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The PAF1 complex differentially regulates cardiomyocyte specification

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Abstract

The specification of an appropriate number of cardiomyocytes from the lateral plate mesoderm requires a careful balance of both positive and negative regulatory signals. To identify new regulators of cardiac specification, we performed a phenotype-driven ENU mutagenesis forward genetic screen in zebrafish. In our genetic screen we identified a zebrafish *ctr9* mutant with a dramatic reduction in myocardial cell number as well as later defects in primitive heart tube elongation and atrioventricular boundary patterning. Ctr9, together with Paf1, Cdc73, Rtf1 and Leo1, constitute the RNA polymerase II associated protein complex, PAF1. We demonstrate that the PAF1 complex (PAF1C) is structurally conserved among zebrafish and other metazoans and that loss of any one of the components of the PAF1C results in abnormal development of the atrioventricular boundary of the heart. However, Ctr9, Cdc73, Paf1 and Rtf1, but not Leo1, are required for the specification of an appropriate number of cardiomyocytes and elongation of the heart tube. Interestingly, loss of Rtf1 function produced the most severe defects, resulting in a nearly complete absence of cardiac precursors. Based on gene expression analyses and transplantation studies, we found that the PAF1C regulates the developmental potential of the lateral plate mesoderm and is required cell autonomously for the specification of cardiac precursors. Our findings demonstrate critical but differential requirements for PAF1C components in zebrafish cardiac specification and heart morphogenesis.

Keywords

cardiac specification; heart tube; PAF1 complex; PAF1C; zebrafish

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Introduction

The specification of an appropriate number of cardiac precursors is essential for the development of a functional heart. In vertebrates, cardiac progenitor cells are specified from the lateral plate mesoderm (LPM) to form a bilaterally symmetric heart field. These precursor cells then migrate medially and fuse at the midline. In zebrafish, the fusion of the heart field generates a cone-like structure, which develops into a pulsatile primitive heart tube via a process of cellular migration and rearrangement.

The size of the zebrafish heart field is regulated by both positive and negative signals originating in the LPM and surrounding tissues (Chen and Fishman, 2000; Yelon, 2001; Yelon et al., 2002; Zaffran and Frasch, 2002). Inductive signals such as *gata4/5/6*, *bmp2b*, *fgf8*, *hand2* and *nkx2.5* in the LPM push mesodermal cells toward a cardiac fate (Chen and Fishman, 1996; Holtzinger and Evans, 2007; Marques et al., 2008; Peterkin et al., 2007; Reifers et al., 2000; Reiter et al., 1999; Reiter et al., 2001b; Trinh et al., 2005; Yelon et al., 2000), while RA signaling negatively regulates cardiac progenitor production by activating the expression of *hoxb5b* in the limb fields (Waxman et al., 2008). Expression of *scl* and *etsrp* in the LPM anterior to the heart field establish the anterior boundary of cardiac progenitors (Schoenebeck et al., 2007), while unknown signals from the notochord establish the posterior heart field boundary (Goldstein and Fishman, 1998). Precise regulation of the expression of all the factors involved in specification of the heart field is vitally important for the development of a functional heart.

The PAF1 complex (PAF1C) is an RNA Polymerase II-interacting protein complex required for the development of the heart and other organs (Akanuma et al., 2007; Nguyen et al., 2010). The PAF1C was originally identified in yeast, and consists of five core proteins: Paf1, Ctr9, Rtf1, Cdc73, and Leo1. Loss of the PAF1C components Paf1 and Ctr9 in yeast prevents transcriptional activation of the G_1 cyclin CLN2, resulting in defective cell cycle control and cell growth (Koch et al., 1999). Yeast Paf1, Ctr9, and Rtf1 are also essential for the Set1-mediated methylation of histone H3 lysine-4 and Paf1 and Rtf1 are additionally required for Dot1- mediated histone H3 lysine-79 methylation (Krogan et al., 2003). Furthermore, Paf1 is necessary for proper poly(A) site utilization of a subset of yeast genes (Penheiter et al., 2005).

In multicellular organisms, the PAF1C is essential for numerous signaling pathways and developmental processes. In *Arabidopsis*, PAF1C proteins are important regulators of flowering-time. Loss of the *Arabidopsis* homologs of Paf1, Ctr9, and Rtf1 results in an early flowering phenotype because of a reduction in histone H3 lysine-4 trimethylation at the promoter of genes required to repress flowering (He et al., 2004; Oh et al., 2004). The Rtf1 subunit of the PAF1C also regulates the Notch signaling pathway in *Drosophila* and zebrafish (Akanuma et al., 2007; Tenney et al., 2006). Loss of Rtf1 in zebrafish causes a reduction in the expression level of Notch-regulated genes in the somites, resulting in aberrant somitogenesis (Akanuma et al., 2007) and loss of Leo1 in zebrafish produces defects in atrioventricular boundary and neural crest development (Nguyen et al., 2010). Interestingly, mutation in zebrafish *cdc73* is able to suppress the defects in erythroid gene transcription elongation of *Transcription Intermediary Factor 1gamma* mutants, resulting in a rescue of blood development (Bai et al., 2010). Lastly, Cdc73 has also been shown to positively regulate the Wnt and Hedgehog signaling pathways in *Drosophila* and mammalian cells via direct interactions with beta-catenin and Gli proteins (Mosimann et al., 2006, 2009). However, the function of each component of the PAF1C has not yet been evaluated systematically in any animal model.

From our ENU mutagenesis screen for genes affecting cardiovascular development, we identified a mutant, *LA961*, with a severe reduction in cardiac cell number. Positional cloning of this mutant revealed a mutation in the zebrafish homolog of *ctr9*, a member of the PAF1C. Key differences are present in the cardiac phenotypes of *ctr9* and *leo1* mutants, suggesting distinct requirements for the individual proteins of the PAF1C during cardiogenesis. By morpholino knockdown, we found that Paf1, Cdc73 and Rtf1, like Ctr9, are important for establishing an appropriate number of cardiomyocytes and for elongation and patterning of the primitive heart tube. PAF1C components are not essential for the formation of LPM, but instead regulate the specification of LPM derivatives. Furthermore, by transplanting Rtf1-deficient cells into wild type embryos, we found that the PAF1C plays a cell-intrinsic role in the specification of cardiomyocytes.

Materials and Methods

Zebrafish and ENU mutagenesis

Zebrafish colonies were cared for and bred under standard conditions. Developmental stages of zebrafish embryos were determined using standard morphological features of fish raised at 28.5°C (Westerfield, 2000). ENU mutagenesis of Tg(kdrl:GFP)^{LA116} male zebrafish was performed as previously described (Nguyen et al., 2010).

Positional cloning

The *LA961* mutation was identified in the AB background and heterozygous carriers of this mutation were crossed to the polymorphic WIK strain for mapping. Embryo lysis and PCR-based bulk segregant analysis were performed as previously described (Nguyen et al., 2010). Using the Massachusetts General Hospital microsatellite map, we found that *LA961* was linked to markers Z1239 and Z41496 on chromosome 7. The markers generated during fine mapping include 1162-5 and 395-1 and their primer sequences are as follows (from 5’ to 3’):

1162-5-F: ACTCCTGTGGGATGTGTGTG;

1162-5-R: CAAATAAATTGTATGGAACCCAAT;

395-1-F: TCAGTTTTGACATTCCTGCATC;

395-1-R: GCTGTGGTTTCATGTTCGTC.

To identify the *ctr9*^{LA961} mutation, total RNA was isolated from 1 day post fertilization (dpf) *LA961* mutants using RNA Wiz (Ambion, Austin, TX) and cDNA was synthesized using the Superscript II Kit (Invitrogen). cDNA fragments were amplified with Phusion polymerase (NEB) and cloned into pCR-Blunt II-TOPO (Invitrogen) for sequencing.

Cardiomyocyte cell counting

Tg(myl7:EGFP) transgenic (Huang et al., 2003) wild type and ctr9^{LA961} mutant embryos were dissociated in Ca^{2+}-free Ringers solution and flat mounted. Dissociated cardiac cells were photographed and counted using a Zeiss Axiovert 200M equipped with a Zeiss Axiocam camera.

Constructs and morpholinos

The *ctr9*^{WT}, *ctr9*^{LA961}, and *leo1* full length cDNAs were amplified from 1 dpf wild-type embryo cDNA using Phusion polymerase (NEB) and cloned into pCS2+myc or pCS2+3XFLAG for tagging with the Myc and FLAG epitopes. Full length cDNA for *cdc73*, *paf1*, and *rtf1* was amplified with KOD polymerase (Novagen) and cloned into pCS2+3XFLAG. Plasmids were cut with *Not*I and SP6 RNA polymerase was used to generate mRNA for injection.

Morpholino oligonucleotides targeting the translation initiation sites of *ctr9*, *cdc73*, *paf1*, and *rtf1* (ctr9MO, cdc73MO, paf1MO, and rtf1MO) were purchased from Gene Tools, LLC and injected at dosages of 0.75 ng (for ctr9MO), 2 ng (for cdc73MO), 1.5 ng (for paf1MO) and 0.25 ng (for rtf1MO). Cdc73MO and paf1MO were coinjected with a p53 morpholino (4 ng and 6.5 ng, respectively) to prevent non-specific cell death. The sequences of the morpholinos used are as follows (from 5' to 3'):

ctr9MO: GATTTCAATGGATCCCCGAGACATG;

cdc73MO: GAAGAACACTCAACACGTCCGCCAT;

paf1MO: CCTGCGTCTGTATGGTAGGAGCCAT;

rtf1MO: CTTTCCGTTTCTTTACATTCACCAT (Akanuma et al., 2007);

p53 morpholino: GCGCCATTGCTTTGCAAGAATTG.

Morpholino efficacy was tested by Western blotting to detect levels of PAF1 complex proteins in 1 day old morpholino injected embryo lysates. Embryos were lysed in Rubinfield's Lysis buffer (20mM Tris pH 8.0, 140mM NaCl, 1% Triton X-100, 10% glycerol, 1mM EGTA, 1.5mM $MgCl_2$, 1mM Na_3VO_4, 50mM NaF, 1mM DTT) with protease inhibitors (Roche), and one embryo equivalent was loaded per lane on a 8% polyacrylamide gel. Electrophoresed proteins were transferred to a nitrocellulose membrane. Antibodies against PAF1 complex proteins include anti-Ctr9 (1:2,500, SAB1100738, Sigma), anti-Paf1 (1:10,000, A300-172A, Bethyl Laboratories, Inc.), anti-Cdc73 (1:5,000, C3620, Sigma), and anti-Rtf1 (1:2,000, ab70690, Abcam). Relative protein levels were determined by densitometry measurements in Adobe Photoshop CS3 using β-actin (1:5,000, A1978, Sigma) as a loading control.

Whole mount in situ hybridization

Embryos for *in situ* hybridization were raised in embryo medium supplemented with 0.2 mM 1-phenyl-2-thiourea to maintain optical transparency (Westerfield, 2000). Whole-mount in situ hybridization was performed as described previously (Chen and Fishman, 1996). The antisense RNA probes used in this study include *cmlc2* (*myl7*), *nkx2.5*, *vmhc*, *amhc* (*myh6*), *bmp4*, *notch1b*, *tbx2b*, *tbx20*, *gata4*, *gata5*, *hand2*, and *ctr9*.

Antibody staining

Embryos injected with 200 pg of RNA encoding Myc-tagged zebrafish *ctr9*, FLAG-tagged zebrafish *cdc73*, FLAG-tagged zebrafish *paf1*, or FLAG-tagged zebrafish *rtf1* were fixed in 4% PFA in PBS at 75% epiboly. The fixed embryos were incubated in primary antibody (1:50 mouse anti-FLAG M2, F1804, Sigma; 1:50 rabbit anti-Myc, 06–549, Upstate) in blocking solution (10% goat serum in PBDT) for 2 hours at room temperature followed by detection with fluorescent secondary antibodies (1:200 anti-mouse IgG_1-R, sc-2084, Santa Cruz Biotechnology; 1:200 anti-rabbit IgG-Cy5, 81-6116, Invitrogen). Nuclei were stained with DAPI (Invitrogen) and embryos were embedded in 1% low-melt agarose and imaged on a Zeiss LSM510 confocal microscope equipped with a 63X water objective.

Coimmunoprecipitations

For coimmunoprecipitation analysis, 60 embryos injected with tagged constructs were lysed using Rubinfeld's Lysis Buffer (20 mM Tris pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 50 mM NaF, 1 mM DTT) with protease inhibitors (Roche). Binding of antibody (mouse anti-Myc 9E10, 05–419, Upstate) was performed overnight on a nutator at 4°C. Protein G sepharose beads (17-0618-01, GE Healthcare) were used to pull down bound proteins. Immunoprecipitated proteins were separated on a 7% polyacrylamide gel and transferred to a nitrocellulose membrane for

Western analysis. Mouse anti-FLAG M2 (1:10,000, F1804, Sigma) and rabbit anti-mouse IgG-HRP (1:20,000, 61–6020, Invitrogen) antibodies were used to detect Flag-tagged proteins.

Transplantations

For experiments requiring MO treatment Tg(myl7:EGFP) donor embryos or Tg(myl7:mCherry) recipient embryos were injected with 0.25 ng rtf1MO and 0.5 ng p53MO. Donor cells were drawn into a glass needle by back pressure and 20–30 cells were transplanted into high stage recipient embryos at the margin in two locations 90° from each other. On 1 or 2 dpf recipient embryos were scored for donor cell contribution to the heart. For imaging, 2 dpf hearts were gently removed from the embryos and mounted in 1% low melt agarose. Images were taken using a Zeiss LSM510 confocal microscope.

Results

Identification of a novel zebrafish cardiac mutant, *LA961*

In a screen for zebrafish cardiovascular mutants, we identified a novel mutant, *LA961*, with severe defects in the morphogenesis of the primitive heart tube. In wild type zebrafish embryos, a beating heart tube is visible on the left side of the body by 24 hours post fertilization (hpf). At 24 hpf, *LA961* mutants lack a visible heart tube. Additionally, *LA961* mutants exhibit a slight pericardial edema and an absence of otoliths (Figure 1A,B). By 35 hpf, *LA961* mutants develop a severely reduced and dysmorphic heart, a complete lack of pigment cells, and a downwardly curved body (Figure 1F), indicating that the *LA961* gene plays a role in multiple developmental processes.

We used the cardiomyocyte marker *cmlc2* (also known as *my17*) to further examine the heart phenotype in *LA961* and found that these mutants have *cmlc2*-expressing cells; however these cardiomyocytes fail to elongate into a tube by 24 hpf and are instead clustered at the midline (Figure 1C,D). The number of cells expressing *cmlc2* also appeared lower in *LA961* mutants. We directly counted the number of cardiomyocytes in *LA961* at 24 hpf by crossing this mutation into a Tg(myl7:EGFP) genetic background and recording the number of cells expressing green fluorescent protein (GFP). We found an average of 134 GFP-positive cells in wild type embryos at 24 hpf (n= 7), whereas *LA961* mutants only possessed an average of 70 GFP-positive cells at the same developmental stage (n= 6; $p = 0.00006$; Figure 1E). This dramatic reduction in myl7:EGFP-positive cells indicates that the *LA961* gene is critical for generating an appropriate number of cardiomyocytes in addition to its role in the morphogenesis of the primitive heart tube.

The *LA961* locus encodes Ctr9

We mapped the *LA961* locus to an 11 centimorgan (cM) region on chromosome 7. Using custom simple sequence length polymorphism (SSLP) markers, we refined the *LA961* interval and found one marker, 1162-5, located 0.1 cM from the *LA961* mutant locus (Figure 1G). Sequencing of the genes near this marker identified a nonsense mutation in the *ctr9* gene. Overexpression of wild type *ctr9* rescued the *LA961* mutant phenotype (out of 40 injected embryos generated by crossing *LA961* heterozygotes, none exhibited an *LA961* mutant phenotype) and wild type embryos injected with 0.75 ng of a morpholino oligonucleotide targeting the translation start site of *ctr9* (ctr9MO) exhibited phenotypes identical to those seen in *ctr9* mutants (n= 80) (Supplemental Figure 1).

Zebrafish Ctr9 contains 3 putative nuclear localization signal (NLS) domains in its C-terminal tail. The $ctr9^{LA961}$ mutation eliminates the third and the majority of the second of these NLSs (Figure 1H). To test if this mutation results in defective localization of the Ctr9

protein, we tagged wild type and mutant Ctr9 protein with a Myc epitope and overexpressed these constructs in zebrafish embryos. Interestingly, we found that both $Ctr9^{WT}$ and $Ctr9^{LA961}$ localize to the nucleus (Supplemental Figure 2A,B), indicating that the final 2 NLSs are not required for the cellular localization of Ctr9. However, examination of endogenous *ctr9* mRNA levels in $ctr9^{LA961}$ mutants by in situ hybridization revealed a dramatic reduction in expression (Supplemental Figure 2C,D), indicating that $ctr9^{LA961}$ transcripts are likely unstable and are degraded by cellular nonsense mediated decay mechanisms. Together these findings suggest that loss of function of Ctr9 is causative to the cardiac and pigment defects observed in *LA961* and that the *LA961* mutation may be hypomorphic.

The PAF1C is evolutionarily conserved in zebrafish

Ctr9 is a member of the multi-protein PAF1C (also consisting of Paf1, Rtf1, Cdc73, and Leo1), which was initially identified in yeast. PAF1C proteins have been conserved through evolution, and are present and interact with one another in *Drosophila* and humans (Adelman et al., 2006; Kim et al., 2010). We questioned whether the PAF1C was structurally conserved in the zebrafish as well. Members of the PAF1C are known to localize to the nucleus and interact with RNA polymerase II (Mueller and Jaehning, 2002; Shi et al., 1997). We have previously shown that zebrafish Leo1 exhibits a nuclear localization (Nguyen et al., 2010). Here we further tested whether the other zebrafish PAF1 component homologs were present in the same subcellular compartment. We found that Ctr9, Cdc73, Paf1, and Rtf1 all localized specifically to the nucleus (Figure 2A–D). This finding is consistent with the localization of the PAF1C in yeast, *Drosophila*, and humans (Adelman et al., 2006; Kim et al., 2010; Lin et al., 2007a; Lin et al., 2007b; Porter et al., 2005), and suggests that all five members of the putative zebrafish PAF1C can potentially physically interact.

To determine if the zebrafish PAF1C homologs physically interacted, we co-expressed different tagged PAF1 components in zebrafish embryos and performed a coimmunoprecipitation assay. We found that Paf1, Cdc73, and Leo1 efficiently coimmunoprecipitated with Ctr9, but that Rtf1 did not, despite significant levels of expressed protein (Figure 2E). This finding is consistent with previous studies showing that while Rtf1 coimmunoprecipitates with other PAF1 components in yeast, its interaction with other PAF1C proteins is comparatively weaker in animals (Adelman et al., 2006; Mueller and Jaehning, 2002; Rozenblatt-Rosen et al., 2005; Yart et al., 2005). This implies that the PAF1C in zebrafish is similar to that of other animals, consisting of at a minimum Ctr9, Paf1, Cdc73, and Leo1, and that the interaction of Rtf1 with this complex may be too weak to detect in a standard coimmunoprecipitation assay.

The PAF1C is required for multiple steps of cardiac formation in zebrafish

In addition to the *ctr9* mutation, we also recently identified a mutation in the zebrafish *leo1* homolog. Interestingly, the specification of cardiomyocytes and heart tube elongation are normal in *leo1* mutants (Nguyen et al., 2010), indicating that loss of different members of the PAF1C can produce different effects on the process of cardiac development.

Because of the disparate roles played by Ctr9 and Leo1 during early cardiac development, we investigated the functions of the zebrafish homologs of the remaining three PAF1C components, Cdc73, Paf1, and Rtf1. We generated morpholino oligonucleotides targeting the translation start site of each gene and confirmed their efficacy by Western blotting (Supplemental Figure 3). Using these morpholinos, we found that knockdown of *cdc73*, *paf1*, and *rtf1* resulted in a reduction in cardiac tissue like that observed in *ctr9* mutants and morphants (Figure 3A–E). The loss of cardiac tissue was most severe in *rtf1* morphants,

with most of these embryos completely lacking cardiomyocytes (Figure 3E). The reduction in cardiac cell number in *ctr9*, *cdc73*, *paf1*, and *rtf1* morphants suggests that the PAF1C plays a critical role in the production of an appropriate number of cardiomyocytes.

Although early cardiac development proceeds normally in *leo1* mutants, our lab has previously identified a role for *leo1* in the differentiation of the atrioventricular (AV) boundary. Loss of *leo1* results in a failure to initiate expression of an AV boundary genetic program including *bmp4*, *versican*, *notch1b*, and *wnt2bb* (Nguyen et al., 2010). We examined the differentiation of the AV boundary in embryos deficient in the other PAF1C components to determine if the PAF1C as a whole plays a role in this developmental process. By 2 days post fertilization (dpf), *rtf1* morphants have no detectable cardiac tissue (data not shown) and *ctr9*-, *cdc73*-, and *paf1*-deficient embryos have small, dysmorphic hearts. Like wild type embryos, *ctr9*-, *cdc73*-, and *paf1*-deficient embryos generate a morphologically distinct ventricle and atrium separated by an AV canal and express *vmhc* and *amhc* in a chamber-specific manner (Figure 4A–H). However, the expression patterns of markers for the AV boundary are highly abnormal in these embryos. In 2-day-old wild type embryos, *bmp4* and *tbx2b* are strongly expressed in the myocardial cells at the AV boundary and sinus venosus (Figure 4I, M). In *ctr9*-, *cdc73*-, and *paf1*-deficient embryos, expression of *bmp4* is not concentrated at the AV boundary (Figure 4J–L) and *tbx2b* is expressed throughout the heart (Figure 4N–P), suggesting that the differentiation of the AV boundary is aberrant in these embryos. In addition, we found that the cardiac expression of *notch1b*, which is strongly localized to the endocardial cells of the AV boundary in wild type embryos, was absent in the hearts of *ctr9*-, *cdc73*-, and *paf1*-deficient embryos (Figure 4Q–T).

Taken together, our data show that all PAF1C components, except for Leo1, are required for establishing appropriate numbers of cardiomyocytes. This reduction in cardiac cell number has the potential to induce secondary defects in later aspects of cardiac development, like the AV boundary gene expression defects present in *ctr9*-, *cdc73*-, and *paf1*-deficient embryos. However, the AV boundary defects in these morphants are very similar to those observed in *leo1* mutants (Nguyen et al., 2010), which have a normal number of cardiac cells, suggesting that all members of the zebrafish PAF1C may play an essential and specific role in the differentiation of the AV boundary myocardium and endocardium.

Loss of PAF1C activity causes defective production and migration of cardiac progenitors

To better understand the role of the PAF1C in early cardiac development, we examined the specification of myocardial precursor cells in the LPM during somitogenesis. We analyzed the expression of *nkx2.5*, a transcription factor involved in regulating the initiation of the cardiogenic differentiation program (Chen and Fishman, 1996), which marks a subset of myocardial progenitors (Schoenebeck et al., 2007).

In wild type embryos, the myocardial precursors are situated in two bilateral groups at the 16S stage (Figure 3F). By the 20S stage, the myocardial cells in wild type embryos have completed their migration to the midline and fused to form the cardiac cone (Figure 3K). In *ctr9* and *paf1* morphants, the expression of *nkx2.5* at the 16S stage is slightly reduced compared to wild type embryos while the expression of *nkx2.5* in *cdc73* morphants is relatively normal (Figure 3F–I). Embryos lacking activity of *ctr9*, *paf1*, and *cdc73* have reduced or slightly reduced *nkx2.5* expression at the 20S stage, and the myocardial precursors in these embryos have failed to fully migrate to the midline and fuse to form a cardiac cone (Figure 3L–N). Despite this delay in migration and fusion, cardiomyocytes in *ctr9*-, *cdc73*-, and *paf1*-deficient embryos do reach the midline by 24 hpf (Figure 3B–D). Remarkably, we found that expression of *nkx2.5* is barely detectable in *rtf1* morphants throughout somitogenesis (Figure 3J,O). These results suggest that the components of the

PAF1C, and especially Rtf1, are required for the initiation of the cardiogenic program and the timely migration of myocardial precursors to the midline.

The PAF1C regulates the specification of cardiomyocytes from the LPM

Because of the profound reduction in cardiac tissue and myocardial precursors seen in PAF1C morphant embryos, we wondered if the PAF1C was required for the formation or gene expression of the anterior lateral plate mesoderm (ALPM), the embryonic origin of cardiomyocytes. To determine if the ALPM was present in embryos lacking activity of PAF1C members, we analyzed the expression patterns of several ALPM markers. We examined the expression of *hand2*, which is expressed in both anterior and posterior compartments of the LPM, and *gata4* and *gata5*, which are expressed in the ALPM and portions of the endoderm (Angelo et al., 2000; Reiter et al., 1999; Reiter et al., 2001a; Serbedzija et al., 1998; Yelon, 2001; Yelon et al., 2000). In wild type embryos, *hand2*, *gata4*, and *gata5* are expressed in the ALPM at the 16S stage (Figure 5A,D,G). These genes are expressed at similar levels in *ctr9* and *rtf1* morphant embryos (Figure 5B,C,E,F,H,I), suggesting that the PAF1C is not required for the formation of the ALPM.

We also examined the expression pattern of the transcription factor *tbx20* in *ctr9* and *rtf1* morphant embryos. Expression of *tbx20* is detected in the ALPM at early stages, and becomes restricted to cardiac progenitors by the 15S stage (Griffin et al., 2000). At the 16S stage, we found that wild type embryos have strong expression of *tbx20* in cardiac progenitors (Figure 5J), which occupy a domain of the ALPM. In contrast, *ctr9* and *rtf1* morphant embryos have reduced *tbx20* expression (Figure 5K,L), indicating that despite the presence of ALPM, these embryos are defective in the process of cardiomyocyte specification. This is also consistent with the reduction in the expression level of the cardiomyocyte progenitor marker *nkx2.5* we detected in PAF1C-deficient embryos, and suggests that the PAF1C is essential for the specification of cardiac tissue from the ALPM.

To test if the earliest steps in the differentiation of mature cardiomyocytes require PAF1C function, we examined expression of *cmlc2* at the 16S stage in PAF1C component morphants. We found that *cmlc2* expression has initiated in wild type embryos at 16S, but does not initiate in *rtf1* morphants (Supplemental Figure 4K–N). In embryos injected with a combination of *ctr9*, *cdc73*, and *paf1* morpholinos, we noted a significant delay in the initiation of *cmlc2* expression (Supplemental Figure 4E,O). This indicates that in addition to the role of Rtf1 in generating cardiac progenitors, Rtf1 and other PAF1C components (excluding Leo1) are essential for the initial expression of the mature cardiomyocyte marker *cmlc2*. These data suggest that the PAF1C may be essential for two distinct phases of cardiac development: cardiac progenitor cell specification and the differentiation of mature cardiomyocytes.

The role of Rtf1 in cardiac specification is cell autonomous

The heart field is patterned by signals originating both inside the LPM and in neighboring tissues (Chen and Fishman, 2000; Yelon, 2001; Yelon et al., 2002; Zaffran and Frasch, 2002), and therefore the activity of the PAF1C may be required in a cell intrinsic or extrinsic manner for the specification of cardiac cells. To determine in which tissue(s) the activity of the PAF1C is required we transplanted cells from *rtf1* morphant embryos into wild type embryos and vice versa and analyzed the ability of the donor cells to contribute to the myocardium. In order to differentiate between cardiomyocytes descended from donor or host cells, we transplanted cells from Tg(myl7:EGFP) transgenic donors in which cardiomyocytes express GFP (green) into Tg(myl7:mCherry) transgenic host embryos in which cardiomyocytes express the red fluorescent protein mCherry. As a control, we first transplanted cells from wild type donor embryos to wild type recipients. When cells are

transplanted from one wild type embryo to another, they robustly contribute to the heart in 51% (73/144) of recipient embryos (Figure 6A,D). Transplanted wild type cells were frequently found in clusters and were fully functional and integrated into the heart, displaying normal morphology and contractility (Figure 6A and data not shown).

On the contrary, we found that *rtf1* morphant cells did not contribute to the heart at a high frequency when transplanted into wild type hosts. Donor *rtf1* morphant cells were found in only 8% (11/133) of recipient hearts (Figure 6D), and unlike transplanted wild type cells, *rtf1* morphant cardiomyocytes were small and dysmorphic and did not appear to contract (Figure 6B and data not shown). When visualized using confocal microscopy, it is apparent that these morphant cells are only loosely associated with the periphery of the myocardial layer and do not fully integrate into the heart (Supplemental Video 1). While this data shows that the activity of the PAF1C is required within potential myocardial precursors for their entry into the cardiogenic program, its activity may also be required in surrounding tissues. To test this we performed a reverse transplantation experiment, placing cells from a wild type embryo into an *rtf1* morphant host. We found that although *rtf1* morphants lack hearts at 2 dpf, wild type donor cells were able to form myocardium in 44% (68/154) of *rtf1* morphant recipients (Figure 6D). Remarkably, wild type donor cells in 15 of these *rtf1* morphant recipient embryos clustered to form a small beating heart-like structure. Taken together, our transplantation data suggest that PAF1C activity is required cell autonomously for the specification of myocardial progenitors, and that activity of the PAF1C in tissues surrounding the myocardium likely plays a lesser role in cardiac specification.

Discussion

The PAF1C is an RNA polymerase II-associated protein complex consisting of five proteins: Ctr9, Cdc73, Paf1, Rtf1, and Leo1. Genetic studies have revealed a requirement for the PAF1C in multiple biological processes including cell cycle regulation, somite and neural crest development, and maintenance of stem cell pluripotency (Akanuma et al., 2007; Ding et al., 2009; Koch et al., 1999; Nguyen et al., 2010). Here we show that the PAF1C is essential for multiple steps in the development of the heart. All PAF1C proteins are critical for specification of the atrioventricular boundary of the heart, and Ctr9, Cdc73, Paf1, and Rtf1 are required for establishing an appropriate number of cardiomyocyte precursors by promoting the specification of cardiac cells from the ALPM (Figure 7A). By transplanting cells between wild type and *rtf1* morphant embryos, we found that Rtf1 functions in a cell autonomous manner. Transplanted wild type cells were capable of organizing into a beating structure in *rtf1* morphants, whereas *rtf1*-deficient cells fail to incorporate into the myocardial layer when transplanted into wild type hosts. Our findings establish the PAF1C as a key regulator of cardiac cell fate.

We found that PAF1C activity is dispensable for formation of the ALPM, but is required for determining the cardiogenic potential of the ALPM, likely via regulation of the expression of a set of cardiac determinants including *nkx2.5* and *tbx20*. Previous studies in yeast, plants, flies and human cells have indicated critical roles of this complex in chromatin remodeling, transcription elongation and mRNA 3'-end formation (Kim et al., 2010; Krogan et al., 2003; Oh et al., 2004; Penheiter et al., 2005; Tenney et al., 2006; Zhang et al., 2009). Thus the PAF1C may regulate the transcription of target cardiac genes by influencing histone modifications or control the elongation and stability of cardiac gene transcripts. It is not yet known which cardiogenic determinants are directly regulated by PAF1C activity. This study paves the way for future examination of the direct binding and epigenetic effects of the PAF1C at cardiac gene promoters.

The reduction in cardiac cell number varied between *ctr9*, *cdc73*, *paf1*, and *rtf1* morphants, with the most severe defect in cardiomyocyte specification being observed in *rtf1* morphants, which usually produced no cardiomyocytes. Based on Western analysis, Rtf1 protein was almost completely absent in *rtf1* morphants, whereas low levels of targeted proteins still existed in *ctr9*, *cdc73* and *paf1* morphants. This, along with the finding that cardiac progenitor cell specification was sensitive to the dosage of Rtf1 suggest that the variation in cardiac specification defects observed among PAF1C component morphants may reflect different efficacies of the morpholinos used in our study. Creating mutant embryos lacking both maternal and zygotic messages of PAF1C should provide further insights into the effect of complete loss of PAF1C function on cardiac development. Alternatively, the distinct cardiac phenotypes observed among PAF1C component morphants and mutants may be indicative of disparate roles of PAF1C proteins in heart development. Interestingly, simultaneous knockdown of *ctr9*, *paf1*, and *cdc73* with the morpholino dosages used in our study induced a significantly weaker phenotype than that seen in *rtf1* morphants. We have also previously shown that *leo1* mutants generate a normal number of cardiomyocytes (Nguyen et al., 2010). These data argue in favor of the possibility that Rtf1 may play a unique role in cardiac development.

In yeast, Ctr9, Cdc73, Paf1, Rtf1 and Leo1 form a stable complex (Mueller and Jaehning, 2002). Our biochemical study shows that zebrafish Cdc73, Paf1, and Leo1, but not Rtf1, coimmunoprecipitate with Ctr9. While it is possible Rtf1 is not a component of the PAF1C in zebrafish, the abundant phenotypic similarities between *rtf1* morphants and embryos deficient in other PAF1C proteins lead us to hypothesize that Rtf1 is an integral component of the PAF1C but its interaction with other members of PAF1C is too weak to detect by coimmunoprecipitation (Figure 7B). This hypothesis is consistent with earlier reports that Rtf1 fails to coimmunoprecipitate with the *Drosophila* and human PAF1Cs (Adelman et al., 2006; Kim et al., 2010; Rozenblatt-Rosen et al., 2005). Our findings suggest that a looser association between Rtf1 and other PAF1C members is a feature that may be conserved universally among metazoans.

Our analysis of the zebrafish PAF1C reveals that while all complex members are required in the specification of the atrioventricular boundary, individual PAF1C components have disparate roles in early cardiogenesis. Loss of each PAF1C member, excluding Leo1, causes a reduction in the number of cardiomyocytes specified from the LPM (Nguyen et al., 2010). The mechanisms responsible for differing roles of individual PAF1C components are not yet known. One potential explanation is that losing individual PAF1C components may have different effects on overall complex stability. Recent studies indicate that loss of human Paf1 or Ctr9, but not Leo1, destabilizes the interactions between other PAF1C members (Kim et al., 2010). This suggests that Paf1 and Ctr9 may serve as a scaffold for the formation of the PAF1C, and that loss of these proteins disrupts the stability of the complex as a whole. This idea is consistent with the severe defects in cardiogenesis noted in *ctr9* and *paf1* morphants compared to the later, mild cardiac defects noted in *leo1* mutants. Another plausible explanation is that each PAF1C member mediates specific protein-protein interactions. Indeed, individual PAF1C members have been shown to interact with a number of different proteins involved in transcription regulation. For example, yeast Paf1 interacts directly with Bre1, linking the PAF1C to histone ubiquitination machinery (Kim and Roeder, 2009). Yeast Rtf1 directly interacts with the chromatin remodeling protein Chd1 (Simic et al., 2003). In mammalian cells, PAF1C interacts with the Wnt and Hh signaling pathways by the direct association of Cdc73 with beta-catenin and Gli proteins (Mosimann et al., 2006, 2009). Therefore, the specific effects on development we noted upon loss of individual PAF1C components could be a result of loss of interaction with particular genes and signaling pathways important for cardiogenesis. Further studies that map out all the interacting partners and target genes of the PAF1C will be needed to uncover the

mechanisms by which this complex regulates specific signaling pathways and developmental processes in vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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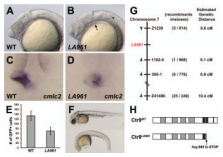


Figure 1. Mutation in zebrafish *ctr9* perturbs primitive heart tube elongation and causes a reduction in cardiomyocyte cell number

(A,B) Lateral views of 24 hpf wild type (A) and *LA961* mutant (B) embryos. *LA961* mutants can be distinguished from wild type embryos based on missing otoliths (arrowhead) and pericardial edema (arrow). (C,D) Wild type and *LA961* mutant embryos analyzed for *cmlc2* expression at 24 hpf. Wild type embryos have an elongated primitive heart tube (C), whereas *LA961* mutants have a clump of cardiomyocytes at the midline (D). (E) Graph of number of GFP-positive cardiomyocytes in wild type (WT) and *LA961* mutant embryos. Error bars indicate standard deviations. (F) Lateral view of wild type (top) and *LA961* mutant (bottom) embryos at 35 hpf. (G) Diagram of *LA961* mapping. Estimated genetic distance is in centimorgans (cM). (H) Diagram of $Ctr9^{WT}$ and $Ctr9^{LA961}$ proteins. Grey regions are predicted tetratricopeptide repeat domains and black regions are predicted nuclear localization domains.

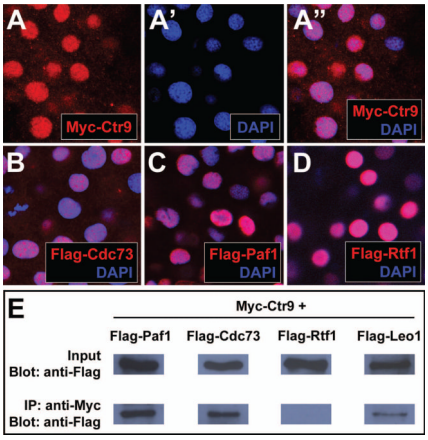


Figure 2. Zebrafish PAF1C components localize to the nucleus and interact with each other *in vivo*

(A-A") Panel (A") shows the colocalization of Myc-tagged Ctr9 protein (red, A) and nuclear DAPI staining (blue, A'). (B) Flag-tagged Cdc73 (red) and DAPI staining (blue) colocalize in the nucleus. (C) Flag-tagged Paf1 (red) and DAPI staining (blue) colocalize in the nucleus. (D) Flag-tagged Rtf1 (red) and DAPI staining (blue) colocalize in the nucleus. (E) Flag-Paf1, Flag-Cdc73, and Flag-Leo1 coimmunoprecipitate with Myc-Ctr9. Input row shows 1 embryo equivalent of total protein lysate. IP row shows immunoprecipitated protein from 20 embryo equivalents of total protein lysate.

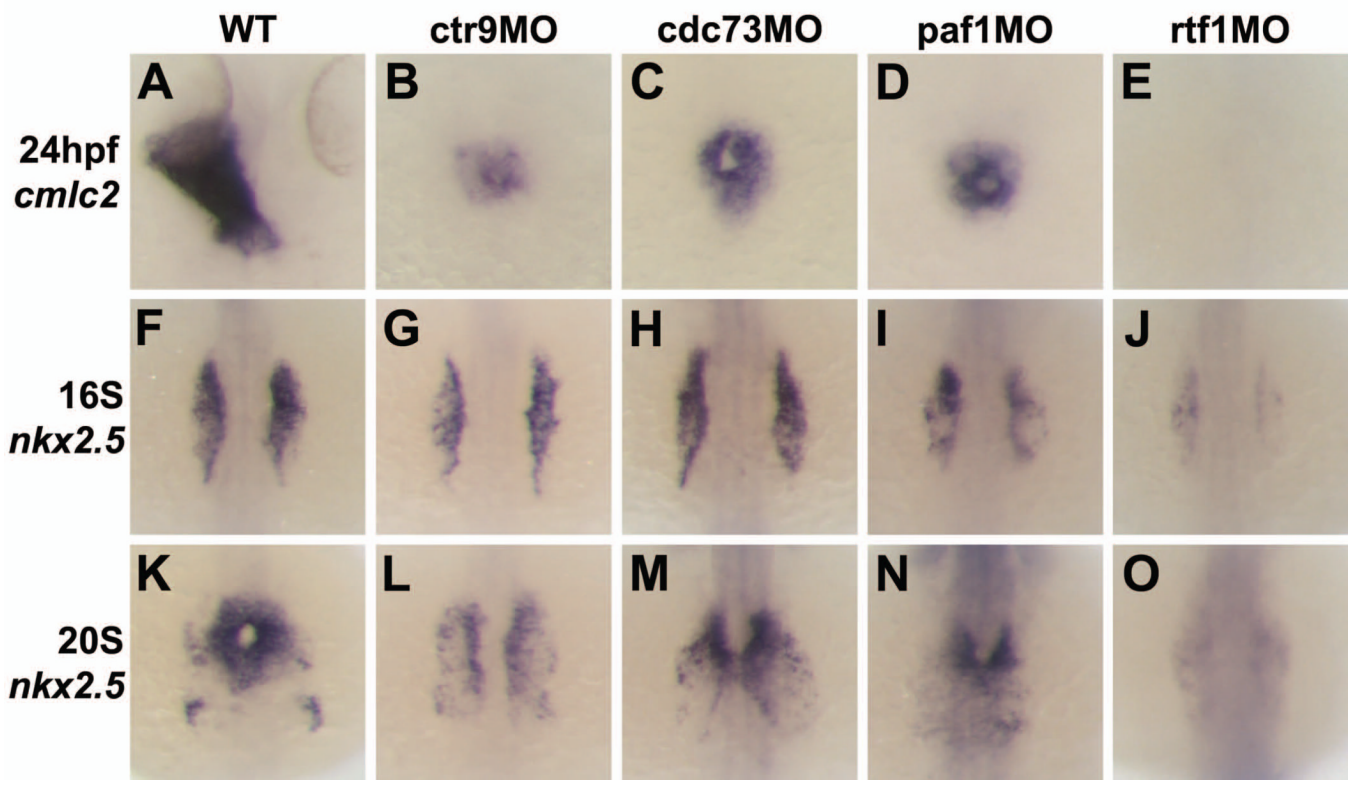


Figure 3. Loss of Ctr9, Cdc73, Paf1, and Rtf1 cause defects in myocardial migration, cardiomyocyte cell number, and primitive heart tube elongation

(A–E) Dorsal views of *cmlc2* expression in 24 hpf wild type and morphant embryos. Wild type embryos have an elongated primitive heart tube at 24 hpf (A), whereas *ctr9* (B), *cdc73* (C), and *paf1* (D) morphants have a small clump of cardiomyocytes situated at the midline. *Rtf1* morphants have no detectable *cmlc2* expression at 24 hpf (E). (F–O) Dorsal views of *nkx2.5* expression in 16S (F–J) and 20S (K–O) stage wild type and morphant embryos. In wild type embryos, myocardial progenitors arise in the LPM, migrate towards the midline, and fuse to form a cardiac cone (F,K). In *ctr9* morphants (G,L), *nkx2.5* expression is reduced and myocardial migration and fusion are delayed. *Cdc73* morphants (H,M) also display delays in the migration and fusion of the myocardial progenitors, but have relatively normal *nkx2.5* expression levels. *Paf1* morphants (I,N) and *rtf1* morphants (J,O), like *ctr9* morphants, display reduced *nkx2.5* expression and delayed migration and fusion of the myocardial progenitors. Expression of *nkx2.5* in *rtf1* morphants is barely detectable.

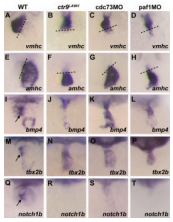


Figure 4. Ctr9, Cdc73, and Paf1 are required for AV boundary specification

(A–D) Cardiac expression of *vmhc* in 48 hpf embryos. In wild type (A), *ctr9*^{LA961} mutant (B), *cdc73* morphant (C), and *paf1* morphant (D) embryos, *vmhc* expression is detected primarily in the ventricle. (E–H) Cardiac expression of *amhc* in 48 hpf embryos. In wild type (E), *ctr9*^{LA961} mutant (F), *cdc73* morphant (G), and *paf1* morphant (H) embryos, *amhc* expression is detected primarily in the atrium. (I–L) Cardiac expression of *bmp4* in 48 hpf embryos. In wild type embryos (I), *bmp4* expression is detected in the myocardium of the ventricle, sinus venosus, and AV boundary (arrow). *Ctr9*^{LA961} mutant (J), *cdc73* morphant (K), and *paf1* morphant (L) embryos display ventricular expression of *bmp4*, but have no discernable AV boundary expression. (M–P) Cardiac expression of *tbx2b* in 48 hpf embryos. In wild type embryos, *tbx2b* is expressed by the myocardium of the AV boundary (arrow, M). *Ctr9*^{LA961} mutant (N), *cdc73* morphant (O), and *paf1* morphant (P) embryos instead aberrantly express *tbx2b* throughout the heart. (Q–T) Cardiac expression of *notch1b* in 48 hpf embryos. *Notch1b* is expressed by the AV boundary endocardium in wild type embryos (arrow, Q), but is not expressed in the hearts of *ctr9*^{LA961} mutants (R), *cdc73* morphants (S), and *paf1* morphants (T).

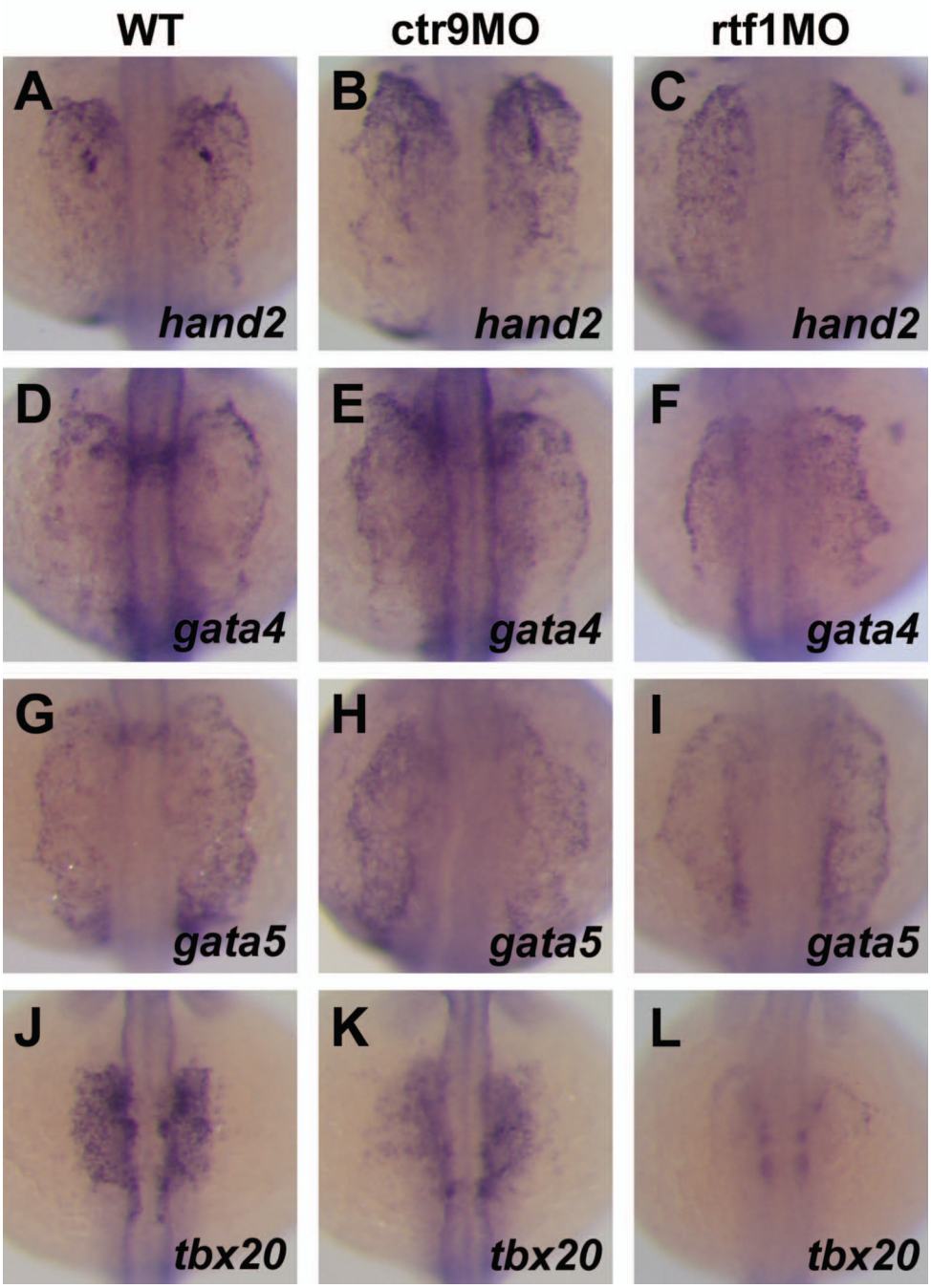
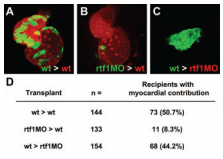


Figure 5. The PAF1C is required for the specification of cardiomyocytes from the anterior lateral plate mesoderm

(A–L) Dorsal views of *hand2* (A–C), *gata4* (D–F), *gata5* (G–I), and *tbx20* (J–L) expression in 16S stage wild type and morphant embryos. In wild type embryos, expression of *hand2* (A), *gata4* (D), and *gata5* (G) is detected in the anterior lateral plate mesoderm. In *ctr9* and *rtf1* morphants, *hand2* (B,C), *gata4* (E,F), and *gata5* (H,I) are expressed in a domain similar to that of wild type embryos. (J–L) In wild type embryos, *tbx20* is expressed by cardiomyocyte progenitors in the ALPM (J). Expression of *tbx20* is reduced in *ctr9* morphants (K) and strongly reduced in *rtf1* morphants (L) at the 16S stage.



D

Transplant	n =	Recipients with myocardial contribution
wt > wt	144	73 (50.7%)
rtf1MO > wt	133	11 (8.3%)
wt > rtf1MO	154	68 (44.2%)

Figure 6. Rtf1 regulates cardiomyocyte specification cell autonomously

(A–C) Confocal projections showing a ventral view of the heart in two day-old transplanted embryos. Wild type donor cells contribute robustly to the heart when transplanted into wild type recipients (A), while *rtf1* morphant cells exhibit limited contribution when transplanted into a wild type recipient (B). Wild type cells are also able to generate myocardial tissue when transplanted into an *rtf1* morphant host. (D) Summary of the results from transplantation experiments showing the contribution of donor cells to the myocardium of recipient embryos.

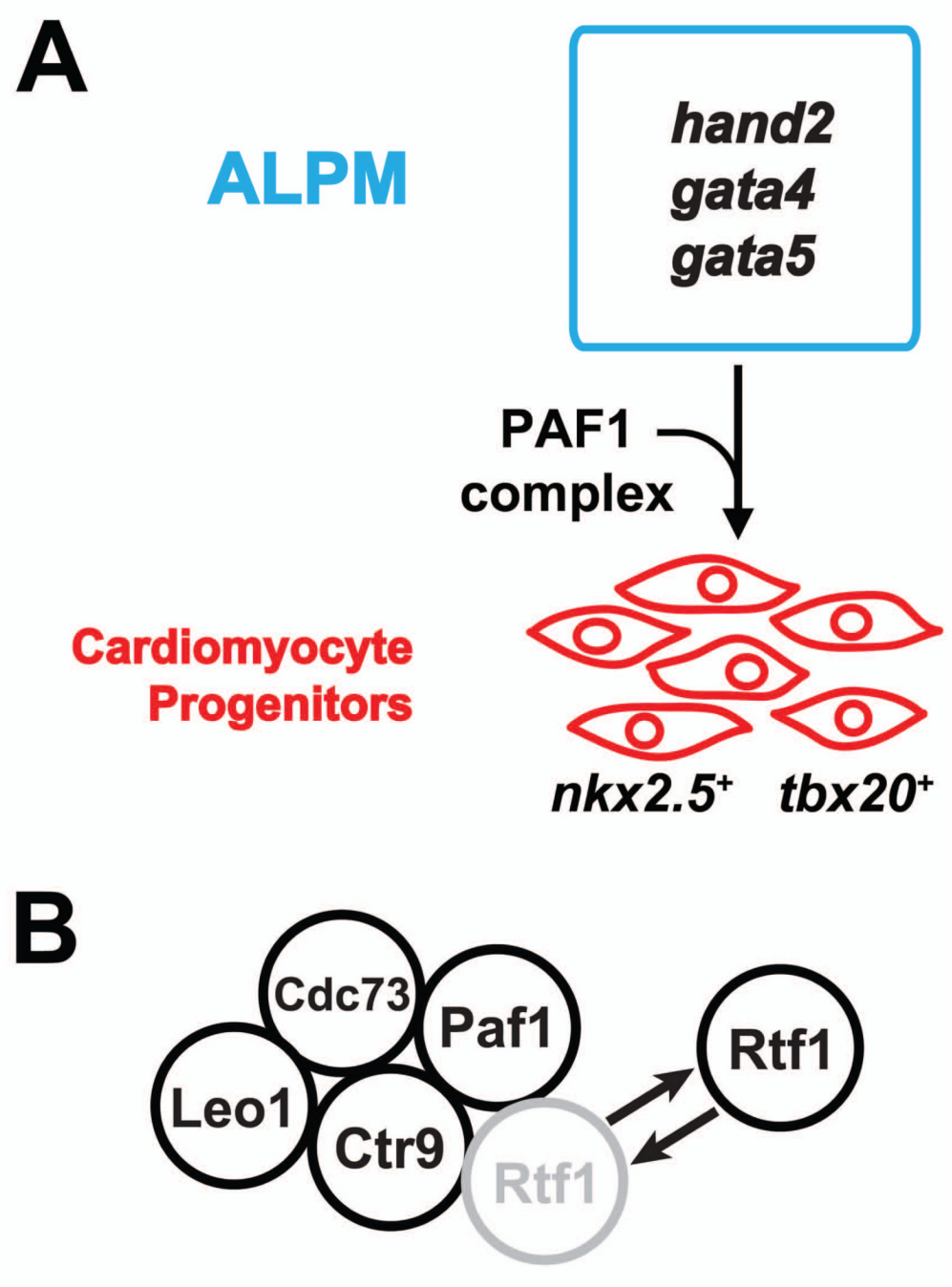


Figure 7. Diagram of zebrafish PAF1C form and function

(A) Cardiomyocyte progenitors (red) are derived from the anterior lateral plate mesoderm (ALPM, blue). The ALPM expresses markers including *hand2*, *gata4*, and *gata5*. The PAF1C is required for the specification of nkx2.5^{+}/tbx20^{+} cardiomyocyte progenitors from the ALPM. (B) The PAF1C in zebrafish consists of at least 4 strongly interacting proteins: Ctr9, Paf1, Leo1, and Ctr9. Based on the phenotypic similarities between embryos lacking Rtf1 and other PAF1C components, we hypothesize that Rtf1 associates with the zebrafish PAF1C, albeit less strongly, as has been suggested in other studies on metazoans.