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Ligase Trapping: An efficient and reliable method to identify ubiquitinated substrates in yeast

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Ligase Trapping: An efficient and reliable method to identify ubiquitinated substrates in yeast

by

Kevin G. Mark

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
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Chapter 1 is excerpted from a solicited review in preparation for submission to *Nature Protocols* written in collaboration with Theresa Loveless and David Toczyski.

Chapter 2 is reproduced from work published in *Molecular Cell* in collaboration with Alessio Maiolica from the Aebersold Lab, Marco Simonetta and Charles Seller:


Chapter 3 is work recently submitted to the *Journal of Biological Chemistry*. 
ABSTRACT

Posttranslational modifications enable cells to regulate their proteomes in a rapid and localized fashion. One such modification is ubiquitination and it is well conserved throughout eukaryotes and plays a role in nearly all intracellular processes. Ubiquitin is covalently attached to protein substrates through an enzymatic cascade in which ubiquitin ligases specify the target to be modified. The SCF superfamily of ubiquitin ligases are multisubunit enzymes that share a common scaffold which includes cullin (Cul1), an adaptor protein (Skp1) and F-box proteins that bind specific targets for degradation. To overcome limitations of current techniques to identify ubiquitinated substrates, we developed a technique in which ubiquitin-binding domains are fused to F-box proteins in order to enhance the stability of ligase-substrate interactions. These “Ligase Trap” constructs were then used to isolate polyubiquitinated substrates via a two-step purification procedure and the substrates identified by tandem mass spectrometry (Chapter 1). In budding yeast, Ligase Traps allowed us to identify both known and novel substrates using eight different F-box proteins. One F-box protein, Saf1, was found to target vacuolar/lysosomal zymogens (Chapter 2). Since it is unusual for a ligase to recognize an entire class of enzymes and because there is no known ubiquitin activity in a cell vacuole, I further examined how Saf1 interacts with its substrates (Chapter 3). My studies showed that SCF\textsuperscript{Saf1} specifically targets the catalytically active form of yeast vacuolar/lysosomal proteases. These results seem to suggest that ubiquitin ligases may interact with zymogens in the ER to regulate their activity or function prior to processing.
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Chapter 1
Isolation of Ubiquitinated Substrates by Tandem Affinity Purification of E3 Ligase-Polyubiquitin Binding Domain Fusions (Ligase Traps)

This chapter is excerpted from a review that is in preparation for submission to Nature Protocols written in collaboration with Theresa Loveless and David Toczyski.

ABSTRACT

Ubiquitination is an essential protein modification activity that may influence many eukaryotic processes ranging from substrate degradation/removal/elimination to nonproteolytic pathway alterations including DNA repair and endocytosis. Previous attempts to analyze substrates via physical association with their respective ubiquitin ligases have had limited success. However, due to the transient nature of enzyme-substrate interactions and rapid protein degradation, detection of many substrates remains a challenge. The present protocol describes a large-scale affinity purification approach in which Ligase Traps, modified ubiquitin ligases fused to a polyubiquitin-binding domain, are used to isolate ubiquitinated substrates. Specifically, immunoprecipitation is used to enrich for proteins bound to the Ligase Trap. Subsequently, affinity purification is used under denaturing conditions to capture proteins conjugated with polyhistidine-tagged ubiquitin. Using this procedure, ubiquitinated substrates specific for a given ligase can be isolated for mass spectrometry analysis to determine their identity. After cells have been collected, the described protocol can be completed in 2-3 d.

INTRODUCTION

Ubiquitin is a highly conserved, small polypeptide that is covalently linked to protein substrates targeted for intracellular modification. Sequential enzymatic cascade transfers ubiquitin to its
target with an E3 ligase catalyzing the eventual covalent linkage to the ε-amino group of a lysine residue or an N-terminal methionine of the substrate (Breitschopf et al., 1998; Finley et al., 2012; Komander and Rape, 2012; Schulman, 2011). Since ubiquitin itself contains seven lysine residues, polyubiquitination can lead to the formation of distinct ubiquitin chains whose topology are recognized by ubiquitin-binding proteins containing ubiquitin-binding domains, such as ubiquitin-associated domains (UBAs) or ubiquitin interacting motifs (UIMs) (Husnjak and Dikic, 2012; Komander, 2009). In general, monoubiquitination can alter protein-protein interaction or protein localization (Hicke, 2001) while polyubiquitination leads to proteasomal degradation or regulation of oligomeric signaling complex assembly (Chen and Sun, 2009; Grabbe et al., 2011).

The identification of ubiquitin ligase substrates has remained a bottleneck in the field for two main reasons. First, the interaction between ligase and substrate is often too weak to permit isolation of substrates based on its physical association with the ligase. Studies have shown that certain ubiquitin ligases dissociate from their substrates on the order of seconds (Pierce et al., 2009). Furthermore, since the frequent outcome of ubiquitination is proteasomal degradation, substrate stability is greatly reduced thereby preventing isolation of the ligase-substrate complex.

Approaches to identifying ubiquitinated substrates in vivo generally consist of two different types of experiments. The Global Protein Stability (GPS) technique and related methods involve comparing changes in steady-state levels of total protein in the presence or absence of a given ligase or an inactivated ligase (Benanti et al., 2007; Emanuele et al., 2011; Yen and Elledge, 2008; Yen et al., 2008). While successful in identifying targets of proteolysis, these techniques do not allow for the detection of non-degradative ubiquitination events or degradation of minor
subpopulations. Also, if the approach involves the utilization of an inactivated ligase, the lack of an E3 ligase can have detrimental effects on cellular physiology thus perturbing the ubiquitin proteome (Barral et al., 1995; Koepp et al., 2006). In addition, some substrates are targeted by more than one ligase; under these conditions, the absence of a single ligase may fail to significantly stabilize the substrate (Pant and Lozano, 2014). Other approaches to identify ligase targets involve the immunoprecipitation of ligase-substrate complexes followed by mass spectrometric analysis of the isolated peptides (Davis et al., 2013; Ho et al., 2002; Kuchay et al., 2013). The main drawback of affinity-based methods is that ligase-substrate interactions may be too weak for copurification of the target protein.

In this paper, we describe a protocol that uses ubiquitin ligases fused to polyubiquitin-binding domains (Ligase Traps) to identify ligase substrates with greater efficacy. The presence of a polyubiquitin-binding domain increases the binding affinity of a ligase to its ubiquitinated substrates, as well as improving the stability of the bound substrate for affinity purification. To validate our approach, we generated Ligase Traps using the UBA domains from the soluble ubiquitin receptor proteins, Rad23 and Dsk2, which deliver ubiquitinated substrates to the 26S proteasome (Elsasser and Finley, 2005; Grabbe and Dikic, 2009). We chose the UBA class of ubiquitin-binding domains because they exhibit high affinity for polyubiquitinated polypeptide chains; the Rad23 UBA displays K48-linkage specificity while the Dsk2 UBA is less selective (Raasi and Pickart, 2003; Raasi et al., 2005; Sims et al., 2009). To increase our ability to identify unambiguously substrates captured by the Ligase Trap, we adopted a two-step tandem affinity purification protocol using hexahistidine-tagged ubiquitin to isolate selectively the ubiquitinated species (Fig. 1). First, we perform an anti-FLAG immunoprecipitation under native conditions to
enrich for the Ligase Trap and its interacting proteins, while a subsequent Ni-NTA pulldown under denaturing conditions selectively captures proteins conjugated with polyhistidine-tagged ubiquitin. This two-step purification allows for the enrichment of ubiquitin-conjugated substrates, including those that constitute only a small fraction of the total cellular protein. We have used this technique to isolate substrates of the SCF family of ubiquitin ligases in both budding yeast and mammalian cells. Purified substrates are subjected to mass spectrometry analysis.

A limitation of this technique is that fusion of the UBA domain onto a particular ligase may interfere with its function. In the case of the essential yeast F-box, Cdc4, C-terminally tagged alleles generated slow growing strains (Mark et al., 2014). Another drawback is that weakly expressed ligases may suffer low yields of material, as Ligase Traps are expressed from their endogenous promoters. In addition, the use of N-terminally tagged ubiquitin in this procedure may preclude the ability to form linear (N-linked) polyubiquitin chains, thus underrepresenting this modification (Tokunaga et al., 2009).

**Experimental design**

In this section, we will highlight some of the critical steps for efficient usage of the Ligase Trap.

**Generation of Ligase Trap yeast strains.** This protocol requires a yeast strain that expresses both (1) galactose-inducible hexahistidine-tagged ubiquitin and (2) an F-box protein fused to a UBA domain via a 3x FLAG linker sequence. To generate the latter construct, we used the integrating vector pRS306 to clone the DNA in the following order: a partial C-terminus of an F-
box protein, a 3x FLAG linker sequence, and either the two C-terminal UBA domains of the RAD23 gene (codons 143-397) or the single UBA domain of the DSK2 gene (codons 327-373). To integrate the Ligase Trap into its endogenous locus, the plasmid was linearized at a unique restriction site in the F-box protein and transformed into yeast cells. Ligase Traps with the UBA domain fused to the N-terminus of the F-box protein were also created (promoter-UBA-3xFLAG-F-box). To overexpress hexahistidine-tagged ubiquitin, we inserted the GAL1 promoter upstream of the UBI4 locus (which contains five ubiquitin sequences in tandem) using standard molecular techniques and selected a strain containing only a single copy of ubiquitin 6xHis-tagged cassette. All strains were verified for expression of the fusion protein and the 6xHis-tagged ubiquitin by western blot analysis using anti-FLAG and anti-6xHis antibodies. All plasmids are available upon request.

**Proteomic conditions.** Care should be taken to avoid keratin contamination of samples, as this will interfere with the detection of substrate peptides. For reagent preparation, we recommend the following precautions:

- Purchase HPLC-grade water for all solutions.
- Use polypropylene supplies for all purification procedures. If you must use durable plastic or glassware, be sure to wash well with Milli-Q water.
- Use dedicated reagents and supplies for mass spectrometry analysis, if possible. Minimize handling. (Autoclaving does not remove keratin.)
- Sterilize buffers by filtration through a 0.2 μm syringe filter.
- Use sterile, disposable consumables, tubes and bottles, for reagent storage.
- Wear a lab coat and change gloves often.
• Wear disposable hair bonnets, if possible.
• Use barrier pipette tips.

Sample monitoring. Prior to mass spectrometry analysis, it is useful to collect a small quantity of each eluate for western blotting and silver staining to estimate the efficiency of purification. This is noted in Steps 10, 13, 16, 17 and 19.

Timing. To produce a protein sample for mass spectrometry (MS) analysis from saturated liquid yeast cultures takes approximately 3 days. Following data collection, processing of the results obtained can take significantly longer.

Future directions. This protocol describes the use of UBA-ligase fusion proteins to identify substrates of the SCF family of ubiquitin ligases. Other ubiquitin-like modifiers, such as SUMO or LC3, also have specific binding domains (McEwan and Dikic, 2011; van Wijk et al., 2011) and they can be used to generate an analogous enzyme “trap” to search for target substrates. At this time, we have not extensively tested this technique on non-SCF ligases. We are also interested in altering/ extending the 3x FLAG linker length or using different linkage-specific ubiquitin-binding domains (i.e. TAB2 NZF for K63, NEMO UBAN for linear) for Ligase Traps to determine their effects on the repertoire of substrates captured (Kulathu et al., 2009; Rahighi et al., 2009).
Materials related to budding yeast culture:

- Yeast strain (*Saccharomyces cerevisiae*): ubi4::GAL1pr-6xHisUb for the generation of Ligase Trap strain (available upon request)
- Plasmid with F-box-UBA fusion for generation of Ligase Trap strain (available upon request)
- Adenine (Sigma-Aldrich, cat. no. A8626)
- Uracil (Sigma-Aldrich, cat. no. U0750)
- Yeast extract (Fisher, cat. no. BP1422)
- Bacto peptone (BD, cat. no. 211677)
- Ammonium sulfate (Fisher, cat. no. A702)
- Succinic acid (Sigma-Aldrich, cat. no. S7501)
- Yeast nitrogen base w/o AA, carbohydrate & w/o AS (US Biological, cat. no. Y2030)
- Raffinose pentahydrate, low glucose (US Biological, cat. no. R1030)
- D-(+)-Galactose (Sigma-Aldrich, cat. no. G0750)
- Water, filtered, HPLC grade (Fisher Scientific, cat. no. W5)
- Dimethyl sulfoxide (Sigma-Aldrich, cat. no. D8418)
- HEPES (Fisher Scientific, cat. no. BP310)
- Potassium acetate (Fisher Scientific, cat. no. BP364)
- Magnesium chloride (Fisher Scientific, cat. no. M33)
- Calcium chloride (Fisher Scientific, cat. no. C77)
- Protease inhibitor tablets, EDTA-free (Roche, cat. no. 04693132001)
- Phosphatase inhibitor cocktail tablets, PhosSTOP (Roche, cat. no. 04906837001)
- Leupeptin (Sigma-Aldrich, cat. no. L2023)
• Bestatin (Sigma-Aldrich, cat. no. B8385)
• Benzamidine HCl (Sigma-Aldrich, cat. no. B6506)
• Pepstatin A (Sigma-Aldrich, cat. no. P5318)
• Phenylmethanesulfonyl fluoride (Sigma-Aldrich, cat. no. 78830)
• Sodium orthovanadate (ACROS Organics, cat. no. 205330500)
• β-glycerophosphate disodium salt hydrate (Sigma-Aldrich, cat. no. G5422)
• Sodium chloride (Fisher Scientific, cat. no. S640)
• Potassium chloride (Fisher Scientific, cat. no. BP366)
• Sodium phosphate (ACROS Organics, cat. no. 424395000)
• Potassium phosphate dibasic (Fisher Scientific, cat. no. BP363)
• Sodium hydroxide (Fisher Scientific, cat. no. S318)
• Deoxyribonuclease I from bovine pancreas (Sigma-Aldrich, cat. no. D4527)
• MG132 (Sigma-Aldrich, cat. no. C2211)
• Anti-FLAG M2 magnetic beads (Sigma-Aldrich, cat. no. M8823)
• 3x FLAG peptide, lyophilized powder (Sigma-Aldrich, cat. no. F4799)
• Nonidet P-40 (US Biological, cat. no. N3500)
• Tris base (Fisher, cat. no. BP152)
• Ni-NTA agarose (Invitrogen, cat. no. R901)
• Urea (Fisher, cat. no. BP169)
• Imidazole (Sigma-Aldrich, cat. no. 56750)
• RapiGest SF Surfactant (Waters, cat. no. 186001186)
• EDTA disodium salt dihydrate (Fisher, cat. no. BP120)
• Glycerol (Fisher, cat. no. BP229)
• 2-mercaptoethanol (Sigma-Aldrich, cat. no. M6250)
  
  o **Caution:** 2-mercaptoethanol is toxic; avoid exposure and handle in a fume hood.

• Pierce BCA Protein Assay Kit (Thermo Scientific, cat. no. 23225)

• Mouse monoclonal ANTI-FLAG M2 antibody (Sigma-Aldrich, cat. no. F1804)

• 6xHis monoclonal antibody, albumin free (Clontech, cat. no. 631212)

• Pierce Silver Stain Kit (Thermo Scientific, cat. no. 24612)

**EQUIPMENT**

• Incubator shaker, 30 °C

• Growing flasks for yeast

• Centrifuge (Avanti J-20 XP) with JLA-9.1 and JA-25.5 rotors

• Nalgene Oak Ridge high-speed centrifuge tubes (Thermo Scientific, cat. no. 05-562-16A)

• Magnetic stir bar

• Stirring hot plate

• 1 liter polycarbonate bottle assemblies (Beckman Coulter, cat. no. A98812)

• 10 ml Luer-Lok Tip Syringe (BD, cat. no. 309604)

• 60 ml Luer-Lok Tip Syringe (BD, cat. no. 309653)

• 0.2 µm Nalgene Syringe filter (Thermo Scientific, cat. no. 190-2520)

• 0.2 µm Nalgene Rapid-Flow Sterile Disposable Bottle Top Filter (Thermo Scientific, cat. no. 291-3320)

• Microcentrifuges (Eppendorf 5430 R and Eppendorf 5415 D)

• Heat block

• 2.0 ml microcentrifuge tubes (Axygen, cat. no. 311-10-051)
• 1.5 ml low adhesion microcentrifuge tubes (USA Scientific, cat. no. 1415-2600)
• 0.5 ml low adhesion microcentrifuge tubes (USA Scientific, cat. no. 1405-2600)
• 15 ml polypropylene conical centrifuge tube (Falcon, cat. no. 352096)
• 50 ml polypropylene conical centrifuge tube (Falcon, cat. no. 352070)
• Screw-cap tubes (Axygen, cat. no. SCT-150-W)
• UV/Vis photometer
• Rocking platform
• Vortex mixer with multiple sample head
• Scissors
• Spatula
• Clean razor blades
• Shaker rotisserie
• Ball mill (Retsch M301) with CryoKit and 25 mm diameter steel grinding ball
• Magnetic bead rack
• 25G x 1 1/2 PrecisionGlide needle (BD, cat. no. 305127)
• GeLoader pipette tips (Eppendorf, cat. no. 022351656)
• 4-20% Criterion Tris-HCl Gel, 26 well, 15 µl (Bio-Rad, cat. no. 345-0034)

REAGENT SETUP

Solutions related to budding yeast culture

100x Adenine

• Mix 2 g adenine powder with 5 ml 4M NaOH in 1 liter water. Autoclave 30 min.

100x Uracil
• Mix 2 g uracil powder with 5 ml 4M NaOH in 1 liter water. Autoclave 30 min.

YM-1 media
• Resuspend 5.5 g yeast extract, 11 g peptone, 5.5 g ammonium sulfate, 11 g succinic acid, 1.61 g yeast nitrogen base w/o amino acids or ammonium sulfate, 5.72 g NaOH, 20 ml 100x adenine, and 20 ml 100x uracil in 1 liter of water. Titrate to pH 5.8 with NaOH. Autoclave 60 min.

20% Raffinose
• Dissolve 200 g raffinose in 1 liter of water. Filter with bottle top filter and autoclave 30 min.

20% Galactose
• Dissolve 200 g galactose in 1 liter of water. Filter with bottle top filter and autoclave 30 min.

Stock solutions

Deoxyribonuclease I
• Resuspend Deoxyribonuclease I in lysis buffer to a final concentration of 10,000 U/ml.
  ○ Critical: Aliquots can be stored at -20°C for up to 2 years. Do not re-freeze.

MG132
• Resuspend MG132 in dimethyl sulfoxide to a final concentration of 50 mg/ml.
  ○ Critical: Can be stored at -20°C for up to 1 month.

10x FLAG peptide
• Resuspend 3x FLAG peptide to a final concentration of 5 mg/ml in PBS.
  ○ Critical: Can be made ahead of time and stored as 100 µl aliquots at -80°C.
Buffers

Lysis buffer

- 25 mM HEPES (pH 8), 150 mM potassium acetate, 10 mM magnesium chloride, 5 mM calcium chloride, 1 mM phenylmethanesulfonyl fluoride, 5 mM sodium fluoride, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml bestatin, 1 µM benzamidine HCl, 1 µg/ml pepstatin A, protease inhibitor cocktail PhosSTOP tablets (2 per 100 ml) and protease inhibitor cocktail tablets, EDTA-free (2 per 100 ml), 15 µg/ml MG132. Filter using a 0.2 µm syringe filter prior to use.
  - Critical: Prepare fresh and keep at 4°C.

PBS

- 2x PBS solution: 270 mM sodium chloride, 5 mM potassium chloride, 17 mM sodium phosphate, and 2 mM potassium phosphate. Titrate to pH 7.4 - 7.5 with 8 M NaOH.

FLAG elution buffer

- 0.5 mg/ml FLAG peptide in PBS with 0.08% Nonidet P-40.
  - Critical: Aliquots can be stored at -20°C for up to 2 years. Do not re-freeze.

Buffer B (9.4 M urea lysis buffer)

- 1.5x solution: 118 mM sodium phosphate, 12 mM tris base, and 9 M urea. Stir overnight and titrate to pH 8 using 8 M NaOH. Filter prior to use (using 0.2 µm syringe filter).
  - Critical: Prepare fresh and keep at room temperature.

Ni-NTA final wash buffer
• 100 mM sodium phosphate, 11 mM tris base, 1 M urea. Adjust to pH 8 using 8 M NaOH. Filter prior to use (using 0.2 µm syringe filter).

Ni-NTA elution buffer

• 0.5x Ni-NTA final wash buffer, 300 mM imidazole, 0.1% RapiGest.

Sina’s sample buffer

• 2x solution: 0.1 M Tris (pH 7.5), 10 mM EDTA, 10% SDS, 20% glycerol, 1% 2-mercaptoethanol, and a sprinkle of bromophenol blue (for color).

PROCEDURE

Step 1: Cell collection and lysis  
Timing: 6-7 h

Critical: This experimental protocol uses a 4 liter yeast culture that can be scaled down proportionally based on need. For example, a 1 liter culture may suffice for mass spectrometry analysis of Ligase Traps that are strongly expressed. For validation experiments, we recommend using extracts from 350 ml of yeast culture.

1| From an overnight culture grown in YM-1 with 2% raffinose, inoculate 4 liters of the same medium to achieve an OD$_{600}$ of 0.3. Incubate with vigorous shaking at 30°C for 1 h.

2| Add 440 ml of 20% galactose and continue incubation with shaking until reaching OD$_{600}$ = 1.0 (about 5-6 h).
3| Centrifuge cells at 17,000g for 10 min at 4°C. Discard supernatant and wash pellet by vortexing in 200 ml water. Resuspend pellet by vortexing in 3.5 ml of lysis buffer.

- **Critical:** Multiple samples can be normalized to each other by OD$_{600}$.

- **Pause point:** Pellets can be stored at -80°C until ready to use.

4| Using scissors or a razor blade, make several non-overlapping 1-cm slits in the cap of a 50 ml Falcon tube. Remove cap and fill tube with about 50 ml of liquid nitrogen. Use a pipet to add yeast sample drop-by-drop into liquid nitrogen containing tube, while refilling with liquid nitrogen to maintain at least ~30 ml volume. Screw cap back on and discard liquid nitrogen through slits.

- **Critical:** Drop sample into liquid nitrogen slowly to avoid clumps. Be careful when performing this procedure as liquid nitrogen can cause severe burns. Use of a face shield is highly recommended.

- **Pause point:** Sample “beads” can be stored at -80°C until required.

- **Troubleshooting**

5| Grind samples with ball mill using 5 cycles of 2 min at 27 Hz. Cool chambers intermittently by submerging them in liquid nitrogen for 2 min between cycles.

- **Critical:** Cool chambers in liquid nitrogen prior to adding samples. Do not close metal chambers too tightly – bubbles should appear when submerged. Apply safety precautions while handling liquid nitrogen.

- **Troubleshooting**
6| Transfer powder with a spatula that has been pre-cooled in liquid nitrogen to a 50 ml Falcon tube and resuspend in 10 ml lysis buffer on a rocking platform at 4°C. Make sure the sample is thoroughly suspended (about 2 h). Transfer to 15 ml Falcon tube. Add 500 µl deoxyribonuclease I (5000 U) and incubate at 4°C on rocking platform for 30 min.

- **Critical:** Precool spatula in liquid nitrogen before use to prevent sample from melting.

7| Preclear the lysate by centrifuging at 6,000g for 10 min at 4°C using an Oak Ridge centrifuge tube. Transfer supernatant to a new Oak Ridge tube and centrifuge at 58,500g for 1 h at 4°C.

- **Troubleshooting**

**Step 2: Purification – stage I (native FLAG)  
Timing: 12-16 h**

8| In a 15 ml Falcon tube, wash 380 µl anti-FLAG magnetic bead slurry three times with 4 ml of lysis buffer containing 0.08% Nonidet P-40.

- **Critical:** Use wide bore tips to avoid damaging beads during pipetting. Mix beads well before removal as they settle quickly.

9| (Optional) Take 5 µl of lysate and check protein concentration with BCA kit using manufacturer’s instructions.

10| Save 30 µl of lysate (0.2%) for quality-control analysis (See Step 21). Add lysate to beads and incubate on a rotating platform overnight at 4°C. Expect ~15 ml total volume.
11| Prepare Buffer B for the Ni-NTA purification step. Keep beads stirring overnight at room temperature.

Step 3: Purification - stage II (denaturing Ni-NTA)                      Timing: 5–6 h

12| Adjust pH of Buffer B to 8.0 with 8 M NaOH and filter sterilize.

13| Wash beads three times with 2 ml PBS containing 0.08% Nonidet P-40. Save 4 µl of FLAG flow-through (0.2%) for quality-control analysis (See Step 21).

14| Transfer sample to a 1.5 ml low-adhesion tube and elute with 570 µl FLAG elution buffer with gentle vortexing for 45 min at room temperature.
   • Critical: Use wide bore pipette tips to avoid damaging beads.

15| Wash 60 µl Ni-NTA agarose slurry three times with 600 µl Buffer B containing 10 mM imidazole, spinning down the slurry each time at 800g in a micentrifuge. Resuspend the Ni-NTA agarose beads in 60 µl of Buffer B with 10 mM imidazole and place into a 2.0 ml tube.
   • Critical: Use wide bore pipette tips to avoid damaging beads. Use a 25G x 1 1/2 needle to aspirate supernatant while avoiding beads.
   • Troubleshooting
16| Save 20 µl of FLAG eluate (3.5%) for quality-control analysis (See Step 21). Transfer the remainder of the FLAG eluate to the tube containing the Ni-NTA agarose beads. Add 1.14 ml 1.5x Buffer B and 17 µl of 1 M imidazole. Incubate at room temperature for 3.5 h on a rotisserie.

• **Critical:** This step and all subsequent steps are done at room temperature.

17| Centrifuge at 800g for 3 min and carefully transfer the beads to a 1.5 ml low protein-binding tube. Wash beads three times with 1 ml of Buffer B containing 10 mM imidazole. Centrifuge at 800g for 3 min. Save 35 µl (3.5%) of the Ni-NTA flow-through for quality-control analysis (See Step 21).

• **Critical:** For each wash, a new tube should be used to reduce background. Use wide bore pipet tips to avoid damaging beads. Use a 25G x 1 1/2 needle to aspirate supernatant to avoid picking up any beads.

• **Troubleshooting**

18| Wash beads twice with 1 ml Ni-NTA wash buffer containing 10 mM imidazole. Centrifuge at 800g for 3 min to collect the beads after each wash.

• **Critical:** For each wash, a new tube should be used to reduce background.

19| Elute beads using 90 µl of Ni-NTA elution buffer. Vortex at room temperature for 20 min. To collect the eluate, place an Eppendorf GeLoader tip into the bead bed and gently remove the liquid by pipetting. Save 10 µl imidazole elution (11%) for quality-control analysis (See Step 21) and freeze the remainder in liquid nitrogen for mass spectrometry analysis.
While some laboratories are capable of performing mass spectrometry studies, most will choose to collaborate with others for this analysis or use the service of a protein chemistry core facility.

**Step 4 – Quality control post-purification**

To determine the recovery efficiency of each purification step, aliquots of saved samples are used for western blotting analysis as well as silver staining. To do this, samples are diluted 1:2 in 2x Sina’s sample buffer and boiled for 5 min prior to loading on 4-20% Criterion Tris-HCl 26-well SDS-PAGE. We generally analyze 0.0008-0.008% of the total cell extract, 0.5% of the FLAG elution and 2% of the imidazole elution. For silver stains, we run 6% of the imidazole elution with 1-10 ng of BSA as a control.

- **Troubleshooting**

**TROUBLESHOOTING**

Troubleshooting advice can be found in Table 1.

**TIMING**

Step 1, cell collection and lysis: 6-7 h

Step 2, purification – stage I (native FLAG): 12-16 h

Step 3, purification – stage II (denaturing Ni-NTA): 5-6 h

Step 4, quality control post-purification: 1 d
ANTICIPATED RESULTS

**Figure 2** shows a representative western blot of Ligase Trap purifications. Polyubiquitinated material exhibits retarded mobility in SDS-PAGE and runs as a ladder of bands or a smear.

By using the protocol above, we identified 17 known substrates and 18 novel substrates of eight F-box proteins in budding yeast (Mark et al., 2014). Our work also demonstrated that Ligase Traps with different UBAs, Rad23 or Dsk2, performed well in identifying target substrates. Furthermore, we showed that UBAs can be fused to either the N- or the C-terminus of the F-box protein with little difference in the ability to capture substrate.

FIGURE LEGENDS

**Figure 1: Overview of the Ligase Trapping procedure**

F box-UBA domain fusion proteins (i.e. Ligase Traps) are expressed in cells at physiological levels along with overexpression of a single copy of the ubiquitin gene containing an N-terminal hexahistidine epitope tag. The UBA of the Ligase Trap interacts with the nascent ubiquitin chain on endogenous SCF substrates, thereby delaying their release (left). Cells are then lysed and subjected to an anti-FLAG coimmunoprecipitation under native conditions to isolate Ligase Trap complexes (center). FLAG eluates are collected and a second purification is performed using Ni-NTA agarose beads under denaturing conditions to capture ubiquitinated substrates (right). This second step eliminates interactors associated with the ubiquitinated species that are not themselves substrates.
Figure 2: Quality-control post purification results

(a) Western blot analysis of different Ligase Traps showing two-step purification of polyubiquitinated species. Western blots were probed with an anti-ubiquitin (P4D1) antibody (gift from E. Wayner) and an anti-FLAG antibody (Sigma-Aldrich). Loaded per lane, relative to input (In), is 500x of the FLAG IP (1st) and 2000x of the Ni-NTA IP (2nd).
Figure 1
Figure 2

Galpr-6xHis-Ub

IB: Ubiquitin

IB: Flag
Chapter 2
Ubiquitin Ligase Trapping Identifies an SCF\textsuperscript{Saf1} Pathway Targeting Unprocessed Vacuolar/Lysosomal Proteins

This work was published in Molecular Cell in 2014 Jan 9; 53(1): 148-61. Co-author Alessio Maiolica performed mass spectrometry and analysis; Charles Seller generated strains and performed experiments; Marco Simonetta generated strains, performed experiments, and co-wrote the paper.

SUMMARY
We have developed a technique, called Ubiquitin Ligase Substrate Trapping, for the isolation of ubiquitinated substrates in complex with their ubiquitin ligase (E3). By fusing a ubiquitin-associated (UBA) domain to an E3 ligase, we were able to selectively purify the polyubiquitinated forms of E3 substrates. Using Ligase Traps of eight different F box proteins (SCF specificity factors) coupled with mass spectrometry, we identified known, as well as previously unreported, substrates. Polyubiquitinated forms of candidate substrates associated with their cognate F box partner, but not other Ligase Traps. Interestingly, the four most abundant candidate substrates identified for the F box protein Saf1 were all vacuolar/lysosomal proteins. Analysis of one of these substrates, Prb1, showed that Saf1 selectively promotes ubiquitination of the unprocessed form of the zymogen. This suggests that Saf1 is part of a pathway that targets protein precursors for proteasomal degradation.

INTRODUCTION
The ubiquitin-proteasome system regulates protein activities through degradation and performs quality control of misfolded, mistranslated, or aggregated peptides (Deshaies and Joazeiro, 2009; Finley et al., 2012; Ravid and Hochstrasser, 2008). The Skp1-Cul1-F box (SCF) complex is one of several families of ubiquitin ligases that mediate the timely proteolysis of regulatory proteins.
(Cardozo and Pagano, 2004; Petroski and Deshaies, 2005). This complex is assembled around a cullin scaffold (Cdc53) that bridges a small RING finger protein (Rbx1) and an adaptor protein (Skp1), which in turn recruits an F box protein. The F box subunit serves as the substrate-recognition module (Bai et al., 1996; Patton et al., 1998). In general, F box proteins recognize their substrates only after they are modified, typically by phosphorylation (Hsiung et al., 2001; Orlicky et al., 2003; Skowyra et al., 1997). Budding yeast encodes 20 putative F box proteins (Willems et al., 2004), whereas humans encode 68 (Jin et al., 2004). Although numerous substrates have been identified for the well-characterized Cdc4 and Grr1 F box proteins (Jonkers and Rep, 2009; Reed, 2003), many of the remaining yeast F box proteins have few, if any, known substrates.

Several strategies have previously been used to search for in vivo substrates of ubiquitin ligases. In most studies, substrates were identified as proteins that were no longer ubiquitinated or were selectively stabilized when a ligase was inactivated (Benanti et al., 2007; Emanuele et al., 2011; Kim et al., 2011; Yen and Elledge, 2008). While this approach has identified some substrates, it has significant drawbacks. First, loss of ligase activity may perturb cellular physiology, causing cell-cycle alterations or DNA damage, indirectly affecting the ubiquitin proteome. In addition, some substrates are targeted by more than one ligase, and the absence of a single ligase may not lead to a significant change in the level of the target protein (Landry et al., 2012). Furthermore, by using protein levels as the indicator of ubiquitination, polyubiquitination events that result in a nonproteolytic outcome or target only a specific subpopulation will not be detected. Other approaches to identifying ligase targets exploit the physical interaction between a ligase and its substrate (Busino et al., 2007; Davis et al., 2013). In these studies, immunoaffinity purification
techniques are used to isolate ligase-substrate complexes. The major challenge in using this strategy is that ligase-substrate interactions are often too weak for successful copurification of the target protein.

In this paper, we describe a method called ubiquitin ligase substrate trapping (“Ligase Trapping”), which we used to identify substrates of ubiquitin ligases. In this approach, a polyubiquitin-binding domain (UBA) is fused to an E3 ligase. The UBA increases the affinity of the ligase for its polyubiquitinated substrate, thereby enhancing ligase-substrate stability and permitting the isolation of polyubiquitinated substrates by affinity purification. We used this approach to look for target substrates of eight different F box proteins and identified 17 known substrates specific to the F box proteins examined. In addition, 18 previously unreported candidates were shown to bind specifically to their targeting F box protein as a polyubiquitinated species and/or were enriched in mutants lacking that F box protein. Interestingly, the most abundant group of candidates isolated from the poorly described F box protein Saf1 was vacuolar/lysosomal proteases. Characterization of one of these proteases, the Prb1 zymogen, indicated that the Saf1 Ligase Trap specifically bound the polyubiquitinated form of the unprocessed protein. This suggests a model in which the SCFSaf1 ligase is part of a pathway that targets incorrectly, or incompletely, processed vacuolar/lysosomal proteins.

RESULTS

Fusion of Ubiquitin-Associated Domains to F Box Proteins Increases Their Binding Affinity to Ubiquitinated Substrates

To increase the binding affinity between F box proteins and their ubiquitinated substrates, we
fused a ubiquitin-associated (UBA) domain from the soluble ubiquitin receptor Dsk2 or Rad23 to F box proteins (Figure 1A). These UBAs have a strong preference for polyubiquitin but bind both the K48- and K63-linked forms (Raasi et al., 2005; Sims et al., 2009). Using three tandem Flag epitopes as a linker, we fused the amino terminus of the F box protein Cdc4 to the tandem UBAs of Rad23 (Rad23-Fl-Cdc4). Polyubiquitinated Cdc6 and Far1, known Cdc4 substrates, copurified with Rad23-Fl-Cdc4 in a manner that required the UBA domain (Figure 1B). To determine whether association of the Ligase Trap with polyubiquitinated substrates required substrate recognition by Cdc4, we mutated Arg 467 of Cdc4’s WD40 domain in the fusion protein (Rad23-Fl-R467A). Since this mutation abolishes the ability of Cdc4 to bind substrates (Nash et al., 2001), it is lethal, and thus the experiments in Figure 1B were performed with a galactose-inducible tagged allele in the presence of wild-type CDC4. The R467A mutation strongly impaired the ability of Rad23-Fl-Cdc4 to interact with ubiquitinated Cdc6 and Far1 (Figure 1B), indicating that the binding of the fusion protein to ubiquitinated substrate depends upon both the binding of Cdc4 to the substrate and the UBA to ubiquitin chains. The single UBA domain of Dsk2 could also increase the binding affinity of Cdc4 for ubiquitinated substrates, as demonstrated using a Dsk2-Fl-Cdc4 fusion (Figure 1C). We named this approach “Ubiquitin Ligase Substrate Trapping.”

A Proteomic Screen for Ubiquitinated Substrates of the SCF Using Ligase Trapping

To identify SCF substrates by Ligase Trapping, we analyzed ubiquitinated proteins bound to the UBA-F box fusions using liquid chromatography-tandem mass spectrometry (LC-MS/MS). A two-step purification procedure was adopted using cells that express hexahistidine (His₆)-tagged ubiquitin. After an initial anti-Flag immunoprecipitation, a second purification was performed
under denaturing conditions (6 M urea) using Ni-NTA beads to enrich for ubiquitinated substrates (Figures 1A and 1D). This second step reduced nonspecific binding and eliminated proteins that were associated with the ubiquitinated species but were not themselves substrates.

To examine whether Ligase Trapping worked for other F box proteins, we produced UBA fusions with Grr1, an F box protein involved in cell-cycle regulation and nutrient response. A Grr1-Fl-Rad23 fusion protein bound ubiquitinated Pfk27, a known substrate of Grr1 (Benanti et al., 2007) (Figure 1E). Interestingly, since the Rad23 UBA domain was fused to the carboxyl terminus of Grr1 (Grr1-Fl-Rad23), the successful isolation of tagged ubiquitinated substrates demonstrates that UBAs work at either terminus. Given these results, we performed a proteomic screen for yeast SCF substrates using UBA fusions of eight different F box proteins (Figure S1). For these and all experiments, except those in Figures 1B and 1C, Ligase Traps were expressed under their endogenous F box promoter and represent the only copy of the F box protein in the cell.

A library of yeast strains, each expressing a galactose-inducible His6-ubiquitin allele and a different UBA-F box fusion protein (Figure 2), was used for our initial Ligase Trapping experiment. For the Cdc4 Ligase Trap, both N-terminal and C-terminal fusions were used. In addition, we generated Ligase Traps with a UBA from both Rad23 and Dsk2 for each F box protein. In the case of Mdm30, Saf1, and Skp2, the addition of the tandem UBAs of Rad23 (but not the single Dsk2 UBA) resulted in reduced expression of that Ligase Trap, and therefore these constructs were not used. We performed a two-step purification of each fusion, followed by LC-MS/MS analysis, and identified 17 known SCF substrates (Figure 2 and Table S1). Two
replicates of each Grr1 Trap were performed identically, and the results of both are shown.

Importantly, Ligase Trapping exhibited a high degree of specificity, as most known substrates copurified uniquely with their expected F box protein. Only 3 of the 17 substrates were found associated with an unrelated F box protein: the Grr1 substrate, Mth1, and the Cdc4 substrate, Far1, were also detected in the Ufo1-Fl-Rad23 purification. While we can’t exclude a functional redundancy between these F box proteins, the Ufo1-Fl-Rad23 Ligase Trap exhibited higher nonspecific binding to ubiquitinated targets than the other seven Traps. Ufo1 is unique among yeast F box proteins for having a C-terminal ubiquitin-interacting motif, another class of ubiquitin binding domain, possibly contributing to this background. The Ufo1 substrate HO was captured not only by the Ufo1-Fl-Dsk2 and Ufo1-Fl-Rad23 traps but also by Met30-Fl-Rad23.

The criteria used to select candidate SCF substrates is based on both an enrichment factor of $>25$-fold over other Ligase Traps and an average spectral count of $\geq 1.8$. The exception was Ufo1, for which we instituted a threshold of $\geq 6$ spectral counts and which was not included in the fold enrichment calculation because of its high background. Hits that appear in more than one F box protein may represent redundant targeting by F box proteins; however, these were put aside for our initial analysis. Thirty-eight candidate substrates met these criteria (Table S1). Since our nonspecific binding was so low, the majority of these showed no binding to the other Ligase Traps analyzed. To determine whether proteins with fewer peptides also represent substrates, we examined several candidates that fell below our 1.8 spectral count cutoff for F box proteins Grr1 and Cdc4.
Validation of Grr1 Candidate Substrates

To assess the specificity of Ligase Trapping, we validated candidate substrates of Grr1 most extensively. First, we determined if the stability of Grr1 candidate substrates was increased in \( grr1\Delta \) cells. Of 12 examined, 6 showed a significant increase in stability in asynchronous populations of \( grr1\Delta \) cells (Bud4, Tis11, Gac1, Ynl144c, Sfg1, and Fir1), three others showed more modest changes (Yhr131c, Dre2, and Sbe2), and three appeared stable (Met2, Npl4, and Ykr045c) (Figure 3A and Table 2). While these nine candidate substrates were stabilized in \( grr1\Delta \) cells relative to \( GRR1 \), some still exhibited significant turnover (Sfg1 and Tis11) (Figure 3A). Destabilization that only occurs during a particular phase of the cell cycle might be less detectable in asynchronous cultures. Therefore, we analyzed five substrates in synchronized \( GRR1 \) and \( grr1\Delta \) cells (Figure 3B). Grr1 was largely responsible for the cell-cycle-dependent expression of Bud4 and Sfg1. Tis11 and Yhr131c also showed some cell-cycle regulation, and this was modestly reduced in \( grr1\Delta \) cells. In contrast, Dre2 was not regulated in a cell-cycle-dependent fashion.

The SCF often targets phosphorylated substrates. Several stabilized substrates (Sfg1, Tis11, Fir1, Ynl144c, and Sbe2) were enriched for an electrophoretically shifted, and likely phosphorylated, form in \( grr1\Delta \) strains (Figure 3A). This is particularly evident for Ykr045c, which shows a species in \( grr1\Delta \) cells with slightly reduced mobility in both G1- and nocodazole-arrested cells (Figure 4A). This form, which is most likely phosphorylated, cannot be detected in \( GRR1 \) cells, possibly due to its selective degradation upon ubiquitination. While bulk levels of Ykr045c do not decrease following addition of cycloheximide, a very high-molecular-weight (and likely ubiquitinated) smear, seen only in the \( GRR1 \) cells, disappears. This finding underscores the fact
that a lack of bulk turnover of a candidate substrate does not necessarily indicate that the F box protein in question is not targeting this substrate. Three other Grr1 candidate substrates (Met2, Npl4, and Sbe2) also showed no significant difference in stability between \( GRR1 \) and \( grr1\Delta \) cells, although Met2 and Sbe2 showed a significant increase in steady-state levels in \( grr1\Delta \) strains. Npl4 is a component of the Cdc48 complex, which is thought to help disassemble the SCF (Yen et al., 2012), and is likely not a Grr1 substrate. We previously found Met2 to be strongly transcriptionally induced by deletion of \( GRR1 \), suggesting that this may not be a direct target (Benanti et al., 2007).

Our Ligase Trapping technology allows not only the initial identification of substrates but also a means by which to examine the ubiquitination of that substrate directly. To validate that SCF\(^{Grr1}\) candidate substrates were ubiquitinated in vivo, we performed two-step purifications of Grr1-Fl-Rad23 Ligase Traps in cells expressing Myc-tagged alleles of four proteins that met our criteria for candidates (Sfg1, Bud4, Tis11, and Yhr131c) and four that did not (Sbe2, Dre2, Gin4, and Mps1) (Table 2). Purifications were performed in Grr1-Fl-Rad23 and two control Ligase Traps. We employed Mfb1-Fl-Rad23 because it used the same UBA and was expressed at similar levels as Grr1. We also chose Ufo1-Fl-Rad23, as this Ligase Trap showed the highest nonspecific binding, and therefore set an upper threshold for background. All four candidates that fell within the 1.8 peptide cutoff copurified as ubiquitinated proteins specifically with Grr1-Fl-Rad23 (Table 2 and Figure 4B). Of these, Yhr131c reproducibly showed less extensive ubiquitination. For the remaining four, Dre2 and Sbe2 showed Grr1-specific ubiquitination (Figure 4B), whereas Mps1 and Gin4 did not (data not shown). Thus, of the ten Grr1 candidates that fell within our cutoff, at least seven appeared to be genuine substrates by at least one criterion. Moreover, these data
suggest that several of the lower abundance hits are likely to be substrates as well. To show that our candidate substrates are direct targets of Grr1, we determined whether each could associate with a Grr1 construct lacking the F box motif, Grr1ΔF, which fails to incorporate into functional SCF complexes (Bai et al., 1996). As shown in Figure 4C, all ten substrates tested copurified strongly with the Flag-tagged Grr1ΔF construct (some in a shifted polyubiquitinated form). While copurification with Grr1ΔF is useful as a follow-up to confirm binding, this technique provides levels of background too high for initial substrate identification.

Examining Substrates of Other F Box Proteins

We also examined candidates obtained for four additional F box proteins: Cdc4, Ufo1, Skp2, and Saf1. Unlike the other Ligase Traps, we generated traps of Cdc4 fused at either terminus. This was carried out because C-terminal tagging of Cdc4 appeared to compromise its function. Moreover, all Cdc4 fusions purified less polyubiquitinated product than other Ligase Traps (Figure S1), consistent with our finding that Cdc4 is particularly difficult to modify while retaining functionality. Since the N-terminally tagged CDC4 strains were healthier, we employed the RAD23-Fl-CDC4 in parallel. Despite its compromised function in vivo, the CDC4-Fl-RAD23 identified known substrates equally as well as, if not better than, the RAD23-Fl-CDC4 allele (Figure 2). We examined seven candidate Cdc4 substrates above the 1.8 peptide cutoff (Atc1, Isr1, Swi1, Amn1, Sac3, Osh3, and Ipt1) and two that were below it (Pcl1 and Rav2). In the case of Atc1, Isr1, and Swi1, stabilization could be observed in the cdc4-1 strain (Figure 5A). Partial stabilization was also observed for Pcl1, a G1 cyclin. Cdc4 and Grr1 have previously been shown to target the G1 cyclin Cln3 redundantly (Landry et al., 2012), but we found that Grr1 contributed only slightly to Pcl1 turnover. Rav2 is a protein component of the RAVE complex,
which functions in assembly of the vacuolar ATPase and binds the SCF core subunit Skp1 (Seol et al., 2001). Interestingly, levels of a smaller form of Rav2 are elevated in cdc4-1 strains, and a shifted form is seen to accumulate. Cdc4 did not affect the stability of Osh3, Ipt1, or Amn1. To better characterize some of these potential Cdc4 substrates, we determined whether their ubiquitinated forms associated with the Rad23-Fl-Cdc4 Ligase Trap (Figure 5B) and two similarly abundant control Ligase Traps. Four of the tested candidates (Swi, Atc1, Isr1, and Pcl1) showed specific association of a ubiquitin smear with the Cdc4 Trap. In contrast, Osh3 and Ipt1 did not (data not shown).

For the F box protein Ufo1, we identified HO as a substrate, as has previously been reported (Kaplun et al., 2006). Because the Ufo1 Traps showed unusually high background, we set the threshold for substrates for this Trap to be six spectral counts and 25-fold enrichment. Despite this, we were able to discern several strongly enriched candidates, many of which had roles in translation. The strongest of these, the polysome-associated Rbg1 protein, is a member of the Obg/CgtA GTP-binding proteins conserved from bacteria to humans (Wout et al., 2009). Although Rbg1 levels were not affected in ufo1Δ strains (Figure 5C), ubiquitinated Rbg1 purified with two forms of the Ufo1 Trap, but not the Grr1-Fl-Rad23 control (Figure 5D). This suggests that only a subset of Rbg1 in the cell is targeted by Ufo1 under these conditions. Alternatively, Rbg1 may be targeted in only a subset of cells.

**Saf1 Promotes Ubiquitination of Proteins of the Secretory Pathway**

All four of the candidate substrates identified for Saf1 were vacuolar/lysosomal enzymes (Figure 6B and Table S1): three proteases (Prb1, Prc1, and the putative Ybr139w) and an alkaline
phosphatase (Pho8), two of which have previously been shown to associate with Saf1 in a large-scale study (Ho et al., 2002). The yeast vacuole is thought to be similar to the mammalian lysosome as it contains a large number of degradative enzymes. The uncharacterized protein Ybr139w has been suggested to be a serine carboxypeptidase (Baxter et al., 2004; Wunschmann et al., 2007) and shows vacuolar localization. Prb1, Prc1, and Pho8 are synthesized as the inactive zymogens preproPrb1, preproPrc1, and proPho8. (The processing of Ybr139w, if any, is uncharacterized.) During ER-to-vacuole progression, precursors are cleaved to generate the active enzymes, which can be distinguished by their lower molecular weight. For Prb1, there are at least four proteolytic steps. The signal sequence is first removed upon translocation into the ER, followed by a second cleavage event to remove P1, generating “proPrb1” (Figure 6A). After exiting the ER, two C-terminal cleavages remove P2 and P3 to generate the mature form (mPrb1).

To show that SCF\textsuperscript{Saf1} ubiquitinates these vacuolar/lysosomal substrates, we C-terminally Myc-tagged each substrate in cells expressing Saf1-Fl-Dsk2 or the control Trap Grr1-Fl-Dsk2. Two-step purifications immunoprecipitated ubiquitinated Prb1-Myc, Prc1-Myc, and Ybr139w-Myc, but not Pho8-Myc (Figure 6B and data not shown), specifically with the Saf1 Ligase Trap. The high-molecular-weight species indicative of ubiquitinated Prb1-Myc are detected almost exclusively above the ~110 kDa band corresponding to preproPrb1-Myc (Figures 6E, 6F, and S3B). This suggests that SCF\textsuperscript{Saf1} ubiquitinates Prb1 prior to proteolytic activation, although C-terminal tagging of Prb1 did delay processing somewhat (Figure S3A). LC-MS/MS data from Saf1 Ligase Trap purifications confirmed that Saf1 interacts with preproPrb1. Of the 29% sequence coverage we obtained for Prb1, most of it (17%) was located in portions of Prb1 that
are removed during proteolytic activation, whereas only 12% represented portions of mPrb1 (Figures 6A and S2). This is despite the fact that mPrb1 represents the vast majority of Prb1 in wild-type cells, with preproPrb1 being barely visible (Figure S3A). Unfortunately, only two distinct peptides, representing 4% coverage, were obtained for Prc1. Given that only a small portion (about 20%) of Prc1 is removed during processing, this data set does not allow us to determine if the Prc1 precursor was targeted. We could not detect Pho8-Myc in Saf1-Fl-Dsk2 purifications by western blot. While we cannot exclude the possibility that Pho8 is a false-positive hit of the LC-MS/MS analysis, it is also possible that the epitope tag on Pho8 interferes with its ubiquitination by SCFSaf1. Since it was surprising that an F box protein acted upon substrates targeted to the vacuole, we examined whether other SCF components were required. Mutations in either the cullin subunit (cdc53-1) or the SCF’s E2 (cdc34-2) eliminated Saf1 binding to polyubiquitinated Prb1 (Figure 6C). The reduced length of the polyubiquitin chains seen is due to the elevated temperature used for this experiment (Figure S3B). However, even at 30°C, Saf1 substrates appeared less ubiquitinated than those of other SCF ligases (Figure S1), suggesting either that SCFSaf1 is less processive or that the UBA fusion interferes with the function of Saf1.

To determine whether the ubiquitination of Prb1 plays a role in the processing and translocation of preproPrb1, we examined the rate of processing of Prb1 in a saf1Δ strain. By pulsing Prb1, expressed under the control of the GAL1 promoter, one can see the initial accumulation of the preproPrb1 form, followed by the rapid accumulation of a size corresponding to proPrb1 (lacking P1), and, finally, the appearance of mPrb1 (Figure 6D). This processing was unaffected in saf1Δ, suggesting that SAF1 is not required for processing or translocation (since the final step of
processing is thought to occur in the vacuole. Purification of ubiquitinated Prb1 was intact in \textit{vam3Δ} mutants, which are defective in translocation to the vacuole (Srivastava and Jones, 1998), suggesting that targeting of Prb1 occurs prior to reaching this organelle (Figures 6E). This was also the case for Prc1, shown using a \textit{vps10Δ} mutant (Figure S3C), which fails to properly deliver Prc1 from the Golgi to the vacuole (Marcusson et al., 1994). Both a \textit{sec65-1} mutant, which blocks entry into the ER, and tunicamycin, which eliminates N-linked glycosylation, blocked Prb1 ubiquitination (Figures 6E and S3E). However, Prb1 could still be ubiquitinated in \textit{sec7-1} and \textit{sec23-1} mutants, both of which are defective in ER-to-Golgi traffic (Novick et al., 1980; Wolf et al., 1998) (Figure 6E).

To determine whether the selective targeting of unprocessed Prb1 requires all portions of the full-length substrate, we made a series of deletion mutants of Prb1. Removal of Prb1’s signal sequence eliminates its ability to efficiently enter the ER, and this strongly reduced Saf1-mediated ubiquitination (Figures S3D and 6F). This is consistent with the absence of ubiquitination in the \textit{sec65-1} mutant (Figure 6E), suggesting that Prb1 must be ER-targeted before it is recognized. Moreover, eliminating either P1 or P2-3 blocked Saf1 targeting. At least in the case of the ΔP1 mutants, this is not due to a disruption in ER localization, as the ΔP1 and ΔP1ΔP2-3 peptides are altered in size after treatment with tunicamycin, suggesting that they have entered the ER (Figure S3D). These findings are consistent with Saf1 selectively targeting the unprocessed form.

The ubiquitination of ER proteins by Saf1 is reminiscent of the ER-associated degradation (ERAD) quality control pathway, a process whereby unfolded ER proteins are targeted for
ubiquitination after their retrotranslocation from the ER into the cytoplasm (Tsai et al., 2002). ER proteins are thought to become ERAD substrates following redox stress, such as that induced by treatment with the reducing agent DTT, heat shock, or tunicamycin treatment. Saf1 ubiquitination of Prb1 was not increased by any of these treatments, but was in fact decreased (Figures S3B and S3E). It is possible that ubiquitination of Prb1 could decrease after this treatment because of competition with increased amounts of other substrates generated under these conditions. However, ubiquitination was decreased even after more modest treatments with these agents (Figure S3E). The Prc1 protein (a.k.a. CPY), identified by the Saf1 Ligase Trap, is a hallmark ERAD substrate when mutated at a single site, G255R, which is thought to cause its partial unfolding (Finger et al., 1993). We will refer to this point mutant as Prc1*. If Saf1 were part of the previously characterized ERAD pathway, its ubiquitination of Prc1 should increase significantly in a Prc1* strain. Despite the fact that there exists consistently higher levels of mutant Prc1* protein than wild-type Prc1, we saw no increase in the percentage of ubiquitinated Prc1 associated with the Saf1 Ligase Trap (Figure 6G). (Note, the higher levels may reflect the fact that Prc1* is the only form of PRC1 expressed in these cells.) Interestingly, however, we do see a qualitative difference, in that, in addition to the less ubiquitinated forms seen associated with Saf1, there is an additional lower mobility form. This suggests that a portion of the Prc1* that is targeted by Saf1 was previously targeted by another more processive (possibly ERAD) ligase. Together, these data suggest that Saf1 is not a component of the previously characterized ERAD quality control system, but rather part of a ubiquitination pathway that specifically targets zymogens that cannot be processed.

DISCUSSION
We have developed a method allowing us to trap a given E3 ubiquitin ligase in association with its substrates. This not only allows us to identify previously unreported ubiquitin ligase substrates but also provides a robust method by which we can determine whether a given ubiquitin ligase targets a particular protein in vivo. This is critical, since many substrates are not quantitatively degraded, and, therefore, one cannot always see a strong selective stabilization of a substrate after mutation of the ligase in question. While we have carried out this proof-of-principle experiment on F box proteins in yeast, this methodology should be readily applicable to other classes of ubiquitin ligases and other organisms.

We have used the Ligase Trapping technique on eight F box proteins and followed up on candidate substrates for four of these in detail (Cdc4, Grr1, Ufo1, and Saf1). Many of our identified Grr1 and Cdc4 substrates are consistent with the known role of Grr1 in nutrient sensing and the role of both proteins in cell-cycle regulation. While Ufo1 appears to target several proteins involved in translation, Saf1 emerges as an F box protein with a focused role in targeting incompletely processed degradative enzymes bound for the vacuole.

The F box substrates fell into three categories with respect to their stability. Some, such as Bud4 and Sfg1, were highly unstable proteins that were very strongly stabilized when the gene for the F box protein targeting them (GRR1) was deleted. This corresponds to the simplest case in which a substrate is quantitatively targeted by a single ubiquitin ligase. Second, there were substrates, such as Tis11 and Pcl1, which were unstable and only partially stabilized in their respective F box mutants, suggesting that they are redundantly targeted. Finally, there was a significant set of substrates that appeared stable even in wild-type cells (e.g., Ykr045c and Rbg1). There are
several possible explanations for this. These proteins may be targeted in only some subcellular locations or contexts (e.g., when part of a specific complex). Alternatively, they may be targeted in only a subset of cells that are either in a particular cell-cycle phase or are under some stress. Importantly, the high-molecular-weight forms of Rbg1 and Ykr045c are lost upon cycloheximide treatment (Figure 4A and data not shown), suggesting that ubiquitination leads to degradation of the modified subset, although it is also possible that deubiquitination accounts for this. An advantage of Ligase Trapping is that, unlike many existing technologies, it allowed us to confirm that a given substrate was targeted by a particular ligase even when the targeting was redundant or did not lead to quantitative turnover.

**Functional Clusters of Substrates Identifies Multilayer Regulatory Roles of Grr1, Cdc4, and Ufo1 in Different Aspects of Cell Biology**

Live fluorescence microscopy showed that Grr1 transiently localizes at the bud neck during mitosis to disappear shortly after cytokinesis is completed (Blondel et al., 2005), where it controls the levels of Hof1 and Gic2. Bud4 also localizes at the bud neck during mitosis, and its localization is required to mark the bud site and recruit downstream regulators of the cytokinetic ring (Kang et al., 2012). We show that Grr1 is responsible for the degradation of Bud4 in G1, suggesting that Grr1 promotes the downregulation of Bud4 activity once this function is accomplished (Figure 3B). Grr1 also targets Sbe2, which is involved in cell-wall integrity and has a putative role in establishing a correct polar budding pattern (Santos and Snyder, 2000) (Figure 3A). Interestingly, Sfg1 is a transcriptional repressor that targets many genes involved in mother-daughter cell separation and is thought to be a substrate of Cdk (White et al., 2009), which may target it for Grr1-mediated turnover.
Grr1 also regulates different metabolic pathways by promoting the degradation of substrates (including Pfk27, Tye7, and Mth1) in response to nutrient availability. We found that the PP1 subunit Gac1, which regulates glycogen storage, is regulated by Grr1. We find that Grr1 may also have a role in iron metabolism, as it targets Tis11 and Dre2. Tis11 is a highly conserved protein that interacts with AU-rich elements in the 3’ UTR of a specific group of mRNAs and promotes their turnover in conditions of iron starvation (Puig et al., 2005). Dre2 is an essential protein involved in the biogenesis of iron-sulfur proteins such as ribonucleotide reductase (Zhang et al., 2011). The regulation of the targeting of Dre2 by Grr1 was not clear, as bulk levels of Dre2 were largely stable. While Dre2 did not exhibit cell-cycle-dependent regulation, Tis11 levels appear to fluctuate in the cell cycle. Thus, Grr1 might have a role in coordinating iron homeostasis with cell-cycle progression, or Tis11 may have an additional role in the targeting of messages encoding cell-cycle-related genes.

Despite the fact that the Cdc4 Ligase Traps resulted in somewhat reduced fitness, they identified several substrates, including the Swi/Snf transcription factor Swi1, the cyclin Pcl1, and the kinase Isr1. Surprisingly, two very strong Cdc4 hits, Osh3 and Ipt1, do not appear to be Cdc4 substrates (J. Lao, personal communication). Myc tagging these proteins may disrupt their ability to associate with Cdc4.

Three of the top four Ufo1 candidates identified were associated with translation: Rbg1, Yef3, and Rps2 (Table S1). Rps2 is a component of the small ribosomal subunit. Rbg1 and the elongation factor Yef3 are both components of polysomes (Fleischer et al., 2006; Hutchison et
al., 1984; Wout et al., 2009). Rbg1 is conserved from E. coli to humans and, in bacteria, has been shown to be involved in the regulation of translation in response to nutrient deprivation (Wout et al., 2009). Moreover, a human homolog of Rbg1 (Drg2) undergoes SCF-mediated turnover (Chen et al., 2012), suggesting that this regulation is conserved.

Met30 is thought to regulate methionine biosynthesis pathways, and the corresponding Ligase Trap only copurified its previously characterized target, Met4. Mdm30 regulates mitochondrial biology, and we identified its known target, Fzo1 (a protein involved in mitochondrial fusion) and another mitochondrial protein, Yjl045w (Table S1). While included in this study, it is unclear if Mfb1 and Skp2 are bona fide SCF components, since, unlike the other six F box proteins examined here, neither of these identified the cullin Cdc53 in our purifications or in previously characterized proteomic or two hybrid analyses (Ho et al., 2002; Krogan et al., 2006; Seol et al., 2001). While Mfb1 identified no substrates, Skp2 did purify several proteins. Predominant among these were Dma1 and Dma2. These were previously known to be strong Skp2 interactors (Ho et al., 2002), but neither appeared to be direct substrates of Skp2 (data not shown). As these are themselves E3s, they are likely autoubiquitinated and thus purified in our second step.

**Saf1 Targets Unprocessed Prb1**

Of the substrates identified in our study, the most surprising by far were the identification of several vacuolar proteins. Because the background was so low with the Ligase Trapping system, we were confident enough to follow up on hits that seemed, at first, very unlikely SCF substrates. All four hits for the F box protein Saf1 were vacuolar/lysosomal hydrolases. This is
unanticipated for several reasons. First, these proteins are not thought to be present in the cytosol and nucleus, where the SCF is located. Second, they are stable proteins. Finally, the peptides corresponding to Prb1 matched the full-length preproPrb1, which is quite rare, suggesting that the unprocessed form was selectively ubiquitinated.

SCFSaf1 appears to target Prb1 that cannot be processed. This appears to require ER entry and is likely to occur after retrotranslocation of the protein back into the cytosol. However, Saf1 does not appear to function as part of an Hrd1- and Doa10-like ERAD pathway, as turnover of Prb1 is not promoted by its unfolding. As is the case for most quality control pathways, it does not appear that Saf1 targets a large percentage of preproPrb1, since steady-state levels of the unprocessed protein are not altered upon deletion of SAF1. Prior to this study, only a single substrate of Saf1 had been identified. A purine-salvage pathway protein, Aah1, was previously shown to be targeted by Saf1 during nitrogen starvation (Escusa et al., 2006). While Aah1 is not thought to be vacuolar, the fact that Aah1 is targeted under conditions that promote autophagy, a process during which nutrients are salvaged by targeting cellular structures to the vacuole/lysosome, is an intriguing connection between these Saf1 substrates. Prb1, Prc1, and Pho8 are all induced upon nutrient limitation (Hansen et al., 1977; Kaneko et al., 1985; Klar and Halvorson, 1975).

Interestingly, Saf1 contains neither LRR nor WD40 repeats, but instead has another β-propeller domain composed of RCC1-like repeats (Escusa et al., 2007). Whether these RCC1-like repeats will, like WD40s and LRRs, recognize substrates only after phosphorylation is not yet known. An intriguing possibility is that Prb1 glycosylation is required. Prb1 is N-glycosylated (Mechler
et al., 1982; Moehle et al., 1987), but while mutation of this site leads to much lower levels of Prb1, its relative level of ubiquitination is not strongly affected (Figure S3F). While there is typically not thought to be a direct correspondence between human and yeast F box proteins, it is interesting to note that \textit{S. cerevisiae}, \textit{S. pombe}, and humans all contain a single F box protein with RCC1-like repeats (Jin et al., 2004). Whether this human F box protein also targets lysosomal targets remains to be determined.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**

Genotypes of yeast strains, including the specific experiments in which each was used, are detailed in Table S2. With the exception of Figures 6, S3, and S4 (W303 background), all strains are in the S288c background. Strains and plasmids were generated using standard techniques.

**Plasmids**

To perform one-step purification, as shown in Figure 2B, Fl-Cdc4, Rad23-Fl-Cdc4 and Rad23-Fl-R467A were expressed using the ARS/CEN plasmid pRS316. A DNA sequence, which encodes three copies in tandem of the Flag epitope (3xFlag), was cloned downstream the \textit{GAL1} promoter in pRS316. The CDC4 gene was amplified from genomic DNA and fused to the 3’ end of 3xFlag sequence to generate the fusion sequence 3xFlag-CDC4 under \textit{GAL1} promoter control (Plasmid pMS1). To express Rad23-Fl-Cdc4, the 3’ terminal sequence of RAD23 gene, encoding the two C-terminal UBA domains (codons 143-397), was amplified by genomic DNA and cloned into pMS1 downstream \textit{GAL1} promoter, and upstream 3xFlag-CDC4 generating the fusion sequence RAD23-3xFlag-CDC4 (Plasmid pMS2). Quick-change site-directed mutagenesis kit
(Stratagene) was used to mutate the codon 467 of CDC4 in pMS2 to encode Alanine instead of the Arginine467 of WT Cdc4 (Plasmid pMS3). DSK2-3xFlag-CDC4 DNA fusion sequence was obtained by exchanging the sequence encoding the two UBAs of Rad23 in pMS2 with the sequence encoding the single C-terminal UBA domain of Dsk2 (codons 327-373; Plasmid pMS4).

All two-step purifications were performed expressing F-box protein-UBA fusions under the endogenous promoter at the genomic loci. Genes encoding F-box proteins were fused to UBAs at genomic loci using the integration plasmid pRS306, which lacks ARS/CEN sequence elements. N-terminal Flag-tagging of Cdc4 at the endogenous locus was obtained cloning the following DNA sequences into pRS306 in this order: Cdc4 promoter sequence (946 bps upstream the ATG of CDC4), 3xFlag, and the partial sequence of the CDC4 containing the first 583 bps (Plasmid pMS5). Plasmid pMS5 was cut using a unique restriction site in the Cdc4 partial sequence and used to transform strains that were selected for the URA+ phenotype. Correct integration was confirmed by PCR and Western blotting. The expression of Rad23-Fl-Cdc4 fusion from the endogenous locus was obtained through the same procedure used for Fl-Cdc4, but this time the integration vector pRS306 contained the following DNA sequences in this order: Cdc4 promoter, sequence encoding the two UBAs of Rad23 (codons 143-397), 3xFlag and CDC4 partial sequence (Plasmid pMS6). Except for Cdc4 all the other F-box proteins were tagged at the carboxy-terminus by cloning a partial sequence of F-box protein genes into the integration plasmid pRS306 in frame with 3xFlag, 3xFlag-RAD23 or 3xFlag-DSK2 sequences followed by the stop codon TAG. The integration was accomplished as described above by cutting the plasmids using a unique restriction site in the F-box protein partial sequence, transforming
strains and selecting for URA+ phenotypes. The same technique was used to tag both known and candidate substrates cloning their 3’ terminal partial sequences into pRS306 fused to 3xFlag or 13xMYC sequences.

To overexpress N-terminally 6xHis-tagged ubiquitin, HIS3MX-GAL1-6xHIS cassette was amplified by PCR performed using plasmid pFA6a-His3MX-PGAL1 and primers containing sequence homology to target the cassette to the genomic locus UBI4, which contains five ubiquitin sequences in tandem. Strains were transformed and selected for HIS3+ phenotype. Colonies were checked by PCR for cassette integration that resulted in a single copy of the ubiquitin (targeting the last ubiquitin sequence of the UBI4 locus) tagged with 6xHis under the GAL1 promoter. For Flag pull-down assays, the pYES2-GRR1dF-FLAG-URA3 expression vector was generated by subcloning out the N-terminal GST epitope tag from pYES2-GST-GRR1dF-FLAG-URA3 (Benanti et al., 2007) and replacing it with a start codon.

Western Blotting

To examine the intracellular protein levels, an equivalent of five OD_{600} were harvested and lysed. Pellets were washed in cold water and resuspended in 200 µl pre-heated SDS buffer (50 mM Tris pH 7.5, 5 mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 0.05% bromophenol blue, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM benzamidine, 17 µg/ml PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate). Resuspension was then incubated for 5 minutes at 95°C and homogenized for 3 minutes in a Mini BeadBeater (Biospec) using 100 µl glass beads. Samples were then clarified by centrifugation at 16,000 x g for 15 minutes. Extracts were analyzed by SDS-PAGE, followed by transfer to nitrocellulose
membranes, and Western blotting with antibodies against Myc (Clone 9E10, Covance), Cdc28 (sc-6709, Santa Cruz Biotechnology), Clb2 (sc-9071, Santa Cruz Biotechnology), Flag (Clone M2, Sigma-Aldrich), HA (Clone 12CA5, Harlan Bioproducts), Ubiquitin (Clone P4D1, gift from E. Wayner), Rad53 (Rabbit anti-Rad53, DAB001, gift from D. Durocher) and Prb1 (Rabbit anti-Prb1, (Moehle et al., 1989)).

**One-step Purification**

One-step purification in Figure 1B was performed using strains carrying pRS316 plasmids (pMS1-pMS4). Cells were grown to mid-log phase in C media lacking uracil (C-Ura) containing 2% raffinose. Two percent galactose was added, and cultures were incubated an additional 3 hr. Cells were harvested from 100 ml culture at an optical density (OD$_{600}$) of ~1.0, lysed in 700 µl HEPES lysis buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 17 µg/ml PMSF, 5 mM sodium fluoride, 80 mM b-glycerophosphate, 1 mM sodium orthovanadate, and a Complete Proteasome Inhibitor Tablet [Roche Diagnostics] by bead beating in a cold block for six cycles of 1.5 min [alternated with 2 min on ice]), and cleared by centrifugation at 4°C. Protein concentrations were then quantified using the Bio-Rad Protein Assay, based on the method of Bradford, and equal amounts of extract (2 mg) were incubated with 30 µl slurry of anti-Flag M2 Magnetic Beads (Sigma-Aldrich) while rotating at 4°C for 3 hr. Beads were collected on a magnetic rack and washed three times with 700 µl lysis buffer. Proteins were eluted by mild vortexing in 1x PBS buffer containing 500 µg/ml 3x Flag peptide (Sigma-Aldrich) for 30 min and analyzed by western blotting against the Flag epitope on the Cdc4 proteins and the Myc tag or HA tag on Cdc6 and Far1.
Two-step Purification

Two-step purifications were performed by harvesting cells at an optical density (OD600) of ~1.0. Cell cultures of 350 ml (small-scale) and 2–4 l (large-scale) were used for western blot analysis and LC-MS/MS analysis, respectively. Cells were grown in YM-1 medium containing 2% raffinose and 2% galactose. Small-scale pellets were resuspended in 1.2 ml lysis buffer and lysed by bead beating, as described in the one-step purification method. Large-scale pellets were resuspended in 3 ml lysis buffer, frozen in liquid nitrogen, grounded in a Retsch M301 ball mill, and resuspended in an additional 7 ml lysis buffer. Lysis buffer for small- and large-scale purifications was composed of 25 mM HEPES (pH 7.5), 150 mM potassium acetate, 1 mM EDTA, 17 µg/ml PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.02 mM MG132 (Sigma-Aldrich), and a Complete Proteasome Inhibitor Tablet (Roche Diagnostics). Cell lysates were cleared by centrifugation and incubated with 100 µl slurry (small-scale) and 200 µl slurry (large-scale) of anti-Flag M2 Magnetic Beads (Sigma-Aldrich) overnight while rotating at 4°C. Beads were collected on a magnetic rack and washed three times with PBS buffer containing 0.1% NP-40. Proteins were eluted by mild vortexing with five times beads volume of PBS buffer containing 0.1% NP-40 containing 500 µg/ml 3xFlag peptide (Sigma-Aldrich) for 45 min at room temperature. Eluted proteins (first step) were denatured by adjusting elution buffer with 40 mM NaH2PO4, 5 mM Tris-Cl, 6M urea (pH 8), and were incubated for 2 hr with 30 µl (small-scale) or 60 µl slurry (large-scale) of Ni-NTA agarose beads (Invitrogen) previously equilibrated with 100 mM NaH2PO4, 10 mM Tris-Cl, and 8M urea (pH 8). Beads were washed three times with 100 mM NaH2PO4, 10 mM Tris-Cl, and 8 M urea (pH 8) and were washed two times with PBS buffer. Proteins were eluted with three times beads volume of PBS buffer (pH 8) containing 300 mM imidazole and 0.1% RapiGest (Waters) by
mild vortexing at room temperature for 30 min (second step). Relative to input, samples for SDS-PAGE were loaded at 170x–800x and 1,300x–2,800x for the Flag (first) and His (second) elutions, respectively.

**Sample Preparation and Mass Spectrometry Analysis**

The purified complexes were reduced by incubation with TCEP (Thermo) at a final concentration of 10 mM at RT for 45 minutes. The produced free thiols were alkylated with 20 mM iodoacetamide (Sigma) at room temperature for 45 min in the dark and digested with sequencing grade-modified trypsin in 1:50 ratio w/w (Promega, Madison, Wisconsin) overnight at 37°C. Peptides were desalted on a C18 Sep-Pak cartridge according to manufacture instructions (Waters, Milford, Massachusetts) and the eluted peptides were dried in SpeedVac and successively reconstitute in 2% AcN, 0.1% FA and analyzed by LC-MS/MS.

Each peptide sample was analyzed on an Eksigent Nano LC system (Eksigent Technologies) connected to a hybrid linear ion trap LTQ Orbitrap XL (Thermo Scientific), which was equipped with a nanoelectrospray ion source (Thermo Scientific). Peptide separation was carried out on a RP-HPLC column (75 micro m inner diameter and 10 cm length) packed in-house with C18 resin (ReproSil-Pur 120 C18-AQ, 3 µm, Dr. Maisch GmbH) and the peptides were eluted from the analytical column with a 60-min gradient ranging from 7% to 35% solvent B followed by a 5-min gradient from 35% to 80% solvent B at a constant flow rate of 300 nl/minute. The solvent for liquid chromatography composition was 0.1% formic acid in water (98%) and acetonitrile (2%), and solvent B consisted of 0.1% formic acid in acetonitrile (98%) and water (2%). The data acquisition mode was set to acquire 1 high resolution MS scan in the ICR cell followed by 5
collision induced dissociation MS/MS scans in the linear ion trap. For high resolution MS scan, 10e6 ions were accumulated over a maximum time of 500 ms and the FWHM resolution was set to 60 000 (at m/z 300). Only MS signals exceeding 500 ion counts triggered a MS/MS attempt and 10e4 ions were acquired for a MS/MS scan over a maximum time of 200 ms. The normalized collision energy was set to 35. Singly charged ions were excluded from triggering MS/MS scans.

Raw data files from the MS instruments were converted with ReAdW into mzXML files and mzXML files were searched with Sorcerer-SEQUEST against a concatenated protein yeast SGD database (Version 20110203), the reversed sequences of all proteins. Statistical analysis of each search result for each LCMS analysis was performed using the Trans-Proteomic Pipeline TPP TPP v4.5 RAPTURE rev 1, Build 201201161611 including PeptideProphet and ProteinProphet. The ProteinProphet probability score was set to 0.9, which resulted in an average protein false discovery rate of less than 1% for all search results estimated by ProteinProphet. The adjusted spectral counts were calculated using the software ABACUS with default settings.

**Turnover Analysis**

Asynchronous cells were grown to mid-log phase in YM-1 containing 2% dextrose and treated with cycloheximide (50 µg/ml, Sigma-Aldrich). For cell-cycle experiments, cycloheximide was added to cells previously arrested with α factor (10 µg/ml, Elim Biopharm) or nocodazole (10 µg/ml, Sigma-Aldrich), both for 2 hr. Cell pellets were collected for western blotting at indicated time points.
**FLAG Pull-down Assays**

Strains carrying either pRS426 or pYES2-GRR1 dF-FLAG-URA3 plasmids were grown to OD600 ~0.3 in 50 ml of synthetic media lacking uracil and containing 2% raffinose. To induce, 2% galactose was added and cultures were grown for two doublings. Cells were lysed in a buffer containing 100 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, 0.2% NP-40 with a Roche Complete protease inhibitor tablet without EDTA (one tablet/25 ml), 1 mM PMSF, and four Roche PhosSTOP Phosphatase Inhibitor Tables (four tablets/25 ml). Lysis was carried out by bead beating, as described in the one-step purification method, and cleared by centrifugation at 4°C. Lysates were incubated with a 25 ml slurry of anti-Flag M2 Magnetic Beads (Sigma-Aldrich) overnight while rotating at 4°C. Beads were washed three times with PBS buffer containing 0.1% NP-40. Proteins were eluted by mild vortexing with five times beads volume of PBS buffer containing 0.1% NP-40 containing 500 µg/ml 3xFlag peptide (Sigma-Aldrich) for 45 min at room temperature. Samples were loaded for SDS-PAGE at 0.06% of the total input and 20% of the Flag elution.

**FIGURE LEGENDS**

**Figure 1. UBA Fusions to Ubiquitin Ligases Increase Their Affinity for Ubiquitinated Substrates**

(A) Schematic representation of Ligase Trapping and purification procedures. The top panel shows that, during normal degradation, ubiquitin ligases like the SCF associate with both substrates and E2s. Ubiquitin-charged E2s then transfer their ubiquitin to the substrate, leading to the formation of a polyubiquitin chain. This is shuttled to the proteasome with the help of ubiquitin receptors, such as Rad23. The bottom panel shows Ligase Trapping. A UBA domain is
fused to a ubiquitin ligase via a Flag linker. The UBA binds the nascent ubiquitin chain while the linker allows a Flag immunoprecipitation of the ligase in complex with the substrate. The expression of His6-tagged ubiquitin allows a second purification step that specifically isolates ubiquitinated species using Ni-NTA agarose beads under denaturing conditions.

(B) Western blots of input whole-cell extract (In) and the first purification step (first). Flag immunoprecipitation of Fl-Cdc4, Rad23-Fl-Cdc4, and Rad23-Fl-Cdc4-R467A (containing a R467A mutation in CDC4) in cells expressing Cdc6-Myc or Far1-Myc. Ligase Traps were under the GAL1 promoter. Top panel: anti-Myc; bottom panel: anti-Flag.

(C) Flag immunoprecipitation was performed, as in (B), using Dsk2-Fl-Cdc4 as bait and Far1 tagged with HA. Top panel: anti-HA; bottom panel: anti-Flag.

(D) Two-step purification of Fl-Cdc4 and Rad23-Fl-Cdc4 expressed from the Cdc4 promoter. His6-tagged ubiquitin was expressed under the GAL1 promoter in strains expressing Cdc6-Myc or Far1-Myc. Input (In), Flag immunoprecipitation (first) followed by Ni-NTA purification (second), as illustrated in (A). Top panel: anti-HA; bottom panel: anti-Flag.

(E) As in (D), using Grr1 and its known substrate, Pfk27-Myc. Unlike Cdc4, Grr1 Traps are C-terminal fusions. Top panel: anti-Myc; bottom panel: anti-Flag.

Figure 2. LC-MS/MS Analysis of Two-Step Purifications of Ligase Traps Identifies Known SCF Substrates

Color-coded matrix showing known SCF substrates identified by Ligase Trapping. Two-step purifications were performed from cell extracts expressing UBA fusions of eight F box proteins. These represent data from initial purifications performed in parallel. In the case of Cdc4, both N- and C-terminal purifications are shown. Dsk2 and Rad23 fusions are shown when both fusions
were well expressed. Repeats of two identical pairs of Grr1 traps are shown for comparison. Colors represent spectral counts for each protein in each purification. For full list of substrates and references, see Table S1.

**Figure 3. Stability of Grr1 Candidate Substrates**

(A) Epitope-tagged candidate Grr1 substrates were expressed in *GRR1* or *grr1Δ* cells. To rescue slow growth, all *grr1Δ* cells are also *rgt1Δ*. Asynchronous cultures were treated with cycloheximide (CHX) for the indicated number of minutes, and anti-Myc western blots were performed on whole-cell extracts. Western blot of Cdc28 is shown as a loading control.

(B) A subset of candidate substrates was analyzed during the cell cycle by western blots of whole-cell extracts. Cells were released from α factor-mediated G1 arrest and collected at different time points. α factor was again added after 60 min to rearrest cells in the subsequent G1. Clb2 and Cdc28 are shown to monitor cell-cycle progression and as a loading control, respectively. Asynchronous cells are shown for comparison.

**Figure 4. Ubiquitination of Grr1 Candidate Substrates**

(A) Short and long exposures of anti-Myc western blots of a CHX chase assay of the uncharacterized ORF YKR045C in *GRR1* and *grr1Δ* cells. Steady-state levels of Ykr045c are shown in G1- and nocodazole-arrested cells.

(B) Six candidate Grr1 substrates were expressed in cells containing Ligase Traps of Grr1, as well as Mfb1 and/or Ufo1 as negative controls. Western blots of two-step purifications, as in Figure 1D, are shown.

(C) Western blots of whole-cell extract (I) and anti-Flag pull-downs (P) from strains expressing
Myc-tagged candidate Grr1 substrates and transformed with either empty vector (pRS426) or pYES2-Grr1ΔF-Fl (a galactose-inducible copy of Grr1-Flag lacking the F box domain).

**Figure 5. Ubiquitination of Cdc4 and Ufo1 Candidate Substrates**

(A) Myc-tagged Cdc4 candidate substrates were expressed in CDC4 cells or cdc4-1 temperature-sensitive mutants. Cultures were simultaneously shifted from 23°C to 37°C and treated with CHX for the indicated number of minutes. Western blots were probed with anti-Myc (substrates) or anti-Cdc28 (loading control). A cdc4-1 grr1Δ double mutant was examined for Pcl1.

(B) Western blots of two-step purifications performed on cell extracts expressing Rad23-Fl-Cdc4 and Myc-tagged Swi1, Atc1, Isr1, or Pcl1, as in Figure 1.

(C) The Ufo1 candidate substrate Rbg1 was Myc-tagged in UFO1 and ufo1Δ cells, treated with CHX for the indicated times, and examined by western blot.

(D) Western blot of two-step purifications performed on cell extracts expressing Ufo1-Fl-Rad23, Ufo1-Fl-Dsk2, or Grr1-Fl-Rad23, together with Myc-tagged Rbg1.

**Figure 6. Saf1 Targets Vacuolar Zymogens that Fail to Properly Mature**

(A) Full-length Prb1 (preproPrb1) is constituted by 635 amino acids. The N-terminal signal peptide (SP; 20 amino acids) is cleaved during translocation into the ER. In the ER, an intramolecular proteolytic cleavage removes P1 (260 amino acids) to yield proPrb1. In the vacuole, P2 (~30 amino acids) and P3 (~30 amino acids) are cleaved off. Mature Prb1 (mPrb1) is roughly 295 amino acids (~31 kDa). Positions of Prb1 peptides identified by LC-MS/MS analysis of the two-step purification of Saf1-Fl-Dsk2 are represented as solid gray bars below.

(B) Two-step purification from extracts expressing Saf1-Fl-Dsk2 and Myc-tagged Prb1, Prc1, or
Ykr139w. Grr1-Fl-Dsk2 was used as a negative control for binding specificity.

(C) Two-step purification was performed as in (B) in wild-type, \( cdc53-1 \), or \( cdc34-2 \) mutants. Cells were maintained at 23°C then shifted to 38°C for 45 min prior to collection.

(D) Strains containing PRB1 under the inducible GAL1 promoter were maintained in 2% raffinose and induced with 2% galactose for 15 min, collected, and resuspended in 2% glucose. Time points were collected, western blotted, and probed with anti-Prb1 antibody, which recognizes all forms of Prb1.

(E) Two-step purification in wild-type, \( sec65-1 \), \( vam3\Delta \), \( sec7-1 \), or \( sec23-1 \) mutants performed as in (B), except strains were grown at 23°C (permissive temp) and shifted to the 38°C (restrictive temp) for 45 min prior to collection.

(F) Prb1 constructs, under the control of the TEF1 promoter, were examined by two-step purification as in (B).

(G) Two-step purification was performed as in (B) with strains expressing either PRC1 or prc1-G255R, which encodes the Prc1* (a.k.a. CPY*) allele.
Figure 2

Number of peptides
- 0
- 1-5
- 6-10
- >10

Known Substrates

F-box-UBA fusions
- Grr1-FL-Dsk2
- Grr1-FL-Dsk2
- Grr1-FL-Rad23
- Grr1-FL-Rad23
- Cdc4-FL-Rad23
- Rad23-FL-Cdc4
- Ufo1-FL-Dsk2
- Ufo1-FL-Rad23
- Met30-FL-Dsk2
- Met30-FL-Rad23
- Mdm30-FL-Dsk2
- Saf1-FL-Dsk2
- Skp2-FL-Dsk2
- Mfb1-FL-Rad23
- Mfb1-FL-Dsk2
Figure 3
Figure 4

A

CHX

WT

grr1Δ

G1

M

WT

Δ

Δ

Ykr045c (light)

Ykr045c (dark)

Cdc28

IB: Myc

IB: Flag

B

Bud4-Myc

Sfg1-Myc

Tis11-Myc

Grr1-Flag

Mbf1-Flag

Ufo1-Flag

IB: Myc

IB: Flag

Yhr131c-Myc

Die2-Myc

She2-Myc

Grr1-Flag

Mbf1-Flag

Ufo1-Flag

C

Bud4-Myc

Fir1-Myc

Gac1-Myc

Npl4-Myc

Sbe2-Myc

Sfg1-Myc

Tis11-Myc

Yhr131c-Myc

Ykr045c-Myc

Ynl144c-Myc

IB: Myc (light)

IB: Myc (dark)
Chapter 3
Prb1 Protease Activity is Required for its Recognition by the F box Protein Saf1

This work was submitted to the Journal of Biological Chemistry as of February 2015. Co-authors Jeffrey Johnson, Billy Newton, and Nevan Krogan performed mass spectrometry and analysis.

ABSTRACT

The SCF ligase targets proteins for destruction by attaching ubiquitin molecules to its substrates. Substrates are typically recognized through a defined phosphodegron by the F box protein. Previously, we showed that the F box protein Saf1 ubiquitinates the zymogen form of several lysosomal/vacuolar proteases in yeast. Here, we characterize the interaction of Saf1 with Prb1, a yeast vacuolar protease. We show that while Saf1 binds the mature protein (mPrb1), it ubiquitinates only the precursor. A lysine is found to be ubiquitinated in a peptide eliminated from the mature protein. Interestingly, the recognition of Prb1 by Saf1 requires Prb1’s proteolytic activity. Mutations that eliminate Prb1 activity block Saf1 targeting of the zymogen precursor. Mutation in the cleavage site between P1 and mPrb1 also eliminates ubiquitination. Our data suggest that Saf1 recognizes zymogens when they are ready to undergo processing but recognition/binding requires the zymogen’s catalytic activity.

INTRODUCTION

The ubiquitin-proteasome system mediates the destruction of regulatory proteins and performs quality control on misfolded or aggregated polypeptides (Finley et al., 2012). A ubiquitin molecule is transferred from an E1 activating enzyme to an E2 conjugase and subsequently to an E3 ligase. The E3 ligase catalyzes covalent attachment of ubiquitin to a lysine
residue on the substrate. Incorporation of additional ubiquitin molecules leads to formation of polyubiquitin chains that result in substrate recognition and elimination by the 26S proteasome (Hershko and Ciechanover, 1998).

The Skp1-Cul1-F box (SCF) E3 ligases are multisubunit enzymes that share a common scaffold (Cdc53), an adaptor protein (Skp1), and different F box proteins that bind specific substrates (Bai et al., 1996; Patton et al., 1998). The C-terminus of the F box protein often contains either a leucine-rich repeat (LRR) or a WD40 repeat domain important for substrate interaction (Willems et al., 2004). In general, F box proteins recognize substrates that contain posttranslational modifications, typically phosphorylation (Feldman et al., 1997; Hsiung et al., 2001; Nash et al., 2001; Orlicky et al., 2003; Skowyra et al., 1997; Wu et al., 2003) though in some cases methylation (Lee et al., 2012) or glycosylation (Yoshida et al., 2002). Degradation motifs (degrons) are normally a single short stretch of amino acids (Nash et al., 2001) or a series of such consensus sites (Koivomagi et al., 2011; Tang et al., 2012). Degrons are usually N- or C-terminal extensions in the target protein and can direct proteasomal degradation when fused to ectopic substrates (Edenberg et al., 2014; Landry et al., 2012; Liu et al., 2005; Melvin et al., 2013; Nishimura et al., 2009; Yaglom et al., 1995).

Saf1 is an F box protein that is poorly characterized in budding yeast (Ho et al., 2002; Krogan et al., 2006; Seol et al., 2001). Saf1 is unique in that it possesses RCC1 repeats rather than LRR or WD40 repeats (Escusa et al., 2007). Until recently, only one substrate has been identified for Saf1: the adenine-deaminating enzyme, Aah1. Degradation of Aah1 occurs during cell entry into quiescence and requires a complete SCF complex (Escusa et al., 2006; Escusa et al., 2007). However, posttranslational modifications of Aah1 have not been observed (Escusa et al., 2007).
Recently, we developed a method called Ligase Trapping to identify novel substrates of SCF ligases (Mark et al., 2014). Using this approach, we identified a set of vacuolar serine proteases as Saf1 targets. These substrates included Protease B, Protease C, and an uncharacterized ORF, Ybr139w, which encodes a putative serine protease. Vacuolar serine proteases play an essential role during nutrient deprivation in yeast (Teichert et al., 1989; Wolf and Ehmann, 1979; Zubenko et al., 1979). Among these, the soluble Protease B (Prb1) and Protease C (Prc1) are synthesized as glycosylated zymogens that undergo proteolytic processing as they transit the secretory pathway (Hemmings et al., 1981; Mechler et al., 1987; Mechler et al., 1982; Stevens et al., 1982; Valls et al., 1987).

PRB1 encodes a 73 kDa protein precursor (preproPrb1) whose maturation involves at least four proteolytic cleavage steps (Mechler et al., 1988; Moehle et al., 1989; Schiffer et al., 1990) (Fig. 1). After removal of the signal peptide, 260 amino acids are removed from the N-terminus in an autocatalytic manner. The third cleavage, catalyzed by another vacuolar protease (Protease A), eliminates a small region of the C-terminus. Finally, a 6 kDa peptide at the C-terminus is removed by autocatalysis to yield the 31-kDa mature protease (mPrb1). The PRC1 gene encodes a 61 kDa zymogen that undergoes similar cleavage events to generate the mature Protease C (Blachly-Dyson and Stevens, 1987; Hasilik and Tanner, 1978; Hemmings et al., 1981; Mechler et al., 1987; Stevens et al., 1982).

In this study, we investigate the interaction of SCF\textsuperscript{Saf1} with Prb1 and the role of ubiquitination in zymogen processing. We find that Saf1 binds the mature, active fragment of Prb1 but ubiquitinates only the unprocessed form of the enzyme. A site of ubiquitination in the C-terminus of the zymogen is identified. In addition, Prb1 catalytic activity is required for Saf1
targeting. These data suggest that Saf1 may recognize unprocessed proteases by virtue of their catalytic domains.

**EXPERIMENTAL PROCEDURES**

_Yeast strains and media_ – Yeast strains were grown in YM-1 media with 2% dextrose at 30°C unless otherwise noted. Strains were made using standard techniques. Unless otherwise noted, all strains are in the W303 background.

_Flag pull-down assays_ – Flag pull-down assays were performed as previously described (Mark et al., 2014).

_Western blotting_ – Western blotting was performed as previously described (Mark et al., 2014).

_UbiScan analysis_ – Prb1 bands excised from a polyacrylamide gel were digested in-gel with trypsin (Hellman et al., 1995). Tryptic peptides were lyophilized and peptides containing a diglycine ubiquitin remnant were purified using a ubiquitin remnant antibody according to the manufacturer’s specifications (Cell Signaling). A portion of tryptic peptides was reserved for mass spectrometry analysis. Samples were analyzed on a Thermo Scientific LTQ Orbitrap Elite MS system equipped with an Easy nLC-1000 HPLC and autosampler system that is capable of maintaining back pressures of up to 10,000 psi for high resolution chromatographic separations. The HPLC interfaces with the MS system via a nanoelectrospray source. Samples were injected onto a C18 reverse phase capillary column (75 µm inner diameter x 25 cm length, packed with 1.9 µm C18 particles). Peptides were then separated by an organic gradient from 5% to 30% ACN in 0.1% formic acid over 112 minutes at a flow rate of 300 nl/min. The mass spectrometer collected data from one full scan in the Orbitrap at 120,000 resolution followed by 20 collision-
induced dissociation MS/MS scans in the dual linear ion trap for the 20 most intense peaks from the full scan. Dynamic exclusion was enabled for 30 seconds with a repeat count of 1. Charge state screening was employed to reject analysis of singly charged species or species for which a charge could not be assigned.

**MS data analysis** – Raw mass spectrometry data were analyzed using the MaxQuant software package (version 1.3.0.5) (Cox and Mann, 2008). Data were matched to the UniProt S. cerevisiae reference protein database (downloaded on 15/2/13, 6,627 protein sequence entries). MaxQuant was configured to generate and search against a reverse sequence database for false discovery rate calculations. Variable modifications were allowed for methionine oxidation, lysine diglycine remnant, and protein N-terminus acetylation. A fixed modification was indicated for cysteine carbamidomethylation. Full trypsin specificity was required. The first search was performed with a mass accuracy of +/- 20 parts per million and the main search was performed with a mass accuracy of +/- 6 parts per million. A maximum of 5 modifications were allowed per peptide. A maximum of 2 missed cleavages were allowed. The maximum charge allowed was 7+. Individual peptide mass tolerances were allowed. For MS/MS matching, a mass tolerance of 0.5 Da was allowed and the top 6 peaks per 100 Da were analyzed. MS/MS matching was allowed for higher charge states, water and ammonia loss events. Data were searched against a concatenated database containing all sequences in both forward and reverse directions with reverse hits indicating the false discovery rate of identifications. The data were filtered to obtain a peptide, protein, and site-level false discovery rate of 0.01. The minimum peptide length was 7 amino acids. Results were matched between runs with a time window of 2 minutes for technical duplicates.
Two-step purification – Two-step purifications were performed as previously described (Mark et al., 2014). Typically, cell cultures of 350 ml (small-scale) and 2–4 l (large-scale) were used for western blot analysis and LC-MS/MS analysis, respectively.

RESULTS

Saf1 interacts with the mature form of Prb1 – The Prb1 protease zymogen contains five domains: a signal sequence (SS), P1, P2, P3, and the mPrb1 peptide. Peptides are sequentially removed by proteolysis to liberate the active mature mPrb1 enzyme (Fig. 1) (Moehle et al., 1987; Moehle et al., 1989). Previously, we showed that the SCF$^{Saf1}$ ligase binds the polyubiquitinated form of preproPrb1 in both immunoprecipitation experiments and mass spectrometry analysis of Saf1-bound substrates (Mark et al., 2014).

To verify that Saf1 selectively ubiquitinates preproPrb1, we performed coimmunoprecipitation experiments using cells expressing epitope-tagged Prb1 and Saf1. Note that C-terminal tagged Prb1 shows a delay in autocatalysis thereby allowing precursor forms to accumulate (Mark et al., 2014). The F box, Grr1, was used as a control. Western blots of cell lysates were probed with an anti-Myc antibody that recognizes both preproPrb1 and proPrb1 and a polyclonal anti-Prb1 antibody that can detect all forms of Prb1 (Moehle et al., 1989). Saf1 bound to the proPrb1 precursor (~75 kDa) at higher levels when compared to the Grr1 control (Fig. 2a, left). Surprisingly, Saf1 also bound significant amounts of mPrb1 (~31 kDa) (Fig. 2a, right).

To demonstrate that Saf1 can bind mPrb1, immunoprecipitation experiments were performed using a set of truncation mutants lacking various precursor fragments (mutants ∆P1, ∆P2-3, and ∆P1ΔP2-3). Our results showed that none of the Prb1 mutants, including the
truncated peptide containing only the sequences found in mature mPrb1 (ΔP1ΔP2-3), were able to bind Saf1 (Fig. 2b, data not shown). The inability of these forms to bind Saf1 is surprising because each of these truncated peptides contain mPrb1, which Saf1 appears to recognize. Of note is that these mutant forms of Prb1 are nonfunctional as they were unable to complete zymogen processing (Mark et al., 2014). We believe that correct processing of preproPrb1 leads to proper folding of mPrb1 and that this is critical for Saf1 binding. Furthermore, that the polyclonal antibody failed to detect mPrb1 in the “input” lysate (Fig. 1b) lends support to this notion.

It is possible that Saf1 recognizes preproPrb1 but that rapid processing during or after immunoprecipitation resulted in only mPrb1 remaining. To test this hypothesis, we performed a pulse-chase experiment by expressing PRB1 under the inducible GAL1 promoter. Prb1 expression was induced with galactose for 15 minutes and immunoprecipitations were then performed at various time points after induction. Western blot results showed an initial accumulation of a peptide corresponding to proPrb1 (40-42 kDa), followed by a conversion of this species into one that contains just P3 (37 kDa), with the eventual appearance of mPrb1 (Fig. 2c). Since detection of mPrb1 occurred only after 30 minute of chase, this mature form of mPrb1 cannot result from autoproteolysis of a precursor form during purification. After 10 minutes of chase, several higher molecular weight species can be seen in the Western blot, suggesting that Prb1 intermediates were undergoing ubiquitination (see Fig. 2c, IP lane). These data indicate processing is not occurring during or after immunoprecipitation and Saf1 binds preferentially to mPrb1 when compared to its precursors (Fig. 2c).

We next investigated whether Saf1 binds mPrb1 in a cellular context or whether the interaction occurs upon cell lysis. To this end, we mixed equal volumes of cell lysates from a
Saf1-3xFlag; prb1Δ strain and a wild type PRB1 strain. As a control, we mixed cell lysates from a Saf1-3xFlag; PRB1 strain with a prb1Δ strain. In this case, the extract mixture contains the same amount of Flag-tagged Saf1 and mPrb1 originating from the same strain. Results from these experiments revealed that Saf1 binds mPrb1 in both cases, suggesting that the Saf1 Prb1 interaction can be reconstituted in extract (Fig. 2d). Although Saf1 can bind mPrb1 in vitro, this does not preclude the possibility that mPrb1 and Saf1 also associate in vivo.

**Prb1 catalytic domain is required for Saf1-dependent ubiquitination** – One explanation that Saf1 cannot bind truncated forms of Prb1 is that autocatalysis is necessary to generate the correct conformation of the mature protease (Mark et al., 2014). To examine this further, we mutated each catalytic residue (Asp325, His357, Ser519) of Prb1 independently and determined if the mutants can be polyubiquitinated by the Ligase Trap (27). Results in Figure 3A show that Saf1 failed to target the ubiquitinated forms of the D325N and the S519A mutants. Surprisingly, we found that the H357A mutant is capable of producing the proPrb1 form (Fig. 3b), suggesting it has residual catalytic activity. Unlike the S519A and D325N mutants, ubiquitinated prb1-H357A purifies with Saf1. These data suggest that the ability to undergo catalytic processing is required for Prb1 recognition and ubiquitination by Saf1.

The fact that Prb1 requires catalytic activity, yet does not have to complete the first catalytic step, suggests that proper ubiquitination might require that P1 is inside the substrate binding pocket in a cleavable form. To test this, we constructed a Prb1 mutant that blocks P1 cleavage by changing both the T280 and E281 residues flanking the cleavage site to prolines. The T280P/E281P mutant failed to bind Saf1 and was not ubiquitinated by the Ligase Trap (Fig. 3a, data not shown). To determine whether this requirement for catalytic activity extends to other serine proteases, we examined the related protease, Prc1. Consistently, Prc1 exhibits reduced
binding to the Saf1 Ligase Trap as a ubiquitinated species when carrying the analogous mutation in its catalytic serine (Fig. 3c).

To determine whether ubiquitination of catalytically inactive Prb1 can be rescued by the presence of wild type Prb1 activity, we generated diploid Saf1 Ligase Trap strains containing wild type PRB1 and a Myc-tagged prb1-S519A mutant. Results in Figure 3D show that Prb1 activity in the same cell did not allow ubiquitination of the catalytically inactive Prb1 mutant zymogen, showing that mutant preproPrb1 cannot be rescued by providing proteolytic activity in trans.

**Prb1 zymogen is ubiquitinated in its C-terminal P2/P3 region** – To determine where ubiquitination occurs on Prb1, we affinity purified Prb1 from yeast cells using a Saf1 Ligase Trap. Following immunoprecipitation and affinity purification of cell lysates using both the Flag and the His tags, ubiquitinated region(s) of Prb1 were mapped by the UbiScan procedure. UbiScan uses an antibody against branched di-glycine (-KGG) motifs to enrich for ubiquitinated peptides in trypsinized extracts prior to LC-MS/MS analysis. By this approach, a single ubiquitinated site was found at K325 of the P2/P3 region (Fig. 4a). We then mutated this lysine residue to arginine (K325R) and performed a Saf1 Ligase Trap experiment. That this mutation did not abrogate Prb1 ubiquitination (data not shown) suggests that other lysines may be ubiquitinated when the K325 lysine is eliminated. Therefore, other residues in preproPrb1 can also serve as targets for Saf1 ubiquitination.

In our initial Ligase Trap studies, we observed ubiquitinated full-length preproPrb1 due to its high abundance in cells carrying the Myc-tagged allele, which undergoes much slower processing. Subsequent analysis using an HA-tagged allele of Prb1 revealed a ladder of
ubiquitinated forms of proPrb1 (Fig. 4b, right). These results would indicate that a significant amount of the ubiquitination occurs on the P2/P3 portion of this precursor molecule.

**DISCUSSION**

Protein quality control plays an essential role in maintaining cellular homeostasis. The ER-associated degradation (ERAD) pathway detects and eliminates defective proteins (Buchberger et al., 2010; Claessen et al., 2012; Schubert et al., 2000) destined for the endoplasmic reticulum. In yeast, the ubiquitin ligase Doa10 and deubiquitinase Ubp1 degrade proteins that fail to insert into the ER and thus are mislocalized to the cytoplasm (Ast et al., 2014; Ast et al., 2013; Chen et al., 1993; Hessa et al., 2011; Levine et al., 2005; Rane et al., 2004; Shao and Hegde, 2011). In the ER lumen or membrane, misfolded or unmodified polypeptides can accumulate and aggregate. In such cases, the yeast transmembrane ubiquitin ligases, Hrd1 and Doa10, retrotranslocate them to the cytosol for proteasomal degradation (Thibault and Ng, 2012). In eukaryotes, five F box proteins (Fbs1, Fbs2, Fbg3, Fbg4, and Fbg5) are thought to recognize N-glycosylated substrates, although the SCF has not been linked to ER quality control (Yoshida, 2007).

The experiments described in this report define a unique mechanism where the SCF$^{Saf1}$ ligase targets a zymogen substrate prior to its processing in the ER. We propose a model in which SCF$^{Saf1}$ targets preproPrb1 in its native conformation poised to perform the first endopeptidase reaction of P1 removal (Fig. 4c, left). Our experiments also show that Saf1 preferentially binds the mPrb1 fragment, which contains the catalytic domain. Mutations that abrogate catalytic activity or disrupt the P1 cleavage site inhibit the recognition and polyubiquitination of preproPrb1 (Fig. 4c, right).
Saf1 may be the first example of an F box protein that targets a specific class of enzymes, namely the vacuolar proteases. Since specific structural domains can be directly recognized by F box proteins, we propose that Saf1 recognizes a conserved protease structure that is shared among Prb1, Prc1 and Ybr139w. While we know of no other F box proteins recognizing a class of molecules by their catalytic site, FBXO4 binds its target via a GTPase-like fold in the absence of posttranslational modification (Li and Hao, 2010; Zeng et al., 2010). Similarly, FBXL3 recognizes CRY1 and CRY2 (cryptochrome 1 and 2) via a conserved pocket on their surfaces (Busino et al., 2007; Xing et al., 2013). Unfortunately, the functional significance of Saf1 binding to such serine proteases remains unknown. We did not observe a phenotype of saf1Δ yeast strains in the presence of protein folding modifiers such as tunicamycin, dithiothreitol, geldenamycin, and radicicol. Mutant saf1Δ strains also exhibited no genetic interaction with mutants in the ERAD pathway (data not shown).

Given that the mature Prb1 protease is present only in the vacuolar lumen, it was an unexpected finding that Saf1 could bind mPrb1. We hypothesize that Saf1 recognizes the active protease domain of Prb1 in the context of the entire zymogen. Consistent with this hypothesis, we identified ubiquitination sites in P2/P3 of the Prb1 precursor (Fig. 4a and 4b). The fact that Saf1 can bind mPrb1 in cell extracts in vitro (Fig. 2d) is consistent with a model wherein mPrb1 is not the actual substrate targeted by Saf1 because it is missing the ubiquitination site and is in the wrong location, but still contains the recognition motif.

Where does SCF_{Saf1} target Prb1 in the cell? To date, there are no known E2 enzymes or SCF components that localize within the lumen of the ER or vacuole. Since most ubiquitin machinery is located in the nucleus and the cytosol, we believe ubiquitination of Prb1 by Saf1 likely occurs in the cytosol. Presumably, SCF_{Saf1} binds Prb1 zymogen after it has
retrotranslocated out of the ER. Immunofluorescence experiments were done to visualize Saf1 in
the yeast cell. Unfortunately, the assays were not sufficiently sensitive to detect Saf1 (data not
shown). The transmembrane ERAD ligase Hrd1 is not required for Prc1 ubiquitination by Saf1
(data not shown). The ER Membrane protein Complex (EMC) may play a role in removal of
unfolded proteins from the ER (Jonikas et al., 2009). However, our Ligase Trapping experiments
in emc1Δ and emc6Δ mutants showed that the EMC is not required for Prb1 ubiquitination (data
not shown). It is possible that Sec61, the anterograde translocation pore, could be used as a
channel for retrograde extraction of ubiquitinated substrates from the ER to the proteasome
(Gillece et al., 2000; Ng et al., 2007; Wiertz et al., 1996). Future experiments will focus on
determining whether Sec61 helps transport the Prb1 zymogen into the cytosol.

In conclusion, our studies on recognition of serine protease precursors by Saf1 suggests
that this F box protein binds its substrates either near the catalytic site, or at a second site whose
conformation is strongly affected by the catalytic site. Mutational analysis of this unusual
recognition is hindered by the fact that so many mutations block Prb1 activity. Thus, a thorough
understanding of this binding event will need to await structural investigation.

FIGURE LEGENDS

FIGURE 1. Prb1 maturation pathway.
The Prb1 zymogen undergoes a series of cleavage reactions to liberate the mature enzyme. The
signal sequence (SS) is first removed upon translocation into the ER to generate “preproPrb1”. A
second cleavage removes P1 yielding “proPrb1” in the ER while two C-terminal cleavages
remove P2 and P3 to generate mature “mPrb1” in the vacuole. (PrA = Protease A; scissors =
autocatalysis)
FIGURE 2. **Saf1 recognizes the protease domain of Protease B (mPrb1).**

*A*, Western blots of whole-cell extracts (In) and anti-Flag pull-downs (IP) from strains expressing Myc-tagged Prb1 and either Grr1-3xFlag or Saf1-3xFlag. Proteins were visualized by probing with anti-Myc, anti-Prb1 or anti-Flag antibodies. Asterisk indicates nonspecific bands. *B*, Western blots of whole-cell extracts (In) and anti-Flag pull-downs (IP) from strains expressing a Myc-tagged Prb1 truncation mutant in either Grr1-3xFlag or Saf1-3xFlag cells. This mutant retains a signal sequence for ER translocation. Proteins were visualized as in (A). *C*, Strains expressing PRB1 under the GAL1 promoter were maintained in 2% raffinose and induced with 2% galactose for 15 min, collected at various time points and cell lysates prepared for anti-Flag pull-downs. Proteins were visualized as in (A). Two exposures of the anti-Prb1 blot are shown. *D*, Whole cell extracts were collected from the following strains: wild type, prb1Δ, Grr1-3xFlag prb1Δ, Saf1-3xFlag prb1Δ or Saf1-3xFlag alone. Different combinations of cell lysates were mixed in equal volume prior to anti-Flag pull-down as indicated. Western blots of whole-cell extracts (In) and anti-Flag pull-downs (IP) were visualized as in (A).

FIGURE 3. **Protease activity is required for ubiquitination by Saf1.**

*A*, Western blot analysis of yeast strains expressing Prb1 mutants with compromised autocatalytic activities. A two-step purification procedure was used to isolate ubiquitinated products from whole-cell extracts. Lanes are Input (In), Flag immunoprecipitation (1st) and Ni-NTA purification (2nd) for ubiquitinated products. Proteins were visualized as in Fig. 2. *B*, Western blot analysis of whole-cell extracts from strains expressing Prb1 mutants with or without a Myc tag. Proteins were visualized by probing with anti-Prb1 or anti-Cdc28 (loading
control) antibodies. C, Comparison of wild type and inactive (S257A) Prc1 protease. A two-step purification procedure was used as in (A). Proteins were visualized as in Fig. 2. Two independently derived isolates of prc1-S257A are shown. D, Prb1 ubiquitination in haploid yeast strains expressing wild type (lanes 1-3) or mutant (lanes 10-12) Myc-tagged Prb1 versus diploid strains expressing wild type (lanes 4-9) or mutant Myc-tagged Prb1 (lanes 13-18) with a second copy of untagged Prb1, Saf1 Ligase Trap construct and 6xHis-tagged ubiquitin. A two-step purification procedure was used as in (A). Proteins were visualized as in Fig. 2. Two isolates of each diploid are shown.

FIGURE 4. Saf1 ubiquitinitates the C-terminus of the Prb1 zymogen.
A, Annotated spectrum for the ubiquitination site identified in the Prb1 precursor. B, Western blot analysis of cell extracts isolated by the two-step purification approach as in Fig. 3A, from a yeast strain expressing HA-tagged Prb1. Proteins were visualized by probing with anti-HA and anti-Flag antibodies. Light (left) and dark (right) exposures are shown. Laddering can be seen coming from both preproPrb1 and proPrb1. C, A model of Prb1 ubiquitination requiring an intact Prb1 protein. (Left) Under normal conditions, the preproPrb1 zymogen is folded in a conformation poised to remove the P1 segment via autocatalytic cleavage. SCF\textsuperscript{Saf1} ligase recognizes this form of the protease via the mPrb1 domain and ubiquitinates its C-terminus. (Right) Mutations that eliminate autocatalytic activity (asterisk) or disrupt the conformation of preproPrb1 (i.e. the P1 cleavage site mutant) prevent recognition and ubiquitination by SCF\textsuperscript{Saf1} ligase.
Figure 1
Figure 2

(A) Prb1-13xMyc

B) TEFPr-Prb1-13xMyc

WT ΔPr1ΔP2-3

C) post-GAL (min): t = 0'  t = 15'  t = 30'  t = 60'

D) Prb1Δ + WT

IP: Flag
IB: Prb1 (Ik)

IP: Flag
IB: Prb1 (dk)
Figure 3

A

B

C

D

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References


## Appendix

### Table 1: Troubleshooting

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<td>Sample aggregates in large clumps</td>
<td>Sample dropped too quickly in liquid nitrogen</td>
<td>Maintain maximum liquid nitrogen volume and introduce sample drops slower</td>
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<td>5</td>
<td>Sample stuck to the inside of the chamber after grinding</td>
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<td>Scrape off as much sample as possible with a cold spatula. It is not necessary to remove all of the sample</td>
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<td>After second centrifugation, the sample is cloudy</td>
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<td>Sample cloudiness does not necessarily compromise sample quality. Proceed with the protocol and monitor sample quality/quantity</td>
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<tr>
<td>15, 17</td>
<td>Bead volume decreases during washing</td>
<td>Beads may be accidentally aspirated</td>
<td>Avoid bead bed when aspirating. Keep needle bore against side of tube. Tilt tube if it helps avoid removing beads</td>
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<td>Protein did not elute from Ni-NTA beads</td>
<td>Inaccurate or wrong pH of elution buffer</td>
<td>Ensure that the pH of the elution buffer is correct</td>
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<td>No visible bands on western blot or silver stain</td>
<td>Insufficient material</td>
<td>Samples may need to be loaded at higher quantities depending on the level of ligase expression. Consider overexpressing the ligase of interest, as this is often the limiting factor for purifying enough material</td>
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Table 2. Substrates Identified for Grr1

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A list of all proteins that were ≥25-fold enriched and had an average of ≥1.8 spectral counts are shown. Also included are three verified candidates that had an average of <1.8 spectral counts. Under t₁/₂, “+,” “+/-”, and “-” indicate a significant, slight, or no change in grr1Δ cells, respectively. The symbol “*” indicates an increase in steady-state levels, but not in half-life. Under “Ubiquit,”
Supplementary Figure S1

Figure S1, related to Figure 2. Purification of polyubiquitinated species by different F-box Ligase Traps.

Western blot of two-step purifications was performed as previously described and probed with an anti-ubiquitin antibody (P4D1).
Figure S2, related to Figure 6. Peptides from Saf1 Ligase Trap purification.

All Prb1 and Prc1 peptides identified from two separate purifications with the Saf1 Ligase Trap. Peptides are indicated with red lettering. Signal sequence is highlighted in fuchsia, whereas proteolytically removed fragments are marked in teal and gray using the best available mapping data.
Figure S3, related to Figure 6. Saf1 targets the vacuolar Prb1 precursor.

(A) Whole cell extracts of strains containing prb1Δ, wild type, sec65-1, Myc-tagged Prb1, or Myc-tagged Prb1 in the sec65-1 background were examined by probing a Western blot with anti-Prb1 antibody, which recognizes all forms of Prb1. (B) Western blot of two-step purification performed as previously described. Logarithmic cultures were grown at 30°C and shifted to either 38°C or 42°C for 45 minutes before cells were collected. (C) Two-step purification was performed as previously described with wild type and vps10Δ strains. (D) Wild type, prb1Δ, or cells expressing PRB1 alleles under the control of the TEF1 promoter were examined by probing Western blots of whole cell extracts with anti-Prb1 antibody. Where indicated, cells were treated with 1 µg/ml tunicamycin for 45 minutes. Rad53 (probed with an anti-Rad53 antibody) was used as a loading control. (E) Western blot of two-step purification performed as previously described. Logarithmic cultures were treated as indicated for 45 minutes prior to collection. (F) Two-step purification was performed as previously described with strains expressing either PRB1 or a mutant prb1(N314Q) allele under control of the TEF1 promoter. Light and dark exposures of the anti-Myc blot are shown for comparison.
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Table S1, related to Figure 2. Candidate substrates for 8 F-box proteins.

All Candidate substrates shown that were enriched 25-fold in the Ligase Trap listed. Candidates blocked out in grey meet the spectral count threshold of 1.8 (six for Ufo1). Average spectral counts shown for all (between two and four) purifications. Known substrates in red: Far1 (Henchoz et al., 2007), Ste5 (Garrenton et al., 2009), Swi5 (Kishi et al., 2008), Hst4 (Tang et al., 2005), Hst3 (E. Edenberg, unpublished data), Cln3 (Landry et al., 2012), Plm2 (Tang et al., 2005), Mth1 (Flick et al., 2003; Spielewoy et al., 2004), Cln2 (Barral et al., 1995; Kishi and Yamao, 1998; Li et al., 1997), Gic2 (Jaquenoud et al., 1998; Spielewoy et al., 2004), Cln1 (Barral et al., 1995; Kishi and Yamao, 1998; Li et al., 1997; Skowyra et al., 1999), Pfk27 (Benanti et al., 2007), Tye7 (Benanti et al., 2007), Fzo1 (Fritz et al., 2003; Neutzner and Youle, 2005), Met4 (Kaiser et al., 2000; Rouillon et al., 2000); Aah1 (Escusa et al., 2006), and HO (Kaplun et al., 2006).
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Supplementary Table S2
Supplementary Table S2, related to Figures 1-6. List of strains used in this study.
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