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Supplemental information

Yeast cell death pathway requiring AP-3 vesicle trafficking leads to vacuole/lysosome membrane permeabilization

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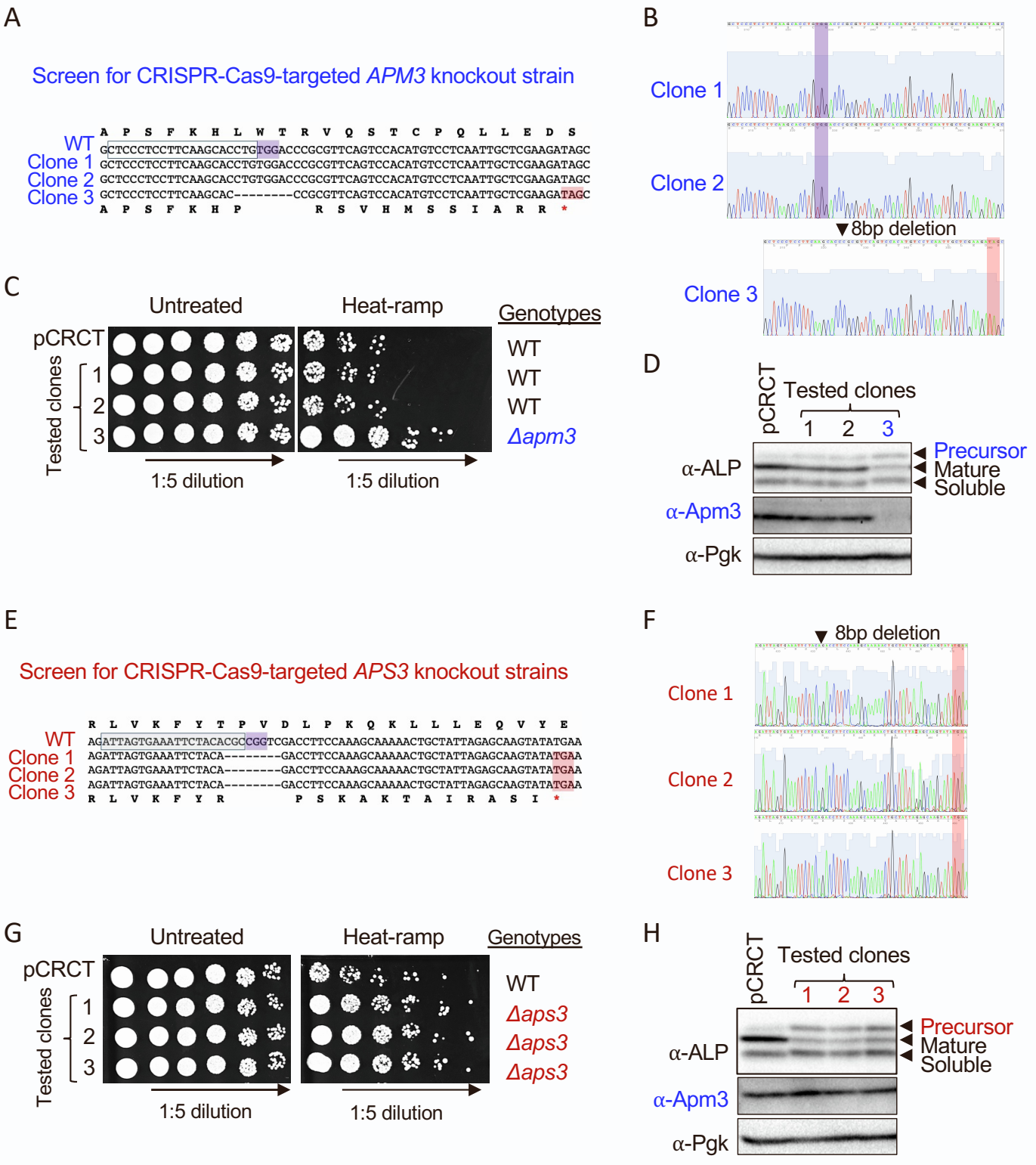


Figure S1. CRISPR-Cas9 design and sequencing for *APM3* and *APS3* knockout strains. [supports Figure 2]
(A and E) Sequence alignments of genomic DNA from three colony-derived yeast clones (BY4709) transformed with gene-targeted or untargeted CRISPR pCRCT plasmids. CRISPR-Cas9 guide RNA sequence (box), PAM sequence (violet), and resulting in-frame stop codon (rose) following 8 bp deletion. (B and F) Sanger sequencing chromatograms for A and E. (C and G) Heat-ramp cell death assays for sequenced clones (30°C to 51°C as described in Figure 1A) confirm that death-resistance correlates with gene disruptions. (D and H) Tests for AP-3 trafficking function by assessing transport and processing of the precursor form of alkaline phosphatase (ALP/Pho8) to mature and soluble forms detected with anti-ALP antibody (from Gregory Payne, UCLA). Apm3 immunoblots confirm lack of expression in *Δapm3*, and evidence for AP-3 complex stability in *Δaps3*, assessed with anti-Apm3 antibodies provided by Dr. Sandra Lemmon, University of Miami (Panek et al., 1997).

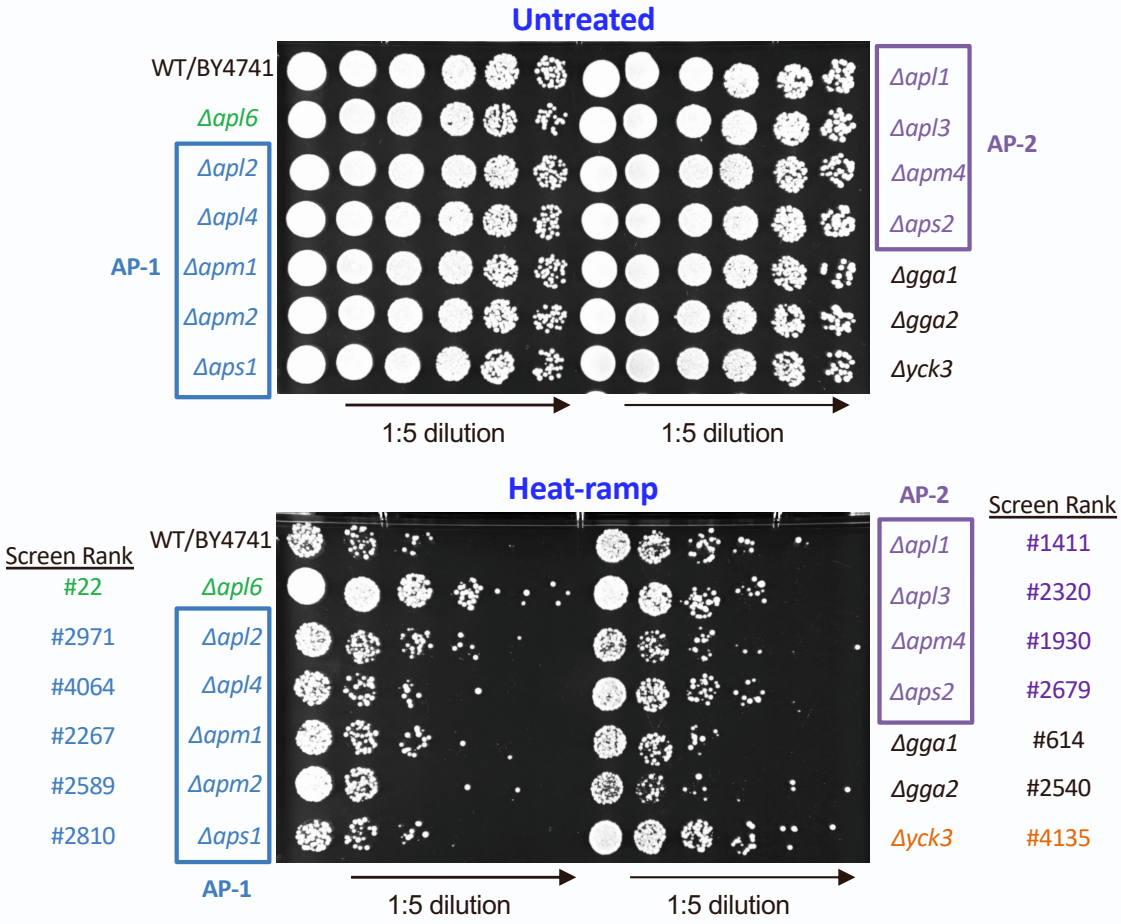


Figure S2. Cell death assay confirming lack of death-resistance for deletion strains of adaptor complexes AP-1 and AP-2. **[supports Figure 2]** Log phase yeast cultures of AP-1 (blue boxes), AP-2 (violet boxes) and other yeast knockout strains (BY4741 *MATa* YKO collection) were spotted on plates before (upper) and after (lower) heat-ramp treatment (20-min, 30°-51°C, from Figure 1A) and compared to the death-resistant AP-3 subunit Apl6 knockout (*Δapl6*). Ranks from the genome-wide heat-ramp screen in Figure 2A and Supplementary Table S1 are shown.

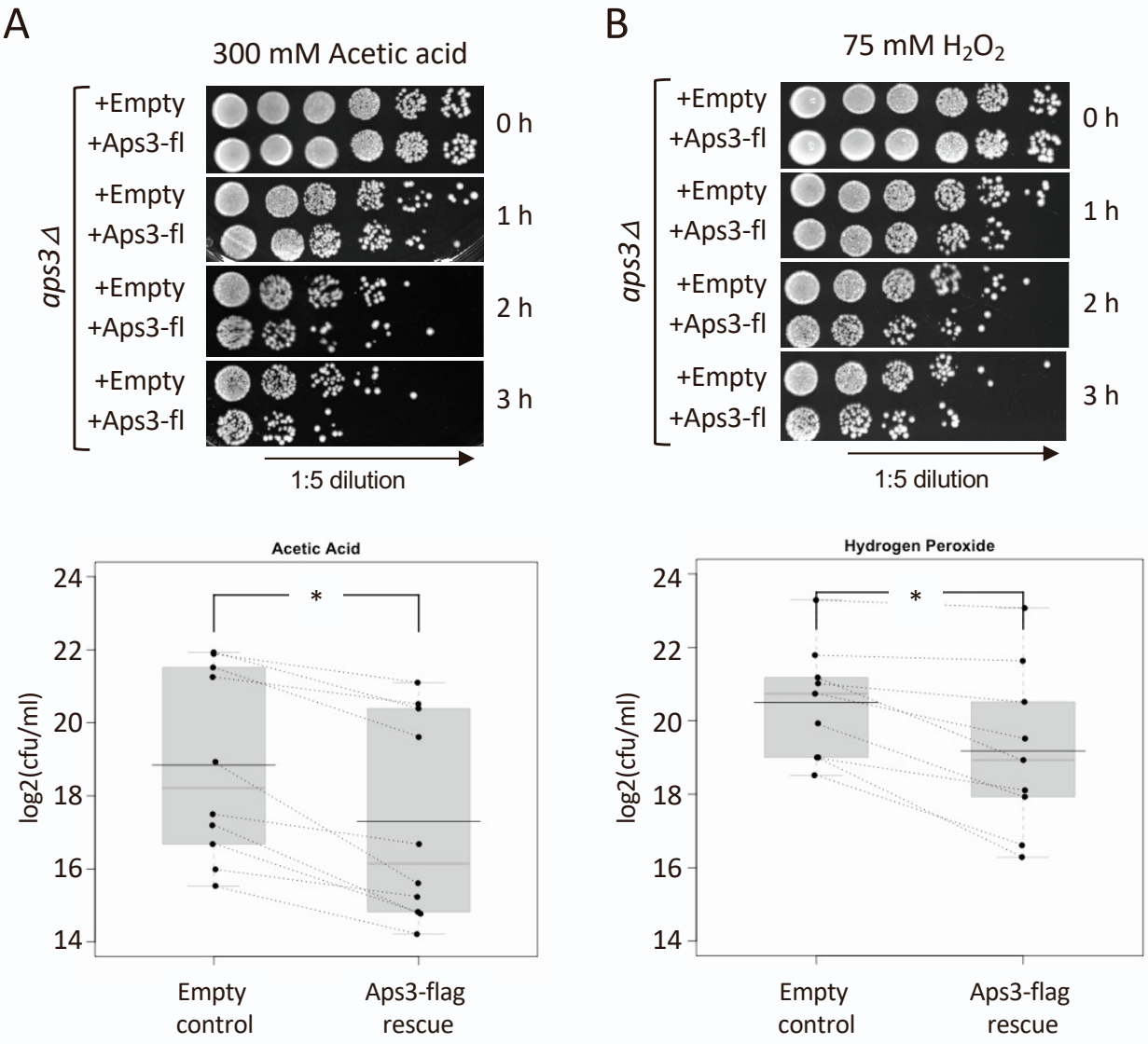


Figure S3. Rescue of *Δaps3* with *APS3-Flag* restores susceptibility to acetic acid and H_2O_2-induced cell death
[supports Figure 3]
(A) Survival of *Δaps3* + untargeted control or APS3-Flag grown to log phase and spotted before and after treatment of *Δaps3* + untargeted control (empty pRS303) or APS3-Flag rescue grown to log phase and spotted before and after treatment with 242-300 mM acetic acid for 1-3 h. Quantified results are from 10 determinations (5 at 2 h and 5 at 3 h) in 5 independent experiments, presented as log2(cfu/ml). Paired, 2-tailed t-test yields *p = 0.00036.
(B) Same as described in A, except treated with 50-75 mM H_2O_2 for 1-3 h. Quantified as for A except for 9 determinations (5 at 2 h and 4 at 3 h) in 5 independent experiments. Paired, 2-tailed t-test yields *p = 0.0029. All data are presented.

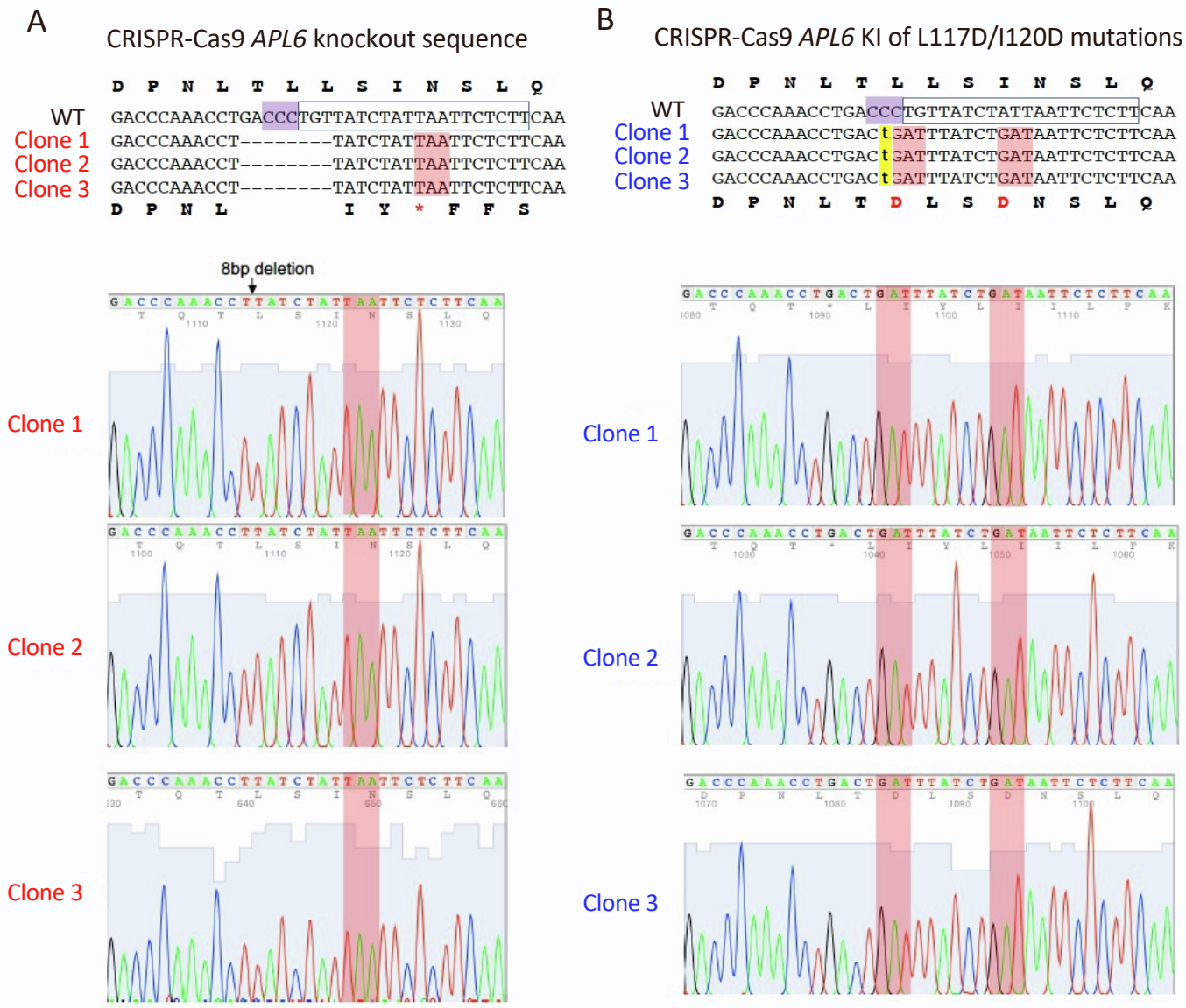


Figure S4. CRISPR-Cas9 design and sequencing for *APL6* knockin and knockout mutant strains. [supports Figure 4]
(A) Genomic DNA sequence alignment of *APL6*-disrupted strains in BY4709 background, showing CRISPR-Cas9 guide RNA sequence (box), PAM sequence (violet), the resulting in-frame stop codon (rose) following 8 bp deletion, and corresponding Sanger sequencing chromatograms.
(B) Genomic DNA sequence alignment of Arf1-interacting site mutations engineered into *APL6* knockin strains (BY4709). CRISPR-Cas9 guide RNA sequence (box); PAM sequence (violet); knockin Leu117Asp/Ile120Asp mutations (rose) based on Ren *et al*. Cell 2013; synonymous C>T nucleotide change engineered to eliminate PAM sequence (lower case, yellow highlight); corresponding Sanger sequencing chromatograms.

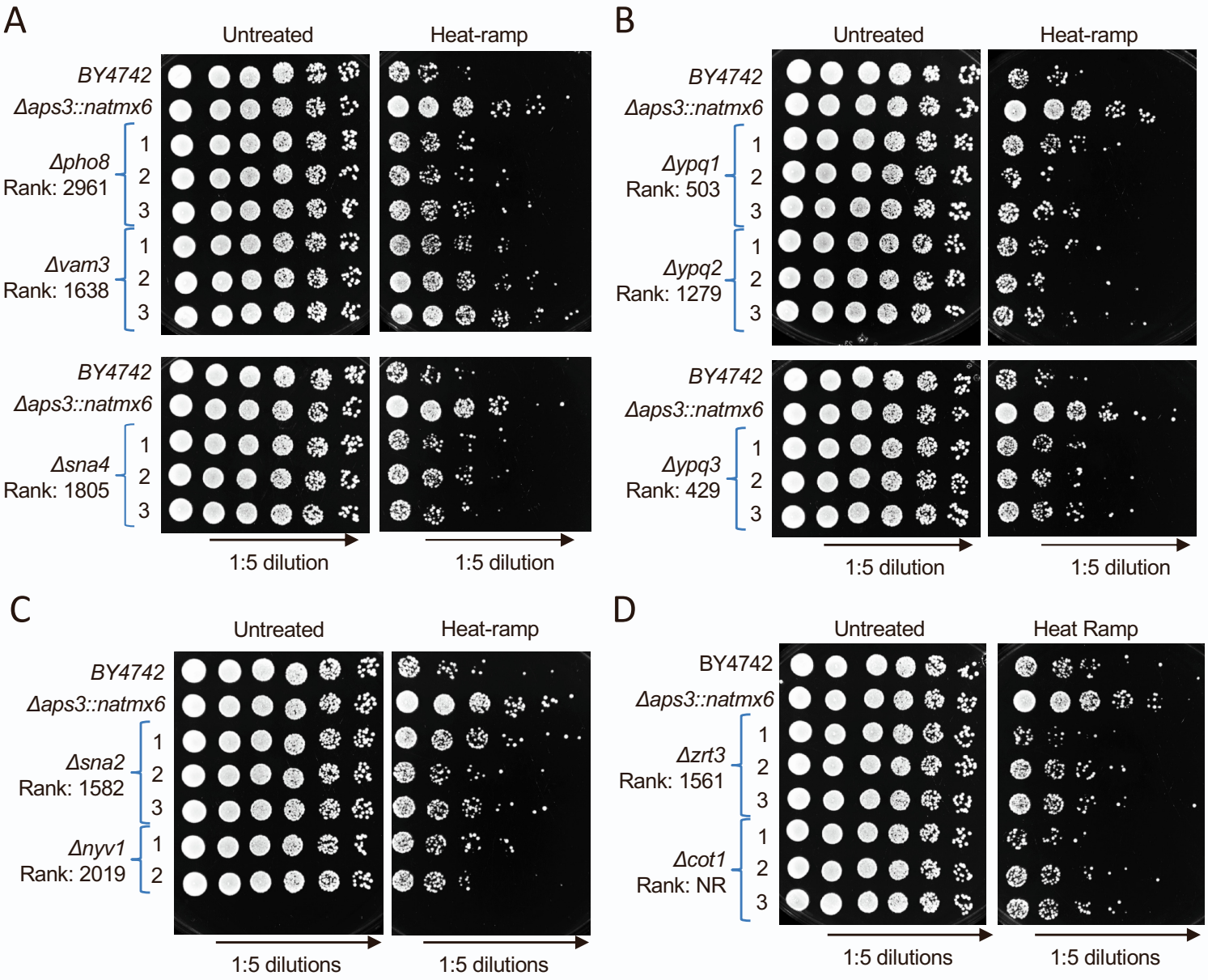
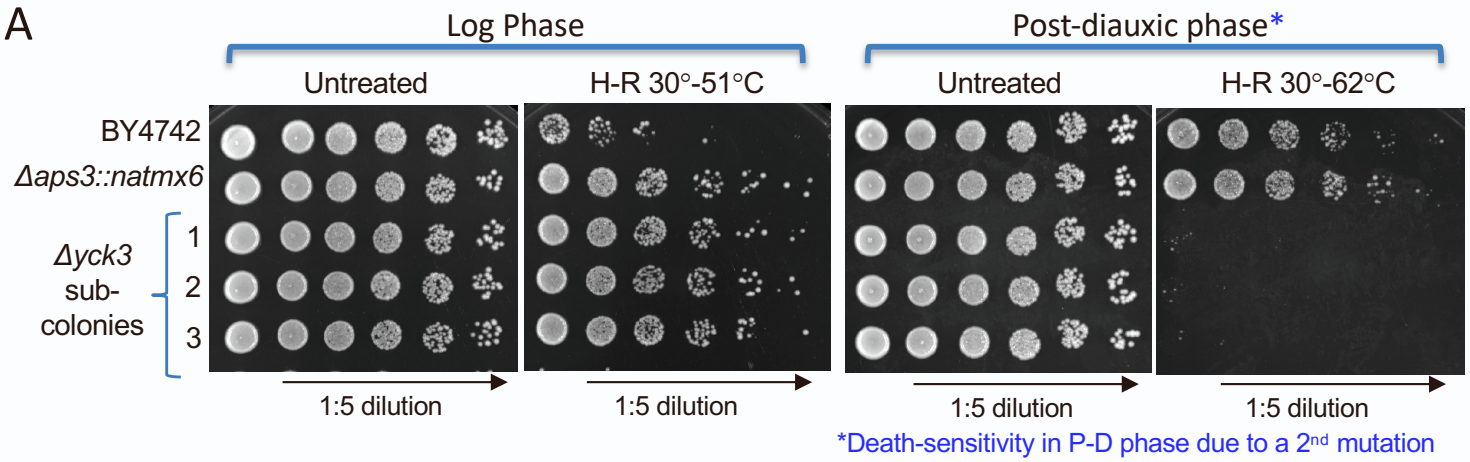


Figure S5. Retesting knockout strains of AP-3 cargo for cell death phenotypes. **[supports Figure 4]** Cell death assays for knockouts of AP-3 cargo proteins that contain a di-leucine motif (A and B), or a tyrosine motif (C), for interacting with AP-3, and other candidate cargo (D). Growth/survival of spotted yeast cultures before and after heat-ramp (20 min, 30°C to 51°C, Figure 1A). Each strain from the BY4741 YKO collection was streaked onto rich (YPD) agar plates and three single cell-derived colonies (substrains #1-3) were tested in parallel with the AP-3 knockout *Δaps3* and its parental wild type (BY4742). Five-fold serial dilutions were plated (5 µL) and imaged after incubating 2-days at 30°C. Rank: z-scores for cell death resistance from the genome-wide screen.

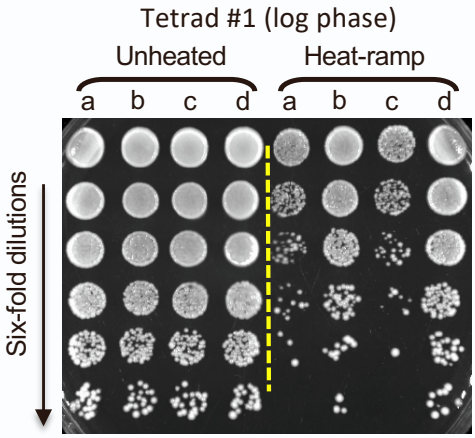
A



B

H-R assay prep

Overnight culture OD_{600} (1:10 dilution)	
a	0.690
b	0.641
c	0.697
d	0.734
3 h OD_{600} (1:2)	
a	0.240
b	0.232
c	0.240
d	0.218
Renormalized for H-R	
a-d	0.4



WT *BY4742alpha* x *YCK3::KANMX* BY4741							
tetrad	spore	*MATa*	*MATα*	*LYS2*	*MET15*	*KAN*	H-R
#1	a	+			+		+
	b		+	+		+	++++
	c		+				+
	d	+		+	+	+	++++

Summary of tetrads from △*yck3* x WT

WT x	2:2 segregation of △*yck3* with **death-Resistance**	segregation of △*yck3-only* with death-**Sensitivity**
△*yck3*	8 of 8 tetrads (log phase)	0 of 5 tetrads (post-dauxic)

C

Summary of tetrads from △*yck3* x △*aps3*

△*aps3* x	2:2 segregation of △*aps3* and/or △*yck3* & **death-R**	2:2 segregation of △'s & **death-Sensitivity**
△*yck3*	8 of 8 tetrads (log phase)	0 of 5 tetrads (post-dauxic)

Figure S6. Deletion of *YCK3* confers death-resistance [supports Figure 4 main text]
(A) Heat-ramp cell death assay for single cell-derived substrains of *Δyck3* (BY4741 YKO collection) and reference strains tested in log-phase (20-min, 30°C to 51°C, as in Figure 1A) and in post-diauxic phase used in the genome-wide screen (20-min, linear 30°C to 62°C). Note this BY4742 strain is more death resistant than most BY4741
(B) Cell death assay results for example tetrad (4 spore-derived strains, a-d) from crossing WT x *Δyck3* and tested as in A. ODs of yeast cultures during sample preparation prior to heat-ramp (left) to minimize effects of metabolic state differences on cell death versus survival following heat-ramp (middle). Tetrad validation markers (upper right), reveal co-segregation of the knockout locus (*KanMX*) with death-resistance, summarized for all tetrads tested (lower right). Death-resistance (R), death-sensitive (S).
(C) Summary of cell death results for all tetrads tested from crossing *Δyck3* x *Δaps3* in log and post-diauxic phases as in A. Cell death sensitivity in post-diauxic phase-only is due to a secondary mutation that segregates independently, and is assumed to be present in the original *Δyck3* strain as it is present in crosses to both *Δaps3*, and to WT (B), while crosses *Δaps3* or other AP-3 knockouts to WT lack this mutation.
Post-diauxic heat-ramp method details. Strains were streaked onto rich YPD agar, incubated 2-days at 30ºC, and approximately matched numbers of cells from individual colonies (substrains) or the population, were grown in liquid YPD on a roller at 30ºC for ~16 h. Starting OD_{600} was recorded, cultures were normalized to 0.2 OD_{600}/mL in 3 mL fresh YPD, and grown for 3 h with rotation at 30°C to reach log phase with closely matched OD_{600} between samples (~0.45 OD_{600}). Cultures were re-normalized to 0.4 OD_{600}, 100 µL was transferred to 0.2 mL tubes for heat-ramp treatment in a thermocycler, and 3 µL of serial dilutions were immediately spotted on YPD agar and incubated 18-24 h for BioSpot imaging, or 2-days for visual imaging on a ChemiDoc® Imager (Bio-Rad, CA, USA), exposure time 0.175 seconds.

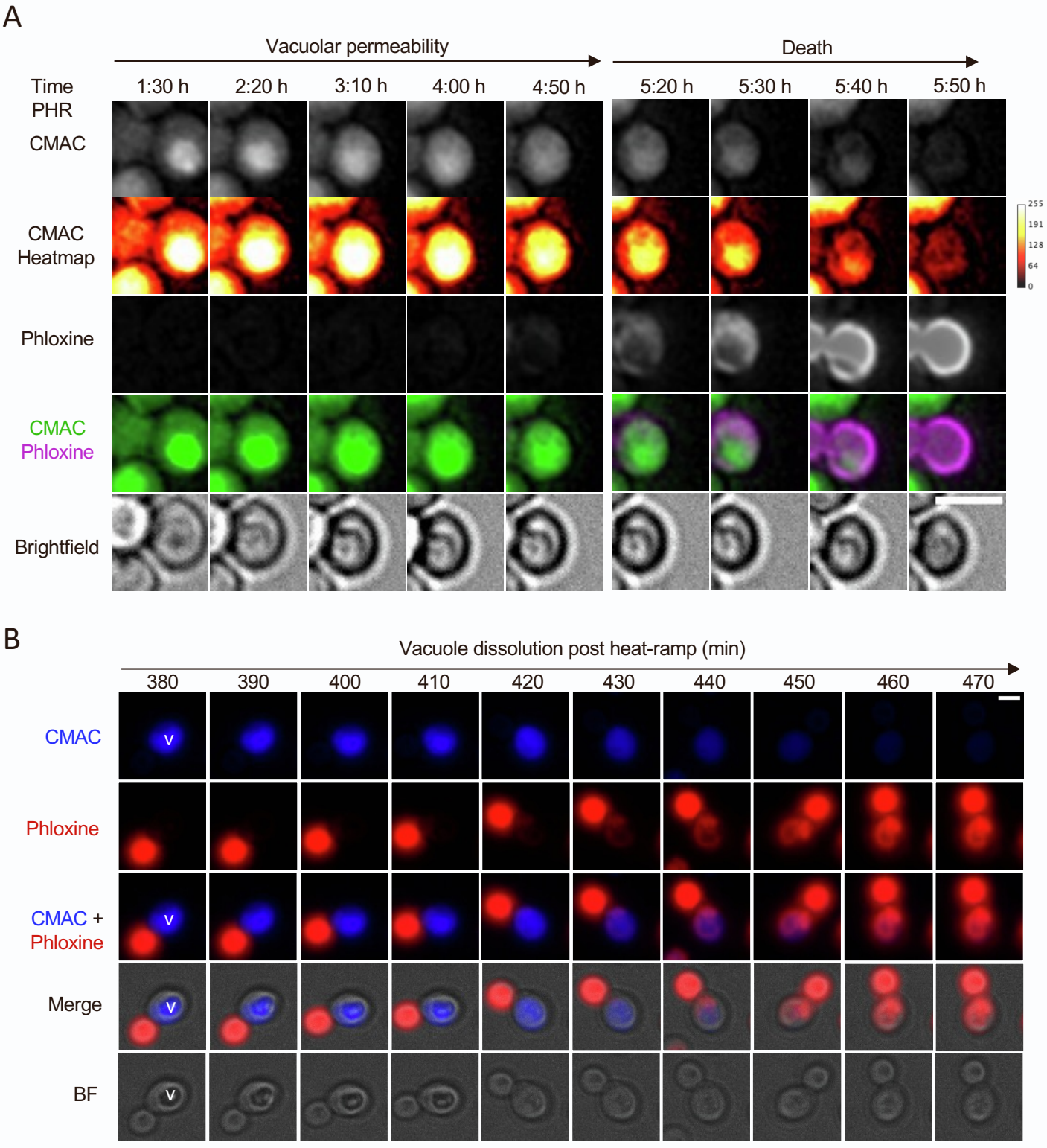


Figure S7. Vacuole membrane permeabilization after heat-ramp treatment. [supports Figure 6A]
(A) A second example as described in Figure 6A, time stamped post heat-ramp (PHR). Scale bar 2.5 μm.
(B) Non-deconvolved micrographs from an independent experiment showing vacuole (v) permeabilization and subsequent phloxine staining of WT cells after heat-ramp. Scale bar 2.5 μm.

Table S2. Primers and CRISPR gene block sequences (related to STAR Methods)

Primer	Use/Description	Sequence
Δaps3 NAT FW	Upstream primer for generation of Δaps3::NatMx6	AGATCTGGGTTACAGAGGCCAGAACACATATAACGTTACACAATGGAC ATGGAGGCCCAGAATACC
Δaps3 NAT REV	Upstream primer for generation of Δaps3::NatMx6	CTTGACGAACGAAAATAACCATCATCCGATTAATTTGTTTGTTTACAGTA TAGCGACCAGCATTCA
APS3 Primer A v2	FWD primer for APS3 Genotyping	TAGTTCTCATTCAGTCTTTTTTCTTTCTTTTTGAATACGATGG
APS3 Primer D v2	Reverse primer for APS3 Genotyping	CCTGTCAAAAAGAAAGAAAACTCATTCTCTAGACACAGCGAGAT
5' NatMX6 REV	5' insertion genotyping primer for Δaps3::NatMx6	GAAAGAAGAACCTCAGTGGCAAATCCTAACCTTTTATATT
3' NatMX6 FWD	3' insertion genotyping primer for Δaps3::NatMx6	TCAGTACTGACAATAAAAAGATTCTTGTTTTCAAGAACTT
APS3 Primer B	5' WT internal REV genotyping primer for APS3	TTGCTCTAATAGCAGTTTTTGCTTT
APS3 Primer C	3' WT internal FWD genotyping primer for APS3	TGAATTGATTTCTCAAAGGAATAGC
APM3 Primer A v2	FWD primer for APM3 Genotyping	ATGTTGCTAAATATATTTCCAATACTATTTGGAACGCTGTTGGTA
APM3 Primer D v2	REV primer for APM3 Genotyping	TTTTGACTGTTTCCCCTCCTGCCATTCAATTAACTCAAATCACTC
APL6 Primer A	FWD primer for APL6 Genotyping	AAGTTACCAAAAGATTCGAAGAGGT
APL6 Primer D	REV primer for APL6 Genotyping	TGTATTAGGCTTGACTTTCTTCACC
VPH1-Envy FWD	FWD primer for generation of VPH1 C' terminally tagged with Envy	ATGGAAGTCGCTGTTGCTAGTGCAAGCTCTTCCGCTTCAAGC GGTGACGGTGCTGGTTTA
VPH1-Envy REV	REV primer for generation of VPH1 C' terminally tagged with Envy	GTACTTAAATGTTTCGCTTTTTTTAAAAGTCCTCAAAATTTATCGAT GAATTCGAGCTCG
PEP4-Envy FWD	FWD primer for generation of PEP4 C' terminally tagged with Envy	TACGATTTGGGCAACAATGCGGTTGGTTTGGCCAAAGCAATTGGTG ACGGTGCTGGTTTA
PEP4-Envy REV	REV primer for generation of PEP4 C' terminally tagged with Envy	GCAGAAAAGGATAGGGCGGAGAAGTAAGAAAAGTTTAGCTCATC GATGAATTCGAGCTCG
APS3 Endo Prom FWD	FWD primer for cloning APS3 under native promoter into pRS303	TAGAACTAGTGGATCTTTCTTTTTTATATTTCTTCTCTT
APS3 FLAG REV	REV primer for cloning APS3-FLAG into pRS303	TAGAACTAGTGGATCTTTCTTTTTTATATTTCTTCTCTT

gBlocks	Sequence
APS3 deletion	CTTTGGTCTCACCAAAACTTGGTATAGTCAATAAGAAATGCCAACCAAGATTAGTGAAATTCTACAGACCTTCCAAAGCAAAAACTGCTATTA GAGCAAGTATATGAATTGATTTCTCATTAGTGAAATTCTACACGCGTTTTAGAGAGAGACCTTTC
APM3 deletion	CTTTGGTCTCACCAAAACCTTATTTTCCAGTATCTTTTAGGTGCAACAGCTCCCTCCTTCAAGCACCCGCGTTCAGTCCACATGTCCTCAATTGC TCGAAGATAGCAGTAGTGATGATCTCCCTCCTTCAAGCACCTGGTTTTAGAGAGAGACCTTTC
APL6 deletion	CTTTGGTCTCACCAAAACAGACTTATTCACCTGTATTTACTTAGATTTGCAGAAAATGACCCAAACCTTATCTATTAATTtTCTTCAAAAATCATT GTCTGATTCGAATTCCGAACTGAAGAGAATTAATAGATAACAGTTTTAGAGAGAGACCTTTC
APL6 L117D/I120D	CTTTGGTCTCACCAAAACAGACTTATTCACCTGTATTTACTTAGATTTGCAGAAAATGACCCAAACCTGACtgatTTATCTgatAATTCTCTTCAAA AATCATTGTCTGATTCGAATTAAGAGAATTAATAGATAACAGTTTTAGAGAGAGACCTTTC