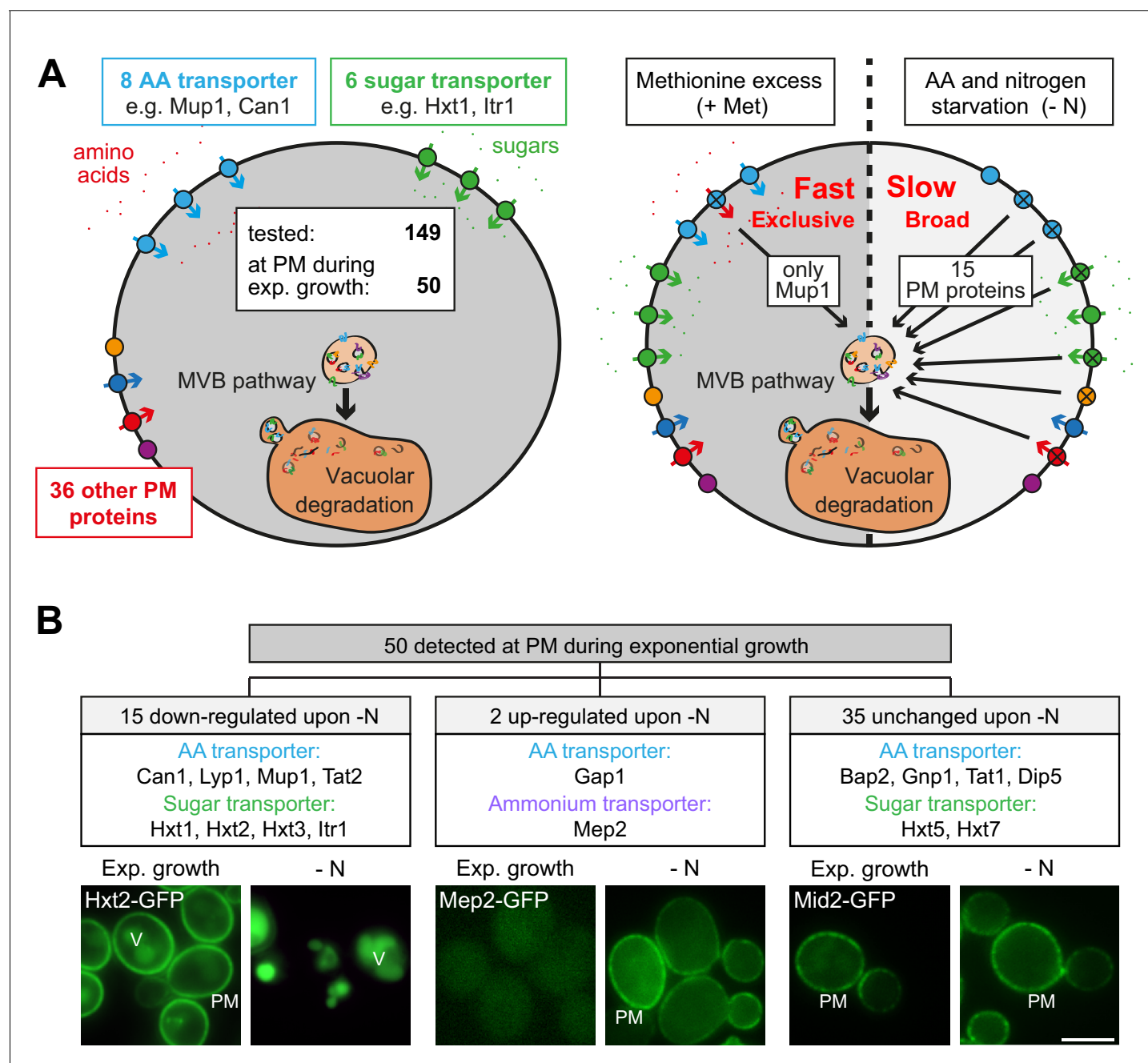


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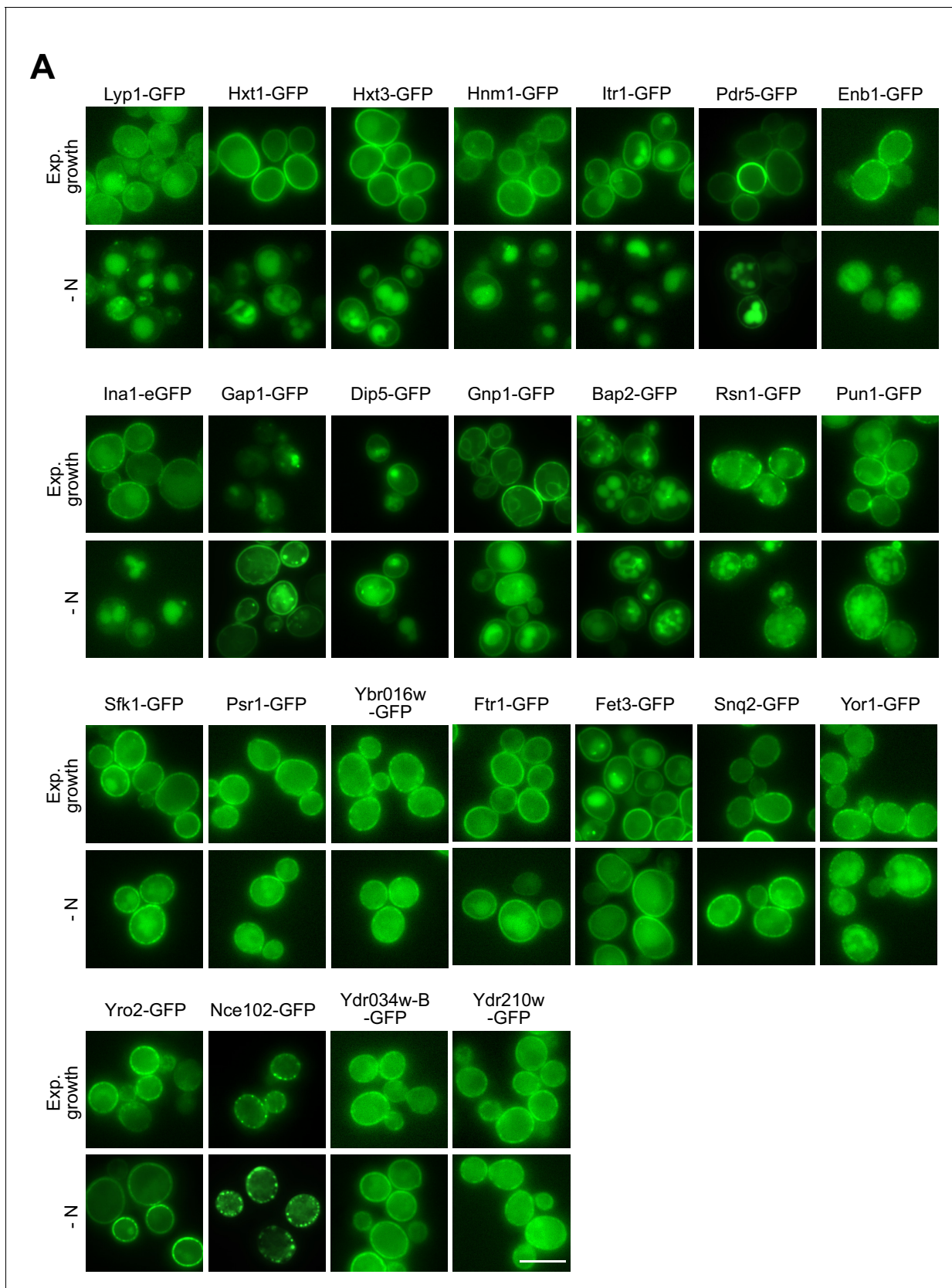
## Figures and figure supplements

Complementary  $\alpha$ -arrestin-ubiquitin ligase complexes control nutrient transporter endocytosis in response to amino acids

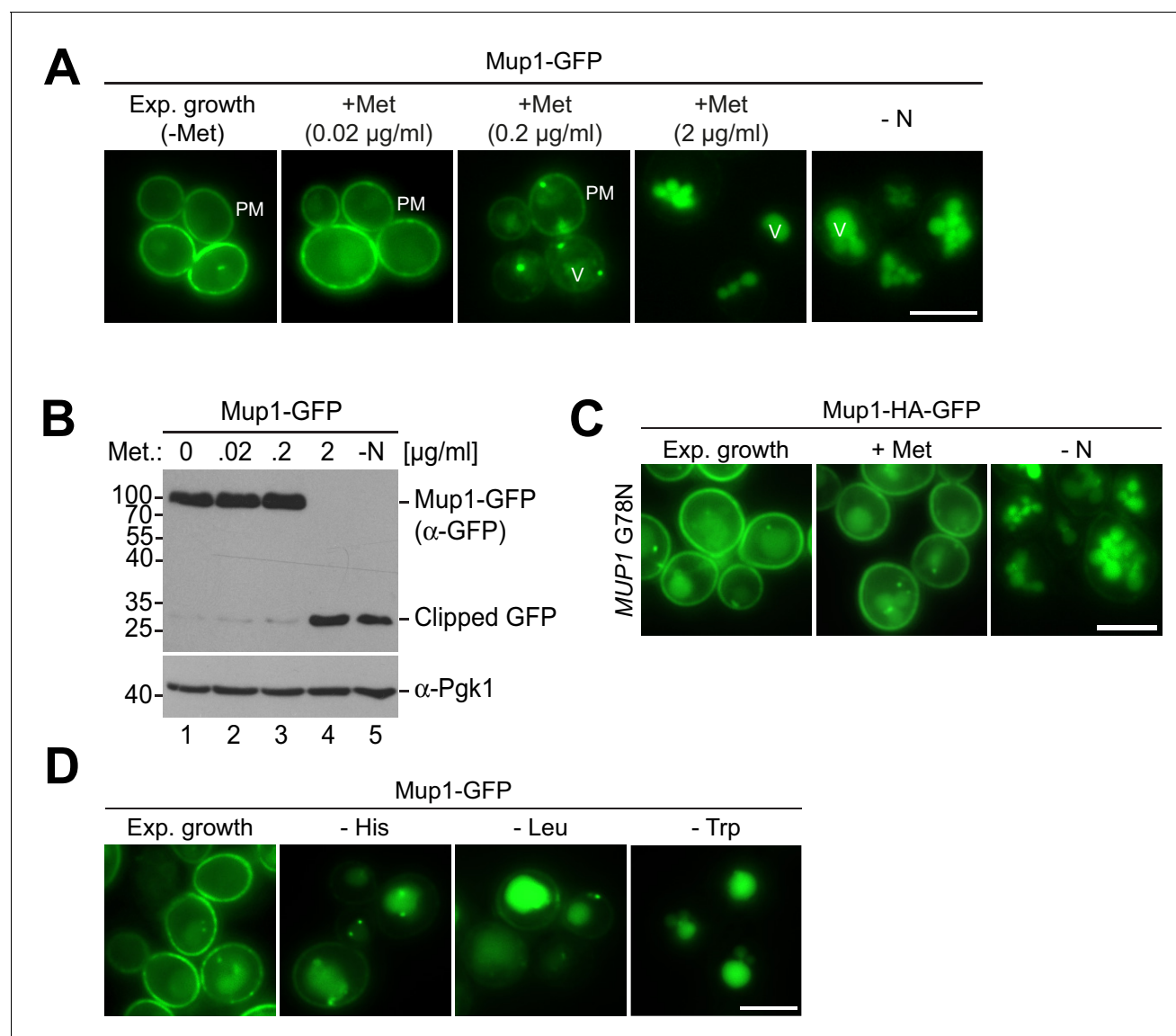
**Vasyl Ivashov et al**



**Figure 1.** Amino acid and nitrogen starvation triggers broad but specific endocytosis and lysosomal degradation of plasma membrane proteins. (A) Left: a library of 149 yeast strains expressing chromosomally GFP-tagged membrane proteins was tested for plasma membrane (PM) localization during nutrient replete exponential growth. Right: verified PM proteins were starved 6–8 hr for amino acids and nitrogen (- N) or treated with 20  $\mu$ g/ml L-methionine (+Met) after 24 hr of exponential growth. The localization of GFP was assayed by fluorescence microscopy. (B) Summary of the phenotypes of GFP-tagged PM proteins during starvation. Indicated are numbers of PM proteins that are down-regulated, up-regulated or unchanged compared to the exponential growth phase, each exemplified by one representative strain. PM: plasma membrane; V: vacuole. Scale bars = 5  $\mu$ m. See also *Figure 1—figure supplements 1 and 2* and *Supplementary file 1*.

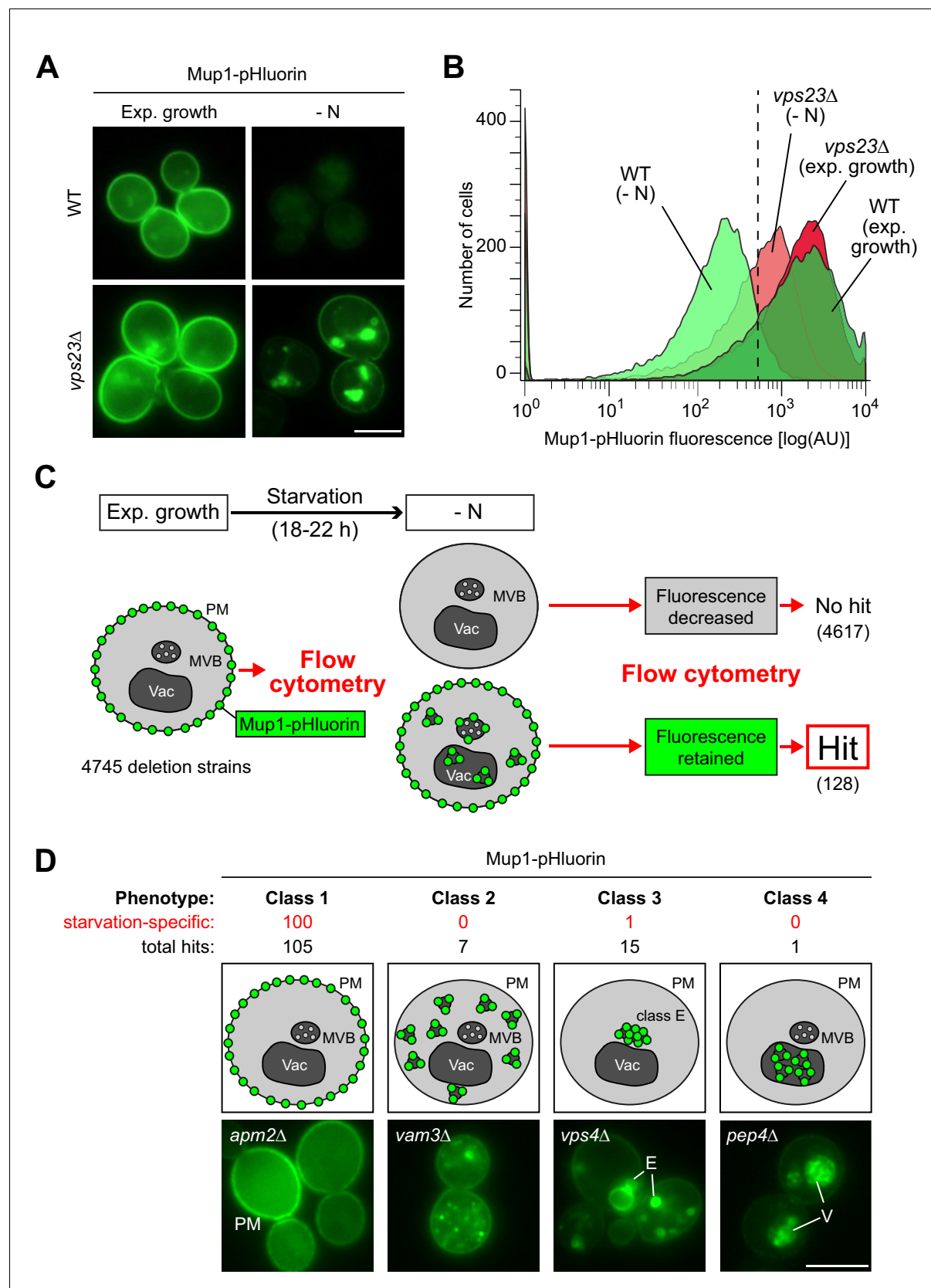


**Figure 1—figure supplement 1.** Localization of PM proteins during exponential, rich growth and starvation. (A) Live-cell fluorescence microscopy analysis of chromosomally GFP-tagged plasma membrane proteins. Cells were starved (- N) for 6–8 hr after 24 hr exponential growth. Scale bar = 5  $\mu$ m.



**Figure 1—figure supplement 2.** Characterization of starvation- and substrate-induced endocytosis of Mup1. (A) Live-cell fluorescence microscopy analysis of WT cells expressing *MUP1-GFP*. Cells were treated with the indicated methionine concentrations (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (B) SDS-PAGE and western blot analysis with the indicated antibodies of WT cells expressing *MUP1-GFP*. Cells were treated with the indicated methionine concentrations (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (C) Live-cell fluorescence microscopy analysis of *MUP1(G78N)-HA-GFP* cells. Cells were treated with 20 µg/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (D) Live-cell fluorescence microscopy analysis of WT cells expressing *MUP1-GFP*. Cells were starved for histidine (- His), leucine (- Leu) or tryptophan (- Trp) for 4.5 hr after in presence of all other amino acids required for growth after 24 hr nutrient replete exponential growth. PM: plasma membrane; V: vacuole. Scale bars = 5 µm.



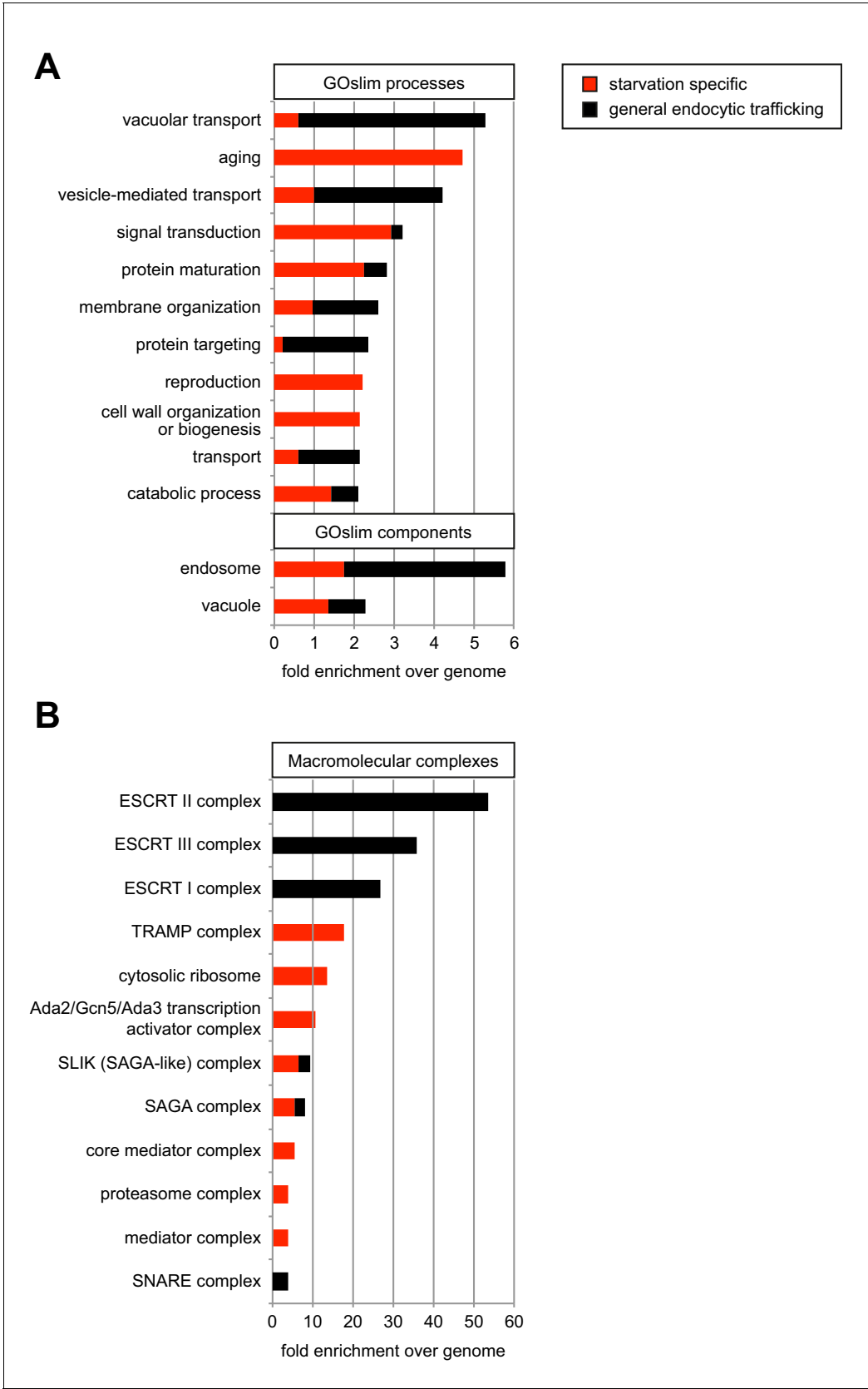


**Figure 2.** A genome wide screen revealed genes affecting Mup1-pHluorin endocytosis during starvation. (A) Live-cell fluorescence microscopy analysis of WT (BY4742) and *vps23Δ* cells expressing *MUP1*-pHluorin from plasmid and starved (- N) for 18–22 hr. The images exemplify quenched pHluorin

Figure 2 continued on next page

## Figure 2 continued

fluorescence in vacuoles of wild type (WT)-like cells and retained fluorescence in mutants with defects in the starvation-induced endocytosis of Mup1-pHluorin. (B) The strains from (A) were exponentially grown in 96-well plates for 5 hr and starved (- N) for 18–22 hr. At least 15,000 cells from each strain and condition were analyzed by flow cytometry. The exemplified histograms display decrease of fluorescence in wild type (WT)-like strains and fluorescence retention in mutants with defects in the starvation-induced endocytosis of Mup1-pHluorin (e.g. *vps23Δ*). (C) Workflow of the flow-cytometry-based genome-wide screen for mutants defective in starvation-induced endocytosis of Mup1-pHluorin. (D) Summary of phenotypes of all mutants scored in the starvation-induced endocytosis screen (C) as determined by fluorescence microscopy. Class one mutants retain Mup1-pHluorin fluorescence at the plasma membrane (PM); class two mutants in small cytosolic objects; class three mutants in class E-like objects (E); class four mutants within vacuoles (V). Each phenotype is exemplified by one representative deletion mutant. Indicated are the numbers of strains that share a similar phenotype and the number of hits specific for starvation-induced endocytosis of Mup1-pHluorin (red). Scale bars = 5  $\mu$ m. See also **Figure 2—figure supplement 1** and **Supplementary file 2**.

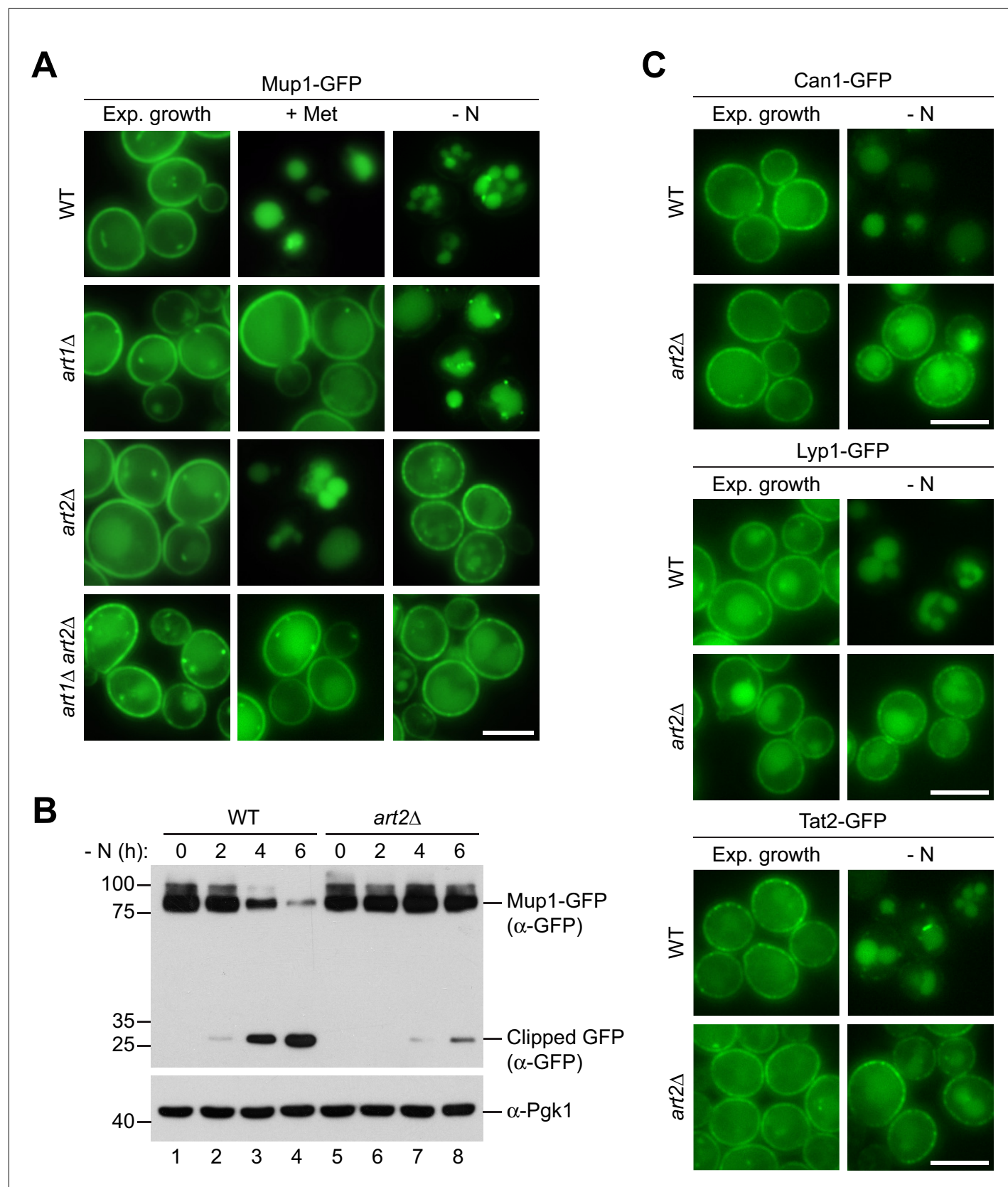


**Figure 2—figure supplement 1.** Gene ontology term analysis of the genome wide screen for genes affecting starvation-induced endocytosis of Mup1-pHluorin. (A) Enrichment of cellular processes and components GO terms in 128 hits from the genome-wide screen for genes involved in the

*Figure 2—figure supplement 1 continued on next page*

*Figure 2—figure supplement 1 continued*

starvation-induced endocytosis of Mup1-pHluorin (**Supplementary file 2**). Data are represented as fold-enrichment over whole genome frequency, with the fraction of starvation-specific genes in red and general endocytic trafficking regulators in black. Only GO terms with more than two-fold enrichment over genome were included. (B) Enrichment of macromolecular complex GO terms in 128 hits from the genome-wide screen for genes involved in the starvation-induced endocytosis of Mup1-pHluorin analyzed as in (A). See also **Supplementary file 3**.

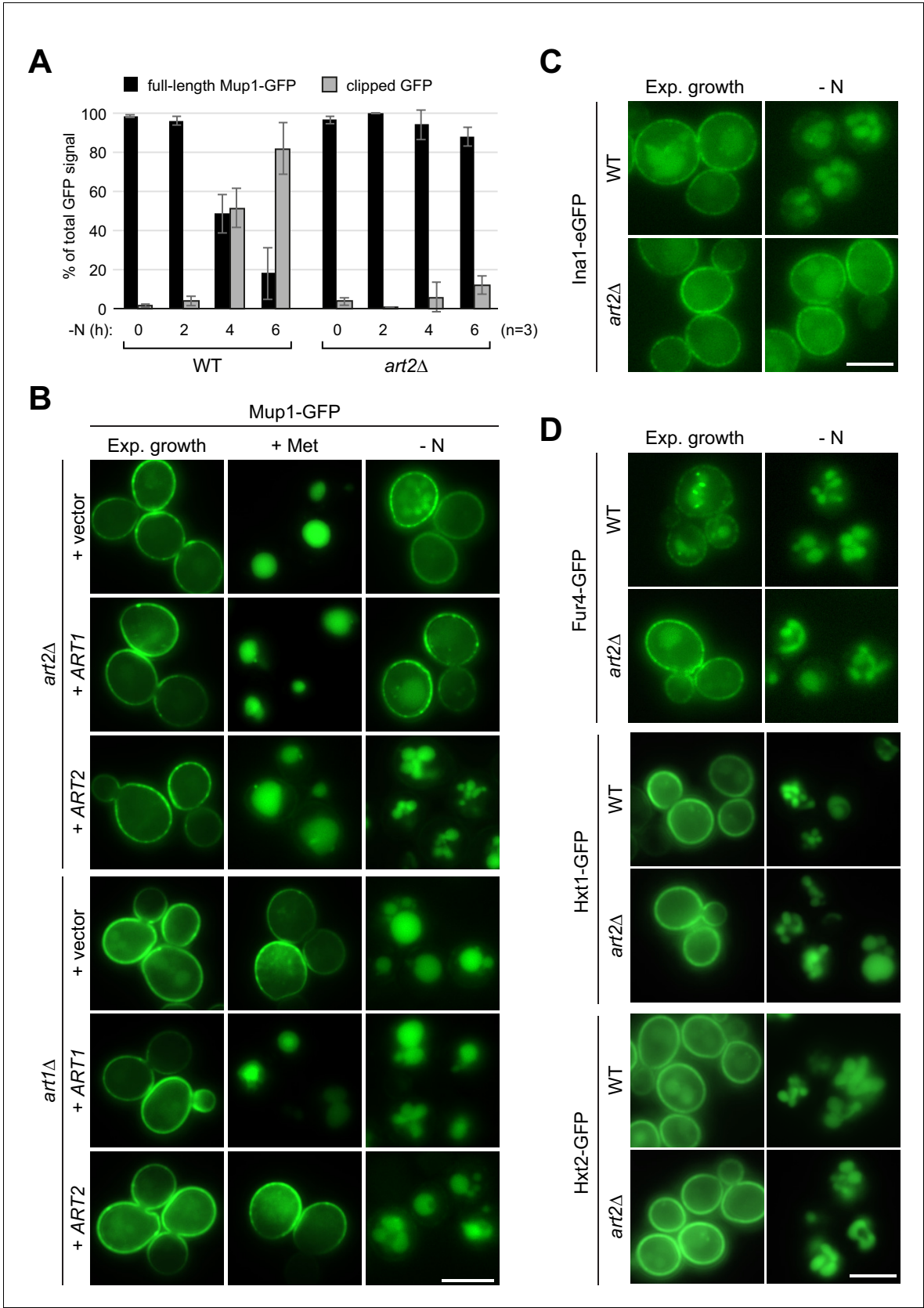


**Figure 3.** Art1 and Art2 are non-redundant in promoting substrate- and starvation-induced endocytosis of amino acid transporters. (A) Live-cell fluorescence microscopy analysis of Mup1-GFP endocytosis in wild type (WT), *art1Δ*, *art2Δ* and *art1Δ art2Δ* cells expressing *MUP1-GFP* from plasmid. Figure 3 continued on next page



*Figure 3 continued*

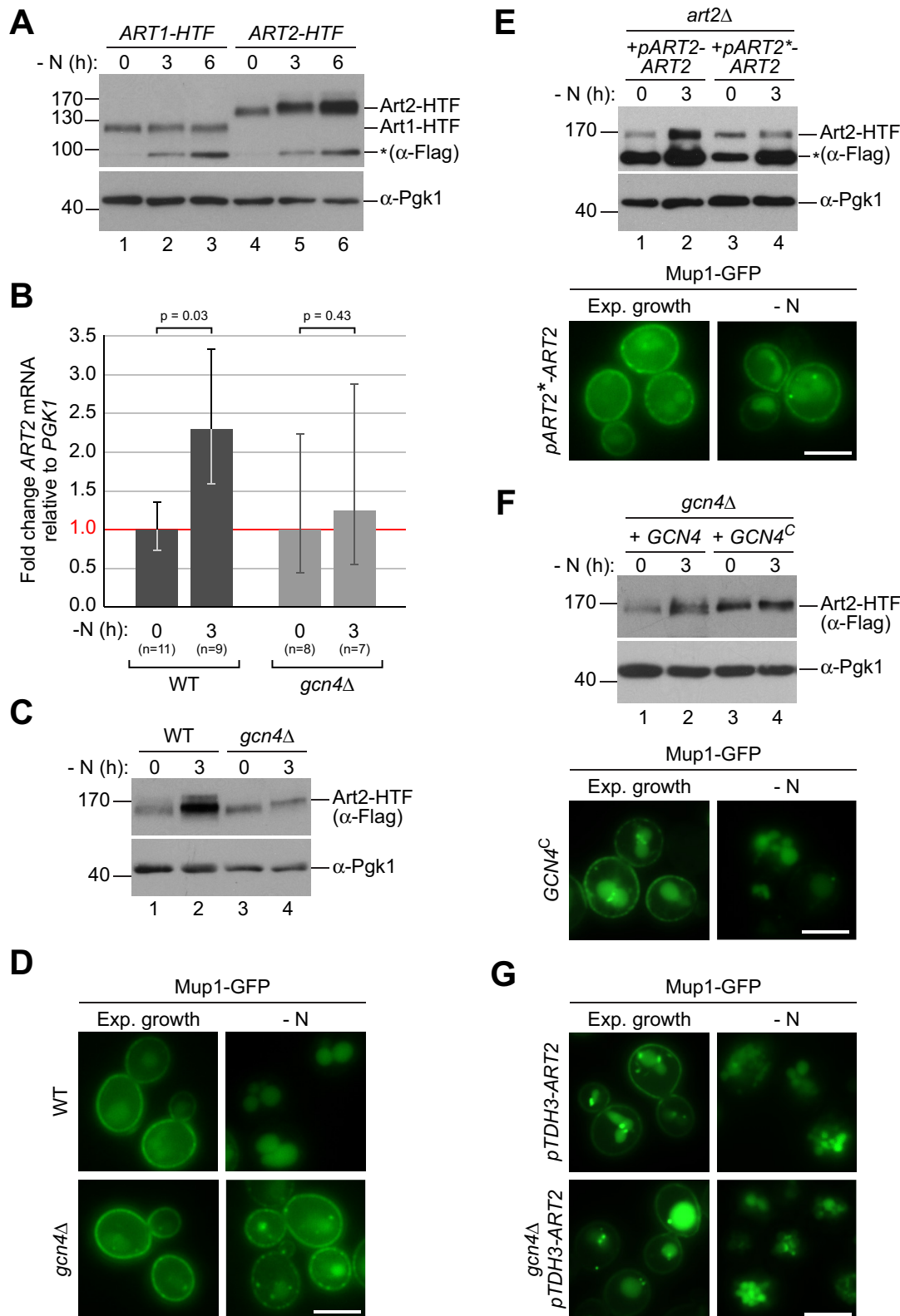
Cells were treated with 20  $\mu$ g/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (B) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from wild type (WT) and *art2 $\Delta$*  cells expressing *MUP1-GFP* that were starved (- N) for the indicated times after 24 hr exponential growth. Quantification in **Figure 3—figure supplement 1A**. (C) Live-cell fluorescence microscopy analysis of wild type (WT) and *art2 $\Delta$*  cells expressing *CAN1-GFP*, *LYP1-GFP* or *TAT2-GFP*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. Scale bars = 5  $\mu$ m. See also **Figure 3—figure supplement 1**.



**Figure 3—figure supplement 1.** Art2-dependent and -independent starvation-induced endocytosis of PM proteins. (A) Densitometric quantification of Figure 3B. Displayed is the distribution of the GFP signal into full length Mup1-GFP and clipped GFP fragment. Mean % of total GFP signal in each Figure 3—figure supplement 1 continued on next page

## Figure 3—figure supplement 1 continued

lane  $\pm$  standard deviation from  $n = 3$  independent experiments. (B) Live-cell fluorescence microscopy analysis of *art2 $\Delta$*  and *art1 $\Delta$*  cells expressing *MUP1-GFP* and plasmids encoding *ART1*, *ART2* or empty vectors as indicated. Cells were treated with 20  $\mu$ g/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (C) Live-cell fluorescence microscopy analysis of wild type (WT) and *art2 $\Delta$*  cells expressing *INA1-eGFP*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. (D) Live-cell fluorescence microscopy analysis of wild type (WT) and *art2 $\Delta$*  cells expressing pRS416-*FUR4-GFP*, *HXT1-GFP* or *HXT2-GFP*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. Scale bars = 5  $\mu$ m.

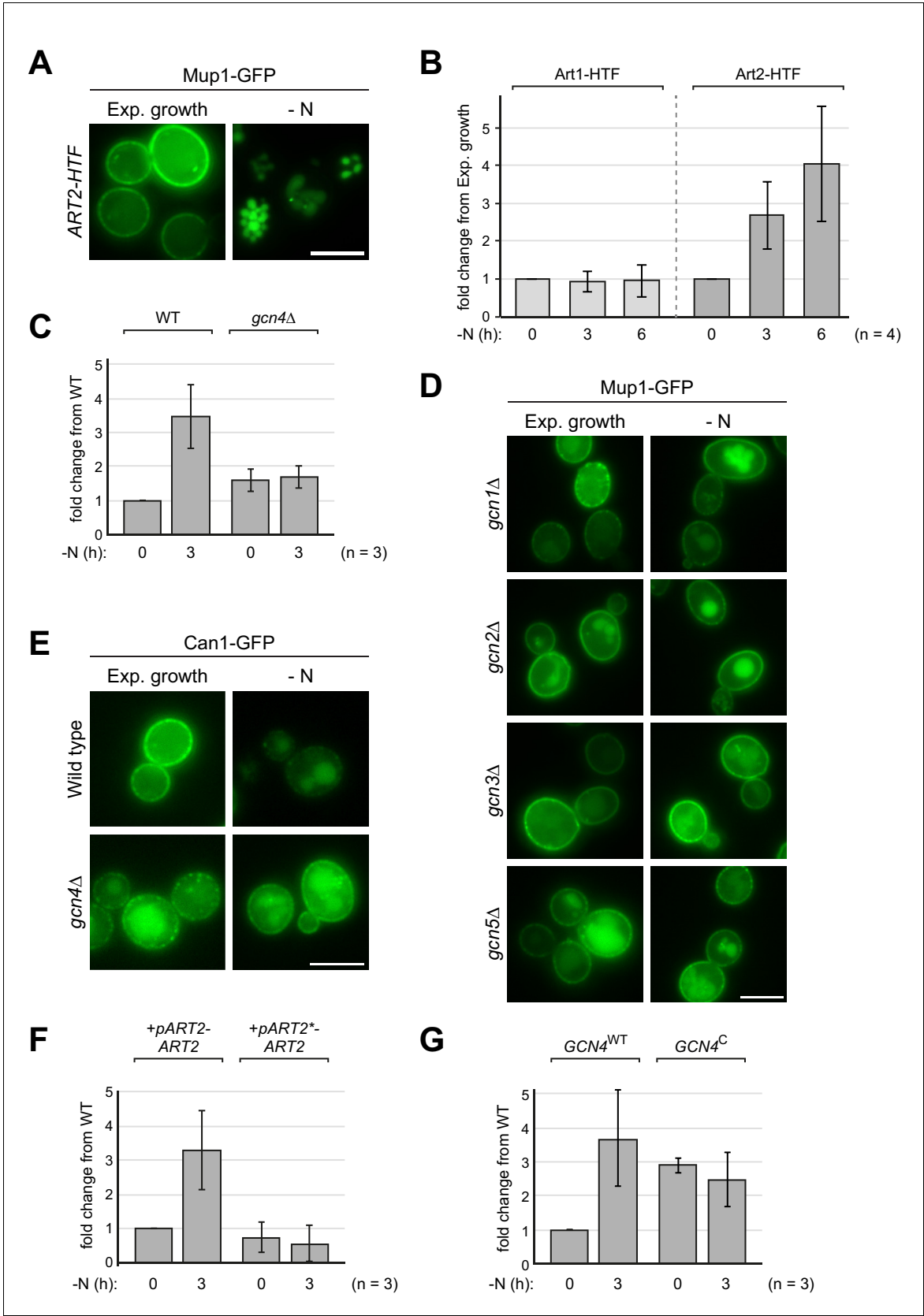


**Figure 4.** The general amino acid control pathway promotes starvation-induced endocytosis Mup1 by up-regulating Art2. (A) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from WT cells expressing ART1-HTF or ART2-HTF. Cells were starved (- N) for Figure 4 continued on next page

## Figure 4 continued

the indicated times after 24 hr exponential growth. The asterisk indicates a non-specific background band of the FLAG antibody. Quantification in **Figure 4—figure supplement 1B**. (B) RT-qPCR analysis of ART2 transcript levels (normalized to the stable *PGK1* transcript) in wild type (WT) and *gcn4Δ* cells. Cells were starved (- N) for 3 hr after 24 hr exponential growth. Values are presented as fold-change of the starting values (t = 0). Error bars represent the standard deviation. Statistical significance was assessed by Student's t-test. (C) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from the indicated strains expressing *ART2-HTF*. Cells were starved (- N) for 3 hr after 24 hr exponential growth. Quantification in **Figure 4—figure supplement 1C**. (D) Live-cell fluorescence microscopy analysis of the indicated strains expressing *MUP1-GFP* from plasmid. Cells were starved (- N) for 6 hr after 24 hr exponential growth. (E), (F) The indicated strains were analyzed as in C) (upper panels) and D) (lower panels). Quantification of western blots in **Figure 4—figure supplement 1F,G**. (G) Live-cell fluorescence microscopy analysis of *art2Δ* or *gcn4Δ* cells expressing pRS415-*MUP1-GFP* and pRS416-*pTDH3-ART2* starved (- N) for 6 hr after 24 hr exponential growth. Scale bars = 5 μm. See also **Figure 4—figure supplements 1** and **2**.

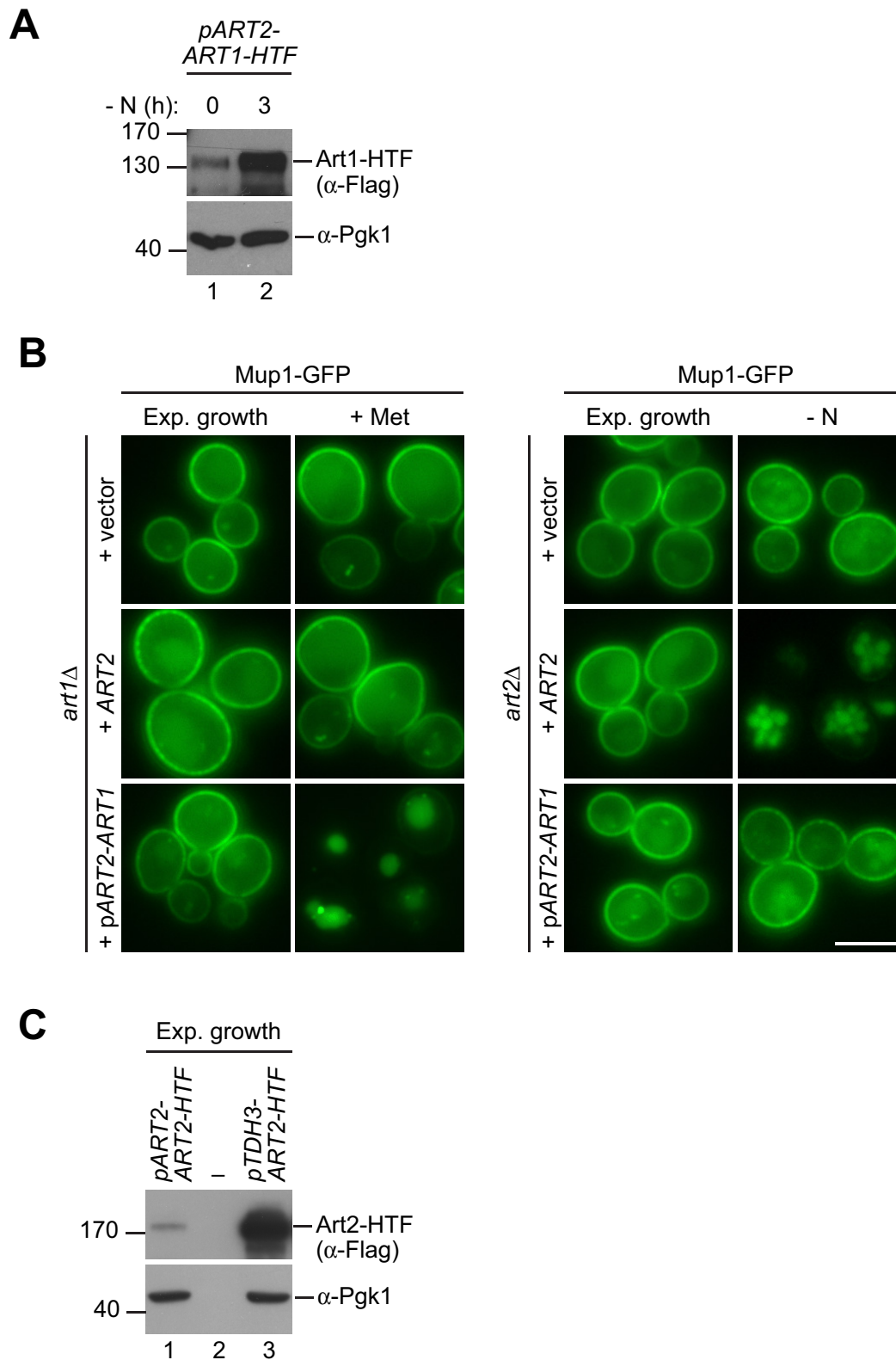




**Figure 4—figure supplement 1.** The general amino acid control pathway promotes starvation-induced endocytosis Mup1 by up-regulating Art2 – supporting experiments and quantifications. **(A)** Live-cell fluorescence microscopy analysis of WT cells expressing chromosomally 6xHis-TEV-3xFLAG-  
Figure 4—figure supplement 1 continued on next page

*Figure 4—figure supplement 1 continued*

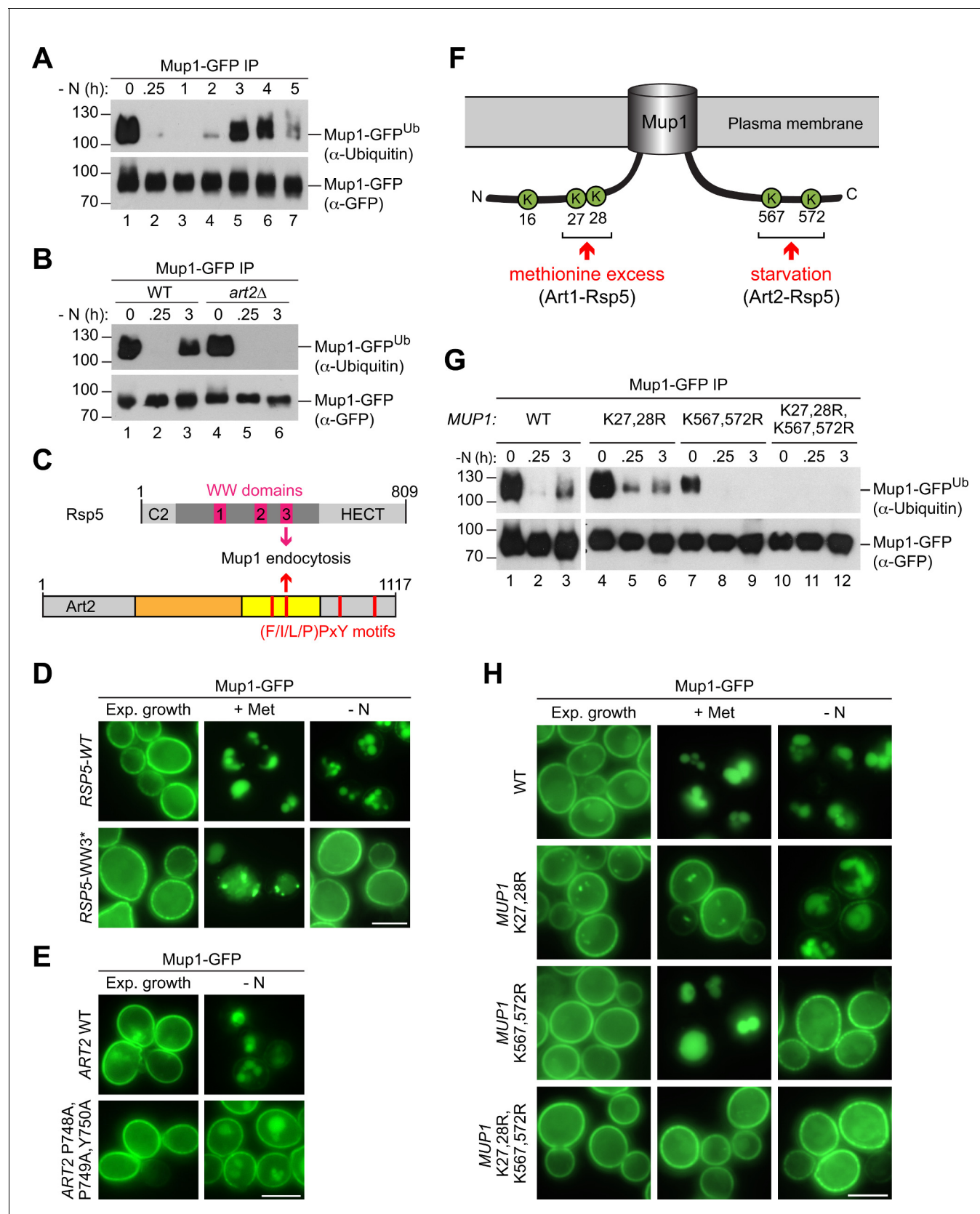
tagged ART2 (*ART2-HTF*) and *MUP1-GFP* starved (- N) for 6 hr after 24 hr exponential growth. (B) Densitometric quantification of Art1-HTF and Art2-HTF protein levels in **Figure 4A**. Data were normalized to Pgk1 loading control and presented as fold-change from expression in exponential growth (0 hr) (mean  $\pm$  standard deviation from  $n = 4$  independent experiments). (C) Densitometric quantification of Art2-HTF protein levels in **Figure 4C**. Data were normalized to Pgk1 loading control and presented as fold-change in expression from WT in exponential growth (0 hr) (mean  $\pm$  standard deviation from  $n = 3$  independent experiments). (D), (E) Live-cell fluorescence microscopy analysis of the indicated strains starved (- N) for 6 hr after 24 hr exponential growth. (F), (G) Densitometric quantification of Art2-HTF protein levels in **Figure 4E and F** analyzed as in C). Scale bars = 5  $\mu$ m.



**Figure 4—figure supplement 2.** Upregulation of Art1 cannot substitute Art2 in starvation-induced endocytosis of Mup1. (A) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from *art2Δ* cells expressing pRS416-pART2-ART1-HTF starved (- N) for 3 hr after Figure 4—figure supplement 2 continued on next page

*Figure 4—figure supplement 2 continued*

24 hr exponential growth. (B) Live-cell fluorescence microscopy analysis of *art1Δ* and *art2Δ* cells expressing *MUP1-GFP* and the indicated plasmids. Cells were treated with 20 μg/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (C) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from WT cells expressing *pART2-ART2-HTF* or *pRS416-pTDH3-ART2-HTF* after 24 hr exponential growth. Scale bars = 5 μm.

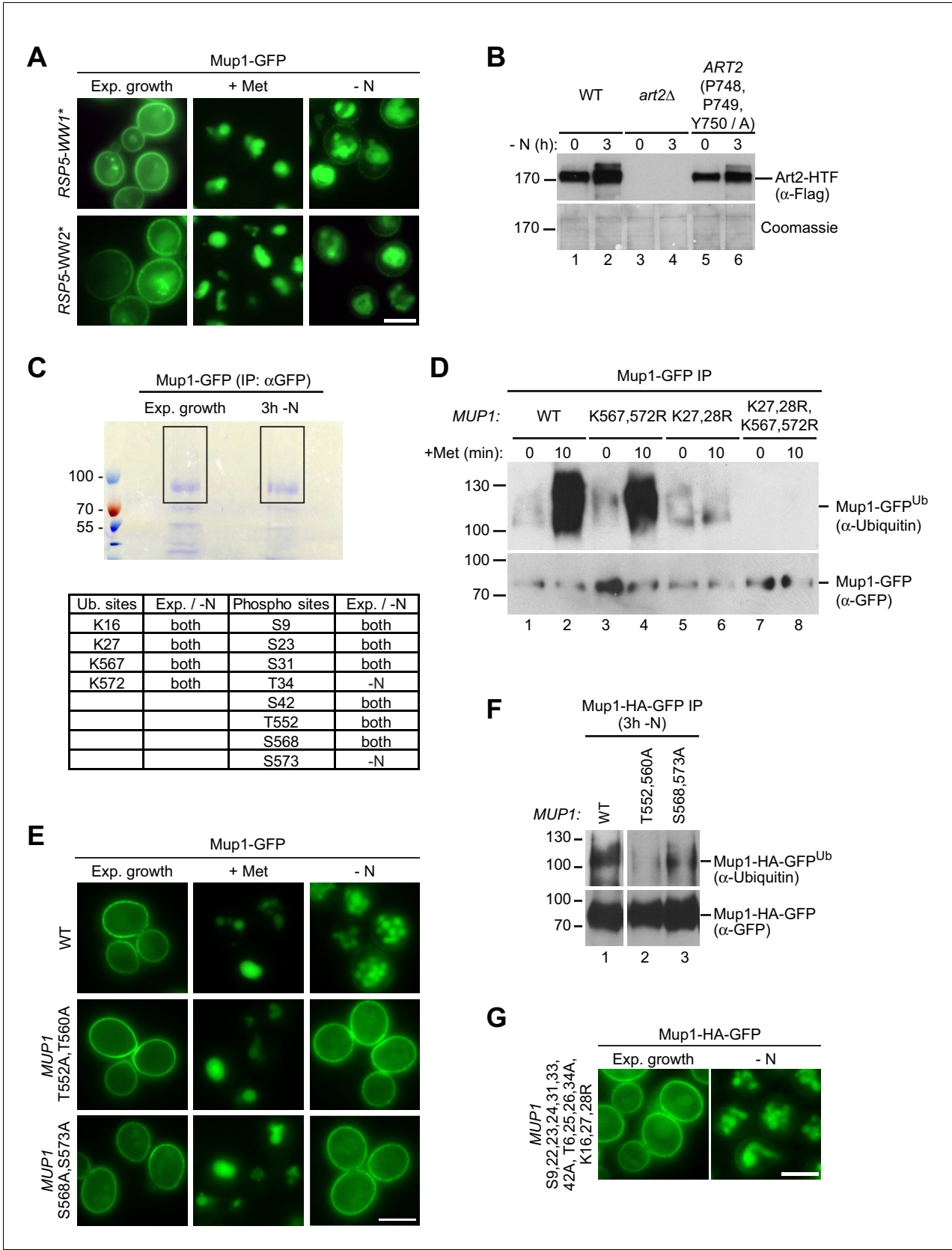


**Figure 5.** Art2-Rsp5 mediates the starvation-induced ubiquitination of Mup1-GFP at two specific C-terminal lysine residues. (A), (B) SDS PAGE and western blot analysis with the indicated antibodies of immunoprecipitated Mup1-GFP from WT cells or *art2Δ* cells starved for the indicated times after Figure 5 continued on next page



## Figure 5 continued

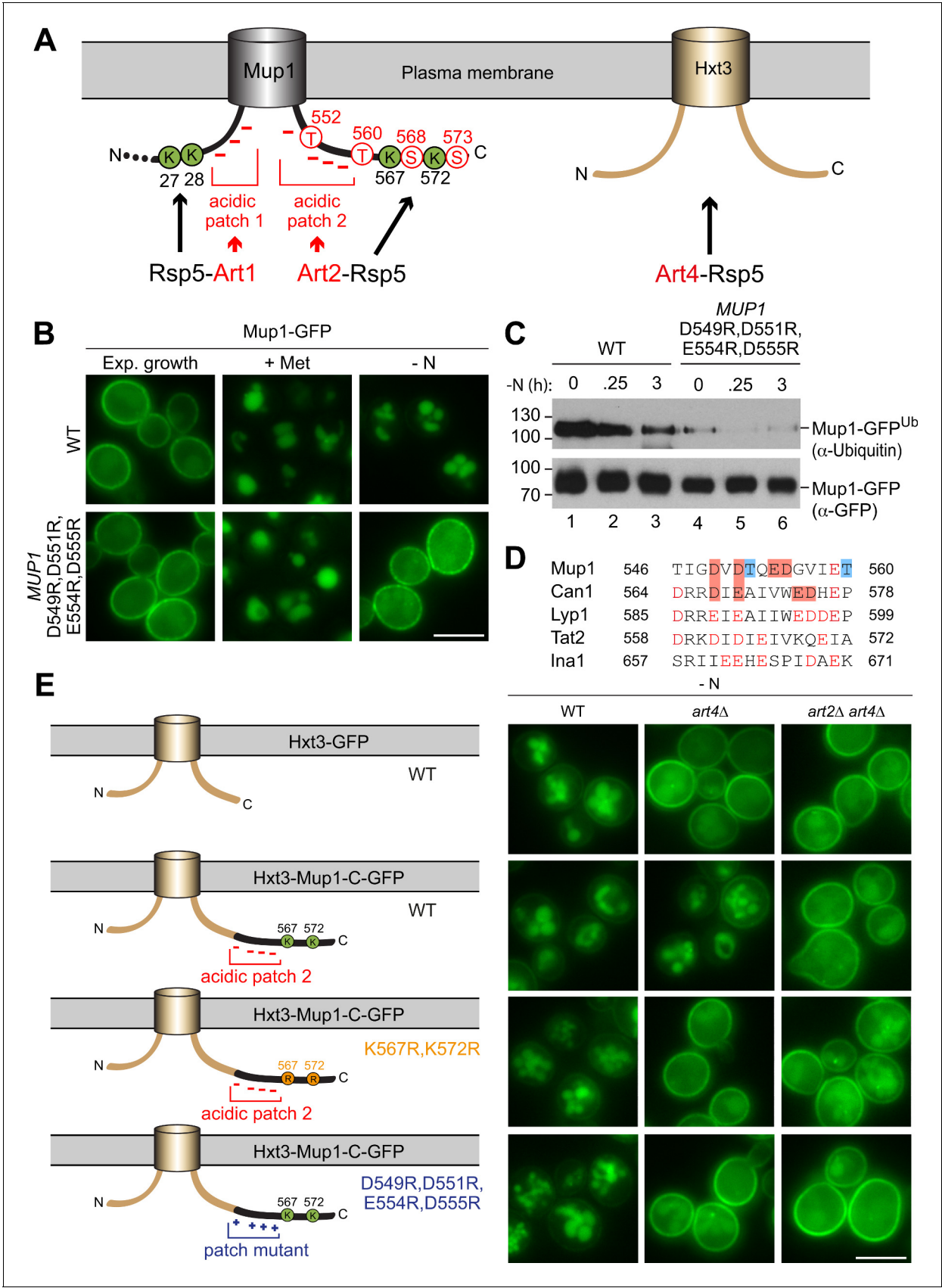
24 hr of exponential growth. Equal amounts of immunoprecipitated Mup1-GFP were loaded to compare the extent of ubiquitination. (C) Scheme depicting the domain arrangement of Rsp5 and Art2, indicating the localization of the WW domains and PY motifs required for starvation-induced endocytosis of Mup1. (D) Live-cell fluorescence microscopy analysis of *rsp5Δ* cells expressing pRS416-*MUP1-GFP* and pRS415-*HTF-RSP5-WT* (wild type) or pRS415-*HTF-RSP5-WW3\**. Cells were treated with 20 μg/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (E) Live-cell fluorescence microscopy analysis of *art2Δ* cells expressing *MUP1-GFP* and pRS416-*ART2* (WT) or pRS416-*ART2 P748A,P749A,Y750A*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. (F) Scheme of Mup1 topology with the N- and C-terminal ubiquitination sites targeted during substrate excess by Art1-Rsp5 and during starvation by Art2-Rsp5. Ubiquitinated lysines (K) shown in green with numbers corresponding to amino acid positions in the Mup1 sequence. (G) WT cells expressing *MUP1-GFP* WT or the indicated *MUP1-GFP* mutants starved for the indicated times after 24 hr of exponential growth analyzed as in B). (H) Live-cell fluorescence microscopy analysis of cells expressing *MUP1-GFP* (wild type (WT)), *MUP1 K27,28R-GFP*, *MUP1 K567,572R-GFP* or *MUP1 K27,28,567,572R-GFP* as in D). Scale bars = 5 μm. See also **Figure 5—figure supplement 1**.



**Figure 5—figure supplement 1.** Post-translational modifications regulating starvation- and substrate-induced endocytosis of Mup1. (A) Live-cell fluorescence microscopy analysis of *rsp5Δ* cells expressing pRS416-MUP1-GFP and pRS415-HTF-RSP5-WW1\* or pRS415-HTF-RSP5-WW2\*. Cells were Figure 5—figure supplement 1 continued on next page

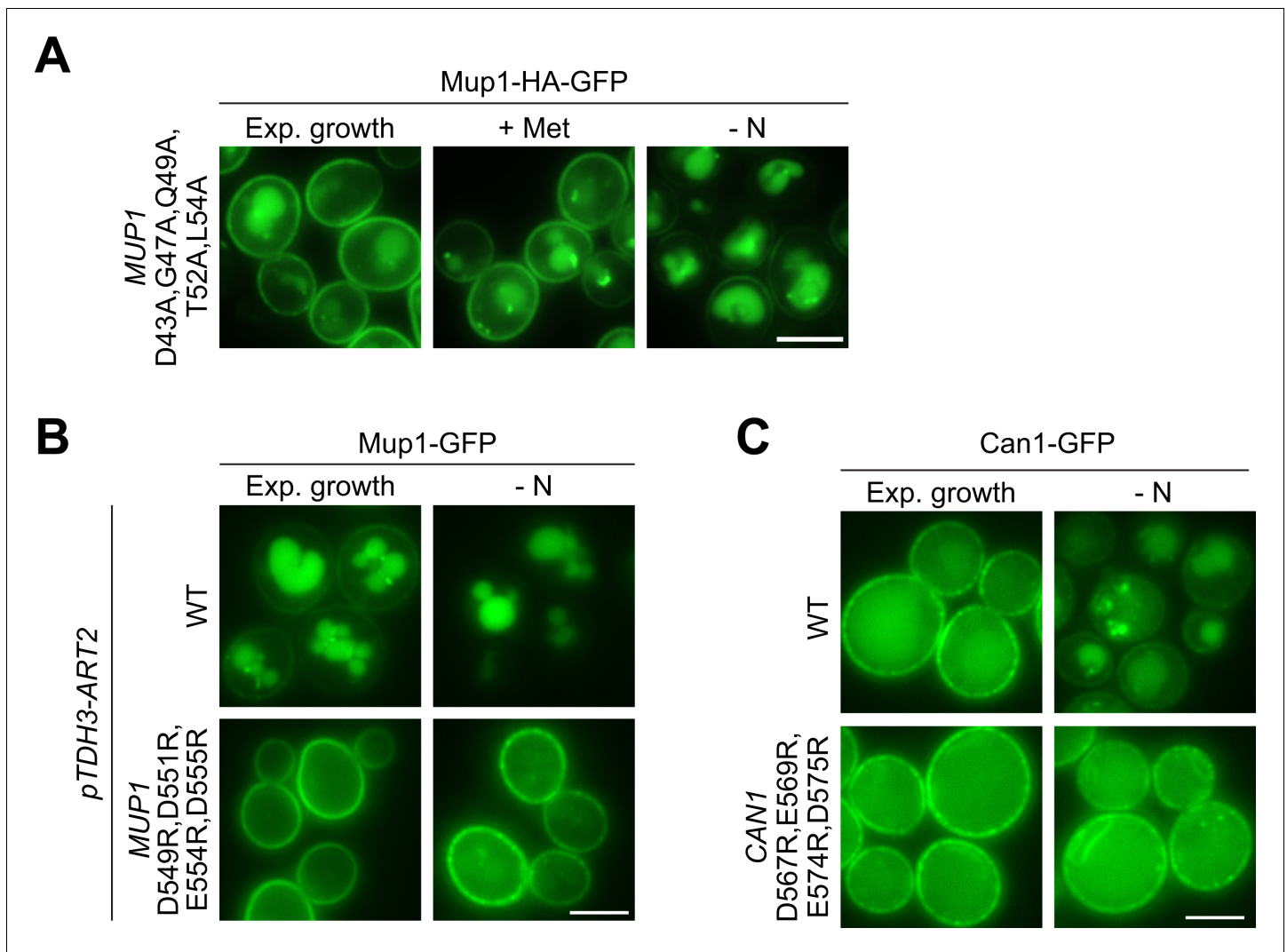
## Figure 5—figure supplement 1 continued

treated with 20  $\mu$ g/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (B) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from *art2 $\Delta$*  cells expressing pRS416-ART2-HTF (WT), pRS416 or pRS416-ART2 P748A,P749A, Y750A-HTF. Cells were starved (- N) for 3 hr after 24 hr exponential growth. (C) Upper panel: Coomassie-stained SDS PAGE of immunoprecipitated Mup1-GFP from WT cells after 24 hr exponential growth or subsequent 3 hr starvation (-N). The black rectangles indicate the regions of the gel analyzed by mass spectrometry. Lower panel: Ubiquitinated lysine (K) and phosphorylated serine (S) and threonine (T) residues of Mup1 identified by mass spectrometry during exponential growth and/or starvation with numbers corresponding to amino acid positions in the Mup1 sequence. (D) SDS PAGE and western blot analysis with the indicated antibodies of immunoprecipitated Mup1-GFP from WT cells or the indicated *MUP1* mutants treated with 20  $\mu$ g/ml L-methionine for 10 min after 24 hr of exponential growth. Equal amounts of immunoprecipitated Mup1-GFP were loaded to compare the extent of ubiquitination. (E) Live-cell fluorescence microscopy analysis of cells expressing *MUP1-GFP* (wild type (WT)), *MUP1 T552,560A-GFP* or *MUP1 S568,573A-GFP*. Cells were treated with 20  $\mu$ g/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (F) *MUP1-HA-GFP* (wild type (WT)), *MUP1 T552,560A-HA-GFP* or *MUP1 S568,573A-HA-GFP* cells were starved (- N) for 3 hr and analyzed as in D). (G) Live-cell fluorescence microscopy analysis of cells expressing *MUP1 T6,25,26,34A,S9,22,23,24,31,33,42A,K16,27,28R-HA-GFP*. Cells were starved (- N) for 3 hr after 24 hr exponential growth. Scale bars = 5  $\mu$ m.

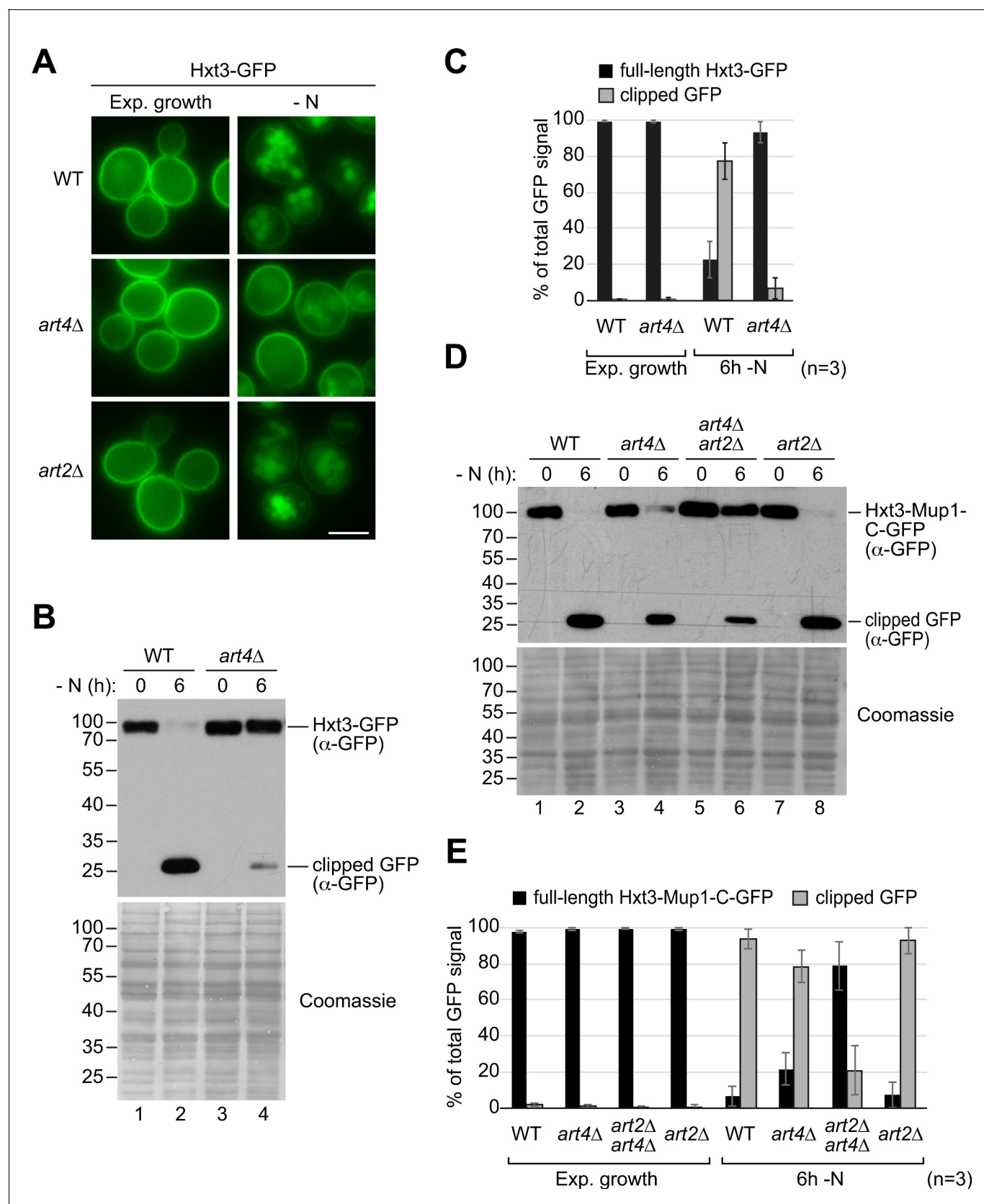


**Figure 6.** The C-terminus of Mup1 harbors a transplantable, starvation-responsive acidic degron. (A) Left: scheme of Mup1 topology with N- and C-terminal ubiquitination sites and acidic patches targeted by Art1-Rsp5 and Art2-Rsp5, respectively, and the C-terminal phosphorylation sites of Mup1 that promote its starvation-induced endocytosis. Ubiquitinated lysines (K) shown in green and phosphorylated serines (S) and threonines (T) in red with numbers corresponding to amino acid positions in the Mup1 sequence. Right: Hxt3 as an Art4-Rsp5 dependent cargo during nitrogen starvation. (B) Live-cell fluorescence microscopy analysis of Mup1-GFP endocytosis in cells expressing *MUP1-GFP* (wild type (WT)) or *MUP1 D549R,D551R,E554R,D555R-GFP*. Cells were treated with 20  $\mu$ g/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. (C) SDS PAGE and western blot analysis with the indicated antibodies of immunoprecipitated Mup1-GFP from cells expressing *MUP1-GFP* (WT) or *MUP1 D549R,D551R,E554R,D555R-GFP* starved for the indicated times after 24 hr of exponential growth. Equal amounts of immunoprecipitated Mup1-GFP were loaded to compare the extent of ubiquitination. (D) Amino acid sequence alignment of the C-terminal acidic patches of Mup1, Can1, Lyp1, Tat2 and Ina1. The boxes indicate acidic residues (red) and phosphorylation sites (blue), which are required for Art2-dependent starvation-induced endocytosis. Red letters illustrate further acidic residues. (E) Live-cell fluorescence microscopy analysis of wild type (WT), *art4 $\Delta$*  and *art2 $\Delta$  art4 $\Delta$*  cells expressing *HXT3-GFP* (top), *HXT3-MUP1-C-GFP* (second row), *HXT3-MUP1-C K567,572R-GFP* (third row) or *HXT3-MUP1-C D549R,D551R,E554R,D555R-GFP* (bottom). Cells were starved (- N) for 6 hr after 24 hr exponential growth. Scale bars = 5  $\mu$ m. See also **Figure 6—figure supplements 1 and 2**.





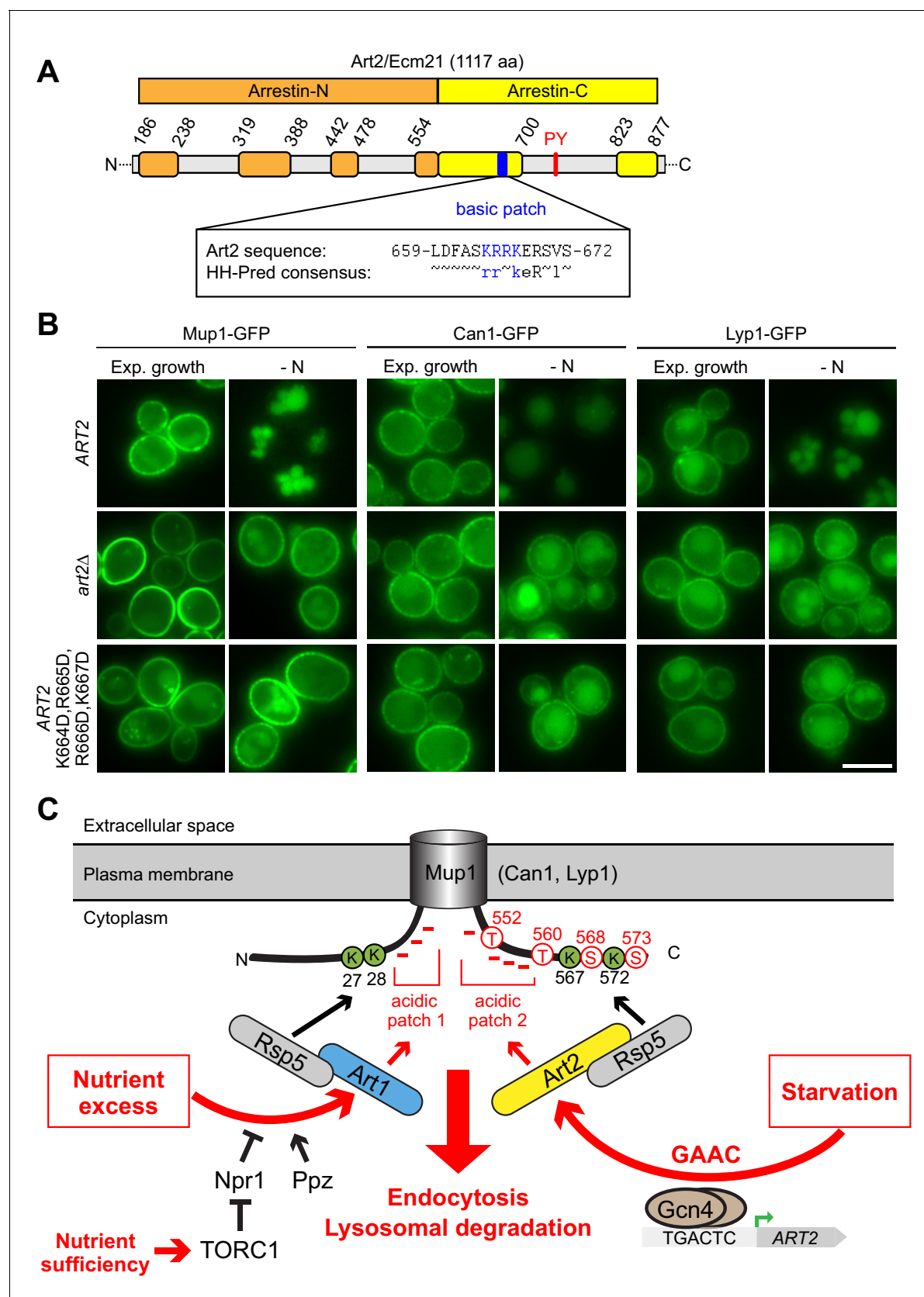
**Figure 6—figure supplement 1.** The role of acidic patches in AATs for starvation-induced endocytosis. (A-C) Live-cell fluorescence microscopy analysis of the indicated strains treated with 20 µg/ml L-methionine (+ Met) for 1.5 hr or starved (- N) for 6 hr after 24 hr exponential growth. Scale bars = 5 µm.



**Figure 6—figure supplement 2.** Art4-dependent starvation-induced endocytosis of Hxt3. (A) Live-cell fluorescence microscopy analysis of the indicated strains starved (- N) for 6 hr after 24 hr exponential growth. (B) SDS PAGE and western blot analysis with the indicated antibodies of whole Figure 6—figure supplement 2 continued on next page

*Figure 6—figure supplement 2 continued*

cell protein extracts from wild type (WT) and *art4Δ* cells expressing *HXT3-GFP*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. Coomassie staining of the membrane serves as loading control. (C) Densitometric quantification of B). Displayed is the distribution of the GFP signal into full length Hxt3-GFP and clipped GFP fragment. Mean % of total GFP signal in each lane  $\pm$  standard deviation from  $n = 3$  independent experiments. (D) Wild type (WT), *art4Δ*, *art2Δ art4Δ* and *art2Δ* cells expressing *HXT3-MUP1-C-GFP* were treated as in B). (E) Densitometric quantification of C). Displayed is the distribution of the GFP signal into full-length Hxt3-Mup1-C-GFP and clipped GFP fragment. Mean % of total GFP signal in each lane  $\pm$  standard deviation from  $n = 3$  independent experiments. Scale bars = 5  $\mu$ m.

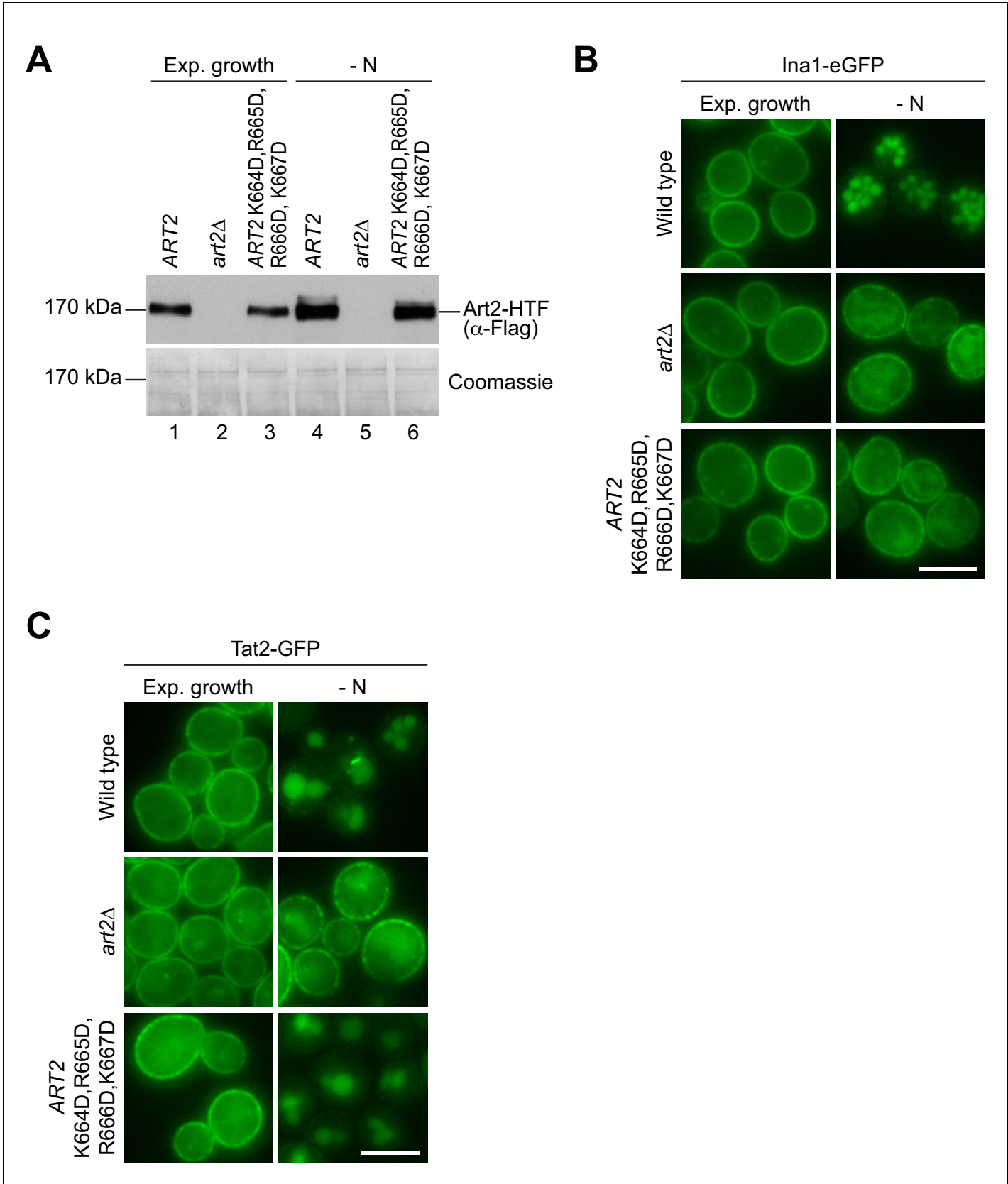


**Figure 7.** A basic patch of Art2 promotes the starvation-induced endocytosis of Mup1, Can1 and Lyp1. (A) Scheme of Art2 topology with arrestin-N domain in orange, arrestin-C domain in yellow and tails and interspersed extended loops (Baile et al., 2019) in light grey. The basic amino acid

Figure 7 continued on next page

## Figure 7 continued

residues shown in blue mediate the starvation-induced endocytosis of Mup1, Can1 and Lyp1 (numbers correspond to amino acid positions in the Art2 sequence). Below is aligned the HHpred consensus sequence for Art2/Ecm21 derived from three HHblits iterations (**Zimmermann et al., 2018**), suggesting conservation of the basic patch in the Art2 protein family. (B) Live-cell fluorescence microscopy analysis of *art2Δ* cells expressing *MUP1-GFP*, *CAN1-GFP* or *LYP1-GFP* and pRS416-ART2-WT, empty vector or pRS416-ART2 K664D,R665D,R666D,K667D. Cells were starved (- N) for 6 hr after 24 hr exponential growth. (C) Scheme for the regulation of the substrate- and starvation-induced endocytosis of Mup1. During substrate excess TORC1 inhibits the Npr1 kinase which otherwise would phosphorylate and inhibit Art1. Art1-Rsp5 becomes dephosphorylated and subsequently binds the acidic patch 1 at the N-terminal tail of Mup1, leading to the ubiquitination of K27 and K28 and degradation of Mup1 (left). During amino acid and nitrogen starvation, the general amino acid control (GAAC) pathway upregulates the ubiquitin ligase adaptor Art2 via the transcriptional regulator Gcn4. The ensuing Art2-Rsp5 complex binds with its basic patch to the acidic patch 2 of Mup1 leading to the ubiquitination of K567 and K572 and degradation of Mup1 (right). At the same time, starvation causes TORC1 inhibition and activation of Npr1 and inhibits Art1-dependent ubiquitination. Ubiquitinated lysines (K) shown in green and phosphorylated serines (S) and threonines (T) in red with numbers corresponding to amino acid positions in the Mup1 sequence. Scale bars = 5 μm. See also **Figure 7—figure supplement 1**.



**Figure 7—figure supplement 1.** Additional characterization of the basic patch mutant of Art2. (A) SDS PAGE and western blot analysis with the indicated antibodies of whole cell protein extracts from *art2Δ* cells expressing pRS416-ART2-HTF, empty vector or pRS416-ART2 K664D,R665D,R666D, Figure 7—figure supplement 1 continued on next page

## Figure 7—figure supplement 1 continued

*K667D-HTF*. Cells were starved (- N) for 3 hr after 24 hr exponential growth. (B) Live-cell fluorescence microscopy analysis of *art2Δ* cells expressing *INA1-eGFP* and *pRS416-ART2*, empty vector or *pRS416-ART2 K664D,R665D,R666D,K667D*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. (C) Live-cell fluorescence microscopy analysis of *art2Δ* cells expressing *TAT2-GFP* and *pRS416-ART2*, empty vector or *pRS416-ART2 K664D,R665D,R666D,K667D*. Cells were starved (- N) for 6 hr after 24 hr exponential growth. Scale bars = 5 μm.