PROSPECT
Journal of Cellular Biochemistry 110:554–563 (2010)

Journal of Cellular Biochemistry

Smurf Control in Bone Cells

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ABSTRACT

The homologous to the E6-assosiated protein carboxyl terminus (HECT) domain E3 ubiquitin ligase Smurf1 is the first E3 ligase to be implicated in regulating bone cell function. The involvement of Smurf1 in multiple signaling pathways and pathological conditions is presently an area of extensive scientific interest. This review highlights recent works exploring Smurf-regulated biological processes in bone cells and highlights recent discoveries surrounding the regulatory mechanisms modulating its catalytic activity and substrate recognition capability. Moreover, we discuss the relevance of targeting the HECT E3s through the development of small-molecule inhibitors as an anticancer therapeutic strategy. J. Cell. Biochem. 110: 554–563, 2010.

KEY WORDS: Smurf; OSTEOBLAST; UBIQUITINATION; PROTEASOME DEGRADATION

Protein ubiquitination is an important regulation mechanism for controlling cell function. This mechanism has also been implicated in the control of bone cell homeostasis. Several key molecules which are essential for osteoblast or osteoclast genesis such as Runx2 [Jonason et al., 2009] and TRAF6 [Darnay et al., 2007] are ubiquitinated. The ubiquitination process is carried out through sequential enzymatic reactions. It is initiated by an E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to one of a family of E2 ubiquitin-conjugating enzymes. The E3 ubiquitin ligases (E3s) transfer ubiquitin from E2 to various lysine residues of target substrates. Ubiquitin is an evolutionarily highly conserved 76 amino acid polypeptide that is abundant in all eukaryotic cells. Ubiquitin is synthesized as a monomer or as a lysine-linked polymer, which is then covalently attached to lysine residues of target proteins. The polymers are often linked through lysine 48 or lysine 63 on ubiquitin protein, leading to Lys-48 or Lys-63 poly-ubiquitination of target protein.

Ubiquitinated proteins have different fates dependent on the location of ubiquitin conjugates and the type of ubiquitin polymers. When intracellular substrates are tagged with ubiquitin through Lys48-linked poly-ubiquitin chains, substrate proteins are generally labeled for 26S proteasome-mediated recognition and proteolysis [Ciechanover, 1998]. In contrast, mono- or poly-ubiquitination by ubiquitin polymers other than Lys48, such as Lys63 [Herman-Bachinsky et al., 2007] leads to a variety of cellular activities in a proteolysis-independent manner. RANKL-induced TRAF6 activation is through Lys63 ubiquitination, which activates the downstream signaling pathway, rather than degradation [Deng et al., 2000].

The E3 ligase is thought to confer substrate specificity in ubiquitination by serving as an adaptor between the ubiquitin–protein conjugation machinery and the target molecule. Because of their substrate specificity, E3 ligases represent potentially attractive drug targets for a variety of pathological disorders that are accompanied by an abnormal expression or dysfunction of E3 ligases. There are about 1000 E3 ligases in the human genome that can be classified into three major types based on domain structure and substrate recognition. The first class comprises N-end rule ubiquitin ligases that target protein substrates bearing specific destabilizing N-terminal residues [Varshavsky, 2003] such as *Drosophila* inhibitor of apoptosis protein [Ditzel et al., 2003]. The second and largest group of E3 ligases is the Really Interesting New Gene (RING) family. TRAF6 contains a RING finger domain, which promotes its own ubiquitination in response to RANKL binding to

Grant sponsor: NIH/NIAMS; Grant numbers: R01 AR051189, R01 AR054465, R01 AR055915, K02 AR052411; Grant sponsor: New York State Department of Health; Grant number: N08G-070; Grant sponsor: Empire State Stem Cell Board.

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Received 12 February 2010; Accepted 17 February 2010 • DOI 10.1002/jcb.22586 • © 2010 Wiley-Liss, Inc.
Published online 5 April 2010 in Wiley InterScience (www.interscience.wiley.com).

RANK on the cell surface [Lamothe et al., 2007]. The third type of E3 ligase is homologous to the E6-assosiated protein carboxyl terminus (HECT), with the first family member being E6-associated protein (E6-AP), which, together with oncoprotein E6, promotes p53 ubiquitination and degradation [Scheffner et al., 1993]. HECT domain E3 ligases contain an ~350 amino acid C-terminal region homologous to that of E6-AP with a conserved active-site cysteine residue near the C-terminus [Bernassola et al., 2008]. N-terminal regions are highly variable and may be involved in substrate recognition [Hershko and Ciechanover, 1998]. The C2-WW-HECT subfamily of E3 ligase has nine mammalian members, including NEDD4-1, NEDD4-2, Itch, WWP1, WWP2, and Smad ubiquitination regulatory factor (Smurf) 1 and 2. All C2-WW-HECT E3 ligases are characterized by the presence of an N-terminal C2 domain, 2–4 serial WW domains as well as the C-terminal enzymatic HECT domain. The C2 domain mediates association with the phospholipid membrane in response to intracellular $Ca2^{+}$ and WW domains are responsible for the interaction with substrate proteins. The cysteine residue in the C-terminal HECT domain form a thiolester bond with ubiquitin and is critical for the catalytic activity of the HECT domain E3 ligases [Bernassola et al., 2008]. Mutation of this highly conserved cysteine residue to Alanine (C710A in Smurf1) or (C716A in Smurf2) will cause total loss of the E3 ligase activity for the mutant Smurf1 and Smurf2 [Zhu et al., 1999; Lin et al., 2000; Zhang et al., 2001]. The WW domain is the other important character of the HECT-domain E3 ligases. WW domains, which contain about 30 amino acids, including two highly conserved tryptophan residues and one proline residue, specifically interacts with the proline-rich (PPXY) motif of substrate proteins. E3 ligases with different WW domains show different selectivity for target proteins. Smurf1, which has two WW domains located at amino acids 236–311, interacts with the PY motif of Smads 1 and 5, and Smurf2, which has three WW domains located at amino acids 248–369, interacts with the PY motif of Smads 2 and 3. Smads with mutations of the PY motif lose the ability to interact with Smurfs and avoid degradation. In addition to Smurfs, recent findings demonstrate that E3 ligase WWP1 interacts with Smad7 and specifically regulates the TGF-β pathway through induction of degradation of Smad7 [Komuro et al., 2004]. Among C2-WW-HECT E3 ligases, Smurfs are the most studied E3 ligases in bone biology. Studies from biochemical analyses and genetically modified mouse models reveal that Smurf1 negatively regulates the function of osteoblast lineage cells, including proliferation, differentiation, and maturation. In contrast, Smurf2 affects mainly chondrocyte function. Smurf2 transgenic mice in which the Smurf2 transgene is driven by the Col2a1 promoter develop an osteoarthritis-like phenotype [Wu et al., 2008a]. Here, we review recent studies of Smurf and bone cell function in normal and pathologic conditions.

Smurf1 TARGETS Smad1 AND Runx2 PROTEINS IN OSTEOBLAST PRECURSOR CELLS

BMP signaling proteins play an essential role in bone development and postnatal bone formation. In cells of osteoblast linage including mesenchymal stem cells (MSCs), osteoblast precursors, and perhaps mature osteoblasts, BMP binds to BMP receptor leading to Smad1 phosphorylation. Phosphorylated Smad1 then binds Smad4 and the Smad1–4 complex translocates to the nucleus to modulate transcription. Smad1 interacts with Runx2 on the promoter of target genes and coordinately controls osteoblast gene expression and differentiation [Jonason et al., 2009]. The expression of Smad1 and Runx2 is tightly regulated at mRNA and protein levels. Over the last decade, ubiquitination and proteasomal degradation was revealed as an important regulatory mechanism to control Smad and Runx2 protein levels in osteoblasts.

Zhu et al. [1999], using Xenopus Smad1 as bait, identified Smurf1 as an E3 ligase interacting with Smad1 and subsequently inducing Smad1 ubiquitination and proteasome degradation. Investigation of *Drosophila* Smurf (DSmurf) demonstrated that mutations of DSmurf result in down-regulation of signals from DPP, the ortholog of BMP2/4, indicating that Smurf1 is likely to regulate BMP signaling pathways in humans [Podos et al., 2001]. In 2003, our group first decribed the function of Smurf1 in Runx2 degradtion and osteoblast function. We demonstrated that overexpression of Smurf1 induces proteasomal degradation of Smad1 and Runx2 proteins in 2T3 osteoblast precursor cells and in C2C12 myoblast/osteoblast precursor cells. Through its WW domain, Smurf1 specifically recognizes the PY motif of Smad1 and Runx2, resulting in their poly-ubiquitination and degradation through 26S proteasome. Smurf1-induced Smad1 and Runx2 degradation is prevented by treating cells with proteasome inhibitor. Thus, Smurf1 targets ubiquitination of Smad1 and Runx2, and is the first E3 ligase identified in the BMP signaling pathway as a negative regulator of bone cell function [Zhao et al., 2003]. Smurf1 also induces the degradation of Smad5, facilitating myogenic differentiation of C2C12 cells at the expense of reduced BMP-induced osteogenic differentiation [Ying et al., 2003].

Smad1 consists of three distinct domains: two highly conserved N- and C-terminal domains, referred to as mad homology 1 (MH1) and MH2, respectively, and a more divergent intervening linker region. In the inactive state, MH1 and MH2 bind to one another, mutually inhibiting the function of each domain. Binding of BMP receptor with BMP triggers C-terminal phosphorylation of MH2 domain of Smad1, which opens up this structure to allow association with Smad4 or with other DNA-binding proteins via the MH2 domain [Tsukazaki et al., 1998; Whitman, 1998] and activates the downstream target genes. In contrast, mitogen-activated protein kinases (MAPKs) catalyze phosphorylation in the linker region leading to an inhibition of Smad1 translocation into the nucleus [Kretzschmar et al., 1997]. Although MAPK-induced inhibition of BMP signal has been known for more than 10 years, the molecular mechanisms involved remain unknown. A recent study demonstrated that MAPK-induced phosphorylation of the linker region restricts Smad1 activity by enabling Smad1 recognition by the Smurf1 leading to Smad1 ubiquitination and degradation. In addition, Smurf1 binding also blocks the interaction of Smad1 with the nuclear translocation factor Nup214. Thus, MAPK-dependent Smurf1 binding has two negative effects on Smad1 activity: proteasomal degradation and cytoplasmic retention [Sapkota et al., 2007]. Interestingly, the phosphorylation of the linker region of Smad1 is triggered also by BMP, which is considered a feedback

control mechanism. In a similar fashion, TGF-β induces C-terminal phosphorylation of Smads 2 and 3 proteins to activate the TGF-β signaling pathway. TGF-β also promotes the phosphorylation of the linker regions of Smads 2 and 3. However, instead of Smurfs, another member of C2-WW-HECT subfamily E3 ligase, Nedd4-2 is responsible for linker region phosphorylation and Smad2/3 polyubiquitination and degradation [Gao et al., 2009]. Nedd4-2 was previously identified as a regulator of renal sodium channels. *Nedd4-2*$^{-/-}$ mice are born normally and survived into adulthood. The animals have hypertension and cardiac hypertrophy. The bone phenotype has not been reported in these mice [Shi et al., 2008]. These new findings indicate that location of phosphorylation of Smad protein determines the fate of Smad proteins, which may explain how cells respond to various stimuli under different conditions.

To determine whether Smurf1 induces Runx2 degradation through the interaction with the PY motif of Runx2, we created a mutant Runx2 with a PY motif deletion and found that Smurf1 retained some of its ability to induce the degradation of the mutant Runx2, suggesting that Smurf1 could also induce Runx2 degradation through an indirect mechanism. Smurf1 has been shown to interact with Smads 1, 5, 6, and 7 [Moren et al., 2005] and Smads 1 and 5 also interact with Runx2. We found that Smads 1 and 5 had no effect on Smurf1-induced Runx2 degradation. Smad6 but not Smad7, binds Runx2, enhancing Smurf1-induced Runx2 degradation. These results demonstrate that in addition to its interaction with the PY motif of Runx2, Smurf1 induces Runx2 degradation in a Smad6-dependent manner [Shen et al., 2006b]. Smad6 does not increase Smurf1-induced JunB degradation and this is probably because that Smad6 does not interact with JunB [Zhao et al., 2010]. Smad6 gene transcription is up-regulated by BMP-2 in osteoblasts through Smad1 and Runx2 binding to the OSE2 sequences in the Smad6 promoter. Chromatin immunoprecipitation demonstrated that Smurf1 binds the OSE2 site through Runx2 and inhibits Smad6 gene transcription. Treatment with BMP-2 and transfection of Smad1 abolish Smurf1 binding to the OSE2 site, suggesting that Smad1 binding excludes Smurf1 interaction with the OSE2 site and promotes Smad6 gene transcription [Wang et al., 2007]. Although the ubiquitin-proteasome system functions in the cytoplasm and in nuclear compartments [von Mikecz, 2006], whether Smurf1 affects Runx2 protein stability in the nucleus is currently unknown.

Runx2 is a member of the Runt domain transcription factor family, which is comprises Runx-1, -2, and -3. The PY motif is conserved among all three Runx proteins, which are targeted by Smurf1 for ubiquitination and degradation in vitro [Jin et al., 2004; Shen et al., 2006b]. Genetic analyses of animals and humans revealed the involvement of Runx1 in hematopoiesis and leukemia, and Runx3 in the development of T-cells and dorsal root ganglion neurons and in the genesis of gastric cancer. There is no information on the involvement of Smurf1 in these systems and the biologic significance of Smurf1-induced Runx-1 and -3 degradation needs to be determined. Our recent results demonstrate that cyclin D1 induces both Runx-2 and -3 degradation through ubiquitination. Parathyroid hormone-related protein (PTHrP) might prevent premature hypertrophy in chondrocytes partially through inducing the degradation of Runx-2 and -3 in a cyclin D1-dependent manner [Zhang et al., 2009]. However, which of the E3 ligases are involved in this process remains unknown.

Smurf1 AND MESENCHYMAL STEM CELLS

Osteoblasts are derived from MSCs in the bone marrow. MSCs are clonal, plastic, adherent cells, characterized by their ability to differentiate into various specific cell lineages, including osteoblasts, adipocytes, and chondrocytes. MSCs were first identified as colony-forming unit-fibroblasts (CFU-Fs) by Friedenstein et al. [1970] because cells in CFU-Fs can give rise to different cell types in a specific culture system. Although MSC research has grown rapidly in the past 15 years, a definitive and positive marker for MSCs is still lacking. However, there is some consensus regarding the expression profile of surface proteins on MSCs. For instance, human MSCs usually express Stro-1, CD106, CD73, and CD90 and do not express the hematopoietic and endothelial markers CD45, CD11b, CD31, or CD14 [Swart et al., 2008]. The surface marker profile is different between human and mouse [Kolf et al., 2007]. Mouse MSCs do not express Stro-1 and CD73, and few of them express CD90 [Kolf et al., 2007]. However, they express CD29, CD44, CD106, and CD105 [Meirelles Lda and Nardi, 2003; Peister et al., 2004]. Since MSCs belong to lineage negative cells that do not express CD45, increasing the $CD45^-$ population represents an enrichment of MSCs [Mukherjee et al., 2008; Morikawa et al., 2009].

MSCs undergo commitment, proliferation, differentiation, and maturation to give rise to osteoblasts in a process named osteogenesis. Osteogenesis is controlled by a series of transcription factors, which have significant impact on bone formation. Treatment of $CD45^-/CD105^+$ MSCs with an FDA-approved proteasome inhibitor, bortezomib, increases their osteogenic potential both in vivo and in vitro, indicating that proteasomal machinery may play an important role during the commitment of MSCs into osteoblasts [Mukherjee et al., 2008]. This is consistent with our previous report in which systemic administration of the proteasome inhibitors epoxomicin and proteasome inhibitor-1 to wild-type mice increases bone volume and bone formation rates [Garrett et al., 2003].

We found that bone marrow mesenchymal progenitor cells from *Smurf1*$^{-/-}$ mice form significantly increased alkaline phosphatase-positive colonies, indicating roles of MSC proliferation and differentiation in bone mass accrual in *Smurf1*$^{-/-}$ mice. While searching for a PY motif, the Smurf1 targeting sequence in proteins known to control cell growth, we found a PPXY sequence in the JunB protein, suggesting that JunB may be an additional target for Smurf1. *Smurf1*$^{-/-}$ cells have an elevated protein level of JunB. Biochemical experiments demonstrate that Smurf1 interacts with JunB through its PY motif and targets JunB protein for ubiquitination and proteasomal degradation. Indeed, Smurf1-deficient MSCs have higher proliferation rates, consistent with the facts that both cyclin D1 and its mRNA are increased in *Smurf1*$^{-/-}$ cells and JunB can induce the *cyclin D1* promoter. Moreover, JunB overexpression induces osteoblast differentiation showing increased expression of osteoblast markers. Silencing of JunB not only decreases osteoblast differentiation but also restores the osteogenic

potential to the wild-type level in *Smurf1*$^{-/-}$ cells. Thus, apart from regulation of BMP signaling proteins and Runx2, Smurf1 negatively regulates MSC proliferation and differentiation by controlling JunB turnover through an ubiquitin-proteasome pathway [Zhao et al., 2010].

THE ROLE OF Smurfs IN CHONDROCYTE DEVELOPMENT

Endochondral bone formation is a tightly regulated process. The process starts from mesenchymal cell condensation, subsequent formation of chondrocytes, to chondrocyte proliferation and differentiation into hypertrophic chondrocytes. A number of growth factors and signaling pathways are involved during chondrogenesis, especially TGF-β and BMPs. BMP is one of the most effective factors to stimulate chondrogenesis and chondrocytes maturation. TGF-β promotes chondrogenesis in cultures of undifferentiated multipotent mesenchymal cells [Leonard et al., 1991] and inhibits chondrocyte hypertrophy [Ballock et al., 1993]. In articular chondrocytes TGF-β maintains cells in an undifferentiated status. Serra et al. generated transgenic mice that express a truncated, kinase-defective TGF-β type II receptor driven by a metallothionein-like promoter (MT-DNIIR). This loss-of-function transgenic mouse model developed progressive skeletal degeneration, articular chondrocyte maturation, and synovium hyperplasia [Serra et al., 1997]. The mutant mice homozygous for a targeted disruption of Smad3 exon 8 show similar phenotype to the dominant-negative TGF-β type II receptor transgenic mice, which resemble human osteoarthritis [Yang et al., 2001]. These findings indicate that TGF-β and BMP pathways play critical roles during chondrogenesis and chondrocyte differentiation and maturation.

It has been demonstrated that Smurf1 binds Smads 1 and 5 and promotes their degradation [Zhu et al., 1999], indicating that overexpression of Smurf1 in chondrocytes may inhibit chondrocyte maturation and delay endochondral bone formation. However, Horiki et al. generated transgenic mice expressing Smurf1 transgene driven by the Col-XI promoter and did not find obvious abnormalities. One possible reason is that the activity of the collagen XI promoter may be relatively weak compared with that of the collagen II promoter. When Smurf1 transgenic mice were crossed with Smad6 transgenic mice, the mineralized area in the femur was significantly shorter and ossification was further delayed in the double-transgenic mice compared with the Smad6 single transgenic mice. This result suggests that the cooperation between Smad6 and Smurf1 may inhibit the BMP signaling pathway more efficiently than Smad6 alone during chondrocyte maturation [Horiki et al., 2004]. In addition to Smurf1, we also investigated the function of Smurf2 during endochondral ossification. We found that Smurf2 is up-regulated in cartilage from patients with osteoarthritis disease. Smurf2 transgenic mice in which the Smurf2 transgene was driven by the Col2a1 promoter exhibit severe cartilage arthropathy characterized by hypertrophy of articular chondrocytes, progressive degradation of the articular cartilage and development of osteophytes and subchondral sclerosis, accompanied with inhibition of TGF-β signaling and induction of phosphorylated Smad3 degradation. The overexpression of Smurf2 showed little effect on the degradation of Smads 1 and 2 and on BMP signaling [Wu et al., 2008a]. In addition to the down-regulation of phosphorylated Smad3, we also found that β-catenin protein levels are significantly increased in articular and growth plate chondrocytes in Smurf2 transgenic mice partially due to enhanced GSK-3β degradation in these cells [Wu et al., 2008b, 2009]. More studies are required to further confirm if GSK-3β is an endogenous substrate of Smurf2 in chondrocytes.

OTHER TARGETS OF Smurf1

Like other E3 ligases, Smurf1 has multiple substrate proteins whose funciton in bone cells has not been studied. For instance, Smurf targets several proteins that play critical role in regulating cell motility and polarity for poly-ubiquitin-dependent degradation. These include GTPases RhoA and Rap1 [Wang et al., 2003; Zhang et al., 2004a], the human homologue of the Ascidian protein Posterior End Mark-2 [Yamaguchi et al., 2008], and Talin [Huang et al., 2009]. The biologic significance of Smurfs in cell mobility is supported by a recent study describing an unexpected role for Smurfs in controlling planar cell polarity, convergence, and extension movements during embryonic development in Smurf1/Smurf2 double knockout mice. The defects are due to Smurf-induced ubiquitin-mediated degradation of Prickle1, core planar cell polarity protein [Narimatsu et al., 2009]. Prickle1 is a component in the noncanonical Wnt signaling pathway. Mutation of *prickle1* gene has been implicated in human epilepsy [Bassuk et al., 2008]. The role of prickle1 in bone cell function has not been investigated.

In a yeast two-hybrid screening using the WW domains through which Smurf1 targets its substrates as the bait, TNF receptor-associated factor 4 (TRAF4) was identified as a candidate substrate for Smurf1. In vitro experiments demonstrated that the PY motifs of TRAF4 mediates the interaction with the second WW domain of Smurf1 [Kalkan et al., 2009; Li et al., 2009]. *Traf4*$^{-/-}$ mice have defects in neural crest development and neural folding, whereas TRAF4 overexpression boosts signaling and expands the neural crest [Heissmeyer and Rao, 2008]. Smurf1 siRNA elevates TRAF4 levels in human embryonic kidney 293 cells, indicating endogenous TRAF4 is regulated by Smurf1. Overexpression of Smurf1 reduced the protein levels of TRAF4 in a Smurf1 E3 ligase activity-dependent and proteasome-dependent manner, suggesting that TRAF4 acts as a Smurf1-regulated mediator of BMP and Nodal signaling that are essential for neural crest development and neural plate morphogenesis.

Other TRAF family members, including TRAFs 1, 2, 5, and 6, stimulate signal transduction, which is induced by RANKL, TNF, interleukin, or Toll-like receptors. TRAF proteins play an important role in regulation of bone cell function, especially in controlling osteoclastogenesis. Interestingly, a recent study found that Smurf1 promotes ubiquitination and proteasome degradation of all six TRAFs. Consequently, Smurf1 interferes with the functions of TRAFs in NF-κB signaling under basal or stimulated condition. These findings suggest an additional role of Smurf1 in inflammation and immunity through controlling the degradation of TRAFs [Li et al., 2009]. Along with this line, we found that Smurf1 overexpression

reduces TGF-β-activated kinase (TAK1) protein levels in 293T cells, TAK1 protein levels are increased in *Smurf1*$^{-/-}$cells, and Smurf1 increases the ubiquitination of TAK1 protein (Fig. 1). Together, these in vitro results suggest that Smurf1 may negatively regulate NF-κB signaling through a similar mechanism mediated by Itch E3 ligase [Heissmeyer and Rao, 2008; Shembade et al., 2008]. Since *Smurf1*$^{-/-}$ mice do not have obvious defect in immune system and osteoclast function, the role of Smurf1-mediated TAK and TRAF degradation in vivo need to be further investigated. Smurf1-targeted substrate proteins are summarized in Table I.

REGULATION OF Smurfs

Like many other posttranscriptional regulatory mechanism, ubiquitination, and conjugation of ubiquitin-like polypeptides to target proteins are tightly regulated by extracellular signals. In many cases, this regulation is dependent upon protein phosphorylation [Gao and Karin, 2005; Yang et al., 2006]. Phosphorylation of either substrate proteins or E3 ligases is a critical regulatory step for the protein ubiquitination [Kramer et al., 2000; Hayami et al., 2005]. Apart from MAPKs- and GSK-3β-induced phosphorylation of linker region of Smad1 protein, little is known if phosphorylation is required for other Smurf1 targeted substrates. We have reported that phosphorylation of Runx2 affects its degradation, but this phosphorylation-dependent degradation of Runx2 seems independent of Smurf1 [Shen et al., 2006b].

Some C2-WW-HECT E3 ligases are regulated by phosphorylation. JNK induces the serine/threonine phosphorylation of the E3 ligase Itch and enhances its E3 ligase activity by phosphorylation-induced confirmation changes [Gao et al., 2004], while the Src kinase Fyn-induced tyrosine phosphorylation of Itch has a negative effect on modulating Itch-promoted ubiquitination, resulting in reduced its interaction with substrate protein [Yang et al., 2006]. Smurf1, but not Smurf2, interacts with casein kinase-2 interacting protein-1 (CKIP-1), resulting in an increase in its E3 ligase activity. CKIP-1 targets specifically to the linker region between the WW domains of Smurf1, thereby augmenting its affinity to the substrate proteins and promoting ubiquitination of the substrate. *CKIP-1*$^{-/-}$ mice develop age-dependent increased bone mass. Bone marrow stromal cells isolated from *CKIP-1*$^{-/-}$ mice have accelerated osteogenesis and decreased Smurf1 activity [Lu et al., 2008]. Thus, the WW linker domains may represent an important regulatory region for controlling Smurf1 E3 activity.

Ubiquitin E3 ligases often undergo auto-ubiquitination and proteasome degradation along with its substrate. The protein expression level of transfected wild-type Smurf1 is always remarkably lower than its catalytic mutant, indicating that loss of Smurf1 expression is due to its E3 ligase activity (unpublished observation). In human breast cancer MDA231 cells, Smurf2 interacts physically with Smurf1 and induces Smurf1 ubiquitination and degradation, whereas Smurf1 fails to induce degradation of Smurf2. Smurf2 siRNA increases levels of the Smurf1 protein, leading to the enhancement of cell migration in vitro and bone metastasis in vivo [Fukunaga et al., 2008]. Whether this mechanism applies to bone cells is currently unknown.

In addition to be targeted as substrates, Smad proteins often serve as adaptor proteins to mediate other protein degradation. For instance, TGF-β treatment induces Smurf2 binding to Smad2 leading to Smurf2 targeting to the transcriptional co-repressor,

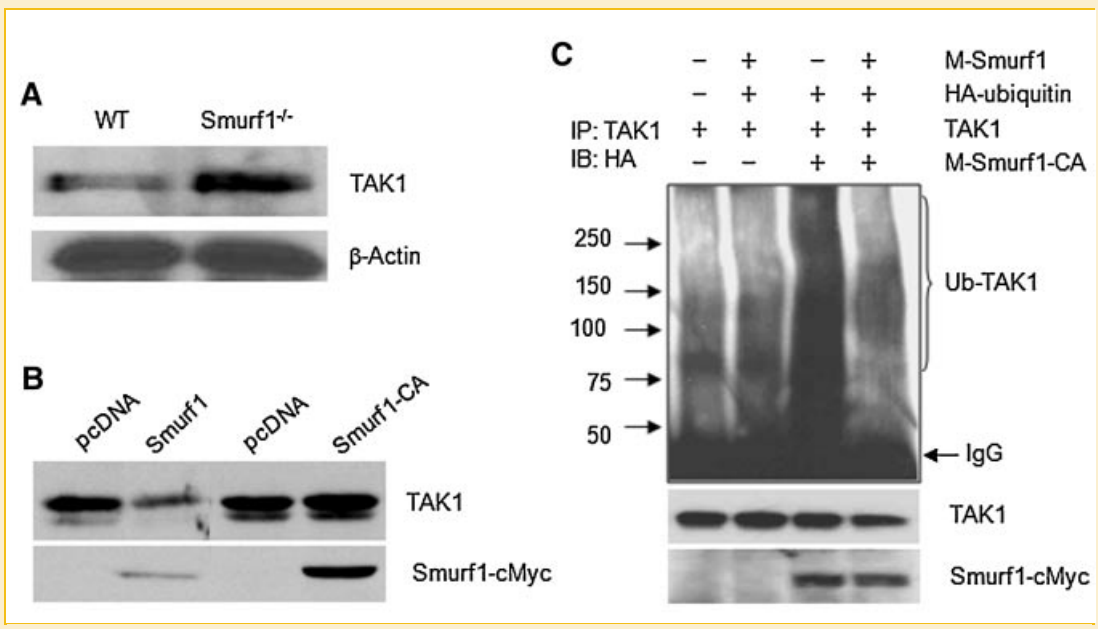

Fig. 1. Smurf1 promotes ubiquitination and proteasome degradation of TAK1 protein. A: TAK1 protein levels were determined in bone marrow stromal cells from wild-type and *Smurf1*$^{-/-}$ mice by Western blot analysis. B: Overexpression of wild-type but not Smurf1 catalytic mutant reduces TAK1 protein levels in 293T cells, which are co-transfected with Smurf1 and TAK1 expression vectors. C: Smurf1 increases ubiquitin conjugated TAK1 in the presence of proteasome inhibitor MG132 in 293T cells.

TABLE I. Smurf1 Substrates

Substrate	Function	Refs.
JunB	AP-1 signaling	Zhao et al. [2010]
Smad1	BMP signaling	Zhao et al. [2003]
Smad4	Co-Smad	Moren et al. [2005]
Smad5	BMP signaling	Ying et al. [2003]
Runx1		Shen et al. [2006a]
Runx2	Osteoblast transcription factor	Zhao et al. [2003]
Runx3		Shen et al. [2006a]
MEKK2	MARK kinase	Yamashita et al. [2005]
TG F-b receptor I,	TGF-β signaling	Ebisawa et al. [2001]
TRAF1	NF-κB and MARK signaling	Li et al. [2009]
TRAF2	NF-κB and MARK signaling	Li et al. [2009]
TRAF3	Negative regular of NF-κB signaling	Li et al. [2009]
TRAF4	Adaptor protein, function is not clear	Kalkan et al. [2009]; Li et al. [2009]
TRAF6	NF-κB and MARK signaling	Li et al. [2009]
TAK1	NF-κB and MARK signaling	Current
RhoA	G-protein	Wang et al. [2003]; Zhang et al. [2004b]
Rap1	G-protein	Wang et al. [2003]; Zhang et al. [2004b]
Ascidian protein	GTPase	Yamaguchi et al. [2008]
Posterior End Mark-2		
Talin	Cytoskeleton	Huang et al. [2009]
Prickle 1	Core planar cell polarity protein	Narimatsu et al. [2009]

SnoN [Bonni et al., 2001; Stroschein et al., 2001]. In this case, Smad2 acts as an adaptor and mediates the interaction between Smurf2 and SnoN leading to SnoN degradation. Thus, assembly of the Smurf2–Smad2 ubiquitin ligase complex induced by TGF-β stimulation plays a positive regulatory role for target protein degradation. Therefore, in addition to serving as signaling proteins in TGF-β Smads also function as adaptors for Smurf E3 ligase complexes that target-specific proteins for degradation in response to TGF-β stimulation. Similarly, Smad6 could also serve as an adaptor to mediate Smurf1-induced Runx2 degradation [Shen et al., 2006b].

Smurf1-induced Smad 1 degradation is blocked by LIM mineralization protein-1 (LMP-1). LMP-1 is an intracellular LIM domain protein that enhances cellular responsiveness to BMP-2 [Sangadala et al., 2006]. LMP-1 stimulates BMP-2-induced differentiation of C2C12 cells into osteoblast lineage cells. LMP-1 promotes osteogenesis by rescuing Smad1 from proteasome degradation through binding to the WW2 domain of Smurf1. A mutant form of LMP-1, that lacks the binding motif to the Smurf1-WW2 domain, has significantly reduced its effect on enhancing BMP-2 activity [Okada et al., 2009].

BONE PHENOTYPE OF Smurf1 GENETICALLY MODIFIED MICE

In 2004, we generated Smurf1 transgenic mice in which a Flag-tagged Smurf1 transgene was under the control of the osteoblast-specific Col1a1 promoter (2.3 kb). Col1a1-Smurf1 transgenic mice have decreased bone volume and a reduced bone formation rates at 3 months of age. BrdU-positive osteoblast-like cells were decreased on the surface of the calvariae of the mice [Zhao et al., 2004].

Smurf1$^{-/-}$ mice were generated in 2005 by Dr. Zhange's group [Yamashita et al., 2005]. *Smurf1*$^{-/-}$ mice are normal at birth, but exhibit an age-dependent increase in bone mass. Bone marrow stromal cells from aged *Smurf1*$^{-/-}$ mice have increased growth and expression of ALP, Smad1, and Runx2 mRNA when they are cultured in an osteoblast-inducing medium. Interestingly, the cells from these mice have an accumulation of phosphorylated MEKK2 and activation of the downstream JNK signaling cascade. Smurf1 overexpression signficiantly increased MEKK2 ubiquitination, indicating that MEKK2 is another important target for Smurf1 in osteoblasts [Viswanathan and Sylvester, 2008]. A striking feature of *Smurf1*$^{-/-}$ mice is their age-related high bone mass phenotype, raising the hypothesis that osteoblasts from aging subjects may have higher expression of Smurf1. We examined Smurf1 mRNA expression in bone marrow stromal cells or bone samples from young (8-week-old) and old (1-year-old) wild-type animals and did not find difference (unpublisehd observation). It will be interesting to determine if ageing increases the Smurf1 enzyme activity in osteblasts.

Patients with chronic inflammatory disorders, such as Crohn's disease, lupus erythematosus, and rheumatoid arthritis (RA), often have severe systemic bone loss, due to increased bone resorption, but they also have impaired osteoblastic bone formation, the basis of which is less well understood [Guo et al., 2008]. We reported that Smurf1 is partially responsible for systemic bone loss in TNF transgenic mice, a model of chronic inflammatory arthritis, by promoting ubiquitination and proteasomal degradation of Smad1 and Runx2 proteins [Kaneki et al., 2006; Guo et al., 2008]. We found that deletion of Smurf1 prevented the TNF-induced reduction in Smad1/5 and Runx2 protein levels in osteoblasts and partially rescued systemic bone loss, suggesting that Smurf1 may play an important role in mediating the degradation of Smad1 and Runx2 proteins under the activated condition. These findings provide the first experimental evidence for a significant role for ubiquitin ligase-mediated protein degradation in mature osteoblasts during inflammatory bone loss.

Osteoblasts are derived from MSCs. Bone marrow MSCs derived from patients or animals with RA have decreased osteoblast differentiation [Kastrinaki et al., 2008; Jian et al., 2009], indicating that the micro-environment of bone marrow inflammation may directly affect the fate of MSCs and lead to the inhibition of

osteoblast differentiation. However, it is not known if this mechanism also applies to osteoblast inhibition of MSCs in RA. To search for the C2-WW-HECT E3 member responsible for the reduced osteogenesis of MSCs during inflammatory disorders, we examined expression levels of E3 ligases in MSCs from TNF-Tg and wild-type mice and found that the levels of WWP1 but not Smurf1 are elevated. WWP1, a member of C2-WW-HECT E3 ligase, is identified first as a novel protein based on its WW modules exhibiting high affinity towards the PY motif [Kasanov et al., 2001; Verdecia et al., 2003]. Although WWP1 has been shown to function as an E3 ubiquitin ligase, only a few substrates have been identified, such as p53, KLFs, and Smad7 [Zhang et al., 2004a; Chen et al., 2005; Moren et al., 2005]. WWP1 is essential for embryonic development in *C. elegans* [Huang et al., 2000], but *Wwp1*$^{-/-}$ mice do not have obvious abnormalities and they are survived into adulthood. The association between WWP1 and bone was first suggested in Schnurri-3 (*Shn3*)$^{-/-}$ mice by Jones et al. [2006, 2007]. *Shn3*$^{-/-}$ mice develop severe osteosclerosis with dramatically increased bone mass. Shn3 enhances Runx2 protein ubiquitination and degradation through recruitment of WWP1 to Runx2 in vitro, leading to a hypothesis that WWP1 mediates osteosclerotic phenotype of *Shn3*$^{-/-}$ mice [Jones et al., 2006, 2007]. However, whether or not WWP1 plays a critical role in physiologic and pathologic bone loss in vivo is currently unknown. Our preliminary findings of increased bone volume in adult *Wwp1*$^{-/-}$ mice suggest that WWP1 is an important regulator of postnatal bone volume (unpublished data).

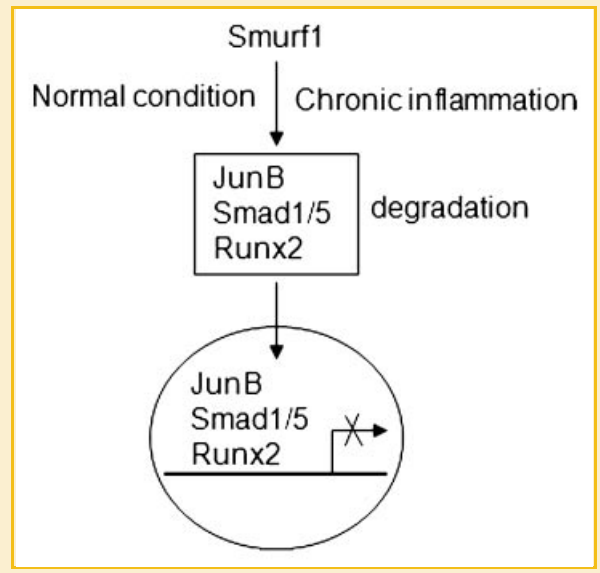

Fig. 2. Smurf1 regulates osteoblast function. A: In osteoblasts and precursors, under normal condition, Smurf1 negatively regulates osteoblast differentiation by limiting the accumulation of BMP Smad, Runx2, and JunB protein in the cytoplasm through proteasome degradation. B: In chronic inflammation where systemic cytokine levels are elevated, Smurf1-medaited protein degradation is increased due to increased expression or activity of Smurf1 and modified substrate sensitivity for ubiquitination, leading to reduced levels of these positive osteoblast factors in the cytoplasm and reduced transcription of osteoblast-specific genes.

PERSPECTIVES AND SUMMARY

Since the identification of ubiquitin E3 ligase Smurf1 functions as a negative regulator of osteoblast activity in 2003 [Zhao et al., 2003], significant progresses have been made to understand the role of Smurf and proteasomal degradation in bone biology: (1) both the BMP (Smad-1 and -5, Runx2) and MARK (MEKK2 and JunB) signaling proteins are targeted by Smurf1 for ubiquitination and proteasome degradation in osteoblast precursor cells; and (2) mice deficient in Smurf1 have increased bone mass along with aging and they do not have osteoclast phenotypes, and Smurf1 depletion prevents animals from bone loss in inflammatory arthritis. Based on these findings, we propose a model to outline the action of Smurf1 in wild-type osteoblasts and in cells exposed to inflammatory cytokines (Fig. 2). Under physiologic conditions, Smurf1 inhibits osteoblast differentiation by limiting the accumulation of BMP Smad, Runx2, and JunB proteins through proteasome degradation. In contrast, under the chronic inflammatory conditions where systemic cytokine levels are elevated, Smurf1-medaited protein degradation is increased due to increased expression or activity of Smurf1 and modified substrate sensitivity for ubiquitination.

However compared to rapid progress made in research of other osteoblast regulators such as Wnt/β-catenin pathway, our understanding of Smurf1 or other E3 ligases in bone cell regulation is rather limited. (1) We still do not know how Smurf1-mediated protein degradation is initiated in bone cells. For instance, we do not know if Smurf1 is phosphorylated in response to growth factor signaling or other stimuli. (2) Although Smurf1 is responsible for the degradation of key osteoblast signaling proteins or transcription factors, we do not know why *Smurf1*$^{-/-}$ mice have relatively minor bone phenotype. Is this due to redundant roles contributed by other E3 ligases? (3) Does Smurf1 promote K63 ubiquitination? (4) Smurf1 has several newly identified substrate proteins. What is their function in bone cells? (5) Is Smurf1 participated in bone disorders in humans? (6) Does Smurf1 affect mature osteoblast function such as matrix synthesis? More importantly, (7) how can we distinguish osteoblast targets from other targets of Smurf1 in order to develop osteoblast-based therapy? It will be important to answer these questions because it is now recognized that osteoblasts and their precursor cells have important unanticipated functions regulating themselves and other cells, such as osteoclasts, chondrocytes, and hematopoietic stem cells during development and in pathologic conditions. Smurf1 may be a new therapeutic target for the pathologic conditions.

ACKNOWLEDGMENTS

This publication was made possible by grant numbers R01 AR48697 and R21 AR53586 (to L.X.) and by grant numbers R01 AR051189, R01 AR054465, R01 AR055915, and K02 AR052411 (to D.C.) from NIH/NIAMS. This work was also supported by grant number N08G-070 from New York State Department of Health and the Empire State Stem Cell Board (to D.C.). We thank Ms. Kimberly A. Napoli for editing of this manuscript.

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