

among these would be polymerizing actin at the neck of a budding vesicle or tubule and forcing it away from the plasma membrane. However, actin polymerization is not universally observed during the budding of clathrin vesicles, and actin depolymerization does not shut down all of endocytosis. A simple explanation for the lack of a uniform requirement for actin for the formation of endocytic vesicles might be that cells have developed multiple mechanisms to form and fission off endocytic carriers. The requirement for a mechanism using actin might be dependent upon whether or not actin and actin regulatory proteins like N-WASP are locally abundant. One can imagine that in locations (such as the adherent plasma membrane) where a dense cortical network is in the way of membrane deformation

and movement, a rearrangement of the actin cytoskeleton might be necessary and has been adapted to move membrane carriers through the obstruction by using its own components. At other locations where an actin cytoskeleton is less abundant, a mechanism using actin might not be required. Since the actin cytoskeleton is dynamic and sensitive to growth states, the requirement for actin in endocytosis could vary for the same cell type under different growth conditions, and might even vary at different locations in the same cell.

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Ubiquitin Ligation without a Ligase

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Classically, ubiquitination requires three enzymes acting in sequence: E1, E2, and E3. E3 ubiquitin ligases typically provide substrate specificity. An article in *Molecular Cell* (Hoeller et al., 2007) now describes the E3-independent monoubiquitination of certain proteins. The mechanism has interesting parallels to SUMO ligation.

Ubiquitin covalently modifies other proteins, either as ubiquitin chains (polyubiquitination) or single ubiquitin moieties (monoubiquitination) (Kerscher et al., 2006). Attachment of ubiquitin to a protein enhances its interaction with ubiquitin receptors containing ubiquitin-binding domains (UBDs). The consequences of such interactions depend on their timing and cellular location and on the type of ubiquitin modification. Generally, a lysine in the substrate is coupled to the C-terminal glycine of ubiquitin by an amide (isopeptide) bond. This requires prior activation of the ubiquitin C terminus by E1, after which the

ubiquitin is passed to an active site cysteine side chain in an E2, creating a thioester-linked E2-ubiquitin complex. An additional factor, the E3 ubiquitin ligase, is usually necessary for efficient ubiquitin transfer from E2 to substrate. E3 enzymes contain substrate- and E2-binding domains and may activate ubiquitin transfer by the E2.

One area of cell regulation that makes extensive use of monoubiquitination is endocytosis (Mukhopadhyay and Riezman, 2007). Ubiquitin attachment to plasma membrane proteins usually causes their downregulation by stimulating endocytosis and traf-

ficking to the lysosome for degradation. Additionally, multiple endocytic adaptor proteins contain UBDs, which allow them to modulate membrane receptor trafficking by binding to the ubiquitinated receptor or to other endocytic factors that have been ubiquitinated. UBDs come in a variety of structural flavors but have in common the ability to bind ubiquitin noncovalently, usually with fairly low affinity (Harper and Schulman, 2006). Interestingly, many of the monoubiquitinated endocytosis factors also bear a UBD. Covalent monoubiquitination of these proteins depends on the ability of the UBD to engage ubiquitin

noncovalently, a process termed “coupled monoubiquitination” (Woelk et al., 2006).

The mechanism of coupled monoubiquitination has been analyzed previously for two E3s, parkin and Nedd4, which belong to different E3 structural classes. Parkin is unusual in that it also contains an N-terminal ubiquitin-like domain, which is capable of binding the UBD of the endocytic adaptor Eps15. In the regulated endocytosis of epidermal growth factor receptor (EGF receptor), EGF stimulation triggers receptor monoubiquitination and the recruitment of Eps15. Parkin is also recruited under these conditions, and it binds to and is activated by Eps15, leading to monoubiquitination of the adaptor (Fallon et al., 2006). This stimulates release of Eps15 from the ubiquitinated EGF receptor by switching it into a “closed” conformation caused by intramolecular ubiquitin-UBD association (Hoeller et al., 2006). The net result of parkin activation is enhancement of EGF signaling at the plasma membrane due to delayed receptor endocytosis.

Another E3 that can stimulate Eps15 monoubiquitination is Nedd4, which forms a ubiquitin-thioester on its HECT domain. A second ubiquitin is attached to the E3 at another site (Woelk et al., 2006). This second ubiquitin binds the UBD in Eps15, facilitating transfer of the ubiquitin from the HECT active site cysteine to a specific Eps15 lysine. Together, these studies indicate that either a ubiquitin-like domain built into the E3 or self-ubiquitination of the E3 can generate binding sites for UBD-substrates, and these interactions stimulate the ubiquitination of the latter proteins.

Thus, it comes as something of a surprise that Eps15 as well as other UBD proteins can be monoubiquitinated in vitro without any E3 ubiquitin ligase at all, based on an article in the current issue of *Molecular Cell* (Hoeller et al., 2007). At first blush, these observations would not appear to be unusual insofar as inefficient E3-independent ubiquitination of proteins in vitro has been seen since the early days of ubiquitin analysis and has often been dismissed as an in vitro artifact. However, in Hoeller et al. (2007) the

E3-independent monoubiquitination requires a functional UBD, which is known to be physiologically relevant for coupled monoubiquitination in vivo. Moreover, results of siRNA knockdown and FRET experiments with transfected tissue culture cells corroborate the interaction of the ubiquitin-E2 conjugate with substrate and suggest that neither this interaction nor the monoubiquitination of UBD-substrate requires an E3. Why E3-independent in vitro monoubiquitination was not seen in an earlier study (Woelk et al., 2006) with the same Eps15 substrate and cognate E2 is unclear.

Hoeller and colleagues checked a battery of different E2s on an array of different kinds of UBD-containing proteins (Hoeller et al., 2007). All the tested E2s can monoubiquitinate one or another UBD protein, but efficiency and specificity vary widely. Collectively, the data suggest that both UBD and non-UBD elements in the substrates are important for E2-ubiquitin binding and orientation. The UBD proteins themselves do not function as ordinary E3 ligases. They promote their own ubiquitination—acting as a kind of “cis ligase”—but they do not appear able to promote transfer of ubiquitin to another protein in *trans*.

While the new results from Hoeller et al. (2007) will likely engender lively discussion among those analyzing ubiquitin conjugation, the story will ring familiar to researchers studying SUMO-protein ligation. Site-specific E3-independent in vitro ligation of substrates to SUMO, a ubiquitin-like protein (Ubl), is commonplace. Notably, this usually requires relatively high E2 concentrations, and the question of full E3 independence in vivo is unresolved. A noncovalent SUMO-interacting motif (SIM) is also found in many proteins, and in the same way that ubiquitin-UBD interactions target ubiquitinated proteins to specific “ubiquitin receptors,” noncovalent SUMO-SIM binding directs sumoylated proteins to SUMO receptors (Kerscher et al., 2006). Moreover, certain SIM-bearing proteins are also subject to covalent SUMO modification, and noncovalent SIM-SUMO interaction is sometimes a prerequisite

for their sumoylation (“coupled monosumoylation”) (Takahashi et al., 2005; Lin et al., 2006). Crystallographic analysis (Baba et al., 2005) of one of these sumoylated SIM proteins revealed an intramolecular SIM-SUMO interaction that might be analogous to the autoinhibitory UBD-ubiquitin interaction of endocytic factors (Hoeller et al., 2006).

Combinations of covalent and noncovalent Ubl associations in the same polypeptide might be a broader element of Ubl regulation than is currently appreciated. A recent study found that the Ubl called Nedd8, which covalently modifies cullin proteins, also interacts noncovalently with a hydrophobic motif in the cullin (Wimutisuk and Singer, 2007). This latter interaction might be able to stimulate in *cis* ligation of Nedd8 to the cullin as well.

In summary, the report of E3-independent monoubiquitination of proteins carrying UBDs suggests deeper mechanistic connections to the conjugation of other UbIs than previously realized. Questions raised include how exactly E3s stimulate coupled monoubiquitination and how this process is regulated. Hoeller et al. (2007) suggest that E3-stimulated monoubiquitination of endocytic adaptors occurs specifically when receptor signaling has been activated. Whether constitutive E3-independent monoubiquitination of such factors is important for regulating endocytosis remains to be determined. If it is, then it may first need to be reversed to bring the affected adaptors into the active pool. Deubiquitinating enzymes would be key to such mechanisms. We can look forward to further developments on these and related issues in the next few years.

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A Complex Solution to a Sexual Dilemma

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The *C. elegans* male sex-determining protein, FEM-1, has been identified as a substrate recognition subunit of a Cullin-2 ubiquitin ligase complex. This complex controls the level of TRA-1A, a Ci/Gli homolog and master regulator of sex determination, by ubiquitin-mediated proteolysis.

Sex determination is a fundamental biological process involving a deceptively simple binary fate decision. Studies over the last several decades have revealed that diversity rules when it comes to elucidating the molecular mechanisms underlying this universal process.

In the nematode *C. elegans*, a chromosomal counting mechanism generates the primary sex determining signal, the X:A ratio; XO animals develop as males and XX animals as hermaphrodites (Figure 1) (Zarkower, 2005). Downstream genes participate in a signal transduction pathway with similarity to the Hedgehog (Hh) signaling pathway (Kuwabara et al., 2000). Significantly, the master regulator of sex determination is the zinc finger transcription factor, TRA-1 (Transformer-1), which is the only *C. elegans* ortholog of *Drosophila* Ci and human Gli proteins—terminal regulators of Hh signaling. Genetic arguments posit that TRA-1 promotes female or represses male somatic development; however, only repressive roles for TRA-1 have been identified so far. The primary mode of *tra-1* regulation is posttranscriptional (Zarkower and Hodgkin, 1992). The *tra-1* locus ex-

presses two isoforms: TRA-1A ($M_r = 135$ kDa) and TRA-1B ($M_r = 37$ kDa), but only TRA-1A binds DNA (Schvarstein and Spence, 2006; Zarkower and Hodgkin, 1993). Like *Drosophila* Ci (Jiang, 2002), proteolytic cleavage of TRA-1A generates a range of C-terminally truncated phosphoisoforms ($M_r = 90$ –110 kDa), which accumulate only in feminized animals (Schvarstein and Spence, 2006). In XO males, the *fem* genes prevent the accumulation of TRA-1A phosphoisoforms; however, the domain architecture of the FEM proteins has provided scant clues to explain how this is achieved. FEM-3 is a novel protein, FEM-1 carries an ankyrin domain, and FEM-2 is a phosphatase in search of a substrate. Importantly, inactivation of a single *fem* gene is sufficient to feminize XO animals inappropriately. So, just how do the *fem* genes negatively regulate *tra-1*?

In this issue of *Developmental Cell*, Starostina and colleagues establish that a Cullin-2 (CUL-2) ubiquitin ligase complex is the missing player in this long-standing puzzle (Starostina et al., 2007). In the multisubunit CBC (for CUL2, Elongin B, Elongin C) (E3) ubiquitin ligase complex, CUL-2 asso-

ciates with an Rbx1/Roc1 RING finger protein at its C terminus and an Elongin C adaptor at its N terminus. Elongin C is also associated with Elongin B and to a variable substrate recognition subunit (SRS) (Kipreos, 2005). With regard to sex determination, light dawned when FEM-1 was uncovered during a proteomic screen for CUL-2 interacting partners. Inspection of the FEM-1 sequence revealed the presence of a VHL box, a domain mediating binding between the SRS and Elongin C. Subsequently, a VHL-box-dependent physical interaction between FEM-1 and Elongin C was shown. Consistent with its proposed role in reducing TRA-1 activity, a reduction in *cul-2* activity produced partially feminized XO intersexes and suppressed the weak masculinization phenotypes of XX *tra-1* hypomorphs. The complete elimination of *cul-2* is lethal, which may explain why *cul-2* was not found in genetic screens for sexual transformation mutants. Once it was established that FEM-1 could function as an SRS subunit, it was short work to demonstrate that TRA-1A was the primary substrate targeted for proteasomal degradation by the CBC^{FEM-1} complex. Thus, *cul-2*