Determining the binding interaction between Cat1 and Any1 by mutational analysis

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Background

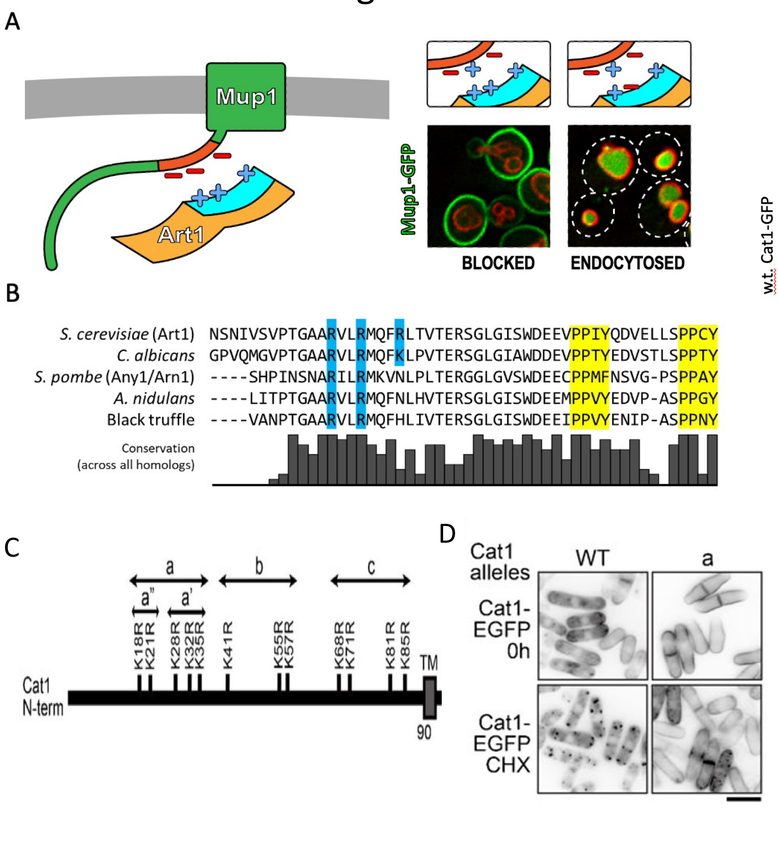

Figure 1. Data supporting hypothesized interaction between *S. pombe* pump Cat1 and adaptor Any1

A: Summarizing Guiney, Klecker Emr, 2016. Methionine pump Mup1 and ubiquitin ligase adaptor Art1 are *S.cerevsiae* homologs of Cat1/Any1. The suspected charge interaction summarized on the left. Right panel illustrates the disruption of ubiquitin mediated endocytosis when charges are unbalanced and the restoration of endocytosis with balanced and inverted charges.

B: From Baile, Guiney, *et al.,* 2019. Blue highlighted regions demonstrate conservation of positively charged region in Art1 and Any1. Yellow highlights mark conserved recruitment region for ubiquitin ligase enzyme. This project tests the hypothesis that this positively charged region mediates binding to pumps across diverse species, starting with the *Schizosaccharomyces pombe* pump/adaptor pair Cat1/Any1.

C,D: Reproduced from Nakashima *et al.,* 2014. Identification of likely ubiquitination site on Cat1. When lysines in region (a) are mutated, Cat1 endocytosis (stimulated by cycloheximide CHX) is blocked. Not shown; Cat1 ubiquitination also requires Any1.

Findings

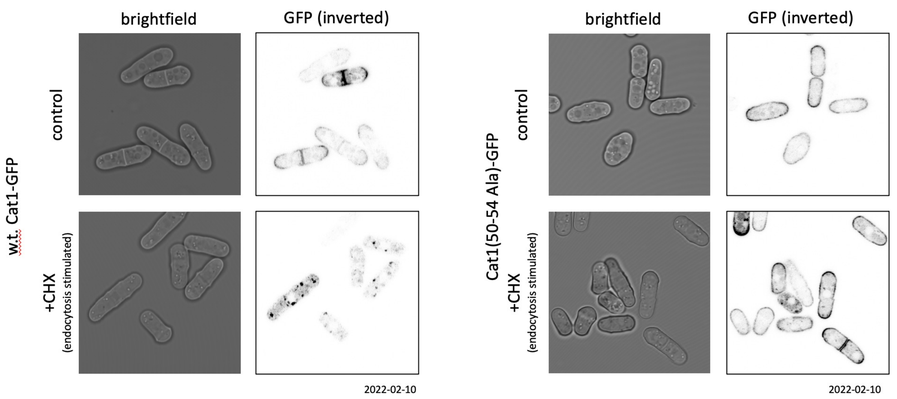

Figure 2: Cat1 endocytosis assay

Left panels show Cat1-GFP at plasma membrane (control) or endocytosed after 1hr treatment with cycloheximide.
Right panels show putative Any1 binding site mutant (residues 50-54 mutated to alanine), with endocytosis blocked.

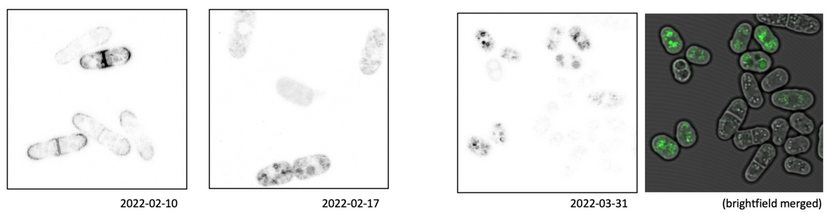

Figure 3: Troubleshooting of Cat1-GFP endocytosis assay

Repeated Cat1-GFP localization assays show substantial variability both in localization (left two panels) and stability of expression (right panel).

Figure 4: Location of key regions on Cat1. Yellow region marks residues 50-54 (see also figure 2), the hypothesized negatively charged "acidic patch" thought to be involved in endocytosis. Purple region marks ubiquitination site as defined by Nakashima *et al.* Red (positively charged) and blue (negatively charged) amino acids are indicated. To test role of acidic patch, we have generated mutants that introduce positively charged arginines across the acidic patch (mutants are p355 through p363).

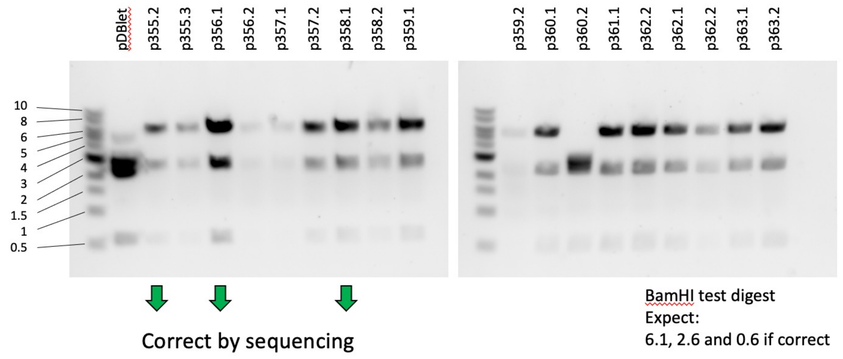

Figure 5: Cloning Cat1 arginine mutants. After cloning Arginine mutants p355-p63, test digest with BamHI help determine if Cat1-arginine mutants were assembled correctly. Expected fragment sizes of 6.1kb, 2.6kb, and 0.6kb if correct. Not shown: DNA sequence analysis showed 3 of 9 mutants were correct and contained only the desired mutation.

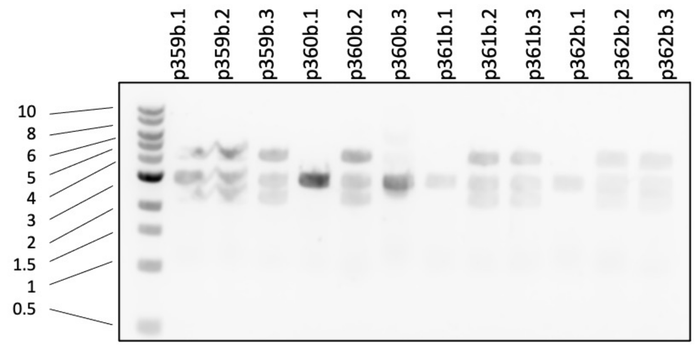

Figure 6: Second round of Cat1 arginine mutant cloning. Incorrectly assembled mutants from Fig 5 were re-cloned. Test digest using EcoRI and XmaI following incorporation of the plasmid into cells. Expected sizes of 2.5kb and 7.5kb if correct.

Future Studies

- Troubleshoot genomic cloning
- Determine appropriate growth conditions for the cells upon culturing
- Send cloning samples for genomic sequencing to determine correctness

Works Cited

Guiney, E.L., Klecker, T. and Emr, S.D., 2016. Identification of the endocytic sorting signal recognized by the Art1-Rsp5 ubiquitin ligase complex. *Molecular biology of the cell*, *27*(25), pp.4043-4054.

Baile, M.G., Guiney, E.L., Sanford, E.J., MacGurn, J.A., Smolka, M.B. and Emr, S.D., 2019. Activity of a ubiquitin ligase adaptor is regulated by disordered insertions in its arrestin domain. *Molecular biology of the cell*, *30*(25), pp.3057-3072.

Nakashima, A., Kamada, S., Tamanoi, F. and Kikkawa, U., 2014. Fission yeast arrestin-related trafficking adaptor, Arn1/Any1, is ubiquitinated by Pub1 E3 ligase and regulates endocytosis of Cat1 amino acid transporter. *Biology open*, *3*(6), pp.542-552.