Ubiquitination: **RING for destruction?**

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Ubiquitination targets proteins for degradation and is a potent regulator of cellular protein function. Recent results implicate the RING finger domain in specific ubiquitination events; it is possible that all RING proteins act as E3 ubiquitin protein ligases, with implications for a variety of biological areas.

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Targeted protein proteolysis is increasingly understood to be an important general mechanism by which cells regulate protein levels — and consequently their functions at specific times. In eukaryotic cells, the main mechanism for such control involves the specific covalent modification by polyubiquitin, which labels target proteins for proteolysis and subsequent degradation. There are many known examples of such events, and ubiquitination is now recognised as a major mechanism for cellular regulation, perhaps

We have already begun to identify multiprotein complexes, known as E3 ubiquitin protein ligases, that function in choosing specific targets for ubiquitination. These E3 proteins bind to both target proteins and the ubiquitin E2 conjugating enzymes, thereby selecting and promoting ubiquitination. These complexes are few and far between, however, but recent studies on several different proteins that contain a domain called the RING finger have now provided significant insights into how ubiquitin targeting can be achieved [1–4]. The RING finger is a small zinc-binding domain found in many functionally distinct proteins, which up until now had no known general function. The new studies have now shown that the RING finger can specifically interact with E2 ubiquitin conjugating enzymes, thereby promoting ubiquitination, and that proteins which contain a RING finger may act as E3 ubiquitin protein ligases.

The RING finger protein sequence motif was first identified nearly ten years ago in the protein product of the human gene *RING1 — Really Interesting New Gene 1* — which is located proximal to the major histocompatibility region on chromosome 6. Although the domain was initially identified in only a few functionally distinct proteins, the set of proteins known to have RING fingers has grown enormously over the last few years, and the motif and its variants have been found in more than 200 proteins from diverse eukaryotes, but interestingly not in any prokaryote protein (reviewed in [5]). Perhaps the most famous RING finger protein is BRCA1, product of a breast cancer-associated gene; point mutations within the RING finger domain of BRCA1 predispose females carrying them to breast cancer. Other family members are also well known, however; for example, the family includes the protooncogene products Cbl, BMI-1 and PML, the immunoglobulin gene recombination enzyme RAG1, the Rbx1 component of the VHL tumour suppressor complex, and the p53 regulator MDM2, to name but a few [5].

The RING finger motif can be defined as a unique linear series of conserved cysteine and histidine residues: Cys–X_2–Cys–$X_{9–39}$–Cys–$X_{1–3}$–His–$X_{2–3}$–Cys/His–X_2–Cys–$X_{4–48}$–Cys–X_2–Cys, where X can be any amino acid, although there are distinct preferences for particular types of amino acid at particular positions [5]. Apart from the absolutely conserved cysteine and histidine residues, there is little sequence conservation, unlike other cysteine-rich motifs. Initial structural and biophysical studies

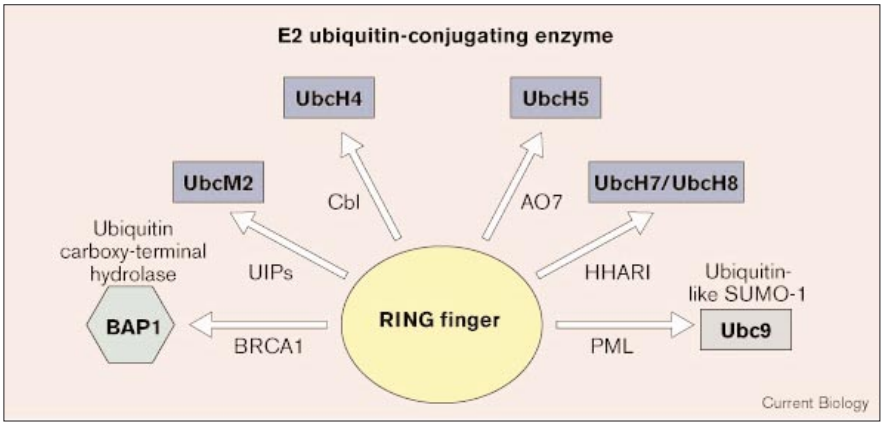
binds two zinc atoms in a unique 'cross-brace' arrangement; the conserved spacing between the second and third pairs of cysteine/histidine residues implies conservation of the distance between the two zinc-binding sites. Indeed this is the case, as shown by several three-dimensional RING finger structures that show the motif to fold into a compact autonomously folded domain comprising a small central β sheet and an α helix in some cases, with an inter-zinc distance of ~14 Å in all cases (reviewed in [5]).

There are a few other interesting features of RING fingers that are worth mentioning. RING fingers and their variants are generally found close to an amino or carboxyl terminus. Interestingly, RING-H2 motifs — where the fourth cysteine is replaced with a histidine — are often found close to a carboxyl terminus, although there are no hard and fast rules. RING fingers can also be associated with other domains to form larger conserved motifs, such as the RING finger–B box–α-helical coiled-coil (RBCC) motif [5].

Despite all the detailed information available on RING fingers, a number of important questions about them have, until recently at least, remained unanswered. The most important of these is the key question of what is the function of the RING finger domain? And why are RING fingers found in such a variety of proteins? Can we attribute

Figure 1

RING finger domains mediate specific interactions with components of the ubiquitination pathway. RING-containing proteins are named alongside arrows that indicate their specific RING-mediated interactions. (See text for details.)



a common function to the RING finger? And if we can, how do we account for the variation in sequence, size and structure of the domain?

Early results did indicate that RING fingers are essential for the normal functioning of the proteins that contain them. For example, studies on the TRAF proteins, which transduce signals from members of the tumour necrosis factor (TNF) receptor superfamily to the transcription factor NF-κB, showed that deletion or mutation of the cysteine residues in their RING finger domains abrogate TNF receptor signalling [6]. Other studies identified protein–protein interactions that depend on the RING finger domain — for example, BRCA1 and the protein BARD1 were shown to interact via the two proteins' RING finger domains [7]. These observations were interesting, but did not provide general insights into the function or mechanism of action of the RING finger. This situation is now changing, as over the last year or so a number of results have indicated that the RING finger is involved in the ubiquitination pathway. This has culminated in several recent studies [1–4] which have for the first time provided direct functional evidence that RING fingers mediate ubiquitination events.

The ubiquitination pathway generally involves three types of enzyme, know as E1, E2 and E3. E1 and E2 are ubiquitin conjugating enzymes, which catalyse formation of thioester bonds directly with the carboxyl terminus of ubiquitin; an E1 enzyme acts first and passes ubiquitin to an E2 enzyme ready for targeting to a protein substrate. The E3 enzymes are ubiquitin protein ligases, responsible for substrate recognition and promoting polyubiquitin ligation to a substrate, marking it for degradation by the 26S proteosome. E3 enzymes often bring together the activated E2~ubiquitin adducts and the target substrates, and they are thought to be the least conserved component of the ubiquitination pathway.

The functional connection between RING fingers and ubiquitination has come primarily from studies on the RING-containing protein Cbl. This protein is an important negative regulator of several signalling pathways that are activated through the receptor for platelet-derived growth factor (PDGF), epidermal growth factor (EGF) or colony stimulating factor. Cbl is of interest to cancer researchers, as the protein becomes oncogenic *in vivo* as a result of mutation or deletion of the RING finger. In addition to the RING, Cbl also has a variant SH2 domain and a proline-rich region, all the hallmarks of an adaptor protein. It is now generally accepted that Cbl works by promoting polyubiquitination of receptor protein tyrosine kinases, although until recently the precise molecular mechanisms have been elusive.

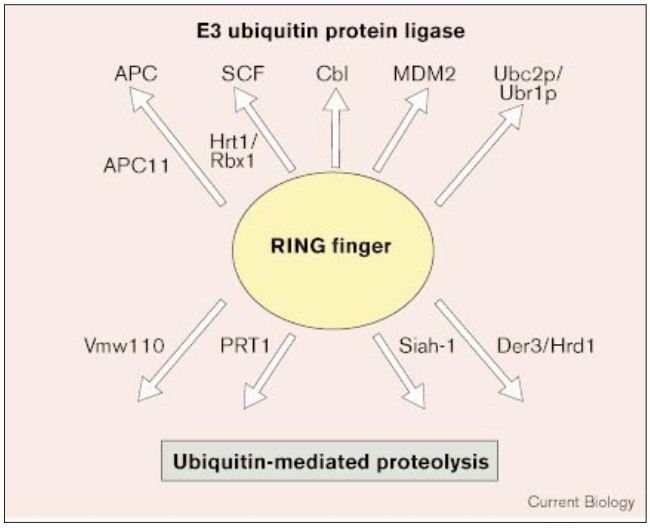
Yarden and colleagues [1] have now shown that the desensitisation of the EGF receptor that normally follows its activation by ligand binding requires the Cbl RING finger. They found that single cysteine mutations of the RING finger produce dominant-negative forms of Cbl, which bind to but do not downregulate activated receptors. Baron and colleagues [2] have gone even further and shown that the Cbl RING finger interacts with the E2 ubiquitin conjugating enzyme UbcH7, thereby mediating the ubiquitination of the EGF receptor *in vivo* in a ligand-dependent manner. Hunter and colleagues [3] have dissected Cbl function further. They have shown that the ubiquitination of receptor tyrosine kinases is directly dependent on both the SH2 and RING finger domains of Cbl. Furthermore, they have demonstrated that the Cbl RING finger specifically recruits, and allosterically activates, the E2 ubiquitin conjugating enzyme UbcH4. From these observations they made the startling conclusion that Cbl actually acts as an E3 ubiquitin protein ligase [3]; that is, Cbl recognises tyrosine phosphorylated substrates, such as PDGF receptor, via its SH2 domain and then recruits and activates E2 ubiquitin conjugating enzymes via its RING finger.

In their study, Weissman and colleagues [4] identified a novel RING-containing protein, AO7, in a yeast two-hybrid screen using as bait the human E2 ubiquitin-conjugating enzyme UbcH5. They then proceeded to show that AO7 itself acts as a substrate for polyubiquitination by binding directly to the E2 enzyme via its RING-H2 domain. More importantly perhaps, they also tested a number of unrelated proteins containing RING fingers for E2-mediated autoubiquitination activity and somewhat surprisingly, they found that in all cases RING and RING-H2 domains appeared functional [4]. More recently Robinson and colleagues [8] have also reported a specific interaction between a RING finger protein and an E2 enzyme. By using the human E2 conjugating enzyme UbcH7 as bait in a yeast two-hybrid screen, they isolated two novel RING-containing proteins, one of which, HHARI, interacts with UbcH7 via its RING finger *in vitro*. By testing other E2s, they found that only the highly related enzyme UbcH8 interacted with HHARI, supporting a specific RING-E2 interaction [8].

Recent studies have thus identified several examples of specific RING-mediated E2 interactions (Figure 1). The obvious question they raise is whether all RING-containing proteins act as E3 ubiquitin-protein ligases? The Cbl story provides direct evidence for a RING-mediated E3 activity, but several other directly relevant observations were reported earlier last year [9–11]. These studies defined a new component of the SCF ubiquitin ligase complex — where SCF derives from the three components Skip1, Cdc53 and F box protein — a modular multiprotein complex that mediates ubiquitination and subsequent degradation of a variety of different proteins, including the G1 cyclins. The newly identified SCF protein in question is Hrt1/Rbx1, which has a RING-H2 domain [9–11]. Hrt1/Rbx1 acts together with F box proteins — substrate-targeting components of SCF complexes — and stimulates the ubiquitin ligase activity of several SCF complexes by promoting association of the E2 conjugating enzyme Cdc34 with Cdc53. Perhaps of more significance is the fact that Hrt1/Rbx1 is strikingly similar to the Apc11 subunit of the anaphase-promoting complex (APC), an E3 ubiquitin ligase complex that plays an important role in cell-cycle progression [9]. Very recently, studies on another E2–E3 complex, Ubc2p–Ubr1p, have shown that the RING-H2 domain of the E3 Ubr1p is necessary for polyubiquitination but interestingly not for interaction with the E2 Ubc2p [12].

An even higher profile player that has a variant RING finger and acts as an E3 ubiquitin protein ligase now becomes very relevant to the discussion. The protooncogene product MDM2 is the critical cellular regulator of p53, determining p53's stability by binding directly to it and mediating its ubiquitination and subsequent degradation. Perhaps not surprising now, but highly relevant, is the observation that MDM2's variant RING finger is essential for its E3 activity [13]. It is very plausible that MDM2 uses the variant RING finger to interact specifically with an E2 enzyme and thereby promote ubiquitination of p53. RING finger proteins have thus now been found in several different E3 complexes (Figure 2), and this may turn out to be true of all RING finger proteins.

Figure 2



RING fingers are required for both E3 ubiquitin protein ligase activities and ubiquitin-mediated proteolysis. The names indicate specific RING-containing proteins, apart from SCF and APC which are multiprotein complexes that contain the RING proteins APC11 and Hrt1/Rbx1. Arrows indicate a direct involvement of the RING finger.

Over the last couple of years or so, a number of other studies have implicated RING fingers in ubiquitination events. For example, members of a novel family of RING finger proteins have been reported to interact directly with the ubiquitin-conjugating enzyme UbcM4 [14]. And the RING-H2 domain of a protein known as Der3/Hrd1 has been implicated in protein degradation associated with the endoplasmic reticulum in yeast [15]. Also of interest is the observation that the RING domain of Siah-1 — the human homologue of Seven in absentia, a protein involved in eye development in *Drosophila* — is required for proteolysis of several target proteins [16]. The RING finger protein PRT1 has been shown to be required in plants for the degradation of short-lived proteins with a particular amino acid at the amino terminus; this amino acid acts as a destabilising tag, an example of the 'N-end rule' ubiquitination pathway [17]. More recently, the RING finger of the HSV protein Vmw110 has been shown to be essential for a virus-induced block in mitosis in infected cells, which is directly correlated with the proteosome-mediated degradation of the CENP-C protein [18].

Taken together, these various observations provide compelling evidence for the view that RING fingers have a general function in ubiquitin-mediated proteolysis (Figure 2). Several important questions still need to be addressed, however. For example, is the role of RING fingers in ubiquitination events limited to that of mediating specific interactions with E2 enzymes? Perhaps not, as it has already been shown that the BRCA1 RING finger mediates a direct interaction *in vivo* with a ubiquitin carboxy-terminal hydrolase named BAP1 [19]. Is the function of the RING finger therefore limited to the general ubiquitination pathway? Again perhaps not, as there is evidence that the acute promyelocytic leukaemia protein PML uses its RING finger to interact with an E2, UbcH9, whose substrate is not ubiquitin, but a ubiquitin-like protein known as SUMO-1 [20]. What is clear, however, is that there is now a significant body of evidence supporting the involvement of a number of different, and functionally distinct, RING finger proteins in ubiquitin or ubiquitin-like pathways and events.

References

1. Waterman H, Levkowitz G, Alroy I, Yarden Y: **The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor.** *J Biol Chem* 1999, **274:**22151-22154.
2. Yokouchi M, Kondo T, Houghton A, Bartkiewicz M, Horne WC, Zhang H, Yoshimura A, Baron R: **Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7.** *J Biol Chem* 1999, **274:**31707-31712.
3. Joazeiro CA, Wing SS, Huang H, Leverson JD, Hunter T, Liu YC: **The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase.** *Science* 1999, **286:**309-312.
4. Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM: **RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination.** *Proc Natl Acad Sci USA* 1999, **96:**11364-11369.
5. Saurin AJ, Borden KL, Boddy MN, Freemont PS: **Does this have a familiar RING?** *Trends Biochem Sci* 1996, **21:**208-214.
6. Takeuchi M, Rothe M, Goeddel DV: **Anatomy of TRAF2. Distinct domains for nuclear factor-kappaB activation and association with tumor necrosis factor signaling proteins.** *J Biol Chem* 1996, **271:**19935-19942.
7. Wu LC, Wang ZW, Tsan JT, Spillman MA, Phung A, Xu XL, Yang MC, Hwang LY, Bowcock AM, Baer R: **Identification of a RING protein that can interact in vivo with the BRCA1 gene product.** *Nat Genet* 1996, **14:**430-440.
8. Moynihan TP, Ardley HC, Nuber U, Rose SA, Jones PF, Markham AF, Scheffner M, Robinson PA: **The ubiquitin-conjugating enzymes UbcH7 and UbcH8 interact with RING Finger/IBR motif-containing domains of HHARI and H7-AP1.** *J Biol Chem* 1999, **274:**30963-30968.
9. Freed E, Lacey KR, Huie P, Lyapina SA, Deshaies RJ, Stearns T, Jackson PK: **Components of an SCF ubiquitin ligase localize to the centrosome and regulate the centrosome duplication cycle.** *Genes Dev* 1999, **13:**2242-2257.
10. Kamura T, Koepp DM, Conrad MN, Skowyra D, Moreland RJ, Iliopoulos O, Lane WS, Kaelin WG Jr, Elledge SJ, Conaway RC, *et al.*: **Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase.** *Science* 1999, **284:**657-661.
11. Skowyra D, Koepp DM, Kamura T, Conrad MN, Conaway RC, Conaway JW, Elledge SJ, Harper JW: **Reconstitution of G1 cyclin ubiquitination with complexes containing SCFGrr1 and Rbx1.** *Science* 1999, **284:**662-665.
12. Xie Y, Varshavsky A: **The E2-E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain.** *EMBO J* 1999, **18:**6832-6844.
13. Honda R, Yasuda H: **Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53.** *EMBO J* 1999, **18:**22-27.
14. Martinez-Noel G, Niedenthal R, Tamura T, Harbers K: **A family of structurally related RING finger proteins interacts specifically with the ubiquitin-conjugating enzyme UbcM4.** *FEBS Lett* 1999, **454:**257-261.
15. Bordallo J, Wolf DH: **A RING-H2 finger motif is essential for the function of Der3/Hrd1 in endoplasmic reticulum associated protein degradation in the yeast *Saccharomyces cerevisiae*.** *FEBS Lett* 1999, **448:**244-248.
16. Hu G, Fearon ER: **Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins.** *Mol Cell Biol* 1999, **19:**724-732.
17. Potuschak T, Stary S, Schlogelhofer P, Becker F, Nejinskaia V, Bachmair A: **PRT1 of *Arabidopsis thaliana* encodes a component of the plant N-end rule pathway.** *Proc Natl Acad Sci USA* 1998, **95:**7904-7908.
18. Lomonte P, Everett RD: **Herpes simplex virus type 1 intermediate-early protein Vmw110 inhibits progression of cells through mitosis and from G1 into S phase of the cell cycle.** *J Virology* 1999, **73:**9456-9467.
19. Jensen DE, Proctor M, Marquis ST, Gardner HP, Ha SI, Chodosh LA, Ishov AM, Tommerup N, Vissing H, Sekido Y, *et al.*: **BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression.** *Oncogene* 1998, **16:**1097-1112.
20. Duprez E, Saurin AJ, Desterro JM, Lallemand-Breitenbach V, Howe K, Boddy MN, Solomon E, de The H, Hay RT, Freemont PS: **SUMO-1 modification of the acute promyelocytic leukaemia protein PML: implications for nuclear localisation.** *J Cell Sci* 1999, **112:**381-393.