CRIPTO PROMOTES A–P AXIS SPECIFICATION INDEPENDENTLY OF ITS STIMULATORY EFFECT ON NODAL AUTOINDUCTION

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Introduction

CRIPTO, a glycophosphatidylinositol (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 (Ciccocioppo 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 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 phosphorylates 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 FooH1 (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 et al., 2006). Furthermore, Cripto has been found to bind specific Nodal antagonists, such as the transmembrane protein tomo-regulin-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...
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 extraembryonic 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.

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).

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Furthermore, rescue experiments in cripto/H11002/H11002 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...
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.

### Results

**Cripto<sup>F78A/F78A</sup> 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 (<sup>F78A</sup>) using Cre/loxP-mediated recombination (Fig. 1, A–C). The resulting heterozygous <sup>cripto<sup>F78A/F78A</sup></sup> mice appeared phenotypically normal and were fertile; however, homozygosity for the <sup>cripto<sup>F78A</sup></sup>-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 <sup>cripto F78A/F78A</sup> 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, <sup>cripto<sup>F78A/F78A</sup></sup> mutants 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.

### A–P axis and mesendoderm formation in <sup>cripto<sup>F78A/F78A</sup></sup> mutants

Loss-of-function analysis has shown that <sup>cripto</sup> converts proximal-distal patterning into an A–P axis and promotes primitive streak formation (Ding et al., 1998; Liguori et al., 2003). To assess...
whether cripto<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, cripto-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 cripto<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 cripto-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 cripto-null mutants (Ding et al., 1998). Krox20 mRNA was clearly detected in cripto<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 cripto-null mutants, was expressed in the posterior region of cripto<sup>F78A/F78A</sup> embryos (Fig. S1, C and C′). These results demonstrate that cripto<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 cripto<sup>F78A/F78A</sup> mutant embryos, we next visualized derivatives of the anterior primitive streak, such as the node, a structure that expresses Nodal (blue). In wild-type embryos, Nodal mRNA is expressed in a group of posterior cells, whereas it is confined to the embryonic–extraembryonic boundary in cripto-null mutants (B′). In cripto<sup>F78A/F78A</sup> embryos (B′), Nodal mRNA is expressed in a group of posterior cells, whereas it is confined to the embryonic–extraembryonic boundary in cripto-null mutants (B′). In contrast, in cripto-null mutants (D′), Chordin (blue) and Brachyury (red) 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
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’, s1), whereas they are missing in cripto-null mutants (Fig. 3 C”). Similarly, Cer-1 was clearly induced in 5 out of 10 cripto_F78A/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/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 Fgf8 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, Fgf8 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 potentely 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
similar to Nodal. Unlike wild-type Cripto, the Cripto<sup>F78A</sup> mutant failed to potentiate GDF1 or 3 signaling in this assay (Fig. 5 A and not depicted).

Cripto<sup>F78A</sup> might activate the Smad2 pathway only in an embryo-like cell context. To test this, 2-d-old Cripto<sup>−/−</sup> ES cell–derived EBs were starved in low serum for 3 h and then stimulated with recombinant soluble Cripto or the F78A mutant protein. Consistent with published data (Watanabe et al., 2007), Cripto without a GPI anchor was poorly active but, when applied at elevated concentrations, it significantly increased the phosphorylation of Smad2 (Fig. 5 B). In contrast, soluble Cripto<sup>F78A</sup> failed to detectably stimulate Smad2 phosphorylation (Fig. 5 B). However, mutation of F78 did not diminish the ability of Cripto to stimulate the MAPK signaling pathway (Fig. 5 C). Thus, we conclude that the F78A mutation selectively impairs Nodal–ALK4–Smad2,3 while leaving intact Smad–independent signals mediated by MAPK.

**Discussion**

 Cripto<sup>F78A/F78A</sup> embryos establish an A–P axis and initiate gastrulation

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<sup>−/−</sup> 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; Ligouri et al., 2003). In this paper, we show that homozygous mutants carrying the novel cripto<sup>F78A</sup> allele display less severe defects than cripto<sup>−/−</sup> 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-midline cells also express the axial marker Chordin, which is consistent with their mesendodermal origin. Likewise, posterior cells expressing Fgf8 and Brachyury that are absent or immobilized in the proximal epiblast of cripto-null embryos (Ding et al., 1998) clearly ingress in the primitive streak of cripto<sup>F78A/F78A</sup> 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<sup>F78A/F78A</sup> embryos establish an A–P axis and initiate gastrulation, suggesting that this mutant allele encodes a functional hypomorph.

The phenotype of cripto<sup>F78A/F78A</sup> 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 initialed 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<sup>F78A/F78A</sup> and cripto-null mutants fail to expand the Nodal expression domain, confirming that Smad-dependent autoinductive Nodal signaling is inhibited. However, interestingly, mutant Cripto<sup>F78A</sup> protein was sufficient to induce or prolong the expression of several other Nodal target genes, including Lefty1, Lefty2, and Fgf8, which were completely silenced in cripto-null mutants at the stages examined.

These results substantiate our conclusion that cripto<sup>F78A</sup> 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<sup>F78A/F78A</sup> 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.
**CriptoF78A 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 CriptoF78A protein fails to stimulate well-characterized Nodal luciferase reporter genes, which specifically rely on ALK4–Smad–FoxH1 signaling. Furthermore, CriptoF78A is also unable to significantly activate Smad2 in ES cells–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 CAGA-luc reporter by native forms of GDF1 and 3 (Andersson et al., 2004). Cripto can also 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.

**Materials and methods**

**Targeting of the cripto locus**

The targeting vector was derived from pFlox vector (Chen et al., 1998) by excision of a Salm–BamHI DNA fragment spanning the loxP site and by removing the BglII–SmaI DNA fragment spanning the hsvg-k gene (Fig. 1A). A 3-kb 5' homologous sequence spanning exons 1 and 2 was inserted upstream of the loxP site–flanked cassette encoding the nea' gene. A 5.6-kb 3' homologous sequence spanning exons 3 to 6 was inserted downstream of the neo' gene (Fig. 1A). The two overlapping PCR primers F78A (5'-GCATCCGGGGCTCCGTGGTGCCTGCCCTC-3') and 3'F78A (5'-GGAGGGCAGGCACAGGGGCCACCGAGATGC-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).

**Mouse breeding and genotyping**

Heterozygous mice for the criptoF78A allele were maintained on a mixed genetic background C57BL/6 x 5v129. Heterozygous mice for the cripto-null allele were maintained on a mixed genetic background C57BL/6 x 5v129 x Black Swiss) and also backcrossed to an inbred C57BL/6 strain. No phenotypic differences were observed between cripto-null embryos on different genetic backgrounds. Timed matings between heterozygotes were used to obtain both criptoF78A/F78A and cripto-null homozygous mutant embryos. Embryos were genotyped by PCR at 7.5 dpc using extraembryonic tissues. At 6.75 dpc, DNA was extracted from whole embryos for genotyping.

**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 PBT (0.1% Tween 20 in PBS), dehydrated through graded methanol, and stored in 100% methanol at −20°C. For immunohistochemistry, embryos were rehydrated in PB (0.25% Triton X-100 in PBS), bleached with 0.5% H2O2 overnight, blocked with PBSAB (10% normal goat serum and 1 mg/ml BSA in PB), 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 PB (1 h, six times) and labeled with biotinylated secondary antibody overnight at 4°C. After six washes in PBX, embryos were incubated with streptavidin complex (AB complex; Vector Laboratories), revealed by incubation for 30 min with 0.5 mg/ml of 3,3′ diaminobenzidine (Sigma-Aldrich), and developed by addition of 0.03% H2O2. 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 (Liguori et al., 2003). Probes for the following genes were used in this study: Brachyury (Wilkinson et al., 1990), Cerberus-like (Belo et al., 1987), Chordin (Bochiller et al., 2000),...**
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 transflecting 293T cells with either the ARE-luc or the pCAGA-luc luciferase reporter constructs and expressing vectors for Cripto, FoxH1, and wild type or a stabilized form of Fgf8.

Online supplemental material

Fig. S1 contains additional information on the embryonic development of either the ARE-luc or the pCAGA-luc luciferase reporter constructs and expression vectors for Cripto, FoxH1, and wild type or a stabilized form of Fgf8.
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