Expanded View Figures

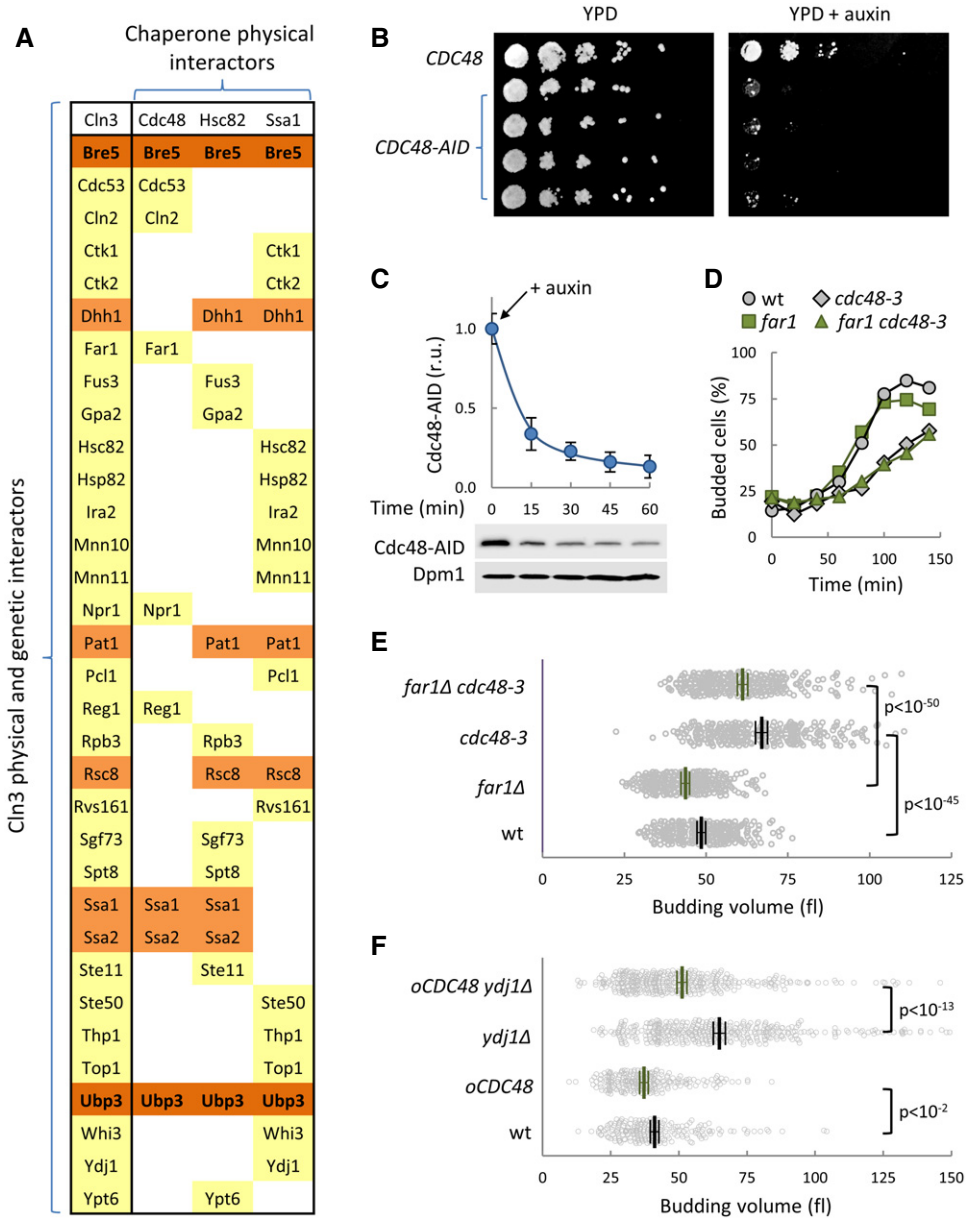


Figure EV1. Chaperone target proteins in the Cln3 interactome and genetic interactions of *CDC48* in cell cycle entry and size determination.

A Physical interactors of Ssa1, Hsc82, and Cdc48 that display genetic or physical interactions to Cln3 (SGD Project. http://www.yeastgenome.org 07/07/2017).

B Serial dilutions of four independent isolates expressing *CDC48-AID* or *CDC48* were plated and incubated for growth at 30°C for 2 days in the presence or absence of auxin.

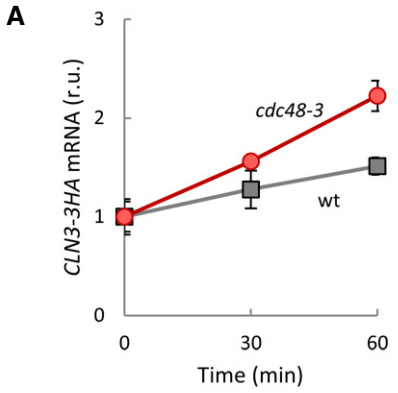
C Cdc48-AID levels in cells at different times from auxin addition. Dpm1 served as loading control and quantified levels with the confidence limits (α = 0.05) for the mean are plotted at the top.

D Budding frequencies of newborn daughter cells with the indicated genotypes during growth at the restrictive temperature (37°C).

E Individual volumes at budding of cells with the indicated genotypes. Mean values (N > 200, thick lines), confidence limits (α = 0.05, thin lines) for the mean and P values obtained from t-tests are also shown.

F Individual volumes at budding of wild-type and *ydj1*Δ cells overexpressing Cdc48 (*oCDC48*) or not. Mean values (N > 200, thick lines), confidence limits (α = 0.05, thin lines) for the mean, and P values obtained from t-tests are also shown.

Source data are available online for this figure.



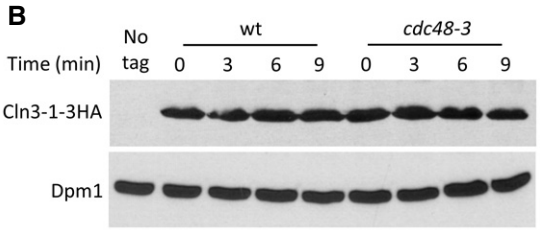
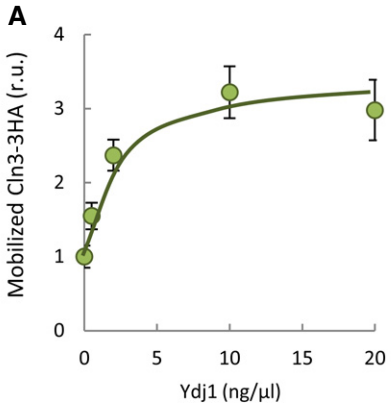


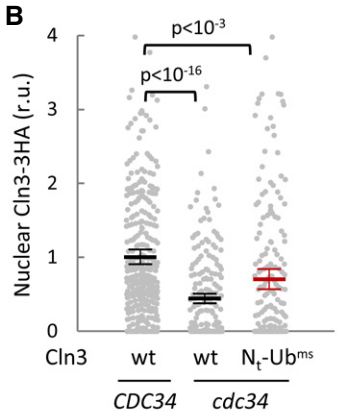
Figure EV2. Cdc48 regulates Cln3 levels at a post-translational level.

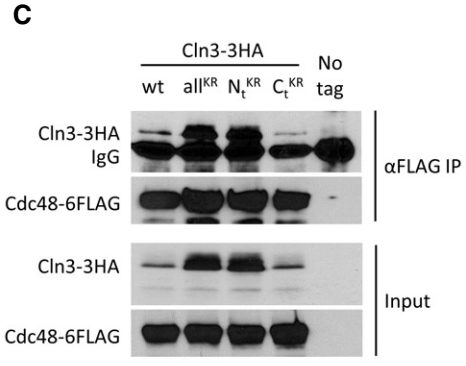
A *CLN3-3HA* mRNA levels in wild-type (wt) and *cdc48-3* cells at the indicated times after transferring cells to the restrictive temperature (37°C). Values were made relative to *HXK1* mRNA and time 0. Mean values (N = 3) and confidence limits (α = 0.05) for the mean are shown.

B Cln3-1-3HA stability in Cdc48-deficient cells. After being transferred to the restrictive temperature (37°C) for 30 min, wild-type (wt) and *cdc48-3* cells expressing the hyperstable *CLN3-1-3HA* allele at endogenous levels were added cycloheximide and collected at the indicated times to determine protein levels. Dpm1 is shown as a loading control.

Source data are available online for this figure.







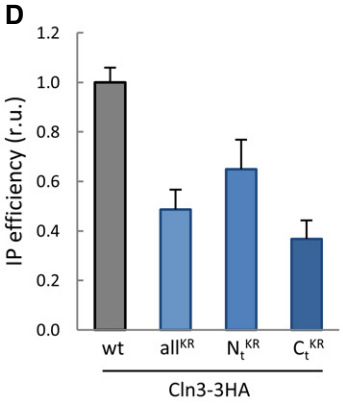


Figure EV3. Mobilization, nuclear accumulation, and interaction of Cln3 with Cdc48.

A Cell extracts from *ydj1Δ CLN3-3HA* cells were incubated for 30 min at 27°C in the presence of an ATP-regeneration system and the indicated concentrations of purified Ydj1. Mobilized soluble fractions were obtained to analyze released Cln3-3HA by immunoblotting. Mean values (N = 3) and confidence limits (α = 0.05) for the mean are shown.

B Nuclear accumulation of wild-type and N_t-Ub^{ms} Cln3-3HA in individual wild-type *CDC34* or thermosensitive *cdc34* cells growing at the restrictive temperature was analyzed by immunofluorescence as in Fig 2E. Relative mean values (N = 500, thick horizontal lines), confidence limits (α = 0.05, thin lines) for the mean and P values obtained from t-tests are also shown.

C Cell extracts (Input) and αFLAG immunoprecipitates (αFLAG IP) of cells expressing Cdc48-6FLAG and wild-type, all^{KR}, N_t^{KR}, or C_t^{KR} Cln3-3HA proteins were analyzed by immunoblotting with either αHA or αFLAG antibodies. The αFLAG heavy chain (IgG) band is also marked.

D Quantification of Cln3-3HA levels from immunoblots in (C). Mean values (N = 3) and confidence limits (α = 0.05) for the mean are shown.

Source data are available online for this figure.

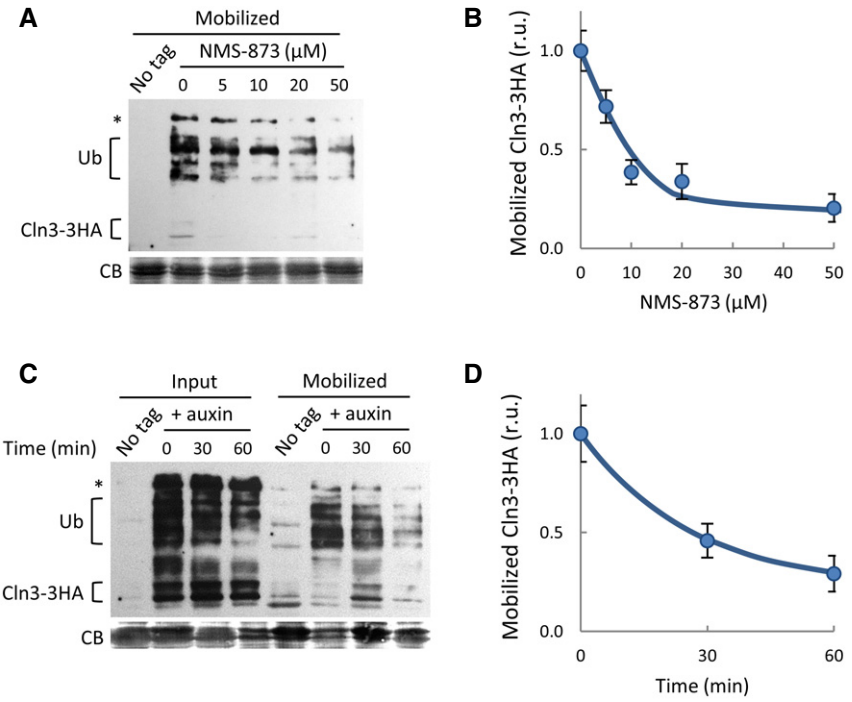


Figure EV4. Cdc48 interacts with Cln3 and plays a key role in release from the ER.

A Mobilized soluble fractions were obtained as in Fig 3A in the presence of NMS-873 at the indicated concentrations to analyze released Cln3-3HA by immunoblotting. High-molecular-weight species corresponding to ubiquitinated Cln3-3HA are indicated (Ub). A high-molecular-weight αHA cross-reacting band is marked (*), and a prominent Coomassie Blue (CB) band is shown as loading control.

B Quantification of mobilized Cln3-3HA as a function of NMS-873 concentration from experiments as in (A). Mean values (N = 3) and confidence limits (α = 0.05) for the mean are shown.

C Mobilized soluble fractions were obtained as in Fig 3A with extracts from Ydj1-deficient cells expressing Cdc48-AID collected at different times after auxin addition. High-molecular-weight species corresponding to ubiquitinated Cln3-3HA are indicated (Ub). A high-molecular-weight αHA cross-reacting band is marked (*), and a prominent Coomassie Blue (CB) band is shown as loading control.

D Quantification of mobilized Cln3-3HA as a function of Cdc48-AID levels from experiments as in (C). Mean values (N = 3) and confidence limits (α = 0.05) for the mean are shown.

Source data are available online for this figure.

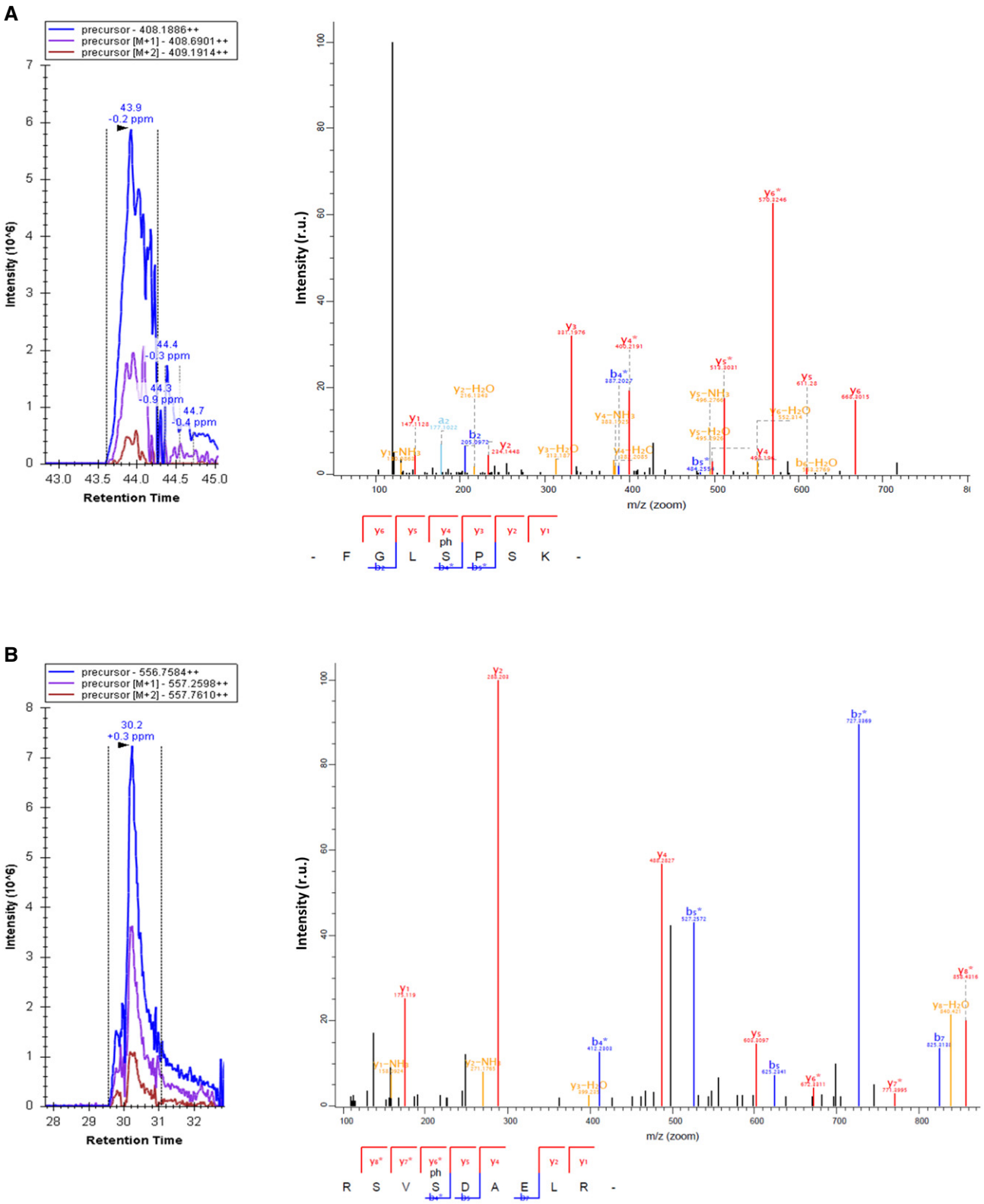


Figure EV5. Identification of Cdc48 peptides containing pS519 and pS770 by mass spectrometry analysis.

A, B Cycling cells expressing GST-Cdc48 at endogenous levels were used to prepare cell extracts and purify GST-Cdc48 for MS analysis. Representative ion chromatograms (left panels) and fragmentation spectra (right panels) of ions corresponding to pS519 (A) and pS770 (B) peptides are shown.

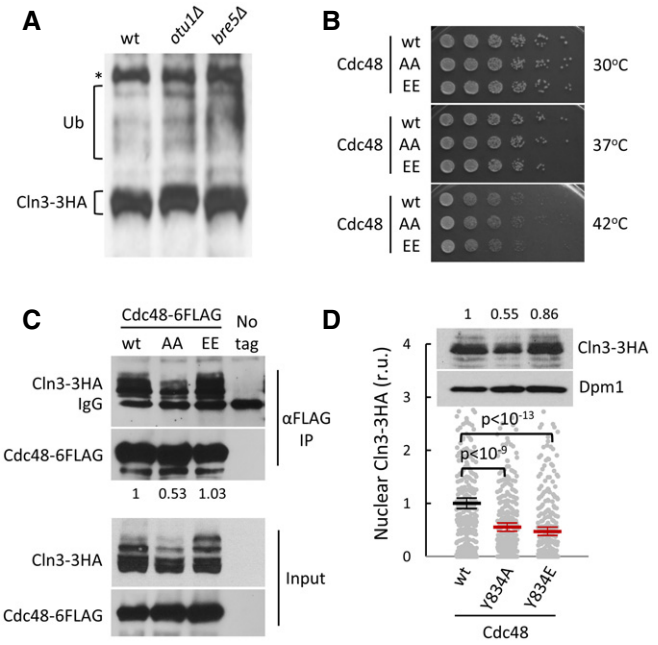


Figure EV6. Cln3 fate and Cdc48 phosphorylation mutant proteins.

A Cell extracts from wild-type, *otu1*Δ and *bre5*Δ cells expressing *CLN3-3HA* were analyzed by immunoblotting. High-molecular-weight species corresponding to ubiquitinated Cln3-3HA are indicated (Ub). A high-molecular-weight αHA cross-reacting band is marked (*).

B Cells expressing wild-type (wt), non-phosphorylatable (AA), or phosphomimetic (EE) Cdc48 proteins at endogenous levels were diluted, spotted onto YPD plates and grown at 30, 37 or 42°C.

C Cell extracts (Input) and αFLAG immunoprecipitates (αFLAG IP) of cells expressing Cln3-3HA and wild-type (wt), non-phosphorylatable (AA), or phosphomimetic (EE) Cdc48-6FLAG proteins from the *GAL1p* promoter as in Fig 6G (same experiment) were analyzed by immunoblotting with either αHA or αFLAG antibodies. The αFLAG heavy chain (IgG) band is also marked. Indicated numbers refer to the relative levels of Cln3-3HA in immunoprecipitated samples as quantified from image.

D Nuclear accumulation of Cln3-3HA in individual cells expressing wild-type (wt), tyrosine non-phosphorylatable (Y834A), or tyrosine phosphomimetic (Y834E) Cdc48 proteins at endogenous levels. Immunofluorescence analysis was performed in cycling cells as in Fig 2E. Relative mean values ($N > 275$, thick horizontal lines), confidence limits ($\alpha = 0.05$, thin lines) for the mean, and P values obtained from t-tests are also shown. An immunoblot showing overall levels of Cln3-3HA in cells from this experiment is shown at the top and relative amounts are indicated. Dpm1 was detected as loading control.

Source data are available online for this figure.

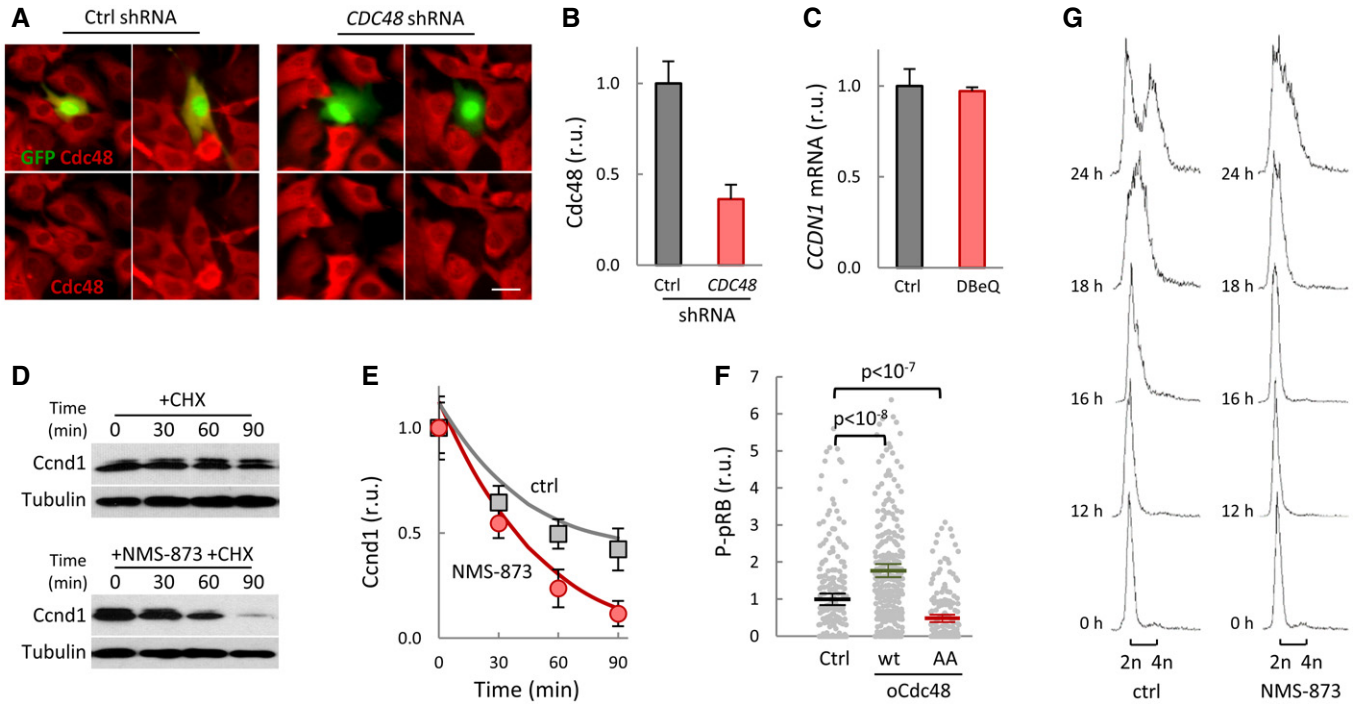


Figure EV7. Cdc48 also controls stability and nuclear functions of cyclin D1 in mammalian cells.

A Immunofluorescence analysis of Cdc48 (red signal) in mouse 3T3 cells transfected with control or Cdc48 shRNAs. GFP (green) was used as a transfection reporter. Scale bar, 20 μm.

B Quantification of Cdc48 levels in 3T3 cells transfected with control or Cdc48 shRNAs as in (A). Values were made relative to the average obtained from control cells. Mean values ($N > 50$) and confidence limits ($\alpha = 0.05$) for the mean are shown.

C *CCND1* mRNA levels in 3T3 cells in the absence (Ctrl) or presence of 10 μM DBeQ for 90 min. Values were made relative to *GAPDH* mRNA and control sample. Mean values ($N = 3$) and confidence limits ($\alpha = 0.05$) for the mean are shown.

D Ccnd1 stability in 3T3 cells. After being treated with or without 2 μM NMS-873 for 2 h, cells were added cycloheximide and collected at the indicated times to determine Ccnd1 levels. Tubulin is shown as a loading control.

E Quantification of Ccnd1 stability in control (squares) and NMS-873 treated (circles) cells from immunoblot analysis as in (D) at the indicated times after cycloheximide addition. Mean values ($N = 3$) and confidence limits ($\alpha = 0.05$) for the mean are shown.

F P-S780-pRB levels in 3T3 cells overexpressing wild-type (wt) or non-phosphorylatable (AA) Cdc48, or none as control. Values were made relative to the average obtained from control cells. Mean values ($N > 200$, thick horizontal lines), confidence limits ($\alpha = 0.05$, thin lines) for the mean, and *P* values obtained from *t*-tests are also shown.

G Serum-starved 3T3 cells were stimulated to enter the cell cycle by the addition of 10% FCS in the presence or absence of 2 μM NMS-873, and samples were taken at the indicated times to obtain DNA content profiles by FACS.

Source data are available online for this figure.