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CICLO XXI

FAM, a deubiquitinating enzyme essential for TGFβ signaling controls Smad4 monoubiquitination

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To my beloved parents & my wife

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ABSTRACT (IN ENGLISH)

How growth factors can direct cell behavior depending on strength and duration of the signal remains a central unanswered question in cell and developmental biology. TGFβ serves as paradigm example, as this pathway induces distinct cell fates depending on the levels of nuclear complexes formed by Smad4 with receptor phosphorylated RSmads. To serve reliably as quantitative signal, the assembly of the Smad4/RSmad complex must be not only positively regulated by receptors, but also actively kept under negative control. Yet, the factors responsible for the latter are only now starting to emerge. We found that Smad4 monoubiquitination is a central mechanism by which cells inhibit Smad4/RSmad complex assembly. By mean of siRNA screen we identified FAM (Usp9x) as a new enzyme acting as essential and evolutionary conserved component in TGFβ and BMP signaling. Smad4 is monoubiquitinated in lysine 519 in vivo and this modification renders Smad4 a latent factor, impeding association with phospho-Smad2. FAM is the DUB that reverts this negative modification, re- empowering Smad4 activity. The activity of FAM is cytoplasmic and opposite to that one of Ectodermin/Tif1g(Ecto), a nuclear factor for which we now clarify a prominent role as Smad4 K519- monoubiquitin ligase. Loss of FAM leads to increased Smad4 monoubiquitination and repression of Smad4-dependent TGFβ and BMP responses, whereas loss of Ecto has opposite effects. Critically, these enzymes operate in the same pathway, being Ecto function required downstream of FAM. Intriguingly, the activity of Ecto on Smad4 is fostered by its association with P-Smad2, indicating that Ecto may also serve in vivo as "disruptase" of the Smad2/Smad4 complex. Thus, we suggest that Smad4 monoubiquitination provides a novel and powerful mechanism by which cells can quantitatively tune their responsiveness to TGFβ.

ABSTRACT (IN ITALIAN)

Come i fattori di crescita possano influenzare il comportamento cellulare sulla base dell'intensità e della durata del segnale rimane una questione irrisolta nel campo della biologia cellulare e dello sviluppo. TGFβ ne è un esempio paradigmatico, in quanto questo segnale induce differenti destini cellulari in dipendenza dei livelli nucleari del complesso formato da Smad4 assieme alle Rsmads fosforilate. Per poter agire quantitativamente, la formazione dei complessi Smad4/RSmad non deve essere regolata solo positivamente dai recettori, ma anche negativamente in modo attivo. I fattori responsabili di questo controllo negativo stanno cominciando solo ora ad essere scoperti. Nel nostro studio abbiamo verificato come la monoubiquitinazione di Smad4 sia un meccanismo centrale nell'inibire la formazione del complesso Smad4/RSmad. Attraverso uno screening con siRNA abbiamo identificato FAM (Usp9x) come una nuova deubiquitinasi (DUB) che agisce come componente essenziale ed evolutivamente conservato nelle vie di segnale TGFβ e BMP. Smad4 è monoubiquitinata in vivo a livello della lisina 519: questo la rende un fattore latente e ne impedisce l'associazione con fosfo-Smad2. FAM è la DUB che rimuove questa modificazione negativa ripristinando l'attività di Smad4. L'attività di FAM è citoplasmatica ed opposta a quella di Ectodermin/Tif1g(Ecto), un fattore nucleare per il quale proponiamo un ruolo come monoubiquitina ligasi di Smad4. La inattivazione di FAM porta all'aumento di monoubiquitinazione ed alla repressione delle risposte a TGFβ e BMP dipendenti da Smad4; la inattivazione di Ecto ha effetti opposti. Questi enzimi agiscono nella stessa pathway, poiché la inattivazione di Ecto è dominante rispetto a quella di FAM. E' interessante notare come l'attività di Ecto su Smad4 sia favorita dalla sua associazione con fosfo-Smad2, indicando un possibile ruolo di Ecto come "disassemblatore" del complesso Smad2/Smad4.Per questi motivi proponiamo che la monoubiquitinazione di Smad4 sia un nuovo e potente meccanismo attraverso cui le cellule possono regolare quantitativamente la loro responsività a TGFβ.

PUBLICATIONS

Sirio Dupont, **Anant Mamidi**, Michelangelo Cordenonsi, Marco Montagner, Luca Zacchigna, Maddalena Adorno, Graziano Martello, Michael J Stinchfield, Sandra Soligo, Leonardo Morsut, Masafumi Inui, Stefano Moro, Nicola Modena, Francesco Argenton, Stuart J Newfeld, Stefano Piccolo, FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. *Cell* 2009 vol. 136 (1) pp.123-35

Graziano Martello, Luca Zacchigna, Masafumi Inui, Marco Montagner, Maddalena Adorno, **Anant Mamidi**, Leonardo Morsut, Sandra Soligo, Uyen Tran, Sirio Dupont, Michelangelo Cordenonsi, Oliver Wessely, Stefano Piccolo MicroRNA control of Nodal signaling, *Nature* 2007 vol. 449 (7159) pp. 183-8.

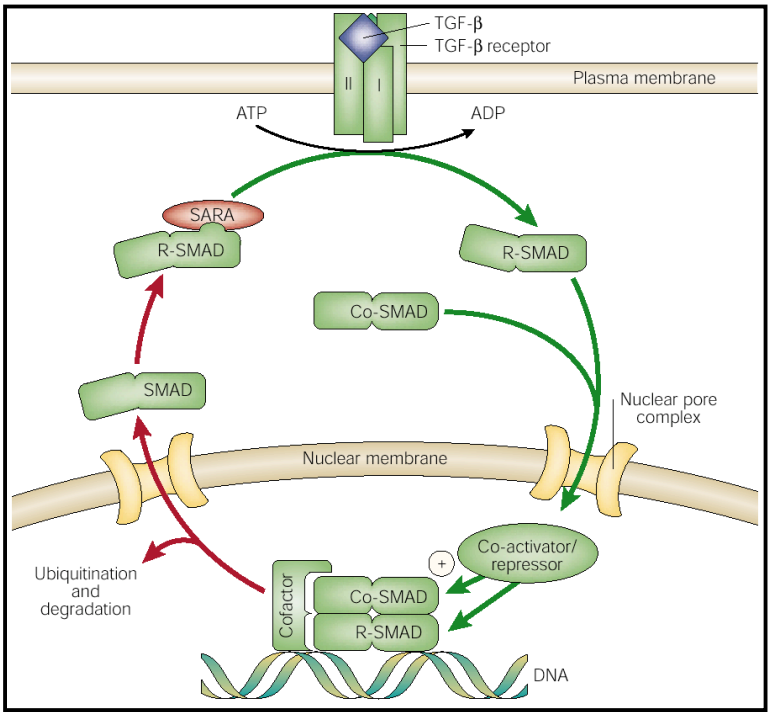
Michelangelo Cordenonsi, Marco Montagner, Maddalena Adorno, Luca Zacchigna, Graziano Martello, **Anant Mamidi**, Sandra Soligo, Sirio Dupont, Stefano Piccolo Integration of TGF-beta and Ras/MAPK signaling through p53 phosphorylation, *Science* 2007 vol. 315 (5813) pp. 840-3.

INTRODUCTION

TGFβ Signaling pathway

Cell proliferation, differentiation and death are controlled by a multitude of cell-cellular signals, and loss of these controls has profound consequences. Prominent among these regulatory signals is the transforming growth factor-β (TGFβ) family of cytokines, which can trigger a bewildering diversity of responses depending on the genetic and epigenetic makeup of the target cell. TGFβ signaling controls a wide set of cellular processes, including cell proliferation, migration, differentiation and apoptosis both during embryogenesis as well as in mature tissues, in species ranging from flies and worms to mammals (Patterson and Padgett, 2000) (Ten Dijke et al., 2002) (Massague and Wotton, 2000)

A TGFβ ligand initiates signaling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface. This allows receptor II to phosphorylate the receptor I, unmasking its kinase domain, which then propogates the signal through phosphorylation of the Smad proteins. There are eight distinct Smad proteins, constituting three functional classes: the receptor-regulated Smad (R-Smad), the Co-mediator Smad (Smad4) and the inhibitory Smad (I-Smad). R-Smads (Smad1, 2, 3, 5 and 8) are directly phosphorylated by the type I receptor kinases and can thus form a heterotrimeric complex with Smad4. The activated Smad complexes are translocated into the nucleus and, in conjuction with other nuclear cofactors, regulate the transcription of target genes (Pardali et al., 2000). The I-Smad, Smad6 and Smad7, negatively regulate TGFβ signaling by competing with R-Smads for receptor or for interaction with Smad4 and also by targeting the receptors for degradation (Moustakas et al., 2001).



The TGFβ family of cytokines includes 42 members in human, 9 in fly, and 6 in worm (Lander et al., 2001). It contains two subfamilies, the TGFβ/Activin/Nodal subfamily and the BMP(bone morphogenetic protein)/GDF(growth and differentiation factor)/MIS(Muellerian inhibiting substance) subfamily, as defined by sequence similarity and by the specific receptors/signaling pathways that they activate. TGFβ ligands can be subdivided according to the type of R-Smad they can activate, with TGFβ/Activin/Nodal activating Smads 2/3 and BMP/GDF activating Smads 1/5/8 (Massague and Wotton, 2000).

TGFβ in development

The TGFβ signaling pathway plays essential roles during embryogenesis. In vertebrates, TGFβ signaling has a key role for the formation and patterning of the primary germ layers, namely the ectoderm, the mesoderm and the endoderm. A decade of work in *Xenopus*, mouse and *zebrafish* model systems provided us with a detailed picture, so far unparalled for other signaling pathways, of how TGFβ signals shape the gastrulating embryo (De Robertis and Kuroda, 2004; Niehrs, 2004). The mesodermal and endodermal germ layers are induced by

endogenous TGFβ ligands, called Nodals (Piccolo et al., 1999). Within the mesoderm, Nodals play not only a general inductive role but also contribute to the localization of the dorsal-most mesoderm, namely the Spenann Organizer in frogs or the Node in mice (Conlon et al., 1994; Whitman, 2001). This tissue is fundamental to coordinate the morphogenetic movements and the patterning of the embryonic tissues during gastrulation, and it is responsible for the establishment of the embryonic body plan, that will be only refined throughout the subsequent developmental phases (Niehrs, 2004).

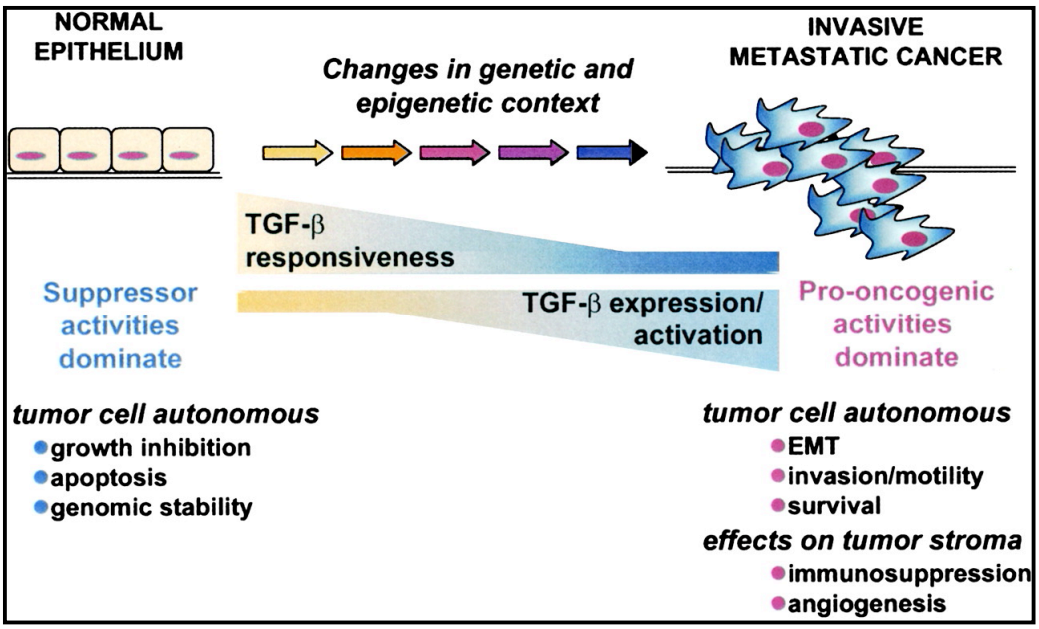
Much of the activity of the Spemann Organizer is related to another class of TGFβ ligands active in embryos, the BMPs. Indeed, seminal work in *xenopus* demonstrated that the Organizer is able to pattern embryonic tissues along the dorso-ventral axis because it is a source of secreted antagonists of BMP signals, like Chordin and Noggin (De Robertis and Kuroda, 2004; Piccolo et al., 1996). BMPs are widely expressed and actively promote a ventral identity of the germ layers, and only in their absence (i.e. in the vicinity of secreted antagonists) the embryonic cells adopt a default dorsal fate. In the case of the ectoderm, this mechanism ensures the correct partitioning of the epidermis (high BMP) from the neural tissue (no BMP).

TGFβ and cystostasis

In adult tissue homeostasis, TGFβ signals have been mainly regarded as tumor suppressors because of their potent inhibitory effects on the proliferation of epithelial cells (Siegel and Massague, 2003). Moreover, components of the TGFβ signaling pathways has been found mutated in human hereditary syndromes predisposing to cancer such as Juvenile Polyposis (caused by inactivation of Smad4 or type I BMP receptor) and HNPCC (hereditary non-polyposis colon cancer – caused by inactivation of type II TGFβ receptor), and somatic loss of these same genes is found at high frequencies in sporadic neoplasms such as pancreatic carcinomas and colon cancers (Akhurst and Derynck, 2001).

The molecular routes that TGFβ uses to trigger these effects are only partially understood, but the TGFβ cytostatic response typically entails the transcriptional activation of the CDK inhibitors p21waf1, p15Ink4a and the repression of c-Myc (Massague, 2008) leading to a specific G1/S cell-cycle arrest before cells enter the S-phase. For this response, TGFβ-activated Smads act in concert with another well-known tumor suppressor, p53, which is required to empower Smad transcriptional activities (Cordenonsi et al., 2003; Cordenonsi et al., 2007).

However, tumors do manage to escape this control: only in specific type of cancers, and anyway with a low frequency, this is attained by inactivating core components of the pathway (10% of colon and 40% of pancreas cancers); much more commonly, either p53 is mutated or genetic/epigenetic changes hit some regulators of the pathway, allowing cancer cells to escape TGFβ mediated tumor suppression but leaving the TGFβ transduction machinery otherwise perfectly functional (Akhurst and Derynck, 2001).



TGFβ and metastasis

Several evidences suggest that TGFβ signaling has a double-edged role in cancer, behaving as tumor suppressor in normal epithelia, but turning into a promoter of malignancy in advanced cancers (Derynck et al., 2001; Wakefield and Roberts, 2002). When cancer cells manage to inactivate or circumvent TGFβ cytostatic effects, they can now exploit TGFβ signaling to foster tumor malignancy, including the gain of invasive properties (i.e., loss of epitheliality, increased motility) and the bypass of the natural barriers normally preserving tissue architecture and function (i.e., degradation of basement membranes or extracellular matrix, and evasion from immune surveillance) (Massague, 2008). Underscoring the importance of TGFβ for metastasis, there is growing interest in anti TGFβ compounds as therapeutic targets, including blocking antibodies and small molecule inhibitors TGFβ receptor kinases (Arteaga et al., 2006; Bierie and Moses, 2006; Wrzesinski et al., 2007).

Features of Smad proteins

The first intracellular mediator of TGFβ signaling, MAD, was identified in *Drosophila* (Sekelsky et al., 1995) which was followed by the discovery of orthologs in worm (SMA mutants) and vertebrates, which were then named "Smad" (Derynck et al., 1996). The R-Smad and Co-Smad proteins contain two conserved structural domains, the N-terminal MH1 (MAD-homology 1) domain and the C-terminal MH2 domain. The R-Smads, but not the Co-Smad Smad4, contain a characteristic SSXS motif at their C-termini, which is the target of receptor kinase phosphorylation. The MH1 domain of Smad4 and most R-Smads exhibits sequence-specific DNA binding activity, although it is generally considered that the affinity of Smads for DNA is low, requiring interaction with additional DNA-binding factors to stably recognize promoter elements and efficiently drive transcription. The MH2 domain is instead required for interaction between Smads. And also found in I-Smads (Massague and Wotton, 2000).

While Smad4 constantly shuttles between the nucleus and the cytoplasm, R-Smads are

actively segregated in the cytoplasm in the absence of TGFβ stimulation; only upon phosphorylation R-Smads are disengaged from cytoplasmic anchors and accumulate in the nucleus, where they form heteromeric complexes with Smad4 (Inman et al., 2002). These complexes, in turn, retain Smad4 within the nucleus. The nuclear Smad complexes, however, are not stable during TGFβ stimulation, but are instead constantly disassembled, followed by the dephosphorylation of R-Smads and their relocalization to the cytoplasm (Schmierer et al., 2008). This mechanism ensures that R-Smads continuously moniter the state of receptor activation, and enables quantitative translation of changing extracellular TGFβ concentrations into graded nuclear Smad activity in time and space. In other words, this mechanism would explain how TGFβ signals can act as morphogens.

Smad4 is a central transducer of TGFβ responses, essential for most of the TGFβ biological effects, including embryonic development, tumor suppression and metastasis (Deckers et al., 2006; Hahn et al., 1996; Yang et al., 1998). Critically, Smad4 is the only shared mediator of the TGFβ and BMP signaling branches. Differently from RSmad, however, Smad4 is not regulated by TGFβ through phosphorylation; this led to consider Smad4 as a ubiquitously/constitutively active cofactor, sidestepping the potential of Smad4 to be also subjected to rounds of activation/inactivation. Given the existence of Smad4 ubiquitin ligases (Izzi and Attisano, 2006), in this thesis work, we have here considered that ubiquitination may represent a mechanism to regulate Smad4 function.

Ubiquitin-dependent regulation of TGFβ signaling

Ubiquitination has been discovered for its role in protein degradation, but in recent years several other mechanisms 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 (DUBs) (Nijman et al., 2005).

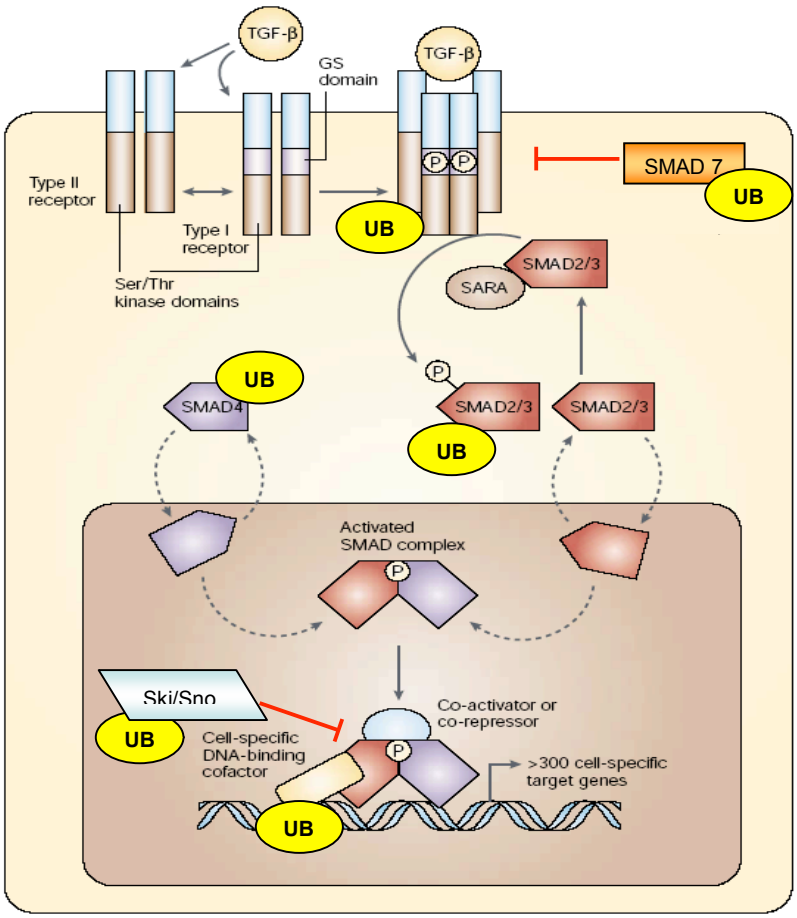
Ubiquitination occurs through a three-step process involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes (Pickart, 2001). The C-terminal Gly-Gly residue of ubiquitin is activated in an ATP-dependent step by a specific activating enzyme, E1. This step involves the covalent binding of ubiquitin to a Cys residue of E1 in a thiolester linkage. Activated ubiquitin is next transferred to an active site Cys residue of a ubiquitin-carrier protein, E2. In the third step, ubiquitin is linked by its C-terminus in an amide isopeptide linkage to an ε-amino group of the substrate protein's Lys residues. This step is catalyzed by an E3 ubiquitin protein ligase, whose main function is to bring the substrate and the E2 in close proximity, providing target-specificity for the ubiquitination reaction.

E3 ubiquitin ligases are generally divided into three classes based on their structure: HECT (homologous to the E6-associated protein C-terminus) type, RING (really interesting gene) type, and U-box type (Pickart, 2001). The major difference between these proteins is that HECT-type ligases form a covalent intermediate with Ub, while the others only recruit E2 enzymes to the substrate. Moreover, E3 ligases can be subdivided in single molecule enzymes (such as HECT and some RING ligases such as MDM2, the E3 ligase for p53) and multi-protein complexes such as the CRL-type of ligases (entailing in the prototypical case of SCF complexes a scaffolding protein, Cullin, a RING-finger protein recruiting the E2, Rbx1, and substrate-recognition complex containing F-box proteins).

Ubiquitination is oppositely regulated by ubiquitin cleavage, which is performed by deubiquitylating enzymes (DUBs). The human genome encodes for approximately 100 DUBs, which can be divided into five classes on the basis of differences in the catalytic domain (Amerik and Hochstrasser, 2004; Nijman et al., 2005). Four classes of DUBs are cysteine proteases– the ubiquitin COOH-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), Machado-Joseph disease protein domain proteases, and ovarian tumor proteases

(OTUs) - whereas the JAMM motif proteases are metalloproteases. Among the five classes of DUBs, the USPs are the largest group with 55 members in humans. They contain a characteristic catalytic core formed by invariant cysteine and histidine residues, which is defined by a small number of motifs flanked by large NH_2 and/or COOH-terminal extensions.

Isolation of several TGFβ regulators endowed with Ub-ligase activity unveiled how TGF-beta is extensively regulated by ubiquitination, both positively and negatively (Izzi and Attisano, 2006). The first Ubiquitin-ligase isolated in the pathway was Smurf1, the ligase responsible for Smad1 degradation, based on its anti-BMP activity in *Xenopus* embryos (Zhu et al., 1999). Subsequently, several other E3 has been proposed to regulate aspects of TGFβ signal transduction, although the physiological significance of most of these findings is still not clear. Importantly, all the ubiquitination events so far described in TGFβ signaling entail polyubiquitination and the regulation of protein degradation/stability, while no monoubiquitination/regulative ubiquitination events have been described so far (Izzi and Attisano, 2006).



Regulation of Smad4 through the Ubiquitin-Dependent Pathway

Being a common intracellular effector of both TGFβ and BMP signaling pathways, Smad4 is a critical point at which both cascades can be modulated to maintain homeostasis. Like R-Smads, Smad4 has been proposed to be regulated by a number or E3 ubiquitin ligases including Smurf1/2, Nedd4-2, SCF/β-TrCP1 and WWP1/Tiul1 (Moren et al., 2005). However, because Smad4 lacks a PY motif, it cannot directly associate with HECT-containing E3 ligases, but rather can recruit the enzymes through adaptors such as I-Smads and R-Smads. Moreover, the endogenous requirement of these ligases for Smad4 regulation has not been addressed, questioning the physiological relevance of these biochemical observations. On the other hand, *Drosophila* Highwire (Hiw), a RING-type E3 ligase regulating BMP signaling at the Drosophila neuro-muscular junction, was shown to interact with the Smad4 homolog *Medea* (*med*) (McCabe et al., 2004). Although ubiquitination and proteosomal degradation of *med* were not directly demonstrated, complete genetic removal of *med* in *hiw* mutants suppresses excessive synaptic growth displayed in *hiw* single mutants. Moreover, in keeping with the proposed ligase activity of *hiw*, the neuronal overexpression of yeast UBP2 (a highly aspecific DUB) or of *Drosophila* Fat Facets (Faf) deubiquitinase mimicked *hiw* mutants, resulting in synaptic overgrowth in wild-type larvae, which was suppressed in *med* mutants (McCabe et al., 2004).

In our lab, we recently described another Smad4 ubiquitin ligase, Ectodermin/ Tif1γ (Ecto) (Dupont et al., 2005). We have identified Ecto in a functional screen for ectoderm determinants in *Xenopus* embryos. Ectodermin protein is asymmetrically localized within the embryo, being enriched in the animal hemisphere where the ectoderm forms. Ecto is essential for the specification of the ectoderm by fulfilling two functions: first it acts by restricting the mesoderm-inducing activity of TGFβ signals to the mesoderm, protecting ectodermal cells from TGFβ ligands emanating from the vegetal hemisphere into extracellular spaces. Second, it prevents excessive BMP signaling in the animal pole, thus allowing for the proper activity

of BMP antagonists secreted by the Spemann Organizer, resulting in efficient neural induction (Dupont et al., 2005). Moreover, we showed that the function of Ectodermin as antagonist of TGFβ and BMP signals is conserved in human cells, representing a constitutive fence against TGFβ cytostatic effects. Mechanistically, we proposed that Ectodermin works as RING-finger ubiquitin ligase for Smad4, regulating Smad4 localization and possibly its stability.

To gain further insight into the role and mechanisms of Smad4 ubiquitination, we aimed at isolating a Smad4 deubiquitinase opposing Ectodermin functions. Surprisingly, still nothing is known on the role of DUBs in regulating TGFβ/BMP signaling. By means of a siRNA screen we had identified FAM (Usp9x), as a deubiquitinase (DUB) acting as essential and evolutionarily conserved component in TGFβ and BMP signaling. FAM/Usp9x, the homologue of Drosophila fat-facets (Wood et al., 1997), acts 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.

COLLABORATIONS AND CONTRIBUTIONS

This work could not be realized without the contributions of several colleagues. In particular, Dr. Sirio Dupont and Dr. Michelangelo Cordenonsi from my lab, who identified Ectodermin and performed the siRNA screening in HaCAT keratinocytes that led to the identification of FAM, and provided data on the functional requirement of FAM for TGFβ signaling in cellines and in Xenopus. Dr. Stuart Newfeld performed Drosophila wing experiments showing effects of Ecto and FAF. Finally, using Molecular Modeling we had the hypothetical three-dimensional model of the Smad4-Ubiquitin covalent complex in collaboration with Dr. Stefano Moro.

RESULTS

We sought to identify deubiquitinating enzymes (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 ubiquitin proteases (Figure 1A and Table 1). Pooled siRNAs (i.e. a mix of two oligos for each gene) were transfected in HaCaT keratinocytes; after 48 hours, cells were treated with TGFβ1 and then harvested for western blot analysis. As read-outs, we used two endogenous responses, namely, the induction of the TGFβ target $p21^{Waf1}$ and Smad3 phosphorylation (Figure 1B).

Out of this screen, 6 siRNA pools inhibited TGFβ effects. Among the corresponding candidate genes, FAM (Usp9x) attracted our attention for two reasons: first, it displayed the most penetrant requirement for TGFβ responses (see below) and, second, genetic evidences in *Drosophila,* albeit scattered, suggested that FAM could be an evolutionarily conserved regulator of Smad4: FAM is in fact the vertebrate homologue of *Drosophila* Fat-facets (Faf); overexpression of *Faf* in fly neurons induces synaptic overgrowth, a BMP-related phenotype, but has no effect in Smad4/Medea mutants (McCabe et al., 2004).

To validate FAM as a general mediator of TGFβ responses, we carried out the following experiments: first, we confirmed these data using additional, independent siRNAs targeting different regions of the gene, ruling out off-target effects (Figure 1C-D-E-F). Second, to rule out gene-specific effects, we extended the molecular characterization from $p21^{Waf1}$ to expression of different endogenous Smad target genes, such as PAI1, $p15^{INK4B}$, Smad7, JunB and Smurf1, analysed at the protein and/or mRNA levels. The results, exemplified in Figures 1C-D-E-F, 2A, 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, (Dennler et al., 1998) (Figure 2B), 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 MDA-MB231, HaCaT, HCT116, HepG2 and Hela, and found that FAM is required for TGFβ signaling in multiple cellular contexts (Figure 1).

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 2C). Similar results were obtained in MDA-MB231 cells transfected with FAM siRNAs.

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 (Korchynskyi and ten Dijke, 2002). Gain-of-FAM enhanced TGFβ and BMP responses (Figure 2D-E). In contrast, overexpression of a catalytically-inactive FAM mutant (i.e. carrying a single Cys-Ser substitution in an essential residue of the protease domain, (Huang et al., 1995) was inactive, indicating that FAM acts as ubiquitin-protease to promote TGFβ activity (Figure 2D). 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. Thus, we challenged FAM depletion in two very different experimental settings. First, we used HCT116 colon cancer cells, for which knockout evidences demonstrated a clear Smad4-dependent

G1/S cell-cycle arrest upon TGFβ treatment (Zhou et al., 1998). Cells were transfected with control- or FAM-siRNAs, treated for 24 hours with or without TGFβ1, and then collected for cell-cycle analysis. As reference, we also used Smad4-siRNA transfected cells. As shown in Figure 3A-B, 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 invasiveness, an effect quantifiable by transwell migration assays (Deckers et al., 2006). We compared MDA-MB231 cells transfected with control-, Smad4- or FAM-siRNA (Figure 3C). Depletion of FAM or Smad4 abolished TGFβ induced migration. Similarly, in wound-healing assays, while control cells migrated efficiently into the wound upon TGFβ treatment, there was no effect on either FAM or Smad4 depleted cells (Figure 3D). These results show that FAM is a critical determinant for TGFβ biological effects, closely recapitulating Smad4 requirements.

FAM is a Smad4 deubiquitinating enzyme

To test for physical interactions between FAM and Smads, we carried out co-immunoprecipitation experiments. We transfected HEK293T cells with Flag-tagged Smad1, Smad2 or Smad4 and V5-tagged FAM; cells were either untreated or treated with TGFβ1 or BMP2. Smads were immunoprecipitated, and the coprecipitating FAM protein visualized by immunoblotting. As shown in Figure 4A, FAM coprecipitated efficiently with Smad4, in a manner independent of signaling; moreover, we could also detect a weaker interaction with RSmads, in particular with Smad2. To test if the binding with RSmads was direct, rather than through endogenous Smad4, we compared FAM/RSmad interactions in wildtype and Smad4-depleted (shSmad4) HEK293T cells. As shown in Figure 4B, RSmads interaction did not occur 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 one hour with TGFβ1 and their lysates immunoprecipitated with an anti-FAM antibody. As shown in Figure 4C, endogenous Smad4 and FAM form a complex in vivo.

We then explored the domain of Smad4 involved in FAM recognition. To this end, we co-expressed FAM together with Smad4 deletions in HEK293 cells and performed coimmunoprecipitation experiments. As shown in Figure 4D, 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. Noteworthy, this biochemical interaction parallels the biological requirement of FAM in both TGFβ and BMP signaling, sharing Smad4 as transducer.

To functionally validate the relevance of Smad4 as key target of FAM, we monitored the role of FAM on Smad4-independent events. To this end, we compared nuclear Smad2 accumulation by immunoflurosescence, and phospho-Smad-dependent degradation of SnoN by TGFβ by western blotting, in cells depleted with control or FAM siRNAs (Nicolas and Hill, 2003; Stroschein et al., 1999). As shown in Figure 4 E-F-G, both these specific read-outs of Smad2/3 activity were not affected by the absence of FAM.

Next, 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 from cell lysates under stringent conditions (to avoid co-purification of contaminants), and its ubiquitination pattern visualized by immunoblotting against HA-Ubiquitin. Remarkably, the major modification of Smad4 in human cells corresponds to a monoubiquitination (Figure 5A, lane 2), also detectable using anti-Smad4 antibodies; only after longer exposures, higher molecular weight bands appear, likely corresponding to oligo-

polyubiquitination (as in (Moren et al., 2003; Dupont et al., 2005). Strikingly, overexpression of wild-type FAM, but not of the catalytically-inactive FAM mutant, inhibited Smad4 monoubiquitination (compare lanes 3 and 4). Ovexpression of FAM also inhibits monoubiquitination of endogenous Smad4 (Figure 5B).

To establish if endogenous FAM is required for regulating Smad4 monoubiquitination, we transfected Smad4 and HA-Ubiquitin plasmids in control and FAM-siRNA depleted MDA-MB231 cells. As shown in Figure 5C, Smad4 monoubiquitination was markedly enhanced upon depletion of FAM. This indicates that Smad4 is a relevant target of FAM in vivo.

Next, we tested whether the deubiquitination of Smad4 by FAM is direct. Indeed, in several cases the DUB is recruited to the substrate through the ligase itself as for example in the case of Fbwx7 and Usp28 targeting c-Myc ubiquitination. To show this, we purified to homogeneity unmodified Smad4, ubiquitinated Smad4 (Ub-Smad4) and FAM proteins by affinity chromatography. First, we compared these purified Smad4 proteins for the ability to interact with FAM, and found that ub-Smad4 binds directly to FAM with similar affinity to non-modified Smad (Figure 5D). Next, to test for direct FAM enzymatic activity, we performed an *in vitro* deubiquitination assay by incubating affinity-purified FAM with Ub-Smad4 in a deubiquitination buffer. This led to hydrolysis of Ub-Smad4 with release of "free" Smad4 and one Ub moiety (Figure 5E), which is visualized using HA antibody against tagged ubiquitin.

In sum, Smad4 is primarily targeted by monoubiquitination in mammalian cells, and FAM is a required as Smad4 DUB. Thus, the turnover of Smad4 monoubiquitination, uncoupled from effects on stability, represents an unprecedented regulatory tier for Smads.

Ectodermin/Tif1γ is the E3 ubiquitin ligase opposed by FAM

Data presented so far imply that, upon FAM depletion, a Smad4 monoubiquitin ligase is left unopposed, leading to inhibition of TGFβ signaling. One should expect that, oppositely to FAM, the loss of this enzyme should markedly enhance TGFβ signaling. We therefore posed 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 (Ecto - (Dupont et al., 2005), β-TrCP1 (Wan et al., 2004), WWP1, Smurfs, NEDD4-2 (Moren et al., 2005), CHIP (Li et al., 2004) and eIF4A (Li and Li, 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 pre-validated siRNA sequences). As shown in Figure 6A, Ecto/Tif1γ stood up as the most powerful natural antagonist of Smad signaling, not only if compared to other Ub ligases, but also to other inhibitors of the pathway such as Smad7, Ski/Sno or TGIF (Itoh and ten Dijke, 2007). This finding is consistent with other independent evidences: 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 (Figure 6 B-C).

We noticed that loss-of-Ecto promotes Smad2/4 activity without stabilizing steady state levels of Smad4 (Figure 6 B-C), suggesting that Ecto inhibits TGFβ signaling primarily by controlling Smad4 *function*, rather than stability; this is compatible with regulative ubiquitination events analogous to those unveiled for FAM. We therefore tested if Ecto may serve as Smad4 monoubiquitin ligase. As shown in Figure 6 D, shRNA-mediated depletion of

Ecto in HEK293T cells leads to the reduction of the Smad4 monoubiquitination band; this effect is specific, as it could be rescued by adding back shRNA-insensitive wild-type Ecto (Ecto*). Similar results were obtained for endogenous Smad4. Thus, Ecto is a required determinant for Smad4 monoubiquitination.

We next sought to determine if this enzymatic activity of Ecto is causal for its antagonistic effects on canonical signaling. Indeed, Ectodermin/Tif1γ has at least two distinct biological activities, as it inhibits Smad4 responses (Dupont et al., 2005; Levy et al., 2007) but can also bind phospho-Smad2 to trigger non-canonical, Smad4-independent TGFβ effects during erythrocyte differentiation (He et al., 2006). Thus, others have proposed that Ectodermin/Tif1γ, rather than acting by a ubiquitin-based mechanism, could, in principle, antagonize canonical signaling by simply binding to Smad2 and Smad3, and sequestering them away from Smad4 ("squelching" model) (He et al., 2006). These observations have contributed to a controversy surrounding Ectodermin/Tif1γ activity (Heldin and Moustakas, 2006), that we are now addressing in detail in this thesis work.

That said, it must be underlined that no *in vivo* functional proof has been provided to buttress the significance of the "squelching" model for canonical TGFβ responses. To address this issue, we compared the activity of wild-type Ecto with that of Ecto-Middle, namely the isolated Smad binding domain (Dupont et al., 2005; He et al., 2006). As shown in Figure 6F-G-H, the Ecto-Middle domain is *per se* void of biological effects in human cells and *Xenopus* embryos, at least as far as these direct, canonical and well-established TGFβ/BMP responses are concerned. In stark contrast, the Ub ligase activity of Ecto/Tif1γ remains absolutely critical for Ecto-mediated antagonism over Smad responses, as deletion of the RING-finger domain (Ecto-ΔTRIM) - or point-mutation of two critical cysteines in this E2 interaction domain (Joazeiro and Weissman, 2000) (Ecto-CAmut) - abolish Ecto activity in TGFβ and BMP responses (Figures 6F-G). As an additional control, we found that Ecto-Middle, Ecto-ΔTRIM

and Ecto-CAmut interact similarly to wild-type Ecto with both Smad2 and Smad4 proteins, and yet are inactive. Of note, in Figure 6F-G wild-type Ecto expressed at just 5 folds its endogenous levels and is sufficient to inhibit canonical TGFβ and BMP responses, without impinging on overall Smad4 stability. This is consistent with Ecto mediating primarily a regulative-type ubiquitination event, such as monoubiquitination of Smad4.

We have also 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 7A), raising the so far unexplored possibility that Ecto may 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 antagonistic and mutually exclusive heterodimers, we should find only Ecto, and not Smad2, as Smad4 partner after this procedure. In contrast, as visualized by immunoblot, both Smad2 and Ecto were copurified (Figure 7B). The results suggest that, upon TGFβ signaling, Ecto, Smad4 and Smad2 form a trimeric complex.

To test the role of Smad2 as modulator of Ecto enzymatic effects, we compared Smad4 monoubiquitination in presence and absence of signaling. As shown in Figure 7C, the presence of TGFβ/Smad2 signaling increases Smad4 monoubiquitination levels. Together, the data suggest that TGFβ fosters Ecto-mediated Smad4 ubiquitination through the incorporation of P-Smad2 into the Ecto/Smad4 complex.

Our biochemical evidences support a model where Ecto promotes Smad4 monoubiquitination, inhibiting its function, and FAM removes this modification, thus reactivating Smad4. As shown in Figure 7D, Ecto-dependent Smad4 monoubiquitination was

inhibited by co-transfection of FAM. As FAM binds to - and directly cleaves - Ub-Smad4 in vitro (Figure 4), these data support the view that FAM works on Smad4 molecules after they have been ubiquitinated by Ecto. Intriguingly, Ecto and FAM act in distinct cellular compartments, nucleus and cytoplasm, respectively (Figure 7E), arguing that mono-Ub Smad4 originates in the nucleus but needs to shuttle back to the cytoplasm to be reactivated by FAM.

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* if phenotypes caused by loss-of-FAM recapitulated those of Smad4 knockdown and Ecto overexpression. In frog embryos, TGFβ/Nodal ligands are critical for inducing the mesoderm in the marginal (equatorial) zone (Niehrs, 2004). Regulation of BMP signaling is instead required for patterning along the dorsoventral axis (De Robertis and Kuroda, 2004). To study these processes, 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 (*Sizzled* is a ventral marker, induced by BMP, whereas *Otx2* is a dorso-anterior marker, whose expression is inhibited by BMP signaling). Smad4 inactivation was carried out either by injecting *Dominant-Negative Smad4* mRNA or Morpholino antisense oligonucleotides targeting the XSmad4 isoforms (XSmad4 MOs, see Experimental Methods) (Howell et al., 1999; Masuyama et al., 1999). Smad4 depletion affected TGFβ-dependent mesoderm formation (Figure 8B). Moreover, as expected from loss of BMP patterning, inhibition of Smad4 expands the *Otx2* staining and reduces *Sizzled* (Figures 8F and 8J).

To study the function of *Xenopus* FAM, we designed an antisense morpholino covering the ATG of the endogenous transcript (FAM MO); this reagent downregulated endogenous FAM protein levels at gastrula stage (Figure 8S). We then tested whether FAM

was required for endogenous TGFβ and BMP signaling. To this end, embryos were depleted of endogenous FAM by radial microinjection of FAM MO. As shown in Figures 8C, 8G and 8K, FAM depletion reduced the *Xbra* and *Sizzled* domains and conversely expanded *Otx2* expression, remarkably recapitulating the loss-of-Smad4. Finally, phenocopying FAM and Smad4 inactivation, Ectodermin gain-of-function by microinjection of *Ecto* mRNA is very effective in inhibiting both TGFβ and BMP responses (Figures 8D, 8H and 8L). These data indicate that FAM and Ecto are 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 signaling for the formation of cross-veins. For example, cross-veins are missing in fly mutant for the BMP-related ligands *dpp* or *gbb* as well as in *Medea* mutants (Hudson et al., 1998; Ray and Wharton, 2001) (see also Figure 8N). Moreover, this read-out has been previously used to demonstrate the specific activity of vertebrate BMP antagonists, such as Noggin and Chordin (Ross et al., 2001; Yu et al., 2000), and to study Smad function (Marquez et al., 2001; Takaesu et al., 2005). To test Ectodermin, 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 (compare Figures 8N, 8O and 8P). Thus, in flies, Ecto behaves as a classical 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 Figure 8Q, 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, as cross-veins development was rescued and ectopic veins continued to form (Figure 8R). Overall, these results strongly support our model for Ecto as signaling antagonist via ubiquitination of Smad4/Medea. Importantly, it also suggests a mechanism by which Faf facilitates BMP signaling - by reversing the negative effects of Smad4/*Medea* ubiquitination.

If FAM and Ecto do work in the same Smad4 pathway, then the lack of monoubiquitinated Smad4, as caused by Ecto-knockdown, should render irrelevant the effects of a concomitant loss-of-FAM (see Figure 9 A-B-C). In other words, if our hypothesis is correct, Ecto function should be epistatic to FAM. For this, we transfected HaCaT cells with control-, FAM- or FAM+Ecto- siRNAs. Cells were treated with TGFβ1 and harvested for $p21^{Waf1}$ immunoblotting. Strikingly, dual depletion of Ecto and FAM rescued TGFβ responsiveness compared to the sole FAM depletion, suggesting that Ecto is required for FAM activity (Figure 9D). Some controls further support this conclusion: first, depletion of Smad4 abolished TGFβ responses but, at difference with FAM, this deficiency was not rescued by coupled Ecto depletion. Second, raising signaling upstream of Smad4 by knocking-down receptor inhibitors, such as Smad7 or Smurfs (Ebisawa et al., 2001; Kavsak et al., 2000), could not rescue FAM depletion (Figure 9D).

Finally, we repeated Ecto and FAM single and double knock-down experiments in MDA-MB231 cells, and visualized phenotypically their epistatic relationship with an established TGFβ assay, namely, TGFβ-induced transwell migration. In this setup, knockdown of FAM abolished migration, but this was dramatically rescued by the compound FAM/Ecto knockdown (Figure 9E). Collectively, these results strongly support the notion that FAM and Ecto regulate TGFβ signaling acting in the same biochemical pathway, i.e. regulation of Smad4 monoubiquitination levels.

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 10A) 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 10A). However, K519 was the most critical residue for Smad4 monoubiquitination, while mutation of K507 had minor effects (Figure 10B, 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.

Finally, we tested if Ecto is the enzyme triggering ubiquitination of lysine 519. As shown in Figure 10C, Ecto promoted ubiquitination of wild-type but not of K519R Smad4 proteins. Interestingly, K519 is one of the most conserved lysine residues across phyla including *Drosophila Medea*; this likely explains the effectiveness of Ecto overexpression in fly transgenic wings.

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 in the structure of Smad4. Biochemical, functional and crystallographic evidences indicate that the Smad transcriptional complex is a

heterotrimer comprising one Smad4 molecule and two phospho-RSmad molecules, designated as A, B and C (see model in Figure 10D, (Chacko et al., 2004). The complex is thus characterized by three non-identical interfaces designated AB (between one RSmad and Smad4), CA (between the two RSmads) and BC (between Smad4 and the second RSmad). Although K519 does not participate directly in RSmad recognition, 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., 2004; Shi et al., 1997), we reasoned that attachment of an ubiquitin moiety to K519 was very likely to generate a similar damage (Figures 10D and 10E). Thus, we modelled in silico the structure of monobiquitinated 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 RSmad recognition (compare Figures 10E and 10F).

These structural hints suggest the idea that K519 monoubiquitination of Smad4 may correspond to a "latent" Smad4, incapable of RSmad recognition. To test this, we used pure preparations of affinity purified unmodified Smad4 and monoubiquitinated Smad4 (Ub-Smad4), and compared their ability to bind immobilized recombinant GST-Smad3-MH2 domain (i.e. the partner of Smad4 in the BC interface) by mean of in vitro GST pull-down assays. Strikingly, only unmodified Smad4 was able to interact with Smad3, but not Ub-Smad4 (Figure 10G). To rule out that differences in the purification procedure might have interfered with these results, we also used a third Smad4 preparation that contained comparable amounts of Smad4 and Ub-Smad4 (indicated as Ub/wt 1:1 in Figure 10G, bottom blot). Also in this case, Smad4 was the only isoform able to complex Smad3. In a more stringent setting, we carried out co-immunoprecipitations assays of purified Smad4 and recombinant in vitro phosphorylated Smad2: here, the monoubiquitinated BC interface had indeed to "compete" with functional AB and AC interfaces, reflecting the endogenous

situation. Yet, even in this case, monoubiquitination disrupted complex formation (Figure 10H). We conclude from these results that Smad4 K519 monoubiquitination inhibits its binding to RSmads.

To directly test if monoubiquitination is a leading inhibitory event in vivo, we tested whether raising the levels of Ecto antagonizes the formation of the endogenous Smad4/Smad2 complex. As shown in Figure 11A, 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, as 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. Of note, these findings closely parallel the lack of TGFβ inhibitory activity displayed by Ecto-mutants in functional assays (see Figures 6F-G-H).

Smads constantly shuttle in and out of the nucleus: TGFβ induces phosphorylation of Smad2, and this promotes nuclear Smad2 accumulation. In turn, Smad2 serves as an anchor to retain Smad4 in the nucleus (De Bosscher et al., 2004; Inman et al., 2002). If monoubiquitination impinges on Smad2/4 complex formation, then this should also impact on subcellular localization of endogenous Smad4. Using this as a read-out, we tested whether loss of FAM - and subsequent raise of Smad4 monoubiquitination - would inhibit Smad4 nuclear accumulation. As shown in Figure 11B, FAM is required in Hela cells for Smad4 nuclear accumulation upon TGFβ stimulation, similarly to Smad2 knockdown. This pairs with the previously published opposite requirement of Ecto for Smad4 nuclear exclusion (Dupont et al., 2005).

If cycles of Smad4 monoubiquitination and de-ubiquitination are the mean by which Ecto and FAM regulate TGFβ signaling in vivo, then loss of FAM should have no effect on cells expressing only the non-ubiquitinated Smad4 K519R mutant (see model in Figure 9 A-B-C). To test this hypothesis, we engineered the Smad4-null MDA-MB468 cell line with

wild-type or K519R Smad4, expressed at near-to-endogenous levels (i.e. those of MDA-MB-231 cells) by retroviral infection. 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* was not detrimental to Smad4 function (Figure 11C). Strikingly, however, while 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 9 A-B-C).

DISCUSSION

The assembly of the Smad complex is the most critical event in TGFβ signaling. Yet, we have so far little clues on the dynamic of the active Smad2/Smad4 complex, on the nature of the "fences" that cells raise against undesired signal activation, and on 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 matters. For the first time, we now establish i) that Smad4 monoubiquitination is a reversible system by which cells inactivate TGFβ signaling cascade and forestall responsiveness to this class of cytokines, ii) the molecular mechanism of this inhibition and iii) how this system is regulated, including the identification of the first DUB essential for Smad function (Figure 13).

A model for TGFβ signaling regulation: turnover of Smad4 monoubiquitination by Ectodermin/Tif1γ and FAM

Ubiquitination is recognized as a key mean to negative regulate TGFβ receptors and Smads, and several E3 ubiquitinating enzymes have been implicated in TGFβ and BMP responses (Itoh and ten Dijke, 2007; Izzi and Attisano, 2006). At difference with polyubiquitination, the conjugation of a single ubiquitin molecule (monoubiquitination) is now emerging as a powerful and versatile device to control protein function (Di Fiore et al., 2003; Salmena and Pandolfi, 2007). In analogy to phosphorylation, a main appeal of regulative ubiquitination events is their reversibility via DUBs (Nijman et al., 2005). Strikingly, however, no DUB has been so far implicated in endogenous TGFβ responses.

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

Upon TGFβ signaling, RSmads accumulate in the nucleus, where they form an active complex with Smad4 to regulate target gene transcription. We propose that Ecto and FAM impinge on Smad4 function from opposite cellular compartments (Figure 7E): in the nucleus, Ecto ubiquitinates either free Smad4 and, to a greater efficiency, the Smad4/Smad2 complex, serving as antagonist and destabilizing factor for the RSmad/Smad4 complex. "Latent" Ub-Smad4 is less retained in the nucleus by RSmads. In the cytoplasm, FAM/Usp9x deubiquitinates and recycles Smad4, re-empowering its competence to mediate TGFβ signaling.

The sole K519R mutation prevents, to a large extent, the endogenous Smad4 monoubiquitination pattern. Thus, although monoubiquitination of Smad4 in another residue (K507) has been suggested to play a positive role for Smad4 activity (Moren et al., 2003), the negative monoubiquitination event in K519 here described appears quantitatively dominant.

Mechanistically, we found that monoubiquitinated Smad4 is inactive because linkage of a ubiquitin moiety sterically impedes the interaction of Smad4 with phospho-RSmads. It is interesting to note that the functional consequences of monoubiquitin linkage in this residue are equivalent to Smad4 mutations in human cancers, that very often hit the same interaction surface disrupted by ubiquitination. In more physiological settings the turnover of Smad4 monoubiquitination by the opposing functions of Ectodermin and FAM may represent economic and yet safe way to place transiently at rest a critical cell regulator. Clearly, the relative levels of Ecto and FAM are critical for this interplay, and it will be important to study how their specific activities can be tuned by other intracellular and extracellular cues. It is in fact plausible that other signaling pathways may exploit the Smad4 monoubiquitination machinery to orchestrate their effects with TGFβ.

We think that our findings on Smad4 present exciting analogies with those of another key tumor suppressor, p53 (Salmena and Pandolfi, 2007). p53 is monoubiquitinated by low

levels of MDM2 in the nucleus, inhibiting, by unknown mechanisms, its activity as transcription factor, and leading to p53 relocalization in the cytoplasm (Li et al., 2003); cytoplasmic monoUb-p53 may be polyubiquitinated by high-doses of mdm2 or other ligases and then degraded, or instead recycled by the activity of the ubiquitin protease HAUSP/USP7 (Li et al., 2002).

It is plausible that, p53 alike, monoubiquitinated Smad4 may serve as target for other "E4" Ub ligases (Hoppe et al., 2004), indeed promoting its degradation in specific contexts (Heldin and Moustakas, 2006). For example, we previously showed, using sensitive pulse-chase assays, that Ecto overexpression at high levels promotes Smad4 instability. It is tempting to speculate that some of the other E3 ligases so far implicated in Smad4 polyubiquitination may serve as "E4" enzyme acting downstream of Ecto. Indeed, in colorectal cancer HCT116 cells, loss-of-FAM does affect Smad4 stability, rescued by concomitant loss-of-Ecto. 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 6E).

Ectodermin/Tif1γ serves as Smad4 monoubiquitin ligase

We identified in Ectodermin/Tif1γ (Ecto) the enzyme responsible for Smad4 monoubiquitination at lysine 519. Similarly to FAM gain-of-function, Ecto-knockdown prevents to a large extent Smad4 monoubiquitination, leading to a potent upregulation of canonical Smad signaling (Figure 6A). In mammalian cells, *Xenopus* and *Drosophila* embryos, Ecto is an inhibitor of Smad4-dependent TGFβ and BMP responses and its effects are opposite to those of FAM. Crucially, for all these antagonistic effects, Ecto requires its RING-finger domain.

In contrast, our evidences argue against the previously proposed "squelching" model, by which Ecto/Tif1γ acted on canonical responses by titrating Smad2 away from Smad4 (He et al., 2006). This is supported by the following data:

1) We confirmed that Ecto independently interacts with Smad4 under basal and induced conditions and, upon signaling, with activated Smad2 (Figures 12A-B) (Dupont et al., 2005; He et al., 2006). However, these do not represent strictly alternative and competitive complexes, as here we show that Ecto, Smad4 and Smad2 can also assemble into a ternary complex upon TGFβ signaling (Figure 7B). We envision the Ecto/Smad4, the Ecto/Smad2 and the ternary complex as three pools in dynamic equilibrium (Figure 12C), of which Smad4 monoubiquitination and the relative activities of FAM and Ecto emerge as critical determinant. In future, it will be important to define what other factors intersect this equilibrium.

We also comparatively quantified the interaction of endogenous Ecto with Smad4 and that one of Ecto with Smad2: the amount of Smad4 co-precipitated by Ecto is comparable to that one of Smad4 co-precipitated by Smad2 after TGFβ signaling (Figure 12D, left panel) whereas the amount of Smad2 co-precipitated by Smad4 is at least 20 fold the amount of Smad2 bound by Ecto (Figure 12D, right panel). The latter represents a very minor fraction of the input. Thus, we argue that even if the interactions of Ecto/Tif1γ with Smad4 or Smad2 were mutually exclusive, the "erosion" of the endogenous pool of Smad2 by this non-catalytic squelching mechanism would be so minor to hardly be relevant for canonical TGFβ signaling. This obviously contrasts with the striking effects of Ecto depletion on Smad transcription and for multiple biological responses (Figure 6A and (Dupont et al., 2005).

2) we could not detect any biochemical titration of Smad2 by the isolated Smad-binding domain of Ecto (Ecto-Middle, at least when this was used up to 50 fold endogenous Ecto levels, see Figure 11A). However, irrespective of any conclusion obtained in vitro, the isolated

Ecto-Middle or Ecto RING-mutants failed to reveal any antagonistic role once tested in vivo (Figures 6F-G-H).

3) We show that Ectodermin/Tif1γ is a general BMP antagonist. For example, even in this strict BMP-dependent heterologous context of *Drosophila* wing cross-veins, Ecto recapitulates classical BMP antagonists. These effects cannot be explained by RSmad titration, given that Ecto is unable to bind Smad1 (Dupont et al., 2005; He et al., 2006).

Importantly, however, while for Ecto antagonism Smad2 binding is *per se* all but irrelevant as stoichiometric/titrating event toward the Smad4/Smad2 complex, it supports Ecto enzymatic properties toward Smad4 (Figure 7C). In this light, our clarification of the mechanisms by which Ecto/Tif1γ inhibits Smad4, namely, not by promoting its degradation but by forestalling its interaction with Smad2 upon ubiquitination at lysine 519, also serves as essential reconciliation of our data with those of (He et al., 2006).

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. Moreover, Smad4 monoubiquitination may also operate as a mean to disrupt the Smad4/Smad2 complex in order to turn-off signaling. Given that Ecto is a nuclear protein, that Smad4 is maintained in the nucleus by phospho-Smad2, and that Smad4 ubiquitination by Ecto is fostered by Smad2 presence, it is plausible that the Smad4/Smad2 complex might be indeed the preferred target of Ecto in vivo.

In the case of RSmads, phosphorylation and nuclear accumulation are maintained only while receptors are active, suggesting that the nucleus is constantly cleared of phospho-RSmads by the activity of phosphatases (i.e. PPM1A (Lin et al., 2006)and others (Schmierer and Hill, 2007)). So far, as 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, as TGFβ/phospho-RSmads 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-RSmads (Schmierer et al., 2008), that is after RSmads 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

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 8A) were obtained by reciprocal swappings between a wild-type cDNA (wt* in Figure 8A) and a complete Lys-Arg mutant Smad4 cDNA (Kall in Figure 8A), 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 & 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). TGF-β1 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) prior to treatment in the same medium. Where indicated, control cells were supplemented of 5μM SB431542 (Tocris) in the medium to quench autocrine TGF-b signaling.

For FAM knockdown the sequences of the siRNA were: #1: GAUGAGGAACCUGCAUUUCtt; #2: GCAGUGAGUGGCUGGAAGUtt. These were used as a 1:1 mix, except otherwise indicated. For complete list of siRNAs, see Table 1.

Immunoprecipitations/GST pulldowns

For in vivo ubiquitination assays, HEK293T cells transfected with the Calcium-Phosphate method were harvested by sonication in Ub-lysis buffer (50mM Hepes pH=7.8, 200mM NaCl, 5mM EDTA, 1% NP40, 5% Glycerol, freshly complemented with 1mM DTT, Protease Inhibitor Cocktail (Roche), Phosphatase Inhibitor Cocktail II (Sigma), 250ng/ml Ubiquitin-aldehyde (Sigma)). Cell lysates were immunoprecipitated 4 hours at 4°C with protein-A sepharose/α-Smad4 (H552) beads in the same buffer supplemented with 2mM MgCl2, followed by three washes of 2min rotating at RT (50mM Hepes pH=7.8, 500mM NaCl, 5mM 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 (25mM Hepes pH=7.8, 400mM KCl, 5mM EDTA, 0,4% NP40, 10% Glycerol freshly supplemented of 1mM DTT, protease and phosphatase inhibitors). Extracts were diluted fourfold to bring KCl concentration to 100mM and NP40 to 0,1%, supplemented of 0,5% BSA (Roche frk.V) and 10mM MgCl2, and subjected to protein-A sepharose immunoprecipitation 4 hours at 4°C. Beads were quickly washed three times at RT with 100mM KCl, 0,05% NP40.

For *in vitro* protein-protein interactions, purified and/or recombinant proteins were diluted in Binding Buffer (25mM Hepes pH=7.5, 100mM KCl, 2mM 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 co-immunoprecipitation, beads were treated for 2 hours at 37°C with PNGaseF (NEB) after the final IP washings to shift Iggs toward lower molecular weights.

Protein purifications and in vitro deubiquitination assay

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 50mM Hepes pH 7,5, 100mM 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 500mM NaCl. Pooled HA eluates were dyalized o.n. against 50mM Hepes pH=7,5, 100mM NaCl, 5% glycerol, 1mM 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), eluted with 0,4 mg/ml V5 peptide (Sigma) in 100mM NaCl, and dyalized o.n. For in vitro deubiquitination assay, purified Ub-Smad4 and FAM were incubated o.n. at 30°C in 50mM Hepes pH=7,5, 100mM NaCl, 5% glycerol, 5mM MgCl2, 1mM ATP, 1mM DTT.

Biological assays in mammalian cells

For cell-cycle analysis, cells were plated in 6cm dishes, transfected and treated as indicated in the figures, trypsinized, washed in PBS, and fixed with ice-cold 70% ethanol

while vortexing. Cells were rehydrated in PBS and stained 30min at RT with propidium iodide (50μg/ml PI, 0,5mg/ml RNAse in PBS) prior to flow-cytometric analysis. Every experiment was repeated at least 2 times independently, with two replicas for each sample.

Transwell assays were performed in 24 well PET inserts (Falcon 8.0 μm pore size). MDA-MB-231 cells were plated and transfected with siRNA and, after 8 hours, serum starved overnight. Then, 50000 or 100000 cells were plated in transwell inserts (at least 3 replicas for each sample) and either left untreated, treated with 5μM SB431542 or 5ng/ml TGFβ1. Cells in the upper part of the transwell were removed with a cotton swab; migrated cells were fixed in 4% PFA and stained with 0.5% Crystal Violet. Filters were photographed and the total number of cells counted. Every experiment was repeated at least 3 times independently.

Gifts of Plasmids

Expression plasmids for Flag-tagged Smads, β-gal, CA-Alk5, CA-Alk3, human wild-type and CA-mutant Flag-Ecto/Tif1γ, *Xenopus* HA-Ecto were as previously described (Dupont et al., 2005). HA-Ubiquitin and Flag-tagged Smad4 domains were from Dr. A. Moustakas. HA-Smad2 was from Dr. J. Massagué. ID1-BRE-lux was from Dr. P. Ten Djike.

RNA interference

Custom Silencer Predesigned siRNA DUB library was purchased from Ambion (see Table 1 for a complete list).

For the other siRNA oligos, synthesized by Invitrogen, the sequences used were:

siRNA	**sense sequence**	**references**
Control	UUCUCCGAACGUGUCACGU dTdT	
FAM #1	GAUGAGGAACCUGCAUUUC dTdT	
FAM #2	GCAGUGAGUGGCUGGAAGU dTdT	

Smad4	GUACUUCAUACCAUGCCGA dTdT	
Ecto #1	GGUAUGUACUAGUUGUGAA dTdT	
Ecto #2	GGAAGAAAGAAGAUGUCUC dTdT	
Smad2 (stealth)	GCUUAGGUUUACUCUCCAAUGUUAA	
Smad3 (stealth)	GAUGCAACCUGAAGAUCUUCAACAA	
WWP1/Tiul1	GGCACGAAUGGAAUAGAUA dTdT	(Seo et al., 2004)
b-trcp1+2	GUGGAAUUUGUGGAACAUC dTdT	(Margottin-Goguet et al., 2003)
CHIP	GCACGACAAGUACAUGGCG dTdT	(Tateishi et al., 2006)
cSki	GCUUCUACUCCUACAAGAG dTdT	(Nagano et al., 2007)
SnoN	GUUGGAGGAGAAAAGAGAC dTdT	(Nagano et al., 2007)
Nedd4-2	CCACAACACAAAGUCACAG dTdT	(Snyder et al., 2004)
eIF4A-I	GCCCAAUCUGGGACUGGGA dTdT	(Ferraiuolo et al., 2004)
eIF4A-II	AGGAGUCUCUCCUUCGUGG dTdT	(Ferraiuolo et al., 2004)
Smad7	CCGCAGCAGUUACCCCAUC dTdT	(Ibarrola et al., 2004)
TGIF	AUCUGGACCAAGUACGAAU dTdT	(Seo et al., 2004)
Smurf1	CCUUGCAAAGAAAGACUUC dTdT	(Ying et al., 2003)

Smurf2 siRNA pool was from Dharmacon, as in (Levy et al., 2007). Except otherwise indicated, FAM, Ecto Smurf1/2 and eIF4A-I/II siRNA were used as a 1:1 mix of two oligos.

Stable shRNA-expressing lines were obtained by stable infection with pSUPER-RETRO-PURO plasmids, whose hairpin sequences were designed based on siRNA FAM #1, Ecto #1 and Smad4; Control (GCAAGCTGACCCTGAAGTTCAT) was a shRNA targeting GFP (Xeragon). All the plasmids were verified by nucleotide sequencing.

Retroviruses were produced by transfecting pSUPER (for shRNA-knockdown) or pBABE (for cDNA expression) plasmids together with a pan-tropic envelope plasmid into

HEK293gp cells. 48 hours after transfection, supernatants were supplemented with 10% FCS, 100mM glutamine and 8ug/ml polybrene (Sigma), and either used to infect cells or stored at -20°C. To obtain stable cell lines, cells were infected at low confluence (10%) for 24 hours with retroviral supernatants diluted 1:1 into normal culture medium. Cells were placed under puromycin selection 48 hours after infection, and selected for one week while passaging before use.

Northern blotting

Total RNA was extracted from cells plated and treated in 6cm or 10cm dishes with Trizol (Invitrogen). Typically, 5-10ug of RNA per sample were loaded and separated in a 6% formaldehyde 1% agarose gel, blotted by upward capillary transfer onto GeneScreenPlus (PerkinElmer) and UV-crosslinked. Membranes were pre-hybridized several hours at 42°C with ULTRAhyb-Oligo solution (Ambion), and hybridized in the same solution with 32P-labeled random-primed DNA probes o.n. at 42°C. Membranes were washed at 68°C with 2xSSC/0,5%SDS and exposed for autoradiography. GAPDH was probed after stripping of the membranes to ensure proper loading of the samples. Each experiment was repeated at least twice.

Probe	**RZPD EST entry**	**Restriction fragment**
p21Waf1	HU3_p983F0138D	EcoRI/NotI (1200bp)
p15INK4B	HU3_p983D1274D2	XhoI/HindIII (650bp)
Smad7	HU3_p983A022D	EcoRI/NotI (850bp)
JunB	HU3_p983B019D	EcoRI/NotI (900bp)
FAM/Usp9x	N-terminal cds fragment	XbaI/EcoRV (500bp)
Id2	HU3_p983A09334	AvaI (500bp)

Luciferase assays

HaCaT-CAGA12-lux cells were siRNA transfected and, after 48 hours, either untreated (normal culture medium) or treated 6 hours with 2ng/ml TGF-β1 before harvesting. Normalization was carried out based on total protein content, as measured by colorimetric Bradford assay. Each sample was transfected in triplicate. Each experiment was repeated twice. Representative data are represented as mean +/- SEM.

HepG2 and HEK293T cells were transfected with Transit-LT1 (MirusBio) and, after 24 hours, the medium was changed to 0,2% FCS. Cells were harvested 48 hours after transfection. Luciferase reporters (25ng/cm2) were cotransfected with CMV-β-gal (40ng/cm2) to normalize for transfection efficiency by CPRG (Roche) colorimetic assay. TGFβ and BMP stimulation was provided by cotransfecting constitutive-active TGFβ (CA-Alk5 4,5ng/cm2) or BMP (CA-Alk3 1,5-4,5-15ng/cm2) receptors. DNA content in all samples was kept uniform by adding pBluescript plasmid. Each sample was transfected in duplicate. Each experiment was repeated twice. Representative data are represented as mean +/- SEM.

Antibodies and Western Blotting

α-Smad4 (H552 and B8), α-lamin (C20), α-tubulin (H235), α-Smurf1 (H60), α-JunB (C11), α-HA polyclonal (Y11) were from SantaCruz; α-b-cat, α-FLAG M2 (Sigma); α-HA monoclonal ascite (Babco), α-V5-HRP conjugate (Invitrogen), α-Smad2/3, α-PAI1, α-p21Cip1/Waf1 (BD Biosciences), α-V5 polyclonal (Bethyl), α-Ecto/Tif1γ (Dupont et al., 2005), α-FAM (1C4 Abnova). Custom α-FAM antibody, used for immunoblotting and immunoprecipitations, was raised by Bethyl in rabbits immunized with GST-FAM-CAT (a kind gift from Dr. Kaibuchi).

To monitor endogenous gene responses, cells were harvested by sonication in Ub-lysis buffer (25μl/cm2). Proteins were loaded according to Bradford quantitation, run in

commercial 4-12% or 10% Nupage MOPS acrylamide gels (Invitrogen) and transferred onto PVDF membranes (Immobilon-P) by wet electrophoretic transfer. In general, blots were blocked one hour at RT with 0,5% non-fat dry milk (BioRad) in PBSw (0,05% Tween) and incubated o.n. at 4°C with primary antibodies. Secondary antibodies were incubated 50min at RT. Washes after antibody incubations were done on an orbital shaker, three times 10 min, with 1X PBS 0,05% Tween-20. For coimmunoprecipitation experiments, we used ExactaCruz HRP-conjugated secondary antibodies; for Smad2 visualization upon coimmunoprecipitation, beads were treated for 2 hours with PNGaseF (NEB) after the final IP washings to shift Iggs toward lower molecular weights. Blots were developed with Pico or Dura SuperSignal West chemiluminescent reagents (Pierce).

Immunofluorescent localizations

Two days after siRNA transfection, cells were trypsinized and replated on Collagen I coated Permanox chamber slides (Nunc), treated as indicated and fixed 10min at RT with 4% PFA in PBS. Slides were permeabilized 10 min at RT with PBS 0,3% TritonX100, blocked one hour at RT with PBST (PBS, 0,1% Triton) 10% goat serum (GS), and incubated overnight at 4°C with primary antibodies in PBST 2% GS: α-Smad4 (H554) 1:150; α-Ecto (Bethyl IHC00216) 1:1000; α-FAM (Abnova 1C4) 1:1000. Secondary antibodies goat anti-rabbit Alexa555 or goat anti-mouse Cy3 were incubated 1,5 hours at RT diluted 1:100 in PBST 0,2% GS. Hoechst staining (Sigma) was used to mark nuclei. Images were obtained with a Leica Axioplan microscope equipped with a DC500 CCD camera. Red-channel images were turned green to allow better visualization upon B&W print.

***Drosophila* assays (In Collaboration with Dr. Stuart J. Newfeld)**

UAS-HA-xEcto and UAS-HA-xEcto-CAmut were constructed by excising the corresponding cDNA sequences from parental pCS2 plasmids with BamHI and SalI and then

inserting them into pUAST cut with BglII and XhoI. Multiple transgenic lines for each construct were generated by standard methods, and the comparable expression of the transgenes checked by HA western blotting. The MS1096-Gal4, gbb^{1}, gbb^{4} and $P[EP]faf^{EP381}$ strains are described in Flybase (flybase.bio.indiana.edu). Crosses of MS1096-Gal4 to lines bearing UAS transgenes and wing scoring of the progeny followed (Takaesu et al., 2005).

***Xenopus* assays**

Xenopus embryos manipulations, in situ hybridization, and capped mRNA preparation were as in (Martello et al., 2007). *Xenopus* FAM-MO, targeting the translation start site, was TCGTGGCTGTCATACTCGACACAGG. To determine the sequence of *Xenopus laevis* FAM/Usp9 mRNA surrounding the ATG, gastrula-stage embryos whole cDNA was PCR amplified with oligos designed on the *Xenopus tropicalis* sequence predicted from the genome, and the PCR product directly sequenced to detect nucleotide polymorphisms (not shown). XSmad4β1-MO was GTAACAACAAGGGCAAAAGATGGCG. XSmad4β2-MO was GGGTCAGAGACATGGCCGGGATCTC XSmad4α-MO was TGTTTGTGATGGACATATTGTCGGT. XSmad4 morpholinos were mixed and injected at 1:1:1 ratio. Control-MO was GeneTools non-targeting morpholino. Embryos injected with MOs were cultivated at 18°C, typically o.n., until reaching the desired developmental stage.

Molecular Modeling (In Collaboration with Dr. Stefano Moro)

The assembly of the hypothetical three-dimensional model of the Smad4-Ubiquitin covalent complex has been carried out following the procedure: a) starting from the crystallographic coordinates of Smad4 MH2 domain (PDB code: 1UF7) and Ubiquitin (PDB code: 1UBQ) a protein-protein docking protocol has been performed to explore the best surface complementarities between Smad4 and Ubiquitin monomers. The program Molfit has been used to perform the protein-protein docking study (Katchalski-Katzir et al., 1992); b) all

energetically stable Smad4-Ubiquitin complexes sample by Molfit have been inspected, and we have selected only those complexes compatible with an Ubiquitin monomer in proximity to K519 of Smad4; c) the missing Smad4/Ubiquitin covalent connection has been create using the Molecular Operating Environment (MOE) software (Chemical Computing Group Inc.); d) Smad4-Ubiquitin covalent complex has been energy minimized using the AMBER99 force field (Cornell et al., 1996) until the energy gradient reached 0.05 kcal/mol.

TABLE 1.

Complete list of the siRNAs targeting 75 human known or predicted deubiquitinating enzymes (DUBs). Where available, Entrez/PubMed Gene ID is provided.

Gene Name	Gene ID	Sense siRNA Sequence	Antisense siRNA Sequence
UCHL1	7345	GGGACAAGAAGUUAGUCCUtt	AGGACUAACUUCUUGUCCCtt
		GGAAAAAGCAGAUUGAAGAtt	UCUUCAAUCUGCUUUUUCCtg
UCHL3	7347	GGCACCAAGUAUAGAUGAGtt	CUCAUCUAUACUUGGUGCCtc
		GGGACAAGAUGUUACAUCAtt	UGAUGUAACAUCUUGUCCCtg
BAP1	8314	GGAGAUCUACGACCUUCAGtt	CUGAAGGUCGUAGAUCUCCtc
		GCAGCUGAUAAGAGUAACAtt	UGUUACUCUUAUCAGCUGCtg
UCHL5/UCH37	51377	GCCAUAGUGAGUGUGUUACtt	GUAACACACUCACUAUGGCtt
		GCAAUUCAGAUGUGAUUCGtt	CGAAUCACAUCUGAAUUGCtc
DUB3	377630	GGCCUACGUCCUCUUUUACtt	GUAAAAGAGGACGUAGGCCtg
		CGUACUUGUGAUUCAUCAAtt	UUGAUGAAUCACAAGUACGtc
DUB1A	n.a.	GGACGACUCACUCUACUUGtt	CAAGUAGAGUGAGUCGUCCtc
		CCAUCAUCCUGAACAGCAAtt	UUGCUGUUCAGGAUGAUGGtt
USP1	7398	CCUAUAAACUGUGAGAAGAtt	UCUUCUCACAGUUUAUAGGtg
		GGCGACUGCUUAACACACUtt	AGUGUGUUAAGCAGUCGCCtt
USP2	9099	GGAUGCUCAGGAGUUCCUUtt	AAGGAACUCCUGAGCAUCCtg
		GGAGUUCCUUCGCUUUCUUtt	AAGAAAGCGAAGGAACUCCtg
USP3	9960	GGUAUGUGAAUGGCCAUGCtt	GCAUGGCCAUUCACAUACCtt
		GGCAUAAGAAAAGAAAACUtt	AGUUUUCUUUUCUUAUGCCtg
USP4	7375	GGCGUGGAAUAAACUACUAtt	UAGUAGUUUAUUCCACGCCtc
		GGUUUAAACCACAUCAUGCtt	GCAUGAUGUGGUUUAAACCtt
USP5	8078	GGAGAAGUUUGAAUUAGACtt	GUCUAAUUCAAACUUCUCCtc
		GGAUGUGAAGAUUGUCAUUtt	AAUGACAAUCUUCACAUCCtc
USP6	9098	GGGAACUAUUCUACAUCCUtt	AGGAUGUAGAAUAGUUCCCtc
		GCACAGUAGCAAACUCAUAtt	UAUGAGUUUGCUACUGUGCtt

USP7/HAUSP	7874	GGCAACCUUUCAGUUCACUtt	AGUGAACUGAAAGGUUGCCtc
		GGCUACGUCGGCUUAAAGAtt	UCUUUAAGCCGACGUAGCCtg
USP8/UBPY	9101	GGAAAGGGCCUAUGUACUAtt	UAGUACAUAGGCCCUUUCCtc
		GGAGUCACUGCUAGUUGGAtt	UCCAACUAGCAGUGACUCCtg
USP9X/FAM	8239	GCAGUGAGUGGCUGGAAGUtt	ACUUCCAGCCACUCACUGCtt
		GAUGAGGAACCUGCAUUUCtt	GAAAUGCAGGUUCCUCAUCtt
USP10	9100	GGCUGUGGAUAAACUACCUtt	AGGUAGUUUAUCCACAGCCtg
		GGCGGAAGUUUUGGAAAAUtt	AUUUUCCAAAACUUCCGCCtc
USP11	8237	GAUAGAAAACGGCGAGAGUtt	ACUCUCGCCGUUUUCUAUCtg
		GGCGAGGAUUAUGUGCUGCtt	GCAGCACAUAAUCCUCGCCtt
USP12	219333	GUUCUUGCGUAUAAGAGUCtt	GACUCUUAUACGCAAGAACtt
		GGUGAAAUCAGAGAUACCAtt	UGGUAUCUCUGAUUUCACCtc
USP13	8975	GCAUUACAGAGACAUGGGCtt	GCCCAUGUCUCUGUAAUGCtc
		GGAGGAAUUCCAAGAUUUUtt	AAAAUCUUGGAAUUCCUCCtt
USP14	9097	GGUAUGCAGGUGCCUUGAGtt	CUCAAGGCACCUGCAUACCtt
		GGCUCAGCUGUUUGCGUUGtt	CAACGCAAACAGCUGAGCCtt
USP15	9958	GGUUGGAAUAAACUUGUCAtt	UGACAAGUUUAUUCCAACCtt
		GGUCAAGAGCCAAUAGCACtt	GUGCUAUUGGCUCUUGACCtt
USP16	10600	GGUGUAUGAGGUUGUAAAUtt	AUUUACAACCUCAUACACCtt
		CCACCUGAUUUGGCAUUAAtt	UUAAUGCCAAAUCAGGUGGtt
USP18	11274	GGAAGAAGACAGCAACAUGtt	CAUGUUGCUGUCUUCUUCCtt
		GGUGUUCGUAAUGAAUGUGtt	CACAUUCAUUACGAACACCtg
USP19	10869	GCGGCACAAGAUGAGGAAUtt	AUUCCUCAUCUUGUGCCGCtg
		GGCCAUUGUGGCGAGUAAGtt	CUUACUCGCCACAAUGGCCtt
USP20	10868	GGGAACCUGUCAGUCGUGUtt	ACACGACUGACAGGUUCCCtt
		GGCAAAAAAGCACAACUUGtt	CAAGUUGUGCUUUUUUGCCtg
USP21	27005	GGACAGCAAGAUUGUGGACtt	GUCCACAAUCUUGCUGUCCtc
		GGUUUUUUGUGACCUGUCCtt	GGACAGGUCACAAAAAACCtc
USP22	23326	GACGGACUAUACUGAGAGCtt	GCUCUCAGUAUAGUCCGUCtg

		GAGCCUAUGACAAUAGCCGtt	CGGCUAUUGUCAUAGGCUCtc
USP24	23358	GGACGAGAAUUGAUAAAGAtt	UCUUUAUCAAUUCUCGUCCtt
		GGUACUACUUGCUCUGUUGtt	CAACAGAGCAAGUAGUACCtc
USP25	29761	GGAGGAGACAACUUACUACtt	GUAGUAAGUUGUCUCCUCCtg
		GGACAGAAAUAGAAAAUGAtt	UCAUUUUCUAUUUCUGUCCtc
USP26	83844	GGAUUAUCCUCCACAGAUGtt	CAUCUGUGGAGGAUAAUCCtt
		GGAGAUGUUACUCUUGAAUtt	AUUCAAGAGUAACAUCUCCtt
USP27X	389856	CCCGAGGAGAAGAAGAAUCtt	GAUUCUUCUUCUCCUCGGGtt
		GCAGAAUGAAUGGACAAUUtt	AAUUGUCCAUUCAUUCUGCtc
USP28	57646	GCCCUGGAUCUAUUAAAGGtt	CCUUUAAUAGAUCCAGGGCtg
		GGACCCUUCCUUUCUCCAUtt	AUGGAGAAAGGAAGGGUCCtg
USP29	57663	GGAAAUUAAACUGGUGGUCtt	GACCACCAGUUUAAUUUCCtt
		GGAAUAUGCUGAAGGAAAUtt	AUUUCCUUCAGCAUAUUCCtg
USP30	84749	GGAAUAUAUGUUAUUUGGGtt	CCCAAAUAACAUAUAUUCCtg
		GCAGCAGUCAGAAAUAACUtt	AGUUAUUUCUGACUGCUGCtc
USP31	57478	GGCCUCUCUAUGUCACUGUtt	ACAGUGACAUAGAGAGGCCtt
		GGCAAAUGUUCUCACUGCAtt	UGCAGUGAGAACAUUUGCCtt
USP32	84669	GGACCUGUGGACUCUCAUAtt	UAUGAGAGUCCACAGGUCCtc
		GGUGAUUUACUGUUCUUUUtt	AAAAGAACAGUAAAUCACCtc
USP33	23032	GGAUUGUAAAGUCCAAGGAtt	UCCUUGGACUUUACAAUCCtg
		GGACCAAAUCUUUGGGCAUtt	AUGCCCAAAGAUUUGGUCCtt
USP34	9736	GCAUAAUACUCACCCUACUtt	AGUAGGGUGAGUAUUAUGCtt
		GCACAUUAUACCUUUUAGGtt	CCUAAAAGGUAUAAUGUGCtg
USP35	57558	GCAUCUUGAUCGAGGUUUCtt	GAAACCUCGAUCAAGAUGCtg
		GCAGGAUGAUUGACUGGGUtt	ACCCAGUCAAUCAUCCUGCta
USP36	57602	GGCUACUACCUUGGUCCAUtt	AUGGACCAAGGUAGUAGCCtg
		GGAUGUAGGCUAUCCGGAAtt	UUCCGGAUAGCCUACAUCCtt
USP37	57695	GGAAAAUGAUUCAUCAUCGtt	CGAUGAUGAAUCAUUUUCCtt
		GCCUAGUAGUUCACUACAAtt	UUGUAGUGAACUACUAGGCtg

USP38	84640	GGAGACAAGUAUUAUCUUUtt	AAAGAUAAUACUUGUCUCCtg
		CGGCCUGAAGCUGAUUAUGtt	CAUAAUCAGCUUCAGGCCGtt
USP39	10713	GGAUCUUCACUAAAAAGCUtt	AGCUUUUUAGUGAAGAUCCtc
		CGUGCUUCAUCAUGGGACAtt	UGUCCCAUGAUGAAGCACGtg
USP40	55230	GGAUAAACCCGAUGCAAAGtt	CUUUGCAUCGGGUUUAUCCtt
		GCUUUGGGUGGACCAGUAAtt	UUACUGGUCCACCCAAAGCtg
USP41	373856	GAUCGCUGAUGUGCACUUGtt	CAAGUGCACAUCAGCGAUCtg
		GGACCAGAUCGCUGAUGUGtt	CACAUCAGCGAUCUGGUCCtt
USP42	84132	GUUGACAAAGCUUCUGAAUtt	AUUCAGAAGCUUUGUCAACta
		GUUCAUCUGUACCUGAUAAtt	UUAUCAGGUACAGAUGAACtc
USP43	124739	GGAAACUCAAGGAAAAUGCtt	GCAUUUUCCUUGAGUUUCCtt
		GGACCUGAAUACCAUCGCAtt	UGCGAUGGUAUUCAGGUCCtc
USP44	84101	GGAGAAGGAUACUAAUGGGtt	CCCAUUAGUAUCCUUCUCCtg
		CGAAGGCCAAUAGUAACUCtt	GAGUUACUAUUGGCCUUCGtt
USP45	85015	GGGAUGUGGUAAAAACUCAtt	UGAGUUUUUACCACAUCCCtg
		GGGAGGAAAAUGCAGAAAUtt	AUUUCUGCAUUUUCCUCCCtt
USP46	64854	GGCAUUGUACUUCUGCCGUtt	ACGGCAGAAGUACAAUGCCtg
		CCAAAGAAGUUCAUUUCAAtt	UUGAAAUGAACUUCUUUGGtg
USP47	55031	GGAGAAUACAGAGUUAAAGtt	CUUUAACUCUGUAUUCUCCtt
		GCUACUCCUACUCAUCUAUtt	AUAGAUGAGUAGGAGUAGCtt
USP48	84196	GGAGAAAAAAGAACUCAUUtt	AAUGAGUUCUUUUUUCUCCtc
		GCAUAUUUGGUUAGGAGAAtt	UUCUCCUAACCAAAUAUGCtc
USP49	25862	GGUAUUAACCAUGGAACCUtt	AGGUUCCAUGGUUAAUACCtg
		GGACUACGUGCUCAAUGAUtt	AUCAUUGAGCACGUAGUCCtt
USP50	373509	GCAUUUACGAAAAAGAUGCtt	GCAUCUUUUUCGUAAAUGCtg
		GCUCAAUUAUAGCAUCGUAtt	UACGAUGCUAUAAUUGAGCtg
USP51	158880	GCUUUAAAGUAGGUAAGAAtt	UUCUUACCUACUUUAAAGCtc
		GGGUUUGAAGACAAGCAAUtt	AUUGCUUGUCUUCAAACCCtg
USP52	9924	GGAAUCAGUGCACAUAAUGtt	CAUUAUGUGCACUGAUUCCtg

		GGUGUCUACUCUGAAUUGCtt	GCAAUUCAGAGUAGACACCtt
USP53	54532	GGUUUUAUGGCAAUUGGAUtt	AUCCAAUUGCCAUAAAACCtg
		CGAUUUUCAGGCAAACUCAtt	UGAGUUUGCCUGAAAAUCGtt
USP54	159195	GGAUAGAAGUUUGUCAGGUtt	ACCUGACAAACUUCUAUCCtt
		GGCUCUAGGUGUGACAACAtt	UGUUGUCACACCUAGAGCCtt
OTUB1	55611	GGACCGAAUUCAGCAAGAGtt	CUCUUGCUGAAUUCGGUCCtg
		CGGCAACUGUUUCUAUCGGtt	CCGAUAGAAACAGUUGCCGtc
CYLD	1540	GCUCCUUAAAGUACCGAAGtt	CUUCGGUACUUUAAGGAGCtt
		GUACCGAAGGGAAGUAUAGtt	CUAUACUUCCCUUCGGUACtt
TNFAIP3/A20	7128	GGACACAGACUUGGUACUGtt	CAGUACCAAGUCUGUGUCCtg
		GGAAACAGACACACGCAACtt	GUUGCGUGUGUCUGUUUCCtt
OTUD1	220213	CCCAGAGUACGACAACUGGtt	CCAGUUGUCGUACUCUGGGtt
		GGAAUAAGUACCGAUUCCAtt	UGGAAUCGGUACUUAUUCCtc
YOD1	55432	GGCCUUAAACGUGUCAUAUtt	AUAUGACACGUUUAAGGCCtc
		GGAGGAUUAAAUGUCUUAAtt	UUAAGACAUUUAAUCCUCCtg
OTUD3	23252	GGAAACAGAUUGAGAGCUGtt	CAGCUCUCAAUCUGUUUCCtg
		GGAGACAGUGGACUACAUGtt	CAUGUAGUCCACUGUCUCCtg
OTUD4	54726	GGUGUUACUGUGUUUUUCAtt	UGAAAAACACAGUAACACCtt
		GGAAUGGGUAGGACAAGUGtt	CACUUGUCCUACCCAUUCCtg
OTUD6A	139562	GCCGAUCAUCCUGGUCUACtt	GUAGACCAGGAUGAUCGGCtt
		GCACUACAACUCCGUGACAtt	UGUCACGGAGUUGUAGUGCtc
OTUD6B	51633	GGAGAUAUGUAUACUCCAGtt	CUGGAGUAUACAUAUCUCCtg
		GGGCAUGAAGAAUGCUGUUtt	AACAGCAUUCUUCAUGCCCtg
OTUD7A	161725	GGACGACAUUGCCCAAGAAtt	UUCUUGGGCAAUGUCGUCCtg
		GGCAAAAACUGGGACCUGAtt	UCAGGUCCCAGUUUUUGCCtt
OTUD7B	56957	GGAAAGAAUUGGGAUGUGAtt	UCACAUCCCAAUUCUUUCCtt
		GGGUUUUCUGACAGAGAGCtt	GCUCUCUGUCAGAAAACCCtt
TRABID	54764	GGCUAAAAGAUGUGUUGUUtt	AACAACACAUCUUUUAGCCtt
		GGAGGAAGUAGUCCUUUGAtt	UCAAAGGACUACUUCCUCCtt

ATXN3	4287	GGUAGUUCCAGAAACAUAUtt	AUAUGUUUCUGGAACUACCtt
		GGAAUGUUAGACGAAGAUGtt	CAUCUUCGUCUAACAUUCCtg
ATX3L	n.a.	GGAAAUAAGGCUUUUAGACtt	GUCUAAAAGCCUUAUUUCCtc
		GCUAGAUGAAGAAGAGAGGtt	CCUCUCUUCUUCAUCUAGCtg
JOSD1	9929	GGUUGUCUCCAAACACCAUtt	AUGGUGUUUGGAGACAACCtc
		GGCUAUGAAGCUGUUUGGUtt	ACCAAACAGCUUCAUAGCCtt
JOSD2	126119	UGAUGUCAAUGUGAUCAUGtt	CAUGAUCACAUUGACAUCAta
		UGUCAAUGUGAUCAUGGCCtt	GGCCAUGAUCACAUUGACAtc
AMSH/STAMBP	10617	GGUGAAUGAAGACAUUCCAtt	UGGAAUGUCUUCAUUCACCtc
		GGAAGGCAACAUUGAACAUtt	AUGUUCAAUGUUGCCUUCCtc
AMSH-LP	57559	GCUUGGUUGUAAUAUCACCtt	GGUGAUAUUACAACCAAGCtt
		GCUAGAAUCGGAGCAGUUUtt	AAACUGCUCCGAUUCUAGCtg

REFERENCES

Akhurst, R.J., and Derynck, R. (2001). TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol *11*, S44-51.

Amerik, A.Y., and Hochstrasser, M. (2004). Mechanism and function of deubiquitinating enzymes. Biochim Biophys Acta *1695*, 189-207.

Arteaga, M.F., Wang, L., Ravid, T., Hochstrasser, M., and Canessa, C.M. (2006). An amphipathic helix targets serum and glucocorticoid-induced kinase 1 to the endoplasmic reticulum-associated ubiquitin-conjugation machinery. Proceedings of the National Academy of Sciences of the United States of America *103*, 11178-11183.

Bierie, B., and Moses, H.L. (2006). Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer *6*, 506-520.

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 TGF-beta signaling. Mol Cell *15*, 813-823.

Conlon, F.L., Lyons, K.M., Takaesu, N., Barth, K.S., Kispert, A., Herrmann, B., and Robertson, E.J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development (Cambridge, England) *120*, 1919-1928.

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.

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

Cornell, W.D., Cieplak, P., Bayly, C.I., Gould, I.R., Merz, K.M., Ferguson, D.M., Spellmeyer, D.C., Fox, T., Caldwell, J.W., and Kollman, P.A. (1996). A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules J. Am. Chem. Soc. 1995, 117, 5179-5197. J Am Chem Soc *118*, 2309-2309.

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.

Deckers, M., van Dinther, M., Buijs, J., Que, I., Lowik, C., van der Pluijm, G., and ten Dijke, P. (2006). The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. Cancer Res *66*, 2202-2209.

Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S., and Gauthier, J.M. (1998). Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. Embo J *17*, 3091-3100.

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

Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C.H., Kern, S.E., Massague, J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B.*, et al.* (1996). Nomenclature: vertebrate mediators of TGFbeta family signals. Cell *87*, 173.

Di Fiore, P.P., Polo, S., and Hofmann, K. (2003). When ubiquitin meets ubiquitin receptors: a signalling connection. Nat Rev Mol Cell Biol *4*, 491-497.

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.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J Biol Chem *276*, 12477-12480.

Ferraiuolo, M.A., Lee, C.S., Ler, L.W., Hsu, J.L., Costa-Mattioli, M., Luo, M.J., Reed, R., and Sonenberg, N. (2004). A nuclear translation-like factor eIF4AIII is recruited to the mRNA during splicing and functions in nonsense-mediated decay. Proceedings of the National Academy of Sciences of the United States of America *101*, 4118-4123.

Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H.*, et al.* (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*, 350-353.

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.

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.

Huang, Y., Baker, R.T., and Fischer-Vize, J.A. (1995). Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene. Science *270*, 1828-1831.

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 (Cambridge, England) *125*, 1407-1420.

Ibarrola, N., Kratchmarova, I., Nakajima, D., Schiemann, W.P., Moustakas, A., Pandey, A., and Mann, M. (2004). Cloning of a novel signaling molecule, AMSH-2, that potentiates transforming growth factor beta signaling. BMC Cell Biol *5*, 2.

Inman, G.J., Nicolas, F.J., and Hill, C.S. (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. Mol Cell *10*, 283-294.

Itoh, S., and ten Dijke, P. (2007). Negative regulation of TGF-beta receptor/Smad signal transduction. Curr Opin Cell Biol *19*, 176-184.

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.

Katchalski-Katzir, E., Shariv, I., Eisenstein, M., Friesem, A.A., Aflalo, C., and Vakser, I.A. (1992). Molecular surface recognition: determination of geometric fit between proteins and their ligands by correlation techniques. Proceedings of the National Academy of Sciences of the United States of America *89*, 2195-2199.

Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., and Wrana, J.L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell *6*, 1365-1375.

Korchynskyi, O., and ten Dijke, P. (2002). Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J Biol Chem *277*, 4883-4891.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W.*, et al.* (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

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.

Li, J., and Li, W.X. (2006). A novel function of Drosophila eIF4A as a negative regulator of Dpp/BMP signalling that mediates SMAD degradation. Nature cell biology *8*, 1407-1414.

Li, L., Xin, H., Xu, X., Huang, M., Zhang, X., Chen, Y., Zhang, S., Fu, X.Y., and Chang, Z. (2004). CHIP mediates degradation of Smad proteins and potentially regulates Smad-induced transcription. Mol Cell Biol *24*, 856-864.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science *302*, 1972-1975.

Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A.Y., Qin, J., and Gu, W. (2002). Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature *416*, 648-653.

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.

Margottin-Goguet, F., Hsu, J.Y., Loktev, A., Hsieh, H.M., Reimann, J.D., and Jackson, P.K. (2003). Prophase destruction of Emi1 by the SCF(betaTrCP/Slimb) ubiquitin ligase activates the anaphase promoting complex to allow progression beyond prometaphase. Dev Cell *4*, 813-826.

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 TGF-beta signal transducers in Drosophila melanogaster suggests new roles and new interactions between family members. Genetics *157*, 1639-1648.

Martello, G., Zacchigna, L., Inui, M., Montagner, M., Adorno, M., Mamidi, A., Morsut, L., Soligo, S., Tran, U., Dupont, S.*, et al.* (2007). MicroRNA control of Nodal signalling. Nature *449*, 183-188.

Massague, J. (2008). TGFbeta in Cancer. Cell *134*, 215-230.

Massague, J., and Wotton, D. (2000). Transcriptional control by the TGF-beta/Smad signaling system. Embo J *19*, 1745-1754.

Masuyama, N., Hanafusa, H., Kusakabe, M., Shibuya, H., and Nishida, E. (1999). Identification of two Smad4 proteins in Xenopus. Their common and distinct properties. J Biol Chem *274*, 12163-12170.

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.

Moren, A., Imamura, T., Miyazono, K., Heldin, C.H., and Moustakas, A. (2005). Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J Biol Chem *280*, 22115-22123.

Moustakas, A., Souchelnytskyi, S., and Heldin, C.H. (2001). Smad regulation in TGF-beta signal transduction. J Cell Sci *114*, 4359-4369.

Nagano, Y., Mavrakis, K.J., Lee, K.L., Fujii, T., Koinuma, D., Sase, H., Yuki, K., Isogaya, K., Saitoh, M., Imamura, T.*, et al.* (2007). Arkadia induces degradation of SnoN and c-Ski to enhance transforming growth factor-beta signaling. J Biol Chem *282*, 20492-20501.

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.

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.

Pardali, K., Kurisaki, A., Moren, A., ten Dijke, P., Kardassis, D., and Moustakas, A. (2000). Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. J Biol Chem *275*, 29244-29256.

Patterson, G.I., and Padgett, R.W. (2000). TGF beta-related pathways. Roles in Caenorhabditis elegans development. Trends Genet *16*, 27-33.

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.

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.

Pickart, C.M. (2001). Mechanisms underlying ubiquitination. Annu Rev Biochem *70*, 503-533.

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

Ray, R.P., and Wharton, K.A. (2001). Context-dependent relationships between the BMPs gbb and dpp during development of the Drosophila wing imaginal disk. Development (Cambridge, England) *128*, 3913-3925.

Ross, J.J., Shimmi, O., Vilmos, P., Petryk, A., Kim, H., Gaudenz, K., Hermanson, S., Ekker, S.C., O'Connor, M.B., and Marsh, J.L. (2001). Twisted gastrulation is a conserved extracellular BMP antagonist. Nature *410*, 479-483.

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. Proceedings of the National Academy of Sciences of the United States of America *105*, 6608-6613.

Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., and Gelbart, W.M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics *139*, 1347-1358.

Seo, S.R., Lallemand, F., Ferrand, N., Pessah, M., L'Hoste, S., Camonis, J., and Atfi, A. (2004). The novel E3 ubiquitin ligase Tiul1 associates with TGIF to target Smad2 for degradation. Embo J *23*, 3780-3792.

Shi, Y., Hata, A., Lo, R.S., Massague, J., and Pavletich, N.P. (1997). A structural basis for mutational inactivation of the tumour suppressor Smad4. Nature *388*, 87-93.

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

Snyder, P.M., Steines, J.C., and Olson, D.R. (2004). Relative contribution of Nedd4 and Nedd4-2 to ENaC regulation in epithelia determined by RNA interference. J Biol Chem *279*, 5042-5046.

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

Takaesu, N.T., Herbig, E., Zhitomersky, D., O'Connor, M.B., and Newfeld, S.J. (2005). DNA-binding domain mutations in SMAD genes yield dominant-negative proteins or a neomorphic protein that can activate WG target genes in Drosophila. Development (Cambridge, England) *132*, 4883-4894.

Tateishi, Y., Sonoo, R., Sekiya, Y., Sunahara, N., Kawano, M., Wayama, M., Hirota, R., Kawabe, Y., Murayama, A., Kato, S*., et al.* (2006). Turning off estrogen receptor beta-mediated transcription requires estrogen-dependent receptor proteolysis. Mol Cell Biol *26*, 7966-7976.

Ten Dijke, P., Goumans, M.J., Itoh, F., and Itoh, S. (2002). Regulation of cell proliferation by Smad proteins. J Cell Physiol *191*, 1-16.

Wakefield, L.M., and Roberts, A.B. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. Curr Opin Genet Dev *12*, 22-29.

Wan, M., Tang, Y., Tytler, E.M., Lu, C., Jin, B., Vickers, S.M., Yang, L., Shi, X., and Cao, X. (2004). Smad4 protein stability is regulated by ubiquitin ligase SCF beta-TrCP1. J Biol Chem *279*, 14484-14487.

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

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.

Wrzesinski, S.H., Wan, Y.Y., and Flavell, R.A. (2007). Transforming growth factor-beta and the immune response: implications for anticancer therapy. Clin Cancer Res *13*, 5262-5270.

Yang, X., Li, C., Xu, X., and Deng, C. (1998). The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. Proceedings of the National Academy of Sciences of the United States of America *95*, 3667-3672.

Ying, S.X., Hussain, Z.J., and Zhang, Y.E. (2003). Smurf1 facilitates myogenic differentiation and antagonizes the bone morphogenetic protein-2-induced osteoblast conversion by targeting Smad5 for degradation. J Biol Chem *278*, 39029-39036.

Yu, K., Srinivasan, S., Shimmi, O., Biehs, B., Rashka, K.E., Kimelman, D., O'Connor, M.B., and Bier, E. (2000). Processing of the Drosophila Sog protein creates a novel BMP inhibitory activity. Development (Cambridge, England) *127*, 2143-2154.

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. Proceedings of the National Academy of Sciences of the United States of America *95*, 2412-2416.

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.

FIGURES

Figure 1: Isolation of FAM/Usp9x, a deubiquitinating enzyme (DUB) required for TGFβ signaling.

A-B) siRNA screen to identify DUBs regulating TGFβ signaling. A) shows a diagram of the screening procedure. **B)** shows the representative effects of selected anti-DUB siRNAs on TGFβ signaling, as monitored by induction of an established target gene ($p21^{Waf1}$) and RSmad activation (phospho-Smad3).

C) Validation of FAM requirement for TGFβ responses. Panels show immunoblots of MDA-MB231 cells transfected with control- (lanes 1 and 2) or two independent FAM-siRNA (lanes 3 and 4). Cells were treated with 5μM SB435412 (-) or with 1ng/ml TGFβ1 (+) for 8 hours.

D) Requirement for FAM in HaCaT cells, HaCaT cells were transiently transfected with control (lanes 1 and 2), two independent si-RNAs of FAM (lanes 3 and 4) and Smad4 siRNA (lane 5). Cells were treated with 5μM SB435412 (-) or with 1ng/ml TGFβ1 (+) for 8 hours. Blotted for TGFb induction of $p21^{WAF1}$.

E) FAM is required for TGFβ induction of JunB in HCT116chr3 colon cancer cells, HCT116chr3 cells were transiently transfected with indicated siRNAs and treated with 5μM SB435412 or with 1ng/ml and 5ng/ml TGFβ 1 for 8hours. Blotted for TGFβ induction of JunB.

F) FAM is required for TGFβ induction of $p21^{Waf1}$ in HepG2 cells, HepG2 cells were checked for p21 levels upon transient transfection of FAM siRNA and treatement with Activin for indicated timepoints.

FIGURE 1

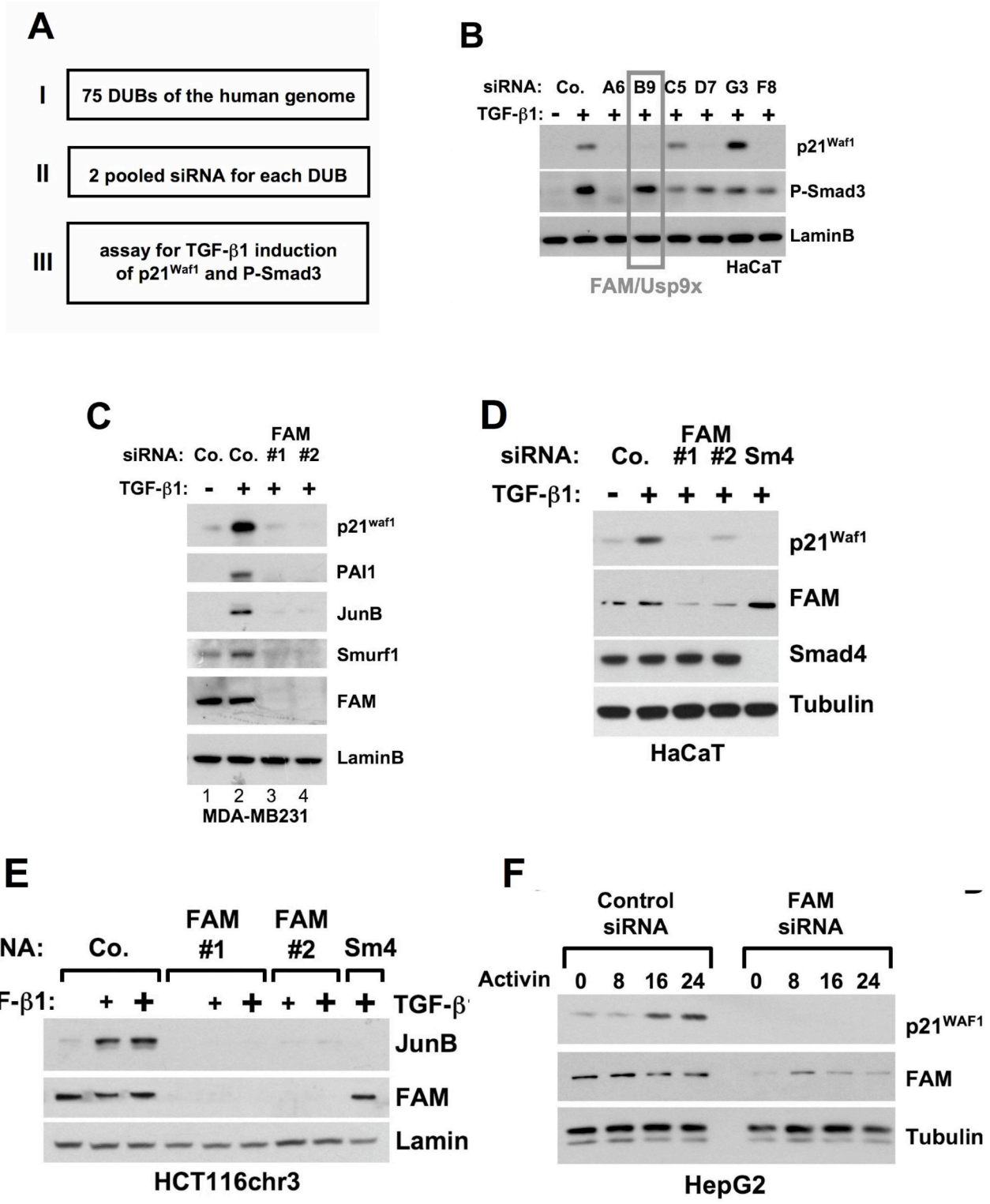

A
I
75 DUBs of the human genome
II
2 pooled siRNA for each DUB
III
assay for TGF-β1 induction of p21Waf1 and P-Smad3
B
siRNA: Co. A6 B9 C5 D7 G3 F8
TGF-β1: - + + + + + + +
p21Waf1
P-Smad3
LaminB
HaCaT
FAM/Usp9x
C
siRNA: Co. Co. FAM #1 #2
TGF-β1: - + + +
p21waf1
PAI1
JunB
Smurf1
FAM
LaminB
1 2 3 4
MDA-MB231
D
siRNA: Co. FAM #1 #2 Sm4
TGF-β1: - + + + +
p21Waf1
FAM
Smad4
Tubulin
HaCaT
E
siRNA: Co. FAM #1 FAM #2 Sm4
TGF-β1: + + + + + + + TGF-β
JunB
FAM
Lamin
HCT116chr3
F
Control siRNA
FAM siRNA
Activin 0 8 16 24 0 8 16 24
p21WAF1
FAM
Tubulin
HepG2

Figure 2: FAM is required for BMP signaling

A) FAM is required for Smad-induced transcription. Panels show northern blots of Hela cells stably expressing control- or FAM-shRNA, either untreated (-) or treated with increasing amounts of TGFβ1 for 2 hours. Knockdown of FAM inhibits induction of direct Smad target genes.

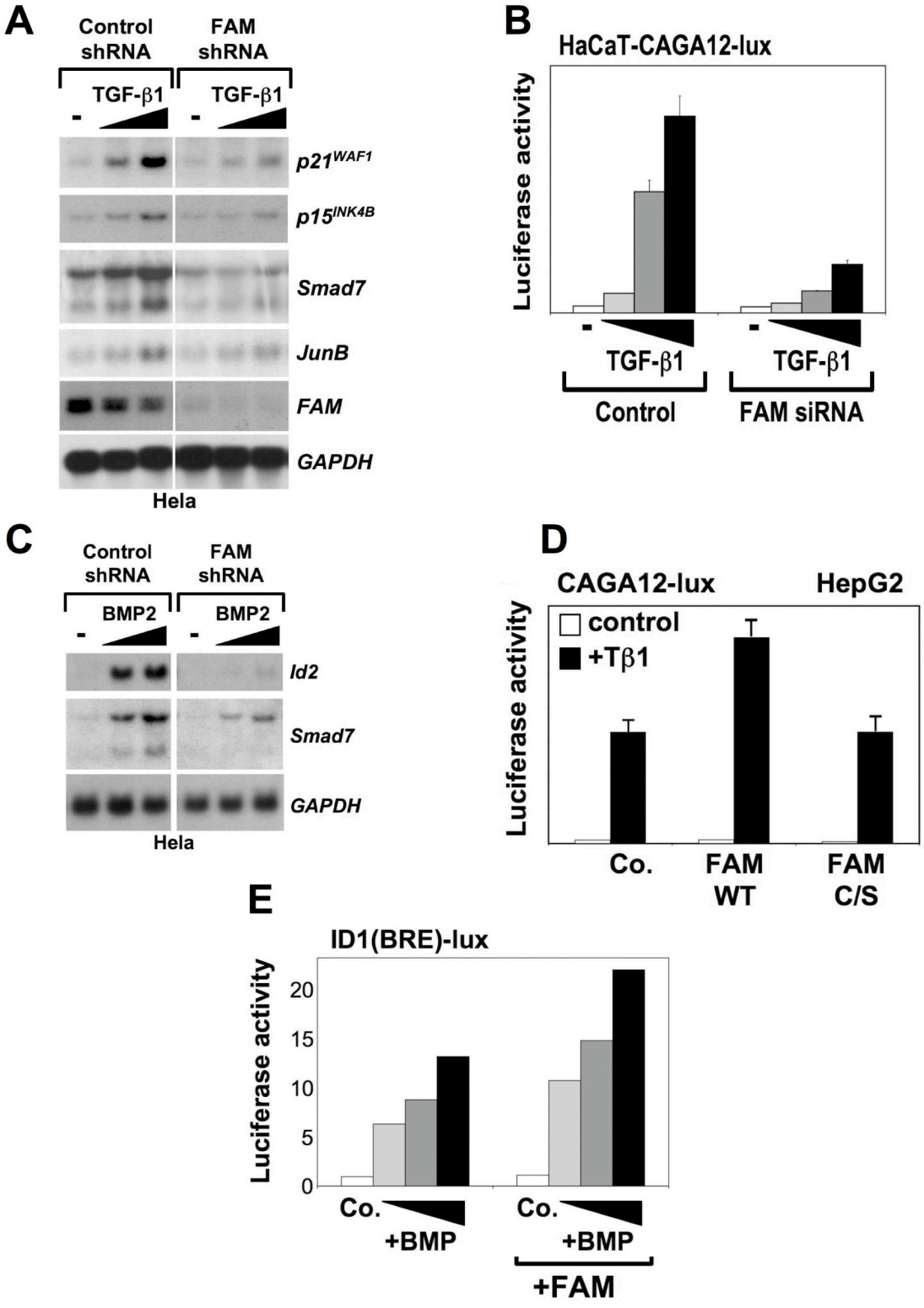
B) FAM affects Smad activity. HaCaT cells stably integrated with the CAGA12-lux reporter (Levy et al., 2007) were transiently transfected with control- (Co.) or FAM-siRNA, and either left untreated (-) or treated with increasing amounts of TGFβ1 for 4 hours. In the absence of FAM, the transcriptional activity of Smads is inhibited.

C) Northern blots of Hela cells stably expressing control- or FAM-shRNA, either untreated (-) or treated with increasing amounts of BMP2 for 2 hours. Knockdown of FAM inhibits induction of established direct Smad1/5 target genes. Conversely, gain-of-FAM fosters BMP responses.

D) FAM sustains Smad activity through its protease-activity. HepG2 cells were transiently transfected with an established reporter of Smad activity, CAGA12-lux, in the presence of an empty vector (Co. 250ng/cm2), with plasmids encoding for wild-type (WT 250ng/cm2) or catalitically-inactive (C/S 250ng/cm2). Overexpression of FAM enhances activation of the luciferase reporter, expression of the protease-dead FAM is ineffective.

E) FAM enhances BMP signaling, HepG2 cells were transiently transfected with an established reporter of Smad1/5 activity, ID1-BRE-lux (50ng/well), in the presence of an empty vector (white bars, 500ng/well), or with a plasmid encoding for wild-type FAM (black bars, 500ng/well), in the absence (-) or presence of increasing BMP stimulation (as obtained by transfecting constitutive-active BMP receptor CA-Alk3).

FIGURE 2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FAM shRNA
TGF-β1
TGF-β1
p21WAF1
p15INK4B
Smad7
JunB
FAM
GAPDH
Hela
B
HaCaT-CAGA12-lux
Luciferase activity
TGF-β1
TGF-β1
Control
FAM siRNA
C
Control shRNA
FAM shRNA
BMP2
BMP2
Id2
Smad7
GAPDH
Hela
D
CAGA12-lux
HepG2
control
+Tβ1
Luciferase activity
Co.
FAM WT
FAM C/S
E
ID1(BRE)-lux
Luciferase activity
20
15
10
5
0
Co.
+BMP
Co.
+BMP
+FAM

Figure 3: FAM/Usp9x is required for TGFβ effects

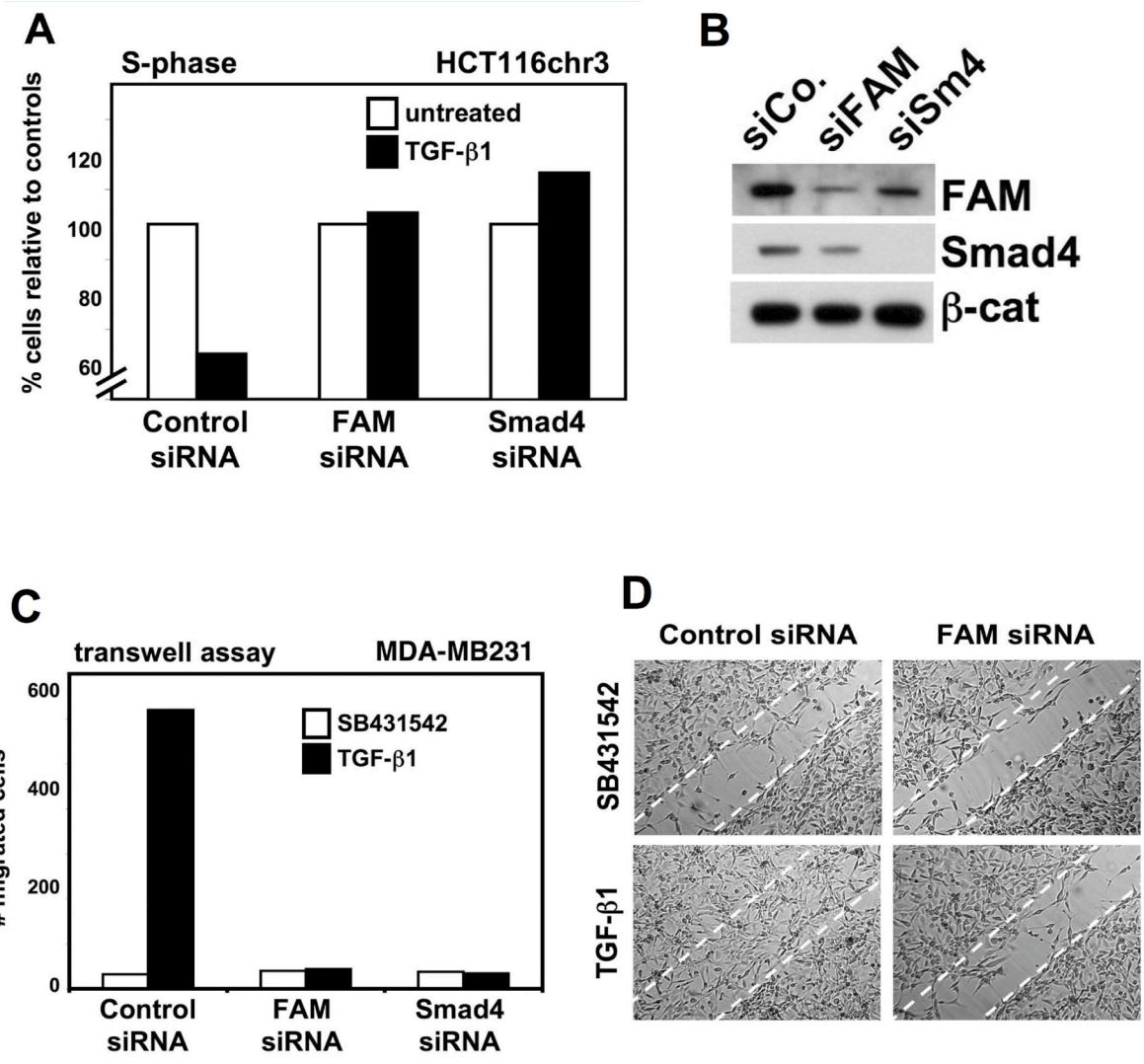
A) FAM is required for TGFβ induced growth-arrest. HCT116chr3 colon cancer cells were transiently transfected with control-, FAM- or Smad4-siRNA, and either left untreated (white bars) or treated with TGFβ1 for 24 hours (black bars) for cell-cycle analysis. The number of cells in each phase for unstimulated cultures was given an arbitrary value of 100%, and all other values are depicted relative to this. TGFβ treatment of control siRNA transfected cells blocked entry of cells in S-phase. Depletion of FAM, similarly to depletion of Smad4, impairs TGFβ cytostatic effects. Immunoblots in Figure 2E show efficient knockdown of target proteins.

B) Western blotting on TGFβ treated samples of Figure 3A showing efficient knockdown of target proteins. β-catenin serves as loading control.

C-D) FAM is required for TGFβ induced cell migration. C) MDA-MB231 breast cancer cells were transfected with the indicated siRNAs, replated onto transwell filters (upper chamber), and treated either with 5μM SB435412 (white bars) or with 4ng/ml TGFβ1 for 24 hours to allow migration of cells through the porous membrane. While TGFβ induced efficient migration across the filter in control cells, it was ineffective in the absence of FAM or Smad4.

D) MDA-MB231 cell cultures, transfected with control or FAM siRNA, were scratched with a pipette tip at confluence, and then either treated with 5μM SB435412 or with 4ng/ml TGFβ1 for 48 hours. The wound edges are indicated by the white dotted lines. In the absence of FAM, cells do not respond to TGFβ and leave the wound empty.

FIGURE 3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S-phase
HCT116chr3
untreated
TGF-β1
% cells relative to controls
120
100
80
60
Control siRNA
FAM siRNA
Smad4 siRNA
B
siCo.
siFAM
siSm4
FAM
Smad4
β-cat
C
transwell assay
MDA-MB231
SB431542
TGF-β1
migrated cells
600
400
200
0
Control siRNA
FAM siRNA
Smad4 siRNA
D
Control siRNA
FAM siRNA
SB431542
TGF-β1

Figure 4: Interaction of FAM with RSmads and Smad4

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 2 hours with TGFβ1 or BMP2, and harvested for immunoprecipitation with anti-Flag affinity resin. Coprecipitating FAM, visualized by anti-V5 immunoblot, indicates a preferential association of FAM with Smad4.

B) Binding of FAM to RSmads is indirect. HEK293T cells, either control or Smad4-shRNA, were transfected with the indicated expression plasmids, and the resulting lysates were subjected to immunoprecipitation/western blotting as in (A). Note how, in the absence of endogenous Smad4, binding of Smad1 or Smad3 to FAM is lost.

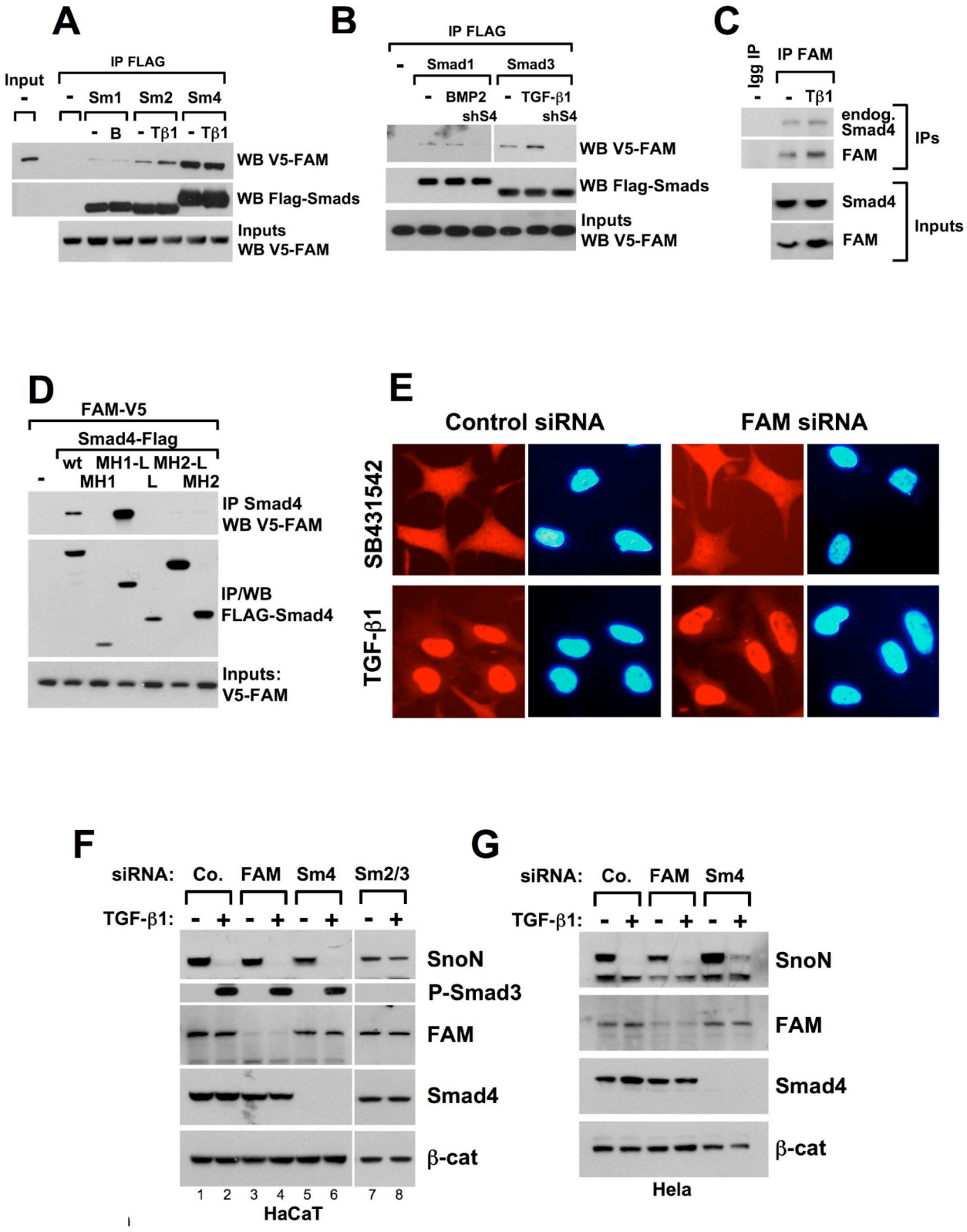
C) FAM and Smad4 form an endogenous protein complex. Extracts of HEK293 cells, untreated (-) or treated (Tβ1) with 2ng/ml TGFβ1 for one hour, were immunoprecipitated with anti-FAM antiserum or an unrelated antiserum (Igg), and the co-precipitating Smad4 visualized by western blotting.

D) Mapping of Smad4 domains required for FAM binding. HEK293T cells were transfected with plasmids encoding for V5-tagged FAM and Flag-tagged Smad4 deletions, containing combinations of the MH1, linker (L) and MH2 domains. Upon anti-Flag immunoprecipitation, only full-length Smad4 and the MH1+linker domains (MH1-L) bound FAM.

E) Knockdown of FAM does not affect Smad2/3 TGFβ1-induced nuclear translocation in Hela cells trasfected with control or FAM siRNA. Red-channel: Smad2/3 imunofluorescence. Blue-channel: Hoechst nuclear counterstaining.

F-G) SnoN degradation in response to TGFβ1 (1 hour, 5ng/ml) is a FAM- and Smad4-independent response in HACAT (F) and Hela (G) while it depends on endogenous Smad2/3 (panel F).

FIGURE 4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Input
IP FLAG
-
- Sm1 Sm2 Sm4
- B - Tβ1 - Tβ1
WB V5-FAM
WB Flag-Smads
Inputs
WB V5-FAM
B
IP FLAG
- Smad1 Smad3
- BMP2 - TGF-β1
shS4 shS4
WB V5-FAM
WB Flag-Smads
Inputs
WB V5-FAM
C
Igg IP
IP FAM
- Tβ1
endog. Smad4
FAM
IPs
Smad4
FAM
Inputs
D
FAM-V5
Smad4-Flag
wt MH1-L MH2-L
- MH1 L MH2
IP Smad4
WB V5-FAM
IP/WB
FLAG-Smad4
Inputs:
V5-FAM
E
Control siRNA
FAM siRNA
SB431542
TGF-β1
F
siRNA: Co. FAM Sm4 Sm2/3
TGF-β1: - + - + - + - +
SnoN
P-Smad3
FAM
Smad4
β-cat
1 2 3 4 5 6 7 8
HaCaT
G
siRNA: Co. FAM Sm4
TGF-β1: - + - + - +
SnoN
FAM
Smad4
β-cat
Hela

Figure 5: FAM is the Smad4 deubiquitinating enzyme

A) FAM reduces Smad4 monoubiquitination in vivo. HEK293T cells transfected with HA-Ubiquitin (8μg/10cm plate) and Smad4-Flag (100ng/10cm plate) plasmids were immunoprecipitated and probed with HA antibodies. Use of an unrelated Igg served as negative control for the purification (lane 1). The two anti-HA blots correspond to 5 seconds (bottom) and 30 seconds (top) exposures. In normal cells (lane 2), Smad4 is mainly monoubiquitinated, as indicated by the presence of a major band migrating at an apparent molecular weight of 84KDa (arrow-heads), also positive with anti-Smad4 (not shown). Other bands likely correspond to oligoubiquitination. Expression of FAM (wt, lane 3), but not enzymatically-inactive FAM (C/S, lane 4), antagonized Smad4 ubiquitination. Immunoblots on the bottom ensure even production of HA-Ubiquitin and FAM isoforms in lysates (inputs).

B) FAM deubiquitinates endogenous Smad4. A major monoubiquitination band is also detectable on immunoprecipitated endogenous Smad4 and FAM overexpression reduces it.

C) FAM is an endogenous restraining factor of Smad4 monoubiquitination. MDA-MB231 cells were transfected first with control (Co.) or FAM-siRNA, and 24 hours later with plasmids encoding for Smad4 and Ubiquitin. Immunoprecipitation followed by western blotting (as in A) confirmed also in these cells the same monoubiquitination pattern of Smad4. In cells lacking FAM, this pattern was significantly increased. Immunoblots on the bottom ensure even production of HA-Ubiquitin and FAM depletion in lysates (inputs).

D) FAM interacts with Smad4 or monoubiquitinated Smad4 (Ub-Smad4) in vitro. FAM, Smad4 and Ub-Smad4, purified to homogeneity from HEK293T cell lysates, were mixed as indicated and subjected to anti-Smad4 immunoprecipitation. Co-precipitating FAM was visualized by immunoblotting.

E) FAM directly deubiquitinates Smad4. Purified Ub-Smad4 and FAM were mixed and allowed to react in vitro. In the presence of FAM, free Smad4 (i.e., non ubiquitinated) and free Ubiquitin were cleaved from Ub-Smad4.

FIGURE 5

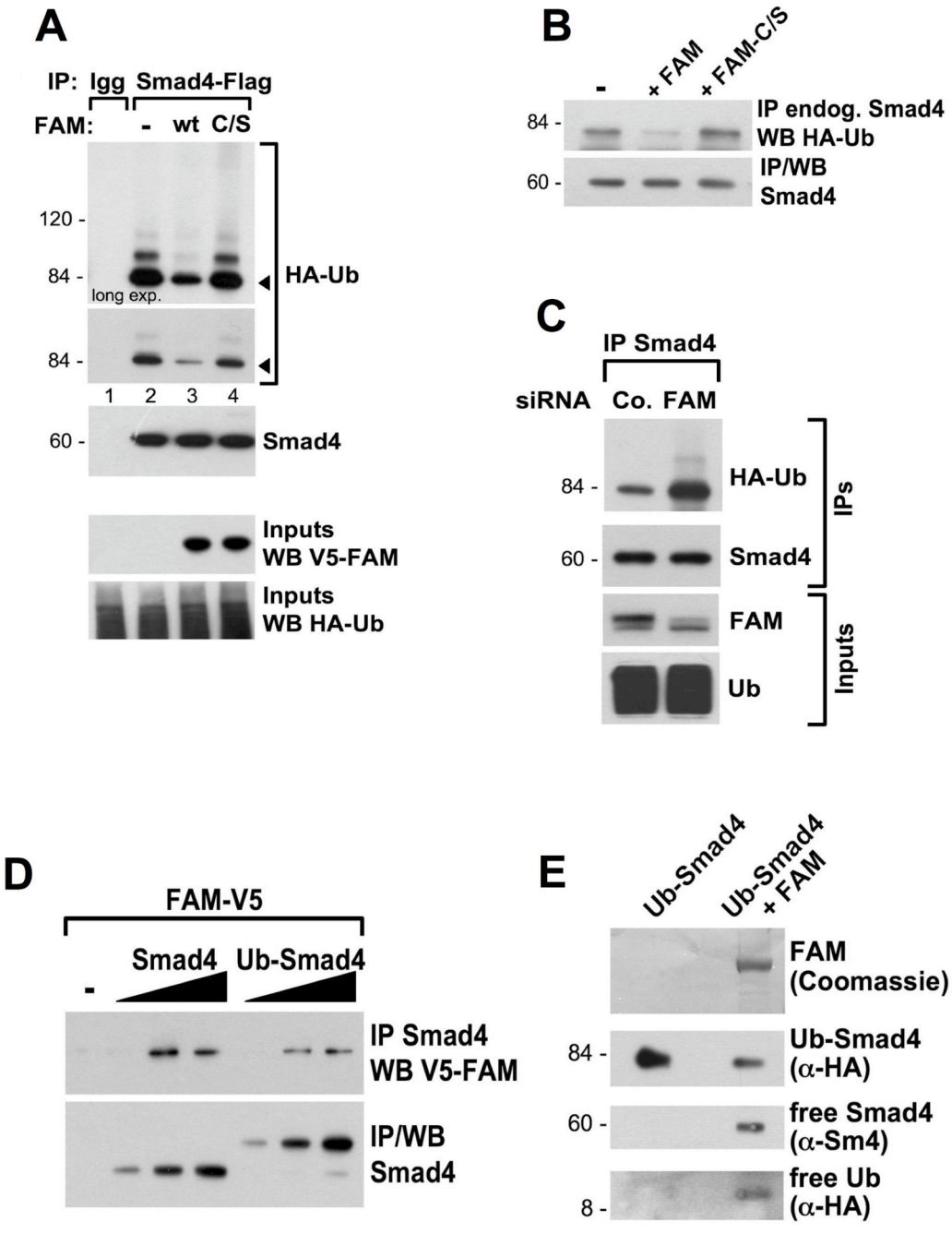
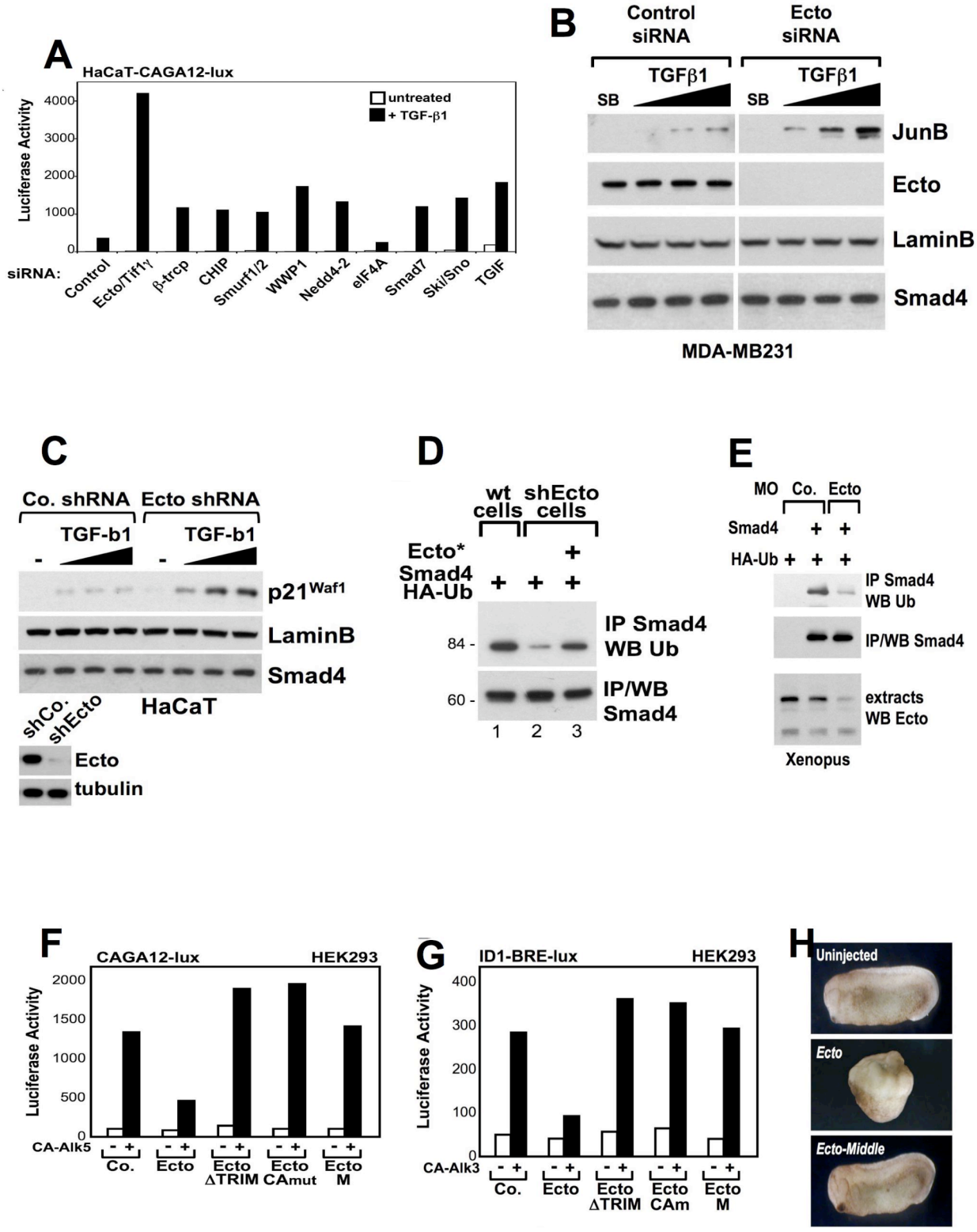

A
IP: Igg Smad4-Flag
FAM: - wt C/S
120 -
84 -
long exp.
HA-Ub
84 -
1 2 3 4
60 -
Smad4
Inputs
WB V5-FAM
Inputs
WB HA-Ub
B
- + FAM + FAM-C/S
84 -
IP endog. Smad4
WB HA-Ub
60 -
IP/WB
Smad4
C
IP Smad4
siRNA Co. FAM
84 -
HA-Ub
60 -
Smad4
IPs
FAM
Ub
Inputs
D
FAM-V5
Smad4 Ub-Smad4
-
IP Smad4
WB V5-FAM
IP/WB
Smad4
E
Ub-Smad4
Ub-Smad4 + FAM
FAM
(Coomassie)
84 -
Ub-Smad4
(α-HA)
60 -
free Smad4
(α-Sm4)
8 -
free Ub
(α-HA)

Figure 6: Ectodermin/Tif1γ is a inhibitor of TGFβ /BMP signaling.

A) Comparison of the biological relevance of Smad4 E3 ligases for TGFβ/Smad4 responses. To search for Smad4 E3 ubiquitin ligase, we compared the endogenous relevance of several known Smad4 ubiquitin ligases (Ecto/Tif1γ, β-trcp, CHIP, Smurfs, Nedd4-2 and eIF4A). As additional comparative control, we also tested the knockdown of other inhibitors of the pathway (Smad7, Ski/Sno and TGIF). HaCaT cells stably integrating the CAGA12-lux reporter were transfected with the indicated siRNA, and either untreated (white bars) or treated (black bars) 4 hours with 2ng/ml TGFβ1.
B) Ectodermin/Tif1γ is an endogenous restraining factor for TGFβ in MDA-MB-231 cells. Cells depleted of Ecto/Tif1γ by siRNA transfection display enhanced activation of JunB in response to suboptimal TGFβ stimulation (50-100-200pg/ml TGFβ1 for 8 hours).
C) Depletion of Ecto in HaCaT cells by stable shRNA enhanced activation of $p21^{Waf1}$ in response to TGFβ stimulation (50-100-200pg/ml TGFβ1 for 6 hours). Immunoblots on the bottom show the levels of Ecto knockdown in shRNA-depleted cells.
D) HEK293T cells, either control or Ecto-shRNA, were transfected with the indicated expression plasmids, and their lysates subjected to immunoprecipitation. In cells lacking of Ecto, the Smad4 monoubiquitination band is strongly inhibited (compare lanes 1 and 2). This effect is specific, as transfection of a plasmid encoding for a shRNA-insensitive Ecto (Ecto*, lane 3) rescues Smad4 ubiquitination to normal levels.
E) *Xenopus* embryos were injected in the animal pole with the indicated combinations of mRNA (coding for HA-Ub and Smad4) and of morpholino oligonucleotides (MO) to knockdown endogenous Ectodermin expression. Lysates were immunoprecipitated with anti-Smad4 antibody and the ubiquitination pattern of Smad4 visualized by HA immunoblotting
F) HEK293T cells were transfected with an established CAGA12-lux reporter of Smad activity, either with empty vector (Co. 50ng/cm2), or with plasmids (50ng/cm2) wild-type Ecto, mutants lacking the RING-finger domain (Ecto-ΔTRIM and Ecto-CAmutant) and Middle portion of Ecto (Ecto-M). The inhibitory activity of Ecto requires the integrity of the RING-finger domain, and it cannot be recapitulated by the simple overexpression of the Smad interacting domain.
G) Ectodermin/Tif1γ inhibits BMP signaling as measured with ID1-BRE-lux reporter. The inhibitory activity of Ecto requires the integrity of the RING-finger domain.
H) Injection of Ectodermin mRNA in the marginal zone of 2-cell stage embryos (300ng/embryo) potently inhibits mesoderm development, leading to development of tadpoles with strongly reduced axial structures, small/absent heads and an excess of epidermis on the ventral side, phenocopying TGFβ/Nodal inhibition. Injection of the same amount of *xEcto-Middle* mRNA has no effects on embryogenesis, indicating that the sole Smad interaction cannot account for Ecto biological activities in vivo.

FIGURE 6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Luciferase Activity
untreated
+ TGF-β1
siRNA:
Control
Ecto/Tif1γ
β-trcp
CHIP
Smurf1/2
WWP1
Nedd4-2
eIF4A
Smad7
Ski/Sno
TGIF
B
Control siRNA
Ecto siRNA
TGFβ1
SB
JunB
Ecto
LaminB
Smad4
MDA-MB231
C
Co. shRNA
Ecto shRNA
TGF-b1
p21Waf1
LaminB
Smad4
shCo.
shEcto
HaCaT
Ecto
tubulin
D
wt cells
shEcto cells
Ecto*
Smad4
HA-Ub
IP Smad4
WB Ub
IP/WB Smad4
84 -
60 -
1 2 3
E
MO
Co.
Ecto
Smad4
HA-Ub
IP Smad4
WB Ub
IP/WB Smad4
extracts
WB Ecto
Xenopus
F
CAGA12-lux
HEK293
Luciferase Activity
CA-Alk5
Co.
Ecto
Ecto ΔTRIM
Ecto CAmut
Ecto M
G
ID1-BRE-lux
HEK293
Luciferase Activity
CA-Alk3
Co.
Ecto
Ecto ΔTRIM
Ecto CAm
Ecto M
H
Uninjected
Ecto
Ecto-Middle

Figure 7: Ectodermin/Tif1γ is a Smad4 monoubiquitin ligase restraining TGFβ signaling.

A) Endogenous Smad4 monoubiquitination is fostered by TGFβ/Smad2 activation. HEK293T cells were transfected with expression plasmid encoding for HA-Ub in the absence or presence (TGFβ1) of Smad2-Flag and overnight treatment with 2ng/ml TGFβ1 in 0.2% FCS as indicated, and their lysates subjected to immunoprecipitation. Elevation of TGFβ/Smad2 levels increases Smad4 monoubiquitination. Immunoprecipitation of HA-Ub expressing lysates with an unrelated Igg served as negative control (first lane).

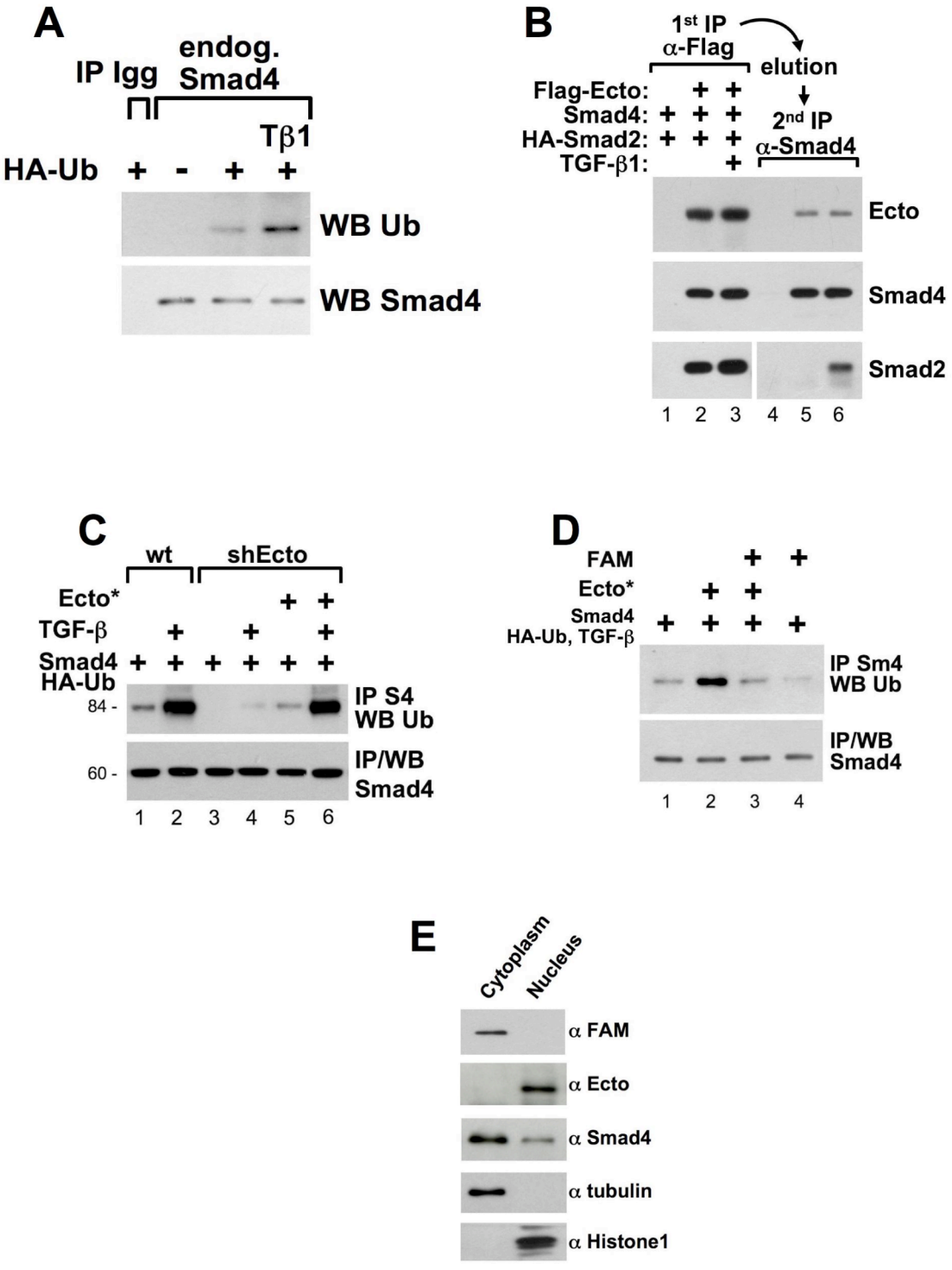
B) Ecto, Smad4 and Smad2 form a trimeric complex. Ecto-containing protein complexes were subjected to sequential affinity purifications with anti-Flag and anti-Smad4 antibodies.

C) Smad2 fosters ubiquitination of Smad4 by Ecto. Smad4 ubiquitination assay in HEK293T cells. TGFβ stimulation (by overexpressing Smad2 and stimulating cells o.n. with 2ng/ml TGFβ1) fosters Smad4 ubiquitination in normal cells (wt, lanes 1 and 2), in an Ecto-dependent manner (shEcto cells, lanes 3 and 4). Together with Ecto*-reconstitution (lanes 5 and 6), this indicates the critical role of Ecto in these events.

D) FAM antagonizes Ecto-mediated ubiquitination of Smad4. Smad4 ubiquitination assay was carrried out as in (E) on Ecto*-reconstituted shEcto-HEK293T cells to monitor Ecto-dependent effects. Smad4 monoubiquitination by Ecto (lane 2) is inhibited by coexpressed FAM (lane 3).

E) FAM is a cytoplasmic protein while Ecto is localized in the nucleus. Panels show western blotting on extracts from the cytoplasmic and nuclear fractions of HepG2 lysates. Histone1 and Tubulin immunoblots served as controls for efficient nuclear and cytoplasmic purifications, respectively.

FIGURE 7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endog. Smad4
Tβ1
HA-Ub + - + +
WB Ub
WB Smad4
B
1st IP
α-Flag
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Flag-Ecto: + +
Smad4: + + +
HA-Smad2: + + +
TGF-β1: +
2nd IP
α-Smad4
Ecto
Smad4
Smad2
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C
wt
shEcto
Ecto* + +
TGF-β + + +
Smad4 + + + + + +
HA-Ub
84 -
IP S4
WB Ub
60 -
IP/WB
Smad4
1 2 3 4 5 6
D
FAM + +
Ecto* + +
Smad4 + + + +
HA-Ub, TGF-β
IP Sm4
WB Ub
IP/WB
Smad4
1 2 3 4
E
Cytoplasm
Nucleus
α FAM
α Ecto
α Smad4
α tubulin
α Histone1

Figure 8: FAM and Ecto operate in the same pathway regulating Smad4.

A-L) FAM and Ecto are antagonistic Smad4 regulators during *Xenopus* early development.

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 dorso-anterior marker *Otx2* (at neurula stages).

Loss-of-Smad4 by injection of XSmad4-morpholino antisense oligonucleotides (Xsmad4-MOs totaling 60ng/embryo) or by overexpressed *dominant-negative Smad4* mRNA (*DN-Smad4*) inhibits endogenous Nodal/TGFβ signaling, as indicated by reduced *Xbra* staining (**B**), and BMP signaling, as shown by reduction of ventral (**F**) and expansion of dorsal (**J**) tissues.Loss-of-FAM (**C, G, K**) and Gain-of-Ecto (**D, H, L**) as attained, respectively, by morpholino (FAM-MO 120ng/embryo) or *Ecto* mRNA (100pg/embryo) microinjections, antagonize TGFβ (*Xbra* down) and BMP signaling (*Sizzled* down and *Otx2* up), phenocopying loss-of-Smad4.

M-R) Ecto works as BMP inhibitor in the fly wing and is antagonized by Fat-facets, the FAM/Usp9x homolog.

M) enlargement of a wing from wild-type *Drosophila*. acv: anterior cross-vein. pcv: posterior cross-vein. L5: the posterior-most longitudinal vein.

N) mutants for *gbb*, a BMP ligand, display missing cross-veins and L5 vein truncation.

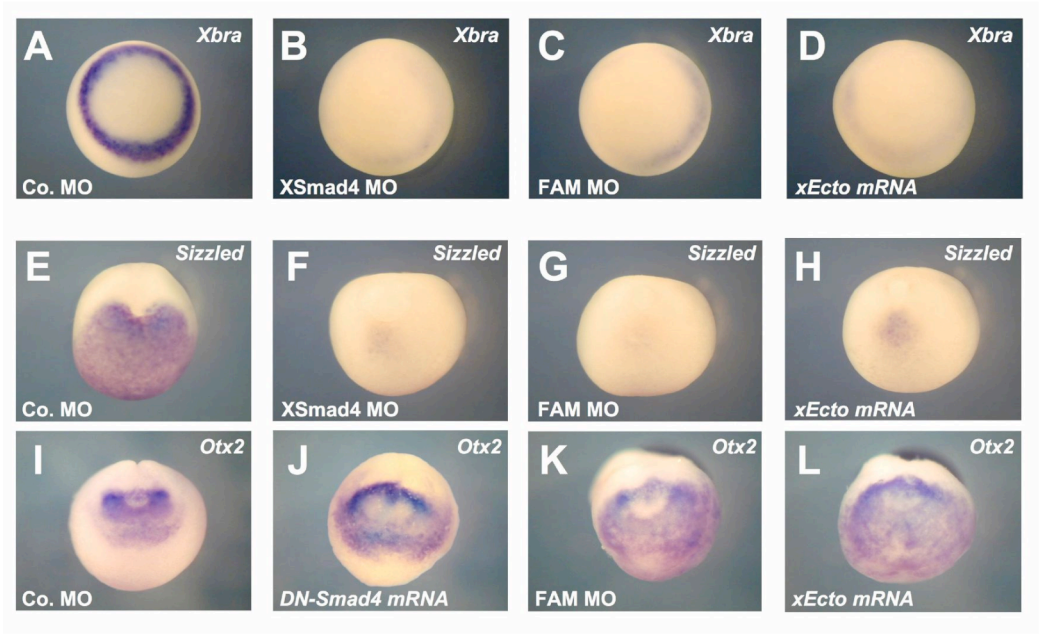
O-P) ectopic expression of Ecto (**O**), but not of the Ecto RING mutant (Ecto-CAmut - **P**), in the wing primordium causes loss of the cross-veins and L5 truncation, phenocopying loss of *gbb/*BMP signaling (compare with **N**).

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) FAM-MO dowregulates endogenous FAM protein in *Xenopus* embryos. Embryos were injected in the animal pole at the 2-cell stage with Control- or FAM-MO, cultivated until the gastrula stage (10+), and explanted of their animal halves. Western blotting revealed depletion of endogenous FAM.

FIGURE 8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Co. MO
B
Xbra
XSmad4 MO
C
Xbra
FAM MO
D
Xbra
xEcto mRNA
E
Sizzled
Co. MO
F
Sizzled
XSmad4 MO
G
Sizzled
FAM MO
H
Sizzled
xEcto mRNA
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Co. MO
J
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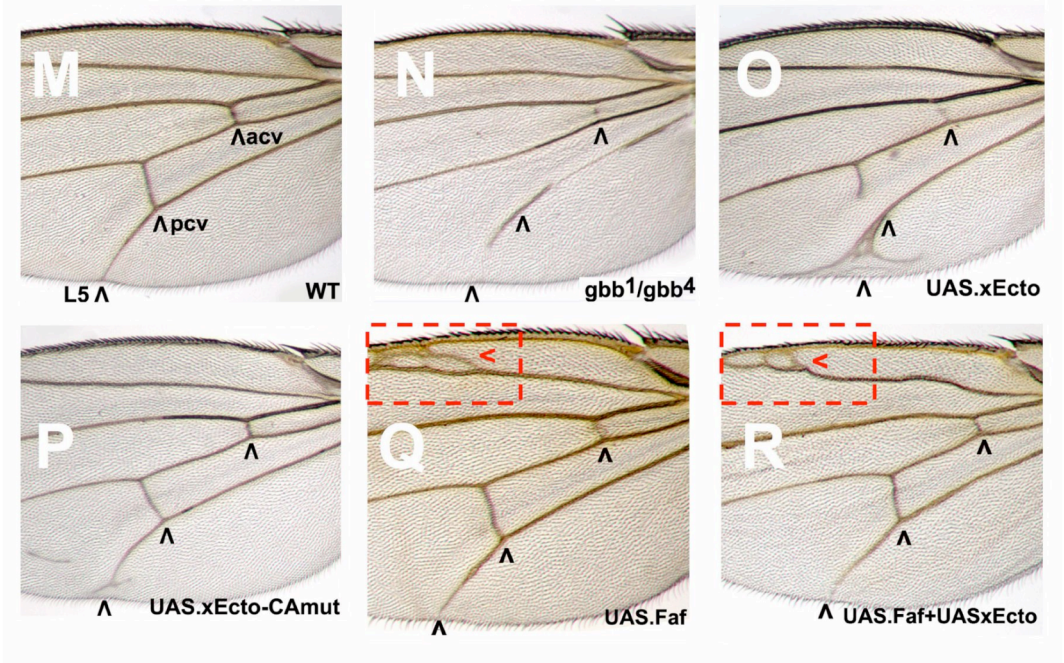
DN-Smad4 mRNA
K
Otx2
FAM MO
L
Otx2
xEcto mRNA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Λacv
Λpcv
L5 Λ
WT
N
Λ
Λ
Λ
gbb1/gbb4
O
Λ
Λ
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UAS.xEcto
P
Λ
Λ
Λ
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Λ
Λ
UAS.Faf+UASxEcto

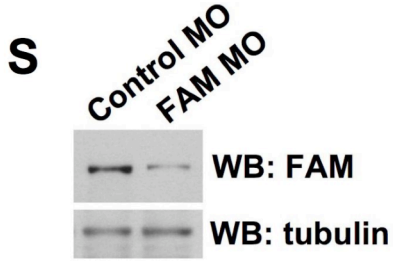

S
Control MO
FAM MO
WB: FAM
WB: tubulin

Figure 9: A model exemplifying the relationships between FAM and Ecto as predicted if they act by oppositely regulating the same process, namely, Smad4 ubiquitination.

A) in normal cells, Smad4 ubiquitination by Ecto is balanced by FAM-mediated deubiquitination.

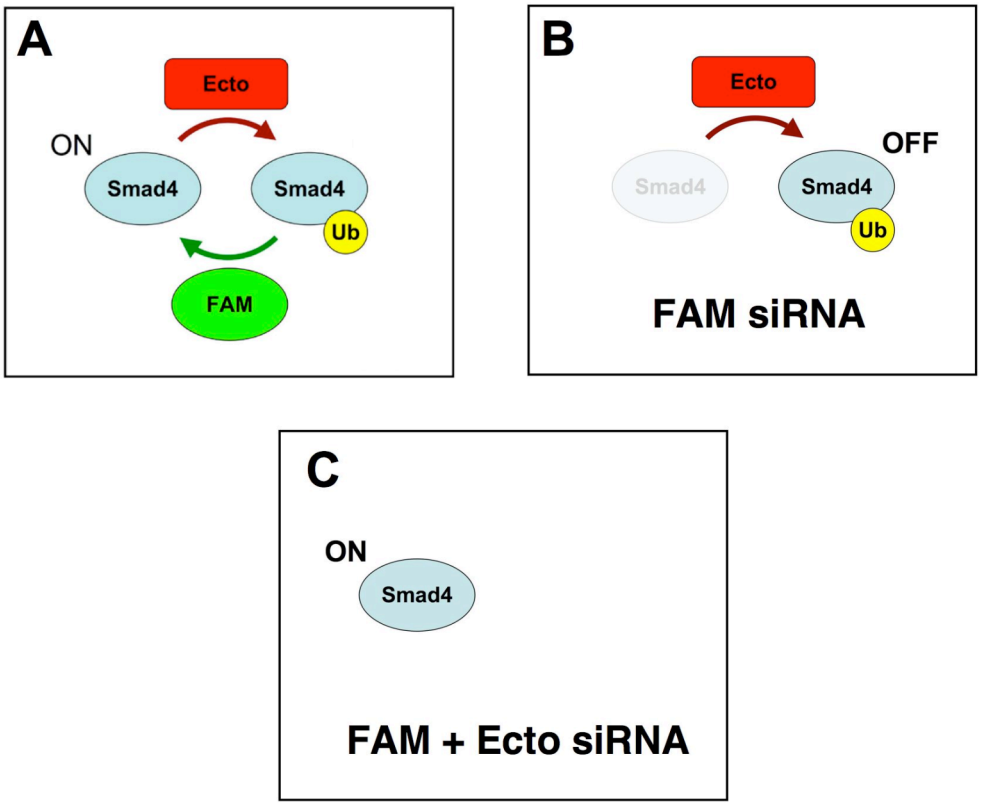
B) in cells lacking FAM, Ecto is left unopposed to ubiquitinate Smad4, leading to Smad4 inactivation.

C) in cells lacking both Ecto & FAM, and thus lacking ubiquitinated Smad4, the activity of FAM is irrelevant for Smad4 activity, and Smad4 is unopposed.

D-E) Ecto is epistatic to FAM. Panels in (**D**) show immunoblots of HaCaT cells transfected with the indicated combinations of siRNA (single siRNA: 7pmol/cm2; double siRNA: 7pmol/cm2+3pmol/cm2 siEcto), untreated or treated (+) with 1ng/ml TGFβ1 for 6 hours, and probed for $p21^{Waf1}$ induction. At this dose, the effect of TGFβ on $p21^{Waf1}$ is at plateau, and cannot be further increased by loss-of-Ecto (compare with 6B). Lanes 3-4: depletion of FAM inhibits TGFβ. Lanes 5-6: cells depleted of both FAM and Ecto regain TGFβ responsiveness. Lanes 7-8: depletion of Smad4 inhibits TGFβ. Lanes 9-10: loss of Ecto does not regain TGFβ responsiveness in Smad4 depleted cells. Lanes 11-14: fostering TGFβ signaling upstream of Smad4 by knockdown of TGFβ receptor inhibitors (Smad7 and Smurfs), cannot rescue FAM depletion.

E) Ecto biological function is required downstream of FAM, as revealed by TGFβ induced migration assays in MDA-MB231 cells.

FIGURE 9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Ecto
ON
Smad4
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Ub
FAM
B
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OFF
Smad4
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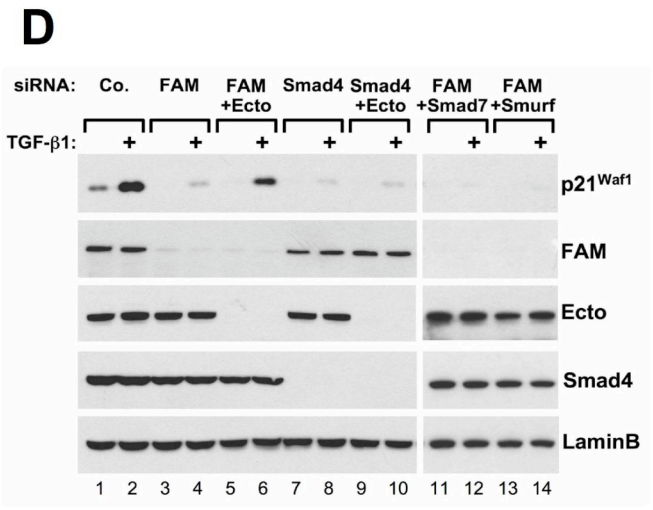
Ub
FAM siRNA
C
ON
Smad4
FAM + Ecto siRNA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FAM
Ecto
Smad4
LaminB
1 2 3 4 5 6 7 8 9 10 11 12 13 14

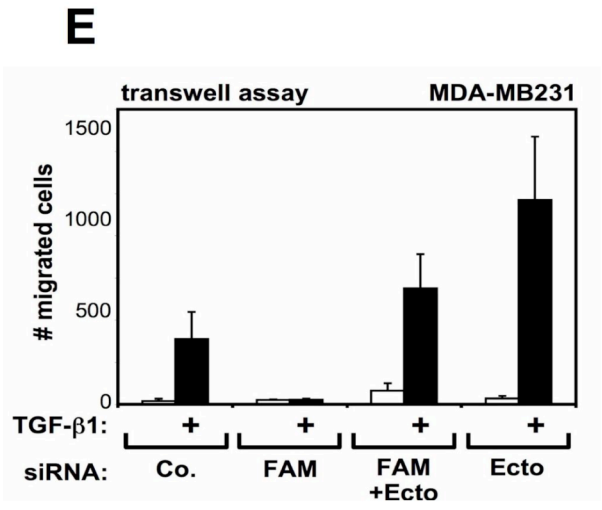

E
transwell assay
MDA-MB231
migrated cells
1500
1000
500
0
TGF-β1: + + + +
siRNA: Co. FAM FAM +Ecto Ecto

Figure 10: Smad4 ubiquitination at lysine 519 inhibits RSmad/Smad4 binding.

A) Mapping of the ubiquitinated lysine on Smad4. HEK293T cells were transfected with plasmids encoding for HA-Ub and a series of Smad4 mutants carrying lysine-arginine substitutions. K-all bears all the lysines mutated. MH1-KR and MH2-KR bear all the lysines of the MH1 or of the MH2 domain mutated. Mutants A-F carry mutations in groups of neighboring lysines (indicated in the diagram on top). Western blotting of immunoprecipitated Smad4 mutants indicates that Smad4 monoubiquitination targets one of the two residues mutated in the F mutant, lysine 507 (K507) or lysine 519 (K519).

B) Lysine 519 is required and sufficient for Smad4 monoubiquitination. Mutation of K519, but not of K507, downregulates Smad4 monoubiquitination. Moreover, reconstitution of K519 in an otherwise mutated MH2-KR background (MH2-KR-519wt) restores monoubiquitination.

C) Ecto ubiquitinates Smad4 on lysine 519. In vivo Smad4 ubiquitination assay in shEcto-HEK293T cells. Mutation of K519 abolishes Ecto-induced Smad4 ubiquitination.

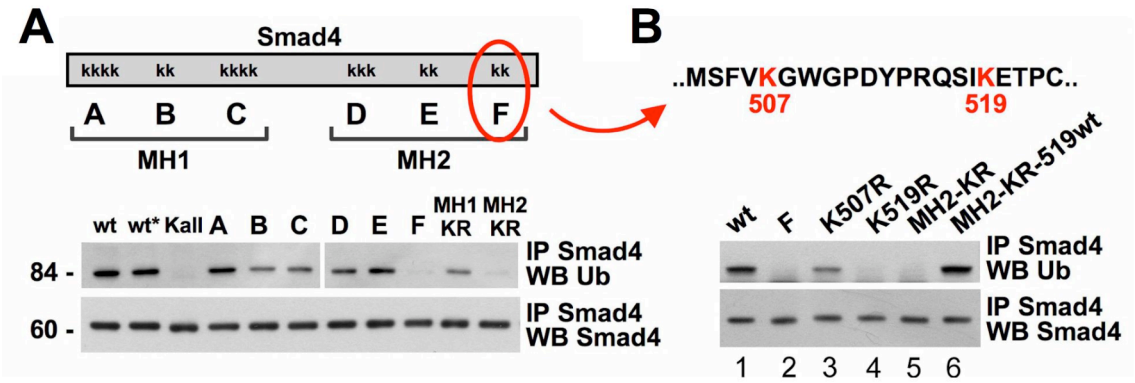
D) Model of the heterotrimeric RSmad/Smad4 complex, formed by the MH2 domains of two RSmad (A and C) and of Smad4 (B) subunits (Chacko et al., 2004). Note how Smad4 is involved in two different interactions, entailing the AB and the BC interfaces. P indicates the phosphorylated c-terminal portion of RSmads. Lysine 519 falls near to the BC interface.

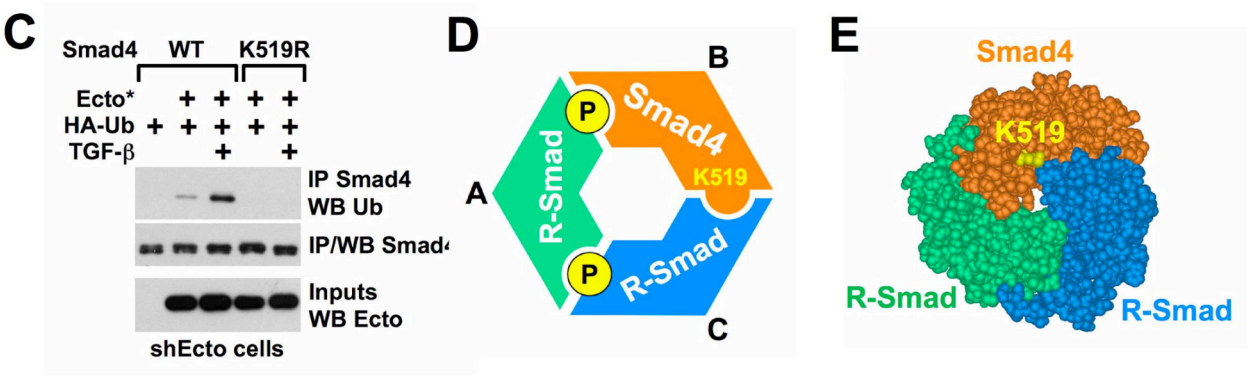
E) Crystallographic structure of the heterotrimeric RSmad/Smad4 complex as in (Chacko et al., 2004). Lysine 519 side chain is higlighted in yellow.

F) Modeling of Smad4 monoubiquitinated on lysine 519. 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 RSmad.

G-H) Ubiquitinated Smad4 is unable to bind Smad3-MH2 domain or phospho-Smad2. Purified non-ubiquitinated Smad4 and monoubiquitinated Smad4 (Ub-Smad4) were compared for the ability to bind recombinant Smad3-MH2 (G) or recombinant phospho-Smad2 (H). Ub/wt 1:1 is a preparation of Ub-Smad4 containing similar amounts of non-ubiquitinated Smad4 as contaminant.

FIGURE 10


A
Smad4
kkkk kk kkkk kkk kk kk
A B C D E F
MH1
MH2
wt wt* KallA B C D E F MH1 KR MH2 KR
84 -
60 -
IP Smad4
WB Ub
IP Smad4
WB Smad4
B
..MSFVKGWGPDYPRQSIKETPC..
507
519
wt F K507R K519R MH2-KR MH2-KR-519wt
IP Smad4
WB Ub
IP Smad4
WB Smad4
1 2 3 4 5 6


C
Smad4 WT K519R
Ecto* + + + +
HA-Ub + + + + +
TGF-β + +
IP Smad4
WB Ub
IP/WB Smad4
Inputs
WB Ecto
shEcto cells
D
A
B
C
P
R-Smad
Smad4
K519
P
R-Smad
E
Smad4
K519
R-Smad
R-Smad

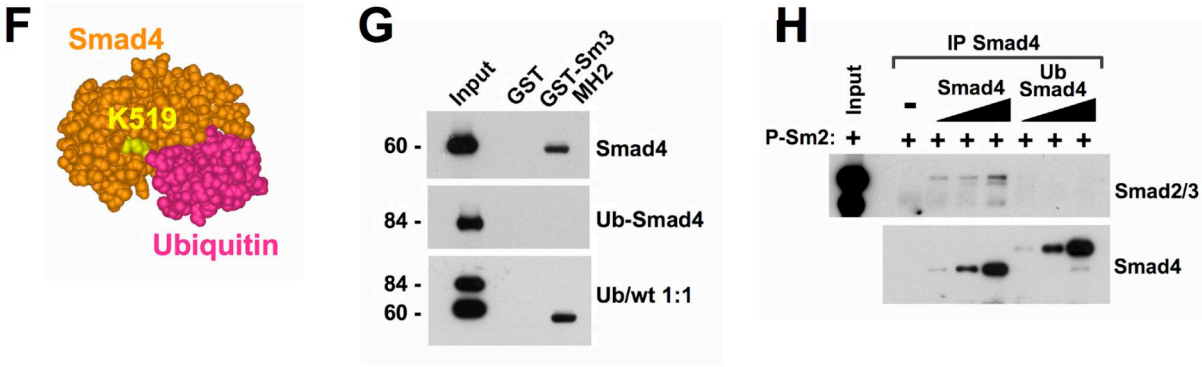

F
Smad4
K519
Ubiquitin
G
Input
GST
GST-Sm3 MH2
60 -
84 -
84 -
60 -
Smad4
Ub-Smad4
Ub/wt 1:1
H
IP Smad4
Input
Smad4
Ub Smad4
P-Sm2: + + + + + + + +
Smad2/3
Smad4

Figure 11: Monoubiquitination of Smad4 affects RSmad/Smad4 complex in vivo

A) Raising Smad4 monoubiquitination antagonizes endogenous Smad2/Smad4 complex. HEK293T cells were transfected with plasmids encoding for HA-Ub together with empty vector (Co.) or with plasmids encoding for Ecto isoforms. After overnight starvation, cells were either left untreated (-) or treated (+) for 2,5 hours with 0,5ng/ml TGFβ1, and harvested for immunoprecipitation. Note how the inhibitory activity of Ecto toward the Smad2/4 complex depends on integrity of the RING-finger domain (lacking in Ecto-ΔTRIM and Ecto-CAmut).

B) Knockdown of FAM destabilizes nuclear Smad2/4 complexes. Immunofluorescence (IF) for endogenous Smad4. Upon TGFβ stimulation, phospho-RSmads accumulate in the nucleus, leading, in turn, to Smad4 nuclear accumulation (note the absence of nuclear Smad4 in cells lacking Smad2/3). In cells lacking FAM, Smad4 is not retained in the nucleus, in line with decreased ability to bind Smad2. Bottom: quantification of the IF stainings and controls of effective knockdowns.

C) FAM sustains TGFβ signaling acting through lysine 519 deubiquitination.

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

FIGURE 11

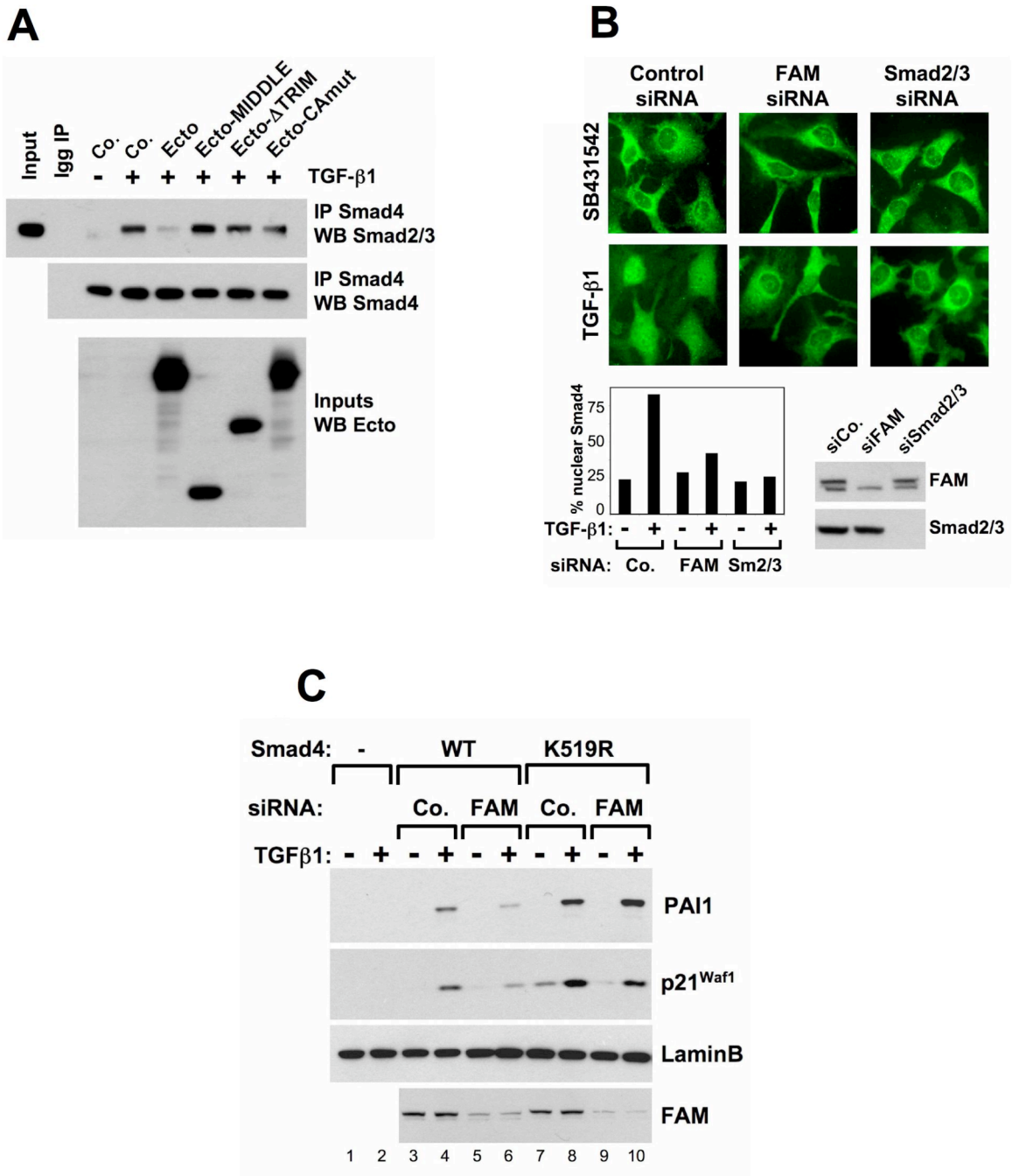

A
Input
Igg IP
Co.
Co.
Ecto
Ecto-MIDDLE
Ecto-ΔTRIM
Ecto-CAmut
- + + + + +
TGF-β1
IP Smad4
WB Smad2/3
IP Smad4
WB Smad4
Inputs
WB Ecto
B
Control siRNA
FAM siRNA
Smad2/3 siRNA
SB431542
TGF-β1
% nuclear Smad4
75
50
25
0
TGF-β1: - + - + - +
siRNA: Co. FAM Sm2/3
siCo.
siFAM
siSmad2/3
FAM
Smad2/3
C
Smad4: - WT K519R
siRNA: Co. FAM Co. FAM
TGFβ1: - + - + - + - + - +
PAI1
p21Waf1
LaminB
FAM
1 2 3 4 5 6 7 8 9 10

Figure 12: Trimeric complex of Ecto with Smad4 and Smad2

A-B) Ecto/Tif1γ can bind Smad2 independently from Smad4. The amounts of Smad2 co-purifying with Ecto were compared in the presence and absence of Smad4 protein. To this end, anti-Ecto immunoprecipitations were carried-out either from MDA-MB231 stably depleted with Smad4-shRNA (A) or from Smad4-null HT29 cells, reconstituted by Smad4 plasmid transfection (B). In both cases, receptor-activated Smad2 interacts with Ecto irrespective of the presence of Smad4 and, *vice versa*, Smad4 bind to Ecto in unstimulated cells, i.e., in the absence of phospho-Smad2. Of note, we could not observe increased Smad2 binding in the absence of Smad4, suggesting the potential for independent associations to Ecto. Asterisk: non-specific Igg band.

C) A model for the dynamic equilibrium between the different pools of Ecto/Smad4/Smad2 complexes. Under basal conditions, Ecto binds Smad4. Upon signaling, the assembly of the ternary complex fosters Ecto ligase activity, leading to inhibition of Smad2/4 complex formation. It is plausible that upon ubiquitination, Smad4 may also lose affinity for Ecto, as the MH2 domain is also used to contact Ecto. This would shift the balance toward Ecto/Smad2 complexes. Perhaps, in some experimental conditions, such as sustained signaling or specific cellular contexts (He et al., 2006), the formation of the Ecto/Smad4 complex might be transient. In contrast, FAM binds the MH1-linker and indeed can associate with Ub-Smad4 (Figure 4D).

D) Ecto/Tif1γ interactions with Smad4 and Smad2. HaCaT cell extracts, untreated or treated with TGFβ1, were immunoprecipitated with anti-Ecto antibodies to directly compare its interactions with endogenous Smad4 (lanes 1-3) or with endogenous Smad2 (lanes 10-12). Smad2/Smad4 complex formation was used as a reference. Ecto/Tif1γ interacts with Smad4 similarly to Smad2 (lanes 3 and 6), while it has much lower affinity than Smad4 for Smad2 (compare lanes 9 and 12). The amount of Smad2 bound to Ecto corresponded to 2-3% of the input. Asterisk: non specific Igg band.

FIGURE 12

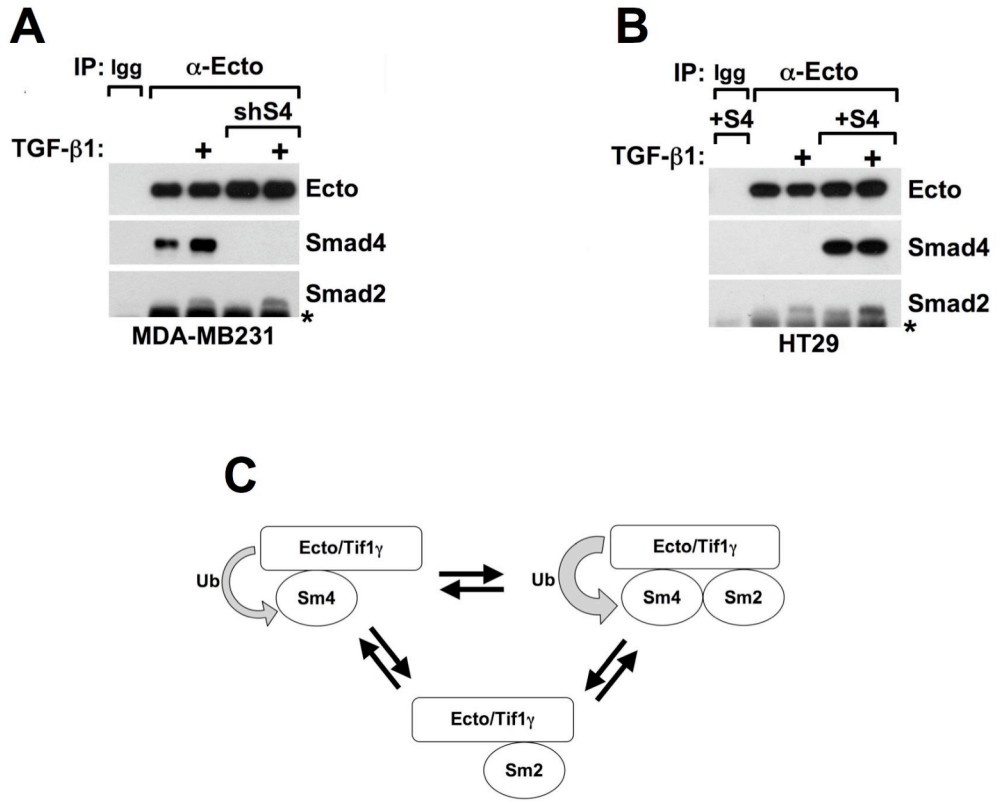
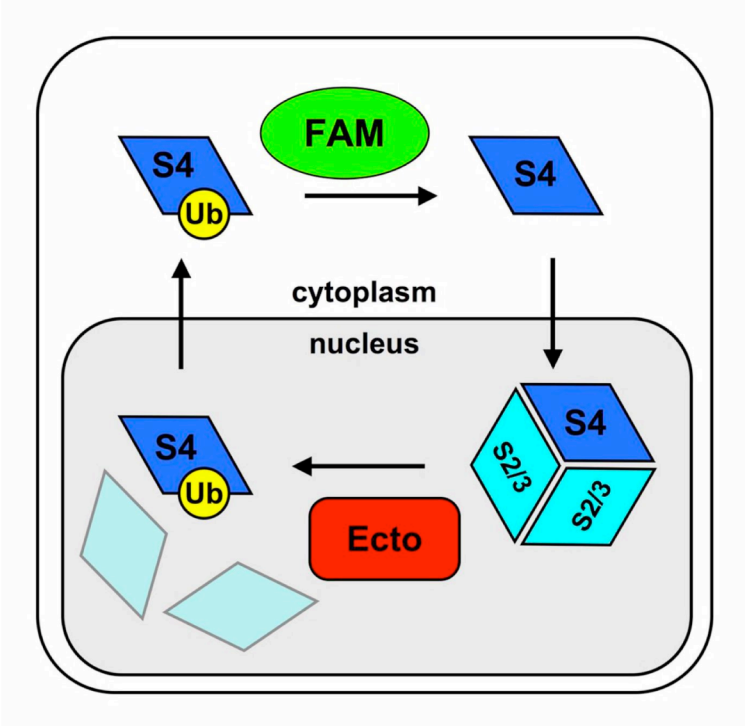

A
IP: Igg
α-Ecto
shS4
TGF-β1:
+
+
Ecto
Smad4
Smad2
*
MDA-MB231
B
IP: Igg
α-Ecto
+S4
+S4
TGF-β1:
+
+
Ecto
Smad4
Smad2
*
HT29
C
Ecto/Tif1γ
Ub
Sm4
Ecto/Tif1γ
Ub
Sm4
Sm2
Ecto/Tif1γ
Sm2
D
IP: Igg Ecto Igg Sm2
TGF-β1: - + - +
Smad4
*
1 2 3 4 5 6
Ecto
Smad2
IP: Igg Sm4 Igg Ecto
TGF-β1: - + - +
Smad2
7 8 9 10 11 12
Smad4
Ecto

Figure 13: A model for Smad4 monoubiquitination turnover mediated by FAM and Ecto.

FIGURE 13



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