Supporting Information

Control of Hsp90 chaperone and its clients by N-terminal acetylation and the N-end rule pathway

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SI Materials and Methods

Antibodies and Other Reagents. Antibodies to the following antigens were used for immunoblotting: anti-hsc82 antibody (Abcam, ab30920); anti-hsv antibody (Sigma, H6030); anti-flag M2 mouse monoclonal antibody (Sigma, F1804); anti-c-Myc-9E10 monoclonal antibody (Sigma, M5546); and anti-ha tag rabbit monoclonal antibody (Sigma, H6908). Secondary antibodies for immunoblotting were Li-Cor IRDye-conjugated goat anti-mouse 800CW (Li-Cor, C60405-05) or anti-rabbit 680RD (Li-Cor, C51104-08). Affinity-purified rabbit antibody to *S. cerevisiae* Ubr1 was produced as described previously (1, 2). Fluorescence patterns were detected and quantified using Odyssey-9120 (Li-Cor, Lincoln, NE).

Affinity-purified rabbit antibody to *S. cerevisiae* Naa10 was produced by immunizing two rabbits (at Covance, Inc., Sherman Oaks, CA) with ~5 mg of purified Naa10. The latter was produced by expressing His_{10}-Ub-Naa10 in *E. coli* from pJO162 (Table S2) and purifying untagged Naa10 by affinity chromatography and the cleavage by a deubiquitylase as described previously (3). Antisera to Naa10 were purified by TALON affinity chromatography (Clontech), with immobilized Naa10. The resulting antibody was then further purified through "negative" selection using a nitrocellulose membrane with immobilized extract from *naa10*Δ *S. cerevisiae* cells. For immunoblotting results with this antibody, see Figure 3A, lanes 11-13.

Other reagents included "complete protease inhibitor cocktail" tablets (Roche, 11697498001); protease inhibitor cocktail "for use with fungal and yeast extracts" (Sigma, P8215); protease inhibitor cocktail "for use in purification of Histidine-tagged proteins" (Sigma, P8849); phenylmethylsulfonyl fluoride ((PMSF) Sigma, P7626); cycloheximide (Sigma, C7698); tetracycline hydrochloride (Sigma, T3383); and DNase I (Roche, 11284932001), human rhinovirus (HRV) 3C protease (Thermo Fisher, 88946).

Yeast Strains, Media, and Genetic Techniques. The *S. cerevisiae* strains used in this work are described in Table S1. Standard techniques (4) were used for strain construction and transformation. *S. cerevisiae* JOY379, JOY439, JOY440, JOY441, JOY442, JOY443, JOY468, JOY487, JOY498, JOY505 and JOY529 were constructed using the PCR-based gene targeting method (5) and pFA6a-KanMX6, HphNT1 or NatNT2 modules (6). *S. cerevisiae* JOY512, JOY513 and JOY514 were constructed by extending, using standard recombination methods (6), the endogenous *CHK1* open reading frame (ORF) in the strains BY4742, CHY271 and JOY379, respectively (Table S1), with a sequence encoding nine consecutive c-myc epitope tags from either pYM20 or pYM18. *S. cerevisiae* media included YPD (1% yeast extract, 2% peptone, 2% glucose; only most relevant components are cited); SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose); and synthetic complete (SC) medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate,2% glucose), plus a drop-out mixture of compounds required by specific auxotrophic strains.

Construction of Plasmids. The plasmids used in this study are described in Tables S2. To construct the DHFR-reference part of the PRT vector pJO622, the multipartite P_{TDH3} promoter, containing three tetracycline (Tc) aptamers, was amplified from P_{TDH3}-tc3-3×ha (Table S2) (7), and the $_{flag}DHFR_{ha}$ ORF was amplified from pJO379. PCR products were digested with *SacI/EcoRI* and *EcoRI/XhoI*, respectively, and ligated into *SacI/XhoI*-cut pRS413-Met25. The PRT-based pJO624 (Table S2), used throughout this study, was constructed by amplifying P_{TDH3}-tc3, *S. Cln3-3-flag*, the *cyc1* terminator, and P_{TDH3}-tc3-$_{flag}DHFR_{ha}$ from pJO622 (Table S2). The resulting DNA was digested with *SacII/AscI*, *AscI/NotI*, *NotI/FseI* and *FseI/XmaI* respectively, and ligated into *SacII/XmaI*-cut pRS313, yielding pJO624 (Table S2).

For constructing a Chk1-expressing plasmid, the *S. cerevisiae CHK1* ORF (amplified from *S. cerevisiae* genomic DNA) was digested with *AscI/BglII* and was ligated into *AscI/BamHI*-cut pJO624, yielding pJO630 (Table S2). Otherwise identical plasmids encoding N-terminal mutants of Chk1 were produced using PCR, with a forward primer that contained a desired N-terminal mutation. Each PCR-amplified DNA fragment was digested with *AscI/BamHI* and ligated into *AscI/BamHI*-cut pJO630. Other ORFs used in this study were

amplified from *S. cerevisiae* genomic DNA and cloned by digesting with *AscI/BamHI* sites, followed by the cloning route described above.

Plasmids expressing Ub^{K48}-Y-eK βgal and Ub^{K48}-H-eK βgal from PRT-based plasmids, were constructed by amplifying DNA segments encoding, respectively, Ub^{K48}-Y-eK βgal and Ub^{K48}-H-eK βgal from pJO746 and pJO747 (Table S2). The resulting DNA fragments were digested with *AscI/BamHI* and ligated into *AscI/BamHI*-cut pJO699, yielding pJO752 and pJO753 (Table S2).

For split-Ub assays, full-length as well as C-terminally truncated Chk1, Hsc82, Ufd4 and Ubr1 ORFs were amplified from *S. cerevisiae* genomic DNA using appropriate primers that contained (asymmetric) *SfiI* sites at both ends. The resulting DNA fragments were digested with *SfiI* and ligated into *SfiI*-cut pDHB1 or pPR3-N (Table S2). To construct plasmids for two-hybrid assays, the full length *S. cerevisiae UBR1* ORF, and either full-length or C-terminally truncated Chk1-encoding ORFs were cloned into the pDonor/Zeo via the BP recombination reaction of the Gateway system (8) that included the Gateway BP clonase II enzyme mix (Invitrogen). The resulting plasmids were pJO781, pJO930, pJO931, and pJO932 (Table S2). Thereafter the LR Gateway reaction was carried out with the above plasmids and pGADCg for Gal4-AD expression or pGBKT7g for Gal4-DBD expression, using the Gateway LR clonase II enzyme mix (Invitrogen). The resulting plasmids were pJO794, pJH024, pJO028, and pJH030, respectively (Table S2).

To construct pJO690, a high copy (2μ-based) plasmid that expressed $Ufd4_{hsv}$ and $_{f}Ubr1$ from the bidirectional $P_{GAL1/10}$ promoter, a three-step procedure was used. First, a *SacI/XhoI* fragment was cut from pRS314-Gal1/10 (which contains $P_{GAL1/10}$), and was ligated into *SacI/XhoI*-cut pRS426-Met25, replacing P_{MET25} of the original plasmid with the $P_{GAL1/10}$. The resulting plasmid was pJO241. Second, the full-length *UBR1* ORF (encoding the N-terminal flag tag as well) was amplified from pNTfUBR1 (Table S2) and digested with *MfeI/XhoI*. The resulting DNA fragment was ligated into *EcoRI/XhoI*-cut pJO241, yielding pJO689. Finally, the *NotI/FseI* fragment of the PCR-amplified *UFD4* ORF that was followed by an hsv tag-coding DNA sequence, and the *FseI/SacII*-produced *CYC1* terminator (amplified from *S. cerevisiae* genomic DNA were triply ligated into *NotI/SacII*-cut pJO689, yielding pJO690 (Table S2).

The plasmids pJO589 and pJO591, which encoded, respectively, *S. cerevisiae* Ubc4 and mono-Ub were constructed by ligating the *NcoI/BamHI*-digested PCR products of Ubc4 and

mono-Ub ORFs into *NcoI/BamHI*-cut pET-15b, yielding pJO589 and pJO591, respectively (Table S2). pJO663, which encoded His_6 followed by the human rhinovirus (HRV) 3C protease site and the *S. cerevisiae* Rad6 moiety, was produced by overlap extension PCR (9). Two overlapping DNA fragments, one of them bearing the *NcoI* site and encoding His_6 as well as the HRV 3C protease site, and the other one bearing the *BamHI* site and encoding Rad6 were denatured and reannealed, so the they formed a partially duplex DNA that encompassed the HRV 3C segment. The resulting PCR-produced fragment was digested with *NcoI*/BamHI and ligated into *NcoI/BamHI*-cut of pET-11d. The resulting plasmid was pJO663 (Table S2). Construction details for other plasmids are available upon request. All final constructs were verified by DNA sequencing.

PRT-based cycloheximide-chases and tetracycline-chases. *S. cerevisiae* were grown to A_{600} of ~ 1.0 in selective liquid media at 30°C, followed by treatment with cycloheximide (CHX) or tetracycline (Tc), at the final concentration of 0.36 mM (0.1 mg/ml) or 0.5 mM, respectively. At indicated times, cell samples (corresponding to 1 ml of cell suspension at A_{600} of ~1.0) were harvested by centrifugation for 5 min at 21,130*g*. The pellet was resuspended in 0.8 ml of 2 M Li-acetate for 5 min on ice, followed by centrifugation for 5 min at 21,130*g* and resuspension in 0.8 ml of 0.4 M NaOH for 5 min on ice. The resulting suspension was centrifuged for 5 min at 21,130*g*. The pellet was resuspended in 50 μl of HU buffer (8 M urea, 5% SDS, 1 mM EDTA, 0.1 M dithiothreitol (DTT), 0.005% bromophenol blue, 0.2 M Tris-HCl, pH 6.8) containing 1×protease inhibitor cocktail (Roche) and 1×protease inhibitor cocktail "for use with fungal and yeast extracts" (Sigma), and heated for 10 min at 70°C. After centrifugation for 1 min at 21,130*g*, 10 μl of each supernatant was used to carry out SDS-4-12% NuPAGE (Invitrogen), followed by immunoblotting, done as described previously (10, 11), using a mixture of anti-ha (1:2,000) and anti-flag (1:2,000) antibodies. Immunoblots were processed using secondary antibodies labeled with different fluorophores. Visualized protein bands were quantified using the Odyssey-9120 Imaging System (Li-Cor, Lincoln, NE).The near-infrared fluorescence range and other features of the Odyssey scanner facilitate quantification of immunoblots.

^{35}S-pulse-chase assays. These assays were performed largely as described (1, 11-13). *S. cerevisiae* were grown at 30°C to A_{600} of ~1 in 10 ml of SC medium. Cells were pelleted by centrifugation, gently resuspended, and washed in 0.8 ml of SD medium with required amino acids but without Met and Cys. Cell pellets were gently resuspended again in 0.4 ml of the same medium and incubated at 30°C for 20 min and thereafter labeled for 2 min at 30°C with 0.16 mCi of ^{35}S-EXPRESS Met/Cys (Perkin-Elmer). Cells were pelleted again and resuspended in 0.3 ml of SD medium with required amino acids as well as unlabeled 10 mM methionine and 5 mM cysteine. Samples (0.1 ml) were taken at indicated time points, followed by preparation of extracts using Mini-Beadbeater-16 (BioSpec) (4 times for 15 sec each, with 5-min intervals on ice). The resulting extracts were clarified by centrifugation at 21,000*g* for 15 min, twice, and supernatants were processed for immunoprecipitation, using anti-flag antibody immobilized on magnetic beads (Sigma). Immunoprecipitates were analyzed by SDS-4-12% NuPAGE (Invitrogen) and autoradiography.

Two-hybrid assays. Yeast-based two-hybrid binding assays (14) were carried out largely as described previously (8). *S. cerevisiae* AH109 (Table S1) was cotransformed with pJO794 (expressing NLS-Ubr1-Gal4^{AD}) and either pGBKT7g (expressing Gal4^{DBD}), or pJH024 (expressing Gal4^{DBD}-Chk1$^{1-527}$), or pJH028 (expressing Gal4^{DBD}-Chk1$^{1-502}$), or pJH030 (expressing Gal4^{DBD}-Chk1$^{1-465}$) using the lithium acetate method. "AD" and "DBD" refer to activation domain and DNA-binding domain, respectively. In both two-hybrid and split-Ub assays (described below), the expression of *HIS3* (the ultimate readout of both assays), in otherwise His$^-$ cells, was a function of affinity between test proteins.

Split ubiquitin assays. Yeast-based split-Ub binding assays (15, 16) were carried out largely as described previously (8). *S. cerevisiae* NMY51 (*MAT***a** *trp1 leu2 his3 ade2 LYS2::lexA-HIS3 ade2::lexA-ADE2 URA3::lexA-lacZ*) (Dualsystems Biotech AG, Schlieren, Switzerland), JOY505 (*naa10Δ::KanMX6* in NMY51) or JOY529 (*naa10Δ::KanMX6, ubr1Δ::HphNT1* in NMY51) were cotransformed with split-Ub-based bait and prey plasmids (Table S2) using the Li-acetate method. Transformants were selected for the presence of bait and prey plasmids during 3 days of growth at 30°C on SC(-Trp, -Leu) medium (minimal medium containing 2% glucose, 0.67% yeast nitrogen base, 2% bacto-agar, and complete amino acid

mixture lacking Leu and Trp). Single colonies of resulting cotransformants were grown in the otherwise identical liquid medium to a near-stationary phase, until A_{600} of ~1.0. The cultures were thereafter serially diluted by 3-fold, and 10 μl samples of cell suspensions were spotted onto either double-dropout SC(-Trp, -Leu) plates or triple-dropout SC(-Trp,-Leu,-His), which were incubated at 30°C for 2-3 days.

Purification of proteins for *in vitro* ubiquitylation assay. The N-terminally flag-tagged $_f$Ubr1, the C-terminally flag-tagged Ufd4$_f$, and the C-terminally myc-flag tagged full-length SL-Chk1$^{1-527}_{m-f}$ as well as its SL-Chk1$^{1-502}_{m-f}$ derivative were overexpressed in protease-deficient *S. cerevisiae* SC295 or BY4742 (Table S1) as described previously (17). The resulting proteins were purified from cell extracts by affinity chromatography, using anti-flag M2 antibody-magnetic beads as described previously (18), with slight modifications. *S. cerevisiae* SC295 that expressed $_f$Ubr1was grown at 30°C to A_{600} of ~4 in 8 l of SC(-Leu) medium. BY4742 cells overexpressed either Ufd4$_f$, or SL-Chk1$^{1-527}_{m-f}$, or SL-Chk1$^{1-502}_{m-f}$ were grown at 30°C to A_{600} of ~4 in 8 l of SGal(-His) medium (the same as SC(-His) but with 2% galactose instead of 2% glucose). Cells were harvested by centrifugation, washed once with cold phosphate-buffered saline (PBS), and frozen in liquid N_2.

Frozen cell pellets (~25 g) were thawed/resuspended in 150 ml of lysis buffer (10% glycerol, 0.5% NP40, 0.2 M KCl, 1 mM PMSF, 5 mM β-mercaptoethanol, 50 mM HEPES, pH 7.5) containing protease inhibitor mixture ("for use with fungal and yeast extracts"; Sigma). The resulting cell suspension was distributed equally into four tubes, and cells were disrupted using Mini-Beadbeater-16 (BioSpec; 10 times for 1 min each, with 5-min intervals on ice). The resulting suspensions were centrifuged at 11,200*g* for 30 min at 4°C, and each supernatant was added to 1 ml of anti-flag-M2 magnetic beads (Sigma), followed by incubation, with rocking, at 4° C for 2 hr. The beads were collected by magnet, and were washed, sequentially, with 10 ml of lysis buffer, with 10 ml of buffer A (10% glycerol, 0.5% NP40, 1 M KCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 50 mM HEPES, pH 7.5), and with 10 ml of buffer B (lysis buffer without NP40). $_f$Ubr1, Ufd4$_f$, SL-Chk1$^{1-527}_{m-f}$, and SL-Chk1$^{1-502}_{m-f}$, bound to the immobilized anti-flag antibody in four different samples, were eluted with 5 ml of buffer C (buffer B containing flag-epitope peptide (Sigma) at 0.5 mg/ml), and the sample was dialyzed at 4°C overnight against

storage buffer (10% glycerol, 0.15 M NaCl, 5 mM β-mercaptoethanol, 50 mM HEPES, pH 7.5), followed by storage of samples at -80°C.

To induce expression of N-terminally His_6-tagged ubiquitin (His_6-Ub) expression, pJO591 (Table S2) was transformed into BL21 (DE3) *E. coli*. 5 ml of overnight culture of transformed cells was inoculated into 0.4 l of LB medium containing 34 μg/ml chloramphenicol and 100 μg/ml ampicillin, followed by incubation at 37 °C for ~1 h to A_{600} of ~0.6. Expression of His_6-Ub was induced with 1 mM isopropyl-D-thiogalactoside (IPTG) at 22°C overnight. Cells were harvested, thereafter, by centrifugation and frozen at -80°C. To overexpress N-terminally His_6-tagged Ubc4 (His_6-Ubc4), pJO589 (Table S2) was transformed into BL21 (DE3) *E. coli*. At A_{600} of ~0.5, IPTG was added to 1.6 l culture to the final IPTG concentration of 1 mM, followed by incubation at 37°C for 4 hr. N-terminally His_6-tagged Rad6 (His_6-Rad6) was similarly overexpressed from *E. coli* BL21 (DE3) transformed with pJO663 by adding 1 mM IPTG to 400 ml of culture at A_{600} of 0.6, then incubated overnight at 22 °C (Table S2).

His_6-Ub and other His_6-tagged overexpressed proteins were purified using TALON affinity resin (Clontech) as described in the manufacturer's protocol, with slight modifications. Briefly, 3-4 g of cell pellet were resuspended in 40 ml of xTractor buffer containing 1×protease inhibitor cocktail ("for bacterial cells"; Sigma) 1×PMSF, 1×DNase I, and 40 mg lysozyme, followed by gentle shaking for 20 min at 4°C. After centrifugation at 12,000*g* for 20 min at 4°C, the supernatant was transferred to a 50-ml tube containing 1 ml of pre-washed TALON resin. After gentle shaking at 4°C for 1 hr, the slurry was transferred to a 10-ml polypropylene column, followed by two washes with equilibration buffer, one wash with wash buffer, and the elution of proteins with 10 ml of elution buffer (Clontech).

His_6-Rad6 purification was done as described above, except that before the elution step, the TALON-bound His_6-Rad6 was incubated, at 4°C overnight, in 0.5 ml of the cleavage buffer (0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 0.05% NP-40, 50 mM Tris-HCl, pH 8.0) containing 30 μl of HRV 3C protease (Thermo Fisher). The resulting flow-through (the released His_6-Rad6) was changed to equilibration buffer using PD-10 column (GE Healthcare). The sample was incubated, then, with 0.5 ml of Talon resin at 4°C for 1 hr and the flow-through was collected. Final protein samples were dialyzed against storage buffer (10% glycerol, 0.15 M NaCl, 5 mM β-mercaptoethanol, 50 mM HEPES, pH 7.5) overnight, followed by storage at -80°C.

***In vitro* ubiquitylation assay.** Purified proteins for the assay were produced as described above. Purified *S. cerevisiae* Uba1 (Ub-activating enzyme, E1) was purchased from Boston Biochem. Purified SL-Chk1$^{1-527}_{m-f}$ or SL-Chk1$^{1-502}_{m-f}$ (5 μM) were incubated at 30°C for 15 min in the total volume of 20 μl containing 4 mM ATP, 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM DTT, 50 mM HEPES, pH 7.5, as well as 80 μM (1.6 nmoles) His_6-Ub and 0.1 μM (2 pmoles) Uba1. The assay also contained conditionally added (in several combinations) 1 μM (20 pmoles) Rad6 and/or purified $_f$Ubr1 (final concentration 0.2 μM (4 pmoles)), and/or purified Ufd4f (final concentration 0.2 μM (4 pmoles)). All reaction components except Uba1 were mixed together on ice for 10 min, followed by the addition of Uba1. Reactions were carried out at 30°C and terminated by adding 8 μl of 4×LDS/PAGE loading buffer (Invitrogen). One half of each sample (14 μl) was heated at 95°C for 5 min, followed by 4-12% NuPAGE and immunoblotting with anti-c-myc antibody (Sigma).

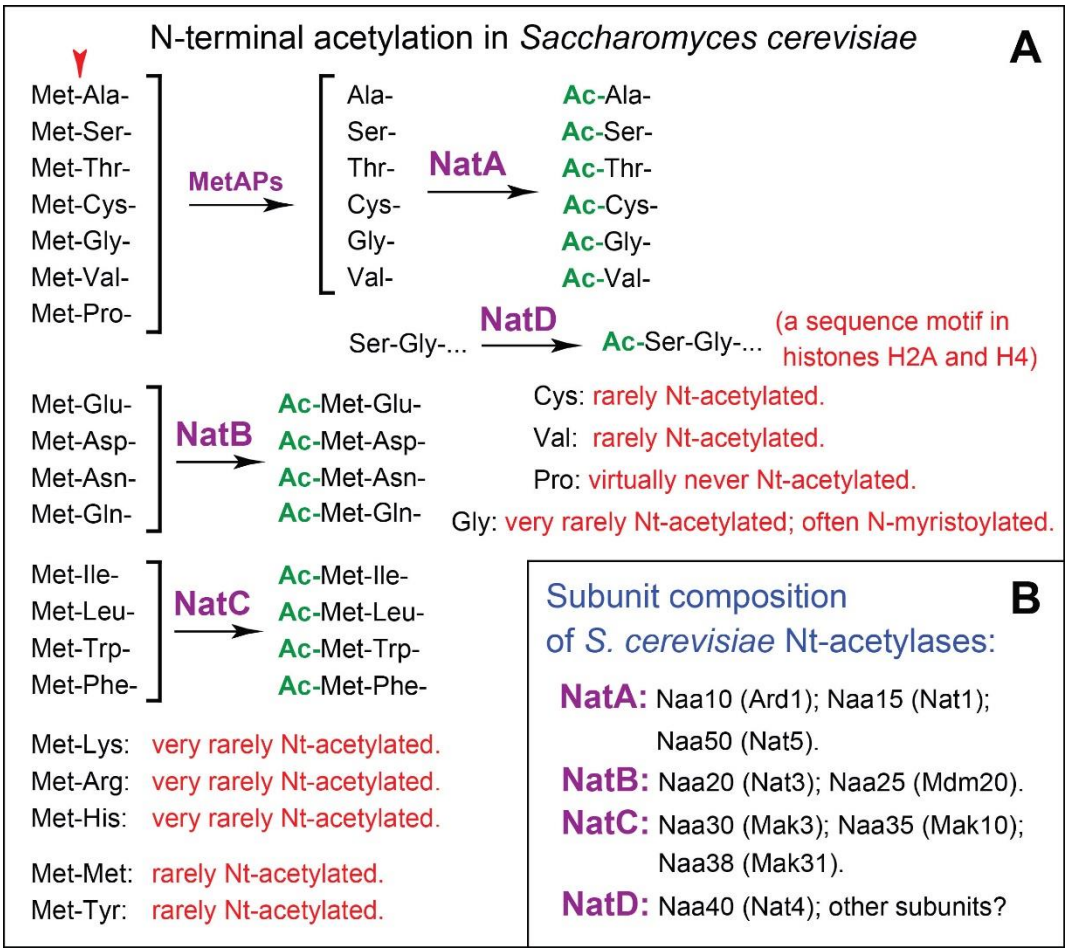

Fig. S1. Substrate specificities and subunit compositions of *S. cerevisiae* Nt-acetylases. Older names of specific subunits are in parentheses. See the main text and references therein.

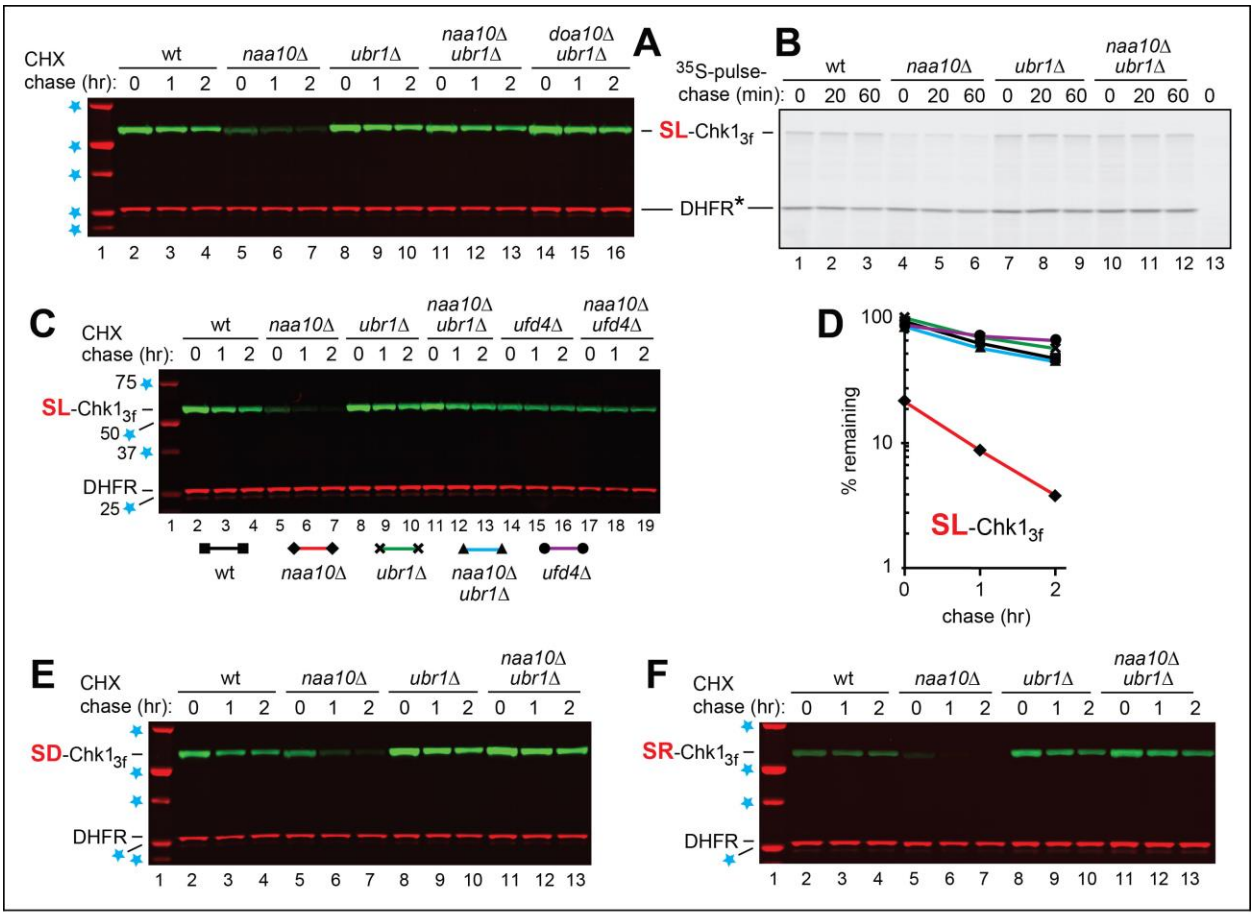

Fig. S2, Oh et al.

Fig. S2. Accelerated degradation of Chk1 in *naa10Δ* cells requires both Ubr1 and Ufd4.

(*A*) Lane 1, blue stars denote 25, 37, 50, and 75 kDa markers, respectively. CHX-chases, using PRT (Figure 2B), with wt SL-Chk1$_{3f}$, for 0, 1 and 2 hr, with wild-type (wt) (lanes 2-4), *naa10Δ* (lanes 5-7), *ubr1Δ* (lanes 8-10), *naa10Δ ubr1Δ* (lanes 11-13), and *doa10Δ ubr1Δ* (lanes 14-16) *S. cerevisiae*. Extracts were prepared from cells withdrawn at indicated times of a chase. Proteins in an extract were fractionated by SDS-PAGE, followed by immunoblotting with anti-flag and anti-ha antibodies. The bands of Chk1$_{3f}$ and $_{f}$DHFR$_{ha}$ are indicated.

(*B*) ^{35}S-pulse-chases, using PRT (Figure 2B), a 2-min pulse with ^{35}S-Met/Cys, and a CHX-chase afterward, with wt SL-Chk1$_{3f}$, for 0, 20 and 60 min, in wt (lanes 1-3), *naa10Δ* (lanes 4-6), *ubr1Δ* (lanes 7-9), and *naa10Δ ubr1Δ* (lanes 10-13) *S. cerevisiae*. Extracts were prepared from cells withdrawn at indicated times of a chase, followed by precipitation of proteins with anti-flag magnetic beads, SDS-PAGE, and autoradiography.

(*C*) Lane 1, kDa markers. CHX-chases, using PRT (Figure 2B), with wt SL-Chk1$_{3f}$, for 0, 1 and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), *ubr1Δ* (lanes 8-10), *naa10Δ ubr1Δ* (lanes 11-13), *ufd4Δ* (lanes 14-16), and *naa10Δ ufd4Δ* (lanes 17-19) *S. cerevisiae*. The bands of Chk1$_{3f}$ and $_{f}$DHFR$_{ha}$ are indicated.

(*D*) Quantification of data in C. For curve designations, see the lower part of C. All chases in this study were performed at least twice, yielding results that differed by less than 10%.

(*E*) Lane 1, blue stars denote 20, 25, 37, 50, and 75 kDa markers, respectively. CHX-chases, using PRT (Figure 2B), with the SD-Chk1$_{3f}$ mutant, for 0, 1 and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), *ubr1Δ* (lanes 8-10), and *naa10Δ ubr1Δ* (lanes 11-13) *S. cerevisiae*. The bands of the SD-Chk1$_{3f}$ mutant and $_{f}$DHFR$_{ha}$ are indicated.

(*F*) Lane 1, blue stars denote 25, 37, 50, and 75 kDa markers, respectively. CHX-chases, using PRT (Figure 2B), with the SR-Chk1$_{3f}$ mutant, for 0, 1 and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), *ubr1Δ* (lanes 8-10), and *naa10Δ ubr1Δ* (lanes 11-13) *S. cerevisiae*. The bands of the SR-Chk1$_{3f}$ mutant and $_{f}$DHFR$_{ha}$ are indicated.

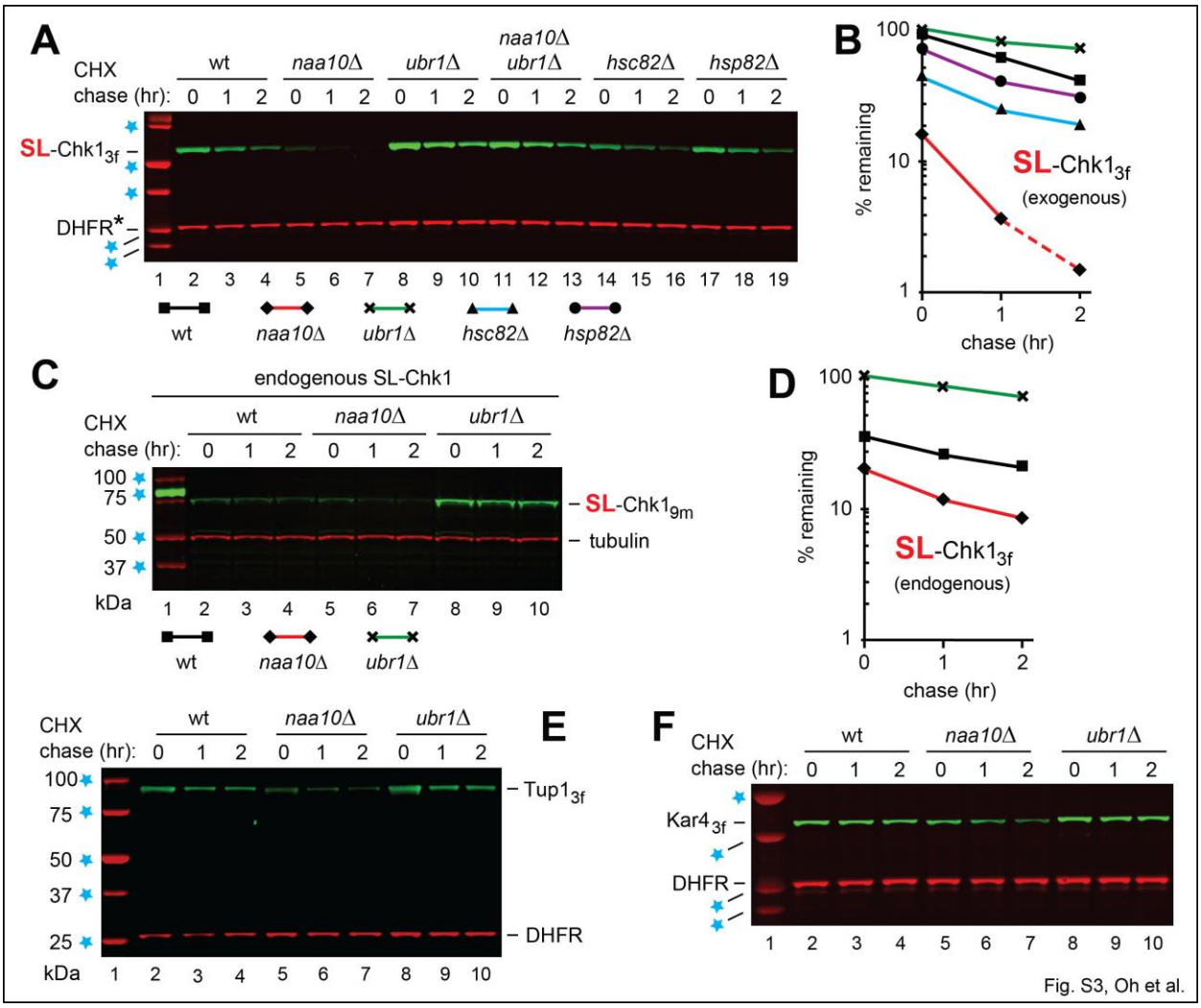

Fig. S3. Faster degradation of Chk1 in cells containing lower levels of Hsp90.

(*A*) Lane 1, blue stars denote 20, 25, 37, 50, and 75 kDa markers, respectively. CHX-chases, using PRT (Figure 2B), with wt SL-Chk1$_{3f}$, for 0, 1 and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), *ubr1Δ* (lanes 8-10), *naa10Δ ubr1Δ* (lanes 11-13), *hsc82Δ* (lanes 14-16), and *hsp82Δ* (lanes 14-16) *S. cerevisiae*. Extracts were prepared from cells withdrawn at indicated times of a chase. Proteins in an extract were fractionated by SDS-PAGE, followed by immunoblotting with anti-flag and anti-ha antibodies. The bands of Chk1$_{3f}$ and $_{f}$DHFR$_{ha}$ are indicated.

(*B*) Quantification of data in A. For curve designations, see the lower part of A. All chases in this study were performed at least twice, yielding results that differed by less than 10%.

(*C*) Ubr1-mediated degradation of the endogenous wt SL-Chk1, C-terminally tagged (at the endogenous *CHK1* gene) with myc epitopes (Chk1$_{9m}$). Lane 1, kDa markers. CHX-chases with wt endogenous Chk1$_{9m}$, for 0, 1 and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), and *ubr1Δ* (lanes 8-10) *S. cerevisiae*. With immunoblots of tubulin as a loading control.

(*D*) Quantification of data in C. For curve designations, see the lower part of C. All chases in this study were performed at least twice, yielding results that differed by less than 10%.

(*E*) Degradation of Tup1. Lane 1, kDa markers. CHX-chases, using PRT (Figure 2B), with the wt $Tup1_{3f}$ protein (see the main text) for 0, 1 and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), and *ubr1Δ* (lanes 8-10) *S. cerevisiae*. The bands of $Tup1_{3f}$ and $_{f}DHFR_{ha}$ are indicated.

(*F*) Degradation of Kar4. Same as in E but with the wt $Kar4_{3f}$ protein (see the main text).

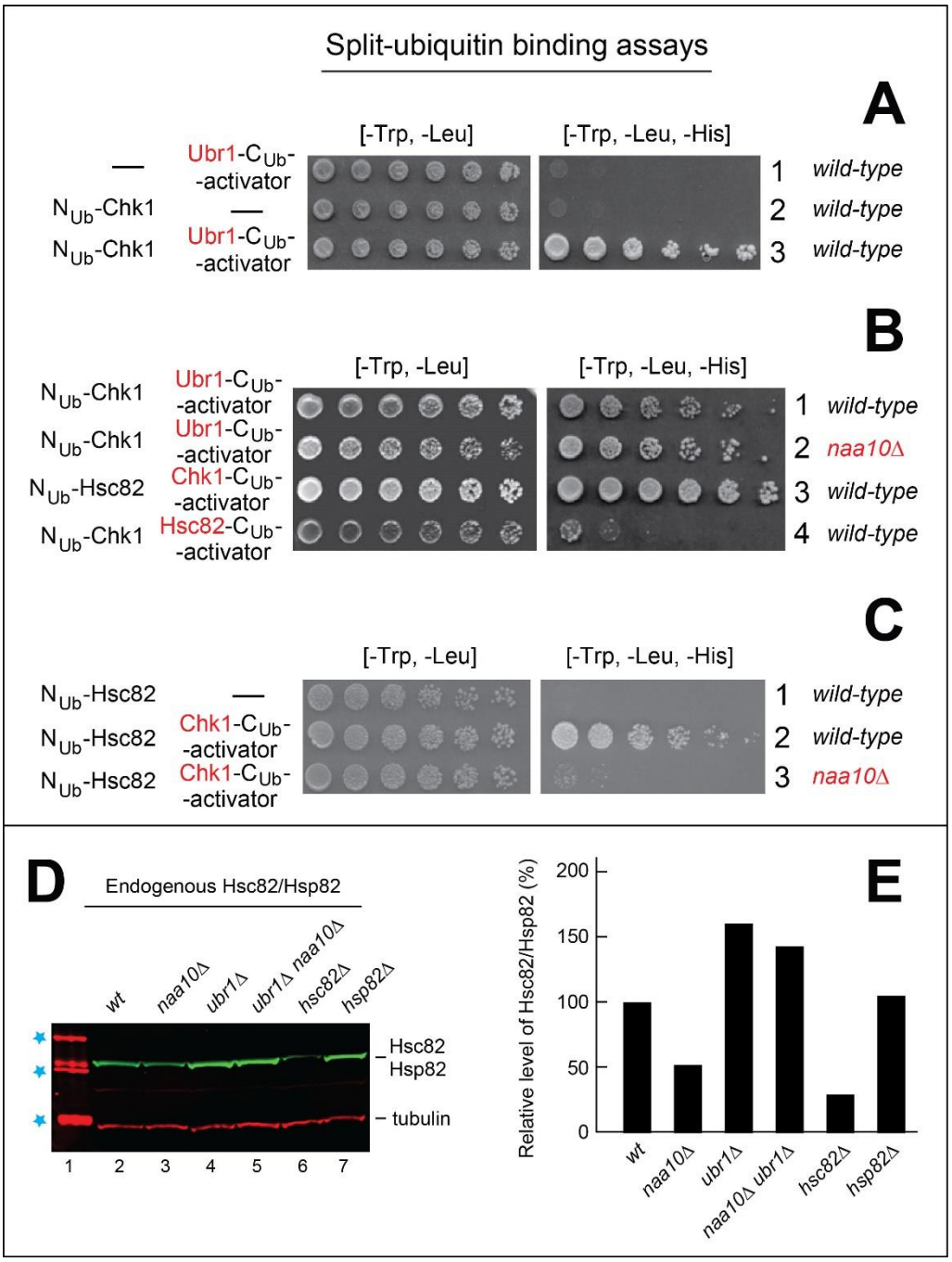

Fig. S4. Use of split-ubiquitin assays to analyze Chk1-Ubr1 and Chk1-Hsc82 Interactions.

(A) Split-Ub-based fusions of Ubr1 vs. full-length Chk1$^{1\text{-}527}$ (see the legend to the main Figure 6 for additional details) in wt *S. cerevisiae*. A1 and A2, either Ubr1 alone or Chk1 alone (negative controls). A3, split-Ub assay with both Chk1 and Ubr1. Expression of *HIS3* (the ultimate readout of split-Ub assay), in otherwise His$^-$ cells, was a function of affinity between test proteins. Left panels in A-C are images of His-containing plates, on which all yeast strains grew. His-lacking plates, on which only His$^+$ cells grew, are on the right (see STAR Methods).

(B) Chk1-Ubr1 and Chk1-Hsc82 interactions. B1, Split-Ub assay with split-Ub-based fusions of full-length Chk1 vs. full-length Ubr1 in wt cells. B2, same as in B1 but in *naa10Δ* cells. B3, split-Ub assay, in wt cells, with split-Ub-based fusions of Chk1 vs. Hsc82, in which the C_{Ub}-activator moiety was located at the N-terminus of the Hsc82 moiety. B4, same as in B3, except that the C_{Ub}-activator moiety was located at the C-terminus of the Hsc82 moiety. Note a dramatically smaller split-Ub signal.

(C) Split-Ub assays with Chk1 and Hsc82 in wt vs. *naa10Δ* cells. C1, Split-Ub assay, in wt cells, with Hsc82 alone (negative control). C2, Hsc82 vs. Chk1, in wt cells. C3, same as C2 but in *naa10Δ* cells (see also the main Figure 6C). Note the near-absence of split-Ub signal in *naa10Δ* cells (C3), in contrast to wt cells (C2).

(D) Immunoblotting-based comparison of the levels of the endogenous (untagged) *S. cerevisiae* Hsc82+Hsp82 in wt and mutant backgrounds using an antibody that recognizes both Hsc82 and Hsp82 (these highly similar proteins practically co-migrated upon SDS-PAGE under conditions used). Lane 1, blue stars denote 50, 75 and 100 kDa markers, respectively. Lanes 2-7, Hsc82+Hsp82 in wt, *naa10Δ, ubr1Δ*, *naa10Δ ubr1Δ*, *hsc82Δ*, and *hsp82Δ* *S. cerevisiae*. With immunoblots of tubulin as a loading control.

(E) Quantification of data in D, with the level of Hsc82+Hsp82 in wt cells taken as 100%. All immunoblotting analyses in this study were performed at least twice, yielding results that differed by less than 10%.

Hsp90 chaperones and their cochaperones are either demonstrated or predicted substrates of the NatA (Naa10) Nt-acetylase				
Proteins	**Nt-acetylases**			
Hsp90 system	**NatA**	**NatB**	**NatC**	**NatD**
Hsc82	(M)ASETF-	–	–	–
Hsp82	(M)AGETF-	–	–	–
Cochaperones of Hsp90:				
Aha1	(M)VVNNP-	–	–	–
Cdc37	(M)AIDYS-	–	–	–
Cns1	(M)SSVNA-	–	–	–
Cpr6	(M)TRPKT-	–	–	–
Cpr7	(M)IQDPL-	–	–	–
Hch1	(M)VVLNP-	–	–	–
Pih1	(M)ADFLL-	–	–	–
Ppt1	(M)STPTA-	–	–	–
Rbv1	(M)VAISE-	–	–	–
Rvb2	(M)SIQTS-	–	–	–
Sba1	(M)SDKVI-	–	–	–
Sse1	(M)STPFG-	–	–	–
Sse2	(M)STPFG-	–	–	–
Sti1	(M)SLTAD-	–	–	–
Tah1	(M)SQFEK-	–	–	–
Sgt1	(M)PVEKD- unacetylated			

Fig. S5. The known components of the *S. cerevisiae* Hsp90 system, save for Sgt1, are either identified or predicted substrates of the NatA (Naa10) Nt-acetylase. See also Fig. S1, the main text, and references therein.

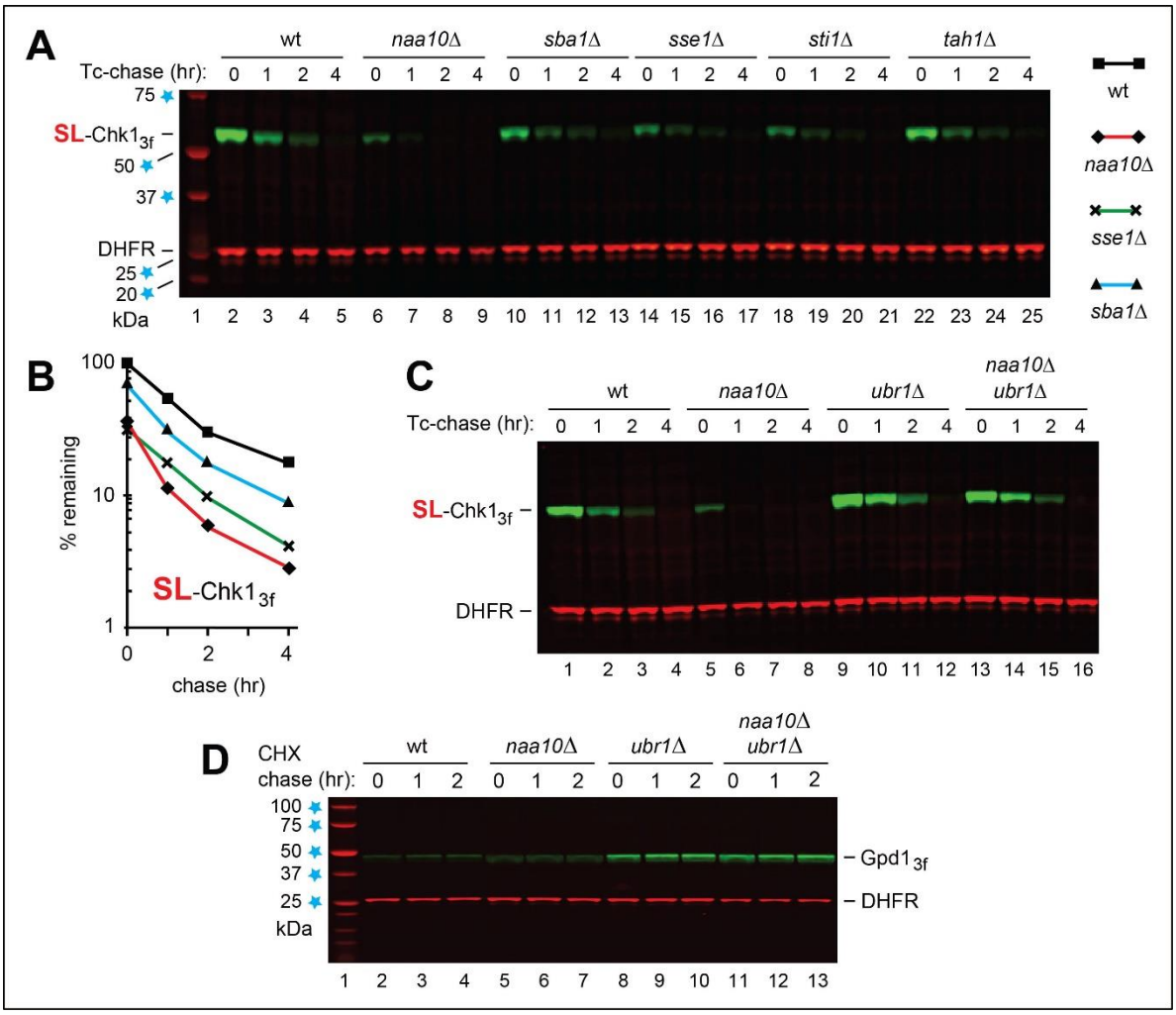

Fig. S6. Ablation of Hsp90 cochaperones can destabilize Chk1.

(A) Lane 1, kDa markers. Tetracycline (Tc)-based chases, using PRT (Figure 2B), with wt SL-Chk1$_{3f}$, for 0, 1, 2, and 4 hr, with wt (lanes 2-5), *naa10Δ* (lanes 6-9), *sba1Δ* (lanes 10-13), *sse1Δ* (lanes 14-17), *sti1Δ* (lanes 18-21), and *tah1Δ* (lanes 22-25) *S. cerevisiae*. Extracts were prepared from cells withdrawn at indicated times of a chase. Proteins in an extract were fractionated by SDS-PAGE, followed by immunoblotting with anti-flag and anti-ha antibodies. The bands of Chk1$_{3f}$ and $_{f}$DHFR$_{ha}$ are indicated.

(B) Quantification of data in A. For curve designations, see the right part of A. All chases in this study were performed at least twice, yielding results that differed by less than 10%.

(C) Tetracycline (Tc)-based chases, using PRT (Figure 2B), with wt SL-Chk1$_{3f}$, for 0, 1, 2, and 4 hr, with wt (lanes 1-4), *naa10Δ* (lanes 5-8), *ubr1Δ* (lanes 9-12), and *naa10Δ ubr1Δ* (lanes 13-16) *S. cerevisiae*.

(D) Degradation of Gpd1. Lane 1, kDa markers. CHX-chases, using PRT (Figure 2B), with wt Gpd1$_{3f}$, for 0, 1, and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), *ubr1Δ* (lanes 8-10), and *naa10Δ ubr1Δ* (lanes 11-13) *S. cerevisiae*. The bands of Gpd1$_{3f}$ and $_{f}$DHFR$_{ha}$ are indicated.

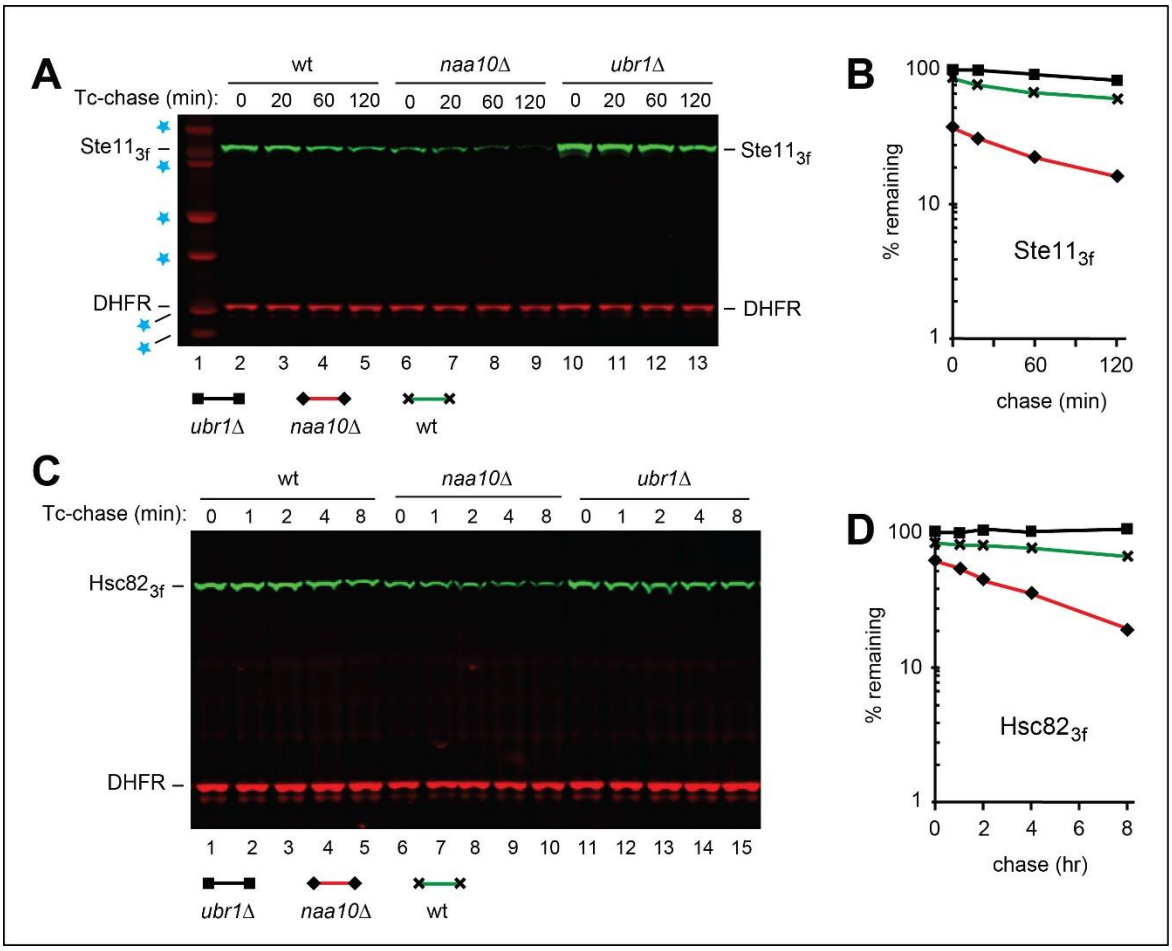

Fig. S7. Degradation of Ste11 and Hsc82 by the Arg/N-end rule pathway in *naa10Δ* cells.

(A) Degradation of Ste11. Lane 1, blue stars denote 20, 25, 37, 50, 75 and 100 kDa markers, respectively. Tetracycline (Tc)-chases, using PRT (Figure 2B), with wt Ste11$_{3f}$, were for 0, 20, 60, and 120 min with wt (lanes 2-5), *naa10Δ* (lanes 6-9), and *ubr1Δ* (lanes 10-12) *S. cerevisiae*. Extracts were prepared from cells withdrawn at indicated times of a chase. Proteins in an extract were fractionated by SDS-PAGE, followed by immunoblotting with anti-flag and anti-ha antibodies. The bands of Ste11$_{3f}$ (see the main text) and $_{f}$DHFR$_{ha}$ are indicated.

(B) Quantification of data in A. For curve designations, see the lower part of A. All chases in this study were performed at least twice, yielding results that differed by less than 10%.

(C) Degradation of Hsc82. Tetracycline (Tc)-chases, using PRT (Figure 2B), with wt Hsc82$_{3f}$, were for 0, 1, 2, 4, and 8 hr, with wt (lanes 2-5), *naa10Δ* (lanes 6-9), and *ubr1Δ* (lanes 10-13) *S. cerevisiae*.

(D) Quantification of data in C. For curve designations, see the lower part of C. All chases in this study were performed at least twice, yielding results that differed by less than 10%.

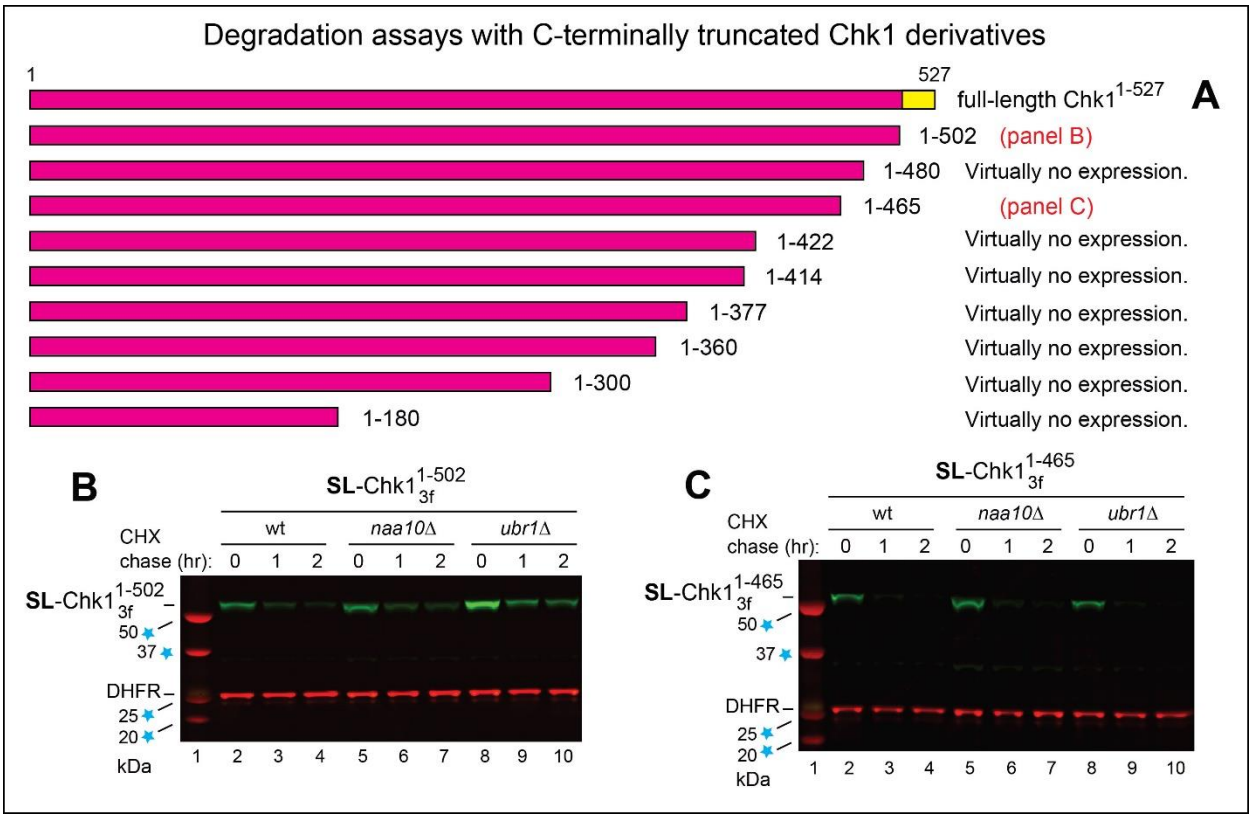

Fig. S8. Mapping Chk1 degron by degradation assays.

(A) A set of C-terminal truncations of the 527-residue Chk1. As indicated on the right (see also the main text), only SL-Chk1$^{1-502}_{3f}$ and SL-Chk1$^{1-465}_{3f}$ (in addition to the full-length SL-Chk1$^{1-527}_{3f}$; see, e.g., Figure 2C) could be expressed at readily detectable levels in reference-based PRT assays (Figure 2B). A yellow rectangle in the diagram of the full-length SL-Chk1$^{1-527}_{3f}$ denotes the 25-residue C-terminal segment of Chk1 that contains the bulk of its Ubr1-specific degron (see the main text).

(B) Lane 1, kDa markers. CHX-chases, using PRT (Figure 2B), with the SL-Chk1$^{1-502}_{3f}$ derivative of the full-length Chk1, for 0, 1, and 2 hr, with wt (lanes 2-4), *naa10Δ* (lanes 5-7), and *ubr1Δ* (lanes 8-10*S. cerevisiae*. The bands of SL-Chk1$^{1-502}_{3f}$ and $_{f}$DHFR$_{ha}$ are indicated.

(C) Same as in B but with SL-Chk1$^{1-465}_{3f}$.

Table S1. *E. coli* and *S. cerevisiae* strains used in this study.

Strains	Relevant genotypes	Sources
E. coli strains:		
DH5a	*F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1*	Invitrogen
BL21-CodonPlus(DE3)-RIL	*F– ompT hsdS(rB – mB –) dcm+ Tetr gal λ(DE3) endA Hte [argU ileY leuW Camr]*	Stratagene
STBL2	*F– endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ–*	Invitrogen
S. cerevisiae strains:		
BY4742	*MATα his3-1 leu2-0 lys2-0 ura3-0 can1-100*	Open Biosystems
CHY271	*naa10*Δ*::KanMX6* in BY4742	Open Biosystems
CHY272	*naa30*Δ*::KanMX6* in BY4742	Open Biosystems
CHY273	*naa20*Δ*::KanMX6* in BY4742	Open Biosystems
JOY499	*sse1*Δ*::KanMX6* in BY4742	Open Biosystems
JOY4501	*hsc82*Δ*::KanMX6* in BY4742	Open Biosystems
JOY4502	*hsp82*Δ*::KanMX6* in BY4742	Open Biosystems
JOY544	*sti1*Δ*::KanMX6* in BY4742	Open Biosystems
JOY545	*tah1*Δ*::KanMX6* in BY4742	Open Biosystems
AH109	*MAT**a**, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4*Δ*, gal80*Δ*, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*	Clontech
NMY51	*MATa trp1 leu2 his3 ade2 LYS2::lexA-HIS3 ade2::lexA-ADE2 URA3::lexA-lacZ*	Dualsystems

CHY346	*ubr1*Δ*::KanMX6, doa10*Δ*::HphNT1* in BY4742	Lab collection
CHY347	*ubr1*Δ*::KanMX6, naa10*Δ*::HphNT1* in BY4742	Lab collection
SC295	*MAT a ura3-52 leu2-3, 112 reg1-501 gal1 pep4-3*	Lab collection
JOY379	*ubr1*Δ*::HphNT1* in BY4742	This study
JOY439	*cup9*Δ*::NatNT2* in BY4742	This study
JOY440	*naa10*Δ*::KanMX6, cup9*Δ*::NatNT2* in BY4742	This study
JOY441	*ubr1*Δ*::KanMX6, naa10*Δ*::HphNT1, cup9*Δ*::NatNT2* in BY4742	This study
JOY442	*ufd4*Δ*::HphNT1* in BY4742	This study
JOY443	*naa10*Δ*::KanMX6, ufd4*Δ*::HphNT1* in BY4742	This study
JOY468	*sba1*Δ*::HphNT1* in BY4742	This study
JOY487	*pdr5*Δ*::NatNT2* in BY4742	This study
JOY498	*naa10*Δ*::KanMX6, pdr5*Δ*::NatNT2* in BY4742	This study
JOY505	*naa10*Δ*::KanMX6* in NMY51	This study
JOY512	*CHK1-9myc::HphNT1* in BY4742	This study
JOY513	*CHK1-9myc::HphNT1, naa10*Δ*::KanMX6* in BY4742	This study
JOY514	*CHK1-9myc::KanMX4, ubr1*Δ*::HphNT1* in BY4742	This study
JOY529	*naa10*Δ*::KanMX6, ubr1*Δ*::HphNT1* in NMY51	This study

Table S2. Plasmids used in this study.

Plasmid	Description	Source or Reference
pDHB1	Split-Ub assay bait vector. Contains the P_{CYC1} promoter. Used to produce Ost4-Cub-LexA-VP16 fusion.	Dualsystems
pPR3-N	Split-Ub assay prey vector. Contains the P_{CYC1} promoter. Produces NubG–HA fusion.	Dualsystems
pDONR/Zeo	Donor vector for Gateway cloning through BP reaction.	Invitrogen
pGADCg	Y2H expression vector. Contains the P_{ADH1} promoter. Used to produce C-terminal Gal4-AD_{ha*} fusion by Gateway cloning. *contains a partial ha epitope sequence.	Addgene
pGBKT7g	Y2H expression vector. Contains the P_{ADH1} promoter. Produces N-terminal Gal4-DBD_{myc} fusion by Gateway cloning.	Addgene
pDONR/Zeo	Donor vector for Gateway cloning via BP reaction	Invitrogen
pET-11d	pET-11d	Novagen
pET-15b	pET-15b	Novagen
pFA6a-KanMX6	pFA6a-KanMX6	(19)
pFA6a-HphNT1	pFA6a-HphNT1	(6)
pFA6a-NatNT2	pFA6a-NatNT2	(6)
pTDH3-tc3-3×HA	P_{TDH3}-tc3-3×HA	(7)
pRS313	pRS313	(20)
pRS314 Gal1/10	pRS314 with $P_{GAL1/10}$	(20)

pRS413 Met25	pRS413 with P_{MET25}	(20)
pRS425 Gal1	pRS425 with P_{GAL1} promoter	(20)
pYM18	9 c-Myc with KanMX4	(6)
pYM20	9 c-Myc with HphNT1	(6)
pNTfUBR1	$_{flag}$Ubr1 in YEPlac181	(21)
pUsp2-cc	$Usp2^{1-45}$ ORF in pET-15b	(3)
pHUE	His_6-Ub fusion in pET-15b	(3)
pJO162	Naa10 in $pH_{10}UE$	This study
pJO241	pRS426 with $P_{GAL1/10}$	This study
pJO267	Naa10 in pRS425 Gal1/10	This study
pJO379	MK-eK-$_{haha}$-Ura3-Ub-$_{flag}$DHFR$_{ha}$ in pRS413 Gal1	This study
pJO589	His_6-Ubc4 in pET-15b	This study
pJO591	His_6-Ub in pET-15b	This study
pJO622	P_{TDH3}-tc3 and $_{flag}$DHFR$_{ha}$ in pRS413	This study
pJO624	Complete set of PRT vector with MAI-Cln3 in pRS313	This study
pJO630	MSL-Chk1 in pJO624	This study
pJO638	MSA-Gpd1 in pJO624	This study
pJO639	MTA-Tup1 in pJO624	This study
pJO641	MSD-Chk1 in pJO630	This study
pJO642	MSK-Chk1 in pJO630	This study
pJO643	MSP-Chk1 in pJO630	This study
pJO644	MSR-Chk1 in pJO630	This study
pJO645	MSF-Chk1 in pJO630	This study

pJO661	MPS-Chk1 in pJO630	This study
pJO663	His_6-HRV 3C protease site-Rad6 in pET-11d	This study
pJO679	$Ufd4_{flag}$ in pRS425 Gal1	This study
pJO684	MAI-Cdc37 in pJO624	This study
pJO689	$_{flag}$Ubr1 in pRS426 Gal1/10	This study
pJO690	$Ufd4_{hsv}$ and $_{flag}$Ubr1 in pRS426 Gal1/10	This study
pJO691	MAG-Hsc82 in pJO624	This study
pJO695	P_{TDH3}-tc3 and P-$_{flag}DHFR_{ha}$ in pRS413	This study
pJO697	$Chk1^{1-527}$ in pJO699	This study
pJO699	P_{TDH3}-tc3 and P-$_{flag}DHFR_{ha}$ in pJO624	This study
pJO723	$Chk1^{1-480}$ in pJO699	This study
pJO724	$Chk1^{1-422}$ in pJO699	This study
pJO725	$Chk1^{1-360}$ in pJO699	This study
pJO726	Chk-1^{1-300} in pJO699	This study
pJO727	$Chk1^{1-180}$ in pJO699	This study
pJO732	$Chk1^{1-502}$ in pJO699	This study
pJO733	$Chk1^{1-465}$ in pJO699	This study
pJO734	$Chk1^{1-414}$ in pJO699	This study
pJO735	$Chk1^{1-377}$ in pJO699	This study
pJO736	MAF- Kar4 in pJO699	This study
pJO746	P_{CUP1}-DHFR-ha-Ub^{K48}-Y-eK-βgal in pRS313	This study
pJO747	P_{CUP1}-DHFR-ha-Ub^{K48}-H-eK-βgal in pRS313	This study
pJO752	Ub^{K48}-Y-eK βgal in pJO699	This study
pJO753	Ub^{K48}-H-eK βgal in pJO699	This study
pJO781	attL-Ubr1 in pDONR/Zeo	This study

pJO794	SV40-NLS-Ubr1-Gal4-AD in pGADCg	This study
pJO829	$Chk1^{1-527}{}_{myc\text{-}flag}$ in pRS423	This study
pJO830	$Chk1^{1-502}{}_{myc\text{-}flag}$ in pRS423	This study
pJO841	Ost4-Chk1-C_{Ub}-LexA-VP16 in pDHB1	This study
pJO842	N_{Ub}-$Chk1^{1-527}$ in pPR3-N	This study
pJO858	N_{Ub}-$Chk1^{1-502}$ in pPR3-N	This study
pJO859	N_{Ub}-$Chk1^{1-510}$ in pPR3-N	This study
pJO860	N_{Ub}-$Chk1^{1-517}$ in pPR3-N	This study
pJO862	Ost4-Hsc82-C_{Ub}-LexA-VP16 in pDHB1	This study
pJO863	N_{Ub}-Hsc82 in pPR3-N	This study
pJO898	MEQ-Ste11 in pJO624	This study
pJO903	Ost4-$_{flag}$Ubr1-C_{Ub}-LexA-VP16 in pDHB1	This study
pJO930	attL-$Chk1^{1-527}$ in pDONR/Zeo	This study
pJO931	attL-$Chk1^{1-502}$ in pDONR/Zeo	This study
pJO932	attL-$Chk1^{1-465}$ in pDONR/Zeo	This study
pJH024	Gal4-DBD_{myc}-$Chk1^{1-527}$ in pGBKT7g	This study
pJH028	Gal4-DBD_{myc}-$Chk1^{1-502}$ in pGBKT7g	This study
pJH030	Gal4-DBD_{myc}-$Chk1^{1-465}$ in pGBKT7g	This study

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