Yeast Dun1 kinase regulates ribonucleotide reductase small subunit localization in response to iron deficiency

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ABSTRACT
Ribonucleotide reductase (RNR) catalyzes the rate-limiting step in the \textit{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 (1-3)). In the budding yeast \textit{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

stimuli, which suggests different Dun1 activating pathways. Importantly, 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 relocalization to the cytoplasm and promote RNR function.

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Ribonucleotide reductase (RNR) catalyzes the rate-limiting step in the \textit{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 (1-3)). In the budding yeast \textit{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 (10, 11)).

In response to genotoxic or replication stress, 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 Dun1 kinase (14-17). Finally, Dun1 promotes RNR function through multiple mechanisms. Dun1 hyperphosphorylates 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, 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 import 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, DNA glycosylases, DNA helicases/nucleases and various DNA repair enzymes, in addition to RNR (28-34). Consequently appropriate iron delivery to enzymes in 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-sulphur 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-sulphur cluster assembly (CIA) machinery, responsible for delivering iron-sulphur 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 Cth2 RNA-binding protein specifically interacts with WTM1 transcript and facilitates its degradation (46). The resulting decrease in Wtm1 protein abundance promotes Rnr2-Rnr4 relocalization 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 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 dif1Δ (dif1::KanMX4), derived from wild-type Y300 (MATa, can1-100, ade2-1, his3-11,15, leu2-3,112, trpl-1, ura3-1, lys12). Yeast precultures were incubated overnight at 30ºC in synthetic complete SC medium lacking uracil (SC-ura), tryptophan (SC-trp) or leucine (SC-leu), and then reinoculated
in SC at OD$_{600\text{nm}}$ = 0.35. To regulate iron availability yeast cells were incubated between 6 and 8 hours in SC (iron-sufficient conditions) or SC supplemented with 100 μM of 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 M hydroxyurea (HU) was performed during the last 2 hours 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 the cytoplasm, or a predominantly cytoplasmic signal. Average and standard deviation were represented.

Protein analyses. Total protein extracts were obtained by using glass beads disruption in 20% trichloracetic acid as described (48). Protein extracts from 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 3Myc-Dif1 protein was detected with monoclonal mouse anti-c-myc antibody (9E10, Roche). Immunoblot was developed with HRP-labeled secondary mouse antibody and Western Lightening Plus ECL kit (Perkin Elmer).

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 ubiquitylated and degraded to facilitate RNR activation under low iron conditions (47). We have also shown that Rnr2-Rnr4 heterodimer is redistributed to the cytoplasm when iron availability is low (46). Given that Dun1 promotes Rnr2-Rnr4 relocalization 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 the control of its own promoter. Yeast transformants were grown under iron-sufficient conditions (SC), iron-deficient conditions achieved by the addition of Fe$^{2+}$-specific chelator BPS, or treated with the DNA alkylating agent methyl methanesulfonate (MMS). Then, RNR small subunit subcellular localization was determined by indirect immunofluorescence (IMF) with specific Rnr2 and Rnr4 antibodies (Figure 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 Figure 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 approximately 60%-70% of cells showed a cytoplasmic pattern (Figure 1B and 1C). As previously reported (24), dun1Δ cells displayed a defect in R2 redistribution to the cytoplasm when treated with MMS (Figure 1D and 1E). 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 (Figure 1D and 1E). 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 D328 residue within Dun1 kinase domain abolishes its catalytic activity and eliminates Dun1-dependent phosphorylation and degradation of Sml1 protein by DNA damage (Figure 2A, (49-51)). To ascertain whether Dun1 kinase catalytic activity was required for R2 redistribution to the cytoplasm during iron scarcity, we expressed
kinase dead \textit{DUN1-D328A} mutant allele in \textit{dun1Δ} cells and determined Rnr2 and Rnr4 subcellular distribution by IMF. As expected, Dun1 kinase activity was essential for proper R2 redistribution by genotoxic stress (Figure 2B and 2C). Importantly, yeast cells expressing \textit{DUN1-D328A} allele showed a defect in Rnr2 and Rnr4 relocation to the cytoplasm similar to that of \textit{dun1Δ} cells when iron was depleted (Figure 1D and 1E; Figure 2B and 2C). This was not a consequence of a reduction in Dun1-D328A protein abundance since 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.

\textbf{Contribution of Dun1 phosphorylation sites to Rnr2 and Rnr4 redistribution under iron deficiency.} Phosphorylation of T380 residue within Dun1 activation loop is essential for Sml1 protein degradation and growth when cells suffer DNA damage or replication stresses (51). To address whether T380 was important for R2 redistribution by iron limitation, we determined Rnr2 and Rnr4 subcellular localization in yeasts expressing \textit{DUN1-T380A} mutant allele. We first studied how 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 (Figure 2D and 2E). Similarly, Dun1-T380A cells displayed a strong accumulation of Rnr2 and Rnr4 proteins in the nucleus after treatment with BPS (Figure 2D and 2E). 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 T380 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, S10 and S139, which are important for optimal growth under DNA replication stress (51). We observed that both \textit{DUN1-S10A} and \textit{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 (Figure 3). Regarding iron deficiency, only \textit{DUN1-S139A} expressing cells exhibited a very slight defect in Rnr2 and Rnr4 redistribution to the cytoplasm, whereas no defect was observed for \textit{DUN1-S10A} allele (Figure 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 Dun1-S139A slight distribution defect is probably a consequence of its diminished abundance. Taken together, these analyses suggest that Dun1 T380 residue (but neither S10 nor S139) is important for small RNR subunit transport to the cytoplasm upon iron limitation.

\textbf{The integrity of Dun1 forkhead-associated domain is only partially required for Rnr2-Rnr4 relocation to the cytoplasm when iron availability decreases.} Dun1 FHA domain is necessary for binding to Rad53 diphosphothreonine motif (52). Thus, mutagenesis of Dun1 R60 or K100 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 \textit{DUN1-R60A} and \textit{DUN1-K100A/R102A} mutant alleles in a \textit{dun1Δ} strain and determined Rnr2 and Rnr4 subcellular distribution by IMF (Figure 4). As expected, both \textit{DUN1-R60A} and \textit{DUN1-K100A/R102A} expressing cells exhibited a significant defect in Rnr2 and Rnr4 relocation to the cytoplasm as compared to wild-type cells when MMS was added to the growth medium (Figure 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 (Figure 4). If we compare R2 distribution pattern of FHA-mutants under low iron conditions (Figure 4C-4F) to that of cells expressing wild-type \textit{DUN1} (Figure 4A and 4B), \textit{dun1Δ} cells (Figure 1), and cells expressing a non-functional \textit{DUN1} allele (Figure 2), we conclude that both \textit{DUN1-R60A} and \textit{DUN1-}
expressing cells exhibit a partial defect in Rnr2 and Rnr4 relocalization to the cytoplasm when iron is scarce. Therefore, these results indicate that Dun1 FHA domain is only partially required for a proper distribution of RNR small subunits during 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 cytoplasmic Rnr2-Rnr4 heterodimer to promote its import into the nucleus (17,27). Upon DNA damage or replicative stress, checkpoint Dun1 kinase phosphorylates Dif1 protein at specific residues within its Sml domain and promotes its degradation, 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 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, which is stabilized in 3Myc-Dif1 protein (Figure 5A). We observed a similar slower-migrating Dif1 in BPS-treated cells (Figure 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 (Figure 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 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 Dif1Δ79-103 deletion protein, and Dif1-T83A/S85A and Dif1-T89A/T92A double mutant proteins were still phosphorylated in response to iron deficiency (Figure 5B). Importantly, Dif1-T102A/S104A/T105A triple mutant protein was not phosphorylated upon iron limitation (Figure 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 S104 and T105 residues to alanine fully abrogated Dif1 phosphorylation under low-iron conditions (Figure 5C). To further ascertain whether Dif1 phosphorylation was caused by iron deficiency and not by a secondary effect of BPS, we determined Dif1 protein pattern in a fet3Δfet4Δ 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 Figure 5D, wild-type Dif1, Dif1-S104A and Dif1-T105A proteins were phosphorylated in fet3Δfet4Δ cells grown under iron-sufficient conditions. Importantly, Dif1 phosphorylation was abolished only when both S104 and T105 residues were mutagenized or DUN1 gene was deleted (Figure 5D). These results indicate that Dun1 kinase equally phosphorylates both S104 and T105 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 DNA alkylating agent MMS or DNA replication-blocking agent HU. As observed for BPS, simultaneous mutagenesis of S104 and T105 residues fully removed Dif1 phosphorylation in HU-treated cells (Figure 6A). However, in this case Dif1-S104A protein exhibited a lower level of phosphorylated Dif1 than wild-type Dif1 and Dif1-T105A proteins (Figure 6A), strongly suggesting that, upon HU treatment, Dif1 protein is preferentially phosphorylated at S104. When Dif1 phosphorylation sites were examined in MMS-treated cells, we observed that the S104A substitution alone abolished Dif1 phosphorylation form (Figure 6B), indicating that S104 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
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 is known since the sixties (reviewed in (33,34)). 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 multiple cancer types, RNR is considered a target for chemotherapeutic treatments (53,54). While many studies have characterized RNR regulation during 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 (reviewed in (11), Figure 8). One strategy involves Cth2 RNA-binding protein, which is specifically expressed in response to iron depletion (39). Cth2 binds to and degrades *WTMI* mRNA inducing a drop in protein levels of the R2 nuclear anchor Wtm1, thereby promoting RNR holoenzyme assembly in the cytoplasm and dNTP synthesis (Figure 8; (46)). Cth2 also binds and slightly diminishes *RNR2* and *RNR4* mRNA abundance, in a likely fine-tuning strategy to save iron cofactor (46). A second player is 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 (Figure 8; (47)). Here, we show that Dun1 kinase activation under iron deficiency also facilitates Rnr2 and Rnr4 redistribution to the cytoplasm (Figure 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 (*DUNI-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 Dun1 kinase domain is required for R2 redistribution when iron availability decreases or cells suffer DNA damage (Figure 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 through 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 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 T380 residue within Dun1 activation loop (14-16,51,52). In this work, we have addressed the contribution of Dun1 FHA
domain and T380 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 Dun1 FHA domain or T380 have been mutagenized (Figures 2 and 4). However, we observe that Dun1 FHA domain plays a minor role in R2 redistribution during iron scarcity (Figure 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 distribution under certain iron-deficient conditions. Furthermore, IMF results shown in this work indicate that Dun1 T380 residue is important for nucleus-to-cytoplasm transport by iron deficiency (Figure 2), suggesting that, in addition to Rad53, various 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 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 T380 residue (47). Taken together, these results suggest that Dun1 kinase is activated though 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 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 CIA 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 ISC synthesis pathway, we propose that various signaling pathways modulate Dun1 kinase function through its FHA domain or T380 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 Dif1 S104 and T105 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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Author contributions: NS and AMR conducted the indirect immunofluorescence experiments. CZ performed western blots. XW and XA constructed yeast strains and plasmids. NS, AMR, CZ, MH and SP designed the experiments and analyzed and interpreted the results. MH and SP wrote the paper.

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**FOOTNOTES**

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The abbreviations used are: BPS, bathophenanthroline disulfonic acid disodium; CIA cytoplasmic iron-sulphur cluster assembly; dNTP, deoxyribonucleotide; FHA, forkhead-associated; HU, hydroxyurea; IMF, Indirect immunofluorescence; ISC, iron-sulphur cluster; MMS, methyl methanesulfonate; RNR, Ribonucleotide reductase.
Dun1 regulates R2 localization in iron deficiency

FIGURE LEGENDS

Figure 1. Dun1 kinase participates in redistribution of Rnr2 and Rnr4 to the cytoplasm in response to iron deficiency. (A) Yeast dun1Δ cells transformed with either pMH80 (DUN1) or pRS416 (dun1Δ) plasmids were grown at 30°C for 6 h to exponential phase in SC or SC with 100 µM BPS. Rnr2 and Rnr4 subcellular localization was determined by IMF with anti-Rnr2 and anti-Rnr4 antibodies, respectively. (B and C) Quantitative analysis of Rnr2 (B) and Rnr4 (C) subcellular localization patterns of dun1Δ cells transformed with pMH80 (DUN1) and grown in SC, SC with 100 µM BPS, or SC with 0.04% MMS added during the last 2 h of incubation. (D and E) Quantitation of Rnr2 (D) and Rnr4 (E) subcellular localization patterns of dun1Δ cells transformed with pRS416 (dun1Δ), and grown as in panels B and C. More than 200 cells from at least 3 independent experiments were scored and percentages of cells with a predominantly nuclear signal (N, black bars), localization in both the nucleus and the cytoplasm (N/C, dark gray bars), or a predominantly cytoplasmic signal (C, light gray) were represented. Average and standard deviation are shown.

Figure 2. Dun1 kinase catalytic activity and T380 phosphorylation site are required for nucleus-to-cytoplasm Rnr2 and Rnr4 redistribution upon iron scarcity. (A) Schematic representation of most relevant Dun1 domains and amino acid residues. Numbers indicate amino acid position. FHA: forkhead-associated domain. (B and C) Dun1 kinase activity is necessary for R2 redistribution by iron deficiency. Yeast dun1Δ cells transformed with pMH80 (DUN1), pRS416 (dun1Δ), or pMH62 (DUN1-D328A) plasmids were cultivated and analyzed as described in Figure 1. Data corresponding to DUN1 and dun1Δ cells are shown in Figure 1. Quantitative analysis of Rnr2 (B) and Rnr4 (C) subcellular localization in cells expressing DUN1-D328A is represented. (D and E) Dun1 phosphorylation site T380 is important for R2 nucleus-to-cytoplasm redistribution by iron deficiency. Yeast dun1Δ cells transformed with pSP692 (DUN1), pRS413 (dun1Δ), or pSP695 (DUN1-T380A) plasmids were cultivated in SC and analyzed as described in Figure 1. Quantitative analysis of Rnr2 (D) and Rnr4 (E) subcellular localization in cells expressing DUN1-T380A is represented. Data corresponding to DUN1 and dun1Δ cells were similar to those displayed in Figure 1 and have not been represented for simplicity. Average and standard deviation are represented.

Figure 3. Dun1 serines 10 and 139 are not essential for Rnr2 and Rnr4 relocation to the cytoplasm promoted by iron depletion. Yeast dun1Δ cells transformed with pSP692 (DUN1), pRS413 (dun1Δ), pSP693 (DUN1-S10A) or pSP694 (DUN1-S139A) plasmids were cultivated and analyzed as described in Figure 1. Quantitative analysis of Rnr2 (A and C) and Rnr4 (B and D) subcellular localization in cells expressing DUN1-S10A (A and B) or DUN1-S139A (C and D) is represented. Data corresponding to DUN1 and dun1Δ cells were similar to those displayed in Figure 1 and have not been represented for simplicity. Average and standard deviation are shown.

Figure 4. Dun1 mutants in FHA-domain display a partial defect in the Rnr2 and Rnr4 relocalization induced by iron deficiency. Yeast dun1Δ cells transformed with pSP684 (DUN1), pRS413 (dun1Δ), pSP685 (DUN1-R60A), or pSP686 (DUN1-K100A/R102A) plasmids were cultivated and analyzed as described in Figure 1. Quantitative analysis of Rnr2 (A, C and E) and Rnr4 (B, D and F) subcellular localization in cells expressing DUN1 (A and B), DUN1-R60A (C and D) or DUN1-K100A/R102A (E and F). Data corresponding to dun1Δ cells were similar to those displayed in Figure 1 and have not been represented for simplicity. Average and standard deviation are represented.

Figure 5. Dif1 protein is phosphorylated at S104 and T105 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. (B) Mutagenesis of T102, S104 and T105 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. (C) Dif1 protein is phosphorylated at S104 and T105 in response to iron deficiency. 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 S104 and T105 residues under genetic iron deficiency in a Dun1-dependent manner. fet3Δfet4Δ and fet3Δfet4Δdun1Δ 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.

**Figure 6. Dif1 phosphorylation upon MMS or HU treatment.** (A) HU promotes Dif1 protein phosphorylation at S104 and T105. Yeast dif1Δ transformed with pMH1494 (DIF1), pXW26 (DIF1-S104A), pXW27 (DIF1-T105A), or pXW30 (DIF1-S104A/T105A) were grown in SC without or with 0.2M HU added for 2 h. (B) MMS promotes Dif1 protein phosphorylation at S104. Yeast dif1Δ mutant cells transformed as in panel C were grown in SC without or with 0.04% MMS added for 2 h. In both panels, proteins were extracted and analyzed as in Figure 5. Each result was repeated at least once, from cell culture treatment to protein blot.

**Figure 7. DIF1 mutants deficient in phosphorylation display defects in Rnr2 and Rnr4 exit from the nucleus during iron scarcity.** Yeast dif1Δ cells transformed with pMH1488 (DIF1, solid black line) or pMH1760 (DIF1-S104A/T105A, dashed black line) plasmids were grown to exponential phase. An aliquot was extracted (time zero), and then BPS was added to 100 µM final concentration. Additional aliquots were isolated after 2 and 6 h of BPS treatment. Rnr2 and Rnr4 subcellular localization was determined by IMF with anti-Rnr2 (A) and anti-Rnr4 (B) antibodies, respectively. More than 100 cells from at least 3 independent experiments were scored, and nuclear signal was represented over time in iron-deficient conditions. Average and standard deviation are shown.

**Figure 8. A model for the regulation of yeast ribonucleotide reductase in response to iron deficiency.** In response to iron deficiency, yeast cells promote RNR function by multiple mechanisms. Aft1 transcription factor activates the expression of 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).
TABLE 1. List of plasmids used in this study.

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Figure 1

(A) **DUN1** and **dun1Δ** subcellular localization of Rnr2 and Rnr4 in SC, BPS, and MMS. 

(B) **DUN1** subcellular localization of Rnr2 and Rnr4 in SC, BPS, and MMS. 

(C) **DUN1** subcellular localization of Rnr2 and Rnr4 in SC, BPS, and MMS. 

(D) **dun1Δ** subcellular localization of Rnr2 and Rnr4 in SC, BPS, and MMS. 

(E) **dun1Δ** subcellular localization of Rnr2 and Rnr4 in SC, BPS, and MMS.
Figure 2
Figure 3

A. **DUN1-S10A**

B. **DUN1-S10A**

C. **DUN1-S139A**

D. **DUN1-S139A**

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Figure 4
Figure 5
Figure 6
Figure 7

A

Rnr2

B

Rnr4

Figure 7
Figure 8