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Transcription Factors Mcm1 and Sfp1 May Affect [*PSI*$^+$] Prion Phenotype by Altering the Expression of the *SUP35* Gene

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Abstract: Mcm1 is an essential Q/N-rich transcription factor. Q/N-rich proteins interact with each other, and many affect the [*PSI*$^+$] prion formed by the translation termination factor Sup35 (eRF3). We found that transient *MCM1* overexpression increased nonsense suppression in [*PSI*$^+$] strains and *SUP35* transcription. As we had discovered similar effects of another Q/N-rich transcription factor, Sfp1, here we focus on the roles of Mcm1 and Sfp1 in *SUP35* expression, as well as on the effects of Sfp1 on the expression of the gene encoding another release factor, Sup45 (eRF1). Mutations in the *SUP35* promoter showed that none of the potential Mcm1 binding sites affected the Sup35 protein level or nonsense suppression, even during *MCM1* overexpression. Mcm1 itself neither formed aggregates in vivo nor affected Sup35 aggregation. In contrast, a mutation in the Sfp1-binding site decreased Sup35 production and [*PSI*$^+$] toxicity of excess Sfp1. Mutation of the Sfp1 binding site in the *SUP45* promoter lowered *SUP45* expression and increased nonsense suppression even more drastically. Our data indicate that the mechanisms of Mcm1 and Sfp1 action differ. While Mcm1 seems unlikely to directly regulate *SUP35* expression, Sfp1 appears to act through its binding sites and to directly activate *SUP35* expression, which in turn may influence the [*PSI*$^+$] prion phenotype and toxicity.

Keywords: [*PSI*$^+$]; prion; Sup35; Sup45; transcription factors; Mcm1; Sfp1

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1. Introduction

Amyloid prions in baker's yeast (*Saccharomyces cerevisiae*) act as heritable cytosolic factors, as the newly synthesized proteins rapidly acquire amyloid conformation, which is then transmitted to the daughter cells and thereby persists in the cell progeny. [*PSI*$^+$] is a prion formed by self-perpetuating amyloid of yeast essential release factor Sup35 (eRF3). Presence of [*PSI*$^+$] leads to almost complete inactivation of Sup35 in an aggregated form, resulting in shortage of release factor and inefficient termination of translation. This in turn causes more frequent stop codon readthrough, and may lead to suppression of nonsense mutations (or nonsense suppression), as reviewed in [1]. Two release factors, Sup35 and Sup45 (eRF1), are involved in the termination of translation. Nonsense suppression may also result from mutations in either of these factors or from their downregulation (reviewed in [2–4]).

Most known yeast prion proteins contain domains that are enriched in asparagine (N) or glutamine (Q) residues, which are necessary for prion maintenance (reviewed in [5,6]). In the case of the Sup35 protein, its N-terminal domain is Q/N-rich and prionogenic, its middle (M) domain is half positively and half negatively charged, aiding in its possible pH-sensor function during phase separation into biomolecular condensates [7], and the C-terminal domain is globular and essential for the eRF3 release factor function [8,9]. Yeast amyloid prions are known to interact with each other; for example, [*PIN*$^+$], a prion form of another Q/N-rich protein, Rnq1, is necessary for the de novo appearance of [*PSI*$^+$],

i.e., it acts as a [*PSI*$^+$]-inducing (Pin$^+$) factor [10]. While [*PIN*$^+$] has no detectable phenotype, its presence along with another prion [*SWI*$^+$] formed by the transcription factor Swi1 results in the [*NSI*$^+$] factor, which manifests in a [*PSI*$^+$]-like nonsense suppressor phenotype caused by *SUP45* downregulation [11,12]. These interactions are not limited to only prion-forming proteins; various other Q/N-rich proteins have been shown to act as Pin$^+$-factors [10] as well as to form nonheritable aggregates [13,14] that can indirectly affect prion properties. Notably, transcription factors are often found among Q/N-rich proteins, with at least four of them forming bona fide prions: Ure2, Swi1, Mot3, and Cyc8 (reviewed in [14,15]).

We previously investigated the possible influence of two Q/N-rich transcription factors, Mcm1 and Sfp1, on [*PSI*$^+$] properties. Both were discovered by screening using a synthetic lethality test, which helped to identify factors that affect the lethality of [*PSI*$^+$] in the presence of *sup45* mutation by influencing nonsense suppression [16–18]. Here, we attempted to find out whether the effects of Mcm1 and Sfp1 on [*PSI*$^+$] are due to their function as transcription regulators.

2. Materials and Methods

2.1. Plasmids

All of the plasmids used in this work are listed in Supplementary Table S1. Plasmids pRS316 and pRS426 have been described before [19,20]. Plasmids pRS426-SFP1 [21] and pU-MCM1 [22] were kindly provided by Tatiana M. Rogoza and Anton A. Nizhnikov, respectively. pUGC-MCM1-GFP is a pUG35-based vector obtained through a series of intermediate vectors and contains the *MCM1* ORF with upstream 50 bp sequence, flanked by BglII and SacII restriction sites (the PsuI-SacII fragment of the pGPD-f1-MCM1-YFP plasmid [17]) under control of the *CUP1* promoter originally derived from pRS316CG [23]. Plasmid pYX242-Nab2NLS-2mCherry [24] was a kind gift from Simon Alberti. The plasmids with mutations and deletions in the *SUP35* and *SUP45* promoters are based on pRSU1 [25] and pRS315-SUP45 [26], respectively. They were constructed using site-directed mutagenesis as described previously in [27]. The primers used for site-directed mutagenesis are listed in Supplementary Table S2. The Δ*Mcm1-2** variant, which has an insertion of an additional C in position −282 as well as a one-nucleotide deletion in the Abf1 binding site (*mutAbf1* variant), was obtained as a PCR-induced mutation during site-directed mutagenesis. All the obtained vectors were verified by Sanger sequencing.

2.2. Strains

The yeast strains used in this work are listed in Supplementary Table S3. The strains used for *MCM1* overexpression studies were isogenic to 74-D694 [28–32]. All other strains, which were used for plasmid shuffling experiments, were isogenic to GT81 [26,27,33–36]. Strains with *LEU2* plasmids with mutant promoter variants were selected via plasmid shuffling using 5-FOA medium as described previously [37].

Yeasts were grown at 30 °C in standard liquid and solid media using conventional methods [38,39]. 1/4YEPD medium was used for the color phenotype detection [40]. To check for the presence of the [*PSI*$^+$] prion, 1/4YEPD medium supplemented with 4 mM GuHCl was used. For the induction of the *CUP1* promoter, $CuSO_4$ was routinely added to the medium at a final concentration of 50 μM unless indicated otherwise. Yeast transformations were performed using a standard protocol [41].

2.3. qPCR

Cells were grown in liquid cultures to the mid-log phase. In the case of *CUP1* promoter induction, the medium was supplemented with $CuSO_4$ at a final concentration of 150 μM. RNA extraction, cDNA synthesis, and qPCR reactions were performed as described previously [32]. The primers used for the qPCR are listed in Supplementary Table S2. *ACT1* was used as a reference. Relative units of expression were calculated as $2^{-\Delta\Delta Ct}$ [42].

2.4. Protein Analysis

The alkaline lysis protocol [43] was used for protein extraction for subsequent SDS-PAGE and Western blot analysis [38]. SDD-AGE and capillary transfer were performed as described [44,45]. The antibodies SE4290 [46], SE-45-2 [47], Anti-GFP (Abcam, Cambridge, UK, #ab290), ADH1A (LsBio, Lynnwood, WA, USA, #LS-C68862), and Anti-α-Tubulin (Sigma-Aldrich, St. Louis, MO, USA, #T6074) were used to detect Sup35, Sup45, GFP, Adh1, and Tub1, respectively. ECL Select Western Blotting Detection Reagent (Cytiva, Marlborough, MA, USA) was used for antibody detection. Images were acquired using GeneGnome (Syngene, Bangalore, India).

2.5. Fluorescence Microscopy

Cells were grown in liquid media until reaching OD_{600} = 0.2–0.3. $CuSO_4$ was then added to a final concentration of 50 μM for the *CUP1* promoter induction. Cells were grown for an additional 3–4 h and then visualized using Zeiss Axioscope A1 equipped with a Zeiss AxioCam 506 Color camera. Images were acquired using Zeiss Zen software, version 3.9.

2.6. Bioinformatic Analysis

Searches for potential TFBSs in the *SUP35* and *SUP45* promoters was carried out using the oPOSSUM-3 online tool [48] (http://cisreg.ca/software/, accessed on 27 March 2024). A similarity threshold of 75% was used. Mutant promoter variants were checked for the absence of pre-existing TFBS in the results, i.e., the similarity of all mutant sites to their respective TFBS profiles should be less than 75%. Transcription factor binding site profiles were taken from the JASPAR 2022 database [49] (https://jaspar2022.genereg.net, accessed on 27 March 2024). Statistical analysis was performed in R v.4.3 (R Core Team, 2023). Boxplots were constructed using the ggplot2 package [50].

3. Results

3.1. Transient Overexpression of MCM1 Enhances Nonsense Suppression in [PSI⁺] Strains

In our previous studies using constructs for the constitutive expression of the *MCM1*, we did not observe any effect on nonsense suppression; however, overexpression of the *MCM1* gene controlled by copper-inducible *CUP1* promoter appeared to enhance the synthetic lethality of [*PSI*⁺] with *sup45* mutations in the test (Supplementary Figure S1). As the enhanced lethality in the test could reflect increased nonsense suppression, we checked whether transient Mcm1 overexpression affected the suppressor phenotype in [*PSI*⁺] strains. Indeed, various [*PSI*⁺] strains demonstrated enhanced suppression (Figure 1A). The C-terminally GFP-tagged variant of Mcm1 (Mcm1-GFP) showed the same effects (Figure 1A). Previously, we observed similar effects on the part of another Q/N-rich transcription factor Sfp1, which was shown to influence both transcription of the release factor genes and Sup35 aggregation [32]. Thus, we checked whether Mcm1 also affected these processes.

First, using qPCR, we assessed changes in *SUP35* and *SUP45* mRNA levels under transient overexpression of *MCM1*. A significant increase in *SUP35* but not *SUP45* mRNA levels was detected (Figure 1B), suggesting that *MCM1* might be involved in the control of *SUP35* transcription. However, no changes in Sup35 protein levels were observed (Figure 1C). These results were dissimilar to those obtained when studying Sfp1 overexpression, as the latter was shown to increase both *SUP35* and *SUP45* mRNA levels even though only Sup35 protein levels were visibly elevated [32]. Nevertheless, our results suggest that both Mcm1 and Sfp1 might be involved in regulating transcription of the release factor genes.

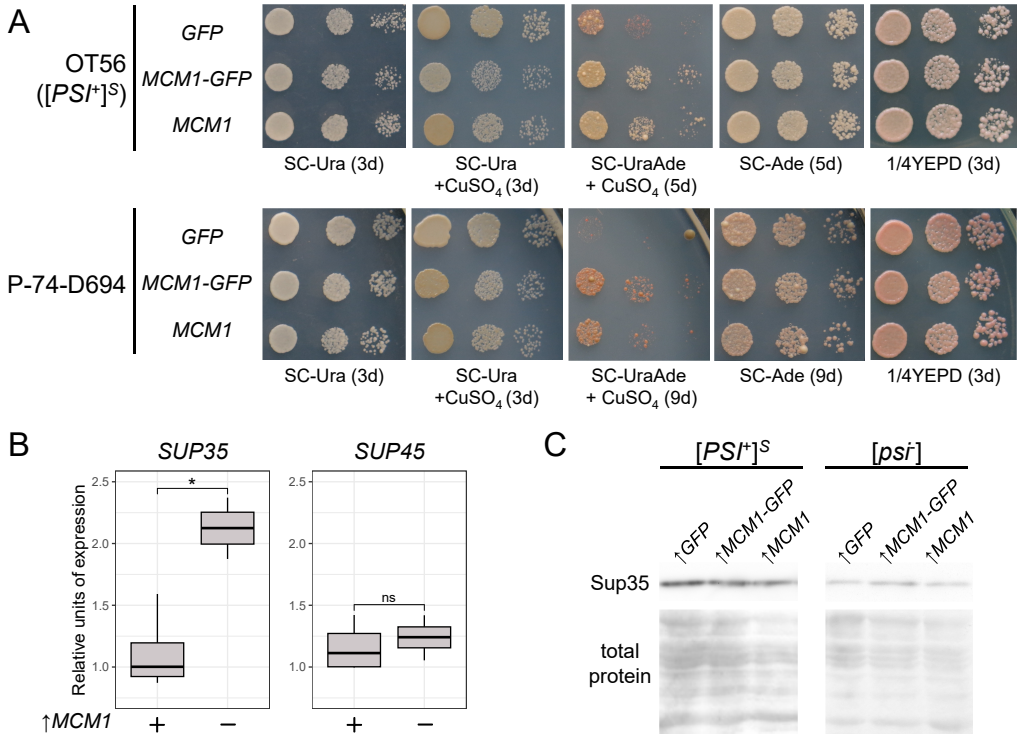

Figure 1. *MCM1* overexpression enhances nonsense suppression in [*PSI*$^+$] strains. (**A**) The OT56 and P-74-D694 strains were transformed with pRS316CG (*GFP*), pUGC-MCM1-GFP (*MCM1-GFP*), or pU-MCM1 (*MCM1*). The phenotypes of the resulting clones were analyzed by plating cells onto various media to assess growth (SC-Ura, SC-Ura+$CuSO_4$) and nonsense suppression (SC-UraAde+$CuSO_4$, SC-Ade, 1/4YEPD). Shown are tenfold serial dilutions of the representative clones. (**B**) qPCR analysis of *SUP35* and *SUP45* mRNA levels in the OT56 ([*PSI*$^+$]^S) clones bearing the pU-MCM1 plasmid. The relative expression from copper-induced cultures is compared to no-induction samples. *, $p < 0.05$ in Wilcoxon Mann–Whitney test; ns, not significant ($p > 0.05$). (**C**) Analysis of the total Sup35 protein levels in OT56 ([*PSI*$^+$]^S) and 74-D694 ([*psi*$^-$]) strains transformed with the plasmids from (**A**) using SDS-PAGE and Western blotting with anti-Sup35 antibodies. Coomassie R-250 staining was used to visualize the total proteins.

3.2. Search for the Potential Mcm1 and Sfp1 Binding Sites in the SUP35 and SUP45 Promoter Regions

We next attempted to determine whether Sfp1 and Mcm1 could directly regulate transcription of the *SUP35* and *SUP45* genes. We performed a bioinformatic analysis of the *SUP35* and *SUP45* promoters in order to find possible transcription factor binding sites (TFBSs) using the oPOSSUM 3.0 tool. We found four potential Mcm1 TFBSs in the *SUP35* upstream region and six in the *SUP45* upstream region. Three and two potential Sfp1 TFBSs were also found in the upstream regions of *SUP35* and *SUP45*, respectively (Table 1). We designed mutations and deletions in the potential Mcm1 and Sfp1 TFBSs. Because no influence of the Mcm1 on *SUP45* mRNA was observed, we chose only Mcm1 TFBSs in the *SUP35* promoter (Figure 2A). We designed complete deletions of two such TFBSs and mutations in one. The latter site overlaps with TFBS of another transcription factor, Spt2, which is known to physically interact with the *SUP35* promoter [51]. We changed the sequence in such a way that Spt2 TFBS would remain while the potential Mcm1 TFBS was lost (Figure 2A,B). We also designed mutations in the two most probable Sfp1 TFBSs, one in the *SUP45*, and one in the *SUP35* promoter. The mutation in the *SUP45* promoter was designated '*flipSfp1*', as part of the sequence was flipped (Figure 2C).

Table 1. Bioinformatic analysis of potential Mcm1 and Sfp1 binding sites in the *SUP35* and *SUP45* promoters Putative transcription factor binding sites (TFBS) of Sfp1 and Mcm1 in the promoter regions of *SUP35* and *SUP45* were identified by oPOSSUM3.0 single-site analysis. The %Score value indicates the similarity of the sequence to the TFBS profile. The optimal sites selected for further analysis are highlighted in bold.

Transcription Factor	Gene	TFBS Start (Rel. to ORF Start)	TFBS End (Rel. to ORF Start)	%Score
Mcm1	*SUP35*	**−315**	**−304**	**81.3%**
		−281	**−270**	**82.7%**
		−169	**−158**	**80.4%**
		−40	−29	75.6%
	SUP45	−43	−32	83.6%
		−67	−56	81.5%
		−165	−154	76.4%
		−388	−377	75.2%
		−435	−424	76%
		−475	−464	76.6%
Sfp1	*SUP35*	−426	−406	75.1%
		−328	−308	81.9%
		−163	**−143**	**93.3%**
	SUP45	−20	1	77.3%
		−174	**−154**	**92.6%**

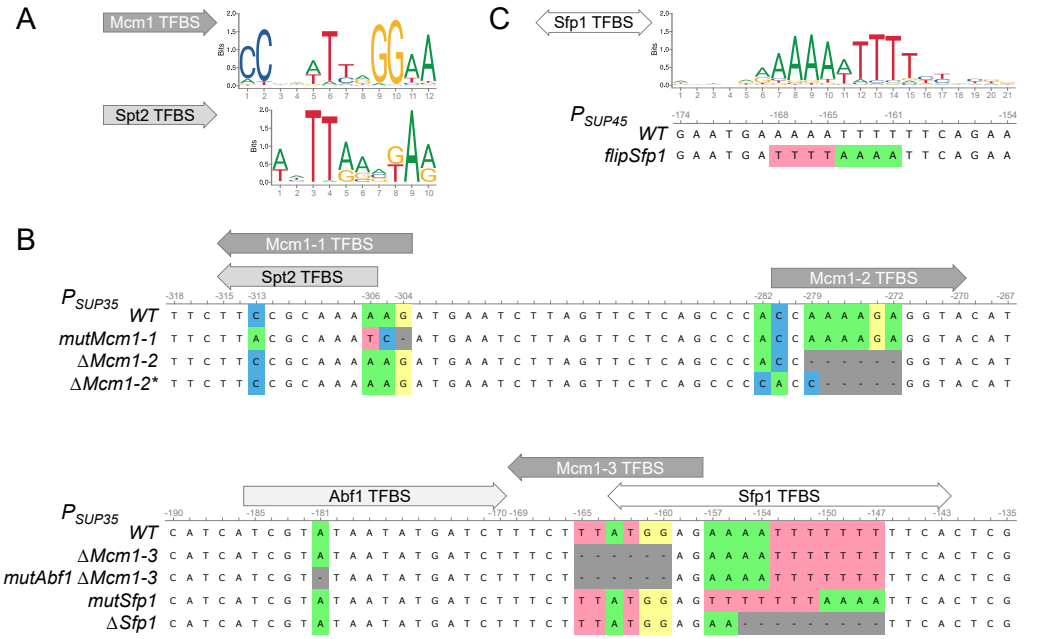

Figure 2. Design of mutations and deletions in the potential Mcm1 and Sfp1 TFBS. (**A**) Profiles for the TFBSs of Mcm1 (MA0331.1) and Spt2 (MA0387.1) according to the JASPAR2022 database. (**B**) Locations of the predicted TFBSs in the *SUP35* promoter and alignments of the deletions and mutations used in this work. (**C**) Profile for the Sfp1 TFBS (MA0378.1) and alignment of the *SUP45* promoter region predicted to contain this site.

3.3. The Potential Mcm1 and Sfp1 Binding Sites in the SUP35 Promoter Regions Do Not Significantly Influence Nonsense Suppression

We obtained a series of vectors based on the centromeric plasmid with the *SUP35* gene pRSU1 [25] with mutations and deletions in the potential Mcm1 and Sfp1 TFBSs. Using the 12-D1682 strain and the isogenic [*PSI*$^+$] strains bearing the only copy of the *SUP35* gene on

a centromeric plasmid, we obtained both [*PSI*$^+$] and [*psi*$^-$] strains with *SUP35* regulated by promoters with the designed mutations and deletions. Phenotypic analysis of such strains did not reveal any visible changes in the nonsense suppression phenotype (Figure 3A; Supplementary Figures S2 and S3). The only exception was the deletion of the Sfp1 TFBS in the [*psi*$^-$] strain, which resulted in a slight change in color on the 1/4YEPD, indicating a slight increase in *ade1-14* suppression. On several occasions we were able to observe very slow growth of this strain on media not containing adenine (Supplementary Figure S2B). We tested various [*PSI*$^+$] strains for possible strain-specific allosuppression, but found none (Supplementary Figure S3). In contrast to changes in the potential Sfp1 and Mcm1 TFBSs, introducing an additional point mutation in the Abf1 TFBS close to the Mcm1-3 TFBS (Figure 2) resulted in suppression of the *ade1-14* mutation (Figures 3A and S2A) at levels comparable to complete deletion of the Abf1 TFBS [34]. Such little or no influence on nonsense suppression by the analyzed promoter mutations suggests that they do not affect *SUP35* expression. To check this, we compared the Sup35 protein levels in our strains. Indeed, mutations and deletions of the potential Mcm1 TFBSs did not affect the Sup35 levels. Surprisingly, however, mutation and deletion of the Sfp1 TFBS significantly reduced Sup35 levels, though not so drastically as mutation of the Abf1 TFBS (Figures 3B and S4). Thus, it is evident that potential Mcm1 TFBSs do not influence production of the Sup35 protein under normal conditions, while Sfp1 does so slightly. In case the Sfp1 TFBS is inactive, the remaining Sup35 production level is probably still sufficient for maintaining nearly normal levels of nonsense suppression.

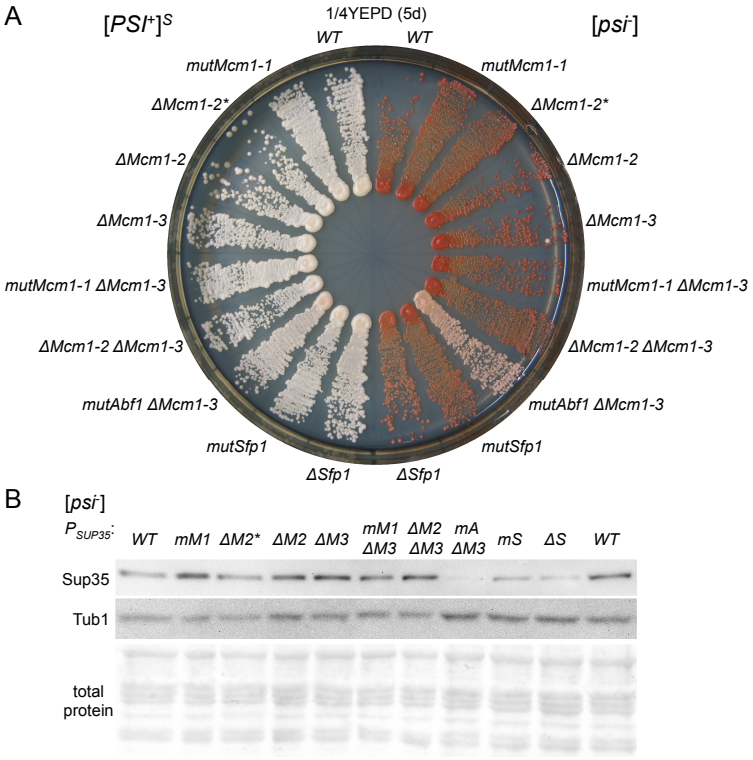

Figure 3. The mutations in the TFBSs of Mcm1 do not affect nonsense suppression or Sup35 production. (**A**) Analysis of phenotypes in strains with mutations in the *SUP35* promoter affecting potential Mcm1 binding sites; growth of the strains derived from U-P^S-A-GT671 ([*PSI*$^+$]^S) and U-12-D1682 ([*psi*$^-$]) on 1/4YEPD medium is shown. (**B**) The results of analysis of the Sup35 protein levels in U-12-D1682 derivatives performed using Western blotting. Coomassie R-250 staining was used to visualize total protein. Promoter variants are denoted as follows: WT, wild-type promoter; *mM1*, Δ*M2*, Δ*M2**, Δ*M3*, *mM1*Δ*M3*, Δ*M2*Δ*M3*, *mA*Δ*M3*, *mS*, and Δ*S* stand for *mutMcm1-1*, Δ*Mcm1-2*, Δ*Mcm1-2**, Δ*Mcm1-3*, *mutMcm1-1*Δ*Mcm1-3*, Δ*Mcm1-2*Δ*Mcm1-3*, Δ*Mcm1-3 mutAbf1*, *mutSfp1*, and Δ*Sfp1*, respectively.

3.4. The Effects of Transient MCM1 Overexpression Do Not Depend on the Potential Mcm1 Binding Sites

We have shown that the potential Mcm1 TFBSs do not affect nonsense suppression when Mcm1 is produced at normal levels. However, because *MCM1* overexpression enhances nonsense suppression, it is possible that additional Mcm1 may use binding sites that are not used otherwise. Thus, we tested whether the potential Mcm1 TFBSs are involved in the increase in nonsense suppression levels caused by *MCM1* overexpression. We used the [*PSI*$^+$] strains with mutant promoter variants and compared their suppressor phenotypes with and without overexpression of *MCM1*. Transient overproduction of Mcm1 resulted in slightly increased suppression in all strains, including those with double mutations in the potential TFBSs (Figure 4). Similar results were obtained in strains with other [*PSI*$^+$] prion variants (Supplementary Figure S5). As the increase in suppression upon Mcm1 overproduction did not depend on the presence of functional Mcm1 TFBSs in the promoter, our results suggest that direct binding of Mcm1 to the *SUP35* promoter is not involved in the effects of Mcm1 on nonsense suppression.

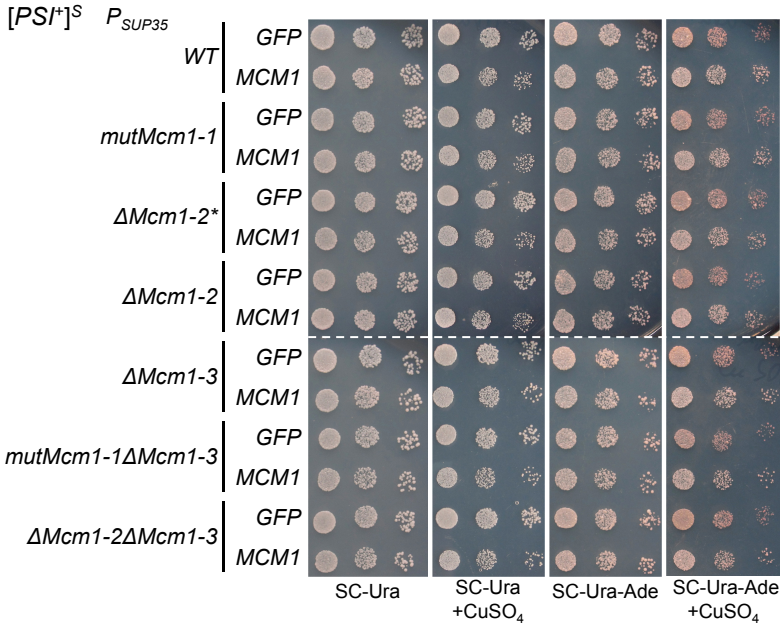

Figure 4. Deletions and mutations of potential Mcm1 binding sites in the *SUP35* promoter do not affect the suppressor effect of Mcm1 overproduction. Derivatives of the U-P^S-A-GT671 strain with *SUP35* under control of the mutant promoters were transformed with pRS316CG (*GFP*) or pU-MCM1 (*MCM1*). Shown are tenfold serial dilutions of the representative clones. Media were supplemented with $CuSO_4$ at a final concentration of 50 μM where indicated.

3.5. Mcm1 Does Not Form Detergent-Resistant Aggregates and Does Not Affect Sup35 Aggregation

Mcm1 is one of the Q/N-rich proteins that might be prone to aggregation, at least according to certain prediction models [13,52]. Taking into account that aggregates of different proteins are known to interact with each other [53], a possible explanation for the effects of Mcm1 on the [*PSI*$^+$] phenotype might be Mcm1 aggregation interfering with Sup35 aggregation. Thus, we first investigated whether Mcm1 could form aggregates in vivo. Transiently overproduced GFP-tagged Mcm1 demonstrated an uneven distribution, forming a single heterogeneous cluster per cell. These clusters were seen to consist of multiple small particles when viewed under high magnification (Figure 5A). This distribution pattern of Mcm1 did not depend on the presence or absence of the [*PSI*$^+$] and [*PIN*$^+$] prions (Supplementary Figure S6A). The clusters were presumably localized in the nucleus, which was confirmed by their colocalization when the nucleus was imaged using NLS-mCherry protein (Figure 5B). To determine whether the fluorescent foci of Mcm1-GFP

corresponded to aggregates, we analyzed protein samples using semi-denaturing detergent agarose gel electrophoresis (SDD-AGE). While we were able to detect the Mcm1-GFP protein using Western blotting, its weight distribution corresponded to the monomeric protein fraction (Figure 5C), indicating that Mcm1-GFP did not form amyloid-like or any other SDS-resistant aggregates. Notably, Mcm1-GFP was stable and produced on sufficient levels (Supplementary Figure S6B).

Even though Mcm1 did not form aggregates in vivo, its overproduction could still interfere with Sup35 aggregation in [*PSI*$^+$] strains. To test this, we assessed Sup35NM aggregation by co-expressing *MCM1* with *SUP35NM-GFP* and then estimating the rate of Sup35NM aggregate appearance. We found no differences between strains overproducing Mcm1 and control strains (Figure 5D,E), indicating that excess Mcm1 does not influence [*PSI*$^+$] aggregates or Sup35 aggregation. The SDD-AGE analysis also showed that Mcm1 overproduction did not alter Sup35 aggregate size distribution in [*PSI*$^+$] strain (Figure 5C). In addition, no effect on the Rnq1 aggregate size distribution in the [*psi*$^-$][*PIN*$^+$] strain was observed (Supplementary Figure S6C). As such, it is evident that Mcm1 does not affect the [*PSI*$^+$] phenotype via changes in Sup35 aggregation.

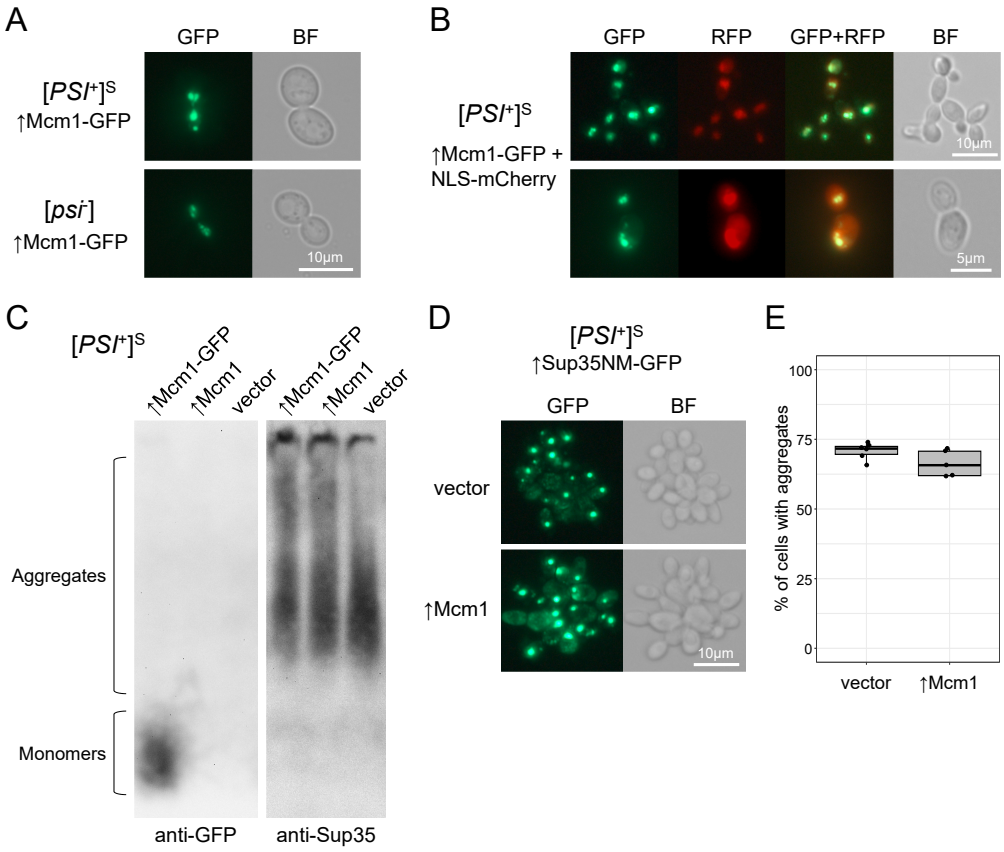

Figure 5. Mcm1 does not form aggregates and does not influence [*PSI*$^+$] aggregates. (**A**) Fluorescence microscopy of OT56 ([*PSI*$^+$]^S) and 74-D694 ([*psi*$^-$]) cells transformed with the pUGC-MCM1-GFP plasmid. BF, bright field. (**B**) OT56 was co-transformed with pUGC-MCM1-GFP and pYX242-Nab2NLS-2mCherry and cells were analyzed with fluorescence microscopy. (**C**) Protein lysates of OT56 cells overproducing either Mcm1 or Mcm1-GFP were subjected to SDD-AGE and Western blotting using anti-GFP and anti-Sup35 antibodies. (**D**) OT56 bearing the pRS315CNMG plasmid was co-transformed with either pU-MCM1 (↑Mcm1) or pRS316 (vector). Aggregates formed by Sup35NM-GFP were visualized with fluorescence microscopy. (**E**) The proportions of cells with visible aggregates were counted for six vector-containing clones and five Mcm1-overproducing clones from the same transformations as in (**D**). No less than 50 cells were counted for each clone. Wilcoxon Mann–Whitney tests showed no significant difference between the proportions of cells with aggregates ($p > 0.05$). In all experiments (**A**–**E**), cells were analyzed after 4 h of copper induction.

3.6. The potential Sfp1 Binding Site in the SUP45 Promoter Affects Its Expression and Nonsense Suppression

As Sfp1 was shown to affect the transcription of both *SUP35* and *SUP45*, we studied its potential binding sites in the promoters of both genes. First, we considered a potential TFBS in the *SUP45* promoter. We introduced a mutation predicted to prevent Sfp1 binding into the centromeric plasmid pRS315-SUP45 [26] and obtained strains bearing this plasmid as a sole source of the *SUP45* expression. Analysis of phenotypes of the obtained strains showed a weak increase in nonsense suppression levels, as evidenced by a slight shift from red to pink color on the 1/4YEPD medium, indicating weak suppression of the *ade1-14* mutation, as well as by slow growth on media lacking tryptophan, indicating suppression of *trp1-289* (Figure 6A). Increased suppression implies a decrease in *SUP45* expression. We checked this first by estimating the *SUP45* mRNA levels using qPCR and second by assessing the Sup45 protein levels with Western blotting. Indeed, we observed a decrease in both mRNA and protein levels of *SUP45* regulated by the promoter with mutant Sfp1 TFBS (Figure 6B,C). We conclude that Sfp1 acts as a transcriptional activator of *SUP45* expression directly via the predicted TFBS. However, *SUP45* upregulation by Sfp1 is not essential, as only slight enhancement in nonsense suppression levels is observed in its absence. The mutation in the Sfp1 TFBS did not significantly alter cell viability under normal conditions. However, excess Sfp1 is known to exacerbate lethality in a [*PSI*$^+$] prion-dependent manner [32]. We tested for possible effects of the mutant Sfp1 TFBS on the growth inhibition caused by *SFP1* overexpression, but found no influence (Supplementary Figure S7). This result corroborates the previously shown independence of the Sfp1-derived toxicity of the Sup45 abundance [32].

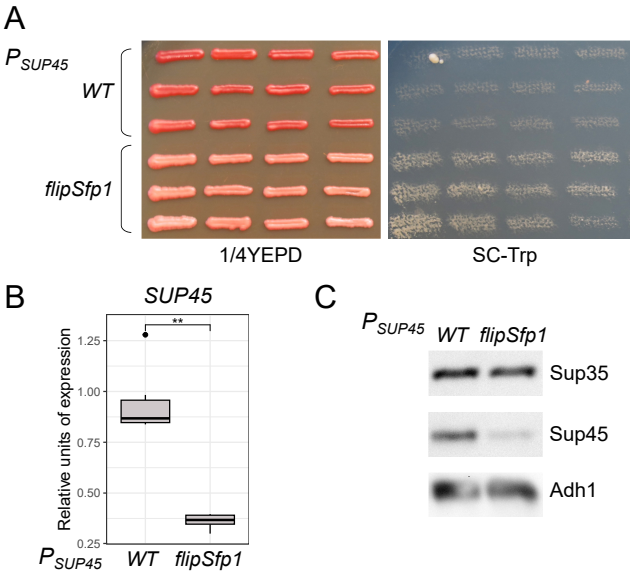

Figure 6. Mutation of a potential Sfp1 binding site in the *SUP45* promoter enhances nonsense suppression by reducing *SUP45* expression. (**A**) Shown are twelve independently-obtained clones obtained from the U-1A-D1628 strain that bear the sole copy of the *SUP45* gene under control of either wild-type (*WT*) or *flipSfp1* promoter variant passaged on 1/4YEPD and replica plated on SC-Trp medium. (**B**) The results of qPCR analysis of the *SUP45* mRNA levels in the strains from panel A. The relative expression of *SUP45* regulated by the wild-type promoter is compared to the *flipSfp1* variant. **, $p < 0.01$ in the Wilcoxon Mann–Whitney test. (**C**) Analysis of the Sup35 and Sup45 protein levels in the strains from panel A using SDS-PAGE and Western blotting. Adh1 levels were used as a reference.

3.7. The Potential Sfp1 Binding Site in the SUP35 Promoter is Important for [PSI$^+$] Prion Toxicity

Similar to the Sfp1 TFBS in the *SUP45* promoter, we checked whether the potential Sfp1 TFBS in the *SUP35* promoter had any influence on the [*PSI*$^+$] prion toxicity caused by excess Sfp1. Overexpression of *SFP1* appeared to be more toxic in the [*PSI*$^+$] strain

with the wild-type *SUP35* promoter compared to strains with mutation or deletion in the Sfp1 TFBS (Figure 7A). Importantly, no such difference could be observed on the [*psi*$^{-}$] background (Figure 7A), suggesting that the presence of the Sfp1 TFBS may contribute to the prion-dependency of the observed lethality. As we previously reported that *SUP35* upregulation is one of the factors responsible for Sfp1-derived [*PSI*$^{+}$] toxicity, we checked whether the defects in the Sfp1 TFBS affect Sup35 production. Not only did both the mutation and deletion of Sfp1 TFBS reduce the amount of the Sup35 protein, they also prevented an increase in its production during *SFP1* overexpression (Figure 7B), which implies that the effect of Sfp1 on the Sup35 levels depends on the intact Sfp1 binding site in the *SUP35* promoter. Consequently, the Sfp1 TFBS is responsible for the elevated *SUP35* expression, which in turn enhances the toxicity in a [*PSI*$^{+}$] prion-dependent manner. However, it should be noted that the decrease in growth during *SFP1* overexpression is more pronounced in [*PSI*$^{+}$] compared to [*psi*$^{-}$] strains even in the absence of the Sfp1 binding site, indicating that *SUP35* upregulation is not the only mechanism of the prion toxicity in this case.

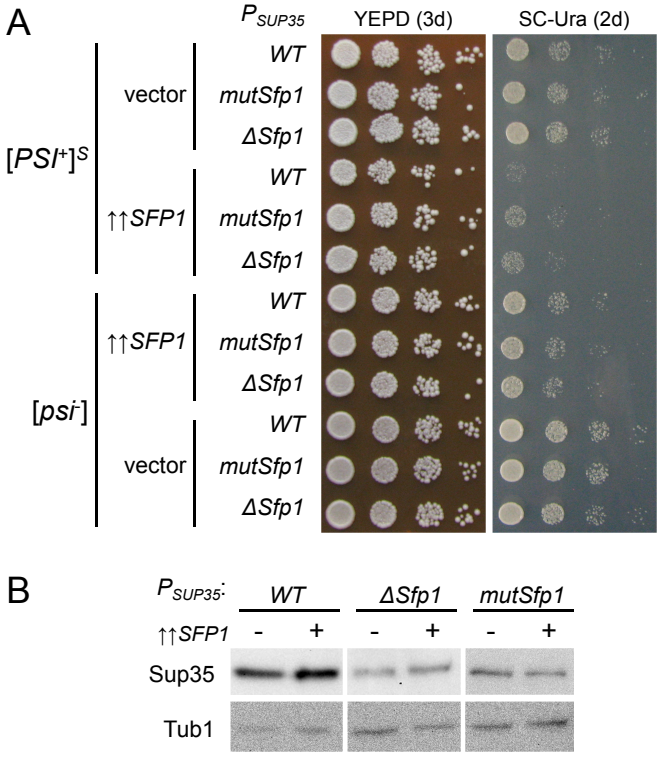

Figure 7. *SUP35* upregulation caused by excess Sfp1 contributes to [*PSI*$^{+}$]-dependent toxicity and requires Sfp1 TFBS in the *SUP35* promoter. (**A**) U-P^{S}-A-GT671 ([*PSI*$^{+}$]^{S}) and U-12-D1682 ([*psi*$^{-}$]) derivatives containing plasmids with the indicated *SUP35* promoter variant as the sole source of *SUP35* were transformed with pRS426-SFP1 (↑↑*SFP1*) or pRS426 (vector). Shown are tenfold serial dilutions of representative clones. (**B**) Assessment of the Sup35 protein levels in strains from (**A**) using SDS-PAGE and Western blotting. Tub1 levels were used as a reference.

4. Discussion

The influence of various Q/N-rich proteins on yeast prion propagation has been addressed in multiple studies. Primarily, this is because they are prone to either amyloid aggregation or phase separation-based inclusion formation (or both); a number of instances in which they may aid in aggregation of each other have been documented [10,13,54]. Among the Q/N-rich proteins, transcription regulators are frequently found (reviewed in [15]). Several attempts ta screening for such factors affecting the [*PSI*$^{+}$] prion or nonsense suppression have been made; however, these have yielded contradictory results. For

example, *SFP1* overexpression was found to enhance nonsense suppression on the [*PSI*$^+$] background, but not in other test systems [17,18,22,32]. Here, in order to uncover new mechanisms of translational accuracy, we concentrated on the effects of Sfp1 and Mcm1 on the transcription of the release factor genes.

Mcm1 is a MADS-box (an acronym of Mcm1, Agamous, Deficiens, and SRF proteins) transcription factor. It operates as a dimer to directly bind DNA in a sequence-specific manner [55]. Mcm1 has been shown to act as both an activator and repressor of genes involved in DNA replication, cell cycle progression, mating type-specific behaviour, stress response, and other processes (reviewed in [56–59]). While able to act by itself, Mcm1 is also known to act in cooperation with various other transcription factors and thereby participate as both an activator and repressor of different regulatory modules. For example, the combinatorial complex formation with α1, α2, and Ste12 governs both up- and downregulation of mating-type specific genes [59], while alternative interaction of Mcm1 with either Fkh2 or Yox1 controls cell-cycle genes expressed late in mitosis [60]. Mcm1 binding sites have also been shown to repeatedly appear in the promoters of ribosomal protein genes in diverse clades of *Ascomycota*, presumably due to an ability of Mcm1 to cooperate with another conservative transcription factor, Rap1, in activating TFIID [61]. Even though regulation of the release factor genes is known to be similar to the ribosomal protein genes, sequences resembling Rap1 binding site consensus are absent in the *SUP35* promoter, and no direct Rap1 binding to the *SUP35* promoter has been found either in vitro or in vivo [62]. Yet, excess Mcm1 somehow leads to an increase in nonsense suppression and enhances *SUP35* transcription. Considering that Mcm1 is often seen interacting with other transcription factors, it is possible that its excess affects the activity of some other as yet unknown factors that might in turn shift the balance of proteins involved in the control of nonsense suppression efficiency, thereby indirectly affecting the [*PSI*$^+$] prion phenotype.

Another possible way in which Mcm1 could influence the [*PSI*$^+$] phenotype is possible interference with Sup35 aggregation. Mcm1 contains a C-terminal domain enriched in Q (42.8%) and N (6%) residues. The role of this domain in Mcm1 function is unclear, as it is not essential and its presence is not required for the functioning of Mcm1 as a transcription factor, at least within the mating type-specific regulatory network [63,64]. While domain requirements for other regulatory activities of Mcm1 remain unknown, considering that it uses a conservative DNA-binding motif in most of its detectable DNA-binding events [62], it would be reasonable to assume that the Q/N-rich domain is also unnecessary, at least for binding promoters of other target genes. The C-terminal domain is preceded by an acidic tract composed almost exclusively of aspartate (D) and glutamate (E) residues [63]. Such a structure resembles that of the Sup35 protein, which contains a Q/N-rich N-terminal domain and D/E-rich part in the middle of the M-domain. Nevertheless, Sup35 forms a prion, while Mcm1 does not seem to form detergent-resistant aggregates in vivo. In a large survey of candidate prion proteins, Q/N-rich Mcm1 fragments showed no signs of aggregation either in vitro or in vivo in all tests performed [13], which is consistent with our results. The reason for this may lie in the composition of the C-terminal domain of Mcm1, as in addition to Q and N it is also enriched in proline (P) residues (9.0%). The presence of prolines is thought to destabilize the amyloid structure.; even single proline substitutions in the residues within the amyloid core of Sup35 aggregates result in the loss of prion [65]. In the case of Mcm1, proline residues flank almost all Q-stretches; the maximum length of polyQ sequence uninterrupted by P or other residues is 10 amino acids (aa), while the longest Q/N-rich sequence between two prolines is 20 aa. Studies of polyQ aggregation in yeast suggest that such stretches are too short for amyloid formation, as polyQ sequences of 20 aa or even 25 aa have been used as no-aggregation controls in studies of aggregation of Huntingtin with larger polyQ tracts [66,67]. However, a 25Q-GFP Huntingtin variant was shown to form insoluble aggregates seeded by polymers of other Q/N-rich proteins, including Sup35 [68]. Even though a similar effect could be expected from Mcm1, we did not detect its aggregates, even in cells with [*PSI*$^+$] and [*PIN*$^+$] amyloids (Figure 5C). One reason might be nuclear localization of Mcm1, which makes its interaction with cytosolic

prion particles highly problematic. In contrast, redirecting Huntingtin to the nucleus by addition of a nuclear localization signal (NLS) led to the formation of nuclear aggregates in the case of NLS-23Q-GFP [69] but not that of NLS-20Q-GFP [70]. Transiently overproduced Mcm1-GFP also formed aggregate-like dots in the nucleus (Figure 5A); however, these these were not detergent-resistant, and consequently unlikely to be amyloid. It is possible that, as in the case of nuclear-localized Huntingtin, uneven nuclear distribution of Mcm1-GFP is caused by cellular protein quality control (PQC) machinery, which is known to be active inside the nucleus [71,72]. Thus, the puncta-like structures of Mcm1-GFP that we observed in the nucleus are likely PQC compartments rather than aggregates.

Sfp1 is an unusual Zn-finger transcription factor in which two Cys2His2-type domains are separated by a sequence of 37-39 aa. The usual distance for such proteins is 7-8 aa, leading to its name, "Split finger protein" [73]. Sfp1 is a nonessential transcription factor; however, its absence results in a substantial decrease in cell growth rate in combination with smaller cell size. Sfp1 is considered a key regulator of multiple processes under normal unstressed conditions. It is known to take part in the regulation of G1/S and G2/M cell cycle progression and DNA-damage response, while its main course of action is activation of the ribosomal protein (RP) and ribosome biogenesis (RiBi) genes [74–77]. Transcriptome analysis of Sfp1-deficient cells has revealed changes in expression of a large number of genes, up to 2000, which is almost third of all yeast genes [78–80]. However, the slow-growth phenotype of the *sfp1*Δ cells and total decrease in cellular translation rates make it difficult to distinguish between direct and indirect effects. The deletion of *SFP1* has been shown to reduce the expression of the *SUP35* and *SUP45* genes [80,81]; again, however, there has been no evidence that these effects are the consequences of direct regulation. Similarly, the transcription of some 2000 genes was affected in response to the overexpression of *SFP1* [81]. The transcription of both *SUP35* and *SUP45* has been shown to be increased during *SFP1* overexpression, even though the difference on the protein level could be detected only for Sup35 [32,82]. Here we present evidence of direct regulation of the *SUP35* and *SUP45* genes by Sfp1. Interestingly, even though the absence of functional Sfp1 TFBSs in the promoters reduces both Sup35 and Sup45 protein levels (Figures 3B and 6C), excess Sfp1 affects only Sup35 [32]. It is possible that under normal conditions the Sup45 protein is already produced at its maximum levels, meaning that there is no capacity for additional production. This could explain why the effects of the mutations in the Sfp1 TFBSs lead to a more pronounced phenotype in the case of *SUP45* (Figures 6A and S2). However, this may also be due to differences in the requirements of Sup35 and Sup45 for the nonsense suppression level control. Alternatively, there might be additional feedback mechanisms for the maintenance of Sup45 but not Sup35 abundance, even though no such mechanism has been discovered yet. In the only described system in which reduction of one release factor led to decrease in another, this worked both ways; however, specific engineered promoters were used for Sup35 and Sup45 production [4], so whether such feedback mechanisms exist for natively expressed genes remains unknown. Nonsense mutations in *SUP35* or *SUP45* do not lead to reduced levels of eRF1 or eRF3, respectively [55].

The targets of Sfp1 were found to be enriched in PAC and RRPE elements [78,83]. The latter was later shown to correspond to the Sfp1 TFBS profile derived from ChIP-chip analysis [84], which is identical to the profile in the JASPAR database that we used in this work. However, it turned out that multiple Sfp1 targets were missed by ChIP-chip and ChIP-seq. Another technique, ChEC-seq, allowed identification of Sfp1 binding to the promoters of RiBi and RiBi-like genes, suggesting several distinct modes of Sfp1 action depending on its cooperation with other transcription factors, such as Swi4 and Ifh1 [85]. The regulation of the expression of the release factor genes has been shown to be similar to that of the RP and RiBi genes; thus, they are also included in the RiBi-like group, even though their promoters do not contain PAC elements [78,85]. The promoters of RiBi-like genes have been shown to be enriched in the RRPE-like sequences. Thus, Sfp1 has been previously shown to physically interact with the *SUP35* and *SUP45* promoters [85];

however, it is not clear whether this interaction has any effect on the functioning of these genes. Here, we show that this mode of Sfp1 action makes it one of the transcription factors that balance the basic level of nonsense suppression. Another such factor is Abf1, which has also been shown to upregulate the release factor genes [86]. Both Abf1 and Sfp1 are non-essential for *SUP35* and *SUP45* expression, in the sense that the absence of upregulation driven by Abf1 or Sfp1 does not result in cell death as a sufficient amount of the release factors is still produced. However, such cells demonstrate elevated levels of nonsense suppression (Figures 3A–B and 6A–C). This effect is more visible in the case of Abf1, as even point mutations in the Abf1 TFBS lead to a moderate nonsense suppression level comparable to that of complete deletion of the TFBS [34]. The effect on phenotype of alterations in the Sfp1 TFBSs is much less pronounced, even though a decrease in both Sup35 and Sup45 protein levels is observed.

Apart from being a transcriptional regulator, Sfp1 also contains Q/N-rich domains. Its transient overexpression was shown to lead to the appearance of detergent-resistant Sfp1 aggregates that co-localize with the Hsp40-Sis1 chaperone. Even though, Sfp1 normally resides in the nucleus, similar to Mcm1, its aggregates are localized in the cytosol. Overproduction of Sfp1 also influences aggregation of Sup35 in the [*PSI*$^+$] cells, leading to an increase in the size of aggregates. Both this influence and the observed toxicity are alleviated by additional Sis1 [32]. As a result, Sfp1 enhances the [*PSI*$^+$] prion phenotype by simultaneously upregulating *SUP35* and promoting Sup35 aggregation.

5. Conclusions

In summary, we investigated the influence of two transcription factors, Mcm1 and Sfp1, both of which enhance the [*PSI*$^+$] suppressor phenotype when overproduced, on [*PSI*$^+$] prion properties and nonsense suppression. While the exact mechanism behind the effects of Mcm1 remains unclear, we have shown that it is unlikely to involve direct binding to the *SUP35* promoter. Mcm1 did not affect Sup35 aggregation either, suggesting that it affects the [*PSI*$^+$] properties indirectly. In contrast, in the case of Sfp1 we found that it is likely to directly activate transcription of both the *SUP45* and *SUP35* genes. The latter is an important factor that contributes to the effects of Sfp1 on [*PSI*$^+$], though it is not the only mechanism behind the enhanced [*PSI*$^+$] toxicity caused by excess Sfp1. Another such mechanism is an influence of Sfp1 on Sup35 aggregation [32], and it seems that both mechanisms contribute equally.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres15020034/s1, Figure S1: The synthetic lethality of [*PSI*$^+$] with *sup45* mutations is increased upon *MCM1* transient overexpression. Figure S2: Alterations in the potential Mcm1 TFBSs do not affect nonsense suppression in [*psi*$^-$] strains, while deletion of the potential Sfp1 binding site in the *SUP35* promoter leads to an extremely small increase in the nonsense suppression. Figure S3: Mutations and deletions of the potential TFBSs of Mcm1 and Sfp1 do not affect nonsense suppression in various [*PSI*$^+$] strains. Figure S4: Analysis of the Sup35 protein levels in U-P^S-A-GT671 derivatives using SDS-PAGE, followed by Western blotting with anti-Sup35 antibodies. Figure S5: Deletions and mutations of potential Mcm1 binding sites in the *SUP35* promoter do not affect the suppressor effect of Mcm1 overproduction. Figure S6: The [*PIN*$^+$] prion does not affect Mcm1 aggregation, and Mcm1 overproduction does not affect the [*PIN*$^+$] prion. Figure S7: Mutation of the potential Sfp1 binding site in the *SUP45* promoter does not affect [*PSI*$^+$] toxicity. Table S1: Plasmids used in this work. Table S2: Oligonucleotides used in this work. Table S3. Yeast strains used in this work. File S1: Zip archive with original images of blots.

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Abbreviations

The following abbreviations are used in this manuscript:

aa	amino acids
bp	base pair
GFP	green fluorescent protein
ChIP	chromatin immunoprecipitation
ChEC	chromatin endogenous cleavage
5-FOA	5-fluoroorotic acid
TFBS	transcription factor binding site
NLS	nuclear localization signal
PQC	protein quality control
RP	ribosomal protein
RiBi	ribosome biogenesis
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SDD-AGE	semi-denaturating detergent agarose gel electrophoresis

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