Supplementary Information

Supplementary Methods

Materials

Yeast cells were grown in rich medium (YPD: 1% yeast extract, 2% peptone, and 2% glucose), lactate medium (YPL: 1% yeast extract, 2% peptone, and 2% lactate), synthetic minimal medium with glucose (SMD: 0.67% yeast nitrogen base, 2% glucose, and amino acids) or synthetic minimal medium with lactate (SML: 0.67% yeast nitrogen base, 2% lactate, and amino acids). Nitrogen starvation experiments were performed in synthetic minimal medium lacking nitrogen (SD-N: 0.17% yeast nitrogen base without amino acids and ammonium sulfate and 2% glucose). Unless otherwise stated, cells were grown at 30°C.

The plasmids for expression of ProA-tagged Atg32 and HA-tagged Atg11 under the control of the *CUP1* promoter have been described previously [1]. To generate an HA-tagged Cka1 and HA-tagged Ckb1 under the control of the *CUP1* promoter, 3HA was first cloned into the *Bam*HI/*Pst*I sites of pCu414 and pCu416 to generate pCu3HA414 and pCu3HA416, respectively, and then the open reading frame of *CKA1* and *CKB1* was cloned into the *Eco*RI/*Xho*1 site of pCu3HA414 and/or pCu3HA416.

Anti-Atg32 antibody [2], anti-HA antibody (Sigma-Aldrich, St. Louis, MO), anti-Pgk1 antibody (Nordic Immunological Laboratories, The Netherlands), anti-protein A antibody (GeneTex Inc., CA), and anti-GFP antibody (Takara Bio, Japan) were used for immunoblotting.

***In vitro* kinase assay**

N-terminal GST-tagged Cka1 and Ckb1, Sko1 (N-terminal 214 amino acids), and Atg32 (N-terminal 250 amino acids; wild-type or serine 114- and/or serine 119-to-alanine mutant) expression vectors were constructed using pGEX-4T-1 (GE Healthcare, UK) and were expressed in *E. coli* BL21 (DE3). Expressed proteins were purified using glutathione Sepharose 4B (GE Healthcare). One microgram of GST-Cka1, 1 μg of GST-Ckb1, and 2.5 μg of GST-Sko1 or GST-Atg32 were mixed in the kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, and 2 mM dithiothreitol) and incubated at 30°C for 30 min together with [γ-^{32}P]ATP (0.2 μCi/μl). The labeled proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel images were visualized by BAS-2500 autoradiography (Fujifilm, Japan).

Supplemental Figure Legends

Supplementary Figure S1

The indicated gene-deleted strains were cultured in YPL medium until the mid-log growth phase and then shifted to SD-N medium for 0 or 1 hour. The phosphorylation status of Atg32 was observed by immunoblotting with anti-Atg32 antibodies. If the immunoblotted bands were unclear, we repeated the experiments and show the result in panels S1–4.

Supplementary Figure S2

The wild-type (WT), *cka1Δ*, *cka2Δ*, *ckb1Δ*, and *ckb2Δ* strains (BY4742) were cultured in YPL medium until the mid-log growth phase and then shifted to SD-N medium for 0, 1, or 4 hours. The phosphorylation status of Atg32 was observed by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies.

Supplementary Figure S3

The wild-type (WT), *cka1Δ*, *cka2Δ*, *ckb1Δ*, *ckb2Δ*, and *ckb1Δ*/*ckb2Δ* strains were cultured in YPD (A) or YPL (B) until the mid-log growth phase and then cells were diluted to an OD_{600} of 0.1 in YPD or an OD_{600} of 0.3 in YPL. Cell growth (OD_{600}) was observed at the indicated time.

Supplementary Figure S4

The wild-type (WT), *cka1Δ*, *cka2Δ*, *ckb1Δ*, *ckb2Δ*, and *ckb1Δ*/*ckb2Δ* strains expressing Om45-GFP were cultured in YPD (A) or YPL (B) to the mid-log growth phase. The localization of GFP was visualized by fluorescence microscopy. DIC, differential interference contrast.

Supplementary Figure S5

(A, B) Wild-type (WT) or *cka1Δ*/*cka2ts* (*CK2ts*) cells expressing ProA-Atg32 and HA-Atg11 were grown in SML medium until the mid-log phase at 28°C, then incubated at 37°C for 30 min, and starved in SD-N at 37°C for 1 hour (A). WT cells expressing ProA-Atg32 and HA-Atg11 were grown in SML medium until the mid-log phase and then starved in SD-N with or without 70 μM TBB for 1 hour (B). ProA-Atg32 was precipitated using IgG-Sepharose from cell lysates. An immunoblot of total cell lysate (input) and the IgG precipitates (pull-down) were probed with anti-HA and anti-Protein A antibodies.
(C) WT cells expressing Om45-GFP with empty vector, the HA-Cka1 vector, or the HA-Cka1 and HA-Ckb1 vectors were cultured in SML medium until the mid-log growth phase and then shifted to SD-N for 0, 4, or 6 hours. GFP processing and CK2 expression were observed by immunoblotting with anti-GFP, anti-HA, and anti-Pgk1 (loading control) antibodies.

Supplementary Figure S6

(A) The wild-type (WT) (TKMY236), *atg1Δ* (TKMY256), *cka1Δ* (TKMY237), and *cka2Δ* (TKMY238) yeast strains were grown in YPD medium and shifted to SD-N for 3 or 6 hours. Samples were collected and protein extracts assayed for Pho8Δ60 activity. The results represent the mean and standard deviation of three experiments.

(B, C) Atg1 temperature-sensitive cells (*atg1Δ* strain with *atg1ts*-expressing vector, positive control) and *CK2ts* cells were cultured in SMD medium at 24°C until the mid-log growth phase and then shifted to 37°C for 4 hours (B). WT cells were cultured in YPD until the mid-log growth phase and then supplemented with 70 μM TBB or 1 mM phenylmethanesulfonyl fluoride (PMSF, positive control) and cultured for 4 hours (C). Cell lysates were analyzed for precursor Ape 1 (p-Ape1) maturation (m-Ape-1) by immunoblotting with anti-Ape1 antiserum.

Supplementary Figure S7

Recombinant GST-Atg32 (1–250) wild-type (WT) or serine 114 and 119-to-alanine mutant (2SA) was phosphorylated by recombinant yeast CK2 supplemented with or without TBB in the presence of [γ-^{32}P]ATP (A). Recombinant GST-Atg32 (1–250), the WT or SA

mutant, was phosphorylated by human CK2 supplemented with or without TBB in the presence of [γ-^{32}P]ATP (B). Recombinant GST-Atg32 (1–250) WT was phosphorylated by recombinant Cka1 with or without recombinant Ckb1 in the presence of [γ-^{32}P]ATP (C). The labeled proteins were resolved by SDS-PAGE, and an autoradiograph image is shown.

Supplementary Figure S8

Wild-type (WT) cells expressing HA-Ckb1 and ProA-Atg32 or ProA only were grown in SMD medium until the mid-log phase and then starved in SD-N for 1 hour (A). WT cells expressing HA-Cka1 and ProA-Atg32 WT or ProA-Atg32 S114A/S119A mutant (2SA) were grown in SMD medium until the mid-log phase and then starved in SD-N for 0 or 1 hour (B). WT cells expressing HA-Cka1 and ProA-Atg32 or ProA only were grown in SML medium until the mid-log phase and then starved in SD-N for 1 hour (C). ProA-Atg32 was precipitated using IgG-Sepharose from cell lysates. The left panel shows an immunoblot of total cell lysate (input) and the right panel shows the IgG precipitates (pull-downs), which were probed with anti-HA and anti-ProA antibodies.

Supplementary Figure S9

(A) The wild-type (WT), *atg32Δ*, *slt2Δ*, and *hog1Δ* strains expressing Om45-GFP were

cultured in YPL medium until the mid-log growth phase and then shifted to SD-N for 0, 4, or 6 hours. GFP processing was monitored by immunoblotting with anti-GFP and anti-Pgk1 (loading control) antibodies.

(**B**) The wild-type (WT), *atg32Δ*, *slt2Δ*, and *hog1Δ* strains were cultured in YPL medium until the mid-log growth phase and then shifted to SD-N medium for 0, 2, or 4 hours. The phosphorylation status of Atg32 was observed by immunoblotting with anti-Atg32 and anti-Pgk1 (loading control) antibodies.

Supplementary Figure S10

Wild-type cells expressing Cka1-GFP, Cka2-GFP, Ckb1-GFP, or Ckb2-GFP were cultured in YPD or YPL medium until the mid-log phase (A). Cells were cultured in YPL medium until the mid-log phase and then shifted to SD-N medium for 1 hour (B). The localization of GFP was visualized by fluorescence microscopy. DIC, differential interference contrast.

Supplementary Figure S11

Wild-type cells expressing Cka1-GFP were cultured in YPL medium until the mid-log phase and then shifted to SD-N medium for 1 hour. Cells were labeled with 4',6-diamidino-2-phenylindole (DAPI, 2.5 μg/ml) that accumulates both on mitochondrial

DNA and nuclear DNA (A) or MitoTracker RedCMXRos (0.5 μM) that stains mitochondria (B), and analyzed by fluorescence microscopy.

Supplementary Figure S12

Wild-type cells expressing Ckb1-GFP were cultured in YPL medium until the mid-log phase and then shifted to SD-N medium for 1 hour. Cells were labeled with DAPI (2.5 μg/ml) that accumulates both on mitochondrial DNA and nuclear DNA (A) or MitoTracker RedCMXRos (0.5 μM) that stains mitochondria (B), and analyzed by fluorescence microscopy.

Supplementary References

1. Kanki T, Wang K, Cao Y, Baba M, Klionsky DJ (2009) Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev Cell* **17:** 98-109
2. Aoki Y, Kanki T, Hirota Y, Kurihara Y, Saigusa T, Uchiumi T, Kang D (2011) Phosphorylation of Serine 114 on Atg32 mediates mitophagy. *Mol Biol Cell* **22:** 3206-3217
3. Robinson JS, Klionsky DJ, Banta LM, Emr SD (1988) Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Mol Cell Biol* **8:** 4936-4948
4. Kanki T, Klionsky DJ (2008) Mitophagy in yeast occurs through a selective mechanism. *J Biol Chem* **283:** 32386-32393
5. Shintani T, Huang WP, Stromhaug PE, Klionsky DJ (2002) Mechanism of cargo selection in the cytoplasm to vacuole targeting pathway. *Dev Cell* **3:** 825-837

Supplementary Table S1: List of genes tested in the manuscript

kinase/component of kinase (including putative kinase)

gene	systematic name
ADK1	YDR226W
ALK1	YGL021W
ALK2	YBL009W
ARK1	YNL020C
ATG1	YGL180W
BCK1	YJL095W
BUB1	YGR188C
BUD32	YGR262C
CDC5	YMR001C
CHK1	YBR274W
CKA1	YIL035C
CKA2	YOR061W
CKB1	YGL019W
CKB2	YOR039W
CKI1	YLR133W
CLA4	YNL298W
CLA4	YNL298W
CMK1	YFR014C
CMK2	YOL016C
CTK1	YKL139W
CTK1	YKL139W
CTK2	YJL006C
CTK3	YML112W
DAK1	YML070W
DAK2	YFL053W
DBF20	YPR111W
DUN1	YDL101C
EKI1	YDR147W
ELM1	YKL048C
FRK1	YPL141C
FUS3	YBL016W
GCN2	YDR283C
GIN4	YDR507C
GUT1	YHL032C
HAL5	YJL165C
HOG1	YLR113W

HRK1	YOR267C
HSL1	YKL101W
IKS1	YJL057C
IME2	YJL106W
IRE1	YHR079C
ISR1	YPR106W
KCC4	CL024W
KDX1	YKL161C
KIN1	YDR122W
KIN2	YLR096W
KIN3	YAR018C
KIN4	YOR233W
KKQ8	YKL168C
KNS1	YLL019C
KSP1	YHR082C
LCB4	YOR171C
MCK1	YNL307C
MEK1	YOR351C
MET14	YKL001C
MKK1	YOR231W
MKK2	YPL140C
MRK1	YDL079C
NNK1	YKL171W
NPR1	YNL183C
PHO85	YPL031C
PKH1	YDR490C
PKH2	YOL100W
PKH3	YDR466W
PKP1	YIL042C
PKP2	YGL059W
PPZ1	YML016C
PRK1	YIL095W
PRO1	YDR300C
PRR1	YKL116C
PRR2	YDL214C
PSK1	YAL017W
PSK2	YOL045W
PTK1	YKL198C
PTK2	YJR059W
RCK1	YGL158W

RCK2	YLR248W
RIM15	YFL033C
RLM1	YPL089C
RTK1	YDL025C
SAT4	YCR008W
SCH9	YHR205W
SCY1	YGL083W
SIP1	YDR422C
SIP2	YGL208W
SKM1	YOL113W
SKS1	YPL026C
SKY1	YMR216C
SLT2	YHR030C
SMK1	YPR054W
SNF1	YDR477W
SPS1	YDR523C
SSK2	YNR031C
SSK22	YCR073C
STE11	YLR362W
STE7	YDL159W
SWE1	YJL187C
TCO89	YPL180W
TDA1	YMR291W
TEL1	YBL088C
THR1	YHR025W
TOR1	YJR066W
TOS3	YGL179C
TPK1	YJL164C
TPK2	YPL203W
TPK3	YKL166C
URK1	YNR012W
VHS1	YDR247W
VIP1	YLR410W
VPS15	YBR097W
YAK1	YJL141C
YCK1	YHR135C
YCK2	YNL154C
YGK3	YOL128C
YPK1	YKL126W
YPK2	YMR104C

YPK3	YBR028C
YPL150W	YPL150W

kinase related protein

gene	systematic name
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CLB2	YPR119W
CLB3	YDL155W
CLB4	YLR210W
CLB5	YPR120C
CLB6	YGR109C
CLN1	YMR199W
CLN2	YPL256C
CLN3	YAL040C
PCL1	YNL289W
PCL1	YNL289W
PCL10	YGL134W
PCL7	YIL050W
PCL8	YPL219W
PCL9	YDL179W
PHO80	YOL001W
SAK1	YER129W
SSN8	YNL025C
STE20	YHL007C
YCK3	YER123W
YPL109C	YPL109C

Supplementary Table S2: Yeast strains used in this study

Strain	Genotype	Source
SEY6210 (WT)	MATα *his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9 GAL*	[3]
TKYM22	SEY6210 *OM45-GFP::TRP1*	[4]
TKYM67	SEY6210 *PEX14-GFP::KanMX6*	[4]
TKYM72	SEY6210 *atg1Δ::HIS5 S.p., PEX14-GFP::KanMX6*	[4]
TKYM140	SEY6210 *atg32Δ::LEU2, OM45-GFP::TRP1*	[2]
TKYM165	SEY6210 *atg32Δ::HIS5 S.p.*	[2]
TKYM222	SEY6210 *ckb1Δ::HIS5 S.p.*	this study
TKYM224	SEY6210 *ckb2Δ::HIS5 S.p.*	this study
TKYM230	SEY6210 *cka1Δ::KanMX6*	this study
TKYM231	SEY6210 *cka2Δ::KanMX6*	this study
TKYM236	SEY6210 *pho8Δ::HIS5 S.p., pho13Δ::LEU2*	[2]
TKYM237	SEY6210 *pho8Δ60::HIS5 S.p., pho13Δ::LEU2, cka1Δ::KanMX6*	this study

TKYM238	SEY6210 *pho8Δ60::HIS5 S.p., pho13Δ::LEU2, cka2Δ::KanMX6*	this study
TKYM250	SEY6210 *hog1Δ::HIS5 S.p., OM45-GFP::TRP1*	[2]
TKYM256	SEY6210 *pho8Δ60::HIS5 S.p., pho13Δ::LEU2, atg1Δ::URA3*	[2]
TKYM261	SEY6210 *pho8Δ60::HIS5 S.p., pho13Δ::LEU2, cka1Δ::TRP1 cka2Δ::KanMX6* [pRS416-CKA2D225N]	this study
TKYM269	SEY6210 *cka1Δ::TRP1 cka2Δ::KanMX6* [pRS416-CKA2WT]	this study
TKYM271	SEY6210 *cka1Δ::TRP1 cka2Δ::KanMX6* [pRS416-CKA2D225N]	this study
TKYM276	SEY6210 *cka1Δ::TRP1 cka2Δ::KanMX6 OM45-GFP::HIS3MX6* [pRS416-CKA2WT]	this study
TKYM277	SEY6210 *cka1Δ::TRP1 cka2Δ::KanMX6 OM45-GFP::HIS3MX6* [pRS416-CKA2D225N]	this study
TKYM314	SEY6210 *OM45-GFP::TRP1 Slt2Δ::KanMX6*	this study
TKYM315	SEY6210 *CKA1-GFP::TRP1*	this study
TKYM316	SEY6210 *CKA2-GFP::TRP1*	this study
TKYM317	SEY6210 *CKb1-GFP::TRP1*	this study
TKYM318	SEY6210 *CKb2-GFP::TRP1*	this study

TKYM333	SEY6210 *cka1Δ::KanMX6 OM45-GFP::TRP1*	this study
TKYM334	SEY6210 *cka2Δ::KanMX6 OM45-GFP::TRP1*	this study
TKYM335	SEY6210 *ckb1Δ::HIS5 S.p. OM45-GFP::TRP1*	this study
TKYM336	SEY6210 *ckb2Δ::HIS5 S.p. OM45-GFP::TRP1*	this study
TKYM342	SEY6210 *ckb1Δ::URA3 ckb2Δ::HIS5 S.p.*	this study
TKYM347	SEY6210 *ckb1Δ::URA3 ckb2Δ::HIS5 S.p. OM45-GFP::TRP1*	this study
TKYM370	SEY6210 *cka1Δ::HIS5 S.p. cka2Δ::URA3* [pRS405-CKA2D225N]	this study
WHY1	SEY6210 *atg1Δ*:: *HIS5 S.p.*	[5]

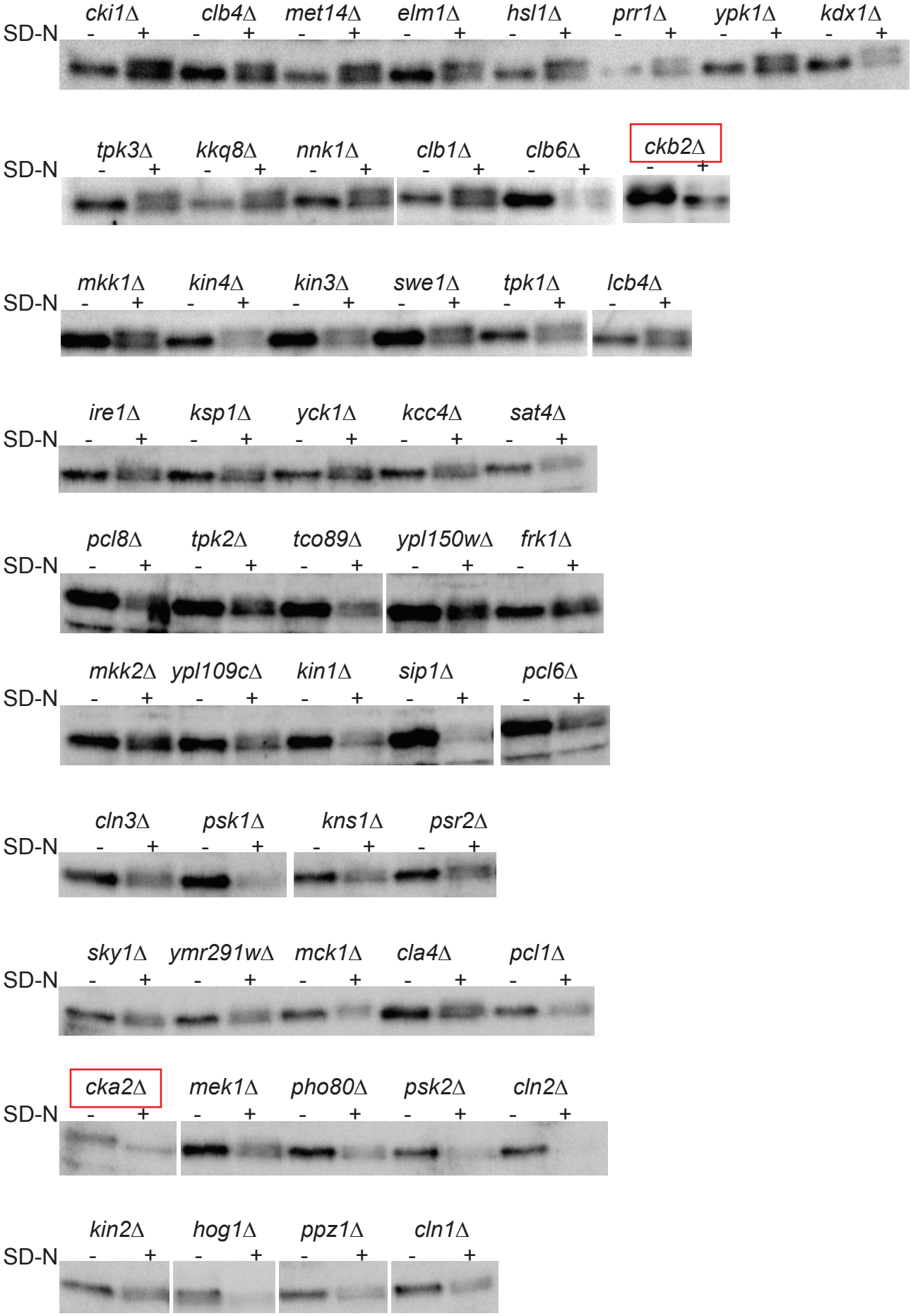
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Figure S1-2

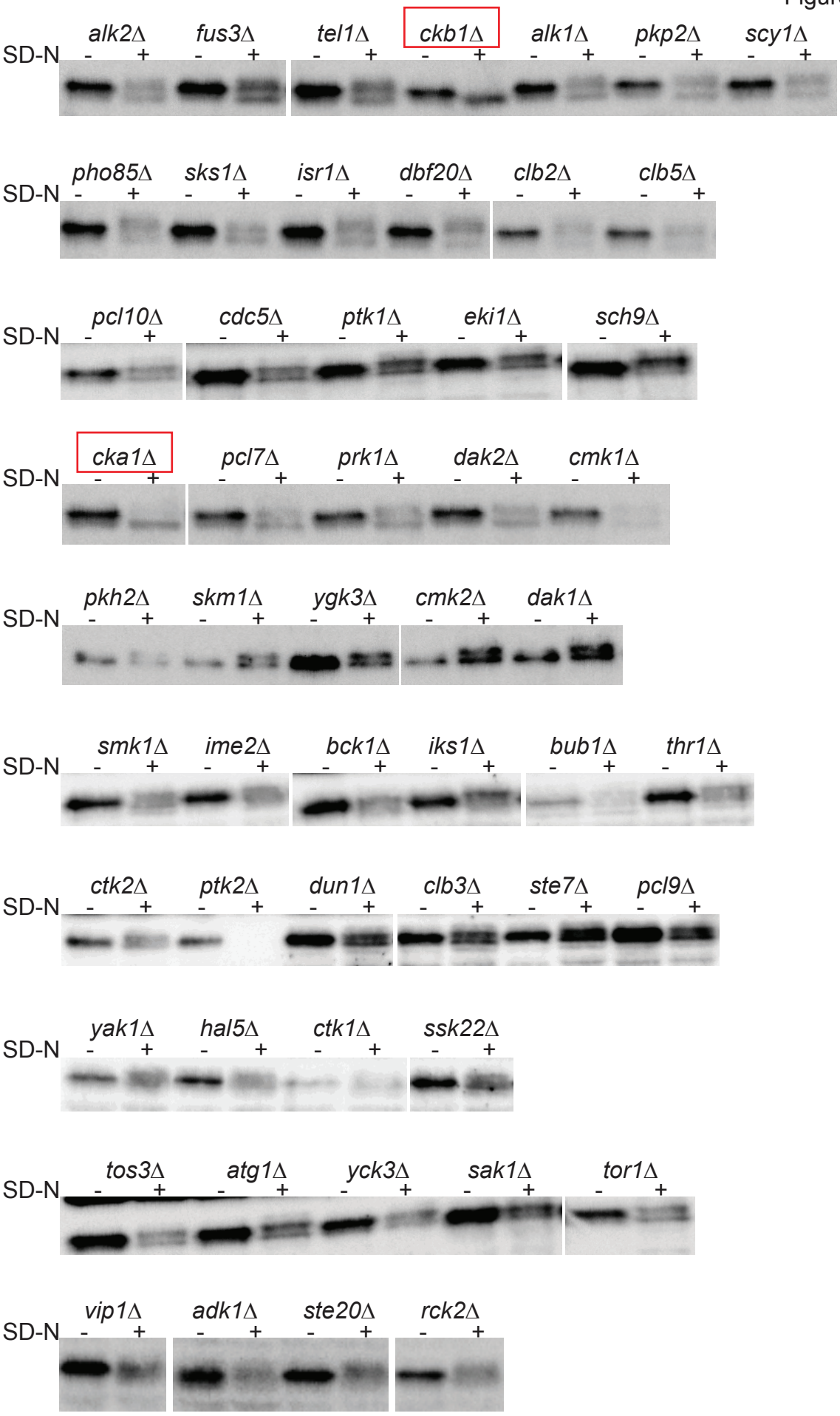
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Figure S1-3

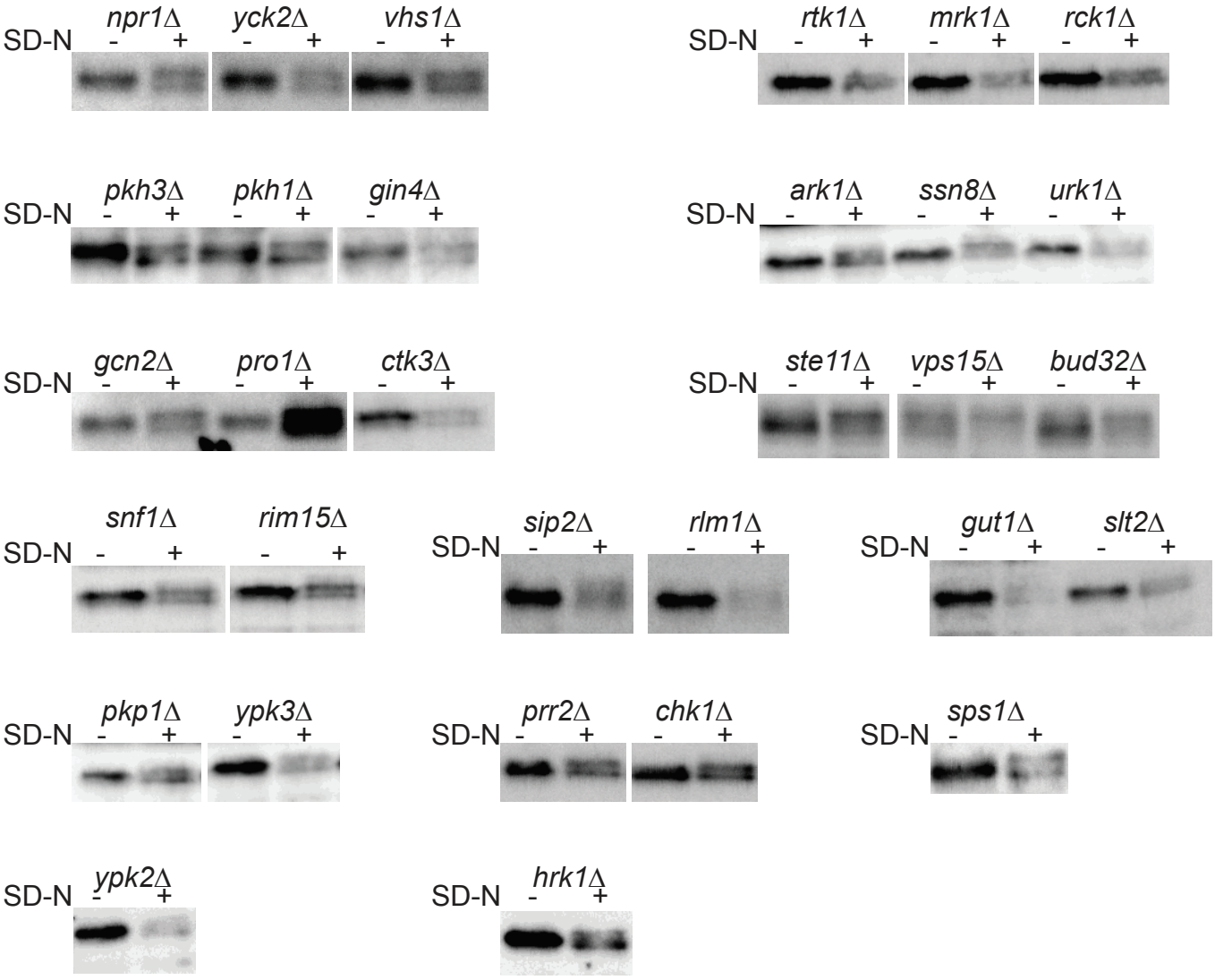


Figure S1-4

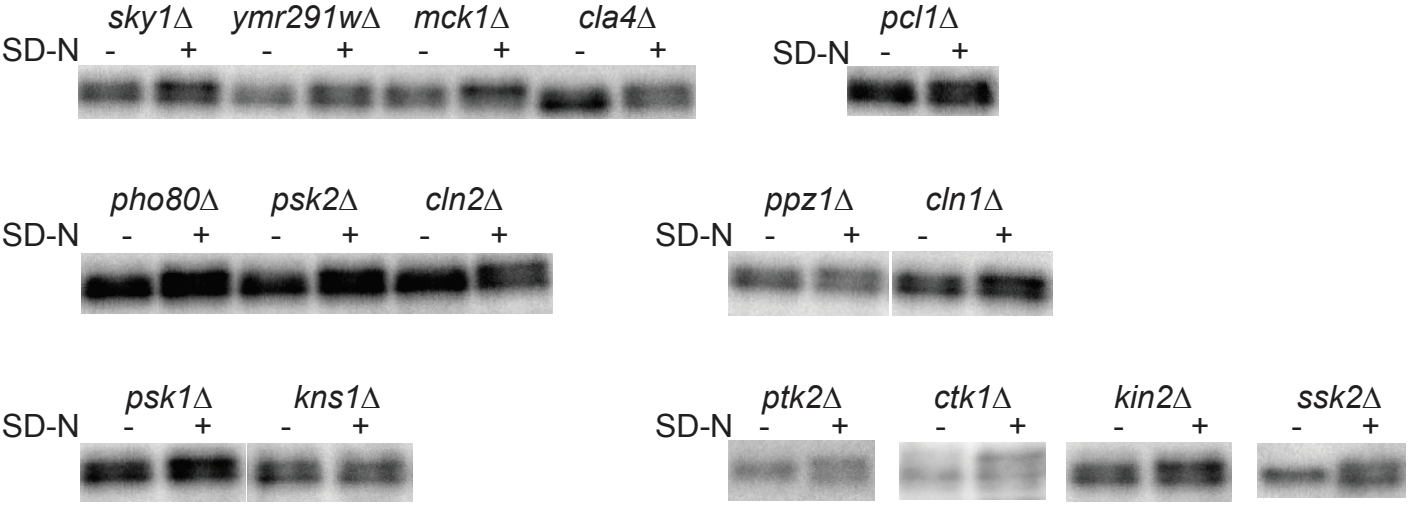
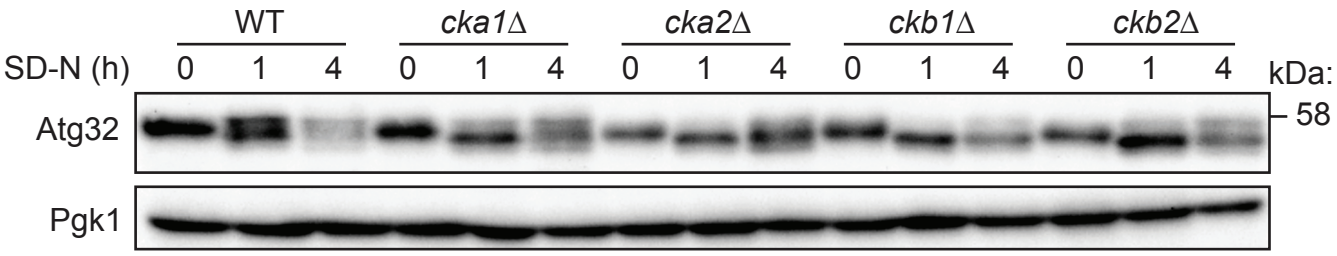


Figure S2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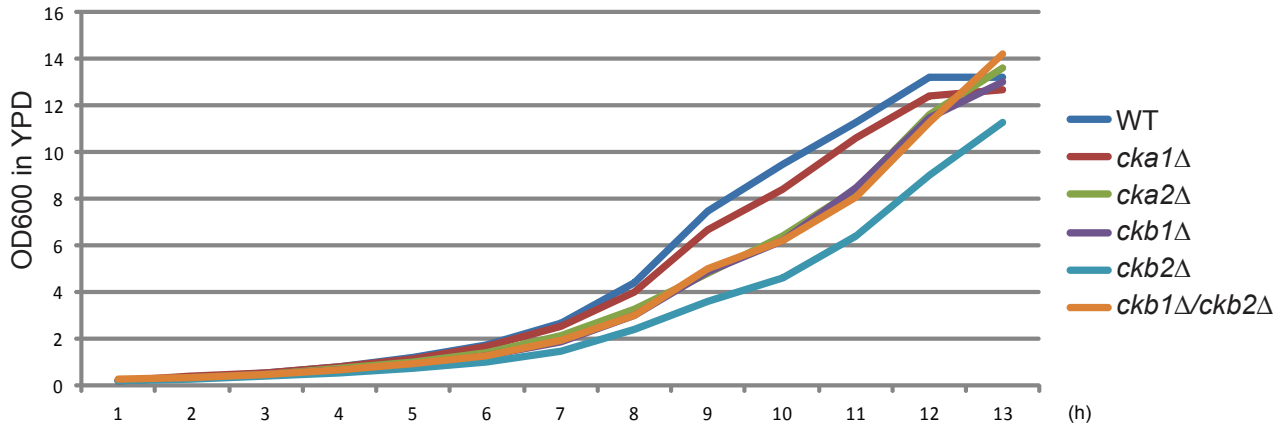
Atg32
Pgk1

Figure S3

A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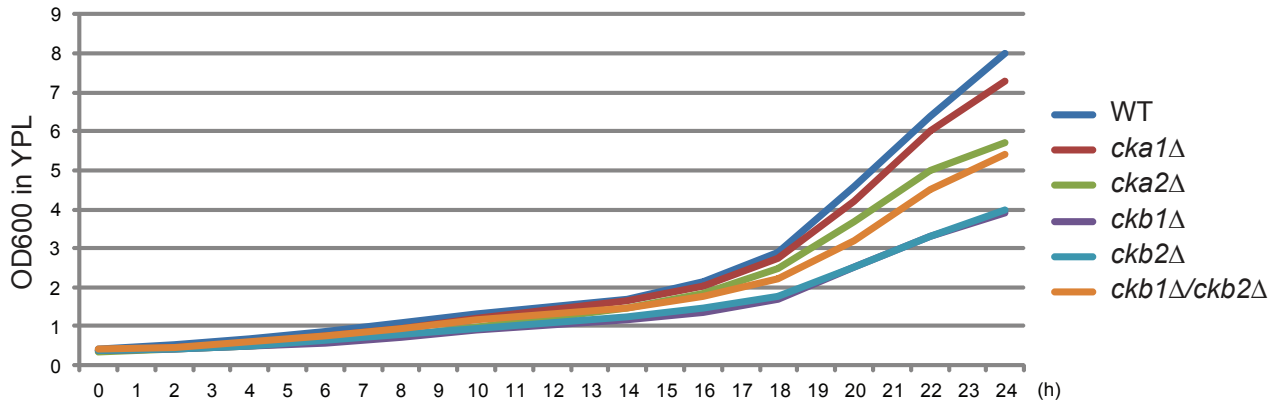
ckb2Δ
ckb1Δ/ckb2Δ

B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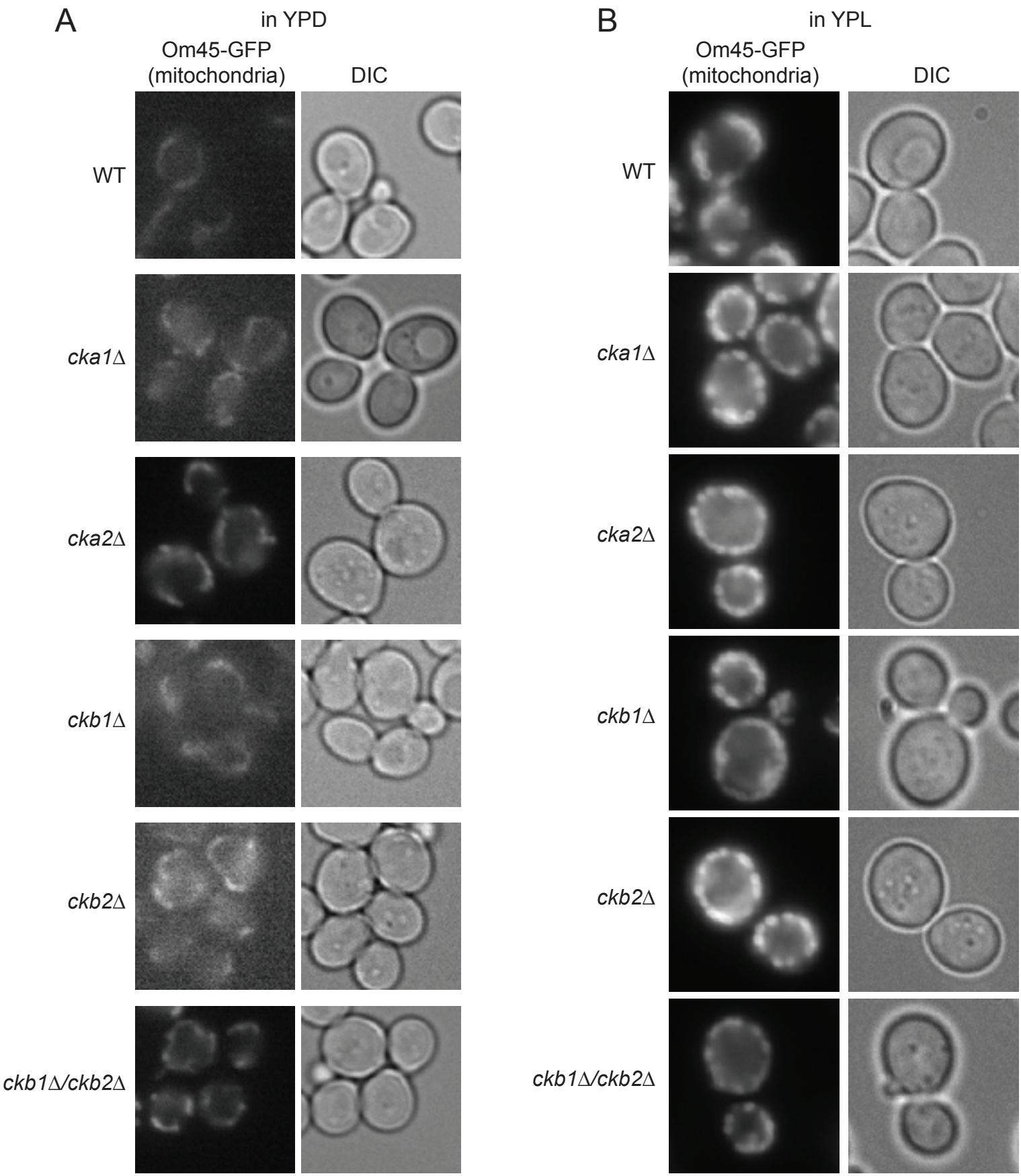
ckb1Δ/ckb2Δ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DIC
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in YPL
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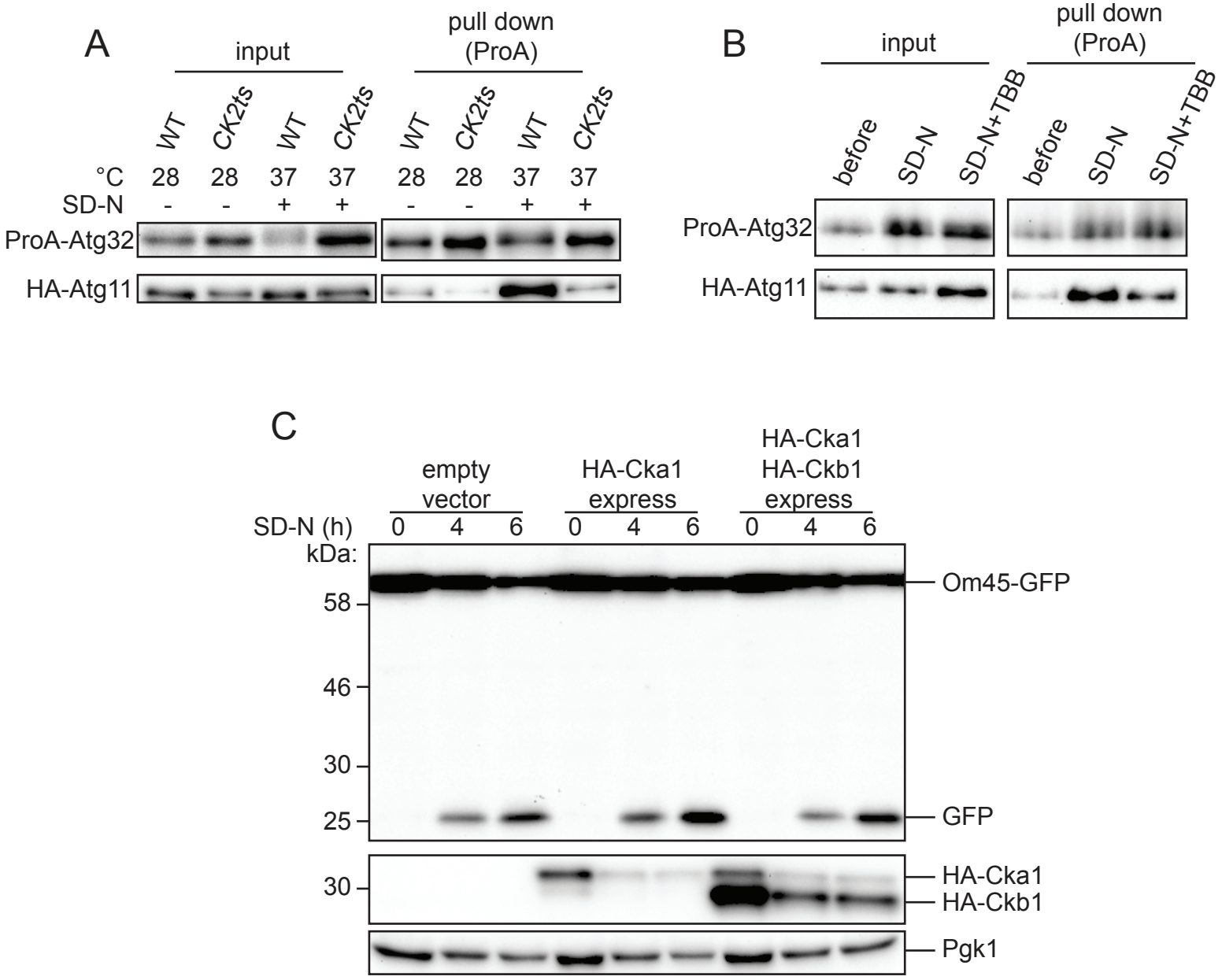
ckb2Δ
ckb1Δ/ckb2Δ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pull down (ProA)
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CK2ts
WT
CK2ts
WT
CK2ts
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SD-N - - + + - - + +
ProA-Atg32
HA-Atg11
B
input
pull down (ProA)
before
SD-N
SD-N+TBB
before
SD-N
SD-N+TBB
ProA-Atg32
HA-Atg11
C
empty vector
HA-Cka1 express
HA-Cka1 HA-Ckb1 express
SD-N (h) 0 4 6 0 4 6 0 4 6
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Om45-GFP
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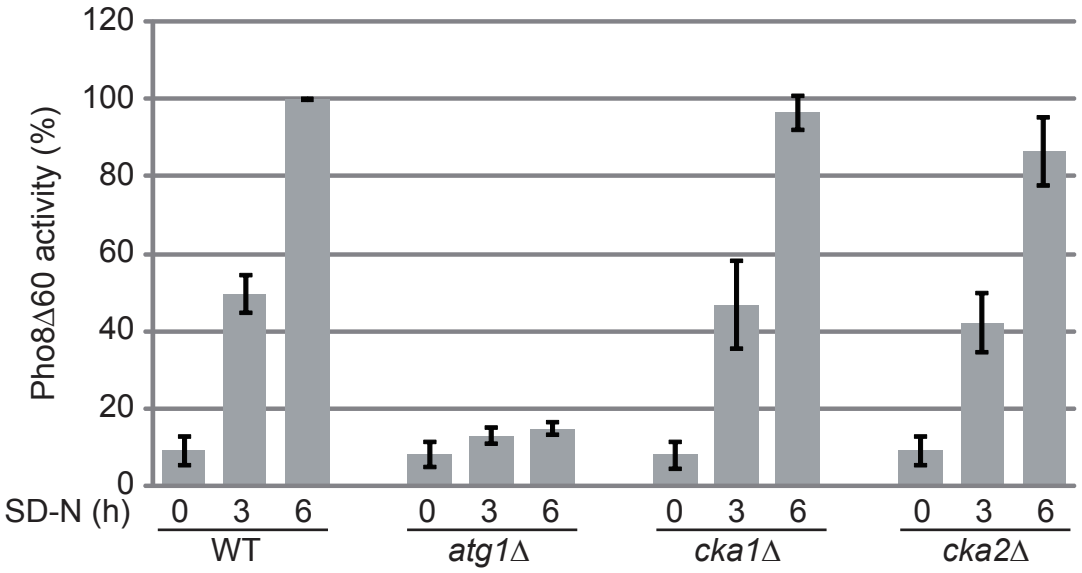
Pgk1

Figure S6

A



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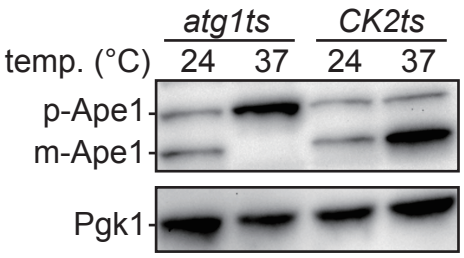

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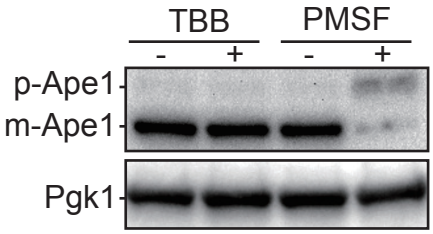
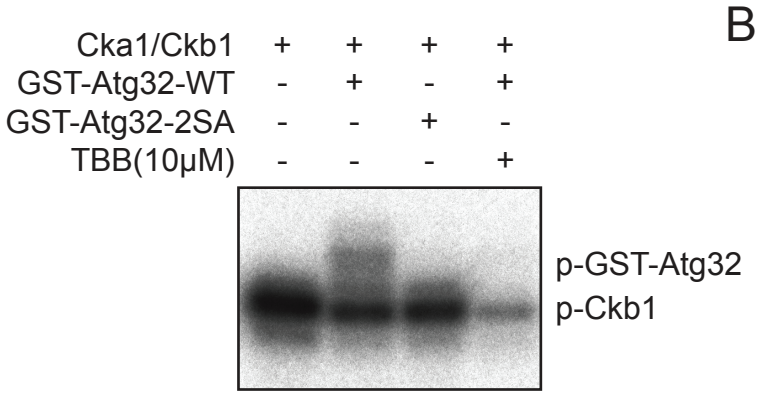
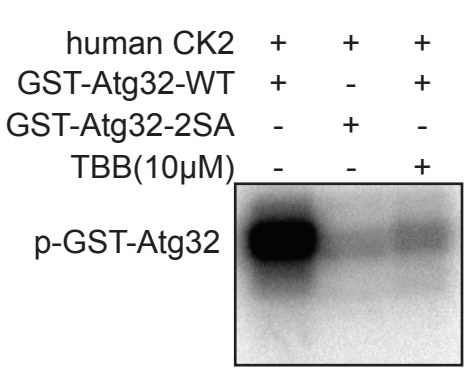


A


Cka1/Ckb1 + + + +
GST-Atg32-WT - + - +
GST-Atg32-2SA - - + -
TBB(10μM) - - - +
p-GST-Atg32
p-Ckb1

B


human CK2 + + +
GST-Atg32-WT + - +
GST-Atg32-2SA - + -
TBB(10μM) - - +
p-GST-Atg32

C

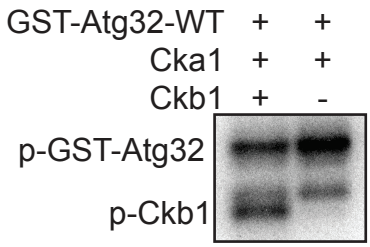
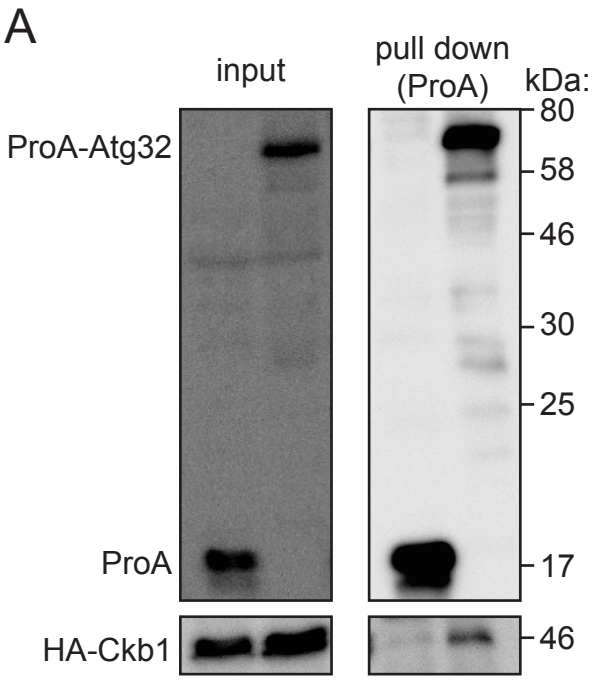
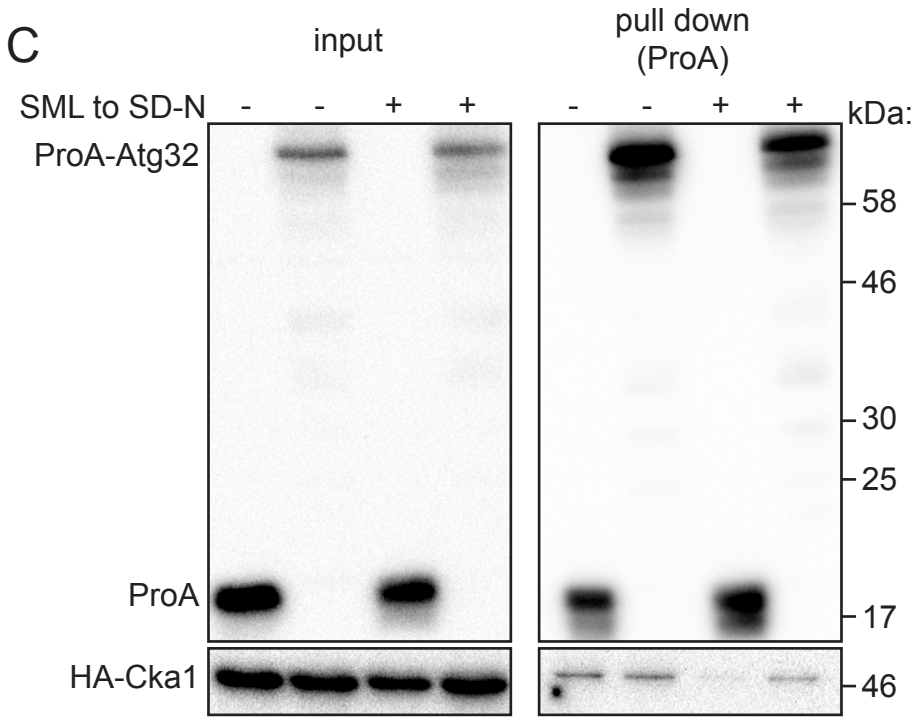

GST-Atg32-WT + +
Cka1 + +
Ckb1 + -
p-GST-Atg32
p-Ckb1

Figure S8


A
input
pull down
(ProA)
kDa:
ProA-Atg32
ProA
HA-Ckb1
80
58
46
30
25
17
46


C
input
pull down
(ProA)
SML to SD-N
- - + +
- - + +
kDa:
ProA-Atg32
ProA
HA-Cka1
58
46
30
25
17
46

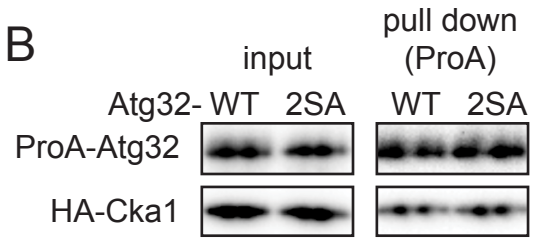
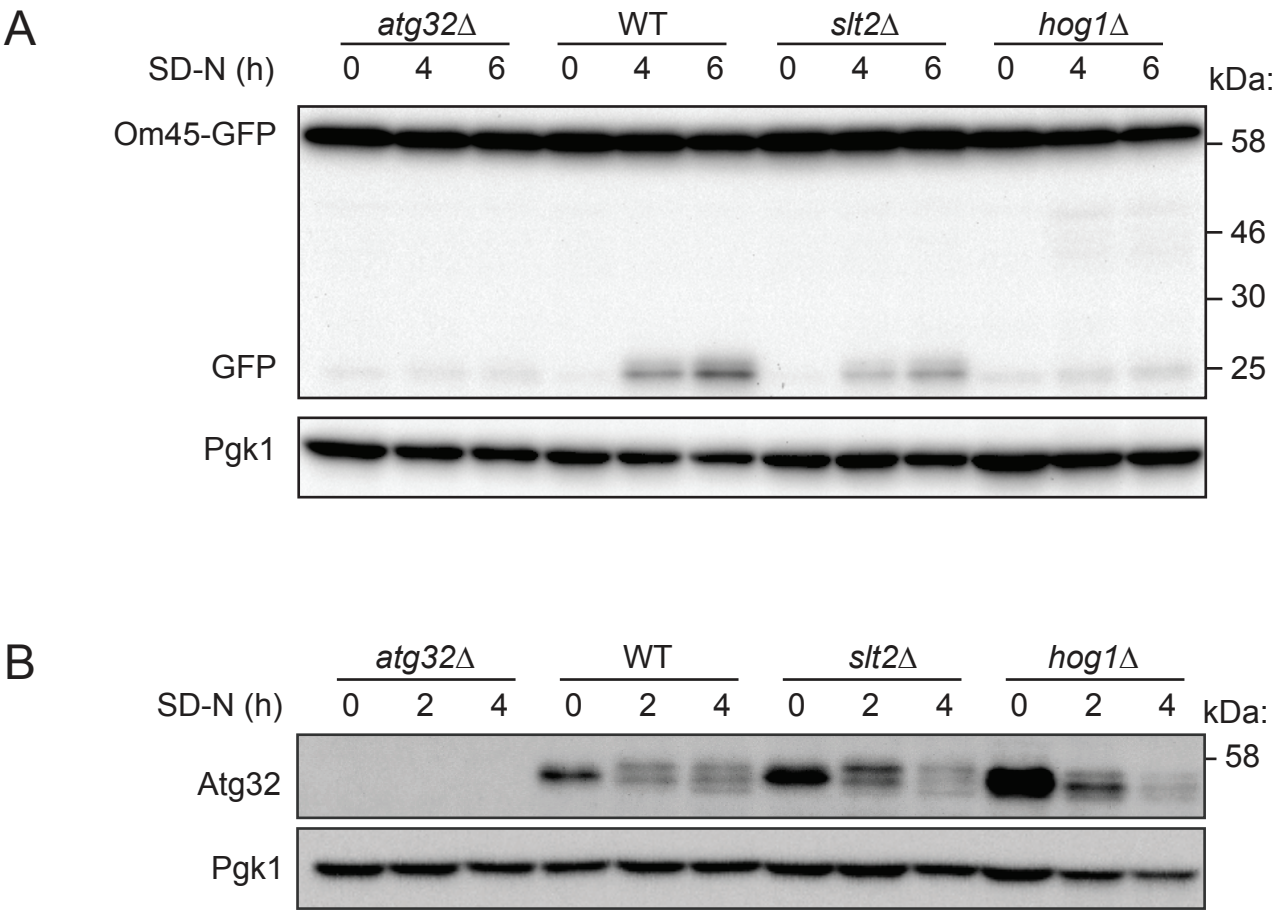

B
input
pull down
(ProA)
Atg32- WT 2SA WT 2SA
ProA-Atg32
HA-Cka1

Figure S9


A
atg32Δ
WT
slt2Δ
hog1Δ
SD-N (h)
0 4 6 0 4 6 0 4 6 0 4 6
kDa:
Om45-GFP
58
46
30
GFP
25
Pgk1
B
atg32Δ
WT
slt2Δ
hog1Δ
SD-N (h)
0 2 4 0 2 4 0 2 4 0 2 4
kDa:
58
Atg32
Pgk1

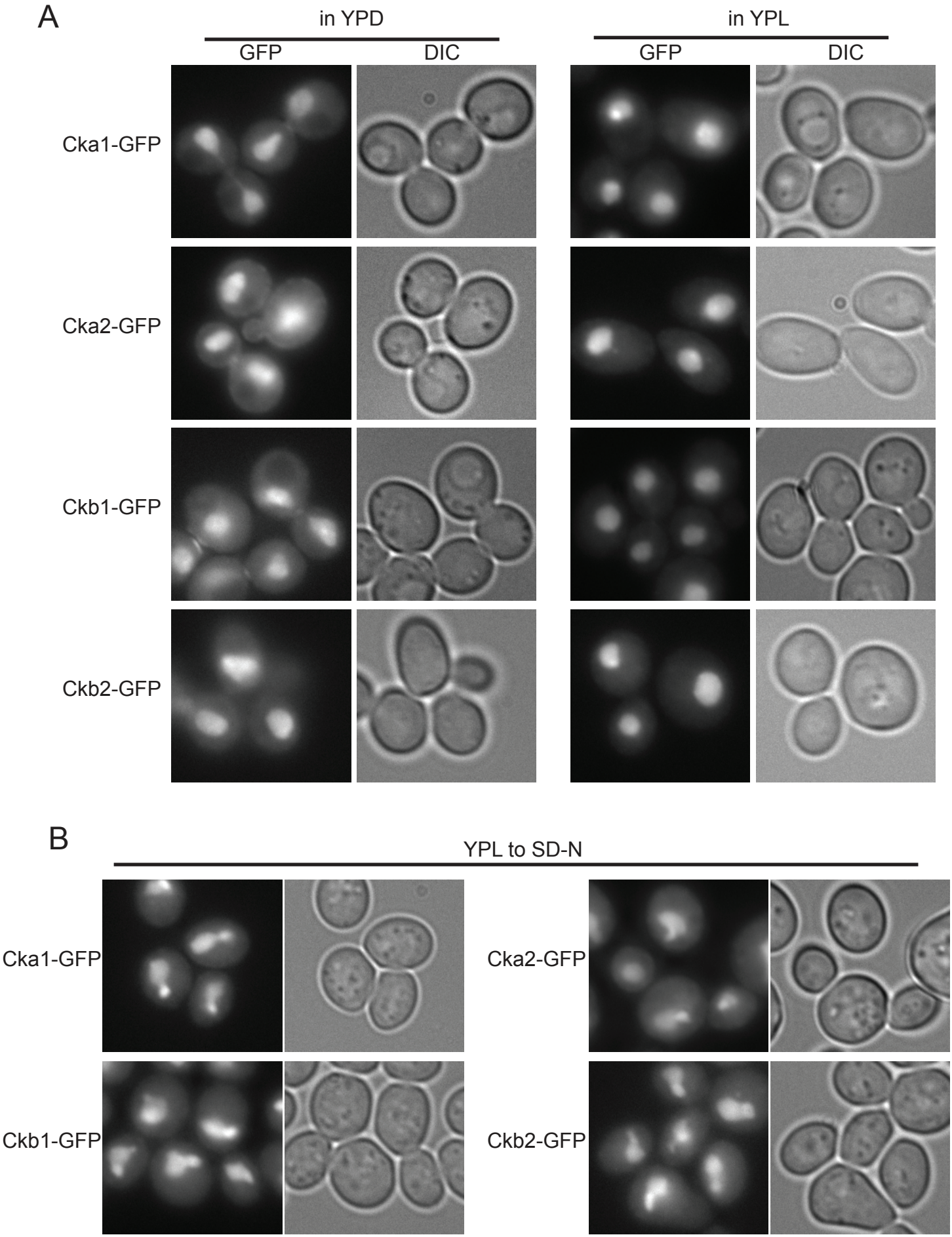

A
in YPD
in YPL
GFP
DIC
GFP
DIC
Cka1-GFP
Cka2-GFP
Ckb1-GFP
Ckb2-GFP
B
YPL to SD-N
Cka1-GFP
Cka2-GFP
Ckb1-GFP
Ckb2-GFP

Figure S11

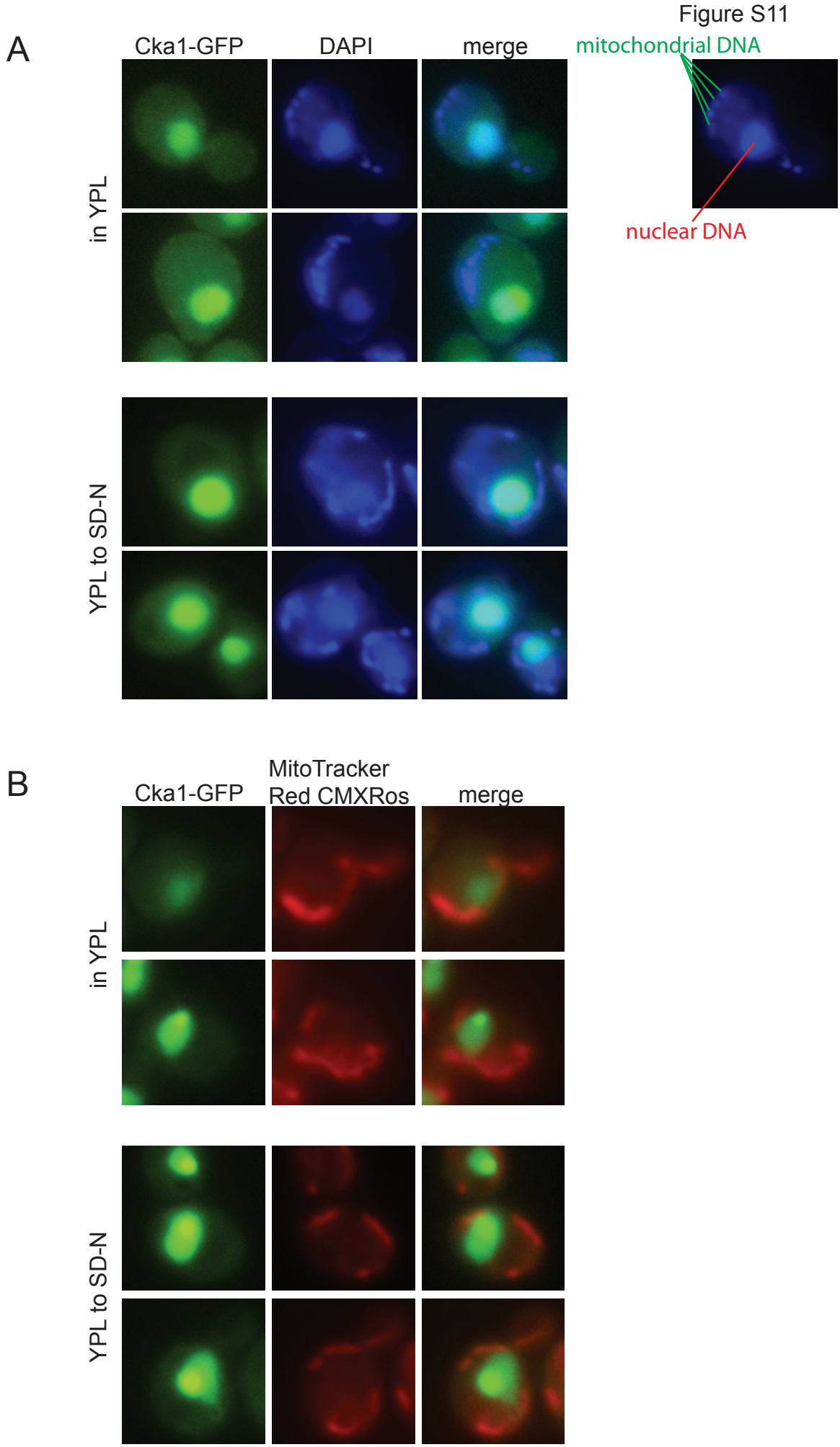
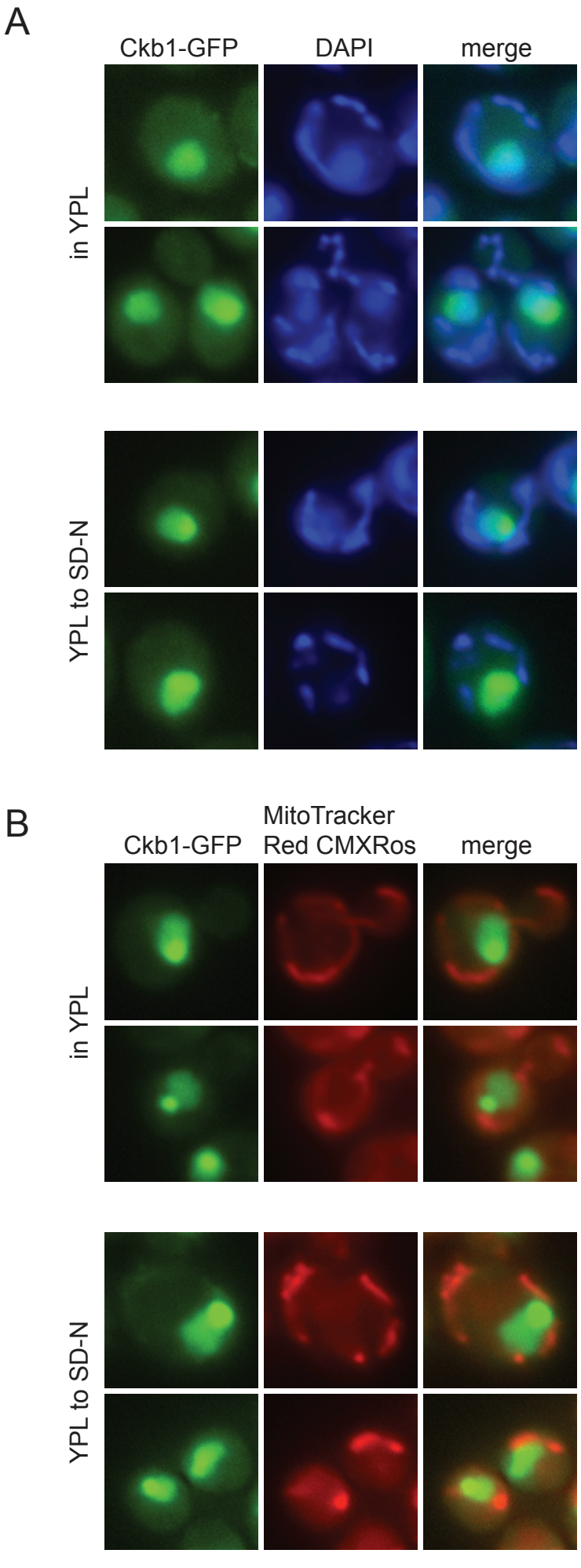

A
Cka1-GFP
DAPI
merge
mitochondrial DNA
nuclear DNA
in YPL
YPL to SD-N
B
Cka1-GFP
MitoTracker
Red CMXRos
merge
in YPL
YPL to SD-N

Figure S12


A
Ckb1-GFP
DAPI
merge
in YPL
YPL to SD-N
B
Ckb1-GFP
MitoTracker
Red CMXRos
merge
in YPL
YPL to SD-N