Brief Communication

Photocrosslinking Activity-Based Probes for Ubiquitin RING E3 Ligases

Highlights
- Photoactivated activity-based probes developed for large class of ubiquitin E3 ligases
- ABPs are compatible with divergent RING E3 activation mechanisms
- Parallelized E3 profiling and detection of growth factor-induced E3 activation

Authors
Sunil Mathur, Adam J. Fletcher, Emma Branigan, Ronald T. Hay, Satpal Virdee

Correspondence
s.s.virdee@dundee.ac.uk

In Brief
Activity-based probes (ABPs) are valuable research tools for studying enzyme function. Ubiquitin E3 ligases are one of the largest enzyme families yet ABPs for this enzyme class do not exist. Mathur et al. developed photocrosslinking ABPs for RING E3s and using activity-based proteomics demonstrate activity-dependent readout of diverse E3 activation.

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Photocrosslinking Activity-Based Probes for Ubiquitin RING E3 Ligases

Sunil Mathur,1 Adam J. Fletcher,1 Emma Branigan,2 Ronald T. Hay,2 and Satpal Virdee1,3,*

1MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, Scotland, UK
2Division of Gene Regulation and Expression, University of Dundee, Scotland, UK
3Lead Contact
*Correspondence: s.s.virdee@dundee.ac.uk
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SUMMARY

Activity-based protein profiling is an invaluable technique for studying enzyme biology and facilitating the development of therapeutics. Ubiquitin E3 ligases (E3s) are one of the largest enzyme families and regulate a host of (patho)physiological processes. The largest subtype are the RING E3s of which there are >600 members. RING E3s have adapter-like activity that can be subject to diverse regulatory mechanisms and have become attractive drug targets. Activity-based probes (ABPs) for measuring RING E3 activity do not exist. Here we re-engineer ubiquitin-charged E2 conjugating enzymes to produce photocrosslinking ABPs. We demonstrate activity-dependent profiling of two divergent cancer-associated RING E3s, RNF4 and c-Cbl, in response to their native activation signals. We also demonstrate profiling of endogenous RING E3 ligase activation in response to epidermal growth factor (EGF) stimulation. These photocrosslinking ABPs should advance E3 ligase research and the development of selective modulators against this important class of enzymes.

INTRODUCTION

Ubiquitination is fundamental posttranslational modification that regulates normal cellular physiology and its dysfunction can lead to disease onset (Rape, 2018). Ubiquitination is carried out by an enzymatic cascade involving the sequential activities of ubiquitin E1-activating (E1), ubiquitin E2-conjugating (E2), and ubiquitin E3 ligases (E3s) (Hershko and Ciechanover, 1998). Ubiquitin (Ub) is covalently transferred from catalytic cysteine in E1 onto catalytic cysteine in E2 forming a thioester-linked E2 intermediate (E2~Ub). Hundreds of E3s are known to exist which recruit E2~Ub and ubiquitinate-specific substrates. Divergence of E3 mechanism has led to two general classes. “Cys E3s,” of which there are ~50, utilize a catalytic cysteine to form a covalent thioester-linked intermediate with the Ub before substrate modification (Scheffner et al., 1995; Wenzel et al., 2011; Pao et al., 2018). However, the largest class are adapter-like E3s of which there are >600 distinct forms (Deshaies and Joazeiro, 2009). Adapter-like E3s are devoid of a catalytic nucleophile and catalyze direct transfer of Ub from E2~Ub to substrate. This adapter-like activity is utilized by multi-subunit Cullin-RING E3s and ~350 single polypeptide RING E3s (hereafter simply referred to as RING E3s). The latter can exist as monomers, homodimers, or heterodimers (Metzger et al., 2014). Activity regulation is a particularly important aspect of E3 biology that ensures cellular homeostasis and adaptive signaling. Dysregulation can lead to disease onset hence RING E3s have become attractive therapeutic targets (Burgess et al., 2016). However, the cellular roles and the regulatory mechanisms for the vast majority of RING E3s remain poorly understood. Furthermore, RING E3s have recently been shown to be compatible with targeted protein degradation strategies (e.g., PROTAC methodology) (Naito et al., 2019; Spradlin et al., 2019; Ward et al., 2019), so tools for determining which are active in clinical contexts are needed to further leverage this potential.

A hallmark of adapter-like E3s is that when in the active state, they shift the dynamic E2~Ub conformational ensemble toward a distinct population where the E2~Ub conjugate adopts a folded back or “closed” conformation (Pruneda et al., 2011, 2012; Dou et al., 2012b; Plechanovová et al., 2012) (Figure 1A). This conformation activates the thioester bond within E2~Ub to nucleophilic attack and is a requisite for efficient aminolysis activity. RING E3 activity can be regulated and switching to an activated state is achieved by the E3 acquiring structural features that engage the Ub component thereby promoting induction of the closed conformation. For example, RING E3s such as RNF4 and BIRC7 are activated by RING domain dimerization where a tail region of the second RING protomer engages the Ub component (Dou et al., 2012b; Plechanovová et al., 2012). Dimerization can be regulated by cellular signals and in the case of RNF4, this is brought about by binding to poly-SUMO chains (Rojas-Fernandez et al., 2014).

For activation of monomeric RING E3s a so-called non-RING element has been shown to play a role in binding the Ub component and, in the case of Cbl-b and c-Cbl, this is a phosphorylated tyrosine residue (Dou et al., 2013). Phosphorylation is carried out by the kinase c-Src in response to growth factor stimulation and Cbl activation triggers the ubiquitination and degradation of receptor and non-receptor tyrosine kinases (Levkowitz et al., 1998, 1999; Yokouchi et al., 2001). RING E3s that require dimerization and the presence of a non-RING element have also been reported (Koliopoulos et al.,...
Additional RING E3 activation mechanisms exist including allosteric binding of accessory proteins or ligands (DaRosa et al., 2015; Dickson et al., 2018; Duda et al., 2012; Wright et al., 2016). Numerous crystal structures of E2–Ub bound to activated RING E3s have been solved revealing a highly conserved binding mode (Dou et al., 2012b, 2013; Koliopoulos et al., 2016; Plechanovova et al., 2012; Wright et al., 2016). Importantly, a consensus region of the Ub component in the closed E2–Ub conjugate becomes proximal to the activated RING. Furthermore, biophysical analysis demonstrates that activated RING E3s studied thus far can have higher free energy of binding for E2–Ub than their inactive forms (Berndsen et al., 2013; Buetow et al., 2016).

Activity-based probes (ABPs) are powerful chemical tools that undergo activity-dependent covalent labeling of enzyme family members (Niphakis and Cravatt, 2014; Hewings et al., 2017). This enables: (1) the study of enzyme regulation, (2) discovery of novel enzyme classes, (3) inhibitor screening, (4) inhibitor selectivity profiling, and (5) stabilization of enzymatic intermediates for structural studies (Hu et al., 2002). We and others have developed ABPs for Cys E3s which have been deployed to dissect E3 activation mechanisms and discover entirely novel E3 classes (Love et al., 2009; Pao et al., 2016, 2018; Mulder et al., 2018; Xu et al., 2019). ABPs that accurately assess RING E3 ligase activity do not currently exist.

We reasoned that the conserved (and activity-dependent) consensus interaction of the Ub component within the closed E2–Ub, coupled with the enhanced free energy of binding for activated RING E3s, could be exploited for the development of ABPs for RING E3s. An ABP could then be based on an engineered E2–Ub conjugate where the labile cysteine thioester has been replaced with a more stable linkage chemistry such as a lysine isopeptide (Plechanovova et al., 2012). However, ABPs typically contain an electrophilic warhead that covalently labels a catalytic nucleophile within the enzyme family under investigation. As RING E3s utilize an adapter-like mechanism, devoid of a catalytic nucleophile, we reasoned that rational placement of a photocrosslinking moiety within the E2–Ub conjugate would compensate for the absence of this catalytic feature (Figure 1B). Such a strategy has been successfully employed for metalloenzymes that also lack a catalytic residue (Saghatelian et al., 2004). Incorporation of photocrosslinking groups into polymeric ubiquitin species have also been reported, where these serve as photoaffinity probes for generic Ub interactors (Chojnicki et al., 2017; Liang et al., 2017). The utility of photocrosslinker incorporation into E2s for E3 study has also been demonstrated. Crosslinker incorporation by chemical labeling has enabled the mapping of Cys E3 catalytic residues (Krist and Statsyuk, 2015) and, more recently, incorporation into an E2 by chemical synthesis has been used to generate a
photoaffinity probe for a SUMO E3 (Zhang et al., 2019). However, the utility of these technologies as ABPs has not been demonstrated.

RESULTS

Design and Assembly of Photocrosslinking RING ABPs

To establish potential positions for photocrosslinker incorporation we generated structural superpositions for solved RING E3:E2−Ub cocryostal structures (Figure S1) (Dou et al., 2012b, 2013; Koliopoulos et al., 2016; Plechanovová et al., 2012). Striking conservation in binding mode was apparent and multiple residues within both Ub and E2 lie proximal to the RING domain(s). To impart activity dependence to the probe we incorporated the photocrosslinking moiety into Ub as unlike the E2, this component only binds proximally to active RING E3s. We chose ten consensus sites within Ub that are proximal to activated RINGs (Figure 1C) and incorporated the photocrosslinking amino acid p-benzoyl-L-phenylalanine (Bpa) using an evolved Methanocaldococcus jannaschii tyrosyl-tRNA synthetase (M/YRS)-tRNA$_{CUA}$ pair (Figures 1B and 1C) (Chin et al., 2002). Efficient incorporation was achieved at all sites yielding ~4–6 mg of protein per liter of culture medium. Ub mutants were purified to homogeneity and characterized by liquid chromatography-mass spectrometry (LC-MS) (Figure S2). All of the mutant Ub variants were then enzymatically conjugated to the promiscuous E2 UBE2D3 (Brzovic and Klevit, 2006), bearing an N-terminal hexahistidine tag (Figure 1D). In addition to facilitating purification, the latter serves as a convenient reporter tag for immunoblot analysis. Conjugation to E2 was carried out with E1 activating enzyme and, to form a more stable linkage between Ub and E2, the catalytic cysteine was mutated to lysine enabling stable isopeptide conjugation (Plechanovová et al., 2012) (Figure 2A). Importantly, structural analysis has shown that the isopeptide is an acceptable structural mimic of the native thioester (Koliopoulos et al., 2016; Plechanovová et al., 2012; Wright et al., 2016). We also introduced an S22R mutation into the E2 component, which disrupts a non-covalent Ub binding site that could result in ABP self-association (Brzovic et al., 2006). All E2−Ub variants were purified to homogeneity by size-exclusion chromatography as determined by SDS-PAGE and LC-MS analysis (Figures 2B–2D and S3A).

Activity-Dependent Profiling of the Dimeric RING E3 RNF4

The RING E3 RNF4 is inactive in the monomeric state, which is predominant at endogenous concentrations. Binding of poly-SUMO chains to SUMO-interacting motifs (SIMs) within RNF4 enhances the local concentration of RNF4 thereby promoting RING domain homodimerization and activation of E3 ligase activity (Rojas-Fernandez et al., 2014). This leads to ubiquitination and degradation of SUMO-modified promyelocytic leukemia protein (Tatham et al., 2008). Strikingly, therapeutic induction of this process leads to remission of acute promyelocytic leukemia in >90% of cases (Massaro et al., 2016). An engineered version of RNF4 that is constitutively active has been designed that consists of full-length protein with an additional RING domain fused to the native C terminus via a flexible linker (RNF4-RING) (Figure 3A) (Plechanovová et al., 2011). To determine the optimum photocrosslinker position we incubated all ten Bpa mutant E2−Ub conjugates with RNF4−RING (Figure S3B) and assessed crosslinking efficiency upon UV irradiation (10 min). Significant and dose-responsive crosslinking was only observed with Bpa incorporation at position 31 (photo-ABP-Bpa31) (Figures 3A and 3B). Importantly, no crosslinking was observed when RNF4−RING was incubated and UV irradiated with unconjugated Ub-Bpa31, indicating that the photocrosslinking was dependent on E2-driven Ub proximity, consistent with the native mechanism. Notably, an additional crosslinking product corresponding to the molecular weight for the addition of two photoABP-Bpa31 molecules was observed (Figure 3B). Structural studies on dimeric RING:E2−Ub complexes has revealed that both faces of the active RING dimer engage and activate a separate E2−Ub conjugate. By virtue of the fused RNF4−RING construct it is possible to disrupt binding to a single E2−Ub molecule, or both, by introducing an M140A protein (Tatham et al., 2008).
Figure 3. Activity-Dependent Profiling of RNF4 E3 Ligase Activity

(A) Constitutively active RNF4-RING fusion protein can productively engage two E2–Ub conjugates.

(B) Probe photoABP-Bpa31 (40 nM) undergoes two crosslinking reactions with RNF4-RING (10 μM).

(C) Engagement of one or both E2–Ub conjugates can be disrupted with a M140A R181A double mutation introduced into one or both RING domains in RNF4-RING.

(D) Probe photoABP-Bpa31 (20 nM) crosslinking is attenuated or abolished depending on whether one or both RING domains are mutated.

(E) Introduction of a F62A mutation into the E2 component should abolish E3 binding.

(F) Crosslinking is abolished with the photoABP-Bpa31 F62A (probe concentration 40 nM).

(G) At concentrations below the $K_d$ for dimerization, RNF4 is inactive. Binding of poly-SUMO chains induces dimerization and E3 ligase activity.

(H) photoABP-Bpa31 (5 μM) undergoes poly-SUMO chain (10 μM) dependent crosslinking of native RNF4 (100 nM) whereas RNF4-RING (50 nM) crosslinks independent of poly-SUMO chains. IB denotes immunoblot and the primary antibody used for detection is adjacent (i.e., anti-RNF4).
R181A double mutation into one or both of the RING domains (RNF4x/RING or RNF4x/RINGx, respectively) (Figure 3C) (Rojas-Fernandez et al., 2014). Consistent with photoABP-Bpa31 profiling this structurally elucidated bipartite mechanism, crosslinking of the second photoABP-Bpa31 molecule was lost with RNF4x-RING and was completely abolished with RNF4x-RINGx (Figure 3D). To further confirm activity-dependent photocrosslinking we prepared a mutant photoABP-Bpa31 control probe. Part of the conserved E2-RING interaction involves the E2 F62 residue (F63 in some model E2s) and mutation to alanine typically impairs/abolishes E3 binding (Figure 3E) (Weissman, 2001). This control probe would further inform on whether observed labeling is consistent with a native E2-RING interaction, thus suitable for screening inhibitors that disrupt the native interaction. Consistent with the probe being engaged in a native manner, the photoABP-Bpa31 F62A probe did not undergo RING crosslinking (Figure 3F). This probe should also serve as a valuable control when profiling RING E3s agnostically.

**ABP Profiling of Poly-SUMO Chain-Induced RNF4 Activation**

Cellular RNF4 is activated by recruitment to poly-SUMO chains via its SIM domains thereby inducing dimerization. The Kd of dimerization is ~180 nM (Rojas-Fernandez et al., 2014) so by working at concentrations below this value we established a biochemical assay to assess whether photoABP-Bpa31 could profile poly-SUMO chain-induced activation of native RNF4 (Figure 3G). As expected, constitutively active RNF4-RING was insensitive to dilution and underwent photoABP-Bpa31 crosslinking but native RNF4 did not (Figure 3H). However, in the presence of a linear amide-linked tetra-SUMO (SUMOx4) fusion protein (10 μM), which recapitulates the activation properties of native isopeptide-linked poly-SUMO chains (Tatham et al., 2008), photoABP-Bpa31 crosslinking was observed with an efficiency comparable with that of RNF4-RING. Insightfully, a crosslinked band was observed for addition of a second photoABP-Bpa31 molecule (Figure 3H). This suggests that natively activated wild-type RNF4 retains its bipartite activity and its associated processivity is presumably utilized in cells. Taken together, the data so far demonstrate that photoABP-Bpa31 undergoes activity-dependent crosslinking of a natively activated RING E3, which is devoid of a catalytic nucleophile.

**Activity-Dependent Profiling of Phosphorylation-Induced RING E3 Activation**

We next tested photoABP-Bpa31 with a RING E3 that activates via a distinct mechanism. The Cbl proteins are multidomain and multifunctional RING E3 ligases consisting of three homologs: c-Cbl, Cbl-b, and Cbl-c (Lyle et al., 2019). The majority of Cbl function is associated with RING E3 activity and involves regulation of angiogenesis and aberrations in Cbl activity have been implicated with a number of cancers. Cbl is overexpressed in many breast cancer cells and tissues and is also found to be downregulated in human myeloid neoplasms, and non-small-cell lung cancers (Kales et al., 2010; Tan et al., 2010). Hence, modulation of Cbl E3 activity is an attractive therapeutic strategy and has attracted considerable interest from the pharmaceutical industry. The most common mutation manifesting in the clinic is at residue Y371. Y371 is subject to growth factor-induced phosphorylation by c-Src kinase and this leads to structural changes that present a non-RING element, enhance affinity for E2−Ub, and stimulate Cbl E3 activity (Buetow et al., 2016; Dou et al., 2013). Indeed, the affinity for Cbl phosphorylated at Y371 (c-Cbl pTyr371) enhances E2−Ub affinity ~30-fold (Buetow et al., 2016).

To assess whether photoABP-Bpa31 can profile Src-dependent activation of c-Cbl E3 activity we incubated recombinant Src with Cbl and Cbl Y371F, the latter expected to be refractory to phosphorylation-induced activation. PhotoABP-Bpa31 crosslinking was observed for c-Cbl in the presence of c-Src but not for c-Cbl Y371F, nor when c-Src was withheld (Figure 4A). Labeling was again abolished with the photoABP-Bpa31 F62A control probe (Figure 3D), and was also ATP dependent (Figure S3E). Therefore, consistent with previous studies, phosphorylation at Y371 specifically is required for activation of E3 activity (Dou et al., 2013). We also tested the panel of engineered E2−Ub conjugates with Bpa incorporation at different positions against c-Src activated c-Cbl and found partial overlap of productive sites with those for RNF4 (Figure S3F). An optimal Bpa position was 31 but, unlike RNF4, position 32 also crosslinked with similar efficiency. This is perhaps reflective of nuances between the monomeric and dimeric activation mechanism exhibited by these two RING E3s (Dou et al., 2013; Plechanovova et al., 2012). Interestingly, photocrosslinking efficiency remained substoichiometric regardless of photoABP-Bpa31 concentration suggestive of a subpopulation of the recombinant protein preparation being active (Figure S3G). Although Phos-tag gel analysis indicated that Cbl was quantitatively phosphorylated (Figure S3H), Src is known to phosphorylate multiple sites within Cbl and the degree of probe labeling may reflect substoichiometric phosphorylation at position Y371 (Dou et al., 2012a). We tested if incubation with elevated concentrations of Src could enhance photocrosslinking efficiency but found that as concentrations approached stoichiometry, photocrosslinking was inhibited; presumably due to Src competition with photoABP-Bpa31 for Cbl binding (data not shown).

**Profiling c-Cbl Activation in a Human Cell Line**

To establish whether we could profile c-Cbl activation in a human cell line we transiently transfected HEK293 cells with GST-tagged c-Src (GST-Src) together with GFP-tagged c-Cbl (GFP-Cbl) or GFP-tagged c-Cbl Y371F (GFP-Cbl Y371F). To prevent potential degradation of activated Cbl due to autoubiquitination we treated cells with the proteasome inhibitor MG132 for 90 min before lysis. Activity-dependent crosslinking was strictly dependent on Src coexpression and the presence of Y371 but absent with the photoABP-Bpa31 F62A control probe (Figures 4B and S4A).

**Profiling Endogenous RING E3 Activation in Response to Growth Factor Stimulation**

We next assessed the ability to carry out parallelized profiling of endogenous RING E3 activation in response to a physiological stimulus. Such experiments would potentially enable poorly understood RING E3s to be ascribed to regulatory functions across a spectrum of both physiological and pathophysiological...
ubiquitination of endogenous Cbl activation could be detected in response to epidermal growth factor (EGF) stimulation, which induces Cbl phosphorylation (Levkowitz et al., 1998, 1999). HEK293T cells were stimulated with EGF and, to prevent potential degradation of activated RING E3s, we prior treated with the proteasome and lysosomal inhibitors MG132 and bafilomycin, respectively. Parallel experiments confirmed EGF responsiveness by immunoblotting for downstream mitogen-activated protein kinase activation, which is a robust marker of EGF receptor activation (Traverse et al., 1992) (Figure 4C).

We prepared a biotinylated variant of photoABP-Bpa31 allowing selective enrichment of crosslinked proteins from complex cellular samples. Bpa was incorporated into N-terminal cysteine-tagged Ub and labeled with idodacetyl-PEG2-biotin (Pao et al., 2018). Biotin labeled UbBpa31 was then enzymatically conjugated to E2 via an isopeptide bond using the procedure for untagged Ub (Figures S4B–S4F). We next tested whether endogenous Cbl activation could be detected in response to epidermal growth factor (EGF) stimulation, which induces Cbl phosphorylation (Levkowitz et al., 1998, 1999). HEK293T cells were stimulated with EGF and, to prevent potential degradation of activated RING E3s, we prior treated with the proteasome and lysosomal inhibitors MG132 and bafilomycin, respectively. Parallel experiments confirmed EGF responsiveness by immunoblotting for downstream mitogen-activated protein kinase activation, which is a robust marker of EGF receptor activation (Traverse et al., 1992) (Figure 4C).

Extracted proteomes were incubated with biotinylated photoABP-Bpa31 and enriched against streptavidin resin (Pao et al., 2018) (Figure 4D). Identification of crosslinked proteins and their probe reactivity was inferred by streptavidin enrichment followed by data-dependent LC-tandem MS (LC-MS/MS) and spectral counting (Pao et al., 2018). Twenty-five RING E3s were detected, including Cbl, and Cbl peptides were only detected in EGF and UV treated samples (Figure 4E). This suggests that the
photocrosslinking probe can detect native RING E3 activation at the endogenous level. Interestingly, there was a notable increase in spectral counts for two other RING E3s, Praja2 and TRIM11 that was EGF and UV dependent (Figure 4E). As both of these E3s have been implicated with growth factor signaling, their detection may also be reflective of their activation or upregulation in response to EGF stimulation (Di et al., 2013; Rinaldi et al., 2016).

As Ub-interacting surfaces are shared across various ubiquitin system enzymes, we unexpectedly obtained UV-dependent enrichment of HECT (11), RBR (1), and RCR (1) E3s, as well as deubiquitinating enzymes (31) and an E1-activating enzyme (Figures S4H and S4I). As a consequence, probe modification of these additional ubiquitin system components could modulate their activity and in turn alter the activation status, or stability, of RING E3s under investigation. However, this is unlikely to pose any limitations beyond those associated with the employment of cellular extracts where the majority of cellular processes would be arrested.

**DISCUSSION**

In summary, we have developed activity-based probes for the adapter-like activity of RING E3 ligases. We demonstrate an activity-dependent signal for RNF4 and c-Cbl in response to their native activation cues and how the ABP-based readout can afford further mechanistic insights. These tools allow direct assessment of RING E3 activity (no dependence on E1, E2, or substrate) in diverse sample types. We also demonstrate parallelized profiling of a subset of endogenous RING E3s in extracted proteomes and detect activation of Cbl in response to growth factor stimulation. Therefore, this technology should find utility in the study of RING E3 regulatory biology, target discovery, biomarker applications, and modulator discovery. Detection of only a subset of RING E3s in our LC-MS/MS experiments might be reflective of many being inactive or beyond the detection limit of our current experimental conditions. Another possibility is that many E3s are not functional with the E2 enzyme photoABP-Bpa31 is based on (UBE2D3). However, the engineered isopeptide conjugation strategy, for stabilizing the labile thioester, has been demonstrated with E2s that are divergent from the E2 UBE2D3 employed herein (Branigan et al., 2015; Orduereau et al., 2015). Hence, our highly modular probe production strategy should be readily applicable to other E2s simply by using distinct recombinant E2 building blocks. This would potentially grant broader RING E3 coverage and also provide insights into cellular E2-E3 interaction networks.

**SIGNIFICANCE**

The ubiquitin system regulates a host of cellular process in both health and disease. Hundreds of single polypeptide RING E3s are encoded by the human genome, yet the cellular roles and how activity is regulated has only been demonstrated for a handful of members. Agnostic assessment of the activation status of RING E3s, using the reported photoABP technology, should facilitate the elucidation of RING E3 biological function and the discovery of novel regulatory mechanisms. RING E3s have also become highly attractive therapeutic targets. However, there is a paucity of methods for measuring RING E3 activity without convoluted reconstitution of the E1-E2-E3 cascade. Assessment of endogenous E3 activity can also be challenging. The photoABPs might allow the development of simplified and direct assays for the discovery of therapeutic RING E3 modulators. The ability to parallelize measurements at the endogenous level by interfacing with mass spectrometry could also facilitate comprehensive selective profiling of therapeutic candidates. RING E3s have also recently been shown to be amenable to targeted protein degradation strategies. To fully realize this potential it is important to establish which RING E3s might be active in certain cell types. Here, the photoABPs may find further utility.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.chembiol.2019.11.013.

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**AUTHOR CONTRIBUTIONS**

S.M., A.J.F., R.T.H., and S.V. designed the research. S.M. constructed probes and validated against purified proteins with assistance from E.B. S.M. and
REFERENCES


### KEY RESOURCES TABLE

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## Experimental Models: Cell Lines

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## RNF4 (WT)


## RNF4-RING

| RNF4-RING | Plechanovova et al., 2011 | https://www.ncbi.nlm.nih.gov/pubmed/21857666 |

## RNF4x-RING

| RNF4x-RING | Plechanovova et al., 2011 | https://www.ncbi.nlm.nih.gov/pubmed/21857666 |

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Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Satpal Virdee (s.s.virdee@dundee.ac.uk). Plasmids generated in this study have been deposited to MRC Reagents and Services with the unique DU number specified in the Key Resources Table (http://mrcppureagents.dundee.ac.uk). All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

H293T cells (donor sex: female) were obtained from ATCC. 293T is a human cell line, derived from the HEK293 cell line, that expresses a mutant version of the SV40 large T antigen (RRID:CVCL_0063). Cells were cultured at 37°C in a humidified incubator under a 5% CO₂ atmosphere. Dulbecco’s modified Eagle medium was used and supplemented with fetal bovine serum and L-glutamine.

Escherichia coli BL21(DE3) or BL21 RosettaTM (DE3) cells used for protein expression in this study were grown in LB media supplemented with 100 μg/mL of ampicillin and 34 μg/mL chloramphenicol (for details see STAR Methods - Expression of Recombinant Proteins).

METHODS DETAILS

Site-Specific Incorporation of pBpa Unnatural Amino Acid into Ubiquitin

pEvol-Bpa plasmid was derived from pEVOL-pBoF (kindly provided by P. Schultz, The Scripps Research Institute). Mutations for incorporation of Bpa were introduced into both copies of M/YRS gene to make the plasmid pEVOL-Bpa (Young et al., 2010; Chin et al., 2002). BL21 cells (50 μL) were co-transformed with the pET-Ubiquitin-6His-TAGx (where x is the Bpa incorporation site) and pEvol-Bpa plasmids using heat shock and recovered in 200 μL SOC media at 37°C for 1 hour and used to inoculate 50 mL Luria-Bertani (LB) containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. 10 mL overnight culture was then used to inoculate 1 L LB broth containing the same concentrations of antibiotics. The cells were grown until OD₆₀₀ reached ~0.6 and the culture was divided into two 500 mL portions. One portion was supplemented with 1 mM p-Benzoyl-L-phenylalanine (Bpa; Bachem) and the other served as a control where Bpa was withheld. The cultures were incubated for 20 mins (37°C, 200 rpm), or until the OD₆₀₀ reached 0.6–0.7, and protein expression was induced by adding 0.02% arabinose and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were incubated for 5 hours (37°C, 200 rpm). The cells were harvested and suspended in 10 mL BugBuster® Protein Extraction (Merck Millipore) reagent before transferring to 50 mL falcon tube. The lysates were incubated for 20 minutes and then clarified by centrifugation before transferring to 50 mL falcon tube containing 1 mL Ni-NTA agarose beads and incubated for 1 hour with gentle shaking. The resin was centrifuged (4°C, 1000 rpm) and washed with wash buffer (20 mM Na₂HPO₄, pH 7.5, 25 mM imidazole). Finally, the protein was eluted with 200 μL elution buffer (20 mM Na₂HPO₄, pH 7.5, 300 mM imidazole). A 20 μL aliquot from the elution fraction was mixed with equal amount of 4X SDS loading buffer and loaded onto 4–12% SDS-PAGE gel. The proteins were separated at 200 V using MES buffer for 30 minutes and detected using Coomassie blue staining. A separate 10 μL sample was analyzed by LC-MS. LC-MS was carried out with an Agilent 1200 LC-MS system fitted with a Max-Light Cartridge flow cell coupled to a 6130 Quadrupole spectrometer. An Agilent ZORBAX 300SB-C3 5 μm, 2.1 x 150 mm column was employed unless otherwise stated. The solvent system consisted of 0.05% trifluoroacetic acid in H₂O as buffer A, and 0.04% TFA acid in acetonitrile as buffer B. Protein UV absorbance was monitored at 214 and 280 nm. MS acquisition was carried out in positive ion mode and total protein masses were calculated by deconvolution within the MS Chemstation software (Agilent Technologies).

Fractions containing the pBpa incorporated-Ub were pooled concentrated with an Amicon Ultra-15 3 kDa MWCO centrifugal filter device (Millipore). The sample was desalted into 10 mM Tris-HCl pH 7.5 using a PD-10 column (GE Life Sciences). DTT (1 mM) was added to the sample, followed by hexahistidine tag cleavage with UCH-L3 (Virdee et al., 2010), at a final concentration of 15 μg mL⁻¹. The sample was incubated at 37°C for 2 hours to remove the N-terminal His tag. Bpa incorporated-Ub was further purified by semi-preparative HPLC and the fractions were lyophilized yielding approximately 4-6 mg of Ub-pBpa.

Expression of UBE2D3(S22R/C85K) Recombinant Protein

S22R and C85K were introduced into UBE2D3 by using site-directed mutagenesis. The cells were grown until OD₆₀₀ reached 0.6–0.7 at 37°C, 200 rpm. Once OD₆₀₀ reached 0.6–0.7, protein expression was induced by adding IPTG (1 mM) and incubated at 37°C for 3 h. The cells were harvested and resuspended in buffer (20 mM Na₂HPO₄, pH 7.5, 150 mM NaCl, 1 mM TCEP, complete protease inhibitor cocktail (EDTA-free, Roche). Lysozyme was added (0.5 mg mL⁻¹) and cells were incubated on ice for 30 min followed by sonication. Clarified lysates containing His₆-tagged UBE2D3(S22R/C85K) were loaded onto Ni-NTA resin and washed with buffer (20 mM pH 7.5, Na₂HPO₄, pH 7.5, 25 mM imidazole, 150 mM NaCl, 1 mM TCEP), followed by elution with elution buffer (20 mM pH 7.5, Na₂HPO₄, pH 7.5, 300 mM imidazole, 150 mM NaCl, 1 mM TCEP). Samples were further purified by size-exclusion chromatography with a HiLoad Superdex-75 16/60 column (GE Healthcare) with running buffer (20 mM Na₂HPO₄, pH 7.5, 150 mM NaCl, 1 mM TCEP).

Preparation of Biotin-UbBpa31

Lyophilized UbBpa31 with an N-terminal MGCSSG cysteine-containing motif (10 mg) was reconstituted in 1 mL 10% DMSO/90% 0.5 mM TCEP (aq) and incubated at 23°C for 45 mins with gentle mixing, followed by the addition of 5 molar equivalents of EZ-link iodo-acetyl PEG2-Biotin (Thermofisher) in reaction buffer (50 mM Na₂HPO₄, 150 mM NaCl, 0.5 mM TCEP). The reaction was incubated at 23°C with gentle shaking for 1 h and monitored to completion by LC-MS. Product was then purified by preparative HPLC at a flow rate and lyophilized yielding biotin-UbBpa31 (6-8 mg).
Preparation of Isopeptide-linked photoABPs
To generate the photoABP-Bpa probes UBE2D3(S22R C85K) (200 μM) was incubated with UbBpa (200 μM) and His6–Uba1 (1 μM) at 35°C for 26 h conjugation buffer (50 mM Tris, pH 10.0, 150 mM NaCl, 5 mM ATP, 5 mM MgCl2, 1 mM TCEP). The E2– UbBpa conjugate was applied onto a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare) (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP). The purified photoABP-Bpa probes were concentrated to 2 mg ml⁻¹, and stored at -80°C. Biotin-photoABP-Bpa31 probe was prepared using the same procedure.

Expression of Recombinant RNF4 Protein
Cloning, expression and purification of linear fusion of two RNF4 RING domains, and associated mutants, has been described previously (Plechanovová et al., 2011). The fusion of two RING domain of RNF4 were expressed in E. coli Rosetta (DE3) cells (Novagen). The cells were grown until OD₆₀₀ reached 0.6–0.7 at 37°C, 200 rpm. Once the OD₆₀₀ reached 0.6–0.7, the protein expression was induced by adding IPTG (1 mM) and incubated overnight at 16°C, 200 rpm.

The cells were harvested and resuspended in lysis buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 10 mM imidazole, 2 mM benzamidine, complete protease inhibitor cocktail (EDTA-free, Roche)) and cells were lysed by sonication. His6-MBP-fusion proteins were purified by Ni-NTA (Qiagen) chromatography, followed by cleavage with TEV protease at 4°C overnight. To remove any uncleaved fusion protein, His6-tagged MBP, as well as His6-tagged TEV protease, material was depleted against fresh Ni-NTA resin followed by size-exclusion chromatography with a HiLoad Superdex 75 16/60 column (GE Healthcare) (20 mM Tris, 150 mM NaCl, 1 mM TCEP, pH 7.5).

Expression of c-Cbl and c-Cbl (Y371F) Recombinant Protein
BL21(DE3) cells (50 μL) were transformed with the pGEX6P-1-Cbl plasmid and recovered in 200 μL SOC media at 37°C for 1 hour and used to inoculate 50 mL Luria-Bertani (LB) containing 100 μg mL⁻¹ ampicillin. 10 mL overnight culture was then used to inoculate LB broth containing the same concentration of antibiotic and 0.2 mM zinc chloride. The cells were grown until OD₆₀₀ reached 0.6–0.7 at 37°C, 200 rpm. Once the OD₆₀₀ reached 0.6–0.7, protein expression was induced by adding 1 mM IPTG and left overnight at 16°C, 200 rpm. The cells were harvested and resuspended in buffer (50 mM Hepes, pH 7.5, 0.5 M NaCl, 1 mM TCEP) and lysed by sonication. The lysates were incubated with glutathione sepharose beads for 1 hour with gentle shaking. The resin was centrifuged (4°C, 1000 rpm) and washed with buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP), followed by cleavage with Rhinovirus 3C protease at 4°C overnight. Cleaved protein was further purified by size-exclusion chromatography with a HiLoad Superdex 200 16/600 column (GE Healthcare) (20 mM HEPES, 150 mM NaCl, 1 mM TCEP, pH 7.5).

c-Cbl Phosphorylation
Purified c-Cbl (3 μM) was phosphorylated by incubating with Src kinase (1.5 μM), 10 mM MgCl₂, 5 mM ATP at 37°C, 45 mins. Samples (15 μl) were collected and mixed well with 4X LDS loading buffer (ThermoFisher), followed by boiling before loading onto 7.5 % acrylamide phos-tag gel. The proteins were separated at 160 V using MOPS buffer for 60 mins and analysed using Coomassie staining and western blot.

Furthermore, ATP-dependent phosphorylation and photo-cross linking of c-Cbl with photoABP-Bpa31 (5 μM) was analysed using Coomassie staining. Samples (15 μl) were collected and mixed well with 4X LDS loading buffer, followed by boiling them for 5 mins at 95°C before loading onto 12% SDS-PAGE gel using MOPS running buffer and analysed using Coomassie staining. Moreover, gels were blotted and analysed using western blot with anti-Cbl (1:5000 dilution) as primary and anti-mouse (1:10000 dilution) as secondary antibodies.

UV Irradiation Conditions for Photo-Cross-Linking
Photo-cross linking reactions (45 μL) were performed in a 24-well plate (Cryshem HR3-158, Hampton Research) in reaction buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP). Samples were divided into two portions. One portion was irradiated at 365 nm on ice at a distance of 2 cm from a handled UV lamp (BLE-8T365, Spectroline), for 10-30 min and the other portion was preserved in the dark. For purified proteins such as RNF4-RING (5-10 μM) and c-Cbl (3 μM) and c-Cbl Y371F (3 μM), photo-cross linking reactions were performed with photoABP-Bpa31 probe (5-40 μM) and irradiated with UV. Samples were resolved by SDS-PAGE and visualized by Coomassie staining or immunoblotting. Control experiments were performed under the same conditions.

Photo-Crosslinking in Cell Extracts
HEK293 cells were transfected with plasmids expressing GFP-Cbl, GST-Src and GFP-Cbl. The cells were lysed in lysis buffer (50 mM Na₂HPO₄, 10 mM Glycerophosphate, 50 mM Sodium Fluoride, 5 mM Sodium Pyrophosphate, 1 mM Sodium Vanadate, 0.25 M Sucrose, 50 mM NaCl, 0.2 mM PMSF, 1 mM Benzamidine, 10 mM TCEP, 1 % NP-40). Probe photoABP-Bpa31 (5-10 μM) was mixed with cell lysate and UV irradiated (10 mins) using the photocrosslinking procedure described in the general method. Samples were analysed by 4-12 % SDS-PAGE gel using MOPS running buffer (160 V, 60 mins) and visualized by immunoblotting with anti-Cbl (1:5000 dilution) as primary and anti-mouse (1:10000 dilution) as secondary antibodies.

Phos-tag™ Gel Electrophoresis
To assess Src-mediated c-Cbl phosphorylation, we poured resolving gels (7.5 % acrylamide/bis-acrylamide, 375 mM Tris-HCl pH 8.8, 0.1% sodium dodecyl sulfate (SDS), 100 μM MnCl₂, 50 μM Phos-tag™, 0.05 % (v/v) ammonium persulphate (APS),
0.0625 % (v/v) tetramethylethylenediamine (TEMED)) and stacking gels (4 % acrylamide/bis-acrylamide, 125 mM Tris-HCl pH 6.6, 0.1 % SDS, 0.05 % (v/v) APS, 0.1 % (v/v) TEMED), degassing with argon, then allowing polymerization at room temperature for three hours. Cell extracts (50 μg) were boiled in LDS-sample buffer and supplemented with 10 mM MnCl₂ before loading. Electrophoresis was performed at 70 V through the stacking gel and 130 V through the resolving gel using running buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS), before staining with Coomassie dye, or washing 3 x 20 min in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 20% methanol) supplemented with 10 mM EDTA and 0.05 % SDS to chelate manganese, followed by 1 x 20 min in transfer buffer supplemented with 0.05 % SDS. Protein was then transferred to 0.45 μm nitrocellulose membrane in transfer buffer at 100 V, 3 hr, 4°C.

**Cell Culture, Transfection and Lysis**

293T cells were cultured (37°C, 5 % CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 2.0 mM L-glutamine and antibiotics (100 units mL⁻¹ penicillin, 0.1 mg mL⁻¹ streptomycin). Cells were seeded at a density of 4 x 10⁶ in 100 mm dishes. 18 hr post seeding, cell transfections (2 μg DNA, empty vector (pcDNA (Thermo Fisher)), pcDNA and GST-Src, pcDNA and GFP-c-Cbl, or GST-Src and GFP-c-Cbl) were performed using 5 μL FuGene-6 (Promega) in 200 μL Eagle’s Minimum Essential Medium (Opti-MEM). MG132 (25 μM) was added to cells 90 min before harvesting. Cells were rinsed and collected with ice-cold PBS, and extracted with ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 10 mM sodium 2-glycerophosphate, 50 mM sodium fluoride, 5.0 mM sodium pyrophosphate, 1.0 mM sodium orthovanadate, 0.27 M sucrose, 50 mM NaCl, 0.2 mM phenylmethanesulfonyl fluoride (PMSF), 1.0 mM benzamidine, 10 μM TCEP, 1% NP-40) on ice for 30 min. Lysates were clarified by centrifugation at 4°C for 20 min at 21,100g. Supernatants were collected and protein concentration was determined by Bradford assay.

**Activity-Based Proteomic Profiling of EGF-Stimulated HEK293 Cells**

293T cells were seeded in 150 mm dishes at a density of 5 x 10⁶ and cultured (37°C, 5 % CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 2.0 mM L-glutamine and antibiotics (100 units mL⁻¹ penicillin, 0.1 mgmL⁻¹ streptomycin). The next day, media was replaced for DMEM lacking FBS. The following day, cells were treated with 20 μM MG132 and 200 nM Bafilomycin for 6 hours at 37°C, then with or without recombinant EGF 100 ngmL⁻¹ (Thermo Fisher Scientific, PHG0311) for 15 minutes at 37°C. Dishes were transferred on to ice, washed, resuspended in ice-cold PBS, and washed twice at 4°C, and lysates extracted in ice-cold lysis buffer. 293T cells were treated with biotinylated probe (biotin-photoABP-Bpa31) (10 μM). Samples were divided and irradiated with UV for 1 hour or UV was withheld. Biotin enrichment was then carried out against streptavidin resin followed by on-resin tryptic digestion and LC-MS/MS analysis and data processing, as previously described, with an exception being hardware parametrization with an inclusion list of theoretical Cbl tryptic peptides (Pao et al., 2018).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data were presented as mean ± standard error determined from technical replicate. Statistical analysis was performed with GraphPad Prism (version 6.0). Figures 4E, S4H, and S4I were filtered against the PFAM domain terms “RING, HECT, IBR, zf-UBR and DUBs”. All biological experiments were performed at least twice.

**DATA AND CODE AVAILABILITY**

The mass spectrometry raw data files have been deposited in the Mendeley database (https://dx.doi.org/10.17632/vv8spnwgyr.1).