Investigating the role of the Hal4 and Hal5 protein kinases in plasma membrane transporter trafficking

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ABSTRACT

The yeast protein kinases Sat4/Hal4 and Hal5 are required for the plasma membrane stability of the K^+ transporter Trk1 and some amino acid and glucose permeases. The transcriptomic analysis of a hal4 hal5 mutant strain indicates that the absence of these genes causes alterations in the general control of the metabolism of both nitrogen and carbon, which correlates with reduced transport of amino acids and glucose, respectively. Here, we investigated whether the reduction in methionine uptake in the double mutant hal4 hal5 was due to a destabilization of the high affinity permease Mup1. We note that, as observed for other plasma membrane permeases such as Hxt1, Can1, Fur4 and Gap1, Mup1 is also internalized and delivered to the vacuole in the absence of potassium supplementation in the double mutant. In order to further analyze the role of these kinases in the regulation of nutrient uptake, we studied their involvement during biosynthesis. Our results suggest that the double mutant hal4 hal5 also presents a defect in the accumulation of Mup1 in the plasma membrane, suggesting that the Hal4 and Hal5 kinases are also involved in regulating the delivery of Mup1 to the membrane. In yeast, the endocytosis of nutrient transporters requires ubiquitination by the E3 ubiquitin ligase Rsp5, which often requires specific adapters to recognize target proteins. So far, 19 proteins have been described as possible adaptors for Rsp5 that target specific PM proteins for endocytic downregulation, including 9 proteins called arrestin related trafficking adaptors, or ARTs. In this work we have also investigated whether a functional connection exists between the Hal4 and Hal5 kinases and ART family adapters in the mechanism of regulation of the PM transport proteins. We confirmed the interaction described in the literature between Mup1 and Art1. We also observed that there is no functional redundancy between Art1 and Art2. However, we failed to detect a physical interaction between the N-terminus of Mup1 (bp 1-180) and Art1 Art2, Art3 or Art4 in the two-hybrid system. Finally we studied whether Art1 is involved in the internalization of Mup1, in the hal4 hal5 mutant strain under conditions of potassium depletion and methionine treatment. The results of the analysis of a triple hal4 hal5 art1 mutant suggest that Art1 is not required for the internalization of Mup1 in the hal4 hal5 strain upon potassium removal, and that removal of ART1 causes a delay in the internalization of Mup1 after methionine treatment. Other transporters, such as Can1, Dip5 and Trk1 are being tested.

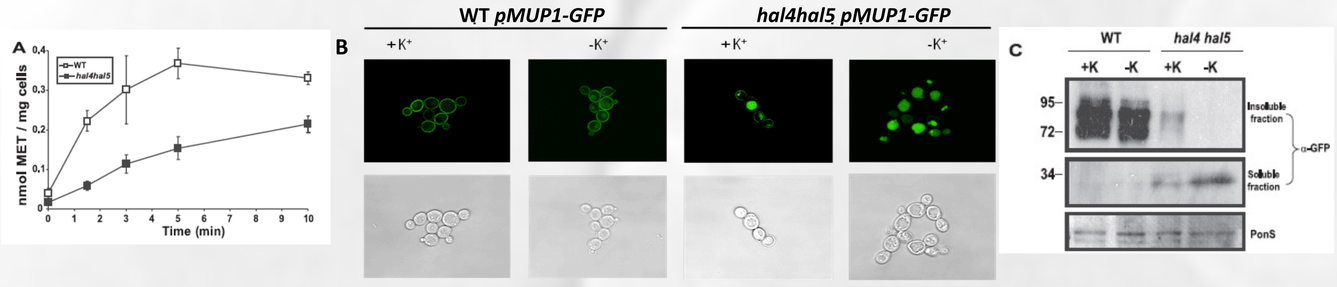

FIG. 1. Methionine uptake is reduced in the *hal4 hal5* mutant. A. The indicated strains (W303 background) were grown to exponential phase in YPD pH 4.5 medium. Cells were harvested and used for methionine uptake assays. Graphs show the median value and the standard deviation from three independent experiments. B. The subcellular localization of Mup1-GFP was analyzed by confocal microscopy of the indicated strains grown to mid-log phase in K-supplemented media ($+K^+$) and after 2 h in low-K media ($-K^+$). Representative images are shown. C. The indicated strains, treated as in panel B, we processed for protein extraction and fractionation. The corresponding Western blots were analyzed with an anti-GFP (αGFP) antibody. The image of a portion of the membrane stained with PonS is shown as a loading control. Molecular mass markers are indicated on the left (kDa). Similar results were observed in three independent experiments.

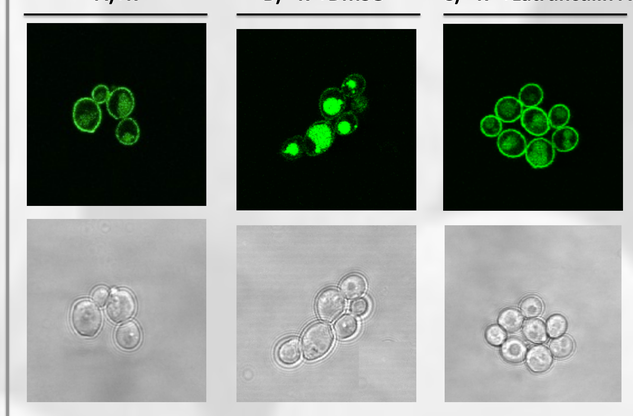

FIG. 2. Inhibition of endocytosis blocks the vacuolar delivery of Mup1 in the *hal4 hal5* mutant. The subcellular localization of Mup1-GFP was analyzed by confocal microscopy in the *hal4 hal5* mutant grown to mid log phase in three different conditions. A) In K^+ supplemented media ($+K^+$), B) after 30 minutes in low K-media ($-K^+$) and C) after 30 minutes in low K-media supplemented with latrunculin A. Representative images are shown.

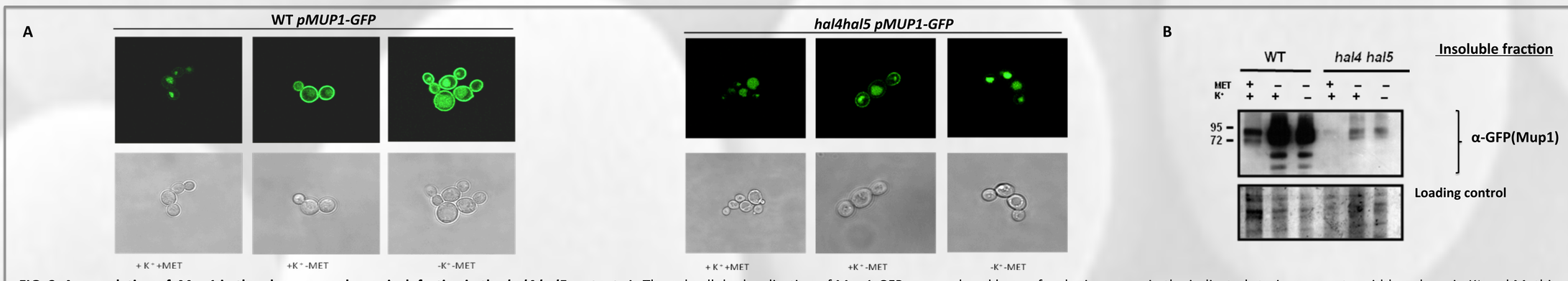

FIG. 3. **Accumulation of Mup1 in the plasma membrane is defective in the *hal4 hal5* mutant**. A. The subcellular localization of Mup1-GFP was analyzed by confocal microscopy in the indicated strains grown to mid-log phase in K^+ and Methionine-supplemented media ($+K^+$ +MET). Methionine was removed from the medium and cells were incubated for 2 h in K^+-supplemented media (+ K^+ -MET) and in low-K^+ media ($-K^+$-MET). Representative images are shown. B. The indicated strains, treated as in panel A, we processed for protein extraction and fractionation. The corresponding Western blots were analyzed with an anti-GFP (αGFP) antibody. The image of a portion of the membrane stained with Direct Blue is shown as a loading control. Molecular mass markers are indicated on the left (kDa). Similar results were observed in three independent experiments.

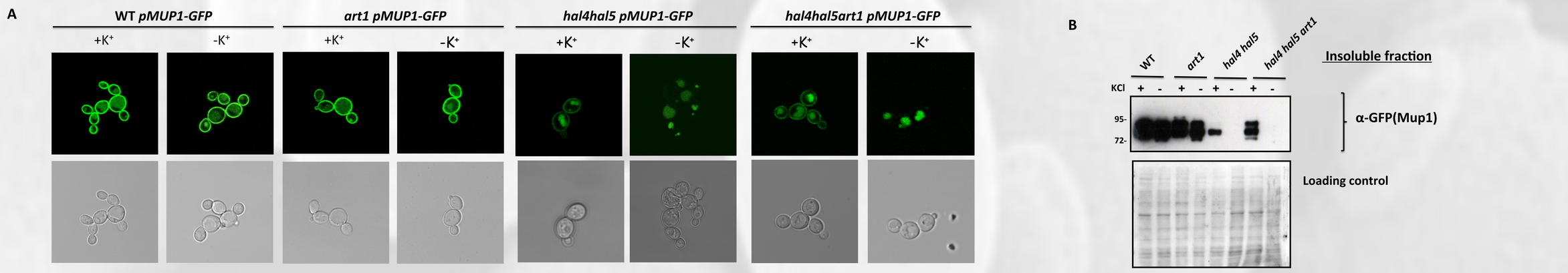

FIG. 4. **Study of Mup1 stability in the *hal4 hal5 art1* mutant during KCl starvation.** A. The subcellular localization of Mup1-GFP was analyzed by confocal microscopy in the indicated strains grown to mid-log phase in K^+-supplemented media ($+K^+$) and after 2 h in low-K^+ media ($-K^+$). Representative images are shown. B. The indicated strains, treated as in panel A, we processed for protein extraction and fractionation. The corresponding Western blots were analyzed with an anti-GFP (αGFP) antibody. The image of a portion of the membrane stained with Direct Blue is shown as a loading control. Molecular mass markers are indicated on the left (kDa). Similar results were observed in three independent experiments.

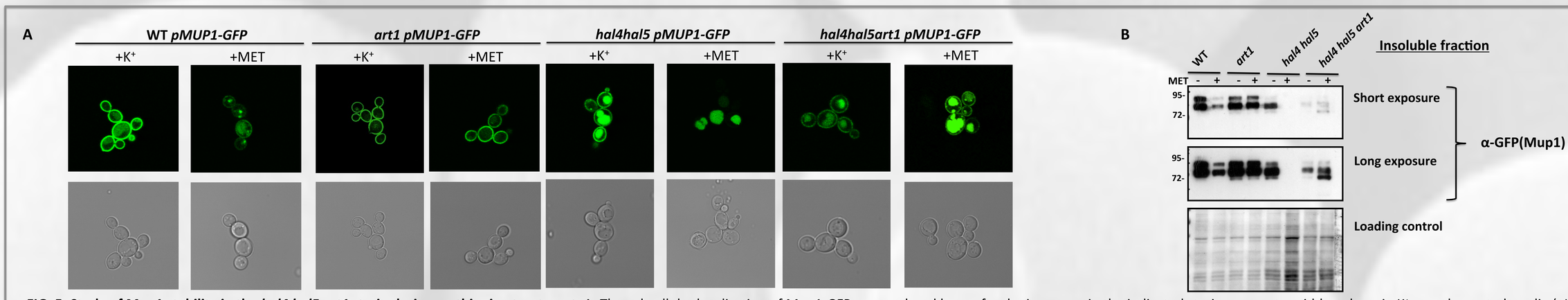

FIG. 5. Study of Mup1 stability in the *hal4 hal5 art1* strain during methionine treatment. A. The subcellular localization of Mup1-GFP was analyzed by confocal microscopy in the indicated strains grown to mid-log phase in K^+ -supplemented media ($+K^+$). Then a concentration of 20µg/ml of methionine was added to the medium and images were taken after 30' of methionine treatment. B. The indicated strains, treated as in panel A, we processed for protein extraction and fractionation. The corresponding Western blots were analyzed with an anti-GFP (αGFP) antibody. The image of a portion of the membrane stained with Direct Blue is shown as a loading control. Molecular mass markers are indicated on the left (kDa). Similar results were observed in three experiments.

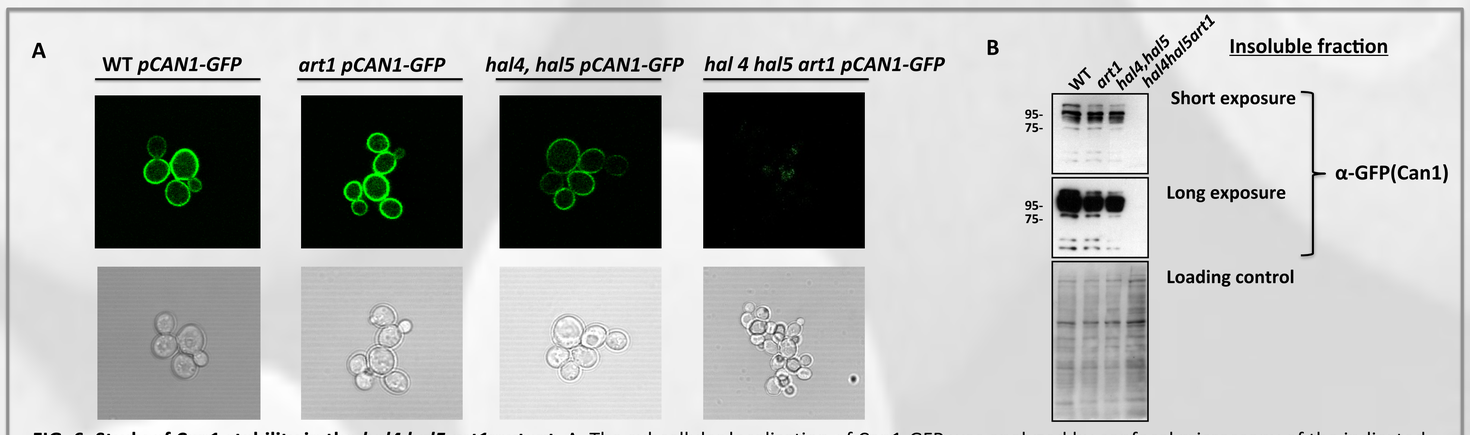

FIG. 6. Study of Can1 stability in the *hal4 hal5 art1*mutant. A. The subcellular localization of Can1-GFP was analyzed by confocal microscopy of the indicated strains grown to mid-log phase in K-supplemented media . Representative images are shown. B. The indicated strains treated as in panel A, were processed for protein extraction and fractionation. The corresponding Western blots were analyzed with an anti-GFP (αGFP) antibody. The image of a portion of the membrane stained with Direct Blue is shown as a loading control. Molecular mass markers are indicated on the left (kDa). Similar results were observed in 4 different clones transformed independently.

1. Our data suggest that induction of methionine biosynthetic genes is due to a decrease in the uptake of methionine, and more specifically due to a mislocalization of the Mup1 high affinity methionine permease.
2. Latrunculin A is an actin monomer-sequestering drug. Within 5 minutes of its addition, endocytosis is completely prevented. Our data shows that vacuolar accumulation of Mup1 is prevented by LatA treatment in the *hal4 hal5* mutant during KCl starvation, indicating that the protein kinases Hal4 and Hal5 are required for the inhibition of Mup1 endocytosis in low K^+ conditions.
3. Mup1 biosynthesis is also defective in strains lacking the *HAL4* and *HAL5* genes
4. Art1 is not required for the internalization of Mup1 in the *hal4 hal5* mutant in low K^+ medium
5. Lack of Art1 impedes the methionine-stimulated endocytosis of Mup1 in both WT and *hal4 hal5* strains. However, alterations in electrophoretic mobility of Mup1 are observed in the *hal4 hal5 art1* mutant, suggesting a functional link between the Hal4/Hal5 and Art1 signaling pathways.
6. Unexpectedly, Can1-GFP fails to accumulate to detectable levels in the *hal4 hal5 art1* mutant, suggesting that Art1 may also be involved in Can1 biosynthesis and reinforcing the notion that trafficking of at least some permeases is regulated by both Art1 and Hal4/Hal5-dependent pathways.