UCSD MOLECULE PAGES

doi:10.6072/H0.MP.A002436.01
Volume 1, Issue 1, 2012

Review Article Open Access

Smurf1

Gerald H Thomsen[1]

The Smad Ubiquitination Regulatory Factor-1, or Smurf1, is an E3 ubiquitin ligase that catalyzes mono- or polyubiquitylation of protein targets which primarily function in TGFβ signaling pathways, but also include a growing list of substrates encompassing other molecular pathways and cellular processes distinct from TGFβ pathways. Smurf1 and a close homolog, Smurf2, are E3 ubiquitin ligases that belong to a small family of proteins distinguished by the presence of a catalytic, C-terminal region that is known as the HECT domain, for Homologous to E6AP C-Terminus. This region of the Smurfs and other HECT E3s form a covalent intermediate with ubiquitin (Ub) and subsequently transfer the Ub moiety to a substrate protein whose selection and targeting are governed by either direct interaction with Smurf1, or in concert with an adaptor protein(s) that bridges Smurf1 and the substrate. Smurf1 can operate throughout the cell and has a wide variety of protein targets, reflecting the diversity of biological processes it regulates, which spans cell proliferation, cell polarity, adhesion, apoptosis, differentiation, stem cell activity, embryonic development, pattern formation, organogenesis and organism physiology. Smurf1 is expressed in a wide range of cell types in developing embryos through adults, and Smurf1 gene orthologs are found in all lineages of the animal kingdom, from sponges through chordates. Defects in Smurf1 gene structure, expression or regulation are implicated in human diseases and birth defects, and Smurf1 is being actively pursued as a therapeutic target.

KEYWORDS
E3 ubiquitin ligase SMURF1; Smad ubiquitination regulatory factor 1; Smurf1

IDENTIFIERS
Molecule Page ID:A002436, Species:Mouse, NCBI Gene ID: 75788, Protein Accession:NP_083714.3, Gene Symbol:Smurf1

PROTEIN FUNCTION
Smurf1 is an E3 ubiquitin ligase possessing intrinsic catalytic activity that covalently modifies lysine residues of its substrate proteins with mono- or poly-Ubiquitin (Ub), and these modifications either alter the affected protein activity or trigger destruction by the 26S Proteasome, respectively. The majority of Smurf1 substrates are components of TGFβ signal transduction pathways, however a wider network of Smurf1 targets have emerged that operate in a variety of signaling pathways and molecular processes, occurring at locations and compartments throughout the cell. Consequently, Smurf1 affects a cornucopia of cellular and developmental functions encompassing cell differentiation, cell shape and polarity, cell adhesion and migration, autophagy, embryonic pattern formation, morphohogenesis, organogenesis and organism physiology and fertility. Not surprisingly, diseases and developmental defects have been attributed to dysfunctions in Smurf1.

Smurf1 and a close relative, Smurf2, are members of the HECT family of E3 ubiquitin ligases that are characterized by a signature C-terminal domain that contains a catalytic active site responsible for conjugating Ub to target proteins (Bernassola *et al*., 2008). This HECT domain (an acronym for "Homologous to E6AP C-Terminus) harbors a key cysteine residue that forms a covalent thiolester intermediate with ubiquitin donated by an associated E2 ubiquitin-conjugating enzyme (UbcH5 or 7). Ub is then transferred from the HECT to a lysine residue on a substrate protein or to an Ub moiety that is being elongated into a polyUb chain. Smurfs, like many HECT E3s, also contain a C2 lipid/Ca2+ binding domain at their N-terminus, and several WW domains, located between the C2 and the HECT domain, that mediate many substrate interactions by binding to proline/tyrosine-rich PY motifs (consensus PPXY). Smurf1 emerged early during animal evolution, with Smurf1 orthologs present in the genomes of cnidarians (e.g. Hydra) through man (Marin, 2010).

The most well studied targets of Smurf1 are components of TGFβ signal transduction pathways, (Inoue and Imamura, 2008). Historically Smurf1 was discovered as a binding partner of Xenopus Smad1 that polyubiquitylates and triggers proteasomal destruction of Smad1 and Smad5, resulting in bone morphogenetic protein (BMP) signaling loss and abnormal Xenopus embryonic development (Zhu *et al*., 1999). Smurf1 was quickly shown to function more broadly in TGFβ signal transduction as additional Smurf1 substrates were identified, including Type I and II BMP and TGF-ß receptors, inhibitory Smads (Smad6, Smad7) and downstream transcription factors (Lönn *et al*., 2009). These studies also revealed that Smurf1 can employ adaptor proteins to target substrates, as illustrated by the ability of Smad6 and Smad7 to stably associate with and recruit Smurf1 to TGFβ and BMP receptors (Ebisawa *et al*., 2001; Suzuki *et al*., 2002; Murakami *et al*., 2003). This ability was first demonstrated by a Smurf1 homolog, Smurf2, which uses Smad7 to target TGFβ receptors (Kavsak *et al*., 2000). Receptor regulation by Smurf1 can also be affected by FKBP12 (FKBP1a), which interacts with both Smad7 and the TGFβ/Activin Type I receptor ALK4 (ActR1) (Yamaguchi *et al*., 2006). This interaction marks FKBP12 and the receptor for proteasomal degradation and suppresses spurious receptor activity. Smad7 can be ubiquitylated by Smurf1 during the process of receptor downregulation (Ebisawa *et al*., 2001) and other contexts (Grönroos *et al*., 2002; Yan *et al*., 2009;Chong *et al*., 2010). Arkadia, a ring finger adaptor-type E3, can indirectly oppose the effects of Smurf1 by regulating the levels of Smad7 (Choi *et al*., 2007).

Smurf1 can operate at the transcriptional level using inhibitory Smads (known as I-Smads, which includes Smad6 and Smad7)

[1]Biochemistry and Cell biology, Stony Brook University (State University of NY), 11794, US.
Correspondence should be addressed to Gerald H Thomsen: gerald.h.thomsen@sunysb.edu
Published online: 24 Jan 2012 | doi:10.6072/H0.MP.A002436.01

as adaptors. Smurf1 can ubiquitylate and downregulate the transcription factors Runx2/3 and Tbx6 using Smad6 as an adaptor (Shen *et al*., 2006; Chen *et al*., 2009a). Similarly, nuclear Smad4 can be targeted by Smurf1 (and Smurf2) via Smad6 or Smad7 acting as adaptors (Moren *et al*., 2005). Although it can act as an adaptor protein, Smad7 in the nucleus also can be ubiquitylated by Smurf1, and this can be modulated by Smad7 acetylation and deacetylation (Grönroos *et al*., 2002; Kume *et al*., 2007). Smad7 deacetylation by SIRT1 promotes Smurf1-dependent ubiquitylation and destruction of Smad7, but ubiquitin modification can be counteracted by p300-dependent acetylation of Smad7, which stabilizes it.

Beyond its historical roots in TGFβ signaling, the actions of Smurf1 continue to expand into an impressive variety of other signaling pathways (Rotin and Kumar, 2009) that include non-canonical Wnt planar cell polarity (PCP) (Narimatsu *et al*., 2009), small GTPases (Wang *et al*., 2003; Zhang *et al*., 2004; Yamaguchi *et al*., 2008), Jun Kinase/JunB (Zhao *et al*., 2010), MEKK2/Jun Kinase (Yamashita *et al*., 2005; Zhao *et al*., 2010), TNF (Guo *et al*., 2008; Kaneki *et al*., 2006), TRAF (Li *et al*., 2010; Kalkan *et al*., 2009), NF-kB (Li *et al*., 2010) and hypoxia (Murakami *et al*., 2010; Liu *et al*., 2007) signaling pathways. Smurf1 also regulates PAR6/RhoA polarity complexes to affect cell morphology, adhesion and migration (Wang *et al*., 2003; Ozdamar *et al*., 2005; Townsend *et al*., 2008, 2011; Vohra and Heuckeroth, 2007; Cheng *et al*., 2011). Smurf1 can target the endoplasmic reticulum to regulate Wolfram syndrome protein (WFS1; Guo *et al*., 2011). Smurf1 is also essential for viral autophagy and mitophagy (Orvedhal *et al*., 2011). Smurf1 has a diverse and growing variety of protein targets beyond TGFβ signal transduction pathways, and Smurf1 is emerging as a governor of a wide network of biological processes.

Abnormalities in Smurf1 expression or regulation are directly implicated in diseases that include cancer and hypertension, and congenital malformations such as dwarfism and abnormal bone development (Chen and Matesic, 2007; Scheffner and Staub, 2007; Bernassola *et al*., 2008; Xing *et al*., 2010) and potentially others (Guo *et al*., 2011).

Of note, Smurf1 and Smurf2 share some biochemical and biological activities (refer to the Smurf2 molecule page).

REGULATION OF ACTIVITY

Most proteins that interact with Smurf1 become ubiquitylated, which suggests that the catalytic activity of Smurf1 is constitutive, but like other HECT E3 ligases, Smurf1 regulators have begun to be identified. These either directly alter Smurf1 substrate affinity and specificity, or function indirectly as adaptor proteins that affect Smurf1-substrate interactions (Kee and Huibregtse, 2007).

The ability of Smurf1 to target substrates which require bridging adaptors, such as Smad6 or Smad7, can be dramatically altered by the levels of such adaptors, as discussed above for receptor or nuclear protein targets, or by proteins that interact with such adaptors. An example of the latter is provided by TGF-β-stimulated clone 22 (TSC-22), a protein that disrupts the ability of Smad7 to interact with the Type I receptor, TßR1 (Yan *et al*., 2011). Therefore, high levels of TSC-22 inhibit the ability of Smurf1 to ubiquitylate and downregulate TGFß receptors. As its name implies, TSC-22 is upregulated by TGFß signals, so its induction provides a positive feedback loop that enhances TGFß signaling, and this can contribute to myocardial differentiation and fibrosis-associated gene expression (Yan *et al*., 2011). Targeting of Smad7-dependent Smurf1 substrates (including TGFß receptors) also can be suppressed by the RING ubiquitin ligase, Arkadia, that targets Smad7 for proteasomal degradation, consequently relieving Smad7-dependent repression of TGFß responsiveness (Koinuma *et al*., 2003), which has been shown to contribute to myocardial fibrosis (He *et al*., 2011).

The interaction of Smurf1 with substrates that contain PY motifs can be affected by CKIP-1, originally identified as a binding protein for Casein Kinase 2. CKIP-1 can interact with the polypeptide region spanning the two WW domains of Smurf1, and this interaction increases the affinity of Smurf1 for substrates that it binds via WW-PY interactions (such as Smad1/5). Binding of CKIP-1 to Smurf1 increases the rate of Smurf1 substrate polyubiquitylation and proteasomal destruction (Lu *et al*., 2008). CKIP interacts with Smurf1 but not Smurf2, reflecting sequence differences between the WW linkers of these two Smurfs. Differences in these linker domains between Smurf1 and Smurf2 contribute to the substrate specificity of the two Smurfs (Chong *et al*., 2010).

The C2 domain located at the end of Smurf1 also affects target selection. The Smurf1 C2 domain is required for Smurf1 to target RhoA, but not Smad5 and Runx2. This specificity is a consequence of the affinity of the C2 domain for phospholipids, which localizes Smurf1 to the plasma membrane in close proximity to RhoA (Lu *et al*., 2011). The Smurf1 C2 domain also interacts directly with RhoA (Tian *et al*., 2011).

Substrate selection by Smurf1 can be regulated by Protein Kinase A (PKA)-dependent phosphorylation, which reduces the affinity of Smurf1 for Par6 while simultaneously increasing Smurf1 ubiquitylation of RhoA. In hippocampal neurons, phosphorylation of Smurf1 by PKA increased axon growth and branching (Cheng *et al*., 2011; Stiess and Bradke, 2011). Targeting of RhoA can also be regulated by synaptopodin, which competitively inhibits the ability of Smurf1 to interact with RhoA, and this consequently promotes stress fiber formation and reduces actin-dependent protrusive activity as well as cell motility (Asanuma *et al*., 2006).

Smurf1 protein concentration can be affected by various ubiquitin ligases, including Smurf1 itself (see Regulation of Concentration). Whether ubiquitylation of Smurf1 alters its catalytic activity, substrate affinity or other aspects of Smurf1 function have not been determined.

Based on findings with Smurf2, the activity of Smurf1 has the potential to be regulated by alterations in the affinity of Smurf1 for its E2 Ubiquitin Conjugating Enzymes (Ogunjimi *et al*., 2005). In the absence of Smad7, the Smurf2 HECT domain interacts weakly with the E2, but the binding of Smad7 to Smurf2 supplies an additional E2 docking surface that stabilizes E2 binding and elevates the rate and extent of substrate ubiquitylation.

Given the important functions and interactions attributed to Smurf1, there is great interest in developing chemical modulators of Smurf1 activity. Small molecule screens have identified two related compounds (SVAK-3 and 12) that bind to the second WW domain of Smurf1 and consequently boost BMP signaling (Kato *et al*., 2011). The exact targets affected by the compounds were not determined, but since the WW2

domain binds Smads 1, 5, and 6 at PY motifs (Sangadala *et a*l., 2006; Sangadala *et a*l., 2007) it is likely that the SVAKs block these interactions.

INTERACTIONS

More than 300 proteins have been found to interact with vertebrate orthologs of Smurf1. These encompass a wide range of biochemical and biological functions, and implicate Smurf1 as a regulator of a wide array of cellular activities. Protein-protein interaction screens, protein microarrays and intuitive testing have netted this multitude of Smurf1 partners (e.g. Zhao *et al*., 2003; Colland *et al*., 2004; Barios-Rodiles *et al*., 2005; Kalkan *et al*., 2009, Andrews *et al*., 2010), yet only a small fraction have been verified as actual substrates, adaptors or regulators *in vivo*. This section focuses on validated Smurf targets, while most other Smurf1 interactors are collated in the automated Interactions link on this Molecule Page.

Most interactions between wild-type Smurf1 and its substrates are transient, so a catalytically-inactivated Smurf1, harboring a Cys to Ala mutation at residue 723 in mouse (699 in human), has been an indispensable reagent for identifying proteins that can be bound by Smurf1. This cysteine is located very close to the C-terminus of Smurf1, in the HECT domain, where it forms the Ub-thiolester intermediate prior to transfer of Ub to a lysine side chain on a target protein or the elongating polyubiquitin chain. This mutant, Smurf1C723A (or SmurfCA), captures otherwise fleeting substrate interactions, allowing easy detection of candidates by co-IP.

As already previewed above, quite a few Smurf1 partners operate in canonical TGF-β/activin/nodal and BMP signaling pathways, with a bias toward the BMP branch. Most other interactors represent a wide and surprising variety of other signaling pathways and cellular functions. However, the vast majority of these have yet to be vetted by deeper biochemical and biological assays. The molecular and biological consequences of their interactions remain to be determined, but verified interacting proteins are summarized below. Most are substrates, but a handful of others function as adaptors or modulators of Smurf1 activity.

Smurf1 ligands that are substrates

Dozens of proteins have been verified to be substrates that interact with Smurf1 and consequently become polyubiquitylated and destroyed by the 26S Proteasome. Smads 1, 5 and 7 were the first to be discovered (Zhu *et al*., 1999; Ebisawa *et al*., 2001; Grönroos *et al*., 2001; Shen *et al*., 2006; Ying *et al*., 2003), and these interactions did not require posttranslational modifications, such as C-terminal phosphorylation by active BMP receptors. However, more recently the interaction of Smurf1 with Smad1 has been shown to be greatly enhanced by phosphorylation of residues in the Smad1 linker domain, mediated by MAP Kinases, cyclin-dependent kinases and GSK3 (Fuentealba *et al*., 2007; Sapkota *et al*., 2007; Alarcon *et al*., 2009; Aragon *et al*., 2011).

Smurf1 can target most Type I receptors (ALKs 2-6) but not Type II receptors in the TGFß superfamily (Ebisawa *et al*., 2001; Murakami *et al*., 2003; Yamaguchi *et al*., 2006), and these interactions require either Smad6 or Smad7 to bridge Smurf1 to the receptor as noted above. Other validated substrates inside or outside of TGFß pathways include the chromatin adaptor Ing2 (Barrios Rodiles *et al*., 2005; Nie *et al*., 2010), the transcription factors JunB/AP-1 (Zhao *et al*., 2010), KLF2 (Xie *et al*., 2011), LMP-1 (Fei *et al*., 2007), Runx 2 and 3 (Bellido *et al*., 2003; Zhao *et al*., 2003; Jin *et al*., 2004) and Tbx6 (Chen *et al*., 2009), the MAP Kinase MEKK2/Map3K2 (Yamashita *et al*., 2005), the small GTPase RhoA (Wang *et al*., 2003, 2006; Ozdamar *et al*.,2005) a GEF for cdc42 named PEM-2 (Yamaguchi *et al*., 2008), the polarity protein PAR6 (Ozdamar *et al*., 2005; Cheng *et al*., 2011), and the ER-specific Wolfram Syndrome Protein 1 (WFS1; Guo *et al*., 2011). Smurf1 can also target TRAFS 1-6 (Kalkan *et al*., 2009; Li *et al*., 2010) and the adaptor protein Myd88 (Lee *et al*., 2011), which affect Nf-κB and associated inflammatory pathway signaling by Tumor Necrosis Factor (TNFR) and Toll-like (TLR) receptors. TRAF4 is implicated in BMP and nodal signaling as well (Kalkan *et al*., 2009).

A protein microarray screen of more than 8000 proteins identified about 90 additional Smurf1 substrates that could be ubiquitylated by a mixture of Smurf1 and the E2 enzymes, UbcH5 or UbcH7. Only a few of these candidates were validated further by alternative *in vitro* reactions or *in vivo* tests (Andrews *et al*., 2010), but the ones that emerged include some very interesting prospects: Protein Kinase C-iota, NEK2 (Never In Mitosis Gene A-related kinase 2), HIP2 (huntingtin-interacting protein 2), JIK (an STE20-like kinase), TPM4 (Tropomysin 4), ATAXIN3 and TOM1 (Target of Myb-1). The latter two were further shown to be polyubiquitylated in cells. The biological consequences of Smurf1 interactions with these newfound substrates are presently unknown. The E2 enzyme that associates with an E3 Ub ligase can affect substrate specificity, so it is worth noting that in this protein microarray screen, Smurf1 combined with Ubch5b captured a larger set of proteins (86) than Smurf1 together with Ubch7 (33), but 90% of the Ubch7/Smurf1 targets were also substrates for Ubch5/Smurf1 (Andrews *et al*., 2010).

Smurf1 ligands that are not substrates

A handful of proteins interact with, but are not ubiquitylated by Smurf1. Instead, they alter Smurf1 activity or facilitate Smurf1-substrates interaction by functioning as adaptors that assemble multiple protein complexes containing Smurf1. These include CCM2, CKIP-1, Par6, Protein Kinase C-zeta (PKC-ζ), Smad6, Smurf2, Trb3 and RNF11.

Cerebral cavernous malformation 2 (CCM2) promotes Smurf1 dependent ubiquitiylation and degradation of RhoA in neurons by mobilizing Smurf1 to the plasma membrane and thus facilitating Smurf1 interactions with RhoA at the peripheral actin cytoskeleton (Crose *et al*., 2009). Casein Kinase Interacting Protein-1 (CKIP-1, or PLEKHO1) can bind Smurf1 and stimulate ubiquitylation of Smad proteins by increasing the affinity or accessibility of Smurf1 WW domains for Smad1/5 PY motifs (Lu *et al*., 2008). Smurf1 can interact with PKC-ζ and selectively binds to Par6 that has been phosphorylated by TGFß Type I receptors, and these interactions ubiquitylate and degrade RhoA (Wang *et al*., 2003, Ozdamar *et al*., 2005; Barrios-Rodiles *et al*., 2005; Townsend *et al*., 2008, 2010). This consequently affects cell shape, polarity and motility.

Smurf1 can also interact with other ubiquitin ligases, and interestingly, Smurf1 directly interacts with Smurf2, its closest homolog in the HECT family. This interaction results in polyubiquitylation and degradation of Smurf1 (Fukunaga *et al*., 2008). Tribbles-like protein 3 (Trb3) interacts with and ubiquitylates Smurf1, in a mechanism that requires BMP receptor activation (Chan *et al*., 2007). In particular, Trb3

associates with the C-terminus of inactive BMP receptors and gets released upon receptor activation, allowing Trb3 to bind to and ubiquitylate Smurf1. The small molecule phenamil enhances this action (Park *et al*., 2009). As noted above, Smad6 can interact Smurf1 (and this facilitates targeting of BMP receptors), but in contrast to Smad7, there is no evidence that Smad6 is a substrate for ubiquitylation by Smurf1. Lastly, a ring finger protein, RNF11, can interact with Smurf1 (Colland *et al*., 2004), and while the consequences of this interaction are not known, RNF11 has been shown to function as an adaptor protein for Smurf2 to target AMSH (associated molecule with the SH3 domain of STAM), a protein that interacts with and antagonizes Smads 6 and 7 (Li and Seth, 2004). Smurf1 has also been shown to interact with MDM2 and MDMX, resulting in increased ubiquyitylation of p53 by MDM2 (Nie *et al*., 2010).

PHENOTYPES

Smurf1 gain or loss-of-function phenotypes have been described in a variety of model organisms and *in vivo* culture systems. Furthermore, abnormal expression of the Smurf1 gene and dysfunction of Smurf1 protein are increasingly implicated in the etiology of several diseases.

Embryonic phenotypes

A Drosophila ortholog, DSmurf1, was isolated as a suppressor of weak Mad (Smad1) alleles, and homozygous DSmurf mutants display phenotypes resembling DPP overexpression, including an early (blastula/gastrula) stage expansion of phospho-MAD signaling domains that result in multiple adult phenotypes mimicking DPP loss of function (Podos *et al*., 2001). DSmurf mutants also show a late, organogenesis stage defect in hindgut morphogenesis, a process controlled partly by DPP signals. Overexpression of DSmurf in the wing imaginal disc causes reduced p-MAD levels and highly defective, rudimentary adult wings (Liang *et al*., 2003). DSmurf1 also regulates DPP signaling in germline stem cells by targeting the DPP receptor, Thickveins (Xia *et al*., 2010).

In Xenopus embryos, overexpression of Smurf1 neuralizes and dorsalizes ectodermal (animal cap) and mesodermal tissues, respectively, by reducing the ability of cells to respond to BMP signals. However, Smurf1 overexpression enhances animal cap responses to the activin/nodal/TGF-ß pathway (Zhu *et al*., 1999; Zhang *et al*., 2001). Smurf1 expression knockdown by morpholino oligos (MOs) or dominant-negative Smurf1 results in head and dorsal neurectodermal defects (Alexandrova and Thomsen, 2006). MO knockdown reduces neural tissue differentiation and incomplete anterior neural plate closure that result from elevated p-Smad1/5 in the neural plate, and loss of actin-rich cells that form neural plate hingepoints.

In the mouse, homozygous Smurf1 knockout causes a mild adult phenotype affecting long bones, but no embryonic phenotypes were observed (Yamashita *et al*., 2005). Adult Smurf1-/- mice have increased bone mass compared to wild type mice or Smurf1 -/+ mice, which look normal. Surprisingly, this increased bone mass is not due to increased BMP/Smad1/5/8 signaling, which would have been predicted. Instead the Smurf1 -/- mice have increased MAPK signaling specifically in their osteoblasts (see below). A double knockout of Smurf1 and Smurf2, however, exhibits early embryonic phenotypes that include defective anterior neural plate closure and abnormal head morphology, as well as inner ear defects at later stages (Narimatsu *et al*., 2009). Unlike the BMP signaling effects resulting from Smurf1/2 knockdown in Xenopus, the double knockout mouse phenotypes appear primarily due to defects in wnt planar cell polarity signaling. Smurf2 interacts with Dishevelled, to target Prickle, a cell polarity protein, for ubiquitylation and proteasomal degradation. These effects are clearly evident in inner ear (cochlea) hair cells, where Smurf2 targets prickle on one side of the cell, thus affecting polarity. Defects in BMP signaling in the double mutant were not noted. In mouse embryo lung epithelia, Smurf1 overepression reduces branching by lowering the Smad1/5 levels and BMP signaling, consequently reducing cell proliferation and differentiation (Shi *et al*., 2004).

Cell adhesion

As noted above (but worth repeating), Smurf1 regulates cellular protrusions (filopodia and lamellipodia), cell polarity, cell adhesion and cell motility, by affecting the levels and location of RhoA, often in association with PAR6 protein complexes. In cell motility and polarity, Smurf1/PAR6/PKCz complexes target RhoA for ubiquitylation and degradation at the cell periphery, resulting in assembly of actin meshworks (via cdc42) rather than stress fibers favored by RhoA (Wang *et al*., 2003; Zhang *et al*., 2004; Ozdamar *et al*., 2005). This results in elevated membrane protrusive activity and motile cell behavior. The GTP-bound and activated form of RhoA is the preferred Smurf1 target (Boyer *et al*., 2006).

Synaptopodin, an actin binding protein, can competitively inhibit Smurf1 binding and ubiquitylation of RhoA (Asanuma *et al*., 2006). In podocytes, the amounts of RhoA and stress fibers directly correlate with the levels of synaptopodin, both of which increase when Smurf1is blocked. Conversely, knockdown of synaptopodin reduces the cellular levels of RhoA, actin stress fibers, oriented cell polarization and cell motility.

Targeting of RhoA at tight junctions (TJ) by Smurf1 can initiate an epithelial-mesenchymal transition (EMT), through TGF-ß or BMP ligand activation of Type I and II receptor complexes. Receptor activation phosphorylates PAR6, resulting in Smurf1 becoming associated with TJs and consequent proteasomal degradation of RhoA at the TJs. This Smurf1-driven EMT mobilize epithelial cells during coronary vessel formation (Sanchez and Barnett, 2011) and heart valve morphogenesis (Townsend *et al*., 2008, 2011). A Smurf1-binding protein, Cerebral Cavernous Malformation 2 (CCM2) protein can regulate Smurf1-driven EMT by promoting the localization of Smurf1 to the cell membrane of endothelial cells, where it targets RhoA and thus promotes EMT as well as protrusive and migratory activity (Crose *et al*., 2009). Smurf1-mediated EMT might be a mechanism to promote metastasis in cancer, although more evidence for such an endogenous role remains to be gathered.

Smurf1 can also affect cell adhesion and migration by regulating Talin, a cytosolic protein that interacts with actin and the C-terminal tail of integrins, to stabilize focal adhesions (Critchley 2009; Huang *et al*., 2009). Cleavage of Talin by Calpain II promotes focal adhesion dissolution by liberating a Talin “head group” that gets targeted for destruction by Smurf1-mediated ubiquitylation. Destruction of the Talin head promotes focal adhesion disassembly and cell migration. These effects can be blocked, however, if the Talin head gets phosphorylated by Cdk5, a cyclin dependent kinase, which inhibits Smurf1 binding to the Talin head, resulting in stabilized focal adhesions that reduce cell motility (Huang *et al*., 2009).

Bone

One of the most clear biological functions of Smurf1 is to regulate bone development and growth. Transgenic overexpression of Smurf1 in mouse embryo osteoblasts reduced bone formation, consistent with parallel findings that Smurf1 overexpression in cultured osteoblasts degraded Smads 1 and 5 and the Runx2 (Cbfa) transcription factor which resulting in failed osteoblast differentiation (Ying *et al*., 2003; Zhao *et al*., 2003, 2004). Similarly, overexpression of Smurf1 in mouse cartilage cells caused hypertrophy and defective bone formation, resulting in dwarf mice with osteopenia (Horiki *et al*., 2004). Smurf1 can interact with Runx2 directly, or indirectly via Smad6, which recruits Smurf1 to Runx2 (Shen *et al*., 2006, Horiki *et al*., 2004), Thus, overexpression of Smurf1 plus Smad6 in mouse embryo cartilage was shown to inhibit chondrocyte differentiation and result in dwarf mice (Horiki *et al*., 2004). In multipotent C2C12 cells treated with BMP ligand, overexpression of Smurf1 inhibits osteoblast differentiation and promotes formation of myoblasts, while knockdown of Smurf1 promotes cartilage differentiation (Ying *et al*., 2003). Smurf1 also can regulate the anti-apoptotic response of osteoblasts to PTH by targeting Runx2 (Bellido *et al*., 2003). The affinity of Smurf1 for some of its substrates can be enhanced by the binding of CKIP-1, and in the context of mouse bone formation, knockout of CKIP-1 enhances osteogenesis, in part due to reduced Smurf1 activity against BMP pathway components in osteoblasts (Lu *et al*., 2008).

The essential nature of Smurf1 in bone development and homeostasis was revealed by Smurf1-/- mice, which develop with greater bone mass than wild-type mice, as noted above. One might reasonably expect the sensitive Smurf1 target(s) to be Smad1/5 or Runx, but surprisingly those are not affected. Instead, MEKK2 protein levels become elevated in the Smurf1-/- mice, consistent with findings that Smurf1 can target MEKK2 for proteasomal degradation (Yamashita *et al*., 2005). In Smurf1-/- embryos, phospho-MEKK2 levels were elevated compared to controls, due to activated Jun Kinase signaling in osteoblasts, This boosted cartilage and calcified bone deposition, raising bone mass.

Heart

As noted above, Smurf1 promotes EMT during development of the vertebrate heart, directing formation of heart valves from the atrioventricular cushion (AVC) of the endocardium, as well as formation of coronary vessels from the developing epicardium. In valve formation, TGF-ß2 or BMP-2 activate the Type I receptor, ALK5, that in turn phosphorylates PAR6 and recruits Smurf1 to the TJs where RhoA becomes degraded and adhesion is lost (Townsend *et al*., 2008, 2011). This pathway also triggers an EMT in mouse epicardial cells to mobilize them to reorganize into coronary vessels, and this process TGFß Type III receptor signaling (Sanchez and Barnett, 2011).

Neurons

Besides effects on neural plate morphogenesis, Smurf1 promotes neuron outgrowth by reducing RhoA levels that cause elevation in actin-based protrusive activity and neurite elongation (Bryan *et al*., 2005). In a similar fashion, enteric neuron axonal elongation and cell polarity are also regulated by Smurf1 in PAR-PKCζ complexes that target RhoA for degradation (Vohra *et al*., 2007). Elevating retinoic acid (RA) appears able to boost Smurf1 levels and promote enteric neuroblast precursor differentiation. But when RA levels are reduced, Smurf1 mRNA and protein levels also become reduced causing RhoA levels to increase, promoting neurite outgrowth (Sato and Heuckeroth, 2008). Smurf1 regulation of neuronal growth and polarity can be regulated by protein kinase A (PKA)-dependent phosphorylation, which causes Smurf1 to shift the level of ubiquitylation and destruction between PAR6 and RhoA (Cheng *et al*., 2011; Stiess and Bradke, 2011). Phosphorylation of Smurf1 at THR-306 promotes RhoA ubiquitylation and degradation (and enhances axon formation), yet reduces PAR6 destruction. Blocking this phosphorylation promotes PAR6 degradation, axon formation and axon polarization.

Genotypes and Phenotypes that influence Smurf1

In a mouse model of Thoracic Aortic Aneurism (TAA) a variety of genes in TGFβ pathways are upregulated, including Smurf1 (Jones *et al*., 2008).

Smurf1 in disease

Dysregulation of Smurf1 is implicated in several diseases, including cancers (Chen and Matesic, 2007), particularly lung cancer (Xie *et al*., 2011; Shi *et al*., 2004), pancreatic cancer (Bashyam *et al*., 2005; Birnbaum *et al*., 2011; Loukopoulos *et al*., 2007; Suzuki *et al*., 2008; Kwei *et al*., 2011), gastric adenocarcinoma (van Dekken *et al*., 2009) and possibly breast cancer (Fukunaga *et al*., 2008). There is emerging evidence that Smurf1-mediated EMT and degradation of RhoA promotes tumor metastasis (Fukunaga *et al*., 2008; Huang *et al*., 2009; Loukopoulos *et al*., 2007; Sahai *et al*., 2007). Besides cancer, Smurf1 is implicated in dwarfism and osteopenia (Horiki et al., 2004), tissue fibrosis (Fukusawa *et al*., 2004; He *et al*., 2011; Yan *et al*., 2011) and Wolfram syndrome (Guo *et al*., 2011) which causes diabetes mellitus and retinal atrophy. Defective Smurf1 has the potential to cause birth defects (e.g. Alexandrova and Thomsen, 2006; Narimatsu *et al*., 2009; Townsend *et al*., 2008).

MAJOR SITES OF EXPRESSION

The Smurf1 gene is widely expressed in embryonic and adult cells and tissues, and enriched expression is observed in particular developmental stages and locations, such as the ectoderm, mesoderm and neurectoderm at blastula and gastrula stages of fly, Xenopus and mouse embryos (Horiki *et al*., 2004; Narimatsu *et al*., 2009; Podos *et al*. 2001; Zhu *et al*., 1999).

In embryos and adults, Smurf1 has been detected in a myriad of developing and fully differentiated tissues and organs, including the brain, nervous system, neurons and neural crest (Alexandrova and Thomsen, 2006; Bryan *et al*., 2005; Cheng *et al*., 2011; Kalkan *et al*., 2009; Vohra and Heuckeroth 2007; Zhu *et al*., 1999), somites (Zhu *et al*., 1999), heart (Townsend *et al*., 2008, 2011; Sánchez and Barnett, 2012), lung (Shi *et al*., 2004), testis (Itman *et al*., 2011), ovary (Casanueva and Ferguson, 2004; Xia *et al*., 2010), as well as cartilage, bone and osteogenic tissues and cells (Horiki *et al*., 2004; Xing *et al*., 2010; Yamashita *et al*., 2005; Zhao *et al*., 2003, 2004, 2010). Expression of Smurf1 mRNA has been detected in many mouse tissues via cDNA cloning and EST profiling:

At the protein level, Smurf1 has been observed in a variety of subcellular locations that include the plasma membrane, endosomes, lysosomes, adherens junctions, tight junctions, cellular projections (filopodia, lamellipodia), actin and tubulin

cytoskeleton, general cytoplasm, nucleus, chromatin and transcription complexes (see section above).

SPLICE VARIANTS

Two isoforms of human Smurf1 have been identified that are generated by alternative splicing. Isoforms 1 and 2 encode proteins that are 757 and 731 amino acids long respectively, differing by an insertion of 26 residues in isoform 1,between the two WW domains of the protein (insertion point residue 268). Since the linker region between these WW domains has been shown to affect substrate binding (Lu *et al*., 2011) the two Smurf1 isoforms have the potential to bind WW-dependent substrates with different affinities. Functional differences between the two isoforms, however, have not been demonstrated. The isoform 1 splice variant corresponds to NIH NCBI reference mRNA sequence NM_020429.1 and reference protein NP_065162.1. Isoform 2 corresponds to NIH NCBI reference mRNA sequence NM_181349.1 and protein reference NP_851994.1. Whether Smurf1 isoforms are differentially expressed in developmental or cell type specific ways is not known.

REGULATION OF CONCENTRATION

Little is known about how Smurf1 protein concentration is regulated in cells, but there is emerging evidence for control at the transcriptional, post-transcriptional and post-translational levels.

At the transcriptional level, inflammatory cytokines, specifically Tumor Necrosis Factor (TNF) and Interleukin 1ß (Il-1ß), can upregulate transcription of the human Smurf1 gene (Kaneki *et al*., 2006; Guo *et al*., 2008; Liu *et al*., 2011). Hypoxia also can induce Smurf1 gene expression and reduce BMP signaling in a rat model of pulmonary artery hypoxia (PAH) (Murakami *et al*., 2010). Lipopolysacharide and ubiquinone have opposing effects on Smurf1 gene expression in human THP monocytes, with the former resulting in elevated and the latter resulting in reduced Smurf1 levels, which may modulate inflammatory and antioxidant responses by these cells (Schmelzer and Döring, 2010). Inflammatory cytokine signals elevate Smurf1 transcription in ligament-derived mesenchymal stem cells (Liu *et al*., 2011).

At the post-transcriptional level, the expression of Smurf1 can be regulated by pre-mRNA splicing, through the action of protein kinase CKαLS, a nuclear protein that stabilizes the splicing component hnRNP C and in turn promotes Smurf1 expression (Panchenko et al., 2010). The micro-RNA mir-17 inhibits Smurf1 mRNA levels in mesenchymal stem cells (MSCs), and mir-17 expression is suppressed by the same inflammatory cytokine signals that can stimulate Smurf1 transcription (Liu *et al*., 2011). These effects modulate the ability of MSCs to undergo osteogenic differentiation.

Smurf1 protein levels can be controlled post-translationally by ubiquitin-dependent proteasomal degradation driven by Smurf1 itself, Smurf2, Trb3 or an SCF ubiquitin ligase complex containing the F-box protein, FBXL15, that recognizes the N-terminal lobe of the Smurf1 HECT domain (Chan *et al*., 2007; Fukunaga *et al*., 2008; Lu *et al*., 2008; Cui *et al*., 2011). Furthermore, ubiquitin-independent degradation of Smurf1 (and also Smurf2) can result from interacting with REGgamma, an activator of the 19S component of the 26S proteosome (Nie *et al*., 2010).

Elevated ER stress can trigger Smurf1 degradation through an unknown mechanism, and as discussed above, Smurf1 can regulate the unfolded protein response of the ER by targeting WFS1 to the proteasome (Guo *et al*., 2011). Stress of the mechanical sort applied to MC3T3-E1 osteoblast cells has been shown to downregulate Smurf1 and upregulate Smad1/5 protein as well as BMP signaling, without affecting changes in the Smad1/5 mRNA levels (Wang *et al*., 2010), but the mechanism is unknown.

ANTIBODIES

Monoclonal and polyclonal antibodies that recognize human Smurf1 are available from various commercial sources such as Abcam, Abgent, Abnova, Boston Biochem, Epigentech, Invitrogen, and others (i.e. there are many sources). A limited amount of anti-Xenopus Smurf1 monoclonal antibody is available from the Thomsen lab (Wang *et al*., 2003). This monoclonal detects human or Xenopus Smurf1 in western blots, but detects only human Smurf1 in cell immunohistochemical staining.

Table 1: Functional States

STATE DESCRIPTION	LOCATION	REFERENCES
native unbound	Unknown	
Smurf1/UbcH5[E2]	Unknown	Andrews PS *et al.* 2010
Smurf1/UbcH7[E2]	Unknown	Andrews PS *et al.* 2010; Ogunjimi AA *et al.* 2010
Smurf1/ubiquitin	Unknown	
Smurf1-polyUb	Unknown	Ogunjimi AA *et al.* 2010
Smurf1-p	Unknown	Cheng PL *et al.* 2011
Smurf1/Smad1	Unknown	
Smurf1/Smad5	Unknown	
Smurf1/Smad6	Unknown	
Smurf1/Smad7	Unknown	
Smurf1/Smad6/ALK2	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK3	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK4	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK5	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK6	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/Runx2	Unknown	Shen R *et al.* 2006
Smurf1/Smad6/Tbx6	Unknown	Chen YL *et al.* 2009
Smurf1/Smad7/ALK2	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK3	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK4	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK5	membrane	Ebisawa T *et al.* 2001
Smurf1/Smad7/ALK6	membrane	Murakami G *et al.* 2003
Smurf1/Ataxin3	Unknown	Andrews PS *et al.* 2010
Smurf1/Ccm2	Unknown	Crose LE *et al.* 2009
Smurf1/CKIP-1	Unknown	Lu K *et al.* 2008
Smurf1/CRM1[Xpo1]	exportin	Tajima Y *et al.* 2003
Smurf1/HIP2[UBE2K]	Unknown	Andrews PS *et al.* 2010
Smurf1/ING2	Unknown	Barrios-Rodiles M *et al.* 2005; Nie J *et al.* 2010
Smurf1/JunB	Unknown	Zhao L *et al.* 2010
Smurf1/JIK[Taok3]	Unknown	Andrews PS *et al.* 2010
Smurf1/LMP-1	Unknown	Sangadala S *et al.* 2006
Smurf1/Mdm2	Unknown	Nie J *et al.* 2010
Smurf1/MEKK2	Unknown	Yamashita M *et al.* 2005
Smurf1/NEK2	Unknown	Andrews PS *et al.* 2010
Smurf1/PAR6	Unknown	Ozdamar B *et al.* 2005; Wang HR *et al.* 2003
Smurf1/PKCiota	Unknown	Andrews PS *et al.* 2010
Smurf1/PKCzeta	Unknown	Bryan B *et al.* 2005; Wang HR *et al.* 2003
Smurf1/RhoA	Unknown	Wang HR *et al.* ; Wang HR *et al.* 2003
Smurf1/RNF11	Unknown	Colland F *et al.* 2004
Smurf1/Runx2	Unknown	Shen R *et al.* 2006
Smurf1/Runx3	Unknown	Shen R *et al.* 2006
Smurf1/Smurf2	Unknown	Fukunaga E *et al.* 2008
Smurf1/Talin	Unknown	Huang C *et al.* 2009
Smurf1/TOM1	Unknown	Andrews PS *et al.* 2010
Smurf1/TRAF[1-6]	Unknown	Li S *et al.* 2010; Kalkan T *et al.* 2009
Smurf1/TriblesLike3	Unknown	Chan MC *et al.* 2007
Smurf1/tropomyosin4	Unknown	Andrews PS *et al.* 2010
Smurf1/UbcH7-Ub	Unknown	Ogunjimi AA *et al.* 2010; Andrews PS *et al.* 2010
Smurf1/Smad1-polyUb	Unknown	Zhu H *et al.* 1999
Smurf1/Smad7-polyUb	Unknown	Grönroos E *et al.* 2002; Kavsak P *et al.* 2000
Smurf1/Smad5-polyUb	Unknown	Zhu H *et al.* 1999
Smurf1/Smad6/ALK2-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK3-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK4-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK5-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad6/ALK6-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK2-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK4-polyUb	membrane	Murakami G *et al.* 2003

Smurf1/Smad7/ALK3-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK5-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Smad7/ALK6-polyUb	membrane	Murakami G *et al.* 2003
Smurf1/Ataxin3-polyUb	Unknown	Andrews PS *et al.* 2010
Smurf1/Smad7/FKBP12	plasma membrane	Yamaguchi T *et al.* 2006
Smurf1/HIP2[UBE2K]-polyUb	Unknown	Andrews PS *et al.* 2010
Smurf1/ING2-polyUb	Unknown	Nie J *et al.* 2010
Smurf1/JunB-polyUb	Unknown	Zhao L *et al.* 2010
Smurf1/JIK[Taok3]-polyUb	Unknown	Andrews PS *et al.* 2010
Smurf1/Mdm2/Mdm4	Unknown	Nie J *et al.* 2010
Smurf1/MEKK2-polyUb	Unknown	Yamashita M *et al.* 2005
Smurf1/NEK2-polyUb	Unknown	Andrews PS *et al.* 2010
Smurf1/PAR6-p	tight junction	Ozdamar B *et al.* 2005
Smurf1/polyubiquitin chain	Unknown	Ogunjimi AA *et al.* 2010
Smurf1/Par6-polyUb	tight junction	Ozdamar B *et al.* 2005; Townsend TA *et al.* ; Wang HR *et al.* 2003
Smurf1/PKCiota-polyUb	Unknown	Andrews PS *et al.* 2010
Smurf1/RhoA-polyUb	tight junction	Cheng PL *et al.* 2011; Crose LE *et al.* 2009; Ogunjimi AA *et al.* 2010; Sahai E *et al.* 2007; Townsend TA *et al.* 2008
Smurf1/Synaptopodin	Unknown	Asanuma K *et al.* 2006
Smurf1/Runx2-polyUb	Unknown	Bellido T *et al.* 2003; Guo R *et al.* 2008; Jeon EJ *et al.* 2006; Kaneki H *et al.* 2006; Shen R *et al.* 2006; Zhao M *et al.* 2003
Smurf1/Smad6/Runx2-polyUb	Unknown	Shen R *et al.* 2006
Smurf1/Runx3-polyUb	Unknown	Choi SH *et al.* 2007; Miyazono K *et al.* 2004
Smurf2/Smurf1-polyUb	Unknown	Fukunaga E *et al.* 2008
Smurf1 autoubiquitylation	Unknown	Andrews PS *et al.* 2010; Lu K *et al.* 2008
Smurf1/Talin-polyUb	Unknown	Huang C *et al.* 2009
Smurf1/Smad6/Tbx6-polyUb	Unknown	Chen YL *et al.* 2009
Smurf1/Tom1-polyUb	Unknown	Andrews PS *et al.* 2010
Smurf1/Traf[1-6]-poly-Ub	Unknown	Kalkan T *et al.* 2009; Li S *et al.* 2010
Trb3/Smurf1-polyUb	Unknown	Chan MC *et al.* 2007
Smurf1/Tropomyosin4-polyUb	Unknown	Andrews PS *et al.* 2010

ACKNOWLEDGEMENTS

The author acknowledges support from NIH grants R01GM080462 and R01GM076599.

SUPPLEMENTARY

Supplementary information is available online.

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This molecule exists in 78 states , has 77 transitions between these states and has 0 enzyme functions.(Please zoom in the pdf file to view details.)