. (E) Adjacent sections from an adult mosaic heart immunostained for Tropomyosin and GFP (left) or stained with AFOG (right). Boxed area shows apex region at higher magnification. (F) Quantification of the percentage of GFP+ myocardium relative to the total ventricular myocardium from mosaic adult hearts (n=19). (G) DNA content of GFP- (mO+) and GFP+ cardiomyocytes from mosaic hearts. (H) Quantification of indicated cardiomyocyte populations from (F) (mean±s.d., n=620 and 520 total cells from 4 and 3 biological replicates per group, respectively; 3 ventricles per replicate; ****P<0.0001; ***P<0.001 by one-way ANOVA followed by Tukey’s multiple comparisons test). (I) Dissociated cardiomyocytes from a mosaic heart immunostained for Tropomyosin and GFP. Boxed regions show DAPI staining. Shown are diploid (top), mononucleated tetraploid (middle) and binucleated tetraploid (bottom) cardiomyocytes. (J) Quantification of cardiomyocyte size from the indicated classes from ventricular dissociations (n=822, 84 and 97 cardiomyocytes; ****P<0.0001; Kruskal-Wallis test followed by Dunn’s multiple comparisons test). Scale bars: 5 mm (D) 100 mm (E), 25 mm (I). CM, cardiomyocyte; ER, early recombination; HS, heat-shocked.
Figure 5.3. continued. percentage of GFP+ myocardium relative to the total ventricular myocardium from mosaic adult hearts (n=19). (G) DNA content of GFP- (mO+) and GFP+ cardiomyocytes from mosaic hearts. (H) Quantification of indicated cardiomyocyte populations from (F) (mean±s.d., n=620 and 520 total cells from 4 and 3 biological replicates per group, respectively; 3 ventricles per replicate; ****P<0.0001; ***P<0.001 by one-way ANOVA followed by Tukey’s multiple comparisons test). (I) Dissociated cardiomyocytes from a mosaic heart immunostained for Tropomyosin and GFP. Boxed regions show DAPI staining. Shown are diploid (top), mononucleated tetraploid (middle) and binucleated tetraploid (bottom) cardiomyocytes. (J) Quantification of cardiomyocyte size from the indicated classes from ventricular dissociations (n=822, 84 and 97 cardiomyocytes; ****P<0.0001; Kruskal-Wallis test followed by Dunn’s multiple comparisons test). Scale bars: 5 mm (D) 100 mm (E), 25 mm (I). CM, cardiomyocyte; ER, early recombination; HS, heat-shocked.

Altogether, our two experimental strategies generated healthy adult zebrafish whose hearts contained a GFP+ cardiomyocyte population with approximately equivalent numbers of diploid and polyploid cardiomyocytes. In one strain, subsets of the myocardium were GFP+ and polyploid enriched (Figures 5.3.A-3H). In the other strain, the entire myocardium was GFP+ and polyploid enriched (Figures 5.4.A-4F). Importantly, we did not uncover evidence that cardiomyocyte polyploidization grossly undermines cellular (Figures 5.3.E, 3I, 4D, S6A-S6C, and S6E-S6F) or animal (Figures 5.3.D and 5.4.C) health when analyzed prior to cardiac injury. Moreover, inducing dnEct2 expression transiently for one week in homeostatic adult diploid myocardium did not elicit signs of toxicity including DNA damage (Figure S6D) or cell death (Figure S6G), suggesting that dnEct2 targets cytokinesis very specifically without impinging on multiple cellular processes. In this experiment, cardiomyocyte ploidy was not altered (Figure S6H-S6K) because cellular proliferation is minimal during homeostasis (Wills et al., 2008).
Figure 5.4. Experimental strategy to generate hearts highly enriched in permanently labeled polyploid cardiomyocytes through transient cytokinesis inhibition. (A,B) Transgenes and experimental strategy employed to maximize the percentage of cardiomyocytes susceptible to polyploidization. (C) External appearance of double-transgenic adult zebrafish, subjected to early recombination during embryogenesis, grown in the absence (top) or presence (bottom) of polyploid-inducing heat-shock treatments. (D) Adjacent sections from a fully recombined adult heart immunostained for Tropomyosin and GFP (left) or stained with AFOG (right). Boxed area shows apex region at higher magnification. (E) DNA content of GFP+ cardiomyocytes from the indicated cohorts. (F) Quantification of indicated cardiomyocyte populations from I (mean±s.d., n=564 and 406 total cardiomyocytes from 4 and 4 pooled ventricles, respectively, ***P<0.001; **P<0.01, by one-way ANOVA followed by Tukey’s multiple comparisons test). Scale bars: 5 mm (C) 100 mm (D). CM, cardiomyocyte; ER, early recombination; HS, heat-shocked.
We studied regeneration in both strains following ventricular amputation. Importantly, animals were never heat shocked during the regenerative window. In other words, dnEct2 expression and experimental cytokinesis inhibition were non-existent, which allowed us to monitor the inherent proliferative capacities of polyploid cardiomyocytes in an otherwise highly permissive environment *in vivo*.

**Cardiomyocyte polyploidization is sufficient to impair regenerative proliferation.**

To evaluate the proliferative potential of polyploid cardiomyocytes relative to their diploid neighbors, we amputated ventricular apices of hearts containing subsets of GFP+ polyploid enriched cardiomyocytes (Figures 5.3.C and 5.3.E). After 45 days, we quantified the percentages of GFP+ polyploid enriched muscle in regenerated and surrounding myocardium. For each heart, we calculated a “GFP+ Cell Contribution Index” (GCCI), which represents the log$_2$-fold change in the percentage of GFP+ myocardium that occurred during proliferative expansion of wound-edge cardiomyocytes. A negative GCCI would reflect an impaired relative contribution made by GFP+ cells. To establish a baseline, we generated adult animals whose hearts contained GFP+ “neutral” diploid cardiomyocytes by recombining $\text{cmlc2:}(i\text{F-S})^{\text{lox}}\text{-mG}$ in a subset of embryonic cardiomyocytes with the $\text{cmlc2:CreER}^T_2$ transgene (Kikuchi et al., 2010). These hearts regenerated normally (Figure 5.5.A) and GFP+ diploid cells contributed robustly to regenerated muscle (Figure 5.5.E). Next, we confirmed that FlpOERT$_2$ was not leaky and that three months of daily heat shocking during growth did not impair regeneration in animals carrying unrecombined transgenes (Figure 5.5.B). Lastly, we documented that a
second population of “neutral” cardiomyocytes, those carrying the hsp:Cre-dnEct2 transgene but never exposed to heat shocking, contributed normally to regenerated muscle (Figures 5.5.C and 5.5.E).

When we injured hearts containing subsets of GFP+ polyploid-enriched myocardium, regeneration proceeded normally as evidenced by a lack of accumulated scar tissue (Figure 5F). In the regenerated area, we observed a striking reduction in GFP+ cardiomyocytes (Figure 5.5.D), which caused the GCCI to become significantly negative (Figures 5.5.E). Importantly, BrdU incorporation analysis revealed that proliferation within the GFP+ polyploid enriched cardiomyocyte population was ~2-fold lower than that in the GFP- diploid fraction (Figures 5G-5I). Therefore, diploid cardiomyocytes outcompeted their polyploid neighbors in producing regenerated muscle through proliferative expansion, indicating that a single cytokinesis failure and resulting polyploidization during cardiac growth are sufficient to significantly reduce cardiomyocyte proliferation during regeneration.
Figure 5.5. Myocardial polyploidization creates a barrier to cardiomyocyte proliferation and heart regeneration. (A-D) Adjacent sections from 45 dpr hearts from the indicated cohorts, immunostained for Tropomyosin and GFP (left) or stained with AFOG (right). $n = 14$ (12), 10 (9), 9 (9), 19 (18) hearts (number of hearts that showed complete regeneration indicated within the brackets), respectively. (E) Relative change in percentage of GFP+ cardiomyocytes in the regenerate compared to the surrounding region in hearts from the indicated cohorts ($n = 12$, 9 and 19, respectively). ***$P<0.001$; **$P<0.01$; Kruskal-Wallis test followed by Dunn’s multiple comparisons test. (F) Quantification of the scar area of hearts from (A-D) normalized to the ventricular area. Measurements were performed on every fifth pair of serial sections from throughout the heart ($n = 14$, 10, 19 hearts; solid black line indicates the mean); n.s., not significant by Kruskal-Wallis test. (G) BrdU pulse-chase strategy used to evaluate cardiomyocyte proliferation during regeneration in experiments from G-H. (H) Section from a 14 dpr mosaic heart composed of diploid (GFP-) and polyploid-enriched (GFP+) cardiomyocyte populations, immunostained for Tropomyosin, GFP, Mef2 and BrdU. BrdU and Mef2/GFP signals of boxed region are shown at higher magnifications. Blue arrowheads indicate BrdU+ cardiomyocyte nuclei. (I) Cardiomyocyte BrdU labeling indices of GFP- and GFP+ populations in injury sites in experiments from F-G. Box-and-whisker plot. $n = 7$ hearts. **$P < 0.01$, Mann–Whitney test. CM, cardiomyocyte; ER, early recombination; HS, heat-shocked. Scale bars: 50 mm.
Myocardial polyploidization creates a barrier to heart regeneration

During the course of analyzing regeneration of mosaic hearts containing a GFP+ polyploid-enriched cardiomyocyte population, we observed scar formation instead of myocardial regeneration in a small number of hearts (**Figure 5.6.A, n=2**). In these hearts, ~80% of the myocardium surrounding the injury area was GFP+. Therefore, the polyploid percentage was ~40% (~80% GFP+ myocardium x ~50% polyploid cells in enriched population= ~40%). This observation suggested that the ploidy composition of wound edge myocardium might determine regenerative competence.

To pursue this observation further, we injured hearts with homogenous GFP expression and 45% polyploid myocardium to determine if reducing the proportion of proliferation-competent diploid cardiomyocytes from 99% to 55% would undermine regenerative potential. First, we documented normal regeneration in animals grown at normal temperature to rule out the possibility that injury alone would activate the hsp:Cre-dnEct2 transgene (**Figure 5.6.B**). Next, we learned that hearts composed of ~50% (45.04 ± 0.76 %, mean ± SD; Figure 4E) polyploid cardiomyocytes were incapable of completely regenerating and consistently scarred (**Figure 5.6.C**). We found that the scar size in the polyploid-enriched hearts was 12-times bigger than that in control animals (Figure 6D). This was accompanied by a 61% reduction in BrdU incorporation between 7 dpr and 14 dpr (**Figures 5.6.E-6F and 5.6.H**). Importantly, heat shocking equivalent animals exclusively during adulthood, when the myocardium is minimally proliferative (Wills et al., 2008), did not undermine subsequent cardiomyocyte BrdU incorporation between 7 dpr and 14 dpr (**Figures 5.6.G and 5.6.H**), demonstrating that failed regeneration depends on dnEct2-
mediated polyploidization that occurs specifically during cardiac growth (Figures 5.4.F and S6J). Taken together, these data demonstrate that the ploidy composition of the myocardium is a critical determinant of regenerative capacity.

Importantly, the myocardium in these hearts naturally upregulated endogenous Ect2 following injury (Figure S7), which suggests that re-expression of Ect2 in polyploid cardiomyocytes is not sufficient to revert cardiomyocyte ploidy and that the proliferative barrier created by polyploidization is largely irreversible. This conclusion was further supported by the observation that overexpression of Ect2 in the highly polyploid myocardium of mice did not boost ventricular myocardial proliferation after experimental myocardial infarction (Figure S8).
Figure 5.6. Increasing the proportion of polyploid cardiomyocytes impairs heart regeneration. 
(A,B) Adjacent sections from 45 dpr hearts from the indicated cohorts, immunostained for Tropomyosin and GFP (left) or stained with AFOG (right). \( n = 5 \) (5), 9 (0) hearts (number of hearts that showed complete regeneration indicated within the brackets), respectively. (C) Quantification of the scar area of hearts from (A,B) normalized to the ventricular area. Measurements were performed on every fifth pair of serial sections from throughout the heart (n= 5, 9 hearts; solid black line indicates the mean); ***\( P < 0.001 \), two-tailed unpaired t-test. (D-F) Sections from 14 dpr hearts of the indicated cohorts, immunostained for Mef2 and BrdU, as described in Figure 5G-5H. White arrows indicate BrdU+ cardiomyocyte nuclei. (G) Cardiomyocyte BrdU labeling index in injury sites in experiments from (D-F). Box-and-whisker plot. \( n = 11, 10, 7 \) ventricles, respectively. ****\( P < 0.0001 \); **\( P < 0.01 \) by one-way ANOVA followed by Tukey’s multiple comparisons test). CM, cardiomyocyte; ER, early recombination; HS, heat-shocked. Scale bars: 50 mm.
Figure 5.7. Model of cardiomyocyte polyploidization as a barrier to heart regeneration. (A) Hearts composed almost exclusively of diploid cardiomyocytes (1x2c), such as those in the adult zebrafish and mouse neonate, regenerate efficiently after amputation through myocardial proliferation. (B) In mosaic hearts composed of diploid (GFP-) and polyploid-enriched (GFP+) cardiomyocyte populations, diploid cardiomyocytes proliferate actively to replace injured muscle, with minor contributions from the polyploid-enriched population. (C) Minimizing the proportion of diploid cardiomyocytes in the zebrafish heart, a situation similar to that in adult mammals, including humans, results in reduced cardiomyocyte proliferation and persistent scarring. Dashed line, plane of amputation; dark area, amputated tissue; blue boxes, regenerated myocardium; red and green cells, diploid and polyploid-enriched cardiomyocyte populations.
DISCUSSION

Since the discovery of heart regeneration in zebrafish 15 years ago, substantial efforts have been expended to identify the signaling pathways required to initiate and achieve myocardial regeneration in this model organism (González-Rosa et al., 2017). However, it remains unclear whether the absence of cardiac regenerative potential in other vertebrate species, including adult mammals, can be ascribed to failed activation of pro-regenerative pathways, to cell-intrinsic properties that might prevent cardiomyocytes from participating in regeneration, or to a combination of both factors. To date, investigators have highlighted unique attributes of highly regenerative species as factors that might be permissive for cardiac regeneration. These include low blood pressure, hypoxic cardiac environment, low cardiac metabolic requirements, unique immune responses (Lai et al., 2017), and immature cardiomyocyte phenotypes including less organized sarcomeres and diploid DNA content (Vivien et al., 2016). Implicit in these arguments is that differing characteristics in non-regenerative species represent barriers to cardiac regeneration. However, these simple correlations do not establish causality. In most cases, experimental evidence has not been provided to demonstrate that modifying these properties in a highly regenerative species is sufficient to dampen cardiac regeneration.

We took advantage of the highly regenerative zebrafish heart to study the potentially negative effect of polyploidization on myocardial regenerative potential. In this regard, we attempted to mimic the natural process of cardiomyocyte polyploidization that occurs from failed cytokinesis during post-natal life in mammals and correlates temporally with the loss of regenerative potential (Porrello et al.,
As designed, we attempted to isolate polyploidization as an initiating event in regenerative decline without directly modifying other properties of the zebrafish heart, which might contribute to the loss of regenerative potential in post-natal mice including immune system maturation (Lai et al., 2017), cardiac fibroblast expansion (Banerjee et al., 2007), elevated production of reactive oxygen species (Puente et al., 2014), and extracellular matrix alterations (Bassat et al., 2017).

Despite the notion that cardiomyocyte polyploidization might be synonymous with cell-cycle exit, several counterexamples have been described in nature where polyploid cells re-enter the cell cycle and divide robustly. This is the case for mammalian hepatocytes during liver growth and regeneration (Duncan et al., 2010) and for rectal papillar cells in Drosophila (Fox et al., 2010). Moreover, polyploidization is required for regeneration of certain structures, such as the abdominal epithelium during Drosophila wound healing (Losick et al., 2013).

Recently, the percentage of mononuclear diploid cardiomyocytes in adult hearts of inbred mouse strains was reported to be variable and positively correlated with functional recovery after myocardial infarction (Patterson et al., 2017). Through genome wide association and genetic loss of function analyses, the authors identified TNNI3 interacting kinase (Tnni3k) activity as a positive regulator of myocardial polyploidization. Accordingly, continuous overexpression of Tnni3k in zebrafish cardiomyocytes elevated the percentage of mononuclear polyploid cardiomyocytes in adult animals from 0 to 7-20%, which correlated with compromised myocardial regeneration after amputation. While these data are consistent with a connection between myocardial polyploidization and reduced regenerative capacity, the potentially negative influence of a modest percentage of
polyploid cardiomyocytes on heart regeneration in these animals cannot be disentangled from that of ongoing Tnni3k expression in spared cardiomyocytes (Vagnozzi et al., 2013). Lastly, this study was not designed to make direct comparisons between the regenerative capacities of diploid and polyploid cardiomyocytes as neither population carried a genetic label.

By establishing that the ploidy composition of zebrafish myocardium is diploid, we provided additional evidence to support the already-strong correlation between diploid myocardium and high regenerative capacity. To move beyond a correlation, we designed transgenic tools to transiently inhibit cytokinesis and induce cardiomyocyte polyploidization in zebrafish. To our knowledge, our experimental approach is unique in its ability to: 1) induce polyploidization through expression of dnEct2 exclusively in cardiomyocytes, without affecting other cell types; 2) enable lineage-tracking of polyploid enriched populations of cardiomyocytes during regeneration; and 3) discontinue dnEct2 expression during regeneration, which allowed us to assess the inherent proliferative capabilities of polyploid cardiomyocytes during heart regeneration. Importantly, our transgenic strategy allowed us to assess whether polyploidization resulting from cytokinesis failure, rather than dnEct2 expression itself, creates a barrier to regeneration. This strategy is therefore superior to alternatives where a factor is expressed under a heat-shock promoter, which allows for temporal control but lacks tissue specificity, or where a factor is expressed from a myocardial specific promoter, which remains active following injury.

While the most obvious consequence of cytokinesis failure is the increase in DNA content, it is important to emphasize that a number of downstream changes are
triggered in polyploid cells. These changes include increases in cell size, centrosome amplification, or metabolic alterations (Schoenfelder and Fox, 2015). Importantly, some of these changes are precisely some of the cell-intrinsic properties that might explain the differences between regenerative and non-regenerative hearts (Vivien et al., 2016). Nonetheless, identifying the molecular and cellular events downstream of polyploidization that impinge on myocardial regeneration will require further investigation.

By exploiting the ability of our transgenic system to generate mosaic hearts composed of GFP- diploid and GFP+ polyploid enriched populations, we learned that decreasing the percentage of diploid cells in the GFP+ fraction, through polyploidization, caused quantitatively similar reductions in proliferation and contributions to regenerated muscle. Because these hearts were mosaic for the GFP+ population, the total percentage of polyploid myocardium was always less than 50% and regeneration was grossly unaffected. Remarkably, when we analyzed the regenerative potential of hearts containing >99% GFP+ myocardium, ~50% of which was polyploid, we observed a profound reduction in BrdU incorporation at 7 days post injury and scarring at later stages (Figure 7). Thus, while hearts composed of ~25% polyploid cardiomyocytes were able to regenerate, increasing the proportion to ~50% drastically reduced the regenerative ability of the zebrafish heart, suggesting that polyploid cardiomyocytes create a barrier to heart regeneration at a threshold within this range.

Further studies are required to understand why the substantial fraction (~50%) of diploid cardiomyocytes in these hearts did not support regeneration. One possibility is that diploid cardiomyocytes are endowed with a limited number cell
divisions following injury. In agreement with this hypothesis, multicolor clonal analysis of the myocardium during zebrafish heart regeneration revealed that clones in the regenerated myocardium are composed of 2-4 cells, suggesting that cardiomyocytes along the wound edge divide 1-2 times (Gupta et al., 2013). As a result, the percentage of diploid cells might become rate limiting when a large enough fraction of cardiomyocytes become proliferation-compromised by polyploidization. Any given maximal number of divisions might be determined by telomere erosion upon division. This hypothesis is consistent with the observation that telomerase reexpression and telomere expansion upon injury is required for heart regeneration in zebrafish (Bednarek et al., 2015). An alternative hypothesis is based on the relative locations of polyploid and diploid cardiomyocyte populations in the mosaic hearts. If the GFP+ border zone myocardium is preferentially enriched in polyploid cells, then diploid cardiomyocytes might not be able to actively invade the injured area and contribute to regeneration. Lastly, it remains possible that regenerative capacity is regulated by non-autonomous signals released by diploid or polyploid cardiomyocytes that are pro- or anti-regenerative, respectively. If true, then increasing the polyploid fraction would tip the balance towards lowered regenerative potential.

In summary, we transiently inhibited cytokinesis to induce polyploidization of zebrafish cardiomyocytes to mimic one aspect of the post-natal maturation of mammalian hearts. Although several factors have been hypothesized to impair heart regeneration in mammals (Vivien et al., 2016), our data provide compelling evidence that cytokinesis failure and polyploidization are the initiating events in the loss of regenerative potential. Myocardial polyploidization appears to have evolved at the
expense of regenerative ability, perhaps as a mechanism to enhance organ growth or physiology. However, the specific advantages of cardiomyocyte polyploidization remain unknown. Lastly, our data provide rationale to better characterize rare diploid cardiomyocytes in mammalian hearts and devise therapies to stimulate their deployment for boosting natural heart regeneration in humans.
METHODS

Experimental Models and Subject Details

Zebrafish

Zebrafish embryos, larvae, and adults were produced, grown, and maintained according to standard protocols approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital. Ethical approval was obtained from the Institutional Animal Care and Use Committees of Massachusetts General Hospital. For experiments with adult zebrafish, animals ranging in age from 3 to 18 months were used. Approximately equal sex ratios were used for experiments. Adult density was maintained at ~3-4 fish·l$^{-1}$ for all experiments in Aquarius racks and fed three times daily. Water temperature was maintained at 28 °C except during heat shock treatments.

Published strains used in this study include: wild-type AB, ect2 mutants (ect2)$^{hi3820aTg}$ (Amsterdam et al., 2004), Tg(cmlc2:nlsDsRed-Express)$^{hsc4}$ (Takeuchi et al., 2011), Tg(cmlc2:mKate-CAAX)$^{sd11}$ (Lin et al., 2012), Tg(-5.1 cmlc2:CreER$^{T2}$, cryaa:DsRed)$^{rd10}$ (Kikuchi et al., 2010), Tg(ubb:Zebrabow-M)$^{a131}$ (Pan et al., 2013). Details of the construction of the new lines generated in this study are described below. At least three independent founders of each line were isolated and tested to confirm the described expression patterns and phenotypes. Transgene sequences are available upon request.

Mice

Experimental mice were generated in an inbred DBA/2J background. Animals were housed under a 14-h light, 10-h dark cycle in the Laboratory Animal Research
Center at the Indiana University Medical School. Experiments were initiated when mice reached 12 weeks of age. Animals were provided food and water ad libitum. All mice treatment, surgery, and euthanasia protocols utilized in this study were performed in accordance with National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee (Study #10881). All surgeries were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

**Method Details**

**Ect2 mutant genotyping**

The $\text{ect2}^{\text{hi3820aTg}}$ allele consists of a retroviral insertion in the first intron of $\text{ect2}$ that interrupts the expression of the gene (Figure S3). Heterozygous animals carrying the $\text{ect2}^{\text{hi3820aTg}}$ allele were identified by PCR using the primers 5'-CGCTTCTCGCTTCTGTTCG-3' and 5'-GACATTGTGGAAGGAAGACACG-3'. Because the first primer anneals in the retroviral sequence, animals carrying the mutant allele produced a 471 bp amplicon.

**Construction of $\text{cmlc2:nucEGFP}$**

To generate the $\text{cmlc2:nucEGFP}$ transgenic line, a construct containing the following DNA elements was assembled by Gibson cloning: (1) a ~0.9-kb $\text{cmlc2}$ promoter to drive specific expression in cardiomyocytes; (2) the coding sequence for a nuclear localized enhanced green fluorescent protein (nls-EGFP). The entire construct was flanked with Tol2 sites to facilitate transgenesis. In this line, all cardiomyocytes constitutively express nls-EGFP. The full name of this line is
Construction of cmlc2:GdnEct2

To generate the cmlc2:GdnEct2 transgenic line, a construct containing the following DNA elements was assembled by Gibson cloning: (1) a ~0.9-kb cmlc2 promoter to drive specific expression in cardiomyocytes; (2) a bicistronic cassette encoding EGFP and a truncated version of Danio rerio ect2 containing the first 337 amino acids (amplified from clone MDR1734-202791320, GE Dharmacon, using the primers 5’-ATGGCTGACAGCAGCATACT-3’ and 5’-ACACAGCTTTCTTCATGGCAGG-3’), separated by a P2A sequence (Kim et al., 2011). Expression of similar truncations have been described in mammals to strongly inhibit cytokinesis due to the lack of GEF activity (Oceguera-Yanez, 2005; Sakata et al., 2000; Tatsumoto et al., 1999). The entire construct was flanked with Tol2 sites to facilitate transgenesis. In this line, all cardiomyocytes constitutively express a dominant negative form of Ect2 and the fluorescent protein EGFP. The full name of this line is Tg(cmlc2:EGFP-P2A-dnEct2)fb19.

Construction of cmlc2:(iF-S)lox-mG zebrafish

To generate the cmlc2:(iF-S)lox-mG transgenic line, a construct containing the following DNA elements was assembled by Gibson cloning: (1) a ~0.9-kb cmlc2 promoter to drive specific expression in cardiomyocytes; (2) a floxed bicistronic nls-mOrange2-P2A-FlpOERT2-polyA cassette that was generated by subcloning mOrange2 from pThy1-Brainbow3.2 (Addgene #45179, (Cai et al., 2013)) and FlpOERT2 from pBS-FlpOER (kindly provided by Drs. Lao and Joyner (Lao et al.,
2012)); and (3) a GFP containing a farnesylation tag to direct the fluorescent protein to the cell membrane (subcloned from pThy1-Brainbow3.2). The entire construct was flanked with Tol2 sites to facilitate transgenesis. In this line, all cardiomyocytes express the fluorescent protein mOrange2 directed to the nucleus and a tamoxifen-inducible Flipase. Upon Cre mediated recombination, the floxed cassette is eliminated and cardiomyocytes (and those cardiomyocytes that derive from them) are permanently labeled by the expression of a membrane-tagged GFP (Figure S5). The full name of this line is \( Tg(cmlc2:loxP-nls-mOrange-P2A-FlpOER^{T2} \cdot STOP-\text{loxP-memGFP})^{tb00}. \)

**Construction of cmlc2:(F-S)\(^{\text{lox}}\)-mG zebrafish**

To generate the \( cmlc2:(F-S)^{\text{lox}}\)-mG transgenic line, the \( cmlc2:(iF-S)^{\text{lox}}\)-mG construct was modified by Gibson cloning as follows: (1) the bicistronic nls-mOrange2-P2A-FlpOER\(^{T2}\)-polyA cassette was substituted with a nls-mOrange2-P2A-FlpO in which the recombinase lacks the \( \text{ER}^{T2} \) domain and is therefore constitutively active; and (2) a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) -- \( \text{Xenopus} \) b-globin polyadenylation signal cassette was added downstream of the FlpO to increase transcript stability. In this line, all cardiomyocytes express the fluorescent protein mOrange2 directed to the nucleus and a constitutively active Flipase. Upon Cre mediated recombination, the floxed cassette is eliminated and cardiomyocytes (and those cardiomyocytes that derive from them) are indelibly labeled by the expression of a membrane-tagged GFP. The full name of this line is \( Tg(cmlc2:loxP-nls-mOrange-P2A-FlpO-STOP-\text{loxP-memGFP})^{tb21}. \)
**Construction of hsp:(P*-S)$^{\text{FRT}}$-Cre-dnEct2 zebrafish**

To generate the *hs:(P*-S)$^{\text{FRT}}$-Cre-dnEct2* transgenic line, a construct containing the following DNA elements was assembled by Gibson cloning: (1) a ~1.5-kb *hsp70l* promoter to drive expression in response to heat shock (Halloran et al., 2000), amplified from p5E-*hsp70l* (Kwan et al., 2007); (2) the coding sequence of a Y65A mutated version (non fluorescent) of the fluorescent protein PhiYFP amplified from pThy1-Brainbow3.2 (Cai et al., 2013) flanked by *FRT* sites; (3) a bicistronic nls-Cre-P2A-dnEct2 cassette generated by subcloning a codon optimized, nuclear directed Cre from pAAV-pgk-Cre (a gift from Patrick Aebischer, Addgene plasmid #24593) and the *Danio rerio* dnEct2 amplified from *cmlc2:GdnEct2*. A *cryaa:ZsYellow* transgenesis control was included in the opposite orientation to facilitate visualization of transgenic animals. The entire construct was flanked with Tol2 sites to facilitate transgenesis. In this line, cells express PhiYFP$^*$ only after heat shock. Upon Flipase mediated recombination, the *FRT*-flanked cassette is eliminated and cells exposed to heat shock then express nls-Cre and dnEct2 (Figure S5). The full name of this line is *Tg(hsp70l:FRT-nucPhiYFP*-STOP-FRT-nlsCre-P2A-dnEct2; cryaa:ZsYellow)$^{\text{b22}}$.

**Generation of MHC-ECT2 mice**

The MHC-ECT2 transgene used the mouse alpha-cardiac myosin heavy chain (MHC) promoter and sequences encoding *Mus musculus* ECT2 (amplified from clone MMM4769-202769099, GE Dharmacon). The SV40 early region transcription terminator/poly-adenylation site (nucleotide residues #2586-2452) was inserted downstream from the ECT2 sequences. Transgene insert was purified and
microinjected into inbred C3HeB/FeJ (Jackson Laboratories, Bar Harbor MA) zygotes as described (Soonpaa et al., 1994). The resulting pups were screened using diagnostic PCR amplification and transgenic lineages were established. Transgene expression was stratified initially via qPCR and then confirmed by anti-Ect2 immunofluorescence (Figure S8).

Zebrafish Cardiac Injuries

Regeneration experiments were conducted using adult zebrafish as described (Poss et al., 2002). Briefly, fishes were anesthetized in tricaine, placed with their ventral side up on a sponge and a small incision was created to expose the apex of the ventricle. Approximately 20% of the ventricle was amputated using iridectomy scissors. A Kimwipe was applied to the bleeding animal with gentle pressure for a few seconds, and the animals were retrieved to a fish tank. After surgery, animals were revived by gently directing water to their gills using a plastic Pasteur pipette.

Myocardial infarction and cardiomyocyte DNA synthesis assay in mouse.

MHC-ECT2 line 1 mice were crossed with MHC-nLAC mice (Soonpaa et al., 1994), which express a nuclear cardiomyocyte-restricted β-galactosidase reporter to assist in cardiomyocyte nuclear identification in tissue sections. MHC-ECT2; MHC-nLAC double transgenic mice and MHC-nLAC single transgenic mice were intubated and ventilated with 2% isoflurane and oxygen. Via left thoracotomy, the left coronary artery was ligated at the inferior border of the left auricle as described previously (Murry et al., 2004). The mice were then implanted with osmotic mini-pumps (Alzet #1002; Cupertino, California) containing bromodeoxyuridine (16 mg/ml in phosphate
buffered saline (PBS); Roche, #280879; Indianapolis, Indiana) as described (Soonpaa and Field, 1994). Tissues were harvested, fixed in cacodylic acid/paraformaldehyde as described (Soonpaa and Field, 1997), and then cryoprotected in 30% sucrose in PBS, and sectioned at 10 microns using standard methodologies. After antigen retrieval (30 minute incubation in sodium citrate buffer at 100°C), samples were processed for β-galactosidase (Invitrogen Lifetechnologies #A-11132) and BrdU (Roche #11296736001; clone BMG 6H8 IgG1) immune reactivity using the Vector Mouse-on-Mouse kit (#BMK-2202). Signal was developed using Alexa 555-conjugated goat anti-rabbit and Alexa 488-conjugated goat anti-mouse secondary antibodies (Invitrogen Lifetechnologies #A21429 and #A11001, respectively). DNA was visualized with Hoechst 33342 (Invitrogen Lifetechnologies).

**Tamoxifen and heat shock treatments**

To induce Flipase-ER\(^{T2}\) mediated recombination, 24-30 hours post-fertilization (hpf) zebrafish larvae were exposed to 10 µM 4-hydroxy-tamoxifen (4-OHT, Sigma) for 12 hours. Treated embryos were washed in E3, raised to adulthood and used in the experiments described below. For mosaic experiments, embryos with highly recombined hearts were selected for growing. Heat-shock induction was conducted by placing embryos into a 39 °C water bath for 1 hour. When required, ~2-3 weeks post fertilization animals were transferred to an automated heat shock rack that exposed them daily to 60 minute elevations in temperature from 28 °C to 39 °C, as described (Lee et al., 2005). Adult heat shock treatments were performed using the same system.
Cardiomyocyte isolation from zebrafish ventricles

To generate single cell suspensions, zebrafish hearts were dissected as described previously (González-Rosa and Mercader, 2012) using ice-cold PBS supplemented with 0.3% bovine serum albumin (BSA, A3059, Sigma) and 20 mM glucose (G7528, Sigma) as dissection buffer. Hearts were collected in a 3 ml Petri Dish (153066, ThermoFisher Scientific) maintained on ice. After removing the atrium and the bulbous, ventricles were bisected to wash out any remaining blood. Tissues then were washed twice in dissection buffer and treated for 15 min in a solution of 0.2% trypsin, 0.8 mM EDTA (25200-056, Gibco) supplemented with 20 mM glucose and 10 mM 2,3-butanedione monoxime (B0753, Sigma). The first digestion was performed on ice and using gentle agitation. Next, the digestion solution was removed, the ventricles were washed three times using dissection buffer supplemented with 10 mM BDM and then digested for 45 min at room temperature in Accumax (SCR006, EMD Millipore) supplemented with 20 mM glucose and 10 mM BDM under mild agitation. Tissue fragments were dissociated by gentle pipetting and cell suspensions were immediately fixed in 10% neutral buffered formalin (HT501128, Sigma) for 1 hour at room temperature. Cells were pelleted by centrifugation at 400 x g for 5 minutes, resuspended in PBS, spread in Superfrost Plus slides (1255015, ThermoFisher Scientific) and air dried.

DNA quantification in cell spreads.

DNA content was determined by quantifying the integrated nuclear density of cells stained with the DNA dye 4',6-diamidino-2-phenylindole (DAPI), by adapting preexisting protocols (Ikenishi et al., 2012; Roukos et al., 2015; Tane et al., 2014).
Briefly, cardiomyocyte spreads were rehydrated in PBS, permeabilized for 45 minutes in 0.1% IGEPAL CA-630 (I8896, Sigma), 3% BSA in PBS and stained for 30 min using 14.3 µM DAPI dihydrochloride (Invitrogen) in permeabilization solution. After washing in PBS, slides were mounted in FluorSave Reagent (345789, Calbiochem) and images were acquired using a Nikon 80i compound microscope (Nikon Instruments) with a 20x lens, a Retiga 2000R high/speed CCD camera (QImaging) and the NIS-Elements image acquisition and analysis system (Nikon Instruments).

For each field (592 µm x 444 µm), fluorescence was captured from adequate channels (e.g. red for mKate-CAAX and/or mCherry, green for nucGFP and blue for DAPI) and saved independently as 12-bit *.nd2 files. Individual channels were merged in ImageJ software (Schneider et al., 2012), exported as a composite *.tiff file and binary masks containing nuclear areas, based on the blue channel (DAPI), were generated manually in Adobe Photoshop (Adobe Systems Incorporated). Only intact cells were analyzed and isolated nuclei or damaged cells were excluded. Independent binary masks were generated according to the expression of fluorescent proteins to distinguish cell populations.

Masks were then processed using the ROI Manager tool of ImageJ and the following macro:

```java
run("RGB Color");
run("8-bit");
setAutoThreshold("Default");
//setThreshold(0, 200);
setOption("BlackBackground", false);
```
run("Convert to Mask");
run("Analyze Particles...", "display exclude clear add");
run("Flatten");

that allowed to recognize individual nuclei and to generate a copy of the binary mask with each nucleus receiving a number for further reference. Integrated density from each nucleus was then calculated using the Multimeasure tool in ImageJ in the original 12-bit *.nd2 DAPI image. Values were exported and collected in an Excel file.

Ploidy measurement in dissociated cardiomyocytes

Ploidy values were determined on a per-nucleus basis by using the following strategy (Figure S1): First, the average raw integrated density value from a reference cell population (i.e., uninjured cardiomyocytes expressing a distinctive fluorescent protein) was determined for every picture. Then, the integrated density value from each cell from the same picture was divided by the average value of the control population and multiplied by 2. Additionally, images were manually inspected to determine the presence of binucleated cells. To that end, binary masks were compared to images containing membrane labeling (i.e., mKate-CAAX) (Figure S1). In the case of binucleated cells, values from both nuclei were then added in Excel. Ploidy values were then transferred to GraphPad Prism 7 to generate frequency histograms using a bin size of 0.25c. As a consensus based on the reference population, we considered diploid “2c”, those cells with values between 1.25c and 2.75c.
Quantification of cardiomyocyte nucleation in adult zebrafish

Cardiomyocyte nucleation was scored manually using cell spreads counterstained with DAPI as described above. To efficiently distinguish bona fide binucleated cells from cell aggregates, cardiomyocytes were isolated from animals carrying a transgene that directed the expression of a fluorescent protein to the cell membrane (i.e., \textit{cmlc2:mKate-CAAX} or \textit{cmlc2:mGFP}) (Figure S1).

Quantification of cardiomyocyte nucleation and ploidy in zebrafish cardiomyocytes

Cardiomyocyte nucleation was analyzed in \textit{ect2} mutant, \textit{Tg(cmlc2:GdnEct2)} and sibling embryos carrying the \textit{Tg(cmlc2:nlsDsRed-Express)} transgene. Embryos were fixed at 30 and 72 hpf overnight in 4% paraformaldehyde (PFA) in PBS at 4°C. Fixed embryos were bleached and permeabilized as described (Jahangiri et al., 2016), and subjected to immunofluorescence staining with anti-DsRed (Clontech, 1:200) and anti-Alcama (Dm-Grasp, clone Zn-8, Developmental Studies Hybridoma Bank; 1:50) antibodies. Alexa conjugated secondary antibodies (Life Technologies, 1:500) were used to detect primary antibody signals. Nuclei were counterstained with DAPI. Stained embryos were mounted in low-melting agarose and imaged using a Nikon A1 confocal microscope equipped with a 40X water immersion lens. Hearts of stained embryos were imaged and analyzed using ImageJ software. To estimate cardiomyocyte nucleation, the number of DsRed+ nuclei per cardiomyocyte (whose borders were Almcam+) were quantified. Nuclear ploidy was calculated based on DAPI integrated density as described above and compared to non-cardiomyocyte populations present in the same optical sections. Cardiomyocyte ploidy combines both nucleation and nuclear ploidy analysis.
Analysis of cardiomyocyte proliferation in zebrafish embryos

Zebrafish embryos were incubated in 5 mg/ml BrdU, 1% DMSO in E3 medium from 48 hpf to 72 hpf at 28°C, rinsed three times in E3 medium and fixed overnight in 4% PFA. Fixed embryos were rinsed in PBST, bleached in the dark for 20 min (using 0.8% KOH, 0.9% H2O2 and 1% Tween-20 in distilled water), permeabilized using 1% Triton-X100 in PBS for 2 h and equilibrated in DNase I buffer (40 mM Tris-HCl, pH 8.0, 10 mM MgSO4, 1 mM CaCl2) for 30 min at 37°C. Equilibrated embryos were treated with DNase I (1:50 in equilibration buffer) for 2 h at 37°C, rinsed three times in PBSTw (PBS+0.1% Tween20) and subjected to immunofluorescent staining with anti-GFP (clone B-2, Santa Cruz Biotechnology; 1:200) and anti-BrdU (clone BMC9318, Roche; 1:100) antibodies. The ventricles of stained embryos were imaged and analyzed as described above. A ventricular proliferation index (number of BrdU+, GFP+ double positive cells divided by the total number of GFP+ cardiomyocytes) was manually calculated for each embryo using ImageJ and averaged.

Zebrafish histological analysis and imaging

Adult zebrafish were euthanized by immersion in 0.16% tricaine (Sigma) and hearts dissected as described (González-Rosa and Mercader, 2012). Samples were fixed overnight at 4°C in 4% PFA, included in paraffin and sectioned following conventional histological procedures.

To detect ect2 transcripts, RNAscope (Advanced Cell Diagnostics) was performed on 7 μm paraffin sections following the manufacturer’s instructions.
Advanced Cell Diagnostic designed an ect2 probe spanning nucleotides 859-1756. Following ect2 detection, sections were incubated overnight with anti-tropomyosin or GFP antibodies (see below) and nuclei were counterstained using DAPI. To confirm ect2 re-expression in polyploid-enriched hearts (Figure S7), a new probe spanning nucleotides 1920-2835 of ect2 NM_001003883.1 was generated to avoid cross-reaction with dnEct2 sequence.

Immunofluorescence and TUNEL in paraffin sections were performed as described (González-Rosa et al., 2011). Primary antibodies used were mouse anti-tropomyosin (clone CH1, Developmental Studies Hybridoma Bank; 1:50), chicken anti-GFP (AVES, 1:500), mouse anti-GFP (clone B-2, Santa Cruz Biotechnology; 1:200), rabbit anti-Mef2 (clone C-21, Santa Cruz Biotechnology; 1:100), rat anti-BrdU (ab6326, Abcam; 1:100), mouse anti-BrdU (clone BMC9318, Roche; 1:100), rabbit anti-γH2A.X phospho-Ser139 (GTX127342, GeneTex; 1:100), rabbit anti-mKate (AB233, Evrogen; 1:500) and rabbit anti-Ect2 (clone C-20, Santa Cruz Biotechnology; 1:100). Alexa conjugated secondary antibodies (Life Technologies, 1:500) were used to detect primary antibody signals. Nuclei were counterstained with DAPI and slides were mounted in FluorSafe. A Nikon A1 confocal microscope was used to image immunostained sections.

Acid fuchsin-orange G (AFOG) stain was used to detect fibrotic tissue. Muscle, fibrin/cell debris and collagen were stained brown-orange, red and blue, respectively.

BrdU pulse-chase experiments during zebrafish heart regeneration

For BrdU pulse-chase experiments (Figures 5G-5I and 6D-6G), adult
zebrafish were used to perform apical ventricular resection. At 7 dpr, animals were injected intraperitoneally with 50 µl of 2.5 mg/ml, at 7 days post-resection (dpr), and hearts were extracted and processed for analysis at 14 dpr. To calculate BrdU cardiomyocyte labeling indices, ventricular sections were immunostained with anti-Mef2, anti-GFP, anti-Tropomyosin and anti-BrdU antibodies (see above). 2-3 ventricular sections containing the largest injury areas were imaged. Mef2+, GFP+Mef2+BrdU+ and GFP−Mef2+BrdU+ cells were counted manually using ImageJ software in defined regions (200 µm x 424.55 µm) that include the injury area and border zone. The percentages of GFP+Mef2+BrdU+/Mef2+ and GFP−Mef2+BrdU+/Mef2+ cells from individual sections were averaged to establish a BrdU labeling index for each animal.

Quantification of GFP+ contribution to regeneration

To quantify the percentage of regenerated myocardium occupied by cells expressing EGFP, histological sections containing regenerated muscle from each heart were systematically identified and analyzed. Every heart was sectioned in its entirety and pairs of consecutive sections were repeatedly distributed across the same five slides. As a result, five slides were created for every heart that contained equally spaced sections across the entire organ. For each heart, one slide was stained with AFOG and all sections were carefully examined under the microscope. Regenerated regions of muscle were identified in sections as localized thickenings of dense muscle containing evidence of residual fibrosis (blue) when compared to surrounding compact muscle. Next, a slide containing adjacent pairs of sections was immunostained with antibodies to recognize GFP and tropomyosin as described
above. Guided by which section(s) contained regenerated muscle on the AFOG-stained slide, an adjacent section was identified on the immunostained slide. In that section, the location of regenerated muscle was identified as a localized thickening of dense muscle, which showed signs of myocardial disarray. This section was imaged by confocal microscopy as described elsewhere. In photoshop, three regions of 140 μm x 140 μm were defined. This region size was empirically determined to encompass the entire regenerated area in all cases. The middle region was centered over the regenerated muscle. Two adjacent, non-overlapping regions were aligned laterally on either side to define regions of spared muscle. The EGFP+ and tropomyosin+ areas were quantified in pixels for each region, and the percentage of EGFP versus tropomyosin was calculated. The average value of EGFP+/tropomyosin+ was calculated for the uninjured regions. To calculate the relative change in proportion of GFP between the regenerate and the surrounding area, the following expression was used: \[
\log_2\left(\frac{\%\text{GFP}_{\text{regenerate}}}{\%\text{GFP}_{\text{surrounding}}}\right).
\]

Quantification of fibrotic tissue in regenerated hearts

To quantify the fibrotic area in regenerated hearts at 45 dpr, images of evenly-spaced AFOG-stained serial sections of the whole heart were captured using a Nikon 80i compound microscope (Nikon Instruments) with a 4x lens and a mounted Excelis AU600HDS HD Camera (Accu-Scope Inc. Commack, NY). On average, ~21 sections were analyzed per heart (min=13, max=30; 1427 total sections analyzed). Masks containing the entire ventricular area and the scar area were manually generated using Adobe Photoshop based on differential staining (uninjured/regenerated muscle=brown/orange; fibrotic area=(fibrin (red) + collagen
Selected areas were measured using ImageJ software. As described previously (Chablais et al., 2011; Schnabel et al., 2011), the fibrotic area was normalized to the total ventricular area to calculate the percentage of the scar size for each heart.

**Quantitative real-time PCR**

RNA from cardiac ventricles or zebrafish larvae was extracted using Trizol reagent (Life Technologies) and the Direct-zol RNA MicroPrep kit (Zymo Research). For adult expression experiments (Figure 1L), 1 ventricle was used per biological replicate and 10 biological replicates were analyzed per time point. For embryonic expression (Figure S3), 10-pooled control or mutant embryos were used per biological replicate. 6 biological replicates were analyzed. RNA was transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Life Technologies). qRT-PCR was performed using Power SYBER Green PCR Master Mix (Applied Biosystems, Life Technologies). Expression of genes was normalized with the arithmetic mean of the expression level of the constitutive gene *rps11* as described (González-Rosa et al., 2014). A complete list of the primers used in this study is provided in the Key Resources Table.

**Quantification and Statistical Analysis**

Sample sizes were chosen based on previous publications and are indicated in each figure legend. No animal or sample was excluded from the analysis unless the animal died during the procedure. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome
assessment. All statistical values are displayed as mean ± standard deviation. Sample sizes, statistical test and P values are indicated in the figures or figure legends. Data distribution was determined before using parametric or non-parametric statistical test. Statistical significance was assigned at P < 0.05. All statistical tests were performed using Prism 7 software.
REFERENCES


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CHAPTER 6

Discussion, Future Directions, and Concluding Statement
DISCUSSION AND FUTURE DIRECTIONS

ruvbl2 is a negative regulator of zebrafish cardiomyocyte proliferation

Discussion of Dissertation Research

While ruvbl1 and ruvbl2 have been implicated in a wide variety of cellular processes, the tissue-specific role for either protein has yet to be fully explored (Mao and Houry, 2017). Previous work by Rottbauer et al. described a novel role for ruvbl1 and ruvbl2 in regulating heart growth in zebrafish during embryogenesis. Specifically, the authors described the ruvbl2\textsuperscript{lik} mutation as a gain of function ruvbl2 mutation which results in an increased total number of ventricular myocytes in the zebrafish. Furthermore, they found that morpholino knockdown of ruvbl1 resulted in enlarged hearts with an overall increase in cell number. From these studies, it was concluded that ruvbl1 and ruvbl2 exerted antagonistic roles in regulating zebrafish heart growth during embryogenesis.

In this thesis, we have characterized the cardiac phenotype of the ruvbl2\textsuperscript{lik} mutants. We have explored ruvbl2\textsuperscript{lik} mutants in more detail, both in order to delineate the organ-specific role of ruvbl1 and ruvbl2 in regulating heart size, and to illustrate the tissue-specific role of ruvbl2 in regulating cardiomyocyte proliferation. Through our studies, we have discovered that ruvbl2\textsuperscript{lik} is in fact a loss of function mutation of ruvbl2, a finding that is in contrast with the initial model presented by Rottbauer et al. Our research further asserts that ruvbl2\textsuperscript{lik} is indistinguishable from the loss of function whole locus deletion allele of ruvbl2, ruvbl2\textsuperscript{Δ/Δ}. Our preliminary results from a whole locus deletion of ruvbl1 reveal an absence of cardiomyocyte proliferation.
These data, together with our *ruvbl2* data, allow us to construct a revised model for the role of *ruvbl1* and *ruvbl2* in regulating cardiomyocyte proliferation.

Through this model, we confirm that *ruvbl1* and *ruvbl2* exert antagonistic roles in regulating myocyte proliferation. Furthermore, we provide novel evidence for *ruvbl2* as a suppressor of myocyte proliferation during embryogenesis and in the context of regeneration after injury. Additionally, we find that *ruvbl1* is an activator of cardiomyocyte proliferation as whole locus deletion mutant cardiomyocytes fail to proliferate (Figure 6.1). These data allow us to revise the original model put forth by Rottbauer et al. (2002) (Figure 6.2).

Broad expression of *ruvbl2* in the developing embryo provided us with the rationale to test the tissue-specific role of *ruvbl2* in regulating cardiomyocyte proliferation. Given that the cardiomyocyte numbers were affected in *ruvbl2*\(^{+/−}\) mutants, we reasoned that *ruvbl2* was acting cell autonomously within cardiomyocytes. Indeed, we confirmed that *ruvbl2* acts within the myocardium to regulate cardiomyocyte proliferation. Interestingly, we also found that *ruvbl2* acts non-autonomously within the endocardium to suppress cardiomyocyte proliferation. In both contexts, we found that overexpression of *ruvbl2* in either tissue compartment was sufficient to dampen proliferation and block regeneration during adulthood. In summary, these results provide novel tissue-specific evidence for *ruvbl2* as a suppressor of cell cycle activity in cardiomyocytes.
Figure 6.1. *ruvbl1* is required for zebrafish cardiomyocyte proliferation. (A) CRISPR/Cas9 strategy for generating whole locus deletion *ruvbl1*Δ/Δ mutants. Guide RNAs (sgRNAs) were designed to target upstream of the endogenous 5’UTR and downstream of the 3’UTR to remove the endogenous *ruvbl1* locus. (B) Wild-type and (C) *ruvbl1*Δ/Δ embryos at 72 hpf. (C-D) Confocal single plane projection images of control (D) and (E) and *ruvbl1*Δ/Δ hearts at 72 hpf. White boxes highlight regions of interest. (D’,E’) Yellow arrows point to proliferating cardiomyocytes and the white arrows points to proliferating endocardial cells in *ruvbl1*Δ/Δ mutants. (F) Quantification of ventricular cardiomyocyte proliferation in control and *ruvbl1*Δ/Δ hearts; means represented as fold change mean±s.d, n=6 embryos per group; *P<0.05 by Student’s T test. V, ventricle; scale bars: 25 µm for confocal images.
Revised Model: \textit{ruvbl1/ruvbl2} antagonistically regulate heart growth during embryogenesis.

\textbf{Figure 6.2.} \textit{ruvbl1/ruvbl2} antagonistically regulate zebrafish cardiomyocyte proliferation. \textit{ruvbl2}^{\text{lik/lik}} and \textit{ruvbl2}^{\Delta/\Delta} loss of function mutants exhibit increased cardiomyocyte proliferation, demonstrating that \textit{ruvbl2} is a suppressor of cardiomyocyte proliferation. \textit{ruvbl1}^{\Delta/\Delta} loss of function mutant cardiomyocytes fail to proliferate, demonstrating that \textit{ruvbl1} is required for cardiomyocyte proliferation. These data revise the original model proposed by Rottbauer et al. 2002 and also confirm that \textit{ruvbl1/ruvbl2} exert antagonistic roles in regulating zebrafish cardiomyocyte proliferation.
Future Directions

The results from this thesis lay the foundation to elucidate the mechanisms by which \textit{ruvbl1} and \textit{ruvbl2} act to regulate cardiomyocyte proliferation. There are several potential avenues that should be explored in order to further understand the autonomous and non-cell autonomous role of \textit{ruvbl2} in regulating cardiomyocyte proliferation.

We must better understand the downstream transcriptional targets which determine how \textit{ruvbl2} acts in the myocardium to suppress myocyte proliferation. This would provide additional evidence describing the molecular pathways that \textit{ruvbl2} regulates as it regulates cardiomyocyte cell cycle. Given the role of \textit{ruvbl1} and \textit{ruvbl2} in regulating cardiomyocyte proliferation, we would hypothesize that there are significant transcriptional changes in cardiomyocytes, changes which critically regulate cell cycle. These data would also allow us to identify potential downstream targets that are critical for driving cell autonomous proliferation of cardiomyocytes during embryogenesis or regeneration.

Of greater interest to researchers are the non-cell autonomous transcriptional changes occurring in endocardial cells to promote myocardial proliferation since there a few endocardial signals known to regulate myocyte proliferation. Previously, the endocardium has been described as a source of secreted mitogenic signals which induce cardiomyocyte proliferation. However, our current understanding of the collective mitogenic signals sufficient to induce myocyte proliferation is still limited (reviewed in Tian and Morrisey, 2012). Understanding the transcriptional changes in the endocardial cells of \textit{ruvbl2} mutants with hyperproliferative hearts would allow us
to identify the molecular targets, and therefore potential mitogenic signals, secreted from the endocardium to induce cardiomyocyte proliferation.

In the adult, we have demonstrated the tissue-specific sufficiency of *ruvbl2* in suppressing cardiomyocyte proliferation and blocking regeneration. However, it is not known whether *ruvbl2* is required for cardiac proliferation in the context of adult cardiac regeneration. It has been shown that *ruvbl2ΔΔ* embryos die by 4 dpf in the zebrafish. Accordingly, one potential avenue to test in the adult would involve generating tissue-specific knockouts of *ruvbl2* in the endocardium or myocardium to see if *ruvbl2* is required for efficient regeneration after injury.

Previous research has indicated that *ruvbl2* exists as part of a number of chromatin remodelling complexes (Hota and Bruneau, 2016). A genetic approach may help us determine through which complex *ruvbl2* acts in regulating cardiomyocyte proliferation. This would likely involve testing the genetic interactions of *ruvbl2* with components of each chromatin remodelling complex. Because *ruvbl2* has been demonstrated to function through INO80 in numerous contexts, we hypothesized that *ruvbl2* functions through the INO80 complex to regulate myocyte proliferation. To this end, we generated whole locus deletions of INO80 to test the genetic interaction of *ruvbl2* with INO80. If INO80 is determined to be a critical regulator of myocyte proliferation, we expect that INO80ΔΔ mutant embryos will exhibit defects in cardiomyocyte proliferation. Furthermore, if we find that *ruvbl2* mutants fail to complement INO80 mutants and exhibit increased cardiomyocyte proliferation, we can conclude that *ruvbl2* acts through INO80 to regulate cardiomyocyte proliferation. Lastly, our discoveries of *ruvbl1* and *ruvbl2* as critical regulators of cardiomyocyte proliferation in the heart may offer novel therapeutic avenues for regulating
proliferation of myocytes after cardiac injury in human patients. Ultimately, boosting myocyte proliferation post-injury could improve patient outcomes and serve as a means by which to combat high rates of death by heart failure found around the world. There are many ways to potentially stimulate myocardial proliferation and achieve a therapeutic advantage, including the knock down of ruvbl2 using RNAi in cardiomyocytes, endocardial cells, or small molecule inhibitors of ruvbl2. Further research on ruv proteins and cardiomyocyte proliferation should focus on transferring what we have learned in the zebrafish to humans more broadly, and using our knowledge to develop new therapies and solutions for cardiac patients.
CONCLUDING STATEMENT

The concept of regeneration has fascinated humans for centuries, with the earliest description of regeneration in lizards from Aristotle in 350 BCE (Aristotle et al. 1965(Aristotle, 2002)). Despite the complexity of the regenerative process, we have come a long way in the field of regenerative biology to improve our understanding of some of the regulatory mechanisms that govern regeneration. This thesis work is a presentation of some of the factors broadly involved in regeneration. Here, we have provided evidence for fosl2 as an important regulator of accurate chamber formation during embryogenesis but a dispensable factor during adult regeneration. Furthermore, we describe the evolutionarily conserved role of the innate immune receptor c5ar1 as a critical regulator of cardiomyocyte proliferation during regeneration. Additionally, we demonstrate that cardiomyocyte DNA content is a barrier to regeneration after injury. Lastly, we identify ruvbl2 as a suppressor of cardiomyocyte proliferation. Further studies are required to dissect the mechanisms regulating cardiomyocyte proliferation in order to develop a deeper understanding of how this process takes place. We believe that the work presented in this thesis offers promising novel therapeutic avenues for improving cardiomyocyte proliferation after injury, with profound implications for human subjects and for cardiac patient outcomes.
REFERENCES


Supplementary Figure S.1. *ruvbl2*<sup>lik</sup> does not increase specification of cardiac progenitors (A) wild-type and (B) *ruvbl2<sup>lik/lik</sup>* embryos at 48 hpf respectively. (C) Quantification of ventricular cardiomyocytes in control and *ruvbl2<sup>lik/lik</sup>* hearts; means represented as fold change mean±s.d, n=6 embryos per group; *P<0.05 by Student's T test. V, ventricle; AT, atrium; scale bars Scale bars: 25 µm.
Supplementary Figure S.2. *fosl2*-/- adults are indistinguishable from siblings with regard to animal size, heart size, heart morphology, and cardiac regenerative capacity. (A,B) Photographs of 6-month old sibling control (CTRL; A; n=6) and *fosl2* null (B; n=6) zebrafish showing their equivalent sizes. (C,D) Photographs of hearts dissected from 6 month old control (C; n=6) and *fosl2* null (D, n=6) zebrafish showing their equivalent sizes and morphologies. (E,F) AFOG-stained cardiac sections from 6 month-old control (E; n=4 hearts) and *fosl2* null (F; n=4 hearts) hearts on day 45 post apical resection. Mutant hearts regenerated (*) resected myocardium normally. A, atrium. V, ventricle. BA, bulbous arteriosus. Scale bars: 1mm in (A,B), 200mm in (C-F).