Cdc1p is a Golgi-localized glycosylphosphatidylinositol-anchored protein remodelase

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ABSTRACT **Glycosylphosphatidylinositol-anchored proteins (GPI-APs) undergo extensive posttranslational modifications and remodeling, including the addition and subsequent removal of phosphoethanolamine (EtNP) from mannose 1 (Man1) and mannose 2 (Man2) of the glycan moiety. Removal of EtNP from Man1 is catalyzed by Cdc1p, an event that has previously been considered to occur in the endoplasmic reticulum (ER). We establish that Cdc1p is in fact a *cis*/medial Golgi membrane protein that relies on the COPI coatomer for its retention in this organelle. We also determine that Cdc1p does not cycle between the Golgi and the ER, and consistent with this finding, when expressed at endogenous levels ER-localized Cdc1p-HDEL is unable to support the growth of *cdc1*Δ cells. Our *cdc1* temperature-sensitive alleles are defective in the transport of a prototypical GPI-AP-Gas1p to the cell surface, a finding we posit reveals a novel Golgi-localized quality control warrant. Thus, yeast cells scrutinize GPI-APs in the ER and also in the Golgi, where removal of EtNP from Man2 (via Ted1p in the ER) and from Man1 (by Cdc1p in the Golgi) functions as a quality assurance signal.**

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INTRODUCTION

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are broadly distributed throughout eukaryotic organisms, where they play key roles in a variety of biological processes. In mammalian cells, for example, GPI-APs are concentrated in lipid rafts that are involved in receptor-mediated signal transduction pathways and membrane trafficking (Paulick and Bertozzi, 2008). In yeast cells, GPI-APs are primarily components of the cell wall and plasma membrane (Pittet and Conzelmann, 2007). Among the ~6000 genes encoded by the budding yeast genome, more than 60 genes are predicted to encode GPI-APs (Caro *et al.*, 1997; Kapteyn *et al.*, 1999), all of which play a role in some aspect of cellular integrity, either by virtue of being localized to the limiting membrane of the cell or through their covalent incorporation into cell walls. GPI-APs likely represent a significant proportion of the yeast proteome (Hamada *et al.*, 1999).

The structure of GPI anchors is complex (Nosjean *et al.*, 1997) and is composed of ethanolamine phosphate (EtNP), three mannose (Man) moieties, one non-N-acetylated glucosamine, and an inositol phospholipid (Kinoshita and Fujita, 2016). The phospholipid tail serves to attach the GPI anchor to the membrane, while the GPI glycan is linked to the C-terminus of the target protein through an EtNP bridge on Man3. The biosynthesis of GPI precursors and the remodeling of GPI anchors are predominantly carried out in the ER (Orlean, 2012; Kinoshita and Fujita, 2016). Upon completion of the biosynthesis and processing of a GPI anchor, the GPI-AP is transported to the Golgi via vesicular mediated anterograde trafficking. The p24 family of ER-resident protein export receptors are required for the efficient packaging of GPI-APs into COPII vesicles, and there is evidence that removal of EtNP from Man2 of GPI-APs is critical for this (Muñiz and Riezman, 2000; Muñiz *et al.*, 2000; Castillon *et al.*, 2009, 2011; Fujita *et al.*, 2011; Theiler *et al.*, 2014). Following posttranslational modification in the Golgi, the extensively glycosylated GPI-APs are transported to the cell surface.

During GPI-AP biosynthesis in the yeast ER, Man1 and Man2 are modified by the addition of EtNP, a reaction that is mediated by the ER resident proteins Mcd4p (Man1) and Gpi7p (Man2) (Benachour *et al.*, 1999; Gaynor *et al.*, 1999; Hong *et al.*, 1999). Following the

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Abbreviations used: CFW, calcofluor white; ER, endoplasmic reticulum; EtNP, ethanolamine phosphate; 5-FOA, 5-fluoro-orotic acid; GPI, glycosylphosphatidylinositol; GPI-AP, glycosylphosphatidylinositol-anchored protein; Man1, mannose 1; Man2, mannose 2; TPI, triose phosphate isomerase; WCE, whole cell extract; WT, wild type; YEPD, yeast extract peptone dextrose.

covalent attachment of the protein substrate to Man3, EtNP is removed from Man1 (via Cdc1p; Vazquez *et al.*, 2014) and Man2 (via Ted1p; Manzano-Lopez *et al.*, 2015). Robust ER export of GPI-APs only occurs when GPI anchors have been subject to extensive remodeling that includes the removal of EtNP from Man2 through the action of the ER-resident GPI-anchor remodelase Ted1p/PGAP5 (Fujita *et al.*, 2009; Manzano-Lopez *et al.*, 2015; Vazquez *et al.*, 2014).

A previous study identified Cdc1p as an ER-localized protein and established that *cdc1* mutants affected Golgi inheritance (Rossanese *et al.*, 2001; Losev *et al.*, 2008). While *CDC1* is essential for yeast cell growth, *TED1* is a nonessential gene, and while *ted1*Δ cells accumulate an ER-localized processing intermediate of the GPI-AP Gas1p, *cdc1* temperature-sensitive mutants do not exhibit any such accumulation (Fujita *et al.*, 2009; Fujita and Kinoshita, 2012; Vazquez *et al.*, 2014). Taken together, these observations lead to several possible conclusions, including that the removal of EtNP from Man1 of GPI-APs is not the essential activity of Cdc1p and is not required for robust ER export of GPI-APs, or alternatively that the removal of EtNP from Man1 occurs later in the transport pathway of these proteins—a prospect that is at odds with the reported localization of Cdc1p to the ER (Losev *et al.*, 2008). In contrast to mammalian cells, it is generally accepted that budding yeast cells do not remodel GPI anchors in the Golgi—principally because of the lack of any evidence for such processing events.

In this report, we have reexamined the localization of Cdc1p in cells, as well as characterizing several novel temperature-sensitive alleles of *CDC1*. Our findings confirm a role for Cdc1p in yeast cell wall integrity and reveal that Cdc1p is localized to and functions in the Golgi, rather than in the ER as previously reported (Losev *et al.*, 2008). Thus, like their mammalian cell counterparts, yeast cells can also remodel GPI-APs in the Golgi.

RESULTS AND DISCUSSION

Cdc1p is a Golgi-localized membrane protein

In yeast cells, GPI-APs are extensively remodeled by a group of resident enzymes before being exported from the ER (Kinoshita and Fujita, 2016; Muñiz and Riezman, 2016; Lopez *et al.*, 2019; Figure 1A). In a previous study, an epitope-tagged form of Cdc1p (Cdc1p-myc) was shown to be localized to the ER (Losev *et al.*, 2008); however, this observation is somewhat difficult to reconcile with the known phenotypes of temperature-sensitive alleles of *CDC1* (Losev *et al.*, 2008; Vazquez *et al.*, 2014). For example, *cdc1* mutants do not accumulate the prototypical GPI-AP Gas1p (pGas1p) in the ER, unlike cells carrying deletions of genes with well-documented roles in remodeling of GPI-APs in the ER, such as *TED1*, *BST1*, *PER1*, and *GUP1* (Vazquez *et al.*, 2014; Figure 1, A and B). However, it is not clear whether Cdc1p-myc is in fact functional, as these data were not included in Losev *et al.* (2008). It is therefore conceivable that the reported localization of Cdc1p to the ER is erroneous. To address these apparent anomalies, we reexamined the localization of Cdc1p in yeast cells (see Supplemental Table S1). Yeast strains in which the endogenous *CDC1* gene was tagged at the N- or C-terminus with the fluorescent mNeon protein (*cdc1::mNeon-CDC1* and *cdc1::CDC-mNeon*, respectively) were viable (Figure 1C). In addition, neither strain was sensitive to calcofluor white (CFW), a compound that exacerbates cell wall defects (Vazquez *et al.*, 2014), or temperature-sensitive for growth (Figure 1C), indicating that these proteins fully recapitulated the function of Cdc1p. However, unlike myc-tagged Cdc1p (Losev *et al.*, 2008), both mNeon-Cdc1p and Cdc1p-mNeon were found in puncta (Figure 1, D and E), rather than in the ER. Additionally, whilst mNeon-Cdc1p and Cdc1p-mNeon did not colocalize appreciably with the *trans* Golgi marker Sec7p-mCherry (1–2% coincidence; Supplemental Figure S1, A and B), they did partially colocalize with the *cis* Golgi marker mCherry-Sed5p (~40% coincidence; Supplemental Figure S1, A and B). Moreover, we find that mNeon-Cdc1p and Cdc1p-mNeon share ~ 60% colocalization with the *cis* Golgi resident protein Mnn9p and ~ 40% colocalization with the medial Golgi resident protein Kre2p (white arrowheads in Figure 1, D and E). On the basis of these data, we concluded that Cdc1p is in fact a Golgi membrane protein that is localized to both the *cis* and medial Golgi, but not to the *trans* Golgi.

Cdc1p does not cycle between the Golgi and the ER but relies on COPI for its Golgi retention

Having established that Cdc1p is a Golgi-resident membrane protein, we next asked if Cdc1p could cycle constitutively between the Golgi and the ER—a phenomenon that applies to several proteins that are localized to the Golgi at steady state, such as Sed5p (Wooding and Pelham, 1998; Gao and Banfield, 2020). To address this, we examined the localization of mNeon-Cdc1p (as both mNeon-Cdc1p and Cdc1p-mNeon localization patterns are indistinguishable; Figure 1, D and E) in cells that display a temperature-sensitive defect in the function of the COPII coat and consequently an impairment in the export of proteins from the ER (*sec23-1*; Figure 2A). As Golgi –ER transport can persist in *sec23-1* cells even when ER export is blocked, proteins retrieved from the Golgi are subsequently prevented from exiting the ER (Wooding and Pelham, 1998). Unlike Sed5p, which does cycle robustly between the Golgi and the ER (Wooding and Pelham, 1998; Gao and Banfield, 2020), mNeon-Cdc1p did not accumulate in the ER, but rather was lost from puncta in *sec23-1* cells (Figure 2A). This observation is consistent with the notion that Cdc1p can be sorted into COPI vesicles but that these vesicles cannot fuse with the ER. Moreover, this finding suggests that once synthesized de novo, Cdc1p exits the ER; the protein does not cycle constitutively between the Golgi and the ER, and consequently Cdc1p's function is most likely restricted to the Golgi.

To seek evidence that Cdc1p's Golgi localization was dependent on the COPI-coatomer, we examined the fate of mNeon-Cdc1p in yeast strains carrying temperature-sensitive mutations in the β′-COP (*sec27-1*) and γ-COP (*sec21-1*) subunits of the COPI-coatomer. We found that even when *sec27-1* and *sec21-1* cells were grown at the permissive temperature (25°C), mNeon-Cdc1p was partially mislocalized to the vacuole, the default location for mislocalized membrane proteins in yeast cells. Mislocalization was absolute when *sec27-1* and *sec21-1* cells were grown at the restrictive temperature (37°C; Figure 2B). The Golgi localization of Cdc1p does not appear to require known sorting receptors or effectors of COPI function, however, as Cdc1p-mNeon remained in Golgi-like puncta in *vps74*Δ and *bre5*Δ cells (Tu *et al.*, 2008, 2012; Wang *et al.*, 2020; Figure 2C). The results of in vitro protein pulldown assays to assess binding to the COPI-coatomer from yeast whole cell extracts (WCEs) did not show particularly robust binding of either the N-terminal cytoplasmically oriented loop of Cdc1p (amino acids 1–45) or the cytoplasmically oriented internal loop of Cdc1p (amino acids 413–468). Nevertheless, we cannot exclude the possibility that the comparatively weak binding of the cytoplasmically oriented internal loop of Cdc1p (relative to the KKXX motif of Wbp1p) is physiologically relevant (Figure 2D).

The observation that Cdc1p is a Golgi-localized GPI-AP remodelase prompted us next to examine the molecular basis by which de novo synthesized Cdc1p exits the ER. Previous studies identified the COPII coat component Sec24p as functioning in the robust export

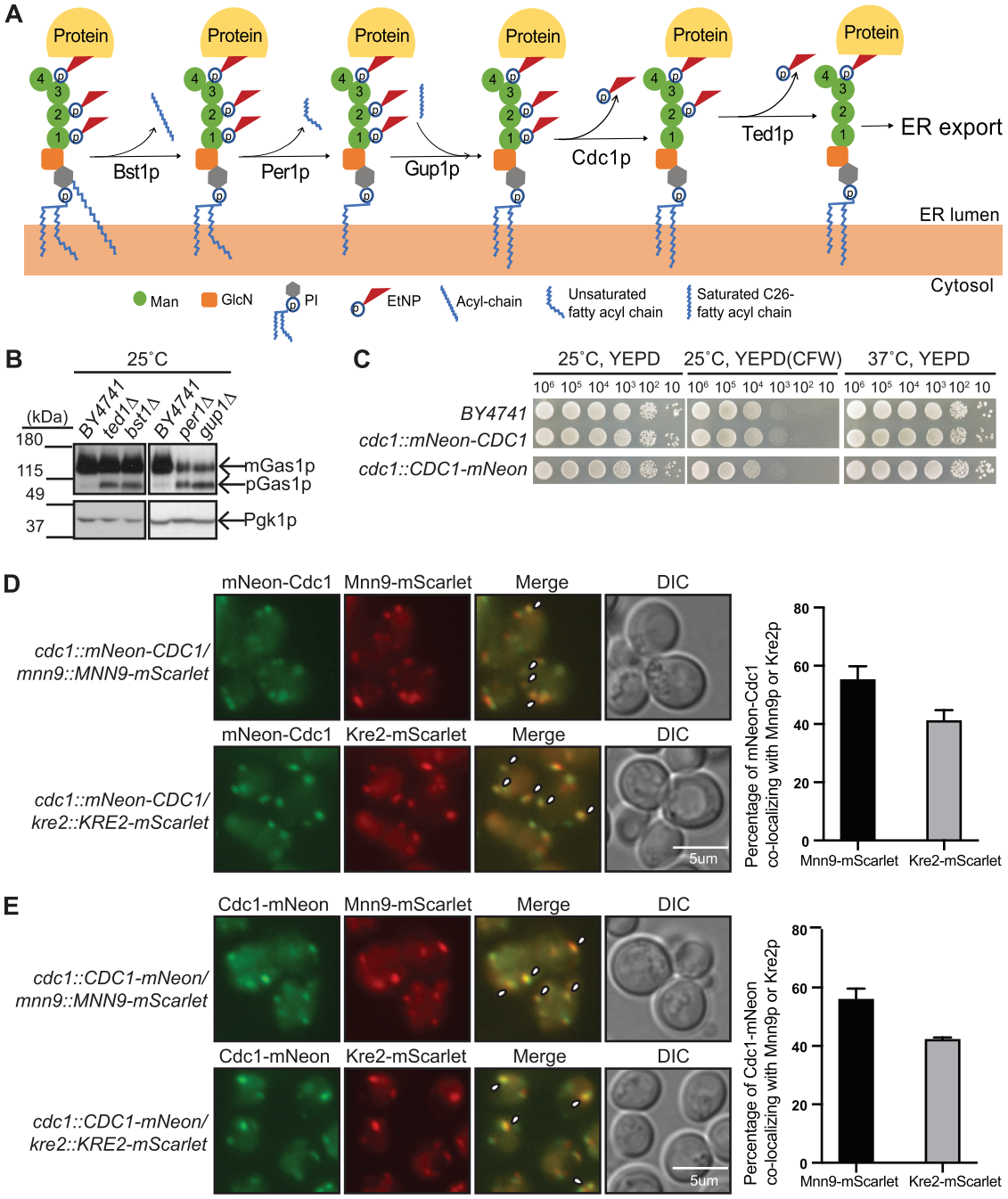

FIGURE 1: Cdc1p is a Golgi-localized membrane protein. (A) Abbreviated depiction of the remodeling of GPI-APs in the yeast ER. (B) The steady-state levels of Gas1p in cells lacking the indicated ER resident GPI-AP remodelases (see A). mGas1p and pGas1p denote the mature (plasma membrane) and precursor (ER) forms of Gas1p, respectively. Pgk1p serves as a protein loading control. (C) mNeon-*CDC1* and *CDC1*-mNeon are functional. The endogenous *CDC1* gene was replaced with a version of the gene in which the protein coding sequence of mNeon was inserted at either the 5′ (mNeon-*CDC1*) or 3′ (*CDC1*-mNeon) end of the gene. Tenfold serial dilutions of the indicated strains were spotted onto YEPD plates with or without 50 µg/mL CFW. (D) mNeon-Cdc1p partially colocalizes with the *cis* Golgi resident protein Mnn9p (~60%) and the medial Golgi resident protein Kre2p (~40%). See *Materials and Methods* for further details. (E) Cdc1p-mNeon partially Cdc1p partially colocalizes with the *cis* Golgi resident protein Mnn9p (~60%) and the medial Golgi resident protein Kre2p (~40%). See *Materials and Methods* for further details. Arrowheads denote colocalizing puncta.

of proteins from the ER (Miller *et al.*, 2003). Using the same GST-fusion proteins described for our COPI-coatomer binding studies (Figure 2D). The purified GST-fusion proteins were mixed with WCEs from a yeast strain expressing an epitope-tagged version of Sec24p (to facilitate detection by immune staining; Sec24p-9myc). Although binding could be detected between the N-terminal cytoplasmic portion of Cdc1p (amino acids 1–45) and Sec24-9myc (Figure 2E), binding was undetectable with the portion of Cdc1p corresponding to its internal cytoplasmically oriented region (amino acids 413–468; Figure 2F). The binding of Cdc1p (amino acids 1–45) to Sec24-9myc

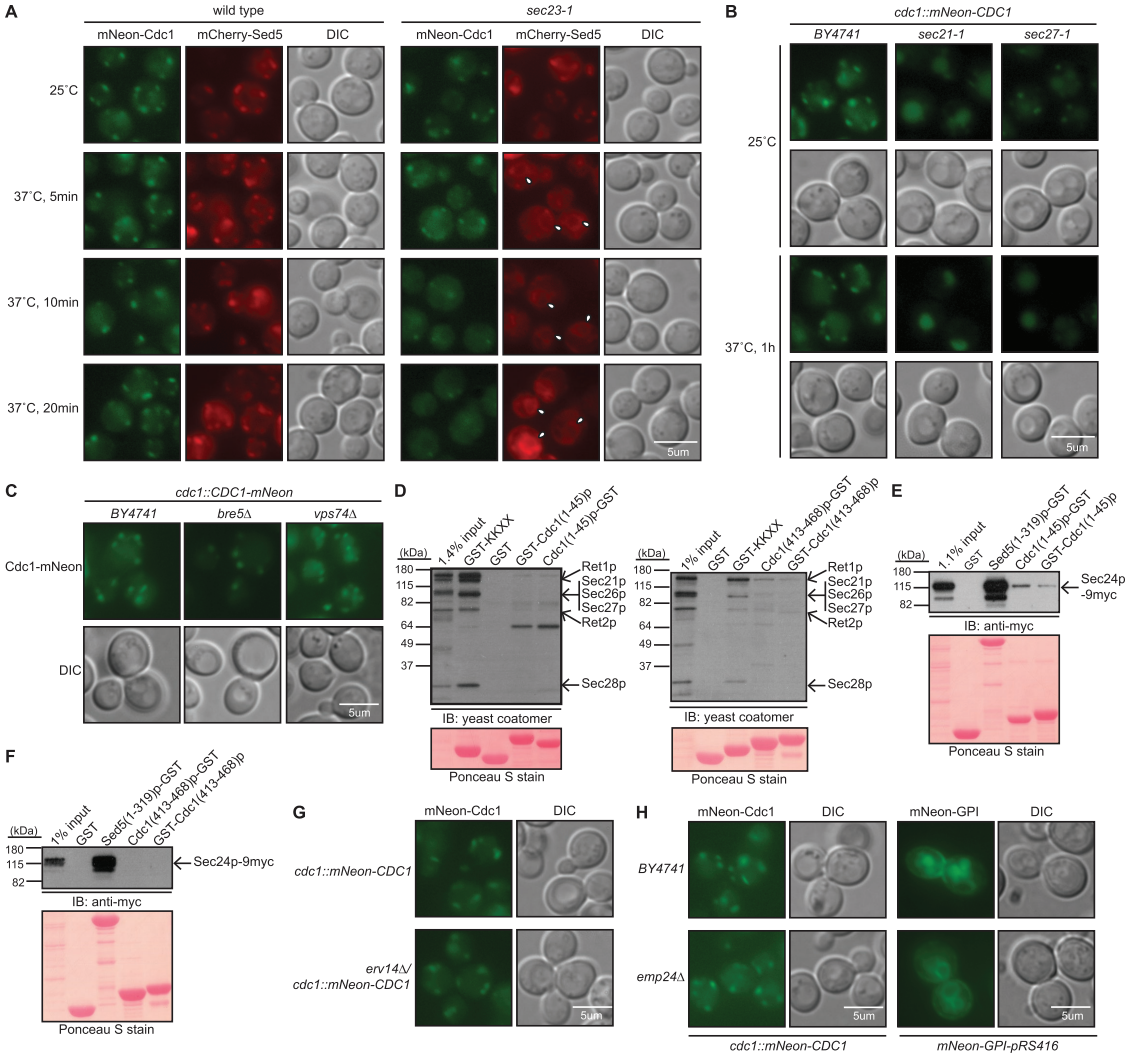

FIGURE 2: Cdc1p does not cycle between the Golgi and ER but relies on COPI for its Golgi retention. (A) Unlike mCherry-Sed5p, mNeon-Cdc1p does not cycle between the Golgi and the ER. Arrowheads indicate the ER membranes that envelop the nucleus. (B) In the COPI-coatomer mutants *sec21-1* (which encodes γ-COP) and *sec27-1* (which encodes β′-COP; see Supplemental Table S1), mNeon-Cdc1p is mislocalized to the vacuole. (C) Cdc1-mNeon remains in the Golgi in cells lacking *VPS74* (*vps74Δ*) or *BRE5* (*bre5Δ*); see text for details. (D) In vitro mixing assays assessing the interaction between the cytoplasmic regions of Cdc1p and COPI-coatomer. WCEs from WT cells were mixed with the indicated bacterially expressed purified Cdc1p peptides and binding assessed by SDS–PAGE and immune staining. GST-KKXX serves as a positive control. (E, F) In vitro mixing assays assessing the interaction between the cytoplasmic regions of Cdc1p and the COPII protein Sec24p. WCEs from cells expressing myc-tagged Sec24p (see Supplemental Table S1) were mixed with the indicated bacterially expressed purified Cdc1p peptides and binding assessed by SDS–PAGE and immune staining. Sed5-GST serves as a positive control (see Supplemental Table S2) (G) mNeon-Cdc1p Golgi localization is unaffected in *erv14Δ* cells. (H) mNeon-Cdc1p Golgi localization is unaffected in *emp24Δ* cells, while mNeon-GPI ER export is delayed. IB denotes the primary antibodies used.

was not as robust as that of Sed5p to Sec24-9myc; nonetheless we cannot exclude the prospect that Cdc1p is a bona fide client of Sec24p in cells. Consistent with this supposition, we found no evidence that Cdc1p's ER export was reliant upon the ER protein export receptor Erv14p (Figure 2G; Herzig *et al.*, 2012) or that Cdc1p was cosorted with GPI-APs into COPII vesicles, as *emp24Δ* cells did not accumulate Cdc1p-mNeon in the ER, while mNeon-GPI did (Figure 2H; Supplemental Table S2). The observation that Cdc1p does not accumulate in the ER in *emp24Δ* cells makes it unlikely that Cdc1p is cotransported from the ER together with GPI-APs, and based on this inference, we assume that Cdc1p is not a client of Lst1p (Manzano-Lopez *et al.*, 2015).

CDC1 temperature-sensitive mutants exhibit cell wall defects and are deficient in the transport of a GPI-anchored protein to the cell surface

Another confounding issue regarding earlier studies on *CDC1* mutants concerns differences in the so-called wild-type (WT) amino acid sequence encoded by this gene. Several previous reports have employed the *cdc1-314* and *cdc1-310* alleles, for example, which were generated with a variant of *CDC1* that differs by one amino acid (P441S) from the amino acid sequence of Cdc1p found in the *Saccharomyces* Genome Database (yeastgenome.org). P441S maps to a cytoplasmically exposed portion of Cdc1p, and thus would not likely be directly involved in binding to GPI-AP substrates. Thus,

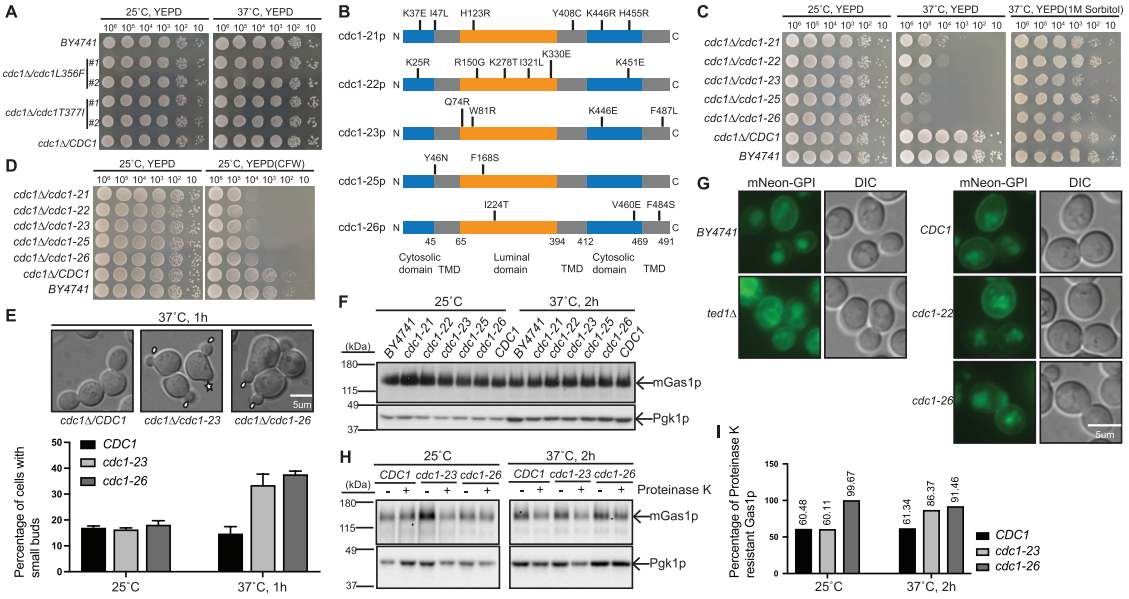

FIGURE 3: CDC1 temperature-sensitive mutants exhibit cell wall defects and are deficient in the transport of a GPI-anchored protein to the cell surface. (A) *cdc1* (L356F) and *cdc1* (T377I) cells are not ts for growth. Tenfold serial dilutions of the indicated strains were spotted onto YEPD plates and incubated at 25 and 37°C for 48 h before being photographed. (B) The distribution of amino acid substitutions in the cdc1-21p–cdc1-26p transmembrane domain. (C) The temperature sensitivity of *cdc1-21–cdc1-26* alleles is osmoremedial. Tenfold serial dilutions of the indicated strains were spotted onto YEPD plates with or without 1M sorbitol and incubated at 25 and 37°C for 48 h before being photographed. (D) *cdc1-21–cdc1-26* cells show increased sensitivity to CFW. Tenfold serial dilutions of the indicated strains were spotted onto YEPD plates with or without 50 µg/mL CFW and incubated at 25°C for 48 h before being photographed. (E) At 37°C ~40% of α-factor-treated *cdc1-23* and *cdc1-26* cells arrest growth with polarization defects and small buds. White arrowheads denote the small bud phenotype and the star symbol denotes a polarization anomaly. The quantification data are derived from three separate experiments in which at least 100 cells were assessed for each strain. (F) *cdc1-21–cdc1-26* cells do not accumulate ER or hypoglycosylated forms of Gas1p at the nonpermissive temperature for growth (37°C). Equivalent amounts of WCEs from the indicated strains (grown at 25 and 37°C) were resolved by SDS–PAGE and Gas1p was detected by immune staining with an anti-Gas1p antibody. The mature form of Gas1p (mGas1p) is indicated with an arrow. Pgk1p serves as a gel loading control. (G) mNeon-Gas1p is localized to the cell surface, vacuole, and internal structures in *cdc1-22* and *cdc1-26* cells grown at 25°C. (H) *cdc1-23* and *cdc1-26* cells accumulate ~25–30% more Proteinase K–resistant Gas1p than WT cells when grown at 37°C. (I) Quantification of the data shown in H; see *Materials and Methods* for details.

cdc1-314 and *cdc1-310* harbor two amino acid substitutions, T377I and P441S (*cdc1-314*) and L356F and P441S (*cdc1-310*) (Losev *et al.*, 2008; Vazquez *et al.*, 2014). However, in the context of our WT *CDC1* gene (which does not contain the P441S substitution; yeastgenome.org), neither the T377I (*cdc1-314*) nor the L356F (*cdc1-310*) substitution (both of which are located in the enzymatic region of Cdc1p and therefore in the lumen of the Golgi) could confer temperature sensitivity (Figure 3A).

A previous study that employed *cdc1-314* revealed that the cell wall and actin polarization defects of this ts mutant were osmoremedial, but that the growth defect was not. This study also showed that the function of Cdc1p was to remove EtNP from Man1 of GPI-APs and presumably, like the reversible modification of Man2 by EtNP by Ted1p, Cdc1p functioned in quality control surveillance of GPI-AP processing. However, unlike the reversible modification of Man2, the reversible modification of Man1 does not affect the export of GPI-APs from the ER, an observation that is consistent with our findings that Cdc1p is a Golgi-resident membrane protein.

Genetic interaction studies with *cdc1-314* (Vazquez *et al.*, 2014), as well as other alleles of *cdc1* (Paidhungat and Garrett, 1998), are also somewhat confounding—as *cdc1* mutants show strong synthetic negative interactions with some ER-resident GPI remodelases but positive interactions with others (Vazquez *et al.*, 2014).

Given the apparently perplexing data for *CDC1*, we chose to reexamine the function of this gene by generating a novel collection of temperature-sensitive alleles in BY4741 cells (*cdc1-21–cdc1-26*; see Figure 3B). In agreement with previous reports, our *cdc1* alleles showed osmoremedial temperature-sensitive phenotypes (unlike *cdc1-314*; Figure 3C) as well as sensitivity to CFW (Figure 3D)—data that are in agreement with the established role for Cdc1p in some facet of cell wall integrity. Our *cdc1* alleles also accumulated a preponderance of cells at the restrictive temperature with small buds and polarization defects (Figure 3E), an observation that is consistent, in part, with the original description of this gene as being required for the cell division cycle (Hartwell, 1971). None of our *CDC1* temperature-sensitive alleles displayed a delay in the export of Gas1p from the ER, unlike deletion of several other genes whose products function in GPI-AP remodeling in the ER (Figures 3F and 1B).

Given that *cdc1* mutants do not display a kinetic delay in the export of Gas1p from the ER, we next sought to address the fate of GPI-APs from which EtNP from Man1 has not been removed. *cdc1-314* cells have been shown to accumulate GPI-APs in puncta, suggesting that improperly modified GPI-APs cannot be transported efficiently to the cell surface (Vazquez *et al.*, 2014). To examine the fate of GPI-APs in our *cdc1* alleles, we employed mNeon-GPI

(Figure 2H; see Supplemental Table S2). Unlike *ted1*Δ cells, which showed the accumulation of mNeon-GPI in the ER (in addition to the vacuole and cell surface), WT cells and our *cdc1* alleles showed predominantly vacuolar and cell surface accumulation of mNeon-GPI when grown at 25°C (Figure 3G)—a temperature at which these mutants show sensitivity to CFW (Figure 3D) and hence exhibit a partial loss of function. For technical reasons, we could not assess the fate of *GAL1*-driven mNeon-GPI at 37°C, as our *cdc1* alleles, as well as WT cells, showed accumulation of mNeon-GPI in the ER—an observation that may be due to saturation of the ER export machinery but in any case is not consistent with the fate of endogenous Gas1p in these mutants (Figure 3F).

To establish the extent to which GPI-APs accumulated within the cell in our *cdc1* alleles, we took a biochemical approach. Two *cdc1* alleles were selected that showed the most robust ts phenotypes (*cdc1-23* and *cdc1-26*). Cells were grown at the permissive and restrictive temperatures and thereafter incubated with Proteinase K in order to degrade any cell surface–localized Gas1p, the assumption being that Gas1p that was resistant to digestion by Proteinase K was inside the cell (Figure 3H). When grown at 25°C, both WT and *cdc1-23* cells showed more or less the same amount of intracellular Gas1p (~60%; Figure 3I). However, *cdc1-26* cells showed a substantially greater amount. The data obtained for *cdc1-26* cells at 25°C are perplexing and difficult to explain (see below), but at the very least, *cdc1-26* cells likely exhibit a partial loss of function at 25°C. In contrast, when grown at the restrictive growth temperature (37°C), both *cdc1* alleles accumulated roughly the same amount of intracellular Gas1p (~86-91%), whereas the level in WT cells was more or less the same at 25° and 37°C (~60%).

How might the data presented in Figure 3, H and I be explained? The Proteinase K–resistant Gas1p is unlikely to represent significant pools of ER or early Golgi-localized protein. Cells deficient in Golgi-resident mannosyltransferases activities typically contain incompletely mannosylated Gas1p, which migrates more quickly on SDS–PAGE gels than Gas1p from WT cells and is distinct from the ER form of the protein (Tu *et al.*, 2008). The lack of pGas1p and the apparent lack of any anomalously glycosylated Gas1p in our immune-staining experiments suggests that this intracellular pool of Gas1p has transited the Golgi. Presumably, then, the Proteinase K–resistant pool of Gas1p corresponds to Gas1p that has left the ER, has transited the Golgi, and is en route to the plasma membrane (Figure 3H). While it is possible that this Proteinase K–resistant pool represents Gas1p that is kinetically delayed in export from the Golgi, unlike a previous report (Vazquez *et al.*, 2014), we were unable to establish whether any of this internal Gas1p localized to puncta in cells.

Additionally, we cannot exclude the possibility that some of this protease-resistant protein represents Gas1p that has been missorted to the vacuole (either from the Golgi or via endocytosis from the plasma membrane). This prospect seems somewhat unlikely to us, however, as Gas1p that had been mislocalized to the vacuole would presumably be susceptible to degradation via vacuolar proteases. A relatively small portion of Gas1p is known to be cleaved and covalently linked to the cell wall (Rolli *et al.*, 2009), and *cdc1* mutants have been shown to be defective in this process (Vazquez *et al.*, 2014). While it is possible that Gas1p that cannot be incorporated into the cell wall is endocytosed, it seems doubtful that such an endosomal pool could entirely account for the differences in protease resistant Gas1p observed between WT cells and our *cdc1* mutants (which is ~30%). Nevertheless, and consistent with an earlier report (Vazquez *et al.*, 2014), it appears that our ts *cdc1* mutants accumulate a pool of intracellular Gas1p. It therefore seems plausible that the removal of EtNP from Man1 contributes to the sorting of GPI-APs in the late secretory pathway.

Importantly, however, our *cdc1* mutants are not completely defective in the delivery of remodeling-defective GPI-APs to the plasma membrane, and therefore the phenotype of the *cdc1* mutants may also be a reflection of the presence of GPI-APs bearing EtNP on Man1 at the plasma membrane rather than the underdelivery of such proteins to the plasma membrane.

Cdc1p's enzymatic activity is restricted to the Golgi

If Cdc1p exclusively processes its substrates in the Golgi, it seems plausible that substrate recognition might require the prior enzymatic action of Ted1p—the ultimate ER-localized remodelase that removes EtNP from Man2 of GPI-APs (Manzano-Lopez *et al.*, 2015). If this were so, then one might expect *ted1*Δ *cdc1* mutants to show a synthetic growth phenotype, and this is precisely what we observe (Figure 4A). This synthetic growth phenotype can be suppressed by supplementing growth media with 1M sorbitol, and therefore seems likely to be due to a cell wall defect caused by the synthetic processing defects of GPI-APs in *ted1*Δ *cdc1* cells. If Cdc1p cannot process GPI-Aps, that have not previously undergone remodeling in the ER, then redistributing the cell's pool of Cdc1p to the ER should result in lethality. To address this prospect, we generated a yeast strain in which the endogenous copy of *CDC1* was modified to encode a C-terminal HDEL motif. When the HDEL receptor (Erd2p) was concomitantly overexpressed in these cells, mNeon-Cdc1p-HDEL was localized to the ER rather than to puncta (Figure 4B); however, these cells were unable to grow in the absence of WT Cdc1p (Figure 4C). When expressed using the triose phosphate isomerase (TPI) promoter, mNeon-Cdc1p-HDEL was localized to the ER and to puncta (Figure 4D), and TPI-driven mNeon-*CDC1*-HDEL could support the growth of *cdc1*Δ cells (although these strains showed a modest ts growth phenotype; Figure 4E). From these results, we surmised that Cdc1p cannot remove EtNP from Man1 of GPI-APs in the ER, either because substrate recognition requires that the ER remodelases have already successfully modified GPI-APs, or alternatively because premature removal of EtNP from Man1 impairs the proper processing of GPI-APs in the ER. In any case, ER-localized Cdc1p seems unlikely to impair processing of ER-localized GPI-APs in general, as we saw no evidence of a synthetic increase of steady state levels of pGas1p in such cells, nor did transformants display any dominant negative growth phenotype (Figure 4F and results not shown). Moreover, Cdc1p's substrate specificity does not appear to extend to removal of EtNP from Man2, as localization of Cdc1p-HDEL to the ER did not result in any reduction in pGas1p levels in *ted1*Δ cells (Figure 4F).

Why do yeast cells only remove EtNP from Man1 of GPI-APs once they have reached the Golgi? The block in transport to the cell surface may provide a clue. Removal of EtNP from Man1 may play a role in sorting of GPI-APs into a distinct pool of exocytic vesicles, much as removal of EtNP from Man2 functions to segregate GPI-AP proteins into p24 protein–containing COPII vesicles (Muñiz and Riezman, 2016). Indeed, an earlier study reported that GPI-APs accumulated in internal puncta-like structures (Vazquez *et al.*, 2014). Our biochemical experiments are consistent with this observation, as *cdc1-23* and *cdc1-26* cells accumulated ~30% more Proteinase K–resistant mGas1p than WT cells when grown at 37°C (Figure 3, H and I); however, the precise location of this pool of intracellular Gas1p remains to be established.

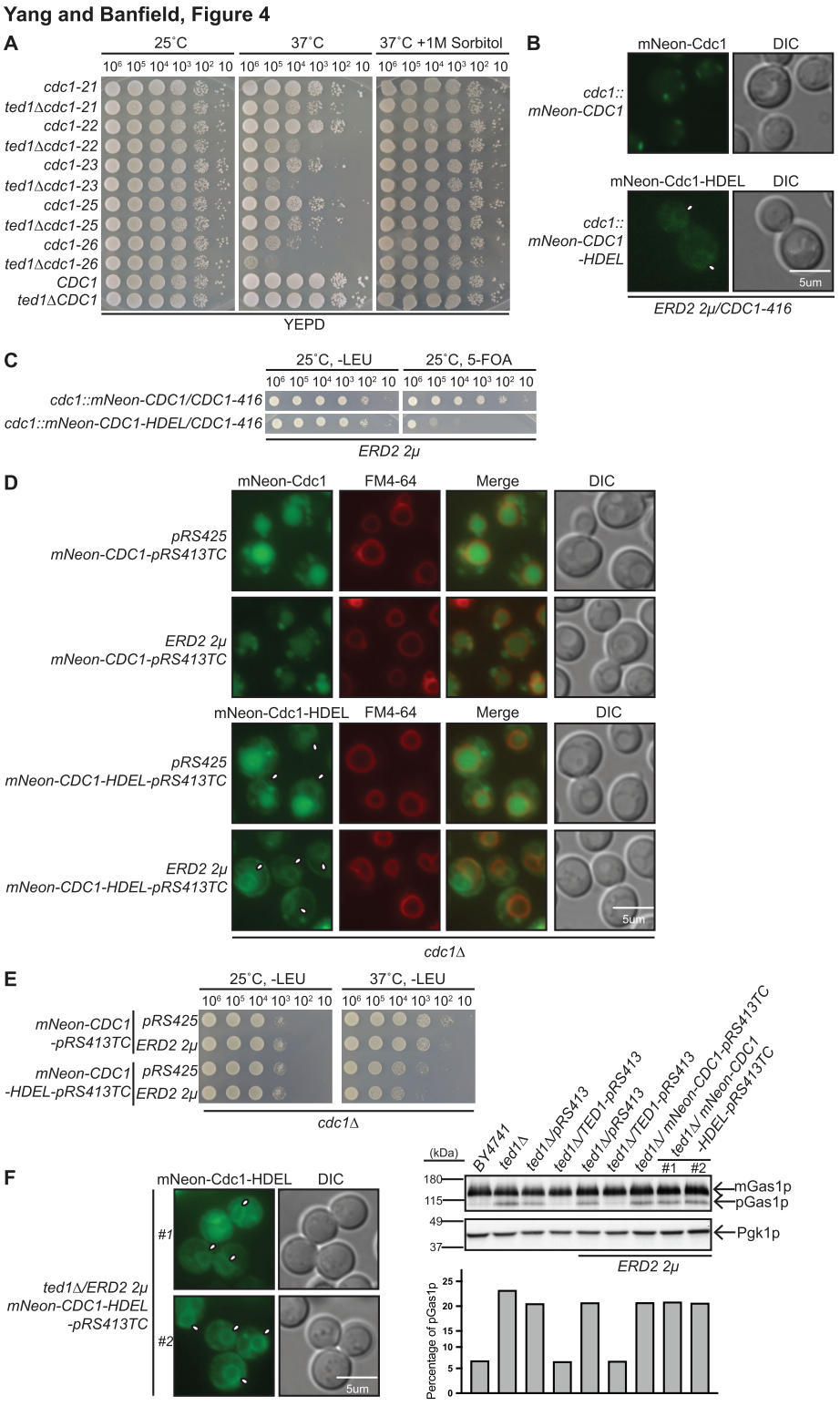

FIGURE 4: Cdc1p's enzymatic activity is restricted to the Golgi. (A) *cdc1 ted1*Δ cells show an osmo-remedial synthetic temperature-sensitive phenotype. Tenfold serial dilutions of the indicated strains were spotted onto YEPD plates with or without 1M sorbitol and incubated at 25 and 37°C for 48 h before being photographed. (B) mNeon-Cdc1-HDEL is localized to the ER in cells that concomitantly overexpress Erd2p (2μ *ERD2*). White arrowheads indicate ER membranes. (C) Cells expressing endogenous levels of mNeon-Cdc1-HDEL that concomitantly overexpress Erd2p (2μ *ERD2*) cannot support the growth *cdc1*Δ cells. Tenfold serial dilutions of the indicated strains were spotted onto plates with or without 1 mg/ml 5-FOA and incubated at 25°C for 72 h before being photographed. (D) When overexpressed, mNeon-Cdc1-HDEL is localized to the ER and puncta. (E) When overexpressed, mNeon-Cdc1-HDEL can support the growth *cdc1*Δ cells. Tenfold serial dilutions of the indicated strains were spotted onto SD-Leu plates and incubated at 25 and 37°C for 48 h before being photographed. (F) ER-localized Cdc1p does not have a dominant negative effect on the processing of Gas1p. Cells expressing mNeon-Cdc1-HDEL from the TPI promoter in the presence of a 2μ *ERD2* plasmid (ER-localized Cdc1p) do not show a synthetic accumulation of the precursor form of Gas1p (pGas1p). WCEs from the indicated strains (including two isolates of mNeon-Cdc1p-HDEL + 2μ *ERD2*-expressing cells) were resolved by SDS–PAGE and Gas1p detected by immune staining with an anti-Gas1p antibody. The mature form (mGas1p) and precursor form (pGas1p) are indicated with arrows. Pgk1p serves as a gel loading control. The presence of pGas1p detected in each strain is represented graphically below the immune-staining experiment. White arrowheads indicate ER membranes.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains used in this study are listed in Supplemental Table S1 and the plasmids used in this study are listed in Supplemental Table S2.

Reagents

Restriction enzymes and Q5 high-fidelity DNA polymerase were purchased from New England Biolabs. 5-Fluoroorotic acid (5-FOA) was purchased from Zymo Research. CFW (Fluorescent brightener 28) and sorbitol were purchased from Sigma. Concanavalin-A was purchased from Sigma. *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl-hexatrienyl) pyridinium dibromide (FM 4-64) was purchased from Invitrogen. EDTA-free protease inhibitor cocktail tablets and Pefabloc SC were purchased from Roche. Glutathione Sepharose 4B was purchased from GE Healthcare. Ponceau S was purchased from Sigma. α-factor was purchased from Sigma. The anti-Pgk1p antibody was purchased from Molecular Probes. The anti-Myc antibody was purchased from Roche. The anti-mouse IgG secondary antibody and the anti-rabbit IgG secondary antibody were purchased from Sigma. The anti-yeast coatomer antibody was kindly provided by Anne Spang (University of Basel). The anti-Gas1p antibody was kindly provided by Howard Riezman (University of Geneva).

Generation of temperature-sensitive alleles of CDC1

Temperature-sensitive alleles of *CDC1* were generated by an error-prone PCR-based approach (Muhlrad *et al.*, 1992). A genomic

DNA fragment comprising the coding sequence plus 226 base pairs (bp) 5′ of the initiator ATG and 93 bp 3′ of the stop codon was cloned into pRS413. The error-prone PCR reaction was conducted using a pair of primers: RGO2 (5′-GTGTGGAATTGTGAGCGGAT -3′) and RGO5 (5′-AGGGTTTTCCCAGTCACGAC-3′). Mutagenized DNA generated by the PCR was cotransformed into SARY4724 cells (see Supplemental Table S1) together with *Bam*HI–*Hind*III digested pRS413, and recombinants were selected on SD-histidine plates following in vivo homologous recombination.

The transformants selected on SD-histidine plates were patched onto plates containing 5-fluoro-orotic acid (5-FOA, 1 mg/ml), in duplicate. One set was incubated at 25°C and the other at 37°C to identify temperature-sensitive mutants.

Calcofluor white sensitivity test

Yeast cells were grown to an OD_{660} of ~0.6–0.8 in appropriate medium, after which the cell number of each strain was adjusted to ~1×10^6 cells/5 μL. Tenfold serial dilutions of cell suspensions were prepared and 5 μL of each strain was spotted onto YEPD plates and YEPD plates containing 50 μg/mL CFW. Plates were then incubated at 25°C for 48–72 h before being photographed.

Quantification of colocalizing puncta for mNeon-Cdc1p and Cdc1p-mNeon

For each colocalization set (e.g., mNeon-Cdc1p/Mnn9p-mScarlet), at least 100 cells were examined ($N = 3$). The distribution of puncta was assessed in each case, and colocalization was scored as positive if puncta overlapped when images were merged.

Proteinase K–mediated protease assay

Cells were grown at 25 or 37°C in YEPD medium to an OD_{660} of ~0.6, harvested, and resuspended in Buffer 1 (200 mM Tris-HCl, 20 mM EDTA, 1% β-mercaptoethanol, 10 mM NaF, 10 mM NaN_3). Cells were incubated at 30°C for 10 min, followed by centrifugation at 500 × *g* for 3 min, after which the supernatant was discarded. Then the cells were washed with Buffer 2 (YEPD with 1M sorbitol, 10 mM NaF, 10 mM NaN_3) and resuspended in Buffer 2 supplemented with 0.15 mg/ml Zymolyase 20T and incubated at 30°C for 1 h to generate spheroplasts. Spheroplasts were washed twice with Buffer 2 and incubated with 200 μg/ml proteinase K (in Buffer 2) at 30°C for 1 h. Protease digestion was arrested by the addition of 1 mM Pefabloc SC and incubation at 4°C for 10 min. Proteins were precipitated by the addition of 15% vol/vol trichloroacetic acid and analyzed by SDS–PAGE and immune staining.

α-Factor treatment

Yeast cells were grown at 25°C to an OD_{660} of ~0.4 in YEPD (pH 4) and then treated with 10 μM α-factor (in YEPD, pH 4) for 3 h at 25°C before the growth temperature was shifted to 37°C for 30 min. Cells were then washed three times with prewarmed YEPD (37°C) and incubated at 37°C in YEPD for 1 h. Cells were mounted onto Concanavalin A–coated slides, examined by epifluorescence microscopy, and photographed immediately thereafter.

Preparation of yeast whole cell extracts for the Gas1p processing assay

Yeast cells were grown to an OD_{660} of ~0.6 –0.8 in appropriate medium from which ~1×10^7 cells were collected by centrifugation and resuspended in 100 μL SDS–PAGE sample buffer (1× EDTA-free protease inhibitors, 5 mM dithiothreitol [DTT], and 1 mM Pefabloc SC) containing ~30 μL of acid-washed glass beads. Yeast cells were lysed on a vortex mixer at 4°C for 5 min and thereafter heated to 95°C for 5 min.

Calculation of the percentage of Proteinase K–resistant Gas1p

Integrated densities of band intensities from immune-staining experiments were obtained using the NIH image processing program (ImageJ). The relative amount of mGas1p present in each lane was standardized by comparing the integrated densities of the bands corresponding to mGas1p with that of Pgk1p. The percentages of the relative amounts of mGas1p in cells with or without Proteinase K treatment were calculated from data presented in Figure 3H, and these values correspond to the percentage of Protease K–resistant Gas1p plotted in Figure 3I.

Immune staining

Proteins were resolved by SDS–PAGE and thereafter transferred to nitrocellulose membranes. Membranes were incubated in PBST (PBS, 0.1% vol/vol Tween, 5% wt/vol milk powder) for 1 h. Membranes were then incubated with appropriately diluted primary and secondary antibodies for 1 h. Membranes were washed three times in PBST following each antibody incubation. Detection was performed using enhanced chemiluminescence and autofluography.

Labelling yeast cell membranes with FM4-64

Yeast cell cultures were grown to an OD_{660} of ~0.5, from which 1 ml of cells were collected by centrifugation and resuspended in 50 μL YEPD containing 30 μM of FM4-64. After incubation at 25°C for 30 min, cells were harvested by centrifugation, washed once with YEPD (to remove excess FM4-64), and then incubated in 1mL YEPD at 25°C for 90–120 min. Following FM4-64 treatment, cells were mounted on Concanavalin A–coated slides, examined by epifluorescence microscopy, and photographed immediately thereafter.

Expression and purification of GST-fusion proteins

GST-fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells. Protein expression was induced when cell cultures reached an OD_{600} of 0.9 by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and incubation at 37°C for 3 h. Following protein induction, bacterial cultures were harvested and resuspended at ~80 OD_{600} per mL in Buffer A (25 mM Tris-HCl, 400 mM KCl, 10% glycerol, 4% Triton-X 100, pH 7.5) supplemented with 1× EDTA-free protease inhibitor, 5 mM DTT, and 1 mM Pefabloc SC. Cells were lysed using a constant cell disruption system (Constant System) at 25 kpsi with a precooled chamber. Bacterial lysates were cleared by centrifugation at 16,000 × *g* for 10 min at 4°C.

In vitro pulldown assays

Yeast cells were grown to an OD_{660} between 0.6 and 0.8 in appropriate medium and resuspended at ~100 OD_{660} units/mL in Buffer P (PBS containing 0.1% Triton-X-100) supplemented with 1× EDTA-free protease inhibitor, 2 mM DTT, and 1 mM Pefabloc SC. Yeast cells were lysed using acid-washed glass beads and a Vortex mixer. Following lysis, homogenates were subjected to centrifugation at 13,000 × *g* for 10 min at 4°C. The resulting supernatant served as the yeast WCEs for the pulldown experiments. Purified GST-fusion proteins prepared as described above were added to a suspension of glutathione Sepharose 4B beads preequilibrated with Buffer A (25 mM Tris-HCl, 400 mM KCl, 10% glycerol, 4% Triton X-100, pH 7.5) supplemented with 1× EDTA-free protease inhibitor, 5 mM DTT, and 1 mM Pefabloc SC at 4°C for 2 h with continuous gentle rotation. The beads were then washed two times with Buffer A and two

times with Buffer P. GST-fusion proteins bound to glutathione Sepharose 4B beads were incubated with yeast WCEs for 2 h at 4°C. After incubation, the beads were washed four times with Buffer P, and bound proteins were identified following SDS–PAGE by immune staining. GST-fusion proteins were visualized by Ponceau S staining of nitrocellulose membranes.

URA3+ counterselection assay using 5-fluoro-orotic acid

To assess the functional consequences of gene variants and/or dosage suppressors of temperature-sensitive alleles, yeast strains were grown to an OD_{660} of ~0.6–0.8 in appropriate medium. Tenfold serial dilutions were prepared thereafter, and cells were spotted onto plates containing 1 mg/ml 5-FOA. The 5-FOA-containing plates were then incubated at either 25 or 37°C (as required) for 48–72 h before being photographed.

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