Degradation of Specific Nuclear Proteins Occurs in the Cytoplasm in *Saccharomyces cerevisiae*

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ABSTRACT The ubiquitin/proteasome system has been characterized extensively, although the site of nuclear substrate turnover has not been established definitively. We report here that two well-characterized nuclear proteins are stabilized in nuclear export mutants in *Saccharomyces cerevisiae*. The requirement for nuclear export defines a new regulatory step in intracellular proteolysis.

THE proteasome plays a vital role in diverse cellular functions (Glickman and Ciechanover 2002). Proteasome subunits and subcomplexes are detected in the nucleus, suggesting that proteolysis can occur within (Reits *et al.* 1997; Enenkel *et al.* 1998; Enenkel *et al.* 1999; Russell *et al.* 1999b; Laporte *et al.* 2008). However, it is unclear if proteasome subunits are assembled into intact complexes that can catalyze degradation, and the site of substrate turnover has not been investigated systematically.

The Sts1 protein traffics proteasomes to the nucleus. Sts1 binds the nuclear import factor Srp1 and the proteasome subunit Rpn11, which together provide a mechanism for targeting proteasomes to the nucleus (Chen *et al.* 2011). A defect in this mechanism is lethal (*sts1-2*) and causes rapid accumulation of multiubiquitinated (multi-Ub) proteins and the stabilization of proteasome substrates (Romero-Perez *et al.* 2007). Sts1 is distantly related to a *Schizosaccharomyces pombe* protein, Cut8, which also promotes the targeting of proteasomes to the nuclear surface (Takeda and Yanagida 2005; Takeda *et al.* 2011). We questioned if nuclear substrates were exported to proteasomes on the nuclear surface. We found that the DNA repair protein Rad4 and DNA polymerase subunit Cdc17 were both stabilized in *sts1-2* and well-characterized nuclear export mutants. In contrast, a cytosolic substrate was efficiently degraded in these export mutants. These studies demonstrate that the nuclear export pathway plays an important role in the degradation of specific nuclear substrates of the proteasome.

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Results and Discussion

Proteasome mislocalization stabilizes nuclear proteins

Proteasome subunits in the regulatory (Rpn11–GFP) and catalytic particles (Pup1–RFP) were both localized to the nucleus in *STS1* at 37°, but were strongly mislocalized in *sts1-2* (Figure 1A), consistent with earlier work (Chen *et al.* 2011). The colocalization of proteasome subunit Rpn11–GFP with the nucleus was confirmed by DAPI staining (Figure 1B). To test the effect of proteasome mislocalization we compared the stabilities of nuclear substrates Rad4–HA and Cdc17–HA. We found that both proteins were degraded efficiently in *STS1*, but were stabilized in *sts1-2* (Figure 1, C and D). Protein levels were quantified (Figure 1, E and F). The stabilities of the shuttle-factor Rad23, proteasome subunit Rpn12, and spindle pole body component Cdc31 are unaffected in *sts1-2*. We also measured the turnover of a nonnuclear protein, Deg1–FLAG–Sec62; an engineered endoplasmic reticulum-associated protein that is degraded by cytosolic proteasomes (Rubenstein *et al.* 2012). Deg1–FLAG–Sec62 was degraded in both *STS1* and *sts1-2* (Figure 1G), indicating that Sts1 specifically promotes the turnover of nuclear proteins.

Nuclear substrates are stabilized in export mutants

The stabilization of nuclear substrates in *sts1-2* could be due to either the lack of proteasomes in the nucleus or the failure to translocate nuclear proteins to cytosolic proteasomes. The rapid stabilization of nuclear proteins in *sts1-2* following

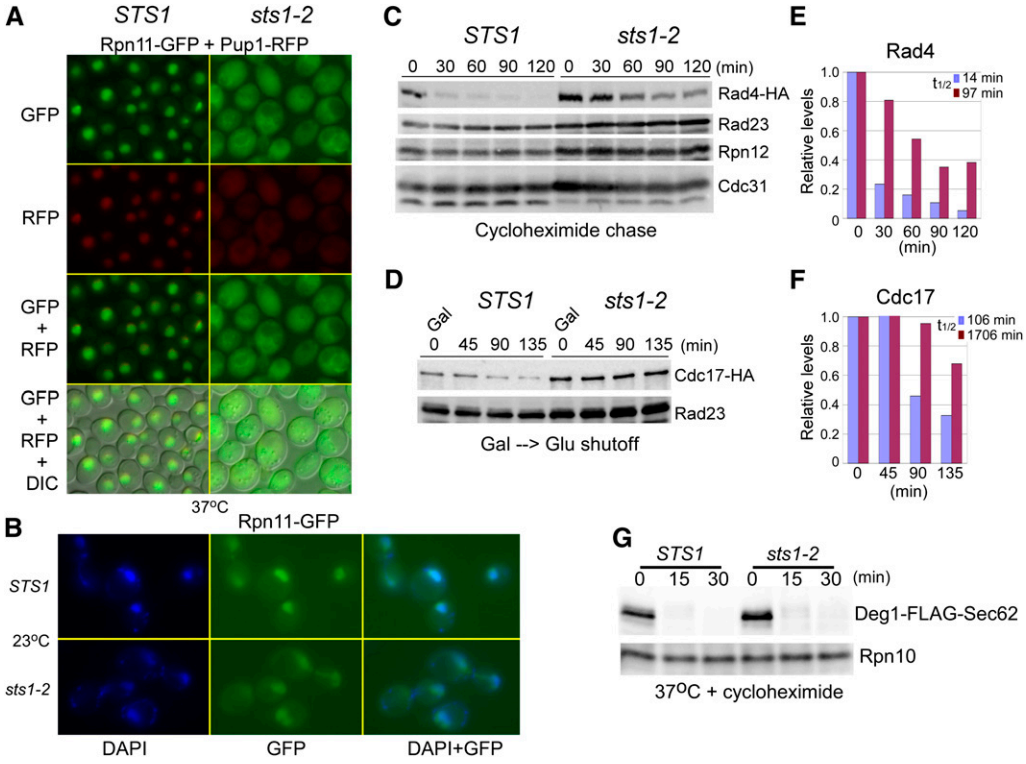

Figure 1 Nuclear substrates are stabilized in *sts1-2*. (A) The localization of proteasome subunits Rpn11–GFP (19*S*) and Pup1–RFP (20*S*) was investigated in *STS1* and *sts1-2*. Both subunits were nuclear localized in *STS1*, but were mislocalized in *sts1-2*. A merged image including DIC is also shown. (B) The colocalization of proteasome subunit Rpn11–GFP with the nucleus was confirmed by staining *STS1* and *sts1-2* cells with DAPI. (C and D) We compared the stability of HA-tagged nuclear proteasome substrates (Rad4–HA; Cdc17–HA) in *STS1* and *sts1-2*. Cultures expressing Rad4–HA were transferred from 23° to 37°, and cycloheximide was added. Protein extracts were prepared at the times indicated and analyzed by immunoblotting. The filter was probed with antibodies against HA, Rad23, Rpn12, and Cdc31. Cdc17–HA was expressed from the regulated *GAL1* promoter at 23°, expression was inhibited after transfer to glucose medium at 37°, and samples were examined at the times indicated. The blot was treated with antibodies against HA and Rad23. The results in C and D were quantified by densitometry and values representing one of three independent studies are shown (E and F). Nuclear substrate levels were standardized to the level of the loading control, and the 0-min value was set to 1.0. (G) The stability of the cytosolic proteasome substrate Deg1–FLAG–Sec62 was examined in *STS1* and *sts1-2* (at 37°), after the addition of cycloheximide.

transfer to the nonpermissive temperature was unexpected, because continued degradation should have occurred if proteasomes had entered the nucleus before the temperature shift. We therefore questioned if substrates were exported out of the nucleus to proteasomes, which may be located at the nuclear surface.

Xpo1/Crm1 can export nuclear proteins that contain a nuclear export sequence (NES) (Stade *et al.* 1997; Maurer *et al.* 2001). The *crm1*^{T539C} allele shows an export deficiency in the presence of the drug leptomycin B (LMB) (Neville *et al.* 1997; Kudo *et al.* 1999), whereas the *xpo1-1* mutant shows an export defect at 37° (Maurer *et al.* 2001). Both Rad4–HA and Cdc17–HA were stabilized in *crm1*^{T539C} after pharmacological inhibition of export (+ LMB; Figure 2, A and B). Both proteasome substrates were efficiently degraded in *CRM1*. Protein levels were quantified by densitometry (Figure 2, C and D). We confirmed that proteasomes were intact and functional in *crm1*^{T539C} (Figure 2E), and as expected no peptidase activity was detected in proteasome mutant *pre1-1 pre2-2*. We also confirmed that proteasomes were efficiently targeted to the nucleus in *crm1*^{T539C} (supporting information, Figure S1A). A nonnuclear protein, Deg1–FLAG–Sec62, was efficiently degraded in *crm1*^{T539C} (Figure 2F), indicating that the export pathway is not required for the turnover of this cytosolic protein. Similarly, Cdc17–HA was stabilized in *xpo1-1* mutant at 37° when nuclear export is rapidly and irreversibly inhibited, but not in *XPO1* (Figure 2G). Protein levels were quantified, and two- to threefold stabilization of Cdc17–HA was seen in *xpo1-1* (Figure 2H). The stability of Deg1–FLAG–Sec63 was unaffected in *xpo1-1* (data not shown).

Nuclear substrates are stabilized if they are not released Into the cytosol

The nuclear pore is irreversibly sealed in *nup116-5* at 37° (Wente and Blobel 1993). Exported proteins become trapped in hernias that are formed around the nuclear pore, by an expansion of the nuclear envelope. If proteasomes operated inside the nucleus, substrates might be expected to be degraded even after the pores were sealed. However, if substrates were exported and degraded outside the nucleus they would be stabilized *nup116-5*. We determined that both Rad4–HA and Cdc17–HA were stabilized in *nup116-5* (Figure 3, A and B). Because significant time (~1 hr) was required to seal the nuclear pores in *nup116-5* we observed weaker stabilization (Figure 3C and D) than seen in *sts1-2* and *crm1*^{T539C} (Figure 1, C and D, and Figure 2, A and B). Nonetheless, the stabilization of Rad4–HA and Cdc17–HA in *nup116-5* raises the possibility that the degradation of other proteins might also involve nuclear export. In agreement, higher levels of multiubiquitinated proteins were detected in *sts1-2* and export mutants (Figure 3E; also Chen *et al.* 2011), demonstrating that the stabilization of Rad4–HA and Cdc17–HA reveals a more widespread defect in overall nuclear proteolysis.

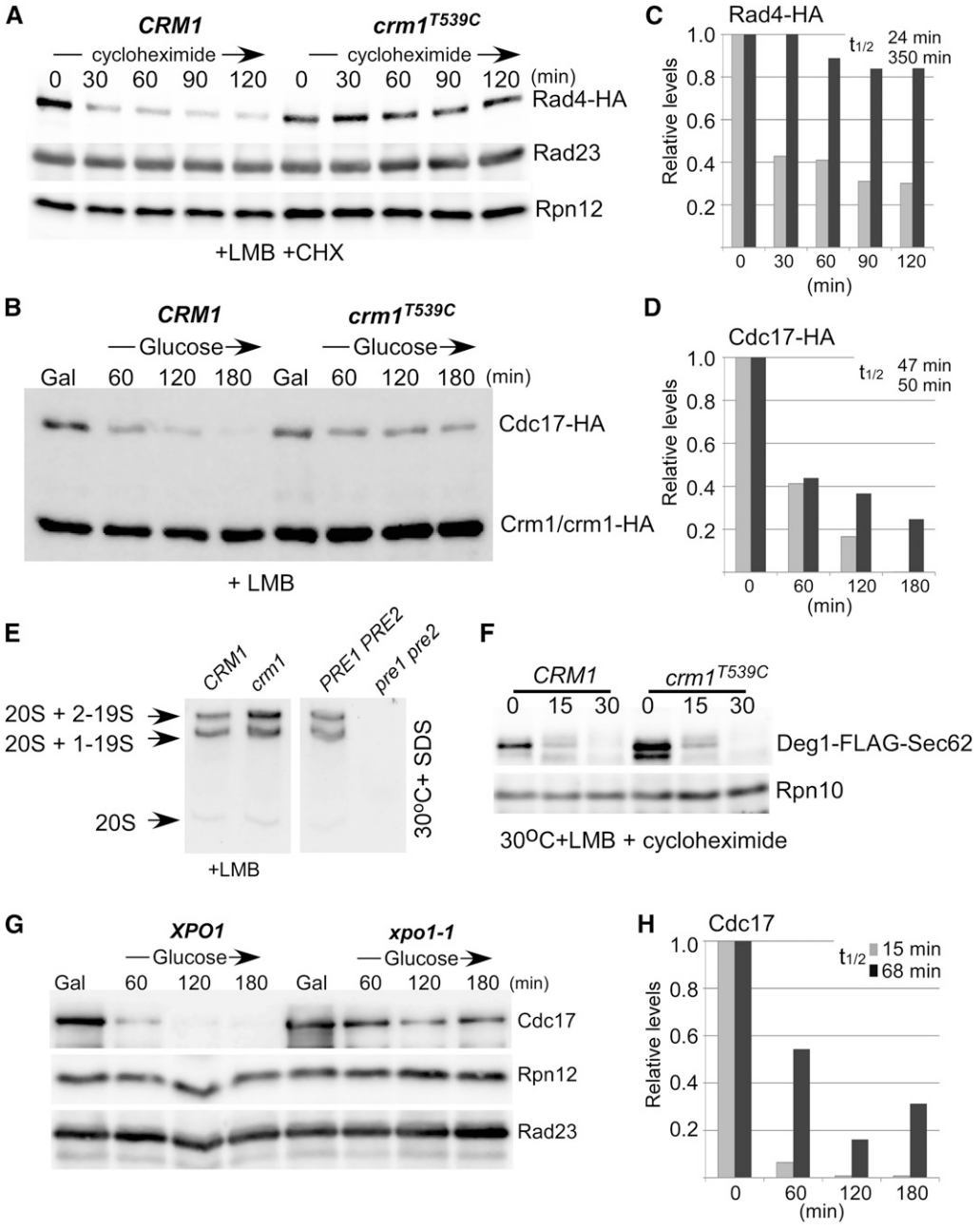

Figure 2 Stabilization of proteasome substrates in nuclear export mutants. (A and B) Epitope-tagged nuclear proteins (Rad4–HA and Cdc17–HA) were expressed in *CRM1* and *crm1*^{T539C}. LMB was added to actively growing cells at 30° and protein stability was determined after addition of cycloheximide to the growth medium. Both nuclear substrates were stabilized following inhibition of nuclear export (+ LMB in *crm1*^{T539C}). The levels of internal controls (Rad23, Rpn12, Crm1–HA) are shown. (C and D) Rad4–HA and Cdc17–HA levels were quantified by densitometry. (E) Proteasome integrity and functionality was confirmed using a native in-gel fluorogenic assay, in which the hydrolysis of a chymotryptic substrate (LLVY–AMC) was examined. Proteasome peptidase activity was similar in *CRM1* and *crm1*^{T539C}. As expected, no chymotryptic activity was detected in the proteasome mutant *pre1-1 pre2-2*. (F) The turnover of Deg1–FLAG–Sec62 was unaffected in *crm1*^{T539C}. (G) The stability of Cdc17–HA was also tested in the temperature-sensitive *xpo1-1* export mutant. Cdc17–HA was expressed from the galactose-inducible P_{GAL1} promoter at 23° and protein stability was examined at the nonpermissive temperature (37°), after transfer to glucose medium. (H) The level of Cdc17–HA was measured by densitometry.

The turnover of proteins by the ubiquitin/proteasome system has been characterized extensively. Nuclear substrates may be degraded inside the nucleus because proteasome subunits are detected in the nucleus and enriched in the nuclear envelope (Enenkel *et al.* 1998; Kruger *et al.* 2001; Savulescu *et al.* 2011). However, the site of nuclear protein turnover has not been examined systematically, and our findings show that the degradation of two well-studied nuclear proteins requires the export pathway.

Pathways that promote export include mechanisms that may (Richards *et al.* 1996) or may not require a NES (Liu and Defranco 2000; Lischka *et al.* 2001; Wiechens and Fagotto 2001). Although the exportin protein encoded by *CRM1/XPO1* plays a central role in nuclear export, other factors may also facilitate the export of specific classes of nuclear substrates of the proteasome. It is noteworthy that a requirement for nuclear export for the degradation of p53 (Freedman and Levine 1998), TRIP–Br2 (Cheong *et al.* 2008), β-catenin (Wiechens and Fagotto 2001), and hMSF5 (Lahaye *et al.* 2010) has been reported. These previous findings, along with our evidence that multi-Ub protein levels increase in export mutants (Figure 3E), indicate that the nuclear export system contributes broadly to the degradation of nuclear proteins. The proteins we examined are well-characterized native substrates of the proteasome. In contrast, the turnover of unfolded and damaged nuclear proteins (Gardner *et al.* 2005) might occur by a distinct mechanism.

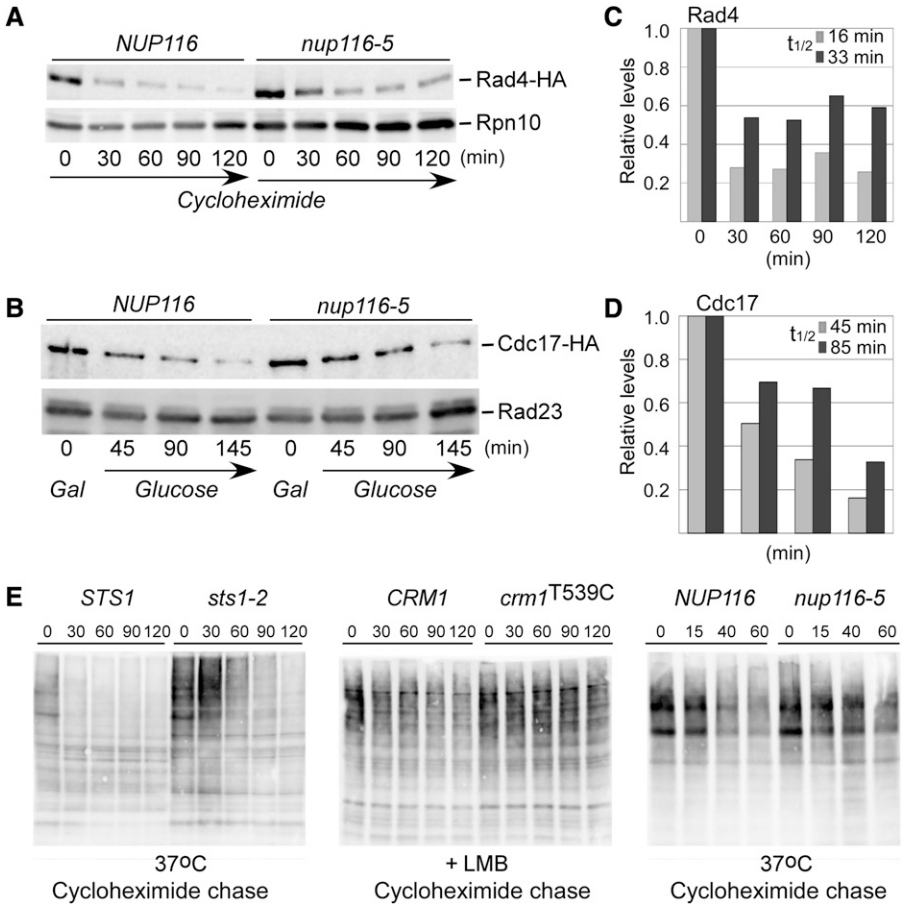

Figure 3 Nuclear substrates are stabilized when the nuclear pore is sealed. (A and B) Rad4–HA and Cdc17–HA were expressed in *NUP116* and *nup116-5*. Cells were transferred from 23° to 37° and stabilization of Rad4–HA and Cdc17–HA was seen in *nup116-5*. Internal controls are shown (Rpn10, Rad23). (C and D) The levels of Rad4–HA and Cdc17–HA were quantified by densitometry, and values were adjusted to the 0-min sample for each protein. (E) The levels of overall multiubiquitinated proteins was determined in *sts1-2* and nuclear transport mutants (*crm1*^{T539C} and *nup116-5*). Protein extracts were prepared from *STS1* and *sts1-2* at 37° after the addition of cycloheximide (left). High levels of multi-Ub species (ranging from <10 to >250 kDa) accumulated in *sts1-2*. Similarly, protein extracts examined after the addition of LMB showed higher levels of multi-Ub proteins in *crm1*^{T539C} (middle; + LMB) and in *nup116-5* at the nonpermissive temperature (right; 37°).

Nuclear substrates may become stabilized in *sts1-2* because proteasomes are unable to enter the nucleus. However, this interpretation is inconsistent with the requirement for nuclear export that we describe here. GFP-tagged proteasome subunits have been detected in the nucleus by many investigators, although their assembly into catalytically active complexes in the nucleus has not been demonstrated. This is an important consideration because proteasome subunits and subcomplexes can perform nonproteolytic roles in the nucleus (Russell *et al.* 1999a; Xie and Varshavsky 2001; Gonzalez *et al.* 2002).

Although the generality of our findings remains to be determined, the accumulation of multiubiquitinated proteins in export mutants indicates that many nuclear proteins may be stabilized and raises questions on the *bona fide* function of proteasome subunits in the nucleus. Our findings are relevant to higher organisms because of the strong conservation in the mechanisms of nuclear export and intracellular proteolysis. The proteasome has emerged as an important target in cancer treatment and its link to the nuclear export pathway offers a new venue for therapeutic drug discovery.

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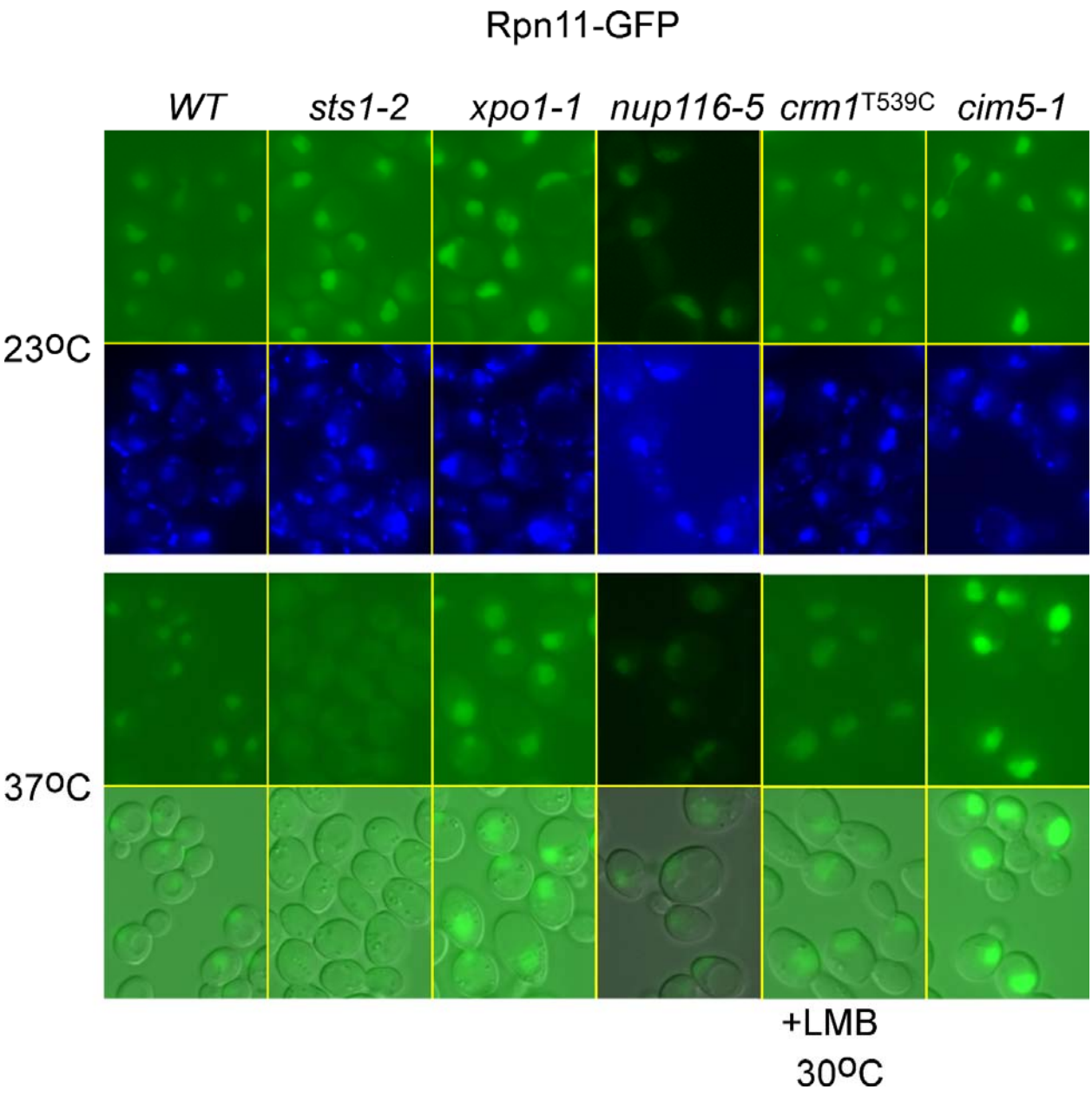

Figure S1 Proteasome localization is unaffected in nuclear export mutants. Genes encoding the 19S proteasome subunit Rpn11-GFP were integrated into the chromosome in the strains indicated, to ensure physiological levels of expression. Actively growing cells were examined by fluorescence microscopy. Temperature sensitive mutants and their corresponding wildtype counterparts were both shifted to the non-permissive temperature (37°C). *crm1*^{T539C} cells were grown at 30°C and either untreated (upper panels), or treated with LMB (lower panels). With the exception of *sts1-2*, proteasome localization to the nucleus was unaffected in all mutants strains examined. The co-localization of Rpn11-GFP with nuclei was confirmed by staining with DAPI at 23°C. (Cells grown at 37°C were not stained with DAPI due to structural fragility of some of the mutant cells).