The PAF1 complex component Leo1 is essential for cardiac and neural crest development in zebrafish

Catherine T. Nguyen a,1, Adam Langenbacher a,1, Michael Hsieh a, Jau-Nian Chen a,b,c,d,*

a Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095, USA
b Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA
c Jonsson Cancer Center, University of California, Los Angeles, CA 90095, USA
d Cardiovascular Research Laboratory, University of California, Los Angeles, CA 90095, USA

ARTICLE INFO

Article history:
Received for publication 7 September 2009
Revised 8 February 2010
Accepted 15 February 2010
Available online 21 February 2010

Keywords:
PAF1 complex
Heart development
Neural crest
Zebrafish

ABSTRACT

Leo1 is a component of the Polymerase-Associated Factor 1 (PAF1) complex, an evolutionarily conserved protein complex involved in gene transcription regulation and chromatin remodeling. The role of leo1 in vertebrate embryogenesis has not previously been examined. Here, we report that zebrafish leo1 encodes a nuclear protein that has a similar molecular structure to Leo1 proteins from other species. From a genetic screen, we identified a zebrafish mutant defective in the leo1 gene. The truncated Leo1LA1186 protein lacks a nuclear localization signal and is distributed mostly in the cytoplasm. Phenotypic analysis showed that while the initial patterning of the primitive heart tube is not affected in leo1LA1186 mutant embryos, the differentiation of cardiomyocytes at the atrioventricular boundary is aberrant, suggesting a requirement for Leo1 in cardiac differentiation. In addition, the expression levels of markers for neural crest-derived cells such as crestin, gch2, dct and mitfa are greatly reduced in leo1LA1186 mutants, indicating a requirement for Leo1 in maintaining the neural crest population. Consistent with this finding, melanocyte and xanthophore populations are severely reduced, craniofacial cartilage is barely detectable, and mbp-positive glial cells are absent in leo1LA1186 mutants after three days of development. Taken together, these results provide the first genetic evidence of the requirement for Leo1 in the development of the heart and neural crest cell populations.

© 2010 Elsevier Inc. All rights reserved.

Introduction

Embryonic developmental processes are controlled spatially and temporally by precise genetic programs. In addition to tissue-specific transcription factors, tightly regulated transcriptional controls involving the recruitment of RNA polymerase II-associated initiation and elongation complexes are required for the initiation of mRNA synthesis and the stability of nascent transcripts. The subsequent recruitment of other RNA polymerase II-associated complexes such as the Srb-Mediator complex or Polymerase-Associated Factor (PAF1) complex promotes chromatin remodeling which further affects transcription efficiency.

While the roles of tissue-specific transcription factors required for regulating cardiac development have been studied extensively, mechanisms by which global transcription regulatory complexes and chromatin remodeling proteins influence cardiac differentiation are just being discovered. The important roles of general transcription complex components in cardiac development have been exemplified by studies of the zebrafish mutants pandora (pan/spt6) and foggy (fog/spt5). Spt6 and Spt5 are elongation factors associated with RNA Polymerase II following the initiation of transcriptional elongation. Both pan and fog mutant embryos display normal expression of the early cardiac markers gata4 and nkd2.5, but terminal differentiation of myocardial precursors is severely affected (Yelon et al., 1999; Keegan et al., 2002), demonstrating a critical regulatory role for general transcription factors in cardiac development.

The PAF1 complex consists of five evolutionarily conserved proteins: Paf1, Ctr9, Leo1, Rtf1, and Cdc73 (Mueller and Jaehning, 2002; Rozenblatt-Rosen et al., 2005; Adelman et al., 2006). The PAF1 complex has been shown to associate with RNA polymerase II, transcription elongation factors and chromatin remodeling proteins (Shi et al., 1996, 1997; Mueller and Jaehning, 2002; Krogan et al., 2003; Simic et al., 2003), but the precise requirements of individual PAF1 complex components in development are just being evaluated. In yeast, mutants deficient in members of the PAF1 complex exhibit pleiotropic phenotypes and changes in a subset of transcripts, indicative of general transcription defects (Betz et al., 2002; Mueller and Jaehning, 2002). Loss of function of Arabidopsis homologs of Paf1 and Ctr9 results in an early flowering phenotype due to a reduction in histone H3 methylation (He et al., 2004; Oh et al., 2004). In Drosophila, RNAi-mediated knockdown of Rtf1 enhances the wing phenotype in...
a Notch hypomorphic background (Tenney et al., 2006) and aberrant expression levels of Cdc73 homologues modulate Wnt and Hh signaling (Mosimann et al., 2006, 2009). Similarly, loss of function of RT1 and Cr9 in zebrafish results in a host of developmental defects including cardiogenesis abnormalities, a reduction of the neural crest population, and reduced Notch signaling in the developing somites (Akanuma et al., 2007). Furthermore, patients carrying mutations in the human CDC73 homologue (the Hyperparathyroidism-jaw tumor syndrome tumor suppressor gene HRPT2) suffer from parathyroid tumors and kidney cysts among other defects (Carpten et al., 2002). Whether mutations in other components of the PAF1 complex are causative to other congenital defects or diseases is not yet known.

Unlike other PAF1 complex proteins, loss of Leo1 function in yeast causes no obvious phenotypes (Magdolen et al., 1994; Mueller and Jaehning, 2002), and the requirements for Leo1 in vertebrate development have not previously been investigated. Here we report the isolation of a zebrafish leo1 mutant from a genetic screen designed to identify genes critical for heart development. The leo1LA1186 mutant embryos have dysmorphic hearts and severely reduced blood circulation due to a differentiation defect in cardiomyocytes particularly in embryos have dysmorphic hearts and severely reduced blood circulation due to a differentiation defect in cardiomyocytes particularly in embryogenesis. Zebrafish colonies were cared for and bred under standard conditions and developmental stages of zebrafish were determined using standard morphological features of zebrafish.

**Materials and methods**

**Zebrafish husbandry and ENU mutagenesis**

Male fish of the Tg(kdr:GFP)LA116 line were mutagenized with ENU as previously described (Mullins et al., 1994; Solnica-Krezel et al., 1994; Choi et al., 2007). LA1186 mutants were identified based on their cardiac defects from a screen that surveyed 900 mutagenized genomes. Zebrafish colonies were cared for and bred under standard conditions and developmental stages of zebrafish embryos were determined using standard morphological features of fish raised at 28.5 °C (Westermfield, 2000).

**Positional cloning**

LA1186 heterozygotes were crossed to the polymorphic WIK strain to generate a hybrid line for mapping. Embryos used for mapping were lysed in embryo lysis buffer (10 mM Tris pH 8.0, 2 mM EDTA, 0.2% Triton X-100, 100 µg/ml Proteinase K) at 55 °C overnight to obtain genomic DNA. Genomic DNA from 24 embryos (wild-type or mutant) was pooled and used for bulk segregant analysis with a panel of 200 microsatellite markers designed at the Cardiovascular Research Center of the Massachusetts General Hospital (Michelmore et al., 1991; Mably et al., 2003). Primer sequences of the custom markers 18-839-3 and 18-189-5 are: 18-839-3-F, 5′-TACCAACTGAGGCGCCATTAC; 18-839-3-R, 5′-ACTTGGTGAGGGATGGATGCT; 18-189-5-F, 5′-CCGACATTTTGTTCACATCTAG; and 18-189-5-R, 5′-CTGACGCGAAATAAATCGTGT.

Total RNA was isolated from day post fertilization (dpf) LA1186 mutants and their wild-type siblings using RNA Wiz (Ambion) and cDNA was synthesized using the Superscript II Kit (Invitrogen). cDNA fragments were amplified with Phusion polymerase (Finnzymes) and cloned into pCR-Blunt II-TOPO (Invitrogen) for sequencing.

**Con structs and injections**

The leo1 cDNA constructs were amplified from 1 dpf wild-type embryo cDNA using Phusion polymerase (Finnzymes) and cloned into pCS2+3XFLAG for tagging with the FLAG epitope. The plasmids were cut with NotI and SP6 RNA polymerase was used to generate mRNA for injection. For rescue experiments, embryos from a cross of LA1186/+ fish were injected with 75 pg leo1 mRNA or leo1LA1186 mRNA at the one-cell stage.

**Histology**

Fixed embryos were dehydrated, embedded in plastic blocks [JB-4, Polysciences], sectioned at 10 µm and stained with 0.1% methylene blue as previously described (Chen and Fishman, 1996).

**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 (Westermfield, 2000). Whole mount in situ hybridization was performed as described previously (Chen and Fishman, 1996). The antisense RNA probes used in this study include leo1, nkd2.5, cmic2 (myf7), vmhc, amhc (myh6), bmp4, notch1b, wnt2b, versican (vcana), crestin, mbp, snai1b, sox9b, foxd3, sox10, dct, gch2, mitfa, tbx1, endothelin-1, flk2, paw2, and hand2. Wnt2b was amplified from 2 dpf wild-type embryo cDNA by PCR with Phusion polymerase (Finnzymes) and cloned into pCR-Blunt II-TOPO (Invitrogen). The plasmid was linearized with NotI and SP6 RNA polymerase was used to generate antisense riboprobe.

**Antibody staining**

Embryos injected with RNA encoding FLAG-tagged zebrafish leo1 (100 pg) were fixed in 4% PFA in PBS at 75% epiboly. The fixed embryos were incubated in primary antibody (1:50 mouse anti-FLAG M2 (Sigma)) in blocking solution (10% goat serum in PBDT) for 2 h at room temperature followed by detection with fluorescent secondary antibody (1:200 anti-mouse IgG1-R (Santa Cruz Biotechnology)). Stained embryos were embedded in 1% low-melt agarose and imaged on a Zeiss LSM510 confocal microscope equipped with a 63× water objective.

**Alcian blue staining**

Embryos were fixed in 4% PFA in PBST after three days of development. Staining was carried out as previously described (Golling et al., 2002). In brief, embryos were stained with 0.1% Alcian blue solution dissolved in 50% ethanol/0.1 M HCL for 2 h. After staining, embryos were rinsed in ethanol and rehydrated gradually into PBST, before being transferred into 0.05% trypsin solution dissolved in saturated sodium tetraborate for 3 h. Embryos were then bleached in 1% KOH/3% H2O2 for 1 h.

**Results**

To identify genes critical for cardiac development, we conducted an ethynylsourousose mutagenesis screen. From this screen we found a mutant, designated LA1186, that exhibits a dysmorphic and dysfunctional heart. The primitive heart tube forms normally in LA1186 mutants and the expression of multiple early cardiac markers, including nkd2.5 and cmic2, are indistinguishable between LA1186 mutant embryos and their wild-type siblings (Figs. 1D–G). However, by 30 h post fertilization (hpf), LA1186 hearts exhibit reduced contractility. As a result, a cardiac edema occurs and the outflow tract, sinus venosus, and ventricle collapse by 2 days post fertilization (dpf) (Fig. 11). These findings suggest that LA1186 is required for maintaining the proper function and morphology of the embryonic heart, but is not involved in the initial steps of cardiac specification or heart tube elongation.
During the segmentation stage, mutant observed in responsible for the cardiac, pigmentation and craniofacial defects of restricted to the eyes, brain, pharyngeal arches, and anterior somites by range of tissues (Figs. 3B,C,E,F). The expression of (Fig. 2B), suggesting that the nonsense mutation in that produces a premature stop codon in good candidate gene for LA1186. An early hybridization analysis as early as the 6-somite (S) stage. We first step toward identifying the molecular lesion of LA1186 encodes Leo1, a PAF1 complex protein. LA1186 encodes Leo1, a PAF1 complex protein.

As the first step toward identifying the molecular lesion of LA1186, we mapped LA1186 to zebrafish linkage group 18 between marker z8343 and the custom marker 18-189-5. We then generated additional custom markers in this region and detected no recombination between 18LA1186 and 18-839-3 (Fig. 2A). The leo1 gene is in close proximity to 18LA1186, making it a good candidate gene for LA1186. We amplified the coding region of leo1 from LA1186 mutant embryos and their wild-type siblings. Sequencing analysis revealed a C to T transition at nucleotide 1744 that produces a premature stop codon in LA1186 mutant embryos (Fig. 2B), suggesting that the nonsense mutation in leo1 may be responsible for the cardiac, pigmentation and craniofacial defects observed in LA1186.

Transcripts of leo1 can be detected in wild-type embryos by whole mount in situ hybridization as early as the 6-somite (S) stage. During the segmentation stage, leo1 transcripts are present in a wide range of tissues (Figs. 3B,C,E,F). The expression of leo1 becomes restricted to the eyes, brain, pharyngeal arches, and anterior somites by 24 hpf, and the transcripts of leo1 are no longer detectable by in situ hybridization in zebrafish embryos by 48 hpf (Figs. 3G,H and data not shown). No significant reduction of leo1 signal was noted in LA1186 mutant embryos (data not shown), suggesting that leo1LA1186 transcripts are not subject to nonsense-mediated decay. Leo1 is a component of the PAF1 complex, which is associated with RNA polymerase II in the nucleus and has an important role in transcription regulation (Betz et al., 2002; Mueller and Jaehning, 2002). The premature stop codon in LA1186 truncates Leo1 prior to its putative nuclear localization sequence and may thereby disturb nuclear localization of Leo1LA1186. To examine the subcellular localization of Leo1, we tagged Leo1wt and Leo1LA1186 with FLAG epitopes and injected these constructs into wild-type embryos at the 1-cell stage. As expected, FLAG-Leo1wt is localized to the nucleus, while FLAG-Leo1LA1186 is present predominantly in the cytoplasm (Figs. 2D–G). Given the previously described role of the PAF1 complex during transcription, the inability of Leo1LA1186 to localize to the nucleus would likely impair the function of the Leo1 mutant protein. To further evaluate the causative relationship between loss of Leo1 activity and the LA1186 phenotypes, we injected leo1 mRNA into one-cell stage embryos collected from a cross of LA1186 heterozygotes. All embryos from the LA1186 heterozygous cross that were injected with 75 pg of leo1 mRNA had normal jaw and cardiac morphology and normal levels of pigmentation (n = 180) (Fig. 1C), even though genotyping these embryos with marker 18-939-3 indicated that one quarter were homozygous for LA1186. On the contrary, injection of leo1LA1186 mRNA to embryos collected from a LA1186 heterozygous cross was unable to rescue LA1186 mutant phenotypes (data not shown). These findings indicate that the Leo1LA1186 protein is not functional and demonstrate that wild-type leo1 mRNA is sufficient to rescue both cardiac and pigmentation phenotypes in LA1186 mutant embryos. Furthermore, overexpression of leo1LA1186 transcript in wild-type or LA1186 heterozygotes did not result in any noticeable morphological defects (data not shown), suggesting that Leo1LA1186 is not acting as a dominant-negative protein in mutant embryos.
Zebrafish Leo1 is a 696 amino acid protein that shares a high level of similarity to yeast Leo1 (55%) and other vertebrate Leo1 proteins (>70%) (Supplementary Fig. 2). As with all the Leo1 proteins analyzed, the zebrafish Leo1 protein contains a unique Leo1 domain and a putative nuclear localization sequence. In addition, zebrafish Leo1 contains an aspartic acid-rich C-terminal region that is not present in the yeast, mouse and human Leo1 proteins (Fig. 2C and Supplementary Fig. 2). To determine whether this C-terminal tail was...
essential for Leo1 function, we generated a truncated form of the zebrafish Leo1 protein lacking sequences after amino acid 647. We injected the transcripts generated from this truncated Leo1 construct into embryos collected from an LA1186 heterozygous cross and found that the truncated protein was able to rescue the LA1186 mutant phenotype \((n = 69)\), suggesting that the aspartic acid-rich tail is not necessary for Leo1 activity.

**Leo1 is required for the differentiation of the atrioventricular boundary of the heart**

LA1186 mutant hearts are unable to establish proper circulation, which is often associated with abnormal morphology and cardiac gene expression in zebrafish. Indeed, the sinus venosus, outflow tract, and ventricle are collapsed in 2 dpf leo1LA1186 mutants (Fig. 1I). We utilized several cardiac markers to assess whether leo1LA1186 mutant hearts differentiate properly. We found that while the atrial marker, amhc, is properly expressed in the atrium, the expression domain of the ventricular marker, vmhc, is expanded to the atrium (Figs. 4A–D), suggesting a defect in the regionalization of cardiac chamber-specific genes. The differentiation defects of the cardiomyocytes are most prominent at the atrioventricular (AV) boundary. In wild-type hearts, expression of bmp4, versican and wnt2bb is restricted to myocardial cells at the AV boundary after two days of development. In leo1 mutant hearts, the expression levels of bmp4, versican and wnt2bb are greatly reduced and the expression domains of bmp4 and versican are no longer restricted to the AV boundary (Figs. 4G–L). Furthermore, expression of notch1b in endocardial cells at the AV boundary is absent in leo1 mutant hearts (Fig. 4F). Taken together, these data indicate an important role for Leo1 in cardiac differentiation, especially for those cells located at the AV boundary.

**Leo1 is required for the development of neural crest cells**

Leo1 mutant embryos exhibit a severe reduction in both the melanophore and xanthophore pigment cell populations after two days of development (Fig. 1B). To further investigate the pigmentation defects in leo1 embryos, we examined the expression of gch2, dct, and mitfa (markers for xanthophores (Knight et al., 2004), melanophores (Lamason et al., 2005) and early melanoblasts (Hong et al., 2005), respectively), at 1 dpf. Consistent with the loss of melanophores and xanthophores in 2-day-old leo1LA1186 mutant embryos, we found that the expression levels of these markers were greatly reduced in leo1LA1186 mutant embryos after one day of development, suggesting that Leo1 may regulate the differentiation of pigment cells (Fig. 5).

In addition, after three days of development defects in craniofacial formation become apparent in leo1LA1186 mutant embryos. Staining

---

**Fig. 4.** Cardiac differentiation phenotypes of leo1LA1186. Normally, vmhc expression is restricted in the ventricle in wild-type embryos (A), but is expanded into the atrium in leo1LA1186 mutants (B). Expression of amhc, however, is restricted properly to the atrial chamber in both wild-type (C) and leo1LA1186 mutant embryos (D). Notch1b transcripts are detected in the endocardium at the AV boundary of wild-type embryos (E), but are absent in leo1LA1186 mutants (F). Expression of bmp4 (G), versican (I), and wnt2bb (K) are detected in the myocardium of the AV boundary in wild-type embryos. Both bmp4 (H) and versican (J) transcripts are no longer restricted in leo1LA1186 mutants, while wnt2bb is not detected in mutant embryos (L). Dashed lines (in A–D) and arrows (in E, G, I, K) mark the boundary of atrium and ventricle.
with Alcian blue showed that the ethmoid plate and all pharyngeal arches are completely absent and only remnants of trabeculae cranii remain in 3-day-old leo1LA1186 mutant embryos (Figs. 6A–D). By 5 dpf, a very reduced craniofacial skeleton has formed in leo1LA1186 mutants (Supplementary Fig. 3). However, the expression of tbx1 and endothelin-1, markers for the pharyngeal arch epithelium and mesodermal core (Piotrowski et al., 2003), are unaffected in leo1 mutant embryos (Figs. 6E–H). These findings indicate that Leo1 activity is not required for the formation of the epithelium or the mesodermal core of the pharyngeal arches, but is required for the differentiation of an appropriate number of craniofacial chondrocytes, or the proliferation or survival of these cells.

Melanophores, xanthophores and the cartilaginous component of the craniofacial skeleton are derived from neural crest cells (Kelsh et al., 1996; Kimmel et al., 2001; Barrallo-Gimeno et al., 2004; Montero-Balaguer et al., 2006). Another cell type that neural crest cells are known to contribute to in zebrafish are glia of the peripheral nervous system (PNS) (Kelsh et al., 2000). To determine if Leo1 is also required for PNS glial cell development, we examined the expression of the glial marker myelin basic protein (mbp) in wild-type and leo1LA1186 mutant embryos. Wild-type embryos display mbp expression in cranial glia of the CNS and the Schwann cells of the trunk. Interestingly, no mbp positive cells were detected in leo1LA1186 mutants after 3 and 5 days of development, indicating a complete loss of both cranial and trunk glia (Supplementary Fig. 4). The loss of peripheral glia (Schwann cells) is consistent with the notion that the development of neural crest-derived tissues is defective in leo1LA1186 mutants and the loss of CNS glia suggests that Leo1 may play a role in the development of glia in addition to its function in neural crest development.

The loss of multiple neural crest-derived cell types in leo1LA1186 mutant embryos suggests that Leo1 may be required for neural crest specification and/or differentiation. To determine if Leo1 is required for the initial specification of the neural crest cell population, we evaluated the expression pattern of early neural crest markers. We found that the expression patterns of pre-migratory neural crest markers (sox9b, sox10, foxd3, and snai1b; (Barrallo-Gimeno et al., 2004; Hong et al., 2005; Yan et al., 2005)) are indistinguishable between wild-type and leo1LA1186 embryos at the 10S stage, indicating that neural crest cells are properly specified in leo1LA1186 mutant embryos (Supplementary Fig. 5). Furthermore, we detected no significant changes in the expression of the pharyngeal arch neural crest markers dlx2 (Figs. 7A,B) and hand2 (data not shown) in leo1LA1186 mutant embryos at the 18S stage, suggesting that the reduction of the craniofacial skeleton is not due to an absence of neural crest progenitors, but rather the inability of a sufficient number of these cells to develop into craniofacial chondrocytes (Hong et al., 2005). Interestingly, at both the 12S stage and 24 hpf, the expression level of the pan-neural crest marker crestin is reduced in leo1LA1186 mutant embryos (Figs. 7C–J). We also noted that the loss of crestin expression is most severe in the cranial neural crest region, the population of neural crest cells anterior to the otic vesicle. Furthermore, we could barely detect any crestin-positive cells in the cranial neural crest region at the 12S stage and only residual crestin-positive
**Fig. 6.** Craniofacial phenotypes of leo\(^{1A1186}\). (A, B, C, D) Alcian blue staining of wild-type (A, C) and leo\(^{1A1186}\) embryos (B, D) at 3 days post fertilization. Leo\(^{1A1186}\) exhibit severe craniofacial defects including absence of the ethmoid plate from the anterior neurocranium and complete loss of pharyngeal arches. (A, B) Lateral view. (C, D) Ventral view. ANC, anterior neurocranium; t, trabecular cranii; m, mandibular arch; h, hyoid arch; P3–7, pharyngeal arches 3–7. (E, F, G, H) Tbx1 and endothelin-1 (edn-1) expression patterns in the pharyngeal arch epithelium and mesenchymal core are indistinguishable between wild-type embryos (E, G) and leo\(^{1A1186}\) mutants (F, H) at 27 hpf.

**Fig. 7.** Development of neural crest cells is dependent upon Leo1 function. (A, B) Dorsal anterior view of dix2 expression in pharyngeal arch neural crest at the 18S stage. The expression pattern of dix2 is normal in wild-type (A) and leo\(^{1A1186}\) embryos (B). (C–J) Pan-neural crest marker, crestin, at the 12 somite stage (C–F) and 24hpf (G–J). Crestin expression is detected in anterior and trunk neural crest of wild-type embryos (C, E, G, I), but is severely reduced in the region anterior to the otic vesicle of leo\(^{1A1186}\) mutants (D, F, H, J). (C, D, G, H) Lateral view. (E, F, I, J) Dorsal anterior view.
cells are detected in this region at 24 hpf ([Fig. 7]). Taken together, these data suggest that Leo1 is not required for the initial specification of neural crest cells, but is required for their subsequent development into derivatives such as pigment cells and craniofacial cartilage.

Discussion

From a forward genetic screen, we isolated the zebrafish LAI186 mutant exhibiting abnormalities in cardiac development as well as craniofacial defects and a reduction in pigmentation. Subsequent molecular cloning showed that the LAI186 locus encodes Leo1, a component of the PAF1 complex. Consistent with previous findings that the PAF1 complex is associated with RNA polymerase II and plays a role in chromatin remodeling (Shi et al., 1996; 1997; Mueller and Jaenicho, 2002; Krogan et al., 2003), the Leo1 protein is localized to the nucleus, and this nuclear localization is required for its biological function. The requirements for Leo1 in the development of multi-cellular organisms, however, have not been previously examined. Our study shows that the leo1LA1186 mutation is embryonic lethal, indicating that Leo1 is an essential gene for vertebrates. Our phenotypic analysis of leo1LA1186 mutant embryos further reveals defects in the differentiation of the heart, especially at the AV boundary, and the development of neural crest-derived tissues, providing the first genetic evidence for the requirements of Leo1 in vertebrate development.

The PAF1 complex is an evolutionarily conserved protein complex. A large number of biochemical studies in yeast and cultured mammalian cells have shown that the PAF1 complex consists of Paf1, Ctr9, Rtf1, Cdc73 and Leo1(Mueller and Jaenicho, 2002; Rozenblatt-Rosen et al., 2005; Adelman et al., 2006). In zebrafish, embryos lacking the activity of Ctr9 or Rtf1 have defects in a wide range of tissues including the heart and neural crest cells (Akanuma et al., 2007). The similarity of the defects in heart development and neural crest-derived tissues among leo1, rtf1 and ctr9 mutants and morphants is in agreement with the fact that these proteins are members of the same protein complex. However, while the cardiac and neural crest phenotypes of ctr9 and rtf1 mutants are highly similar (Akanuma et al., 2007), the effect of loss of leo1 function is significantly milder, suggesting that each component of the PAF1 complex may be differentially required for various biological processes. This notion is further supported by the findings that embryos deficient in Ctr9 and Rtf1 are small and have pleiotropic defects. It has been carefully documented that both ctr9 morphants and rtf1 mutants are defective in somite and otic vesicle formation in addition to the aforementioned cardiac and neural crest abnormalities (Akanuma et al., 2007). The leo1LA1186 mutant embryos, on the contrary, exhibit normal body size and have defects that are restricted to the heart and some neural crest-derived tissues. Future studies on the precise requirements of each component of the PAF1 complex would provide insights into mechanisms by which the PAF1 complex regulates vertebrate development.

Leo1 mutant embryos have a severe reduction of craniofacial cartilage, melanophores, and xanthophores indicating a requirement for Leo1 activity in neural crest-derived tissues. The expression patterns of all early neural crest markers analyzed are normal in leo1LA1186 mutant embryos suggesting that the specification of neural crest cells is not affected. However, the severe decrease in the expression of dct, gch and mtfu in leo1LA1186 mutants suggests that Leo1 may be important for the survival or differentiation of neural crest cells. The loss of function of Leo1 affects a subset of anterior neural crest cells most severely. When analyzing the expression of crestin, a member of a family of retroelements that is expressed in neural crest cells in zebrafish (Rubinstein et al., 2000), we found that while the overall crestin expression level is reduced in leo1LA1186 mutant embryos, crestin expression is barely detectable in the region anterior to the otic vesicle. This observation is consistent with the severe loss of craniofacial cartilage observed in leo1LA1186 mutant embryos. Interestingly, the expression of other neural crest markers, such as dbx2 and hand2, is indistinguishable between leo1LA1186 mutant embryos and their wild-type siblings suggesting that the neural crest population does exist in the anterior region of leo1 mutant embryos. It is possible that Leo1 directly regulates the expression of crestin and other, as of yet unknown, genes that are critical for the development of neural crest-derived cell types, especially in the population of neural crest cells located anterior to the otic vesicle. Alternatively, the anterior neural crest might consist of crestin-positive and crestin-negative populations, with the formation of the crestin-positive neural crest cell population requiring Leo1 activity. Furthermore, if two distinct neural crest cell populations exist, our finding that the craniofacial cartilage formation is severely defective even though a substantial amount of dbx2 and hand2-positive, crestin-negative neural crest cells remain in leo1LA1186 mutant embryos would suggest that crestin-positive neural crest cells are major contributors to the cartilage component of the craniofacial skeleton.

Leo1 is also required for proper cell differentiation in the heart. The initial patterning of the primitive heart tube appears normal in leo1LA1186 mutant embryos as evidenced by their normal morphology and the proper expression pattern of multiple cardiac markers analyzed. However, the heart begins to show signs of dysmorphism and dysfunction after 30 hpf. Genes that are normally expressed in cardiomyocytes at the AV boundary, such as bmp4 and versican fail to be properly regionalized and the expression of wnt2bb is absent in leo1LA1186 mutant hearts, indicating an essential role for Leo1 in the differentiation of cardiomyocytes. The mechanisms underlying the cardiac differentiation defect observed in leo1LA1186 embryos are not yet clear. It is conceivable that the failure of the AV boundary to be properly regionalized in leo1LA1186 mutants is secondary to defects in cardiac contractility, since proper cardiac function is required for normal gene expression and cellular morphology of the AV boundary endocardium (Bartman et al., 2004; Beis et al., 2005). Alternatively, Leo1 may function as an intrinsic factor to drive cardiomyocyte differentiation. One caveat of this possibility is that we have not been able to detect significant leo1 expression in the lateral plate mesoderm and leo1 expression appeared to be excluded from the heart in embryos 24 hpf and older. It is formally possible that the expression of leo1 in cardiac progenitors or cardiomyocytes is below the level that can be detected by whole mount in situ hybridization. However, a more exciting possibility is that Leo1 influences heart development via regulation of the differentiation of neural crest cells. In mouse and chick, neural crest cells have a pivotal role in the development of the cardiac outflow tract septum, aorticopulmonary septum, and semilunar and atrioventricular valves (for review see (Hutson and Kirby, 2003; Nakamura et al., 2006; Hutson and Kirby, 2007)). The contribution of neural crest cells to the development of the two-chambered zebrafish heart is less well understood. Lineage studies have demonstrated that a subset of neural crest cells (located in the region from the midbrain–hindbrain boundary to the first somite at the 10-somite stage) migrate to the heart and differentiate into cardiomyocytes in the outflow tract, ventricle, and AV boundary and to a lesser extent, the atrium (Li et al., 2003; Sato and Yost, 2003). Removal of these cells by laser ablation results in cardiac ventricular dysfunction and failure of cardiac looping (Li et al., 2003). Intriguingly, the differentiation of the anterior neural crest population, which would include a substantial portion of cardiac neural crest, is most affected in the leo1LA1186 mutant and the cardiac defects of the leo1LA1186 mutants are similar to those observed in cardiac neural crest-ablated embryos. Like cardiac neural crest-ablated embryos, leo1LA1186 mutants exhibit reduced cardiac contractility shortly after the heart begins to beat and subsequently develop pericardial edema and abnormal cardiac looping. Further studies on the causative relationship between the neural crest and cardiac defects in leo1LA1186 mutant embryos using transgenic approaches to drive expression of leo1 specifically in the neural crest may help to expand our
understanding of the role of neural crest contribution in zebrafish heart development.

Acknowledgements

We thank Drs. A. Sagasti, A. T. Look, T. Schilling, I. Dawid, S. Hong, T. Pitrowski, K. Cheng and J. Talbot for sharing plasmids for in situ hybridization analysis. We are grateful to members of the Chen lab for experimental suggestions and critiques and to Yuan Dong for participating in the genetic screen. This work was supported by NIH R01 HL081700 to JNC, a predoctoral fellowship from the Training Program of Genetic Mechanisms to CTN and an NSF Graduate Fellowship to ADL.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.02.020.

References


