**Cripto is required for mesoderm and endoderm cell allocation during mouse gastrulation**

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**ABSTRACT**

During mouse gastrulation, cells in the primitive streak undergo epithelial–mesenchymal transformation and the resulting mesenchymal cells migrate out laterally to form mesoderm and definitive endoderm across the entire embryonic cylinder. The mechanisms underlying mesoderm and endoderm specification, migration, and allocation are poorly understood. In this study, we focused on the function of mouse Cripto, a member of the EGF-CFC gene family that is highly expressed in the primitive streak and migrating mesoderm cells on embryonic day 6.5. Conditional inactivation of Crypt1 during gastrulation leads to varied defects in mesoderm and endoderm development. Mutant embryos display accumulation of mesenchymal cells around the shortened primitive streak indicating a functional requirement of Cripto during the formation of mesoderm layer in gastrulation. In addition, some mutant embryos showed poor formation and abnormal allocation of definitive endoderm cells on embryonic day 7.5. Consistently, many mutant embryos that survived to embryonic day 8.5 displayed defects in ventral closure of the gut endoderm causing cardiia bifida. Detailed analyses revealed that both the Fgf8–Fgfr1 pathway and p38 MAP kinase activation are partially affected by the loss of Cripto function. These results demonstrate a critical role for Cripto during mouse gastrulation, especially in mesoderm and endoderm formation and allocation.

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**Introduction**

Before gastrulation, mouse embryonic cells are organized into a cylindrical structure called the egg cylinder that consists of two layers, the inside epiblast (also known as embryonic ectoderm) and the outside visceral endoderm (Beddington and Robertson, 1999; Tam and Loebel, 1997). At the onset of gastrulation, cells on one side of the epiblast delaminate and undergo epithelial–mesenchymal transition (EMT) leading to the formation of a morphologically distinguishable area called the primitive streak (Beddington and Robertson, 1999; Tam and Loebel, 2007). The emerging primitive streak expands distally and forms a characteristic structure termed the node on the distal end (Beddington, 1994; Tam and Loebel, 2007). The anterior tip of the primitive streak (APS), and later the node, serves as the trunk organizer capable of inducing the posterior region when transplanted onto a host embryo (Beddington, 1994). The anterior visceral endoderm (AVE), located in the anterior region of the embryo, is essential for anterior neural formation and patterning (Beddington, 1998; Beddington and Robertson, 1998, 1999; Kimelman, 2006; Shawlot et al., 1999; Thomas et al., 1998). While expanding toward the distal end of the embryo, the primitive streak cells also migrate out laterally to form an intervening layer of mesoderm between the visceral endoderm and epiblast, that will be further specified to cardiac and lateral plate mesoderm, axial mesoderm and paraxial mesoderm (Harvey, 2002; Lawson et al., 1991; Shawlot et al., 1999; Tam and Behringer, 1997; Tam and Tan, 1992). Following the migration and allocation of mesoderm cells, definitive endoderm cells also migrate out of the primitive streak to form endoderm-derived tissues and organs such as the gut (Tam et al., 2007). In addition, cells from the primitive streak also move proximally to form extra-embryonic mesoderm (Downs et al., 2004).

Numerous signaling and regulatory factors have been found to be involved in early embryonic induction and patterning. Among them, the components of the Nodal and Wnt pathways are particularly important. Nodal, a member of TGF-β superfamily, and its intracellular transducers, Smad2 and Smad3, are required for various aspects of early embryonic events such as A-P establishment, mesoderm and endoderm induction and patterning, and axial midline formation (Brennan et al., 2001; Csonlon et al., 1994; Dunn et al., 2004; Lowe et al., 2001; Nomura and Li, 1998; Schier and Shen, 2000; Varlet et al., 1997; Vincent et al., 2003; Waldrip...
et al., 1998; Weinstein et al., 1998; Zhou et al., 1993). Consistently, loss of Nodal inhibitors such as Drap1 and Lefty1 results in elevated Nodal activity and an expanded primitive streak as well as excess mesoderm in mice (Iratni et al., 2002; Meno et al., 1999). Loss of β-catenin function in mice affects the formation of AVE as well as the primitive streak and its derivatives (Huelsken et al., 2000), whereas mutation of the Wnt3 gene abolishes only the primitive streak without affecting AVE formation (Liu et al., 1999). Interestingly, the AVE induces anterior neural formation by producing Nodal and Wnt antagonists such as Cer1 and DKK1 indicating that suppression of Nodal and Wnt activities in the anterior epiblast is essential for head formation and patterning (Glinka et al., 1998; Mao et al., 2001; Piccolo et al., 1999; Semenov et al., 2001). Therefore, precise spatial and temporal regulation of Nodal and Wnt activity is critical for embryonic induction and patterning. Additional pathways and factors important for early embryogenesis in the mouse include Shh (Chiang et al., 1996), BMPs and their antagonists Chordin and Noggin (Bachiller et al., 2000; Fujiwara et al., 2002; Hogan, 1996), transcription factors Lhx1 (Shawlot and Behringer, 1995; Shawlot et al., 1999), Foxa2 (Ang and Rossant, 1994), Mix1 (Hart et al., 2002) and many others (Tam and Loebel, 2007).

Compared with mesoderm induction and patterning, little is known about the migration of mesoderm and endoderm. The Fgf8–Fgf1 pathway plays a central role in driving mesoderm cell migration and is mediated, at least in part, by transcription factors Snai1 and Tbx6 (Ciruna and Rossant, 2001; Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994). In this pathway, Snai1 is mainly responsible for EMT, because it is able to repress E-Cadherin gene expression (Barrallo-Gimeno and Nieto, 2005; Batlle et al., 2000; Cano et al., 2000) and more importantly, Snai1 mutant mouse embryos form a layer between the epiblast and the visceral endoderm, of which the cells are more epithelial than mesenchymal in character and retain E-Cadherin expression, which is consistent with previous reports that p38-dependent EMT. Studies with p38 interacting protein (p38IP) revealed that the activation of p38 MAP kinase is required for efficient EMT in the primitive streak and subsequent lateral migration of mesoderm cells (Zohn et al., 2006). This observation is consistent with previous reports that p38α MAP kinase is required for embryonic branching angiogenesis, a process involving endothelial cell migration (Adams et al., 2000; Mudgett et al., 2000). Activation of the p38-mediated pathway in the primitive streak requires p38IP and NCK interacting kinase (NIK), but is independent of Fgf8 signaling. Moreover, Eomesoderm is required for EMT during mouse gastrulation that is independent of the Fgf8 pathway (Arnold et al., 2008). However, the relationship between Eomesoderm and the p38 pathway has not been reported.

Cripto is the founding member of the EGF-CFC family and functions as an essential co-factor for several TGF-β superfamily members such as Nodal during vertebrate embryogenesis (Dono et al., 1993; Schier and Shen, 2000; Shen, 2007; Shen and Schier, 2000; Shen et al., 1997). Cripto acts as both ligand and co-receptor in facilitating Nodal signaling (Yan et al., 2002). In addition, Cripto can block the function of Activin, also a member of TGF-β superfamily, by forming a complex with its type II receptor (Gray et al., 2003). During mouse development, Cripto is initially expressed in the epiblast before gastrulation (Ding et al., 1998). During gastrulation, Cripto is highly expressed in the primitive streak, migrating mesoderm, the node and axial mesendoderm (Chu et al., 2005; Ding et al., 1998). In Cripto-null mutant embryos, the prospective AVE is specified in the distal end, but is incapable of anterior migration (Ding et al., 1998). Similarly, the prospective trunk organizer is initiated in the proximal end of the epiblast, but fails to translocate to the posterior region, resulting in the lack of primitive streak, embryonic mesoderm and endoderm (Ding et al., 1998). These results indicate that Cripto is capable of regulating embryonic cell migration at pre-gastrulation stage. Experiments with a Cripto hypomorphic allele revealed that it is also required for the formation of axial midline structures, particularly the anterior definitive endoderm and prechordal mesoderm (Chu et al., 2005). However, the function of Cripto during early gastrulation, particularly in the formation of mesoderm layer, remains elusive.

Materials and methods

Mouse lines

The Cripto<sup>lox</sup> allele used in this study has been described before (Ding et al., 1998). The Cripto<sup>lox<sup>fl</sup></sup> allele was generated by removing the floxed PGK-Neo region in the previously reported Cripto<sup>fl/fl</sup> allele (Chu et al., 2005) using the Ella-Cre line.

The generation of the Cripto<sup>fl<sup>lox</sup></sup> allele is schematically illustrated in supplementary Fig. S1. The following PCR primer pairs were used to genotype the Cripto<sup>fl</sup> allele: 5′-GTG GTA AGT ATT TCC TCT TTC-3′ (forward) and 5′-AGG AAC ATT CCA ATG GCC TTC TG3′ (reverse) detect the second lox<sup>P</sup> site yielding a 500 bp band for the floxed allele and a 400 bp band for the wild type allele; 5′-AGC CAT CTC ACC ACC CTT CA-3′ (forward) and 5′-ACC CCA CCA TCC A-3′ (reverse) were used to determine the first lox<sup>P</sup> site producing a 450 bp band for the floxed allele and a 580 bp band for the wild type allele.

The Sox2-Cre line was purchased from the Jackson Laboratory (Hayashi et al., 2002) and the Fgf8<sup>lox<sup>fl</sup></sup> null mutant strain generated by Dr. Gail Martin’s group, was purchased from MMRRC.

In situ hybridization

Whole mount in situ hybridization was carried out according to Shen (Shen, 2001) and double in situ hybridization was based on Cai (Cai et al., 2003). The post-hybridized embryos were sectioned and counter-stained with nuclear fast red from Vector Laboratories (catalog # H-3403). For each probe, more than 10 embryos were analyzed.

Immunostaining

Activation of p38 MAP kinase was detected by immunostaining using a rabbit monoclonal antibody from Cell Signaling that specifically recognizes phospho-p38 (P-p38) (catalog # 4631L). E7.5 embryos were fixed with 4% paraformaldehyde at 4 °C for 2–3 h followed by OCT embedding for cryo-sectioning and stored at −80 °C until use. Sections were incubated with primary antibody overnight using a dilution of 1:10 or 1:20 followed by incubation with Cy3-conjugated goat anti-rabbit secondary antibody from Jackson ImmunoResearch Laboratories.

Results

Loss of Cripto function causes mesoderm cell accumulation around the primitive streak area during gastrulation

Cripto is highly expressed in the primitive streak and migrating mesoderm during early gastrulation (Ding et al., 1998) suggesting that Cripto may function during this process. However,


171
development of Cripto-null mutant embryos is arrested at the pre-gastrulation stage due to failure in anterior–posterior polarity establishment (Ding et al., 1998). We therefore generated a floxed allele of Cripto as shown in supplementary Fig. S1. The resulting Cripto$^{lox/lox}$ mice are viable and develop normally. Crosses of Cripto$^{lox/lox}$ male mice with [Cripto$^{lacZ/wt}$; Sox2-Cre] female mice, which contain a high level of maternal Cre activity (Hayashi et al., 2003; Vincent and Robertson, 2003), gave rise to a severe phenotype that was identical to Cripto-null mutant embryos, indicating the deletion of the floxed region disrupts the entire function of Cripto (supplementary Fig. S2). In addition, this Cripto$^{lox}$ allele has been used in a recent study examining the function of Cripto in skeletal muscle regeneration (Guadiala et al., 2012; Michael Shen, personal communication).

To investigate the function of Cripto during mouse gastrulation, we utilized the Cripto$^{lox}$ and Cripto$^{lacZ}$ lines in combination with the Sox2-Cre transgenic line, which expresses Cre activity throughout the epiblast and has been successfully used to study the function of Nodal during gastrulation (Hayashi et al., 2003). To avoid the high maternal Cre activity in the Sox2-Cre line as mentioned above (Hayashi et al., 2003; Vincent and Robertson, 2003), we crossed [Cripto$^{lacZ/wt}$; Sox2-Cre] males with Cripto$^{lox/lox}$ females. The majority of the resulting [Cripto$^{lacZ/lox}$; Sox2-Cre] embryos survived to gastrulation and post-gastrulation stages. As shown in Fig. 1, using in situ hybridization, we examined the expression of Brachyury, a primitive marker, on E7.5. Compared with wild type embryos (Fig. 1A), [Cripto$^{lacZ/lox}$; Sox2-Cre] embryos formed an abnormal primitive streak area that was shortened and widened (Fig. 1D). Cross-sections of these embryos revealed that the mesoderm cells in wild type embryos migrated out of the posterior region and were spread over the entire embryo (Fig. 1B). In marked contrast, mesoderm cells in the conditional mutant embryos accumulated in the posterior region within the abnormal primitive streak area (Fig. 1E), indicating a defect in lateral migration of mesoderm. This defect was detectable as early as E6.5. On E6.5, the expression of Lim1 marks the anterior visceral endoderm (AVE) and newly formed migrating mesoderm cells that have moved out of the posterior region (Fig. 1C and G). However, in Cripto conditional mutant embryos on E6.5, Lim1-positive mesoderm cells were restricted to the posterior region, even though the Lim1-expressing visceral endoderm cells had already migrated from the distal end to the anterior region (Fig. 1F and I). Compared to the epithelial embryonic ectoderm cells, the cells adjacent to the abnormal primitive streak region in the conditional mutant embryos were typical mesenchymal cells with loose cell–cell adhesion, indicating that the cells had undergone EMT (Fig. 1E). Consistently, the expression of E-Cadherin was down-regulated in mesoderm cells in these conditional mutant embryos (Fig. 1H and J). In addition, Cripto-null mutant embryos can still form mesenchymal mesoderm cells in the extra-embryonic area (Ding et al., 1998; Kimura et al., 2001). We also found extensive formation of blood islands in Cripto-null mutant embryos (data not shown), indicating that even Cripto-null mutants undergo EMT. Therefore, the defects observed here were not due to deficient EMT.

**Fig. 1.** Mesoderm cell allocation defects in Cripto conditional mutant embryos. In situ hybridization showing the expression of Brachyury (A, B, D and E), Lim1 (C, F, G and I) and E-Cadherin (H and J) in wild type (Wt) and Cripto conditional knock (cKO) embryos. On E7.5, wild type embryos form the primitive streak, marked by Brachyury expression, in a narrow line across the embryo from the proximal to distal end (arrow in A). Mesenchymal mesoderm cells were found over the entire embryo (arrows in B). In contrast, Cripto conditional mutant embryos formed a shortened and widened patch of abnormal primitive streak (arrow in D) and mesenchymal mesoderm cells accumulated in the posterior region around the abnormal primitive streak (arrow in E). On E6.5, the migrating mesoderm cells, marked by Lim1 expression, in wild type embryo (C) have migrated out of the posterior region (arrows in G), whereas the Lim1-expressing cells in Cripto conditional mutant embryos (F) remained in the posterior region (arrow in I), although the expression of Lim1 in the visceral endoderm has moved to anterior region in both cases (arrow heads in G and I). Wt: wild type; cKO: conditional knock-out. The dashed lines in panels A, C, D and F represent the positions of cross-sections shown in panels B, E, G and I, respectively.
**Cripto function**

Previous studies with Fgf8 and Fgfr1 mutant embryos revealed their critical roles in EMT and mesoderm cell migration during gastrulation (Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994). Subsequent studies demonstrated that this function is mediated, at least in part, through regulation of Snai1 and Tbx6 expression (Ciruna and Rossant, 2001). Therefore, Fgf8 acts through Fgfr1 to regulate the expression of downstream target genes such as Snai1 and Tbx6 to control EMT and mesoderm cell migration during gastrulation (Ciruna and Rossant, 2001; Zohn et al., 2006). Because the Cripto conditional mutant embryos displayed defects in mesoderm cell migration that, to some degree, resembled the phenotype found in Fgf8 mutant embryos, we examined Fgf8 expression in Cripto conditional mutant embryos, but found no significant changes (Fig. 2A and D), except that the expression in Cripto conditional mutant embryos tended to be more wide and proximally located due to the shortened and widened primitive streak. We then tested whether Cripto is a downstream target of Fgf8 in the primitive streak and migrating mesoderm. For this, we examined the expression of Cripto in Fgf8-null mutant (FgflacZ/lacZ) embryos, and found that Cripto expression was highly retained in the accumulated mesoderm cells in FgflacZ/lacZ mutant embryos on E7.5, indicating that Cripto expression is not controlled by Fgf8 (Fig. 2B and E). In contrast, we found that the expression of Fgfr1 was severely affected in the mesoderm cells in the Cripto conditional mutant embryos. In wild type embryos, Fgfr1 was highly expressed throughout the entire embryo on E7.5 including the epithelial embryonic ectoderm and mesenchymal mesoderm cells, with stronger expression in the mesoderm compared to ectoderm (Fig. 2C and G). The expression in mutant embryos was reduced overall, and the sections showed that the remaining Fgfr1 expression was found largely in the embryonic ectoderm, with little expression in mesoderm cells (Fig. 2F and J). In wild type embryos, the expression in the mesoderm is even higher than that in the ectoderm (Fig. 2C and G). We also examined Fgfr1 expression in Cripto null mutant embryos on E7.5 and found that Fgfr1 expression was retained in the ectoderm of Cripto null embryos (data not shown). Therefore, the expression of Fgfr1 is differentially down-regulated in mesoderm cells in these Cripto conditional mutant embryos. We then examined the expression of Snai1 and Tbx6, two downstream target genes of Fgf8/Fgfr1 and found that the expression of Tbx6 was completely abolished in all the [Cripto^lacZ/lacZ;Sox2-Cre] embryos examined with abnormal streak morphology (n > 10) (Fig. 2I and L), whereas Snai1 was still expressed in the mutant embryos (Fig. 2H and K). Moreover, we found Snai1 was also

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**Fig. 2.** The Fgf8–Fgfr1 pathway is partially affected in Cripto conditional mutant embryos. (A and D) Fgf8 expression on E7.5 does not show significant differences between wild type (A) and Cripto conditional mutant (D) embryos, except that mutant embryos (D) show more expression in the proximal end due to the shortened primitive streak. (B and E) Cripto is highly expressed in Fgf8 null mutant embryos on E7.5 including the accumulated mesoderm cell mass in the posterior region (arrow in E). (C, F, G and J) On E7.5, Fgfr1 is highly expressed in wild type embryos (arrow in C), including both mesoderm cells (arrow in G) and embryonic ectoderm cells (arrow head in G). Notably, the expression in the mesoderm is higher than the expression in the embryonic ectoderm. However, in Cripto conditional mutant embryo, the expression is largely reduced (arrow in F). Compared to the expression in the embryonic ectoderm (arrow head in J), the expression in the mesoderm is much weaker (arrow in J). (H, I, K and L) In situ hybridization showing the expression of Snai1 (H and K) and Tbx6 (I and L), two known Fgf8–Fgfr1 downstream genes, in wild type and conditional mutant embryos on E7.5. The expression of Snai1 in the mutant embryo (K) is unchanged compared to wild type embryos, whereas the expression of Tbx6 is completely abolished (L). Wt: wild type; cKO: conditional knock-out; Fgf8^lox/lox: Fgf8 null mutant. The dashed lines in panels B, C, and F represent the positions of cross-sections shown in panels E, G and J, respectively.
expressed in Cripto null mutant embryos on E7.5 (data not shown), indicating that even the complete loss of Cripto function does not abolish Snail1 expression. These results indicated that even the complete loss of Cripto function does not abolish Snail1 expression. These results indicated that even the complete loss of Cripto function does not abolish Snail1 expression.

Cripto function regulates p38 MAP kinase activation in embryonic ectoderm

During our search for genes responsible for the migration defect in Cripto conditional mutant embryos, we found that the expression of Mesp1, a primitive streak- and mesoderm-expressed gene responsible for cardiac mesoderm cell migration (Saga et al., 1999) was completely abolished at the early streak stage in all the [Cripto^lacZ/fox_Sox2-Cre] embryos examined with abnormal streak morphology (n > 10) (Fig. 3A, B, E and F). Consistently, we found that Mesp1 expression was undetectable in Cripto null mutant embryos on E6.5 (Fig. 3C and G), whereas the expression of other primitive streak markers such as Fgfr5, Lim1 and goosecoid was still retained, although in the proximal end (Ding et al., 1998). Therefore, Cripto is required for Mesp1 expression at both the pre-gastrulation and gastrulation stages. We then examined the expression of Mesp1 in Fgfr5-null mutant embryos to determine if Mesp1 expression is Fgfr5 dependent, and found that Mesp1 was

**Fig. 3.** Loss of Cripto function abolishes Mesp1 expression and dramatically reduces p38 MAP kinase activation in the embryonic ectoderm. (A-H) In Situ hybridization showing high expression of Mesp1 in wild type embryos on E7.5 (A and B) and E6.5 (C) including the primitive streak (arrow head in B) and mesoderm cells (arrows in B), whereas no expression is detected in E7.5 conditional mutant embryos (E and F) in the primitive streak (arrow head in F) and the accumulated mesoderm cells (arrow in F). In addition, the expression is completely abolished in E6.5 Cripto null mutant embryo (C). In contrast, Mesp1 is still highly expressed in Fgfr5 null mutant embryos (arrows in D and H). (I-R) Immunostaining using P-p38 specific antibody showing p38 MAP kinase activation in E7.5 wild type (I-K and M-O) and Cripto null mutant (L, P, Q,R) embryos. Panel S is the whole mount view of a Cripto null embryo corresponding to the staining shown in panels Q and R. Note that the distal end of this embryo invaginated upwards as indicated by the lines. The dashed line indicates the position and direction of section Q and R. Since the Cripto null embryos do not form embryonic mesoderm and endoderm, the tissues shown here are embryonic ectoderm cells. In wild type embryos, as shown by both transverse (I, J, M, and N) and frontal sections (K and O), embryonic ectoderm cells are uniformly positive for P-p38 (arrow heads in I and M). In distal areas, the entire mesoderm layer is P-p38 negative (J and N). In Cripto null mutant embryos, some transverse sections show a complete lack of P-p38 cells in the embryonic ectoderm (arrows in I and F), except for a small region around the ectoderm on distal end (arrow heads in Q and R). WT: wild type; cKO: conditional knock-out; Fgfr5^−/−: Fgfr5 null mutant; Null: Cripto null mutant. The dashed lines in panels A, D and E represent the positions of cross-sections shown in panels B, H and F, respectively. Note: Since invaginations always occur in the distal ectoderm of E7.5 Cripto null mutant embryo as reported before and also shown by the arrowheads in (U), the proximal–distal axes in (R, T) were indeed oriented in top–bottom manner, although they look like bottom–top.
highly expressed in these mutant embryos on E7.5 indicating that 
Mesp1 expression is not dependent on the Fgf8–Fgfr1 pathway
(Fig. 3D and H). Therefore, Cripto may function through other
pathways in addition to Fgf8–Fgfr1. The p38 MAP kinase pathway
is another major pathway reported to play critical roles during
EMT and mesoderm cell migration (Zohn et al., 2006). Loss of p38
interacting protein (p38IP) negatively affects the activation of p38
MAP kinase in the embryonic ectoderm and mesoderm cells on
E7.5 and leads to mesoderm migration defects and a delay in EMT
(Zohn et al., 2006). We investigated the activation of p38 MAP
kinase in wild type and Cripto conditional mutants by immuno-
staining using a phospho-p38 (P-p38) specific antibody. In E7.5
wild type embryos, we found that positive signals were uniformly
present in all embryonic ectoderm cells examined by both cross
and frontal sections (Fig. 3I–K and M–O). However, the signals in
mesoderm cells were less uniform and were position dependent.
In the proximal region, the majority of mesoderm cells were P-p38
positive with only a few negative cells (Fig. 3I and M). In contrast
the signals were absent in mesoderm cells in the distal region
(Fig. 3J) and N). This is slightly different from a previous report
which showed uniform p38 activation in mesoderm cells on E7.5
(Zohn et al., 2006). We believe that this discrepancy is probably
due to stage differences as well as the positional differences
mentioned above. To better evaluate the function of Cripto in
activating p38 MAK kinase in embryonic ectoderm cells, we
examined the activation of p38 in Cripto-null mutant embryos on
E7.5. If loss of Cripto function indeed affects p38 activation in
embryonic ectoderm cells, it should be more pronounced in Cripto
null mutant embryos. We found that in most of the cross sections
of Cripto null mutant embryo sections did not contain P-p38
positive cells on E7.5 (Fig. 3L and P). Consistently, the frontal
sections showed that P-p38 positive cells were present only in a
small area on the distal end, whereas most areas along proximal–
distal axis were devoid of P-p38 (Fig. 3Q and R). Therefore, loss of
Cripto function can significantly affect the activation of p38 MAP
kinase, at least in embryonic ectoderm cells. The remaining
activation of p38 in Cripto null mutants could be due to functional
compensation from Cryptic (Chu and Shen, 2010). We also exam-
ined the expression of p38IP mRNA in Cripto mutant embryos and
found no changes compared to wild type embryos (data not shown).

Consistent with our observation, a recent study revealed that
Nodal affects AVE differentiation by activating p38 MAP kinase in
visceral endoderm before gastrulation (Clements et al., 2011).
These investigators also noticed that P-p38 was undetectable in the
visceral endoderm of Cripto null mutant embryo by

![Image](https://example.com/image.png)
immunostaining on E5.5 (Clements et al., 2011), demonstrating that Cripto can affect p38 activation in visceral endoderm.

**Cripto is required for embryonic endoderm development**

In addition to mesoderm, cells that migrate out of the primitive streak also form the definitive endoderm layer that is essential for many organs such as the gut (Lawson et al., 1991; Nagy, 2003; Tam et al., 2007; Tam and Loebel, 2007). Some of the Cripto conditional mutant embryos survived to late stage and displayed cardia bifida, in which cardiac progenitors failed to migrate towards the midline to form a linear heart tube, a defect also seen in the zebrafish Oep mutant embryos (Griffin and Kimelman, 2002) (Fig. 4A, B, D and E). To ensure that these mutant embryos underwent gastrulation, it is necessary to examine the cardia bifida in relation to A-P polarity establishment, mesoderm and endoderm formation. We therefore carried out in situ hybridization analyses using four probes simultaneously: Mlx2a for myocytes; Shh for axial mesoderm, ventral midline and endoderm; Meox1 for paraxial mesoderm; and Otx2 for anterior neural tissue. Mlx2a expression is shown in red and the other three are dark purple. As shown in Fig. 4, mutant embryos at E8.5 stained positively for all four marker genes, indicating that they underwent gastrulation and mesoderm formation, although Otx2 expression showed midline defects in anterior region (Fig. 4A, B, D and E). Similar to what occurs in Zebrafish Oep mutants, the cardia bifida in these mutants was probably caused by foregut endoderm defects, in particular defects in the ventral closure of the foregut (Fig. 4E). At early E7.5, the conditional mutant embryos expressed Sox17, a key regulatory gene in endoderm cell formation (Kanai-Azuma et al., 2002; Seguin et al., 2008), indicating the formation of endoderm cells. However, Sox17-expressing cells were missing in a region corresponding to the ventral midline region including the future gut (Tam et al., 2007) (Fig. 4C and F). Therefore, the endoderm cells were formed in the mutant embryos, but the regional allocation was perturbed, especially with regard to the future gut region. This endoderm cell allocation defect could be directly due to the cell migration defects in the primitive streak. Consistently, we found that the expression of Mxi1 was altered in these mutant embryos. Mxi1 is highly expressed in the primitive streak and the adjacent mesendoderm cells (Fig. 4G and H). However, in severe mutants that resemble Cripto-null mutants with no sign of the primitive streak and embryonic mesendoderm, Mxi1 expression was exclusively found in the extra-embryonic region (Fig. 4I). In embryos that formed an abnormal primitive streak and mesendoderm, no expression of Mxi1 was found in the primitive streak region and the adjacent mesendoderm except for a narrow band near the extra-embryonic region (Fig. 4K and L). Considering the importance of Mxi1 in the formation and allocation of endoderm cells (Hart et al., 2002; Tam et al., 2007), this mis-expression of Mxi1 may be responsible for the endoderm defects found in Cripto conditional mutant embryos. The importance of Oep in Zebrafish endoderm formation has been well documented (Griffin and Kimelman, 2002; Schier et al., 1997), and previous studies have reported the function of Cripto in mouse anterior definitive endoderm formation (Chu et al., 2005). The findings reported here extend previous studies and reveal a conserved role of EGF-CFC genes in general endoderm development, especially the regional allocation of endoderm cells.

**Discussion**

During mesoderm formation, epithelial epiblast cells undergo EMT and the resulting mesenchymal cells migrate out of the primitive streak area. It has been demonstrated that Snai1 is critical for EMT during mouse gastrulation (Carver et al., 2001). Snai1 mutant mouse embryos display defects in EMT and the cells originating from the primitive streak are more epithelial than mesenchymal in character and retain E-Cadherin expression (Carver et al., 2001). Surprisingly, these epithelial cells do not accumulate around the primitive streak area, but migrate out and form an intervening cell layer between the epiblast and the visceral endoderm (Carver et al., 2001). Therefore, it appears that an EMT defect is not sufficient to cause cell migration failure. In Fgf1 and Fgf8 mutants, cells accumulated around the primitive streak area and retained E-Cadherin expression, indicating defects in both EMT and mesoderm cell migration (Ciruna and Rossant, 2001; Deng et al., 1994; Sun et al., 1999). Since an EMT defect is not sufficient to cause cell accumulation around the primitive streak as shown by Snai1 mutants (Carver et al., 2001), the cell migration defect in Fgf8 and Fgf1 mutants may not be caused by the EMT defect. Snai1 and Tbx6 are two downstream targets of the Fgf8–Fgf1 pathway during gastrulation, and down-regulation of Snai1 expression is responsible for the EMT defect in Fgf1 and Fgf8 mutants (Ciruna and Rossant, 2001; Sun et al., 1999).

In this study, we investigated the function of Cripto during mouse gastrulation by conditional deletion of Cripto allele using Sox2-Cre. Sox2-Cre drives Cre gene expression throughout the epiblast, and the resulting the [Cripto(fl/fl):Sox2-Cre] embryos should, in principle, display pre-gastrulation defects like Cripto null mutant embryos. However, we obtained only a few [Cripto(fl/fl):Sox2-Cre] embryos that look like null mutant embryos, the majority of [Cripto(fl/fl):Sox2-Cre] embryos displayed late phenotype or no phenotype. This was probably due to the variation of Cre activity among [Cripto(fl/fl):Sox2-Cre] embryos and the non-cell autonomous nature of mouse Cripto function (Chu et al., 2005). Analysis of the resulting [Cripto(fl/fl):Sox2-Cre] revealed that conditional inactivation of Cripto function during gastrulation caused cells to accumulate around the primitive streak area. Unlike the Fgf8 and Fgf1 mutants, these accumulated cells were typical mesenchymal cells in morphology, with loose cell–cell adhesion and no expression of E-Cadherin, indicating EMT occurred normally in Cripto conditional mutants. Furthermore, previous studies revealed that Cripto null mutant embryos can still form mesenchymal mesoderm cells in the extra-embryonic region (Ding et al., 1998; Kimura et al., 2001), and we also found extensive blood cell formation in Cripto null mutant embryos (data not shown), indicating the EMT process is not affected even by the complete loss of Cripto function. We also found that the expression of Fgfr1 was down-regulated in mesoderm/mesenchymal cells in Cripto conditional mutant embryos. Moreover, the expression of Tbx6, a target of the Fgf8–Fgf1 pathway, was abolished in Cripto conditional mutants, whereas the expression of Snai1, another target of the Fgf8–Fgf1 pathway was unaffected. In addition, we showed that Snai1 expression was retained in Cripto null mutant embryos, further confirming that loss of Cripto does not down-regulate Snai1 expression. Therefore, the Fgf8–Fgf1 pathway was partially affected by the loss of Cripto function. Considering the importance of the Fgf8–Fgf1 pathway for mesoderm cell migration out of the primitive streak area as discussed above, disruption of this pathway may contribute to the cell accumulation in Cripto conditional mutants.

In addition to the Fgf8–Fgf1 pathway, our results showed that loss of Cripto function affects activation of p38 MAP kinase, and activation of p38 is essential for mesoderm cell migration during mouse gastrulation (Zohn et al., 2006). Interestingly, a recent report demonstrated that p38 activation in visceral endoderm by Nodal is required for anterior visceral endoderm differentiation and migration (Clements et al., 2011). AVE is first specified on the distal end before migration to the anterior end (Thomas et al., 1998). Clements and colleagues demonstrated that this process requires Nodal stimulated p38 activation in visceral endoderm on E5.5 (Clements et al., 2011). They also found that p38 activation in
visceral endoderm was undetectable in Cripto null mutant embryos as determined by P-p38 immunostaining, indicating that Cripto function is required for p38 activation in visceral endoderm at the pre-gastrulation stage (Clements et al., 2011). However, in Cripto null mutant embryos, the AVE is still specified in the distal end, but fails to migrate to anterior end (Ding et al., 1998). Because the differentiation of AVE still occurs in Cripto null mutant embryos, there should be a basal level of p38 activation maintained in the visceral endoderm of Cripto null mutant embryos. It is possible that in Cripto null mutant embryos, activation of p38 in the visceral endoderm is significantly reduced to a level below the sensitivity of P-p38 immunostaining, but sufficient to promote AVE differentiation in the distal end. In other words, the differentiation of AVE requires a low level of p38 activation, whereas the migration of AVE from the distal end to the anterior region requires a higher level of p38 activation, and Cripto is required only for high level activation of p38 in visceral endoderm. This situation may also applies to the epiblast or embryonic ectoderm, where loss of Cripto function leads to a low level of p38 activation sufficient only for EMT, a process of cell differentiation, but inadequate to promote the subsequent mesoderm cell migration. In p38IP mutant embryos, p38 activation may be reduced to a greater extent than in Cripto mutants and leads to defects in both EMT and cell migration as reported (Zohn et al., 2006). Therefore, Cripto interacts with both the Fgβ–Fgfr1 and p38 MAP kinase pathways to regulate mesoderm migration during gastrulation.

The phenotype described here resembles Nodal−/− embryos, a severe Nodal hypomorph mutant embryo, which displays massive accumulated mesoderm cells expressing Snai1 within a wider and shorter region (Ben-Haim et al., 2006). This is consistent with the notion that Cripto acts as a co-factor for Nodal during early embryogenesis (Chu and Shen, 2010; Shen and Schier, 2000).

During mouse gastrulation, cells from the primitive streak also contribute the definitive endoderm (Lawson et al., 1991; Nagy, 2003; Tam et al., 2007; Tam and Loebel, 2007). The defects in primitive streak cell migration should, in principle, affect the formation of embryonic endoderm. Consistently, we observed that a large portion of Cripto conditional mutant embryos that survived to late stage displayed defects in the ventral closure of foregut endoderm. Interestingly, these mutant embryos also suffered cardio bifida, a defect caused by ventral migration failure of cardiac progenitor cells. It is known that Mesp1 is required for this process (Saga et al., 1999), and Cripto function is required for its expression as shown by the current study. Therefore, the heart malformation observed here could be caused by direct defects in cardiac progenitor cell migration. In addition, the formation of a linear heart tube along the ventral midline is also dependent on the ventral foregut development, which was disrupted in these mutants. Therefore, the cardio bifida could be due to multiple mechanisms.

In conclusion, Cripto plays a critical role during mouse gastrulation, especially in mesoderm and endoderm formation. This function of Cripto is partially mediated through pathways involving Fgf receptor 1 and p38 MAP kinase.

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Appendix A. Supporting information

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References


