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Molecular basis and regulation of flocculation in *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* – a review

Flocculation is an important facet of yeast behaviour and widely exploited in the brewing industry as an effective, low cost and natural mechanism for yeast removal from beer. Phenotypic characterisation has been conducted for decades and has revealed several different flocculation types based on their inhibition by different types of sugars. However, recent research has unveiled that not all adhesin proteins involved in flocculation fit into these two categories. Over the last 10 years, the crystal structures of the sugar-binding domains of flocculins Flo1p and Lg-Flo1p have been elucidated and gave important insights into the underlying molecular basis of the different flocculation types. Together with the vast research into the cellular regulation and the intrinsic genetic instability of these two major flocculation proteins, it has become clear that a purely descriptive characterisation of flocculation is insufficient and needs to be amended with molecular biological and genetic research techniques. This is especially true in light of aiming to control flocculation during beer fermentation. Therefore, this review aims to summarise the current understanding of the underlying molecular biology and cellular signalling mechanisms involved in flocculation to give brewing scientists an adequate overview of the current state of research.

Descriptors: flocculation, *Saccharomyces cerevisiae*, *Saccharomyces pastorianus*, gene regulation, cell signalling, FLO gene family

1 Introduction

Flocculation describes the asexual, reversible aggregation of yeast cells towards the end of fermentation, causing the cells either to rise to the top (top-fermenting) or to sink to the bottom of the fermentation vessel (bottom-fermenting) (reviewed in [1]). This behaviour is distinct from other adhesive/aggregative behaviours, e.g. sexual aggregation which is dependent on pheromones or pseudo-hyphal and biofilm formation (reviewed in [2]). Flocculation is being thought of as a survival mechanism where cells within the flocs lyse to provide nutrients [3]. Further, it is linked to cell conjugation and mating efficiency [4] and it has been shown that yeast flocs from mixed cultures were preferentially composed of *FLO1* expressing cells [5]. It has therefore been suggested that *FLO1* is a rare "green-beard" gene, driving cooperation and altruism in yeast [5].

Flocculation is exploited in beer production, as it enables efficient separation of yeast cells from the final product, while being low-cost and environmentally friendly. However, exact control of flocculation is difficult as it can be influenced by a plethora of technical and chemical parameters. The importance of these factors on the technology of the brewing process have been subject to numerous reviews [1, 6–8] and thus the technological aspects are mostly skipped here. It is crucial to note that the (ongoing) selection of specific yeasts by breweries has created considerable genetic variation within the family of brewing yeasts, especially in the *FLO* genes [9]. The occurrence of Lg-*FLO1* in ale yeasts gives evidence that co-occurrent usage of lager and ale yeasts might lead to further interspecific hybridization [9]. Also, the inherent genetic instability of these genes [10, 11] can contribute to changes in flocculation behaviour over time, which can lead to problems with yeast used for re-pitching. This poses a challenge for the brewing process as an altered flocculation behaviour is unwanted. A loss of flocculation ability will increase production cost as it necessitates the usage of other yeast removal procedures, while a too early flocculation is associated with problems in beer flavour quality and microbial stability due to a premature end of fermentation. The crucial influence of the underlying cellular signalling and genetic mechanisms necessitates not only a phenotypical characterization, but also molecular biological and genomic research.

The aim of this review is thus to detail the molecular basis and cellular signalling mechanisms involved in and leading to flocculation. Cell signalling is part of a system which enables organisms to react to intracellular and extracellular events. Protein phosphorylation/dephosphorylation is a major signal transduction mechanism [12] central to establishment of a flocculation phenotype and involves the enzymatic action of protein kinases and

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-phosphatases (see further in this review). Typical amino acids in proteins acting as phosphate group acceptors are the side-chain hydroxyl groups of serine, threonine and tyrosine. The addition of ionic phosphate group(s) onto the acceptor site by kinases causes a conformational change in the 3-dimensional structure of the such modified protein, leading to activation/deactivation of the protein function and/or change of protein interaction partners [13]. These phosphate group(s) can then be subsequently removed by phosphatases to restore the basal state of the protein [14]. This "on/off-switching" is exploited by the cell to transduce signals, e.g. about environmental changes, which then lead to a cellular reaction e.g. by a change in gene expression [15]. Thus, via protein phosphorylation/dephosphorylation the cell is able to react in a regulated manner to intra- and extracellular events. Examples of such events are alerting the cellular machinery to DNA damage [16] or adjustment of gene expression in relation to changes in nutrient availability [17].

However, (de)phosphorylation is not the only mechanism by which proteins are modified in the process of cellular reactions. Over the course of this review, a further modification will be important: Acetylation. Acetylation/deacetylation in the here discussed cases is a central mechanism by which histones are modified to render DNA accessible for gene transcription [18]. Histones are a group of proteins responsible for the spatial organization of DNA. DNA in eukaryotic cells is linear, however way too long to fit into the cell as such and is therefore wound around histone complexes for better organization. Depending on the acetylation state of certain histones this organizational structure is either tight (heterochromatin, deacetylated) or loose (euchromatin, acetylated). The loose structure initiated by acetylation of histones renders DNA regions accessible and is also involved in recruiting further proteins e.g. RNA polymerases responsible for the transcription of genes [18]. Histones are acetylated on specific lysines and the reactions are catalysed by histone acetyl transferases (HATs) while the reverse reactions are catalysed by histone deacetylases (HDACs) [19].

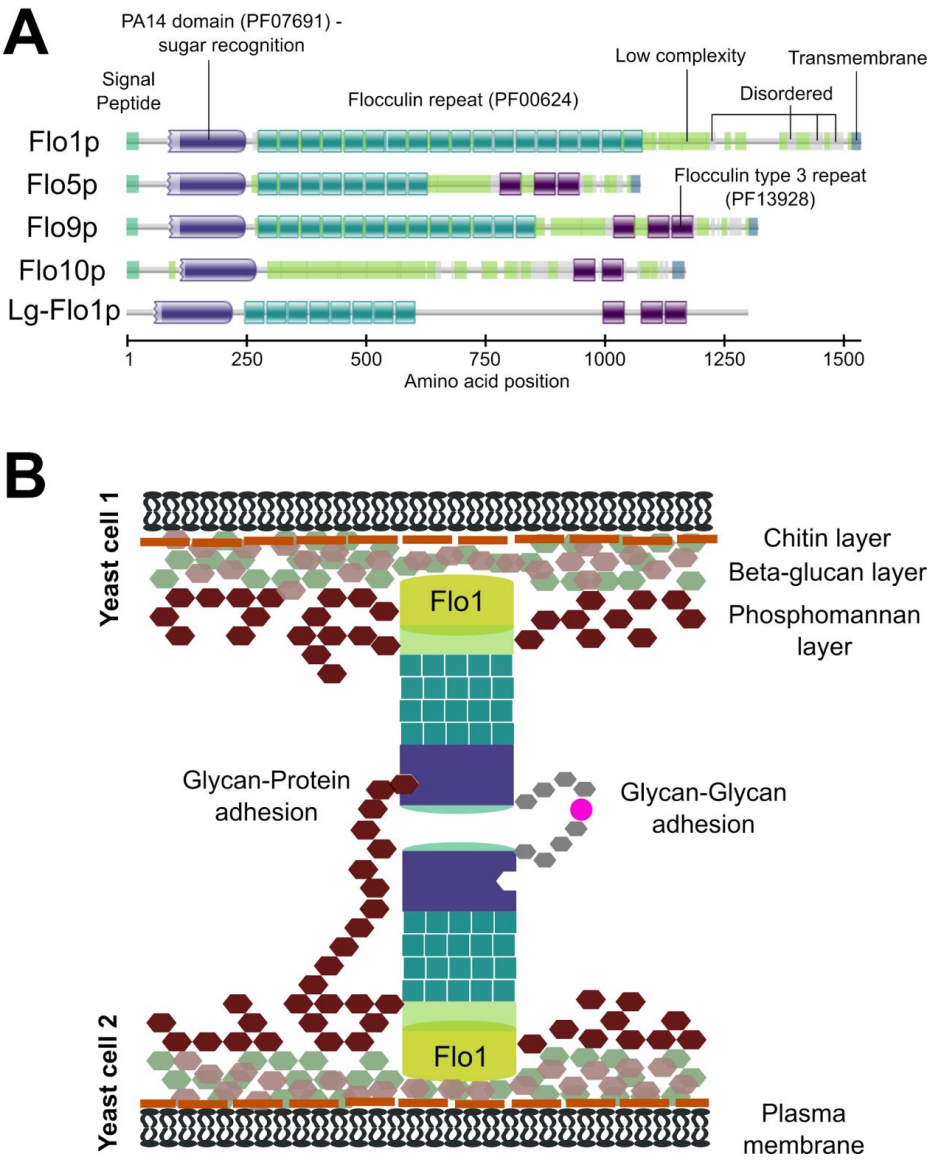

Fig. 1 **(A) Protein domain structure as per Pfam database [121]. Protein sequence data to generate the graphs were downloaded from Uniprot on 05/01/2021. Following Uniprot accession numbers were used: P32768 (Flo1p), P38894 (Flo5p), P39712 (Flo9p), P36170 (Flo10p) and B3IUA8 (Lg-Flo1p). Colours indicate identified protein domains: Light turquoise: Signal peptide, purple: PA14 domain, teal: flocculin repeats, dark purple: flocculin repeats type 3, light green: regions of low complexity, light grey: disordered regions, blue: transmembrane domain. PF numbers indicate the specific Pfam domain assigned. (B) Schematic representation of Flo1p adhesion mechanisms in the yeast cell wall. Flo1p is bound to the extracellular beta-glucan network. Two modes of adhesion are shown: Glycan-protein adhesion between a mannan chain of a yeast cell and Flo1p of another yeast cell. Glycan-glycan adhesion by Ca^{2+} (pink sphere) mediated bridging of N-linked phospho-mannan of the N-terminal domain of Flo1p**

The following chapters will summarize the molecular inventory and mechanisms conferring a flocculation phenotype in *S. cerevisiae* and *S. pastorianus*.

2 Molecular basis of flocculation

Flocculation in yeasts depends on the expression of so-called flocculins, genes belonging to the *FLO* family [20]. Flocculins belong to the family of adhesins and are, as the name suggests, involved in cell-cell, cell-surface and cell-substrate adhesion. Structurally, adhesin proteins consist of three major domains which will be described: An N-terminal domain in which the so called PA14 domain [21], conferring sugar binding ability, is located. This is followed by a serine/threonine rich central domain dominated by flocculin repeat units which are N- and O-glycosylated [22]. The third domain is a partially conserved C-terminal transmembrane domain which

contains a binding site for a glycosyl phosphatidylinositol (GPI) anchor (see Fig. 1A). GPI anchors are usually involved in fixing proteins into the cell plasma membrane [23], however in case of the Flo proteins this GPI anchor is further processed and only a remnant is retained. The Flo proteins are fixed in place via cross-linking to cell wall beta glucans [24, 25] (see Fig. 1B).

The *FLO* gene family consists of 11 members, of which *FLO1, FLO5, FLO8, FLO9, FLO10* and *FLO11* are dominant [26] (and reviewed in [27]). The gene *FLO3* is semi-dominant and the genes flo6 and flo7 are recessive, while *FLO2* and *FLO4* are alleles of *FLO1* (reviewed in [27]). In *S. cerevisiae* the proteins Flo1, Flo5, Flo9 and Flo10 confer the ability to flocculate [28] and exhibit 96 % (*FLO5*), 94 % (*FLO9*) and 58 % (*FLO10*) gene sequence homology to *FLO1* [27]. The *S. pastorianus* gene Lg-*FLO1* is a dominant gene on chromosome VIII and shows a 83 % identical gene sequence to *FLO1* of *S. cerevisiae*. Interestingly, while *FLO1* is located on chromosome I in *S. cerevisiae*, Lg-*FLO1* is located on chromosome VIII in *S. pastorianus* [29].

2.2 Sugar binding and the N-terminal domain of flocculins

The sugar binding ability of the flocculins form the basis of one of the central mechanisms behind flocculation in yeast: Binding of Flo proteins to mannose residues in the cell wall of adjacent yeast cells is leading to the formation of multicellular flocs. A further mechanism is the Ca^{2+} mediated bridging of terminal phosphates of mannose N-linked to the N-terminal domain of Flo1p [4] (see Fig. 1B). Flocculation types are distinguished based on their inhibition by different sugars which compete for receptor binding. The Flo1-type of flocculation is inhibited exclusively by the addition of mannose to the culture medium [30]. This stands in contrast to the NewFlo-type of flocculation which is additionally inhibited by glucose, maltose and sucrose (but not by galactose). Two further, minor types of flocculation are characterised by their insensitivity to mannose addition [31] and the requirement of ethanol to flocculate [32].

The NewFlo flocculation type described in lager type yeasts (*S. pastorianus*) is caused by a *FLO1* homologue termed Lg-*FLO1* (lager type *FLO1*). This gene has amino acid exchanges in the N-terminal sugar binding domain which confer a broader substrate specificity to Lg-Flo1p. The responsible changes were determined by exchanging amino acids in Flo1p to amino acids corresponding in Lg-Flo1p [33]. A proline to threonine exchange at amino acid position 202 (P202T), V226K, W228L and T236V were reported [33]. The amino acids at position 226 and 228 were reported as crucial for sugar recognition and lie within a pentapeptide which markedly differs between Flo1p and Lg-Flo1p: VSWGT (Flo1p) and KVLAR (Lg-Flo1p) (see Fig. 2A). It is of note that the reported amino acid sequence for the KVLAR pentapeptide in Lg-Flo1p by Kobayashi in 1998 differs from the currently published sequence KALAR as submitted by [29] and as reported in the NCBI assembly ASM1102231v1 [34]. However, this might be related to the analysis of differing yeast strains and does not seem crucial as both amino acids are hydrophobic.

However, there are potential problems with a flocculation classification into Flo1- and NewFlo-types, as exemplified in the study of *Van Mulders* et al. [28]. The proteins Flo5, Flo9 and Flo10 were found to be strongly inhibited by mannose and slightly by other sugars. They therefore follow neither Flo1- nor the NewFlo- (or other) types of flocculation classification [28]. A caveat in the study of Van Mulders et al. is that the respective *FLO* genes were over-

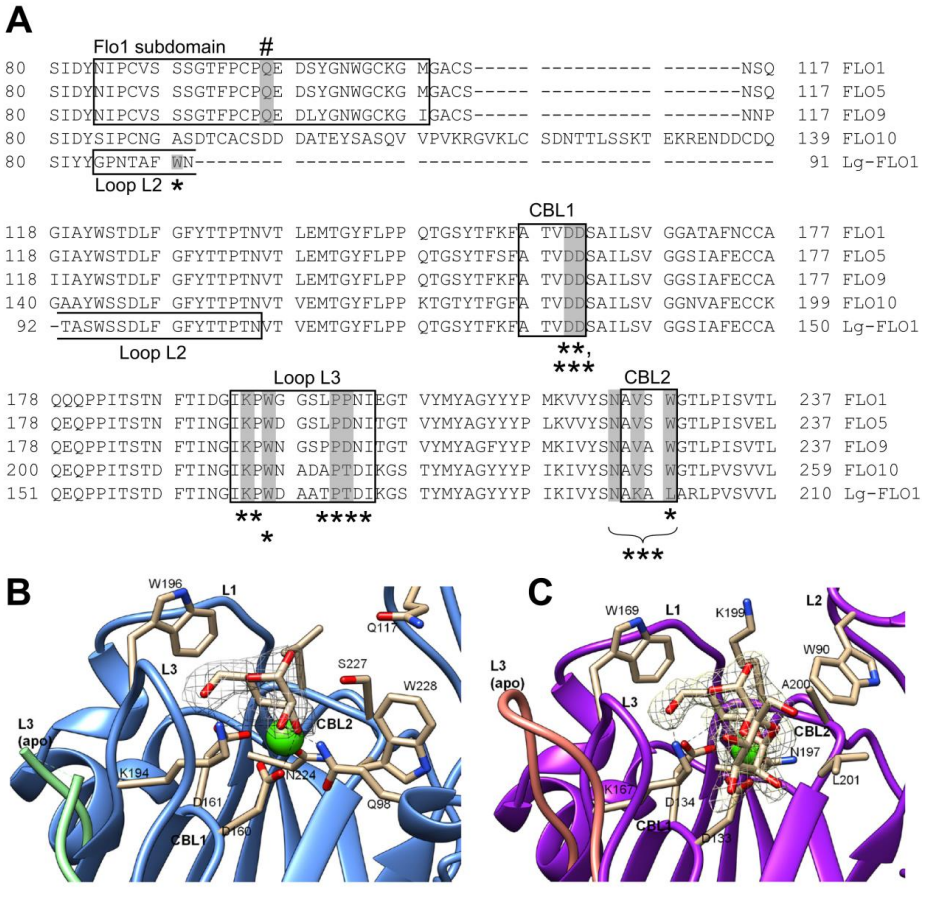

Fig. 2 **(A) Multiple Sequence Alignment using ClustalW [122]. Identical protein sequences as indicated in Figure 1 were used. Gene names are indicated on the right hand side of the graph, with numbers on the left/right of the sequence indicating the position of the first/last amino acid of the respective sequence. Sequence features are outlined, symbols mark amino acids underlied in grey involved in *: Hydrophobic interaction with ligand; **: Hydrogen bond formation with ligand; *** Ca^{2+} coordination; **** Loop flexibility; #: Discrimination between mannose and glucose. CBL: Carbohydrate binding loop (B) N-terminal portion of Flo1p (N-Flo1p) carbohydrate-binding site (PDB code 4LHN), in complex with calcium and mannose. The side chains of the main residues participating in ligand binding are shown and labeled. Ca^{2+} is depicted as a green sphere. The position of the L3 loop for the unbound state is indicated in light green. (C) The N-Lg-Flo1p (PDB code 4LHK) carbohydrate-binding site, in complex with calcium and 1,2-mannobiose. The position of the L3 loop for the unbound state is indicated in salmon. Image parts B) and C) with respective legends have been reproduced/modified from *Goossens* et al. [4]**

expressed to varying amounts which might cause experimental artefacts. Nonetheless, this study highlights the limits of classical flocculation type designation based on inhibition experiments.

The first amino acids in the sequence of the *FLO* genes are composed of a signal peptide domain, which prompts the cell to funnel the new synthesised protein into the secretory pathway [35]. Interestingly, the protein domain analysis performed in this review did not identify a signal peptide for Lg-Flo1p (see Fig. 1A), although such a sequence is annotated in earlier literature [7].

The elucidation of the crystal structure of the N-terminal domains of Flo1p and Lg-Flo1p (see Fig. 2B and 2C) has crucially furthered the understanding of the sugar recognition and binding mechanisms by Flo1p and Lg-Flo1p [4, 36]. The study by Goossens et al. revealed several structural domains in the N-terminus (see Fig. 2A). Amino acids 193-204 of Flo1p (which were also under investigation by Kobayashi et al., see above) are part of a loop called L3 and residue 202 is likely crucial for loop flexibility [4]. This is reflected in the crystallography data, as a high flexibility of the L3 loop due to a proline at position 202 of Flo1p led to an undefined structure during X-ray data collection (see the broken L3 loop structure in Fig. 2B). This did not occur during Lg-Flo1p measurement, which has a threonine at this position, and hints to a diminished L3 flexibility [4].

The establishment of flocculation is dependent on Ca^{2+} which was found to be coordinated between two aspartic acid residues at position 160 and 161 of the designated carbohydrate binding loop 1 (CBL1, amino acids 157-161), asparagine 224 and Valine 226 and Tryptophan 228 of CBL2 (amino acids 225-228). Upon binding of the 3' and 4' hydroxyl groups of the mannose ligand, the coordination shell in the carbohydrate binding pocket of Flo1p is completed. The 3'- and 4'- hydroxyl groups of mannose establish hydrogen bonds with D160 and D161, the 2'-oxygen with Q98. This Q98 side chain is part of the so-called Flo1 subdomain (amino acids 84-110) and confers the high specificity of Flo1p for mannose as the C2 hydroxyl groups between mannose and glucose are differently positioned (mannose: axial, glucose: equatorial). This Flo1 subdomain is missing in Lg-Flo1p which thus has a substrate specificity for both mannose and glucose. Ultimately, upon carbohydrate binding the L3 loop repositions towards CBL1, acting as a lid for the active site.

The binding mechanism for Lg-Flo1p is similar to Flo1p, here D133 and D134 of CBL1, N197 and K199, L201 of CBL2 coordinate Ca^{2+}. However, as mentioned above the Flo1 subdomain and any Q98 equivalents are missing in Lg-Flo1p. The Flo1 subdomain is spatially replaced in Lg-Flo1p by the L2 loop (G84-N108) where W90 establishes hydrophobic interactions (together with A200 and L201 of CBL2) to trap the ligand. Further hydrogen bonds and hydrophobic interactions with the ligand are established through K167 and W169 of the L3 loop in Lg-Flo1p. It is of note that the Flo1 subdomain is also absent in Flo10p (see Fig. 2A) and would explain the decreased sugar specificity and NewFlo flocculation conferred by this type of adhesin [37].

The N-terminal domain of Flo1p is heavily glycosylated containing three N-glycosylation sites at N135, N187 and N262 and three O-glycosylation sites. These N-glycosylations also play a role in cell-cell adhesion. A previous study by Goosens et al. suggested that the N-terminal domain of Flo1p is responsible for two different carbohydrate binding mechanisms [38]: A high affinity mode likely derived from the VSWGT motif and a low affinity mechanism. The low affinity mechanism showed a preference for chained mannose carbohydrates, while the high affinity mode preferred monomannose [38]. The suggested binding model in flocculation was thus the initial binding of a mannan chain via low affinity binding and subsequent binding of the terminal mannose to the high affinity site to establish a strong interaction between cells [38]. The nature of the low affinity binding mechanism was later revealed to be homophilic carbohydrate-carbohydrate interactions of the N-glycans attached to asparagines at amino acid position 135, 187 and 262 in Flo1p [4]. Interestingly, an additional role of calcium was reported in these strongly ionic carbohydrate-carbohydrate interactions, where Ca^{2+} mediates the interaction of mannosyl-phosphate groups from the N-linked glycans, uniting the lectin hypothesis with an earlier Ca^{2+}-bridging hypothesis [4].

2.2 The central domain of flocculins

The central domain of Flo1p consists of flocculin repeat units which are rich in serine/threonine and correspond to internal tandem repeats of the gene sequence [27]. Tandem repeats are recombination hotspots during DNA replication and repair (reviewed in [39]) conferring genomic instability to the *FLO1* gene, which ultimately leads to changes in the number of these repeats over generation time [11]. A further factor contributing to this instability is the location of the *FLO* genes at the subtelomeres (with exception of *FLO11*) [2], known recombinational hotspots [40]. In case of *FLO1* the underlying mechanism for the occurrence of loss or gain of the tandem repeats was suggested to be replication slippage [11]. The mutational frequency of *FLO1* tandem repeats is rather high with approx. 1×10^{-5} of which about 15 % result in a gain of repeats, while in the rest of mutations a loss occurs. It is remarkable that the loss/gain of repeats in *FLO*1 are in-frame and do therefore lead to an unaltered translation of the down-stream codons [11]. The number of tandem repeats (and therefore the number of repeats in the Flo1 protein) is positively correlated with the strength of flocculation [10, 11]. It was suggested that the higher number of tandem repeats lead to a longer protein with a better exposure of the N-terminal sugar binding domain [11, 41–43]. The higher percentage of loss of tandem repeats would suggest a loss of flocculation ability over time and this is indeed a well known phenomenon in bottom-fermenting brewing yeasts [10, 44–46]. However, it was also shown that in yeast mother cells the ability to flocculate increases with the number of cell divisions [47]. This hints that the complete mechanisms behind the flocculation ability/strength are not completely understood yet.

A surprising finding was reported by *Liu* et al. where it was shown that the number of internal repeats in the central domain of *FLO1* could cause conversion from a Flo1- to a NewFlo flocculation type with additional inhibition of flocculation by galactose related to a C-terminal domain [48].

Most research so far was concentrated on the sugar binding ability of the N-terminal domain. However, the lectins responsible for

flocculation exhibit only weak affinities (ranging in the milli and high micromolar region [4, 49]) which has led to the question how this leads to the stable formation of flocs [50, 51]. A recent study illuminated this further and reported that under mechanical stress the hydrophobic tandem repeats of Flo1 are getting exposed. These unfolding forces together with the adhesin binding are suggested to confer the observed strong cell-cell interaction [51].

2.3 The C-terminal domain of flocculins

The C-terminal domain of Flo1p, Flo5p, Flo9p and Flo10p is characterised by a transmembrane domain, which is not identified in Lg-Flo1p (see Fig. 1A). The transmembrane domain contains a consensus sequence for the C-terminal attachment of a GPI anchor. These GPI anchors can be thought of as an alternative form of a transmembrane domain, fixing proteins facing the extracellular matrix into the membrane. This is achieved by hydrophobic interaction between membrane lipids and the anchor [23]. In yeast, however, the fatty acid and inositol components of the GPI anchor are removed before extracellular cross-linking of the protein to beta-1,6-glucans of the yeast cell wall [25, 52]. This means no direct plasma membrane integration of Flo proteins occurs (see Fig. 2B).

The following parts of this review will focus on the regulatory mechanisms controlling the most dominant flocculation genes *FLO1* and Lg-*FLO1* [26, 28, 33]. We will additionally explore the role of Flo8p as transcriptional activator of *FLO1* and *FLO11*. Although a regulatory target of Flo8p, *FLO11* is not involved in flocculation in *S. cerevisiae*, but is crucial for pseudo-hyphae formation [53], invasive growth [53], surface attachment [54] and biofilm formation [55]. However, it should be mentioned that it was shown to be involved in a Flo1-type flocculation in *S. cerevisiae* var. *diastaticus*, which might be an effect of a strain specific mannosylation of Flo11p and homophilic interaction [56, 57].

3 Genetic regulation of flocculation

The following part focuses on *FLO1* gene regulation in *S. cerevisiae*: In the presence of glucose *FLO1* is repressed, whilst the absence of glucose results in the *FLO1* expression. In *S. pastorianus*, the exact control mechanism of the Lg-*FLO1* expression is mostly unknown. Due to a chromosomal recombination in *S. pastorianus* Lg-*FLO1* is translocated from chromosome I onto chromosome VIII and is placed under control of a promoter highly similar to the *FLO5* promoter found in *S. cerevisiae* [29]. So far it was only shown that Lg-*FLO1* expression is not influenced by changing sugar levels, but by lack of nitrogen availability which involves the regulatory action of the protein Gln3 [58]. Therefore an involvement of the cAMP-PKA pathway via the mTORC1 nutritional sensing axis [59] can be suspected.

3.1 *FLO1* gene expression

The gene expression of *FLO1* is positively dependent on the rate of *FLO8* gene transcription [60]. The encoded transcriptional activator Flo8p is additionally involved in the regulation of the genes *FLO9*, *STA1* and *FLO11* [61]. Consequently, a nonsense mutation (premature stop codon) of *FLO8* in the laboratory yeast strain S288c and the lack of a Flo8p binding site in the *FLO1* promoter sequence in the strain Σ1278b, prevent a flocculation phenotype in these strains [26, 60–62]. Although a DNA binding protein, Flo8 does not contain typical DNA binding motifs like zinc fingers, or helix-loop-helix, leucine zipper or highly hydrophobic domains [60].

Flo8p itself is under the control of the cyclic adenosine monophosphate - protein kinase A (cAMP-PKA) pathway [63]. The cAMP-PKA pathway can be summarised as follows: Following an internal or external stimulus e.g., nitrogen or glucose starvation, the membrane protein adenylate cyclase converts ATP into cyclic adenosine monophosphate (cAMP). cAMP binds to the regulatory subunit of PKA, upon which the regulatory subunit changes its conformation. This confirmation change releases the regulatory subunit from the now active catalytic subunit which can now phosphorylate other kinases or transcription factors [64–67]. Flo8p has five consensus PKA phosphorylation sites and is a direct target of PKA [68]. Depending on the PKA subunit performing the phosphorylation of Flo8p, this transcription factor is either inhibiting or inducing flocculation.

Additionally, the regulation of *FLO1* by Flo8p is supported by the transcription factor Mss11 [69]. In the Mss11 protein the first 148 amino acids show similarities to the sequence of Flo8p and this region is required for the inhibition of flocculation. The amino acid regions 148–340 and position 604–641 both contain glutamine and asparagine repeats and appear to be crucial for a proper induction of flocculation [69].

Mss11p is not exclusively involved in flocculation, it also regulates invasive growth, pseudohyphal differentiation, adhesion and starch metabolism based on nutrient availability [70–75]. In regards of changing the flocculation phenotype, the primary target of Mss11p and Flo8p is *FLO1* [69]. Nevertheless both proteins are also involved in the regulation of *FLO11* (among other genes) [75, 76]. There are two upstream activation sequences before the *FLO11* core promoter. In the first step to activate the *FLO11* transcription, the DNA binding protein Ste12p and the Ty transcription activator Tec1p bind at the upstream activation sequence closer to the promoter of *FLO11*. These two proteins recruit the coactivator Swi-Snf complex, which is capable of nucleosome rearrangement. Mss11p and Flo8p bind to the upstream activation sequence farther away from the promoter. Further, Mss11p and Flo8p bind to the coactivator complex Swi-Snf so that the DNA forms a loop, and the RNA polymerase II is recruited by Mss11p and Flo8p to the promoter sequence of *FLO11* [53, 61, 76, 77].

Since, the Swi-Snf complex is involved in the transcriptional activation of both *FLO1* and *FLO11*, a similar mechanism governing the expression of both genes is suspected. However, an upstream activation sequence similar to *FLO11*, where the transcript activators could bind to the DNA, was not found within 3 kilo base pairs upstream of *FLO1* [69].

Previous research suggested that the coactivator complex Swi-Snf and the corepressor complex Cyc8-Tup1 organise the nucleosome upstream of *FLO1*. The activity of the *FLO1* promoter is determined by the nucleosome organisation [77]. In absence of

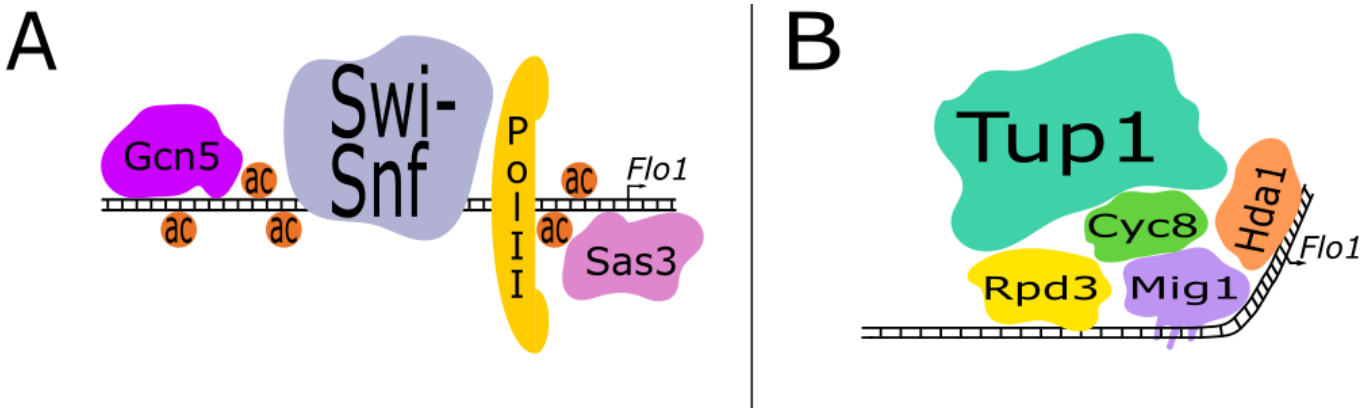

Fig. 3 **Depending on the nucleosome arrangement, RNA transcription is performed. (A) In the absence of glucose, *FLO1* is expressed. The phosphorylated Mig1 protein does not enter the nucleus and therefore cannot bind to the *FLO1* promoter. Instead, the coactivator Swi-Snf complex re-arranges the nucleosome upstream of *FLO1*. Subsequently, the DNA gets acetylated by the two histone acetyltransferase Gcn5 and Sas3. If the open reading frame and the promoter region of *FLO1* is acetylated, the RNA polymerase II transcribes the RNA. (B) In the repressed state of *FLO1*, the DNA is bound by the zinc finger protein Mig1 upstream of the *FLO1* gene. Mig1p recruits the transcript repressor complex Cyc8-Tup1 to the promoter region of *FLO*1. The two histone deacetylases Rpd3 and Hda1 bind as well and deacetylate the DNA causing repression of *FLO1* transcription**

corepressor complex Cyc8-Tup1, the coactivator Swi-Snf complex is not blocked from access to the promoter site of *FLO1* [78]. The Swi-Snf complex binds and rearranges the nucleosome 2.5 kb upstream of the *FLO1* gene [77] (see Fig. 3A). Important for the nucleosome rearrangement is the acetylation of lysine at amino acid sequence position 9 and 14 of the histone H3. When the *FLO1* transcription is active the gene is marked by the acetylated lysine 14 of histone H3 and the methylated lysine 36 of histone H3 [79] (see Fig. 3A). This acetylation is performed by the two histone acetyltransferase Gcn5p and Sas3p [78]. Gcn5p is part of the Ada-Hat complex as well as the SAGA complex [80], while Sas3p is part of the multisubunit Nua3 complex [81]. It has been proposed that Gcn5 and Sas3 are recruited to the actively transcribed *FLO1* gene coding region by the methylated lysines 4 and 36 of histone H3, which are located at the 5' and 3' open reading frame [79]. Only if the DNA is acetylated at the promoter region of *FLO1* and at the open reading frame, the RNA polymerase II is initiated and elongates the RNA transcription of *FLO1* [78]. It has been hypothesised that the acetylation of lysine 14 of histone H3, lying within the *FLO1* gene coding region, drives histone eviction from the DNA, which allows the RNA polymerase II to progress across the open reading frame of the gene [79].

An overview of the DNA structure during *FLO1* expression is seen in Figure 3A.

3.2 *FLO1* gene repression

Early studies have shown that a disruption of the two genes *TUP1* and *CYC8* results in a *FLO1* expression [82] which suggests the Cyc8-Tup1 complex regulates the *FLO1* gene repression.

Previous data suggests that glucose induces the down-stream cascade for activation of protein phosphatase 1 (PP1) by post-translational modifications. These modifications are most likely induced by the glucose dependent activation of the cAMP-PKA pathway [83, 84]. Protein phosphatase 1 plays a major role in protein dephosphorylation in eukaryotic cells. The *Saccharomyces cerevisiae* homologue of human *PP1* is *GLC7* [85]. The serine/threonine protein phosphatase Glc7p and the regulatory subunit Reg1p form the Glc7-Reg1 complex [86, 87]. The Reg1 protein acts as a regulatory subunit of the Glc7-Reg1 complex and Reg1p is itself also a Glc7 substrate [88]. Glc7-Reg1 complex interacts with Snf1 kinase to form a repressor complex, which is promoted by hexokinase 2 (which is usually involved in the phosphorylation of glucose during glycolysis) [67][89]. The Snf1 kinase complex is involved in the adaptation to environmental and nutritional stress as well as cell ageing process [90–93]. In response to an increase in glucose concentration, Glc7-Reg1 dephosphorylates Threonine 210 of the Snf1 kinase complex, deactivating the catalytic capability of Snf1 [94] (see Fig. 4A).

The Snf1 kinase complex consists of the so-called beta subunit, which binds Snf1 kinase and the complex activating subunit Snf4p. The beta subunit is one of the three proteins Sip1 (Snf1 protein kinase subunit beta-1), Sip2 (Snf1 protein kinase subunit beta-2) or Gal83 (Snf1 protein kinase subunit beta-3). The kinase subunit of the complex, Snf1, contains a regulation domain and a catalytic domain [95, 96] (see Fig. 4).

Under low glucose concentration the Snf1 kinase is activated by the phosphorylation of the amino acid threonine at position 210 by three different Snf1p activating kinases: Pak1, TopS3 and Elm1 [97, 98] (see Fig. 4A). Snf1p activating kinases are constitutively active independent of the glucose concentration [99]. The phosphorylation of Snf1p releases the catalytic domain (CD) from autoinhibition by the regulatory domain (RD). The Snf4p subunit binds to the regulatory domain instead [100, 101] (see Fig. 4A). The now activate Snf1 kinase phosphorylates the Reg1 protein, the beta subunit and the additional substrate multicopy inhibitor of gal gene expression (Mig1p) [93, 97, 102]. Dephosphorylation of threonine 210 of Snf1p promotes the closure of the Snf1 complex into the autoinhibited form [100, 102] (see Fig. 4B).

Depending on the phosphorylation status, Mig1p is either imported to or exported from the nucleus. Dephosphorylated Mig1p is imported in the nucleus, whilst phosphorylated Mig1p is exported. It has been suggested that Msn5p is involved in the nuclear export,

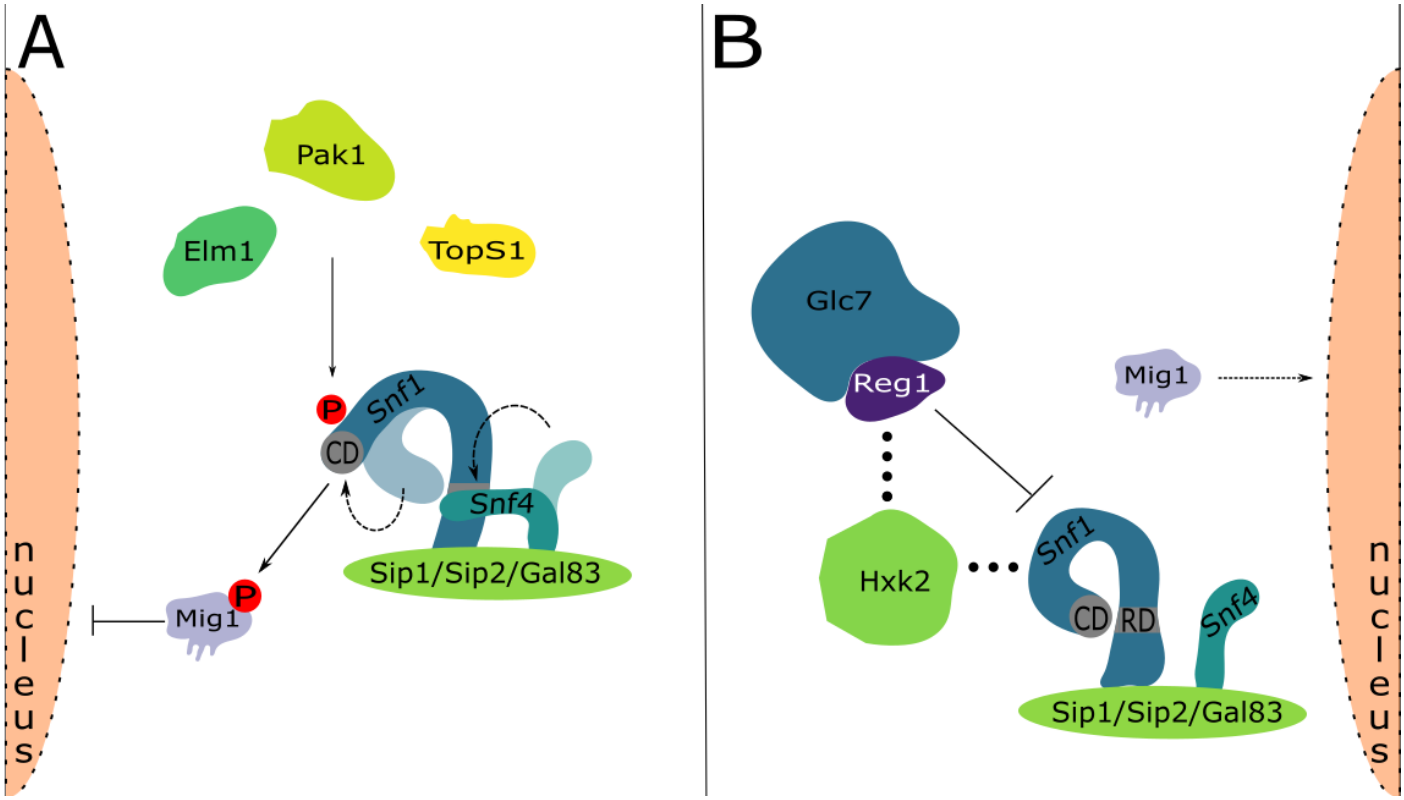

Fig. 4 **Activity of the Snf1 kinase complex is depending on the phosphorylation state of threonine 210 of Snf1p. The kinase complex consists of Snf1p and Snf4p, both connected to a beta subunit backbone consisting of either Sip1p, Sip2p or Gal83p. (A) In the absence of glucose, the Snf1 complex is phosphorylated by the kinases Pak1, TopS3 and/or Elm1. Phosphorylated Snf1p undergoes a conformational change, the catalytic domain (CD) is released from the regulatory subunit (RD) to which Snf4p now binds. The active Snf1 complex is able to phosphorylate Mig1p, which is prevented from translocation into the nucleus, causing a de-repressed *FLO1* gene. (B) In the presence of glucose, the protein phosphatase complex Glc7-Reg1, supported by the Hxk2p, dephosphorylates Threonine 210 of the Snf1 kinase complex. The CD of Snf1p will re-connect with the RD of Snf1p. The complex is now inactive and therefore cannot phosphorylate Mig1p, which will translocate into the nucleus inducing *FLO1* repression**

while Cse1p might be involved in the nuclear import of Mig1p [103, 104]. As described above, the dephosphorylation and inactivation of Snf1 kinase by Glc7-Reg1 results in a dephosphorylation of Mig1p followed by Mig1p import into the nucleus and gene repression [99, 105]. However, Mig1p is not exclusively involved in the *FLO1* repression: *FLO5*, 9 and 11 also respond to different Mig1p levels [106].

Functionally, Mig1 belongs to the zinc finger protein family which mediates DNA binding [107]. First, Mig1p binds to a guanine/cysteine rich DNA sequence located closely to the TATA box [108]. The TATA box is an adenosine and thymine rich DNA consensus sequence found in the promoter sequence [109]. After Mig1p has bound to the GC rich sequence, the TATA box bends around the Mig1 protein [108] rendering the DNA inaccessible for transcription by RNA polymerase II. Furthermore, Mig1p recruits the transcript repressor complex Cyc8-Tup1 to the promoter region of *FLO1*. Hereby, Cyc8p mediates the association between Tup1p and Mig1p [110]. In the repressor complex Cyc8-Tup1, the protein Cyc8 is interacting with a DNA-binding protein, which is promoter specific and guides the complex to the correct sequence. Whilst Tup1p, inhibits the transcriptional apparatus and is responsible for the gene repression [111, 112].

Depending on the presence of Cyc8-Tup1 complex at the promoter of *FLO1* the histone deacetylases Rpd3 and Hda1 bind to the promoter as well, leading to histone deacetylation and nucleosome re-organisation. The combination of the Cyc8-Tup1 complex and the histone deacetylases Rpd3 and Hda1 result in a strong repression of *FLO1* transcription [113].

It is important to note that the *FLO1* gene expression is not based on the absence of the corepressor Cyc8-Tup1 complex, but is dependent on the presence of the coactivator Swi-Snf complex. While Cyc8-Tup1 and Swi-Snf organise the nucleosome upstream of *FLO1*, the nucleosome organisation determines the activity of the *FLO1* promoter [77] (see Fig. 3B).

3.3 Additional pathways of *FLO1* regulation

A recent study has reported that environmental factors to cell wall stress can be linked to the activation of cell wall integrity mitogen-activated protein (MAP) kinase pathway. Hereby lead the involvement of the phosphorylates the MAP protein kinase Slt2 and transcription factor Rlm1 leads to the expression of *FLO1* [114]. The MAP kinase pathway is a cascade of three protein kinases, which are part of common signalling pathways. Furthermore, the MAP kinase pathway engages with the cell integrity pathway and responds to numerous cell indicators like cell cycle regulation, osmosis and growth temperature [115].

Besides MAP kinase influence on *FLO1*, it is also involved in the

FLO11 regulation [63]. Step12p, which recruits the coactivator Swi-Snf complex to the upstream activation sequence closer to *FLO11* promoter, is a target of the MAP kinase pathway [116].

A further regulation mechanism involving the gene silencing COMPASS (complex proteins associated with Set1) histone methylation complex was identified by Dietvorst et al. [117]. In this study it was shown that yeast strains defective in COMPASS have increased expression levels of *FLO1*, *FLO5* and *FLO9* with an increase in flocculation capability. Further, the expression of the MAL1 - MAL4 genes was elevated and a higher maltose fermentation efficiency was observed [117]. A subsequent study additionally reported that absence of Hda1 or Gnc5 also plays a role in improved maltose utilisation in high-gravity fermentations (in addition to their role in flocculation discussed above) [118].

On another note it has been shown that with a higher ethanol concentration, originating from a higher starting sugar content in the media, flocculation increases in *S. cerevisiae* [5]. It has been speculated that the effect of ethanol on flocculation is via quorum sensing (QS) [5]. This was based on the observation that the known QS molecule tryptophol also causes strong yeast flocculation [5]. Interestingly, tryptophol was shown to induce *FLO11* via combined action of Tpk2p and Flo8p [119], for which the latter would provide a potential link to *FLO1* induction.

4 Conclusion

This review detailed the molecular basis for flocculation in *S. cerevisiae*, including the current understanding of the sugar binding mechanism of Flo1p and Lg-Flo1p as well as the regulation of the expression of their genes. In the case of *S. cerevisiae*, several pathways originating in glucose or stress sensing converge at the Snf1 complex which regulates the localization of the Mig1 protein. Phosphorylation of Mig1p by the Snf1 complex causes Mig1p retention in the cytoplasm, *FLO1* is de-repressed. In the case of *S. pastorianus*, Lg-*FLO1* is translocated onto the locus of *FLO5* on chromosome VIII and flocculation is induced by nitrogen starvation under the control of gln3. The gene product of *FLO1* is the protein Flo1, an extracellular cell wall protein with the main function of binding mannose. Depending on the amino acid composition, the ability to bind different sugars can change. In case of Lg-Flo1p the occurrence of a KALAR pentapeptide (instead of a VSWGT sequence in Flo1p) in concert with the loss of a glutamine due to deletion of the Flo1 subdomain enables Lg-Flo1p to bind mannose and glucose. These changes confer the two main phenotypes observed in flocculation: Flo1 in which flocculation is inhibited exclusively by mannose and NewFlo in which additional sugars inhibit flocculation as well. However, flocculation is not a pure glycan-flocculin binding process. The flocculins are glycosylated themselves and the terminal phosphate groups on the mannose chains can ionically interact by bridging through Ca^{2+}. The evolutionary advantage of flocculation is being thought of as conferring enhanced survival, increased mating efficiency and sporulation. However, in the brewing industry flocculation is valuable for different reasons, where this process is exploited as an energy and cost saving mechanism for efficient yeast removal from the final product.

Although vast progress has been achieved in flocculation research over the last 30 years, the understanding of the regulation of the *FLO* genes is far from complete. This is especially true in light of the application of flocculation in the brewing industry where yeast is subject to a quite unique environment during fermentation. The fact that flocculation is governed by cellular environmental sensing, the direct transfer of experimental results conducted in environments not resembling the brewing praxis using laboratory yeast strains which are genetically far removed from brewing yeast strains [120] is therefore a risky approach.

Thus, future research avenues should build on this fundament and explore the question how the changing wort environment during beer production influences the cellular signalling cascades leading to flocculation as well as the effect of the brewing environment on the genetic setup. This will pave the way to realize refined technological control of flocculation, especially on the basis of the variable flocculation behaviours exhibited (e.g. over re-pitching cycles) while accounting for the specific yeast strains employed by the different breweries.

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