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Section: *Biochemistry*

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Supporting Information (SI):

Five enzymes of the Arg/N-degron pathway form a targeting complex: the concept of superchanneling

Jang-Hyun Oh, Ju-Yeon Hyun, Shun-Jia Chen, and Alexander Varshavsky*

Division of Biology and Biological Engineering,
California Institute of Technology,
Pasadena, CA 91125, USA

*To whom correspondence should be addressed: avarsh@caltech.edu

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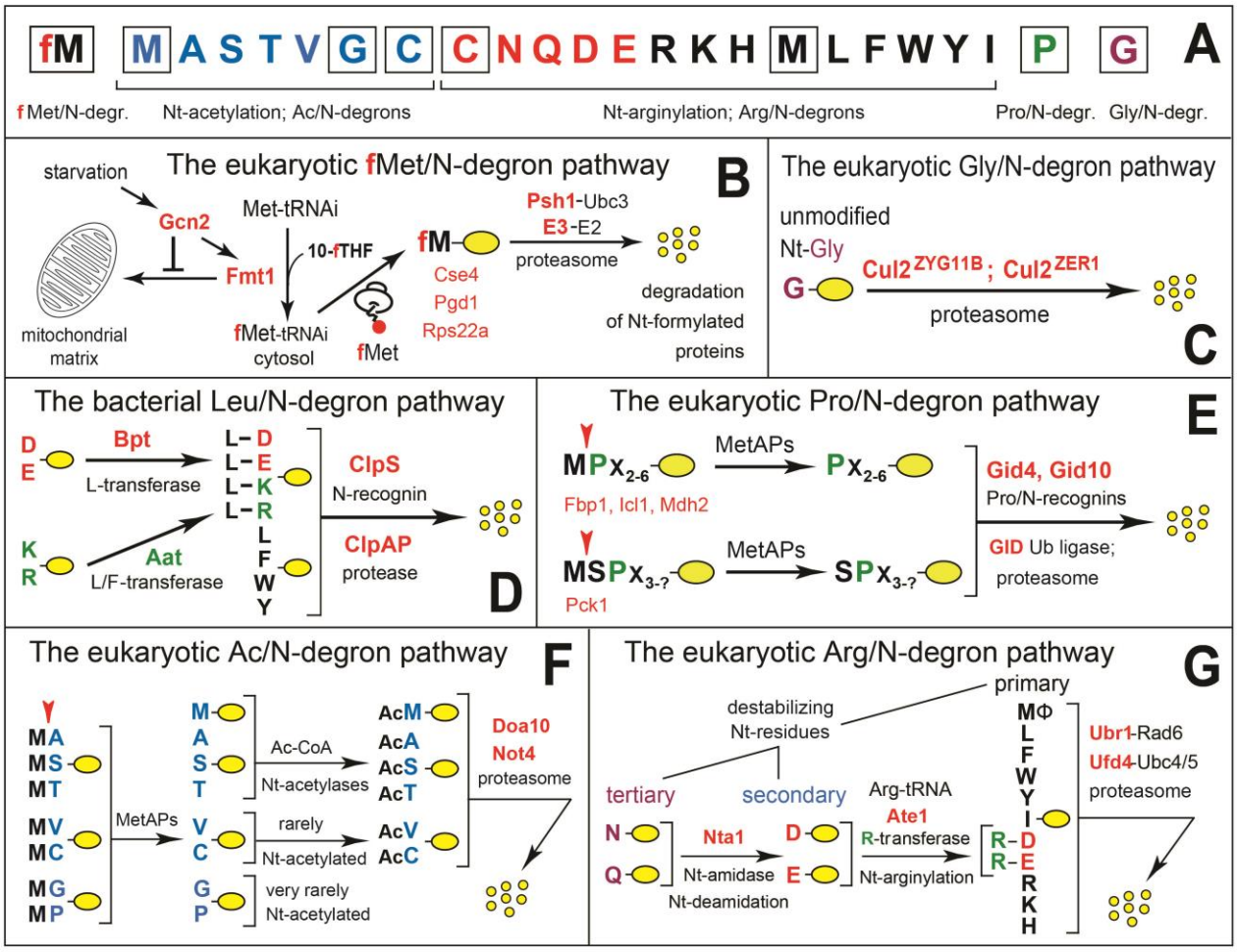

Fig. S1. N-degron pathways. N-terminal (Nt) residues are indicated by single-letter abbreviations. A yellow oval denotes the rest of a protein substrate. (*A*) Twenty amino acids of the genetic code are arranged to delineate specific N-degrons. Nt-Met is cited thrice, since it can be recognized by the Ac/N-degron pathway (as Nt-acetylated Ac-Met), by the Arg/N-degron pathway (as unacetylated Nt-Met), and by the fMet/N-degron pathway (as Nt-formylated fMet). Nt-Cys is cited twice, since it can be recognized by the Ac/N-degron pathway (as Nt-acetylated Cys) and by the Arg/N-degron pathway (as arginylatable Nt-Cys sulfinate or Nt-Cys-sulfonate, formed in multicellular eukaryotes but apparently not in unstressed *S. cerevisiae*). (*B*) The eukaryotic (*S. cerevisiae*) fMet/N-degron pathway. 10-fTHF, 10-formyltetrahydrofolate. (*C*) The bacterial (*E. coli*) fMet/N-degron pathway. (*D*) The bacterial (*V. vulnificus*) Leu/N-end rule pathway. (*E*) The eukaryotic (*S. cerevisiae*) Pro/N-degron pathway. (*F*) The eukaryotic (*S. cerevisiae*) Ac/N-degron pathway. (G*)* The eukaryotic (*S. cerevisiae*) Arg/N-degron pathway. See *Introduction* for references and other details.

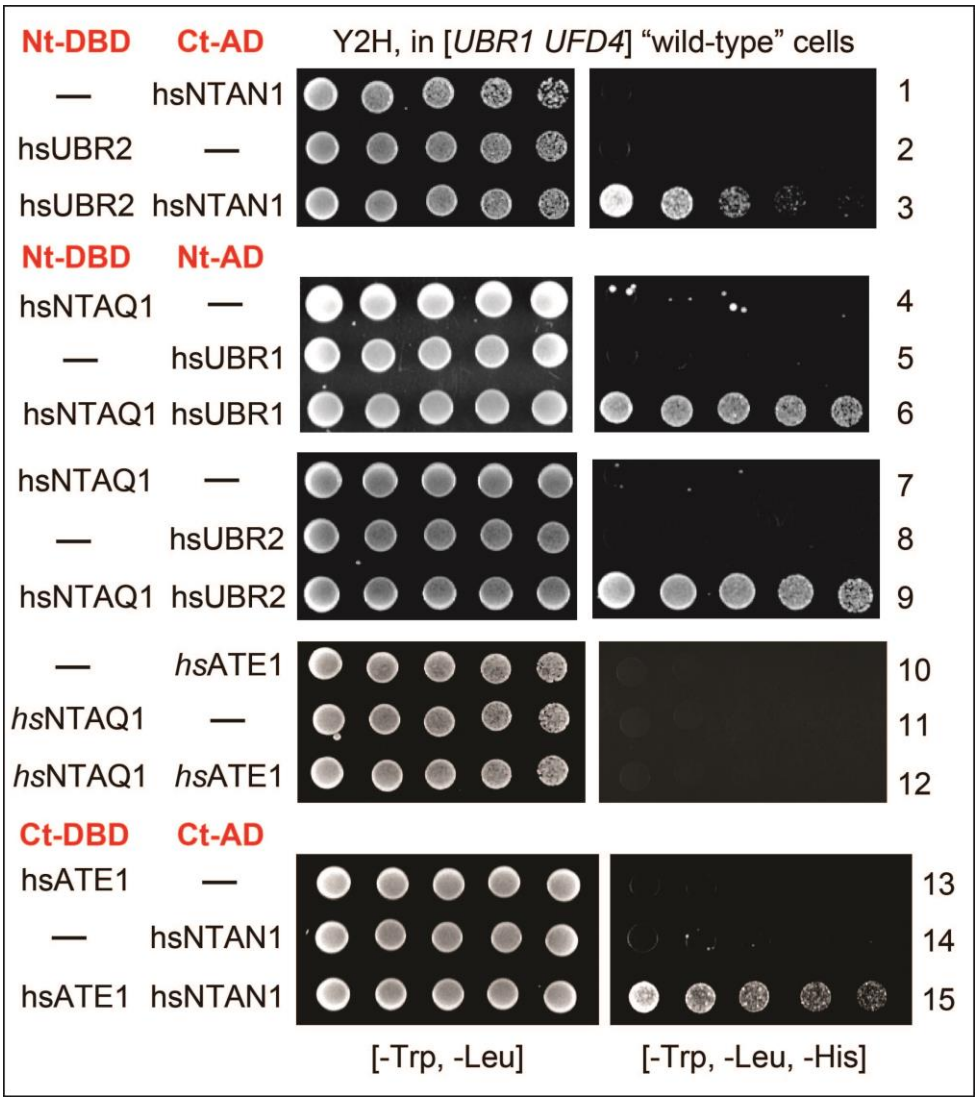

Fig. S2. Y2H binding assays, in wild-type [*UBR1 UFD4*] *S. cerevisiae*, with enzymes of the human Arg/N-degron pathway. Expression of *HIS3*, the assays' readout in otherwise His^- cells, is a function of binding affinity between test proteins (1). Histidine-lacking plates (shown on the right) were incubated for ~2 days at 30°C to detect the growth of His^+ cells. Nt and Ct Y2H-specific domains in Y2H-based protein fusions are marked in red. Row 1, *hs*NTAN1 vs. vector alone. Row 2, *hs*UBR2 vs. vector alone. Row 3, *hs*UBR2 vs. *hs*NTAN1. Row 4, *hs*NTAQ1 vs. vector alone. Row 5, *hs*UBR1 vs. vector alone. Row 6, *hs*NTAQ1 vs. *hs*UBR1. Row 7, *hs*NTAQ1 vs. vector alone. Row 8, *hs*UBR2 vs. vector alone. Row 9, *hs*NTAQ1 vs. *hs*UBR2. Row 10, *hs*ATE1 vs. vector alone. Row 11, *hs*NTAQ1 vs. vector alone. Row 12, *hs*ATE1 vs. *hs*NTAQ1. Row 13, *hs*ATE1 vs. vector alone. Row 14, *hs*NTAN1 vs. vector alone. Row 15, *hs*NTAN1 vs. *hs*ATE1.See *SI Appendix*, Materials and Methods for details.

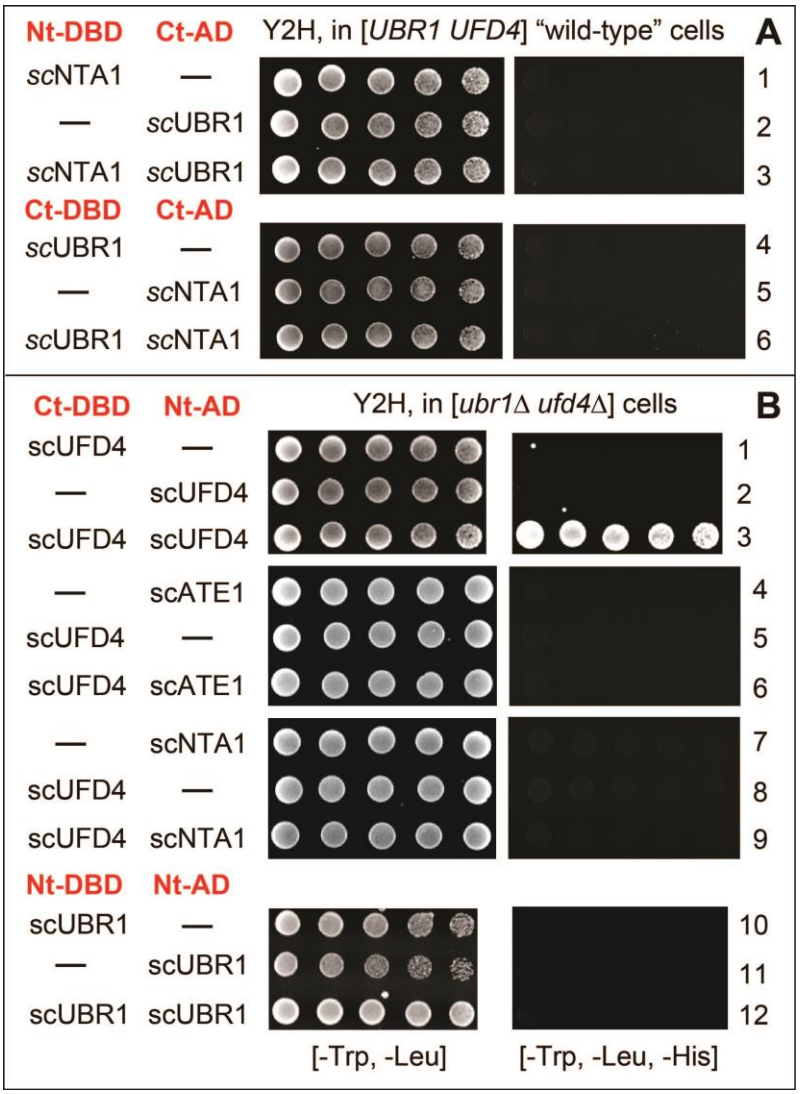

Fig. S3. (*A*) Y2H binding assays, in wild-type (*UBR1* UFD4) *S. cerevisiae*, with enzymes of the *S. cerevisiae* Arg/N-degron pathway. Expression of *HIS3*, the assays' readout in otherwise His^- cells, is a function of binding affinity between test proteins (1). Histidine-lacking plates (shown on the right) were incubated for ~2 days at 30°C to detect the growth of His^+ cells. Nt and Ct Y2H-specific domains in Y2H-based protein fusions are marked in red. Row 1, *sc*NTA1 vs. vector alone. Row 2, *sc*UBR1 vs. vector alone. Row 3, *sc*NTA1 vs. *hs*UBR1. Row 4, *sc*UBR1 vs. vector alone Row 5, *sc*NTA1 vs. vector alone. Row 6, *sc*UBR1 vs. *sc*NTA1 (the indicated configuration of DBD/AD domains is different from those in rows 1-3). (*B*) Y2H assays in double-mutant (*ubr1*Δ *ufd4*Δ) *S. cerevisiae*. Row 1, *sc*UFD4-DBD vs. vector alone. Row 2, AD-*sc*UFD4 vs. vector alone. Row 3, *sc*UFD4-DBD vs. AD-*sc*UFD4. Row 4, *sc*ATE1 vs. vector alone. Row 5, *sc*UFD4 vs. vector alone. Row 6, *sc*UFD4 vs. *sc*ATE1. Row 7, *sc*NTA1 vs. vector alone. Row 8, *sc*UFD4 vs. vector alone. Row 9, *sc*UFD4 vs. *sc*NTA1. Row 10, DBD-*sc*UBR1 vs. vector alone. Row 11, AD-*sc*UBR1 vs. vector alone. Row 12, DBD-*sc*UBR1 vs. AD-*sc*UBR1. See *SI Appendix*, Materials and Methods for details.

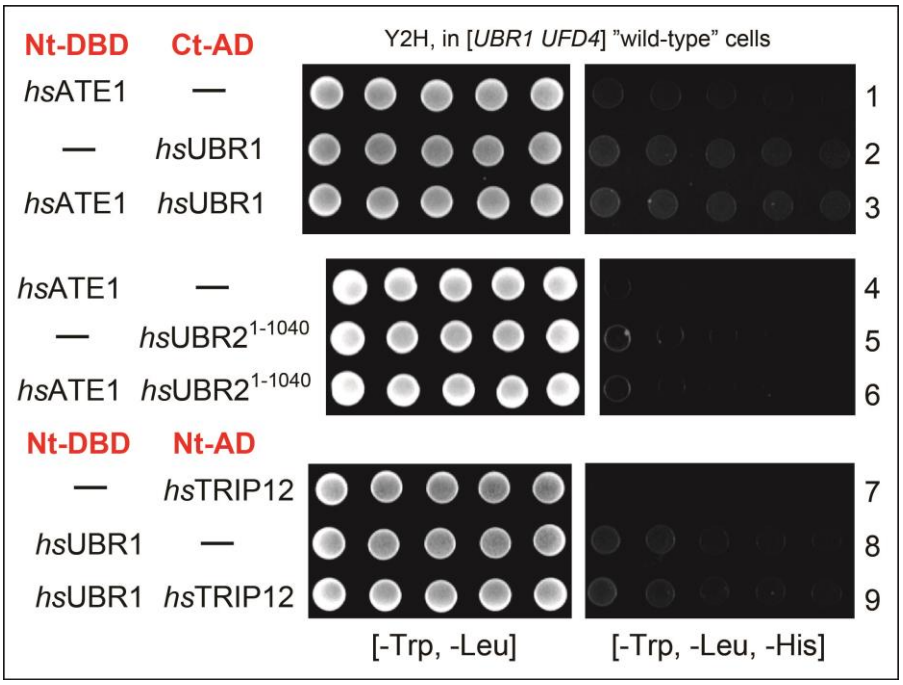

Fig. S4. Y2H binding assays, in wild-type (*UBR1 UFD4*) *S. cerevisiae*, with human proteins. Expression of *HIS3*, the assays' readout in otherwise His^- cells, is a function of binding affinity between test proteins (1). Histidine-lacking plates (shown on the right) were incubated for ~2 days at 30°C to detect the growth of His^+ cells. Nt and Ct Y2H-specific domains in Y2H-based protein fusions are marked in red. Row 1, *hs*ATE1 vs. vector alone. Row 2, *hs*UBR1 vs. vector alone. Row 3, *hs*UBR2 vs. *hs*NTAN1. Row 4, *hs*ATE1 vs. vector alone. Row 5, *hs*UBR2$^{1-1040}$ (Nt-fragment of *hs*UBR2) vs. vector alone. Row 6, *hs*ATE1 vs. *hs*UBR2$^{1-1040}$. Row 7, *hs*TRIP12 vs. vector alone. Row 8, *hs*UBR1 vs. vector alone. Row 9, *hs*UBR1 vs. *hs*TRIP12. See *SI Appendix*, Materials and Methods for details.

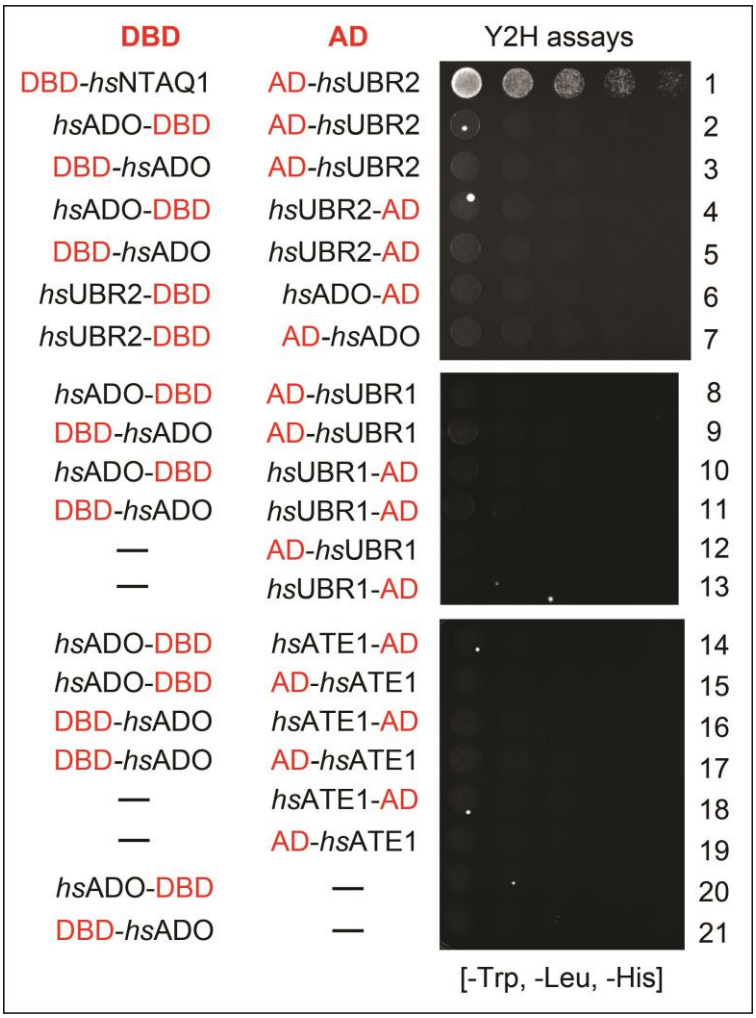

Fig. S5. Y2H binding assays, in wild-type (*UBR1 UFD4*) *S. cerevisiae*, with human proteins. Expression of *HIS3*, the assays' readout in otherwise His^- cells, is a function of binding affinity between test proteins (1). Histidine-lacking plates (the only ones shown here) were incubated for ~2 days at 30°C to detect the growth of His^+ cells. Nt and Ct Y2H-specific domains (AD and DBD) in Y2H-based protein fusions are marked in red. The names of test proteins are cited together with (varying) locations of their DBD or AD domains. Row 1, DBD-*hs*NTAQ1 vs. AD-*hs*UBR2 (positive control). Row 2, *hs*ADO-DBD vs. AD-*hs*UBR2. Row 3, DBD-*hs*ADO vs. AD-*hs*UBR2. Row 4, *hs*ADO-DBD vs. *hs*UBR2-AD. Row 5, DBD-*hs*ADO vs. *hs*UBR2-AD. Row 6, *hs*UBR2-DBD vs. *hs*ADO-AD. Row 7, *hs*UBR2-DBD vs. AD-*hs*ADO. Row 8, *hs*ADO-DBD vs. AD-hsUBR1. Row 9, DBD-*hs*ADO vs. AD-*hs*UBR1. Row 10, *hs*ADO-DBD vs. *hs*UBR1-AD. Row 11, DBD-*hs*ADO vs. *hs*UBR1-AD. Row 12, AD-*hs*UBR1 vs. vector alone. Row 13, *hs*UBR1-AD vs. vector alone. Row 14, *hs*ADO-DBD vs. *hs*ATE1-AD. Row 15, *hs*ADO-DBD vs. AD-*hs*ATE1. Row 16, DBD-*hs*ADO vs. *hs*ATE1-AD. Row 17, DBD-*hs*ADO vs. AD-*hs*ATE1. Row 18, *hs*ATE1-AD vs. vector alone. Row 19, AD-*hs*ATE1 vs. vector alone. Row 20, *hs*ADO-DBD vs. vector alone. Row 21, DBD-*hs*ADO vs. vector alone. See *SI Appendix*, Materials and Methods for details.

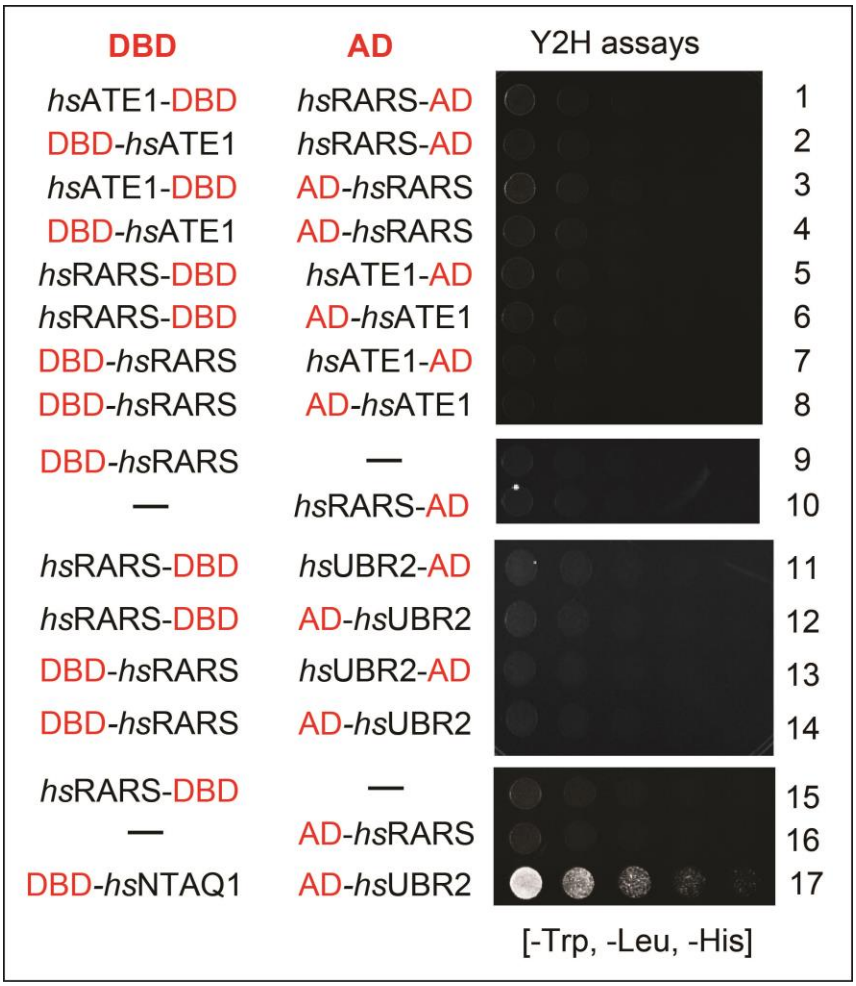

Fig. S6. Y2H binding assays, in wild-type (*UBR1 UFD4*) *S. cerevisiae*, with human proteins. Expression of *HIS3*, the assays' readout in otherwise His^- cells, is a function of binding affinity between test proteins (1). Histidine-lacking plates (the only ones shown here) were incubated for ~2 days at 30°C to detect the growth of His^+ cells. Nt and Ct Y2H-specific domains (AD and DBD) in Y2H-based protein fusions are marked in red. The names of test proteins are cited together with (varying) locations of their DBD or AD domains. Row 1, hsATE1-DBD vs. *hs*RARS-AD. Row 2, DBD-*hs*ATE1 vs. *hs*RARS-AD. Row 3, DBD-hsATE1 vs. AD-*hs*RARS. Row 4, DBD-*hs*ATE1 vs. AD-*hs*RARS. Row 5, *hs*RARS-DBD vs. *hs*ATE1-AD. Row 6, *hs*RARS-DBD vs. AD-*hs*ATE1. Row 7, DBD-*hs*RARS vs. *hs*ATE1-AD. Row 8, DBD-*hs*RARS vs. AD-*hs*ATE1. Row 9, DBD-*hs*RARS vs. vector alone. Row 10, *hs*RARS-AD vs. vector alone. Row 11, *hs*RARS-DBD vs. *hs*UBR2-AD. Row 12, *hs*RARS-DBD vs. AD-*hs*UBR2. Row 13, DBD-*hs*RARS vs. *hs*UBR2-AD. Row 14, DBD-*hs*RARS vs. AD-*hs*UBR2. Row 15, *hs*RARS-DBD vs. vector alone. Row 16, AD-*hs*RARS vs. vector alone. Row 17, DBD-*hs*NTAQ1 vs. AD-*hs*UBR2 (positive control). See *SI Appendix*, Materials and Methods for details.

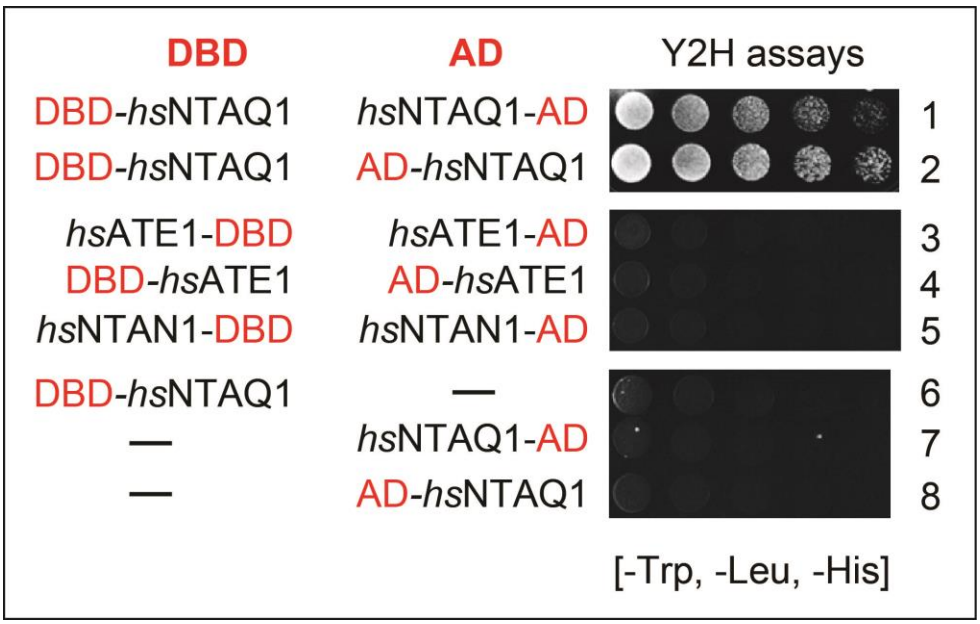

Fig. S7. Y2H binding assays, in wild-type (*UBR1 UFD4*) *S. cerevisiae*, with human proteins. Expression of *HIS3*, the assays' readout in otherwise His^- cells, is a function of binding affinity between test proteins (1). Histidine-lacking plates (shown on the right) were incubated for ~2 days at 30°C to detect the growth of His^+ cells. Nt and Ct Y2H-specific domains in Y2H-based protein fusions are marked in red. Row 1, DBD-*hs*NTAQ1 vs. *hs*NTAQ1-AD. Row 2, DBD-*hs*NTAQ1 vs. AD-*hs*NTAQ1. Row 3, *hs*ATE1-DBD vs. *hs*ATE1-AD. Row 4, DBD-*hs*ATE1 vs. AD-*hs*ATE1. Row 5, *hs*NTAN1-DBD vs. *hs*NTAN1-AD. Row 6, DBD-*hs*NTAQ1 vs. vector alone. Row 7, *hs*NTAQ1-AD vs. vector alone. Row 8, AD-*hs*NTAQ1 vs. vector alone. See *SI Appendix*, Materials and Methods for details.

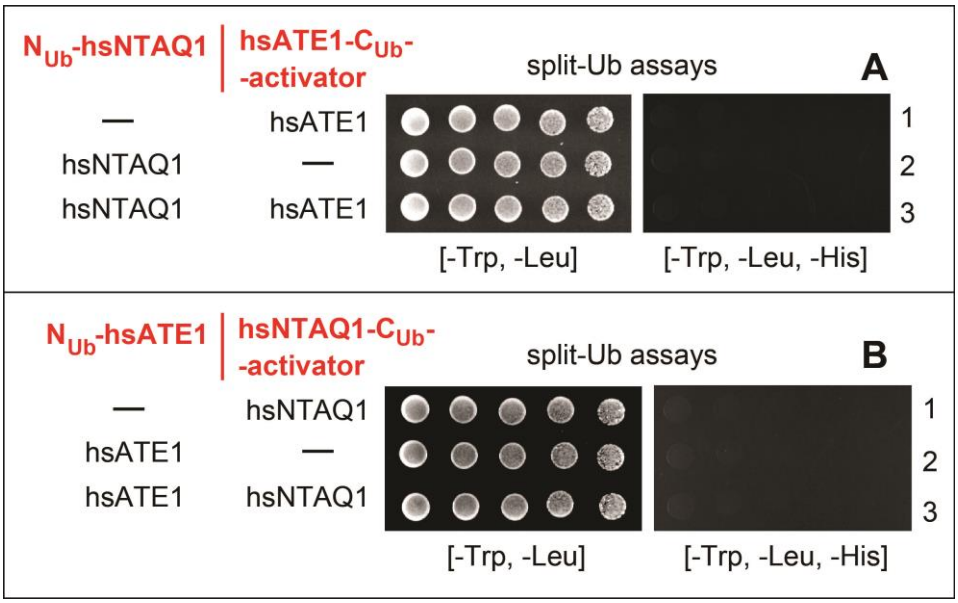

Fig. S8. *S. cerevisiae*-based split-Ub binding assays (2, 3) with components of human Arg/N-degron pathway. In split-Ub, test proteins are expressed as fusions, respectively, to a Ct-half of Ub (C_{Ub}) and to its mutant Nt-half (N_{Ub}). Interactions between test proteins would reconstitute a quasi-native Ub moiety from C_{Ub} and mutant N_{Ub}, causing the cleavage of a C_{Ub}-containing fusion by deubiquitylases downstream from the (reconstituted) Ub moiety. This cleavage acts, through additional steps, as a readout of split-Ub assays (growth or absence of growth of cells in the absence of histidine; panels on the right) (2, 3). (*A*) Row 1, *hs*ATE1-C_{Ub} vs. vector alone. Row 2, N_{Ub}-*hs*NTAQ1 vs. vector alone. Row 3, N_{Ub}-*hs*NTAQ1 vs. hsATE1-C_{Ub}. (*B*) Row 1, , *hs*NTAQ1-C_{Ub} vs. vector alone. Row 2, N_{Ub}-*hs*ATE1 vs. vector alone. Row 3, N_{Ub}-*hs*ATE1 vs. *hs*NTAQ1-C_{Ub}. No interactions between *hs*NTAN1 and *hs*ATE1, and between *hs*NTAQ1 and *hs*ATE1 were detected by split-Ub assays, in agreement with Y2H data (see *Results*).

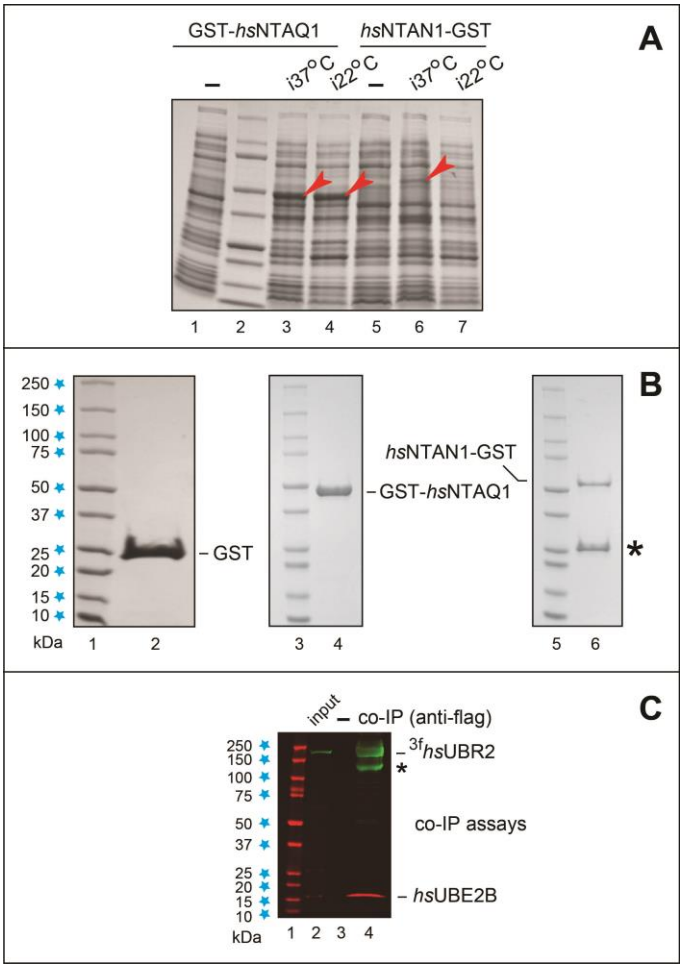

Fig. S9. Expression of GST, GST-*hs*NTAQ1 and *hs*NTAN1-GST in *E. coli* (*A*, *B*) and coimmunoprecipitation of *hs*^{3f}UBR2 E3 and *hs*UBE2B E2 *(C)*. (*A*) Expression of indicated GST-containing proteins in *E. coli*. (*A*) Lane 1, "uninduced" *E. coli* carrying a Lac repressor-regulated plasmid that expressed GST-*hs*NTAQ1. Lane 2, kDa markers. Lane 3, the same as lane 1, but from *E. coli* cells "induced" (denoted by "i") by treating them with IPTG at 37°C. Lane 4, same as lane 3, but IPTG treatment at 22°C. Lanes 5-7, the same as lanes 1, 3, and 4, but with *E. coli* carrying a plasmid expressing *hs*NTAN1-GST. Red arrowheads point to the bands of GST-*hs*NTAQ1 and *hs*NTAN1-GST fusions in a Coomassie-stained gel. (*B*) Lanes 1, 3 and 5, kDa markers. Lanes 2, 4 and 6, purified GST and GST fusions, which were used in GST-pulldown assays (see Fig. 4*D*). The asterisk denoted a GST-containing proteolytic fragment of *hs*NTAN1-GST. (*C*) Coimmunoprecipitation of *hs*^{3f}UBR2 E3 and *hs*UBE2B E2 from *S. cerevisiae* strain stably coexpressing these human proteins. Lane 1, kDa markers. Lane 2, detection by immunoblotting, using anti-flag antibody, of ^{3f}*hs*UBR2 in an input sample of extract from *S. cerevisiae* that coexpressed ^{3f}*hs*UBR2 and *hs*UBE2B. Lanes 3 and 4, anti-flag antibody-lacking (lane 3) and anti-flag-containing beads (lane 4) were used to immunoprecipitate proteins in the extract, followed by SDS-PAGE and immunoblotting with both anti-flag antibody and antibody to (untagged) *hs*UBE2B. Note the band of co-IP-ed *hs*UBE2B in the lane 4 but not in the lane 3. See *SI Appendix*, Materials and Methods for details.

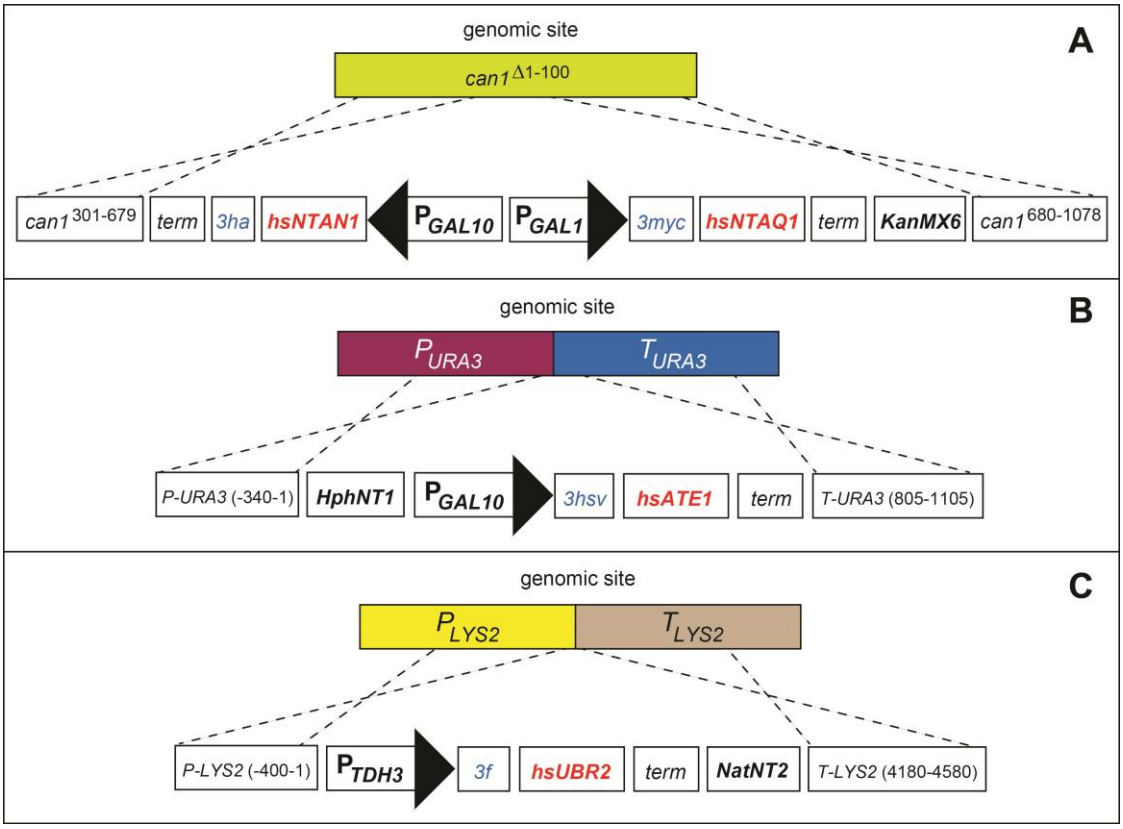

Fig. S10. Construction of *S. cerevisiae* strains that stably express (or coexpress) specific human proteins that mediate substrate targeting in the Arg/N-degron pathway. (*A*) Integrated DNA fragment that expressed ^{3myc}*hs*NTAQ1 Gln/Nt-amidase and *hs*NTAN1^{3ha} Asn/Nt-amidase from the bidirectional $P_{GAL1\text{-}GAL10}$ promoter. (*B*) Integrated DNA fragment that expressed ^{3hsv}*hs*ATE1 R-transferase from the P_{GAL10} promoter. (*C*) Integrated DNA fragment that expressed ^{3f}*hs*UBR2 E3 from the P_{TDH3} promoter. Not shown is an integrated DNA fragment that expressed the untagged *hs*UBE2B E2 from the P_{TDH3} promoter. DNA elements other than the open reading frames (ORFs) of the above proteins, the making of relevant plasmids, and the construction of *S. cerevisiae* strains are described in *SI Appendix*, Materials and Methods.

SI Materials and Methods

Antibodies and Other Reagents

Antibodies to the following antigens were used for immunoblotting: anti-hsv rabbit polyclonal antibody (Sigma, H6030); rabbit polyclonal antibody to human UBE2B (Sigma, HPA003875); anti-flag M2 mouse monoclonal antibody (Sigma, F1804); anti-myc-9E10 mouse monoclonal antibody (Santa Cruz, SC-40); and anti-ha rabbit polyclonal 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). Fluorescence patterns were detected and quantified using Odyssey-9120 (Li-Cor, Lincoln, NE).

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 His-tagged proteins" (Sigma, P8849); phenylmethylsulfonyl fluoride ((PMSF) (Sigma, P7626); DNase I (Roche, 11284932001); glutathione-Sepharose-4B (GE healthcare, 17075601); glutathione magnetic agarose beads (Thermo Fisher, 78601), Dynabeads Protein A (Invitrogen, 10001D); Dynabeads Protein G (Invitrogen, 10003D); 3×FLAG peptide (Sigma, F4799-4MG); Zirconia/silica beads (0.5 mm) (Biospec, 11079105Z).

Yeast Strains, Media, and Genetic Techniques

S. cerevisiae media included YPD (1% yeast extract, 2% peptone, 2% glucose; only most relevant components are cited); YPG (same as YPD but 2% galactose instead of glucose); 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. *S. cerevisiae* strains used in this work are described in Table S1. Standard techniques were used for strain construction and transformation (4, 5). JOY541, and JOY542 (Table S1) were constructed using the PCR-based gene targeting method (6) and pFA6a-*KanMX6* or *HphNT1* modules (7). For constructing *S cerevisiae* strains used in coimmunoprecipitation assays (Fig. 4A, and *SI Appendix*, Fig. S9), the relevant plasmids described in Table S2 were modified to yield DNA fragments that were used for site-specific genomic integration, as described below.

To construct a strain, JOY579 (Table S1), that overexpressed ^{3f}*hs*UBR2, the plasmid pJO1306 (Table S2) was digested with *NotI/SalI.* The resulting (relevant) DNA fragment was purified by agarose gel electrophoresis, and was used to transform, using the lithium acetate method (6), *S. cerevisiae* BY4742 (Table S1), which lacked the *LYS2* open reading frame (ORF) but contained *LYS2* promoter and *LYS2* terminator DNA segments (*SI Appendix*, Fig. S9*C*). Transformants were selected for resistance to nourseothricin, an antibiotic selection marker in the integration cassette (*SI Appendix*, Fig S9 and its legend).

To integrate a *hs*UBE2B-expressing DNA cassette, the *S. cerevisiae* strain JOY579 was used (Table S1). It lacked the *HIS3* ORF but contained *HIS3* promoter and *HIS3* terminator DNA segments. The plasmid pJO1317 (Table S2) was digested with *NotI/SalI*. The plasmid's resulting (relevant) fragment, which contained the *LEU2* selection marker, was transformed into JOY579, with selection for relevant (and verified) transformants on SD(-Leu) plates, yielding the strain JOY580 (Table S1).

To integrate a DNA cassette that could express *hs*NTAN1^{3ha} and ^{3myc}*hs*NTAQ1 from the galactose-inducible $P_{GAL10\text{-}GAL1}$ bidirectional promoter, the plasmid pJO1309 (its construction is

briefly described below) was digested with *NotI/SalI*. The plasmid's resulting (relevant) fragment that expressed the *KanMX6* gene (conferring resistance to the geneticin antibiotic), was transformed into JOY580 (Table S1), with transformants selected for resistance to geneticin, yielding the strain JOY581.

For expression of either h*sNTAN1*^{3ha} or ^{3myc}*NTAQ1*, *NotI/SalI*-digested pJO1368 or pJO1383, were transformed, respectively, into JOY584 (with homologous recombination at the *CAN1* locus), followed by selection for geneticin resistance on YPD plates. These procedures yielded, respectively, the strains JOY589 and JOY590 (Table S1).

To construct *S. cerevisiae* strains that expressed ^{3hsv}*hs*ATE1 (either alone or together with other human proteins), the pJO1330 plasmid (Table S2) was digested with *NotI/SalI*. The plasmid's resulting (relevant) fragment contained, in particular, the *HphNT1* gene, conferring resistance to the antibiotic hygromycin. That DNA fragment was used to transform *S. cerevisiae* JOY581, JOY579, JOY580, or BY4742 (Table S1), which lacked the *URA3* ORF but contained *URA3* promoter and *URA3* terminator DNA segments (*SI Appendix*, Fig. S9*B*). Transformants were selected for resistance to hygromycin. These procedures yielded the strains JOY582, JOY585, JOY586 and JOY584 respectively (Table S1).

Construction of Plasmids

The plasmids used in this study are described in Table S2. To construct plasmids for two-hybrid (Y2H) assays, the full length *S. cerevisiae UFD4*, *ATE1*, and *NTA1* open reading frames (ORFs) were amplified by PCR from *S. cerevisiae* genomic DNA. The full-length human *UBR1*, *ATE1*, *NTAN1*, *NTAQ1*, *TRIP12*, *ADO*, and *RARS* ORFs, as well as DNA encoding the Nt-half of the human *UBR2* ORF (UBR2$^{1\text{-}1140}$) were amplified by PCR from corresponding human cDNAs, which were from MRC PPU (U.K.), GenScript or Dharmacon (DU24040, OHu12263, OHu07250, OHu11849, 40083165, 5295674, OHS1770-202316102, and OHu14207).The resulting DNA fragments were purified by agarose gel electrophoresis, and were cloned into the pDonor/Zeo via BP recombination reactions of the Gateway system (8) that included the Gateway BP clonase II enzyme mix (Invitrogen). The resulting plasmids were pJO788, pJO1228, pJO1229, pJO1156, pJO980, pJO977, pJO1025, pJO1081, pCSJ1047, pJO968, and pJO1367 (Table S2). Thereafter the LR Gateway reaction, using the Gateway LR clonase II enzyme mix (Invitrogen), was carried out with the above plasmids and either pGADCg/pGADT7g for Y2H-based plasmids bearing the *GAL4*-AD domain or pGBKT7g for Y2H-based plasmids bearing the GAL4-DBD domain. The resulting plasmids were pJH011 or pJH012 for *scUFD4*, pJO1232 for *scATE1*, pJO1234 or pJO1235 for *scNTA1*, pJO1168, pJO1169 or pJO1171 for *hsUBR1*, pJO1000, pJO1001 or pJO1002 for *hsATE1*, pJO993 for *hsNTAN1*, pJO1036, pJO1037 or pJO1039 for *hsNTAQ1*, pJO1083 for *hsTRIP12*, pCSJ1048, pCSJ1049, pCSJ1054, and pCSJ1055 for *hsADO*, pCSJ1056, pCSJ1057, pCSJ1058, and pCSJ1059 for *hsRARS*, pJO970 or pJO972 for the full-length *hsUBR2*, and pJO1379 for the N-terminal half of *hsUBR2* (encoding *hs*UBR2$^{1\text{-}1040}$) (Table S2).

For split-Ub assays, the full-length human *UBR2*, *ATE1*, *NTAN1*, and *NTAQ1* ORFs were amplified, by PCR, from the corresponding human cDNAs (obtained from Dharmacon), using appropriate oligonucleotide 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, yielding pJO967, pJO989 or pJO990, pJO978, and pJO1026 or pJO1027, respectively (Table S2).

GST-pulldown assays employed the plasmids pJO1127, pJO1112, and pJO1126 (Table S2), which could express, respectively, GST, GST-*hs*NTAQ1 and *hs*NTAN1-GST in

E. coli. To construct the GST-*hs*NTAQ1-expressing plasmid pJO1112, the *GST* ORF was amplified by PCR from pGEX 4T-1 (GE Healthcare), followed by digestion with *NcoI/NdeI*. Thereafter the PCR-amplified DNA fragment that encoded *hs*NTAQ1 was digested with *NdeI/NotI*. The resulting DNA fragment was ligated into *NcoI/NotI*-cut pHUE1, yielding pJO1112 (Table S2). To construct pJO1126, which could overexpress *hs*NTAN1-GST (Table S2), a DNA fragment encoding *hs*NTAN1 was amplified by PCR from the above *hsNTAN1* cDNA-containing plasmid (Table S2). GST-coding and hsNTAN1-coding DNA fragments were digested, respectively, with *BamHI/NotI* and *NcoI/BamHI* (correct?), followed by their ligation into *NcoI/NotI*-cut pHUE1 (Table S2). To construct pJO1073, a high copy (2μ-based) plasmid that expressed ^{f}hsUBR2 in *S. cerevisiae*, the corresponding (*^{f}hsUBR2*-coding) ORF was produced using PCR from cDNA OHu14207 (see above). The resulting DNA fragment was digested with *XmaI/XhoI* and ligated into *XmaI/XhoI*-cut pRS426-Gal1 (containing the P_{GAL1} promoter), yielding pJO1073 (Table S2).

Plasmids expressing human components of the Arg/N-degron pathway that were used to construct *S. cerevisiae* strains for coimmunoprecipitation (co-IP) assays, contained DNA segments that comprised the following elements: DNA sequences used for integrating linear DNA fragments at specific *S. cerevisiae* genomic loci; a galactose inducible promoter (either the unidirectional P_{GAL1} promoter or the bidirectional $P_{GAL10/GAL1}$ promoter) or the constitutive P_{TDH3} promoter for expression of specific human cDNAs; and either a drug-resistance marker (including genes coding for resistance to geneticin, hygromycin or nourseothricin) or an auxotrophic marker such as *LEU2* for selection of cognate transformants.

To construct pJO1309, which encoded *hs*NTAN1^{3ha} and ^{3myc}*hs*NTAQ1, and expressed them from the bidirectional $P_{GAL1/10}$ promoter (*SI Appendix*, Fig. S9*A*), the following steps were carried out. First, a PCR-amplified, *NotI/SalI*-digested DNA fragment that contained the relevant region of the *CAN1* gene (301-1078 bp), was ligated into *NotI/SalI*-cut pGEM-T-easy (Promega) to yield pJO147 (Table S2). Then a PCR-generated DNA fragment containing the *KanMX6* gene was amplified using primers with multiple restriction enzyme sites (*MfeI*, *FseI*, *XmaI*, *BglII*, *SacII*, *EcoRI* and *MfeI*). That fragment was digested with *MfeI* and ligated into *EcoRI*-cut pJO147 to yield pJO180 (Table S2). Finally, the plasmid pJO1309 was constructed, through sequential ligations, into the above (*FseI/SacII*-cut) pJO180 plasmid, of a PCR-amplified, *FseI/XhoI*-digested DNA fragment that contained the inverted *CYC1* transcriptional terminator; of a PCR-amplified, *AscI/XhoI*-digested DNA fragment that contained an inverted sequence coding for the C-terminal triple ha-tagged hsNTAN1^{3ha}; of a PCR-amplified, *AscI/BamHI*-digested DNA fragment that contained the $P_{GAL1\text{-}GAL10}$ promoter; of a PCR-amplified, *BglII/XmaI*-digested DNA fragment that encoded ^{3myc}hsNTAQ1; and a PCR-amplified, *XmaI/SacII*-digested DNA fragment that contained the *CYC1* terminator. The resulting pJO1309 plasmid was used to construct, as described in the preceding section the *S. cerevisiae* strain JOY581 (Table S1), containing the expression cassette shown in *SI Appendix*, Fig. S9*A*.

pJO1306, which encoded the N-terminally triple flag-tagged ^{3f}*hs*UBR2, was constructed by sequential ligation, into *NotI/SalI*-cut pGEM-T-easy (Promega), of the following DNA fragments: a PCR-amplified, *NotI/FseI*-digested DNA fragment that encompassed 400 bp of the P_{LYS2} promoter for a site-specific integration at that site in the *S. cerevisiae* strain BY4742 that lacked the *LYS2* ORF but contained both *LYS2* promoter and transcriptional terminator (Table S1); a PCR-amplified, *FseI/XmaI*-digested DNA fragment containing the P_{TDH3} promoter; a PCR-amplified, *XmaI/SacII*-digested DNA fragment encoding *^{3f}hsUBR2*; a PCR-amplified,

S*acII/EcoRI*-digested DNA fragment containing the *CYC1* transcriptional terminator; a PCR-amplified, *EcoRI/MfeI*-digested DNA fragment containing the *NATNT2*-encoded positive selection marker (resistance to nourseothricin) (7), and a PCR-amplified, *EcoRI/SalI*-digested DNA fragment containing the *LYS2* transcriptional terminator. The *S. cerevisiae* strain JOY579 (Table S1), which expressed ^{3f}*hs*UBR2 from the P_{TDH3} promoter, was constructed as described in the preceding section.

The pJO1368 plasmid (Table S2), which expressed *hs*NTAN1^{3ha} alone (without ^{3myc}hsNTAQ1), was constructed by digesting pJO1309 with *BamHI/SmaI*, gap-filling by Klenow DNA polymerase, and self-ligation. Similarly, to construct pJO1383 (Table S2) which expressed ^{3myc}hsNTAQ1 (without *hs*NTAN1^{3ha}), pJO1309 was digested with *AscI/XhoI*, followed by gap-filling by the Klenow DNA polymerase, and self-ligation.

The plasmid pJO1387 (Table S2), which expressed *hs*UBE2B, was constructed, through sequential ligations into *NotI/SalI*-cut pGEM-T-easy (Promega), of the following DNA elements: a PCR-amplified, *NotI/FseI*-digested DNA fragment containing DNA sequence of the P_{HIS3} promoter (for integration at that site; see the preceding section about construction of specific *S. cerevisiae* strains); a PCR-amplified, *FseI/BamHI*-digested DNA fragment containing the P_{TDH3} promoter (which directed expression of *hs*UBE2B); a PCR-amplified, *BamHI/XmaI*-digested DNA fragment containing the *hsUBE2B* ORF; a PCR-amplified, *XmaI/HindIII*-digested DNA fragment containing the *CYC1* transcriptional terminator; a PCR-amplified, *HindIII/SacII*-digested DNA fragment encoding the *scLEU2* auxotrophic marker; and a PCR-amplified, *SacII/SalI*-digested DNA fragment containing DNA sequence of the P_{HIS3} terminator (i.e., the second "flanking" DNA region for integration at the *HIS3* ORF-lacking *HIS3* site; see the preceding section about construction of specific *S. cerevisiae* strains).

Construction details for other plasmids are available upon request. All final constructs were verified by DNA sequencing.

Two-Hybrid Assays

Yeast-based two-hybrid (Y2H) binding assays (1) were carried out largely as described previously (8-11). *S. cerevisiae* AH109 or JOY542 (*ubr1Δ::HphNT1, ufd4Δ::KanMX6* in AH109) (Table S1) were cotransformed, using the lithium acetate method (12), with specific Y2H-based plasmids, constructed as described above and cited in Table S2. Y2H-based fusions encoded by these plasmids are described in *Results and Discussion*. "AD" and "DBD" refer to the activation domain and DNA-binding domain, respectively. In both Y2H and split-Ub assays (described below), the expression of *HIS3* (the 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 (2, 3) were carried out largely as described previously (8, 9). *S. cerevisiae* NMY51 (*MAT***a** *trp1 leu2 his3 ade2 LYS2::lexA-HIS3 ade2::lexA-ADE2 URA3::lexA-lacZ*) (Dualsystems Biotech AG, Schlieren, Switzerland) (Table S1) was cotransformed with split-Ub-based bait and prey plasmids (Table S2) using the lithium 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 that lacked Leu and Trp). Single colonies of resulting cotransformants were grown in SC(-Trp, -Leu) liquid medium to A_{600} of ~1.0 (a near-stationary phase). 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) or triple-dropout SC(-Trp,-Leu,-His) plates, which were incubated at 30°C for 2-3 days.

GST Fusions and GST-Pulldown Assays

To overexpress GST as well as a GST fusion (GST-*hs*NTAQ1 and *hs*NTAN1-GST), encoded by the plasmids pJO1127, pJO1112, or pJO1126 (Table S2), one of these plasmids was transformed into *E. coli* CodonPlus (DE3)-RIL (Agilent). Transformants were inoculated into 0.6 l of LB medium containing chloramphenicol (34 µg/ml) and ampicillin (100 µg/ml), followed by incubation at 37°C for ~2 hr, until the culture reached A_{600} of ~0.6. Expression of GST (or a GST fusion) was then induced with isopropyl-D-thiogalactoside (IPTG; 1 mM) at 37°C for 5 hrs. Cells were harvested by centrifugation at 12,000 *g* for 20 min, washed once with phosphate-buffered saline (PBS), and thereafter frozen at -80°C.

To purify GST-*hs*NTAQ1, *hs*NTNA1-GST, or GST, 2-3 grams of *E. coli* cell pellet was resuspended in 40 ml of xTractor buffer (Clontech) containing 1×protease inhibitor cocktail ("for bacterial cells"; Sigma), 40 µl of PMSF, and DNase I (10 µl/ml), 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 glutathione-Sepharose-4B suspension. 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 (Clontech), one wash with wash buffer, and the elution of proteins with 10 ml of TNGT buffer (50mM Tris pH8.0, 100mM NaCl, 10 mM of reduced glutathione, and 1 mM DTT). The resulting protein samples were dialyzed against storage buffer (10% glycerol, 0.15 M NaCl, 5 mM β-mercaptoethanol, 50 mM HEPES, pH 7.5) overnight, then concentrated by approximately 15-fold, using Amicon ultra-15 (Millipore), and stored at -80°C.

GST-pulldown assays were carried out largely as described previously (13), with slight modifications. 5µg of purified GST, GST-*hs*NTAQ1, or *hs*NTNA1-GST were incubated with glutathione/magnetic agarose beads (Pierce) in 0.1 ml of 50 mM NaH_2PO_4/Na_2HPO_4 (pH 8.0) at 4°C. The beads were washed once with 0.5 ml of GST-loading buffer (0.5 M NaCl, 10% glycerol, 1% Nonidet-P40, 1 mM EDTA, 50 mM Tris-HCl, pH 8.0) and once with 0.5 ml of GST-binding buffer (50 mM NaCl, 10% glycerol, 0.05 % Nonidet-P40, 50 mM HEPES, pH 7.8). Purified GST-fusion proteins on magnetic beads, in 0.1 ml of GST-binding buffer containing 1% bovine serum albumin (BSA) were incubated, at 4°C for 1 hr, with 0.2 ml of extract from *S. cerevisiae* that expressed ^{f}hsUbr2. The extract (0.2 ml) was prepared from a 100-ml yeast culture grown to A_{600} of ~1.0. The beads were washed three times with 0.4 ml of binding buffer and beads-bound proteins were eluted by adding 15 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) and stored at -80°C, followed by analysis by SDS-PAGE and immunoblotting (Fig. 4D).

Coimmunoprecipitation (co-IP) Assays

Construction of *S. cerevisiae* strains stably expressing up to 5 human proteins that mediate the targeting by the Arg/N-degron pathway is described above (see also Fig. 4*A* and *SI Appendix*, Fig. S9). For co-IP assays, these yeast strains (Table S1) were grown at 30°C to A_{600} of ~1 in 150 ml of YPG medium. Cells were harvested by centrifugation at 6,000 *g* for 15 min, washed once with cold PBS, and stored at -80°C. Frozen cell pellets were thawed/resuspended in 1.2 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 cell suspension was distributed equally

into three tubes, and cells were disrupted using Mini-Beadbeater-16 (BioSpec; 10 times for 1 min each, with 5-min intervals on ice).

The resulting samples were centrifuged at 11,200*g* for 30 min at 4°C. 15 µl of each supernatant was taken and added to the same volume of 2 × LDS (lithium dodecyl sulphate) buffer (4 × LDS: invitrogen), followed by storage of 30 µl from each sample at -80°C (future "input" samples; see Fig. 4). The rest of each supernatant was mixed with 10 µl of protein A/G (Dynabeads, Invitrogen) sample that had been preequilibrated with lysis buffer, followed by incubation at 4°C for 30 min for preclearing. After separation of protein A/G by DynaMag (ThermoFisher), the resulting supernatants were then incubated with 10 µl of anti-flag-M2 magnetic beads (Sigma) or, alternatively, with 10 µl of protein A/G mixture together with 1.5 µl of anti-hsv rabbit polyclonal antibody (Sigma), with rocking, at 4°C for 1 hr. The beads were collected, and washed three times with 1 ml of lysis buffer, and the bound proteins were eluted by adding 15 µl of elution buffer (lysis buffer containing 150 ng/µl of 3×flag peptide (Sigma)) or HU buffer. The latter buffer was used for elution of proteins bound to anti-hsv-beads. In the case of elution with 3×flag peptides, 5 µl of 4×LDS buffer was added to the eluted sample, followed by heating at 95°C for 3 min. Samples eluted with HU buffer were heated at 70°C for 10 min. Proteins in the resulting samples were fractionated by 4-12% NuPAGE, followed by immunoblotting with antibodies described above.

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
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
SC295	*MAT a ura3-52 leu2-3, 112 reg1-501 gal1 pep4-3*	Lab collection
JOY541	*ubr1*Δ*::HphNT1* in AH109	This study
JOY542	*ufd4*Δ*::KanMX6* in JOY541	This study
JOY579	*lys2*Δ*::TDH3-^{3flag}hsUbr2-NatNT2* in BY4742	This study
JOY580	*his3*Δ*::TDH3-hsUbe2b-Leu2* in JOY579	This study
JOY581	*can1*Δ*::^{3ha}hsNtan1-GAL10/GAL1-^{3myc}hsNtaq1-KanMX6* in JOY580	This study
JOY582	*ura3*Δ*::HphNT1-GAL10-^{3hsv}hsAte1* in JOY581	This study

JOY584	*ura3*Δ*::HphNT1-GAL10-*^{3hsv}*hsAte1* in BY4742	This study
JOY585	*ura3*Δ*::HphNT1-GAL10-*^{3hsv}*hsAte1* in JOY579	This study
JOY586	*ura3*Δ*::HphNT1-GAL10-*^{3hsv}*hsAte1* in JOY580	This study
JOY589	*can1*Δ*::*^{3ha}*hsNtan1-GAL10/ -KanMX6* in JOY584	This study
JOY590	*can1*Δ*::/GAL1-*^{3myc}*hsNtaq1-KanMX6* in JOY584	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
pGADT7g	Y2H expression vector. Contains the P_{ADH1} promoter. Used to produce N-terminal Gal4-AD^{ha} fusion by Gateway cloning.	Addgene
pGBKCg	Y2H expression vector. Contains the P_{ADH1} promoter. Produces C-terminal Gal4-DBD^{myc} fusion by Gateway cloning.	Addgene
pGBKT7g	Y2H expression vector. Contains the P_{ADH1} promoter. Produces N-terminal Gal4-DBD^{myc} fusion by Gateway cloning.	Addgene
pGEM-T easy	Cloning vector	Promega
pGEX 4T-1	GST fusion vector	GE healthcare
pHUE1	Ub fusion vector	(14)
pBS-hADO	hsADO in pBluescriptR	Dharmacon
pDONR223-hRARS	hsRARS in pDONR	Dharmacon

pFA6a-KanMX6	pFA6a-KanMX6	(15)
pFA6a-HphNT1	pFA6a-HphNT1	(7)
pFA6a-NatNT2	pFA6a-NatNT2	(7)
pFA6a-NatNT2	pFA6a-NatNT2	(7)
pRS314 Gal1/10	pRS314 with $P_{GAL1/10}$	(16)
pRS426 Gal1	pRS426 with P_{GAL1} promoter	(16)
pJO147	$CAN1^{301-1078}$ in pGEM-T-easy	This study
pJO180	$CAN1^{301-679}$-KanMX6-$CAN1^{680-1078}$ in pGEM-T-easy	This study
pJO781	attL-scUbr1 in pDONR/Zeo	(9)
pJO788	attL-scUfd4^{flag} in pDONR/Zeo	This study
pJO794	SV40-NLS-scUbr1-Gal4-AD in pGADCg	(9)
pJO919	Gal4-DBD_{myc}-scUbr1 in pGBKT7g	This study
pJO920	Gal4-DBD^{myc}-scUfd4^{flag} in pGBKT7g	This study
pJO967	N_{Ub}-hsUbr2 in pPR3-N	This study
pJO968	attL-hsUbr2 in pDONR/Zeo	
pJO970	SV40-NLS- Gal4-AD- hsUbr2 in pGADT7g	This study
pJO972	Gal4-DBD^{myc}-hsUbr2 in pGBKT7g	This study
pJO977	attL-hsNtan1 in pDONR/Zeo	This study
pJO978	Ost4-hsNtan1-C_{Ub}-LexA-VP16 in pDHB1	
pJO980	attL-hsAte1 in pDONR/Zeo	This study
pJO989	Ost4-hsAte1-C_{Ub}-LexA-VP16 in pDHB1	This study

pJO990	N_{Ub}-hsAte1 in pPR3-N	This study
pJO993	SV40-NLS-hsNtan1-Gal4-AD in pGADCg	This study
pJO1000	SV40-NLS- Gal4-AD- hsAte1 in pGADT7g	This study
pJO1001	hsAte1-Gal4-DBD^{myc} in pGBKCg	This study
pJO1002	Gal4-DBD^{myc}-hsAte1 in pGBKT7g	This study
pJO1025	attL-hsNtaq1 in pDONR/Zeo	This study
pJO1026	Ost4-hsNtaq1-C_{Ub}-LexA-VP16 in pDHB1	This study
pJO1027	N_{Ub}-hsNtaq1 in pPR3-N	This study
pJO1036	SV40-NLS-hsNtaq1-Gal4-AD in pGADCg	This study
pJO1037	SV40-NLS- Gal4-AD- hsNtaq1 in pGADT7g	This study
pJO1039	Gal4-DBD^{myc}-hsNtaq1 in pGBKT7g	This study
pJO1073	P_{GAL1}-^{flag}hsUbr2 in pRS426	This study
pJO1081	attL-hsTrip12 in pDONR/Zeo	This study
pJO1083	SV40-NLS- Gal4-AD- hsTrip12 in pGADT7g	This study
pJO1112	GST-PreScission-hsNtaq1 in pET15b	This study
pJO1126	hsNtan1-PreScission-GST in pET15b	This study
pJO1127	GST in pET15b	This study
pJO1156	attL-hsUbr1 in pDONR/Zeo	This study
pJO1168	SV40-NLS-hsUbr1-Gal4-AD in pGADCg	This study
pJO1169	SV40-NLS- Gal4-AD- hsUbr1in pGADT7g	This study
pJO1171	Gal4-DBD^{myc}-hsUbr1 in pGBKT7g	This study
pJO1228	attL-scAte1 in pDONR/Zeo	This study
pJO1229	attL-scNta1 in pDONR/Zeo	This study
pJO1232	scAte1-Gal4-DBD^{myc} in pGBKCg	This study
pJO1234	SV40-NLS-scNta1-Gal4-AD in pGADCg	This study

pJO1235	SV40-NLS- Gal4-AD-scNta1in pGADT7g	This study
pJO1237	Gal4-DBD^{myc}-scNta1 in pGBKT7g	This study
pJO1238	SV40-NLS- Gal4-AD- scUbr1in pGADT7g	This study
pJO1306	$P_{TDH3}\rightarrow{}^{3flag}$hsUbr2 in pGEM-T easy for targeted integration	This study
pJO1309	^{3ha}hsNtan1$\leftarrow P_{GAL10}$/ $P_{GAL1}\rightarrow{}^{3myc}$hsNtaq1 in pGEM-T easy for targeted integration	This study
pJO1330	$P_{GAL10}\rightarrow{}^{3hsv}$hsAte1 in pGEM-T easy for targeted integration	This study
pJO1367	attL-hsUbr2$^{1-1040}$ in pDONR/Zeo	This study
pJO1368	^{3ha}hsNtan1$\leftarrow P_{GAL10}$ in pGEM-T easy for targeted integration	This study
pJO1379	SV40-NLS-hsUbr2$^{1-1040}$-Gal4-AD in pGADCg	This study
pJO1383	$P_{GAL1}\rightarrow{}^{3myc}$hsNtaq1 in pGEM-T easy for targeted integration	This study
pJO1387	$P_{TDH3}\rightarrow$hsUbe2b in pGEM-T easy for targeted integration	This study
pJH009	scUbr1-Gal4-DBD^{myc} in pGBKCg	This study
pJH010	SV40-NLS- Gal4-AD- scUfd4^{flag} in pGADT7g	This study
pJH011	scUfd4^{flag}-Gal4-DBD^{myc} in pGBKCg	This study
pJH012	SV40-NLS-scUfd4^{flag}-Gal4-AD in pGADCg	This study
pCSJ1047	attL-hsADO in pDONR/Zeo	This study
pCSJ1048	SV40-NLS-hsADO-Gal4-AD in pGADCg	This study
pCSJ1049	SV40-NLS- Gal4-AD- hsADO in pGADT7g	This study
pCSJ1054	hsADO -Gal4-DBD^{myc} in pGBKCg	This study
pCSJ1055	Gal4-DBD^{myc}- hsADO in pGBKT7g	This study
pCSJ1056	SV40-NLS-hsRARS-Gal4-AD in pGADCg	This study

pCSJ1057	SV40-NLS- Gal4-AD- hsRARS in pGADT7g	This study
pCSJ1058	hsRARS -Gal4-DBD^{myc} in pGBKCg	This study
pCSJ1059	Gal4-DBD^{myc}- hsRARS in pGBKT7g	This study

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