Roles of mono-ubiquitinated Smad4 in the formation of Smad transcriptional complexes

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Abstract

TGF-β activates receptor-regulated Smad (R-Smad) through phosphorylation by type I receptors. Activated R-Smad binds to Smad4 and the complex translocates into the nucleus and stimulates the transcription of target genes through association with co-activators including p300. It is not clear, however, how activated Smad complexes are removed from target genes. In this study, we show that TGF-β enhances the mono-ubiquitination of Smad4. Smad4 mono-ubiquitination was promoted by p300 and suppressed by the c-Ski co-repressor. Smad4 mono-ubiquitination disrupted the interaction with Smad2 in the presence of constitutively active TGF-β type I receptor. Furthermore, mono-ubiquitinated Smad4 was not found in DNA-binding Smad complexes. A Smad4-Ubiquitin fusion protein, which mimics mono-ubiquitinated Smad4, enhanced localization to the cytoplasm. These results suggest that mono-ubiquitination of Smad4 occurs in the transcriptional activator complex and facilitates the turnover of Smad complexes at target genes.

(138 words)

Keywords: **TGF-β, Smad, mono-ubiquitination, p300, c-Ski, transcription**

Introduction

The transforming growth factor-β (TGF-β) family of extracellular growth factors regulates cell proliferation, differentiation, apoptosis, and morphogenesis [1, 2]. Upon ligand binding, type II receptors activate type I receptors which in turn transduce intracellular signals through phosphorylation of Smad proteins. Smad proteins are classified into three groups: receptor-regulated Smad (R-Smad), common partner Smad (Co-Smad), and inhibitory Smad (I-Smad). Smad1, Smad5 and Smad8 are phosphorylated by bone morphogenetic protein (BMP) receptors, whereas Smad2 and Smad3 are phosphorylated by TGF-β/activin receptors. Phosphorylated R-Smad forms a functional signaling complex with Smad4 (Co-Smad) and translocates into the nucleus to regulate the expression of ligand-responsive genes [3]. Activated Smad complexes bind directly to the AGAC box or related DNA sequences in target genes and recruit coactivators, including p300/CBP [4, 5]. p300/CBP stimulates transcription through acetylation of histones. However, it is not clear how activated Smad complexes dissociate from target genes. Proteasome-mediated degradation of Smad3 following ubiquitination by the ROC1-SCF^{Fbw1a} E3 ubiquitin ligase complex was found to arrest TGF-β signaling [6]. Although Smad4 mono-ubiquitination has been reported [7], the functional significance of this modification has not been determined.

Recent studies have suggested a link between the activator function of many transcription factors and their ubiquitination/degradation [8]. Sequences targeted by the degradation machinery ("degrons") and those required for transcriptional activation often overlap and can, in fact, be functionally equivalent [9]. For example, Gal4 ubiquitination and destruction are required for transcriptional activation by Gal4 in yeast. Ubiquitination occurs at a post-initiation step in transcription [10]. In mammalian cells, ubiquitination of c-Myc, Tat and CIITA transcription factors stimulates transcriptional activation [11-14]. In this study, we show that TGF-β stimulates the mono-ubiquitination of Smad4. Smad4 mono-ubiquitination was positively and negatively regulated by co-activator p300 and co-repressor c-Ski, respectively.

Materials and methods

Cells. 293T and COS-7 cells were obtained from the American Type Culture Collection. HaCaT cells were obtained from Dr. N.E. Fusenig. These cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10 % fetal bovine serum (FBS) and penicillin-streptomycin solution (Gibco). HaCaT cells stably expressing FLAG-Ubiquitin were selected and maintained in medium containing 1 μg/ml puromycin (Sigma).

DNA constructs and transfection. Expression constructs encoding ALK-5(TD), Ubiquitin, Smads, p300 and c-Ski were described previously [6, 15]. FLAG-Ubiquitin was cloned into the pCAG-IP vector [16] and used to establish stable transfectants. The Smad4-Ubiquitin fusion protein was generated using a PCR-based method. Cells were transfected using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer's recommendations.

Immunoprecipitation, DNA affinity precipitation (DNAP) and immunoblotting. For immunoprecipitation, cells were solubilized in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Nonidet P-40, 1.5 % Trasylol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were cleared by centrifugation. Lysates or immunoprecipitates were subjected to SDS-PAGE. Proteins were electrotransferred to PVDF membranes (ProBlott, Applied Biosystems) and subjected to immunoblotting. Anti-Myc (9E10, Calbiochem), anti-hemagglutinin (HA) (3F10, Roche), anti-FLAG (M2, Sigma), anti-Smad4 (B8, Santa Cruz) and anti-phospho-Smad2 [17] antibodies were used as primary antibodies. Reacted antibodies were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For re-blotting, membranes were stripped according to the manufacturer's protocol.

DNA affinity precipitation was performed as reported [18] using biotinylated oligo-DNA (3×CAGA probe) and streptavidin-agarose beads (Sigma) for precipitation. Sequence of 3×CAGA probe was as follows:

Sense strand,

5'-TCGAGAGCCAGACAAGGAGCCAGACAAGGAGCCAGACACTCGAG-3'

Antisense strand,

5'-CTCGAGTGTCTGGCTCCTTGTCTGGCTCCTTGTCTGGCTCTCGA-3'

Immunofluorescence. Immunohistochemical staining of FLAG-Smad4 and FLAG-Smad4-Ub in transfected COS-7 cells was performed using anti-FLAG (M2) antibody followed by incubation with Alexa 488-labeled goat anti-mouse IgG (Molecular Probes). Nuclei were stained with Hoechst33342 (Sigma). Intracellular localization was observed by fluorescence microscopy (Axiovert 200, Zeiss).

Results and Discussion

TGF-β stimulates mono-ubiquitination of Smad4.

Mono- or poly-ubiquitination of Smad4 was reported to be important for Smad-mediated transcriptional activation [7]. To further investigate the roles of Smad4 mono-ubiquitination in TGF-β signaling, FLAG-Smad4 and HA-Ubiquitin cDNAs were transfected in 293T cells and the mono-ubiquitination of Smad4 was examined in the absence or presence of TGF-β (Fig. 1A). As reported previously [7], Smad4 mono-ubiquitination occurred in the absence of TGF-β, and was further enhanced by TGF-β (Fig. 1A). We could not detect the ubiquitination of Smad2 with or without TGF-β stimulation (data not shown). We next established HaCaT cells stably expressing FLAG-tagged Ubiquitin (Fig. 1B). Using this cell line, we investigated the ubiquitination of endogenous Smad4. Following TGF-β treatment, Smad4 was immunoprecipitated and subjected to immunoblot analysis with anti-FLAG antibody. Mono-ubiquitinated Smad4 was detected at 1 - 2 hours after treatment with TGF-β and

decreased in abundance at 4 hours (Fig. 1C). Phosphorylation of Smad2 peaked at 1 hour after treatment and gradually declined. These results indicate that TGF-β signaling stimulates mono-ubiquitination of Smad4.

Effects of p300 and c-Ski on the mono-ubiquitination of Smad4.

p300 enhances ubiquitination of Smad3 by recruiting the ROC1-SCF^{Fbw1a} E3 ubiquitin ligase complex [6]. We examined the effects of p300 on the mono-ubiquitination of Smad4. p300 was coexpressed with Smad4, FLAG-Ubiquitin, and a constitutively active form of TGF-β type I receptor (ALK-5(TD)-HA) in 293T cells (Fig. 2A). Coexpression of p300 induced a shift in Smad4 mobility consistent with Smad4 mono-ubiquitination. In contrast, the mobility of Smad2 did not change (Fig. 2A). We observed non-ubiquitinated Myc-Smad4 and Myc-Smad2 in FLAG-Ubiquitin immunoprecipitates, suggesting that Ubiquitin forms non-covalent associations with these proteins in the absence of p300, and that covalent Ubiquitin linkages are promoted by p300. The mobility shift of Smad4 induced by p300 was also detected using an anti-Smad4 antibody (Fig. 2B). The binding of c-Ski to Smad2/3 and Smad4, dissociates the p300/CBP transcriptional coactivator proteins from Smad complexes [19]. We previously showed that c-Ski enhances the DNA binding affinity of Smad4 and forms a stable c-Ski-Smad complex on Smad binding elements (SBE) [20]. The formation of a stable c-Ski-Smad complex on DNA is thought to prevent the recruitment of p300 [19]. Therefore, we examined the effects of c-Ski on Smad4 mono-ubiquitination. As shown in Fig. 2B, c-Ski suppressed the p300-induced mono-ubiquitination of Smad4 in a dose dependent manner.

Ubiquitination of transcription factors including c-Myc and CIITA has been shown to be important for transcriptional activation. Skp2-mediated ubiquitination and proteasome-mediated degradation of c-Myc are required for c-Myc-mediated transcription [13, 14]. The impact of CIITA on transcription is also enhanced by its poly- and mono-ubiquitination [12]. Several histone acetyltransferases (HATs) including

p300/CBP, PCAF, and TAF1 have been reported to have intrinsic Ubiquitin ligase activities [21]. PCAF, one of HATs, enhances the ubiquitination of CIITA. Furthermore, trichostatin A, a broad inhibitor of histone deacetylases (HDACs), enhances the ubiquitination of CIITA, indicating that CIITA ubiquitination was controlled by HATs and HDACs [12]. As shown in Fig. 2, Smad4 mono-ubiquitination was positively and negatively regulated by p300 and c-Ski, respectively. These data suggest that ubiquitination of transcription factors including Smad4 can be regulated by co-activators and co-repressors, although a mechanism for this regulation has not yet been determined.

Mono-ubiquitinated Smad4 does not form a stable complex with Smad2 and is released from target DNA elements.

Smad4 mono-ubiquitination occurs at Lys507 [7], which is located at the interaction interface with phosphorylated R-Smad [22]. Thus, we predicted that mono-ubiquitination would release Smad4 from R-Smad. To address this possibility, FLAG-Smad4 was coexpressed with Myc-Smad2, HA-Ubiquitin and ALK-5 (TD)-V5 (Fig. 3A). Mono-ubiquitinated Smad4 was detected after immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-HA antibody (Fig. 3A, lane 2). Although an equivalent amount of FLAG-Smad4 was co-immunoprecipitated with anti-Myc antibody (Fig. 3A, lane 4, second panel), mono-ubiquitinated Smad4 was not found in the Smad2-Smad4 complex (Fig. 3A, lane 4, top panel). This result indicates that mono-ubiquitinated Smad4 does not interact with Smad2. Moreover, mono-ubiquitinated Smad4 was not found in the Smad complex that is affinity precipitated by 3×CAGA probe (Fig. 3B). Mono-ubiquitinated Smad4 was clearly detected in the Smad4 immunoprecipitate in the presence of constitutively active ALK-5 (Fig. 3B, lane 2, top panel). Although a similar amount of Smad4 was captured onto 3×CAGA probe, mono-ubiquitinated Smad4 was not observed (Fig. 3B, lane 3, 4, top panel). It is not clear whether mono-ubiquitination of Smad4 occurs in the

Smad4/R-Smad complex or in monomeric Smad4. Because Smad4 has less affinity for p300 than does R-Smad [23, 24], p300-mediated mono-ubiquitination of Smad4 is thought to occur after the formation of the Smad4/R-Smad complex.

Subcellular localization of Smad4-Ubiquitin fusion protein.

Mounting evidence suggests that protein mono-ubiquitination can modulate cellular localization, transcription, and DNA repair [25-27]. To investigate the function of Smad4 mono-ubiquitination, we constructed a Smad4-Ubiqutin fusion protein (Smad4-Ub) (Fig. 4A, B). Smad4 has been shown to shuttle rapidly and continuously between the cytoplasm and nucleus [28]. In transfected COS-7 cells, Smad4 was distributed throughout the cytoplasm and nucleus. In contrast, Smad4-Ub was predominantly localized in the cytoplasm. Because leptomycin B (LMB), which inhibits chromosomal region maintenance 1 (CRM1)-dependent nuclear export, caused Smad4-Ub to accumulate in the nucleus, we concluded that the nuclear import of Smad4-Ub was intact (Fig. 4C). These results suggest that Smad4-Ub is subject to export from the nucleus. In a similar manner, p53-Ubiquitin was found to accumulate in the cytoplasm [29], indicating that mono-ubiquitination regulates Smad4 and p53 trafficking. More studies are needed to investigate how mono-ubiquitinated Smad4 is processed (either degraded or deubiquitinated).

The removal of activated Smad complexes from *cis*-regulatory elements may regulate transcription of target genes. An activated Smad complex stimulates transcription by binding to the gene-regulatory elements and recruiting co-activators including p300 [1, 2]. At the same time, promoter clearance may play a role in transcription [8, 9]. Activated Smad proteins can potentially be removed from transcription complexes by several mechanisms including Ubiquitin-mediated degradation [6] and dephosphorylation [30, 31] of R-Smad.

In conclusion, TGF-β stimulates mono-ubiquitination of Smad4. The mono-ubiquitination of Smad4 may, in turn, dissociate the Smad4/R-Smad complex and

remove Smad4 from DNA. The proposed scheme of Smad4 mono-ubiquitination is shown in Fig. 4D. These results provide novel insight into the mechanism of turnover of Smad complexes on target genes.

Acknowledgements

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Figure legends

Fig 1. TGF-β stimulates mono-ubiquitination of Smad4.

A. 293T cells were transfected with DNA constructs as indicated. Cells were treated with TGF-β (100 pM) and cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with antibodies as indicated. *B*. Establishment of HaCaT cells stably expressing FLAG-tagged Ubiquitin. Cell lysates from mock- and FLAG-Ubiquitin-transfected cells were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-FLAG antibody. *C*. HaCaT cells stably expressing FLAG-tagged Ubiquitin were treated with TGF-β (100 pM) as indicated. Cell lysates were immunoprecipitated with anti-Smad4 antibodies, followed by immunoblotting as indicated. Immunoblots of total cell lysates were performed using anti-Smad2 and phospho-Smad2 antibodies.

Fig 2. Effects of p300 and c-Ski on the mono-ubiquitination of Smad4.

A - B. 293T cells were transfected with DNA constructs as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with

antibodies as indicated. Total cell lysates were subjected to immunoblotting with antibodies as indicated.

Fig 3. Mono-ubiquitinated Smad4 is not found in Smad4 complexes containing Smad2 or Smad4 complexes binding target DNA.

A. 293T cells were transfected with DNA constructs as indicated. Cell lysates were immunoprecipitated with anti-FLAG (lanes 1 and 2) or anti-Myc (lanes 3 - 5) antibodies, followed by immunoblotting with antibodies as indicated. *non-specific band. *B*. 293T cells were transfected with DNA constructs as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody (lanes 1 and 2) or affinity precipitated onto a 3x CAGA DNA probe (lanes 3 - 5), followed by immunoblotting with antibodies as indicated.

Fig 4. Subcellular localization of Smad4-Ubiquitin fusion protein.

A. Schematic representation of Smad4 and Smad4-Ubiquitin fusion proteins. *B*. COS-7 cells were transfected with DNA constructs as indicated. Immunoblot analysis of total cell lysates was performed using anti-FLAG antibody. *C*. COS-7 cells were transfected with DNA constructs as indicated. Cells were cultured in the presence or absence of leptomycin B (LMB) (2 ng/ml) for 1 h. Subcellular localization of Smad4 and Smad4-Ub was analyzed using anti-FLAG antibody. Nuclei were stained with Hoechst33342. Graph shows the percentage of cells containing the indicated grade of nuclear staining (right). N=C, nuclear staining as strong as cytoplasmic staining; N>C, nuclear staining stronger than cytoplasmic staining; N<C, cytoplasmic staining stronger than nuclear staining. *D*. Proposed scheme of mono-ubiquitination of Smad4. TGF-β stimulation phosphorylates C-terminus of R-Smad. Phosphorylated R-Smad forms a complex with Smad4. The Smad complex translocates into the nucleus and binds to the target gene. p300 associates with the Smad complex on the DNA, acetylates histones and activates transcription. At the same time, p300 induces mono-ubiquitination of

Smad4 causing dissociation from R-Smad and DNA. c-Ski competes with p300 for binding to the Smad complex and suppresses Smad4 mono-ubiquitination and turnover of the complex.

Figure 1

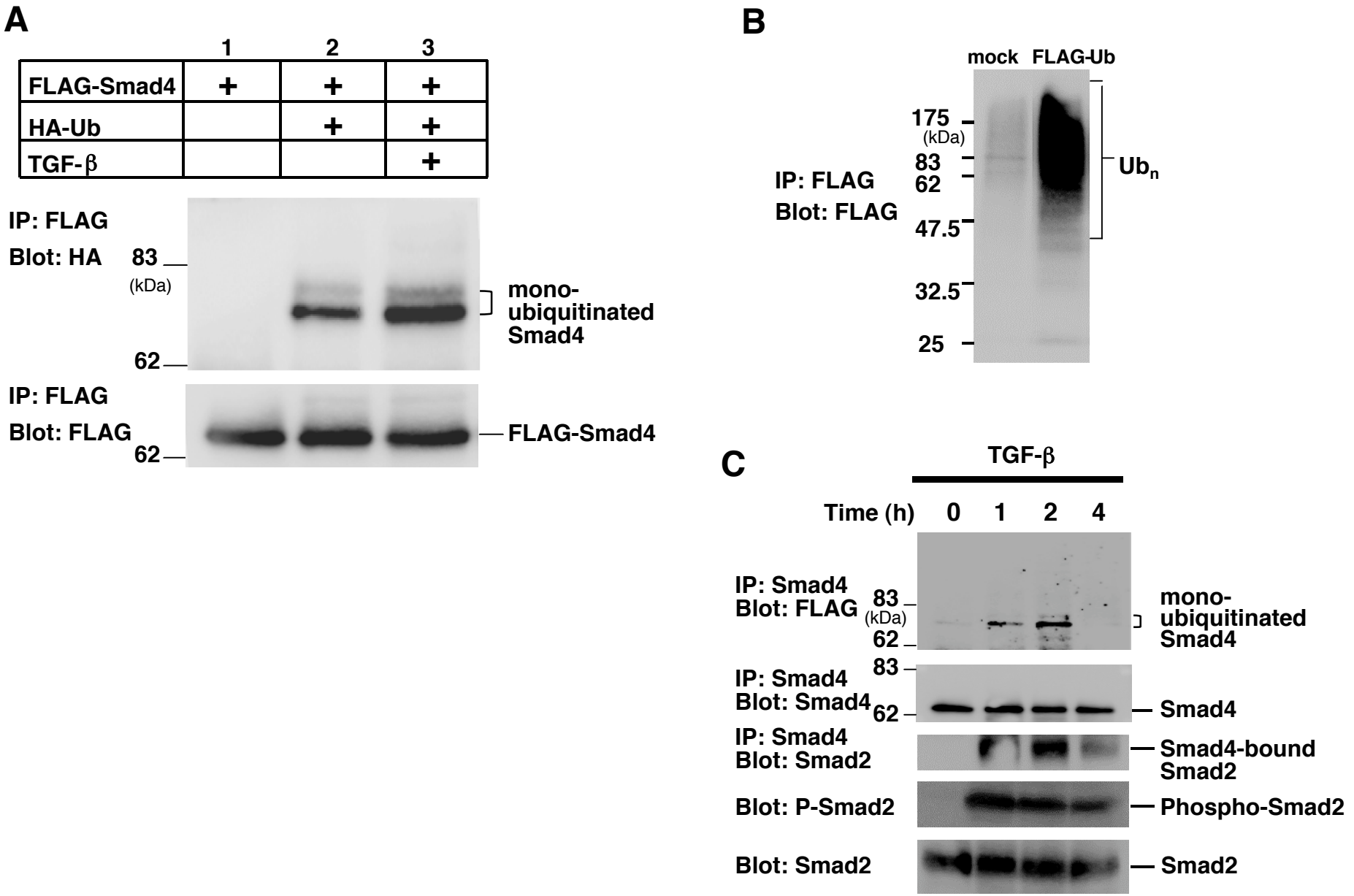
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Figure 2

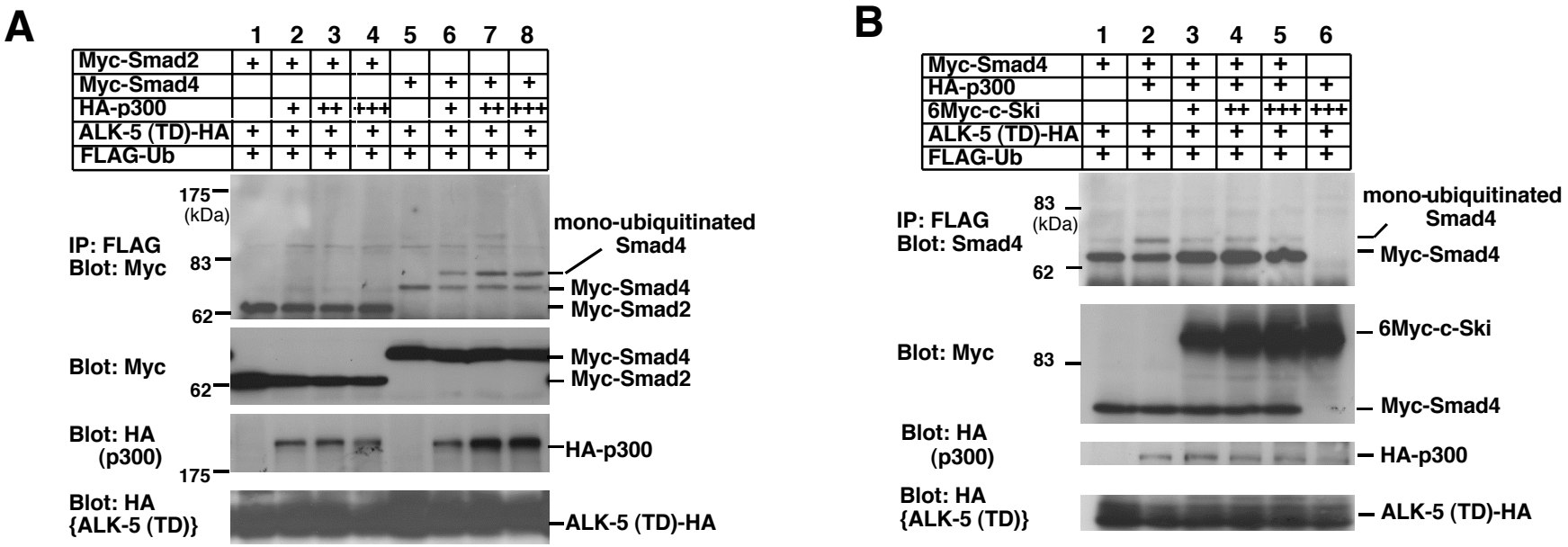
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Figure 3

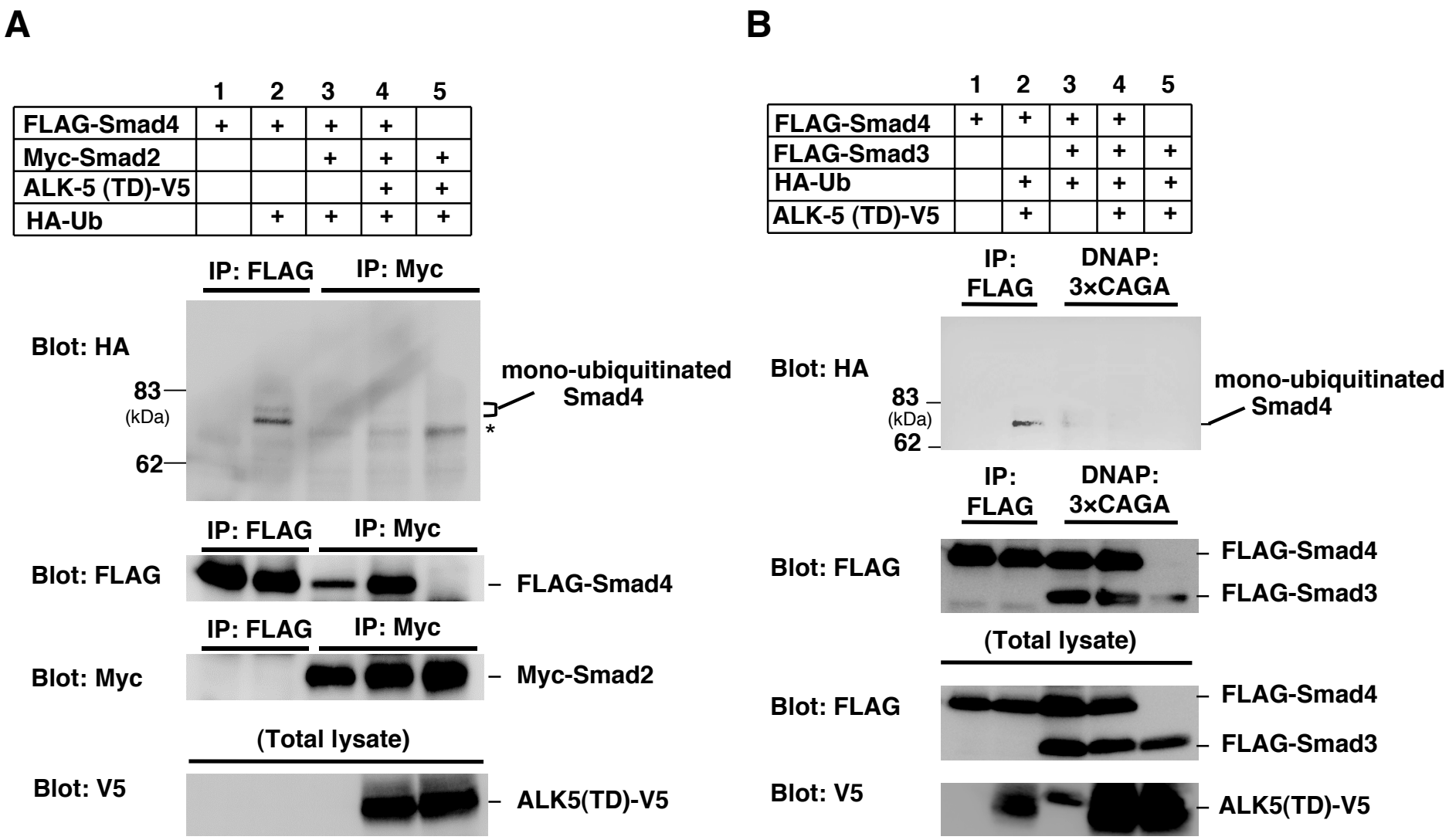
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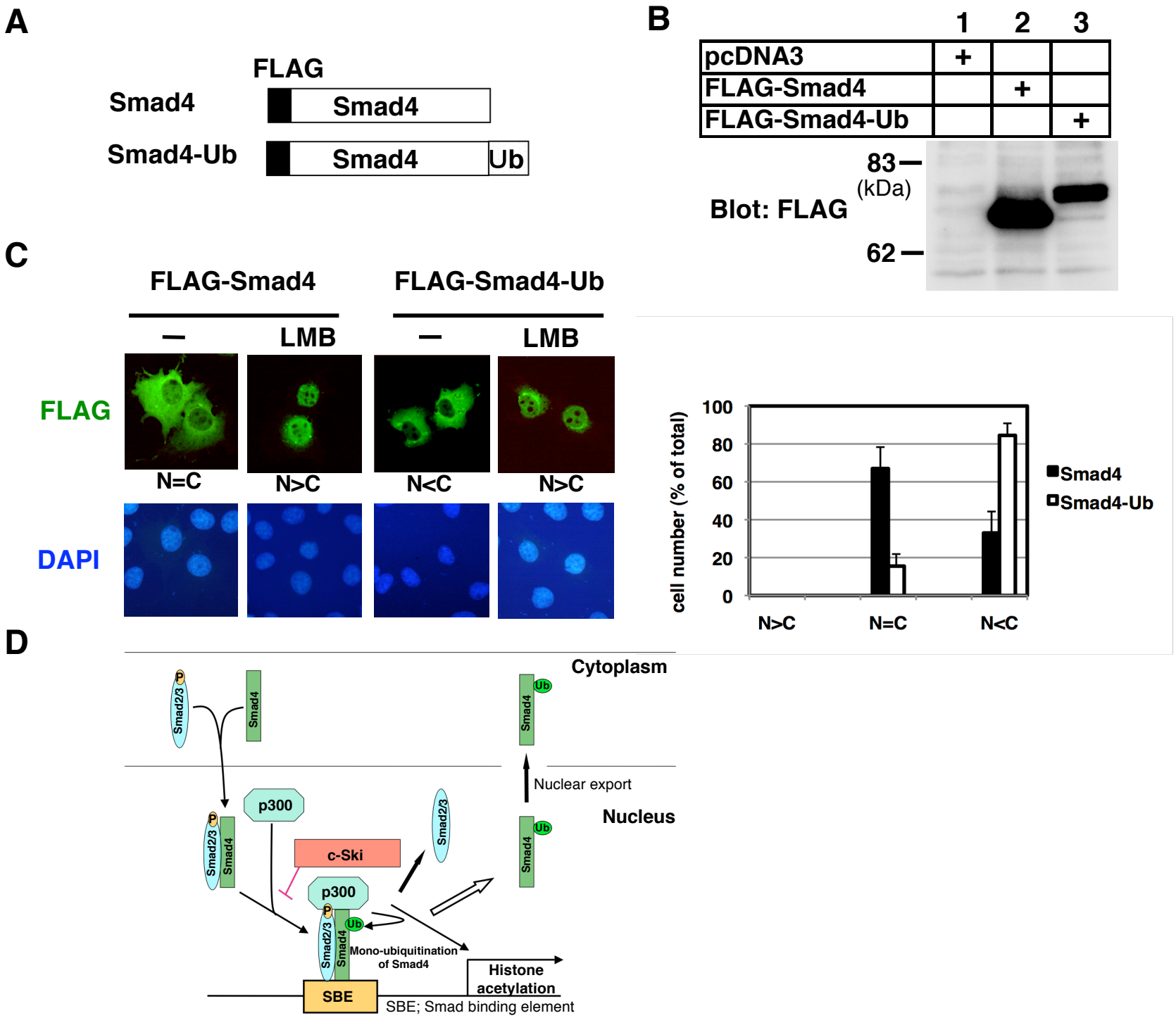
Figure 4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40
20
0
N>C
N=C
N<C
Smad4
Smad4-Ub
D
Cytoplasm
Nuclear export
Nucleus
Smad2/3
Smad4
p300
c-Ski
Ub
Mono-ubiquitination of Smad4
Histone acetylation
SBE
SBE; Smad binding element