Cell

FAM/USP9x, a Deubiquitinating Enzyme Essential for TGFβ Signaling, Controls Smad4 Monoubiquitination

Sirio Dupont,[1] Anant Mamidi,[1] Michelangelo Cordenonsi,[1] Marco Montagner,[1] Luca Zacchigna,[1] Maddalena Adorno,[1] Graziano Martello,[1] Michael J. Stinchfield,[2] Sandra Soligo,[1] Leonardo Morsut,[1] Masafumi Inui,[1] Stefano Moro,[4] Nicola Modena,[3] Francesco Argenton,[3] Stuart J. Newfeld,[2] and Stefano Piccolo[1,*]

[1]Department of Histology, Microbiology, and Medical Biotechnologies, University of Padua School of Medicine, viale Colombo 3, 35131 Padua, Italy

[2]School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA

[3]Department of Biology, University of Padua, via Bassi 58/B, 35131 Padua, Italy

[4]Molecular Modeling Section, Department of Pharmaceutical Sciences, University of Padua, via Marzolo 5, 35131 Padua, Italy

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

DOI 10.1016/j.cell.2008.10.051

SUMMARY

The assembly of the Smad complex is critical for TGFβ signaling, yet the mechanisms that inactivate or empower nuclear Smad complexes are less understood. By means of siRNA screen we identified FAM (USP9x), a deubiquitinase acting as essential and evolutionarily conserved component in TGFβ and bone morphogenetic protein signaling. Smad4 is monoubiquitinated in lysine 519 in vivo, a modification that inhibits Smad4 by impeding association with phospho-Smad2. FAM reverts this negative modification, re-empowering Smad4 function. FAM opposes the activity of Ectodermin/Tif1γ (Ecto), a nuclear factor for which we now clarify a prominent role as Smad4 monoubiquitin ligase. Our study points to Smad4 monoubiquitination and deubiquitination as a way for cells to set their TGFβ responsiveness: loss of FAM disables Smad4-dependent responses in several model systems, with Ecto being epistatic to FAM. This defines a regulative ubiquitination step controlling Smads that is parallel to those impinging on R-Smad phosphorylation.

INTRODUCTION

Transforming growth factor beta (TGFβ) is a family of cytokines regulating a vast array of biological processes (Akhurst and Derynck, 2001; Niehrs, 2004). The ligand-activated receptor phosphorylates the cytoplasmic transducer of the pathway, R-Smad, which forms an active nuclear transcriptional complex upon association with Smad4 (Schmierer and Hill, 2007). The simplicity of this pathway is only apparent: cells can read TGFβ in different ways, turning on specific programs depending on the cellular context as well as strength and duration of the signal. The most striking example of this occurs in embryonic development, where TGFβ and bone morphogenetic protein (BMP) ligands act on pluripotent cells as morphogens, able to induce different gene-expression programs according to ligand concentration and time of exposure (Niehrs, 2004). Although the activation of a specific gene set can be explained by the restricted expression (or activity) of a specific DNA-binding cofactor of Smads, it is less clear how cells integrate TGFβ inputs quantitatively (Schmierer and Hill, 2007).

To translate quantitative differences of extracellular TGFβ into proportional levels of Smad activity, mechanisms must exist to continuously inactivate nuclear Smad complexes; this avoids saturation over time and guarantees that Smads keep monitoring receptor activation. One mechanism by which this is achieved is by controlling R-Smad phosphorylation through the opposing functions of the receptors' kinase activity and R-Smad nuclear phosphatases (Lin et al., 2006; Schmierer and Hill, 2007). However, distinct and hitherto unexplored types of regulation must exist. For example, it has been recently shown that the nuclear R-Smad phosphatases target monomeric R-Smad, but not the Smad4/R-Smad complex (Schmierer et al., 2008), which raises the possibility that Smad4 is also a target of regulation.

Smad4 is a central transducer of TGFβ responses and is essential for most TGFβ biological effects, including embryonic development, tumor suppression, and metastasis. Critically, Smad4 is the only shared mediator of the TGFβ and BMP signaling branches. Unlike R-Smad, however, Smad4 is not regulated by phosphorylation; this has so far sidestepped the potential of Smad4 to also be subjected to rounds of activation/inactivation. Given the existence of Smad4 ubiquitin ligases (Izzi and Attisano, 2006), we have here considered that ubiquitination may represent a mechanism to regulate Smad4 function.

Ubiquitination has been discovered for its role in protein degradation, but in recent years several other mechanisms have emerged by which ubiquitination can regulate protein function, including regulation of subcellular localization, protein-protein interactions, and activity (Salmena and Pandolfi, 2007). Just like phosphorylation, which is constantly opposed by dephosphorylation, ubiquitination is also a reversible modification, as indicated by the existence of a whole family of deubiquitinating enzymes

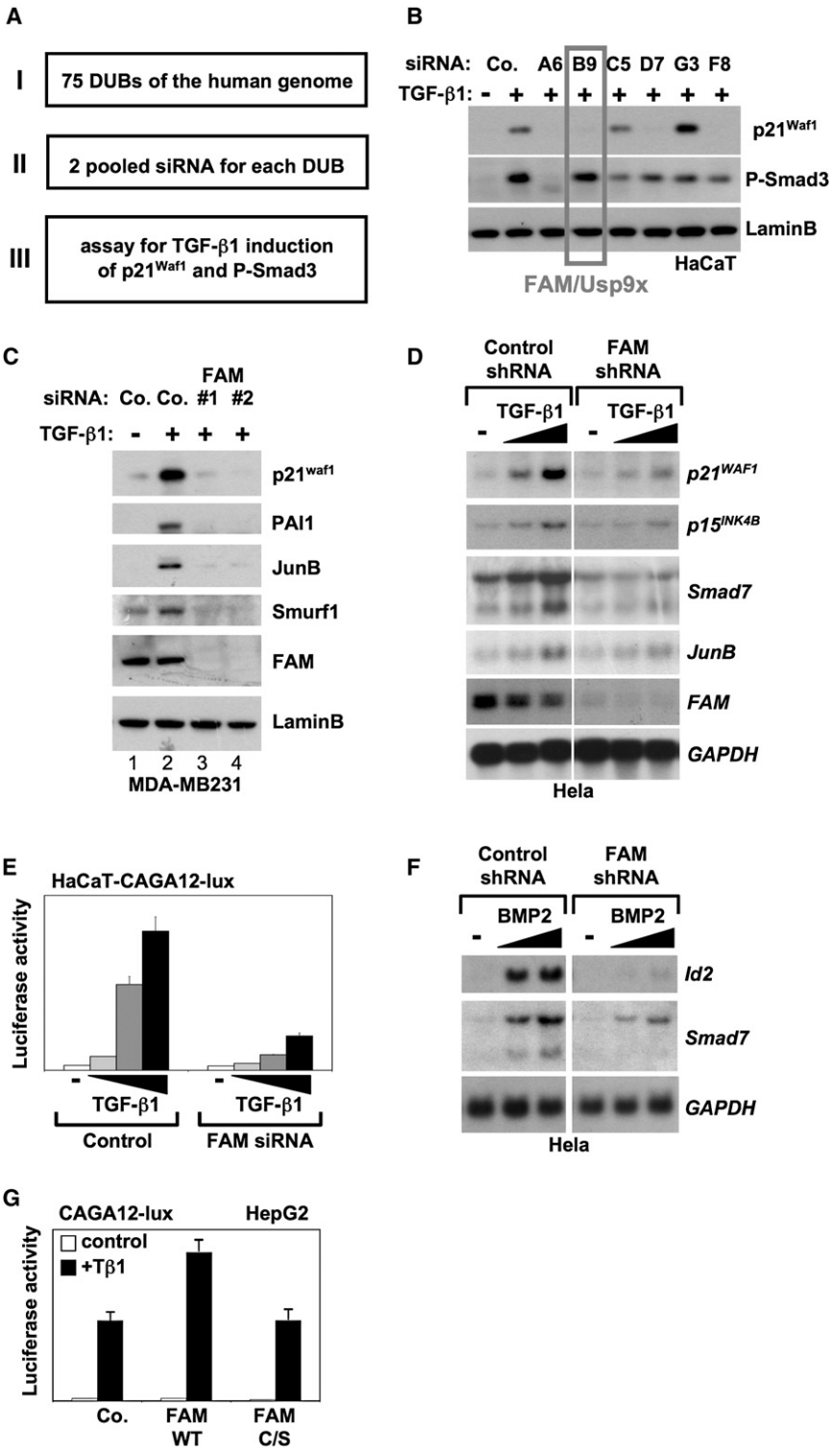

Figure 1. Isolation of FAM/USP9x, a Deubiquitinating Enzyme Required for TGFβ Signaling

(A and B) siRNA screen to identify DUBs regulating TGFβ signaling. (A) Panel shows a diagram of the screening procedure (see text). (B) Panel shows the representative effects of selected anti-DUB siRNAs on TGFβ signaling. Immunoblot for laminB serves as a loading control.

(C) Validation of FAM requirement for TGFβ responses in MDA-MB231 cells. Panels show immunoblots of cells transfected with control (lanes 1 and 2) or two independent FAM-siRNAs (lanes 3 and 4).

(D) FAM is required for Smad-induced transcription in stably depleted HeLa cells, as visualized by northern blotting.

(E) Depletion of FAM in HaCaT cells stably integrated with the CAGA12-lux reporter (Levy et al., 2007) inhibits Smad activity. Data are represented as mean and standard deviation (SD).

(F) FAM is required for BMP signaling in stably depleted HeLa cells, as visualized by northern blotting.

(G) FAM sustains Smad activity through its DUB activity. Data are represented as mean and SD.

(DUBs) (Nijman et al., 2005). Little is known about the role of DUBs in the regulation of TGFβ/BMP signaling.

Here we identified FAM/Usp9x, the homolog of *Drosophila fat facets* (Wood et al., 1997), as a DUB critical for TGFβ and BMP responsiveness in human cells and *Xenopus* embryos. Biochemically, FAM interacts with and deubiquitinates monoubiquitinated Smad4, opposing the activity of Ectodermin/Tif1γ (Ecto), for which we now revise the function as a monoubiquitinating factor that reversibly blocks Smad4 activity, rather than stability (Dupont et al., 2005; He et al., 2006). Monoubiquitination of Smad4, which occurs in lysine 519, hampers the ability of Smad4 to form a stable complex with activated Smad2/3, resulting in pathway inhibition.

RESULTS

FAM/USP9x Is a New Component of the TGFβ Pathway

We sought to identify DUBs involved in TGFβ signaling. To this end, we designed an unbiased loss-of-function screen using siRNAs to inhibit the expression of 75 known or predicted human DUBs (Figure 1A and Table S1 available online). Pooled siRNAs (i.e., a mix of two oligos for each gene) were transfected in HaCaT keratinocytes; after 48 hr, cells were treated with TGFβ1 and then harvested for western blot analysis. As read-outs, we used two endogenous TGFβ responses, namely, the induction of $p21^{Waf1}$ and Smad3 phosphorylation (Figure 1B).

Out of this screen, six siRNA pools inhibited TGFβ effects. Among the corresponding candidate genes, FAM/Usp9x

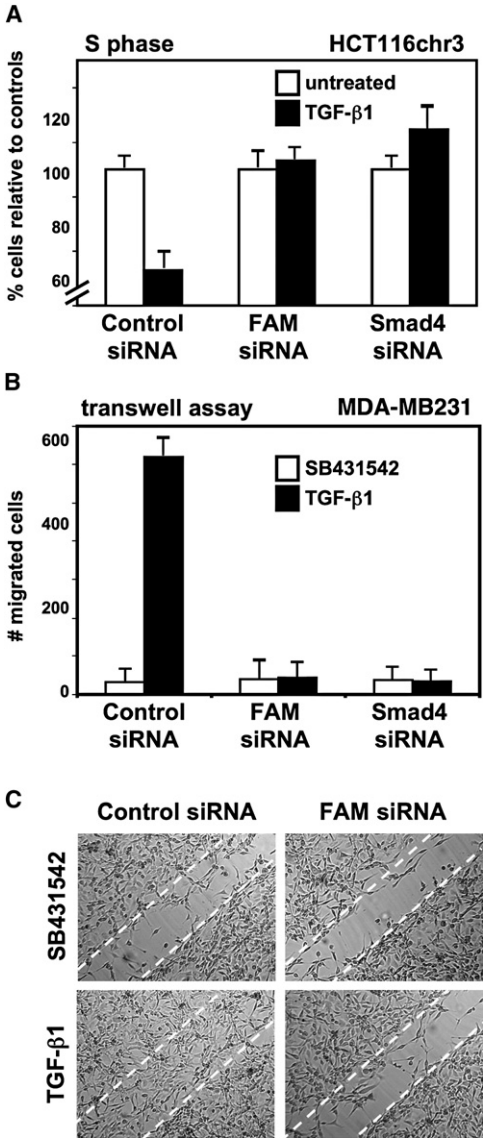

Figure 2. FAM/Usp9x Is Required for TGFβ Effects

(A) FAM is required for TGFβ-induced growth arrest in HCT116chr3 (Wang et al., 2004) colon cancer cells. The number of cells in each phase for unstimulated cultures was given an arbitrary value of 100%, and all other values are depicted relative to this. Data are represented as mean and SD. Immunoblots in Figure S1E show efficient knockdown of target proteins.

(B and C) FAM is required for TGFβ-induced cell migration as assayed in MDA-MB231 breast cancer cells by transwell (B) or scratch-assay (C). The wound edges, at the beginning of the experiment, are indicated by the white dotted lines. Data are represented as mean and SD.

attracted our attention because it displayed the most penetrant requirement for TGFβ responses (see below). FAM is the human homolog of *Drosophila fat facets* (*faf*) gene (FAM stands for *Fa*t facets in *m*ammals), which is essential for fly early embryonic development and, at later stages, plays a role in cell fate specification in the eye (Fischer-Vize et al., 1992). Little is known about the function of FAM. Intriguingly, however, overexpression of *faf* in fly neurons induces synaptic overgrowth, a BMP-related phenotype, but *faf* is unable to do so in Smad4/Medea mutants (McCabe et al., 2004). This finding suggested that FAM could be an evolutionarily conserved regulator of TGFβ responses.

To validate whether FAM is a general mediator of TGFβ responses, we carried out the following experiments. First, we confirmed the initial results using additional, independent siRNAs targeting different regions of the gene, ruling out off-target effects (Figures 1C, S1A, and S1B and data not shown). Second, we extended the molecular characterization to expression of additional endogenous Smad target genes, such as PAI1, $p15^{INK4B}$, Smad7, JunB, and Smurf1, analyzed at the protein and/or mRNA levels. The results, exemplified in Figures 1C, 1D, and S1, show that loss of FAM abolishes multiple TGFβ gene responses. FAM knockdown also blocks TGFβ-mediated induction of a synthetic Smad promoter fused to luciferase (CAGA12-lux, Figure 1E), in line with the notion that FAM is a critical factor for Smad signaling per se. Finally, we cross-validated the requirement of FAM in different cells, such as HaCaT, MDA-MB231, HeLa, HCT116, and HepG2, and found that FAM is required for TGFβ signaling in multiple cellular contexts (Figures 1 and S1).

We next sought to define the requirement of FAM for BMP responses. To this end, we monitored the induction of endogenous *Id2* and *Smad7*, two established direct targets of Smad1/5. BMP2 induced these genes in control cells (shGFP) but not in FAM-depleted (shFAM) HeLa cells (Figure 1F). Similar results were obtained in MDA-MB231 cells transfected with FAM siRNAs (data not shown).

Next, we tested the effects of FAM gain-of-function. For this, cells were transiently transfected with synthetic luciferase reporters either monitoring Smad3 activity (CAGA12-lux), or Smad1/5 activation (ID1-BRE-lux). Gain-of-FAM enhanced TGFβ and BMP responses (Figures 1G and S1D). In contrast, overexpression of a catalytically inactive FAM C/S mutant (i.e., carrying a single Cys-Ser substitution in an essential residue of the protease domain, Nijman et al., 2005) was inactive, indicating that FAM acts as a deubiquitinase to promote TGFβ activity (Figure 1G). Taken together, the data suggest that FAM is a novel element in TGFβ/BMP signal transduction.

FAM Is Required for TGFβ Biological Effects

Canonical TGFβ/Smad2/Smad4 signaling has pleiotropic functions: growth-arrest is the dominant response induced in normal epithelia or early neoplasms, whereas promotion of migratory/invasive behaviors prevails in advanced tumors (Akhurst and Derynck, 2001). If FAM targets Smad activity, then it should be required for both types of responses. We first challenged FAM depletion in HCT116 colon cancer cells, which undergo Smad4-dependent growth arrest upon TGFβ stimulation (Zhou et al., 1998). Cells were transfected with control- or FAM-siRNAs, treated for 24 hr with or without TGFβ1, and then collected for cell-cycle analysis. As reference, we also used Smad4-siRNA-transfected cells. As shown in Figure 2A, TGFβ1 treatment blocked entry in S-phase, and this effect was lost by FAM depletion.

We then used metastatic MDA-MB231 breast cancer cells; these respond to canonical TGFβ signaling by increasing their motility, an effect quantifiable by transwell migration assays and by scratch-assay, in which a "wound" is introduced in a confluent monolayer with a pipette tip. We compared

MDA-MB231 cells transfected with control-, Smad4-, or FAM-siRNA. Depletion of FAM or Smad4 abolished TGFβ-induced migration (Figures 2B and 2C). Taken together, these results show that FAM is a critical determinant for TGFβ biological effects, closely recapitulating Smad4 requirements.

FAM Is a Smad4 Deubiquitinating Enzyme

We aimed to gain insight into the molecular mechanisms underlying FAM function. In *Drosophila*, zygotic fat facets has been implicated in regulating endocytosis in photoreceptor precursors (Cadavid et al., 2000). Receptor trafficking is also important for TGFβ signaling (Di Guglielmo et al., 2003). However, two direct read-outs of receptor activity, namely the levels of phospho-Smad2/3 and phospho-p38, were not significantly affected in FAM-depleted cells (Figure S2). This suggests that FAM intercepts TGFβ signaling primarily downstream of receptor activation.

To test for physical interactions between FAM and Smads, we first performed coimmunoprecipitation experiments with overexpressed, tagged proteins. As shown in Figure 3A, FAM coprecipitated efficiently with Smad4, in a manner independent of signaling; moreover, we could also detect a weaker interaction with R-Smads, in particular with Smad2. To test if the binding with R-Smads was direct, we compared FAM/R-Smad interactions in wild-type and Smad4-depleted (shSmad4) HEK293T cells. As shown in Figure 3B, R-Smads interaction was inhibited in the absence of Smad4.

To demonstrate that the interaction between FAM and Smad4 occurs at physiological levels of these proteins, HEK293T cells were treated for 1 hr with TGFβ1 and their lysates immunoprecipitated with an anti-FAM antibody. As shown in Figure 3C, endogenous Smad4 and FAM form a complex in vivo. To functionally validate the relevance of Smad4 as key target of FAM, we monitored the role of FAM on Smad4-independent events, namely, nuclear accumulation of Smad2 and the degradation of SnoN by TGFβ (Nicolas and Hill, 2003; Stroschein et al., 1999). As shown in Figure S3, these specific read-outs of Smad2/3 activity were not affected by the absence of FAM.

We then explored the domains of Smad4 involved in FAM recognition. To this end, we coexpressed FAM together with Smad4 deletions. As shown in Figure 3D, FAM binds the Smad4 MH1+linker, but not the isolated MH1, MH2, or linker domains alone. Taken together, the data suggest that FAM is a novel Smad4-interacting partner. It is noteworthy that this biochemical interaction parallels the biological requirement of FAM in both TGFβ and BMP signaling, sharing Smad4 as transducer.

In light of the association of FAM with Smad4, we tested if FAM regulates Smad4 ubiquitination. To reveal Smad4 modifications, we transfected HEK293T cells with expression plasmids encoding for Smad4 and HA-tagged ubiquitin, either alone or in combination with FAM. Smad4 was immunoprecipitated and its ubiquitination pattern visualized by immunoblotting against HA-Ubiquitin. Remarkably, the major modification of Smad4 corresponds to a monoubiquitination (Figure 3E, lane 2), also detectable using anti-Smad4 antibodies (not shown). After longer exposures, minor higher-molecular-weight bands appear, including a diubiquitinated band corresponding to branching of another ubiquitin on K48 (Figure S4). Strikingly, overexpression of wild-type FAM, but not of the catalytically inactive FAM mutant, inhibited Smad4 monoubiquitination in vivo (Figure 3E, compare lanes 3 and 4). Overexpression of FAM also inhibits monoubiquitination of endogenous Smad4 (Figure 3F). Conversely, Smad4 monoubiquitination was markedly enhanced upon depletion of FAM, indicating that FAM is a required DUB for Smad4 (Figure 3G). Notably, steady-state levels of Smad4 are unaffected by gain or loss of FAM, consistent with a regulative-type ubiquitination (Figures S1, S2, and 3).

The deubiquitination of Smad4 by FAM is direct. To show this, we purified to homogeneity unmodified Smad4, ubiquitinated Smad4 (Ub-Smad4), and FAM proteins by affinity chromatography. In coimmunoprecipitation assays, both unmodified and Ub-Smad4 bind directly to FAM (Figure 3H). When proteins were allowed to react in vitro, FAM cleaved Ub-Smad4, releasing "free" Smad4 and one Ub moiety (Figure 3I).

In sum, Smad4 is primarily targeted by monoubiquitination in mammalian cells (Figure S6), and FAM is a required Smad4 DUB.

Ectodermin/Tif1γ Is the E3 Monoubiquitin Ligase Opposed by FAM

The data presented so far imply that, upon FAM depletion, a Smad4 monoubiquitin ligase is left unopposed, leading to inhibition of TGFβ signaling. In contrast to FAM, the loss of this molecule should markedly enhance TGFβ signaling. We therefore sought to identify this enzyme using a candidate gene approach.

So far, several proteins have been proposed as Smad4 Ub-ligases in mammals, including Ectodermin/Tif1γ/TRIM33, β-TrCP1, WWP1, Smurfs, NEDD4-2, CHIP, and eIF4A (Izzi and Attisano, 2006). We tested the specific requirement of these genes as endogenous regulators of Smad activity by transfecting their corresponding siRNAs in HaCaT cells carrying the Smad reporter CAGA12-lux (using published or prevalidated siRNA sequences; see Supplemental Experimental Procedures). As shown in Figure 4A, Ecto stood out as a powerful endogenous antagonist of Smad signaling.

We noticed that loss-of-Ecto promotes Smad activity without stabilizing steady state levels of Smad4 (Figures S7A and S7B), suggesting that Ecto inhibits TGFβ signaling primarily by controlling Smad4 *function*, rather than stability. This is compatible with a requirement of Ecto in regulative ubiquitination events, analogous to those unveiled for FAM, and provides a revision of our previous conclusions, based on data obtained with overexpressed Ecto and pulse-chase assays, that Ecto promoted Smad4 instability (Dupont et al., 2005). We therefore tested if Ecto may serve as a Smad4 monoubiquitin ligase. As shown in Figure 4B, shRNA-mediated depletion of Ecto in HEK293T cells leads to the reduction of the Smad4 monoubiquitination band; this effect is specific, because it could be rescued by adding back shRNA-insensitive wild-type Ecto (Ecto*). Similar results were obtained for endogenous Smad4 (not shown). Thus, Ecto is a required determinant for Smad4 monoubiquitination.

We think that our findings on Smad4 present interesting analogies with those of another key tumor suppressor, p53. p53 is monoubiquitinated by low levels of MDM2 in the nucleus, inhibiting its activity and leading to p53 relocalization in the cytoplasm; cytoplasmic monoubiquitinated-p53 may be polyubiquitinated by high doses of mdm2 (or other ligases) and then degraded, or instead recycled by the activity of DUBs (Salmena

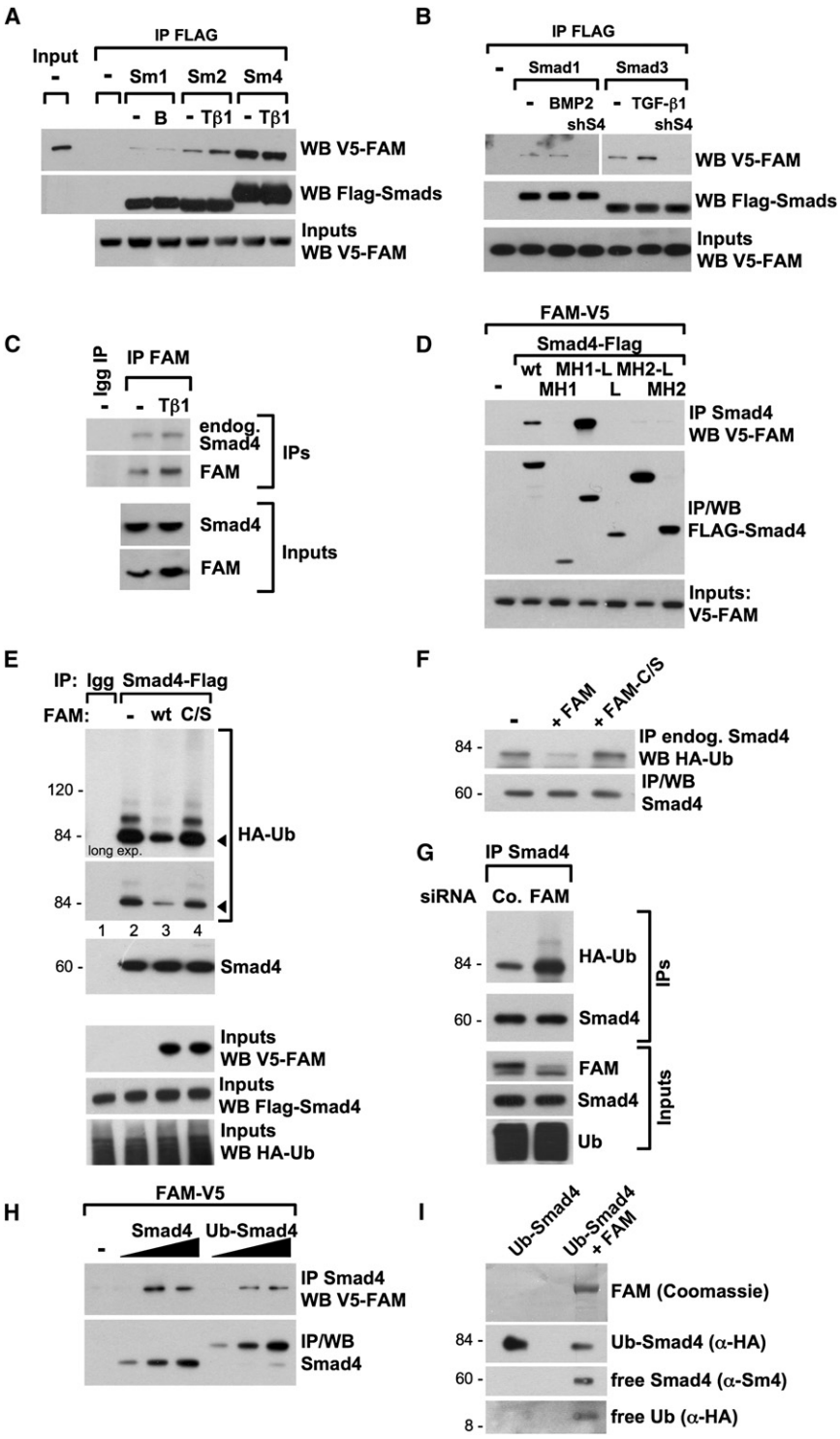

Figure 3. FAM Is a Smad4 Deubiquitinating Enzyme

(A) FAM is a novel Smad4-interacting protein. HEK293T cells were transfected as indicated with expression plasmids encoding V5-tagged FAM and Flag-tagged Smad1, Smad2, or Smad4. Cells were left untreated (–) or treated for 2 hr with TGFβ1 or BMP2, and harvested for immunoprecipitation with anti-Flag affinity resin.

(B) Depletion of Smad4 (shS4 cells) diminishes FAM/R-Smad interaction.

(C) FAM and Smad4 form an endogenous protein complex in HEK293 cells.

(D) Mapping of Smad4 domains required for FAM binding using combinations of the MH1, Linker (L), and MH2 domains.

(E) Expression of FAM (wild-type, lane 3), but not enzymatically inactive FAM (C/S, lane 4), deubiquitinates Smad4. The two anti-HA panels on the top represents longer and shorter exposures of the same blot, respectively. Immunoblots on the bottom ensure even production of HA-ubiquitin, Smad4, and FAM isoforms in lysates (Inputs).

(F) A major Smad4 monoubiquitination band is also detectable on immunoprecipitated endogenous Smad4, and FAM overexpression reduces it.

(G) FAM depletion (siRNA #2) enhances Smad4 monoubiquitination in MDA-MB231 cells.

(H) FAM interacts with Smad4 or monoubiquitinated Smad4 (Ub-Smad4) in vitro. See Figure S5 for SDS-PAGE/Coomassie analysis of the purified proteins.

(I) FAM directly deubiquitinates Ub-Smad4, clipping away Smad4 from ubiquitin, when the two purified proteins were allowed to react in vitro.

and Pandolfi, 2007). It is plausible that, p53 alike, some of the other E3 ligases so far implicated in Smad4 polyubiquitination might serve as "E4" enzymes acting downstream of Ecto-mediated monoubiquitination, indeed promoting its degradation in specific contexts (Hoppe et al., 2004; Heldin and Moustakas, 2006). Indeed, in colorectal cancer HCT116 cells, loss-of-FAM does affect Smad4 stability, an effect rescued by concomitant loss-of-Ecto (data not shown). However, even in contexts in which polyubiquitination is easily detectable by western blotting, such as in *Xenopus* embryos (Dupont et al., 2005), Ecto-dependent monoubiquitinated Smad4 remains a dominant isoform (Figure S8).

To establish the causality between the requirement of Ecto as Smad4 inhibitor and its role as Smad4 monoubiquitin ligase, we compared the activity of wild-type Ecto with that one of mutants containing deletion of the RING-finger domain (Ecto-ΔTRIM), or point mutation of two critical cysteines in this E2 interaction domain (Ecto-CAmut) (Joazeiro and Weissman, 2000). As shown in Figures 4C and S7C, the Ub-ligase activity of Ecto is critical for Ecto-mediated antagonism over canonical Smad responses turned on by both TGFβ and BMP. Of note, the isolated Ecto-Middle, namely the isolated Smad binding domain (Dupont et al., 2005; He et al., 2006), is void of biological effects in human cells and *Xenopus* embryos

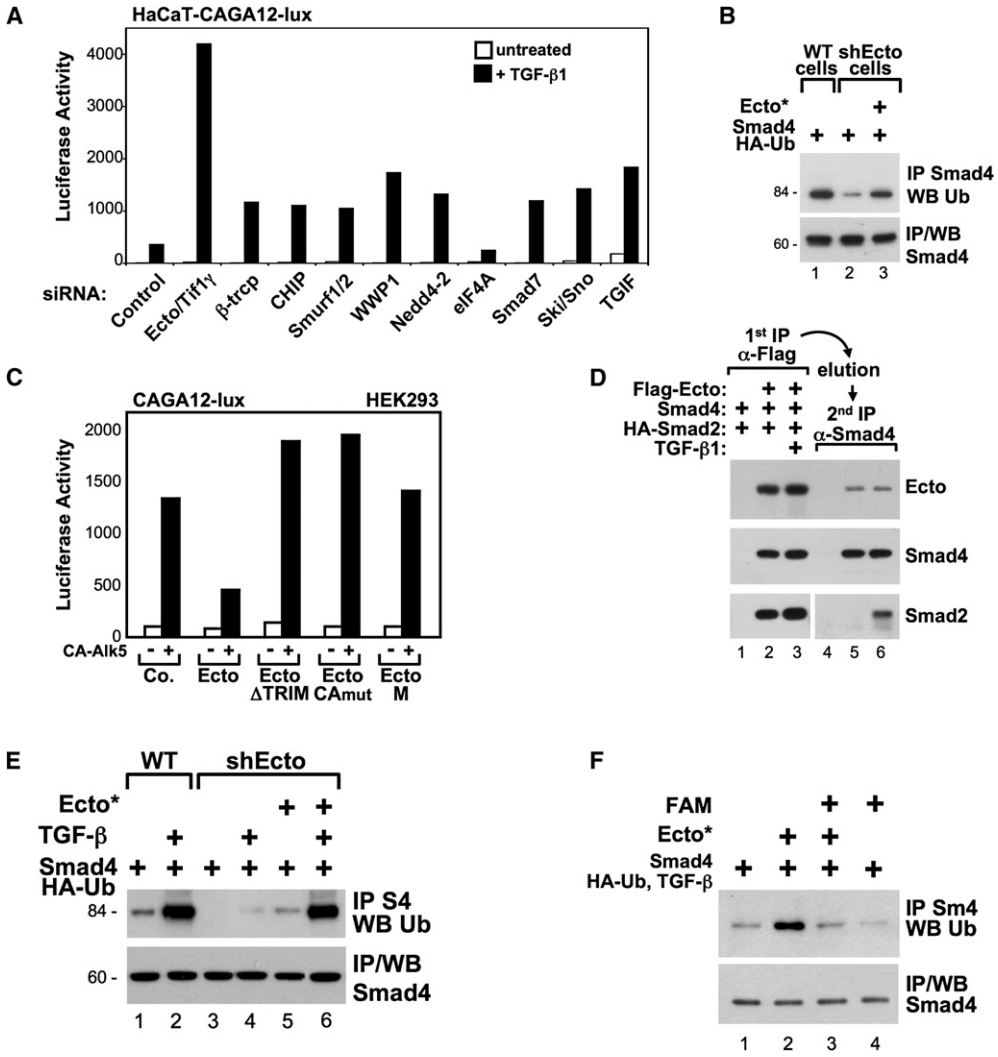

Figure 4. Ectodermin/Tif1γ Is a Smad4 Monoubiquitin Ligase Restraining TGFβ Signaling

(A) Comparative analysis of the requirement of distinct Smad4 ubiquitin ligases for canonical TGFβ responses. Data are represented as mean of a representative experiment (SD was below 5%).

(B) Ecto is required in vivo for Smad4 monoubiquitination in HEK293T cells (compare lanes 1 and 2). Adding back a shRNA-insensitive Ecto plasmid rescues Smad4 ubiquitination to normal levels (Ecto*, lane 3). A similar requirement can be also observed in *Xenopus* embryos (Figure S8).

(C) RING-dependent activity of Ecto for inhibition of TGFβ/Smad4 signaling. Data are represented as mean of a representative experiment (SD was below 10%).

(D) Ecto, Smad4, and Smad2 form a trimeric complex. See also a repetition of the same experiment with tagged Smad4 in Figure S9.

(E) Smad2 fosters ubiquitination of Smad4 by Ecto. TGFβ stimulation was delivered using transfected Smad2 and 2 ng/ml TGFβ treatment overnight.

(F) FAM antagonizes Ecto-mediated ubiquitination of Smad4. The ubiquitination assay was performed as in (E).

(Figure S7D), excluding that Ecto could antagonize canonical TGFβ signaling by simply titrating/squelching Smads.

Ectodermin/Tif1γ has at least two distinct biological activities: it inhibits Smad4 responses (Dupont et al., 2005; Levy et al., 2007) and binds phospho-Smad2 (He et al., 2006). Thus, we investigated whether binding of phospho(P)-Smad2/3 to Ecto could also serve as potential modulator of Ectodermin for Smad4 monoubiquitination. Indeed, we noticed that monoubiquitination of endogenous Smad4 is enhanced by TGFβ signaling (Figure S6), raising the so far unexplored possibility that Ecto might form a trimeric complex with both Smad2 and Smad4. To test this, we used HEK293T cell extracts containing Flag-Ecto, Smad4, and HA-Smad2. Ecto was first affinity purified on a Flag resin and then eluted together with its coprecipitating proteins by incubation with Flag peptide. The eluted complexes were then subjected to a second affinity purification procedure on an anti-Smad4 matrix. If Ecto/Smad2 and Ecto/Smad4 were segregated in just mutually exclusive heterodimers, we should find only Ecto, and not Smad2, as a Smad4 partner after this procedure. In contrast, as visualized by immunoblot, both Smad2 and Ecto were copurified (Figures 4D and S9). The results suggest that, upon TGFβ signaling, Ecto, Smad4, and Smad2 can form a trimeric complex. To test the role of Smad2 as modulator of Ecto, we compared Smad4 monoubiquitination in the presence

and absence of signaling. The presence of TGFβ/Smad2 signaling increases Smad4 monoubiquitination levels (Figure 4E) and overexpression of FAM removes this modification (Figure 4F).

Ecto and FAM act in distinct cellular compartments: nucleus and cytoplasm, respectively (Figure S10A). This argues that monoubiquitinated Smad4 originates in the nucleus but needs to shuttle back to the cytoplasm to be reactivated by FAM. Accordingly, we could detect the interaction of Smad4 with FAM and Ecto in the corresponding cytoplasmic and nuclear fractions of HaCaT cells (Figures S10B and S10C). Moreover, cell fractionation analyses indicate that ubiquitinated Smad4 originates in the nucleus, and that this pool is enhanced upon a pulse of TGFβ signaling (Figure S10D). Finally, the timing of Smad4 monoubiquitination closely follows the kinetics of Smad4/Smad2 association and Smad2 phosphorylation (Figures S10E and S10F).

Ecto and FAM Operate in the Same Pathway Controlling Cycles of Smad4 Inhibition and Reactivation

The relevance of the Ecto-Smad4-FAM loop is supported by biological evidences in three independent model systems: *Xenopus* embryos, *Drosophila*, and mammalian cells. We first compared in *Xenopus* embryos if phenotypes caused by loss-of-FAM recapitulated those of Smad4 knockdown and Ecto overexpression. During development, TGFβ/Nodal ligands are critical for inducing the mesoderm, whereas regulation of BMP signaling is required for patterning along the dorsoventral axis (Niehrs, 2004; De Robertis and Kuroda, 2004). To study these processes in frog embryos, we monitored the expression of *Xbra*, a direct TGFβ target in mesoderm, and the complementary expressions of *Sizzled* and *Otx2* as read-outs of BMP signaling.

To study the function of *Xenopus* FAM, we microinjected in embryos an antisense morpholino covering the start codon of the endogenous transcript (FAM-MO); this reagent downregulated endogenous FAM protein levels at the gastrula stage (Figure S11). Phenotypically, FAM depletion reduced the *Xbra* and *Sizzled* domains and, conversely, expanded *Otx2* expression, remarkably recapitulating the loss-of-Smad4 or Ecto overexpression (Figures 5A–5L). These data are consistent with the idea that FAM and Ecto act as antagonistic Smad4 regulators during embryonic development.

We next decided to challenge the opposing functions of FAM and Ecto using a completely heterologous assay. The development of the *Drosophila* wing offered an ideal playground: the fly has no Ectodermin homolog but requires BMP/Smad4 signaling for the formation of cross-veins (Figure 5N and Hudson et al., 1998). Thus, we generated *Drosophila* strains expressing Ecto under the control of UAS sites, and used GAL4 under a wing-specific promoter (MS1096-Gal4) to direct Ecto expression throughout the presumptive wing blade. Crucially, expression of Ecto, but not of Ecto-CAmut (i.e., RING-finger mutant), generated adult wings that phenocopy those of *gbb* mutants lacking a BMP ligand (compare Figures 5N and 5O with 5P). Thus, also in flies, Ecto behaves as a BMP antagonist.

Taking this experimental system one step further, we then asked if overexpression of Fat Facets (Faf), the *Drosophila* FAM homolog, could antagonize Ecto activity. As shown in Figures 5Q, when Faf was expressed in the wing blade, we observed appearance of ectopic veins between L1 and L2 (red inset), a phenotype related to increased BMP signaling (Marquez et al., 2001). Most importantly, when we overexpressed both Faf and Ecto, the Faf phenotype dominated over Ecto activity, because crossveins development was rescued and ectopic veins continued to form (Figure 5R).

Remarkably, in human cells, depletion of Ecto rescued TGFβ responsiveness of FAM depleted cells, supporting the notion that FAM and Ecto work in the same biochemical pathway (Figure 5S). Some controls further substantiate this conclusion. First, depletion of Smad4 abolished TGFβ responses but, unlike with FAM, this deficiency was not rescued by coupled Ecto depletion, which would be expected if Smad4 is a downstream mediator of Ecto activity. Second, raising signaling upstream of Smad4 by knocking down receptor inhibitors, such as Smad7 or Smurfs, could not rescue FAM depletion (Figure 5S). The epistatic relationship between Ecto and FAM was confirmed in TGFβ-induced migration assays: knockdown of FAM abolished migration, but this was dramatically rescued by the compound FAM/Ecto knockdown (Figure 5T).

Smad4 Is Monoubiquitinated at Lysine 519

Next, we turned our attention to Smad4 monoubiquitination and its functional consequences. To map the lysine responsible for the Smad4 monoubiquitination pattern, we prepared a series of Smad4 mutants bearing lysine-to-arginine substitutions. We mutated all the lysines of Smad4 (Smad4-Kall), only those of the MH1 domain (MH1-KR), or those of the MH2 domain (MH2-KR). Then, we progressively narrowed down the mapping to groups of neighboring lysines (Smad4 mutants dubbed A–F, Figure 6A) and finally to individual lysines. When we visualized the monoubiquitination pattern of these Smad4 mutants in lysates of transfected HEK293T cells, we found that, in addition to Kall, the MH2-KR and the F mutant (containing only K507R and K519R mutations) displayed strongly reduced monoubiquitination (Figure 6A). However, K519 was the most critical residue for Smad4 monoubiquitination, whereas mutation of K507 had minor effects (Figure 6B, lanes 3 and 4). Conversely, adding back the sole wild-type K519 in the background of MH2-KR Smad4 mutant was sufficient to restore the monoubiquitination pattern (lanes 5 and 6), suggesting that K519 is a primary/direct target of ubiquitination, rather than being required for activity/recognition of the ubiquitination complex. Monoubiquitination of Smad4 in another residue (K507) has been suggested to play a positive role for Smad4 activity (Moren et al., 2003); however, the negative monoubiquitination event in K519 here described appears quantitatively dominant.

Finally, we investigated whether Ecto is instrumental for ubiquitination of lysine 519. As shown in Figure 6C, Ecto promoted ubiquitination of wild-type but not of K519R Smad4 proteins in vivo. Further direct evidence of this event was obtained using an in vitro cell-free ubiquitination assay with purified Smad4 and Ecto proteins (Figure S13). Interestingly, K519 is the only Smad4-specific lysine that is evolutionarily conserved across phyla (Konikoff et al., 2008).

Ubiquitination of Smad4 Inhibits Smad2/3 Complex Formation

How can Smad4 K519 monoubiquitination inhibit TGFβ gene responses? To answer this question, we first searched for hints

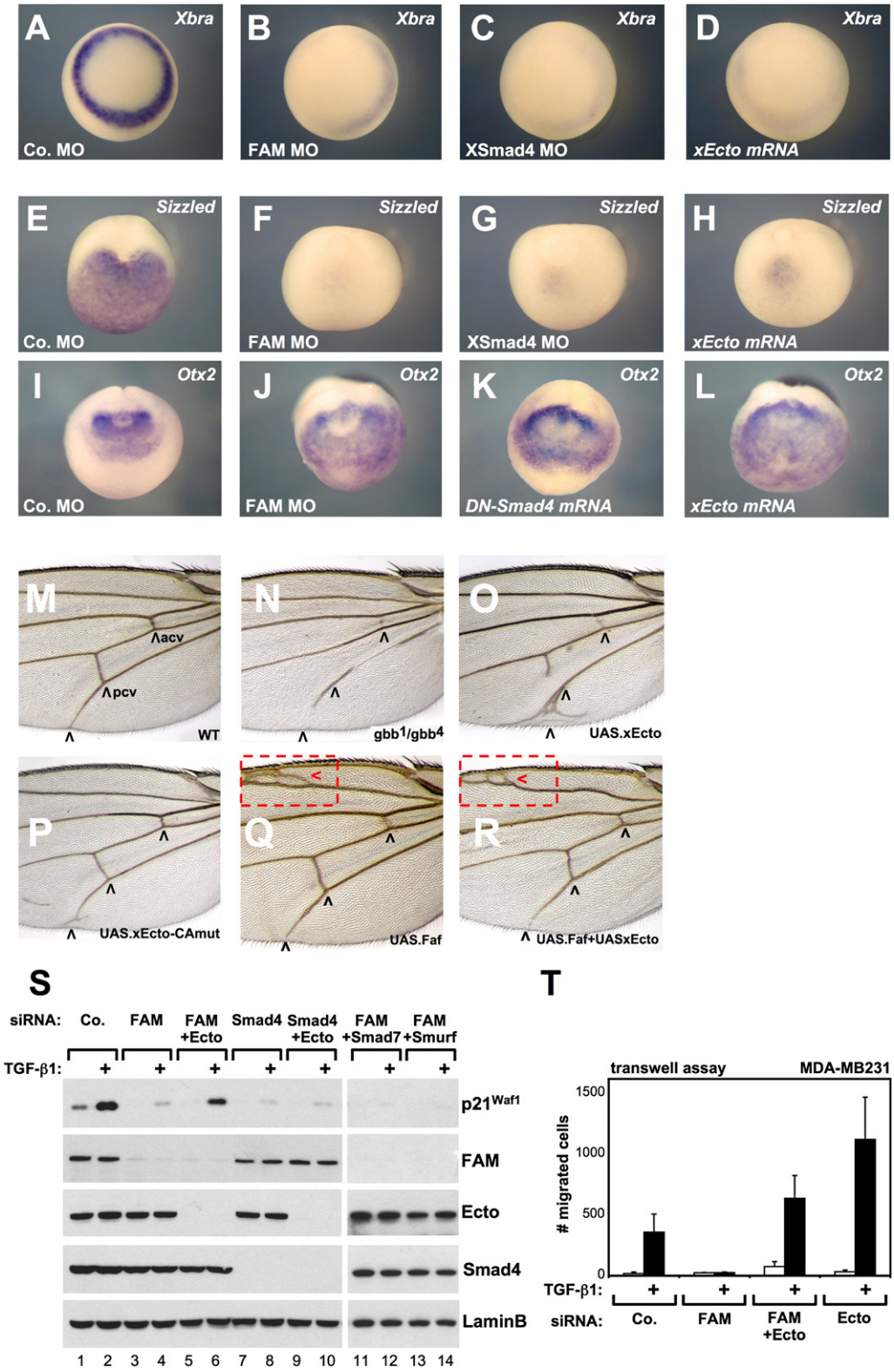

Figure 5. FAM and Ecto Operate in the Same Pathway Regulating Smad4

(A–L) Panels show in situ hybridizations on *Xenopus* embryos for the pan-mesodermal marker *Xbra* (at gastrula stage), for the ventral marker *Sizzled* and for the dorsoanterior marker *Otx2* (at neurula stages). Embryos were microinjected either with morpholino antisense oligonucleotides (MOs), dominant-negative Smad4 mRNA (DN-Smad4), or Ecto mRNA.

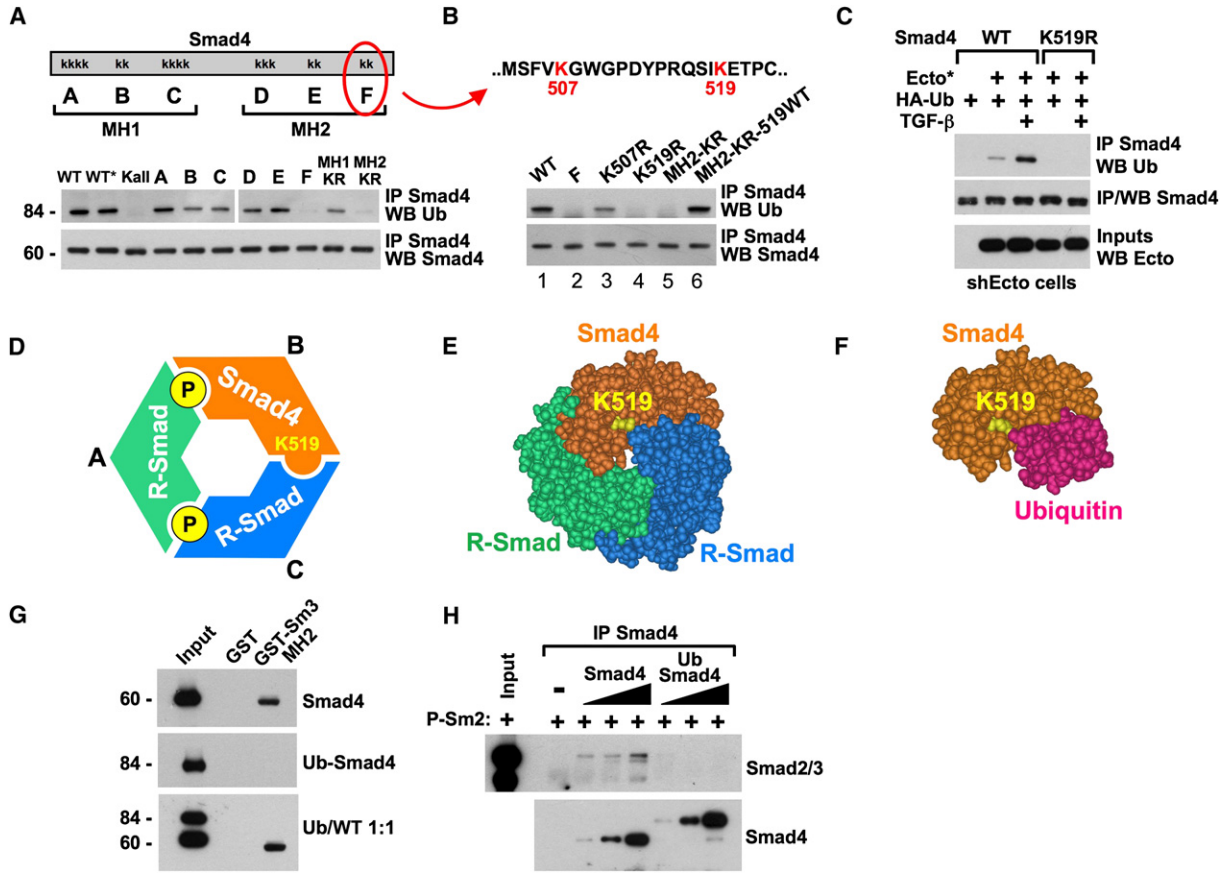

Figure 6. Smad4 Ubiquitination at Lysine 519 Inhibits R-Smad/Smad4 Binding

(A and B) Top: Diagram and partial sequence of Smad4 lysine mutants. Bottom: Immunoblots of immunoprecipitated Smad4 mutants, mapping the ubiquitination site on lysine 519.

(C) Mutation of K519 abolishes Ecto-induced Smad4 ubiquitination, without modifying levels of Smad4 sumoylation or acetylation (Figure S12). The ubiquitination assay was performed as in Figure 4E.

(D) Model of the heterotrimeric R-Smad/Smad4 complex (Chacko et al., 2004). Note how Smad4 is involved in two different interactions, entailing the AB and the BC interfaces. P indicates the phosphorylated C-terminal portion of R-Smads. Lysine 519 falls near to the BC interface.

(E) Crystallographic structure of the heterotrimeric R-Smad/Smad4 complex as described previously (Chacko et al., 2004). Lysine 519 side chain is highlighted in yellow.

(F) In silico modeling of the tridimensional structure of Smad4-MH2 bearing K519-linked ubiquitin. Note how the ubiquitin moiety completely occupies the Smad4 surface involved in the BC interface with R-Smad.

(G and H) Purified ubiquitinated Smad4 (Ub-Smad4) is unable to bind recombinant Smad3-MH2 domain (G) or phospho-Smad2 (H). In (G) Ub/wt 1:1 is an independent preparation of Ub-Smad4 containing similar amounts of nonubiquitinated Smad4 as contaminant.

in the structure of Smad4. Biochemical, functional, and crystallographic evidence indicates that the Smad transcriptional complex is a heterotrimer comprising one Smad4 molecule and two phospho-R-Smad molecules, designated as A, B, and C (see model in Figure 6D; Chacko et al., 2004). The complex is thus characterized by three nonidentical interfaces designated AB (between one R-Smad and Smad4), CA (between the two R-Smads), and BC (between Smad4 and the second R-Smad). Although K519 does not participate directly in R-Smad recognition, it is positioned near the BC interface; given that missense mutations in the vicinity of K519 are sufficient to destroy heterotrimeric complex formation (Chacko et al., 2001), we reasoned that attachment of an ubiquitin moiety to K519 was very likely to generate a similar damage (Figures 6D and 6E). Thus, we

(M–R) Close-up views of *Drosophila* wings showing anterior (acv) and posterior (pcv) cross-veins.

(N) Mutants for *gbb*, a BMP ligand, display missing cross-veins.

(O and P) Ectopic expression of Ecto (O), but not of the Ecto RING mutant (Ecto-CAmut [P]), in the wing primordium causes loss of the cross-veins.

(Q) Expression of *Drosophila* Fat facets (Faf) induces ectopic wing veins (red box).

(R) Expression of Fat facets antagonizes Ecto, rescuing the formation of the cross-veins.

(S and T) Ecto is epistatic to FAM. Panels in (S) show immunoblots of HaCaT cells transfected with the indicated combinations of siRNA. Ecto biological function is required downstream of FAM, as also revealed by TGFβ induced transwell migration assays in MDA-MB231 cells (T). Data are represented as mean and SD.

modeled in silico the structure of monoubiquitinated Smad4 by docking the structure of a single ubiquitin bound to K519 over the Smad4 MH2 domain, and we found that this masks the BC interface, potentially interfering with R-Smad recognition (compare Figures 6E and 6F).

These structural hints suggest that K519 monoubiquitination of Smad4 might correspond to a ''latent'' Smad4 that is incapable of R-Smad recognition. To test this, we used pure preparations of affinity-purified unmodified Smad4 and monoubiquitinated Smad4 (Ub-Smad4), and compared their ability to bind recombinant GST-Smad3-MH2 domain or in vitro phosphorylated Smad2. Strikingly, only unmodified Smad4 could interact with R-Smads (Figure 6G).

To directly test if monoubiquitination is inhibitory in vivo, we tested whether raising the levels of Ecto antagonizes the formation of the endogenous Smad4/Smad2 complex. As shown in Figure 7A, overexpression of Ecto decreases the ability of Smad4 to interact with Smad2 upon TGFβ treatment; importantly, this effect is not simply due to the ability of Ecto to interact with Smads, because overexpression of either the Middle domain of Ecto (Ecto-M) or RING-deficient mutants (Ecto-ΔTRIM and Ecto-CAmut) were unable to inhibit Smad4/Smad2 complex formation.

Upon TGFβ signaling, Smad2 has been proposed to anchor Smad4 in the nucleus (De Bosscher et al., 2004). Because Smad4 monoubiquitination inhibits Smad2/4 complex, increasing Smad4 monoubiquitination should affect Smad4 subcellular localization similarly to loss of R-Smad. Indeed, Smad4 nuclear accumulation upon TGFβ stimulation is lost upon Smad2/3 knockdown and this is phenocopied by loss of FAM (Figures 7B and 7D). This pairs with the previously published requirement of Ecto for Smad4 nuclear exclusion (Dupont et al., 2005). As a control, inhibition of the Smad4 nuclear exporting factor CRM-1 with leptomycin B caused nuclear retention of Smad4 in both control and FAM-knockdown cells (Figure 7B).

If cycles of Smad4 monoubiquitination and deubiquitination are the means by which Ecto and FAM regulate TGFβ signaling in vivo, then loss of FAM should have no effect on cells expressing only the nonubiquitinated Smad4 K519R mutant. To test this hypothesis, we engineered the Smad4-null MDA-MB468 cell line with wild-type or K519R Smad4, expressed by retroviral infection at near-to-endogenous levels (i.e., those of MDA-MB-231 cells, data not shown). Both wild-type and K519R Smad4 were equally able to rescue TGFβ responsiveness, as monitored by immunoblotting for $p21^{Waf1}$ and PAI1, confirming that K519 mutation per se is compatible with Smad4 activity (Figure 7C). Strikingly, whereas wild-type Smad4-expressing cells were sensitive to FAM knockdown, K519R-reconstituted cells were insensitive to FAM depletion. Thus, FAM works through deubiquitination of K519-ubiquitinated Smad4. This pairs with the biological requirement of Ecto for FAM function (Figure 5).

DISCUSSION

The assembly of the Smad complex is the most critical event in TGFβ signaling, yet we have few clues regarding the dynamic of the active Smad2/Smad4 complex, the nature of the ''fences'' that cells raise against undesired signal activation, and the mechanisms used to shut-off Smad2/4 nuclear accumulation and to re-empower it in case of repeated ligand/receptor activation. The present work represents a contribution to these investigations. We now establish that Smad4 monoubiquitination is a reversible system by which cells forestall responsiveness to TGFβ, and define a novel set of enzymatic activities mediating this inhibition, including the identification of the first DUB essential for Smad function (Figure 7D).

A Model for TGFβ Signaling Regulation: Turnover of Smad4 Monoubiquitination by Ectodermin/Tif1γ and FAM

By means of a siRNA-based screen and subsequent validations, we identified FAM/Usp9x as DUB required for Smad4 activity. FAM depletion disables canonical Smad responses in mammalian cells, including TGFβ-induced growth arrest and migratory behavior.

We propose that Ecto and FAM impinge on Smad4 function from opposite cellular compartments: in the nucleus, Ecto monoubiquitinates in lysine 519 free-Smad4 and, to a greater efficiency, the Smad4/Smad2 complex. Thus, Ecto serves as Smad4 antagonist and destabilizing factor for the R-Smad/Smad4 complex. We propose that ''latent'' Ub-Smad4 is less retained in the nucleus by R-Smads. Additionally, ubiquitination might more actively regulate Smad4 shuttling, for example by recruiting still-unknown ubiquitin binding proteins, that might assist nuclear export or anchor Smad4 in cytoplasm. In the cytoplasm, FAM/Usp9x deubiquitinates and recycles Smad4, re-empowering its competence to mediate TGFβ signaling.

Ectodermin/Tif1γ Serves as Smad4 Monoubiquitin Ligase

We identified in Ecto a critical factor for Smad4 monoubiquitination at lysine 519. Ecto knockdown prevents to a large extent Smad4 monoubiquitination, leading to upregulation of canonical Smad signaling (Figure 4A). In our assays, Ecto is an inhibitor of Smad4-dependent TGFβ and BMP responses and its effects are opposite and epistatic to those of FAM. Crucially, for all these antagonistic effects, Ecto requires its RING-finger domain. These findings are consistent with other independent evidence: (a) the recent identification of Ecto/Tif1γ as most critical restraining factor of TGFβ responses in genome-wide screen for E3 Ub-ligases (Levy et al., 2007); (b) the developmental requirement of *Xenopus* Ecto as natural barrier to Nodal and BMP signaling for ectoderm pluripotency in frog embryos (Dupont et al., 2005; Pinho and Niehrs, 2007); and (c) the requirement of Ecto/Tif1γ to restrain endogenous gene responses in human cells (Figures S7A and S7B).

Others have proposed that Ecto/Tif1γ binds to Smad2 and Smad3, competing them away from Smad4, and functions as signaling factor with them (He et al., 2006). This biochemical interaction could, in principle, provide an alternative explanation for the inhibitory activity of Ecto toward canonical TGFβ signaling (''squelching'' model). These observations have contributed to a controversy surrounding Ecto/Tif1γ activity (Heldin and Moustakas, 2006). As addressed in detail in the Supplemental Results and Discussion, a ''squelching'' model cannot explain the activity of Ecto against well-established Smad2/Smad4 dependent TGFβ read-outs in several cell and developmental set-ups; however, the finding that Smad2 binding might support Ecto monoubiquitinase activity, and, in so doing, destabilize the

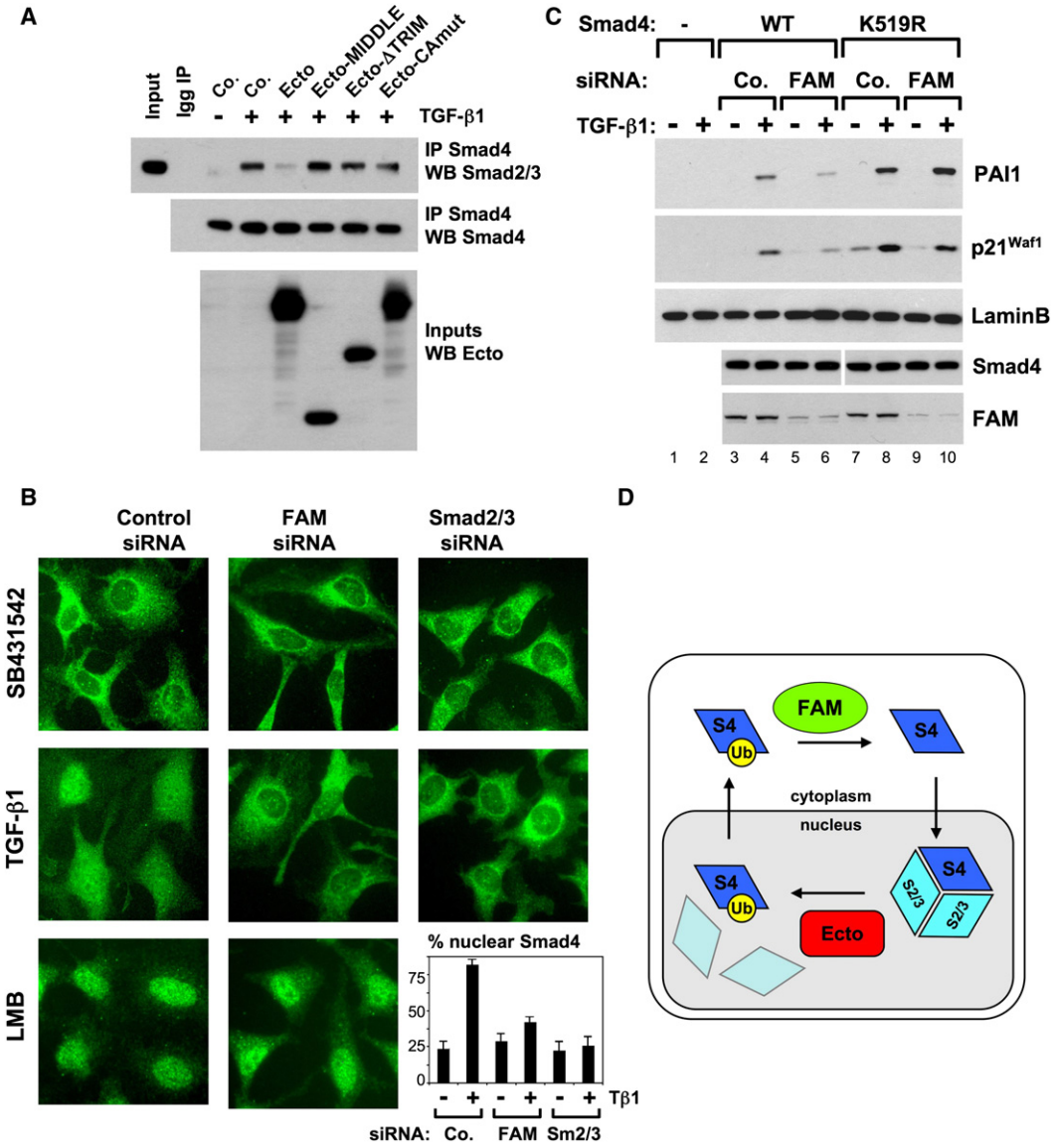

Figure 7. Monoubiquitination of Smad4 Affects R-Smad/Smad4 Complex In Vivo

(A) Coimmunoprecipitation of Smad2/Smad4 complexes. Smad2 association to Smad4 is inhibited only by overexpressed wild-type Ecto. Ecto-ΔTRIM is an N-terminal deletion of Ecto; Ecto-CAmut is mutated in the RING-finger; Ecto-MIDDLE contains the sole Smad binding domain. Thirty-six hours after transfection, cells were serum starved overnight and then treated for 2.5 hr with 0.5 ng/ml TGFβ1.

(B) Immunofluorescence (IF) for endogenous Smad4 in HeLa cells transfected with the indicated siRNAs and treated as indicated with the TGFβ-receptor inhibitor SB431542, TGFβ1, or leptomycin B (LMB). Graph shows quantification of the IF stainings (n = 2 ± SD). For control of effective knockdowns and nuclear counterstains, see Figure S14.

(C) MDA-MB468 are Smad4-null cells unable to respond to TGFβ stimulation (lanes 1 and 2), but regain TGFβ responsiveness, as monitored by $p21^{Waf1}$ and PAI1 immunoblotting, after retroviral expression of wild-type (lanes 3 and 4) or K519R (lanes 7 and 8) Smad4. Upon loss-of-FAM, however, only wild-type Smad4 and not the nonubiquitinatable K519R mutant is inhibited (compare lanes 5 and 6 with lanes 9 and 10).

(D) A model for Smad4 monoubiquitination turnover mediated by FAM and Ecto.

interaction between Smad4 and Smad2, also serves as essential reconciliation of our data with the biochemical observations of He et al. (2006) (see also the model in Figure S16C).

A Smad4/2 Complex "Disruptase"

Smad4 monoubiquitination can be envisioned as a barrier against undesired activation of the pathway; in this scenario, it precedes signaling, raising the thresholds of responsiveness to both TGFβ and BMP ligands. Additionally, Smad4 monoubiquitination might also operate as a mean to disrupt the Smad4/Smad2 complex in order to turn-off signaling. Indeed, our data collectively suggest that the Smad4/Smad2 complex might be the preferred target of Ecto in vivo; whereas the mechanistic details of this inhibition must await better structural characterizations and the cloning of

additional cofactors, it is tempting to speculate that Ecto binding to nuclear Smad2/4 complex serves as an intermediate step to transiently dissociate Smad2 and Smad4 so that the Ub moiety can be finally added, locking away Smad4 from Smad2. Perhaps this explains the residual anti-TGFβ activity of RING-deficient *Xenopus* Ectodermin once injected at high doses in *Xenopus* embryos (data not shown).

In the case of R-Smads, phosphorylation and nuclear accumulation are maintained only while receptors are active, suggesting that the nucleus is constantly cleared of phospho-R-Smads by the activity of phosphatases and degradative ubiquitin ligases, such as PPM1A (Lin et al., 2006) and Smurf-1 (Fuentealba et al., 2007). So far, because Smad4 is not phosphorylated by receptors, the existence of a conceptually similar clearing mechanism for Smad4 has gone unnoticed. We now show that the monoubiquitination/deubiquitination cycle of Smad4 is required for TGFβ signaling, because TGFβ/phospho-R-Smads are ineffective in the absence of FAM. More crucially, elegant work by Hill and colleagues has shown that Smad phosphatases operate only on homomeric phospho-R-Smads (Schmierer et al., 2008); that is, *after* R-Smads have been disengaged from Smad4. This implies the existence of a Smad-complex "disruptase," whose nuclear activity must be reverted by an opposing activity in the cytoplasm. Our work suggests that these functions are fulfilled by the interplay between Ecto and FAM through Smad4 monoubiquitination.

EXPERIMENTAL PROCEDURES

Additional methods can be found in the Supplemental Experimental Procedures.

Plasmids

pEF-DEST V5-tagged mouse FAM/Usp9x, wild-type and C/S mutant, were a kind gift from Dr. Stephen Wood. Expression plasmids for human Flag-Ecto-Middle (449–885), human Flag-Ecto-ΔTRIM (449–1121), and *Xenopus* HA-tagged Ecto-Middle (370–811) were generated by PCR amplification of the indicated protein segments from full-length Ecto cDNA and cloned in pCS2. Human Ecto siRNA-insensitive (Ecto*) was obtained by targeted mutagenesis at wobble codons, preserving the natural protein sequence. Human Smad4-Flag lysine mutants (A–F mutants, MH1-KR, and MH2-KR in Figure 6A) were obtained by reciprocal swappings between a wild-type cDNA (wt* in Figure 6A) and a complete Lys-Arg mutant Smad4 cDNA (Kall in Figure 6A), both engineered to bear unique restriction sites surrounding each group of neighboring lysines, without altering the encoded protein (GeneScript). K507R and K519R single mutants were obtained by targeted mutagenesis. For retroviral infections, untagged Smad4 and Smad4-K519R cDNAs were subcloned in pBABE-PURO. All the plasmids were verified by nucleotide sequencing.

Cell Cultures and Transfections

HaCaT, HCT116chr3, HEK293T, and HeLa cells were cultivated in DMEM 10%FCS, MDA-MB231, and MDA-MB468 cells in DMEM/F12 10% FCS (HepG2 in MEM 10% FCS supplemented with NEA). DNA transfections were performed with calcium phosphate or Transit-LT1 reagent (MirusBio); for siRNA transfections we used Lipofectamine-RNAiMax (Invitrogen) in all cell lines but MDA-MB468 and HepG2 cells, for which we used Transit-TKO (MirusBio). HaCaT cells expressing HA-ubiquitin were obtained by stable transfection as described previously (Cordenonsi et al., 2007). TGFβ1 or BMP2 cytokines (Peprotech and R&D) were diluted in normal medium for HaCaT and HCT116chr3 cells; for the remaining cell lines, cells were starved overnight with 0.2% (HEK293T, HeLa, HepG2), 0.5% (MDA-MB468), or no serum (MDA-MB231) before treatment in the same medium. Where indicated, control cells were supplemented with 5 μM SB431542 (Tocris) in the medium to quench autocrine TGFβ signaling.

For FAM knockdown, the sequences of the siRNA were as follows: #1: GAU GAGGAACCUGCAUUUCtt; #2: GCAGUGAGUGGCUGGAAGUtt. These were used as a 1:1 mix, except as otherwise indicated. A complete list of siRNAs can be found in Supplemental Experimental Procedures.

Immunoprecipitations/GST Pulldowns

For in vivo ubiquitination assays, HEK293T cells were transfected with the calcium-phosphate method with HA-ubiquitin (8 μg/10 cm dish), Smad4-Flag (100 ng/dish), FAM (8 μg/dish), and/or Ecto (4 μg/dish) plasmids as indicated. DNA content was kept uniform by adding pBluescript plasmid. Forty-eight hours later, cells were harvested by sonication in Ub-lysis buffer (50 mM HEPES [pH 7.8], 200 mM NaCl, 5 mM EDTA, 1% NP40, 5% glycerol, freshly complemented with 1 mM DTT, protease inhibitor cocktail [Roche], phosphatase inhibitor cocktail II [Sigma], 250 ng/ml ubiquitin-aldehyde [Sigma]). Cell lysates were immunoprecipitated 4 hr at 4°C with protein-A sepharose/α-Smad4 (H552) beads in the same buffer supplemented with 2 mM MgCl2, followed by three washes of 2 min rotating at room temperature (RT) (50 mM HEPES [pH 7.8], 500 mM NaCl, 5 mM EDTA, 1% NP40, 5% glycerol).

For protein-protein interaction studies, cells were treated as indicated in the text and lysed by sonication in Marais' lysis buffer (25 mM HEPES [pH 7.8], 400 mM KCl, 5 mM EDTA, 0.4% NP40, 10% glycerol freshly supplemented with 1 mM DTT, protease, and phosphatase inhibitors). Extracts were diluted fourfold to bring KCl concentration to 100 mM and NP40 to 0.1%, supplemented with 0.5% BSA (Roche frk.V) and 10 mM $MgCl_2$, and subjected to protein-A sepharose immunoprecipitation 4 hr at 4°C. Beads were quickly washed three times at RT with 100 mM KCl, 0,05% NP40. For cell-fractionation studies, see Cordenonsi et al. (2003). For in vitro protein-protein interactions, purified and/or recombinant proteins were diluted in binding buffer (25 mM HEPES [pH 7.5], 100 mM KCl, 2 mM MgCl2, 0.1% NP40, 5% glycerol), immunoprecipitated with protein-A sepharose beads, and washed three times with the same buffer. To visualize coprecipitating proteins in immunoprecipitation experiments, we used ExactaCruz HRP-conjugated secondary antibodies; for endogenous Smad2 visualization upon coimmunoprecipitation, beads were treated for 2 hr at 37°C with PNGaseF (NEB) after the final IP washings to shift Iggs toward lower molecular weights.

Protein Purifications and In Vitro Ub/Deubiquitination Assays

To obtain purified monoubiquitinated-Smad4, Smad4-Flag, and HA-Ub expression, plasmids were calcium-phosphate transfected in HEK293T. Cell lysates (Ub lysis buffer) were immunoprecipitated overnight with α-Flag-M2 resin (Sigma), followed by two sequential elutions with Flag peptide (Sigma, 1 mg/ml in 50 mM HEPES [pH 7.5], 100 mM NaCl, 0.1% NP40, 5% glycerol). Pooled Flag eluates were subsequently immunoprecipitated with α-HA resin, followed by two sequential elutions with HA peptide (Sigma) in 500 mM NaCl. Pooled HA eluates were dialyzed overnight against 50 mM HEPES (pH 7.5), 100 mM NaCl, 5% glycerol, and 1 mM DTT. For purification of FAM protein from mammalian cells, FAM-V5-transfected HEK293T cell lysates (without protease or DUB inhibitors) were immunoprecipitated overnight with α-V5 resin (Bethyl) and eluted with 0.4 mg/ml V5 peptide (Sigma) in 100 mM NaCl, and dialyzed overnight Flag-Ecto protein was obtained similarly.

For in vitro deubiquitination assay, purified Ub-Smad4 and FAM were incubated overnight at 30°C in 50 mM HEPES (pH 7.5), 100 mM NaCl, 5% glycerol, 5 mM MgCl2, 1 mM ATP, and 1 mM DTT. For in vitro ubiquitination assay, purified proteins were mixed in a total volume of 50 ul, containing 10 ul rabbit reticulocyte lysates supplemented with Ubch5 (500 ng) and Ub-aldehyde, and incubated at 30°C for 1.5 hr (as described in Shenoy et al., 2001). The reactions contained the indicated combinations of Flag-Ecto (500 ng), Smad4-Flag (wild-type and mutants, 200 ng), and recombinant human ubiquitin (Boston Biochem, 5 μg).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Results and Discussion, Supplemental Experimental Procedures, 16 figures, and 1 table and are available with this article online at http://www.cell.com/supplemental/S0092-8674(08)01445-1.

ACKNOWLEDGMENTS

We are indebted to Caroline Hill for thoughtful discussions. We are grateful to S. Wood, K. Kaibuchi, F. Francis, A. Moustakas, P. Ten Dijke, C. Hill, M. Brattain, J. Massague, and C. Chang for gifts of reagents and Angela Bachi for comments. A special thanks to Anna Cabrelle for technical assistance with the FACS. Thanks to Oliver Wessely and Giorgio Bressan for reading the manuscript. We are also grateful to Chemical Computing Group and in particular to Dr. Andrea Bortolato for his assistance in molecular modeling. This work is supported by TELETHON-Italy (GGP07218), Cariparo Foundation Excellence Grant, ASI, and Swissbridge grants (to S.P.), and by an AIRC grant (to S.D.). A.M. is recipient of a Marie Curie Epiplast Carcinoma RTN network fellowship. S.J.N. is supported by the NIH (grant CA095875) and SFAz (grant CAA0285-08); F.A. is supported by EU grant LSH-CT-2003-503496.

Received: June 8, 2008
Revised: September 29, 2008
Accepted: October 29, 2008
Published: January 8, 2009

REFERENCES

Akhurst, R.J., and Derynck, R. (2001). TGFbeta signaling in cancer—a double-edged sword. Trends Cell Biol. *11*, S44–S51.

Cadavid, A.L., Ginzel, A., and Fischer, J.A. (2000). The function of the Drosophila fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. Development *127*, 1727–1736.

Chacko, B.M., Qin, B., Correia, J.J., Lam, S.S., de Caestecker, M.P., and Lin, K. (2001). The L3 loop and C-terminal phosphorylation jointly define Smad protein trimerization. Nat. Struct. Biol. *8*, 248–253.

Chacko, B.M., Qin, B.Y., Tiwari, A., Shi, G., Lam, S., Hayward, L.J., De Caestecker, M., and Lin, K. (2004). Structural basis of heteromeric Smad protein assembly in TGFbeta signaling. Mol. Cell *15*, 813–823.

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

Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S., and Piccolo, S. (2007). Integration of TGFβ and Ras/MAPK signaling through p53 phosphorylation. Science *315*, 840–843.

De Bosscher, K., Hill, C.S., and Nicolas, F.J. (2004). Molecular and functional consequences of Smad4 C-terminal missense mutations in colorectal tumour cells. Biochem. J. *379*, 209–216.

De Robertis, E.M., and Kuroda, H. (2004). Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu. Rev. Cell Dev. Biol. *20*, 285–308.

Di Guglielmo, G.M., Le Roy, C., Goodfellow, A.F., and Wrana, J.L. (2003). Distinct endocytic pathways regulate TGFbeta receptor signalling and turnover. Nat. Cell Biol. *5*, 410–421.

Dupont, S., Zacchigna, L., Cordenonsi, M., Soligo, S., Adorno, M., Rugge, M., and Piccolo, S. (2005). Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell *121*, 87–99.

Fischer-Vize, J.A., Rubin, G.M., and Lehmann, R. (1992). The fat facets gene is required for Drosophila eye and embryo development. Development *116*, 985–1000.

Fuentealba, L.C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E.M., and De Robertis, E.M. (2007). Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell *131*, 980–993.

He, W., Dorn, D.C., Erdjument-Bromage, H., Tempst, P., Moore, M.A., and Massague, J. (2006). Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. Cell *125*, 929–941.

Heldin, C.H., and Moustakas, A. (2006). A new twist in Smad signaling. Dev. Cell *10*, 685–686.

Hoppe, T., Cassata, G., Barral, J.M., Springer, W., Hutagalung, A.H., Epstein, H.F., and Baumeister, R. (2004). Regulation of the myosin-directed chaperone UNC-45 by a novel E3/E4-multiubiquitylation complex in C. elegans. Cell *118*, 337–349.

Hudson, J.B., Podos, S.D., Keith, K., Simpson, S.L., and Ferguson, E.L. (1998). The *Drosophila medea* gene is required downstream of dpp and encodes a functional homolog of human Smad4. Development *125*, 1407–1420.

Izzi, L., and Attisano, L. (2006). Ubiquitin-dependent regulation of TGFbeta signaling in cancer. Neoplasia *8*, 677–688.

Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. Cell *102*, 549–552.

Konikoff, E.C., Wisotzkey, R.G., and Newfeld, S.J. (2008). Lysine conservation and context in TGFb and Wnt signaling suggest new targets and general themes for posttranslational modification. J. Mol. Evol., in press. 10.1007/s00239-008-9159-4.

Levy, L., Howell, M., Das, D., Harkin, S., Episkopou, V., and Hill, C.S. (2007). Arkadia activates Smad3/Smad4-dependent transcription by triggering signal-induced SnoN degradation. Mol. Cell. Biol. *27*, 6068–6083.

Lin, X., Duan, X., Liang, Y.Y., Su, Y., Wrighton, K.H., Long, J., Hu, M., Davis, C.M., Wang, J., Brunicardi, F.C., et al. (2006). PPM1A functions as a Smad phosphatase to terminate TGFbeta signaling. Cell *125*, 915–928.

Marquez, R.M., Singer, M.A., Takaesu, N.T., Waldrip, W.R., Kraytsberg, Y., and Newfeld, S.J. (2001). Transgenic analysis of the Smad family of TGFbeta signal transducers in Drosophila melanogaster suggests new roles and new interactions between family members. Genetics *157*, 1639–1648.

McCabe, B.D., Hom, S., Aberle, H., Fetter, R.D., Marques, G., Haerry, T.E., Wan, H., O'Connor, M.B., Goodman, C.S., and Haghighi, A.P. (2004). Highwire regulates presynaptic BMP signaling essential for synaptic growth. Neuron *41*, 891–905.

Moren, A., Hellman, U., Inada, Y., Imamura, T., Heldin, C.H., and Moustakas, A. (2003). Differential ubiquitination defines the functional status of the tumor suppressor Smad4. J. Biol. Chem. *278*, 33571–33582.

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

Niehrs, C. (2004). Regionally specific induction by the Spemann-Mangold organizer. Nat. Rev. Genet. *5*, 425–434.

Nijman, S.M., Luna-Vargas, M.P., Velds, A., Brummelkamp, T.R., Dirac, A.M., Sixma, T.K., and Bernards, R. (2005). A genomic and functional inventory of deubiquitinating enzymes. Cell *123*, 773–786.

Pinho, S., and Niehrs, C. (2007). Dkk3 is required for TGFbeta signaling during Xenopus mesoderm induction. Differentiation *75*, 957–967.

Salmena, L., and Pandolfi, P.P. (2007). Changing venues for tumour suppression: balancing destruction and localization by monoubiquitylation. Nat. Rev. Cancer *7*, 409–413.

Schmierer, B., and Hill, C.S. (2007). TGFbeta-SMAD signal transduction: molecular specificity and functional flexibility. Nat. Rev. Mol. Cell Biol. *8*, 970–982.

Schmierer, B., Tournier, A.L., Bates, P.A., and Hill, C.S. (2008). Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system. Proc. Natl. Acad. Sci. USA *105*, 6608–6613.

Shenoy, S.K., McDonald, P.H., Kohout, T.A., and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. Science *294*, 1307–1313.

Stroschein, S.L., Wang, W., Zhou, S., Zhou, Q., and Luo, K. (1999). Negative feedback regulation of TGFbeta signaling by the SnoN oncoprotein. Science *286*, 771–774.

Wang, J., Sergina, N., Ko, T.C., Gong, J., and Brattain, M.G. (2004). Autocrine and exogenous transforming growth factor beta control cell cycle inhibition through pathways with different sensitivity. J. Biol. Chem. *279*, 40237–40244.

Wood, S.A., Pascoe, W.S., Ru, K., Yamada, T., Hirchenhain, J., Kemler, R., and Mattick, J.S. (1997). Cloning and expression analysis of a novel mouse gene with sequence similarity to the Drosophila fat facets gene. Mech. Dev. *63*, 29–38.

Zhou, S., Buckhaults, P., Zawel, L., Bunz, F., Riggins, G., Dai, J.L., Kern, S.E., Kinzler, K.W., and Vogelstein, B. (1998). Targeted deletion of Smad4 shows it is required for transforming growth factor beta and activin signaling in colorectal cancer cells. Proc. Natl. Acad. Sci. USA *95*, 2412–2416.