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Analysis of *Cripto* expression during mouse cardiac myocyte differentiation

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ABSTRACT Vertebrate cardiac progenitor cells are initially allocated in two distinct domains, the first and second heart fields. It has been demonstrated that first heart field cells give rise to the myocardial cells in the left ventricle and part of the atria, whereas second heart field cells move into the developing heart tube and contribute to the myocardium of the outflow tract and right ventricle and the majority of atria. In this study, we have examined the expression of the mouse *Cripto* gene and the lineage of *Cripto*-expressing cells, focusing on its relationship with cardiac myocyte differentiation. The mouse *Cripto* gene is initially expressed at late head fold (LHF) stages in the cardiac crescent region, known as the first heart field; later in the medial region of the early heart tube, and by embryonic day 8.5, it is localized to the outflow tract. Using a *Cripto-LacZ* allele, we found that *Cripto*- expressing progeny cells contribute to the myocardium of the entire outflow tract and right ventricle, as well as to a majority of cells within the left ventricle. In contrast, no *Cripto*- expressing progeny cells were found in the atria or atrio-ventricular canal. Therefore, *Cripto* is transiently expressed in early differentiating myocardial cells of the left ventricle, right ventricle and outflow tract between LHF stages and E8.5. *Cripto* expression is subsequently downregulated as cells undergo further differentiation.

KEY WORDS: *cripto, heart development, hear fields, myocyte differentiation*

Introduction

The heart is the first functional organ formed during vertebrate embryogenesis. Heart formation involves a series of complex morphogenetic and differentiation events. Late in gastrulation, inductive signals from the anterior endoderm such as BMPs and Fgf8 specify cells in the anterior lateral mesoderm to become cardiac progenitor cells (Alsan and Schultheiss, 2002, Harvey, 2002, Ilagan *et al.*, 2006, Schultheiss *et al.*, 1997).

Cardiac precursor cells have been ascribed to two heart fields: the first and the second heart field (Laugwitz *et al.*, 2008, Srivastava, 2006). Cells of the first heart field are the first to differentiate at cardiac crescent and early heart tube stages, and will give rise to the first heart lineage which largely contributes to the left ventricle and parts of the atria. The second heart field is located dorsal-medial to the first heart field at crescent stages and will give rise to second heart lineages which comprise the outflow tract, right ventricle, and a majority of cell within the atria (Evans *et al.*, 2010, Laugwitz *et al.*, 2008, Srivastava, 2006, Watanabe and Buckingham, 2010). As development proceeds, cells within the cardiac crescent migrate toward the midline and fuse, forming an anterior-posterior oriented linear heart tube that subsequently undergoes rightward looping (Fishman and Chien, 1997, Harvey, 2002, Laugwitz *et al.*, 2008, Olson and Srivastava, 1996, Srivastava, 2006, Srivastava and Olson, 2000). Following heart tube stages, the heart grows by addition of second heart field cells at both anterior and posterior poles of the heart (Abu-Issa and Kirby, 2008, Cai *et al.*, 2003, Kelly *et al.*, 2001, Laugwitz *et al.*, 2008, Srivastava, 2006). The contribution of pharyngeal mesoderm cells to the outflow tract was first demonstrated by dye labeling experiments in chick embryos (Waldo *et al.*, 2001), and later in mouse embryos by genetic approaches using *isl1-Cre*, *isl-nlacZ*, and *Mlc1-lacZ* mice (Cai *et al.*, 2003, Kelly *et*

Abbreviations used in this paper: LHF, late head fold; LV, left ventricle; RV, right ventricle.

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al., 2001, Sun *et al.*, 2007). *Isl1* mRNA is expressed only in the second heart field and pharyngeal mesoderm (Cai *et al.*, 2003). Studies with [β-*actin-LacZ*;*isl1-Cre*] mouse embryos showed that over 90% of the cells in the outflow tract and the right ventricle are β-galactosidase positive demonstrating the contribution of second heart field cells to the outflow tract and right ventricle (Cai *et al.*, 2003).

EGF-CFC proteins function as essential co-factors for several transforming growth factor-β (TGF-β) superfamily members such as Nodal, GDFs and Vg-1, to signal through Activin receptors and control various aspects of vertebrate embryogenesis including the formation and patterning of the body plan (Chen *et al.*, 2006, Cheng *et al.*, 2003, Gritsman *et al.*, 1999, Yeo and Whitman, 2001). The human *CRIPTO* gene, the founding member of *EGF-CFC* gene family, was first indentified in human teratocarcinoma cells (Ciccodicola *et al.*, 1989). Subsequently, the mouse *Cripto* gene was cloned and shown to be expressed in embryonic mesoderm and developing heart (Dono *et al.*, 1993, Johnson *et al.*, 1994, Minchiotti *et al.*, 2000). Studies with *Cripto* mutant mouse embryos demonstrated that *Cripto* plays critical roles in axial patterning, mesoderm and endoderm formation, and ventral midline formation (Chu *et al.*, 2005, Ding *et al.*, 1998, Xu *et al.*, 1999). In addition, loss of *Cripto* function during ES cell differentiation blocks cardiac myocyte differentiation and promotes neural differentiation (Parisi *et al.*, 2003, Xu *et al.*, 1999). Further studies showed that *Cripto* acts through apelin/APJ to control cardiac lineage in ES differentiation (D'Aniello *et al.*, 2009). These ES cell based studies strongly suggested that *Cripto* may play critical roles in heart development during embryogenesis *in vivo*. Previous studies on *Cripto* expression in heart development *in vivo* were performed mainly before the current two heart field model was formulated. To understand the precise role of *Cripto* in heart development, it is important to determine the *Cripto* expression during cardiogenesis in more detail. For example, *Cripto* was shown previously to express in the outflow tract on E8.5, and it is now known that outflow tract cells are derived from second heart field, and therefore, it is important to find out whether *Cripto* is expressed at early stage such as crescent stage, if so, it is in the first or second heart field. In addition, cells in the growing heart tube including the outflow tract region are very dynamical, it is critical to know where the *Cripto*-expressing progeny cells populate. In the present study, we attempted to address these questions, at least to some extent.

Results and Discussion

Expression of Cripto *during mouse cardiac development*

As shown in Fig. 1, *Cripto* mRNA expression was detected at the late head fold stage (LHF), specifically in a region possibly corresponding to the cardiac crescent (Fig. 1 A,E). At the 3-4 somite stage, expression was observed in a region consistent with the medial cardiomyocytes in the early heart tube (Fig. 1 B,F). Further examination using double *in situ* hybridization of *Cripto* with *Mlc2a* demonstrated that these cells are *Mlc2a* positive, confirming they are indeed differentiating cardiomyocytes (see section 3 of the results and figure 3). At the 6-7 somite stage, *Cripto* is expressed in the dorsal region of the developing heart tube (Fig. 1 C,G). On E8.5, when embryos contain 9-10 somites and have undergone cardiac looping, *Cripto* expression is restricted to outflow tract (Fig. 1 D,H), consistent with previous studies showing a restricted *Cripto* expression in the outflow tract on E8.5 (Dono *et al.*, 1993, Johnson *et al.*, 1994, Minchiotti *et al.*, 2000). At this stage, no *Cripto* mRNA is observed in pharyngeal mesoderm or foregut endoderm (Fig.1D). After E8.5, *Cripto* mRNA is no longer detectable by *in situ* hybridization.

The progeny of Cripto *expressing cells contribute to derivatives of the first heart field and anterior second heart field*

To locate the progeny of *Cripto*-expressing cells, we utilized a *Cripto-Lacz* allele, in which the *LacZ* gene is under the control of the endogenous *Cripto* promoter and recapitulates *Cripto* expression pattern (Ding *et al.*, 1998). As β-galactosidase activity is stable, it has been widely used for lineage studies, including the fate of *Cripto*-expressing cells in axial mesendoderm (Chu *et al.*, 2005, Ding *et al.*, 1998). From the early somite stage to embryonic day 8.5 (E8.5), β- galactosidase is present in a broader domain than that observed for *Cripto* mRNA (Fig. 2 A -D). Following heart looping, high levels of β- galactosidase activity are observed in the outflow tract and right and left ventricles, whereas *Cripto* mRNA is restricted to outflow tract only as shown by previous studies (Dono *et al.*, 1993, Johnson *et al.*, 1994, Minchiotti *et al.*, 2000) and the present study (Fig.1 D,H), indicating that the progeny of *Cripto*-expressing cells contribute to the ventricular region and to the outflow tract. On E9.5, when *Cripto* mRNA is no longer expressed in the heart, β- galactosidase activity is retained in the outflow tract and right and left ventricles (Fig. 2 H,I). However, no β-galactosidase positive cells are present in the atria or atrio-ventricular canal regions

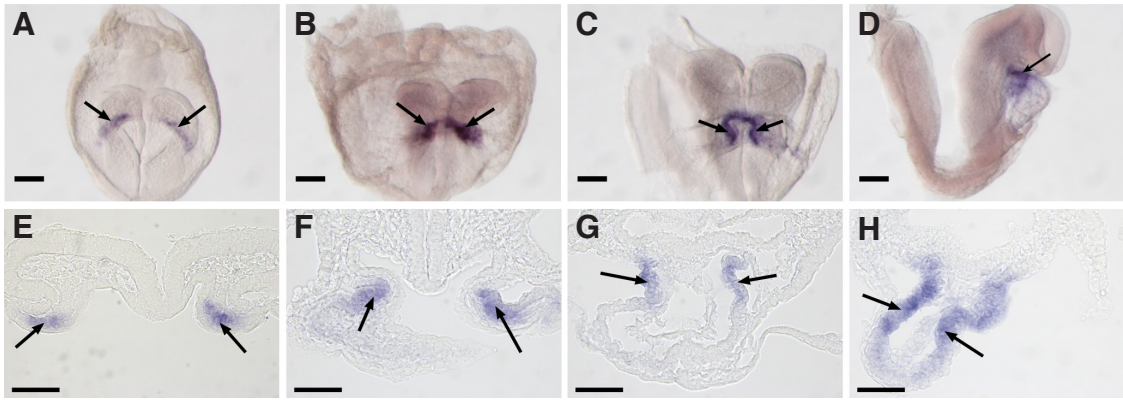

Fig. 1. Expression of *Cripto* mRNA during mouse heart formation. *RNA* in situ *hybridization reveals* Cripto *expression (arrows) in mouse embryos at late head fold stage (LHF)* **(A,E)**, *3-4 somites stage* **(B,F)**, *6-7 somites stage* **(C,G)** *and 9-10 somites stage* **(D,H)**. *Panels (E, F, G and H) are transverse sections corresponding to whole embryos in panels (A, B, C and D), respectively. The expression of* Cripto *was restricted to myocardial cells, and no expression was found in endocardial cells* **(G,H)**. *Scale bars represent 200 μm; (A-D) and 50 μm (E-H).*

(Fig. 2J). Serial sections revealed that β-galactosidase positive cells populate the majority of the myocardium in the outflow tract and in the right and left ventricle ventricles at E8.5 and E9.5 (Fig.2 E-G and K-L). It is worth noting that the β-galactosidase staining in the left ventricle is weaker than the staining in the outflow tract and right ventricle (Fig. 2 D,H,I). Since the left ventricle is derived from the first heart field, the β- galactosidase in the left ventricle is inherited from the cardiac crescent cells on E7.5, whereas the β-galactosidase staining in the outflow tract and right ventricle represent the progeny of *Cripto* expressing cells from the early heart tube stage to E8.5.

Cripto *expressing cells and their progeny are always localized within* Mlc2a *expression domain*

To determine the *Cripto* expression domain in relation to first and second heart fields at the LHF/cardiac crescent stage, we performed double whole-mount *in situ* hybridization for *Cripto* and *Mlc2a*, an marker for early differentiating myocytes of the cardiac crescent (Cai *et al*., 2003, Laugwitz *et al*., 2008), and found that *Cripto*-expressing cells (purple) are exclusively localized within the *Mlc2a* expression domain (red) in the cardiac crescent (Fig. 3 A-F). Fig. 3A is a mouse embryo at the LHF stage showing *Cripto* expression only, and the same embryo was subjected to detection for *Mlc2a* expression, as shown in Fig. 3B. *Cripto*-expressing and *Mlc2a*- expressing cells were co-localized in the crescent region as revealed by section analysis (Fig. 3C). It is important to note that not all the cells in the crescent were *Cripto* positive. Instead, only the region immediately adjacent to the second heart field were positive for both *Cripto* and *Mlc2a*, whereas crescent cells distal to the second heart field were positive only for *Mlc2a* expression. At early somite stages when the linear heart tube starts to form, *Cripto* positive cells were restricted to the medial domain of *Mlc2a* expression (Fig. 3 D-F).

We next investigated the location of the progeny of *Cripto* expressing cells in relation to differentiated myocytes within the early looping heart using β- galactosidase staining in combination with *Mlc2a in situ* hybridization in *Cripto*$^{Lacz/Wt}$ embryos at early heart looping stages. Fig. 3G shows a β- galactosidase stained *Cripto*$^{Lacz/Wt}$ embryo. The same embryo was subjected to *Mlc2a in situ* hybridization and the strong *Mlc2a* signal masks the previous β-galactosidase signal (Fig. 3H). In sections, β-galactosidase signal is also completely masked by signal from *Mlc2a* mRNA *in situ* (Fig. 3I), with the exception of scattered β-galactosidase signals in the endoderm, which are likely to be remnants of early *Cripto* expression in axial mesoendoderm (Chu *et al*., 2005). Therefore, *Cripto*-expressing cardiac progenitor cells and their progeny are restricted to differentiating myocyte cells of the first and anterior second heart fields.

We next sought to examine the relationship between *Cripto*-expressing cells and the second heart field by double *in situ* hybridization for *Cripto* (in red) and *Isl1* (dark purple) at crescent stages, and found that *Isl1* mRNA was located dorsal-medial to the *Cripto* expression domain with no overlap (Fig. 3J). We also investigated the location of the progeny of *Cripto* expressing cells in relation to the second heart field by β-galactosidase staining in combination with *Isl1 in situ* hybridization in *Cripto*$^{Lacz/Wt}$ embryos at later stages, and found that at linear heart tube stages, *Isl1* mRNA was expressed in endoderm and pharyngeal mesenchymal cells distinct from β-gal positive cells (Fig. 3 K,L).

The foregoing observations are consistent with *Cripto* being expressed in distinct subpopulations of cardiomyocytes as they first differentiate.

The *Cripto* expression domain is immediately adjacent to the second heart field. It has been reported previously that this region is positive for Isl1 protein expression, although it is negative for *Isl1* mRNA expression (Prall *et al*., 2007, Sun *et al*., 2007). To test

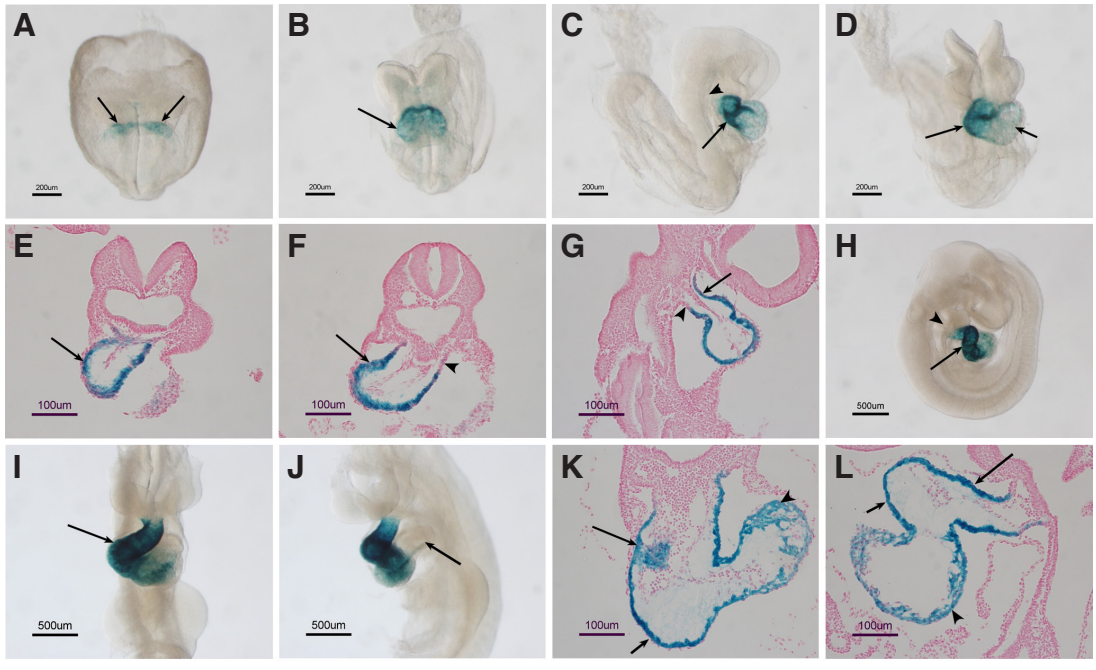

Fig. 2. β-galactosidase staining pattern in *Cripto*$^{LacZ/Wt}$ embryos. *At the 3-4 somite stage* **(A)**, *6-7 somite stage* **(B)**, *11-12 somite stage* **(C-G)** *and E9.5* **(H-L)**. **(D,I)** *frontal views;* **(C,H)** *right side view, whereas* **(J)** *is left side view.* **(E, F,K)** *Cross sections, whereas* **(G,L)** *sagittal sections. At early somite stages, β-galactosidase staining positive cells were found in the entire myocardium (arrows in A and B). On E8.25-8.5 with 11-12 somites, intense staining was found in the outflow tract and right ventricle (arrows in C and D) with slightly weak signals in the left ventricle (shot arrow in D). E9.5 embryos showed a similar pattern in the outflow tract, right ventricle and left ventricle regions (arrows in H and I). However, no signal was found in the atria or atrio-ventricular canal (arrow in J). The majority of myocardial cells in the outflow tract (arrows in E, F, G, K and L), right ventricle (short arrows in K and L) and left ventricle (arrowheads in K and L) were blue and only a few unstained cells were found (arrowheads in F, G,). No signal was found in the pharyngeal mesoderm (arrowheads in C and H). Scale bars represent 200 μm (A-D), 100μm (E-G and K-L), 500 μm (H-J).*

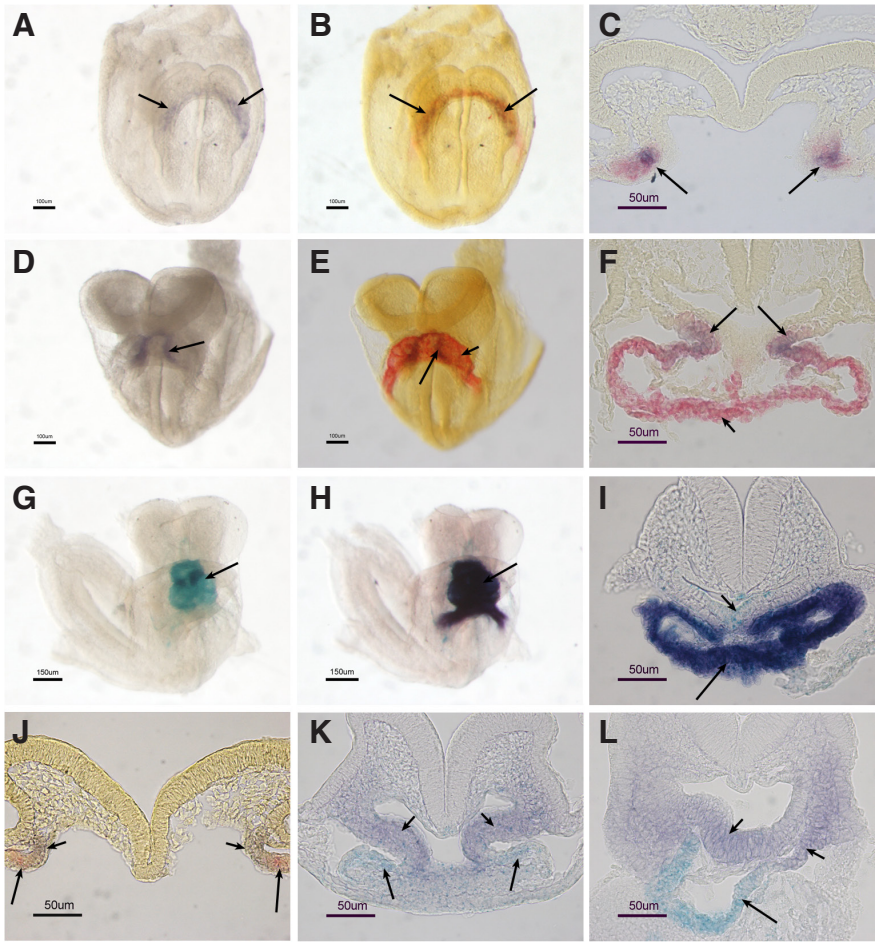

Fig. 3. *Cripto*-expressing cells and their progeny are located within the *Mlc2a* expression domain and are distinct from the domain of *Isl1* expression area. (A-F) *Double* in situ *hybridization of* Cripto *(purple) and* Mlc2a *(red) in wild type embryos showing* Cripto*-expressing cells are localized within the* Mlc2a *expression region in the cardiac crescent (arrows in A-C). Once the heart tube is formed, they are co-localized in the anterior heart tube (arrows in D-F), whereas the rest heart tube was positive only for* Mlc2a *(short arrows in E and F).* **(G-I)** *β-galactosidase staining (G) followed by* Mlc2a in situ *hybridization (H and I) in* Cripto$^{LacZ/Wt}$ *embryos at the early heart looping stage showing that the* Mlc2a in situ *hybridization signal completely overlays the β-galactosidase staining (arrows in G-I), only a few scattered β-galactosidase positive cells were observed in the foregut endoderm (short arrow in I).* **(J)** *Double* in situ *hybridization showing* Isl1 *expression in purple (short arrows) and* Cripto *expression domain in red (arrows) in the cardiac crescent of wild type embryos.* **(K,L)** *β-galactosidase staining followed by* Isl1 in situ *hybridization showing* Cripto*-lineage cells in blue (arrows) are distinct from the* Isl1*-expressing pharyngeal mesenchymal cells (short arrows).*

whether *Cripto* expressing cells are Isl1 protein positive, we carried out Isl1 immunostaining and *Cripto in situ* hybridization in adjacent sections and found that *Cripto* mRNA expression domain is more broad than Isl1 protein expression domain in the heart tube, and the two domains partially overlap in the dorsal portion of the heart tube (see Supplementary Figure).

In conclusion, the *Cripto* gene has two phases of expression during mouse cardiac development from the LHF stage to E8.5. At the LHF stage, *Cripto* is highly expressed in differentiating myocytes in the cardiac crescent that will give rise to the cells of the future left ventricle, but not the atria. From early heart tube stages, cells in the second heart field migrate into the developing heart tube through anterior and posterior poles of the tube and differentiate into myocytes. Previous studies have demonstrated that second heart field cells that migrate through anterior pole will contribute to the myocardial cells in the outflow tract and right ventricle, whereas the cells that move via the posterior pole will contribute to the majority of the atria (Cai *et al.*, 2003, Evans *et al.*, 2010, Waldo *et al.*, 2001). During this process, expression of *Cripto* mRNA is initially restricted to the medial region of early heart tube, and later (E8.5) to the outflow tract of the heart tube. In contrast, the progeny of *Cripto* expressing cells populate the right and left ventricles as well as the outflow tract at E8.5 and E9.5. Since *Cripto* mRNA is not expressed in the right ventricle, it appears that *Cripto* expression specifically marks differentiating myocytes of the anterior developing heart tube, namely, when second heart field cells are migrating through the anterior pole of the developing heart tube and differentiating to myocytes, these cells express *Cripto*, but only transiently, as the expression soon disappears once the cells undergo further differentiation. Since *Cripto*-expressing cells and their progeny cells are not detected in the atrial region, second heart field cells that migrate through the posterior pole of the developing heart tube do not express *Cripto* as they are differentiating into myocytes.

Materials and Methods

Mouse strains and mutant alleles

The *Cripto^{lacZ}* allele has been reported previously (Ding *et al.*, 1998). In this allele, the expression of *Lacz* gene is controlled by the endogenous *Cripto* promoter, which recapitulates *Cripto* expression in vivo. In addition, we performed *in situ* hybridization using a *LacZ* gene probe on *Cripto$^{lacz/wt}$* embryos and found the expression of *LacZ* mRNA was identical to the expression of *Cripto* mRNA (data not shown).

Embryos between E7.5-E8.0 were staged according to Downs and Davis (Downs and Davies, 1993) and by somite number. Embryos from E8.5 to E9.5 were staged by gestation days, the day a plug observed was designated as day 0.5 (E0.5). For any given stage, at least 10 embryos are used for each experiment.

In situ *hybridization and β-galactosidase staining*

Single probe whole-mount *in situ* hybridization was carried out as previously described (Shen, 2001). A plasmid containing full length *Cripto* cDNA

was used to generate the probe for *Cripto in situ* (Ding *et al.*, 1998). Probes for *Mlc2a* and *Isl1* were provided by Dr. Sylvia Evans at UCSD (Cai *et al.*, 2003). Double color whole-mount *in situ* hybridization protocol was based on(Cai *et al.*, 2003). Briefly, Digoxigenin and Fluorescein labeled antisense RNA probes were hybridized simultaneously, followed by first blue color reaction using anti-Digoxigenin-AP Fab fragments and NBT/BCIP as the conventional single color *in situ* hybridization. The second red color detection was visualized by anti-Fluorescein-POD Fab fragments (Roche) and Fast Red. Following the first color development, the embryos were incubated at high temperature followed by treatment with 4% paraformaldehyde and glycine in order to completely inactivate alkaline phosphatase activity. For comparison and documentation, we usually photographed embryos individually before the second color reaction.

Whole-mount β-galactosidase staining was performed using standard procedure. Briefly, E7.5-9.5 embryos were fixed in 4% paraformaldehyde at 4°C for 50-60 minutes and incubated with staining solution (phosphate buffered saline, pH 7.4, 2 mM $MgCl_2$, 0.02% Nonidet P -40(v/v), 5mM potassium ferricyanide, 5mM potassium ferrocyanide, and1 mg/ml X-gal) for 4-6 hours at 37°C. Stained embryos were post-fixed with 10% formalin for at least 24 hours. To combine *in situ* hybridization with β-galactosidase staining, embryos were first stained for β-galactosidase under RNase free conditions and then fixed overnight in 4% paraformaldehyde at 4°C followed by washes with PBT (PBS with 0.1% Tween-20) and stored at -20°C in methanol until processed for *in situ* hybridization.

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