REVIEW ARTICLE

# Choosing the right lifestyle: adhesion and development in *Saccharomyces cerevisiae*

Stefan Brückner & Hans-Ulrich Mösch

Department of Genetics, Philipps-Universität Marburg, Marburg, Germany

**Correspondence:** Hans-Ulrich Mösch, Department of Genetics, Philipps-Universität Marburg, Karl-von-Frisch-Straße 8, D-35043 Marburg, Germany. Tel.: +49 6421 282 3013; fax: +49 6421 282 3032; e-mail: moesch@staff.uni-marburg.de

Received 7 February 2011; revised 21 March 2011; accepted 4 April 2011.
Final version published online 20 May 2011.

DOI:10.1111/j.1574-6976.2011.00275.x

Editor: Gerhard Braus

**Keywords**
adhesins; biofilms; microbial development; cell surface; social behavior; signaling.

## Abstract

The budding yeast *Saccharomyces cerevisiae* is a eukaryotic microorganism that is able to choose between different unicellular and multicellular lifestyles. The potential of individual yeast cells to switch between different growth modes is advantageous for optimal dissemination, protection and substrate colonization at the population level. A crucial step in lifestyle adaptation is the control of self- and foreign adhesion. For this purpose, *S. cerevisiae* contains a set of cell wall-associated proteins, which confer adhesion to diverse biotic and abiotic surfaces. Here, we provide an overview of different aspects of *S. cerevisiae* adhesion, including a detailed description of known lifestyles, recent insights into adhesin structure and function and an outline of the complex regulatory network for adhesin gene regulation. Our review shows that *S. cerevisiae* is a model system suitable for studying not only the mechanisms and regulation of cell adhesion, but also the role of this process in microbial development, ecology and evolution.

## Introduction

Adhesion of cells to each other and to foreign surfaces is key for multicellular development, colonization and pathogenesis. Adhesive properties are predominantly conferred by specific cell surface proteins, the adhesins. In metazoa, cell adhesion is mediated by several classes of diverse cell adhesion molecules, whose structures and functions during development have been studied in detail (Beckerle, 2002; Abedin & King, 2010). In bacteria, the central role of adhesins during biofilm formation and host–microorganism interactions is becoming more and more evident and is at the focus of many investigations that include diverse bacterial species (Kline *et al*., 2009; Flemming & Wingender, 2010). In fungi, functional studies in the budding yeast *Saccharomyces cerevisiae* and in pathogenic *Candida* species have shown that adhesins allow agglutination of sexual partners before cell fusion, enable the formation of protective and invasive multicellular growth forms, mediate adherence to foreign biotic and abiotic surfaces and confer interactions with solid substrates and host cells (Hoyer, 2001; Kaur *et al*., 2005; Verstrepen & Klis, 2006; Dranginis *et al*., 2007). Comparative genomics fueled by the growing number of genome sequences has helped to identify putative adhesin genes and families in other fungal species as well (Verstrepen *et al*., 2004; Galagan *et al*., 2005; Linder & Gustafsson, 2008; Ramage *et al*., 2009). However, many fungi exhibit only a poor experimental tractability. Therefore, detailed insights into fungal adhesin structure and function as well as into the regulatory networks involved must rely on studies with tractable model systems.

*Saccharomyces cerevisiae* is a formidable organism to investigate almost every aspect of eukaryotic molecular and cell biology. This also includes adhesion of sexual partner cells as well as nonsexual, vegetative adhesion. Sexual adhesion of budding yeast is well understood and is mediated by cell-type-specific adhesins called agglutinins, which are produced by mating partners after exchange of pheromones and confer cell–cell adherence by high-affinity heterotypic protein–protein interactions (Lipke & Kurjan, 1992; Chen *et al*., 2007; Dranginis *et al*., 2007). In contrast, detailed molecular insights into vegetative adhesion in *S. cerevisiae* have been lacking, most likely because many laboratory strains have acquired mutations that suppress this process (Liu *et al*., 1996). On the other hand, the process of flocculation, the reversible adhesion of vegetative cells, has been widely studied by classical genetics in industrial production strains (Bauer *et al*., 2010). However, detailed biochemical and structural studies of the responsible adhesins (flocculins) have been performed only recently



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(Douglas *et al.*, 2007; Veelders *et al.*, 2010; Goossens *et al.*, 2011). The 'rediscovery' that *S. cerevisiae* is able to develop diverse multicellular growth forms such as filaments and biofilms (Gimeno *et al.*, 1992; Roberts & Fink, 1994; Reynolds & Fink, 2001) has led to a significant boost in the research of vegetative adhesion in this organism. These efforts have uncovered a highly complex regulatory network for the control of vegetative adhesion and multicellular development and provided detailed insights into adhesin structure and function. In addition, these studies provided new clues for the ecophysiological significance of adhesion.

In this review, we summarize the current knowledge on the role of vegetative adhesins in the establishment of multicellular lifestyles in *S. cerevisiae*. We first discuss the diverse growth forms with respect to their importance for basic research and industrial applications. Here, we also focus on the potential ecological significance of different *S. cerevisiae* lifestyles and their role as models for virulent growth forms of human pathogenic yeasts. In a second part, we present new insights into the structure and function of *S. cerevisiae* vegetative adhesins and we discuss their impact on our understanding of the molecular mechanisms that underlie cell–cell and cell–substrate adhesion. Finally, we will describe the complex regulatory network that controls adhesin gene expression in *S. cerevisiae* through numerous conventional and epigenetic mechanisms. Here, we also discuss the mechanisms that enable individual cells or whole populations to adapt specific adhesive properties in response to changing environments. Our review will not cover other aspects of multicellular development in *S. cerevisiae* such as intercellular communication, cell cycle control or regulation of cell polarity and morphogenesis, as these topics have been reviewed recently (Rua *et al.*, 2001; Palkova & Vachova, 2003; Bähler, 2005; Arkowitz, 2009; Piel & Tran, 2009; Slaughter *et al.*, 2009; Wang, 2009; Enserink & Kolodner, 2010; Moore & Cooper, 2010; Perez & Rincon, 2010).

## Lifestyles of *S. cerevisiae*

The observation that vegetative cells of *S. cerevisiae* can adopt a multicellular growth form was made more than 100 years ago by Emil Hansen, who described the formation of large yeast aggregates ('skins') in industrial strains after fermentation (Hansen, 1883). Later on, additional multicellular forms were described using diverse industrial and laboratory strains and they were named as flocs (Lindquist, 1952), flors (Allan, 1939), biofilms (Cruess, 1938; Reynolds & Fink, 2001) and pseudohyphal filaments (Morris, 1958; Gimeno & Fink, 1992; Gimeno *et al.*, 1992). The principal features of these growth forms are shown in Fig. 1, with a focus on the adhesion properties involved. Basically, development of the different lifestyles requires adhesion of yeast cells to each other (self-adhesion) or adhesion of cells to foreign biotic or abiotic surfaces (foreign adhesion). In this context, foreign surfaces are defined as structures not present on *S. cerevisiae* cells. Multicellular development is further influenced by the nature of the environment, which in principle can be a solid substrate directly exposed to air or a liquid medium with interfaces to the air and to the solid bottom.

As shown in Fig. 1, *S. cerevisiae* can choose between a number of different lifestyles. (1) On solid substrates exposed to air, cells that do not produce adhesins are sessile and will develop nonadhesive colonies. This is the typical growth form of many laboratory strains on solid agar media. (2) When expressing genes for self-adhesion, yeast cells can aggregate and form nondissolvable colonies that do not adhere to the substrate. (3) In order to develop nonremovable biofilms, adhesins must be produced that confer foreign adhesion. Typically, cells in biofilms adhere to each other and to the foreign surface. Foreign adhesion is also important for the formation of invasive filaments, which are able to penetrate solid substrates. A simple test for self- and foreign adhesion is a wash assay, in which colonies or larger cell patches are exposed to a stream of water (Roberts & Fink, 1994). This will dissolve nonadhesive colonies and remove cells from the agar surface. (4) In a liquid medium, nonadhesive yeast cells are planktonic and will produce turbid cultures of individual cells. This is the classical growth form of most laboratory strains in liquid media. (5) When producing proteins for self-adhesion, yeast cells can form aggregates that may sediment to the bottom (flocs) or that can float on the liquid surface (flor). These growth forms are observed in diverse industrial strains during the production of alcoholic beverages. (6) In liquid substrates, biofilms and/or filaments may also develop if yeast cells are able to adhere to the surface at the bottom.

Although multicellular growth forms of *S. cerevisiae* have been observed since the 19th century, the molecular basis for adhesion remained unclear until the isolation of the responsible genes. Initial studies uncovered genes for floc formation, which encoded proteins that were accordingly named flocculins (Miki *et al.*, 1982a, b; Stratford, 1989a, b, 1992). To date, at least eight different adhesins have been identified in diverse industrial and laboratory strains that confer vegetative adhesion (Fig. 2). They include *FLO1*, *FLO5*, *FLO9*, *FLO10*, *FLO11* (or *MUC1*), *FIG2* and *AGA1*, which all encode cell wall-associated surface proteins that belong to the yeast adhesin family (Dranginis *et al.*, 2007). Although the expression of *S. cerevisiae* adhesin genes is highly heterogeneous depending on the genetic background and the environmental conditions, they have been found to lead to the formation of flocs, flors, biofilms or filaments when sufficiently expressed. Thus, the encoded proteins are likely to be involved in the binding of specific ligands present on other yeast cells or on foreign surfaces.

To understand in detail how *S. cerevisiae* uses adhesin genes to choose the appropriate lifestyle, at least three key

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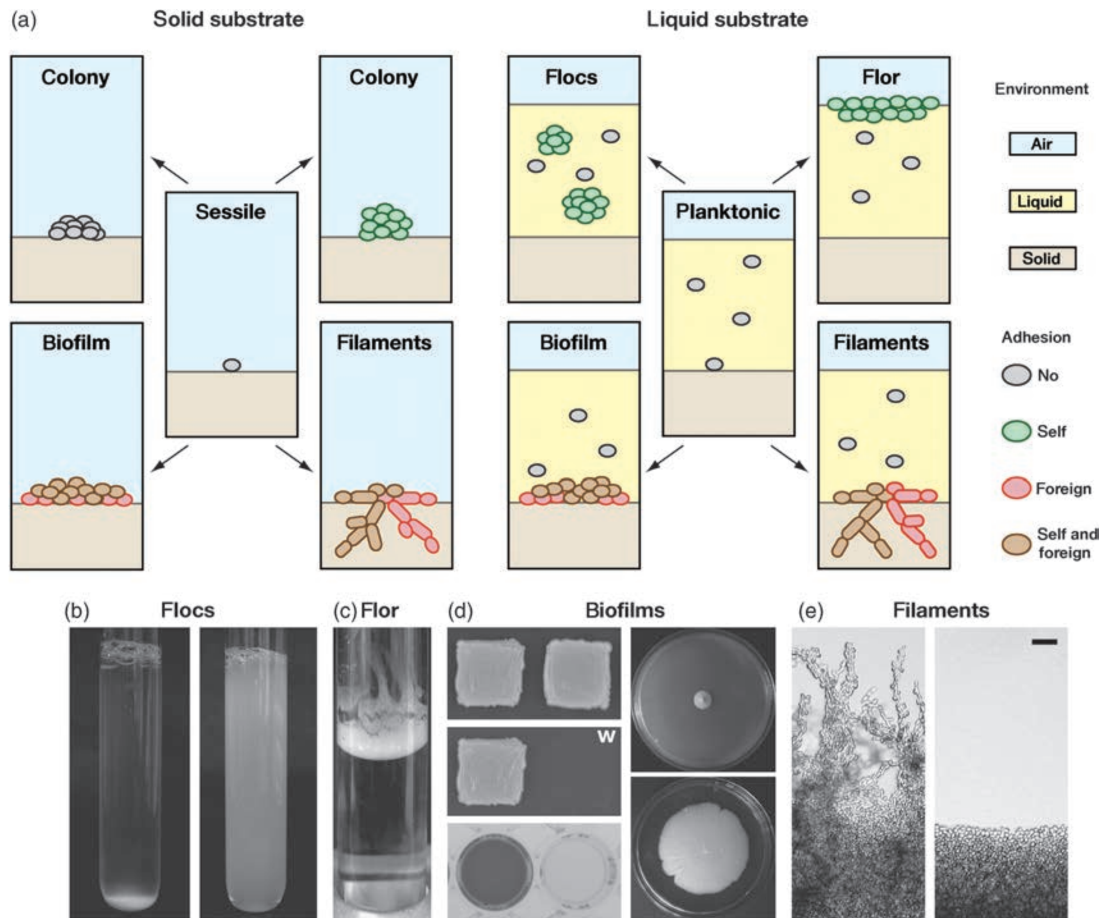


**Fig. 1.** Lifestyles of *Saccharomyces cerevisiae*. (a) Scheme showing the development of colonies, biofilms, filaments, flocs and flor by cell–cell (self) and cell–substrate (foreign) adhesion from sessile or planktonic cells on solid or in liquid media. The adhesive properties of cells and the physical condition of the environment are depicted in different colors and explained on the right. (b) Floc formation and sedimentation of a flocculent (left) and a nonflocculent yeast strain (right) is shown 15 s after extensive mixing of cultures. (c) Flor formation of a *FLO11*-expressing yeast strain at the top of a liquid culture was photographed after 4 days of growth. (d) Biofilm formation on solid (top), semi-solid (right bottom) and in liquid media (bottom left). On solid agar media, *FLO11*-expressing strains form adhesive biofilms (top left, left patch) or colonies (top right). Nonadhesive cells of a *flo11* mutant strain (top left, right patch) can be removed by washing (w). In liquid medium, *FLO11*-expressing strains (bottom left, left) form visible biofilms on polystyrene, whereas cells of a *flo11* mutant strain (bottom left, right) can be removed from plastic surfaces. The formation of a mat is shown for a *FLO11* strain after 10 days of growth on a semi-solid agar (bottom left). (e) Formation of invasive filaments at the border of a colony is shown for a diploid *FLO11*-expressing strain (left) 4 days after growth on a solid agar. No filaments are formed by a *flo11* mutant strain (right; scale bar = 20 μm).

issues must be addressed. (1) The specific requirement of adhesin genes for the development of the diverse growth forms must be determined. This should also involve a more detailed characterization of the known growth forms and even aim at the discovery of as yet unknown lifestyles. (2) The structural and functional properties of the encoded proteins must be characterized in detail. Here, the domains at the cell surface are of special interest as well as the ligand molecules that are specifically bound. (3) The regulation of adhesin genes and proteins by the environment must be studied. Specifically, the signaling pathways and the exact mechanisms that control adhesin gene expression and protein function must be uncovered in detail.

The following paragraphs will summarize the current knowledge on the diverse growth forms, whereas structure–function relationships and regulation of adhesins will be discussed in the subsequent chapters.

## Flocs

The classic definition of flocculation is the asexual, reversible and $Ca^{2+}$-dependent aggregation of thousands of vegetative cells into flocs (Lindquist, 1952; Mill, 1964; Miki *et al.*, 1982a, b; Stratford, 1989a, b). Depending on the specific density, which is influenced by the amount of entrapped gas, the resulting clumps of cells either sediment to the

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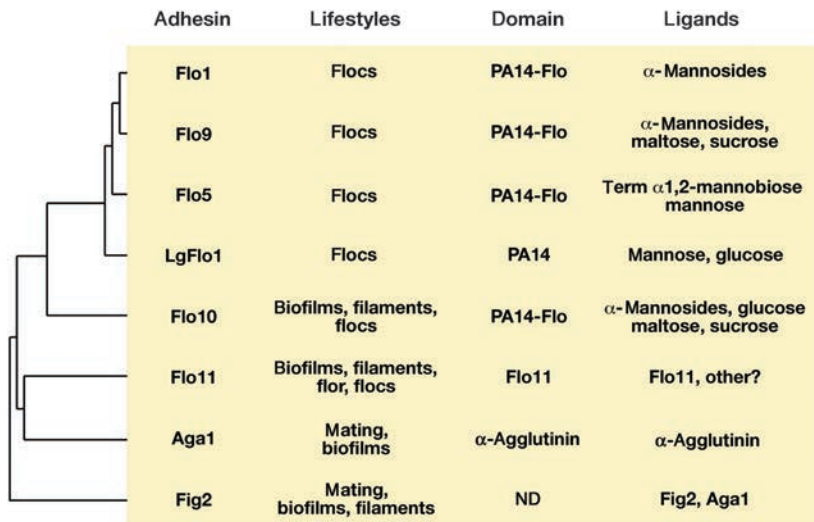


**Fig. 2.** Relationship and properties of *Saccharomyces cerevisiae* adhesins. The dendrogram depicting the distances between the indicated proteins is based on their amino acid sequence and was created using the program CLUSTAL X and the NJ algorithm (Larkin *et al*., 2007). The yellow box summarizes the involvement of adhesins in different vegetative lifestyles and sexual development, the known domains and the ligands recognized (ND, not determined). Further details and references are mentioned in the text.

bottom of the liquid growth substrate or they might rise to the surface. To understand the phenomenon of flocculation, initial studies proposed the lectin hypothesis, which suggested that flocculating yeast strains express cell-adhesion molecules that are able to bind mannose polymers located on the surface of adjacent cells in the presence of $Ca^{2+}$ (Eddy & Rudin, 1958; Taylor & Orton, 1973, 1975; Nishihara *et al*., 1977; Miki *et al*., 1982a, b; Stratford, 1989a, b). This theory was further supported by the finding that floc formation is reversibly inhibited by the presence of mannose, but in other cases also by glucose, maltose, sucrose or galactose (Eddy, 1955; Taylor & Orton, 1978; Miki *et al*., 1982a, b; Nishihara & Toraya, 1987; Stratford, 1989a, b; Masy *et al*., 1992).

A large number of studies have shown that the highly related *FLO1*, *FLO5* and *FLO9* genes encode for cell surface proteins that are able to confer flocculation by acting as lectins (Miki *et al*., 1982a, b; Stratford, 1989a, b; Stratford & Assinder, 1991; Stratford & Carter, 1993; Teunissen *et al*., 1993a, b; Bidard *et al*., 1994, 1995; Watari *et al*., 1994; Bony *et al*., 1997; Kobayashi *et al*., 1998; Javadekar *et al*., 2000). More recent studies directly show that these proteins recognize specific mannose oligomers and use a unique $Ca^{2+}$-binding motif (Veelders *et al*., 2010; Goossens *et al*., 2011). The *FLO10* and *FLO11* genes are also able to confer vegetative cell–cell adhesion (Guo *et al*., 2000; Bayly *et al*., 2005). However, floc formation mediated by these proteins is either $Ca^{2+}$ independent or mannose insensitive or it requires a specific genetic background (Bayly *et al*., 2005; Van Mulders *et al*., 2009). Therefore, cell–cell aggregation conferred by Flo10 and Flo11 seems to differ from classical flocculation and might involve mannan- or even lectin-independent mechanisms that operate through, for example cell surface hydrophobicity (Masy *et al*., 1992; Smit *et al*., 1992; Jin *et al*., 2001).

Flocculation has been studied extensively in industrial *S. cerevisiae* strains, because of its relevance for many biotechnological applications. Specifically, flocculation is a fast, cost-effective and environment-friendly way to remove yeast cells at the end of fermentation processes in the production of, for example beer, wine, ethanol or biodiesel (Bauer *et al*., 2010; Soares, 2010). In addition, flocculent yeast strains that effectively bind $Ca^{2+}$ ions have been used in bioremediation to remove other divalent ions, for example heavy metals from contaminated sites (Wang & Chen, 2006). Industrial strains have been widely analyzed with respect to flocculin gene variability and expression as well as with regard to other factors that influence flocculation, such as nutrient availability, pH, temperature, oxygen, cell density, agitation, ions, sugars, ethanol and cultural age. Brewing yeast strains for instance have been historically classified according to their ability to form flocs that either rise to the surface or sediment to the bottom (Dengis *et al*., 1995; Dengis & Rouxhet, 1997). Top-fermenting (ale) yeasts were predominantly classified as *S. cerevisiae*, whereas bottom-fermenting (lager) yeast strains are natural hybrids between *S. cerevisiae* and *Saccharomyces bayanus* and were named *Saccharomyces pastorianus* (Yamagishi & Ogata, 1999; Dunn & Sherlock, 2008; Ogata *et al*., 2008). Initial genetic analysis revealed that *S. pastorianus* strains carry a special type of flocculin gene called *Lg-FLO1* (Kobayashi *et al*., 1998). In contrast to Flo1 present in many laboratory strains, Lg-Flo1 is sensitive not only to mannose, but also to glucose. This mannose–glucose-sensitive adhesion has been named NewFlo phenotype and is of considerable industrial importance, because the relaxed sugar specificity of Lg-Flo1 ensures that flocculation occurs only at the end of fermentation, when all sugars that inhibit floc formation are consumed (Stratford, 1989a, b; Stratford & Assinder, 1991; Verstrepen *et al*., 2003). In recent

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years, a remarkable flocculation gene variability in different industrial brewing yeast strains has been revealed (Sato *et al*., 2001, 2002; Ogata *et al*., 2008; Van Mulders *et al*., 2010). This explicit genetic variation has been attributed to the extraordinary mutational frequency observed for flocculin genes and might hamper the stability of flocculation in industrial processes (Verstrepen *et al*., 2005; Rando & Verstrepen, 2007). Therefore, present and future strategies for optimized control of flocculation include global genetic analysis of diverse yeast strains and their targeted genetic improvement by, for example controlled expression of flocculin genes that confer specific adhesion properties (Govender *et al*., 2008, 2010; Bauer *et al*., 2010; Saerens *et al*., 2010). Comparative genomics of diverse industrial strains might also help to reveal the molecular basis for other factors that determine whether flocs rise to the surface or sediment to the bottom, for example the inclusion of carbon dioxide or the adsorption to rising gas bubbles (Soares, 2010).

The relevance of flocculation in industrial applications has long been recognized, but the ecophysiological significance of this phenomenon has not been investigated at the molecular level until recently. In principle, the formation of multicellular aggregates will protect cells in the center against a harmful environment, but concomitantly, these cells can be deprived from nutritional supply. Thus, the ability to flocculate can provide a yeast population with an evolutionary advantage, but it also comes at the cost of a reduced efficiency to couple nutrient availability to growth. Indeed, it has been shown that the expression of single flocculin genes, for example *FLO1*, is sufficient to confer the formation of flocs that are protective to environmental stresses such as ethanol or fungicides (Smukalla *et al*., 2008). This social protection comes at an individual cost, because *FLO1*-expressing cells grow significantly slower than *flo1* cells. In order to prevent nonexpressing individuals from acting as cheaters and taking advantage of the protection by aggregates without paying the cost associated with *FLO1* expression, flocs formed by mixed populations are coated with *flo1* cells that act as a first line of defense against harmful environments (Smukalla *et al*., 2008). As such, flocculin genes fulfill the definition of greenbeard genes, which direct cooperation towards other carriers of the same gene (Hamilton, 1964; Dawkins, 1976). Floc formation of *S. cerevisiae* also represents a social behavior (Brown & Buckling, 2008; Queller, 2008), which can be observed in other microorganisms, for example the bacteria *Proteus mirabilis* (Gibbs *et al*., 2008) or the slime mold *Dictyostelium discoideum* (Queller *et al*., 2003). The molecular mechanism underlying this behavior is cooperative binding of flocculin gene-expressing cells to each other by binary self-recognition, whereas binding of nonexpressing cells to flocs can occur only by one-way interaction via their surface oligosaccharides. The structural basis for flocculin-mediated social behavior of *S. cerevisiae* has been elucidated recently through the example of Flo5 (Veelders *et al*., 2010). This study shows that cooperativity is mainly achieved by heterophilic interactions between the Flo5 protein and specific oligomannoside structures present in the cell wall of adjacent cells. This mode of self-recognition of *S. cerevisiae* differs from that observed in *D. discoideum*, where cooperative binding is provided by a direct homophilic interaction between csA adhesin molecules present at the surface of neighboring cells (Benoit *et al*., 2000). However, the resulting multicellular aggregates appear to fulfill similar ecological functions in both of these microorganisms by providing a mechanism for protection to harmful environments and a strategy for long-time survival (Palkova & Vachova, 2006; Brown & Buckling, 2008; Williams, 2010).

## Flor

A yeast flor is defined as an air–liquid interfacial layer of floating cells that are attached to each other and form a biofilm, which has also been named as yeast velum or flotation (Cruess, 1938; Hohl & Cruess, 1939; Freiberg & Cruess, 1955; Martinez *et al*., 1995; Zara *et al*., 2005). Similar to flocculation, flor formation has long been recognized to be of considerable importance for the production of certain alcoholic beverages, particularly sherry wines (Allan, 1939; Pretorius, 2000). At the end of alcoholic fermentation, yeast strains involved in sherry maturation rise to the surface of the wine and form a flor that is exposed to air (Martínez *et al*., 1997). This layer of yeast cells has access to higher amounts of oxygen and is therefore able to produce metabolites such as acetaldehyde from ethanol and to consume other nonfermentable carbon sources such as glycerol and ethyl acetate (Cortes *et al*., 1999; Zara *et al*., 2010). The resulting compounds from aerobic metabolism are essential for the characteristic aroma of flor sherry wines and include dozens of different metabolites (Berlanga *et al*., 2001; Moyano *et al*., 2002; Peinado *et al*., 2004). The characterization of natural yeast strains from flors of sherry wine revealed that they belong to different races of *S. cerevisiae* that can grow and survive in the presence of high concentrations of ethanol (over 15% v/v), but that show a huge heterogeneity with regard to the nuclear and mitochondrial genome (Martinez *et al*., 1995). A common feature of flor-forming yeast strains is that they are able to adapt their hydrophobicity to allow cells to aggregate (Iimura *et al*., 1980a, b; Martínez *et al*., 1997). As a result, the aggregates retain gas bubbles that originated in the respiration process and become less dense than the wine (Zara *et al*., 2005).

Initially, the molecular basis for cell surface hydrophobicity during flor formation has been attributed to an elevated unsaturation level and mean chain length of fatty acid residues

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in membranes (Iimura *et al.*, 1980a, b; Farris *et al.*, 1993). However, the addition of ergosterol and/or oleic acid has no effect on flor formation, whereas incubation with proteases was found to break up the velum, suggesting that the process might depend on the synthesis of hydrophobic proteins rather than lipids (Martínez *et al.*, 1997). A classic genetic study indicated that flor formation in *S. cerevisiae* is under the control of a single Mendelian gene (Santa Maria & Vidal, 1973). Later on, molecular genetic analysis has shown that in many industrial strains, flor formation depends on the efficient expression of the *FLO11* gene (Ishigami *et al.*, 2004, 2006; Zara *et al.*, 2005, 2009; Fidalgo *et al.*, 2008; Govender *et al.*, 2008). These studies also indicate that *FLO11* confers flor formation by inducing increased cell surface hydrophobicity, which is in agreement with the finding that Flo11 is a hydrophobic cell wall protein (Reynolds & Fink, 2001; Mortensen *et al.*, 2007; Purevdorj-Gage *et al.*, 2007). In contrast to *FLO11*, the dominant expression of *FLO1* or *FLO5* in a laboratory strain was not sufficient to induce flor formation, although these proteins are able to significantly increase cell surface hydrophobicity (Govender *et al.*, 2008; Van Mulders *et al.*, 2009). This indicates that efficient flor formation might depend on factors other than hydrophobicity, which might be provided by Flo11, but not by other flocculins. Other non-flocculin proteins have also been described that confer cell surface hydrophobicity and are involved in velum formation in French wine yeast (Alexandre *et al.*, 2000) and foam formation in sake-producing yeast (Shimoi *et al.*, 2002). In addition, the formation of flors might be further stabilized by an extracellular matrix (ECM) of unknown composition that has been observed in certain yeast strains (Kuthan *et al.*, 2003; Zara *et al.*, 2009).

As in the case of flocculation, the ecological role of yeast flor formation in natural environments is not really known (Palkova, 2004; Palkova & Vachova, 2006). However, it seems reasonable that such biofilms at the air–liquid interface might be used as a strategy to bring part of a yeast population to a zone of higher oxygen concentration after planktonic cells have fermented sugars in a liquid substrate (Martinez *et al.*, 1995, 1997; Berlanga *et al.*, 2001). *Saccharomyces cerevisiae* is well known to be able to rapidly convert more complex carbohydrates into ethanol as a possible means to eliminate competitors (Herman, 2002; Borneman *et al.*, 2007a, b; Ding *et al.*, 2009). After fermentation, ethanol can then be used as a rich energy source by respiration, which is more efficient at the air–liquid interface. In support of such an ecological view of flors, their formation is favored in media that lack fermentable sugars such as glucose and that contain high amounts of ethanol (Martinez *et al.*, 1995, 1997; Berlanga *et al.*, 2001). Also, these conditions have been found to induce the expression of *FLO11* (Reynolds & Fink, 2001; Kuchin *et al.*, 2002; Palecek *et al.*, 2002; Zara *et al.*, 2005; Ishigami *et al.*, 2006).

## Biofilms

Biofilms can be defined as communities of microorganisms attached to a surface (Monds & O'Toole, 2009). In bacteria and fungi, biofilm formation is a complex developmental behavior that includes adhesion of cells to foreign surfaces, biofilm growth and maturation and the production of a protective ECM (Blankenship & Mitchell, 2006; Verstraeten *et al.*, 2008; Harding *et al.*, 2009; Karatan & Watnick, 2009; Moons *et al.*, 2009; Nadell *et al.*, 2009; Ramage *et al.*, 2009; Finkel & Mitchell, 2010; Flemming & Wingender, 2010; Wintermute & Silver, 2010). With respect to adhesion, biofilm-forming microorganisms must therefore produce surface cues that allow binding of foreign molecular structures (Pendrak & Klotz, 1995; Verstrepen & Klis, 2006; Dranginis *et al.*, 2007; Kline *et al.*, 2009). *Saccharomyces cerevisiae* has also been found to adhere to foreign biotic and abiotic surfaces including agar and plastic, which has made these organisms a valuable model to study the molecular basis of biofilm formation (Roberts & Fink, 1994; Reynolds & Fink, 2001).

On solid or semi-solid agar surfaces exposed to air, *S. cerevisiae* can form biofilms in the form of adhesive colonies or mats (Fig. 1). In addition, adhesive colonies can adopt different morphologies (Kuthan *et al.*, 2003; Casalone *et al.*, 2005; Vopalenska *et al.*, 2005; St'ovicek *et al.*, 2010) and even build stalk-like structures (Engelberg *et al.*, 1998; Scherz *et al.*, 2001). A number of genetic studies have shown that agar adhesion can be mediated by *FLO11*, *FLO10* and *FIG2*, but not by *FLO1*, *FLO5* or *FLO9* (Lambrechts *et al.*, 1996a, b; Lo & Dranginis, 1998; Guo *et al.*, 2000; Govender *et al.*, 2008; Van Mulders *et al.*, 2009). How exactly the Flo11, Flo10 and Fig2 proteins confer agar binding is not known. With respect to biofilm development, the clonal formation of mats from single cells on semi-solid agar is an interesting model. A growing mat will form two visually distinct populations of cells at the edge of the mat (rim) and in the interior (hub), whose development depends on the creation of concentric glucose and pH gradients in the medium (Reynolds & Fink, 2001). Based on their agar adhesion properties, these structures can be physically separated from one another. Interestingly, *FLO11* is expressed in both portions of mats, indicating that the difference in adhesion between the rim and the hub might stem from reduced Flo11 activity that is caused by an elevated pH at the rim (Reynolds *et al.*, 2008). In addition, processing of Flo11 protein might provide a fluid layer that surrounds yeast mats to enable spreading growth by sliding motility similar to biofilm-forming bacteria (Karunanithi *et al.*, 2010). In natural environments, mat formation might be a lifestyle that is adopted by *S. cerevisiae* on semi-solid substrates and help the organism to more rapidly colonize preferred habitats. It should be pointed out, though, that ecologically important surface structures that are recognized by the

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organism to initiate biofilm formation are not yet known. It is also not clear whether *S. cerevisiae* biofilm formation includes the production of a protective ECM, as has been observed for bacterial biofilms. Transmission electron microscopy revealed that flocculin gene-expressing yeast strains secrete a mixture of glucose and mannose polysaccharides that surrounds the cells (Beauvais *et al.*, 2009). Although this matrix does not seem to play a role in the resistance of flocs against drugs and ethanol, it indicates that *S. cerevisiae* is in principle able to produce ECM-like structures that may be important in natural habitats.

When grown in liquid media, *S. cerevisiae* can also adhere to diverse plastic surfaces including polystyrene, polypropylene and polyvinylchloride. This ability was attributed to the expression of *FLO11* and the hydrophobic properties conferred by this adhesin (Reynolds & Fink, 2001). A correlation between cell surface hydrophobicity and the ability to mediate adhesion to the polystyrene surface was also found in the case of Flo1, Flo5, Flo9 and Flo10 (Van Mulders *et al.*, 2009). This indicates that the molecular basis for plastic adhesion might be more unspecific. However, laboratory strains expressing *FLO11* readily adhere to both liquid-hydrophobic and liquid-hydrophilic solid interfaces and are able to grow as a biofilm monolayer in a flow system (Purevdorj-Gage *et al.*, 2007). This suggests that additional features of adhesins might be involved in adhesion to certain plastic surfaces. The ability of *S. cerevisiae* to adhere to plastic surfaces is also of medical importance, because diverse strains have been found to cause opportunistic infections in humans (Eschete & West, 1980; Aucott *et al.*, 1990; Nyirjesy *et al.*, 1995; Bassetti *et al.*, 1998; McCullough *et al.*, 1998; Xu *et al.*, 1999; Wheeler *et al.*, 2003; de Llanos *et al.*, 2004). It is well known that human pathogenic bacteria and fungi are able to persist as biofilms on medical plastic devices, for example catheters (Marrie & Costerton, 1984; Costerton *et al.*, 1999; Kojic & Darouiche, 2004; Fux *et al.*, 2005; Donlan, 2008; Finkel & Mitchell, 2010). Thus, the study of plastic adhesion of *S. cerevisiae* might also reveal important insights into microbial persistence in humans and hospitals.

## Filaments

*Saccharomyces cerevisiae* does not belong to the classical filamentous fungi that grow in the form of true hyphae, which are defined as long and branching filamentous structures that arise by continuous tips growth of hyphal cells and subsequent fission of cells through the formation of septa (Gow, 1994; Carlile, 1995; Borkovich & Ebbole, 2010). However, diploid strains of *S. cerevisiae* are able to produce filaments in the form of pseudohyphae, which are defined as chains of attached, elongated cells that are formed from one another by budding (Lodder *et al.*, 1958; Gimeno & Fink, 1992; Gimeno *et al.*, 1992; Kron & Gow, 1995; Gow, 1997; Mösch, 2000, 2002; Gancedo, 2001). A major difference between hyphal and pseudohyphal filaments is the mode of origin and not the end-product, which, in both cases, is a mycelium (Scherr & Weaver, 1953; Shepherd, 1988). In addition, numerous studies using other ascomycetes, for example *Candida albicans*, have shown that further significant differences exist between hyphae and pseudohyphae including the organization of the polarisome or the regulation of cell cycle events (Sudbery *et al.*, 2004). Therefore, *S. cerevisiae* ranks among the dimorphic yeasts that are able to interconvert between unicellular and filamentous growth phases (Kron, 1997; Ernst & Schmidt, 2000). In order to produce a filament, pseudohyphal cells of *S. cerevisiae* adhere to each other and to the substrate. In addition, linear chain formation depends on oriented cell division, which is not necessary for the formation of, for example flocs or flors. For this purpose, pseudohyphal cells maintain a unipolar budding pattern, where buds continuously emerge from the site opposite to the birth end, the distal cell pole (Chant & Herskowitz, 1991; Gimeno *et al.*, 1992; Zahner *et al.*, 1996; Taheri *et al.*, 2000; Krappmann *et al.*, 2007). Pseudohyphal cells also adapt the timing of their cell cycle to divide symmetrically and to acquire an elongated morphology by targeting the actin cytoskeleton (Kron *et al.*, 1994; Kron, 1997). Genetically, the processes of substrate adhesion, bud site selection and cell morphogenesis, which together are required for filament formation in *S. cerevisiae*, can be dissected from each other (Mösch & Fink, 1997).

Adhesion to foreign surfaces is one of the key factors that enable fungal filaments to penetrate solid substrates and to grow invasively (Carlile, 1995; Borkovich & Ebbole, 2010). In *S. cerevisiae*, adhesion to agar enables pseudohyphal cells to act as an anchor for the cell at the apex (Gimeno *et al.*, 1992). Anchored filaments in combination with the force that is produced by unipolar cell divisions might be the key to propel pseudohyphae or hyphae through a solid substrate. It has been shown that nonadhesive diploid yeast strains are able to produce filaments by unipolar cell division, but they remain at the surface of the substrate (Mösch & Fink, 1997). In the laboratory, agar invasion is mediated by *FLO11*, *FLO10* and *FIG2* that confer adhesion to this substrate (Lambrechts *et al.*, 1996a, b; Lo & Dranginis, 1998; Guo *et al.*, 2000). In the case of *FLO11*, filament formation well correlates with expression of the gene. Numerous conditions have been found that induce both *FLO11* expression and agar invasion by filaments, for example starvation for nitrogen, glucose or amino acids (Gimeno *et al.*, 1992; Ljungdahl *et al.*, 1992; Cullen & Sprague, 2000). In natural environments, *S. cerevisiae* might therefore use substrate adhesion to penetrate solid surfaces and to explore substrates in the third dimension (Palkova & Vachova, 2006; Vopalenska *et al.*, 2010). However, neither the molecular structure of natural substrate surfaces nor


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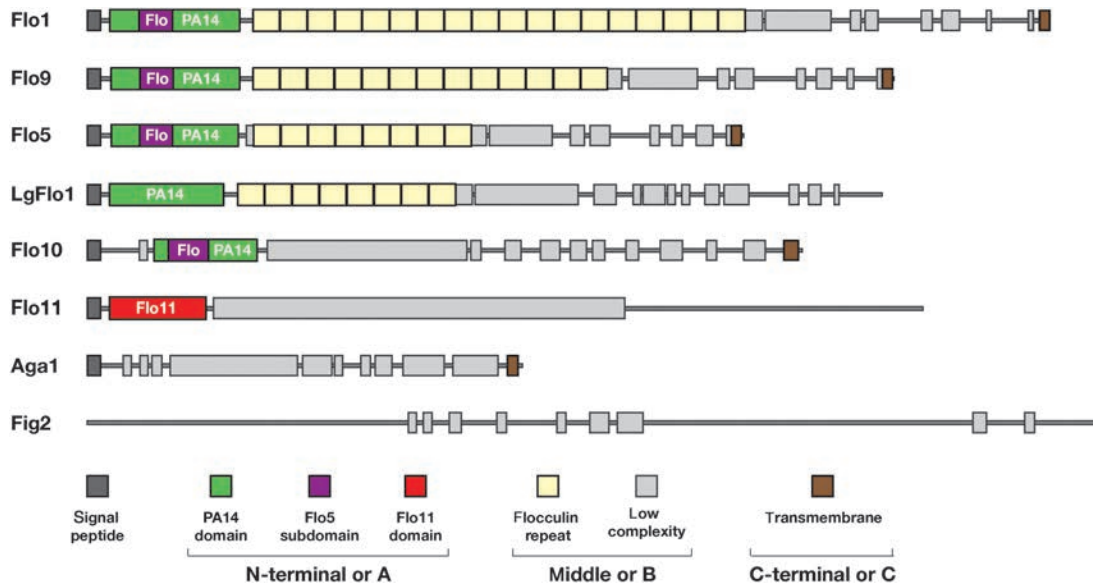


**Fig. 3.** Domain organization of *Saccharomyces cerevisiae* adhesins. Proteins were analyzed using the Pfam protein families' database at http://pfam.sanger.ac.uk (Finn *et al*., 2010). Known domains are shown in different colors. Flo5-like subdomains were determined according to Veelders *et al*. (2010). The broad partition into N-terminal (A), middle (B) and C-terminal (C) domains is indicated.

their interaction with different members of the *S. cerevisiae* adhesin family is known so far.

Finally, the study of *S. cerevisiae* filament formation has medical relevance. Similar to human pathogenic yeasts, pseudohyphal cells of *S. cerevisiae* not only become adhesive, they also secrete enzymes such as hydrolases and proteinases that support invasion of the substrate (Odds, 1994; Madhani *et al*., 1999; Murphy & Kavanagh, 1999; Kaur *et al*., 2005; Zhu & Filler, 2010). The precise molecular mechanisms for substrate adhesion and invasion are likely to differ between *S. cerevisiae* and human pathogenic yeasts. However, central signaling pathways that control dimorphism in *S. cerevisiae* have also turned out to be relevant for filament formation in pathogenic yeasts, for example in *C. albicans* (Lo *et al*., 1997). In fact, investigation of dimorphism in *S. cerevisiae* has been instrumental to uncover many of the signaling routes that control hyphal growth and virulence in a growing number of human pathogenic fungi (Lengeler *et al*., 2000; Bahn *et al*., 2007).

## Structure and function of *S. cerevisiae* adhesins

### General architecture

The vegetative adhesins of *S. cerevisiae* belong to the large family of fungal glycosylphosphatidylinositol-linked cell-wall glycoproteins (GPI-CWPs) (Verstrepen *et al*., 2004; Verstrepen & Klis, 2006; Dranginis *et al*., 2007; Linder & Gustafsson, 2008). These secreted proteins confer unique adhesion properties and their primary amino acid sequence often shares only a low degree of similarity. However, GPI-CWPs have a common overall architecture and consist of at least three different domains (Fig. 3). The N-terminal or A domain that follows the secretion signal is exposed at the cell surface and confers the recognition and binding of ligand molecules presented *in trans*. This domain is followed by a segment of variable length (middle or B domain) that is extremely rich in serine and threonine residues and that is highly glycosylated. At the carboxy-terminal region (or C domain), a GPI anchor is added for localizing adhesins to the cell wall. Of the proteins known to confer vegetative adhesion in *S. cerevisiae*, the flocculins Flo1, Flo5, Flo9, Flo10 and Flo11 fulfill these structural criteria (Dranginis *et al*., 2007; Linder & Gustafsson, 2008). In contrast, Fig2 and Aga1 do not fully match the general architecture of GPI-CWPs, with the most obvious difference of lacking a clearly defined A domain (Verstrepen *et al*., 2004).

### N-terminal adhesion domains

With respect to understanding the process of establishing contact between the yeast cell surface and other solid surfaces, the N-terminal A domain of GPI-CWPs appears to be the most interesting one, as it has been shown to play a major role in specific binding of *trans* ligands. In general, fungal adhesion can been divided into sugar-sensitive (lectin-like) and sugar-insensitive adhesion (Verstrepen & Klis, 2006; Dranginis *et al*., 2007). In the case of Flo1, Flo5, Flo9 and Flo10, it has been shown that these adhesins confer

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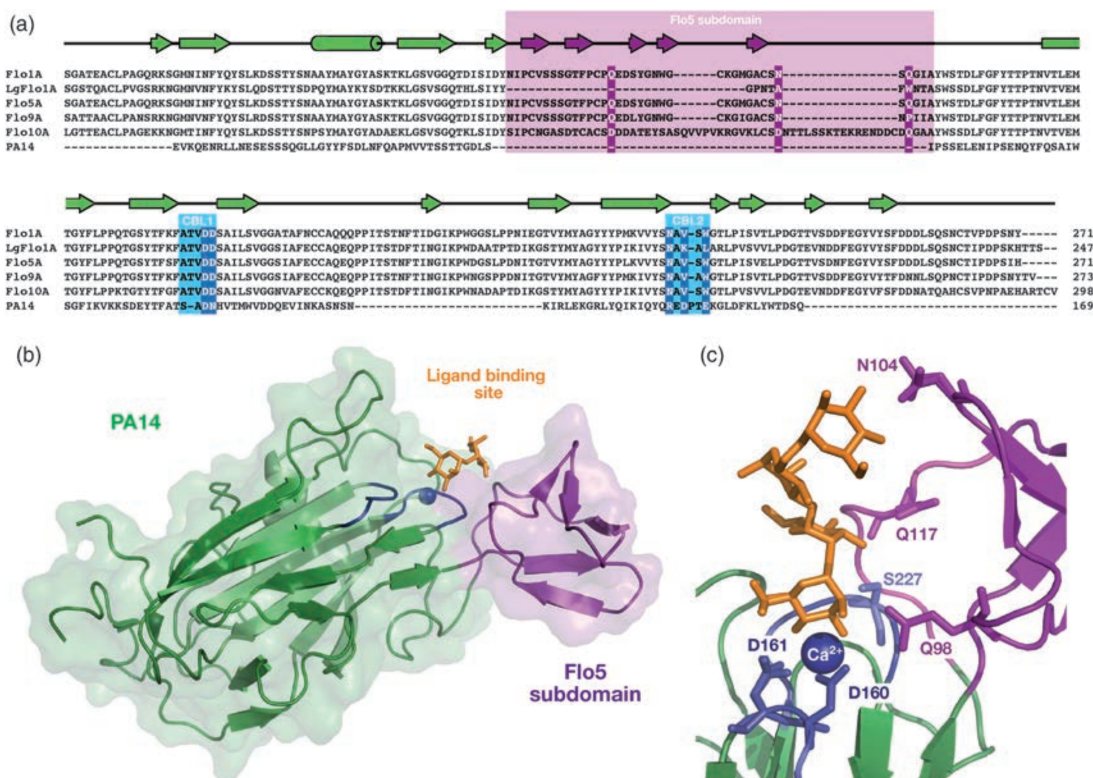


**Fig. 4.** PA14/Flo5-type proteins of *Saccharomyces cerevisiae*. (a) Sequence alignment of A domains of Flo1, Lg-Flo1, Flo5, Flo9 and Flo10 in comparison with PA14 was performed using the T-COFFEE method (Notredame *et al*., 2000). The common secondary structure of indicated proteins was determined using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred) and is shown above indicating α-helices (barrels) and β-sheets (arrows). The Flo5 subdomain is colored in light purple and residues known to be critical for ligand binding-specificity in Flo5 are indicated in purple (Veelders *et al*., 2010). The CBL1 and CBL2 loops involved in $Ca^{2+}$ binding are indicated in light blue, with critical residues highlighted in dark blue. Numbering refers to the amino acid sequences (http://www.uniprot.org). (b) Three-dimensional structure of Flo5A showing the PA14 domain (green), the Flo5 subdomain (purple), $Ca^{2+}$ (blue sphere), $Ca^{2+}$-binding loops CBL1 and CBL2 (blue), and the primary ligand-binding site bound to α1,2-mannobiose (orange). (c) Detailed view of the primary ligand-binding site of Flo5A bound to mannopentaose mimicking a yeast *N*-linked core oligosaccharide (orange). Residues D160, D161 and S227 involved in $Ca^{2+}$ binding (dark blue) and amino acids Q98, N104 and Q117 involved in carbohydrate binding (purple) are indicated. Structures shown in (b) and (c) were generated using the program PYMOL (DeLano, 2002).

dominant, $Ca^{2+}$-dependent floc formation that can be inhibited by specific hexoses, suggesting that they are lectins (Miki *et al*., 1982a, b; Stratford & Assinder, 1991; Bidard *et al*., 1994, 1995; Teunissen & Steensma, 1995; Teunissen *et al*., 1995a, b; Guo *et al*., 2000; Govender *et al*., 2008). Flo11 is thought to mediate sugar-insensitive adhesion to agar and plastic by increasing the cell surface hydrophobicity (Lambrechts *et al*., 1996a, b; Lo & Dranginis, 1996, 1998; Reynolds & Fink, 2001). *In silico* analysis reveals that the A domains of these flocculins comprise approximately 250 amino acids, which are highly similar to sequence identities of at least 64% (Fig. 4). Moreover, the C-terminal half of their A domain exhibits weak sequence similarity to the PA14 domain from the anthrax-protective antigen. This β-barrel domain has been identified in many eubacterial and eukaryotic proteins including bacterial toxins, glycosidases, glycosyltransferases, proteases, signaling molecules and yeast adhesins (Rigden *et al*., 2004). However, the N-terminal halves of the A domains of Flo1, Flo5, Flo9 and Flo10 lack any similarity to known domain types including the structurally well-characterized animal or plant lectins (Loris, 2002) or the fungal fruit-body lectins/galectins (Walser *et al*., 2004; Leonidas *et al*., 2007). It was thus suggested that the A domains of these flocculins represent a special type of the PA14 domain family (Goossens & Willaert, 2010).

With respect to understand the precise function of Flo proteins during self-recognition and cell–cell adhesion, long-standing issues have been the identification of domains and residues that confer carbohydrate binding and discrimination between closely related hexoses. Carbohydrate competition studies with strains expressing *FLO1*, *FLO5*, *FLO9* or *FLO10* and different hexoses have suggested that the encoded Flo proteins bind to mannose-containing carbohydrate structures present at the cell surface of

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neighboring cells (Stratford, 1989a, b; Stratford & Assinder, 1991; Van Mulders *et al*., 2009; Veelders *et al*., 2010). Specific variants of *FLO1* such as *Lg-FLO1* were found to confer not only mannose-, but also glucose-inhibitable flocculation (Kobayashi *et al*., 1998; Ogata *et al*., 2008). These studies uncovered a conserved VSWGT motif encompassing residues 226–230 of Flo1 to be involved in carbohydrate binding and pointed towards the tryptophan residue at position 228 to be participating in discriminating between mannose and glucose. Notably, the VSWGT motif can be structurally and functionally related to the EYDGA motif, which is found in the N-terminal PA14 domain of the epithelial adhesin Epa1 of the human pathogenic yeast *Candida glabrata* and is involved in sugar recognition (Zupancic *et al*., 2008; Goossens & Willaert, 2010). Furthermore, the molecular role of $Ca^{2+}$ has remained unclear. It has been proposed that $Ca^{2+}$ is either involved in the formation of semi-rigid rod-like superstructures of the heavily *O*-glycosylated B region of Flo proteins (Jentoft, 1990) or plays a crucial role in carbohydrate recognition as exemplified by classical C-type lectins (Miki *et al*., 1982a, b; Stratford, 1989a, b; Kuriyama *et al*., 1991).

## Flo5

The first high-resolution insight into Flo proteins was recently provided by solving the three-dimensional structure of the Flo5A domain complexed to its cognate ligands derived from yeast high-mannose oligosaccharides at resolutions of up to 0.95 Å (Veelders *et al*., 2010). This study has provided answers to many of the long-standing questions regarding domain organization, the role of $Ca^{2+}$ and ligand binding/discrimination. The overall structure of Flo5A shows a bipartite organization comprising a large β-sandwich domain that is topologically related to the PA14 domain and an additional insertion, the Flo5 subdomain (Fig. 4). This Flo-specific subdomain (N84-A120) consists of five short β-strands that are stabilized by two disulfide bridges. A second region unique for Flo5A is formed by its N- and C-termini, which extend as an L-shaped stretch from the core domain and are fixed to it by disulfide bridges to two consecutive cysteines. This loop (G168-T189) is considerably longer than in the PA14 domain and, together with a second loop (Y46-T68), seals the surface of the underlying β-sheet from solvent access.

The structural and biochemical analysis of the Flo5A domain revealed that $Ca^{2+}$ is directly involved in carbohydrate binding (Veelders *et al*., 2010). The crystal structure of the mannose/$Ca^{2+}$ complex of Flo5A shows a C-lectin-type mode of carbohydrate binding via $Ca^{2+}$-mediated recognition of the 2′- and 3′-hydroxyl groups, where the $Ca^{2+}$ ion is bound between the sugar and the protein (Fig. 4). The $Ca^{2+}$-binding site of Flo5A is a unique and characteristic hallmark of the PA14/Flo5-like protein family. The $Ca^{2+}$ ligands belong to the carbohydrate-binding loops, CBL1 and CBL2. CBL1 is unusual by bearing a rare *cis*-peptide between the nonproline residues D160 and D161. So far, a *DcisD* motif has only been found in the nucleotide-binding site of the ATP synthase (Abrahams *et al*., 1994) as well as in a $Zn^{2+}$-dependent aminoprotease (Chevrier *et al*., 1994). This motif is present throughout the PA14/Flo5-like family, but is missing in the PA14 domain itself. The crucial role of the unique *DcisD* motif in the PA14/Flo5-like family of $Ca^{2+}$- and carbohydrate-dependent adhesins is reflected by a complete lack of flocculation, when either of the two D residues is mutated (Veelders *et al*., 2010). Multiple sequence alignment shows that 45% of the known PA14 domains, including human proteins like galactosyltransferases and fibrocystin, contain a double D at the equivalent position (Sonnhammer *et al*., 1997).

The study with Flo5A also suggests that terminal arms of yeast high-mannose oligosaccharides act as cognate ligands *in vivo*. The yeast cell wall has a complex architecture (Klis *et al*., 2006; Lesage & Bussey, 2006) and contains a large proportion of mannoproteins with either *N*- or *O*-linked oligosaccharides (Lehle *et al*., 2006; Lommel & Strahl, 2009). These carbohydrates have long been discussed to be involved in flocculation (Stratford, 1992), but the exact structures of the ligands that are recognized by flocculins were not known. Fluorescence-titration and crystal soaking experiments revealed that only α1,2-linked mannobiosides, but neither α1,3- nor α1,6-linked mannoses, are bound. Moreover, α1,2-linkage to a second mannose increased the affinity ninefold (KD = 3.5 mM) when compared with mannose. The recognition of the second mannose moiety is achieved by hydrogen bonds between its 3′-hydroxyl group and S227 from CBL2 and, in addition, Q117 residing in the Flo5 subdomain (Fig. 4). Interestingly, mutation of S227 to alanine was found to increase the affinity towards mannose without affecting specificity. α1,2-α1,2-Mannotriose shows an affinity and binding mode that is comparable to α1,2-mannobiose. The same is true for a synthetic mannopentaose, which mimics a yeast *N*-linked core oligosaccharide (Fig. 4). Apparently, the Flo5 subdomain supports discrimination between the branches of the core oligosaccharide by steric hindrance or the contribution of a hydrogen bond between the fourth mannoside and residues N104.

The high-resolution view of Flo5A allows to explain the behavior of NewFlo-type flocculins with broadened sensitivity against different sugars like glucose (Stratford & Assinder, 1991). Initial work suggested that NewFlo-type variants like D202T and W228L are directly affected in carbohydrate binding (Kobayashi *et al*., 1998). However, the Flo5A structure reveals that these residues are not directly involved in carbohydrate recognition and indirectly affect the structure of the primary binding site. Lg-Flo1, which also promotes NewFlo-type flocculation, lacks the

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complete Flo5A subdomain and therefore exhibits relaxed carbohydrate recognition and less efficient sugar binding. Here, the Flo5A structure allows to predict and engineer novel NewFlo-type flocculins with unaltered flocculation efficiency. A prominent example is the Q98A variant, for which a loss of interaction with the axial 2-hydroxyl group of mannose and therefore a reduced ligand specificity can be predicted. Indeed, the Q98A variant confers unaltered flocculation efficiency, but completely lacks discrimination between mannose and glucose.

Finally, the crystal structure of Flo5A provides a possible answer to a problem that is posed by the fact that high concentrations of mannose ligands are not only present on the surface of neighboring cells (*trans* position), but also on the site of the flocculin (*cis* position). An interesting question is how flocculins preferentially interact with ligands presented in *trans* without being compromised by *cis* ligand interactions, which would prevent cell–cell adhesion. Interestingly, a secondary carbohydrate-binding site is observed at the back of the Flo5A domain, although molar concentrations of mannose or glucose are required and binding is $Ca^{2+}$ independent (Veelders *et al*., 2010). It is therefore possible that the secondary carbohydrate-binding site may help to fix the A domain at the cell surface by interacting with *cis* ligands and thereby allow the primary binding site to preferentially bind to *trans* ligands (Fig. 5). A similar mechanism is observed in the case of certain cell-adhesion molecules of higher eukaryotes, for example the human neural cell adhesion molecule (NCAM), where secondary binding with adhesion molecules in *cis* ensures efficient primary binding with the adhesin molecules present in *trans* (Kiselyov *et al*., 2005).

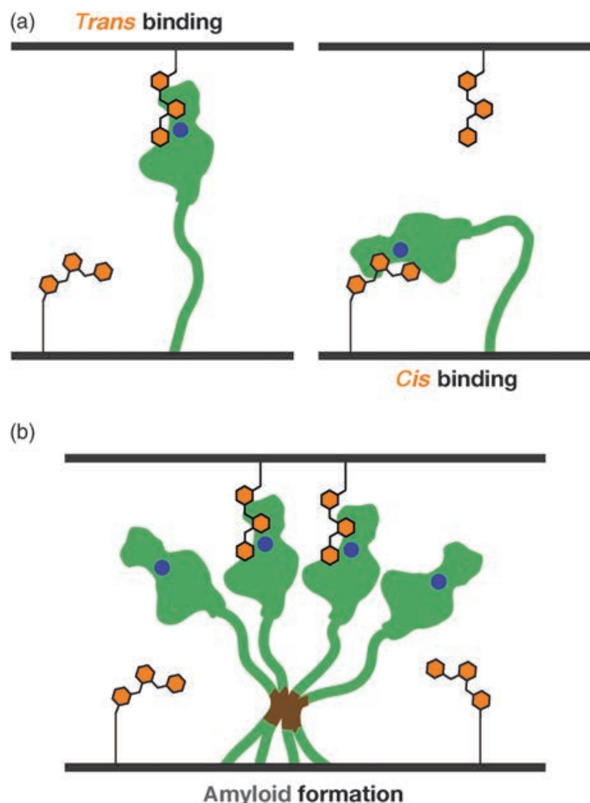


**Fig. 5.** Ligand binding by adhesins. (a) Comparison of *trans* and *cis* ligand-binding modes. For *trans* binding the A domain (green) interacts with carbohydrates (orange) present at the surface of neighboring cells resulting in cell–cell adhesion. Interaction of the adhesin with ligands at the surface of the same cell results in nonproductive *cis* binding. $Ca^{2+}$ in the primary binding site is indicated as a blue dot. (b) Multimerization of adhesins by amyloid formation. Bundling of several adhesins by interaction of β-aggregation-prone sequences (brown) might prevent *cis* ligand binding and favor cell–cell adhesion by directing the binding sites towards carbohydrates present on neighboring cells. Models in (a) and (b) are adapted from Veelders *et al*. (2010) and Ramsook *et al*. (2010), respectively.

## Flo1, Flo9 and Flo10

The Flo5A structure allows to model the structure of the A domains of the closely related Flo1 (94% identical), Flo9 (89% identical) and Flo10 (66% identical) proteins and to predict functional characteristics. In the case of Flo1A, all residues that are crucial for $Ca^{2+}$ and ligand binding in Flo5A are conserved, suggesting identical functions (Watari *et al*., 1994; Groes *et al*., 2002; Veelders *et al*., 2010). An interesting difference is observed in Flo9A, which carries an alanine at position 227 instead of a serine. This suggests that Flo9A might confer enhanced floc formation as has been observed for the S227A variant of Flo5A. The most striking differences can be found in Flo10A, which has an extra insert in the Flo-specific subdomain and carries aspartate residues at positions 98 and 104. However, the $Ca^{2+}$-binding site is conserved in Flo10A, and computer modeling predicts a similar overall structure when compared with Flo5A. This suggests that Flo10A has an altered carbohydrate-binding spectrum when compared with Flo1A and Flo5A, which might explain its special adhesion properties *in vivo*. Strikingly, *FLO10* not only confers flocculation when overexpressed, but also agar and plastic adhesion (Guo *et al*., 2000; Van Mulders *et al*., 2009). It will be interesting to determine as to which of these functions are mediated by the Flo10A domain.

## Flo11

In contrast to Flo5 and its close relatives, the exact structure and molecular function of the Flo11 N-terminal domain is not known. The Flo11 A domain is conserved and can be found in related species within the Saccharomycotina as well as in *Schizosaccharomyces pombe* (Linder & Gustafsson, 2008). Because Flo11 confers hydrophobic properties to yeast cells, it has been suggested that this flocculin functions

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similar to fungal hydrophobins (Reynolds & Fink, 2001; Ishigami *et al*., 2006). However, whether hydrophobicity is conferred by the A domain of Flo11 has not been investigated. Interestingly, *FLO11* has been found to mediate strain-specific phenotypes. In strains of *S. cerevisiae* var. *diastaticus*, *FLO11* expression confers $Ca^{2+}$-dependent and mannose-sensitive flocculation, but no agar adhesion or filament formation (Bayly *et al*., 2005; Douglas *et al*., 2007). In contrast, strains of the Σ1278b genetic background are nonflocculent, although they express *FLO11* at high levels (Guo *et al*., 2000). Instead, these strains require *FLO11* for agar adhesion and filamentation (Lo & Dranginis, 1998; Rupp *et al*., 1999), and genetic analysis suggests that adhesive functions are conferred by the A domain (Veelders *et al*., 2010). Biochemical studies with secreted Flo11 proteins from the two strains attached to microscopic beads have shown that it confers the ability to bind to *S. cerevisiae* var. *diastaticus* cells, but not to Σ1278b cells (Douglas *et al*., 2007). Moreover, this function of Flo11 is mannose sensitive and depends on the expression of *FLO11* in the *S. cerevisiae* var. *diastaticus* target cells. These findings suggest homotypic adhesive mechanisms that might involve the binding of Flo11 to mannose residues presented by other Flo11 molecules *in trans*. It has been suggested that these differences in Flo11 functions might arise from strain-specific mannosylation of the adhesin (Bayly *et al*., 2005; Douglas *et al*., 2007). However, functional variance might also be attributed to the structural differences observed in the two A domains. Interestingly, Flo11A from Σ1278b strains carries an insert of an extra 15 amino acids when compared with *S. cerevisiae* var. *diastaticus* and lacks two of the highly conserved residues of the Flo11 family. Thus, Flo11A might have diverged in Σ1278b strains to recognize new ligands and to confer alternate adhesion properties. In the future, high-resolution structure–function analysis of different Flo11A variants will be required to answer these questions.

## Middle domains

In contrast to the N-terminal domains, much less is known about the structure and function of the middle parts of *S. cerevisiae* adhesins. Bioinformatic analysis reveals that they have a length of several hundred amino acids and are highly enriched in threonine and serine residues predicting extensive *N*- and *O*-linked glycosylation (Lehle *et al*., 2006; Lommel & Strahl, 2009). In addition, middle domains are often composed of conserved tandem repeats of variable length (Verstrepen *et al*., 2004; Dranginis *et al*., 2007; Linder & Gustafsson, 2008) and they contain sequences enriched for β-branched aliphatic amino acids, indicating a high potential for β-aggregation and amyloid formation (Ramsook *et al*., 2010). Although the functions of these diverse structural features are not yet understood in full detail, a number of studies provide initial insights.

Middle domains appear to play an important role in properly presenting the N-terminal A domains at the cell surface. In the case of Flo1 and Flo11, it has been shown that the length of the middle domain directly correlates with the functionality of the proteins during flocculation and biofilm formation (Verstrepen *et al*., 2005; Zara *et al*., 2009). These findings are in agreement with studies on *Candida* adhesins (Frieman *et al*., 2002; Frieman & Cormack, 2004) and they show that longer middle domains usually enable more efficient adhesion, while smaller domains confer less avid adherence. This suggests that longer middle parts are required to expose A domains to neighboring cells during flocculation or to foreign surfaces during biofilm formation, while shorter parts cause A domains to remain buried in the cell wall. A further interesting twist comes from the observation that tandem repeats allow for rapid changes in repeat numbers by triggering frequent recombination events, causing a variation in the size and composition of middle domains (Verstrepen *et al*., 2005; Fidalgo *et al*., 2006, 2008). This mechanism might enable fungal populations to rapidly adapt adhesion properties to the environment (Levdansky *et al*., 2007; Verstrepen & Fink, 2009).

Vegetative adhesins of *S. cerevisiae* contain a large number of residues susceptible to *N*- and *O*-linked glycosylation. It has been shown that Flo1 and Flo11 are heavily glycosylated, although their exact glycan profile has not yet been determined (Straver *et al*., 1994; Bony *et al*., 1997; Douglas *et al*., 2007; Karunanithi *et al*., 2010). The functional role of this glycosylation for adhesion is not clear. It has been suggested that *O*-linked oligosaccharide side-chains enable the middle domains to obtain a rod-like structure that is further stabilized by $Ca^{2+}$ ions (Jentoft, 1990). The resulting semi-rigid stalks may help to project the protein through the exterior of the cell wall and enable the A domains to interact efficiently with ligands (Frieman *et al*., 2002; Verstrepen & Klis, 2006). In addition, the glycans provided by the middle domains of adhesin might affect the hydrophobicity. In *C. albicans*, for example, this correlation between the cell wall mannosylation and cell surface hydrophobicity has been demonstrated (Masuoka & Hazen, 1997, 2004). In *S. cerevisiae*, hydrophobicity has initially been linked to the expression of *FLO11* (Reynolds & Fink, 2001). However, a more recent study shows that strains expressing *FLO1*, *FLO5*, *FLO9* or *FLO10* also become highly hydrophobic (Van Mulders *et al*., 2009). This implicates that hydrophobicity is conferred by glycan structures, because the amino acid composition of adhesin middle domains can be highly divergent. Similarly, surface adhesion observed for strains that produce adhesins without an obvious N-terminal A domain, for example Fig2, might be conferred by oligosaccharides (Guo *et al*., 2000).

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Bioinformatic screening has uncovered that a number of *S. cerevisiae* adhesins contain one or more sequences with a high β-aggregation potential including Flo1, Flo11, Aga1, Fig2 and adhesins from *C. albicans* (Otoo *et al*., 2008; Frank *et al*., 2010; Ramsook *et al*., 2010). Functional studies show that Flo1 and Flo11 proteins form amyloids *in vivo* and *in vitro* and that cell–cell aggregation mediated by these adhesins is sensitive to amyloid-binding dyes. This suggests that amyloid formation of β-aggregation-prone sequences in the middle domains leads to the generation of adhesin multimers. These structures might affect the functionality of the N-terminal A domains by increasing the avidity or the potential for ligand binding *in trans* (Fig. 5).

### C-terminal domains

The C-terminal domains of *S. cerevisiae* adhesins contain an attachment site for a GPI anchor that is added in the endoplasmic reticulum (Caro *et al*., 1997; De Groot *et al*., 2003). This modification either enables membrane anchoring of proteins by incorporation of the GPI moiety into the lipid bilayer or it confers covalent attachment to the β-1,6-glucans of the cell wall by further processing steps (Udenfriend & Kodukula, 1995; Frieman & Cormack, 2003, 2004). In the case of the *S. cerevisiae* adhesins Aga1, Flo1, Flo9 and Flo11, GPI-attachment sites have been demonstrated to be required for cell wall association and functionality of the proteins (Lu *et al*., 1994, 1995; Bony *et al*., 1997; Frieman & Cormack, 2004; Douglas *et al*., 2007; Karunanithi *et al*., 2010). Although the biochemistry of covalent cell wall attachment is not known in full detail for most of these proteins, studies with Aga1 and cell wall proteins from other fungi suggest that adhesins are covalently linked by different mechanisms. This includes transglycosylation of a truncated GPI-glycan to β-1,6-glucan (Lu *et al*., 1995; Frieman *et al*., 2002; Klis *et al*., 2006), glutamine-dependent transesterification (Ecker *et al*., 2006) and disulfide bond formation (Huang *et al*., 2003).

## Regulation of adhesion

To understand how individual yeast cells and whole populations adapt their surface properties in response to the environment, it is essential to understand the mechanisms underlying the regulation of adhesin genes and proteins. More specifically, the signaling pathways and transcription factors that target the different promoter regions must be uncovered as well as the post-transcriptional and post-translational mechanisms that control adhesin production and function. In addition, the integration of regulatory responses must be studied under diverse environmental conditions to understand how individual cells control adhesin production and how phenotypic heterogeneity is achieved in yeast populations. A large number of studies have addressed the control of *FLO11* expression and revealed a highly complex promoter structure and regulatory pattern for this gene (Rupp *et al*., 1999; Mösch, 2000, 2002; Gancedo, 2001; Gagiano *et al*., 2002; Verstrepen & Klis, 2006; Zaman *et al*., 2008). In contrast, the regulation of *FLO1*, *FLO5*, *FLO9* and *FLO10* is less well understood, especially at the molecular level (Teunissen & Steensma, 1995; Verstrepen *et al*., 2003). This situation might stem from the fact that these *FLO* genes are not active in many laboratory strains, for example of the S288c or the Σ1278b genetic background (Liu *et al*., 1996; Halme *et al*., 2004). Finally, it is important to understand how *S. cerevisiae* can adapt adhesin gene structure on a longer evolutionary time scale.

### Chromosomal organization of adhesin genes

The *FLO1*, *FLO5*, *FLO9* and *FLO10* genes are carried in subtelomeric loci, whereas *FLO11* is located further away from the telomere region (Teunissen & Steensma, 1995; Verstrepen *et al*., 2004). *AGA1* is located in the middle between the telomere and the centromere and *FIG2* is present near the centromere region (Cherry *et al*., 1997). The subtelomeric location has an influence on both the evolution and the genetic regulation of *FLO* genes (Verstrepen & Fink, 2009). It has been shown that the *FLO* gene family has evolved and expanded extraordinarily fast (Hahn *et al*., 2005). This finding is partially attributable to the increased recombination frequencies found for (sub)telomeric loci, which tend to cluster together (Ricchetti *et al*., 2003). In addition, *FLO* genes contain up to 20 tandemly repeated sequences of approximately 100 nucleotides in their middle region (Verstrepen *et al*., 2004, 2005). Such tandem repeats are unstable and can induce intragenic mutations by replication slippage or recombination events between different genes that lead to changes in the number of repeats. Frequent recombination of *FLO* genes is thought to be an important mechanism for the rapid adaptation of adhesion properties of natural yeast in, for example changing environments (Infante *et al*., 2003; Rando & Verstrepen, 2007). Indeed, *FLO* genes are extraordinarily diverse in different laboratory and industrial *S. cerevisiae* strains (Verstrepen *et al*., 2005; Fichtner *et al*., 2007; Damas-Buenrostro *et al*., 2008; Dowell *et al*., 2010; Van Mulders *et al*., 2010). In addition, frequent recombination frequency has led to several *FLO*-derived pseudogenes that do not appear to be functional (Verstrepen & Fink, 2009). The chromosomal location of *FLO* genes also has an important influence on their expression, because (sub)telomeric loci are susceptible to stochastic gene silencing mechanisms (Gottschling *et al*., 1990; Renauld *et al*., 1993; Rusche *et al*., 2003; Raj & van Oudenaarden, 2008; Ehrentraut *et al*., 2010). As a consequence, epigenetic regulation of *FLO* genes

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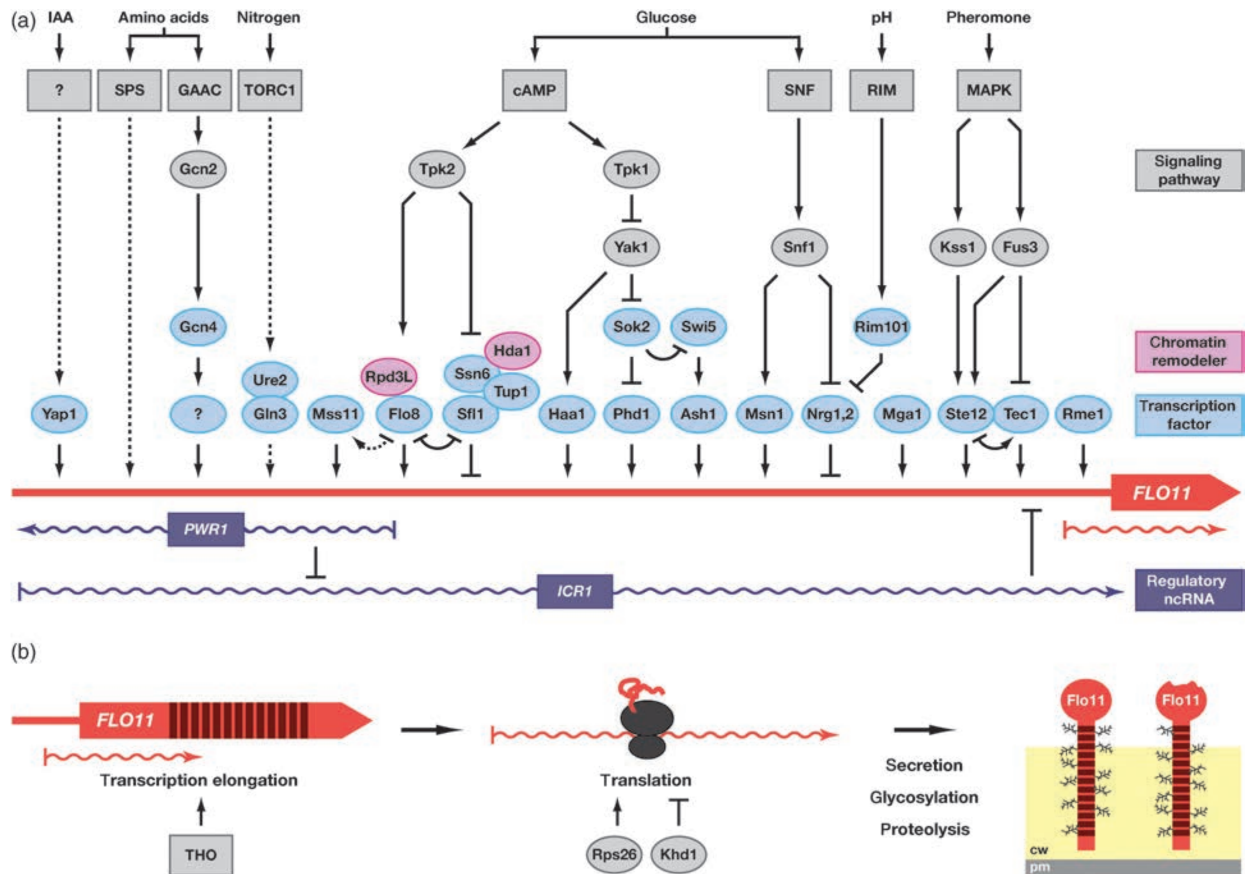


**Fig. 6.** Regulation of *FLO11* expression. (a) Wiring diagram showing the complex regulation of the *FLO11* promoter by conventional and epigenetic mechanisms. Arrows indicate positive regulation and inhibition is shown by bars. The different stimuli and corresponding signaling pathways targeting *FLO11* are indicated at the top (IAA = indole acetic acid; ? = unknown pathway). Downstream acting protein kinases are shown in gray. Chromatin remodelers found in the *FLO11* promoter region are shown in magenta. Transcription factors targeting the *FLO11* promoter (red line) are shown in light blue (? = unknown transcription factor). The input of the different transcription factors is only shown schematically and does not correspond to the positions of known binding sites. Regulatory ncRNAs (dark blue) affecting *FLO11* expression and *FLO11* mRNA (red) are shown as waved arrows. A detailed description of the signaling pathways controlling *FLO11* can be found in the text. (b) Regulation of *FLO11* during transcription elongation and at the post-transcriptional level. The positive effect of the THO complex on transcription elongation of *FLO11* at repetitive sequences (dark red stripes) is shown at the left. The positive influence of Rps26 and the negative effect of Khd1 on translation of the *FLO11* mRNA are depicted in the middle. On the right, glycosylated Flo11 protein carrying complex carbohydrate moieties (black) and inserted into the cell wall (cw) is illustrated. The plasma membrane (pm) is shown in gray. Both an unshedded and a proteolytically processed version of Flo11 are shown.

leads to highly variable adhesion properties of cells within clonal populations (Halme *et al*., 2004).

## *FLO11*

The *FLO11* promoter spans more than 3 kb and contains many upstream activation sequences (UASs) and elements for repression (Lo & Dranginis, 1996; Rupp *et al*., 1999). Numerous genetic studies in combination with genome-wide transcription factor binding analysis have revealed detailed insights into the topology of regulatory pathways at the promoter and its responsiveness to many external and internal signals (summarized in Fig. 6). In addition, single-cell analysis and mathematical modeling have provided a first picture of population-level heterogeneity of *FLO11* expression through the integration of conventional and epigenetic regulatory mechanisms (Halme *et al*., 2004; Vinod *et al*., 2008; Octavio *et al*., 2009).

### Fus3/Kss1 mitogen-activated protein kinase (MAPK) cascade

Under conditions of ample nutritional supply, cell-surface adhesion and *FLO11* expression depend on specific elements of the Cdc42-regulated Fus3/Kss1 MAPK cascade. This signaling module is highly conserved in all eukaryotes and has initially been identified to control sexual mating of haploid yeast cells (Bardwell, 2005; Chen & Thorner, 2007).

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Execution of vegetative adhesion and mating is under the control of shared and program-specific components. The shared components include the Rho-type GTPase Cdc42, the scaffold protein Ste50, the p21-activated protein kinase (PAK) Ste20, the MAPK kinase kinase (MAPKKK) Ste11, the MAPK kinase (MAPKK) Ste7, the MAPKs Fus3 and Kss1 as well as the transcription factor Ste12 (Liu *et al.*, 1993; Roberts & Fink, 1994; Mösch *et al.*, 1996, 2001; Cook *et al.*, 1997; Madhani *et al.*, 1997; Mösch & Fink, 1997; Roberts *et al.*, 1997; Ramezani Rad *et al.*, 1998; Rupp *et al.*, 1999). These elements control the expression of *FLO11* during vegetative growth and they are required for the activation of mating genes in response to peptide pheromones. The mating-specific components include the pheromone receptors, the three subunits for the heterotrimeric G protein and the scaffold protein Ste5, all of which are not required for *FLO11*-dependent adhesion during vegetative growth (Liu *et al.*, 1993; Roberts & Fink, 1994).

The adhesion-specific components include the upstream acting signaling mucin Msb2, which interacts with Cdc42 (Cullen *et al.*, 2004; Pitoniak *et al.*, 2009), and the downstream acting transcription factor Tec1, which belongs to the TEA domain (TEAD) family of regulators that control cellular development in many eukaryotes including several fungi (Andrianopoulos & Timberlake, 1991; Bürglin, 1991; Schweizer *et al.*, 2000; Anbanandam *et al.*, 2006). In *S. cerevisiae*, Tec1 is required for *FLO11* expression and for vegetative adhesion (Gavrias *et al.*, 1996; Mösch & Fink, 1997; Lo & Dranginis, 1998; Rupp *et al.*, 1999). In contrast, Tec1 is not required for pheromone stimulation of mating-specific genes, for example the sexual agglutinin encoding *FUS1* (Oehlen & Cross, 1998; Zeitlinger *et al.*, 2003). Tec1 and Ste12 can interact with each other and have become a paradigm for studying the mechanisms of combinatorial and promoter-specific target gene control (Madhani & Fink, 1997; Köhler *et al.*, 2002; Zeitlinger *et al.*, 2003; Borneman *et al.*, 2007a, b; Heise *et al.*, 2010). The TEA DNA-binding domain of Tec1 is composed of a three-helix bundle, which binds to conserved TEA consensus sequence (TCS) elements (Madhani & Fink, 1997; Anbanandam *et al.*, 2006; Heise *et al.*, 2010). Ste12 binds to pheromone response elements (PREs), and two or more of these elements are necessary to confer pheromone-responsive transcriptional control by the Fus3/Kss1 MAPK pathway (Fields & Herskowitz, 1985; Dolan *et al.*, 1989; Errede & Ammerer, 1989; Hagen *et al.*, 1991; Yuan & Fields, 1991). For combinatorial target gene control, Tec1 and Ste12 form a complex, enabling cooperative binding to combined filamentation and invasion response elements (FREs), which consist of a TCS and a PRE (Madhani & Fink, 1997; Chou *et al.*, 2006; Heise *et al.*, 2010). However, Tec1–Ste12 complexes regulate many target genes via single TCS elements, with Tec1 providing the DNA binding and Ste12 the transcriptional activation domain (Zeitlinger *et al.*, 2003; Borneman *et al.*, 2006, 2007a, b; Chou *et al.*, 2006), and Tec1 is even able to activate target genes independent of Ste12 (Köhler *et al.*, 2002; Heise *et al.*, 2010).

The *FLO11* promoter contains both FREs and TCS elements and is bound by Tec1 and Ste12 *in vivo* (Borneman *et al.*, 2006, 2007a, b; Chou *et al.*, 2006). Moreover, Tec1 is able to activate *FLO11* expression by Ste12-dependent and -independent mechanisms (Köhler *et al.*, 2002; Heise *et al.*, 2010). During vegetative growth, *FLO11* is under the negative control of Dig1 and Dig2, because these proteins directly inhibit the transcriptional activity of Ste12 and Tec1–Ste12 complexes (Cook *et al.*, 1997; Pi *et al.*, 1997; Tedford *et al.*, 1997; Bardwell *et al.*, 1998; Chou *et al.*, 2006). In response to pheromone, the inhibitory functions of Dig1 and Dig2 are relieved by phosphorylation through the MAPKs Fus3 and Kss1, enabling the maximal activation of Ste12 target genes, for example the sexual agglutinin genes or *FIG2*. However, *FLO11* expression declines drastically during mating, because the MAPK Fus3 phosphorylates Tec1 and triggers rapid ubiquitin-mediated degradation of the transcription factor (Bao *et al.*, 2004; Brückner *et al.*, 2004; Chou *et al.*, 2004). This control mechanism ensures that *FLO11* is not expressed during mating and sexual adhesin gene expression is efficiently prevented during vegetative growth (Zeitlinger *et al.*, 2003; Chou *et al.*, 2006, 2008; Heise *et al.*, 2010). This indicates that vegetative and sexual adhesins may constrain each other if present simultaneously at the cell surface.

### cAMP-PKA pathway

A further important signaling route that controls *FLO11*-dependent adhesion and filamentation is the cAMP-dependent protein kinase A (PKA) pathway (Robertson & Fink, 1998; Pan & Heitman, 1999; Rupp *et al.*, 1999). Central components of this glucose-responsive pathway include the adenylyl cyclase Cyr1, the phosphodiesterases Pde1 and Pde2 and the PKA itself that is composed of the regulatory subunit Bcy1 and any of the catalytic subunits Tpk1, Tpk2 or Tpk3 (Tamaki, 2007). All three Tpk proteins are redundant for viability (Toda *et al.*, 1987), but they differentially regulate *FLO11*-dependent adhesion (Pan & Heitman, 1999; Robertson *et al.*, 2000). Tpk2 stimulates *FLO11* expression by activating the transcription factor Flo8 and inhibiting the repressor Sfl1, which bind to the *FLO11* promoter at overlapping sites (Robertson & Fink, 1998; Rupp *et al.*, 1999; Conlan & Tzamarias, 2001; Pan & Heitman, 2002). Interestingly, Flo8 has been genetically inactivated in S288c strains, which fail to express *FLO11* (Liu *et al.*, 1996). In contrast, Σ1278b strains or EM93 (progenitor of S288c) have a functional *FLO8* gene and are therefore competent for agar and plastic adhesion (Liu *et al.*, 1996; Fichtner *et al.*, 2007). Unlike Tpk2, Tpk1 and Tpk3 negatively control *FLO11* and filamentation probably via feedback inhibition of cAMP

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production (Pan & Heitman, 1999; Robertson *et al.*, 2000). Tpk1 also phosphorylates and inhibits the DYRK family protein kinase Yak1, which is required for filamentation and *FLO11* expression (Garrett & Broach, 1989; Garrett *et al.*, 1991; Zhu *et al.*, 2000; Zhang *et al.*, 2001; Budovskaya *et al.*, 2005; Deminoff *et al.*, 2006, 2009; Malcher *et al.*, 2011). In turn, Yak1 targets *FLO11* via a transcription factor module that includes Sok2 and Phd1, which control adhesion and filamentation by directly binding to the *FLO11* promoter (Gimeno & Fink, 1994; Ward *et al.*, 1995; Pan & Heitman, 2000; Borneman *et al.*, 2006; Malcher *et al.*, 2011). Finally, Sok2 also affects *FLO11* and filamentation via the regulators Swi5 and Ash1 (Chandarlapaty & Errede, 1998; Pan & Heitman, 2000).

The PKA pathway is regulated by cAMP-dependent and -independent mechanisms. The adenylyl cyclase Cyr1 can be stimulated by activated forms of the small GTP-binding proteins Ras1 and Ras2 (Broek *et al.*, 1985; Toda *et al.*, 1985), which thereby positively control *FLO11* and filamentation (Gimeno *et al.*, 1992; Mösch *et al.*, 1996, 1999; Rupp *et al.*, 1999). In parallel, the transmembrane receptor Gpr1 and the Gα-like protein Gpa2 control PKA activity and *FLO11*, probably also by activation of Cyr1 (Kübler *et al.*, 1997; Lorenz & Heitman, 1997; Xue *et al.*, 1998; Yun *et al.*, 1998; Ansari *et al.*, 1999; Donzeau & Bandlow, 1999; Kraakman *et al.*, 1999). Further control on PKA is exerted by the kelch repeat proteins Gpb1 and Gpb2 that act via Ras2 or Gpa2 or that directly antagonize PKA (Harashima & Heitman, 2002; Batlle *et al.*, 2003; Lu & Hirsch, 2005; Harashima *et al.*, 2006; Peeters *et al.*, 2006; Phan *et al.*, 2010).

Glucose is generally accepted to activate PKA and *FLO11*-mediated adhesion via Ras2 and Gpa2, but the exact means by which this environmental signal stimulates the G proteins are still elusive (Gancedo, 2008; Zaman *et al.*, 2008). In addition, the secreted aromatic alcohols tryptophol and phenylethanol stimulate *FLO11* expression through a Tpk2-dependent mechanism, suggesting that PKA might be part of a quorum-signaling pathway that links environmental sensing to adhesion (Chen & Fink, 2006; Wuster & Babu, 2010). A further signal that controls PKA is ammonium, which acts via Mep2, a low-affinity ammonium permease required for filamentation (Lorenz & Heitman, 1998a, b; Van Nuland *et al.*, 2006). This indicates that PKA might affect the stimulation of *FLO11* in response to nitrogen starvation. Finally, it has been shown that the PKA pathway is connected to the Fus3/Kss1 MAPK cascade, which enables cross-pathway control towards *FLO11* (Mösch *et al.*, 1996, 1999; Vinod & Venkatesh, 2007; Chavel *et al.*, 2010).

### Snf1 pathway

Glucose starvation has been found to drastically induce agar and plastic adhesion as well as *FLO11* expression in haploid strains (Cullen & Sprague, 2000; Reynolds & Fink, 2001). Upregulation of *FLO11* depends on the Snf1 protein kinase pathway (Kuchin *et al.*, 2002; Palecek *et al.*, 2002; Van de Velde & Thevelein, 2008), which is part of a primary signaling pathway for the adaptation of yeast cells to glucose limitation and growth on less preferred carbon sources (Hedbacker & Carlson, 2008). The Snf1 catalytic subunit forms a heterotrimeric complex with one of the β-subunit isoforms Gal83, Sip1 or Sip2 and with the activating g-subunit Snf4, which prevents autoinhibition of Snf1 in response to glucose starvation (Yang *et al.*, 1994; Jiang & Carlson, 1997). The different Snf1 complexes can be activated by the upstream kinases Elm1, Sak1 and Tos3 and they are inactivated by the Reg1-Glc7 protein phosphatase (Hedbacker & Carlson, 2008). Of the three different β-subunits, Gal83 seems to be specifically involved in the regulation of *FLO11* regulation (Vyas *et al.*, 2003). Moreover, Snf1-Gal83 controls *FLO11* by inactivating the transcriptional repressors Nrg1 and Nrg2 (Vyas *et al.*, 2003; Ishigami *et al.*, 2004, 2006). How exactly these repressors control the *FLO11* promoter is not known, but studies with the related *STA1* promoter in strains of *S. cerevisiae* var. *diastaticus* suggest that binding of Nrg1 negatively acts on a UAS element (Kim *et al.*, 2004a, b). In addition, Nrg1 and Nrg2 are under the control of the pH-responsive Dfg16-Rim101 pathway, which affects agar adhesion and *FLO11* expression, indicating that these regulators might also be involved in pH control of *FLO11* (Lamb & Mitchell, 2003; Barwell *et al.*, 2005; Barrales *et al.*, 2008).

### Nitrogen and amino acids

Several studies have shown that *FLO11* expression, adhesion and filamentation are activated by nitrogen or amino acid starvation (Gimeno *et al.*, 1992; Ljungdahl *et al.*, 1992; Braus *et al.*, 2003). Genetic evidence indicates the involvement of Mep2-PKA and possibly Snf1 in connecting nitrogen starvation to *FLO11* expression (Lorenz & Heitman, 1998a, b; Kuchin *et al.*, 2002; Vyas *et al.*, 2003; Van Nuland *et al.*, 2006). As discussed above, these signaling pathways might control the *FLO11* promoter by targeting the transcription factors Flo8, Sfl1, Nrg1 and Nrg2. In addition, the target of rapamycin complex 1 (TORC1) has been implicated in nitrogen control of *FLO11* and filamentation (Cutler *et al.*, 2001; Vinod *et al.*, 2008). These studies indicate a requirement of TORC1 activity under nitrogen-limiting conditions. However, the connection between TORC1 and the *FLO11* promoter is not clear. TORC1 is known to control the nitrogen discrimination pathway (NDP) that involves the transcription factor Gln3 and its regulator Ure2 (Blinder *et al.*, 1996; Beck & Hall, 1999; Cardenas *et al.*, 1999; Cooper, 2002; Feller *et al.*, 2006). In addition, Gln3 and Ure2 are known to positively regulate filamentation in response to nitrogen starvation (Lorenz & Heitman, 1998a, b). Whether

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these regulators directly act on *FLO11* expression, however, needs to be further investigated.

Amino acid starvation has been demonstrated to activate adhesive growth and *FLO11* in the presence of glucose and ammonium (Braus *et al*., 2003; Kleinschmidt *et al*., 2005). This effect depends on the sensor kinase Gcn2 and the transcription factor Gcn4, which are central elements of the general amino acid control (GAAC) system (Hinnebusch, 2005). However, Gcn4 seems to regulate *FLO11* indirectly by controlling an as yet unknown signaling pathway that confers derepression of *FLO11* in response to amino acid starvation (Braus *et al*., 2003; Valerius *et al*., 2007). A further connection between amino acid availability and *FLO11* comes from the observation that strains mutated for *SSY1* or *PTR3*, which encode elements of the SPS amino acid-sensing pathway, are hyperadhesive (Klasson *et al*., 1999; Ljungdahl, 2009). However, no connection has been made between downstream regulators of the SPS system, for example the transcription factors Stp1 and Stp2 (Andreasson & Ljungdahl, 2004), and *FLO11*. Finally, exposure of yeast cells to the plant hormone indole acetic acid (IAA) stimulates the expression of *FLO11* by involving members of the Avt family of IAA and amino acid transporters and the transcription factor Yap1 (Prusty *et al*., 2004). This suggests that *S. cerevisiae* seems to be able to adapt its adhesion properties in response to plant hormone and nutritional signals, but the exact signaling routes leading to *FLO11* remain to be elucidated.

### Transcription factors Mss11, Msn1, Mga1, Rme1 and Haa1

*FLO11* expression is under further control of several transcription factors including Mss11, Msn1, Mga1, Rme1 and Haa1, which operate through not well-understood mechanisms. *MSS11* and *MSN1* were initially identified as activators of *FLO11* and filamentation, but also as multicopy suppressors of *mep2* and *ras2* mutations (Gimeno & Fink, 1994; Lambrechts *et al*., 1996a, b; Vivier *et al*., 1997; Gagiano *et al*., 1999). Later studies showed that Mss11 is required for the activation of *FLO11* by many other regulators including Tec1, Flo8, Phd1, Nrg1, Nrg2, Sok2 and Sfl1 (Gagiano *et al*., 2003; van Dyk *et al*., 2005). How exactly Mss11 operates at the *FLO11* promoter is not clear, but its function depends on a conserved glutamine-rich activation domain and may involve physical association with Flo8 (Kim *et al*., 2004a, b). *MSN1* (also known as *PHD2* or *MSS10*) was originally isolated as a multicopy suppressor of an *snf1* mutation, indicating that this regulator might function downstream of the Snf1 kinase (Estruch & Carlson, 1990). It was also suggested that Msn1 may act at longer distances to destabilize chromatin (Sidorova & Breeden, 1999). A further regulator of *FLO11* is Mga1, a protein with similarity to heat shock transcription factors. The *MGA1* gene was originally isolated as a multicopy suppressor of pseudohyphal growth defects of *mep2* mutants (Lorenz & Heitman, 1998a, b). Genome-wide location analysis demonstrated that Mga1 – along with Ste12, Tec1, Sok2, Phd1 and Flo8 – binds to the *FLO11* promoter *in vivo* (Borneman *et al*., 2006). This study also revealed that the promoters of *MGA1* and *PHD1* were bound by all of these transcription factors, identifying them as master regulators of *FLO11* and as key target hubs in the complex regulatory network for adhesion and filamentation. The transcriptional regulator Rme1, a DNA-binding protein that represses entry into meiosis, was also identified to positively regulate *FLO11* and adhesion (van Dyk *et al*., 2003). The expression of *RME1* is under direct control of the a1-$\alpha$2 repressor and is therefore efficiently expressed in haploids, but not in diploids (Covitz *et al*., 1991). This might at least in part explain why *FLO11* expression is significantly higher in haploid than in diploid strains (Rupp *et al*., 1999). However, Rme1 does not seem to be involved in the ploidy-specific expression of *FLO11*, which is conferred by as yet unknown mechanisms (Galitski *et al*., 1999). Finally, *FLO11* has recently been found to be under control of the transcription factor Haa1, which is also required for adaptation of yeast to acidic stress (Keller *et al*., 2001; Aranda & del Olmo, 2004; Fernandes *et al*., 2005). *HAA1* is required for *FLO11* expression, but only under acid stress conditions, and it acts as a multicopy suppressor of a *yak1* mutation (Malcher *et al*., 2011). Whether Haa1 is a direct target of the Yak1 kinase and how Haa1 controls the *FLO11* promoter is not known.

### Epigenetic regulation

In addition to the many conventional regulatory mechanisms that target the *FLO11* promoter, the expression of *FLO11* is also under epigenetic control. In response to nitrogen starvation, diploid yeast strains express *FLO11* and initiate filamentation in a highly heterogeneous manner (Halme *et al*., 2004; Vinod *et al*., 2008). The high degree of cell-to-cell variation observed is caused by metastable and inheritable silencing of the *FLO11* promoter and is based on both promoter and genomic positional information. At least two distinct mechanisms contribute to epigenetic control of *FLO11*. One mechanism depends on the regulator Sfl1 and the histone deacetylase (HDAC) Hda1, which is part of a complex implicated in transcriptional control (Rundlett *et al*., 1996; Vogelauer *et al*., 2000; Lee *et al*., 2009). In the case of *FLO11*, Sfl1 has been suggested to provide promoter specificity for silencing by direct binding (Halme *et al*., 2004). Hda1 is likely to integrate both genome positional information and promoter-specific signals that stem from Sfl1, which could recruit Hda1 via the Tup1/Ssn6 corepressor (Conlan & Tzamarias, 2001). The nature of the

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positional determinants, however, remains unclear. A second mechanism involves a pair of *cis*-interfering noncoding RNAs (ncRNAs) and Rpd3L, another HDAC complex involved in transcriptional regulation (Yang & Seto, 2008; Bumgarner *et al.*, 2009). Interestingly, Rpd3L is an activator of *FLO11*, which is unexpected, given the fact that the complex is present at the *FLO11* promoter (Barrales *et al.*, 2008; Bumgarner *et al.*, 2009). However, Rpd3L seems to act via chromatin condensation at the *FLO11* promoter at an upstream site that includes the binding sites for Sfl1 and Flo8. This event blocks the access of Sfl1, but promotes Flo8 binding and enables the expression of the ncRNA *PWR1*, whose transcription is initiated upstream of and in a direction opposite to *FLO11*. As a consequence, *FLO11* expression is promoted because the *PWR1* ncRNA interferes with the transcription of *ICR1*, a second ncRNA. The expression of *ICR1* is promoted by Sfl1 binding and represses *FLO11* in *cis* by a 'promoter occlusion' mechanism (Martens *et al.*, 2004, 2005; Bumgarner *et al.*, 2009). Because transcription of *ICR1* occurs in the same direction as *FLO11* and covers most of the *FLO11* promoter region, it probably blocks access of the *FLO11* core promoter to general transcription factors and to chromatin remodelers required for nucleosome rejection (Martens & Winston, 2003). Additional chromatin remodeling at the *FLO11* promoter might be provided by the SWI/SNF complex, a multisubunit DNA-dependent ATPase that regulates transcription by altering chromatin structure and that is involved in *FLO11* activation (Peterson & Workman, 2000; Barrales *et al.*, 2008).

Epigenetic mechanisms provide a circuitry for toggling between a silenced and a transcriptional competent state (Bumgarner *et al.*, 2009). In the case of *FLO11*, they contribute to the observed variegated and bistable expression and the resulting variation of cell surface properties within a yeast population. The observed *cis*-acting mechanisms are likely to contribute to the finding that two copies of the *FLO11* locus present within a single cell switch between a silenced and a competent promoter state in a random and independent manner (Octavio *et al.*, 2009). Epigenetic mechanisms help to classify conventional *trans*-acting activators of *FLO11* expression, based on their ability to stabilize the competent state. It has been shown that Tec1, Ste12 and Phd1 belong to a class of activators, which only weakly stabilize the competent state and cannot effectively activate transcription at a silenced promoter (Octavio *et al.*, 2009). These class I activators regulate fast promoter fluctuations and destabilize the competent state to increase the burst frequency (Bar-Even *et al.*, 2006; Newman *et al.*, 2006). In contrast, Flo8 belongs to the class II of activators that primarily regulate slow promoter fluctuations and stabilize the competent state. At medium levels, Flo8 'opens' the silenced promoter state and enables activation by class I activators. At high levels, Flo8 fully disrupts silencing to induce a homogenous high-level expression of *FLO11* within yeast populations.

### Transcriptional elongation and post-transcriptional regulation

In addition to the plethora of control mechanisms that act on the promoter of *FLO11*, the expression of the gene is regulated during transcription elongation and at the post-transcriptional level. It has been found that *FLO11* transcription elongation is hindered in the region of the tandem repeats in *tho* mutants (Voynov *et al.*, 2006). In *S. cerevisiae*, the multisubunit THO complex has been identified as a possible elongation complex, which is recruited to actively transcribed genes and is involved in the cotranscriptional formation of messenger ribonucleoparticles (mRNP) that are competent to be exported from the nucleus (Jimeno *et al.*, 2002; Strasser *et al.*, 2002; Jimeno & Aguilera, 2010). In the case of *FLO11* (and also *FLO1*), regulation by THO depends on the internal repeats and is partially uncoupled when *TOP1* encoding topoisomerase is overexpressed (Voynov *et al.*, 2006). Interestingly, mutations that reduce THO or topoisomerase activity induce the formation of DNA : RNA hybrids (R loops) (Masse *et al.*, 1997; Huertas & Aguilera, 2003; Drolet, 2006). This indicates that the THO complex might help to overcome such inhibitory hybrid structures and thereby allow efficient transcription elongation at internal repeats.

Several studies have reported post-transcriptional control mechanisms for *FLO11* expression. Comparison of *FLO11* transcript levels with the activity of a coexpressed *FLO11-lacZ* reporter gene suggests that translation of the *FLO11* mRNA is upregulated in response to amino acid starvation and that efficient translation and adhesion requires the ribosomal protein Rps26 at levels that are sufficient for viability (Strittmatter *et al.*, 2006; Fischer *et al.*, 2008). Thus, yeast cells seem to be able to adapt adhesion properties by adjusting *FLO11* translation efficiency. A further post-transcriptional mechanism for *FLO11* expression involves the RNA-binding protein Khd1, which regulates the asymmetric expression of *FLO11* indirectly via the transcriptional regulator Ash1, which is required for the expression of *FLO11* and filamentation (Chandarlapaty & Errede, 1998; Pan & Heitman, 2000; Wolf *et al.*, 2010). In addition, Kdh1 directly binds to the *FLO11* mRNA and inhibits its translation (Wolf *et al.*, 2010). This regulation allows changes in *FLO11* expression between mother and daughter cells to establish the asymmetry that is required for the transition between yeast form and filamentous growth. Finally, Flo11 also seems to be regulated at the post-translational level. It has been shown that Flo11 is shed from cells and involves the protease Kex2, which is required for the cleavage and maturation of the Flo11 protein (Karunanithi *et al.*, 2010). This study also

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found that Flo11 shedding contributes to the overall balance in adherence properties that is optimal for filamentation and mat formation. Moreover, shed Flo11 is a component of a fluid layer that surrounds mats, which may have functions analogous to the mucus secretions of higher eukaryotes. Whether and how shedding is regulated in response to environmental changes remains to be investigated.

### *FLO1, FLO5, FLO9* and *FLO10*

When compared with *FLO11*, less information is available on the regulation of other *FLO* genes. In industrial strains, *FLO* gene expression has been found to be regulated by a number of environmental stimuli (Bauer *et al*., 2010; Soares, 2010). In brewing strains for instance, *FLO1* is regulated by carbon and/or nitrogen starvation (Sampermans *et al*., 2005), pH (Soares & Seynaeve, 2000) and ionic strength (Jin & Speers, 2000). However, detailed analysis of the regulatory pathways and control mechanisms in production strains has been hampered by their limited experimental tractability.

In the most commonly used laboratory strain S288c, flocculation and adhesion to foreign surfaces are impaired due to the acquisition of a nonsense mutation in the *FLO8* gene, which is required for the expression of not only *FLO11*, but also *FLO1* (Liu *et al*., 1996; Kobayashi *et al*., 1999; Bester *et al*., 2006; Fichtner *et al*., 2007). In this strain background, *FLO1* expression is also under control of the activator Mss11 (Bester *et al*., 2006; Shen *et al*., 2006; Fichtner *et al*., 2007) and the Tup1/Ssn6 corepressor (Teunissen *et al*., 1995a, b; Fleming & Pennings, 2001; Zhang & Reese, 2004). Furthermore, the RNA polymerase II mediator complex tail component Sin4, but not Sfl1, controls *FLO1* in S288c strains. In contrast, *FLO5*, *FLO9* and *FLO10* are not induced by the deletion of different repressor genes including *SFL1*, *TUP1*, *SSN8*, *SIN4* or *SRB8* (Fichtner *et al*., 2007). Finally, *FLO1* is induced in S288c strains lacking the HDAC Hda1 or the mediator subunit Srb10 (Green & Johnson, 2004; Zhang & Reese, 2004). Thus, not only *FLO11*, but also *FLO1* appears to be under complex control of conventional and epigenetic mechanisms, although detailed insights are still lacking.

In Σ1278b laboratory strains, *FLO* gene expression patterns differ from the S288c genetic background. Under standard growth conditions, Σ1278b *FLO1*, *FLO5*, *FLO9* and *FLO10* are epigenetically silenced (Guo *et al*., 2000; Halme *et al*., 2004; Reynolds *et al*., 2008). *FLO1* expression is further impaired due to the absence of an efficient Flo8-binding site in the promoter (Fichtner *et al*., 2007). In Σ1278b, *FLO1* has also been found to be regulated during transcription elongation by the THO complex (Voynov *et al*., 2006). *FLO10* expression in Σ1278b was found to be derepressed by the deletion of *SFL1* or in the absence of either Ira1 or Ira2, two Ras2 GTPase-activating proteins that negatively control the cAMP-PKA pathway (Halme *et al*., 2004). The same study also uncovered that *FLO10* is regulated by the same transcription factors that control *FLO11*, Sfl1 and Flo8, but is silenced by a distinct set of histone deacetylases, Hst1 and Hst2. Further epigenetic control is inferred on *FLO10* by a mechanism that involves a pair of *cis*-interfering ncRNAs (Bumgarner *et al*., 2009). Similar to *FLO11*, these transcripts are encoded upstream of *FLO10* and are likely to control gene expression by a promoter occlusion mechanism. Finally, none of the mutations that derepress *FLO10* in Σ1278b are sufficient to induce significant expression of *FLO5* or *FLO9*, indicating that they are regulated by distinct mechanisms. Genome-wide transcription factor-binding studies in Σ1278b strains have revealed that, for example the *FLO9* promoter is bound by diverse regulators including Phd1, Sok2 or Nrg1 (Harbison *et al*., 2004). However, whether these regulators indeed control *FLO9* remains to be investigated.

### *AGA1* and *FIG2*

*AGA1* and *FIG2* play well-established roles in sexual agglutination and the expression of both genes is highly induced during mating (Roy *et al*., 1991; Cappellaro *et al*., 1994; Erdman *et al*., 1998; Zhao *et al*., 2001; Jue & Lipke, 2002; Zhang *et al*., 2002). Accordingly, *AGA1* and *FIG2* are under control of the Fus3/Kss1 MAPK cascade and their promoters are bound by the transcription factor Ste12 *in vivo* (Oehlen *et al*., 1996; Zeitlinger *et al*., 2003; Harbison *et al*., 2004). In addition, both of these genes also confer vegetative adhesion. In Σ1278b *flo11* mutant strains for instance, the expression of *FIG2* from the strong *GAL1* promoter is sufficient to induce agar adhesion and filamentation (Guo *et al*., 2000). It has therefore been suggested that successful mating not only depends on sexual adhesion of partner cells, but might also require a more complex response before agglutination (Erdman & Snyder, 2001). Such an early response could be induced by low levels of pheromone and enhance the ability of cells to search for mating partners by directed surface growth, which requires vegetative adhesion by Fig2 and Aga1. Further stimuli and regulatory pathways, which in principle could exist to control *AGA1* and *FIG2*, have not yet been discovered.

## Concluding remarks

### *Saccharomyces cerevisiae* as a model for fungal adhesion

The formation of multicellular aggregates by *S. cerevisiae* has been observed already in the 19th century, but a precise picture of the molecular mechanisms that underlie yeast adhesion has only recently become available. In this review, we have summarized a substantial number of publications that provide first detailed insights into the structure and

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function of adhesins and reveal a highly complex regulatory network for adhesion in *S. cerevisiae*. These studies have further allowed to uncover new concepts in gene evolution, to investigate the interplay of conventional and epigenetic mechanisms in gene regulation and to analyze the importance of self-recognition for cooperative cell interaction and social behavior of microorganisms. Thus, cell adhesion of *S. cerevisiae* is a valuable system to study not only fundamental cellular processes, but also the general principles of microbial development.

So far, many of the concepts for cell adhesion that have been elaborated in *S. cerevisiae* are based on only a small number of selected examples and were mostly obtained under laboratory conditions using non-natural test systems. This stands in contrast to many human and plant pathogenic fungi, where the ecological niches are often well known, a fact that allows to study adhesion under natural conditions and on specific host surfaces. In fungal pathogens, adhesion is a crucial step for the development of infection structures such as germ tubes and appressoria (Tucker & Talbot, 2001; Kaur *et al*., 2005; Kumamoto & Vinces, 2005; Meng *et al*., 2009; Torto-Alalibo *et al*., 2009). Moreover, a number of different adhesins have been identified that confer specific attachment of pathogenic fungi to host cells. In the human pathogenic yeasts *C. albicans* and *C. glabrata*, for example, the *ALS* and *EPA* gene families play crucial roles in the process of adhesion to host surfaces (Hoyer, 2001; Sundstrom, 2002; Kaur *et al*., 2005; Filler, 2006; Hoyer *et al*., 2008). Both families are related to the adhesins of *S. cerevisiae* and are GPI-CWPs (Verstrepen & Klis, 2006; Dranginis *et al*., 2007). Functional studies have revealed that Als and Epa adhesins confer adhesion to diverse carbohydrates and proteins present on host cell surfaces and that binding specificity is governed by their N-terminal A domains (Cormack *et al*., 1999; Frieman *et al*., 2002; Hoyer *et al*., 2008). In plant pathogenic fungi, for example *Magnaporthe grisea* or *Phytophthora parasitica*, adhesins have been identified that confer attachment to host cells and might act as lectins or hydrophobins (Talbot *et al*., 1996; Gaulin *et al*., 2002; Ahn *et al*., 2004; Liu *et al*., 2009). The exact structure and function of these molecules, however, is largely unknown.

In pathogenic fungi, analysis of adhesin function and regulation is often hampered by their elaborate or limited experimental tractability. Here, *S. cerevisiae* has proven to be a valuable model for the characterization of adhesins from other fungi. A prominent example is the functional analysis of Epa adhesins from *C. glabrata* in *S. cerevisiae* cells to study domain structures and ligand-binding specificity (Cormack *et al*., 1999; Frieman *et al*., 2002; Zupancic *et al*., 2008). Similar strategies might be used for studying adhesin molecules from other pathogens. Furthermore, several lessons learned from studying the regulatory network for adhesion in *S. cerevisiae* have turned out to be true for other fungi (Lengeler *et al*., 2000). For instance, the finding that the cAMP-PKA and MAPK pathways control *S. cerevisiae* adhesion has allowed to directly test whether the same is the case in human pathogenic fungi. Indeed, both pathways also act in parallel in *C. albicans* to control adhesion and virulence (Liu *et al*., 1994; Lo *et al*., 1997; Feng *et al*., 1999). In addition, many of the transcription factors that control *FLO11* in *S. cerevisiae* are related to regulators of adhesion and virulence in fungal pathogens including Tec1, Ste12, Phd1, Sok2 and Mss1 (Liu *et al*., 1994; Stoldt *et al*., 1997; Schweizer *et al*., 2000; Su *et al*., 2009). Finally, high-resolution structural analysis of *S. cerevisiae* adhesins allows to produce more precise models for related proteins from pathogenic fungi, for example PA14 domain-related adhesins (de Groot & Klis, 2008; Veelders *et al*., 2010). These findings are promising indicators that future knowledge gained by studying the *S. cerevisiae* adhesion model will have an important impact on the research directed in other fungi.

## Future perspectives

Owing to the fact that we have only limited information on the true ecological significance of *S. cerevisiae* adhesion, future research in this field must include a detailed analysis of a large number of adhesin genes and proteins and it must also aim at the establishment of more natural test systems. More specifically, such studies must include high-resolution structure and function analysis of many more yeast adhesins, for example members of the Flo10 and Flo11 subfamily, with a clear focus on uncovering the precise mechanisms for ligand recognition and discrimination. A helpful step towards this goal will be the comparative analysis of adhesin genes in a large number of diverse laboratory and industrial *S. cerevisiae* strains. In addition, such studies should include a wide variety of *Saccharomyces sensu stricto* strains isolated from diverse natural environments or clinical origin (Fay *et al*., 2004; Carreto *et al*., 2008; Klingberg *et al*., 2008). Uncovering the biodiversity of yeast adhesin structure and function may also provide new insights into the evolutionary adaptation of fungal cell adhesion in general and further develop *S. cerevisiae* into an attractive model for ecology and evolution (Replansky *et al*., 2008). A second future effort should be the elucidation of the physiologically relevant conditions and the corresponding regulatory pathways that control adhesin gene expression in natural environments. Clearly, the mechanisms that control adhesin genes in the subtelomeric regions are far from understood. Here, new lessons learned from *S. cerevisiae* may also contribute to a better understanding of adhesin gene regulation in human pathogenic yeasts, for example *C. glabrata*, which often carry important epithelial adhesin genes close to telomeres (Castano *et al*., 2006). A further and very challenging future

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goal is the identification of ecophysiologically relevant surfaces structures and ligand molecules that are bound by *S. cerevisiae* adhesins in natural environments and habitats. There is only limited information about the distribution and population structure of wild strains in natural environments (Fay & Benavides, 2005; Aa *et al*., 2006). *Saccharomyces cerevisiae* has been isolated from cultivated plants, for example damaged or rotting fruits, but also from uncultivated habitats, for example the bark and fluxes of oaks in natural woodlands (Mortimer *et al*., 1994; Polsinelli *et al*., 1996; Naumov *et al*., 1998; Mortimer & Polsinelli, 1999; Sniegowski, 1999; Sniegowski *et al*., 2002; Fay *et al*., 2004). In this context, it is interesting to note that adhesin genes and genes for hydrolytic enzymes, for example pectinases and glucoamylases, are coregulated in *S. cerevisiae*, indicating a competence for plant substrate adhesion and invasion (Vivier *et al*., 1997; Madhani *et al*., 1999). *Saccharomyces cerevisiae* and other *Saccharomyces sensu stricto* species have also been found to be associated with insects, for example wasps, honey bees and fruit flies (Phaff & Knapp, 1956; Phaff *et al*., 1956; Stevic, 1962; Lachance *et al*., 1995; Naumov *et al*., 1995, 1996). It has therefore been discussed that *S. cerevisiae*, which is not an airborne microorganism, may use insects as a vector for dissemination (Stevic, 1962; Mortimer & Polsinelli, 1999). Although these observations point towards possible foreign surfaces that might be specifically recognized by *S. cerevisiae*, it remains to be determined whether plants or insects have specific surface structures that are recognized by *S. cerevisiae* adhesin. The future identification of naturally relevant adhesin ligands will also crucially depend on the development of new *in vivo* and *in vitro* test systems for *S. cerevisiae* cell adhesion. These approaches will ultimately allow to understand the ecophysiological roles of adhesins not only for the development of protective multicellular structures, but also for the colonization of substrates and the distribution of *S. cerevisiae* in natural habitats.

## Acknowledgements

Work in our laboratory was supported by grants from the Deutsche Forschungsgemeinschaft (DFG), MO 825/3-1 and GRK 1216, by the International Max-Planck Research School (IMPRS) for Environmental, Cellular and Molecular Microbiology and by the Marburg Center for Synthetic Microbiology (SYNMIKRO).

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