Supplementary Figure 1: Bioinformatic analysis of Lag2 and Cand1-like sequences from different species

The alignment was produced by the MAFFT program, using the L-INS-I algorithm (Katoh et al. 2005). Similar residues (Sim) are coloured depending on the level of similarity as follows: Sim>3.0 cyan, Sim>1.5 midblue and Sim>0.5 lightgray. The following species are included in the alignment: Yeast: *S. cerevisae*, CANGA: *C. glabrate*, VANPO: *V. polyspora*, SCHPO: *S. pombe*, NEUCR: *N. crassa*, CAEEL: *C. elegans*; HUMAN: *H. sapiens*, DROME: *D. melanogaster*, ARATH: *A. thaliana*, CANAL: *C. albicans*

Supplementary Figure 2: Cdc53 and Lag2 interact by two-hybrid assay, and Lag2 does not co-immunoprecipitate with Skp1

A: The interaction between Cdc53 fused to LexA-DNA binding domain and Lag2 fused to activation domain was assessed by two-hybrid assay using the LexA-LacZ reporter assay. An empty plasmid (ev) served as a negative control. β-galactosidase activity was normalized in Miller Units; the average of at least three experiments with standard deviations is shown.

B: Extracts prepared from wild type cells were immunoprecipitated with either control IgG or Skp1-specific antibodies, and bound proteins were immunoblotted as indicated with Cdc53, Lag2 or Skp1 antibodies. One percent of total extracts were loaded as input (sup).

Supplementary Figure 3: Analysis of the purified components by SDS-PAGE

A: Coomassie-stained SDS-PAGE gel showing 1.5 micrograms of the purified *S. cerevisiae* proteins used for *in vitro* assays. The Molecular Weight Marker (BioRad) is shown on the left (lane 1).

B: Purified proteins were mixed in the indicated molar ratios and incubated for 30 minutes on ice in 25mM Tris pH=7.6 50mM NaCl 8% Glycerol 1mM DTT. Samples were ran at 130V on native gels for 100 minutes, and visualized by coomassie blue staining.

Supplementary Figure 4: Wild type and non-neddylatable Lag2-K16R prevent Cdc53 neddylation *in vitro* with comparable efficiency

Purified Cdc53-Hrt1 complexes were subjected to neddylation reactions as described in Material and Methods in the presence of [^{32}P]-Rub1, and analyzed by autoradiography after the times indicated. Where indicated, the complexes were pre-incubated for 30 minutes with purified wild type Lag2 or non-neddylatable Lag2^{K16R}. The neddylation efficiency of Cdc53 was normalized by phosphorimager analysis from at least three experiments and plotted against time (minutes). Diamonds: no addition; squares: pre-incubation with wild type Lag2; diamonds: pre-incubation with Lag2^{K16R}.

Supplementary Figure 5: Dcn1 interacts with the Cdc53/Hrt1/Lag2 complex *in vitro*, but does not trigger Lag2 removal *in vitro* or *in vivo*

A: Purified Dcn1 or the Dcn1 PONY domain does not bind to Lag2 directly, but may bind to Cdc53/Hrt1/Lag2 complexes *in vitro* as analyzed by native gel shift analysis. Proteins were mixed in the indicated molar ratios and incubated for 30 minutes on ice in 25mM Tris pH=7.6 50mM NaCl 8% Glycerol 1mM DTT. Samples were ran on native gels for 30 minutes, and visualized by coomassie blue staining. Note that Dcn1 and Lag2 do not appear to bind by native gel shift analysis, but due to the size and PI of Dcn1 and Lag2 they run at nearly identical positions in the native gels. We thus also analyzed the PONY domain of Dcn1, which fully complements the Dcn1 function when expressed in *dcn1D* cells *in vivo* (Kurz et al., 2008). The PONY domain of Dcn1 has a lower PI than full-length and hence migrates more rapidly in the native gels. Indeed, Dcn1 and the Dcn1 PONY domain may form a trimeric complex with Cdc53 and Lag2 *in vitro*.

B: Purified Cdc53-Hrt1 complexes were subjected to neddylation reactions as described in Material and Methods in the presence of [^{32}P]-Rub1, and analyzed by autoradiography after the times indicated (minutes). Where indicated (+), the complexes were pre-incubated (pre-incubate) for 30 minutes with purified Dcn1 or Lag2. The neddylation reaction was started by addition of [^{32}P]-Rub1 in the presence (+) or absence (-) of purified Dcn1 (inject). Note that neither pre-incubation nor initiation of the rubylation reaction with Dcn1 affected the inhibitory effect of Lag2 *in vitro*.

C: Total cell extract prepared from wild type (wt), *lag2Δ*, *dcn1Δ*, and *dcn1Δ lag2DΔ*cells were analyzed by immunoblotting with antibodies against Cdc53 (upper panel) and Lag2

(lower panel). Note that deletion of Lag2 does not significantly alter the neddylation state of Cdc53 in wild type and *dcn1Δ* cells.

Supplementary Figure 6: Genetic interaction of Lag2 and Skp1

Five fold serial dilutions of an equal number of *skp1-12* (upper plates) and *skp1-12 lag2Δ* (lower plates) transformed with an empty control plasmid (ev) or plasmids allowing as indicated overexpression of wild type Lag2 or $Lag2^{GN(551)}$ from the regulatable *GAL1,10*-promoter, were spotted on media containing galactose (*GAL*-promoter on). The plates were photographed after three days at the semi-permissive temperatures of 30^0C (left plates) or 33^0C (right plates). Note that overexpression of functional Lag2 is toxic in cells compromised for Skp1 activity.

Supplementary Figure 7: Lag2 specifically interacts with the Cul4-type cullin Rtt101 by GST-pull-down

Extracts prepared from cells expressing Myc-tagged Rtt101 were incubated with beads coated with GST alone or Lag2-GST. After extensive washing, bound proteins were eluted with gel-loading buffer and visualized by immunoblotting with antibodies recognizing the Myc-tag. An aliquot of the input (sup) controls for the presence of Rtt101 in the extract.

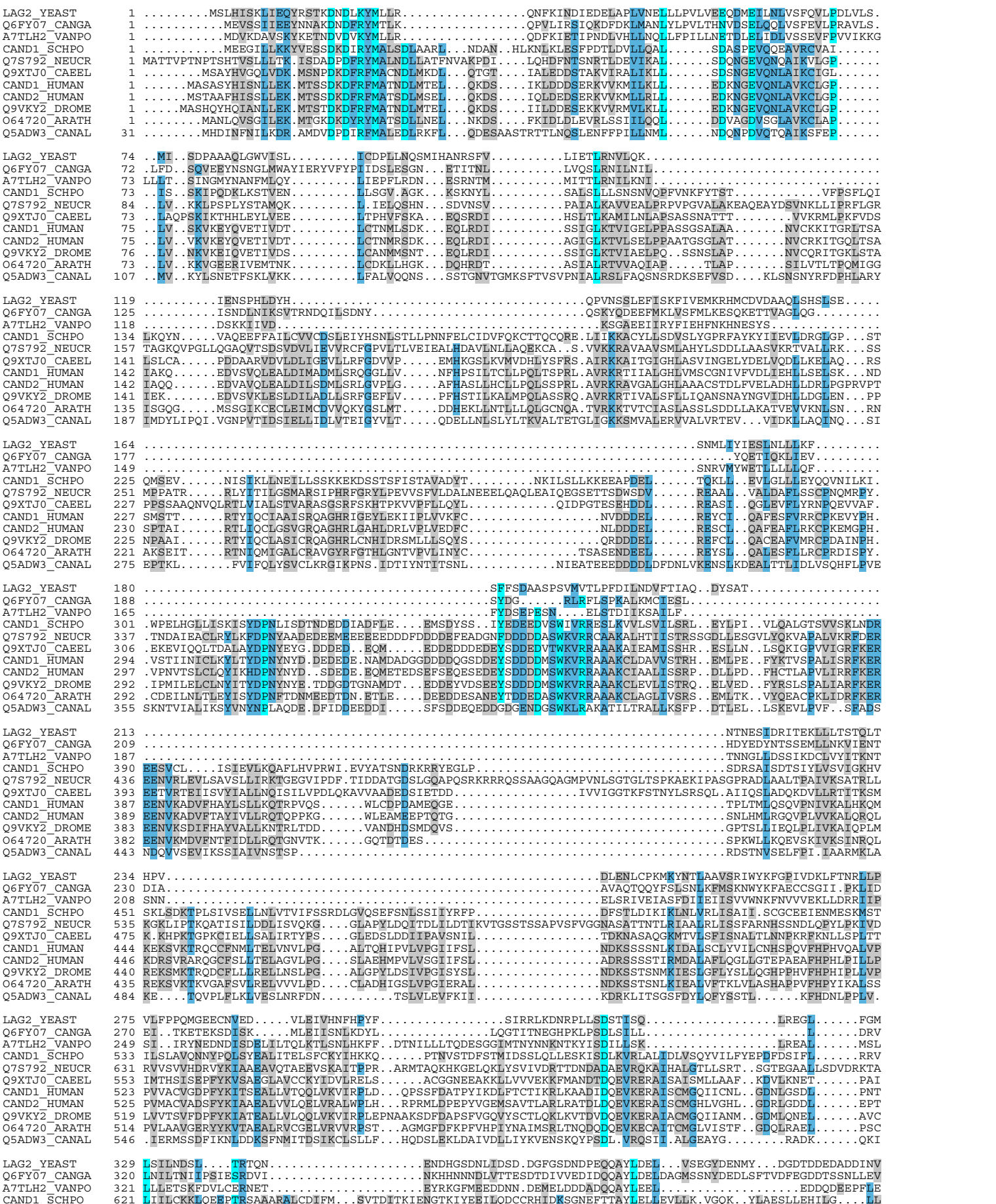
Supplementary Figure 8: Only a small fraction of Lag2 co-fractionates with Cdc53 in rapidly growing yeast cells

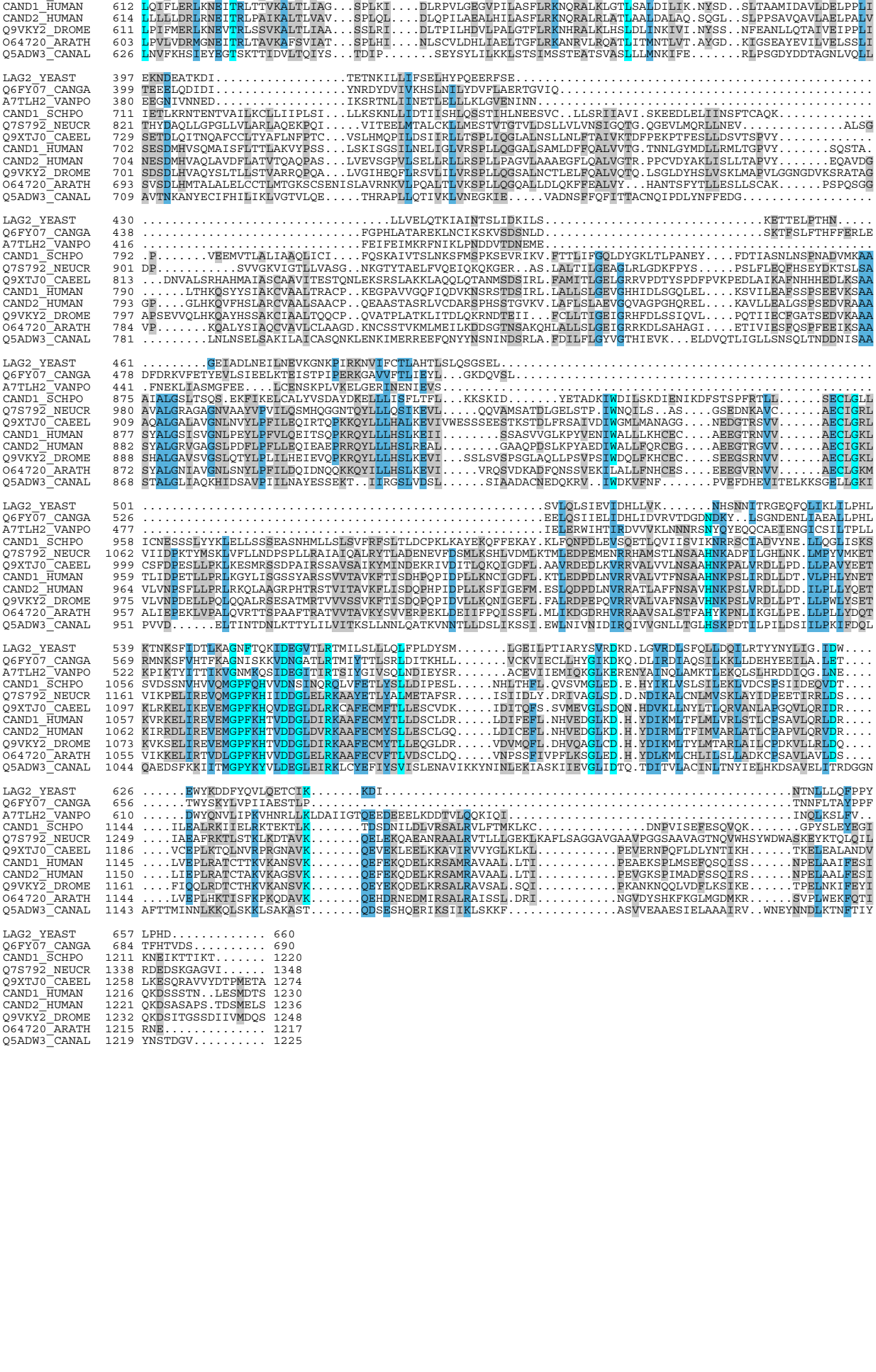
Extracts from exponentially growing wild type cells were separated on a Sepharose 6 gel filtration column, and selected fractions (1 – 13) analyzed by immunoblotting with antibodies against Cdc53 (upper panel), Lag2 (middle panel) and Skp1 (lower panel). An aliquot of the input (sup) was included for control of the extract. The position of the molecular weight markers is indicated on top.

Supplementary Table 1: Bioinformatic analysis of selected species for the presence of Lag2 or CAND1-like proteins

The indicated genomes were analyzed by multiple sequence alignments (Katoh et al., 2005) for the presence of Lag2 or CAND1-like proteins. Depicted in gray are yeast species. The analysis suggests that many fungal species carry a Lag2-like protein, while other yeast species, *C. elegans*, *D. melanogaster*, and mammalian species encode a CAND1-like protein instead.

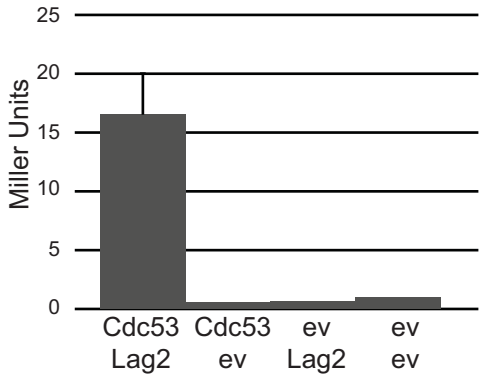
Katoh, K., K. Kuma, H. Toh & T. Miyata (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res,* 33**,** 511-8.


demo.msf
LAG2_YEAST
Q6FY07_CANGA
A7TLH2_VANPO
CAND1_SCHPO
Q7S792_NEUCR
Q9XTJ0_CAEEL
CAND1_HUMAN
CAND2_HUMAN
Q9VKY2_DROME
O64720_ARATH
Q5ADW3_CANAL

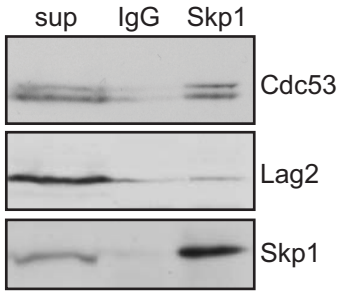


Supplementary Figure 2.

A.

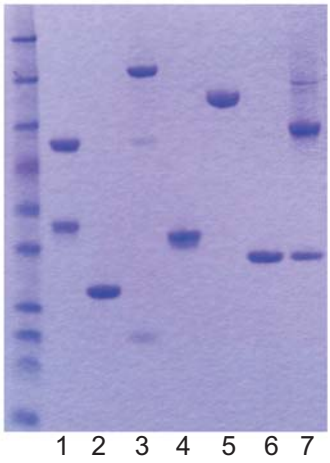


B.



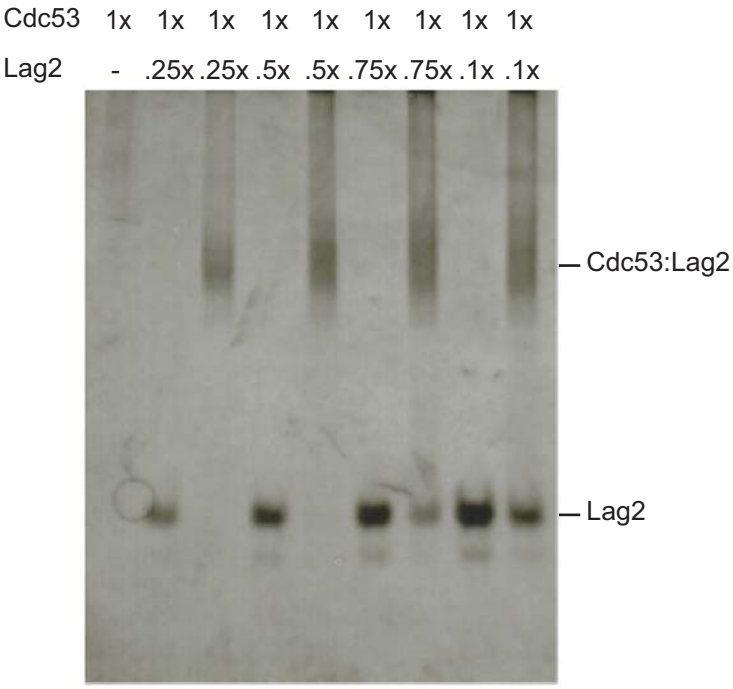
Supplementary Figure 3.

A.

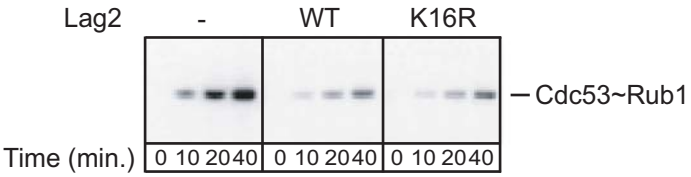


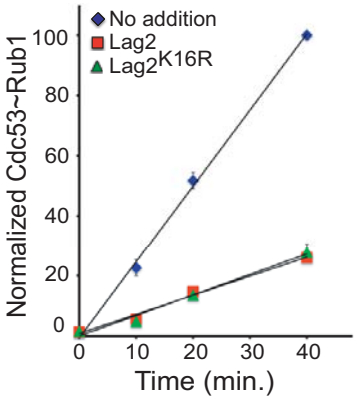
1. Markers
2. Sc Uba3/Ula1
3. Sc Ubc12
4. Sc Cdc53/Hrt1
5. Sc Dcn1
6. Sc Lag2
7. Sc Skp1
8. Sc Cdc4/Skp1

B.



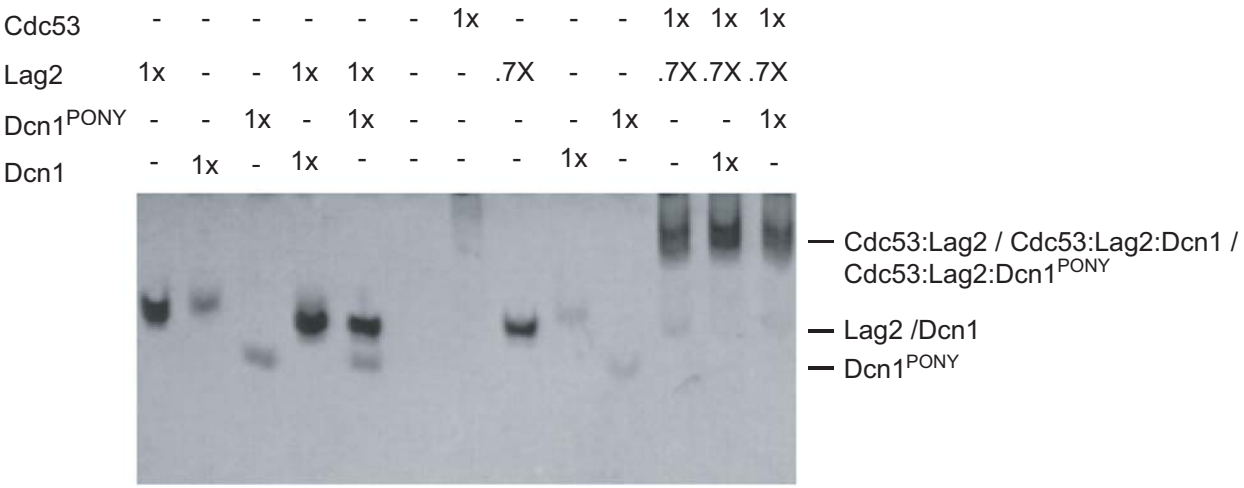
Supplementary Figure 4.



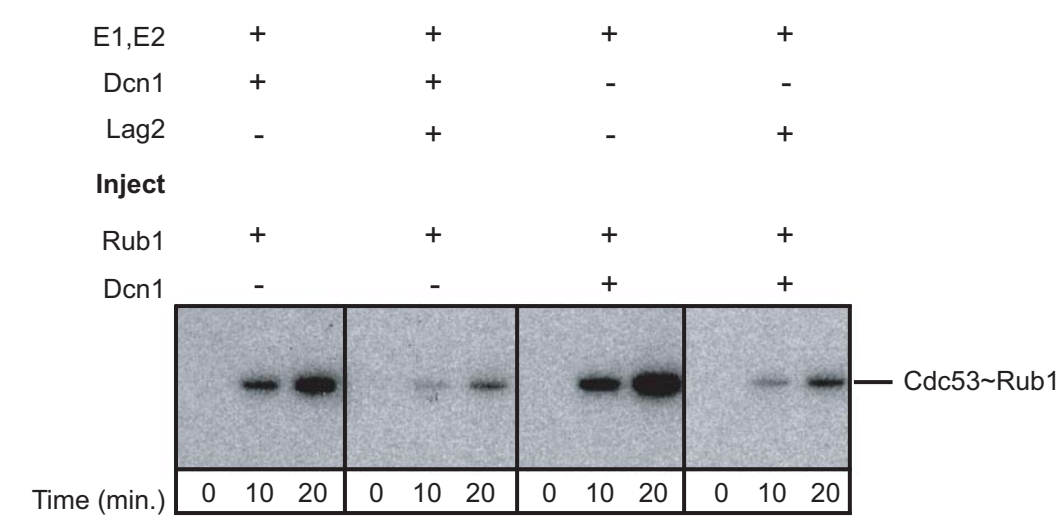


Supplementary Figure 5.

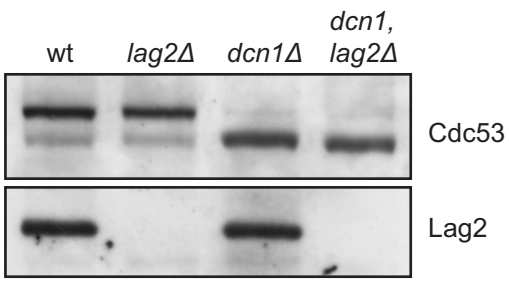
A.



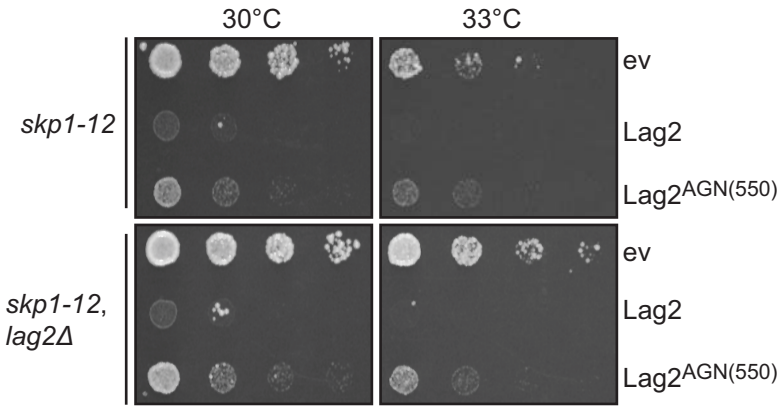
B.



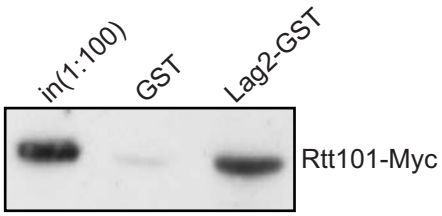
C.



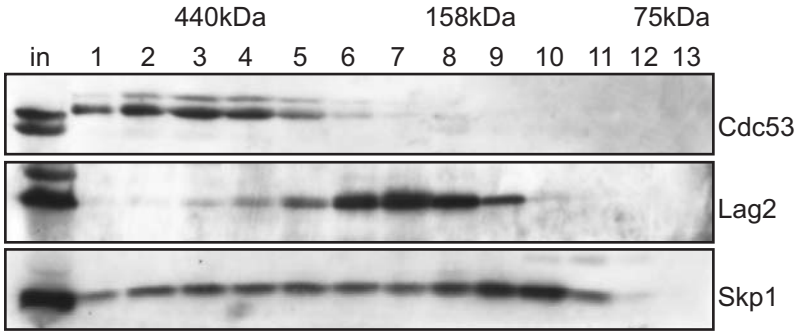
Supplementary Figure 6.



Supplementary Figure 7.



Supplementary Figure 8.



Supplementary Tables

Supplementary Table I. Genomes with Lag2- or Cand1-like sequence

Genome Contains Lag2 but not Cand1	Genome Contains Cand1 but not Lag2
Saccharomyces (e.g. cerevisiae, castelli)	Zygomycetes
Vanderwaltozyma polyspora	Basidiomycetes
Zygosaccharomyces rouxii	Schizosaccharomyces pombe
Candida glabrata	Candida albicans
Kluyveromyces thermotolerans	Homo sapiens
Kluyveromyces waltii	Mus musculus
Kluyveromyces lactis	Drosophila melanogaster
Ashbya gossypii	Caenorhabditis elegans
	Arabidopsis thaliana

Supplementary Table II. Plasmids used in this study

Plasmid ID	Plasmid name	Vector name	Reference
pES31	Lag2	pRS426Gal1	this study
pES30	$Lag^{GN(551)}$	pRS426Gal1	this study
pES32	$Lag2^{DDYM(17)}$	pRS426Gal1	this study
	$Lag2^{K16R}$	pRS426Gal1	this study
pES63	Lag2	P41eHIS3 (pGR49)	this study
pES64	$Lag2^{DDYM(17)}$	P41eHIS3 (pGR49)	this study
pES66	$Lag2^{GN(551)}$	P41eHIS3	this study

		(pGR49)	
	$Lag2^{K16R}$	P41eHIS3 (pGR49)	this study
pES46	HA-Dcn1	pRD54Gal1	Kurz et al., 2008
pES47	HA-$Dcn1^{DAD}$-	pRD54Gal1	Kurz et al., 2008
	HA-Rub1	pRD54Gal1	this study
pMT704	Cdc53	pRS424	(Willems et al., 1996)
pMT2166	Cdc53 K760R	pRS424	(Willems et al., 1996)
pTK260	GST-Hrt1, Lag2, 6xHis-Cdc53		this study
pTK261	GST-Hrt1, Skp1, 6xHis-Cdc53	pST39	this study
	ScSkp1	pGEX(TEV)	this study
	Lag2	pGEX(TEV)	this study
	$Lag2^{K16R}$	pGEX(TEV)	this study
	$Lag2^{GN(551)}$	pGEX(TEV)	this study
	$Lag2^{DDYM(17)}$	pGEX(TEV)	this study
	ScUbc12	pGEX(TEV)	this study
	ScDcn1	pGEX(TEV)	this study
	Rub1GG	pGEX2TK	this study
	His-$Cdc4^{267-774}$	pRSFDuet	this study
	His-$Cdc4^{267-774}$	pRSFDuet	this study

	Cdc53	pFASTBAC	this study
	Hrt1$^{8\text{-}C}$	pFASTBAC	this study
pDH447	Uba3/Ula1	pGEX	this study
	Cul1NTD	pAL	(Zheng et al., 2002b)
	Rbx1-rbs-Cul1CTD	pGEX	(Zheng et al., 2002b)
	CAND1	pGEX	(Duda et al., 2008)
pMH43	Lag2	pJG4-6	this study
pBL54	Cdc53	pEG203	(Kurz et al., 2005)
pFR28	Lag2	pGEX	this study

Table III. Yeast strains used in this study

Strain ID	Strain name	Background	Reference
yES47	his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	S288C	
yES49	MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}	W303	
yES48	*lag2::kan*	S288C	this study
yES81	*lag2::kan*	W303	this study
yES58	*rub1::kan*	S288C	heterozygous diploid deletion collection (Euroscarf)
yES62	*rub1::kan*	W303	this study

yES59	*rri1::kan*	S288C	diploid deletion collection (Euroscarf)
yES142	*lag2^{wt}::HIS3*	S288C	this study
yES168	*lag2$^{GN(551)::}$HIS3*	S288C	this study
yES170	*lag2$^{DDYM(17)}$::HIS3*	S288C	this study
yES171	*lag2$^{K16::}$HIS3*	S288C	this study
MTY871	*cdc53-1*	W303	(Willems et al., 1996)
MTY706	*cdc53::ADE2 <Cdc53 CEN>*	W303	(Willems et al., 1996)
MTY1752	*cdc53::ADE2 <Cdc53-K760R CEN TRP1>*	W303	(Kurz et al., 2008)
Y553	*skp1-11*	W303	(Bai et al., 1996)??
Y555	*skp1-12*	W303	(Bai et al., 1996)??
yES73	*skp1-11 lag2::kan*	W303	this study
yES74	*skp1-12 lag2::kan*	W303	this study
YTK28	*dcn1::KanMX4 his3*Δ *leu2*Δ *met15*Δ *ura3*Δ	S288C	(Kurz et al., 2005)
yES102	*dcn1::nat; lag2::kan*	S288C	this study
yES77	*ubc12::kan*	S288C	diploid deletion collection (Euroscarf)
yES78	*ubc12::kan, rri1::nat*	S288C	(Kurz et al., 2005)

yES80	*dcn1::kan, rri1::nat*	S288C	(Kurz et al., 2005)
yGR97	*rtt101:: MYC-RTT101-HIS*	S288C	this study