

Differential Ubiquitination of Smad1 Mediated by CHIP: Implications in the Regulation of the Bone Morphogenetic Protein Signaling Pathway

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Smad1, a downstream regulator of the bone morphogenetic protein (BMP) receptors, is tightly regulated by the ubiquitin-proteasomal degradation system. To dissect the mechanisms that underlie the regulation of Smad1, it is important to investigate the specific ubiquitination site(s) in Smad1. Here we report that the α -NH₂ group of the N terminus and the ϵ -NH₂ groups of internal lysine residues 116, 118 and 269 (K116, K118 and K269) of Smad1 are ubiquitin acceptor sites mediated by the carboxyl terminus of Hsc70-interacting protein (CHIP). The *in vitro* degradation assay indicates that ubiquitination at the N terminus partially contributes to the degradation of Smad1. Furthermore, we demonstrate that the ubiquitination level of pseudo-phosphorylated Smad1 by CHIP is stronger than that of wild-type Smad1 and can be strongly inhibited by a phosphorylated tail of Smad1, PIS (pS)V(pS). Third, our results indicate that Hsp70 facilitates CHIP-mediated poly-ubiquitination of Smad1 whereas it attenuates CHIP-mediated mono-ubiquitination of Smad1. Finally, consistent with the *in vitro* observation, we show that CHIP preferentially mediates the degradation of phospho-Smad1/5 *in vivo*. Taken together, these results provide us a hint that CHIP might preferentially regulate phosphorylated Smad1 and thus the BMP signaling.

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Introduction

The ubiquitin-proteasome pathway is highly conserved in all eukaryotes. Ubiquitin (Ub)-mediated degradation of regulatory proteins plays important roles in the control of key cellular

processes, including signal transduction, cell cycle progression, transcriptional regulation and endocytosis.¹ The transforming growth factor- β (TGF- β) and bone morphogenetic protein (BMP) pathways have been reported to be tightly regulated by the ubiquitin-proteasomal degradation system.^{2,3} Smad proteins are members of these signaling pathways that function downstream of type I and II serine/threonine kinase receptors. The structurally related Smad proteins can be divided into three classes, based on their sequences and functions: receptor-regulated Smads (R-Smads), common-mediator Smad (Co-Smad, Smad4), and inhibitory Smads (I-Smads).^{3–6} R-Smads are phosphorylated by activated receptors at their C-terminal SXS sequence and dictate the nature of the receptor-induced responses. Smad1, Smad5, and Smad8 are phosphorylated by the activated BMP receptors and mediate BMP responses, whereas Smad2 and Smad3 are activated by activin and TGF- β receptors. Once activated, these R-Smads form hetero-

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Abbreviations used: TGF- β , transforming growth factor- β ; BMP, bone morphogenetic protein; CHIP, carboxyl terminus of Hsc70-interacting protein; TPR, tetratricopeptide repeat; R-Smads, receptor-regulated Smads; Smurf1, Smad ubiquitination regulatory factor-1; WT, wild-type; Ubiquitin(K0), all lysine residues of ubiquitin were mutated to arginine; Ub, ubiquitin; GST, glutathione S-transferase; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase.

meric complexes with Co-Smad, Smad4. The Smad complexes then translocate into the nucleus where they regulate transcription of various target genes together with transcriptional factors and co-activators.^{3,6} Among the I-Smads, which include Smad6 and Smad7, Smad6 preferentially inhibits BMP signaling, whereas Smad7 preferentially inhibits activin and TGF- β signaling.^{7,8} Both the size of the Smad pool in unstimulated cells and the Smad protein levels subsequent to the activation of the pathway are finely controlled by ubiquitination.^{2,3}

It is now known that ubiquitination is carried out by a cascade of three enzymes: an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. Typically, the E2 enzyme associates with an E3 ubiquitin ligase, which is primarily responsible for recognizing the substrate. As the E3 ligase action determines the life or death of a protein, it is critical to understand how these enzymes select and regulate their targets.¹ Several E3 ligases have been reported to mediate Smads ubiquitination to date. Smad ubiquitination regulatory factor-1 (Smurf1) has been identified as an E3 ubiquitin ligase for Smad1, which regulates the basal level of Smad1.⁹ A second Smurf1-related E3 ligase, termed Smurf2, has also been described in targeting unactivated and activated Smad1, 2 and 3 for ubiquitination and proteasomal degradation.^{10–12} Recent studies have shown that the degradation of Smad4 is controlled by Smurfs, WWP1, or NEDD4-2, *via* formation of ternary complexes between Smad4 and Smurfs, and is mediated by R-Smads (Smad2) or I-Smads (Smad6/7) which act as adaptors.¹³ The SCF-Roc1 E3 ligase complex has been shown to be responsible for triggering the ubiquitination of phosphorylated Smad3.¹⁴ It has been reported that activated-Smad1 is targeted to the proteasome for degradation by interacting with ornithine decarboxylase antizyme (AZ) and the 20 S proteasome β -subunit HsN3.¹⁵ The carboxyl terminus of Hsc70-interacting protein (CHIP), which was originally identified as a co-chaperone protein and a U-box-containing E3 ligase,^{16–26} has been shown to regulate the TGF- β /BMP signaling pathway by targeting Smads for ubiquitination and degradation in our previous studies.^{27–29} Recent crystal structures have shown further details of the mechanism by which CHIP ubiquitinates its substrates *via* interacting with other chaperones and Ubc13-Uev1a.^{30,31}

Although many reports have demonstrated the importance of R-Smad proteins ubiquitination, little is known about their specific ubiquitination sites. Our previous study shows that CHIP can mediate the degradation of Smad1,²⁷ while the degradation of phosphorylated Smad1 by CHIP remains unclear. Here we first identify the N terminus and the internal lysine residues 116, 118 and 269 (K116, K118 and K269) of Smad1 as ubiquitin acceptor sites mediated by CHIP *in vitro*. We further demonstrate that CHIP might preferentially regulate the phospho-Smad1 level *in vitro* and *in vivo* and Hsp70 plays crucial roles in regulating CHIP-mediated Smad1 ubiquitination.

Results

CHIP efficiently ubiquitinates Smad1 *in vitro*

In order to understand the molecular mechanisms of Smad1 ubiquitination, we performed the *in vitro* ubiquitination experiments by using the purified components. We found that the purified CHIP exhibits self-ubiquitinating activity (data not shown). The ubiquitination of Smad1 was detected as high molecular mass smear bands by Western blotting with an anti-Smad1 monoclonal antibody after separation by SDS-PAGE when E1, E2 (UbcH5a) and E3 (CHIP) were added to the reaction system (Figure 1(a), lane 5). To further confirm that these multiple smear bands indeed represent ubiquitinated Smad1, we used glutathione S-transferase (GST)-Ubiquitin to replace His-Ubiquitin. Anti-Smad1 reactive bands shifted to higher molecular mass positions (Figure 1(a), lane 10), demonstrating that the ubiquitination of Smad1 indeed occurs under the *in vitro* system by E1, UbcH5a and CHIP. Interestingly, we observed that Smad1 was ubiquitinated without addition of CHIP in either His-Ubiquitin or GST-Ubiquitin reaction systems (Figure 1(a), lanes 3 and 8), while it was not ubiquitinated in the absence of E1, E2 or ubiquitin (Figure 1(a), lanes 1, 2, 4, 6, 7 and 9). These results indicate that E1 and UbcH5a might mainly mediate mono-ubiquitination of Smad1 directly, while CHIP might promote the poly-ubiquitination of Smad1 *in vitro*. The ubiquitination pattern of His-Smad1 was similar to that of Smad1, albeit with slightly higher bands in corresponding positions (Figure 1(b)).

In order to test whether the ubiquitination site selection of Smad1 is pH-dependent, we performed *in vitro* ubiquitination assays at five different pH values (from pH 6.0 to pH 10.0) (Figure 1(c)). The results show that the bands corresponding to different ubiquitination sites are almost identical at different pH values, indicating that the ubiquitination site selection of Smad1 is pH-independent *in vitro*.

Mapping the ubiquitin acceptor region(s) in Smad1

In most cases, target proteins are ubiquitinated at internal lysine residues. Human Smad1 contains 24 lysine residues. To identify which region(s) of Smad1 contain(s) ubiquitination site(s), we constructed two truncated mutants of Smad1, MH1 and MH2+Linker (MH2-L). Purified mutant proteins were used for *in vitro* ubiquitination assays. As shown in Figure 2(b), both MH1 and MH2-L were ubiquitinated, suggesting that both regions contain ubiquitination site(s). As we previously demonstrated that CHIP interacts with the MH2 domain rather than the MH1 domain of Smad1 *in vitro*,²⁷ it is interesting to find that the MH1 domain alone can also be ubiquitinated by E1/UbcH5a/CHIP here. We postulate that UbcH5a might interact with

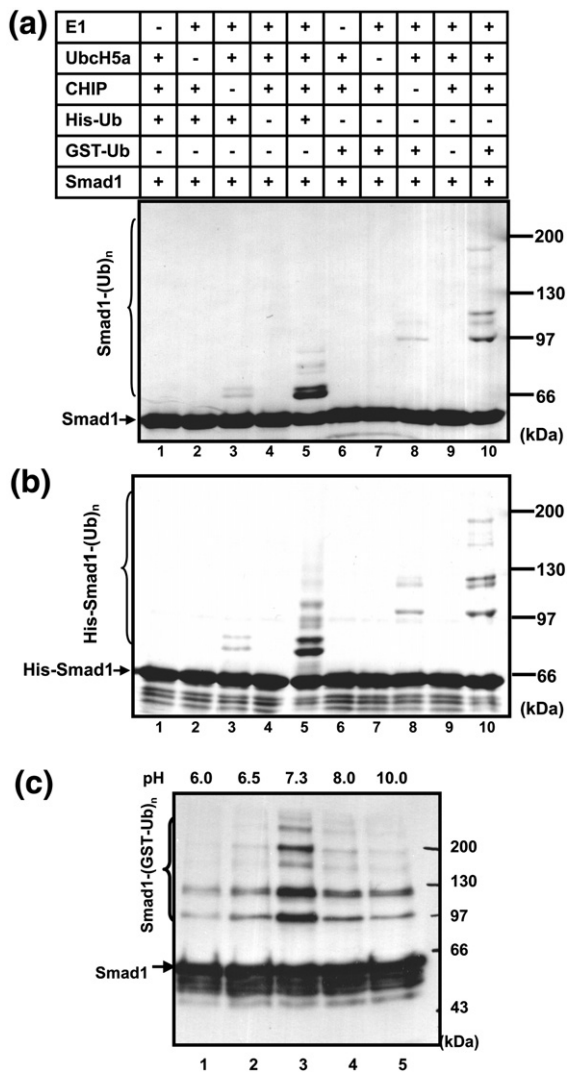


Figure 1. Efficient ubiquitination of Smad1 mediated by CHIP *in vitro*. (a) E1, E2(UbcH5a), E3(CHIP) and ubiquitin dependent poly-ubiquitination of Smad1. *In vitro* ubiquitination assays were performed at 30 °C for 2 h with the indicated combinations of E1, E2(UbcH5a), E3(CHIP), Ubiquitin and Smad1. The reaction mixture was resolved under reducing conditions by SDS-PAGE, and the separated proteins were subjected to immunoblot analysis with antibodies against Smad1(A-4). (b) *In vitro* ubiquitination assays were performed as for (a), except that His-Smad1 was used as substrate. (c) *In vitro* ubiquitination assays were performed with E1, E2, CHIP, GST-Ubiquitin and Smad1 at five different pH values as indicated.

the MH1 domain of Smad1, which is similar to the interaction between Ubc9 and the MH1 domain of Smad4.³²

In order to identify the specific lysine(s) required for Smad1 ubiquitination, we generated series of lysine to arginine (K/R) mutants. We mutated all the lysine residues in each of the four different regions, as indicated in Figure 3(a). The ubiquitination experiment results show that two slower migrating bands disappeared when all the lysine residues in the K53–269 region were mutated to arginine (K53–269R) (Figure 3(b), lanes 4, upper and lower panels),

indicating that there are at least two ubiquitination sites among these seven lysine residues. Surprisingly, none of these mutants abolished the faster migrating bands of the ubiquitinated Smad1. These results remind us to address whether the free α -NH₂ group of the N terminus is modified by ubiquitin.

Smad1 can be ubiquitinated at three internal sites: K116, K118 and K269

To determine which of the seven lysine residues (K53, K81, K104, K116, K118, K128 and K269) in the K53–269 region is ubiquitinated, we further mutated the lysine residues in different combinations. As indicated in Figure 3(b), when all four lysine residues were mutated to arginine (K81–118R) one ubiquitination band disappeared (Figure 3(b), lanes 6, upper and lower panels) compared with the wild-type Smad1, Smad1(WT) (Figure 3(b), lanes 1, upper and lower panels), indicating that at least one ubiquitination site was located among K81, K104, K116 and K118. Therefore, we constructed four plasmids, in each of which a single lysine residue of the K81–118R mutant was restored (Figure 3(b), lanes 7–10, upper and lower panels). Interestingly, restoration of a single lysine at K116 or K118 restored the ubiquitination band of Smad1. Our data also suggest that

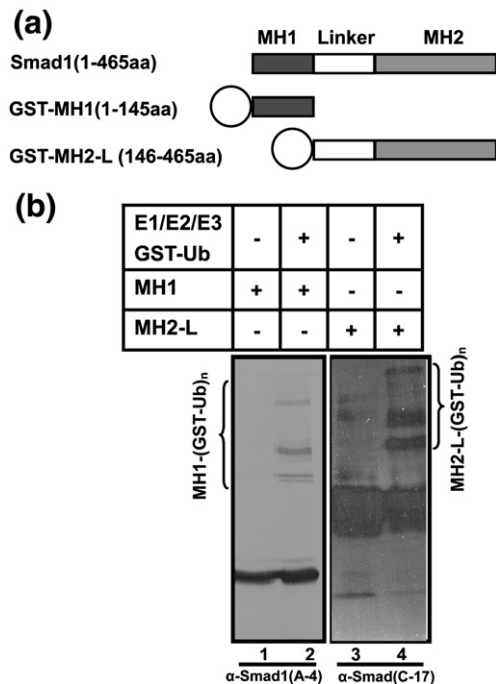


Figure 2. Mapping of the ubiquitin acceptor region(s) in Smad1. (a) Diagram depicting the three domains: MH1, linker region, MH2 within Smad1. GST is shown as a circle and the expression constructs are shown on the left. (b) Both MH1 domain and MH2-L were ubiquitinated *in vitro*. Ubiquitination reactions of MH1 and MH2-L were performed as described. The reaction mixture was separated by SDS-PAGE and subjected to immunoblot analysis with antibodies against Smad1(A-4) and Smad(C-17) for MH1 and MH2-L, respectively. The positions of ubiquitinated MH1 and MH2-L((MH1-(GST-Ub)_n and MH2-L-(GST-Ub)_n) are indicated.

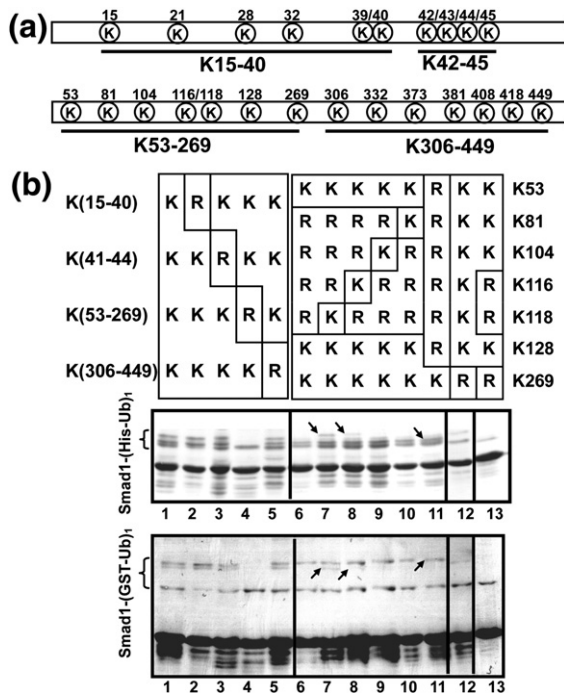


Figure 3. Identification of major Smad1 ubiquitination sites. (a) Diagram showing the distribution of 24 lysine residues (K) in Smad1. (b) Identification of three internal ubiquitination sites of Smad1, K116, K118 and K269, by combinatorial mutation analysis. A series of Smad1 mutants were created by PCR and purified mutants proteins were subjected to *in vitro* ubiquitination reactions as described in Materials and Methods. His-Ubiquitin(K0) and GST-Ubiquitin(K0) were used for the upper panel and lower panel, respectively. The positions of mono-ubiquitinated Smad1 are marked in parentheses. Lanes 13 were grouped from different gels.

ubiquitination at K118 is stronger than that at K116 *via* comparing the band density (Figure 3(b), lanes 7 and 8, upper and lower panels, marked by arrows). Thus we conclude that the two ubiquitination sites are K116 and K118. We also observed that one band of K269R mutant disappeared (Figure 3(b), lanes 12, upper and lower panels), while restoration of K269 of the K53–269R mutant restored the corresponding band (Figure 3(b), lanes 11, upper and lower panels, marked by arrows). These data indicate that Smad1 can be ubiquitinated at K269. Two slower migrating bands disappeared when lysine residues 116, 118 and 269 were mutated to arginine simultaneously (K116/118/269R) (Figure 3(b), lanes 13, upper and lower panels). All of these results indicate the presence of three internal ubiquitination sites in Smad1: K116, K118 and K269.

The α -NH₂ group of Smad1 at the N terminus can also be ubiquitinated

Based on previous a observation (Figure 3), we sought to examine the possibility that the N terminus of Smad1 can be targeted for ubiquitination. First, we prepared an expression construct, pET28a-Smad1(K0), in which all lysine residues

were replaced by arginine. Unfortunately, this protein was expressed as inclusion bodies in the *Escherichia coli* strain Rosetta(DE3). Subsequently, we performed an *in vitro* ubiquitination assay using GST-tagged Ubiquitin and His-tagged Smad1 (with thrombin cleavage site). After the reaction, we performed glutathione-Sepharose purification to pull-down the ubiquitinated species. The ubiquitinated species were eluted and divided into two aliquots, one of which was treated with thrombin. If Smad1 is indeed ubiquitinated at the N terminus, we would expect to observe the appearance of the mono-ubiquitinated form of the N-terminal His-tag after thrombin cleavage (Figure 4(a), top). As indicated in Figure 4(b) (right panel, lane 2), we did observe a band for GST-Ub-His-tag recognized by anti-His-tag antibody, which had a slightly higher position than that of GST-Ubiquitin and was also recognized by an antibody against HA-tag (a tag in GST-Ubiquitin) (data not shown). In addition, we also observed that some bands of lane 2 shift to lower positions compared with lane 1 (Figure 4(b), left panel, marked by asterisks). These results further indicate that Smad1 possesses internal ubiquitination sites (Figure 4(a), bottom).

In order to rule out the possibility that N-terminal ubiquitination of His-Smad1 was an artifact caused by His-tag at the N terminus, we generated a His-Ubiquitin(K0)-Smad1 fusion protein (His-Ub(K0)-Smad1) and a tag-free Smad1 by thrombin cleavage of GST-Smad1. Since all of the internal lysine residues of ubiquitin were mutated to arginine, His-Ub(K0)-Smad1 was expected to block the addition of ubiquitin to the N terminus of Smad1 (Figure 4(c)). As shown in Figure 4(d), lanes 1 and 3, the faster migrating band corresponding to ubiquitinated Smad1 disappeared when His-Ub(K0)-Smad1 was used, while the slower migrating band(s) shifted to a slightly higher position compared with that of Smad1. These results indicate that the faster migrating band represents N-terminal mono-ubiquitinated Smad1, while the slower migrating form(s) represents ubiquitinated Smad1 at internal sites.

To unequivocally demonstrate that Smad1 is ubiquitinated at the N terminus, we purified ubiquitinated Smad1 from *in vitro* ubiquitination reaction mixture by immunoprecipitation and SDS-PAGE (Figure 5(a)), and then we analyzed whole tryptic digests of purified Smad1 and mono-ubiquitinated Smad1 by MALDI-TOF-MS (Figure 5(b) and (c)). Inspection of the detected peptide masses of mono-ubiquitinated Smad1 indeed identified a peptide corresponding to a GG peptide on the N terminus of Smad1 (GG-GSMNVTSLSFSTSPAVK; *m/z* 1886.91) (Figure 5(c), top). These data provide the direct evidence that Smad1 can be ubiquitinated at the N terminus.

N-terminal ubiquitination partially contributes to the degradation of Smad1

To investigate the function of N-terminal ubiquitination of Smad1, we developed an *in vitro* assay for the degradation of Smad1. Wild-type Smad1 and

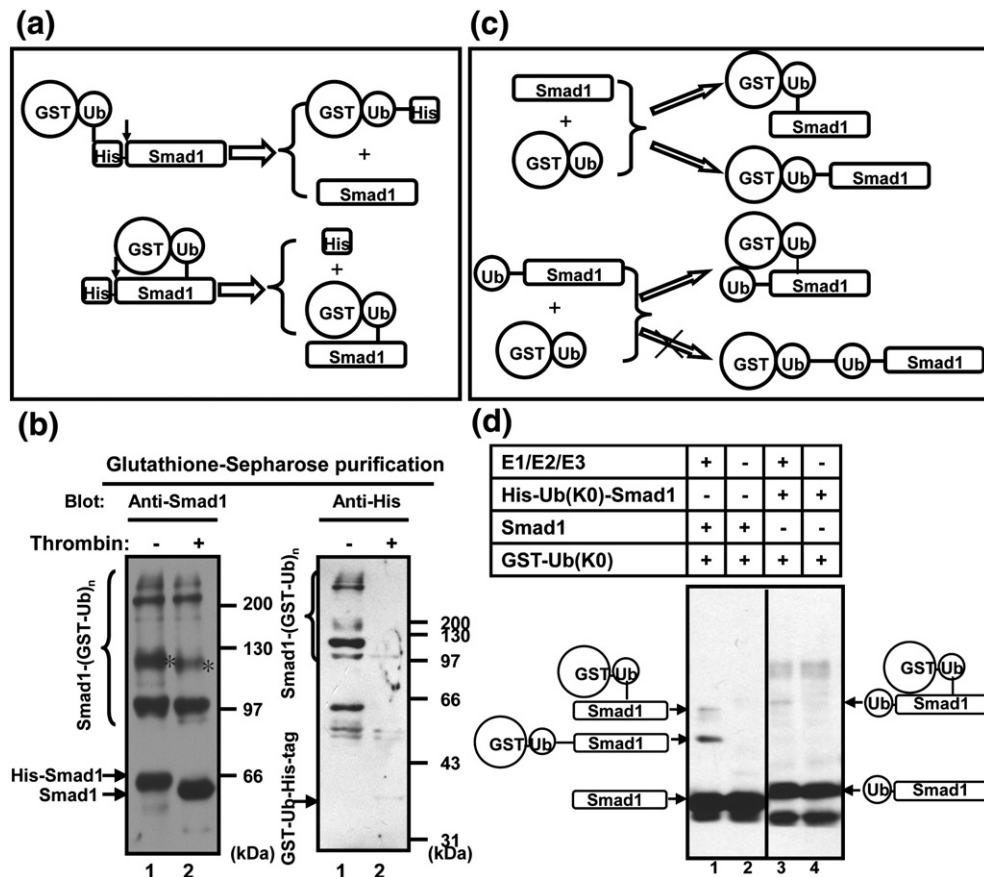


Figure 4. Smad1 can be ubiquitinated at the N-terminal α -NH₂ group *in vitro*. (a) Schematic representation of two types of ubiquitin modifications (N-terminal and internal) and the predicted results after thrombin cleavage. (b) Thrombin cleavage of ubiquitinated Smad1. Samples treated with or without thrombin were separated by SDS-7% PAGE (left panel) and SDS-10% PAGE (right panel) and subjected to immunoblot analysis with antibodies against Smad1 (A-4) and His-tag (left panel and right panel, respectively). The positions of ubiquitinated Smad1 (Smad1-(GST-Ub)_n) and GST-Ub-His-tag are indicated. (c) Schematic representation of the ubiquitination of Smad1 and His-Ub(K0)-Smad1. (d) Fusion of a long tag (His-Ubiquitin(K0)) to the N-terminal residue of Smad1 blocks the ubiquitination of Smad1 on its N terminus. Smad1 and His-Ub(K0)-Smad1 were subjected to *in vitro* ubiquitination assays. The bands and their corresponding identities are indicated.

Smad1(K116/118/269R) were incubated at 37 °C in the presence of purified 26 S proteasomes without (Figure 6(a) and (b), lanes 1–5) or with MG132 (Figure 6(a) and (b), lanes 6–10). We observed that the degradation of Smad1(WT) and Smad1(K116/118/269R) was in a time-dependent manner (Figure 6(a) and (b), lanes 1–5) and this process can be inhibited by proteasomes inhibitor MG132 (Figure 6(a) and (b), lanes 6–10). These results show that a functional ubiquitin system is necessary for the degradation of Smad1(WT) and Smad1(K116/118/269R) *in vitro*. Interestingly, the degradation rate of Smad1(K116/118/269R) was slower than that of Smad1(WT) (Figure 6(c) and (d)), indicating that N-terminal ubiquitination partially contributes to the degradation of Smad1.

Enhanced ubiquitination level of the Smad1(3E) mutant mediated by CHIP

Based on previous studies,³⁵ we postulate that CHIP might preferentially mediate the ubiquitination of phosphorylated Smad1. We created a pseudo-

phosphorylated Smad1, Smad1(3E), in which all three C-terminal serine residues of Smad1 were mutated to glutamic acid residue (SSVS → EEEV), to mimic the structural and electrostatic properties of phosphorylation.^{33,34} The CHIP-mediated ubiquitination level of Smad1(3E) was greatly enhanced, compared with Smad1(WT) (Figure 7(a) and (b), lanes 1 to 6). In contrast, the ubiquitination of Smad1(3E) mediated by Smurf1 was hardly changed compared with Smad1(WT) (Figure 7(a) and (b), lanes 7 to 12). Interestingly, we also observed that one ubiquitination band induced by CHIP (Figure 7(a) and (b), lanes 2, 3, 5 and 6, marked by arrows) do not exist when Smurf1 was used as E3 ligase (Figure 7(a) and (b), lanes 8, 9, 11, 12, and unpublished data). The band position corresponds to mono-ubiquitination at the K269 residue of Smad1 (Figure 3(b), lanes 11, upper and lower panels, marked by arrows). These results implicate that CHIP and Smurf1 might play different roles in the regulation of Smad1: CHIP might preferentially regulate the level of activated-Smad1 while Smurf1 predominately controls the basal level of Smad1.⁹

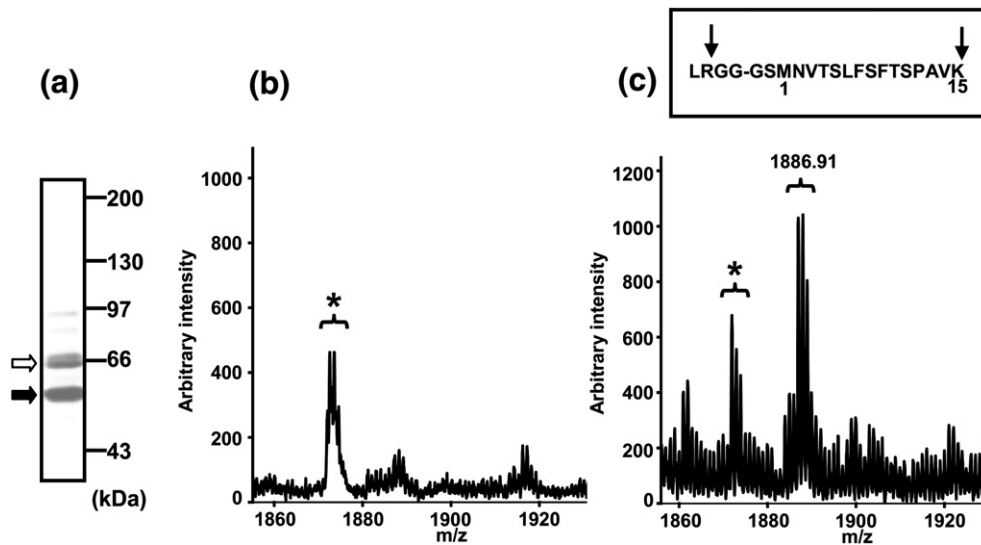


Figure 5. Direct identification of N-terminally ubiquitinated Smad1 by mass spectrometry. (a) Preparation of ubiquitinated Smad1. Ubiquitinated Smad1 was isolated from *in vitro* ubiquitination reaction mixture by immunoprecipitation and SDS-PAGE. An immunoblot of duplicate samples to those used for mass spectrometry is shown after the immunoprecipitation. The protein bands used for mass spectrometry are marked by an open arrow (Smad1) and filled arrow (ubiquitinated-Smad1). (b) and (c) Mass spectrometric profile of the isolated non-ubiquitinated (b) and mono-ubiquitinated Smad1 (c). In-gel tryptic digests of the control Smad1 and the mono-ubiquitinated Smad1 were analyzed by MALDI-TOF-MS (see Materials and Methods). Brackets with asterisks mark a characteristic mass peak cluster that remains unaltered between unmodified and mono-ubiquitinated proteins. A bracket with indicated mass peak cluster (m/z 1886.91) corresponds to the ubiquitin-Smad1 peptide conjugate. The corresponding peptide sequences are shown on top of the spectra, and arrows indicate sites of trypsin cleavage.

Inhibition of CHIP-mediated Smad1(3E) ubiquitination by polypeptide, PIS(pS)V(pS)

Surprisingly, the C-terminal IEEVE sequence of the Smad1(3E) mutant is almost identical with those of Hsp90 (MEEVD) and Hsp70 (IEEVD), which can

bind directly the tetratricopeptide repeat (TPR) domain of CHIP.^{31,36} Therefore, we speculate that the enhanced ubiquitination level of Smad1(3E) mediated by CHIP might result from the direct interaction between the IEEVE motif and the TPR domain of CHIP. For phosphorylated Smad1, we hypothe-

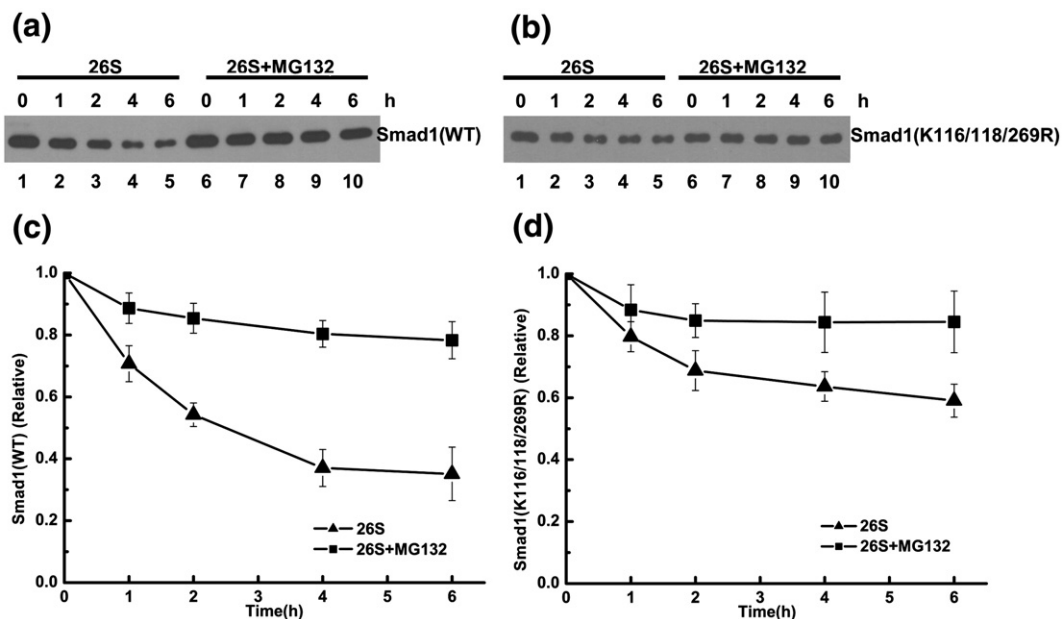


Figure 6. Wild-type Smad1 and Smad1(K116/118/269R) mutant are degraded *in vitro*. (a) Degradation of Smad1(WT) was carried out in the presence of purified 26 S proteasomes without (lanes 1–5) or with MG132 (lanes 6–10). Protein levels were assessed by SDS-PAGE followed by immunoblot analysis. (b) The same as for (a) except that Smad1(K116/118/269R) was used as substrate. (c) and (d) Quantitative analysis of replicate experiments of (a) and (b), respectively. Error bars represent \pm SD.

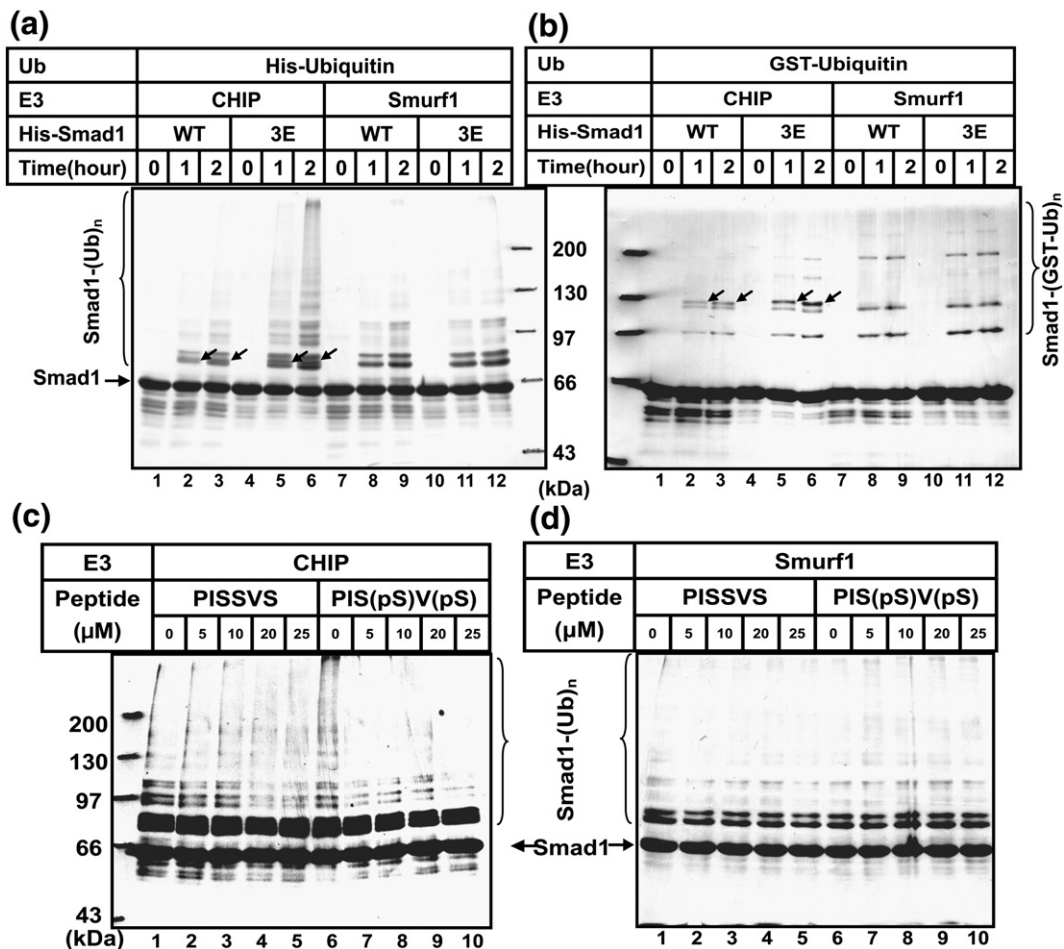


Figure 7. CHIP preferentially mediates poly-ubiquitination of Smad1(3E). (a) Enhanced ubiquitination levels of the Smad1(3E) mutant mediated by CHIP. Smad1(WT) and Smad1(3E) were subjected to *in vitro* ubiquitination assays in the combinations as indicated. The positions of poly-ubiquitinated Smad1 are labeled by Smad1-(Ub)_n. (b) The same as for (a) except that GST-Ubiquitin was used to replace His-Ubiquitin. (c) Dose-dependent inhibition of Smad1(3E) ubiquitination by the PIS(pS)V(pS) polypeptide. Smad1(3E) was subjected to a ubiquitination assay and two polypeptides, PISSVS and PIS(pS)V(pS), were added to the reaction mixture simultaneously with increasing concentrations as indicated. The positions of poly-ubiquitinated Smad1(3E) are marked in parentheses. (d) The same as for (c) except that Smurf1 served as the E3 ligase.

size that the phosphorylated PIS(pS)V(pS) tail might bind to the TPR domain of CHIP, thus enhancing the ubiquitination of phosphorylated Smad1.

To test this hypothesis, we synthesized two polypeptides PISSVS (unphosphorylated) and PIS(pS)V(pS) (phosphorylated). If CHIP indeed interacts with the phosphorylated tail of Smad1, addition of the phosphorylated polypeptide into the *in vitro* ubiquitination reaction system would competitively inhibit the ubiquitination level of Smad1(3E). Consistent with this idea, we did observe stronger inhibition of Smad1(3E) ubiquitination by PIS(pS)V(pS) than PISSVS when CHIP was used as the E3 ligase (Figure 7(c)). In contrast, none of the polypeptides influenced the Smad1(3E) ubiquitination induced by Smurf1, which was used as a control (Figure 7(d)).

These data together suggest that the phosphorylation of Smad1 at its C-terminal tail is able to enhance its association with CHIP, thus increasing the ubiquitination of phosphorylated Smad1.

Hsp70 facilitates CHIP-mediated poly-ubiquitination of Smad1 and inhibits CHIP-mediated mono-ubiquitination of Smad1

Given the importance of Hsp70 in CHIP function, we investigated the roles of Hsp70 in CHIP-mediated ubiquitination of Smad1. As shown in Figure 8(a), with the increasing amounts of Hsp70 in *in vitro* ubiquitination reactions, the smear band density corresponding to poly-ubiquitinated Smad1 was greatly increased, while the band density corresponding to mono-ubiquitinated Smad1 was decreased. In order to examine the influence of Hsp70 on CHIP-mediated mono-ubiquitination of Smad1, we used His-Ubiquitin(K0) for *in vitro* ubiquitination reactions to inhibit poly-ubiquitination of Smad1. We observed that the band density corresponding to CHIP-mediated mono-ubiquitination of Smad1 was decreased in the presence of Hsp70 (Figure 8(b), lanes 1 to 3), while the band density corresponding to Smurf1-mediated mono-

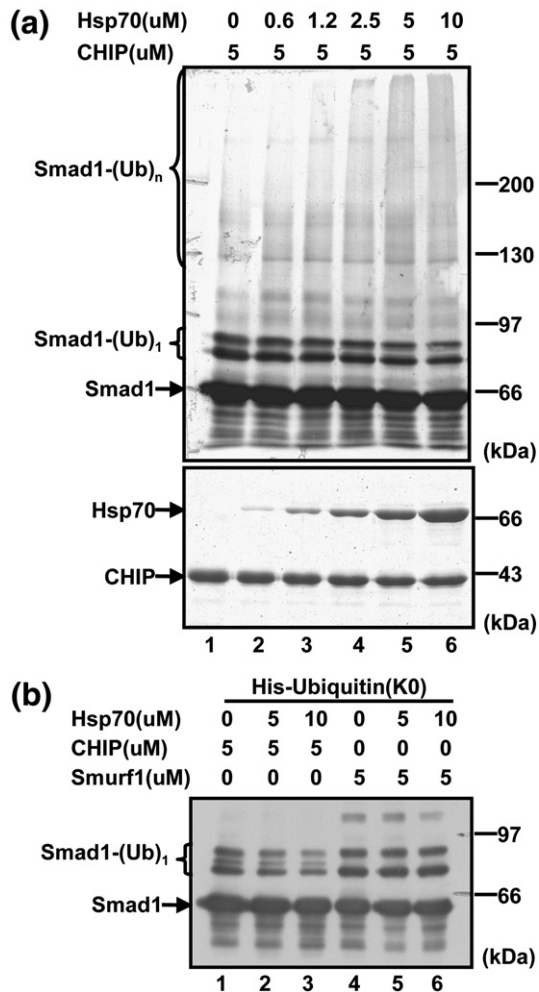


Figure 8. Hsp70 facilitates CHIP-mediated poly-ubiquitination of Smad1 and inhibits CHIP-mediated mono-ubiquitination of Smad1. (a) Dose-dependent enhancement of CHIP-mediated Smad1 poly-ubiquitination by Hsp70. Hsp70 was added to the *in vitro* ubiquitination mixtures containing E1, E2 (UbcH5a), CHIP, Smad1, and His-Ubiquitin for 2 h at 30 °C. The samples were separated by SDS-7.5% PAGE and detected by anti-Smad1 antibody (upper panel). Purified Hsp70 and CHIP used in each reaction were separated by SDS-10% PAGE and visualized by Coomassie brilliant blue staining (lower panel). The positions of poly and mono-ubiquitinated Smad1 are labeled by Smad1-(Ub)_n and Smad1-(Ub)₁, respectively. (b) Dose-dependent inhibition of CHIP-mediated Smad1 mono-ubiquitination by Hsp70. The same as for (a) except for the usage of His-Ubiquitin (K0) and other different combinations as indicated.

ubiquitination of Smad1 was not influenced by Hsp70, which was used as control (Figure 8(b), lanes 4 to 6). The influence of Hsp70 on Smad1(3E) ubiquitination was very similar to that of wild-type Smad1 (data not shown). Our results suggest that Hsp70 might facilitate CHIP-mediated poly-ubiquitination of Smad1 while it attenuates CHIP-mediated mono-ubiquitination of Smad1. The results also show that the positions of those bands corresponding to different ubiquitination sites, in spite of the changes of the band density, were un-

changed in the presence or absence of Hsp70 (Figure 8(b), lanes 1 to 3), indicating that Hsp70 might not influence CHIP-mediated ubiquitination site selection of Smad1.

CHIP preferentially mediates the degradation of endogenous phospho-Smad1/5

In order to further support the notion that CHIP preferentially triggers the ubiquitination of activated Smad1, we examined the degradation of endogenous Smad1/5 and phospho-Smad1/5 of HEK293T cells induced by CHIP. Consistent with our *in vitro* observation, overexpression of CHIP indeed significantly decreased the phospho-Smad1/5 protein levels in a manner sensitive to the proteasomes inhibitor MG132 (Figure 9(a), the second panel and Figure 9(c)), while CHIP did not influence the total Smad1/5 protein levels (Figure 9(a), the first panel and Figure 9(b)).

Furthermore, we investigated the degradation kinetics of phospho-Smad1/5 with or without the overexpression of CHIP (Figure 10(a)). We found that the degradation rate of the phospho-Smad1/5 was increased when cells were transfected with CHIP (Figure 10(b) and (c)). These results implicate that CHIP might preferentially induce the proteasomal degradation of endogenous phospho-Smad1/5.

Discussion

Post-translational modifications, such as ubiquitination, phosphorylation and acetylation, play an important role in regulating many key cellular processes. Recent reports have revealed that Smad4 is regulated by SUMO (small ubiquitin-related modifier) and ubiquitin modifications at the same sites, lysine 113 and lysine 159 in the MH1 domain,^{32,37-40} and that Smad4 has one mono-ubiquitinated site (lysine 507) in the MH2 domain.⁴¹ Smad7 stability is regulated by competition among ubiquitination, acetylation and deacetylation at two lysine residues in the N-terminal region.^{42,43} However, the specific R-Smad ubiquitination site(s) has not yet been identified. Here we provide evidence for the identification of Smad1 ubiquitination sites, and we propose a mechanism whereby phosphorylated Smad1 is preferentially ubiquitinated by CHIP *in vitro* and *in vivo*.

We first identified three sites for ubiquitin conjugation as lysine 116, 118 and 269 (K116, K118 and K269) by combinational mutation analysis (Figure 3). Subsequently, we demonstrated that the N terminus of Smad1 is ubiquitinated by thrombin cleavage assay and ubiquitination inhibition assay *via* fusion of a long tag (His-Ubiquitin(K0)) to the N-terminal residue of Smad1 (Figure 4). N-terminal ubiquitination of Smad1 was further confirmed by mass spectrometric analysis (Figure 5). Crystal structures of the MH1 domain of Smad3^{44,45} and MH2 domain of Smad1³⁴ indicate that all of these ubiquitination sites are located in loop regions,

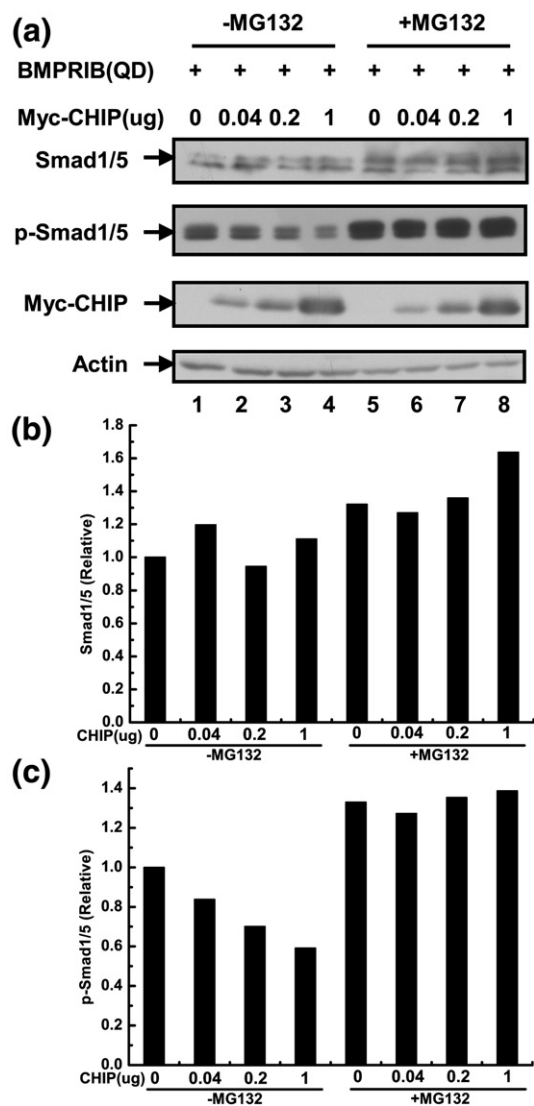


Figure 9. CHIP preferentially mediates the degradation of endogenous phospho-Smad1/5 (p-Smad1/5). (a) Overexpression of CHIP results in degradation of phospho-Smad1/5 in a dose-dependent manner. HEK293T cells were co-transfected with 0.5 μg of constitutively active receptor BMPRIB(QD) and Myc-CHIP in the presence or absence of MG132 as indicated. Endogenous Smad1/5 and phospho-Smad1/5 were detected by anti-Smad1/5 and anti-phospho-Smad1/5 antibodies, respectively. The dose dependently increased CHIP level was detected by anti-Myc antibody. The level of β -actin was used as an internal control. (b) Graphical representation shows relative levels of Smad1/5 in the transfection of different doses of Myc-CHIP in the presence or absence of MG132. The Smad1/5 level of the first lane was set as 1. (c) The same as for (b), except that phospho-Smad1/5 levels were quantified.

suggesting that they are readily accessible for ubiquitin conjugation by E3 ligase. Although it is believed, in most cases, that the first ubiquitin moiety is transferred to an ϵ -NH₂ group of an internal lysine residue in the substrate, proteins with no internal lysine residues or with mutations of all internal lysine residues were still ubiquitinated and

degraded in a proteasome dependent pathway.^{46–56} In some cases, the attachment of ubiquitin to the free α -NH₂ group at the N terminus has been directly evidenced by mass spectrometry.⁵³ Recently, it has been reported that a protein complex, HOIL-1L and HOIP, possesses the unique function to assemble a novel head-to-tail poly-ubiquitin chain *via* linkages between the C and N termini of ubiquitin.⁵⁷ Cdt1 degradation was also mediated by N-terminal ubiquitination.⁵⁸ N-terminal ubiquitination is a controversial process, but emerges as an important novel mode of modification as supported by the increasing number of proteins that are reported to be modified by this pathway. However, the specific E3 ligase for N-terminal ubiquitination has not yet been identified. Our results presented here provide evidence that the N terminus of Smad1 is ubiquitinated. In addition, our data indicate that N-terminal ubiquitination partially contribute to the degradation of Smad1 (Figure 6).

TGF- β and BMP signaling pathways are tightly controlled by positive and negative regulators *via* the accumulation and subsequent degradation of the phosphorylated R-Smads. The down-regulation of phosphorylated R-Smads must be a critical mechanism for effectively terminating signaling in order to avoid excess stimulation. To date, only a few E3 ligases have been identified to participate in termination of TGF- β and BMP signaling. Among them, the SCF-Roc1 E3 ligase complex and Smurf2 have been shown to be responsible for triggering the ubiquitination of phosphorylated Smads.^{11,14} The specific E3 ligase for activated Smad1 has not yet been identified. Here, we observed that CHIP-mediated ubiquitination of pseudo-phosphorylated Smad1, Smad1(3E) (mimicking the effect of physiological phosphorylation^{33,34}), was greater than that of Smad1(WT) (Figure 7(a) and (b)). Furthermore, the ubiquitination of Smad1(3E) was greatly inhibited by an *in vitro* synthesized polypeptide PIS(pS)V (pS) (Figure 7(c)). Similar to our results, Rees *et al.* has reported that CHIP binds the androgen receptor in a phosphorylation and sequence-dependent manner.³⁵ Therefore, we propose that the phosphorylation of Smad1 can enhance the binding affinity between phosphorylated Smad1 and CHIP, thus increasing the ubiquitination of phosphorylated Smad1.

Hsp70 plays vital roles in CHIP-mediated substrate ubiquitination.^{17–20,22,25,26} It has been reported that poly-ubiquitin chain can be pre-assembled on the catalytic cysteines of many E2 enzymes, such as Ube2g2 and Ubc7, before being transferred to substrates and the transfer of poly-ubiquitin chain to substrates is likely to be faster in the presence of E3 ligase.^{61,62} Our results demonstrate that Hsp70 strongly facilitates the Smad1 poly-ubiquitination and attenuates Smad1 mono-ubiquitination induced by CHIP (Figure 8). We speculate Hsp70 might directly interact with Smad1 and CHIP simultaneously and possibly promotes CHIP-mediated poly-ubiquitin chain formation and transferring from E2 (UbcH5a) to Smad1, therefore facilitating

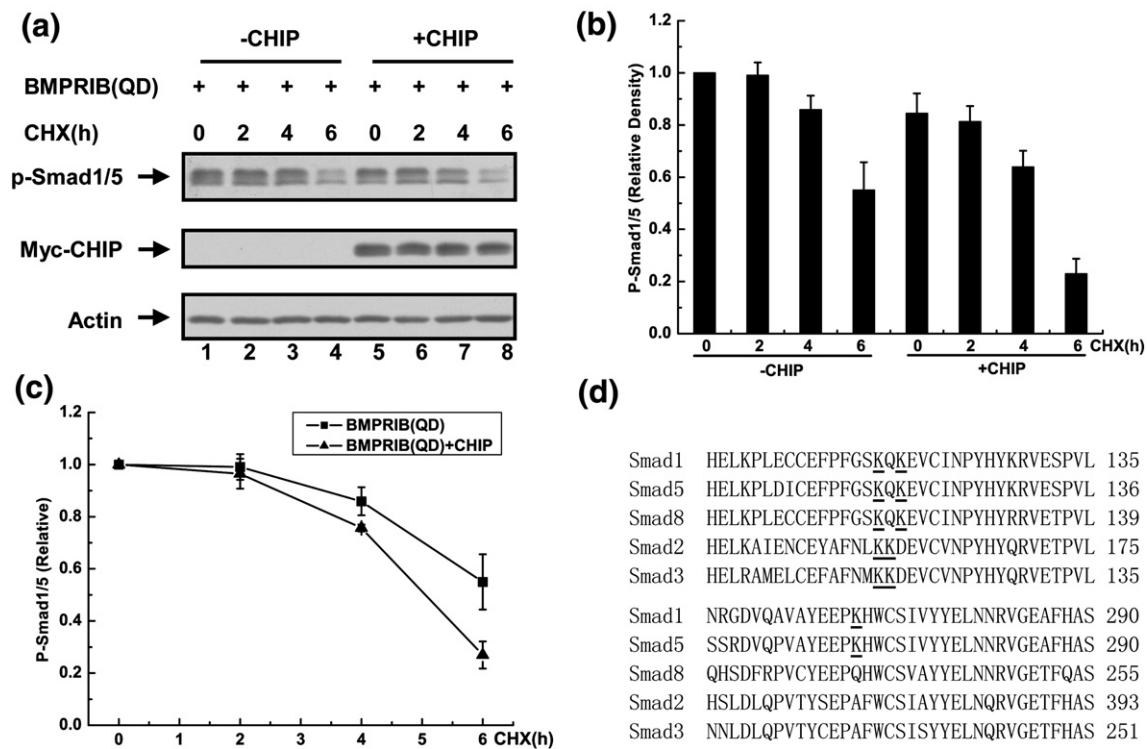


Figure 10. The degradation rate of phospho-Smad1/5 (p-Smad1/5) is increased by overexpressed CHIP. (a) The phospho-Smad1/5 levels at different time points after treatment with cycloheximide (CHX) (5 μ M) were shown by Western blot using an anti-phospho-Smad1/5 antibody. (b) and (c) Graphical presentation shows the relative abundance of the phospho-Smad1/5 levels after normalization with actin. The phospho-Smad1/5 level of the first lane was set as 1 (b). Phospho-Smad1/5 levels were quantified and valued relative to that at 0 h (c). The experiments were carried out in triplicate. The data are represented as the average with \pm SD. (d) Sequence alignment of the R-Smad proteins. K116, K118 and K269 of Smad1 and the corresponding positions in other R-Smads are marked by underlines.

the poly-ubiquitination of Smad1. The decreased mono-ubiquitination of Smad1 caused by Hsp70 might result from Hsp70 competitively binding to CHIP. In fact, Hsp70 is able to interact with Smad3 directly and Hsp70/Smad3/CHIP forms a ternary complex *in vivo* (unpublished data). Similar to Smad3, our preliminary results also indicate Smad1 might directly interact with Hsp70 and form a ternary complex with Hsp70 and CHIP. Thus, although Hsp70 might compete with Smad1 to bind the similar motif of CHIP, CHIP might also ubiquitinate Smad1 presented by Hsp70.

Our previously published data indicate that CHIP decreases the level of overexpressed Smad1 in a dose-dependent manner²⁷ and CHIP mediates the basal level of unactivated Smad3,²⁹ while our data shown here implicate that CHIP does not influence the basal level of endogenous Smad1/5 and CHIP preferentially regulates the degradation of phospho-Smad1/5 (Figure 9 and Figure 10(a)–(c)). These differences might result from the overexpression of Smad1 in the previous studies and different regulations of Smad1 and Smad3 mediated by CHIP. In addition, sequence alignment of the R-Smad proteins shows that K116 and K118 of Smad1 are relative conserved among all R-Smads, but K269 is only conserved among Smad1 and Smad5 and absent from other members of the R-Smads family (Figure 10(d)). This indicates that ubiquitination at

the K269 is specific for Smad1 and Smad5, which are members of the BMP signaling pathway.

In summary, CHIP-mediated Smad1 ubiquitination occurs mainly at four sites: the N terminus, K116, K118 and K269. Differing from Smurf1, an E3 ligase that mainly regulates the basal level of Smad1, CHIP might preferentially regulate the ubiquitination and proteasomal degradation of phosphorylated Smad1 and thus the BMP signaling *via* increasing association of CHIP and the phosphorylated tail of Smad1.

Materials and Methods

Plasmids and constructs

cDNAs encoding N-terminal His-tagged E1 and UbcH5a were kindly provided by Dr Dieter A Wolf and Dr Kazuhiro Iwai, respectively. MH1 and MH2-L fragments of Smad1 (from pcDNA6/V5-MH1 and MH2-L) were sub-cloned into the pGEX4T-1 vector to generate GST-tagged Smad1(MH1) and Smad1(MH2-L) constructs, respectively. His-CHIP and Myc-CHIP were constructed as described.^{27,29} BMPRIB(QD) was kindly provided by Dr Xiaofan Wang. pGEX6P1-HA-Ubiquitin(WT) and pGEX6P1-HA-Ubiquitin(K0) plasmids were kindly provided by Drs Keiichi Nakayama and Shigetsugu Hatakeyama. HA-Ubiquitin(WT) and HA-Ubiquitin(K0) were sub-cloned into the pET28a(+) vector to create His-

Ubiquitin(WT) and His-Ubiquitin(K0) constructs, respectively. GST-tagged Smad1 and His-tagged Smad1 expression vectors were constructed by PCR based on pKR5-Smad1. pET28a-Ubiquitin(K0)-Smad1 was constructed by inserting Ubiquitin(K0) into pET28a-Smad1. His-tagged Smurf1 was generated by PCR based on pBS2-KS-Flag-hSmurf1 (a gift from Dr Gerald H. Thomsen). Other mutated pET28a-Smad1 plasmids (Smad1-(K15–40R), (K42–45R), (K53–269R), (K306–449R), (K81–118R), (K81/104/116R), (K81/104/118R), (K81/116/118R), (K104/116/118R), (K53–128R), (K269R), (K116/118/269R), (K0), (3E)) were created by multiple instances of three-step PCR, and the identities of individual clones were verified by sequencing. Detailed information is available upon request.

Protein expression and purification

His-tagged proteins (E1, UbcH5a and CHIP) and GST-tagged proteins (Smad1, Smad1(MH1), Smad1(MH2-L), Ubiquitin(WT), Ubiquitin(K0)) were expressed and purified as described.^{21,28,59,60} Briefly, *E. coli* BL21(DE3) cells, transformed with the appropriate expression vectors, were cultured in 2×YT or LB medium at 37 °C, and 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to induce at 30 °C for 6 h when A_{600nm} reached 0.6. Bacteria were harvested and then lysed by sonication. His-tagged proteins were purified by Ni²⁺-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer's instructions. GST fusion proteins were purified by affinity chromatography on glutathione-Sepharose 4B (GE Healthcare Biosciences) according to the manufacturer's instructions. To generate Smad1, E1 and CHIP, the GST-tag and His tag were cleaved by thrombin (GE Healthcare Biosciences) for 16 h at 4 °C. Other His-tagged proteins were expressed in *E. coli* Rosetta(DE3) cells and purified as described above.

In vitro ubiquitination and ubiquitination/degradation assays and immunoblot analysis

The ubiquitination assay was performed as described^{21,59} with some modifications. In brief, the reaction mixture (20 μl) containing 5 μM Smad1 or Smad1 mutants, 0.1 μM E1, 2.5 μM UbcH5a, 5 μM CHIP or 1 μM Smurf1, 2 μg/μl of His-Ubiquitin or 5 μg/μl of GST-Ubiquitin and 2 μl of 10×ATP regenerating system (10 mM ATP, 100 mM creatine phosphate (Fluka), 40 mM magnesium acetate, 100 unit/ml creatine kinase (Sigma)) in 50 mM Tris-HCl (pH 7.3), 100 mM NaCl, 2 mM dithiothreitol was incubated for 2 h at 30 °C. It should be noted that ATP regenerating system is very important for repeatability of all experiment results. For degradation of bacterial Smad1, 5 μl of *in vitro* ubiquitination reaction mixture and 50 nM 26 S proteasomes (Boston Biochem.) were incubated at 37 °C with or without 50 μM MG132. The reaction was terminated by the addition of 5×SDS sample buffer and heating at 100 °C for 5 min. Samples were separated on SDS-7% (w/v)PAGE and then subjected to immunoblot analysis with a mouse monoclonal antibody against Smad1(A-4) (Santa Cruz) (or other specific primary antibodies where indicated) and horseradish peroxidase-conjugated goat polyclonal antibodies to mouse immunoglobulin (Santa Cruz). Immune complexes were detected by enhanced chemiluminescence (ECL kit; Santa Cruz).

Sources of other antibodies were as follows: His-tag monoclonal antibody, Novagen; Smad(C-17) polyclonal

antibody, Santa Cruz; antihemagglutinin(HA) probe mouse monoclonal antibody, Santa Cruz; horseradish peroxidase-conjugated rabbit polyclonal antibodies to goat immunoglobulin, Santa Cruz; anti-CHIP polyclonal antibody, generated in our laboratory; anti-phospho-Smad1/5/8 rabbit polyclonal antibody, Cell Signaling Technology; anti-Myc (9E10) antibody, Santa Cruz; anti-β-actin antibody, Santa Cruz.

GST pull-down and thrombin cleavage assay

His-Smad1 was subjected to an *in vitro* ubiquitination assay at 30 °C for 2 h with E1, E2(UbcH5a), E3(CHIP) and GST-Ubiquitin. The reaction mixture after ubiquitination was incubated with glutathione-Sepharose 4B at 4 °C for 4 h, then the beads were washed six times. The ubiquitinated species were eluted and then treated with or without thrombin at 25 °C for 16 h.

Mass spectrometric analysis

Non-tagged Smad1 was subjected to *in vitro* ubiquitination assays. After the reaction, Smad1 and ubiquitinated Smad1 were immunoprecipitated by anti-Smad1 antibody, the beads were washed six times with Tris-HCl buffer (50 mM Tris-HCl (pH 7.3), 100 mM NaCl) and then boiled in 1×SDS sample buffer for 5 min. The proteins obtained were resolved by SDS-PAGE and stained by Coomassie brilliant blue. The non-ubiquitinated and mono-ubiquitinated bands were cut out and digested in the gel. The mass spectra were recorded by using a time-of-flight delayed extraction MALDI mass spectrometer (Bruker Autoflex, Center of Proteomics Research, Beijing Genomics Institute).

The search for ubiquitinated peptides was accomplished by FindPept tool (ExPASy Proteomics Server), by scanning for the expected addition of either of the two most C-terminal tryptic peptides of human ubiquitin (GG or LRGG, of 114.05 or 383.22 Da, respectively) onto a N-terminal residue or a Lys residue in the tryptic digest of human Smad1 plus a GS peptide at the N terminus.

Peptide inhibition assay

Two polypeptides, PISSVS and PIS(pS)V(pS) (synthesized by GL Biochem (Shanghai) Ltd), were added to the ubiquitination reaction mixture simultaneously to inhibit the poly-ubiquitination of Smad1(3E).

Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) fetal bovine serum (Invitrogen), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. Cell transfections were performed using VigoFect (Vigorous Biotech.) with the indicated amounts of plasmid DNA according to manufacturer's instructions. Cells were lysed in RIPA buffer (20 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1%(v/v) NP40, 1%(w/v) deoxycholate, 0.1%(w/v) SDS, 1 mM EDTA, 10 mM NaF, 1 mM sodium vanadate, 1 mM PMSF and protease inhibitors) followed by centrifugation. The cell extracts were separated by SDS-10% PAGE and analyzed by immunoblotting with a specific antibody according to manufacturer's instructions. In some cases, transfected

cells were treated with 50 μ M MG132 (Calbiochem) for 4 h before harvest.

In vivo degradation assay

HEK293T cells were seeded in 12-well plates and were transfected with 0.5 μ g of BMPRIB(QD) and with or without 0.5 μ g of CHIP. Protein lysates were prepared at indicated time points after addition of cycloheximide (CHX) (5 μ M). Equal amounts of protein were separated by SDS-PAGE. Levels of phospho-Smad1/5 were determined by immunoblotting and quantified at indicated time points.

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