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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 is 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 *Cripto* 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 cardia 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.

Keywords

Cripto; mouse gastrulation; cell migration; mesoderm; endoderm; Fgf and p38

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 Behringer, 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

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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; Conlon 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), Mixl1(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-Fgfr1 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, demonstrating a functional requirement of Snai1 for EMT during gastrulation (Carver et al., 2001). Since an intervening epithelial cell layer still forms in *Snai1* mutant mouse embryos, it appears that cell migration out of the primitive streak is not necessarily dependent on 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 p38-α 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, Eomesodermin is required for EMT during mouse gastrulation that is independent of the Fgf8 pathway (Arnold et al., 2008). However, the relationship between Eomesodermin 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 $Cripto^{LacZ}$ null allele used in this study has been described before (Ding et al., 1998). The $Cripto^{flox}$ allele was generated by removing the floxed *PGK-Neo* region in the previously reported $Cripto^{3loxP}$ allele (Chu et al., 2005) using the *EIIa-Cre* line.

The generation of the *Cripto*$^{flox/flox}$ allele is schematically illustrated in supplementary figure 1. The following PCR primer pairs were used to genotype the *Cripto*^{flox} allele: 5′-GTG GTA AGT AAT TCC TCT TTC-3′ (forward) and AGG AAC ATT CCA ATG GCC TTG (reverse) detect the second *loxP* 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 AGC CTT CA-3′ (forward) and 5′-ACC TCC CCA CCA TCC A-3′ (reverse) were used to determine the first *loxP* 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*^{LacZ}, a *Fgf8* 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 hours 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 Jack Immuno 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, 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 figure 1. The resulting *Cripto*$^{flox/flox}$ mice are viable and develop normally. Crosses of *Cripto*$^{flox/flox}$ 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 figure 2). In addition, this *Cripto*^{flox} allele has been used in a recent study examining the function of *Cripto* in skeletal muscle regeneration (Guardiola et al., 2012; Michael Shen, personal communication).

To investigate the function of *Cripto* during mouse gastrulation, we utilized the *Cripto*^{flox} 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*$^{flox/flox}$ females. The majority of the resulting [*Cripto*$^{lacz/flox}$; *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 streak marker, on E7.5. Compared with wild type embryos (Fig.1A), [*Cripto*$^{lacz/flox}$; *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.

Fgf8-Fgfr1 pathway in mesoderm cells is partially affected by the loss of *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 (*Fgf8*$^{lacz/lacz}$) embryos, and found

that *Cripto* expression was highly retained in the accumulated mesoderm cells in *Fgf8*$^{lacz/lacz}$ 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*$^{lacz/flox}$:*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 *Snail1* was also 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 the Fgf8-Fgfr1 pathway is partially affected by the conditional inactivation of *Cripto*.

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*$^{lacz/flox}$:*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 *Fgf8, 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 *Fgf8*-null mutant embryos to determine if *Mesp1* expression is Fgf8 dependent, and found that *Mesp1* was 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 immunostaining 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 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 examined 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 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: *Mlc2a* for myocytes; *Shh* for axial mesoderm, ventral midline and endoderm; *Meox1* for paraxial mesoderm; and *Otx2* for anterior neural tissue. *Mlc2a* 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 *Mixl1* was altered in these mutant embryos. *Mixl1* 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, *Mixl1* expression was exclusively found in the extra-embryonic region (Fig.4I). In embryos that formed an abnormal primitive streak and mesendoderm, no expression of *Mixl1* was found in the primitive streak region and the adjacent mesoendoderm except for a narrow band near the extra embryonic region (Fig.4K and L). Considering the importance of *Mixl1* in the formation and allocation of endoderm cells (Hart et al., 2002; Tam et al., 2007), this mis-expression of *Mixl1* 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 *Fgfr1* 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 *Fgfr1* mutants may not be caused by the EMT defect. *Snai1* and *Tbx6* are two downstream targets of the Fgf8-Fgfr1 pathway during gastrulation, and down-regulation of *Snai1* expression is responsible for the EMT defect in *Fgfr1* 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 [$Cripto^{lacz/flox}$:*Sox2-Cre*] embryos should, in principle, display pre-gastrulation defects like *Cripto* null mutant embryos. However, we

obtained only a few [*Cripto*$^{lacz/flox}$:*Sox2-Cre*] embryos that look like null mutant embryos, the majority of [*Cripto*$^{lacz/flox}$; *Sox2-Cre*] embryos displayed late phenotype or no phenotype. This was probably due to the variation of Cre activity among [*Cripto*$^{lacz/flox}$:*Sox2-Cre*] embryos and the non-cell autonomous nature of mouse *Cripto* function (Chu et al., 2005). Analysis of the resulting [*Cripto*$^{lacz/flox}$:*Sox2-Cre*] revealed that conditional inactivation of *Cripto* function during gastrulation caused cells to accumulate around the primitive streak area. Unlike the *Fgf8* and *Fgfr1* 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-Fgfr1 pathway, was abolished in *Cripto* conditional mutants, whereas the expression of *Snai1*, another target of the Fgf8-Fgfr1 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-Fgfr1 pathway was partially affected by the loss of *Cripto* function. Considering the importance of the Fgf8-Fgfr1 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-Fgfr1 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 Fgf8-Fgfr1 and p38 MAP kinase pathways to regulate mesoderm migration during gastrulation.

The phenotype described here resembles $Nodal^{nr/nr}$ embryos, a severe *Nodal* hypomorphic 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 venture closure of foregut endoderm. Interestingly, these mutant embryos also suffered cardia 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 cardia bifida could be due to multiple mechanisms.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

Adams RH, Porras A, Alonso G, Jones M, Vintersten K, Panelli S, Valladares A, Perez L, Klein R, Nebreda AR. Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. Mol Cell. 2000; 6:109–116. [PubMed: 10949032]

Ang SL, Rossant J. HNF-3 beta is essential for node and notochord formation in mouse development. Cell. 1994; 78:561–574. [PubMed: 8069909]

Arnold SJ, Hofmann UK, Bikoff EK, Robertson EJ. Pivotal roles for eomesodermin during axis formation, epithelium-to-mesenchyme transition and endoderm specification in the mouse. Development. 2008; 135:501–511. [PubMed: 18171685]

Bachiller D, Klingensmith J, Kemp C, Belo JA, Anderson RM, May SR, McMahon JA, McMahon AP, Harland RM, Rossant J, De Robertis EM. The organizer factors Chordin and Noggin are required for mouse forebrain development. Nature. 2000; 403:658–661. [PubMed: 10688202]

Barrallo-Gimeno A, Nieto MA. The Snail genes as inducers of cell movement and survival: implications in development and cancer. Development. 2005; 132:3151–3161. [PubMed: 15983400]

Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol. 2000; 2:84–89. [PubMed: 10655587]

Beddington R. Cripto-analysis of embryonic codes. Nature. 1998; 395:641, 643. [PubMed: 9790182]

Beddington RS. Induction of a second neural axis by the mouse node. Development. 1994; 120:613–620. [PubMed: 8162859]

Beddington RS, Robertson EJ. Anterior patterning in mouse. Trends Genet. 1998; 14:277–284. [PubMed: 9676530]

Beddington RS, Robertson EJ. Axis development and early asymmetry in mammals. Cell. 1999; 96:195–209. [PubMed: 9988215]

Ben-Haim N, Lu C, Guzman-Ayala M, Pescatore L, Mesnard D, Bischofberger M, Naef F, Robertson EJ, Constam DB. The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev Cell. 2006; 11:313–323. [PubMed: 16950123]

Brennan J, Lu CC, Norris DP, Rodriguez TA, Beddington RS, Robertson EJ. Nodal signalling in the epiblast patterns the early mouse embryo. Nature. 2001; 411:965–969. [PubMed: 11418863]

Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, Evans S. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell. 2003; 5:877–889. [PubMed: 14667410]

Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol. 2000; 2:76–83. [PubMed: 10655586]

Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol Cell Biol. 2001; 21:8184–8188. [PubMed: 11689706]

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996; 383:407–413. [PubMed: 8837770]

Chu J, Ding J, Jeays-Ward K, Price SM, Placzek M, Shen MM. Non-cell-autonomous role for Cripto in axial midline formation during vertebrate embryogenesis. Development. 2005; 132:5539–5551. [PubMed: 16291788]

Chu J, Shen MM. Functional redundancy of EGF-CFC genes in epiblast and extraembryonic patterning during early mouse embryogenesis. Dev Biol. 2010; 342:63–73. [PubMed: 20346354]

Ciruna B, Rossant J. FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell. 2001; 1:37–49. [PubMed: 11703922]

Clements M, Pernaute B, Vella F, Rodriguez TA. Crosstalk between Nodal/Activin and MAPK p38 Signaling Is Essential for Anterior-Posterior Axis Specification. Curr Biol. 2011; 21:1289–1295. [PubMed: 21802298]

Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, Robertson EJ. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development. 1994; 120:1919–1928. [PubMed: 7924997]

Deng CX, Wynshaw-Boris A, Shen MM, Daugherty C, Ornitz DM, Leder P. Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. 1994; 8:3045–3057. [PubMed: 8001823]

Ding J, Yang L, Yan YT, Chen A, Desai N, Wynshaw-Boris A, Shen MM. Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. Nature. 1998; 395:702–707. [PubMed: 9790191]

Dono R, Scalera L, Pacifico F, Acampora D, Persico MG, Simeone A. The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. Development. 1993; 118:1157–1168. [PubMed: 7916676]

Downs KM, Hellman ER, McHugh J, Barrickman K, Inman KE. Investigation into a role for the primitive streak in development of the murine allantois. Development. 2004; 131:37–55. [PubMed: 14645124]

Dunn NR, Vincent SD, Oxburgh L, Robertson EJ, Bikoff EK. Combinatorial activities of Smad2 and Smad3 regulate mesoderm formation and patterning in the mouse embryo. Development. 2004; 131:1717–1728. [PubMed: 15084457]

Fujiwara T, Dehart DB, Sulik KK, Hogan BL. Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. Development. 2002; 129:4685–4696. [PubMed: 12361961]

Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature. 1998; 391:357–362. [PubMed: 9450748]

Gray PC, Harrison CA, Vale W. Cripto forms a complex with activin and type II activin receptors and can block activin signaling. Proc Natl Acad Sci U S A. 2003; 100:5193–5198. [PubMed: 12682303]

Griffin KJ, Kimelman D. One-Eyed Pinhead and Spadetail are essential for heart and somite formation. Nat Cell Biol. 2002; 4:821–825. [PubMed: 12360294]

Guardiola O, Lafuste P, Brunelli S, Iaconis S, Touvier T, Mourikis P, De Bock K, Lonardo E, Andolfi G, Bouche A, Liguori GL, Shen MM, Tajbakhsh S, Cossu G, Carmeliet P, Minchiotti G. Cripto regulates skeletal muscle regeneration and modulates satellite cell determination by antagonizing myostatin. Proc Natl Acad Sci U S A. 2012; 109:E3231–3240. [PubMed: 23129614]

Hart AH, Hartley L, Sourris K, Stadler ES, Li R, Stanley EG, Tam PP, Elefanty AG, Robb L. Mixl1 is required for axial mesendoderm morphogenesis and patterning in the murine embryo. Development. 2002; 129:3597–3608. [PubMed: 12117810]

Harvey RP. Patterning the vertebrate heart. Nat Rev Genet. 2002; 3:544–556. [PubMed: 12094232]

Hayashi S, Lewis P, Pevny L, McMahon AP. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Gene Expr Patterns. 2002; 2:93–97. [PubMed: 12617844]

Hayashi S, Tenzen T, McMahon AP. Maternal inheritance of Cre activity in a Sox2Cre deleter strain. Genesis. 2003; 37:51–53. [PubMed: 14595839]

Hogan BL. Bone morphogenetic proteins in development. Curr Opin Genet Dev. 1996; 6:432–438. [PubMed: 8791534]

Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W. Requirement for beta-catenin in anterior-posterior axis formation in mice. J Cell Biol. 2000; 148:567–578. [PubMed: 10662781]

Iratni R, Yan YT, Chen C, Ding J, Zhang Y, Price SM, Reinberg D, Shen MM. Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. Science. 2002; 298:1996–1999. [PubMed: 12471260]

Kanai-Azuma M, Kanai Y, Gad JM, Tajima Y, Taya C, Kurohmaru M, Sanai Y, Yonekawa H, Yazaki K, Tam PP, Hayashi Y. Depletion of definitive gut endoderm in Sox17-null mutant mice. Development. 2002; 129:2367–2379. [PubMed: 11973269]

Kimelman D. Mesoderm induction: from caps to chips. Nat Rev Genet. 2006; 7:360–372. [PubMed: 16619051]

Kimura C, Shen MM, Takeda N, Aizawa S, Matsuo I. Complementary functions of Otx2 and Cripto in initial patterning of mouse epiblast. Dev Biol. 2001; 235:12–32. [PubMed: 11412024]

Lawson KA, Meneses JJ, Pedersen RA. Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development. 1991; 113:891–911. [PubMed: 1821858]

Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. Nat Genet. 1999; 22:361–365. [PubMed: 10431240]

Lowe LA, Yamada S, Kuehn MR. Genetic dissection of nodal function in patterning the mouse embryo. Development. 2001; 128:1831–1843. [PubMed: 11311163]

Mao B, Wu W, Li Y, Hoppe D, Stannek P, Glinka A, Niehrs C. LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature. 2001; 411:321–325. [PubMed: 11357136]

Meno C, Gritsman K, Ohishi S, Ohfuji Y, Heckscher E, Mochida K, Shimono A, Kondoh H, Talbot WS, Robertson EJ, Schier AF, Hamada H. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. Mol Cell. 1999; 4:287–298. [PubMed: 10518210]

Mudgett JS, Ding J, Guh-Siesel L, Chartrain NA, Yang L, Gopal S, Shen MM. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc Natl Acad Sci U S A. 2000; 97:10454–10459. [PubMed: 10973481]

Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the mouse embryo: a laboratory manual (3rd edition). 2003 3rd ed.

Nomura M, Li E. Smad2 role in mesoderm formation, left-right patterning and craniofacial development. Nature. 1998; 393:786–790. [PubMed: 9655392]

Piccolo S, Agius E, Leyns L, Bhattacharyya S, Grunz H, Bouwmeester T, De Robertis EM. The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature. 1999; 397:707–710. [PubMed: 10067895]

Saga Y, Miyagawa-Tomita S, Takagi A, Kitajima S, Miyazaki J, Inoue T. MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development. 1999; 126:3437–3447. [PubMed: 10393122]

Schier AF, Neuhauss SC, Helde KA, Talbot WS, Driever W. The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. Development. 1997; 124:327–342. [PubMed: 9053309]

Schier AF, Shen MM. Nodal signalling in vertebrate development. Nature. 2000; 403:385–389. [PubMed: 10667782]

Seguin CA, Draper JS, Nagy A, Rossant J. Establishment of endoderm progenitors by SOX transcription factor expression in human embryonic stem cells. Cell Stem Cell. 2008; 3:182–195. [PubMed: 18682240]

Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, He X. Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr Biol. 2001; 11:951–961. [PubMed: 11448771]

Shawlot W, Behringer RR. Requirement for Lim1 in head-organizer function. Nature. 1995; 374:425–430. [PubMed: 7700351]

Shawlot W, Wakamiya M, Kwan KM, Kania A, Jessell TM, Behringer RR. Lim1 is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse. Development. 1999; 126:4925–4932. [PubMed: 10529411]

Shen MM. Identification of differentially expressed genes in mouse development using differential display and in situ hybridization. Methods. 2001; 24:15–27. [PubMed: 11327798]

Shen MM. Nodal signaling: developmental roles and regulation. Development. 2007; 134:1023–1034. [PubMed: 17287255]

Shen MM, Schier AF. The EGF-CFC gene family in vertebrate development. Trends Genet. 2000; 16:303–309. [PubMed: 10858660]

Shen MM, Wang H, Leder P. A differential display strategy identifies Cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. Development. 1997; 124:429–442. [PubMed: 9053319]

Sun X, Meyers EN, Lewandoski M, Martin GR. Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes Dev. 1999; 13:1834–1846. [PubMed: 10421635]

Tam PP, Behringer RR. Mouse gastrulation: the formation of a mammalian body plan. Mech Dev. 1997; 68:3–25. [PubMed: 9431800]

Tam PP, Khoo PL, Lewis SL, Bildsoe H, Wong N, Tsang TE, Gad JM, Robb L. Sequential allocation and global pattern of movement of the definitive endoderm in the mouse embryo during gastrulation. Development. 2007; 134:251–260. [PubMed: 17151016]

Tam PP, Loebel DA. Gene function in mouse embryogenesis: get set for gastrulation. Nat Rev Genet. 2007; 8:368–381. [PubMed: 17387317]

Tam PP, Tan SS. The somitogenetic potential of cells in the primitive streak and the tail bud of the organogenesis-stage mouse embryo. Development. 1992; 115:703–715. [PubMed: 1425350]

Thomas PQ, Brown A, Beddington RS. Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. Development. 1998; 125:85–94. [PubMed: 9389666]

Varlet I, Collignon J, Robertson EJ. nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. Development. 1997; 124:1033–1044. [PubMed: 9056778]

Vincent SD, Dunn NR, Hayashi S, Norris DP, Robertson EJ. Cell fate decisions within the mouse organizer are governed by graded Nodal signals. Genes Dev. 2003; 17:1646–1662. [PubMed: 12842913]

Vincent SD, Robertson EJ. Highly efficient transgene-independent recombination directed by a maternally derived SOX2CRE transgene. Genesis. 2003; 37:54–56. [PubMed: 14595840]

Waldrip WR, Bikoff EK, Hoodless PA, Wrana JL, Robertson EJ. Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. Cell. 1998; 92:797–808. [PubMed: 9529255]

Weinstein M, Yang X, Li C, Xu X, Gotay J, Deng CX. Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppressor smad2. Proc Natl Acad Sci U S A. 1998; 95:9378–9383. [PubMed: 9689088]

Yamaguchi TP, Harpal K, Henkemeyer M, Rossant J. fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev. 1994; 8:3032–3044. [PubMed: 8001822]

Yan YT, Liu JJ, Luo Y, E, C. Haltiwanger RS, Abate-Shen C, Shen MM. Dual roles of Cripto as a ligand and coreceptor in the nodal signaling pathway. Mol Cell Biol. 2002; 22:4439–4449. [PubMed: 12052855]

Zhou X, Sasaki H, Lowe L, Hogan BL, Kuehn MR. Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. Nature. 1993; 361:543–547. [PubMed: 8429908]

Zohn IE, Li Y, Skolnik EY, Anderson KV, Han J, Niswander L. p38 and a p38-interacting protein are critical for downregulation of E-cadherin during mouse gastrulation. Cell. 2006; 125:957–969. [PubMed: 16751104]

Highlights

- Mesoderm migration is a fundamental, but poorly understood question.
- Only a limited genes and pathways identified such as Fgf and p38 MAP.
- We found mouse *Cripto* gene is required for this process.
- Loss of *Cripto* function affects both the Fgf8-Fgfr1 and p38 MAP kinase pathway.
- Therefore, Cripto could be a part of the link connecting the two pathways.

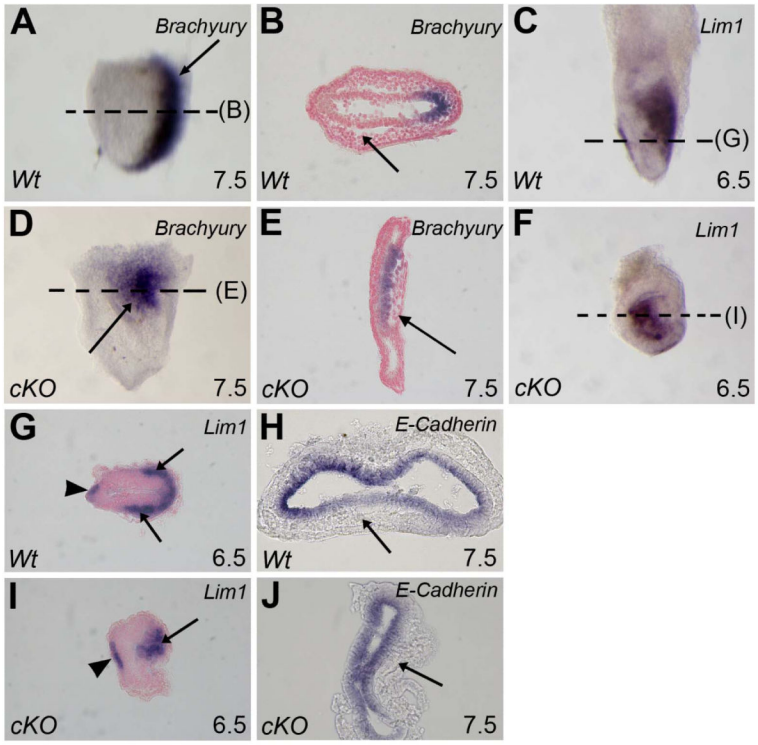

Figure 1. Mesoderm cell migration defects in *Cripto* conditional mutant embryos

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.

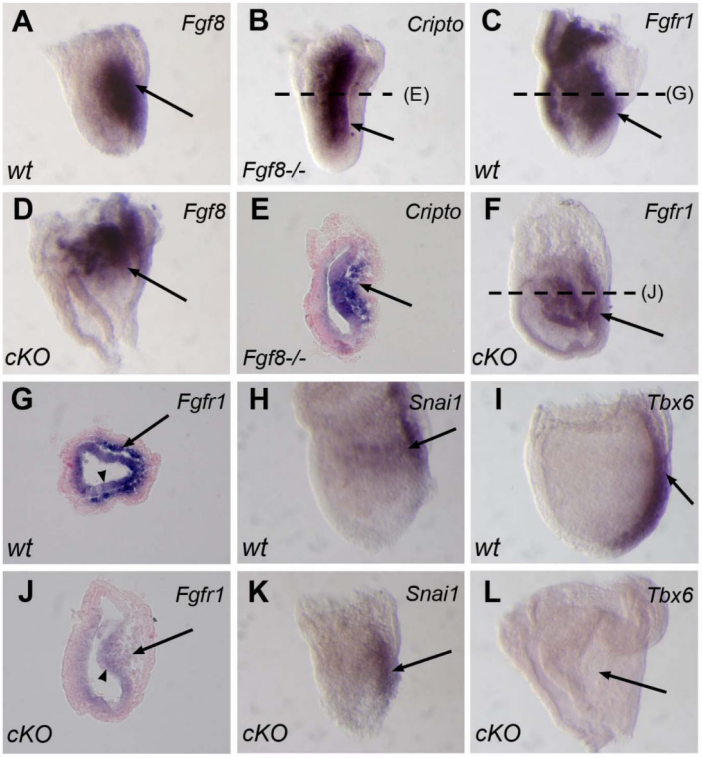

Figure 2. The Fgf8-Fgfr1 pathway is partially affected in *Cripto* conditional mutant embryos

(**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***$^{-/-}$: *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.

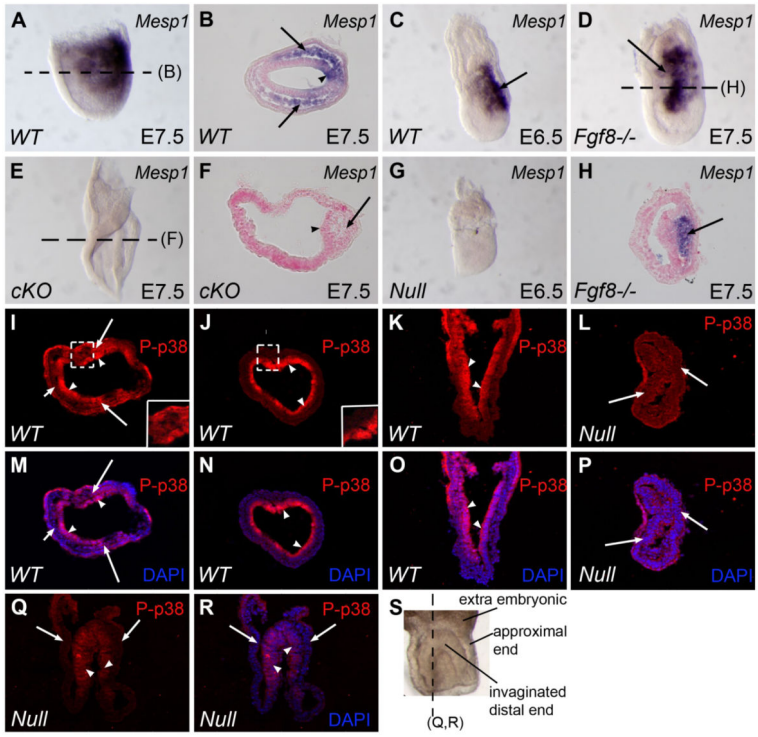

Figure 3. Loss of *Cripto* function abolishes *Mesp1* expression and dramatically reduces p38 MAP kinase activation in the embryonic ectoderm

(**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 (G). In contrast, *Mesp1* is still highly expressed in *Fgf8* 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-K and M-O). In some areas, most of the mesoderm cells are positive for P-p38 (arrows in I and M), but a few mesoderm cells are negative (short arrows 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 L and P), and frontal sections show that most of the ectoderm cells are P-p38 negative (arrows in Q and R), except for a small region around the ectoderm on distal end (arrow heads in Q and R). ***Wt***: wild type; ***cKO***: conditional knock-out; ***Fgf8***$^{-/-}$: *Fgf8* 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.

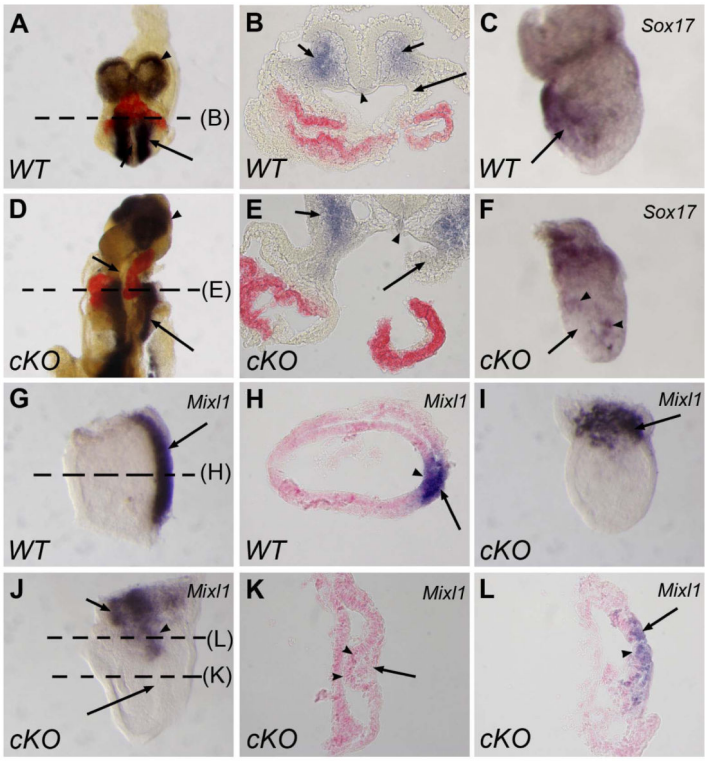

Figure 4. Loss of *Cripto* function affects definitive endoderm cell allocation during gastrulation

(**A** and **D**) whole-mount view of wild type (A) and *Cripto* conditional mutants (D) embryos on E8.5 showing expression of *Otx2* (arrowheads), *Meox-1* (arrows), *Shh* (short arrows) and *Mlc2a* (red). (**B** and **E**) Cross sections of (A) and (D) showing *Meox-1* positive paraxial mesoderm tissue, somites (short arrows), *Shh* positive axial midline structure, notochord (arrowheads) and foregut endoderm cells expressing *Shh* (arrows). (**C** and **F**) whole-mount view showing *Sox17*-expressing endoderm cells form a patch in wild type embryo on early E7.5 including the area corresponding to the future ventral foregut (arrow in C), whereas *Sox17*-expressing cells are present in E7.5 *Cripto* conditional mutant embryos (arrow heads in F), but were missing from the area corresponding to the future ventral gut (arrow in F). (**G**-**L**) *Mixl1* is highly expressed in the E7.5 wild type embryos (G) including the primitive streak (arrow head in H) and the adjacent mesendoderm cells (arrow in H). For severely affected E7.5 *Cripto* conditional mutant embryos that have no primitive streak and mesendoderm cells, the expression of *Mixl1* was found only in the proximal extra-embryonic region (arrow in I), and for less severely affected embryos that do form mesendoderm layer (arrow in J), only a trace level of *Mixl1* expression was found in the primitive streak (arrow heads in K) and no signals were found in the adjacent mesendoderm (arrow in K). Only the extra-embryonic region (short arrow in J) and the area near the edge of the embryonic region (arrow head in J) showed positive signals in mesendoderm cells (arrow in L). ***Wt***: wild type; ***cKO***: conditional knock-out. The dashed lines in panels A, D,G and J represent the positions of cross-sections shown in panels B, E, H and (L,K), respectively.