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Twist1a Limits Myocardial Differentiation in Zebrafish

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by

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The Thesis of Kristina Marie Garske is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This thesis is dedicated to my parents, Wayne and Marla Garske, and my grandmother, Judy Turner. Without their support, encouragement, and advice, I would not be where I am today.

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ABSTRACT OF THE THESIS

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Congenital heart disease can result from defects in heart cell differentiation and morphogenesis during the early stages of cardiac development. Importantly, differentiation of the correct number of cardiomyocytes during these early stages ensures the proper function of the

heart, as too few or too many cells can lead to a dysfunctional organ. While a few transcription factors have been shown to have roles in promoting myocardial differentiation, our knowledge of the important regulators of this process is incomplete. Here, we show that a bHLH transcription factor, Twist1a, functions to limit the production of myocardial cells in the early embryo. Reducing the function of *twist1a* in zebrafish embryos with an anti-*twist1a* morpholino (MO) results in expanded expression of *cmlc2*, a myocardial marker, suggesting a repressive role for Twist1a during myocardial differentiation. This role for Twist1a contrasts with that of Hand2, a bHLH transcription factor that is known to promote myocardial differentiation. We show that reducing the function of *twist1a* in *hand2* hypomorphic mutants results in expanded *cmlc2* expression. However, *hand2* null mutants injected with the *twist1a* MO have no apparent increase in the expression of *cmlc2*. The idea that the function of Twist1a in limiting myocardial differentiation is dependent upon the presence of Hand2 supports our conclusion that the two transcription factors interact genetically during this process. Together, our findings suggest that the relative doses of Twist1a and Hand2 play an important role in setting the scale for the amount of myocardial differentiation in the zebrafish embryo.

INTRODUCTION

Moderate to severe congenital heart defects affect about 1% of live births worldwide (Hoffman and Kaplan, 2002). These defects arise mainly as a result of improper regulation during embryogenesis, in the early developmental stages of cardiac differentiation and morphogenesis (Olson, 2006). Thus, understanding the mechanisms of heart development is essential to discovering the underlying factors that contribute to the malformation or improper function of the heart.

In the developing embryo, organ fields contain progenitor cells that will eventually differentiate into the different types of cells required for the formation of that organ. The regulation of the boundaries and size of these fields, along with the correct positioning of the progenitors is extremely important to guaranteeing that an organ will develop properly. Cardiac progenitors are found in bilateral regions of the anterior lateral plate mesoderm (ALPM) (Fig. 1A) (Schoenebeck et al., 2007). After differentiation into their respective cardiac cell types, medial migration of these heart fields and subsequent fusion and elongation along the ventral midline results in a linear heart tube, which later loops to form the chambers of the heart (Srivastava, 2006).

A complex network of transcription factors and secreted signals ensures that the cardiac progenitors are directed to the proper cell fate, differentiation and patterning to form the complete heart. Disruption of these mechanisms can lead to a wide variety of cardiac defects. Here, we focus on the importance of producing the proper number of differentiated cardiomyocytes. This process is

critical to the development of a healthy heart, as too few or too many cells can have detrimental effects on the morphology of the developing heart. Considering this, understanding how cardiac progenitors ultimately decide to differentiate into functional cardiomyocytes can prove to be extremely valuable knowledge.

In this thesis, we utilize zebrafish as our model organism to study cardiac development, specifically in terms of determining myocardial cell number. There are many advantages to using zebrafish as our model. They are easy to manage because of their small size, and they mature quickly, becoming fertile around three months of age (Beis and Stainier, 2006). Zebrafish embryos are externally fertilized and transparent, making it easy to observe their inner morphology during the first few days of their development. Their hearts form a linear tube by 24 hours post fertilization (hpf) that can be seen beating under a microscope. Zebrafish embryos can survive for 3-5 days with severe cardiac defects. This makes it possible for us to analyze the function of important transcription factors involved in myocardial differentiation.

Hand2 is required to promote myocardial differentiation in zebrafish

Hand2 is a transcription factor that is expressed by myocardial progenitors within the ALPM (Schoenebeck et al., 2007). However, the entire ALPM does not contribute to the myocardium. Blood and vessel cells also originate from this mesodermal territory. Construction of an ALPM fate map has shown that blood

and vessel lineages arise from the rostral portion of the ALPM, while the relatively caudal territories contain myocardial progenitors (Fig. 1B, C). The transcription factor *gata4* marks the entire ALPM, whereas the expression of *nkx2.5* and *hand2* is restricted to the caudal region of ALPM (Schoenebeck et al., 2007). The latter two transcription factors, particularly *hand2*, mark a broad region of cells that have the potential to become myocardium. However, not all cells that express *hand2* go on to become fully differentiated cardiomyocytes. This study focuses on the mechanisms by which this group of cardiac progenitors decides how many cells will become myocardium.

Hand2 is required for the differentiation of the correct number of myocardial cells (Yelon et al., 2000). *hand2* mutants display extremely reduced amounts of myocardium, and these mutants also exhibit failure of the bilateral regions of myocardial tissue to fuse and form a cardiac tube at the midline (Yelon et al, 2000). Interestingly, the vascular progenitor cell boundary does not extend caudally in these mutants. *nkx2.5* expression is also normal, suggesting that the progenitor fields are not compromised. The fact that very few of these progenitors differentiate into *cmlc2*-expressing cardiomyocytes is a strong indication that Hand2 plays a specific role in the differentiation of myocardial tissue (Schoenebeck et al., 2007).

Interactions between Hand2 and the Twist family of bHLH transcription factors

Hand2 is part of a large class of transcription factors with a basic helix-loop-helix (bHLH) domain, which is composed of a short stretch of basic amino acids followed by two α-helices connected by a loop (Conway et al., 2010). The helix-loop-helix portion allows for the dimerization of different bHLH proteins. Following this dimerization, the combined basic domains of the two proteins can then bind DNA and exert their effects on gene transcription (Dai and Cserjesi, 2002; Conway et al., 2010). The formation of the dimer complex between two bHLH proteins is thought to be a requirement for DNA binding to occur (Barnes and Firulli, 2009). Thus, dimer choice is an important factor in determining which target genes are affected through homo- or heterodimer DNA binding (Conway et al., 2010). Hand2 has been shown to dimerize with many bHLH proteins, including at least three of the four ubiquitously expressed class A bHLH E-proteins, which are important for cell growth and differentiation (Murakami et al., 2004; Dai and Cserjesi, 2002). However, specific interactions between these proteins and Hand2 in the heart have not been described (Murakami et al., 2004).

Hand2 belongs to a subclass of class B bHLH proteins called the Twist family (Cai and Jabs, 2005). These transcription factors play important roles in many aspects of embryonic development. They are grouped into this family

based on the highly conserved serine and threonine residues in helix 1, where they can be phosphorylated (Barnes and Firulli, 2009). Phosphorylation is important in the regulation of these proteins, as it can affect their dimer partners (Cai and Jabs, 2005). This then alters their DNA binding affinities in different tissues and the resulting effects on gene transcription. Twist family proteins are broadly expressed throughout development, and their overlapping expression is common. While it has been shown that two or more Twist proteins can have roles in the same tissues, these roles are not necessarily redundant (Zhang et al., 2012; Yang et al., 2011; Cai and Jabs, 2005; Barnes and Firulli, 2009). Thus, the levels of these proteins relative to each other need to be tightly regulated. When the amount of one protein is altered, it can affect the available interactions amongst others within the vicinity (Firulli et al., 2010).

The interactions between Twist1 and Hand2 in the limbs of chick and mouse are particularly enlightening in this regard. The two Twist family proteins participate in an antagonistic relationship that ensures the proper patterning of the limbs (Firulli et al., 2005). *Twist1* loss-of-function mutations are dominant, known to cause Saethre-Chotzen syndrome in humans, which is characterized by craniofacial defects and limb abnormalities. Similar limb defects arise in *Twist1* heterozygous null mice, which have been shown to exhibit polydactyly. Early indications of a relationship between Twist1 and Hand2 stemmed from observations that ectopically expressed *Hand2* produced polydactylous phenotypes that were similar to those produced by *Twist1* haploinsufficient

mutations in mice. Moreover, Firulli and colleagues have shown that the polydactyly seen in *Twist1* heterozygous null mice can be rescued when these mice are crossed with *Hand2* heterozygous null mice (Firulli et al., 2005). The opposing functions of the two proteins are thus dose-dependent, which indicates that genetic interactions exist between them. The well-established role of Hand2 in cardiac development, along with these genetic interactions between Twist1 and Hand2 demonstrated in the limbs of chick and mouse led us to wonder if the two transcription factors might interact in the zebrafish heart.

Phosphorylation of Twist1 is necessary for proper cardiac morphogenesis

Recently, Twist1 has been implicated in having some role in cardiac morphogenesis in mice. Transgenic mice mutated in the threonine and serine residues of helix 1 of Twist1 are unable to be phosphorylated (Lu et al., 2011). Recall that phosphorylation is a posttranslational modification that can affect Twist family protein dimer partners. These mice exhibit hypertrophic hearts with atrial septal defects. Thus, misregulation of Twist1 has some effect on cardiac morphogenesis, although specific roles in the early stages of myocardial differentiation are unknown. We sought to study the early role of one of the mammalian *Twist* orthologues in zebrafish, as well as its possible interactions with Hand2 in cardiac development.

Twist1a opposes Hand2 function in zebrafish

Our data suggest that a zebrafish *Twist1* ortholog, *twist1a*, functions to limit the number of myocardial cells that differentiate from the ALPM. In addition, Twist1a-Hand2 genetic interactions are implicated in knockdown experiments of *twist1a* in *hand2* mutant backgrounds. Reducing Twist1a function in *hand2* hypomorphic mutants results in a significant increase in myocardial cells compared to the limited numbers of cardiomyocytes normally observed in *hand2* mutants. Interestingly, similar experiments reducing *twist1a* function in *hand2* null mutants does not improve their myocardium-deficient phenotype. We propose a model in which Hand2 presence is necessary for Twist1a to function in restricting cardiomyocyte numbers.

RESULTS

twist1a is expressed in the ALPM

In order to determine whether any of the zebrafish Twist proteins have the potential of interacting with Hand2 during myocardial differentiation, we compared their expression patterns. We conducted in situ hybridization experiments using probes for the four zebrafish *twist* genes: *twist1a, twist1b, twist2* and *twist3* (Germanguz et al., 2001). We were most interested in determining whether any of the *twist* genes are expressed in the anterior lateral plate mesoderm (ALPM), where cardiac progenitors are known to reside (Schoenebeck et al. 2007). We found and were able to confirm previous reports (Germanguz et al., 2001) that *twist1a* is expressed in the posterior LPM and reaches anteriorly into the ALPM (Fig. 2). The other three *twist* genes displayed overlapping expression with *twist1a* in some areas, such as the pectoral fin bud primordia, and the head mesenchyme. *twist1b* shares expression with *twist1a* in the somites and neural crest as well (data not shown; Germanguz et al., 2001). The expression patterns for the four genes are broad. However, *twist1a* is the only gene for which we could detect expression in the ALPM. Thus, we chose to focus on *twist1a* for our study.

Knockdown of *twist1a* results in expanded *cmlc2* expression

The first step we took to elucidate whether *twist1a* has a role in cardiac development was to examine the effects of reducing the amount of protein in the

developing embryo. We knocked down *twist1a* function using a translation-blocking antisense morpholino (MO) oligonucleotide (MO#1; see Materials and Methods). The use of a splice-blocking MO was not an option, as *twist1a* has only one exon. Because the use of an anti-*twist1a* MO had not yet been published, we tested the effects of a range of concentrations, from 2-18 ng. It was immediately clear that doses above 8 ng were highly toxic, resulting in very limited survival of injected embryos. Injection of 2, 4, 6 or 8 ng allowed the embryos to survive and develop into later stages. However, cell death was evident in the head and somites of the embryos injected with 6 or 8 ng concentrations and was occasionally observed in embryos injected with 4 ng of MO. This led us to consider whether the use of an anti-*p53* MO would be valuable for our experiments. p53 is a known apoptosis inducer (reviewed by Haupt et al., 2003). Previous studies have shown that knocking down its function can reduce the amount of non-specific apoptosis in MO-injected embryos (Robu et al., 2007). Injection of 2 ng of the *p53* MO along with the *twist1a* MO markedly reduced the cell death in injected embryos. The tissue in the head and somites was clear and healthy, comparable to the wild-type embryos (data not shown). Thus, we decided to employ the use of a zebrafish MO directed against *p53* (Robu et al., 2007) combined with the *twist1a* MO in all future injections.

We first wanted to see whether Twist1a has an impact on the myocardial cells that form from the bilateral heart fields. We analyzed the effect of the *twist1a* MO by comparing expression of the myocardial marker *cmlc2* in injected

versus uninjected wild-type (WT) embryos at 17 somites. This stage was chosen because the expression of *cmlc2* is strong compared to earlier stages when it has just begun to be expressed (Huang et al., 2003). In addition, because our assay would be based on the amount of *cmlc2* staining seen, we preferred to study a stage at which the heart fields still lay across one plane as opposed to later stages, once fusion has begun and multiple layers of cells have formed. We gathered data sets for injections of 2, 4 and 6 ng of *twist1a* MO. *cmlc2* expression did not appear to be any different in the embryos injected with 2 ng of the *twist1a* MO compared to the uninjected wild-type embryos (data not shown). However, embryos injected with 4-6 ng of the *twist1a* MO seemed to have expanded expression of *cmlc2* (see Figure 3C, D). Because repeated experiments resulted in similar phenotypes and healthy embryos at this range of concentrations, we concluded that 4-6 ng of the *twist1a* MO was the optimal dose for our injections.

We tested the effects of a second, non-overlapping anti-*twist1a* MO (MO#2; see Materials and Methods) to confirm that the effects we were seeing from MO#1 were the result of specific knockdown of *twist1a* function. Injected embryos displayed similar phenotypes with both MOs in all experiments (data not shown). However, our MO#1 seemed to produce higher percentages of embryos displaying a clear and easy to qualify phenotype (Fig. 3E). From this point on, all references to injected embryos should be assumed to be injections of 4-6 ng *twist1a* MO#1 (see Materials and Methods) together with 2 ng *p53* MO.

Our results from these experiments led us to believe that knockdown of *twist1a* function leads to an increased number of cardiomyocytes, based on our observations of expanded expression of the myocardial marker *cmlc2*. Wild-type embryos at 17 somites express *cmlc2* in distinct bilateral areas with rather smooth boundaries. Interestingly, we observed clear differences in this expression in slightly over one quarter of all injected embryos (Fig. 3E). It is easiest to describe these differences by categorizing the embryos based on the shape of the bilateral populations of cardiomyocytes. However, it is important to note that both categories represent embryos that visibly displayed more *cmlc2*-expressing cells compared to the uninjected siblings.

Injected embryos displaying expanded *cmlc2* expression were placed into category A if the smooth boundaries typical of wild-type embryos were still present. The bilateral fields in these injected embryos appeared much larger those seen in the uninjected wild-type embryos (Fig. 3A, C). We called this type of expansion “general expansion,” because the overall pattern of *cmlc2* expression was maintained. Around one half of the visibly affected injected embryos fit into this category (Fig. 3E). The remaining half of the injected embryos that exhibited expanded *cmlc2* expression were placed into category B. These embryos had noticeably misshapen patterns of *cmlc2* expression (Fig. 3B, D). This phenotype was the result of extra cells that were grouped into areas lateral or medial to the typical wild-type bilateral regions of *cmlc2*-expressing cells. These cells would appear to be removed from the rest of the group, and at

times there would even be cells completely disconnected from the bilateral fields (Fig. 3D).

Although the phenotypes observed in our injected embryos were clear and seen in repeated experiments, we were concerned about the seemingly low penetrance of visibly evident myocardial defects (Fig. 3E). We considered the possibility that the remaining uncategorized injected embryos had more *cmlc2*-expressing cells as well, but weren't as visibly extreme as those we were able to place into category A. Perhaps the expansion we saw was not major at all times, but more of a shift toward an increased number of myocardial cells overall. If this were the case, our qualitative assessments up to this point would not include these embryos. A more quantitative approach could confirm that what we observed in our injected embryos was real and not the result of the subjectivity of the assay.

We decided to perform area measurements to compare the relative area of *cmlc2*-expressing cells in uninjected wild-type versus injected embryos. Our measurements showed an increase of 24% in the average area of *cmlc2* expression in our injected compared to uninjected embryos (Fig. 4). Our data show that the area of *cmlc2* expression covers a wide range, as can be seen from the standard deviation. We feel that the large range of data is not an indication of imprecise measurements, but rather, the nature of our assay (see Discussion). While *cmlc2* has somewhat predictable morphology throughout

development, the area of expression is not fixed at any time point, even in wild-type embryos. However, our measurements show an increase in the average area of *cmlc2* expression taking into account all embryos, and these data are statistically significant (Fig. 4). Thus, these data suggest a model in which Twist1a normally functions to restrict the number of cells that differentiate into myocardium. This observation is consistent with the idea that Twist1a and Hand2 have opposing functions in the regulation of cardiomyocyte differentiation.

Reduction of *twist1a* function in *han*$^{s6/c99}$ transheterozygotes results in expanded *cmlc2* expression

It is interesting that our observations up to this point suggest similarities to the antagonistic relationship between Hand2 and Twist1 seen in the limbs of chick and mouse (Firulli et al., 2005). Firulli and colleagues have shown that reducing the dosage of *Hand2* in *Twist1* heterozygous mice can rescue their polydactylous phenotype. This dosage-dependency suggests a mechanism in which the opposing function of the two transcription factors results from a genetic interaction between them. We hypothesized that a similar relationship exists between Hand2 and Twist1a in the regulation of myocardial differentiation. Reducing *twist1a* function in embryos already deficient in *hand2* could be highly revealing in this regard.

We have two mutant alleles at the *han* locus, both of which result in loss-of-function mutations in *hand2* (Yelon et al., 2000). The *s6* mutation is a deletion that removes the *hand2* gene, resulting in a null allele. The amount of *cmlc2* expression in han^{s6} mutants is much reduced, and the heart fields fail to fuse at the midline, greatly disrupting cardiac morphogenesis. The *c99* mutation is a ~5 kb insertion in the 5' UTR of *hand2* that causes missplicing, leading to the deletion of some of the 5' UTR and some coding sequence. This mutation results in a hypomorphic allele, as some of the normally spliced *hand2* mRNA sequence is detectable in han^{c99} homozygous mutants. These mutants display an intermediate phenotype between that of the null han^{s6} mutation and the wild-type heart (Yelon et al., 2000).

To test whether there is some sort of dosage-dependency between Hand2 and Twist1a, we next performed injections of our *twist1a* MO into *hand2* mutant embryos. The MO does not reduce *twist1a* function completely, or measurably, as there is not an antibody for zebrafish Twist1a available. Thus, in order to substantially reduce the amount of Hand2 present while ensuring it is not eliminated completely, we first chose to test the effects of our *twist1a* MO in $han^{s6/c99}$ transheterozygotes.

We injected the *twist1a* MO into transheterozygotes and fixed the embryos at 24 hpf. This time point was chosen in order to simplify our assay. Embryos that are wild-type or heterozygous for *hand2* mutations form a linear heart tube

by 24 hpf (Fig. 5C). *han*$^{s6/c99}$ transheterozygotes exhibit a range of phenotypes, from completely separate bilateral heart fields to some sort of fusion across the midline (Fig. 5A, B). However, linear heart tubes are never seen at 24 hpf in these mutants (Yelon et al., 2000). Thus, this time point allows us to pick out the mutant embryos from our crosses easily (see Fig. 5G), and also allows us to continue to perform area measurements of *cmlc2* expression.

Our injections into the transheterozygotes resulted in an expansion of *cmlc2* expression (Fig. 5A, B, D, E). This expansion was much more obvious than what we had observed in our wild-type injection experiments. In our initial qualitative assessment of the *cmlc2* expression in the transheterozygotes, we found that 68% of our injected embryos displayed obviously expanded expression of the myocardial marker (Fig. 5G). This number was much more compelling than the 27% of embryos displaying clear phenotypes in our injections of wild-type embryos (see Fig. 3E). Subsequent area measurements again confirmed our observations. The average area of *cmlc2* expression in our injected embryos was 50% larger than in our uninjected transheterozygotes (Fig. 6). Again, this was a significantly larger increase in area compared to the 24% increase we observed in our wild-type injection experiments (see Fig. 4).

An unexpected observation in our injected transheterozygotes was the presence of more embryos that displayed the capability for their bilateral populations of cardiomyocytes to fuse across the midline (Fig. 7C). While the

majority of the fusion occurred in a fashion similar to that of uninjected transheterozygotes, a different type of fusion was observed that is not seen in our uninjected transheterozygotes at this time point (Fig. 7A, B). We called this phenotype “bridge fusion,” because of the morphology of the myocardial cells that connect the two heart fields. This type of fusion was seen in 19% of our injected transheterozygotes, and in none of our uninjected transheterozygotes (Fig. 7C). This phenotype was intriguing, especially when taken together with the fact that *hand2* mutants do not only exhibit limited numbers of myocardial cells, but inability or difficulty fusing across the midline. We considered the possibility that these embryos represented some sort of rescue of this other *hand2* mutant phenotype.

It is important to note that the increased amount of fusion seen in our injected transheterozygotes did not account for the increase in area of *cmlc2* expression. In the injected embryos, those displaying two separate heart fields as well as those that had fused across the midline averaged to comparable area measurements. The same was true for the uninjected embryos (compare Fig. 5A and B; Fig. 5C and D).

Injecting the *twist1a* MO into *han*$^{s6/c99}$ transheterozygotes resulted in more readily visible expansion of *cmlc2* expression, as well as an increase in average area of *cmlc2* expression that was twice as large as that seen in the wild-type injected embryos. These results are consistent with our hypothesis that there is

a genetic interaction between Twist1a and Hand2. The antagonistic relationship between the two transcription factors appears to be dose-dependent, such that reducing the function of both results in a shift toward a wild-type phenotype from the mutant *hand2* phenotype.

Reducing *twist1a* function in *han^{s6}* mutants has no apparent effect on myocardial cell number

Until now, we have tested the effect of reducing *twist1a* function in embryos that have at least some functional Hand2 protein. In an attempt to further characterize the interactions between Twist1a and Hand2, we injected the *twist1a* MO into *han^{s6}* mutant embryos, which do not have any Hand2 protein. Again, we used 24 hpf as our time point to allow for quick identification of the mutants and to aid our observational assays (Fig. 8).

Our initial observations indicate that there is no apparent increase in *cmlc2* expression in our injected versus uninjected *han^{s6}* mutants (Fig. 8A, C). In our prior experiements, area measurements could be made rather reliably, as we were measuring areas of *cmlc2* expression that were generally in a single layer of cells. However, studies have shown that the same idea may not apply to *han^{s6}* mutants. Myocardial progenitors in *han^{s6}* mutants are incapable of producing polar epithelia (Trinh et al., 2005). This is associated with the improper

organization of fibronectin, which is normally localized to the basal side of epithelia. Images of transverse sections of the ALPM in *han*^{s6} mutants indicate that the irregular fibronectin deposition is accompanied by the arrangement of myocardial cells into multiple layers, rather than in one single layer. Thus, it seems reasonable to conclude that we cannot reliably estimate the number of cardiomyocytes based on the area of *cmlc2* expression seen from a dorsal view. Area measurements were therefore not performed on the *han*^{s6} embryos, although we believe there was no indication of an increase in *cmlc2*-expressing cells in our injected *han*^{s6} mutants compared to the uninjected mutants (compare Fig. 8A and C). These data imply that the presence of Hand2 is necessary for the function of Twist1a in repressing myocardial differentiation. One possible explanation for this that correlates with our model is that the two are involved in the same pathway in myocardial differentiation, whether directly or indirectly.

Surprisingly, we observed fusion of the bilateral populations of cardiomyocytes in 46% of our injected *han*^{s6} mutants (Fig. 9B, C). This was unexpected, as *han*^{s6} mutants do not just have difficulty fusing like the *han*^{c99} mutants; instead, they do not exhibit any fusion at 24 hpf (Yelon et al., 2000). In addition, some of the fusion phenotypes in our injected *han*^{s6} mutants were comparable to the “bridge fusion” phenotype seen in our injected transheterozygotes (Fig. 9C). Consistent with our previous observation of myocardial cell number in the injected versus uninjected *han*^{s6} mutants, these embryos did not seem to have more *cmlc2*-expressing cells. Rather, the small

population of myocardial cells appeared to be spread out over a wider area (compare Fig. 9A to B and C). These results contrast with what we see with the effects of the *twist1a* MO on myocardial cell number. Thus, it seems that Hand2 presence is not necessary for the partial rescue of the fusion phenotype through reduction of *twist1a* function. Therefore, Twist1a may have separate, unrelated roles in restricting myocardial cell number and cardiac fusion.

DISCUSSION

Our data suggest a compelling model for the genetic interactions between Twist1a and Hand2 during the process of myocardial differentiation. Our experiments show that when *twist1a* function is reduced, more myocardial cells are able to differentiate. Thus, we can propose that Twist1a is an inhibitor of myocardial differentiation. Taken together with our knowledge of Hand2 and its role in promoting the differentiation of cardiomyocytes, the two transcription factors can be said to have antagonistic roles. Evidence of a genetic interaction between the two transcription factors is revealed when we reduce *twist1a* function in *hand2* hypomorphic mutants. Here, we see that the increased number of myocardial cells is much more evident than in wild-type embryos. We can imagine a model in which reduction of *twist1a* in a mutant background with reduced amounts of Hand2 partially rescues the *hand2* mutant phenotype, as is seen in previous studies in the limbs of chick and mouse (Firulli et al., 2005). Interestingly, we did not observe a visible expansion in *cmlc2* expression when we knocked down *twist1a* function in *han*^{s6} mutants. We interpret this result to indicate that at least some Hand2 presence is necessary for Twist1a to function in repressing myocardial differentiation.

If Twist1a functions to reduce the number of cardiomyocytes only in the presence of Hand2, it would seem that a similar relationship could occur when considering the fusion of the bilateral populations of cardiomyocytes. However, this does not seem to be the case. Here, we show that even in the absence of

Hand2, Twist1a can still have some effect on the morphology of the myocardium. Certainly, while this fusion is not quite comparable to wild-type cardiac fusion, it is a step closer to what needs to occur for proper cardiac morphogenesis. It is possible that Twist1a has a separate function from Hand2 in the restriction of myocardial cell movement.

Twist1a functions to restrict myocardial differentiation

The identification of a negative regulator of myocardial differentiation is exciting, as we are still unclear of the exact mechanisms that occur during this process in early cardiac development. Obtaining clear results, in which simply reducing the function of *twist1a* led to increased numbers of cardiomyocytes, has the potential to uncover many important concepts as further studies are conducted. Certainly, in these early experiments, the increase we observed in myocardial cells was rather modest. After performing area measurements on the expression of *cmlc2* in our embryos, we noticed that our data covered a wide range of total area, with the increase in average area in our injected embryos being 24%. Although our standard deviation bars (see Fig. 4) were quite large, our data were statistically significant. In wild-type embryos, the expression of *cmlc2* expands dynamically during the 17-19 somite stage. While this could be a source of variability within our area measurements, we feel that our data reflects much more. The size of our error bars may be an indication of the variability in

cmlc2 expression during these stages, as they are similar in both the uninjected and injected data. However, the average area we see in our injected embryos is increased. This increase, while not as large as we would hope, is significant. Thus, we favor the idea that knockdown of *twist1a* results in a marked shift toward increased numbers of myocardial cells, rather than a major jump. To confirm this further, we plan to do cell-counting experiments at ~24 hpf and compare reliable integers, rather than the arbitrary units of our area measurements.

While we are confident in our results, we continue to question possible reasons for the seemingly limited expansion we see in *cmlc2* expression after knocking down *twist1a*. One factor could be that we are unable to knock down *twist1a* enough with our MO. We cannot determine the degree to which we knock down translation without an antibody for Twist1a, and our injection concentrations were limited by the toxicity of the MO in the embryos. This leads us to consider other ways we can increase the effectiveness of our knockdown experiments.

Recently, Das and Crump published a paper in which they analyzed the effects of knocking down both *twist1a* and *twist1b* together. When studying the role of Twist1 in cardiac neural crest cells (cNCCs), they report that injection of just a *twist1a* MO into zebrafish embryos resulted in a subtle increase in the expression of the cNCC marker they studied (Das and Crump, 2012). This

increase was much more pronounced when they injected both *twist1a* and *twist1b* MOs concurrently. This led us to consider the possibility that we would obtain similar results in our experiments. If *twist1a* and *twist1b* have redundant roles in one aspect, they may in others. It would be interesting to see if the effects on myocardial differentiation, fusion, or both, are more pronounced when *twist1b* is knocked down along with *twist1a*.

With the identification of a potential regulator of myocardial differentiation, we would also attempt to elucidate the mechanism behind this regulation. One question our experiments have not addressed is the meaning behind the phenotype we have observed. We have considered a few possible reasons that *cmlc2* expression could have been expanded upon the knockdown of *twist1a*. Firstly, it is possible that more cardiac progenitors are capable of differentiating in the absence of Twist1a. This could occur through basic or complex transcriptional regulation at the level of fate decision-making during differentiation. Another possible reason for expanded *cmlc2* expression is that the myocardial cells are proliferating more once they have differentiated into fully functioning cardiomyocytes. Finally, the progenitor fields themselves could potentially be expanded, leading to more myocardial differentiation. It has been shown that reducing *twist1a* function in zebrafish induces the occurrence of ventralized embryos (Yang et al., 2011). It is possible that global changes in the mesoderm could expand the regions from which cardiac progenitors differentiate.

It may be informative to look at the effect of *twist1a* knockdown on an ALPM marker, such as *gata4*, and see if there does seem to be expansion of this region. In addition, we could test whether there is any effect on the boundaries between the endocardial or myocardial progenitors by examining the effects of *twist1a* knockdown on *hand2, nkx2.5,* and *scl* (see Fig. 1; Schoenebeck et al., 2007). Further experiments are required to test what is happening in the injected embryos to cause expanded *cmlc2* expression. Additionally, a different approach that we have already begun to pursue is to push the genetic influences of our injection experiments further by testing our *twist1a* MO in backgrounds other than wild-type embryos.

Genetic interactions between Twist1a and Hand2

When the amount of *twist1a* is reduced in *han*$^{s6/c99}$ transheterozygotes, the increase in the area of *cmlc2* expression is two times greater than that of reducing Twist1a in wild-type embryos (Compare Fig. 4 and 6). This result is reminiscent of previous reports of Hand2-Twist1 genetic interactions in the limbs of chick and mice. In those cases, complete rescue of polydactyly phenotypes was seen in crosses between *Hand2* heterozygotes and *Twist1* heterozygotes (Firulli et al., 2005). Our results were not as clear-cut as this complete rescue, as we did not observe a heart tube at 24 hpf like we would expect to see in wild-type embryos. One reason for this could be that we are not reducing the amounts of

Hand2 and Twist1a equally. We are not able to measure the amounts of Twist1a and Hand2 knockdown, since no specific antibodies are currently available. However, the fact that the increase in *cmlc2* expression was much more pronounced in our transheterozygotes than in wild-type embryos was exciting. We would like to perform future experiments to see the effect of *twist1a* knockdown in transheterozygotes at later stages in development. It is possible that development of the heart tube and chambers can proceed further and/or faster in injected compared to uninjected transheterozygotes.

The function of Twist1a in myocardial differentiation is dependent on Hand2 presence

The results of our *twist1a* MO injections into *han*^{s6} homozygous mutants imply that the presence of at least some Hand2 protein is necessary for Twist1a to function in its role in repressing myocardial differentiation. Although we have not confirmed with area measurements or cell counts, our preliminary results suggest that knocking down Twist1a function in these mutants does not result in expanded *cmlc2* expression as it did in the wild-type and transheterozygote embryos. This is interesting, because even in *han*^{s6} mutants, some cardiomyocytes are capable of differentiating. If Twist1a normally functioned to repress a general mechanism for myocardial differentiation, we could suppose that in its absence, more myocardial cells could differentiate, whether or not

Hand2 was present. However, our data suggest otherwise. In the complete absence of Hand2, reducing Twist1a may not lead to an increase in myocardial cells. Thus, is seems as though Twist1a normally represses a similar pathway to one in which Hand2 potentiates myocardial differentiation. This would imply that the two proteins interact genetically in some way, either directly or indirectly. Future cell-counting experiments can help verify our conclusions that *cmlc2* expression is not expanded in injected *han*^{s6} mutants.

Twist1a has a role during cardiac morphogenesis that is independent of the presence of Hand2

The fact that we observed increased amounts of cardiac fusion in our injected transheterozygotes and *han*^{s6} mutants is interesting, because knockdown of *twist1a* did not appear to have the same effects on myocardial differentiation in *han*^{s6} mutants as in the transheterozygotes. Thus, it seems as if the role Twist1a has in myocardial cell migration and fusion across the midline is not dependent on Hand2 presence. The fusion we observed in both the injected transheterozygotes and *han*^{s6} mutants did not resemble exactly what should be seen in wild-type cardiac fusion. However, the morphology of the cardiomyocytes in these fused embryos did seem quite organized. Thus, we feel that the phenotype could be considered a shift toward what needs to occur for a functional heart to develop, and that this result represented a partial rescue in

cardiac fusion. It would be interesting to determine what role Twist1a plays in this process. One future experiment that could be instructive would be to analyze the effect of *twist1a* knockdown on the expression of *natter*, the gene that encodes fibronectin (Trinh and Stanier, 2004). We could hypothesize from our results here that if reducing *twist1a* function leads to reduced *natter* expression in *han*^{s6} mutants (and an improvement in myocardial migration), then Twist1a normally plays a role in promoting fibronectin deposition, separate from its role and relationship with Hand2 in myocardial differentiation.

A model for antagonistic interactions between Twist1a and Hand2

Taken together, we feel our data support a general model in which Twist1a and Hand2 have opposing roles in myocardial differentiation. Because the presence of Hand2 is implicated to be necessary for Twist1a to function in this regard, we propose that they interact within the same pathway to regulate the differentiation of cardiomyocytes from cardiac progenitors. Because reducing *twist1a* function does not result in an extreme expansion of *cmlc2* expression in wild-type embryos, we can imagine a case in which some other protein "X" is also involved in this pathway. In one possible model, a Hand2-X dimer would act to promote myocardial differentiation, while Twist1a can also bind X, creating Twist1a-X dimers and sequestering the availability of X to bind Hand2. When there is less Twist1a available, more Hand2-X dimers can form, but that does not

mean that there is an infinite capability for myocardial cells to differentiate in wild-type embryos. However, when Twist1a is reduced in *hand2* hypomorphic mutants, more protein X can bind the already limited amount of Hand2, and a more robust increase in myocardial cells is seen than in injected wild-type embryos. Lastly, in the absence of Hand2, the reduction of Twist1a releases protein X, but the pathway in which X binds Hand2 to promote myocardial differentiation cannot be induced, as no Hand2 is present. Of course, one possibility for the identity of protein X is Hand2, such that Hand2-Hand2 homodimers promote myocardial differentiation, while Twist1a-Hand2 heterodimers limit Hand2 availability.

Further experiments testing the molecular interactions between Twist1a and Hand2 could be useful in studying this model. For example, injecting synthetic Hand2-Twist1a or Hand2-Hand2 dimers into *hand2* mutants could indicate whether a physical interaction between the proteins inhibits or encourages myocardial differentiation. We could also test what promoters are being bound by the transcription factors. This could be useful in identifying which dimers are DNA-binding and whether others are bystanders that indirectly regulate transcription. Combining future biochemical techniques with our genetic experimental approach can help us further define a model for the regulation of myocardial differentiation through interactions between Twist1a and Hand2.

MATERIALS AND METHODS

Zebrafish

We used wild-type fish as well as fish heterozygous for a mutation in the *han* locus, which encodes the transcription factor Hand2. Heterozygotes contained either a null (han^{s6}) mutation or hypomorphic (han^{c99}) mutation (Yelon et al, 2000).

In situ hybridization

Whole-mount in situ hybridization was performed as previously described (Yelon et al., 1999). Embryos were fixed from a range of 9 to 24hpf and stained with *cmlc2* (Yelon et al., 1999) or *twist1a* (Germanguz et al., 2001) RNA probe. In situs were cleared in a 2:1 benzyl benzoate/benzyl acetate solution and imaged on a Zeiss Axioplan using a Zeiss AxioCam. Images were processed using Axiovision software and Adobe photoshop. Area measurements of *cmlc2* expression were done using ImageJ (http://rsb.info.nih.gov/ij/index.html).

Morpholinos

Embryos were injected at the one-cell stage with 4-6 ng of an antisense morpholino oligonucleotide (MO) against *twist1a* to block translation and knock down the expression of the protein. We used an MO targeting the 5' UTR (MO#1). Because *twist1a* has only one exon, using an MO against a splice site was not an option. We utilized another MO (MO#2) that targeted the start codon

to confirm that the observed effects of MO#1 could be attributed to the specific knockdown of Twist1a function. In addition, 2ng of anti-*p53* MO (Robu et al., 2007) was injected to suppress apoptosis and reduce the amount of cell death evident from *twist1a* MO injection alone.

anti-*Twist1a* MO#1: 5'-CGTGCATCGCCTCTTCCTCAAACAT-3'

anti-*Twist1a* MO#2: 5'-TCGTGGCTTCCCTGAGTCCGACGAA-3'

anti-*p53* MO: 5'-GCGCCATTGCTTTGCAAGAATTG-3'

REFERENCES

Barnes RM, Firulli AB. (2009). A twist of insight – the role of Twist-family bHLH factors in development. J Dev Biol. *7*, 909-924.

Beis D, Stainer DY. (2006). In vivo cell biology: following the zebrafish trend. Trends Cell Biol. *2*, 105-112.

Cai J, Jabs EW. (2005). A twisted hand: bHLH protein phosphorylation and dimerization regulate limb development. BioEssays. *11*, 1102-1106.

Conway SJ, Firulli B, Firulli AB. (2010). A bHLH code for cardiac morphogenesis. Pediatr Cardiol. *3*, 318-324.

Das A, Crump JG. (2012) Bmps and id2a act upstream of twist1 to restrict ectomesenchyme potential of the cranial neural crest. PLoS Genet. *5*, e1002710.

Dai YS, Cserjesi P. (2002). The basic helix-loop-helix factor, Hand2, functions as a transcriptional activator by binding to E-boxes as a heterodimer. J Biol Chem.*15*, 12604-12612.

Firulli BA, Krawchuk D, Centonze VE, Virshup DM, Conway SJ, Cserjesi P, Laufer E, Firulli AB. (2005). Altered Twist1 and Hand2 dimerization is associated with Saethre-Chotzen syndrome and limb abnormalities. Nat Genet. *4*, 373-381.

Firulli BA, McConville DP, Byers JS, Vincentz JW, Barnes RM, Firulli AB. (2010). Analysis of a Hand2 hypomorphic allele reveals a critical threshold for embryonic viability. Dev Dyn.*10*, 2748-2760.

Germanguz I, Lev D, Waisman T, Kim CH, Gitelman I. (2001). Four twist genes in zebrafish, four expression patterns. Dev Dyn. *9*, 2615-2626.

Haupt S, Berger M, Goldberg Z, Haupt Y. (2003). Apoptosis – the p53 network. J of Cell Sci. *116*, 4077-4085.

Hoffman JI, Kaplan S. (2002). The incidence of congenital heart disease. J Am Coll Cardiol. *12*, 1890-1900.

Huang CJ, Tu CT, Hsaio CD, Hsieh FJ, Tsai HJ. (2003). Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. Dev Dyn. *1*, 30-40.

Lu S, Nie J, Luan Q, Feng Q, Xiao Q, Chang Z, Shan C, Hess D, Hemmings BA, Yang Z. (2011). Phosphorylation of the Twist1-family basic helix-loop-helix transcription factors is involved in pathological cardiac remodeling. PLoS One. *4*, e19251.

Murakami M, Katoaka K, Tominaga J, Nakagawa O, Kurihara H. (2004). Differential cooperation between dHAND and three different E-proteins. J Biochem Biophys Res Commun. *1*, 168-174.

Olson EN. (2006). Gene regulatory networks in the evolution and development of the heart. Science. *5795*, 1922-1927.

Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, Ekker SC. (2007). p53 activation by knockdown technologies. PLos Genet. *5*, e78.

Schoenebeck JJ, Keegan BR, Yelon D. (2007). Vessel and blood specification override cardiac potential in anterior mesoderm. Dev Cell. *3*, 254-267.

Srivastava D. (2006). Making or breaking the heart: from lineage determination to morphogenesis. Cell. *6*, 1037-1048.

Trinh LA, Stainier DY. (2004). Fibronectin regulate epithelial organization during myocardial migration in zebrafish. Dev Cell. *3*, 371-382.

Trinh LA, Yelon D, Stanier DY. (2005). Hand2 regulates epithelial formation during myocardial differentiation. Curr Biol. *15*, 441-446.

Yang D-C, Tsai C-C, Liao Y-F, Fu H-C, Tsay H-J, Huang T-F, Chen Y-H, Hung S-C. (2011). Twist controls skeletal development and dorsoventral patterning by regulating runx2 in zebrafish. PLoS One. *11*, e27324.

Yelon D, Horne SA, Stainier DY. (1999). Restricted expression of cardiac myosin genes reveals regulated aspects of the heart tube assembly in zebrafish. Dev Biol. *1*, 23-37.

Yelon D, Ticho B, Halpern ME, Ruvinsky I, Ho RK, Silver LM, Stainier DY. (2000). The bHLH transcription factor hand2 plays parallel roles in zebrafish heart and pectoral fin development. Development. *12*, 2573-2582.

Zhang Y, Blackwell EL, McKnight MT, Knutsen GR, Vu WT, Ruest LB. (2012). Specific inactivation of Twist1 in the mandibular arch neural crest cells affects the development of the ramus and reveals interactions with Hand2. Dev Dyn. *5*, 924-940.

FIGURES

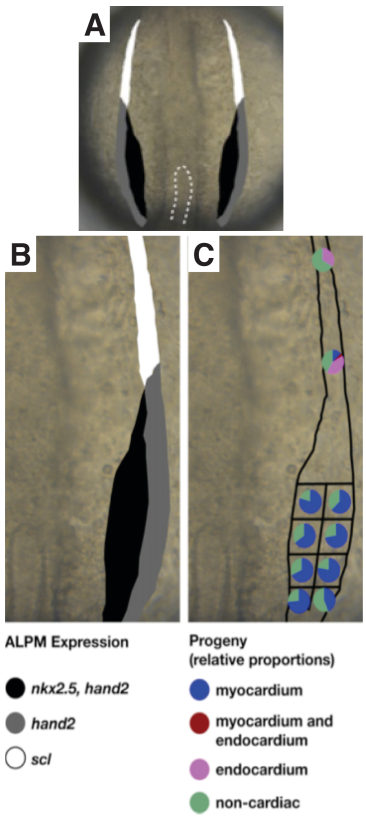


Figure 1. Fate map of cardiac progenitors in ALPM.
(A) Bilateral regions of ALPM where cardiac progenitors are located. Dashed line represents the tip of the notochord. Different colors in ALPM represent territories of gene expression. White is rostral, black is medial, and grey is lateral area of ALPM. Dorsal view, anterior to the top. (B) Expression pattern of transcription factors in the ALPM. *nkx2.5* and *hand2* are expressed together in the medial region of the ALPM, while only *hand2* is expressed in the lateral region. *scl* is expressed in the rostral region of the ALPM. Dorsal view of the right side of the ALPM, anterior to the top. (C) Fate map of cardiac progenitors in ALPM. The progeny of labeled cells within each boundary tested were analyzed for cardiac cell identity. Pie charts depict the proportions of labeled cells that went on to differentiate into myocardium (blue), endocardium (purple), both myocardium and endocardium (maroon) or non-cardiac cells (green). Labeled cells that differentiated into myocardium originated mostly from the medial and lateral territories. Those that differentiated into endocardium originated mostly from the rostral territory of the ALPM. Thus, myocardial progenitors are found mainly in the medial and lateral regions, where *nkx2.5* and *hand2* are expressed, and endocardial progenitors are found in the rostral region of the ALPM, where *scl* is expressed. Adapted from Schoenebeck et al., 2007.

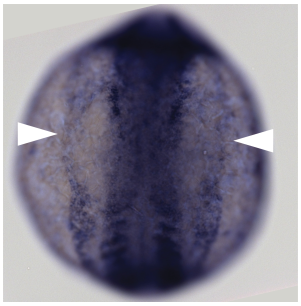
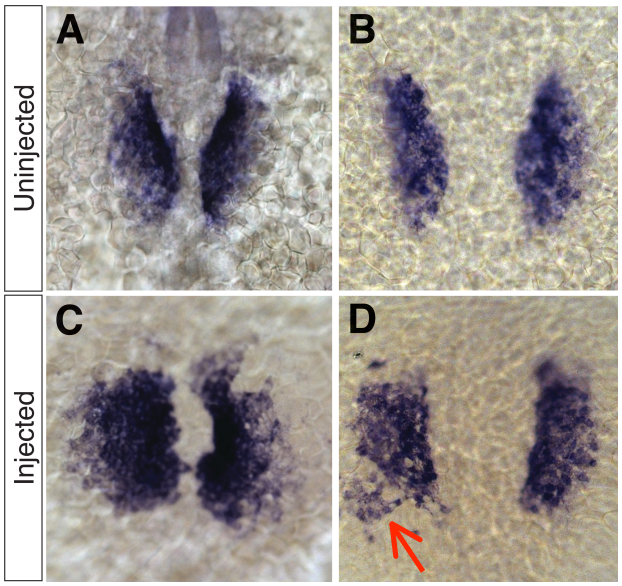


Figure 2. *twist1a* is expressed in the ALPM.
In situ hybridization depicts expression of *twist1a* in the posterior lateral plate mesoderm that extends into the ALPM (arrowheads) at 10 somites. Dorsal view, anterior to the top.



E

	MO#1 n=120	MO#2 n=134
Category A	12.5%	10.0%
Category B	15.0%	7.5%

Figure 3. Knockdown of *twist1a* in WT embryos results in expanded *cmlc2* expression.
In situ hybridization shows *cmlc2* expression at 17-18 somites. Dorsal views, anterior to the top. Embryos in (A) and (C) are at the same point in cardiac fusion. (C) *cmlc2* expansion is evident. The generally smooth boundaries of *cmlc2* expression seen in wild-type are observed, so embryo is placed in Category A. Embryos in (B) and (D) are at the same point in cardiac fusion. (D) *cmlc2* expression pattern is abnormal. There are more cardiomyocytes, and the boundaries are not maintained. Embryo represents the type placed in Category B. (E) We tested the effects of two non-overlapping anti-*twist1a* morpholinos. While injections of either at doses of 4-6 ng resulted in similar phenotypes, our first MO seemed to give a higher percentage of embryos exhibiting the phenotype at easily observable levels.

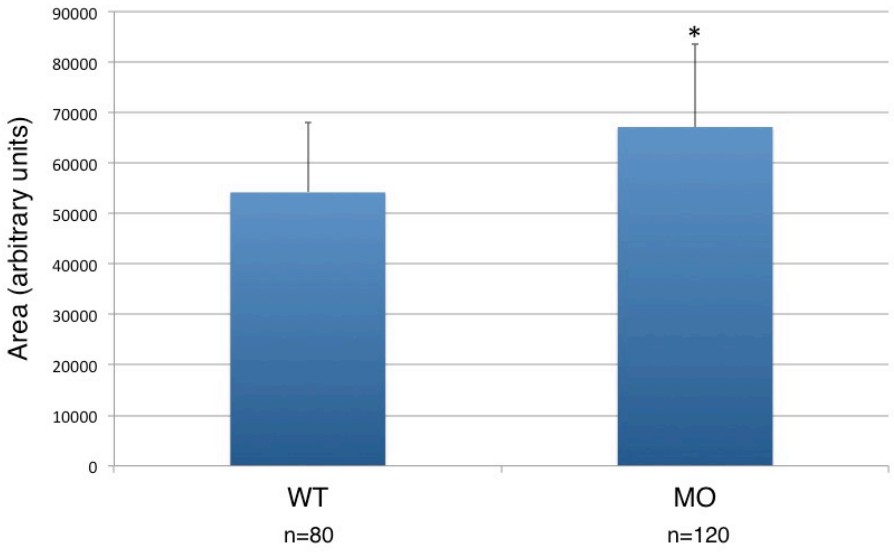
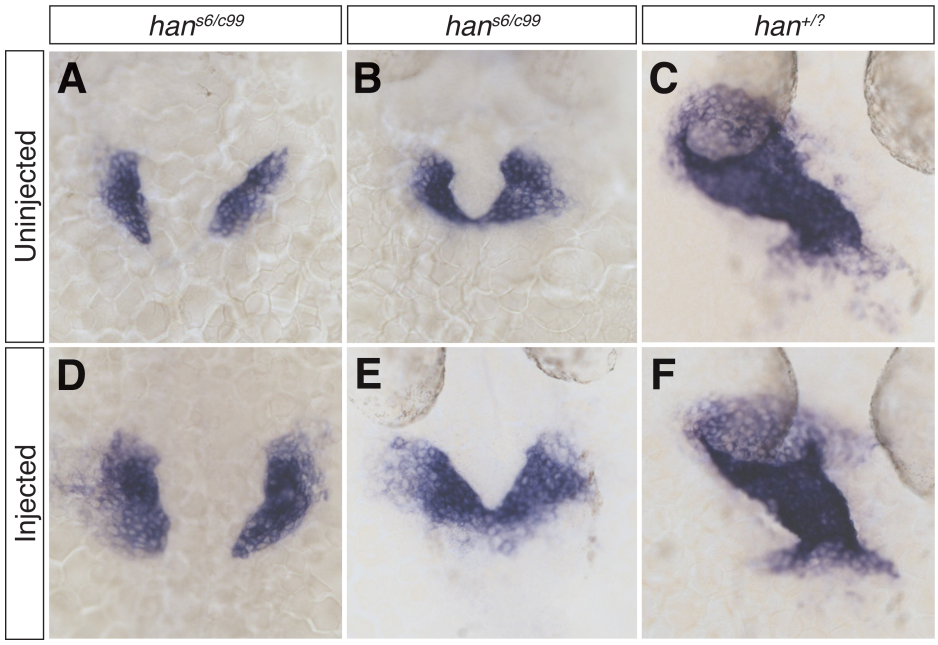


Figure 4. Knockdown of *twist1a* in WT embryos results in a larger average area of *cmlc2* expression.
Area measurements of *cmlc2* expression in WT embryos at 17 somites in arbitrary units. Error bars represent standard deviation. Reducing Twist1a function results in an average increase of 24% in area of *cmlc2* expression in injected embryos. (*$p<0.01$, Student's T Test)



G	WT phenotype	Transheterozygote phenotype	Obvious *cmlc2* expansion
Uninjected	115/152 (76%)	37/152 (24%)	-
Injected	91/122 (75%)	31/122 (25%)	21/31 (68%)

Figure 5. Knockdown of *twist1a* function in *han*$^{s6/c99}$ transheterozygotes results in expanded *cmlc2* expression.
In situ hybridization depicts expression of *cmlc2* at 24 hpf. Dorsal views, anterior to the top. (A and B) *han*$^{s6/c99}$ transheterozygotes have variable phenotypes, ranging from two separate heart fields to some type of fusion at the midline. (D and E) *han*$^{s6/c99}$ transheterozygotes injected with *twist1a* MO show expanded *cmlc2* expression. (C and F) *han*$^{+/?}$ embryos do not show detectable phenotypes in uninjected or injected embryos. *cmlc2* expression depicts a linear heart tube typical of WT embryos at 24 hpf. (G) Embryos were characterized as transheterozygotes based on the presence of the obvious phenotype exhibited at 24 hpf compared to a WT linear heart tube. Both uninjected and injected groups showed the mutant phenotypes in one quarter of the total embryos. 68% of the injected transheterozygotes displayed obvious expansion of *cmlc2* in comparison to the uninjected siblings.

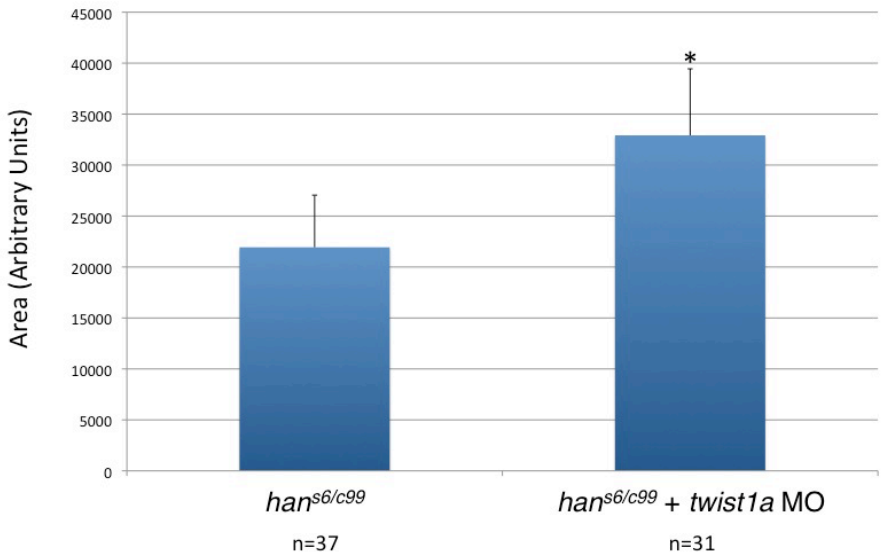
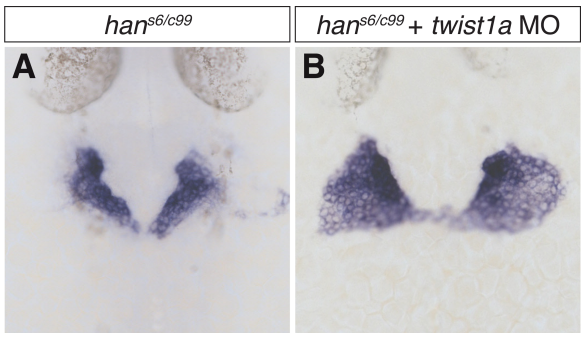
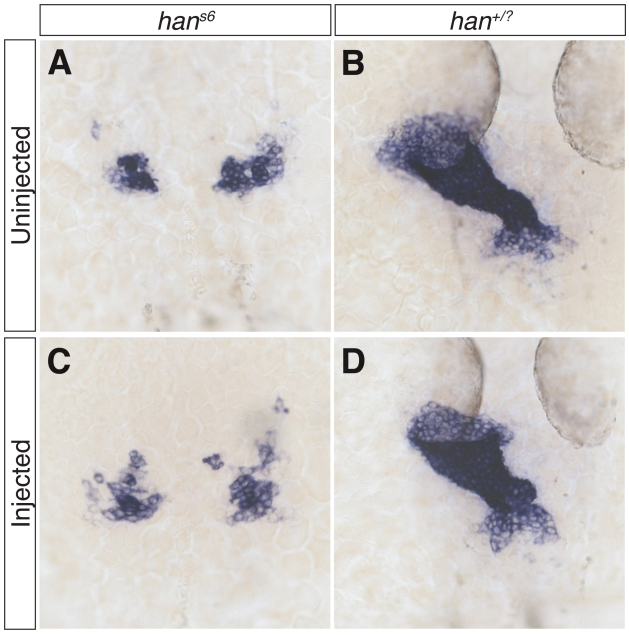


Figure 6. Knockdown of *twist1a* in *han*$^{s6/c99}$ transheterozygotes results in an increase in the average area of *cmlc2* expression.
Area measurements of *cmlc2* expression in transheterozygotes at 24 hpf in arbitrary units. Error bars represent standard deviation. Reducing Twist1a function results in an average increase of 50% in area of *cmlc2* expression in injected embryos. (*$p<0.01$, Student's T Test)



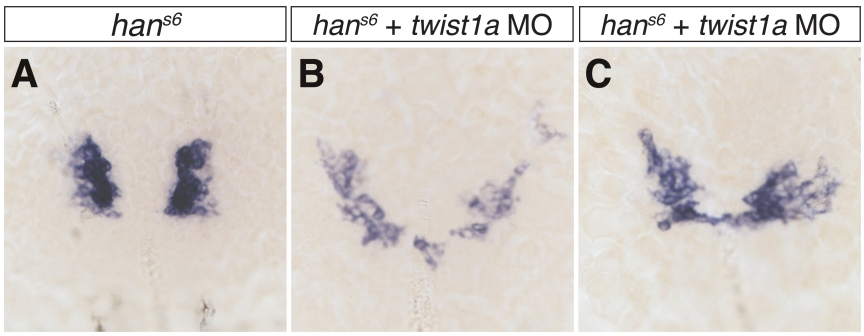
C	2 separate fields	Fused across midline	'Bridge' fusion
Uninjected transhets	12/37 (32%)	25/37 (68%)	-
Injected transhets	5/31 (16%)	20/31 (65%)	6/31 (19%)

Figure 7. Fusion occurs more often in transheterozygotes injected with *twist1a* MO.
In situ hybridization shows expression of *cmlc2* at 24 hpf. Dorsal views, anterior to the top. (A) *han*$^{s6/c99}$ transheterozygotes display the ability for the bilateral populations of cardiomyocytes to fuse across the midline of the embryo. (B) *han*$^{s6/c99}$ transheterozygotes injected with *twist1a* MO sometimes exhibit abnormal 'bridge' fusion not seen in uninjected embryos at this time point. Neither injected nor uninjected transheterozygote embryos display fusion comparable to WT fusion. (C) Injected transheterozygotes exhibit fusion in 26/31 (84%) of the total mutants, while uninjected siblings only fused across the midline in 68% of the total mutants. The morphology of fusion in injected transheterozygotes was distinct from uninjected embryos at this time point in 6/31 of the total injected mutants.



E	WT phenotype	*han*^{s6} mutant phenotype	Obvious *cmlc2* expansion
Uninjected	69/89 (78%)	20/89 (22%)	-
Injected	39/52 (75%)	13/52 (25%)	-

Figure 8. Knockdown of *twist1a* function in *han*^{s6} mutants has no apparent effect on the amount of *cmlc2*-expressing cells.
In situ hybridization depicts expression of *cmlc2* at 24 hpf. Dorsal views, anterior to the top. (A) *han*^{s6} mutants have reduced numbers of cardiomyocytes compared to WT and do not migrate toward midline. (B) *han*^{s6} mutants injected with *twist1a* MO do not seem to exhibit a change in number of cardiomyocytes compared to the uninjected mutants. (B and D) *han*$^{+/?}$ embryos do not show detectable phenotypes in uninjected or injected embryos. *cmlc2* expression depicts a linear heart tube typical of WT embryos at 24 hpf. (E) Embryos were characterized as *han*^{s6} mutants based on the presence of the obvious phenotype exhibited at 24 hpf compared to a WT linear heart tube. Both uninjected and injected groups showed the mutant phenotypes in about one quarter of the total embryos. None of the injected *han*^{s6} mutants displayed obvious expansion of *cmlc2* expression compared to uninjected siblings.



D	2 separate fields	Fused or almost fused
Uninjected *han*^{s6} mutants	19/20 (95%)	1/20 (5%)
Injected *han*^{s6} mutants	7/13 (54%)	6/13 (46%)

Figure 9. Abnormal morphology of *cmlc2* expression seen in *han*^{s6} mutants injected with *twist1a* MO.
In situ hybridization depicts expression of *cmlc2* at 24 hpf. Dorsal views, anterior to the top. (A) *han*^{s6} mutants do not fuse across the midline at 24hpf. (B and C) *han*^{s6} mutants injected with *twist1a* MO display abnormal morphology in 6/13 of injected mutants. Bilateral populations of cardiomyocytes appear to be capable of crossing the ventral midline. (C) is very similar in appearance to the 'bridge' fusion sometime seen in injected transheterozygotes. (D) About half of the injected *han*^{s6} mutant embryos exhibit capability of bilateral populations of cardiomyocytes to move toward the midline.