

Consistent with this model, a Skp2 mutant lacking the N-terminal 90 amino acids formed a SCF complex more efficiently than full-length Skp2 (ref. 2).

In contrast to the findings of Lin *et al.* that Ser 72 phosphorylation is required for SCF-Skp2 assembly and activity<sup>2</sup>, others found recently that simultaneous mutation of amino acids 64 and 72 (S64D/S72D and S64A/S72A) did not affect SCF assembly or its activity<sup>10</sup>. In these experiments, Skp2 and all other SCF subunits, as well as the cofactor Cks1, were overexpressed<sup>10</sup>, whereas Lin *et al.* only expressed Skp2. Several experimental differences may explain the opposite outcome. For example, overexpression of all SCF subunits may favour SCF-Skp2 complex formation and ligase activity even in the absence of Ser 72 phosphorylation.

Adding to this complexity, Gao *et al.* and Lin *et al.* both found that Ser 72 phosphorylation also translocates the protein to the cytoplasm. Again, two different mechanisms seem to contribute to this. First, Ser 72 is located within a putative nuclear localization sequence (NLS) and its phosphorylation impairs Skp2 binding to nuclear import receptors<sup>3</sup>. Second, Lin *et al.* and Gao *et al.* found that Ser 72 phosphorylation facilitates Skp2 binding to 14-3-3 proteins<sup>2,3</sup>. Skp2 cytoplasmic localization required 14-3-3 $\beta$ <sup>3</sup>.

Overexpressed Skp2<sup>S72D</sup> shows only partial cytoplasmic localization<sup>2,3</sup>, and a Skp2<sup>S64D/S72D</sup> double mutant was mainly nuclear<sup>10</sup>; however, endogenous Ser 72-phosphorylated Skp2 was predominantly cytosolic, but not nuclear<sup>2</sup>.

Akt1-phosphorylated Skp2 is bound in 14-3-3 complexes, which anchors the protein in the cytoplasm<sup>2</sup>. Overexpression of Skp2 may exceed the 14-3-3 pool, permitting partial nuclear localization. The cytoplasmic localization of Skp2 raises a number of interesting questions. For example, can Skp2/14-3-3 integrate into active SCF complexes? If so, does this alter target substrate selection and what are central substrates of cytosolic SCF-Skp2? If nuclear, how can Ser 72-phosphorylated Skp2 escape 14-3-3 and how is the protein imported? Of note, a Skp2-NES (nuclear export signal) fusion protein was predominantly cytosolic but unable to form an active SCF or to ubiquitylate p27 (ref. 2). As Cdh1 is usually nuclear, is inhibition of Cdh1 binding physiologically significant as long as most Ser 72-phosphorylated Skp2 resides in the cytoplasm?

Skp2 overexpression by gene amplification is frequently observed in metastatic tumours<sup>5</sup>. Lin *et al.* found that Skp2<sup>-/-</sup> MEFs showed a profound defect in cell migration, which could be compensated by Skp2<sup>S72D</sup> but not Skp2<sup>S72A</sup> (ref. 2), and a predominantly cytosolic Skp2-NES fusion protein rescued migration of null MEFs. These findings suggest that cytoplasmic Skp2 has a potential function in metastasis. Although p27 has a well-established role in cell migration<sup>4</sup>, regulation of cell motility by cytoplasmic Skp2 seems to be independent of its ability to ubiquitylate p27, as Skp2-NES fails to form a ubiquitin ligase<sup>2</sup>. Further studies should elucidate mechanisms by which cytoplasmic Skp2 affects cell motility.

Taken together, these studies provide compelling evidence that Skp2 phosphorylation on Ser 72 has a central role in tumorigenesis. Skp2 phosphorylation seems to affect Skp2 localization and activity by several complementary mechanisms. The cluster of three phosphorylation sites of different phylogenetic conservation located within a region of Skp2 required for Cdh1 binding and adjacent to the F-box suggests possible redundant functions that could explain the variable molecular consequences observed in response to phosphorylation. It is interesting that although mouse Skp2 lacks Ser 72, most molecular consequences of Akt phosphorylation are also observed in mice<sup>2</sup> suggesting that the Akt-Skp2 axis is functionally conserved but may use distinct mechanisms.

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## Targeting protein ubiquitylation: DDB1 takes its RING off

Sarah Jackson and Yue Xiong

**Ubiquitin E3 ligases of the RING and HECT families are distinct not only in their catalytic mechanisms but also in targeting substrates. Now it seems that one heterodimeric complex can target substrates to both types of E3 ligase.**

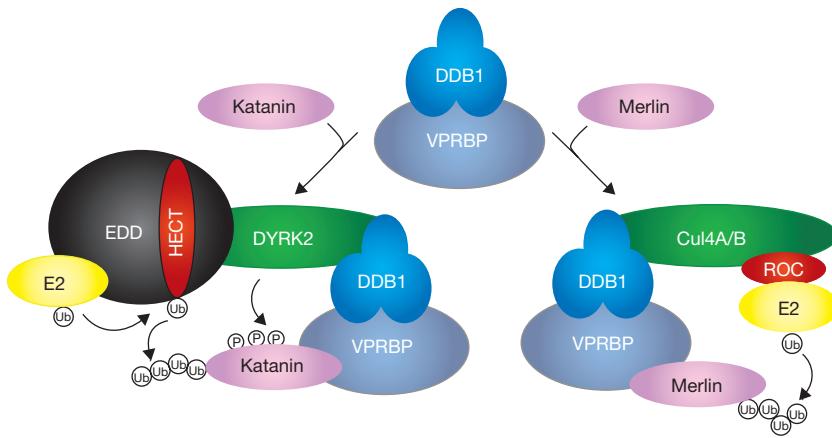
Protein ubiquitylation has a broad and critical role in regulating a wide range of cellular processes. The addition of Lys 48-linked polyubiquitin chains to specific substrate

proteins regulates timely degradation by the 26S proteasome. In addition, like other covalent modifications, ubiquitylation can modulate the function of a substrate by causing a conformational change. Ubiquitylation begins with the ATP-dependent activation of ubiquitin by the E1 enzyme, and is followed by the subsequent transfer of ubiquitin to one of a small family of E2 ubiquitin-conjugating

enzymes; finally, an E3 ubiquitin ligase is responsible for recognizing a specific substrate and promoting ubiquitin ligation. More than 1,000 distinct E3 ligases are predicted to exist, either as individual proteins or multi-subunit complexes, in mammalian cells.

There are two major families of E3 ligases distinguished by their active domains: the HECT family ('homologous to the E6-AP

Sarah Jackson and Yue Xiong are in the Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, North Carolina 27599, USA. e-mail: yxiong@email.unc.edu



**Figure 1** DDB1–VPRBP targets substrates to distinct E3 ubiquitin ligase complexes. The DDB1–VPRBP heterodimer can target different substrate to a DYRK2–HECT or a Cul4–ROC1 E3 ligase complex. DYRK2 is required for assembly of the E3 complex and for phosphorylation of its substrate katanin, but not for the initial binding of katanin with VPRBP. Ub, ubiquitin.

carboxy terminus') and the RING family (first recognized in the human 'really interesting new gene product')<sup>1,2</sup>. The HECT domain mediates interaction with the cognate E2 and, through an evolutionarily conserved cysteine residue, forms a thioester linkage with ubiquitin. Human cells contain as many as 28 HECT proteins and most, if not all, are believed to function as E3 ligases. Unlike the HECT domain, the RING domain promotes a direct transfer of ubiquitin from the E2 to the substrate without forming an intermediate with ubiquitin. Human cells express more than 450 RING proteins, and E3 ligase activity has been experimentally demonstrated for many of them. In addition, although not containing a RING domain themselves, members of the evolutionarily conserved cullin family can bind a small RING protein, either ROC1 or ROC2 (also known as Rbx). A remarkable feature of cullin proteins is that the amino-terminal sequence in each of the six classical human cullin family members interacts selectively with a different motif such as an F-box, a SOCS box, a BTB domain and a WD40 repeat. These common motifs are present in many proteins, suggesting the potential assembly of as many as 300–500 distinct cullin–RING ligase (CRL) complexes *in vivo*<sup>3</sup>, making cullins the largest subfamily of E3 ligases.

Not only do HECT and CRL E3 ligases use different catalytic mechanisms in catalysing the transfer of ubiquitin from E2 to the substrate, they are also thought to have unique means of assembly, regulation and substrate targeting. On page 409 of this issue, Maddika and Chen<sup>4</sup> identify and characterize a novel E3 ligase that uses DYRK2 as a scaffold for the assembly

of a HECT E3 complex and a heterodimeric complex consisting of DDB1 and VPRBP for recruiting substrate. This finding is particularly unexpected because DYRK2 is a protein kinase and DDB1 is established as a key adaptor protein for recruiting substrate to the Cul4–RING ligases (CRLs)<sup>5–8</sup>.

DYRK2 is a member of evolutionarily conserved dual-specificity tyrosine (Y)-regulated kinases, whose function has been broadly linked to DNA repair, cell proliferation, differentiation and apoptosis. Maddika and Chen identify a novel DYRK2 complex that contains EDD, DDB1 and VPRBP. EDD (E3 identified by differential display) is a large protein containing multiple domains linked to ubiquitylation, including an N-terminal ubiquitin associated (UBA) domain, a UBR box (a motif important for the targeting of N-end rule substrates) and a C-terminal HECT domain. No known substrate has previously been identified for EDD. DDB1 (damaged-DNA-binding protein) serves as a key linker to bridge a subset of WD40-containing proteins to Cul4–RING ligases<sup>5–8</sup>. As many as one-third of the 300 WD40 proteins found in human cells could interact with DDB1 (ref. 5). VPRBP, a WD40-containing protein that binds DDB1, was initially identified as the human HIV Vpr-binding protein. The significance of VPRBP–Vpr interaction remains unclear, especially whether Vpr, like E6, hijacks a VPRBP complex or exploits normal substrate ubiquitylation to benefit HIV propagation. So far, only one candidate substrate, the cytoplasmic localized neurofibromatosis type 2 (NF2) tumor suppressor gene product, Merlin, has been reported to be targeted by VPRBP to the DDB1–Cul4–ROC1 ligase for degradation<sup>9</sup>.

However, there are reasons to believe that VPRBP may target additional proteins, because VPRBP can bind to chromatin and is required for normal DNA replication, and genetic disruption of *VPRBP* causes early embryonic lethality in mouse and various developmental defects in plants<sup>10,11</sup>.

In *Caenorhabditis elegans*, the DYRK2 homolog MBK-2 phosphorylates and regulates the meiotic protein, MEI-1/katanin, the catalytic subunit of the microtubule-severing AAA ATPase complex. Maddika and Chen<sup>4</sup> therefore tested whether mammalian katanin was a substrate for the newly identified DYRK2 E3 complex, referred to as EDVP (EDD–DDB1–VPRBP). *In vitro* binding and *in vivo* ubiquitylation assays demonstrated that katanin associates with and is polyubiquitylated by the EDVP E3 ligase complex. VPRBP binds directly to, and is required for, bringing katanin to the EDVP E3 ligase; notably, no Cul4 or ROC1 is detected in the complex. Silencing individual components of EDVP, but not Cul4A and Cul4B, severely impaired katanin polyubiquitylation. Maddika and Chen show that DYRK2 acts as a scaffold to assemble the complex components, but this scaffold function does not rely on its kinase activity. However, phosphorylation by DYRK2 is required for subsequent katanin polyubiquitylation: coexpression of either a catalytically inactive DYRK2 or a triple phospho-mutant of katanin inhibits katanin polyubiquitylation. Supporting the physiological relevance of this ubiquitylation, ectopic expression of katanin causes mitotic defects (as determined by the increase in cells with 4N DNA content and positive for phospho-histone H3) that can be largely alleviated by co-expression with wild-type, but not kinase-dead, DYRK2. Knocking down either DYRK2 or EDD causes katanin accumulation and a similar increase in G2/M cells, which can be rescued by simultaneous silencing of katanin. Hence, the EDVP E3 complex is capable of phosphorylation and subsequent ubiquitylation of its substrate.

This study raised two interesting questions whose resolution may shed new light on mechanisms of ubiquitylation and substrate targeting. First, how does DYRK2-mediated phosphorylation of substrate katanin contribute to subsequent ubiquitylation by EDD? Substrate phosphorylation is known to have a key function in the initial recognition by some E3s, as best documented for several substrates whose phosphorylation triggers the binding

with specific F-box proteins and subsequent ubiquitylation by the SCF/CRL1 complex. Unlike phosphorylation-dependent binding between substrate and the F-box, there is no evidence that DYRK2-mediated phosphorylation is required for katanin to bind with VprBP-DDB1. However, the phospho-mutant katanin cannot be efficiently ubiquitinated. Similarly, the catalytic mutant DYRK2 does not seem to have any defect in assembling EDD, DDB1 and VPRBP but fails to promote katanin polyubiquitylation. Could phosphorylation have a function in orienting the substrate towards or closer to the ubiquitin-linked catalytic Cys in the HECT domain of EDD? It has been deduced from structural analysis of several E3s that the distance between the active Cys residue either in the E2 bound to the RING finger or in the HECT domain is too far away for transfer of ubiquitin to the substrate. For example, the Cys in the active site of E2 is 41 Å away from the active site in the HECT domain in E6AP, and 50 Å away from the nearest amino-acid F-box protein in the SCF/CRL1 complex<sup>12,13</sup>.

Second, how does the DDB1-VPRBP heterodimer determine which substrate is targeted to which E3? Among the estimated 90-plus DWD (DDB1-binding WD40) proteins, VPRBP is unique in that it is a particularly large protein that is abundantly expressed in many cell types and, like DDB1, has an essential function for cell growth and embryo development. Do these properties make the DDB1-VPRBP heterodimer a unique complex in recruiting different substrates to different E3 ligases? Are there other DWD proteins, in addition to VPRBP, that are also capable of shuttling between both families of E3 ligases? DYRK2 was not detected by several previous proteomic screens of proteins associated with DDB1 and VPRBP, suggesting that we may still be underestimating the reach of adaptor proteins and substrate receptor complexes in targeting substrate proteins for ubiquitylation. We have already seen that individual F-box proteins can target multiple substrates to specific CRLs. For example, the SKP2 and β-TrCP F-box proteins have each been linked to the ubiquitylation of

nearly 30 proteins<sup>14</sup>. These current findings demonstrate even more versatility in targeting substrates for ubiquitylation than previously realized, and indicate the potential to expand the repertoire of specific protein substrates ubiquitylated by E3 ligases.

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## SOC: now also store-operated cyclase

James W. Putney Jr

**Depletion of Ca<sup>2+</sup> from intracellular stores has long been known to signal to and activate plasma membrane ‘store-operated’ channels. We now learn that store depletion also controls the formation of cyclic AMP (cAMP) through the regulation of adenylyl cyclase (A-Cyclase). These findings substantially broaden the scope and biological significance of Ca<sup>2+</sup> store-regulated signalling.**

The generation of intracellular Ca<sup>2+</sup> signals by hormones, neurotransmitters and other extracellular ligands represents a major mechanism for the regulation of rapid to long-term cellular responses. Typically, these Ca<sup>2+</sup> signals comprise a combination of intracellular discharge of Ca<sup>2+</sup> from stores and influx of Ca<sup>2+</sup> across the plasma membrane. Intracellular messengers, most typically inositol trisphosphate (InsP<sub>3</sub>), are responsible for intracellular Ca<sup>2+</sup> release. Although there are several mechanisms underlying the activation of plasma membrane Ca<sup>2+</sup> channels, the most common involves signalling

from the depleted endoplasmic reticulum (ER) to the channels, a process long referred to as ‘capacitative’ or ‘store-operated’ Ca<sup>2+</sup> entry<sup>1</sup>. On page 433 of this issue, Lefkimiatis *et al.*<sup>2</sup> provide convincing evidence that the same store-operated pathway can also signal to and activate A-Cyclase, thus resulting in the formation of the second messenger cAMP.

The concept of store-operated Ca<sup>2+</sup> entry is now over 20 years old<sup>1</sup>. However, it is only in the past few years that modern high-throughput genetic screening techniques have identified two of the key molecular players in this pathway. Signalling from the ER to the plasma membrane is initiated by the Ca<sup>2+</sup> sensor proteins STIM1 and STIM2. These proteins are single-pass membrane proteins, with Ca<sup>2+</sup>-binding EF-hand motifs directed to the lumen of the endoplasmic reticulum. Dissociation

of Ca<sup>2+</sup> causes the proteins to aggregate and accumulate in regions just beneath the plasma membrane<sup>3</sup>. There, they communicate with proteins of the Orai (also known as CRACM) family (Orai1–3; refs 3, 5), resulting in channel activation and the appearance of the highly Ca<sup>2+</sup>-selective current *I*<sub>crac</sub> (calcium-release-activated calcium current)<sup>1</sup>.

The original idea of store-operated calcium entry came from studies of the mechanism by which intracellular stores were replaced following their release<sup>1</sup>. Initially, it was unclear whether this mode of entry represented a true signalling function, or a housekeeping role ensuring adequate ER Ca<sup>2+</sup> levels for proper protein synthesis and folding<sup>6</sup>. The discovery of the signalling proteins STIM1 and STIM2 clearly indicates that STIM1-activated entry functions primarily as a signalling pathway<sup>7,8</sup>,

James W. Putney Jr is in the Laboratory of Signal Transduction, National Institute of Environmental Health Sciences–NIH, Department of Health and Human Services, PO Box 12233, Research Triangle Park, NC 2770, USA.  
e-mail: putney@niehs.nih.gov