**Supplemental Table 1: Experimental Models: Organisms/Strains, Related to STAR Methods.**

**Supplemental Table 2: Oligonucleotides, Related to STAR Methods.**

**Supplemental Table 3: Recombinant DNA, Related to STAR Methods.**

**Supplemental Figure 1. The truncation sites in the NBD2 reside in a predicted β-sheet and the Q394X truncation chromatographs in a large complex, Related to Figures 1 and 2.** (A) The relative half-lives of wild type chimera and Q394X were examined by cycloheximide chase analysis and pulse-chase analysis. For pulse chase analyses, cells were pulsed for 15 min with $^{35}$S-Met, then chased for 1 hr. Cells were lysed and the substrates were immunoprecipitated with an anti-HA antibody and Protein A-Sepharose. Representative images from each condition are shown, but a total of 6 (for cycloheximide chase analyses) and 7 (for pulse chase analyses) independent experiments were performed and the data were averaged for $t_{1/2}$ calculations. (B) A structure for the closest homologous domain to Ste6 NBD2 was selected by querying the Research Collaboratory for Structural Bioinformatics Protein Data Bank, www.rcsb.org (Berman et al., 2002). The mouse P-glycoprotein (PDB ID: 4M1M, (Li et al., 2014)) was chosen based on 35% sequence identity in the NBD2 sequence. Homology models were then constructed with Moddeller 9v13 (Sali and Blundell, 1993). The predicted model of the cytosolic NBD is shown in the absence of the transmembrane domains. A magnified view of the region containing the truncations in the Ste6 and wild type chimera NBD2 is also shown (amino acids 1240-1268 in Ste6). The structure and predicted sites of each truncation were visualized and labeled using Pymol (The PyMOL Molecular Graphics System, Version 1.7.4.5 Schrödinger, LLC.). Shown in red is the C-terminus of the NBD2. Sites labeled in blue represent truncations with similar half-lives to the wild type protein while those labeled in orange are degraded more quickly. (C) ER-enriched microsomes containing Q394X were treated with DDM and chromatographed through a size exclusion column. The residence of Q394X and Sec61 were determined by western blotting. The graph represents the quantification of these data. The void volume ($V_o$) and positions of the standards (x $10^3$) are also shown.

**Supplemental Figure 2. The chimera truncation mutants display varying levels of detergent solubility, Related to Figure 3.** *BY4742* (wild type) yeast expressing the indicated truncations were harvested and ER-enriched microsomes were prepared as described in the Methods. The microsomes were then treated with 1.8 mM DDM to solubilize the substrates. As controls, reactions were conducted in the absence of detergent ("0 mM") or in the presence of the strong, ionic detergent sodium dodecyl sulfate (SDS). Samples were centrifuged at 18,000 *x g* and the percent of solubilized (S) and membrane-retained (P) protein was determined after immunoblotting with an anti-HA antibody. These values are reported in Figure 3C. Sec61 solubilization was also monitored with an anti-Sec61 antibody to control for variations in membrane solubilization between samples. The images are representative for N = 4 independent experiments.

**Supplemental Figure 3. The cytosolic Hsp70 chaperone, Ssa1, Hsp40 chaperone, Ydj1, and nucleotide exchange factor (NEF), Fes1, are required for maximal degradation of Q394X, Related to Figure 4.** Cultures expressing Q394X were shifted to 37°C for 30 min prior to the addition of cycloheximide and were chased for 1 hr at 37°C. Mutants tested correspond to (A) the cytosolic Hsp70, Ssa1 (this strain expresses a plasmid-borne mutant Ssa1 species in an *ssa1Δssa2Δssa3Δssa4Δ* background), and (B) the cytosolic Hsp40, Ydj1, and (C) the NEF for Ssa1, Fes1. N = 3-9 individual experiments. +/- SEM. **P < 0.01, ***P < 0.001.

**Supplemental Figure 4. The ERAD of Q394X is unaffected by deletions in the genes encoding the small heat shock proteins, the TriC complex, a component of the Tail-Anchor insertion complex, or the Rad23-Dsk2 Ubl/Uba-domain containing proteins, Related to Figure 4.** All cycloheximide chases were performed at 37°C after a 30 min pre-shift. The stability of Q394X was investigated in (A) the absence of the small heat shock proteins, Hsp26 and Hsp42 (N = 3), (B) in yeast containing a temperature-sensitive chaperonin subunit, Cct1 (N = 16), (C) a strain deleted for the Tail-Anchor insertion pathway component, Sgt2 (N = 6), and (D) cells lacking the ubiquitinated protein shuttle factors, Rad23 and Dsk2 (N = 6). All data represent the means of the indicated number of independent experiments, +/- SEM.

**Supplemental Figure 5. Hsp104 acts downstream of Q394X ubiquitination and requires ATPase activity to degrade an aggregation-prone ERAD substrate, Related to Figures 4 and 6.** (A) A plasmid engineered for the expression of the ATPase-inactive Hsp104 (K218T K620T) mutant or an empty vector (EV) control was transformed into wild type or *hsp104Δ* cells expressing Q394X. Cultures were shifted to 37°C for 30 min prior to the addition of cycloheximide and subsequently chased for 1 hr at 37°C. N = 4 individual experiments, +/- SEM. (B) *BY4742* cells expressing either Q394X or L385X and that contained either an empty vector control (EV) or an Hsp104 over-expression plasmid (*HSP104*) were chased at 37°C for 15 min in the absence of a 37°C preshift. (C) Q394X ubiquitination was examined in the indicated yeast strains after cells were shifted to 37°C for 1.5 hrs, lysed, and the Q394X substrate was immunoprecipitated using anti-HA-conjugated agarose beads. Q394X was detected (HA) as well as total ubiquitin. Strain-specific variations in ERAD and ubiquitination efficiency have previously been noted and likely arise due to different background genotypes and transcriptomic profiles (Zhang et al., 2001).

**Supplemental Figure 6. Characterization of an aggregation-prone, Hsp104-dependent ERAD substrate, TM-Ubc9$^{ts}$, Related to Figure 4.** (A) A schematic for the topology of TM-Ubc9$^{ts}$ is presented. The structure used to depict Ubc9 is modified from Uniprot (2GJD). (B) ER-enriched microsomes expressing TM-Ubc9$^{ts}$ were isolated from wild type yeast shifted to 37°C for 1.5 hrs. These microsomes were then used to investigate the residence of TM-Ubc9$^{ts}$ in the ER membrane by carbonate extraction. As controls, the residence of the soluble ER resident chaperone Pdi1 and the ER membrane protein Sec61 were also determined. (C) ERAD dependence was determined by performing cycloheximide chase analyses in *DOA10* and *doa10Δ* cells expressing TM-Ubc9$^{ts}$ at 37°C . Shown is a representative of one of 3 independent experiments. (D) *HSP104* and *hsp104Δ* cells expressing TM-Ubc9$^{ts}$ were preshifted to 37°C for 30 min prior to the addition of cycloheximide and subsequently chased for 1 hr. N = 6 independent experiments, +/- SEM. ***P < 0.001. (E) ER-enriched microsomes were purified from *HSP104* and *hsp104Δ* cells expressing TM-Ubc9$^{ts}$ that were treated as described in panel (B). These microsomes were then incubated with 14.4 mM DDM or 1% SDS and TM-Ubc9$^{ts}$ solubilization was analyzed by western blotting after centrifugation. Polyacrylamide gel percentage used to resolve TM-Ubc9$^{ts}$ is stated next to the blots. Note that the signal for monomeric TM-Ubc9$^{ts}$ is slightly lower in *HSP104* vs *hsp104Δ* samples. This correlates with decreased ERAD in the absence of Hsp104, which can be seen in (D). The asterisk represents a moderately aggregated population of TM-Ubc9$^{ts}$. The arrowhead marks the presence of a high molecular weight population of TM-Ubc9$^{ts}$. The "W" represents the population of TM-Ubc9$^{ts}$ that resides in the wells of the polyacrylamide gels. (F) Indirect immunofluorescence was performed with *HSP104* and *hsp104Δ* cells expressing TM-Ubc9$^{ts}$. Cells were shifted to 37°C for 1.5 hrs prior to fixation and indirect immunofluorescence microscopy. Note the formation of puncta only in the *hsp104Δ* strain.

**Supplemental Figure 7, Related to Figure 7. The retrotranslocation of ubiquitinated Q394X is unaffected by the absence of the small heat shock proteins or Sse1.** *In vitro* ubiquitination assays were

performed with cytosol and microsomes isolated from (A) *HSP26 HSP42* and *hsp26Δ hsp42Δ* and (B) *SSE1* and *sse1Δ* strains. After centrifugation, the retrotranslocated (S) and membrane integrated (P) populations of ubiquitinated Q394X were immunoprecipitated with an anti-HA antibody and visualized by SDS PAGE and phosphorimage analysis. The native molecular weight of Q394X is marked by an arrowhead.

## Supplemental

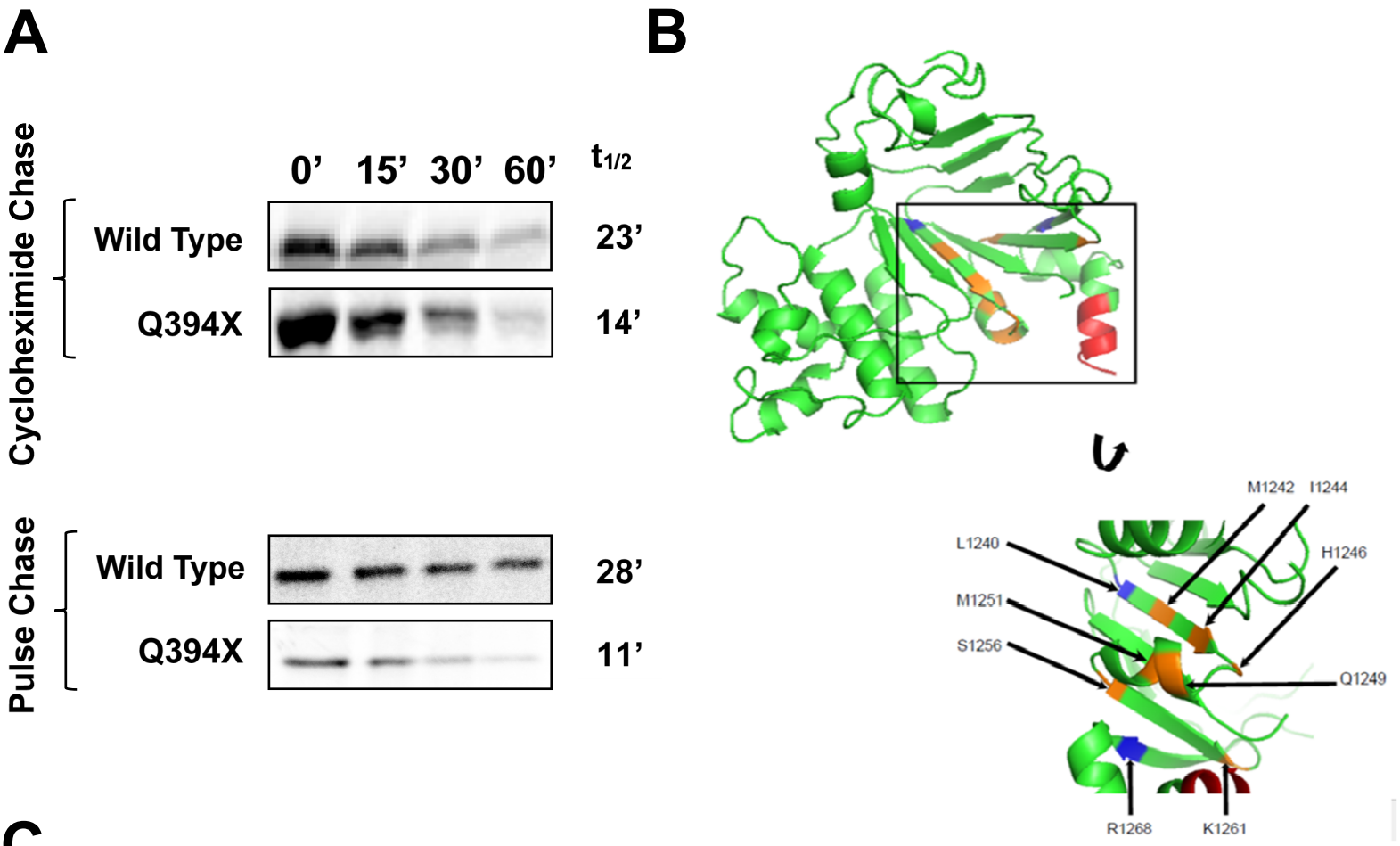
| Table S1: Experimental Models: Organisms/Strains. Related to STAR Methods. | | |
|---|---|---|
| *S. cerevisiae*: *SM2721 (MAT**a** trp1 leu2 ura3 his4 can1 ste6-Δ5(8-1290))* | (Berkower et al., 1994) | N/A |
| *S. cerevisiae*: *BY4742 (MAT**α** his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0)* | Invitrogen | |
| *S. cerevisiae*: *pdr5Δ (MAT**α** his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0 pdr5Δ::KAN)* | Open Bio Systems | |
| *S. cerevisiae*: *sgt2Δ (MAT**α** his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0 sgt2::KAN)* | Open Bio Systems | |
| *S. cerevisiae*: *HSP104 (MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1)* | (Sikorski and Hieter, 1989) | N/A |
| *S. cerevisiae*: *hsp104Δ (MAT**a** ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 hsp104::LEU2)* | (Glover and Lindquist, 1998) | N/A |
| *S. cerevisiae*: *SSA1 (MAT**α** his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 SSA1 ssa2- 1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2)* | (Becker et al., 1996) | N/A |
| *S. cerevisiae*: *ssa1-45 (MAT**α** his3-11,15 leu2-3,112 ura3-52 trp1-Δ1 lys2 ssa1-45 ssa2-1::LEU2 ssa3-1::TRP1 ssa4-1::LYS2)* | (Becker et al., 1996) | N/A |
| *S. cerevisiae*: *W303-1B (MAT**α** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100)* | (Caplan et al., 1992) | N/A |
| *S. cerevisiae*: *ydj1-151 (MAT**α** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 ydj1-2::HIS3 ydj1-151::LEU2)* | (Caplan et al., 1992) | N/A |
| *S. cerevisiae*: *sse1Δ (MAT**α** ade2-1 his3-11,15 leu2-3,112 ura3-1 trp1-1 can1-100 sse1::KAN)* | (Shaner et al., 2004) | N/A |
| *S. cerevisiae*: *CCT1 (MAT**α** leu2-3,112 ura3-52 trp1-7 tcp::LEU2 (YCpMS38; TCP1::TRP1)* | (Ursic and Culbertson, 1991) | N/A |
| *S. cerevisiae*: *cct1-2 (MAT**α** leu2-3,112 ura3-52 trp1-7* | (Ursic and Culbertson, 1991) | N/A |

| | | |
|---|---|---|
| *tcp::LEU2 (YCpMS38; tcp1-2::TRP1)* | | |
| *S. cerevisiae*: *HSP42 HSP26 (MAT**a** ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL)* | (Haslbeck et al., 2004) | N/A |
| *S. cerevisiae*: *hsp42Δ hsp26Δ (MAT**a** ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL hsp26::HIS3 hsp42::LEU2)* | (Haslbeck et al., 2004) | N/A |
| *S. cerevisiae*: *rad23Δ dsk2Δ (MATa, his3Δ1, leu2Δ0, ura3Δ0, rad23Δ::KanMX, dsk2Δ::KanMX)* | (Huyer et al., 2004) | N/A |
| *S. cerevisiae: HSP104-GFP: MATa, pdr5Δ::kanMX4 can1Δ::STE2pr-Sp_his5, lyp1Δ, ura3Δ0, met15Δ0, HSP104-GFP-LEU2* | Nystrom Lab | N/A |
| *S. cerevisiae: HSP104-GFP pSM3149: MATa, pdr5Δ::kanMX4 can1Δ::STE2pr-Sp_his5, lyp1Δ, ura3Δ0, met15Δ0, HSP104-GFP-LEU2, mCherry-Scs2-Tm::NAT* | This study | N/A |
| *S. cerevisiae: FES1: MATa, ura3-52 leu2-3,112 trp1-Δ901 ade2-101* | Brodsky Lab | N/A |
| *S. cerevisiae: fes1Δ: MATa, ura3-52 leu2-3,112 trp1-Δ901 ade2-101, fes1::KanMX4* | Brodsky Lab | N/A |

| Table S2: Oligonucleotides. Related to STAR Methods. | | |
|---|---|---|
| oMP01 (GCGCCCGGGATGAACTTTTTAAGTTTTAAGAC) | This study | N/A |
| oMP02 (CGCCCGCGGTTATAGAGCAGGTGGACCTTTTTTGACC) | This study | N/A |
| oMP03 (CGCCCGCGGTTATGTTAGTAGAGCAGGTGGACC) | This study | N/A |
| oMP04 (CGCCCGCGGTTAAACCATTGTTAGTAGAGCAGGTGGACC*)* | This study | N/A |
| oMP05 (CGCCCGCGGTTACGTTATAACCATTGTTAGTAGAGCAGG) | This study | N/A |
| oMP06 (CGCCCGCGGTTACATTTGTTCACTATGCGTTATAACC) | This study | N/A |
| oMP07 (CGCCCGCGGTTAGTTACAAGACCTCATCATTTGTTCAC) | This study | N/A |
| oMP08 (CGCCCGCGGTTAAAGAACTGCAATCGAGTTACAAGACC) | This study | N/A |
| oMP09 (CGCCCGCGGTTACTCAACCACTTTACCATCTTTAAG) | This study | N/A |
| oMP10 (GAAGTAATTATCTACTTTTTACAACAAATCTAGAATTCCTGCAGCCCGGGATGAACTTTTTAAGTTTTAA) | This study | N/A |
| oMP11 (CGCGGATCCATGAACGACCAAA) | This study | N/A |
| oMP12 (CGCCTGCAGTTAATCTAGGTCATCATCAATT TCC) | This study | N/A |
| oMP13 (AAATCTAGAATTCCTGCAGCATGAACTTTTTAAGTTTTAAGACTACAAAACAC) | This study | N/A |
| oMP14 (AACTACTCATAAGCTTAGAACCACCACCACCAG) | This study | N/A |
| oMP15 (TTCTAAGCTTATGAGTAGTTTGTGTCTACAGCG) | This study | N/A |
| oMP16 (AATTGGAGCTCCACCGCGGCCTATTTAGAGTACTGTTTAGCTTGAAG) | This study | N/A |

| Table S3: Recombinant DNA. Related to STAR Methods. | | |
|---|---|---|
| pSM694 *(2µ LEU2 STE6::HA)* | (Berkower et al., 1994) | N/A |
| pSM1175 (*2µ LEU2 ste6-L1240X::HA)* | This study | N/A |
| pSM1176 *(2µ LEU2 ste6-M1242X::HA)* | This study | N/A |
| pSM1177 *(2µ LEU2 ste6-I1244X::HA)* | This study | N/A |
| pSM1178 *(2µ LEU2 ste6-H1246X::HA)* | This study | N/A |
| pSM1179 *(2µ LEU2 ste6-M1251X::HA)* | This study | N/A |
| pSM1180 *(2µ LEU2 ste6-S1256X::HA)* | This study | N/A |
| pSM1264 *(2µ LEU2 ste6-R1268X::HA)* | This study | N/A |
| pSM1400 *(2µ LEU2 ste6-K1261X::HA)* | This study | N/A |
| pSM2319 *(2µ LEU2 ste6-Q1249X::HA)* | This study | N/A |
| pSM3149 (*p404 TRP P-GPD mCherry-Scs2TM-NatMax)* | (Zhou et al., 2014) | N/A |
| pCG19 *(2µ URA3 P-PGK chimera::HA)* | (Guerriero et al., 2017) | N/A |
| pCG12 *(2µ URA3 P-PGK chimera-Q394X::HA)* | (Guerriero et al., 2017) | N/A |
| pKN31 *(2µ HIS3 Pcup1-mycUb-Tcyc1)* | (Nakatsukasa et al., 2008) | N/A |
| pMP01 *(2µ URA3 P-PGK chimera-L385X::HA)* | This study | N/A |
| pMP02 *(2µ URA3 P-PGK chimera-M387X::HA)* | This study | N/A |
| pMP03 *(2µ URA3 P-PGK chimera-I389X::HA)* | This study | N/A |
| pMP04 *(2µ URA3 P-PGK chimera-H391X::HA)* | This study | N/A |
| pMP05 *(2µ URA3 P-PGK chimera-M396X::HA)* | This study | N/A |
| pMP06 *(2µ URA3 P-PGK chimera-S401X::HA)* | This study | N/A |
| pMP07 *(2µ URA3 P-PGK chimera-K406X::HA)* | This study | N/A |
| pMP08 *(2µ URA3 P-PGK chimera-R413X::HA)* | This study | N/A |
| pMP09 *(2µ LEU2 HSP104)* | This study | N/A |
| pMP10 *(2µ LEU2 P-GPD HSP104)* | This study | N/A |

| | | |
|---|---|---|
| pMP11(*CEN LEU2 P-TEF HSP104*) | This study | N/A |
| pMP12 (*2µ URA3 P-PGK TM-Ubc9$^{ts}$::HA)* | This study | N/A |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Cycloheximide Chase
0’ 15’ 30’ 60’
t1/2
Wild Type
23’
Q394X
14’
Pulse Chase
Wild Type
28’
Q394X
11’
B
M1242 I1244
L1240
H1246
M1251
S1256
Q1249
R1268
K1261
C
Vo 660 158 43 13.7
Q394X
Sec61
% of Total Protein
Fraction Number
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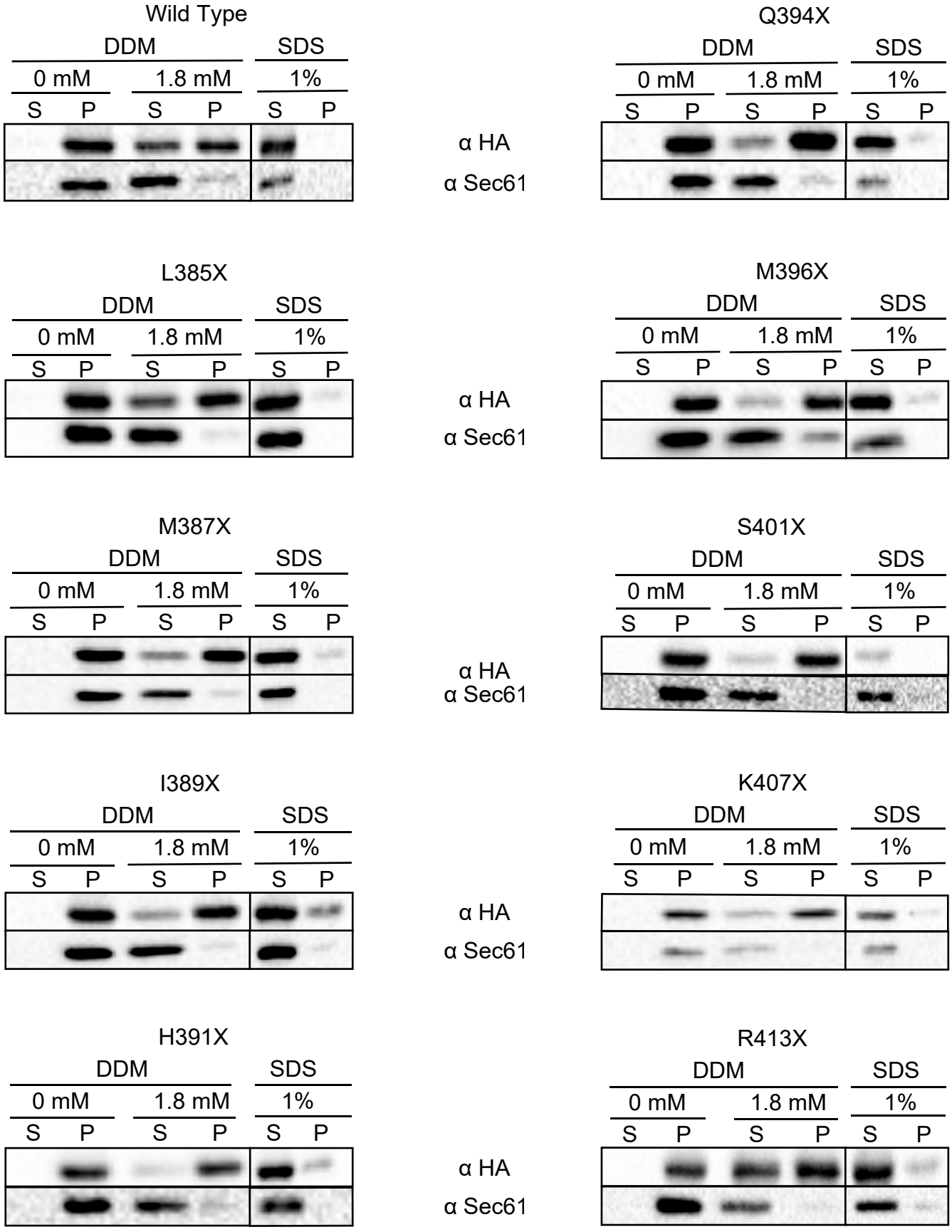
**Figure S1**


Wild Type
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SDS
0 mM
1.8 mM
1%
S P S P S P
α HA
α Sec61
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L385X
M396X
M387X
S401X
I389X
K407X
H391X
R413X

**Figure S2**

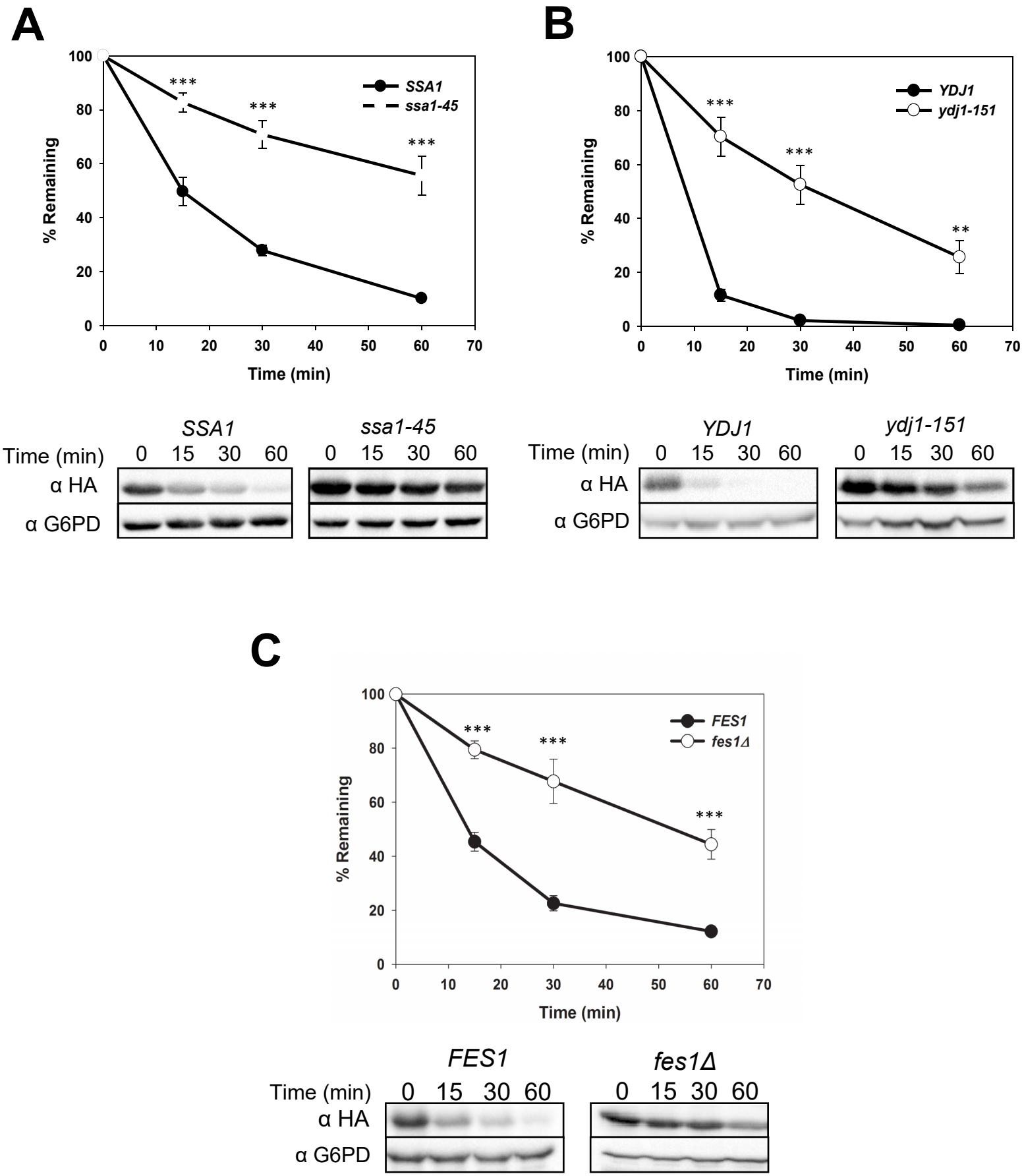
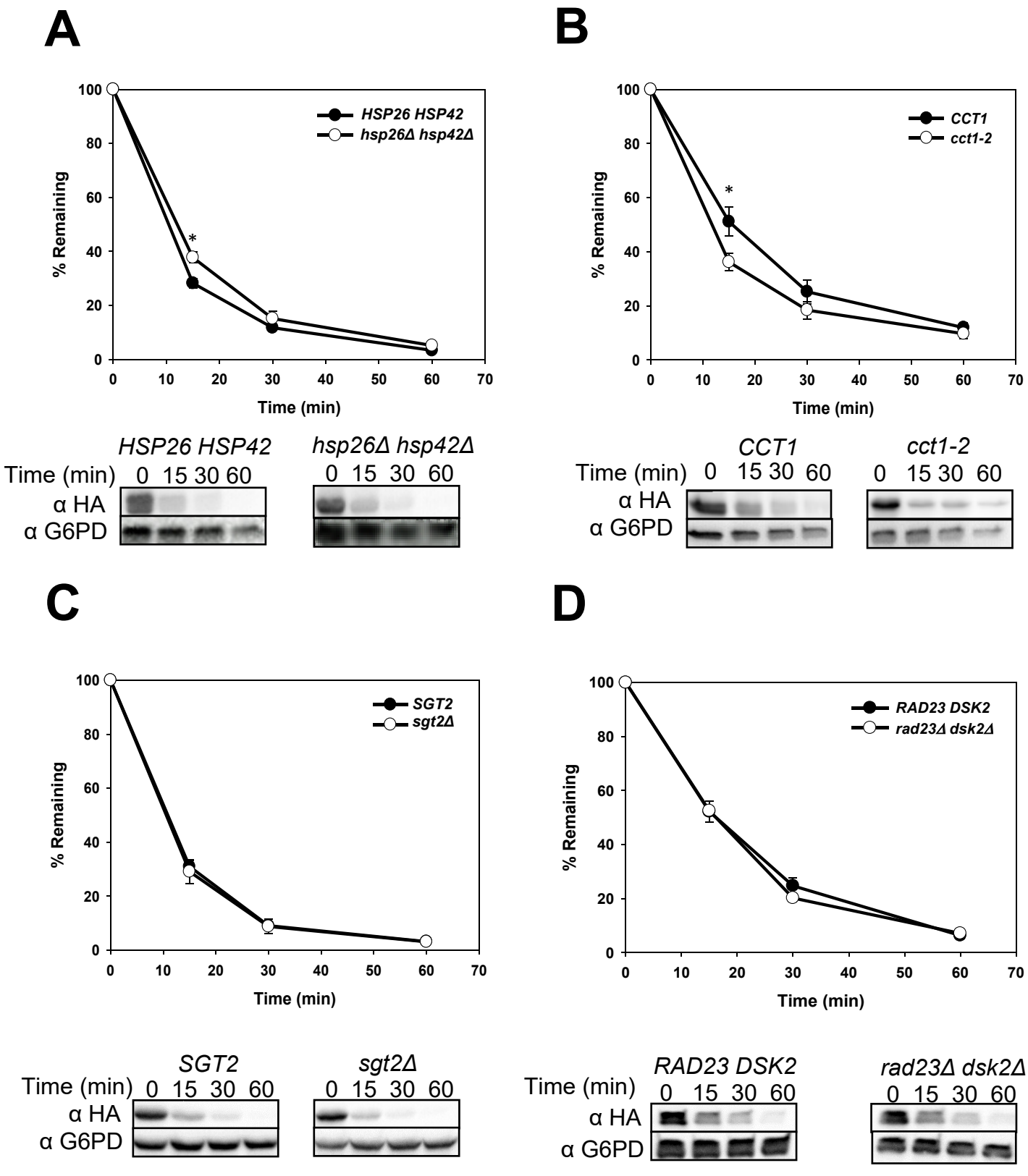

A
B
C
% Remaining
Time (min)
SSA1
ssa1-45
YDJ1
ydj1-151
FES1
fes1Δ
***
**
Time (min)
0 15 30 60
α HA
α G6PD

**Figure S3**


A
HSP26 HSP42
hsp26Δ hsp42Δ
% Remaining
Time (min)
*
HSP26 HSP42
hsp26Δ hsp42Δ
Time (min) 0 15 30 60
α HA
α G6PD
B
CCT1
cct1-2
% Remaining
Time (min)
*
CCT1
cct1-2
Time (min) 0 15 30 60
α HA
α G6PD
C
SGT2
sgt2Δ
% Remaining
Time (min)
SGT2
sgt2Δ
Time (min) 0 15 30 60
α HA
α G6PD
D
RAD23 DSK2
rad23Δ dsk2Δ
% Remaining
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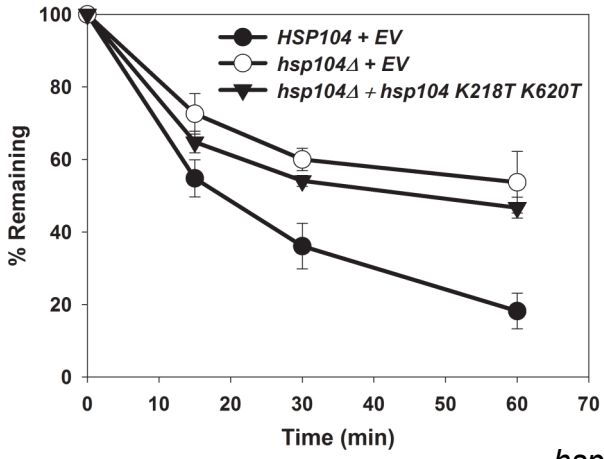
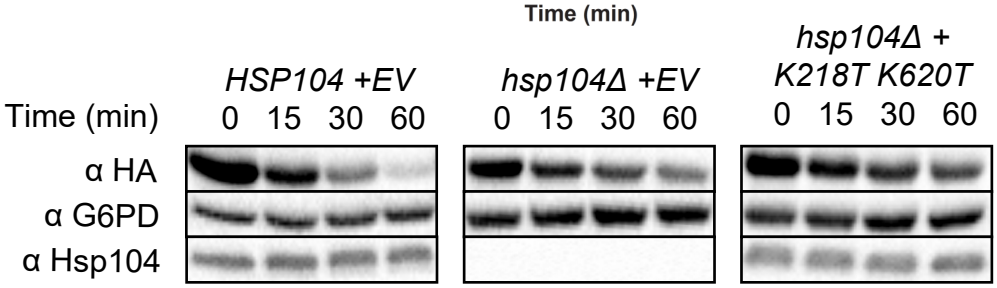
RAD23 DSK2
rad23Δ dsk2Δ
Time (min) 0 15 30 60
α HA
α G6PD

**Figure S4**

**A**

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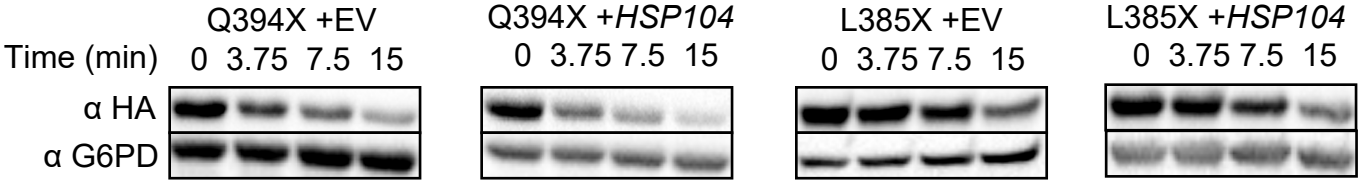


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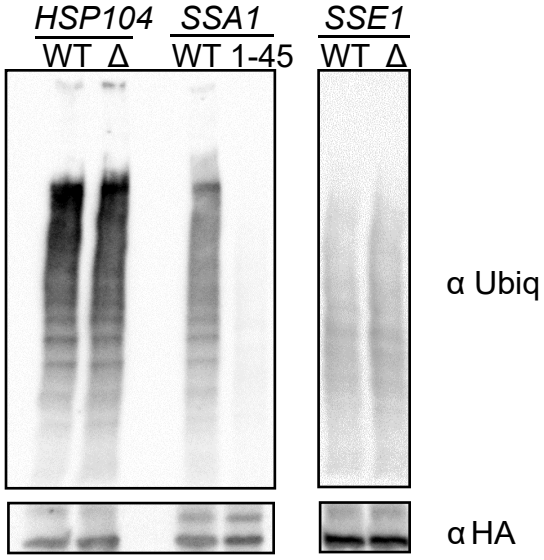
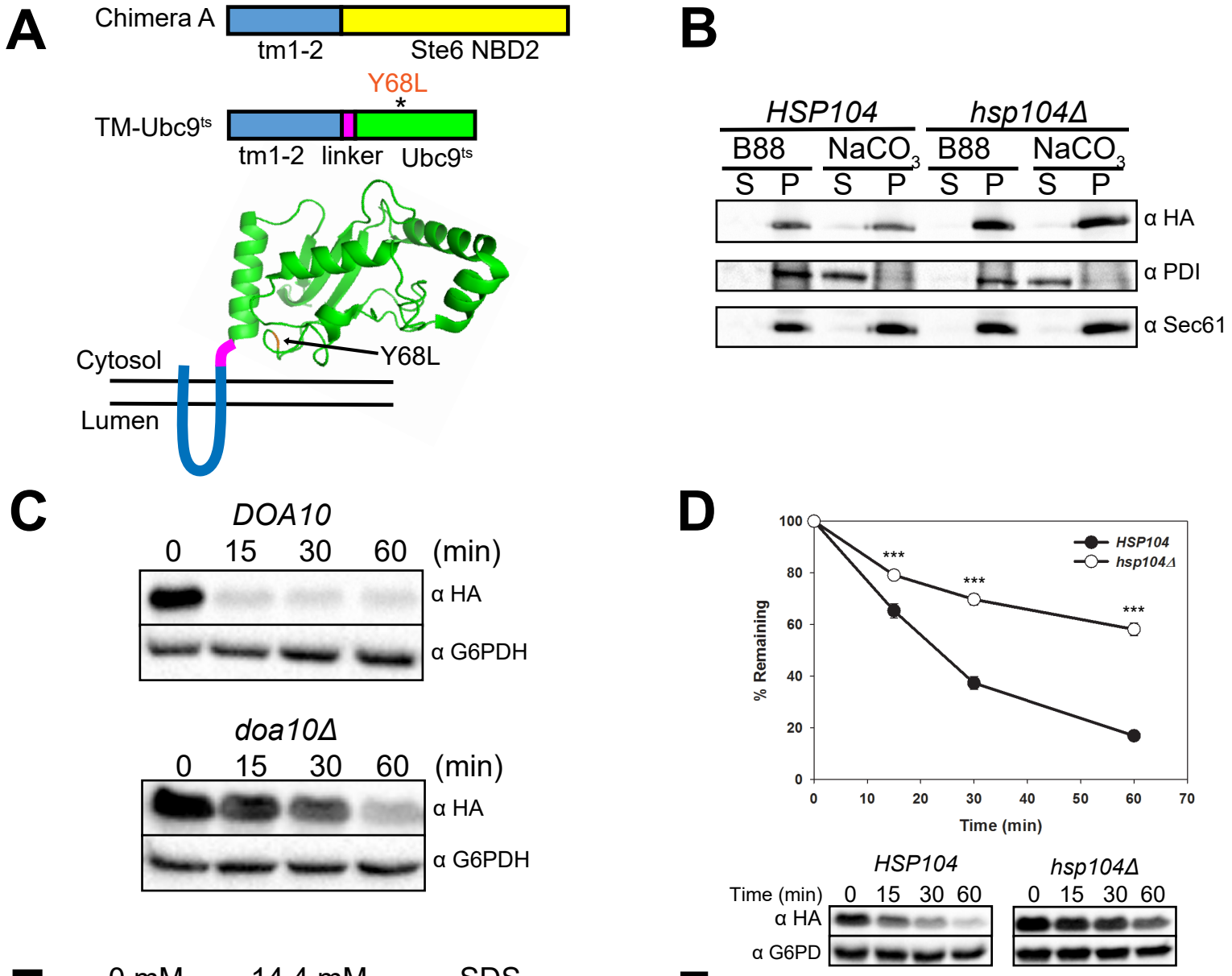



**Figure S5**


A
Chimera A
tm1-2
Ste6 NBD2
Y68L
TM-Ubc9ts
tm1-2 linker Ubc9ts
Cytosol
Lumen
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B
HSP104
hsp104Δ
B88
NaCO3
S P S P S P S P
α HA
α PDI
α Sec61
C
DOA10
0 15 30 60 (min)
α HA
α G6PDH
doa10Δ
0 15 30 60 (min)
α HA
α G6PDH
D
% Remaining
Time (min)
HSP104
hsp104Δ
***
Time (min) 0 15 30 60
α HA
α G6PD
E
0 mM
14.4 mM
SDS
WT Δ WT Δ WT Δ
S P S P S P S P S P S P
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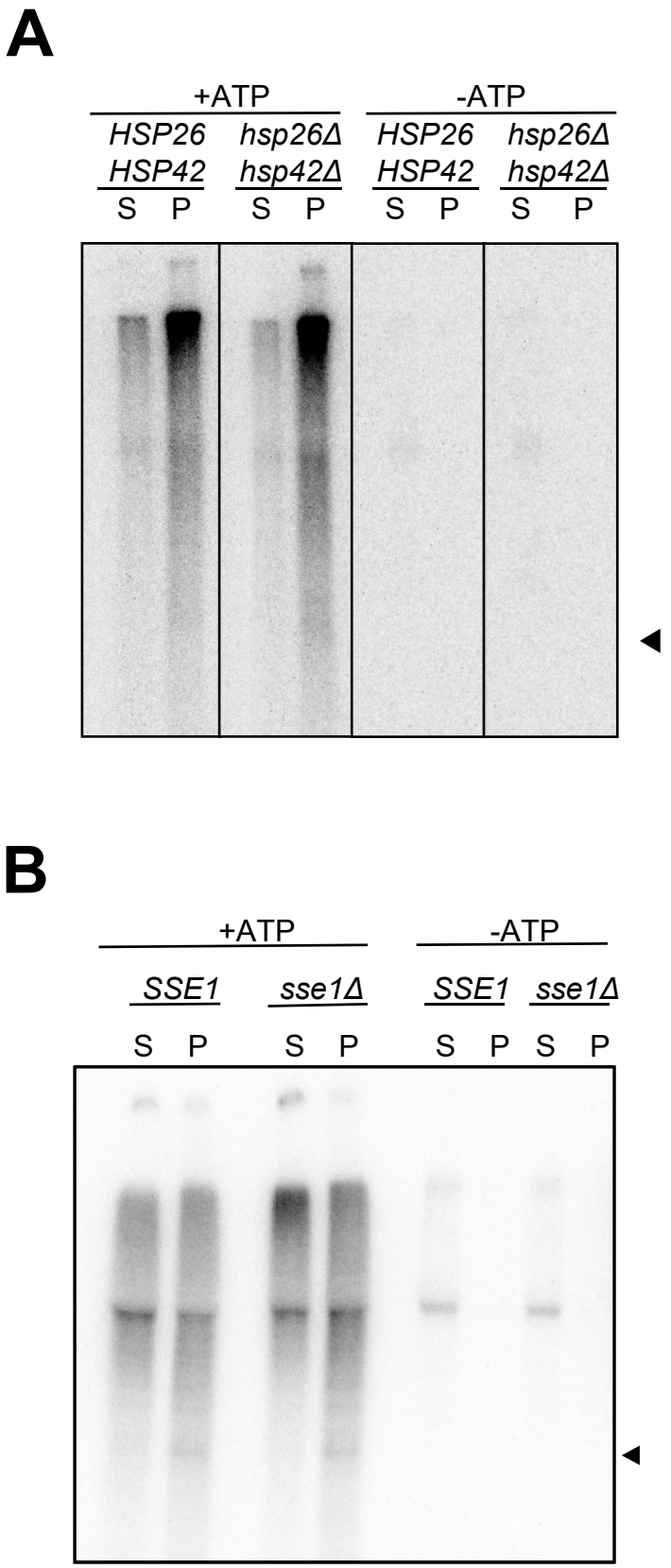
α Sec61
F
HA
Kar2
HSP104
hsp104Δ

**Figure S6**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+ATP
-ATP
HSP26
HSP42
hsp26Δ
hsp42Δ
HSP26
HSP42
hsp26Δ
hsp42Δ
S P S P S P S P
B
+ATP
-ATP
SSE1
sse1Δ
SSE1
sse1Δ
S P S P S P S P

**Figure S7**