

# Conditional regulation of Puf1p, Puf4p, and Puf5p activity alters *YHB1* mRNA stability for a rapid response to toxic nitric oxide stress in yeast

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**ABSTRACT** Puf proteins regulate mRNA degradation and translation through interactions with 3' untranslated regions (UTRs). Such regulation provides an efficient method to rapidly alter protein production during cellular stress. *YHB1* encodes the only protein to detoxify nitric oxide in yeast. Here we show that *YHB1* mRNA is destabilized by Puf1p, Puf4p, and Puf5p through two overlapping Puf recognition elements (PREs) in the *YHB1* 3' UTR. Overexpression of any of the three Pufs is sufficient to fully rescue wild-type decay in the absence of other Pufs, and overexpression of Puf4p or Puf5p can enhance the rate of wild-type decay. *YHB1* mRNA decay stimulation by Puf proteins is also responsive to cellular stress. *YHB1* mRNA is stabilized in galactose and high culture density, indicating inactivation of the Puf proteins. This condition-specific inactivation of Pufs is overcome by Puf overexpression, and Puf4p/Puf5p overexpression during nitric oxide exposure reduces the steady-state level of endogenous *YHB1* mRNA, resulting in slow growth. Puf inactivation is not a result of altered expression or localization. Puf1p and Puf4p can bind target mRNA in inactivating conditions; however, Puf5p binding is reduced. This work demonstrates how multiple Puf proteins coordinately regulate *YHB1* mRNA to protect cells from nitric oxide stress.

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## INTRODUCTION

Cells must rapidly adapt to various types of environmental signals and stresses. Efficient methods to rapidly alter gene expression in response to such signals include the posttranscriptional regulation of mRNA translation and decay rates. Some of the most familiar cases of decay control come from mammalian proto-oncogenes,

cytokines, and transcription factors, whose mRNAs are targeted for rapid decay in response to environmental stimuli (Shim and Karin, 2002). Changes in decay rates of specific transcripts are also involved in circadian clock control (Lidder *et al.*, 2005), the cell cycle (Penelova *et al.*, 2005), oxidant stress response by cystic fibrosis transmembrane conductance regulator (CFTR; Cantin *et al.*, 2006), and differentiation (Jack and Wabl, 1988). The regulatory elements for these changes are often found within the 3' untranslated region (UTR), where regulatory RNA-binding proteins attach and perform repressive functions.

*YHB1* encodes the only known defense protein against nitric oxide (NO) stress in *Saccharomyces cerevisiae* (Liu *et al.*, 2000). To counteract the toxic effects of NO, expression of the flavohemoglobin Yhb1p is induced, which metabolizes NO into nitrate or dinitrogen oxide, depending on the aerobic or anaerobic conditions (Foster *et al.*, 2009). Concomitant with its function, Yhb1p is localized to both the cytosol and the mitochondrial matrix (Cassanova *et al.*, 2005). The absence of *YHB1* causes growth inhibition when cells are treated with the aerobic NO donor DETA NONOate (Liu *et al.*, 2000; Foster *et al.*, 2009). The regulation of *YHB1* expression occurs at both transcriptional and posttranscriptional levels and by

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Abbreviations used: 2  $\mu$ , episomal high-copy vector; CEN, centromere low-copy vector;  $C_q$ , quantification cycle; DIC, differential image contrast; EV, empty vector; FL, full length; GAL UAS, galactose upstream activating sequence; GFP, green fluorescent protein; GST, glutathione S-transferase; IP, immunoprecipitation; mut, mutant; NO, nitric oxide; nts, nucleotides; OD, optical density; OE, overexpression; ORF, open reading frame; PRE, Puf recognition element;  $\Delta$ puf1-5, quintuple Puf mutant; qPCR, quantitative PCR; RD, repeat domain; RT, reverse transcriptase;  $T_{1/2}$ , half-life; TAP, tandem affinity purification; UTR, untranslated region; WT, wild type.

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multiple environmental signals. Transcriptional up-regulation occurs in response to both NO exposure and the available sugar source through independent transcription factor mechanisms, and Yhb1p levels have been shown to decrease immediately upon the addition of glucose (Zhu *et al.*, 2006). Within yeast cells, mitochondrial cytochrome oxidase produces NO, and the amount of NO production is dependent on the amount of respiration in the cell (Li *et al.*, 2011). The addition of glucose limits the need for respiration in yeast cells, which limits NO production and the need for Yhb1p. In addition to transcriptional control, regulation of mRNA decay and/or translation rates offers a rapid response to such environmental stresses.

One class of eukaryotic RNA-binding proteins involved in environmental responses by regulating mRNA decay and translation is the Puf protein family (Wickens *et al.*, 2002; Miller and Olivas, 2011). Puf proteins have a diverse set of roles, including stem cell maintenance (Forbes and Lehmann, 1998; Parisi and Lin, 1999; Crittenden *et al.*, 2002; Moore *et al.*, 2003), development and differentiation, (Murata and Wharton, 1995), neuronal plasticity (Menon *et al.*, 2004), and stress response (Foat *et al.*, 2005; Miller *et al.*, 2014). At the molecular level, Puf proteins repress mRNAs by interacting with sequence elements typically located in the 3' UTR of target mRNAs. Once bound to an mRNA, Puf proteins elicit repression either through protein interactions that inhibit cap-binding events of translation initiation or interactions with mRNA decay machinery to stimulate deadenylation and decapping steps of decay (Goldstrohm *et al.*, 2006, 2007; Lee *et al.*, 2010; Miller and Olivas, 2011).

The Puf family is characterized by a conserved RNA-binding domain consisting of eight imperfect repeats of a 36-amino acid sequence plus short flanking regions. Crystal structure analysis of multiple Puf-mRNA complexes revealed a primarily modular binding method in which conserved amino acids within each repeat contact and stack with successive bases along the RNA, although binding specificity and flexibility involve some RNA bases flipping out from the protein binding surface (Edwards *et al.*, 2001; Wang *et al.*, 2001, 2002, 2009; Miller *et al.*, 2008; Zhu *et al.*, 2009). The conserved Puf recognition element (PRE) contains a UGU sequence followed by an AU-rich region, although different Pufs bind specific variants of the AU-rich region (Murata and Wharton, 1995; Wreden *et al.*, 1997; Zamore *et al.*, 1997, 1999; Souza *et al.*, 1999; Nakahata *et al.*, 2001; Tadauchi *et al.*, 2001; Wang *et al.*, 2001, 2002; Gerber *et al.*, 2004; Jackson *et al.*, 2004).

*S. cerevisiae* contains six Puf proteins (Puf1p–Puf6p). All except Puf2p have been shown to stimulate mRNA decay and/or repress translation via 3' UTR interaction (Olivas and Parker, 2000; Tadauchi *et al.*, 2001; Gu *et al.*, 2004; Goldstrohm *et al.*, 2006; Hook *et al.*, 2007; Ulbricht and Olivas, 2008). The function of several bona fide and putative targets of Puf regulation in yeast relates to stress response (Foat *et al.*, 2005; Garcia-Rodriguez *et al.*, 2007; Miller *et al.*, 2014). For example, our previous work demonstrated that Puf3p stimulates mRNA decay of nucleus-encoded mitochondrial transcripts in fermentative growth conditions (in glucose) when mitochondrial respiration is not required. However, Puf3p activity is inhibited in ethanol, galactose, and raffinose conditions that use mitochondria, thereby stabilizing the mitochondrial transcripts for increased translation. The response to carbon source by Puf3p is rapid and not due to altered Puf3p expression (Miller *et al.*, 2014). The ability of other Pufs to respond to stress by altering regulatory capacity for target mRNAs was unknown.

In this work, we evaluate *YHB1* mRNA as a target of condition-specific Puf-mediated decay stimulation in yeast. *YHB1* was originally identified in a microarray analyzing altered RNA levels in a yeast strain deleted of *PUFs* 1–5 (Olivas and Parker, 2000). The *YHB1*

3' UTR contains two overlapping PREs that could potentially be used by multiple Puf proteins, although not simultaneously. We determined that Puf1p, Puf4p, and Puf5p all play a role in stimulating *YHB1* mRNA decay through these two overlapping PREs. This Puf-mediated decay stimulation is inhibited under stress conditions when Yhp1p production is required, with reduced Puf5p-binding activity contributing to decay inhibition. However, overexpression of Pufs can override such inactivation, and Puf overexpression during NO exposure can destabilize *YHB1* mRNA, resulting in decreased cell growth. Taken together, these results advance our understanding of the multifaceted mechanisms controlling *YHB1* mRNA stability and cell fitness in rapid response to stress.

## RESULTS

### *YHB1* mRNA is destabilized by multiple Puf proteins

The yeast *YHB1* transcript was first identified as a potential target of Puf protein-mediated decay stimulation based on its increased expression in a Puf deletion strain ( $\Delta puf1-5$ ) versus a wild-type strain (Olivas and Parker, 2000). *YHB1* mRNA contains two overlapping candidate PREs located in its 3' UTR (Figure 1A). To evaluate *YHB1* mRNA for control by Puf proteins, we performed decay analysis in a wild-type *PUF* strain (WT), strains deleted of individual *PUFs* 1–5, or a quintuple mutant ( $\Delta puf1-5$ ). To ascertain the direct role of Puf protein regulation through the *YHB1* 3' UTR, we created a reporter vector in which the 3' UTR of *YHB1* is cloned downstream of the stable coding region of *PGK1*. Prior studies showed that the 3' UTRs of Puf-regulated targets are sufficient to confer rapid decay to otherwise stable mRNAs (Jackson *et al.*, 2004). Half-lives were determined after transcriptional repression by two simultaneous methods. First, strains containing the temperature-sensitive RNA polymerase II allele (*rpb1-1*) were used to inhibit transcription by shifting to a nonpermissive temperature (Herrick *et al.*, 1990). Second, promoter-specific transcriptional repression of the *PGK1/YHB1* 3' UTR reporter under the control of the *GAL* UAS was achieved by shifting from galactose to dextrose in the media (Decker and Parker, 1993).

In WT yeast, the *PGK1/YHB1* 3' UTR reporter mRNA decayed with a half-life of  $10.7 \pm 1.3$  min (Figure 1B). In the *puf2Δ* and *puf3Δ* strains, the reporter half-life was similar to that of WT; however, in the *puf1Δ*, *puf4Δ*, and *puf5Δ* strains, the reporter half-life was significantly extended by 1.5- to 2-fold, suggesting that Pufs 1, 4, and 5 all contribute to the destabilization of *YHB1* mRNA (Figure 1B). To evaluate the full extent of Puf destabilization on our reporter, we determined the half-life in the  $\Delta puf1-5$  strain to be  $24.3 \pm 2.2$  min (Figure 1B). Both the *puf4Δ* and *puf5Δ* strains showed shorter half-lives than  $\Delta puf1-5$ , suggesting that Puf4p and Puf5p do not act redundantly and the level of active Pufs may be limiting the decay rate of *YHB1* mRNA. These data also indicate that Puf4p and Puf5p contribute equally to decay stimulation of *YHB1* mRNA, whereas Puf1p plays less of a role.

### A single flexible binding site is required for Puf1p, Puf4p, and Puf5p destabilization of *YHB1* mRNA

Previously identified targets of multiple Puf regulation, such as *HO*, *HXX1*, and *TIF1* mRNAs, contain multiple, nonoverlapping PREs in their 3' UTRs (Hook *et al.*, 2007; Ulbricht and Olivas, 2008). In contrast, whereas the *YHB1* mRNA 3' UTR is also regulated by multiple Pufs, it potentially contains two overlapping PREs, such that only one Puf protein could bind this region at one time. To determine whether one or both PREs are necessary for Puf-mediated decay, we mutated the conserved UGU element within each PRE, which has been shown to be required for Puf binding (Jackson *et al.*, 2004; Ulbricht and Olivas, 2008). If a particular UGU element is necessary

A.

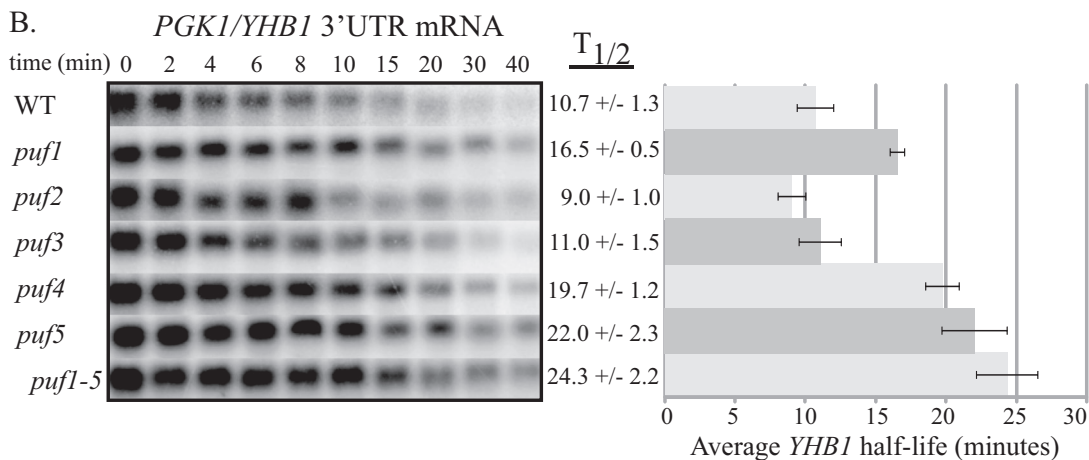
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 stop  
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AGACCAUUGC UAAUCUUU UGUUGUUAUACUG UGUUAGAAAUCCUCGUGAAAUCCUUG

UGAAAUCCUAUGUGGAAUAUUUAGAUAGUAAGUAAAGAUGUGUAUGUAUUUAUAAGA  
 site #1 site #2

UGAAUAAGCGCCAGGUGCUAAACAGAGUUCACUUCACGUUAACGCCAGUCCAAUUUU

UGAUUGCAUUC CCGUUUUU



**FIGURE 1:** Puf1p, Puf4p, and Puf5p regulate *YHB1* mRNA stability. (A) Sequence of the *YHB1* 3' UTR. Canonical binding sequences of Puf proteins are in bold. Underlined regions are candidate sites of Puf interaction (site #1 and #2). UGUN sequences are shaded gray. (B) Decay of *PGK1/YHB1* 3' UTR fusion mRNA in wild-type (WT), individual *PUF* deletion, and  $\Delta puf1-5$  yeast strains grown to an  $OD_{600}$  of 1.0 in galactose, then transcription repressed at time 0 with addition of dextrose. Left, representative Northern blots, with average half-life ( $T_{1/2}$ ) listed to the right of each blot. Right, graphical representation of  $T_{1/2}$ . Minutes after transcriptional shut-off are indicated above blots and along the x-axis. The error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ).

for Puf-mediated decay, its mutation will extend the mRNA's half-life in a wild-type *PUF* strain. To test this hypothesis, we either mutated the first UG in the first PRE while leaving the second PRE intact (site #1 mutant) or mutated the UGU elements in both PREs (site #2 mutant; Figure 2A). We did not make mutations in the second UGU alone because this would also disrupt the first PRE. The site #1 mutant mRNA, *PGK1/yhb1-1*, decayed with a half-life 1.5-fold longer than the WT mRNA, suggesting that disruption of the first PRE inhibits optimal regulation of the reporter, although the second PRE can still function to mediate decay in the absence of the first PRE (Figure 2B). The site #2 mutant mRNA, *PGK1/yhb1-2*, decayed with a half-life 2.4-fold longer than the WT mRNA, similar to the half-life in the  $\Delta puf1-5$  strain, indicating that all Puf-mediated decay stimulation acts through these overlapping PREs. Unlike other known targets of multiple Puf decay regulation, *YHB1* mRNA is unique because only one binding site containing overlapping PREs is necessary and sufficient to confer destabilization by three Puf proteins.

To evaluate whether differential RNA binding contributes to the differential roles of Puf1p, Puf4p, and Puf5p in decay stimulation, as well as the decreased decay stimulation with the mutant sites, we performed *in vitro* binding and gel mobility shift assays. Glutathione S-transferase (GST)-tagged Puf proteins were purified from *Escherichia coli* and incubated with radiolabeled RNA encompassing the *YHB1* 3' UTR PRE region (33 nucleotides [nts]) with the wild-type, site #1 mutant, or site #2 mutant sequence (Figure 2A). The resulting complexes were separated on a native polyacrylamide gel. As

shown in Figure 2C and graphically in Figure 2D, Pufp + RNA complex formation was reduced with the site #1 mutant RNA relative to wild-type and even further reduced with the site #2 mutant RNA for all three Puf proteins, with Puf5p binding being most dramatically reduced. Thus decreased decay stimulation of the mutant sites correlates with decreased binding capacity of the Puf proteins. To compare binding between the Puf proteins, we incubated increasing concentrations of Puf proteins with either the wild-type or site #1 radiolabeled RNA sequence and analyzed complexes on native polyacrylamide gels. As shown in Figure 2E, relative levels of Puf5p binding with wild-type RNA was significantly larger than with Puf1p or Puf4p, with Puf1p showing the lowest levels of binding. This pattern of differential binding was also seen with the site #1 RNA, but the relative differences between the Pufs were not as large. These experiments support the idea that differential binding capacity between Puf1p, Puf4p, and Puf5p likely contributes to their differential roles in decay stimulation, especially for the lesser role of Puf1p, although Puf4p appears to have a greater role in decay than its binding capacity would suggest.

#### Overexpression of Puf4p or Puf5p enhances decay of *YHB1* mRNA

To determine whether overexpression of Puf proteins can increase the decay rate of *PGK1/YHB1* 3' UTR mRNA, we coexpressed our reporter construct with Puf overexpression constructs consisting of either the repeat domain (RD) or full-length (FL) Puf protein in the

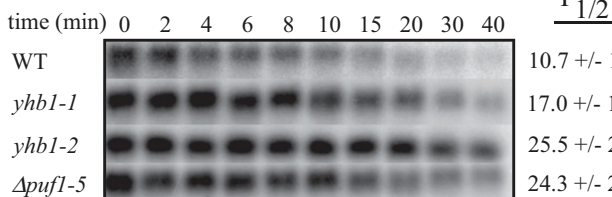
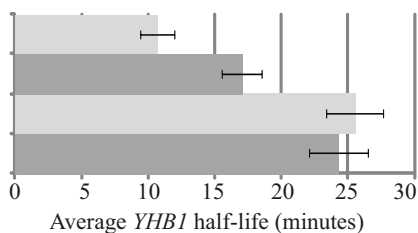
A.

*YHB1* 3'UTR Puf recognition element

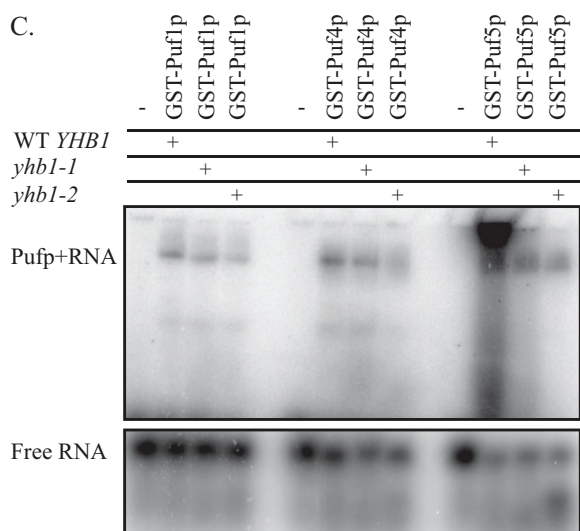
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site #1 mutant (*yhb1-1*)site #2 mutant (*yhb1-2*)UAG...GUAAGAUUG **ACU**UAUGUAUUUAAGAUGAAUAUAG...GUAAGAUUG **ACAACA**AUUUAAGAUGAAUA

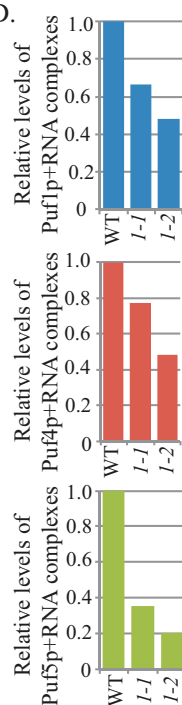
B.

*PGK1/YHB1* 3'UTR mRNA $T_{1/2}$ 

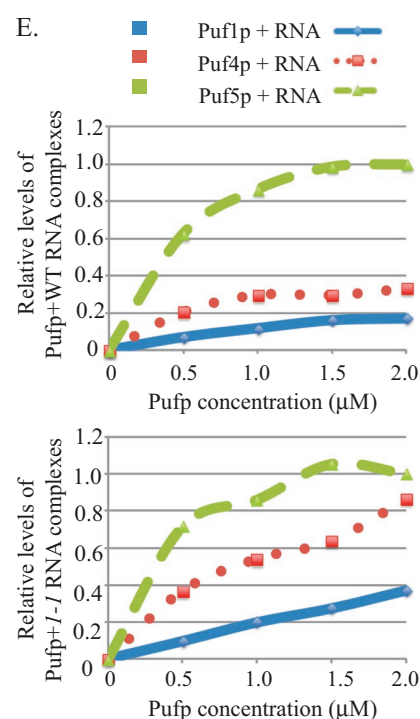
C.



D.



E.

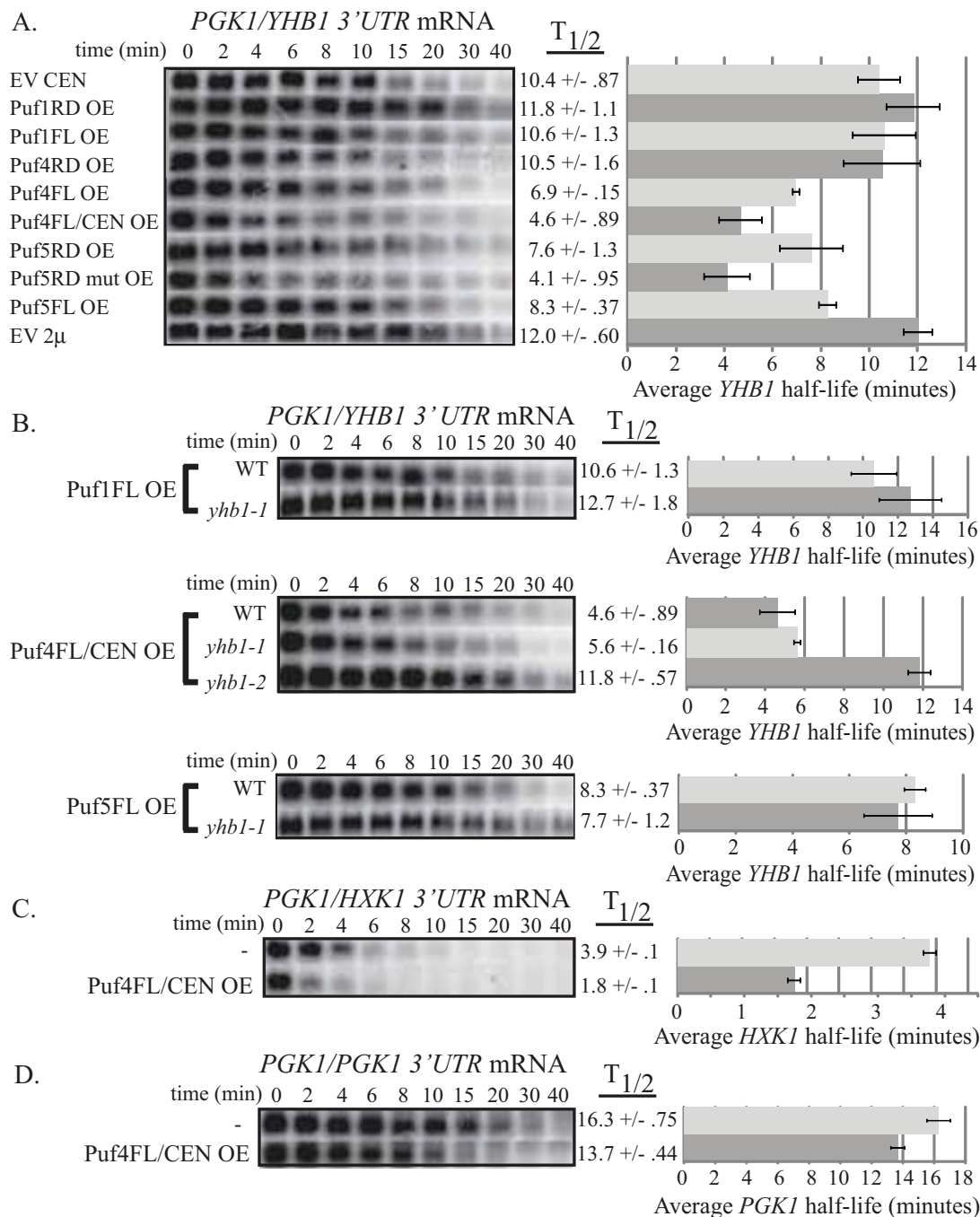


**FIGURE 2:** A single, flexible Puf recognition element in the *YHB1* 3' UTR is required for Puf1p, Puf4p, and Puf5p regulated decay and binding. (A) Sequence of the Puf recognition element (bolded and underlined) in wild-type *YHB1* 3' UTR. Left, mutation of the first UGU to ACU, site #1 mutant, (*yhb1-1*); right, mutation of both UGU elements to ACA, site #2 mutant, (*yhb1-2*). Mutated sequences are boxed. (B) Decay of WT, *yhb1-1* mutant, and *yhb1-2* mutant *PGK1/YHB1* 3' UTR fusion mRNA in WT yeast or WT *PGK1/YHB1* 3' UTR in  $\Delta$ *puf1-5* yeast. Left, representative Northern blots, with average half-life ( $T_{1/2}$ ) listed to the right of each blot. Right, graphical representation of  $T_{1/2}$ . Minutes after transcriptional shut-off are indicated above blots and along the x-axis. The error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ). (C) Representative gel mobility shift assay of the WT *YHB1*, *yhb1-1* mutant, or *yhb1-2* mutant RNA sequence shown in A in the presence or absence of 1.5  $\mu$ M GST-Puf1p, GST-Puf4p, or GST-Puf5p. Positions of free radiolabeled RNA and RNA bound to a Puf protein (Pufp + RNA) are indicated. (D) Graphical representation of the data from C, with relative levels of Pufp + RNA complexes for each Puf protein on the y-axis. Data are an average of two experiments. (E) Graphical representation of gel mobility shift assays in which increasing concentrations of Puf proteins were incubated with either WT RNA (top) or *yhb1-1* mutant RNA (bottom). Relative levels of Pufp + RNA complexes are indicated on the y-axis, with Puf protein concentration on the x-axis. Data are an average of two experiments.

WT strain. All FLAG-tagged constructs were validated for expression by Western blot analysis (Supplemental Figure S1). As expected from the deletion analysis, overexpression of Puf2p or Puf3p had no effect on the decay of our reporter (Supplemental Figure S2). Unexpectedly, overexpression of either Puf1RD or Puf1FL had no effect

on decay (Figure 3A), suggesting that Puf1p cannot enhance the normal decay stimulation by Pufs on this mRNA, likely due to its inferior binding. In contrast, overexpression of Puf4FL, Puf4FL/CEN vector, Puf5FL, or Puf5RD enhanced decay of the reporter, with Puf4FL/CEN having the greatest effect by decreasing the half-life





**FIGURE 3:** Overexpression of Puf4p or Puf5p in WT yeast stimulates a more rapid decay of target mRNAs. (A) Decay of WT *PGK1/YHB1* 3' UTR fusion mRNA in WT yeast coexpressed with empty vector (EV CEN or EV 2  $\mu$ ), Puf1 full-length (FL), Puf1 repeat domain (RD), Puf4FL, Puf4RD, Puf4FL/CEN, Puf5FL, Puf5RD, or Puf5RD mutant (mut). Left, representative northern blots, with average half-life ( $T_{1/2}$ ) listed to the right of each blot. Right, graphical representation of  $T_{1/2}$ . Minutes after transcriptional shut-off are indicated above blots and along the x-axis. Overexpression (OE) constructs used are indicated to the left of the blots. (B) Decay of WT *PGK1/YHB1*, site #1 mutant (*yhb1-1*), or site #2 mutant (*yhb1-2*) in the presence of Puf1FL, Puf4FL/CEN, or Puf5FL overexpression. (C) Decay of *PGK1/HXK1* 3' UTR fusion mRNA in the absence (-) or presence of Puf4FL/CEN. (D) Decay of *PGK1/PGK1* 3' UTR fusion mRNA in the absence (-) or presence of Puf4FL/CEN. For A, B, and D, the error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ). For C, the error for each  $T_{1/2}$  is the SEM ( $n = 2$ ).

greater than twofold (Figure 3A). These data indicate that the levels of Puf4p and Puf5p normally limit the decay rate of *YHB1* mRNA and/or these Pufs can outcompete wild-type levels of Puf1p for binding/decay stimulation. It is unclear why Puf4RD did not enhance decay. It is possible that sequences outside the repeat domain are

important for activity. Alternatively, all overexpression constructs were made in a 2  $\mu$  vector except the Puf4FL/CEN, which was made in a CEN vector, and this Puf4FL/CEN showed greater activity in enhancing decay than Puf4FL from the 2  $\mu$  vector. Although both the CEN and 2  $\mu$  vectors use the constitutive GPD promoter to

express Puf4FL, it is possible that expression from the high-copy 2  $\mu$  vector adversely affects Puf activity, perhaps through aggregation of the larger pool of Puf proteins. In contrast, the low-copy CEN vector may overexpress enough Puf4FL to enhance decay but not so much as to aggregate. Moreover, Puf4RD is expressed approximately sevenfold higher than Puf4FL from the 2  $\mu$  vector (and approximately threefold higher than Puf5RD), which may contribute to its further aggregation and loss of activity (Supplemental Figure S1). During the construction of the Puf5RD overexpression vector, a mutant was randomly generated during PCR at residue 177 of Puf5p, with asparagine changed to aspartic acid. This residue is directly downstream of a known phosphorylated residue at 176 (Bodenmiller *et al.*, 2010; PhosphoPep). Overexpression of this Puf5RD mutant decreased the half-life of our reporter greater than twofold, showing stronger activity than the wild-type Puf5RD protein (Figure 3A). Because expression of this mutant was not significantly different from the wild-type Puf5RD (Supplemental Figure S1), this result suggests that the local charge and perhaps phosphorylation in this region enhance Puf5 activity.

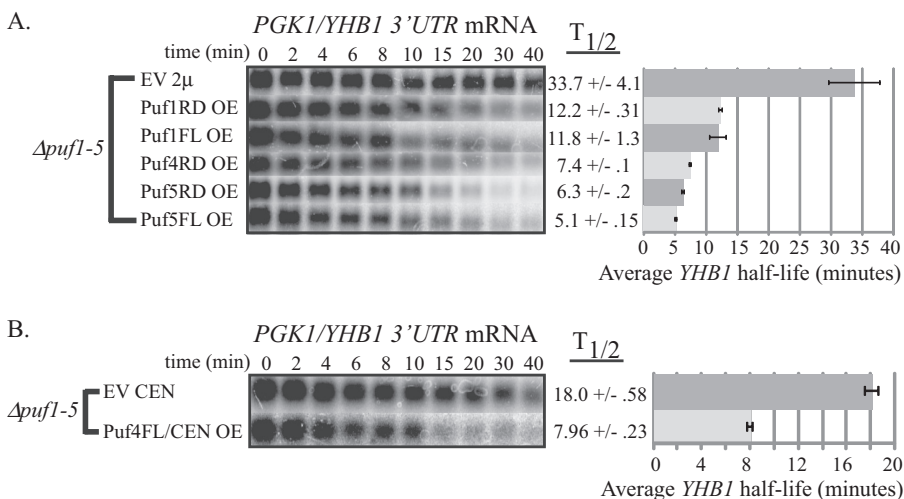
### Overexpression of Puf4p requires a functional PRE to enhance decay

We next sought to determine whether the enhanced decay resulting from the overexpression of Puf proteins specifically acts through a functional PRE. Using our mutant reporter constructs, we performed transcriptional repression assays in the presence of Puf1FL, Puf4FL/CEN, or Puf5FL overexpression and evaluated reporter half-life. With the site #1 mutant (*yhb1-1*), which normally decays with a half-life of  $17.0 \pm 1.5$  min (Figure 2B), both Puf4FL/CEN and Puf5FL could stimulate rapid decay to the same extent as with the wild-type *YHB1* 3' UTR (Figure 3B). These results indicate that Puf4p or Puf5p overexpression facilitates enhanced decay by using the second UGU element. Puf1FL overexpression also rescued decay of the *yhb1-1* mRNA to near-WT *YHB1* levels. Thus, whereas Puf1FL

overexpression cannot enhance decay beyond the endogenous rate, it can facilitate decay of the mutant through the second UGU site. In contrast, when both UGU sites are mutated (*yhb1-2*), Puf4FL/CEN overexpression could not fully rescue decay, as the mutant *yhb1-2* showed a nearly threefold increase in half-life compared with WT (Figure 3B). These data suggest that the enhanced decay mediated by Puf overexpression depends on binding to a functional PRE. We also examined *HXK1* mRNA, a second known target destabilized by Puf1p, Puf4p, and Puf5p (Ulbricht and Olivas, 2008). In the presence of Puf4FL/CEN overexpression, the half-life of the *PGK1/HXK1* 3' UTR construct was decreased twofold, indicating that the enhanced decay facilitated by overexpression of Puf4p is not limited to a single target mRNA (Figure 3C). Whereas Puf4FL/CEN overexpression was unable to enhance decay of the *yhb1-2* mutant to the level of the WT construct, the decay of the *yhb1-2* mutant was still faster in the presence of Puf4FL/CEN overexpression than with endogenous levels of Puf proteins. It is possible that increased levels of Puf4p may allow for use of other UGU elements in the *YHB1* 3' UTR that are not consensus PREs (Figure 1A). To examine off-target effects of Puf4FL/CEN overexpression, we examined the decay rate of a *PGK1/PGK1* 3' UTR construct that is not under Puf protein regulation and contains no 3' UTR UGU elements (Ulbricht and Olivas, 2008). Puf4FL/CEN overexpression did not substantially decrease the half-life of this nonspecific target, indicating that enhanced decay by Puf overexpression is specific to target mRNAs (Figure 3D).

### Expression of Puf1p, Puf4p, or Puf5p rescues decay of *YHB1* mRNA in the absence of other Pufs

To evaluate the ability of individual Puf proteins to stimulate *YHB1* mRNA decay in the absence of other Puf proteins, we performed individual Puf rescue studies in the  $\Delta$ *puf1-5* strain by coexpressing our reporter construct with constructs containing the full-length or repeat domain of Pufs 1, 4, or 5. In the absence of *PUF* 1-5, exogenous overexpression of Puf1FL or its RD was able to rescue decay to levels similar to the  $10.7 \pm 1.3$  min half-life seen in the wild-type *PUF* strain (Figure 4A). Thus, whereas Puf1p overexpression was not able to enhance decay faster than wild-type rates when other Puf proteins are present, it is able to facilitate decay of *YHB1* on its own. Moreover, the repeat domain of Puf1p is sufficient for this activity. Exogenous expression of either Puf4FL or its RD, or Puf5FL or its RD, was able to rescue decay to levels faster than that seen in the wild-type *PUF* strain (Figure 4, A and B). These data illustrate that either the full-length or the repeat domain of Puf4p or Puf5p is individually sufficient to facilitate enhanced decay of *YHB1*, demonstrating no need for different Pufs to act synergistically when overexpressed and further supporting the idea that endogenous levels of Puf proteins are limiting *YHB1* decay. Even alone, Puf4p and Puf5p are more active than Puf1p, likely due to their enhanced binding capacity. It is interesting that the Puf4RD facilitated enhanced decay in the absence of other Pufs, whereas it did not enhance the endogenous rate of decay in the presence of other Pufs. The Puf4RD



**FIGURE 4:** Expression of Puf1p, Puf4p, or Puf5p or the corresponding repeat domain (RD) is sufficient to rescue decay of *YHB1* mRNA in a  $\Delta$ *puf1-5* strain. (A) Decay of WT *PGK1/YHB1* fusion mRNA in a  $\Delta$ *puf1-5* strain coexpressed with EV 2  $\mu$ , Puf1FL, Puf1RD, Puf4RD, Puf5FL, or Puf5RD. Left, representative northern blots, with average half-life ( $T_{1/2}$ ) listed to the right of each blot. Right, graphical representation of  $T_{1/2}$ . Minutes after transcriptional shut-off are indicated above blots and along the x-axis. Overexpression (OE) constructs used are indicated to the left of the blots. (B) Decay of WT *PGK1/YHB1* fusion mRNA in a  $\Delta$ *puf1-5* strain coexpressed with EV CEN or Puf4FL/CEN. For A, the error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ) except for EV 2  $\mu$ , for which  $T_{1/2}$  is the SEM ( $n = 2$ ). For B, the error for Puf4FL/CEN  $T_{1/2}$  is the SEM ( $n \geq 3$ ) and the error for EV CEN  $T_{1/2}$  is the SEM ( $n = 2$ ).

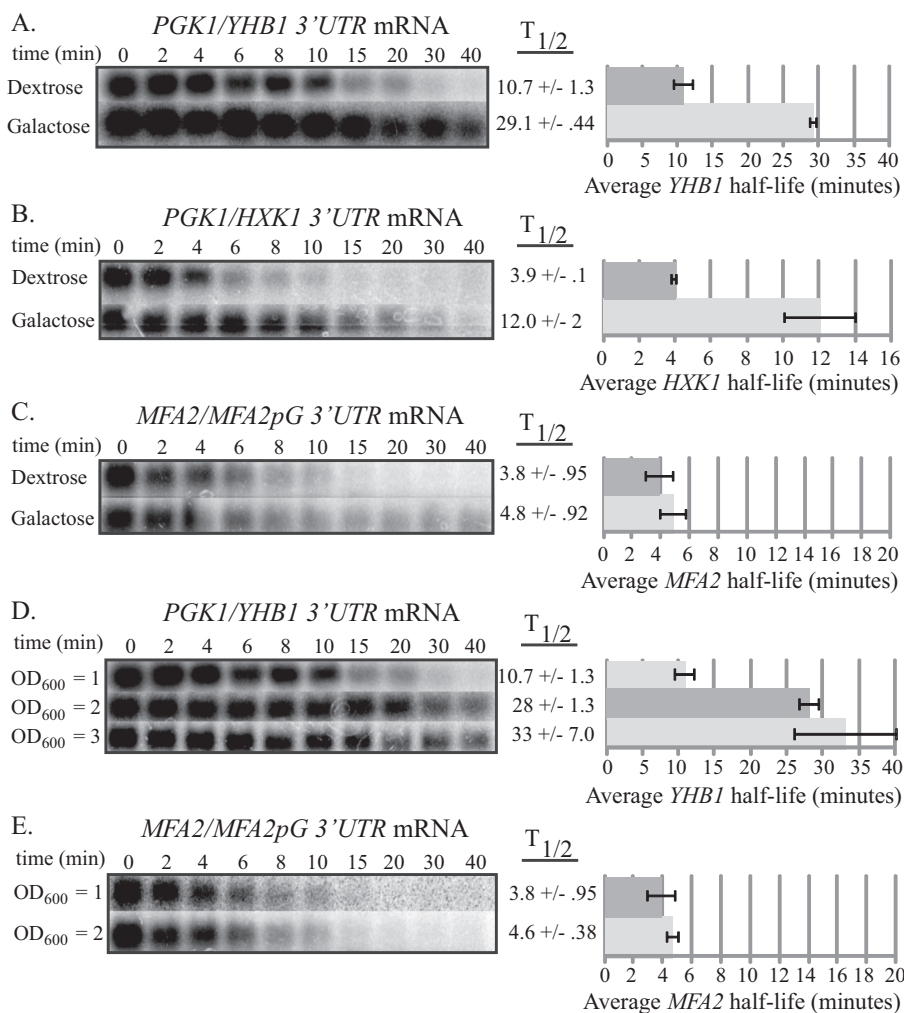
that is available for binding (and not potentially aggregated) may be less able to compete with endogenous Puf4p and Puf5p for RNA binding.

### Stimulation of *YHB1* mRNA decay by Puf proteins is dependent on the available carbon source

Steady-state expression profiles of mRNAs containing putative PREs show altered expression levels in response to carbon source, suggesting that Puf protein regulation of these mRNAs is dependent on the available carbon source (Foat et al., 2005). Yhb1p levels have been shown to decrease immediately upon glucose addition (Zhu et al., 2006). This result is consistent with reduced mitochondrial respiration in the presence of glucose, which would decrease the production of NO and the need for Yhb1p. Posttranscriptional regulation of *YHB1* mRNA decay could contribute to such rapid changes in protein production.

To assess the effects of carbon source on Puf-mediated decay of *YHB1* mRNA, we performed transcriptional repression assays of our

reporter constructs using only temperature shift to mediate repression in the continual presence of galactose, as compared with all of our prior analyses of *YHB1* decay, in which dextrose was added at the time of transcriptional repression. In the presence of continual galactose, the *YHB1* half-life was extended threefold from that found in dextrose (Figure 5A), similar to the half-life of the  $\Delta puf1-5$  strain. This result suggests that Puf-mediated decay is inhibited in galactose. To further examine the effect of carbon source on a target of Puf1p, Puf4p, and Puf5p destabilization, we examined our *HXX1* reporter. Similar to *YHB1*, the *HXX1* reporter showed a fourfold extension of half-life in the presence of galactose as compared with dextrose (Figure 5B). To verify that this extension is not a global effect on mRNA decay and confirm that transcriptional repression of the *GAL* UAS is complete with only temperature shift, we evaluated the *MFA2/MFA2pG* 3' UTR reporter, which is not under the control of Puf proteins. This reporter decayed similarly in both dextrose and galactose conditions, suggesting that changes in carbon source do not affect global mRNA decay (Figure 5C). This negative control also



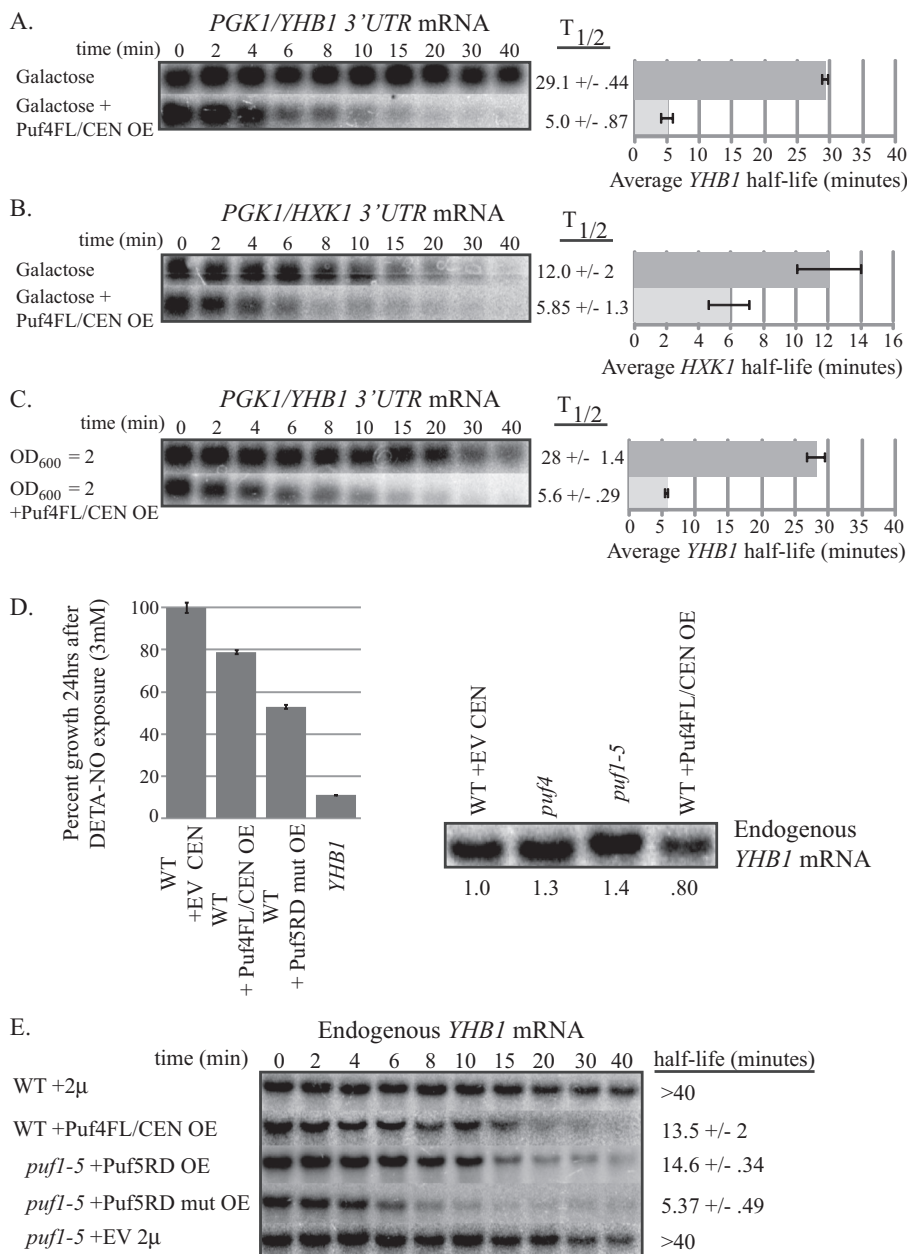
**FIGURE 5:** Regulation of *YHB1* mRNA stability by multiple Puf proteins is dependent on environmental conditions. Decay of *PGK1/YHB1* 3' UTR fusion mRNA (A), *PGK1/HXX1* 3' UTR fusion mRNA (B), or *MFA2/MFA2pG* 3' UTR fusion mRNA (C) in cultures grown in dextrose or galactose carbon sources. Decay of *PGK1/YHB1* 3' UTR fusion mRNA (D) or *MFA2/MFA2pG* 3' UTR fusion mRNA (E) grown to different final culture densities. Left, representative Northern blots, with average  $T_{1/2}$  listed to the right of each blot. Right, graphical representation of  $T_{1/2}$ . Minutes after transcriptional shut-off are indicated above blots and along the x-axis. For A–E, the error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ).

eliminates the possibility that the *GAL* UAS promoter is leaky when performing temperature-mediated transcriptional repression in the presence of galactose. In addition, the *YHB1* half-life was similar in the  $\Delta puf1-5$  strain in galactose or dextrose conditions, mirroring galactose conditions in a WT strain (Supplemental Figure S2). These data suggest that decay stimulation by Puf1p, Puf4p, and Puf5p is inhibited in respiration conditions (galactose) and active in fermentation conditions (dextrose). The results also imply that the switch from galactose to dextrose performed in all of our prior transcriptional repression assays leads to a very rapid activation of Puf-mediated decay. Given the rapid activation of Puf activity upon dextrose addition at the time of transcriptional repression, we hypothesize that a posttranslational mechanism such as phosphorylation is responsible for changes in Puf activity. Such mechanisms may affect protein localization or mRNA binding affinity.

### Stimulation of *YHB1* mRNA decay by Puf proteins is dependent on the culture density

Global expression studies suggest that culture density may also alter Puf regulation (Foat et al., 2005). To test this possibility, we performed transcriptional repression assays at elevated  $OD_{600}$  values as compared with an  $OD_{600}$  of 1, which was used in our prior analyses. In cell density conditions with  $OD_{600}$  of 2 and 3, we observed an extension of half-life of  $\geq 3$ -fold, similar to that observed in galactose conditions (Figure 5D). To control for any global effects of elevated  $OD_{600}$  on mRNA decay, we examined the *MFA2/MFA2pG* reporter and observed identical half-lives at  $OD_{600}$  of 1 and 2, indicating that the increase in mRNA stability is specific to targets under the control of Puf proteins (Figure 5E).





**FIGURE 6:** Overexpression of Puf proteins bypasses conditional regulation of *YHB1* mRNA stability. Decay of *PGK1/YHB1* 3' UTR fusion mRNA (A) or *PGK1/HXK1* 3' UTR fusion mRNA (B) in galactose in the absence or presence of Puf4FL/CEN overexpression. Left, representative Northern blots, with average  $T_{1/2}$  listed to the right of each blot. Right, graphical representation of  $T_{1/2}$ . Minutes after transcriptional shut-off are indicated above blots and along the x-axis. (C) Decay of *PGK1/YHB1* 3' UTR fusion mRNA at OD<sub>600</sub> of 2 in the absence or presence of Puf4FL/CEN overexpression. (D) Left, percentage cell growth of WT yeast expressing EV CEN, Puf4FL/CEN, or Puf5RD mut or *yhb1* $\Delta$  yeast 24 h after exposure to 3 mM DETA-NO in galactose conditions. Percentage growth is normalized to the growth of strains in the absence of DETA-NO. Right, Northern blot analysis of endogenous, steady-state *YHB1* mRNA levels in WT yeast expressing EV CEN or Puf4FL/CEN or *puf4* $\Delta$  or  $\Delta$ *puf1-5* yeast. Levels of *YHB1* mRNA relative to the level in WT yeast expressing EV CEN and normalized for loading against scRI RNA are shown below each lane. (E) Decay of endogenous *YHB1* mRNA. Representative northern blots are shown, with the average  $T_{1/2}$  listed to the right of each blot. Yeast strain and expression vectors are indicated to the left of the blots. Minutes after transcriptional shut-off are indicated above the blots. For A–C, the error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ). For D, the error is the SEM ( $n \geq 3$ ). For E, the error for each  $T_{1/2}$  is the SEM ( $n \geq 3$ ).

### Overexpression of Puf proteins abrogates condition-specific inhibition of mRNA decay

To investigate whether overexpression of Puf protein levels can rescue Puf-mediated decay of target mRNAs in normally inactivating conditions, we first performed transcriptional repression assays in continual galactose in the presence of Puf4FL/CEN overexpression. The *YHB1* reporter decayed rapidly, resulting in a half-life sixfold shorter than without Puf4FL/CEN overexpression (Figure 6A). Similar results were observed for Puf5RD overexpression (Supplemental Figure S2). To validate this phenomenon with other Puf targets, we analyzed decay of our *HXK1* reporter. Again, rapid decay was rescued in continual galactose when Puf4FL/CEN was overexpressed (Figure 6B). We next examined whether the inhibition of Puf-mediated decay at elevated culture density could be overcome by Puf overexpression. At the elevated OD<sub>600</sub> of 2, Puf4FL/CEN overexpression resulted in a fivefold shorter half-life than without overexpression. These results suggest that the condition-specific Puf inactivation signal is limiting in cells (Figure 6C).

The regulation of *YHB1* expression occurs at both transcriptional and posttranscriptional levels and by multiple environmental signals. Transcriptional up-regulation occurs in response to both NO exposure and the available carbon source through independent transcription factor mechanisms (Zhu *et al.*, 2006). We have shown that Puf1p, Puf4p, and Puf5p destabilize *YHB1* mRNA posttranscriptionally in a carbon source- and culture density-dependent manner. To evaluate the biological relevance of Puf regulation, we examined the effects of altering *YHB1* mRNA decay on cell fitness when exposed to exogenous NO. Previous studies showed that a *yhb1* $\Delta$  strain exhibits severe growth defects when exposed to 3 mM DETA NONOate (Liu *et al.*, 2000). We hypothesized that overexpression of Puf4p would lead to a decrease in the pool of Yhb1p needed to combat exogenous NO stress, resulting in a growth defect. We first recapitulated the severe growth defect observed by (Liu *et al.*, 2000) in a *yhb1* $\Delta$  strain exposed to 3 mM DETA NONOate (Figure 6D). As predicted, overexpression of Puf4FL/CEN resulted in a significant reduction in growth upon DETA NONOate exposure (Figure 6D). Furthermore, overexpression of the hyperactive mutant Puf5RD during DETA NONOate exposure resulted in a stronger growth defect



than for Puf4FL/CEN (Figure 6D). Analysis of steady-state levels of endogenous *YHB1* mRNA showed reduced levels with Puf4FL/CEN overexpression and increased levels in a *puf4Δ* or *Δpuf1-5* strain compared with wild type (Figure 6D). Finally, we evaluated endogenous *YHB1* mRNA decay in response to Puf overexpression. In a wild-type strain with empty vector, the half-life of endogenous *YHB1* mRNA was >40 min (Figure 6E). In contrast, Puf4FL/CEN overexpression in the wild-type strain showed a half-life of  $13.5 \pm 2$  min. Overexpression of Puf5RD in the *Δpuf1-5* strain similarly increased decay from a >40 min half-life to  $14.6 \pm 0.34$  min. To gain insight into our hyperactive Puf5RD mutant, we overexpressed it in the *Δpuf1-5* strain and observed a threefold decrease in half-life beyond that observed with the wild-type Puf5RD (Figure 6E). This hyperactivity likely contributes to the stronger growth defect observed during DETA NONOate exposure compared with Puf4FL (Figure 6D). These data demonstrate that altered mRNA levels resulting from changes to *YHB1* decay by Puf proteins influence the cell's response to toxic NO.

### Puf protein-inactivating condition alters Puf5p mRNA binding

To begin to dissect the mechanism by which Puf1p, Puf4p, and Puf5p activity is altered by conditions, we first examined whether the expression levels of these Puf proteins are altered in different carbon sources. We used endogenously tandem affinity purification (TAP)-tagged Puf strains to evaluate steady-state Puf protein levels in cells grown in dextrose and galactose. Puf1p, Puf4p, and Puf5p levels did not change between dextrose and galactose conditions, eliminating the possibility that decreased Puf activity in galactose is due to decreased protein levels (Figure 7A). This result supports our hypothesis that changes in Puf activity are due to a posttranslational mechanism. Next we investigated condition-specific localization of Puf1p-green fluorescent protein (GFP), Puf4p-GFP, and Puf5p-GFP. No changes in localization of Puf1p, Puf4p, or Puf5p were observed between dextrose and galactose conditions, eliminating altered localization as being responsible for Puf inactivation in galactose (Figure 7B). Finally, we assessed whether mRNA binding was inhibited in galactose conditions. We used quantitative PCR (qPCR) to quantitate the amount of mRNA that copurified with TAP-tagged Puf1p, Puf4p, and Puf5p from cells grown in dextrose or galactose. Both *YHB1* and *HXX1* mRNAs were analyzed as positive binding targets, whereas *CBS1* mRNA was analyzed as a negative control target to ensure that we enriched for specific targets after Puf protein immunoprecipitation (IP). As shown in Figure 7C (top), *YHB1* and *HXX1* were both enriched after IP with Puf1p, Puf4p, and Puf5p from both dextrose and galactose conditions as compared with control reactions without reverse transcriptase (albeit to different levels), whereas there was no enrichment of *CBS1* mRNA. We next directly compared levels of *YHB1* and *HXX1* mRNAs that copurified with Puf proteins from dextrose or galactose conditions after normalizing for differences in IP efficiency of the Puf proteins and setting the levels found in galactose arbitrarily to 1 (Figures 7, C, bottom, and D). For both Puf1p and Puf4p, no significant changes in binding to either target mRNA could be detected between conditions, especially given the large difference in values obtained from the two trials with *YHB1* mRNA, as shown by the large error bars. In contrast, Puf5p binding to each target mRNA was consistently decreased in galactose conditions. Thus condition-dependent binding to target RNAs by Puf5p may contribute to its altered decay activity.

## DISCUSSION

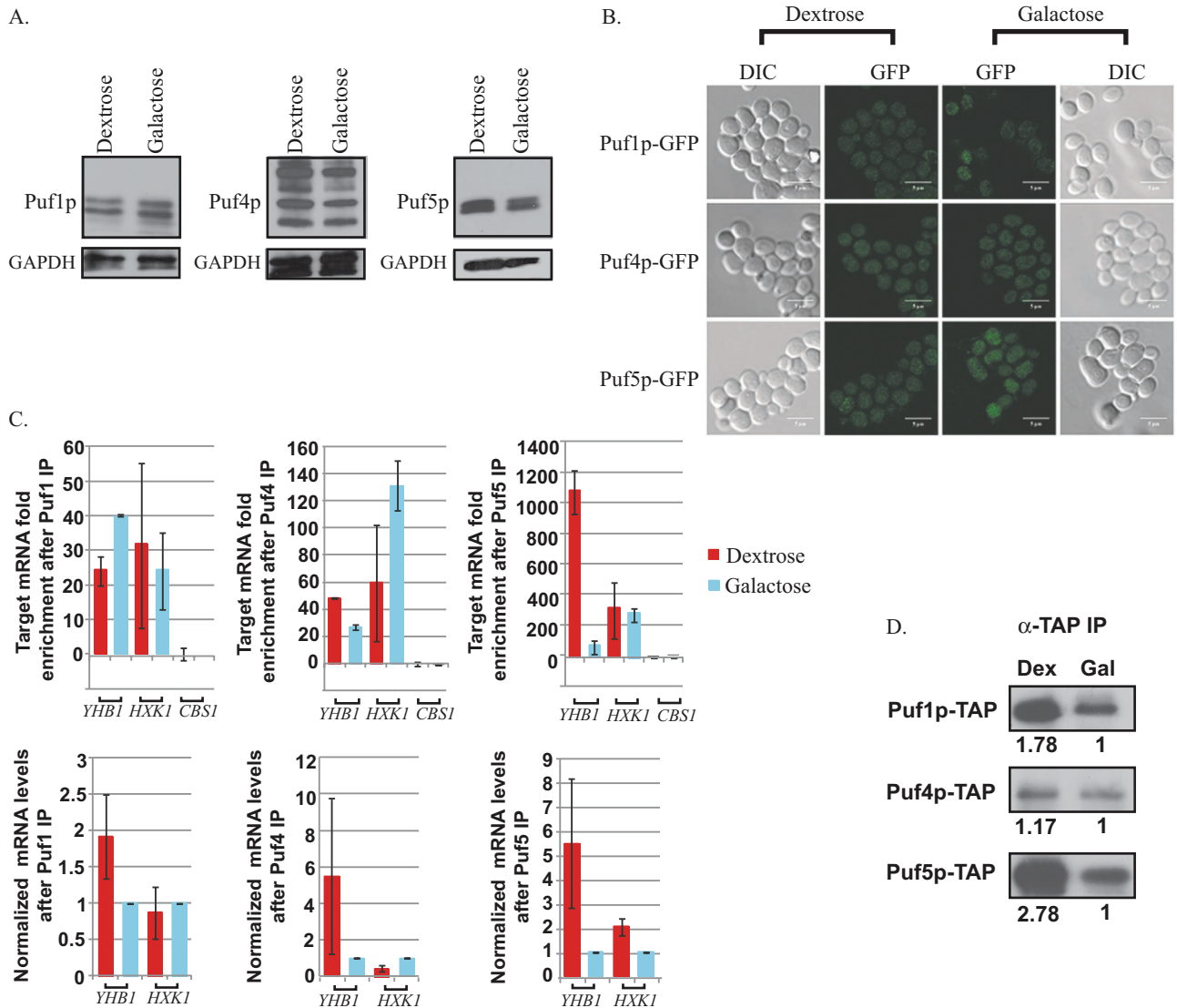
Gene expression is regulated by a multitude of mechanisms to ensure the careful balance of protein production for cell maintenance

and growth in any particular environment. However, cells must rapidly alter protein production in order to respond to perturbations and stresses in their environment. Transcriptional changes are a key component of stress response pathways, but alterations in mRNA decay and translation rates also play important roles and allow more rapid responses to environmental changes. For example, the stability and translation of growth factor transcripts containing AU-rich elements are regulated in a condition-specific manner via differential protein binding (Vasudevan and Peltz, 2001; Vasudevan et al., 2005). In *S. cerevisiae*, Yhb1p is the only known defense against NO stress (Liu et al., 2000). On exposure to NO, *YHB1* expression is increased due to transcriptional activation, as well as to posttranscriptional regulation (Foster et al., 2009). This work demonstrates that three Puf proteins coordinately mediate such posttranscriptional regulation by promoting decay of *YHB1* mRNA in nonstress conditions, whereas Puf activity is abrogated in stress conditions, resulting in stabilization and increased levels of *YHB1* mRNA to combat the stress.

The flexibility of Puf proteins to regulate target mRNAs is increasingly evident. Systematic analyses identified 90 yeast transcripts that copurified with more than one Puf protein (Gerber et al., 2004). Several established targets of multiple Puf regulation contain multiple, nonoverlapping PREs, allowing simultaneous Puf binding (Hook et al., 2007; Ulbricht and Olivas, 2008). Some of these PREs are also flexible enough to bind two different Puf proteins, including Puf1p and Puf5p alternatively binding a single site in *TIF1* mRNA (Ulbricht and Olivas, 2008) and Puf4p and Puf5p alternatively binding a single site in *HO* mRNA (Hook et al., 2007). Because different Puf proteins favor binding to distinct PREs, the basis for this flexibility derives from the ability of Puf proteins to flip out one or more bases from the binding surface to accommodate noncanonical/extra bases within the 8-nt binding site (Gupta et al., 2008; Miller et al., 2008; Koh et al., 2009; Lu et al., 2009; Valley et al., 2012).

This work investigates a novel target of multiple Puf regulation, *YHB1* mRNA, containing two overlapping PREs in its 3' UTR such that only one Puf protein can bind at one time. The first PRE in *YHB1* does not conform to either a Puf4p or a Puf5p binding site, whereas the second PRE conforms to the canonical binding site for Puf5p (UGUANNNUA; Gerber et al., 2004). Nonetheless, *YHB1* is destabilized by Puf1p, Puf4p, and Puf5p (Figure 1). Specifically, all three Pufs can bind and act through the second PRE, although both PREs contribute to full Puf-mediated decay stimulation (Figures 2 and 3). It is proposed that Puf4p and Puf5p both bind to the sequence (UGUANNAUA) where the N bases at nts 5 and 6 flip out from the Puf binding surface, whereas only Puf4p binds the sequence (UGUANANUA) where nts 5 and 7 flip out (Valley et al., 2012). However, neither overlapping PRE in *YHB1* conforms to either of these sequences, illustrating the uniqueness of *YHB1* as a target of multiple Puf regulation and implicating additional modes of base recognition and base flipping by Puf1p, Puf4p, and Puf5p than previously described.

The binding and destabilization of *YHB1* mRNA by Puf1p, Puf4p, and Puf5p indicate competition between Pufs. The extension of half-life in the *puf1Δ* strain was smaller than in the *puf4Δ* or *puf5Δ* strains, implying different binding and/or regulatory properties between Pufs. Our in vitro binding data suggest that inferior binding of Puf1p to the *YHB1* PREs likely contributes to its lesser role in decay stimulation, whereas the superior binding of Puf5p likely contributes to its larger role in decay. We also demonstrate that endogenous levels of Puf proteins are limiting, as overexpression of Puf4p or Puf5p in a wild-type strain facilitates more-rapid decay of *YHB1* mRNA than



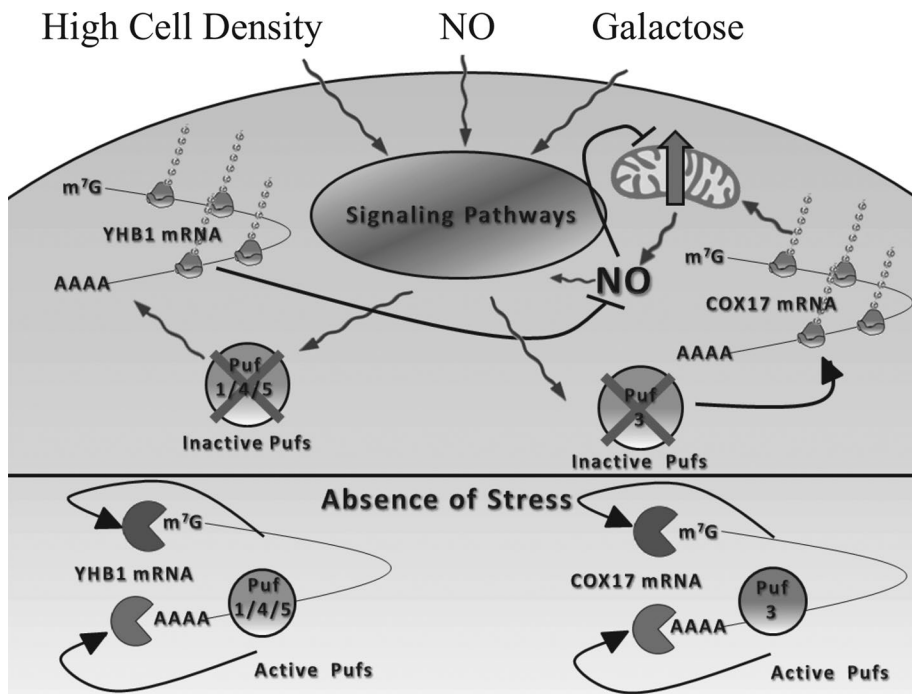
**FIGURE 7:** Effects of carbon source on Puf protein expression level, localization, and mRNA binding. (A) Representative Western blot of Puf1p-TAP, Puf4p-TAP, or Puf5p-TAP levels in cultures grown in YEP media with 2% dextrose or galactose. GAPDH was detected as a loading control for all analyses. (B) Yeast expressing endogenously GFP-tagged Puf1p, Puf4p, and Puf5p were grown in YEP media with 2% dextrose or galactose. Each image represents 10 flattened Z-plane slices through fixed cells using confocal microscopy. Puf1-GFP, Puf4-GFP, and Puf5-GFP are shown in green. Differential image contrasts (DICs) are shown for reference. Bar, equals 5  $\mu$ m. (C) Endogenously TAP-tagged Puf1p, Puf4p, and Puf5p were immunoprecipitated from yeast grown in YEP medium with 2% dextrose (red bars) or galactose (blue bars).  $C_q$  values after IP were compared with no–reverse transcriptase (–RT)  $C_q$  values for each mRNA to acquire fold enrichment (top graphs).  $C_q$  values after IP in dextrose were compared with  $C_q$  values after IP in galactose and normalized to protein levels after IP to acquire mRNA levels bound after IP (bottom graphs). (D) Representative Western blot of protein levels after conditional IPs. Numbers below blots represent relative levels of protein after normalization. For A and B, experiments were done in triplicate. For C and D, experiments were done in duplicate.

with wild type (Figure 3A). In contrast, Puf1p overexpression in a wild-type strain had no effect on *YHB1* mRNA decay, which is likely a result of its inferior binding activity. However, any single deletion of *PUF1*, *PUF4*, or *PUF5* results in a decay phenotype, demonstrating that each Puf is important for fine-tuned regulation of *YHB1*.

Overexpression of a mutant form of the Puf5RD enhanced *YHB1* decay better than WT Puf5RD (Figure 3). This N  $\rightarrow$  D mutation at residue 177 neighbors a known phosphorylated serine at 176 (Bodenmiller *et al.*, 2010; PhosphoPep). We hypothesize that the increased activity is due to the constitutive charge mimic in this region. Phosphorylation of Puf proteins has been shown to either

stimulate activity, as for human Pum1 (Kedde *et al.*, 2010), or inhibit activity, as seen with yeast Puf6 (Deng *et al.*, 2008). Future research will evaluate the potential role of Puf5p phosphorylation in activity. Differential phosphorylation could lead to altered RNA binding or altered interactions with other proteins.

In the absence of other Pufs, overexpression of Puf1p, Puf4p, or Puf5p is sufficient to rescue decay of *YHB1* mRNA (Figure 4). Thus, in the absence of competition from other Pufs, Puf1p is capable of binding and stimulating decay, even though it is not normally as active as Puf4p or Puf5p. The repeat domains of Puf1p, Puf4p, and Puf5p are also sufficient to rescue decay of *YHB1*



**FIGURE 8:** Model for Puf protein activity in the presence or absence of environmental stress. During exposure to environmental stresses, including high cell density, NO, and galactose, unknown signaling pathways inactivate Puf1p, Puf4p, and Puf5p, resulting in up-regulation of *YHB1* mRNA levels and protein production, allowing for protection from toxic NO levels. In addition, Puf3p activity is inhibited during exposure to galactose, resulting in increased mRNA stability of *COX17* mRNA and other mRNAs involved in mitochondrial development and function to accommodate the respiration demand. In the absence of stress, Puf proteins are active and repress target mRNAs.

mRNA, demonstrating that all sequences necessary for both binding and decay stimulation are present (Figure 4).

Inhibition of Puf-mediated decay contributes to the stabilization and increased levels of *YHB1* mRNA in respiratory conditions (Figure 5). During respiration, mitochondrial cytochrome oxidase produces NO proportional to the cellular respiration level (Li *et al.*, 2011). We previously showed that decay stimulation by Puf3p, but not mRNA binding, is inhibited by respiratory conditions such as galactose (Miller *et al.*, 2014). This inactivation allows stabilization of Puf3p target mRNAs encoding mitochondrial proteins, therefore increasing mitochondrial production under respiratory conditions (Figure 8). As with Puf3p, we propose that Puf1p, Puf4p, and Puf5p are also inactivated in respiratory conditions. Such inactivation would allow for the increased production of Yhb1p to rapidly combat toxic NO levels accumulating in the cell (Figure 8). The stress of high cell density also inactivates Puf1p, Puf4p, and Puf5p, resulting in stabilization of *YHB1* mRNA to combat this otherwise toxic event (Figure 5). In fermentation conditions or without exogenous stress, Puf1p, Puf3p, Puf4p, and Puf5p are active to stimulate rapid turnover of their target mRNAs, whose protein products are not needed under these conditions.

Overexpression of Puf4p could stimulate rapid decay of *YHB1* mRNA even in galactose and high cell density, suggesting that the high levels of Puf4p were overwhelming normal inactivation of Puf regulatory function (Figure 6). These results highlight the importance of the precise balance of Puf proteins normally in the cell to promote decay or respond to inactivation signals, possibly phosphorylation changes, under stress conditions. We further demonstrate the biological relevance of this mechanism by overexpression

of Puf4p or Puf5RD (hyperactive mutant) during exposure to exogenous NO, which resulted in decreased cell growth (Figure 6). To correlate this growth phenotype with levels of *YHB1* mRNA, we determined that steady-state levels of endogenous *YHB1* mRNA increase in *puf4Δ* and *Δpuf1-5* strains but decrease with Puf4p overexpression (Figure 6). Overexpression of either Puf4p or Puf5p also increased the decay rate of endogenous *YHB1* mRNA. Specifically, hyperstimulation of decay by the Puf5RD mutant beyond that by either wild-type Puf4p or Puf5p likely contributes to the more impaired growth observed with the Puf5RD mutant during exposure to DETA NONOate (Figure 6).

Condition-specific inhibition of Puf protein activity could be due to decreased Puf expression, altered localization, reduced mRNA binding capacity, disruption of interactions with other proteins, or altered activity of protein cofactors. We found that the inhibition of Puf1p, Puf4p, and Puf5p activity in galactose is not due to decreased expression or altered localization. Examination of Puf-mRNA binding interactions *in vivo* demonstrated that Puf1p and Puf4p bind target mRNAs in both activating and inactivating conditions. However, Puf5p showed reduced binding in inactivating conditions, which likely contributes to the condition-specific inhibition of Puf-mediated decay stimulation of *YHB1*, given that Puf5p plays the largest role in *YHB1* decay. Such reduced binding by Puf5p is a unique mechanism of inactivation, as previous studies with Puf3p demonstrated that its mRNA binding was enhanced in galactose conditions (Miller *et al.*, 2014). Further research will dissect the mechanism of Puf5p binding inhibition, as well as possible changes to the interactions with or activity of protein cofactors.

In this work, we elucidated a key role for Puf proteins in response to elevated levels of NO by which stabilization of a specific target mRNA (*YHB1*) leads to increased cell fitness. Puf proteins may also play a role in translational inhibition of *YHB1* mRNA. Previous work demonstrated that Puf4p acts exclusively through stimulation of deadenylation, whereas Puf5p can repress mRNAs through additional mechanisms, including translational inhibition (Hook *et al.*, 2007; Chritton and Wickens, 2010). A global study to identify mRNAs whose translation is dependent on Eap1p identified *YHB1*, and Puf activity has been shown to be dependent on Eap1p (Cridge *et al.*, 2010; Blewett and Goldstrohm, 2012). It is plausible that Puf proteins also translationally repress *YHB1* mRNA through Eap1p, which would provide an additional mechanism for the cell to limit production of this stress response factor in the absence of stress while allowing a rapid respond to NO stress by inactivation of Puf activity and corresponding up-regulation of protein production.

## MATERIALS AND METHODS

### Oligonucleotides, plasmids, and yeast strains

All yeast strains, plasmids, and oligonucleotides are listed in Supplemental Tables S1–S3, respectively. Strain yWO268 was made by transforming yWO204 with pWO183 after digestion with *Bam*HI to

replace *URA3* with *KanMX3*. Transformants were plated on yeast extract/peptone/dextrose plates containing 300 µg/ml Geneticin. Colonies were isolated and backplated on media lacking uracil to verify loss of *URA3*. Strain yWO269 was made by replacement of *YHB1* with *URA3* in yWO7. *URA3* was amplified from pWO15 with *YHB1* flanking regions using primers oWO77 and oWO78. The PCR product was transformed into yWO7, with selection on medium lacking uracil. Deletion of *YHB1* was verified by colony PCR and Northern blot analysis.

### PUF overexpression plasmids

The *PUF1* open reading frame (ORF) was amplified from genomic DNA using primers oWO466 and oWO467. The *PUF1RD* was amplified from pWO48 with primers oWO468 and oWO145. The PCR products were inserted into the *Bam*HI and *Sall* sites of pWO15 3' of the FLAG tag and under the control of the constitutive *GPD* promoter to create pWO114 (*PUF1* full-length) and pWO115 (*PUF1RD*).

*PUF2RD* was amplified from genomic DNA using primers oWO570 and oWO571. The PCR product was inserted into the *Bam*HI and *Sall* sites of pWO15 to create pWO192.

pWO16 was created by removing the *PUF3RD* from pWO14 using *Bam*HI and *Sall* and inserting into the respective sites in pWO15.

The *PUF4* ORF was amplified from genomic DNA using primers oWO610 and oWO611. The *PUF4RD* was amplified from genomic DNA using primers oWO638 and oWO611. The PCR products were inserted into the *Bam*HI and *Sall* sites of pWO15 to create pWO193 (*PUF4RD*) and pWO194 (*PUF4* full-length). pWO116 (pRS415-*GPD-PUF4*) was a kind gift from Marvin Wickens (University of Wisconsin–Madison).

The *PUF5* ORF was amplified from pWO18 using primers oWO612 and oWO613. The *PUF5RD* was amplified from pWO18 using primers oWO568 and oWO569. The PCR products were inserted into the *Bam*HI and *Sall* sites of pWO15 to create pWO195 (*PUF5RD*), pWO196 (*PUF5* full-length), and pWO200 (*PUF5RDmut*).

All constructs were verified by sequencing.

### Protein expression and purification

The GST-*PUF1* fusion construct (pWO201) was made by PCR amplification of Puf1 with *Bam*HI and *Not*I restriction sites from yeast genomic DNA using primers oWO865 and oWO866. The resulting fragment was then inserted into pGEX-3X (Amersham Biosciences/GE Healthcare, Pittsburgh, PA) and validated by sequencing and Western blot. The GST-*PUF4* fusion construct (pWO202) was made similarly, with the modification of using restriction sites *Eco*RI and *Not*I and primers oWO867 and oWO868. The GST-PUF fusion constructs pWO18, pWO201, and pWO202 were transformed into the protease-deficient *E. coli* strain BL-21, and GST fusion proteins were purified as recommended by the manufacturer. Protein eluates were dialyzed into 50 mM Tris-HCl (pH 8.0), and expression products were verified by Western blot analysis with anti-GST antibody.

### PGK1-YHB1 3' UTR reporter plasmids

pWO127 (*URA3* marker) and pWO128 (*LEU2* marker) were made by PCR amplification of the *YHB1* 3' UTR from genomic DNA using primers oWO262 and oWO263. For pWO127, the PCR product was inserted into pRS227 (Heaton *et al.*, 1992) 3' of the *PGK1* ORF between the *Sac*I and *Hind*III sites. For pWO128, the *PGK1-YHB1* 3' UTR insert was cut from pWO127 and inserted into pWO61 between the *Pvu*II sites.

pWO129 (*URA3* marker) was created by PCR-based in vitro site-directed mutagenesis of the *YHB1* 3' UTR PRE sequence UGUAUGUA to ACAACAA using primers oWO480 and oWO481,

in accordance with manufacturer's recommendations (QuikChange XL Site-Directed Mutagenesis Kit; Stratagene/Agilent Technologies, Santa Clara, CA). pWO197 (*URA3* marker) was created by site-directed mutagenesis of the PRE sequence UGUAUGUA to ACUAUGUA using primers oWO513 and oWO514. pWO199 (*LEU2* marker) was made by performing a *URA3*-to-*LEU2* marker swap on pWO197 using pWO162 (product #87552; ATCC) according to supplier's instructions.

All constructs were verified by sequencing.

### In vivo YHB1 decay analysis of Puf deletion mutants

Decay of reporter mRNAs was monitored in strains harboring the temperature-sensitive *rbp1-1* RNA polymerase II allele, in which transcription is rapidly halted after a shift from 24 to 37°C. All yeast transformations were performed by LiOAc high-efficiency transformation (Gietz *et al.*, 1995).

Transcriptional shut-offs were performed as described (Caponigro *et al.*, 1993) in yeast strains containing pWO127 or pWO128, which express a fusion RNA containing the *PGK1* Δ82 ORF and *YHB1* 3' UTR, with transcription regulated by the GAL UAS. pWO127 was transformed into yWO7 (WT), yWO102 (*puf1Δ*), yWO43 (*puf3Δ*), yWO105 (*puf4Δ*), and yWO268 (*Δpuf1-5*). pWO128 was transformed into yWO48 (*puf2Δ*), yWO49 (*puf5Δ*), and yWO204 (*Δpuf1-5*). Transcriptional shut-offs of *PGK1/yhb1* mRNA mutants were performed in yWO7 (WT) containing pWO129 or pWO197. Strains were grown as 200-ml cultures in selective minimal medium containing 2% galactose at 24°C to an OD<sub>600</sub> of 1.0. Half of each culture was harvested and resuspended in 20 ml of selective medium containing 8% dextrose at 37°C, effectively shutting off transcription by both the temperature-sensitive inactivation of RNA pol II and carbon source inactivation of the GAL UAS. Time course samples were taken over a 40-min time period at 37°C. Total RNA was isolated from yeast samples as described (Caponigro *et al.*, 1993), followed by Northern blot analysis (BioBond plus nylon membrane; Sigma-Aldrich, St. Louis, MO). Northern blots were probed with <sup>32</sup>P-end-labeled oWO105, oWO159, or oWO447 to detect *HXK1* 3' UTR, *YHB1* 3' UTR, or *PGK1* 3' UTR, respectively. Loading was normalized using *scR1* RNA, a constitutively expressed RNA polymerase III transcript, and all imaging and quantification of half-lives were determined by ImageQuant software (Molecular Dynamics).

### In vitro binding assays

RNAs representing the wild-type *YHB1* 3' UTR, *yhb1-1* mutant 3' UTR, and *yhb1-2* mutant 3' UTR were purchased from IDT (oWO 898, oWO899, and oWO900, respectively). The RNAs were subjected to <sup>32</sup>P end labeling for detection in the binding assay. Binding reactions included radiolabeled RNA (100,000 cpm) and 1× binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 50 mM KCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], 200 U/ml RNasin ribonuclease inhibitor, 0.1 mg/ml bovine serum albumin [BSA], 0.01% Tween-20, 0.1 mg/ml poly(rU), and 10 µg/ml yeast tRNA) in the presence or absence of GST-Puf1p, GST-Puf4p, or GST-Puf5p in a total of 20 µl. Reactions were incubated for 30 min at 24°C, 5 µg of heparin was added, and reactions were incubated for a further 10 min at 24°C; then the reactions were electrophoresed on 8% nondenaturing polyacrylamide gels for 2.5 h at 200 V at 4°C. To calculate relative binding, storage phosphor signals were first determined for each bound complex, and background signal from the no-protein lane was subtracted from each. For comparison of binding between RNA targets, values for each set of Puf + RNA complexes were divided by the corresponding Puf + WT RNA value; then normalized values were averaged between trials. For comparison of



binding between Puf proteins on a single RNA target, values for each set of Puf-RNA complexes were divided by the 2  $\mu$ M Puf5p + RNA value; then normalized values were averaged between trials.

### In vivo *YHB1* decay analysis with Puf overexpression

Transcriptional shut-offs were performed as described in yWO7 cotransformed with plasmid pWO100, pWO102, pWO127, pWO128, pWO129, pWO197, or pWO199 and Puf overexpression plasmid pWO15, pWO16, pWO58, pWO114, pWO115, pWO116, pWO192, pWO193, pWO194, pWO195, pWO196, or pWO200.

Transcriptional shut-offs were also performed in yeast strains yWO204 ( $\Delta$ puf1-5) and yWO268 ( $\Delta$ puf1-5) cotransformed with plasmid pWO127 or pWO128 and Puf overexpression plasmid pWO15, pWO16, pWO58, pWO114, pWO115, pWO116, pWO192, pWO193, pWO194, pWO195, or pWO196. Similar detection and quantification methods were used as described.

### In vivo decay analysis in alternate carbon sources or culture densities

Transcriptional shut-offs were performed in yWO7 (WT) transformed with pWO61, pWO100, or pWO127; or yWO204 ( $\Delta$ puf1-5) transformed with pWO128. To examine decay in cultures grown continuously in galactose, strains were grown as 200-ml cultures in selective minimal medium containing 2% galactose at 24°C to an OD<sub>600</sub> of 1.0. Half of each culture was harvested and resuspended in 20 ml of selective medium containing 8% galactose at 37°C, effectively shutting off transcription by the temperature-sensitive inactivation of RNA pol II. To examine decay in cultures grown to higher cell densities, strains were grown as 200-ml cultures in selective minimal medium containing 2% galactose at 24°C to an OD<sub>600</sub> of 2.0 or 3.0. Either 50 ml (OD<sub>600</sub> of 2.0) or 25 ml (OD<sub>600</sub> of 3.0) of cells was harvested and resuspended in 20 ml of selective medium containing 8% dextrose at 37°C, effectively shutting off transcription by both the temperature-sensitive inactivation of RNA pol II and carbon source inactivation of the GAL UAS. Similar detection and quantification methods were used as described earlier. Northern blots were probed with <sup>32</sup>P-end-labeled oWO105, oWO159, or oWO238 to detect *HXK1* 3' UTR, *YHB1* 3' UTR or *MFA2pG* 3' UTR, respectively.

### In vivo endogenous *YHB1* mRNA decay analysis

Transcriptional shut-offs were performed in yWO7 or yWO268 transformed with overexpression plasmids pWO15, pWO58, pWO116, pWO195, or pWO200. Strains were grown as 200-ml cultures in selective minimal medium containing 2% galactose at 24°C to an OD<sub>600</sub> of 1.0. Half of each culture was harvested and resuspended in 20 ml of selective medium containing 8% dextrose at 37°C, effectively shutting off transcription by the temperature-sensitive inactivation of RNA pol II. Similar detection and quantification methods were used as described earlier.

### Growth inhibition study

Mid log-phase cells (OD<sub>600</sub> of 0.4–0.6) were diluted to an OD<sub>600</sub> of 0.01 and allowed to grow at 24°C for 24 h during exposure to 3 mM DETA NONOate. Cell growth was monitored every 4 h by OD<sub>600</sub> measurements. Growth percentage represents growth with 3 mM DETA NONOate exposure normalized against growth with no added DETA NONOate for each individual strain (yWO7 transformed with pWO58 or pWO116, or yWO269).

### Steady-state detection of endogenous *YHB1* mRNA

Mid log-phase cells (OD<sub>600</sub> of 0.4–0.6) were harvested, and total RNA was extracted from yWO7 transformed with pWO58 or

pWO116, yWO105, or yWO204. Northern analysis and detection of *YHB1* mRNA with oWO159 were performed. Similar detection and quantification methods were used as described earlier.

### Puf1p, Puf4p, and Puf5p Western analysis

Protein extracts were prepared from 20-ml yeast cultures of endogenously TAP-tagged strains TAP-Puf1p, TAP-Puf4p and TAP-Puf5p (Ghaemmaghami *et al.*, 2003; Thermo-Fisher Scientific) grown in yeast extract/peptone (YEP) containing 2% dextrose or galactose at 30°C to an OD<sub>600</sub> of 1.0. Harvested cells were resuspended in 0.25 ml of sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 2% glycerol, 10%  $\beta$ -mercaptoethanol) and lysed with glass beads, and extract was collected by poking a hole in the bottom of the microfuge tube and spinning into a 15-ml centrifuge tube. Equal OD<sub>600</sub> units of total protein were loaded onto a 10% Tris-glycine polyacrylamide gel (Bio-Rad, Hercules, CA). Resulting gels were blotted to nitrocellulose and probed with anti-TAP antibodies. Blots were also probed with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Thermo Scientific, Waltham, MA) antibodies as a loading control.

### Confocal fluorescence microscopy

Endogenously GFP-tagged Puf1p, Puf4p, and Puf5p (yWO200, yWO203, yWO199; Huh *et al.*, 2003) were grown at 30°C to an OD<sub>600</sub> of 1.0 in YEP supplemented with 2% dextrose or galactose. Cells were then fixed with 3.7% formaldehyde for 1 h. The cells were washed twice with 1 $\times$  phosphate buffered-saline (PBS) and resuspended in 1 ml of 1 $\times$  PBS. A 10-ml amount of cells was loaded onto a circular glass slide coated with 1% polyethyleneimine (P3143-100ML; Sigma-Aldrich), and cells were allowed to settle for 10 min. The cell solution was aspirated from the edge of the slide, and the slide was then dipped in 1 $\times$  PBS twice to remove nonadherent cells. A coverslip was applied, and the cells were immediately visualized with a Zeiss LSM-700 confocal microscope on the 100 $\times$  oil immersion objective for enhanced GFP fluorescence. Ten slices were taken through the Z-plane of the cells. The slices were flattened using Fiji Is Just ImageJ (FIJI) Z-projection at maximum intensity, and the intensity of GFP signal was adjusted such that the maximum signal for cells grown in galactose was set as the maximum for cells grown in dextrose.

### Quantitative real-time PCR of RNA associated with Puf1p, Puf4p, and Puf5p

RNA IP was performed essentially as described (Gerber *et al.*, 2004), with minor alterations. Endogenously TAP-tagged Puf1p, Puf4p, and Puf5p cells (yWO272, yWO271, and yWO270) were grown at 30°C in 1 l of YEP supplemented with 2% dextrose or galactose to an OD<sub>600</sub> of 1.0. The cells were pelleted, washed twice with 25 ml of ice-cold buffer A (20 mM Tris-HCl, pH 8.0, 140 mM KCl, 1.8 mM MgCl<sub>2</sub>, 0.1% Nonidet P-40, 0.02 mg/ml heparin), and frozen at –80°C. The next day, the cells were thawed on ice and resuspended in 5 ml of buffer B (buffer A with 0.5 mM DTT, 1 $\times$  Complete Mini Protease Inhibitors [11-836-153-001; Roche Diagnostics, Indianapolis, IN], 40 U/ml RNasin ribonuclease inhibitor, 0.2 mg/ml heparin). Cells were vortexed in the presence of glass beads for 1 min and placed on ice for 1 min for a total of five times. Lysates were clarified by centrifuging at 7000  $\times$  g for 5 min. The supernatant was collected, and protein concentration was measured using a standard Bradford assay. Lysates were normalized to contain 1.625 mg in a volume of 5 ml, and BSA to 1% and 50  $\mu$ g yeast tRNA were added. The lysates were incubated with 400  $\mu$ l of 50% immunoglobulin G-Sepharose 6 Fast Flow (17-0969-010; GE Healthcare) that had been blocked for 1 h in 1 ml of buffer B plus 1% BSA and

50 µg of yeast tRNA. Beads were incubated at 4°C for 2 h and washed once with 5 ml of buffer B for 15 min and three times with 5 ml of buffer C (20 mM Tris-HCl, pH 8.0, 140 mM KCl, 1.8 mM MgCl<sub>2</sub>, 0.01% Nonidet P-40, 0.5 mM DTT, 12 U/ml RNasin ribonuclease inhibitor) for 15 min each. Beads were resuspended in 400 µl of buffer C, and 80 U of ProTEV Plus (V6101; Promega, Fitchburg, WI) was added. Beads were incubated for 2 h at 16°C. The resultant supernatant was drawn off, and total RNA was isolated via hot phenol extraction. Glycogen (20 µg) was added to the final aqueous phase to assist precipitation of RNA before the addition of 1/10 volume of 3 M NaOAc and 2.5 volumes of ethanol. The entirety of immunoprecipitated RNA was subjected to Turbo DNase treatment via manufacturer's instructions (Ambion, Grand Island, NY). Reverse transcription of RNA was performed according to manufacturer's specifications (Bio-Rad iScript). qPCR was optimized and performed on a Bio-Rad CFX96 Real-Time system using SYBR Green detection chemistry (Bio-Rad SsoAdvanced). Gene-specific qPCR primers are listed in Supplemental Table S3. All RT-qPCR experiments were conducted in technical triplicates and biological duplicates. RNA fold enrichment after IP was calculated as  $2^{(\text{No RT} - \text{RT})}$ , where No RT is the reaction performed without reverse transcriptase and RT is the reaction with reverse transcriptase. Normalized mRNA levels between carbon sources were calculated as  $2^{(\text{galactose IP} - \text{dextrose IP})}$  and then multiplied by the normalized protein levels after IP.

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