The mRNA Decay Pathway Regulates the Expression of the Flo11 Adhesin and Biofilm Formation in *Saccharomyces cerevisiae*

Tricia L. Lo, Yue Qu, Nathalie Uwamahoro, Tara Quenault, Traude H. Beilharz, and Ana Traven¹

Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Victoria 3800, Australia

ABSTRACT Regulation of the *FLO11* adhesin is a model for gene expression control by extracellular signals and developmental switches. We establish that the major mRNA decay pathway regulates *FLO11* expression. mRNA deadenylation of transcriptional repressors of *FLO11* by the exonuclease *Ccr4* keeps their levels low, thereby allowing *FLO11* transcription.

CELL-wall adhesins mediate attachment between cells and to abiotic substrates. Adhesion is key for the ability of unicellular yeasts to change morphology, mate, invade substrates and host cells, and associate into protective multicellular communities, such as biofilms and flocs (reviewed in Bruckner and Mosch 2012). These pathways are important for industrial applications and in the context of human disease caused by fungal pathogens (Hoyer *et al.* 2008; Finkel and Mitchell 2011; Liu and Filler 2011; Bruckner and Mosch 2012). In the model yeast *Saccharomyces cerevisiae*, the cell-wall adhesin Flo11 mediates many such adhesion-related phenotypes, such as attachment to polystyrene and formation of multicellular structures called “mats” (Lo and Dranginis 1998; Guo *et al.* 2000; Reynolds and Fink 2001). Mats form when yeast cells spread over a semisolid agar substrate and have a defined “floral” architecture, suggestive of a developmental pathway (Reynolds and Fink 2001). The regulation of the expression of the *FLO11* gene has long served as a model for understanding how extracellular signals, developmental pathways, and epigenetic mechanisms impinge on gene expression (reviewed in Bruckner and Mosch 2012). The expression of *FLO11* depends on the environmental context (e.g., nutrient limitation, quorum sensing) and is regulated by a range of transcriptional activators and repressors under the control of signaling pathways. For example, the cAMP/PKA path-

way, the mitogen-activated protein kinase pathway, and the pH-responsive Rim101 pathway all regulate the expression of *FLO11* through transcriptional activators such as Flo8, Ste12, Tec1, and Rme1 and repressors such as Sfl1, Nrg1, Nrg2, and Sok2 (Lo and Dranginis 1998; Kuchin *et al.* 2002; Braus *et al.* 2003; Vyas *et al.* 2003; Chen and Fink 2006; Bruckner and Mosch 2012). Epigenetic mechanisms also control *FLO11* expression (Halme *et al.* 2004).

The mRNA decay pathway is important for the control of mRNA stability and translation (Goldstrohm and Wickens 2008). The components of this pathway include the Ccr4-NOT mRNA deadenylase complex, which shortens the mRNA poly(A) tail as the first step leading to mRNA decay; the decapping enzyme Dcp1/Dcp2 catalyzing 5' cap hydrolysis following deadenylation; the exonuclease Xrn1 that degrades the mRNA after decapping; and decapping activators such as the RNA helicase Dhh1 (Parker and Song 2004; Goldstrohm and Wickens 2008). While deadenylation and decay act on all transcripts during their life cycle, it is thought that additional, gene-specific effects also occur (Beilharz and Preiss 2007; Lackner *et al.* 2007). This is manifested in transcripts displaying different steady-state distribution of poly(A) tails: mRNAs subject to gene-specific deadenylation by Ccr4 are preferentially “short-tailed,” while those that are not display longer tails (Beilharz and Preiss 2007; Lackner *et al.* 2007; Dagley *et al.* 2011). In the absence of Ccr4, the poly(A) tails become longer on all mRNAs, but the expression of transcripts that are “short-tailed” in the wild type is likely to be most affected by the change in poly(A) tail length. These gene-specific effects could be mediated by RNA-binding proteins, such as the PUF proteins that bind to recognition elements in the 3'

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¹Corresponding author: Monash University, Clayton Campus, Bldg. 76 (STRIP2), Melbourne, Victoria 3800, Australia. E-mail: ana.traven@monash.edu

Figure 1 The mRNA decay pathway is necessary for adherence to plastic and wild-type *FLO11* expression. (A) The $\Sigma 1278b$ strain (*MAT α ura3-52 his3::hisG leu2::hisG*) was used for all experiments. The wild-type strain and the *flo11Δ* mutant were a generous gift from Todd Reynolds and are described in Reynolds and Fink (2001). Deletion mutants in the mRNA decay pathway components were constructed by standard methods. Growth of the indicated strains was assessed on 2% agar YPD plates, using 10 $\times$ serial dilutions (starting from OD₆₀₀ = 0.5). Plates were photographed after 3 days of growth at 30°. (B) For testing adherence to polystyrene, overnight cultures were grown in 2% glucose synthetic media and then diluted to OD₆₀₀ $\approx$ 1 into 0.2% glucose media before inoculation into 96-well polystyrene plates. The cells were allowed to adhere for 90 min (at 30°, 75 rpm), followed by washes in PBS. Fresh 0.2% glucose media was then dispensed into each of the wells, and the adherent cells further incubated for 6 and 24 hr. Quantification was performed by crystal violet staining and reading OD₅₉₅ after washing and destaining in 95% ethanol. Shown are the averages of three biological repeats assayed in quadruplicate and the standard error. Independent deletion clones of the mutants (two to three) were tested and gave analogous results. For the *ccr4Δ*, *pop2Δ*, and *dhh1Δ* strains, the *P*-values were <0.001 at all time points. For the *puf5Δ* strain, statistically significant differences were

observed at 90 min (*P* = 0.017) and at 24 hr (*P* < 0.001). At 6 hr, a slight reduction in adherence was observed, but this was not statistically significant (*P* = 0.38). (C) Mat formation was assessed on 0.3% agar YPD plates at room temperature as described previously (Reynolds and Fink 2001). The mutants were assayed against the wild type and the *flo11Δ* negative control on several separate occasions, and a composite of the performed experiments is shown. Two to three independently constructed deletion clones for each of the mutants were tested and gave similar results, except for *ccr4Δ*, for which independent clones displayed defective mat formation, although clone 1 was more affected than clone 2. Four other *ccr4Δ* clones were tested, of which two behaved as clone 1, one as clone 2, and one had an intermediate phenotype between clones 1 and 2 (Figure S1). The variability of *ccr4Δ* clones 1 and 2 was not observed in quantitative assays of attachment to plastic or *FLO11* expression, with both strains displaying comparable defects (see B and D). (D) The expression levels of *FLO11* were determined by quantitative real time PCR (qPCR) from cells grown in YPD at 30°. RNA was prepared by the hot phenol method. Reverse transcription was performed, and the resulting cDNA was used as the template in qPCRs essentially as described (Dagley *et al.* 2011). The qPCR data were analyzed with the LinReg PCR program (Ruijter *et al.* 2009; Tuomi *et al.* 2010). *ACT1* levels were used for normalization. All values were expressed relative to the average of the wild type. Shown are averages of at least three independent cultures and the standard error. The *P*-value was <0.001 for all mutants.

untranslated regions (3' UTRs) of mRNAs, and recruit *Ccr4*-NOT and *Dhh1* (Goldstrohm *et al.* 2006, 2007; Beilharz and Preiss 2007; Hook *et al.* 2007; reviewed in Quenault *et al.* 2011).

We and others have previously established a role for *Ccr4*-NOT, *Dhh1*, and the PUF protein *Puf5* in cell-wall integrity and regulation of cell morphology (Kaeberlein and Guarente 2002; Prinz *et al.* 2007; Stewart *et al.* 2007; Traven *et al.* 2009, 2010; Dagley *et al.* 2011). The close links between cell-wall structure, morphogenesis, and fungal adhesion prompted us to investigate the role of the mRNA decay pathway in *Flo11*-dependent phenotypes. To that end, we inactivated *CCR4* and *POP2* (which together encode the exonuclease activity of *Ccr4*-NOT) and *DHH1* and *PUF5* in the $\Sigma 1278b$ strain that expresses *FLO11* and is capable of *Flo11*-dependent phenotypes. The *dhh1Δ* and *puf5Δ* mutants displayed wild-type growth, while the *ccr4Δ* and *pop2Δ* strains grew slower on standard YPD plates (Figure 1A). First, we tested adherence to polystyrene in response to low (0.2%) glucose, a phenotype that depends on *Flo11* (Reynolds and Fink 2001). In this assay, particularly at earlier time points, adherence occurs in the absence of growth:

the wild type is able to adhere substantially already after 30 min when there is very little growth in the low-glucose synthetic media (Reynolds and Fink 2001 and data not shown). The *ccr4Δ*, *pop2Δ*, and *dhh1Δ* mutants were all severely compromised for adherence and even after 24 hr did not reach levels that the wild type was able to reach after 90 min (Figure 1B). The phenotype of the *puf5Δ* mutant was mild, but statistically significant differences were observed at the 90-min and 24-hr time points (Figure 1B).

We also tested other phenotypes for which *Flo11* is required: mat formation on semisolid (0.3% agar) plates and adhesion and invasion on standard 2% agar plates. The *ccr4Δ*, *pop2Δ*, and *dhh1Δ* mutants were defective for mat formation on low agar plates (Figure 1C). The *puf5Δ* mutant did not show a measurable defect in this assay. The growth defect of the deadenylase mutants was less pronounced than the mat formation defect, suggesting that the inability to form mats is not solely due to slower growth (compare Figure 1, A and C). Interestingly, we noted in the mat assay that independent clones of *ccr4Δ* showed a different degree of defect in regards to the size of the mat and the presence or absence of "floral" structure (Figure 1C; see also Supporting

Figure 2 The mRNA decay pathway negatively regulates the expression of the *FLO11* repressors *NRG1* and *NRG2*. (A) qPCRs were performed as described in Figure 1. Shown are averages of at least three independent biological replicates and the standard error. *P*-value was <0.001 for all mutants. (B) mRNA poly(A) analysis was performed using a modified version of the poly(A) test (PAT) assay (Beilharz and Preiss 2007; Traven et al. 2009; Dagley et al. 2011; Jänicke et al. 2012). This is a reverse transcription/PCR assay where the size of the PCR products reflects the size of the mRNA poly(A) tail. The shortest tail detected in this assay is ~12 bp [shown by the TVN sample in B, for which a primer binding to the 3' UTR–poly(A) junction is used]. The samples were spiked before reverse transcription with human HeLa RNA, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assayed as a control, showing that the assay works equally well between samples for detecting the length of the poly(A) tail. Gene-specific PCR products were analyzed by 2% high-resolution agarose gel, prestained with SYBR safe, and imaged against a 100-bp ladder using an LAS 3000 imager and multiguage software (Fujifilm). Whether the tails are preferentially shorter or longer was determined by quantifying the signals corresponding to the longer or shorter forms in the wild-type samples (the dotted boxes in the second set of samples indicate how the quantification was performed). A ratio of long/short poly(A)-tailed forms of <1 is typical of a short-tailed transcript (Beilharz and Preiss 2007). (C) The 3' UTR regions of *NRG1* and *NRG2* (200 bp downstream of the STOP codon) were searched for putative PUF-binding sites. The *NRG1* 3' UTR contains a sequence corresponding to the PUF5-1 motif identified by bioinformatic searches, which includes a core PUF consensus sequence UGUR starting 87 bp after the stop codon (Riordan et al. 2011). The 3' UTR of *NRG2* did not contain a consensus PUF-binding site. (D) The experiments testing adherence to polystyrene were performed as in Figure 1B. The yeast strain overexpressing *NRG1* and *NRG2* (bottom, red bars) was constructed by placing the genes under the

control of the strong constitutive promoter *TEF1* at the endogenous genomic locus using PCR and homologous recombination and with the plasmids pYM-N18 and pYM-N19 as PCR templates (Janke et al. 2004). qPCRs were used to confirm elevated expression levels, which were 7.2-fold (± 0.28 SE) for *NRG1* and 2.8-fold (± 0.38 SE) for *NRG2*. Deletion of *NRG1* and *NRG2* caused increased adherence in the wild-type strain, consistent with a previous report (Kuchin et al. 2002), but the effect was more pronounced in the *ccr4Δ* background (statistical significance at all points was with $P \leq 0.05$). For example, at 90 min, the fold difference between the wild type and the *nrg1Δ nrg2Δ* mutant was 1.23-fold, whereas the difference between the *ccr4Δ* strain and the triple mutant was 3.65-fold. (E) qPCR experiments were performed as described in Figure 1. The cells were grown in conditions used to assay adherence to polystyrene, and *FLO11* expression was assayed after 2 hr in 0.2% glucose synthetic complete media. Deletion of the repressors *NRG1* and *NRG2* in the *ccr4Δ* strain rescued *FLO11* levels by 9.4-fold ($P < 0.001$), while, in the wild type, background deletion of the two repressors caused a 2.4-fold upregulation of *FLO11* ($P = 0.005$).

Information, Figure S1, which shows four other independently constructed *ccr4Δ* strains). When we tested the two clones for adherence to polystyrene and *FLO11* expression, they showed comparable, severe defects (see Figure 1, B and D). This indicates that *Ccr4* has a complex role in regulating the expression of *FLO11*, perhaps influencing some of the epigenetic mechanisms that operate on this gene (Halme et al. 2004). All mutants were compromised for adherence to 2% agar plates, albeit to a different degree, and *pop2Δ* and *dhh1Δ* also for invasion (Figure S2). In agreement with our data, defective adherence to agar for the *dhh1Δ* strain has been previously observed (Park et al. 2006).

Consistent with the adherence defect, transcript levels for *FLO11* were lower in all mutants compared to the wild type (Figure 1D). The magnitude of the effect on *FLO11* tran-

script levels in the mutants correlated with the magnitude of their adherence defect (e.g., *FLO11* levels and the ability to adhere were the lowest for the *pop2Δ* mutant, while *puf5Δ* was the least affected for both phenotypes) (Figure 1, B and D).

The mRNA decay pathway negatively regulates gene expression, and thus a possible explanation for reduced *FLO11* transcript levels in the mutants is that this pathway inhibits the expression of *FLO11* repressors. Higher levels of *FLO11* repressors in the mutants would cause reduced *FLO11* transcription. To test this hypothesis, we assayed mRNA levels for *NRG1* and *NRG2*, two transcriptional repressors of *FLO11* that control adherence to plastic in response to low glucose (Kuchin et al. 2002). In the *ccr4Δ*, *dhh1Δ*, and *puf5Δ* mutants, *NRG1* and *NRG2* transcript

Figure 3 Model for the control of *FLO11* expression by the mRNA decay pathway. The mRNA decay pathway keeps the levels of the *NRG1* mRNA low by targeted Ccr4-dependent deadenylation and decay. Puf5 provides specificity by binding to the *NRG1* 3' UTR and recruiting the Ccr4-NOT-Dhh1 complex. Dhh1 acts on the mRNA 5' cap as an activator of decapping and translational inhibitor. The length of the mRNA poly(A) tail positively correlates with translation, and thus targeted deadenylation by Ccr4 not only causes decay, but likely also limits translation of the *NRG1* mRNA. In the mutants inactivated for the components of the mRNA decay pathway, the mRNA and protein levels for *NRG1* are higher, causing increased repression of *FLO11*. A similar mechanism might be operating on the *NRG2* transcript.

levels were upregulated (Figure 2A). The mRNA levels of another Flo11 repressor, *SOK2*, were not affected (with a cutoff of 1.5-fold change) (Figure S3A). *NRG1* displayed preferentially short mRNA poly(A) tails in the wild type (long/short ratio of 0.67 ± 0.03 SD). The *NRG1* poly(A) tail was long in *ccr4Δ* cells. In the *puf5Δ* mutant, the *NRG1* poly(A) tail shifted toward longer tails compared to the wild type (long/short = 1.12 ± 0.26 SD) (Figure 2B). These results are consistent with *NRG1* being a target of gene-specific deadenylation by Ccr4, which is in part mediated by Puf5. Consistent with Ccr4 and Puf5 acting in the same pathway to regulate *NRG1*, the level of the *NRG1* mRNA in the double *ccr4Δ puf5Δ* mutant was equivalent to the single *ccr4Δ* mutant (Figure S3B). A motif corresponding to the computationally predicted Puf5-1-binding motif (Riordan *et al.* 2011) is present within the annotated *NRG1* 3' UTR; instead of the 3' UA that is common in PUF-binding sites (Gerber *et al.* 2004), an AA is found in the *NRG1* 3' UTR, indicating a noncanonical motif (Figure 2C). The presence of a putative Puf5-binding site in the 3' UTR suggests direct regulation of the *NRG1* mRNA by Puf5. *NRG2* displayed an equal amount of shorter and longer poly(A) tails in the wild type (long/short = 0.99 ± 0.067 SD) (Figure 2B), and no putative PUF sites were identified in 3' UTR, although its tails tended to be longer in the *puf5Δ* mutant (long/short = 1.43 ± 0.32 SD). Given that the effect of Puf5 on *FLO11* mRNA levels and adherence is substantially milder than that of Ccr4, Ccr4 must have additional Puf5-independent roles in these phenotypes. The

Puf5-independent roles of Ccr4 could be mediated by other RNA-binding proteins or deadenylase-independent roles of Ccr4 [notably, complete deletion of the *CCR4* gene causes stronger phenotypes than the exonuclease-dead allele in several assays, such as DNA damage responses and changes to cell morphology (Traven *et al.* 2005, 2009)].

To test whether elevated expression of *NRG1* and *NRG2* is causative of low adherence and low *FLO11* levels in the *ccr4Δ* strain, we deleted the two genes in the *ccr4Δ* background and tested for suppression of phenotypes. Figure 2, D and E, shows that deletion of *NRG1* and *NRG2* substantially rescued *FLO11* expression (9.9-fold) and adherence to polystyrene in the *ccr4Δ* strain (between 2.2- and 3.7-fold, depending on the time point). That deletion of the repressors does not fully rescue the *ccr4Δ* mutant indicates that Ccr4 has additional roles in *FLO11* expression. Conversely, overexpression of *NRG1* and *NRG2* in the wild type by placing the genes under the strong constitutive promoter *TEF1* resulted in low *FLO11* levels and low ability to adhere (Figure 2, D and E), thus mimicking inactivation of Ccr4.

Conclusion

Our data identify a role for the mRNA decay pathway in the expression of the *FLO11* adhesin and Flo11-dependent adherence phenotypes. We propose a model in which gene-specific, targeted deadenylation by the Ccr4-NOT complex lowers the mRNA (and likely also protein levels) of the *FLO11* repressors *NRG1* and *NRG2*, thereby allowing wild-type levels of *FLO11* transcription (Figure 3). Puf5 might provide the specificity by recruiting Ccr4-NOT to the *NRG1* transcript. Other, Puf5-independent mechanisms are also at play. This represents a new means for the control of *FLO11* expression and sets the scene for investigating the functions of the mRNA decay pathway in fungal adhesion.

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Supporting Information

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Figure S1 The effect of *CCR4* on mat formation. Four independently constructed *ccr4*Δ clones of the Σ 1278b strain were tested for mat formation on 0.3% agar plates. The plates were grown at room temperature and photographs taken after 10 days of growth.

Figure S2 The mRNA decay pathway and agar adhesion and invasion. Cells of the indicated strains were inoculated onto YPD + 2% agar plates (100 μ l of a $OD^{600}=0.5$ cell suspension) and grown for 6 days at 30°C. To assay adhesion, cells were washed under tap water. Invasion was assessed after rubbing off the adherent cells with a gloved finger. Invasion by the strains into the agar was photographed with a stereo dissecting microscope (Olympus SZX16) under 16X magnification.

Figure S3 Transcript levels of *FLO11* repressors in mRNA decay pathway mutants. A) Transcript levels for *SOK2* were assayed as described in the legend to Figure 1. Shown are averages of three independent cultures for each of the strains and the standard error. B) Levels of *NRG1* were detected by qPCR as in A. For this experiment, the *S. cerevisiae* KY803 strain background was used and the strains are described in (TRAUVEN *et al.* 2010).