Cripto promotes A–P axis specification independently of its stimulatory effect on Nodal autoinduction

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The EGF-CFC gene cripto governs anterior–posterior (A–P) axis specification in the vertebrate embryo. Existing models suggest that Cripto facilitates binding of Nodal to an ActRII–activin-like kinase (ALK) 4 receptor complex. Cripto also has a crucial function in cellular transformation that is independent of Nodal and ALK4. However, how ALK4-independent Cripto pathways function in vivo has remained unclear. We have generated cripto mutants carrying the amino acid substitution F78A, which blocks the Nodal–ALK4–Smad2 signaling both in embryonic stem cells and cell-based assays.

In criptoF78A/F78A mouse embryos, Nodal fails to expand its own expression domain and that of cripto, indicating that F78 is essential in vivo to stimulate Smad-dependent Nodal autoinduction. In sharp contrast to cripto-null mutants, criptoF78A/F78A embryos establish an A–P axis and initiate gastrulation movements. Our findings provide in vivo evidence that Cripto is required in the Nodal–Smad2 pathway to activate an autoinductive feedback loop, whereas it can promote A–P axis formation and initiate gastrulation movements independently of its stimulatory effect on the canonical Nodal–ALK4–Smad2 signaling pathway.

Introduction

Cripto, a glycophaspatidylinositol (GPI)-linked membrane protein, is the founding member of a family of vertebrate signaling molecules, the EGF–Cripto-FRL1-Cryptic (CFC) family, which includes human, mouse, and chick Cripto (Ciccodicola et al., 1989; Dono et al., 1993; Colas and Schoenwolf, 2000), human and mouse Cryptic (Shen et al., 1997), Xenopus laevis FRL-1/XCR1, XCR2, and XCR3 (Kinoshita et al., 1995; Dorey and Hill, 2006; Onuma et al., 2006), and zebrafish one-eyed pinhead (oep; Zhang et al., 1998). During development, members of the EGF-CFC family are required for mesoderm and endoderm formation and patterning of the anterior–posterior (A–P) and left–right axes (Shen and Schier, 2000). Genetic studies and cell-based assays have shown that the EGF-CFC proteins stimulate signaling by the TGF-β–related Nodal (Shen and Schier, 2000). Moreover, receptor reconstitution experiments and coimmunoprecipitation assays suggest that Cripto interacts with Nodal and the activin type IB receptor (activin-like kinase [ALK] 4), thereby activating a complex with the activin type IIB serine/threonine kinase (ActRIIB) receptor (Reissmann et al., 2001; Yeo and Whitman, 2001; Bianco et al., 2002; Sakuma et al., 2002; Yan et al., 2002). Upon receptor activation, the intracellular kinase domain of the type I receptor phosphorlylates Smad2 and/or Smad3, which form a hexameric complex with the common Smad4 and translocate into the nucleus to regulate gene expression in conjunction with other transcription factors such as FoxH1 (Massague and Chen, 2000; Adkins et al., 2003; Gray et al., 2003; Harrison et al., 2005). Similarly, Cripto can sensitize a complex of ActRIIB and ALK7 to Nodal (Reissmann et al., 2001), and it also interacts with a subset of related ligands such as GDF1 and 3 (Cheng et al., 2003; Chen and Shen, 2004). Furthermore, Cripto has been found to bind specific Nodal antagonists, such as the transmembrane protein tomor-gulin-1 (TMEFF-1; Harms and Chang, 2003) or the TGF-β–related Lefty proteins (Chen and Shen, 2004). However, the structural determinants that mediate these diverse protein–protein interactions and their relative influence on specific signaling pathways in the embryo are poorly defined.

Consistent with an important role for cripto in Nodal signaling, loss-of-function analysis in the mouse has shown that cripto is essential for both primitive streak formation and conversion of the initial proximal-distal patterning into the A–P
like tyrosine kinase (Bianco et al., 2003). However, without reagents that prevent endogenous Cripto from activating canonical ALK signaling, it has remained difficult to directly assess the physiological role of ALK-independent pathways.

Several structural determinants have been identified in the EGF and the CFC domains that regulate Cripto activity in cell transfection and X. laevis injection assays. Specifically, the CFC domain is essential for ALK4 interaction (Yeo and Whitman, 2001; Adkins et al., 2003), whereas threonine 72 in the EGF domain is O-fucosylated (Schiffer et al., 2001) and, apparently, promotes Nodal binding (Yeo and Whitman, 2001; Yan et al., 2002). It is worth noting that recent data indicate that the threonine residue that carries fucose, but not fucose per se, is required for Cripto to facilitate Nodal signaling (Shi et al., 2007). Furthermore, rescue experiments in cripto−/− mouse embryonic stem cells and in oep mutant zebrafish established that recombinant Cripto protein also relies on the conserved amino acid F78 (Minchiotti et al., 2001; Parisi et al., 2003). However, whether axis during gastrulation (Ding et al., 1998; Liguori et al., 2003). However, cripto-null embryos express posterior markers, such as Brachyury and Fgf8, and form anterior neural structures and extra-embryonic mesoderm, whereas Nodal mutants do not (Brennan et al., 2001). Thus, Nodal promotes anterior and posterior fates through both Cripto-dependent and -independent pathways.

Cripto has also been implicated in stimulating the progression of a broad spectrum of tumors (Salomon et al., 1999). Expression of cripto is increased severalfold in human colon, gastric, pancreatic, and lung carcinomas and in a variety of different types of mouse and human breast carcinomas (Ciardiello et al., 1991; Baldassarre et al., 1997). Although a specific receptor for Cripto has not yet been identified in mammary gland or cancer cells, mouse and human Cripto can activate a ras-raf-MAP kinase signaling pathway. This response may depend on the ability of Cripto to transactivate erbB-4 and/or FGF receptor 1 or to specifically bind to a membrane-associated heparan sulfate proteoglycan, glypican 1, leading to the activation of a Src-like tyrosine kinase (Bianco et al., 2003). However, without reagents that prevent endogenous Cripto from activating canonical ALK signaling, it has remained difficult to directly assess the physiological role of ALK-independent pathways.

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were enlarged (Fig. 2, B and B'), apparently at the expense of mesodermal structures, because somites and a beating heart were absent. These results show that residue F78 of Cripto is essential for postimplantation development.

A–P axis and mesendoderm formation in \textit{cripto} F78A/F78A mutants

Loss-of-function analysis has shown that \textit{cripto} converts proximal-distal patterning into an A–P axis and promotes primitive streak formation (Ding et al., 1998; Liguori et al., 2003). To assess

\begin{table}
\caption{Genotypes of embryos and viable offspring from heterozygous intercrosses}
\begin{tabular}{lllll}
\hline
Stage & Number of litters analyzed & Number of animals obtained & Genotype (%) \\
\hline
E 6.5 & 35 & 220 & +/+ & 32 & +/F78A & 48 & F78A/F78A & 20 \\
E 7.5 & 45 & 304 & +/+ & 20 & +/F78A & 49 & F78A/F78A & 31 \\
E 8.5 & 33 & 221 & +/+ & 20 & +/F78A & 47 & F78A/F78A & 33 \\
E 9.5 & 4 & 30 & +/+ & 22 & +/F78A & 51 & F78A/F78A & 27 \\
E 10.5 & 3 & 26 & +/+ & 18 & +/F78A & 48 & F78A/F78A & 34 \\
E 11.5 & 4 & 40 & +/+ & 25 & +/F78A & 70 & F78A/F78A & 5 \\
E 12.5 & 4 & 40 & +/+ & 33 & +/F78A & 67 & F78A/F78A & 0 \\
From newborn to adult & 100 & 602 & +/+ & 35 & +/F78A & 65 & F78A/F78A & 0 \\
\hline
\end{tabular}
\textit{E}, embryonic day.
\end{table}

F78 is essential for all Cripto activities or whether it specifically promotes Nodal signaling has remained unclear.

In this paper, we provide direct evidence that Cripto$^{F78A}$ is unable to activate detectable amounts of Smad2 in embryonic stem (ES) cell–derived embryoid bodies (EBs) and that it fails to stimulate canonical Nodal–ALK4–Smad2 signaling in cell-based luciferase reporter assays. Further analysis of \textit{cripto} F78A/F78A mutant embryos confirms that residue F78 is essential to potentiate autoregulatory feedback signaling mediated by the Nodal–ALK4–Smad–FoxH1 pathway. We show that, unlike \textit{cripto}-null mutants, \textit{cripto} F78A/F78A embryos clearly establish an A–P axis and initiate germ layer formation and gastrulation movements. A subset of known Nodal effector genes that are down-regulated in \textit{cripto}-null mutants are significantly induced in \textit{cripto} F78A/F78A embryos. Collectively, these results suggest that Cripto can promote axis formation and gastrulation movements independently of its known stimulatory effect on the canonical Nodal–ALK4–Smad2 pathway.

\section*{Results}

\textit{cripto} F78A/F78A mutants are embryonic lethal

To unravel the complex network of molecular interactions of Cripto with its target proteins in vivo, the amino acid residue F78, which is located in the EGF-like domain, was substituted by alanine (F78A) using Cre/loxP-mediated recombination (Fig. 1, A–C). The resulting heterozygous \textit{cripto}$^{F78A}$ mice appeared phenotypically normal and were fertile; however, homozygosity for the \textit{cripto}$^{F78A}$-targeted allele resulted in embryonic lethality. We first verified the expression of the mutated allele in vivo by whole-mount immunohistochemistry analysis. Cripto protein was consistently detected in homozygous \textit{cripto} F78A/F78A embryos, although its expression remained confined to the proximal epiblast (Fig. 1 D). Although this result indicates that the alanine substitution does not abolish the synthesis or stability of Cripto protein, expansion of the expression domain to the distal epiblast is clearly compromised. Upon dissection, \textit{cripto} F78A/F78A embryos were recovered at the expected mendelian ratio until 10.5 d past confluence (dpc) and later were resorbed (Table I). However, at 7.5 dpc they already displayed ectopic folds in the embryonic region (Fig. 2, A and A'). At 8.5 dpc, mutant embryos failed to turn and the neural folds were enlarged (Fig. 2, B and B'), apparently at the expense of mesodermal structures, because somites and a beating heart were absent. These results show that residue F78 of Cripto is essential for postimplantation development.

\begin{figure}
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\includegraphics[width=0.5\textwidth]{figure2.jpg}
\caption{Homozygous mutant \textit{cripto}$^{F78A/F78A}$ embryos initiate gastrulation but subsequently arrest development. Morphology of wild-type embryos (A–D) and \textit{cripto}$^{F78A/F78A}$ mutant litter mates (A'–D') dissected at 7.5 (A and A'), 8.5 (B and B'), 9.5 (C and C'), and 10.5 (D and D') dpc. Both anterior and posterior structures that are absent in \textit{cripto}-null mutants (Ding et al., 1998; Liguori et al., 2003) can be clearly recognized at up to 8.5 dpc but subsequently deteriorate (C' and D'). Bars, 40 μm.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure3.jpg}
\caption{Homozygous mutant \textit{cripto}$^{F78A/F78A}$ embryos initiate gastrulation but subsequently arrest development. Morphology of wild-type embryos (A–D) and \textit{cripto}$^{F78A/F78A}$ mutant litter mates (A'–D') dissected at 7.5 (A and A'), 8.5 (B and B'), 9.5 (C and C'), and 10.5 (D and D') dpc. Both anterior and posterior structures that are absent in \textit{cripto}-null mutants (Ding et al., 1998; Liguori et al., 2003) can be clearly recognized at up to 8.5 dpc but subsequently deteriorate (C' and D'). Bars, 40 μm.}
\end{figure}
whether crypto<sup>F78A/F78A</sup> embryos have defects in axis formation, we examined the expression of asymmetrically expressed marker genes such as Brachyury and Otx2 at 7.5 dpc. In normal embryos, Brachyury marks the primitive streak, whereas expression of the anterior neural marker Otx2 by this stage is restricted to the opposite pole (Fig. 3 A; Wilkinson et al., 1990; Simeone et al., 1993). By comparison, crypto-null mutants largely consist of anterior neuroectoderm (Ding et al., 1998; Liguori et al., 2003) and, therefore, ectopically express Otx2 throughout the distal embryonic region (Fig. 3 A'; Ding et al., 1998; Liguori et al., 2003), whereas the mesodermal marker Brachyury is only activated in a few cells along the embryonic–extraembryonic boundary (Fig. 3 A''; Ding et al., 1998). In contrast, in crypto<sup>F78A/F78A</sup> mutant embryos, Brachyury expression was normally posteriorized and persisted until 8.5 dpc, indicating the presence of posterior mesoderm populations that are missing in crypto-null mutants (Fig. 3 A'; and Fig. S1, A, A', and A'', available at http://www.jcb.org/cgi/content/full/jcb.200709090/DC1). In addition, Otx2 mRNA was consistently localized in the anterior region (Fig. 3 A'; and Fig. S1, A and A'), suggesting that A–P patterning is relatively normal. To monitor posterior neuroectoderm, we also analyzed the expression of Krox20, a marker of rhombomeres three and five, which is absent in crypto-null mutants (Ding et al., 1998). Krox20 mRNA was clearly detected in crypto<sup>F78A/F78A</sup> embryos at 8.5 dpc (Fig. S1, B and B'). In addition, Mox1, a marker of paraxial mesoderm that fails to be induced in crypto-null mutants, was expressed in the posterior region of crypto<sup>F78A/F78A</sup> embryos (Fig. S1, C and C'). These results demonstrate that crypto<sup>F78A/F78A</sup> homozygotes establish an A–P axis and arrest development at a later stage compared with null mutants.

To characterize gastrulation defects in crypto<sup>F78A/F78A</sup> mutant embryos, we next visualized derivatives of the anterior primitive streak, such as the node, a structure that expresses Nodal and Cer-1 mRNAs (Fig. 3, B–E; Monaghan et al., 1993; Conlon et al., 1994; Biben et al., 1998; Bachiller et al., 2000). In crypto-null mutants, expression of Foxa2 was absent and Nodal expression was confined to the proximal epiblast, confirming that node formation is inhibited (Fig. 3 B''). In crypto<sup>F78A/F78A</sup> mutants, Nodal and Foxa2 expression were clearly detected in the distal tip of the rudimentary
primitive streak, suggesting that anterior primitive streak derivatives are specified (Fig. 3, B’ and C’). Moreover, Foxa2 mRNA staining revealed that axial mesendoderm populations are also present more anteriorly (Fig. 3 C’), whereas they are missing in cripto-null mutants (Fig. 3 C’’). Similarly, Cer-1 was clearly induced in 5 out of 10 cripto/F78A mutants, even though the mRNA level was reduced and its expression domain extended to more posterior regions compared with wild-type controls (Fig. 3, D and D’). Likewise, Chordin was undetectable in cripto-null embryos but expressed in 3 out of 10 of the cripto/F78A mutants that were analyzed (Fig. 3, E and E’ [arrowhead]). Thus, compared with a cripto-null mutation, the F78A substitution has only relatively mild inhibitory effects on mesendoderm and primitive streak formation.

**Nodal signaling is impaired in cripto/F78A/F78A embryos**

Several studies in mice, X. laevis, and zebrafish link Cripto to the Nodal pathway (Shen and Schier, 2000). Therefore, to assess the role of residue F78 of Cripto, we analyzed the expression pattern of Nodal and its target genes, Lefty1 and 2, in cripto/F78A/F78A and cripto-null mutants at 6.75 dpc. At this stage, Nodal is expressed throughout the primitive streak and posterior mesoderm in wild-type embryos (Fig. 4 A; Conlon et al., 1994; Collignon et al., 1996). In contrast, in both cripto/F78A/F78A and cripto-null mutants, Nodal expression was reduced and remained at the rim of the proximal epiblast (Fig. 4, A’ and A’’). Next, to assess whether Nodal signaling was induced, we analyzed the expression of Lefty1 and 2. In wild-type embryos at 6.75 dpc, Lefty1 is expressed in the anterior visceral endoderm, whereas Lefty2 marks the nascent mesoderm generated from the primitive streak (Fig. 4 B; Meno et al., 1997). Expression of both Lefty1 and 2 was absent in cripto-null mutants (Fig. 4 B’). Interestingly, both genes were induced in cripto/F78A/F78A embryos, although below normal levels (Fig. 4 B’’). To determine whether Nodal signaling is also maintained at later stages in cripto/F78A/F78A embryos, we analyzed the expression pattern of Lefty2, a direct Nodal target gene, and Fgfl8 at 7.5 dpc. As expected, both genes were readily detectable in the primitive streak of wild-type embryos (Fig. 4 C) but not in cripto-null mutants (Fig. 4 C’’). In contrast, Lefty2 mRNA was detected in a subset of cells in the posterior side of cripto/F78A/F78A embryos (Fig. 4 C’). Furthermore, Fgfl8 was expressed in cripto/F78A/F78A mutants and its expression domain was even enlarged and extended into the extraembryonic region (Fig. 4 C’’). Collectively, these data strongly suggest that the strength or duration of Nodal signaling in cripto/F78A/F78A embryos is perturbed compared with wild-type embryos, although it significantly exceeds that observed in cripto-null mutants.

**Cripto/F78A fails to potentiate Nodal signaling in cell culture but retains MAPK activity**

Previous analysis of ES cell–derived EBs suggested that F78 is essential for Cripto to stimulate the in vitro differentiation of cardiomyocytes (Parisi et al., 2003). Similarly, substitution of F78 by alanine entirely blocks the ability of Cripto to rescue gastrulation of oep mutant zebrafish embryos (Minchiotti et al., 2001). Given these reports, it was surprising that substitution of F78 by alanine only partially inhibited Cripto activity in the mouse embryo. To determine whether Cripto/F78A can stimulate Nodal signaling in cell culture, we monitored its effect on CAGA-luc, a well-characterized and sensitive luciferase reporter of ALK4–Smad3 signaling. Although transfection of wild-type cripto potently stimulated the activity of Nodal, Cripto/F78A was completely inactive in this assay (Fig. 5 A). Analogous results were obtained using the activin response element (ARE)–luc reporter construct in conjunction with wild-type Nodal or a more potent supercleaved and stabilized derivative (Nsc-g; Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709090/DC1; Yan et al., 2002; Le Good et al., 2005; Chen et al., 2006; Andersson et al., 2007). These results suggest that Cripto/F78A is unable to activate a Nodal–ALK4–Smad signaling complex.

Cripto can also potentiate growth/differentiation factor (GDF) 1 and 3 signaling (Andersson et al., 2007), raising the question of whether these activities rely on F78 in a manner...
Thus, we conclude that the F78A mutation selectively impairs Western blot analysis using anti-phospho-Smad2 antibody. Levels of total P-Smad2 and total Smad2. (C) F78A recombinant Cripto retains its ability to activate Smad2 phosphorylation, is shown at the bottom. Secreted forms of wild-type and F78A Cripto lacking the GPI anchor were inactive in this assay. Error bars represent SD of three experiments. (D) Effect of recombinant soluble Cripto or the F78A mutant on Nodal–ALK4–Smad2,3 while leaving intact Smad-independent signals mediated by MAPK.

**Discussion**

**Cripto**

Understanding how Cripto stimulates Nodal-dependent cell movements in the visceral endoderm and epiblast is fundamental to our understanding of how the A–P body axis is established in mammalian embryos. In *cripto*−/− embryos, distal visceral endoderm do not move, and the vast majority of cells in the epiblast adopt a neuroectodermal character because mesendoderm progenitors, which form the primitive streak, are either absent or remain confined to the proximal epiblast (Ding et al., 1998; Liguri et al., 2003). In this paper, we show that homozygous mutants carrying the novel *cripto* allele display less severe defects than *cripto*−/− embryos. In particular, definitive endoderm and axial mesoderm populations marked by Cer-1 and Foxa2 transcripts are readily detectable, and neural progenitors expressing Otx2 mRNA consistently localize to the anterior region. In some instances, anterior-most midline cells also express the axial marker Chordin, which is consistent with their mesendodermal origin. Likewise, posterior cells expressing Fgfg and Brachyury that are absent or immobilised in the proximal epiblast of *cripto*-null embryos (Ding et al., 1998) clearly ingress in the primitive streak of *cripto*−/− mutants, even though this structure remains abnormally short and eventually fails to form a morphologically distinguishable node or notochord. Thus, in sharp contrast to *cripto*-null mutants, *cripto* embryos establish an A–P axis and initiate gastrulation, suggesting that this mutant allele encodes a functional hypomorph.

The phenotype of *cripto* embryos is reminiscent of patterning defects that arise when Nodal autoinduction is inhibited (Hoodless et al., 2001; Yamamoto et al., 2001; Norris et al., 2002). During normal development, Nodal expression is initiated in the proximal epiblast and, upon activation of an autoregulatory enhancer by FoxH1, spreads to the visceral endoderm and distal epiblast (Brennan et al., 2001; Norris et al., 2002). In this paper, we show that both *cripto* and *cripto*-null mutants fail to expand the Nodal expression domain, confirming that Smad-dependent autoinductive Nodal signaling is inhibited. However, interestingly, mutant Cripto protein was sufficient to induce or prolong the expression of several other Nodal target genes, including Lefty1, Lefty2, and Fgfg, which were completely silenced in *cripto*-null mutants at the stages examined.

These results substantiate our conclusion that *cripto* is a hypomorphic allele that is sufficient to mediate Alk4–Smad–FoxH1–independent Nodal signaling. They can also explain why primitive streak and posterior mesoderm formation are relatively mildly perturbed in *cripto* mutants, because previous analysis of FoxH1 mutants (Hoodless et al., 2001; Yamamoto et al., 2001) and hypomorphic alleles of Nodal (Lowe et al., 2001; Norris et al., 2002; Vincent et al., 2003; Ben-Haim et al., 2006) established that posterior mesoderm formation requires lower levels of Nodal signaling compared with axial midline structures.
Cripto<sup>F78A</sup> fails to potentiate Nodal signaling

Previous studies have shown that Cripto strictly depends on residue F78 to rescue mutant zebrafish embryos lacking the Nodal coreceptor oep. Using cell-based activity assays, we confirmed in this paper that Cripto<sup>F78A</sup> protein fails to stimulate well-characterized Nodal luciferase reporter genes, which specifically rely on ALK4–Smad–FoxH1 signaling. Furthermore, Cripto<sup>F78A</sup> is also unable to significantly activate Smad2 in ES cell–derived EBs, a model that more closely mimics a physiological environment. Coimmunoprecipitation experiments in transfected cells previously established that Cripto can directly bind both Nodal and ALK4 to potentiate Nodal signaling (Reissmann et al., 2001; Yeo and Whitman, 2001). However, a triple mutant of the EGF-like domain comprising the F78 residue completely abolished the ability of Cripto to stimulate the induction of a Nodal luciferase reporter in mammalian tissue culture cells (Yan et al., 2002). Moreover, chemical cross-linking experiments in 293T cells, followed by coimmunoprecipitation, suggest that this triple mutant fails to bind Nodal, whereas it interacts with the ALK4 receptor in a manner similar to that of the wild type (Yan et al., 2002). The present results are thus consistent with a model in which the F78 residue of Cripto is essential to assemble functional Cripto–ALK4 receptor complexes and thereby potentiate a Nodal autoregulatory feedback loop.

Luciferase reporter assays and coimmunoprecipitation experiments suggest that Cripto can also potentiate Nodal signaling through ALK7 (Reissmann et al., 2001). Therefore, it is formally possible that the loss of F78 selectively blocks the ability of Cripto to activate ALK4 without affecting Nodal signaling via the ALK7 receptor. However, it has previously been shown that ALK7 is dispensable and unable to compensate for the loss of ALK4 in the mouse embryo (Gu et al., 1998; Jornvall et al., 2004). Cripto can also stimulate the induction of CAGLuc reporter by native forms of GDF1 and 3 (Andersson et al., 2003) and develop by addition of 0.03% H<sub>2</sub>O<sub>2</sub>. Stained embryos were examined and photographed using a stereomicroscope (MZ12; Leica). All images were processed in Photoshop 5.0 (Adobe).

Whole mount immunohistochemistry and in situ hybridization

Embryos were dissected in PBS and fixed in 4% paraformaldehyde in PBS at 4°C for 2–16 h, washed in PB (0.1% Tween 20 in PBS), dehydrated through graded methanol, and stored in 100% methanol at −20°C. For immunohistochemistry, embryos were rehydrated in PBtx (0.25% Triton X-100 in PBS), bleached with 0.5% H<sub>2</sub>O<sub>2</sub> overnight, blocked with PBtbs (10% normal goat serum and 1 mg/ml BSA in PBtx), and incubated overnight at 4°C with 2 μg/ml of affinity-purified α-Cripto antibodies. To remove the unbound antibody, embryos were extensively washed in PBtx (1 h, six times) and labeled with biotinylated secondary antibody overnight at 4°C. After six washes in PBtx, embryos were incubated with biotin–streptavidin complex (AB complex; Vector Laboratories), revealed by incubation for 30 min with 0.5 mg/ml of 3–3′ diniaminobenzidine (Sigma-Aldrich) and developed by addition of 0.03% H<sub>2</sub>O<sub>2</sub>. Stained embryos were examined and photographed using a stereomicroscope (MZ12; Leica). All images were processed in Photoshop 5.0 (Adobe).

Whole mount immunohistochemistry and in situ hybridization was performed according to standard procedures (Ligouri et al., 2003). Probes for the following genes were used in this study: Brachyury (Wilkinson et al., 1990), Cerberus-like (Belo et al., 1997), Chordin (Bochill et al., 2000), Kidney-specific factor 2 (Kd-f2; reference), ankyrin G (GlcA 2 [null]), X. laevis Slit-2, and X. laevis Hoxa-4.

Materials and methods

Targeting of the cripto locus

The targeting vector was derived from pFlx vector (Chen et al., 1998) by excision of a Smal–BamHI DNA fragment spanning the lox<sup>P</sup> site and by removing the BglII–Smal DNA fragment flanking the ne<sup>o</sup> gene (Fig. 1A). A 3.5kb 5′ homologous sequence spanning exons 1 and 2 was inserted upstream of the lox<sup>P</sup> site–flanked cassette encoding the neo<sup>o</sup> gene. A 5.6kb 3′ homologous sequence spanning exons 3 to 6 was inserted downstream of the neo<sup>o</sup> gene (Fig. 1A). The two overlapping PCR primers 5′ F78A (5′-GGACATTCTGGGTCGCCGCTGGCCTGGCCTC-3′) and 3′ F78A (5′-GGAGGGCCAGGCACAGGGGACCCCCCCAGATGC-3′) were used to introduce the F78A point mutation in the targeting vector (underlining in primers indicates the nucleotide sequence that was modified to insert the F78A mutation).

RI ES cells were transfected with the targeting vector and selected in G-418. DNA prepared from individual drug-resistant colonies was digested with EcoRV for Southern blot analysis using a 600-bp 5′ external probe (RH5 in Fig. 1A and B), a 500-bp 3′ internal probe (BE6 in Fig. 1A and B), and a neo<sup>o</sup> probe. After identification of the targeted clones, the presence of the point mutation was verified by PCR amplification and sequence analysis. Selected ES cell lines were used to generate germine chimera mice that were subsequently bred to C57BL/6 females (Charles River Laboratories). F1 cripto<sup>F78Aneo</sup> heterozygotes were crossed with a pgk-Cre deletion strain.

Mouse breeding and genotyping

Heterozygous mice for the cripto<sup>F78A</sup> allele were maintained on a mixed genetic background Cs7BL/6 × Sv129 × Black Swiss) and also backcrossed to an inbred C57BL/6 strain. No phenotypic differences were observed between Cripto<sup>F78A</sup> heterozygotes and C57BL/6 females (T. Yeo and J. Whitman, unpublished observations). Therefore, Cripto<sup>F78A</sup> is dispensable and unable to compensate for the loss of ALK4 in the mouse embryo (Gu et al., 1998; Jornvall et al., 2004). Cripto can also stimulate the induction of CAGLuc reporter by native forms of GDF1 and 3 (Andersson et al., 2003) and develop by addition of 0.03% H<sub>2</sub>O<sub>2</sub>. Stained embryos were examined and photographed using a stereomicroscope (MZ12; Leica). All images were processed in Photoshop 5.0 (Adobe).

Whole mount immunohistochemistry and in situ hybridization

Embryos were dissected in PBS and fixed in 4% paraformaldehyde in PBS at 4°C for 2–16 h, washed in PB (0.1% Tween 20 in PBS), dehydrated through graded methanol, and stored in 100% methanol at −20°C. For immunohistochemistry, embryos were rehydrated in PBtx (0.25% Triton X-100 in PBS), bleached with 0.5% H<sub>2</sub>O<sub>2</sub> overnight, blocked with PBtbs (10% normal goat serum and 1 mg/ml BSA in PBtx), and incubated overnight at 4°C with 2 μg/ml of affinity-purified α-Cripto antibodies. To remove the unbound antibody, embryos were extensively washed in PBtx (1 h, six times) and labeled with biotinylated secondary antibody overnight at 4°C. After six washes in PBtx, embryos were incubated with biotin–streptavidin complex (AB complex; Vector Laboratories), revealed by incubation for 30 min with 0.5 mg/ml of 3–3′ diniaminobenzidine (Sigma-Aldrich) and developed by addition of 0.03% H<sub>2</sub>O<sub>2</sub>. Stained embryos were examined and photographed using a stereomicroscope (MZ12; Leica). All images were processed in Photoshop 5.0 (Adobe).

Whole mount immunohistochemistry and in situ hybridization was performed according to standard procedures (Ligouri et al., 2003). Probes for the following genes were used in this study: Brachyury (Wilkinson et al., 1990), Cerberus-like (Belo et al., 1997), Chordin (Bochill et al., 2000), Kidney-specific factor 2 (Kd-f2; reference), ankyrin G (GlcA 2 [null]), X. laevis Slit-2, and X. laevis Hoxa-4.
Cripto activity assay in transiently transfected 293T cells

Cripto activity assays were performed as previously described (Yan et al., 2002; Anderson et al., 2007) by transiently transfecting 293T cells with either the ARE-luc or the pCAGA-luc reporter constructs and expression vectors for Cripto, FoxH1, and wild type or a stabilized form of Nodal (Nuc-g; Le Good et al., 2005).

ES cell differentiation assay and Western blot

The ES cells lines Rit and cripto-/-DE7 were used throughout the study and differentiation assays were performed as previously described (Paris et al., 2003). Western blot analysis was performed as previously described (Paris et al., 2003). Anti–phospho-Smad2 (Ser465/467), total Smad2 (Wilkinson et al., 1989), Fgf8 expression vectors for Cripto, FoxH1, and wild type or a stabilized form of Nodal (Le Good et al., 2005).

Online supplemental material

Fig. S1 contains additional information on the embryonic development of cripto F78A/F78A mutants at the head-fold stage. Fig. S2 contains additional information on Cripto activity in the 293 cell ARE-luc reporter assay and shows that F78A mutant is inactive in this assay. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709090/DC1.

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