Mutant Membrane Protein of the Budding Yeast Spindle Pole Body Is Targeted to the Endoplasmic Reticulum Degradation Pathway

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ABSTRACT

Mutation of either the yeast *MPS2* or the *NDC1* gene leads to identical spindle pole body (SPB) duplication defects: The newly formed SPB is improperly inserted into the nuclear envelope (NE), preventing the cell from forming a bipolar mitotic spindle. We have previously shown that both *MPS2* and *NDC1* encode integral membrane proteins localized at the SPB. Here we show that *CUE1*, previously known to have a role in *c*oupling *u*biquitin conjugation to *E*R degradation, is an unusual dosage suppressor of mutations in *MPS2* and *NDC1*. Cue1p has been shown to recruit the soluble ubiquitin-conjugating enzyme, Ubc7p, to the cytoplasmic face of the ER membrane where it can ubiquitinate its substrates and target them for degradation by the proteasome. Both *mps2-1* and *ndc1-1* are also suppressed by disruption of *UBC7* or its partner, *UBC6*. The Mps2-1p mutant protein level is markedly reduced compared to wild-type Mps2p, and deletion of *CUE1* restores the level of Mps2-1p to nearly wild-type levels. Our data indicate that Mps2p may be targeted for degradation by the ER quality control pathway.

IN the budding yeast *Saccharomyces cerevisiae*, the spindle pole body (SPB) functions as the sole microtubule-organizing center (Byers *et al.* 1978; Hyams and Borisy 1978). The SPB is a disc-like structure composed of six major layers, with the central layer, or plaque, in the same plane as the nuclear envelope (NE; Bullitt *et al.* 1997), which remains intact throughout the yeast cell cycle. From its position in the NE, the SPB nucleates microtubules from its cytoplasmic and nuclear faces. To set up a proper bipolar mitotic spindle, the SPB must be precisely duplicated during G1 of the cell cycle (Byers and Goetsch 1975). The process of SPB duplication is thought to occur through a conservative mechanism (Vallen *et al.* 1992; Pereira *et al.* 2001). The newly formed SPB, or duplication plaque, is assembled next to the existing SPB on the cytoplasmic side of the NE and then inserted into the NE by an unknown mechanism (Adams and Kilmartin 1999; O'Toole *et al.* 1999).

The *NDC1*, *MPS2*, and *BBP1* genes are required for a late step in SPB duplication (Winey *et al.* 1991, 1993; Schramm *et al.* 2000). Cells mutant for any of these genes contain duplicated SPBs, but the nascent SPB is aberrant. As a consequence, a bipolar spindle cannot be formed, and the spindle assembly checkpoint is activated (Hardwick *et al.* 1996). The cells arrest in mitosis with large buds and unsegregated DNA, all of which is associated with the functional SPB. The defective SPB lies on the cytoplasmic face of the NE, unable to nucleate nuclear microtubules. The aberrant structure is very similar to the SPB duplication plaque (Adams and Kilmartin 1999; O'Toole *et al.* 1999). It was originally proposed that Mps2p and Ndc1p function to insert the nascent SPB into the NE, allowing the inner plaque to form and nucleate microtubules on the nuclear face (Winey and Byers 1993). Recently, Ndc1p was shown to be a shared component of SPBs and nuclear pore complexes (NPCs; Chial *et al.* 1998), consistent with a direct role for Ndc1p in the insertion event. Similarly, the *MPS2* gene encodes an integral membrane protein that is localized at SPBs (Wigge *et al.* 1998; Munoz-Centeno *et al.* 1999). Mps2p physically interacts with Bbp1p, which appears to connect the SPB to the NE (Schramm *et al.* 2000).

Interestingly, *MPS2* was also isolated in a screen for genes that were toxic when overexpressed in cells harboring a mutation in the *CIM5* gene (Munoz-Centeno *et al.* 1999). *CIM5* encodes a proteasome subunit required for the G2/M transition (Ghislain *et al.* 1993). This screen was performed with the intention of finding substrates of Cim5p-containing proteasomes; however, the levels of Mps2p remained constant and did not fluctuate during the cell cycle. It was recently reported that green fluorescent protein (GFP)-Cim5p-labeled proteasomes are enriched in the NE/endoplasmic reticulum (ER) in living yeast cells, indicating that proteasomal degradation is concentrated at this compartment in yeast (Enenkel *et al.* 1998). Because Mps2p is also localized at the NE, it is possible that Mps2p may interact with the proteasome, even though it does not appear that Mps2p is a proteasome substrate (Munoz-Centeno *et al.* 1999).

To gain a better understanding of Mps2p function, we screened for dosage suppressors of the *mps2-1* tem-

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perature-sensitive growth defect. The results of this screen and subsequent experiments reveal that the Mps2-1p mutant protein may be a substrate of the ubiquitin-proteasome pathway associated with the ER. ER-assisted degradation (ERAD) is a mechanism by which misfolded, unassembled, and downregulated secretory and membrane proteins are transported in a retrograde manner via a translocon from the ER back into the cytosol, where they are ubiquitinated and degraded by a cytosolic proteasome (for reviews, see Kopito 1997; Sommer and Wolf 1997). How the cell achieves specificity for shuttling substrates through the translocon in a retrograde manner is under intense scrutiny. It is believed that the retrotranslocon pore contains Sec61p (Pilon *et al.* 1997; Plemper *et al.* 1997, 1998), a cognate member of the normal translocon observed in mammalian and yeast systems (Gorlich *et al.* 1992; Hartmann *et al.* 1994; Panzner *et al.* 1995). In addition, it has been postulated that the membrane proteins Der3p/Hrd1p, which is a ubiquitin ligase (Hampton *et al.* 1996; Bordallo *et al.* 1998; Bays *et al.* 2001), and Hrd3p (Hampton *et al.* 1996) form a dynamic complex around the Sec61p pore, rendering it a retrotranslocon distinct from the translocon (Plemper *et al.* 1999). Three other proteins, Cue1p, Ubc7p, and Ubc6p, act downstream of retrotranslocation, at the ER surface, to ubiquitinate the substrates (reviewed in Sommer and Wolf 1997). In this article, we report the finding that deletions of *CUE1*, *UBC7*, or *UBC6* suppress mutations in the NE integral membrane proteins Ndc1-1p and Mps2-1p. We also show that Mps2-1p abundance increases in cells lacking Cue1p and postulate that Mps2-1p may be targeted for degradation by the ERAD pathway.

MATERIALS AND METHODS

Yeast strains and media: The yeast strains used in this study are listed in Table 1. Yeast media, growth conditions, and genetic and molecular techniques were as previously described (Sambrook *et al.* 1989; Guthrie and Fink 1991). Yeast were transformed with plasmids using the EZ transformation kit (Zymo Research, Orange, CA). All yeast strains were grown in YPD media (1% yeast extract, 2% bactopeptone, and 2% glucose) or in synthetic media supplemented with the appropriate amino acids and 2% glucose. 5-Fluoroorotic acid (5-FOA)-containing plates were prepared as previously described (Boeke *et al.* 1987). Copper-containing plates were made by adding cupric sulfate (final concentration 100 μM) to autoclaved synthetic media, as described previously (Finley *et al.* 1994).

Strains SMY20-8c and -8b were isolated as the meiotic products of the *mps2-1, cue1*Δ*::HIS3* strain (SMY1655) crossed to a wild-type S288c strain (WX257-4c). The *cue1*Δ*::HIS3* strain (SMY20-8b) was crossed to *ndc1-1* (HC7-31c) to generate *ndc1-1, cue1*Δ*::HIS3* (SMY23-11c). The presence of the *ndc1-1* mutation (Thomas and Botstein 1986) was confirmed by sequencing a PCR product from the genomic DNA prepared from this strain. The *mps2-1* strain (SMY8-4b) was crossed to *ubc6::LEU2* to generate *mps2-1, ubc6::LEU2* (SMY33-1a). Identification of *mps2-1, ubc6::LEU2* doubles [which are not temperature sensitive (ts)] was determined by complementation of *UBC6* with a plasmid-borne copy and scoring temperature sensitivity. The *mps2-1* strain (SMY8-3a) was crossed to *ubc7::LEU2* to generate *mps2-1, ubc7::LEU2* (SMY31-1c). Identification of *mps2-1, ubc7::LEU2* doubles (which are not ts) was determined as above. The *ndc1-1* strain (HC5-8c) was crossed to *ubc7::LEU2* to generate *ndc1-1, ubc7::LEU2* (SMY32-4d). The *ndc1-1, ubc6::LEU2* double mutant (SMY59-2d) was generated by crossing *ndc1-1* (SMY47-2a) and *ubc6::LEU2*. Double mutants were scored as above.

Yeast strains harboring GFP-tagged versions of Ndc1p and Ndc1-1p were constructed by transforming HC14-10c with pRS304-*NDC1-GFP* and pRS304-*ndc1-1-GFP*, respectively, linearized with *Sph*I to target integration at the *ndc1*Δ*::kanMX* locus.

Mps2p and Mps2-1p were tagged with GFP at the C terminus by transforming BY4733 (wild type) and SMY72 (*mps2-1*), respectively, with a PCR product containing GFP, kanMX, and *MPS2*-flanking DNA, using pYM12 for a template (Knop *et al.* 1999) and primers S2-MPS2 and S3-MPS2. Transformants were selected on YPD plates containing G418.

High-copy suppressor screen: The *mps2-1* strain SMY6-4b (Table 1) was transformed with a 2μ, *URA3*-based yeast genomic library (Connelly and Hieter 1996) and plated on synthetic media lacking uracil. Approximately 16,000 transformants were replica plated onto YPD plates at 37°. Transformants that grew at 37° were grown in the presence of 5-FOA to determine which strains were dependent on the presence of the plasmid. Each strain was cured of the *URA3* plasmid by growth on 5-FOA and then challenged at the restrictive temperature. Plasmids were rescued into *Escherichia coli* from the 57 yeast strains that no longer grew at 37°, and it was determined by restriction digest which plasmids harbored unique inserts. Unique plasmids were reintroduced into the original strain, SMY6-4b, and plasmids that supported growth at the restrictive temperature (36°) were sequenced using the T7 and T3 primers by the DNA Sequencing Facility at the Molecular, Cellular, and Developmental Biology Department at the University of Colorado. Three unique plasmids conferred growth at 36°, one of which is reported here.

Disruption of *CUE1* and *HRD1*: The entire *CUE1* gene was replaced with either the *HIS3* or the *TRP1* genes by the one-step method (Baudin *et al.* 1993), using primers SUP2UP, 5′-GCATTACAATCTACGATCGCGCAAACTTTTTTCTTTTGGCCAGATTGTACTGAGAGTGCAC-3′ and SUP2DP, 5′-GCATTATGGGCACACTTGCGTGTTCCCGACAAGCACTTAAGCGTCTGTGCGGTATTTCACACCG-3′ and pRS423 or pRS424 as a template, respectively. The *HIS3* PCR product was transformed into a *mps2-1* strain harboring a wild-type copy of *MPS2* on a CEN-based *URA3* plasmid (SMY8-4b). Histidine and uracil prototrophs were selected and PCR was used to detect the desired gene replacement. The ploidy of the *mps2-1-GFP* strain (2832, described above) spontaneously increased from haploid to diploid (a known *mps2-1* phenotype, Munoz-Centeno *et al.* 1999); therefore *CUE1* was deleted on both chromosomes with *HIS3* and *TRP1*, respectively, generating strain 2927 (*mps2-1-GFP/mps2-1, cue1*Δ*::HIS3/cue1*Δ*::TRP1*). Likewise, *HRD1* was deleted on both chromosomes with *URA3* and *TRP1*, using primers HRDONE F1, 5′-CAATTGCAATTTGTAAGAGAAGGGGAGAAAGACAAAATAATAATAGATTGTACTGAGAGTGCAC-3′ and HRDONE R1, 5′-CCAGTAGTTTTTTTCTTTAAAAAAAACTATGTATAATATAAAACATGCAATCTGTGCGGTATTTCACACCG-3′, using pRS426 or pRS424 as a template, respectively, generating strain 3090 (*mps2-1-GFP/mps2-1, hrd1*Δ*::TRP1/hrd1*Δ*::URA3*).

Plasmids: The plasmids used in this study are listed in Table 1. Plasmid pRS426-YMR263-265 was constructed by digesting pRS202-13 with *Kpn*I and *Sac*I to isolate three complete ORFs: *CUE1* and adjacent ORFs, YMR263 and YMR265. This frag-

TABLE 1

Yeast strains and plasmids

Yeast name	Relevant genotype	Reference
SMY20-8b	*MAT*α *cue1*Δ*::HIS3 his3*Δ*200*	This study
SMY20-8c	*MAT*α *cue1*Δ*::HIS3 mps2-1 his3*Δ*200*	This study
SMY23-11c	*MAT*α *cue1*Δ*::HIS3 ndc1-1 his3*Δ*200*	This study
SMY33-1a	*MAT***a** *ubc6::LEU2 mps2-1 leu2-3,112*	This study
SMY31-1c	*MAT***a** *ubc7::LEU2 mps2-1 leu2-3,112*	This study
SMY32-4d	*MAT***a** *ubc7::LEU2 ndc1-1 leu2-3,112*	This study
SMY59-2d	*MAT*α *ubc6::LEU2 ndc1-1 leu2-3,112*	This study
SMY6-4b	*MAT*α *mps2-1 ura3-52 leu2-3,112 his3*Δ*200*	This study
SMY8-3a	*MAT*α *mps2-1 ura3-52 leu2-3,112* + *pRS316MPS2*	This study
SMY8-4b	*MAT***a** *mps2-1 ura3-52 leu2-3,112* + *pRS316MPS2*	This study
SMY1655	*MAT***a** *mps2-1 cue1*Δ*::HIS3 ura3-52 his3*Δ*200* + *pRS316MPS2*	This study
WX257-4c	*MAT*α *ura3-52 leu2-3,112 his3*Δ*200*	This study
BY4733	*MAT***a** *his3*Δ*200 leu2*Δ*0 met15*Δ*0 ura3*Δ*0 trp1*Δ*63*	Brachmann *et al.* (1998)
2740	*MAT***a** *MPS2-GFP::KanMX4 his3*Δ*200*	This study
SMY72	*MAT***a** *mps2-1 his3*Δ*200 leu2*Δ*0 trp1*Δ*63*	This study
2832	*MAT***a**/**a** *mps2-1-GFP::KanMX4/mps2-1 his3*Δ*200/his3*Δ*200 trp1*Δ*63/trp1*Δ*63*	This study
2927	*MAT***a**/**a** *mps2-1-GFP::KanMX4/mps2-1 cue1*Δ*::HIS3/cue1*Δ*::TRP1 his3*Δ*200/his3*Δ*200 trp1*Δ*63/trp1*Δ*63*	This study
3090	*MAT***a**/**a** *mps2-1-GFP::KanMX4/mps2-1 hrd1*Δ*::URA3/hrd1*Δ*::TRP1 ura3*Δ*0/ura3*Δ*0 trp1*Δ*63/trp1*Δ*63*	This study
3161	*MAT***a** *mps2-1-GFP::KanMX4*	This study
HC7-31c	*MAT***a** *ndc1-1 his3*Δ*200 leu2-*Δ*1*	Chial *et al.* (1999)
HC14-5d	*MAT*α *ura3-52 ade2-*Δ*426 ade3*Δ *his3*Δ*200 leu2-*Δ*1 lys2-801*	Chial *et al.* (1999)
HC5-8c	*MAT*α *ndc1-1 leu2-*Δ*1*	Chial *et al.* (1999)
SMY47-2b	*MAT***a** *ndc1-1 ubc6::LEU2 leu2-*Δ*1*	This study
SMY47-2a	*MAT*α *ndc1-1 ura3-52 leu2-*Δ*1*	This study
1981	*MAT***a** *ndc1*Δ*::kanMX-TRP1-NDC1-GFP trp1-*Δ*63*	This study
1984	*MAT***a** *ndc1*Δ*::kanMX-TRP1-ndc1-1-GFP trp1-*Δ*63*	This study
HC14-10c	*MAT***a** *ndc1*Δ*::kanMX trp1-*Δ*63* + pALR10-*NDC1*	Chial (1998)

Plasmids name	Genotype	Reference
pRS202-13	2μ (high-copy) *URA3*-rescuing clone containing *CUE1*	This study
pRS426	2μ-*URA3*	Christianson *et al.* (1992)
pRS423	2μ-*HIS3*	Christianson *et al.* (1992)
pRS423-*CUE1*	2μ-*HIS3-CUE1*	This study
pRS425	2μ-*LEU2*	Christianson *et al.* (1992)
pRS425-*CUE1*	2μ-*LEU2-CUE1*	This study
pRS425-*MPS2*	2μ-*LEU2-MPS2*	Monterrosa (1997)
pRS425-*NDC1*	2μ-*LEU2-NDC1*	Chial (1998)
pRS315	CEN-*LEU2*	Sikorski and Heiter (1989)
pRS315-*CUE1*	CEN-*LEU2-CUE1*	This study
pRS316-*MPS2*	CEN-*URA3-MPS2*	This study
pRS424	2μ-*TRP1*	Christianson *et al.* (1992)
pRS424-*NDC1*	2μ-*TRP1-NDC1*	Chial (1998)
pALR10-MPS2	CEN-*URA3-ADE3-MPS2*	This study
YEp9	CEN-*TRP1-Ub* (wild type)	Finley *et al.* (1994)
pUB203	CEN-*TRP1-Ub* (K48R)	Finley *et al.* (1994)
pUB202	CEN-*TRP1-Ub* (G76A)	Finley *et al.* (1994)
pRS304-*NDC1-GFP*	Integrating *TRP1-NDC1-GFP*	Chial (1998)
pRS304-*ndc1-1-GFP*	Integrating *TRP-ndc1-1-GFP*	Chial (1998)

ment was ligated with the multicopy vector pRS426 (Christianson *et al.* 1992) digested with *Kpn*I and *Sac*I. pRS423-*CUE1* was created by digesting pRS426-YMR263-265 with *Bst*BI and *Eco*RV to generate a 1.7-kb fragment, which was ligated with pRS423 digested with *Cla*I and *Eco*RV. pRS425-*CUE1* and pRS424-*CUE1* were constructed by digesting pRS423-*CUE1* with *Sma*I and *Sal*I, isolating a 1.7-kb fragment containing the *CUE1* ORF, and ligating this with pRS424 and pRS425 cut with the same enzymes. pRS315-*CUE1* was constructed by digesting pRS425-*CUE1* with *Xho*I and *Spe*I and isolating a 1.7-kb fragment, which was ligated to pRS315 digested with the same enzymes. pRS316-*MPS2* was created by using plasmid E150

(Munoz-Centeno *et al.* 1999) as a template for PCR using primers flanking the *MPS2* ORF at positions −342 (the A of the start codon is designated as +1) and +1369. The primer at −342 was designed with a *Bam*HI restriction site at the 5′ end and the +1369 primer was designed with a *Kpn*I site to ligate the resulting PCR fragment into the pRS316 polylinker. pRS304-*NDC1*-GFP was constructed by ligating the *Nhe*I-*Sph*I fragment from pRS315-*NDC1*-GFP no. 7 (Chial 1998), containing the GFP tag, with the large *Nhe*I-*Sph*I fragment (excluding the myc tag) of pRS304-*NDC1*-3xmyc (Chial 1998). pRS304-*ndc1-1*-GFP was constructed by ligating the *Nhe*I-*Sph*I GFP-containing restriction fragment (above) with pRS304-*ndc1-1* (Chial 1998) cut with *Nhe*I and *Sph*I.

Flow cytometry: Flow cytometry was carried out as described (Hutter and Eipel 1979) using propidium iodide to stain the DNA. Stained cells were analyzed using a FACScan flow cytometer and Cell Quest software package for data analysis (Becton-Dickinson, San Jose, CA).

Cytology: GFP-tagged Ndc1p and Ndc1-1p proteins were visualized in cells that had been fixed with 3% formaldehyde for 5 min. GFP-tagged Mps2p and Mps2-1p proteins were visualized in living cells. Briefly, an aliquot of log phase cells was pelleted and rinsed with PBS. The DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. The cells were rinsed once with PBS and then spotted onto poly-l-lysine-coated microscope slides (Polysciences, Warrington, PA). Standard fluorescence microscopy was performed using a Leica DMRXA/RF4/V automated microscope equipped with a digital camera (SensiCam CCD camera; Cooke, Tonawanda, NY). Images were acquired and deconvolved using the Slidebook software package (Intelligent Imaging Innovations, Denver).

Immunoprecipitation and immunoblotting: Yeast strains were grown to an OD_{600} of 0.5 at 23°. A 25-ml aliquot of cells was removed for time 0 and the remaining culture was divided into two different flasks. Cycloheximide was added to a final concentration of 50 μg/ml. The flasks were incubated for 1 hr at 23° or 36° and 25-ml samples were removed after 30 and 60 min. Cell pellets were lysed with 200 μl lysis buffer (50 mm Tris-HCl pH 8.0, 10 mm EDTA, 1 mm EGTA, 200 mm NaCl, 5% glycerol, 2.25 m urea, 0.5% SDS), a protease inhibitor cocktail (Complete; Boehringer Mannheim, Germany), and 200 μm *N*-ethyl-maleimide, 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF), and 200 μl glass beads (0.5-mm diameter; BioSpec Products, Bartlesville, OK) by vortexing two times for 5 min on a multivortexer at top speed. The lysates were centrifuged at 3000 rpm for 3 min at room temperature and the supernatants were diluted 2.5-fold with immunoprecipitation (IP) buffer (lysis buffer without NaCl, urea, or SDS), bringing the final concentrations to 80 mm NaCl, 1 m urea, and 0.2% SDS. The protein concentration was determined and 200 μg of total protein was mixed with 5 μl of Protein A-Sepharose (Sigma, St. Louis) for 1 hr at 4° to remove nonspecifically bound proteins. The beads were pelleted at 3000 rpm and the supernatant was mixed with 0.6 μl of a polyclonal antibody to full-length GFP [Living Colors Full-Length Aequorea victoria (A.v.) GFP polyclonal antibody; CLONTECH, Palo Alto, CA] and 7.5 μl of Protein A-Sepharose for 3 hr at 4°. The Protein A-Sepharose beads were pelleted at 3000 rpm and washed three times in 1 ml phosphate-buffered saline. A total of 10 μl of 2× sample buffer was added and the samples were heated for 7 min at 95° before separating the proteins by 10% SDS-PAGE. The separated proteins were transferred to polyvinyldifluoride (PVDF) membrane and probed with either a monoclonal antibody to GFP (provided by P. O'Farrell, University of California, San Francisco) and a goat anti-mouse antibody conjugated to an infrared dye (anti-mouse IgG-IRDye800; kindly provided by Amy Geschwender, LI-COR, Lincoln, Nebraska) or the polyclonal antibody to GFP (CLONTECH) and a goat anti-rabbit antibody conjugated to infrared dye (IRDye800; provided by Amy Geschwender, LI-COR). The blot was scanned on a LI-COR infrared imaging system using their Odyssey software.

RESULTS

Dosage suppressors of *mps2-1*: To further understand the role of Mps2p during spindle pole body duplication, a screen was performed to search for genes that, when overexpressed, could suppress the temperature-sensitive growth of the *mps2-1* mutant (Winey *et al.* 1991). We transformed a *mps2-1* strain with a library of 2μ-based plasmids harboring inserts of yeast genomic DNA and looked for growth at 37°. One plasmid that conferred growth at the restrictive temperature was analyzed and the suppressor was identified as *CUE1* (Figure 1A). The *mps2-1* and *ndc1-1* mutations exhibit identical defects in SPB duplication. Therefore, the *CUE1* plasmid was also transformed into the *ndc1-1* strain to determine if higher levels of *CUE1* could suppress the *ndc1-1* cold-sensitive (cs) growth phenotype. We found that 2μ levels of *CUE1* could rescue the *ndc1-1* cs growth as well (Figure 1B). *CUE1* encodes a protein that *c*ouples *u*biquitin conjugation to *E*R degradation (Biederer *et al.* 1997). The analysis of *CUE1*-mediated suppression is the main focus of this study.

Genetic interactions between *mps2-1*, *ndc1-1*, and components of the ER-associated degradation pathway: Several enzymes, E1, E2, and E3, act sequentially in the ubiquitin-proteasome pathway to target proteins for degradation (reviewed in Varshavsky 1997). Following activation of a ubiquitin molecule by the E1, any one of several E2s [ubiquitin-conjugating enzymes (UBCs)] is able to transfer ubiquitin from E1 to the substrate that is bound to a ubiquitin-protein ligase, or E3. The *CUE1* gene encodes a small integral membrane protein that sequesters a soluble E2, Ubc7p, to the cytoplasmic side of the ER membrane (Biederer *et al.* 1997). The specificity of Ubc7p for some of its substrates is provided by Hrd1p, a membrane-bound ubiquitin ligase (Hampton *et al.* 1996; Bordallo *et al.* 1998; Wilhovsky *et al.* 2000; Bays *et al.* 2001), or Doa10p, another membrane-bound ubiquitin ligase (Swanson *et al.* 2001). Together with another E2, Ubc6p, an integral ER membrane protein, Cue1p-bound Ubc7p ubiquitinates various polypeptides destined to be degraded by the proteasome (Biederer *et al.* 1996; Hiller *et al.* 1996). *CUE1*, *UBC6*, *UBC7*, *HRD1*, and *DOA10* are nonessential genes, but when deleted result in stabilization of proteins that they target for degradation.

We reasoned that the overexpression of Cue1p in the previous screen may have disrupted its normal function. On the basis of these observations and the physical interaction of Cue1p with Ubc7p, we determined whether deleting *CUE1*, *UBC6*, or *UBC7* would suppress the growth

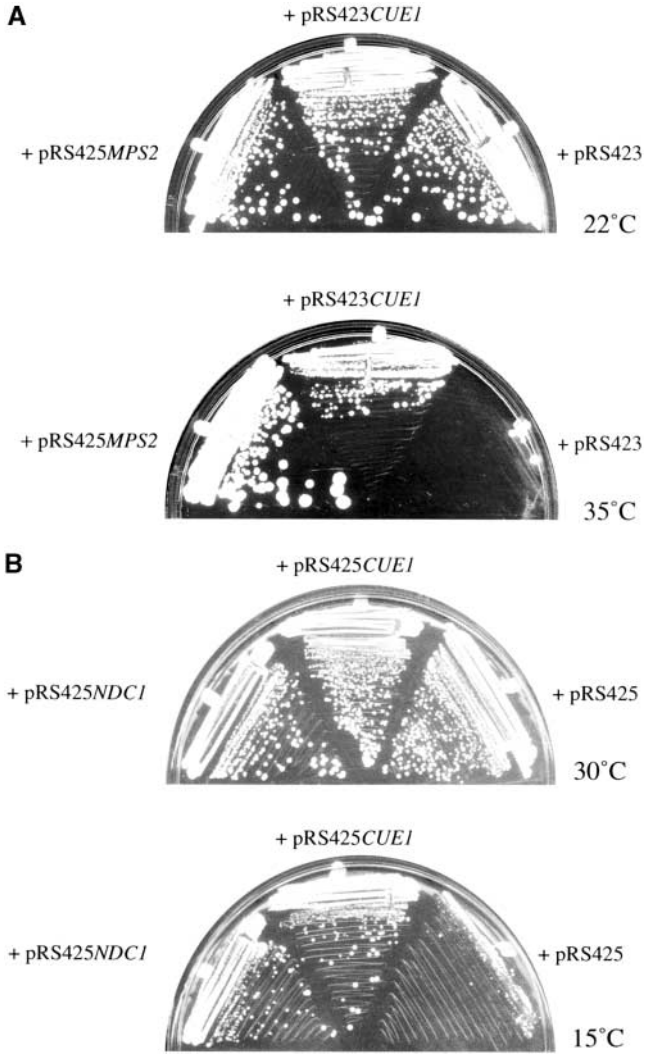

FIGURE 1.—*CUE1* is a high-copy suppressor of *mps2-1* and *ndc1-1*. (A) *mps2-1* temperature-sensitive growth is suppressed by a high-copy plasmid expressing *CUE1*. *mps2-1* cells (SMY6-4b) were transformed with pRS423, pRS425*MPS2*, or pRS423*CUE1*. Individual transformants were grown on YPD at either 22° or 35°. (B) *ndc1-1* cold sensitivity is suppressed by high-copy *CUE1*. *ndc1-1* cells (HC7-31c) were transformed with pRS425, pRS425*NDC1*, or pRS425*CUE1*. Transformants were grown on YPD at either 30° or 15°.

defect of *mps2-1* and *ndc1-1* cells. We observed that deletion of *CUE1*, *UBC6*, or *UBC7* restored the growth of *mps2-1* to wild-type levels at the restrictive temperature (Figure 2A). We confirmed that the growth of these double-mutant strains is specifically due to the disrupted gene by reintroducing the respective gene on a CEN-based plasmid, which restored the temperature sensitivity due to the *mps2-1* mutation (Figure 2B and our unpublished results). Similarly, we found that *ndc1-1* is also suppressed by disrupting *CUE1*, *UBC6*, and *UBC7* (Figure 2, C and D, and our unpublished results). As noted in Table 2, expression of *UBC6* or *UBC7* from a 2μ plasmid did not suppress either *mps2-1*- or *ndc1-1*-containing cells.

To determine the degree of *cue1*Δ suppression, we synchronized *mps2-1*, *cue1*Δ cells at the G1 phase of the yeast cell cycle (before SPB duplication is completed) and then shifted the culture to 36°, a restrictive temperature for *mps2-1*. After 3 hr at 36°, there is an approximately equal number of cells with a G1 (1c) and a G2 (2c) DNA content (Figure 3). For comparison, we shifted the *mps2-1* single-mutant strain and observed that a large proportion of cells have a G2 DNA content and ~80% of the cells in the population are large budded. This is characteristic of the mitotic arrest normally observed for *mps2-1* (WINEY *et al.* 1991). Thus, removing Cue1p from the cell allows the Mps2-1p mutant protein to function properly during SPB duplication and the cells proceed normally through mitosis.

Suppression of *ndc1-1* by a mutant ubiquitin: Eliminating components of the ER degradation pathway suppresses two different mutated genes, suggesting that either one or both of the expressed mutant proteins may be degraded by the ubiquitin-proteasome pathway under restrictive conditions. A key step in targeted degradation by the proteasome is the multiubiquitination of substrates. We examined whether blocking polyubiquitination with a mutant ubiquitin would suppress *mps2-1* and *ndc1-1*. To test this, we used plasmids containing the wild-type ubiquitin gene and two mutant ubiquitin genes under the control of the inducible *CUP1* promoter. The K48R mutant contains an arginine residue at position 48 instead of the lysine residue in wild type. This lysine residue is the site for the attachment of the next ubiquitin molecule to the growing ubiquitin chain, and the mutant acts as a chain terminator (FINLEY *et al.* 1994). The glycine-to-alanine mutation in the G76A mutant ubiquitin inhibits deubiquitination (HODGINS *et al.* 1992). However, FINLEY *et al.* (1994) showed that the G76A mutation leads to a time-dependent bias toward short chain lengths. Both of these ubiquitin mutants were able to suppress a mutation in a kinetochore protein (KOPSKI and HUFFAKER 1997). When *mps2-1* and *ndc1-1* cells were transformed with these constructs and plated onto media in the absence or presence of 100 μM copper, only *ndc1-1*-containing cells were able to grow at the restrictive temperature when either of the two mutant ubiquitin genes were expressed (our unpublished results and Figure 4). The mutant ubiquitins did not suppress *ndc1-1* to the same extent as *NDC1*-containing strains (*ndc1-1* transformed with *NDC1* on a plasmid), but they did suppress it to a significantly greater extent than the wild-type ubiquitin gene or vector alone.

In addition, the *mps2-1* and *ndc1-1* conditional phenotypes are suppressed by transforming the mutant strains with a CEN plasmid harboring the *mps2-1* or *ndc1-1* allele, respectively, suggesting that the levels of the mutant proteins are important for survival (our unpublished observations). A summary of all the genetic interactions tested is shown in Table 2.

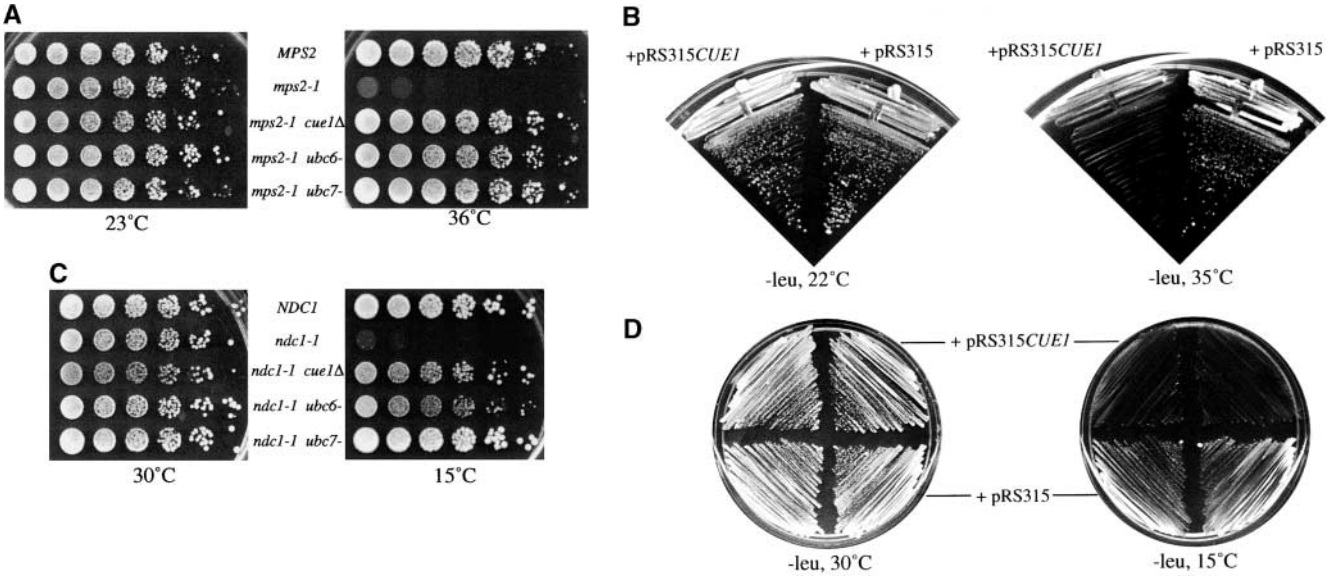

FIGURE 2.—*mps2-1* and *ndc1-1* are suppressed by deleting *CUE1*, *UBC6*, or *UBC7*. (A and C) The indicated strains were grown to saturation, and then 3 OD_{600} was serially diluted (fivefold) and grown on YPD plates at the indicated temperatures. For A, the strains are SMY31-1a (wild type), SMY6-4b (*mps2-1*), SMY20-8c (*mps2-1, cue1*Δ), SMY33-1a (*mps2-1, ubc6*$^-$), and SMY31-1c (*mps2-1, ubc7*$^-$). For C, the strains are HC14-5d (wild type), HC7-31c (*ndc1-1*), SMY23-11c (*ndc1-1, cue1*Δ), SMY59-2d (*ndc1-1, ubc6*$^-$), and SMY32-4d (*ndc1-1, ubc7*$^-$). (B and D) The rescue of *mps2-1* and *ndc1-1* growth is specific for deletion of *CUE1*. (B) SMY20-8c (*mps2-1, cue1*Δ) and (D) SMY23-11c (*ndc1-1, cue1*Δ) were transformed with either *CUE1* on a CEN-based plasmid or vector alone and incubated at the restrictive temperature. Transformation with pRS315-*CUE1* recapitulates the temperature sensitivity of both strains. Two different transformants of *ndc1-1, cue1*Δ are shown for each plasmid.

Abundance of Ndc1-1p and Mps2-1p mutant proteins: The genetic analysis suggested a role for protein stability in the conditional phenotypes of *mps2-1* and *ndc1-1* cells. Therefore, we examined Ndc1-1p and Mps2-1p mutant proteins at the nonpermissive temperature. Previously, we have shown that Ndc1p-GFP is localized at NPCs and SPBs at all stages of the cell cycle (CHIAL *et al.* 1998). Here, we synchronized *ndc1-1* cells in G1, then shifted the culture to 13.5° for 16 hr. An aliquot of cells was analyzed by fluorescence microscopy at 1-hr intervals (for 6 hr) following release from G1. The Ndc1p-GFP wild-type and the Ndc1-1p-GFP mutant proteins localized at NPCs and SPBs at all time points (Figure 5A, the 6-hr time point is shown). By 6 hr at 13.5°, the cells have completed DNA synthesis and SPB duplication and are in mitosis (see morphology of the wild-type cell shown in Figure 5A; also determined by DNA content and budding index, our unpublished observations). Despite the *ndc1-1* cells having failed in SPB duplication, or insertion of the nascent SPB into the NE, Ndc1-1p-GFP localizes to both SPBs (Figure 5A, bottom, arrowheads). The GFP signal at the SPB (Figure 5A, arrowheads) is always brighter than the signal of the NPCs along the nuclear envelope (CHIAL *et al.* 1998). Ndc1-1p-GFP was still detected at SPBs after 16 hr at 13.5° (our unpublished observation). We also found that the stability of the Ndc1-1p mutant protein fused with Protein A was indistinguishable from wild type at the restrictive temperature on the basis of the decay rate upon exposure to cycloheximide (immunoblot analysis, our unpublished observations). In addition, the half-life of Ndc1-1p-ProA appeared similar to Ndc1p-ProA in a pulse-chase experiment (our unpublished observations). Because of these results, we did not pursue an analysis of Ndc1-1p-ProA in a *cue1* null strain.

Mps2-1p mutant protein was analyzed in a similar manner by fusing GFP at the C terminus of mutant Mps2-1p and wild-type Mps2p. The GFP-tagged strains were grown at the permissive temperature of 23° and then shifted to the restrictive temperature of 36°. Mps2p-GFP is an integral membrane protein localized at SPBs (MUNOZ-CENTENO *et al.* 1999). An example of this localization for the wild-type protein (fused to GFP) is shown in Figure 5B (top row, $t = 0$). Mps2-1p-GFP is also localized at SPBs at the permissive temperature (middle row, $t = 0$). However, the amount of Mps2-1p-GFP at the SPBs is markedly reduced upon shift to the nonpermissive temperature. Strikingly, the mutant protein is almost undetectable at SPBs by 80 min at the restrictive temperature (middle row, $t = 80$ min, see arrow) and is virtually absent by 180 min (middle row, $t = 180$ min). Since the *mps2-1* conditional phenotype is suppressed by deleting *CUE1* from the genome, we asked whether the localization of the mutant Mps2-1p-GFP at the SPB could be restored by removing Cue1p. In the double mutant (*mps2-1-GFP, cue1*Δ), Mps2-1p-GFP is detected at SPBs at 23° and 36° (Figure 5B, bottom). It is clear that the overall level of Mps2-1p-GFP at the SPBs is lower than that of wild type, even at the permissive temperature. Removing Cue1p enhances the level of Mps2-1p-GFP at SPBs at both temperatures; however, it does not reach wild-type levels (Figure 5B, compare top and bottom).

TABLE 2

Summary of genetic interactions

	mps2-1	*ndc1-1*
Plasmids		
2μ *CUE1*	+[a]	+
2μ *NDC1*	−[b]	+
2μ *MPS2*	+	−
2μ *BBP1*	−[c]	−
2μ *UBC6*	−	−
2μ *UBC7*	−	−
2μ Ub-K48R	−	+
2μ Ub-G76A	−	+
2μ Ub (wt)	−	−
Double mutants		
*cue1*Δ[d]	+	+
ubc6⁻	+	+
ubc7⁻	+	+
*hrd1*Δ	−	NT

NT, not tested; wt, wild type.

[a] The + sign represents wild-type or nearly wild-type growth at the restrictive temperature.

[b] The − sign represents no growth at the restrictive temperature.

[c] Published in Schramm *et al.* (2000).

[d] *cue1*Δ does not suppress the *mps2* null allele; *cue1*Δ cannot rescue the haploinsufficiency of *ndc1*Δ*/NDC1*; *i.e.*, *cue1*Δ*/cue1*Δ *ndc1*Δ*/NDC1* + p*URA3-ADE3-NDC1* cannot grow on 5-FOA.

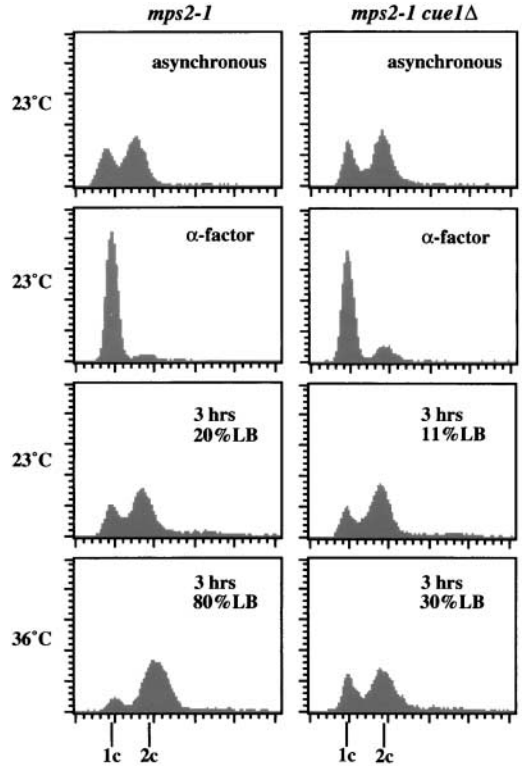

Figure 3.—Deletion of *CUE1* rescues the cell cycle defect in *mps2-1* cells. *mps2-1* and *mps2-1, cue1*Δ cells were grown to log phase and synchronized with α-factor. The cells were released from α-factor into media at either 23° or 36° and allowed to grow for 4 hr. The percentage of large-budded (LB) cells is indicated. Only the 3-hr time point is shown at 23° and 36°.

The percentage of cells with GFP foci (regardless of intensity) that colocalized with the DNA is shown in Table 3.

The localization data indicated to us that the mutant Mps2-1p mutant protein could be degraded at both the permissive and nonpermissive temperature. To investigate this further, we immunoprecipitated either wild-type Mps2p-GFP or mutant Mps2-1p-GFP from lysates of cells grown at either 23° or 36° in the presence of cycloheximide, a protein synthesis inhibitor. With this experiment, we can examine the stability of the entire pool of wild-type or mutant Mps2p proteins at regular intervals after blocking protein synthesis (Hampton and Rine 1994). The results of this experiment are shown in Figure 6. Mps2p-GFP remains stable up to 60 min at 23° or 36°. In contrast, the mutant Mps2-1p-GFP is barely detected even at the permissive temperature and is undetectable by 30 min at 23° and 36°. Strikingly, deleting *CUE1* from the genome allows the mutant Mps2-1p to accumulate to nearly wild-type levels. These results indicate that *cue1*Δ restores Mps2-1p-GFP levels, possibly by blocking protein turnover by ER-assisted degradation.

Mps2-1p mutant protein abundance is Hrd1p independent: The ER degradation of some substrates, including Hmg2p (Hampton *et al.* 1996; Wilhovsky *et al.* 2000), a mutant form of Pdr5p (Plemper *et al.* 1998), a mutant form of carboxypeptidase Y (Bordallo *et al.* 1998), and Sec61-2p mutant protein (Bordallo *et al.* 1998), is dependent on Ubc7p and Hrd1p, a membrane-bound ubiquitin ligase (Hampton *et al.* 1996; Bordallo *et al.* 1998; Wilhovsky *et al.* 2000; Bays *et al.* 2001). However, other ER quality control substrates that are degraded in a Ubc7p-dependent manner are not dependent on Hrd1p, including a mutant form of uracil permease encoded by the Fur4-403Np mutant allele (Galan *et al.* 1998; Wilhovsky *et al.* 2000). Mps2-1p would appear to be an ER quality control substrate in which a mutation leads to degradation in a Ubc7p-Cue1p-dependent manner. We tested whether Mps2-1p-GFP degradation is dependent on *HRD1* by deleting the gene from the *mps2-1-GFP* strain and monitoring the disappearance of the mutant protein in the presence of cycloheximide. As shown in Figure 7, Mps2-1p-GFP mutant protein is undetectable even in the *hrd1* null mutant. Furthermore, the *hrd1* null mutation does not suppress the *mps2-1* mutation (our unpublished results). We conclude from these experiments that Mps2-1p-GFP degradation is Hrd1p independent.

DISCUSSION

The *MPS2* and *NDC1* genes encode integral membrane proteins that are localized at SPBs. Both Mps2p and Ndc1p are thought to be required at the same step during the insertion of the nascent SPB into the NE.

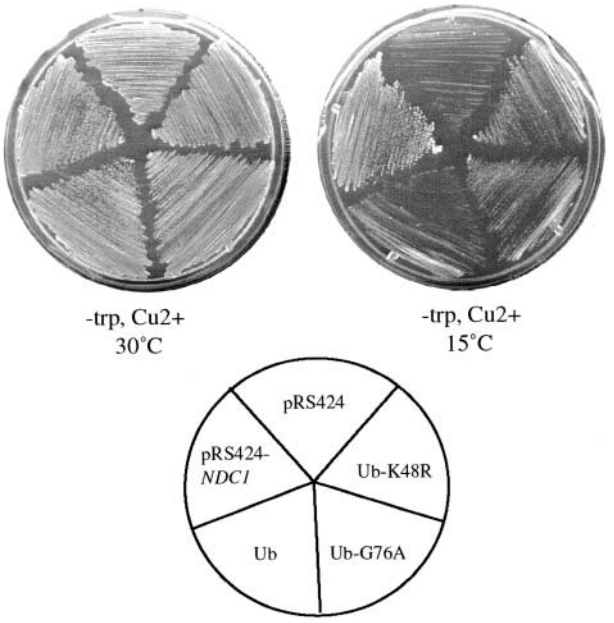

FIGURE 4.—*ndc1-1* is suppressed by expression of mutant forms of ubiquitin. The *ndc1-1* strain HC7-31c was transformed with the indicated plasmids and allowed to grow on synthetic media plates lacking tryptophan, but containing 100 μM cupric sulfate.

To identify proteins that might function with Mps2p during SPB duplication, we looked for dosage suppressors of the *mps2-1* mutation. *CUE1* was found as a dosage suppressor of *mps2-1* and also suppresses *ndc1-1*, but *CUE1* does not suppress the temperature-sensitive growth of *bbp1-1*, an SPB duplication mutant. Bbp1p is a component of the SPB that physically interacts with Mps2p (SCHRAMM *et al.* 2000). We also determined that removing components of the ER-associated degradation pathway restored *mps2-1* and *ndc1-1* cell growth to nearly normal levels. Finally, we showed that Mps2-1p-GFP is present at low levels and that its abundance increases by removing Cue1p, but not by removing Hrd1p, an E3 ubiquitin ligase of the ER that has specificity for Ubc7p and Ubc1p (BAYS *et al.* 2001).

Previous work has shown that removing components of the ER-associated degradation pathway causes stabilization of mutant forms of Sec61p, a component of the multisubunit translocation apparatus (DESHAIES and SCHEKMAN 1987; DESHAIES *et al.* 1991), carboxypeptidase Y (CPY*), and a β-Gal protein fused to the *Deg1*-degradation signal of the short-lived transcriptional repressor matα2 (BIEDERER *et al.* 1996). In addition, both *ubc6*Δ and *ubc7*Δ suppress the temperature-sensitive growth of *sec61-2* (SOMMER and JENTSCH 1993; BIEDERER *et al.* 1996) and *ndc10-2* (KOPSKI and HUFFAKER 1997), which encodes a mutant kinetochore component. Interestingly, *UBC6*, but not *UBC7*, was also identified as a high-copy suppressor of the *ndc10-2* mutation (KOPSKI and HUFFAKER 1997). It appears that suppression of ER-assisted degradation is a common genetic theme among divergent organisms. In *Caenorhabditis elegans*, mutations in SEL-1, which encodes the homolog of *S. cerevisiae HRD3*, suppress *lin-12* hypomorphic alleles, presumably by blocking degradation of the lin-12 protein (GRANT and GREENWALD 1997). Hrd3p is an integral membrane protein of the ER required for ER-assisted degradation (BORDALLO *et al.* 1998; PLEMPER *et al.* 1999; GARDNER *et al.* 2000).

Initially, we expected that Ndc1-1p stability would be compromised at the restrictive temperature since expression of mutant ubiquitin molecules could suppress *ndc1-1*. However, the Ndc1-1p mutant protein at the restrictive temperature appears as stable as the wild-type protein and is localized at SPBs and NPCs. Yeast cells are acutely sensitive to levels of the *NDC1* and *ndc1-1* genes (CHIAL *et al.* 1999, 2000). In fact, haploid yeast cells containing two chromosomal copies of *ndc1-1* are no longer cs (CHIAL *et al.* 2000). Therefore, the conditional phenotype could be the result of a small, difficult to detect reduction in the level of Ndc1-1p due to the *ndc1-1* mutation. Likewise, only a minor increase in the mutant protein level, permitted by perturbation of the ubiquitination mechanism (as in the mutant ubiquitin transformants), would be required to restore viability at the restrictive temperature. In summary, due to the sensitivity of cells to *NDC1* and *ndc1-1* dosage, we are unable to show directly that the Ndc1-1p mutant protein is targeted to the ER degradation pathway. Identification of other mutant alleles of *NDC1* that are suppressed by removing Cue1p could be useful in studying the metabolism of this NE integral membrane protein.

Conversely, a dramatic difference between the Mps2p and Mps2-1p levels at the SPB was observed at the nonpermissive temperature for *mps2-1*. The suppression of *mps2-1* by *cue1*Δ, *ubc6*Δ, and *ubc7*Δ suggests a role for ubiquitin-mediated degradation in the manifestation of the *mps2-1* phenotype. Therefore, it was surprising to us that the *mps2-1* allele was not suppressed by expressing the mutant forms of ubiquitin. It is possible that the *mps2-1* mutation causes such a severe defect in the protein that it is ubiquitinated and degraded immediately after being translated, presumably via retrograde transport into the ER-associated degradation machinery (*i.e.*, Cue1p, Ubc6p, and Ubc7p), consistent with the low levels of Mps2-1p even at the permissive temperature. Thus, expression of the mutant ubiquitin is apparently insufficient to rescue the fate of Mps2-1p by allowing it to accumulate. Removing Cue1p, however, sufficiently restores the level of Mps2-1p in the cell to allow the mutant protein to accumulate and localize at the SPB. In comparing Figure 5B to Figure 6, Mps2-1p is visible at the SPB at the permissive temperature; however, it is barely detectable by immunoblotting. It is also possible that Mps2-1p is more susceptible than wild-type Mps2p to proteolytic degradation once the cells are lysed. Finally, we cannot rule out that Mps2-1p is affected indirectly by the ERAD pathway. Perhaps altering the

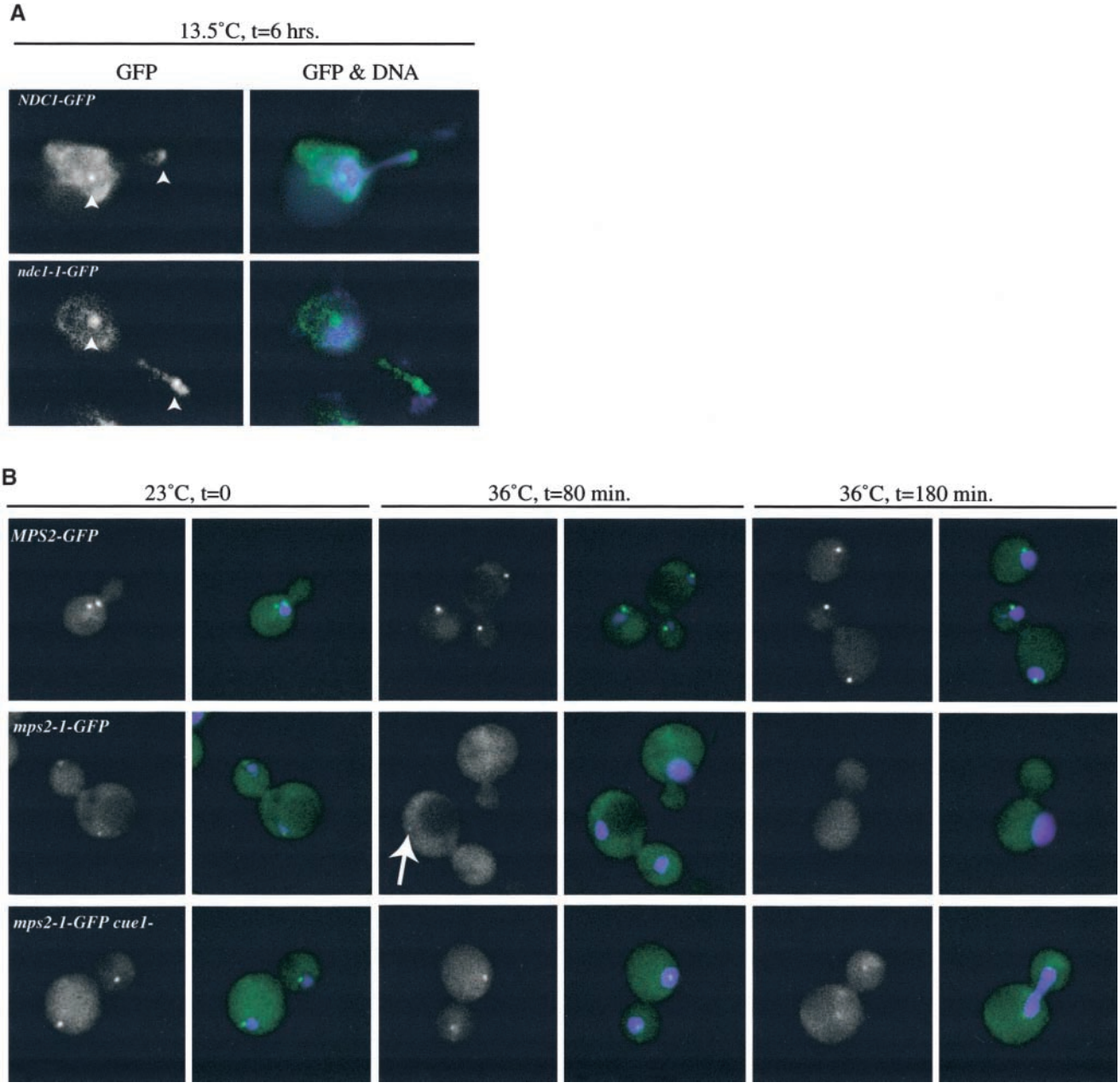

Figure 5.—Localization of Ndc1-1p and Mps2-1p mutant proteins at the restrictive temperature. (A) Cells expressing either Ndc1p-GFP or Ndc1-1p-GFP were grown at 30° and synchronized with α-factor. The cells were released from α-factor after ~90 min and then shifted to 13.5°. Aliquots were taken every hour and fixed with formaldehyde; the 6-hr time point is shown. The DNA was visualized with DAPI. A single plane of focus is shown for each image that was deconvolved using the Slidebook software package from Intelligent Imaging Systems. (B) Cells expressing Mps2p-GFP, Mps2-1p-GFP, or Mps2-1p-GFP in a *cue1*Δ strain were shifted to 36° from a 23° log phase culture. Aliquots of cells were removed at the indicated times and live cells were analyzed by fluorescence microscopy. Each image was captured with the same exposure time and deconvolved as in A. The arrow in the middle points to a faint focus of GFP fluorescence.

ERAD pathway changes the stability of a different protein, which in turn affects Mps2-1p abundance. However, on the basis of the genetic interactions shown here and for proven ERAD substrates elsewhere, we propose that Mps2-1p abundance is affected directly by ERAD. These data point to the mechanism of suppression in the *mps2-1* mutant by deletion of *CUE1*. However, the *mps2-1* mutation is also suppressed by overexpressing Cue1p from a high-copy-number plasmid. One possibility is that elevated levels of Cue1p disrupt its function and/or disrupt its interaction with Ubc7p, thereby interrupting the normal flow of ERAD. Alternatively, overexpression of the membrane-bound Cue1p might target Cue1p itself for degradation, competing with Mps2-1p and Ndc1-1p as a substrate for ubiquitination by Ubc6p and Ubc7p.

TABLE 3

Percentage of cells with GFP coincident with DNA

	Time		
Strain	23°, $t = 0$	36°, $t = 80$ min	36°, $t = 180$ min
MPS2-GFP	98 ($n = 67$)	94 ($n = 100$)	93 ($n = 100$)
mps2-1-GFP	78 ($n = 100$)	39 ($n = 100$)	10 ($n = 73$)
*mps2-1-GFP cue1*Δ	93 ($n = 76$)	86 ($n = 100$)	90 ($n = 100$)

The extremely low levels of Mps2-1p have made it impossible to show directly that the reduction of Mps2-1p is due to polyubiquitination and subsequent degradation. Mps2-1p mutant protein remained undetectable in a proteasome mutant (*pre1-1*; Heinemeyer *et al.* 1991, 1993) after immunoprecipitation and immunoblotting fractionated yeast lysates (our unpublished observations). One possibility for this apparent lack of proteasome dependency is that the *pre1-1* mutation may not completely inactivate the proteolytic function of the proteasome, thereby preventing Mps2-1p from accumulating to detect it sufficiently. We also investigated Mps2-1p in the presence of the proteasome inhibitor MG-132 in a mutant strain (*erg6*Δ; Graham *et al.* 1993) that is permeable to the drug. Again, the levels of Mps2-1p were insufficient to detect (our unpublished observations).

Despite these observations, the genetic interactions with *CUE1*, *UBC6*, and *UBC7* support our view that Mps2-1p is degraded through the ER-associated ubiquitin proteasome pathway. As noted in Table 2, a *cue1* null mutation does not suppress the marginal viability of an *mps2* null allele (Munoz-Centeno *et al.* 1999) or the haploinsufficiency of *NDC1* (Chial *et al.* 1999), indicating that the deletion of *CUE1* does not affect activities downstream of Ndc1p and Mps2p. Interestingly, Mps2-1p degradation is independent of the ubiquitin ligase, Hrd1p, which exhibits specificity for Ubc7p and Ubc1p (Bays *et al.* 2001). Recently, a second ER membrane-localized ubiquitin ligase, Doa10p, was discovered (Swanson *et al.* 2001) that also exhibits specificity for Ubc7p. It is possible that Doa10p functions in targeting Mps2-1p for degradation.

In summary, perhaps less severe mutations in Mps2p may provide insight into the mechanism of Mps2p protein turnover. In fact, a different *mps2* mutant generated in our lab is not suppressed by altering *CUE1* dosage (our unpublished observations). The possibility that defective Mps2p proteins are processed differently depending on the severity of the defect is not unprecedented. A study of various mutant Ste6p proteins revealed that certain mutant proteins were degraded rapidly by the ER-coupled ubiquitin-proteasome pathway, whereas other mutant proteins were retained in the ER by the protein-folding machinery (Loayza *et al.* 1998).

The genetic interactions described here do not neces-

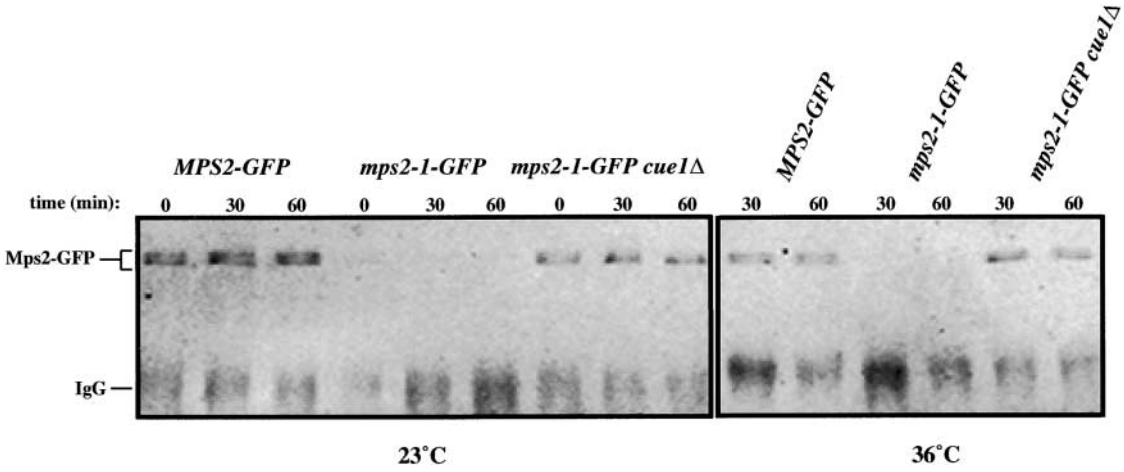

Figure 6.—Mps2-1p is unstable in the presence of Cue1p. The indicated yeast strains were grown for 1 hr at 23° or 36° in the presence of cycloheximide. Aliquots of cells were removed at times 0, 30, and 60 min after addition of cycloheximide and lysed (see materials and methods). GFP-tagged proteins were immunoprecipitated with a rabbit polyclonal antibody against GFP and Protein A-Sepharose, separated by SDS-PAGE, transferred to PVDF, and detected on the blot with a mouse monoclonal antibody to GFP. There is some cross-reactivity of the secondary antibody (goat anti-mouse) with the rabbit IgG on the blot. Mps2p-GFP migrates according to its estimated size (72 kD) with a 70-kD molecular weight marker from Bio-Rad (Richmond, CA; markers not shown here).

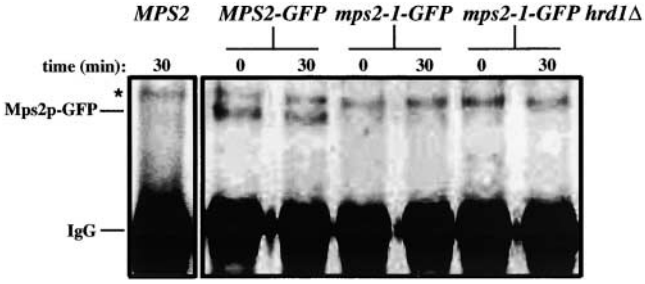

FIGURE 7.—Mps2-1p instability is Hrd1p independent. The indicated yeast strains were grown at 23° in the presence of cycloheximide. An aliquot of cells was removed at times 0 and 30 min after addition of cycloheximide and lysed. GFP-tagged proteins were isolated as described in MATERIALS AND METHODS. The GFP-tagged proteins were detected on the blot with the rabbit polyclonal antibody used in the immunoprecipitation and a goat anti-rabbit secondary antibody. The asterisk marks a band from untagged (first lane) and GFP-tagged immunoprecipitates that cross-reacts with the secondary antibody.

sarily reflect an event occurring in wild-type yeast cells and therefore would not affect SPB duplication. Wild-type Mps2p (this article and MUNOZ-CENTENO *et al.* 1999) and Ndc1p (our unpublished observations) are very stable, and immunoprecipitates of these proteins do not appear to contain ubiquitinated Mps2p or Ndc1p (our unpublished observations), suggesting that the turnover of Mps2p and Ndc1p is not regulated by ERAD. The data here describe newly discovered genetic interactions between two NE integral membrane proteins and several components of ERAD. In addition, we have shown that the mutant Mps2-1p abundance is restored to near wild-type levels when Cue1p function is disrupted. We believe that the two mutant membrane proteins of the SPB, Mps2-1p and Ndc1-1p, are specifically targeted to the ERAD pathway. To our knowledge, this is the first example of specialized NE integral membrane proteins being affected by a degradation pathway of the ER.

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