RESEARCH ARTICLE

Figures and figure supplements

A protein quality control pathway at the mitochondrial outer membrane

Meredith B Metzger *et al*

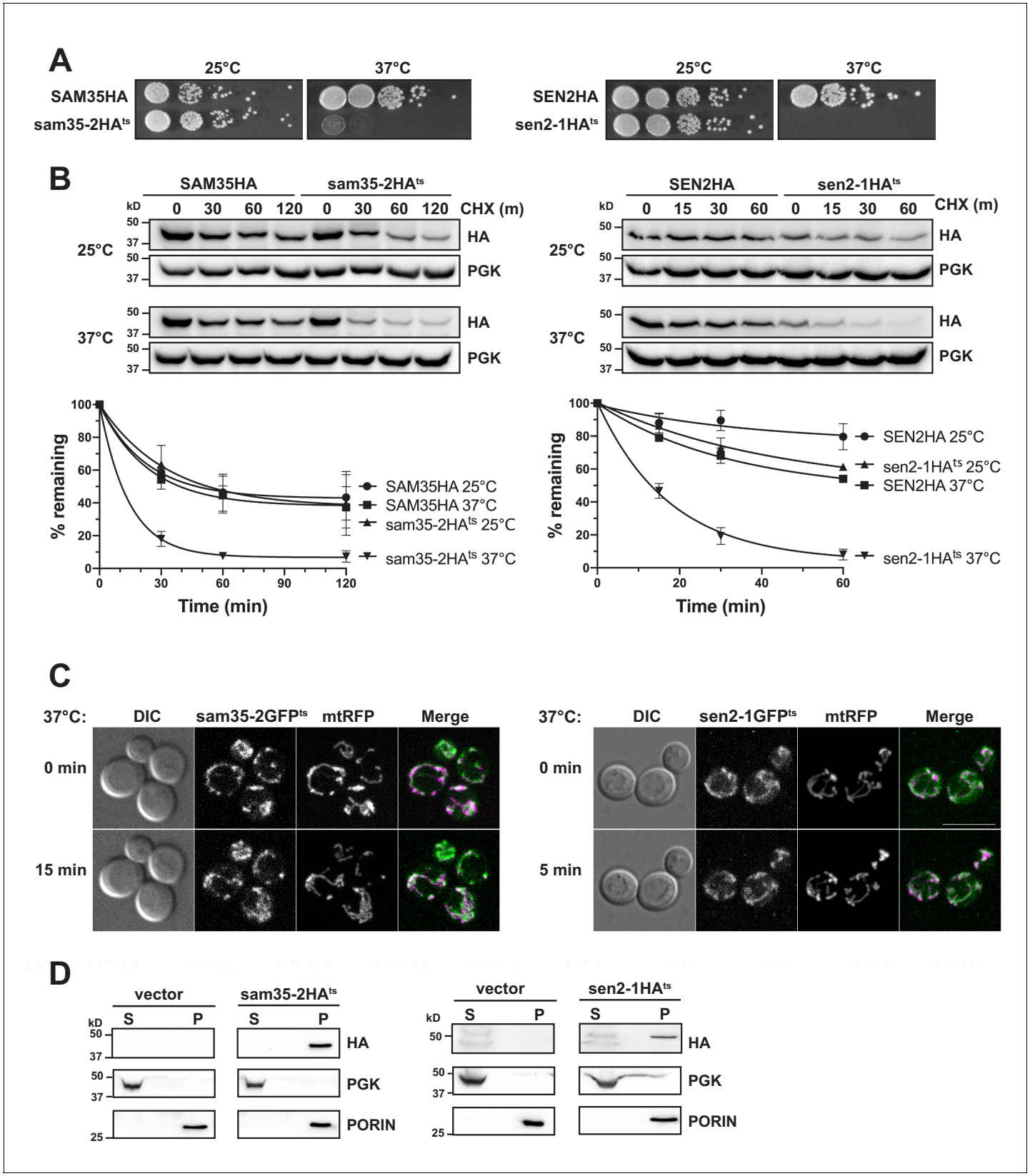

Figure 1. The temperature sensitive proteins sam35-2HA^{ts} and sen2-1HA^{ts} are novel thermosensitive substrates for mitochondrial quality control. (**A**) Spot growth assay of cells expressing chromosomal SAM35HA, sam35-2HA^{ts}, SEN2HA, or sen2-1HA^{ts} (yMM36, 37, 40, and 41, respectively) at

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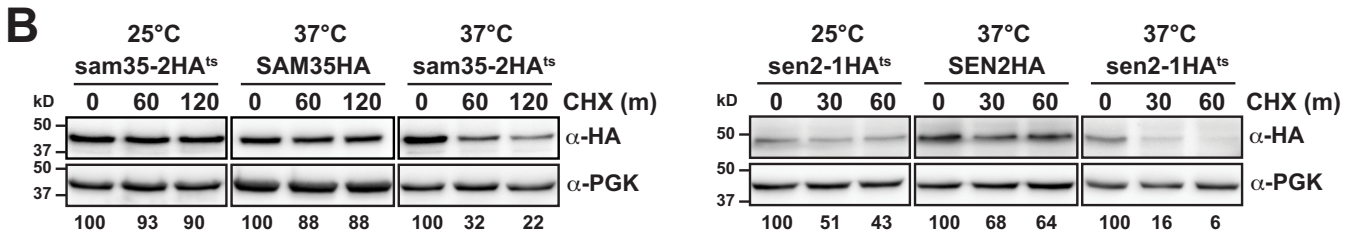
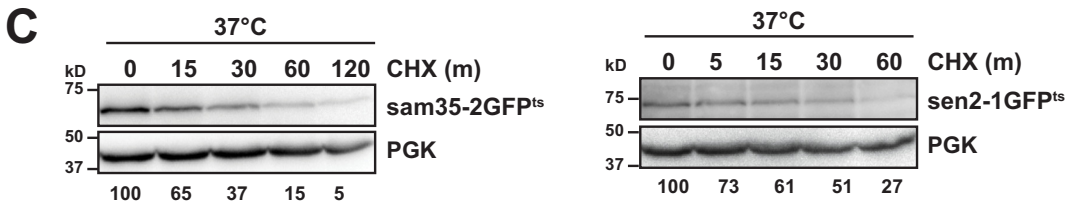
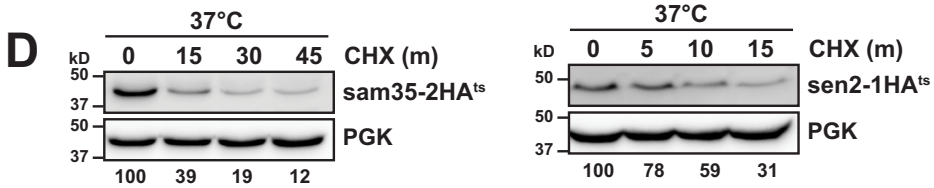
permissive (25°) or non-permissive (37°) temperatures. (**B**) Wild type (WT; WCG4a) yeast were treated with cycloheximide (CHX) at 25°C or 37°C and analyzed at the indicated times to assess the degradation of centromeric (CEN) plasmid-expressed SAM35HA, sam35-2HA^{ts}, SEN2HA, or sen2-1HA^{ts} (pMM158, 157, 159, 160, respectively). The ts- proteins were detected by immunoblotting with HA antibody. Phosphoglycerate kinase (PGK) served as a protein loading control. Graphed below is the mean and standard deviation (SD) of the PGK-normalized HA signal at each time point for three biological replicates. (**C**) Live-cell microscopy analysis of agarose-embedded WT cells (WCG4a) co-expressing a mitochondrial-matrix targeted RFP (mtRFP; pMD12) and either sam35-2GFP^{ts} (pMD1) or sen2-1GFP^{ts} (pMD4) at the indicated times after temperature shift to 37°C. CHX was also added at 0 min, although CHX diffusion through agarose is likely problematic. 'Merge' of GFP (green) and RFP (magenta) channels and differential interference contrast (DIC) are shown; Scale bar = 10 μm. (**D**) Lysates of spheroplasted yeast from the strains used in B were fractionated at 12,000*xg* at 37°C into mitochondrial pellet (P) and post-mitochondrial supernatant (S). Fractions were subject to immunoblotting with antibodies to HA, PGK (cytosolic protein control), and PORIN (mitochondrial protein control).

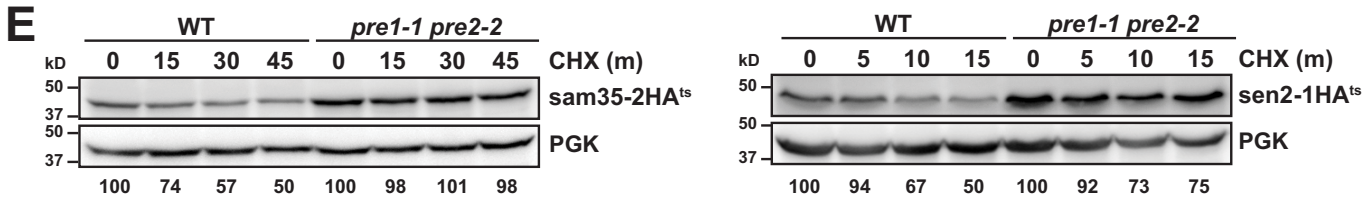
A

sam35-2	L35S E53G L54S G58E W82R K136E M150T L155P Y245C E315G
sen2-1	M72I Q134R L220P F300C









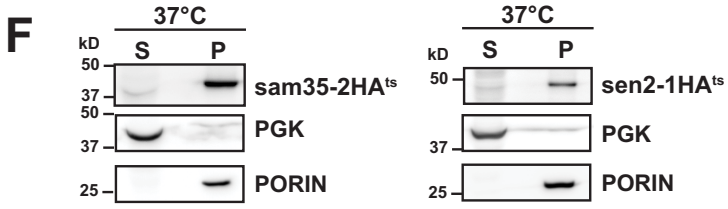


Figure 1—figure supplement 1. The temperature sensitive proteins sam35-2HA^{ts} and sen2-1HA^{ts} are novel thermosensitive substrates for mitochondrial quality control. (**A**) Amino acid substitutions in the sam35-2^{ts} and sen2-1^{ts} proteins compared to the WT proteins. (**B**) Cells with chromosomal SAM35HA, sam35-2HA^{ts}, SEN2HA, or sen2-1HA^{ts} (yMM36, 37, 40, and 41, respectively) were treated with cycloheximide (CHX) at 25°C or 37°C and analyzed at the indicated times to assess the degradation of genomic SAM35HA, sam35-2HA^{ts}, SEN2HA, or sen2-1HA^{ts}. Ts- proteins were

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detected by immunoblotting with HA antibody. Phosphoglycerate kinase (PGK) served as a protein loading control. Percentage of each ts- substrate remaining is labeled for each time point under blots. (**C**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2GFP^{ts} or sen2-1GFP^{ts} (pMD1 or 4, respectively) in WT yeast cells (WCG4a). (**D**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in WT (WCG4a) cells. (**E**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in spheroplasts generated from WT (WCG4a) and pre1-1 pre2-2 (WCG4-11/21a) yeast cells. (**F**) Lysates from strains expressing genomic sam35-2HA^{ts} or sen2-1HA^{ts} (yMM37 and 41, respectively) were fractionated at 12,000*xg* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN.

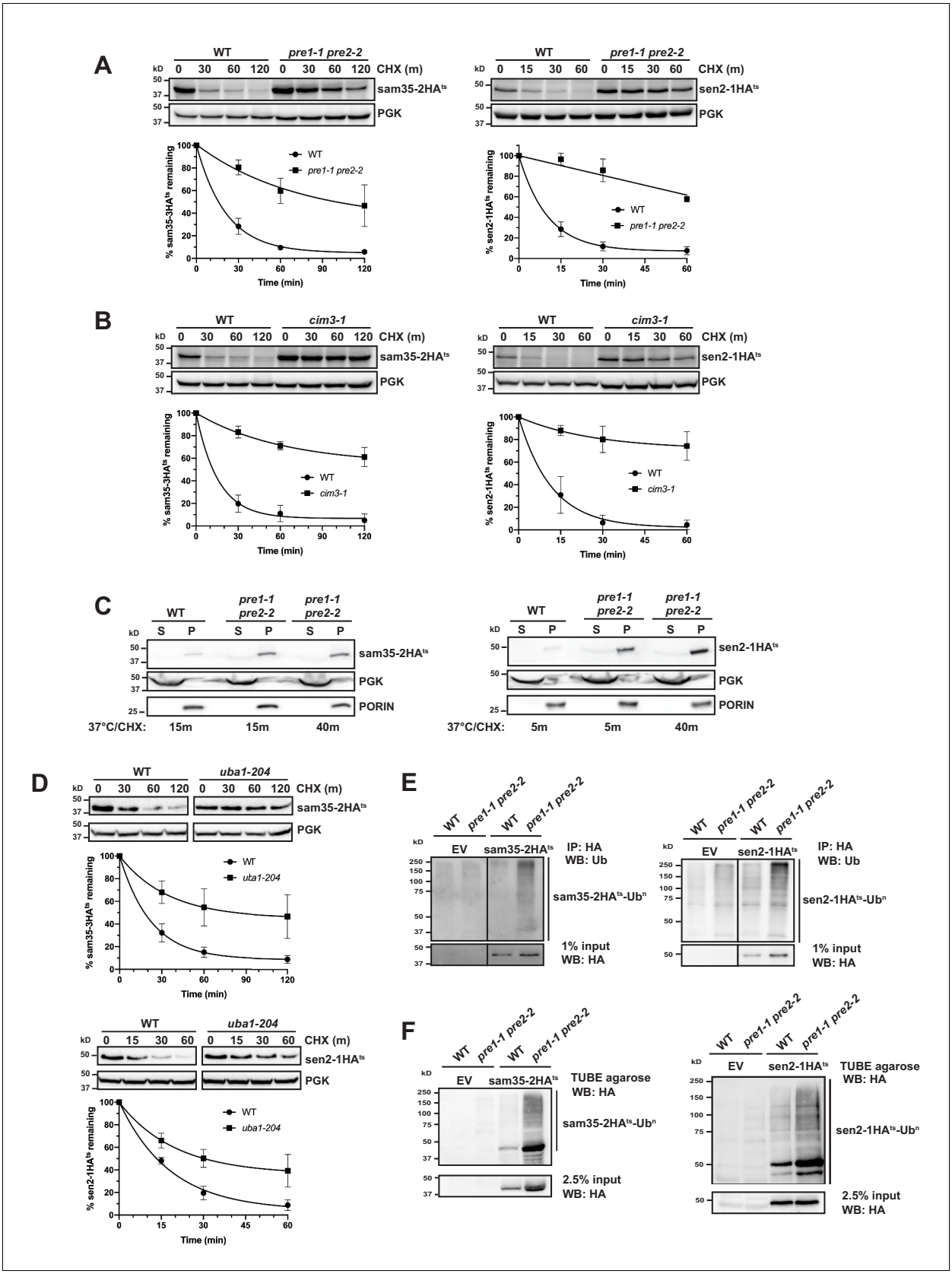

Figure 2. The degradation of MAD QC substrates requires the ubiquitin-proteasome system. (**A, B**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in WT (WCG4a) and *pre1-1 pre2-2* proteasome mutant (WCG4-11/

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21a) cells (**A**) or WT (CIM) and *cim3-1* proteasome mutant cells (**B**). Proteins were detected by immunoblotting. Graphed below is the mean and SD of the PGK-normalized HA signal at each time point for three biological replicates. (**C**) Lysates from the strains used in A were fractionated at 12,000*xg* into mitochondrial pellets (P) and post-mitochondrial supernatants (S) after incubation at 37°C for the indicated times. Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN. (**D**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in a *uba1-204* strain relative to its isogenic WT strain. (**E**) Ubiquitination of sam35-2HA^{ts} and sen2-1HA^{ts} was assessed by immunoprecipitation (IP) from lysates of the strains used in A with anti-HA agarose, followed by immunoblotting with ubiquitin antibodies. 1% of IP input lysate was reserved and also analyzed by immunoblotting. (**F**) Ubiquitination of sam35-2HA^{ts} and sen2-1HA^{ts} was assessed by IP from lysates of the strains used in A using tandem ubiquitin-binding entities (TUBE) agarose, followed by immunoblotting with HA antibody. 2.5% of the TUBE input lysate was reserved and analyzed by immunoblotting.

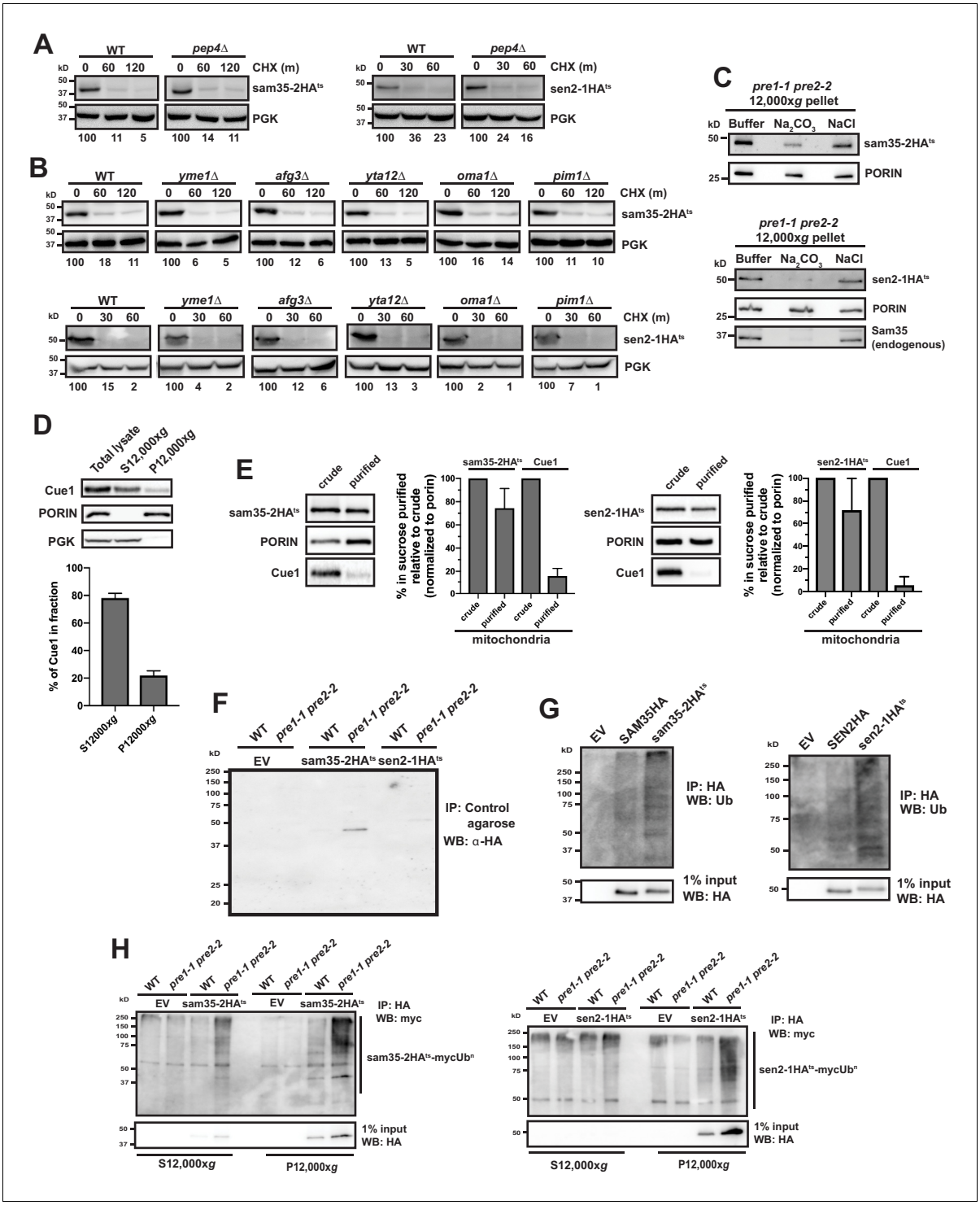

Figure 2—figure supplement 1. The degradation of MAD QC substrates requires the ubiquitin-proteasome system. (**A, B**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in WT (BY4741) and *pep4*Δ (**A**) or *yme1*Δ, *afg3*Δ, *yta12*Δ, *oma1*Δ, or *pim1*Δ (**B**) cells. Proteins were detected by immunoblotting. Percentage of each ts- substrate remaining is labeled for each time point under blots. (**C**) Sodium carbonate (Na_2CO_3) extraction of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 and 160, respectively) from the 12,000x*g* mitochondrial pellet of *pre1-1 pre2-2* (WCG4-11/21a) cells. Control treatments of sodium chloride (NaCl) and buffer are shown for comparison. Proteins were detected with immunoblotting for HA, PORIN, and Sam35 (in analysis of sen2-1HA^{ts} only). (**D**) Total lysate from *pre1-1 pre2-2* (WCG4-11/21a) cells expressing sam35-2HA^{ts} (pMM157) were fractionated at 37°C into mitochondrial pellets (P12,000x*g*) and post-mitochondrial supernatants (S12,000x*g*). Fractions were subject to immunoblotting with antibodies to Cue1, PGK, and PORIN. The Cue1 signal in each fraction was quantified and the mean

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plus standard deviation (SD) of the percentage of the total Cue1 signal is graphed below (N = 3). (**E**) P12,000xg mitochondrial fractions ('crude') isolated from *pre1-1 pre2-2* (WCG4-11/21a) cells expressing sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 and 160, respectively) were further purified by sucrose gradient fractionation ('purified'). The HA (sam35-2HA^{ts} or sen2-1HA^{ts}) and Cue1 signals in crude and purified mitochondrial fractions were quantified and normalized to the PORIN signal. The crude mitochondrial signal was set to 100% and the mean plus SD of the percentage in purified mitochondria relative to crude is graphed (N = 3). (**F**) Negative control experiment for ***Figure 2E***. Cell lysates from *pre1-1 pre2-2* (WCG4-11/21a) cells expressing sam35-2HA^{ts}, sen2-1HA^{ts}, or empty vector (EV; pMM157, pMM160, or pRS315, respectively) were precipitated with unconjugated TUBE control agarose (containing no ubiquitin-binding domains), followed by immunoblotting with HA antibody. (**G**) Ubiquitination was assessed by IP with anti-HA agarose from lysates of WT (BY4741) cells expressing empty vector (EV; pRS315), SEN2HA (pMM159), sen2-1HA^{ts} (pMM160), SAM35HA (pMM158), or sam35-2HA^{ts} (pMM157), followed by immunoblotting with ubiquitin antibody. 1% of the IP was reserved and analyzed by immunoblotting for the unmodified proteins. (**H**) Ubiquitination in the 12,000x*g* mitochondrial pellet (P12,000x*g*) or post-mitochondrial supernatant (S12,000x*g*) was assessed by IP from each fraction with anti-HA agarose from WT (WCG4a) and *pre1-1 pre2-2* (WCG4-11/21a) cells expressing myc-Ub (pSM3666) and either empty vector (EV; pRS315), sam35-2HA^{ts} (pMM157), or sen2-1HA (pMM160), followed by immunoblotting with c-myc antibody. 1% of IP input lysate was reserved and analyzed by immunoblotting.

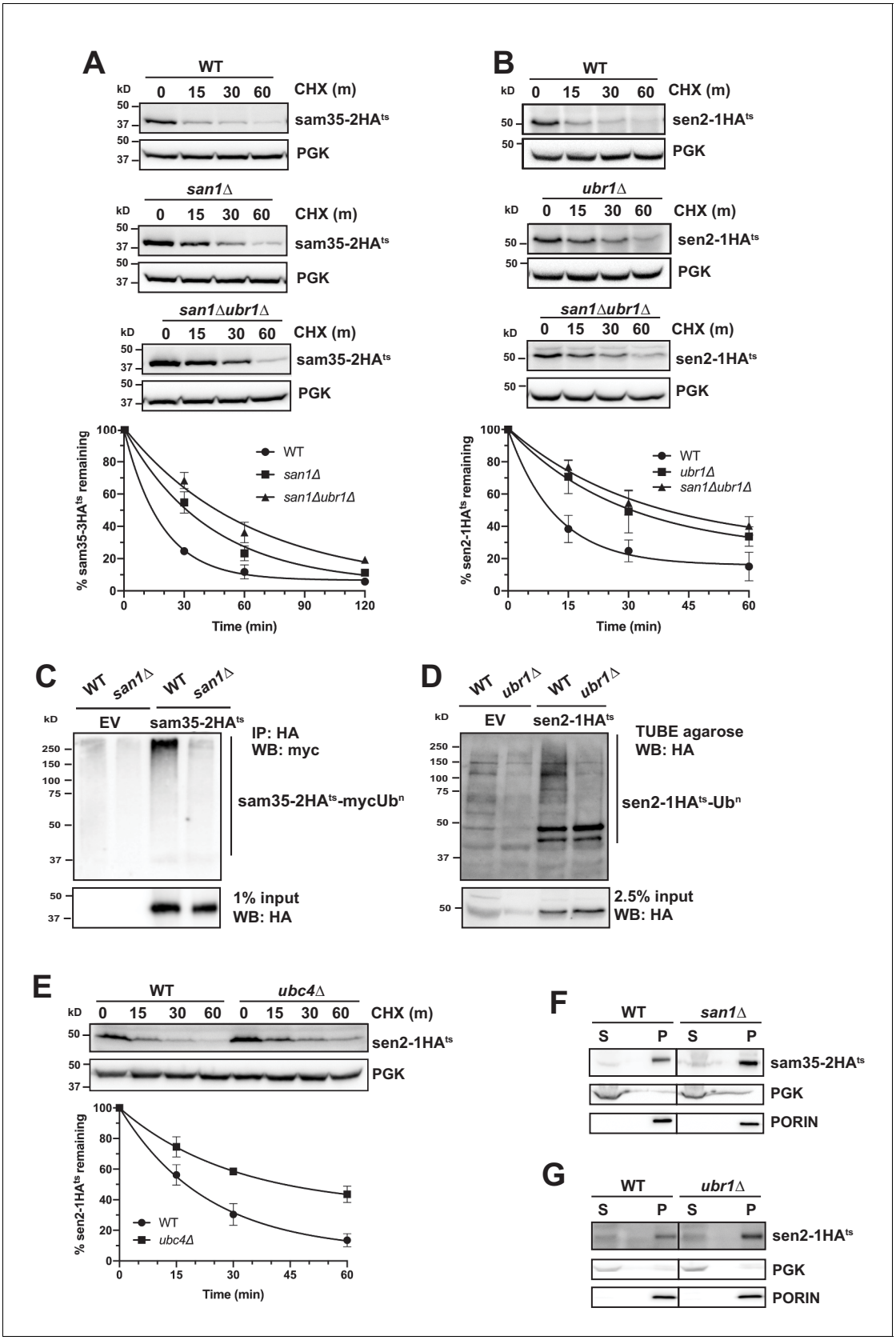

Figure 3. Distinct E3 ubiquitin ligases act on sam35-2HA^{ts} and sen2-1HA^{ts}. (**A**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} (pMM157) in WT (BY4741), *san1*Δ, and *san1*Δ *ubr1*Δ (SM5770) cells. Proteins were detected by immunoblotting. Graphed below is the

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Figure 3 continued

mean and SD of the PGK-normalized HA signal at each time point for three biological replicates. (**B**) CHX chase for the indicated times at 37°C assessing the turnover of sen2-1HA^{ts} (pMM160) in WT (BY4741), *ubr1*Δ (yMM149), and *san1*Δ *ubr1*Δ (SM5770) cells, as in A. (**C**) Ubiquitination of sam35-2HA^{ts} was assessed by IP with anti-HA agarose of lysates from WT (BY4741) and *san1*Δ cells expressing myc-Ub (pSM3666) and either empty vector (EV; pRS315) or sam35-2HA^{ts} (pMM157), followed by immunoblotting with c-myc antibody. 1% of IP input lysate was reserved and analyzed for sam35-2HA^{ts} by immunoblotting. (**D**) Ubiquitination of sen2-1HA^{ts} was assessed by IP of lysates from WT and *ubr1*Δ strains expressing either EV (pRS315) or sen2-1HA^{ts} (pMM160) using TUBE agarose, followed by immunoblotting with HA antibody. 2.5% of the TUBE input lysate was reserved and analyzed by immunoblotting for sen2-1HA^{ts}. (**E**) CHX chase for the indicated times at 37°C assessing the turnover of sen2-1HA^{ts} (pMM160) in *ubc4*Δ cells compared to isogenic WT (BY4741). (**F**) Lysates from the WT and *san1*Δ strains used in A expressing sam35-2HA^{ts} (pMM157) were fractionated at 12,000*xg* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN. (**G**) Lysates from the WT and *ubr1*Δ strains used in B expressing sen2-1HA^{ts} (pMM160) were fractionated at 12,000*xg* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN.

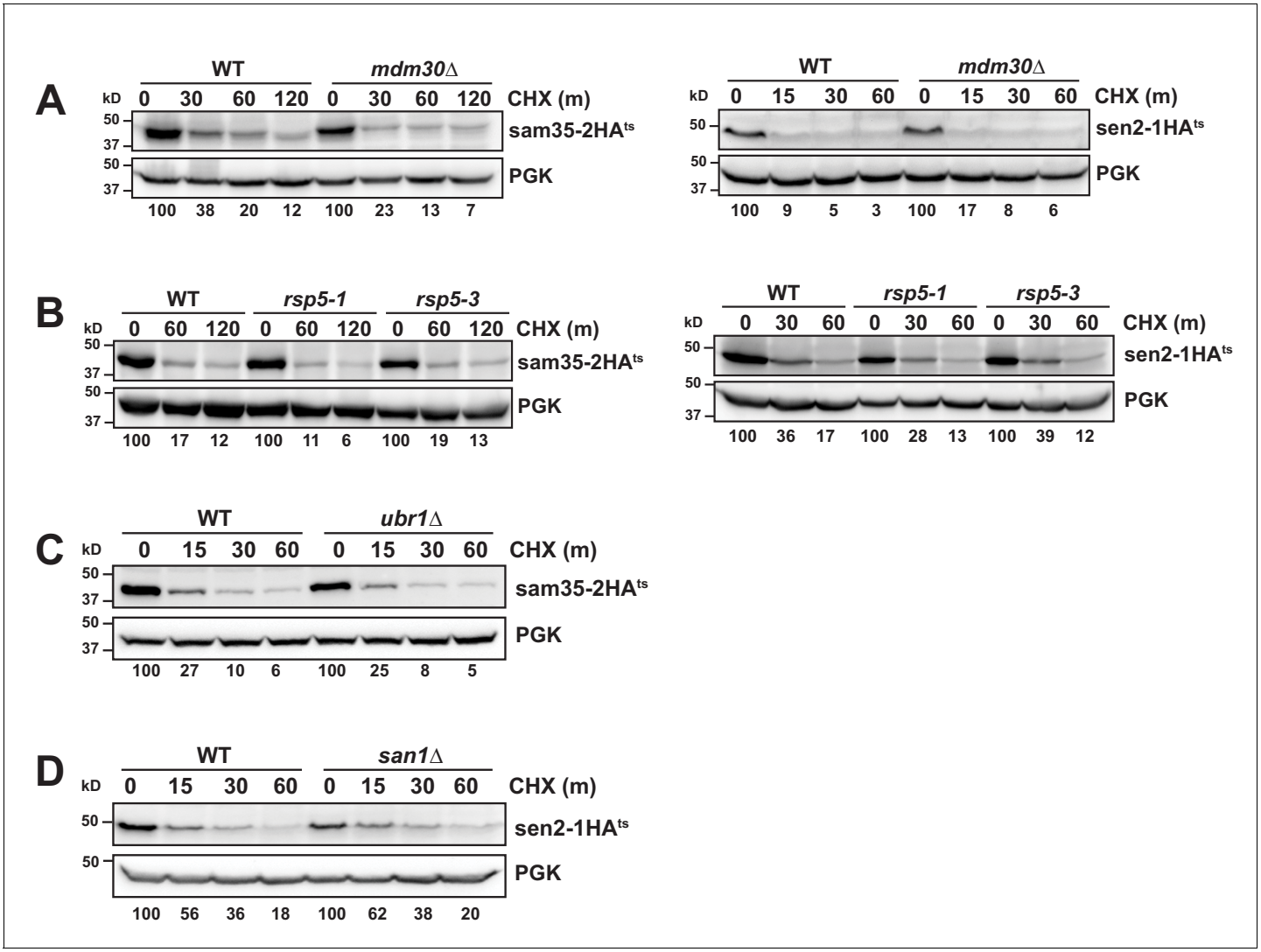

Figure 3—figure supplement 1. Distinct E3 ubiquitin ligases act on sam35-2HA^{ts} and sen2-1HA^{ts}. (**A–D**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in *mdm30*Δ (**A**); *rsp5-1* and *rsp5-3* (**B**); *ubr1*Δ (yMM149; **C**); or *san1*Δ (**D**) strains relative to isogenic WT strains. Proteins were detected by immunoblotting. Percentage of each ts- substrate remaining is labeled for each time point under blots.

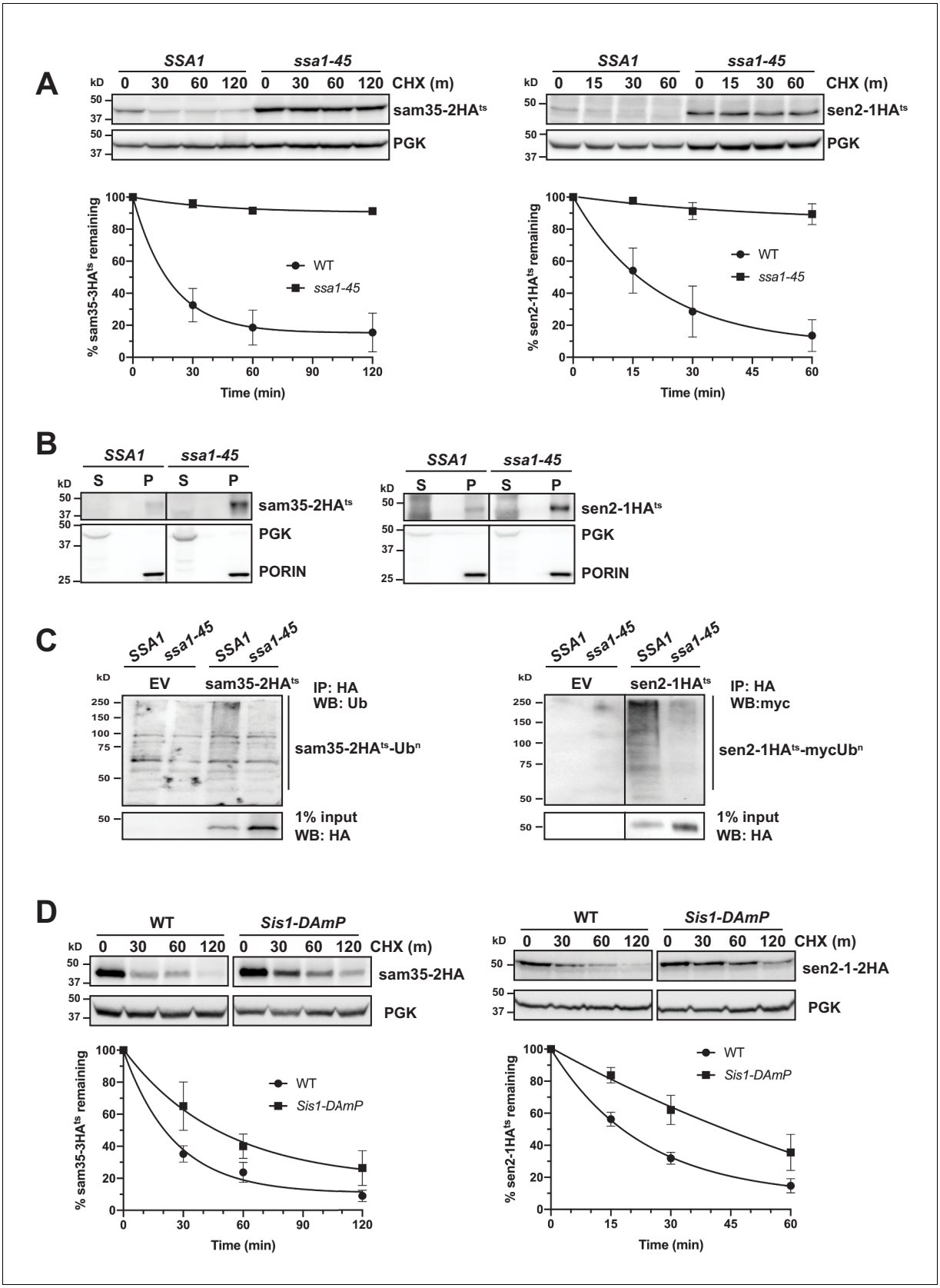

Figure 4. Cytosolic chaperones are required for the degradation of sen2-1HA^{ts} and sam35-2HA^{ts}. (**A**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} (pMM231) and sen2-1HA^{ts} (pMM234) in WT (*SSA1*) and *ssa1-45*^{ts} cells. Proteins were detected by

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Figure 4 continued

immunoblotting. Graphed below is the mean and SD of the PGK-normalized HA signal at each time point for three biological replicates. (**B**) Lysates from WT and *ssa1-45*^{ts} strains expressing sam35-2HA^{ts} (pMM231) or sen2-1HA^{ts} (pMM234) were fractionated at 12,000x*g* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN. (**C**) Ubiquitination of sam35-2HA^{ts} and sen2-1HA^{ts} was assessed by IP with anti-HA agarose from lysates from WT and *ssa1-45*^{ts} cells expressing either empty vector (EV; pRS316), sam35-2HA^{ts} (pMM231), or sen2-1HA^{ts} (pMM234), followed by immunoblotting with either ubiquitin or c-myc antibody. 1% of IP input lysate was reserved and analyzed by immunoblotting. (**D**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} (pMM157) and sen2-1HA^{ts} (pMM160) in WT (*yTHC*) and *Sis1-DAmP* cells treated with 10 µg/mL doxycycline for 18 hr at 25°C to decrease Sis1 mRNA abundance prior to the addition of CHX.

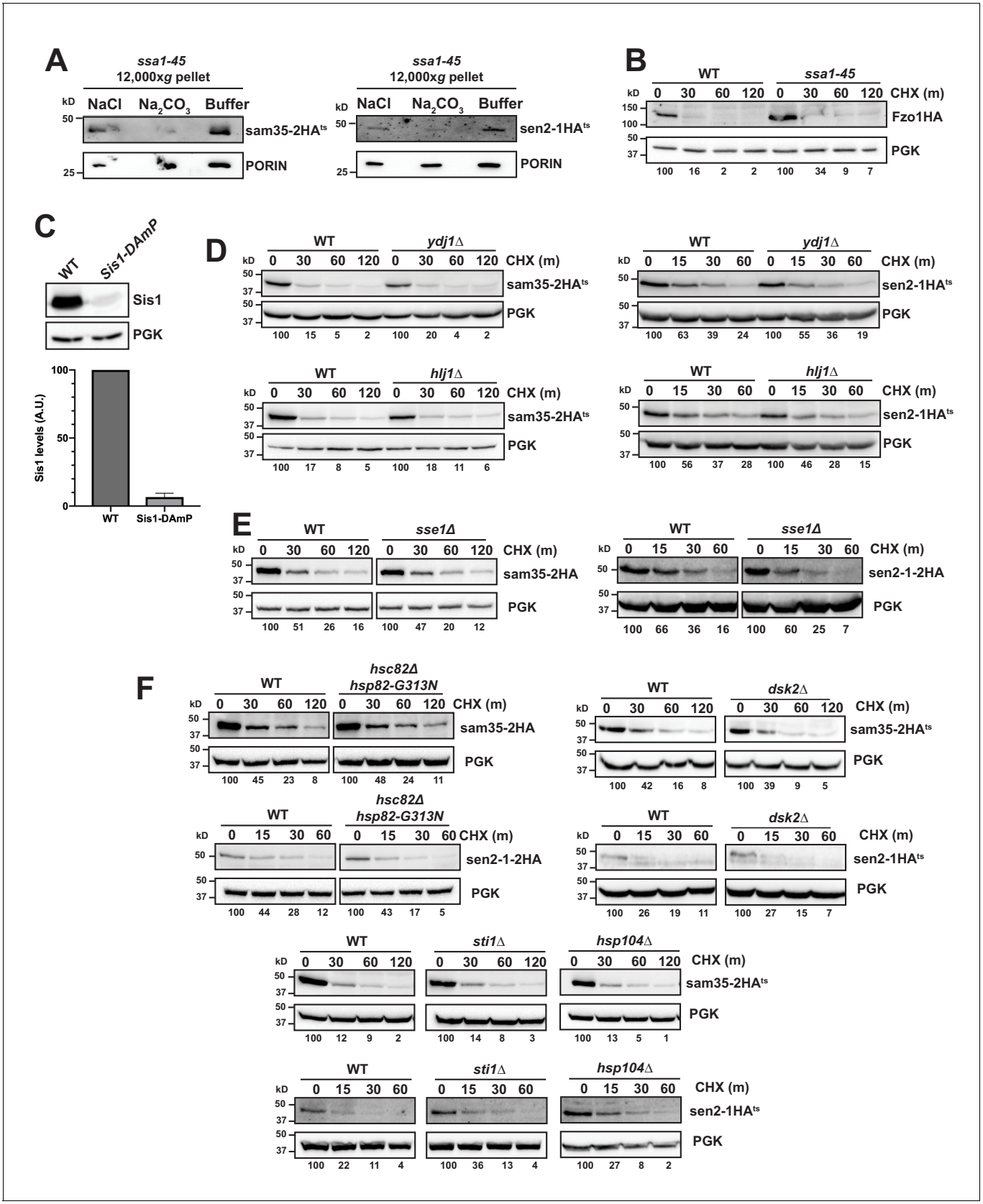

Figure 4—figure supplement 1. Cytosolic chaperones are required for the degradation of sen2-1HA^{ts} and sam35-2HA^{ts}. (**A**) Sodium carbonate (Na_2CO_3) extraction of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 and 160, respectively) from the 12,000*xg* mitochondrial pellet of *ssa1-45* cells. Control

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treatments of sodium chloride (NaCl) and buffer are shown for comparison. Proteins were detected with immunoblotting with HA and PORIN antibodies. (**B**) CHX chase for the indicated times at 37˚C assessing the turnover of Fzo1HA (pADH1-Fzo1pHA) in *SSA1* WT and *ssa1-45* mutant strains. Proteins were detected by immunoblotting. Percentage of each ts- substrate remaining is labeled for each time point under blots. (**C**) Sis1 protein levels in WT (*yTHC*) and *Sis1-DAmP* cells treated with 10 µg/mL doxycycline for 18 hr at 25˚C were quantified and normalized to PGK levels. The mean and SD were graphed (N = 3). (**D**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in *ydj1*Δ and *hlj1*Δ. (**E, F**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in *sse1*Δ (**E**) or chaperone mutant strains (*hsc82*Δ *hsp82-G313N, sti1*Δ, *hsp104*Δ, *dsk2*Δ; **F**), relative to their isogenic WT strains.

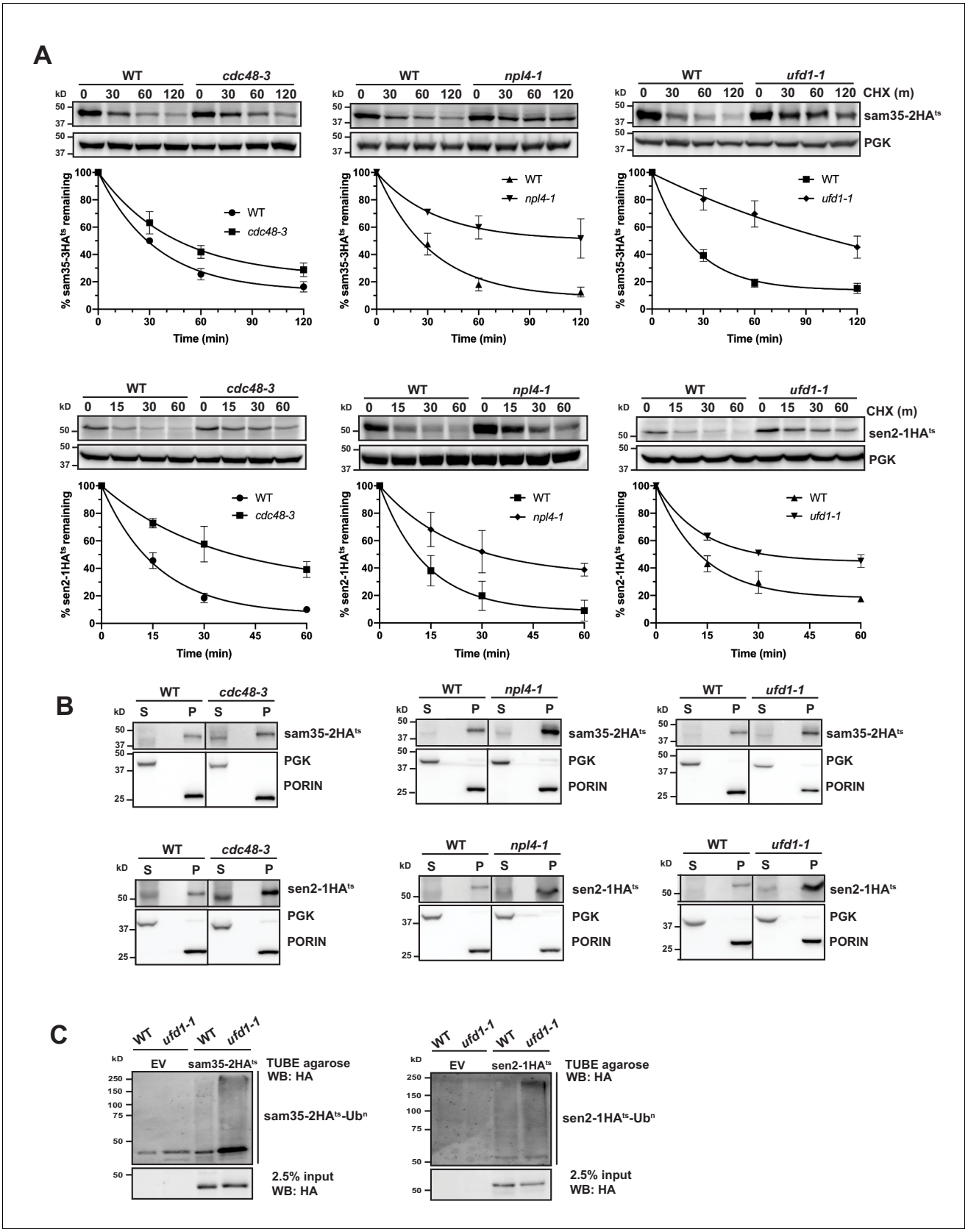

Figure 5. The Cdc48-Npl4-Ufd1 complex is required for degradation of MAD substrates. (**A**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} (pMM157) or sen2-1HA^{ts} (pMM160) in *cdc48-3*, *npl4-1*, and *ufd1-1* mutant strains compared to isogenic WT strains. Proteins

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were detected by immunoblotting. Graphed below is the mean and SD of the PGK-normalized HA signal at each time point for three biological replicates. (**B**) Lysates from the strains used in A expressing sam35-2HA^{ts} (pMM157) or sen2-1HA^{ts} (pMM160) were fractionated at 12,000*xg* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN. (**C**) Ubiquitination of sam35-2HA^{ts} and sen2-1HA^{ts} was assessed by IP from lysates of the *ufd1-1* mutant and isogenic WT strain used in A using TUBE agarose, followed by immunoblotting with HA antibody. 2.5% of the TUBE input lysate was reserved and analyzed by immunoblotting.

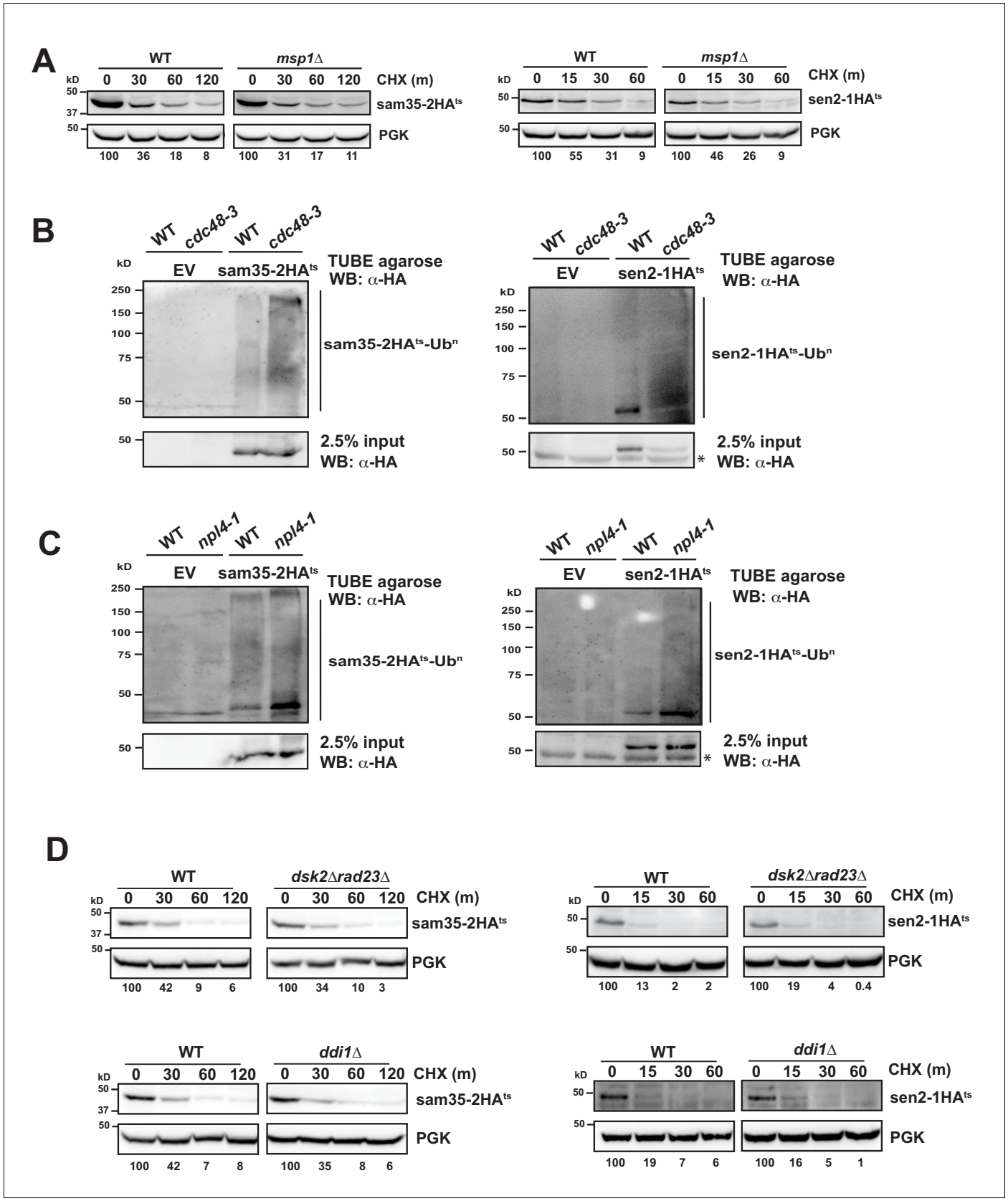

Figure 5—figure supplement 1. The Cdc48-Npl4-Ufd1 complex is required for degradation of MAD substrates. (**A**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in WT (BY4741) and *msp1*Δ cells. Proteins were detected by

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immunoblotting. Percentage of each ts- substrate remaining is labeled for each time point under blots. (**B, C**) Ubiquitination of sam35-2HA^{ts} and sen2-1HA^{ts} was assessed by immunoprecipitation (IP) using tandem ubiquitin-binding entities (TUBE) agarose from *cdc48-3* (**B**) *and npl4-1* (**C**) mutant and isogenic WT lysates expressing EV (pRS315), sam35-2HA^{ts} (pMM157), or sen2-1HA^{ts} (pMM160), followed by immunoblotting with HA antibody. 2.5% of the TUBE input lysate was reserved and analyzed by immunoblotting. Asterisk indicates a cross-reactive band seen in some exposures. (**D**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in WT (BY4741), *dsk2Δ rad23Δ* (SM5186), or *ddi1Δ* cells.

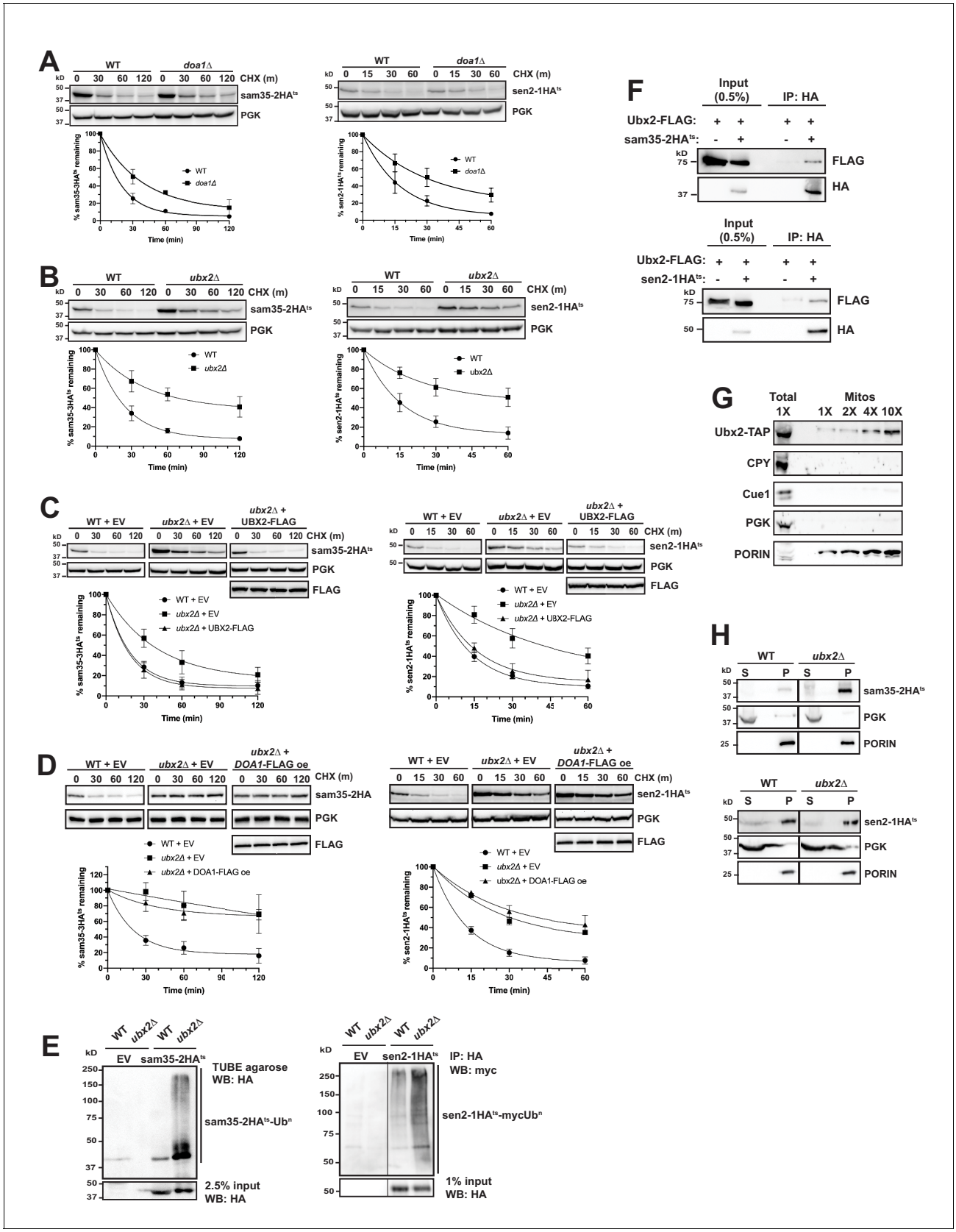

Figure 6. The Cdc48 co-factors Ubx2 and Doa1 are implicated in MAD. (**A**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} (pMM157) and sen2-1HA^{ts} (pMM160) in WT (BY4741) and *doa1*Δ cells (yJS208). Proteins were detected by immunoblotting. Graphed below is the

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mean and SD of the PGK-normalized HA signal at each time point for three biological replicates. (**B**) CHX chase as in A for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} (pMM157) and sen2-1HA^{ts} (pMM160) in WT (BY4741) and *ubx2*Δ cells (yJS155). (**C**) CHX chase as in A for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} (pMM157) and sen2-1HA^{ts} (pMM160) in WT (BY4741) and *ubx2*Δ (yJS155) cells co-expressing either empty vector (EV; pRS315) or *CEN* Ubx2-FLAG (pMM242). (**D**) CHX chase as in A for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} (pMM231) or sen2-1HA^{ts} (pMM234) in WT (BY4741) cells or *ubx2*Δ (yJS155) cells expressing either EV (pRS315) or Doa1-FLAG (pMM254) from a high copy 2μ plasmid. (**E**) Ubiquitination of sam35-2HA^{ts} and sen2-1HA^{ts} was assessed by IP using TUBE agarose or anti-HA agarose from *ubx2*Δ (yJS155) and WT (BY4741) lysates expressing EV (pRS315), sam35-2HA^{ts} (pMM157), or sen2-1HA^{ts} (pMM160), followed by immunoblotting with HA or c-myc antibody. 2.5% or 1% of the IP input lysate was reserved and analyzed by immunoblotting. (**F**) Co-IP of Ubx2-FLAG (pMM242) with sam35-2HA^{ts} or sen2-1HA^{ts} (pMM231 and 234, respectively) from *pre1-1 pre2-2* (WCG4-11/21a) cells was assessed by immunoblotting with the indicated antibodies. IP of Ubx2-FLAG from cells co-expressing EV (pRS316) in place of HA-tagged substrates and 0.5% of the input lysate are shown for comparison. (**G**) Lysate ('Total') and increasing amounts of mitochondria purified by 12,000x*g* and sucrose gradient fractionation ('Mitos') from Ubx2-TAP-expressing cells were examined by immunoblotting with the indicated antibodies. (**H**) Lysates from WT (BY4741) and *ubx2*Δ (yJS155) cells expressing sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 and 160, respectively) were fractionated at 12,000x*g* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN.

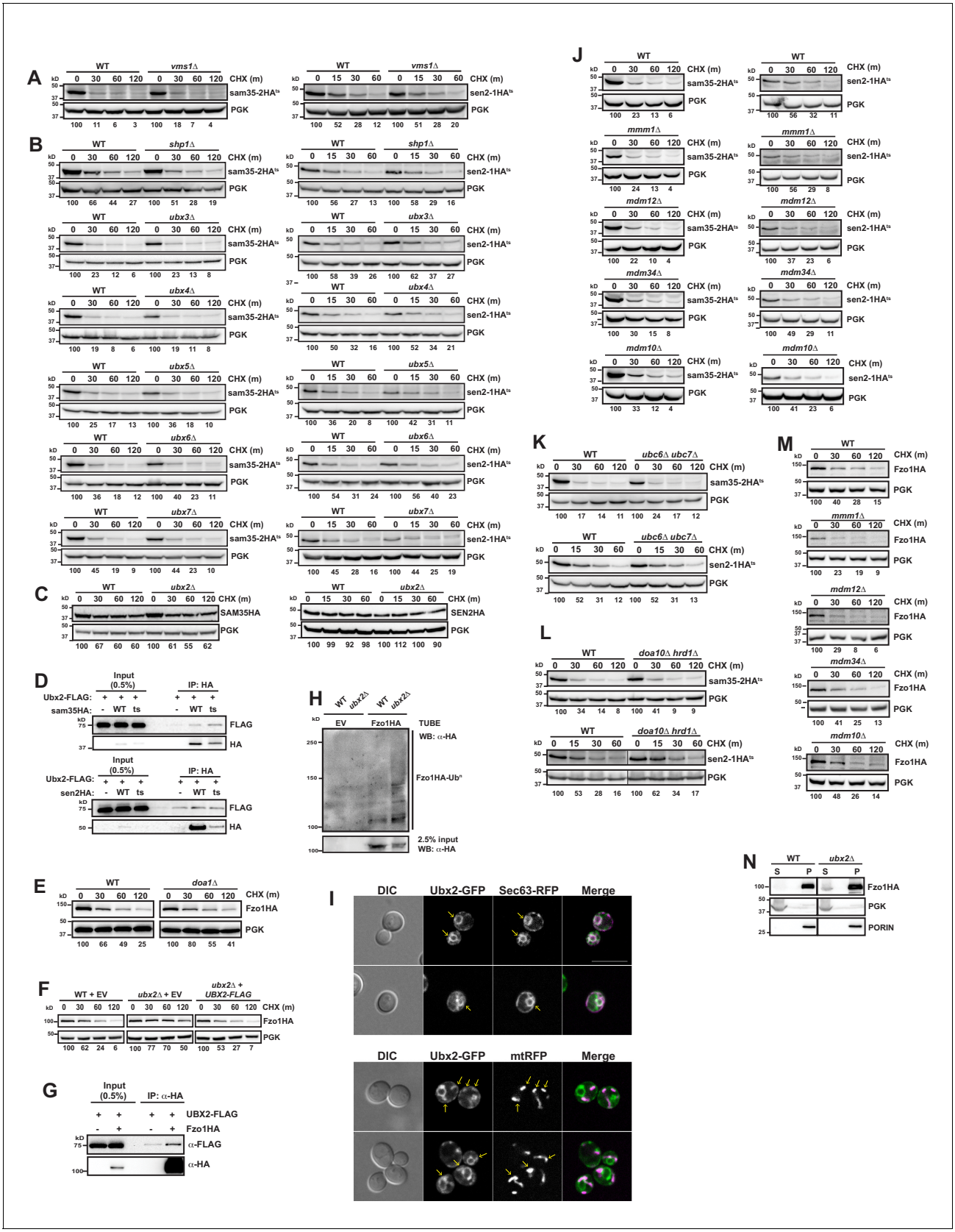

Figure 6—figure supplement 1. The Cdc48 co-factors Ubx2 and Doa1 are required for MAD. (**A**) CHX chase for the indicated times at 37˚C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in *vms1Δ* compared to isogenic WT (BY4741). Proteins were detected by

Figure 6—figure supplement 1 continued on next page

Figure 6—figure supplement 1 continued

immunoblotting. Percentage of each ts- substrate remaining is labeled for each time point under blots. (**B**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in the indicated UBX protein deletion strains compared to isogenic WT (BY4741). (**C**) CHX chase for the indicated times at 37°C assessing the turnover of SAM35HA or SEN2HA (pMM158 or 159, respectively) in *ubx2*Δ (yJS155) compared to isogenic WT (BY4741). (**D**) Co-IP of Ubx2-FLAG (pMM242) with SAM35HA, sam35-2HA^{ts}, SEN2HA, or sen2-1HA^{ts} (pMM232, pMM231, pMM233, and 234, respectively) from *pre1-1 pre2-2* (WCG4-11/21a) cells was assessed by immunoblotting with the indicated antibodies. IP of Ubx2-FLAG from cells co-expressing EV (pRS316) in place of HA-tagged substrates and 0.5% of the input lysate are shown for comparison. (**E**) CHX chase for the indicated times at 30°C assessing the turnover of Fzo1HA (pADH1-Fzo1pHA) in WT (BY4741) and *doa1*Δ (yJS208) strains. (**F**) CHX chase for the indicated times at 30°C assessing the turnover of Fzo1HA (pADH1-Fzo1pHA) in WT (BY4741) and *ubx2*Δ (yJS155) cells co-expressing either EV (pRS315) or Ubx2-FLAG (pMM242). (**G**) Co-IP of Ubx2-FLAG (pMM242) with Fzo1HA (pADH1-Fzo1pHA) from *pre1-1 pre2-2* (WCG4-11/21a) cells was assessed by immunoblotting with the indicated antibodies. Negative control of IP of Ubx2-FLAG from cells co-expressing empty vector (pRS316) in place of HA-tagged substrates is shown, as is 0.5% of the input lysate. (**H**) Ubiquitination of Fzo1HA was assessed by IP using TUBE agarose from *ubx2*Δ (yJS155) and WT (BY4741) lysates expressing EV (pRS316) or Fzo1HA (pADH1-Fzo1pHA) followed by immunoblotting with HA antibody. 2.5% of the TUBE input lysate was reserved and analyzed by immunoblotting. (**I**) Microscopy analysis of immobilized Ubx2-GFP cells co-expressing either mtRFP (pMD12) to label mitochondria or SEC63-RFP (pSM1959) to label the ER. Some regions of co-localization are highlighted with yellow arrows. 'Merge' of GFP (green) and RFP (magenta) channels and differential interference contrast (DIC) are shown; scale bar = 10 um. (**J**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in WT (BY4741) and *mmm1*Δ, *mdm12*Δ, *mdm34*Δ, and *mdm10*Δ strains. (**K, L**) CHX chase for the indicated times at 37°C assessing the turnover of sam35-2HA^{ts} or sen2-1HA^{ts} (pMM157 or 160, respectively) in a *ubc6*Δ*ubc7*Δ (K; SM5364) or *doa10*Δ*hrd1*Δ (**L**, SM5360) strains relative to their isogenic WT strain (BY4741). (**M**) CHX chase for the indicated times at 30°C assessing the turnover of Fzo1HA (pADH1-Fzo1pHA) in WT (BY4741) and *mmm1*Δ, *mdm12*Δ, *mdm34*Δ, and *mdm10*Δ strains. (**N**) Lysates from WT (BY4741) and *ubx2*Δ (yJS155) cells expressing Fzo1HA (pMM190) were fractionated at 12,000*xg* at 37°C into mitochondrial pellets (P) and post-mitochondrial supernatants (S). Fractions were subject to immunoblotting with antibodies to HA, PGK, and PORIN.

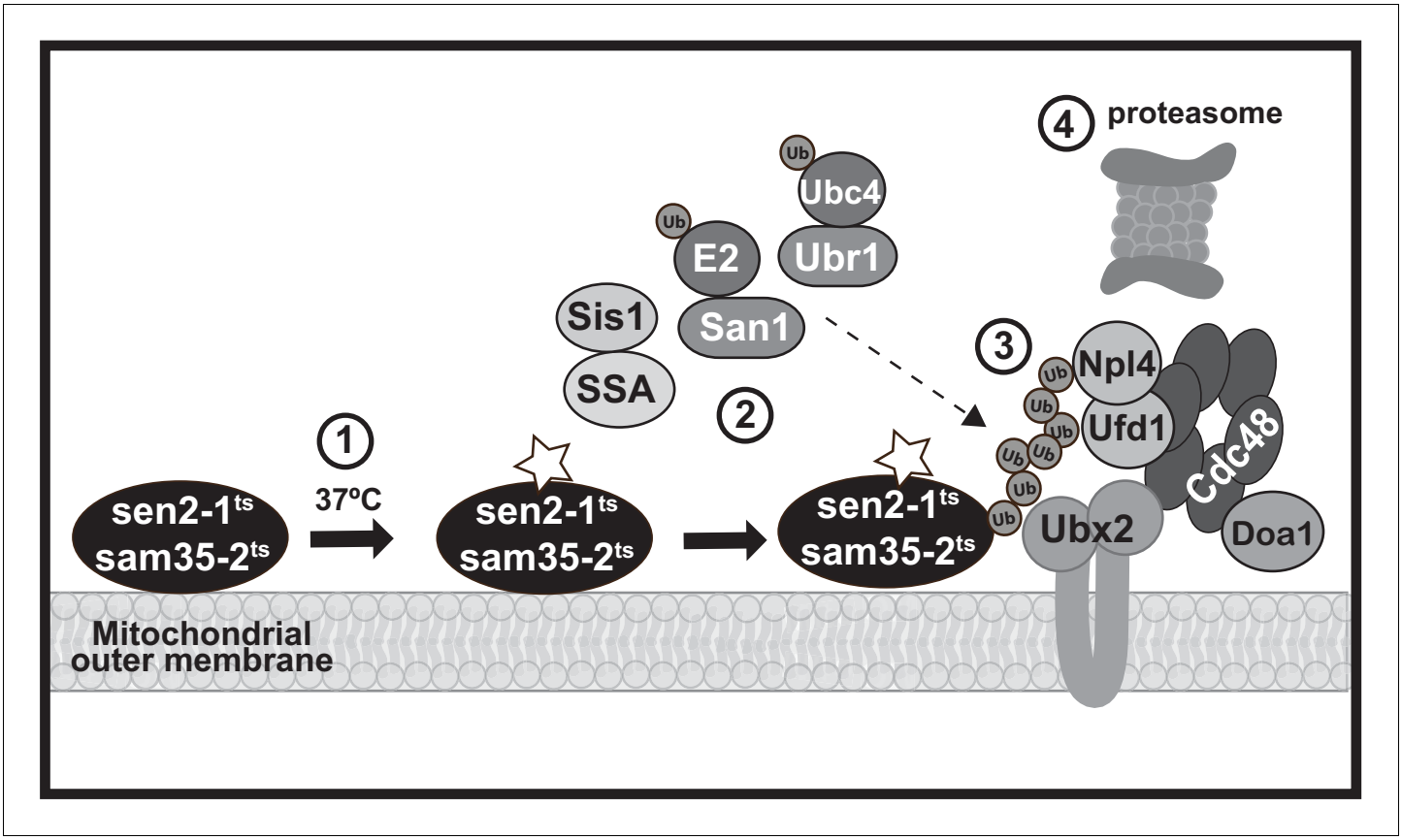

Figure 7. A model MAD QC pathway based on the present study. When the temperature is increased to 37°C, the peripheral MOM ts- proteins sam35-2HA^{ts} and sen2-1HA^{ts} become non-functional (denoted by a star) yet remain at the mitochondrial outer membrane (step 1). They are recognized as quality control substrates and ubiquitinated (step 2), which requires cytosolic chaperones (Ssa1 and Sis1) and the ubiquitin ligase San1 (for sam35-2HA^{ts}) or Ubr1 and the ubiquitin conjugating enzyme Ubc4 (for sen2-1HA^{ts}). Once ubiquitinated, the Cdc48-Npl4-Ufd1 unfoldase, along with its co-factor Doa1 and a mitochondria-localized pool of its co-factor Ubx2 (step 3), act to direct them to the 26S proteasome for degradation (step 4).