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**M.Sc. PHU NGUYEN VAN**

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

**Doctoral dissertation**

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**M.Sc. PHU NGUYEN VAN**

# Antagonistická regulace pomocí globálních transkripčních faktorů Tup1p a Cyc8p u Flo11 a Flo11-dependentních fenotypů u divokých kvasinek

**Dizertační práce**

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**STATEMENT**

This is to certify that no material appearing in this dissertation has previously been submitted and approved for the award of a degree by this or any other university. I furthermore certify that the work presented here is my own, conducted under the supervision of Prof. Zdena Palkova and Dr. Libuse Vachova.

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# ACKNOWLEDGEMENT

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| | |
|---|---|
| ALS | Aglutinin like sequence |
| $A_{600}$ | Absorbance at a wavelength of 600 nm |
| $A_{570}$ | Absorbance at a wavelength of 570 nm |
| ATF | Activating transcription factor |
| ATP | Adenosine triphosphate |
| cAMP | Cyclic adenosin-monophosphate |
| COMPASS | Complex of proteins associated with set1 |
| CREB | Cyclic AMP response element binding protein |
| ECM | Extracelullar matrix |
| EPA | Epithelial adhesin |
| FLO | Flocculins – a family of cell wall proteins of *S. cerevisiae* |
| GFP | Green fluorescent protein |
| GM | Glycerol medium |
| GPI | Glycosylphosphatidylinositol |
| HDACs | Histone deacetylases |
| HOG | High osmolarity glycerol |
| IAA | Indole acetic acid |
| kDa | Kilodalton |
| LC-MS/MS | Liquid chromatography with tandem mass spectrometry |
| MAPK | Mitogen activated protein kinase |
| ORF | Open reading frame |
| PCR | Polymerase chain reaction |
| pKA | Protein kinase A |
| ssDNA | Salmon sperm DNA |
| SDS-PAGE | Sodium dodecyl sulphate–polyacrylamide gel electrophoresis |
| SSN6 (CYC8) | Suppressor of snf1 protein or cytochrome c 8 |
| SWI/SNF | Switch/sucrose non-fermentable |
| TAE | Tris base/acetic acid/EDTA buffer |
| TBP | TATA-box binding protein |
| TPR | Tetratricopeptide repeat |
| TOR | Target of rapamycin |
| TUP1 | Deoxythymidine monophosphate uptake 1 |
| UAS | Upstream activation sequence |
| URS | Upstream repression sequence |
| UTY | Ubiquitously transcribed y chromosome |
| UTX | Ubiquitously transcribed x chromosome |
| WD40 repeat | a short structural motif of approximately 40 amino acids, usually ending with a tryptophan-aspartic acid (W-D). |

# ABSTRACT

Biofilms are a common mode of yeast growth in which cells adhere to each other and adhere to biotic and abiotic surfaces to form complex multicellular structures. Living together in biofilms provides cells with several benefits, compared to planktonic cells such as protection and resistance to antimicrobials, environmental stresses and host immune attacks. Biofilms may play many important roles in commercial industries. But they are considered to be extremely dangerous in clinical settings. There is thus great interest in studying biofilms and how to eliminate them.

In this study, we used wild yeast *Saccharomyces cerevisiae* colony biofilm as an ideal system to investigate potential functions of the yeast Cyc8p-Tup1p transcriptional corepressor complex in the regulation of yeast adhesion and biofilm formation on agar and at solid-liquid interfaces. Unexpectedly, we found that Cyc8p and Tup1p antagonistically control *FLO11* expression and the formation of structured biofilm colonies on agar. Cyc8p itself acts as a key repressor of *FLO11* and biofilm colony formation, whereas Tup1p promotes the formation of biofilm colonies and induces *FLO11* expression by inhibiting the repressive function of Cyc8p and preventing Flo11p degradation possibly by inhibiting an extracellular protease. Other typical features of biofilm colonies such as formation of fibers inter-connecting the cells and cell invasiveness, are inversely regulated by Cyc8p and Tup1p as well. On the other hand, both proteins in concert repress cell flocculation as reduced expression of either *CYC8* or *TUP1* led to the production of macroscopic flocs (clusters of cells).

The antagonistic actions of Cyc8p and Tup1p were also exhibited in the formation of solid-liquid interface biofilms. We have provided experimental evidence that Cyc8p and Tup1p are key regulators in two steps of the *S. cerevisiae* biofilm developmental life cycle-cell adhesion (followed by

biofilm formation) and biofilm dispersal. The first step, adhesion and biofilm organization, is conversely regulated by Tup1p (activator) and Cyc8p (repressor), whereas biofilm dispersal is controlled by Cyc8p and is dependent on the level of environmental glucose. We show that even a low level of glucose is sufficient to disrupt the biofilm and release planktonic cells.

Our proteomic data not only identified hundreds of genes known to be regulated by Cyc8p and Tup1p, but identified for the first time several extra sets of genes encoding proteins that are involved in processes such as protein refolding and protein complex assembly, chronological cell ageing and apoptosis. The data indicated that global effects of Cyc8p and Tup1p on the regulation of gene expression in yeast. Where Cyc8p and Tup1p may act together or act independently to control gene expression. Even more interesting, they can act oppositely in regulation of several target genes such as *FLO11, MET17* and *URA2.*

Findings in this study confirmed that Flo11p is a key factor in abiotic adhesion and biofilm formation and other typical features of biofilm colonies that are positively regulated by Tup1p and negatively controlled by Cyc8p.

# ABSTRAKT

Biofilmy jsou běžným způsobem růstu kvasinek, při kterém buňky adherují jak k sobě navzájem tak i k abiotickým povrchům za vzniku složitých mnohobuněčných struktur. Společné soužití v biofilmech poskytuje buňkám několik výhod ve srovnání s planktonními kulturami. Mezi ně patří nepochybně ochrana a odolnost vůči antimikrobiálním látkám, stresovým faktorům prostředí nebo imunitnímu napadení hostitele. Biofilmy se nacházejí v mnoha prostředích a hrají důležité role v komerčních průmyslových odvětvích. Mohou však být také extrémně nebezpečné v klinickém prostředí. Existuje tedy velký zájem o studium biofilmů a o to, jak je eliminovat.

V této studii jsme použili biofilm divokého kmene kvasinky *Saccharomyces cerevisiae* jako ideální systém pro zkoumání potenciálních funkcí komplexu transkripčních korepresorů Cyc8-Tup1 při regulaci buněčné adheze a tvorby biofilmu na agarovém médiu a na rozhraní pevné látky a kapaliny. Neočekávaně jsme zjistili, že Cyc8p a Tup1p antagonisticky řídí tvorbu strukturovaných biofilmových kolonií na pevných médiích prostřednictvím modulace expresí genu *FLO11*. Samotný Cyc8p působí jako klíčový represor *FLO11*, zatímco Tup1p podporuje tvorbu biofilmových kolonií a indukuje expresi *FLO11* inhibicí represivní funkce Cyc8p a zároveň zabraňuje degradaci Flo11p možnou inhibicí extracelulární proteázy. Kromě toho jsou pomocí Cyc8p a Tup1p nepřímo regulovány i další vlastnosti typické pro biofilmové kolonie jako je tvorba vláken propojujících buňky a invazivní růst. Na druhou stranu oba proteiny shodně potlačují flokulaci buněk, protože snížená exprese *CYC8* a delece *TUP1* vedly k produkci makroskopických vloček (shluků buněk).

Antagonistická role Cyc8p a Tup1p popsaná výše v souvislosti s regulací tvorby biofilmu na agarovém médiu byla také pozorována při tvorbě biofilmů na rozhraní mezi pevným podkladem a kapalinou. Poskytli jsme

experimentální důkazy o tom, že Cyc8p a Tup1p jsou klíčovými regulátory ve dvou krocích vývojového cyklu biofilmu *S. cerevisiae* - adheze buněk následovaná tvorbou biofilmu a disperze biofilmu. První krok adheze a organizace biofilmu je protichůdně regulován Tup1p (aktivační funkce) a Cyc8p (represorová funkce), zatímco disperze biofilmu je řízena pouze Cyc8p a je závislá na hladině glukózy v prostředí. Ukázali jsme, že i nízká hladina glukózy (srovnatelná například s hladinou glukózy v krvi) je dostatečná pro narušení biofilmu a uvolnění planktonických buněk.

Naše proteomická data nejen identifikovala stovky genů, o kterých je známo, že jsou regulovány pomocí Cyc8p a Tup1p, ale poprvé identifikovala několik dalších sad genů kódujících proteiny, které se podílejí na procesech, jako je opětovné skládání proteinů a sestavení proteinového komplexu, chronologické stárnutí buněk a apoptóza. Data ukázala, že globální účinky Cyc8p a Tup1p na regulaci genové exprese v kvasinkách. Kde Cyc8p a Tup1p mohou působit společně nebo působit nezávisle na řízení genové exprese. Ještě zajímavější je, že mohou působit opačně při regulaci několika cílových genů, jako jsou FLO11, MET17 a URA2.

Zjištění v této práci potvrdila, že Flo11p je klíčovým faktorem abiotické adheze a tvorby biofilmu a dalších typických rysů biofilmových kolonií, které jsou pozitivně regulovány pomocí Tup1p a negativně pomocí Cyc8p.

# GENERAL INTRODUCTION

Non-*Candida* yeast species, such as *Saccharomyces cerevisiae*, are increasingly being considered as opportunistic pathogens since they have recently been reported to infect immunosuppressed or critically ill patients (de Groot *et al.,* 2013). *S. cerevisiae* is relatively resistant to amphotericin B and azoles (Barchiesi *et al.,* 1998), so it is able to inhabit niches, left empty after the elimination of azole-sensitive *Candida albicans* and other yeasts (Bojsen *et al.,* 2014). Two important virulence factors involved in yeast infection by either *Candida* species or clinical *S. cerevisiae* strains are adhesion and biofilm formation (de Groot *et al.,* 2013). Adhesion, the first step in pathogenic biofilm formation, is mediated by a group of cell wall proteins knows as adhesins (Lipke *et al.,* 2018).

Previous findings suggested that *FLO11* is a key player in adhesion to abiotic surfaces (Reynolds and Fink, 2001) as well as in biofilm colony development in yeast *S. cerevisiae* (Stovicek *et al.,* 2010; Vopalenska *et al.,* 2010). Deletion of the *FLO11* gene triggered a switch from structured to smooth colonies and the mRNA levels of Flo11p are much higher in structured colonies of wild yeast than in smooth colonies of domesticated strains (Stovicek *et al.,* 2010; Stovicek *et al.,* 2014).

At 2.8 kb in length, the *FLO11* promoter is much longer than most other yeast promoters, facilitating the integration of multiple signals from diverse signaling cascades including Ras-cAMP-PKA, MAPK and major pathways of glucose repression. These induce or repress Flo11p expression in response to growth stage and nutritional conditions (Rupp *et al.,* 1999; Verstrepen and Klis, 2006; Vinod *et al.,* 2008). In addition, epigenetic mechanisms such as histone deacetylation, chromatin-remodeling and non-coding RNA expression also regulate the expression of Flo11p (Conlan and Tzamarias, 2001; Halme *et al.,* 2004; Bumgarner *et al.,* 2009; Barrales *et al.,* 2012).

The corepressor complex, Cyc8p-Tup1p (formerly Ssn6p-Tup1p) consisting of four molecules of Tup1p and one of Cyc8p is highly conserved among eukaryotes (Smith and Johnson, 2000). This complex controls the expression of many *S. cerevisiae* genes and regulates a wide range of pathways, from glucose, starch and oxygen use to osmostress responses, mating, meiosis and sporulation and also flocculation (DeRisi *et al.,* 1997; Smith and Johnson, 2000; Green and Johnson, 2004; Malave and Dent, 2006). It has been shown that Flo11p may be regulated directly or indirectly by the Cyc8p-Tup1p complex through interaction with Mig1p and Nrg1p at the promoters of glucose repressed genes (Wilson *et al.,* 1996; Verstrepen and Klis, 2006).

Several mechanisms for repression by the Cyc8p-Tup1p complex have been proposed: such as repression of target genes by changing chromatin, altering nucleosome positioning, or recruiting histone deacetylases (HDACs) by interacting with under acetylated isoforms of histones H3 and H4, or even that the Cyc8p-Tup1p complex can inhibit the general transcriptional machinery or block and/or inhibit activators at the activation domains of target genes (Wong and Struhl, 2011).

This Cyc8p-Tup1p complex may also act as transcriptional co-activators which promote the expression of various genes under a variety of conditions, including *HAP1* (Zhang and Guarente, 1994; Hickman and Winston, 2007), *GRE2* (Proft and Struhl, 2002), *FRE2* (Fragiadakis *et al.,* 2004), *ARG1* and *ARG4* through Gcn4p (Kim *et al.,* 2005) and Stp1/2p (Tanaka and Mukai, 2015).

Moreover, several independent roles of Tup1p and Cyc8p have been reported in *C. albicans*. For example, Cyc8p plays a critical role in filamentous growth and virulence, independently of Tup1p (Hwang *et al.,* 2003), whereas some, hypha-specific genes are suppressed by Tup1p- Nrg1p, in a Cyc8p independent manner (Garcia *et al.,* 2005). Cyc8p was identified as a core regulator of white-opaque switching (Hernday *et al.,* 2016). While

Tup1p has been reported as a key repressor of the opaque state and coordinates with Wor1p, to control the opaque switch under different conditions (Alkafeef *et al.,* 2018).

Deletion of *TUP1* in other pathogenic yeast, such as in *Cryptococcus neoformans, Magnaporthe grisea* and*, Ustilago maydis* resulted in defects in quorum sensing, morphogenesis and pathogenicity of the fungus (Lee *et al.,* 2007; Elias *et al.,* 2011; Chen *et al.,* 2015). Thus, Tup1p and Cyc8p may be considered as virulence factors, contributing to the regulation of metabolic, morphological and transcriptional responses to varied nutritional and environmental signals and stresses in pathogenic fungal species.

The aim of my PhD research is to uncover functions of Cyc8p and Tup1p in regulation of structured biofilm development on agar as well as of solid-liquid interface biofilm formation, Flo11p expression, adhesion to plastic surfaces and other features, specific to complex biofilms. We also aim to discover the global effects of these regulators on gene expression at the protein level.

To do this, we used as our model a wild strain of yeast *S. cerevisiae* with well-defined characteristics and behavior (Stovicek *et al.,* 2010; 2014; Vopalenska *et al.,* 2010). We used a combination of inducible promoters ($p_{GAL}$, $p_{CUP}$), fluorescent protein tagging methods and techniques such as LC-MS/MS, electron microscopy and two photon confocal microscopy.

We provide evidence that Tup1p is a driver of *FLO11* expression and Flo11p-dependent phenotypes such as adhesiveness and structured biofilm development on agar and at solid liquid interfaces, whereas Cyc8p is a repressor of *FLO11* expression and inhibits biofilm formation and cell adhesion. Interestingly, our results also show that Cyc8p stimulates biofilm dispersal and that Cyc8p expression is promoted by glucose. Moreover, the antagonistic effects of Cyc8p and Tup1p also extend to other target genes such as *MET17* and *URA2*. The global impact of Cyc8p and Tup1p on the

proteome of cells in yeast colonies is also described. Many sets of genes were repressed by both regulators. However, dozens of genes were independently regulated by either Cyc8p or Tup1p since these genes were upregulated in the *cyc8* mutant, but not in the *tup1* mutant or vice versa. Excitingly, some sets of genes have been shown for the first time to be repressed by Cyc8p and/or Tup1p. These findings, described in detail in chapter III, provide new insights into the regulatory functions of the Cyc8p-Tup1p complex and could make a significant contribution towards targeting the twin problems of biofilm formation and dispersal in medicine and industry. The data reported in this dissertation has resulted so far in one paper in *PLOS Genetics* and another in *npj Biofilms and Microbiomes*. These papers are included in the Attachments.

This dissertation is organized as follows: chapter I, a literature review. Chapter II presents material and methods that were used in this work. Results and discussion are presented in chapter III. Finally, conclusions make up chapter IV.

# CHAPTER I. LITERATURE REVIEW

## 1.1 Biofilm

In nature, many microorganisms prefer to grow together on surfaces to yield colonies, biofilms and other structured, multicellular communities (Palkova *et al.,* 2004). The major advantage of biofilm formation is that microorganisms within biofilms are safe from environmental insults such as desiccation as well as antifungal therapeutics and host immune responses. Cells in biofilm are highly resistant to antibiotics, they differentiate and exhibit different phenotypes that are associated with the persistence of infections (Sharma *et al.,* 2019), whereas these characteristics are not found in planktonic cells or non-biofilm forming microorganisms (Martinez and Fries, 2010; Fanning and Mitchell, 2012).

Biofilms have been found in a wide range of environments from rivers, lakes and soil to the skin and mucus membranes of animals. They can form on catheters, needles and other medical devices (Viudes *et al.,* 2002), in industrial pipes and in drinking or waste-water plants (Torregrossa *et al.,* 2012). Biofilms can live in severe conditions such as extremes of heat and pH and even in frozen glaciers (Bogino *et al.,* 2013; Desai *et al.,* 2014).

Biofilm may have economic benefits in some cases, such as in bioremediation, heavy metals can be removed efficiently by biofilm-forming microbes (Basak *et al.,* 2014; Grujic *et al.,* 2017; Nguyen *et al.,* 2020). Biofilms grown on filters help to remove organic waste and play a vital role in the clean-up of seawater, contaminated with petroleum oil (Lee *et al.,* 2010; Lee *et al.,* 2014).

But biofilms also cause serious issues for human healthcare, since mature biofilms can tolerate a wide range of antibiotics and immune responses (Bryers *et al.,* 2008). For example, it is extremely challenging to prevent pathogenic bacteria from forming biofilm on surgical devices and

cystic fibrosis patients are at risk from biofilm-forming bacteria such as *Pseudomonas aeruginosa* (Lynch and Robertson, 2008). Infections involving biofilms are more often associated with pathogens both in plants and in animals (including man), such as in bacterial vaginosis urinary tract infections and formation of dental plaque, causing dental caries (Viudes *et al.,* 2002; Pfaller and Diekema, 2007; Arendrup *et al.,* 2011).

**1.2 *Saccharomyces cerevisiae* as a model for studying biofilm**

Many fungal pathogens such as *Candida* (Finkel and Mitchell, 2011), *Cryptococcus* (Martinez and Casadevall 2007), *Trichosporon* (Bonaventura *et al.,* 2006), *Coccidioides* (Davis *et al.,* 2002) and *Pneumocystis* (Cushion *et al.,* 2009) spp., can form biofilms with extracellular matrix that exhibit high resistance to antifungal agents and that adhere strongly to medical devices and implants (Fanning and Mitchell, 2012). Therefore, preventing and/or disrupting biofilms in clinical and industrial settings is of great importance. Using *Candida* species or other pathogenic yeast to investigate the mechanism of biofilm formation is relatively complicated and a health risk. Moreover, genetic modifications of this species are more challenging due to incomplete characterization of genes and limited availability of strains and genetic markers, and other molecular biology tools.

*S. cerevisiae* is much simpler to genetically manipulate and has long been a preferred eukaryotic model for biological research since it has several advantageous characteristics, including both asexual and sexual reproductive life cycles (Liti *et al.,* 2015). *S. cerevisiae* was the first eukaryote to have its genome sequenced in full (Goffeau *et al.,* 1996), genes and proteins have been extensively characterized. Resequencing of the reference and related *S. cerevisiae* strains has given the species great utility as a model organism (Engel *et al.,* 2014). Moreover, there is a large number of useful tools such as deletion, overexpression and GFP tagging libraries that make *S. cerevisiae* such a tractable research tool, compared with other yeasts (Ryan *et al.,* 2012). So, this strain has been widely used as a biofilm model in various studies.

### 1.3 Yeast biofilm development

Four main stages of biofilm development have been proposed as shown in Fig. 1.1, which include: (1) Adhesion: initial adhesion to a foreign surface is the first step in biofilm formation by planktonic cells; (2) Initiation: following adhesion, the cells progressively grow and form aggregates; (3) Proliferation: yeast cells proliferate and colonize available abiotic and biotic surfaces, begin phenotypic switching and secrete extracellular matrix (ECM); (4) Maturation: the formation of mature, highly structured biofilm with high production of ECM. Cells may be dispersed from the biofilm with the ability to colonize new sites and repeat the cycle (Santos *et al.,* 2018; Fanning and Mitchell, 2012).
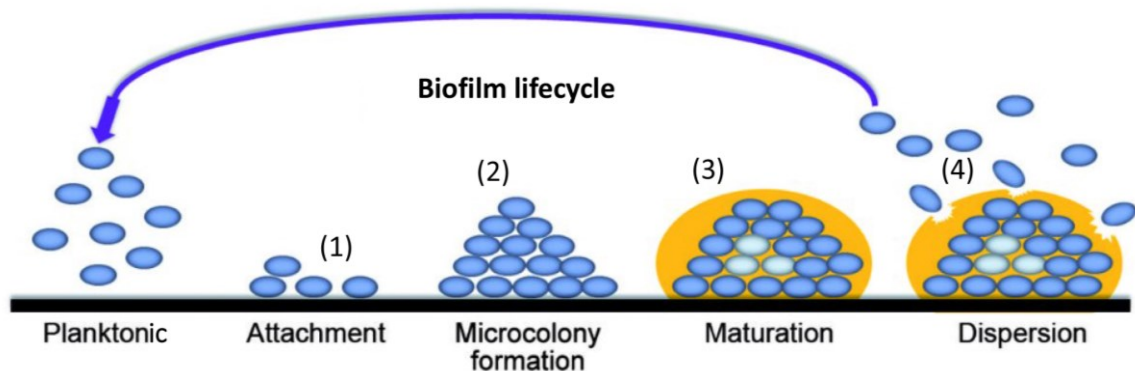


**Figure 1.1. Biofilm development steps produced by microorganisms.**

*The image shows the adhesion of microbes to a surface, followed by microcolony formation, maturation of the biofilm 3D architecture and, finally, its dispersion, which can lead to the colonisation and formation of a new biofilm structure in a distinct place, as well as the persistence of the infectious disease. Note the occurrence of specialized cells, including persisters (depicted in light blue oval) and the presence of extracellular polymeric matrix (depicted in orange). Blue ovals are microbial cells. This figure was adapted from (Santos et al., 2018).*

### 1.4 Yeast adhesion

Yeast cells are able to adhere to a variety of biotic or abiotic surfaces since they possess specialized surface proteins named adhesins (Douglas *et al.,* 2007; de Groot *et al.,* 2013) that are present on the surface of the cell wall, where they contribute to mating, phenotypic switching, biofilm formation and interaction with other cells, as well as with mammalian and

plant hosts and nonspecific binding to abiotic surfaces (Douglas *et al.,* 2007). It has been established that yeast adhesins are major virulence factors of pathogenic yeast such as *C. albicans* and *C. glabrata* (Bernhardt *et al.,* 2001), but they also confer useful qualities on industrial yeast such as *S. cerevisiae*. Understanding how yeast cells adhere to one-another, to surfaces and how environmental factors influence these processes is important to industry, medicine and environmental biology (de Groot *et al.,* 2013).

Yeast adhesion is the first and crucial step in pathogenic biofilm formation because it prevents cells being flushed from a nourishing environment and enables them to establish biofilms and infect hosts (Klemm *et al.,* 2010; de Groot *et al.,* 2013). This contributes to yeast cell development through nutrient availability and protection from hostile environments. For instance, *C. albicans* cells, adhered to an abiotic dental prosthetic, exhibited greater tolerance towards a variety of antifungals than those growing planktonically (Ramage *et al.,* 2005; Taff *et al.,* 2013).

Although yeast adhesin genes display broad variation, they also exhibit certain structural and functional similarities with those of related species. For example, the PA14 domain-related adhesins, which are conserved in both non-pathogenic and pathogenic yeasts (Timmermans *et al.,* 2018). Introducing the gene for Als1p (an adhesion protein of *C. albicans* and a key virulence factor) into *S. cerevisiae*, cells enable the latter to attach to human epithelial and endothelial cells (Fu *et al.,* 1998), while *S. cerevisiae* cells expressing Als5p (another adhesion protein of *C. albicans*) could adhere to ECM and then undergo cell-cell aggregation (Gaur *et al.,* 2002). In addition, *S. cerevisiae* cells efficiently adhered to human epithelial cells when expressing the *C. glabrata* gene *EPA1* (Cormack *et al.,* 1999).

In the same manner, *S. cerevisiae* cells display the ability to adhere to one another (to flocculate) and to plastic surfaces (Verstrepen and Klis, 2006). Five dominant lectin-encoding genes have been identified that may play a role

in *S. cerevisiae* adhesion. While four adhesion proteins, Flo1p, Flo5p, Flo9p and Flo10p mediate interaction between adjacent cells, leading to aggregation of cultured cells (flocs), which sediment out of suspension (Teunissen and Steensma, 1995; Govender *et al.,* 2008), Flo11p expression does not contribute to flocculation at all (Guo *et al.,* 2000). On the contrary, Flo11p is responsible for various forms of cell/substrate adhesion, such as adhesion to surfaces (plastics, glass, etc.), biofilm formation (fluffy colonies), mat formation, flor formation, pseudohyphal and invasive growth (Lambrechts *et al.,* 1996; Rupp *et al.,* 1999; Reynolds and Fink, 2001; Reynolds *et al.,* 2008; Vopalenska *et al.,* 2010).

In regard to environmental changes, yeast cells express different types of adhesins (Fig. 1.2), which allow the yeast to quickly adapt to unfavourable environments. For example, when glucose is present, Flo11p is not produced, but under glucose depletion or nitrogen starvation, Flo11p is activated, allowing yeast cells to attach to and invade substrates to seek nutrients and to evade a range of stresses (Kron 1997; Gagiano *et al.,* 2002; Verstrepen and Klis, 2006). Increasing Flo11p expression from basal to high level adhesion possibly permits yeast to accommodate stresses. In the main, *FLO* genes involved in adhesion are triggered by diverse environmental factors such as nitrogen or carbon starvation and changes in pH, with tight regulation by many transcription factors (Verstrepen *et al.,* 2003; Sampermans *et al.,* 2005).
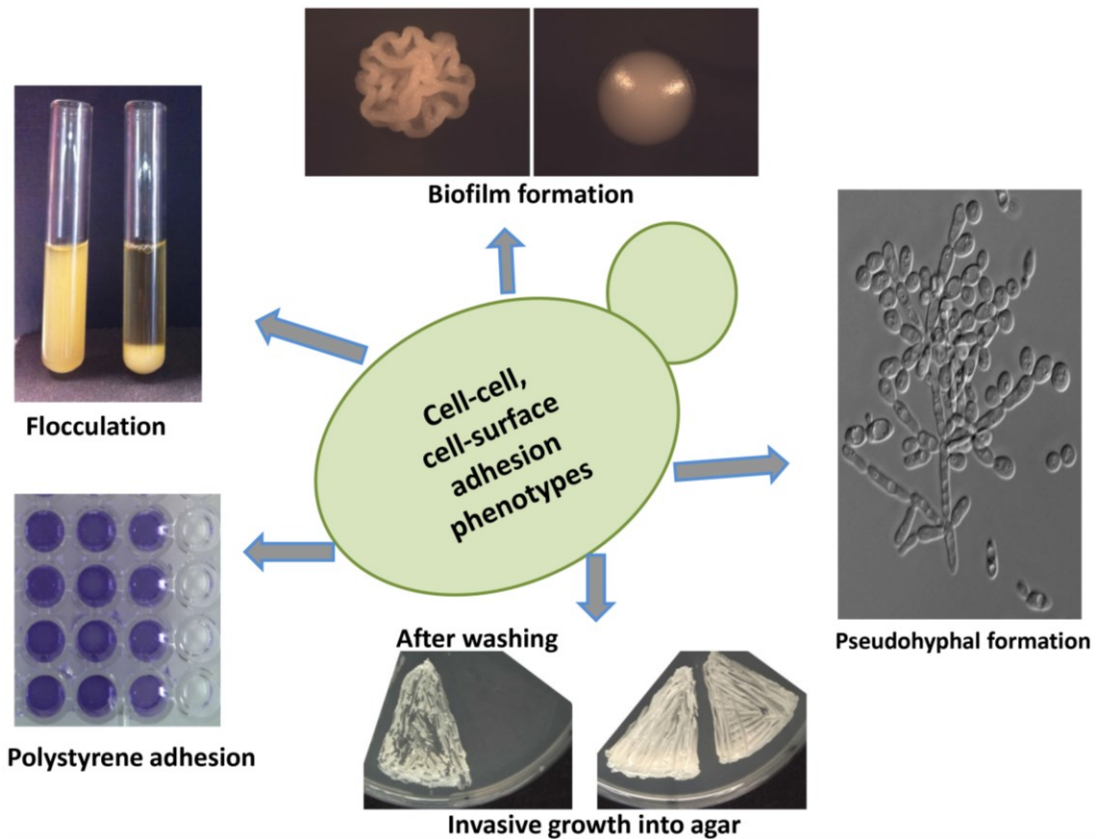
**Figure 1.2. Yeast cells exhibit different adhesion phenotypes**

## 1.5 *FLO* gene family

Flo1p, Flo5p, Flo9p and Flo10p, encoding specific surface proteins have a similar binding domain at the N-terminus of the lectin, which binds to mannose residues on the surface of other cells. This binding is dependent on the presence of $Ca^{2+}$ ions and allows cell flocculation, i.e. non-sexual cell clustering accompanied by sedimentation of these aggregates in fluid medium (Verstrepen and Klis, 2006; Goossens and Willaert, 2010). Genes coding for these adhesins are located in subtelomeric regions, whereas *FLO11* is located more distant from the telomere and Flo11p-dependent adhesion is not dependent on saccharides (Teunissen and Steensma, 1995; Lo and Dranginis, 1996).

The agglutinin-like sequence (ALS) in *C. albicans* and epithelial adhesion (EPA) genes in *C. glabrata* are orthologous to flocculin (*FLO*) genes in *S. cerevisiae* (de Groot *et al.,* 2013; Desai *et al.,* 2014). They are cell wall associated adhesins attached to the plasma membrane by a

glycophosphatidylinositol (GPI) anchor sequence. They share common architecture involved in surface activity and biological function. Adhesins can be divided into three domains designated as A, B and C see Fig. 1.3 (Goossens and Willaert, 2010).

The amino terminal domain (A): this region possesses carbohydrate groups, mediates adhesion to cells and surfaces, binds calcium and extends from the surface of the cell. Except for Flo11p, the A domain of all flocculins (Flo1p, Flo5p, Flo9p and Flo10p) consists of the PA14 domain, a conserved β-barrel structure, which is responsible for interaction with the sugar (Rigden *et al.,* 2004; Veelders *et al.,* 2010). The A domain of Flo5p has been shown to directly interact with complex mannose oligomers stabilized by $Ca^{2+}$ (Veelders *et al.,* 2010). However, they all have a short hydrophobic signal sequence of 20 to 30 amino acids that ensures efficient targeting to the endoplasmic reticulum (ER), and entry into the secretory pathway. Once inside the ER this sequence is cleaved by a signal protease (Verstrepen and Klis, 2006; Dranginis *et al.,* 2007). The three dimensional structure of the N-terminal domain was first described in detail for Flo5p and discovery of the structure allowed researchers to model the A domain of other flocculins including Flo1p (sharing 94% sequence identity with Flo5p at the protein level), Flo9p (89% identity) and Flo10p (64% identity) (Veelders *et al.,* 2010).

The central domain (B): this domain is composed of multiple serine and threonine rich repeats (which account for 60% of the Flo11p sequence). *FLO* genes in different strains have variable lengths of tandem repeats, which are vulnerable to N-or O-linked glycosylation (Douglas *et al.,* 2007). In addition, repeated sequences facilitate recombination events, inducing deletions and insertions, causing changes in the amount and lengths of repeats (Verstrepen *et al.,* 2005). The tandem repetitions therefore play a key role in the stabilization of flocculins (Loza *et al.*, 2004).

The carboxy terminal domain (C) of adhesins contains a high proportion

of hydrophobic amino acids and, like the central domain, undergoes an important post-translational modification in the endoplasmic reticulum: the addition of a glycosyl phosphatidylinositol (GPI) anchor, which helps to connect the adhesin to the plasma membrane (Hamburger *et al.,* 1995; Bony *et al.,* 1997; Verstrepen *et al.,* 2001; Verstrepen and Klis, 2006). A short signal sequence at the end of the protein is responsible for recognition during these steps and is replaced by this anchor. After arrival in the plasma membrane, the adhesin is excised from the GPI anchor and the adhesin is covalently bound to β-1,6 glucans (Verstrepen *et al.,* 2004b; Verstrepen and Klis, 2006; Dranginis *et al.,* 2007).
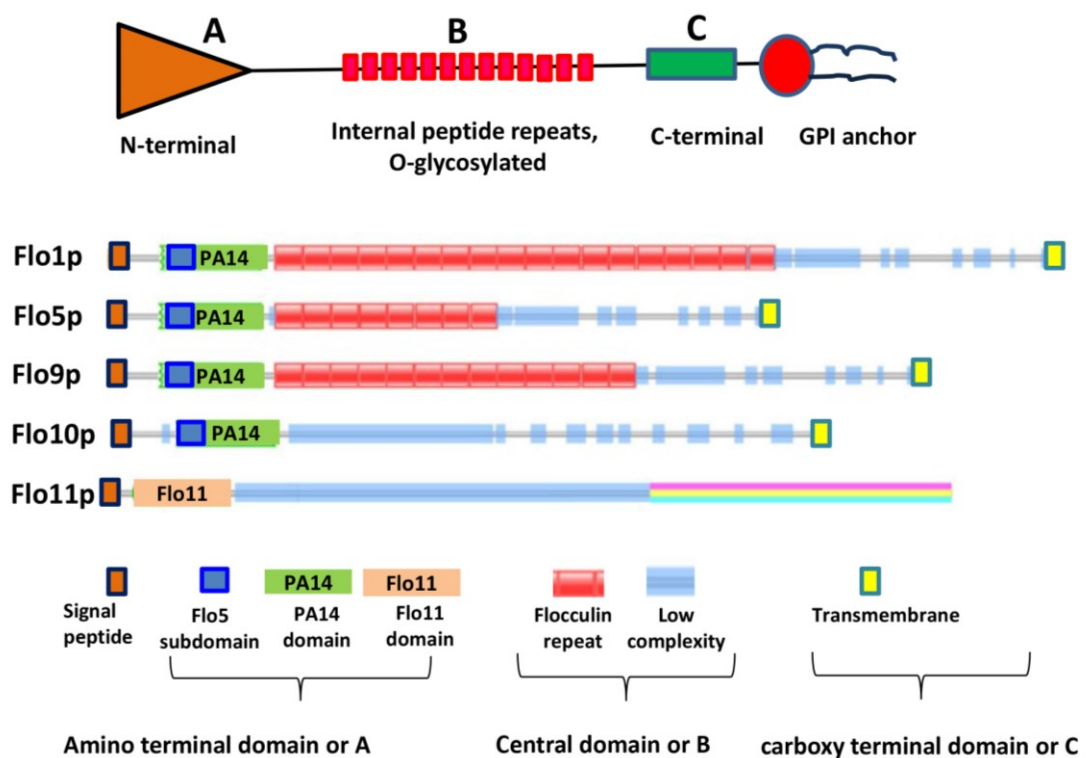


**Figure 1.3.** *S. cerevisiae* **strain S288c: flocculin domains** *as explained in the text, adapted from (Goossens and Willaert, 2010).*

The expression of *FLO* genes results in a flocculation phenotype, this helps yeast cells respond to some stresses, such as limited nutrients, hypoxia, pH changes and temperature changes (Gibson *et al.,* 2007). Flocculation may shield cells within flocs from heat, cold and chemical reagents (Smukalla *et*

*al.,* 2008). It is a beneficial phenotype of brewing strains, removing yeast cells at the end of the fermentation process (Verstrepen *et al.,* 2003; Soares *et al.,* 2011). However, some common laboratory strains do not flocculate readily, because of a nonsense mutation in *FLO8* (Liu *et al.,* 1996).

*FLO* genes are subject to complicated regulation by the Ras-cAMP-PKA pathway and numerous upstream genetic elements, such as Flo8p, Mss11p (Bester *et al.,* 2006), the COMPASS methylation complex (Dietvorst and Brandt, 2008), histone deacetylase (Hda1p) and histone acetyl transferase (Gcn5p). The *FLO1* gene is expressed in the absence of Had1p, while *FLO1* and *FLO9* are induced in the absence of Gcn5p (Dietvorst and Brandt, 2010). In contrast to Flo11p, the expression of Flo1p mediates cell-cell adhesion rather than filamentation or adhesion to other surfaces (Guo *et al.,* 2000). However, Flo1p and Flo11p may share regulators of expression (Fichtner *et al.,* 2007) detailed in Fig. 1.4.
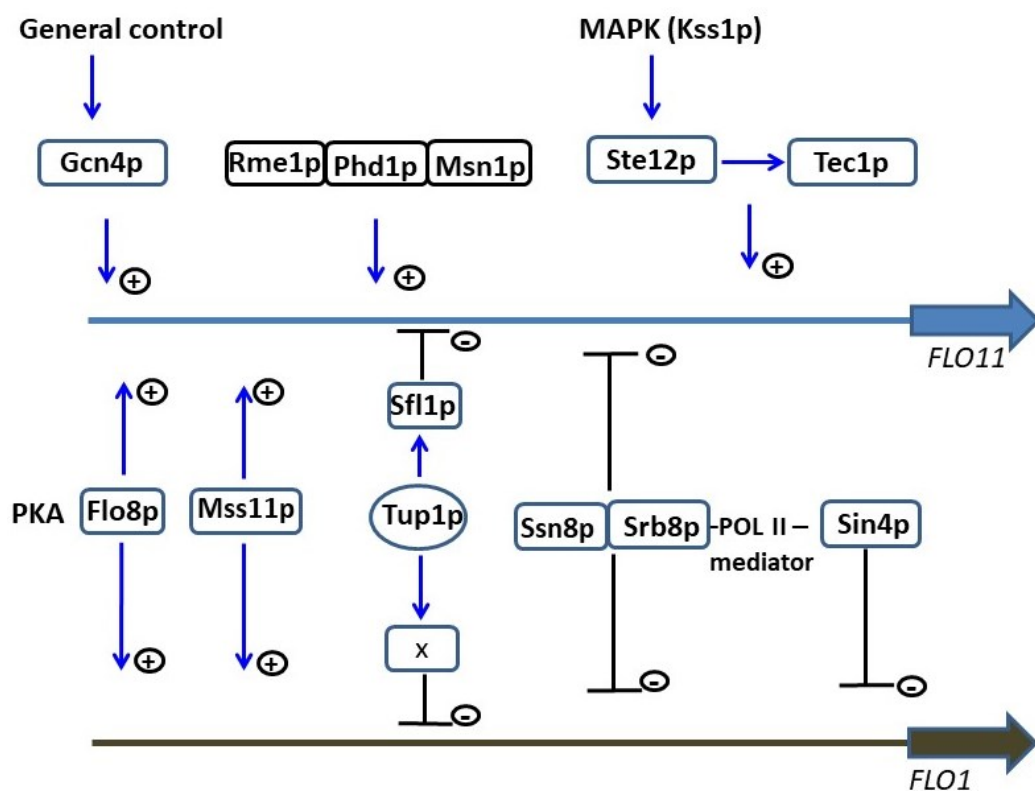


**Figure 1.4. *FLO11* and *FLO1* transcriptional regulation in *S. cerevisiae* S288c**.

## 1.6 Flo11p

### *1.6.1 Structure of Flo11p*

*S. cerevisiae* Flo11p was first described in 1996 as a putative membrane-bound protein, for which similarities to mammalian membrane-bound mucins were demonstrated. Therefore, it was originally called *MUC1* (Lambrechts *et al.,* 1996). Then it became more widely known as a factor in invasive growth and the development of pseudohyphae. Because of its role in flocculation, the structural similarity to other members of the *FLO* family and the localization of the protein on the cell surface, it was later referred to as *FLO11* (Lo and Dranginis, 1996).

Flo11p has a length of 1367 amino acids, a molecular weight of 136 kDa, an isoelectric point of 3.98 and similar three-domain-like composition to other fungal adhesins (Fig. 1.3) (Lo and Dranginis, 1996; Goossens and Willaert, 2012). In *S. cerevisiae* S288c *FLO11* is located on Chromosome IX and includes multiple repeat sequences (Marinangeli *et al.,* 2004; Verstrepen *et al.,* 2005). More recently, Timo Kraushaar and coworkers described the N-terminal Flo11p A domain structure at high resolution (Kraushaar *et al.,* 2015).

### *1.6.2 FLO11 regulation*

Complex signaling controls the expression of *FLO11* and signaling is integrated via an unusually large promoter of *FLO11,* containing a large number of transcription factor binding sites (Fig. 1.5)
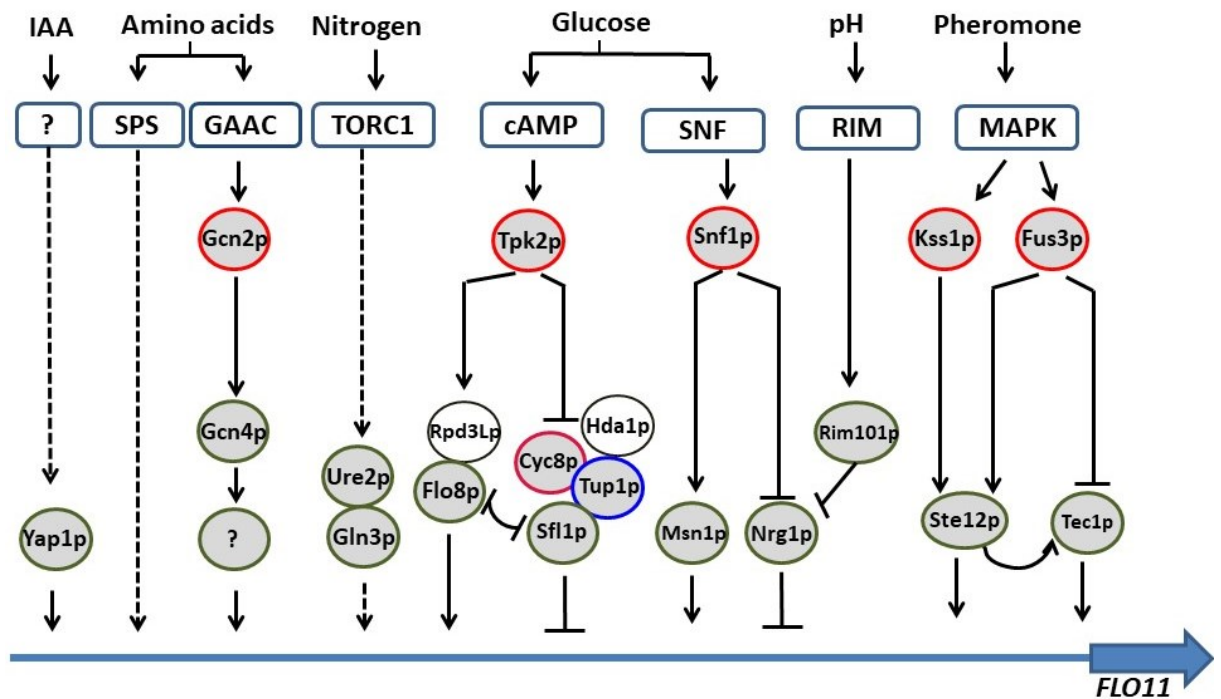
**Figure 1.5. Regulation of *FLO11* expression.**

*Complex character of FLO11 regulation under the control of genetic and epigenetic mechanisms. Arrows indicate positive regulation, inhibition is shown by bars. Different stimuli and corresponding signaling pathways targeting FLO11 are indicated at the top (IAA – indole acetic acid; ?– unknown pathway). Rectangles – signal transduction pathways, red bordered circles – kinases involved in regulatory cascades. Transcription factors targeting the FLO11 promoter are shown as circles with tan borders, Cyc8p and Tup1p are indicated as dark red and blue bordered circles, respectively. The input of the different transcription factors is shown schematically and does not correspond to the positions of known binding sites. Reproduced with modifications from Bruckner and Mosch (2012).*

**1.6.2.1 MAPK pathway**

In *S. cerevisiae* the mitogen-activated protein kinase (MAPK) is a highly conserved signaling cascade, it is triggered by changes in the extracellular environment eliciting physiological responses that allow the cell to quickly adapt to changing conditions (Gustin *et al.,* 1998; Chen and Thorner, 2007). It comprises a variety of kinases which phosphorylate each other sequentially in order to convey a signal (Chen and Thorner, 2007).

Pheromone stimulates haploid cells, triggering arrest of the cells in G1 phase and preparing them for mating (Bardwell *et al.,* 2005). Rather than

inducing mating, growing yeast cells on poor nutrients, induces Flo11p production through the MAPK cascade (Dohlman and Slessareva, 2006), Ras2p (Gimeno *et al.,* 1992) and Cdc42p (Mosch *et al.,* 1996) (Fig. 1.6). In fact, Cdc42p, a GTPase of the Rho subfamily (Johnson *et al.,* 1999) activates the MAPK component via interaction with Ste20p, which is the first protein kinase in the cascade. Each component phosphorylates the next in turn: Ste20p → Ste11p → Ste7p → Kss1p (Chen and Thorner, 2007). Kss1p then activates Ste12p (Cook *et al.,* 1996; Chou *et al.,* 2006), which induces Tec1p expression (Kohler *et al.,* 2002). Coordinated binding of Ste12p and Tec1p at the *FLO11* promoter activates *FLO11* transcription (Madhani and Fink, 1997). On the other hand, inactive Kss1p (the unphosphorylated form) binds to the Dig1/2p repressor complex, which interacts with Ste12p/Tec1p and represses *FLO11* transcription (Madhani and Fink, 1997; Pan and Heitman, 2002).
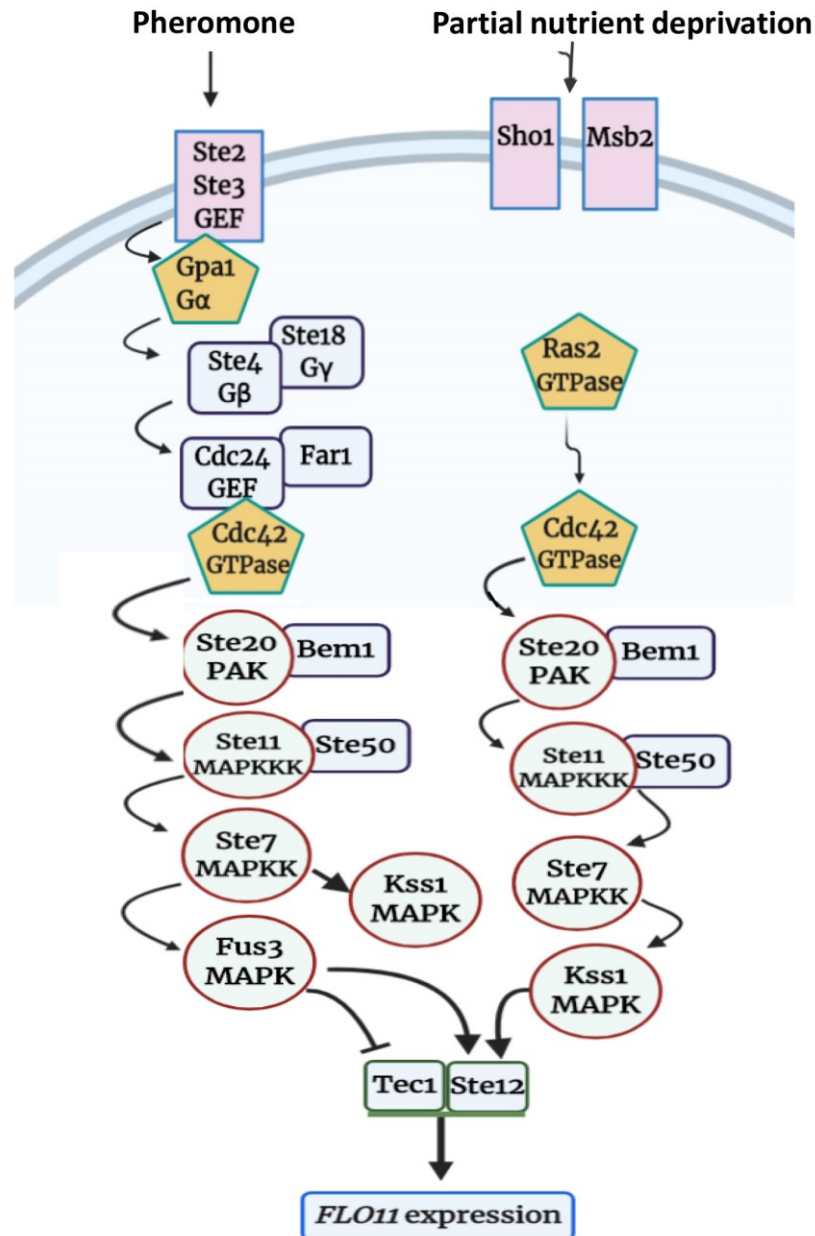
**Figure 1.6. The MAPK pathway in regulation of Flo11p expression.**
*Symbols: protein kinases, ovals; GTP-binding proteins, pentagons; scaffold, adaptor, and activating proteins, rectangles; cell surface proteins, vertical rectangles; activation, arrows; inhibition, T-bars. Adapted from Chen and Thorner (2007).*

### 1.6.2.2 TORC1 pathway

The TORC1 signaling pathway allows cells to sense and respond to intra- and extracellular hypoxia, other stresses, nitrogen availability, etc. (Evans *et al.,* 2011). The TOR pathway is negatively regulated by the antibiotic rapamycin that binds to TORC1 (TOR complex 1 consisting of the

Tor1p or Tor2p protein kinase and the Kog1p, Lst8p and Tco89p proteins) (Conrad *et al.,* 2014). Rapamycin suppression of filamentous growth is mediated via FKBP12 and TOR in response to nitrogen limitation (Conrad *et al.,* 2014). Filamentous growth is controlled by the TOR pathway under starvation in various ways.

Firstly, it controls the transcription of the *FLO11* gene by the transcription factor Gcn4p (Braus *et al.,* 2003). *FLO11-LacZ* expression was significantly decreased in *gcn4* deletion strains. Conversely, increased expression of *GCN4* led to increase expression of *FLO11-LacZ* and induced filamentous growth. Moreover, Gcn4p together with protein kinase Gcn2p promotes filamentous growth and *FLO11* expression in the absence of amino acids (Braus *et al.,* 2003). Gcn4p is regulated by the Tap42p and Sit4p phosphatases (Song and Kumar, 2012). The overexpression of Tap42p restores filamentous growth in rapamycin treated cells. In contrast, deletion of Sit4p phosphatase leads to reduced filamentous growth and increased sensitivity to rapamycin (Fig. 1.7). Gcn4p, which regulates *FLO11* expression, is itself regulated by the TOR pathway (Braus *et al.,* 2003; Smets *et al.,* 2008). TOR regulation of filamentous growth is independent of RAS/PKA and MAPK signaling. This conclusion arises from nitrogen-starvation-induced inhibition of filamentous growth by rapamycin, which is mediated by Tap42p and Sit4p TOR phosphatases (Conrad *et al.,* 2014).
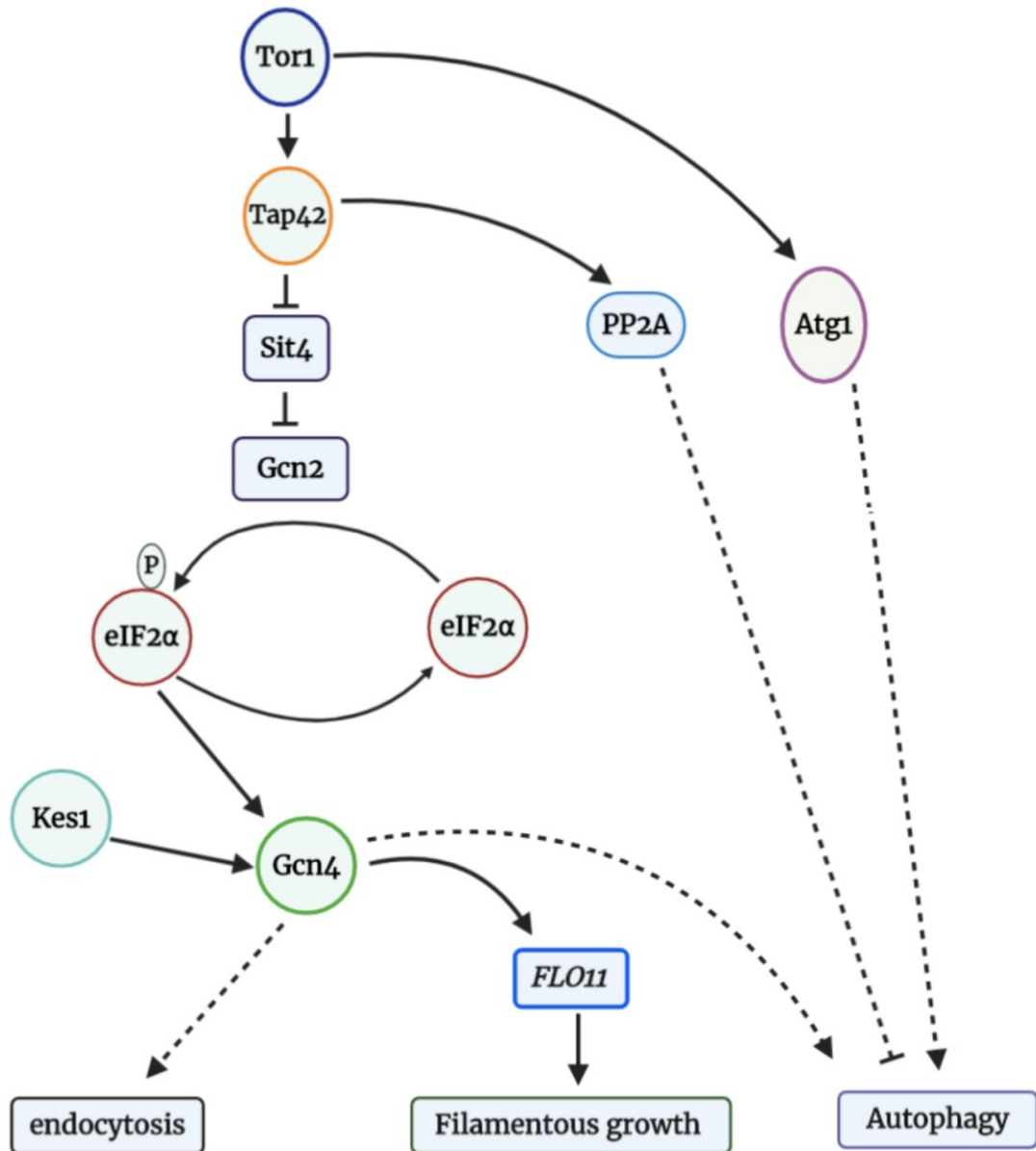
**Figure 1.7. TORC1 signaling pathways in regulation of Flo11p.**

*Highlighted relevant regulators contribute to yeast pseudohyphal growth, autophagy, and endocytosis. Activation, arrows; inhibition, T-bars, dashed lines indicate effects that encompass additional unlisted proteins. (adapted from Song and Kumar (2012).*

**1.6.2.3 cAMP-PKA pathway**

Protein kinase A (PKA) plays a pivotal role in the response of yeast cells to glucose (Ptacek *et al.,* 2005; Portela *et al.,* 2006; Livas *et al.,* 2011). Control of *FLO11* dependent adhesion and filamentation by cAMP-dependent

protein kinase A has been extensively studied (Robertson and Fink, 1998; Pan and Heitman, 1999; Rupp *et al.,* 1999).

CAMP binds to its regulatory subunit Bcy1p, which leads to release of three catalytic subunits: Tpk1p, Tpk2p and Tpk3p (Toda *et al.,* 1987). These three subunits of protein kinase A have overlapping functions in *S. cerevisiae* viability but distinct functions in the regulation of *FLO11*. Tpk2p is an activator of *FLO11* since deletion of the *TPK2* gene results in loss of filamentous growth. Indeed, the Tpk2p subunit activates the Flo8p transcription factor and inhibits the Sfl1p repressor (Robertson and Fink, 1998). Flo8p and Sfl1p share a binding site in the *FLO11* promoter (Pan and Heitman, 2002). Since activator and repressor compete for *FLO11* promoter binding, *FLO11* expression is positively or negatively regulated by Flo8p or Sfl1p respectively, depending on the balance of transcription factor binding. The effect of Tpk1p on *FLO11* expression is dependent on genetic background and experimental conditions. Deletion of *TPK1* did not affect filamentous growth (Robertson and Fink, 1998) see (Fig. 1.8). However, other authors observed an increase in filamentous growth following the deletion of *TPK1,* indicating that subunit Tpk1p inhibits formation of pseudohyphae (Pan and Heitman, 1999). According to newer work Tpk1p represses *FLO11* expression by inhibiting Yak1p (Malcher *et al.,* 2011).
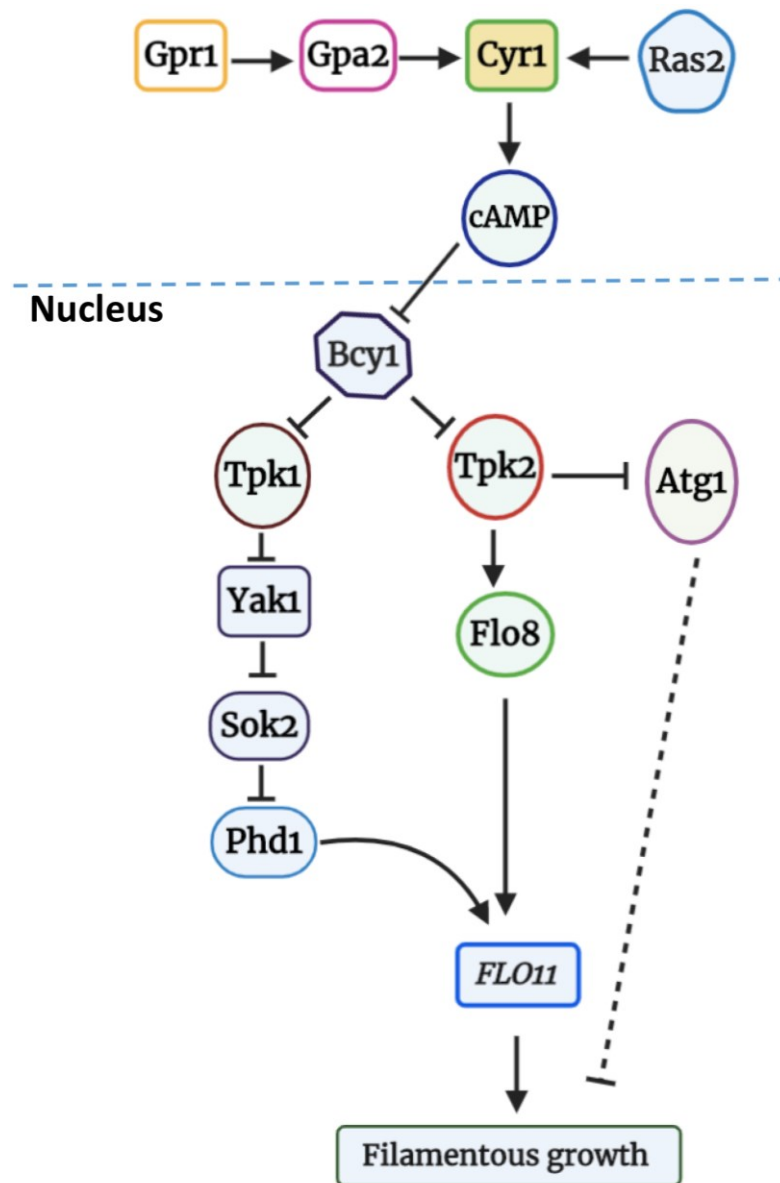
**Figure 1.8. The PKA pathway and relevant regulators in regulation of Flo11p and yeast pseudohyphal growth,** *adapted from Song and Kumar (2012).*

### 1.6.2.4 Epigenetic regulation of *FLO11*

Besides the regulatory networks described above, *FLO11* expression also appears to be subject to epigenetic control mechanisms. Two regulators, Flo8p and Sfl1p regulate *FLO11* in an opposing manner through epigenetic regulation of *FLO11*, turning gene expression "ON" or "OFF" (Halme *et al.,* 2004). This control is in fact governed by two histone deacetylases Rpd3Lp, Hda1p and the two long non-coding RNAs (lncRNAs) *PWR1* and *ICR1,* which are transcribed by RNA polymerase II from loci that overlap the

upstream promoter region of *FLO11*. *ICR1* is a tandem, upstream locus, overlapping the upstream promoter of *FLO11* in the sense direction while *PCR1* overlaps both the *FLO11* promoter and the 5' end of *ICR1* in the antisense direction (Martens *et al.,* 2005; Bryers 2008). Sfl1p excludes Flo8p from the "toggle" sites, repressing *PWR1* and inducing *ICR1*, thus blocking *FLO11* transcripts (i.e. via promoter exclusion). Rpd3Lp excludes Sfl1p from the *PWR1* promoter. Flo8p can then bind to toggle sites, activating *PWR1* and inhibiting *ICR1* (Fig. 1.9). Thus, Rpd3Lp and Flo8p activate *FLO11* expression by modulating the transcription of a pair of overlapping, cis-interfering ncRNAs located on opposite strands (Bumgarner *et al.,* 2009; Bruckner and Mosch, 2012).
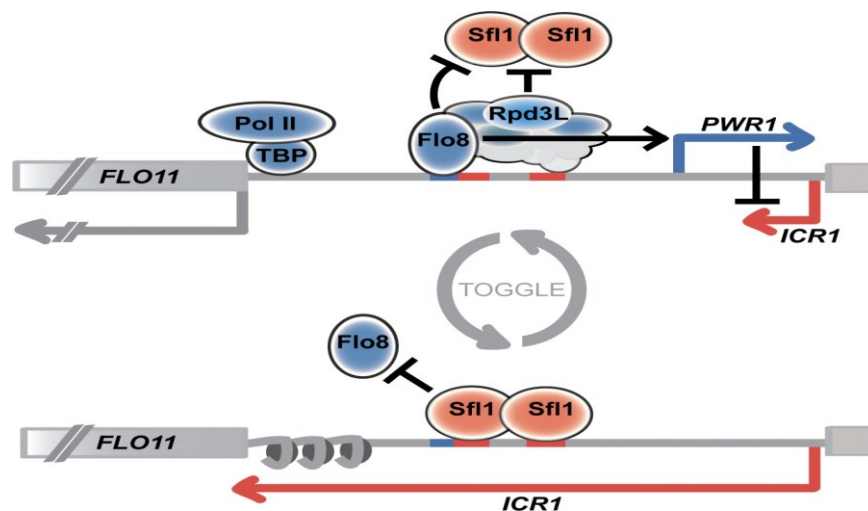


**Figure 1.9. Sfl1p/Flo8p competition determines which lncRNA is transcribed.**

*Flo8p binding induces PWR1 expression, thus repressing ICR1 expression and facilitating FLO11 expression. However, Sfl1p binding suppresses FLO11 expression. Adapted from Bumgarner et al (2009).*

**1.7 Cyc8p-Tup1p complex**

*1.7.1 Structure of Tup1p and Cyc8p*

Originally, Cyc8p and Tup1p were identified independently, but experiments later demonstrated that they have overlapping functions. Similar phenotypes of mutants *tup1* and *cyc8*, including poor growth, flocculation, mating type defects, sporulation defects and loss of glucose repression (Roth

*et al.,* 1995; Smith and Johnson, 2000), implied a similar function in regulation of various pathways. Immunoprecipitation studies showed that their products can build a complex, which consists of one molecule of Cyc8p and four molecules of Tup1p (Fig. 1.11) (Williams *et al.,* 1991; Varanasi *et al.,* 1996).

Cyc8p is a large 107 kDa protein, with ten tandem copies of a tetratricopeptide repeat (TPR) at the N-terminus, which contains the Tup1p binding sites and is rich in glutamine and proline amino acid residues (Papamichos *et al.,* 2000; Sprague *et al.,* 2000; Tartas *et al.,* 2017). The TPR motifs of Cyc8p also help it interact with different DNA-binding proteins to negatively regulate certain genes (Tzamarias and Struhl, 1995).

Tup1p (78 kDa) contains seven conserved copies of a WD-40 repeat at the C-terminus, each consisting of 40 amino acid residues followed by tryptophan-aspartate. WD40 repeats of Tup1p (Fig. 1.10) are probably responsible for protein-protein interactions (Williams and Trumbly 1990). The C-terminus of Tup1p is highly conserved and has been found in a wide range of protein families. In addition, Tup1p is glutamine rich and contains a repression domain at the N-terminus (Tzamarias and Struhl, 1994; Zhang *et al.,* 2002).

Conserved WD-40 and TPR repeats are believed to be major mediators of interactions between Cyc8p and Tup1p as well as between this complex and other proteins (Williams *et al.,* 1991; Varanasi *et al.,* 1996; Gounalaki *et al.,* 2000). For example, the WD-40 and TPR motifs are required for the interaction between Cyc8p-Tup1p and sequence specificity of alpha 2 proteins (Komachi *et al.,* 1994; Smith *et al.,* 1995).

### 1.7.2 Evolution of Cyc8p and Tup1p homologs

Tup1p sequence homology has been found in the *Drosophila* Groucho and the mammalian transducin-like enhancer of split (TLE) proteins which are involved in Notch signaling and neural development and segmentation (Chen and Courey, 2000). The Tup1 and Groucho/TLE repressors are structurally similar and are characterised by a WD40 repeat structure in their

C-termini (Causier *et al.,* 2012) (Fig. 1.10). It has been reported that Groucho/TLEs also interact with the tails of histones (Palaparti *et al.,* 1997) and histone deacetylase Rpd3p and that interaction of Rpd3p with the N-terminus of Groucho results in repression of transcription (Chen *et al.,* 1999), hence the repressive functions of Groucho/TLEs may resemble that by Tup1p (Chen and Courey, 2000).

A Tuplp homolog was also found in the human pathogenic fungus *C. albicans* (Braun and Johnson, 1997), *Ca*Tup1p was thought to play a role in repression of genes, responsible for initiating filamentous growth. Tup1p homologs have been identified in different yeast species based on sequence identity with Tup1p of *S. cerevisiae*. These include homologs in *Kluyveromyces lactis* (Lamas *et al.,* 2011), *Ustilago maydis* (Elias *et al.,* 2015), *Talaromyces marneffei* (Todd *et al.,* 2003) and *Cryptococcus neoformans* (Lee *et al.,* 2005).

Cyc8p (Ssn6p) is a highly conserved protein, found in both higher eukaryotes and lower eukaryotes (Fig. 1.10A). Cyc8p homologs include a *C. albicans* homolog (Ssn6p) and eukaryotic posterior development (HOX) genes (Smith *et al*., 1995; Tzamarias and Struhl, 1995; Agger *et al*., 2007). Moreover, results of a yeast two hybrid assay showed the interaction of mammalian TLE proteins with yeast Cyc8p and mammalian UTY/X (genes on Y/X chromosomes) (Grbavec *et al.,* 1999). It should be noted that all male human tissues express UTY protein, which has 8 TPR repeats in the N-terminus. Sequence alignment of the TPR domain of UTY shows a strong similarity with the TPR domain of Cyc8p protein (Das Ak *et al.,* 1998). Moreover, the UTY and Cyc8p proteins are extremely rich in glutamine and proline residues, which may indicate a possible functional similarity (Grbavec *et al.,* 1999).
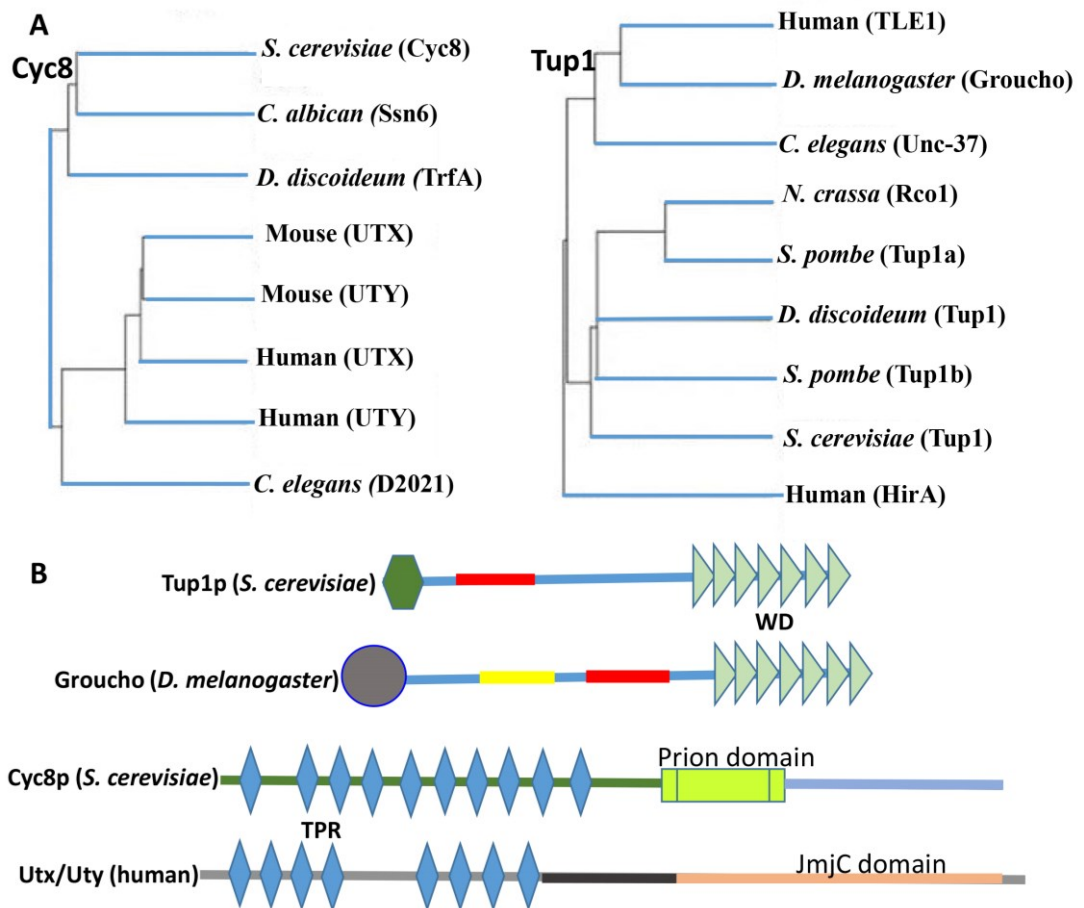
**Figure 1.10. Cyc8p and Tup1p structure and function is highly conserved.**

*A, evolutionary relationships among eukaryotic Cyc8p (Ssn6p) and Tup1p-homologs; B, protein structures showing key domains, adapted from Smith and Johnson (2000).*

### 1.7.3 Regulation mechanisms of Cyc8p-Tup1p

Neither Cyc8p nor Tup1p bind directly to DNA, instead they are brought to promoters through interactions with sequence-specific DNA-binding proteins which synchronize the expression of a large number of specific genes (Smith and Johnson, 2000). For example, Cyc8p-Tup1p is recruited to specific promoters involved in hypoxia and DNA damage inducible genes by Rox1p and Crt1p (Deckert *et al.,* 1995; Huang *et al.,* 1998).
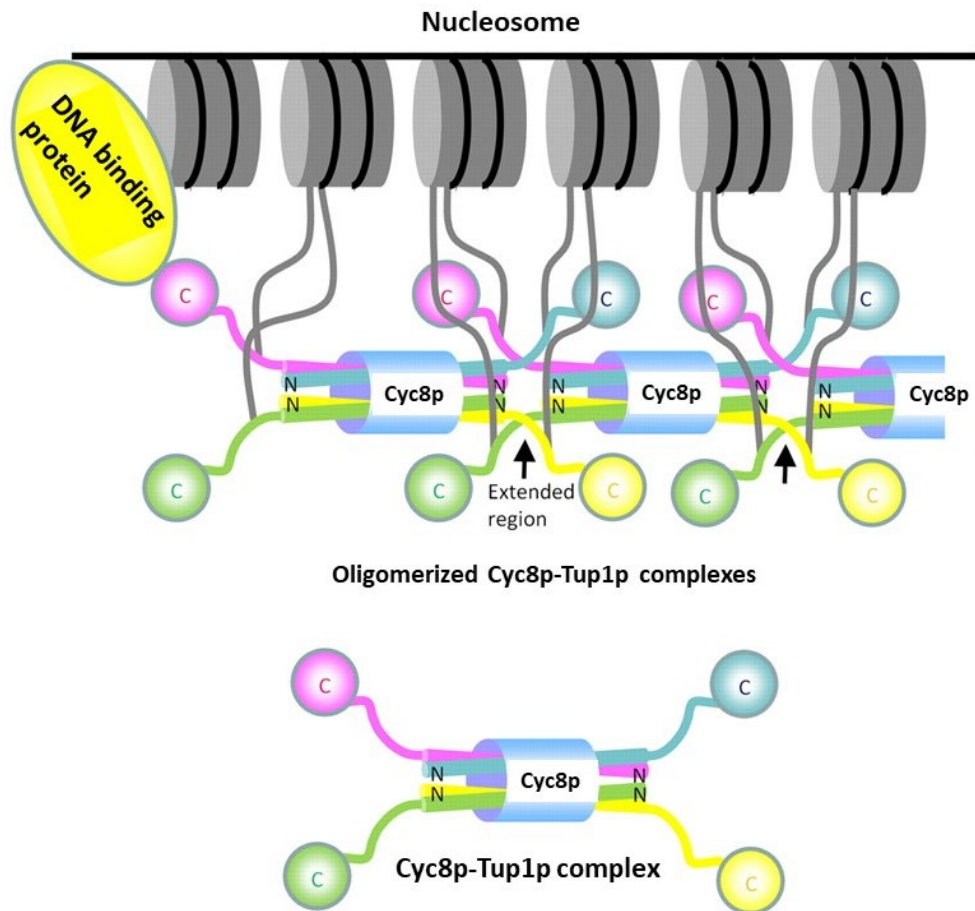
**Figure 1.11. Four Tup1p and one Cyc8p molecules form a complex,** *adapted from Matsumura et al (2012).*

It has been reported that Tup1p acts as a main repressor in many cases and Cyc8p as an adaptor protein, which links Tup1p and specific transcription factors. Cyc8p was believed unable to perform transcriptional suppression without Tup1p, but Tup1p may control transcription if it interacts with target promoters via the DNA binding proteins (Keleher *et al.,* 1992; Tzamarias and Struhl, 1995). However, recent studies indicated that Cyc8p may play more direct and independent roles in repression (Malave and Dent, 2006; Chen *et al*., 2013; Nguyen *et al.,* 2018).

To date, at least three models (Fig. 1.12) have been proposed to describe how the Cyc8p-Tup1p complex orchestrates the repression of transcription once it is recruited to the promoters of target genes by specific repressor proteins in yeast.
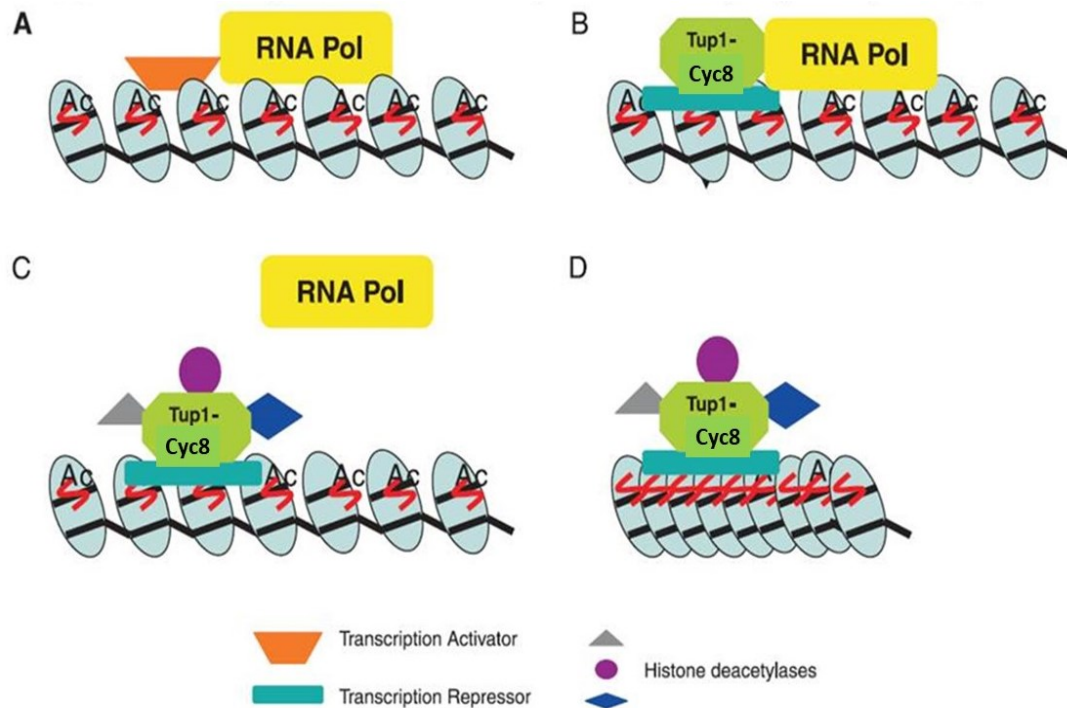
**Figure 1.12. Model of the mechanisms of Cyc8p-Tup1p mediated repression.**

*Via repressor interaction, Cyc8p-Tup1p is recruited to active promoters and interacts with the RNA polymerase II mediator complex to stop transcription (B). The Cyc8p-Tup1p complex then recruits HDACs (C) to deacetylate histones and the resulting chromatin compaction leads to complete gene repression (D), adapted from Malave and Dent (2006).*

**1.7.3.1 Nucleosome positioning at target promoters**

This model is proposed based on the discovery of well-positioned nucleosomes in many promoter regions of target genes such as *STE6* (Cooper *et al.,* 1994), *SUC2* (Gavin and Simpson, 1997), hypoxia gene *ANB1* (Kastaniotis *et al.,* 2000), osmotic stress response gene *ENA1* (Wu *et al.,* 2001; Davie *et al.,* 2002) and the DNA repair *RNR2, RNR3* genes (Li and Reese, 2001).

When nucleosomes are in position, the TATA box of these promoters is usually protected by nuclease digestion. Findings suggest that Tup1p blocks the transcription initiation site and TATA box, thus eliminating binding of the TATA-binding protein (TBP) and inhibiting the assembly of the preinitiation complex (PIC) (Kuras and Struhl, 1999). Moreover, Tup1p can repress a1-alpha2 of haploid specific genes via direct interaction with hypoacetylated

histone H3 and H4 tails (Edmondson *et al.,* 1996; Huang *et al.,* 1997; Davie *et al.,* 2002). Similar results were obtained on deletion of *CYC8* and *TUP1,* which caused re-positioning of nucleosomes within the *STE6* gene (Cooper *et al.,* 1994) and disruption of nucleosome positioning at the promoter region (operator). In addition, it has been reported that at the upstream repression sequences of some target genes such as DNA damage (*RNR2, RNR3*) and osmotic stress response (*ENA1*) genes, nucleosomes are repositioned by Tup1p (Wu *et al.,* 2001; Davie *et al.,* 2002).

**1.7.3.2 Changing chromatin structure by recruitment of histone deacetylase complexes (HDACs)**

Transcription of target genes may be repressed by the interaction between Cyc8p-Tup1p corepressor and histone deacetylases (HDACs), leading to histone deacetylation at specific promoters and compaction of chromatin. It was shown that reduced acetylation of histones H3 and H4 is involved in Cyc8p-Tup1p repression at promoters in vivo (Davie *et al.,* 2002).

Previous findings have indicated that concomitant deletion of genes, encoding class I HDACs (*RPD3/HOS1/HOS2*) or class II HDACs (*HDA1*) abrogates Cyc8p-Tup1p repression of several genes, after which increased promoter histone acetylation and decreased Tup1p association leads to de-repression (Watson *et al.,* 2000; Bone and Roth, 2001; Deckert and Struhl, 2001; Wu *et al.,* 2001; Fleming *et al.,* 2014). These findings suggest that the activity of Cyc8p–Tup1p repression is adjusted by alterations in histone acetylation. The results of acetylation microarray analysis indicate that the role of Hda1p in regulation of gene expression is associated with Cyc8p-Tup1p repression (Robyr *et al.,* 2002). Moreover, direct interaction between the N-terminal domain of Tup1p and Hda1p has been reported (Wu *et al.,* 2001). In contrast, the function of the Rpd3p group of HDACs does not relate to the Cyc8p-Tup1p function, instead it cooperates with Ume6p (Robyr *et al.,* 2002; Malave and Dent, 2006).

**1.7.3.3 Interaction with activator or basal transcription machinery**

Cyc8p-Tup1p could block activator binding and inhibit gene activation by nucleosome positioning at target promoters, or it could prevent the activator from binding to the promoter. For example, Cyc8p-Tup1p mediates repression of transcription by masking the Gal4p activation domain (Redd *et al.,* 1996; Wong and Struhl, 2011).

Interference with transcription machinery has also been proposed since mutations within subunits of the RNA polymerase II mediator complex result in relief of the Cyc8p-Tup1p repression function at the promoters of plasmid reporter systems (Carlson *et al.,* 1997; Lee *et al.,* 2000). By that, interactions between Cyc8p-Tup1p and mediator components Med3p, Srb7p, Srb8p, Srb10p and Srb11p were demonstrated in vitro in genetic experiments (Gromoller and Lehming, 2000; Papamichos *et al.,* 2000; Han *et al.,* 2001; Zaman *et al.,* 2001). Findings have shown that deletion of either *SRB10* or *SRB11* abolishes repression by Tup1p (Zaman *et al.,* 2001), thus indicating that increased Tup1p- holoenzyme interaction leads to Srb10/11p-dependent repression.

*1.7.4 Cyc8p-Tup1p can act as a coactivator complex*

Cyc8p-Tup1p is well known as a repressor complex, but evidence has suggested that this complex could also act as a co-activator, antagonizing its own repression activities. The first such observation indicated that deletion of either *TUP1* or *CYC8* results in decreased function of Hap1p in activation of oxygen and heme regulation (Zhang and Guarente, 1994). Later, it was observed that the N-terminus of Cyc8p interacted with the activation domain of Rgt1p, a transcriptional activator of *CIT2*. Thus, deletion of *CYC8* led to the inactivation of *CIT2* (Conlan *et al.,* 1999). Other work demonstrated that the Cyc8p-Tup1p complex may interact with the SAGA (histone acetylation) and SWI/SNF (chromatin remodeling) complexes. These interactions may be necessary for maximal recruitment of SAGA and SWI/SNF to target genes and therefore for positive regulation of transcription (Papamichos *et al.,* 2002;

Proft and Struhl, 2002). Moreover, Aft1p activation of *FRE2* is also dependent on Cyc8p and, to some extent, Tup1p. Via interaction with Aft1p, Cyc8p is recruited to the *FRE2* promoter, collaborating with Nhp6p to remodel promoter nucleosomes (Fragiadakis *et al.,* 2004). Importantly, Cyc8p-Tup1p promotes binding of Gcn4p at *ARG1* and *ARG4* upstream activation sequence (UAS) elements (Fig. 1.13) (Kim *et al.,* 2005). More recently, N. Tanaka and Y. Mukai (Tanaka and Mukai, 2015) showed that when overexpressed *TAT1* and *TAT2* (Tryptophan transporter genes) suppressed the poor growth of a Δ*cyc8* strain. More interestingly, growth of Δ*cyc8* cultures was partly restored by adding tryptophan. *TAT1* and *TAT2* expression were lower after deletion of *CYC8* or *TUP1*. *CYC8* and *TUP1* function was also needed for the transcription of the other Stp1/2p-dependent amino acid transporter (AAT) genes (Tanaka and Mukai, 2015).

Collectively, these data provide an explanation of how Cyc8p-Tup1p might function as a coactivator under certain circumstances.
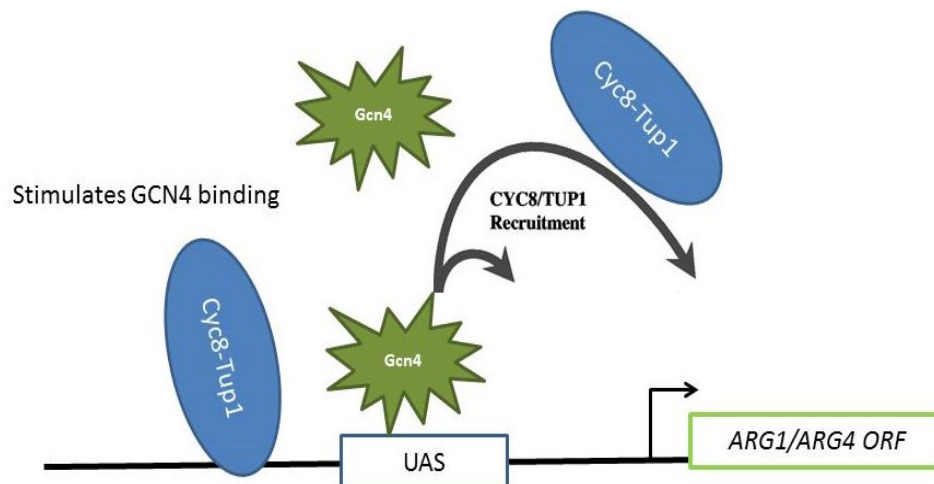


**Figure 1.13. Cyc8p-Tup1p functions to stimulate Gcn4p binding to the Upstream Activation Sequence (UAS) elements at *ARG1* and *ARG4*,** *adapted from Kim et al (2005).*

### *1.7.5 Targets of Cyc8p-Tup1p*

#### 1.7.5.1 Glucose repression genes

When glucose is present, cells accumulate high ATP levels and simultaneously repress the expression of genes associated with utilization of

other sugars such as sucrose, maltose and galactose. Mig1p is the main glucose repressor, which represses transcription of the *SUC*, *MAL* and *GAL* genes (Treitel and Carlson, 1995; Schuller *et al.,* 2003). The mechanistic model of this regulation has been proposed as shown in Fig. 1.14.

First, at high glucose levels (repressive conditions), Mig1p is dephosphorylated in the cytoplasm by the Reg1p-Glc7p protein phosphatase complex and then brought into the nucleus where it binds, via its zinc finger domain, the promoters of glucose-repressed genes. Here, it represses specific genes via Cyc8p-Tup1p complex recruitment (Roy *et al.,* 2014).

When cells become limited for glucose (glucose depleted), Mig1p is phosphorylated by the Snf1p kinase complex and releases from the Cyc8p–Tup1p complex. Mig1p is then sent back to the cytoplasm (Schuller *et al.,* 2003; Roy *et al.,* 2014).
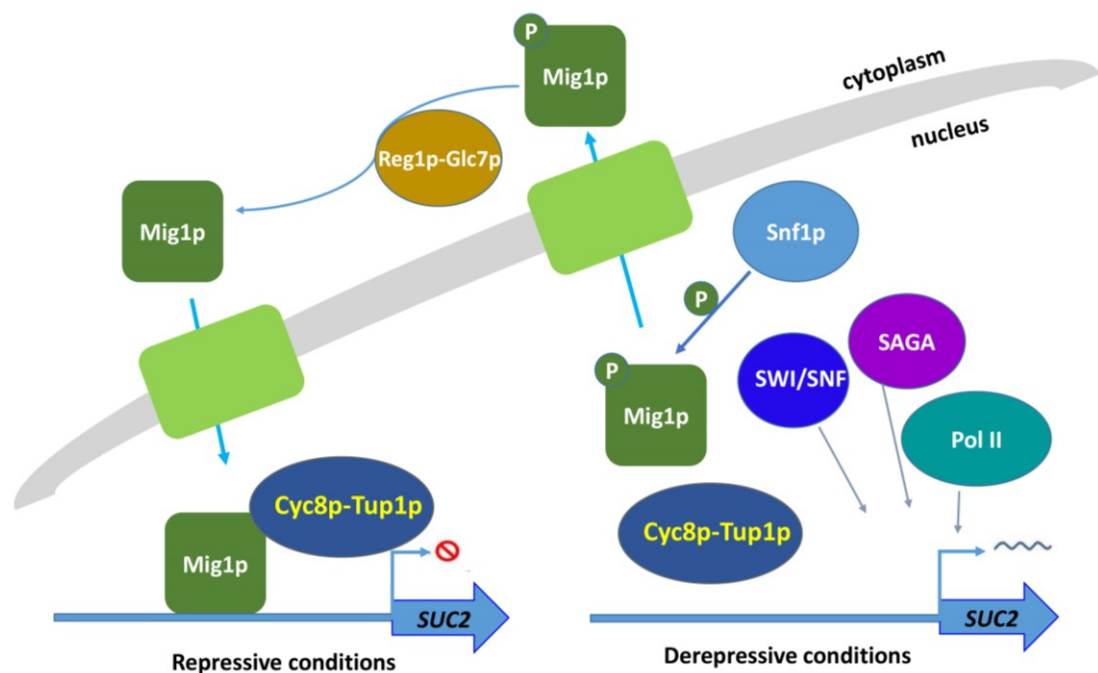


**Figure 1.14. Mig1p recruits the Cyc8p-Tup1p complex to promoter regions of specific, glucose-repressible genes.**

*When glucose is low, the Snf1p kinase blocks the interaction between Mig1p and Cyc8p-Tup1p, leading to phosphorylation/repression of Mig1p. When glucose is high, (and Snf1p inactive), dephosphorylated Mig1p recruits Cyc8p-Tup1p leading to target gene repression (adapted from (Roy et al., 2014)).*

### 1.7.5.2 DNA damage induced genes

The ribonucleotide reductase (RNR) complex catalyzes the conversion of nucleotides into their deoxynucleotide (dNTP) form. RNR complex activity is induced to the maximum level in early S-phase and upon DNA damage to increase dNTP pools required for accurate replication and repair. In *S. cerevisiae*, *RNR3* and the other *RNR* genes (*RNR1, RNR2* and *RNR4*) are regulated by the Cyc8p-Tup1p complex (Fig. 1.15) (Li and Reese, 2001), which is recruited to the *RNR* gene promoters by the sequence-specific DNA-binding protein Rfx1p (Crt1p), which binds DNA damage response elements (DREs) within the upstream repression sequence (URS) (Huang *et al.,* 1998). Nucleosome positioning maintains a repressed state in *RNR3* and treatment with methyl methanesulfonate (MMS, a DNA-damaging agent) leads to release, from the URS, of the Rfx1p-Cyc8p-Tup1p complex and then to remodeling of chromatin, implicating chromatin structure in *RNR3* gene regulation (Huang *et al.,* 1998).
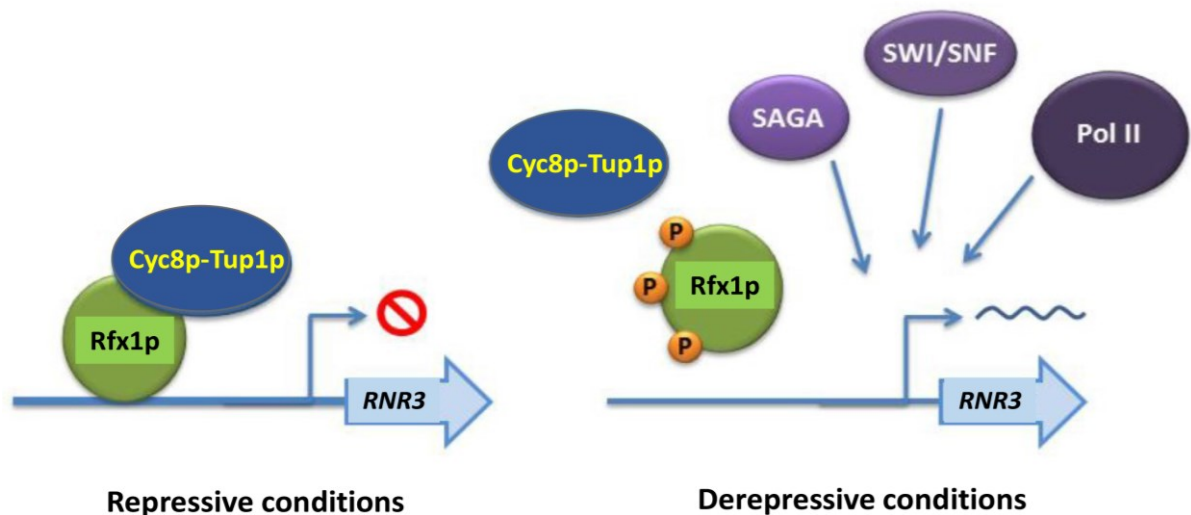


**Figure 1.15. The regulation of Cyc8p-Tup1p in expression of DNA damage regulated genes.**

*The Cyc8p-Tup1p complex interacts with Rfx1p to repress target gene expression. Mec1p kinase is activated during the DNA damage response and phosphorylates Rfx1p, leading to Cyc8p-Tup1p dissociation (adapted from (Huang et al., 1998)).*

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### 1.7.5.3 Osmotic-stress genes

In response to hyperosmotic stress, *S. cerevisiae* cells first activate a complex adaptive program which includes temporary interruption of cell cycle progression, altering gene expression patterns and inducing expression of genes involved in biosynthesis of the osmolyte, glycerol. The high osmolarity glycerol (HOG) pathway controls many of these adaptations. In *S. cerevisiae*, Sko1p is a basic leucine zipper transcription factor of the ATF/CREB family. Sko1p mediates osmotic stress signaling, acts downstream of the HOG pathway and suppresses the expression of genes that help cells to cope with stressful conditions, e.g. *ENA1* and *HAL1* (Proft and Serrano, 1999; Pascual *et al.,* 2001).

During osmotic stress, Hog1p kinase phosphorylates the amino terminus of Sko1p (Proft *et al.,* 2001), converting the Sko1p-Tup1p-Cyc8p complex from a repressor to an activator (Proft and Struhl, 2002). Mutation of either *TUP1* or *CYC8* abrogates the osmotic sensitivity of the Δ*hog1* strain. In these mutant strains, the expression of DNA binding protein-regulated genes was normal except for genes regulated by Sko1p. Derepression of Sko1p-regulated *GRE2* and *AHP1* occurred during non-stress conditions, indicating that Tup1p and Cyc8p mutants might be defective in Sko1p-dependent gene repression. Chromatin immunoprecipitation analyses of the *GRE2* promoter revealed that the Sko1p–Tup1p–Cyc8p complex in these mutants was present at the promoter with Gcn5p/SAGA, raising the possibility that defective SAGA recruitment caused the derepression (Kobayashi *et al.,* 2008).
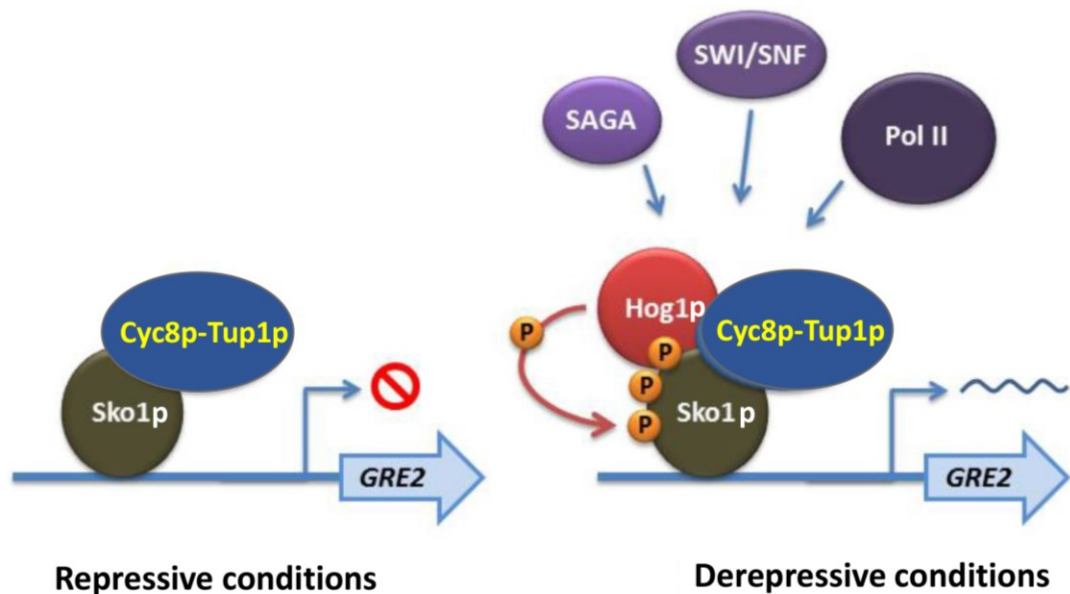
**Figure 1.16. The Cyc8p-Tup1p complex regulates osmotic stress induced genes.**

*Sko1p recruits Cyc8p-Tup1p to target promoters, repressing GRE2 and other genes that are regulated by DNA damage. Hog1p kinase is activated by osmotic shock and other stresses, phosphorylates Sko1p and thus converts the latter from a repressor to an activator of gene transcription, adapted from Proft and Struhl (2002).*

**1.7.5.4 Flocculation-related genes**

Flocculation is a cell-cell interaction and mediated by flocculin genes. It has been reported that *FLO1* and other *FLO* genes are repressed by the Cyc8p-Tup1p complex together with Hda1p and Rpd3p (histone deacetylases). Disruption of Cyc8p-Tup1p complex by mutating either *CYC8* or *TUP1* leads to derepress strongly flocculation phenotype (Verstrepen and Klis, 2006; Chen *et al.,* 2013; Fleming *et al.,* 2014; Paul *et al.,* 2015).

# CHAPTER II. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals and equipment

| Chemical, equipment | Manufacturer/country |
| --- | --- |
| Ampicillin | Werner Bioagents GmbH, Germany |
| G418 | GoldBio, USA |
| Hygromycin | GoldBio, USA |
| Nourseothricin | Werner Bioagents GmbH |
| GFP-antibody | GFP (sc-9996 HRP, Santa Cruz) |
| LA taq polymerase | Takara, Japan |
| OneTaq® 2X Master Mix | New England Biolabs, England |
| PPP master mix | Top-Bio, Czech Republic |
| L303i balance | BEL Engineering |
| Camera Jenoptic ProgRes CT3 | Jenoptik, Germany |
| Equipment for SDS PAGE and blotting | Bio-Rad, USA |
| Fluorescent microscope Leica DMR | Leica, Germany |
| Fluorescent microscope Zeiss | Zeiss, Germany |
| Sartorius Weighing Scales | Sartorius, Germany |
| Incubation shaker for Eppendorf tubes | Eppendorf, Germany |
| Precision water bath | N-BIOTEK, Korea |
| Microcentrifuge (model 5424) | Eppendorf, German |
| Centrifuge (model 320) | Hettich, Germany |
| PCR thermocycler Minicycler | Bio-Rad, USA |
| PCR thermocycler | Bioer technology, China |

| | |
|---|---|
| pH-meter | Mettler Toledo, USA |
| Spectrophotometer nanodrop1000 | Thermo Scientific, USA |
| Spectrophotometer optize1412v | Mecasys, K Lab, Korea |
| Epoch™ Microplate Spectrophotometer | Biotek instruments, USA |
| UV transilluminator | Herolab GmbH, Germany |
| Vortex Genie 2 | Scientific Industries, USA |
| EchoTherm™ Benchtop Incubator | Fisher Scientific, USA |
| DNA electrophoresis | Thermo Scientific, USA |
| Agarose | Sigma, USA |
| Scilogex ms7 -stirrer | M2 Scientifics, USA |

## 2.1.2 Bioinformatics resources

Yeast genome database:

 - http://www.yeastgenome.org/

- http://www.yeastract.com/tfsbindingsites.php

- http://www.rothsteinlab.com/tools/

- http://string-db.org/cgi/network.pl

Oligocalculator:

- https:// thermo-scientific-web-tools/tm-calculator.html

PubMed database:

- https://www.ncbi.nlm.nih.gov/geo/query/

Sequence manipulation:

- http://www.bioinformatics.org/sms2/

## 2.1.3 Primers and plasmids list

## Table 2.1. List of the primers

| Primer | Sequence | Purpose |
|---|---|---|
| CYC8-del-forward | AACAACAACAAACAAAACACGACTGGAAAAAAAAATTAGGAAAACAGCTGAAGCTTCGTACGC | deletion of *CYC8* gene |
| CYC8-del- | GATTATAAATTAGTAGATTAATTT | deletion of *CYC8* |

| | | |
|---|---|---|
| reverse | TTTGAATGCAAACTTTGCATAGG CCACTAGTGGATCTG | gene |
| TUP1-del-forward | TGATAAGCAGGGGAAGAAAGAA ATCAGCTTTCCATCCAAACCAATC AGCTGAAGCTTCGTACGC | deletion of *TUP1* gene |
| TUP1-del-reverse | GTTTAGTTAGTTACATTTGTAAAG TGTTCCTTTTGTGTTCTGTTCGCA TAGGCCACTAGTGGATCTG | deletion of *TUP1* gene |
| GAL1_CYC 8 forward | AACAACAAACAAAACACGACTGG AAAAAAAAAATTAGGAAAAATG CGTACGCTGCAGGTCGAC | Insertion of $p_{GAL}$ or $p_{CUP}$ in front of *CYC8* coding sequence |
| GAL1_CYC 8-reverse | CTGTTGAGCGGGTTGTTCCATTAT TGTTTGTTCACCGCCCGGATTCAT CGATGAATTCTCTGTCG | Insertion of $p_{GAL}$ or $p_{CUP}$ in front of *CYC8* coding sequence |
| TEF1_CYC8 -forward | AACAACAAACAAAACACGACTGG AAAAAAAAAATTAGGAAAAATG CGTACGCTGCAGGTCGAC | Insertion of $p_{TEF}$ in front of *CYC8* coding sequence |
| TEF1_CYC8 -reverse | CTGTTGAGCGGGTTGTTCCATTAT TGTTTGTTCACCGCCCGGATTGGA TCCACTAGTTCTAGA | Insertion of $p_{TEF}$ in front of *CYC8* coding sequence |
| GAL1_TUP 1 -forward | TAAGCAGGGGAAGAAAGAAATC AGCTTTCCATCCAAACCAATATG CGTACGCTGCAGGTCGAC | Insertion of $p_{GAL}$ or $p_{CUP}$ in front of *TUP1* coding sequence |
| GAL1_TUP 1- reverse | GCTCATTCAGCTTATTCTGCGTAT TCGAAACGCTGGCAGTCATCGAT GAATTCT | Insertion of $p_{GAL}$ or $p_{CUP}$ in front of *TUP1* coding sequence |
| NRG1-del-forward | TTCCTCTCGACCAGCATATTACTA CCCTTCGCAAACTTTCAGGCACA GCTGAAGCTTCGTACGC | deletion of *NRG1* gene |
| NRG1-del-reverse | CGGAATAGTAGTACTGCTAATGA GAAAAACACGGGTATACCGTCAA GCATAGGCCACTAGTGGATCTG | deletion of *NRG1* gene |
| MIG1-del- | ACACGAGAGTTGAGTATAGTGGA | deletion of *MIG1* |

| forward | GACGACATACTACCATAGCCCAG CTGAAGCTTCGTACGC | gene |
|---|---|---|
| MIG1-del-reverse | CTATTGTCTTTTGATTTATCTGCA CCGCCAAAAACTTGTCAGCGTAG CATAGGCCACTAGTGGATCTG | deletion of *MIG1* gene |
| SFL1-del-forward | GGTCCTAAGACAGCACAAATCAG TTATATAGAAAAAAGAAGAAAA AATC CAGCTGAAGCTTCGTACGC | deletion of *SFL1* gene |
| SFL1-del-reverse | GAGGTGCTTTGAACTTTTAGACA ACTAGAGATTAAAAAGGCAAAGA GCATAGGCCACTAGTGGATCTG | deletion of *SFL1* gene |
| CYC8-EGFP-forward | AAAATGTAGTAAGGCAAGTGGAA GAAGATGAAAACTACGACGAC GGTGACGGTGCTGGTTTA | C-terminal fusion of *CYC8* with GFP |
| CYC8-EGFP-reverse | TCTCGTTGATTATAAATTAGTAGA TTAATTTTTTGAATGCAAACTTTT TATCGATGAATTCGAGCTCG | C-terminal fusion of *CYC8* with GFP |
| TUP1-EGFP-forward | TAAAGCAAGGATTTGGAAGTATA AAAAATAGCGCCAAATGGTGACG GTGCTGGTTTA | C-terminal fusion of *TUP1* with GFP |
| TUP1-EGFP-reverse | GTTAGTTACATTTGTAAAGTGTTC CTTTTGTGTTCTGTTCTCGATGAA TTCGAGCTCG | C-terminal fusion of *TUP1* with GFP |
| CYC8 ver-REV | GAGCGAACCATATCTGTCAT | Verification of deletion of *CYC8* |
| CYC8 intFW C-terminus | CGCTGCTACAACGATAACTG | Verification of *CYC8* tagging construct |
| CYC8 intRV | TGGAAGTGTGTTCTCCTGTG | Verification of deletion of *CYC8* |
| CYC8 intFW | CCACACTTTAGTAGATGCCG | Verification of deletion of *CYC8* |
| CYC8-UTRev | CTAATTCACGTTACCCACCT | Verification of removal of *CYC8* by |

| | | cre-LoxP |
|---|---|---|
| TUP1 verFW | AGCTCTCCCGTCAAAGCAA | Verification of deletion of *TUP1* |
| TUP1 verRV | GTTGCGCAACTGGAACAGAT | Verification of deletion of *TUP1* |
| TUP1 inter FW | CACTTCGTCTTCCCCATCAT | Verification of deletion of *TUP1* |
| TUP1 inter REV | ATAACGGATTGCCGGATTTC | Verification of deletion of *TUP1* |
| TUP1intFW C-terminus | CCGGCAATCCGTTATTGATG | Verification of *TUP1* tagging construct |
| TUP1-UTRev | CCGCAATATTCAGAAACACAGG | Verification of removal of *TUP1* by cre-LoxP |
| NRG1 verify fw | TTTCTACAGTCTGGCTGCAG | Verification of deletion of *NRG1* |
| NRG1 verify Rev | TTGGCTCCCACTTTTCAGAG | Verification of deletion of *NRG1* |
| SFL1 verify FW | GTGCACACAAAGGGTTGTTG | Verification of deletion of *SFL1* |
| SFL1 verify REV | GCCTTACTAAACCGTTCCAC | Verification of deletion of *SFL1* |
| MIG1 verify FW | ACCTGGAGTGATGGTAAAGG | Verification of deletion of *MIG1* |
| Mig1 verify REV | GTCTGATGTTCCAGTCTGTG | Verification of deletion of *MIG1* |
| GAL proFW | CCCCACAAACCTTCAAATGA | Verification of pGAL construction |
| TEF proFW | CCTCTAGGGTGTCGTTAATT | Verification of pTEF1 construction |
| CUP proFW | GAAGCAAAAAGAGCGATGCG | Verification of pCUP1 construction |

**Table 2.2. List of plasmids**

| Plasmid | Application | Reference |
|---|---|---|
| pAG25 | Deletion cassette (nat1 marker) | (Goldstein and McCusker, 1999) |
| pUG6 | deletion cassette (KanMX marker) | (Gueldener *et al.,* 2002) |
| pYM-N23 | p$_{GAL1}$ cassette | (Janke *et al.,* 2004) |
| pYM-N20 | p$_{TEF1}$ cassette | (Janke *et al.,* 2004) |
| pYM-N1 | P$_{CUP1}$ cassette | (Janke *et al.,* 2004) |
| pSH69 | Cre expression plasmid (HygR marker) | (Hegemann and Heick, 2011) |
| pSH66 | Cre expression plasmid (nat1 marker) | (Hegemann and Heick, 2011) |
| pUG6-25 | deletion cassette (nat1 marker, with Cre-loxP site) | Modified from pUG6 |
| pUG6-32 | Deletion cassette (HygR marker, with Cre-loxP site) | Modified from pUG6 |
| pKT127 | C-terminal GFP fussion | (Sheff and Thorn, 2004) |

**Table 2.3. List of strains used in this study**

| | Name | Genotype | Colony morphology* | Source |
|---|---|---|---|---|
| 1 | BR-F | *MATa/MATα* | structured | (Kuthan *et al.,* 2003) |
| 2 | *tup1* | *MATa/MATα, tup1Δ::KanMX, tup1Δ::nat1* | smooth | this study |
| 3 | p$_{TEF}$-*CYC8* | *MATa/MATα, nat1-TEF1-* | smooth | this study |

| | | *CYC8/CYC8* | | |
|---|---|---|---|---|
| 4 | p<sub>GAL</sub>-*CYC8* | *MATa/MATα, cyc8Δ::KanMX, nat1-GAL1-CYC8* | structured** | this study |
| 5 | p<sub>TEF</sub>-*TUP1* | *MATa/MATα, nat1-TEF1-TUP1/TUP1* | structured | this study |
| 6 | p<sub>GAL</sub>-*TUP1* | *MATa/MATα, tup1Δ::KanMX, nat1-GAL1-TUP1* | smooth** | this study |
| 7 | BR-F/Flo11p-GFP | *MATa/MATα, FLO11-GFP/FLO11* | structured | (Stovicek *et al.,* 2014) |
| 8 | Flo11p-GFP/p<sub>TEF</sub>-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8* | smooth | this study |
| 9 | Flo11p-GFP/p<sub>TEF</sub>-*TUP1* | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-TUP1/TUP1* | structured | this study |
| 10 | Flo11p-GFP/p<sub>GAL</sub>-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11, cyc8Δ::KanMX, nat1-GAL1-* | structured** | this study |

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| | | *CYC8* | | |
|---|---|---|---|---|
| 11 | Flo11p-GFP/p$_{GAL}$-*TUP1* | *MATa/MATα, FLO11-GFP/FLO11, tup1Δ::KanMX, nat1-GAL1-TUP1* | smooth** | this study |
| 12 | p$_{GAL}$-*TUP1*/ p$_{CUP}$-*CYC8* | *MATa/MATα, tup1Δ::loxP, cyc8Δ::loxP, nat1-GAL1-TUP1, KanMX-CUP1-CYC8* | smooth** | this study |
| 13 | p$_{CUP}$-*TUP1*/ p$_{GAL}$-*CYC8* | *MATa/MATα, tup1Δ::loxP, cyc8Δ::loxP, KanMX-CUP1-TUP1, nat1-GAL1-CYC8* | smooth**[$] | this study |
| 14 | Flo11p-GFP/ p$_{GAL}$-*TUP1*/ p$_{CUP}$-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11, tup1Δ::loxP, cyc8Δ::loxP, nat1-GAL1-TUP1, KanMX-CUP1-CYC8* | smooth** | this study |
| 15 | Flo11p-GFP/ p$_{CUP}$-*TUP1*/ p$_{GAL}$-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11,* | smooth** [$] | this study |

| | | | | |
|---|---|---|---|---|
| | | *tup1Δ::loxP, cyc8Δ::loxP, KanMX-CUP1-TUP1, nat1-GAL1-CYC8* | | |
| 16 | *sfl1*/CYC8/p<sub>TEF</sub>-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8, sfl1Δ::KanMX, sfl1Δ::HygR* | smooth | this study |
| 17 | *nrg1*/CYC8/p<sub>TEF</sub>-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8, nrg1Δ::KanMX, nrg1Δ::HygR* | smooth | this study |
| 18 | *mig1*/CYC8/p<sub>TEF</sub>-*CYC8* | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8, mig1Δ::KanMX, mig1Δ::HygR* | smooth | this study |
| 19 | *CYC8*/Cyc8p-GFP | *MATa/ MATα CYC8-EGFP-kanMX/ CYC8* | structured | This study |
| 20 | *TUP1*/Tup1p-GFP | *MATa/ MATα* | structured | This study |

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| | | *TUP1-EGFP-kanMX/ TUP1* | | |
|---|---|---|---|---|
| 21 | BR-S | *MATa/MATα,* | smooth | (Kuthan *et al.,* 2003) |
| 22 | BR-S/*cyc8*/p<sub>GAL</sub> -*CYC8* | *MATa/MATα, cyc8Δ::KanMX, nat1-GAL1-CYC8* | Semi-structured** | This study |
| 23 | BR-S/*tup1* | *MATa/MATα, tup1Δ::KanMX, tup1Δ::nat1* | smooth | This study |
| 24 | BY4742 | *MATα, his3Δ, leu2Δ, lys2Δ and ura3Δ* | smooth | Euroscarf.de |

*For colonies grown on GMA medium

** Without induction; $ Semi-structured when medium contains traces of $Cu^{2+}$.

## *2.1.4 Media and solutions*

### 2.1.4.1 Solid media

YEPDA     1% yeast extract, 1% peptone, 2% agar, 2% glucose

After autoclaving, the following were added:

YEPDA + antibiotics     G418 - 400 mg/L

Hygromycin B - 400 mg/L

Nourseothricin - 200 mg/L

GMA     1% yeast extract, 3% glycerol, 2% agar

### 2.1.4.2 Liquid media

YEPD     1% yeast extract, 1% peptone, 2% glucose

YD     1% yeast extract, 2% glucose

GM     1% yeast extract, 3% glycerol

### 2.1.5 Other solutions

| | |
|---|---|
| 1M LiAc | 10.2 g $CH_3COOLi$ in 100 ml $H_2O$ |
| 1000x EtBr | 10 mg ethidium bromide in 1 ml $H_2O$ |
| 1% agarose gel | 500 mg boiled in 50 ml 0.5 x TBE buffer |
| 50% PEG | 10 g PEG 3500 in 20 ml $H_2O$ |
| 10 mM MES | 192 mg in 100 ml $H_2O$, (pH 6, HCl) |
| 100 mM PMSF | 17.4 mg Phenylmethylsulfonyl fluoride in 1 ml isopropanol |
| 1 mM AEBSF | 24 mg 4- (2- aminoethyl) benzenesulfonyl fluoride in 1 ml deionized $H_2O$ |
| 10 mM Tris-HCl | 121 mg Tris in 100 ml $H_2O$ |
| 30% Acrylamide | 30 g Acrylamide in 100 ml $H_2O$ |
| 3 M $CH_3COONa$ | 24.6 g $CH_3COONa$ in 100 ml $H_2O$ |
| RIPA buffer (Sigma-Aldrich) | 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0. |

## 2.2 Methods

### 2.2.1 Preparation of cassettes for different types of constructs

Strains were constructed from wild type strain BR-F (Stovicek *et al.,* 2010) via homologous recombination using cassettes created by PCR. Cassettes for gene deletion, GFP fusion and insertion of inducible promoters GAL1 ($p_{GAL}$) and CUP1 ($p_{CUP}$) were generated via PCR amplification of resistance markers within plasmids pUG6, pK127, pYM-N23 and pYM-N1 respectively (except where otherwise stated), using primers with homology to regions up and downstream of target regions on both plasmid and genome target sites (Gueldener *et al.,* 2002; Sheff and Thorn, 2004). For *CYC8* and *TUP1* disruption cassettes were created, using forward and reverse primers, containing flanking regions, homologous to a 45 bp sequence, just upstream and downstream (respectively) of the coding region of the target gene. For C-terminal fusions with GFP, the GFP-KanMX integrative cassettes were amplified by PCR using forward and reverse primers with regions of

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homology to sites just upstream and just downstream of the stop codon, respectively. PCR program and components for making cassettes as described:

| PCR program preparation | PCR reaction components |
| --- | --- |
| 95 $^{\circ}$C – 5 min | 12.5 µl – PCR mix |
| 95 $^{\circ}$C – 30 sec ⎤ | 11 µl – deionised $H_2O$ |
| 55 $^{\circ}$C – 30 sec ⎬ 30x | 0.5 µl – forward primer (100 pmol/µl) |
| 72 $^{\circ}$C – 2.30 min ⎦ | 0.5 µl – reverse primer (100 pmol/µl) |
| 72 $^{\circ}$C – 15 min | 0.5 µl – template DNA (plasmid) |
| 4 $^{\circ}$C – ∞ | |

## 2.2.2 Yeast transformation

The LiAc/SS Carrier DNA/PEG method was used for transforming yeast cells with cassettes (Gietz and Woods, 2002). 100 µl of yeast cells (approx. $2x10^9$ cells/ml) were mixed with the transformation mixture (36 µl 1M LiAc, 240 µl PEG 50%, 54 µl $H_2O$, 10 µl boiled ssDNA, 20 µl cassette reaction), incubated at 28 $^{\circ}$C for 10 min, heat shocked at 42 $^{\circ}$C for 20 min and left at 28 $^{\circ}$C for 10 min. The transformation mixture was spun down and the pellets were re-suspended in 1 ml YPD and incubated for 3-4 hours at 28 $^{\circ}$C. Subsequently, culture cells were collected by centrifugation, resuspended in 0.3 ml distilled water and plated on selective plates supplemented with appropriate antibiotics (G418, Hygromycin B - 400 mg/L, Nourseothricin - 200 mg/L).

## 2.2.3. Verification of transformation

After 4 days, colonies (transformants) that survived on selective plates, were transferred to new plates with appropriate antibiotics. Individual colonies were verified by PCR using specific primers that are listed in Table 2.1.

Altered genes, promoters and GFP fusions were sequenced in all strain constructs to ensure there were no mistargeted cassettes and strains were checked for growth morphology and growth rate. Transformants were then

stored in glycerol stocks at -75 ºC as previously described (Stovicek *et al.,* 2010; Nguyen *et al*., 2018; 2020).

### 2.2.4 Inducible promoter

The *GAL1/CUP1* inducible promoter was inserted in front of each coding gene ($p_{GAL}$-*CYC8* and $p_{GAL}$-*TUP1* or $p_{CUP}$-*CYC8* and $p_{CUP}$-*TUP1*) under the control of the $p_{GAL}$ or $p_{CUP}$ promoter rather than its native promoter but at their natural genomic positions. In galactose medium (or copper-supplement medium) the promoters were induced and these genes were overexpressed, whereas genes were not transcribed or underwent limited transcription in the absence of galactose/copper.

Induction experiments were performed on colonies that were grown for 3 days on GMA plates. Galactose and/or copper ($Cu^{2+}$) was added to a well at the centre of the agar plate to the desired concentrations: 2% of galactose and/or 3 mM $Cu^{2+}$ for 4 h incubation before analysis by northern blot or LC-MS/MS, or 0.1% of galactose and/or 0.25 mM $Cu^{2+}$ for 18 h incubation to analyze changes in colony morphology and Flo11p-GFP production (by two photon confocal microscopy and western blot). We used a high concentration of inducer (s) for 4 h for mRNA analysis since 4 h is sufficient to induce transcription (as determined in previous experiments (Stovicek *et al.,* 2010, Vopalenska *et al.,* 2010) while minimizing secondary effects. However, altering colony morphology requires a longer time period, and high concentrations of inducer cannot be used since the inducer (s) either cause toxicity (high $Cu^{2+}$) or affect colony morphology (galactose, as described by Granek and Magwene, 2010).

### 2.2.5 Cre-loxP induction

To generate multiple genetic modifications in one background, selective marker recycling is necessary and widely used. In this case, the genetic construct contains a selective marker, sandwiched between recombinase recognition sequences. This facilitates removal of markers via recombinase

gene expression and thus marker recycling. For example, in *S. cerevisiae*, the loxP flanked G418 resistance marker (loxP-kanMX-loxP) was used for gene deletion and then removed via transformation with a plasmid containing a different selectable marker (pSH66, with nat1 marker) and the galactose-induced Cre recombinase gene (Gueldener *et al.,* 2002).

### 2.2.6 Monitoring growth and development by colony imaging

Microcolonies were grown at densities from $10^3$ to $6x10^3$ per plate. Images of colonies in the incident and/or transmitted light were captured using a ProgRes® CT3 CMOS camera with a Navitar objective and NIS Elements software (Laboratory Imaging, s.r.o, Prague, CZ)

### 2.2.7 Microscopic analysis of cells within the colony structure

Two-photon excitation confocal microscopy (2PE-CM) (Figures 3.3, and 3.6) was performed according to (Vachova *et al*., 2009; Vachova *et al*., 2011). In brief, colonies were embedded in agarose and cut vertically down the middle. The cut surface was placed on a coverslip, and colony side views were obtained by 2PE-CM. A true confocal scanning microscope (SP2 AOBS MP; Leica, Germany) was used, fitted with a mode-locked laser (Ti:Sapphire Chameleon Ultra; Coherent Inc., USA), for two-photon excitation with 20×/0.70 and 63×/1.20 water immersion plan Apochromat objectives. An excitation wavelength of 920 nm was used for GFP. The emission bandwidth was set to 480-595 nm for GFP. An overview of the morphology of colonies was obtained simultaneously with green GFP fluorescence as autofluorescence in the 600-740 nm wavelength range. Images of whole colonies were composed of two or three stitched fields of view.

Internal structure of colonies and colony biofilms (Figure 3.2) was visualized by fluorescence microscopy of thin sections as described in (Cap *et al.* 2012). In brief, a 3 or 4-day-old microcolony, grown on a GMA plate was embedded in 2% agarose and vertical cross-sections of colonies/colony biofilms were prepared using an automatic microtome with a vibrating blade

(Leica VT1200S) to yield 20-50 μm-thick sections. Sections were transferred to a glass microscope slide and covered with a coverslip. Cells in these thin sections were observed under a Leica DMR fluorescence microscope with 10x, 40x and 100x lenses (immersion lens HCX PL fluotar 100x /1.3). Green fluorescence in GFP cells was observed using a GFP fluorescent cube (BP 470/40, BP 525/50) and cubes I3 (BP 450-490, LP 515), filter sets for GFP (excitation 450-490 nm; emission 500-550 nm). The exposure time of each image is stated in the relevant images. Images were captured with a JENOPTIK Progres® monochrome camera and NIS Elements software.

### 2.2.8 Flocculation and invasion assay

The flocculation assay was performed according to Bester *et al.,* (2006). Briefly, cells were harvested after 2 days of growth in GM medium. Flocs were dispersed using EDTA (pH 8, 50 mM final concentration) and cell density was measured using an optize1412v spectrophotometer (K-Lab, Korea) at a wavelength of 600 nm ($A_{600}$), [reading A]. After two washes with $H_2O$, cells were suspended in $CaCl_2$ (30 mM). After one minute, the $A_{600}$ of the upper cell suspension layer was measured [reading B]. Flocculation (%) was quantified using the formula: 100*(A-B)/A. Mean of 4 independent assays +/- SD is shown. Flocs were imaged at 30s, 60s and 3 min after preparation of the cell suspensions. Individual cells and cell aggregates were observed using light microscopy.

The invasive growth assay was modified from that used in a previous study (Roberts and Fink, 1994). Cells were harvested from colonies on fresh plates (YEPD), streaked onto GMA plates and incubated for 3 days at 28 °C. After vigorous washing, the plates were photographed.

### 2.2.9 Cell preparation for adhesion assays

Biofilm cultures (static cells): yeast cells were grown in either GM or YD liquid medium with shaking overnight at 28 °C, then harvested by centrifugation for 3 min at 3000 rpm at room temperature.

Then they were suspended in fresh GM or YD at a final concentration of 0.3mg/ml and 150 µl of suspended cells were pipetted into each well of a 96 polystyrene well flat-bottomed microtiter plate (V400917, GAMA group Inc., CZ) and incubated at 28 °C with shaking (150 rpm). Cell growth was assessed by measuring the $A_{600}$ immediately after seeding of the wells and after 4, 8, 12, 16, 20 and 24 h of incubation.

Shaken cultures (planktonic cells): biomass from each strain was collected from the overnight culture of yeast cells grown at 28 °C in either GM or YD liquid medium. Cells were then suspended in GM or YD, 10 mL of cell suspension (with a concentration of 0.3 mg/ml) was grown in an Erlenmeyer flask in liquid YD or GM media at 28 °C with vigorous shaking (150rpm). At chosen time points, yeast cells were harvested, washed in sterile water and suspended in either YD or GM media. The $A_{600}$ of the cell suspension was measured and the cell concentration adjusted to achieve a final $A_{600}$ value of 1. Following this, 150µl of cell suspension of $A_{600}$=1 was pipetted into each well of a 96 well plate and incubated for 3 hrs with shaking (150 rpm) at 28 °C before determining cell adhesion.

Experiments were carried out independently in quadruplicate for each strain and condition. Cultures were supplemented with specified concentrations of galactose and/ or copper to yield induction conditions. After specific periods of culture, cell density was determined via measurement of cell absorbance ($A_{600}$). Meanwhile, cell adhesion was also assessed ($A_{570}$).

### 2.2.10 Adhesion assays

The adhesion assay was carried out according to Reynolds and Fink, (2001) and Mowat *et al.,* (2007) with some modifications. Wells with liquid (medium or water) were washed three times with water and stained with 150 µl of 1% crystal violet dye. Following that, plates were incubated at room temperature with vigorous shaking for 15 min. The plates were then washed gently with water and crystal violet solution was eluted with 150 µl of 95% ethanol per well. 100 µl of the crystal violet eluted from each well was

pipetted into a well of a fresh microtiter plate and measured at 570 nm by absorption spectrophotometer (Epoch Microplate, Biotek). The adhesion capability and the amount of adhered cells were determined by absorption value ($A_{570}$). Control measurements, throughout the protocol were carried out in microtiter plates without cells. 0.02 was established as the background absorbance (BA) value and is also indicated in the graphs.

To identify the influence of glucose on adhered cells, glucose was added to a well with intact biofilm that had been grown in GM or glucose. Alternatively, an appropriate concentration of glucose was added after non-adhered cells were washed off. After 4 h incubation with glucose, non-adhered cells were removed and washed. Finally, the numbers of adhered cells were determined as stated. Control wells were without glucose supplementation.

## 2.2.11 DNA phenol-chloroform extraction

The DNA suspension was mixed with phenol (1:1). After vortexing, the mixture was centrifuged (10 min at 12000 rpm). After transfer to a fresh tube, the water phase was mixed with 0.5 volumes of phenol and 0.5 volumes of chloroform and centrifuged (10 min at 12000 rpm). Then the water phase was transferred to a fresh tube and mixed (1:1) with chloroform, vortexed and centrifuged (10 min at 12000 rpm). After mixing with ice-cold ethanol (2.5 vols) and 1/10 volume of 3 M $CH_3COONa$ pH 5.4 (0.1 vol) the water phase was stored for 1 hour at -75 °C. DNA was centrifuged (30 min at 14000 rpm, 4 °C). After washing with 80% ethanol, the pellet was dried and dissolved in deionised $H_2O$. DNA concentration was checked using a nanodrop before dilution to desired concentration for further experiments.

## 2.2.12 RNA extraction

Total RNA was isolated by the hot phenol method (Palkova *et al.,* 2002). In brief: microcolonies (3-5 days old) were collected and re-suspended in TES buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS). 400 µl/100 mg of biomass, before adding approximately 200 µl of acid-washed glass beads and

400 µl of phenol:chloroform (5:1, from Sigma ref P-1944). Incubated at 65 ºC for 30 min and vortexing vigorously for 30 seconds every 5 min, the mixture was immediately placed in a deep freeze (-75 ºC) for at least 60 min. After centrifugation at RT for 15 min at 13000 rpm, the upper aqueous phase was transferred to a new tube, already containing 400 µl of phenol: chlorofom 5:1. The RNA mixture was extracted 3 times in phenol: chlorofom 5:1, before a final wash with chloroform.

After removal of chloroform, the upper aqueous layer was transferred into a new tube with 30 µl of 3 M sodium acetate pH 5.3, before adding approx 600 µl of 100% ethanol and incubating overnight at -20 ºC for precipitation of RNA.

## 2.2.13 Determination of Flo11p-GFP levels by Western blots and of Tup1p and Cyc8p by LC-MS-MS

The detection of GFP tagged Flo11p in the cell lysates or the extracellular fluid was carried out by western blots according to (Vachova et al., 2009). In brief, 70-100 mg biomass of 3 days old colonies was harvested and disrupted by glass beads in the presence of protease inhibitors, and proteins from cell lysates (quantified using a protein detection kit, Bio-Rad) were subjected to SDS-PAGE (25 µg/lane). After the proteins were transferred to a PVDF membrane, GFP was detected by mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-GFP antibody (Santa Cruz). The peroxidase signal was visualized using Super Signal West Pico (Pierce) on Super RX medical X-ray film (Fuji). The levels of Flo11p or free GFP were evaluated by UltraQuant 6.0. To minimize the effect of band saturation, less exposed WBs were used for quantification. Membranes stained by Coommassie blue were used as loading controls (Figure S5). Samples of extracellular proteins were prepared from 3-day-old colonies suspended in phosphate-buffered saline (PBS) containing protease inhibitors. After centrifugation, proteins of the supernatant were precipitated by methanol/chloroform treatment (Wessel et al., 1984). Extracellular proteins

extracted from 50 mg of wet biomass were loaded into each slot.

For LC-MS-MS analysis, biomass of 3-day-old colonies from the wild type strain and strains expressing different levels of Cyc8p and Tup1p were harvested. The cells were disrupted in 100 mM triethyl-ammonium bicarbonate buffer without protease inhibitors using glass beads. Total protein concentration was determined by the bicinchoninic acid assay (Sigma). Protein aliquots (30 µg) were solubilized using sodium deoxycholate (1% (w/v) final concentration), reduced with tris (2-carboxyethyl) phosphine, alkylated with S-methyl methanethiosulfonate, digested sequentially with trypsin and extracted with ethyl acetate saturated with water (Masuda *et al*., 2008). Samples were desalted using C18 sorbent (Supelco pn: 66883-U, Sigma) and eluents were dried and resuspended in 20 ul of 1% trifluoroacetic acid. Peptides (2 µg) from each sample were separated on Nano reverse phase columns (EASY-Spray column a 50 cm × 75 µm ID, PepMap C18, 2 µm particles using a 1 h elution gradient and analyzed in DDA mode on an Orbitrap Fusion Tribrid (Thermo Scientific, Waltham, MA, USA) mass spectrometer. Three biological replicates were performed for each strain and condition. Resulting raw files were processed in MaxQuant (v. 1.5.8.3) (Cox *et al*., 2014). Searches were performed against the latest version of the *S. cerevisiae* Uniprot database and common contaminant database Perseus (v.1.6.1.1.) and Excel 2013 were used for further analysis. Protein abundances for the mutant strains with altered levels of *CYC8* or/and *TUP1* were normalized to the abundance of that protein in the wild type BR-F strain to identify fold differences in protein expression. Next data were filtered for proteins that were significantly differentially expressed in mutant strains versus wild type with a p-value threshold of 0.05 (student's t-test, corrected for multiple testing using the Benjamini Hochberg method) and that were upregulated or downregulated in mutant versus wild type strain at least 1.5-fold. The gene ontology (GO) analyses were performed using FUNSPEC as described (Robinson et al. 2002).

# CHAPTER III. RESULTS AND DISCUSSION

## 3.1 Global effect of Cyc8p and Tup1p on protein expression in yeast colony

In this part, a range of functions of Tup1p and Cyc8p were investigated and described, based on proteomic data. Several of the identified targets were validated to confirm the roles of these regulators. The data in this chapter has not yet been published. The author constructed strains, prepared cultures and carried out total protein extraction and measurement, following which LC-MS/MS was performed at the proteomics core facilities, Biocev. Raw data analysis was performed by Prof. Zdena Palkova. Author carried out data analysis by excel 2013 with advice from Prof. Zdena Palkova and Dr. Derek Wilkinson.

This part contains supplementary Tables S1 to S4 which are available on pages 74 to 78.

### 3.1.1 The impact of Cyc8p and Tup1p on proteome of yeast cell colonies

Several studies have investigated differences in mRNA expression upon deletion of *CYC8* or *TUP1,* but little is known about changes in the protein levels of Cyc8p and Tup1p gene targets. To identify the effect of Cyc8p and Tup1p on yeast cell proteome, we used LC-MS/MS analysis for proteomic quantification to examine changes in the proteome of p$_{GAL}$ -*CYC8* (BR-F/*cyc8*/p$_{GAL}$ -*CYC8*) and p$_{GAL}$ -*TUP1* (BR-F/*tup1*/p$_{GAL}$ -*TUP1*) cells compared to that of the parental BR-F cells strain, as described previously (Nguyen *et al*., 2018).

Approximately 3000 proteins were detected in at least one of the samples, out of a total 5858 proteins, known to be expressed in yeast (Ho *et al*., 2018). 265 proteins (4.52% of the proteome) were upregulated in non-induced p$_{GAL}$ -*TUP1* cells and 242 proteins (4.13% of the proteome) were upregulated in non-induced p$_{GAL}$ -*CYC8* cells compared to wild type BR-F

cells. In theory, *S. cerevisiae* genes that are repressed by Cyc8p or Tup1p could be expected to be overexpressed in *cyc8* and *tup1* mutant cells, respectively. Moreover, Cyc8p is considered to be a corepressor with Tup1p, so their targets could be expected to overlap. As shown in Figure 3.1A, a substantial fraction (71.9% or 174 proteins) of the 242 proteins significantly upregulated (1.5 times) when *CYC8* expression was reduced to basal level (p$_{GAL}$ -*CYC8*) were also upregulated in reduced *TUP1* expression (p$_{GAL}$ -*TUP1* noninduced). Overlapping Cyc8p and Tup1p repression profiles, as demonstrated in this analysis, suggest that these two transcriptional regulators co-repress a large number and wide variety of genes, many of which are involved in carbohydrate metabolism, ATP synthesis, DNA repair response, protein refolding and replicative cell ageing, as shown in Table 3.1.
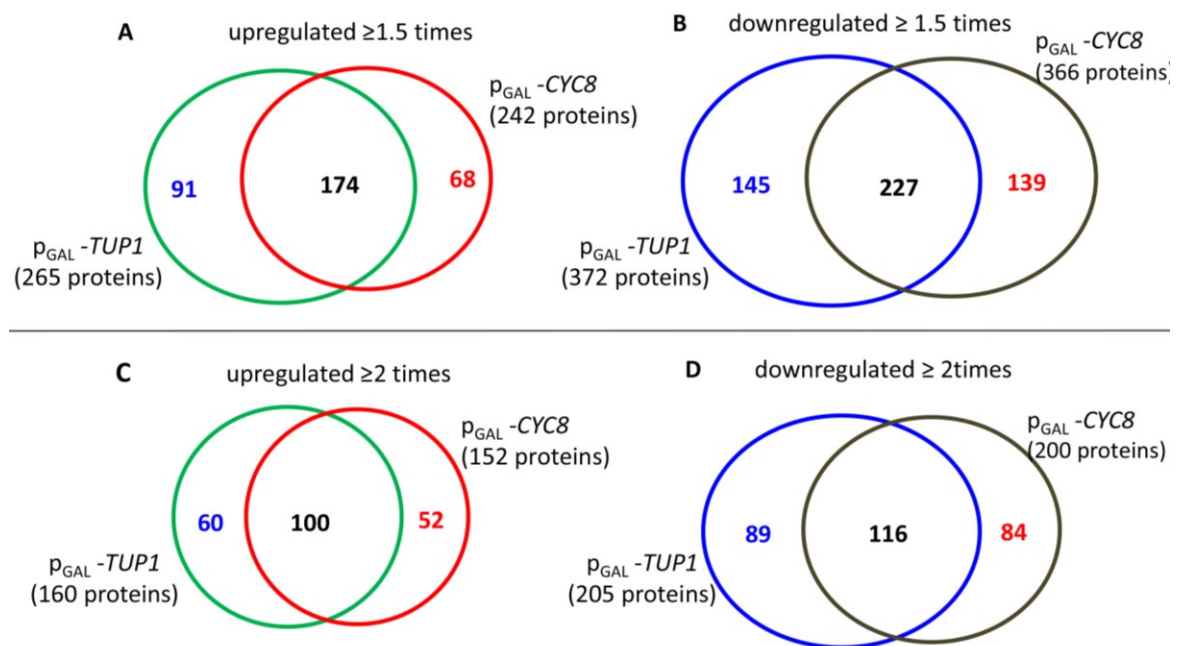


**Figure 3.1. Venn diagrams showing the number of overlapping and distinct proteins in p$_{GAL}$ -*TUP1* and p$_{GAL}$ -*CYC8*.**

*A-B, with a fold difference threshold of 1.5, and C-D, with fold difference threshold of 2.*

**Table 3.1. Genes whose protein products were upregulated in p<sub>GAL</sub> -*TUP1* or p<sub>GAL</sub> -*CYC8* strain versus wild type (P <0.05 and difference >1.5)**

| Category | p-value | In Category from Cluster |
|---|---|---|
| oxidation-reduction process [GO:0055114] | <1e-14 | *PRX1 MIS1 PDB1 HBN1 IDP1 MDH3 NDE2 HEM13 MRP1 DIT2 YEL047C DSF1 HEM14 RNR1 LPD1 ERG4 RNR4 AIM17 OYE2 KGD1 YIR035C RNR2 MDH1 YKL107W SDH1 AHP1 IMD3 NDI1 YML131W FET3 ADH3 ADH6 IDH1 GOR1 IDH2 FDH1 ALD6 OYE3 FSH1* |
| ATP synthesis coupled proton transport [GO:0015986] | 2.07E-12 | *ATP6 ATP1 ATP3 ATP5 ATP2 ATP7 ATP14 ATP4 ATP15 ATP20* |
| mitochondrial translation [GO:0032543] | 6.67E-09 | *MRPL36 MRPL11 MRP1 MRP13 MRPL6 MRPL49 MEF1 MRPL4 YML6 MRPS8 MRPL24 MRPL33 MRPL19 TUF1 MRP51* |
| thiamine biosynthetic process [GO:0009228] | 1.801e-05 | *THI20 THI6 THI12 SNZ2 SNO2* |
| apoptosis [GO:0006915] | 1.61E-05 | *PET9 OYE2 FIS1 NUC1 POR1 OYE3* |
| deoxyribonucleotide biosynthetic process [GO:0009263] | 0.0003393 | *RNR1 RNR4 RNR2* |
| protein refolding [GO:0042026] | 0.0003615 | *MDJ1 SSC1 HSP60 HSP10* |
| chronological cell ageing [GO:0001300] | 0.000415 | *ACB1 FIS1 MDH1 NDI1* |
| sporulation | 0.000518 | *DIT2 EMI2 SPS100 SGA1* |
| aerobic respiration [GO:0009060] | 0.0006137 | *PET9 MNP1 QCR8 MDH1 COQ9 COQ5* |
| protein complex assembly | 0.0007206 | *SCO1 ATP7 YTA12 SAM50 ATP20* |

| [GO:0006461] | | |
|---|---|---|
| glycolysis [GO:0006096] | 0.0007206 | *PDB1 EMI2 HXK1 HXK2 KGD1* |
| iron ion homeostasis [GO:0055072] | 0.001546 | *FIT1 FET3 FET4 FIT2 FIT3* |
| maltose metabolic process [GO:0000023] | 0.002539 | *IMA1 IMA5 IMA2* |

*) genes in red text were further verified

In contrast to previously reported transcriptomic data (Green *et al*., 2004; Rizzo *et al*., 2011, Chen *et al*., 2013; Chujo *et al*., 2015), the *FLO* and *PAU* gene families and the cell wall mannoprotein-encoding *TIR* genes, were not identified in our proteomic data as differentially expressed, despite these subtelomeric genes being well-known targets of the Cyc8p-Tup1p complex ( (Malave and Dent, 2006).

Nevertheless, in this study, many genes whose protein products displayed the highest level of repression by Tup1p or Cyc8p in LC-MS/MS data were previously identified as Tup1p- and/or Cyc8p-repressed at the transcriptomic level (Green *et al*., 2004; Rizzo *et al*., 2011, Chen *et al*., 2013; Chujo *et al*., 2015). These include genes with roles in nucleotide reduction to deoxynucleotides (*RNR2, RNR4*); sporulation (*DIT2, EMI2, SPS100, SGA1)*; use of alternative carbon sources: isomaltases (*IMA1, IMA5, IMA2*), thiamine, glycogen and mannitol metabolism (*THI16, THI20; GSY1, UGP1; DSF1*). Hence, these genes were targets of the Cyc8p-Tup1p complex, which regulates the expression of these genes at both transcription and translation levels.

Remarkably, several sets of Cyc8p/Tup1p-repressed genes, described in our study were not reported in the previous transcriptomic studies (Green *et al*., 2004; Rizzo *et al*., 2011; Chen *et al.,* 2013; Chujo *et al.,* 2015), including genes involved in ATP synthesis, protein refolding and protein complex assembly, chronological cell ageing and apoptosis as shown in Table 3.2

**Table 3.2. Genes, not previously recognized as being regulated by Cyc8p, and/or Tup1p whose proteins were upregulated in either *cyc8* or *tup1* mutant strain.**

| Category | p-value | In Category from Cluster |
|---|---|---|
| ATP synthesis coupled proton transport [GO:0015986] | 4.108e-14 | *ATP6 ATP1 ATP3 ATP5 ATP2 ATP7 ATP14 ATP4 ATP15 ATP20* |
| mitochondrial translation [GO:0032543] | 3.348e-11 | *MRPL36 MRPL11 MRP1 MRP13 MRPL6 MRPL49 MEF1 MRPL4 YML6 MRPS8 MRPL24 MRPL33 MRPL19 TUF1 MRP51* |
| glutamate biosynthetic process [GO:0006537] | 6.536e-05 | *IDP1 ACO1 IDH1 IDH2* |
| protein complex assembly [GO:0006461] | 0.0001282 | *SCO1 ATP7 YTA12 SAM50 ATP20* |
| protein refolding [GO:0042026] | 0.0001595 | *MDJ1 SSC1 HSP60 HSP10* |
| chronological cell ageing [GO:0001300] | 0.0001595 | *ACB1 FIS1 MDH1 NDI1* |
| apoptosis [GO:0006915] | 0.0005891 | *PET9 FIS1 NUC1 POR1* |
| replicative cell ageing [GO:0001302] | 0.0006405 | *PNC1 HXK2 MDH1 ACS2 RAS1* |

A significant number of *S. cerevisiae* proteins was downregulated in $p_{GAL}$-*TUP1* colonies (372 proteins) and in $p_{GAL}$-*CYC8* colonies (366 proteins) compared to wild type colonies with a p-value cutoff of 0.05 and a fold change threshold of 1.5. As shown in Figure 3.1B, 62.02% (227 proteins) of 366 proteins whose expression was decreased in the $p_{GAL}$-*CYC8* strain were also reduced in the $p_{GAL}$-*TUP1* strain. Genes with roles in 'amino acid metabolism' and 'RNA polymerase II transcriptional preinitiation' ontology

groups were significantly enriched among the set of genes encoding proteins that were downregulated in *cyc8* and *tup1* mutant strains, as shown in Table 3.3.

**Table 3.3. Significantly enriched GO biological process and cellular component terms of genes whose proteins were downregulated in p$_{GAL}$ -CYC8 or p$_{GAL}$ -TUP1 strain versus wild type on GMA (P <0.05 and fold enrichment >1.5)**

| | | |
|---|---|---|
| metabolic process [GO:0008152] | 2.494e-07 | *GDH3 GAL1 ZTA1 DUR1,2 PCS60 HIS4 GPM2 BPL1 GLT1 DPP1 TRP4 PHO8 SER3 TMT1 ERG26 PMA1 LEU1 ADE3 GND2 PAN5 SER33 IRC24 BNA1 ARO7 YKL027W GFA1 OXP1 YLL056C BNA5 TAL1 FBP1 YMR090W GDH1 PMA2 ATH1* |
| cellular amino acid biosynthetic process [GO:0008652] | 8.997e-07 | *CYS3 LEU2 HIS4 GLT1 TRP4 HIS1 SER3 LEU1 ADE3 ARG4 SER33 ARG3 ARG1 ARO7 ASN1 PUT1 PUT4* |
| oxidation-reduction process [GO:0055114] | 4.601e-05 | *COX2 GDH3 ZTA1 LEU2 HIS4 GLT1 SER3 ERG26 ADE3 GND2 PAN5 SER33 IRC24 BNA1 YJR096W SOD1 GPX1 SRX1 PUT1 HMG1 ADH1 GCY1 GDH1* |
| ATP catabolic process [GO:0006200] | 0.0003467 | *ADP1 PMA1 PXA2 BPT1 PMA2 PDR12 PXA1* |
| RNA polymerase II transcriptional preinitiation complex assembly [GO:0051123] | 0.001729 | *TAF12 SPT15 CRT6 NHP6A* |
| fatty acid transport [GO:0015908] | 0.00339 | *PXA2 PXA1* |

However, when we increased stringency by employing a fold change threshold of 2 (p-value 0.05), the number of proteins, downregulated in p$_{GAL}$ -TUP1 colonies was 205 (two-fold change) compared to 372 proteins (1.5

fold-change) and in p$_{GAL}$ -*CYC8* colonies was 200 (2-fold change) compared to 366 proteins (1.5-fold change). 116 proteins (58% of those, downregulated in at least one mutant strain) were downregulated in both the *cyc8* and *tup1* mutant strains. While 100 upregulated proteins (65.8%) were shared between the 160 proteins upregulated in p$_{GAL}$ -*TUP1* and the 152 proteins upregulated in p$_{GAL}$ -*CYC8* colonies, as shown in Fig. 3.1C and D, (for more information see Table S1-4). These results indicate there is greater overlap among proteins, upregulated than among those, downregulated in *cyc8* and *tup1* mutant strains, which is consistent with the major reported functions of Cyc8p and Tup1p as repressors (Chen *et al.,* 2013). Moreover, reduced *CYC8* and *TUP1* expression strains resulted in very poor growth that could indirectly cause decreasing levels of gene expression (Wong *et al.*, 2011).

In particular, genes whose protein products were only upregulated when *CYC8* expression was reduced (as shown in the upper half of Table 3.4) mainly function in cell wall organization (*CWP1, SMI1*, *FKS1, UTR2*) proteolysis (*PHB1 PHB2, YFR006W*), O-linked glycosylation (*PMT6, MNN1*), glycine metabolism (*GCV3, SHM2, GCV2*), phosphate transport (*PHO88, PIC2, DIC1*), amino acid (*MET8, MET10, MET17, CYS4, ADI1*) and lipid biosynthesis (*CEM1, ERG25, NCP1*). In comparison, dozens of genes encoding proteins that were only upregulated when *TUP1* expression was kept at a basal level, were involved in electron transport (*COR1, QCR6, SDH2, CYB5, CYT1, QCR2),* mitochondrial citrate transport (*CTP1, YHM2*), heme synthesis (*HEM3, HEM1*), cristae formation (*TIM11, FCJ1*) and proteins import into the mitochondrial matrix (*TIM44, TIM23, MGE1*) as shown in the upper half of Table 3.4.

Interestingly, many genes whose protein levels only decreased in p$_{GAL}$ -*TUP1* colonies (lower half of Table 3.4) are related to the proteasome (*PUP3, SCL1, PRE9, PUP2, PRE3, CPS1, PRE8, PRE5*), methionine metabolism (*STR3, MET3, MET5, MET14, MET1, MET17*), transcription from RNA polymerase III (*SFP1, GTR1, RPB8*) and vesicle-mediated transport (*ERV46,*

*APM3, CHC1, CLC1, VTI1, SEC12, RUD3, APL5*). Notably, expression of Gas protein family members Gas1p, Gas3p and Gas5p, which are crucial for cell wall assembly and remodeling (Lesage *et al*., 2006), was also downregulated compared to the wild type strain. Others with roles in mRNA transport (*HEK2, NUP170, SAC3, NUP157, NUP192, NSP1, NUP2*), transcription control (*REB1, MAF1, SAC3, HMO1, THO1, RAI1, ASK10, MGA1, DOT5, NOT3, STH1, SWI3, SWI6, RPC31, RAP1, SIN3, HIR2, ETT1, AZF1, TOA1, MBF1, HAP5*), steroid biosynthesis (*ERG7, ERG12, ERG8, MVD1*) and *de novo* pyrimidine biosynthesis (*URA2, URA6*) were downregulated in $p_{GAL}$-*CYC8* colonies.

These data suggest that Cyc8p (but not Tup1p) represses the expression of a set of genes encoding proteins with roles in cell wall organization, protein O-linked glycosylation, amino acid and lipid biosynthesis. On the other hand, Tup1p (but not Cyc8p) negatively regulates a second set involved in electron transport and mitochondrial citrate transport. Moreover, Cyc8p may have positive functions in the expression of genes whose proteins are involved in mRNA transport, transcriptional control and pyrimidine synthesis, while Tup1p may act as an activator of proteins with roles in methionine synthesis, cellular transport and cell wall assembly and organization. This highlights the independent roles of each factor in the regulation of target gene as shown in Table 3.4.

**Table 3.4. List of independent genes whose protein abundances were significantly altered in the limited Cyc8p or Tup1p strain (P <0.05 and fold enrichment >1.5)**

|  | Reduced *CYC8* | Reduced *TUP1* |
|---|---|---|
| **Upregulated** | *CWP1  SUL1  GCV3  CCP1 PHB1  ADI1  AFG1  AIM45 FKS1  SMI1  PHB2  ALT1 YSC83 POL1 RSM23 PEX25 NCP1  GCV2  PEX11  DLD2 YFR006W    RSM19    SOP4* | *YIH1  TRM1  PBS2  CIT1  SDH2 CTR3  HSP150  MTC1  GDH2 MSY1    VAN1    PSA1    NDE1 HSP32  PRS1  MRPL15  TRP1 QCR2    YHB1    PSP1    ATP18 COR1    GAR1    IDP2    CYT1* |

| | | |
|---|---|---|
| | *GPH1 MNN1 MET10 RDL1 CYS4 ERG25 SHM2 SPE3 MET8 PMT6 CTA1 MPM1 MSC1 POS5 MPD1 RSM24 UBC8 SND3 COQ6 CEM1 MET17 FET4 GPD1 RPS0A SDH5 MRPL8 PIC2 TOM40 FCY1 FDH2 SNZ1 UTR2 OM14 GSY2 CMC4 ECI1 EUG1 MAL12 DIC1 DCI1 FMP46 MPC3 ADE17 YPL113C* | *YMR31 WTM2 EFM4 DNM1 FMP25 QCR6 TIM22 MAM33 CYB5 MCP2 MCR1 KGD2 NGL3 RPS14B RPE1 GTS1 TIM44 LCB3 LCB1 MRPL38 YKR070W HEM3 MRPL35 UTR4 AGX1 FMP32 YPL088W ATP25 SLF1 MCY1 STM1 ATP12 MGE1 MED11 YHM2 RIX1 MRPL40 GRE2 TIM23 FMP10 CTP1 PHO86 URA2 FUB1 MRP7 RRP12 SSA2 SIP5 TIM11 MRPL17 ATP17 PAA1 HNT1 YLR287C MIC60 HEM1 RPL34A TOS1* |
| **Downregulated** | *DIP5 RCR1 DAL2 SIM1 YCL019W RSE1 HXT5 CLP1 YLR173W MSH2 SOK1 GTO1 GYL1 VAM7 ENP1 ETT1 SPC97 RAP1 TKL2 KRI1 EIS1 VPS5 CIT2 HHF1 CNB1 MSC3 KIN2 NYV1 UBX5 HHT1 SEC10 MAF1 SEC3 HIR2 SIS1 SWI6 VID27 CWH41 UBX3 VNX1 THO1 STH1 MTR10 CIA2 GAD1 SRC1 FOL2 RRP4 REB1 RPC31 HAP5 DBP1 YAR1 NUP157 ERG12 ARD1 PSR1 HEH2 ICL1 IGO1 NSP1 PFK26 RHO2 UBA4 NUP170 YPL247C MVD1 SAM4 YCP4 KTR4 CEG1 NUP192 ROD1 HEK2 SWI3 NUP2 CDC55* | *PDC6 MET3 MET14 YGR125W MET1 YBR287W BLM10 COX8 SOL4 ERG1 ZPR1 TRX2 MET17 ARG8 ERG6 HXT7 APL5 CRM1 YCH1 AIM46 URA4 NAS6 RSC3 YDR109C STR3 MAM3 MET7 YDR415C AMS1 DBP9 CLC1 PDR11 YNL115C BIO5 CTF4 LOS1 CAB4 TAF14 ALD5 MLS1 SFP1 TAP42 PBP1 VTI1 ABP140 YSC84 TFG2 MAG1 XKS1 RRP8 YMR295C PRE5 ARG5,6 LAS17 BNA4 IGO2 GTR1 ERV46 NVJ2 FAT3 DDP1 APM3 BGL2 HIS5 HOM3 ARA2 PFK2 YGR266W ENA5 PRE9 APE3 HSE1 TRR2 RPB8 GAP1 CHC1 CHO2 EMP47* |

| | | |
|---|---|---|
| | KTR6 SAC3 DOT5 NAT3 GSF2 USA1 NMA1 EMW1 YMR315W AMD1 ASK10 FBP26 PIB2 MPD2 COX1 ECM29 SIN3 NOT3 IGD1 MRT4 SPG4 VAC14 PDC1 *URA2* EXG1 GLO3 EMP70 ERG8 CYR1 EAP1 PIL1 NAT1 URA6 RFA3 RAI1 MTR4 MYO3 DYS1 PRS5 SUR7 ERG7 HIS7 RHO3 AZF1 CDC42 GBP2 MCM2 ADD66 YCK1 UGA1 TOA1 MGA1 MBF1 IST2 VTC3 NPR1 IMO32 HMO1 TGL4 PKC1 KRR1 | MET5 TFG1 SOR2 PRE3 SPB1 PUP2 ADE1 GAS5 VPS35 SUM1 AAT1 CIC1 PXP1 YER134C VHR1 PAP1 SHS1 PUP3 NOP4 SSF1 ADE12 ALE1 TYS1 YHR138C BAT2 BRO1 PRP19 ERG11 COX12 PKH2 TGL3 NHP6B RTT103 PRS2 PRE8 YBR137W TFA1 PFK1 ABD1 PAL2 DSE4 DUT1 MPC1 NCR1 APE2 LSO2 PGC1 YGL242C RIE1 DFR1 CPS1 PGA2 CGI121 SEC12 RTF1 LSB3 RBG1 GPM1 SCL1 RUD3 YGR017W TDH1 YMR196W BUD14 SOR1 ENA2 |

\*) genes in red text were further verified.

### 3.1.2 Validated target genes of Cyc8p and Tup1p

We identified a high confidence set of genes encoding proteins that were consistently upregulated in both mutant strains. To validate these results, we employed GFP tagging of some Cyc8p and Tup1p target genes including *FSH1*, *RNR4, OYE2, URA2*, and *MET17* (shown in red text in Table 3.1 and Table 3.3). These genes were selected because of their involvement in different Cyc8p-Tup1p-regulated pathways.

In agreement with the proteomic data (Table 3.1) and literature (Li *et al*., 2000; Klinkenberg *et al*., 2006) Cyc8p and Tup1p act together to repress *RNR4* as shown in Fig 3.2, Rnr4p-GFP signal was significantly increased in either the $p_{GAL}$ -*CYC8* or $p_{GAL}$ –*TUP1* strain. Rnr4p is a factor that catalyzes the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides and therefore directly involved in the pathway of DNA

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replication. Cyc8p-Tup1p may repress *RNR4* via a chromatin remodeling mechanism as described by Li and Reese (2001), who found that Cyc8p-Tup1p interacts with the N terminus of Crt1p to establish a nucleosomal array over the promoter of *RNR3*, a minor isoform of the large subunit of ribonucleotide-diphosphate reductase. Deletion of *CRT1*, *CYC8*, or *TUP1* causes the loss of nucleosome positioning and transcription activation of *RNR2, RNR3* and *RNR4* (Li and Reese, 2001).

Fig.3.2 also shows that the Fsh1p-GFP signal was faint in the wild type, but potent in either the *cyc8* or *tup1* mutant. Fsh1p is repressed possibly via Cyc8p-Tup1p interacting with Crt1p as previously findings (Zaim et al., 2005) have shown that Fsh3p, another *S. cerevisiae* lysophospholipase was increased in *Δcrt1*. Fsh1p, which has a role in lipid homeostasis and is a lysophospholipase that hydrolyzes lysophosphatidylserine to release fatty acid (Gowsalya *et al.*, 2020). It shares biochemical properties with human OVCA2 (Bun *et al.*, 2020), therefore further study into the mechanistic functions of Cyc8p and Tup1p in the regulation of Fsh1p and lipid homeostasis, may have wider relevance.

Moreover, reducing either *CYC8* or *TUP1* expression resulted in increasing *OYE2* expression as shown in Fig.3.2. *OYE2* a conserved NADPH oxidoreductase has a role in the general stress response since its expression is induced to response to linoleic acid hydroperoxide (LoaOOH), $H_2O$ and diamide (Alic *et al.*, 2004; O'Doherty *et al.*, 2013). Cyc8p-Tup1p represses *OYE2* may be via *YAP* family proteins, in which *YAP4* and *YAP6* physically interact with Cyc8p and/or Tup1p as previous described (Hanlon *et al.*, 2011) or via other unknown mechanisms.
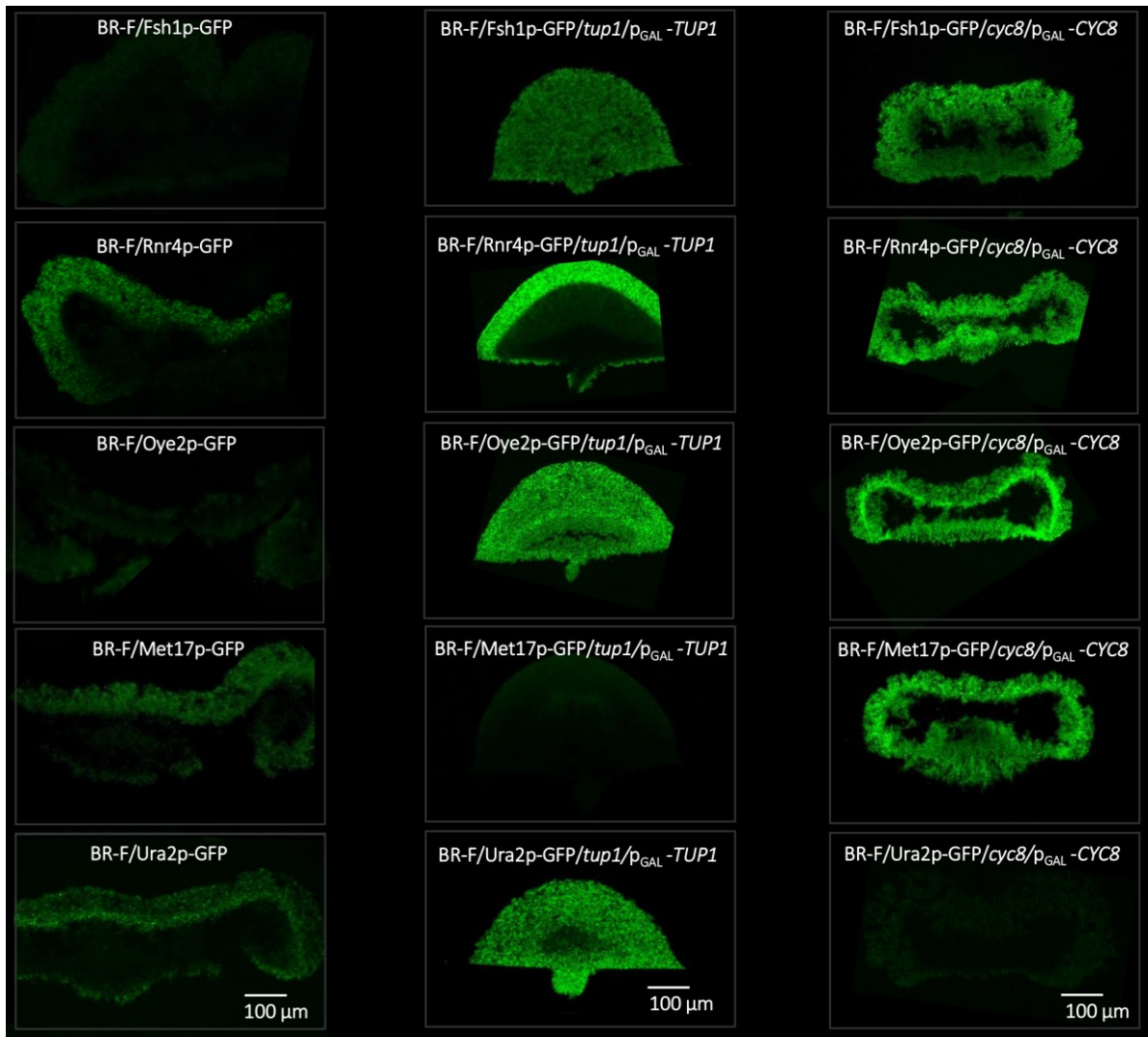
**Figure 3.2. Validation of expression data for some targeted genes via fusion with GFP in the wild-type strain and in strains expressing different levels of Cyc8p or Tup1p.**

*Cross-sectioned colonies of strains under Leica DMR fluorescence microscope. Scale bar; 100 μm.. Colonies were grown on GMA plates for 3 days.*

Interestingly, Cyc8p and Tup1p exhibit antagonistic functions in the regulation of methionine biosynthesis. Limiting Cyc8p expression led to significantly increased Met17p-GFP, whereas limiting Tup1p expression resulted in reduced expression of Met17p-GFP (Fig. 3.2). Excitingly, the data also indicated converse roles in regulating *URA2* expression. A basal level of Tup1p expression enhanced the Ura2p-GFP signal (Fig. 3.2), whereas limiting Cyc8p expression caused a significant decrease in the expression of this

protein. These GFP tagging results are consistent with the proteomic data (Table 3.3), where Ura2p was downregulated in $p_{GAL}$ -*CYC8* but upregulated in $p_{GAL}$ -*TUP1*. While Met17p was upregulated in $p_{GAL}$ -*CYC8*, but downregulated in $p_{GAL}$ -*TUP1*.

### *3.1.3 Discussion-1*

Hundreds of genes were identified as being repressed by either Cyc8p or Tup1p. 265 proteins were identified as significantly upregulated when *TUP1* expression was reduced by the $p_{GAL}$ promoter. In comparison, reduced *CYC8* expression led to significantly upregulated expression of 242 proteins, possibly implying derepression of the encoding genes. Our results are broadly consistent with previous transcriptomic studies (Green *et al*., 2004; Rizzo *et al*., 2011, Chen *et al*., 2013; Chujo *et al*., 2015) in which significantly upregulated genes in either *cyc8* or *tup1* mutants were enriched for those, mapping to the Gene Ontology terms stress response, hexose transport, thiamine biosynthesis and sporulation.

Interestingly, many genes, not previously reported to be regulated by Cyc8p/Tup1p (Green *et al*., 2004; Rizzo *et al*., 2011, Chen *et al.,* 2013; Chujo *et al.,* 2015), were identified in our study as significantly derepressed in either the *cyc8* or *tup1* mutant as shown in Table 3.2. These included genes encoding proteins with roles in protein refolding and protein complex assembly, chronological cell ageing and apoptosis. Some Cyc8p-Tup1p controlled genes involved in mitochondrial translation and glutamate metabolism fall into the same functional categories as those of the previously reported Cyc8p-Tup1p target genes (Green *et al*., 2004; Rizzo *et al*., 2011, Chen *et al.,* 2013). We therefore believe this represents an expansion of the previously identified set of Cyc8p-Tup1p targeted genes rather than the identification of new networks. The difference may arise from different methods used, growth stage during which gene expression was measured, and differences in the expression profile of planktonic cells versus biofilm cells,

as previously reported in *C. albican*s (Uppuluri *et al*., 2018). In addition, the strains used in this study involve a single gene knockout in a diploid strain background, whereas previous reports used haploid strains. The Cyc8p-Tup1p complex may repress genes, involved in chronological cell ageing and apoptosis, possibly by masking the activation domain of Mig1p until the Snf1p complex phosphorylates Mig1p, uncovering the activation domain and turning the Tup1p-Cyc8p-Mig1p complex from a repressor to an activator of genes involved in maintaining chronological lifespan (Maqani *et al*., 2018). Proteins mediating respiration and generation of energy (ATP) were also upregulated in $p_{GAL}$ -*CYC8* and $p_{GAL}$ -*TUP1* strains. These findings are consistent with those of Wang *et al.,* (2017) who identified shared gene targets of the Dbp2p and Cyc8p-Tup1p complexes that include cellular respiration genes. In agreement with previous reports that the Cyc8p-Tup1p complex regulates processes involved in translation (Parnell *et al*., 2011; Germayel *et al*., 2015). In this study, differential regulation of genes involved in mitochondrial translation was also identified in the mutant strains as shown in Table 3.1.

However, many cell wall mannoproteins were not detected in our LC-MS/MS including Pau, Tir and Flo proteins. This may be due to limitations in the detection of cell wall proteins using LC-MS/MS and a more specific technique for analyzing cell wall protein preparations might be needed (Yin *et al*., 2008; Hsu *et al*.,2015).

Nevertheless, a high-confidence set of genes repressed by Cyc8p and/or Tup1p was identified in LC-MS/MS data. Gene ontology analysis identified biological process term enrichment in the high-confidence data set. The use of alternative carbon sources (*EMI2, GLC3, IMA1, SGA1, SUC2, IMA5, YNR071C, IMA2, THI20, THI6, THI12, SNZ2, SNO2, OYE2*) and upregulation of oxidative phosphorylation (*PRX1, MIS1, PDB1, HBN1, IDP1, MDH3, NDE2, HEM13, MRP1, DIT2, YEL047C, DSF1, HEM14, RNR1, LPD1, ERG4,*

*RNR4, AIM17, RNR2, MDH1, YKL107W, SDH1, AHP1, IMD3, NDI1, YML131W, FET3, ADH3, ADH6, IDH1, GOR1, IDH2, FDH1, ALD6*) are indicative of Cyc8p-/Tup1p-dependent cellular reprogramming in response to a scarcity of good sources of carbon or nitrogen (Broach *et al*., 2012). Tup1p and Cyc8p repress genes involved in alternative carbon source utilization in the presence of glucose in order to make use of this ideal carbon source. Glycolysis/fermentation is less efficient in terms of ATP generation from carbohydrates but supports rapid growth and replication with low generation of reactive oxygen species, which are often derived from mitochondrial respiration (Nilsson *et al*., 2016). When glucose is limited, these genes are derepressed to make use of alternative carbon sources (e.g. via beta-oxidation of fatty acids), to conserve carbon (the glyoxylate cycle avoids two decarboxylation steps of the TCA cycle) and to maximize energy extraction from those sources via oxidative phosphorylation (RoyA *et al*., 2013).

In accordance with our findings, Martinez-Moya et al (2020) reported that the most highly represented functional category among differentially abundant proteins found in *cyc8* and/or *tup1* mutants in *Xanthophyllomyces dendrorhous* yeast are alternative carbon sources and that protein abundances in *cyc8* and *tup1* mutants were more similar than those in the *mig1* mutant.

Many of the genes identified were co-repressed by Tup1p and Cyc8p, but some were determined in this study to be antagonistically regulated by these factors, including *URA2* and *MET17*. In response to methionine depletion, yeast cells activate Gcn4p, resulting in the induction of several methionine (*MET*) genes including *MET17* (Natarajan *et al*., 2001). It has been reported that Tup1p and Cyc8p interact with Gcn4p and promote the expression of several amino acid biosynthetic genes (Kim *et al*., 2005). However, no direct evidence indicates that Cyc8p and Tup1p function in *MET17* expression. Our results indicated that Tup1p activates *MET17* expression, whereas Cyc8p inhibits this protein's expression. How Tup1p and

Cyc8p regulate *MET17* in yeast colony cells needs to be studied further. It may be that Tup1p interacts with Gcn4p or other factors and, together they enhance *MET17* expression, while Cyc8p may inhibit the interaction between Gcn4p and Tup1p by neutralizing Tup1p molecules, as shown in our proposed model (Nguyen *et al.*, 2018).

Ura2p which catalyzes the first step of UTP synthesis is upregulated when pyrimidine is limited (Broach *et al.*, 2012). Our results indicated that Tup1p negatively regulates Ura2p, whereas Cyc8p positively regulates Ura2p. The function of Tup1p in the regulation of *URA2* possibly via interaction with, and regulation of Ppr1p, a zinc-finger transcription factor. Ppr1p expression is repressed by Tup1p (Patzold *et al.*, 2001). Limiting Tup1p leads to increased *PPR1* expression and Ppr1p activates *URA2* expression and *URA3* expression is regulated in a similar manner (Patzold *et al.*, 2001). On the other hand, limiting Cyc8p results in an increase in free Tup1p (as per our proposed model (Nguyen *et al.*, 2018)) and consequently, Tup1p represses *URA2* expression via inhibition of Ppr1p. The antagonistic functions of the two regulators may extend to other yeast genes and may be due to differences in the regulation of chromatin by Cyc8p and Tup1p (Chen *et al.,* 2013).

*Supplementary data-1*

**Table S1. List of genes encoding proteins that were significantly upregulated in colonies of p$_{GAL}$ -*TUP1* strain versus wild type on GMA**

| 1.5-2 fold (106 or 40% of 265) | 2-4 fold (130 or 49.05% of 265 | >4 fold (29 or 10.94% of 265) |
|---|---|---|
| *YMR31 GCV1 YJL045W LPD1 WTM2 MDJ1 EFM4 MRPL36 SAM50 DNM1 FMP25 RGI1 QCR6 TIM22 RPS0B HSP60 FMP40 MAM33 CYB5 EGD1 MCP2 MCR1 AAP1 MAS1 TFS1 ECM19 KGD2 NGL3 RPE1 ACB1 RPS14B SRP21 HBN1 GTS1 PNC1 TIM44 LCB3 MRPL38 MRPL11 PDB1 YKR070W LCB1 MRPL19 DDR48 HEM14 MRPS8 AIM17 POT1 SRO9 HEM3 MRPL35 PYC2 UTR4 AGX1 FMP32 YPL088W ATP25 SLF1 ELO2 MCY1 MRPL4 YTA12 YKL107W STM1 MRP1 ATP12 GLC3 MGE1 MED11 GAL3 RDL2 GPT2 YHM2 TIM9 RIX1 MRPL40 GRE2 MEF1 IDP1 TIM23 FMP10 OM45 CTP1 PHO86 SCO1 FUB1 MRP7 RRP12 SSA2 GPP2 SIP5 TIM11 CWH43 MRPL17 ATP17 PAA1 HNT1 YLR287C RNR1 WTM1* | *GSY1 IDS2 CYC1 ATP2 SPS100 AHP1 ATP16 CAF20 ATP5 ILV6 DIT2 INO1 ATP6 NUC1 REE1 ACO1 MIC10 RPP1A EMI2 MSC6 GTT1 MRP51 FET3 YMR244W ACH1 RGI2 RPS21B TUF1 RCF2 COQ5 FRD1 SUC2 MRPL49 HXK2 SDH1 FPS1 KGD1 MGM1 KAP120 GUA1 ATP3 DAP1 GRE1 EFM1 THI20 YIH1 SHO1 TRM1 MIR1 UGP1 IDH2 PRX1 ANB1 MDH1 ATG33 PBS2 ATP7 CIT1 GOR1 NDI1 ATP20 FUM1 SDH2 HXK1 FIS1 HSP30 HSP26 PTR2 RNR2 CTR3 HSP10 APT2 HSP150 ATP1 ATP4 ECM15 TOM22 MTC1 YML6 MRPL33 RNR4 SFC1 YML131W SDH3 PIM1 GDH2 PET9 MSY1 ACS2 VAN1 SGA1 IDH1 ZRC1 PSA1 POR1 MRP13 URA2 NDE1 HSP32 MDH3 PRS1 ALD6 ERG4 NDE2 MRPL15 THI6 YIL055C COQ9 TRP1 QCR2 SAM1 MGR2 MRPL24 YOR131C RTK1 IMD3 YHB1 PSP1 MRPL6 YDL218W* | *DSF1YNR071C IMA2 OYE3 FSH1 IMA5 HSP31 ATP15 HEM13 PCK1 FDH1 CRC1 ATP14 ARO9 YJL218W IMA1 MNP1 RAS1 NRE1 TMH11 YLR179C HXT2 GRX5 OYE2 PDR15 MIS1 CYC7 ADH6 GLY1* |

| YJR142W  TOS1  YNL208W MIC60  HEM1 RPL34A | ATP18 SSC1 ADH3 PDE2 COR1 GAR1 QCR8 IDP2 TIM50 CYT1 | |
|---|---|---|

**Table S2. List of genes encoding proteins that were significantly upregulated in colonies of p<sub>GAL</sub> -*CYC8* strain versus wild type on GMA**

| 1.5-2 fold (90 or 37.19% of 242) | 2-4 fold (112 or 46.28% of 242) | >4 fold (40 or 16.53% of 242) |
|---|---|---|
| SOP4    ADH3    YIL055C GPH1 EGD1 FET4 MRPL6 ANB1 ATP7 MNN1 MET10 ECM19 PDE2 TIM50 RDL1 DCI1 CYS4 SDH3 CWH43 YMR244W  KGD1  FMP46 MGR2 MAS1 MGM1 ERG25 HSP60 SHM2 SPE3 IMD3 MRPL19 MDJ1 PIM1 QCR8 MET8 NDI1 CAF20 POR1 PMT6 PDB1 GLY1 MRPL4 MRPL11    CTA1    MPM1 PYC2 WTM1 RDL2 MRP13 ATP1 LPD1 MSC1 ERG4 POS5 MPD1 RSM24 MPC3 DDR48 UBC8 PNC1 MRP1 SND3 COQ6 GPP2 ATP3 MRPL49   MRPS8   CEM1 ATP4 RPS0B RPS0A SDH5 YPR127W   MRPL8   APT2 PIC2 TOM40 IDH1 SAM50 YJR142W   MEF1   FCY1 ATP20 ADE17 IDP1 ZRC1 SRP21 YTA12 GPD1 ACS2 | COQ5  SUC2  THI20  ATP14 HBN1 ACO1 CRC1 INO1 SNZ1 UGP1   MRP51   FPS1   RCF2 AIM17  FET3  ALD6  HSP26 UTR2  MIC10  OM14  GCV3 ELO2 SFC1 GSY2 AAP1 GOR1 YML131W   SPS100   YNL208W TUF1   ATP6   CMC4   EFM1 MRPL33 RPS21B HXK2 HXK1 SAM1  SCO1  YPL113C  CCP1 GRX5   COQ9   REE1   ILV6 YKL107W PHB1 CYC1 ATP2 ADI1 SRO9 MSC6 AFG1 RTK1 AIM45  ACB1  MRPL24  FKS1 ATG33   POT1   SMI1   SDH1 HSP10 OM45 FIS1 PHB2 ECI1 NDE2  NUC1  ALT1  MAL12 YSC83  DAP1  HEM14  MIR1 TFS1 RNR1 SSC1 EUG1 FMP40 ATP15 POL1 RSM23 PEX25 IDH2 RGI2 YOR131C NCP1 MDH3  ATP5  PRX1  GCV2 ACH1 PET9 YDL218W FMP16 MRPL36 YML6 PEX11 RPP1A RGI1 GLC3 DIC1 DIT2 MDH1 TOM22 GPT2 DLD2 MET17 YFR006W ECM15 THI6 RSM19 | DSF1 YNR071C IMA2 NRE1 IMA1 HXT2 HEM13 FSH1 CWP1 HSP31 PDR15 IMA5 YLR179C EMI2 GTT1 YJL218W FDH1 FDH2 PCK1 GSY1 SGA1 CYC7 TMH11 RNR4 FUM1 GCV1 SUL1 MIS1 ADH6 SHO1 HSP30 PTR2 RAS1 FRD1 ARO9 MNP1 AHP1 OYE3 RNR2 OYE2 |

**Table S3. List of genes encoding proteins that were significantly downregulated in colonies of p$_{GAL}$ -*CYC8* strain versus wild type on GMA**

| 1.5-2 fold (166 or 45.35% of 366) | 2-4 fold (169 or 46.17% of 366) | >4 fold (31 or 8.47% of 366) |
|---|---|---|
| THO1 STH1 SMD3 MTR10 ATO3 ARO7 CIA2 YNL194C MAK21 GAD1 PCS60 COX13 SRC1 FOL2 PPN1 PXA1 GPX1 HAM1 RRP4 REB1 HPT1 ADE3 ENT2 HXT6 IST1 SMD2 SER33 CCC1 RPC31 KIN1 DBP1 RIB4 YAR1 NUP157 ERG12 ARD1 PSR1 VIP1 YCR016W BPL1 PHM7 GGA1 PMA2 VID30 HEH2 ICL1 IGO1 GFA1 NSP1 PFK26 RHO2 UBA4 CPR2 NUP170 TUB3 YPL247C MVD1 CTI6 SAM4 YCP4 MLP1 KTR4 CEG1 NUP192 ECM14 SEH1 ROD1 MES1 HEK2 YGR210C SWI3 VAC8 NUP2 SCD6 DUR1,2 CDC55 KTR6 SAC3 DOT5 NCB2 NAT3 GSF2 NMA1 PMA1 NET1 DCP2 EMW1 YMR315W AMD1 OPI10 TRP4 ASK10 VPH1 SOD1 EFG1 FBP26 PIB2 MPD2 COX1 ECM29 SSO2 SIN3 NOT3 NNR2 MET22 ATH1 IGD1 RAD52 YBL029C-A | EGO4 HMG1 YGP1 SIP18 BNA5 ACP1 NHP10 ASN1 PRP22 URA2 DSD1 APE1 YCL019W LDO45 YHI9 PUT4 YGR161C-D NUS1 CAR1 UIP4 TAF12 RSE1 SRX1 HXT5 PHO8 TCD2 CLP1 KNS1 YLL056C GTT3 PAI3 YCL042W TAN1 YJR096W CRP1 GRX6 PAN5 HHO1 YMR114C YLR173W FBP1 DPP1 YGL082W ARG1 BOI2 LSP1 CYS3 MSH2 MRH1 SOK1 GTO1 PXA2 YVC1 BNA3 WHI2 OXP1 RIM15 LEU1 GYL1 GRX8 PUS1 FMP42 VAM7 ENO2 ENP1 NHP6A NPC2 RAS2 BPT1 NAS2 TBF1 WWM1 YGR130C GLO4 LAM1 NUP57 LEU2 ETT1 CBF1 SPC97 RAP1 ARG3 DAP2 YHR112C NSG2 SEA4 NOP9 PUT1 SSS1 PRM15 SHB17 RPD3 YBR085C-A TKL2 BIO3 FCP1 ARG4 AIM29 SEG1 STE20 SSA4 KSS1 SFH5 KRI1 TGL1 | YSP3 GTF1 EGO4 SCW4 GLN1 DSD1 YGR201C SSK1 HIS4 SER3 GLT1 PDR12 ARG1 GDH1 PDC6 ASN1 YLL056C YJR096W ARG3 AVT1 YHR202W YKL091C COX2 ACP1 YHR112C ADH1 GTB1 ARG4 GRX8 NHP10 LDO45 MET3 GND2 CYC8 |

| | | |
|---|---|---|
| MRT4 YDR262W PGK1 SPG4 VAC14 PDC1 YPR089W EXG1 YRA2 GLO3 EMP70 ERG8 CYR1 EAP1 PIL1 NSR1 NAT1 URA6 RFA3 RAI1 MTR4 MYO3 DYS1 PRS5 SUR7 ERG7 HIS7 RHO3 AZF1 CDC42 COX17 GBP2 MCM2 ADD66 YCK1 UGA1 GSH1 TOA1 MGA1 MBF1 IST2 PRE2 VTC3 NPR1 IMO32 HMO1 ABF1 TGL4 PKC1 KRR1 BCH1 USA1 TMT1 LYS9 TAF6 HAP5 TAL1 | ADP1 EIS1 RGT1 VPS5 CHD1 EFM5 YFR016C ARE2 YER010C CIT2 LSB6 NCE102 RTC3 CET1 DIS3 DIP2 NOP12 YTA7 BZZ1 HHF1 CNB1 MSC3 RER1 ZTA1 KIN2 NYV1 BNI4 COX2 UBX5 TUB1 SPT15 HHT1 RNY1 ATG42 RSC8 SEC10 MAF1 DCS2 ERG26 CDC10 GPM2 BFR2 PLN1 SEC3 HIR2 YBR241C GLC8 YNL108C CAB3 CSE1 BNA1 PNS1 SIS1 PAF1 SWI6 ERC1 VID27 YGR201C HIS1 CWH41 RTT102 UBX3 SRP40 VNX1 | |

**Table S4. List of genes encoding proteins that were significantly downregulated in colonies of p<sub>GAL</sub> -*TUP1* strain versus wild type on GMA**

| 1.5-2 fold (167 or 44.89% of 372) | 2-4 fold (172 or 46.23% of 372) | >4 fold (33 or 8.87% of 372 |
|---|---|---|
| TGL1 GTR1 ERV46 NVJ2 YCR016W FAT3 DDP1 SMD3 PMA2 GLC8 APM3 CCC1 BGL2 CSE1 HIS5 COX17 BCH1 HOM3 ARA2 CPR2 RIM15 OPI10 PFK2 YGR266W NSG2 ENA5 RAD52 VIP1 PRE9 MLP1 APE3 HSE1 TRR2 RPB8 NHP6A YFR016C ABF1 CHD1 DCP2 YCL042W ARE2 SFH5 YER079W | FMP42 GAS3 DPP1 APE1 PHO8 YLR257W SSA4 NUS1 MET14 YGR125W BNA3 YGP1 HMG1 SER33 PUT4 GAL1 PAN5 MET1 MET22 HIS1 YBR287W PUT1 CSS1 COX13 BNA1 BLM10 ATO2 RNY1 ADE3 TAN1 COX8 BNA5 YGL082W SOL4 ERC1 PHM7 FMP45 YBR016W ERG1 DUR1,2 ZPR1 YER010C TRX2 NSR1 SEG1 | YSP3 GTF1 EGO4 SCW4 GLN1 DSD1 YGR201C SSK1 HIS4 SER3 GLT1 PDR12 ARG1 GDH1 PDC6 ASN1 YLL056C YJR096W ARG3 AVT1 YHR202W YKL091C COX2 ACP1 YHR112C |

| | | |
|---|---|---|
| CHC1 PRP22 DIS3 CHO2 ENO2 SRP40 EMP47 MET5 AIM29 CAR1 TFG1 BZZ1 SOR2 YBR085C-A PRE3 SPB1 CBF1 PUP2 YGR130C GPX1 NCE102 ADE1 GAS5 NUP57 VPS35 SUM1 ADP1 AAT1 SPT15 TAF6 RTC3 ATG42 YER134C NCB2 ERG26 SHB17 KIN1 VHR1 PAP1 MAK21 SHS1 NET1 CDC10 PUP3 NOP4 SSF1 ADE12 YDR098C-B ALE1 TYS1 YHR138C TUB1 BAT2 PUS1 BRO1 PRP19 TAL1 ERG11 COX12 PKH2 TGL3 NHP6B RTT103 PRS2 PRE8 CTI6 FBP1 YBR137W TFA1 YGR210C DIP2 PFK1 ABD1 PAL2 PXA1 NAS2 PAF1 RIB4 DSE4 DUT1 MPC1 PPN1 NCR1 YVC1 VAC8 APE2 GSH1 LSO2 PGC1 YRA2 SEH1 SSO2 OXP1 YGL242C PGK1 CET1 RIE1 GRX6 DFR1 VPH1 NNR2 CPS1 PGA2 PNS1 CGI121 VID30 SEC12 RTF1 LSB3 RBG1 GPM1 STE20 SCL1 RUD3 DCS2 YDR262W YGR017W TDH1 YMR196W BUD14 CIC1 SOR1 PXP1 ENA2 | GCY1 DAP2 YBL029C-A YNL108C MET17 ARG8 TCD2 HHO1 GTT3 YMR090W TBF1 UIP4 SOD1 RTT102 ERG6 TMT1 NPC2 PLN1 HXT7 YBR241C WHI2 ENT2 BOI2 YHI9 APL5 CRM1 SSS1 YMR114C PRM15 PMA1 LEU2 SRX1 IRC24 YCH1 FCP1 MRH1 YNL194C NOP9 TAF12 CYS3 AIM46 KSS1 URA4 NOP12 NAS6 BPL1 RER1 SEA4 RGT1 RSC3 SMD2 YDR109C PXA2 STR3 YTA7 MAM3 GFA1 BFR2 PAI3 MET7 LAM1 LSB6 YDR415C BPT1 AMS1 EFM5 ECM14 DBP9 ZTA1 WWM1 CLC1 PDR11 YNL115C PRE2 GDH3 BIO5 GPM2 KNS1 CTF4 LOS1 RSC8 CAB4 PCS60 TAF14 ALD5 MLS1 SIP18 BNI4 SCD6 SFP1 ATO3 RAS2 MES1 YGR161C-D HPT1 TAP42 PBP1 LEU1 VTI1 ATH1 LSP1 BIO3 ABP140 GGA1 YSC84 TUB3 TFG2 HAM1 MAG1 XKS1 CRP1 ARO7 HXT6 TRP4 RRP8 GLO4 IST1 YMR295C PRE5 ARG5,6 RPD3 LAS17 EFG1 BNA4 IGO2 CAB3 | ADH1 GTB1 ARG4 GRX8 NHP10 LDO45 MET3 GND2 TUP1 |

## 3.2 Antagonistic regulation of global transcription factors Cyc8p and Tup1p on yeast colony complexity

Most data present in this part, were published in 2018 in *PLOS Genetics* under the title ***"Cyc8p and Tup1p Transcription Regulators Antagonistically Regulate Flo11p Expression and Complexity of Yeast Colony Biofilms"***.

Author designed and performed experiments under Prof. Palkova and Dr. Vachova supervision. Some experiments were carried out in collaboration with other Lab-members: Electron microscopy was carried out by MSc. J. Marsikova, confocal microscopy by Prof. Z. Palkova and Dr. L. Vachova, western blots by Alexandra Pokorná and northern blots by Dr. O. Hlavacek, as detailed in figure legends.

This part contains supplementary data which are available on pages 103 to 106.

### 3.2.1 Cyc8p and Tup1p inversely affect biofilm colony architecture

It is known that wild type *S. cerevisiae* can switch from structured (fluffy) colonies (BR-F) to smooth (BR-S) colonies (Stovicek *et al.,* 2014) with post-switch characteristics resembling those of colonies of laboratory strains. This process of domestication is accompanied by changes in the expression of hundreds of genes, including many with putative roles in colony morphology, such as *FLO11* (Kuthan *et al.,* 2003; Stovicek *et al.,* 2014). These alterations in gene expression may depend on the antagonistic actions of regulators. Global transcriptional regulators, Cyc8p and Tup1p are thought to be involved in *FLO11* regulation (Wilson *et al.,* 1996; Conlan and Tzamarias, 2001; Barrales *et al.,* 2008). Taken together, these findings led us to question whether Cyc8p-Tup1p is involved in the regulation of colony morphology and biofilm development.

A series of strains (listed in Table 2.3), expressing different levels of *CYC8* and *TUP1*, was prepared in the BR-F background (which forms structured biofilm colonies), to determine the effects of these regulators on colony morphology. We successfully constructed strain BR-F/*tup1/tup1* by

deleting both alleles of *TUP1* but could not produce a *cyc8/cyc8* strain as the second allele was resistant to deletion each time (further detail see in supplementary Fig. S1). This indicated that *CYC8* may be an essential gene in the BR-F strain background, as it is in the Σ1278 strain-background that has several similar features with wild yeast strains (Dowell *et al.,* 2010). To overcome the *CYC8* problem, we therefore constructed strain BR-F/*cyc8*/p$_{GAL}$ -*CYC8* with reduced *CYC8* expression by deleting one copy of the *CYC8* gene and expressing the second from the *GAL1*-inducible promoter (p$_{GAL}$), which keeps *CYC8* expression at negligible level compared to the BR-F strain in the absence of galactose as indicated in Fig. S2 and LC-MS/MS results in Fig.3.7B. We also constructed strain BR-F/*CYC8*/p$_{TEF}$ -*CYC8* overexpressing *CYC8* from the *TEF1* promoter (p$_{TEF}$). We then analyzed the development of colonies of these strains and examined colony architecture using two-photon excitation confocal microscopy (2PE-CM).

Surprisingly, although Tup1p and Cyc8p build a repressor complex together, deletion of *TUP1* and reduced *CYC8* expression resulted in completely different colony morphologies. Loss of *TUP1* caused conversion of well-structured, fluffy colonies to smooth ones, similar to those formed when *CYC8* was overexpressed (Fig. 3.3A). This smooth colony morphology is similar to the colony morphology of *S. cerevisiae* laboratory strains and strains lacking *FLO11* (Stovicek *et al.,* 2010). On the other hand, reduced *CYC8* expression in BR-F did not significantly change colony morphology, which gradually exhibited (with prolonged growth) a fluffy colony, similar to the structured architecture of wild type strain biofilm (Fig. 3.3A). Furthermore, as indicated in Fig. 3.3B, 3-day-old p$_{GAL}$-*CYC8* colonies have similar architecture to and resemble, younger (40-h-old) structured biofilms formed by the wild type strain and 5-day-old p$_{GAL}$-*CYC8* colony biofilms exhibit architecture, resembling that of 3-day-old biofilms of the wild type strain (Fig. 3.3B). Moreover, strains with deletion of *TUP1* or reduced *CYC8*

expression led to very poor growth compared to the wild type. Similar results were observed in previous studies (Smith and Johnson, 2000; Chujo *et al.,* 2015).
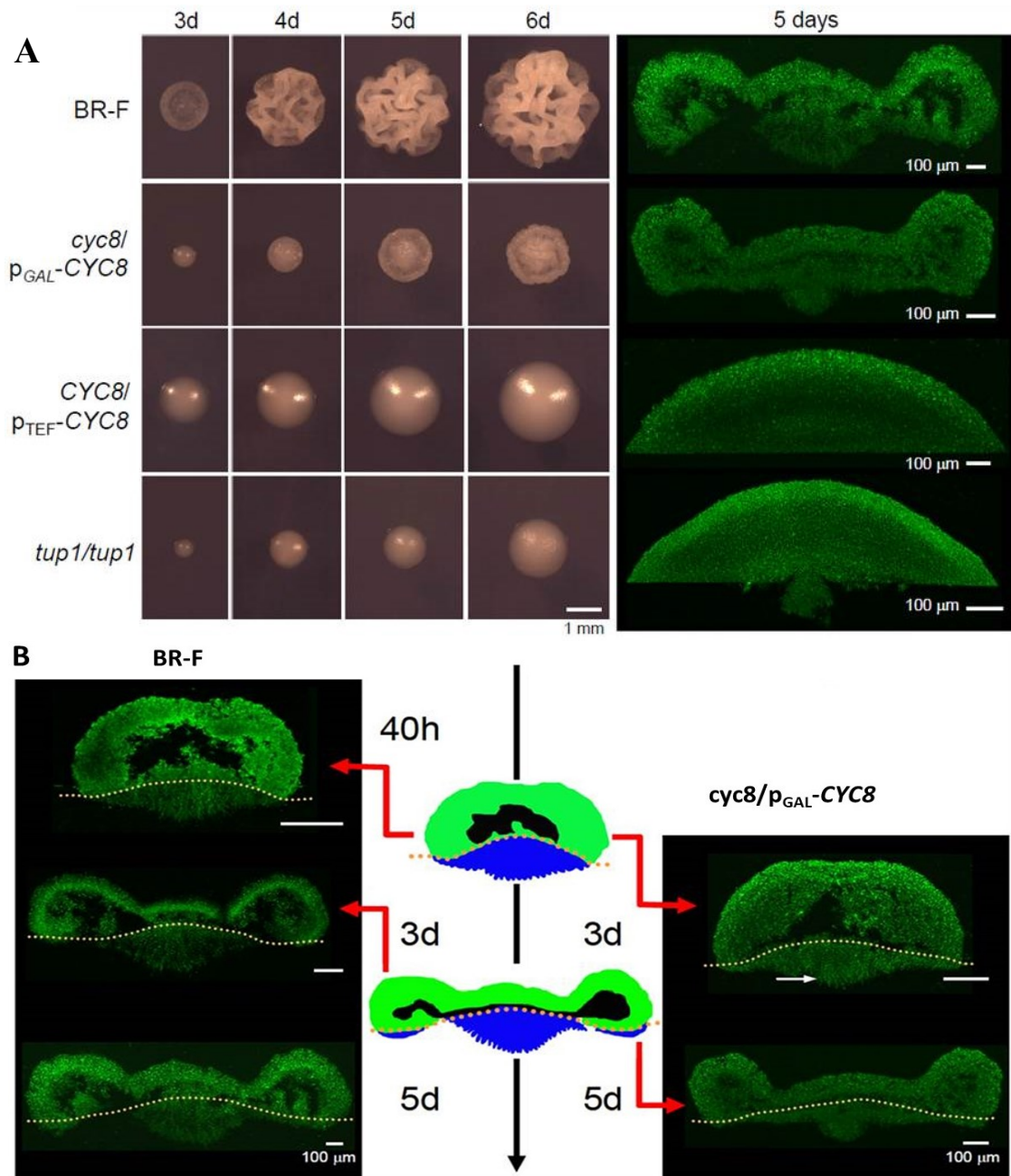


**Figure 3.3. Colony morphology of strains expressing varied levels of Cyc8p and Tup1p.**

*100 µL aliquots of strains BR-F, BR-F/cyc8/p_GAL -CYC8, BR-F/CYC8/p_TEF -CYC8 and BR-F/tup1/tup1 strains at a density of approximate $10^4$ cells/mL were inoculated onto glycerol medium agar. Colonies were grown at 28 °C for the time periods stated. A, comparison of colony morphology of mutant strains with wild*

*type strain, rightmost column is cross-sectioned colonies with calcofluor white (false green color) by 2PE-CM, white bar, 1 mm. B, comparison of biofilm development of BR-F and more slowly growing cyc8/p_GAL-CYC8 colony biofilm, red arrows indicate similar morphologies on different days of development of wild type (BR-F) and mutated (cyc8/p_GAL-CYC8) colony biofilms, white bar, 100 μm. This figure was adapted from figure 1A and S1B in published paper (Nguyen et al., 2018).*

### 3.2.2 Cyc8p and Tup1p conversely regulate the FLO11 expression

There is abundant documented evidence indicating that Flo11p is an essential factor for biofilm formation. Based on the above results, it has been shown that Tup1p promotes fluffy colony morphology, whereas Cyc8p represses the formation of fluffy colonies. Therefore, to clarify the function of Cyc8p and Tup1p in the context of *FLO11* expression during the development of colony morphology, first we carried out an analysis of *FLO11* mRNA expression in reduced *CYC8* or *TUP1* knock-out strains using northern blotting. As expected, the results show that *FLO11* mRNA level is variable and consistent with the colony morphologies. In strains, exhibiting smooth colony morphology (overexpression of *CYC8* or deletion of *TUP1*) *FLO11* mRNA was not detected (Fig. 3.4A, lane 3, 4 respectively), whereas *FLO11* mRNA was strongly expressed in wild type and the reduced Cyc8p strains (Fig. 3.4A, lane 1, 2 respectively). The slightly lower levels of *FLO11* mRNA in the BR-F/*cyc8*/p_GAL-*CYC8* compared with the BR-F colonies was possibly due to the slower growth rate of colonies with reduced levels of Cyc8p as shown in Fig. 3.3B. Thus, these results showed that a higher level of Tup1p correlates with a higher level of *FLO11* mRNA, whereas a higher level of Cyc8p results in a lower level of *FLO11* mRNA.
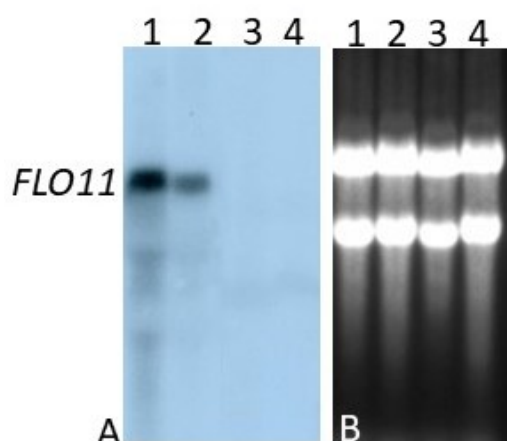
**Figure 3.4. Northern blot analysis of *FLO11* mRNA.**

*A, detection of Flo11 mRNA, lane 1. BR-F; 2. BR-F/cyc8/p_{GAL}- CYC8; 3. BR-F/CYC8/p_{TEF}- CYC8; 4. BR-F/tup1/tup1. B, total RNA loading control stained by Ethidieum bromide, lane number as in panel A. Northern blotting was carried out by Otakar Hlavacek.*

Second, we analyzed the expression of *FLO11* at protein level in the context of colony biofilm morphology of strains derived from the BR-F/Flo11p-GFP (Stovicek *et al.,* 2014), in which Cyc8p and Tup1p production were induced by galactose via p_{GAL} promoter. Because galactose is also a fermentative carbon source, GMA containing galactose may affect colony morphology (Granek and Magwene, 2010). We therefore grew BR-F/Flo11p-GFP/*tup1*/p_{GAL} -*TUP1* and BR-F/Flo11p-GFP/*cyc8*/p_{GAL} -*CYC8* colonies first on GMA plates for 3 days followed by 18 h induction of p_{GAL}-regulated gene expression via addition of galactose to wells in the agar media. The BR-F/Flo11p- GFP/*tup1*/p_{GAL} -*TUP1* strain forms smooth colonies when growing on GMA plates but turned into structured colonies when growing in the presence of galactose (the p_{GAL} inducer) (Fig. 3.5). While the BR-F/Flo11p-GFP/*cyc8*/p_{GAL} -*CYC8* strain forms structured colonies on GMA plates but converts to smooth ones when *CYC8* expression is induced by galactose (Fig. 3.5). Colonies held their original morphologies when located further from the inducer (at the margins of plates) where neither *CYC8* nor *TUP1* was induced. In addition, strain BR-F/Flo11p-GFP was used as control and the morphology did not change after galactose treatment (Fig. 3.5).
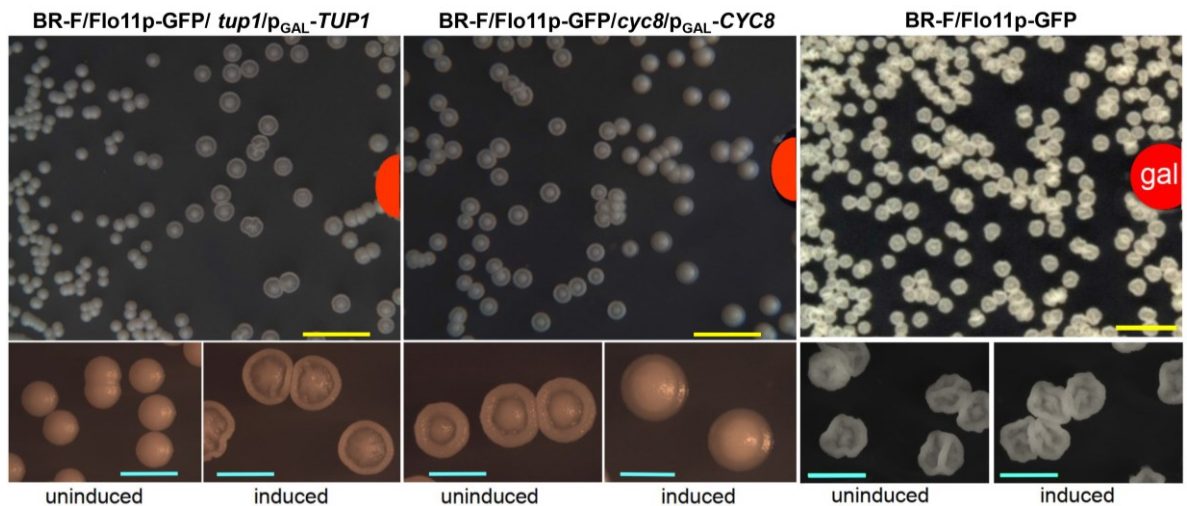
**Figure 3.5. Induction of two transcription factors by galactose resulted in changes in colony morphology.**

*100 μL aliquots of strains BR-F/Flo11p-GFP, BR-F/Flo11p-GFP/cyc8/p$_{GAL}$-CYC8 and BR-F/Flo11p-GFP/tup1/p$_{GAL}$-TUP1 strains at a density of approximate $10^4$ cells/mL were inoculated onto glycerol agar plates for 3 days at 28 °C, following galactose (gal) supplement into a well on the agar plate and incubation for 18 h. Yellow bar, 5 mm; turquoise bar, 2 mm. This figure was adapted from figure 2A and S5 in published paper (Nguyen et al., 2018).*

After 18 h induction, vertical cross-sections of colonies were analyzed by 2PE-CM, in BR-F colonies, cells on the colony surface and cells invading the agar express Flo11p-GFP at a higher level than aerial (upper) cells (Fig. 3.6C). This pattern was also observed in structured BR-F/Flo11p-GFP/ *tup1*/p$_{GAL}$ -*TUP1* colonies close to the inducer source and in structured BR-F/Flo11p-GFP/*cyc8*/p$_{GAL}$ -*CYC8* colonies that are not affected by the inducer (further away from the galactose source) (Fig. 3.6A, B respectively). However, the Flo11p-GFP signal rapidly decreased or even became undetectable in smooth colonies (absence of Tup1p on GMA plates, non-induced Tup1p), or when Cyc8p was overexpressed in the presence of the galactose inducer (Fig. 3.6A, lower part and 6B upper part, respectively).

The fluorescent signal of Flo11p-GFP was consistent with western blot analysis, in which Flo11p-GFP was strongly expressed in the induced Tup1p strain (BR-F/Flo11p-GFP/ *tup1*/p$_{GAL}$ -*TUP1*, lane 4 Fig. 3.6D), whereas it was markedly diminished in the induced Cyc8p (p$_{GAL}$ -*CYC8*) strain close to the

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inducer (Fig. 3.6D, lane 7). In agreement with this, the Flo11p-GFP level when *TUP1* was overexpressed (lane 1) was as high as that of wild type colonies (Fig. 3.6D, lane 2). But Flo11p-GFP was detected neither in colonies constitutively overexpressing *CYC8* (Fig. 3.6D, lane 5) nor in those without induction of Tup1p or deleted for *TUP1* (Fig. 3.6D, lane 3 and lane 8, respectively).
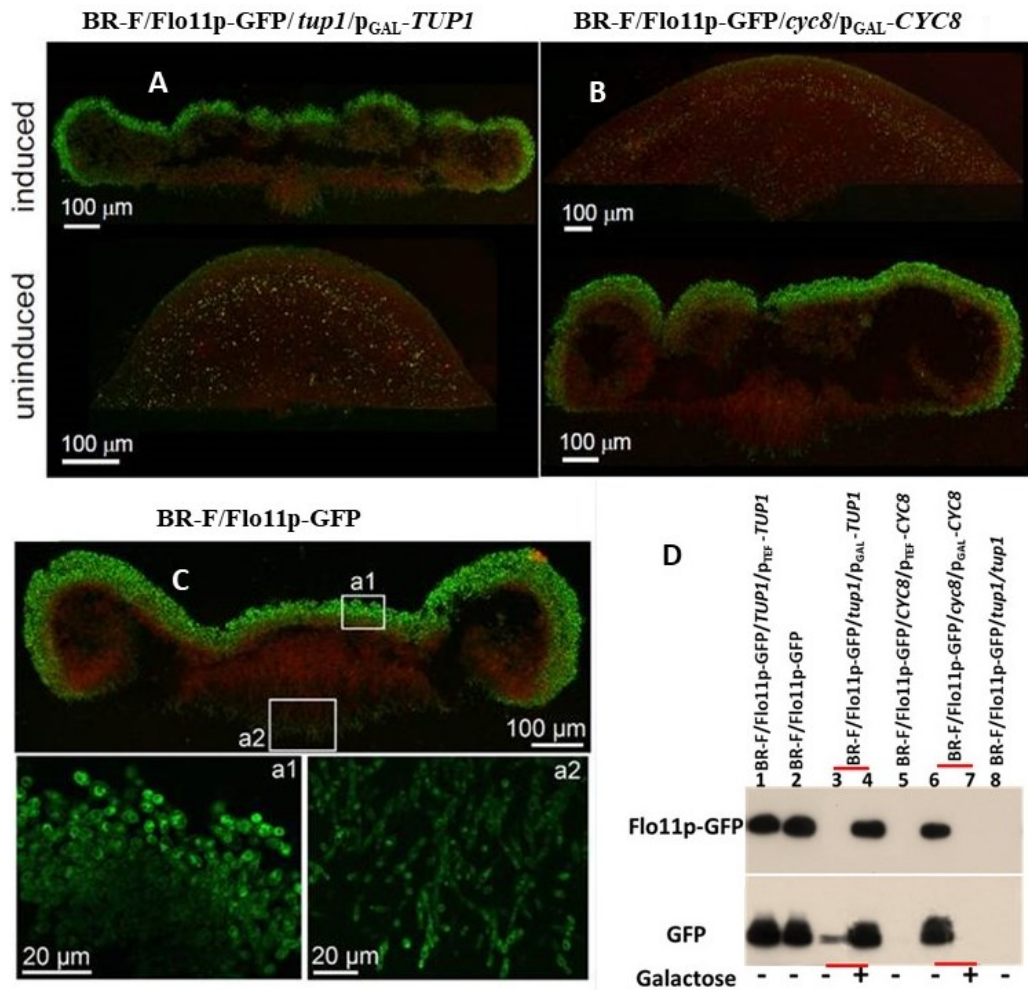


**Figure 3.6. Flo11p-GFP detection by two-photon confocal microscopy (2PE-CM) and western blot analysis.**

*A, B, cross-sectioned colonies from plates with diffusing galactose were analyzed by 2PE-CM. Induced colonies; uninduced colonies of either Tup1p or Cyc8p, respectively. C, 2PE-CM analysis of cross-sectioned colonies of BR-F/Flo11p-GFP grown on GMA for 90 h (3 days + 18 h treatment). Green, Flo11p-GFP; red, cell autofluorescence. D, western blot detection of Flo11p-GFP in colonies grown on GMA for 90 h (3 days + 18 h treatment), loading control in Fig. S6 (A). Following microcolony cultivation with or without galactose by author, 2PE-CM was carried out by Zdena Palkova and Libuse*

### 3.2.3 Mutual effects of Cyc8p and Tup1p on colony development and FLO11 expression

To further identify regulatory functions of Tup1p and Cyc8p, we analyzed the expression of *TUP1* and *CYC8* at both mRNA and protein levels in wild type colonies and colonies of strain constructs, producing different levels of Cyc8p or Tup1p. Colonies of strains: BR-F; BR-F/*Δtup1*; BR-F/*tup1*/p$_{GAL}$ -*TUP1*; BR-F/*cyc8*/p$_{GAL}$ -*CYC8*; BR-F/*CYC8*/p$_{TEF}$ -*CYC8*; BR-F/*TUP1*/p$_{TEF}$ -*TUP1* were grown for 3 days on GMA plates and then induced by galactose (or treated with distilled water). After 4 hours of induction, northern blot was used to analyze mRNA level and results shows in Fig. 3.7A indicated that the induction significantly enhanced the mRNA levels of *TUP1* and *CYC8*, respectively, in p$_{GAL}$-*TUP1* and p$_{GAL}$-*CYC8* strains (Fig. 3.7A, lanes 4 and 6, respectively). On the other hand, the mRNA levels of both *CYC8* and *TUP1* were only slightly raised when expressing the gene from the constitutive p$_{TEF}$ promoter (Fig. 3.7A, lanes 7 and 8, respectively). To quantify expression levels of Tup1p and Cyc8p, we constructed strains expressing Tup1p, and Cyc8p fused with GFP or 6HA. But the GFP or 6HA fusion proteins, of Tup1p and Cyc8p were not fully functioning and the strain required a functional (wild type) allele (as seen in Fig. S3). Primary antibodies, raised against Tup1p and Cyc8p, were also obtained from a commercial company (Abmart, Shanghai, China) but were found to be unsuitable (as shown in Fig. S4). To overcome this problem, we therefore used a label-free LC-MS/MS to quantify Tup1p and Cyc8p in cells from 3-day-old colonies, which were treated (induced) with galactose for 4 hours, or with distilled water (non-induced control: Fig. 3.7B). *TUP1* and *CYC8* mRNA level was increased in a similar pattern when the expression was induced by galactose (Fig. 3.7A, lanes 4 and 6 respectively), while at the protein level there were differences

between Tup1p and Cyc8p induction. Cyc8p was elevated only by 40% (1.4 times), whereas Tup1p was enhanced over 5-fold as compared to wild type colonies (Fig. 3.7B). It is noteworthy that, in the absence of galactose induction, neither Cyc8p nor Tup1p were detected in p$_{GAL}$ -*CYC8* and p$_{GAL}$ -*TUP1* colonies, respectively (Fig. 3.7B). These results are in agreement with those at the mRNA level mentioned above.
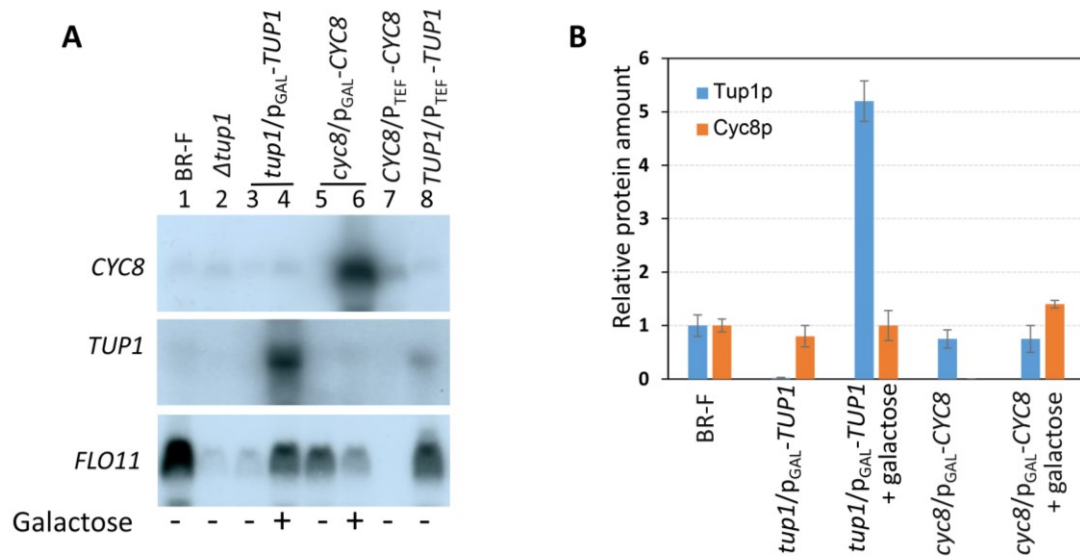


**Figure 3.7. Levels of *CYC8, TUP1* and *FLO11* in strains expressing varied levels of Cyc8p and Tup1p regulators.**

*A, detection of mRNA of CYC8, TUP1 and FLO11 by northen blot, loading control in Fig.S6A; B, quantification of Cyc8p and Tup1p by LC-MS/MS. Following strain preparation, growth and treatment, northern blotting was carried out by Otakar Hlavacek; LC-MS/MS was carried out at the proteomics core facilities, Biocev and initial processing of raw data, and preparation of this graph was carried out by Prof. Zdena Palkova. This figure was adapted from figure 3A, B in published paper (Nguyen et al., 2018).*

Completely different results were obtained when we compared the effects of Cyc8p and Tup1p on *FLO11* expression at both mRNA (Fig. 3.4A and Fig. 3.7A) and protein levels (Fig. 3.6). Overexpression of *CYC8* from the constitutive *TEF* promoter caused completely defective *FLO11* expression at both the mRNA and protein levels (Fig. 3.7A, lane 7 and Fig. 3.6D lane 5). Similar results were observed in *TUP1* deletion strain, in which a small

amount of *FLO11* mRNA was still detectable (Fig. 3.7, lane 2), but Flo11p was not detected (Fig. 3.6D, lane 8). Moreover, the *FLO11* mRNA level strongly decreased after 4 h of galactose induction in the p$_{GAL}$ -*CYC8* strain (Fig. 3.7A, compare lane 5 and 6) but was significantly elevated after 4 h of galactose induction in the p$_{GAL}$ -*TUP1* strain (Fig. 3.7A, compare lane 3 and lane 4). Remarkably, p$_{GAL}$ -*TUP1* colonies did not produce Flo11p, but after 18 h of galactose induction, Flo11p protein levels increased from an undetectable level to reach as high as the level in the wild type strain (Fig. 3.6D, compare lanes 3 and 4) and decreased from wild type-like level to a non-detectable level in induced p$_{GAL}$ -*CYC8* colonies (Fig. 3.6D, compare lanes 6 and 7). In general, the results presented thus far demonstrate that Cyc8p is a repressor of the *FLO11* gene, whereas Tup1p plays a role in the activation of *FLO11* expression.

We next checked further mutual effects of the two regulators. To do this, we generated more strains, derived from BR-F and BR-F/Flo11p-GFP strains, in which the expression of both regulators was induced by either galactose or copper (strain number 12-15 in the list of strains on Table 2.3). In this case, on standard media GMA, colonies exhibited smooth morphology when Cyc8p was expressed from the *CUP1* promoter and *TUP1* from the *GAL1* promoter. However, they exhibit semi fluffy morphology in the case of Tup1p induced by copper (under *CUP1* promoter) and Cyc8p induced by galactose. This likely occurred because of traces of copper in the GMA plates that induced either *TUP1* or *CYC8* expression (Fig. S5). Colonies of strains p$_{GAL}$-*CYC8*/p$_{CUP}$ -*TUP1* (*cyc8*/p$_{GAL}$ -*CYC8*/*tup1*/p$_{CUP}$ -*TUP1*) and p$_{GAL}$ -*TUP1*/p$_{CUP}$ -*CYC8* (*tup1*/p$_{GAL}$ -*TUP1*/*cyc8*/p$_{CUP}$ -*CYC8*) were grown on GMA plates for 3 days. Thereafter, galactose or copper, or both were added to plates and were incubated either for 4 hours for northern blot analysis or 18 hours for western blot. We then collected biomass and evaluated the expression of *FLO11* at both mRNA and protein levels in varying levels of Tup1p and Cyc8p. As shown in lanes 2-9 of Fig. 3.8A, induced expression of *CYC8* and *TUP1*

mRNA resembled that of strains in which only one of these regulators was expressed from an inducible promoter (either *CUP1* or *GAL1*) and the second expressed under the native promoter (Fig. 3.7A, lanes 3-6).
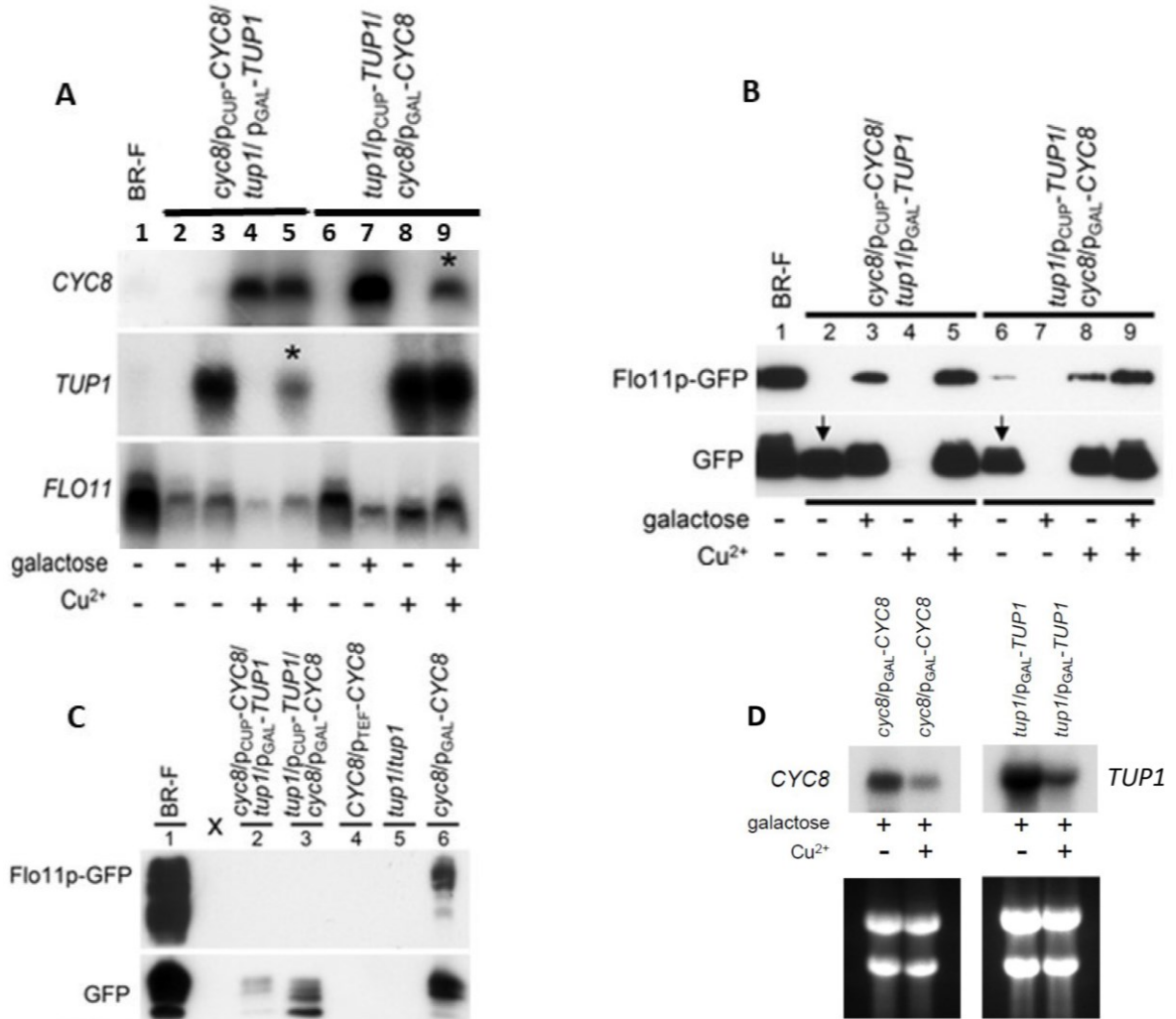


**Figure 3.8. Mutual effect of Cyc8p and Tup1p on Flo11p.**

*100 µL aliquots of BR-F, BR-F/Flo11p-GFP strains and strains expressing different levels of Cyc8p and Tup1p at a density of approximate 10⁴ cells/mL were inoculated on glycerol plates. Colonies were grown at 28 °C for 3 days following 4 h or 18 h treatment with inducer (galactose or copper or both) before RNA and protein extraction. A, northern blot detection of FLO11, CYC8, TUP1 in strains with both regulators expressed from inducible promoters. Asterisks indicate negative effects of copper on p<sub>GAL</sub> promoter transcription. B, western blot detection of Flo11p-GFP in strains both regulators expressed from inducible promoters. Arrows: degradation of Flo11p-GFP to GFP. C, biomass (50 mg wet weight) of colonies grown for 3 days on GMA without galactose or copper induction was harvested and extraction of the extracellular fluid carried out to monitor levels of Flo11p-GFP. D, effect of*

Increased *CYC8* expression resulted in decreased *FLO11* expression at the mRNA level (Fig. 3.8A, lanes 4 and 7) and abolished Flo11p expression at the protein level (Fig. 3.8B, lanes 4 and 7) in $p_{GAL}$ -*CYC8*/$p_{CUP}$ -*TUP1* and $p_{GAL}$ -*TUP1*/$p_{CUP}$ -*CYC8* colonies.

When neither Cyc8p nor Tup1p was induced, a basal level of *FLO11* mRNA was detected (Fig. 3.8A, lanes 2 and 6). This basal level of *FLO11* mRNA was even stronger than under conditions in which Cyc8p expressed under the native promoter and Tup1p was kept at basal level or no Tup1p (*tup1* or $p_{GAL}$ -*TUP1* colonies without galactose, Fig. 3.7A, lanes 2 and 3).

4 h-induction of Tup1p by either copper or galactose did not stimulate *FLO11* mRNA expression (Fig. 3.8A, lanes 3 and 8) beyond a basal level determined when both inducers were absent (Fig. 3.8A lanes 2 and 6). To be specific, this basal level was higher in the $p_{GAL}$ -*CYC8*/$p_{CUP}$ -*TUP1* strain and much lower in the $p_{CUP}$ -*CYC8*/$p_{GAL}$ -*TUP1* strain (Fig. 3.8A, compare lanes 2 and 6), possibly due to the medium containing trace copper (as shown on Fig. S5) which can promote *CYC8* expression, resulting in increased Cyc8p accumulation from the onset of colony growth.

Fig. 3.8A also revealed that mRNA levels of both *TUP1* and *CYC8* under $p_{GAL}$ regulation were partially decreased during simultaneous galactose and copper induction (Fig. 3.8A; compare lanes 3 and 5 for *TUP1* and lanes 7 and 9 for *CYC8*. Reduced level of mRNA is marked by asterisk). The reduction of transcription from the $p_{GAL}$ promoter seems to be partially affected by copper. To check whether copper interferes with $p_{GAL}$ promoter transcription, we added copper to galactose-induced strains, in which *CYC8* is expressed from the $p_{GAL}$ promoter and *TUP1* from its native promoter. We also tested the reverse arrangement, with *TUP1* expressed from the $p_{GAL}$ promoter and *CYC8* from its native promoter. As expected, (Fig. 3.8D) copper

has a negative effect and the expression of either *TUP1* mRNA or *CYC8* mRNA was reduced in the presence of copper (at high concentration; 3 mM) in p$_{GAL}$-*TUP1* and p$_{GAL}$-*CYC8* colonies, respectively.

### 3.2.4 Tup1p appears to inhibit Flo11p degradation

Full-size Flo11p-GFP (approximately 163 kDa) was almost not detected when both regulators were at the basal level, whereas free GFP was strongly detected in these samples which demonstrate that Flo11p-GFP was synthesized at a significant level (in agreement with higher basal *FLO11* mRNA, Fig. 3.8A, lane 2 and 6), but Flo11p-GFP was then effectively degraded (Fig. 3.8B, lanes 2 and 6 - arrows mark free GFP). These findings argue that Tup1p plays dual roles, in terms of regulating Flo11p concentration and biofilm complexity. Tup1p may counteract Cyc8p repression of *FLO11* gene expression and it may also prevent Flo11p degradation, possibly via repression of a gene, encoding a specific protease.

Flo11p is a GPI-anchored cell wall protein of yeast *S. cerevisiae* and can be partially shed from cells to the extracellular space (Karunanithi *et al.,* 2010). We next sought to determine if Flo11p-GFP outside the cell is degraded and whether this degradation is inhibited by Tup1p. Unsurprisingly, Flo11p-GFP and free GFP were not identified in extracellular fluid from colonies of *TUP1* deletion (Δ*tup1*) and *CYC8* overexpression (p$_{TEF}$ -*CYC8*) strains (Fig. 3.8C, lanes 4, 5), in which Cyc8p represses *FLO11* expression. In contrast, in extracellular fluid from wild type and p$_{GAL}$ -*CYC8* colonies (Fig. 3.8C, lanes 1, 6 respectively), high levels of free GFP and partially degraded Flo11p-GFP were identified. This demonstrates that some Flo11p-GFP is degraded, possibly via shedding from the cell surface. Moreover, Flo11p protein was not detected in colonies of either p$_{GAL}$ -*TUP1*/p$_{CUP}$ -*CYC8* or p$_{CUP}$ -*TUP1*/p$_{GAL}$ -*CYC8* strains without any inducers, while high level of only free GFP was detected in extracellular fluid (Fig. 3.8C, lanes 2 and 3). These data demonstrate that Tup1p forestalls extracellular Flo11p-GFP degradation probably by repressing expression of a protease that localizes to the cell wall or is secreted or shed into the extracellular region. At least four dibasic sites

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in the N-terminus of Flo11p, are targeted by proteases, causing differences in Flo11p processing, which were identified in a strain expressing a nonfunctional version of kexin Kex2p (Karunanithi *et al.,* 2010), a serine peptidase that catalyzes cleavage of precursors of secreted proteins in the late compartment trans-Golgi network. On the other hand, Bader and co-workers (2008) did not find Flo11p to be a possible substrate of Kex2p and did not locate predominant Kex2p cleavage site(s) (Lys-Arg at P1 and P2 position) (Bader *et al.,* 2008). Therefore, Flo11p might be an indirect target of Kex2p. The latter may participate in the secretion/processing of another protease that then acts on Flo11p.

### *3.2.5 Cyc8p and Tup1p levels influence length of the fibers connecting the cells within the colonies*

Relatively long fibrous interconnections among cells were observed in biofilm colonies but not in BR-F/*flo11Δ* strain smooth colonies. These fibers have been proposed to be important in formation of biofilm colonies (Vachova *et al.,* 2011). As shown in Fig. 3.9A, fibrous structures were present on surfaces of cells in both structured and smooth colonies and help to connect adjacent cells. However, fibers present on surfaces of cells from smooth $p_{TEF}$-*CYC8*, Δ*tup1* and Δ*flo11* colonies were much (20-30%) shorter and less organized than fibers connecting cells in structured colonies of BR-F and BR-F/*cyc8Δ*/$p_{GAL}$-*CYC8* (non-induced) strains (Fig. 3.9B).

Interestingly, at contact sites among cells in biofilm colonies, fibrous materials were visible with highly regular, Velcro-like structure (Fig. 3.9C-a1), while less structured material was exhibited in cell-cell contact sites in smooth colonies (Fig. 3.9C-d1). Although direct evidence of the existence of Flo11p in these fibers is not yet available, Velcro-like connections may reflect the interaction of N-terminal Flo11A domains of Flo11p as described in (Kraushaar *et al.,* 2015) (Fig. 3.9C, represented by red dots).
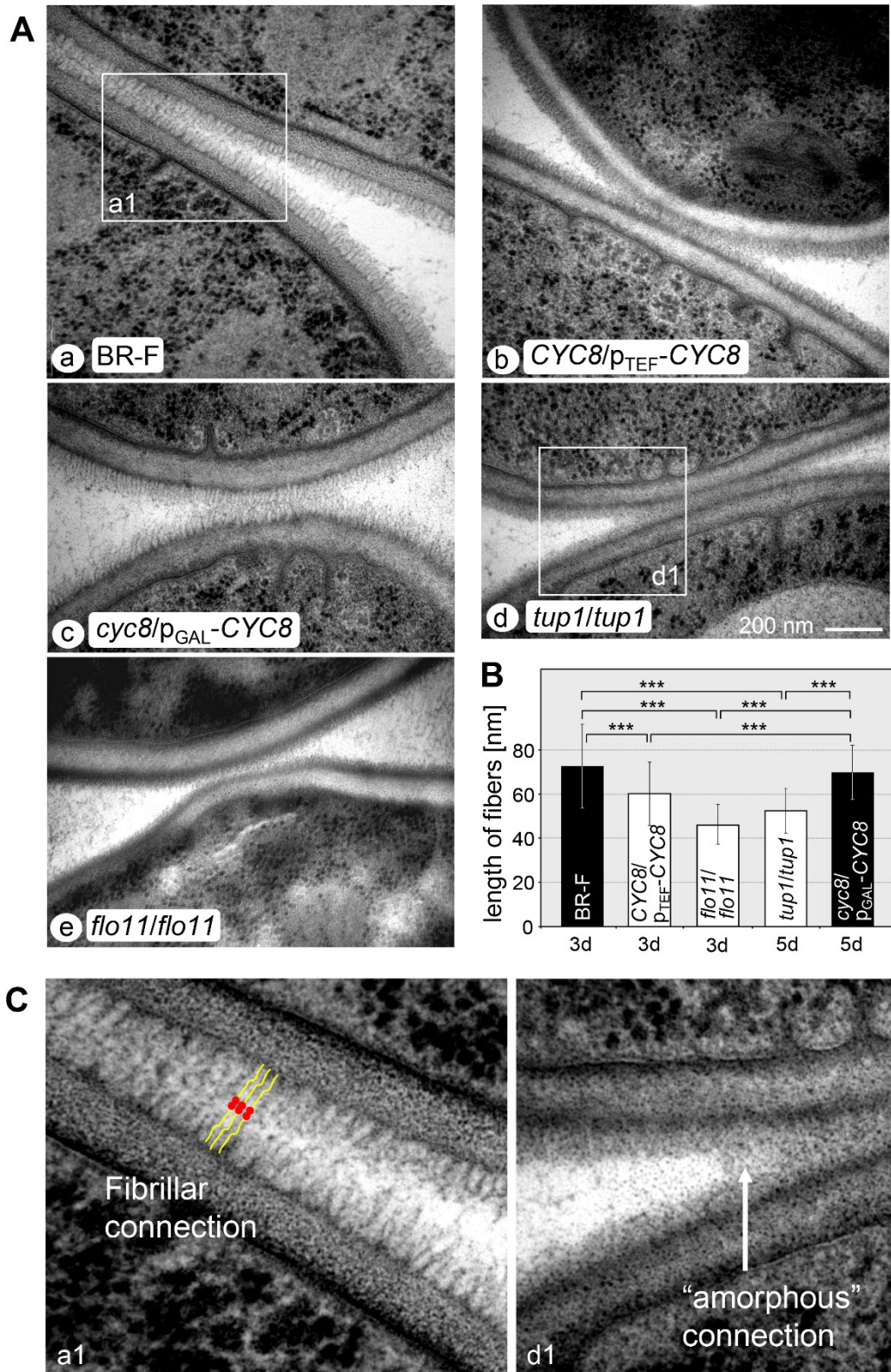
**Figure 3.9. Extracellular fibrous interconnections among cells of colonies formed by strains expressing different levels of Cyc8p and Tup1p.**

*A, fibrous connections between cells under electron microscopy, cells from colonies grown on GMA: 3-day-old (a, b, e) and 5-day-old (c, d). B, graph shows average fiber length. Black columns, structured colony biofilms; white columns, smooth*

*colonies; bars, SD; \*\*\*, P<0.001. C, Examples of extracellular fibrous connections in colony biofilms (BR-F, inset a1 from 3.9A) and smooth colonies (tup1, inset d1 from 3.9D) shown at higher magnification. BR-F fibrous connections shown in yellow, the red circles represent N-terminal Flo11A domains (Kraushaar et al., 2015), potentially associated with the interaction. Figure 3.9 was prepared by Jana Maršíková. This figure was adapted from figure 5 in published paper (Nguyen et al., 2018).*

### 3.2.6 Converse regulation of agar penetration by Cyc8p and Tup1p

Adhesion to solid surfaces and invasive growth are typical features of fungal biofilms as well as of biofilm colonies (Reynolds *et al*., 2001), being evident particularly in the area of colonial roots of wild yeast (Vachova *et al*., 2011). We next examined the agar adhesion capability and invasive growth of strains with altered levels of Cyc8p and Tup1p. Fig. 3.10A showed that smooth colonies of BR-F/*CYC8*/p$_{TEF}$ -*CYC8* or BR-F/*tup1/tup1* strains were severely compromised for invasive growth and poorly adhered to the agar. In contrast, even when robustly washed, the BR-F/cyc8/p$_{GAL}$-*CYC8* strain exhibited strong adhesion to the agar. Similar results were obtained in the BR-F parental strain. Under microscope observation, we found that biofilm colonies of the BR-F strain were built from both elongated and oval cells in the surface regions and by elongated cells (pseudohyphae) in subsurface regions (Fig. 3.10B and Fig. S7). On the other hand, elongated cells were not found in strains forming smooth colonies with negligible levels of Tup1p (Δ*tup1*) and were uncommon when there were increased levels of Cyc8p (p$_{TEF}$-*CYC8*) and in colony-biofilm forming strain (p$_{GAL}$-*CYC8*) with a basal level of Cyc8p. This is consistent with previous findings (Stovicek *et al.,* 2010), which revealed that some wild *S. cerevisiae* strains exhibit structured colony biofilm but could not form typical pseudohyphae consisting of elongated cells.

In C. *albicans,* both Tup1p and Ssn6p (a functional homolog of *S. cerevisiae* Cyc8p) have been identified as repressors of invasive/filamentous growth (Hwang *et al.,* 2003; Garcia-Sanchez *et al.,* 2005; Lee *et al.,* 2015). The present findings suggest that in *S. cerevisiae*, cell filamentation is determined, not by the presence or absence of Cyc8p and Tup1p, but by the balance between these two regulators.
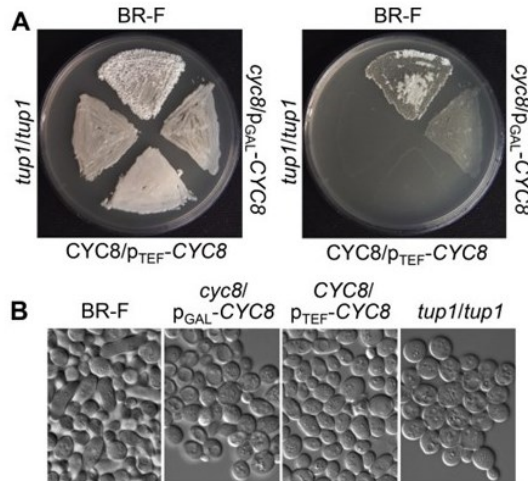
**Figure 3.10. Characteristic of agar penetration and cell morphology of strains in varied levels of Cyc8p and Tup1p.**

*A, invading cells (right) displayed after cells from 3-day-old colonies, grown on GMA agar, were strongly rinsed (left). B, cell morphology from the aerial parts of 3-day-old colonies grown on GMA. This figure was adapted from figure 6A and 6B in published paper (Nguyen et al., 2018).*

### 3.2.7 Cyc8p and Tup1p act in concert to repress flocculation

Cyc8p-Tup1p has been implicated in the repression of flocculation genes and flocculation (Chen *et al*., 2013; Chujo *et al*., 2015). In an effort to verify whether Cyc8p and Tup1p repress flocculation, we used a standard flocculation test (Bester *et al.,* 2006) with some modifications (see method 2.2.8). We found that either deletion of *TUP1* or a significant decrease of *CYC8* expression (p$_{GAL}$ -*CYC8* without galactose) led to appearance of macroscopic flocs (cell clusters), that sedimented efficiently. Similar results were obtained whether expression of *TUP1* or *CYC8* was reduced (p$_{CUP}$ -*CYC8*/p$_{GAL}$ -*TUP1* or p$_{CUP}$ -*TUP1*/p$_{GAL}$ -*CYC8* strains without inducer), indicating that limiting either Tup1p or Cyc8p profoundly de-repressed flocculation genes (Fig. 3.11A and B). In extreme contrast, the wild type strain- BR-F containing native levels of Tup1p and Cyc8p and the *CYC8-* or *TUP1-* overexpressing strains (p$_{TEF}$ -*CYC8* or p$_{TEF}$ -*TUP1* strains) were not able to form cell clusters (Fig. 3.11A). These data above support the hypothesis that Cyc8p and Tup1p together repress flocculation and flocculation related genes.

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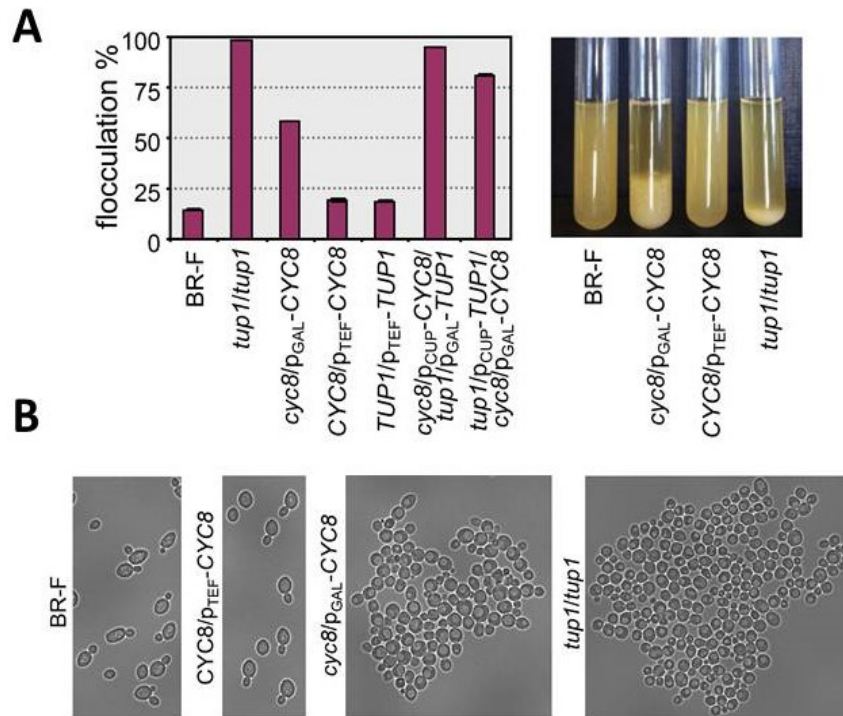
**Figure 3.11. Flocculation and cell morphology in flocs of strains expressing varied levels of Cyc8p and Tup1p.**

*A, flocculation analysis of cell cultures grown in GM for 2 days (graph); examples of flocculation in tubes (right). B, free cells and flocs were observed under microscopy. This figure was adapted from figure 6C and 6D in published paper (Nguyen et al., 2018).*

### 3.2.8 Discussion-2

Wild yeast *S. cerevisiae* is able to form fluffy or biofilm colonies with a high level of structuring and organization due, in part, to the production of extracellular matrix (ECM) and expression of adhesion proteins, especially Flo11p (Vachova *et al.,* 2011). Interestingly, a common phenomenon in pathogenic yeasts is phenotypic switching, which allows yeast cells to efficiently infect the host -i.e. increasing their virulence (Soll *et al.,* 2002). The phenotypic switching is also observed in wild yeast *S. cerevisiae,* which can switch from fluffy colonies to smooth ones under favourable conditions (rich nutrients), turning off ECM production and limiting adhesion protein expression (Flo11p) (Kuthan *et al.,* 2003). In contrast, when nutrients are limited (unfavourable conditions) smooth colonies of yeast cells can convert

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to fluffy ones, recover ECM production and express Flo11p (Stovicek *et al.,* 2014). ECM production and adhesion enhance survival under starvation and during stressful conditions. However, the expression of adhesins and secretion of ECM is costly in terms of resources and unnecessary under favourable conditions, when nutrients are freely available (Stovicek *et al*., 2010; Marsikova *et al*., 2020).

*FLO11* is a central player of phenotypic switching, permitting the yeast *S. cerevisiae* to exhibit many different phenotypic characteristics such as biofilm formation and invasive and filamentous growth. As a consequence, *FLO11* expression is targeted by many transcription factors.

Cyc8p-Tup1p is thought to act as a transcriptional repressor in *S. cerevisiae* for a wide range of genes, including *FLO11*. While Cyc8p has been reported as a repressor of *FLO11* expression (Conlan and Tzamarias, 2001), reports concerning the relationship between Tup1p and *FLO11* expression are not consistent. Both positive (Gromoller and Lehming, 2000; Conlan and Tzamarias, 2001; Barrales *et al.,* 2008) and negative (Barrales *et al.,* 2008) effects have been reported.

We present here an unexpected and important finding that Cyc8p and Tup1p conversely regulate biofilm formation and Flo11p accumulation at different steps in its expression. Deletion of *TUP1* or reduced Tup1p expression diminishes the expression of *FLO11* mRNA (Fig. 3.4A, lane 4 and Fig. 3.7A, lane 2-3) and blocks biofilm formation (Fig.3.3 and Fig. 3.5). Remarkably, the same effect was obtained by overexpression of Cyc8p (Fig. 3.6A and Fig. 3.7A, lane 7). This is in line with the findings of Barrales and co-workers (Barrales *et al.,* 2008) who deleted *TUP1* in the 133d strain resulted in decreasing *FLO11* mRNA expression. Nevertheless, it contrasts with previous reports, suggesting that defects in *TUP1* result in the induction of *FLO11* mRNA (Gromoller and Lehming, 2000; Conlan and Tzamarias, 2001; Fichtner *et al.,* 2007) and the reports of Barrales *et al.,* (2008) whose experiments were carried out in the *L5684* strain.

Conversely, *FLO11* transcripts were detected in the reduced *CYC8* expression strain (Fig. 3.4A, lane 2 and Fig. 3.7A, lane 5), which exhibits biofilm colony (fluffy) architecture similar to the wild type phenotype (Fig.3.3 and Fig. 3.5). matching previous results (Conlan and Tzamarias, 2001). These results are consistent at the protein level, a strong Flo11p-GFP signal was produced in biofilm colonies in line with the high level of Tup1p and low level of Cyc8p, whereas very little Flo11p-GFP signal was detected in high levels of Cyc8p or the absence of Tup1p (Fig. 3.6 and Fig. 3.8B).

Vopalenska and co-workers (2010) showed that Flo11p production is highly dynamic during the development of biofilm colonies, with the Flo11p-GFP signal initially at the bud-neck regions of dividing cells of young biofilm colonies and then spreading to cover the whole cell surface at later stages. When biofilm colonies were fully developed (after 4 days), the Flo11p-GFP signal gradually decreased and the GFP signal relocalized to the vacuole as the GFP was targeted for degradation (Vopalenska *et al.,* 2010). In support of this, our analysis (Fig. 3.8B and C) revealed that the band pattern of Flo11-GFP from cell extracts and extracellular fluid derived from the smooth colonies (deletion of *TUP1*), completely differed from that obtained from fluffy colonies formed by wild type and reduced Cyc8p strains. These findings suggested that Tup1p may have a role in counteracting Flo11p degradation by preventing a specific protease from being expressed or secreted.

Flo11p has been attributed with an important role in building the fiber connections, which helps to strengthen colony architecture (Vachova *et al.,* 2011). In addition, Kraushaar (2015) reported that the N-terminus of Flo11p harbours a fibronectin type III-like domain which mediates interconnections between cells of biofilm colonies (Kraushaar *et al.,* 2015). Our results demonstrated that Flo11p is not essential for fiber connection, since after deletion of *FLO11,* the fiber connections were still present but with a shorter and simpler structure, as formed in strains lacking *TUP1* or with *CYC8* expressed from the constitutively active $p_{TEF}$ promoter. In contrast, strains

with high levels of *TUP1* expression, or expressing *CYC8* at the basal level, exhibited structured biofilm that possessed much longer and more organized fiber connections (Fig. 3.9).

In accord with the above data, regarding the antagonistic effects of Cyc8p and Tup1p on invasive growth regulation, smooth colonies resulting from deletion of *TUP1* or overexpression of *CYC8* (due to low level of *FLO11*) lost the ability to invade agar plates (Fig. 3.10A). This effect is seen upon deletion of several transcription factor genes, including *TUP1* (Barrales *et al.,* 2008), and in *S. cerevisiae flo11Δ* colonies (Piccirillo *et al*., 2010). By contrast, fluffy colonies with the low level of Cyc8p (with high Flo11p) resemble those formed in wild type strains strongly adhering to the agar (Fig. 3.10A). Similar results were obtained in the double deletion mutant strain *nrg1/nrg2* with induction of *FLO11* expression and invasive growth (*Kuchin et al.,* 2002), or in the *FLO11*-overexpression strain that adhered strongly to agar (Van Mulders *et al.,* 2009). In contrast, deletion of *TUP1* in the S288c background resulted in induction of *FLO1* and *FLO11* mRNA expression and strong adhesion to plastic surface and to agar (Fichtner *et al*., 2007). Importantly, invasive growth allows yeast cells to respond to environmental stresses, including glucose depletion, amino acid starvation and the presence of alcohols (Braus *et al*., 2003; Cullen and Sprague, 2000). That may reflect the ability of fluffy strains of the non-mobile wild yeast *S. cerevisiae* to invade and forage for scarce nutrients. In addition, it serves as a foundation for the establishment of a structured colony with primitive multicellular characteristics, including coordinated growth, cell differentiation and division of labour (Marsikova *et al*., 2020). While some cell subtypes secrete ECM, others upregulate multidrug efflux pumps, nutrient transporters and/or adhesins, maximizing colony efficiency (Stovicek *et al*., 2010; Vachova *et al*., 2011).

Importantly, unlike the regulation of *FLO11* expression by Cyc8p and Tup1p, these two transcription repressors together repress genes mediating flocculation. Any mutant affecting formation of the complex, either through reduced expression of Cyc8p or via deletion of *TUP1*/reduced Tup1p

expression, resulted in induction of *FLO* genes expression and promoted flocculation more than five-fold and this flocculation was dependent on the presence of $Ca^{2+}$ (Fig. 3.11A). These results are consistent with previous findings in the literature that mutation of either *CYC8* or *TUP1* causes derepression of *FLO* genes and induces a flocculation phenotype (Patel *et al.,* 2009; Chen *et al.,* 2013; Fleming *et al.,* 2014; Chujo *et al.,* 2015).

Taken together, the results described here support the novel finding that Tup1p and Cyc8p appear to act antagonistically with respect to colony biofilm formation, *FLO11* expression and Flo11p-related events as shown in Fig. 3.12. Tup1p is an indispensable factor for the formation of biofilm colonies, whereas Cyc8p suppresses formation of colony biofilms - overexpression of Cyc8p results in formation of smooth colonies similar in morphology to laboratory strains. Tup1p and Cyc8p also conversely regulate other features typical of the yeast biofilm life-style including invasive growth and cell-cell adhesion by fibrous material.
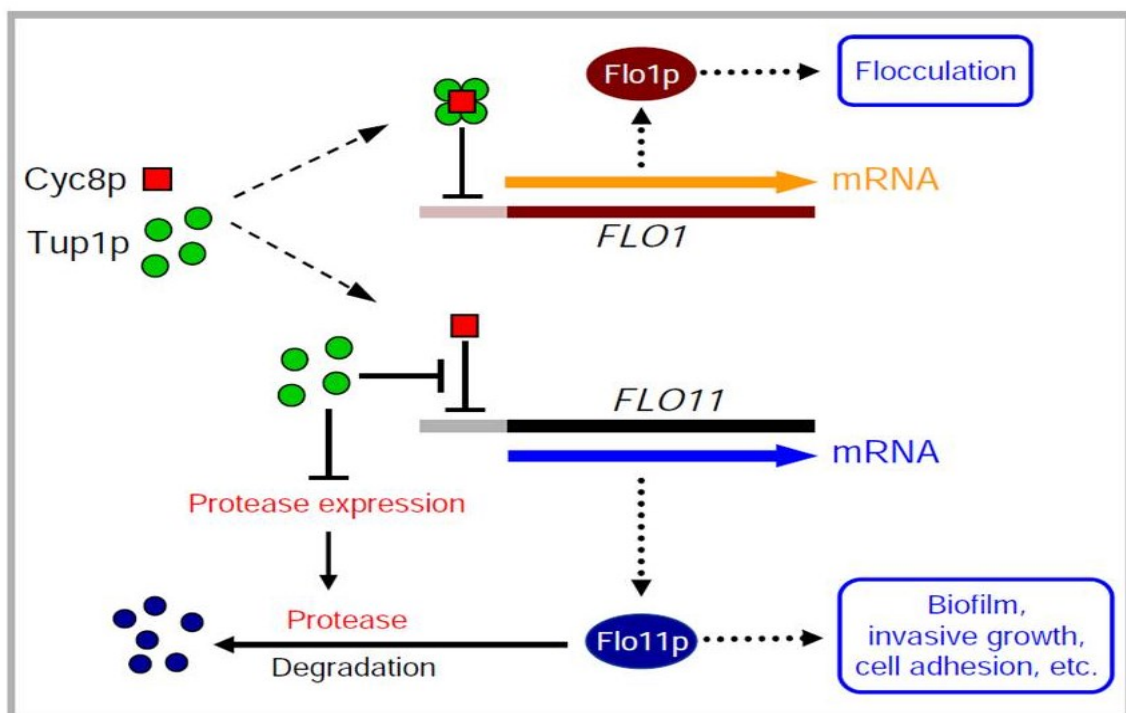


**Figure 3.12. Model schematic of the different functions of Cyc8p and Tup1p in colony biofilm formation.**

*In wild type cells forming colony biofilms, the level of Cyc8p and Tup1p is balanced so*

*that the Cyc8p-Tup1p complex is formed and free Tup1p is present, which contributes to inhibiting Cyc8p repressor and also represses a putative extracellular proteinase that degrades Flo11p. The Cyc8p-Tup1p complex is responsible for repression of other cellular functions, such as cell flocculation. This figure was adapted from figure 4 in published paper (Nguyen et al., 2018).*

Cyc8p itself inhibits transcription of *FLO11*, whereas Tup1p counters Cyc8p function, thus promoting *FLO11* expression. One molecule of Cyc8p in concert with four molecules of Tup1p creates a complex. Hence, the level of free Cyc8p (no interaction with Tup1p) influences *FLO11* repression efficiency. The biological relevance of the balance between these two transcription factors might influence *FLO11* expression and other typical features of colony biofilm. Our data also support the possibility that Tup1p promotes Flo11p accumulation in colony biofilm by inhibiting its degradation. Tup1p may suppress an extracellular protease as its orthologs do in other yeasts such as *C. albicans* and *Aspergillus nidulans* (Naglik *et al.,* 2004; Schachtschabel *et al.,* 2013). Our results were supported by other experiments performed in the BR-S background: the domesticated strain derived from BR-F. Deletion of *TUP1* did not change BR-S colony morphology, whereas keeping *CYC8* expression at basal level converted smooth colonies to semi-fluffy ones (Fig. 3.13A).

Interestingly, the opposing effects of Tup1p and Cyc8p do not extend to other important features such as flocculation and cell morphology (elongation), confirming that biofilm colony formation is different from flocculation, which is repressed by both regulators (Fleming *et al.,* 2014).

In line with this, deletion of genes known for *FLO11* repression, that are involved in mediating the effects of the Cyc8p-Tup1p complex such as *NRG1, MIG1* and *SFL1*(Conlan and Tzamarias, 2001; Govender *et al.,* 2008) did not inhibit the repressive functions of Cyc8p in colony biofilm formation in the p$_{TEF}$-*CYC8* strain (Fig. 3.13B). These results suggest a central role of Cyc8p in repression of *FLO11* expression and biofilm formation.
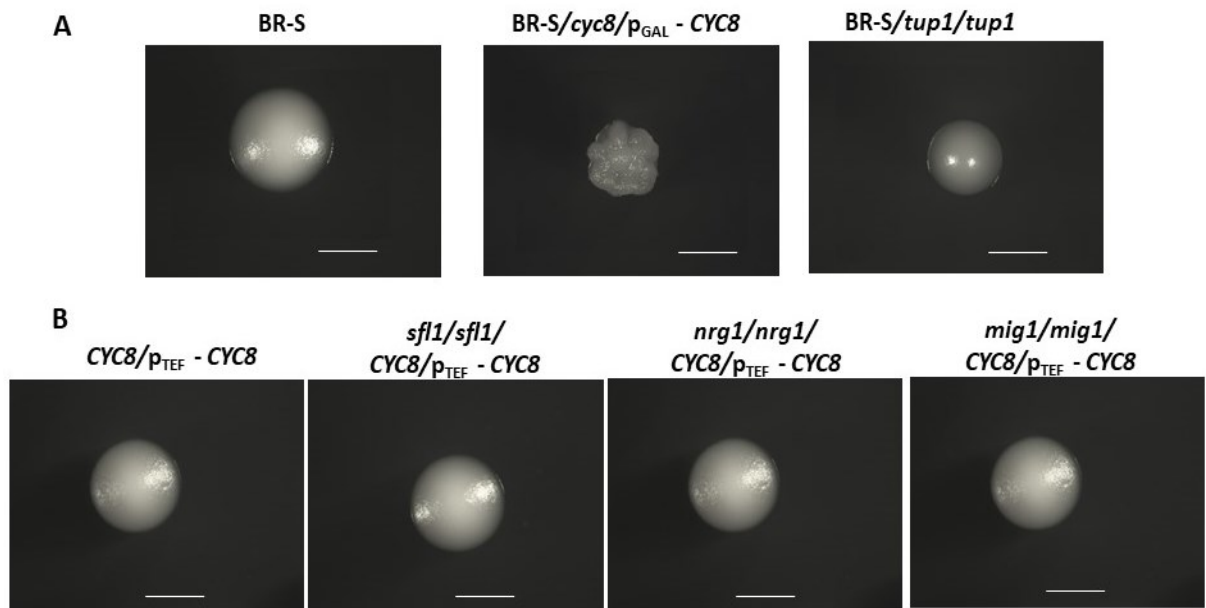
**Figure 3.13. Comparing colony morphology:**

*A, of BR-S, Δtup1 and p$_{GAL}$ -CYC8 strains, B, of p$_{TEF}$ -CYC8 strain and strains with deletions of SFL1, NRG1 and MIG1 genes, respectively, were derived from p$_{TEF}$ - CYC8 strain in BR-F background. All strains were grown on GMA for 4 days. Bar, 1mm. Fig 3.13B was adapted from figure S3 in published paper (Nguyen et al., 2018).*
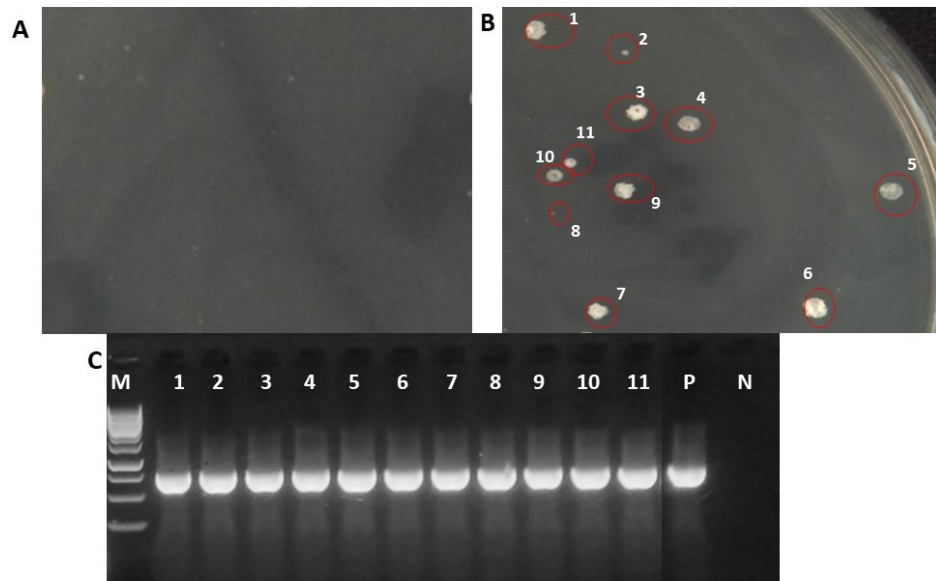
**Figure S1. Results of transformation of *CYC8* cassettes.**

*A, no transformant. B, putative transformant colonies for further verification. C, PCR verification of putative transformant colonies (clones 1 to 11) using primers located in the CYC8 ORF, M is GeneRuler 1 kb DNA Ladder (Thermo Scientific™), 1-11 colonies from number 1 to 11, P is positive control with DNA from wild type, N is negative control without template. CYC8 deletion cassettes were prepared via standard methods (see method 2.2.1) and transformed into the single deletion cyc8Δ/CYC8/BR-F strain, a wild type allele remained in each transformant. A wide range of transformation conditions were tried, different incubation temperatures, various nutrient compositions (e.g. high content of peptone), both fermentative (glucose, fructose and galactose) and respiratory carbon sources (glycerol). Transformation consistently yielded no transformants (Figure S1) or colonies that had retained one or more allele of CYC8 as verified by PCR using primer pairs within the coding region (Figure S1-B,C).*
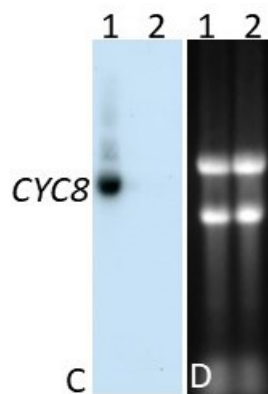


**Figure S2. Northern blot analysis of *CYC8* expression.**
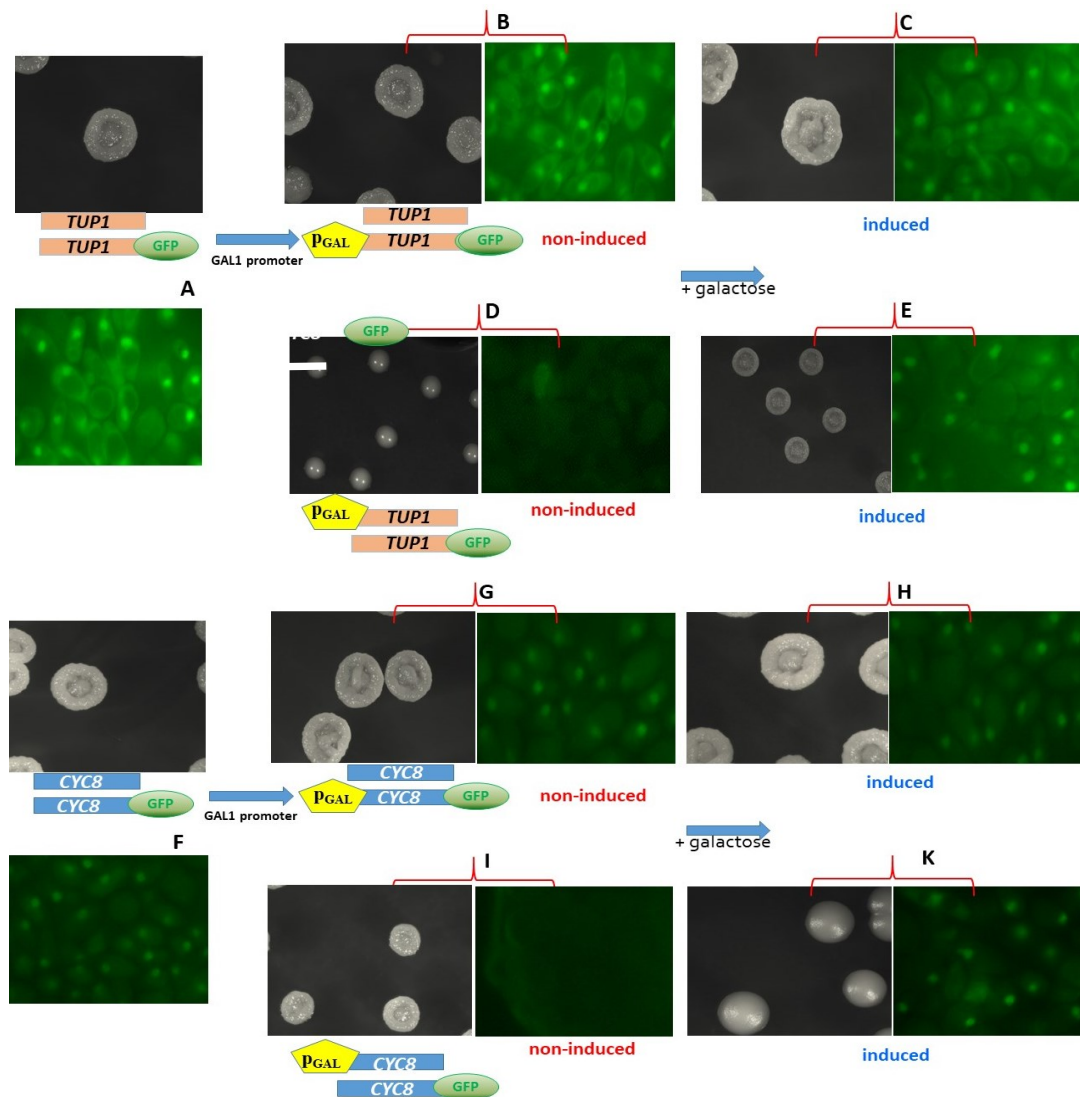
**Figure S3. Localization and validation of function of GFP tag of Cyc8p and Tup1p expressed from the GAL promoter in 3 day old colonies on GM.**

*A-E, Tup1p-GFP; F-K, Cyc8p-GFP. Bar, 10 μm. When Tup1p-GFP or Cyc8p-GFP was expressed from the GAL promoter and the untagged allele from its native promoter, colony morphology remained fluffy and induced colonies showed normal nuclear localization (B and C, G and E). When the untagged allele was expressed from the GAL promoter and the tagged allele from its native promoter, uninduced colonies lost the GFP signal and induced colonies had strong nuclear GFP signals as expected (D and E, I and K). In addition, inducing CYC8 resulted in smooth colony morphology instead of the normal fluffy whereas pGAL-TUP1 colonies produced smooth colonies when not induced but fluffy ones when induced. This is consistent with the theory that Tup1p and Cyc8p regulate colony morphology (via FLO11 expression) antagonistically.*
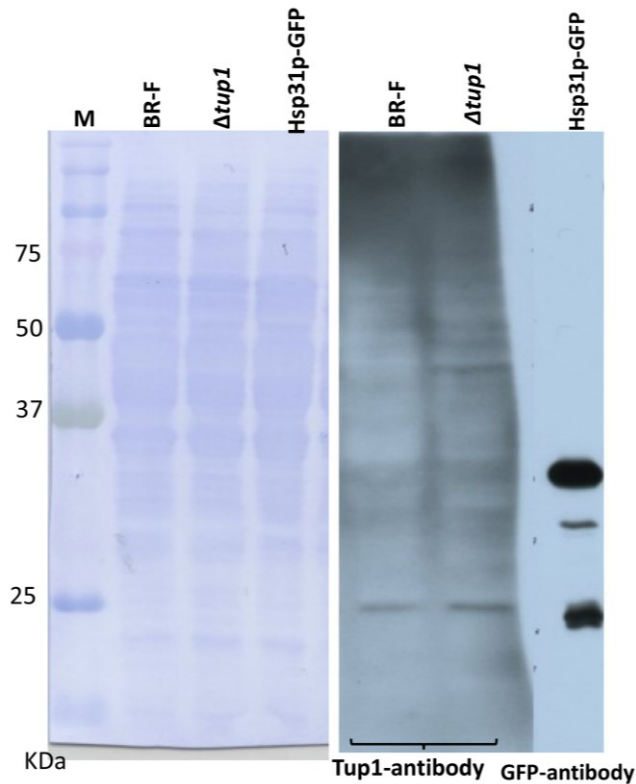
**Figure S4. Detection of Tup1p by commercial antibody.**

*On the left is the Coomassie blue-stained load control, on the right, western blot analysis of Tup1p with Hsp31p-GFP as positive control. Commercial antibodies for detection of Tup1p and Cyc8p were from Abmart (Shanghai, China), all antibodies were diluted and stored as per instructions of the provider. But we were unable to detect the two regulators using these antibodies. Despite all attempts to optimize conditions, the western blot showed very poor results with extremely high background, compared to control detection of GFP (Hsp31p-GFP). Similar results were observed for Cyc8p antibodies (data not shown).*
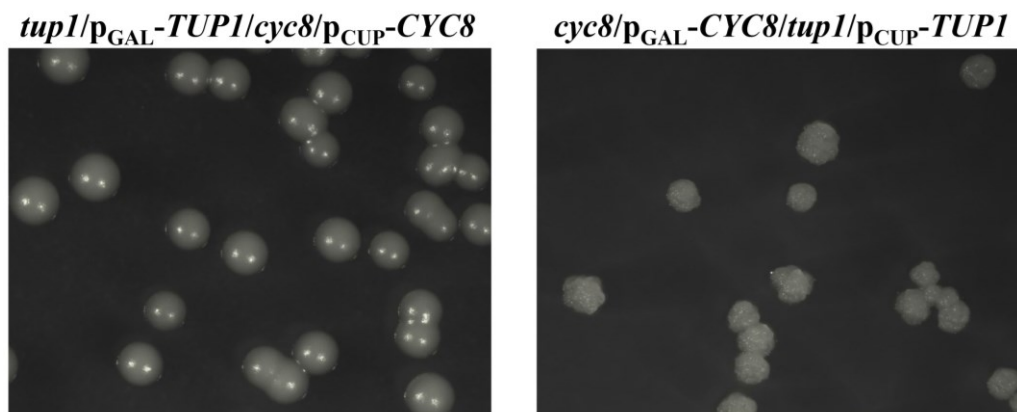


**Figure S5. Trace copper affects colony morphology in GMA plates.**

*Trace copper induces TUP1 or CYC8 expression when either TUP1 or CYC8 are regulated from the CUP promoter. Colonies were grown on GMA for 3 days.*
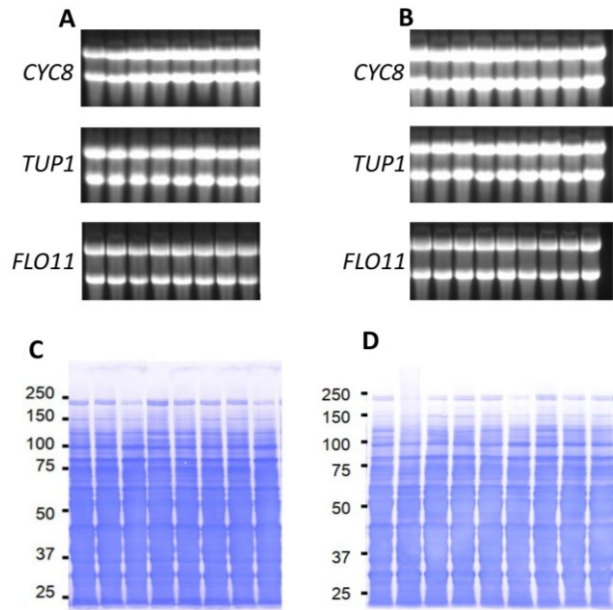
**Figure S6. Loading controls,** *for northern blots in Figure 3.7 (A) and Figure 3.8A (B); for western blots in Figure 3.6D (C) and Figure 3.8B (D).*
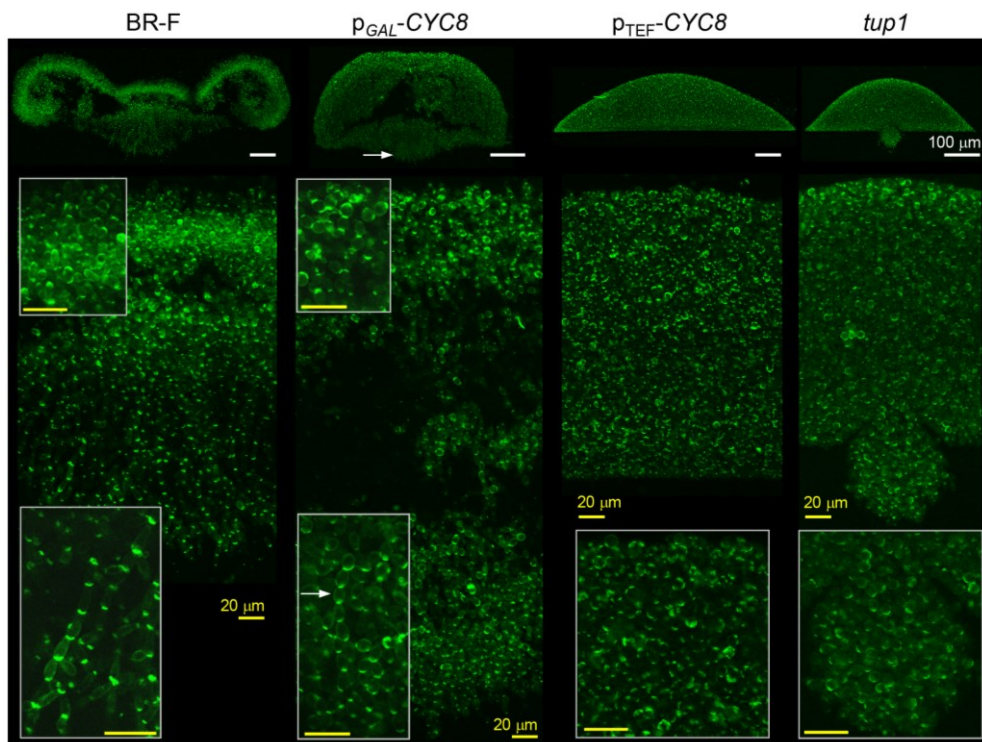


**Figure S7. 2PE-CM of colony cross-sections stained with Calcofluor white (false green color).** *The Upper part, cross-section whole colonies (20x objective); the lower part, observation of central colony parts at higher magnification (63x objective), insets: details of aerial and subsurface cells (strains BR-F and pGAL-CYC8); detail of central part (pTEF-CYC8); detail of the colony bottom (tup1). White bar, 100 μm; yellow bar, 20 μm. Arrow shows chains of rounded cells invading the agar. This figure was adapted from Fig. 1B in published paper (Nguyen et al., 2018).*

### 3.3 Regulation of biofilm formation and dispersal in wild yeast *Saccharomyces cerevisiae* by global transcription factors Cyc8p and Tup1p

Data present in this part, was published in 2020 in *npj Biofilms and Microbiomes* under the title **"*Glucose, Cyc8p and Tup1p regulate biofilm formation and dispersal in wild Saccharomyces cerevisiae*"**

Author contributed to experimental design, performed all experiments and analyzed raw data of all adhesion assays as well as carrying out sample preparation for Fig. 3.19, Fig. 3.20 and Fig. 3.22. Contributed to writing the manuscript.

This part contains supplementary data which are available on page 131.

### 3.3.1 Different *S. cerevisiae* strains exhibit varying adhesion ability to plastic surfaces

To determine factors involved in *S. cerevisiae* adhesion to abiotic surfaces and subsequent formation of biofilm, we first examined adhesion of non-isogenic *S. cerevisiae* strains BY4742 (derivative of S288c laboratory strain), BR-F (wild strain) and its domesticated derivative BR-S (Stovicek *et al.,* 2014) to polystyrene surface, using 96-well polystyrene plates. Attention was focused on this material since it is widely used in everyday life in packaging, for bottles, working surfaces, as well as in industry and medicine, etc. Two culture methods (as described in material and methods 2.2.9) were used for investigating the adhesion ability of these strains.

In the static assay, the cells were cultured directly in wells in microtiter plates for 44 hours, the cell growth rates were determined at time points as shown in Figure 3.14A, by measuring the absorbance of cultures at 600 nm wavelength ($A_{600}$). It was clear that cells grew better in YD media than GM (compare Fig. 3.14B and 14A). Strains bearing either mutated *TUP1* (both $\Delta tup1$ and $p_{GAL}$ -*TUP1*) or $p_{GAL}$ -*CYC8* were accompanied by poor growth, compared to the BR-F parent strain. After 44 hours of growth, non-adhered cells were removed and adhered cells were then stained with crystal violet dye

and absorbance was measured at wavelength 570 nm ($A_{570}$). The results are shown in Fig. 3.14C-D.
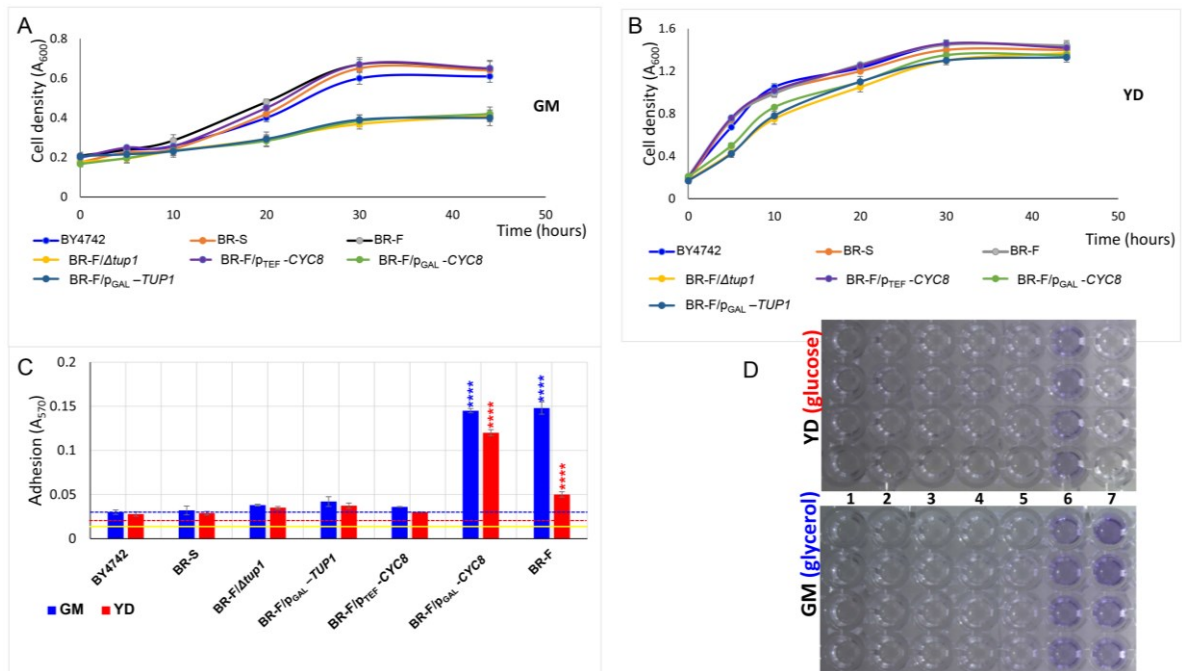


**Figure 3.14. Adhesion of wild, domesticated and laboratory strains and *tup1* and *cyc8* mutant strains to polystyrene.**

*A, growth curve of 44-h static cells in GM; B, growth curve of 44-h static cells in YD; C, Adhesion of cells ($A_{570}$) on polystyrene, normalized to the cell density ($A_{600}$). Blue and red dotted lines demonstrate $A_{570}$ value, which was measured from non-adhesive strain BY4742. Yellow line shows background absorbance (BA). D, crystal violet stained cells adhering to wells, 1-7 named of strains BY4742, BR-S, BR-F/tup1, BR-F/tup1/$p_{GAL}$ -TUP1, BR-F/CYC8/$p_{TEF}$ -CYC8, BR-F/cyc8/$p_{GAL}$ -CYC8 and BR-F, respectively. Experiments were replicated 4 times independently for each strain and condition, with the results displayed as the means and standard deviation (S.D.S). The statistical significance of the variation relative to the non-adhesive BY4742 was determined using an unpaired two-tailed t-test and GraphPad Prism6 software; ****p-value < 0.0001. Fig 3.14 C and D were adapted from figure 1a in published paper (Nguyen et al., 2020).*

In the second approach, after 18 h culture in Erlenmeyer flask, planktonic cells (diluted approx. $A_{600} = 1$) were applied to wells of microtiter plates. The adhered cells ($A_{570}$) were determined after incubating for 3 hours with shaking at 150 rpm at 28 ºC (Fig. 3.15A). Adhesion was normalized to cell density as shown in Fig. 3.15A.
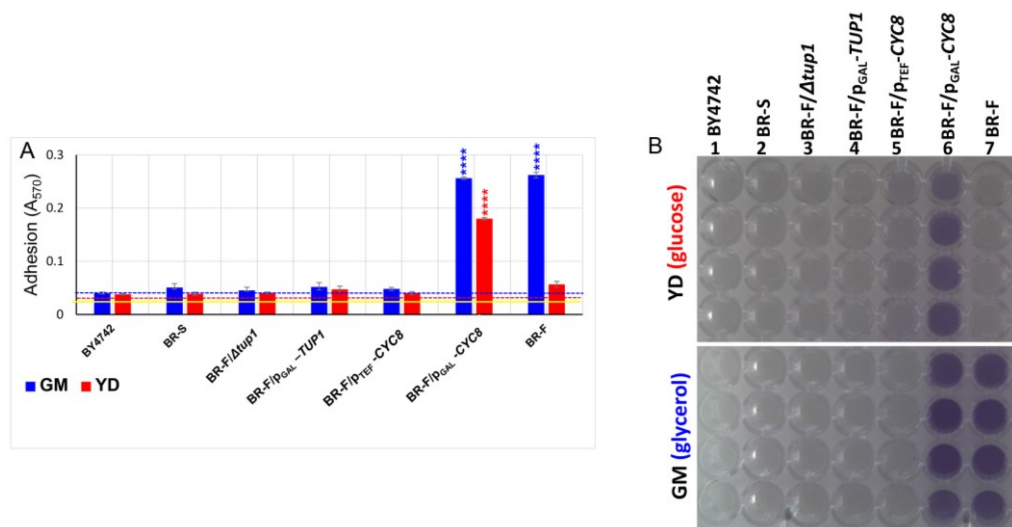
**Figure 3.15. Adehsion capability of wild, domesticated and laboratory strains and *tup1* and *cyc8* mutant strains on polystyrene according to the second protocol.**

*A, adhesion of cells ($A_{570}$) on polystyrene, normalized to the cell density ($A_{600}$). Blue and red dotted lines demonstrate $A_{570}$ value, which was measured from non-adhesive strain BY4742. Yellow line shows background. B, crystal violet assay. Experiments were replicated 4 times independently for each strain and condition with the results displayed as the means and standard deviation (S.D.S). The statistical significance of the variation relative to the non-adhesive BY4742 strain was determined using an unpaired two-tailed t-test and GraphPad Prism6 software; \*\*\*\*p-value < 0.0001. Fig 3.15A was adapted from figure 1b in published paper (Nguyen et al., 2020).*

As shown in Fig. 3.14 and Fig. 3.15 differences in adhesion were observed between strains and cell adhesion depended strongly on strain - only the BR-F strain exhibited a strong adhesion ability, whereas adhesion of BY4742 and the BR-S strains to plastic surfaces of wells (in either of the test conditions) was negligible. The adhesion ability of both static (Fig. 3.14C) and planktonic (Fig. 3.15A) BR-F cells to the plastic surface was stronger in glucose-free (GM) media than in glucose-rich YD media. This is in good agreement with the colorimetric assay (Fig. 3.14D and Fig. 3.15B), which produced only a faint colour in YD and a strong violet colour in GM. The reduced adhesion of BR-F static cells in YD compared to in GM (Fig. 3.14C) could arise from utilization of glucose and thus its reduced level as a result of extended duration of cultivation.

### 3.3.2 Cyc8p and Tup1p regulate adhesion in the opposite manner

Previously, we indicated that Cyc8p and Tup1p antagonistically regulate colony complexity with features typical of the yeast biofilm lifestyle, such as cell invasiveness and cell-cell adhesion via cell wall fibers (Nguyen *et al.,* 2018). More importantly, based on primary results of adhesion ability of strains: those with deletion of *TUP1*, or basal level of *TUP1* expression (BR-F/*tup1*/p$_{GAL}$-*TUP1*), or constitutive overexpression of *CYC8* (BR-F/*CYC8*/p$_{TEF}$-*CYC8*) resulted in only minor adhesion to polystyrene in both GM and YD media based on statistical analysis (Fig. 3.14C and 3.15A) and colorimetric assay (Fig. 3.14D and Fig. 3.15B). In contrast, strain exhibiting reduced *CYC8* (BR-F/*cyc8*/p$_{GAL}$-*CYC8*) expression levels did not alter adhesion of BR-F in GM but significantly enhanced adhesion of both static and planktonic cells in the presence of high-glucose (YD, Fig. 3.14C and Fig. 3.15A). We therefore wondered whether such adhesion is controlled by Cyc8p and Tup1p in a similar manner to the regulation of colony biofilm-specific processes. To test this, we took advantage of two strains BR-F/*tup1*/p$_{GAL}$-*TUP1* and BR-F/*cyc8*/p$_{GAL}$-*CYC8* in which the expression of either Tup1p or Cyc8p is controlled by the p$_{GAL}$ promoter. Yeast cells were cultured in 96 well microtiter plates as static culture with different galactose concentrations (0-1%) for 24 h of shaking incubation (at 150 rpm). Plates were then washed to remove non-adhering yeasts and finally, adhering yeasts were stained with crystal violet and determined using spectrophotometry A$_{570}$.

Along with increasing galactose concentration as a result of the induction of *CYC8* or *TUP1* expression, the growth rate of two strains BR-F/*cyc8*/p$_{GAL}$-*CYC8* and BR-F/*tup1*/p$_{GAL}$ -*TUP1*, was dramatically induced by 0-1% galactose in GM medium (Fig. 3.16A and Fig. 3.16C, respectively). However, the adhesion capability of these two strains differed completely (compare Fig. 3.16B and Fig. 3.16D). Adhesive strength was substantially increased in line with induction of Tup1p as galactose concentration increased from 0-0.1% in GM medium and was constant in a range of 0.1-1% galactose (Fig. 3.16D). Interestingly, inducing Tup1p expression appeared to promote the formation

of colony biofilm (wrinkled biofilm) and can be observed by the naked eye in the presence of 0.05 to 1% galactose (as shown in Fig. 3.16G). In contrast, adhesion of $p_{GAL}$ -*CYC8* was strong in both GM and YD media without galactose, but subsequently decreased when Cyc8p was induced by 0.025-1% galactose in both GM and YD media (Fig. 3.16B) and colony biofilm was not observed (Fig. 3.16G).
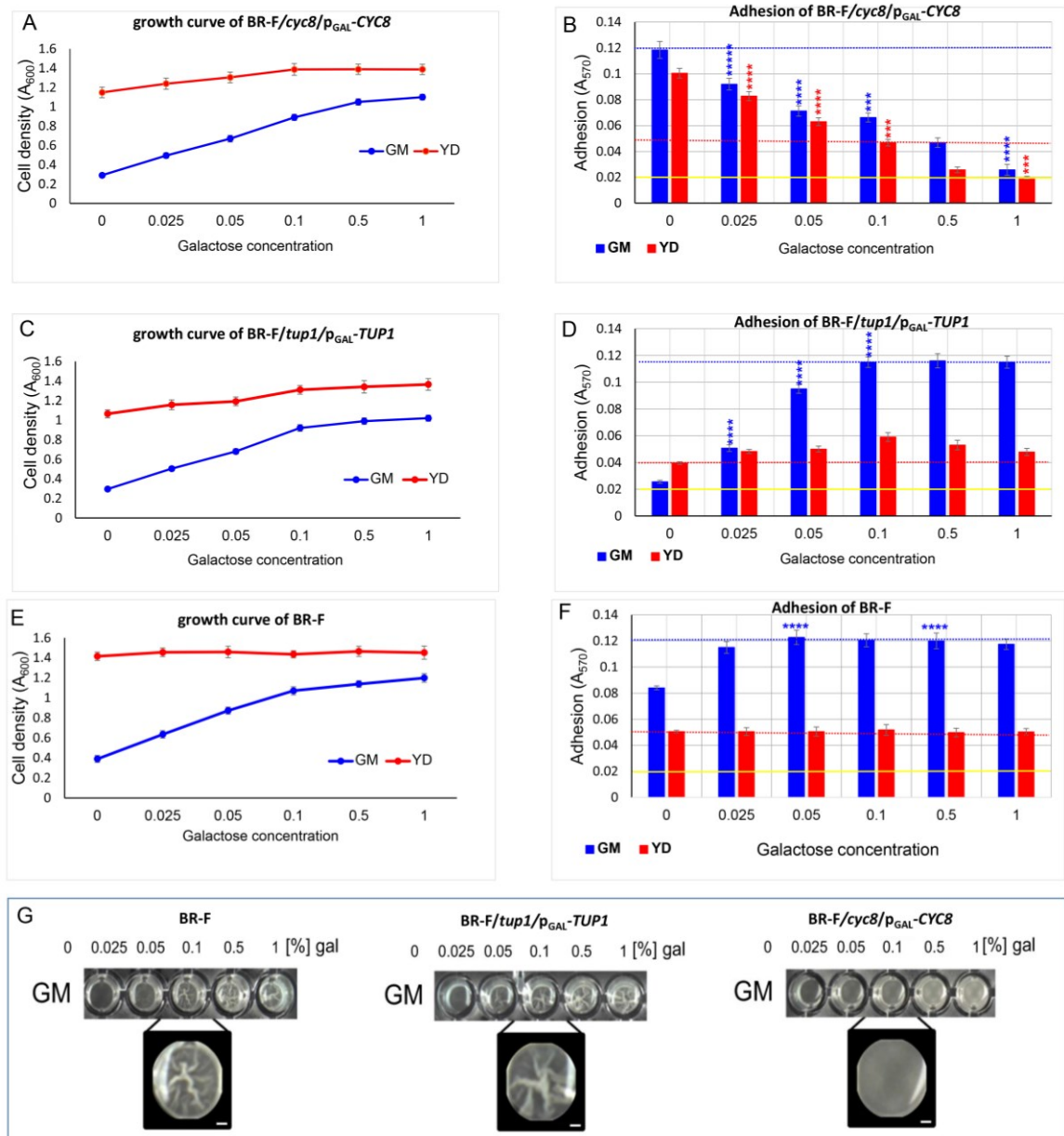


**Figure 3.16. Impact of galactose on adhesion and biofilm formation of static BR-F, BR-F/*tup1*/$p_{GAL}$-*TUP1* and BR-F/*cyc8*/$p_{GAL}$-*CYC8* cells in GM and YD.**

*Adhesion of 24 hour old static cultures in GM (blue bars) or YD (red bars) induced by*

*0-1% galactose. Biomass yield ($A_{600}$) shown as blue curve (in GM) and as red curve (in YD) in the left side:BR-F(A), BR-F/cyc8/$p_{GAL}$ -CYC8 (C), BR-F/tup1/$p_{GAL}$ -TUP1 (E). Maximal adhesion of BR-F (absorbance at $A_{570}$ of crystal violet-stained adhered cells) is represented by blue and red dotted lines, while yellow line demonstrates background absorbance. The formation of solid-liquid interface biofilm by BR-F and galactose-induced BR-F/tup1/$p_{GAL}$ -TUP1 in GM (G). This figure was adapted from figure 2a in published paper (Nguyen et al., 2020).*

Unlike the $p_{GAL}$ -*CYC8* (BR-F/*cyc8*/$p_{GAL}$-*CYC8*) strain, the adhesion capability of BR-F was not influenced (Fig. 3.16F), while that of the $p_{GAL}$ -*TUP1* (BR-F/*tup1*/$p_{GAL}$-*TUP1*) strain was slightly enhanced by galactose in the YD media (Fig. 3.16D). As shown in Fig. 3.16E, BR-F biomass increased with galactose concentration in GM medium. However, adhesion ability of BR-F was moderated in GM without galactose and slightly enhanced by a concentration ranging from 0.025- 0.05% galactose (Fig. 3.16F). This may be due to incomplete coverage of the well surface by cells in poor GM. In the range 0.05-1% of galactose, cell adhesion is uniform and independent of growth rate. The growth and adhesion of BR-F in YD medium were not affected by galactose (Fig. 3.16E-F).

The $p_{GAL}$ promoter is galactose-induced and glucose repressed, so a modest rise in $p_{GAL}$-dependent expression at high (2%) glucose concentration could be expected (Meurer *et al.,* 2017). To elucidate negative effects of glucose on the $p_{GAL}$ promoter in our set up, we therefore used strains BR-F/*tup1*/$p_{CUP}$ -*TUP1* and BR-F/*cyc8*/$p_{CUP}$ -*CYC8*, in which Tup1p and Cyc8p were induced by copper to estimate the correlation of *TUP1* and *CYC8* induction and cell adhesion in YD (glucose media). As shown in Figure 3.17 (red bars) adhesion was dramatically decreased in the $p_{CUP}$ -*CYC8* strain, whereas the BR-F, and $p_{CUP}$ -*TUP1* strains exhibited minor adhesion when induced by copper in presence of glucose. The results therefore were consistent with those obtained by galactose induction in YD, proving that galactose induction may not be strong enough to overcome the glucose repression mechanism. In addition, the adhesion was increased when Tup1p expression was induced and stable in the wild type strain in glucose free-media (Fig. 3.17).
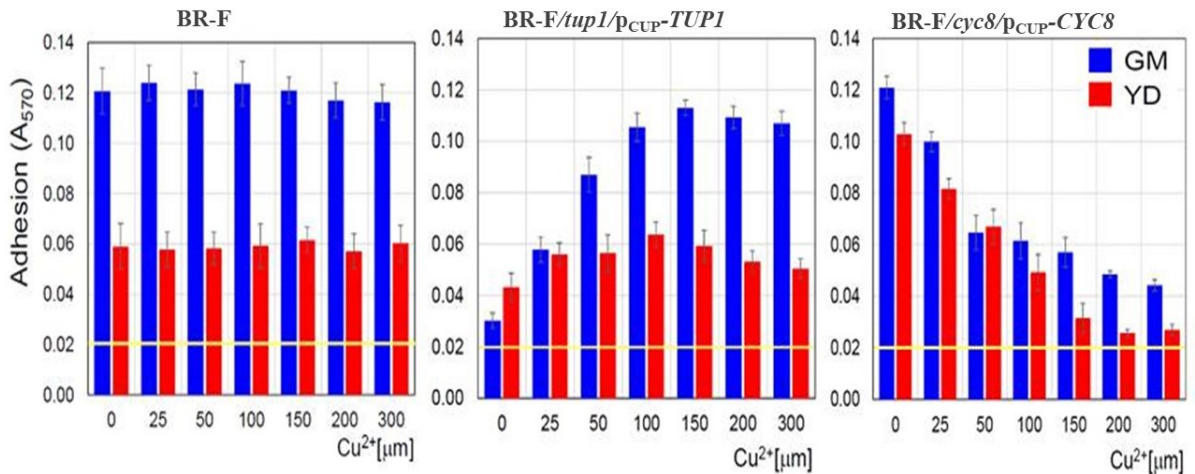
**Figure 3.17. Effect of Copper on adhesion of static BR-F, BR-F/*tup1*/p<sub>CUP</sub>-*TUP1* and BR-F/*cyc8*/p<sub>CUP</sub>-*CYC8* cells in GM and YD.**

*Adhesion was analyzed after 24-h old static cultures in GM (blue bars) or YD (red bars) were treated with 0-300 μM Cu²⁺. Yellow line indicates BA. This figure was adapted from figure S1 in published paper (Nguyen et al., 2020).*

To investigate the mutual effects of Cyc8p and Tup1p on cell adhesion, strains in which both regulators were controlled by $p_{CUP}$ and $p_{GAL}$ inducible promoters, BR-F/*tup1*/$p_{GAL}$-*TUP1*/*cyc8*/$p_{CUP}$-*CYC8* and BR-F/*cyc8*/$p_{GAL}$-*CYC8*/*tup1*/$p_{CUP}$-*TUP1* were cultured in GM and YD medium for 24 hours in 96 well microtiter plates (static cells). It is known that galactose is a fermentable carbon source, which drives rapid growth and replication, but it also inhibits biofilm properties (Granek and Magwene, 2010) at high concentration: consistent with our results, shown above. To minimize the possible negative effects of galactose on cell adhesion, a threshold galactose concentration of 0.1% was selected for subsequent experiments, in order to yield uniform BR-F adhesion, maximal BR-F/*tup1*/$p_{GAL}$-*TUP1* adhesiveness and a fall in BR-F/*cyc8*/$p_{GAL}$- *CYC8* adhesiveness to below the BR-F threshold.

In glucose-free medium (GM), the adhesion of strains in which *CYC8* and *TUP1* were induced by galactose and copper, respectively (or vice-versa) or in which *TUP1* (but not *CYC8*) was induced by either galactose or copper (Cyc8p kept at basal level) (Fig. 3.18A, B) is as pronounced as the adhesion of the BR-F strain (Fig. 3.18C). Conversely, adhesion was totally abolished when Cyc8p was induced by either galactose or copper (Fig. 3.18A-B).

Without induction (no galactose, no copper), adhesion of the two strains greatly diminished. However, it was still greater than that in the presence of high levels of Cyc8p (compare Fig. 3.18A and Fig. 3.18B). In glucose medium (YD), the adhesion of strain BR-F is generally poor/weak (Fig. 3.18C). Similar results were observed when expression of both regulators was either induced (high Cyc8p and Tup1p) or non-induced (low Cyc8p and Tup1p) (Fig. 3.18A and B). Adhesion was thoroughly eradicated when only Cyc8p was induced (high Cyc8p) and Tup1p was kept as basal level (non-induced), whereas when Tup1p was induced (high Tup1p) and Cyc8p was low (non-induced), adhesion increased (Fig. 3.17 and Fig. 3.18). Thus, adhesion was increased in YD when Cyc8p was absent and Tup1p was present.
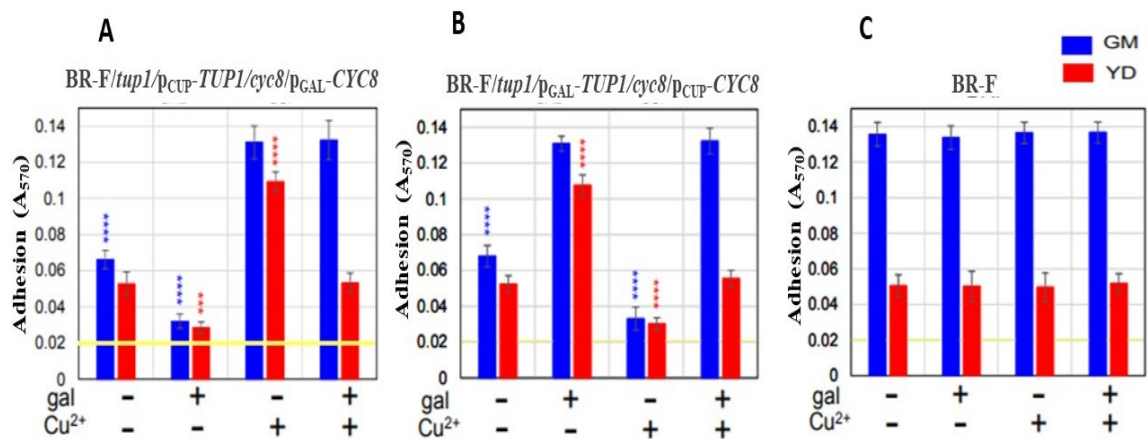


**Figure 3.18. Impacts of copper and galactose on adhesion of static: A, BR-F/*cyc8*/p$_{GAL}$-*CYC8*/*tup1*/p$_{CUP}$-*TUP1*; B, BR-F/*tup1*/p$_{GAL}$-*TUP1*/*cyc8*/p$_{CUP}$-*CYC8*; C, BR-F cells in GM and YD.**

*Adhesion was measured after 24-h static cultures in GM (blue) or YD (red) with or without 0.1% galactose and 300μM Cu$^{2+}$. Yellow line indicates background (BA). This figure was adapted from figure S2 in published paper (Nguyen et al., 2020).*

### 3.3.3 Flo11p is a key player of yeast cell adhesion to plastic surface

Flo11p promotes the formation of fluffy colonies (Stovicek *et al.,* 2010; Vopalenska *et al.,* 2010), pseudohyphal growth (Lo and Dranginis, 1998) and adhesion to agar (Verstrepen *et al.,* 2004; Nguyen *et al.,* 2018). It has also been reported that Flo11p enhances yeast cell adhesion to plastic surfaces or polystyrene since the deletion of *FLO11* in Σ1278b, and *S. cerevisiae var. diastaticus* strains resulted in defective adhesion to polystyrene (Douglas *et*

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*al.*, 2007). We therefore examined the relationship between Flo11p expression and strain adhesion of wild yeast in our set up. The three strains BR-F/Flo11p-GFP, BR-F/Flo11p-GFP/*tup1*/p~GAL~-*TUP1* and BR-F/Flo11p-GFP/*cyc8*/p~GAL~-*CYC8* were grown as static culture in GM or YD with 0.1% galactose or without galactose then cells were harvested to measure the levels of Flo11p-GFP. As shown in Fig. 3.19, Flo11p-GFP expression correlated strictly with adhesiveness, was high in BR-F/Flo11p-GFP grown in GM (whether galactose is present or absent, Fig. 3.19A) and in BR-F/Flo11p-GFP/*cyc8*/p~GAL~-*CYC8* grown in either GM or YD without galactose (*CYC8* expression uninduced, Fig. 3.19C-c1,2). The signal of Flo11p-GFP was also high in BR-F/Flo11p-GFP/*tup1*/p~GAL~-*TUP1* grown in GM with galactose (where *TUP1* expression was induced, Fig. 3.19B-b1). Flo11p-GFP fluorescence was negligible in all other conditions tested. These results demonstrated that Cyc8p and Tup1p oppositely regulate Flo11p-dependent cell-plastic adhesion. Furthermore, Flo11p expression is strongly de-repressed in the presence of high levels of glucose, with the presence of Tup1p but without Cyc8p (Cyc8p expression is kept at the basal level, without galactose), as shown in Fig. 3.19C-c2.
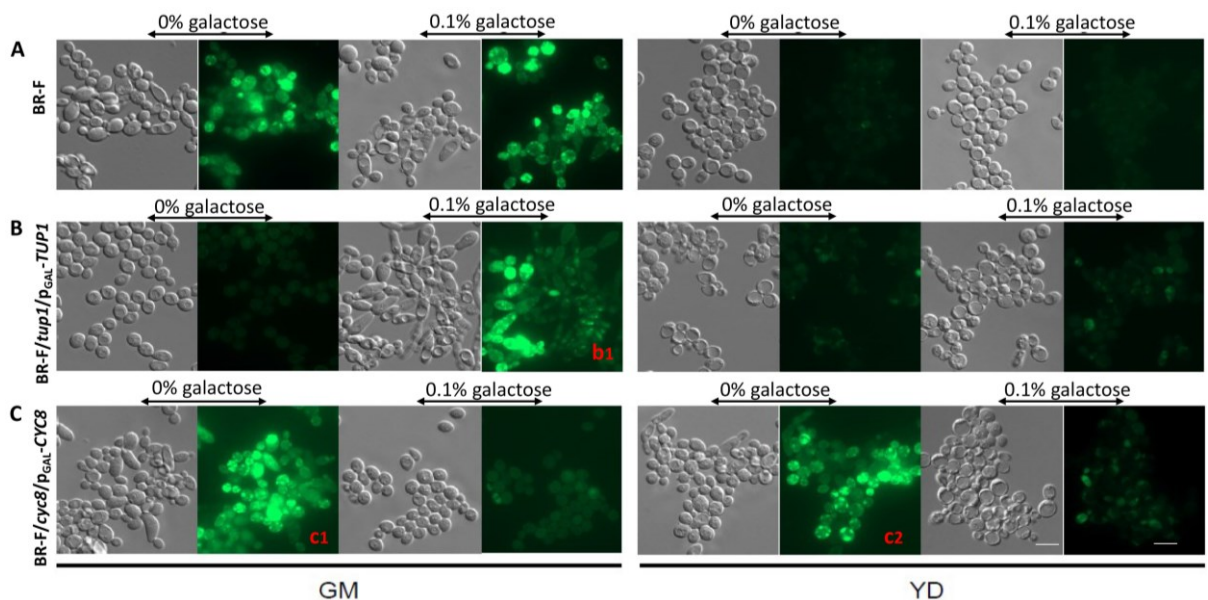


**Figure 3.19. Effect of galactose on Flo11p-GFP production in static BR-F (A), BR-F*/tup1*/p~GAL~-*TUP1* (B) and BR-F/*cyc8*/p~GAL~-*CYC8* (C) cells in GM and YD.**

*Cell morphology and distribution of Flo11p-GFP in cells of relevant strains grown either in GM or YD with/without galactose. Bar, 10 μm. b1 is Flo11p-GFP signal in Tup1p induction (BR-F/tup1/p<sub>GAL</sub>-TUP1) in GM; c1, c2 are Flo11p-GFP in basal Cyc8p (non-induced) in GM and YD, respectively. Author prepared cell culture and induction conditions, following microscopy was performed by Vítězslav Plocek. This figure was adapted from figure 2b in published paper (Nguyen et al., 2020).*

### 3.3.4 Formation of wrinkled solid liquid biofilm was oppositely controlled by Cyc8p and Tup1p

To test whether inverse functions of Cyc8p and Tup1p in the regulation of adhesion, were also implicated in the formation of wrinkled solid-liquid biofilms. Strains of BR-F, BR-F/*tup1*/p$_{GAL}$ -*TUP1* and BR-F/*cyc8*/p$_{GAL}$ -*CYC8* were cultured in GM and YD in the presence and absence of galactose. After 24 and 48 hours, the variation of thickness of biofilms (wrinkled structures) and non-adhered cell layers from strains above was measured. In agreement with cell adhesion results, Tup1p induced, whereas Cyc8p repressed the formation of the three-dimensional structured biofilm on solid-surfaces (Fig. 3.20). Induction of Tup1p (BR-F/*tup1*/p$_{GAL}$ -*TUP1*), BR-F and BR-F/*cyc8*/p$_{GAL}$ -*CYC8* (non-induced) strains in GM (Fig. 3.20A) generated structured biofilms within 24 h with greater thickness than that of biofilm formed by BR-F/*cyc8*/p$_{GAL}$ -*CYC8* (non-induction) in YD (Fig. 3.20B). The result demonstrates that a basal level of *CYC8* expression is enough to enhance adhesion and initiate formation of biofilm in the presence of glucose, but completely structured biofilm development possibly needs other factors that are not present in the presence of high glucose. Extended culture did not greatly affect the thickness of the biofilm since 48-hour old biofilms exhibited thickness, similar to that of 24-hour old biofilm (compare Fig. 3.20 A,B and Fig. 3.20 C,D).
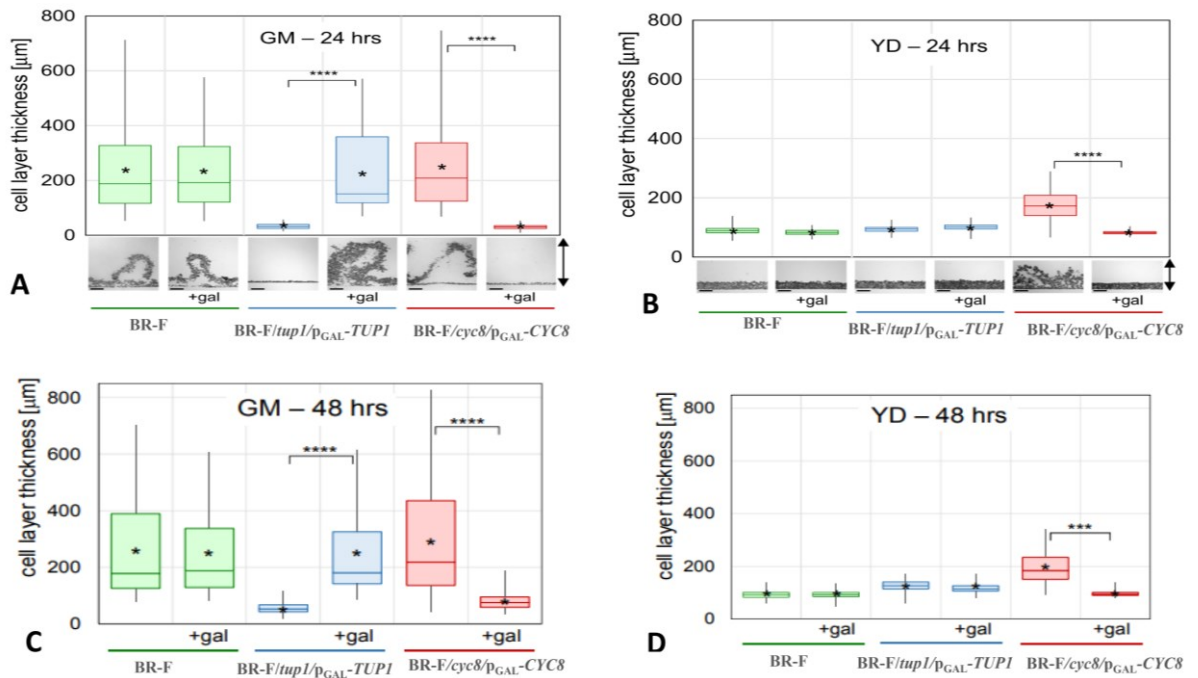
**Figure 3.20. Quantification of thickness of biofilms and non-adhered cell layers from strains:**

*BR-F, BR-F/tup1/p_{GAL} -TUP1 and BR-F/cyc8/p_{GAL} -CYC8 by vertically sectioned 24 and 48 h-old static cultures in GM (A, C) or YD (B, D) in the absence of galactose or treated with 0.1% galactose (gal). The thickness distribution at various positions (deep and shallow wrinkles) within non-adhered cell layers and biofilms was measured and displayed as box plots (box: 25th to 75th percentiles; centerline: median; whiskers: Min to Max; asterisk: mean). Over 200 positions with 3 to 5 different sections of each plot were measured by ImageJ. An unpaired two-tailed t-test and GraphPad Prism6 software were used to identify the probability value of the difference between biofilm formation with and without galactose treatment; \*\*\*\* p-value <0.0001. Vertically cross-sectioned (black arrows) biofilm and non-adhered cell layers are shown as inserts at the bottom. Bar, 100 μm. Data in Figure 3.20 were obtained by Vítězslav Plocek. This figure was adapted from figure 3 and figure S3 in published paper (Nguyen et al., 2020).*

### 3.3.5 Glucose interferes with biofilm formation and releases adhered cells

Glucose has been reported to be involved in biofilm formation and biofilm dispersal (Jackson *et al.,* 2002; Uppuluri *et al.,* 2010). Moreover, our data above also suggested that glucose somehow affect yeast adhesion so it is interesting to clarify whether glucose affects biofilm formation and plastic adhesion in our set up. The BR-F strain first was cultured in 96 well plates as

static cultivation for 44 h, then different amounts of glucose were added either to a fully developed biofilm of the BR-F strain or to adhesive cells after removal of original medium with non-adhesive cells, after incubation for 4 h. As shown in Figure 3.21, biofilm was significantly disrupted and adhesive cells were released by glucose. Even a low concentration of 0.1% glucose subsequently caused adhesion reduction by 15% (treated intact biofilm) and 30% (treated adhered cells) respectively. Almost 70% of adhered cells were released in treatment with 2% glucose, after removal of the medium, (Fig. 3.21). These results might be in line with previous findings that poor nutrient sources and low pH promote biofilm formation in *C. albicans*, while media-rich in nutrients accelerates biofilm dispersal (Uppuluri *et al.,* 2010). In addition, neither galactose nor maltose (as carbon source) triggers biofilm dispersal as effectively as glucose (Blankenship and Mitchell, 2006; Uppuluri *et al.,* 2010).
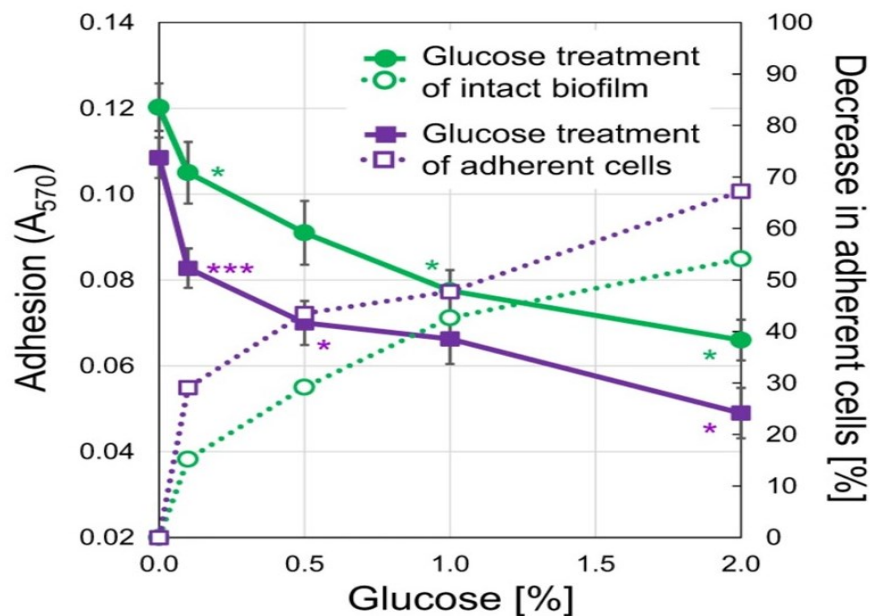


**Figure 3.21. The effect of glucose on biofilm formation and adhesion.**

*The intact biofilm in GM (green) or adhered cells after removal of the medium and treatment with glucose (violet). Solid lines indicate adhered cells post-treatment; dotted lines show reduced adhered cell ratio due to glucose treatment. This figure was adapted from figure 4a in published paper (Nguyen et al., 2020).*

### *3.3.6 Glucose promotes accumulation of Cyc8p in cell nuclei*

Interestingly, the results above indicate that reduction of *CYC8* expression induces cell adhesion and biofilm formation even in the presence of glucose (in YD). To understand the association of glucose with Cyc8p function, we used strains with GFP fused to the C-terminal of either Cyc8p or Tup1p (strain number 21 and 22 in the Table 2.2 list of strains), these strains were then cultured in GM (without glucose) and YD (with glucose) 96 well plates and the levels of two regulators were assayed. After 24 h cultured in microtiter plates, biofilms and non-adhered cells were cross-sectioned and investigated by fluorescence and bright-field microscopy. Cells in non-adhered cell layers in YD (in the presence of glucose) produced significantly higher amount of Cyc8p-GFP than cells in biofilm on GM (free-glucose) (Fig. 3.22). The distribution of Cyc8p-GFP was irregular, being high in the nuclei of surface cell layers, especially in YD, and lower in internal regions, potentially because the glucose is already spent in internal layers where cells are not in direct contact with the medium. Thus, the large difference in the level of Cyc8p between non-adhered cells and biofilms is constrained to surface areas. In contrast to Cyc8p, the level of Tup1p-GFP in biofilms and non-adhered cells were similar (Fig. S8 supplementary).
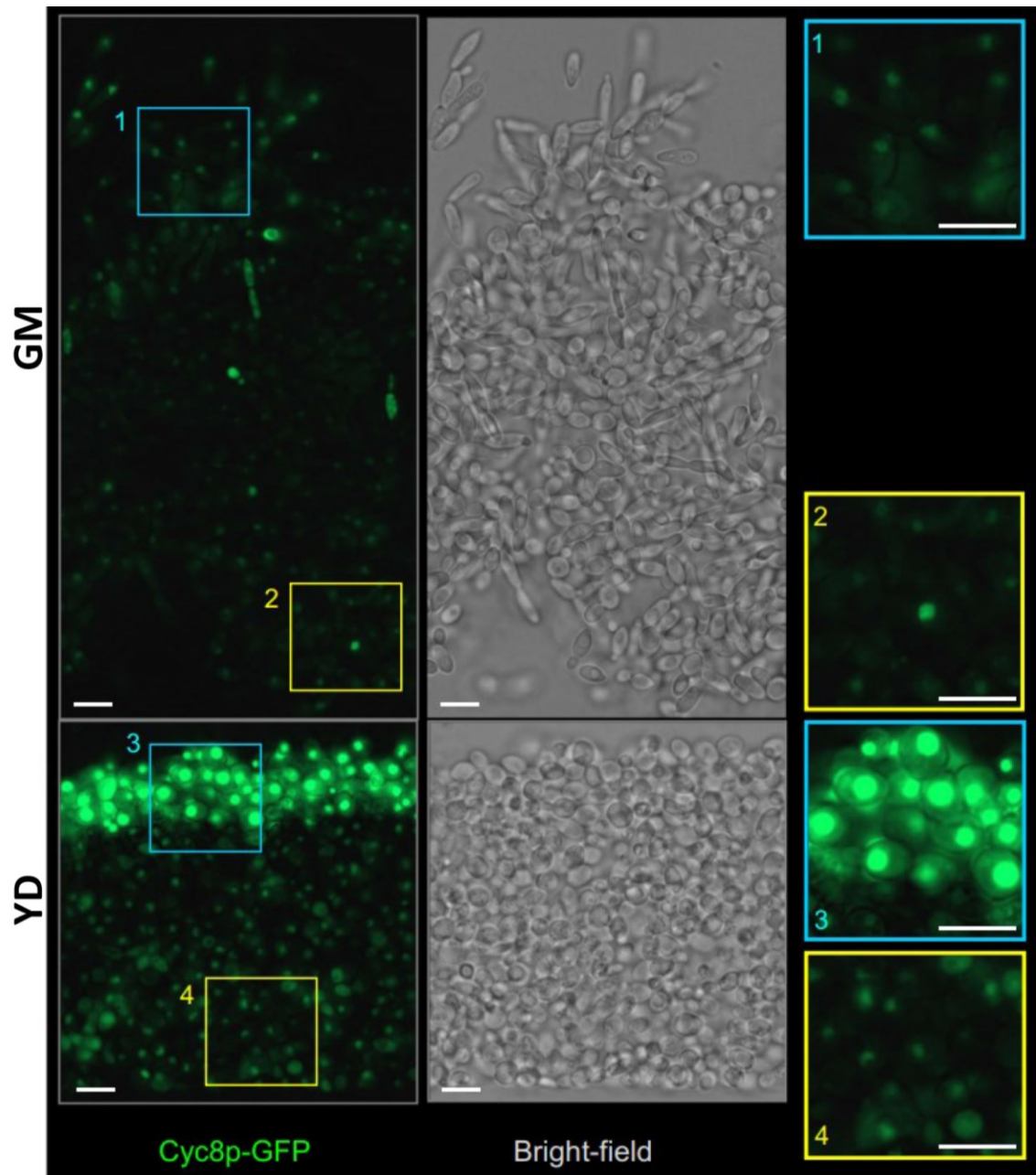
**Figure 3.22. The effect of glucose on Cyc8p-GFP signal from strains cultured in GM or YD medium**.

*Cyc8p-GFP production from 24 h-old static cultures of BR-F/Cyc8p-GFP in glucose-free (GM) and in glucose-rich medium (YD) by vertical cross-sections. Cyc8p-GFP fluorescence is shown in green, cells shown in bright-field. Insets "1-4": cells at higher magnification, showing different intensities of Cyc8p-GFP signal in biofilm and non-adhered cells. Author prepared strains, cultured biofilms and non-adhered cells, following microscopy was performed by Vítězslav Plocek. This figure was adapted from figure 4b in published paper (Nguyen et al., 2020).*

### 3.3.7 Planktonic and static cell adhesion is independent of growth phase under Tup1p and Cyc8p regulation

We wondered if Tup1p and/or Cyc8p dependent adhesion is affected by the growth phase of static and/or planktonic cells. Two assays were set up as per the method description.

First, in the "static" setup, cells of BR-F/$p_{GAL}$ -*TUP1*, BR-F/$p_{GAL}$ -*CYC8* and BR-F strains were cultured in GM or YD in wells of 96 well plates, following which the expression of either *TUP1* or *CYC8* was induced by galactose supplement at certain time points for either 6 h (Fig. 3.23) or 10 h (Fig. 3.24) and adhesion ability was then analyzed. As shown in Fig. 3.23, the growth of BR-F cells was induced when GM medium was supplemented with galactose and cell density ($A_{600}$) reached ~ 0.4 at 17h (Fig. 3.23A) resulted in maximum adhesion ability and this level remained constant, notwithstanding continued growth of the strain (Fig. 3.23D). As expected, BR-F/$p_{GAL}$ -*TUP1* adhesion was absent in GM without galactose (Tup1p was kept at basal level). After 6-hours induction by galactose (Tup1p induced), adhesion increased rapidly but still did not reach BR-F maximal level (compare Fig. 3.23D and Fig.3.23E), even though cell density was similar to, or higher than, that of BR-F ($A_{600} = 0.4$). Similar to BR-F, adhesion of BR-F/$p_{GAL}$-*TUP1* young cells was a little lower than that of older populations. This observation is consistent with previous findings that adhesion of yeast cells from older cultures is significantly stronger than that of cells from younger populations (Bowen *et al.,* 2001; Kregiel *et al.,* 2012). The maximal level of adhesion was observed after 10 hours induction of *TUP1* expression, which is as same as BR-F maximal adhesion (Fig. 3.24E). In accordance, in YD, BR-F cells adhered poorly to polystyrene and independently of galactose treatment (Fig. 3.23D and Fig. 3.24D). BR-F/$p_{GAL}$-*TUP1* adhesion in YD with or without galactose, also displayed basal adhesion (Fig. 3.23E and Fig. 3.24E).
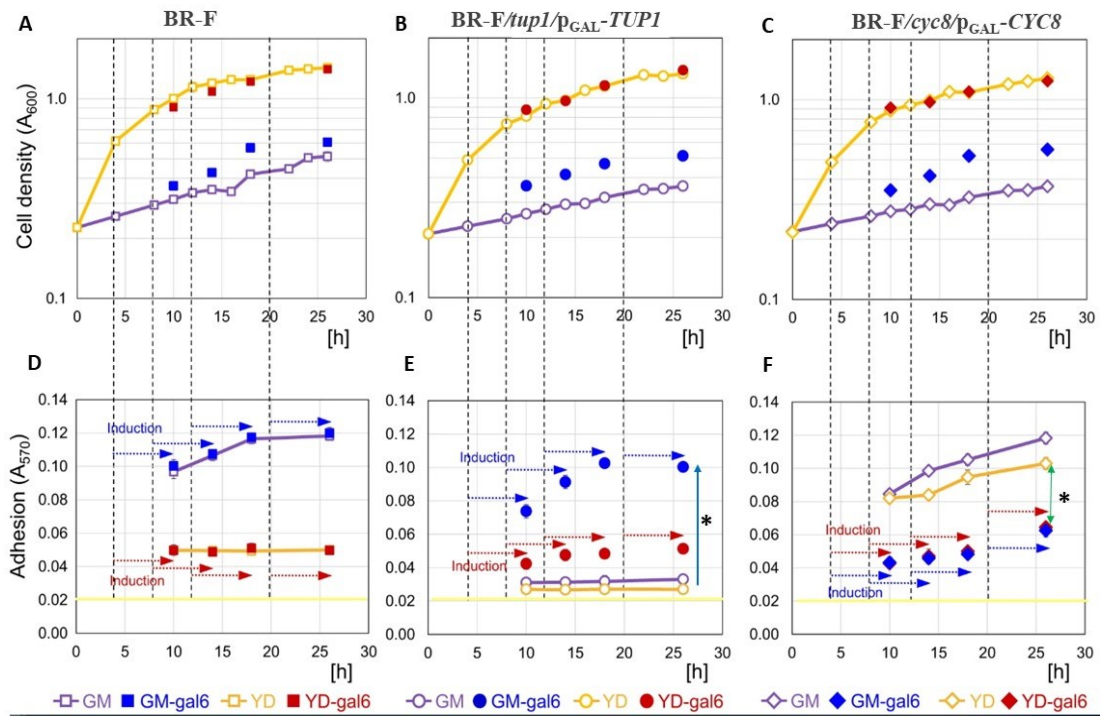
**Figure 3.23. Adhesion capability of cells in static cultures during different growth phases with either Tup1p or Cyc8p induction for 6 hours.**

*A-C, dynamic growth curves of strains in GM and YD. Media was supplemented with galactose for 6 h before measurement of the biomass ($A_{600}$). D-F, adhesion capability of strains without induction and with induction for 6 h (with galactose). Arrows (D-F) demonstrate interval of galactose treatment relative to time points at which cell adhesion was identified. Background absorbance (BA) is shown by yellow line. Arrow marked with asterisks: change in adhesion. Results shown are the mean and s.d.s from quadruplicate experiments. This figure was adapted from figure 5 in published paper (Nguyen et al., 2020).*

In contrast, without galactose, BR-F/$p_{GAL}$ -*CYC8* cells exhibit strong adhesion to polystyrene similar to that of BR-F cells in GM. Subsequently, adhesion dramatically diminished almost independently of induction period of galactose treatment (Fig. 3.23F and Fig. 3.24F, green arrow, marked with asterisk). Interestingly, adhesion efficiency of BR-F/$p_{GAL}$ -*CYC8* in YD is similar to that in GM. Moreover, a similar pattern of adhesion reduction was also observed in YD and GM when *CYC8* expression was induced by galactose (Fig. 3.23F and Fig. 3.24F).
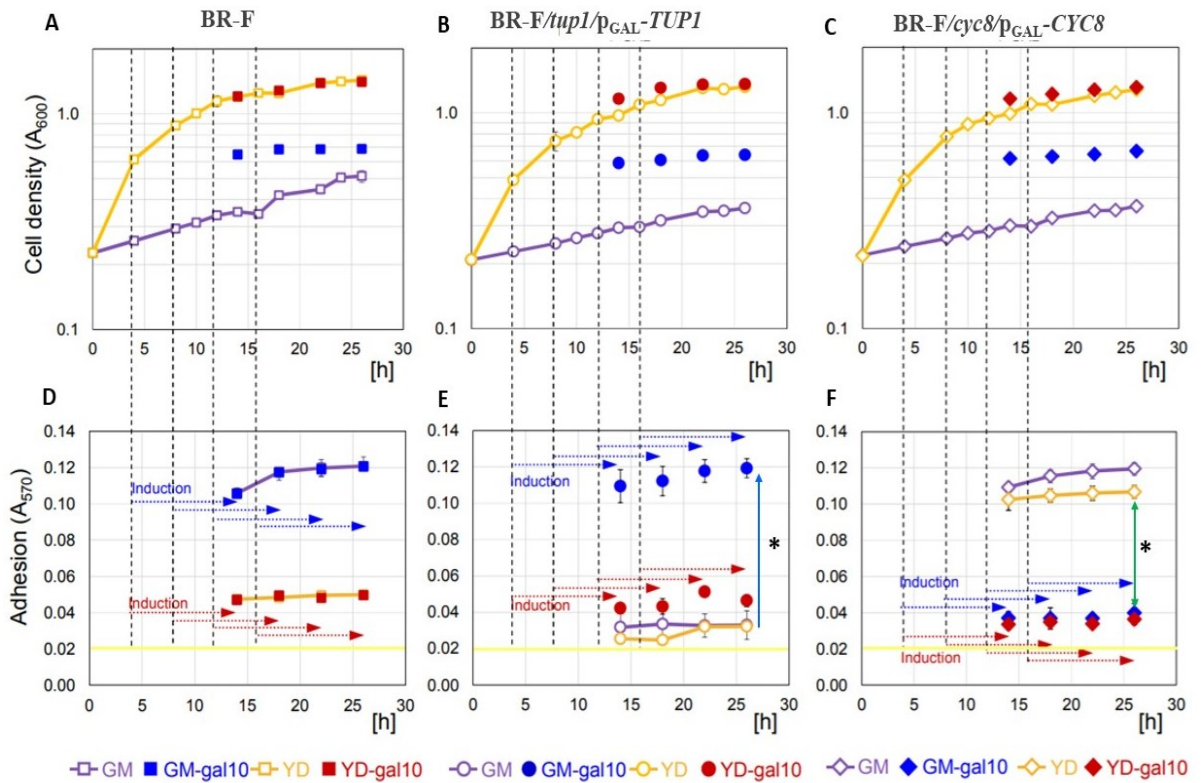
**Figure 3.24. Adhesion capability of cells in static cultures during different growth phases with either Tup1p or Cyc8p induction for 10 hours.**

*A-C, dynamic growth curves of strains in GM and YD. Media was supplemented with galactose for 10 h before measurement of the biomass ($A_{600}$). D-F, adhesion capability of strains without induction and with induction for 6 h (with galactose). Arrows (D-F) demonstrate interval of galactose presence with regard to time points in which cell adhesion was observed. Background absorbance is shown by yellow line. Arrows marked with asterisks: change in adhesion. This figure was adapted from figure S5 in published paper (Nguyen et al., 2020).*

In the second protocol (planktonic setup) biomass of each strain was harvested from cultures and diluted to $A_{600} = 1$, before the adhesion assay was conducted at specific time points. A standard amount of planktonic cells was pipetted into wells of the 96 well plates and incubated for 3 hours before checking adhesion. Unlike static cultures, cell density on the wells did not affect the adhesion efficiency of any strain tested (see Fig. 3.25D-F and Fig. 3.26D-F). In fact, adhesion achieved its highest level under assay conditions, allowing adhesion and was not dependent upon cell growth phase. This was consistent with the finding that in static cultures, galactose did not affect

adhesion of planktonic BR-F cells: adhesion was low in YD and high in GM (Fig. 3.25D and Fig. 3.26D). Adhesion of BR-F/p$_{GAL}$-*TUP1* was very low in GM and YD, but dramatically increased along with induction of Tup1p by galactose in GM only. Maximum adhesion was observed after 10 h compared to 6 h of galactose induction (Fig. 3.25E and Fig. 3.26E, as indicated by blue arrow, marked with asterisk). In contrast to static cell adhesion, even after 10 h induction, adhesion was slightly lower than that of BR-F. Adhesion was unchanged when BR-F, or Tup1p-induced cells, were grown in YD media in both time points (Fig. 3.25D,E and Fig. 3.26D,E). Unlike Tup1p results, adhesion of BR-F/p$_{GAL}$-*CYC8* was as high as BR-F in GM but, again, considerably higher in YD (Fig. 3.25F and Fig. 3.26F). By comparison 6-h of *CYC8* galactose induction in static cells caused a smaller reduction in adhesion than 10 h-treatment (compare Fig. 3.25F and Fig. 3.26F) and in neither case, did adhesion decrease to BR-F at basal level (compare Fig. 3.25D, F and Fig. 3.26F, D).
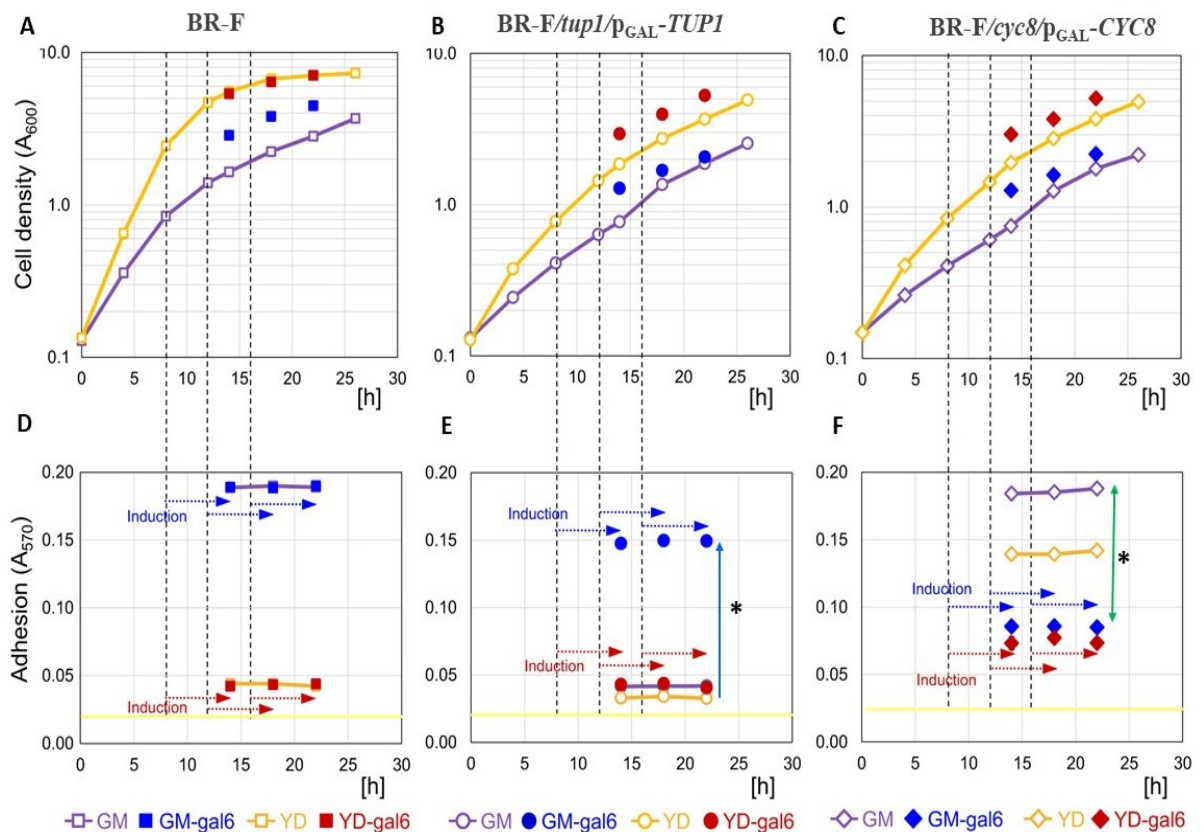


**Figure 3.25. Adhesion capability of cells in different growth phases of planktonic shaken cultures with Tup1p or Cyc8p induction for 6 hours.**

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*A-C, dynamic growth curves of strains in GM and YD. Media was supplemented with galactose for 6 h induction before checking biomass ($A_{600}$). D-F, adhesion capability of strains without treatment and with induction for 6 h (with galactose). Arrows (D-F) demonstrate interval of galactose treatment with regard to time points at which cell adhesion was identified. Yellow line indicates background absorbance (BA). Arrows marked with asterisks: change in adhesion. This figure was adapted from figure 6 in published paper (Nguyen et al., 2020).*
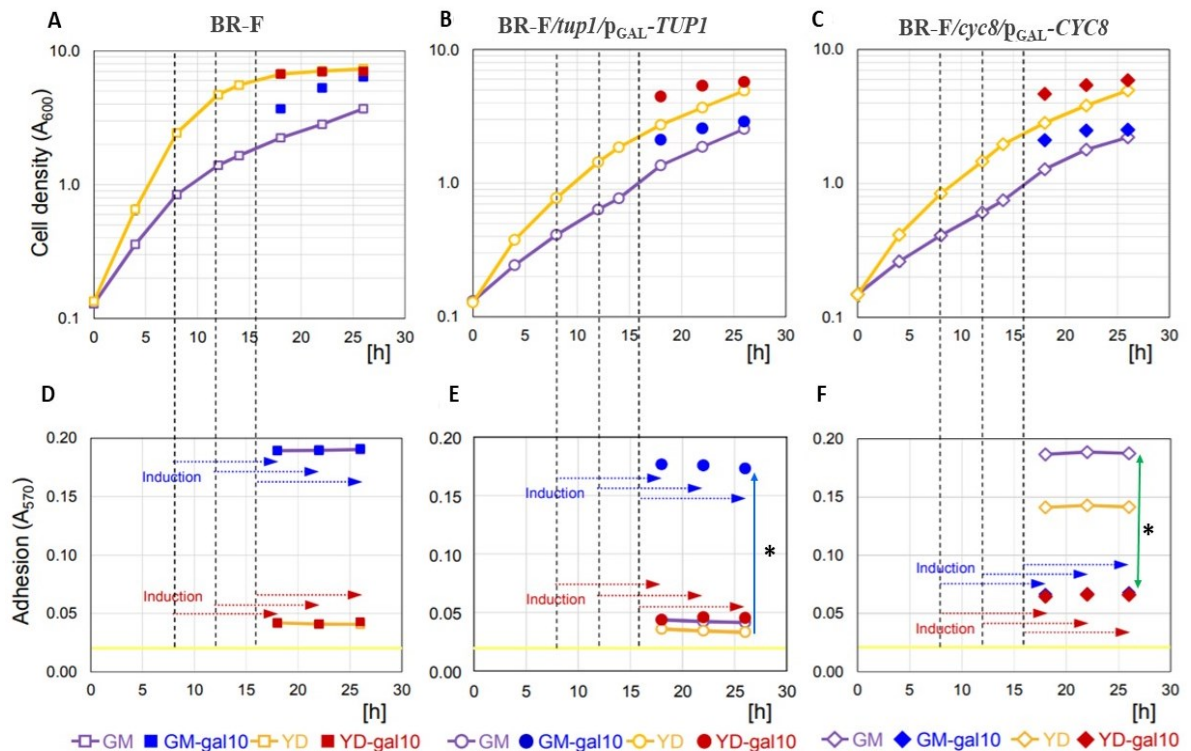


**Figure 3.26. Cells in different growth phases of planktonic shaken cultures with Tup1p or Cyc8p induction for 10 hours.**

*A-C, dynamic growth curves of strains in GM and YD. Media was supplemented with galactose for 6 h induction before measurement of the biomass ($A_{600}$). D-F, adhesion capability of strains without induction and with induction for 10 h (with galactose). Arrows (D-F) demonstrate period of galactose treatment with regard to time points at which cell adhesion was identified. Yellow line indicates BA. Arrows marked with asterisks: change in adhesion. This figure was adapted from figure S6 in published paper (Nguyen et al., 2020).*

In brief, regulation of cell adhesion by Cyc8p and Tup1p is not dependent on lifestyle, being similar in static and planktonic culture and largely independent of growth phase. In both static and planktonic cultivation, the effect of Cyc8p induction was earlier, at 6 h than that of Tup1p induction, which peaked after 10 h.

### 3.3.8 Discussion-3

Adhesion is the first step of biofilm formation, while dispersal allows cells to spread within a host (Kaplan *et al.,* 2010). These events are controlled by numerous physical and biochemical factors. Unlike the widely studied subjects of adhesion and biofilm dispersal in bacteria, relatively little is known about yeast adhesion and dispersal. *S. cerevisiae* has been used as a model for studying molecular mechanisms in pathogenic yeasts since more than 80% of genes are conserved between *S. cerevisiae* and *C. albicans* (Reynolds and Fink, 2001). Thus, improved knowledge of adhesion and dispersal of yeast is of great significance to efficient cleaning and disinfection strategies for biofilm removal in industry and medicine.

In this study, our research has highlighted that in contrast to BY4742 and BR-S strains which neither produce Flo11p nor adhere to plastic surfaces, wild yeast strain BR-F exhibits strong adhesion to an abiotic solid surface (polystyrene). Adhesion is connected with Flo11p expression and formation of biofilm at solid-liquid interfaces is regulated oppositely by Tup1p and Cyc8p. Generally, Tup1p promotes adhesion, whereas Cyc8p inhibits adhesion. Moreover, Tup1p induction in GM medium is sufficient to enhance adhesion, while in YD medium (with 2% of glucose) Tup1p induction is inadequate to induce adhesion. On the other hand, Cyc8p represses adhesion completely in both GM and YD medium. Thus, in the *GAL CYC8* strain (Cyc8p at basal level) there is strong adhesion and Flo11p expression of cells grown in YD medium, similar to that when grown in GM. Cyc8p and Tup1p regulate the adhesion of both static and planktonic cells, with only minor differences. Relatively homogeneous planktonic cells adhered independently of growth phase and more effectively than their static counterparts. The level of adhesion mediated by Tup1p was slightly more pronounced in older static cultures. This demonstrates that both strongly and weakly adhesive cells are present in static cultivation and that strongly

adhesive cell frequency increases as the biofilm develops. Adhesive cells, close to the plastic surface, may then adhere. Static cells respond more rapidly than planktonic cells to upregulated Cyc8p expression, resulting in the release of huge numbers of cells from the plastic surface. While *TUP1* induction has a less rapid effect on cells, this response however is faster in static than in planktonic cells.

The capability of BR-F cells to adhere and form biofilm was strictly dependent on the presence of glucose, being high without glucose (permissive conditions) and low in high glucose (non-permissive) conditions. These findings are in line with previous findings (Jackson *et al.,* 2002; Sauer *et al.,* 2004), that biofilm formation by bacterial species like *Pseudomonas aeruginosa* and the pathogenic yeast *C. albicans*, is dependent on available nutrients, particularly of glucose, which is known to inhibit biofilm formation by increasing biofilm dispersal (Blankenship and Mitchell, 2006; Uppuluri *et al.,* 2010). Adhesion was significantly increased by *TUP1* induction without glucose, but increased Tup1p in the presence of glucose had only a minor impact on adhesion. In contrast, Cyc8p seems to mediate an important effect of glucose by inhibiting Tup1p functions, potentially via mechanisms as previously described (Nguyen *et al.,* 2018). Hence, glucose may promote Cyc8p level and/or repressor activity (Fig. 3.22). Nrg1p, Mig1p and Sfl1p play key roles related to glucose repression via the Cyc8p-Tup1p complex (Wilson *et al.,* 1996; Conlan and Tzamarias, 2001; Verstrepen and Klis, 2006). However, deletion of any of the genes, encoding these three proteins, did not affect antagonistic regulatory functions of Cyc8p and Tup1p (Nguyen *et al.,* 2018). Little is known about environmental regulation of *CYC8* transcription. However, some preliminary work was carried out and genome-wide transcriptomic screening revealed that the level of *CYC8* mRNA was approximately 2.4-fold greater after 15 min treatment with 2% glucose than with 0.05% glucose (Casamayor *et al.,* 2012) and moderately declined over a longer period of growth in high glucose YD medium (Gasch *et al.,* 2000). In

this study, we also demonstrated that Cyc8p-GFP is present at higher levels in nuclei of cells at surface layers of non-adherent cells grown in glucose-YD than in those of cells in colony biofilms grown in glucose-free medium (Fig. 3.22). The evidence from this study points towards the possibility that glucose may regulate Cyc8p through an, as yet unidentified factor, which affects repressive functions of Cyc8p on target genes. The level of Cyc8p is influenced by glucose mainly in surface cells, including cells which are in touch with the medium. This is consistent with the efficient release at solid-liquid interfaces, observed upon glucose supplementation. Broadly speaking, in this manner, individual cells or even whole biofilms, may be efficiently released from solid/semi-solid surfaces.

Previous findings together with our evidence support a model (Fig. 3.27A) for wild yeast *S. cerevisiae*, in which, a glucose-responsive adhesion/biofilm formation factor has key functions in yeast virulence, markedly affecting systemic and biofilm infections by pathogenic yeast. A yeast cell settles in an available niche (e.g. a particular organ or tissue) where available glucose is low and the level or activity of Cyc8p is reduced, allowing Tup1p to enhance cell adhesion and invasiveness, promoting the formation of a new biofilm (Fig. 3.27 B, Biofilm 1). When glucose concentration is high (such as in blood/plasma) it induces Cyc8p expression. Exposing the biofilm to high glucose causes reduced adhesion and release of free planktonic cells, which can be dispersed. When Cyc8p level/function is suppressed again after a dispersed cell reaches another low glucose niche (another organ/tissue) where the cell can attach and form biofilm (Fig. 3.27 B, Biofilm 2).
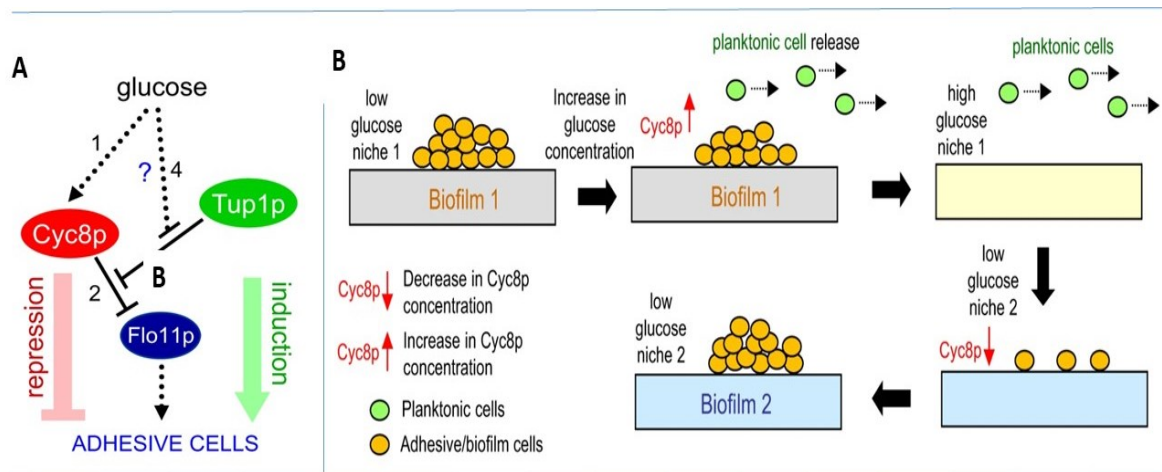
**Figure 3.27. Glucose coordinates with Cyc8p and Tup1p in regulating wild *S. cerevisiae* adhesion and formation of solid-liquid interface biofilm: model and experimental data.**

*A, glucose promotes Cyc8p expression/function "1", Cyc8p suppresses FLO11 expression "2" and obstructs cell adhesion. This effect may be increased if glucose was to additionally negatively influence Tup1p function "4". In glucose absence, Cyc8p level is reduced and its repressive function is blocked via Tup1p "3" as described (Nguyen et al., 2018). Afterwards, Flo11p is expressed and mediates cell adhesion. Arrows indicate induction pattern, whereas blunt line is repression pattern. Dotted line, other proteins that may be involved. B, environmental glucose level may influence the switch between adhesion/biofilm formation and release of planktonic cells in a structured, heterogeneous environment (a living organism). This figure was adapted from figure 4c and figure 7 in published paper (Nguyen et al., 2020).*

Via this mechanism, planktonic cells and biofilms can exist and disperse in diverse conditions in heterogeneous environments such as host organisms. Since 0.1% glucose (5.5 mM) is enough for the release of 15-30% of biofilm cells (Fig. 3.21), our model is associated with physiological conditions. The glucose level of the blood is commonly less than 7.8 mM 2 hours after a meal. Transient glycosuria in those with diabetes or during pregnancy may facilitate the spread of yeast biofilm to other niches.

Invasive fungal infections are highly opportunistic infections in diabetic and anti-cancer immunosuppressed patients (Rodrigues *et al.,* 2019). For example, Enache and Hennequin (2005) identified 92 cases of *S. cerevisiae* infection and fungemia (bloodstream infections) were common (72 of 92

patients). Extremely high glucose levels have been identified in blood and urine, serum, mucus, sweat as well as in other body fluids such as saliva and is a high-risk factor for fungal infections because of two main reasons: Glucose promotes yeast cell development and reduces some antifungal effects since glucose binds with high affinity to antifungal agents such as voriconazole (Bruen *et al.,* 2017, Mandal *et al.,* 2014). We speculate that higher glucose levels in particular niches could trigger the release of adhered cells and dispersal throughout the organism, possibly seeding new biofilms and leading to new outbreaks of infection. We have demonstrated that Cyc8p is a key player in this process and, along with inverse Cyc8p and Tup1p regulation operates in adherent and planktonic cells and is largely independent of growth phase. We also showed that increased glucose concentration results in release of adhesive cells from structured biofilm. Cyc8p and Tup1p are conserved in yeasts, but knowledge concerning their function in Candida spp. cell adhesion is rare. It has been reported that adhesion of *C. albicans* to keratinocytes decreased in a tup1 mutant strain (Mandal et al., 2014). Thus, as well as being potential risk factors and potential therapeutic targets in (thus far) rare *S. cerevisiae* infections, Cyc8p and Tup1p orthologs may also mediate adhesion/biofilm formation/cell release in *Candida spp.* and in *C. glabrata*, (a close relative of *S. cerevisiae*). Therefore, a better understanding of the processes by which glucose and these two regulators control cell adhesion, biofilm formation and dispersal in biofilm-forming *S. cerevisiae* could contribute towards identifying promising targets for drugs that block biofilm formation or dispersal by dangerous fungal pathogens.
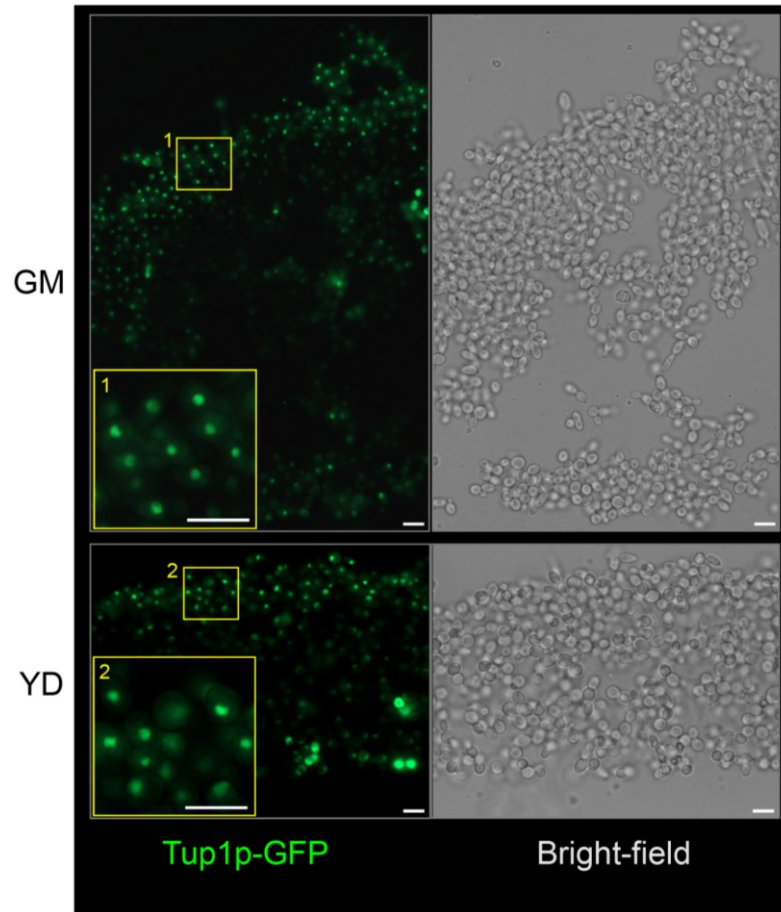
**Figure S8. The effect of glucose on Tup1p-GFP signal from strains cultured in GM or YD medium.**

*24-hrs old static cultures of BR-F/Tup1p-GFP cells in biofilms and non-adhered layers produce Tup1p-GFP, fluorescent signal is in green, cells shown in bright-field. Insets "1-2": cells at higher magnification. Bar, 10 µm. Representative experiment of three (n=3) independent experiments carried out. Figure 3.22 was prepared in collaboration with Vítězslav Plocek. This figure was adapted from figure S4 in published paper (Nguyen et al., 2020).*

# CHAPTER IV. CONCLUSION

In this study, we reported the global effects of Cyc8p and Tup1p in *S. cerevisiae* gene regulation at the proteomic level. Our results are largely consistent with previous findings that the Cyc8p-Tup1p complex represses hundreds of genes that are associated with various pathways. Interestingly, some novel sets of genes were identified for the first time in this study, connected with protein refolding and protein complex assembly, chronological cell ageing and apoptosis that were not reported previously. Importantly, we found that many genes are regulated by Cyc8p independently of Tup1p, and *vice versa:* Cyc8p negatively regulates genes mediating cell wall organization, amino acid and lipid biosynthesis, while Tup1p represses genes involved in electron transport and mitochondrial citrate metabolism. Excitingly, we identified the antagonistic functions of Cyc8p and Tup1p in the regulation of methionine and pyrimidine synthesis. Cyc8p represses, whereas Tup1p promotes, Met17p expression. But Tup1p inhibits *URA2* expression, while Cyc8p promotes Ura2p expression possibly via counteracting Tup1p. Our results suggested that there may be other target genes, that are conversely regulated by Cyc8p and Tup1p merit further investigation.

It has been widely believed that Tup1p and Cyc8p act together as co-repressor in *S. cerevisiae* for a wide range of genes including *FLO11*, a key player in biofilm colony formation. Using a combination of deletion and overexpression experiments, in this study we showed unexpected results: that these factors act in an opposing manner (antagonistically) with respect to Flo11p expression and to colony biofilm formation and development on agar and at solid-liquid interfaces. Deletion of *TUP1* results in lowered *FLO11* expression and diminished biofilm formation on agar. Overexpression of Cyc8p has the same effect. Along with colony morphology, the opposing

effects of Cyc8p and Tup1p on agar adhesion and the formation of extracellular fibers were also revealed. We proposed that Cyc8p itself represses *FLO11,* thus suppressing biofilm formation and that Tup1p, by binding to Cyc8p, blocks it from repressing this gene. In addition, Tup1p may promote *FLO11* by repressing an extracellular protease, inhibiting Flo11p degradation.

In agreement with this colony biofilms (formed on agar) are regulated antagonistically by Cyc8p and Tup1p. Divergent effects of these regulators on cell adhesion (followed by biofilm formation) and biofilm dispersal at solid-liquid interfaces were also observed. Tup1p promotes yeast cell adhesion to plastic surfaces, Flo11p expression and biofilm formation in glucose-free media, whereas Cyc8p inhibits Flo11p expression and adhesion. Strikingly, Cyc8p-mediated cell dispersal from biofilm is stimulated by glucose. Tup1p and Cyc8p antagonistically regulate *FLO11* expression and Flo11-dependent phenotypes by counteracting each other in certain conditions, but they act in concert to co-repress classical flocculation that is driven by other flocculation genes, such as *FLO1*.

Studying the adhesion, formation and development of biofilms and molecular mechanisms involved in these processes in *S. cerevisiae* is an important prerequisite for further study of these processes in pathogenic fungi. This is particularly true as there are increasing reports of *S. cerevisiae* infection and as it is increasingly recognized as an opportunistic pathogen in immunocompromised patients. This study therefore provides valuable information to help better understand the adhesion behaviour of yeasts and other factors contributing to the formation of yeast biofilms. Finding ways to inhibit adhesion, and dispersal of biofilms to reduce the risk of widespread yeast infections are major priorities in treating pathogenic fungi. One goal is to better understand the role of the Cyc8p-Tup1p complex, which is conserved among yeasts, including pathogenic yeasts. Mechanisms involved

in biofilm formation and biofilm dispersal in particular, are key subjects for further investigation. This study provided evidence that the two factors may merit more attention as potential targets for antifungal design, and biofilm removal.

Cyc8p and Tup1p may interact and form complexes with various additional regulators including chromatin-related proteins, so that each of these proteins can alter the functions of Cyc8p and/or Tup1p in specific conditions (Vachova *et al.,* 2019). Future studies may explore more deeply the mechanisms underlying these genetic interactions, for example, which specific transcription factors are involved in mediating the antagonistic interaction between Cyc8p and Tup1p, to find out how the Cyc8p-Tup1p complex generates distinct interactions with *FLO11* in the context of different phenotypic responses (flocculation vs biofilm formation). Moreover, it would be interesting to identify which protease, if any, is involved in the degradation of Flo11p and is apparently suppressed by Tup1p.

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# ATTACHED PUBLICATIONS

***List of attached publications:***

1. Phu Van Nguyen, Otakar Hlaváček, Jana Maršíková, Libuše Váchová, Zdena Palková (2018). *Cyc8p and Tup1p transcription regulators antagonistically regulate Flo11p expression and complexity of yeast colony biofilms*. PLoS Genet. 14(7).

2. Phu Van Nguyen, Vítězslav Plocek, Libuše Váchová, Zdena Palková (2020). *Glucose, Cyc8p and Tup1p regulate biofilm formation and dispersal in wild Saccharomyces cerevisiae.* npj Biofilms and Microbiomes. 6(1):7.

# Cyc8p and Tup1p transcription regulators antagonistically regulate Flo11p expression and complexity of yeast colony biofilms

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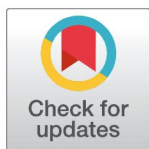
## Abstract

Yeast biofilms are complex multicellular structures, in which the cells are well protected against drugs and other treatments and thus highly resistant to antifungal therapies. Colony biofilms represent an ideal system for studying molecular mechanisms and regulations involved in development and internal organization of biofilm structure as well as those that are involved in fungal domestication. We have identified here antagonistic functional interactions between transcriptional regulators Cyc8p and Tup1p that modulate the life-style of natural *S. cerevisiae* strains between biofilm and domesticated mode. Herein, strains with different levels of Cyc8p and Tup1p regulators were constructed, analyzed for processes involved in colony biofilm development and used in the identification of modes of regulation of Flo11p, a key adhesin in biofilm formation. Our data show that Tup1p and Cyc8p regulate biofilm formation in the opposite manner, being positive and negative regulators of colony complexity, cell-cell interaction and adhesion to surfaces. Notably, in-depth analysis of regulation of expression of Flo11p adhesin revealed that Cyc8p itself is the key repressor of *FLO11* expression, whereas Tup1p counteracts Cyc8p's repressive function and, in addition, counters Flo11p degradation by an extracellular protease. Interestingly, the opposing actions of Tup1p and Cyc8p concern processes crucial to the biofilm mode of yeast multicellularity, whereas other multicellular processes such as cell flocculation are co-repressed by both regulators. This study provides insight into the mechanisms regulating complexity of the biofilm lifestyle of yeast grown on semisolid surfaces.

## Author summary

Yeast biofilms have become an increasingly important clinical problem over the years. Biofilms are often associated with infections resistant to antifungals, which are particularly prevalent in immunosuppressed patients. High resistance is mediated by cell reprogramming leading to the specific organization of internal biofilm structure and development of numerous protective mechanisms including extracellular matrix formation. Colony biofilms, with architecture and protective mechanisms similar to natural biofilms, represent an ideal model for studying molecular mechanisms and regulations behind biofilm

development and organization. Here, we describe a new mechanism of antagonistic regulation of biofilm-specific processes and formation of complex colony biofilm structure by Cyc8p and Tup1p transcriptional regulators. Both these regulators are widespread and conserved among yeasts forming clinically important biofilms, including pathogenic yeasts of *Candida* spp. The identification of Tup1p as a positive regulator of biofilm formation makes Tup1p-orthologs in yeast pathogens potential targets for the design of new strategies of treatment of biofilm infections.

## Introduction

In nature, microorganisms preferentially live within multicellular communities such as different types of biofilms and colonies [1–5]. Yeast biofilms are complex uniquely organized structures, in which cells are protected from hostile environments, including antifungals, host immune systems, and starvation. Active multidrug resistance transporters and protective extracellular matrix that are produced by subpopulations of differentiated cells in colony biofilms formed by wild *Saccharomyces cerevisiae* strains [6], contribute to this protection. Despite the identification of various genes and processes (including chromosome reorganization) involved in formation of colony biofilm structure [1,7,8], many of these processes seem to be specific to particular wild strains. An exception is the Flo11p cell wall adhesin, a key protein involved in several developmental processes including cell adhesion [9] and the formation of colony biofilms [10], flor biofilms [11], and mats [12]. Deletion of *FLO11* results in the formation of smooth colonies in various non-isogenic wild strains isolated from different habitats [13] as well as in Σ1278-derived strains [10,14]. *FLO11* mRNA levels are elevated in colony biofilms and lowered after phenotypic switching called domestication, during which cells are reprogrammed to form smooth colonies similar to colonies of laboratory strains, in which key features of biofilm-life style are switched off [13,15]. The *FLO11* promoter extends to 3 kb and contains at least four upstream activation sequences and nine elements involved in repression [16]. Thus, *FLO11* gene expression integrates signals from diverse signaling cascades, including the Ras-cyclic AMP-dependent kinase, mitogen-activated protein kinase (which controls filamentous growth) and the main glucose repression pathways. These pathways positively or negatively regulate *FLO11* expression in accordance with growth stage and nutritional conditions [1,16–18]. The expression of *FLO11* is also controlled by epigenetic mechanisms, including histone deacetylation, chromatin remodeling, non-coding RNAs and prion formation [1,19–23].

The Cyc8p (Ssn6p)-Tup1p complex is mostly known to function as a transcriptional co-repressor that is conserved in eukaryotic organisms including mammals [24]. Four molecules of Tup1p in concert with one molecule of Cyc8p form this complex [25] with a tendency to oligomerize [26], which regulates hundreds of *S. cerevisiae* genes involved in diverse pathways, such as glucose, starch and oxygen utilization, the response to osmotic stress, DNA repair, mating, sporulation, meiosis and flocculation [24,27,28]. Cyc8p-Tup1p does not bind directly to DNA but is brought to promoters *via* interactions with sequence-specific regulatory binding proteins, which coordinate the expression of specific subsets of genes [24]. Some data indicate that Cyc8p may play a more direct role in the repression [29]. Cyc8p-Tup1p can interact with Mig1p and Nrg1p, which bind to the promoters of glucose-repressed genes, such as *FLO11*, in the presence of glucose [17,30]. Cyc8p-Tup1p can also act as a transcriptional co-activator of various genes such as *HAP1* [31,32], *FRE2* [33], *ARG1* and *ARG4* in cooperation with Gcn4p [34], *TAT1* and *TAT2* [35], and genes induced by Hog1p in cooperation with Sko1p [36].

Genome-wide profiling of changes in nucleosome organization and gene expression that occur following the loss of *CYC8* or *TUP1* in *S. cerevisiae* laboratory strains show significant overlap, but additional changes result from the absence of either *TUP1* or *CYC8* [37]. Thus, the major function of Cyc8p and Tup1p in *S. cerevisiae*, identified so far, is the repression of pleiotropic gene targets mostly in the form of the Cyc8p-Tup1p co-repressor complex. In addition, several mutually independent repressor functions of Tup1p and Ssn6p (a functional homologue of *S. cerevisiae* Cyc8p) have been reported in *Candida albicans*, in which filamentous growth and hypha-specific genes are repressed by Tup1p independently of Ssn6p, whereas Ssn6p may act as a repressor of phenotypic switching independently of Tup1p [38]. Ssn6p was recently identified as a negative regulator of the opaque cell transcription program in white *C. albicans* cells and of the white cell transcription program in opaque cells [39]. Tup1p was reported to be a repressor of the opaque state and, together with its negative regulator Wor1p, has been proposed to control the opaque switch under different circumstances [40]. In addition to its interaction with Tup1p, Ssn6p interaction with histone deacetylase Rpd31p has been reported in *C. albicans* [41]. In this study, Ssn6p appeared to be a repressor of filamentation as well as of wrinkled colony morphology under particular conditions, independently of Tup1p, and some of these repressive effects were enhanced by deletion of *RPD31*. MoTUP1 was recently identified in *Magnaporthe oryzae* (a rice pathogen), and its deletion causes decreased pathogenicity of the fungus [42]. These studies suggest that Tup1p and Cyc8p play important roles in the pathogenicity of different fungi and that, in addition, these factors could have independent roles.

In this study, we provide clear evidence of the functions of the Tup1p and Cyc8p regulators in biofilm colony formation. We present evidence that Cyc8p itself is a repressor of *FLO11* gene expression and of the formation of the structured architecture of colony biofilms, whereas Tup1p counteracts Cyc8p, being a positive regulator of *FLO11* expression and colony complexity. Furthermore, we show that Tup1p regulates Flo11p accumulation at two different levels—gene expression and Flo11p stability. In addition to Flo11p, other features that are important for colony biofilm formation, such as cell invasiveness, adhesion to solid surfaces and presence of fibers connecting the cells, are also antagonistically regulated by Cyc8p and Tup1p. Conversely, features that are related to other types of multicellularity, such as cell flocculation, are co-repressed by both regulators.

## Results and discussion

### Cyc8p and Tup1p exhibit antagonistic effects on the architecture of colony biofilms

A series of strains was constructed producing different levels of Cyc8p and Tup1p regulators (Table 1) derived from the parental BR-F strain (wt strain; [43]), which forms structured colony biofilms [6]. The *tup1* strain (*tup1/tup1*) was prepared by deleting both alleles of *TUP1*, but we did not succeed in preparing a *cyc8* strain (*cyc8/cyc8*). As the *CYC8* gene is essential in the Σ1278 strain-background [44], resembling in several aspects wild yeast strains, this gene may also be essential in the BR-F strain. Therefore, we constructed strain $p_{GAL}$-*CYC8* (*cyc8*/ $p_{GAL}$-*CYC8*), in which one *CYC8* allele is deleted and the second placed under the control of the *GAL1*-inducible promoter ($p_{GAL}$), which provides only very low (basal) level of *CYC8* expression in the absence of galactose. The decreased level of *CYC8* mRNA and level of Cyc8p in this $p_{GAL}$-*CYC8* strain (grown without galactose), compared with the BR-F strain, was confirmed by northern blot (S1A Fig) and LC-MS/MS (see below), respectively. We also prepared strain $p_{TEF}$-*CYC8* (*CYC8*/$p_{TEF}$-*CYC8*) constitutively over-expressing *CYC8* from the *TEF1* promoter ($p_{TEF}$).

**Table 1. Yeast strains.**

| Name | Genotype | Colony morphology* | Source |
|---|---|---|---|
| BR-F | *MATa/MATα* | structured | [43] |
| BR-F-*flo11* | *MATa/MATα flo11Δ::kanMX/flo11Δ::ble* | smooth | [6] |
| *tup1* | *MATa/MATα, tup1Δ::KanMX, tup1Δ::nat1* | smooth | this study |
| p$_{TEF}$-CYC8 | *MATa/MATα, nat1-TEF1-CYC8/CYC8* | smooth | this study |
| p$_{GAL}$-CYC8 | *MATa/MATα, cyc8Δ::KanMX, nat1-GAL1-CYC8* | structured** | this study |
| p$_{TEF}$-TUP1 | *MATa/MATα, nat1-TEF1-TUP1/TUP1* | structured | this study |
| p$_{GAL}$-TUP1 | *MATa/MATα, tup1Δ::KanMX, nat1-GAL1-TUP1* | smooth** | this study |
| BR-F-Flo11p-GFP | *MATa/MATα, FLO11-GFP/FLO11* | structured | [15] |
| Flo11p-GFP/p$_{TEF}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8* | smooth | this study |
| Flo11p-GFP/p$_{TEF}$-TUP1 | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-TUP1/TUP1* | structured | this study |
| Flo11p-GFP/p$_{GAL}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, cyc8Δ::KanMX, nat1-GAL1-CYC8* | structured** | this study |
| Flo11p-GFP/p$_{GAL}$-TUP1 | *MATa/MATα, FLO11-GFP/FLO11, tup1Δ::KanMX, nat1-GAL1-TUP1* | smooth** | this study |
| *sfl1*/CYC8/p$_{TEF}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8, sfl1Δ::KanMX, sfl1Δ::HygR* | smooth | this study |
| *nrg1*/CYC8/p$_{TEF}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8, nrg1Δ::KanMX, nrg1Δ::HygR* | smooth | this study |
| *mig1*/CYC8/p$_{TEF}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, nat1-TEF1-CYC8/CYC8, mig1Δ::KanMX, mig1Δ::HygR* | smooth | this study |
| p$_{GAL}$-TUP1/p$_{CUP}$-CYC8 | *MATa/MATα, tup1Δ::loxP, cyc8Δ::loxP, nat1-GAL1-TUP1, KanMX-CUP1-CYC8* | smooth** | this study |
| p$_{CUP}$-TUP1/p$_{GAL}$-CYC8 | *MATa/MATα, tup1Δ::loxP, cyc8Δ::loxP, KanMX-CUP1-TUP1, nat1-GAL1-CYC8* | smooth** | this study |
| Flo11p-GFP/p$_{GAL}$-TUP1/ p$_{CUP}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, tup1Δ::loxP, cyc8Δ::loxP, nat1-GAL1-TUP1, KanMX-CUP1-CYC8* | smooth** | this study |
| Flo11p-GFP/p$_{CUP}$-TUP1/ p$_{GAL}$-CYC8 | *MATa/MATα, FLO11-GFP/FLO11, tup1Δ::loxP, cyc8Δ::loxP, KanMX-CUP1-TUP1, nat1-GAL1-CYC8* | smooth** $ | this study |

*For colonies grown on GMA medium

** Without induction

$ Semi-structured when medium contains traces of $Cu^{2+}$

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Unexpectedly, deletion of *TUP1* and *CYC8* over-expression resulted in a similar, very prominent change in colony architecture indicating opposing roles of Tup1p and Cyc8p in biofilm formation (Fig 1A). In both cases, the strains formed smooth colonies. Conversely, although reduced *CYC8* expression slowed the growth of the p$_{GAL}$-*CYC8* strain, this strain formed structured colony biofilms that gradually developed morphology similar to that of wt strain biofilms (S1B Fig). Hence, 3-day-old p$_{GAL}$-*CYC8* colonies exhibited an architecture (Fig 1B) with features typical of younger (40-h-old) structured biofilms formed by the wt strain and 5-day-old p$_{GAL}$-*CYC8* colony biofilms resemble 3-day-old biofilms of the wt strain (S1B Fig and [6]).
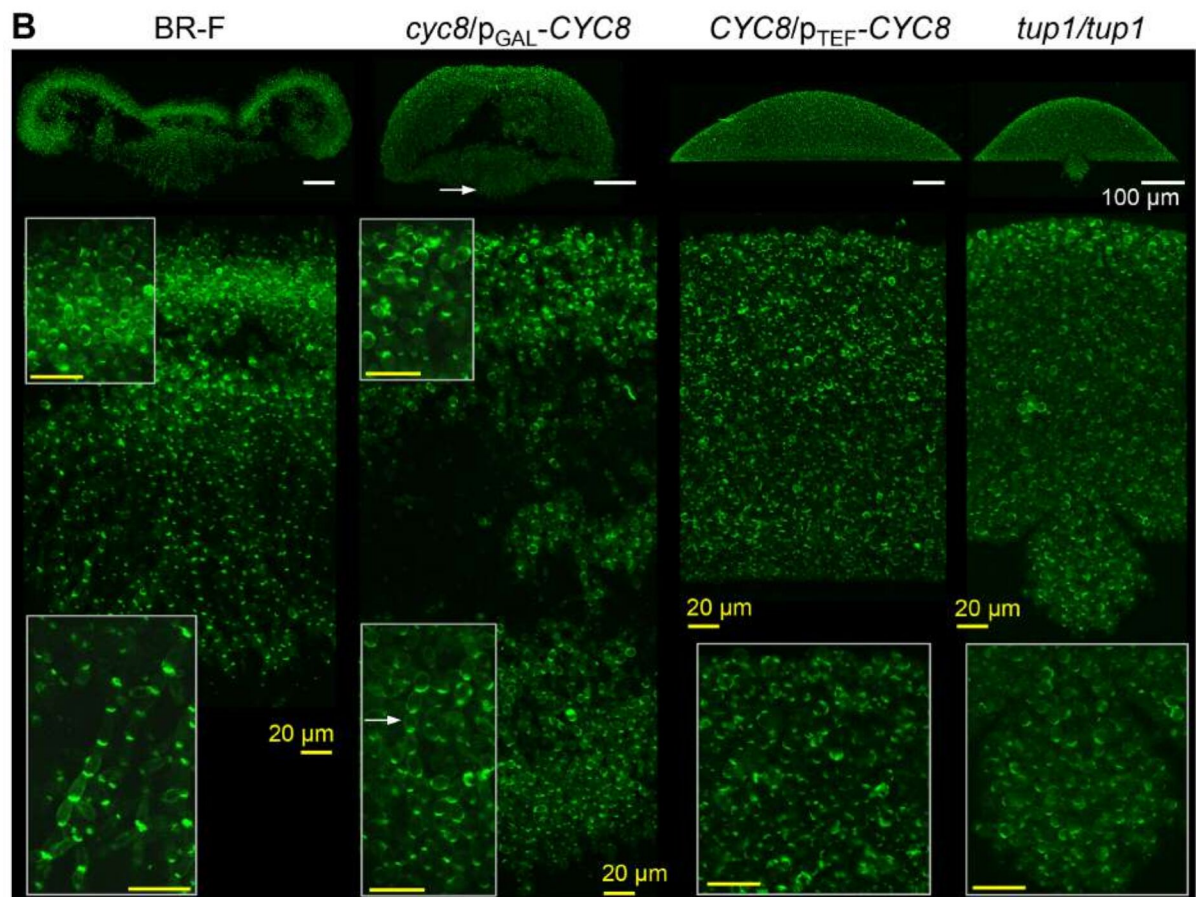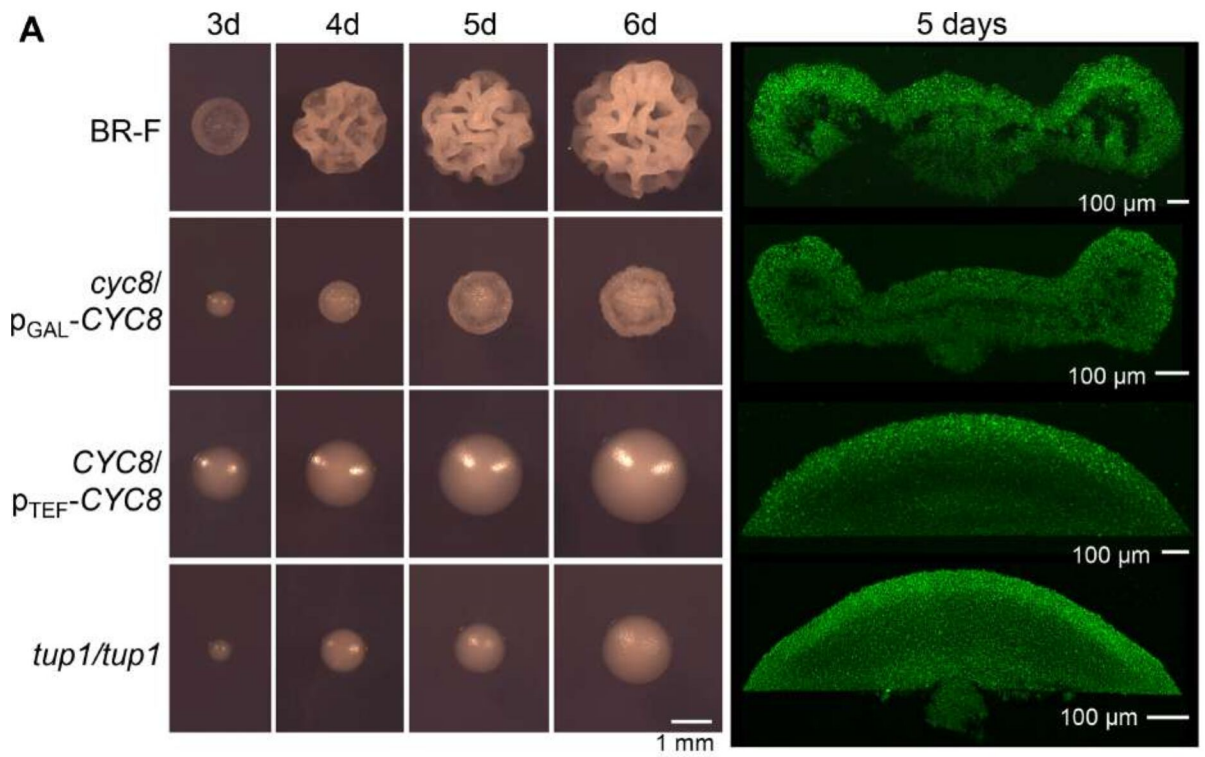
**Fig 1. Development and architecture of colonies with altered levels of Cyc8p and Tup1p.** A, Development of colonies grown on GMA at a density of ~$10^3$ colonies per plate (left panel). Two photon excitation confocal microscopy (2PE-CM) of colony cross-sections stained with Calcofluor white (false green color); colonies were grown for 5 days on GMA at a density of ~$3 \times 10^3$ colonies per plate (right panel). B, 2PE-CM of colony cross-sections stained with Calcofluor white (false green color). Colonies were grown for 3 days on GMA at a density of ~$3 \times 10^3$ colonies per plate. Upper part, whole colonies (20x objective); lower part, central colony parts shown at a higher magnification (63x objective), insets: details of aerial and subsurface cells (strains BR-F and $p_{GAL}$-CYC8); detail of central part ($p_{TEF}$-CYC8); detail of the colony bottom (tup1). White bar, 100 μm; yellow bar, 20 μm. Arrow indicates chains of rounded cells invading the agar.

https://doi.org/10.1371/journal.pgen.1007495.g001

## Cyc8p and Tup1p regulate the production of Flo11p adhesin in an opposite manner

Flo11p is essential for colony biofilm formation. Therefore, we investigated the potential role of Tup1p and Cyc8p in Flo11p expression. We prepared strains $p_{GAL}$-CYC8-Flo11p-GFP (cyc8/$p_{GAL}$-CYC8-Flo11p-GFP) and $p_{GAL}$-TUP1-Flo11p-GFP (tup1/$p_{GAL}$-TUP1-Flo11p-GFP) (derived from the BR-F-Flo11p-GFP strain; [15]), in which CYC8 and TUP1 expression is inducible by galactose, to monitor Flo11p-GFP levels in the context of colony biofilm morphology. Presence of galactose in GMA partially affects the colony appearance, slightly reducing the structured morphology, as has similarly been shown for glucose YEPD medium [45]. $p_{GAL}$-TUP1-Flo11p-GFP and $p_{GAL}$-CYC8-Flo11p-GFP colonies were, therefore, first grown on GMA plates without galactose for 3 days and then expression of $p_{GAL}$-controlled genes was induced for ~18 h by adding galactose to wells in the agar. Fig 2A shows that in areas of higher galactose concentration (near the wells), colony morphologies changed due to the induction of TUP1 (smooth → structured) or CYC8 (structured → smooth), whereas colonies located far from the galactose source retained their original morphologies. Western blots showed that Flo11p-GFP is produced in high levels in $p_{GAL}$-TUP1-Flo11p-GFP colonies induced by galactose (Fig 2B, lane 4), whereas Flo11p-GFP production was totally abolished when CYC8 overexpression was induced by galactose in $p_{GAL}$-CYC8-Flo11p-GFP colonies (lane 8). In accordance, Flo11p-GFP was undetectable in $p_{TEF}$-CYC8-Flo11p-GFP (CYC8/$p_{TEF}$-CYC8-Flo11p-GFP) colonies constitutively overexpressing CYC8 (lane 5), whereas Flo11p-GFP level in $p_{TEF}$-TUP1-Flo11p-GFP (TUP1/$p_{TEF}$-TUP1-Flo11p-GFP) (lane 1) colonies was similar to that of wt colonies (lane 2). Two photon excitation confocal microscopy (2PE-CM) showed that in 3-day-old wt colonies, Flo11p-GFP is present at higher levels in cells at the aerial surface of wt colonies and in cells forming the tips of "roots" invading the agar (Fig 2C). A similar pattern of Flo11p-GFP was observed in structured $p_{GAL}$-TUP1-Flo11p-GFP colonies near the galactose source and in structured $p_{GAL}$-CYC8-Flo11p-GFP colonies that were localized far from the galactose source and were thus not induced (Fig 2D). However, Flo11p-GFP was hardly detectable in smooth colonies of both strains.

To further clarify regulatory functions of Tup1p and Cyc8p, we compared amounts of TUP1 and CYC8 mRNAs and proteins in wt colonies and colonies of above described strains with differently manipulated levels of Cyc8p or Tup1p (Fig 3A, left part, and 3B). Colonies were grown for 3 days on GMA and then induced by galactose (or treated with distilled water as a control) for 4 hours. This induction greatly increased TUP1 and CYC8 mRNA levels, respectively, in $p_{GAL}$-TUP1 and $p_{GAL}$-CYC8 strains (Fig 3A, lanes 4 and 6, respectively). Conversely, amounts of both CYC8 and TUP1 mRNAs were only slightly increased, as compared with mRNA levels in wt colonies, when expression was controlled by the moderate, constitutive $p_{TEF}$ promoter (lanes 7 and 8). Labeling of both Tup1p and Cyc8p proteins with GFP or 6HA tags resulted in dysfunctional proteins and commercial anti-Tup1p and anti-Cyc8p primary antibodies generated high unspecific background. Therefore, we quantified Tup1p and Cyc8p approximate concentrations in cells from 3-day-old colonies induced/non-induced by galactose for 4 hours by label free LC-MS/MS (Fig 3B). Contrary to TUP1 and CYC8 mRNA

**A** *tup1*/p_GAL-*TUP1*-Flo11p-GFP    *cyc8*/p_GAL-*CYC8*-Flo11p-GFP

uninduced    induced    uninduced    induced

**B**

**C** BR-F-Flo11p-GFP

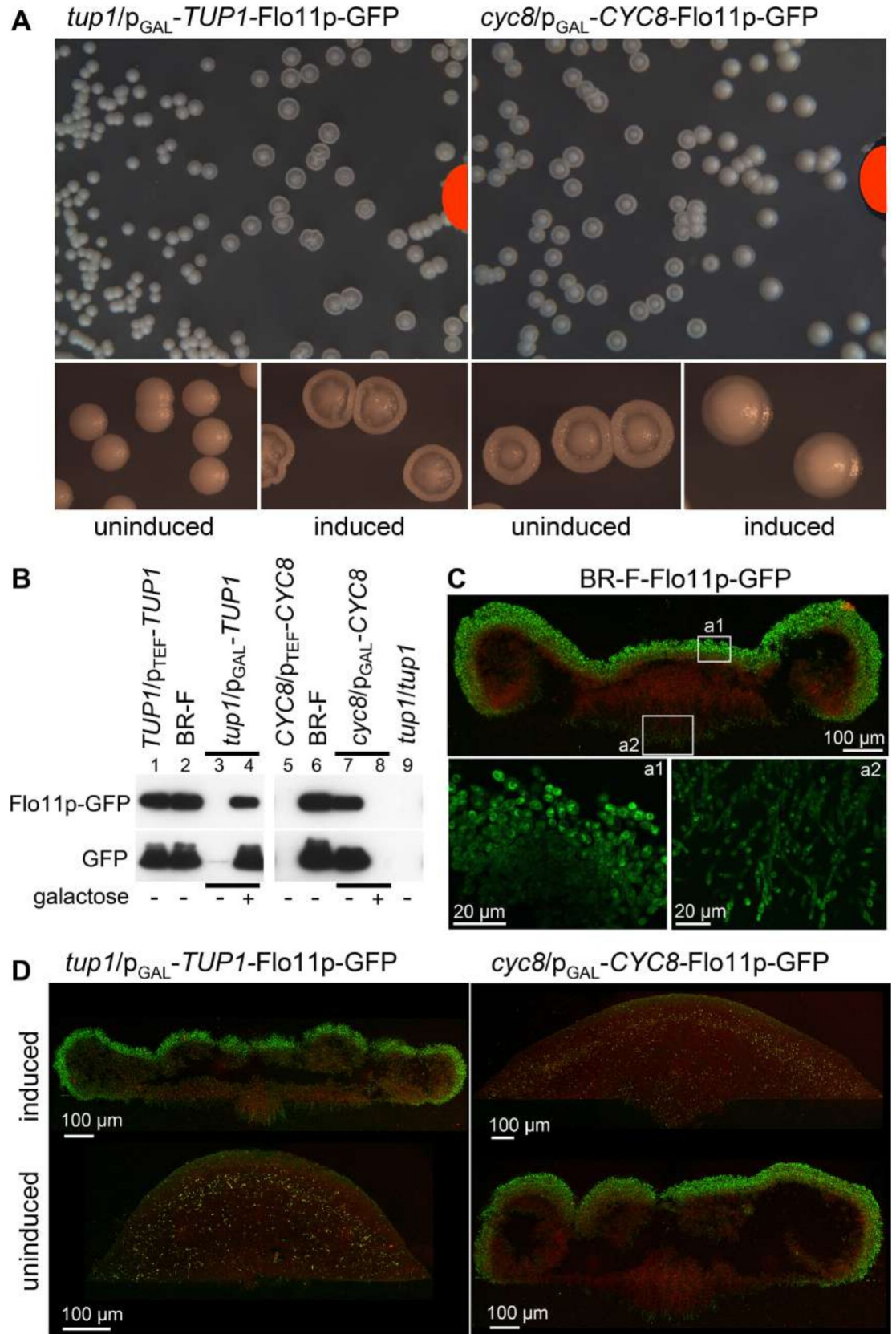**D** *tup1*/p_GAL-*TUP1*-Flo11p-GFP    *cyc8*/p_GAL-*CYC8*-Flo11p-GFP

**Fig 2. Expression of Flo11p adhesin in colonies with altered levels of Cyc8p and Tup1p.** A, Effects of *TUP1* (left) and *CYC8* (right) expression induced by galactose diffusing from the wells in the agar (marked in red) on colony morphology. 120 μl of 10% galactose was applied to each well. Morphologies of wt colonies treated in the same way are shown in S5 Fig. B, Flo11p-GFP in 3-day-old colonies of Flo11p-GFP strains grown on GMA. Representative result of the 4 experiments is shown. Western blot loading controls are in S4 Fig. C, 2PE-CM of cross-sections of 3-day-old colonies of BR-F-Flo11p-GFP strain grown on GMA. Green, Flo11p-GFP; red, cell autofluorescence (in upper picture). Plating density ~4–6 x $10^3$ colonies per plate. D, 2PE-CM of cross-sections of colonies from the plates with galactose induction (panel A). Induced colonies localized close to the galactose source and uninduced colonies localized to the plate margin are shown. Green, Flo11p-GFP; red, cell autofluorescence. Plating density ~4–6 x $10^3$ colonies per plate.

levels, which both were highly elevated when expression was induced by galactose (Fig 3A, lanes 4 and 6), differing enhancement of Cyc8p and Tup1p protein concentration was observed in $p_{GAL}$-*CYC8* and $p_{GAL}$-*TUP1* 3-day-old colonies. Whereas Cyc8p level increased only by 40% (~1.4 times), Tup1p level increased more than 5 times as compared with wt colonies (Fig 3B). In accordance with mRNA analysis (Fig 3A, lines 5 and 3), neither Cyc8p nor Tup1p were detected without galactose induction in $p_{GAL}$-*CYC8* and $p_{GAL}$-*TUP1* colonies, respectively (Fig 3B).

Analyses of *FLO11* mRNA levels (Fig 3A) showed that Cyc8p and Tup1p affect *FLO11* gene expression in opposite ways and with differing efficiencies. *CYC8* constitutive overexpression in $p_{TEF}$-*CYC8* colonies resulted in absence of *FLO11* mRNA (Fig 3A, lane 7) and of Flo11p-GFP protein (Fig 2B, lane 5), thus confirming Cyc8p as a *FLO11* gene repressor. *TUP1* deletion in the presence of functional Cyc8p (*tup1* strain) resulted in the absence of Flo11p (Fig 2B, lane 9), but a small amount of *FLO11* mRNA was still detectable (Fig 3A, lane 2). This result confirmed that *FLO11* transcription is enhanced by Tup1p, but when the *TUP1* gene was deleted, some transcription of *FLO11* still occurred. In accordance, the level of *FLO11* mRNA was significantly reduced after 4 h of galactose induction of $p_{GAL}$-*CYC8* colonies and the level of *FLO11* mRNA was significantly increased after 4 h of induction of $p_{GAL}$-*TUP1* colonies (Fig 3A, lane 6 and 4, respectively). 18 h after galactose induction, Flo11p protein levels increased from non-detectable to a level comparable with the wt strain in $p_{GAL}$-*TUP1* colonies (Fig 2B, compare lanes 3 and 4; Fig 2D) and decreased from a wt-like to non-detectable level in $p_{GAL}$-*CYC8* colonies (Fig 2B, compare lanes 7 and 8; Fig 2D).

To further examine mutual effects of both regulators, we constructed an additional set of strains derived from the BR-F and BR-F-Flo11p-GFP strains, in which amounts of both regulators were adjustable by the inducing compound (galactose or copper) (Table 1, last four strains). We then evaluated levels of *TUP1*, *CYC8* and *FLO11* gene expression (mRNA) and levels of respective proteins. Results of *CYC8* and *TUP1* mRNA analysis after 4 h of galactose and/or copper induction of colonies of strains $p_{GAL}$-*CYC8*/$p_{CUP}$-*TUP1* (*cyc8*/$p_{GAL}$-*CYC8*/ *tup1*/$p_{CUP}$-*TUP1*) and $p_{GAL}$-*TUP1*/$p_{CUP}$-*CYC8* (*tup1*/$p_{GAL}$-*TUP1*/*cyc8*/$p_{CUP}$-*CYC8*) (Fig 3A, lanes 9–16) were consistent with results of induction experiments performed with strains, in which expression of only one of the regulators was adjustable and the second was controlled by its native promoter (Fig 3A, lanes 3–6). Only the level of $p_{GAL}$-regulated mRNA (of both *TUP1* and *CYC8*) was partially diminished when copper was also present during galactose induction (Fig 3A; compare lanes 14 and 16 for *CYC8* and lanes 10 and 12 for *TUP1*, decreased level of mRNA is marked by asterisk). Since $p_{GAL}$-regulated expression of *CYC8* and *TUP1* was also diminished by copper in $p_{GAL}$-*CYC8* and $p_{GAL}$-*TUP1* colonies, respectively (S2 Fig), copper seem to partially reduce transcription from the $p_{GAL}$ promoter. Consistently with $p_{GAL}$-*CYC8* and $p_{GAL}$-*TUP1* induction experiments, increased level of Cyc8p caused a decrease in *FLO11* mRNA (Fig 3A, lanes 11 and 14) and in Flo11p concentrations (Fig 3C, lanes 4 and 7) in $p_{GAL}$-*CYC8*/$p_{CUP}$-*TUP1* and $p_{GAL}$-*TUP1*/$p_{CUP}$-*CYC8* colonies. However, the basal *FLO11* mRNA level when neither Cyc8p nor Tup1p was induced (Fig 3A, lanes 9 and 13) was higher
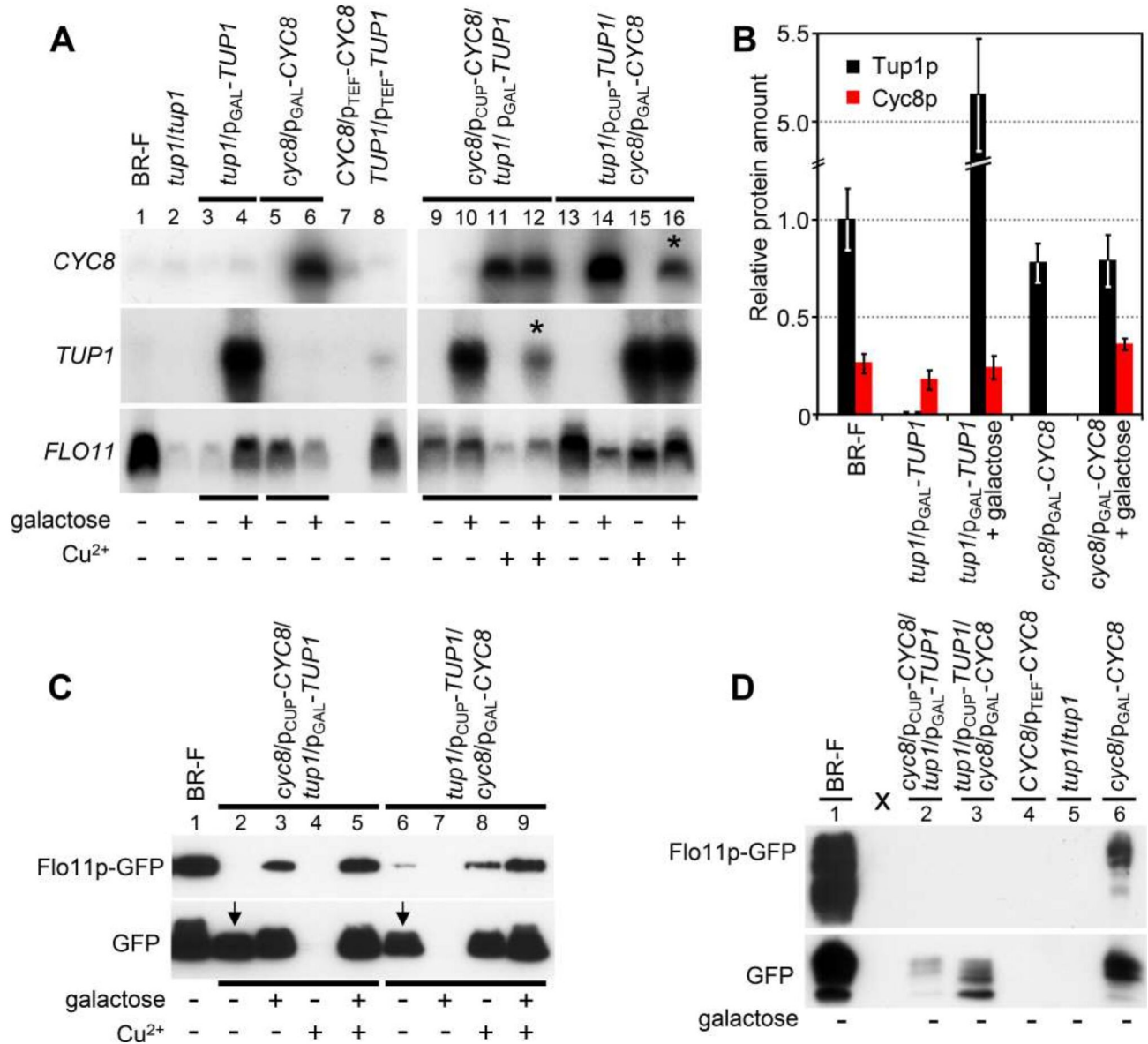
**Fig 3. Levels of *CYC8*, *TUP1* and *FLO11* mRNAs and respective proteins in strains with differently altered levels of Cyc8p and Tup1p regulators.** A. Northern blot of *CYC8*, *TUP1* and *FLO11* mRNAs from 3-day-old colonies grown on GMA and then induced/uninduced 4 h with galactose and/or copper. Representative result of the 4 experiments is shown. Northern blot loading controls are in S4 Fig. Asterisks indicate reduction of transcription from $p_{GAL}$ promoter in the presence of copper. B. Levels of Tup1p and Cyc8p quantified by LC-MS/MS. Colonies were grown 3 days on GMA and then were induced/uninduced by galactose for 4 h. Protein amounts were related to the amount of Tup1p in BR-F colonies. C. Levels of Flo11p-GFP and free GFP in lysates from 3-day-old colonies (prepared from whole colony biomass including extracellular material) of respective strains grown on GMA, induced/uninduced for 18 h by galactose and/or copper. Arrows points to total degradation of Flo11p-GFP to GFP in particular samples. D. Levels of Flo11p-GFP and free GFP in the extracellular fluid extracted from 50 mg of wet weight biomass of 3-day-old colonies grown on GMA without galactose or copper induction.

than under conditions where a wt-level of Cyc8p was present (*tup1* or $p_{GAL}$-*TUP1* colonies without galactose, Fig 3A, lanes 2 and 3). 4 h-induction of Tup1p by either inducing compound did not significantly increase the *FLO11* mRNA level (Fig 3A, lanes 10 and 15) above the basal level identified in the absence of both inducing compounds (lanes 9 and 13). In fact this basal level was lower in the $p_{CUP}$-*CYC8*/$p_{GAL}$-*TUP1* strain than in $p_{GAL}$-*CYC8*/$p_{CUP}$-*TUP1*

**Fig 4. Model schematic of the regulatory functions of Cyc8p and Tup1p.** In colony biofilms, levels of Cyc8p and Tup1p are balanc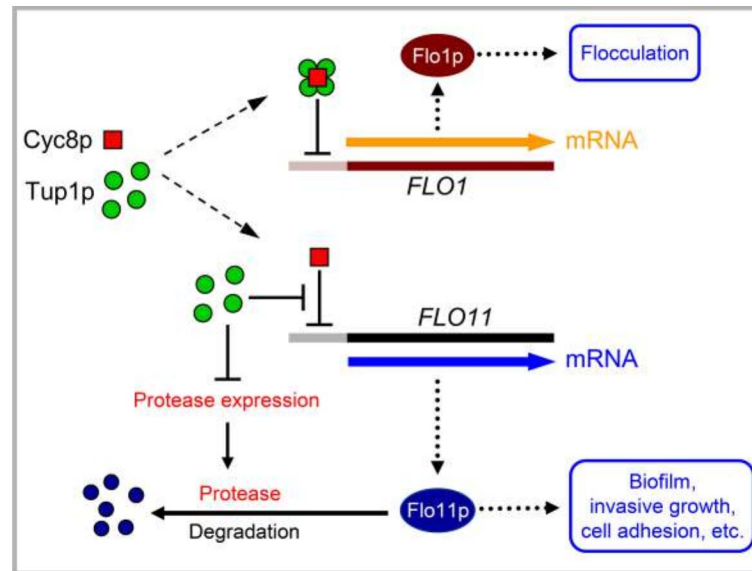ed in such a way that Tup1p inhibits Cyc8p repressor (by forming Cyc8p-Tup1p complex), thus preventing *FLO11* repression. In addition, free Tup1p contributes to repression of putative extracellular protease that degrades Flo11p. The Cyc8p-Tup1p complex represses other cellular functions, such as cell flocculation.

strain (Fig 3A, compare lanes 9 and 13), possibly because of traces of copper in the medium which can slightly increase *CYC8* expression and thus the amount of Cyc8p repressor from the onset of colony growth. Altogether, these data further confirmed that Cyc8p is the main repressor of expression of the *FLO11* gene and indicated that Tup1p modulates the level of Cyc8p repressor, potentially via formation of Tup1p-Cyc8p complex (Fig 4). Analysis of Flo11p-GFP protein levels however suggested an additional function of Tup1p. Flo11p-GFP full length protein was almost undetectable in the absence of both regulators, whereas a high level of free GFP was present in these samples indicating that Flo11p-GFP synthesis was relatively high (in accordance with high basal level of *FLO11* mRNA, Fig 3A, lane 9 and 13), but that the protein was efficiently degraded (Fig 3C, lanes 2 and 6, arrows mark free GFP). These data indicate dual roles of Tup1p in regulation of Flo11p concentration in colonies and thus in regulation of colony biofilm complexity: counteracting *CYC8* repression of *FLO11* gene expression and preventing Flo11p degradation, possibly by repressing expression of a specific protease.

Flo11p is associated with the cell wall and it is partially shed from cells into the extracellular space [46]. We therefore examined further whether extracellular Flo11p-GFP is degraded and whether presence of Tup1p influences such degradation. As expected, neither Flo11p-GFP nor free GFP was detected in extracellular fluid from colonies of p$_{TEF}$-*CYC8* and *tup1* strains (Fig 3D, lanes 4 and 5), in which *FLO11* expression is repressed by Cyc8p. In extracellular fluid from biofilm colonies of wt strain and p$_{GAL}$-*CYC8* strain without galactose, both partially degraded Flo11p-GFP and high level of free GFP were detected (Fig 3D, lanes 1 and 6), indicating degradation of a fraction of Flo11p-GFP, perhaps during its shedding. In colonies of both p$_{GAL}$-*TUP1*/p$_{CUP}$-*CYC8* and p$_{CUP}$-*TUP1*/p$_{GAL}$-*CYC8* strains with high basal levels of *FLO11* gene expression and protein production in the absence of both inducing compounds, free GFP only was present in extracellular fluid (Fig 3D, lanes 2 and 3). Consistently, colonies of these strains are smooth. These data indicate that Tup1p prevents degradation of extracellular Flo11p-GFP possibly via repression of expression of a cell wall associated or extracellularly

localized protease. Differences in Flo11p processing (at several positions within the protein) were found in a strain defective in the kexin Kex2p [46], serine protease which cleaves precursors of secreted proteins in the trans-Golgi network. However, Flo11p was not identified in the screen of possible Kex2p substrates and does not contain prominent Kex2p cleavage sites (Lys-Arg at P1 and P2 position) [47]. Hence, Flo11p is probably not a direct target of Kex2p, but it could be cleaved by another secreted protease, the secretion and/or processing of which requires Kex2p.

## Cyc8p and Tup1p antagonistically regulate other colony biofilm specific processes

Next, we examined Cyc8p and Tup1p roles in regulation of other processes that are specific to colony biofilms, such as cell-substrate adhesion and agar penetration and cell-cell interaction via cell wall fibers.

Long fibers forming Velcro-like structures in contact sites between the cells were identified in colony biofilms, but not in smooth colonies of the BR-F-*flo11* strain, by transmission electron microscopy (TEM) of chemically fixed cells [6]. Here we used high-pressure freezing and freeze substitution TEM that improves identification of these structures and revealed some less abundant, extracellular fibrillar material even on the surface of cells within BR-F-*flo11* colonies. Fig 5A thus shows that cells in both structured and smooth colonies are covered on their surface by extracellular fibrillar material, which connects adjacent cells. However, the fibers in this material were significantly (20–30%) longer in the structured colony biofilms of the BR-F and non-induced $p_{GAL}$-*CYC8* strains than in the smooth colonies of $p_{TEF}$-*CYC8*, *tup1* and *flo11* strains (Fig 5B), in which shorter fibers are occasionally visible despite the material appearing to be less structured. The differences are evident also in cell-cell contact sites, where Velcro-like connections were visible among cells in colony biofilms, whereas less structured material was present at contact sites in smooth colonies (Fig 5C). Velcro-like connections may be caused by interaction of N-terminal Flo11A domains of Flo11p as reported in [48] (Fig 5C, indicated by red mark), although direct proof of the presence of Flo11p in these fibers is still lacking. Adhesion to solid surfaces and invasive growth are typical features of fungal biofilms [12] as well as of colony biofilms [13], which are evident particularly in the area of the colony roots [6]. Cell adhesion and agar invasion are absent in *S. cerevisiae flo11* colonies [10,13,49]. Figs 1 and 6A show that the $p_{TEF}$-*CYC8* and *tup1* strains exhibited defects in invasive growth and adhered poorly to the agar. However, similar to the wt strain, cells of the non-induced $p_{GAL}$-*CYC8* strain adhered to the agar even with robust washing. These data show that both organization of extracellular fibrillar material involved in cell-cell contact and cell adhesion to surfaces are antagonistically regulated by Cyc8p and Tup1p.

Adhesion to, and invasiveness into agar did not correlate with cell morphology. BR-F colony biofilms were formed by both oval and elongated cells in the aerial part and by pseudohyphae consisting of elongated cells in the subsurface part (Figs 1B and 6B). In contrast, there was a failure to form elongated cells by, not only smooth colony-forming strains with decreased level of Tup1p (*tup1*) and increased level of Cyc8p ($p_{TEF}$-*CYC8*), but also a colony-biofilm forming strain with reduced level of Cyc8p ($p_{GAL}$-*CYC8*). Thus, although the $p_{GAL}$-*CYC8* strain formed (in the absence of galactose) a structured colony biofilm, its root part was formed by chains of rounded cells that divided by monopolar budding and invaded the agar (Fig 1B). Consistently, some wild *S. cerevisiae* strains form structured colony biofilms despite being unable to form typical pseudohyphae composed of elongated cells [13]. Tup1p and Ssn6p (Cyc8p) are repressors of invasive/filamentous growth in *C. albicans* [38,41,50]. Our data show that in *S. cerevisiae*, an imbalance in the Cyc8p and Tup1p levels, rather than the
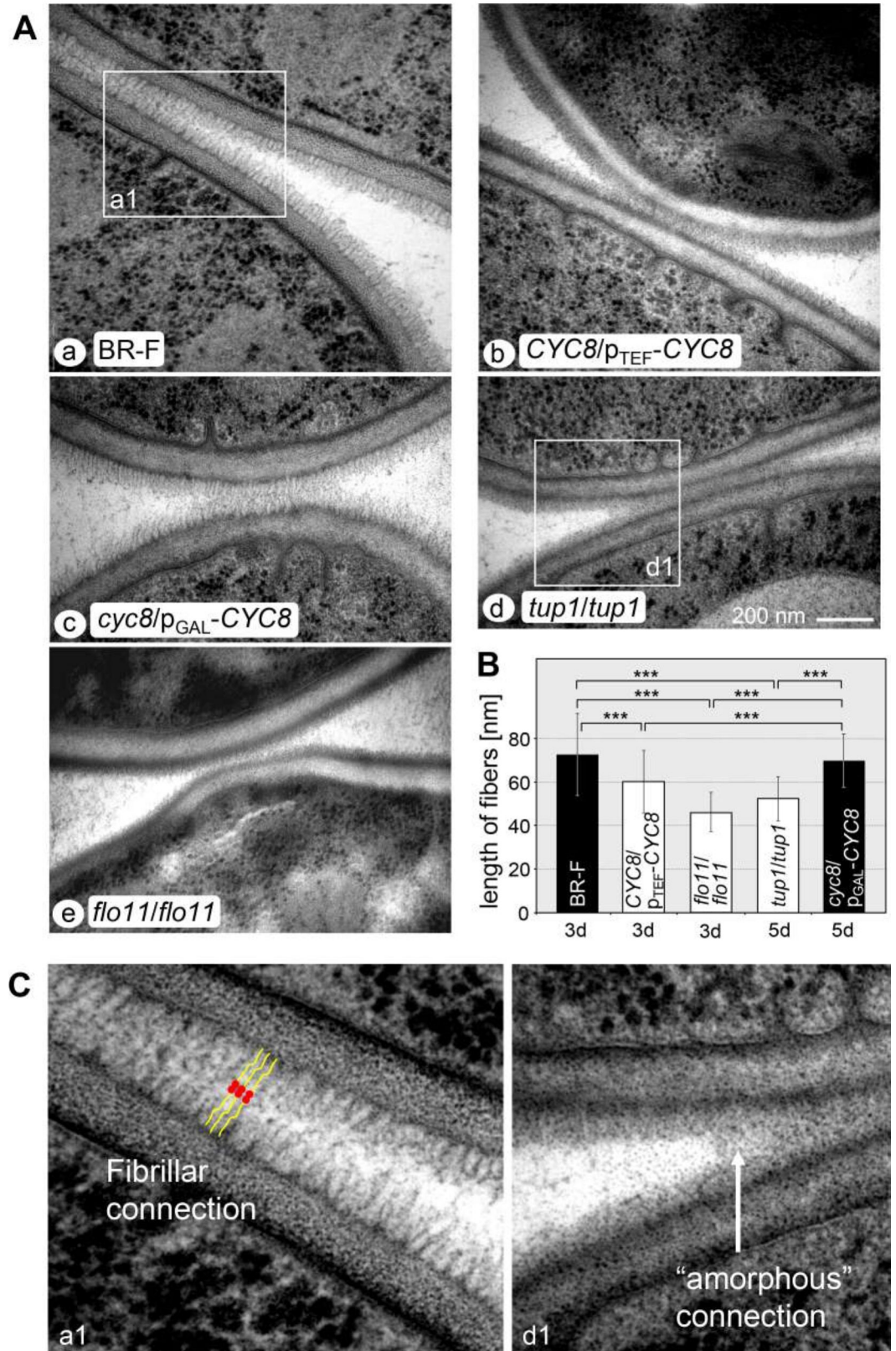
Fig 5. Extracellular fibrillar material between cells within colonies of strains with altered levels of Cyc8p and Tup1p. A, Electron microscopy of Velcro connections between cells from 3-day-old (a, b, e) and 5-day-old (c, d) colonies grown on

GMA. B, The average length of the fibers is shown in the graph. Black columns, structured colony biofilms; white columns, smooth colonies; bars, SD; ***, P<0.001. C, Examples of extracellular material in higher magnification from colony biofilms (BR-F, inset a1 from Aa) and from smooth colonies (*tup1*, inset d1 from Ad). Velcro-like connections in BR-F are schematically indicated in yellow color, the red color indicates N-terminal Flo11A domains [48], potentially involved in the interaction.

https://doi.org/10.1371/journal.pgen.1007495.g005

presence or absence of an individual regulator, diminishes cell elongation. This defect, however, does not influence the colony biofilm morphology.
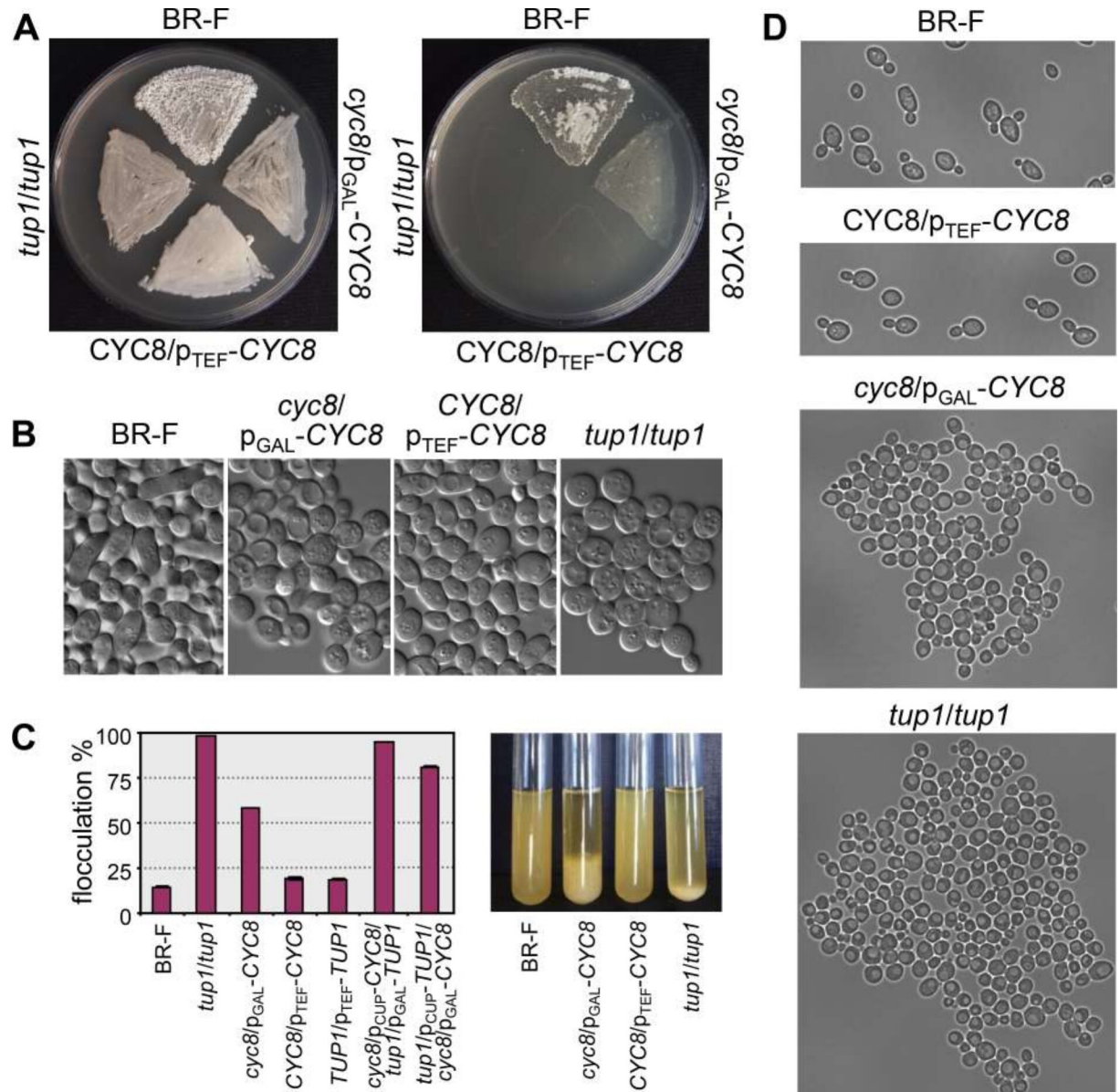


**Fig 6. Cell morphology and adhesion characteristics within colonies of strains with altered levels of Cyc8p and Tup1p.** A, Cell invasion (right picture) shown after washing the cells grown on GMA plates for 3 days (left picture). B, Morphology of cells from the aerial parts of 3-day-old colonies grown on GMA. C, Cell flocculation in liquid cultures grown for 2 days in GM (graph); examples of flocculation in tubes (right) and D, microscopic pictures of free cells and flocs.

https://doi.org/10.1371/journal.pgen.1007495.g006

## Cyc8p and Tup1p co-repress cell flocculation

The Cyc8p-Tup1p complex has been implicated in the repression of genes involved in cell floc-culation, such as *FLO1* [17,51]. Consistent with the literature [52–54], either deletion of *TUP1* (*tup1* strain) or substantial reduction of *CYC8* expression (p$_{GAL}$-*CYC8* without galactose) or reduction of *TUP1* and *CYC8* expression (p$_{CUP}$-*CYC8*/p$_{GAL}$-*TUP1* or p$_{CUP}$-*TUP1*/p$_{GAL}$-*CYC8* strain without inducing compound) resulted in the formation of macroscopic flocs (clusters of cells) that sedimented efficiently (Fig 6C and 6D), indicating de-repression of the flocculation genes. In striking contrast, the wt strain BR-F and the *CYC8*- or *TUP1*- over-expressing strains (p$_{TEF}$-*CYC8* or p$_{TEF}$-*TUP1* strains) did not form cell clusters. These data show that in contrast to the antagonistic functions of Cyc8p and Tup1p in processes involved in colony biofilm for-mation, Tup1p and Cyc8p in concert repress other flocculation genes such as *FLO1* in the BR-F strain. These results are in agreement with findings showing that i) in contrast to *FLO11*, the expression of other flocculation genes (*FLO1*, *FLO9* and *FLO10*) is equivalent in colony biofilms and in smooth domesticated colonies and ii) citrate buffer treatment and the presence of mannose in the medium (both of which eliminate the flocculation caused by Flo1p but not that of Flo11p) affect BR-F cell flocculation in liquid culture but not the adhesiveness of cells from BR-F colonies [43] and iii) *FLO1* and *FLO5* play roles in cell aggregation and flocculation, whereas *FLO11* expression promotes invasive growth and biofilm formation [55].

## Conclusions

Our findings highlight a previously unknown antagonistic function of Tup1p and Cyc8p in the regulation of complexity of yeast colony biofilms. While Tup1p is essential for the forma-tion of colony biofilms, increased levels of Cyc8p prevent formation of colony biofilms leading to formation of smooth colonies similar to those of laboratory strains. The antagonistic func-tions of Tup1p and Cyc8p are specific to features typical of yeast biofilm life-style, such as cell invasiveness, adhesion to semisolid surfaces and cell-cell adhesion by cell-wall fibers. Proper-ties important for other types of multicellularity, such as cell flocculation [51], are regulated differently, being repressed by both regulators. In accordance, deletion of genes *NRG1*, *MIG1* or *SFL1* for repressors recruiting the Cyc8p-Tup1p co-repressor complex to promoters [56–58], did not prevent Cyc8p-mediated repression of colony biofilm formation in p$_{TEF}$-*CYC8* strain (S3 Fig).

Flo11p adhesin is key protein in colony biofilm formation affecting most of the above men-tioned biofilm-specific processes [10,13,14]. We therefore tested a hypothesis that Cyc8p and Tup1p regulate biofilm-specific processes via regulation of Flo11p. Indications exist in previous research of a possible relationship between Cyc8p/Tup1p and *FLO11* expression, but findings were not consistent. Both positive [59–61] and negative [59] effects of *TUP1* deletion on *FLO11* mRNA levels have been reported and deletion of the *CYC8* gene has been shown to increase *FLO11* mRNA levels [56]. Our in depth analyses revealed that Tup1p and Cyc8p regulate the level of Flo11p adhesin in the opposite manner and at different steps in its expression (Fig 4). Firstly, Cyc8p itself represses *FLO11* gene transcription, whereas Tup1p counteracts Cyc8p function, thus contributing positively to *FLO11* expression. Efficiency of Cyc8p-based *FLO11* gene repression depends on the comparative levels of Cyc8p and Tup1p proteins and we hypothesize that Tup1p can balance the level of free Cyc8p by forming a Cyc8p-Tup1p complex, which apparently does not regulate biofilm specific processes, but can regulate other cellular properties such as expression of flocculins. Four molecules of Tup1p interact with one molecule of Cyc8p [26], which means that, in accordance with our data, a smaller change in Cyc8p than in Tup1p levels has a stronger effect on *FLO11* expression. Secondly, Tup1p also positively regu-lates the level of Flo11p protein in colony biofilms by preventing its degradation. Flo11p is

targeted to the cell wall via the secretory pathway and is partially shed into the extracellular space [46]. The mechanism of its degradation and involvement of specific protease(s) are currently unknown. Our data showed that Tup1p prevents degradation of extracellular Flo11p-GFP. Tup1p thus may repress expression of a gene coding for a cell wall protease that is involved in Flo11p degradation. Interestingly, Tup1p represses a set of secreted aspartyl proteinases (SAPs) in *C. albicans* [62] and derepression of genes coding for extracellular proteases was observed in an *Aspergilus nidulans* strain deleted in the TUPA gene (an ortholog of *TUP1*) [63].

In summary, our study identifies Cyc8p and Tup1p as important regulators of Flo11p gene expression and protein stability, both affecting the final Flo11p amount in cells and the extracellular space of yeast multicellular structures. According to Flo11p concentration, the structures then acquire different levels of complexity ranging from smooth colonies to colony biofilms. Fine tuning of the amounts and mutual effects of Cyc8p and Tup1p regulators in colony/biofilm cell subpopulations could also provide a mechanism for balancing Flo11p levels and related cellular properties at different positions within the structure and, potentially, at different times during its development. In this respect, further studies are required to uncover potential role of Cyc8p and Tup1p in regulating the amount of Flo11p in different parts of colony biofilms, such as in the internal colony parts, where Flo11p seem not to be present. Orthologs of both Tup1p and Cyc8p are present in different yeast and other fungal species and their role in filamentation, phenotypic switching and virulence of yeast/fungal pathogens has been recently suggested [39–41,50,64]. Identification of Tup1p as an important positive regulator of the formation of colony biofilms brings practical advantages, making *TUP1* orthologs in pathogenic yeasts prospective gene targets for new antifungal treatment strategies.

## Materials and methods

### Yeast strains and media

All strains prepared in this study (Table 1) were derived from the wild yeast strain BR-F from a collection at the Institute of Chemistry (Slovak Academy of Sciences) and its derivative BR-F-Flo11p-GFP [15]. Colonies were grown on GMA (3% glycerol, 1% yeast extract, and 2% agar) at 28°C unless otherwise indicated, at densities ranging from $10^3$ to 6 x $10^3$ cells per plate. For the flocculation tests, the strains were grown in liquid GM (GMA without agar). For the strain constructions, G418 and nurseothricin concentrations in GMA were 200 and 100 μg/ml.

In galactose/$Cu^{2+}$ induction experiments, colonies were grown 3 days on GMA. Then, the agar was supplemented by galactose and/or $Cu^{2+}$ to a final concentrations of either 2% galactose and/or 3 mM $CuSO_4$ for colony incubation for 4 h (for Northern blot and LC-MS/MS) or 0.1% galactose and/or 0.25 mM $CuSO_4$ for longer 18 h incubations used for morphology experiments and determination of Flo11p-GFP levels by Western blots (lower concentrations of both galactose and $Cu^{2+}$ were needed to avoid artificially affecting colony morphology in longer incubations).

### Colony imaging

Colony images were captured in incident and/or transmitted light. A ProgRes CT3 CMOS camera with a Navitar objective and NIS Elements software (Laboratory Imaging, s.r.o, Prague, CZ) were used.

### Strain constructs

Strains with gene deletions and with genes under the control of an artificial promoter ($p_{CUP}$, $p_{GAL}$ and $p_{TEF}$) were prepared according to [65–67]; primers and plasmids are listed in S1

Table. Yeast cells were transformed as described in [68]. Correct genomic integration of cassettes was verified by PCR using specific primers and by sequencing. Cre-lox system was used to remove antibiotic resistance genes in strains subjected to multiple manipulations [66].

## 2PE-CM of microcolonies

The internal architecture of the microcolonies was visualized by two photon excitation confocal microscopy (2PE-CM) according to [6,69]. In brief, colonies were embedded in agarose and cut vertically down the middle. The cut surface was placed on a coverslip, and colony side views were obtained by 2PE-CM. When required, the cross-sections were stained with 1 µg/ml Calcofluor white. Excitation wavelengths of 920 nm and 790 nm and the emission bandwidths of 480–595 nm and 400–550 nm were used for GFP and Calcofluor white. An overview of the morphology of colonies was obtained simultaneously with green GFP fluorescence as autofluorescence in the 600-740-nm wavelength range. Images of the whole colonies (Figs 1A, 1B, 2C and 2D) and the central parts of the colonies (Fig 1B) were obtained by combining two or three images from neighboring fields of view.

## Determination of Flo11p-GFP levels by western blots and of Tup1p and Cyc8p by nanoLC-MS-MS

The detection of GFP tagged Flo11p (in cell lysates or the extracellular fluid) by western blots was performed as described [70]. In brief, cells harvested from colonies were broken by glass beads in the presence of protease inhibitors, and proteins (25 µg/lane) of cell lysates were subjected to SDS-PAGE. GFP was detected by mouse monoclonal horseradish peroxidase (HRP)-conjugated anti-GFP antibody (Santa Cruz). Membranes stained by Coomassie blue were used as loading controls (S4 Fig). Extracellular proteins were extracted by phosphate-saline buffer from 3-day-old colonies. After centrifugation, proteins of the supernatant were precipitated by methanol/chloroform treatment [71]. Extracellular proteins extracted from 50 mg of wet biomass were loaded to each slot.

For nanoLC-MS-MS analysis, the cells were disrupted in 100 mM triethylammonium bicarbonate buffer using glass beads. Protein aliquots (30 µg; determined by the bicinchoninic acid assay, Sigma) were solubilized using sodium deoxycholate (1% (w/v) final conc.), reduced with tris(2-carboxyethyl)phosphine, alkylated with S-methyl methanethiosulfonate, digested sequentially with trypsin and extracted with ethylacetate saturated with water [72]. Samples were desalted using C18 sorbent (Supelco pn: 66883-U) and eluents were dried and resuspended in 20 µl of 1% trifluoroacetic acid. Peptide (2 µg) from each sample were separated on 50-cm C18 column using 2.5 h elution gradient and analyzed in a DDA mode on Orbitrap Fusion Tribrid (Thermo Scientific) mass spectrometer. Three biological replicates were run for each strain and condition. Resulting raw files were processed in MaxQuant (v. 1.5.8.3) [73]. Searches were performed against latest version of *S. cerevisiae* Uniprot database and common contaminant database. Further analysis was performed in Perseus (v. 1.5.5.3) [74].

## Cell adherence and invasiveness

The cell-cell adherence (flocculation) assay was performed according to [75]. In brief: 2-day-old cell cultures grown in GM medium were harvested, flocculation disrupted by EDTA (pH 8, 50 mM final concentration) and $OD_{600}$ of the cell suspension determined (reading A). Then, cells were washed twice by $dH_2O$ and suspended in 30 mM $CaCl_2$. After 60 s, $OD_{600}$ at upper layers of the cell suspension was measured (reading B). Flocculation (%) was calculated according to the formula: $100*(A-B)/A$. Average of 4 independent measurements +/- SD is shown. The flocs or free cells were photographed using transmission light microscopy

(Microscope DMR, Leica, Germany). In the invasive growth assay [76] cells were streaked onto standard GMA plates and grown at 28°C for 3 days. Plates were vigorously washed with water and photographed.

## Electron microscopy

Samples were prepared according to [77] with some modifications. Briefly, 3-day- and 5-day-old colonies were frozen in an EM PACT2 high-pressure freezer (Leica, Germany). The samples were freeze-substituted in an automatic FS machine (Leica, Germany) in 100% acetone containing 2% osmium tetroxide as follows: -90°C for 96 h, 5°C increase per hour for 14 h, -20°C for 24 h, 3°C increase per hour for 8 h, and 4°C for 18 h. The substituted samples were embedded in pure Epon. Ultrathin sections were cut using a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. The sections were examined using a JEM-1011 transmission electron microscope (JEOL, Japan) operating at 80 kV. Fine structure measurements were performed using a Veleta camera and iTEM 5.1 software (Olympus Soft Imaging Solution GmbH).

## RNA isolation and northern blotting

Colonies were suspended in TES buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 0.5% SDS) and total RNA isolated using the hot phenol method [78]. Fifteen micrograms of total RNA was denatured in loading buffer with formamide, separated in 1.5% agarose gel and transferred to a positively charged nylon membrane (Amersham Hybond-XL, GE Healthcare Ltd). The membranes were hybridized with specific DNA probes prepared using a random primer labeling kit (Takara). The rRNA content was visualized by ethidium bromide staining of gels and used as a loading control (S2 Fig).

## Supporting information

**S1 Fig. Level of *CYC8* mRNA and architecture of colony biofilms formed by *cyc8*/p$_{GAL}$-*CYC8* strain.** A, Northern blot (NB) showing level of *CYC8* mRNA in BR-F and *cyc8*/p$_{GAL}$-*CYC8* colony biofilms (without galactose). B, Development of architecture of BR-F colony biofilm and more slowly growing *cyc8*/p$_{GAL}$-*CYC8* colony biofilm.
(PDF)

**S2 Fig. Level of p$_{GAL}$-regulated *CYC8* and *TUP1* mRNA in presence/absence of copper.**
(PDF)

**S3 Fig. Comparison of colonies of p$_{TEF}$-*CYC8* strain and KO strains.**
(PDF)

**S4 Fig. Loading controls.** Loading controls for western blots in Fig 2B (A) and Fig 3C (C) and for northern blots in Fig 3A (B).
(PDF)

**S5 Fig. Galactose does not affect BR-F colony morphology in the used set-up.** A, 3-day-old BR-F colonies grown on GMA plates were treated by galactose (120 µl of 10% galactose) applied to the wells and cultivated for 18 h. B, Untreated BR-F colonies of the same age grown on GMA plates.
(PDF)

**S1 Table. Primers and Plasmids.**
(PDF)

## Acknowledgments

## Author Contributions

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# ARTICLE  OPEN

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# Glucose, Cyc8p and Tup1p regulate biofilm formation and dispersal in wild *Saccharomyces cerevisiae*

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*Saccharomyces cerevisiae* is a mainly beneficial yeast, widely used in the food industry. However, there is growing evidence of its potential pathogenicity, leading to fungemia and invasive infections. The medical impact of yeast pathogens depends on formation of biofilms: multicellular structures, protected from the environment. Cell adhesion is a prerequisite of biofilm formation. We investigated the adherence of wild and genetically modified *S. cerevisiae* strains, formation of solid–liquid interface biofilms and associated regulation. Planktonic and static cells of wild strain BRF adhered and formed biofilms in glucose-free medium. Tup1p and Cyc8p were key positive and negative regulators, respectively. Glucose caused increased Cyc8p levels and blocked cell adhesion. Even low glucose levels, comparable with levels in the blood, allowed biofilm dispersal and release of planktonic cells. Cyc8p could thus modulate cell adhesion in different niches, dependently on environmental glucose level, e.g., high-glucose blood versus low-glucose tissues in host organisms.

## INTRODUCTION

Fungal infections have become an increasing problem due to high associated mortality in immunosuppressed patients and limited availability of effective drug treatment, including an absence of biofilm-specific drugs. *Candida* and *Cryptococcus* spp. are major human opportunistic yeast pathogens. However, *Saccharomyces cerevisiae* (a close relative of *C. glabrata*) that is widely used in the food industry, has begun to be considered as an opportunistic pathogen in recent years, having been implicated in a variety of infections ranging from vaginitis and cutaneous infections, to systemic bloodstream and organ infections in immunocompromised patients.[1,2] *Saccharomyces* invasive infections are often clinically similar to invasive candidiasis.[3] Clinical *S. cerevisiae* strains (and strains of non-clinical origin with virulence features) are distinct from laboratory strains. They are often resistant to factors such as oxidative stress, copper and high temperature[4,5] and they can better survive in blood infection models. Clinical isolates often retain the ability to perform dimorphic switching between yeast form and pseudohyphae.[6] As *S. cerevisiae* usually has a low susceptibility to amphotericin B and azoles,[7] it can occupy niches, cleared of *C. albicans* and other yeasts in azole-treated patients.

Wild *S. cerevisiae* and *Candida* spp. adhere to different biotic and abiotic surfaces.[8,9] Adhesion efficiencies depend on surface properties of adherent cells, such as the presence of specific proteins, adhesins, mediating cell-cell or cell-biotic/abiotic surface interactions. Adhesion is a key step allowing cells to occupy new niches in the host, and to establish multi-layered biofilm structures, providing yeast cells with multiple protection[10,11] against the immune system and drug treatment. Hence, cell adhesion is an important factor in yeast virulence.[8] For example, *C. albicans* cells adhered to an abiotic dental prosthetic were significantly more resistant to a range of antifungals than planktonic cells.[12] Adhesins, mediating yeast cell adhesion to biotic (e.g., host tissues during infections) and/or abiotic (e.g., plastic) surfaces, include Epa adhesins of *C. glabrata*, Als adhesins of *C. albicans* and Flo11p adhesin of *S. cerevisiae*.[13–16] Flo11p is

also involved in other processes, including invasive growth and formation of complex structure of colony biofilms.[9,17–19] Flo11p production is controlled by numerous factors that operate at different levels of Flo11p expression and function.[20] Cyc8p (Ssn6p) and Tup1p are conserved factors regulating numerous processes, mostly as a co-repressor complex.[21] In addition, Cyc8p and Tup1p antagonistically regulate Flo11p level and complexity of colony biofilms. Cyc8p represses the *FLO11* gene, preventing the formation of colony biofilms, whereas Tup1p antagonizes Cyc8p-mediated *FLO11* repression and, in addition, stabilizes the Flo11p protein by preventing its degradation.[22]

Here we show that Cyc8p negatively and Tup1p positively control adhesion of *S. cerevisiae* strains to plastic surfaces and subsequent formation of structured solid–liquid interface biofilm. The regulators influence adhesion of both shaken planktonic and static (sedimented) cells at any growth phase. In contrast to the wild strain, which is adhesive only in the absence of glucose, decreased level of Cyc8p also stimulates cell adhesion at high glucose concentrations. Glucose modulates cell adhesion and allows the release of planktonic cells from biofilms.

## RESULTS

### Adhesion to plastic varies in different *S. cerevisiae* strains

We first examined adhesion (the first step in biofilm formation) of non-isogenic strains BY4742 (a derivative of laboratory strain S288c), BRF (a wild strain) and domesticated strain BRS (Table 1), to polystyrene wells of microtiter plates. Two adhesion assays were performed. In the first assay, cells were inoculated directly into wells of microtiter plates and grown for 44 h (henceforth referred to as "static" cells) and then adherent cells were stained using crystal violet dye and quantified (Fig. 1a). In this assay, structured biofilm can develop (see below). In the second assay (Fig. 1b), planktonic cells were grown for 18 h in liquid medium with vigorous shaking (henceforth referred to as "planktonic" cells) and, after dilution to $A_{600} = 1$, were transferred to microtiter plate

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wells. After 3 h, adherent cells were stained as above and quantified. In this assay only the initial phase of biofilm formation, cell adhesion, was investigated. In both assays, strains were grown either in complete respiratory medium (GM) or in fermentative glucose medium (YD) (Fig. 1).

Only wild strain BRF was significantly adhesive. Neither BY4742 nor the BRS strain adhered to the plastic surface in any of the conditions tested (Fig. 1). Adhesion efficiency of both static and planktonic BRF cells was much higher in glucose-free GM than in glucose-rich YD. Moderate adhesion of BRF static cells in YD (Fig. 1a) could be due to glucose consumption and thus its decreased level during prolonged cultivation. Adhesion of planktonic GM-pre-grown BRF cells was on average twice as high as that of static cells (compare Fig. 1a, b).

### Cyc8p and Tup1p conversely regulate cell adhesion

Next, we asked whether Cyc8p and Tup1p, which regulate formation of structured colony biofilms[22] are also involved in BRF cell adhesion to plastic. We therefore assayed the adhesion capability of BRF-derived strains with modified levels of Cyc8p and Tup1p (Table 1). Deletion of gene CYC8 is lethal in the BRF strain, therefore we used strain BRF with one CYC8 allele deleted and the second allele placed under the control of the $p_{GAL}$ inducible promoter (BRF-$p_{GAL}$-CYC8) generating only a low basal level of CYC8 expression in the absence of galactose. Similarly, in order to control the level of TUP1 expression, we used strain BRF-$p_{GAL}$-TUP1 with one TUP1 allele deleted and the second controlled by $p_{GAL}$. As in the case of BRF-$p_{GAL}$-CYC8, the BRF-$p_{GAL}$-TUP1 strain expressed only a low basal level of TUP1 in the absence of galactose. We also used a strain, deleted in both TUP1 alleles (BRF-tup1). Loss of expression or low basal expression of TUP1, or constitutive over-expression of CYC8, completely eliminated the adhesion capability of the BRF strain (Fig. 1; strains BRF-tup1, BRF-$p_{GAL}$-TUP1, and BRF-$p_{TEF}$-CYC8). In contrast, a decreased level of CYC8 did not change adhesion capability of BRF in GM but greatly increased cell adhesion of both planktonic and static cells in high-glucose YD (Fig. 1; strain BRF-$p_{GAL}$-CYC8).

Expression of either TUP1 or CYC8 is inducible by galactose in BRF-$p_{GAL}$-TUP1 and BRF-$p_{GAL}$-CYC8, respectively. Galactose, like glucose, is a fermentative sugar that increases growth rate, and could reduce cell adhesion, as it does other biofilm properties,[23] at high concentration. Therefore, we first estimated biomass yield and cell adhesion after 24 h cultivation in GM or YD with different galactose concentrations. As expected, increased BRF biomass yield in static cultivation correlated with galactose concentration in GM (Fig. 2a). BRF adhesion in GM increased slightly up to 0.05% galactose, possibly because of incomplete well surface coverage by cells in poor GM. Cells in 0.05–0.5% galactose exhibited uniform adhesion, independently of enhanced growth. Even the highest initial galactose concentration (1%) did not significantly decrease cell adhesion and so structured biofilm was formed (Fig. 2a). As expected, galactose did not influence BRF growth and adhesion in YD medium.

BRF-$p_{GAL}$-TUP1 and BRF-$p_{GAL}$-CYC8 biomass accrual in GM with galactose was only slightly lower than that of BRF, but adhesion of these two strains varied greatly, depending to galactose concentration: due to the induction of TUP1 or CYC8 expression (Fig. 2a). BRF-$p_{GAL}$-TUP1 adhesion increased in GM as galactose

**Table 1.** Yeast strains.

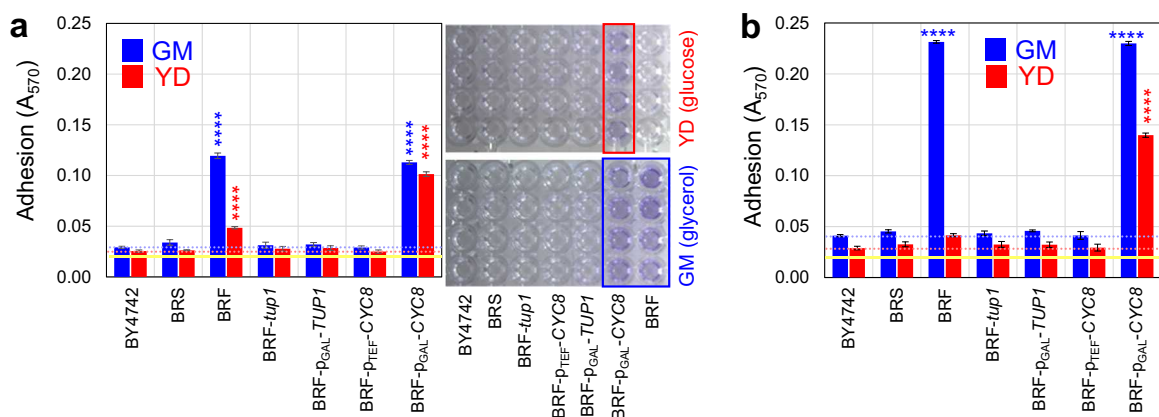| Strains | Genotypes | References |
|---|---|---|
| BRF | MATa/MATα, wild strain isolate | 34 |
| BRF-Flo11p-GFP | MATa/MATα, FLO11-GFP/FLO11 | 41 |
| BRS | MATa/MATα | 34 |
| BY4742 | MATα, his3Δ, leu2Δ, lys2Δ, ura3Δ | Euroscarf.de |
| BRF-$p_{TEF}$-CYC8 | MATa/MATα, FLO11-GFP/FLO11, nat1-$p_{TEF1}$-CYC8/CYC8 | 22 |
| BRF-$p_{GAL}$-CYC8 | MATa/MATα, FLO11-GFP/FLO11, cyc8Δ::KanMX, nat1-$p_{GAL1}$-CYC8 | 22 |
| BRF-$p_{GAL}$-TUP1 | MATa/MATα, FLO11-GFP/FLO11, tup1Δ::KanMX, nat1-$p_{GAL1}$-TUP1 | 22 |
| BRF-$p_{CUP}$-CYC8 | MATa/MATα, FLO11-GFP/FLO11, cyc8Δ::KanMX, nat1-$p_{CUP1}$-CYC8 | This study |
| BRF-$p_{CUP}$-TUP1 | MATa/MATα, FLO11-GFP/FLO11, tup1Δ::KanMX, nat1-$p_{CUP1}$-TUP1 | This study |
| BRF-Cyc8p-GFP | MATa/MATα CYC8-EGFP-kanMX/CYC8 | This study |
| BRF-Tup1p-GFP | MATa/MATα TUP1-EGFP-kanMX/TUP1 | This study |
| BRF-$p_{GAL}$-CYC8-$p_{CUP}$-TUP1 | MATa/MATα, tup1Δ::loxP, cyc8Δ::loxP, KanMX-$p_{CUP1}$-TUP1, nat1-$p_{GAL1}$-CYC8 | 22 |
| BRF-$p_{GAL}$-TUP1-$p_{CUP}$-CYC8 | MATa/MATα, tup1Δ::loxP, cyc8Δ::loxP, nat1-$p_{GAL1}$-TUP1, KanMX-$p_{CUP1}$-CYC8 | 22 |



**Fig. 1 Adhesion to plastic of wild, domesticated and laboratory strains and strains with changed level of Tup1p and Cyc8p regulators.** Adhesion of 24-h old static (**a**) or 18-h old shaken (**b**) cultures in GM (glycerol) in blue or YD (glucose) in red. Picture of plastic microtiter plate used for **a** measurements is shown. Blue and red dotted lines indicate $A_{570}$ value measured with non-adhesive laboratory strain BY4742. Yellow line indicates background absorbance (BA). Four distinct experimental replicates ($n = 4$) were measured for each strain and condition with results expressed as the means and s.d.'s. The statistical significance of the variation relative to the non-adherent BY4742 was determined using an unpaired two-tailed $t$-test and GraphPad Prism6 software; ****$p$-value < 0.0001.
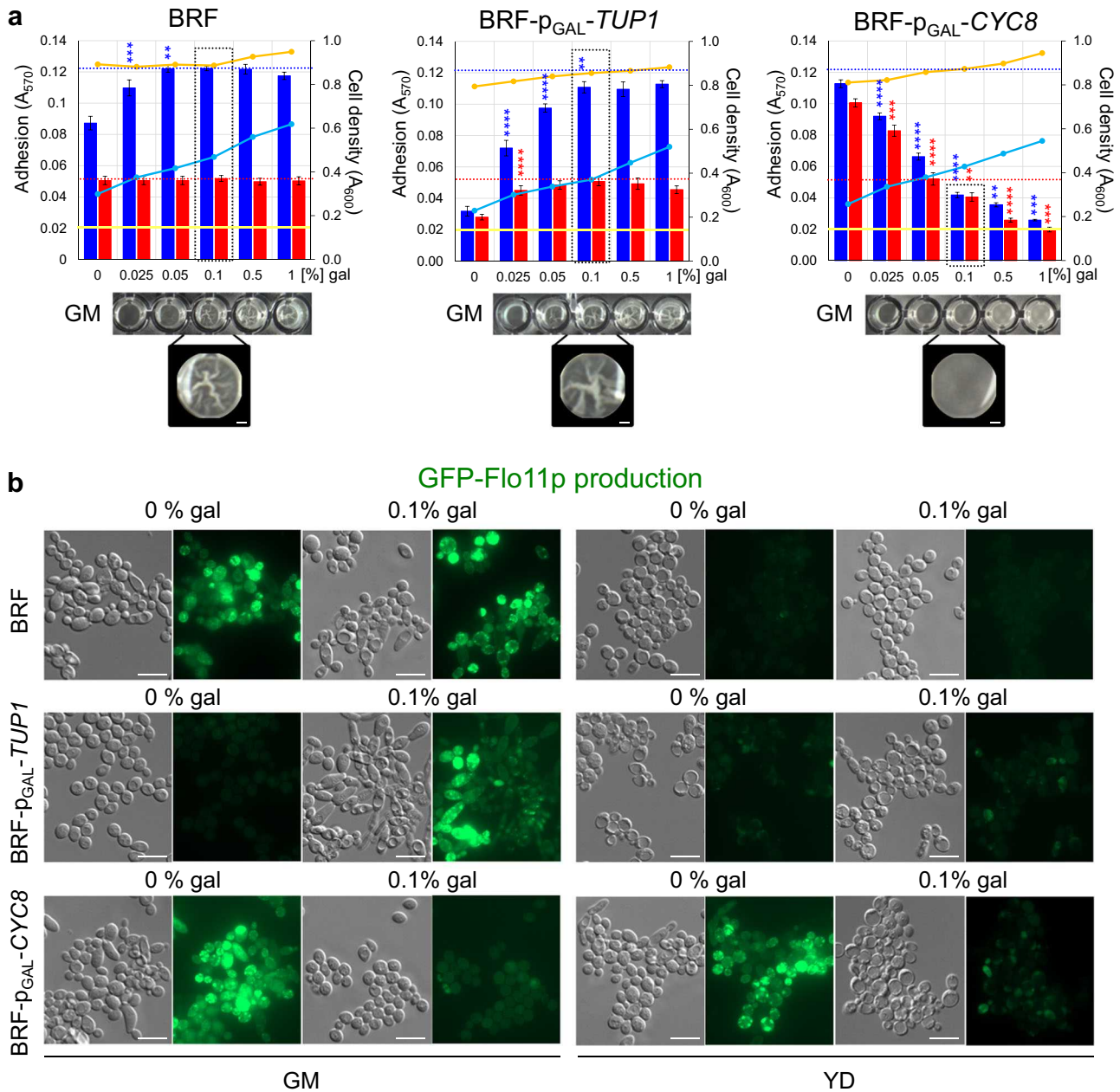
**Fig. 2 Effect of galactose on adhesivity, biofilm formation and Flo11p-GFP production of static BRF, BRF-p$_{GAL}$-TUP1 and BRF-p$_{GAL}$-CYC8 cells in GM and YD. a** Adhesivity of 24-h old static cultures in GM (blue bars) or YD (red bars) treated with 0–1% galactose (gal) was analyzed. Biomass yield ($A_{600}$) in GM (blue curve) and in YD (orange curve). Blue and red dotted lines indicate maximal $A_{570}$ value measured with BRF. Yellow line indicates BA. Solid-liquid interface biofilm formed by BRF and galactose-induced BRF-p$_{GAL}$-TUP1 in GM is shown at the bottom; bar, 1 mm. Four distinct experimental replicates ($n = 4$) were measured for each strain and condition with results expressed as the means and s.d.'s. The statistical significance of the variation between two succeeding galactose concentrations was determined using an unpaired two-tailed $t$-test and GraphPad Prism6 software; ****$p$-value < 0.0001, ***$p$-value < 0.001, and **$p$-value < 0.01. **b** Cell morphology (differential interference contrast, DIC) and presence of Flo11p-GFP in cells of respective strains grown in GM or YD and induced/non-induced by 0.1% galactose. Bar, 10 μm.

concentration ranged from 0 to 0.1% and was constant between 0.1 and 1% galactose. Wrinkled biofilms, visible to the naked eye, were formed over a range of 0.05–1% galactose. BRF-p$_{GAL}$-CYC8 adhesion decreased over the entire concentration range (0.025–1% galactose). In contrast to GM, the presence of galactose in YD did not affect BRF background adhesion and only moderately increased adhesion of BRF-p$_{GAL}$-TUP1. On the other hand, adhesion of BRF-p$_{GAL}$-CYC8 (non-induced) in YD was as high as in GM and was diminished by galactose induction of CYC8 with the same concentration-dependent profile in both media. The

p$_{GAL}$ promoter is inducible by galactose but also repressible by glucose, which together could result in a merely moderate increase in p$_{GAL}$-driven expression at high (2%) glucose concentration.[24] Therefore, we also assayed the effect of TUP1 and CYC8 induction on cell adhesion in GM and YD, using strains BRF-p$_{CUP}$-TUP1 and BRF-p$_{CUP}$-CYC8, inducible by copper. The results (Supplementary Fig. 1) were comparable to those obtained by galactose induction, demonstrating that galactose induction is not deficient in the presence of glucose. To minimize the effect of cell accrual, 0.1% galactose was chosen for further experiments,
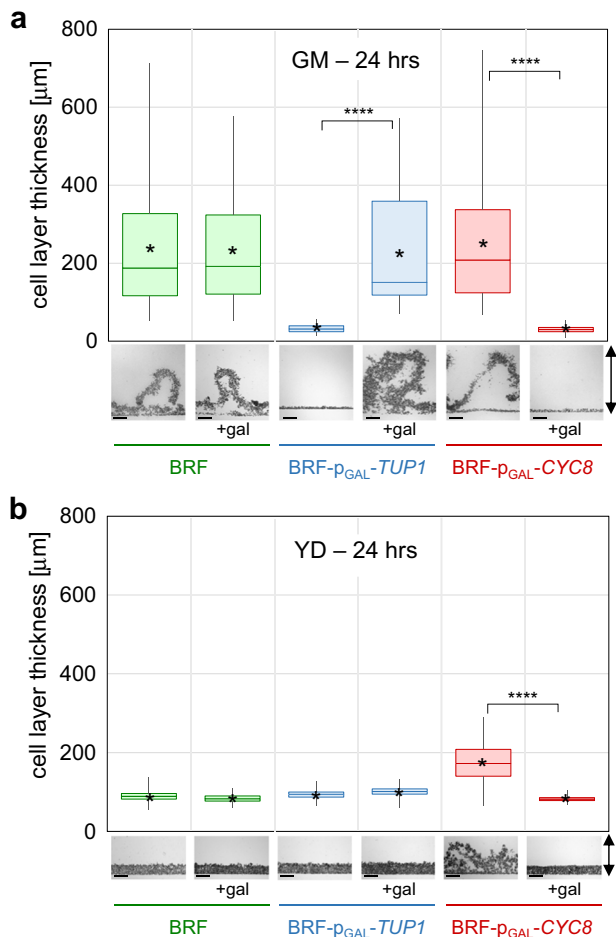
**Fig. 3** **Thickness of biofilms and non-adherent cell layers formed by BRF, BRF-p$_{GAL}$-TUP1 and BRF-p$_{GAL}$-CYC8 cells.** Vertical cross-sections of 24-h-old static cultures in GM (**a**) or YD (**b**) without galactose or treated with 0.1% galactose (gal) were used for measurement of thickness of biofilms and non-adherent cell layers. The box plots show distribution of thickness measured at different positions (deep and shallow wrinkles) within biofilms and non-adherent cells (box extends from the 25th to 75th percentiles with center line representing the median and whiskers from Min to Max, asterisk indicates the mean). >200 different positions from 3 to 5 distinct cross-sections were measured for each plot using ImageJ. The statistical significance of the variation between galactose-treated and untreated samples was determined using an unpaired two-tailed $t$-test and GraphPad Prism6 software; ****$p$-value < 0.0001. Pictures at bottom show examples of cross-sections used for the measurement; black arrows indicate vertical direction of cross-sections. Bar, 100 μm.

producing uniform adhesion of BRF, maximal adhesion of BRF-p$_{GAL}$-TUP1 and a drop in BRF-p$_{GAL}$-CYC8 adhesion to below the BRF-adhesion threshold.

Next, we used strains BRF-p$_{GAL}$-TUP1-p$_{CUP}$-CYC8 and BRF-p$_{GAL}$-CYC8-p$_{CUP}$-TUP1 (Table 1), in which both CYC8 and TUP1 were controlled by inducible promoters to assay the balancing effect of Cyc8p and Tup1p levels on cell adhesion in 24-h-old static cultures in GM and YD (Supplementary Fig. 2). In GM medium, induction of CYC8 and TUP1 (using galactose plus Cu$^{2+}$), or of TUP1 (by galactose or Cu$^{2+}$) in the absence of Cyc8p, lead to adhesion comparable with BRF adhesion. In contrast, induction of CYC8 (by galactose or Cu$^{2+}$) in the absence of Tup1p eliminated adhesion almost completely. Adhesion of both strains without induction of either regulator (no galactose, no Cu$^{2+}$) was also strongly reduced, but was still higher than in the presence of high Cyc8p. In YD

medium, low adhesion, comparable with BRF, was found when strains were induced by galactose and Cu$^{2+}$ (high Tup1p and Cyc8p) as well as in the absence of their induction (low Tup1p and Cyc8p). Adhesion was almost completely eliminated when Cyc8p was high and Tup1p low (galactose or Cu$^{2+}$ induction of only CYC8). When Cyc8p was low and Tup1p high (galactose or Cu$^{2+}$ induction of only TUP1), adhesion increased as in the case of non-induced strain BRF-p$_{GAL}$-CYC8, in which TUP1 is controlled by its native promoter (compare Supplementary Fig. 2 and Fig. 1a). Hence, increased adhesion in YD due to the absence of CYC8 also requires the presence of Tup1p.

We further determined the correlation between strain adhesion and Flo11p expression. Cells of the BRF, BRF-p$_{GAL}$-TUP1 and BRF-p$_{GAL}$-CYC8 strains grown in GM or YD with 0 or 0.1% galactose in the wells were collected to estimate Flo11p-GFP presence (Fig. 2b). Flo11p-GFP level strictly correlated with strain adhesion ability, being high in BRF in GM (with or without galactose), BRF-p$_{GAL}$-TUP1 in GM with galactose (induced TUP1 expression) and BRF-p$_{GAL}$-CYC8 in either GM or YD without galactose (diminished CYC8 expression). In all other conditions, Flo11p-GFP fluorescence was negligible.

These data showed that Cyc8p and Tup1p conversely regulate cell adhesion involving the Flo11p adhesin, which is important in abiotic surface adhesion. Moreover, as with cell adhesion, Flo11p expression is strongly de-repressed in high glucose when Cyc8p level drops (YD without galactose) and Tup1p is present.

### Cyc8p and Tup1p conversely regulate formation of structured biofilm

Next, we determined whether differences in adhesion mediated by Cyc8p and Tup1p, were reflected in formation of wrinkled solid–liquid interface biofilms. BRF, BRF-p$_{GAL}$-TUP1 and BRF-p$_{GAL}$-CYC8 strains were grown for 24 and 48 h in GM and YD with and without galactose and the minimum and maximum thickness of structured biofilm wrinkles and of non-adherent cell layers, respectively, were measured. In accordance with cell adhesion results, the formation of the three-dimensional structure of the wrinkled biofilm was also dependent on the presence of Tup1p and was inhibited by increased Cyc8p expression (Fig. 3). The thickness of 24-h-old biofilm formed by BRF, BRF-p$_{GAL}$-TUP1 (induced by galactose) and BRF-p$_{GAL}$-CYC8 (non-induced) was greater in GM medium (Fig. 3a) than that of biofilm formed by BRF-p$_{GAL}$-CYC8 (non-induced) in YD (Fig. 3b). This indicates that low Cyc8p is sufficient to induce efficient adhesion and initiation of biofilm formation in the presence of glucose, while the development of fully structured biofilm probably requires other factors that are absent in high glucose conditions. Further cultivation did not significantly influence biofilm thickness as 48 h-old biofilms exhibited a thickness, similar to that of 24 h-old biofilms (compare Fig. 3 and Supplementary Fig. 3).

### Glucose disrupts biofilm and releases adhered cells

Intriguingly, decreased level of Cyc8p greatly increased cell adherence and biofilm formation in the presence of glucose (in YD). We therefore tested the hypothesis that level of glucose could modulate release of biofilm cells. Different concentrations of glucose were applied for 4 h either to intact biofilm of strain BRF (formed during 44 h static cultivation in GM) or to the same biofilm, but after gentle removal of liquid medium with non-adherent cells. In both cases, glucose caused significant biofilm disruption and release of adherent cells (Fig. 4a). Even a concentration of 0.1% glucose decreased adhesion by ~15% and ~30%, respectively. Two percent glucose, added after removal of the medium, caused release of almost 70% of adherent cells (Fig. 4a).
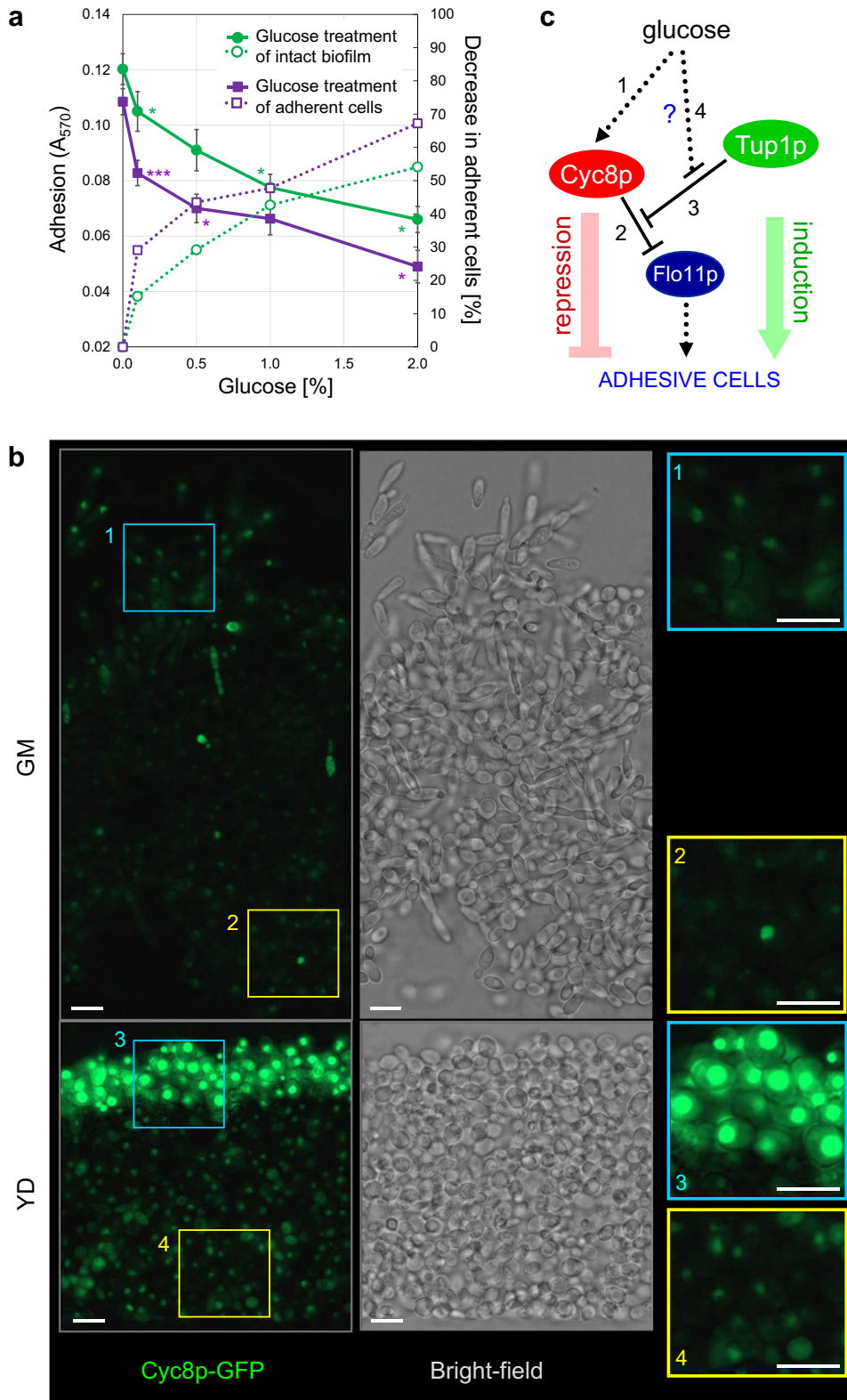
Glucose increases amount of Cyc8p in cell nuclei

To further clarify the relationship between glucose and Cyc8p function, we constructed BRF strains with either Cyc8p or Tup1p tagged with GFP (strains BRF-Cyc8p-GFP and BRF-Tup1p-GFP, Table 1). Both Cyc8p-GFP and Tup1p-GFP localize to the nucleus. We then used these strains to estimate levels of Cyc8p and Tup1p in non-adherent static cells grown in the presence of glucose (YD) and in biofilms grown in the absence of glucose (GM). Biofilms and non-adherent cells were grown in microtiter plates for 24 h and vertical cross-sections analyzed by fluorescence and bright-field microscopy. The level of Cyc8p-GFP was significantly higher in non-adherent cell layers in YD, than in biofilms in GM (Fig. 4b).

**Fig. 4 Role of glucose in Cyc8p and Tup1p regulation of yeast adhesion and biofilm formation: experimental data and scheme. a** Glucose was added to the intact biofilm in GM (green) or to the adherent cells after medium removal (violet). Solid lines, cell adhesion after glucose treatment; dotted lines, decrease in percentage of adhesive cells due to glucose treatment. Experiments were conducted in quadruplicate (distinct samples, $n = 4$) with results expressed as the means and s.d.'s. The statistical significance of the variation between two succeeding galactose concentrations was determined using an unpaired two-tailed t test and GraphPad Prism6 software; ***$p$-value < 0.001 and *$p$-value < 0.05. **b** Vertical cross-sections of 24-h-old static cultures of BRF-Cyc8p-GFP strain in GM or YD; Cyc8p-GFP fluorescence in green, cells are visible in bright-field. Cells in Insets "1–4" are shown at higher magnification, indicating distribution and intensity of fluorescence of Cyc8p-GFP in different biofilm/non-adherent cell regions. Bar, 10 μm. A representative experiment of three ($n = 3$) independent experiments is shown. **c** Model of glucose function: Glucose induces Cyc8p expression/function "1", subsequently Cyc8p represses *FLO11* expression "2" and cell adhesion is blocked. This effect would be enhanced if glucose also negatively affects Tup1p function "4". In glucose absence, Cyc8p level is decreased and its function is inhibited via Tup1p "3" as described.[22] Subsequently Flo11p is produced and contributes to cell adhesion. Arrow, induction; blunt line, repression. Dotted line, additional proteins may participate in the effect. Light-color blunt line (red) and arrow (green) indicate effects of the regulators on cell adhesion.

In addition, the Cyc8p-GFP level was high in nuclei of surface cell layers, in particular in YD, and lower in internal regions. This may be because the glucose had already been spent in lower cells that were not in direct contact with the medium. Hence, the major difference in Cyc8p level between biofilms and non-adherent cell layers concerns surface areas. In contrast to Cyc8p, no visible difference in Tup1p-GFP level was observed between biofilms and non-adherent cell layers (Supplementary Fig. 4).

**Tup1p and Cyc8p regulate adhesion of both planktonic and static cells independently of growth phase**

Next we asked whether cell adhesion is regulated by Tup1p and Cyc8p identically or differently, depending on the cell state, such as different cell growth phases (exponential, diauxic, and stationary phase) or different lifestyle (static versus planktonic cells). We also tested the speed of the cellular response (cell adhesion or release) to increased Tup1p or Cyc8p levels by treating cells with inducer (galactose) for 6 or 10 h.

In the "static" setup, BRF-$p_{GAL}$-*TUP1*, BRF-$p_{GAL}$-*CYC8*, and BRF were grown in GM or YD in wells, *TUP1* or *CYC8* expression was induced by galactose at specific time-points for either 6 (Fig. 5d–f) or 10 h (Supplementary Fig. 5d–f) and adhesion analyzed. In parallel, we measured growth curves of all strains (Fig. 5a–c and Supplementary Fig. 5a–c). Adhesion of BRF in GM reached a similar level, independently of galactose presence and also of cell density, which is higher in galactose-treated cultures. The BRF adhesion maximum in GM was reached at 17 h at a cell density, corresponding to $A_{600} \sim 0.4$ and it remained constant later (Fig. 5d), despite continued growth of the strain (Fig. 5a). As expected, BRF-$p_{GAL}$-*TUP1* with low *TUP1* expression exhibited only basal adhesion in GM. Induction of *TUP1* expression by 6-h-galactose treatment at any time-point was not sufficient to induce the maximal level adhesion reached by BRF (compare Fig. 5d, e). As with BRF, adhesion of younger BRF-$p_{GAL}$-*TUP1* populations was slightly lower than that of older ones (Fig. 5e). Adhesion, similar to that of BRF, was reached only after 10-h induction of *TUP1* expression (Supplementary Fig. 5e). Adhesion of BRF-$p_{GAL}$-*CYC8* with low *CYC8* expression in GM (Fig. 5f and Supplementary Fig. 5f) resembled that of BRF (Fig. 5d and Supplementary Fig. 5d). Induction of *CYC8* expression by galactose diminished BRF-$p_{GAL}$-*CYC8* adhesion almost independently of treatment period (Fig. 5f and Supplementary Fig. 5f). As expected, in YD, BRF exhibited only basal adhesion, independently of galactose treatment (Fig. 5d and Supplementary Fig. 5d). A low level of Tup1p in BRF-$p_{GAL}$-*TUP1* caused a slight decrease in this basal adhesion in YD, which reverted back after galactose induction of *TUP1* (Fig. 5e and Supplementary Fig. 5e). A low level of Cyc8p always increased adhesion efficiency in YD almost to the same level as in GM and galactose induction of *CYC8* diminished adhesion similarly in YD and GM (Fig. 5f and Supplementary Fig. 5f).

In the "planktonic" setup (Fig. 6 and Supplementary Fig. 6), the same number of planktonic cells was applied to the wells in each time-point and thus the adhesion efficiency was not influenced by cell numbers in wells. In contrast to static cultures, adhesion efficiency was completely independent of cell growth phase and reached highest levels under conditions, permitting adhesion (Fig. 6d–f and Supplementary Fig. 6d–f). Adhesion of planktonic BRF cells was independent of galactose presence (similarly to static cultures), being high in GM and basal in YD (Fig. 6d and Supplementary Fig. 6d). BRF-$p_{GAL}$-*TUP1* adhesion was basal in GM and YD and increased only in GM due to galactose-mediated *TUP1* induction, reaching higher values after 10 than 6 h of galactose treatment (Fig. 6e and Supplementary Fig. 6e). BRF-$p_{GAL}$-*CYC8* adhesion was comparable with BRF in GM but, again, significantly higher in YD (Fig. 6f and Supplementary Fig. 6f). In contrast to static cells, 6-h-galactose induction of *CYC8* led to a smaller decrease in adhesiveness (Fig. 6f) than 10 h-treatment (Supplementary Fig. 6f) and neither reduced adhesion to BRF basal level (compare Fig. 6d, f and Supplementary Fig. 6f, d).

In summary, these data indicate that Cyc8p and Tup1p mediated regulation is robust, functioning similarly in static and planktonic cells and being only moderately influenced by cell culture growth phase. In both static and planktonic cultivation, the effect of Cyc8p induction is similar after 6 and 10 h and is thus quicker than that of Tup1p which is higher after 10 than after 6 h. However, the decrease in adhesion, compared with BRF, is higher in static than in planktonic cells upon Cyc8p induction. These observations are consistent with other data indicating that an increase in Cyc8p level causes release of adherent cells from already formed biofilms.

**DISCUSSION**

We have shown that adhesion of wild strain BRF to an abiotic solid surface, the related Flo11p expression, and the formation of structured biofilm at a solid–liquid interface, are regulated conversely by Tup1p and Cyc8p. Furthermore, we have revealed that Tup1p-induced adhesion is efficient in glucose-free conditions, whereas Tup1p presence is necessary but not sufficient to induce adhesion of cells grown in the presence of glucose. In contrast, Cyc8p-mediated repression of adhesion is efficient and comparable in both conditions, thus demonstrating that Cyc8p is an important player in glucose-regulated cell adhesion and biofilm formation.

Adhesion of both static and planktonic cells is regulated by Cyc8p and Tup1p, with only slight differences. Relatively homogeneous planktonic cells adhered independently of growth phase and with higher efficiency than their static counterparts. Tup1p-mediated adhesion efficiency slightly increased in older static cultivations. This indicates that more adhesive and less adhesive cells are present near to the plastic surface in static cultivations and that the frequency of more adhesive cells slightly increases as the biofilm develops. Static biofilm cells also respond faster than planktonic cells to an increase in Cyc8p by massive release from
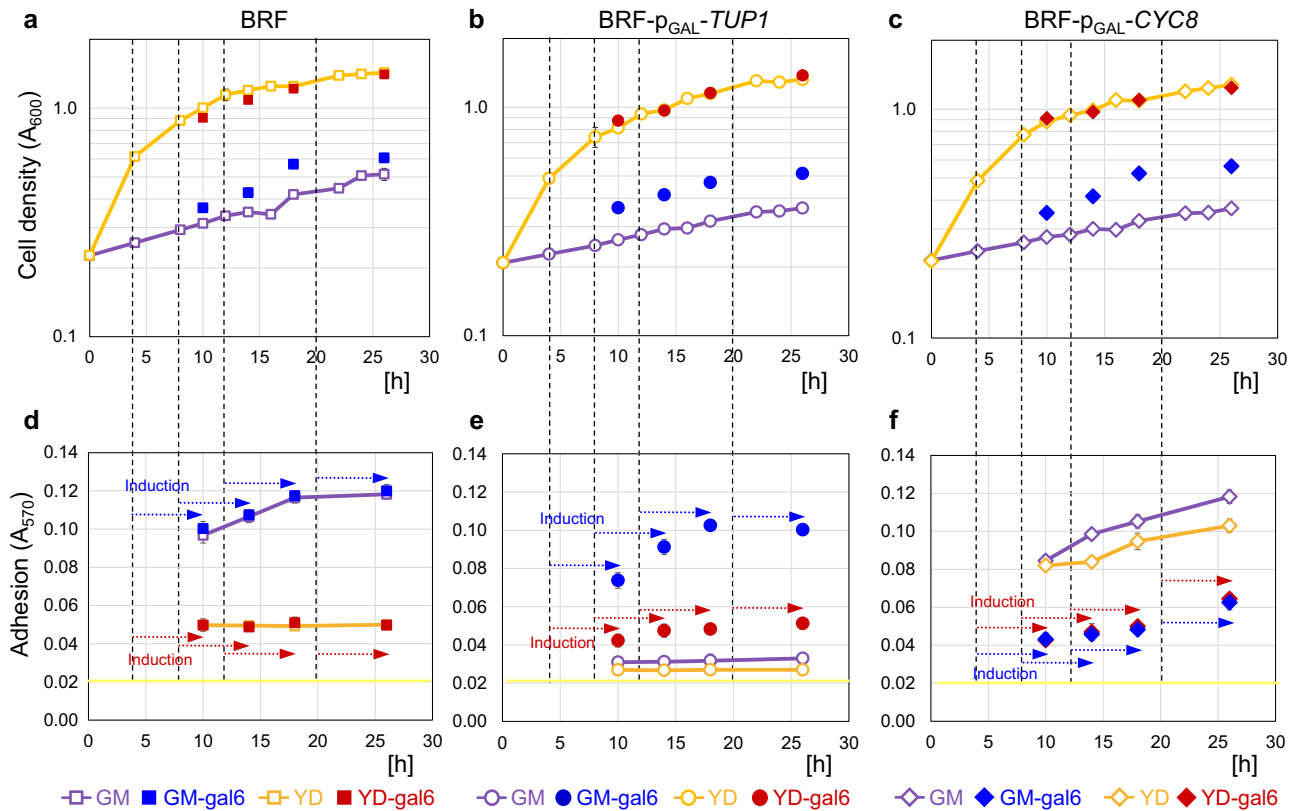
**Fig. 5  Effect of Tup1p or Cyc8p induction on adhesivity of cells in different growth phases of static cultures. a–c** Strain growth curves in GM and YD. gal6, galactose was added 6 h before measurement of the biomass ($A_{600}$). **d–f** Adhesivity of the strains without induction and after 6 h of galactose induction. Arrows (**d–f**) indicate interval of galactose presence in relation to time-points (indicated by dashed lines), in which galactose was added. Yellow line indicates BA. Experiments were conducted in quadruplicate (distinct samples, $n = 4$) with results expressed as the means and s.d.'s.

the plastic surface. Cell response to *TUP1* induction was slower, but still quicker in static than in planktonic cells.

BRF cell adhesion and biofilm formation is strictly dependent on medium composition, being high in the absence of glucose ("permissive") and low in high glucose ("non-permissive") conditions. Tup1p is a key activator of cell adhesion under permissive conditions, but even increased Tup1p levels had negligible effect under non-permissive conditions when Cyc8p was expressed from its native promoter. In contrast, increased Cyc8p level seems to exert a strong effect under non-permissive conditions, counteracting the Tup1p and Flo11p functions, potentially via mechanisms, described recently.[22] Glucose may thus regulate Cyc8p level and/or repressor activity (Fig. 4c). Several factors participating in glucose regulation, such as Nrg1p, Mig1p, and Sfl1p, were shown to interact with, and influence the function of, the Cyc8p-Tup1p complex.[11,25,26] However, neither of these factors is involved in antagonistic Cyc8p and Tup1p regulations.[22] Little information is available on environmental regulation of the *CYC8* gene at the transcription level, but genome-wide transcriptomic screens showed the level of *CYC8* mRNA to be ~2.4 times higher after 15 min treatment in 2% glucose than in 0.05% glucose[27] and *CYC8* mRNA level gradually decreased during prolonged cultivation in glucose YPD medium,[28] possibly due to glucose consumption. Here we show that the level of Cyc8p-GFP is much higher in nuclei of cells at surface layers of non-adherent cells grown in glucose-rich YD medium than in nuclei of cells in biofilms grown in glucose-free GM. Altogether, these data support the hypothesis that Cyc8p level itself is regulated by glucose (Fig. 4c), though the existence of a specific, as yet unidentified, factor influencing repressive Cyc8p function, cannot be excluded. Cyc8p level is affected by glucose mainly in surface cells, which are in contact

with the fluid (medium). This is in agreement with the observed efficient release of solid–liquid interface biofilm upon the addition of glucose. In principle, in this way, whole biofilms and/or individual planktonic cells could be efficiently released from solid/semi-solid supports.

Involvement of a glucose-responsive factor in adhesion and biofilm formation could play an important role in yeast virulence and in systemic and biofilm infections (Fig. 7). When a yeast cell settles in a low-glucose niche (tissue), the level/function of Cyc8p is lowered, allowing Tup1p to mediate cell adhesion and biofilm development (Fig. 7, "Biofilm 1"). Contact of biofilms with higher glucose environments (such as blood/plasma) causes an increase in Cyc8p level/function and subsequent decrease in adhesion and release of free planktonic cells. These cells may then be dispersed until they reach another low glucose niche (another tissue), in which Cyc8p level/function is again repressed, allowing yeast to adhere and form biofilm (Fig. 7, "Biofilm 2") therein. By this mechanism, planktonic cells and biofilms are able to spread and survive in a heterogeneous environment, such as a host organism. As even 0.1% (=~5.5 mM) glucose concentration is sufficient to release 15–30% of cells from the biofilm (Fig. 4a), our model is relevant under physiological conditions, since normal blood glucose concentration is below 7.8 mM (2 h post-prandial).[29] Transient glycosuria in diabetics or some pregnant women may also cause yeast biofilm to spread to the genitourinary tract.

It has been shown that, in addition to anticancer treated immunocompromised patients, diabetic patients represent another group, at high-risk of invasive fungal infections[30] and examples of *S. cerevisiae* infection have been reported in these patients.[1] An increase in glucose level has been detected in blood and other body niches such as urine, intestinal fluid, mucus,
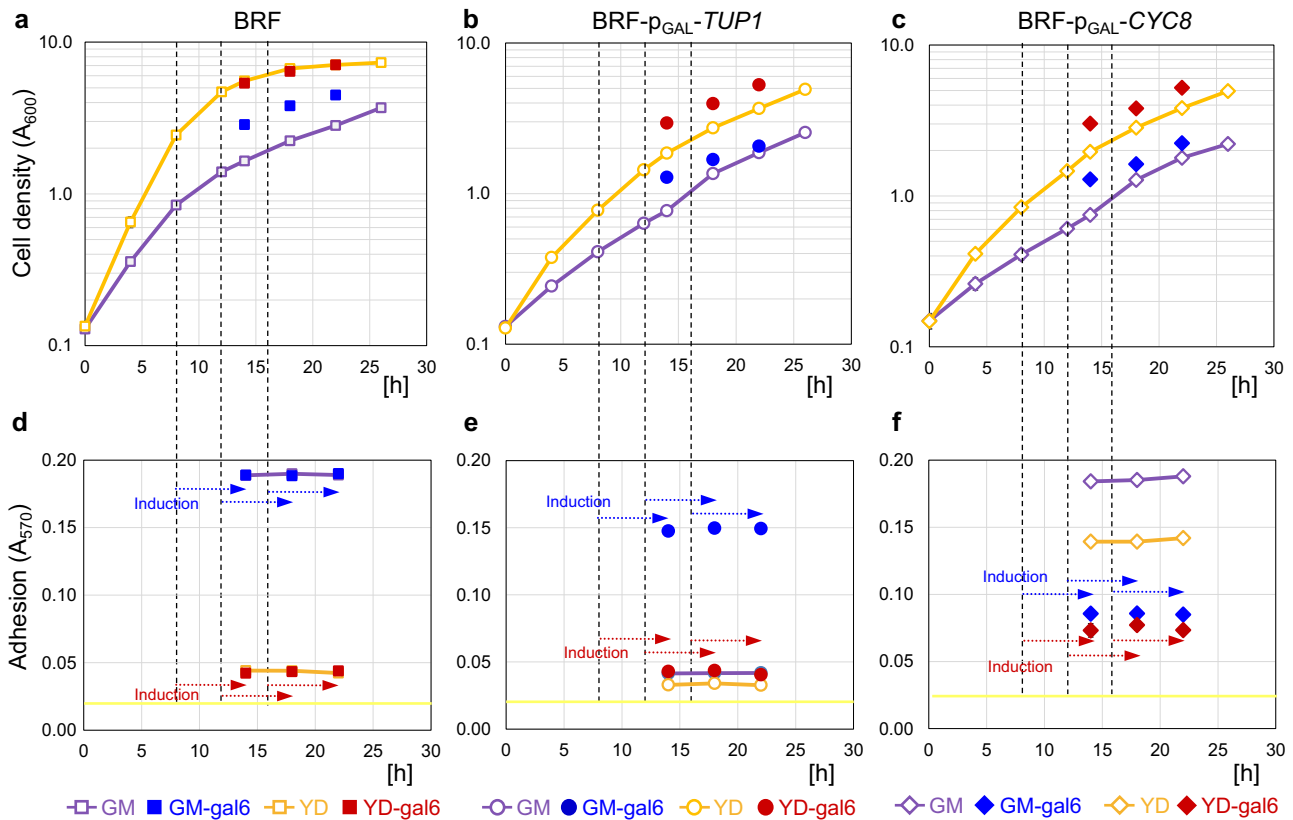
**Fig. 6  Effect of Tup1p or Cyc8p induction on adhesivity of planktonic cells from different growth phases of shaken cultures. a–c** Strain growth curves in GM and YD. gal6, galactose was added 6 h before measurement of the biomass ($A_{600}$). **d–f** Adhesivity of the strains without induction and after 6 h of galactose induction. Arrows (**d–f**) indicate period of galactose treatment in relation to time-points (indicated by dashed lines) in which galactose was added. Yellow line indicates BA. Experiments were conducted in quadruplicate (distinct samples, $n = 4$) with results expressed as the means and s.d.'s.

sweat/perspiration and saliva[31] and is a known risk-increasing factor in fungal infections for two main reasons: Increased glucose benefits yeast cell growth and decreases the effect of some antifungal drugs.[30,32] Here we speculate about a third glucose-related risk—increase of glucose in some niches could lead to release of adherent cells and their dispersal within the organism, potentially followed by development of new biofilms as new outbreaks of infection. We show that Cyc8p is a key factor in this process and, together with Cyc8p and Tup1p mediated anti-regulation, is effective in both adherent and planktonic cells and mostly independent of growth phase. We also proved that an increase in glucose concentration causes release of adherent cells from complex biofilm structures. Both Cyc8p and Tup1p are conserved among yeast and information concerning their function in *Candida* spp. cell adhesion is scarce. One indication of a positive role for Tup1p in adhesion of *C. albicans* comes from findings that a *tup1Δ* strain forms only pseudohyphae with reduced adhesion to keratinocytes.[33] Hence, besides Cyc8p and Tup1p being potential risk factors and therapeutic targets in (so far) less frequent *S. cerevisiae* infections, Cyc8p and Tup1p orthologues may be involved in adhesion, biofilm formation and cell release in *Candida* spp., in particular *C. glabrata*, a close relative of *S. cerevisiae*.

## METHODS
### Yeast strains and media
Strains used in this study (Table 1) were derived from wild strain BRF[18,34] from the collection of the Institute of Chemistry, Slovak Academy of Sciences (collection number CCY 21-4-97). Strain BY4742 was obtained from Euroscarf (collection number Y10000). For adhesion assays, strains were grown in GM (3% glycerol, 1% yeast extract) or YD (2% glucose, 1% yeast extract) media without any additives, or supplemented with galactose and/or $Cu^{2+}$ ($CuSO_4$) in final concentration as described in the Result section.

### Strain constructions
*CYC8* and *TUP1* gene knock-outs were performed by transforming the cells with deletion cassettes generated by PCR from plasmid pUG6.[35] Strains with C terminal GFP fusions were constructed using a GFP-KanMX integrative cassette, amplified by PCR from plasmid pKT127.[36] Strains expressing *TUP1* or *CYC8* under the control of inducible promoter $p_{CUP}$ were constructed by integration of $p_{CUP1}$-natNT2 cassettes amplified from the pYM-N2 plasmid.[37] Yeast cells were transformed using a standard lithium acetate/polyethylene glycol method.[38] Positive transformants were selected on GMA (GM, 2% agar) supplemented with G418 (200 mg/l) or nourseothricin (100 mg/l). Correct genomic integration of cassettes was verified by PCR using specific primers and by sequencing. The primers and plasmids are listed in Supplementary Table 1.

### Cell cultivation for adhesion assays
Biofilm cultures (static cells). Cells from overnight cultures in GM or YD (28 °C, with shaking) were harvested. Biomass was inoculated into fresh GM or YD, respectively, to a concentration of 0.3 mg wet weight/ml. For biofilm cultivation, 150 µl of cell suspension was pipetted into each well of a polystyrene 96 well microtiter plate V400917 from GAMA group Inc., CZ (four independent replicates per strain and condition) and incubated at 28 °C. If needed, galactose and/or $Cu^{2+}$ ($CuSO_4$) was added to the required final concentration. Cell absorbance ($A_{600}$) was determined at indicated time-points. In parallel, cell adhesion was determined.

Shaken cultures (planktonic cells): 10 ml of cell suspension was cultivated at 28 °C in an Erlenmeyer flask with vigorous shaking (150 rpm) and cell density determined as $A_{600}$. If needed, galactose and/
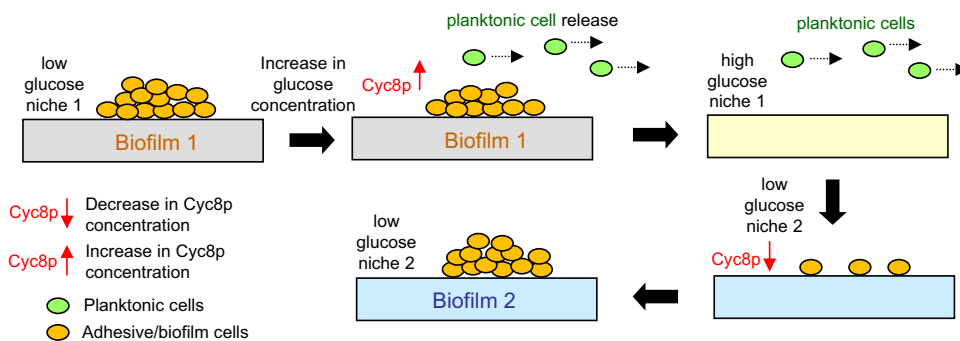
**Fig. 7  Model of role of glucose in formation of solid–liquid interface biofilm.** Level of environmental glucose can regulate switch between cell adhesion/biofilm formation and release of planktonic cells in structured heterogeneous environment (organism). Biofilm 1, primary biofilm from which planktonic cells may be released when glucose concentration increases. Released cells may then settle in new low-glucose niches, in which they form secondary biofilm (Biofilm 2).

or $Cu^{2+}$ (CuSO$_4$) was added to the required final concentration. At appropriate time-points, cells were harvested, washed and resuspended in water to $A_{600} = 1$. Then 150 µl of the cell suspension (approximately $1–3 \times 10^7$ cells/ml) was pipetted into the wells of a 96 well microtiter plate (four independent replicates per strain and condition) and incubated at 28 °C. Adhesion was determined after 3 h incubation.

### Adhesion assays
The adhesion assay[9,39] was performed with modifications. The liquid (medium or water) was removed and the microtiter plates thoroughly washed by submerging three times in water. One hundred and fifty microliter of 1% crystal violet dye was added to each well and plates were incubated at room temperature with gentle shaking. After 15 min, the dye solution was removed and the plates were washed three times with distilled water. Afterwards, 150 µl of 95% ethanol was added to each well to elute the dye from attached cells. Hundred microliter of the crystal violet eluate from each well was transferred to a new microtiter plate and the absorbance ($A_{570}$) measured by Epoch Microplate Spectrophotometer (Biotek). $A_{570}$ value reflects the number of adherent cells and was taken as a measure of relative efficiency of adhesion. In control measurements, the complete procedure was performed in a microtiter plate without cells. The measured value, 0.02 was indicated in the graphs as the background absorbance (BA).

### Release of adherent cells by glucose
Glucose was added (i) directly to the well containing the intact biofilm in GM or (ii) to the biofilm after removal of GM with non-adhered cells and washing, to desired final concentration. After 4 h incubation, all non-adherent cells were removed by washing and adhesive cells quantified as above.

### Biofilm imaging and cell microscopy
Images of biofilms in wells of microtiter plate were captured in incident and/or transmitted light. A ProgRes® CT3 CMOS camera with a Navitar objective and NIS Elements software (Laboratory Imaging, s.r.o, Prague, CZ) were used. For thickness analyses of biofilms and non-adherent cell layers and analyses of Tup1p-GFP and Cyc8p-GFP expression, biofilms and non-adherent cell layers were fixed with 4% agarose and sectioned using a Leica VT1200S vibrating microtome.[40] Re-suspended cells or sections of biofilms/non-adherent cell layers were observed using Carl Zeiss Axio Observer.Z1 fluorescence microscope equipped with Axiocam 506 and a C-Apochromat 10×/0.45 W objective (for thickness analyses) or a C-Apochromat 63×/1.20 W (for GFP expression) using ZEN 2012 (blue edition) software. Filter sets for GFP (excitation 450–490 nm; emission 500–550 nm), differential interference contrast (DIC) or bright field were used. The thickness of biofilms/non-adherent cell layers was measured from images using ImageJ (version 1.52a).

### Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## DATA AVAILABILITY
The data that support the findings of this study are included in the article, its supplementary information files, or are available from the corresponding author upon reasonable request.

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## AUTHOR CONTRIBUTIONS

Z.P. and L.V. devised the study and experimental design. P.N. and V.P. conducted the experiments and processed the data. Z.P. and L.V. analyzed and interpreted the data and wrote the manuscript with contribution by P.N. and V.P. All authors read and approved the final manuscript.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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