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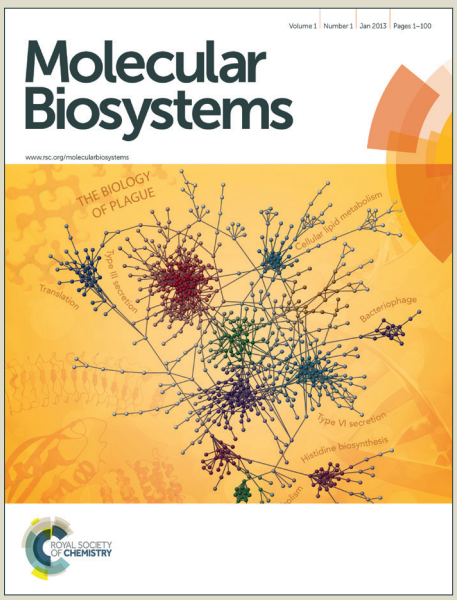

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Crosstalk between Kinases and Nedd4 family Ubiquitin Ligases

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A dazzling array of human biological processes achieves coordination and balance through the posttranslational modification of protein residues with phosphate (95 Da) or ubiquitin (8565 Da). Over the past years, a reciprocal communication has become recognized between phosphorylating (kinases) and ubiquitinating (E3 ligases) enzymes. Such crosstalk occurs when a kinase acts on a ligase or vice versa to modify the catalytic activity, substrate specificity, or subcellular localization of the modified enzyme. In this review, we focus on the crosstalk between the nine members of the Nedd4 family E3 ubiquitin ligases with kinase signal transducers such as cell surface receptors, cytosolic kinases, phosphatases, and transcription factors. Since protein kinases are well explored and established therapeutic targets, we hypothesize that mapping E3 ligases onto kinase signalling networks will provide clues to the full therapeutic potential of pharmacologically targeting E3 ligases.

Introduction

The dynamic and tunable behavior of cell signalling networks is controlled by a large number of posttranslational modifications (PTMs). Among the cacophony of >400 known PTMs, three major PTM classifications stand out: 1) covalent modifications of large proteins with small molecules such as acetyl, methyl, phosphate, glycosyl, and farnesyl groups, 2) covalent modification of proteins with large molecules such as the ~20 known ubiquitin-like proteins (UBLs), and 3) covalent modification of proteins by oligomeric chains such as branched and linear poly-ubiquitin chains or poly-glycosides.[1-4] Increasing evidence hints toward a profoundly complex landscape of crosstalk between different PTMs that regulate processes such as endocytosis, signal transduction, nuclear shuttling, and gene transcription.

Like other PTMs, covalent modification by ubiquitin affects protein conformation, localization, and stability.[5] The possibility for target proteins to undergo mono-, multi- and poly-ubiquitination adds an additional layer of complexity where poly-ubuiquitin chains can be linked through seven ubiquitin lysine residues (Lys^{6}, Lys^{11}, Lys^{27}, Lys^{29}, Lys^{33}, Lys^{48} and Lys^{63}) to dictate physiological consequences through different chain topologies. Canonically, covalent modification of proteins with Lys^{48}-linked poly-ubiquitin chains is usually associated with proteasomal degradation, while chains linked through Lys^{63} signal lysosomal degradation, regulate endocytosis, and mediate protein trafficking.[6]

In contrast to protein phosphorylation, ubiquitination is biochemically more

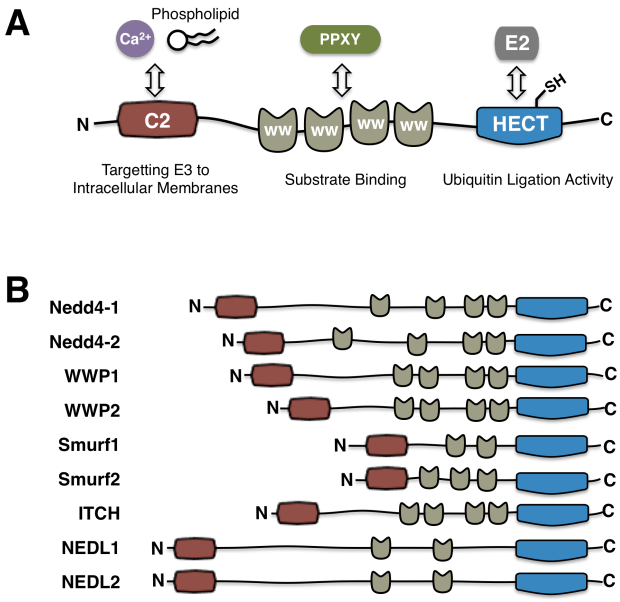

Figure 1. Structure of Nedd4-like E3 ligase Family. (A) Nedd4 family members have an N-terminal C2 domain, 2 to 4 WW domains and a catalytic HECT domain. The C2 domain binds Ca^{2+} and phospholipid, which targets Nedd4 ligases to intracellular membranes. The WW domains (Trp-Trp) bind conserved PPXY sequences (PY motif) on substrates, therefore controlling substrate selectivity. The HECT domain contains a catalytic cysteine which forms an active thioester complex with ubiquitin. (B) Structural domains of the nine Nedd4-like E3 ligases.

complex and requires cooperation between three sequential enzymes: ubiquitin-activating E1 enzymes, ubiquitin-conjugating E2 enzymes, and E3 ligases. Initially, E1 enzyme activates the C-terminal glycine of ubiquitin by forming a doubly loaded E1~Ub/Ub-AMP complex. Subsequently, one ubiquitin molecule is transferred from the E1~Ub/Ub-AMP intermediate onto the catalytic cysteine of E2 via transthiolation. The subsequent E2~Ub thioester works with an E3 ligase to catalyze the direct transfer of ubiquitin to either a protein substrate lysine or to the E3's catalytic cysteine prior to transfer to the protein substrate. Conformational flexibility of E1 and E3 enzymes is critical for the macromolecular juggling of relatively large ubiquitin molecules during the cycles of enzymatic reactions.[7]

We classify E3 enzymes into five different groups based on structure and enzymatic mechanism. The first three types of E3s (type I-III) recruit an E2~Ub thioester and stimulate the direct transfer of ubiquitin onto the lysine of the protein substrate. Type I includes the 15-subunit APC/C E3, type II includes the 4-subunit Cullin-RING E3s, and type III includes single subunit RING E3 ligases. Type IV and V E3 enzymes include RING-between-RING (RBR) and homologous to E6AP C-terminus (HECT) ligases, respectively. Both Type IV and V have a catalytic cysteine and form E3~Ub thioester intermediates prior to transferring ubiquitin to the lysine of a bound protein substrate.

Of the ~30 HECT type ligases known to date, nine (Nedd4-1, Nedd4-2, WWP1, WWP2, Smurf1, Smurf2, ITCH, NEDL1 and NEDL2) are classified as Nedd4-like E3 ligase family based on a shared domain structure comprised of an N-terminal C2

domain, two to four tandem WW domains and a C-terminal catalytic HECT domain (Fig. 1). Originally identified as a calcium-binding domain in protein kinase C family[8], the N-terminal C2 domain binds to phospholipids in a calcium dependent manner to mediate the translocation of Nedd4-like E3 ligases to subcellular membranes such as plasma membrane, endosomes and lysosomes.[9-11] The WW domains mediate the binding of Nedd4-like E3 ligases to their specific substrate proteins. Two signature tryptophan residues in WW domains specifically interact with conserved PY motifs (PPXY or LPXY sequences) or proline rich regions of proteins. The C-terminal HECT domain contains a catalytic cysteine residue that accepts ubiquitin from an E2~ubiquitin thioester complex via transthiolation resulting in an E3~ubiquitin thioester complex, which transfers ubiquitin onto the protein substrate.[12]

Accumulating evidence suggests that cellular signalling cross talk between kinase and Nedd4-like E3 ligases is tightly interwoven. For example, phosphorylation can release Nedd4-like E3 ligases from auto-inhibitory conformations or it can modulate HECT E3/substrate interactions. In turn, the activity of kinases is also regulated by protein ubiquitination which regulates the activity of the kinase.[13] Ultimately, the interplay between protein phosphorylation and ubiquitination modulates various cellular functions that are involved in kinase and ubiquitin signalling. Taken together understanding cross-talk between HECT E3 ligases and protein kinases is critical to understand and to treat human diseases.

Here we map protein kinases and E3 ligases onto cell signalling networks to assess how pharmacological inhibition of these enzymes could be used to understand and to treat human diseases, and whether rationally designed combination therapies can be envisioned. We also propose that mapping E3 ligases and kinases onto cell signalling pathways can provide insights onto the understanding of resistance mechanisms to anticancer therapeutics.

ITCH

Physiological Roles

The ITCH E3 was first discovered in mutant mice that developed a spectrum of immunological disorders including pulmonary chronic interstitial inflammation, inflammation of the glandular stomach, hyperplasia of lymphoid cells, and constant itching in the skin.[14] Perry *et al.* linked the itchy phenotype to a paracentric inversion of genes that produces a null *ITCH* allele due to disruption of the promoter region. To date, accumulating evidence suggests that ITCH is critical for regulating T-cell differentiation.[15] The abnormal autoimmune functions, inflammatory defects, and enlarged spleen and lymph nodes in itchy mice partly result from excessive cytokine (IL-4) production and chronic activation of T-helper2 (Th2) cells.[16] Importantly, recent genetic studies show that Amish children possessing genetically truncated ITCH are diagnosed with multisystem autoimmune diseases, dysmorphic features, and developmental delay. [17] Such broad human phenotypes resulting from ITCH deficiency emphasize the central role of ITCH E3 ligase in human development and inflammation.

Regulation of ITCH Activity

ITCH is not a constitutively active HECT E3 ligase since intramolecular binding

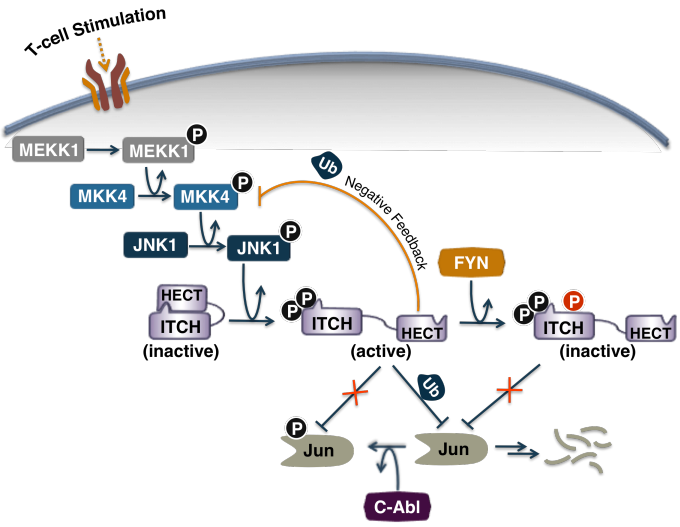

Figure 2. Crosstalk Between ITCH and JNK1 Signalling Pathways. T-cell stimulation activates JNK1 signalling, which activates ITCH by disrupting its auto-inhibitory structure. Activated ITCH ubiquitinates c-Jun and JunB for subsequent degradation. JNK1-ITCH signalling can be terminated by three distinct pathways: (1) ITCH-mediated degradation of MKK4 as a part of a negative feedback loop, (2) c-Abl mediated phosphorylation of c-Jun, which disrupts binding between ITCH and c-Jun, (3) FYN kinase-mediated phosphorylation of ITCH, which decreases the substrate binding affinity of ITCH.

between the ITCH HECT and WW domains auto-inhibits ITCH (Fig. 2).[18] Notably, the auto-inhibition of E3 ligase activity through intramolecular domain interaction is very well precedented, and is also observed in Nedd4-1, Nedd4-2 and WWP2 E3 enzymes, suggesting a general control mechanism of Nedd4-like ligases.[19] ITCH can be activated by JNK1 kinase, which phosphorylates ITCH on its proline rich region (Ser^{199}, Thr^{222} and Ser^{232}) to disrupt the auto-inhibitory conformation of ITCH by releasing the HECT domain for ligase activity.[18] Current evidence also suggests that ITCH can self-regulate its activity via intermolecular auto-ubiquitination with Lys^{63}-linked poly-ubiquitin chains that do not induce the degradation of ITCH. [20]

Cross-talk between ITCH and JNK1 Signalling Pathway

ITCH undergoes extensive cross-talk with the JNK1 kinase signalling pathway to tightly regulate cytokine production and T-cell differentiation. In fact, the immunological defects of itchy mice arise from the disruption of T-cell signalling caused by the inability of mutant *ITCH* to regulate the JNK1 signalling cascade. The JNK1 signalling cascade is classified as one of the most evolutionarily conserved pathway composed of mitogen-activated protein (MAP) kinases. The canonical MAP kinase pathway transduces signals from the cell surface to the nucleus through the three types of kinases: MAP3K, MAP2K, and MAPK. In the JNK1 pathway, the extracellular signal is transduced from the membrane receptor to MEKK1, to MKK4, and then to JNK1.

In mouse T-cells, extracellular stress activates JNK1 through the MEKK1-MKK4-JNK1 cascade, which then phosphorylates and activates ITCH.[18] Active ITCH binds and ubiquitinates substrates such as cFlip, LATS1, p73, PKC-θ, Notch, and the

transcription factors c-Jun and JunB. Notably, the ITCH-induced reduction in JunB levels attenuates IL4 production and decelerates Th2 cell differentiation.[16] This process correlates with the itchy mouse phenotype, where significantly increased IL4 production led to inflammation.

It is interesting to point out that c-Jun and JunB are also direct targets of JNK1. While ITCH poly-ubiquitinates c-Jun and JunB to promote their degradation, JNK1 phosphorylates c-Jun and JunB on their transcriptional activation domains to enhance their activity. The c-Jun mutant lacking its JNK1 phosphorylation sites was ubiquitinated and degraded to a similar degree as wild type c-Jun, thus suggesting that the phosphorylation of c-Jun and its ITCH-dependent degradation can be two independent events. Current data suggests that JNK1 can upregulate or downregulate c-Jun and JunB utilizing the combination of phosphorylation and ubiquitination, and this regulation is cell context dependent.

Activated JNK1/ITCH signalling can be turned off through three distinct mechanisms. First, prolonged T-cell stimulation with CD3 and CD28 antibodies can induce ITCH to mark the upstream activator of JNK1, MKK4, for degradation by ubiquitinating MKK4 at Lys^{140} and Lys^{143}.[21] Loss of MKK4 prevents activation of JNK1, and thus attenuates the activating phosphorylation of ITCH. Second, activation of a T-cell receptor induces translocation of c-Abl, a cytoplasmic tyrosine kinase, to the nucleus where it can phosphorylate c-Jun on its PPXY motif to inhibit its binding to the ITCH WW domains.[22] This protects c-Jun from ITCH-mediated degradation. Finally, Fyn, a tyrosine kinase, phosphorylates ITCH WW3 domain at Tyr^{371} to block the interaction between ITCH and JunB, thus preventing the ubiquitination and degradation of JunB.[23] In support of this rationale, the ITCH Y371F mutant ubiquitinated JunB, Notch and SMAD2 to a greater extent than wild type ITCH. Intriguingly, this mutation did not affect ITCH auto-ubiquitination, suggesting that Tyr^{371} affects substrate ubiquitination but not auto-ubiquitination. It is intriguing to notice that JNK1-mediated phosphorylation of ITCH disrupts auto-inhibitory intramolecular interactions to activate ITCH, while Fyn-mediated phosphorylation decreases the binding affinity between ITCH and its protein substrates. Taken together, Ser/Thr kinases and a Tyr kinase oppositely regulate the activity of ITCH by directly regulating ITCH conformation, in addition to regulating binding interactions between ITCH and its substrates. Thus, selective kinase inhibitors are promising tools to regulate the activity of ITCH ligase and the stability of its substrates.

Smurf1

Physiological Roles

In 1999, Zhu *et al.* identified SMAD ubiquitin regulatory factor 1 (Smurf1) through a yeast two-hybrid screen that used Xenopus SMAD1 in order to find new components of the SMAD regulatory pathway.[24] Smurf1 appears to be an essential negative regulator of osteoblasts since Smurf1 deficient mice experienced an age-dependent bone mass increase.[25]

Regulation of Smurf1 activity

Alternative splicing of the Smurf1 gene results in three protein isoforms (one long isoform and two short isoforms) that vary in the length of the linker between the two

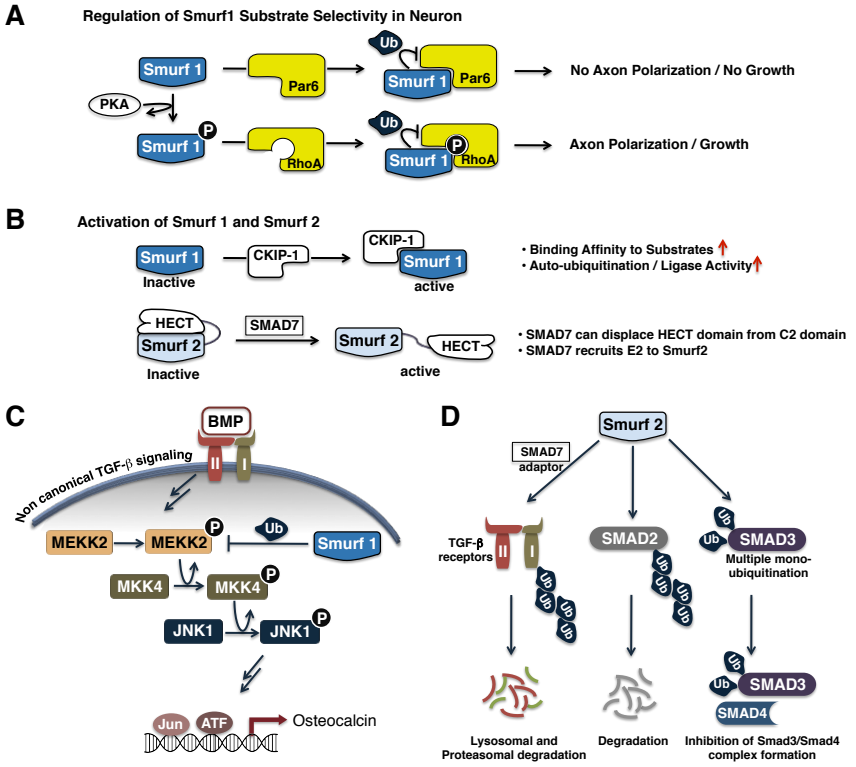

Figure 3. Cross-talk Between Smurf1/2 and Kinase Signalling Pathways. (A) PKA-mediated phosphorylation of Smurf1 changes the substrate preference of Smurf1 from Par6 to RhoA resulting in axon growth. (B) Smurf1 is activated by an auxiliary protein CKIP-1, which increases the Smurf1 binding affinity for substrates. Smurf2 can be activated by SMAD7, which disrupts auto-inhibitory conformation of Smurf2, by disrupting intramolecular C2-HECT domain interaction in Smurf2. SMAD7 also activates Smurf2 by recruiting E2 enzyme (UbcH7) to Smurf2. (C) Smurf1 downregulates osteoblast activity via ubiquitin-mediated degradation of MEKK2. (D) Smurf2 downregulates TGF-β signalling by inducing the degradation of TGF-β receptors and SMAD2. Also, Smurf2 induces multiple mono-ubiquitination on SMAD3 that disrupts SMAD3/SMAD4 complex formation.

WW domains.[26] It is proposed that this splicing modulates Smurf1 specificity since the long Smurf1 isoform shows reduced binding to substrate PY motifs.

In neurons, Smurf1 regulates two proteins that differentially act on axon growth: a growth inhibitory GTPase, RhoA, and a neuronal polarization protein, Par6. While wildtype Smurf1 ubiquitinates and degrades both proteins, PKA dependent phosphorylation of Smurf1 at Thr^{306} directs Smurf1 to preferentially bind to and ubiquitinate RhoA over Par6.[27] Therefore, PKA induces Smurf1-mediated degradation of RhoA and reduced degradation of Par6 to simultaneously stimulate both axon growth and polarization in the course of proper axon formation (Fig. 3A).

Lastly, Smurf1 can also be regulated by an adaptor protein, casein kinase-2 interacting protein-1 (CKIP-1), which binds Smurf1 WW domain linker region and enhances the interaction between Smurf1 and its substrates (Fig. 3-B).[28] Likewise, overexpression of CKIP-1 and wild type Smurf1 in HEK293T cells accelerated the auto-ubiquitination and degradation of Smurf1 leading to a significantly reduced

Smurf1 half-life compared to catalytically inactive Smurf1. Overall, CKIP-1 activates the binding of Smurf1 to its downstream substrates while also accelerating Smurf1 auto-ubiquitination.

Regulation of TGF-β signalling by Smurf1

TGF-β signalling can be activated by approximately 42 TGF-β ligands including the bone morphogenic protein (BMP) family, the growth and differentiation factors (GDF) and the TGF-β family members. The canonical TGF-β signalling pathway is initiated by the binding of TGF-β ligands to its type II transmembrane receptor kinase (TβR-II) which then phosphorylates and activates its type I transmembrane receptor kinase (TβR-I). Activated TβR-I phosphorylates receptor SMAD proteins (R-SMAD: SMAD1-3, 5, 8) that subsequently form heterodimers with SMAD4 prior to nuclear translocation and transcription of target genes when assisted by other cofactors. Non-canonical TGF-β signalling is not transduced through SMAD proteins, but through MAP kinase pathways, Rho-like GTPase signalling pathways, and PI3K-AKT pathways, which are well reviewed by Zhang et al.[29] Due to the large number of TGF-β ligands and signalling pathways, TGF-β signalling controls a variety of cellular processes such as proliferation, differentiation, apoptosis and survival. Consequently, the malfunction of TGF-β signalling is frequently observed in cancer progression and metastasis, immune disorders, and tissue fibrosis. [30-32]

The SMAD proteins consist of two conserved globular domains (MH1 and MH2 domains) connected through a PY motif-bearing linker that can bind the WW domain of a Nedd4 family ligase. Indeed, six of the eight SMAD proteins (1-3 and 5-7) are potential targets of Nedd4 family E3 ligases. A majority of Nedd4 family ligases such as Smurf 1/2, NEDD4-2, ITCH and WWP1/2 are known to bind to and to ubiquitinate SMAD proteins. [31, 33]

Bone morphogenic protein (BMP) is primarily responsible for controlling bone formation and differentiation through canonical TGF-β/SMAD signalling. BMP transduction is mediated by the positive regulation of SMAD1, SMAD5 and SMAD8 proteins, as well as by negative regulation of SMAD6 and SMAD7. Smurf1 negatively regulates BMP signalling by selectively interacting with R-SMAD proteins in BMP signalling (SMAD1, 5, 8).[24] With SMAD1, di-phosphorylation of its linker PY motif (Ser^{206} and Ser^{214}) is required for its binding to Smurf1 and subsequent ubiquitination.[34, 35] Such phosphorylation is carried out by a BMP-triggered MAP kinase cascade that induces Smurf1-dependent degradation of SMAD1. This constitutes another example of how negative feedback of a kinase signalling cascade is mediated by the activation of protein ubiquitination.

While canonical BMP signalling is transduced by SMAD proteins, non-canonical signalling depends on activation of JNK and p38 MAPK kinases. Studies have proven that JNK plays a role in promoting the expression of extracellular matrix (ECM) components and osteoblast specific markers to control osteoblast function.[36, 37] Therefore, both canonical and non-canonical BMP signalling pathways regulate osteoblast activity. Smurf1-mediated downregulation of the non-canonical BMP/JNK pathway requires that its target, MEKK2, be phosphorylated (Fig. 3-C).[25] Since the treatment of *Smurf1*$^{-/-}$ osteoblasts with the JNK specific inhibitor SB600125 rescued the abnormal acceleration of osteoblast activity, it is speculated that the enhanced osteoblast activity in *Smurf1*$^{-/-}$ mice is caused by the deregulation of Smurf1-mediated MEKK2-JNK signalling rather than canonical BMP signalling.

Smurf2

Physiological Roles

Studies showed that mice deficient in either Smurf1 or Smurf2 are viable and survive to adulthood. However, knockout of both genes leads to embryonic lethality.[38] These results suggest that some functions of Smurf2 are redundant and can be compensated by Smurf1. Nevertheless, silencing Smurf2 but not Smurf1 enhanced the activity of the TGF-β/SMAD-pahtway.[25, 39] Thus, Smurf2 may be a more potent inhibitor of TGF-β/SMAD signalling than Smurf1. Smurf1 and Smurf2 share 80% sequence homology (Smurf1 contains two WW domains while Smurf2 contains three WW domains). Remarkably however, Smurf1 and Smurf2 are regulated through significantly different mechanisms, and have distinct protein substrate specificities.

Regulation of Smurf2 activity

While the intracellular localization and substrate specificity of Smurf1 can be regulated by an auxiliary factor CKIP-1 and/or phosphorylation on Thr^{306}, the activity of Smurf2 are regulated via an auto-inhibition mechanism.[40] The resting-state solution structure of Smurf2 indicates that its N-terminal C2 domain binds to the HECT domain proximal to the catalytic cysteine to presumably inhibit Smurf2~Ub thioester complex formation (Fig. 3-B). This intra-molecular C2-HECT interaction inhibits both the auto-ubiquitination of Smurf2 and the ubiquitination of Smurf2 substrates. Scaffolding proteins, such as SMAD7, can activate Smurf2 by disrupting the C2-HECT interaction.[40, 41] Ogujimi et al. also reported that SMAD7 can increase Smurf2 activity by enhancing binding between the E2 enzyme UbcH7 and Smurf2.[41] This is because the interaction between Smurf2 and UbcH7 is weak, and the N-terminal domain of SMAD7 recruits UbcH7 to Smurf2, thereby activating Smurf2.

Regulation of TGF-β Signalling by Smurf2

Smurf1 and Smurf2 not only have distinct modes of regulation, but they also have distinct substrate specificities despite high sequence homology. For example, Smurf1 and Smurf2 interact with distinct protein substrates of the TGF-β signalling pathway, leading to different outcomes. In addition to recruiting UbcH7 to Smurf2, SMAD7 also recruits Smurf2 to the TGFβR-I receptor kinase to facilitate the proteasomal and lysosomal degradation of TGFβR-I (Fig. 3-D). In contrast to Smurf1, Smurf2 regulates the activity of SMAD2 and SMAD3 via different mechanisms. Smurf2 regulates the steady state level of SMAD2 through poly-ubiquitination and proteasomal degradation,[42] but induces multiple mono-ubiquitination on the MH2 domain (mainly Lys^{333} and/or Lys^{378}) of SMAD 3.[39] The multi-ubiquitinated SMAD3 has less binding affinity for its partner SMAD4, and is thus unable to form transcriptionally active SMAD3-SMAD4 complex. Therefore, Smurf2 regulates SMAD3 not by ubiquitin-dependent degradation but by inhibiting the formation of SMAD3 containing protein complexes (Fig. 3D).

Mentioned earlier, the binding affinity of E3s and their substrates can be modulated by phosphorylation of either E3s or their substrates. As di-phosphorylation near the PY motif of SMAD1 primes its binding to Smurf1,

phosphorylation of SMAD3 immediately upstream of its PY motif (Thr^{179}) leads to the ten folds increase in binding of Smurf2 to SMAD3.[39] Since the phosphorylation of SMAD3 at Thr^{179} is induced by TβR-I, the existence of a negative feedback loop is proposed.

Another study showed that an adaptor protein Pin1 binds to the phosphorylated linker region of SMAD2/3, and induces conformational changes that enhance the binding of Smurf2 to the SMAD PY motifs.[43] In this study, the protein levels of both SMAD2 and SMAD3 decreased, when cells were co-transfected with Pin1 and SMAD2 or SMAD3 in a proteasome inhibitor-dependent manner. As expected, knockdown of Pin1 led to increased levels of SMAD 2/3.

Smurf2 Regulates Sensitivity to MEK Inhibitors in Melanoma Cells

Following FDA approval of the B-Raf inhibitor vemurafenib, and the MEK1/2 inhibitor trametinib to treat patients with metastatic melanoma, components of the MAP signalling pathway have received significant attention as therapeutic targets. A recent study recognized Smurf2 as a melanoma specific protein marker that counteracts MEK inhibitor-induced cytotoxic effects.[44] Despite the efficient inhibition of the target kinases *in vitro*, *in vivo* efficacy of MEK inhibitors is often low, requiring higher drug dosage to induce apoptosis in melanoma cells. Smith and coworkers found that Smurf2 is upregulated in MEK inhibitor resistant melanoma cells. The overexpression of Smurf2 inhibited TGF-β signalling, and up-regulated the expression of PAX3 and MITF proteins, which protect melanocytes from MEK inhibitor induced apoptosis. Intriguingly, Smurf2 deficient melanoma cells were 100 times more sensitive to the MEK inhibitor selumetinib compared to the Smurf2 expressing cells. These studies suggest that a combination therapy of MEK1/2 and Smurf2 inhibitors could serve as a useful strategy to treat melanoma. This case also provides a lesson that the effective development of therapeutics targeting kinase or ubiquitin systems requires not only an in-depth understanding of each separate signalling pathway, but also an intimate knowledge of the cross-talk between these two systems.

WWP1

Physiological Roles

In 1997, WWP1 and WWP2 were identified as members of the Nedd4 ligase family based on the presence of tandem WW domains and domain architecture similar to that of Nedd4-1.[45] WWP1 knockout mice showed increased bone mass and bone formation as they aged.[46] These phenotypes were accompanied by significantly elevated levels of the WWP1 target proteins JunB, Runx2, and CXCR4 that are important for osteoblast differentiation in mesenchymal stem cells. In addition to down-regulating osteoblast activity, WWP1 also plays oncogenic roles in human cancers: WWP1 mRNA expression is up-regulated in 58% of human breast cancer samples and 60% of the prostate cancer samples. [47, 48,49, 50] Increased protein levels of WWP1 in tumor cell lines were also observed. Accordingly, RNAi knockdown of WWP1 suppresses the proliferation of breast and prostate cancer cell lines.

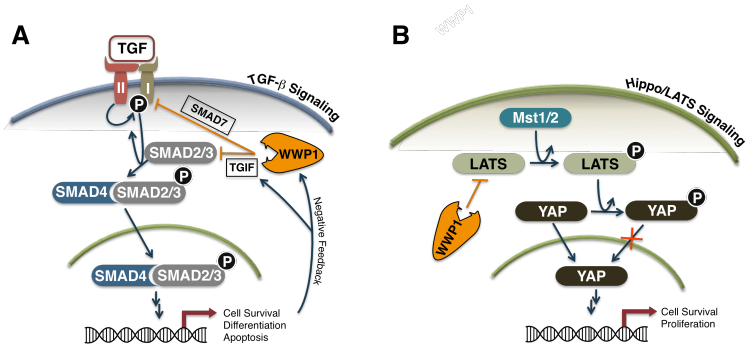

Figure 4. Cross-talk Between WWP1 and Kinase Signalling Pathways. (A) WWP1 inhibits TGF-β signalling by ubiquitinating TβR-I and SMAD2 to target them for degradation. The activation of TGF-β signalling increases transcription of WWP1 and TGIF, suggesting a negative feedback loop. (B) WWP1-mediated ubiquitination induces degradation of the tumor suppressor protein LATS1/2. Such a role for WWP1 can potentially lead to tumorigenesis because of the increase in YAP-mediated transcription of cell survival genes.

Regulation of WWP1 activity

Alternative RNA splicing generates at least six isoforms of WWP1 from the human 8q21 gene. The isoforms have different C2 domain sub-structures, including a variant that lacks two terminal beta strands in the C2 domain, implicating different functions.[51] Given that different isoforms of WWP1 are expressed in different tissues, tissue-specific investigations on the role of each splice form will provide important information on WWP1-mediated signalling pathways. A WWP1 stability study suggests that WWP1 is degraded by the proteasome following auto-ubiquitination.[52] Here, half-life of WWP1 was ~3 h in 22Rv1 cells compared to the longer ~8h half-life of ITCH.

Regulation of TGF-β Signalling by WWP1

Aberrant WWP1 regulation of kinases contributes to the oncogenic role of WWP1 in human cancers by negatively regulating tumor suppressors involved in cell growth pathways. These growth pathways include TGF-β signalling, Hippo/LATS signalling, and ErbB4 receptor signalling.[53-55]

WWP1 serves as a negative regulator of TGF-β kinase signalling by ubiquitinating the TβR-I receptor, which leads to TβR-I degradation.[53] However, the TβR-I receptor lacks a PY motif. Therefore, it is thought that SMAD7 acts as an adaptor protein that binds WWP1 through PY-WW interactions and recruits WWP1 to TβR-I. Such cooperativity between WWP1 and SMAD7 is supported by the observation that TβR-I is not degraded when HEK293 cells are transfected with catalytically inactive WWP1 C890A or when cells are transfected with active WWP1 in the absence of SMAD7.[53] WWP1 also binds and ubiquitinates SMAD2 in the nucleus in association with an adaptor protein TGIF. Interestingly, TGF-β ligands induce the mRNA expression of WWP1 and TGIF to ultimately down-regulate TβR-I and SMAD2 via a negative feedback loop. In this case, the negative feedback is activated through the elevated transcription of WWP1 with its adaptor protein, while the negative feedback via Smurf2 is achieved by the post-translational activation of Smurf2 upon its phosphorylation.

Regulation of Hippo/LATS Signalling by WWP1

Highly conserved across species, the Hippo-LATS signalling pathway is another tumor suppressor pathway regulated by WWP1. Hippo-LATS signalling controls organ size by timely mediating cell proliferation and apoptosis. When disregulated, Hippo-LATS causes uncontrolled cell growth, contributing to the development of human cancers. This is supported by previous studies in which loss of the LATS1 gene in mice led to ovarian or soft tissue sarcomas.[56] The central players in the Hippo-LATS signalling pathway are the Mst1/2-LATS1/2-YAP proteins. YAP acts as a co-activator to stimulate the transcription of genes that induce cell growth. LATS1/2 is a Ser/Thr kinase that serves as a tumor suppressor by negatively regulating YAP through phosphorylation.[57] Once phosphorylated, YAP is sequestered to the cytoplasm where it cannot initiate gene transcription.

WWP1 binds to LATS through interactions between WW domains 1-3 in WWP1 and the $PPXY^{376}$ and $PPXY^{559}$ motifs in LATS1.[54] Upon binding, WWP1 ubiquitinates and targets LATS1/2 for degradation. The down-regulation of LATS1 in breast cancer cell-lines activates YAP, which leads to cell proliferation.[54] It is interesting to notice that LATS1 utilizes its PPXY motif to bind to the WW domain of YAP.[58] It is therefore possible that WWP1 and YAP may compete for binding to LATS1.[59]

WWP2

Physiological Roles

100% of mice deficient in the *Wwp2* gene showed craniofacial anomalies that caused infant mortality and morbidity.[60] Knockdown of WWP2 in zebrafish also showed palatal malformation.[61] WWP2 is abundantly expressed in cartilage tissue where it regulates craniofacial development.[60, 62]

Regulation of WWP2 activity

There are three isoforms generated from the *Wwp2* gene locus, a full length WWP2 (880 aa), an N-terminal isoform (containing the N-terminal C2 domain and the WW1 domain, 336 aa), and a C-terminal isoform (containing the C-terminal WW4 domain and the HECT domain, 440aa).[33] The activity of full length WWP2 can be enhanced by the N-terminal isoform that lacks the catalytic HECT domain. Soond et al. suggested that the N-terminal isoform could relieve the auto-inhibitory conformation of WWP2 by interacting with the HECT domain of full length WWP2 in an intermolecular fashion, thus competing with the intramolecular C2-HECT domain interaction. The interaction between full length WWP2 and the N-terminal isoform is inhibited upon TGF-β ligand stimulation, suggesting that the WWP2-N-terminal isoform dimer is a critical determinant of WWP2 activity.

Regulation of PTEN/PI3K Pathway by WWP2

To date, only a limited number of substrates have been reported for WWP2. The tumor suppressor PTEN is one of the most significant WWP2 protein targets.[63] PTEN is one of the most affected tumor suppressors in the post-p53 era. Numerous studies have shown that the intracellular PTEN concentration and its nuclear

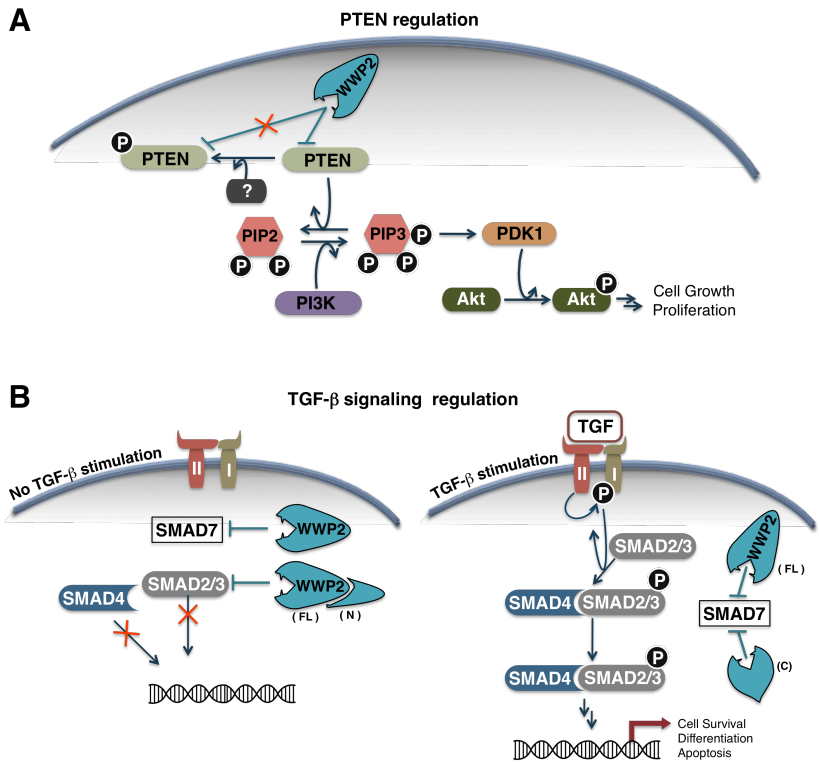

Figure 5. Regulation of Kinase Signalling Pathways by WWP2 (A) WWP2 regulates protein stability of the tumor suppressor PTEN. WWP2-mediated degradation of PTEN leads to cell growth and proliferation. (B) Three isoforms of WWP1 show different substrate specificity in TGF-β signalling. Full-length WWP2 and the N-terminal isoform cooperatively regulate steady state levels of SMAD2/3 and SMAD7. Upon prolonged TGF-β stimulation, full-length WWP2 and the C-terminal isoform selectively bind SMAD7 to induce its degradation.

localization are essential for its tumor suppressive properties. PTEN is a lipid phosphatase which converts PIP_3 to PIP_2 and counteracts the action of PI3K lipid kinase.[64] PTEN is a haploinsufficient tumor suppressor, and small changes in the dosage of PTEN have been shown to cause cancer development in mice. This property of PTEN led to the hypothesis that therapeutic agents that upregulate the stability and nuclear localization of PTEN should be effective in treating PTEN dependent malignancies.

Accordingly, knockdown of WWP2 with siRNA in DU145 prostate cancer cells was characterized by an increased steady state level of PTEN and a subsequent increase in cell death upon doxorubicin treatment.[63] WWP2-mediated ubiquitination of PTEN induces its degradation, yet it does not change PTEN localization in the nucleus where PTEN regulates chromosome stability, DNA repair and cell cycle.[65] Similar to how c-Abl phosphorylates c-Jun to protect it from ITCH-induced degradation, phosphorylation of PTEN on Tyr^{155} by an unknown kinase can prevent PTEN from binding WWP2 and thus increase its stability. This model is supported by recent clinical observations where non-small cell lung cancer and brain tumor patients harbor a PTEN Tyr155Phe mutation, which may preclude phosphorylation and

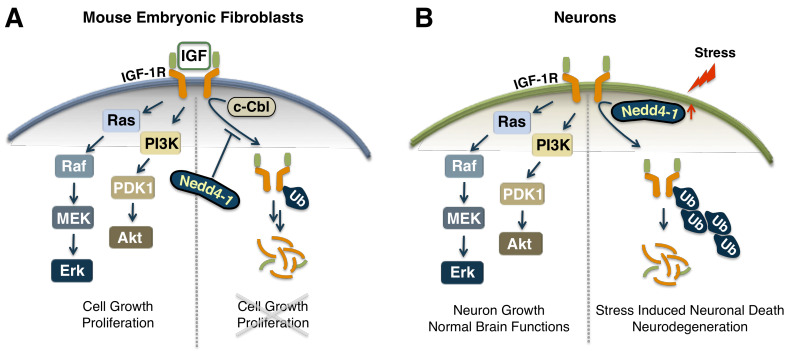

Figure 6. Opposite roles of Nedd4-1 in regulating IGF-1R Signalling Pathway. (A) During the development, Nedd4-1 positively regulates IGF-1R by inhibiting its degradation, leading to cell growth and proliferation (B) In neurons, external stresses upregulate Nedd4-1 expression, and Nedd4-1 directly ubiquitinates IGF-1R causing its degradation. Nedd4-1 is therefore a candidate drug target for neurodegenerative diseases.

stabilization of PTEN in these tumors. [66, 67]

Regulation of TGF-β Signalling by WWP2

WWP2 is also involved in the regulation of TFG-β signalling pathways via its interaction with SMAD2, SMAD3 and SMAD7 proteins. It is remarkable that the three isoforms of WWP2 (one full-length isoform and two short isoforms) interact with SMAD proteins with different selectivity.[33] For example, the WWP2-C isoform interacts only with SMAD7, while the WWP2-N isoform preferentially binds SMAD2 and SMAD3 in HEK-293 cells that were transfected with WWP2 isoforms and SMAD proteins. Intriguingly, the WWP2-N isoform lacking a HECT domain forms a heterodimer with full-length WWP2 and enhances the ability of WWP2 to degrade SMAD2 and SMAD3 proteins in the absence of TGF-β stimuli. Upon TGF-β stimulation, however, the WWP2-N isoform rapidly dissociates from the full-length WWP2. Prolonged TGF-β stimulation also causes preferential ubiquitination and degradation of SMAD7 by full-length WWP2 and WWP2-C. Therefore, different isoforms of WWP2 modulate different arms of TGF-β signalling in an intricate manner that is yet to be elucidated.

Nedd4-1

Physiological Roles

The *Nedd4-1* gene was first discovered from mouse neuronal precursor cells in 1992 as a gene down-regulated during brain development.[68] The dominant phenotype in *Nedd4-1*$^{-/-}$ mice is neonathal lethality, growth retardation during embryogenesis, and reduced body weight.[69] Nedd4-1 is a positive regulator of insulin-like growth factor-1 (IGF-1)/Akt kinase signalling in mouse embryonic fibroblasts (MEFs). *Nedd4-1*$^{-/-}$ MEFs show decreased abundance of the cell surface insulin-like growth factor 1 receptor (IGF1R), reduced mitogenic activity, and reduced cell growth. Nedd4-1 is frequently overexpressed in many different types of cancers including non-small cell lung cancers, gastric carcinomas, and colorectal carcinomas and is thought to be a

promising anti-cancer drug-target.

Regulation of Nedd4-1 activity

Nedd4-1 is known to form an auto-inhibited conformation via intramolecular interaction between the C2 and HECT domains. Accordingly, the catalytic activity of Nedd4-1 can be activated by calcium.[19] Wang et al. suggested that the binding of calcium to the C2 domain recruits Nedd4-1 to the lipid membrane, and subsequently relieves auto-inhibitory conformation of Nedd4-1, thereby activating the enzyme. The auto-inhibited conformation of Nedd4-1 can also be relieved by adaptor proteins called Nedd4 family-interacting proteins, Ndfip1 and Ndfip2.[70] Mund et al. suggested that PY motifs in Ndfip proteins bind Nedd4-1 WW domains to directly control Nedd4-1 ligase activity. Ndfip proteins are also known to control Nedd4 HECT E3 family members by localizing them to endosomal membranes and by serving as adaptors for protein substrates.

Cross-talk between Nedd4-1 and IGF signalling

Ubiquitously expressed IGF-1R forms heterotetrameric complexes that contain cytoplasmic tyrosine kinase domains. Upon ligand binding, the IGF-1R undergoes auto-phosphorylation and activates downstream cell growth pathways such as PI3K-PDK1-Akt and Ras-Raf-Mek-Erk pathways. Similar to how JNK1 can activate or inactivate c-Jun and JunB, Nedd4-1 can also activate or inactivate IGF-1R signalling pathways. It is known that Nedd4-1 positively regulates IGF-1R in mouse embryonic fibroblast (MEF) during development where it can down-regulate the degradation process of IGF-1R mediated by the RING E3 ubiquitin ligase c-Cbl (Fig. 6-A). Here, Nedd4-1 inhibits the ubiquitin-mediated degradation of IGF-1R during embryonic development, thus positively controlling IGF-1R signalling.[69] This agrees with the growth retardation phenotype of *Nedd4-1* $^{-/-}$ mice.

In contrast to MEFs, stressed neuronal cells seem to use Nedd4-1 as a major E3 ligase for the degradation of IGF-1R.[71] Kwak et al. reported that neurotoxic zinc or hydrogen peroxide treatment promotes the expression of Nedd4-1 in cultured neurons to induce the poly-ubiquitination and proteasomal degradation of IGF-1R. Based on the facts that 1) fine tuning of IGF-1R regulation is critical to brain function, 2) neuronal stress upregulates Nedd4-1 expression resulting in the downregulation of IGF-1R, and 3) Nedd4-1 is mis-regulated in neurodegenerative diseases, Nedd4-1 is a promising drug-target to treat these diseases. However, the exact mechanisms that influence the Nedd4-1 regulation of IGF-1R remain to be determined.

Regulation of PTEN by Nedd4-1 : Controversy

One controversy surrounding the growth regulatory functions of Nedd4-1 is whether it poly-ubiquitinates and degrades the tumor suppressor PTEN. Nedd4-1 was initially identified in cell-based assays as the E3 ligase responsible for the poly-ubiquitination and proteasomal degradation of PTEN.[72] The same group also reported that Nedd4-1-mediated mono-ubiquitination of PTEN at Lys^{289} induces the nuclear translocation of PTEN which is essential for its tumor suppressive activity. The physiological relevance of Lys^{289} mono-ubiquitination is further confirmed by

the germline K289E mutations of PTEN in patients with Cowden Syndrome. [73] Furthermore, recent work showed that tyrosine kinase and tumor suppressor Rak phosphorylates Tyr^{336} residue of PTEN, and protects PTEN from polyubiquitination by Nedd4-1 in breast cancer.[74]

Contrary to these results, PTEN in *Nedd4-1*$^{-/-}$ MEFs demonstrated normal stability and nuclear localization, which questions if Nedd4-1 is a true regulator of PTEN.[69, 75] More recently however, Nedd4-1 has been suggested to target PTEN for lysosomal degradation in T cells by forming a Lys^{63}-linked ubiquitin chain on PTEN at Lys^{13} following TCR stimulation. Another study showed that binding of the adaptor protein Ndfip1 to Nedd4-1 stimulates ubiquitination of PTEN at Lys^{13} to signal the exosomal secretion of PTEN.[76] Intriguingly, secreted PTEN is internalized by neighboring cells and inhibits PI3K signalling in the recipient cells.

Given that PTEN is suggested to be ubiquitinated by several E3 enzymes, the development of selective small molecule inhibitors of Nedd4-1 would greatly facilitate the deconvolution of Nedd4-1's physiological roles. Following the example of nutlin, small molecule inhibitors of E3 enzymes that ubiquitinate the tumor suppressor PTEN hold great promise to develop new classes of anticancer therapeutics. The key challenge in developing such inhibitors is the need to inhibit PTEN polyubiquitination, but not its mono-ubiquitination to a) stabilize PTEN, and b) promote PTEN nuclear localization, which is essential for PTEN tumor suppressive activity.

Nedd4-2

Physiological Roles

Nedd4-2 is a major negative regulator of ion channels including ENaC,[77] CFTR,[78] NCC, [79] hERG,[80] EAAT2[81] in a variety of organs such as the brain, lungs, heart, and kidneys. Not surprisingly, misregulation of Nedd4-2 and its interactions has been implicated in cystic fibrosis, hypertensive disorders (Liddle's Syndrome), epilepsy,[82] diseases relating to repolarisation of heart tissue (Long QT Syndrome), and neurological disorders stemming from maligned glutamate transport. Remarkably, Nedd4-2 sits at the axis of at least four separate kinase pathways which orchestrates fluid and salt homeostasis by phosphorylating a set of residues that serve diverse and complex functions.

Regulation of Nedd4-2

Alternate splicing of the *NEDD4L* gene on chromosome 18q21.31 produces eight Nedd4-2 isoforms. Four isoforms contain an auto-inhibitory C2 domain, 4 WW domains, and a HECT domain, while two isoforms lack the C2 domain, and two lack the second WW domain. Interestingly, human isoform 3 lacks two of three phosphorylation sites that are critical to regulating the Nedd4-2/ENaC interaction. [83]

Calcium can activate Nedd4-2 by mediating an interaction between plasma membrane and the Nedd4-2 C2 domain, which binds the HECT domain inter- or intra-molecularly to auto-inhibit this cytosolic ligase.[19] Nedd4-2 also appears to be auto-inhibited by a weak interaction between its WW domains and a PY motif (LPXY) proximal to the catalytic cysteine on the HECT domain. [84] Binding of Nedd4-2 WW domains to substrate releases this inhibition and results in a reduced Nedd4-2 half-life. After ubiquitinating substrate, Nedd4-2 is unencumbered to mark

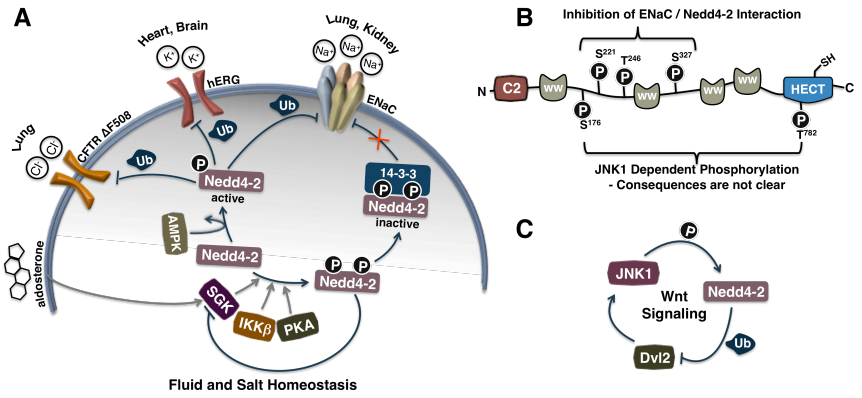

Figure 7. Nedd4-2 Negatively Regulates a Diversity of Ion Channels. (A) Phosphorylation of Nedd4-2 can inactivate or stimulate the ubiquitination of ion channels in a range of cell types. The phosphorylative activation of Nedd4-2 occurs through an unclarified mechanism, while binding of a 14-3-3 heterodimer to phosphorylated residues blocks certain Nedd4-2/substrate interactions. The colocalization of Neddd4-2, SGK1, and ENaC at the plasma membrane through the CNK3 scaffolding protein (not pictured) prolongs the half-life of ENaC. (B) The domain map of Nedd4-2 with key phosphorylation sites is numbered according to human isoform 4. JNK1 phosphorylation of Nedd4-2 is suggested to be stimulatory. (C) Nedd4-2 has been implicated in a Wnt pathway negative feedback loop where activation of JNK1 through Dvl2 results in the stimulatory phosphorylation of Nedd4-2, which may target Dvl2 for degradation and dampen the Wnt signal.

itself for degradation through auto-ubiquitination.

Cross-talk between Nedd4-2 and Hormone-Activated Kinases

The amiloride-sensitive epithelial Na^+ channel (ENaC) facilitates the movement of Na^+ into epithelial cells and is the best-studied target of Nedd4-2. This ion transporter is comprised of three subunits (α-β-γ), each containing a cytoplasmic C-terminus that bears a PY motif (PPXYXXL) to specify interaction with WW domains 3 and 4 of Nedd4-2.[85] Deletion of this PY motif is observed in patients with Liddle's Syndrome, a hereditary hypertensive disorder. Phosphorylation of Nedd4-2 at the linker between WW domains at Ser^{221}, Thr^{246}, and Ser^{327} inhibits the ENaC/Nedd4-2 interaction. For inhibition to occur, Ser^{327} and at least one of the other two residues must be phosphorylated.[86] A known activator of ENaC, the serum and glucocorticoid-induced kinase (SGK) is transcriptionally activated by aldosterone, binds Nedd4-2, and phosphorylates Ser^{327} and Thr^{246}. Independently, protein kinase A (PKA) can be activated by a vasopressin-induced elevation of cAMP to phosphorylate Nedd4-2 at Ser^{327} and Ser^{221}.[87] A third kinase, IκB kinase-β (IKKβ), phosphorylates Nedd4-2 at Ser^{327}, and likely allows a separate kinase to phosphorylate a second Nedd4-2 residue.[88, 89]Once Nedd4-2 is di-phosphorylated, a heterodimer of 14-3-3 proteins binds and inhibits the PY motif-WW domain interaction.[87, 90] Interestingly, phosphorylation of Nedd4-2 by SGK induces the Nedd4-2-mediated degradation of SGK, such that Nedd4-2 and SGK negatively regulate each other (Fig. 7-A, B).[91]

Stimulation of the glucocorticoid receptor induces transcription not only of the SGK1 target gene, but also of the scaffold protein Connector Enhancer of Kinase Suppressor of Ras Isoform 3 (CNK3), which colocalizes Nedd4-2 with SGK1 and ENaC as part of a 1.0 – 1.2 MDa multiprotein complex at the plasma membrane.[92]

Aldosterone-induced expression of CNK3 prolonged ENaC surface expression and amplified Na^+ current in mouse kidney cortical collecting duct cells to suggest that CNK3 assembles an ENaC stimulatory complex that drives the phosphorylation of key Nedd4-2 residues.

Oppositely, phosphorylation of Nedd4-2 has also been shown to accelerate the degradation of ENaC. Hallows and coworkers demonstrated that AMP-activated kinase phosphorylates Nedd4-2 to downregulate ENaC.[93] Presumably, the phosphorylation of Nedd4-2 enhances its ability to ubiquitinate ENaC, however, this mechanism and the Nedd4-2 residue(s) that are phosphorylated have not been identified.

A Potential Drug Target for Cystic Fibrosis

Nedd4-2 down-regulates the mutant form of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR-ΔF508) that is found in 70-90% of cystic fibrosis (CF) patients.[78] Loss of this mutant from the plasma membrane results in the overexpression of ENaC, which augments the proinflammatory phenotype of CF lungs through dehydration of the airway. In both CFPAC-1 and IB3-1 cells, Caohuy et al. showed that the cell surface expression of CFTR ΔF508 can be rescued by the activation of SGK1 with the dexamethasone to abrogate a Nedd4-2/CFTR ΔF508 complex and subsequent ubiquitination of CFTR. Nedd4-2 targets ENaC through a WW domain-PY motif interaction, however, CFTR does not bear a conventional PPXY motif, leaving the mode of interaction between Nedd4-2 and CFTR to be discovered.

Rescuing genetically defective CFTR is an emerging therapy for cystic fibrosis patients as evidenced by the 2012 FDA approval of ivacaftor.[94] This compound corrects the CFTR gating deficiency caused by a G551D mutation. Unlike the G551D mutant, CFTR ΔF508 experiences attenuated residence at the plasma membrane, a defect that has been difficult to address pharmacologically. Pedemonte and Galietta explain that CFTR undergoes multiple proteostasis checkpoints as it translocates from the endoplasmic reticulum to the plasma membrane.[95] Before Nedd4-2 targets CFTR ΔF508 for degradation, the E3 ligase RMA1 and CHIP may also ubiquitinate CFTR ΔF508 marking it for degradation. Combining a drug that corrects CFTR ΔF508 gating with siRNA silencing of RMA1 led to a 13-fold increase of CFTR ΔF508 stability at the plasma membrane.[96] Thus, combining the gating corrector with a Nedd4-2 inhibitor stands as a potential combinational therapy for a majority of cystic fibrosis patients. Such an approach would become even more dynamic with the development of RMA1 and CHIP inhibitors.

A Key Mediator of Development?

Interestingly, observations suggest that JNK1 phosphorylation of Nedd4-2 induces proteasomal degradation of the Wnt transducer Dishevelled2 (Dvl2) (Fig. 7-C).[97] Prior to this study, phosphorylation and phosphopeptide mass spectrometry suggested that Nedd4-2 Ser^{176} and Thr^{782} are JNK1 targets.[98] Mutation of these residues to alanine abrogates the majority of Nedd4-2 phosphorylation by JNK1, and inhibits the ability of these mutants to undergo auto-ubiquitination. However, the necessity for JNK1 to phosphorylate Nedd4-2 at these sites to activate the ligase for Dvl2 ubiquitination has not been rigorously demonstrated. Nevertheless, this study

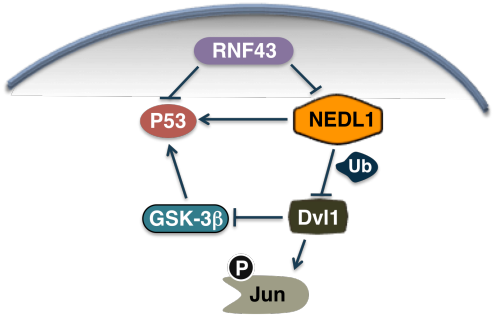

Figure 8. NEDL1 Targets the Kinase Scaffold Dvl1 for Degradation. In the context of amyotrophic lateral sclerosis (ALS), NEDL1 ubiquitinates Dvl1 within an aggregation of mutant SOD1, TRAP-δ, Dvl1, and NEDL1. This was evidenced by attenuated c-Jun phosphorylation upon overexpression of mutant SOD1, which stimulates formation of the aggregation complex. Also, NEDL1 stimulates p53 through a mechanism independent of its E3 ligase activity and RNF43 was found to target both p53 and NEDL1 for degradation.

proposes an intriguing negative feedback loop where Wnt/Dvl-stimulated JNK1 downregulates Dvl2 through Nedd4-2. Also of note, Nedd4-2 point mutations associated with epileptic photosensitivity (S112L, E150A, H414P) slightly relieved the Nedd4-2 degradation of Dvl2, thus implicating malfunctioning Nedd4-2 in neurodegenerative disease.

NEDL1

NEDL1 (NEDD4-like ubiquitin protein ligase-1) has been implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) by down-regulating the kinase scaffold, Dishevelled-1 (Dvl-1). Nakagawara and coworkers first identified NEDL1 in 2004 as a component of Lewy body-like hyaline inclusions along with the translocon-associated protein-δ (TRAP-δ), Dvl-1, and mutant forms of superoxide dismutase-1 (SOD1).[99] In this study, NEDL1 was shown to ubiquitinate wild type Dvl1 and also mutant SOD1 according to the ALS severity associated with the given mutation (SOD1 mutations linked to more aggressive cases of the disease were ubiquitinated to a greater extent). The same aggressive ALS SOD1 mutants bound Dvl1 to a greater extent and resulted in attenuated Ser^{63} phosphorylation of c-Jun when Dvl1 and SOD1 mutants were coexpressed in COS-7 cells. Such experiments suggest that NEDL1 aggregates with TRAP-δ, Dvl1, and uncleared SOD1 mutants to inhibit the normal progression of Dvl1-mediated transduction. In addition to mediating Wnt/β-catenin signalling, Dvl1 also functions within the JNK/c-Jun pathway, and inhibits glycogen synthase kinase-3β (GSK-3β) to moderate microtubule stability (Fig. 8).

Since the sequestration of Dvl1 by mutant SOD1 was enhanced by the overexpression of NEDL1, pharmacological inhibitors of NEDL1 could prove an effective ALS therapy. In 2010, the Nakagawara group observed transgenic mice with human NEDL1.[100] These mice experienced muscular atrophy with neuron degeneration and an increased number of microglial cells in the spinal cord. A variety of anti-inflammatory and antioxidants have prolonged survival in ALS mouse models, but the targets of these small molecules remain to be identified. Development of a NEDL1-specific inhibitor would certainly provide clarity in these

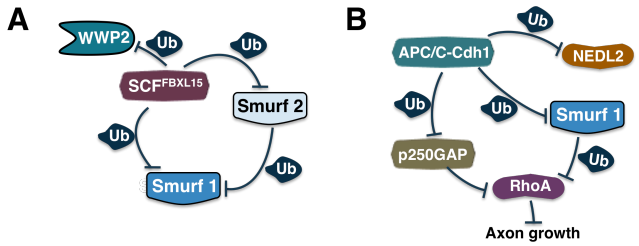

Figure 9. The Stability of Nedd4 Family Ligases is Regulated within an E3 Interaction Network. (A) SCF^{FBXL15} mediates the stability of WWP2, Smurf2, and Smurf1. Also, Smurf2 negatively regulates Smurf1. (B) The APC/C-Cdh1 E3 complex induces the degradation of NEDL2 and Smurf1 during the cell cycle.

studies.

Interestingly, NEDL1 was shown to increase the stability of p53 independent of its HECT domain through an unknown mechanism.[101] It was also demonstrated that siRNA knockdown of NEDL1 significantly increased the expression of the transmembrane tyrosine kinase ErbB4 in T47D breast cancer cells.[55] Since the dynamic control of ErbB4 turnover appears complex regarding cancer pathology, small molecule inhibitors that target specific C2-WW-HECT ligases would allow spatial-temporal disambiguation of each ligase's role in controlling ErbB4 activities.

Degradation of Nedd4 Family Ligases

As the pathways of Nedd4 family-mediated signalling come to light, an understanding of the mechanisms that govern ligase abundance is critical. To date, isolated experiments have suggested that *in trans* and *in cis* auto-ubiquitination, as well as ubiquitination by exogenous ligases mark these enzymes for degradation.[102] Smurf1, for example, appears susceptible to down-regulation following ubiquitination *in cis*, or exogenously by Smurf2,[103] SCF^{FBXL15},[104] or APC/C-Cdh1.[105] Questions thus arise: In what context is Smurf1 marked for degradation by each of these enzymes and are they mutually exclusive? While decades of work have traced intricate kinase pathways, organized schemes for the ubiquitination of E3 ligases are just now becoming possible. This section serves to highlight what is known about the degradation of Nedd4 family ligases and the challenges to deeper understanding.

The Nedd4 family ligases have demonstrated the ability to undergo auto-ubiquitination. WWP2, Nedd4-2, and Smurf1 undergo intramolecular auto-ubiquitination, while ITCH and Smurf2 are capable of transferring ubiquitin intermolecularly. The consequences for these auto-ubiquitinated ligases are diverse ranging from enhanced affinity for substrates, to ligase endocytosis, and to unknown outcomes as in the case of ITCH.[20] WWP2 undergoes *in cis* auto-ubiquitination with Lys^{63}-linked poly-ubiquitin chains to induce its proteasomal degradation.[106] This is somewhat unexpected since Lys^{63}-linked chains are usually bound by ESCRT0 to shuttle tagged substrates to lysosomes.[107] It is speculated that an abundance of protein tagged by Lys^{63}-linked poly-ubiquitin chains may overwhelm the ESCRT machinery and select a proteasomal degradation pathway. For investigators to accurately distinguish these degradation pathways, the use of proteasome and lysosome inhibitors will be useful in a model that preserves the endogenous copy number of the proteins in question. Since overexpression and siRNA experiments are not ideal for examining protein turnover, the development of inhibitors for

Nedd4 family ligases will be critical to elucidating the degradation pathways of these enzymes.

The targeting of Nedd4 family ligases for degradation by exogenous ligases implies a signalling network between E3 ligases. In particular, the E3 ligase SCF^{FBXL15} targets Smurf1, Smurf2 and WWP2 for degradation (Fig. 9-A). The transfection of HEK293T cells with Smurf1 and SCF^{FBXL15} suggested that the SCF ligase can target Smurf1 in a proteasome dependent manner to antagonize the Smurf1-induced degradation of SMAD1 and SMAD5.[104] Interestingly, the abundance of Smurf1 and Smurf2 in synchronized HeLa cells was high into S and G2 cell cycle phases, but then diminished going into M phase. The cell cycle dependence of Smurf abundance is fascinating regarding the spatial-temporal regulation of Nedd4 ligases.

The cell cycle-dependent regulation of HECT ligases is also found in work exploring the proteasomal degradation of Smurf1 by the APC/C-Cdh1 to allow myelin-stimulated inhibition of axon growth through the RhoA GTPase (Fig. 9-B).[108] Here, cerebella from $Cdh^{+/-}$ mice showed enhanced stabilization of Smurf1 compared to wild type tissues. Also, treatment of granule neuron lysates with the proteasome inhibitor lactacystin enhanced Smurf1 stability. It would be interesting to examine the relationship between ubiquitination of Smurf1 by APC/C-Cdh1 vs SCF^{FBXL15}. Although relatively little is known about the ligase NEDL2, it was recently shown to reach peak abundance in synchronized HeLa cells at mitosis and was lowest in G1 phase.[109] The level of NEDL2 mRNA was constant throughout the experiment, and the reduced protein level was rescued by treating cells with Cdh-1-specific siRNA or the proteasome inhibitor MG132. Immunofluorescent staining showed NEDL2 to associate with α-tubulin and to assemble on the mitotic spindle throughout mitosis, and siRNA knockdown of NEDL2 delayed the onset of anaphase.

Although several mechanisms by which Nedd4 family ligases are degraded are known, the contexts in which these mechanisms operate are just now coming to light. The distinction between proteasomal and lysosomal degradation is important and likely depends on the baseline abundance of the ligase and potential trafficking partners such as ESCRT0. Moreover, the emergence of cell cycle dependence for several ligases highlights the temporal dependence of their abundance and hints toward the discovery of new substrates and cell cycle roles.

Conclusions

The ubiquitin system plays critical roles in controlling many cellular processes through protein degradation and non-proteolytic signalling. Since aberrant poly-ubiquitination of tumor suppressors such as p53, PTEN, and LATS by E3 ligases contributes to cancer development, pharmacological inhibitors of E3 enzymes may offer tremendous therapeutic benefit. Furthermore, the need to understand E3-kinase crosstalk is critical given the finding that inhibition of Smurf2 sensitizes melanoma cells toward MEK inhibitors. While the mechanism by which this sensitization occurs requires further clarification, a combination therapy appears promising. Not only do E3 enzymes regulate kinase-signalling cascades, but they also regulate ion channels and cell membrane receptors, representing novel drug targets to treat cystic fibrosis, and other diseases resulting from ion channel deficiencies, such as Liddle's Syndrome.

Since the activity of E3 ligases and E3/substrate interactions are frequently regulated by phosphorylation, kinase inhibitors may gain some of their therapeutic power by impacting the activities undergone by E3 ligases which have been elusive drug targets. A prominent example is dexamethasone-induced activation of SGK1 kinase which rescues CFTR ΔF508, by inhibiting Nedd4-2 ligase and abrogating the formatoin of Nedd4-2/CFTR ΔF508 complex and subsequent ubiquitination of CFTR.

Finally, we would like to draw attention to the emerging realization of E3 ligase signalling networks, where E3 ligases mediate the degradation of one another to stabilize protein substrates. The elucidation of these networks is critical to the accurate pharmacological assessment of E3 enzyme inhibitors. The spatial-temporal dependence of these networks and their physiological implications represent impactful areas of new discovery. In summary, understanding the interplay between protein kinases and E3 ligases is important since kinase signalling networks may provide hints on future therapeutic applications of E3 enzyme inhibitors.

References

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

1. K. W. Moremen, M. Tiemeyer and A. V. Nairn, *Nature reviews. Molecular cell biology*, 2012, **13**, 448-462.
2. B. Fierz, C. Chatterjee, R. K. McGinty, M. Bar-Dagan, D. P. Raleigh and T. W. Muir, *Nature chemical biology*, 2011, **7**, 113-119.
3. H. D. Ulrich, *Molecular cell*, 2008, **32**, 301-305.
4. G. A. Khoury, R. C. Baliban and C. A. Floudas, *Scientific reports*, 2011, **1**.
5. D. Komander and M. Rape, *Annual review of biochemistry*, 2012, **81**, 203-229.
6. L. Hicke and R. Dunn, *Annual review of cell and developmental biology*, 2003, **19**, 141-172.
7. S. Lorenz, A. J. Cantor, M. Rape and J. Kuriyan, *BMC biology*, 2013, **11**, 65.
8. J. H. Luo and I. B. Weinstein, *The Journal of biological chemistry*, 1993, **268**, 23580-23584.
9. R. Dunn, D. A. Klos, A. S. Adler and L. Hicke, *The Journal of cell biology*, 2004, **165**, 135-144.
10. A. Marchese, C. Raiborg, F. Santini, J. H. Keen, H. Stenmark and J. L. Benovic, *Developmental cell*, 2003, **5**, 709-722.
11. A. Angers, A. R. Ramjaun and P. S. McPherson, *The Journal of biological chemistry*, 2004, **279**, 11471-11479.
12. M. Scheffner and S. Kumar, *Biochimica et biophysica acta*, 2013.
13. Z. Lu and T. Hunter, *Annual review of biochemistry*, 2009, **78**, 435-475.
14. W. L. Perry, C. M. Hustad, D. A. Swing, T. N. O'Sullivan, N. A. Jenkins and N. G. Copeland, *Nature genetics*, 1998, **18**, 143-146.
15. Y. C. Liu, *Seminars in immunology*, 2007, **19**, 197-205.
16. M. Gao, T. Labuda, Y. Xia, E. Gallagher, D. Fang, Y. C. Liu and M. Karin, *Science (New York, N.Y.)*, 2004, **306**, 271-275.

17. N. J. Lohr, J. P. Molleston, K. A. Strauss, W. Torres-Martinez, E. A. Sherman, R. H. Squires, N. L. Rider, K. R. Chikwava, O. W. Cummings, D. H. Morton and E. G. Puffenberger, *American journal of human genetics*, 2010, **86**, 447-453.
18. E. Gallagher, M. Gao, Y. C. Liu and M. Karin, *Proceedings of the National Academy of Sciences of the United States of America*, 2006, **103**, 1717-1722.
19. J. Wang, Q. Peng, Q. Lin, C. Childress, D. Carey and W. Yang, *The Journal of biological chemistry*, 2010, **285**, 12279-12288.
20. F. Scialpi, M. Malatesta, A. Peschiaroli, M. Rossi, G. Melino and F. Bernassola, *Biochemical pharmacology*, 2008, **76**, 1515-1521.
21. Y. H. Ahn and J. M. Kurie, *The Journal of biological chemistry*, 2009, **284**, 29399-29404.
22. B. Gao, S. M. Lee and D. Fang, *The Journal of biological chemistry*, 2006, **281**, 29711-29718.
23. C. Yang, W. Zhou, M. S. Jeon, D. Demydenko, Y. Harada, H. Zhou and Y. C. Liu, *Molecular cell*, 2006, **21**, 135-141.
24. H. Zhu, P. Kavsak, S. Abdollah, J. L. Wrana and G. H. Thomsen, *Nature*, 1999, **400**, 687-693.
25. M. Yamashita, S. X. Ying, G. M. Zhang, C. Li, S. Y. Cheng, C. X. Deng and Y. E. Zhang, *Cell*, 2005, **121**, 101-113.
26. P. A. Chong, H. Lin, J. L. Wrana and J. D. Forman-Kay, *Proceedings of the National Academy of Sciences of the United States of America*, 2010, **107**, 18404-18409.
27. P. L. Cheng, H. Lu, M. Shelly, H. Gao and M. M. Poo, *Neuron*, 2011, **69**, 231-243.
28. K. Lu, X. Yin, T. Weng, S. Xi, L. Li, G. Xing, X. Cheng, X. Yang, L. Zhang and F. He, *Nature cell biology*, 2008, **10**, 994-1002.
29. Y. E. Zhang, *Cell research*, 2009, **19**, 128-139.
30. B. Bierie and H. L. Moses, *Cytokine & growth factor reviews*, 2010, **21**, 49-59.
31. M. A. Al-Salihi, L. Herhaus and G. P. Sapkota, *Open biology*, 2012, **2**, 120082.
32. W. A. Border and N. A. Noble, *The New England journal of medicine*, 1994, **331**, 1286-1292.
33. S. M. Soond and A. Chantry, *Oncogene*, 2011, **30**, 2451-2462.
34. G. Sapkota, C. Alarcon, F. M. Spagnoli, A. H. Brivanlou and J. Massague, *Molecular cell*, 2007, **25**, 441-454.
35. C. Alarcon, A. I. Zaromytidou, Q. Xi, S. Gao, J. Yu, S. Fujisawa, A. Barlas, A. N. Miller, K. Manova-Todorova, M. J. Macias, G. Sapkota, D. Pan and J. Massague, *Cell*, 2009, **139**, 757-769.
36. M. E. Engel, M. A. McDonnell, B. K. Law and H. L. Moses, *The Journal of biological chemistry*, 1999, **274**, 37413-37420.
37. A. V. Bakin, C. Rinehart, A. K. Tomlinson and C. L. Arteaga, *Journal of cell science*, 2002, **115**, 3193-3206.
38. M. Narimatsu, R. Bose, M. Pye, L. Zhang, B. Miller, P. Ching, R. Sakuma, V. Luga, L. Roncari, L. Attisano and J. L. Wrana, *Cell*, 2009, **137**, 295-307.
39. L. Y. Tang, M. Yamashita, N. P. Coussens, Y. Tang, X. Wang, C. Li, C. X. Deng, S. Y. Cheng and Y. E. Zhang, *The EMBO journal*, 2011, **30**, 4777-4789.
40. S. Wiesner, A. A. Ogunjimi, H. R. Wang, D. Rotin, F. Sicheri, J. L. Wrana and J. D. Forman-Kay, *Cell*, 2007, **130**, 651-662.
41. A. A. Ogunjimi, D. J. Briant, N. Pece-Barbara, C. Le Roy, G. M. Di Guglielmo, P. Kavsak, R. K. Rasmussen, B. T. Seet, F. Sicheri and J. L. Wrana, *Molecular cell*, 2005, **19**, 297-308.
42. R. Tan, W. He, X. Lin, L. P. Kiss and Y. Liu, *American journal of physiology. Renal physiology*, 2008, **294**, F1076-1083.
43. A. Nakano, D. Koinuma, K. Miyazawa, T. Uchida, M. Saitoh, M. Kawabata, J. Hanai, H. Akiyama, M. Abe, K. Miyazono, T. Matsumoto and T. Imamura, *The Journal of biological chemistry*, 2009, **284**, 6109-6115.
44. M. P. Smith, J. Ferguson, I. Arozarena, R. Hayward, R. Marais, A. Chapman, A. Hurlstone and C. Wellbrock, *Journal of the National Cancer Institute*, 2013, **105**, 33-46.
45. G. Pirozzi, S. J. McConnell, A. J. Uveges, J. M. Carter, A. B. Sparks, B. K. Kay and D. M. Fowlkes, *The Journal of biological chemistry*, 1997, **272**, 14611-14616.
46. L. Shu, H. Zhang, B. F. Boyce and L. Xing, *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*, 2013, **28**, 1925-1935.

47. A. Komuro, T. Imamura, M. Saitoh, Y. Yoshida, T. Yamori, K. Miyazono and K. Miyazawa, *Oncogene*, 2004, **23**, 6914-6923.
48. C. Chen, X. Sun, P. Guo, X. Y. Dong, P. Sethi, X. Cheng, J. Zhou, J. Ling, J. W. Simons, J. B. Lingrel and J. T. Dong, *The Journal of biological chemistry*, 2005, **280**, 41553-41561.
49. C. Chen, X. Sun, P. Guo, X. Y. Dong, P. Sethi, W. Zhou, Z. Zhou, J. Petros, H. F. Frierson, Jr., R. L. Vessella, A. Atfi and J. T. Dong, *Oncogene*, 2007, **26**, 2386-2394.
50. N. S. Nguyen Huu, W. D. Ryder, N. Zeps, M. Flasza, M. Chiu, A. M. Hanby, R. Poulsom, R. B. Clarke and M. Baron, *The Journal of pathology*, 2008, **216**, 93-102.
51. M. Flasza, P. Gorman, R. Roylance, A. E. Canfield and M. Baron, *Biochemical and biophysical research communications*, 2002, **290**, 431-437.
52. C. Chen and L. E. Matesic, *Cancer metastasis reviews*, 2007, **26**, 587-604.
53. S. R. Seo, F. Lallemand, N. Ferrand, M. Pessah, S. L'Hoste, J. Camonis and A. Atfi, *The EMBO journal*, 2004, **23**, 3780-3792.
54. B. Yeung, K. C. Ho and X. Yang, *PloS one*, 2013, **8**, e61027.
55. Y. Li, Z. Zhou, M. Alimandi and C. Chen, *Oncogene*, 2009, **28**, 2948-2958.
56. M. A. St John, W. Tao, X. Fei, R. Fukumoto, M. L. Carcangiu, D. G. Brownstein, A. F. Parlow, J. McGrath and T. Xu, *Nature genetics*, 1999, **21**, 182-186.
57. Y. Hao, A. Chun, K. Cheung, B. Rashidi and X. Yang, *The Journal of biological chemistry*, 2008, **283**, 5496-5509.
58. C. Webb, A. Upadhyay, F. Giuntini, I. Eggleston, M. Furutani-Seiki, R. Ishima and S. Bagby, *Biochemistry*, 2011, **50**, 3300-3309.
59. Z. Salah, G. Melino and R. I. Aqeilan, *Cancer research*, 2011, **71**, 2010-2020.
60. W. Zou, X. Chen, J. H. Shim, Z. Huang, N. Brady, D. Hu, R. Drapp, K. Sigrist, L. H. Glimcher and D. Jones, *Nature cell biology*, 2011, **13**, 59-65.
61. Y. Nakamura, K. Yamamoto, X. He, B. Otsuki, Y. Kim, H. Murao, T. Soeda, N. Tsumaki, J. M. Deng, Z. Zhang, R. R. Behringer, B. Crombrugghe, J. H. Postlethwait, M. L. Warman, T. Nakamura and H. Akiyama, *Nature communications*, 2011, **2**, 251.
62. A. Chantry, *Cell cycle (Georgetown, Tex.)*, 2011, **10**, 2437-2439.
63. S. Maddika, S. Kavela, N. Rani, V. R. Palicharla, J. L. Pokorny, J. N. Sarkaria and J. Chen, *Nature cell biology*, 2011, **13**, 728-733.
64. L. D. Mayo, J. E. Dixon, D. L. Durden, N. K. Tonks and D. B. Donner, *The Journal of biological chemistry*, 2002, **277**, 5484-5489.
65. S. M. Planchon, K. A. Waite and C. Eng, *Journal of cell science*, 2008, **121**, 249-253.
66. G. Jin, M. J. Kim, H. S. Jeon, J. E. Choi, D. S. Kim, E. B. Lee, S. I. Cha, G. S. Yoon, C. H. Kim, T. H. Jung and J. Y. Park, *Lung cancer (Amsterdam, Netherlands)*, 2010, **69**, 279-283.
67. H. Kato, S. Kato, T. Kumabe, Y. Sonoda, T. Yoshimoto, S. Kato, S. Y. Han, T. Suzuki, H. Shibata, R. Kanamaru and C. Ishioka, *Clinical cancer research : an official journal of the American Association for Cancer Research*, 2000, **6**, 3937-3943.
68. S. Kumar, Y. Tomooka and M. Noda, *Biochemical and biophysical research communications*, 1992, **185**, 1155-1161.
69. X. R. Cao, N. L. Lill, N. Boase, P. P. Shi, D. R. Croucher, H. Shan, J. Qu, E. M. Sweezer, T. Place, P. A. Kirby, R. J. Daly, S. Kumar and B. Yang, *Science signalling*, 2008, **1**, ra5.
70. T. Mund and H. R. Pelham, *EMBO reports*, 2009, **10**, 501-507.
71. Y. D. Kwak, B. Wang, J. J. Li, R. Wang, Q. Deng, S. Diao, Y. Chen, R. Xu, E. Masliah, H. Xu, J. J. Sung and F. F. Liao, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 2012, **32**, 10971-10981.
72. X. Wang, L. C. Trotman, T. Koppie, A. Alimonti, Z. Chen, Z. Gao, J. Wang, H. Erdjument-Bromage, P. Tempst, C. Cordon-Cardo, P. P. Pandolfi and X. Jiang, *Cell*, 2007, **128**, 129-139.
73. L. C. Trotman, X. Wang, A. Alimonti, Z. Chen, J. Teruya-Feldstein, H. Yang, N. P. Pavletich, B. S. Carver, C. Cordon-Cardo, H. Erdjument-Bromage, P. Tempst, S. G. Chi, H. J. Kim, T. Misteli, X. Jiang and P. P. Pandolfi, *Cell*, 2007, **128**, 141-156.
74. E. K. Yim, G. Peng, H. Dai, R. Hu, K. Li, Y. Lu, G. B. Mills, F. Meric-Bernstam, B. T. Hennessy, R. J. Craven and S. Y. Lin, *Cancer cell*, 2009, **15**, 304-314.
75. F. Fouladkou, T. Landry, H. Kawabe, A. Neeb, C. Lu, N. Brose, V. Stambolic and D. Rotin, *Proceedings of the National Academy of Sciences of the United States of America*, 2008, **105**, 8585-8590.

76. U. Putz, J. Howitt, A. Doan, C. P. Goh, L. H. Low, J. Silke and S. S. Tan, *Science signalling*, 2012, **5**, ra70.
77. E. Kamynina, C. Debonneville, M. Bens, A. Vandewalle and O. Staub, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 2001, **15**, 204-214.
78. H. Caohuy, C. Jozwik and H. B. Pollard, *The Journal of biological chemistry*, 2009, **284**, 25241-25253.
79. J. P. Arroyo, D. Lagnaz, C. Ronzaud, N. Vazquez, B. S. Ko, L. Moddes, D. Ruffieux-Daidie, P. Hausel, R. Koesters, B. Yang, J. B. Stokes, R. S. Hoover, G. Gamba and O. Staub, *Journal of the American Society of Nephrology : JASN*, 2011, **22**, 1707-1719.
80. J. Guo, T. Wang, X. Li, H. Shallow, T. Yang, W. Li, J. Xu, M. D. Fridman, X. Yang and S. Zhang, *The Journal of biological chemistry*, 2012, **287**, 33132-33141.
81. C. Boehmer, M. Palmada, J. Rajamanickam, R. Schniepp, S. Amara and F. Lang, *Journal of neurochemistry*, 2006, **97**, 911-921.
82. L. M. Dibbens, J. Ekberg, I. Taylor, B. L. Hodgson, S. J. Conroy, I. L. Lensink, S. Kumar, M. A. Zielinski, L. A. Harkin, G. R. Sutherland, D. J. Adams, S. F. Berkovic, I. E. Scheffer, J. C. Mulley and P. Poronnik, *Genes, brain, and behavior*, 2007, **6**, 750-755.
83. O. A. Itani, J. R. Campbell, J. Herrero, P. M. Snyder and C. P. Thomas, *American journal of physiology. Renal physiology*, 2003, **285**, F916-929.
84. M. C. Bruce, V. Kanelis, F. Fouladkou, A. Debonneville, O. Staub and D. Rotin, *The Biochemical journal*, 2008, **415**, 155-163.
85. O. Staub, S. Dho, P. Henry, J. Correa, T. Ishikawa, J. McGlade and D. Rotin, *The EMBO journal*, 1996, **15**, 2371-2380.
86. P. M. Snyder, D. R. Olson, R. Kabra, R. Zhou and J. C. Steines, *The Journal of biological chemistry*, 2004, **279**, 45753-45758.
87. T. Ichimura, H. Yamamura, K. Sasamoto, Y. Tominaga, M. Taoka, K. Kakiuchi, T. Shinkawa, N. Takahashi, S. Shimada and T. Isobe, *The Journal of biological chemistry*, 2005, **280**, 13187-13194.
88. R. S. Edinger, J. Lebowitz, H. Li, R. Alzamora, H. Wang, J. P. Johnson and K. R. Hallows, *The Journal of biological chemistry*, 2009, **284**, 150-157.
89. P. M. Snyder, *Science signalling*, 2009, **2**, pe41.
90. X. Liang, M. B. Butterworth, K. W. Peters, W. H. Walker and R. A. Frizzell, *The Journal of biological chemistry*, 2008, **283**, 27418-27425.
91. R. Zhou and P. M. Snyder, *The Journal of biological chemistry*, 2005, **280**, 4518-4523.
92. R. Soundararajan, T. Ziera, E. Koo, K. Ling, J. Wang, S. A. Borden and D. Pearce, *The Journal of biological chemistry*, 2012, **287**, 33014-33025.
93. V. Bhalla, N. M. Oyster, A. C. Fitch, M. A. Wijngaarden, D. Neumann, U. Schlattner, D. Pearce and K. R. Hallows, *The Journal of biological chemistry*, 2006, **281**, 26159-26169.
94. B. W. Ramsey, J. Davies, N. G. McElvaney, E. Tullis, S. C. Bell, P. Drevinek, M. Griese, E. F. McKone, C. E. Wainwright, M. W. Konstan, R. Moss, F. Ratjen, I. Sermet-Gaudelus, S. M. Rowe, Q. Dong, S. Rodriguez, K. Yen, C. Ordonez and J. S. Elborn, *The New England journal of medicine*, 2011, **365**, 1663-1672.
95. N. Pedemonte and L. J. Galietta, *Frontiers in pharmacology*, 2012, **3**, 175.
96. D. E. Grove, M. F. Rosser, H. Y. Ren, A. P. Naren and D. M. Cyr, *Molecular biology of the cell*, 2009, **20**, 4059-4069.
97. Y. Ding, Y. Zhang, C. Xu, Q. H. Tao and Y. G. Chen, *The Journal of biological chemistry*, 2013, **288**, 8289-8298.
98. K. R. Hallows, V. Bhalla, N. M. Oyster, M. A. Wijngaarden, J. K. Lee, H. Li, S. Chandran, X. Xia, Z. Huang, R. J. Chalkley, A. L. Burlingame and D. Pearce, *The Journal of biological chemistry*, 2010, **285**, 21671-21678.
99. K. Miyazaki, T. Fujita, T. Ozaki, C. Kato, Y. Kurose, M. Sakamoto, S. Kato, T. Goto, Y. Itoyama, M. Aoki and A. Nakagawara, *The Journal of biological chemistry*, 2004, **279**, 11327-11335.
100. L. Zhang, S. Haraguchi, T. Koda, K. Hashimoto and A. Nakagawara, *Journal of biomedicine & biotechnology*, 2011, **2011**, 831092.
101. Y. Li, T. Ozaki, H. Kikuchi, H. Yamamoto, M. Ohira and A. Nakagawara, *Oncogene*, 2008, **27**, 3700-3709.
102. P. de Bie and A. Ciechanover, *Cell death and differentiation*, 2011, **18**, 1393-1402.
103. E. Fukunaga, Y. Inoue, S. Komiya, K. Horiguchi, K. Goto, M. Saitoh, K. Miyazawa, D. Koinuma, A. Hanyu and T. Imamura, *The Journal of biological chemistry*, 2008, **283**, 35660-35667.

104. Y. Cui, S. He, C. Xing, K. Lu, J. Wang, G. Xing, A. Meng, S. Jia, F. He and L. Zhang, *The EMBO journal*, 2011, **30**, 2675-2689.
105. M. Kannan, S. J. Lee, N. Schwedhelm-Domeyer and J. Stegmuller, *Development (Cambridge, England)*, 2012, **139**, 3600-3612.
106. B. Liao and Y. Jin, *Cell research*, 2010, **20**, 332-344.
107. J. A. Nathan, H. T. Kim, L. Ting, S. P. Gygi and A. L. Goldberg, *The EMBO journal*, 2013, **32**, 552-565.
108. M. Kannan, S. J. Lee, N. Schwedhelm-Domeyer, T. Nakazawa and J. Stegmuller, *PloS one*, 2012, **7**, e50735.
109. L. Lu, S. Hu, R. Wei, X. Qiu, K. Lu, Y. Fu, H. Li, G. Xing, D. Li, R. Peng, F. He and L. Zhang, *The Journal of biological chemistry*, 2013.