Endoplasmic Reticulum Exit of Golgi-resident Defective for SREBP Cleavage (Dsc) E3 Ligase Complex Requires Its Activity*

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Sterol regulatory element-binding proteins (SREBPs) are membrane-bound, basic helix-loop-helix leucine zipper transcription factors that regulate lipid homeostasis in mammalian cells (1). Inactive SREBP is retained in the ER. Upon stimulation, SREBP is transported to the Golgi where its cytosolic N-terminal transcription factor domain is released by proteolysis, allowing it to enter the nucleus and activate transcription. Fission yeast Schizosaccharomyces pombe has two SREBP homologs, Sre1 and Sre2, and the SREBP pathway is conserved in S. pombe with two key differences. First, Sre1 is an oxygen-responsive transcription factor required for hypoxic adaptation, and low oxygen stimulates Sre1 activation (2, 3). Second, fission yeast SREBP proteolytic activation requires the Golgi-localized, multi-subunit Dsc E3 ligase (4, 5).

The Dsc E3 ligase is a Golgi complex consisting of five integral membrane proteins, Dsc1–Dsc5 (see Fig. 1A). Dsc1 is a candidate RING E3 ligase homologous to Saccharomyces cerevisiae Tul1, which functions in Golgi quality control and uses Ubc4 as its cognate E2 ubiquitin-conjugating enzyme (5, 6). Dsc2 is a homolog of mammalian UBAC2, containing an N-terminal rhomboid pseudoprotease domain and a C-terminal ubiquitin-associated (UBA) domain (7, 8). Dsc5 is a UBX domain-containing protein with homology to mammalian UBXD8 (4), whereas Dsc3 and Dsc4 are less well characterized. Importantly, deletion of any dsc gene, mutation of the Dsc1 RING domain, or conditional inactivation of ubc4 prevents proteolytic activation of SREBP, establishing a genetic requirement for the Dsc E3 ligase in this pathway (4, 5, 9).

The Dsc E3 ligase complex shares an overall architecture with mammalian gp78, an ER-localized, E3 ligase that functions in ER-associated degradation (8, 10). How these related enzymes achieve their different subcellular localizations is unknown. Here, we demonstrate that Dsc1 is a functional E3 ligase and that Dsc1 E3 ligase activity is required for ER exit of the ligase complex, thus establishing enzyme activity-dependent sorting as a new mechanism for protein trafficking.

**Background:** Proteolytic activation of fungal SREBP requires the five-subunit Golgi Dsc E3 ligase.

**Results:** Dsc1 is an active E3 ligase; loss of Dsc E3 ligase activity leads to ER localization of the Dsc complex.

**Conclusion:** ER exit of the Dsc E3 ligase requires E3 ligase activity.

**Significance:** This is the first example of enzyme activity-dependent protein sorting in the secretory pathway.

Experimental Procedures

**Materials**—We obtained all fine chemicals from Fisher.

Edinburgh minimal medium and amino acids were from Q-BioGen; oligonucleotides were from Integrated DNA Technologies; GelCode Blue, West Pico ECL, and Dura ECL kits were from Pierce; and HRP-conjugated, goat anti-rabbit, and goat anti-mouse antibodies were from Jackson ImmunoResearch Laboratories. Protease inhibitors were from Sigma (1000× stock; leupeptin (10 mg/ml), aprotinin (28 trypsin inhibitor units/ml), pepstatin A (5 mg/ml), and PMSF (0.5 mM)).

Protein sequence homology analysis was performed using ExPASy LALIGN.

**Media and Growth Conditions**—S. pombe cells were grown in YES (5 g/liter yeast extract, 30 g/liter glucose) or Edinburgh minimal medium, both supplemented with 225 μg/ml each of uracil, adenine, leucine, histidine, and lysine at 30 °C. Temperature-sensitive strains were grown at 25 °C and transferred to non-permissive 36 °C for the indicated times. For anaerobic...
growth, growth, and growth. The ade6-M21 allele is either ade6-M210 or ade6-M216. h？ indicates that the mating type is either h- or h+.

| S. pombe strains | Genotype | Reference or source | Figure |
|------------------|----------|---------------------|--------|
| KGY245           | h？ leu1-32 ura4-D18 ade6-M210 his3-D1 | ATCC    | 2, 3, 4, 7 |
| PEY522           | h？ leu1-32 ura4-D18 ade6-M210 his3-D1Δser1-D1::kanMX6 | Hughes et al. (5) | 2 |
| PEY1474          | h？ leu1-32 ura4-D18 ade6-M21?his3-D1 dsc1-1 | Stewart et al. (5) | 2, 3 |
| PEY1448          | h？ leu1-32 ura4-D18 ade6-M210 his3-D1Δdsc1-D1::kanMX6 | Stewart et al. (5) | 2, 3, 4, 7 |
| PEY1747          | h？ leu1-32 ura4-D18 ade6-M210 his3-D1Δdsc1-his3 Δdsc1-D1::kanMX6 | This study | 2, 3, 4 |
| PEY1743          | h？ leu1-32 ura4-D18 ade6-M210 his3-D1Δdsc1-L675D-his3 Δdsc1-D1::kanMX6 | This study | 2, 3, 4, 7 |
| PEY1742          | h？ leu1-32 ura4-D18 ade6-M210 his3-D1Δdsc1-D1::kanMX6 | This study | 2, 3, 4 |
| PEY1547          | h？ leu1-32 ura4-D18 ade6-M210 his3-D1Δdsc2-D1::kanMX6 | Seino et al. (23) | 3, 4 |
| PEY1567          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1 ubc4-P61S::ura4+ | Matynia et al. (27) | 3 |
| PEY1078          | h？ leu1-32 ura4-D18 ade6-M216 | Matynia et al. (27) | 3 |
| PEY1079          | h？ leu1-32 ura4-D18 ade6-M210 sar1-1 | Matynia et al. (27) | 3 |
| PEY1735          | h？ leu1-32 ura4-D18 ade6-M216 h？ his3-D1::dsc2-6 × mGFP-his3 Δdsc2-D1::kanMX6 ost1-mCherry::ura4+ | This study | 5 |
| PEY1736          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3 Δdsc1-D1::kanMX6 Δdsc2-D1::kanMX6 ost1-mCherry::ura4+ | This study | 5 |
| PEY1737          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3 Δdsc3-D1::kanMX6 Δdsc2-D1::kanMX6 ost1-mCherry::ura4+ | This study | 5 |
| PEY1738          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3 Δdsc3-D1::kanMX6 Δdsc2-D1::kanMX6 ost1-mCherry::ura4+ | This study | 5 |
| PEY1739          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3 Δdsc4-D1::kanMX6 Δdsc2-D1::kanMX6 ost1-mCherry::ura4+ | This study | 5 |
| PEY1740          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3+ Δdsc5-D1::kanMX6 Δdsc2-D1::kanMX6 ost1-mCherry::ura4+ | This study | 5 |
| PEY1741          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3+ Δdsc2-D1::kanMX6 ubc4-P61S::ura4+ | This study | 6 |
| PEY1746          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3+ Δdsc2-D1::kanMX6 | This study | 6 |
| PEY854           | h？ leu1-32 ura4-D18 ade6-M216 anp1-mCherry::ura4+ | Vjestica et al. (39) | 7 |
| PEY1744          | h？ leu1-32 ura4-D18 ade6-M216 anp1-mCherry::ura4+ | This study | 7 |
| PEY1745          | h？ leu1-32 ura4-D18 ade6-M216 anp1-mCherry::ura4+ | This study | 7 |
| PEY1761          | h？ leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6 × mGFP-his3+ Δdsc1-D1::mGFP Δdsc2-D1::kanMX6 anp1-mCherry::ura4+ | This study | 7 |

Yeast manipulations and transformations were done using standard protocols. dsc1-integrating plasmid (pES215) (5) was subjected to site-directed mutagenesis to generate pES216 (dsc1-C634A), pES217 (dsc1-H668A), pSR14 (dsc1-I636D), and pSR15 (dsc1-L675D). These plasmids were linearized and transformed to strains for integration at the his3 locus. Transformants were selected for growth on Edinburgh minimal medium lacking histidine. Correct plasmid integration was confirmed by DNA sequencing of PCR-amplified genomic DNA, and protein expression was checked by Western blotting. ESY462 (h？leu1-32 ura4-D18 ade6-M216 his3-D1::dsc2-6×GFP-his3+ Δdsc2-D1::kanMX6) was generated using dsc2-6×GFP plasmid (5). Strains expressing Dsc2-6×GFP and mCherry-tagged Ost1 were generated by standard mating and random spore analysis using ESY462 as a parent strain. Wild-type Dsc1 RING domain (aa 608–695) and the corresponding mutants (C634A, H668A, I636D, and L675D) were PCR-amplified from dsc1 integrating plasmids and cloned into XbaI and HindIII sites of pGEX-KG as N-terminal GST fusion proteins. Recombinant wild-type and mutant Dsc1 RING domains were expressed in Escherichia coli BL21 Codon Plus® (DE3)-RIPL (Stratagene), induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h and purified using GST-agarose beads (Sigma Aldrich). Briefly, E. coli cell pellets expressing the recombinant proteins were lysed by sonication in phosphate-buffered saline (pH 7.4) containing 1× protease inhibitors, 1% Triton X-100, and 0.1% Nonidet P-40. The lysate was cleared by centrifugation at 25,000 × g for 20 min and allowed to bind to GST-agarose beads for 1 h at 4°C. Bound GST fusion proteins were eluted with 10 mM reduced glutathione. The purity of the protein was checked by SDS-PAGE and staining with GelCode Blue. S. pombe strains are listed in Table 1.
alcohol. The plasmid containing full-length ubc4 was transformed into *E. coli* BL21 Codon Plus® (DE3)-RIPL (Stratagene) and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. In both cases, *E. coli* cell pellets were lysed by sonication in buffer A (50 mM NaHPO₄ pH 8.0, 300 mM NaCl), and cell debris was removed by centrifugation at 20,000 × g for 20 min. The supernatant was allowed by bind to nickel-nitrotriacetic acid-agarose beads (Qiagen) and washed with buffer A containing 20 mM imidazole. Bound protein was eluted with buffer A containing 250 mM imidazole. Protein purity was checked by SDS-PAGE and GelCode Blue.

**Antibodies**—Polyclonal antibodies against Sre1, Dsc1, Dsc2, Dsc3, Dsc4, and Dsc5 used in this study were described previously (3, 5). Dsc1 antiserum was affinity-purified by passing Dsc3, Dsc4, and Dsc5 used in this study were described previously (3, 5). Dsc1 antiserum was affinity-purified by passing through a column containing Dsc1 N terminus (aa 1–300) coupled to agarose beads (AminoLink Plus immobilization kit, Thermo Scientific) following HRP-conjugated Dsc antibodies were prepared using the EZ-link Plus activated peroxidase kit (Thermo Scientific) following the manufacturer's instructions. Anti-GST antibody was from Covance, and anti-ubiquitin (P4D1) and anti-FLAG (M2) were from Sigma.

**In Vitro Ubiquitination Assay**—In vitro ubiquitination assays were performed as described (12). Briefly, purified *S. pombe* E1 (Uba1), E2 (Ubc4), wild-type Dsc1 RING domain (aa 608–695), and Dsc1 mutants (C634A, H668A, L636D, and L675D) were used in this assay. Purified RING domain (500 nm) was mixed with 125 nm E1, 400 nm E2, and 5 μm ubiquitin (*S. cerevisiae*, Boston Biochem) in the presence of 2 mM ATP in ubiquitination buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 2 mM NaF, and 0.6 mM DTT). The reaction was incubated at 37 °C for 1 h and stopped by adding equal amount of 2 × SDS-DTT buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, 0.2% bromphenol blue). After boiling for 5 min, proteins were separated using 12% SDS-PAGE, ubiquitination was detected using anti-GST antibody (P4D1), and expression of the proteins was determined using anti-FLAG antibody.

Sre1 and Sre2 Cleavage Assays—Sre1 and Sre2 cleavage assays were performed as described previously (3, 9). Briefly, for Sre1 cleavage, cells were grown to exponential phase under normoxic conditions and then shifted to anaerobic chamber (Invivo² 400 work station) for the indicated times. Cells were collected and washed, and lysates were prepared under denaturing conditions as described (3). Processing of Sre1 was detected by Western blot analysis using anti-Sre1 antibody. Anti-Dsc5 antibody was used as a loading control. For quantification purposes, blots were developed using IRDye secondary antibodies and an Odyssey CLx imager from LI-COR. Signals for Sre1 were normalized to Dsc5, and data were quantified from three independent experiments using Image Studio software from LI-COR.

For Sre2 cleavage assays, strains expressing 3×FLAG-Sre2 (aa 423–793) from the cauliflower mosaic virus promoter (9) were grown at 30 °C to exponential phase, and whole cell lysates were assayed by immunoblotting using anti-FLAG antibody. Immunoblots were developed using chemiluminescence except where noted in the figure legends.

**Deglycosylation Assay**—Cells (5 × 10⁶) were grown to exponential phase and collected by centrifugation at 500 × g for 5 min. Cell pellets were washed once and resuspended in B88 buffer (20 mM HEPES, pH 7.2, 150 mM KOAc, 5 mM Mg(OAc)₂, 250 mM sorbitol) and supplemented with 1× protease inhibitors and with an additional 1× Complete, EDTA-free protease inhibitor (Roche Diagnostics). Cell lysis was performed by vortexing with glass beads (Sigma, 400 – 600 μm) for 10 min, and debris was removed by centrifugation at 500 × g. The resulting supernatant was subjected to 20,000 × g centrifugation for 20 min. The membrane pellet was resuspended in B88 buffer containing 1% Nonidet P-40 and incubated at 4 °C for 1 h followed by centrifugation at 20,000 × g for 20 min. Supernatant was collected and used as Nonidet P-40-solubilized membrane. Solubilized membrane proteins (30 μg) were denatured by adding SDS and 2-mercaptoethanol to final concentrations of 0.5 and 1%, respectively, in 15 μl and heating at 37 °C for 30 min. Peptide-N-glycosidase F (500 units) (New England Biolabs) was added to the denatured membrane protein and incubated at 37 °C for 1 h. The reaction was stopped by the addition of 5× SDS-PAGE loading dye (150 mM Tris-HCl, pH 6.8, 15% SDS, 25% glycerol, 0.02% bromphenol blue, and 12.5% 2-mercaptoethanol) and heating at 37 °C for an additional 30 min. Dsc1 mobility was assayed by immunoblotting using affinity-purified Dsc1 antiserum.

**Cycloheximide Treatment**—Temperature-sensitive *sar1-1* or *ubc4-P61S* strains were grown overnight at permissive 25 °C to exponential phase. Cycloheximide (100 μg/ml) was added to the cells and shifted to non-permissive 36 °C for the indicated times before harvesting. Cell membranes were processed as described above or treated cells were directly used for live cell microscopy.

**Microscopy**—*S. pombe* cells expressing fluorescently tagged proteins were immobilized on 2% agarose as described previously (5) and imaged using a Marianas/Yokogawa CSU22 spinning disk confocal microscope (3i) equipped with Axio Observer (Zeiss), Photometrics Cascade II EM-CCD camera (Roper Scientific), and environmental chamber (Tokai HIT). Cells were imaged at room temperature, 25 °C, or 36 °C as indicated in the figure legends. Images were captured using 100× oil objective with 1.0 NA. We used SlideBook 5.0 for data acquisition. Fifteen Z-images (0.34-μm step size) were collected, and three-dimensional reconstitution of the confocal slices was performed by ImageJ (National Institutes of Health). All the images described in this study were acquired and processed in an identical manner.

**Co-immunoprecipitation**—Co-immunoprecipitation assays were performed as described previously (8). Briefly, cells (6 × 10⁶) from exponentially growing wild-type or mutant strains were collected and lysed using glass beads in digitonin lysis buffer (50 mM HEPES, pH 6.8, 1% (w/v) digitonin, 50 mM KOAc, 2 mM Mg(OAc)₂, 1 mM CaCl₂, 200 mM sorbitol, 1 mM NaF, 0.3 mM Na₃VO₂, supplemented with 1× protease inhibitors). Cellular debris was removed by centrifugation at 100,000 × g for 10 min, and 0.75 mg of lysate supernatant was incubated with 5 μl of Dsc2 antiserum for 15 min at 4 °C followed by the addition of 40 μl of protein A-agarose beads. Binding reaction was rotated overnight at 4 °C, and unbound proteins were removed by three washes with digitonin lysis buffer. The bound fraction was eluted by boiling 5 min in SDS-lysis buffer (10 mM Tris-HCl, pH
6.8, 100 mM NaCl, 1% SDS and 1 mM EDTA) and analyzed by Western blotting using HRP-conjugated Dsc antibodies.

**Results**

The fission yeast Golgi Dsc E3 ligase complex contains five integral membrane proteins, Dsc1–Dsc5. Bioinformatic analysis identified *S. pombe* Dsc1 as a homolog of the *S. cerevisiae* Golgi Tul1 E3 ligase (6). Although Dsc1 and Tul1 have low sequence similarity (less than 30%), both are predicted to contain a large luminal N-terminal domain (about 300–400 aa long), followed by seven transmembrane segments, and a C-terminal, cytosolic RING domain. The RING domain is a well characterized zinc finger in which a total of eight cysteine and histidine residues coordinate two zinc atoms (13). Dsc1 contains an H2-type RING domain (C3H2C3) that differs from the classical RING domain (C3HC4) by having two histidines in the fourth and fifth positions (Fig. 1B) (14). RING domains interact directly with E2 ubiquitin-conjugating enzymes to ubiquitinate protein substrates (13, 15, 16). Although the Dsc1 RING domain contains residues required for E2 binding (Fig. 1B), not all predicted RING domains possess E3 ligase activity (13). To test whether the Dsc1 RING domain is a functional E3 ligase, we performed an *in vitro* ubiquitination assay. RING domains will auto-ubiquitinate, allowing assessment of E3 ligase activity in the absence of substrate (17, 18). Purified Dsc1 RING domain (aa 608–695) fused to GST was incubated with ubiquitin-activating enzyme E1 (Uba1) and cognate ubiquitin-conjugating enzyme E2 (Ubc4) (Fig. 1C). GST-Dsc1 RING was efficiently ubiquitinated in a reaction that required ATP, E1 and E2 enzymes, and Dsc1 RING domain (Fig. 1D). The Dsc1 RING domain lacks lysine residues, indicating that ubiquitination occurred on lysine(s) in GST or on non-lysine residues in Dsc1. The E3 ligase activity of Dsc1 RING required both zinc-coordinating residues (C634A and H668A) and E2-interacting residues (I636D or L675D) (Fig. 1D, lanes 6–9). These results demonstrate that Dsc1 is a functional RING E3 ligase.

Fission yeast SREBP proteolytic activation requires Dsc1 (5). Previously, we demonstrated that mutation of a zinc-coordinating histidine (Dsc1-H668A) or a partial truncation of the RING domain (Dsc1-Q673X) abrogated SREBP activation (5). These mutations may cause misfolding of the RING domain, so we...
ER Exit of Dsc E3 Ligase Requires Enzyme Activity

A  Western Blot

| Strain   | WT | dsc1Δ | dsc1-1 | dsc1-C634A | dsc1-L675D | sre1Δ |
|----------|----|-------|--------|------------|------------|-------|
| O₂ (4h)  | +  | -     | -      | +          | -          | +     |
| Lane     | 1  | 2     | 3      | 4          | 5          | 6     |

B  Western Blot

|    | dsc1 | WT | L675D | C634A | dsc1-1 | dsc1Δ |
|----|------|----|-------|-------|--------|-------|
|    | CaMV-SRE2 | -  | +     | -     | +      | -     |
| Lane | 1    | 2  | 3     | 4     | 5      | 6     |

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0.18

0.14

Sre1P

Normalized (AU)

0.12

0.08

Sre1N

Normalized (AU)

3.0

2.5

2.0

1.5

1.0

0.5

0.0

WT dsc1Δ dsc1-1 dsc1-C634A dsc1-L675D sre1Δ

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50

40

30

anti-FLAG

- P

- N

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50

40

30

anti-FLAG

- P

- N
ER Exit of Dsc E3 Ligase Requires Enzyme Activity

A Western Blot

| Strain  | WT   | dsc2Δ | dsc1Δ | dsc1-C634A | dsc1-I675D | WT   | dsc2Δ | dsc1Δ | dsc1-C634A | dsc1-I675D |
|---------|------|-------|-------|------------|------------|------|-------|-------|------------|------------|
| Lane 1  | 1    | 2     | 3     | 4          | 5          | 6    | 7     | 8     | 9          | 10         |
| kD     | 100  | 80    | 60    | 40         | 30         | 20   | 10    | 8     | 6          | 5          |

anti-Dsc1

B Western Blot

| Strain  | ubc4-P61S | dsc1Δ | WT |
|---------|-----------|-------|----|
| Lane 1  | 1         | 2     | 3  |
| 36°C (min) | 0   | 30   | 60 |
| 80    | 60        | 40    |    |

anti-Dsc1

C Western Blot

| Strain  | WT   | sar1-1 | CHX | 36°C (h) | WT   | sar1-1 |
|---------|------|--------|-----|----------|------|--------|
| Lane 1  | 1    | 2      | 3   | 4        | 5    | 6      |
| CHX     | 0    | 1      | 0   | 1        | 0    | 1      |
| 36°C (h) | 1    | 1      | 1   | 1        | 1    | 1      |
| Lane 2  | 7    | 8      | 9   | 10       | 11   | 12     |

anti-Dsc1

D Western Blot

| Strain  | anti-Dsc1 |
|---------|-----------|
| Lane 1  | 1         |
| 36°C (min) | 0   |
| 80    | 60        |

FIGURE 3. Dsc1 glycosylation requires RING activity. A, detergent-solubilized membranes from wild-type, dsc2Δ, dsc1Δ RING mutants, and dsc1Δ were treated without glucose or with the glycosidase peptide-N-glycosidase F (PNGase F) and then immunoblotted using Dsc1 antiserum. B, wild-type, dsc1Δ, and temperature-sensitive ubc4-P61S cells were grown at 25°C to exponential phase and then shifted to 36°C. Cells were harvested at the indicated times, and detergent-solubilized membranes were immunoblotted using anti-Dsc1 antibody. C, wild-type, temperature-sensitive sar1-1, and dsc1Δ cells were grown at 25°C to exponential phase and then shifted to 36°C in the absence or presence of cycloheximide (CHX, 100 μg/ml) for 1 h. Cells were harvested at the indicated times, and detergent-solubilized membranes were treated without or with the glycosidase peptide-N-glycosidase F and immunoblotted using anti-Dsc1 antibody. D, wild-type, sar1-1, and dsc1-L675D cells were cultured for 2 h at the indicated temperatures. Isolated membranes were immunoblotted with anti-Dsc1 serum. In each panel, differentially glycosylated forms of Dsc1 are denoted: mature (M), intermediate (I), deglycosylated (D).

Further tested whether SREBP activation required a RING domain surface residue essential for E2 binding (Dsc1-L675D) and E3 ligase activity (Fig. 1D) (15). In addition, we tested a second zinc-coordinating residue (Dsc1-C634A). We assayed proteolytic cleavage of both fission yeast SREBPs, Sre1 and Sre2. Consistent with previous results (5), mutation of either residue blocked SREBP activation (Fig. 2, A and B), further demonstrating that SREBP activation requires Dsc1 E3 ligase activity.

Quantification of Sre1 cleavage showed an ~20-fold increase in Sre1N in wild-type cells shifted to low oxygen (Fig. 2A). Interestingly, we observed an ~4-fold decrease in Sre1 precursor in dsc1Δ cells under normoxic conditions. This decrease likely results from reduced levels of the Sre1-binding protein Scp1 in dsc1Δ cells (19). Scp1 stabilizes Sre1 precursor, and in the absence of Scp1, Sre1 precursor is degraded via ER-associated degradation (20). In addition, Sre1 precursor decreased under low oxygen in dsc mutant cells. Low oxygen stimulates ER-to-Golgi transport of the Sre1-Scp1 complex for Sre1 cleavage in the Golgi. In dsc mutant cells, Sre1N is not made (Fig. 2A, lanes 3–12), the Sre1 precursor is likely degraded, and the supply of...
Sre1 precursor is not replenished by positive feedback activation of the sre1 promoter (21). Sre2 does not bind Scp1, and Sre2 cleavage is Scp1-independent (3, 5).

The N-terminal luminal domain of Dsc1 contains five potential N-glycosylation sites and glycosylation of three sites (Asn-52, Asn-115, and Asn-220) has been confirmed by mass spectrometry (22). Interestingly, complete glycosylation of Dsc1 requires Dsc2, Dsc3, and Dsc4 (8). In the absence of Dsc2, Dsc3, or Dsc4, Dsc1 migrated with an intermediate mobility as compared with the mature and deglycosylated forms (Fig. 3) (8). Unexpectedly, we observed that Dsc1 mutants lacking E3 ligase activity also displayed incomplete glycosylation (Fig. 3A, lane 2) (8). Changes in mobility were due to differential N-linked glycosylation because Dsc1 mutants showed the same mobility as wild type after treatment with peptide-N-glycosidase F (Fig. 3A, lanes 6–9). These data indicate that Dsc1 E3 ligase activity is required for complete Dsc1 glycosylation.

Dsc1 E3 ligase activity requires the E2 enzyme Ubc4 in vitro (Fig. 1D), and SREBP cleavage activation requires ubc4, demonstrating that Ubc4 is the cognate E2 enzyme for Dsc1 (5). UbC4 is an essential gene in fission yeast (23); thus we used a temperature-sensitive mutant (ubc4-P61S), or do not coordinate zinc (C634A) (Fig. 4A, lanes 7–12), indicating that complex assembly is unaffected by RING domain mutations. We further investigated Dsc1 ligase complex assembly in ubc4-P61S cells defective for the cognate E2 ubiquitin-conjugating enzyme Ubc4. We observed above that after shifting to non-permissive temperature, Dsc1 accumulates as a faster migrating, intermediate form in ubc4-P61S cells (Fig. 3B). When purified with Dsc2, the Dsc1 intermediate form co-purified with other Dsc subunits as in wild-type cells (Fig. 4B, lanes 7–12). These data demonstrate that the incompletely glycosylated Dsc1 assembles into the Dsc E3 ligase complex and that E3 ligase activity is not required for complex assembly.
Alternatively, incomplete Dsc1 glycosylation could result from a failure of Dsc1 to interact with Golgi glycosylation enzymes. The Dsc E3 ligase complex has a hierarchical organization where Dsc2-Dsc3-Dsc4 define the core, and Dsc1 and Dsc5 are peripheral subunits (Fig. 1A) (8). To assay Dsc E3 ligase localization, we determined the localization of the core subunit Dsc2 in mutant strains using live cell fluorescence confocal microscopy. As reported previously in wild-type cells, these results correlated with the presence of incompletely glycosylated Dsc1 observed previously in dsc3Δ and dsc4Δ cells, but not dsc5Δ cells (8). In addition, Dsc1 is incompletely glycosylated in dsc2Δ cells (Fig. 3A). Thus, assembly of subunits Dsc1–Dsc4 is required for proper Golgi localization of the Dsc E3 ligase, and Dsc5 is not required for ER exit. Often, failure of a multi-protein complex to assemble or exit the ER results in subunit degradation by quality control pathways like ER-associated degradation or autophagy (28). Notably, Dsc complex subunits were stable when retained in the ER, for example, in dsc1Δ cells (Fig. 4A, lane 2) (8).

Interestingly, Dsc2 also accumulated in the ER in dsc1–1 cells that assemble the Dsc complex normally (Fig. 4A), but lack Dsc E3 ligase activity due to a RING domain truncation (Fig. 4) (5).

This result suggested that Dsc1 E3 ligase activity itself might be required for Golgi localization. To test this directly, we examined Dsc E3 ligase localization in ubc4-P61S cells conditionally defective for Dsc E3 ligase activity. Importantly, the Dsc E3 ligase is not mutated in these cells and assembles normally at
the non-permissive temperature when E3 ligase activity is inhibited (Fig. 3B). As expected, incompletely glycosylated Dsc1 accumulated in \textit{ubc4-P61S} cells (Fig. 6A, lanes 1–3). Cycloheximide treatment specifically blocked production of the Dsc1 intermediate form upon Ubc4 inactivation, indicating that Dsc1 fails to receive Golgi-dependent carbohydrate modifications in the absence of Ubc4 activity (Fig. 6A).

Next, we examined Dsc E3 ligase localization in \textit{ubc4-P61S} cells. At permissive temperature, the majority of cells showed Dsc2 localization only in Golgi puncta (88%) (Fig. 6B, left panel, magenta arrowhead), and a small number of cells showed both ER and Golgi localization (green arrowhead). Upon incubation at non-permissive temperature for 2 h, Dsc2 localization changed with 82% of cells showing mixed ER and Golgi localization and 18% displaying only Golgi localization (Fig. 6B, middle panel). Treatment with cycloheximide when shifting to the non-permissive temperature prevented ER accumulation of Dsc2 (88% Golgi only, 12% ER and Golgi) (Fig. 6B, right panel). Dsc2 localized to the Golgi in wild-type cells under each of the three conditions, showing that ER localization required Ubc4 inactivation (Fig. 6A).

To test whether Dsc E3 ligase activity is required specifically for Dsc complex ER exit, we assayed secretory pathway function in \textit{dsc} mutant strains. ER-to-Golgi protein trafficking is essential for cell viability (29). We hypothesized that growth at elevated temperature will stress cells and may reveal \textit{dsc}-dependent defects in ER-to-Golgi transport. We...
compared growth of wild-type and dsc mutant cells at 37 °C and observed no growth difference in exponentially growing cells (Fig. 7A). Next, we looked at the localization of cis-Golgi resident, mannosyltransferase subunit Anp1. Inhibition of COPII-dependent vesicle formation results in Anp1 mislocalization to the ER (5). Anp1-GFP localized to Golgi puncta in wild-type, dsc1Δ, and dsc2Δ cells (Fig. 7B), indicating that COPII vesicle transport does not require Dsc E3 ligase activity. In an independent experiment, we co-localized Dsc2 and Anp1 in dsc1Δ cells, confirming that Golgi-resident Anp1 localizes normally under conditions in which the Dsc E3 ligase is mislocalized to the ER (Fig. 7C).

Discussion

Multiple lines of evidence demonstrate that Dsc1 E3 ligase activity is required for ER exit of the Dsc complex, thus ensuring that only fully functional Dsc E3 ligase moves to the Golgi where it acts in SREBP activation. Although E3 ubiquitin ligases are known to regulate their abundance through auto-ubiquitination (30–32), in this instance, enzyme activity controls protein localization. Multi-subunit membrane complexes such as MHC class I and Kir potassium channels require proper assembly prior to ER exit (33–35). However, even when the Dsc E3 ligase complex assembles properly, ER exit requires E3 ligase activity. To our knowledge, this represents the first example of enzyme activity-dependent protein sorting in the secretory pathway.

How ligase activity controls ER exit is unknown, but potential mechanisms exist. First, the Dsc E3 ligase complex may assemble with an ER retention protein whose removal and possibly degradation require Dsc1-dependent ubiquitination. Alternatively, the Dsc1 E3 ligase may ubiquitinate one or more COPII components whose modification is required for Dsc E3 ligase ER exit. Indeed, packaging of pro-collagen requires CUL3-KLHL12-mediated mono-ubiquitination of the COPII component Sec31 (36), and mono-ubiquitination of Sec23 has been detected in budding yeast (37). Interestingly, Dsc1 E3 ligase utilizes the E2 Ubc4 that preferentially adds mono-ubiquitin to substrates (38). Finally, a Dsc subunit could be a direct substrate, and the addition of ubiquitin may alter Dsc complex conformation or recruit an effector protein to facilitate ER exit. Although the mechanistic details are under investigation, these findings add to the growing complexity of ER cargo sorting and open up an opportunity to investigate the role of ubiquitination in this process.

Lastly, these findings have important implications for the current model of SREBP activation in fungi in which we proposed that SREBP activation requires its ubiquitination. The fact that the Dsc E3 ligase mislocalizes to the ER when inactive necessitates additional studies of the role for SREBP ubiquitination in its proteolytic activation.

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