Yeast Dun1 Kinase Regulates Ribonucleotide Reductase Small Subunit Localization in Response to Iron Deficiency*

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Ribonucleotide reductase (RNR) is an essential iron-dependent enzyme that catalyzes deoxyribonucleotide synthesis in eukaryotes. Living organisms have developed multiple strategies to tightly modulate RNR function to avoid inadequate or unbalanced deoxyribonucleotide pools that cause DNA damage and genome instability. Yeast cells activate RNR in response to genotoxic stress and iron deficiency by facilitating redistribution of its small heterodimeric subunit Rnr2-Rnr4 from the nucleus to the cytoplasm, where it forms an active holoenzyme with large Rnr1 subunit. Dif1 protein inhibits RNR by promoting nuclear import of Rnr2-Rnr4. Upon DNA damage, Dif1 phosphorylation by the Dun1 checkpoint kinase and its subsequent degradation enhances RNR function. In this report, we demonstrate that Dun1 kinase triggers Rnr2-Rnr4 redistribution to the cytoplasm in response to iron deficiency. We show that Rnr2-Rnr4 relocation by low iron requires Dun1 kinase activity and phosphorylation site Thr-380 in the Dun1 activation loop, but not the Dun1 forkhead-associated domain. By using different Dif1 mutant proteins, we uncover that Dun1 phosphorylates Dif1 Ser-104 and Thr-105 residues upon iron scarcity. We observe that the Dif1 phosphorylation pattern differs depending on the stimulus, which suggests different Dun1 activating pathways. Importantly, the Dif1-S104A/T105A mutant exhibits defects in nucleus-to-cytoplasm redistribution of Rnr2-Rnr4 by iron limitation. Taken together, these results reveal that, in response to iron starvation, Dun1 kinase phosphorylates Dif1 to stimulate Rnr2-Rnr4 relocation to the cytoplasm and promote RNR function.

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Ribbonucleotide reductase (RNR) catalyzes the rate-limiting step in the de novo deoxyribonucleotide (dNTP) synthesis by converting ribonucleoside diphosphates to the corresponding deoxy forms. In eukaryotes, the RNR holoenzyme is composed of a large or R1 subunit that contains the catalytic and allosteric sites, and a small or R2 subunit that harbors a di-iron center, which is responsible for generating and keeping a tyrosyl radical required for catalysis (reviewed in Refs. 1–3). In the budding yeast Saccharomyces cerevisiae, RNR large subunit is composed of Rnr1 homodimers and the small subunit is an heterodimer made up of structurally homologous Rnr2 and Rnr4 proteins (4–7). Failures to adjust intracellular dNTP levels lead to DNA damage and genomic instability, both hallmarks of cancer and aging (8–10). Thus, cells have developed multiple strategies to tightly modulate RNR function under different conditions (reviewed in Refs. 10 and 11).

In response to genotoxic or replication stress, the yeast Mec1/Rad53/Dun1 DNA damage checkpoint kinase cascade activates RNR. First, Mec1 kinase phosphorylates and activates Rad53 (12, 13). Then, Dun1 forkhead-associated (FHA) domain recognizes a diphosphothreonine motif in hyperphosphorylated Rad53 kinase that facilitates Rad53-mediated phosphorylation and activation of the Dun1 kinase (14–17). Finally, Dun1 promotes RNR function through multiple mechanisms. Dun1 hyperphosphorylates the Crt1 transcriptional repressor, which is released from the promoter regions of RNR2–4 genes resulting in transcriptional derepression (18). Genotoxic stress also increases Rnr1 protein levels through a Rad53-dependent but Dun1-independent transcriptional RNR1 activation mechanism (19). Moreover, after DNA damage or during S phase, the Mec1/Rad53/Dun1 signaling cascade relieves Sml1 inhibition of RNR by promoting Sml1 phosphorylation, ubiquitylation, and degradation by the 26S proteasome (20–23). Finally, another checkpoint-dependent mechanism facilitates redistribution of Rnr2 and Rnr4 from the nucleus to the cytoplasm, where Rnr1 resides, in response to genotoxic stress (24). In this case, Dun1 kinase promotes Rnr2-Rnr4 heterodimer dissociation from its nuclear anchor protein Wtm1, and in the meantime prevents Rnr2-Rnr4 nuclear import by phosphorylating its importer protein Dif1 targeting it for degradation (17, 25–27).
Iron is an essential cofactor for many key enzymes in DNA replication and repair, which include replicative DNA polymerases, DNA primase, and various DNA repair enzymes, in addition to RNR (28–34). Consequently appropriate iron delivery to enzymes in the DNA metabolism is critical to avoid nuclear genome instability (29, 30, 35, 36). S. cerevisiae is widely used as a model organism to study the response of eukaryotic cells to iron deficiency. Upon iron depletion, yeast Aft1 transcription factor activates the expression of genes encoding high-affinity iron transport systems and Cth2, an RNA-binding protein that facilitates the coordinated degradation of many mRNAs encoding proteins implicated in iron-consuming pathways (37–42). Many studies have demonstrated that Aft1 does not directly perceive intracellular or environmental iron concentration. Instead Aft1 activity is inhibited by an iron-compound synthesized by the mitochondrial iron-sulfur cluster (ISC) biogenesis core and exported to the cytoplasm (43). Mutants defective in components of the mitochondrial ISC biogenesis core activate Aft1-dependent responses to iron deficiency, whereas no activation is observed in cells defective in components of the cytoplasmic iron-sulfur cluster assembly machinery, responsible for delivering iron-sulfur cofactors to other iron-dependent proteins (43–45). During the past years, we have used S. cerevisiae to characterize RNR regulation by iron availability. We have demonstrated that the Cth2 RNA-binding protein specifically interacts with the WTM1 transcript and facilitates its degradation (46). The resulting decrease in Wtm1 protein abundance promotes Rnr2-Rnr4 relocation to the cytoplasm and dNTP synthesis (46). Furthermore, we have reported that, in response to iron deficiency, Dun1 checkpoint kinase induces degradation of the Rnr1 inhibitor protein Sml1, promoting RNR activity (47).

In this study, we uncover novel mechanisms that eukaryotic cells utilize to optimize RNR function when iron bioavailability diminishes. We show that the Dun1 checkpoint kinase contributes to Rnr2-Rnr4 redistribution to the cytoplasm when iron bioavailability is limited. Furthermore, we decipher that Dun1 modulates Rnr2-Rnr4 subcellular localization during iron deficiency by phosphorylating specific Dif1 residues.

**Experimental Procedures**

**Yeast Strains, Plasmids, and Growth Conditions**—In this study, we have used dun1Δ (dun1::KanMX4), fet3Δfet4Δ (fet3::URA3, fet4::KanMX4), and fet3Δfet4Δdun1Δ (fet3::URA3, fet4::KanMX4, dun1::KanMX4) S. cerevisiae strains derived from wild-type BY4741 (MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0) and diffΔ (diff1::KanMX4) derived from wild-type Y300 (MATa, can1–100, ade2–1, his3–11,15, leu2–3,112, trp1–1, ura3–1, lys12). Yeast precultures were incubated overnight at 30 °C in synthetic complete SC medium lacking uracil (SC-ura), tryptophan (SC-trp), or leucine (SC-leu), and then cultured over-night at 30 °C in synthetic complete SC medium lacking uracil (SC-ura), tryptophan (SC-trp), or leucine (SC-leu), and then incubated 6 h in SC (iron-deficient conditions) or SC supplemented with 100 μM Fe^{2+}-specific chelator bathophenanthroline disulfonic acid disodium (BPS) (iron-deficient conditions) before processing. Treatment with either 0.04% methyl methanesulfonate (MMS) or 0.2 mM hydroxyurea (HU) was performed during the last 2 h of SC incubation. Plasmids expressing different Dif1 alleles were constructed as previously described (27). All plasmids used in this study are listed in Table 1.

**Fluorescence Microscopy**—Indirect immunofluorescence (IMF) was performed as described previously (24, 46). Cells were analyzed in an Axioskop 2 microscope (Zeiss) and images captured with a SPOT camera (Diagnostic Instruments). In all cases, more than 200 cells from at least 3 independent experiments were scored as cells with a predominantly nuclear signal, localization in both the nucleus and cytoplasm, or a predominantly cytoplasmic signal. Average and S.D. were represented.

**Protein Analyses**—Total protein extracts were obtained by using glass beads disruption in 20% trichloroacetic acid as described (48). Protein extracts from an equal number of cells were separated in SDS-PAGE gels and transferred onto nitrocellulose membranes. Ponceau S staining was used to assess protein transfer and loading. Epitope-tagged 3Myb-Dif1 protein was detected with monoclonal mouse anti-c-myc antibody (9E10, Roche Applied Science). Immunoblots were developed with HRP-labeled secondary mouse antibody and Western Lightening Plus ECL kit (PerkinElmer Life Sciences).

**Results**

**Dun1 Promotes Rnr2 and Rnr4 Redistribution to the Cytoplasm in Response to Iron Deficiency**—We have previously shown that iron deficiency activates Dun1 protein kinase (47). Then, Dun1 phosphorylates Sml1 protein, which is ubiquitinated and degraded to facilitate RNR activation under low iron conditions (47). We have also shown that the Rnr2-Rnr4 heterodimer is redistributed to the cytoplasm when iron availability is low (46). Given that Dun1 promotes Rnr2-Rnr4 relocation in response to genotoxic stress, we decided to study whether Dun1 also modulates subcellular distribution of Rnr2 and Rnr4 proteins under iron-deficient conditions. For this purpose, we transformed dun1Δ cells with empty vector or the same plasmid expressing wild-type Dun1 under control of its
own promoter. Yeast transformants were grown under iron-sufficient conditions (SC), iron-deficient conditions achieved by the addition of Fe\textsuperscript{2+}-specific chelator BPS, or treated with the DNA alkylating agent MMS. Then, RNR small subunit subcellular localization was determined by IMF with specific Rnr2 and Rnr4 antibodies (Fig. 1A). Yeast cells were classified according to R2 distribution as cells with a predominantly nuclear signal (N), cells with R2 localization in both the nucleus and the cytoplasm (N/C), or cells with a predominantly cytoplasmic signal (C). As shown in Fig. 1, under iron-sufficient conditions, >80% of yeast cells exhibited nuclear Rnr2 and Rnr4 IMF signals. However, under iron-deficient conditions or after MMS treatment, less than 20% of cells expressing DUN1 displayed a nuclear Rnr2 and Rnr4 IMF signal, and ~60–70% of cells showed a cytoplasmic pattern (Fig. 1, B and C). As previously reported (24), dun1Δ cells displayed a defect in R2 redistribution to the cytoplasm when treated with MMS (Fig. 1, D and E). Importantly, after BPS treatment around 60% of dun1Δ cells still accumulated Rnr2 and Rnr4 in the nucleus, whereas only 30% displayed a predominant cytoplasmic pattern (Fig. 1, B and C).
These results uncover that Dun1 protein plays an important role in the subcellular redistribution of RNR small subunit from the nucleus to the cytoplasm that occurs upon iron limitation.

Dun1 Kinase Activity Is Essential for Subcellular Redistribution of Ribonucleotide Reductase Small Subunits to the Cytoplasm in Response to Iron Limitation—Cellular Dun1 function in DNA damage response depends on its capacity to autophosphorylate itself and downstream target proteins (49, 50). Thus, mutagenesis of the Asp-328 residue within the Dun1 kinase domain abolishes its catalytic activity and eliminates Dun1-dependent phosphorylation and degradation of Sm1 protein by DNA damage (Fig. 2A) (49–51). To ascertain whether Dun1 kinase activity was required for R2 redistribution to the cytoplasm during iron scarcity, we expressed a kinase-dead \textit{DUN1-D328A} mutant allele in \textit{dun1}/H9004 cells and determined the subcellular localization of Rnr2 and Rnr4 by IMF. As expected, Dun1 kinase activity was essential for proper R2 redistribution by genotoxic stress (Fig. 2, B and C). Importantly, yeast cells expressing the \textit{DUN1-D328A} allele showed a defect in Rnr2 and Rnr4 relocalization to the cytoplasm similar to that of \textit{dun1}/H9004 cells when iron was depleted (Figs. 1, D and E, and 2, B and C). This was not a consequence of a reduction in Dun1-D328A protein abundance because its expression levels under low iron conditions were similar to those of wild-type Dun1 protein (47). These results reveal that Dun1 kinase activity is required for efficient nucleus-to-cytoplasm redistribution of RNR small subunit in response to iron deprivation and DNA damage.

**CONTRIBUTION OF DUN1 PHOSPHORYLATION SITES TO RNR2 AND RNR4 REDISTRIBUTION UNDER IRON DEFICIENCY**—Phosphorylation of the Thr-380 residue within the Dun1 activation loop is essential for Sm1 protein degradation and growth when cells suffer DNA damage. As expected, Dun1 kinase activity was essential for proper R2 redistribution by genotoxic stress (Fig. 2, B and C). Importantly, yeast cells expressing the \textit{DUN1-T380A} allele showed a defect in Rnr2 and Rnr4 relocalization to the cytoplasm similar to that of \textit{dun1}/H9004 cells when iron was depleted (Figs. 1, D and E, and 2, B and C). This was not a consequence of a reduction in Dun1-D328A protein abundance because its expression levels under low iron conditions were similar to those of wild-type Dun1 protein (47). These results reveal that Dun1 kinase activity is required for efficient nucleus-to-cytoplasm redistribution of RNR small subunit in response to iron deprivation and DNA damage.

FIGURE 2. Dun1 kinase catalytic activity and Thr-380 phosphorylation site are required for nucleus-to-cytoplasm Rnr2 and Rnr4 redistribution upon iron scarcity. A, schematic representation of the most relevant Dun1 domains and amino acid residues. Numbers indicate amino acid positions. \textbf{B} and \textbf{C}, Dun1 kinase activity is necessary for R2 redistribution by iron deficiency. Yeast \textit{dun1}/H9004 cells transformed with pMH80 (\textit{DUN1}), pRS416 (\textit{dun1}), or pMH62 (\textit{DUN1-D328A}) plasmids were cultivated and analyzed as described in the legend to Fig. 1. Data corresponding to \textit{DUN1} and \textit{dun1}/H9004 cells are shown in Fig. 1. Quantitative analysis of Rnr2 (\textbf{B}) and Rnr4 (\textbf{C}) subcellular localization in cells expressing \textit{DUN1-D328A} is represented. \textbf{D} and \textbf{E}, Dun1 phosphorylation site Thr-380 is important for the R2 nucleus-to-cytoplasm redistribution by iron deficiency. Yeast \textit{dun1}/H9004 cells transformed with pSP692 (\textit{DUN1}), pRS413 (\textit{dun1}), or pSP695 (\textit{DUN1-T380A}) plasmids were cultivated in SC and analyzed as described in the legend to Fig. 1. Quantitative analysis of Rnr2 (\textbf{D}) and Rnr4 (\textbf{E}) subcellular localization in cells expressing \textit{DUN1-T380A} is represented. Data corresponding to \textit{DUN1} and \textit{dun1}/H9004 cells were similar to those displayed in Fig. 1 and have not been represented for simplicity. Average and S.D. are represented.
To address whether Thr-380 was important for R2 redistribution by iron limitation, we determined Rnr2 and Rnr4 subcellular localization in yeasts expressing the DUN1-T380A mutant allele. We first studied how the T380A mutation affected R2 subcellular localization upon genotoxic stress. Consistent with lack of Sml1 protein degradation (51), we observed that Dun1-T380A expressing cells barely transported Rnr2 and Rnr4 out of the nucleus when treated with MMS (Fig. 2, D and E). Similarly, Dun1-T380A cells displayed a strong accumulation of Rnr2 and Rnr4 proteins in the nucleus after treatment with BPS (Fig. 2, D and E). We have previously shown that Dun1-T380A protein levels diminish to 60% in iron-deficient conditions (47). However, this decrease in protein abundance can only partially, but not fully, explain the strong Rnr2-Rnr4 nuclear retention displayed by Dun1-T380A expressing cells. These results suggest that the Thr-380 residue is required for R2 transport to the cytoplasm upon iron scarcity and DNA damage.

To further explore potential Dun1 residues required to facilitate R2 redistribution in response to iron limitation, we checked two Dun1 autophosphorylation sites, Ser-10 and Ser-139, which are important for optimal growth under DNA replication stress (51). We observed that both DUN1-S10A and DUN1-S139A mutant alleles displayed a slight defect in Rnr2 and Rnr4 movement to the cytoplasm when treated with MMS, which could contribute to cellular sensitivity to DNA damage and replicative stress (Fig. 3). Regarding iron deficiency, only DUN1-S139A expressing cells exhibited a very slight defect in Rnr2 and Rnr4 redistribution to the cytoplasm, whereas no defect was observed for the DUN1-S10A allele (Fig. 3). Given that Dun1-S139A protein levels decrease to 60% of those of wild-type Dun1 protein under low iron conditions (47), we concluded that the Dun1-S139A slight distribution defect is probably a consequence of its diminished abundance. Taken together, these analyses suggest that the Dun1 Thr-380 residue (but neither Ser-10 nor Ser-139) is important for small RNR subunit transport to the cytoplasm upon iron limitation.

The Integrity of Dun1 Forkhead-associated Domain Is Only Partially Required for Rnr2-Rnr4 Relocalization to the Cytoplasm When Iron Availability Decreases—Dun1 FHA domain is necessary for binding to the Rad53 diphosphothreonine motif (52). Thus, mutagenesis of Dun1 Arg-60 or Lys-100 residues, which are necessary for Dun1 binding to the first or second Rad53 phosphothreonine residue, respectively, abolishes Dun1 activation (52). To ascertain whether Dun1 FHA domain functions in R2 transport to the cytoplasm in response to iron deficiency, we expressed DUN1-R60A and DUN1-K100A/R102A mutant alleles in a dun1Δ strain and determined Rnr2 and Rnr4 subcellular distribution by IMF (Fig. 4). As expected, both DUN1-R60A and DUN1-K100A/R102A expressing cells exhibited a significant defect in Rnr2 and Rnr4 relocalization to the cytoplasm upon iron limitation.
cytoplasm as compared with wild-type cells when MMS was added to the growth medium (Fig. 4). Upon BPS treatment, around 60% cells displayed a predominant cytoplasmic Rnr2 and Rnr4 distribution, but between 20 and 40% of cells still retained both small RNR subunits in the nucleus (Fig. 4). If we compare the R2 distribution pattern of FHA mutants under low iron conditions (Fig. 4, C–F) to that of cells expressing wild-type DUN1 (Fig. 4, A and B), dun1Δ cells (Fig. 1), and cells expressing a non-functional DUN1 allele (Fig. 2), we conclude that both DUN1-R60A and DUN1-K100A/R102A expressing cells exhibit a partial defect in Rnr2 and Rnr4 relocalization to the cytoplasm when iron is scarce. Therefore, these results indicate that the Dun1 FHA domain is only partially required for a proper distribution of RNR small subunits during iron deficiency.

Dun1 Regulates R2 Localization in Iron Deficiency

Dun1 Kinase Specifically Phosphorylates Dif1 Protein in Response to Iron Limitation—Under normal conditions, Dif1 protein inhibits RNR function by direct binding to the cytoplasmic Rnr2-Rnr4 heterodimer to promote its import into the nucleus (17, 27). Upon DNA damage or replicative stress, checkpoint Dun1 kinase phosphorylates the Dif1 protein at specific residues within its Sml domain and promotes its deg-
FIGURE 5. Dif1 protein is phosphorylated at Ser-104 and Thr-105 residues in response to iron deficiency. A, Dif1 protein is phosphorylated in a Dun1-dependent manner when iron is scarce. Wild-type (WT) and dun1Δ cells transformed with pMH1494 (DIF1) were grown to exponential phase in SC alone, SC with 100 μM BPS for 6 h, or SC with 0.04% MMS for 2 h. Mutagenesis of Thr-102, Ser-104, and Thr-105 residues abolishes Dif1 phosphorylation in low iron conditions. Yeast dif1Δ cells transformed with pXW15 (DIF1Δ79–103), pXW16 (DIF1-T83A/S85A), pXW17 (DIF1-T102A/S104A/T105A), pXW19 (DIF1-T89A/T92A), or pMH1494 (DIF1) were grown and analyzed as in panel A. D, Dif1 protein is phosphorylated at Ser-104 and Thr-105 in response to iron deficiency. Dif1Δ cells transformed with pMH1494 (DIF1), pXW25 (DIF1-T102A), pXW26 (DIF1-S104A), pXW27 (DIF1-T105A), pXW28 (DIF1-T102A/S104A), pXW29 (DIF1-T102A/T105A), or pXW30 (DIF1-S104A/T105A) were grown and analyzed as in panel A. D. Dif1 protein is phosphorylated at Ser-104 and Thr-105 residues under genetic iron deficiency in a Dun1-dependent manner. fet3Δ/fet4Δ and fet3Δ/fet4Δ dun1Δ cells transformed with pMH1488 (DIF1), pMH1764 (DIF1-S104A), pMH1765 (DIF1-T105A), or pMH1760 (DIF1-S104A/T105A) were grown in SC to exponential phase. In all panels yeast proteins were obtained and analyzed by Western blot. Epitope-tagged 3Myc-Dif1 protein was detected with anti-c-myc antibody. Each result was repeated at least once, from cell culture treatment to protein blot.

radation, thus relieving RNR inhibition (17, 27). Given that Dun1 also promotes Rnr2 and Rnr4 protein redistribution to the cytoplasm in response to iron deficiency, we decided to explore whether Dun1 altered the Dif1 phosphorylation state under these conditions. For this purpose, we grew wild-type and dun1Δ cells expressing a Myc epitope-tagged Dif1 protein under iron sufficiency (SC), iron deficiency (BPS), and MMS-treated conditions, and detected Dif1 protein by Western blot. Consistent with previous studies (17, 27), wild-type cells treated with MMS led to a slower migrating Dif1 protein representation of the phosphorylated form, which is stabilized in 3Myc-Dif1 protein (Fig. 5A). We observed a similar slower-migrating Dif1 in BPS-treated cells (Fig. 5A), indicating that Dif1 protein is phosphorylated in response to iron deficiency as it occurs upon DNA damage. Importantly, Dif1 phosphorylation was not observed in dun1Δ mutant cells treated with BPS or MMS (Fig. 5A). Together, these results indicate that iron deficiency promotes a Dun1-dependent phosphorylation of Dif1 protein.

Our previous study has mapped DNA damage-induced Dif1 phosphorylation to its Sml domain (residues 76–114) enriched with serine and threonine residues (27). To map specific residues targeted by Dun1 kinase under low iron conditions, we determined the Dif1 migrating pattern in yeast cells expressing different DIF1 alleles with either deletions or alanine substitutions of specific serine/threonine residues within the Sml domain. We observed that the Dif1Δ79–103 deletion protein, and Dif1-T83A/S85A and Dif1-T89A/T92A double mutant proteins were still phosphorylated in response to iron deficiency (Fig. 5B). Importantly, the Dif1-T102A/S104A/T105A triple mutant protein was not phosphorylated upon iron limitation (Fig. 5B). To further ascertain which of these three Dif1 residues were Dun1 kinase targets under low iron conditions, we constructed single and double alanine substitution mutants. Only simultaneous substitutions of Ser-104 and Thr-105 residues to alanine fully abrogated Dif1 phosphorylation under low iron conditions (Fig. 5C). To further ascertain whether Dif1 phosphorylation was caused by iron deficiency and not by a secondary effect of BPS, we determined a Dif1 protein pattern in a fet3Δ/fet4Δ yeast strain, which is genetically deficient in iron due to the lack of Fet3 and Fet4 genes required for high- and low-affinity iron transport, respectively. As shown in Fig. 5D, wild-type Dif1, Dif1-S104A, and Dif1-T105A proteins were phosphorylated in fet3Δ/fet4Δ cells grown under iron-sufficient conditions. Importantly, Dif1 phosphorylation was abolished only when both Ser-104 and Thr-105 residues were...
mutagenized or the DUN1 gene was deleted (Fig. 5D). These results indicate that Dun1 kinase equally phosphorylates both Ser-104 and Thr-105 residues in response to nutritional or genetic iron deficiency.

A Different Pattern of Dif1 Phosphorylation Is Observed Upon Genotoxic Stress—By using this set of Dif1 mutants, we explored Dif1 phosphorylation sites in response to either the DNA alkylating agent MMS or DNA replication-blocking agent HU. As observed for BPS, simultaneous mutagenesis of Ser-104 and Thr-105 residues fully removed Dif1 phosphorylation in HU-treated cells (Fig. 6A). However, in this case the Dif1-S104A protein exhibited a lower level of phosphorylated Dif1 than wild-type Dif1 and Dif1-T105A proteins (Fig. 6A), strongly suggesting that, upon HU treatment, Dif1 protein is preferentially phosphorylated at Ser-104. When Dif1 phosphorylation sites were examined in MMS-treated cells, we observed that the S104A substitution alone abolished the Dif1 phosphorylation form (Fig. 6B), indicating that Ser-104 is the predominant phosphorylation site in response to MMS. Taken together, the different Dif1 phosphorylation patterns obtained for BPS, HU, and MMS-treated cells suggest distinct activity or substrate preference by Dun1 kinase under these different stress conditions.

Dif1 Phosphorylation Is Required for Rnr2 and Rnr4 Exit from the Nucleus during Iron Scarcity—We have previously shown that MMS-treated cells expressing the Dif1-T102A/S104A/T105A mutant protein display a slight delay in Rnr4 exit from the nucleus (27). To ascertain whether Dif1 protein phosphorylation contributes to regulate yeast R2 subcellular localization in response to low iron, we performed a kinetic assay to determine the rate of Rnr2 and Rnr4 exit from the nucleus in exponentially growing cells that expressed either wild-type Dif1 or Dif1-S104A/T105A protein. After addition of BPS, Rnr2 and Rnr4 exit from the nucleus was more efficient in cells expressing wild-type Dif1 than Dif1-S104A/T105A protein (Fig. 7; and data not shown). These results indicate that phosphorylation of Dif1 Ser-104 and Thr-105 residues under low iron conditions is required for efficient Rnr2 and Rnr4 transport to the cytoplasm.

Discussion
Iron has emerged as a crucial cofactor in multiple enzymes required for DNA replication and repair including DNA polymerases, various DNA repair enzymes, and RNR, whose iron dependence has been known since the sixties (reviewed in Refs. 33 and 34). Recent studies have uncovered that defects in iron cofactor biosynthesis or delivery to iron-dependent proteins leads to genome instability and DNA damage (29, 30, 35, 36). Furthermore, due to the increase in RNR activity observed in
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Yeast cells sense iron deficiency indirectly through a decrease in iron cofactor availability (reviewed in Ref. 11) (Fig. 8). One strategy involves the Cth2 RNA-binding protein, which is specifically expressed in response to iron deficiency, yeast cells promote RNR function by multiple mechanisms. Aft1 transcription factor activates expression of the Cth2 protein, which specifically binds to WTM1 mRNA, promoting its degradation. As a consequence, Wtm1 protein levels diminish facilitating Rnr2-Rnr4 redistribution to the cytoplasm (46). Dun1 kinase activation by low iron enhances RNR function by promoting phosphorylation and degradation of Sml1 and Dif1 proteins. Whereas Dif1 phosphorylation facilitates Rnr2-Rnr4 accumulation in the cytoplasm (this study), Sml1 phosphorylation relieves Rnr1 inhibition (47).

multiple cancer types, RNR is considered a target for chemotherapeutic treatments (53, 54). Although many studies have characterized RNR regulation during the normal cell cycle and in response to genotoxic stress, little is known about RNR regulation in response to iron deficiency, the most common and widespread nutritional disorder (55). We have used S. cerevisiae to decipher molecular mechanisms that control eukaryotic RNR function when iron concentrations are low. We have observed that iron scarcity initiates multiple mechanisms directed to compensate the reduction in RNR activity caused by a decrease in iron cofactor availability (46). A second player is the Dun1 DNA damage checkpoint kinase, which has also been implicated in regulating RNR function in response to iron deprivation by promoting the degradation of R1 inhibitor protein Sml1 (Fig. 8) (47). Here, we show that Dun1 kinase activation under iron deficiency also facilitates Rnr2 and Rnr4 redistribution to the cytoplasm (Fig. 8), reinforcing the central role of Dun1 kinase in regulating RNR at multiple levels when iron cofactor availability diminishes.

Upon DNA damage, activated Dun1 kinase phosphorylates downstream targets including two RNR inhibitors, Sml1 and Dif1, which are then degraded to relieve RNR (17, 20–23, 27). By using a kinase-dead allele (DLN1-D328A), previous studies have demonstrated that Dun1 catalytic kinase activity is required for Sml1 protein phosphorylation and degradation in response to both DNA damage and iron deficiency (47, 50, 51). We showed here that the Dun1 kinase domain is required for R2 redistribution when iron availability decreases or cells suffer DNA damage (Fig. 2). From these studies, we conclude that the multiple functions that Dun1 exerts in both low iron conditions and genotoxic stress depend on its kinase catalytic activity.

Genotoxic and DNA replication stresses can activate Dun1 kinase through the Mec1/Rad53/Dun1 checkpoint kinase cascade and Rad53-independent pathways (16). For instance, suppression of gross chromosomal rearrangements, silencing gene expression in telomeres, and transcriptional activation of SNM1, a gene required for repair of DNA cross-links, in response to DNA damage depend on Dun1 kinase but are Rad53-independent (56–58). Two consecutive events dictate Dun1 activation by Rad53 kinase within the DNA damage Mec1/Rad53/Dun1 checkpoint kinase cascade: first, a specific interaction between a diphosphothreonine motif in Rad53 kinase and Dun1 FHA domain; and second, Rad53 phosphorylation of the Thr-380 residue within the Dun1 activation loop (47). In this work, we have addressed the contribution of the Dun1 FHA domain and Thr-380 phosphorylation site to R2 redistribution by iron deficiency and DNA damage. Consistent with a Rad53-dependent mechanism, MMS-treated cells fail to transport Rnr2 and Rnr4 to the cytoplasm when the Dun1 FHA domain or Thr-380 have been mutagenized (Figs. 2 and 4).

However, we observe that the Dun1 FHA domain plays a minor role in R2 redistribution during iron scarcity (Fig. 4), suggesting that Dun1 activation by low iron can occur in the absence of Rad53 protein. Consistent with this notion, we have previously reported that yeast cells lacking either RAD53 or MEC1 genes do not display significant defects in R2 redistribution to the cytoplasm when cultivated in the same iron-deficient conditions used here (46). These results do not discard that Rad53 or Mec1 could partially contribute to R2 redistribution under certain iron-deficient conditions. Furthermore, IMF results shown in this work indicate that the Dun1 Thr-380 residue is important for nucleus-to-cytoplasm transport by iron deficiency (Fig. 2), suggesting that, in addition to Rad53, other signaling proteins activated under low iron conditions may use this residue to promote Dun1 function. A different situation was observed when we looked at Dun1-dependent degradation of the Sml1 protein during iron deficiency (47). In that case, Sml1 targeting by Dun1 in response to low iron conditions depended on its FHA domain but not on its Thr-380 residue (47). Taken together, these results suggest that Dun1 kinase is activated through various upstream signaling mediators when iron cofactor availability decreases.

Yeast cells sense iron deficiency indirectly thought a decrease in the rate of ISC biosynthesis (43). Thus, iron deficiency responses are frequently activated when ISC biosynthesis is down-regulated. We previously observed that when we repress members of the core mitochondrial ISC synthesis pathway, such as the essential cysteine desulfurase Nfs1, Sml1 protein levels decrease in a Dun1-dependent manner (47). Consistent with those results, a recent study has shown that cells lacking Grx5 glutaredoxin, another member of the core mitochondrial...
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ISC synthesis pathway, promote Sml1 protein degradation through a Dun1-dependent but Mec1/Rad53-independent mechanism (59). However, cells lacking non-core ISC member Iba57, which functions in transferring 4Fe-4S clusters to mitochondrial targets (60, 61), or cells in which expression of Npb35, an essential component of cytoplasmic iron-sulfur cluster assembly machinery (62), has been repressed, induce Sml1 protein degradation through a mechanism that requires both Dun1 and Mec1 kinase proteins (59). Given that iron deficiency leads to down-regulation of the ISC synthesis pathway, we propose that various signaling pathways modulate Dun1 kinase function through its FHA domain or Thr-380 residue in response to iron limitation. Moreover, the different Dun1 phosphorylation patterns observed here in response to iron deficiency, DNA damage, or replicative stress also support different activating modulators mediating each response. The molecular bases for these differences and the nature of upstream transducers are currently unknown.

In this study, we demonstrate that Dun1 kinase promotes Rnr2 and Rnr4 redistribution to the cytoplasm in response to iron deficiency by phosphorylating Diff Ser-104 and Thr-105 residues. The physiological relevance of Dun1-dependent regulation of RNR function is highlighted by the decrease in dNTP levels observed in iron-deficient dun1Δ cells (47). Further studies are necessary to decipher how upstream factors perceive, transduce, and finally activate Dun1 kinase in response to iron deficiency and other stresses.

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