PERSPECTIVES: BIOCHEMISTRY

# Ubiquitination—More Than Two to Tango

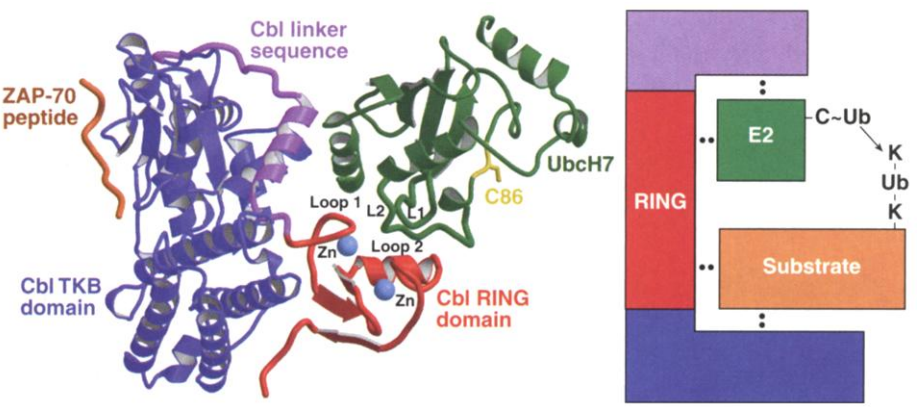
Claudio A. P. Joazeiro and Tony Hunter

The addition of a ubiquitin tag to proteins (ubiquitination), which targets them for degradation, is an essential step in many cellular processes including signal transduction, transcription, and control of the cell cycle. Three enzymes are required for ubiquitination—E1 (the ubiquitin-activator), E2 (the ubiquitin-conjugator), and E3 (the ubiquitin-protein ligase). Many E3 ligases are RING finger proteins containing a zinc-stabilized RING finger motif that binds to E2 and a domain that binds to the protein substrate to be degraded (*1*). One particularly interesting RING E3 is the c-Cbl proto-oncoprotein, which shuts down the signaling of activated growth factor receptor tyrosine kinases by inducing their ubiquitination. Exactly how c-Cbl interacts with both E2 and the receptor tyrosine kinase to be ubiquitinated is unclear. Now, in a recent issue of *Cell*, Pavletich's group (*2*) reports the structure of a c-Cbl fragment—containing the RING finger motif and the variant SH2 (TKB) domain—bound to both UbcH7 (an E2) and a tyrosine-phosphorylated peptide (that mimics the binding site in the protein substrate). The structure reveals new features of c-Cbl family proteins and suggests how other RING E3s may bind to E2 enzymes.

In mammalian cells, the c-Cbl RING E3 is a rate-limiting enzyme for the ubiquitination of epidermal growth factor receptor, platelet-derived growth factor receptor, and colony-stimulating factor–1 receptor. This activity requires the presence of the highly conserved RING finger motif and SH2 domain: The former recruits the E2 and the latter targets activated receptor tyrosine kinases for ubiquitination and degradation (*3*–*5*). Indeed, the 17–amino acid deletion found in the 70Z3 Cbl oncogene (isolated from a pre–B cell lymphoma), which includes the first cysteine in the RING motif, abolishes the ability of c-Cbl to promote ubiquitination of receptor tyrosine kinases.

The c-Cbl–UbcH7 E2 structure is very compact (*2*), with the RING motif itself and an α-helical SH2-RING linker packed tightly against the SH2 domain and the E2 through multiple contacts (see the figure). The RING finger of c-Cbl is folded in the typical ββαβ pattern, with the conserved RING amino acid residues chelating two zinc ions and making up the domain's hydrophobic core (*1*). Five of these conserved or partially conserved residues in the c-Cbl RING participate in direct binding to the UbcH7 E2 and could be involved in E2 recognition. These five residues, together with four nonconserved residues, form a shallow groove lying between loops 1 and 2 and the α helix of the RING finger (see the figure). The three residues at the tips of loops L1 and L2 in the UbcH7 E2 (which are conserved in other E2s that bind to c-Cbl) are inserted into this groove. The structure also suggests how less-conserved residues in both the RING finger of E3s and the E2 may determine specific interactions. For example, a tryptophan at the edge of the groove is present in only a subset of RING E3s; mutation of this residue abolishes c-Cbl's E3 activity and its binding to E2 (*3*). Although these and other observations help to explain the preference of c-Cbl as well as other RING finger proteins for specific E2s, their predictive value remains to be verified experimentally.

Surprisingly, the Pavletich work reveals that the way in which an E2 binds to the RING motif of c-Cbl is very similar to the way in which it binds to the HECT domain (the signature motif of non-RING E3s)—the E2 uses the same two loops inserted into the shallow groove. However, the primary sequence and tertiary structure of the RING and HECT motifs are unrelated and these domains seem to operate differently in the ubiquitination process. (The HECT domain forms a thioester bond with ubiquitin before the ubiquitin is transferred to its protein target.) In addition to the interaction between E2 and the RING finger motif, further interactions are known to contribute to high-affinity binding between E2s and other RING finger proteins. One example is the high-affinity binding between two budding yeast DNA repair proteins, Rad18 and Ubc2/Rad6. This binding depends on a helix-loop-helix (HLH)-like domain in Rad18 (a RING finger protein) and on part of the carboxyl-terminal sequence of Ubc2 (which is not conserved among other E2s) (*6*). Secondary sites that bind E2s may also be provided by additional proteins in E3s that are composed of multiple subunits. For example, Hrt1/Roc1/Rbx1, the small RING finger subunit of the E3 ligase SCF (Skp1/cullin-1/F-box protein), requires an accessory cullin protein both for binding to E2 and for E3 activity. This cullin protein may also be responsible for the versatile interaction of the RING fin-




**Fingering protein degradation.** **(Left)** Interactions between c-Cbl E3 ligase, UbcH7 E2, and a protein substrate. The structure shows the variant SH2 (TKB) domain of c-Cbl E3 in blue, the c-Cbl linker sequence in purple, c-Cbl's RING finger motif in red, the UbcH7 E2 in green, and phosphorylated ZAP-70 peptide (which mimics the binding site for E3 in the protein substrate) in orange. The UbcH7 E2 active-site cysteine is in yellow. Zinc ions are represented as blue spheres. **(Right)** A model depicting how RING finger E3s may participate in ubiquitination. The E3 ligase is shown as a continuous block in purple, red, and blue. This E3 interacts with its protein substrate (orange) and an E2 (green). The primary binding site for the protein substrate (for example, the SH2 domain of c-Cbl) is shown in blue. This might be a separate polypeptide from the RING finger in multisubunit E3 complexes. A secondary binding site for the protein substrate may exist in the RING finger (red). The RING finger of E3s has been defined as a primary binding site for E2; possible secondary sites for E2 binding are shown in purple. The E2 active-site cysteine (C) is shown forming a thioester bond with ubiquitin (Ub). Lysine residues (K) in the protein substrate and in ubiquitin are involved in formation of a polyubiquitin chain, which can target the substrate for degradation once it is attached.

CREDIT: NING ZHENG AND NIKOLA P. PAVLETICH

The authors are at the Molecular Biology and Virology Laboratory, Salk Institute, La Jolla, CA 92037, USA. E-mail: cjoazeiro@aim.salk.edu; hunter@salk.edu

ger cullin complex with E2s from distantly related families (*7–12*).

The c-Cbl–UbcH7 structure shows how a region amino-terminal to the RING finger can contribute to the association of the E3 with its E2. The α-helical linker of c-Cbl (see the figure), which is not conserved among RING finger proteins, nonetheless establishes multiple contacts with an α-helix in UbcH7. Although not essential for E2 binding, the linker contributes to the affinity of c-Cbl for UbcH7 (*5*). Pavletich and colleagues propose that the interactions between the linker, the SH2 domain of c-Cbl, and the UbcH7 E2 may be important structurally, helping to properly orient other sections of this multiprotein ubiquitinating complex. In this regard, it has been suggested that phosphorylation of tyrosine 371 in the linker is essential for c-Cbl's E3 activity (*4*); however, according to the Pavletich structure, this tyrosine is packed against the SH2 domain and so is not readily available for phosphorylation. Certainly, the part played by the c-Cbl linker in the ubiquitination process requires further study.

In the absence of ubiquitin, there is no significant difference in the conformation of the free and c-Cbl–bound UbcH7 E2. Furthermore, none of the cysteines in the c-Cbl RING finger are likely to form an intermediate thioester bond with ubiquitin without disrupting the structure. The structure of the c-Cbl–UbcH7 complex is thus consistent with a model in which RING finger E3s act as adapters that bring the E2 and the protein substrate together. Pavletich and co-workers propose, however, that c-Cbl may play a more active role in ubiquitination than simply increasing the local concentration of reacting species. First, all the domains seem to be rigidly arranged. Second, and somewhat unexpectedly, the UbcH7 active-site cysteine is on the opposite side of the complex relative to the binding site for tyrosine-phosphorylated peptides. The provocative finding of a continuous surface "channel" extending between these two sites, whose lining residues are particularly well conserved among c-Cbl proteins, led the authors to speculate that this channel may guide the polypeptide chain of the protein substrate toward the E2 catalytic cysteine. Thus, c-Cbl and other RING E3s may serve as scaffolds that position and orient the protein substrate and E2 optimally for ubiquitin transfer.

We think that it may be possible to identify regions in RING finger proteins that independently confer E2 and substrate specificity (see the figure). Direct evidence for RING finger–substrate interactions in vitro has recently been reported. In one case, the RING finger of the apoptosis inhibitor cIAP2—but not those of c-Cbl or Apc11, a subunit of the anaphase-promoting complex (APC)—was sufficient for ubiquitination of caspase 7 (an enzyme that promotes apoptosis) (*13*). In the other case, Apc11—but not c-Cbl's RING—sufficed for ubiquitination of APC substrates (*14*, *15*). These in vitro assays use high protein concentrations, and so the specific RING-substrate interactions that they unmask are not necessarily the primary mechanisms for substrate recognition in the cell (see the figure). Indeed, in the cases where it is known, the primary interaction with a protein substrate involves distinct regions of the E3 (for example, the SH2 domain of c-Cbl). The primary substrate binding sites in E3s provide a high-affinity interaction, perhaps enabling the synthesis of a polyubiquitin chain to be completed without release of the substrate (polyubiquitin chains are more efficiently recognized and degraded by the proteasome). In turn, the weaker, secondary binding site in the RING may allow flexibility for changes in position and orientation between the substrate and the E2 catalytic site either as the synthesis of a polyubiquitin chain progresses or as multiple lysines in the substrate are modified with ubiquitin.

In the picture that we have now of a complex between c-Cbl's RING finger and UbcH7 E2, a critical ingredient is missing: ubiquitin. Future structural work on the complex engaged in the ubiquitination process should provide explanations for the other functions of RING finger E3 ligases in ubiquitination besides their E2 binding activity. Importantly, one would like to know how polyubiquitin chains are synthesized by apparently rigid E2-E3 complexes.

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PERSPECTIVES: AGING

# Aging, Chromatin, and Food Restriction—Connecting the Dots

**Judith Campisi**

What causes aging? Current hypotheses generally fall into one of two categories. The first category invokes extrinsic or intrinsic factors that damage intracellular or extracellular molecules; the second invokes changes in gene expression that are either programmed or that are brought about by nonmutational changes in DNA structure. To what extent these hypotheses overlap or intersect is not known. Regardless of the hypothesis, however, caloric restriction (CR) has been an important tool for testing ideas about causes of aging in animals. Caloric restriction—reducing the food intake of animals (normally fed ad libitum) by 50 to 70%—reliably extends the mean and maximum life-spans of several species, including mammals (*1*). It postpones most age-related pathology and alters many, but not all, age-related processes. It is thought to do this primarily by reducing oxidative stress and damage caused by reactive oxygen species (*2*). Yet, despite more than half a decade of research, the major pathways through which CR acts remain enigmatic. Now, on page 2126 of this issue, Lin *et al.* (*3*) describe intriguing results that may link CR to the control of gene expression and to the suppression of DNA damage (loss or rearrangement of DNA) caused by mitotic recombination. These studies were carried out in a model organism, the yeast *Saccharomyces cerevisiae*, from which much basic cellular and molecular information has been gleaned, including several revelations about the genetics and physiology of aging (*4*).

Yeast undergo only a finite number of divisions, after which they die; thus, their life-span is defined by the number of divisions each cell completes (*4*). Lin *et al.* induced CR in yeast by limiting glucose availability or by genetically crippling their ability to sense and respond to glucose. Caloric restriction extended yeast longevity by 20 to 40%, similar to the relative life-span extension induced by CR in mammals. Of importance, this extension required the yeast genes *NPT1* and *SIR2*. *NPT1* encodes one of two enzymes that produce NAD (nicotinamide adenine dinu-

The author is at the Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. E-mail: jcampisi@lbl.gov