Mechanisms of Signal Transduction:

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Atrophin-1-interacting Protein 4/Human Itch Is a Ubiquitin E3 Ligase for Human Enhancer of Filamentation 1 in Transforming Growth Factor-β Signaling Pathways*

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Atrophin-1-interacting protein 4 (AIP4) is the human homolog of the mouse Itch protein (hItch), an E3 ligase for Notch and JunB. Human enhancer of filamentation 1 (HEF1) has been implicated in signaling pathways such as those mediated by integrin, T cell receptor, and B cell receptor and functions as a multidomain docking protein. Recent studies suggest that HEF1 is also involved in the transforming growth factor-β (TGF-β) signaling pathways, by interacting with Smad3, a key signal transducer downstream of the TGF-β type I receptor. The interaction of Smad3 with HEF1 induces HEF1 proteasomal degradation, which was further enhanced by TGF-β stimulation. The detailed molecular mechanisms of HEF1 degradation regulated by Smad3 were poorly understood. Here we report our studies that demonstrate the function of AIP4 as an ubiquitin E3 ligase for HEF1. AIP4 forms a complex with both Smad3 and HEF1 through its WW domains in a TGF-β-independent manner and regulates HEF1 ubiquitination and degradation, which can be enhanced by TGF-β stimulation. These findings reveal a new mechanism for Smad3-regulated proteasomal degradation events and also broaden the network of cross-talk between the TGF-β signaling pathway and those involving HEF1 and AIP4.

The transforming growth factor-β (TGF-β)[1] signaling is involved in a broad range of cellular functions, including proliferation, adhesion, apoptosis, differentiation, and specification of developmental fate (1, 2). The extracellular signals were transduced to the nucleus by the sequential association of type II and type I receptors and the Smad protein cascades (3, 4). The binding of ligands to the receptors leads to the phosphorylation of Smad2 and Smad3 at their SS*X*S motif within the COOH termini. The phosphorylated Smad2 or Smad3 forms complexes with Smad4 and translocates into the nucleus, where they function as DNA-binding transcription factors. Recently, Smad3 was discovered to have the novel ability of regulating the proteasomal degradation of the nuclear proto-oncoproteins SnoN and Ski (5, 6) as well as the human enhancer of filmentation 1 (HEF1) (7).

HEF1 is a member of a multiple domain docking protein Cas family including $p130^{cas}$ and Efs that have been implicated as signaling mediators of diverse processes including cellular attachment, motility, growth factor responses, apoptosis, and oncogenic transformation (8). HEF1 was first isolated in a screen for human proteins with the ability to alter *Saccharomyces cerevisiae* morphology from round to filamentous hyperpolarized cells (9). Based upon its homology to $p130^{cas}$, another group independently isolated HEF1, named Cas-L (10). Members of this family share similar domains, with an amino-terminal Src homology 3 domain that binds polyproline-containing protein, a large central domain encompassing multiple tyrosine motifs that are recognized by the Src homology 2 domain protein upon phosphorylation, a serine-rich domain, and a carboxyl-terminal domain containing a helix-loop-helix motif (9, 11).

Earlier studies have showed that HEF1 is predominantly expressed in epithelial cells and lymphocytes, whereas $p130^{cas}$ is abundant in many cell types (8, 10). The expression of HEF1 is cell cycle-regulated, with $p105^{HEF1}$ and $p115^{HEF1}$ accumulating at the focal adhesion sites when cells go through S and G_2 phase, whereas $p55^{HEF1}$ is specifically produced and localized to the mitotic spindle during mitosis (8). So far, HEF1 has been implicated in integrin, T cell antigen receptor, B cell antigen receptor, and the G-protein coupled calcitonin receptor signaling pathways (10, 12–15). Molecular and genetic studies indicated that HEF1 overexpression leads to an increase in cell motility and apoptosis, consistent with a role of HEF1 in regulating integrin signaling (16–19).

Protein ubiquitination is the type of post-translational modification in which a highly conserved 76-amino acid polypeptide, ubiquitin, is attached to proteins. A cascade of three enzymes mediates this process, the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzymes and the E3 ubiquitin ligase. E1 activates ubiquitin by generating a high energy E1-thiol ester-ubquitin intermediate. E2s transfer the activated ubiquitin to the cysteine residue on E3 before conjugating ubiquitin to the target proteins (20). Ubiquitin E3 ligase plays a vital role in substrate recognition specificity and subsequent protein degradation by the 26 S proteasome, which is a large multisubunit proteolytic complex. There are three major types of E3 ligase: homology to E6-AP carboxyl-terminal (HECT) domain E3s, RING finger motif-containing E3s, and E4/U box-containing proteins.

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; HEF1, human enhancer of filamentation 1; IOD, integrated optical density; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; AIP4, atrophin-1-interacting protein 4; HECT, homology to E6-AP carboxyl-terminal; Smurf1 and -2, Smad ubiquitination regulatory factor 1 and 2, respectively; HA, hemagglutinin; Ub, ubiquitin; GST, glutathione *S*-transferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; MG132, N^{α}-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal; SARA, Smad anchor for receptor activation; hItch, human Itch.

HEF1 was found to be involved in the TGF-β signaling pathway. It was isolated as a Smad3-specific interactor in a yeast two-hybrid screen using Smad3 as bait (7). Further studies indicate that Smad3 interaction with HEF1 enhances HEF1 degradation in a proteasome-dependent fashion and that the activation of TGF-β signaling further enhances HEF1 degradation (7). In epithelial cell lines and a T lymphoid cell line (H9), HEF1 protein level was demonstrated to undergo rapid reduction in responding to TGF-β stimulation followed by a negative feedback-type increase of HEF1 mRNA (7). Zheng and McKeown-Longo (21) also reported that TGF-β and cell adhesion regulate HEF1 expression and phosphorylation in dermal fibroblasts, adding more evidence to the functional link between TGF-β signaling pathways and HEF1 protein expression. Such a link provides a new molecular mechanistic explanation for the ability of TGF-β to regulate myriad biological functions through cross-talk to many of the HEF1-involved signaling pathways. However, the exact nature of the TGF-β signaling events associated with HEF1 degradation is still not fully understood, and the detailed molecular mechanisms of HEF1 degradation also need to be uncovered.

Here we report the biochemical studies of the physical and functional interaction between HEF1 and atrophin-1-interacting protein 4 (AIP4), which is the human homolog of the mice Itch protein (hItch) (22), an HECT family E3 ligase for Notch and JunB (23, 24). Lack of the Itch protein in non-agouti mice contributes to the autoimmune phenotypes of the Itch mice (22). AIP4 was originally cloned as an interactor of atrophin-1, the protein implicated in the neurodegenerative disease dentatorubral pallidoluysian atrophy (25, 26). Its protein sequence suggests that it belongs to the C2-WW subfamily within the HECT domain-containing E3 ligase family. The C2-WW subfamily is characterized with a calcium-dependent phospholipid-binding domain (or C2 domain) at the NH_2 terminus followed by 2–4 WW domains and then the COOH-terminal HECT domain. The HECT domain is a ~350-residue region that harbors a strictly conserved cysteine residue that forms an essential thiol ester intermediate during catalysis at the COOH terminus. Two known C2-WW subfamily members are Smad ubiquitination regulatory factor 1 (Smurf1) and Smad ubiquitination regulatory factor 2 (Smurf2), both of which function as constitutive E3 ligases for the bone morphogenetic protein pathway-restricted Smads (Smad1 and Smad5) and TGF-β pathway-restricted Smads (Smad2 and Smad3), respectively (27, 28). In addition, Smurf2, when bound to Smad7, is recruited to the activated TGF-β receptors to form a complex in which Smurf2 ubiquitinates Smad7 (29). In response to TGF-β stimulation, Smurf2 can be recruited by Smad2/Smad3 to form a complex with and ubiquitinate SnoN (28). The later observation suggests that E3 ligases not only mediate the ubiquitination of Smads but also can be recruited by Smads to mediate the ubiquitination of Smad-interacting proteins. AIP4 was first isolated as an interactor of Smad3 but does not mediate the ubiquitination of Smad3.[2] Recently, it has been demonstrated that AIP4 does mediate the ubiquitination of atrophin-1 as well as the two scaffold proteins MAGI-1 and GAGI-2 that were previously shown to bind atrophin-1.[3] Since Smad3 interacts with HEF1 and regulates HEF1 degradation, we tested the possibility of AIP4 to be recruited by Smad3 to mediate the ubiquitination of HEF1. Our results show that AIP4 is an E3 ligase for HEF1 and, together with Smad3, regulates proteasomal degradation of HEF1.

[2] S. Guedes, J. Farley, X. Liu, and T. Wang, manuscript in preparation.

[3] J. D. Wood, Z. A. Keminsky, Y. Kim, S. Guedes, T. Wang, and C. A. Ross, submitted for publication.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-Myc (9E10) and anti-HEF1 (N-17) were purchased from Santa Cruz Biotechnology. Anti-p130^{cas} 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 Applied Science. N^{α}-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (MG132) (c2211), *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL) (A6185), phosphatase inhibitor (P5726), protease inhibitor mixture (P8340), and ubiquitin (U6253) were all purchased from Sigma. Cycloheximide (100183) was purchased from ICN Biomedicals Inc. MG132 and LLnL were dissolved in Me_2SO and added directly into cell culture medium to a final concentration of 50 μM for 5 h before harvest.

Constructs—The construction of full-length Myc-AIP4 has been described previously.[3] The pCMV-HEF1 expression vector has been described previously (8). All of the other mammalian expression constructs for HEF1 were constructed in our laboratory previously (7). Smad3 and HEF1 were subcloned into EcoRI/XhoI sites, and AIP4 was suncloned with BamHI and NotI sites in pGEX-5X-1 (Amersham Biosciences) using standard procedures (30). Smad3 and HEF1 were subcloned into EcoRI/XhoI sites, and AIP4WT/CA were subcloned into SalI/NotI in pCS2+ vector containing a SP6 promoter for *in vitro* translation. Myc-Smurf1 and Myc-Smurf2 constructs were obtained from the laboratory of J. Wrana. The HA-Ub construct was a kind gift from Dr. M. Treier.

Mammalian Cell Line—293 cells (human kidney cells transformed with adenovirus 5 DNA) were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 25,000 units of penicillin, 25 mg of streptomycin, and 5 ml of 200 mM L-glutamine at 37 °C in the presence of 5% CO_2. Mink lung epithelial cells (Mv1Lu or ML) were cultured in the same medium and under the same conditions as description above except using active fetal bovine serum.

Transfection—293 cells were transfected using the standard $CaPO_4$ procedure (30), and cells were harvested 24 h after transfection.

Immunoprecipitation and Western Blotting—Cells were incubated 30 min on ice with Hepes-buffered saline-lysis buffer (50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100 supplemented with protease and phosphatase inhibitors just prior to use). Cell debris was pelleted by spinning in a microcentrifuge at 14,000 × *g* at 4 °C for 10 min, 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 a 50% slurry of protein G-Sepharose 4 Fast Flow (Amersham Biosciences). Beads were then washed once using lysis buffer and three times with modified lysis buffer (lysis buffer containing 0.1% Triton X-100). The precipitated proteins were eluted in 2× SDS loading buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) plus 10% β-mercaptoethanol, loaded on SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (Millipore Corp.). Membranes were analyzed by Western blot (30). Antibodies were diluted as follows: α-HEF1 (1:1000), α-p130^{cas} (1:1000), α-FLAG (1:4000), α-T7 (1:10,000), α-Myc (1:1000), α-HA (1:2000).

GST Pull-down Assay—GST-AIP4 and GST-HEF1 were expressed and purified from *Escherichia coli* strain BL-21. Briefly, the culture was induced at OD ~0.6 with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 2–3 h. Cell were collected by spinning at 5,000 rpm at 4 °C for ~15 min. The pellet was then resuspended in Prep buffer (100 mM NaCl, 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2% Triton X-100) supplemented with 2 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Lysis occurred using 10 mg/ml lysozyme (catalog no. BP 535-1; Fisher) in Prep buffer for 30 min on ice, and debris was spun down by spinning 30 min at 4,000 × *g* at 4 °C. Cell lysates were then incubated with a 50% slurry of glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences) for 1 h at 4 °C and washed three times with ice-cold PBS. About 4 μg of GST fusion proteins that were immobilized on beads were incubated with extracts 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 lysate system (Promega). The *in vitro* translated product (8 μl) was incubated with the GST beads in 200 μl of modified lysis buffer supplemented with protease inhibitors and washed as previously described.

Yeast Two-hybrid Tests—Protein-protein interaction tests using the yeast two-hybrid system were carried out as described before (7). Briefly, full-length HEF1 was subcloned into the bait construct pEG202 and fused in frame with the DNA binding domain LexA. AIP4 and its

deletions were subcloned into the prey construct pJG4-5 and fused in frame with the transcriptional activation domain B42. Yeast strain *EGY48* was transformed with the bait construct first and then transformed with the prey construct. The interaction was monitored on glucose plate and galactose plate supplemented with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Protein Degradation Assays—Mink lung epithelial cell (Mv1 Lu or ML) extracts were made as described before (5). Briefly, mink lung epithelial cells were cultured for 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% CO_2. To stimulate cells with TGF-β, they were treated with 100 pM TGF-β (a gift from Anita Roberts). After 1 h, cells were washed with phosphate-buffered saline and harvested by scraping. Approximately 1×10^8 harvested cells were resuspended in 500 μl of hypotonic buffer (20 mM HEPES (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol), 1× protease mixture (Roche Applied Science), and an energy regeneration mixture (31) for 30 min to allow cells to swell. Cells were frozen by liquid nitrogen, thawed in a 37 °C water bath, and homogenized with 10 strokes using a Dounce homogenizer. Cell lysates were spun in an Eppendorf microcentrifuge at 14,000 rpm at 4 °C for 1 h. The clear supernatant was collected using a syringe needle and used directly for protein degradation assays. For protein degradation assays, ~10 ng of ^{35}S-labeled HEF1 synthesized in the TNT expression system and 8 ng of unlabeled *in vitro* translated Smad3 or AIP4CA or AIP4 were added to 20 μl of fresh Mv1Lu extracts supplemented with the degradation mixture (1.25 mg/ml ubiquitin, 1× energy regeneration, and 0.1 mg/ml cycloheximide). Aliquots were removed at different times and resolved by SDS-PAGE and autoradiography.

RESULTS

AIP4 Interacts Directly with HEF1—Our previous studies have suggested that the Cas family multidomain docking protein HEF1 was subjected to rapid proteasomal degradation upon its interaction with the key signal transducer of the TGF-β pathway, Smad3. It was shown that the interaction between Smad3 and HEF1 via their amino-terminal domains is important for HEF1 degradation (7). However, it is not clear how Smad3 interaction with HEF1 can lead to HEF1 degradation by proteasome. The HECT family E3 ligase AIP4 was isolated together with HEF1 from the yeast two-hybrid system as a strong and specific interactor of Smad3 (7). Initially, we tested whether AIP4 is an E3 ligase for Smad3. However, unlike Smurf1 or Smurf2, AIP4 does not ubiquitinate Smad3.[2] Thus, we tested whether AIP4 mediates HEF1 ubiquitination by interacting with Smad3 and HEF1.

The interaction between AIP4 and HEF1 was first tested in the yeast two-hybrid system. The full-length HEF1 was cloned into pEG202 to generate a LexA fusion protein. A truncated version of AIP4, AIP4Δ65, fused with the transcription activation domain B42, was obtained directly from the screen using Smad3 as bait. AIP4Δ65 contains four WW domains and part of HECT domain. After both constructs were made and transformed into the yeast, the transformants were selected on a glucose $U^-H^-W^-$ plate. The transformants were then spotted onto a galactose/raffinose-X-gal plate, which allows the tested clone to turn blue within 24 h if an interaction occurs between the two fusion proteins. The same transformants were also spotted onto a control plate of glucose/X-gal, which allows the tested clone to remain white due to the repression of the expression of the B42 fusion protein by glucose. As shown in Fig. 1*A*, the yeast clone containing LexA-HEF1 and B42-AIP4Δ65 turned strong blue on the galactose/raffinose X-gal plate but remained white on glucose X-gal plate, suggesting strong and specific interaction between these two proteins.

To further confirm the interaction observed in the yeast two-hybrid system, we carried out the GST pull-down assays to test the interaction between HEF1 and AIP4 *in vitro*. FLAG-tagged Smad3 and Myc-tagged AIP4 were transiently expressed in the 293 cells respectively or together. Cell lysates were made and tested for the expression of both proteins (Fig. 1*B*, *lanes 1–3*) and incubated with GST bead-bound GST-HEF1 expressed and purified from *E. coli* BL21. After eluting the bound proteins from the beads, Western blot was performed to detect Myc-AIP4 and FLAG-Smad3 using anti-Myc antibody and anti-FLAG antibody, respectively (Fig. 1*B*, *lanes 4–6*). Both Myc-AIP4 and FLAG-Smad3, respectively, were found to bind HEF1 (Fig. 1*B*, *lanes 4* and *5*). When both Myc-AIP4 and FLAG-Smad3 were co-expressed, both proteins were detected to bind GST-HEF1 (Fig. 1*B*, *lane 6*). The FLAG-Smad3 band was even more intense than that detected when FLAG-Smad3 alone was incubated with GST-HEF1 (Fig. 1*B*, *lane 6* compared with *lane 5*), suggesting that there is no competition between Smad3 and AIP4 to bind HEF1, but instead the interaction appears to be simultaneous or even cooperative. The specificity of such interactions was further tested by including HEF1 itself in the lysates (Fig. 1*C*). Excess of HEF1 expression together with FLAG-Smad3 and Myc-AIP4 totally blocked both of these proteins from binding to GST-HEF1 (Fig. 1*C*, compare *lanes 5* and *6* with *lanes 7* and *8*).

The above *in vitro* pull-down assay demonstrates that both AIP4 and Smad3 can form a complex, possibly a ternary complex with HEF1. We then tested via *in vitro* binding assay whether the interaction between AIP4 and HEF1 or between AIP4 and Smad3 is direct. ^{35}S-labeled *in vitro* translated HEF1 or Smad3 proteins (Fig. 1*D*, *lanes 1* and *2*) were incubated with GST-AIP4 (Fig. 1*D*, *lanes 3* and *4*). GST alone was use as a negative control (*lanes 5* and *6*). ^{35}S-Labeled Smad3 and HEF1 were found to bind GST-AIP4 (Fig. 1*D*, *lanes 3* and *4*). A separate study has shown that HEF1 interacts with Smad3 directly.[4] These data pointed out that HEF1, AIP4, and Smad3 perform mutual direct interaction.

Next we tested whether AIP4 and HEF1 interact in mammalian cells. Myc-tagged full-length AIP4 protein was transiently co-expressed with HEF1 protein in the 293 cells. HEF1 protein was immunoprecipitated with anti-p130^{cas} antibody, and the co-precipitated Myc-tagged AIP4 was detected by Western blot using anti-Myc antibody. Myc-AIP4 was detected to co-precipitate with HEF1 (Fig. 1*E*, *lane 4*). A conserved cysteine residue in the HECT domain of AIP4 (C830) is considered to form an essential thiol ester intermediate during catalysis. To determine whether the ligase activity of AIP4 regulates the interaction, as shown to be the case for the Smurfs (27), we mutated this conserved cysteine and created the ligase-dead mutant AIP4 (C830A) and tested its interaction with HEF1. No change was detected; thus, the ligase activity of AIP4 does not affect AIP4 binding to HEF1 (Fig. 1*E*, *lane 5*). The very weak band of AIP4 detected in *lane 3* most likely represents the small amount of Myc-AIP4 co-precipitated with the endogenous HEF1.

HEF1 Interacts with AIP4 through Its Carboxyl Terminus and Requires at Least Two WW Domains of AIP4—We carried out domain mapping analyses on AIP4 and HEF1 to identify critical domains for their binding. First, in the yeast two-hybrid system, a set of AIP4 deletion mutants were made and expressed as the B42 fusion proteins (Fig. 2*A*, *top panel*). They were tested against the LexA fusion protein of full-length HEF1 (LexA-HEF1). The mutant containing only the first WW domain (AIP4WW1) was not sufficient to bind to HEF1, whereas the mutants containing two, three, and four WW domains (AIP4WW2, AIP4WW3, and AIP4Δ65) exhibited strong interaction with HEF1 (Fig. 2*A*, *bottom panel*, *lanes 3–5*).

To determine the AIP4 binding domain on HEF1, various deletions of HEF1 (Fig. 2*B*, *top*, *schematic diagram*) were cloned into mammalian expression vector with T7 or HA epitope tags placed at the NH_2 termini. They were co-trans-

[4] Nourry, C., Maksumova, L., Liu, X., and Wang, T. (2004) *BMC Cell Biol.* **5,** 20.

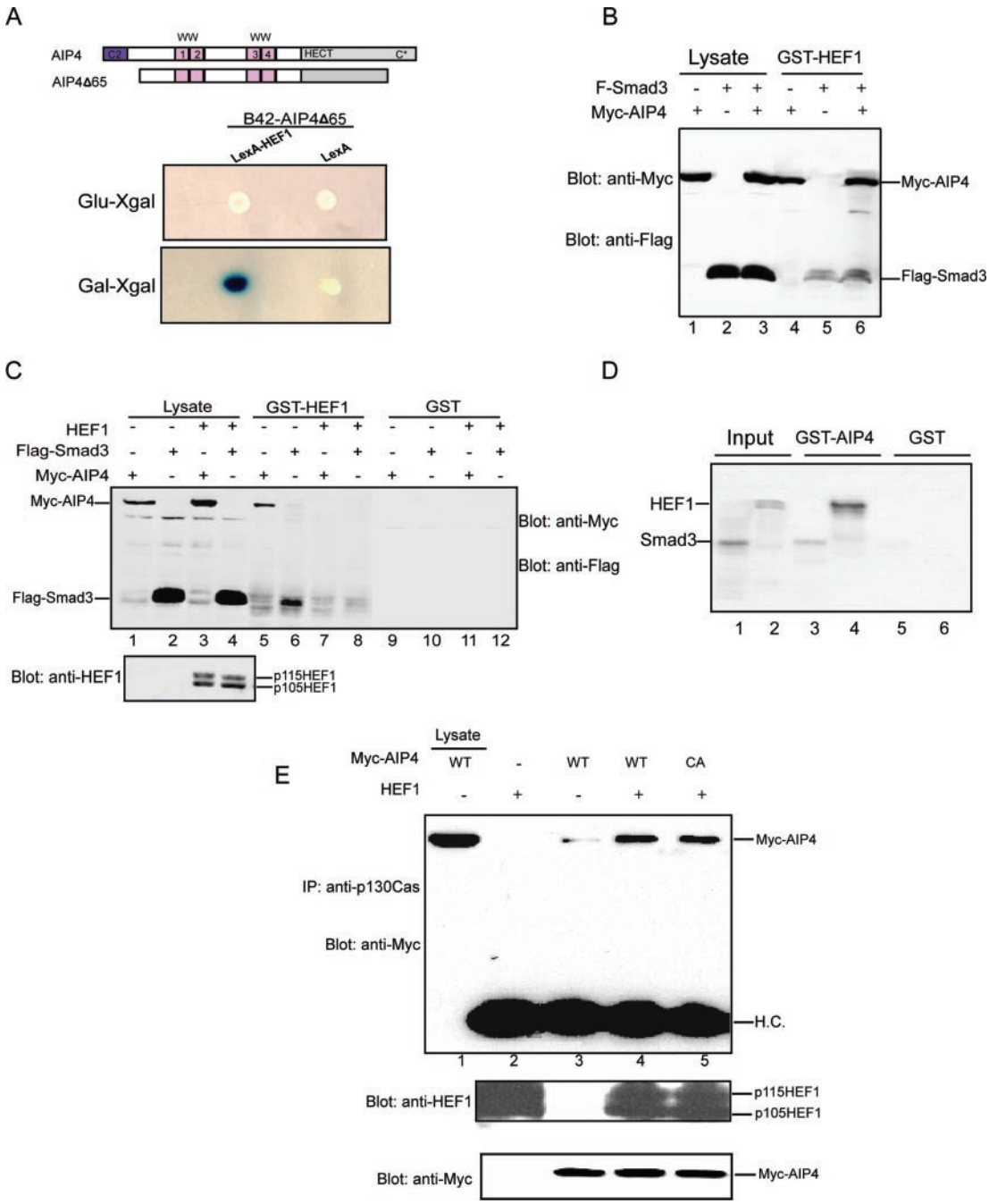

FIG. 1. **HEF1 interacts with AIP4 and Smad3.** *A*, AIP4 interacts with HEF1 in the yeast two-hybrid system. The transcription activation domain B42 was fused to AIP4Δ65 originally isolated from the yeast two-hybrid screen. The DNA-binding domain of LexA was fused to full-length HEF1. Yeast strain EGY48 (*leu2, trp1, his3, ura3*, *LexAop-Leu2* integrated, *LexAop-LacZ* on 2μ plasmid with a *Ura3*-selective marker) was transformed with both fusion constructs and grown on yeast-selective plates lacking uracil (U), histidine (H), and tryptophan (W) ($U^-H^-W^-$ glucose plates). As a negative control, LexA-HEF1 was replaced with LexA alone. The transformants were tested for interaction on a pair of yeast X-gal-selective plates: $U^-H^-W^-$ glucose X-gal plate and $U^-H^-W^-$ galactose X-gal plate. The expression of LexA or LexA fusion protein is constitutive on both plates, whereas the B42 fusion protein is only expressed on the galactose plate. Specific interaction between HEF1 and AIP4 is indicated by the inducible *blue color* of the yeast transformants of LexA-HEF1 and B42-AIP4 on the galactose X-gal plate. *WW*, WW domain. *B*, Smad3 and AIP4 bind to GST-HEF1 in the *in vitro* GST pull-down assay. FLAG-Smad3 and Myc-AIP4 were either transfected alone or co-transfected pairwise, as indicated, into 293 cells, and the lysates (*lanes 1–3*) were incubated with purified GST-HEF1 (*lanes 4–6*). The bound FLAG-Smad3 and Myc-AIP4 were eluted from the beads, separated on SDS-PAGE, and detected by Western blot with α-FLAG and α-Myc antibody. *C*, specific interaction between AIP4 and GST-HEF1 and between Smad3 and GST-HEF1. FLAG-Smad3 and Myc-AIP4 were either transfected alone or co-transfected with HEF1, as indicated, into 293 cells, and the lysates (*top*, *lanes 1–4*) were incubated with purified GST-HEF1 (*top*, *lanes 5–8*) or with GST as a control (*top*, *lanes 9–12*). The bound FLAG-Smad3 and Myc-AIP4 were eluted from the beads, separated on SDS-PAGE, and detected by Western blot with α-FLAG and α-Myc antibody. The excess HEF1 from the lysates blocked such interactions (*top*, *lanes 7* and *8*). The expression level of the transfected HEF1 was detected by Western blot using α-HEF1 antibody (*bottom*). *D*, HEF1 and Smad3 directly interact with GST-AIP4 in the *in vitro* binding test. *In vitro* translated ^{35}S-labeled HEF1 and Smad3 (*lanes 1* and *2*) were incubated with purified GST-AIP4 fusion proteins, which were absorbed on the GST beads (*lanes 3* and *4*). GST alone served as a negative control (*lanes 5* and *6*). ^{35}S-Labeled HEF1 and Smad3 bound to GST-AIP4 were eluted from the beads, separated onto SDS-PAGE, and detected by autoradiography. *E*, HEF1 co-precipitates with AIP4 in mammalian cells transiently co-transfected with both proteins. The 293 cells were transiently transfected with HEF1, either alone or together with Myc-tagged AIP4WT or with AIP4CA (a ligase-dead mutant of AIP4, with cysteine 830 mutated to alanine). Cell lysates were immunoprecipitated (*IP*) with α-p130^{cas} antibody (cross-reactive with HEF1) followed by Western blot with α-Myc antibody (*top*, *lanes 2–5*). The expression levels of the transfected HEF1 and AIP4/AIP4CA were detected by Western blot using α-HEF1 and α-Myc antibodies, respectively (*middle* and *bottom*). *H.C.*, heavy chain; *WT*, wild type; *CA*, C to A point mutation.

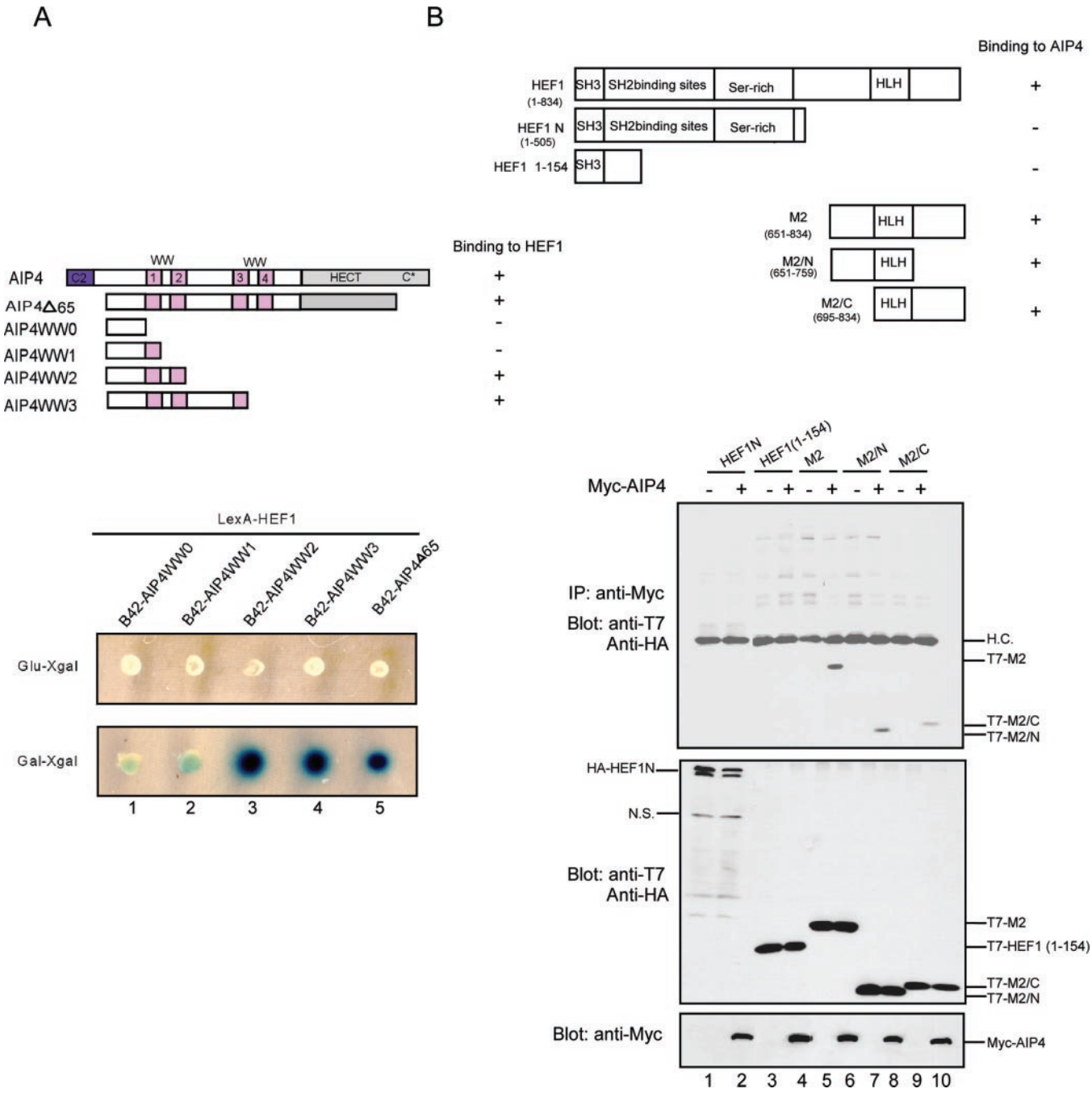

FIG. 2. **The WW domains of AIP4 and the COOH terminus domain of HEF1 are required for AIP4 interaction with HEF1.** *A*, at least two WW domains of AIP4 are necessary and sufficient for binding to HEF1 in the yeast two-hybrid system. *Top*, a *schematic diagram* to illustrate a set of AIP4 deletion constructs made to determine the role of the carboxyl terminus HECT domain and the four WW domains (*WW*) in binding to HEF1. The results obtained from the *bottom* are summarized to the *right* of each construct. *Bottom*, yeast two-hybrid test to determine the ability of the AIP4 deletions to bind to HEF1. B42 fusion proteins of AIP4 deletions were tested against the LexA fusion protein of HEF1 (LexA-HEF1). The transformants were plated onto the two types of selective yeast X-gal plates (glucose X-gal at the *top* and galactose X-gal at the *bottom*). The *blue color* on the galactose X-gal plate indicates specific interaction. *B*, HEF1 interacts with AIP4 through its carboxyl-terminal domain. *Top*, a *schematic diagram* to illustrate various HEF1 deletion mutants. *Bottom*, HEF1 deletions (all are tagged with T7 epitope except HEF1 N, which is tagged with HA epitope) were co-transfected with Myc-tagged AIP4 into 293 cells. Cell lysates were immunoprecipitated with α-Myc antibody followed by Western blot with α-T7 antibody and α-HA antibody, respectively (*top*). The expression of the HEF1 deletions was detected by Western blot of the total lysates (*middle*). The expression of Myc-AIP4 was detected by Western blot with α-Myc antibody (*bottom*). *HLH*, helix-loop-helix. *N.S.*, non-specific; *H.C.*, heavy chain.

fected with full-length Myc-AIP4 into the 293 cells. The expression levels of the transfected HEF1 deletions and Myc-AIP4 were determined by Western blot with the respective antibodies (Fig. 2*B*, *middle* and *bottom*). Myc-AIP4 was then immunoprecipitated from these cell lysates, and the co-precipitated HEF1 deletion mutants were detected by Western blot using anti-T7 antibody and anti-HA antibody (Fig. 2*B*, *top*). HEF1 1–154 and HEF1 N (amino acids 1–505) both failed to bind AIP4 (Fig. 2*B*, *top*, *lanes 1–4*). All three mutants of HEF1 containing the COOH-terminal region co-precipitated with AIP4 (Fig. 2*B*, *top*, *lanes 5–10*), suggesting that this region contains the AIP4 binding domain.

AIP4 Ubiquitinates HEF1 in Vivo—Since AIP4 has been shown to be a ubiquitin E3 ligase for its interacting proteins

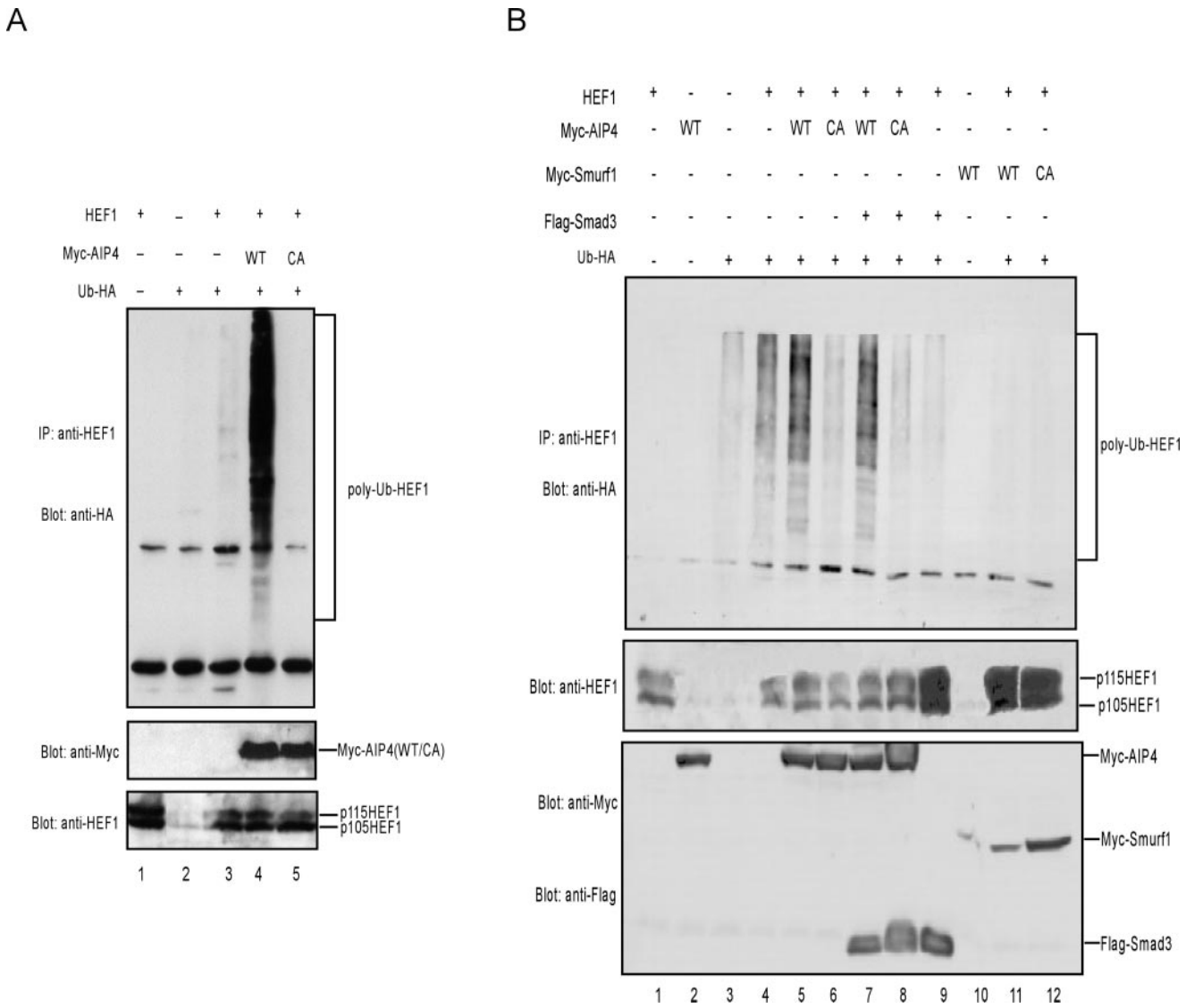

FIG. 3. **AIP4 ubiquitinates HEF1 in 293 cells.** *A*, the 293 cells were transfected with HEF1, Myc-tagged AIP4WT/CA, and HA-tagged ubiquitin. HEF1 proteins from the cell lysates were immunoprecipitated (*IP*) with α-HEF1 antibody. The ubiquitinated HEF1 was detected by Western blot with the α-HA antibody (*top*). The ubiquitinated HEF1 was detected as high molecular weight ladders, which are indicated by a *bracket* to the *right* of the *top panel*. The expression levels of the transfected Myc-AIP4WT/CA and HEF1 were determined by Western blot with the α-Myc antibody (*middle*) and α-HEF1 antibody (*bottom*), respectively. *B*, the 293 cells were transfected with HEF1, FLAG-tagged Smad3, Myc-tagged AIP4WT/CA, Myc-tagged Smurf1, and HA-tagged ubiquitin, as indicated. HEF1 proteins from the cell lysates were immunoprecipitated with α-HEF1 antibody. The ubiquitinated HEF1 was detected by Western blot with the α-HA antibody (*top*). The expression level of the transfected HEF1 was detected by Western blot with the α-HEF1 antibody (*middle*). The expression levels of the transfected FLAG-Smad3, Myc-AIP4, and Myc-Smurf1 were determined by Western blot with the α-FLAG and α-Myc antibody (*bottom panel*). *WT*, wild type; *CA*, C to A point mutation.

such as atrophin 1, North, and JunB (23, 24),[3] we tested whether it also serves as a ubiquitin E3 ligase for HEF1. The *in vivo* ubiquitination assay was carried out in 293 cells. The full-length wild type AIP4 and the ligase-dead mutant AIP4 (C830A) was co-transfected with HEF1 and HA-tagged ubiquitin (HA-Ub) into the 293 cells. The expression of HEF1, AIP4, and AIP4 (C830A) was detected by Western blot using anti-HEF1 and anti-Myc, respectively (Fig. 3*A*, *middle* and *bottom*). HEF1 was immunoprecipitated from cell lysates with an anti-HEF1 antibody, and the ubiquitinated HEF1 was detected by immunoblot with anti-HA, the epitope tagged on the ubiquitin protein. In the absence of AIP4WT protein, HEF1 exhibited a low level ubiquitination (Fig. 3*A*, *lane 3*). The co-expression of HEF1 with AIP4WT resulted in a marked increase in the polyubiquitination of HEF1 (Fig. 3*A*, *lane 4*). Upon the co-expression of HEF1 with AIP4CA, however, no increase of HEF1 ubiquitination was detected, and furthermore, even the low level constitutive ubiquitination of HEF1 was completely blocked (Fig. 3*A*, *lane 5*). The co-expression of Smad3 with either wild type or ligase-dead AIP4 led to no significant changes of HEF1 ubiquitination (Fig. 3*B*, *lanes 7* and *8*). Because Smurf1 is also a HECT family member as well as a Smad-interacting Ub E3 ligase, we tested whether Smurf1 also can ubiquitinate HEF1. The result showed that Smurf1 did not ubiquitinate HEF1 (Fig. 3*B*, *lanes 10–12*). Thus, AIP4 specifically enhances the ubiquitination of HEF1 in a ligase-dependent fashion and therefore is probably an E3 ligase for HEF1.

The Wild Type AIP4 Regulates HEF1 Protein Level via Proteasome-mediated Degradation in a Ligase-dependent Fashion—The ubiquitinated substrates have several destinies, the best known of which is destruction by the 26 S proteasome. To investigate whether AIP4 regulates HEF1 degradation by the proteasome after mediating HEF1 ubiquitination, we performed a series of experiments. First, we tested whether the steady state level of HEF1 protein was down-regulated by

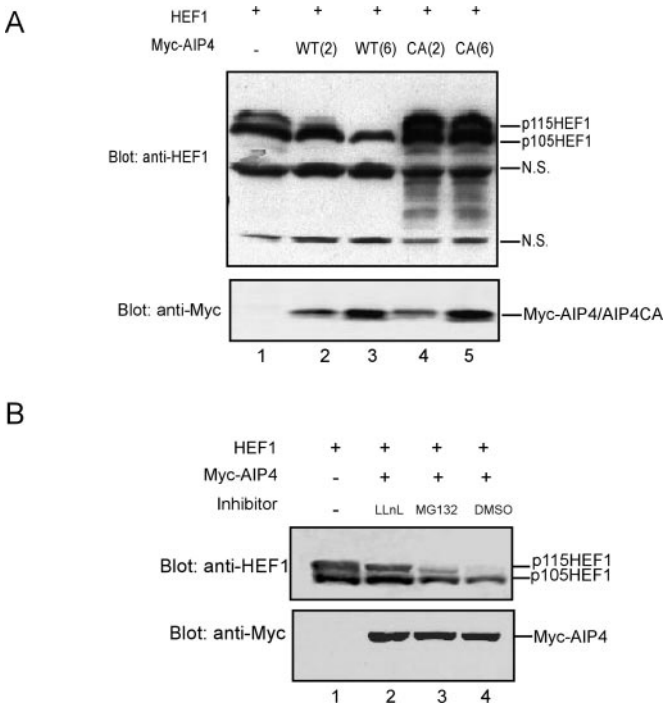

FIG. 4. **AIP4WT but not ligase-dead mutant AIP4CA regulates HEF1 protein level via proteasome-mediated degradation.** *A*, AIP4 exhibits dose- and ligase-dependent down-regulation of the protein level of HEF1. HEF1 was transfected alone or co-transfected with different doses of Myc-tagged AIP4WT and Myc-tagged AIP4CA. Cell lysates were subjected to Western blot to detected HEF1 expression levels using α-HEF1 antibody (*top*). The two forms of HEF1, $p115^{HEF1}$ and $p105^{HEF1}$, were detected. AIP4 expression levels were detected with α-Myc antibody (*bottom*). This figure is representative of multiple experiments. *N.S.*, non-specific; *WT*, wild type; *CA*, C to A point mutation. *B*, proteasome inhibitors block the reduction of HEF1 protein level. HEF1 was transfected alone or co-transfected with AIP4WT into the 293 cells. Cells were either treated with 50 μM LLnL or 50 μM MG132 or Me_2SO as control for 5 h before cells were harvested. Cell lysates were prepared and then subjected to Western blot with α-HEF1 antibody (*top*). The expression of AIP4 was detected by Western blot with α-Myc antibody. This figure is representative of multiple experiments.

co-transfecting HEF1 with the wild type AIP4 in the 293 cells. Increasing the amount of AIP4 caused a dose-dependent decrease of the HEF1 protein level (Fig 4*A*, *top*, *lanes 2* and *3*). Whereas both $p115^{HEF1}$ and $p105^{HEF1}$ were reduced, the $p115^{HEF1}$ exhibited much more reduction. Such a selective effect on $p115^{HEF1}$ was also observed previously for Smad3-induced reduction of the HEF1 protein level (7). The ability of expressed AIP4 to reduce HEF1 protein level is dependent upon its ligase activity, since the ligase-dead mutant AIP4CA had no effect on HEF1 protein level (Fig. 4*A*, *top*, *lanes 4* and *5*). To test whether the decrease is due to proteasomal degradation, we used two types of proteasome inhibitors, the peptidy1 aldehyde proteasome inhibitors LLnL and MG132. As a negative control, cells were treated with Me_2SO, since it was used to dissolve the two inhibitors. Both inhibitors exhibited inhibition, whereas LLnL appeared to be more efficient (Fig. 4*B*, *top panel*, *lanes 2* and *3*). These data suggested that the reduction of HEF1 protein level upon AIP4 coexpression involves proteasome-mediated degradation.

The Ligase Activity of AIP4 Is Essential for AIP4-induced HEF1 Degradation as Well as Smad3-induced HEF1 Degradation in the in Vitro Degradation System—The above experiments are mostly based upon overexpression systems and also cannot rule out the possible involvement of additional regulatory mechanisms such as transcriptional regulation. To directly demonstrate the effect of AIP4 on HEF1 degradation and determine the role of AIP4 ligase activity in Smad3-regulated HEF1 degradation, we applied a reconstructed protein degradation assay using cell extracts from mink lung epithelial cells (Mv1Lu), which have been shown previously to exhibit efficient protein degradation in response to TGF-β (5). Mink lung epithelial cells (Mv1Lu) were either treated or not treated with 100 pM TGF-β for 1 h before cells were harvested. ^{35}S-labeled *in vitro* translated HEF1 was added to extracts from these cells. As shown in Fig. 5*A*, HEF1 was rapidly degraded in extracts from cells stimulated by TGF-β (Fig. 5*A*, *lanes 1–4*). In contrast, no obvious degradation of HEF1 was observed in the extracts from cells with no TGF-β stimulation (Fig. 5*A*, *lanes 5–8*). These results confirm the ability of TGF-β to induce rapid HEF1 degradation. To test the ability of Smad3 to mimic such an effect of TGF-β stimulation, the *in vitro* translated Smad3 was added to the HEF1 degradation reaction system. As shown in Fig. 5*C*, the addition of *in vitro* translated Smad3 induced HEF1 degradation even in the absence of TGF-β stimulation (Fig. 5*C*, *lanes 4–6*). We then tested the ability of AIP4 to mimic such an effect of TGF-β stimulation by adding *in vitro* translated AIP4 to the *in vitro* degradation system. Like Smad3, the *in vitro* translated AIP4 also induced HEF1 degradation (Fig. 5*C*, *lanes 10–12*). To test whether the ligase activity of AIP4 is necessary for such an ability of AIP4, the *in vitro* translated AIP4CA was added to the reaction system, as shown in *lanes 13–15*. The ligase-dead mutant completely failed in inducing HEF1 degradation. When being added with Smad3, AIP4CA even suppressed Smad3-induced HEF1 degradation (Fig. 5*C*, *lanes 7–9*). Data with a similar pattern of changes have been obtained in several other experiments (data not shown). These results confirmed the ability of AIP4 to induce the degradation of HEF1 in a ligase-dependent manner and also suggested a critical role of AIP4 as an E3 ligase in Smad3-regulated proteasomal degradation of HEF1. Together with the data on the complex formation of Smad3, HEF1, and AIP4, these functional data suggest that Smad3 may regulate HEF1 ubiquitination and proteasomal degradation via recruiting AIP4 as an E3 ligase for HEF1.

DISCUSSION

The Itch protein, initially identified in the genetic characterization of non-agouti mice that exhibit autoimmune phenotypes and constant itching in the skin, has been reported to serve as the ubiquitin E3 ligase for several important signaling proteins, such as Notch and JunB (23, 24). The human Itch protein (Itch) is also known as AIP4, which was originally cloned as an interactor of atrophin-1, the protein implicated in the neurodegenerative disease dentatorubral pallidoluysian atrophy (25, 26). As an E3 ligase of JunB and Notch, hItch plays a direct role in T cell development and differentiation (24). In addition, hItch can be recruited by the latent membrane protein 2A of Epstein-Barr virus to mediate the ubiquitination and degradation of Lyn and Syk kinase, thus down-regulating B cell signaling (32). Our studies reported here demonstrate a new role of AIP4/hItch as a ubiquitin E3 ligase for the multidomain docking protein HEF1, which has been implicated to function as an adapter protein in many signaling pathways such as those of integrin, T cell antigen receptor, and B cell antigen receptor (10, 12–15). In our previous studies, HEF1 was also found to be involved in TGF-β signaling pathways, by interacting with Smad3, a key signal transducer in the TGF-β signaling pathway (7). It was demonstrated that the complex formation of Smad3 and HEF1 is closely associated with HEF1 ubiquitination and proteasomal degradation. The underlying mechanism, however, was not known. The new studies revealed that AIP4/hItch interacts with both HEF1 and Smad3 directly and functions as an E3 Ub ligase for HEF1, thereby

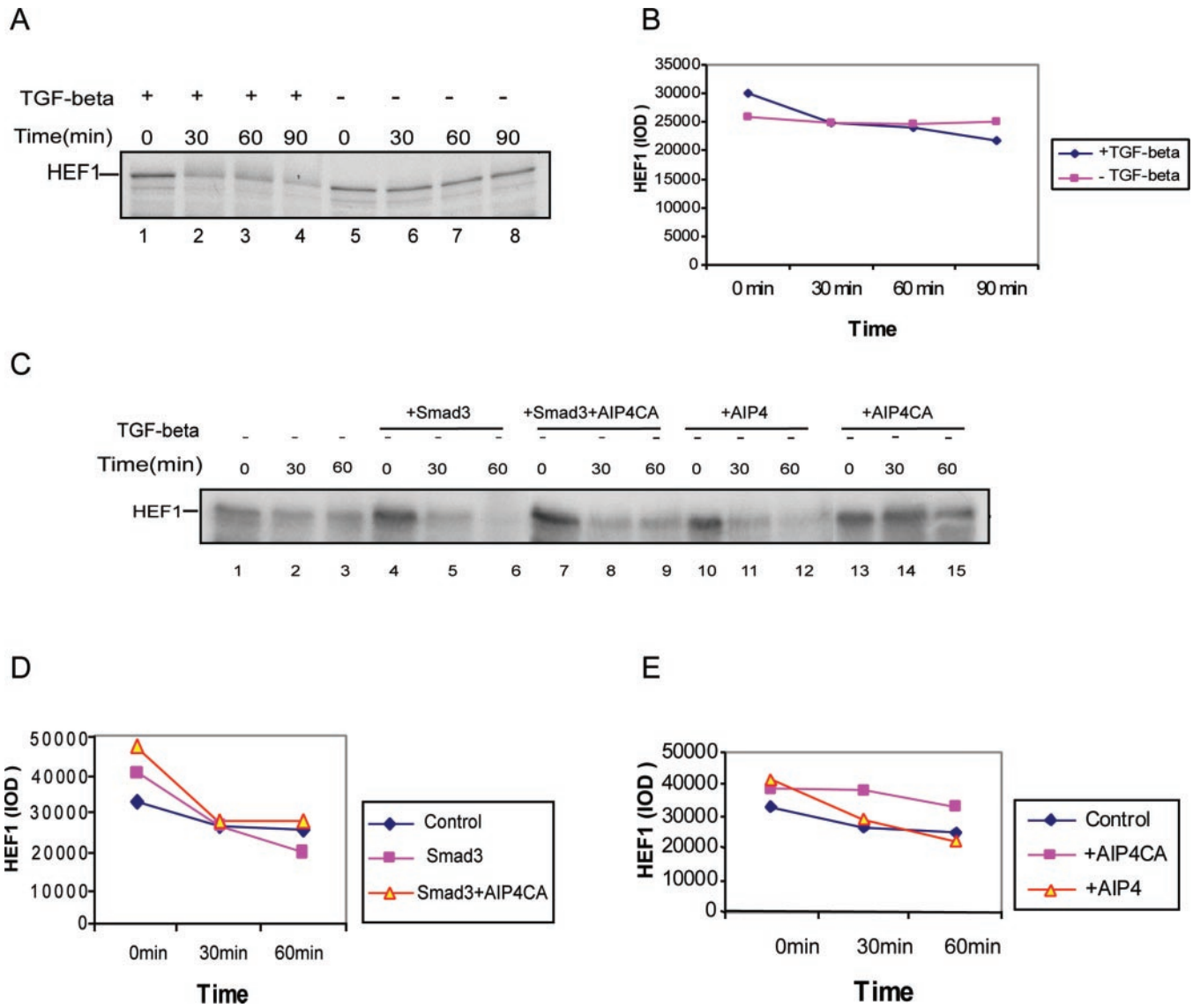

FIG. 5. **The ligase activity of AIP4 is essential for AIP4-induced HEF1 degradation as well as Smad3-induced HEF1 degradation in the *in vitro* degradation system.** *A*, TGF-β stimulation enhances HEF1 degradation in an *in vitro* degradation assay. Mink lung epithelial cells were either treated (*lanes 1–4*) or not treated (*lanes 5–8*) with 100 pM TGF-β for 1 h, and cell extracts were prepared. [^{35}S]methionine-labeled *in vitro*-translated HEF1 was added to the extracts and supplemented with the degradation mixture as described before (5). Aliquots were removed at the indicated time and resolved by SDS-PAGE, and the radiolabeled HEF1 was detected by autoradiography. This figure is representative of multiple experiments. *B*, the HEF1 protein levels in *A* were quantified using densitometry and presented as the IOD. *C*, the addition of *in vitro* translated Smad3 and AIP4WT both enhanced HEF1 degradation, whereas the addition of *in vitro* translated AIP4CA completely blocked the constitutive HEF1 degradation. Approximately 8 ng (8 μl) of *in vitro* translated Smad3 protein (*lanes 4–6*), alone or together with 8 ng (8 μl) of *in vitro* translated AIP4CA protein (*lanes 7–9*) or 8 ng (8 μl) of *in vitro* translated AIP4WT (*lanes 10–12*) or 8 ng (8 μl) of *in vitro* translated AIP4CA (*lanes 13–15*), were added into 20 μl of Mv1Lu extracts. Then ^{35}S-labeled *in vitro* translated HEF1, as the degradation substrate, was added to the Mv1Lu extracts together with the degradation mixture (5). The aliquots were removed at the indicated time and resolved by SDS-PAGE, and the levels of HEF1 were detected by autoradiography. This figure is representative of multiple experiments. *D* and *E*, densitometry analyses of the altered protein levels of HEF1 detected in *C* are presented as the IOD. *D*, the IOD values of HEF1 protein signals from extracts treated with Smad3 alone were compared with those from extracts treated with both Smad3 and AIP4CA. *E*, the IOD values of HEF1 proteins from extracts treated with AIP4 were compared with those from extracts treated with AIP4CA.

regulating the ubiquitination and proteasomal degradation of HEF1.

The interaction between AIP4 and HEF1 was first tested in the yeast two-hybrid system, in which AIP4 was found to exhibit strong interaction with HEF1. The interaction between AIP4 and HEF1 was further confirmed by an *in vitro* GST pull-down assay. The *in vitro* binding assay with purified GST-AIP4 and *in vitro* translated HEF1 and Smad3 was carried out to show that AIP4 interacts with HEF1 and Smad3 directly. Since a separate experiment showed that Smad3 interacts with HEF1 directly,[4] it is likely that AIP4, HEF1, and Smad3 form a ternary complex. Finally, the interaction between AIP4 and HEF1 was confirmed in the 293 cells when both proteins were co-expressed.

Domain mapping studies of AIP4 revealed that the two NH_2-terminal WW domains of AIP4 are sufficient for AIP4 to bind HEF1. The WW domain is one of the smallest protein modules. Its 40 amino acids form a compact triple-stranded, antiparallel β sheet (33). The two highly conserved tryptophan (W) residues are spaced 20–22 amino acids apart and play an important role in its structure and function. These domains are implicated in mediating protein-protein interactions by binding to proline-rich PPXY (PY) motifs or phosphoserine- and phosphothreonine-containing elements in their binding partners (34, 35). HEF1 has a PPXY motif at its NH_2 terminus and a serine-rich domain at its middle region. Surprisingly, AIP4 WW domains did not bind to these sequences. Rather, the COOH terminus of

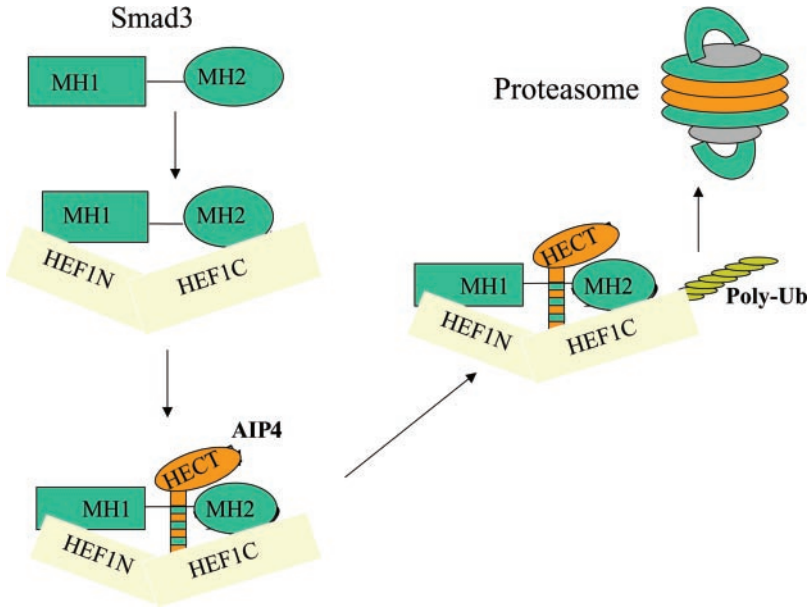

FIG. 6. ***Schematic diagram*** **to illustrate the complex formation of Smad3, HEF1, and AIP4 in AIP4-mediated ubiquitination of HEF1 followed by HEF1 degradation in proteasome.**

HEF1 with a central helix-loop-helix motif appeared to be involved in binding to AIP4 (Fig. 2*B*). This result is consistent with reported observations of Notch interaction with Itch (23). Instead of binding to the COOH terminus proline-, serine-, and threonine-rich PEST sequence of Notch, the Itch WW domain binds to the NH_2 terminus of Notch, a region containing the ankyrin repeats, which are known to mediate protein-protein interactions (36). These studies suggested that AIP4 binds to the COOH-terminal domain of HEF1 via its WW domains. Since Smad3 also binds to AIP4 via the WW domains, AIP4 may use the four WW domains differentially for binding to HEF1 and Smad3.

Since AIP4/hItch exhibited a Ub E3 ligase activity for two substrates, JunB and Notch, we performed *in vivo* ubiquitination assays to test the ability of AIP4 to ubiquitinate HEF1. In the absence of AIP4 coexpression, a low level constitutive ubiquitination of HEF1 was detected. The expression of AIP4 with HEF1 markedly enhanced the Ub conjugation to HEF1, whereas mutating the conserved cysteine at the HECT domain to inactivate AIP4 ligase activity abolished such an effect. In fact, the ligase-dead mutant AIP4CA even reduced the constitutive ubiquitination of HEF1, suggesting that it can function as a dominant negative mutant to compete with the endogenous E3 ligase for HEF1. To determine whether the ligase activity of AIP4 is specific, we also tested the ability of Smurf1 in the same assay. Smurf1 failed to ubiquitinate HEF1, indicating that the observed Ub ligase activity of AIP4 toward HEF1 is specific. Although we did not observe significant changes of HEF1 ubiquitination when Smad3 was co-expressed, a functional role of Smad3 in AIP4-regulated HEF1 ubiquitination was not ruled out, since it is possible that the endogenous Smad3 proteins in 293 cells were sufficient to assist AIP4-regulated HEF1 ubiquitination.

To directly evaluate whether the effect of AIP4 on HEF1 protein level is due to proteolysis, we applied a reconstituted *in vitro* degradation assay. Cell extracts from the Mv1Lu cells, either treated or not treated with TGF-β, were found to contain differential ability to mediate proteasomal degradation of the oncoprotein SnoN, which binds to Smad3 and is degraded by proteasome (5). We observed a similar effect of these extracts on HEF1. In extracts derived from cells not exposed to TGF-β, HEF1 was relatively stable, whereas extracts from cells exposed to TGF-β caused rapid degradation of HEF1. The addition of the *in vitro* translated Smad3 protein to extracts not exposed to TGF-β was sufficient to mimic the TGF-β treatment in causing HEF1 degradation. The addition of *in vitro* translated AIP4, but not AIP4 ligase-dead mutant AIP4CA, also triggered HEF1 degradation in extracts not exposed to TGF-β. The addition of AIP4CA mutant together with Smad3 caused a reduced degradation rate in comparison with adding Smad3 alone. These data directly demonstrate the ability of AIP4 and Smad3 to regulate HEF1 degradation and also suggest that the effect of Smad3 on HEF1 is probably mediated by AIP4 and involves the ligase activity of AIP4.

Thus, our studies revealed the involvement of the Ub E3 ligase, AIP4, in Smad3-regulated HEF1 degradation by forming a complex with both Smad3 and HEF1 and mediating the ubiquitination of HEF1. Based upon our domain mapping data, we propose a model for the complex formation of these three proteins, as illustrated in the schematic diagram in Fig. 6. Smad3 and HEF1 interlock with each other via their amino-terminal domains and carboxyl-terminal domains (7). AIP4, via its WW domains, binds to the Smad3 linker region. The carboxyl-terminal domain of HEF1 also harbors a binding site for separate WW domain(s) on AIP4. In this complex, AIP4 efficiently ubiquitinates HEF1, which is then targeted to proteasome for degradation.

A similar case is the involvement of the Ub E3 ligase Smurf2 in Smad2/3-regulated proteasomal degradation of SnoN (28). Besides the involvement of different Ub E3 ligases, Smad3/AIP4-regulated HEF1 degradation differs from Smad2/Smurf2-regulated or Smad3/Smurf2-regulated SnoN degradation in other aspects. Whereas the former does not depend upon Smad2/3 phosphorylation, the later requires Smad2/3 phosphorylation. Since SnoN is a nuclear protein and Smad2/3 phosphorylation is required for Smad2/3 to enter into the nucleus, the dependence of SnoN degradation on Smad2/3 phosphorylation could be at least partially due to the dependence of Smad2/3 phosphorylation for Smad2/3 accumulation into the nucleus. In the *in vitro* degradation assay, however, despite the lack of the nuclear translocation issue for Smad3, Smad3 phosphorylation was still required for SnoN degradation, suggesting that additional phosphorylation-dependent mechanisms exist for Smad3-regulated SnoN degradation (5). One such mechanism is the enhancing effect of Smad3 phosphorylation on its interaction with SnoN. HEF1, on the other hand, is predominantly a cytoplasmic protein and also does not require Smad3 phosphorylation to interact with Smad3. It has yet to be

determined whether the proteasomal degradation of SnoN occurs in the nucleus, whereas HEF1 degradation occurs in the cytoplasm. In both cases, Smad3 appears to function as an ancillary protein for the E3 ligases. Although our studies have showed that AIP4 directly binds to HEF1, the presence of Smad3 may increase the affinity of the HEF1·AIP4 complex or change HEF1 conformation in favor of the subsequent binding of AIP4 or other components.

If Smad3 phosphorylation is not necessary for HEF1 ubiquitination and degradation, then why can TGF-β stimulation rapidly increase the rate of HEF1 degradation? It is currently considered that inactive unphosphorylated Smad3 is bound to Smad anchor for receptor activation (SARA) protein anchored on the cytoplasmic side of the cell membrane, whereas phosphorylation of Smad3 decreases its affinity for SARA. The release of Smad3 from the Smad3·SARA complex could underlie the phenomenon of TGF-β-induced rapid degradation of HEF1. It also remains a possibility that HEF1 degradation requires other components that are phosphorylation-dependent.

In conclusion, the studies reported here identified AIP4/hItch as a Ub E3 ligase for HEF1 by forming a complex with both Smad3 and HEF1. This finding places AIP4 as an important signaling protein along the Smad3-mediated signaling pathways and broadens the network of cross-talk between TGF-β signaling pathways and those involving HEF1 and AIP4.

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