SYNTHETIC CONTROL OF MITOTIC EXIT FOR STUDYING MULTICELLULAR DRUG RESISTANCE IN YEAST

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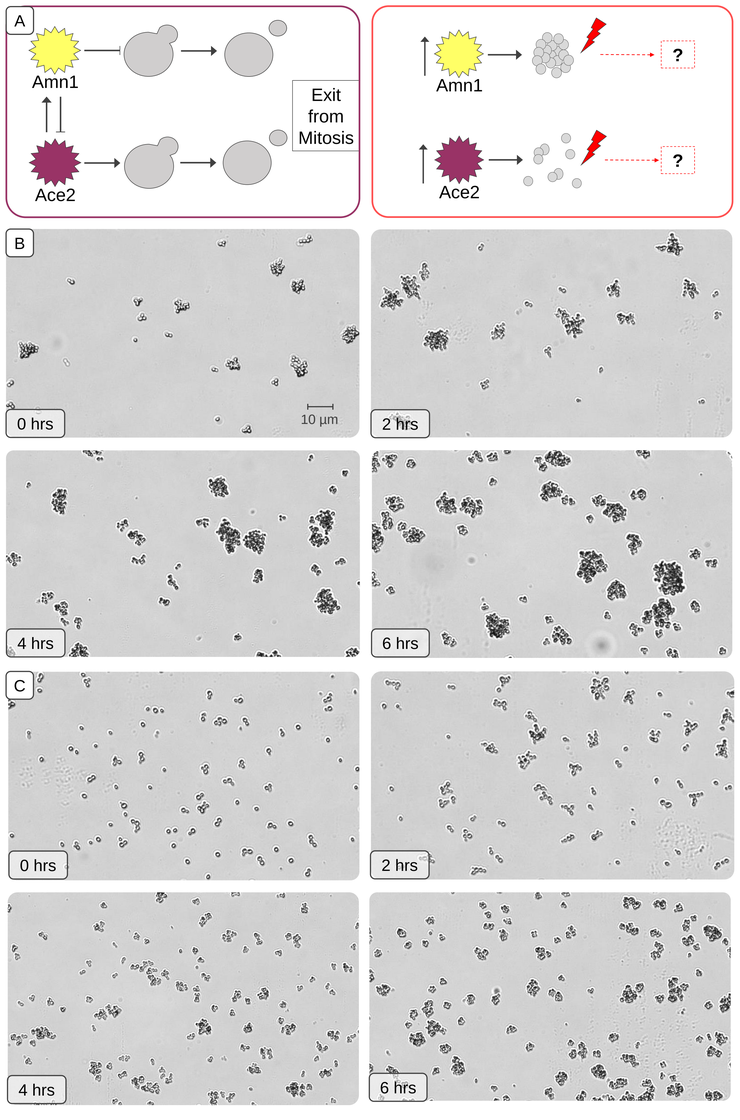
Abstract. Environmental stress plays a crucial role in evolution by both posing a selective pressure on the existing populations and shaping the survival traits of their descendants. The emergence of multicellularity is arguably one of the most descriptive examples of evolutionary decision making. Multicellularity in yeast is proposed to have evolved as a result of stress exposure and thus serves as a good model to study uni- to multicellular phenotype transition. Unlike flocculation, clumping phenotype in yeast is formed by the descendants of one mother cell and is controlled by Mitotic Exit Network (MEN), therefore allowing for its synthetic regulation. Here we investigate MEN, the innate cellular program that regulates mitotic cells separation that is far from being fully described as of today. Specifically, we show that the deletion of a single mitotic gene *AMN1* in *Saccharomyces cerevisiae* Σ1278b (also referred to as TBR1) strain causes a unicellular phenotype. Based on the previous overexpression studies, we hypothesize a dose-response relation between Amn1 levels and clumping. Negative feedback regulation of the synthetic copy of the gene is designed to address this hypothesis. Drug exposure will be included in the stress factors. In our future studies we seek to address the question whether the circuit response and phenotypic change under drug exposure mimics other stress responses and what genetic mechanisms regulate these responses.

Figure 1. A) Schematic illustration of Amn1 and Ace2 antagonistic functions during the mitotic exit. Ace2 is a transcription factor that activates AMN1 expression[1]. ACE2 overexpression has been shown to cause accelerated mitosis progression, whereas Amn1 abundancy prevents cells separation potentially through degradation of Ace2[2]. This project aims to deduce the stress and drug resistance mechanisms that involve these two genes. **B)** Bright-field time-course microscopy of the TBR1 strain forming clumps of the increasing size. **C)** Bright-field time-course microscopy of the sonicated TBR1 strain population showing that low-amplitude sonication decreases the size of the clumps allowing for more accurate image analysis and absorbance measurements while not provoking stress response.



Results.

➢*Δamn1* Mutation

To estimate the contribution of Amn1 to clumping phenotype of a pseudohyphal yeast strain TBR1, we knocked out*AMN1* gene which resulted in unicellular phenotype **(Fig. 2A).** All cells after selection were grown in YPD medium and synchronized by nocodazole.

➢Assessing the Clumping Frequency

Clump-to Single Cell Ratios showed decrease in clumping frequency up to 16-fold in the TBR1 *Δamn1* clone 4 population compared to a parental strain (**Fig. 2B**). For volumetric measurements of cell and cell clumps sizes, three different pattern-recognition image analysis modes were designed (**Fig. 2D**).

➢Synthetic Regulation of *AMN1* gene expression

To fully explore the dynamics of *AMN1* gene expression, we designed a negative feedback-based synthetic gene expression system that utilizes a user-controlled *AMN1* induction. The design includes Tet-repressor (TetR), *AMN1* intact sequence, and ymCherry reporter gene, separated by 2A "self-cleaving" peptides sequences, all expressed under pGAL1-T123 promoter[4].

➢Stress Resistance Profile

We designed stress factor titration experiments to elucidate the stress resistance profile of the strains created. Specifically, we exposed the model strains to mechanical (sonication), physical (freeze/thaw), and chemical (H_2O_2, ethanol) stressors. Among the parameters analyzed were growth kinetics (OD 600 curve slope and maximal value), microscopy-based volumetric measurements (cell and clump size etc.). To elucidate drug tolerance profiles, we suggest to apply the developed experimental setting for antifungal agents as well.

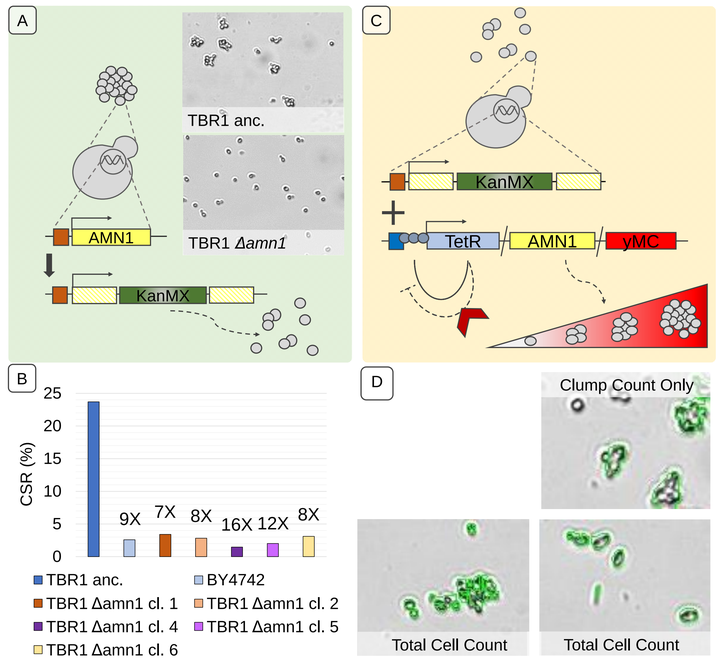
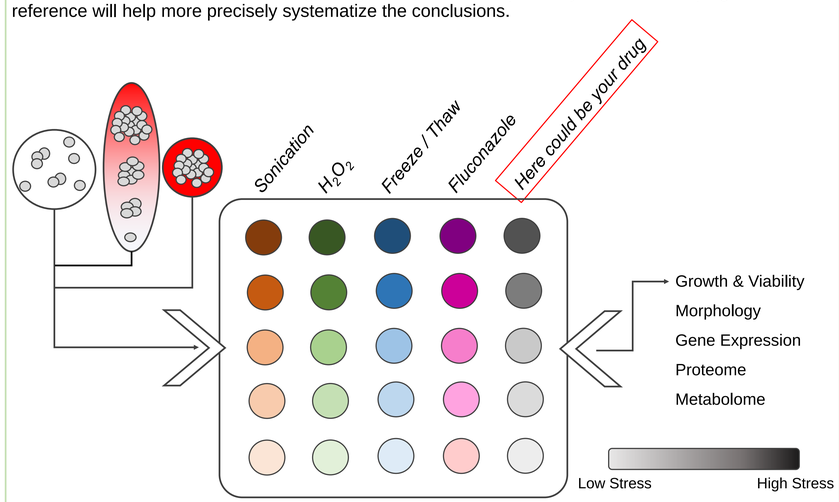


Figure 2. A) Homologous recombination of the *AMN1* deletion cassette (containing KanMX) with the *AMN1* gene locus produces unicellular phenotype in parental TBR1 anc. strain. **B)** Clump-to-Single Cell Ratios (CSR) of different TBR1 *Δamn1* monoclonal populations with the fold change, compared to the parental strain, indicated above each bar. **C)** Tet-repressible negative feedback circuit for *AMN1* expression fine tuning with doxycycline (dark red) as inducer with the hypothesized output, including ymCherry as a fluorescent reporter. **D)** Image-based cytometry modes used to characterize unicellular and clumpy populations.

$$CSR = \frac{n_{Clumps}}{n_{Cells}} * 100\%$$

Materials and Methods. Yeast Strains used in this study: *S. cerevisiae* Sigma 1278b (a.k.a TBR1Aanc.) MATα *ura3-52, his3::hisG, leu2::hisG*; Sigma 1278b (a.k.a. TBR1 *Δamn1*) MATα *ura3-52, his3::hisG, leu2::hisG*, *amn1::kanMX6;* BY4742 MATα *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0.* All molecular cloning procedures were carried out following QIAGEN® protocols and using correspondent kits. Yeast transformation was performed using MP Biomedicals™ EZ-Yeast™ Transformation Kit and G418 for selection. Image acquisition and preliminary image analysis was done using Cellometer® Vision CBA Image Cytometer (Nexcelom Bioscience LLC). Cell culture growth was characterized by Spectrophotometer UNICO® S-1205 and Tecan® Infinite 200 Pro microplate reader. Statistical analysis and image analysis were performed using Microsoft Excel and MATLAB R2018b. Negative feedback circuit regulation was designed following the protocol for the 'Linearizer' circuits[3,4].

Figure 3. Schematic illustration of stress factor titration experiment. The unimodal clumpy or unicellular populations, as well as those with synthetic regulation of clumping, are exposed to a dosage of environmental stress factor. Utilizing the yeast strain with precisely tunable clumping levels as a reference will help more precisely systematize the conclusions.



Discussion.

- **Synthetic Biology & Genetic Engineering.** Complementing the existing synthetic biology toolset for gene expression tuning with the system designed for an endogenous cell cycle gene will provide a new level of precision to fundamental biology discovery. Engineered yeast strains with controlled mitotic progression can serve as model and a reference object for drug resistance studies.
- **Evolution.** Elucidating the genetic background of multicellularity-induced drug resistance in yeast has a range of applications ranging from fundamental biology to drug design.
- **Drug Discovery.** We developed a robust and user-friendly algorithm for detecting the origin of yeast drug resistance. In this experimental setting, input, processing, and output parameters can be adjusted, depending on the question of the study (mechanisms of drug response, effective range of the drug, drug target and system of targets etc.).

Literature Cited

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