

Figure S1, related to Figure 2. Purification of polyubiquitinated species by different F-box Ligase Traps.

Western blot of two-step purifications was performed as previously described and probed with an anti-ubiquitin antibody (P4D1).

Prb1

MKLENTLFTLGALGSISAALVIPNL ENAADHHELINKEDHHERPRKVEFT
KDDDEEPSDSEDKEHGKFHKKGRKG QDKESPEFNGKRASGSHGSAHEGGK
GMKPKHESNDDNDKDKKKPHHKG GCHENKVEEKKMKGKKVKGKKHHEK
TLEKGRHHNRLAPLVSTAQFNDAI SKIIPNRYIIVFKRGAPQEEIDFHK
ENVQQAQLQSVENLSAEDAFFISTK DTSLSTSEAGGIQDSFNIDNLFSGY
IGYFTQEIVDLIRQNPLVDFVERDS IVEATEFDTQNSAPWGLARISHRER
LNLGSFNKYLYDDDAGRGVTSYVID TGVNINHKKDFEKRAIWGKTIPLNDE
DLDGNGHGTHCAGTIASKHYGVAKN ANVVAVKVLRSNGSGTMSDVVKGVE
YAAKAHQKEAQEKKGFKGSTANMS LGGGKSPALDLAVNAAVEVGIHFAV
AAGNENQDACNTSPASADKAITVGA STLSDDRAYFSNWGKCVDFVAPGLN
ILSTYIGSDDATATLSGTSMASPHV AGLLTYFLSLQPGSDSEFFELGQDS
LTPQQLKKKLIHYSTKDILFDIPED TPNVLIYNGGGQDLSAFW~~ND~~TKKSH
SSGFKQELNMDEFIGSKTDLIFDQV RDILDKLNII

Prcl

MKAFTSLCGLGLSTTLAKAISLQR PLGLDKDVLLQAAEKFGLDLDDLHL
LKELDSNVLDAWAQIEHLYPNQVMS LETSTKPKFPEAIKTKKDWFVVK
DAIENYQLRVNKIKDPKILGIDPNV TQYTGyLDVEDEDKHFFFWTFESRN
DPAKDPVILWLNGGPGCSSLTGLFF ELGPSSIGPDLKPIGNPYSWNSNAT
VIFLDQPVNVGFSYSGSSGVSNTVA AGKDVYNFLELFFDQFPEYVNGQD
FHIAGESYAGHYIPVFASEILSHKD RNFNLTSVLIGNGLTDPLTQYNYYE
PMACGEGGEPVLPSEECSAMEDSL ERCLGLIESCYDSQSVWSCVPATII
CNNAQLAPYQRTGRNVYDIRKDCEG GNLCYPTLQDIDDYLNQDYVKEAVG
AEVDHYESC~~N~~FDINRNFLFAGDWMK PYHTAVTDLLNQDLPILVYAGDKDF
ICNWLGNKAWTDVLPWKYDEEFASQ KVRNWTASITDEVAGEVKS~~Y~~KHFTY
LRFVNGGHMVPFDV~~P~~ENALSMVNEW IHGGFSL

Figure S2, related to Figure 6. Peptides from Saf1 Ligase Trap purification.

All Prb1 and Prc1 peptides identified from two separate purifications with the Saf1 Ligase Trap. Peptides are indicated with red lettering. Signal sequence is highlighted in fuchsia, whereas proteolytically removed fragments are marked in teal and gray using the best available mapping data.

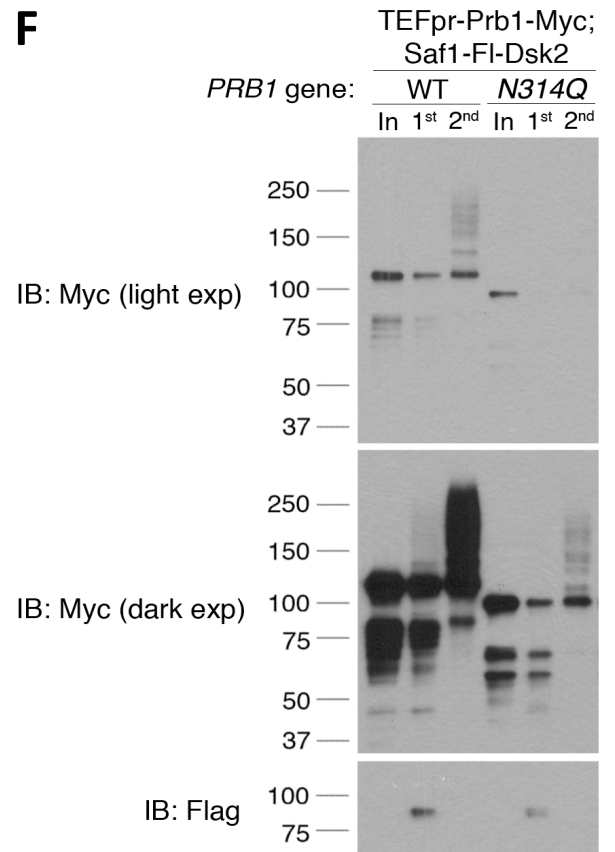
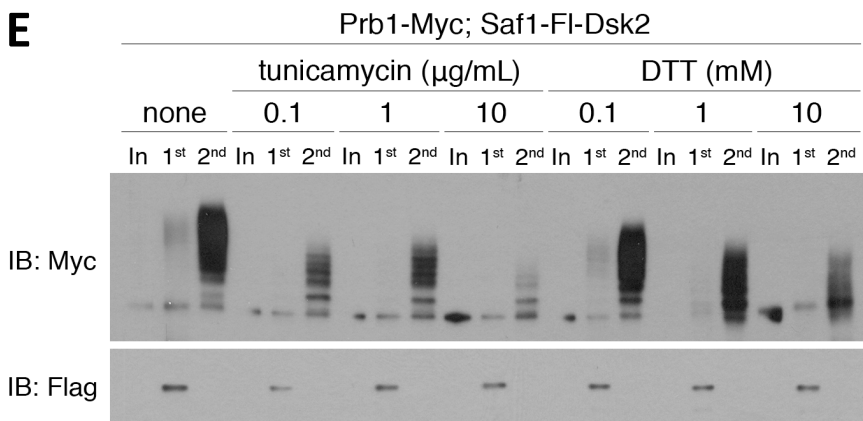
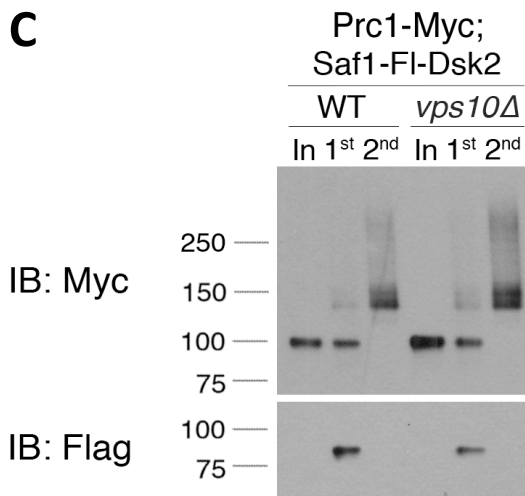
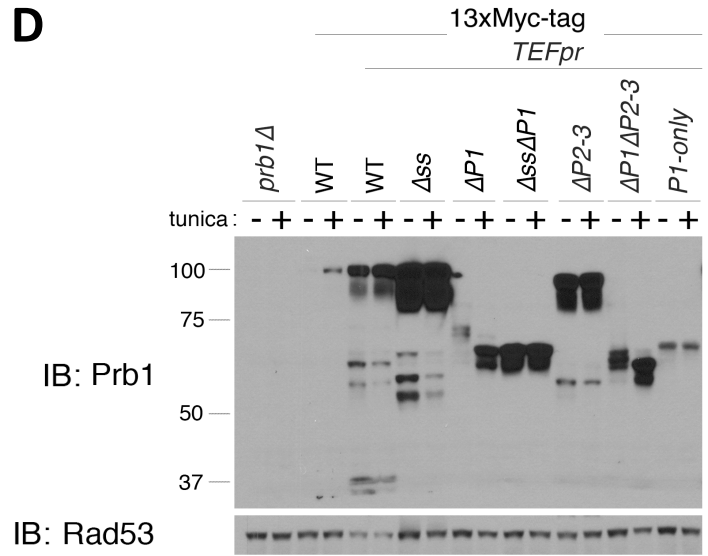
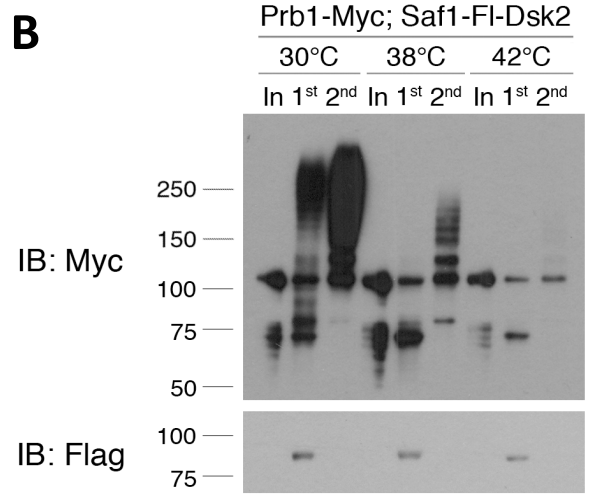
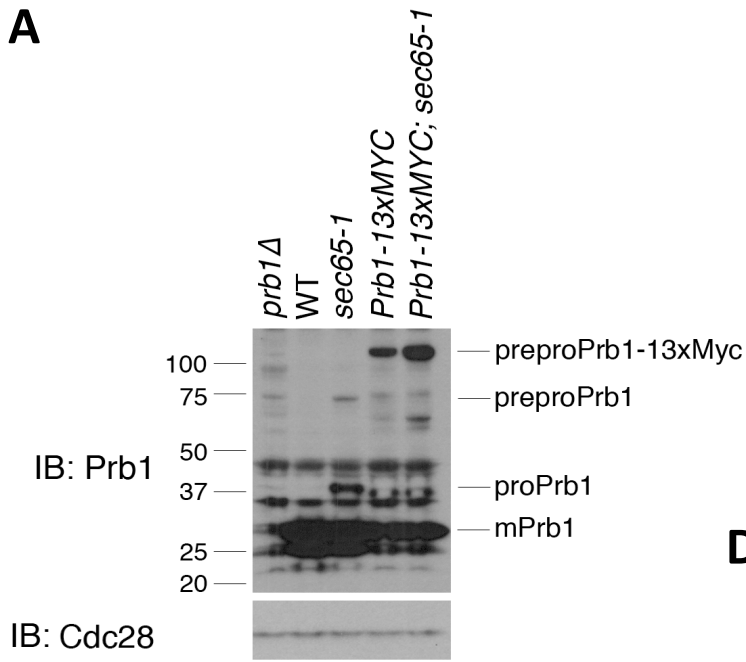


Figure S3, related to Figure 6. Saf1 targets the vacuolar Prb1 precursor.

(A) Whole cell extracts of strains containing *prb1* Δ , wild type, *sec65-1*, Myc-tagged Prb1, or Myc-tagged Prb1 in the *sec65-1* background were examined by probing a Western blot with anti-Prb1 antibody, which recognizes all forms of Prb1. (B) Western blot of two-step purification performed as previously described. Logarithmic cultures were grown at 30°C and shifted to either 38°C or 42°C for 45 minutes before cells were collected. (C) Two-step purification was performed as previously described with wild type and *vps10* Δ strains. (D) Wild type, *prb1* Δ , or cells expressing *PRB1* alleles under the control of the *TEF1* promoter were examined by probing Western blots of whole cell extracts with anti-Prb1 antibody. Where indicated, cells were treated with 1 μ g/ml tunicamycin for 45 minutes. Rad53 (probed with an anti-Rad53 antibody) was used as a loading control. (E) Western blot of two-step purification performed as previously described. Logarithmic cultures were treated as indicated for 45 minutes prior to collection. (F) Two-step purification was performed as previously described with strains expressing either *PRB1* or a mutant *prb1(N314Q)* allele under control of the *TEF1* promoter. Light and dark exposures of the anti-Myc blot are shown for comparison.

Table S1, related to Figure 2. Candidate substrates for 8 F-box proteins.

All Candidate substrates shown that were enriched 25-fold in the Ligase Trap listed. Candidates blocked out in grey meet the spectral count threshold of 1.8 (six for Ufo1). Average spectral counts shown for all (between two and four) purifications. Known substrates in red: Far1 (Henchoz et al., 2007), Ste5 (Garrenton et al., 2009), Swi5 (Kishi et al., 2008), Hst4 (Tang et al., 2005), Hst3 (E. Edenberg, unpublished data), Cln3 (Landry et al., 2012), Plm2 (Tang et al., 2005), Mth1 (Flick et al., 2003; Spielewoy et al., 2004), Cln2 (Barral et al., 1995; Kishi and Yamao, 1998; Li et al., 1997), Gic2 (Jaquenoud et al., 1998; Spielewoy et al., 2004), Cln1 (Barral et al., 1995; Kishi and Yamao, 1998; Li et al., 1997; Skowyra et al., 1999), Pfk27 (Benanti et al., 2007), Tye7 (Benanti et al., 2007), Fzo1 (Fritz et al., 2003; Neutzner and Youle, 2005), Met4 (Kaiser et al., 2000; Rouillon et al., 2000); Aah1 (Escusa et al., 2006), and HO (Kaplun et al., 2006).

Strain	Relevant Genotype	Background	Figure
YKM192	MATa; FARI-13xMYC::NatMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1B
YKM193	MATa; CDC6-13xMYC::NatMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1B
JS Far1-HA	MATa; Far1-3xHA::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1C
MS155_1	MATa; CDC6-13xMYC::URA3; 3xFLAG-CDC4; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1D
MS155_9	MATa; CDC6-13xMYC::URA3; Rad23-3xFLAG-CDC4; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1D
MS155_5	MATa; FARI-13xMYC::URA3; 3xFLAG-CDC4; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1D
MS155_13	MATa; FARI-13xMYC::URA3; Rad23-3xFLAG-CDC4; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1D
MS74 PC2 Grr1-Flag_2	MATa; PFK27-13xMYC::HygMX; CDC6-3xHA::HIS3MX; GRR1-3xFLAG::URA3; ubi4::GAL1pr-6xHis-Ub::NatMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1E
MS50 PC2 GR3	MATa; PFK27-13xMYC::KanMX; CDC6-3xHA::HIS3MX; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::NatMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	1E
MS88 Grr1-FI-Dsk2_1, Gal-6xHisUb	MATa; GRR1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2
MS88_Grr1-FI-Rad23_1, Gal-6xHisUb	MATa; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS50 PC2 CR5	MATa; PFK27-13xMYC::KanMX; CDC6-3xHA::HIS3MX; CDC4-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::NatMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS149-18	MATa; Rad23-3xFLAG-CDC4; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2
MS88_Met30-FI-Dsk2_1, Gal-6xHis-Ub	MATa; MET30-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS104_Met30-FI-Rad23_1, Gal-6xHis-Ub	MATa; MET30-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2
MS88_Mdm30-FI-Dsk2_1, Gal-6xHis-Ub	MATa; MDM30-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS88_Saf1-FI-Dsk2_1, Gal-6xHis-Ub	MATa; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS88_Skp2-FI-Dsk2_1, Gal-6xHis-Ub	MATa; SKP2-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS104_Mfb1-FI-Rad23_1, Gal-6xHis-Ub	MATa; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2
MS88_Mfb1-FI-Dsk2_1, Gal-6xHis-Ub	MATa; MFB1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS160_2	MATa; UFO1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2
MS88_Ufo1-FI-Dsk2_1, Gal-6xHis-Ub	MATa; UFO1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	2, S1
MS135_5	MATa; BUD4-3xFLAG::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A, 3B
MS135_18	MATa; BUD4-3xFLAG::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; his3Δ1; ura3Δ0; leu2Δ0; lys2Δ0; met15Δ0	S288C	3A
MS135_1	MATa; TIS11-3xFLAG::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A, 3B
MS135_13	MATa; TIS11-3xFLAG::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; his3Δ1; ura3Δ0; leu2Δ0; lys2Δ0; met15Δ0	S288C	3A
MS135_7	MATa; YHR131C-3xFLAG::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A, 3B
MS135_19	MATa; YHR131C-3xFLAG-URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; his3Δ1; ura3Δ0; leu2Δ0; lys2Δ0; met15Δ0	S288C	3A
YCAS059	MATa; GAC1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS053	MATa; GAC1-13xMYC::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
MS135_3	MATa; DRE2-3xFLAG::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A, 3B
MS135_15	MATa; DRE2-3xFLAG::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; his3Δ1; ura3Δ0; leu2Δ0; lys2Δ0; met15Δ0	S288C	3A
YCAS057	MATa; MET2-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS051	MATa; MET2-13xMYC::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS055	MATa; YNL144C-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS061	MATa; YNL144C-13xMYC::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS121-1	MATa; FIR1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS123-1	MATa; FIR1-13xMYC::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS011	MATa; SBE2-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
YCAS021	MATa; SBE2-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A
MS135_11	MATa; SFG1-3xFLAG::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	3A, 3B
MS135_23	MATa; SFG1-3xFLAG::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; his3Δ1; ura3Δ0; leu2Δ0; lys2Δ0; met15Δ0	S288C	3A
YCAS012	MATa; YKR045C-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4A
YCAS022	MATa; YKR045C-13xMYC::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4A
MS94_2 (GR2_4)	MATa; BUD4-13xMYC::KanMX; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS137_1	MATa; BUD4-13xMYC::KanMX; MFB1-3xFLAG-Rad23-URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS146_13	MATa; BUD4-13xMYC::KanMX; UFO1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
MS94_6 (Gr7_10)	MATa; SFG1-13xMYC::KanMX; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS145_1	MATa; SFG1-13xMYC::KanMX; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
MS146_9	MATa; SFG1-13xMYC::KanMX; UFO1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
MS94_7 (Ub8_8)	MATa; TIS11-13xMYC::KanMX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS138_1	MATa; TIS11-13xMYC::KanMX; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS146_18	MATa; TIS11-13xMYC::KanMX; UFO1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS142_15	MATa; YHR131C-13xMYC::KanMX; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
MS142_9	MATa; YHR131C-13xMYC::KanMX; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
MS142_10	MATa; YHR131C-13xMYC::KanMX; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; lys2Δ0	S288C	4B
MS142_5	MATa; DRE2-13xMYC::KanMX; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
MS142_1	MATa; DRE2-13xMYC::KanMX; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4B
MS146_29	MATa; DRE2-13xMYC::KanMX; UFO1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0; lys2Δ0	S288C	4B
YCAS094-1	MATa; SBE2-13xMYC::URA3; GRR1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	4B
YKM241	MATa; SBE2-13xMYC::URA3; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	4B
YKM562	MATa; BUD4-13xMYC::KanMX +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM563	MATa; DRE2-13xMYC::KanMX +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM564	MATa; FIR1-13xMYC::LEU2 +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM565	MATa; GAC1-13xMYC::LEU2 +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM566	MATa; SBE2-13xMYC::LEU2 +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM567	MATa; SFG1-13xMYC::KanMX +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM568	MATa; TIS11-13xMYC::KanMX +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM569	MATa; YHR131C-13xMYC::KanMX +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM570	MATa; YKR045C-13xMYC::LEU2 +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM571	MATa; YNL144C-13xMYC::LEU2 +pRS426; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM537	MATa; BUD4-13xMYC::KanMX +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM555	MATa; DRE2-13xMYC::KanMX +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C

YKM560	MATa; FIR1-13xMYC::LEU2 +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM539	MATa; GAC1-13xMYC::LEU2 +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM545	MATa; SBE2-13xMYC::LEU2 +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM547	MATa; SFG1-13xMYC::KanMX +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM549	MATa; TIS11-13xMYC::KanMX +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM561	MATa; YHR045C-13xMYC::KanMX +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM551	MATa; YKR045C-13xMYC::LEU2 +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YKM553	MATa; YNL144C-13xMYC::LEU2 +pYES2[GAL1pr-GRR1dF-Flag::URA3]; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	4C
YCAS173-1	MATa; SWI1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS174-1	MATa; SWI1-13xMYC::URA3; cdc4-1::HygMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS169-1	MATa; ATC1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS170-1	MATa; ATC1-13xMYC::URA3; cdc4-1::HygMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS175-2	MATa; ISR1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS176-2	MATa; ISR1-13xMYC::URA3; cdc4-1::HygMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS068-1	MATa; RAV2-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS113-1	MATa; RAV2-13xMYC::URA3; cdc4-1::HygMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS159-1	MATa; PCL1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS160-1	MATa; PCL1-13xMYC::URA3; cdc4-1::HygMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS161-1	MATa; PCL1-13xMYC::URA3; grr1::NLS-RFP-LEU2; rgt1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YCAS163-1	MATa; PCL1-13xMYC::URA3; cdc4-1::HygMX; grr1::NLS-RFP-LEU2; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5A
YKM233-1	MATa; ATC1-13xMYC::URA3; GRR1-3xFLAG-Rad23::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	5B
YKM234-2	MATa; ISR1-13xMYC::URA3; GRR1-3xFLAG-Rad23::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	5B
YKM235-2	MATa; PCL1-13xMYC::URA3; GRR1-3xFLAG-Rad23::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	5B
YKM236-2	MATa; SWI1-13xMYC::URA3; GRR1-3xFLAG-Rad23::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	5B
YKM238	MATa; ATC1-13xMYC::URA3; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	5B
YKM239-2	MATa; ISR1-13xMYC::URA3; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5B
YKM242-2	MATa; PCL1-13xMYC::URA3; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5B
YKM243-2	MATa; SWI1-13xMYC::URA3; MFB1-3xFLAG-Rad23::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0	S288C	5B
YCAS035	MATa; RBG1-13xMYC::URA3; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5C
YCAS045	MATa; RBG1-13xMYC::URA3; ufo1::KanMX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5C
MS164_3	MATa; RBG1-13xMYC::URA3; GRR1-3xFLAG-Dsk2::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5D
MS164_5	MATa; RBG1-13xMYC::URA3; UFO1-3xFLAG-Rad23::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5D
MS164_7	MATa; RBG1-13xMYC::URA3; GRR1-3xFLAG-Rad23::LEU2MX; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	5D
YCAS075-1	MATa; PRB1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	6B
YCAS088-1	MATa; PRB1-13xMYC-URA3; GRR1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	6B
MS192_1	MATa; PRC1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	6B
MS192_3	MATa; PRC1-13xMYC::URA3; GRR1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	6B
YCAS076-1	MATa; YBR139W-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	6B
YCAS083-1	MATa; YBR139W-13xMYC::URA3; GRR1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ura3Δ0; leu2Δ0; his3Δ0; met15Δ0	S288C	6B
YKM253-1	MATa; PRB1-13xMYC::LEU2; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6C, 6E, S3B, S3E
YKM312	MATa; cdc5-1; PRB1-13xMYC::LEU2; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1, psi+	W303	6C
YKM313-1	MATa; cdc34-2; PRB1-13xMYC::LEU2; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1, psi+	W303	6C
YKM226	MATa; Galpr-PRB1::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6D
YKM228	MATa; Galpr-PRB1::URA3; saf1::KanMX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6D
YKM253-2	MATa; PRB1-13xMYC::LEU2; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6E
YKM254	MATa; PRB1-13xMYC::LEU2; vam3::KanMX; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6E
YKM255-2	MATa; PRB1-13xMYC::LEU2; sec65-1; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6E
YKM506-1	MATa; sec7-1; PRB1-13xMYC::LEU2; SAF1-3xFLAG-UBADsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6E
YKM507-1	MATa; sec23-1; PRB1-13xMYC::LEU2; SAF1-3xFLAG-UBADsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6E
YKM293-1	MATa; TEfpr-PRB1::HygMX; PRB1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F, S3F
YKM294-1	MATa; TEfpr-(Δ2-18)PRB1::HygMX; PRB1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F
YKM295-1	MATa; TEfpr-(Δ19-280)PRB1::HygMX; PRB1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F
YKM296-1	MATa; TEfpr-(Δ2-280)PRB1::HygMX; PRB1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F
YKM379-1	MATa; TEfpr-PRB1::HygMX; PRB1(Δ575-635)-13xMYC::URA3; SAF1-3xFLAG-UBADsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F
YKM378-1	MATa; TEfpr-(Δ19-280)PRB1::HygMX; PRB1(Δ575-635)-13xMYC::URA3; SAF1-3xFLAG-UBADsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F
YKM380-1	MATa; TEfpr-PRB1::HygMX; PRB1(1-280)-13xMYC::URA3; SAF1-3xFLAG-UBADsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6F
YKM303-2	MATa; PRC1-13xMYC::URA3; SAF1-3xFLAG-Dsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6G, S3C
YKM318-2	MATa; CPY*-13xMYC::URA3; SAF1-3xFLAG-Dsk2::URA3; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	6G
ADR21	MATa; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3A
MS179-1	MATa; sec65-1; ade2-1; his3-11,15; leu2-3,112; trp1-1; ura3-53	W303	S3A
YKM210	MATa; prb1::KanMX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3A, S3D
YKM201	MATa; PRB1-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3A, S3D
MS191-3	MATa; PRB1-13xMYC::URA3; sec65-1; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3A
YKM475-1	MATa; vps10::KanMX; PRC1-13xMYC::URA3; SAF1-3xFLAG-UBADsk2::LEU2; ubi4::GAL1pr-6xHis-Ub::HIS3MX; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3C
YKM275	MATa; TEfpr-PRB1::HygMX; PRB1-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D
YKM276	MATa; TEfpr-(Δ2-18)PRB1::HygMX; PRB1-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D
YKM288-1	MATa; TEfpr-(Δ19-280)PRB1::HygMX; PRB1-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D
YKM289-1	MATa; TEfpr-(Δ2-280)PRB1::HygMX; PRB1-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D
YKM307-1	MATa; TEfpr-(Δ19-280)PRB1::HygMX; PRB1(Δ575-635)-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D
YKM345-1	MATa; TEfpr-PRB1::HygMX; PRB1(Δ575-635)-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D
YKM346-1	MATa; TEfpr-PRB1::HygMX; PRB1(1-280)-13xMYC::URA3; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1	W303	S3D

Table S2, related to Figures 1-6. List of strains used in this study.

Supplemental Experimental Procedures

Plasmids

To perform one-step purification, as shown in Figure 2B, FI-Cdc4, Rad23-FI-Cdc4 and Rad23-FI-R467A were expressed using the ARS/CEN plasmid pRS316. A DNA sequence, which encodes three copies in tandem of the Flag epitope (3xFlag), was cloned downstream the *GAL1* promoter in pRS316. The CDC4 gene was amplified from genomic DNA and fused to the 3' end of 3xFlag sequence to generate the fusion sequence 3xFlag-CDC4 under *GAL1* promoter control (Plasmid pMS1). To express Rad23-FI-Cdc4, the 3' terminal sequence of RAD23 gene, encoding the two C-terminal UBA domains (codons 143-397), was amplified by genomic DNA and cloned into pMS1 downstream *GAL1* promoter, and upstream 3xFlag-CDC4 generating the fusion sequence RAD23-3xFlag-CDC4 (Plasmid pMS2). Quick-change site-directed mutagenesis kit (Stratagene) was used to mutate the codon 467 of CDC4 in pMS2 to encode Alanine instead of the Arginine⁴⁶⁷ of WT Cdc4 (Plasmid pMS3). DSK2-3xFlag-CDC4 DNA fusion sequence was obtained by exchanging the sequence encoding the two UBAs of Rad23 in pMS2 with the sequence encoding the single C-terminal UBA domain of Dsk2 (codons 327-373;Plasmid pMS4).

All two-step purifications were performed expressing F-box protein-UBA fusions under the endogenous promoter at the genomic loci. Genes encoding F-box proteins were fused to UBAs at genomic loci using the integration plasmid pRS306, which lacks ARS/CEN sequence elements. N-terminal Flag-tagging of Cdc4 at the endogenous locus was obtained cloning the following DNA sequences into pRS306 in this order: Cdc4 promoter sequence (946 bps upstream the ATG of CDC4), 3xFlag, and the partial sequence of the CDC4 containing the first 583 bps (Plasmid pMS5). Plasmid pMS5 was cut using a unique restriction site in the Cdc4 partial sequence and used to transform strains that were selected for the URA⁺ phenotype. Correct integration was confirmed by PCR and Western blotting. The expression of Rad23-FI-Cdc4 fusion from the endogenous locus was obtained through the same procedure used for FI-Cdc4, but this time the integration vector pRS306 contained the following DNA

sequences in this order: Cdc4 promoter, sequence encoding the two UBAs of Rad23 (codons 143-397), 3xFlag and CDC4 partial sequence (Plasmid pMS6). Except for Cdc4 all the other F-box proteins were tagged at the carboxy-terminus by cloning a partial sequence of F-box protein genes into the integration plasmid pRS306 in frame with 3xFlag, 3xFlag-RAD23 or 3xFlag-DSK2 sequences followed by the stop codon TAG. The integration was accomplished as described above by cutting the plasmids using a unique restriction site in the F-box protein partial sequence, transforming strains and selecting for URA⁺ phenotypes. The same technique was used to tag both known and candidate substrates cloning their 3' terminal partial sequences into pRS306 fused to 3xFlag or 13xMYC sequences.

To overexpress N-terminally 6xHis-tagged ubiquitin, HIS3MX-GAL1-6xHIS cassette was amplified by PCR performed using plasmid pFA6a-His3MX-PGAL1 and primers containing sequence homology to target the cassette to the genomic locus UBI4, which contains five ubiquitin sequences in tandem. Strains were transformed and selected for HIS3⁺ phenotype. Colonies were checked by PCR for cassette integration that resulted in a single copy of the ubiquitin (targeting the last ubiquitin sequence of the UBI4 locus) tagged with 6xHis under the *GAL1* promoter.

For Flag pull-down assays, the pYES2-GRR1dF-FLAG-URA3 expression vector was generated by subcloning out the N-terminal GST epitope tag from pYES2-GST-GRR1dF-FLAG-URA3 (Benanti et al., 2007) and replacing it with a start codon.

Western blotting

To examine the intracellular protein levels, an equivalent of five OD₆₀₀ were harvested and lysed. Pellets were washed in cold water and resuspended in 200 µl pre-heated SDS buffer (50 mM Tris pH 7.5, 5 mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, 0.05% bromophenol blue, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM benzamidine, 17 µg/ml PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate).

Resuspension was then incubated for 5 minutes at 95°C and homogenized for 3 minutes in a Mini BeadBeater (Biospec) using 100 µl glass beads. Samples were then clarified by centrifugation at 16,000 x g for 15 minutes. Extracts were analyzed by SDS-PAGE, followed by transfer to nitrocellulose membranes, and Western blotting with antibodies against Myc (Clone 9E10, Covance), Cdc28 (sc-6709, Santa Cruz Biotechnology), Clb2 (sc-9071, Santa Cruz Biotechnology), Flag (Clone M2, Sigma-Aldrich), HA (Clone 12CA5, Harlan Bioproducts), Ubiquitin (Clone P4D1, gift from E. Wayner), Rad53 (Rabbit anti-Rad53, DAB001, gift from D. Durocher) and Prb1 (Rabbit anti-Prb1, (Moehle et al., 1989)).

Sample preparation and mass spectrometry analysis

The purified complexes were reduced by incubation with TCEP (Thermo) at a final concentration of 10 mM at RT for 45 minutes. The produced free thiols were alkylated with 20 mM iodoacetamide (Sigma) at room temperature for 45 min in the dark and digested with sequencing grade-modified trypsin in 1:50 ratio w/w (Promega, Madison, Wisconsin) overnight at 37°C.

Peptides were desalted on a C18 Sep-Pak cartridge according to manufacture instructions (Waters, Milford, Massachusetts) and the eluted peptides were dried in SpeedVac and successively reconstitute in 2% AcN, 0.1% FA and analyzed by LC-MS/MS.

Each peptide sample was analyzed on an Eksigent Nano LC system (Eksigent Technologies) connected to a hybrid linear ion trap LTQ Orbitrap XL (Thermo Scientific), which was equipped with a nanoelectrospray ion source (Thermo Scientific). Peptide separation was carried out on a RP-HPLC column (75 µm inner diameter and 10 cm length) packed in-house with C18 resin (ReproSil-Pur 120 C18-AQ, 3 µm, Dr. Maisch GmbH) and the peptides were eluted from the analytical column with a 60-min gradient ranging from 7% to 35% solvent B followed by a 5-min gradient from 35% to 80% solvent B at a constant flow rate of 300 nl/minute. The solvent for liquid chromatography composition

was 0.1% formic acid in water (98%) and acetonitrile (2%), and solvent B consisted of 0.1% formic acid in acetonitrile (98%) and water (2%).

The data acquisition mode was set to acquire 1 high resolution MS scan in the ICR cell followed by 5 collision induced dissociation MS/MS scans in the linear ion trap. For high resolution MS scan, 10^6 ions were accumulated over a maximum time of 500 ms and the FWHM resolution was set to 60 000 (at m/z 300). Only MS signals exceeding 500 ion counts triggered a MS/MS attempt and 10^4 ions were acquired for a MS/MS scan over a maximum time of 200 ms. The normalized collision energy was set to 35. Singly charged ions were excluded from triggering MS/MS scans.

Raw data files from the MS instruments were converted with ReAdW into mzXML files and mzXML files were searched with Sorcerer-SEQUEST against a concatenated protein yeast SGD database (Version 20110203), the reversed sequences of all proteins. Statistical analysis of each search result for each LC-MS analysis was performed using the Trans-Proteomic Pipeline TPP TPP v4.5 RAPTURE rev 1, Build 201201161611 including PeptideProphet and ProteinProphet. The ProteinProphet probability score was set to 0.9, which resulted in an average protein false discovery rate of less than 1% for all search results estimated by ProteinProphet. The adjusted spectral counts were calculated using the software ABACUS with default settings.

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