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COMMENTARIES

Ubiquitin Ligase: What Is Your True Substrate?

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Commentary on: Yamashita M, Ying SX, Zhang GM, Li C, Cheng SY, Deng CX, Zhang YE. Ubiquitin ligase Smurf1 controls osteoblast activity and bone homeostasis by targeting MEKK2 for degradation. *Cell* 2005;121(1):101-13.

Ubiquitination and control of protein stability have emerged as a significant biological mechanism for modulation of receptor downstream signaling and fine-tuning of transcriptional responses (1-3). A recent study (4) emphasized the relevance of this mode of control in osteoblasts and highlighted the functional crosstalk between key effectors acting downstream from membrane receptors. As in a whodunit, the real culprit was not initially obvious.

Yamashita *et al*. (4) targeted the ubiquitin ligase Smad ubiquitin regulatory factor 1 (Smurf1) by homologous recombination in embryonic stem cells to engineer mice deficient in Smurf1. Previous studies have shown that Smurf1 and its related family member, Smurf2, modulate signaling downstream from bone morphogenetic protein (BMP) and transforming growth factor β (TGFβ) receptors by ubiquitination of Smad1 and Smad5, thus targeting these transcriptional regulators for proteasome-mediated degradation (5-7). Additional data have shown that TGFβ or BMP type I receptors could themselves be targets of Smurf1/2 activity through ligand-activated binding of inhibitory Smad6 or Smad7 as intermediates (8-10). These mechanisms are relevant to bone cells, as ectopic expression of Smurf1 in pluripotent myoblasts or osteoblast progenitors prevents BMP-induced osteoblast differentiation (11;12). Moreover, expression of a Smurf1 transgene in osteoblasts inhibits bone formation *in vivo* (12). In both cases, the stability of effector Smads was reduced (11;12). Thus, a safe wager would have been that targeted deletion of Smurf1 would impact BMP signaling in mice by increasing Smad1 and/or Smad5 expression levels. A gambling mind might also have placed money on a mechanism involving the stability of the BMP type I or TGFβ receptors themselves, or even the recently identified additional Smurf1 substrates, such as the osteoblast transcription factor Runx2 (13) or the small GTPase RhoA (14). These bets would not have returned any gains.

The mutation engineered by Yamashita and colleagues (4) deleted two functional protein domains within the Smurf1 sequence, thus creating a true null allele that prevented Smurf1 activity in all tissues. Mutant mice were born with the expected Mendelian ratio, developed without obvious abnormalities, enjoyed a normal life span, and furthermore, remained fertile. Histological examination, however, revealed a bone phenotype: an age-dependent increase in bone mass that affected cortical and trabecular bone at the diaphysis. Blood biochemistry, resorption parameters, and the number of osteoblasts were unaffected, whereas the expression of osteoblast differentiation markers and bone-forming rates were increased (4). Disruption of Smurf1 clearly increased osteoblast activity, thus indicating that Smurf1 acts normally as a negative regulator of osteoblast function.

As could be predicted, BMP-mediated signaling was increased in Smurf1-deficient osteoblasts, although TGFβ-dependent responses were unaffected. This was assessed using transient transfection assays with relevant reporter constructs and by measuring alkaline phosphatase activity in BMP- or TGFβ-treated primary calvarial cultures. The surprise came upon evaluation of canonical Smad-mediated responses: the loss of Smurf1 had no impact on TFGβ-induced Smad2 phosphorylation or the steady-state levels of endogenous Smad2 and Smad3. BMP2-induced Smad1/5 phosphorylation and the steady-state levels of the endogenous total Smad1/5 and type I BMP receptors (BMPRIA and BMPRIB) were similarly unaffected. Thus, even though Smurf1-deficient osteoblasts showed increased BMP responsivity, Smad-dependent signaling was not affected *per se*. Runx2 and RhoA stability were also not changed in the absence of Smurf1. What then is the mechanism responsible for the observed phenotype?

TGFβ and BMP also signal via Smad-independent pathways that involve mitogen-activated protein kinases (MAPKs), such as JNK and p38 MAPK (15;16). Yamashita *et al*. (4) measured an increase in AP-1-mediated transcription, an accumulation of activated phospho-JNK, and an increase in JunB in Smurf1-deficient osteoblasts. Blocking JNK activity with specific inhibitors suppressed the increased extracellular matrix production and alkaline phosphatase activity, as well as the augmented sensitivity to BMP treatment, observed in Smurf1-mutant osteoblasts. Because JNK, c-Jun, and JunB do not interact with Smurf1 (4), the authors looked at kinases acting upstream of JNK and observed an accumulation of the phosphorylated form of the mitogen-activated protein kinase MEK kinase 2 (MEKK2) in osteoblasts that lacked Smurf1. It was demonstrated that Smurf1 physically interacts with MEKK2 and controls the ubiquitination-dependent turnover of MEKK2 (4).

These exciting results show that bone is a physiologically relevant target tissue of Smurf1 action and identify MEKK2 as a novel *in vivo* substrate of Smurf1. The results reveal that Smurf1 normally acts to negatively regulate osteoblast activity and dampen their responses to BMP through hitherto unrecognized mechanisms. The data raise a few interesting questions and have several implications.

Although the knockout phenotype was a mirror image of the bone-specific transgenic phenotype (12), the molecular mechanisms turned out to be quite different. This serves as a strong reminder that the obvious molecular targets cannot always explain an observed phenotype and that *in vitro* data cannot always be extrapolated to the *in vivo* context.

It is noteworthy that global inactivation of Smurf1 resulted in a postnatal bone-specific phenotype, despite the roles of Smurf1 during development in other model systems (7). There was a compensatory increase in Smurf2 expression measured in Smurf1-deficient mice (4), and this increase probably accounted for the normal embryonic development of Smurf1$^{-/-}$ mice. Although the authors mentioned preliminary data revealing that Smurf1 and Smurf2 compound mutants die prior to embryonic day 9.5, it would be interesting to determine if Smurf1 can play a functionally redundant role in Smurf2-deficient tissues or whether the physiological role of Smurf1 is tightly restricted to bone. At any rate, the late-onset bone-specific phenotype of Smurf1$^{-/-}$ mice suggests that Smurf1 could represent an interesting target for pharmacological intervention in the treatment of age-related bone loss.

The results of Yamashita *et al*. (4) emphasize the biological importance of the Smad-independent, MAPK-driven pathways operating downstream from BMP receptors. Crosstalk between the two pathways likely mediates cooperative transcriptional responses by Smads and MAPK downstream substrates, such as AP-1 family members and activating transcription factors (ATFs). The key role of these transcription factors in osteoblast biology has been confirmed in knockout and transgenic studies: mice that lack Fra-1 have decreased bone formation rates (17); JunB targeted deletion affects osteoblast

proliferation and differentiation (18); overexpression of Fra-1 or ΔFosB induces osteosclerosis in transgenic mice (19;20); and ablation of ATF4 affects osteoblast differentiation and function (21). In the case of AP-1 family members, the upstream signals controlling the activity of the transcription factors remain unclear. The study of Yamashita *et al*. (4) pointed to BMP receptor-mediated signaling as a physiological input culminating in Jun-mediated transcriptional responses in bone. The authors suggested that Smurf1's normal role is to prevent the Smad-independent pathway from overactivating matrix production and bone formation by osteoblasts after they have received a BMP signal.

The question remains as to which transcription factor dimers mediate the MEKK2-JNK signal downstream from BMP in osteoblasts *in vivo*. Yamashita *et al*. (4) observed an increase in steady-state JunB levels in Smurf1-deficient osteoblasts. JunB-deficient osteoblasts have reduced proliferation, as well as a differentiation defect (18), and it is thus possible that JunB homodimers alone mediate the increased MEKK2-JNK signal in Smurf1-null osteoblasts. The documented propensity of Jun family members to heterodimerize with Fos or ATF family members (22), however, raises the possibility that a Fos/Jun or ATF/Jun dimer could be involved. Similarly, Jun family members can interact with Runx2 (23;24) and Smads (25;26), and it remains a formal possibility that Jun-Runx2 or Jun-Smad dimers could mediate the observed response. Chromatin immunoprecipitation assays at salient target gene promoters could identify the functional transcriptional effectors in Smurf1-ablated osteoblasts. Thus, once more, a novel observation emphasizes that the transcriptional control of osteoblast activity is a tightly regulated phenomenon and confirms that protein-protein interactions play a critical role in the precise regulation of gene expression in bone-forming cells.

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