Cope *et al*. 1075901
Supplementary Material

Supplemental Materials and Methods:

The following yeast strains were used in this study:

Strain	Genotype	Source
RDY 385	MAT a, *cdc4-1*, his7, ura1	(*S1*)
RDY 597	MAT a, *cdc34-2*, *his3Δ*, *ura3-52*, GAL+	(*S2*)
RDY 690	MAT a, ura3, trp1, ade2, *cdc53-1*	(*S3*)
RDY 1377	MAT a, *can1-100, ade2-1, leu2-3,-112, trp1-1, ura3-1, skp1-12*	(*S4*)
RDY 1716	MAT a, ubc12::KanMX, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	(*S5*)
RDY 1721	MAT a, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	(*S5*)
RDY 1735	MAT a, rri1::KANMX, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*	(*S5*)
RDY 1832	MAT α, RRI1::KanMX, *can1-100, leu2-3,-112, his3-11,-15, trp1-1 ,ura3-1, ade2-1*	This study
RDY 1835	MAT a, rri1::KanMX, *can1-100, leu2-3,-112, his3-11,-15, trp1-1, Ura3-1, ade2-1*, pep4::TRP1, bar1::LEU2, skp1::HIS3, SKP1::Myc9	This study
RDY 1914	MAT a, rri1::KanMX, ura3, trp1, ade2, *cdc53-1*	This study
RDY 1915	MAT a, rri1::KanMX, *cdc34-2*, *his3Δ*, *ura3-52*, GAL+	This study
RDY 1916	MAT a, rri1::KanMX, *cdc4-1*, his7, ura1	This study
RDY 1917	MAT a, rri1::KanMX, *can1-100, ade2-1, leu2-3,-112, trp1-1, ura3-1, skp1-12*	This study
RDY 2096	MAT a, pci8::KanMX, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	(*S5*)
RDY 2097	MAT a, YOL117W::KanMX, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0	(*S5*)

The following *S. pombe* strains were used in this study: csn5Δ (RDY 1712)(*S6*); Pcu1^{myc13} (RDY 1568)(*S7*); *csn5Δ*, Csn1^{myc13} (RDY 2092)(*S8*); *csn5Δ*, Csn2^{myc13} (RDY 2093)(*S8*).

The following Plasmids were used in this study:

Plasmid	RDB number
pREP41	1549
pREP41-FLAG-Csn5	1469
pREP41-FLAG-Csn5 (H118A)	1487
pREP41-FLAG-Csn5 (H120A)	1488
pREP41-FLAG-Csn5 (D131N)	1489
pEG(KT)	1572
pEG(KT)-Rri1	1471
pEG(KT)-Rri1 (H179A)	1478
pEG(KT)-Rri1 (H181A)	1491
pEG(KT)-Rri1 (D192A)	1494

Fission Yeast Extracts: Cells were grown to mid-log phase in complete media (YES), pelleted by centrifugation, and washed in ice cold STOP buffer (50mM NaF, 25mM TRIS pH7.2, 0.02% Sodium Azide). Cells were resuspended in an equal volume of lysis buffer (25mM TRIS pH 7.2, 150mM NaCl, 0.3% Triton X-100, 50mM NaF, 1mM EDTA, 1mM DTT, 1mM PMSF, and 1X protease inhibitor cocktail (5ug/mL of Aprotinin, Chymostatin, and Pepstatin A, 1ug/mL Leupeptin)). An equal volume of Glass Beads (average 500 microns, Sigma) was added and cells were vortexed for ten invervals of 40 seconds at max (cell suspension was incubated on ice for one minute between each vortex interval). Lysates were cleared by centrifugation (12Krpm).

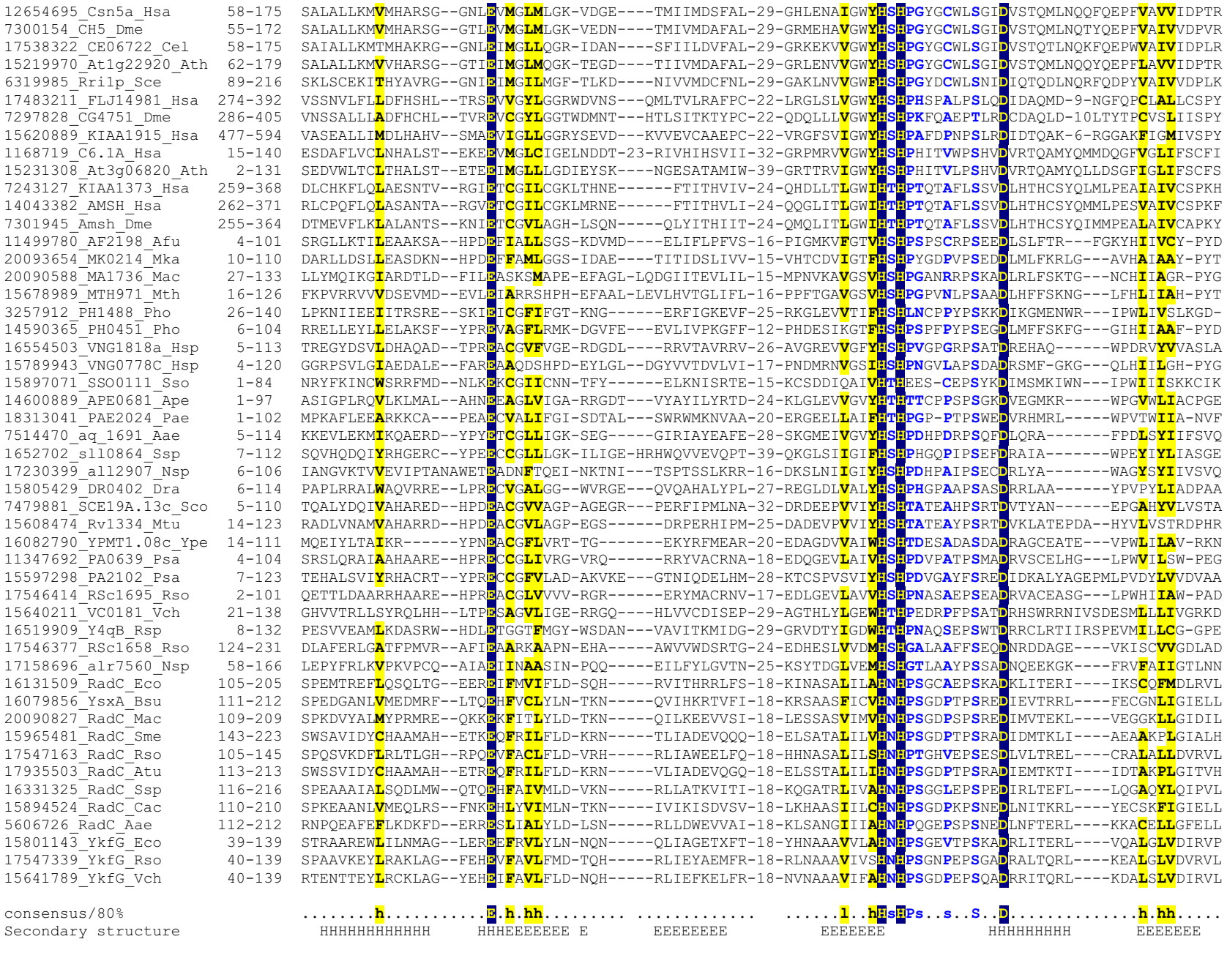
***S. cerevisiae* native extracts and Cul1^{nedd8}/Cdc53^{Nedd8} conjugate purification**: Cells were grown to mid-log phase in complete media (YPD) and processed exactly as described above for S. pombe lysates. For deneddylation assays, clarified Skp1^{myc9} *rri1*Δ lysates were incubated 1 hour in the presence of protein A beads conjugated to α-myc (9E10) antibody. Beads were washed 3 times in buffer A (20mM TRIS (7.2), 150mM NaCl, and 0.3% Triton X-100) and 10uL of beads were used per reaction. psCSN was added to the beads and volume was brought to 10μL with Buffer A. Beads were incubated for 60 minutes at 30ºC.

***S. cerevisiae* denatured extract**: Cells were manipulated as described in the text, pelleted by centrifugation, and washed in ice cold STOP buffer. Cell pellets were frozen in liquid nitrogen (LN_2) and stored at –80ºC. Pellets were thawed and an equal volume of SDS lysis buffer (25mM TRIS pH 7.5, 1% SDS, 1mM EDTA, 1mM PMSF, and 1mM DTT) and Glass beads (average 500 microns, Sigma) were added. Cells were briefly vortexed (2 seconds) and than boiled for three minutes. Cells were vortexed 3 minutes, boiled 2 minutes, than vortexed 2 minutes. Lysates were cleared by centrifugation (12 Krpm) in a microfuge.

***Drosophila* Larvae Extracts**: Third instar larvae were frozen in LN_2 and ground to a powder with a pestle. 20μL of lysis buffer (25mM TRIS (7.2), 150mM NaCl, 5mM DTT, 1mM PMSF, and 2mM $MgCl_2$) was added and mixed briefly. Extracts were cleared by centrifugation and seperated by SDS-PAGE followed by western blot analysis.

***Drosophila* Heat Shock Rescue:** Heat shock rescue of lethality was performed by mating w;*CSN5*^{N}/TM6C to w;P[w+,hs-*CSN5*] line 2/+; *Df(3R)RK6-3*/TM6C. The transgene was followed by eye color. While the cross was maintained at 25°C, heat shocks were at 37°C for 30 min every 12 hr. Rescue of lethality was assessed by comparing white eye and red/orange eye progeny of each genotype raised with and without heat shock. The genotypes of adult progeny with red/orange eye color (with the transgene) were as follows: (with heat shock) *CSN5*^{N}/TM6C, 35 (36.8%); *Df(3R)RK6-3*/TM6C, 31 (32.6%); *CSN5*^{N}/*Df(3R)RK6-3*, 29 (30.5%); and (without heat shock) *CSN5*^{N}/TM6C, 42(51.2%); *Df(3R)RK6-3*/TM6C, 40 (48.8 %); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%). The genotype of progeny with white eye color (without the transgene) were as follows: (with heat shock) *CSN5*^{N}/TM6C, 27 (49.1%); *Df(3R)RK6-3*/TM6C, 28 (50.9%); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%); and (without heat shock) *CSN5*^{N}/TM6C, 43 (55.1%); *Df(3R)RK6-3*/TM6C, 35 (44.9%); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%).

Similarly, heat shock rescue experiment was carried out with hs-*CSN5* (D148N). w;*CSN5*^{N}/TM6C was crossed to w;P[w+, hs-*CSN5* (D148N)] line 2/+; *Df(3R)RK6-3*/TM6C. The transgene was followed by eye color as described above and the condition of heat shocks was same as described above The genotypes of adult progeny with red/orange eye color (with the transgene) were as follows: (with heat shock) *CSN5*^{N}/TM6C, 65 (53.7%); *Df(3R)RK6-3*/TM6C, 56(46.3%); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%); and (without heat shock) *CSN5*^{N}/TM6C, 39(52.0%); *Df(3R)RK6-3*/TM6C, 36 (48.0%); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%). The genotype of progeny with white eye color (without the transgene) were as follows: (with heat shock) *CSN5*^{N}/TM6C, 61 (47.7%); *Df(3R)RK6-3*/TM6C, 67 (52.3%); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%); and (without heat shock) *CSN5*^{N}/TM6C, 37 (51.4%); *Df(3R)RK6-3*/TM6C, 35 (48.6%); *CSN5*^{N}/*Df(3R)RK6-3*, 0 (0%).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17158696_alr7560_Nsp 58-166
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16079856_YsxA_Bsu 111-212
20090827_RadC_Mac 109-209
15965481_RadC_Sme 143-223
17547163_RadC_Rso 105-145
17935503_RadC_Atu 113-213
16331325_RadC_Ssp 116-216
15894524_RadC_Cac 110-210
5606726_RadC_Aae 112-212
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15641789_YkfG_Vch 40-139
consensus/80%
Secondary structure
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HHHEEEEEEE E
EEEEEEEE
EEEEEEE
HHHHHHHH
EEEEEEE

Figure S1: Alignment of predicted active JAMM domains.

The multiple alignment was constructed using the T-Coffee (*S9*) program and modified manually to ensure the correct superposition of the conserved motifs. The alignment includes all detected human JAMM proteins predicted to possess metal-dependent protease activity and their orthologs from the fruit fly *Drosophila melanogaster*, nematode *Caenorhabditis elegans*, and budding yeast *Saccharomyces cerevisiae*, whenever detectable. Additionally included are predicted active JAMM proteases from archaea and a representative set of bacteria, and a subset of bacterial RadC proteins. The sequences of Rpn11 and its orthologs are not included and neither are proteins containing apparently inactivated JAMM domains. For each protein, the position of the aligned region in the sequence is shown by numbers; poorly conserved spacers are not shown and are designated by numbers. The consensus includes amino acid residues conserved in 80% of the aligned sequences; l indicates aliphatic residues (A,I,L,V; yellow shading), h indicates hydrophobic residues (F,Y,W, A,I,L,V,M; yellow shading), and s indicates small residues (G,A,C,S,D,N,V,P; blue). The predicted metal-chelating and catalytic residues (see text) are shown in yellow against a dark-blue background. Secondary structure prediction was made using the PHD program with the multiple alignment submitted as the query (*S10*); E indicates extended conformation (β-strand) and H indicates α-helix. Each protein is denoted by the GenBank identifier (GI) followed by the

gene name and an abbreviated species name. Species abbreviations: Hsa, *Homo sapiens*, Dme, *Drosophila melanogaster*, Cel, *Caenorhabditis elegans*, Ath, Arabidopsis thaliana, Sce, *Saccharomyces cerevisiae*; Afu, Archaeoglobus fulgidus, Mka, *Methanopyrus kandleri*, Mca, *Methanosarcina acetivorans*, Mth, *Methanothermobacter thermoautotrophicus*, Pho, *Pyrococcus horikoshii*, Hsp, *Halobacterium* sp., Sso, *Sulfolobus solfataricus*, Ape, *Aeropyrum pernix*, Pae, *Pyrobaculum aerophylum* (archaea); Aae, *Aquifex aeolicus*, Ssp, *Synechosystis* sp., Nsp, *Nostoc* sp., Dra, *Deinococcus radiodurans*, Sco, *Streptomyces coelicolor*, Mtu, *Mycobacterium tuberculosis*, Ype, *Yersinia pestis*, Psa, *Pseudomonas aeruginosa*, Rso, *Ralstonia solanaraceum*, Vch, *Vibrio cholerae*, Rsp, *Rhizobium* sp., Eco, *Escherichia coli*, Bsu, *Bacillus subtilis*, Sme, *Sinorhizobium meliloti*, Atu, *Agrobacterium tumefaciens*, Cac, *Clostridium acetobutilicum* (bacteria).

S2

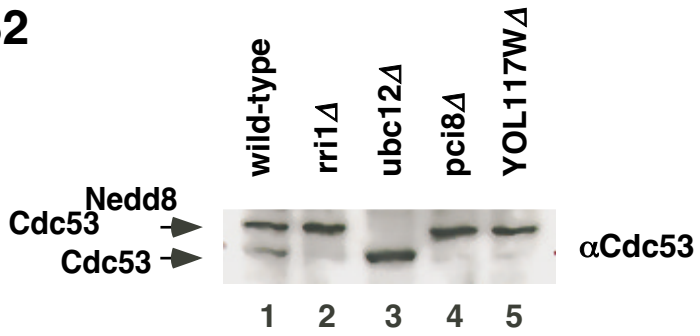
wild-type
rri1Δ
ubc12Δ
pci8Δ
YOL117WΔ
Nedd8
Cdc53
Cdc53
αCdc53
1
2
3
4
5

Figure S2: Deletion of *PCI8* and *YOL117W* in *S. cerevisiae* alters Cdc53 neddylation pattern. Cell lysates (see supplemental methods) from the indicated strains were evaluated by western blot using antibodies specific for Cdc53.

References and Notes:

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