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Claire Nourry (claire_nourry@hotmail.com)
Lola Maksumova (lmaksumo@interchange.ubc.ca)
Mona Pang (mpang@benaroyaresearch.org)
Xiaohong Liu (liaohongliu_99@yahoo.com)
Tongwen Wang (wangt@benaroyaresearch.org)

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Direct interaction between Smad3, APC10, CDH1 and HEF1 in proteasomal degradation of HEF1

Claire Nourry[1, 2], Lola Maksumova[1,3], Mona Pang[1], Xiaohong Liu[4], and Tongwen Wang[1]

[1] Benaroya Research Institute at Virginia Mason, 1201 Ninth Avenue, Seattle, WA 98101,USA, email: wangt@vmresearch.org

[2] INSERM U119 - Molecular Oncology, 27 boulevard Leï Roure, 13009 Marseille, FRANCE, email: claire_nourry@hotmail.com

[3] Department of Pediatrics, British Columbia Research Institute, UBC, 950 28th Avenue West, Vancouver, BC, V5Z4H4, email: farlol@hotmail.com

[4]Serono Reproductive Biology Institute, One Technology Place, Rockland, MA 02370, USA, liaohongliu_99@yahoo.com

Corresponding Author:

Dr. Tongwen Wang

Benaroya Research Institute at Virginia Mason

1201 9th Ave

Seattle, WA 98101

Phone: (206)-223-6842 (office); Fax: (206)-223-7543 ; *E-mail:*

wangt@vmresearch.org

Abstract

Background: The Transforming Growth Factor-β (TGF-β) regulates myriad cellular events by signaling through members of the Smad family signal transducers. As a key signal transducer of TGF-β, Smad3 exhibits the property of receptor-activated transcriptional modulator and also the novel ability of regulating the proteasomal degradation of two Smad3 interacting proteins, SnoN and HEF1. It has been shown that Smad3 recruits two types of Ub E3 ligases, Smurf2 and the Anaphase Promoting Complex (APC), to mediate SnoN ubiquitination, thereby enhancing SnoN degradation. The molecular mechanisms underlying Smad3-regulated HEF1 degradation are not well understood. Furthermore, it is not clear how Smad3 recruits the APC complex.

Results: We detected physical interaction between Smad3 and an APC component APC10, as well as the interaction between HEF1 and CDH1, which is the substrate-interacting component within APC. Detailed domain mapping studies revealed distinct subdomains within the MH2 domain of Smad3 for binding to APC10 and HEF1 and suggests the formation of a complex of these four proteins (Smad3, HEF1, APC10 and CDH1). In addition, the protein levels of HEF1 are subjected to the regulation of overexpressed APC10 and CDH1.

Conclusions: Our data suggests that Smad3 may recruit the APC complex via a direct interaction with the APC subunit APC10 to regulate the ubiquitination and degradation of its interactor HEF1, which is recognized as an ubiquitination substrate by the CDH1 subunit of the APC complex.

Background

The Transforming Growth Factor β (TGF-β) superfamily consists of a large group of structurally related polypeptides including various forms of TGF-β, bone morphogenic proteins (BMPs), activins, growth and differentiation factors (GDFs) and the Anti-Mullerian Hormone (AMH, or MIS) [1,2]. Members of the TGF-β subfamily are molecular organizers for tissue and organ morphogenesis during embryonic development and play key roles in maintaining the homeostasis of various developed systems [3,4]. At the cellular level, diverse processes including cell proliferation, differentiation, adhesion and apoptosis are subjected to TGF-β regulation [5]. The intracellular signaling events are initiated upon TGF-β binding to a pair of Ser/Thr kinase receptors known as the Type I receptor (TβR1) and the Type II receptor (TβRII), which are structurally similar but functionally distinct [6,7]. Upon binding to TGF-β, the Type II receptor recruits and activates the type I receptor by releasing the immunophilin FKBP12 from the type I receptor and also by mediating the trans-phosphorylation of the type I receptor at the GS domain, a highly conserved thirty-amino acid region containing a SGSGSG sequence [8-10]. The GS domain phosphorylation allows the Type I receptor to recruit and phosphorylate the cytoplasmic proteins belonging to the family of the Smad proteins [11,12].

The Smad proteins are the vertebrate homologues of the mothers against Dpp in *Drosophila* and the *C. elegans* Sma proteins [5,13-16]. Based upon their functional properties, Smad proteins are divided into three classes: 1) the receptor-regulated Smads, or R-Smads, which are phosphorylated respectively by TGF-β/activin receptors for Smad2 and Smad3 and by BMP receptors for Smad1, Smad5 and Smad8; 2) the co-mediator Smad (Co-Smads), namely Smad4 in mammals and Smad10 in *Xenopus* and 3) the inhibitor Smads (I-Smads) Smad6 and Smad7 which prevent the phosphorylation of the R-Smads and sometimes the formation of a complex between R-Smads and Co-

Smads. Smad proteins have two conserved globular domains, namely N or Mad homology 1 (MH1) domain and C or MH2 domain, separated by a linker region of variable length and which can associate with one another in the inactive state. Upon phosphorylation by TGF-β type I receptor, Smad2 and Smad3 form a complex with Smad4 and translocate into the nucleus where they function as transcription factors. However, a novel activity of Smad3 in regulating the proteasomal degradation of the nuclear proto-oncoprotein SnoN and of the human enhancer of filamentation 1 (HEF1) has recently been reported. The data also suggest possible roles of proteasomal degradation of HEF1 and SnoN in a negative feedback mechanism of the TGF-β signaling pathway [17-19].

HEF1 was first isolated in a screen for human proteins capable of inducing pseudohyphal growth in *Saccharomyces cerevisiae* [20]. HEF1, also known as CasL, is a cytoplasmic docking protein belonging to the Cas family and structurally related to p130Cas and Efs. HEF1 is predominantly expressed in epithelial cells and lymphocytes. It contains multiple protein-protein interaction domains including a N-terminal SH3 domain that binds polyproline-containing protein, a domain containing multiple SH2 binding sites, a Serine-rich domain and a conserved C-terminal domain containing a helix-loop-helix (HLH) motif [21]. HEF1 is processed in a complex manner since at least four protein species ($p55^{HEF1}$, $p65^{HEF1}$, $p105^{HEF1}$ and $p115^{HEF1}$) can result from a single cDNA expressed *in vivo* in a cell cycle-dependent manner. $p105^{HEF1}$ and $p115^{HEF1}$ represent different phosphorylation states of the full length HEF1 and are more predominantly cytoplasmic whereas $p55^{HEF1}$ arises through cleavage of the full length HEF1 during mitosis [17,22]. HEF1 has been implicated in many pathways including the signaling pathway of integrin, T-cell antigen receptor (TCR), B-cell receptor (BCR), the G protein coupled calcitonin receptor, cell adhesion as well as in the progression of the cell cycle through mitosis [22-26]. HEF1 has also recently been

described as an apoptotic mediator at focal adhesion sites [21]. However the exact nature of the signaling events associated with HEF1 is still unknown.

In TGF-β induced signaling events mediated by Smad3, it has been shown that Smad3 interacts with both the N-terminus and the C-terminus domains of HEF1 via its MH1 and MH2 domains respectively [17]. Such an interaction appears to trigger rapid proteasomal degradation of HEF1. A similar example is the ability of Smad3 to bind to the nuclear transcriptional co-repressor SnoN, which is also degraded by the proteasome pathway upon binding to Smad3 [18,19].

The 26S proteasome is a large proteolytic complex present both in the nucleus and the cytosol of eukaryotic cells. Most known proteasome substrate proteins require polyubiquitination to be targeted to the proteasome for degradation. Ubiquitination occurs through the sequential action of three enzymes [27]. First, the activating enzyme E1 activates ubiquitin. Ubiquitin is then transferred to an E2 ubiquitin conjugating enzyme or Ubc. Finally, an ubiquitin protein ligase or E3 enzyme covalently attaches the ubiquitin to lysine residues present on the substrate protein. There are several classes of E3 ligase which appear to be responsible for substrate selectivity by binding directly or indirectly to the protein substrate: 1) N-end rule E3s; 2) the HECT (Homologous to E6-AP C-terminus) domain family which includes the Smurf family and 3) the RING finger containing E3s. The latest are often part of a large protein complex such as the SCF (Skp1 cullin F-box protein) or the APC (Anaphase-promoting complex) which both belong to the cullin subfamily [28].

The mechanism of Smad3-binding-dependent proteasomal degradation of HEF1 is unknown. For SnoN, it has been shown to involve at least two different mechanisms. First, Smad3 has been shown to recruit the HECT family E3 ligase Smurf2 to regulate SnoN ubiquitination [18]. Smad3 has also been shown to recruit the APC complex to regulate SnoN degradation [18,19]. The APC or cyclosome is a cell cycle-regulated

complex composed of at least eleven subunits in mammalian cells, APC11 being the RING finger containing subunit. It is required for the metaphase-anaphase transition and cyclin degradation. Two WD-40 proteins, namely CDC20 and CDH1, activate APC by direct binding during mitosis and G1 phase respectively [29]. CDC20 only interacts with proteins containing a destruction box or D-box whereas CDH1 can recognize either the D-box or the KEN-box motifs present both in cell-cycle specific and non cell-cycle proteins [30]. Most of the APC subunits are conserved from yeast to humans. Preliminary data from the yeast two-hybrid system suggests that Smad3 interacts with APC10. APC10/Doc1 is a one-domain protein that has a conserved core, the Doc domain, which is homologous to domains found in several other putative E3 ligases [31,32]. Doc domain-containing proteins may mediate ubiquitination because they contain combinations of RING finger, cullin or HECT domains [31]. Moreover, additional data suggest that APC10 is essential for APC function in mammals. Indeed, rearrangements in the APC10 gene locus appear to underlie the mitotic arrest phenotype observed in mice homozygous for the embryonic-lethal developmental mutation oligosyndactylism [33]. Structural analyses of APC10 also suggest that APC10 could mediate or modify the ubiquitination reaction of the APC [32,34].

Here we report the biochemical studies of the interaction between Smad3 and APC10, between HEF1 and CDH1, and the evidence for the functional roles of APC10 and CDH1 in Smad3-regulated HEF1 degradation. These studies suggest a new mechanism of Smad3 in regulating the proteasomal degradation of its interacting proteins via recruiting the cell cycle-linked E3 ligase APC.

Results

Smad3 directly interacts with the Anaphase Promoting Complex (APC) subunit APC10

The yeast two-hybrid system developed in Dr. Roger Brent's laboratory was applied to search for Smad3 interacting proteins, as described previously [17]. Like HEF1, APC10 was isolated as a strong interactor for Smad3 (data not shown). APC10 is an integral component of the E3 ligase complex APC and has been suggested to be essential for APC function in mammals [31,32]. Recent observations made by others have suggested that the APC complex is able to mediate ubiquitination of SnoN in a Smad3-dependent fashion [18,19]. However, it is not clear how Smad3 recruits the APC complex. The ability of Smad3 to bind APC10 thus suggests a mechanism for Smad3 to recruit APC complex and point out a possible role of APC complex in Smad3-regulated HEF1 degradation. We therefore carried out more detailed biochemical studies of this pair of interaction in various systems, as described blow.

First, we tested the ability of APC10 to interact with different Smads in a mammalian overexpression system. HA-tagged Smad proteins (Smad1, Smad2, Smad3, Smad4) were transiently expressed in 293 cells together with T7-tagged APC10. The Smads were immunoprecipitated with anti-HA antibody and the co-precipitated T7-tagged APC10 was detected by Western blot using anti-T7 antibody. T7-APC10 was detected to co-precipitate with Smad2, Smad3, but not with Smad1 or Smad4 (Fig. 1A). Thus, the interaction between Smad3 and APC10 is specific and can occur in mammalian cells.

Next we tested the interaction between APC10 and the Smad proteins by GST pull-down assay using GST-APC10 expressed and purified from *Escherichia coli* BL21 and Flag-tagged Smads expressed in 293 cells. In this assay, APC10 again binds to the two R-Smads involved in the TGF-β signaling pathway, Smad2 and Smad3 (Fig. 1B, lane 2 & 3), but not with the Co-Smad, Smad4 (Fig. 1B, lane 4).

The interactions were further tested via *in vitro* binding assays. ^{35}S-labeled *in vitro* translated Smad3 or APC10 proteins were incubated with GST-APC10 or GST-

Smad3, respectively. GST alone was used as a negative control. ^{35}S-labeled Smad3 was detected to bind GST-APC10 (Fig. 1C, lane 6); ^{35}S-labled APC10 was detected to bind GST-Smad3 (Fig. 1C, lane 5). Labeled HEF1 also binds to GST-Smad3 (Fig. 1C, lane 4). These data suggests that Smad3 can interact with both HEF1 and APC10 *in vitro*, most likely via direct interaction.

Smad3 interacts with APC10 via Smad3 MH2 domain and requires both N- and C-terminus of APC10

To understand how APC10 interacts with Smad3, we carried out deletion analyses to determine the domains of interaction on both Smad3 and APC10. First, GST pull down assays were carried out to test the interaction between purified GST-APC10 and Flag-tagged Smad3 and Flag-tagged Smad3 deletion mutants expressed in 293 cells. The tested Smad3 deletion mutants include Smad3NL, Smad3LC, Smad3 MH1 (Smad3N) and Smad3 MH2 (Smad3C) (Fig. 2 A, top panel). Only Smad3LC and Smad3C exhibited the ability to bind APC10 (Fig. 2A, bottom left panel, lanes 2 & 4). The interaction was also tested in a mammalian overexpression system 293 cells, which were co-transfected with T7-APC10 and Flag-Smad3 deletions. APC10 was immunoprecipitated from the cell lysates and the co-precipitated Smad3 and its deletion mutants were detected by Western blot with anti-Flag antibody. Again, only Smad3LC and Smad3C were detected to co-precipitate T7-APC10 (Fig. 2A, bottom right panel, lanes 7 & 9). Thus, the MH2 domain of Smad3 is necessary and sufficient to bind APC10, although a possible role of the linker region of Smad3 in assisting the interaction between Smad3 and APC10 is not ruled out.

Further experiments were carried out to determine Smad3 binding domain on APC10. A set of deletion constructs of APC10 (Fig. 2B, top panel) were made and tested for expression in 293 cells (Fig. 2B, bottom left panel). All of the three N-terminal deletion constructs (D2, D3, & D4) and one C-terminal deletion construct (D7)

failed to express stable proteins (Fig. 2B, bottom left panel, lanes 2-5), while two C-terminal deletion constructs (D8 & D9) were expressed (Fig. 2B, bottom left panel, lanes 6 & 7). Thus, the N-terminal 41 amino acids and an internal domain (a.a.60-a.a 82) play roles in maintaining the stability of APC10. Both APC10 D2 and APC10 D9, when expressed in yeast as fusion proteins of B42, failed to bind the LexA fusion protein of Smad3 in the yeast-two hybrid system (data not shown). GST pull-down assay using GST-Smad3 fusion protein against APC10 D9 confirmed the defect of this deletion mutant of APC10 in binding to Smad3 (Fig. 2B, bottom right panel, lane 4). These studies revealed that the C-terminal 66 amino acids of APC10 is required for Smad3 interaction while the N-terminal 82 amino acids of APC10 is required for the protein stability.

Different subdomains within the MH2 domain of Smad3 are involved in binding to APC10 and HEF1

In order to map more precisely the domain on Smad3 necessary for APC10 binding, we tested several Smad3 deletion mutants within the MH2 domain against GST-APC10. In addition, we also tested the binding property of a Smad3/Smad1/Smad3 chimera, which contains N-terminus a.a.1-236 and C-terminus a.a. 277-424 but with part of the MH2 region of Smad3 (a.a. 237-276) replaced with the co-responding region of Smad1 (a.a. 276-317), as previously described [18]. Since Smad1 does not interact with APC10 in this assay system, any binding to APC10 is due to the Smad3C portion of the chimera. These deletion constructs of Smad3 are illustrated in Figure 3A. These deletion constructs were transfected into the 293 cells and the expression of each protein was monitored by Western blot analyses with anti-Flag monoclonal antibody, since they are all tagged with the Flag epitope (Fig. 3B, top panels). The cell lysates were incubated with purified GST-APC10 proteins, or control GST proteins both of which were absorbed onto Glutathione Sepharose 4 Fast Flow

beads. The bead bound proteins were eluted and analyzed by Western blot (Fig. 3B, middle and bottom panels). Smad3 d2, which contains a deletion of the C-terminal a.a.362-424, still binds strongly with APC10 (Fig. 3B, lane 1). Further deletion of a.a.330-362 (Smad3 d6) led to a significant reduction of interaction (Fig. 3B, lane 3), and more deletion of a.a. 301-330 (Smad3 d4) further weakened the interaction (Fig. 3B, lane 2). Densitometry analyses of the protein signals were used to calculate the relative percent of APC10-bound proteins in total input proteins, by dividing the protein signals in GST-APC10 panel by those in lysates panel. The derived relative percentages are indicated at the top right side of Fig. 3B. An abrupt drop of percentage occurs between Smad3 d2 (95%) and Smad3 d6 (34%), with an additional small reduction between Smad3 d6 (34%) to Smad3 d4 (17%). These data suggests that the amino acid residues 301 to 362, especially 301 to 330, are important for Smad3/APC10 binding. The Smad3/Smad1/Smad3 chimera (20%) exhibited much reduced binding ability compared with Smad3 d2, suggesting that the region of Smad3 replaced by Smad1, a. a. 237 to 276, is also critical for APC10 binding. Taken all these data together, we have mapped residue 237 to 362 within Smad3MH2 to be involved in APC10 binding.

Since Smad3 MH2 domain has also been shown to bind HEF1 [17], we decided to compare Smad3 interaction with both APC10 and HEF1, in order to better understand the way APC10, Smad3 and HEF1 form a potential ternary complex. Thus, the same set of cell lysates from 293 cells transfected with Smad3 deletion constructs were tested against bead-bound, purified GST-HEF1 and GST, the later served as a negative control. The bead-bound proteins were eluted and detected by Western blot (Fig. 3C, middle and bottom panels). The percentage of bound-protein signals over the lysate protein signals was also calculated and presented at the top right side of Figure 3C. The interaction between Smad3d2 with GST-HEF1 (21%) is not as strong as the interaction between Smad3d2 and GST-APC10 (95%). Also, there is almost no difference between Smad3 d2 (21%) and Smad3 d6 (23%) in binding to GST-HEF1. Thus, the Smad3

MH2 region between a. a. 330 to 362 is not critical for HEF1 interaction. However, there is a major difference between Smad3d6 (23%) and Smad3 d4 (13%) in binding to HEF1, suggesting that the MH2 region between a.a.301 and 330 is very important for Smad3 interaction with HEF1. As for the Smad3/Smad1/Smad3 chimera, its binding to HEF1 (24%) is as strong as that for Smad3 d2 (21%), thus suggesting that the region between a.a. 236 and 276 is not involved in the interaction between HEF1 and Smad3. By comparing the different interaction properties of these Smad3MH2 domain mutants exhibited during their binding to APC10 and HEF1, we concluded that different subdomains within Smad3 MH2 domain are involved in Smad3 binding to APC10 and HEF1. For binding to APC10, the entire region between residues 237 to 362 might be involved (Fig. 3B), while a more localized region between residues 301 to 330 appears to bind HEF1 (Fig. 3C). Combined with the information reported in our previous studies [17], we propose a ternary complex formation between HEF1, Smad3 and APC10, as illustrated in Fig. 3D.

The CDH1 protein in APC complex binds to HEF1

To be ubiquitinated and thus degraded by the proteasome pathway, APC substrates need first to be recognized by either CDC20 or CDH1 which targets the ligase to specific substrates during metaphase-anaphase transition as well as during late anaphase, respectively [29,35,36]. Recent studies have demonstrated the direct binding between APC substrates and CDC20 or CDH1 [37]. CDH1 has also been shown to bind to the other Smad3 interactor SnoN in Smad3-regulated SnoN ubiquitination [18,19]. We thus also examined whether CDH1 interacts with HEF1, by the direct binding test *in vitro.* Either GST-HEF1 or GST alone as a control were incubated with *in vitro* translated ^{35}S-labeled CDH1. As shown in Figure 4A, *in vitro* translated CDH1 binds to GST-HEF1, suggesting a possible role of CDH1 in targeting HEF1 for APC-mediated ubiquitination of HEF1.

CDH1 binds to HEF1 C-terminal M2 domain containing two D boxes

Upon examining HEF1 amino acids sequence, we found five putative D box [RxxLxxxx(N)]. These putative D boxes are located within the SH3 domain (box 1), the SH2 binding sites domain (box 2), after the Ser rich domain (box 3) and within the M2 domain of HEF1 (box 4 & 5) (Fig. 4 B). D box and KEN motif are the two motifs recognized by CDH1 on APC substrates [30].

To determine which D box interacts with CDH1, full-length HEF1 as well as T7-tagged HEF1 deletions, as illustrated in a cartoon in Figure 4C, were co-transfected with myc-tagged CDH1 into 293 cells. Cell lysates were subjected to immunoprecipitation using anti-myc antibody. Full-length HEF1, HEF1 d113 (114-834) and HEF1 M2 domain (654-834) were found to bind to CDH1 (Fig. 4D, top panel, lanes 2, 4, & 5). HEF1 1-154 did not show the ability to bind to CDH1 (Fig. 4D, top panel, lane 3). Therefore, we predict that CDH1 binds to a D box located within the M2 (C-terminal) domain that contains two putative D box, one located between residues 705 and 714 within the HLH motif, the other between amino acids 826 and 834. Since CDH1 is a substrate recognition component for APC ligase complex, the ability of CDH1 to bind to HEF1 suggests that HEF1 is a substrate for the APC ligase. The detected interactions between Smad3, HEF1, APC10 and CDH1 is illustrated in a cartoon in Figure 4E.

Smad3 interaction with APC10 is regulated by TGF-β type I receptor activation while CDH1 interaction with HEF1 is constitutive

To further test the effect of TGF-β receptor activation on the interaction between Smad3 and APC10 or between CDH1 and HEF1, these proteins were expressed in 293 cells in the presence or absence of a constitutively activated TGF-β type I receptor

R4T204D (R4TD). As shown in Figure 5A, APC10 was detected only in the immunoprecipitates of Smad3 from cells with the coexpression of both Smad3 and R4TD (Fig. 5A, lane 6). We noted that anti-Smad3 precipitates two forms of Smad3 in lane 6 while only one form of Smad3 in lane 5. The higher molecular weight form is a phosphorylated version of Smad3, as confirmed by Western blot with anti-phosphoserine (data not shown). Thus, the data suggests that TGF-β receptor-induced Smad3 phosphorylation may enhance its interaction with APC10. This appears to be not the case for HEF1 interaction with CDH1. As shown in Figure 5B, the complex formation of CDH1 and HEF1 occurs in the absence of R4TD and the coexpression of R4TD did not alter the formation of the complex (Fig. 5B, compare lanes 9 & 10 in top panel). The coexpression of Smad3 with CDH1 and HEF1 caused significant reduction of HEF1 protein levels, as expected, but also did not alter the complex formation of CDH1 and HEF1 (Fig. 5A, lanes 11 & 12). When HEF1 was coexpressed with CDH1, APC10 and the MH2 domain of Smad3, which harbors the binding sites for both HEF1 and CDH1, we detected al four proteins in the immunoprecipitates of HEF1 (Supplementary Fig. 1). These data further confirms the complex formation of Smad3, HEF1, CDH1 and APC10 in mammalian cells and also suggest that TGF-β signaling may regulate the complex formation via regulating the interaction between Smad3 and APC10.

APC10 and CDH1 both regulate Smad3-regulated HEF1 degradation

Since APC10 binds to Smad3, which binds to HEF1 and induces proteasomal degradation of HEF1 [17], we tested whether APC10 plays a role in Smad3-regulated proteasomal degradation of HEF1. In 293 cells, HEF1 was co-expressed with Smad3 in the presence or absence of APC10. The Smad3-binding defective APC10 D8 was used as a negative control. As shown in Fig. 6A, the co-expression of Smad3 alone with

HEF1 drastically reduced the protein level of HEF1, as shown previously (Fig. 6A, lanes 1 & 2). The co-expression of APC10 alone with HEF1 also caused the reduction of HEF1 level (Fig. 6A, lane 3). Interestingly, the co-expression of APC10 slightly reduced the ability of Smad3 in causing reduction of HEF1 (Fig. 6A, lane 4). The effect of overexpressing APC10 alone on reducing HEF1 protein level appears to be dependent upon the ability of APC10 to interact with Smad3, since the Smad3-binding deficient APC10 D8 caused much less reduction of HEF1 protein level (Fig. 6A, lane 5). It also failed to interfere with Smad3-induced HEF1 reduction (Fig. 6A, lane 6). Thus, the overexpression of full length APC10 or Smad3 alone was sufficient to induce enhanced HEF1 degradation, while the overexpression of both proteins appears to cause an interference of each protein's function. Considering the nature of the multimeric complex formation of Smad3, HEF1, APC10 and other APC components, we would expect this to be the case since excess of any protein in a multimeric complex would lead to the un-coupling of the complex formation and therefore blocking the normal function of the complex.

To confirm a functional role of CDH1 in proteasomal degradation of HEF1, we also tested the effect of CDH1 on HEF1 protein level, in the presence or absence of Smad3 and APC10, in 293 cells. Like APC10, overexpression of CDH1 alone reduced the protein levels of both forms of HEF1 (Fig. 6B, compare lane 5 with lane 11). The co-expression of the constitutively activated TGF-β type I receptor mutant R4T204D (R4TD) caused further reduction of the p115HEF level in the absence of overexpressed CDH1 or APC10 (Fig. 6B, compare lanes 11 & 12), but such a reduction was not very apparent when CDH1, Smad3, or APC10 was co-expressed either alone or in different combinations (Fig. 6B, compare lanes 1 & 2, 3 & 4, 5 & 6, 7 & 8, 9 & 10). When CDH1 was co-expressed with Smad3, or with Smad3 and APC10, the p105 HEF1 form appeared to be less reduced (Fig. 6B, compare lane 5 with lanes 7, 8 & 9). These data

pointed out that Smad3, APC10 and CDH1 are all capable of altering the protein levels of HEF1 when they are coexpressed with HEF1.

Discussion

HEF1 has been implicated in many different signaling pathways such as those mediated by integrin, TCR and BCR where it plays the role of an adaptor protein. Recent studies have shown that HEF1 degradation is regulated by Smad3 via the proteasomal degradation pathway and is further enhanced by TGF-β stimulation [17]. These findings reveal a novel ability for Smad3, which has been primarily considered as a DNA-binding transcriptional factor, and also suggest a novel cross-talk mechanism between TGF-β/activin pathways and multiple HEF1-involved pathways. The molecular mechanisms underlying Smad3-regulated proteasomal degradation are not clear. Our current studies revealed the ability of Smad3 to bind to APC10, which is a regulatory component of the APC ligase core complex required for substrate interaction, as well as the ability of HEF1 to bind to CDH1, which is a co-activator of APC ligase for specific substrate recognition. The interaction between Smad3 and APC10 is subjected to the regulation by the TGF-β type I receptor, while the interaction between CDH1 and HEF1 is constitutive. Both APC10 and CDH1 exhibit the ability to regulate the steady state levels of HEF1 upon co-expression with HEF1. These data suggests a novel mechanism for Smad3 to regulate the proteasomal degradation of HEF1 via assisting the recognition of HEF1 by the APC E3 ligase.

The interaction between APC10 and Smad3 was first observed in the yeast two-hybrid system. This interaction was confirmed in mammalian over-expression system by co-immunoprecipitation, then by *in vitro* GST pull-down assay, and finally via *in vitro* binding assays to demonstrate a potential direct interaction between Smad3 and APC10. Domain mapping studies showed that Smad3 MH2 domain is necessary and

sufficient to bind APC10, whose C-terminal domain is required for Smad3 binding, while the N-terminal domain for APC10 stability. These data strongly suggested a direct and domain-specific interaction between Smad3 and APC10. Previously Smad3 has been shown to recruit APC complex to ubiquitinate its nuclear interactor SnoN, but it was not clear how Smad3 recruits APC complex [18,19]. The ability of Smad3 to bind directly to APC10 suggests that this interaction could be the missing link for Smad3 to recruit APC complex in the ubiquitination of SnoN. Future studies will be carried out to directly test this possibility.

Our previous studies have shown that Smad3 interlocks with HEF1, with the MH1 domain binds to the N-terminal domain of HEF1, while the MH2 domain of Smad3 binds to the C-terminal domain of HEF1 [17]. Considering the involvement of the MH2 domain of Smad3 in binding to APC10, we compared the ability of various deletion constructs of Smad3 MH2 domain in their ability to bind to APC10 and HEF1. These studies showed that residues located within 237 to 362 on Smad3 are involved in Smad3 binding to APC10, while a more localized region between residues 301 to 330 is involved in binding to HEF1. These data suggests that Smad3, APC10 and HEF1 can potentially co-exit in one single complex.

Since APC10 is known to play a role in regulating substrate recognition and ubiquitination by mammalian APC {Hartmut C. et al., Current Biology 13:1459, 2003} the interaction between Smad3, APC10 and HEF1 suggests that HEF1 and Smad3 are potential ubiquitination substrates for APC ligase. Our finding of the direct interaction between HEF1 and the WD40 repeat protein CDH1 further qualifies HEF1 as a substrate for APC, since interaction with CDH1 or CDC20 has been shown to be a prerequisite for APC substrates [37]. The role of APC10 and CDH1 in regulating HEF1 degradation was confirmed by the ability of overexpressed APC10 and CDH1 in enhancing Smad3-regulated HEF1 degradation in 293 cells (Figure 6). The physical

interaction between HEF1 and CDH1, a regulator at late anaphase of cell cycle, could be functionally linked to a previous observation of the ability of a processed form of HEF1, p55HEF1, in interaction with mitotic spindles [22]. The exact role of HEF1 in cell cycle regulation and how Smad3, via interacting with the co-activators of APC complex to regulate such a role is also an important subject for future studies.

While Smad3 can regulate the proteasomal degradation of both HEF1 and SnoN, phosphorylation of Smad3 by the activated type I receptor is required only for SnoN degradation but not for HEF1 degradation [17,18, 19]. The mechanism for such a difference warrants future investigation. One apparent difference between HEF1 and SnoN is their intracellular localization. While HEF1 is predominantly cytoplasmic, SnoN is primarily a nuclear protein. It has been shown that the inactive Smad3 is primarily cytoplasmic, and its nuclear translocation is triggered upon its phosphorylation by the type I receptor of TGF-β at the C-terminal SSVS motif. Thus, the dependence of SnoN degradation on Smad3 phosphorylation at the SSVS motif could be at least partially due to the dependence of Smad3 phosphorylation for Smad3 accumulation into the nucleus. However, a recent study (Feng L. et al., manuscript submitted) indicated that the *in vitro* translated Smad3 can induce HEF1 degradation in an *in vitro* degradation assay, which does not involve the issue of nuclear localization, thus suggesting that additional component (s) involving phosphorylation-dependent structural changes of Smad3 may be involved in regulating SnoN ubiquitination and degradation. One such structural change has been recently revealed. Previously it was shown that inactive unphosphorylated Smad3 is bound to proteins such as SARA (Smad anchor for receptor activation) in the cytoplasm and that phosphorylation of Smad3 by the type I receptor decreases its affinity for SARA thus allowing Smad3 to interact with the Co-Smad Smad4 before entering the nucleus [38]. In a recent study, molecular details were revealed regarding the interaction between inactive Smad3, SARA and the TGF-β type I receptor. The inactive Smad3 exists in a monomer form in a complex

with SARA and the TGF-β type I receptor, at the cell membrane or at early endosome [39,40]. The phosphorylation of Smad3 releases it from SARA and allows Smad3 to adopt a different conformation that favors trimmer formation. Interestingly, the later conformation is preferred by the nuclear oncoprotein Ski [41]. Thus, SARA serves as a molecular guardian of Smad3 to prevent it from forming aberrant trimmers for constitutive activation. Therefore, one possible explanation for the dependence of Smad3 phosphorylation for SnoN degradation, is that SnoN, like Ski, only interacts with Smad3 in oligomers, which could be either Smad3 homo-trimmers or Smad3 (2)/Smad4 (1) hetero-trimmers, and that the ubiquitination of SnoN occurs in such a complex. For HEF1, since neither the interaction nor the degradation is dependent upon Smad3 phosphorylation, we therefore consider it likely that Smad3 binds to HEF1 in monomeric form before its phosphorylation and that such a complex is sufficient for assisting HEF1 ubiquitination and degradation. It also remains a possibility that HEF1 could function like SARA to keep Smad3 in an inactive conformation, thereby assisting Smad3 recognition by the type I receptor kinase and directly regulating Smad3 trimmer formation. Our preliminary data have suggested such an inhibitory role of HEF1 [17]. Future studies of the crystal structures of the complex of Smad3 and HEF1 in the presence or absence of TGF-β type I receptor cytoplasmic domain, as well as the structural studies of the complex of Smad3, HEF1 APC10 and CDH1, as demonstrated here, will significantly advance our current understandings of the mechanism and regulation of HEF1 degradation in TGF-β pathway.

The physiological regulation of the complex formation between Smad3, APC10, HEF1 and CDH1 is likely very dynamic and complex. Since all these observations are made via *in vitro* systems, we do not know the detailed regulation of these interactions in specific cell types under different conditions. The physiological complex formation between Smad3, HEF1 and APC10 is likely subjected to constant changes, depending upon the expression levels of these proteins. It is also possible that the formation of the

complex involves sequential steps. For example, Smad3 could first binds to the APC10 before binding to HEF1 or vise versa and such binding could stabilizes Smad3 in a favorable conformation for subsequent interaction with other proteins, such as CDH1. The regulation of the formation of this complex could also via phosphorylation of HEF1 and Smad3 and via competition involving other interacting proteins of Smad3 and HEF1. Here we gain a glimpse of the complexity of the regulation of HEF1 ubiquitination and degradation *in vivo*. In 293 cells, we observed an enhancement effect of TGF-β receptor activation on Smad3 interaction with APC10, but not on HEF1 interaction with CDH1 (Fig. 5).

In Figure 7, a cartoon is presented to summarize our data and present a model for the role of Smad3 interaction with APC10 in recruiting APC complex for ubiquitination and degradation of HEF1, downstream of TGF-β type I receptor activation. In this model, we propose three possible ways for HEF1 to be ubiquitinated by APC ligase via the formation of the multimeric complex of Smad3, HEF1, APC10, CDH1, the latter two of which bring in the entire APC ligase. The first scenario is that the phosphorylated Smad3, released from SARA, forms a complex with APC10, while CDH1 interacts with HEF1 constitutively. The two complexes come together via Smad3 interaction with HEF1. APC10 assists CDH1 to bring Smad3-bound HEF1 to APC ligase for the subsequent ubiquitination. The second scenario is that HEF1 tethers Smad3 in a cytoplasmic complex and the type I receptor activation leads to phosphorylation of Smad3 and the subsequent conformational changes that can enhance its interaction with APC10, which recruits the APC ligase and assists CDH1 to bind the substrate HEF1. The third scenario is that monomeric unphosphorylated Smad3 and HEF1 forms a constitutive complex to further recruit APC10 and CDH1. Such a complex may mediate some basal level of constitutive ubiquitination of HEF1. We recognize that the current observations need to be followed up in more

physiological relevant conditions, such that the physiological functions of the observed interactions between these proteins can be validated

Conclusions

The studies presented here revealed a physical link between a key signal transducer of TGF-β (Smad3) and a component (APC10) of the cell cycle regulatory E3 ligase APC, and further suggest a molecular mechanism via which such a physical interaction contributes to the regulation of the protein stability of a multi-domain cytoplasmic docking protein HEF1, which is involved in a large network of signaling events. Since Smad3 interacts with many other nuclear and cytoplasmic proteins, this observation may have broad implications for the regulation of these other Smad3 interactors in a similar fashion. While previous studies of the APC complex have been primarily limited to cell cycle control, the observations reported recently extended the role of APC into non-cell cycle events associated with SnoN [18,19]. Our studies here provide the detailed link between Smad3 and APC and now add a cytoplasmic signaling adapter onto the list of APC substrates. The data imply that the cellular functions previously viewed as separate are intimately interwoven in a complex fashion: a cell cycle regulatory E3 ligase is physically and functionally connected to the vast signaling networks involving Smad3 and HEF1. Furthermore the biological consequences of the TGF-β-induced HEF1 degradation in lymphocytes where HEF1 is predominantly expressed have yet to be studied and their understanding could lead to new insights in the molecular events involved in the onset of complex diseases such as cancers.

Methods

Mammalian cell line

293 cells (human kidney cells transformed with adenovirus 5 DNA) were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO/BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS), 25,000 units of penicillin, 25mg of streptomycin and 5mL of 200mM L-Glutamine at 37ºC in the presence of 5% CO2.

Antibodies and reagents

Anti-myc (9E10), anti-Smad1/2/3 (H-2), anti-HEF1 (N-17) and anti-TGF-β RI (V-22) were purchased from Santa Cruz biotechnology. Anti-p130Cas monoclonal antibody was purchased from Transduction Laboratories. Anti-T7 (69522-4) was purchased from Novagen. Anti-Flag was obtained from Sigma and anti-HA was purchased from Roche. Phosphatase inhibitor (P5726) and protease inhibitor cocktail (P8340) were purchased from Sigma. MG132 was dissolved in DMSO and added directly into cell culture medium to a final concentration of 50μM for 6 hours before harvest.

Constructs

The pCMV-HEF1 expression vector has been described previously [22]. All the other mammalian expression constructs for HEF1 and Smad3 deletions as well as T7-Ub were constructed in our lab previously [17]. The mutant type I receptor R4TD was described previously. Smad3, APC10 and HEF1 were subcloned into EcoRI/XhoI sites in pGEX-5X-1 (Amersham) using standard procedures [42]. Smad3 and APC10 were subcloned into EcoRI/XhoI sites in pCS2+ vector containing a SP6 promoter.

Transfection

293 cells were transfected using the standard $CaPO_4$ procedure [42] and cells were harvested 24h after transfection into ice-cold phosphate-buffered saline (PBS).

Immunoprecipitation and Western blotting

Cells were incubated 30min on ice in HBS-Lysis buffer (50mM HEPES, 5mM EDTA, 50mM NaCl, 1% TritonX-100 supplemented with protease and phosphatase inhibitors just prior to use). Cell debris was pelleted by spinning in a microcentrifuge at 14,000g at 4˚C for 10min and supernatant was saved for immunoprecipitation and western blot analysis. For immunoprecipitation, cell lysates were incubated with 2 μg of primary antibody for 2 h at 4˚C followed by an additional 2 h-incubation with 40 μL of 50% slurry of protein G-sepharose 4 Fast Flow (Amersham). Beads were then washed once using lysis buffer and 3 times with modified lysis buffer (lysis buffer containing 0.1% of TritonX-100). The precipitated proteins were eluted in 2x SDS loading buffer (100mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromophenol blue and 20% glycerol) + 10% β-mercaptoethanol, loaded on SDS-PAGE and transferred onto PVDF membrane (Millipore). Membranes were analyzed by Western blotting [42]. Antibodies were diluted as followed: α-HEF1 (1:500), α-p130Cas (1:1000), α-cdc27 (1:500), α-cdc16 (1:500), α-Flag (1:4000), α-T7 (1:10000), α-myc (1:1000), α-HA (1:1000) and α-TGF-β RI (1:4000).

GST pull-down assay

GST-APC10, GST-Smad3 and GST-HEF1 were expressed and purified from *Escherichia coli* strain BL-21. Briefly, the culture was induced at O.D.~ 0.6 with 0.4 mM IPTG for 2 to 3 hours. Cells were collected by spinning at 5,000 rpm at 4ºC for ~ 15 min. The pellet was then resuspended in Prep buffer (100 mM NaCl, 100 mM Tris-HCl pH8.0, 50 mM EDTA, 2% TritonX-100) supplemented with 2mM DTT and 1mM

PMSF. Lysis occurred using10mg/mL lysozyme (Fisher BP 535-1) in Prep buffer for 30 min on ice and debris was pelleted by spinning 30 min at 14,000g at 4ºC. Cell lysates were then incubated with a 50% slurry of Glutathione Sepharose 4 Fast Flow (Amersham) for 40 min at 4ºC and washed 3 times with ice-cold PBS. About 4 μg of GST fusion proteins that were immobilized on beads were incubated with extract in lysis buffer, washed three times with modified lysis buffer and resuspended in SDS loading buffer. To test a direct protein-protein interaction, proteins were translated *in vitro* and ^{35}S-labeled by using the TNT reticulocyte lysates system (Promega). 7 μL of the *in vitro* translated product and GST beads were incubated in 200 μL of modified lysis buffer complete with protease inhibitors and washed as previously described. The GST beads were resolved by SDS-PAGE followed by Western blot or dried and subjected to autoradiography.

Abbreviations

- TGF-β: Transforming Growth Factor-β; TCR: T-cell receptor
- BCR: B-cell receptor
- HEF1: Human Enhancer of Filamentation I
- APC: Anaphase-Promoting Complex
- CDC20: Cell division cycle 20
- CDH1: Cdc20 homolog 1
- MG132: N^{α}-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal [Z-Leu-Leu-Leu-H]
- SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
- Mv1Lu cell: Mink Lung cell
- MH1, MH2 domain: Mad homology-1, -2 domain

Authors' contributions

Claire Nourry was responsible for carrying out all key experiments, data collection and organizing the data into a thesis format. Lola Maksumova was in charge of the in vivo interaction tests of the interaction between Smad3 and APC10. Xiaohong Liu carried out the initial yeast two-hybrid test to isolate APC10 as a Smad3 interactor. Mona Peng contributed to Figure 4D and Figure 5A, after Claire Nourry left our lab. Tongwen Wang was responsible for providing advice for all the experiments, co-ordination between members who worked on this project and for the editing and submission of the text and figures.

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Figure legends

Figure 1. Smad3 interacts with APC10 specifically.

(A) Co-immunoprecipitation of Smad3 and APC10 from mammalian overexpression system. 293 cells were transiently transfected with T7-APC10 and Smad proteins as indicated. The cell lysates were subjected to Western blot analyses directly or immunoprecipitation followed by Western blot as indicated. (B) Smad3 binds to APC10 in GST pull down assays. Flag-tagged Smad constructs were transfected into 293 cells and tested against GST-APC10 purified from E. Coli. Total input Smad protein levels (100 ug) were detected by immunoblotting of the cell lysates with anti-Flag antibody (top panel). Smad proteins bound to the GST-APC10 beads were eluted and analyzed by immunoblotting with anti-Flag antibody (middle panel). As a control, F-Smad proteins were incubated with the GST alone (bottom panel). (*) We noted that GST-APC10 was recognized by the anti-Flag antibody on Western blot. (C) Smad3 binds to HEF1 and APC10 in *in vitro* binding assay. In vitro translated ^{35}S-labeled HEF1, ^{35}S Smad3 and ^{35}S APC10 were incubated with GST-Smad3, GST-APC10 or GST as indicated. The in vitro translated proteins before and after binding were detected by electrophoresis and autoradiography.

Figure 2. Domain mapping studies of the interaction between Smad3 and APC10 reveal distinct domains involved in binding.

(A) The Smad3C (MH2 domain) is necessary and sufficient for binding to APC10. Top panel: a cartoon to illustrate the deletion constructs of Smad3. The APC10 binding activities of each truncated Smad3 as detected by assays in the bottom panels are summarized at the right side of the panel. Bottom left panel: MH2 domain is necessary and sufficient for Smad3 to bind APC10 in GST pull down assay. Flag-tagged full-length or truncated Smad3, as indicated, was transfected into 293 cells and tested

against GST-APC10 and GST (negative control). Bottom right panel: MH2 domain is necessary and sufficient for Smad3 to bind APC10 in 293 cells. Different Smad3 truncations tagged with Flag were co-transfected with T7-APC10 into 293 cells. The expression of these proteins was detected by Western Blot using anti-Flag and the interaction between APC10 and Smad3 truncations was detected by immunoprecipitation of T7-APC10 followed by Western blot using anti-Flag. "L.C." represents the antibody light chain. (B) The C-terminal domain of APC10 is necessary for binding to Smad3. Top panel: a cartoon that illustrates the deletion mutants of APC10. The top three deletion constructs are amino-terminal deletion mutants. The number of amino acids deleted in each construct is indicated. For example, the D2 construct lacks the N-terminal 41 amino acids, thus it is also labeled as D41N. Bottom left panel: the six deletion constructs of APC10, each tagged with Flag, were transfected into 293 cells. The expression of these deletion mutants was detected by Western blot using anti-Flag. Stable protein expression was detected only in cells transfected with two C-terminal deletion constructs (D8 and D9). Bottom right panel: the C-terminal 66 amino acids of APC10 are required for Smad3 binding. T7-tagged APC10 and Flag-tagged APC10 D9 were transfected into 293 cells. Cell lysates were subjected to immunoblotting with anti-T7 (lane 1) and anti-Flag (lane 2) antibody. APC10 and APC10 D9 were tested against GST-Smad3 (lanes 3 & 4) and GST alone as a control (lanes 5 & 6).

Figure 3. Smad3 MH2 domain contains overlapping but distinct binding sites for HEF1 and APC10.

(A) A Cartoon to illustrate the deletion constructs of Smad3 (Smad3d2, d4 and d6) as well as a hybrid protein S3/S1/S3. (B) GST pull-down assay to map APC10 binding site on Smad3C. Flag-tagged Smad3 deletion mutants were transfected into 293 cells and the cell lysates were tested against GST-APC10. Cell lysates (top panel) and

proteins bound to the beads (middle and bottom panels) were analyzed by Western blot with anti-Smad1/2/3 from Santa Cruz. The percentage listed at the right side of the listed constructs is derived from dividing the signals (measured by ImageQuant) of bound proteins in the middle panel by the signals of lysate proteins in the corresponding lane of the top panel and then times 100 percent. (C) GST pull-down assay to map HEF1 binding site on Smad3C. Same as in (B), with GST-APC10 replaced by GST-HEF1. (*) endogenous Smad1 and Smad3 recognized by anti-Smad1/2/3 antibody. (D) A cartoon to illustrate the complex of Smad3, HEF1 and APC10 and their potential interaction with the APC ligase core complex.

Figure 4. CDH1 interacts with HEF1 at the HEF1 C-terminal M2 domain.

(A) In vitro binding test. In vitro translated ^{35}S labeled CDH1 was incubated with GST-HEF1 or GST alone as a control in modified lysis buffer. ^{35}S labeled CDH1 was separated by electrophoresis and detected by autoradiography. (B) A cartoon that illustrates putative D boxes on HEF1. (C) A cartoon that illustrates the deletion constructs of HEF1 used in (D). (D) Co-immunoprecipitation of HEF1/HEF1 deletions with CDH1 in 293 cells. Full-length HEF1 and T7-tagged HEF1 deletions were co-transfected with myc-tagged CDH1 into 293 cells. Myc-CDH1 was immunoprecipitated with anti-myc antiboby and CDH1 bound HEF1 or HEF1 deletions were detected by immunoblot with either anti-p130Cas antibody (top panel, lanes 1 & 2) or anti-T7 antibody (top panel, lanes 3-5). The expression level of CDH1 was detected with anti-myc antibody (second panel). The amount of HEF1 (third panel) or T7-HEF1 deletions (bottom panel) expressed was detected by anti-p130Cas for HEF1 or anti-T7 antibody. (E) A cartoon that illustrate the putative complex of HEF1, Smad3, APC and CDH1.

Figure 5. TGF-β type I receptor activation enhances Smad3 interaction with APC10 but does not alter CDH1 interaction with HEF1.

(A) Smad3 interaction with APC10 is positively regulated by the activation of TGF-β type I receptor. Flag-Smad3 and T7-APC10 were co-expressed in the presence or absence of a constitutively active TGF-β type I receptor mutant R4T204D (R4TD). The interaction between Smad3 and APC10 was detected by immunoprecipitation of Smad3 with an anti-Smad3 polyclonal antibody followed by Western blot with anti-T7 (Top panel, lanes 4-6). The immunoprecipitated Flag-Smad3 was detected by anti-Flag antibody (middle panel). The expression of R4TD was detected by anti-TGF-β RI polyclonal antibody from Santa Cruz (Bottom panel). R4TD-p represents a potential cleavage product of R4. (B) HEF1 interaction with CDH1 Is not regulated by TGF-β type I receptor activation. The 293 cells were transiently transfected with myc-CDH1 and HEF1 in the presence or absence of Smad3 and R4TD, as indicated. The interaction between HEF1 and CDH1 was detected by immunoprecipitation with anti-p130Cas followed by Western blot with anti-myc (Top panel, lanes 7-13). The expression of myc-CDH1 and Flag-Smad3 was detected by Western blot by anti-myc and anti-Flag (Top panel, lanes 1-6). The expression levels of HEF1 was detected by anti-HEF1 (middle panel) and the levels of R4TD detected by Western blot with anti-TGF-β RI antibody (bottom panel).

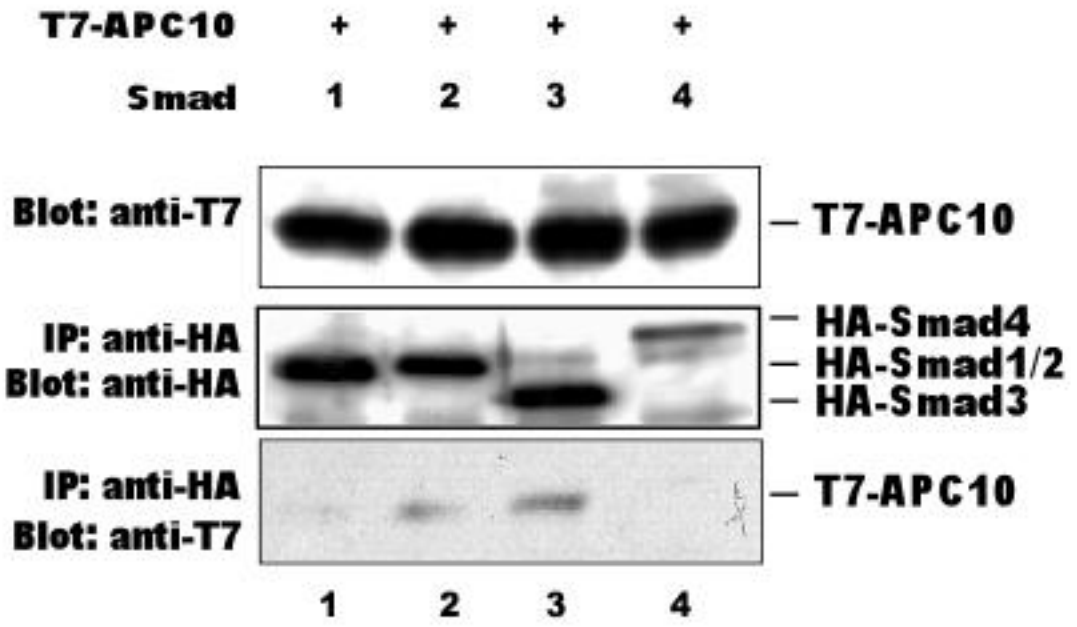
Figure 6. Both APC10 and CDH1 can alter the steady state protein levels of HEF1

(A) Overexpressed APC10 caused the reduction of the steady-state protein levels of HEF1 and such an activity is compromised in a deletion mutant of APC10 (APC10 D8) that is defective in binding to Smad3. HEF1 was co-transfected with ubiquitin, Flag-Smad3, T7-APC10 or T7-APC10 deletion mutant APC10 D8. The expression level of HEF1 was detected by Western blot using anti-p130Cas antibody, as shown in the top panel; the expression of Smad3, APC10 and APC10D8 were detected by Western blot using anti-Flag and anti-T7, as shown in the bottom panel. (B) CDH1 enhances Smad3-regulated HEF1 degradation. HEF1 was co-expressed in different combinations with myc-CDH1, T7-APC10, Flag-Smad3 and R4T204D (R4TD), a constitutively active type I receptor in 293 cells. The steady state levels of HEF1 were detected by anti-HEF1 antibody (top panel) and the expression levels of Smad3 and APC10 were detected with anti-Flag and anti-T7 antibodies, respectively (second and third panels), while the expression levels of myc-CDH1 were detected by anti-Myc (fourth panel). To detect R4-TD, cell lysates were blotted with anti-TGF-β RI antibody (bottom panel).

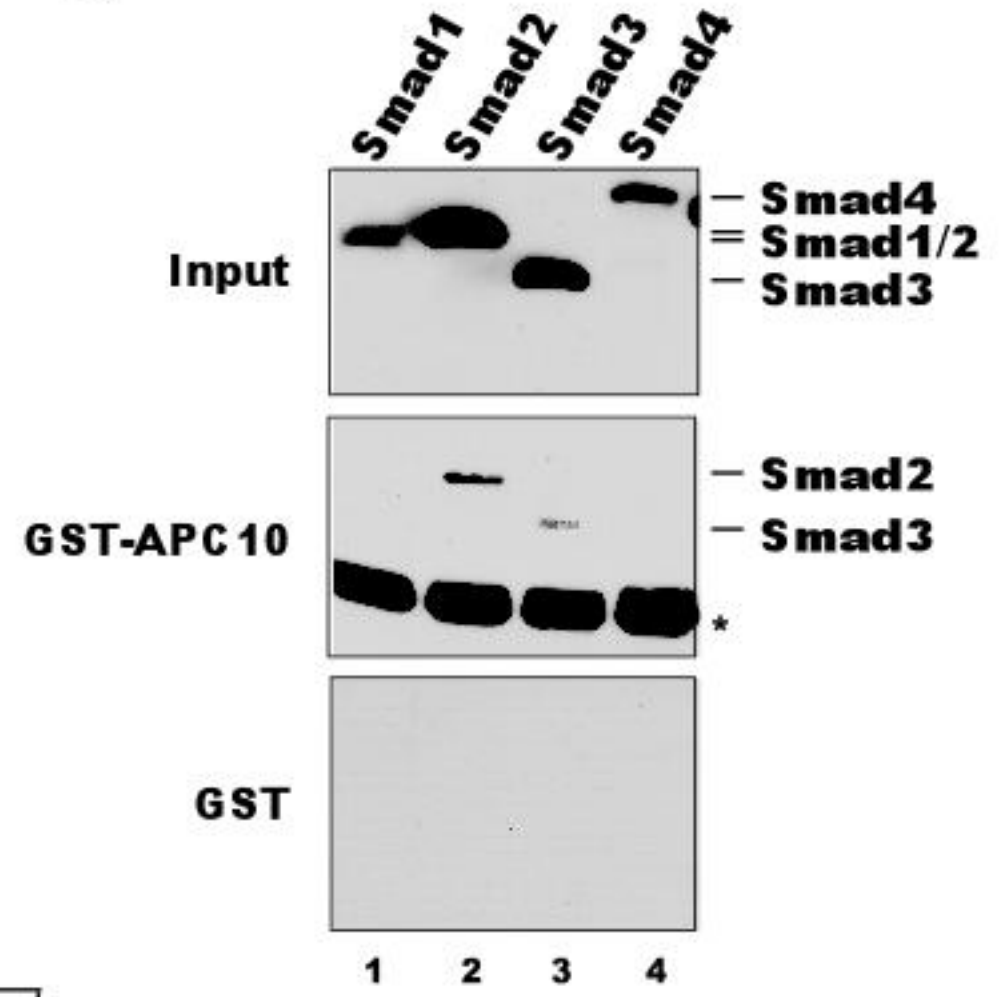
Figure 7. A cartoon for possible signaling pathways that involve Smad3 interaction with APC10.

A cartoon that illustrates possible pathways for Smad3-induced HEF1 degradation involving APC10 and CDH1. Please see text for details.

A


T7-APC10 + + + +
Smad 1 2 3 4
Blot: anti-T7
T7-APC10
IP: anti-HA
Blot: anti-HA
HA-Smad4
HA-Smad1/2
HA-Smad3
IP: anti-HA
Blot: anti-T7
T7-APC10
1 2 3 4

B

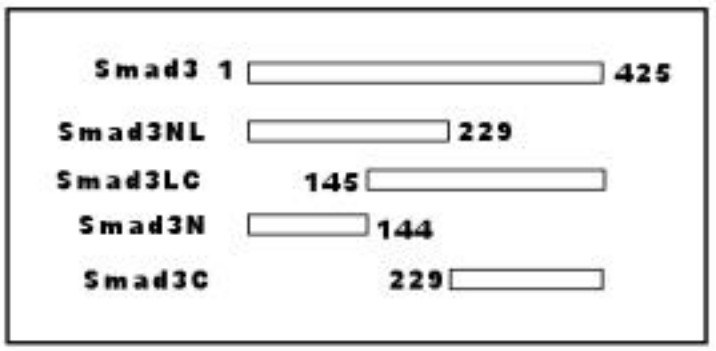

Smad1
Smad2
Smad3
Smad4
Input
Smad4
Smad1/2
Smad3
GST-APC10
Smad2
Smad3
*
GST
1 2 3 4

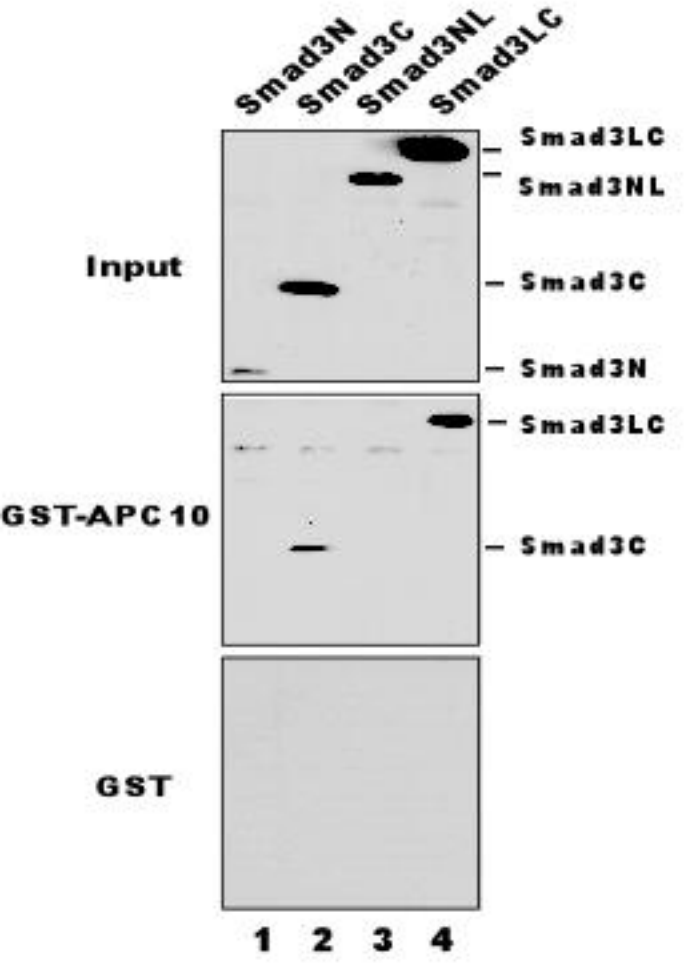
C

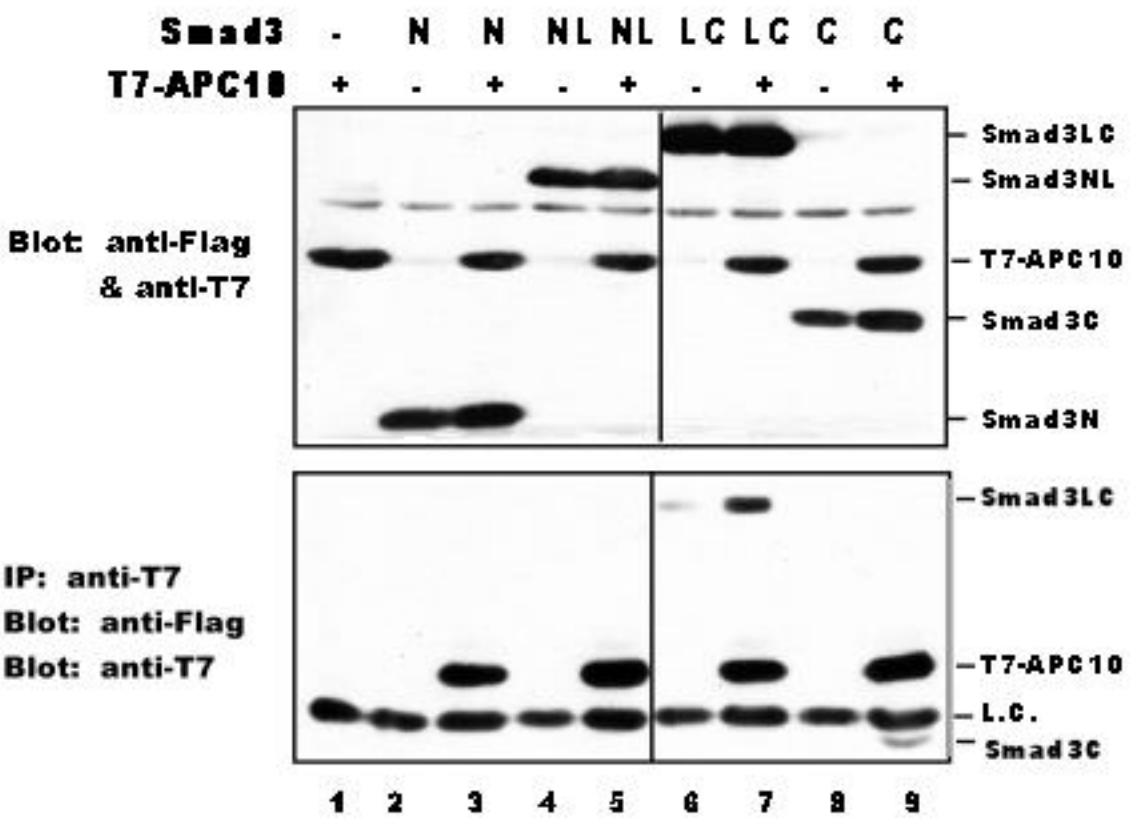
INPUT
GST-Smad3
GST-APC10
GST
HEF1 + - - + - - + + - -
Smad3 - + - - - + - - + -
APC10 - - + - + - - - - +
HEF1
Smad3
APC10
1 2 3 4 5 6 7 8 9 10

Figure 1

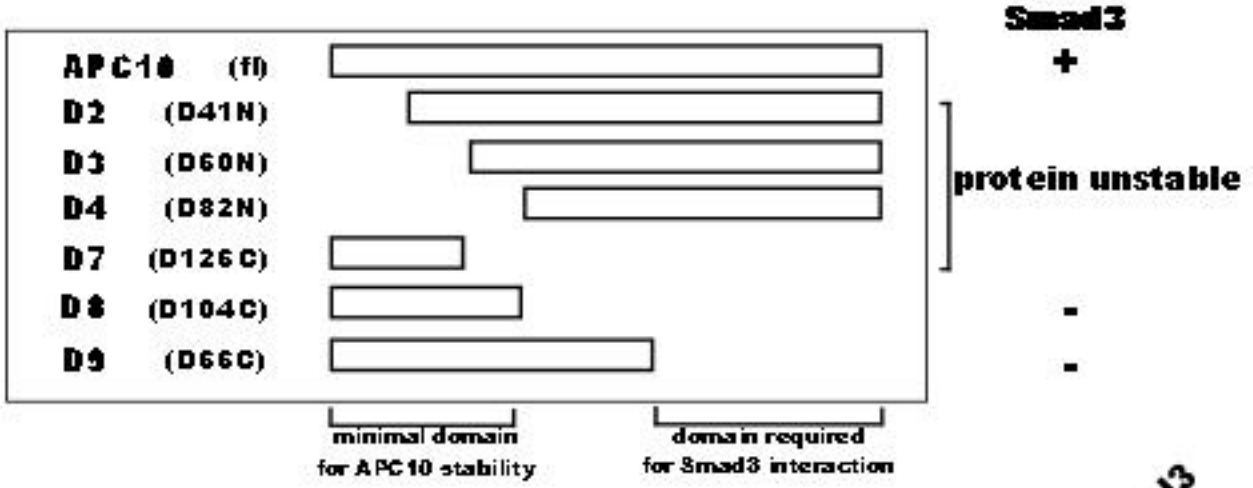
A

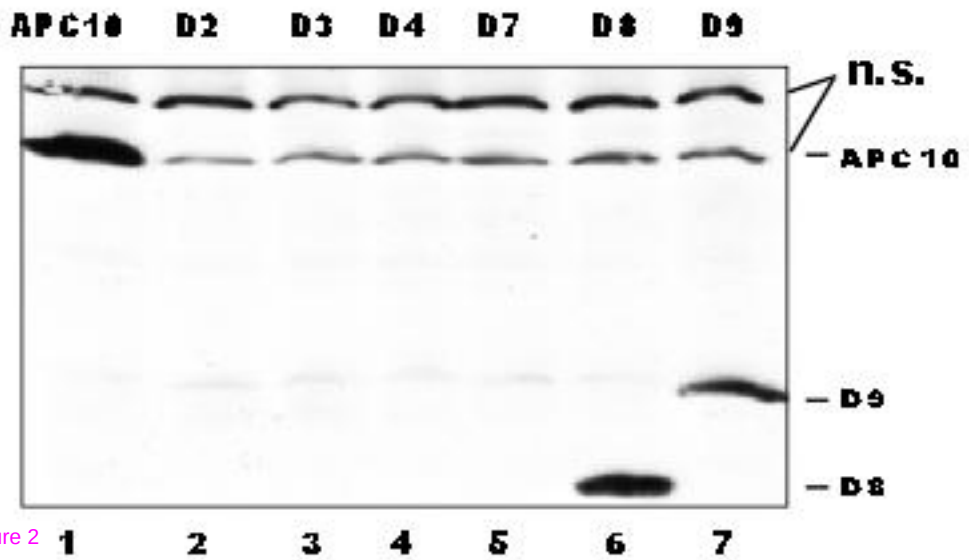

Binding to APC10
Smad3 1 425 +
Smad3NL 229 -
Smad3LC 145 +
Smad3N 144 -
Smad3C 229 +


Smad3N
Smad3C
Smad3NL
Smad3LC
Input
GST-APC10
GST
Smad3LC
Smad3NL
Smad3C
Smad3N
1 2 3 4


Smad3 - N N NL NL LC LC C C
T7-APC10 + - + - + - + - +
Blot: anti-Flag & anti-T7
IP: anti-T7
Blot: anti-Flag
Blot: anti-T7
Smad3LC
Smad3NL
T7-APC10
Smad3C
Smad3N
L.C.
1 2 3 4 5 6 7 8 9

B


Binding to Smad3
APC10 (fl) +
D2 (D41N)
D3 (D60N)
D4 (D82N)
protein unstable
D7 (D126C) -
D8 (D104C)
D9 (D66C) -
minimal domain for APC10 stability
domain required for Smad3 interaction


APC10 D2 D3 D4 D7 D8 D9
n.s.
APC10
D9
D8
1 2 3 4 5 6 7

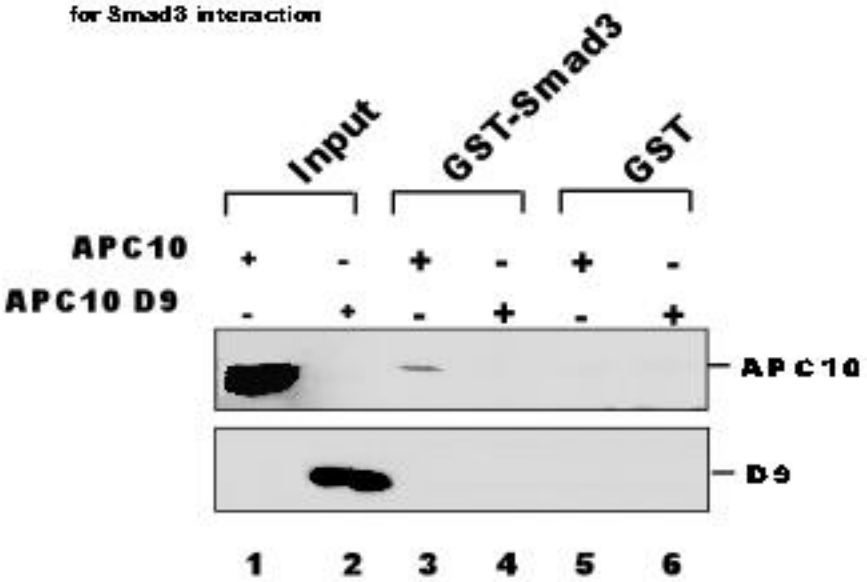
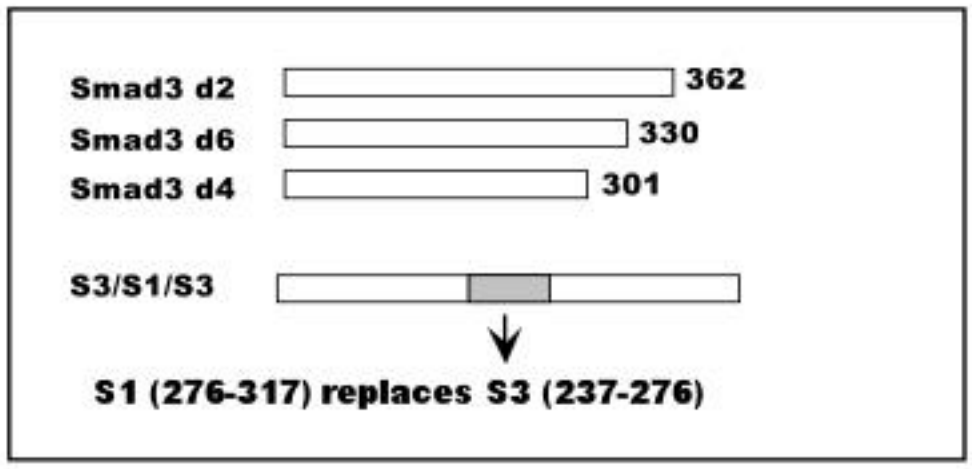

Input
GST-Smad3
GST
APC10 + - + - + -
APC10 D9 - + - + - +
APC10
D9
1 2 3 4 5 6

Figure 2

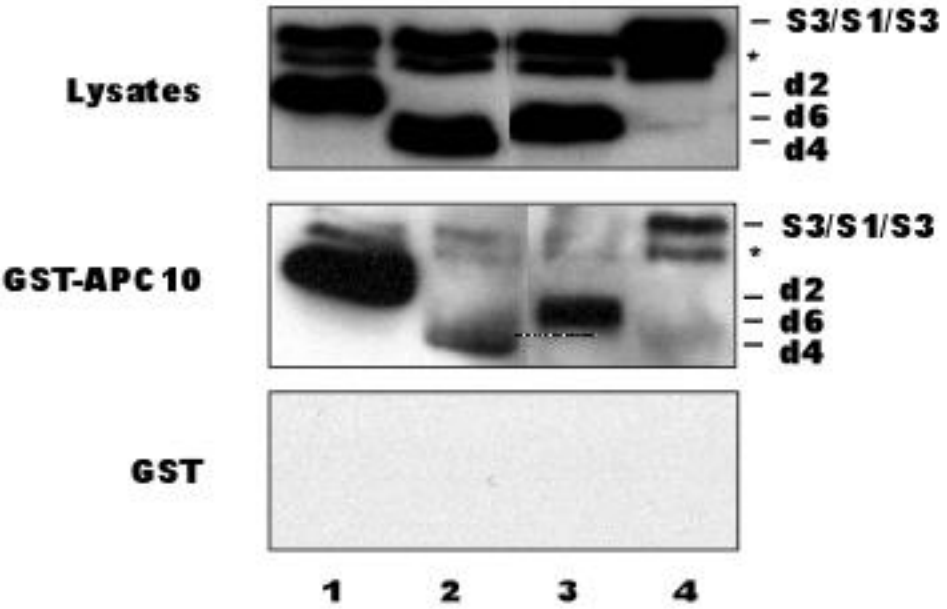
A



B

Binding to APC 10

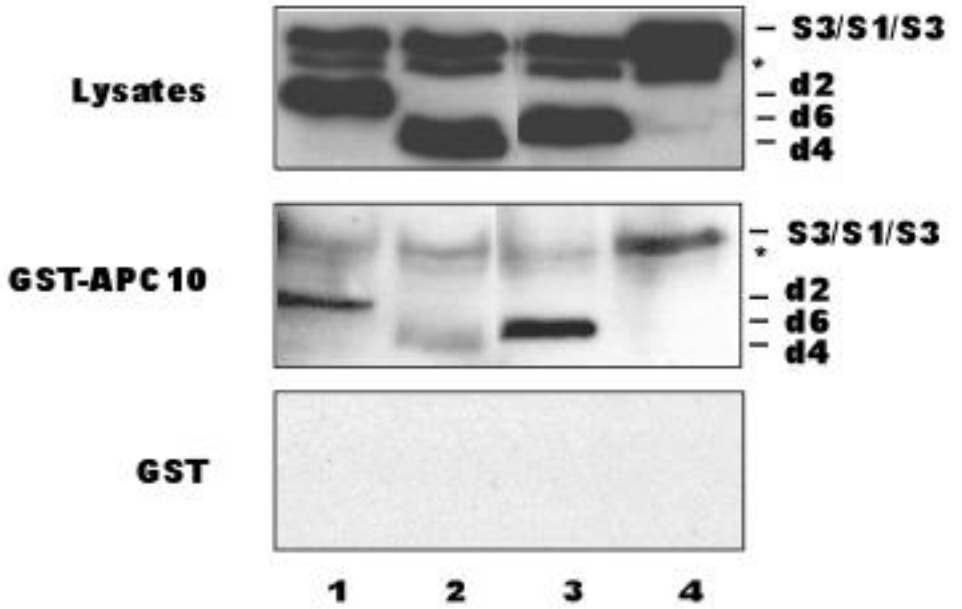
Smad3 d2	+	-	-	-	95%
Smad3 d4	-	+	-	-	34%
Smad3 d6	-	-	+	-	17%
S3/S1/S3	-	-	-	+	20%



C

Binding to HEF 1

Smad3 d2	+	-	-	-	21%
Smad3 d4	-	+	-	-	13%
Smad3 d6	-	-	+	-	23%
S3/S1/S3	-	-	-	+	24%



D

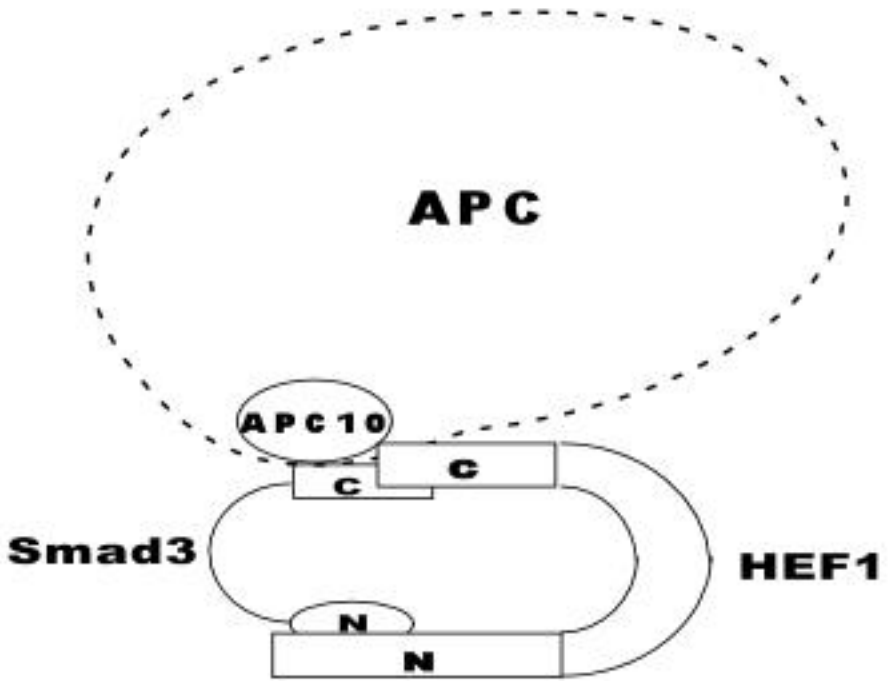
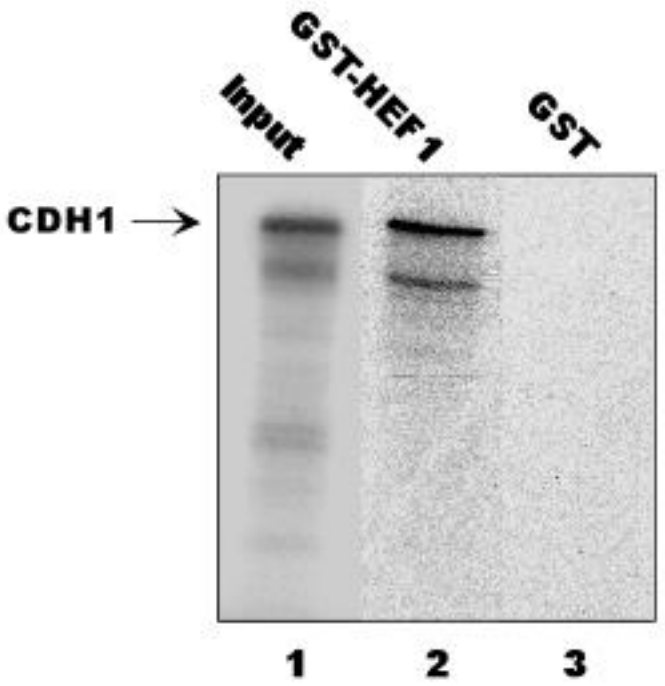
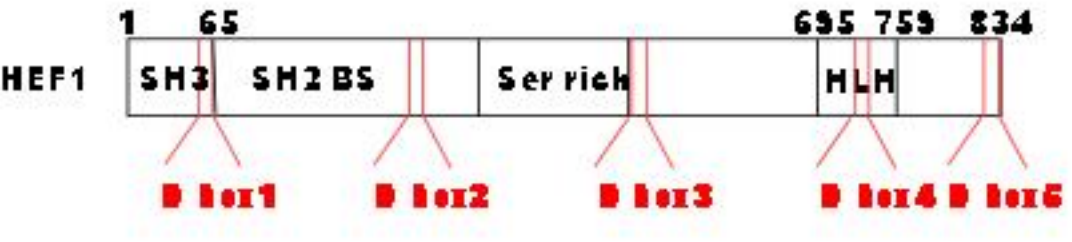


Figure 3

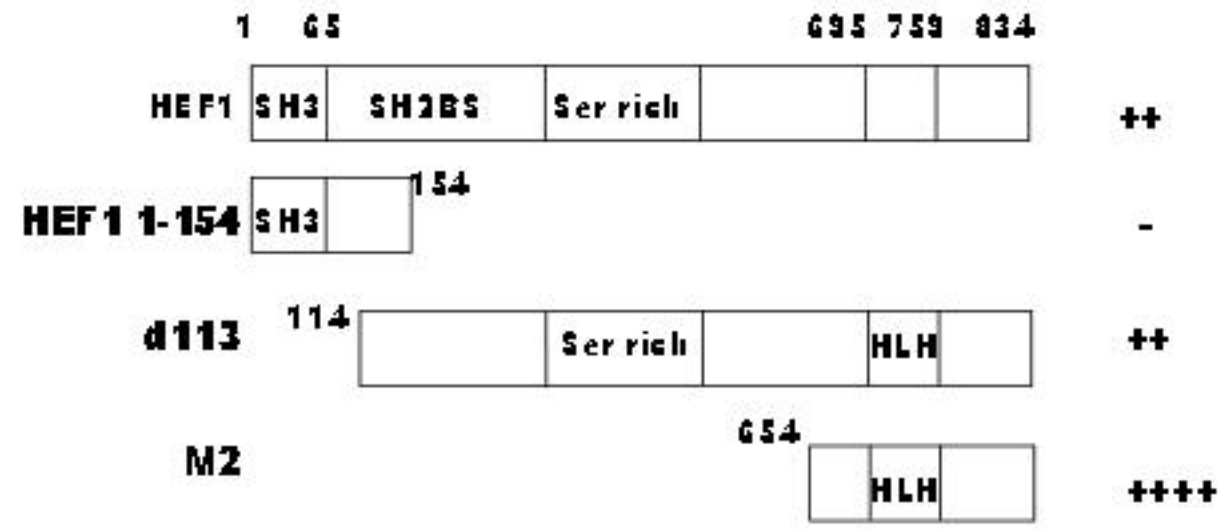
A


Input
GST-HEF1
GST
CDH1
1
2
3

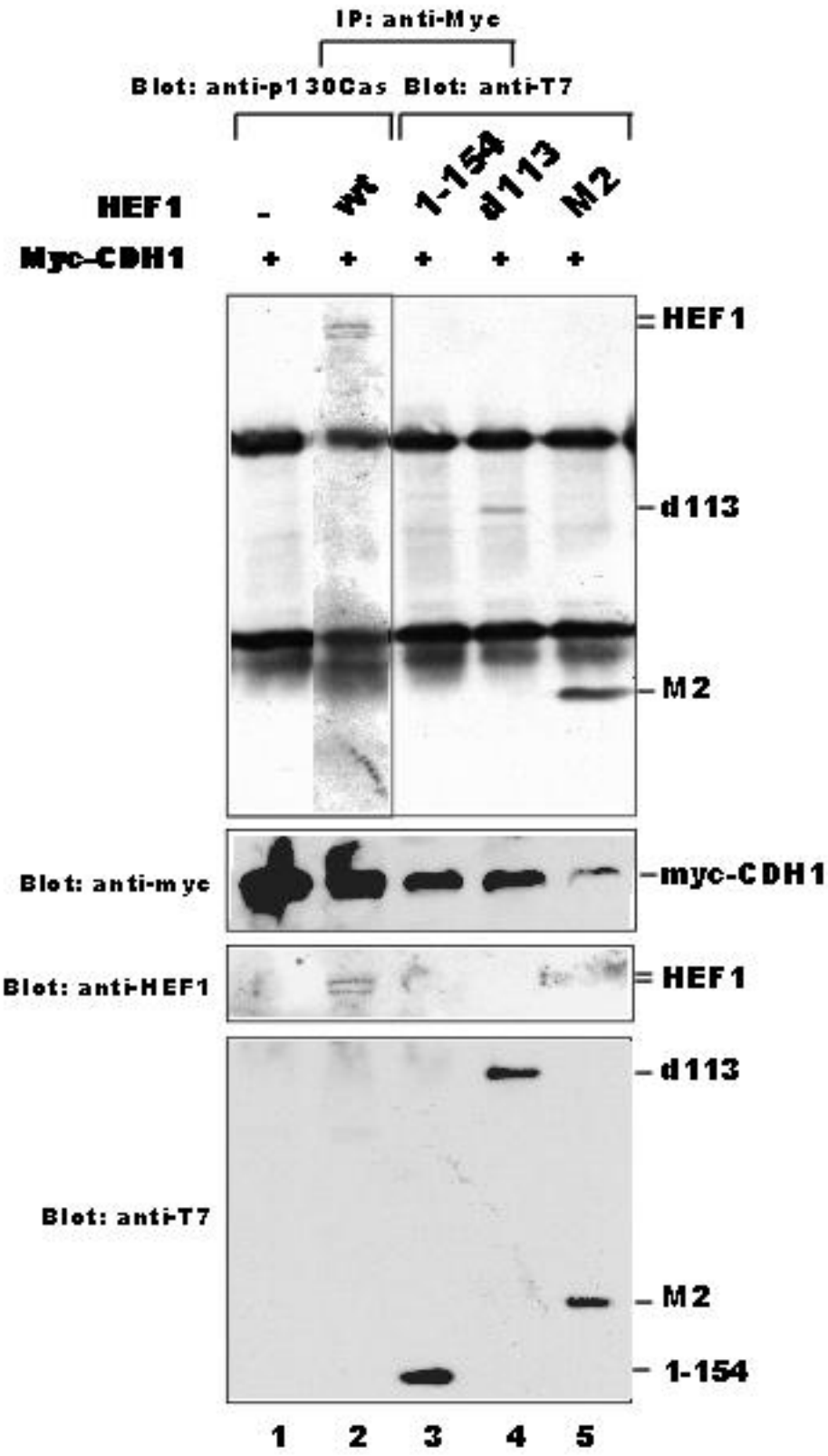
B


1
65
695
759
834
HEF1
SH3
SH2 BS
Ser rich
HLH
D box1
D box2
D box3
D box4
D box6

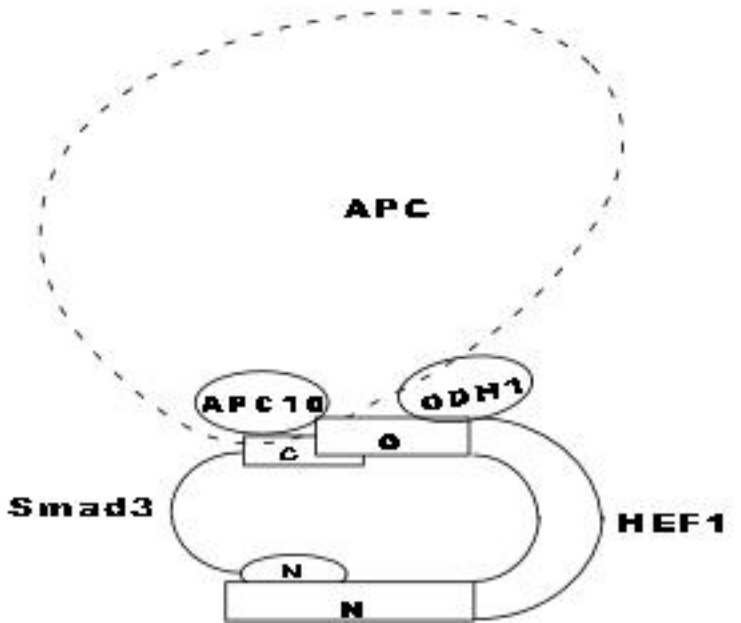
C


Binding to CDH1
1
65
695
759
834
HEF1
SH3
SH2BS
Ser rich
++
HEF1 1-154
SH3
154
-
d113
114
Ser rich
HLH
++
M2
654
HLH
++++

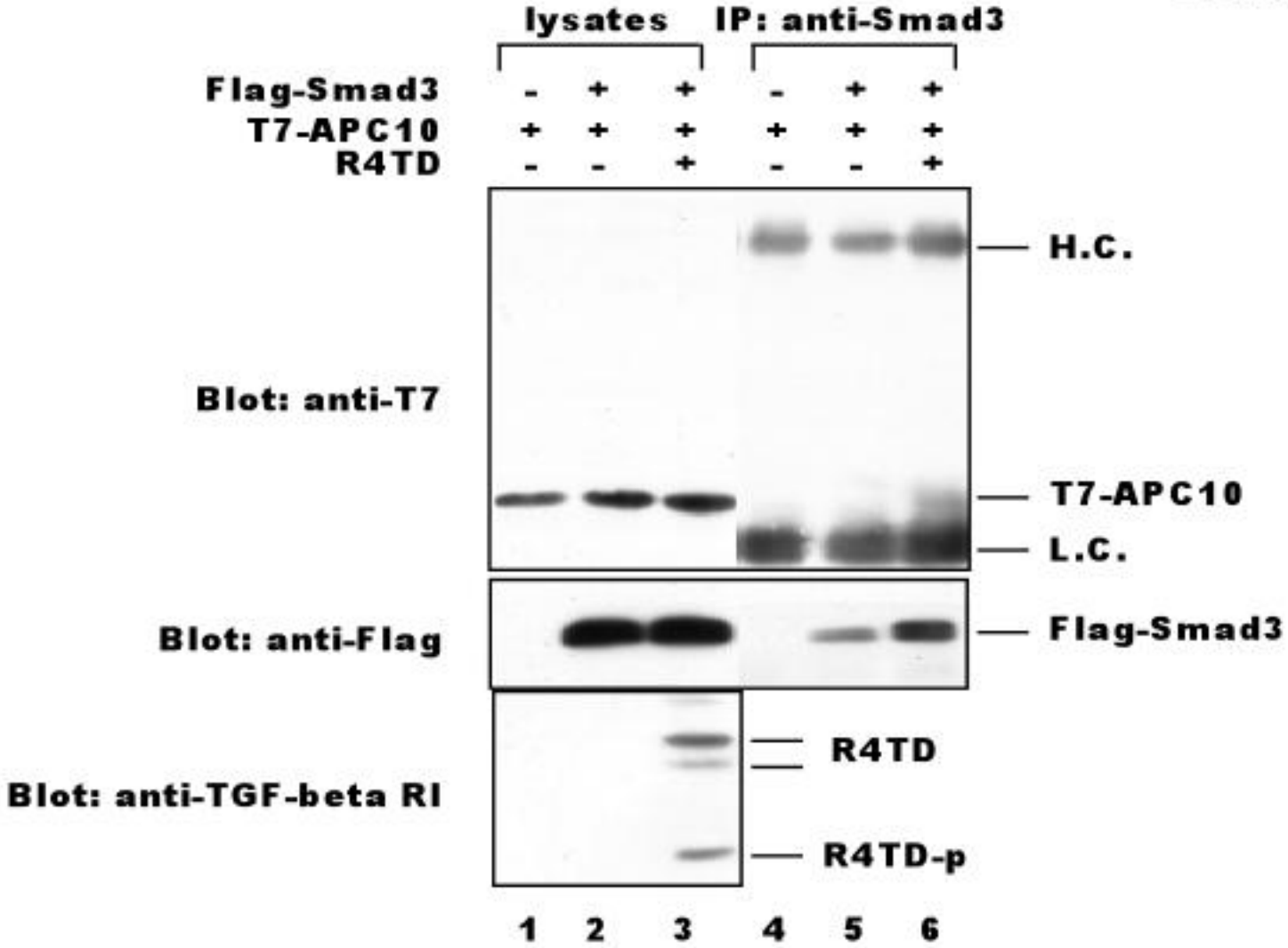
D


IP: anti-Myc
Blot: anti-p130Cas
Blot: anti-T7
HEF1
-
wt
1-154
d113
M2
Myc-CDH1
+
+
+
+
+
HEF1
d113
M2
Blot: anti-myc
myc-CDH1
Blot: anti-HEF1
HEF1
Blot: anti-T7
d113
M2
1-154
1
2
3
4
5

E


APC
APC10
CDH1
C
D
Smad3
HEF1
N
N

A


lysates
IP: anti-Smad3
Flag-Smad3 - + + - + +
T7-APC10 + + + + + +
R4TD - - + - - +
H.C.
Blot: anti-T7
T7-APC10
L.C.
Blot: anti-Flag
Flag-Smad3
R4TD
Blot: anti-TGF-beta RI
R4TD-p
1 2 3 4 5 6

B

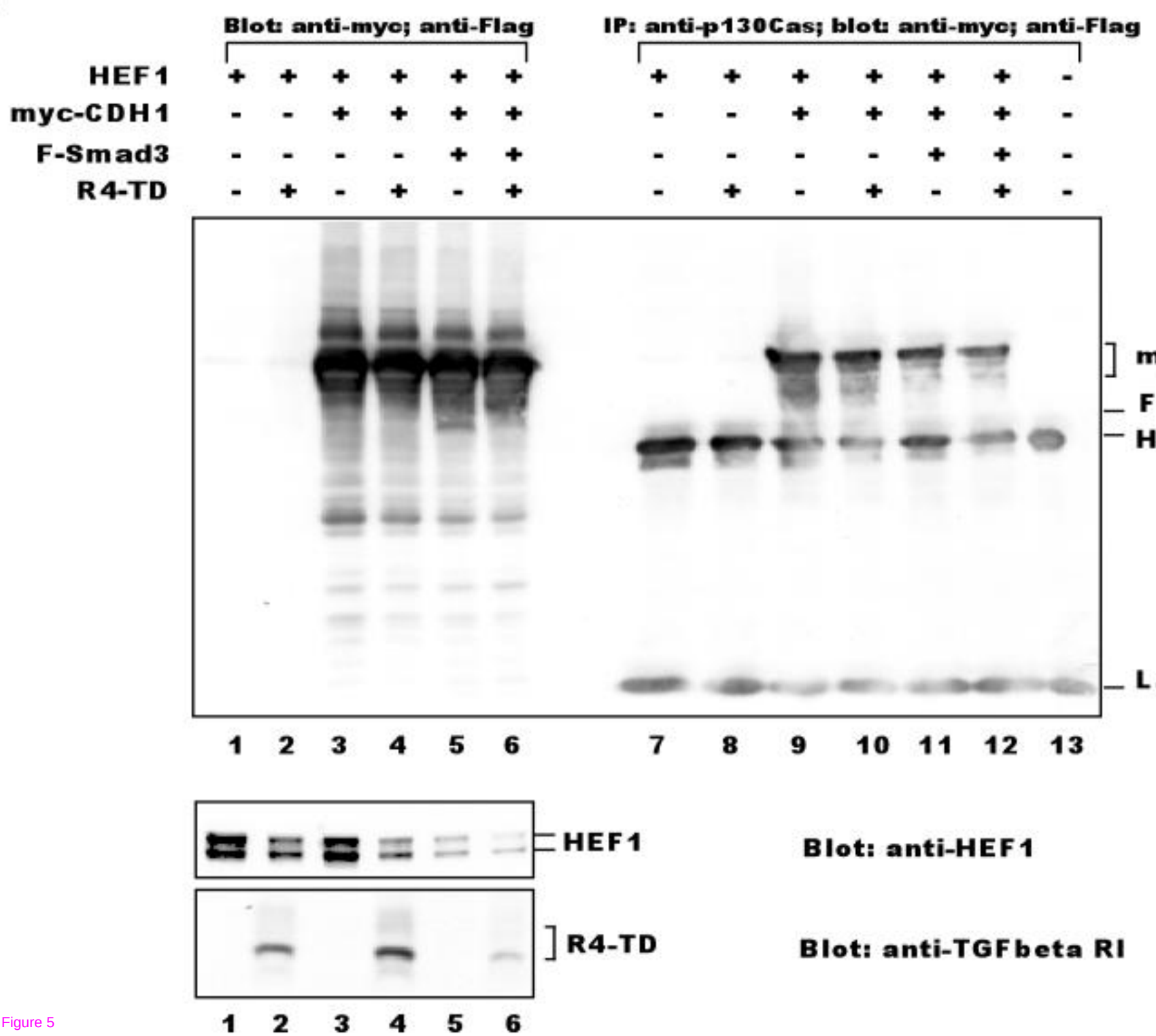
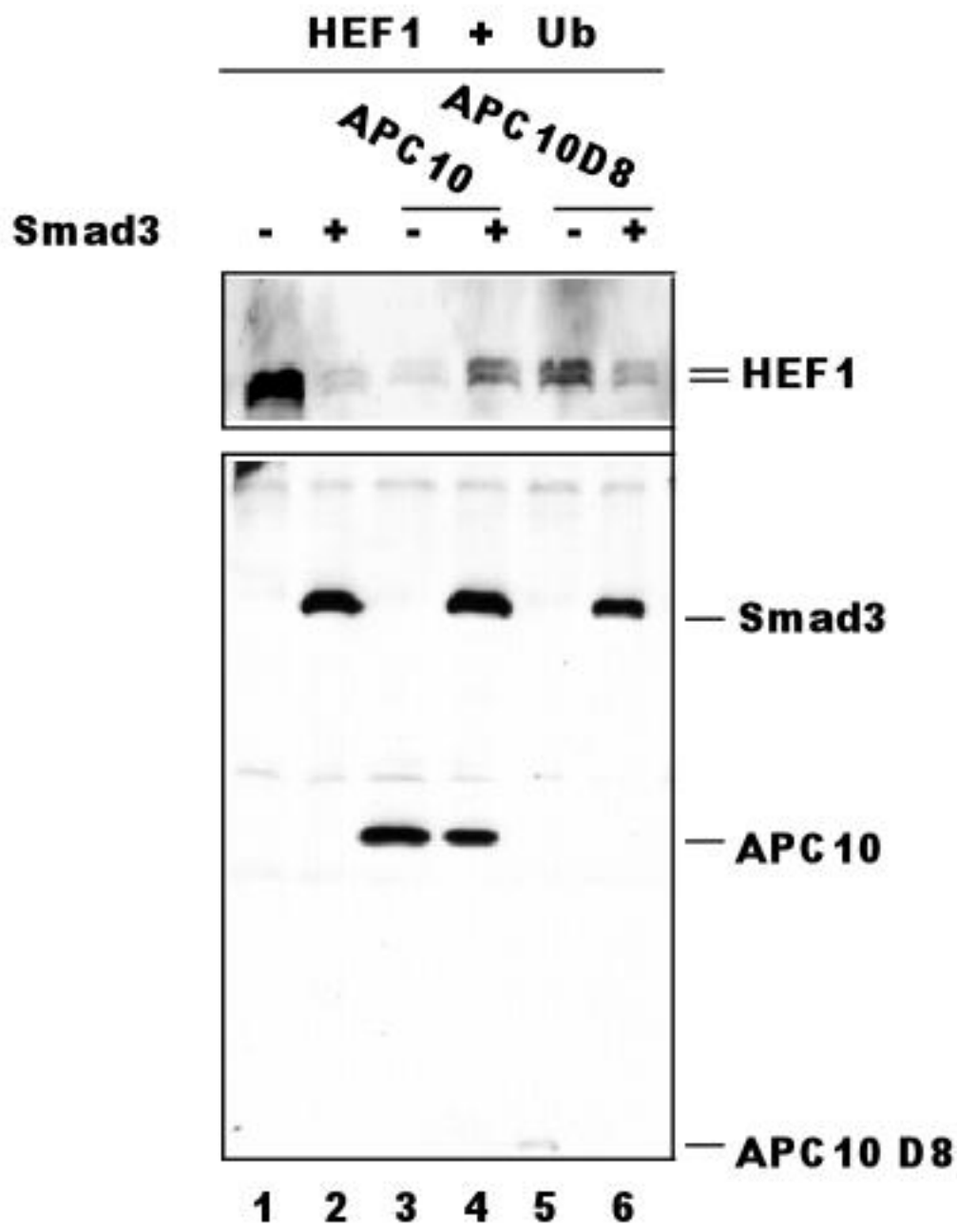

Blot: anti-myc; anti-Flag
IP: anti-p130Cas; blot: anti-myc; anti-Flag
HEF1 + + + + + + + + + + + + + -
myc-CDH1 - - + + + + - - + + + + - -
F-Smad3 - - - - + + - - - - + + - -
R4-TD - + - + - + - + - + - + - -
myc-CDH1
F-Smad3
H.C.
L.C.
1 2 3 4 5 6 7 8 9 10 11 12 13
HEF1
Blot: anti-HEF1
R4-TD
Blot: anti-TGFbeta RI
1 2 3 4 5 6

Figure 5

A


HEF1 + Ub
APC10
APC10D8
Smad3
- + - + - +
HEF1
Smad3
APC10
APC10 D8
1 2 3 4 5 6

B

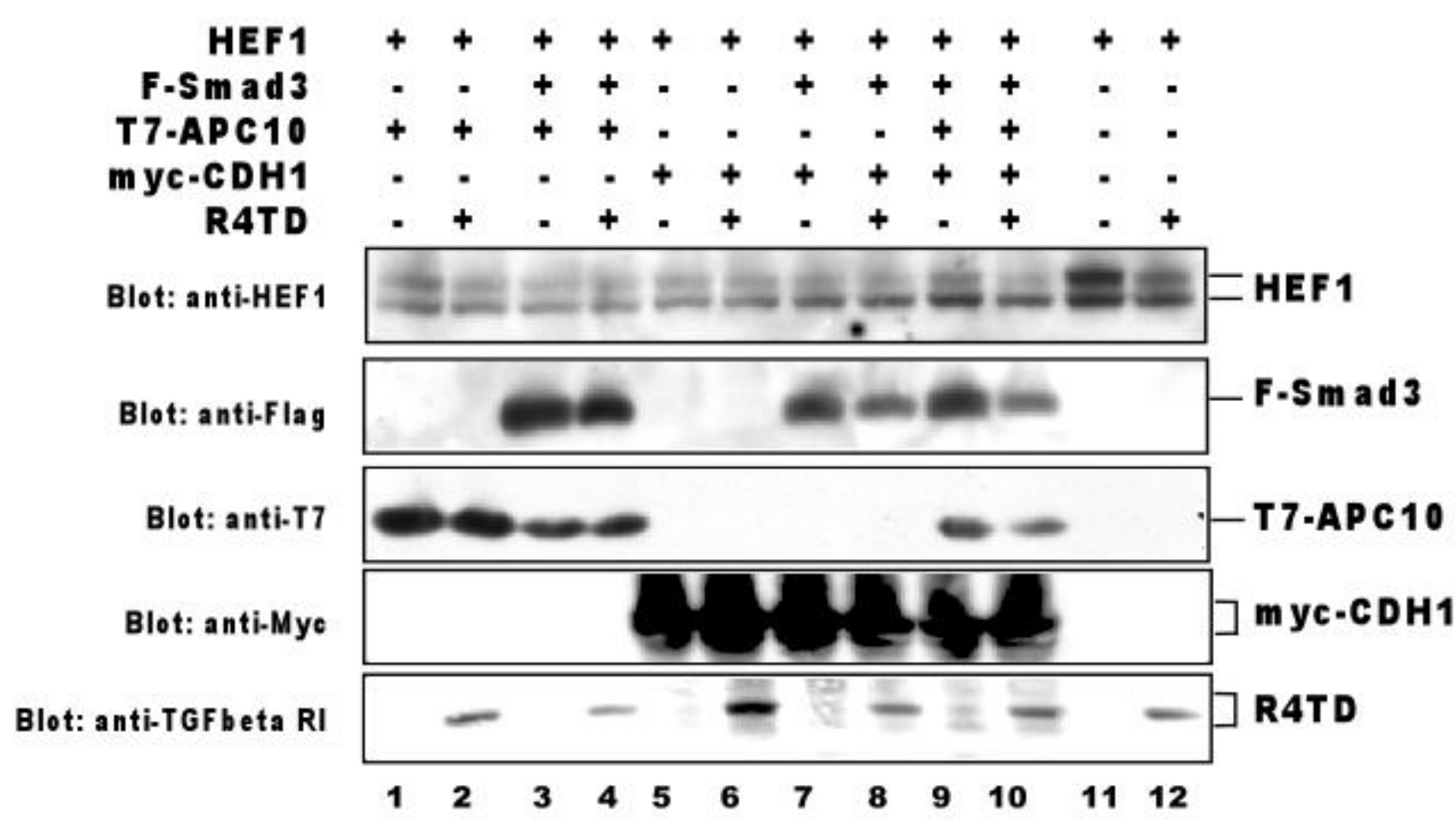

HEF1 + + + + + + + + + + + +
F-Smad3 - - + + - - + + + + - -
T7-APC10 + + + + - - - - + + - -
myc-CDH1 - - - - + + + + + + - -
R4TD - + - + - + - + - + - +
Blot: anti-HEF1
HEF1
Blot: anti-Flag
F-Smad3
Blot: anti-T7
T7-APC10
Blot: anti-Myc
myc-CDH1
Blot: anti-TGFbeta RI
R4TD
1 2 3 4 5 6 7 8 9 10 11 12

Figure 6

Nourry C. et al., Fig. 7

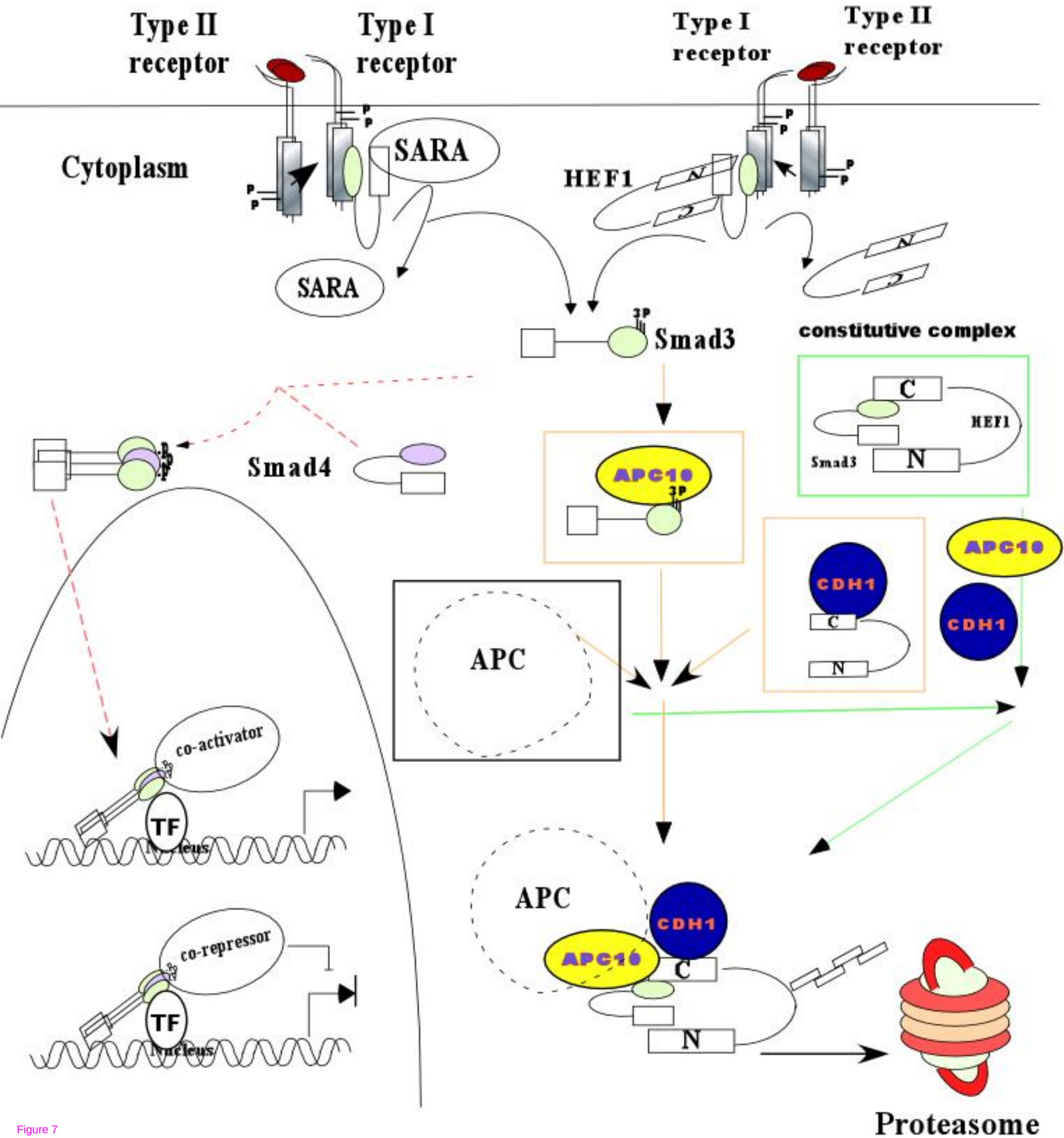


Figure 7