Inactivation of fission yeast Erh1 de-represses pho1 expression: evidence that Erh1 is a negative regulator of prt IncRNA termination

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ABSTRACT

Fission yeast Erh1 exists in a complex with RNA-binding protein Mmi1. Deletion of erh1 up-regulates the phosphate homeostasis gene pho1, which is normally repressed by transcription in cis of a 5′ flanking prt IncRNA. Here we present evidence that de-repression of pho1 by erh1Δ is achieved through precocious 3′-processing/termination of prt IncRNA synthesis, to wit: (i) erh1Δ does not affect the activity of the prt or pho1 promoters per se; (ii) de-repression by erh1Δ depends on CPF (cleavage and polyadenylation factor) subunits Ctf1, Dis2, Ssu72, Swd22, and Ppn1 and on termination factor Rhn1; (iii) de-repression requires synthesis by the Asp1 IPP kinase of inositol 1-pyrophosphates (1-IPPs); (iv) de-repression is effaced by mutating Thr4 of the RNA polymerase II CTD to alanine; and (v) erh1Δ exerts an additive effect on pho1 de-repression in combination with mutating CTD Ser7 to alanine and with deletion of the IPP pyrophosphatase Aps1. These findings point to Erh1 as an antagonist of lncRNA termination in the prt–pho1 axis. In contrast, in mmi1Δ cells there is a reduction in pho1 mRNA and increase in the formation of a prt–pho1 read-through transcript, consistent with Mmi1 being an agonist ofprt termination. We envision that Erh1 acts as a brake on Mmi1′s ability to promote CPF-dependent termination during prt IncRNA synthesis. Consistent with this idea, erh1Δ de-repression of pho1 was eliminated by mutating the Mmi1-binding sites in the prt IncRNA.

Keywords: Erh1; Mmi1; Schizosaccharomyces pombe

INTRODUCTION

Fission yeast Erh1 is a 104-amino acid nuclear protein identified initially by virtue of its homology to the metazoan protein Enhancer of Rudimentary (Krzyzanowski et al. 2012). Schizosaccharomyces pombe erh1Δ cells are viable but display abnormal morphology in stationary phase and sensitivity to several stressful growth conditions (Krzyzanowski et al. 2012). Subsequent studies implicated Erh1 in preventing the deleterious expression of meiotic genes during vegetative growth, which it accomplishes via its physical association with Mmi1, a YTH-family RNA binding protein that recognizes DSR (determinant of selective removal) sequences in target RNAs and promotes their elimination by the nuclear exosome (Yamashita et al. 2013; Sugiyama et al. 2016; Shichino et al. 2018). Mmi1 and Erh1 colocalize in nuclear foci in vegetative cells and are recovered as a stoichiometric Mmi1–Erh1 complex after Erh1-affinity purification from fission yeast cell extracts (Sugiyama et al. 2016). Mmi1 consists of: (i) a carboxy-terminal YTH-fold RNA binding domain with a distinctive mode of recognition of the DSR element UNAAAC and (ii) a low-complexity unstructured amino-terminal domain that is needed for formation of nuclear foci and mediates binding to Erh1 (Chatterjee et al. 2016; Wang et al. 2016; Shichino et al. 2018; Stowell et al. 2018; Xie et al. 2019). A crystal structure of Erh1 (expressed as a fusion in cis to a 28-aa segment of Mmi1 that suffices for binding to Erh1) showed that Erh1 is a homodimer and that each protomer has a Mmi1 peptide bound on its surface (Xie et al. 2019). In addition to its role in targeting RNA decay, Erh1 has imputed functions in the assembly of facultative heterochromatin (Sugiyama et al. 2016; Xie et al. 2019).

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Our interest in Erh1 was prompted by the results of transcription profiling of an erh1Δ mutant whereby it was found that the phosphate homeostasis gene pho1 was up-regulated by sevenfold vis-à-vis a wild-type erh1+ control (Sugiyama et al. 2016). Fission yeast phosphate homeostasis entails a transcriptional response to phosphate availability that affects three genes that comprise a PHO regulon encoding proteins involved in extracellular phosphate acquisition, these being a cell surface acid phosphatase Pho1, an inorganic phosphate transporter Pho84, and a glycerophosphate transporter Tgp1 (Carter-O’Connell et al. 2012). Expression of the pho1, pho84, and tgp1 genes is actively repressed during growth in phosphate-rich medium by the transcription in cis of a long noncoding (lnc) RNA from the respective 5′ flanking genes prt, prt2, and nc-tgp1 (Lee et al. 2013; Ard et al. 2014; Shah et al. 2014; Chatterjee et al. 2016; Garg et al. 2018a; Sanchez et al. 2018a).

Our studies have shown that the repressive action of the IncRNAs on the PHO genes is governed by the RNA polymerase II (Pol2) CTD code, the 3′ cleavage/polyadenylation factor complex CPF, transcription termination factor Rhn1, and synthesis of inositol 1-pyrophosphates (1-IPPs) (Schwer et al. 2014, 2015; Chatterjee et al. 2016; Sanchez et al. 2018b, 2019). Current evidence supports a model for the repressive arm of phosphate homeostasis whereby transcription of the upstream lncRNA interferes with expression of the downstream mRNA genes by displacing the activating transcription factor Pho7 from its binding site(s) in the mRNA promoters that overlap the lncRNA transcription units (Carter-O’Connell et al. 2012; Schwer et al. 2017; Garg et al. 2018b, 2019). Genetic manipulations of the aforementioned governors can elicit either hyper-repression or de-repression of the PHO genes, as depicted in Figure 1. For example, a CTD-T4A mutation that precludes installation of the Pol2 Thr4-PO4 mark hyper-represses the PHO genes under phosphate-rich conditions, as do deletions or loss-of-function mutations in the Ctf1, Dis2, Ssu72, Swd22, and Ppn1 subunits of CPF, deletion of termination factor Rhn1, and ablation of the IPP kinase Asp1 that synthesizes 1,5-IP8 and 1-IP7 from 5-IP7 and IP6, respectively. In contrast, a CTD-S7A mutation that eliminates the Pol2 Ser7-PO4 mark de-represses the PHO genes in phosphate-replete cells, as do IPP pyrophosphatase-inactivating mutations that increase IP8. RNA analyses, transcriptomics, and genetic epistasis analyses cohere around a model whereby: (i) hyper-repression is the result of reduced lncRNA termination and hence increased transcription across the mRNA promoter; and (ii) de-repression results from precocious termination of lncRNA transcription prior to the mRNA promoter and thus relief from transcription interference.

In the present study, we find that ablating Erh1 de-represses pho1 expression in phosphate-replete cells via a pathway that depends on CTD-Thr4, CPF subunits Ctf1, Dis2, Ssu72, Swd22, and Ppn1, termination factor Rhn1, and synthesis of 1-IPPs. Deletion of erh1 exerts an additive effect on pho1 de-repression in combination with CTD-S7A and deletion of the IPP pyrophosphatase Aps1. These genetic interactions, and the findings that erh1Δ has no impact on the activity per se of the prt IncRNA and pho1 mRNA promoters, point to Erh1 as a negative influence on lncRNA termination. In mmi1Δ cells there is a reduction in pho1 mRNA and increased production of a prt–pho1 read-through transcript, consistent with Mmi1 being a positive effector of lncRNA termination. We suggest that Erh1 acts as a damper on Mmi1 such that when Erh1 is absent Mmi1 is unrestrained in eliciting precocious prt termination and thus de-repression of pho1 mRNA synthesis.

**FIGURE 1.** Synthesis of prt lncRNA represses pho1 mRNA transcription. Models for the pho1 hyper-repressed (A) and de-repressed (B) states of the prt–pho1 locus under phosphate-replete conditions. The genetic perturbations that elicit these states are indicated at right.
RESULTS AND DISCUSSION

Deletion of Erh1 de-represses Pho1 expression

We constructed a fission yeast erh1Δ strain and tested the effects of Erh1 loss on growth on YES agar medium at 20°C to 37°C (Fig. 2A) and on pho1 expression during exponential growth at 30°C in liquid culture under phosphate-replete conditions (Fig. 2B). The erh1Δ strain grew as well as the wild-type control strain at 30°C to 37°C, as gauged by colony size, but displayed cold-sensitivity, manifest as small colony size at 25°C and failure to thrive at 20°C (Fig. 2A). Acid phosphatase activity (a gauge of Pho1 enzyme level that correlates with Pho1 mRNA levels) was quantified by incubating suspensions of serial dilutions of the phosphate-replete cell cultures for 5 min with p-nitrophenylphosphate and assaying colorimetrically the formation of p-nitrophenol. The basal Pho1 activity of wild-type cells was de-repressed by sixfold in erh1Δ cells (Fig. 2B).

The loss of Erh1 might de-repress pho1 expression either by (i) reducing the activity of the prt IncRNA promoter that drives transcription interference with the pho1 promoter or (ii) increasing the intrinsic activity of the pho1 mRNA promoter independent of IncRNA synthesis. To address these issues, we used a plasmid-borne prt–pho1 reporter (Fig. 3A) that was introduced into fission yeast cells in which the chromosomal pho1 gene was deleted. This reporter faithfully reflects known homeostatic controls on the native pho1 locus (Chatterjee et al. 2016; Sanchez et al. 2019). We found that the prt–pho1 reporter was responsive to ablation of Erh1, whereby Pho1 levels under phosphate-replete conditions were de-repressed by eightfold in erh1Δ cells compared to the wild-type erh1+ control (Fig. 3D). We then tested a mutated version of the prt–pho1 reporter construct in which the prt promoter is inactivated by nucleotide changes in the HomolD and TATA box elements that drive prt IncRNA synthesis (Fig. 3B; Chatterjee et al. 2016). This mutant reporter provides a readout of the intrinsic activity of the pho1 promoter, absent interference by transcription of the flanking prt IncRNA. The Pho1 activity of the mutant plasmid in wild-type cells is high (i.e., de-repressed) and is not different from the Pho1 activity in erh1Δ cells (Fig. 3E), thereby indicating that the de-repressive effect of erh1Δ on pho1 expression from the wild-type prt–pho1 locus is not caused by up-regulation of the pho1 promoter per se. The effect of erh1Δ on the prt promoter was assessed using a different plasmid reporter (Fig. 3C) in which the prt promoter directly drives expression of the pho1 ORF. Pho1 expression from this plasmid was the same in wild-type and erh1Δ cells (Fig. 3F), signifying that the de-repression of native pho1 by erh1Δ is not caused by decreased activity of the prt promoter. These results suggest that erh1Δ affects pho1 expression by enhancing precocious termination of prt IncRNA synthesis, à la the Pol2 rpb1-CTD-S7A allele (Fig. 1).

FIGURE 2. De-repression of Pho1 in erh1Δ cells requires the synthesis of inositol 1-pyrophosphates by Asp1 kinase. (A) Growth of S. pombe strains with the indicated erh1, asp1, and aps1 alleles. Cells were inoculated in YES broth and grown at 30°C. Exponentially growing cultures were adjusted to 10^6 cells/mL, and aliquots (3 µL) of serial fivefold dilutions were spotted on YES agar and then incubated at the temperatures specified. (B, C) S. pombe strains with the indicated erh1, asp1, and aps1 alleles were grown to OD500 of 0.5 to 0.8 in liquid culture in YES medium at 30°C. Cells were then harvested, washed with water, and assayed for Pho1 acid phosphatase activity by conversion of p-nitrophenylphosphate to p-nitrophenol. Activity is expressed as the ratio of A133 (p-nitrophenol production) to A400 (input cells). Each datum in the bar graph is the average of assays using cells from at least three independent cultures ± SEM. (D) Structures of the 5-IP7, 1-IP7, and 1,5-IP8 are shown. Asp1 kinase converts 5-IP7 to IP8 (and also IP6 to 1-IP7) and the Asp1 pyrophosphatase reverses this process.
Intronic IncRNA control of phosphate homeostasis

**Figure 3.** Does erh1Δ affect the prt IncRNA or pho1 mRNA promoters? (A) Schematic of the plasmid-borne prt-pho1 reporter in which pho1 expression is repressed by prt IncRNA transcription. (B) A reporter of pho1 promoter activity in which prt IncRNA transcription is abolished by mutations (indicated by X) in the HomolD and TATA box elements in the prt promoter (Chatterjee et al. 2016). (C) A reporter of prt promoter activity in which the prt promoter directly drives transcription of the pho1 gene. (D–F) The indicated reporter plasmids were transfected into erh1Δ (WT) or erh1Δ strains in which the chromosomal pho1 locus was deleted. Transformants were selected and single colonies of individual transformants were pooled (>20) and grown in plasmid-selective liquid medium to A600 of 0.5–0.8. Aliquots were harvested for acid phosphatase activity measurements. Each datum in the bar graph is the average of assays using cells from three independent cultures ± SEM.

erh1Δ de-repression of Pho1 expression depends on 1-IPP synthesis

Previous studies showed that de-repression of Pho1 elicited by the rpb1-CTD-S7A allele depends on the activity of the IPP kinase Asp1 (Sanchez et al. 2019). Asp1 is a bifunctional enzyme composed of an amino-terminal IPP kinase domain that converts 5-IPP7 to 1,5-IPP8, and a carboxy-terminal IPP pyrophosphatase domain that converts 1,5-IPP8 back to 5-IPP7 (Fig. 2D; Pascual-Ortiz et al. 2018; Dollins et al. 2020). Asp1 can also phosphorylate IP6 to yield 1-IPP7 and de-phosphorylate 1-IPP7 back to IP6. The in vivo effect of an asp1Δ null allele or a kinase-dead asp1Δ-D333A allele is to eliminate intracellular IP8 and 1-IPP7 and to increase the level of 5-IPP7; the in vivo effect of a pyrophosphatase-defective asp1-H397A allele is to increase the level of IP8 (Pascual-Ortiz et al. 2018). To see if IPP status affects Pho1 de-repression by erh1Δ, we crossed erh1Δ to an asp1Δ-D333A strain. As noted previously (Sanchez et al. 2019), asp1Δ-D333A cells formed slightly smaller colonies than the wild-type control strain (Fig. 2A). The lack of IP8 in asp1Δ-D333A cells resulted in a fivefold hyper-repression of Pho1 acid phosphatase activity (Fig. 2B). We obtained viable erh1Δ asp1Δ-D333A haploids after mating and sporulation; the double-mutant was slow-growing on YES agar and cold-sensitive (Fig. 2A). The instructive finding was that the de-repression of Pho1 activity by erh1Δ was erased in the asp1Δ-D333A background (Fig. 2B), signifying a requirement for 1-IPP synthesis, presumably via 1-IPP’s role as an agonist of 3′ processing/termination during prt transcription (Sanchez et al. 2019).

Pho1 is de-repressed in IP8 pyrophosphatase-defective asp1-H397A cells by virtue of precocious termination of prt IncRNA transcription when IP8 levels are elevated (Fig. 2C; Sanchez et al. 2019). Pho1 is also de-repressed in asp1Δ cells (Fig. 2C; Sanchez et al. 2019) that lack the Nudix-family IPP pyrophosphatase Aps1, which converts 1,5-IPP8 to 1-IPP7 (Fig. 2D; Safrany et al. 1999). Here we tested the genetic interactions of erh1Δ with asp1-H397A and asp1Δ by pairwise mating and recovery of viable erh1Δ asp1-H397A and erh1Δ asp1Δ haploids after sporulation (Fig. 2A). The 11-fold de-repression of Pho1 activity elicited by asp1-H397A vis-à-vis wild-type asp1Δ+ was virtually unaffected by the erh1Δ deletion (Fig. 2C). In contrast, the fivefold de-repression of Pho1 in the asp1Δ strain was enhanced additively to 12-fold in the erh1Δ asp1Δ background (Fig. 2C).

De-repression of pho1 expression by erh1Δ depends on CPF subunits and Rhn1

To test our hypothesis that loss of Erh1 leads to precocious termination during prt IncRNA synthesis, we introduced the erh1Δ mutation into knockout strains lacking the Dis2, Ctf1, Ppn1, or Swd22 subunits of the CPF complex, a strain with a catalytically dead (C13S) version of the Ssu72 protein phosphatase subunit of CPF, and a strain that lacks the transcription termination factor Rhn1. Viable double-mutant haploids were recovered after pairwise matings and sporulation of the diploids (Fig. 4A). As noted previously (Sanchez et al. 2018b), the CPF and
Rhn1 mutations per se resulted in hyper-repression of Pho1 in phosphate-replete cells (Fig. 4B). The instructive findings were that the de-repression of Pho1 by erh1Δ was effaced in rhn1Δ, ssu72-C13S, ctf1Δ, ppp1Δ, and swd22Δ cells and was attenuated in dis2Δ cells (Fig. 4B). Thus, the increase in Pho1 expression in erh1Δ cells requires CPF subunits and Rhn1, consistent with the pre-co-cisous termination model (Fig. 1B).

Genetic interactions of erh1Δ with Pol2 CTD phospho-site mutants

The de-repressive effect of erh1Δ on Pho1 expression and its genetic reliance on IP8 synthesis, CPF subunits, and Rhn1 reported above is similar to the IP8/CPF/Rhn1-dependent de-repression of pho1 observed in rpb1-CTD-S7A cells and contrasts with the hyper-repression of Pho1 in rpb1-CTD-T4A cells (Chatterjee et al. 2016; Sanchez et al. 2018b, 2019). To query epistasis relationships, we mated erh1Δ with the CTD phospho-site mutants and recovered viable haploid erh1Δ CTD-S7A and erh1Δ CTD-T4A progeny after sporulation (Fig. 5A). Assays of acid phosphatase activity showed that the S7A allele enhanced the de-repression of Pho1 by erh1Δ, whereas the T4A allele completely erased Pho1 de-repression (Fig. 5B). Thus, T4A “wins out” over erh1Δ with respect to Pho1 expression, just as it does over other mutations (S7A, asp1-H397A, or asp1Δ) that de-repress the prt-pho1 locus.

Distinctive effects of erh1Δ and mmi1Δ on pho1 expression

Erh1 per se is a homodimer; it forms a 2:2 heterotetramer with Mmi1 (Sugiyama et al. 2016; Xie et al. 2019; Hazra et al. 2020). A pertinent question here is whether the pho1 de-repressive effects of erh1Δ are recapitulated in the absence of Mmi1. To address this issue, we took advantage of the observation that Mmi1, which is essential for vegetative growth of fission yeast (by virtue of its ability to promote elimination of detrimental meiotic RNAs) can be deleted in a genetic background in which mei4 is inactivated, for example, in the mmi1Δ strain SP1111 (Sugiyama and Sugioaka-Sugiyama 2011) where we find there is a Cys125-Stop nonsense mutation in the mei4 gene that truncates the 517-aa Mei4 protein. Figure 6A shows a northern blot analysis of total RNA from phosphate-replete wild-type, erh1Δ, and mmi1Δ cells probed with a radiolabeled DNA oligonucleotide complementary to the pho1 ORF. Consistent with the Pho1 activity assays shown in the preceding sections, we find that the ~1.6 kb pho1 mRNA is increased in erh1Δ cells vis-à-vis wild-type. In contrast, the pho1 mRNA level is decreased slightly in
De-repression of \textit{pho1} by \textit{erh1}\textsuperscript{Δ} depends on DSR elements in the \textit{prt} lncRNA

The \textit{prt} lncRNA contains two clusters of DSR elements—\textit{DSRx3-1} and \textit{DSRx3-2}—each composed of three DSR
hexanucleotide elements (Fig. 7). The DSR clusters in the nascent lncRNA are binding sites for Mmi1 (Kilchert et al. 2015; Chatterjee et al. 2016). Previous studies highlighted the role of the DSR clusters in prt-promoted pho1 repression, via tracking the effects of compound mutations (mut1 and mut2) introduced into each of the hexanucleotide motifs comprising the DSRx3-1 and DSRx3-2 sequences of the prt-pho1 reporter plasmid (Fig. 7; Chatterjee et al. 2016). As recapitulated here, mut1 and mut2 reduced acid phosphatase activity in phosphate-replete cells to 75% and 22% of the wild-type prt DSR control, respectively (Fig. 7). Combining the mut1 and mut2 DSR cluster mutations had an additive effect, reducing Pho1 activity to 11% of the wild-type prt level. The hyper-repressive effect of the prt DSR mut1+mut2 changes on Pho1 expression were shown previously to “win out” over the de-repressive effects of CTD-S7A and asp1-1H397A (i.e., increased IP8), suggesting that DSRs are key to establish the precocious termination of prt IncRNA synthesis that underlies Pho1 de-repression (Chatterjee et al. 2016; Sanchez et al. 2019). Here we queried the effect of DSR mutations on de-repression of the prt-pho1 reporter in erh1Δ pho1Δ cells. The mut1, mut2, and mut1+mut2 changes reduced Pho1 expression in erh1Δ cells to 52%, 11%, and 2%, respectively, of the wild-type DSR reporter control, such that the level of Pho1 expression from the mut1+mut2 reporter was virtually identical in the wild-type and erh1Δ strains (Fig. 7). We conclude that erh1Δ de-repression of pho1 depends on the DSR elements in the prt lncRNA.

Synthetic genetic interactions of mmi1Δ with CPF and Rhn1 mutants

There is significant functional redundancy built into the fission yeast 3' processing/termination machinery, whereby the effects of ablating one inessential component are buffered by other inessential components, such that inactivation of both elicits synthetic lethality or severe synthetic sickness (Sanchez et al. 2018b, 2020). Because precission is one of several processes in which Mmi1 is implicated, we performed a synthetic genetic array analysis entailing pairwise mating of the mmi1Δ strain with ctf1Δ, ppn1Δ, swd2Δ, dis2Δ, ssu72-C13S, and rhn1Δ cells and screening large populations of random spores for the drug-resistance markers linked to the respective mutant alleles. We thereby found that mmi1Δ was synthetically lethal with dis2Δ (Supplemental Fig. S1). The viable mmi1Δ CPF/rhn1 strains were grown in YES liquid medium and then spot-tested for growth on YES agar in parallel with the mmi1Δ single mutant (which grew well at 25°C to 37°C) (Supplemental Fig. S1). We found that: (i) mmi1Δ was synthetically sick at all temperatures with ssu72-C13S, swd22Δ, and ppn1Δ; and (ii) mmi1Δ exacerbated the temperature sensitivity of rhn1Δ such that mmi1Δ rhn1Δ cells failed to grow at 30°C (compare Supplemental Fig. S1 and Fig. 4A).

Probing the structural requirements for Mmi1’s essential function in vegetative cells

The 488-aa Mmi1 protein (Fig. 8A) consists of carboxy-terminal YTH-fold RNA binding domain that suffices for DSR recognition (Chatterjee et al. 2016; Wang et al. 2016) and an unstructured amino-terminal domain that mediates protein–protein interactions. The Mmi1 segment from aa 95–122 (underlined in Fig. 8A) suffices for interaction with Erh1 and forms an extensive hydrophobic and hydrogen-bonding interface with Erh1 in the cocystal structure (Xie et al. 2019). The Mmi1•Erh1 complex interacts physically in vivo with the multisubunit Ccr4-Not deadenylase complex (Sugiyama et al. 2016), and Mmi1 (in the absence of Erh1) is reconstituted as a stoichiometric component of a highly purified recombinant Mmi1•Ccr4-Not complex produced by coexpression using a baculovirus vector (Stowell et al. 2016). Deletion of the amino-terminal 56-aa of Mmi1 abolished interaction with Ccr4-Not (Stowell et al. 2016). In order to interrogate the contributions of the amino-
terminal region to Mmi1’s essential function in vegetative cells, we introduced cDNAs for full-length mmi1 and a series of mmi1 amino-terminal truncation alleles, each marked by a 3’ flanking hygMX drug-resistance cassette, in lieu of one chromosomal mmi1+ locus of a diploid fission yeast strain. The diploids were sporulated and large populations of random haploid progeny were screened for hygromycin resistance linked to the alleles of interest. We recovered viable haploid truncation mutants mmi1-(31-488), mmi1-(63-488), and mmi1-(94-488) at ∼50% frequency in the random spore analysis. Spot testing for growth on YES agar showed that these three truncation strains grew as well as the marked wild-type mmi1-hygMX control strain (Fig. 8B). The inessential 93-aa amino-terminal segment includes the module required for Mmi1 binding to Ccr4-Not, suggesting that this protein–protein interaction is dispensable for Mmi1 function during vegetative growth. In contrast, we were unable to recover any viable hygromycin-resistant haploids bearing the mmi1-(129-488), mmi1-(166-488), mmi1-(222-488), or mmi1-(301-488) alleles (Fig. 8). The loss of viability coincided with deletion of Mmi1 aa 94–128, which embraces the Erh1 interaction motif. Yet the lethality of mmi1-(129-488) is unlikely to reflect necessity for Erh1•Mmi1 interaction, insofar as (i) Erh1 per se is not essential and (ii) a single Mmi1 missense mutation W112A within the Erh1 binding motif that eliminates binding to Erh1 in vitro is able to support vegetative growth (Xie et al. 2019). It is conceivable that the Mmi1-(94–128) segment has a salutary effect on Mmi1 activity separate from its interaction with Erh1.

We also queried the role of RNA binding in Mmi1 vegetative function by alanine scanning of selected amino acids at the interface of the YTH domain with the DSR element 5′-U1U2A3A4A5C6 (Wang et al. 2016). We targeted RNA-binding residues Tyr352 (which makes hydrogen bonds to the A3 nucleobase), Lys436 (which makes hydrogen bonds to the U1pU2 and U2pA3 phosphates), and Tyr466 (which makes a hydrogen bond to the A4 nucleobase). As a control, we mutated Trp372, a residue conserved among YTH proteins and a key component of the aromatic cage that recognizes the modified m6A nucleobase, but which is not involved in the binding of Mmi1 to DSR elements (Chatterjee et al. 2016; Wang et al. 2016). The salient findings were that alanine substitutions for DSR-binding residues Tyr352 and Lys436 were lethal and that a Y466A mutant was viable but extremely sick (Fig. 8B). In contrast, the aromatic cage mutant W372A grew as well as wild-type at 20°C–34°C and formed larger colonies than the WT control at 37°C (Fig. 8B). Thus, DSR recognition is essential for Mmi1 to sustain vegetative growth.

**MATERIALS AND METHODS**

**Deletion of erh1**

Using PCR amplification and standard cloning methods, we constructed a plasmid in which the erh1 gene from nucleotides +1 to
Allelic exchange at the mmi1 locus

Strains harboring marked wild-type and mutated mmi1 alleles were constructed as follows. We first built an integration cassette for wild-type mmi1 that consisted of five elements in series from 5′ to 3′: (i) a 843-bp segment of genomic DNA 5′ of the mmi1″ start codon; (ii) a 1467-bp cDNA fragment encoding wild-type Mmi1, flanked by BamH I and HindIII restriction sites immediately adjacent to the translational start and stop codon, respectively; (iii) a 270-bp segment including poly(A)/termination signals from the mmi1″ gene; (iv) a hygMX gene conferring resistance to hygromycin; and (v) a 815-bp segment of genomic DNA 3′ of the mmi1″ stop codon. Amino-terminal truncation variants were generated by PCR using forward primers that introduced a BamH I site followed by a Met residue in lieu of the wild-type mmi1″ start codon. Allelic exchange was confirmed by Southern blotting after transformation of haploids. The heterozygous diploids were selected and correct integrations at the target locus were confirmed by Southern blotting. The heterozygous diploids were then sporulated and hygromycin-resistant haploids were isolated. A segment of the mmi1-hygMX locus was amplified by PCR and sequenced to verify that the desired mutations were present and to ascertain that the crossovers had occurred upstream of the translational start codon (i.e., that none of the introns were retained).

Acid phosphatase activity

Cells were grown at 30°C in YES liquid medium. Aliquots of exponentially growing cultures were harvested, washed with water, and resuspended in water. To quantify acid phosphatase activity, reaction mixtures (200 μL) containing 100 mM sodium acetate (pH 4.2), 10 mM p-nitrophenyl phosphate, and cells (ranging from 0.01 to 0.1 A600 units) were incubated for 5 min at 30°C. The reactions were quenched by addition of 1 mL of 1 M sodium carbonate, the cells were removed by centrifugation, and the absorbance of the supernatant at 410 nm was measured. Acid phosphatase activity is expressed as the ratio of A410 (p-nitrophenol production) to A600 (cells). The data shown in graphs are averages ± SEM of at least three assays using cells from three independent cultures.

Prt–pho1 reporter plasmids and assays

The prt–pho1 reporter plasmids, marked with a kanamycin-resistance gene (kanMX), were transfected into pho1Δ cells (Chatterjee et al. 2016) or pho1Δ erh1Δ cells and transformants were selected on YES agar medium containing 150 μg/mL G418. Single colonies of individual transformants were pooled (≥20) and grown in liquid YES + G418 medium to A600 of 0.5–0.8. Aliquots were harvested by centrifugation for acid phosphatase activity measurements as described above.

RNA analyses

Total RNA was extracted via the hot phenol method (Herrick et al. 1990) from 20 A600 units of yeast cells that had been grown exponentially to A600 of 0.6 to 0.8 at 30°C. Aliquots (15 μg) of total RNA were used as templates for M-MuLV reverse transcriptase-catalyzed extension of 5′ 32P-labeled oligodeoxynucleotide primers complementary to the pho1 ORF. The primer extension reactions were performed as described previously (Schwer et al. 1998) and the products were analyzed by electrophoresis of the reaction mixtures through a 22-cm 8% polyacrylamide gel containing 7 M urea in 80 mM Tris-borate, 1.2 mM EDTA. The 32P-labeled primer extension products were visualized by autoradiography of the dried gel. The primer sequences were as follows: act1 5′-GATTTCCTTCTCCATGGTCTTGTC and pho1 5′-GTGGGCACAAACGGCGCC. For northern blotting, aliquots (10 μg) of total RNA were resolved by electrophoresis through a 1.2% agarose/formaldehyde gel. After photography under UV light to visualize ethidium bromide-stained rRNAs and tRNAs, the gel contents were transferred to a Hybond-XL membrane (GE Healthcare) and hybridization was performed with a randomly primed 32P-labeled DNA fragment spanning nucleotides 590–1293 of the pho1 ORF.

Mutational effects on fission yeast growth

 Cultures of S. pombe strains were grown in YES liquid medium until A600 reached 0.6–0.8. The cultures were adjusted to A600 of 0.1 and 3 μL aliquots of serial fivefold dilutions were spotted on YES agar. The plates were photographed after incubation for 2 d at 34°C, 2.5 d at 30°C and 37°C, 4 d at 25°C, and 6 d at 20°C.

Tests of mutational synergies

Standard genetic methods were used to generate haploid strains harboring mutations/deletions in two differently marked genes. In brief, pairs of haploids with missense or null mutations were mixed on malt agar to allow mating and sporulation and then the mixture was subjected to random spore analysis. Spores (>1000) were plated on YES agar and on media selective for marked mutant alleles; the plates were incubated at 30°C for up to 5 d to allow slow growing progeny to germinate and form colonies. At least 500 viable progenies were screened by replica-plating for the presence of the second marker gene, or by sequentially replica-plating from YES to selective media. A finding that no haploids with both marker genes were recovered after 6 to 8 d of incubation at 30°C was taken to indicate synthetic lethality. By sequentially replica-plating and gauging the numbers
of colonies at each step, we ensured that wild-type (unmarked) and the differentially marked single mutant alleles were recovered at the expected frequencies. Growth phenotypes of viable double-mutants were assessed in parallel with the individual mutants and wild-type cells at different temperatures (20°C–37°C) as described above.

SUPPLEMENTAL MATERIAL
Supplemental material is available for this article.

ACKNOWLEDGMENTS
We thank Dr. Tomoyasu Sugiyama for generously providing the mmi1Δ strain SP1111. This work was supported by National Institutes of Health, National Institute of General Medical Sciences (NIH-NIGMS) grants R01-GM52470, R01-GM134021, and R35-GM126945. Received May 16, 2020; accepted June 1, 2020.

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