Cell, Vol. 121, 87–99, April 8, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2005.01.033

Germ-Layer Specification and Control of Cell Growth by Ectodermin, a Smad4 Ubiquitin Ligase

Sirio Dupont,[1] Luca Zacchigna,[1] Michelangelo Cordenonsi,[1] Sandra Soligo,[1] Maddalena Adorno,[1] Massimo Rugge,[2] and Stefano Piccolo[1,*]

[1]Department of Histology, Microbiology and Medical Biotechnologies
Section of Histology and Embryology
[2]Department of Oncology and Surgical Sciences
Pathology Section
University of Padua
35121 Padua
Italy

Summary

TGF-β signaling is essential for development and proliferative homeostasis. During embryogenesis, maternal determinants act in concert with TGF-β signals to form mesoderm and endoderm. In contrast, ectoderm specification requires the TGF-β response to be attenuated, although the mechanisms by which this is achieved remain unknown. In a functional screen for ectoderm determinants, we have identified Ectodermin (Ecto). In *Xenopus* embryos, Ecto is essential for the specification of the ectoderm and acts by restricting the mesoderm-inducing activity of TGF-β signals to the mesoderm and favoring neural induction. Ecto is a RING-type ubiquitin ligase for Smad4, a TGF-β signal transducer. Depletion of Ecto in human cells enforces TGF-β-induced cytostasis and, moreover, plays a causal role in limiting the antimitogenic effects of Smad4 in tumor cells. We propose that Ectodermin is a key switch in the control of TGF-β gene responses during early embryonic development and cell proliferation.

Introduction

During early vertebrate development, the fate of initially pluripotent cells becomes progressively restricted to more and more limited developmental choices. A fundamental step in this process is the allocation of cells to the primary germ layers: ectoderm, mesoderm, and endoderm.

Seminal work in *Xenopus* has shown that, through uneven cell divisions, maternal "determinants" located asymmetrically in the egg are differentially inherited by distinct groups of cells; such initial differences are amplified and ultimately fixed by growth-factor signaling (De Robertis et al., 2000; Harland and Gerhart, 1997; Zhang et al., 1998). This represents an attractive paradigm of cell-fate decision in which intrinsic and extrinsic cues interact with each other to generate a coherent embryonic patterning. Over the last decade, substantial progress has been made in the identification of key events leading to mesoderm and endoderm formation. In *Xenopus* embryos, maternal determinants, such as VegT or Vg1, are localized in the vegetal hemisphere, where they activate the transcription of TGF-β/nodal signals responsible for inducing the mesoderm in the overlying marginal-zone cells at the equator of the embryo (Figure 1A) (Piccolo et al., 1999; Whitman, 2001).

Very little is known about genes essential for the specification of the ectoderm germ layer. The existence of determinants important for ectoderm development is suggested by the phenotype of embryos depleted of VegT (Zhang et al., 1998), where the marginal zone differentiates as ectoderm instead of mesoderm. Thus, a specific set of signals is required for ectoderm development, including molecules able to antagonize mesoderm formation (Wessely and De Robertis, 2000).

Embryological experiments indicate that ectoderm cells remain pluripotent until gastrulation (Snape et al., 1987); at this stage, the ectoderm would generate, by "default," neural tissue unless induced to form epidermis by BMP ligands (Piccolo et al., 1996; Zimmerman et al., 1996). These data strongly suggest that ectoderm development requires a strict control of the signaling and gene responses triggered by TGF-β superfamily members.

TGF-β ligands transmit the signal intracellularly to the receptor-Smad family of signal transducers (R-Smads). The TGF-β and BMP signaling branches make use of distinct R-Smads but converge in the common mediator Smad4, which forms a complex with R-Smads in the nucleus to regulate transcription of target genes. In adult tissues, TGF-β signaling is crucial for maintaining proliferative homeostasis, and lack of responsiveness to TGF-β antimitogenic effects is a hallmark of cancer (Siegel and Massague, 2003). How cells escape from TGF-β cytostasis is unknown; genetic studies in pancreatic and colorectal cancers pinpoint that a blunted Smad4 function is a main tool used by tumor cells to disable this antiproliferative response (Takaku et al., 1999; Woodford-Richens et al., 2001; Xu and Attisano, 2000). Since Smad4 or other TGF-β pathway components are rarely directly inactivated by mutations, alterations in unknown Smad regulators could account for loss of TGF-β responsiveness in the majority of tumors (Siegel and Massague, 2003).

Here we describe the identification by functional expression cloning of Ectodermin (Ecto), a RING-type ubiquitin ligase for Smad4 that controls TGF-β/BMP responses. In *Xenopus* embryos, Ectodermin restricts the mesoderm-inducing activity of TGF-β signals to the mesoderm and favors neural induction and is therefore essential for the specification of the ectoderm germ layer. The function of Ectodermin is not restricted to early embryogenesis; it is expressed in human adult cells, where it is an intrinsic limiting factor for TGF-β/Smad4-induced cytostasis. We argue that Ectodermin is a potent endogenous negative regulator of Smad responses in vertebrate cells.

*Correspondence: piccolo@civ.bio.unipd.it

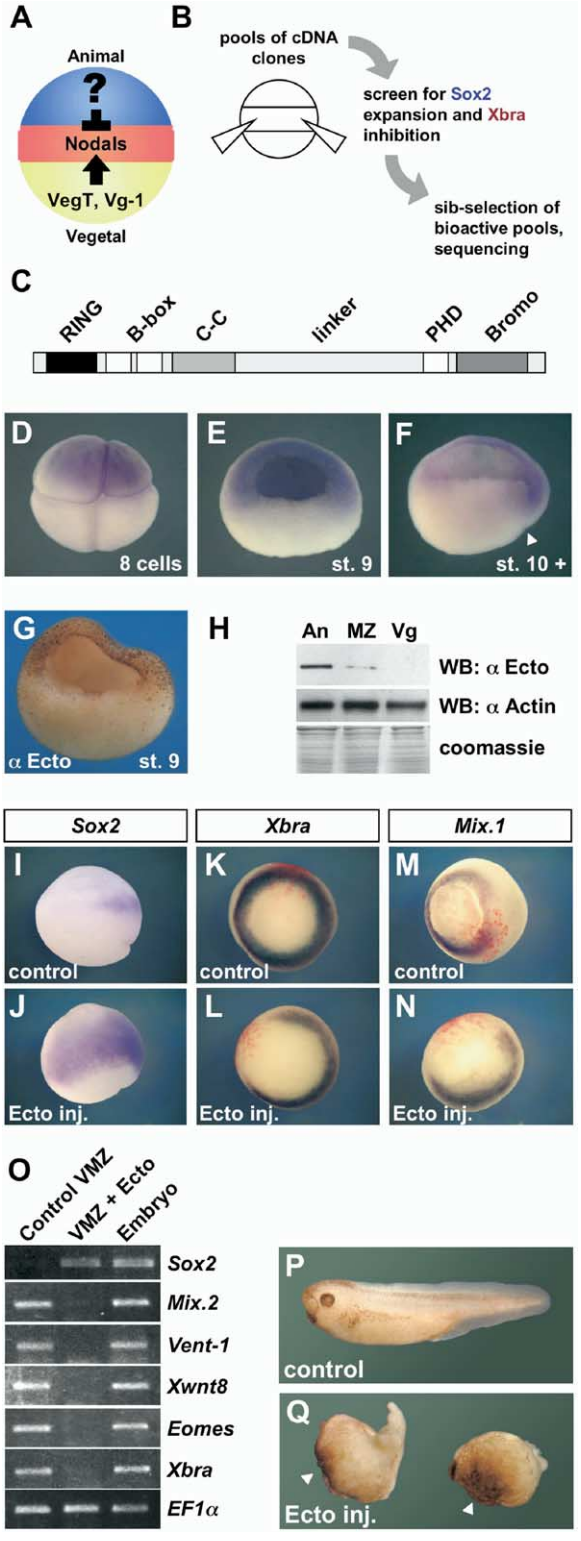

Figure 1. Functional Cloning of Ectodermin, a Maternal Determinant Regulating Cell Fates in Early *Xenopus* Embryos

(A) Current model for the induction of primitive germ layers in *Xenopus* embryos.

(B) Functional cloning strategy for candidate ectoderm determinants.

(C) Schematic representation of the Ectodermin protein. The linker sequence is unique to *Xenopus* and mammalian Ectodermin (Ecto). Domains at the N terminus (RING finger, B boxes, coiled coil) and the C terminus (PHD, bromodomain) are shared with other members of the Tif1/TRIM protein family.

(D–F) Expression analysis of Ectodermin by in situ hybridization. Arrowhead in (F) points to the dorsal lip. Embryos shown in (E) and (F) were bisected before in situ hybridization to allow penetration of the probe in the more vegetal regions. Controls for the in situs are provided in Figure S1.

(G and H) Ectodermin protein is a localized determinant in the prospective ectoderm germ layer. (G) shows whole-mount immunolocalization of the endogenous Ectodermin protein on bisected blastula-stage embryos. Magnifications showing specific nuclear staining and additional controls are provided in Figure S1. (H) shows quantitative analysis of Ectodermin protein distribution along the animal-vegetal axis. Anti-Ectodermin immunoblot shows high enrichment of Ectodermin protein in explanted animal cells (An), low expression in marginal explants (MZ), and no expression in vegetal base (Vg), all explanted from blastula-stage embryos. Sibling explants were pretested by RT-PCR to verify their identity according to molecular markers.

(I–N) Molecular characterization of the biological activity of Ectodermin by in situ hybridization. *Sox2* is an ectoderm and neural plate marker; *Xbra* and *Mix.1* are mesoderm markers. (I and J) Embryos at the four-cell stage were radially injected with *Ecto* mRNA (350 pg/blastomere) and harvested at the early gastrula stage (stage 10+). (K–N) Eight-cell-stage embryos were injected with *Ecto* mRNA (1 ng) in a single blastomere (marginal zone), together with *lacZ* mRNA (200 pg), to identify injected cells (in red). Embryos were harvested at the gastrula stage (stage 11).

(O) RT-PCR analysis of marginal-zone explants (VMZ) expressing control (*GFP* mRNA, 1 ng) and *Ecto* mRNA (1 ng). Cells normally fated to express mesendoderm markers (*Mix.2*, *Vent-1*, *Xwnt8*, *Eomes*, *Xbra*) now change their fate, upregulating the ectoderm-specific marker *Sox2*.

(P and Q) Long-term phenotypic effects of *Ecto* mRNA overexpression. Arrowheads indicate ruffled ventral epidermis.

Results

Functional Expression Cloning of Ectodermin, a Maternal Determinant Promoting Ectoderm Development

We used an unbiased screening strategy to identify molecules involved in the specification of the embryonic ectoderm. To do this, we searched for genes that, once overexpressed in *Xenopus* embryos, could transform mesoderm cells into ectoderm precursors. The rationale of this approach relies on embryological observations indicating that, by the blastula stage, unidentified ectodermal determinants are located in the embryo's animal hemisphere and can direct the differentiation of ectoderm derivatives (Wessely and De Robertis, 2000; Zhang et al., 1998) (Figure 1A).

We cloned a blastula-stage cDNA library into an RNA expression plasmid. Synthetic mRNA was prepared from pools of 50 clones and injected in the equatorial region of four-cell-stage embryos, that is, in the prospective mesoderm (Figure 1B). Injected embryos were assayed by in situ hybridization at the early gastrula stage for reduction of the mesoderm marker *Xbra* and expansion of the early ectoderm marker *Sox2* (Mizuseki et al., 1998).

One positive pool was sib selected, and the isolated cDNA corresponded to a new *Xenopus* ORF named *Ectodermin* (*Ecto*). The Ectodermin protein has an N-terminal TRIM domain composed of a RING finger, two B

boxes, and a coiled coil, followed by a central linker region of unique sequence and a C-terminal PHD/bromodomain (Figure 1C). Ectodermin is the homolog of a mammalian gene with unknown functions, cited in the literature with different names, *TRIM33*, *Tif1*γ, *Ret-fused gene 7*, and *PTC7* (Venturini et al., 1999); for clarity, we refer to it herein as human Ectodermin (hEcto), as we found that it shares similar functions and comparable biochemical activities with *Xenopus* Ectodermin.

We then determined the expression pattern of Ectodermin during *Xenopus* development by in situ hybridization and immunohistochemistry. *Ectodermin* RNA is present in the animal pole of unfertilized eggs and throughout cleavage stages (Figure 1D and data not shown). At the blastula stage, *Ectodermin* mRNA and protein are localized in the animal pole, extending, at lower levels, up to the marginal zone (Figures 1E, 1G, and 1H; see also Figure S1). At the onset of gastrulation, *Ectodermin* RNA level has already dramatically declined, but, intriguingly, it remains asymmetrically enriched in the dorsal side of the early gastrula (Figure 1F). As gastrulation proceeds, *Ectodermin* expression progressively fades.

To test the biological activity of Ectodermin in more detail, we injected *Ecto* mRNA in *Xenopus* embryos. As shown in Figures 1I and 1J, forced expression of *Ecto* mRNA leads to an expansion of *Sox2* into the prospective mesoderm; concomitantly, expressions of *Xbra*, *Eomes*, and *Vent-1*, as well as of the mesendoderm markers *Mix.1* and *Mixer*, are downregulated in injected cells (Figures 1K–1N and data not shown). RT-PCR analysis of explanted ventral marginal zones (VMZ) indicates that misexpression of Ectodermin can change the fate of mesoderm precursors into ectoderm (Figure 1O). In agreement with such molecular characterization, overexpression of Ectodermin has powerful long-term phenotypic effects. Embryos were injected at the two-cell stage in the marginal zone with 1 ng of *Ecto* mRNA and then monitored for appearance of morphological defects. Despite cleaving normally, part of the *Ecto* mRNA-injected embryos (n = 275, 33%) failed to complete gastrulation (data not shown). The surviving embryos displayed severe defects (93%), including shortened axis, head reduction, and a ventral cyst of an extensively folded epidermis (Figures 1P and 1Q). In sum, Ectodermin is a molecule endowed with powerful biological activity that promotes ectoderm development at the expense of other germ layers.

Ectodermin Regulates Germ-Layer Identity along the Animal-Vegetal Axis

The functional cloning, expression pattern, and biological activity of Ectodermin make it an attractive candidate as an endogenous ectoderm determinant. As key test for the developmental relevance of Ectodermin, we carried out the knockdown of Ecto by targeting its expression with a morpholino oligonucleotide (Ecto-MO) designed to cover the initial codons of *Ectodermin* mRNA. As specificity control, we used a mutant version of the same morpholino (control-MO). Ecto-MO was specifically effective in reducing the level of endogenous Ectodermin protein in morpholino-injected embryos ("Ecto-morphants") (Figure 2A).

We investigated the molecular effects of Ectodermin knockdown by monitoring expression of marker genes in animal cap cells isolated at the late blastula stage. Control explants never expressed markers of other germ layers. Remarkably, Ecto-depleted cells displayed the activation of a mesoderm-specific gene expression profile, as monitored by *Eomes*, *VegT*, and *Mix.2* expression, and the downregulation of the ectoderm marker *Sox2* (Figure 2B and Figure S2A).

Next we tested to what extent Ectodermin is required for patterning of the whole embryo. Ecto-MO and control-MO were injected in the prospective marginal zone of one-cell embryos and were analyzed by whole-mount in situ hybridization when embryos reached the gastrula stage. In Ecto-morphants, the mesoderm marker *VegT* was upregulated and expanded toward the animal pole (compare Figures 2C and 2D). Expression of *Mixer*, which marks in the endoderm the border with the adjoining mesoderm, displayed an abnormal expansion into the prospective mesodermal mantle (compare Figures 2G and 2H; see also Figure S2B). Morphologically, Ecto-morphants cleaved normally but displayed a severely retarded gastrulation; once past the midgastrula stage, they stopped developing (data not shown), likely reflecting the changes in cell fate and motility caused by the aberrant patterning of these embryos. Crucially, expansion of *VegT* and *Mixer* is rescued by adding back Ectodermin, as achieved by injection of a morpholino-insensitive *Ecto* mRNA in the animal blastomeres of Ecto-morphant embryos (Figures 2E and 2I). These data support the view that Ectodermin is a critical switch for establishing germ-layer identity along the animal-vegetal axis of the embryo.

Ectodermin Prevents TGF-β Signaling

A wealth of data indicates that the primary mesoderm-inducing signal in vertebrate embryos is delivered by growth factors of the TGF-β superfamily (Whitman, 2001). To test whether Ectodermin antagonizes the activity of TGF-β ligands, we assayed in animal caps the effect of increased levels of Ectodermin on the gene responses triggered by TGF-β signaling (Figure 3A). We found that coinjection of *Ecto* mRNA inhibits in a dose-dependent manner the induction of *Xbra* and *Mixer* by *Activin* mRNA (Figure 3A, lanes 2–5). Similar results were obtained with an activated TGF-β receptor (CA-Alk5) and Smad2 (lanes 6–9). As specificity control, *Xbra* induction triggered by overexpression of an active FGF receptor is not blocked by *Ecto* mRNA (lanes 10 and 11). Furthermore, human Ectodermin, but not the related TRIM family member KAP1 (Venturini et al., 1999), recapitulates the anti-TGF-β activity of *Xenopus* Ectodermin (Figure S3).

Since overexpression of Ectodermin inhibited TGF-β signaling, we tested whether the depletion of Ectodermin enhanced gene responses triggered in vivo by TGF-β ligands. To do so, we treated animal cap cells injected with a low dose of Ecto-MO (25 ng) with a suboptimal concentration of Activin protein. As shown in Figure 3B, Ecto knockdown sensitized the response to Activin. This finding suggests that the expansion of

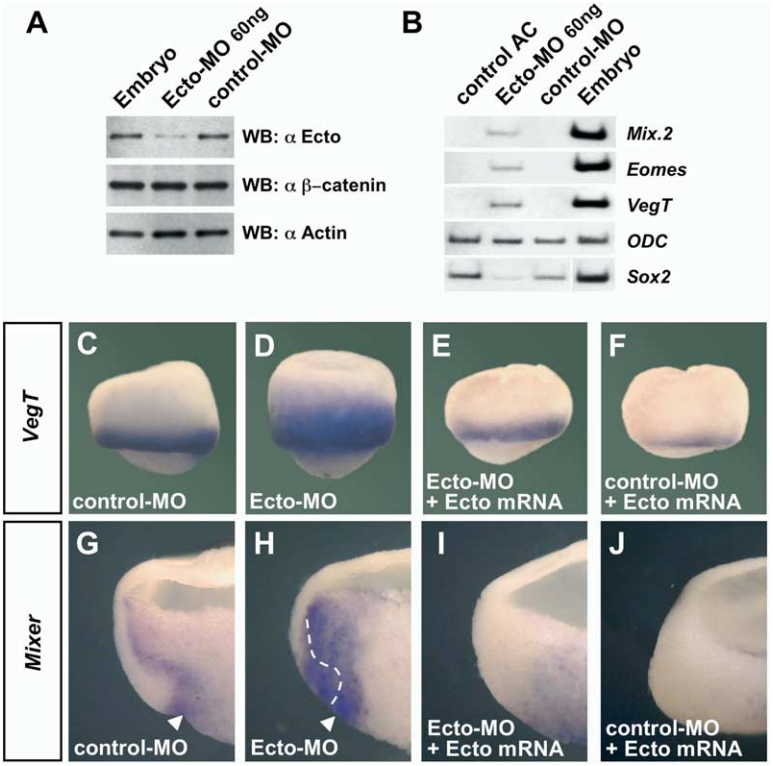

Figure 2. Depletion of Ectodermin Causes a Shift in Cellular Fates along the Animal-Vegetal Axis

(A) Ecto morpholino oligonucleotides specifically knockdown the translation of endogenous Ectodermin protein in whole embryos (control-MO and Ecto-MO, 60 ng/embryo).

(B) Animal caps were injected with 60 ng of control-MO or Ecto-MO, explanted at the late blastula stage (stage 9.5), and analyzed by RT-PCR. Note the induction of mesoderm markers in Ecto-depleted cells. (C–J) Molecular characterization of Ecto knockdown by in situ hybridization on whole embryos (midgastrula stage). (C and D) Zygotic *VegT*, a mesoderm-specific marker, spreads toward the animal pole. (G and H) *Mixer* expression at the boundary between endoderm and mesoderm (dashed line) is expanded. Arrowheads point to the dorsal lip. A morpholino-insensitive *Ecto* mRNA (1.5 ng) is biologically active (F and J) and reverses the knockdown phenotypes (E and I). Targeted injection of Ecto-MO in marginal explants confirms ectopic expression of *Mixer* and *Mix.2* in the mesoderm (see Figure S2B).

mesoderm-specific gene expression observed in whole embryos and explants upon Ectodermin knockdown (shown in Figure 2) is due to an unrestrained response to endogenous TGF-β/nodal ligands. Consistently, the induction of mesoderm markers detected by RT-PCR in Ecto-MO-injected (60 ng) cells was blocked by coinjected Cer-S, an extracellular nodal antagonist (Piccolo et al., 1999) (Figure 3C).

Ectodermin Attenuates BMP Signaling and Favors Neural Development

We next tested whether Ectodermin antagonizes BMP signaling. As shown in Figure 3D, induction of *Vent-1* by *constitutive-active BMP receptor* (*CABR*) and *Smad1* mRNAs is blocked by overexpression of Ectodermin. In whole-embryo assays, while injection of low doses of *Smad1* mRNA produces headless embryos, the coinjection of *Smad1* and *Ecto* mRNAs rescues normal development (Figure S4). In agreement with these observations, the long-term overexpression of *Ectodermin* mRNA in animal cap cells leads to the activation of neural markers (Figure 3E).

As Ectodermin is highly expressed in prospective ectoderm cells, these data suggest that Ecto would favor neural development in vivo by inhibiting BMP signaling. We first tested this hypothesis in isolated ectoderm explants. Neural induction was triggered by overexpression of the BMP antagonist Chordin; notably, in cells explanted from Ecto-morphant embryos, inductions of the neural markers *NCAM* and *Sox2* were downregulated (Figure 3F, see legend). Next we validated this conclusion in whole embryos (Figures 3G–3L). Embryos were injected in the animal hemisphere with Ecto-MO (30 and 60 ng) and control-MO (60 ng) and analyzed in advanced neurulae for expression of *Sox2* and *cytokeratin (Ker)*, which, at this stage, mark the neural plate and epidermis, respectively. Injection of Ecto-MO caused reduction of neural tissue and concomitant expansion of epidermis despite presence of normal expression of *Chordin* in Ecto-morphants (data not shown). Thus, reduction of Ectodermin levels leads to an attenuated response to neural inducers, likely reflecting unrestrained BMP signaling. Accordingly, we found that suboptimal doses of *Ecto* and *Chordin* mRNAs cooperate in neural induction (Figure S5).

In vivo, expression of Ectodermin temporally precedes the appearance of neural inducers in the Spemann Organizer (Figure 1). Taken together, the data presented so far suggest a model for the biological function of Ectodermin (Figure 3M): Ectodermin acts in early development to protect the primary ectodermal territory from mesoderm-inducing signals delivered by TGF-β/nodal cytokines emanating from the embryo's vegetal hemisphere. Such intrinsic anti-TGF-β-signaling activity also plays a role in preventing an excess of BMP signaling prior to gastrulation.

An Ectodermin-Smad4 Complex

We found that human Ectodermin is expressed in a variety of cell lines from adult tissues (data not shown). To validate the antagonism between Ectodermin and TGF-β signaling in human cells, we assayed the effect of human Ectodermin on the transcriptional activation of Smad-dependent enhancers. As direct readout of TGF-β signaling, we used the $CAGA_{12}$ luciferase synthetic reporter, which is activated by a complex be-

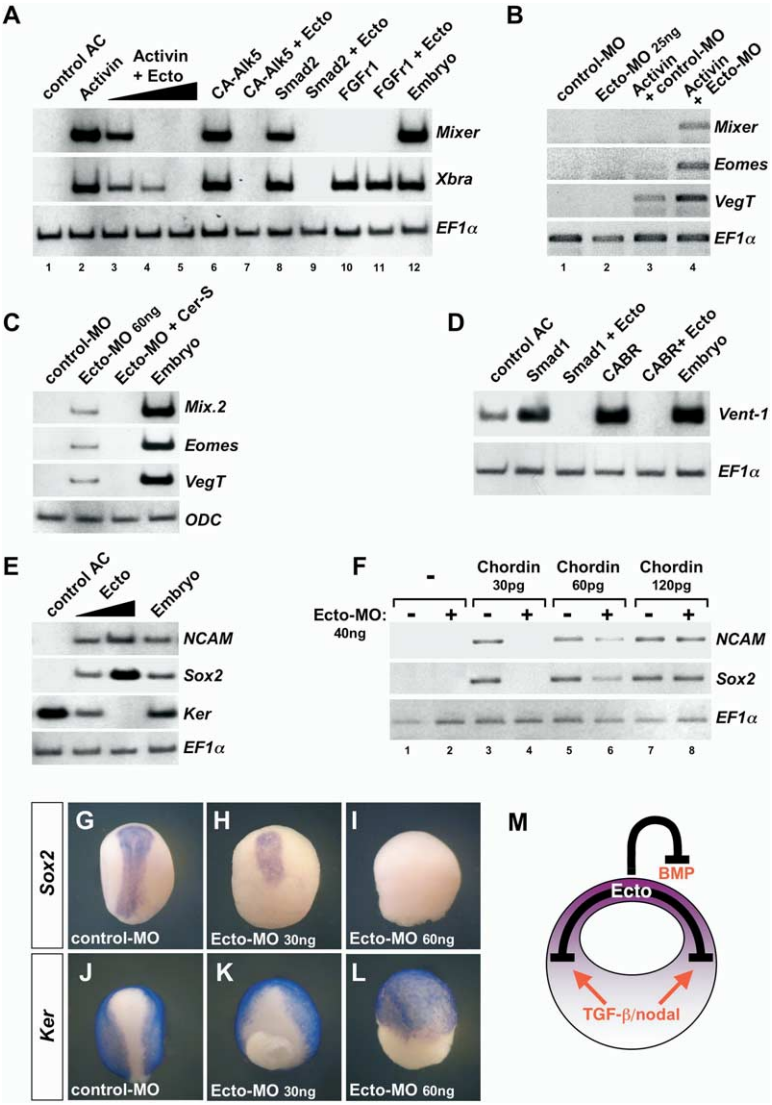

Figure 3. Ectodermin Prevents TGF-β and BMP Signaling

(A) RT-PCR analysis of animal caps expressing the indicated mRNAs (dose in parenthesis). Lanes 2–5: the inductions by *Activin* (40 pg) are downregulated by increasing doses of *Ecto* (lane 3, 200 pg; lane 4, 400 pg; lane 5, 800 pg). Lanes 6–11: *Ecto* (500 pg) blocks inductions by *CA-Alk5* (200 pg) and *Smad2* (300 pg) but not by *FGFr1* (1 ng).

(B) RT-PCR analysis of injected animal caps shows that a low dose of Ecto-MO (25 ng), which is inactive by itself, can induce mesoderm very effectively in conjunction with suboptimal doses of Activin protein (compare lane 4 with lanes 2 and 3).

(C) Ectopic mesoderm gene expressions triggered by Ecto-MO (60 ng) are blocked by coinjection of the TGF-β antagonist *Cer-S* mRNA (300 pg).

(D) Activation of the BMP target *Vent-1* by *Smad1* mRNA (1 ng) and *CABR* mRNA (700 pg) is antagonized by *Ecto* mRNA (800 pg).

(E) Forced expression of *Ecto* mRNA (400 pg and 800 pg) in cultured animal caps (harvested at stage 27) leads to upregulation of neural markers (*NCAM* and *Sox2*) and downregulation of epidermis (*Ker*).

(F) Ecto-depleted animal cap cells display a reduced responsiveness to neural induction triggered by increasing amounts of Chordin mRNA. Ecto-depleted animal cap cells are resistant to weak BMP antagonism (lanes 3 and 4) but still undergo neural default differentiation upon complete BMP blockade (lanes 7 and 8). To prevent any interference with the mesoderm-inducing effects of Ecto-MO (+), animal caps were explanted at the early blastula stage (stage 8–8.5), when they have not yet received any mesoderm-inducing signal. (–) stands for Control-MO.

(G–L) Downregulation of Ectodermin by doses of Ecto-MO parallels with reduction of neural tissue (*Sox2*) and concomitant expansion of epidermis (*ker*).

(M) A model for the function of Ectodermin during embryogenesis.

tween Smad3 and Smad4. For BMP signaling, we monitored transcription from the minimal *Vent-2* promoter, whose activation relies on Smad1/Smad4 complexes. As shown in Figures 4A and 4B, raising the levels of Ectodermin in human cells inhibits the transcriptional response to TGF-β/BMP ligands on these reporters. These studies suggest that Ectodermin operates within the TGF-β/BMP signaling cascades.

We investigated whether the biological antagonism between Ectodermin and TGF-β signals reflected a physical interaction between Ectodermin and the Smads. Indeed, coimmunoprecipitation experiments using epitope-tagged proteins from HEK293T cells showed that Ectodermin binds Smad4 but not other Smads (Figure 4C). To determine whether this interaction occurred at physiological protein levels, we immunoprecipitated endogenous Ectodermin from HEK293T cells and monitored for copurified Smad4 by Western blot and vice-versa. As shown in Figures 4D and 4E, Ectodermin and Smad4 form a complex in vivo. By using a GST-Smad4 affinity matrix and in vitro-translated Ectodermin fragments, we mapped the interaction domain with Smad4 in the linker region of Ectodermin (Figure 4F). Notably, the Ectodermin/Smad4 biochemical interaction is consistent with the biological effects of Ectodermin, being that Smad4 is shared by the BMP and TGF-β signaling branches.

Next we tested whether the antagonism between Ectodermin and TGF-β/BMP signals is mediated by its specific interaction with Smad4. As shown in Figure 4G, expression of Smad4 rescues the activation of neural markers by injected *Ecto* mRNA (lanes 2–4). Conversely, expression of Ectodermin counterbalances mesoderm expansion by increased Smad4 levels, as attained by mRNA injection in the marginal zone of the embryo (Figure 4H). Previous work has shown that ectopic expression of Smad4 in animal cap cells is a quite inefficient inducer of mesoderm (Howell et al., 1999); given the high level of Ectodermin expression in the animal cap, we speculated this may account, at least in part, for the low efficacy of Smad4 in this tissue. Accordingly, we found that partial depletion of Ectodermin

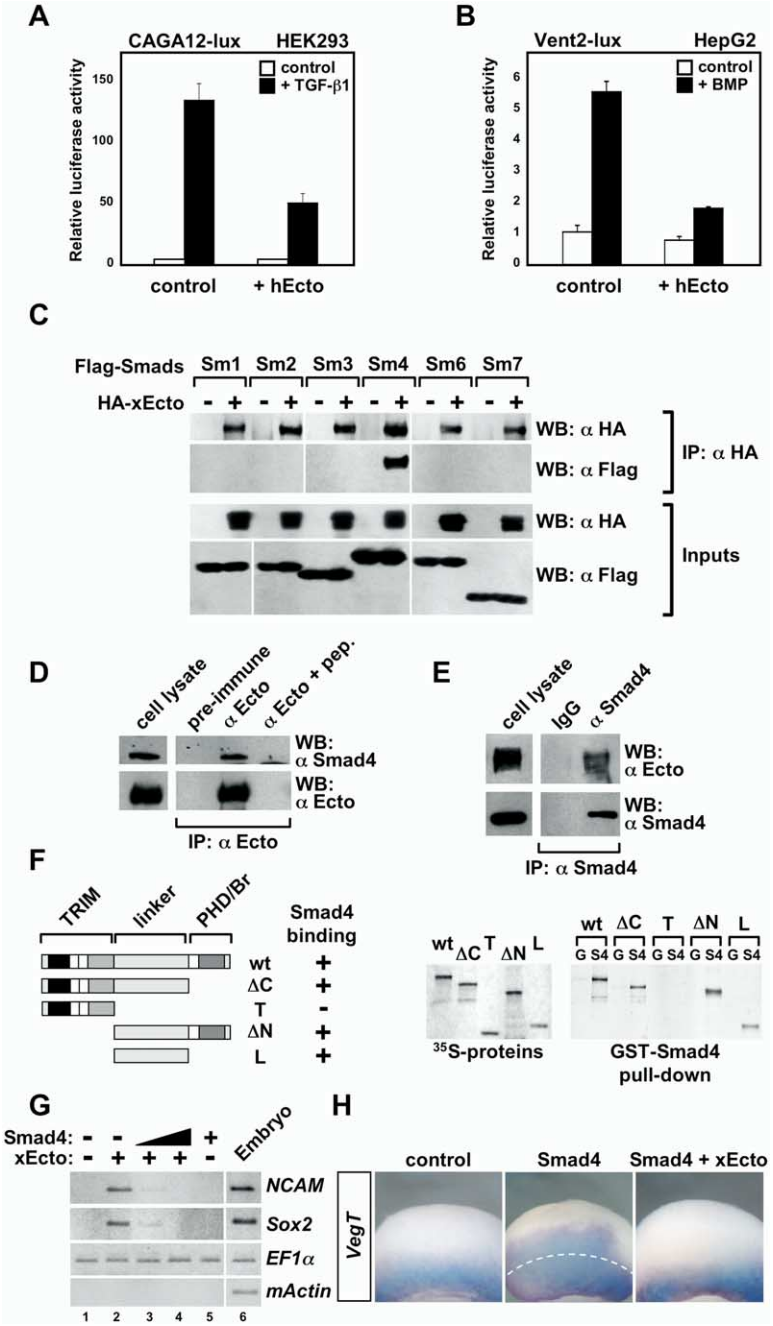

Figure 4. Ectodermin Binds Smad4

(A) Human Ectodermin inhibits TGF-β signaling. The $CAGA_{12}$ luciferase reporter (100 ng) was transfected in HEK293T cells alone or in combination with human Ectodermin expression plasmid (500 ng) as indicated. After transfection, cells were either left untreated (white bars) or treated with 100 pM TGF-β1 (black bars). Doses are for transfection of 2 cm^2 wells. Error bars are standard deviations.

(B) Human Ectodermin inhibits BMP signaling. The Vent-2 BMP minimal responsive element (150 ng) was transfected in HepG2 cells alone or in combination with human Ectodermin expression plasmid (500 ng) as indicated. BMP signaling was provided by cotransfected CA-Alk3 and Smad1 expression plasmids.

(C) Ectodermin interacts with Smad4 in vivo. Shown is immunoprecipitation (anti-HA antibody) of HEK293T cell lysates transfected with the indicated expression plasmids for different Flag-tagged Smad proteins and HA-tagged *Xenopus* Ecto.

(D and E) A physiological complex between endogenous Ectodermin and Smad4 in HEK293T cells. pep indicates the Ectodermin immunogenic peptide competing for the antibody.

(F) In vitro interaction between Ectodermin deletion mutants and Smad4. In vitro-translated ^{35}S-Ecto fragments were incubated with bacterially expressed Smad4 as Sepharose bound GST fusion. Copurifying Ecto fragments were resolved by SDS-PAGE and visualized by autoradiography. The linker region of Ectodermin contains the Smad4 interaction domain.

(G) RT-PCR analysis of cultured animal caps (harvested at stage 27) expressing the indicated mRNAs (dose in parenthesis). Lanes 2–4: the neural markers' inductions (*Sox2* and *NCAM*) by *Ecto* (400 pg) are downregulated by increasing doses of *Smad4* (lane 3, 3 ng; lane 4, 6 ng).

(H) In situ hybridization for the mesoderm marker *VegT* on embryos uninjected (control) or injected in a single blastomere at the four-cell stage with *Smad4* mRNA (2.5 ng) alone or in combination with *Ecto* mRNA (800 pg). Pictures are lateral close-up views, with blastopore lip at the bottom. Dashed line indicates the normal upper limit of *VegT* expression.

enables mesoderm induction by doses of *Smad4* mRNA that are otherwise inactive (Figure S6).

Two different Smad4s have been described in *Xenopus*: XSmad4α is the homolog of mammalian Smad4; the second Smad4 (XSmad4β) displays mesoderm-inducing abilities as well as additional TGF-β/BMP-independent effects during neural development (Howell et al., 1999; LeSueur et al., 2002). Coinjection of *Ecto* mRNA inhibits mesoderm induction triggered in animal caps by both *XSmad4* mRNAs (Figure S7).

Ectodermin Ubiquitinates Smad4

Ectodermin might antagonize Smad4 by any of several mechanisms. One possibility that retained our attention was that Ecto might affect Smad4 stability, given that Ectodermin contains an N-terminal RING finger, a domain found in several E3 ubiquitin ligases. To test this hypothesis, we prepared a point mutation in the RING-finger domain of Ectodermin, creating a catalytically inactive mutant, Ecto-CAmut (C97A/C100A). This mutant is unable to interact within the ubiquitination machinery, resulting in a protein void of any E3 ligase activity (Zhu et al., 1999). Importantly, when compared to wild-type Ectodermin, Ecto-CAmut was biologically inactive in frog assays (Figure 5A). Of note, Ecto-CAmut protein retains normal Smad4 binding capability (Figure S8). These results argue that the biological activity of Ectodermin as TGF-β antagonist primarily stems from

the enzymatic activity of its RING finger rather than from other mechanisms, i.e., Smad4 sequestration.

The potential for Ectodermin to modulate the stability of Smad4 was tested in *Xenopus* cells. In the presence of coexpressed Ectodermin, the steady-state levels of Smad4 were severely decreased (Figure 5B, lanes 5 and 6), whereas Ectodermin had no effect on the steady-state levels of Smad2 or Smad3 (Figure 5B, lanes 1–4). To investigate whether Ectodermin promotes Smad4 degradation, we analyzed the turnover of overexpressed Smad4 by pulse-chase experiments in human HEK293T cells. Figure 5C shows that, although the level of newly synthesized Smad4 is similar in all samples, with increasing incubation times the disappearance of Smad4 was accelerated in cells transfected with human or *Xenopus* Ectodermin expression plasmids. Overexpression of Ectodermin leads also to decreased steady-state levels of endogenous Smad4 (Figure 5D, lanes 1–3), an effect reversed by treatment of transfected cells with MG132, an inhibitor of the 26S proteasome (Figure 5D, lanes 4–6). Together, these data indicate that Ectodermin enhances Smad4 degradation via the ubiquitin-proteasome pathway.

Next we tested whether Ectodermin operates as a Smad4 ubiquitin ligase. As shown in Figure 5E, combinations of ubiquitin, Smad4, and Ecto expression constructs were transfected in HEK293T cells. We monitored the in vivo formation of ubiquitin-conjugated products of Smad4 by assaying immunopurified Smad4 with anti-Smad4 immunoblots. We found that Ectodermin markedly increased ubiquitin-conjugated Smad4, as demonstrated by migration of Smad4 as a ladder of high-molecular-weight bands (Figure 5E, compare lanes 3 and 6), which correspond to ubiquitin-conjugated products (data not shown). As a negative control, the RING-finger mutant hEcto-CAmut (C125A/C128A) was not effective in this assay (Figure 5F).

To test the in vivo relevance of Ectodermin in the process of Smad4 ubiquitination, we compared the ubiquitination pattern of Smad4 in wild-type and Ecto-MO-injected frog embryos. As shown in Figure 5G, the endogenous machinery ubiquitinating Smad4 is well active in *Xenopus* embryos; however, Smad4 ubiquitination is dramatically reduced upon Ectodermin knockdown. Similarly, targeting translation of human Ectodermin in HEK293T cells by transfection of morpholino antisense oligonucleotides inhibited the basal level of Smad4 ubiquitination (Figure S9). These findings prompted us to further test the relevance of Ectodermin on Smad4 stability in *Xenopus* embryos: as shown in Figure 5H, depletion of Ectodermin increases the steady-state levels of injected Myc-tagged XSmad4α. Together these results suggest that Ectodermin is an essential mediator of Smad4 ubiquitination and stability in vertebrate cells.

As originally proposed by Attisano and colleagues, several Smad4 mutant alleles found in human cancers code for proteins that become heavily ubiquitinated and whose instability undermines the tumor-suppressive properties of Smad4 (Xu and Attisano, 2000). We sought to determine whether endogenous Ectodermin plays a role in these events. For this, we transfected the unstable Smad4(R100T) mutant in HEK293T cells together with control or anti-Ecto morpholinos. Down-regulation of endogenous Ectodermin protein reflected a concomitant rise of Smad4(R100T) to levels comparable to those obtained by treatment with the proteasome inhibitor MG132 (Figure 5I). This finding raises the interesting possibility that, during tumor progression, Ectodermin may cooperate with Smad4 mutations to attenuate TGF-β responsiveness in cancer cells.

Ectodermin Antagonizes Nuclear Accumulation of Smad4

We found that Ectodermin is an exclusive nuclear protein (Figure 1G and Figures S1E and S10), whereas Smad4 constantly shuttles in and out of the nucleus (Nicolas and Hill, 2003). Evidence indicates that this aspect of Smad4 regulation is intimately connected to Smad4 ubiquitination. For instance, ubiquitinated Smad4 mutant proteins found in human cancers are defective in nuclear accumulation (Moren et al., 2000). Moreover, sumoylation, a treatment that antagonizes ubiquitination, promotes Smad4 nuclear accumulation and metabolic stability (Lin et al., 2003). Given these precedents, we investigated whether Ectodermin regulates the nuclear versus cytoplasmic localization of Smad4. To do so, we verified the localization of endogenous Smad4 in HeLa cells transfected with control-siRNA or with Ecto-siRNA. In the absence of TGF-β stimulation (Figure 6A), Smad4 was typically evenly distributed in nucleus and cytoplasm and found mostly nuclear only in a minority of cells, as previously reported (Lin et al., 2003). As expected, treatment with TGF-β shifted part of the cytoplasmic pool of Smad4 within nuclei (Figure 6B). Remarkably, however, the number of untreated cells with predominantly nuclear Smad4 increased markedly upon Ecto depletion, and TGF-β further enhanced this phenomenon (Figures 6C and 6D, quantitations in Figure 6F). The results suggest that a physiological function of Ectodermin is to enforce the nuclear clearance of Smad4.

Ectodermin Restrains TGF-β-Induced Cytostasis

By setting an attenuated Smad response, Ectodermin might favor an escape from TGF-β antiproliferative effects. To test this hypothesis, we used BrdU incorporation to assay DNA synthesis/cell cycle progression in control- and Ecto-siRNA-treated HepG2 cells. Depletion of Ectodermin inhibited the basal rate of BrdU incorporation and potently enhanced growth arrest induced by exogenous Activin (Figure 6G).

To investigate molecularly the relevance of Ectodermin on the regulation of cell growth, we monitored the protein levels of the CDK inhibitor $p21^{WAF1}$, a pivotal target of TGF-β-mediated tumor suppression (Siegel and Massague, 2003). Treatment with Ecto-siRNA upregulated the basal levels and dramatically enhanced the induced levels of $p21^{WAF1}$ as a response to Activin (Figure 6H). Together, these loss-of-function studies support the view that Ectodermin has growth-promoting functions in mammalian cells by relaxing the restraints on cell proliferation imposed by TGF-β. Of note, these data remarkably recapitulate the enhanced sensitivity to TGF-β signals previously observed in Ecto-depleted *Xenopus* cells.

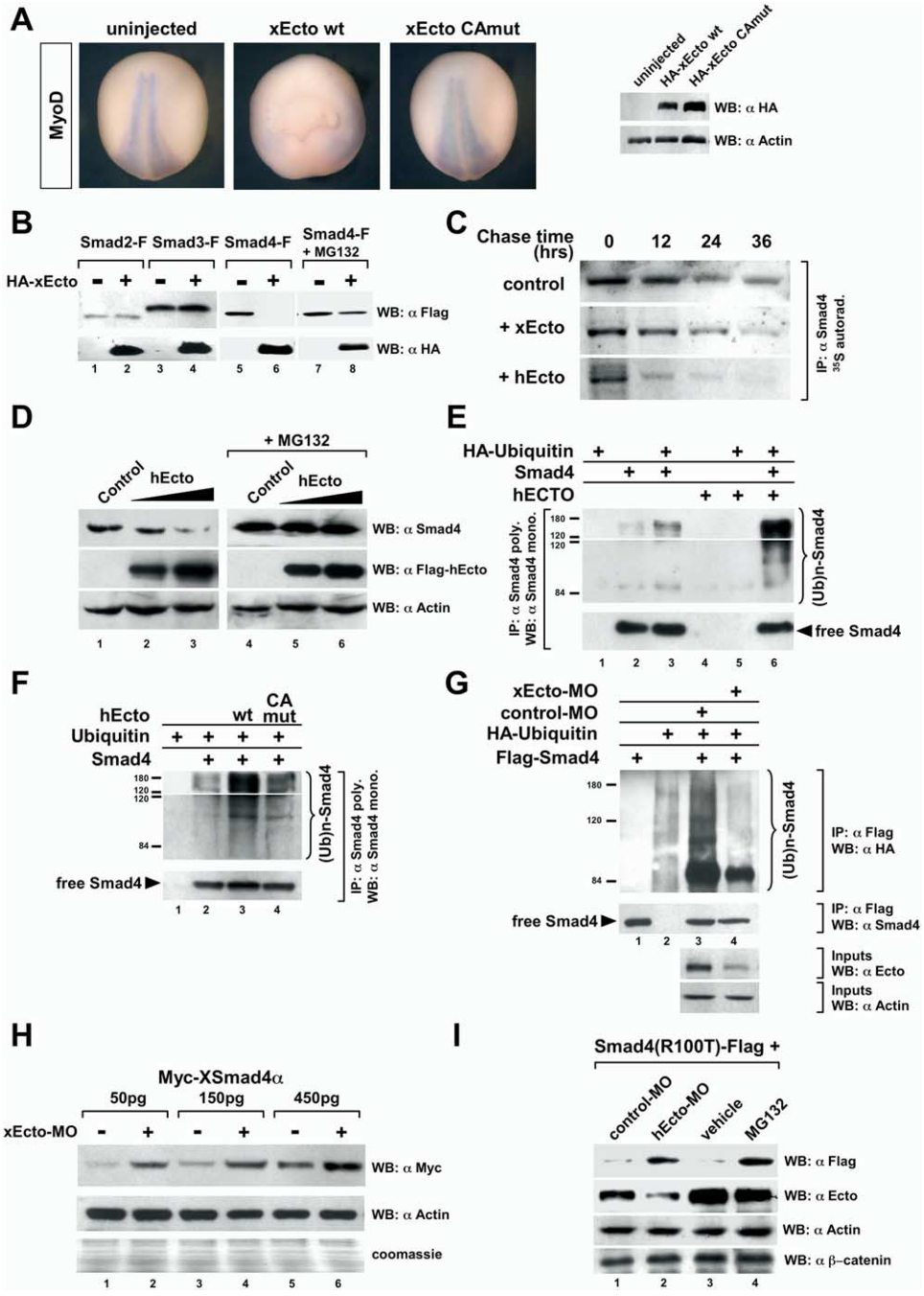

Figure 5. Ectodermin is a Ubiquitin Ligase for Smad4

(A) The biological activity of Ectodermin relies on an enzymatically active RING finger. Two-cell-stage embryos were injected in the marginal zone with mRNAs (800 pg) encoding wild-type or RING-finger point mutant (EctoCAmut) Ecto proteins and processed by in situ hybridization for paraxial mesoderm marker *MyoD*. On the right is Western blotting of embryo lysates showing similar protein levels.

(B) Expression of Ectodermin decreases the steady-state level of Smad4 via the proteasome pathway. *Xenopus* embryos were radially injected with 200 pg mRNAs coding for Flag-tagged Smads together with 1 ng of mRNAs for β-gal (–) or xEcto (+). Steady-state protein levels were determined by Western blotting of total embryo lysates. Lanes 7 and 8: mRNAs were coinjected with the proteasome inhibitor MG132 (10 μM/embryo volume). Note that the inhibition of proteasome reverts the effect of Ectodermin on Smad4.

(C) Overexpression of Ectodermin causes an increased rate of Smad4 protein degradation. HEK293T cells were transfected with HA-tagged Smad4 expression plasmid alone (1 μg/10 cm dish) or in combination with human or *Xenopus* Ectodermin vectors (10 μg). After labeling with ^{35}S-Met/Cys and chasing for the indicated times, cell lysates were immunoprecipitated with anti-HA antibody and labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

(D) Endogenous Smad4 protein is downregulated by raising the level of Ectodermin in human cells. HEK293T cells were transfected with pCS2-hEcto (lanes 2 and 5: 7 μg; lanes 3 and 6: 20 μg) and treated for 6 hr with vehicle (DMSO, lanes 1–3) or 30 μM MG132 (lanes 4–6), and Smad4 steady-state levels were determined by Western blotting. Note that downregulation of Smad4 by hEcto (compare lanes 1–3) is inhibited in lanes 5 and 6.

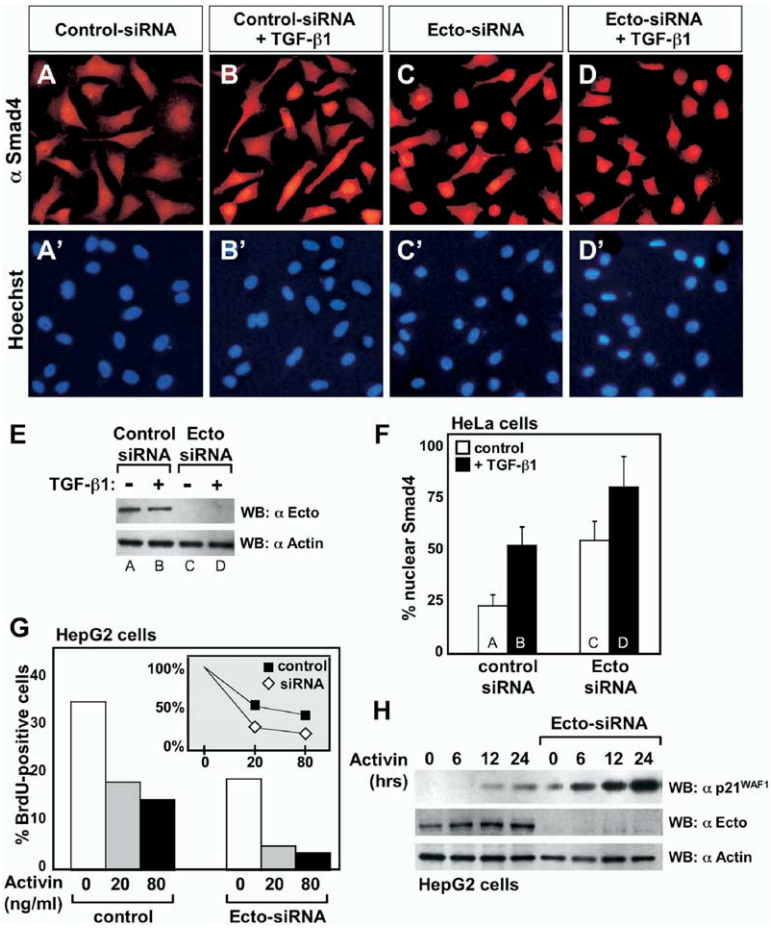

Figure 6. Ectodermin Limits Smad4 Nuclear Accumulation and TGF-β-Induced Growth Arrest

(A–D) Immunofluorescence localization of endogenous Smad4 in control-siRNA- and Ecto-siRNA-depleted HeLa cells, untreated or treated for 90 min with 5 ng/ml TGF-β1 (see Experimental Procedures). Nuclei of the same cells were stained with Hoechst (A′–D′).

(E) Western blotting for samples shown in (A)–(D) to demonstrate quantitative depletion of endogenous Ecto by RNAi.

(F) Quantitations of HeLa cells displaying nuclear Smad4 (same as pictures shown in [A]–[D]). (A) 18%, n = 266; (B) 46%, n = 536; (C) 49%, n = 305; (D) 79%, n = 965. Error bars are standard deviations.

(G) Growth-inhibitory effect of Activin on mock and Ecto-siRNA-treated HepG2 cells, as measured by BrdU incorporation. Columns show the percentage of cells in active DNA synthesis. Inset: the number of BrdU-positive cells in unstimulated cultures was given an arbitrary value of 100%; all other values (Activin treatments) are shown relative to this. Activin is more effective upon Ecto knockdown.

(H) Time course of $p21^{WAF1}$ induction by Activin (20 ng/ml) in mock and Ecto-depleted HepG2 cells. t = 0 is 48 hr after transfection of siRNA. Note that induction of $p21^{WAF1}$ has an earlier onset and reaches higher levels upon Ecto knockdown.

Smad4 Is Epistatic to Ectodermin in Regulating Cell Proliferation

A wealth of genetic studies link the inactivation of Smad4 to the escape from TGF-β/BMP growth-inhibitory responses in several malignancies, especially those of the pancreas and colon (Howe et al., 1998; Takaku et al., 1999). As Ectodermin is a regulator of Smad4 function, we determined the expression of Ectodermin by immunohistochemistry in the human intestine. Strikingly, Ectodermin is localized in the nuclei of the stem cells at the bottom of the crypts (i.e., proliferative compartment) (Figures 7A and 7C), and this staining declines in the higher portion of the gland, becoming undetectable in the superficial (i.e. differentiated) compartment (Figure 7B) (n = 8, 100%; normal mucosa obtained from the right colon). It has been proposed that the biology of intestinal tumors closely recapitulates developmental and homeostatic aspects of their

(E) Ectodermin enhances ubiquitination of Smad4. HEK293T cells were transfected with combinations of pCS2-HA-ubiquitin (1 μg), pCDNA-Smad4 (1 μg), and pCS2-hEcto (15 μg). Cells were harvested after overnight treatment with 10 μM MG132, and lysates were boiled in 1% SDS before immunoprecipitation with anti-Smad4 polyclonal antibody in 0.1% SDS. Ubiquitin-conjugated Smad4 [(Ub)n-Smad4] was detected by Western blotting with anti-Smad4 monoclonal antibody. The panel is composed by three parts (>120 KDa, 84–120 KDa, and free Smad4 at the bottom) corresponding to different exposition times. Plasmid doses are for transfection of 10 cm petri dish.

(F) Ectodermin ubiquitin ligase activity depends on a catalytically active RING finger. Experimental settings were as described in (E).

(G) The endogenous machinery that ubiquitinates Smad4 in *Xenopus* relies on Ectodermin. Two-cell-stage embryos were injected with the indicated combinations of morpholinos (60 ng) and mRNAs (*HA-ubiquitin*, 1 ng; *Flag-Smad4*, 1 ng) and harvested at the gastrula stage for immunoprecipitation with anti-Flag antibody. Ubiquitin-conjugated Smad4 [(Ub)n-Smad4] was detected by Western blotting with anti-HA antibody (upper IP panel). Equal levels of precipitated free Smad4 were checked by anti-Flag immunoblotting (lower IP panel). At the bottom, anti-Ecto and anti-actin Western blots of total lysates used for IPs in lanes 3 and 4.

(H) Depletion of Ectodermin increases the steady-state levels of injected XSmad4α in *Xenopus* embryos. Increasing doses of Myc-tagged *XSmad4*α mRNA were coinjected with 50 ng of control-MO (–) or Ecto-MO (+). Embryos were harvested at the gastrula stage for Western blotting. Epitope-tagged Smad4 was used because antibodies against *Xenopus* Smad4 are not available.

(I) Ectodermin knockdown in human cells rescues the stability of cancer-derived mutant Smad4(R100T). HEK293T cells were transfected with Flag-tagged Smad4(R100T) expression plasmid (4 μg/10 cm dish) and treated as follows. Lane 1: cotransfection of control-MO; lane 2: cotransfection of hEcto-MO; lanes 3 and 4: treatment with vehicle (DMSO) or MG132. Western blotting of total cell lysates shows that depletion of Ecto, just like inhibition of the proteasome, raises Smad4(R100T) level.

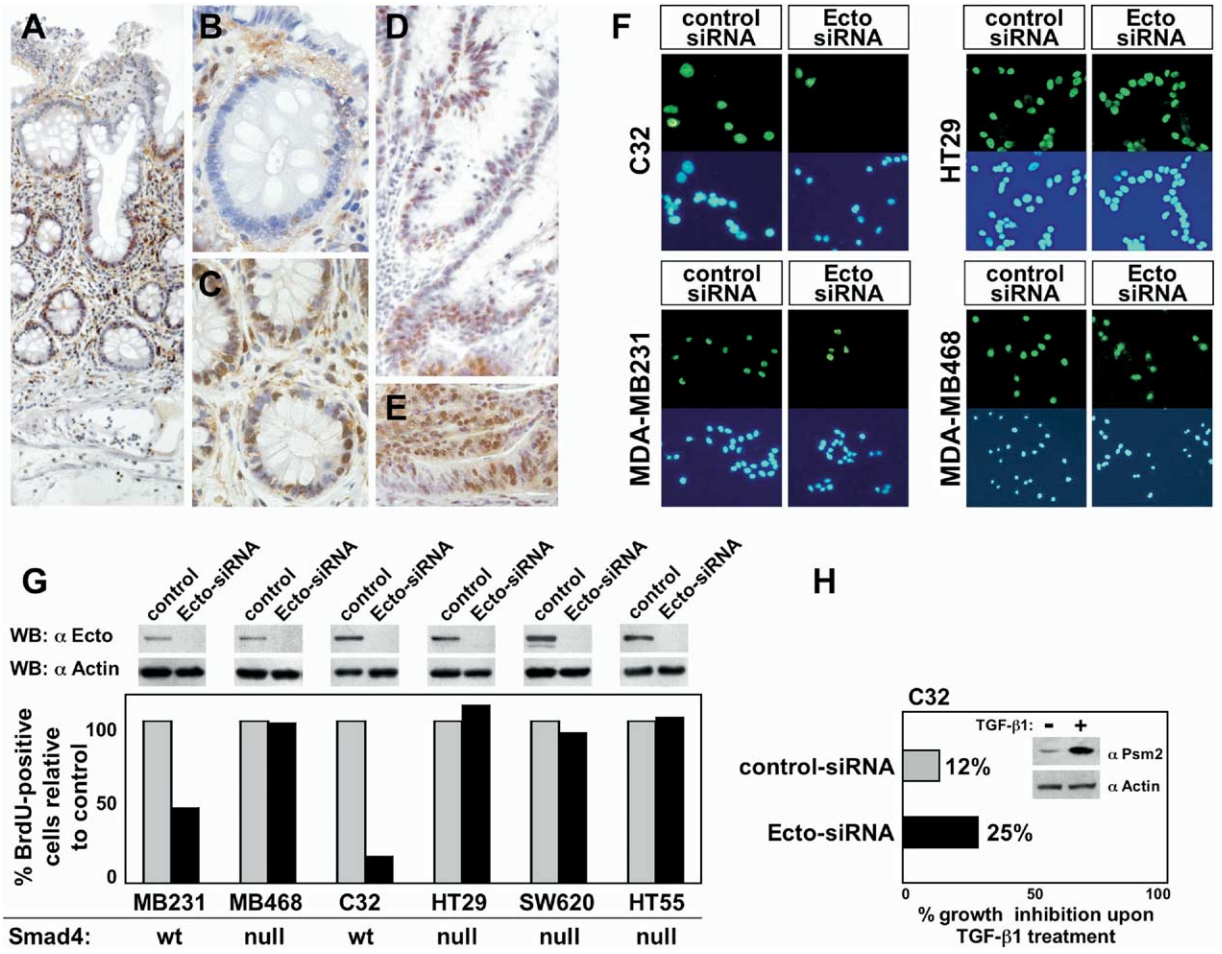

Figure 7. Ectodermin Controls Cell Proliferation via Smad4

(A–E) Immunohistochemical nuclear expression of Ectodermin in normal (A–C) and neoplastic (D, adenomas; E, carcinomas) human colon.

(F and G) Effects of Ectodermin depletion on cell proliferation as measured by BrdU incorporation. Green is anti-BrdU; blue is Hoechst. (G) Smad4 is epistatic to Ectodermin. Columns show the effect of Ecto-siRNA depletion on cell proliferation (black bars) relative to control-siRNA-treated cultures (gray bars). The immunoblot lanes on the top confirm the quantitative knockdown of Ecto in each cell line. Only cells wild-type for Smad4, but not Smad4-null cells, are growth arrested upon Ecto downregulation.

(H) Depletion of Ecto partially rescues the TGF-β1 (5 ng/ml) growth-suppressing activity in C32 cells. Inset: anti-phospho-Smad2 immunoblot shows that TGF-β1 signaling is effective in C32 cells.

progenitor stem cells (van de Wetering et al., 2002). According to this hypothesis, the immunohistochemical expression of Ectodermin was tested in a pilot series of eight cases of advanced colonic precancerous lesions (adenomas with low- and high-grade dysplasia) and in four fully developed, well-differentiated, epithelial colonic malignancies. A noncompartmentalized overexpression of Ectodermin was found in all cases of both colonic adenomas and adenocarcinoma (Figures 7D and 7E).

Next we sought to determine whether Ectodermin plays a causative role in proliferation of colorectal cancer (CRC) and breast cancer cells by keeping blunted Smad4-dependent growth inhibition. We used C32 cells as a CRC model system, as these cells express high amounts of Ectodermin and display an attenuated TGF-β-induced growth inhibition (Figure 7H), despite expression of wild-type Smad4 protein (Woodford-Richens et al., 2001) and capacity to transduce TGF-β signals (see the phospho-Smad2 immunoblot in Figure 7H). In parallel, we also used the MDA-MB231 breast cancer cell line, which retains low levels of wild-type Smad4 and displays attenuated TGF-β responses (Chen et al., 2001). As shown in Figure 7F, transient but quantitative depletion of Ectodermin by RNAi drastically inhibited cell proliferation in both C32 and MDA-MB231 cells, as assayed by BrdU incorporation. Crucially, this inhibition operates via Smad4, as depletion of Ectodermin in Smad4 mutant cells (colon HT29, SW620, and HT55, and breast MDA-MB468 cell lines) was void of any effect on cell proliferation (Figure 7G). As Smad4 appears epistatic to Ectodermin, this finding strengthens the notion that Smad4 regulation is the primary biological target of Ectodermin. Interestingly, in

Ecto-depleted C32 cells, we observed a partial rescue of TGF-β1-induced growth arrest (Figure 7H). Collectively, the results suggest that Ectodermin is critical for the control of cell proliferation in some human cancers.

Discussion

An Ectoderm Determinant in Xenopus Embryos

We identified Ectodermin (Ecto) in an unbiased screen for regulators of early embryonic development. Ectodermin is a localized factor essential for correct formation of the embryonic germ layers. Depletion of Ectodermin from the marginal zone of the embryo causes a shift in the cellular fates along the animal-vegetal axis. The endoderm is expanded well within the prospective mesoderm, whereas the mesoderm, which normally entails a ring of cells at the equator of the embryo, now spreads toward the animal pole at the expense of the ectoderm. Such loss-of-function phenotype of Ectodermin is recapitulated by overexpression of Smad4 in the marginal zone, in agreement with the identification of Smad4 as the main target of Ectodermin. This indicates that, by keeping TGF-β/nodal signaling under control, Ectodermin ensures that ectoderm cells do not adopt a mesodermal fate. Thus, the early frog embryo is patterned by two maternal factors located at the antipodes of the egg: Ectodermin at the animal pole and VegT at the vegetal pole (Zhang et al., 1998). The fine balance between these opposite activities regulates the correct allocation of cells to the primary germ layers (Figure 3M).

In contrast to mesoderm, ectoderm can form in the absence of cell-cell contact, differentiating as neural tissue whenever BMP ligands are antagonized (Piccolo et al., 1996; Wilson and Hemmati-Brivanlou, 1995; Zimmerman et al., 1996); this suggests that maternal, cell-autonomous factors do exist to favor the default neural fate. Knockdown of Ectodermin impairs neural induction triggered by BMP inhibition; conversely, raising the level of Ecto causes expression of neural markers in animal cap cells, and adding back Smad4 reverses these inductions. Thus, Ectodermin generates a bias toward neural differentiation. The need for a control over BMP signaling in the differentiating ectoderm is not unprecedented since other TGF-β-negative regulators expressed in this tissue have been described, such as Smurf-1, the E3 ubiquitin ligase for Smad1, the secreted factor Coco, and the BMP decoy receptor BAMBI (Bell et al., 2003; Onichtchouk et al., 1999; Zhu et al., 1999). Indeed, BMP signaling is capable of an efficient feed-forward loop, with BMP fostering its own expression (Schmidt et al., 1996), raising the need for multiple inhibitory control mechanisms to ensure its strict spatial and temporal control in early embryos, at least until the Organizer's antagonists ensure a BMP-free zone in dorsal ectoderm.

Ectodermin Limits Smad4 Function

Although Smad4 plays a fundamental role in TGF-β signaling during development, homeostasis, and cancer, our knowledge on its regulation remains limited (Siegel and Massague, 2003). Recent evidence, however, has highlighted a central role for Smad4 ubiquitination (Lin et al., 2003; Moren et al., 2000; Xu and Attisano, 2000). Yet the molecular effectors of this activity remain enigmatic. Ectodermin binds Smad4 at the endogenous protein level and is required for Smad4 ubiquitination in vivo. In so doing, Ectodermin promotes Smad4 metabolic instability by increased proteasomal degradation. Our findings suggest a model in which Ectodermin constrains TGF-β/BMP gene responses by promoting Smad4 ubiquitination in the nucleus and favoring Smad4 relocalization in the cytoplasm, where it can be efficiently degraded. In support of this notion, Ecto-depleted cells display an increased nuclear accumulation of Smad4.

One intriguing possibility is that, in some cellular contexts, Ectodermin may mostly promote nuclear exclusion of Smad4 rather than its degradation. This would represent an economic but nevertheless effective mechanism to make cells transiently incompetent to activate TGF-β gene responses, without depleting cells of a key tumor suppressor. In other cases, especially in cancers, Ectodermin may also favor Smad4 degradation, leading to a more stable escape from the TGF-β antiproliferative response.

Ectodermin Is a Determinant for Cell Proliferation

We found that Ectodermin is an endogenous limiting factor for cytostasis induced by TGF-β/Smad4 signaling in human cells. When Ecto is disabled by RNAi, cells respond more potently to TGF-β signals, displaying a marked exit from the cell cycle. It has been shown that the activation of the CDK inhibitor $p21^{WAF1}$ is central in TGF-β-induced cytostasis and requires persistent Smad accumulation in the nucleus (Nicolas and Hill, 2003). Notably, in Ecto-depleted cells, $p21^{WAF1}$ is promptly activated by TGF-β signals without delay; also, $p21^{WAF1}$ is now induced at much higher levels than in control cultures. Such robust antiproliferative effects and endogenous gene responses indicate that Ectodermin acts as a rate-limiting step for TGF-β signaling.

During normal tissue homeostasis, escape from TGF-β/Smad4-induced cytostasis is crucial in a subset of progenitor cells devoted to ensuring epithelial renewal (Howe et al., 1998; Siegel and Massague, 2003). In the colon, these stem cells lie at the bottom of the intestinal crypts (Bienz and Clevers, 2000). Intriguingly, we have shown that Ectodermin is expressed in these cells and is downregulated as cells enter the differentiation compartment. This finding suggests that Ectodermin may behave as a switch, limiting TGF-β/BMP signaling in normal crypt progenitors and being turned off as cells need to exit from the cell cycle and differentiate.

Lack of responsiveness to TGF-β antiproliferative effects is a hallmark of cancer. Yet cancer cells specifically disable the antimitogenic effects and remain free to exploit with impunity other TGF-β responses to foster their own growth or malignant behavior (Derynck et al., 2001). As for the relevance of Smad4 in these processes, genetic data indicate that loss of Smad4 may

correlate not only with the specific loss of the TGF-β cytostatic response but also with the acquisition of an invasive phenotype (Takaku et al., 1998). Notably, we found that depletion of Ectodermin is by itself sufficient to trigger growth inhibition in colorectal and breast cancer cell lines, despite the presence of multiple oncogenic mutations in these cells. Crucially, this depends on the presence of wild-type Smad4, as quantitative depletion of Ectodermin is void of any effect in Smad4-null cell lines. Given that the majority of tumors do retain wild-type Smad4, it will be interesting to test whether functional inactivation of Smad4 promoted by Ectodermin would not only favor proliferation but also unleash the transforming potential of TGF-β. Whether the interference with Smad4 ubiquitination or Ectodermin-Smad4 protein-protein interaction might represent a novel therapeutic strategy remains to be investigated.

Experimental Procedures

cDNA Library Construction, Functional Screen, and Biological Assays in Xenopus

A full-length enriched cDNA library (3.5 kb average size) cloned in a pCS2-derivative plasmid (Cordenonsi et al., 2003) was prepared from *Xenopus* blastulae and contained 40,000 primary transformants. A total of 7500 primary clones were assayed as described in the text. *Xenopus* embryo manipulations, in situ hybridization, and capped mRNA preparation were as in Piccolo et al. (Piccolo et al., 1999). RT-PCR primers and cycling conditions are described in http://www.hhmi.ucla.edu/derobertis/, except in experiments entailing control-MO/Ecto-MO injections in animal caps for mesoderm induction assays (*VegT*, *Eomes*, and *Mix.2* markers), for which we added two cycles to each sample.

The morpholino antisense oligonucleotide (GeneTools) directed against *Xenopus* Ectodermin (Ecto-MO) was CACCTCCTCCTTTGT TATCCGCCAT, and its mismatching control (control-MO) was CAGCTCCTGCTTTCTGATGCGCGAT.

Cell Culture and Transfections in Mammalian Cells

See Supplemental Data for technical details. For experiments entailing morpholino-mediated knockdown, HEK293T cells were first transfected with expression plasmids as above and 24 hr later treated twice with control or hEcto morpholino/EPEI complexes (GeneTools) in Optimem (Gibco), as detailed by GeneTools. Sequence of hEcto-MO was CCGCCGCCTTTGTTTTCCGCCATGT.

For siRNA treatments, cells were plated onto collagen I-coated dishes to avoid clumping. dsRNA oligos (Ambion, 75 ng/cm^2) were transfected twice using the Oligofectamine protocol (Invitrogen), with the second transfection performed 24 hr after the first one. Control-siRNA was the unrelated sequence 5′-UUCUCCGAACGU GUCACGUtt-3′; the coding strands of siRNA duplexes targeting hEcto, used as a 1:1 mix, were 5′-GGUAUGUACUAGUUGUGAAtt-3′ and 5′-GGAAGAAAGAAGAUGUCUCtt-3′.

Ubiquitination Experiments

Total cell lysates were prepared by sonication in 25 mM HEPES (pH 7.9), 0.2 M KCl, 1% NP40, 5 mM EDTA, 1 mM DTT, and 10% glycerol with protease, phosphatase, proteasome (100 μM MG132, Sigma), and 0.25 μg/ml Ubiquitin-aldehyde (Sigma) inhibitors. Injected *Xenopus* embryos were lysed in 10 mM HEPES (pH 7.9), 0.2 M NaCl, 0.1% NP40, 0.1 mM EGTA, 1 mM DTT, 5% glycerol with inhibitors as above. Prior to immunoprecipitation, lysates were boiled in 1% SDS for 5 min and then diluted to 0.1% SDS with 0.5% NP40 in phosphate-buffered saline (PBS).

Please refer to Supplemental Data for a list of plasmids and descriptions of the experimental procedures for immunoprecipitations and GST pull-downs, pulse-chase, BrdU analysis, immunofluorescence microscopy, immunohistochemistry, and antibodies and Western blotting.

Supplemental Data

Supplemental Data include 10 figures, Experimental Procedures, and References and are available with this article online at http://www.cell.com/cgi/content/full/121/1/87/DC1/.

Acknowledgments

We thank A. Ballabio, C. Chang, K. Cho, R. Derynck, C.H. Heldin, C. Hill, N. Minsky, K. Miyazono, A. Moustakas, F.J. Rauscher III, A.J. Rowan, M. Taira, P. ten Dijke, G.H. Thomsen, I. Tomlinson, and N. Ueno for gifts of plasmids, protocols, and cell lines. We are grateful to Professor W. Bodmer (Cancer Research UK) for supplying C32 cells. A special thanks to Graziano Martello and Marco Montagner for enthusiastic support with *Xenopus* experiments and Dr. Cristiano Lanza for technical assistance in the optimization of the immunohistochemical reaction. We are indebted to D. Volpin, K. McCullagh, G. Bressan, B. Amati, O. Wessely, and S. Schiaffino for thoughtful advice and critical reading of the manuscript. This work is supported by grants from MIUR, "Cittadella Contro il Cancro" ONLUS, and Special Programs of Public Health Minister #12712bis/B (Veneto region) to M.R. and from AIRC, TELETHON-Italy (grant GGP04030), MIUR, and ASI to S.P.

Received: October 20, 2004
Revised: December 29, 2004
Accepted: January 15, 2005
Published: April 7, 2005

References

Bell, E., Munoz-Sanjuan, I., Altmann, C.R., Vonica, A., and Brivanlou, A.H. (2003). Cell fate specification and competence by Coco, a maternal BMP, TGFbeta and Wnt inhibitor. Development *130*, 1381–1389.

Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. Cell *103*, 311–320.

Chen, C.R., Kang, Y., and Massague, J. (2001). Defective repression of c-myc in breast cancer cells: a loss at the core of the transforming growth factor beta growth arrest program. Proc. Natl. Acad. Sci. USA *98*, 992–999.

Cordenonsi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C., and Piccolo, S. (2003). Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. Cell *113*, 301–314.

De Robertis, E.M., Larrain, J., Oelgeschlager, M., and Wessely, O. (2000). The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat. Rev. Genet. *1*, 171–181.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. Nat. Genet. *29*, 117–129.

Harland, R., and Gerhart, J. (1997). Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol. *13*, 611–667.

Howe, J.R., Roth, S., Ringold, J.C., Summers, R.W., Jarvinen, H.J., Sistonen, P., Tomlinson, I.P., Houlston, R.S., Bevan, S., Mitros, F.A., et al. (1998). Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Science *280*, 1086–1088.

Howell, M., Itoh, F., Pierreux, C.E., Valgeirsdottir, S., Itoh, S., ten Dijke, P., and Hill, C.S. (1999). Xenopus Smad4beta is the co-Smad component of developmentally regulated transcription factor complexes responsible for induction of early mesodermal genes. Dev. Biol. *214*, 354–369.

LeSueur, J.A., Fortuno, E.S., 3rd, McKay, R.M., and Graff, J.M. (2002). Smad10 is required for formation of the frog nervous system. Dev. Cell *2*, 771–783.

Lin, X., Liang, M., Liang, Y.Y., Brunicardi, F.C., Melchior, F., and Feng, X.H. (2003). Activation of transforming growth factor-beta

signaling by SUMO-1 modification of tumor suppressor Smad4/DPC4. J. Biol. Chem. *278*, 18714–18719.

Mizuseki, K., Kishi, M., Matsui, M., Nakanishi, S., and Sasai, Y. (1998). Xenopus Zic-related-1 and Sox-2, two factors induced by chordin, have distinct activities in the initiation of neural induction. Development *125*, 579–587.

Moren, A., Itoh, S., Moustakas, A., Dijke, P., and Heldin, C.H. (2000). Functional consequences of tumorigenic missense mutations in the amino-terminal domain of Smad4. Oncogene *19*, 4396–4404.

Nicolas, F.J., and Hill, C.S. (2003). Attenuation of the TGF-beta-Smad signaling pathway in pancreatic tumor cells confers resistance to TGF-beta-induced growth arrest. Oncogene *22*, 3698–3711.

Onichtchouk, D., Chen, Y.G., Dosch, R., Gawantka, V., Delius, H., Massague, J., and Niehrs, C. (1999). Silencing of TGF-beta signalling by the pseudoreceptor BAMBI. Nature *401*, 480–485.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E.M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell *86*, 589–598.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E.M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature *397*, 707–710.

Schmidt, J.E., von Dassow, G., and Kimelman, D. (1996). Regulation of dorsal-ventral patterning: the ventralizing effects of the novel Xenopus homeobox gene Vox. Development *122*, 1711–1721.

Siegel, P.M., and Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat. Rev. Cancer *3*, 807–821.

Snape, A., Wylie, C.C., Smith, J.C., and Heasman, J. (1987). Changes in states of commitment of single animal pole blastomeres of Xenopus laevis. Dev. Biol. *119*, 503–510.

Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M.F., and Taketo, M.M. (1998). Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. Cell *92*, 645–656.

Takaku, K., Miyoshi, H., Matsunaga, A., Oshima, M., Sasaki, N., and Taketo, M.M. (1999). Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. Cancer Res. *59*, 6113–6117.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241–250.

Venturini, L., You, J., Stadler, M., Galien, R., Lallemand, V., Koken, M.H., Mattei, M.G., Ganser, A., Chambon, P., Losson, R., and de The, H. (1999). TIF1gamma, a novel member of the transcriptional intermediary factor 1 family. Oncogene *18*, 1209–1217.

Wessely, O., and De Robertis, E.M. (2000). The Xenopus homologue of Bicaudal-C is a localized maternal mRNA that can induce endoderm formation. Development *127*, 2053–2062.

Whitman, M. (2001). Nodal signaling in early vertebrate embryos: themes and variations. Dev. Cell *1*, 605–617.

Wilson, P.A., and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. Nature *376*, 331–333.

Woodford-Richens, K.L., Rowan, A.J., Gorman, P., Halford, S., Bicknell, D.C., Wasan, H.S., Roylance, R.R., Bodmer, W.F., and Tomlinson, I.P. (2001). SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. Proc. Natl. Acad. Sci. USA *98*, 9719–9723.

Xu, J., and Attisano, L. (2000). Mutations in the tumor suppressors Smad2 and Smad4 inactivate transforming growth factor beta signaling by targeting Smads to the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA *97*, 4820–4825.

Zhang, J., Houston, D.W., King, M.L., Payne, C., Wylie, C., and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. Cell *94*, 515–524.

Zhu, H., Kavsak, P., Abdollah, S., Wrana, J.L., and Thomsen, G.H. (1999). A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. Nature *400*, 687–693.

Zimmerman, L.B., De Jesus-Escobar, J.M., and Harland, R.M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell *86*, 599–606.

Accession Numbers

The sequence of *Xenopus Ectodermin* mRNA has been deposited in GenBank with the accession number AY781409.