The yeast Aft1 transcription factor activates ribonucleotide reductase catalytic subunit *RNR1* in response to iron deficiency

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Abstract

Eukaryotic ribonucleotide reductases are iron-dependent enzymes that catalyze the rate-limiting step in the *de novo* synthesis of deoxyribonucleotides. Multiple mechanisms regulate the activity of RNRs in response to genotoxic stresses and iron deficiency. Upon iron starvation, the *Saccharomyces cerevisiae* Aft1 transcription factor specifically binds to iron-responsive *cis* elements within the promoter of a group of genes, known as the iron regulon, activating their transcription. Members of the iron regulon participate in iron acquisition, mobilization and recycling, and trigger a genome-wide metabolic remodeling of iron-dependent pathways. Here, we describe a mechanism that optimizes the activity of yeast ribonucleotide reductase when iron is scarce. We demonstrate that Aft1 and the DNA-binding protein Ixr1 enhance the expression of *RNR1* in response to iron limitation, leading to an increase in both mRNA and protein levels. By mutagenesis of the Aft1-binding sites within *RNR1* promoter, we conclude that *RNR1* activation by iron depletion is important for Rnr1 protein and deoxyribonucleotide synthesis. Remarkably, Aft1 also activates the expression of *IXR1* upon iron scarcity through an iron-responsive element located within its promoter. These results provide a novel mechanism for the direct activation of ribonucleotide reductase function by the iron-regulated Aft1 transcription factor.

Highlights

- 1) Aft1 and Ixr1 activate the expression of *RNR1* in response to iron deficiency.
- 2) Both *AFT1* and *IXR1* contain functional FeRE sites within their promoter regions.
- 3) *RNR1* activation is important for Rnr1 protein and deoxyribonucleotide synthesis.
- 4) Aft1 activates the expression of *IXR1* upon iron scarcity.

1. Introduction

Ribonucleotide reductase (RNR) is an essential enzyme that catalyzes the rate-limiting step in the *de novo* deoxyribonucleotide diphosphate (dNDP) synthesis by reducing ribonucleoside diphosphates to the corresponding deoxy forms. The class Ia of RNR is conserved from yeasts to mammals, and it is composed of a large R1 subunit, which contains the catalytic site and two allosteric effector binding sites, and a small R2 subunit, which harbors a di-iron center that is responsible for generating and maintaining a tyrosyl radical required for catalysis (reviewed in [1-3]). In the budding yeast *Saccharomyces cerevisiae*, the large R1 subunit is mainly composed of an essential Rnr1 homodimer, and the small R2 subunit is formed by an Rnr2-Rnr4 heterodimer. Yeast expresses an alternative non-essential R1 component called Rnr3, which can associate to an Rnr1 monomer forming the large R1 heterodimer Rnr1-Rnr3 [4, 5]. Recent studies have shown that Rnr1, but not Rnr3, promotes the sustained elongation of short telomeres [6, 7].

Failures to achieve adequate and balanced intracellular deoxyribonucleotide triphosphate (dNTP) levels lead to DNA damage and genomic instability, both hallmarks of cancer and aging [8, 9]. Thus, cells have developed multiple strategies to tightly control RNR activity under different conditions (reviewed in [10, 11]). First, allosteric regulation controls RNR overall activity and substrate specificity. While the allosteric specificity site regulates the balance among the four dNTP pools, the allosteric activity site controls the total dNTP pool size by monitoring the dATP/ATP ratio (reviewed in [12]). In response to DNA damage or DNA replication stress, the Mec1-Tel1/Rad53/Dun1 checkpoint kinase cascade promotes yeast RNR activity. The

Mec1 and Tel1 kinase sensors activate Rad53 central transducer, and hyperphosphorylated Rad53 activates Dun1 effector kinase by phosphorylation. Then, Dun1 checkpoint kinase enhances RNR function by multiple mechanisms including the degradation of Sml1 R1 inhibitor, the subcellular redistribution of R2 subunit, and the transcriptional activation of *RNR2-4* genes (reviewed in [10, 11]). Remarkably, *RNR1* expression is induced by DNA damage in a Dun1-independent manner. Instead, in response to genotoxic stress, Mec1 and Rad53 activate *RNR1* expression via Ixr1 protein [13]. Ixr1 was first identified by its ability to bind DNA modified by the anticancer drug cisplatin [14]. Further studies have demonstrated that Ixr1 functions as a high-mobility group transcription factor implicated in ribosomal gene transcription and in the response to hypoxia and oxidative stress [15-17]. Importantly, Ixr1 constitutively interacts with the *RNR1* promoter and enhances its expression upon genotoxic stress [13]. Consistently with a reduced *RNR1* expression, *ixr1Δ* cells result in decreased dNTP levels [13]. RNR activity is also modulated during the progress of cell cycle to adjust dNTP needs (reviewed in [11, 18]). *RNR1* mRNA levels increase more than ten-fold during the transition from G1 to S phase due to the contribution of the MBF transcription factor, a heterodimeric complex composed of the DNA-binding protein Mbp1, which binds to the MluI cell cycle box (MCB) sequence motif within the promoter of *RNR1*, and Swi6, a regulatory transactivating protein. The MBF complex coordinately controls the expression of many genes required for early cell cycle functions, including DNA replication and repair, to restrict their expression to the G1/S transition [19, 20]. Outside the G1/S phase, Stb1, a Swi6-interacting protein, mediates MBF-dependent transcriptional repression [21, 22]. Moreover, the cell cycle regulatory SBF

complex, composed of Swi6 and DNA-binding protein Swi4 participates in the transcriptional activation of the *RNR2* and *RNR3* genes in response to DNA damage [23].

Iron is an essential micronutrient for all eukaryotic organisms. Despite its abundance, the extremely low solubility of ferric iron at physiological pH frequently leads to human iron deficiency anemia, especially in pregnant women and children, and constitutes the most common and widespread nutritional disorder in the world [24]. Given that iron is an indispensable cofactor for key enzymes in DNA replication and repair, such as ribonucleotide reductase, replicative DNA polymerases, DNA primase, and various DNA repair enzymes (reviewed in [18, 25, 26]), the iron status of an organism plays a pivotal role in both the severity of infections and in the progress of DNA-related diseases such as cancer and aging. The yeast *S. cerevisiae* has been widely used to study how eukaryotic cells adapt to iron depletion. In response to iron deficiency, yeast iron-sensing Aft1 transcription factor, and to a minor extent Aft2, directly activate the expression of a group of ~35 genes known as the iron regulon, which functions include iron acquisition (e. g. Fet3-Ftr1 high-affinity iron transport complex), iron mobilization and recycling, and remodeling of iron metabolism (Cth2 mRNA-binding protein) (reviewed in [27-30]). Upon iron depletion, Aft1 protein recognizes and binds to the specific *cis* sequence PyPuCACCCPu (Py: pyrimidine; Pu: purine) called FeRE (Iron Responsive Element) found in the promoter region of iron regulon genes, and activates their transcription [31]. Upon iron sufficiency, Aft1 dissociates from DNA and the iron-deficiency response deactivates. The iron-regulated DNA-binding ability of Aft1

and Aft2 is abolished in the *AFT1-1^{up}* and *AFT2-1^{up}* alleles, which constitutively activate the iron regulon [31-33].

Multiple mechanisms optimize the activity of the iron-dependent enzyme RNR during the adaptation of yeast cells to iron deficiency. Under normal conditions, Sml1 protein directly interacts with Rnr1 to inhibit its function [34-36]. However, in response to iron deficiency, Dun1 checkpoint kinase promotes RNR function by stimulating Sml1 phosphorylation and degradation [37]. The subcellular localization of RNR subunits constitutes another crucial level for regulation. Under iron replete conditions, the large R1 catalytic subunit resides in the cytosol, whereas its small R2 subunit composed of the heterodimer Rnr2-Rnr4 is trapped in the nucleus by two different proteins: Wtm1 and Dif1. The nuclear WD40-containing protein Wtm1 interacts with the R2 subunit, anchoring it to the nucleus [38, 39], whereas the cytosolic Dif1 protein associates to the R2 subunits and drives its import to the nucleus [40, 41]. Upon iron scarcity, two mechanisms facilitate the redistribution of the Rnr2-Rnr4 heterodimer to the cytoplasm, where it assembles with the large Rnr1 homodimer, optimizing RNR function [42]. Firstly, phosphorylation of Dif1 by the Dun1 checkpoint kinase decreases the nuclear retention of R2 subunit [43]. Secondly, the RNA-binding protein Cth2, which expression is specifically activated by Aft1 in response to iron deficiency, binds to AU-rich elements within *WTM1* mRNA and promotes its degradation, facilitating to R2 relocation to the cytoplasm [42]. Therefore, both Cth2 and Dun1 dependent pathways contribute to dNTP synthesis during iron starvation [42, 43]. We have also observed that Rnr1 protein levels increase in response to iron deficiency in a Dun1 and Rad53 independent manner [37]. However, the molecular mechanisms that activate Rnr1

expression when iron is scarce are currently unknown. In this study, we characterize a novel mechanism that yeast cells utilize to optimize RNR function when iron becomes scarce. Specifically, we reveal that the transcriptional activation of *RNR1* gene by Aft1 is required to provide adequate intracellular dNTP levels during the adaptation to iron deficiency.

2. Materials and Methods

2.1. Yeast strains, plasmids and growth conditions. The yeast strains and plasmids used in this study are listed in Supplemental Tables S1 and S2, respectively. The mutagenesis of *RNR1* FeREs (315mt, 470mt, 315/470mt) and *IXR1* FeRE (464mt) was generated by the overlap extension method [44]. All PCR amplifications were performed with the Expand High Fidelity PCR System (Sigma-Aldrich), and the cloned inserts were sequenced. In most cases, yeast cell precultures were incubated overnight at 30°C in synthetic complete SC medium lacking specific requirements when necessary. Then, they were reinoculated at OD_{600nm} = 0.2-0.4 and incubated for the indicated time in SC (+Fe) or SC supplemented with 100 µM of the Fe²⁺-specific chelator bathophenanthroline disulfonate or BPS (iron-deficient conditions: -Fe) to exponential phase before processing. For long-term iron-deficient experiments, yeast precultures were reinoculated at OD_{600nm} = 0.002 in iron-sufficient conditions (+Fe) and at OD_{600nm} = 0.02 in SC supplemented with 100 µM BPS and were cultivated for the indicated time. The treatments with 0.02% MMS, 0.2 M HU and 0.2 mg/L 4-NQO were performed during the last 2 hours of SC incubation.

2.2. RNA analyses. Total yeast RNA isolation, reverse transcription and real-time quantitative PCR (RT-qPCR) were performed as previously described [37]. An oligo-dT primer was used for the reverse transcription and specific primer pairs were used for the RT-qPCR (listed in Supplemental Table S3).

2.3. Protein analyses. Total protein extractions were performed with the alkali method as described [45]. Proteins were resolved in SDS-PAGE gels and transferred to nitrocellulose membranes. Rnr1 protein was detected with anti-Rnr1 (AS16 3639; Agrisera) primary and HRP-labeled anti-rabbit secondary (GE Healthcare Life Sciences) antibodies. Pgk1 protein, used as a loading control to normalize Rnr1 protein levels, was detected with anti-Pgk1 (22C5D8; Invitrogen) primary and HRP-labeled anti-mouse secondary (GE Healthcare Life Sciences) antibodies. ECL Select Western blotting detection kit was used (GE Healthcare Life Sciences). Images were obtained with an ImageQuant LAS 4000 mini Biomolecular Imager (GE Healthcare Life Sciences) and signals were quantified with ImageQuant TL analysis software (GE Healthcare Life Sciences). The Rnr1 and Pgk1 signals for many exposition times were determined and only the average of those within a linear range were used to obtain the final Rnr1/Pgk1 signal corresponding to each biological replicate.

2.4. Miscellaneous. Polyribosome gradient profiles and analysis [46], dATP and dCTP measurements cytoplasm [42], and β -galactosidase assays [47] were performed as previously described.

2.5. Statistical analyses. The data and error bars represent the average and standard deviations of at least three independent biological samples. Tailed t-student tests were applied to evaluate statistical significance. In Figures 1, 8

and S1, the asterisk (*) above the bars indicates statistically significant differences with p -value < 0.05 . The bars in the rest of Figures are labeled with different letters when the statistical test indicates that they are significantly different from each other (p -value < 0.05). When at least one letter over two bars is the same, there are no significant differences.

3. Results

3.1. *RNR1* is transcriptionally induced at long-term iron deficiency

We have previously described that the protein levels of the catalytic subunit of yeast ribonucleotide reductase Rnr1 increase in response to iron depletion [37]. To investigate the mechanisms that regulate the abundance of Rnr1 protein when iron bioavailability is altered, we performed a kinetic in iron-deficient conditions. For this purpose, we cultivated the wild-type BY4741 yeast strain in synthetic complete (SC) medium supplemented with 100 μ M of the Fe^{2+} -specific chelator bathophenanthroline disulfonate (BPS) and collected cells up to 24 hours. Then, we extracted total RNA and determined the mRNA levels of *RNR1*, *FET3* (a multicopper ferroxidase implicated in iron acquisition and transcriptionally activated in response to iron deficiency), and *ACT1* (actin) by RT-qPCR. The expression of *ACT1* suffered a slight decline at the latter times of iron limitation (Figure 1). As expected, the expression of the iron-regulated gene *FET3* showed a significant increase after 6 hours of iron depletion (Figure 1). Although no consistent changes in *RNR1* expression were observed during the initial hours of iron starvation, the abundance of the *RNR1* mRNA increased after a sufficiently prolonged iron deficiency, reaching its highest level around 15 hours after BPS addition (Figure 1). These results suggest that the increase in *RNR1* mRNA levels observed at long-term iron deficiency could explain the up-regulation of Rnr1 protein previously reported [37].

Figure 1. *RNR1* mRNA levels increase in response to severe iron deficiency. The wild-type BY4741 yeast strain was cultivated at 30°C for 24 hours in SC with 100 μ M BPS (-Fe). Cells were collected at the following times (hours): 0, 6, 9, 12, 15, 18, 21 and 24, total RNA was extracted as described in Materials and Methods, and *ACT1* (blue), *RNR1* (orange) and *FET3* (purple) mRNA levels were determined by RT-qPCR using specific primers. Mean values and standard deviations from at least three independent experiments are shown and referred to those obtained at time zero. Each colored asterisk (*) indicates statistically significant differences (p -value < 0.05) to the corresponding time zero.

To investigate whether the increase in *RNR1* mRNA levels upon iron starvation occurred at the transcriptional level, we fused the *RNR1* promoter region to the *lacZ* reporter gene (*P_{RNR1}-lacZ* construct) and we determined β -galactosidase activity. We observed that wild-type cells transformed with *P_{RNR1}-lacZ* increased β -galactosidase activity under low iron conditions, suggesting that *RNR1* up-regulation depends on its promoter region, probably at the

transcriptional level (Figure 2A). Previous studies have shown that the levels of Rnr1 protein increase in response to multiple genotoxic stresses [13]. To compare the expression levels of *RNR1* under iron deficiency to other activation conditions, we incubated wild-type cells transformed with *P_{RNR1}-lacZ* for 2 hours in the presence of 0.2 M of the replication-blocking agent hydroxyurea (HU) or 0.02% and 2 mg/L of the DNA-damaging agents methyl methanesulfonate (MMS) and 4-nitroquinoline 1-oxide (4-NQO), respectively (Figure 2A). β -galactosidase activities indicated that the degree of transcriptional up-regulation of *RNR1* expression that occurs in response to iron depletion is similar to the activation achieved by genotoxic stresses (Figure 2A).

We decided to investigate the potential transcription factors that could be implicated in the up-regulation of *RNR1* when iron is scarce. At the G1/S phase of the cell cycle, the transcription of *RNR1* is controlled by MBF, a dimeric transcription factor composed of Swi6 and Mbp1 [19, 20], whereas the cell cycle regulated SBF complex activates *RNR2* and *RNR3* transcription by DNA damage [21, 22]. To ascertain whether these transcription factors contributed to the activation of *RNR1* in response to iron starvation, we determined the β -galactosidase activity of *mbp1* Δ , *swi6* Δ and *swi4* Δ mutants expressing *P_{RNR1}-lacZ* under iron-sufficient and deficient conditions. We observed that *RNR1* expression was induced in all the mutants, suggesting that these regulatory factors were not required for *RNR1* activation by iron depletion (Figure 2B). Curiously, *RNR1* induction was higher in the *swi6* Δ mutant than in the wild-type cells (Figure 2B). *RNR1* expression is also transcriptionally up-regulated in response to genotoxic stress via the Mec1-Tel1/Rad53/Dun1 checkpoint kinase pathway [13]. Our previous studies had shown that the increase in Rnr1 protein

observed in iron-depleted conditions was independent of *DUN1* and *RAD53* [37]. Therefore, we now assayed *RNR1* activation in the absence of Mec1 and Tel1 kinases, which function upstream of Rad53. For this purpose, we used an *sml1Δmec1Δtel1Δ* triple mutant, since the *mec1Δ* mutant is lethal but rescued by *SML1* deletion [35]. The *sml1Δ* single mutant was also used as a control strain. No defect in *RNR1* induction by low iron was observed for *sml1Δ* or *sml1Δmec1Δtel1Δ* mutants (Figure 2C).

Figure 2. The *RNR1* promoter facilitates its induction by iron deficiency in an Mbp1, Swi4, Swi6, Mec1 and Tel1 independent manner. (A) Wild-type BY4741 cells transformed with *P_{RNR1}-lacZ* plasmid (pSP985) were cultivated at 30°C for 15 hours in SC-Ura (+Fe) or in SC-Ura with 100 µM BPS (-Fe). The treatment with 0.02% MMS, 0.2 M HU or 2 mg/L 4-NQO was performed during the last 2 hours of SC-Ura incubation. **(B)** Wild-type BY4741, *mbp1*Δ (SPY857), *swi4*Δ (SPY758), and *swi6*Δ (SPY859) cells transformed with *P_{RNR1}-lacZ* (pSP985) were cultivated at 30°C for 15 h in SC-Ura (+Fe) or SC-Ura with 100 µM BPS (-Fe). **(C)** Wild-type BY4741, *sml1*Δ (SPY571), and *sml1*Δ*mec1*Δ*tel1*Δ (SPY573), cells transformed with *P_{RNR1}-lacZ* (pSP985) were cultivated as in panel B. In all cases, β-galactosidase activity of the *lacZ* reporter was determined, and the mean values and standard deviations from two to four independent experiments are shown and referred to the wild-type in +Fe conditions. Different letters above the bars represent significant differences among groups (*p*-value < 0.05). When there are no significant differences, at least one letter over the two bars is the same.

Collectively, these results strongly suggest that previously described activators of *RNR1*, including the MBF and SBF complexes and the checkpoint DNA damage cascade components are not implicated in its up-regulation by iron deficiency.

3.2. Aft1 and Ixr1 activate the expression of *RNR1* gene in response to iron deficiency

In response to iron starvation, the yeast Aft1 transcription factor accumulates to the nucleus and activates the expression of the iron regulon

genes [31, 48, 49]. To test whether Aft1 was implicated in the up-regulation of *RNR1* in response to iron limitation, we determined *RNR1* transcript levels in *aft1*Δ mutant cells under both iron sufficiency and deficiency. Interestingly, the increase in *RNR1* mRNA levels that occurred upon iron starvation was dramatically affected in the *aft1*Δ mutant (Figure 3A). A similar result was obtained for β-galactosidase activity when the *aft1*Δ mutant was transformed with *P_{RNR1}-lacZ* (Figure 3B). Although to a minor extent, the Aft2 transcription factor also contributes to the transcription of the iron regulon upon iron starvation, especially on genes implicated in intracellular iron transport [33, 50, 51]. Therefore, we explored the involvement of Aft2 in the regulation of *RNR1* by iron by expressing *P_{RNR1}-lacZ* in *aft2*Δ and *aft1*Δ*aft2*Δ cells. Remarkably, we observed that the expression of *RNR1* was higher in the *aft2*Δ mutant than in wild-type cells, which is probably due to a more severe iron-deficiency perceived by the *aft2*Δ cells (Figure S1). No *RNR1* induction was observed in the *aft1*Δ*aft2*Δ double mutant (Figure S1). We included cells expressing the constitutively active alleles *AFT1-1^{up}* and *AFT2-1^{up}*, which activate the iron regulon independently of iron bioavailability [33, 48]. Strickingly, both alleles were able to up-regulate *RNR1* under either iron-sufficient or deficient conditions (Figure S1). Altogether, these results indicate that Aft1 plays the major role in the transcriptional activation of *RNR1* in response to iron deficiency, whereas Aft2 provides a minor contribution.

Figure 3. Aft1 and Ixr1 transcription factors activate *RNR1* expression in response to iron deficiency. Wild-type BY4741, *aft1*Δ (SPY28), *ixr1*Δ (SPY741), and *aft1*Δ*ixr1*Δ (SPY945) strains transformed with *P_{RNR1}-lacZ* plasmid (pSP985) were cultivated as described in Figure 2B. **(A)** mRNA levels. Total RNA was extracted and *RNR1* and *ACT1* mRNA levels were analyzed by RT-qPCR. **(B)** β-galactosidase

activity was determined. **(C)** Total protein extracts were obtained and Rnr1 and Pgk1 protein levels were determined by Western blot. In all cases the mean values and standard deviations from two or three independent experiments are shown and referred to the wild-type in +Fe conditions. Different letters above the bars represent significant differences among groups (p -value < 0.05). When there are no significant differences, at least one letter over the two bars is the same.

Previous studies had shown that the DNA-binding protein Ixr1 participates in the basal and transcriptional activation of *RNR1* in response to genotoxic stresses [13]. Therefore, we wondered whether Ixr1 would also participate in the regulation of *RNR1* by iron limitation. With this purpose, we assessed *RNR1* mRNA levels in *ixr1* Δ and *aft1* Δ *ixr1* Δ cells grown under iron-sufficient and deficient conditions. We observed that the *ixr1* Δ mutant displayed a defect in the induction of *RNR1* by low iron similar to the *aft1* Δ mutant (Figure 3A). Moreover, *RNR1* induction was completely abolished in the *aft1* Δ *ixr1* Δ double mutant (Figure 3A). Similar results were obtained with cells expressing *P_{RNR1}-lacZ*, although in this case a slight decrease in the basal expression of *RNR1* was observed for cells lacking *IXR1*, as previously described ([13]; Figure 3B). Finally, we determined Rnr1 protein levels in these strains under both plus and minus iron. Consistent with the *lacZ* and mRNA data, Rnr1 protein levels increased in wild-type cells cultivated in iron-deficient conditions (Figure 3C). Both *aft1* Δ and *ixr1* Δ mutants showed a significant decrease in Rnr1 protein abundance in iron deficiency, which was more pronounced in the *aft1* Δ *ixr1* Δ double mutant (Figure 3C). The basal levels of Rnr1 protein decreased in the cells that lacked *IXR1*, as previously described ([13]; Figure

3C). Taken together, these results highlight that both Aft1 and Ixr1 are essential for the proper activation of *RNR1* in response to iron-limiting growth conditions.

3.3. Two iron regulatory elements within the *RNR1* promoter enable its transcriptional activation in response to iron deficiency

To investigate the molecular mechanisms that enable the Aft1 transcription factor to activate the expression of *RNR1* in response to iron deficiency, we examined its promoter looking for potential Aft1-binding sites (FeREs) with the consensus sequence PyPuCACCCPu (where Py is a pyrimidine and Pu a purine). Although they did not completely fulfill a consensus FeRE, we found the AACACCCA and GGCACCCA sequences at 315 and 470 nucleotides upstream of *RNR1* start codon, respectively. To ascertain whether these sequences participated in the up-regulation of *RNR1*, we mutagenized both sites separately and simultaneously (Figure 4A). Then, we cloned the different *RNR1* promoter mutants (*RNR1-315mt*, *RNR1-470mt* and *RNR1-315/470mt*) upstream of the *lacZ* reporter gene and expressed them in yeast cells. We observed that the mutagenesis of either one or both sites did not alter *RNR1* expression under iron replete conditions (Figure 4B). More important, the mutagenesis of either site similarly decreased β -galactosidase activity upon iron starvation (Figure 4B). Importantly, the double 315/470mt mutant construct displayed the lowest *lacZ* expression levels under iron-deficient conditions, although a slight induction was still observed (Figure 4B). From these results, we conclude that both *cis* elements participate in the up-regulation of *RNR1* when iron is scarce.

Figure 4. *RNR1* expression in response to iron deficiency depends on two FeRE sites within its promoter. (A) Schematic representation of the constructs containing the original (white box) and mutated (black box) versions of the FeRE sites in *RNR1* promoter fused to *lacZ*. Two potential FeRE sites located 315 and 470 nucleotides upstream of *RNR1* start codon were separately or simultaneously mutagenized to obtain the $P_{RNR1-315mt}$ -*lacZ* (315mt), $P_{RNR1-470mt}$ -*lacZ* (470mt), and $P_{RNR1-315/470mt}$ -*lacZ* (315/470mt) constructs. Pu means purine and Py pyrimidine. **(B)** Wild-type BY4741 cells transformed with P_{RNR1} -*lacZ* (WT: pSP985), 315mt (pSP1028), 470mt (pSP1015) or 315/470mt (pSP1038) constructs were cultivated and analyzed as described in Figure 2B. The mean values and standard deviations from three independent

experiments are shown and referred to the wild-type in +Fe. Different letters above the bars represent significant differences among groups (p -value < 0.05).

3.4. *IXR1* expression is induced in response to iron deficiency

Since *lxr1* is implicated in the up-regulation of *RNR1* when iron levels are low (Figure 3), we decided to study *IXR1* gene regulation by iron availability. For this purpose, we analyzed *IXR1* mRNA levels both in iron-sufficient and iron-deficient conditions. Interestingly, we discovered that *IXR1* transcript abundance increased 2-fold upon iron depletion (Figure 5A). Moreover, we observed that a strain lacking *AFT1* was unable to properly induce *IXR1* expression in response to iron deficiency, strongly suggesting that Aft1 transcription factor was responsible for *IXR1* activation (Figure 5A). This finding prompted us to look for potential Aft1-binding sites within the *IXR1* promoter. We identified a CACACCCA sequence at 464 nucleotides upstream of *IXR1* start codon, which perfectly matched the FeRE consensus. Next, we constructed a plasmid containing the wild-type and the FeRE mutated (464mt) *IXR1* promoter fused to the *lacZ* coding sequence (Figure 5B). We measured β -galactosidase activity in wild-type (*AFT1/IXR1*) and *aft1* Δ (*aft1* Δ /*IXR1*) strains transformed with *P_{IXR1}-lacZ*, and wild-type cells expressing *P_{IXR1-464mt}-lacZ* (*AFT1/464mt*). All the strains were cultivated under iron sufficiency and deficiency conditions. As shown in Figure 5C, the induction of *IXR1* expression by low iron was not obtained in cells lacking either *AFT1* (*aft1* Δ /*IXR1*) or the *IXR1* FeRE (*AFT1/464mt*). These results firmly suggest that the Aft1 transcription factor activates the expression of *IXR1* gene in response to iron starvation via a FeRE within its promoter region.

Figure 5. Aft1 and a FeRE site are necessary for *IXR1* activation by low iron. (A)

Wild-type BY4741 and *aft1*Δ (SPY28) cells were cultivated as described in Figure 2B and total RNA was extracted. *IXR1/ACT1* mRNA levels were analyzed by RT-qPCR.

(B) Schematic representation of the plasmids containing the original (white box) and the mutated (black box) version of the FeRE site in *IXR1* promoter fused to *lacZ*. A

potential FeRE site located 464 nucleotides upstream of *IXR1* start codon was mutagenized to obtain the *P_{IXR1-464mt}-lacZ* (464mt) construct. **(C)** Wild-type cells transformed with either *P_{IXR1}-lacZ* (pSP1029) plasmid (*IXR1-AFT1*) or the 464mt (pSP1030) construct (*464mt-AFT1*), and the *aft1*Δ mutant (SPY28) transformed with *P_{IXR1}-lacZ* (*IXR1-aft1*Δ), were cultivated and analyzed as described in Figure 2B. In all cases the mean values and standard deviations from three independent experiments are shown and referred to the wild-type in +Fe. Different letters above the bars represent significant differences among groups (*p*-value < 0.05). When there are no significant differences, at least one letter over the two bars is the same.

3.5. A general decrease in bulk translation limits Rnr1 protein levels under iron deficiency

To further ascertain the relevance of the FeRE sites in *RNR1* induction by iron depletion, we mutated both sequences in the yeast genome to obtain the RNR1-315/470mt strain. Furthermore, we constructed an RNR1-WT control strain by inserting the same selection marker in an identical position upstream *RNR1* promoter. Both strains were cultivated in iron-sufficient and iron-deficient conditions, and *RNR1* mRNA levels were determined. In addition to 15 hours, we decided to analyze a less severe iron depletion (6 hours). We observed a 2.5-fold and 17-fold increase in *RNR1* transcript abundance after 6 and 15 hours of iron limitation, respectively (Figure 6A). As expected, the RNR1-315/470mt strain displayed an important defect in the up-regulation of the *RNR1* mRNA by iron deficiency (Figure 6A). This result confirms the critical role that the FeRE sites play in the up-regulation of *RNR1* mRNA when iron becomes scarce. Then, we studied how the FeREs influenced Rnr1 protein synthesis in

these growth conditions. As previously reported, Rnr1 protein levels increased in response to iron deficiency ([37]; Figure 6B). Importantly, we observed that the RNR1-315/470mt strain displayed decreased levels of Rnr1 protein under iron-deficient conditions as compared to wild-type cells (Figure 6B). Taken together, these results demonstrate that the mutagenesis of the Aft1-binding sites within the *RNR1* promoter significantly diminish Rnr1 protein abundance when iron availability is limited.

Figure 6. The FeRE sites within *RNR1* promoter are important for Rnr1 protein synthesis in iron-deficient conditions. The wild-type FeRE strain (RNR1-WT: SPY985) and the 315/470 mutated FeRE strain (RNR1-315/470mt: SPY986) were cultivated at 30°C in SC (+Fe), and 6 and 15 hours in SC with 100 μ M BPS (-Fe). **(A)** mRNA levels. *RNR1/ACT1* mRNA levels were analyzed by RT-qPCR. **(B)** Rnr1 protein

levels. A specific Rnr1 antibody was used to determine protein levels. All values are represented as relative to those of the RNR1-WT strain grown under +Fe conditions. The average and standard deviation of at least three independent experiments is represented. Different letters indicate significant differences (p -value < 0.05), whereas at least one letter over the two bars is the same when there are no significant differences.

During the progress of iron deficiency, we noticed that *RNR1* mRNA abundance dramatically increased from 6 to 15 hours of iron deficiency (Figure 6A), whereas the corresponding Rnr1 protein levels diminished between these two time points (Figure 6B). We have recently observed that the efficiency of global translation gradually decreases during the progress of iron deficiency [52]. To investigate if the decrease in Rnr1 protein abundance was due to defects in bulk translation, we grew BY4741 wild-type cells under iron sufficiency and iron depletion, and obtained polysome profiles (Figure 7A-7C). After 6 hours of iron deficiency, we observed a decrease in polysome abundance, which was more severe after 15 hours of depletion (Figure 7A-C). Quantification of the polysomes/monosomes (40S + 60S + 80S) ratio (P/M ratio) corroborated this gradual decrease (Figure 7D). Then, we determined the distribution of the *ACT1* mRNA among the different polysomal fractions. Our results showed that *ACT1* was highly associated with polyribosomes under both iron sufficiency and 6 hours of iron deficiency, but shifted to lighter polysomal fractions when the iron starvation persisted (Figure 7E). Importantly, under iron-sufficient conditions, *RNR1* mRNAs were mostly associated with heavy polysomal fractions (Figure 7F), which is indicative of an efficient translation.

However, after 6 hours of iron depletion, *RNR1* transcript shifted to lighter polysomal fractions, and accumulated at the monosomal 80S fraction upon 15 hours of iron limitation (Figure 7F). Taken together, these results indicate that the general drop in protein synthesis that yeast cells experience in response to severe iron depletion affects Rnr1 protein levels.

Figure 7. The translation of *RNR1* mRNA decreases in severe iron-deficient conditions. The wild-type BY4741 yeast strain was cultivated as described in Figure 6.

Polysomal fractionation was carried out as described in Materials and Methods. The absorbance at 260 nm (A_{260nm}) profiles after gradient fractionation in +Fe (**A**), 6 hours in –Fe (**B**) and 15 hours in –Fe conditions (**C**) are represented. The ribosomal subunits (40S and 60S), monosomes (80S) and polysomes are indicated. A representative polysome profile from at least three biologically independent replicates is shown. The average polysomes/monosomes (40S + 60S + 80S) ratio (P/M) is indicated in each condition with its respective standard deviation (**D**). The RNA of individual fractions was extracted and *ACT1* (**E**) and *RNR1* (**F**) mRNA levels were analyzed by RT-qPCR as described in Materials and Methods.

3.6. *RNR1* activation by iron depletion is important for dNTP synthesis

To explore the physiological relevance of *RNR1* activation by iron depletion, we first determined the expression levels of *RNR3*, the additional RNR catalytic subunit. We observed that *RNR3* transcript levels under iron deficiency were slightly (1.3 increase) but significantly higher in the *RNR1*-315/470mt mutant than in wild-type cells (Figure S4). This observation suggests that yeast cells lacking *RNR1* FeRE sites could be perceiving a defect in Rnr1 function or expression when iron is scarce that is being compensated by increasing *RNR3* expression. Therefore, we decided to determine the levels of dATP and dCTP during the progress of iron deficiency in cells expressing either the wild-type or the 315/470mt *RNR1* allele. As previously reported, we observed that 6 hours of iron depletion increased dATP and dCTP levels by approximately 2-fold (Figure 8), probably due to multiple mechanisms previously described that optimize yeast RNR activity under these growth conditions [37, 42, 43]. Consistently with Rnr1 protein abundance, dATP and

dCTP levels decreased at a more severe iron starvation (Figure 8). Importantly, the up-regulation in dATP and dCTP levels observed for wild-type cells at 6 hours of iron deficiency did not occur in the RNR1-315/470mt mutants, which exhibited a defect in dATP and dCTP abundance (Figure 8). These results demonstrate that the up-regulation of *RNR1* via FeREs is required to properly synthesize dNTPs under iron-deficient conditions.

Figure 8. The *RNR1* FeRE sites are important for appropriate dNTP synthesis under iron deficiency. The wild-type FeRE strain (RNR1-WT: SPY985) and the 315/470 mutated FeRE strain (RNR1-315/470mt: SPY986) were cultivated at 30°C in SC (+Fe), and 6 and 15 hours in SC with 100 μ M BPS (-Fe). dATP and dCTP levels were determined by the DNA polymerase-based enzymatic assay. All values are represented as relative to those of the RNR1-WT strain under +Fe conditions. The average and standard deviation of at least three independent experiments is represented. The asterisk (*) indicates statistically significant fold induction (p -value < 0.05).

4. Discussion

Although class Ia RNRs use iron as an essential cofactor for catalysis, little is known about the mechanisms that control RNR function upon iron deficiency. In this manuscript, we explore the mechanisms that activate yeast Rnr1 expression when iron is scarce. We first established that RNR activation by low iron occurs at the mRNA level through a mechanism that involves *RNR1* promoter region and Aft1 transcription factor (Figures 1 to 3). However, no perfect PyPuCACCCPu FeRE sites were observed in the promoter region of *RNR1* gene. A visual analysis of *RNR1* promoter revealed two non-canonical Aft1-binding sites, which lacked the first pyrimidine residue, located at 315 and 470 nucleotides upstream *RNR1* start codon, which turned out to be required for *RNR1* up-regulation and dNTP synthesis during iron starvation (Figures 4, 6 and 8), suggesting a direct implication of Aft1 in *RNR1* activation. Yeast Aft1 targets have been thoroughly studied over the years (reviewed in [30]). However, *RNR1* has not been previously reported as an Aft1-target gene probably because its up-regulation only occurs upon a severe iron deficiency, which could be a consequence of the presence of imperfect FeREs. Therefore, *RNR1* can be considered now a new member of the iron regulon set of genes, which are activated in response to iron depletion in an Aft1/Aft2-dependent manner.

The basal and DNA-damage induced expression of *RNR1* depends on the DNA-binding protein Ixr1 [13]. We observed that Ixr1 also participates in the up-regulation of Rnr1 upon iron limitation at the level of mRNA and protein via *RNR1* promoter (Figure 3). Remarkably, *IXR1* mRNA levels increase in response to iron deficiency in a manner dependent of Aft1 transcription factor

and a perfect FeRE (CACACCCA) located 464 nucleotides upstream *IXR1* ATG (Figure 5). These results suggest that Aft1 activates *RNR1* function at multiple levels: indirectly via (1) Cth2, which down-regulates Wtm1 protein levels allowing the relocalization of R2 subunit to the cytoplasm [42] and (2) *lxr1*, which increases *RNR1* expression (Figure 3); and (3) directly, by activating *RNR1* transcription (Figure 3). Interestingly, the *lxr1* effect on *RNR1* expression by iron deficiency is not only due to its Aft1-dependent up-regulation since the *aft1Δlxr1Δ* double mutant displays a more severe defect than the *aft1Δ* and *lxr1Δ* single mutants (Figure 3). We propose the following model for the activation of *RNR1* expression in response to iron deficiency (Figure 9). Upon iron scarcity, the Aft1 transcription factor binds to FeREs within *IXR1* and *RNR1* promoter regions and activates their expression. The increased levels of *lxr1* protein further contribute to *RNR1* expression. Finally, the elevated Rnr1 protein levels improve the assembly of functional RNR holoenzymes that, in combination with other regulatory mechanisms, leads to optimized dNTP synthesis.

Figure 9. Proposed model for *RNR1* transcriptional activation in response to iron deficiency. In response to iron-deficient conditions, the iron-regulated Aft1 transcription factor activates the expression of *IXR1* and *RNR1* genes. Novel FeRE sites within *RNR1* and *IXR1* promoter regions facilitate the Aft1-dependent activation of *RNR1* and *IXR1* expression. Ixr1 contributes to *RNR1* activation in low iron conditions. The RNR small subunit formed by Rnr2 and Rnr4 assembles with the large Rnr1-Rnr1 subunit to obtain a cytosolic and functional RNR holoenzyme. NTP: nucleoside triphosphate; dNTP: deoxiribonucleotide triphosphate.

During the analysis of *RNR1* expression, we observed that the up-regulation of *RNR1* transcript levels at 15 hours of iron depletion did not translate into an equivalent increase in Rnr1 protein abundance (Figure 3). Therefore, we decided to study *RNR1* mRNA and protein levels at shorter times of iron deficiency. We observed that, at 6 hours of treatment, the increase in *RNR1* mRNA levels rendered a parallel increase in Rnr1 protein (Figure 6). Recent studies have shown that bulk protein synthesis diminishes during the

progress of iron deficiency [52, 53]. Consequently, we used polyribosome profiling to monitor the stage of global translation during the progress of iron deficiency. The polyribosome profiles of cells cultivated under iron-limited conditions accumulated monosomes and lowered the abundance of polysomes, which is indicative of global translation repression (Figure 7; [52]). The distribution of *RNR1* transcripts was also consistent with a decrease in its translation efficiency as the iron deficiency proceeded (Figure 7). Previous reports have shown that the tRNA methyltransferase Trm9 selectively improves the translation of mRNAs enriched with specific arginine and glutamic acid codons such as *RNR1* transcript [54]. This could be a possible explanation for the increase in Rnr1 protein levels observed at 6 hours of iron deficiency, when only a slight rise in mRNA levels is observed. However, it is currently unknown whether this or other specific mechanisms function to improve the translation of *RNR1* under iron deficient conditions. In any case, the Aft1-dependent up-regulation of *RNR1* expression increases the final concentration of Rnr1 and dNTPs when the bioavailability of iron is limited.

Iron is an essential requirement for the survival of all eukaryotic organisms, and a competition for iron acquisition is established between hosts and pathogens. As a defense mechanism, mammals dramatically decrease the bioavailability of circulating iron during infections (reviewed in [55, 56]). The human opportunistic pathogen *Candida glabrata*, which is responsible for both superficial and systemic infections, is evolutionarily close to baker's yeast. Multiple aspects of the iron homeostasis network seem to be conserved between *C. glabrata* and *S. cerevisiae* (reviewed in [56]). Interestingly, we have observed that the promoter of *C. glabrata RNR1* gene contains a perfect

CACACCCA sequence located at 305 nucleotides upstream its start codon. Moreover, recent genome-wide studies indicate that *C. glabrata* *RNR1* expression is induced in response to iron deficiency in an Aft1/Aft2-dependent manner [57, 58]. These observations suggest that the mechanisms that regulate RNR upon changes in iron availability could be conserved between *S. cerevisiae* and *C. glabrata*, being an excellent target for the development of new strategies to combat infections caused by this pathogenic yeast.

The results presented in this report uncover two additional targets for the yeast iron-sensing transcription factor Aft1, *RNR1* and *IXR1*, and provide a novel mechanism for the regulation of yeast RNR under low iron conditions. Further studies would be necessary to decipher how the multiple pathways that regulate RNR function coordinate in response to iron deficiency, and whether these mechanisms are conserved in fungal pathogens or human cells.

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6. Author Contributions

Cristina Ros-Carrero: Investigation, Writing – review and editing, visualization; Analysis and interpretation of data. Lucía Ramos-Alonso: Investigation, Writing – review and editing. Antonia M. Romero: Investigation, Writing – review and editing. M. Carmen Bañó: Investigation, Writing – review and editing. María T. Martínez-Pastor: Writing – review and editing, Project administration. Sergi Puig: Conceptualization, Resources, Writing - original draft, Funding acquisition, Project administration, visualization.

7. References

- [1] M. Kolberg, K.R. Strand, P. Graff, K.K. Andersson, Structure, function, and mechanism of ribonucleotide reductases, *Biochim Biophys Acta*, 1699 (2004) 1-34.
- [2] P. Nordlund, P. Reichard, Ribonucleotide reductases, *Annu Rev Biochem*, 75 (2006) 681-706.
- [3] J.A. Cotruvo, J. Stubbe, Class I ribonucleotide reductases: metallocofactor assembly and repair in vitro and in vivo, *Annu Rev Biochem*, 80 (2011) 733-767.
- [4] S.J. Elledge, R.W. Davis, Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase, *Genes Dev*, 4 (1990) 740-751.
- [5] V. Domkin, L. Thelander, A. Chabes, Yeast DNA damage-inducible Rnr3 has a very low catalytic activity strongly stimulated after the formation of a cross-talking Rnr1/Rnr3 complex, *J Biol Chem*, 277 (2002) 18574-18578.
- [6] A. Maicher, I. Gazy, S. Sharma, L. Marjavaara, G. Grinberg, K. Shemesh, A. Chabes, M. Kupiec, Rnr1, but not Rnr3, facilitates the sustained telomerase-dependent elongation of telomeres, *PLoS Genet*, 13 (2017) e1007082.
- [7] A. Maicher, M. Kupiec, Rnr1's role in telomere elongation cannot be replaced by Rnr3: a role beyond dNTPs?, *Curr Genet*, 64 (2018) 547-550.
- [8] H. Niida, M. Shimada, H. Murakami, M. Nakanishi, Mechanisms of dNTP supply that play an essential role in maintaining genome integrity in eukaryotic cells, *Cancer Sci*, 101 (2010) 2505-2509.
- [9] A. Chabes, B. Georgieva, V. Domkin, X. Zhao, R. Rothstein, L. Thelander, Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase, *Cell*, 112 (2003) 391-401.
- [10] E. Guarino, I. Salguero, S.E. Kearsey, Cellular regulation of ribonucleotide reductase in eukaryotes, *Semin Cell Dev Biol*, 30 (2014) 97-103.
- [11] N. Sanvisens, R. de Llanos, S. Puig, Function and regulation of yeast ribonucleotide reductase: cell cycle, genotoxic stress and iron availability, *Biomed J*, 36 (2013) 51-58.
- [12] P. Reichard, Ribonucleotide reductases: substrate specificity by allostery, *Biochem Biophys Res Commun*, 396 (2010) 19-23.
- [13] O. Tsaponina, E. Barsoum, S.U. Astrom, A. Chabes, Ixr1 is required for the expression of the ribonucleotide reductase Rnr1 and maintenance of dNTP pools, *PLoS Genet*, 7 (2011) e1002061.
- [14] S.J. Brown, P.J. Kellett, S.J. Lippard, Ixr1, a yeast protein that binds to platinated DNA and confers sensitivity to cisplatin, *Science*, 261 (1993) 603-605.
- [15] J.R. Lambert, V.W. Bilanchone, M.G. Cumsky, The ORD1 gene encodes a transcription factor involved in oxygen regulation and is identical to IXR1, a gene that confers cisplatin sensitivity to *Saccharomyces cerevisiae*, *Proc Natl Acad Sci U S A*, 91 (1994) 7345-7349.
- [16] A. Vizoso-Vazquez, M. Lamas-Maceiras, M. Becerra, M.I. Gonzalez-Siso, E. Rodriguez-Belmonte, M.E. Cerdan, Ixr1p and the control of the *Saccharomyces cerevisiae* hypoxic response, *Appl Microbiol Biotechnol*, 94 (2012) 173-184.
- [17] A. Vizoso-Vazquez, M. Lamas-Maceiras, M.I. Gonzalez-Siso, M.E. Cerdan, Ixr1 Regulates Ribosomal Gene Transcription and Yeast Response to Cisplatin, *Sci Rep*, 8 (2018) 3090.
- [18] S. Puig, L. Ramos-Alonso, A.M. Romero, M.T. Martinez-Pastor, The elemental role of iron in DNA synthesis and repair, *Metallomics*, 9 (2017) 1483-1500.
- [19] C. Koch, T. Moll, M. Neuberg, H. Ahorn, K. Nasmyth, A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase, *Science*, 261 (1993) 1551-1557.
- [20] V.R. Iyer, C.E. Horak, C.S. Scafe, D. Botstein, M. Snyder, P.O. Brown, Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF, *Nature*, 409 (2001) 533-538.

- [21] Y. Ho, M. Costanzo, L. Moore, R. Kobayashi, B.J. Andrews, Regulation of transcription at the *Saccharomyces cerevisiae* start transition by Stb1, a Swi6-binding protein, *Mol Cell Biol*, 19 (1999) 5267-5278.
- [22] M. Costanzo, O. Schub, B. Andrews, G1 transcription factors are differentially regulated in *Saccharomyces cerevisiae* by the Swi6-binding protein Stb1, *Mol Cell Biol*, 23 (2003) 5064-5077.
- [23] Y. Ho, S. Mason, R. Kobayashi, M. Hoekstra, B. Andrews, Role of the casein kinase I isoform, Hrr25, and the cell cycle-regulatory transcription factor, SBF, in the transcriptional response to DNA damage in *Saccharomyces cerevisiae*, *Proc Natl Acad Sci USA*, 94 (1997) 581-586.
- [24] M.B. Zimmermann, R.F. Hurrell, Nutritional iron deficiency, *Lancet*, 370 (2007) 511-520.
- [25] C. Zhang, Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control, *Protein Cell*, 5 (2014) 750-760.
- [26] J.O. Fuss, C.L. Tsai, J.P. Ishida, J.A. Tainer, Emerging critical roles of Fe-S clusters in DNA replication and repair, *Biochim Biophys Acta*, 1853 (2015) 1253-1271.
- [27] N. Sanvisens, S. Puig, Causes and consequences of nutritional iron deficiency in living organisms, in: T.C. Merkin (Ed.) *Biology of starvation in humans and other organisms*, Nova Science Publishers, Place Published, 2011, pp. 245-276.
- [28] C.C. Philpott, O. Protchenko, Response to iron deprivation in *Saccharomyces cerevisiae*, *Eukaryot Cell*, 7 (2008) 20-27.
- [29] C.C. Philpott, S. Leidgens, A.G. Frey, Metabolic remodeling in iron-deficient fungi, *Biochim Biophys Acta*, 1823 (2012) 1509-1520.
- [30] C.D. Kaplan, J. Kaplan, Iron acquisition and transcriptional regulation, *Chem Rev*, 109 (2009) 4536-4552.
- [31] Y. Yamaguchi-Iwai, R. Stearman, A. Dancis, R.D. Klausner, Iron-regulated DNA binding by the Aft1 protein controls the iron regulon in yeast, *EMBO J*, 15 (1996) 3377-3384.
- [32] R. Ueta, N. Fujiwara, K. Iwai, Y. Yamaguchi-Iwai, Iron-induced dissociation of the Aft1p transcriptional regulator from target gene promoters is an initial event in iron-dependent gene suppression, *Mol Cell Biol*, 32 (2012) 4998-5008.
- [33] J.C. Rutherford, S. Jaron, E. Ray, P.O. Brown, D.R. Winge, A second iron-regulatory system in yeast independent of Aft1p, *Proc Natl Acad Sci USA*, 98 (2001) 14322-14327.
- [34] A. Chabes, V. Domkin, L. Thelander, Yeast Sml1, a protein inhibitor of ribonucleotide reductase, *J Biol Chem*, 274 (1999) 36679-36683.
- [35] X. Zhao, E.G. Muller, R. Rothstein, A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools, *Mol Cell*, 2 (1998) 329-340.
- [36] X. Zhao, B. Georgieva, A. Chabes, V. Domkin, J.H. Ippel, J. Schleucher, S. Wijmenga, L. Thelander, R. Rothstein, Mutational and structural analyses of the ribonucleotide reductase inhibitor Sml1 define its Rnr1 interaction domain whose inactivation allows suppression of *mec1* and *rad53* lethality, *Mol Cell Biol*, 20 (2000) 9076-9083.
- [37] N. Sanvisens, A.M. Romero, X. An, C. Zhang, R. de Llanos, M.T. Martinez-Pastor, M.C. Bano, M. Huang, S. Puig, Yeast Dun1 kinase regulates ribonucleotide reductase inhibitor Sml1 in response to iron deficiency, *Mol Cell Biol*, 34 (2014) 3259-3271.
- [38] Z. Zhang, X. An, K. Yang, D.L. Perlstein, L. Hicks, N. Kelleher, J. Stubbe, M. Huang, Nuclear localization of the *Saccharomyces cerevisiae* ribonucleotide reductase small subunit requires a karyopherin and a WD40 repeat protein, *Proc Natl Acad Sci USA*, 103 (2006) 1422-1427.
- [39] Y.D. Lee, S.J. Elledge, Control of ribonucleotide reductase localization through an anchoring mechanism involving Wtm1, *Genes Dev*, 20 (2006) 334-344.
- [40] X. Wu, M. Huang, Dif1 controls subcellular localization of ribonucleotide reductase by mediating nuclear import of the R2 subunit, *Mol Cell Biol*, 28 (2008) 7156-7167.
- [41] Y.D. Lee, J. Wang, J. Stubbe, S.J. Elledge, Dif1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase, *Mol Cell*, 32 (2008) 70-80.

- [42] N. Sanvisens, M.C. Bano, M. Huang, S. Puig, Regulation of ribonucleotide reductase in response to iron deficiency, *Mol Cell*, 44 (2011) 759-769.
- [43] N. Sanvisens, A.M. Romero, C. Zhang, X. Wu, X. An, M. Huang, S. Puig, Yeast Dun1 Kinase Regulates Ribonucleotide Reductase Small Subunit Localization in Response to Iron Deficiency, *J Biol Chem*, 291 (2016) 9807-9817.
- [44] S. Puig, E. Askeland, D.J. Thiele, Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation, *Cell*, 120 (2005) 99-110.
- [45] V.V. Kushnirov, Rapid and reliable protein extraction from yeast, *Yeast*, 16 (2000) 857-860.
- [46] L. Ramos-Alonso, A.M. Romero, M.A. Soler, A. Perea-Garcia, P. Alepuz, S. Puig, M.T. Martinez-Pastor, Yeast Cth2 protein represses the translation of ARE-containing mRNAs in response to iron deficiency, *PLoS Genet*, 14 (2018) e1007476.
- [47] S. Puig, M. Lau, D.J. Thiele, Cti6 is an Rpd3-Sin3 histone deacetylase-associated protein required for growth under iron-limiting conditions in *Saccharomyces cerevisiae*, *J Biol Chem*, 279 (2004) 30298-30306.
- [48] Y. Yamaguchi-Iwai, A. Dancis, R.D. Klausner, Aft1: a mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*, *EMBO J*, 14 (1995) 1231-1239.
- [49] Y. Yamaguchi-Iwai, R. Ueta, A. Fukunaka, R. Sasaki, Subcellular localization of Aft1 transcription factor responds to iron status in *Saccharomyces cerevisiae*, *J Biol Chem*, 277 (2002) 18914-18918.
- [50] P.L. Blaiseau, E. Lesuisse, J.M. Camadro, Aft2p, a novel iron-regulated transcription activator that modulates, with Aft1p, intracellular iron use and resistance to oxidative stress in yeast, *J Biol Chem*, 276 (2001) 34221-34226.
- [51] M. Courel, S. Lallet, J.M. Camadro, P.L. Blaiseau, Direct activation of genes involved in intracellular iron use by the yeast iron-responsive transcription factor Aft2 without its paralog Aft1, *Mol Cell Biol*, 25 (2005) 6760-6771.
- [52] A.M. Romero, L. Ramos-Alonso, P. Alepuz, S. Puig, M.T. Martinez-Pastor, Global translational repression induced by iron deficiency in yeast depends on the Gcn2/eIF2alpha pathway, *Sci. Rep.*, 10 (2020) 233.
- [53] A.M. Romero, L. Ramos-Alonso, S. Montella-Manuel, J. Garcia-Martinez, M.A. de la Torre-Ruiz, J.E. Perez-Ortin, M.T. Martinez-Pastor, S. Puig, A genome-wide transcriptional study reveals that iron deficiency inhibits the yeast TORC1 pathway, *Biochim Biophys Acta Gene Regul Mech*, 1862 (2019) 194414.
- [54] U. Begley, M. Dyavaiah, A. Patil, J.P. Rooney, D. DiRenzo, C.M. Young, D.S. Conklin, R.S. Zitomer, T.J. Begley, Trm9-catalyzed tRNA modifications link translation to the DNA damage response, *Mol Cell*, 28 (2007) 860-870.
- [55] G. Bairwa, W. Hee Jung, J.W. Kronstad, Iron acquisition in fungal pathogens of humans, *Metallomics*, 9 (2017) 215-227.
- [56] F. Devaux, A. Thiebaut, The regulation of iron homeostasis in the fungal human pathogen *Candida glabrata*, *Microbiology*, 165 (2019) 1041-1060.
- [57] F. Gerwien, A. Safyan, S. Wisgott, F. Hille, P. Kaemmer, J. Linde, S. Brunke, L. Kasper, B. Hube, A Novel Hybrid Iron Regulation Network Combines Features from Pathogenic and Nonpathogenic Yeasts, *MBio*, 7 (2016).
- [58] M. Benchouaia, H. Ripoche, M. Sissoko, A. Thiebaut, J. Merhej, T. Delaveau, L. Fasseu, S. Benaissa, G. Lorieux, L. Jourden, S. Le Crom, G. Lelandais, E. Corel, F. Devaux, Comparative Transcriptomics Highlights New Features of the Iron Starvation Response in the Human Pathogen *Candida glabrata*, *Front Microbiol*, 9 (2018) 2689.

SUPPLEMENTAL TABLES

Supplemental Table S1. Yeast strains used in this study.

Strain	Description	Source/Reference
BY4741	<i>Mata, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0</i>	Research Genetics
SPY28	BY4741 <i>aft1::KanMX4</i>	Research Genetics
SPY741	BY4741 <i>ixr1::KanMX4</i>	Research Genetics
SPY945	BY4741 <i>ixr1::KanMX4 aft1::hphB</i>	This study
SPY857	BY4741 <i>mbp1::KanMX4</i>	Research Genetics
SPY758	BY4741 <i>swi4::KanMX4</i>	Research Genetics
SPY759	BY4741 <i>swi6::KanMX4</i>	Research Genetics
SPY985	loxP-KanMX-loxP-prom950-FeRE-WT-RNR1	This study
SPY986	loxP-KanMX-loxP-prom950-FeRE-315/470mt-RNR1	This study
SPY387	CRY1 (W303a): <i>Mata, can1-100, ade2-1, his3-11, leu2-3,112, trp1-1, ura3-1</i>	Mingxia Huang
SPY571	RCY337-26a: W303a <i>sml1::HIS3 RAD5 CAN1</i>	Mingxia Huang
SPY573	RCY337-3d: W303a <i>tel1::Kan mec1::HIS3 sml1::HIS3 RAD5 CAN1</i>	Mingxia Huang
W303-1A	<i>Mata, ura3-1, ade2-1, trp1-1, his3-11,15, leu2-3,112</i>	Enrique Herrero
MML348	W303-1A <i>aft1::URA (aft1Δ5)</i>	Enrique Herrero
MML1086	W303-1A <i>aft2::KanMX4</i>	Enrique Herrero
MML1088	W303-1A <i>aft1::URA (aft1Δ5) aft2::KanMX4</i>	Enrique Herrero

Supplemental Table S2. Plasmids used in this study.

Plasmid	Description	Source/Reference
pSP985	<i>P_{RNR1}-lacZ</i>	This study
pSP1028	<i>P_{RNR1-315mt}-lacZ</i>	This study
pSP1015	<i>P_{RNR1-470mt}-lacZ</i>	This study
pSP1038	<i>P_{RNR1-315/470mt}-lacZ</i>	This study
pSP1029	<i>P_{IXR1}-lacZ</i>	This study
pSP1030	<i>P_{464mt-IXR1}-lacZ</i>	This study
P177	pRS313-AFT1-1 ^{up}	Jerry Kaplan
P178	pRS313-AFT2-1 ^{up}	Jerry Kaplan

Supplemental Table S3. Primers used for RT-qPCR in this study.

Primer	Sequence (5' to 3')
<i>RNR1-F</i>	CTGTGACTGCAAACGCAACTA
<i>RNR1-R</i>	AATGAGTGGCTACCTGTTGGA
<i>FET3-F</i>	TGACCGTTTTGTCTTCAGGT
<i>FET3-R</i>	CTCCACGATTTTCATCCTTCTC
<i>IXR1-F</i>	CAACTGGGAGAAGTACAGAG
<i>IXR1-R</i>	GACGGTAGGTCTAATCTCCT
<i>RNR3-F</i>	CTGAACAAAAGGCGGCATC
<i>RNR3-R</i>	GGGGCAACACTATCTTCCAA
<i>ACT1-F</i>	TCGTTCCAATTTACGCTGGTT
<i>ACT1-R</i>	CGGCCAAATCGATTCTCAA
<i>lys-qPCR-F</i>	CGAGCAAAGCATTCTCATCA
<i>lys-qPCR -R</i>	AGCTCTCTCCGGATACGACA
<i>phe-qPCR -F</i>	CATGGATGCTGTTTTTCCAT
<i>phe-qPCR 1-R</i>	GCACCTGACCTATCCTCCAA

SUPPLEMENTAL FIGURES

Figure S1. The up-regulation of *RNR1* mRNA by iron deficiency depends on both *AFT1* and *AFT2*. Wild-type W303-1A, *aft1*Δ (MML348), *aft2*Δ (MML1086), and *aft1*Δ*aft2*Δ (MML1088), strains were cultivated in SC as described in Figure 2B. *aft1*Δ*aft2*Δ cells transformed with *AFT1-1*^{up} or *AFT2-1*^{up} plasmids were cultivated in SC-His as described in Figure 2B. Total RNA was extracted and *RNR1* and *ACT1* mRNA levels were analyzed by RT-qPCR. The average and standard deviation of three independent experiments is represented and referred to wild-type cells under iron-sufficient (+Fe) conditions. Each asterisk (*) indicates statistically significant differences (p -value < 0.05) with wild-type cells under +Fe.

Figure S2. Western blots corresponding to Rnr1 and Pgk1 proteins from Figure 3C. Protein extracts from wild-type BY4741, *aft1* Δ , *ixr1* Δ , and *aft1* Δ *ixr1* Δ strains cultivated in iron sufficiency (+Fe; panel A) and 15 hours of iron deficiency (-Fe ,15 h; panel B) were analyzed in different membranes. Common WT +Fe samples were used to correlate protein levels between different blots.

Figure S3. Western blots corresponding to Rnr1 and Pgk1 proteins from Figure 6B. Protein extracts from RNR1-WT and RNR1-315/470 strains cultivated in iron sufficiency (+Fe; panel A), 6 hours of iron deficiency (-Fe, 6 h; panel B) and 15 of hours iron deficiency (-Fe, 15 h; panel C) were analyzed in different membranes. A common RNR1-WT +Fe sample was used to correlate protein levels between different blots.

Figure S4. *RNR3* expression is up-regulated in RNR1-315/470mt iron-deficient cells. The wild-type FeRE strain (RNR1-WT: SPY985) and the 315/470 mutated FeRE strain (RNR1-315/470mt: SPY986) were cultivated at 30°C in SC with 100 μ M BPS for 15 hours, and *RNR3/ACT1* mRNA levels were determined by RT-qPCR. Values are represented as relative to those of the RNR1-WT strain grown under +Fe conditions. The average and standard deviation of three independent experiments is shown. Different letters indicate significant differences (p -value < 0.05).