Single Turnover RING/U-box E3-mediated Lysine Discharge Assays

Lori Buetow, Mads Gabrielsen, Danny T. Huang*

Cancer Research UK Beatson Institute, Garscube Estate, Switchback Road, Glasgow, G61 1BD, United Kingdom; Institute of Cancer Sciences, University of Glasgow, Glasgow, G61 1BD, United Kingdom

*Corresponding author: d.huang@beatson.gla.ac.uk

Running title: Single-turnover lysine discharge assays

Summary

RING and U-box ubiquitin ligases promote ubiquitin (Ub) transfer by priming Ub-conjugated E2 in a closed conformation to optimize the thioester bond for nucleophilic attack by substrate lysine. Here, we describe a single-turnover lysine discharge assay for direct assessment of the activity of any RING/U-box E3-E2~Ub complex.

Key words

Ubiquitination, lysine discharge, RING or U-box E3 ligases, radiolabeling

1. Introduction

Ubiquitination is a post-translational modification whereby the small 76 amino acid protein modifier ubiquitin (Ub) is covalently attached to a protein substrate [**1**]. Ubiquitination is achieved by the sequential actions of three enzymes: Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2) and a Ub ligase (E3) [**2, 3**]. Initially, E1 activates Ub in a Mg^{2+}-ATP-dependent manner and a thioester bond is formed between the catalytic cysteine of E1 and the diglycine motif at the C-terminus of Ub to produce E1~Ub complex, where ~ denotes a thioester (Fig. 1a). E1~Ub subsequently recruits an E2, and Ub is transferred to the active site cysteine of E2 via its tail, thereby forming an E2~Ub intermediate (Fig. 1b). Next, an E3 recruits E2~Ub and substrate to mediate the transfer of Ub to substrate (Fig. 1c). In this final step, an isopeptide/amide link is formed between the Gly 76 of Ub and a free amino group from a lysine side chain or the N-terminus of a substrate protein (Fig. 1d).

[INSERT FIGURE 1 NEAR HERE]

The human genome encodes two E1s, approximately 40 E2s and over 600 putative E3s [**4**]. E3s are categorized into three families based on their Ub transfer mechanism: HECT, RING-between-RING (RBR), and RING/U-box [**5**]. HECT and RBR E3s form an E3~Ub complex prior to transferring Ub to substrate whereas RING/U-box E3s catalyze the transfer of Ub directly from E2~Ub to substrate (Fig. 1e). A shared feature among all RING/U-box E3s is the presence of a RING or U-box domain that recruits E2~Ub and promotes Ub transfer. Simple RING/U-box E3s encode a RING/U-box domain and substrate-binding domain within a single polypeptide chain whereas multisubunit E3s like cullin RING ligases encode these domains on multiple polypeptide chains. Some simple RING E3s like RNF38 function as monomers, but others, like XIAP or BRCA1/BARD, are only functional as dimers [**5**].

Although binding is observed for many E2-E3 pairs *in vitro*, this does not always translate to function. For example, BRCA1/BARD binds the E2 UbcH7 [**6**] but does not enhance its reactivity [**7**]. E2s have an intrinsic reactivity that generally involves Ub transfer to a thiol as in a cysteine residue or to an amine as in a lysine residue or the N-terminus of a protein [**8**]. Based on these properties, it is essential to select an E2 for lysine discharge assays that has intrinsic reactivity toward lysine. Some E2s, like UbcH7, are only reactive toward cysteine [**7**] whereas others, like Ube2W, only react with the N-terminal amino group of a protein or peptide [**9, 10, 11**].

The chemical reaction profile of RING/U-box mediated Ub transfer involves a number of steps with several species of intermediates. Initially, both E2~Ub and a protein substrate are recruited to form a RING/U-box-substrate-E2~Ub complex (Fig. 2a). The substrate lysine side chain or N-terminal amino group is then deprotonated to

produce a nucleophile (Fig. 2b). This activated nucleophile subsequently attacks the carbonyl group of the E2~Ub thioester to form an oxyanion intermediate in which E2 is covalently linked to both Ub and substrate (Fig. 2c). In the final steps, a thiol group is eliminated (Fig. 2d and 2e), thereby forming an amide/isopeptide bond between substrate and ubiquitin (Fig. 2f).

[INSERT FIGURE 2 NEAR HERE]

To promote Ub transfer, RING/U-box E3s bring E2~Ub together with substrate and optimize the orientation of the E2~Ub thioester for nucleophilic attack [**5**]. In addition, some RING/U-box E3s have been shown to impose conformational restraints that regulate which substrate lysines can access the active site of E2~Ub [**12, 13, 14**]. In this chapter, we describe how to perform a single-turnover lysine discharge assay in which we use SDS-PAGE to follow Ub transfer from E2~Ub to free lysine in solution (Fig. 3). [INSERT FIGURE 3 NEAR HERE]. This removes any dependency on substrate recognition and binding and facilitates analysis of the mechanism used by RING/U-box E3s to prime E2~Ub and promote Ub transfer. By using this reductionist approach combined with mutagenesis, we can explore the role of the E2 in coordinating donor Ub (the thioesterified Ub) in the presence and absence of E3, the role of the RING or U-box domain in coordinating donor Ub and priming E2~Ub for transfer, and the role of additional components in stimulating or inhibiting the actions of the RING or U-box domain in mediating Ub transfer.

A single-turnover lysine discharge reaction consists of a charge step in which Ub is loaded onto E2 by E1, a stop step in which reagents are added to inactivate E1 and prevent additional loading of Ub onto E2, and a final step in which lysine alone or a

mixture of lysine with E3 and/or additional components is added to initiate discharge of E2~Ub (Fig. 3). Each step requires empirical optimization. E2-charging efficiency depends on the E1-E2 pair and can be influenced by a number of factors including changing the duration and/or temperature of the charging step, the source organism for E1, and relative concentrations of E1, E2 and Ub. In most cases, adding EDTA quenches the charge reaction. However, for some E1-E2 pairs, inclusion of apyrase together with EDTA seems to work more effectively. For a clear, visually convincing result, the disappearance of the E2~Ub band should be readily apparent over the time points selected and can be affected by adjusting factors like the concentration of lysine and/or E3, or running the assay at a different temperature for the discharge step.

We routinely use *Arabidopsis thaliana* Uba1 as our E1, human UbcH5B as our E2 and radiolabeled Ub (^{32}P-Ub). We have devised a method to purify relatively large quantities of active *Arabidopsis* Uba1 from *E. coli* compared to other species [**15**] and found it to be fairly robust with respect to storage, handling, and E2-charging efficiency. We use UbcH5B when possible/relevant because almost 100% of UbcH5B can be loaded with Ub in our charge reaction. Additionally, most RING/U-box E3s react with UbcH5B~Ub and substrate lysine modification is fairly promiscuous with this E2 *in vitro* [**16**]. In cases where we want to avoid stimulation of Ub transfer by non-covalent binding of Ub to the surface of UbcH5B opposite the active site Cys [**17, 18**], we use an S22R substitution on UbcH5B. Although lysine discharge assays can be performed using Coomassie staining alone, radiolabeled assays are more sensitive and prevent detection/interpretation problems that arise due to overlapping bands on a gel, whether these bands arise from similarly sized assay components or

contaminants. Other methods like fluorescent labeling of Ub are also suitable but the labeling and detection processes require additional optimization and/or standardization such as what fraction of Ub requires labeling to obtain a detectable signal, how stable is the label, and how much gel-to-gel variation is present. Hence, when possible, we use ^{32}P-Ub in our assays.

In the following method, we describe the procedure used to scout for suitable lysine discharge conditions to investigate Ub transfer mediated by the RING domain of RNF38 (RNF38$_{RING}$, residue 389–C). "Suitable conditions" for these assays depend on the type of comparison being made. Previously, to demonstrate that RNF38$_{RING}$-mediated Ub transfer proceeded via the same general mechanism as other RING E3s, we introduced mutations into RNF38$_{RING}$, UbcH5B or Ub and investigated their effect on lysine discharge reactions compared to wild-type [**18**]. For this type of comparison in which the mutations are expected to slow discharge, "suitable conditions" involve lysine/E3 concentrations and a time frame where UbcH5B~Ub is discharged within the first few time points. In contrast, when we used RNF38$_{RING}$ to demonstrate that non-covalent binding of Ub to UbcH5B stimulates E3-mediated Ub transfer, the lysine/E3 concentrations and time frame were adjusted so that UbcH5B~Ub was incomplete at the later time points in the absence of excess non-covalent Ub.

2. Materials

Prepare all solutions with ultrapure water. Purification protocols for *Arabidopsis* E1, 2TK-Ub (see **Note 1**), UbcH5B, and RNF38$_{RING}$ are available in the indicated references [**15, 18**].

2.1 Preparation of buffers and solutions.

1. 5 M NaCl.
2. 1 M Tris-HCl, adjusted to pH 7.6.
3. 2 M $MgCl_2$.
4. 1 M NaOH.
5. 0.5 M ATP, adjusted to pH 7-8 with NaOH and stored at -20 °C in 100 μL aliquots.
6. 10x Charge Buffer: 500 mM Tris-HCl, pH 7.6, 50 mM ATP, 50 mM $MgCl_2$. When mixing the buffer, $MgCl_2$ is added after all the other components including water to avoid producing an insoluble Mg-ATP complex. This buffer is prepared fresh weekly and stored at 4 °C.
7. 1.5 M L-lysine, pH 7.6: Add 2.46 g L-lysine monohydrate (Formedium) to ~4-5 mL water, adjust pH to 7.6 with NaOH and then add water to a final volume of 10 mL. This solution is stored in 0.5-1 mL aliquots at -20 °C and can be repeatedly frozen and thawed.
8. TBS buffer: 150 mM NaCl, 25 mM Tris-HCl, pH 7.6.
9. 0.5 M EDTA, pH 8.0.
10. 1 U/μL Apyrase (Sigma A6535) solubilized in a buffer containing 50 mM NaCl, 25 mM HEPES, pH 7.0, 1 mM $MgCl_2$, and 1 mM DTT. Snap freeze in liquid nitrogen and store at -80 °C in 5-10 μL aliquots. Each aliquot is thawed only once and used fresh on the day of the experiment.
11. 20 mg/mL BSA (Sigma A3294) in water. Store at -20 °C in 200 μL aliquots. Can be repeatedly frozen and thawed.

12. 10x γ-^{32}P-ATP labeling buffer: 1 M NaCl, 150 mM Tris-HCl, pH 7.6, 120 mM $MgCl_2$, 10 mM DTT. Store at -20 °C in 100 μL aliquots and re-frozen/thawed as needed.

2.2 Sample preparation and analysis.

1. 1.5 mL Eppendorf tubes.
2. NuPAGE 4-12% Bis Tris gels (Invitrogen Thermo Fisher Scientific) or similar
3. 4x NuPAGE LDS sample buffer (Invitrogen Thermo Fisher Scientific)
4. 2x SDS-PAGE sample buffer (non-reducing): Equal volumes of non-reducing 4x NuPAGE LDS sample buffer and water.
5. 1x MES Running buffer: 50 mM MES, 50 mM Tris-HCl, pH 7.3, 0.1 % SDS, 1 mM EDTA (in our case, prepared from a 20x solution purchased from Invitrogen Thermo Fisher Scientific).
6. Plastic containers with dimensions suitable to accommodate the gel.
7. Fixing/gel drying buffer: 5% methanol, 8% acetic acid, 5 % glycerol.
8. Whatman paper, cut into pieces slightly bigger than gel.
9. Whatman paper, cut to the size of the gel dryer.
10. Gel dryer.
11. Film or phosphor storage cassette.
12. Film developer or phosphor imager.

3. **Methods**

3.1 Preparation of 100 μM radioactively labeled ^{32}P-Ub (see **Note 2**).

1. Calculate the volume of 2TK-Ub (see **Note 1**) to achieve a concentration of 100 μM Ub in 200 μL labeling buffer. This volume is 2.9 μL based on our current 2TK-Ub stock concentration of 6.96 mM.
2. Mix 20 μL 10x γ-^{32}P-ATP labeling buffer with 169.9 μL water, 2.9 μL 2TK-Ub, 6.2 μL γ-^{32}P-ATP (6000 Ci/mmol 10 mCi/mL), and 1 μL of cAMP-dependent Protein Kinase, catalytic subunit (NEB P6000L). Incubate at room temperature for 1-4 hours. Store at 4 °C.

3.2 Preparation of lysine discharge solutions.

Prepare discharge solution(s) just prior to running assays. With the exception of BSA (see **Note 3**), the concentration of lysine, E3 and/or additional components should be twice the final desired concentration (see **Note 4**). The stability of the E3 and any additional components will determine the concentrations of NaCl, buffer and DTT required in the discharge solution. For RNF38$_{RING}$, NaCl and buffer (Tris-HCl, pH 7.6) concentrations can range between 50-150 mM and 10-50 mM, respectively, and no DTT is required.

1. Lysine dilution buffer/lysine-only discharge buffer: 20 mM lysine, 1 mg/mL BSA, 50 mM NaCl, 25 mM Tris-HCl, pH 7.6 (200 μL).
2. Lysine/E3 discharge solution: 20 mM lysine, 1 mg/mL BSA, 800 nM RNF38$_{RING}$ (100 μL, see **Note 5**).

3.3 Preparation of stop solution.

Stop solution should be prepared just prior to running assays (See **Note 6**).

1. Stop solution: 250 mM NaCl, 500 mM Tris-HCl, pH 7.6, 150 mM EDTA, and 0.1 U/μL apyrase. After charging and stopping, a reaction contains 0.5 units of apyrase for every 0.1 μmol of ATP.

3.4 Preparation of charge stock solution.

Charge stock solution should be prepared just prior to running assays.

1. Per 25 μL charge reaction: 1x charge buffer, 12.5 μM UbcH5B, 12.5 μM ^{32}P-Ub (see **Note 7**), ~75-100 mM NaCl, minimal amounts of reducing agent , i.e. only what is carried over from stock solutions. (see **Note 8**).
2. Calculate the volume of each reagent required per reaction. The relative amounts of water and TBS buffer will vary depending on the stock concentrations of E1, E2 and ^{32}P-Ub and the NaCl content of each of these solutions. For these assays, both the stocks of UbcH5B and ^{32}P-Ub are at 100 μM and contain 150 mM NaCl. The stock of *Arabidopsis* E1 is at 30 μM and contains ~200 mM NaCl. In this case, the volume required to bring the solution to 25 μL can be split equally between water and TBS buffer. If one of the components is very dilute or very concentrated, then the ratio will need adjusting. Each 25 μL reaction requires 2.5 μL 10x charge buffer, 3.125 μL 100 μM UbcH5B, 3.125 μL 100 μM ^{32}P-Ub, 0.833 μL 30 μM *Arabidopsis* Uba1, 1.25 μL BSA (20 mg/mL), 7.1 μL water, and 7.1 μL TBS buffer.
3. There are six reactions in total (see below), but the stock volume is calculated based on eight to account for handling errors. Mix the components in the following order:
    a. 20 μL 10x Charge Buffer.
    b. 56.8 μL water.

c. 56.8 μL TBS buffer.

d. 10 μL BSA (20 mg/mL).

e. 25 μL 100 μM UbcH5B.

f. 6.7 μL 30 μM *Arabidopsis* Uba1 (See **Note 9**).

4. Flick tube and microfuge briefly (~10 sec). Add 21.9 μL to each of six Eppendorf tubes.

3.5 Assay assembly and execution.

1. Prepare sample tubes. Based on an assay with five time points including a zero time point (T_0), each assay requires 25 μL of a charged E2~Ub solution, 6.125 μL of a stop solution and 30 μL of a discharge solution.
2. Add 6 μL 2x SDS-PAGE sample buffer to Eppendorf tubes for T_0 for each reaction. Make sure there is no reducing agent in the sample buffer.
3. Add 4 μL of 4x sample buffer to the tubes for assay time points following addition of discharge solution. For the assays described here, four time points are taken per reaction.
4. Dilute 30 μL of lysine/E3 discharge solution from 3.2.1 with 30 μL of lysine dilution buffer prepared in step 3.2.2.
5. Dilute 30 μL of lysine/E3 discharge solution from step 4 with 30 μL of lysine dilution buffer prepared in step 3.2.1. Repeat 2-fold E3/lysine discharge buffer serial dilution a total of four times. The discharge solutions for the reactions are as follows:

    a. Reaction 1: 20 mM lysine, 1 mg/mL BSA.

    b. Reaction 2: 20 mM lysine, 800 nM $RNF38_{RING}$, 1 mg/mL BSA.

    c. Reaction 3: 20 mM lysine, 400 nM $RNF38_{RING}$, 1 mg/mL BSA.

d. Reaction 4: 20 mM lysine, 200 nM $RNF38_{RING}$, 1 mg/mL BSA.

e. Reaction 5: 20 mM lysine, 100 nM $RNF38_{RING}$, 1 mg/mL BSA.

f. Reaction 6: 20 mM lysine, 50 nM $RNF38_{RING}$, 1 mg/mL BSA.

6. Add 3.125 μL 100 μM ^{32}P-Ub to 21.9 μL charge stock aliquoted in step 3.4.4. Start the timer.
7. Flick tube to mix and spin down briefly (~5 sec) in a benchtop mini centrifuge.
8. Incubate for 15 minutes (See **Note 10**).
9. Add 6.125 μL stop solution. Flick the tube to mix and spin down briefly (~5 sec) in a benchtop mini centrifuge (See **Note 11**).
10. About 15-30 sec after adding stop solution, remove 6 μL and add to T_0 sample tube and vortex. (See **Note 12**).
11. About 1-2 min after adding stop solution, add 25.125 μL discharge solution from step 3.5.5. Pipette up and down 5-10 times.
12. Flick the tube to mix and spin down briefly (~5 sec) in a benchtop mini centrifuge.
13. Take 12 μL time points at 30, 60, 90 and 120 seconds after adding discharge solution for each reaction. Vortex immediately upon adding to gel sample buffer. (See **Note 13**).
14. Microfuge all samples for ~15 seconds prior to loading onto SDS-PAGE gels (See **Notes 14** and **15**).
15. Load samples onto SDS-PAGE gels by reaction with T_0 followed by the time course. Use non-reducing buffers when running gels.
16. Run gels at 200 V for approximately 35 minutes so that the ^{32}P-Ub band almost reaches the bottom of the gel.

17. Transfer the gels to plastic containers filled with fixing/gel drying buffer. Trim off the foot and most of the stacker, leaving ~3 mm of each well. Leave in buffer for 2-5 minutes.
18. Prepare a stack of at least three pieces of gel-sized Whatman filter paper. Label top piece with a unique gel identifier.
19. Pre-wet the labeled filter paper in fixing/gel drying buffer and return to top of stack. Place gel on the labeled filter paper.
20. Place gel stack on three pieces of Whatman filter paper that are cut to a size slightly smaller than your gel dryer.
21. Dry gels. We use cling film to avoid contaminating the gel-drying equipment with radioactive material.
22. Expose gels to film in a darkroom or a phosphor storage cassette to capture the signal from ^{32}P-Ub. Try different exposure times to ensure an optimal and accurate visual representation of the results (See **Notes 16** and **17**). The exposure time is dependent upon the concentration of the radioactive material and the age of the ^{32}P used (half-life is about 14 days).
23. Analyze results based upon the disappearance of the E2~Ub band over time (see Figure 4) to determine if a suitable condition has been found or further screening is required.

[INSERT FIGURE 4 NEAR HERE]

Notes

1. Labeling in our experiment relies on a 2TK tag (RRASV) at the N-terminus of Ub. The gene encoding Ub has been cloned into the BamHI and EcoRI sites of a modified pGEX-2TK GST expression vector (GE Healthcare) in which the

thrombin cleavage site has been replaced with TEV and a non-cleavable His_6 tag precedes the GST tag.

2. The final labeled concentration of 2TK-Ub and amount of γ-^{32}P-ATP can be varied.
3. Some E3s adhere to the surface of the Eppendorf tubes, which reduces apparent activity and affects assay reproducibility. When possible, BSA is added to discharge solutions prior to adding E3 or additional protein/peptide components to prevent adherence.
4. Although we do not fit kinetic parameters with lysine discharge assays, the concentrations of various reactants are determined as if a kinetic analyses were intended. Under ideal conditions, the maximum concentration of E3 (the enzyme) used in any reaction is at least ten-fold lower than E2~Ub (the substrate).
5. Protein stocks are stored at -80 °C at a minimum concentration of 1 mg/mL. Diluted working E3 stocks are prepared in TBS containing 1 mg/mL BSA to minimize losses from surface adherence. For the discharge assays presented here, the -80 °C stock of $RNF38_{RING}$ was at 8.7 mg/mL (937 μM). A 5 μM working stock in TBS containing 1 mg/mL BSA was prepared to make the discharge solution.
6. Although apyrase is inhibited by EDTA, we have found that using a combination of the two is slightly more effective at stopping charging for certain E1-E2 pairs. The concentrations of the other reagents in the stop solution are unchanged regardless of whether apyrase is used.
7. When using a new E2, initial experiments needs to be done to characterize the level of ubiquitin charging onto E2 that can be achieved with a given E1. An

ideal charge reaction will convert all the E2 to E2~Ub without producing byproducts like diUb, E2–Ub or E1–Ub, where – denotes an isopeptide bond between the C-terminal diglycine motif of Ub and the side chain of a lysine residue on the protein. The species of origin of the E1 plays a significant role in the E2-conjugating efficiency as well as the duration, temperature of the charge reaction, and molar ratio of ^{32}P-Ub to E2. For a given E2, we optimize charging conditions by using SDS-PAGE to analyze time courses of several charge reactions in which ^{32}P-Ub:E2:E1 ratios are varied. Almost all of UbcH5B can reliably be converted to UbcH5B~Ub up to concentrations of 20 μM UbcH5B using molar ratios of ^{32}P-Ub:UbcH5B between 1:1 and 2:1 with 1 μM *Arabidopsis* E1.

8. Because the E2~Ub thioester is labile, it is important that the concentration of reducing agent is kept to a minimum.
9. At concentrations less than ~100 nM, there is a pronounced reduction in Uba1 activity due to surface adherence. Hence, when possible, BSA is included in the charge reaction and Uba1 is added after BSA. If BSA is not used, Uba1 is always added after E2.
10. Ub mutants can influence the rate of the E1-E2 transthiolation reaction. In reference [**15**], some charge reactions with Ub mutants were run for 30 minutes instead of 15 minutes to produce equivalent amounts of E2~Ub to the corresponding wild-type reaction.
11. Stopping the reaction is critical. New E1-E2 combinations should be tested by setting up a charge reaction, adding the stop solution, and collecting samples over 10 minutes to check that the reaction has stopped charging and to

investigate the stability of E2~Ub. For UbcH5B, once the charge reaction is stopped, a reduction in UbcH5B~Ub is evident in as little as 5 minutes.

12. For each reaction in an assay, the time between adding stop, removing T_0 and adding discharge solution should be the same and kept to a minimum.
13. Thioester bonds are labile at higher temperature, so do NOT boil the samples.
14. The viscosity of the gel sample buffer from Invitrogen frequently causes problems with reproducibly pipetting accurate volumes. Hence, all the sample volume for each time point is loaded onto the gel.
15. Because of the labile nature of E2~Ub, samples are sensitive to discharge even after denaturation with gel loading buffer. Hence, gels should be run promptly after completing an assay and there are no benefits to holding back "extra" sample in the event that there is a problem with a gel. In addition, when running multiple reactions in an assay, it is best to stagger the reactions to minimize the time between completing the first reaction and loading the gels.
16. When working with new systems, overexposure to check for byproducts is advised to ensure that the concentration of lysine is saturating under the conditions tested. When working with a new system, films should be exposed for a long time to check that Ub transfer occurs to free lysine and not lysine residues on the surface of any of the proteins in the system. We have previously observed formation of diUb and E3–Ub (where – indicates an isopeptide linkage) when lysine concentrations were too low.
17. When multiple gels are run to compare reactions in an assay, the gels should be exposed to film at the same time and onto the same film.

Acknowledgments

This work was supported by Cancer Research UK (C596/A23278) and European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement n° 647849*).*

References

1. Hershko A, Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67:425-479
2. Pickart CM, Eddins MJ (2004) Ubiquitin: structures, functions, mechanisms. Biochim Biophys Acta 1695:55-72
3. Dye BT, Schulman BA (2007) Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. Annu Rev Biophys Biomol Struct 36:131-150
4. Komander D, Rape M (2012) The ubiquitin code. Annu Rev Biochem 81:203-229
5. Buetow L, Huang DT (2016) Structural insights into the catalysis and regulation of E3 ubiquitin ligases. Nat Rev Mol Cell Bio 17:626-642
6. Brzovic PS, Keeffe JR, Nishikawa H et al. (2003) Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. Proc Natl Acad Sci U S A 100:5646-5651
7. Wenzel DM, Lissounov A, Brzovic PS et al. (2011) UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. Nature 474:105-108

8. Stewart MD, Ritterhoff T, Klevit RE et al. (2016) E2 enzymes: more than just middle men. Cell Res 26:423-440
9. Tatham MH, Plechanovova A, Jaffray EG et al. (2013) Ube2W conjugates ubiquitin to alpha-amino groups of protein N-termini. Biochem J 453:137-145
10. Scaglione KM, Basrur V, Ashraf NS et al. (2013) The ubiquitin-conjugating enzyme (E2) Ube2w ubiquitinates the N terminus of substrates. J Biol Chem 288:18784-18788
11. Vittal V, Shi L, Wenzel DM et al. (2015) Intrinsic disorder drives N-terminal ubiquitination by Ube2w. Nature chemical biology 11:83-89
12. Scott DC, Sviderskiy VO, Monda JK et al. (2014) Structure of a RING E3 trapped in action reveals ligation mechanism for the ubiquitin-like protein NEDD8. Cell 157:1671-1684
13. Mcginty RK, Henrici RC, Tan S (2014) Crystal structure of the PRC1 ubiquitylation module bound to the nucleosome. Nature 514:591-596
14. Chang L, Zhang Z, Yang J et al. (2015) Atomic structure of the APC/C and its mechanism of protein ubiquitination. Nature 522:450-454
15. Dou H, Buetow L, Sibbet GJ et al. (2012) BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer. Nat Struct Mol Biol 19:876-883
16. Brzovic PS, Klevit RE (2006) Ubiquitin transfer from the E2 perspective: why is UbcH5 so promiscuous? Cell Cycle 5:2867-2873
17. Brzovic PS, Lissounov A, Christensen DE et al. (2006) A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. Mol Cell 21:873-880

18. Buetow L, Gabrielsen M, Anthony NG et al. (2015) Activation of a primed RING E3-E2-ubiquitin complex by non-covalent ubiquitin. Mol Cell 58:297-310

19. Marvin (version 17.21.0) (2017) ChemAxon (http://www.chemaxon.com).

Figure captions

Figure 1: Schematic overview of the ubiquitination cascade. (**a**) Ub is activated by E1 and (**b**) transferred to E2 conjugating enzyme. (**c**) E3 ligase mediates the transfer of Ub from E2~Ub, onto substrate. (**d**) Close up of final isopeptide bond between Gly 76 of Ub and a Lys sidechain on substrate. (**e**) RING E3 recruits E2~Ub and substrate to facilitate direct transfer of Ub from E2 onto the substrate.

Figure 2: Enzymatic mechanism of substrate ubiquitination by RING/U-box E3 ligases. (**a**) RING/U-box E3 ligases recruit substrate and E2~Ub, thereby forming a RING/U-box-substrate-E2~Ub complex; spatial restraints imposed by the E3 frequently play a key role in substrate lysine selectivity. (**b**) A residue from the E2 (D117 in UbcH5B) deprotonates the lysine side chain amine, thereby converting it to a nucleophile. (**c**) This nucleophile attacks the carbonyl of the E2~Ub thioester, forming an oxyanion intermediate in which covalent interactions are present between substrate, the catalytic Cys of E2 (C85 in UbcH5B) and Ub. The oxyanion intermediate is stabilized by N77 in UbcH5B. (**d**) Deprotonation of the intermediate, which may be performed by a residue from the E2, followed by (**e**) elimination of a thiol, (**f**) thereby freeing the catalytic Cys of E2 to accept another Ub and producing

an isopeptide bond between the side chain of a substrate lysine and Gly 76 of Ub. MarvinSketch was used for drawing chemical structures and reactions [**19**].

Figure 3: Schematic overview a lysine discharge reaction. (**a**) Charge: E1, E2 and Ub are mixed to produce E2~Ub; (**b**) Stop: Chemical and/or enzymatic reagents are added to inactivate E1, thereby preventing production of more E2~Ub or recharging of the E2 after discharge; (**c**) Discharge: L-lysine, E3 and/or additional components are added and (**d**) the discharge of E2~Ub over time is monitored by SDS-PAGE.

Figure 4: Non-reduced autoradiograms of reactions 1–6 as described in the methods section showing the discharge of UbcH5B~^{32}P-Ub over 2 min with final concentrations of 10 mM L-lysine and 0-400 nM $RNF38_{RING}$ as indicated. For assays in which comparisons are made with a stimulating component like non-covalent Ub [**18**], 25-50 nM $RNF38_{RING}$ and 10 mM L-lysine are a suitable starting point provided that the stimulatory component/effect is strong enough to drive the reaction to completion in two minutes. For comparisons across reactions involving UbcH5B mutations that slow discharge, a suitable starting concentration of $RNF38_{RING}$ would be between 200-400 nM, depending on the extent that mutants disrupt discharge. A possible next step in the optimization process would be to test a narrower concentration range of $RNF38_{RING}$ with a mutation that abolishes E3-mediated discharge.

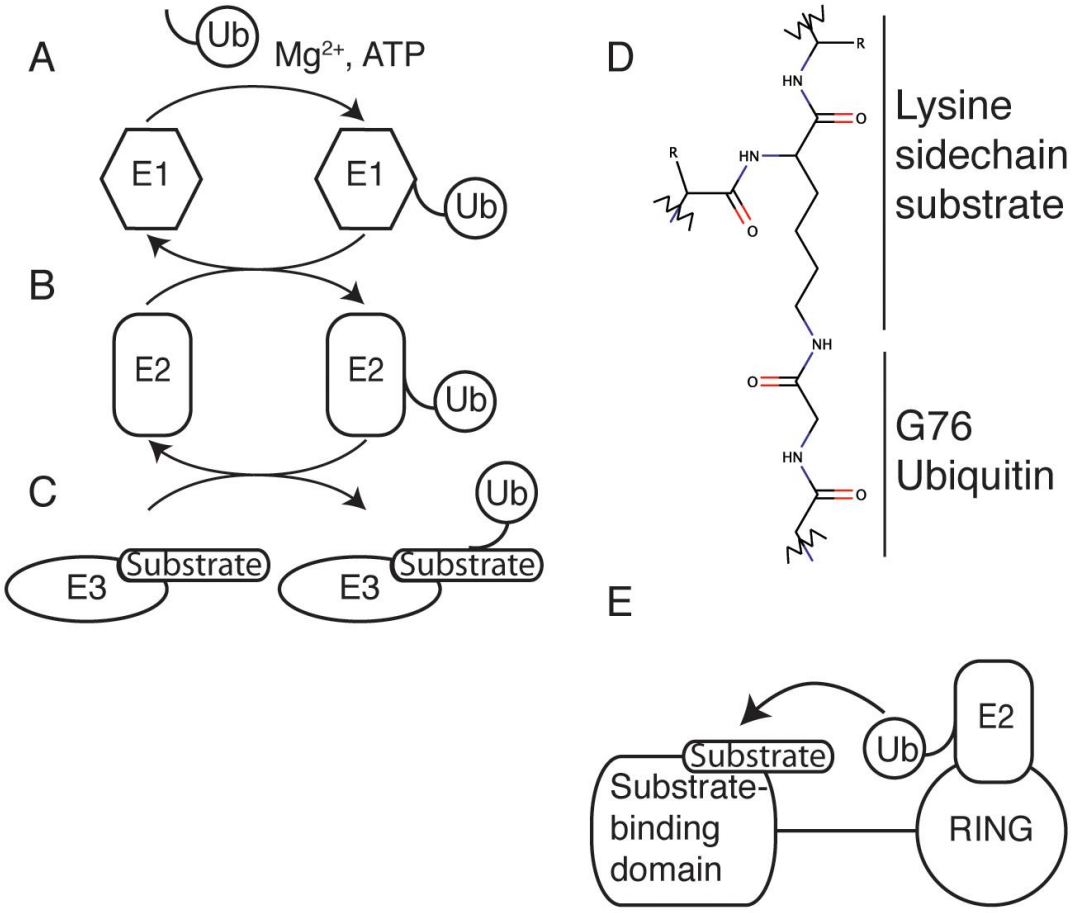
A
Ub
Mg2+, ATP
E1
E1
Ub
B
E2
E2
Ub
C
Ub
E3
Substrate
E3
Substrate
D
R
HN
O
R
HN
O
NH
O
HN
O
Lysine
sidechain
substrate
G76
Ubiquitin
E
Substrate
Ub
E2
Substrate-
binding
domain
RING

Figure 1

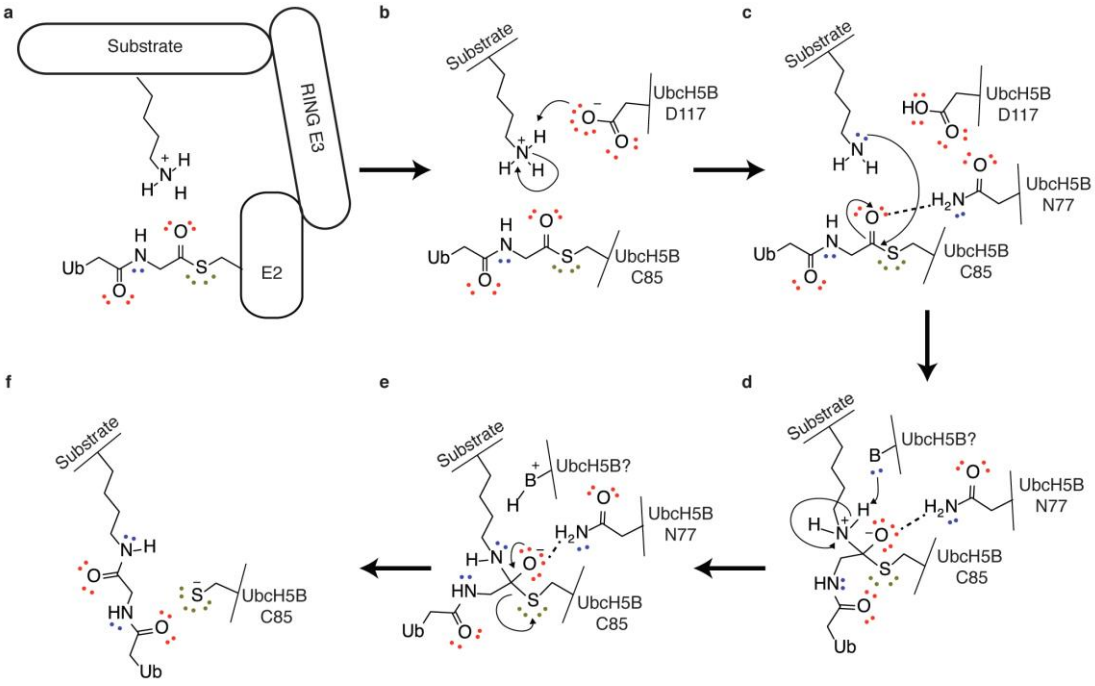
a
Substrate
RING E3
E2
Ub
b
Substrate
UbcH5B
D117
UbcH5B
C85
c
Substrate
UbcH5B
D117
UbcH5B
N77
UbcH5B
C85
d
Substrate
UbcH5B?
UbcH5B
N77
UbcH5B
C85
e
Substrate
UbcH5B?
UbcH5B
N77
UbcH5B
C85
f
Substrate
UbcH5B
C85

Figure 2

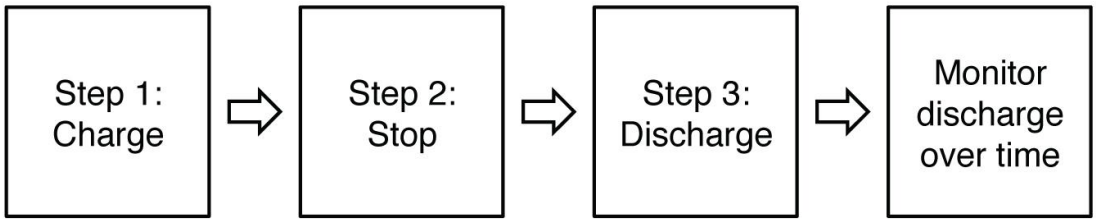
Step 1:
Charge
Step 2:
Stop
Step 3:
Discharge
Monitor
discharge
over time

Figure 3

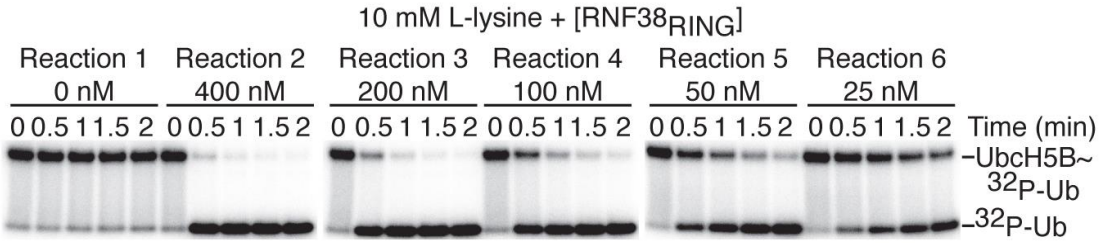
10 mM L-lysine + [RNF38RING]
Reaction 1
0 nM
Reaction 2
400 nM
Reaction 3
200 nM
Reaction 4
100 nM
Reaction 5
50 nM
Reaction 6
25 nM
0 0.5 1 1.5 2 0 0.5 1 1.5 2 0 0.5 1 1.5 2 0 0.5 1 1.5 2 0 0.5 1 1.5 2 0 0.5 1 1.5 2
Time (min)
UbcH5B~32P-Ub
32P-Ub

Figure 4