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TITLE:

Functional Characterization of RING-Type E3 Ubiquitin Ligases In Vitro and In Planta

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SUMMARY:

The goal of this manuscript is to present an outline for the comprehensive biochemical and functional studies of the RING-type E3 ubiquitin ligases. This multistep pipeline, with detailed protocols, validates an enzymatic activity of the tested protein and demonstrates how to link the activity to function.

ABSTRACT:

Ubiquitination, as a posttranslational modification of proteins, plays an important regulatory role in homeostasis of eukaryotic cells. The covalent attachment of 76 amino acid ubiquitin modifiers to a target protein, depending on the length and topology of the polyubiquitin chain, can result in different outcomes ranging from protein degradation to changes in the localization and/or activity of modified protein. Three enzymes sequentially catalyze the ubiquitination process: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin ligase. E3 ubiquitin ligase determines substrate specificity and, therefore, represents a very interesting study subject. Here we present a comprehensive approach to study the relationship between the enzymatic activity and function of the RING-type E3 ubiquitin ligase. This four-step protocol describes 1) how to generate an E3 ligase deficient mutant through site-directed mutagenesis targeted at the conserved RING domain; 2–3) how to examine the ubiquitination activity both in

vitro and in planta; 4) how to link those biochemical analysis to the biological significance of the tested protein. Generation of an E3 ligase-deficient mutant that still interacts with its substrate but no longer ubiquitinates it for degradation facilitates the testing of enzyme-substrate interactions in vivo. Furthermore, the mutation in the conserved RING domain often confers a dominant negative phenotype that can be utilized in functional knockout studies as an alternative approach to an RNA-interference approach. Our methods were optimized to investigate the biological role of the plant parasitic nematode effector RHA1B, which hijacks the host ubiquitination system in plant cells to promote parasitism. With slight modification of the in vivo expression system, this protocol can be applied to the analysis of any RING-type E3 ligase regardless of its origins.

INTRODUCTION:

The vast majority of E3 ubiquitin ligases belong to RING (Really Interesting New Gene)-type proteins. The RING-finger domain was originally identified by Freemont et al.[1] and functionally described as a domain mediating protein-protein interaction[2]. The canonical RING finger is a special type of zinc coordinating domain defined as a consensus sequence of eight conserved Cys (C) and His (H) specifically spaced by other amino acid residues (X), $C\text{-}X_2\text{-}C\text{-}X_{9–39}\text{-}C\text{-}X_{1–3}\text{-}H\text{-}X_{2–3}\text{-}C/H\text{-}X_2\text{-}C\text{-}X_{4–48}\text{-}C\text{-}X_2\text{-}C$. Two Zn^{2+} ions are stabilized by core C and H residues through unique "cross-brace" topology with C_1/C_2 and $C/H_5/C_6$ coordinating the first Zn^{2+} ion whereas C_3/H_4 and C_7/C_8 bind the second (**Figure 1A**)[3,4]. Depending on the presence of either C or H in the fifth Zn^{2+}-coordination site, two canonical subclasses of RING-finger proteins were defined: C3HC4 and C3H2C3 (RING-HC and RING-H2, respectively). Because the RING domain of E3 ubiquitin ligase mediates the interaction between E2 conjugating enzymes and substrates, mutation of these essential C and H residues has been shown to disrupt the ligase activity[5]. An additional five less common subclasses of RING E3 ligases have been described (RING-v, RING-C2, RING-D, RING-S/T, and RING-G)[6]. The RING-type E3 ubiquitin ligases can be further subdivided into simple and complex E3 enzymes. The simple single subunit RING E3 ligases contain both the substrate recognition site and the E2-binding RING domain. By contrast, the multisubunit RING-type E3 complex either recruit's substrate or mediates binding of the E2-ubiquitin intermediate to the E3 complex. The RING domain Lys residue(s) that serves as a primary ubiquitin attachment site(s) for self-ubiquitination might also be important for the E3 ligase activity.

Not all RING-containing proteins function as E3 ligases. Thus, the bioinformatic prediction of RING-finger domain and the capacity for E2-dependent protein ubiquitination must be biochemically validated and linked to the biological role of the tested protein. Here, we describe a step-by-step protocol outlining how to detect and functionally characterize the enzymatic activity of RING-type E3 ubiquitin ligases, both in vitro and in planta, through a site-directed mutagenesis approach. The representative results from this pipeline are shown for the RING-type E3 ligase RHA1B. RHA1B is an effector protein produced by the plant parasitic cyst nematode *Globodera pallida* to suppress plant immunity and manipulate the morphology of plant root cells. To protect themselves from pathogen/parasite invasion, plants have evolved nucleotide-binding domain and leucine-rich repeat (NB-LRR) type immune receptors that detect the presence of a pathogen or parasite and, as a consequence, develop the hypersensitive response (HR), which is a form of rapid and localized cell death occurring at the infection site to arrest colonization of

pathogens. One such immune receptor is the potato Gpa2 protein that confers resistance to some isolates of *G. pallida* (field populations D383 and D372)[7].

Using the presented protocols, it has been recently found that RHA1B interferes with plant immune signaling in an E3-dependent manner by targeting the plant Gpa2 immunoreceptor for ubiquitination and degradation[8].

PROTOCOL:

1. Site-directed mutagenesis (Figure 1)

1.1. Identify the conserved Cys and His amino acids in the RING domain (**Figure 1A**) and design primers carrying the substitution codon of interest flanked by 15 base pairs on either side of the mutation site (**Figure 1B**).

1.2. Introduce the desired mutation by PCR-based amplification of the plasmid harboring the gene of interest using mutagenic primers and high-fidelity DNA polymerase containing *Pfu* in 50 µL of total PCR reaction volume as shown in **Table 1** and **Table 2** according to the manufacturer's protocol.

1.3. Digest the *Escherichia coli*-derived parental methylated and semi-methylated DNA by adding 3 µL of *DpnI* restriction enzyme directly to the PCR reaction (step 1.2) and incubating at 37 °C for 2 h.

NOTE: Methylation is a posttranscriptional protein modification that is added to the plasmid produced and isolated from bacteria. New copies of PCR-generated plasmid lack methylation, therefore, the new copies will remain intact during *DpnI* treatment.

1.4. Purify the mutagenized plasmids using a commercial DNA extraction kit based on spin column technology and elute the DNA with 50 µL of water.

1.5. Transform DH5α *E. coli* chemically competent cells with 0.5 µL of the recovered mutagenized plasmid DNA according to the manufacturer's protocol. In brief, incubate competent cells with DNA on ice for 30 min, then heat-shock them for 20 s at 42 °C, and place tubes again on ice for 2 min. Incubate cells with 500 µL of LB media at 37 °C for 1 h at 250 rpm and then spread them on selective pates.

1.6. Verify the desired mutation by Sanger sequencing the DNA plasmids isolated from *E. coli*.

2. Recombinant protein purification and in vitro ubiquitination assay

2.1. Clone the wild type RING and mutated RING genes of interest into the pMAL-c2 vector (follow the manufacturer's protocol; **Table 3**) to fuse these genes with the MBP epitope tag that permits one-step purification using amylose resin. Introduce the resulting constructs into the *E.*

coli BL21 strain as described in step 1.5.

2.2. Grow the *E. coli* strain BL21 harboring the desired construct in 50 mL LB liquid medium at 37 °C for 2–3 h until it reaches the logarithmic phase (OD_{600} of 0.4–0.6).

2.3. Add IPTG to a final concentration of 0.1–1 mM to induce the expression of MBP-tagged recombinant protein of interest and incubate the *E. coli* culture for 2–3 h at 28 °C. Place the culture on ice after incubation.

NOTE: Perform steps 2.4–2.13 on ice to protect proteins from degradation.

2.4. To check for the induction efficiency, collect 1.5 mL of induced cells, spin them down at 13,000 x *g* for 2 min, remove the supernatant, and resuspend the cells in 20 µL of 2x SDS-PAGE loading buffer (24 mM Tris-HCl pH 6.8, 0.8% SDS, 10% (v/v) glycerol, 4 mM DTT, 0.04% (w/v) bromophenol blue).

2.5. Boil the samples for 5 min and run them on a 10% SDS-PAGE gel. To visually evaluate accumulation of MBP-fusion protein (molecular weight of protein of interest + 42.5 kDa MBP), stain the gel for 20 min by agitating with Coomassie staining buffer (50% methanol, 10% acetic acid, 0.1% Coomassie blue) and destaining overnight with the destaining buffer (20% methanol, 10% acetic acid).

2.6. Harvest the remaining *E. coli* cells by centrifugation at 1,350 x *g* for 6 min, discard the supernatant, and resuspend the cell pellet with 5 mL of column buffer (20 mM Tris HCl, 200 mM NaCl, 1 mM EDTA, bacterial protease inhibitor).

NOTE: This is a good place to stop the protocol overnight. The frozen cells can be stored up to 1 week at -20 °C.

2.7. Break down *E. coli* cells by placing the tube containing the bacteria in an ice-water bath and applying 10 sonication cycles: 10 s sonication at 30% amp followed by 20 s breaks.

2.8. Centrifuge sample at 13,000 x *g* at 4 °C for 10 min and save the supernatant (crude extract).

2.9. Prepare 500 µL of amylose resin in a 15 mL tube. Wash the resin by adding 10 mL of cold column buffer and centrifuging at 1,800 x *g*, 4 °C for 5 min. Do this 2x.

2.10. Add 5 mL of crude extract to the tube with the amylose resin and incubate overnight at 4 °C.

2.11. Centrifuge at 1,800 x *g* at 4 °C for 5 min and discard the supernatant.

2.12. Add 10 mL of column buffer to the resin pellet and incubate for 20 min. Then centrifuge at 1,800 x *g* at 4 °C for 5 min. Repeat this step 2x.

2.13. Elute the fusion protein with 0.5 mL of column buffer containing 10 mM maltose by incubating a sample for 2 h at 4 °C. Centrifuge at 1,800 x *g* at 4 °C for 5 min and collect the eluted protein. Repeat this step 2x.

2.14. Dialyze 1 mL of the protein fraction against the cold PBS. Aliquot protein into single-use tubes (10–20 µL) to avoid freeze-thawing and store at -80 °C until needed.

2.15. Measure the protein concentration using the Bradford assay[9].

2.16. Set up the in vitro ubiquitination reaction in a total volume of 30 µL by mixing up 40 ng of E1 (e.g., AtUBA1), 100 ng of E2 (e.g., AtUBC8, SlUBC1/4/6/7/12/13/17/20/22/27/32), 1 µg MBP-RING type protein, and 2 µg FLAG-Ub (or HA-Ub) in the ubiquitination buffer (50 mM Tris-HCl pH 7.5, 2 mM ATP, 5 mM $MgCl_2$, 30 mM creatine phosphate, 50 µg/mL creatine phosphokinase). Incubate the mixture at 30 °C for 2 h.

NOTE: Premake 20x ubiquitination buffer and store it up to 6 months at -20 °C in small aliquots for a single use. The creatine phosphokinase easily loses its enzymatic activity when the buffer is thawed and frozen repeatedly.

2.17. Terminate the reaction by mixing the 30 µL samples with 7.5 µL of 5x SDS-PAGE loading buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 25% (v/v) glycerol, 10 mM DTT, 0.1% (w/v) bromophenol blue) and boiling for 5 min.

2.18. Separate the proteins with 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transfer onto the PDVF membrane, and detect ubiquitination by Western blotting using the anti-FLAG (or anti-HA).

2.19. Stain the PVDF membrane with Coomassie blue to verify the equal loading of tested MBP-RING-type protein.

3. *Agrobacterium*-mediated transient protein expression in *Nicotiana benthamiana* leaves and in planta ubiquitination assay

3.1. Streak appropriate *Agrobacterium tumefaciens* strains carrying the epitope-tagged gene of interest (e.g., HA-RHA1B, HA-RHA1B^{C135S}, HA-RHA1B^{K146R}, HA-Ub) and empty vector as a control on the LB medium containing the appropriate selection antibiotics.

3.2. After 2 days of growth at 28 °C, pick up single colonies and grow them in LB liquid medium with the appropriate antibiotics at 28 °C/250 rpm for another 24 h.

3.3. Transfer 100 µL of agrobacterial culture to 3 mL fresh LB with the appropriate antibiotics and incubate the culture for an additional 4–6 h at 28 °C with rotation (250 rpm) to the late exponential growth phase.

3.4. Spin down agrobacterial cells at 1,800 x *g* for 6 min, discard the supernatant, and resuspend cells with 3 mL of wash buffer (50 mM MES pH 5.6, 28 mM glucose, 2 mM NaH_2PO_4). Repeat this step 2x.

3.5. After the second wash, resuspend the cells in the induction buffer (50 mM MES pH 5.6, 28 mM glucose, 2 mM NaH_2PO_4, 200 µM acetosyringone, 37 mM NH_4Cl, 5 mM $MgSO_4.7H_2O$, 4 mM KCl, 18 µM $FeSO_4.7H_2O$, 2 mM $CaCl_2$). Incubate the cells with induction buffer for an additional 10–12 h at 28 °C.

NOTE: Acetosyringone induces T-DNA transfer.

3.6. Centrifuge the cells at 1,800 x *g* for 6 min, discard the supernatant, and resuspend the cells with 2 mL of infiltration buffer (10 mM MES pH 5.5, 200 µM acetosyringone).

NOTE: If *Agrobacteria* aggregate after incubation with induction buffer, let the aggregated cells sink to the bottom of the tube by leaving it on the bench for a few minutes, and transfer the clear *Agrobacterium* suspension to a new tube before proceeding with step 3.6.

3.7. Measure the concentration of bacteria using the OD_{600} value (the optical density at absorbance of 600 nm). Adjust OD_{600} values to the desired ones.

NOTE: Usually an OD_{600} value between 0.2–0.4 works best for a single agrobacterial stain expression. If a combination of different agrobacterial strains is applied, the total OD_{600} values of Agrobacterial strains should not exceed 1.

3.8. Agroinfiltrate 4-week-old *N. bethamiana* leaves by gently pricking them with a needle, followed by hand-injecting *Agrobacterium* with a syringe without the needle. Circle the infiltrated leaf area with the marker (usually 1–2 cm in diameter).

3.9. Collect the infiltrated leaf tissues 36 h post-infiltration. Grind the tissue to a fine powder with liquid nitrogen.

3.10. Resuspended tissue powder with 300 µL of protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM DTT, 10% glycerol, 1% polyvinylpolypyrrolidone, 1 mM PMSF, plant protease inhibitor cocktail) and centrifuge at 15,000 x *g* for 15 min at 4 ˚C.

3.11. Transfer the supernatant to a new tube. Add 5x SDS-PAGE loading buffer to a final concentration of 1x and boil for 5 min.

3.12. Separate crude proteins on 10% SDS-PAGE gels, transfer onto PVDF membranes, and probe with anti-HA to detect in planta ubiquitination.

4. Establishing the link between enzymatic activity and function in planta

NOTE: For example, RHA1B promotes degradation of resistant protein Gpa2 to suppress HR cell death. This step shows how to verify that those virulent activities of RHA1B are E3-dependent.

4.1. Streak appropriate *Agrobacterium tumefaciens* strains carrying tagged genes of interest (in this example HA-RHA1B, HA-RHA1B^{C135S}, HA-RHA1B^{K146R}, myc-Gpa2, RBP1) and empty vector as a control. Follow steps 3.1–3.8 for *Agrobacterium* preparation and injection on *N. bethamiana* leaves.

4.2. For the E3-dependent substrate protein degradation, follow steps 3.9–3.12 and perform the Western blotting using appropriate antibodies to detect the protein accumulation in plant cells (e.g., anti-HA and anti-MYC).

4.3. For the E3-dependent hypersensitive response (HR) mediated cell death inhibition, monitor the agroinfiltrated leaves for HR cell death symptoms 2–4 days post-infiltration.

REPRESENTATIVE RESULTS:

In this section, representative results are provided for the protocol used for examination of a single subunit E3 ubiquitin ligase RHA1B that has a PROSITE-predicted RING-H2 type domain (132–176 amino acids)[10]. As shown in **Figure 1**, in order to obtain an E3-deficient mutant protein, at least one of the eight conserved Cs or Hs in the RING domain (**Figure 1A**) needs to be mutagenized (**Figure 1B**). Thus, as a first step, two mutant versions of RHA1B, RHA1B^{C135S} (a substitution of Cys by Ser in the conserved C_3 of RING domain) and RHA1B^{K146R} (a substitution of Lys by Arg in the only Lys present in RHA1B) were generated. Although single subunit E3 ligases mediate ubiquitin transfer from ubiquitin harboring E2 to the substrate rather than directly interacting with ubiquitin, self-ubiquitination of E3 at Lys might be required for its maximal enzymatic activity.

The Western blotting results in **Figure 2A** show a typical positive in vitro ubiquitination assay outcome, with a multibanding smear starting at the molecular weight of the tested protein (e. g., MPB-fused RHA1B ~100 kDa) and progressing upwards. The anti-HA antibody recognized HA-tagged Ub incorporated into the poly-ubiquitination chain of different lengths, creating this typical ubiquitin-associated ladder-like smear. To validate the positive results, **Figure 2A** also presents all important negative controls missing individual components (E1, E2, Ub, or MBP-RHA1B) or using MBP as control and lacking the smeared ubiquitination signal. Furthermore, the Coomassie blue staining of the PVDF membrane showed equal loading of MBP-RHA1B or MBP in all controls.

Figure 2B shows how in vitro ubiquitination results varied depending on the specific E2/E3 combination. In this example, 11 different E2s representing 10 different E2 families were tested. The detected ubiquitination activity ranged from no signal (no smear) to a multibanding smear starting at different molecular weights, indicating different ubiquitination patterns.

Figure 3 shows ubiquitination assay outcomes for RING- and K-mutant versions of tested protein.

Lack of enzymatic activity for RHA1B^{C135S} is supported by its inability to either generate a multibanding smear in vitro (**Figure 3A**) or promote poly-ubiquitination signal in planta (**Figure 3B**). It is notable that overexpression of HA-tagged Ub in planta on its own gave basal level ubiquitination in all tested samples, including the vector control, in contrast to the strong ubiquitination signal conferred by the enzymatic activity of wild type RHA1B. Furthermore, the analysis on the RHA1B^{K146R} mutant suggests that the K146 residue is also essential for the E3 activity of RHA1B. Although a marginal self-ubiquitination signal was detected in vitro (**Figure 3A**), the in planta assay determined the mutant is E3-deficient (**Figure 3B**, only background ubiquitination signal detected).

After generating and biochemically validating the E3-deficient mutant, functional studies can be designed to determine the E3-associated biological role of the tested RING E3 ubiquitin ligase. In the case of RHA1B, this nematode effector suppresses plant immune signaling, as manifested by suppression of the Gpa2-triggered HR cell death. As presented in **Figure 4A**, unlike the wild type RHA1B, the RHA1B^{C135S} mutant lacking E3 ligase activity did not interfere with HR cell death. Given that the most common outcome of protein ubiquitination is its proteasome-mediated degradation, mutations residing in the RING domain can also be used to verify an E3-dependent ability to trigger degradation of their direct and/or indirect substrates. Thus, significantly, Western blotting results in **Figure 4B** confirm that Gpa2 did not accumulate in the presence of wild type RHA1B but RHA1B^{C135S} had no impact on Gpa2 protein stability.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the principle and steps involved in site-directed mutagenesis. (**A**) RING-CH/H2 domain with conserved Cys and His amino acids highlighted. (**B**) An example of mutagenic primers design. (**C**) Steps of site-directed mutagenesis.

Figure 2: Representative in vitro ubiquitination assay. (**A**) Top gel shows ubiquitination assay including all negative controls, and bottom gel shows equal loading. (**B**) The range of expected results depending on E2 enzymes. This figure has been modified from Kud et al[8].

Figure 3: Ubiquitination assay results for RING- and K- mutants (RHA1B^{C135S} and RHA1B^{K146R}) (**A**) In vitro ubiquitination results for RHA1B^{C135S} and RHA1B^{K146R}. (**B**) In planta ubiquitination assay results for RHA1B^{C135S} and RHA1B^{K146R}. This figure has been modified from Kud et al[8].

Figure 4: Representative functional study for E3 dependent biological functions. An example of functional studies showing the E3-dependent biological function. (**A**) E3-dependent HR cell death suppression and (**B**) degradation of a plant immunoreceptor Gpa2. This figure has been modified from Kud et al[8].

Table 1: PCR reaction set up

Table 2: PCR thermocycler program

Table 3: Ligation reaction set up for the RHA1B example.

DISCUSSION:

Elucidating the biochemical and mechanistic basis of RING type E3 ubiquitin ligases can contribute greatly to our understanding of their biological significance in development, stress signaling, and maintenance of homeostasis. The protocol described here couples a mutagenesis approach with in vitro and in planta functional studies. By introducing a single amino acid substitution in the conserved residues of the RING domain through site-direct mutagenesis, the resulting E3-deficient mutant can be tested in parallel with wild type protein to link enzymatic activity with functionality.

It is critical to properly identify the RING domain, particularly its conserved Cys and His residues. Online tools such as PROSITE can be used to do so[10]. To destabilize the RING domain responsible for recruiting the E2 enzyme, Cys is normally substituted with Ser, which is its closest structural replacement lacking the ability to create a disulfide bond used for zinc coordination. Lorick et al. showed that the mutation in any of those critical Cys residues would abolish the ubiquitination activity of the single subunit RING-type E3 ligases[5]. Although some Cys residues are also important for multiunit E3 ligase complexes containing RING-type proteins, due to the multifaceted and dynamic three-dimensional structure of those ubiquitination complexes and different role of RING-type proteins, single substitutions of conserved residues in the RING domain in multiunit E3 ligase has not been successful in generating a ligase deficient phenotype[11].

For the site-directed mutagenesis, we found that using smaller plasmid vectors and lower amplification cycles usually yielded higher efficiency for mutagenesis. The Pfu enzyme can be substituted with any other high-fidelity and high processivity DNA polymerase. Furthermore, if the gene of interest contains rare codons, another *E. coli* stain, Rosetta, can be used to achieve higher yield of the recombinant protein. Additionally, both incubation time and temperature for IPTG induction can be further optimized. Lower temperatures reduce *E. coli* division rate, which might be favorable for expression of certain proteins. Although higher concentration of IPTG could improve protein expression, it also inhibits *E. coli* division processes and is not recommended.

Single subunit RING type E3 ligases not only function as a molecular scaffold that positions the E2-Ub intermediate in close proximity to the substrate but also stimulate the ubiquitin transfer activity of their cognate E2s. Furthermore, given that an E2/E3 combination is important for the length and linkages of the polyubiquitin chain that determines the fate of a modified substrate, any consideration of RING-type E3s must include their enzymatic partners, E2s[12]. As shown in **Figure 3B**, not all tested E2s are compatible with the RHA1B ligase. Therefore, in vitro ubiquitination assays should be carried in parallel with multiple E2 enzymes representing different E2 classes to avoid false negative results.

Presented here is the in vitro enzymatic assay that detects the self-ubiquitination ability of tested RING-type proteins. However, with small modifications, this protocol can be easily adapted to detect in vitro ubiquitination of substrates. To this end, the in vitro ubiquitination mixture from step 2.15 should be supplemented with the recombinant protein of the potential E3 ligase

substrate (500 ng). Following a 2 h incubation at 30 °C, ubiquitinated protein should be captured using 15 µL of anti-HA affinity matrix (if HA-Ub is used, or anti-FLAG affinity matrix if FLAG-Ub is used) by agitation for 2 h at 4 °C. After washing the beads 4x times with the cold Ub wash buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 0.05% Tween 20, 1x PMSF), discard all but 40 µL of the buffer and move to step 2.16. The ubiquitination signal, detected by antibodies specific to the epitope-tagged Ub and substrate, respectively, emerging from molecular weight of the substrate protein, confirms the substrate/enzyme specificity.

Furthermore, identification of E3 ligase substrates in vivo is usually associated with multiple challenges due to transient enzyme-substrate interaction and rapid degradation of ubiquitinated target protein. Using an E3 ligase deficient mutant, which still interacts with its target but no longer ubiquitinates it[13], is a very useful alternative to addition of proteasomal inhibitor MG132, which does not always sufficiently interfere with 26S proteasome function.

A common characteristic of RING-type E3 ligases is a tendency to form and function as homo- and/or heterodimers. Interestingly, the substitution in the conserved residues of the RING domain is usually associated with a dominant negative phenotype where mutated RING-type E3 ligase blocks enzymatic activity of a native wild type protein[13]. Thus, overexpression of RING mutants in planta may be an alternative approach to knocking out the E3 ligase gene.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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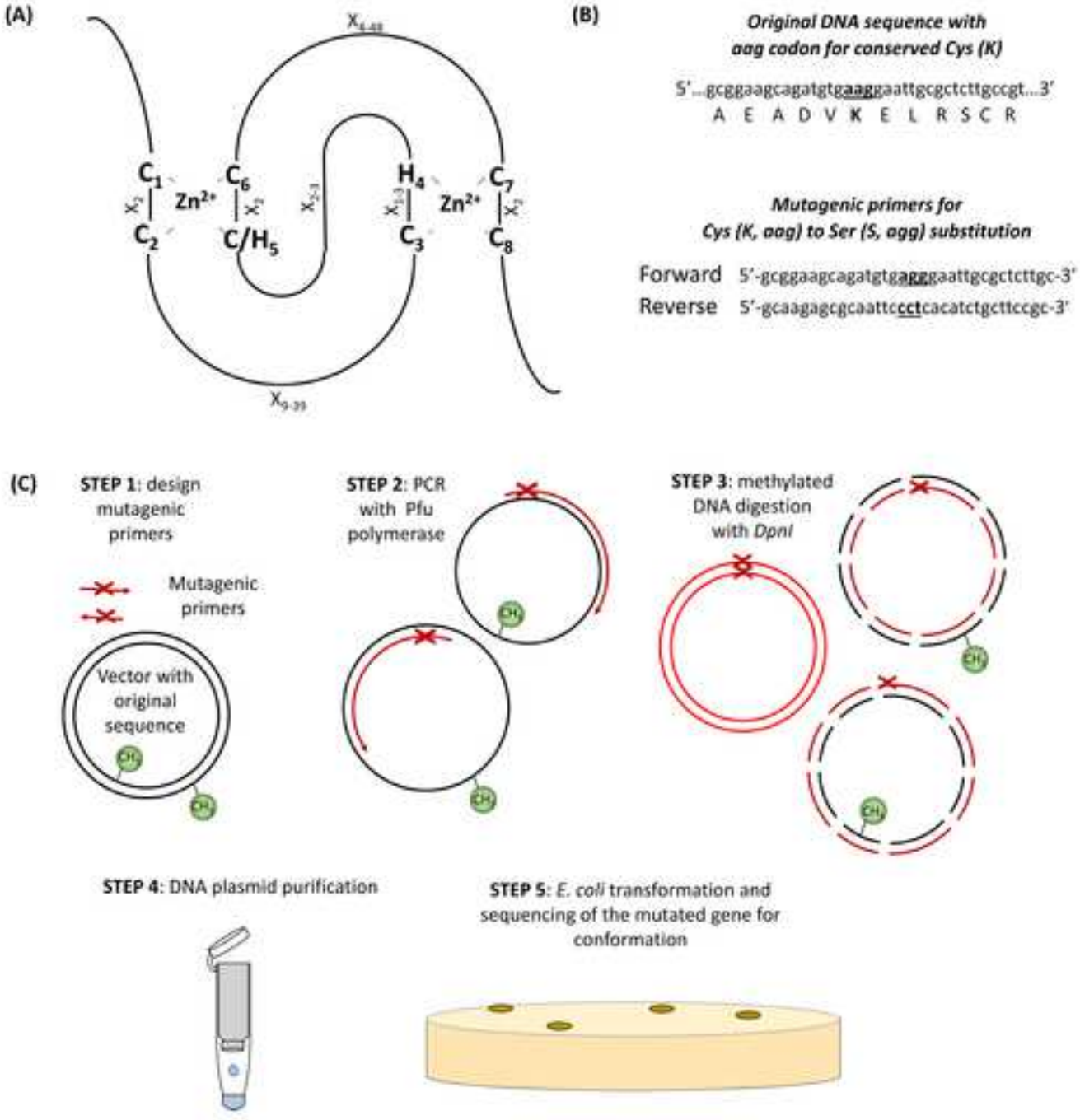

Figure 2

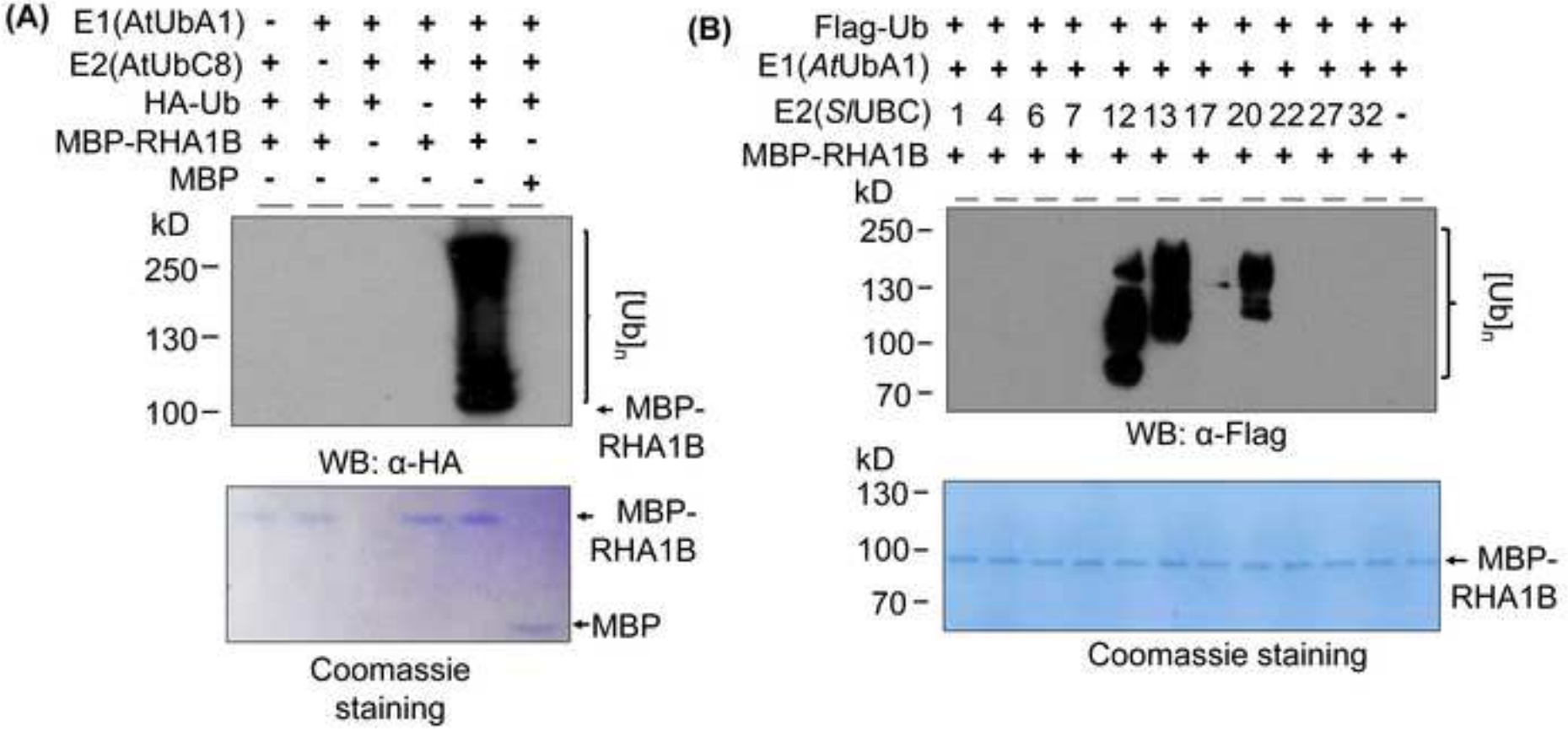

Figure 3

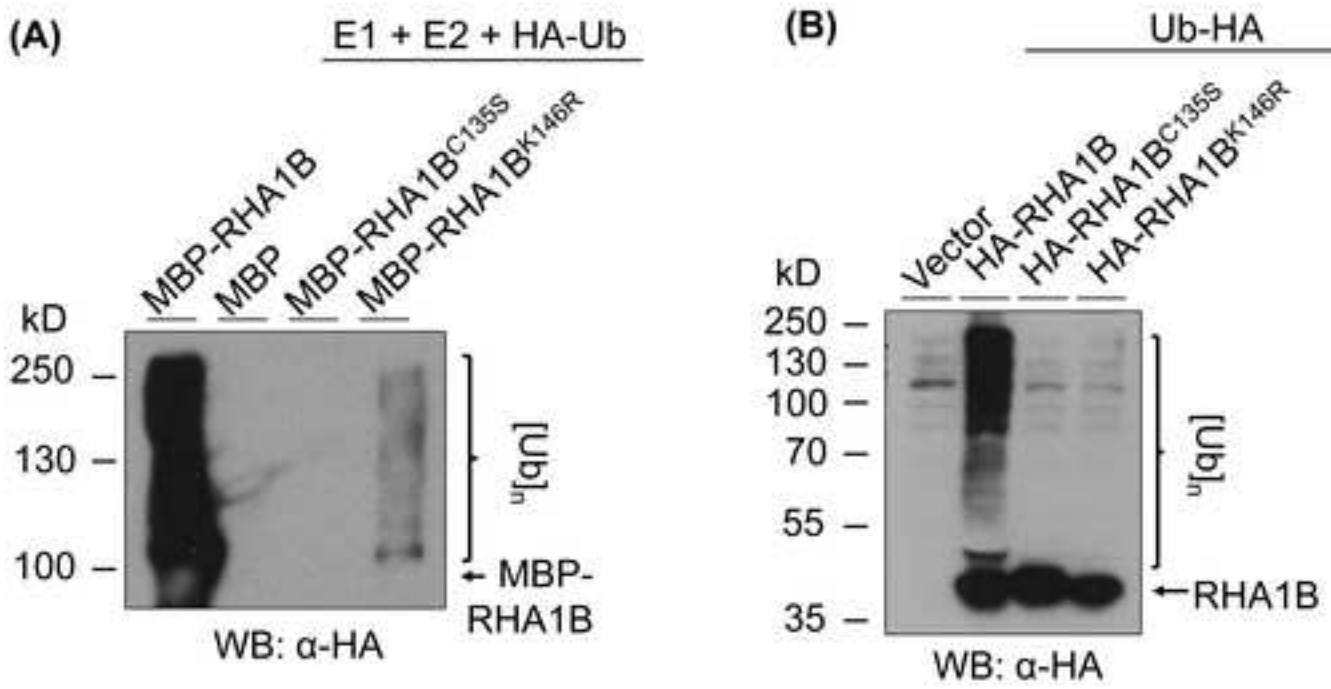

Figure 4

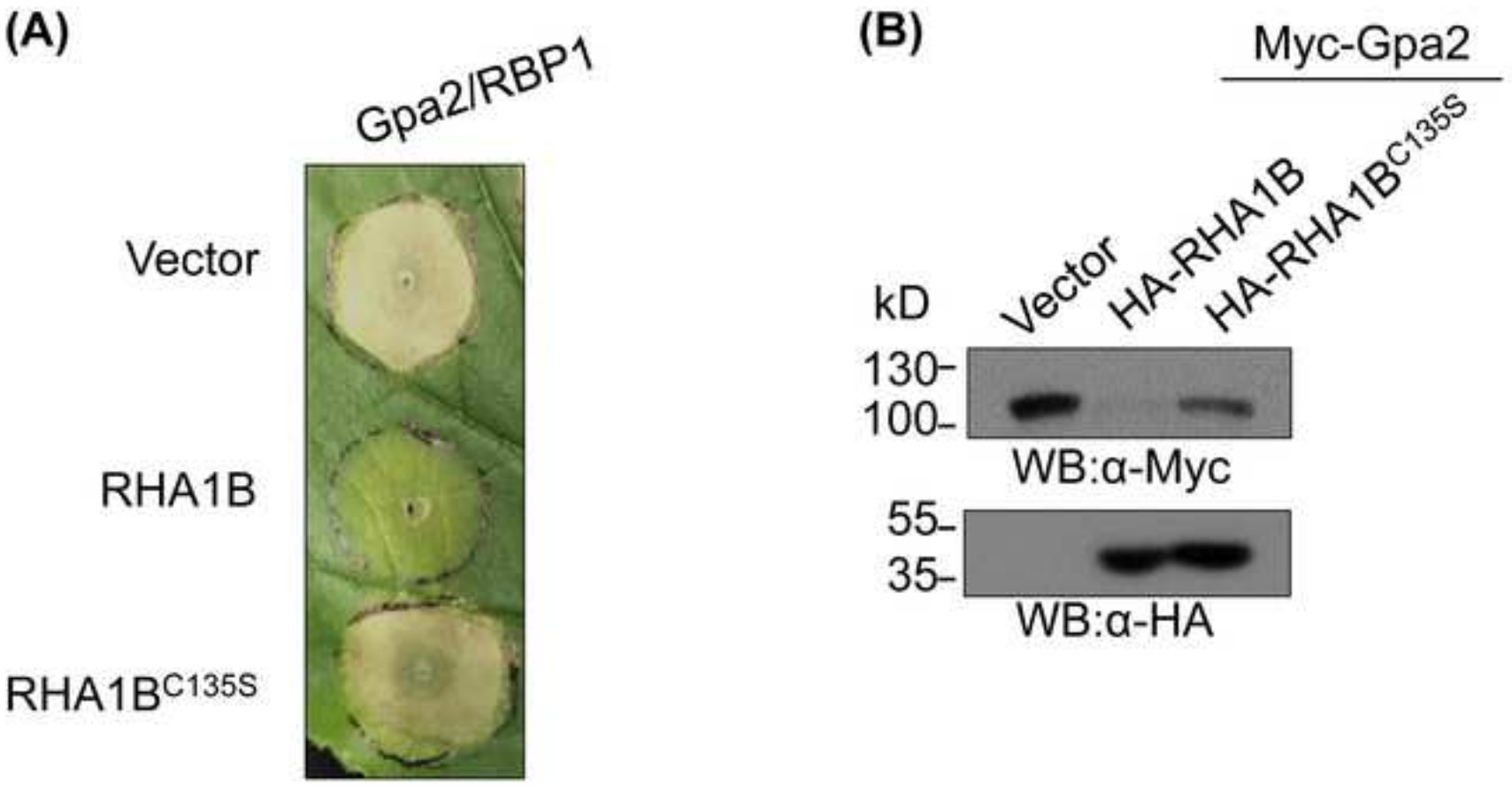

Table 1

PCR set up	
1 µL	plasmid (~100 ng)
1.5 µL	F mutagenic primer (10 µM)
1.5 µL	R mutagenic primer (10 µM)
1 µL	dNTPs (10 mM)
5 µL	buffer (10x)
1 µL	Ultra Pfu polymerase (2.5 U/µl)
39 µL	ddH_2O
50 µL	**TOTAL VOLUME**

thermocycler program			
1	95 ˚C	30 s	
2	95 ˚C	30 s	
3	60 ˚C	30 s	
4	72 ˚C	5 min	repeat 2-4 30 times
5	72 ˚C	5 min	

Table 3

ligation reaction set up for the RHA1B example	
1.5 µL	pMAL-c2::MBP linearized vector by digestion with BamHI and SalI (60 ng)
7 µL	RHA1B/RHA1BC135S or RHA1BK146R insert digested with BamHI and SalI (25 ng)
1 µL	T4 ligase buffer (10x)
0.5 µL	T4 ligase (400 U/µL)
10 µL	**TOTAL VOLUME**

Materials Table

Click here to access/download;Table of Materials;Materials table - revision.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetic acid	Sigma-Aldrich	A6283	
Acetosyringone	Sigma-Aldrich	D134406	
Amylose resin	NEB	E8021S	
ATP	Sigma-Aldrich	A1852	
Bacterial protease inhibitor	Sigma-Aldrich	P8465	
Bromphenol Blue	VWR	97061-690	
$CaCl_2$	Sigma-Aldrich	C1016	
Centrifuge	Beckman Coulter	model: Avanti J-25	
Commassie Blue	VWR	97061-738	
Creatine phosphate	Sigma-Aldrich	P7936	
Creatine phosphokinase	Sigma-Aldrich	C3755	
DNA clean & concentrator Kit	ZYMO RESEARCH	D4029	
DpnI	NEB	R0176S	
DTT	Sigma-Aldrich	D0632	
E. coli BL21	Thermo Fisher Scientific	C600003	
E. coli DH5α competent cells	Thermo Fisher Scientific	18265017	
EDTA	Sigma-Aldrich	324504	
$FeSO_4$ $7H_2O$	Sigma-Aldrich	F7002	
FLAG-Ub	BostonBiochem	U-120	
Glucose	VWR	188	
Glycerol	Sigma-Aldrich	G5516	
HA-Ub	BostonBiochem	U-110	
Heat block	VWR	model: 10153-318	
Incubator	VWR	model: 1525 Digital Incubator	
Incubator shaker	Thermo Fisher Scientific	model: MaxQ 4000	
IPTG	Roche	10724815001	
KCl	Sigma-Aldrich	P9333	
LB Broth	Sigma-Aldrich	L3022	
Liquide nitrogen	university chemistore		
Maltose	Sigma-Aldrich	63418	
MES	Sigma-Aldrich	M3671	
Methanol	Sigma-Aldrich	34860	
$MgCl_2$	Sigma-Aldrich	63138	
$MgSO_4$ $7H_2O$	Sigma-Aldrich	63138	
Microcentrifuge	Eppendorf	model: 5424	
Miniprep plasmid purification kit	ZYMO RESEARCH	D4015	
monoclonal anti-FLAG antibody	Sigma-Aldrich	F3165	
monoclonal anti-HA antibody	Sigma-Aldrich	H9658	
monoclonal anti-MYC antibody	Sigma-Aldrich	WH0004609M2	
Mortar	VWR	89038-144	
NaCl	Sigma-Aldrich	S7653	
NaH_2PO_4	Sigma-Aldrich	S8282	
NanoDrop	Thermo Fisher Scientific	model: 2000 Spectrophotometer	
Needle	Thermo Fisher Scientific	14-826-5C	
NH_4Cl	Sigma-Aldrich	A9434	
PCR machine	Bio-Rad	model: C1000	
Pestle	VWR	89038-160	
Pfu Ultra	Agilent Technologies	600380	
Plant protease inhibitor coctail	Sigma-Aldrich	P9599	
pMAL-c2	NEB	N8076S	
PMSF	Sigma-Aldrich	P7626	
Polyvinylpolypyrrolidone	Sigma-Aldrich	P6755	
SDS	Sigma-Aldrich	1614363	
Sonicator	Qsonica Sonicators	model: Q125	
Syringe	Thermo Fisher Scientific	22-253-260	
Tris	Sigma-Aldrich	T1503	
T4 ligase	NEB	M0202S	

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Author(s):	Joanna Kud, Wenjie Wang, Yulin Yuan, Allan Caplan, Joseph C. Kuhl, Louise-Marie Dandurand, Fangming Xiao

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CORRESPONDING AUTHOR

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Title:	Associate Professor
Signature:	Fangming Xiao
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The manuscript has been proofread.

2. Please revise the title to be more concise. Please remove the words "A guide for"… from the title.

The title has been changed accordingly.

3. Please ensure the Introduction contains all of the following with citation:

a) A clear statement of the overall goal of this method

From our introduction "Here, we describe a step-by-step protocol outlining how to detect and functionally characterize the enzymatic activity of RING-type E3 ubiquitin ligases, both *in vitro* and *in planta*, through a site-directed mutagenesis approach."

b) The rationale behind the development and/or use of this technique

From our introduction "Not all RING-containing proteins function as E3 ligases. Thus, the bioinformatic prediction of RING-finger domain and the capacity for E2-dependent protein ubiquitination must be biochemically validated and linked to the biological role of the tested protein."

c) The advantages over alternative techniques with applicable references to previous studies

N/A

d) A description of the context of the technique in the wider body of literature

From our manuscript "Ubiquitination, as a posttranslational modification of proteins, plays an important regulatory role in homeostasis of eukaryotic cells. The covalent attachment of 76 amino-acid ubiquitin modifier to a target protein, depending on the length and topology of the polyubiquitin chain, can result in different outcomes ranging from protein degradation to changes in the localization and/or activity of modified protein." "Elucidating the biochemical and mechanistic basis of RING type E3 ubiquitin ligases can contribute greatly to our understanding of their biological significance in development, stress signaling and maintenance of homeostasis."

e) Information to help readers to determine whether the method is appropriate for their application

From our manuscript "This four-step protocol describes 1) how to generate an E3 ligase deficient mutant through a site-directed mutagenesis targeted at the conserved RING domain; 2-3) how to examine the ubiquitination activity both *in vitro* and *in planta*; 4) how to link those biochemical analysis to the biological significance of the tested protein."

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

The manuscript has been revised accordingly.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The following Notes have been moved from the Protocol section to the Discussion:

"Note: Using smaller plasmid vectors and lower amplification cycles usually yields higher efficiency for mutagenesis. The Pfu enzyme can be substituted with any other high-fidelity and high processivity DNA polymerase.

Note: If your gene of interest contains rare codons, another *E. coli* stain, Rosetta, can be used to achieve higher yield of the recombinant protein.

Note: The expression level of recombinant protein might vary. If a low yield is observed, both incubation time and temperature for IPTG induction can be optimized. Lower temperatures reduce *E. coli* division rate, which might be favorable for expression of certain proteins. Although higher concentration of IPTG could improve protein expression, it also inhibits *E. coli* division processes and is not recommended."

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

The manuscript has been revised accordingly.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of

text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

The protocol has been revised to contain only 2-3 actions per step.

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

All centrifugation speeds have been converted from rpm to x g.

9. Please ensure you answer the "how" question, i.e., how is the step performed? Please include all specific details associated with the protocol.
10. 1.1: What are the primer sequences used in your experiment

The information about primers has been added to Fig.1 with appropriate reference in the Step 1.

11. 1.2: What was the desired mutation in your case? Please also provide a reaction setup and how to perform the thermocycler protocol for the amplification. This can be provided as a table in .xlsx file, uploaded separately to the editorial manager account and the table can be referred here. Please do not embed any table in the manuscript.

Details about the desired mutation have been included in the Fig 1B and Step 1.1. The PCR reaction setup and the thermocycler setup were created according to the manufacturer protocol and the related information has been added in the Step 1.2.

12. 1.3: Please include the reaction set up, temperature at which this is done and for how long.

The DpnI reaction setup and digestion conditions have been added to the Step 1.3

13. 1.4: If this needs filming, please briefly describe how is this done. In what volume do you perform the recovery?

The information has been added.

14. 2.1: How is the cloning performed?

The information has been added.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that

identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

2.75 pages for the video have been highlighted.

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19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

From our manuscript "It is critical to properly identify the RING domain, particularly its conserved Cys and His residues. Online tools such as PROSITE can be used to do so [10]. To destabilize RING domain responsible for recruiting E2 enzyme, Cys is normally substituted with Ser, which is its closest structural replacement lacking the ability to create a disulphide bond used for zinc coordination. *Lorick et al*. (1991) showed that mutation in any of those critical Cys would abolish ubiquitination activity of single subunit RING-type E3 ligases [5]. Although some Cys residues are also important for multiunit E3 ligase complexes containing RING-type proteins, due to multifaceted and dynamic 3D structure of those ubiquitination complexes and different role of RING-type

proteins, single substitution of conserved residues of RING domain in such multiunit E3 ligase has not been successful in generating ligase deficient phenotype [11].”

b) Any modifications and troubleshooting of the technique

This paragraph has been added to the Discussion by combining Notes previously present in the Protocol section “For the site-directed mutagenesis, we found that using smaller plasmid vectors and lower amplification cycles usually yields higher efficiency for mutagenesis. The Pfu enzyme can be substituted with any other high-fidelity and high processivity DNA polymerase. Furthermore, if your gene of interest contains rare codons, another *E. coli* stain, Rosetta, can be used to achieve higher yield of the recombinant protein. Additionally, both incubation time and temperature for IPTG induction can be further optimized. Lower temperatures reduce *E. coli* division rate, which might be favorable for expression of certain proteins. Although higher concentration of IPTG could improve protein expression, it also inhibits *E. coli* division processes and is not recommended.”

c) Any limitations of the technique

From our manuscript “Single subunit RING type E3 ligases not only function as a molecular scaffold that position the E2-Ub intermediate in close proximity to the substrate but also stimulate ubiquitin transfer activity of their cognate E2s. Furthermore, given that an E2/E3 combination is important for the length and linkages of polyubiquitin chain that determines the fate of modified substrate, any consideration of RING-type E3s must include their enzymatic partners, E2s [12]. As shown in Fig 3B, not all tested E2s are compatible with the RHA1B ligase. Therefore, *in vitro* ubiquitination assays should be carried in parallel with multiple E2 enzymes representing different E2 classes to avoid false negative results.”

d) The significance with respect to existing methods

N/A

e) Any future applications of the technique

From our manuscript “Furthermore, identification of E3 ligase substrates *in vivo* is usually associated with multiple challenges due to transient enzyme-substrate interaction and rapid degradation of ubiquitinated target protein. Using E3 ligase deficient mutant, which still interacts with its target but no longer ubiquitinates it [13], is a very useful alternative to addition of proteasomal inhibitor MG132 which not always sufficiently interferes with 26S proteasome function.”

"A common characteristic of RING-type E3 ligases is a tendency to form and function as homo- and/or heterodimers. Interestingly, the substitution in the conserved residues of RING domain is usually associated with dominant negative phenotype where mutated RING-type E3 ligase blocks enzymatic activity of a native wild type protein [13]. Thus, overexpression of RING mutant *in planta* may be an alternative approach to knockout the E3 ligase gene."

20. Please do not abbreviate the journal titles in the references section.

All the abbreviations in the reference section have been spelled out.

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No trademark (™) and registered (®) symbols are present the Table of Equipment and Materials. The Table content bas been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript presents a clear protocol to assay the ubiquitination activity of RING-type ubiquitin ligase via in vitro and in vivo experiments. Using wild-type and inactive RING protein that lacks a metal ligand of the RING domain, authors provide a helpful approach to test a protein containing RING domain as E3 ubiquitin ligase. I feel this manuscript will be interest and appropriated to publish at JoVe.

Minor Concerns:

1) Line 67, I feel that this passage should be mention the RING-type ligases such as RING-v, RING-C2, RING-D and RING-G (Jiménez-López et al 2018 doi: 10.1371/journal.pone.0203442Jimenez; Stone et al 2005 Plant Physiol).

A statement describing other rare RING type ligases and related citation have been added.

2) Line 73, replace "multiunit" by multisubunit

The word has been replaced.

3) Line 96, Figure 1 should be modified to fit into step 1 since step 2 calls for protein purification.

Figure 1 has been modified to match the step 1 of the Protocol section.

4) I feel that the Note presented in line 144 should be set after Line 123.

The Note has been moved after Line 123 as suggested.

Reviewer #2:

Manuscript Summary:

This is a nice protocol to describe the method of functional characterization of RING-type E3 ubiquitin ligases in vitro and in planta, including point mutation to generate E3 ligase deficient mutants; examine the E3 ligase activity in vivo and in vitro; and test their functional importance. The protocol was well written, and the experimental procedure was clearly laid out. It will be very useful for the community as a guide for ubiquitination assays.

IN THIS SECTION

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