UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Istologia, Microbiologia e Biotecnologie Mediche – Sezione di Istologia ed Embriologia

SCUOLA DI DOTTORATO DI RICERCA IN : BIOMEDICINA
CICLO XXIV

USP15 regulates receptor-activated Smads by opposing a regulative monoubiquitylation

Direttore della Scuola : Ch.mo Prof. Giorgio Palù

Supervisore : Ch.mo Prof. Stefano Piccolo

Dottorando : Andrea Manfrin

INDEX

ABSTRACT

The TGFβ pathway is critical for embryonic development and adult tissue homeostasis. Upon ligand stimulation, the TGFβ/BMP receptors phosphorylate the Receptor-activated Smads (R-Smads), which then associate with Smad4 to form a transcriptional complex that regulates gene expression through site-specific DNA recognition. Several ubiquitin ligases serve as inhibitors of R-Smads, yet no deubiquitylating enzymes (DUBs) for these molecules have so far been identified. This contributed to leave unexplored the possibility that ubiquitylation of R-Smads is reversible and engaged in regulating Smad function, in addition to degradation. Here we identify USP15 as a DUB for R-Smads. USP15 is required for TGFβ and BMP gene responses and biological effects in mammalian cells and *Xenopus* embryos. At the biochemical level, USP15 primarily opposes regulative ubiquitylation of R-Smads, that hits their DNA binding domain and is incompatible with promoter recognition. As such, USP15 is critical for the occupancy of endogenous target promoters by the Smad complex. These data identify a new layer of control by which the ubiquitin system regulates TGFβ biology.

ABSTRACT (ITALIANO)

La via di segnale TGFβ svolge un ruolo critico durante lo sviluppo embrionale e nell'omoestasi dei tessuti nella vita adulta. In seguito alla stimolazione da ligando i recettori per TGFβ/BMP fosforilano le R-Smads che si associano così a Smad4 per formare un complesso trascrizionale in grado di regolare l'espressione genica mediante il riconoscimento di specifiche sequenze di DNA. Molteplici ubiquitina-ligasi agiscono come inibitori delle R-Smads, ma ad oggi non sono ancora stati identificati enzimi deubiquitinanti (DUBs) per queste molecule. Questo lascia aperta la possibilità che l'ubiquitinazione delle R-Smads sia reversibile e che possa essere coinvolta nella regolazione delle funzioni delle R-Smads con modalità diverse dalla degradazione. In questo lavoro USP15 è stata identificata come una DUB per le R-Smads. USP15 è richiesta per le risposte geniche indotte da TGFβ e BMP e per gli effetti biologici mediati da questi segnali in cellule di mammifero e in embrioni di *Xenopus laevis*. A livello biochimico USP15 principalmente si contrappone ad una forma regolativa di ubiquitinazione delle R-Smads, la quale inibisce la loro capacità di legarsi al DNA ed è quindi incompatibile con il riconoscimeto del promotore. Tramite questo meccanismo USP15 è fondamentale nel determinare la persistenza del complesso delle Smads sui promotori dei geni endogeni. Questi risultati evidenziano un nuovo meccanismo con cui l'ubiquitina regola gli effetti biologici di TGFβ e BMP.

PUBLICATIONS

Martello, G., Rosato, A., Ferrari, F., **Manfrin, A.**, Cordenonsi, M., Dupont, S., Enzo, E., Guzzardo, V., Rondina, M., Spruce, T., Parenti, A.R., Daidone, M.G., Bicciato, S., and Piccolo, S. (2010): A microRNA targeting Dicer for metastasis control. Cell 141, 1195-1207.

Inui, M., **Manfrin, A.**, Mamidi, A., Martello, G., Morsut, L., Soligo, S., Enzo, E., Moro, S., Polo, S., Dupont, S., Cordenonsi, M., and Piccolo, S. (2011): USP15 is a deubiquitylating enzyme for receptor-activated SMADs. Nature Cell Biology 13, 1368-1375.

This was realized with the main contributions from the colleague Dr. Masafumi Inui. I primarily contributed to the biochemistry and gene expression studies. Dr. Stefano Moro carried out the modelling analysis. Dr. Simona Polo prepared recombinant E3-ligases.

INTRODUCTION

Biological relevance of TGFβ and BMP signaling

Transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) pathways are well conserved during evolution. They regulate diverse cellular functions such as growth, adhesion, migration, apoptosis, and differentiation (Wu and Hill, 2009). Through these functions, TGFβ and BMP signaling are essential for embryonic development, especially in germ-layer specification and pattern formation during embryogenesis, and for adult tissue homeostasis (Schmierer and Hill, 2007; Morsut et al., 2010). As such, the dysregulation of these pathways has been linked to pathological conditions such as fibrosis, wound-healing disorders, several hereditary conditions and cancer (Schmierer and Hill, 2007).

TGFβ and BMP pathways

The TGFβ superfamily of ligands contains over 30 members including TGFβs, BMPs, growth and differentiation factors (GDFs), Activins, and Nodal (Wu and Hill, 2009). The process of signal transduction from membrane-bound receptors into the nucleus is surprisingly straightforward for the TGFβ and BMP cascades (Schmierer and Hill, 2007). The ligands bind to type II and type I serine-threonine kinase receptors, forming a complex in which type II phosphorylates and activates type I receptor. This, in turn, phosphorylates the receptor-activated Smads (R-Smads) inducing R-Smads to complex with Smad4 and to accumulate into the nucleus. Once in the nucleus, R-Smad/Smad4 active transcriptional complexes regulate the expression of the target genes by binding to DNA directly either alone or in concert with other transcription factors (Shi and Massaguè, 2003; Hill, 2009). Both TGFβ and BMP pathways follow this scheme, only that ligands of the TGFβ branch (TGFβs, Activins and Nodal) activate type I receptors different from those activated by the BMP branch (BMPs and GDFs). Also, different R-Smads serve the BMP and TGFβ branches; Smad1, Smad5 and Smad8 are used by BMP, while Smad2 and Smad3 are used by TGFβ pathway. These two groups

of R-Smads recognize different promoter elements thus regulating distinct sets of genes (Fig. 1a; Schmierer and Hill, 2007).

The biological consequences of TGFβ/BMP stimulation are strictly dependent on strength and duration of the signal; thus, mechanisms that oppose or terminate signaling are just as relevant as those initiating the pathway (Itoh and ten Dijke, 2007). The proposed mechanisms for signal termination are the dephosphorylation of activated receptors and R-Smads (Lin et al., 2006, Shi et al., 2004) or their degradation through ubiquitylation (Ebisawa et al., 2001, Kavsak et al., 2000).

The ubiquitin system

Ubiquitin is a conserved 76-residue polypeptide that fulfills essential functions in eukaryotes through its covalent conjugation to the lysine residues of intracellular proteins and receptors. Conjugation of ubiquitin molecules to the substrate requires the sequential actions of three enzymes: an activating enzyme (E1) that forms a thiol ester thereby activating the C-terminus of ubiquitin, a conjugating enzyme (E2) that transiently carries the activated ubiquitin molecule and a ligase (E3) that transfers the activated ubiquitin to the lysine residue of the substrate (Pickart, 2001). Ubiquitylation comes with different flavors. Polyubiquitylation means that a lysine residue of the substrate is conjugated with a chain of ubiquitin moieties while with monoubiquitylation only a single molecule of ubiquitin is conjugated to the lysine. Ubiquitin posses seven lysine residues to which other ubiquitin molecules can be bound, thus forming chains with different structure and branches (Ikeda and Dikic, 2008). The prevailing role for ubiquitylation is the proteasome-dependent degradation of the conjugated substrate, which relies on polyubiquitin chains built on lys-48 of ubiquitin. In contrast other forms of polyubiquitylation (i.e. branching on lys-63) act as a regulative type of modification for example modifying the activity of the substrate (Iwai and Tokunaga, 2009; Ikeda and Dikic, 2008).

Also monoubiquitylation works as a regulative type of modification in a manner that is not dissimilar, at least conceptually, from phosphorylation. Also in this

case the newly-monoubiquitylated protein acquires new characteristics that affect its activity, cellular localization and interaction with other proteins, including ubiquitin-binding proteins (Salmena and Pandolfi, 2007). The relevance of monoubiquitylation has been reported in protein trafficking (Ahearn et al., 2012), in DNA-repair mechanism (Yan et al., 2009) and in the regulation of signaling pathways (Da Silva-Ferrada et al., 2011; Dupont et al., 2009).

Regulation of TGFβ and BMP pathways by the ubiquitin system

Several reports demonstrated an important role for ubiquitylation in the regulation of TGFβ and BMP pathways (Fig. 1a). It has been reported that E3-ligases Smurf1, Smurf2 and Nedd4-L polyubiquitylate TGFβ receptors and R-Smads, causing their degradation, thus terminating the signaling (Ebisawa et al., 2001; Kavsak et al., 2000; Lo and Massaguè, 1999; Kuratomi et al., 2005). Although it is true from a biochemical standpoint that these E3-ligases polyubiquitylate R-Smads, the degradation of R-Smads as sole mechanism for signal termination can be hardly reconciled with other equally valid and reproducible observations, such as the apparent stability of nuclear R-Smads and their continuous shuttling in and out of the nucleus (Inman et al., 2002). This suggests that ubiquitylation may well occur, but that its role in signal termination is primarily regulative rather than degradative. An example of such a kind of mechanism has been reported for another component of TGFβ/BMP pathway: Smad4 monoubiquitylation results in the disruption of Smad4/R-Smads complex, leading to signal termination (Dupont et al., 2009). If a similar regulation exists also for R-Smads is not known.

Deubiquitylating enzymes

One of the main appeals of monoubiquitylation, as mean to regulate protein function, is that it is a reversible modification mediated by deubiquitylating enzymes (DUBs), that are the proteases responsible for deubiquitylation (Amerik and Hochstrasser, 2004). Informatic analysis indicated that the human genome encodes approximately 95 putative DUBs, divided in five subclasses according to the homology of their ubiquitin-protease domain. DUBs target specific substrates and their biological activity is intimately linked to the function of the

ubiquitylation they counteract (Nijman et al., 2005). It is worth noting that the duality between E3s and DUBs allows a direct control on the activity of the protein, for example through a cycle of inhibition and re-activation. This system is more dynamic than degradation and re-synthesis of the same protein (Dupont et al., 2009; Sacco et al., 2010). Such a kind of dynamicity nicely suits the needs of signal transduction pathways, that need to monitor continuously the presence and the intensity of the stimuli. An example for TGFβ and BMP pathways is the cycle of inactivation and re-activation of Smad4 that relies on monoubiquitylation by the E3-ligase Ectodermin/Tif1γ and deubiquitylation by the DUB FAM/USP9x (Fig. 1b) (Dupont et al., 2009). On the basis of this previous mechanism centered on Smad4 we asked if other DUBs were involved in the regulation of TGFβ and BMP pathways.

RESULTS

USP15 controls TGFβ and BMP signaling

To identify deubiquitylating enzymes (DUBs) involved in the control of both TGFβ and BMP signaling, we performed an unbiased siRNA screen. Pools of siRNAs targeting 75 known or predicted human DUBs were introduced in HaCaT and MDA-MB-231 (MDA231) cells that had been stably transfected respectively with the TGFβ reporter pCAGA12-lux (HaCaT-CAGA12) or the BMP reporter ID1BRE-lux (MDA231-ID1BRE). Cells were treated with TGFβ1 or BMP2 and then assayed for luciferase activity (Fig. 2a). Out of this screen, only two siRNAs were effective at inhibiting both TGFβ and BMP responses (Fig. 2b). One was FAM/USP9x, which we already knew it was required for TGFβ and BMP signaling through the control of SMAD4 activity (Dupont et al., 2009). The second hit corresponded to USP15, a DUB previously identified as component of the COP-9 signalosome and as regulator of HPV-E6 (Baker et al., 1999; Hetfeld et al., 2005; Vos et al., 2009). The results of this screen suggested a novel role for USP15 in TGFβ and BMP pathways.

USP15 is required for TGFβ and BMP gene responses

To exclude siRNA off-targets effects we confirmed the result of the screen using three independent siRNAs against USP15 in HaCaT-CAGA12 cells (Fig. 2c). Moreover the fact that pCAGA12-lux is a pure Smad-responding reporter (i.e. it does not require other co-factors) suggested that USP15 is critical for Smads activity *per se*. To rule out the possibility of general effects on the transcriptional machinery we also tested USP15 siRNAs on reporters specific for other pathways: serum-induced activation of the SRF-luciferase reporter nor β-catenin-induced transcription of the TOP-flash reporter were affected (Fig. 2d and Fig. 2e).

To address to what extent USP15 is effective for endogenous Smad target genes, we monitored in HaCaT and MDA231 cell lines the expression of the TGFβ target genes $p21^{Waf1}$, PAI1, *cMyc* and of the BMP targets *ID2* and *Smad7* by western blotting or quantitative retro-transcription PCR (qPCR). Smad4

depletion was used as positive control. Loss-of-USP15 curtailed all these TGFβ/BMP gene responses (Fig. 3a, Fig. 3b and Fig. 3c). Of note TGFβ-induced repression of *cMyc* mRNA was blunted by USP15 siRNA in line with the fact that USP15 is not involved in the control of general transcription machinery (Fig. 3d). Collectively, these data suggested a requirement for USP15 in Smad-dependent transcription.

USP15 is required for TGFβ and BMP biological effects

Next, we tested the requirement of USP15 for established TGFβ and BMP biological effects. It is well-documented that TGFβ/Smad signaling results in dramatically different outcomes, depending on the cellular context. This includes the dual role of TGFβ as tumor suppressor in normal epithelial cells, and as promoter of aggressive cell behaviors in tumor cells. For example, TGFβ induces growth arrest in epithelia, but is a potent inducer of cell migration in cancer cells (Akhurst and Derynck, 2001). If USP15 targets Smad activity, then it should be required for both types of responses. We first tested the requirement of USP15 for Smad-dependent growth arrest in HaCaT keratinocytes. Cells were transfected with control or USP15 siRNAs, treated for 24 hours with or without TGFβ1, and then assayed for BrdU incorporation. As positive control, cells were transfected with Smad4 siRNAs. As shown in Fig. 4a, TGFβ treatment inhibited S-phase entry in control cells but this response was blunted upon depletion of USP15. As for TGFβ-induced cell migration, we monitored by a scratch-assay the motility of MDA231 breast cancer cells (Dupont et al., 2009). In response to TGFβ, cells depleted of USP15 or Smad4 displayed a reduced migratory capacity compared to control MDA231 cells (Fig. 4b).

To test BMP-mediated biological effects we took advantage of multipotent mesenchymal stem cells which differentiate into osteoblasts in a BMP-dependent manner (Miyazono et al., 2004). Human mesenchymal stem cells (hMSC) were transfected with two independent USP15 siRNAs. BMP2 treatment increased the expression of the early osteoblast marker alkaline phosphatase, but depletion of USP15 prevented this induction (Fig. 4c). Taken together, these results suggest

that USP15 is a novel critical factor for TGFβ and BMP signaling in mammalian cells. Finally, we wanted to assay the role of USP15 *in vivo*. During *Xenopus laevis* embryonic development, TGFβ and BMP ligands induce and pattern the embryonic germ layers (Niehrs, 2004; De Robertis et al., 2000). TGFβ/Nodal signals are relevant for inducing mesodermal fates (marked by expression of the *Xbra* gene), with highest phospho-Smad2 levels being required on the dorsal side of the embryo for induction of the Spemann Organizer (defined by *Chordin* expression); at slightly later stages, BMP activity peaks on the ventral side of the embryo (turning on targets such as *Sizzled*). As USP15 is expressed in early embryos (data not shown), we hypothesized a role of USP15 in these processes. To test this, embryos were injected at the 4-cell stage with antisense morpholinos oligonucleotides (MO) targeting *XUSP15* and its closely homologue *XUSP4*. Morphant embryos displayed severe inhibition in the expression of the *Xbra*, *Chordin* and *Sizzled* markers, ultimately causing gastrulation failure (Fig. 4d and Fig. 4e). These effects were reverted by overexpression of Smad1 or Smad3 suggesting that they were due to attenuation of Smads activity (Fig. 4d and Fig. 4e).

USP15 interacts with R-Smads

Next we aimed to better understand the mechanisms by which USP15 sustains TGFβ/BMP signaling. To assess if USP15 activity depends on its deubiquitylating function, we compared wild-type USP15 and a mutant USP15 (CA-mut, carrying a point mutation in one of the key residues of its catalytic domain; Nijman et al., 2005) for capacity to foster Smad2/3 activity in cells transfected with the pCAGA12-lux reporter. As shown in Fig. 5a, only wild-type USP15 could enhance TGFβ responsiveness, indicating that USP15 indeed acts as a deubiquitiylating enzyme in this pathway. What is the substrate of USP15? Since USP15 requirement for TGFβ and BMP effects is maintained in such diverse contexts as different as mammalian cell lines and *Xenopus laevis* embryos, it likely regulates one of the conserved core components of these pathways. We could exclude an activity of USP15 on TGFβ receptors, since the levels of total-

and phosho-Smad2/3 were not significantly affected in USP15-depleted, or USP15-overexpressing cells (Fig. 5b and data not shown). This suggests that USP15 operates downstream of R-Smads activation by receptors. We tested if USP15 directly targets Smad function, first by analyzing if USP15 and Smads could physically interact with each other. By co-immunoprecipitation (Co-IP) and western blots, Flag-tagged Smad1, Smad2, Smad3 and Smad4 all interacted with V5-tagged USP15 (V5-USP15; Fig. 5c). To test if this occurs also at physiological levels of these proteins, we performed Co-IP assays from lysate of HaCaT cells and found that USP15 indeed forms a complex with Smad2/3 (Fig. 5d). Conversely, we have been unable to detect an interaction between Smad4 and USP15 at the endogenous protein levels, suggesting that Smad4 is not a primary endogenous target of USP15 (data not shown). We then tested if the interaction between USP15 and R-Smads is direct. For this, GST-Smad3 was purified from bacteria and V5-USP15 was affinity purified to homogeneity from transfected HEK293T cells. As shown in Fig. 5e, the two proteins formed a direct complex and - based on GST-Smad3 deletion mutants - the USP15 binding domain was localized to the Smad3 MH1-linker domain (Fig. 5e).

USP15 deubiquitylates R-Smads

Since USP15 is a DUB that binds R-Smads, we hypotesized that it could deubiquitylate R-Smads. First we decided to analyze the ubiquitylation pattern of R-Smads. To this end, HEK293T cells were transfected with expression plasmids encoding for HA-ubiquitin (HA-Ub) and Flag-tagged Smad1 or Smad3; Smad proteins were then affinity purified and their ubiquitylation pattern visualized by immunoblotting against HA-ubiquitin. Monoubiquitylation appeared as a major modification of Smad1 and Smad3 (Fig. 6a, lanes 2 and 4), the latter also revealing a robust di-ubiquitylation band. The same ubiquitylation pattern of R-Smads was visualized when the same experiments were repeated under stringent immunoprecipitation conditions (i.e., boiling of the extract in SDS before immunoprecipitation, a set-up that avoids confounding background from ubiquitylated-proteins co-precipitating with SMADs); moreover, bands appearing positive with anti-HA (ubiquitin) in immunoblots were also positive after re-

probing with anti-Flag antibody (R-Smad) (data not shown). Of note, monoubiquitylation was a major modification of R-Smads also in other cell lines tested (i.e., HepG2, MEFs) and in *Xenopus* embryos (data not shown). Interestingly polyubiquitylated forms of Smad1 and Smad3 were less abundant and often only visible after co-transfection of the ubiquitin ligase Smurf1 (Fig. 6a, lane 3) (Zhu et al., 1999; Fuentealba et al., 2007; Sapkota et al., 2007). Yet, the presence of Smurf1 also robustly increased mono- and di-ubiquitylation.

Overexpression of wild-type USP15, but not of the catalytically inactive USP15 mutants, inhibited R-Smads ubiquitylation (Fig. 6b, Fig. 6c, Fig. 6d and data not shown), and opposed Smurf-induced polyubiquitylation (Fig. 6d). Crucially, loss-of-USP15 enhanced Smad3 or Smad1 mono- and di-ubiquitylation, as well as Smurf-induced polyubiquitylation (Fig. 6e and Fig. 6f). This was observed also on endogenous R-Smads (Fig. 6g), whereas no effect of USP15 knockdown could be detected on Smad4 monoubiquitylation (Fig. 6h). Using affinity-purified proteins, we found that USP15 de-ubiquitylated Smad3 in vitro, suggesting that the enzymatic reaction previously detected in cell lysates was likely direct (Fig. 6i).

Monoubiquitylation regulates R-Smads activity independently from polyubiquitylation/degradation

Previous reports on the link between the ubiquitin system and TGFβ/BMP signaling focused on R-Smad polyubiquitylation and degradation. Because USP15 can regulate Smurf-induced polyubiquitylation, we tested if it may also regulate R-Smad stability. As shown in Fig. 7a, loss-of-USP15 strongly cooperates with Smurf1 overexpression in triggering proteasomal-dependent degradation of Smad1. This suggests that, in some cellular contexts, USP15 can also impinge on Smad stability. That said, under our experimental conditions, R-Smad monoubiquitylation could be uncoupled from polyubiquitylation/degradation: first, the levels of total R-Smads and of TGFβ-activated phospho-Smads are largely unaffected by gain- or loss-of-USP15 (Fig. 5b and data not shown); second, in absence of E3-ligase overexpression, R-Smad are mainly mono- and di-ubiquitylated (Fig. 6a); third, the levels of these isoforms do not accumulate in cells treated with the proteasomal inhibitor MG132 (Fig. 7b), and yet are

regulated by USP15. Taken together, while these results are consistent with a role of USP15 in the well-established regulation of R-Smad stability by polyubiquitylation, at the same time they highlight an unanticipated modality of R-Smad inhibition by the sole monoubiquitylation, that is a regulative type of modification.

Monoubiquitylation of R-Smads occurs in the nucleus

A wealth of evidences indicate that a fundamental layer in the control of Smads activity is their nucleo-cytoplasmic localization: Smads are continuously shuttling between these compartments and ligand stimulation favors nuclear accumulation of the Smad complexes (Hill, 2009). Here we obtained several evidences suggesting that monoubiquitylation occurs primarily, albeit not exclusively, in the nucleus, and that deubiquitylation by USP15 can target both nuclear and cytoplasmic ubiquitylated-R-Smads. First, under basal/unstimulated conditions, nucleo-cytoplasmic fractionation of cell lysates indicated that monoubiquitylated Smad3 is enriched in the nuclear fraction (Fig. 7c); moreover, artificial manipulation of Smad3 nuclear localizing properties - as obtained by engineering Smad3 with ectopic nuclear localization or nuclear export signals (NLS- Smad3 and NES-Smad3 respectively, Fig. 7d and see Experimental Procedures) - revealed that the levels of Smad3 monoubiquitylation follow closely its nuclear distribution (compare in Fig. 7e and Fig. 7f lanes 2 vs. lanes 4). Second, to induce Smad nuclear accumulation in a more physiological manner, HEK293T cells were treated with TGFβ or BMP; as shown in Fig. 7g and Fig. 7h, these treatments promoted monoubiquitylation of R-Smad. This could also be detected on the receptor-phosphorylated pool of R-Smads (Fig. 8a). Third, interestingly TGFβ-induced Smad3 monoubiquitylation was inhibited in Smad4-depleted cells (Fig. 8b) and in cells treated with the transcriptional inhibitors flavopiridol or α-amantin (Fig. 8c). Collectively, these results indicate that R-Smad regulative ubiquitylation is primarily originated in the nucleus; although this event does not absolutely require R-Smad activation, it is clearly induced by Smad assembly into transcriptional complexes.

As for USP15-dependent deubiquitylation, we found that endogenous USP15 could be detected in both nuclear and cytoplasmic fractions (Fig. 8d) and that TGFβ/BMP treatment did not affect USP15 levels or sub-cellular distribution (Fig. 5b, lanes 1 and 2 and data not shown). R-Smad/USP15 interaction occurs in both nucleus and cytoplasm (Fig. 8e) leading, in USP15 overexpressing cells, to an even reduction of ubiquitylated-Smad3 in the two compartments (Fig. 8f). Upon TGFβ-treatment, we could detect a robust association of USP15 with the activated phospho-Smad3 pool (Fig. 8g), and this correlates with a reduction of monoubiquitylated phospho-Smad3 in USP15 overexpressing cells treated with TGFβ (data not shown). Collectively, these results indicate that while monoubiquitylation of R-Smad occurs mainly in the nucleus, deubiquitylation can target cytoplasmic, nuclear and activated Smad pools.

USP15 is required for R-Smads binding to the promoter

Given the above results, we sought to determine how enhanced R-Smads ubiquitylation in USP15-depleted cells inhibits R-Smads activity. We considered several possibilities. We first examined if R-Smad ubiquitylation regulated by USP15 alters nuclear-cytoplasmic shuttling. For this, HaCaT cells were treated with TGFβ for one hour, washed, treated with the TGFβ receptor inhibitor SB505124 and fixed for immunofluorescence at different time points. As shown in Fig. 9a, neither nuclear accumulation nor export dynamics appear overtly affected by USP15 depletion. Moreover, USP15 depletion does not affect the endogenous association between ligand-activated Smad2/3 and Smad4 (Fig. 9b). In the nucleus the activated Smad complexes function as transcription factors by recognizing Smad-binding-elements (SBE, 5'-CAGA-3'). To test if USP15 modulates binding of Smads to DNA, endogenous Smad complexes were affinity selected on biotinylated-SBE oligonucleotides from control or USP15-depleted HaCaT cells. As shown in Fig. 9c, USP15 is crucial for the association of the Smad complex to DNA upon TGFβ stimulation. It is noteworthy that also the DNA interaction of Smad4 is severely affected in USP15-depleted cells (Fig. 9c, second panel), probably reflecting a “lift-off” of the whole Smad complex from

DNA once R-Smads are ubiquitylated. Similar results were obtained by gel-shift assays (data not shown).

If Smad-DNA recognition is the primary event regulated by USP15, then it should be possible to uncouple TGFβ responsiveness from USP15 requirement by forcing Smads to recognize a promoter sequence other than a SBE. To test this idea, we used a GAL4-Smad3 fusion construct that can recognize a UAS-Gal4 enhancer: as shown in Fig. 9d, USP15 depletion (black bars) affects TGFβ responsiveness in cells transfected with the pCAGA-lux reporter (right panel) but does not affect the activity of GAL4-Smad3 on a UAS-lux reporter (left panel). The UAS/GAL4-Smad3 system remains fully dependent on Smad4 for transcriptional activity (data not shown); thus, as specificity control, we checked whether GAL4-Smad3 remained dependent to a different route for Smad inhibition, that is, the weakening of the R-Smad-Smad4 protein-protein interaction through FAM/USP9x depletion (Dupont et al., 2009). Indeed, induction of both pCAGA-lux and UAS/GAL4-Smad3 is FAM-dependent (Fig. 9d, dotted bars). To support the notion that USP15 operates at the level of R-Smad/DNA-recognition, we tested if USP15 regulates the association of the Smad complex with a natural enhancer in which R-Smads do not contact DNA directly. This is the case of the ARE (Activin-Responsive-Element) of the *Xenopus* Mix.2 promoter: this element is sensitive to a Smad2/Smad4 complex in which Smad2 does not bind DNA directly and promoter recognition is provided by FoxH1 (Chen et al., 1996). As shown in Fig. 9e, the binding of Smad2/Smad4 complex to ARE is insensitive to USP15 dosage.

To validate the requirement of USP15 for promoter occupancy *in vivo*, we measured the recruitment of the Smad complex on TGFβ target genes (*PAI-1*, *JunB*, $p21^{Waf1}$) by chromatin immunoprecipitation (ChIP) from HaCaT cell lysates. Stably integrated pCAGA12-lux served as positive control. As shown in Fig. 9f, the recruitment of the TGFβ-activated Smad complex on chromatin was lost upon transfection of USP15 siRNAs. Thus, USP15 is essential to empower transcriptional capacity to Smads.

Monoubiquitylation of R-Smads opposes to DNA binding

The fact that USP15 controls the level of ubiquitylated R-Smads and regulates R-Smads binding to DNA, prompted us to think that monoubiquitylation plays a role in the DNA binding capacity of R-Smads. To corroborate this hypothesis, we decided to map the lysine residues of R-Smads that are targets of monoubiquitylation, and then integrate these data with the structural information available for Smad3 (Shi, 2001). For this, a series of Smad3 mutants bearing lysine-to-arginine substitutions in evolutionary conserved residues were designed and analyzed for monoubiquitylation upon transfection into HEK293T cells. As shown in Fig 10a, mutations in the MH2 domain (KMH2R) had no effect. This observation is consistent with the result shown in Fig. 9b indicating that gain-of-ubiquitylation in USP15-depleted cells does not affect the association of Smad3 with Smad4, an interaction occurring through the MH2 domain. As for the MH1, we screened only single or double mutants and found that mutations in K81 or, to a lesser extent, in K33 and K53, displayed reduced monoubiquitylation levels (Fig. 10a). From a structural perspective, all these lysines are located on the external surface of Smad3; notably, K81 is a key residue in the highly conserved β-hairpin DNA-binding domain of Smads, that forms specific hydrogen bonds with the SBE. K33 is also reported to contact the phosphate backbone of DNA (Chai et al., 2003). Because missense substitutions in K81 or K33 are *per se* sufficient to inhibit sequence-specific DNA binding (see Shi et al., 2001 and Fig. 10b), we reasoned that attachment of an ubiquitin moiety was very likely to generate a similar result. Supporting this notion, we modeled *in silico* the structure of ubiquitylated Smad3 by docking the structure of a single ubiquitin molecule bound to either K81 or K33, and found that this is structurally incompatible with DNA recognition (Fig. 10c and data not shown). To provide direct experimental evidence that ubiquitylation of Smad3 affects its binding to DNA, we used HEK293T cell lysates to purify first Smad3 and then enrich for ubiquitylated-Smad3 (with sequential anti-Flag and anti-HA affinity chromatography and elution). This gave a prep with approximately a 2:1 mix of unmodified vs. monoubiquitylated Smad3 (Fig 10d, input). DNA pull-down with

SBE-oligonucleotides confirmed that only unmodified Smad3 is able to contact DNA (Fig 10d, right panel). These findings suggest that regulative monoubiquitylation tackles Smads at the heart of their transcriptional function.

The facts that a significant proportion of regulative ubiquitylation occurs as a consequence of R-Smad assembly in transcriptional complexes, and that, mechanistically, this event opposes DNA binding, raised the tempting possibility that DNA-bound Smads may be ubiquitylated to cause their dissociation from DNA. To provide a proof-of-principle for this hypothesis, we purified Smurf2 and Nedd4 HECT ubiquitin ligases (Maspero et al., 2011) and first tested their activity on Smad3 off-DNA; these *in-vitro* ubiquitylation reactions generated mono-, di-, tri- and poly-ubiquitylated Smad3 ladders (Fig. 10e and data not shown). Strikingly, when E3 enzymes were applied to DNA-bound Smad3, this caused a quantitative dissociation of Smad3 from DNA (Fig. 10f, compare lanes 1 and 2), and concomitant appearance of ubiquitylated forms in the supernatant (Fig. 10f, lane 4). This effect was specific, as lack of ATP or ubiquitin in the reaction or addition of the enzymatic inhibitor NEM (N-ethylmaleimide) opposed Smad3 lift-off (data not shown).

Altogether these results demonstrate that monoubiquitylation of R-Smads opposes to the DNA binding, and suggest that this modification can actively dissociates R-Smads from the DNA.

DISCUSSION

By identifying USP15, this work shows that R-Smad deubiquitylation is required for full TGFβ responsiveness. The data suggest the following model for a "Smad signaling cycle" (Fig. 11): TGFβ activated receptors induce R-Smad C-terminal phosphorylation, association with Smad4, nuclear accumulation and Smad-dependent transcription. Several molecules then concur to terminate the signaling, including multiple R-Smad ubiquitin ligases (Lonn et al., 2009), the Smad2/4 complex disruptase Ecto/Tif1γ (Dupont et al., 2005; Dupont et al., 2009; Morsut et al. 2010) and R-Smad phosphatases (Lin et al., 2006; Inman et al., 2002). Here we characterized regulative ubiquitylation of R-Smads as a critical step to terminate Smad activity by abrogating their DNA binding. R-Smad monoubiquitylation is clearly induced by Smad transcriptional activity but, at the same time, this modification can lift-off the Smad complex from DNA (Fig. 11). In this perspective, monoubiquitylation serves as self-limiting step during pathway activation, placing R-Smad in a sort of “transcriptional latency”, with USP15-mediated reversal of this modification as relevant to re-empower a new round of Smad-dependent transcription (Fig. 11). As such, USP15 may be particularly relevant for target genes that require enduring Smad activity, as for example, the growth arrest response (Nicolas and Hill, 2003). At least in part, R-Smad monoubiquitylation occurs off-DNA, as monoubiquitylation can be readily detected even in unstimulated or in Smad4-depleted cells. In this respect, USP15-regulated ubiquitylation can be relevant as competence factor setting thresholds of intensity for ligand-dependent transcriptional effects; for example, high levels of USP15 may sensitize otherwise refractory cells to TGFβ/BMP stimulation.

Another aspect deserving discussion is the role of USP15 on the reversal of R-Smad polyubiquitylation and stability. Indeed, E3 ligases are biologically relevant R-Smads inhibitors (Gao et al., 2009; Kuratomi et al., 2005; Alarcon et al., 2009; Dupont et al., 2009; Alexandrova and Thomsen, 2006; Podos et al., 2001) but, at the biochemical level, R-Smad ubiquitylation has so far been investigated almost exclusively in the context of polyubiquitylation and degradation (Lonn et al.,

2009). However, how this could be reconciled with the apparent stability and recycling of the activated pool of R-Smad (Inman et al., 2002; Dai et al., 2009; Hill, 2009) is a matter of debate. The identification of USP15 sheds some light on this issue: loss-of-USP15 indicates that, in addition to polyubiquitylation, endogenous ubiquitin-ligases mediate regulative-ubiquitylation, effectively inhibiting R-Smads function even in absence of overt effects on stability. That said, USP15 can also operate on the pool of polyubiquitylated R-Smads (Fig. 11), and this is remarkably relevant to regulate Smad stability under experimental conditions characterized by enhanced E3-ligase activation. How R-Smads polyubiquitylation and monoubiquitylation relate to each other is not known, but several possibilities exist: 1) polyubiquitylation can be a parallel input to R-Smads monoubiquitylation, occurring at different lysine residues depending on the context; 2) the same ligase responsible for monoubiquitylation can polyubiquitylate R-Smads on the same residue in condition of enhanced activity; 3) a ligase different from the one responsible for monoubiquitylation, activated in particular contexts, extends the polyubiquitin chain on the monoubiquitylated residue.

The balance between polyubiquitylation and monoubiquitylation of R-Smads could be influenced by other post-translational modifications. For example, phosphorylation of the R-Smad linker domain has been reported as only partially affecting monoubiquitylation, whereas this is fully required for polyubiquitylation downstream of CDKs' activation, or of Smad antagonistic pathways, such as Wnt and RTK signaling (Alarcon et al., 2009; Fuentealba et al., 2007; Sapkota et al., 2007). In these instances, by intercepting the pool of polyubiquitylated R-Smads *en route* to degradation, USP15 may oppose the effects of these developmentally-regulated or oncogenic cues and rescue TGFβ responses. Importantly, although in this study we refer to mono-/di-ubiquitylation as regulative, and to polyubiquitylation as degradative modifications, it should be also considered that R-Smad polyubiquitylation may, in some cases, also serve as regulative modification, depending on the structure and branching pattern of the ubiquitin-chain (see Introduction).

One main question on R-Smads monoubiquitylation remains open: which is the responsible E3 ligase? Upon Smurf1 overexpression USP15 is both sufficient and required for opposing R-Smads polyubiquitylation and degradation (Fig. 6d and Fig. 7a). Moreover a mixture of purified Smurf2/Nedd4 could promote Smad3 ubiquitylation in-vitro and promote its detachment from DNA (Fig. 10e and Fig. 10f) that well correlates with the decreased DNA-binding activity of purified monoubiquitylated Smad3 (Fig. 10d). Although these evidences suggest a relationship between these HECT E3-ligases and R-Smads monoubiquitylation, in loss-of-function experiments for Smurf1, Smurf2 or Nedd4 we couldn't observe any effect on the physiological R-Smads monoubiquitylation (data not shown). This apparent discrepancy can be explained in two ways. First these ligases are not involved in Smad ubiquitylation *in-vivo*. In this view, their overexpression or use as recombinant proteins in *in-vitro* assays, provides nothing more than a proof-of-principle, a mockery of the real endogenous situation. The other possibility is that R-Smads monoubiquitylation depends on the redundant activity of an unspecified number of E3-ligases, including HECT family members. Intriguingly, a recent work reinvestigated the biochemical requirements for Smurf2 in Smurf2-/- MEFs and found an essential role for Smad3 monoubiquitylation, rather than polyubiquitylation (Tang et al., 2011).

Another question to address in the future is whether USP15 is regulated. USP15 has been identified as an ATM phosphorylation target in cells upon irradiation (Mu et al., 2007), opening the possibility that DNA damage might modulate the activity of USP15. In this work we showed that USP15 is required for the expression of genes that directly recruit R-Smads to their promoter. Genes that recruit R-Smads indirectly, through other DNA-binding co-factors, do not require USP15 (Fig. 9c, Fig. 9d and Fig. 9e). In this way a putative regulation of USP15 should selectively affects the expression of only a subset of TGFβ/BMP target genes modifying in this way the outcome of the stimulation.

In conclusion in this work we found USP15 as an important new regulator of TGFβ and BMP pathways required for their full responsiveness. Moreover we demonstrated that R-Smads ubiquitylation is mainly a monoubiquitylation that

opposes to R-Smads binding to the promoter of target genes. USP15 reverses this modification and re-empowers R-Smads with transcriptional activity. In this way the cycle of monoubiquitylation of R-Smads and deubiquitylation by USP15 might determine the permanence of the activated R-Smads on the promoter.

In the future the possibility to manipulate USP15 activity could be used in therapeutic intervention for the multiple diseases associated with unbalanced TGFβ/BMP activity.

EXPERIMENTAL PROCEDURES

Plasmids and siRNAs

pCS2-V5-USP15 was generated by PCR amplification of the coding region of human USP15 from pCMV-Tag3B-Myc-USP15 (gift from Dr. W. Dubiel) and subcloning into pCS2 expression vector. Catalytically deficient C269A (CA) and C269S (CS) USP15 mutants were generated by targeted mutagenesis. Human Smad3-Flag lysine mutants (K13R, K19/20R, K29R, K33R, K40/41R, K43/44R, K53R, K81R, K116/117R and KMH2R, see Fig. 10a) were obtained by targeted mutagenesis. SV40 NLS sequence (PKKKRKV) and NES sequence of human MAPKK (MEK) (ALQKKLEELELDE) were introduced into N- terminus of Flag-Smad3 using PCR. Stable shRNA-expressing cell lines were obtained by stable retroviral infection with pSUPER-RETRO-PURO plasmid encoding shRNAs corresponding to the sequence of siRNA USP15#1.

Control shRNA (GCAAGCTGACCCTGAAGTTCAT) is targeting GFP. All plasmids were verified by nucleotide sequencing. Sequences of the siRNAs used to screen human DUBs are reported in Dupont et al., 2009.

List of siRNAs:

USP15#1 siRNA: GGUUGGAAUAAACUUGUCAdtdt;

USP15#2 siRNA: GCACCUUGGAAGUUUACUUdtdt;

USP15#3 siRNA: CCAGUCACUUAAGGAACAUdtdt;

Smad4 siRNA: GUACUUCAUACCAUGCCGAdtdt;

Smad2 siRNA: GCUUAGGUUUACUCUCCAAUGUUAA (Stealth, Invitrogen);

Smad3 siRNA: GAUGCAACCUGAAGAUCUUCAACAA (Stealth, Invitrogen);

FAM siRNAs: 1:1 mix of GAUGAGGAACCUGCAUUUCdtdt and GCAGUGAGUGGCUGGAAGUdtdt (Dupont et al., 2009).

Control siRNA is a scramble sequence: UUCUCCGAACGUGUCACGUdtdt. USP15 #1 siRNA was used in most experiments, unless otherwise indicated.

Cell Cultures and Transfections

HaCaT, HCT116chr3, HEK293T and Hela cells were cultured in DMEM 10%FCS, MDA-MB231 cell in DMEM/F12 10% FCS. hMSC were cultured in growth media (GM Lonza). DNA transfections were performed with calcium phosphate or Transit-LT1 reagent (MirusBio); for siRNA transfections we used Lipofectamine-RNAiMax (Invitrogen). TGFβ1 or BMP2 cytokines (Peprotech and R&D) were diluted in complete medium for HaCaT and HCT116chr3 cells; for the remaining cell lines, cells were starved overnight with 0.2% (HEK293T), or no serum (MDA-MB231) before TGFβ1 or BMP2 addition to the same medium. Where indicated, control cells were supplemented with 5 μM SB505124 (ALK4; ALK5 inhibitor; Tocris) or 5 μM Dorsomorphin (Yu et al., 2008) (BMP-Receptor inhibitor, SIGMA-Aldrich) to quench autocrine TGFβ or BMP signaling. MG132 (10 μM, SIGMA-Aldrich), was added to inhibit the activity of Proteasome, Flavopiridol (0.3 μM, SIGMA-Aldrich) and α-Amanitin (10 μM, SIGMA-Aldrich) were added to block transcriptional elongation.

Biological assays in mammalian cells and Xenopus embryos

For luciferase siRNA screen, HaCaT-CAGA12-lux cells (gift from Caroline Hill; Levy et al., 2007) were siRNA transfected and, after 48 hours, either untreated (normal culture medium) or treated for 4-6 hours with 1 ng/ml TGFβ1 before harvesting. MDA-MB231-ID1BRE-lux cells were obtained by cotransfection of ID1-BRE-lux (gift from Peter ten Dijke) (25 ng/cm^2) and pBABE-Puro plasmids, followed by puromycin selection. For siRNA screen, normalization was carried out based on total protein content, as measured by colorimetric Bradford assay. Each siRNA was transfected in triplicate. Each experiment was repeated at least twice and multiple times with independent USP15 oligonucleotides.

For osteoblast differentiation, hMSC cells were switched from growth (GM) to differentiation medium (ODM, Lonza) and cultured for 6 days in absence or presence of 200 ng/ml BMP2 before staining with Leukocyte Alkaline Phosphatase detection kit (SIGMA-Aldrich).

HEK293T (Fig. 2e), Hela (Fig. 2d), MDA-MB-231 (Fig. 9d) and HCT116-Chr3 (Fig. 5a) 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. TOP-FLASH, SRF-lux, CAGA12-lux and UAS-lux luciferase reporters were cotransfected with CMV-β-gal (each at 40 ng/cm^2) to normalize for transfection efficiency by CPRG (Roche) colorimetic assay. UAS-lux was cotransfected with GAL4-Smad3 expression vector (40 ng/cm^2). DNA content in all samples was kept uniform by adding pBluescript plasmid. Each sample was transfected in duplicate. Each experiment was repeated at least twice. For gene expression analyses, cells were transfected with siRNAs, 48 hours post-transfection treated with TGFβ1 (4 hr, HaCaT; 8 hr MDA-MB231) or 2 hr BMP2 and then collected for protein analysis or real-time PCR as previously described (Adorno et al., 2009).

Oligos for qPCR of BMP targets were:

Smad7	fw	GGCCGGATCTCAGGCATTC
	rev	GAGTCGGCTAAGGTGATGGG
ID2	fw	GACCCGATGAGCCTGCTATAC
	rev	AATAGTGGGATGCGAGTCCAG
GAPDH	fw	AGCCACATCGCTCAGACAC
	rev	GCCCAATACGACCAAATCC
c-Myc	fw	CAAGAGGCGAACACACAACGTCT
	rev	AACTGTTCTCGTCGTTTCCGCAA

For 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay, 24 hr after siRNA transfection, cells where plated on Permanox chamber slides (Nunc), and treated with 4ng/ml TGFβ1 overnight or left untreated. The day after, 10μM BrdU (Roche) was added to cells and left to incorporate for 2 hr. After wash with PBS, cells were fixed in 70% ethanol + 50 mM glycine for 30 min. at -20 °C. After cells re-hydration with PBS α-BrdU antibody in incubation buffer (66mM Tris, 0,66mM $MgCl_2$, 1mM β-mercaptoethanol and nucleases for DNA denaturation) (Roche) was added to the cells. Cells were then washed and α-mouse IgG

fluorescein-conjugated (Roche) was added for 1,5 hr to the samples for detection of BrdU staining. DAPI staining (SIGMA-Aldrich) was used to mark nuclei. Images were obtained with a fluorescence microscope (Zeiss). BrdU positive cells were counted in 10 random/independent fields for each sample. Each experiment was performed at least three times.

For wound-healing assays, cells were seeded in 6-well plates. 24 hours after siRNA transfection, cells were placed in medium without serum; confluent cell monolayers were scratched with a P200 pipette tip to obtain two parallel "wounds" in each well, washed with medium and put in new medium containing no serum and either 5 μM SB505124 (SB) or 4 ng/ml TGFβ1, fixed after 48 hr with 4% PFA and photographed.

Xenopus embryo manipulations, capped mRNAs and Morpholinos injection and in situ hybridization were as previously described (Dupont et al., 2005; Martello et al., 2007).

Control Morpholinos and Morpholinos targeting *XUSP15* and *XUSP4* were:

XUSP4 5'- TACCTCCACCTCCTCCCTCTGCAT -3'

XUSP15 5'- CGCCCTCCGCCATCTTACTCACTT -3'

Control 5'- CCTCTTACCTCAGTTACAATTTATA -3'

All morpholinos were purchased from Gene Tools. MOs were resuspended in Hepes 0.5 mM, pH 7.6 (25 μg/μl stock). MOs were heated to 70 °C for 5 min prior to microinjection. Smad1 and Smad3 mRNAs were transcribed from hSmad1-pCS2 (gift from E. De Robertis) and hSmad3-Flag-pCS2 (Addgene Plasmid #14052). Embryos at the four cell stage were microinjected radially in each blastomere with 4 nl, containing a quarter of the per embryo amount of MOs and/or mRNA, and cultivated at 18°C until reaching the desired developmental stage.

Immunofluorescent Localizations

Two days after siRNA transfection, cells were trypsinized and replated on Permanox chamber slides (Nunc), treated as indicated and fixed 10 min at RT with 4% PFA in PBS. Slides were permeabilized 10 min at RT with PBS 0.3% Triton X-100, 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-Smad2/3 1:200 BD-Biosciences (cat. 610843). Secondary antibodies, i.e., goat α-rabbit Alexa555 or goat α-mouse Alexa-488, were incubated 1.5 hr at RT diluted 1:200 in PBST 2% GS. DAPI staining (SIGMA-Aldrich) was used to mark nuclei. Images were obtained with an SP5 confocal microscope (Leica). Each experiment was performed at least twice. Figures show representative fields, considering at least 10 random/independent fields for each sample with comparable results.

Coimmunoprecipitation, Ubiquitylation and DNA pull-down assays

HEK293T cells were transfected with the calcium-phosphate method with plasmids encoding for HA-ubiquitin (gift from A. Moustakas, 8 μg/10 cm dish), Flag-Smads (100 ng/10 cm dish), V5-USP15 (10 μg/10 cm dish), and/or myc-Smurf1 (5 μg/10 cm dish) as indicated. DNA content was kept uniform by adding pBluescript plasmid. For protein-protein interaction studies, cells were treated as indicated and lysed by sonication in Marais' lysis buffer (25 mM HEPES [pH 7.8], 400 mM KCl, 5 mM EDTA, 0.4% NP40, 10% glycerol freshly supplemented with 1 mM DTT, protease, and phosphatase inhibitors). Extracts were diluted eightfold to bring KCl concentration to 50 mM and NP40 to 0.05%, supplemented with 10 mM $MgCl_2$, and subjected to protein-A sepharose immunoprecipitation 4 hr at 4°C.

For ubiquitylation assays, cells were harvested 48 hr post-transfection by sonication in Ub-lysis buffer (50 mM HEPES [pH 7.8], 200 mM NaCl, 5 mM EDTA, 1% NP40, 5% glycerol, freshly complemented with 1 mM DTT, protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail II (SIGMA-Aldrich), 250 ng/ml ubiquitin-aldehyde (Biomol)). Cell lysates were immunoprecipitated 4 hr at 4°C with protein-A sepharose beads with α-Smad2 (A20: SantaCruz) or α-phosphoSmad3 (Epitomics), or α-FLAG M2 Affinity Gel (SIGMA-Aldrich) in Ub-lysis buffer supplemented with 2 mM $MgCl_2$, followed by three washes of 2 min rotating at room temperature (RT) (50 mM HEPES [pH 7.8], 500 mM NaCl, 5 mM EDTA, 1% NP40, 5% glycerol). Alternatively, cell lysates were boiled

with 0.5% SDS for 5 min and diluted 5 times with PBS before the immumoprecipitation to avoid any co-precipitation of unspecific proteins with R-Smads. For Lysine mapping the protocol for Smad3 ubiquitylation was implemented as follows: HEK293T cells were transfected with LT1 to ensure more uniform and comparable expression of Smad3 between different samples and mutants; each mutant was transfected in triplicate and lanes in Fig. 10a are representative examples; finally, to remain close to the linear range in epitope detection, we employed anti-FlagM2-HRP conjugated antibody (SIGMA-Aldrich).

Nuclear and cytoplasmic fractions of HEK293T or HaCaT cells were prepared as follows: confluent cells were washed with PBS and allowed to swell with ice-cold hypotonic buffer (20 mM HEPES [pH 7.6], 20% glcerol 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.1% NP40) for 10 min at 4°C, gently scraped and collected into falcon tube. Cells were spun at 500 rpm for 10 min and supernatant was used as "Cytoplasmic" fraction. Cell pellet was resuspended with Ub-lysis buffer, sonicated, spun and supernatant was used as "Nuclear" fraction.

Western blot was carried out as previously described (Dupont et al., 2009). Antibodies for western blotting were: α-Smad4 (H552 and B8), α-laminB (C20), α-tubulin (H235), α-GST (1C9), α-JunB (C11), α-SnoN (H317), α-HA polyclonal (Y11) monoclonal (F7) and α-Myc antibodies were all from SantaCruz; α-βcatenin, α-FLAG-M2, α-FLAG-M2-HRP, α-FLAG-M2 agarose-conjugated, α-HA agarose-conjugated and α-V5 agarose-conjugated were from SIGMA-Aldrich; α-Smad2/3, α-PAI1, α-$p21^{Cip1/Waf1}$ were from BD Biosciences; α-HA monoclonal ascite (covance), α-V5-HRP conjugate (Invitrogen), α-P-Smad3 (Epitomics), α-USP9x/FAM, α-Ectodermin, α-USP15 (Abnova), α-GAPDH and α-phosphoSmad1 were from Millipore. For endogenous Smad2 visualization upon coimmunoprecipitation, beads were treated for 2 hr at 37°C with PNGaseF (NEB).

For DNA pull-down with biotinylated double-stranded oligonucleotides nuclear extracts were prepared from cells. Extracts were incubated with 600 ng of biotinylated double-stranded oligonucleotides and 5 μg of poly(dI-dC) at 4°C for

16 hr in DB Buffer (60 mM NaCl, 20 mM HEPES (pH 7.8), 5 mM MgCl2, 0.2 mM EDTA, 10% glycerol, 1 mM DTT, 2% BSA, and 0.1% of NP-40). DNA bound proteins were collected with streptavidin-agarose beads (Pierce) for 1 hr, washed extensively with DB, separated on a SDS-polyacrylamide gel, and identified by Western blotting. In Fig. 9c, control oligo DNA is an oligonucleotide corresponding to the sequence of the Smad-target *Mix.2* promoter, but mutant in the SBE binding element.

SBE Probe sequences for DNA pull-down was: Bio- TCGATAGCCAGACAG GTAGCCAGACAGGTAGCCAGACAGGTAGCCAGACAGG;

ARE probe was: Bio- TATCTGCTGCCCTAAAATGTGTATTCCATGGA AATGTCTGCCCTTCTCTCGAG.

Protein purifications and in vitro ubiquitylation/deubiquitylation assays

To obtain purified monoubiquitylated-Smad3, Flag-Smad3, 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-Aldrich), followed by two sequential elutions with Flag peptide (SIGMA-Aldrich, 1 mg/ml in Ub lysis buffer). Pooled Flag eluates were subsequently immunoprecipitated with α-HA resin (SIGMA-Aldrich), followed by two sequential elutions with HA peptide (SIGMA- Aldrich) in Ub lysis buffer. Pooled HA eluates were dialyzed overnight against 20 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol, 0.1% NP-40 and 1 mM EDTA. For purification of USP15 protein from mammalian cells, V5-USP15-transfected HEK293T cell lysates (without protease or DUB inhibitors) were immunoprecipitated overnight with α-V5 resin (SIGMA-Aldrich) and eluted with 1 mg/ml V5 peptide (SIGMA-Aldrich) in PBS.

For in vitro deubiquitylation assay, purified monoubiquitylated Smad3 and USP15 were incubated overnight at 30°C in 50 mM HEPES (pH 7.5), 100 mM NaCl, 5% glycerol, 5 mM $MgCl_2$, 1 mM ATP and 1 mM DTT. For in vitro ubiquitylation assay, purified Smad3 (1μg per reaction) was first bound to α-Flag-M2 resin and treated with recombinant CDK9/CyclinT1 (invitrogen)

according to manufacturer's instruction (200 ng of Enzymes in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.5 mM EGTA, 2.5 mM DTT, 0,01% TritonX-100 and 100 μM ATP and incubated at 30°C for 2 hr). Indeed it has been previously shown that CDK9-mediated phosphorylation of the linker domain of R-Smad serves to facilitate R-Smad recognition by HECT ubiquitin ligases (Gao et al., 2009). Consistently, we found that addition of CDK9, albeit not absolutely required in vitro, does increase the efficacy of ubiquitylation assays. Beads were briefly washed with binding buffer and subjected to ubiquitylation reaction (25 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM ATP, 0.2 μM DTT) in a total volume of 50 μl, containing 20 nM recombinant E1 (Maspero et al., 2011), 100 nM recombinant E2 (Ubch7, BostonBiochem), 500 μM recombinant Methylated Ubiquitin (BostonBiochem) and incubated at 37°C for 3 hr. For the R-Smad DNA dissociation assay (Fig. 10f), purified Smad3 was first bound to SBE oligo DNA and purified on streptavidin beads; beads (DNA bound) and supernatant (dissociated) fractions were separated after ubiquitylation reaction and analyzed by western blotting analysis.

Chromatin immunoprecipitation (ChIP)

HaCaT-CAGA12 cells were treated with TGFβ1 for 2 hr. DNA and proteins were cross-linked by the addition of formaldehyde (1% final concentration) 10 min before harvesting, and cross-linking was stopped by the addition of glycine pH 2.5 (125 μm final concentration) for 5 min at room temperature. Cells were scraped off the plates, resuspended in hypotonic buffer. Nuclei were spun down, resuspended in 300 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, and protease inhibitors), and sonicated to generate 500–2000-bp fragments. After centrifugation, the cleared supernatant was diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40). The cell lysate was precleared by incubation at 4°C with 15 μl of protein A-sepharose beads preabsorbed with sonicated salmon sperm DNA and bovine serum albumin. The cleared lysates were incubated overnight with α-Smad4 polyclonal antibody (H552) (Santa Cruz) or pre-immune rabbit IgG.

Immune complexes were precipitated with 30 µl of protein A-sepharose beads preabsorbed with sonicated salmon sperm DNA and bovine serum albumin. After centrifugation the beads were extensively washed and the antigen was eluted with 1% SDS, 100 mM sodium carbonate. DNA-protein cross-links were reversed by heating at 65°C for 4–5 hr, and DNA was phenol-extracted and ethanol-precipitated. Levels of CAGA-12, *p21*, *PAI1*, *JunB* promoter DNAs were determined by PCR using oligonucleotides encompassing the Smad binding sites. The following oligonucleotides were used:

CAGA12	fw	CGAGCTCTTACGCGTGCTA
	rev	CCGGGCCTTTCTTTATGTTT
p21 SBE1	fw	GAGGAAAAGCATCTTGGAG
	rev	AAATAGACGGGAGCAACG
p21 SBE2	fw	ACTTGTCCCTAGGAAAATCC
	rev	GAAAACGGAGAGTGAGTTTG
PAI1	fw	GCAGGACATCCGGGAGAGA
	rev	CCAATAGCCTTGGCCTGAGA
Jun B	fw	CAGTCCAGACACACAAACC
	rev	CTGTGTCCATGTGTGACAGT

Molecular Modeling

The assembly of the hypothetical three-dimensional model of the Smad3-ubiquitin covalent complex has been carried out following the procedure: a) starting from the crystallographic coordinates of Smad3-MH1 domain (PDB code: 1OZJ) and ubiquitin (PDB code: 1UBQ) a protein-protein docking protocol has been performed to explore the best surface complementarities between Smad3-MH1 domain and ubiquitin monomers. The program Hex (ver.6.3) has been used to carry out the protein-protein docking simulation; b) all energetically stable Smad3-ubiquitin complexes sample by Hex have been inspected, and we have selected only those complexes compatible with an ubiquitin monomer in proximity to K33, K53, and K81 of Smad3; c) the missing Smad3/ubiquitin covalent connection has been create using the Molecular Operating Environment

(MOE) software (Chemical Computing Group Inc.); d) Smad3-ubiquitin covalent complex has been energy minimized using the AMBER99 force field until the energy gradient reached 0.05 kcal/mol.

REFERENCES

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., Parenti, A.R., Rosato, A., Bicciato, S., Balmain, A., and Piccolo S. (2009). A mutant p53/Smad complex opposes p63 to empower TGFβ-induced metastasis. Cell 137, 87-98.

Ahearn, I.M., Haigis, K., Bar-Sagi, D., and Philips, M.R. (2012). Regulating the regulator: post-translational modification of RAS. Nat Rev Mol Cell Biol 13, 39-51.

Akhurst, R.J., and Derynck, R. (2001). TGF-β signaling in cancer – a double-edged sword. Trends Cell Biol 11(11), 44-51.

Alarcon, C., Zaromytidou, A.I., Xi, Q., Gao, S., Yu, J., Fujisawa, S., Barlas, A., Miller, A.N., Manova-Todorova, K., Macias, M.J., Sapkota, G., Pan, D., and Massaguè, J. (2009). Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-β pathways. Cell 139, 757-769.

Alexandrova, E.M., and Thomsen, G.H. (2006). Smurf1 regulates neural patterning and folding in *Xenopus* enbyos antagonizing the BMP/Smad1 pathway. Dev Biol 299, 398-419.

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

Baker, R.T., Wang, X.W., Woollatt, E., White, J.A., and Sutherland, G.R. (1999). Identification, functional characterization, and chromosomal localization of USP15, a novel human ubiquitin-specific protease related to the UNP oncoprotein, and a systematic nomenclature for human ubiquitin-specific proteases. Genomics 59, 264-274.

Chai, J., Wu, J.W., Yan, N., Massaguè, J., Pavletich, N.P., and Shi, Y. (2003). Features of a Smad3 MH1-DNA complex – Roles of water and zinc in DNA binding. J Biol Chem 278(22), 20327- 20331.

Chen, X., Rubock, M.J., and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGF-β signalling. Nature 383, 691-696.

Da Silva-Ferrada, E., Torres-Ramos, M., Aillet, F., Campagna, M., Matute, C., Rivas, C., Rodriguez, M.S., and Lang, V. (2011). Role of monoubiquitylation on the control of IκBα degradation and NF-κB activity. Plos ONE 6(10), 1-9.

Dai, F., Lin, X., Chang, C., and Feng, X.H. (2009). Nuclear export of Smad2 and Smad3 by RanBP3 facilitates termination of TGF-β signaling. Dev Cell 16, 345-357.

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

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.

Dupont, S., Mamidi, A., Cordenonsi, M., Montagner, M., Zacchigna, L., Adorno, M., Martello, G., Stinchfield, M.J., Soligo, S., Morsut, L., Inui, M., Moro, S., Modena, N., Argenton, F., Newfeld, S.J., and Piccolo S. (2009). FAM/USP9x, a deubiquitylating enzyme essential for TGFβ signaling, controls Smad4 monoubiquitination. Cell 136, 123-135.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-β type I receptor through Smad7 and induces receptor degradation. J Biol Chem 276(16), 12477-12480.

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

Gao, S., Alarcon, C., Sapkota, G., Rahman, S., Chen, P.Y., Goerner, N., Macias, M.J., Erdjument-Bromage, H., Tempst, P., and Massaguè, J. (2009). Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-β signaling. Mol Cell 36, 457-468.

Hetfeld, B.K.J., Helfrich, A., Kapelari, B., Scheel, H., Hofmann, K., Guterman, A., Glickman, M., Schade, R., Kloetzel, P.M., and Dubiel W. (2005). The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1. Current Biology 15, 1217-1221.

Hill, C.S. (2009). Nucleocytoplasmic shuttling of Smad proteins. Cell Res 19, 36-46.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. EMBO Rep 9(6), 536-542.

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

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

Iwai, K., and Tokunaga, F. (2009). Linear polyubiquitination: a new regulator of NF-κB activation. EMBO Rep 10(7), 706-713.

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 TGFβ receptor for degradation. Mol Cell 6, 1365-1375.

Kuratomi, G., Komuro, A., Goto, K., Shinozaki, M., Miyazawa, K., Miyazono, K., and Imamura, T. (2005). NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively regulates TGF-β (transforming growth factor-β) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-β type I receptor. Biochem J 386, 461-470.

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

Lin, X., Duan, X., Liang, Y.Y., Su, Y., Wrighton, K.H., Long, J., Hu, M., Davis, C.M., Wang, J., Brunicardi, F.C., Shi, Y., Chen, Y.G., Meng, A., and Feng, X.H. (2006). PPM1A functions as a Smad phosphatase to terminate TGFβ signalling. Cell 125, 915-928.

Lo, R.S., and Massaguè, J. (1999). Ubiquitin-dependent degradation of TGF-β-activated Smad2. Nat Cell Biol 1, 472-478.

Lonn, P., Moren, A., Raja, E., Dahl, M., and Moustakas, A. (2009). Regulating the stability of TGFβ receptors and Smads. Cell Research 19, 21-35.

Martello, G., Zacchigna, L., Inui. M., Montagner, M., Adorno, M., Mamidi, A, Morsut, L., Soligo, S., Tran, U., Dupont, S., Cordenonsi, M., Wessely, O., and Piccolo S. (2007). MicroRNA control of Nodal signalling. Nature 449, 183-189.

Maspero, E., Mari, S., Valentini, E., Musacchio, A., Fish, A., Pasqualato, S., and Polo, S. (2011). Structure of HECT:ubiquitin complex and its role in ubiquitin chain elongation. EMBO Reports 12(4), 342-349.

Miyazono, K., Maeda, S., and Imamura, T. (2004). Coordinate regulation of cell growth and differentiation by TGF-β superfamily and Runx proteins. Oncogene 23, 4232-4237.

Morsut, L., Yan, K.P., Enzo, E., Aragona, M., Soligo, S.M., Wendling, O., Mark, M., Khetchoumian, K., Bressan, G., Chambon, P., Dupont, S., Losson R., and Piccolo, S. (2010). Negative control of Smad activity by Ectodermin/Tif1γ patterns the mammalian embryo. Development 137, 2571-2578.

Mu, J.J., Wang, Y., Luo, H., Leng, M., Zhang, J., Yang, T., Besusso, D., Jung, S.Y., and Qin, J. (2007). A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. J Biol Chem 282(24), 17330-17334.

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

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

Nijman, S.M.B., Luna-Vargas, M.P.A., Velds, A., Brummelkamp, T.R., Dirac, A.M.G., Sixma, T.K., and Bernards, R. A genomic and functional inventory of deubiquitylating enzymes. Cell 123, 773-786.

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

Podos, S.D., Hanson, K.K., Wang, Y.C., and Ferguson, E.L. (2001). The DSmurf ubiquitin-protein ligase restricts BMP signaling spatially and temporally during *Drosophila* embryogenesis. Dev Cell 1, 567-578.

Sacco, J.J., Coulson, J.M., Clague, M.J., and Urbè, S. (2010). Emerging roles of deubiquitinases in cancer-associated pathways. IUBMB Life 62(2), 140-157.

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

Sapkota, G., Alarcon, C., Spagnoli, F.M., Brivanlou, A.H., and Massaguè, J. (2007). Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25, 441-454.

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

Shi, W., Sun, C., He, B., Xiong, W., Shi, X., Yao, D., and Cao, X. (2004). GADD34-PP1c recruited by Smad7 dephosphorylates TGFβ type I receptor. J Cell Biol 164(2), 291-300.

Shi, Y. (2001). Structural insights on Smad function in TGFβ signaling. Bioessays 23(3), 223-232.

Shi, Y., and Massaguè, J. (2003). Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell 113, 685-700.

Tang, L.Y., Yamashita, M., Coussens, N.P., Tang, Y., Wang, X., Li, C., Deng, C.X., Cheng, S.Y., and Zhang, Y.E. (2011). Ablation of Smurf2 reveals an inhibition in TGF-β signalling through multiple mono-ubiquitination of Smad3. EMBO J 30, 4777-4789.

Vos, R.M., Altreuter, J., White, E.A., and Howley, P.M. (2009). The ubiquitin-specific peptidase USP15 regulates human Papillomavirus type 16 E6 protein stability. J Virol 83(17), 8885-8892.

Wu, M.Y., and Hill, C.S. (2009). TGFβ superfamily signaling in embryonic development and homeostasis. Dev Cell 16, 329-343.

Yan, Q., Dutt, S., Xu, R., Graves, K., Juszczynski, P., Manis, J.P., and Shipp, M.A. (2009). BBAP monoubiquitylates histone H4 at lysine 91 and selectively modulates the DNA damage response. Mol Cell 36(1), 110-120.

Yu, P.B., Hong, C.C., Sachidanandan, C., Babitt, J.L., Deng, D.Y., Hoyng, S.A., Lin, H.Y., Bloch, K.D., and Peterson, R.T. (2008). Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 4(1), 33-41.

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

FIGURES

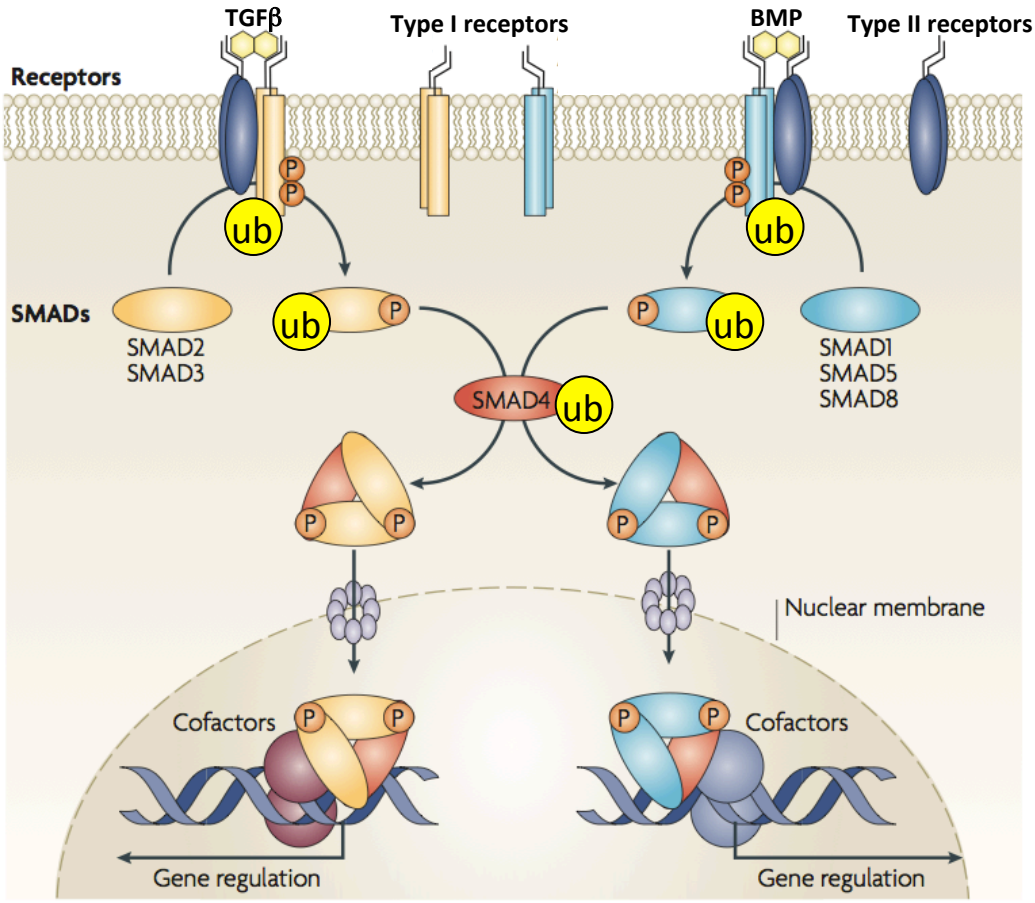
Figure1. Introduction

a. TGFβ and BMP pathways components are regulated through ubiquitylation.
TGFβ and BMP ligands bind to type II and type I receptors, forming a complex in which type II phosphorylates and activate type I receptor. This, in turn, phosphorylates the receptor-activated Smads (R-Smads) inducing them to complex with Smad4 and to accumulate into the nucleus. Once in the nucleus, R-Smad/Smad4 active transcriptional complexes regulate the expression of the target genes by binding to DNA directly either alone or in concert with other transcription factors. BMP and TGFβ signaling activate different type I receptors, thus recruiting different R-Smads: Smad1, Smad5 and Smad8 for BMP, Smad2 and Smad3 for TGFβ pathway. These two groups of R-Smads control the expression of different sets of target genes (also see Schmierer and Hill, 2007).
Receptors, R-Smads, Smad4 are regulated through ubiquitylation.
Ub: ubiquitylation; P: phosphorylation.

b. Dynamic regulation of the activity of Smad4 through monoubiquitylation and deubiquitylation.
In the nucleus, the E3-ligase Ectodermin/Tif1γ (Ecto) monoubiquitylates Smad4 destabilizing R-Smad/Smad4 complex, thus terminating the signal. Once exported in the cytoplasm, the "latent" Ub-Smad4 is targeted by the DUB FAM/USP9x that deubiqutylates it, re-empowering Smad4 competence to mediate TGFβ/BMP signaling (see Dupont et al., 2009).

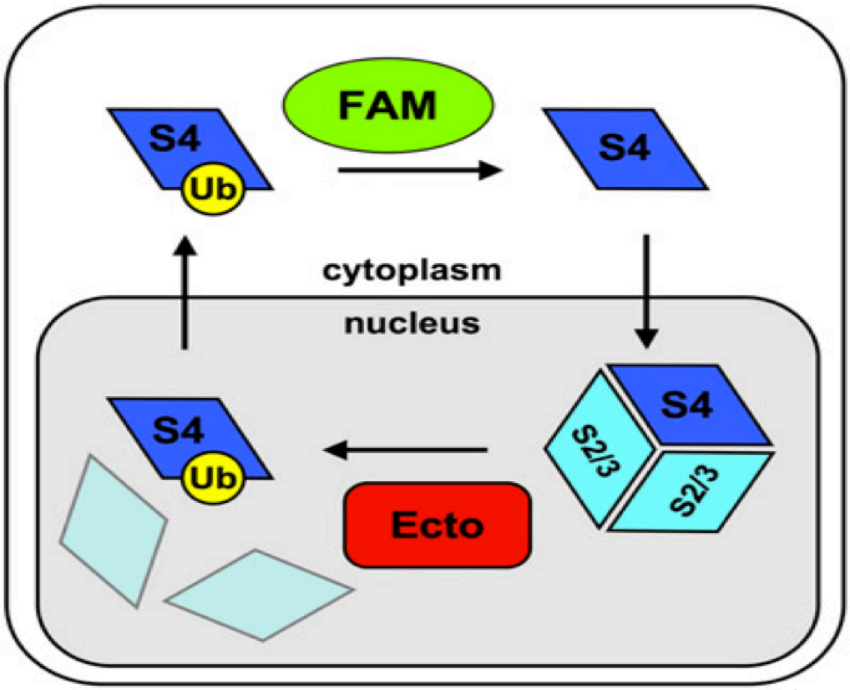
FIGURE 1

a



Modified from Schmierer and Hill, 2007

b



From Dupont et al., 2009

Figure2. USP15 controls TGFβ and BMP signaling

a. Diagram of the screening procedure to identify general/common regulators of TGFβ and BMP signaling. For sequences of siRNAs in our library see Dupont et al., 2009.

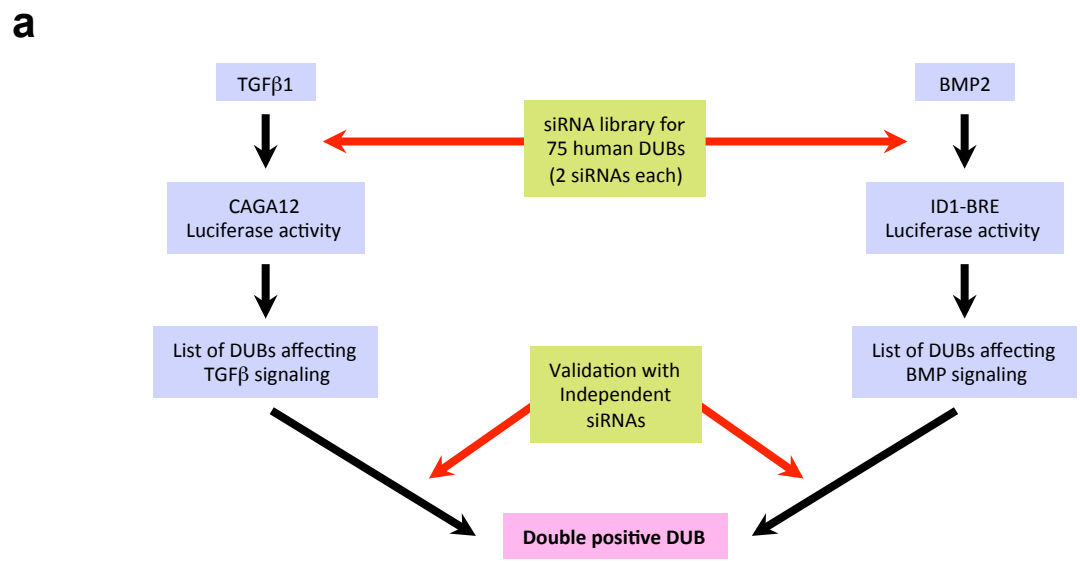
b. Graphs show the ligand-mediated inductions of TGFβ and BMP reporters compared to control cells (lanes 1 and 2) and the effects of a representative set of anti-DUB siRNAs, including FAM and USP15 siRNAs. Data are represented as mean and standard deviation (SD).

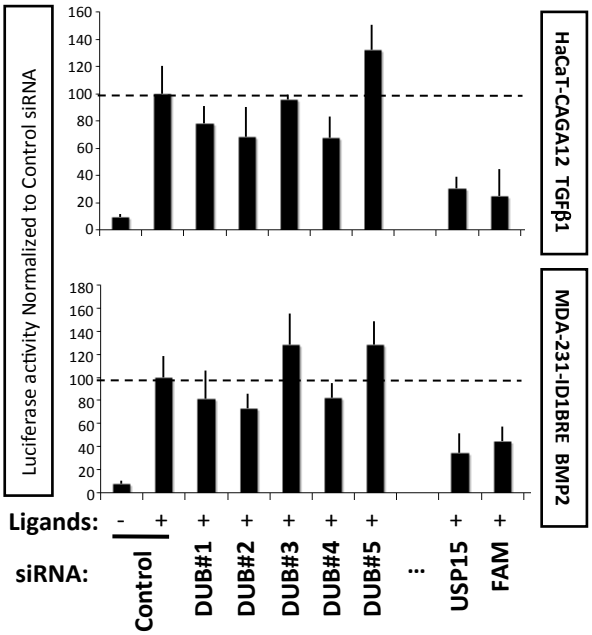
c. Validation of the initial screen with distinct individual siRNAs for USP15 inhibiting TGFβ responsiveness in HaCaT-pCAGA12-lux. Data are represented as mean and SD.

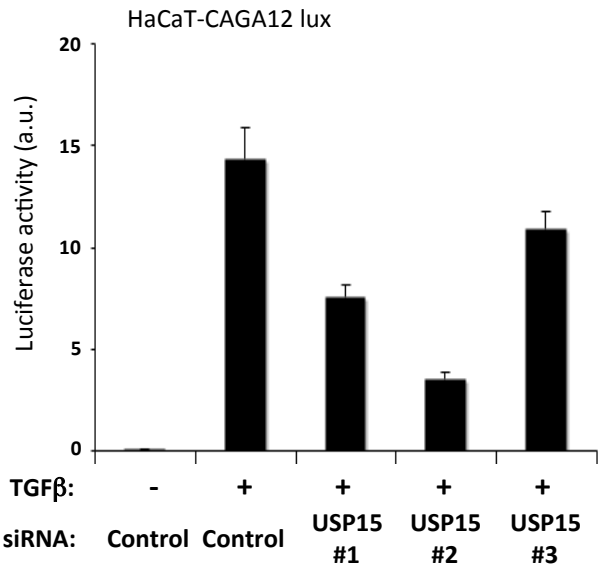
d. Graphs show a comparable activation of a MAL/SRF luciferase reporter in control or USP15 siRNA transfected Hela cells upon serum-treatment. Data are shown as mean and SD.

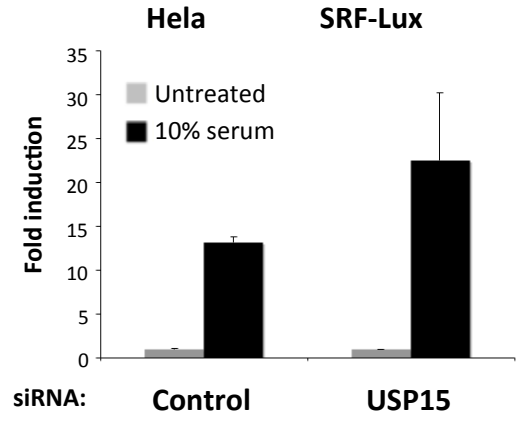
e. Graphs show a comparable β-catenin induced activation of TOP FLASH luciferase reporter in control or USP15 siRNA transfected HEK293T cells. Data are shown as mean and SD.

FIGURE 2


a
TGFβ1
BMP2
siRNA library for 75 human DUBs (2 siRNAs each)
CAGA12 Luciferase activity
ID1-BRE Luciferase activity
List of DUBs affecting TGFβ signaling
List of DUBs affecting BMP signaling
Validation with Independent siRNAs
Double positive DUB


b
Luciferase activity Normalized to Control siRNA
HaCaT-CAGA12 TGFβ1
MDA-231-ID1BRE BMP2
Ligands:
siRNA:
Control
DUB#1
DUB#2
DUB#3
DUB#4
DUB#5
USP15
FAM


c
HaCaT-CAGA12 lux
Luciferase activity (a.u.)
TGFβ:
siRNA:
Control
Control
USP15 #1
USP15 #2
USP15 #3


d
Hela
SRF-Lux
Untreated
10% serum
Fold induction
siRNA:
Control
USP15

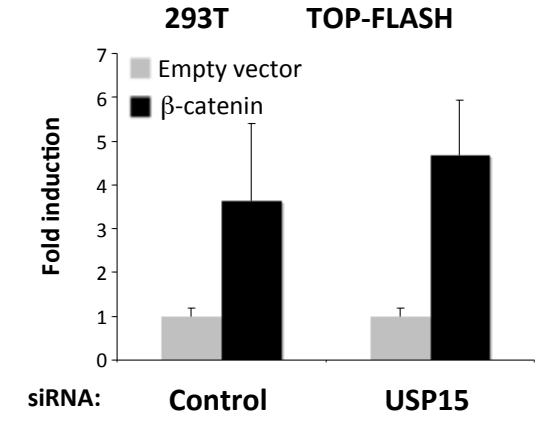

e
293T
TOP-FLASH
Empty vector
β-catenin
Fold induction
siRNA:
Control
USP15

Figure 3. USP15 is required for TGFβ and BMP gene responses

a. Immunoblots for PAI1 and $p21^{Waf1}$, whose TGFβ induction is inhibited by USP15 depletion (USP15 #1 siRNA) in HaCaT cells. Smad4 siRNA is used as a positive control for inhibition. β-catenin serves as loading control.

b. Immunoblots for PAI1 and $p21^{Waf1}$, whose TGFβ induction is inhibited by two independent USP15 siRNAs in MDA-MB-231 cells. LaminB serves as loading control.

c. Graphs show fold induction of *Smad7* and *ID2* mRNAs in MDA-MB-231 cells transfected with the indicated siRNAs and treated with BMP2 vs. dorsomophin (a BMP-Receptor inhibitor that prevents the background from autocrinally expressed BMP ligands). Data are represented as mean and SD.

d. Graph shows fold induction of *c-Myc* mRNAs in HaCaT cells transfected with the indicated siRNAs and treated with TGFβ1 vs. SB505124 (SB). TGFβ-mediated repression of *c-Myc* mRNA occurs in control cells, but not in Smad4- or USP15-depleted cells. Data are represented as mean and SD.

FIGURE 3

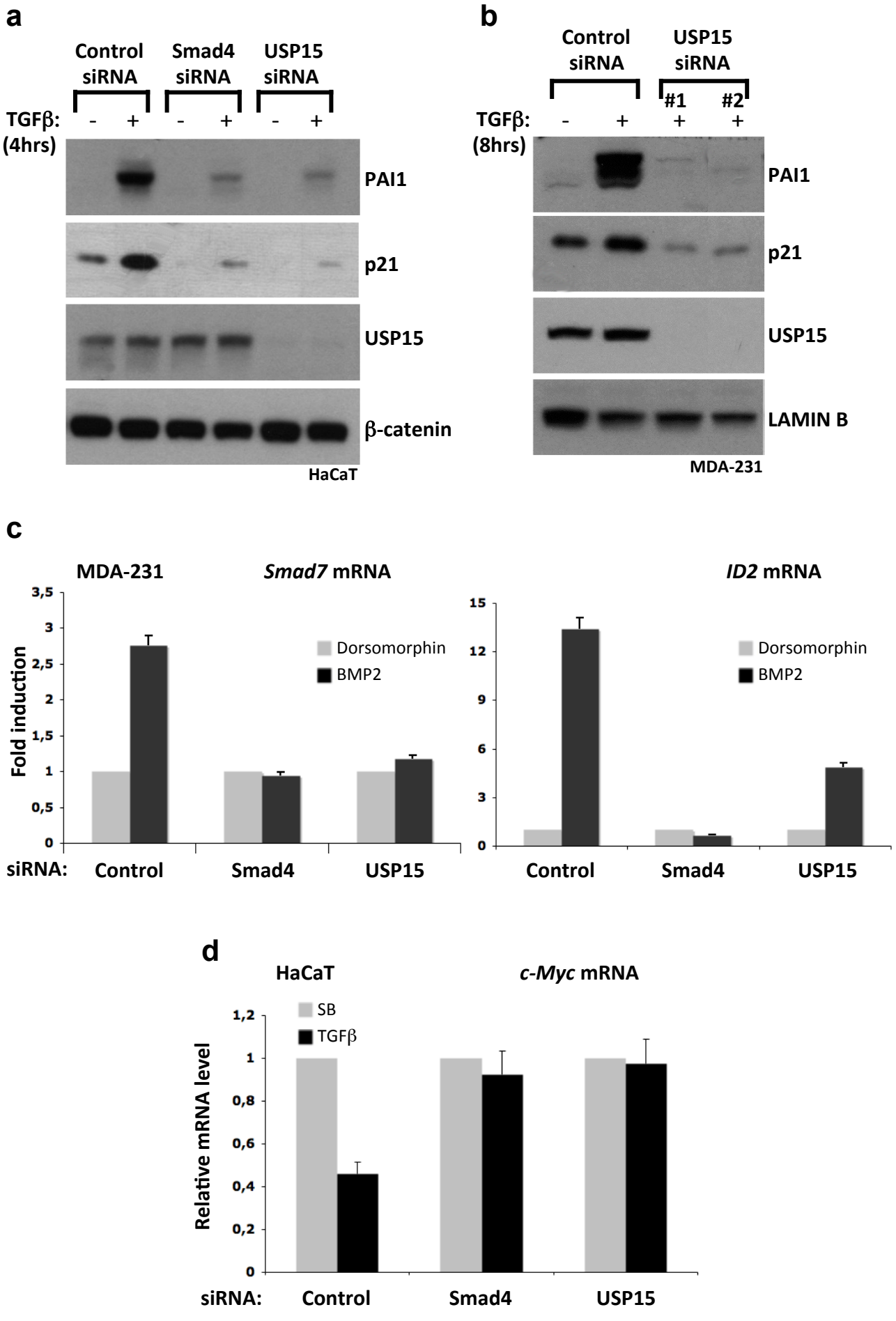

a
Control siRNA
Smad4 siRNA
USP15 siRNA
TGFβ:
(4hrs)
- + - + - +
PAI1
p21
USP15
β-catenin
HaCaT
b
Control siRNA
USP15 siRNA
#1 #2
TGFβ:
(8hrs)
- + + +
PAI1
p21
USP15
LAMIN B
MDA-231
c
MDA-231
Smad7 mRNA
ID2 mRNA
Fold induction
Dorsomorphin
BMP2
siRNA: Control Smad4 USP15
d
HaCaT
c-Myc mRNA
SB
TGFβ
Relative mRNA level
siRNA: Control Smad4 USP15

Figure 4. USP15 is required for TGFβ and BMP biological effects

a. USP15 is required for TGFβ-induced growth arrest in HaCaT cells as assayed by BrdU incorporation. The number of cells in S phase in untreated control cultures was given an arbitrary value of 1 and all other values are depicted relative to this. Smad4 siRNA is used as positive control. Data are represented as mean and SD.

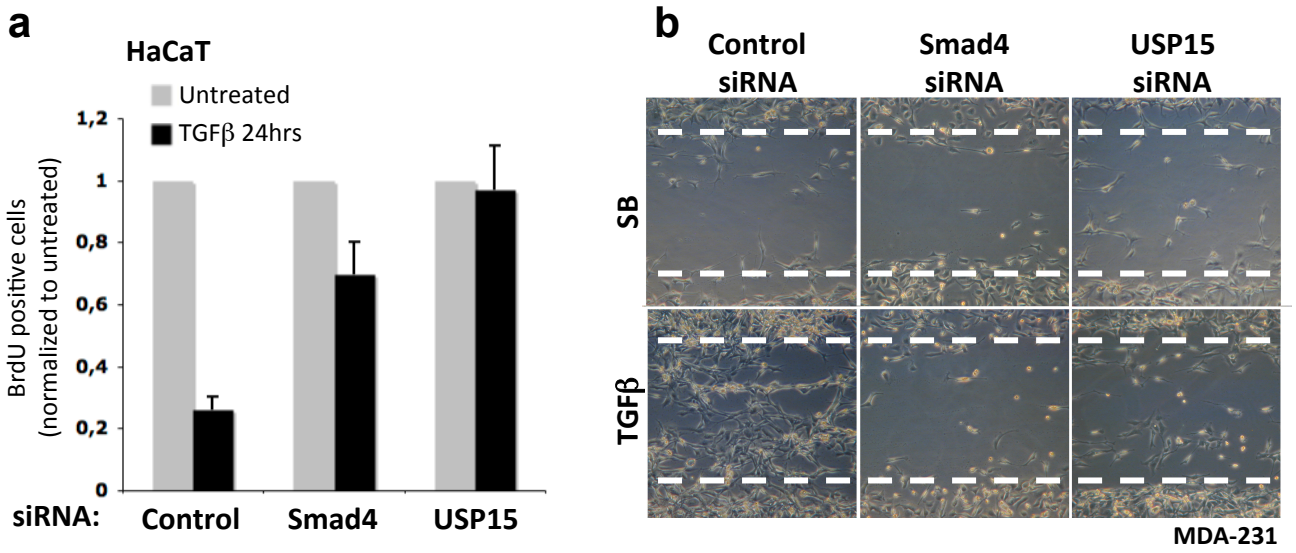
b. Panels show representative pictures of control, Smad4- or USP15- depleted MDA-231 cells migrated into a scratch introduced in confluent monolayers. Dots indicate the edges of the wound at the beginning of the experiment. USP15 is required for TGFβ-induced cell migration. Similar results were obtained with USP15 #2 siRNA (data not shown).

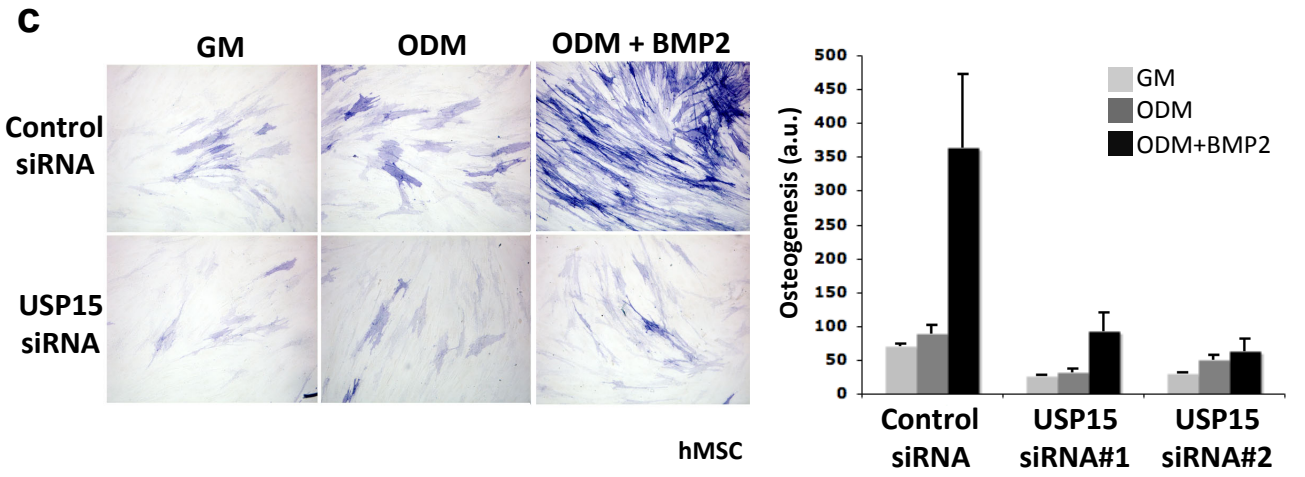
c. USP15 is required for BMP-dependent osteoblast differentiation of hMSC cells, as visualized by alkaline phosphatase (ALP) staining of cells transfected with USP15#1 siRNA. Similar results were obtained with USP15#2 siRNA (right graphs, and data not shown). ODM is hMSC osteodifferentiation media; GM is growth media. Left: representative fields of cells stained for ALP; right: ALP staining in different fields was quantified by ImageJ and normalized over cell number. Data are mean of different fields and SD.

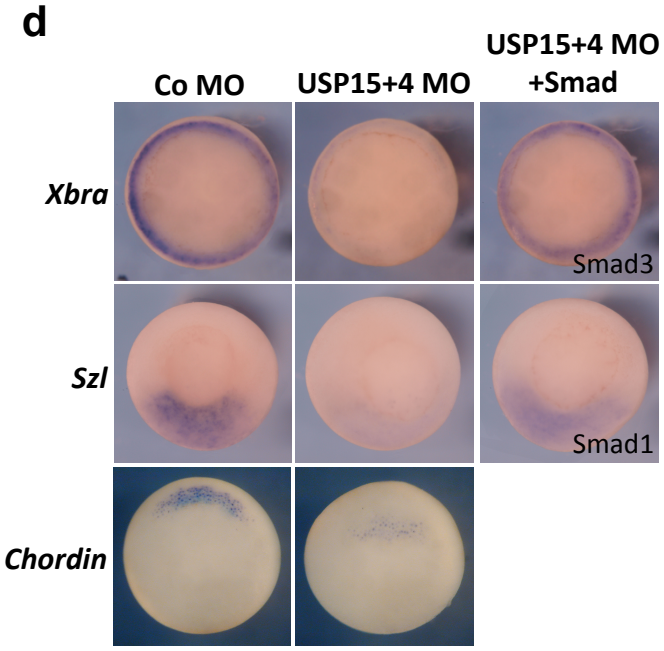
d. Panels show representative in situ hybridizations of *Xenopus* embryos for the Spemann Organizer marker *Chordin* (early gastrula), the pan-mesodermal marker *Xbra* (mid-gastrula) and the ventral marker *Sizzled* (late gastrula). Left panels are embryos injected with control Morpholinos (80 ng); embryos on central panels are USP15/USP4 morphants (40 ng each); embryos on right panels are co-injected with USP15/USP4 Morpholinos and 300 pg of *hSmad3* mRNA or 1000 pg of *hSmad1* mRNA. Dorsal is up, embryos are shown as vegetal views.

e. The table shows quantification of reduced expression of *Chordin*, *Xbra* and *Sizzled* caused by morpholino-mediated knockdown in whole *Xenopus* embryos. Embryos were injected with indicated Morpholino oligonucleotides (MO) and mRNAs.

FIGURE 4







e

Morpholino (ng/embryo) and mRNA (pg/embryo)	Embryos with decreased expression (%) Xbrachyury (n)	Sizzled (n)	Chordin (n)
Control MO (40)	3 (43)	4 (25)	2,8 (39)
Control MO (80)	4,3 (23)	2,5 (44)	4,75 (21)
USP15/4 MO (20+20)	81 (42)	91,7 (24)	93,9 (33)
USP15/4 MO (40+40)	100 (30)	90,2 (41)	88,9 (18)
USP15/4 MO (20+20) + Smad3 mRNA (300)	42 (19)		
USP15/4 MO (20+20) + Smad1 mRNA (1000)		12,5 (16)	

Figure 5. USP15 interacts with R-Smads

a. Gain-of-USP15 enhances, dose-dependently, TGFβ signaling; this requires its enzymatic domain. HCT116-Chr3 cells were transfected with CAGA12-luciferase reporter and 100, 300 or 900 ng of expression plasmids coding for wild-type or C269A- mutant USP15.

b. Induction of phospho-Smad3 by TGFβ is not affected by USP15 depletion. Smad2/3 and Smad4 steady-state levels are also not affected. Western blots showing PAI1 downregulation and USP15 depletion are controls for this experiment. β-catenin is a loading control. Note that USP15 levels are not affected by TGFβ (third panel, lanes 1 and 2).

c. Overexpressed USP15 binds to Smads. HEK293T cells were transfected with the indicated expression plasmids encoding V5-USP15 and Flag-Smads. Cells were left untreated or treated with TGFβ1. Immunoprecipitation (IP) was carried out with anti-Flag resin followed by western blot (IB) with anti-Flag or anti-V5 antibody.

d. Endogenous USP15 protein binds to Smads. HaCaT cells were left untreated or treated with TGFβ1. Immunoprecipitation (IP) was carried out with anti-Smad2/3 antibody or preimmune mouse IgG followed by western blot (IB) with anti-USP15 or anti-Smad2/3 antibody. Asterisk indicates an aspecific band.

e. USP15 directly interacts with the MH1-linker domain of Smad3 by GST-pulldown of recombinant proteins.

FIGURE 5

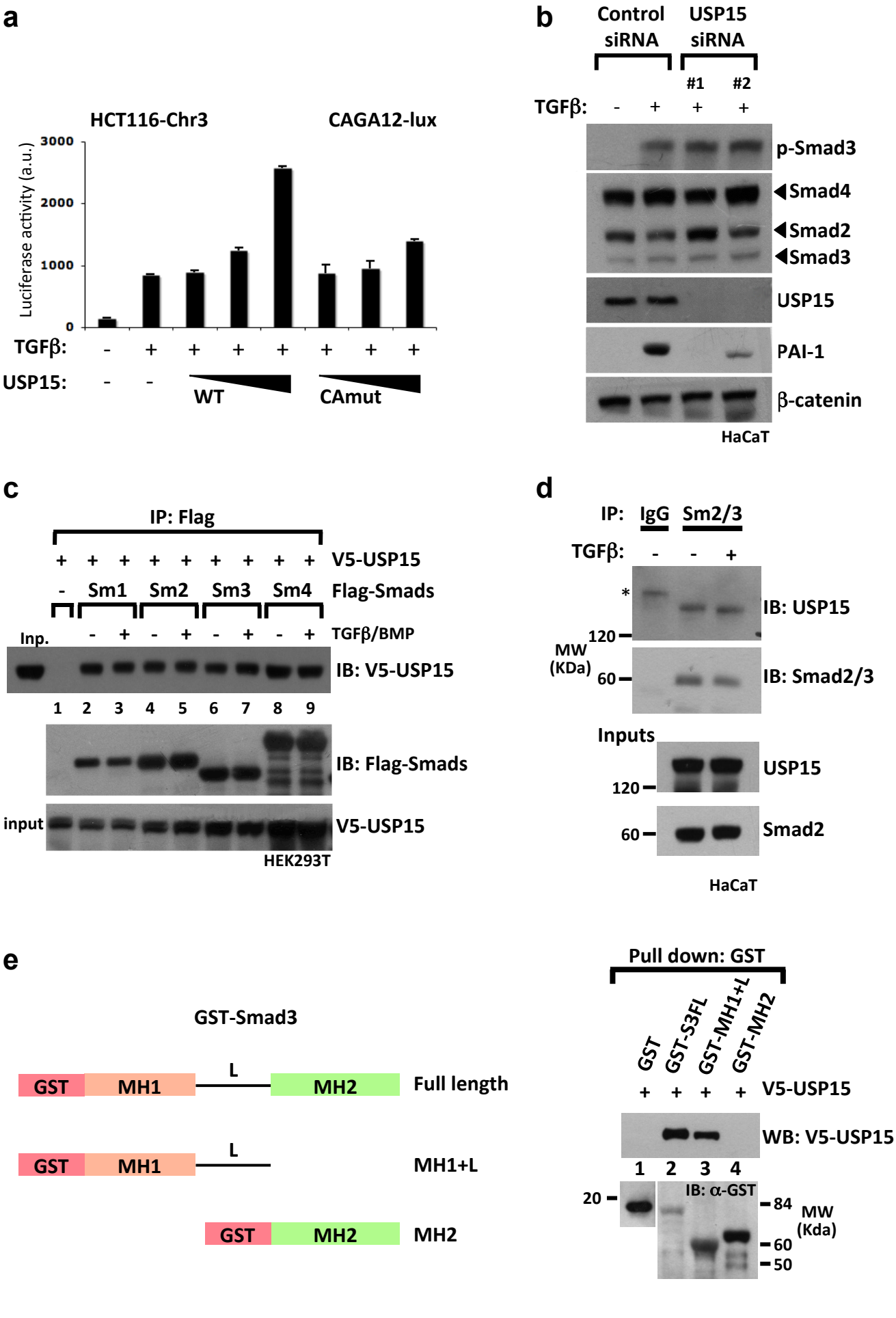

a
HCT116-Chr3
CAGA12-lux
Luciferase activity (a.u.)
3000
2000
1000
0
TGFβ: - + + + + + + +
USP15: - - WT CAmut
b
Control siRNA
USP15 siRNA
#1
#2
TGFβ: - + + +
p-Smad3
Smad4
Smad2
Smad3
USP15
PAI-1
β-catenin
HaCaT
c
IP: Flag
V5-USP15
Sm1 Sm2 Sm3 Sm4
Flag-Smads
Inp.
TGFβ/BMP
IB: V5-USP15
1 2 3 4 5 6 7 8 9
IB: Flag-Smads
input
V5-USP15
HEK293T
d
IP: IgG Sm2/3
TGFβ: - - +
*
IB: USP15
120
MW (KDa)
60
IB: Smad2/3
Inputs
USP15
120
60
Smad2
HaCaT
e
GST-Smad3
GST
MH1
L
MH2
Full length
MH1+L
MH2
Pull down: GST
GST
GST-S3FL
GST-MH1+L
GST-MH2
V5-USP15
WB: V5-USP15
1 2 3 4
20
IB: α-GST
84
60
50
MW (Kda)

Figure 6. USP15 deubiquitylates R-Smads

a. R-Smads are primarily mono/oligo ubiquitylated. HEK293T cells were transfected with the indicated expression plasmids encoding HA-ubiquitin, Flag-Smads and Myc- Smurf1. Immunoprecipitation (IP) was carried out with anti-Flag resin followed by western blot (IB) with anti-Flag or anti-HA antibody. Monoubiquitylated (Ub1-Smad1, Ub1-Smad3), diubiquitylated (Ub2) or polyubiquitylated isoforms are indicated. Asterisk indicates the IgG band.

b. Gain of USP15 reverts Smad2 mono/di-ubiquitylation. Expression of USP15 (WT: lane3), but not enzymatically inactive C269A-USP15 (CA) or C269S-USP15 (CS) (lanes 4 and 5), deubiquitylates Smad2.

c. Gain of USP15 reverts Smad3 mono- and diubiquitylation.

d. Effect of USP15 gain of function on Smurf1-induced Smad1 mono-, di- and polyubiquitylation.

e. USP15 depletion enhances Smad3 mono/di-ubiquitylation. Compared to control siRNA (lane 3), transfection of two independent siRNAs against USP15 (USP15si #1 and #2) leads to increased Smad3 ubiquitylation (lanes 4 and 5). Asterisk indicates the band of IgG.

f. USP15 depletion promotes Smad1 mono-, di- and polyubiquitylation triggered by Smurf1.

g. Effects of USP15 gain and loss of function on endogenous Smad2 mono- and diubiquitylation.

h. Transfection of USP15 siRNAs does not affect the level of monoubiquitylated-Smad4 (Ub1-Smad4). Flag-tagged Smad4 protein was immunoprecipitated with anti-Smad4 antibody.

i. USP15 deubiquitylates Smad3 in vitro. Upper panel: input is a preparation of Flag- affinity purified Smad3 from HEK293T cell lysates, that contains monoubiquitylated Smad3 (as visualized by anti-HA western blotting and molecular weight). This preparation was used as substrate for in vitro deubiquitylation reaction (see Experimental Procedures). Bottom panel: V5 affinity-purified USP15 (WT), but not C269A mutant (CA), deubiquitylates Smad3.

FIGURE 6

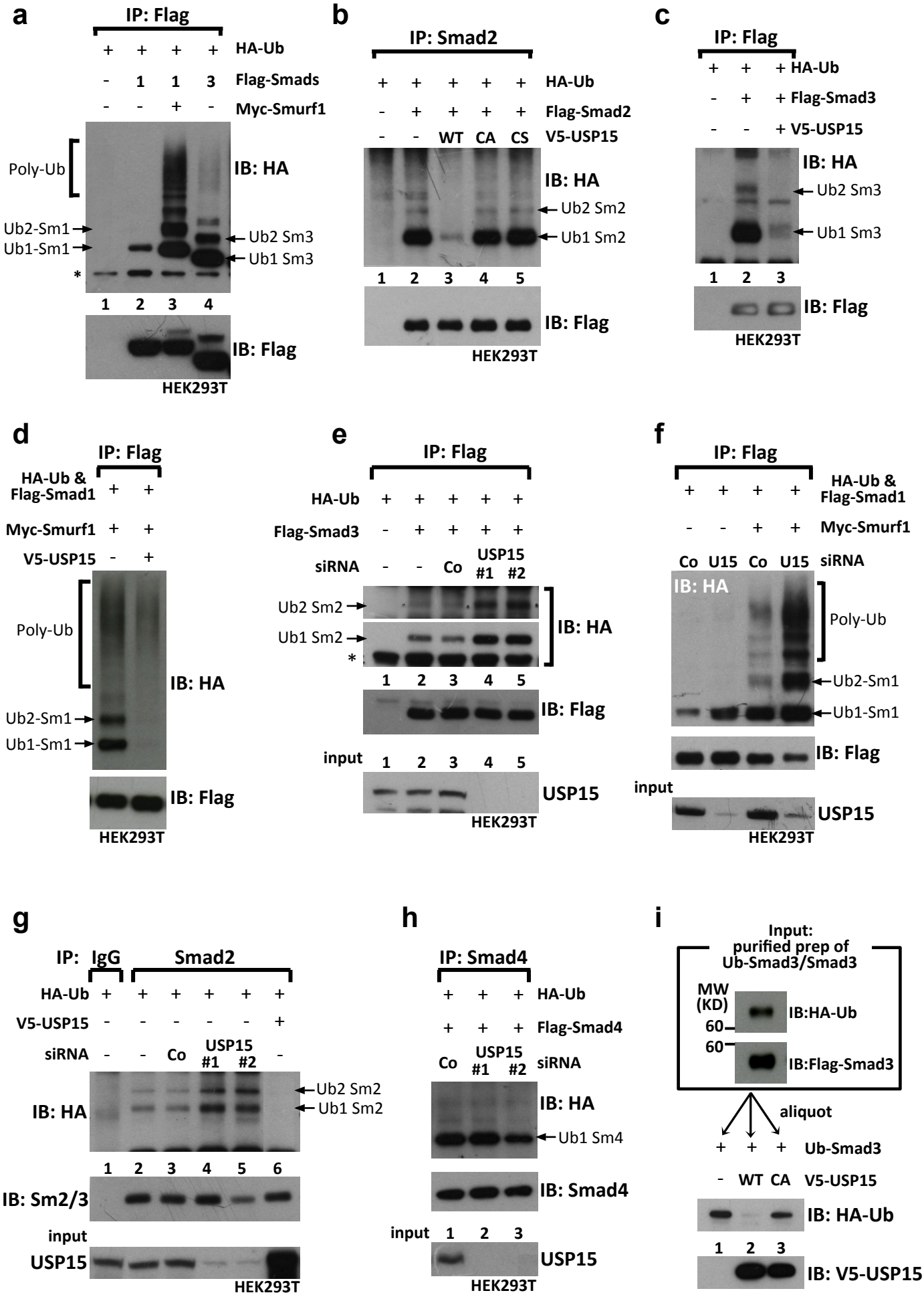

a
IP: Flag
HA-Ub
Flag-Smads
Myc-Smurf1
Poly-Ub
IB: HA
Ub2-Sm1
Ub1-Sm1
Ub2 Sm3
Ub1 Sm3
IB: Flag
HEK293T
b
IP: Smad2
HA-Ub
Flag-Smad2
V5-USP15
WT CA CS
IB: HA
Ub2 Sm2
Ub1 Sm2
IB: Flag
HEK293T
c
IP: Flag
HA-Ub
Flag-Smad3
V5-USP15
IB: HA
Ub2 Sm3
Ub1 Sm3
IB: Flag
HEK293T
d
IP: Flag
HA-Ub &
Flag-Smad1
Myc-Smurf1
V5-USP15
Poly-Ub
IB: HA
Ub2-Sm1
Ub1-Sm1
IB: Flag
HEK293T
e
IP: Flag
HA-Ub
Flag-Smad3
siRNA
Co
USP15 #1 #2
Ub2 Sm2
Ub1 Sm2
IB: HA
IB: Flag
input
USP15
HEK293T
f
IP: Flag
HA-Ub &
Flag-Smad1
Myc-Smurf1
Co U15 Co U15 siRNA
IB: HA
Poly-Ub
Ub2-Sm1
Ub1-Sm1
IB: Flag
input
USP15
HEK293T
g
IP: IgG Smad2
HA-Ub
V5-USP15
siRNA
Co
USP15 #1 #2
IB: HA
Ub2 Sm2
Ub1 Sm2
IB: Sm2/3
input
USP15
HEK293T
h
IP: Smad4
HA-Ub
Flag-Smad4
Co
USP15 #1 #2 siRNA
IB: HA
Ub1 Sm4
IB: Smad4
input
USP15
HEK293T
i
Input:
purified prep of
Ub-Smad3/Smad3
MW (KD)
60
60
IB:HA-Ub
IB:Flag-Smad3
aliquot
Ub-Smad3
WT CA V5-USP15
IB: HA-Ub
IB: V5-USP15

Figure 7. Monoubiquitylation regulates R-Smads activity independently from polyubiquitylation and occurs in the nucleus

a. HEK293T cells were transfected with indicated siRNAs, 30 ng of Flag-Smad1 and 100 ng or 1000 ng of Myc-Smurf1. Cells were treated with BMP2 (50 ng/ml) for 2 hr and subjected to western blotting. Cells were treated with MG132 for 8 hr where indicated.

b. Proteasome inhibitor treatment (MG132, 8 hrs) does not stabilize monoubiquitylated form of Smad1 (compare lane 2 and 3) or Smad3 (compare lane 4 and 5) whereas polyubiquitylated form of Smad4 R100T mutant (Smad4*) is significantly stabilized (lane 6 and 7).

c. Upon nuclear-cytoplasmic fractionation of HEK293T cell lysates, monoubiquitylated-Smad3 is enriched in the nuclear fraction (see methods). C: cytoplasmic N: nuclear.

d. Ectopic addition of nuclear localization signal (NLS) or nuclear exporting signal (NES) efficiently shifted the subcellular distribution of overexpressed Flag-Smad3. FAM and Ecto serve as control of proper fractionation. C: cytoplasmic N: nuclear.

e. Increased monoubiquitylation in NLS-Smad3 compared to wild-type Smad3. Note that the addition of NLS leads to reduced electrophoretic mobility of the protein in SDS-PAGE.

f. Decreased monoubiquitylation in NES-Smad3 compared to wild-type Smad3. Note that the addition of NES leads to reduced electrophoretic mobility of the protein in SDS-PAGE.

g. TGFβ treatment promotes Smad3 monoubiquitylation.

h. BMP treatment promotes Smad1 monoubiquitylation.

FIGURE 7

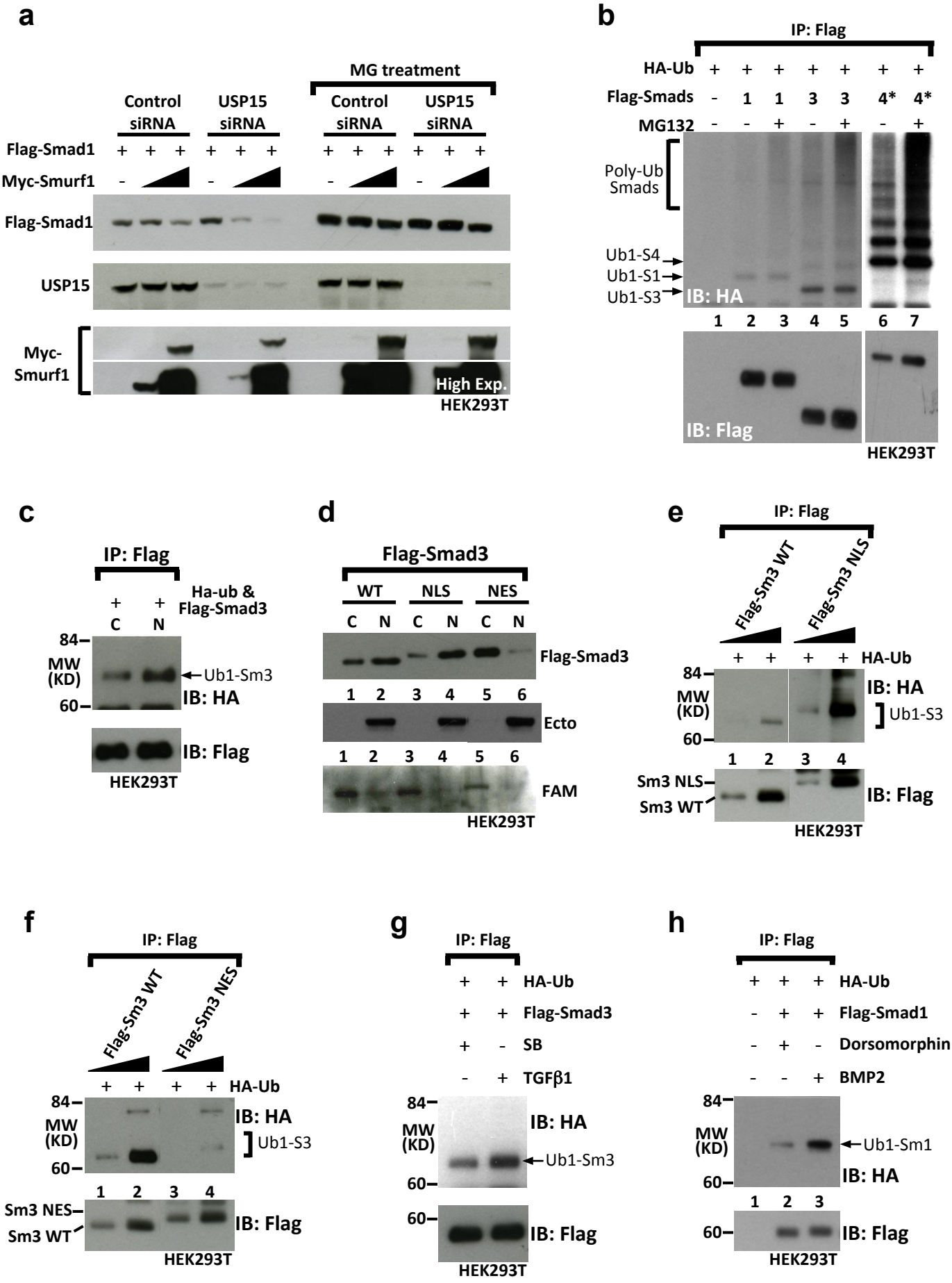

a
MG treatment
Control siRNA
USP15 siRNA
Control siRNA
USP15 siRNA
Flag-Smad1
Myc-Smurf1
Flag-Smad1
USP15
Myc-Smurf1
High Exp.
HEK293T
b
IP: Flag
HA-Ub
Flag-Smads
MG132
Poly-Ub Smads
Ub1-S4
Ub1-S1
Ub1-S3
IB: HA
IB: Flag
HEK293T
c
IP: Flag
Ha-ub & Flag-Smad3
C N
MW (KD)
84
60
Ub1-Sm3
IB: HA
IB: Flag
HEK293T
d
Flag-Smad3
WT NLS NES
C N C N C N
Flag-Smad3
Ecto
FAM
HEK293T
e
IP: Flag
Flag-Sm3 WT
Flag-Sm3 NLS
HA-Ub
MW (KD)
84
60
IB: HA
Ub1-S3
Sm3 NLS
Sm3 WT
IB: Flag
HEK293T
f
IP: Flag
Flag-Sm3 WT
Flag-Sm3 NES
HA-Ub
MW (KD)
84
60
IB: HA
Ub1-S3
Sm3 NES
Sm3 WT
IB: Flag
HEK293T
g
IP: Flag
HA-Ub
Flag-Smad3
SB
TGFβ1
MW (KD)
84
60
IB: HA
Ub1-Sm3
IB: Flag
HEK293T
h
IP: Flag
HA-Ub
Flag-Smad1
Dorsomorphin
BMP2
MW (KD)
84
60
Ub1-Sm1
IB: HA
IB: Flag
HEK293T

Figure 8. USP15 deubiquitylates R-Smads both in the cytoplasm and in the nucleus

a. Phosphorylated Smad3 is monoubiquitylated and USP15 gain of function reverts phospho-Smad3 monoubiquitylation.

b. TGFβ treatment promotes Smad3 mono-ubiquitylation in control cells (lane 3) but this does not occur in Smad4-depleted cells (lane 5).

c. Treatment with transcriptional inhibitors Flavopiridol and α-Amanitin inhibits Smad2 monoubiquitylation. HEK293T cells were transfected with indicated plasmid, pre-treated with Flavopiridol or α-Amanitin for 2 hr and treated with TGFβ1 (1 ng/ml, 1 hr).

d. USP15 is detected in both nuclear and cytoplasmic fractions of HaCaT cell. LaminB and FAM serve as control of proper fractionation.

e. USP15 and Smad3 interact both in cytoplamic and nuclear fractions. HEK293T cells were transfected with the indicated plasmids. After nuclear/cytoplasmic fractionation the fractions were subjected to Flag affinity purification and western blot. Ecto and FAM serve as control of proper fractionation.

f. Gain of USP15 reduces monoubiquitylated form of Smad3 in both cytoplasmic (Cyto. compare lane 1 and 2) and nuclear (compare lane 3 and 4) fractions.

g. USP15 binds nuclear phosphorylated pool of Smad3. C: cytoplasmic N: nuclear.

FIGURE 8

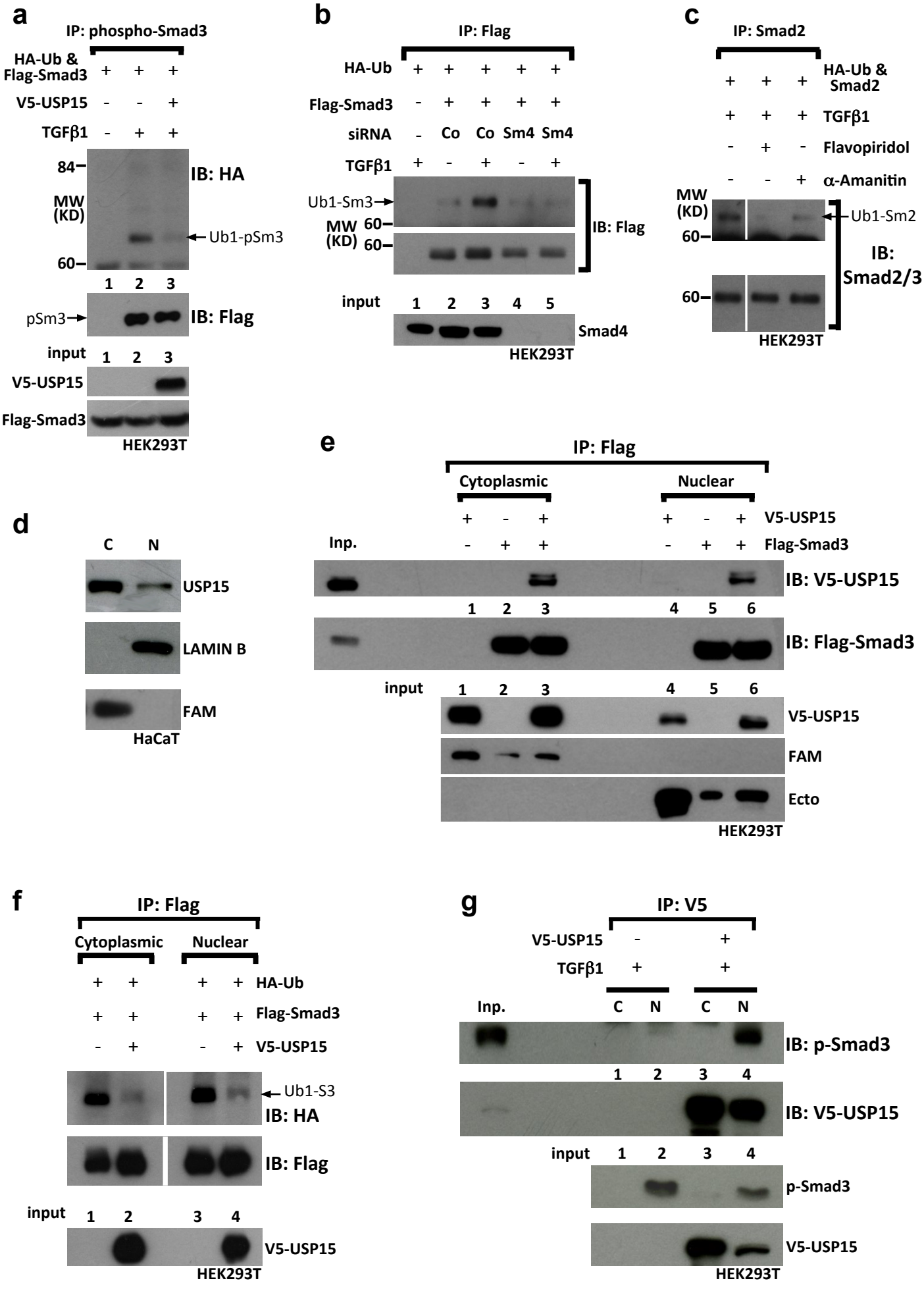

a
IP: phospho-Smad3
HA-Ub & Flag-Smad3 + + +
V5-USP15 - - +
TGFβ1 - + +
84
MW (KD)
60
IB: HA
Ub1-pSm3
1 2 3
pSm3
IB: Flag
input 1 2 3
V5-USP15
Flag-Smad3
HEK293T
b
IP: Flag
HA-Ub + + + + +
Flag-Smad3 - + + + +
siRNA - Co Co Sm4 Sm4
TGFβ1 + - + - +
Ub1-Sm3
MW (KD) 60
60
IB: Flag
input 1 2 3 4 5
Smad4
HEK293T
c
IP: Smad2
+ + + HA-Ub & Smad2
+ + + TGFβ1
- + - Flavopiridol
- - + α-Amanitin
MW (KD)
60
Ub1-Sm2
IB: Smad2/3
60
HEK293T
d
C N
USP15
LAMIN B
FAM
HaCaT
e
IP: Flag
Cytoplasmic
Nuclear
+ - + + - + V5-USP15
Inp. - + + - + + Flag-Smad3
IB: V5-USP15
1 2 3 4 5 6
IB: Flag-Smad3
input 1 2 3 4 5 6
V5-USP15
FAM
Ecto
HEK293T
f
IP: Flag
Cytoplasmic
Nuclear
+ + + + HA-Ub
+ + + + Flag-Smad3
- + - + V5-USP15
Ub1-S3
IB: HA
IB: Flag
input 1 2 3 4
V5-USP15
HEK293T
g
IP: V5
V5-USP15 - +
TGFβ1 + +
Inp. C N C N
IB: p-Smad3
1 2 3 4
IB: V5-USP15
input 1 2 3 4
p-Smad3
V5-USP15
HEK293T

Figure 9. USP15 is required for R-Smads binding to the promoter

a. Localization of Smad2/3 was visualized by immunofluorescence. In control cells, Smad2/3 protein (red signal) localizes in both cytoplasm and nuclei, and accumulates into nuclei upon TGFβ1 treatment. Nuclear accumulation and exit of Smad2/3 occurs equally in control and USP15-depleted cells. DAPI staining (blue) indicates nuclei. After a pulse of TGFβ treatment, cells were washed and incubated with the SB505124 TGFβ receptor inhibitor for the indicated time.

b. USP15 is not required for Smad2/3-Smad4 complex formation. HaCaT cells were transfected with indicated siRNAs and left untreated or treated with 1 ng/ml TGFβ1 for 1 hour. Immunoprecipitation (IP) was performed with anti-Smad4 antibody or pre-immune mouse IgG (IgG).

c. USP15 is required for the association of the Smad complex to DNA after TGFβ stimulation. Panels are western blots of endogenous Smad complexes. Smad4 and R- Smads were affinity purified from extracts of TGFβ treated cells using biotinylated DNA oligonucleotides corresponding to a multimerized SBE sequence.

d. USP15 is required for TGFβ signaling through its activity on the DNA binding domain of Smad2/3 (see Results).

e. Endogenous Smad2/4 complex is co-purified with ARE oligo DNA upon TGFβ signaling, both from control (lane 2) and USP15-depleted (lane 4) MDA231 cells lysate. *Xenopus* Fast1 expression plasmid was transfected in all samples. Note that using same cell extract we have observed that loss of USP15 inhibited the binding of Smad3/4 complex to SBE oligo DNA (data not shown).

f. Left panels are chIP assays to measure the occupancy of the active Smad complexes on endogenous targets (two independent SBEs for the $p21^{Waf1}$ promoter). Right panels are the inputs of chip assays.

FIGURE 9

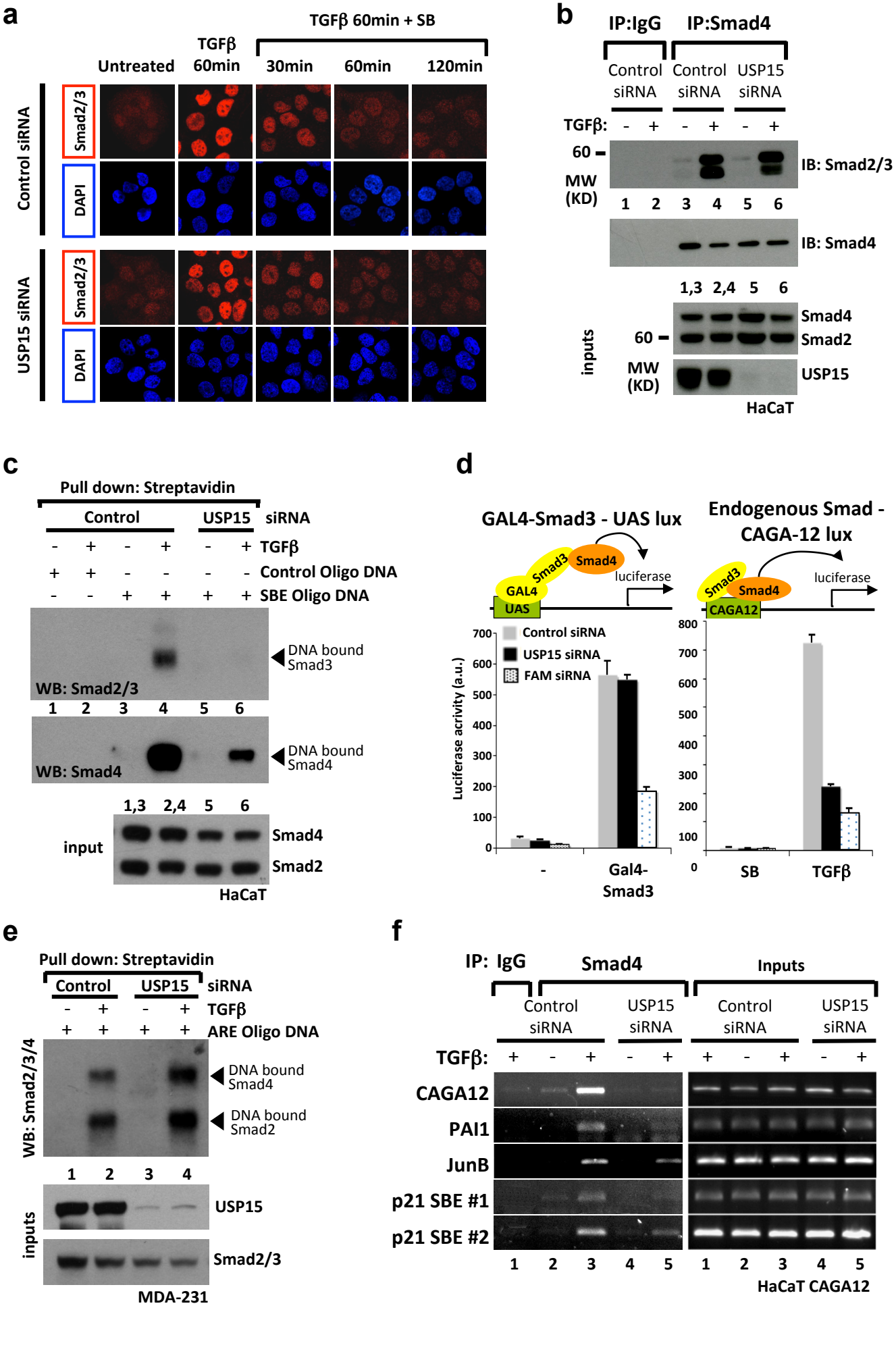

a
TGFβ 60min + SB
TGFβ 60min
Untreated
30min
60min
120min
Control siRNA
USP15 siRNA
Smad2/3
DAPI
b
IP:IgG
IP:Smad4
Control siRNA
Control siRNA
USP15 siRNA
TGFβ: - + - + - +
60
MW (KD)
1 2 3 4 5 6
IB: Smad2/3
IB: Smad4
1,3 2,4 5 6
inputs
Smad4
Smad2
USP15
HaCaT
c
Pull down: Streptavidin
Control
USP15
siRNA
TGFβ
Control Oligo DNA
SBE Oligo DNA
DNA bound Smad3
WB: Smad2/3
DNA bound Smad4
WB: Smad4
1,3 2,4 5 6
input
Smad4
Smad2
HaCaT
d
GAL4-Smad3 - UAS lux
Endogenous Smad - CAGA-12 lux
Smad3
Smad4
GAL4
UAS
CAGA12
luciferase
Control siRNA
USP15 siRNA
FAM siRNA
Luciferase acrivity (a.u.)
-
Gal4-Smad3
SB
TGFβ
e
Pull down: Streptavidin
Control
USP15
siRNA
TGFβ
ARE Oligo DNA
WB: Smad2/3/4
DNA bound Smad4
DNA bound Smad2
1 2 3 4
inputs
USP15
Smad2/3
MDA-231
f
IP: IgG
Smad4
Inputs
Control siRNA
USP15 siRNA
Control siRNA
USP15 siRNA
TGFβ: + - + - + + - + - +
CAGA12
PAI1
JunB
p21 SBE #1
p21 SBE #2
1 2 3 4 5 1 2 3 4 5
HaCaT CAGA12

Figure 10. Monoubiquitylation of R-Smads opposes to DNA binding

a. Top panel: diagram of Smad3 lysine mutants. Bottom panel: monoubiquitylation of Smad3 was used to screen individual or combined lysine-to arginine (KR) mutants.

b. HEK293T cells were transfected with CAGA12-luciferase reporter and the indicated plasmids. Cells were left unstimulated or stimulated with TGFβ1 (1ng/ml, 8 hr). Smad3 bearing mutations in lys-33 and lys-81 does not induce the reporter. Data are shown as mean and SD.

c. Crystallographic structure of the Smad3 MH1 domain bound to DNA, as previously described (Chai et al., 2003) (top panel). The location of the three lysines found positive in the ubiquitylation mapping of Fig. 10a is shown in yellow. Bottom panel: in silico modeling of K81 ubiquitylated Smad3 (ubiquitin in green). Note that this structure is incompatible with DNA major groove recognition. A model of K33 ubiquitylated Smad3 (data not shown) leads to the same conclusion. The functional significance of K53 ubiquitylation remains to be determined.

d. Ubiquitinated Smad3 does not bind DNA. Left panel: input is a preparation of purified monoubiquitylated Smad3/non-ubiquitylated Smad3 eluted as a 1:2 mix after dual anti- Flag and anti-HA purification. 20 μl, 10 μl and 5 μl of this preparation are loaded for α-Flag immunoblot (IB). Right panel: DNA pulldown with the indicated oligonucleotides shows that unmodified Smad3 binds to DNA but ubiquitylated Smad3 does not.

e. In vitro Smad3 ubiquitylation by recombinant HECT E3 ligases. Affinity purified Smad3 bound on anti-Flag resin was subjected to in vitro ubiquitylation assay with purified Smurf2. Similar results were obtained with recombinant NEDD4 (not shown).

f. In vitro ubiquitylation dissociates Smad3 from DNA. Smad3 bound on SBE DNA (on streptavidin beads) was subjected to ubiquitylation reaction (see Experimental Procedures). Lane 1: detection of Smad3 that remains bound to the DNA beads after incubation in ubiquitylation buffer (but in absence of E3); lane 2: upon addition of purified E3, Smads dissociates from DNA. The experiment here shown was carried out with a 1:1 mix of Smurf2 and NEDD4, but similar results were obtained using individual enzymes (not shown); lane 3 and 4 are western blots of the DNA-beads supernatants. Lane 3: residual non-ubiquitylated Smad3 that passively diffuses from DNA; lane 4: mono, oligo and polyubiquitylation status of Smad3 dissociated from DNA.

FIGURE 10

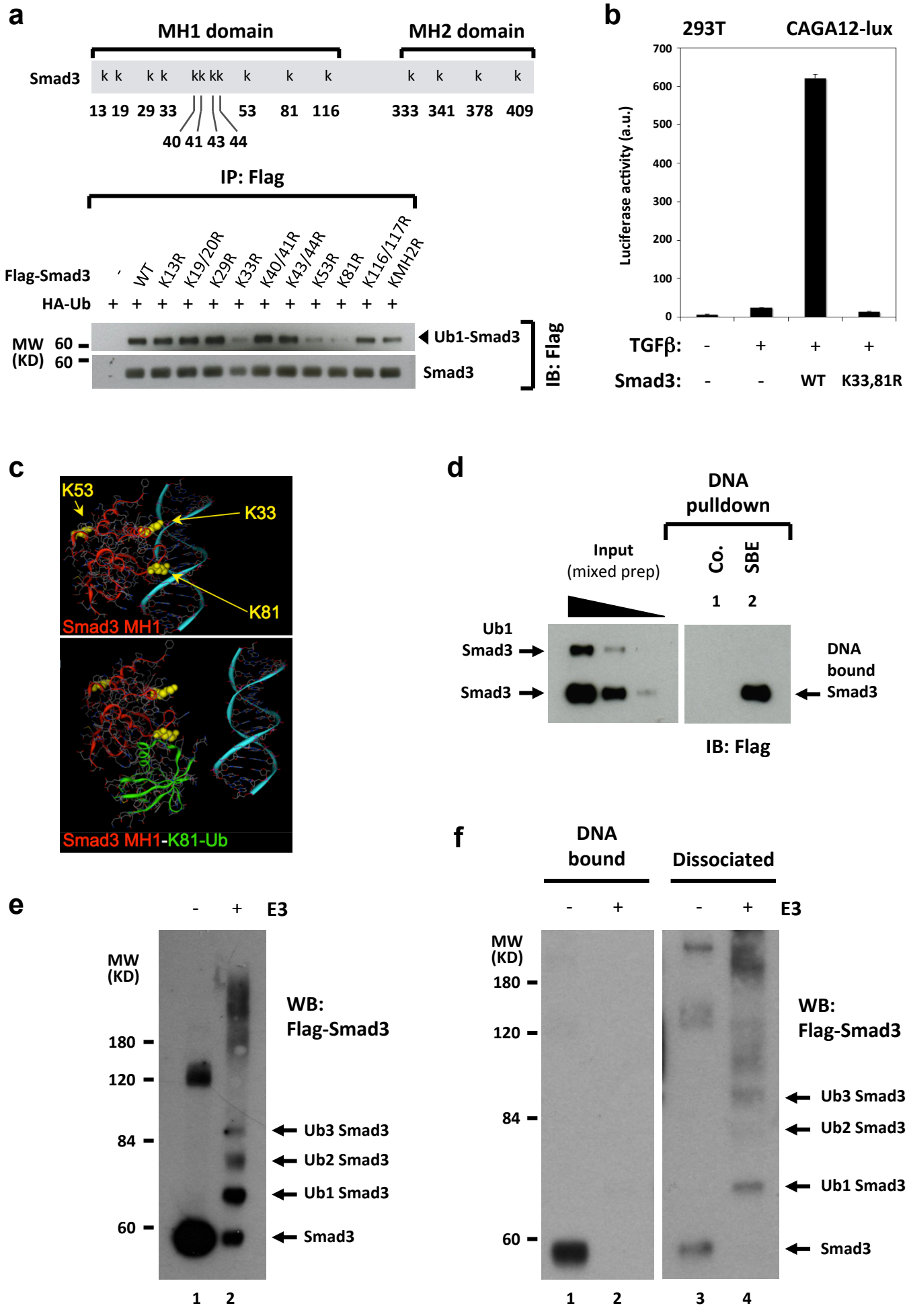
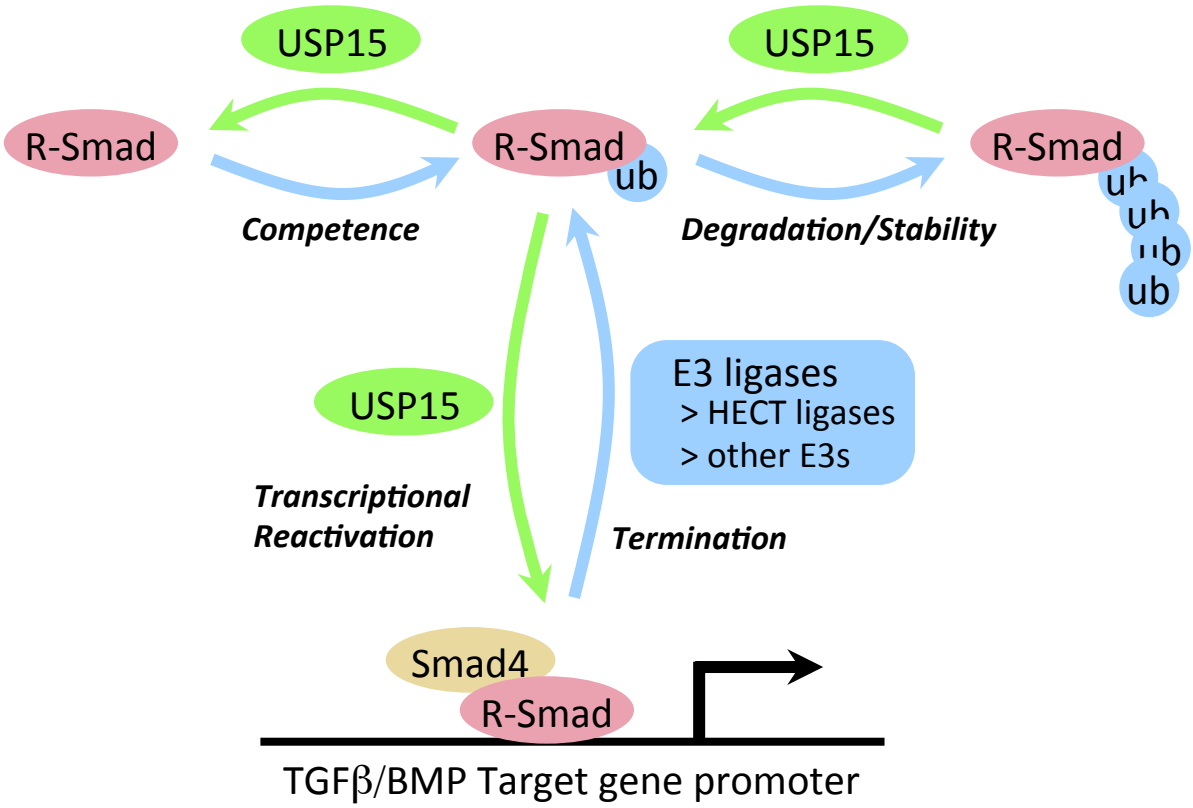

a
MH1 domain
MH2 domain
Smad3
k k k k kk kk k k k k k k k
13 19 29 33 40 41 43 44 53 81 116 333 341 378 409
IP: Flag
Flag-Smad3
WT K13R K19/20R K29R K33R K40/41R K43/44R K53R K81R K116/117R KMH2R
HA-Ub + + + + + + + + + + + +
MW (KD) 60 60
Ub1-Smad3
Smad3
IB: Flag
b
293T CAGA12-lux
Luciferase activity (a.u.)
700 600 500 400 300 200 100 0
TGFβ: - + + +
Smad3: - - WT K33,81R
c
K53
K33
K81
Smad3 MH1
Smad3 MH1-K81-Ub
d
DNA pulldown
Input (mixed prep)
Co.
SBE
1 2
Ub1 Smad3
Smad3
DNA bound Smad3
IB: Flag
e
- + E3
MW (KD)
180 120 84 60
WB: Flag-Smad3
Ub3 Smad3
Ub2 Smad3
Ub1 Smad3
Smad3
1 2
f
DNA bound
Dissociated
- + - + E3
MW (KD)
180 120 84 60
WB: Flag-Smad3
Ub3 Smad3
Ub2 Smad3
Ub1 Smad3
Smad3
1 2 3 4

Figure 11. A model for R-Smad regulation by USP15

R-Smads can be monoubiquitylated by E3-ligases (such as members of the HECT family, as Smurf1, Smurf2 and Nedd4), when they are on-DNA for a round of transcription, or when they are off-DNA.
In the first case monoubiquitylation works as a **termination** mechanism detaching active R-Smad from DNA. In the second case monoubiquitylation opposes to R-Smads DNA binding thus limiting the number of R-Smads available for transcription; this defines the **competence** of the system to respond to signal.
Acting in proximity of DNA USP15 deubiquitylates R-Smads modified on the promoters, thus leading to R-Smads **transcriptional reactivation**. Alternatively USP15 deubiquitylates R-Smads modified off-DNA contributing to set the **competence** of the system.
In particular contexts (see Discussion) **polyubiquitylation** and subsequent **degradation** of R-Smads can eventually become relevant. In these contexts, by opposing R-Smads polyubiquitylation, USP15 will play a role also in the regulation of R-Smads stability.

FIGURE 11


USP15
USP15
R-Smad
R-Smad
ub
R-Smad
ub
ub
ub
ub
Competence
Degradation/Stability
USP15
E3 ligases
> HECT ligases
> other E3s
Transcriptional
Reactivation
Termination
Smad4
R-Smad
TGFβ/BMP Target gene promoter

ACKNOWLEDGMENTS

I would like to thank Prof. Stefano Piccolo for giving me the opportunity to join his lab and to learn a lot over the past four years.
Thank Masa for all your help and suggestions, you've been really a great master.
Thanks to all the lab members for sharing with me the everyday life in the lab.

Grazie ai miei genitori e ad Alice per avermi sempre supportato e dato forza con il loro amore.