Doctoral Thesis (revised version) Review

Title: Antagonistic regulation by global transcription factors Tup1p and Cyc8p of Flo11 and Flo11-dependent phenotypes in wild yeast

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The thesis of M.Sc. Phu Nguyen Van concerns processes related to colony differentiation and biofilm formation of wild yeast strains, a topic representing the long-term interest of the host laboratory. Understanding wild yeast strains' adhesion and biofilm formation regulation is very important regarding human health. Studies described in this thesis can help understand similar processes in pathogenic yeast strains.

The thesis is conceived as a full-version thesis presenting the results summarized in two publications where M.Sc. Nguyen is the first author and some yet unpublished data. The papers are attached, compared to the original submission, however, without the supplemental data. The thesis contains all the necessary parts, including the List of abbreviations missing in the first version. The text of the thesis was much improved in all the chapters, and the revised version of the thesis as a whole is comprehensible. Rarely there are some missing words or incomplete sentences, but this does not hamper the understanding of the topic by the reader.

The Literature review chapter was re-written, and it reads very well. On 30 pages, it describes biofilm formation studied in *Saccharomyces cerevisiae* as a model and so far known regulatory principles. The author emphasizes the positive implications of biofilm formation and flocculation in industrial processes like in breweries compared to their negative influences in medical care with impact on human health. Known functions of various adhesins/flocculins, including Flo11, are addressed, and pathways regulating the expression of genes encoding them are described. The last part of the literature overview is devoted to the characteristics of Tup1 and Cyc8 proteins, how they evolved, how they are regulated, and their roles in regulating various metabolic pathways. All my concerns about this part stated in my first review were addressed in this revised version.

Chapter Material and Methods is quite detailed, including strains, plasmids, and primers tables. The majority of my concerns raised in the first review were addressed. Conditions for fluorescence microscopy, either regular or two-photon, were added and are described in detail. Sample preparation for LC-MS/MS was also included. However, dilutions of antibodies used for immunodetection of proteins and specification of centrifuges when using rpm in the method description are still absent. The methodology used is adequate for the range of performed experiments. However, some quantification of mRNAs detected by Northern blots or quantitative analysis by qPCR would allow for more precise comparison of the expression of the two analyzed genes – *TUP1* and *CYC8* and their influence on the expression of the *FLO11* gene.

The third chapter is devoted to Results and Discussion and is divided into three parts. This chapter was reorganized and re-written. Its first part describes newly added and yet unpublished data on proteomic analysis of strains with reduced production of Cyc8 and Tup1 proteins individually or in combination. Mass spectrometry analysis revealed that 70 % of proteins found upregulated in a *cyc8* variant were affected in the same way in a *tup1* variant. Among those, proteins identified in previous transcriptomic analyses involved in glucose and alternative carbon metabolism and sporulation. Surprisingly, the Flo11 protein was not identified by this analysis as being affected by the transcriptional repressors Cyc8 and Tup1 as

declared by various previous analyses of transcriptomes. New sets of proteins were found upregulated, like those ensuring ATP production and mitochondrial translation, proteins implicated in protein folding, or cell aging. Some downregulated proteins in individually analyzed *cyc8* and *tup1* variant strains and some differentially affected proteins were also identified. However, it is not always clear what carbon source/cultivation medium colonies used for the analysis were grown on. Only in the headings of one table in the main text, the medium GMA was mentioned, and the GMA medium is stated in all supplemental tables. Since this is essential information for the validity of comparison with previous findings and correct conclusions, it should be defined in the description of the experiment. It is not clear whether studies obtained data were compared with, were performed with the same or similar type (non-fermentable) carbon source.

The following section describes results on the analysis of the inverse regulation of the *FLO11* gene expression and biofilm formation by Tup1 and Cyc8 proteins published in PLOS Genetics. Wild yeast strains with various combinations of deletions and regulated expression of *TUP1* and *CYC8* genes were constructed, and their mRNA and protein content was analyzed by Northern blots and SDS-PAGE. The obtained results suggest that Cyc8 is a repressor of the *FLO11* gene expression, and this repression is counteracted by the Tup1 protein probably by binding to Cyc8 in a 4:1 ratio. Changes in the amount of both proteins, therefore, affect the regulation of the *FLO11* promoter. However, underlying DNA-binding proteins mediating the action of the Cyc8/Tup1 complex stay to be discovered. Results obtained within this section further suggest that Tup1 may inhibit the degradation of the Flo11 protein. The author also investigated the mutual roles of Tup1 and Cyc8 proteins in the regulation of flocculation and found out that both proteins repress adhesins/flocculins. The author also made an interesting observation that copper reduces the *GAL1* promoter induction by galactose. My comments on this part in the first review were addressed, and western blot images were replaced.

The third part of the Result chapter brings new results on the regulation of biofilm formation and dispersal in wild *Saccharomyces cerevisiae* by glucose, Tup1, and Cyc8 proteins that were also recently published. Experiments documented in this part corroborate findings from the first paper and include analyses of adhesion of various strains to plastic and the formation of biofilms in static and shaken cultures. As in previous parts, some results come from the author's collaborators from the host laboratory. This section has also been re-written and much improved. It brings new findings on the regulation of biofilms in the presence of high and low concentrations of glucose that are applicable to the field of pathogenic yeast infections and thus are important for human health.

The last chapter is devoted to Conclusions. It summarizes the main results obtained in this thesis's experimental work and places them into a broader context, emphasizing pathogenic yeasts, including pathogenic *Saccharomyces cerevisiae* variants. The fact that the organism long time believed to be safe is now recognized as an opportunistic pathogen gives us food for thought.

M.Sc. Phu Nguyen Van proved his ability to conduct scientific research and obtain good results publishable in high-impact journals. The first version of his thesis was not carefully prepared, and the author's inability to answer the questions of reviewers and members of the board led to the failure of the defense. Even though there are still some misspelling errors, missing words, and forgotten references, the overall impression after having read the revised version of the thesis is entirely different. This time, M.Sc. Phu Nguyen Van prepared a good manuscript with clearly defined goals that were achieved during the experimental work. He fulfilled the requirements of the Ph.D. program in Genetics, Molecular biology, and Virology by presenting two publications. I genuinely hope that M.Sc. Phu Nguyen Van will be successful at the defense, after which he can receive a Ph.D. degree.

Comments, questions, and suggestions for the discussion:

1/ You tried to construct multiple *cyc8* deletion strains with cassettes containing one cre/lox site (loxP). Have you not met a problem with cassettes replacement? Did you check whether the deletion cassette present in the putative transformants is the original one – what antibiotic marker was in? There are cases of recombination between the cassette already present in the genome and that being introduced reported. Other types of cre/lox sites recognized by the Cre recombinase exist and are recommended for multiple deletions within one strain.

2/ You state that the *CYC8* gene might be essential in the BR-F strain because you were not able to prepare a double deletion strain. However, mRNA and protein analysis under non-inducing conditions did show barely detectable *CYC8* mRNA and no protein in BR-F/ *cyc8*Δ/pGAL1-CYC8 (Fig. 6A, B). Provided that the *CYC8* gene is essential, how can the strain cyc8Δ/pGAL1-CYC8 grow in the absence of galactose? Is the GFP fusion of Cyc8 still considered unfunctional? If yes, how to interpret that the strain having one copy under the *GAL1* promoter and the other fused to GFP is vital in uninduced conditions (Fig S3 p.104). Please can you comment on this? I want to ask you to explain the mentioned figure S3 since there seems to be an exchange of fluorescence images B and D and G and I.

3/ I wonder how microscopy images of biofilms adherent to the bottom of wells in 96-well plates were acquired? The information on sample preparation is missing.

4/ Can you suggest a pathway regulating Cyc8 and Tup1 proteins in cells grown on glycerol in the context of the *FLO11* gene promoter and compare it with glucose-dependent regulation? How do your results from proteomic studies revealing the implication of Cyc8 and Tup1 in the repression of proteins involved in mitochondrial translation and ATP production go with the fact that cells with the reduced Tup1 level grow poorly on glycerol-containing media?

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