Function and Regulation of Yeast Ribonucleotide Reductase: Cell Cycle, Genotoxic Stress, and Iron Bioavailability

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Ribonucleotide reductases (RNRs) are essential enzymes that catalyze the reduction of ribonucleotides to desoxyribonucleotides, thereby providing the building blocks required for *de novo* DNA biosynthesis. The RNR function is tightly regulated because an unbalanced or excessive supply of deoxyribonucleoside triphosphates (dNTPs) dramatically increases the mutation rates during DNA replication and repair that can lead to cell death or genetic anomalies. In this review, we focus on *Saccharomyces cerevisiae* class Ia RNR as a model to understand the different mechanisms controlling RNR function and regulation in eukaryotes. Many studies have contributed to our current understanding of RNR allosteric regulation and, more recently, to its link to RNR oligomerization. Cells have developed additional mechanisms that restrict RNR activity to particular periods when dNTPs are necessary, such as the S phase or upon genotoxic stress. These regulatory strategies include the transcriptional control of the RNR gene expression, inhibition of RNR catalytic activity, and the subcellular redistribution of RNR subunits. Despite class Ia RNRs requiring iron as an essential cofactor for catalysis, little is known about RNR function regulation depending on iron bioavailability. Recent studies into yeast have deciphered novel strategies for the delivery of iron to RNR and for its regulation in response to iron deficiency. Taken together, these studies open up new possibilities to explore in order to limit uncontrolled tumor cell proliferation via RNR. *(Biomed J 2013;36:51-58)*

Key words: cell cycle, genotoxic stress, iron deficiency, ribonucleotide reductase, *Saccharomyces cerevisiae*, yeast

All eukaryotic organisms require an adequate, balanced concentration of deoxyribonucleoside triphosphates (dNTPs) in order to assure accurate DNA replication and repair, and to maintain genomic integrity. The rate-limiting step in dNTP synthesis is catalyzed by ribonucleotide reductase (RNR), an essential enzyme mediating the reduction of ribonucleotides to desoxyribonucleotides, thereby providing the building blocks required for DNA synthesis. Consistent with its important role in cell proliferation, a significant increase in RNR activity has been associated with tumor cells and resistance to chemotherapy. Indeed, since the utilization of hydroxyurea in the 70s to the current development of sophisticated RNR inhibitors, RNR as been used as an important target for the chemotherapeutic treatment of numerous cancer types.[1] Therefore, understanding the molecular mechanisms that cells utilize to regulate RNR function in response to different stresses is critical for the development of new and efficient anticancer therapies. In this review, we focus on the yeast *S. cerevisiae* as a eukaryotic model to advance in our understanding of mechanisms regulating the function of eukaryotic RNRs during cell cycle progress and in response to environmental cues, including genotoxic stress and low iron bioavailability.

RNR structure, assembly, and allosteric regulation

In eukaryotes, class Ia RNRs are oxygen-dependent enzymes composed of a large R1 (α_2) and a small R2 (β_2 or $\beta\beta'$) subunit. The R1 subunit contains the catalytic site and two allosteric effector binding sites that

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determine substrate preference and overall activity. The R2 subunit harbors a stable diferric tyrosyl radical cofactor (Fe^{3+}_2-Y·) required for catalysis. Briefly, substrate binding to R1 initiates a ~35 Å proton-coupled electron transfer pathway from R1 to the Fe^{3+}_2-Y in R2 that leads to ribonucleotide reduction. At each catalytic site, the regeneration of an active RNR requires the reduction of a conserved pair of oxidized cysteines in R1 by thioredoxin or glutaredoxin.[2-4]

One unique feature of *S. cerevisiae* RNR relies on the heterodimeric ββ′ nature of its active small R2 subunit, composed of two different proteins denoted Rnr2 and Rnr4. Whereas Rnr2 contains the indispensable Fe^{3+}_2-Y· cofactor, the structurally homologous Rnr4 lacks essential ligands for iron binding and cannot, therefore, form the canonical tyrosyl radical.[5-8] Despite this situation, deletion of *RNR4* leads to lethality or severe growth impairment, which highlights its important role in the RNR function.[5,6] Interestingly, *in vitro* and *in vivo* studies suggest that Rnr4 contributes to the correct folding and assembly of the Fe^{3+}_2-Y· cofactor in Rnr2, although the underlying mechanism remains unknown[9-13] (for a recent review on cofactor assembly into class I RNRs, see Ref. 4). Recent evidence demonstrates that conserved cytosolic monothiol glutaredoxins Grx3 and Grx4 function in delivering iron to multiple iron-containing proteins, including Rnr2.[14] Consistently with this, the yeast cells depleted of Grx4 exhibit reduced RNR-specific activity due to the inefficient incorporation of iron into Rnr2.[14] Furthermore, depletion of Fe–S cluster protein Dre2 also diminishes both Y levels and RNR activity, suggesting that Dre2 functions in providing reducing equivalents in order to deliver iron in its reduced state.[13] These results, and the genetic interactions among mutants *RNR4*, *grx3 grx4* and *Dre2,* suggest a model in which Grx3–Grx4 and Dre2 collaborate in delivering iron ion in its reduced state for Fe^{3+}_2-Y· cluster formation in the Rnr2 partner of the Rnr2–Rnr4 heterodimer.[13]

The RNR large subunit is composed of an Rnr1 homodimer, which is essential for mitotic viability.[15] In addition to *RNR1*, yeast cells express a second RNR large subunit, called *RNR3,* at very low levels, whose deletion does not cause any phenotype, but whose overexpression rescues the lethality of *RNR1* null mutants.[15,16] Despite low Rnr3 specific activity, experiments with a catalytically inactive *RNR1* mutant have indicated that Rnr3 increases its endogenous activity by associating with Rnr1.[16] However, further studies are required to elucidate the physiological function of yeast Rnr3.

Sophisticated allosteric regulations in the R1 large subunit contribute to maintain a balanced pool of dNTPs, which is essential to guarantee DNA synthesis fidelity.[17] Each R1 monomer contains two distinct regulatory sites: The specificity site (or S site) and the activity site (or A site). The S site, which is located at the dimer interface, acts as a sensor of each dNTP's individual concentration and determines which substrate is reduced at the catalytic site. When the allosteric effector binds to the S site, it alters the conformation of a flexible loop, which transmits the specific signal to the catalytic site to make it more amenable to discriminate between substrates, thus maintaining a balance among the four dNTP pools. Therefore, adenosine triphosphate (ATP) and deoxyadenosine triphosphate (dATP) promote the reduction of cytidine diphosphate (CDP) and uridine diphosphate (UDP), deoxythymidine triphosphate (dTTP) increases the guanosine diphosphate (GDP) reduction rate, and deoxyguanosine triphosphate (dGTP) up-regulates adenosine diphosphate (ADP) reduction. The A site, which is located in a small ATP amino-terminal cone domain, acts as a master switch, which controls overall enzyme activity by monitoring the dATP (inhibitor)/ATP (activator) ratio. Despite ATP being more abundant in the cell, dATP has a higher affinity for the A site. dATP displays 10-20 times lower affinity for the A site than for the S site. Thus, at concentrations below micromolar, dATP only functions as an S site regulator. When the dATP pool reaches higher concentrations, RNR activity is turned off by dATP feedback inhibition. However, an unusual relaxed dATP feedback inhibition of yeast Rnr1 leads to an expansion of yeast dNTP pools in response to DNA damage, which does not occur in mammalian cells.[18] Lack of Rnr3 inhibition by dATP suggests that an Rnr1–Rnr3 heterodimer may be advantageous in DNA-damaged cells, as it allows larger dNTP pools to accumulate; however, no decrease in dNTPs is observed in *rnr 3*Δ mutants upon DNA damage.[16,18]

Studies on mammalian and yeast cells suggest that without nucleotide effectors, R1 exists as an inactive α monomer. Upon the binding of ATP, dATP, thymidine triphosphate (TTP), or dGTP to the S site, R1 forms α_2 dimers, which can assemble as active $\alpha_2\beta_2$ ($\alpha_2\beta\beta'$ in yeast) heterotetramers.[19] Structural and functional data indicate that the binding of dATP to the A site promotes the association of three α_2 dimers to form an α_6 hexamer, which can interact only with a single R2 subunit to form an inactive $\alpha_6\beta_2$ complex ($\alpha_6\beta\beta'$ in yeast).[20] According to this model, the conformational changes accompanying dATP-induced hexamerization probably disrupt the proper interaction between the R1 and R2 subunits, leading to the disruption of the protein-coupled electron transport chain. On the other hand, when dATP levels decrease, ATP binding to the A site may promote structurally different conformational changes in the $\alpha_6\beta_2$ complex, which increase RNR catalytic activity. Additional RNR oligomeric structures, including active $\alpha_6\beta_6$ complexes, have also been proposed.[17]

Regulation of RNR during the cell cycle and in response to genotoxic stress

In all eukaryotic cells, including yeast, RNR activity is tightly controlled in order to avoid undesirable increases in dNTP pools, which raise mutation rates. Thus, the up-regulation of RNR activity is highly restricted to particular periods when dNTPs are required. The Mec1–Rad53–Dun1 DNA damage and replication checkpoint kinase cascade is responsible for promoting yeast RNR activity in response to DNA damage, DNA replication stress, or when cells enter the S phase of the cell cycle. The Mec1 kinase sensor activates the Rad53 central transducer. Both *MEC1* and *RAD53* are essential genes that can be rescued only by increasing dNTP pools. Hyperphosphorylated Rad53 activates the Dun1 effector kinase by phosphorylation. Then, the Dun1 checkpoint kinase enhances the RNR function by multiple mechanisms [Figure 1a].

One mechanism involves Crt1, also known as Rfx1, a DNA-binding protein that recognizes 13-nucleotide long cis-regulatory elements known as X-box motives, present in the promoter of damage-inducible genes, including *RNR2*, *RNR3*, and *RNR4*, and represses their transcription by recruiting the general repressor complex Tup1–Ssn6.[21] The robust repression of *RNR* genes by the Crt1 protein is achieved by a synergic corepression with Rox1 and Mot3, two Ssn6–Tup1-recruiting proteins that repress hypoxic genes, including *RNR2–4*.[22] Detailed studies on the nucleosomal structure of the *RNR3* promoter have shown that the Ssn6–Tup1 corepressor requires the collaboration of the ISW2 complex to establish a regular array of positioned nucleosomes that represses the *RNR3* expression.[23,24] In response to DNA damage or replication blocking, Crt1 becomes hyperphosphorylated via the Mec1–Rad53–Dun1 signaling pathway and moves away from DNA, thus preventing the formation of the repressor complex and leading to the activation of damage-inducible genes [Figure 1a].[21] Genetic data indicate that *CRT10* functions as a positive regulator in the *CRT1* pathway in response to DNA damage.[25] Despite the Rox1–Mot3-mediated repression still being functional during genotoxic stress, a substantial part of its repression is lost from lack of a synergy with Crt1.[22] Before dissociating from the *RNR3* promoter, Crt1 recruits the Transcription Factor IID (TFIID), the SWItch/Sucrose NonFermentable SWI/SNF chromatin remodeling complex, and histone deacetylases to promote transcription activation.[26,27] An additional level of regulation is possible because of the X-box motifs present in the *CRT1* promoter permitting an autoregulatory mechanism on the *CRT1* gene expression, which mediates its induction upon genotoxic stress and rapidly restores its repressed state after eliminating damage.[21]

Regulation of *RNR* genes upon genotoxic stress is not completely dependent on Dun1 kinase. For instance in *dun1*Δ mutants, *RNR* genes continue to be significantly induced in response to DNA damage.[21] Furthermore, recent results have demonstrated that the *RNR1* expression is induced upon DNA damage in a Dun1- and Crt1-independent manner. Instead, in response to genotoxic stress, Mec1 and Rad53 activate Ixr1, a DNA-binding protein that interacts with the *RNR1* promoter and which activates its transcription[28] [Figure 1a]. Consistently with a reduced *RNR1* expression, *IXR1* deletion results in lower dNTP levels, sensitivity to DNA damage agents, a Mec1–Rad53–Dun1–Crt1-dependent elevation of Rnr3 and Rnr4, and the down-regulation of Sml1 levels, which explain why *DUN1* is indispensible in *irx1*Δ mutants.[28]

The transcriptional regulation of *RNR* genes also contributes to fluctuating RNR activity during the cell cycle. Whereas *RNR1* mRNA levels increase more than tenfold in the G1/S phase, *RNR2* transcripts display only a slight twofold up-regulation.[15] No cell cycle regulation has been observed for *RNR3* and *RNR4* transcripts.[5,15] *RNR1* transcriptional activation during the transition from G1 to S phase is regulated by the MBF transcription factor, which coordinately controls the expression of many genes required for early cell cycle functions, including DNA replication and repair. MBF is an heterodimeric complex composed of regulatory transactivating protein Swi6, which is tethered to MCB elements at the promoter of *RNR1* and to other G1/S genes via its DNA-binding partner, Mbp1 [Figure 1b].[29,30] The MBF complex restricts the expression of its target genes to the G1/S transition. A Swi6-interacting protein, denoted Stb1, mediates MBF-dependent transcriptional repression, probably via Sin3, prior to its Cln/Cyclin-Dependent Kinase (Cln/CDK)-dependent phosphorylation, which occurs when cells enter G1.[31-33] In a late G1 phase, Stb1 is released from MBF promoters by an unknown mechanism, while a protein called Nrm1 accumulates, interacts with MBF, and promotes MBF-dependent transcriptional repression in the S phase.[34] Interestingly, recent results have demonstrated that in the S phase, genotoxic stress induces the activation of the Rad53 checkpoint kinase, which directly phosphorylates the Nrm1 corepressor in a Dun1-independent manner and prevents it binding to MBF, thereby permitting the specific transcriptional activation of G1/S cell cycle targets, including *RNR1*.[35,36] Moreover, the cell cycle regulatory SBF complex, composed of Swi6, and DNA-binding protein Swi4 have also been implicated in the transcriptional activation of *RNR2* and *RNR3* genes in response to DNA damage.[37]

As a result of the relaxed dATP feedback inhibition of RNR, yeast cells have developed an additional RNR repression mechanism. The Sml1 protein binds through its carboxy-terminal tail to the yeast R1 large subunit by inhib-

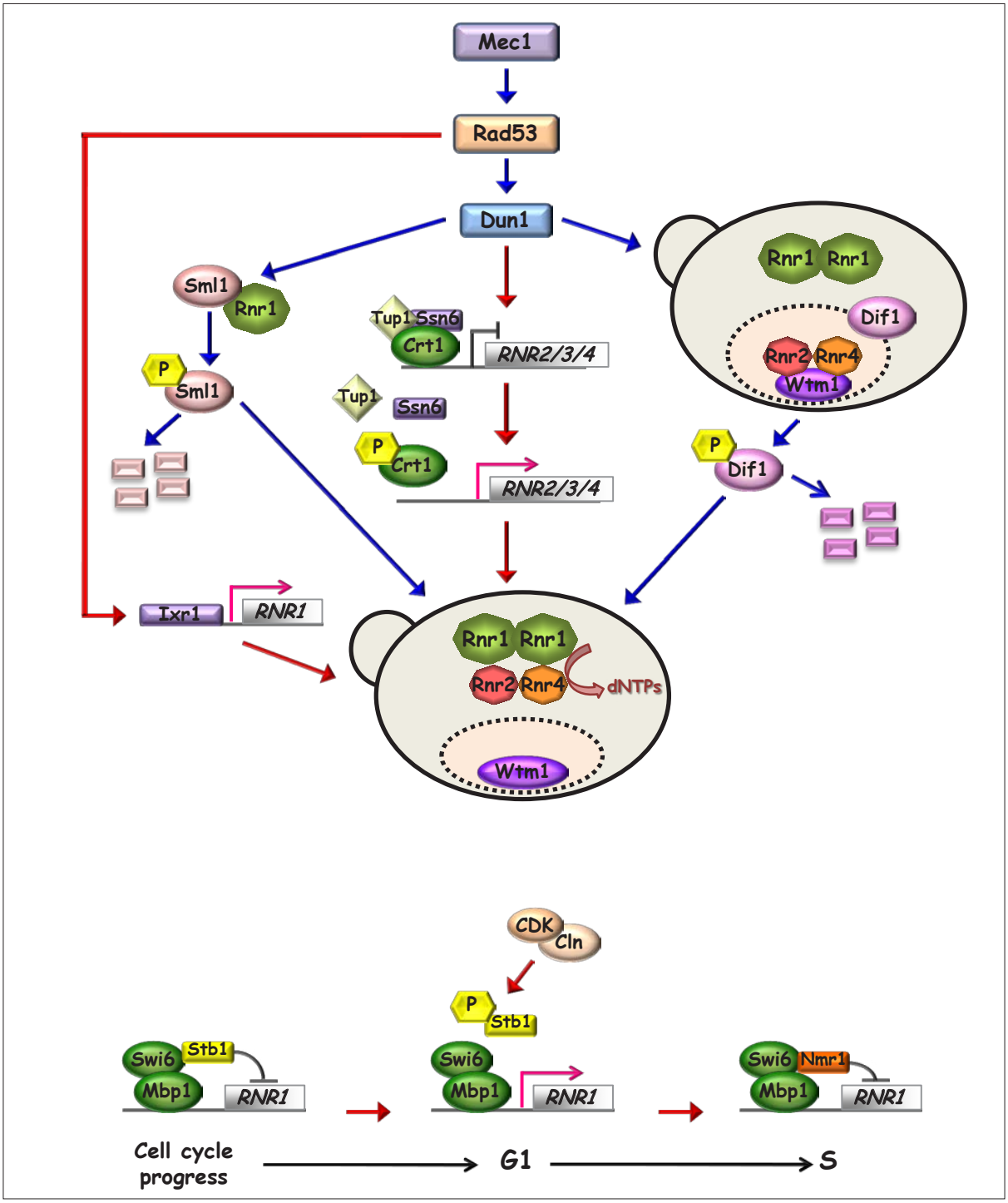

Figure 1: Model for regulating RNR activity in the S phase and in response to genotoxic stress. In addition to allosteric regulation mechanisms, RNR is tightly regulated in the S phase and after DNA damage or DNA replication blocks. (a) In response to genotoxic stress and in the S phase, the Mec1–Rad53–Dun1 kinase pathway activates RNR activity by (1) promoting the phosphorylation and degradation of the Sml1 R1-inhibitor and (2) enhancing R2 relocalization to the cytoplasm via Wtm1 and Dif1 modifications. Upon genotoxic stress, the Mec1–Rad53–Dun1 checkpoint pathway phosphorylates Ctr1, leading to the derepression of *RNR2/3/4* transcription. Recent results show that *RNR1* transcription is activated via Ixr1 in a Mec1–Rad53-dependent, but Dun1-independent manner, in response to genotoxic stress. Blue arrows refer to the pathways activated in genotoxic stress and in the S phase, whereas red arrows indicate the pathways only activated in response to genotoxic stress. (b) The transcription of *RNR1* in the G1/S cell cycle transition phase is regulated by the MBF heterodimeric complex, which is composed of the regulatory transactivating protein Swi6 and its DNA-binding partner Mbp1. Several proteins participate in the repression of *RNR1* outside the G1/S phase. Stb1 mediates MBF-dependent transcriptional repression prior to its Cln/CDK-dependent phosphorylation occurring at G1, whereas Nrm1 promotes the MBF-dependent transcriptional repression of *RNR1* as cells exit the G1 phase

iting its activity when DNA synthesis is not required.[38-40] Elegant *in vivo* experiments have shown that when each catalytic cycle is completed, reduction of cysteine residues at the R1 active site in the amino-terminal part of an α protomer is performed by a couple of cysteines located in the carboxy-terminal domain of the other α protomer.[41] Since Sml1 also binds to the amino-terminal region of R1, it competes with the carboxy-terminal cysteines and inhibits cysteine reduction at the active site and, hence, the enzyme turnover.[41] In the S phase, in response to DNA lesions, Sml1 protein levels dramatically lower, leading to an increase in dNTP levels. The underlying mechanism involves the activation of the checkpoint signaling pathway, which results in the direct phosphorylation of Sml1 by the Dun1 kinase.[42-45] Sml1 phosphorylation triggers its release from the R1 subunit and its degradation by the 26S proteasome to allow RNR activation [Figure 1a]. Recent data have demonstrated that the targeted destruction of phosphorylated Sml1 depends on a multimeric complex which includes E2 ubiquitin-conjugating enzyme Rad6, E3 ubiquitin ligase Ubr2, and E2–E3-interacting protein Mub1.[44]

Another mode of RNR regulation is the differential localization of its subunits. Under normal conditions, the yeast R1 large subunit localizes to the cytoplasm, whereas the R2 small subunit is predominantly localized to the nucleus. Upon DNA damage, replication blockage, or in the S phase, the majority of the R2 subunit is redistributed from the nucleus to the cytoplasm, resulting in an active RNR complex [Figure 1a].[46] Intriguingly, R2 redistribution to the cytoplasm is fully dependent on the Mec1–Rad53–Dun1 kinase pathway upon treatment with methyl methanesulfonate (MMS), but is only partially dependent when hydroxyurea (HU) is added.[46] In any case, both proteins Rnr2 and Rnr4 in R2 are cotransported between the nucleus and the cytoplasm as one heteromeric complex.[47] The subcellular localization of the R2 small subunit is simultaneously controlled by two different proteins: Wtm1 and Dif1. Under normal growth conditions, the WD40-containing protein, Wtm1, physically interacts with the R2 subunit by anchoring it to the nucleus. When cells undergo genotoxic stress, this interaction diminishes and the Rnr2–Rnr4 heterodimer relocalizes from the nucleus to the cytoplasm [Figure 1a].[48,49] The nature of this interaction and the underlying release mechanism upon DNA damage have not been characterized to date. The subcellular localization of R2 is also regulated by Dif1, a primary cytoplasmic protein containing a Hug domain conserved in Spd1, a *Schizosaccharomyces pombe* protein that interacts and sequesters the fission yeast R2 subunit into the nucleus away from its catalytic subunit.[50] Under normal conditions, Dif1 binds directly to R2 via the Hug domain and drives its import to the nucleus.[51,52] Dif1 protein levels are regulated during the cell cycle with a maximum increase at the end of the S phase when the R2 small subunit returns to the nucleus.[51] In response to DNA damage or replicative stress, the Dun1 checkpoint kinase directly phosphorylates Dif1 at specific residues within its Sml domain and promotes its degradation relieving RNR inhibition.[51,52] Thus, compartmentalization of the R2 small subunit contributes to modulate RNR activity and dNTP pools to ensure accurate, efficient DNA synthesis.

Regulation of RNR in response to low iron bioavailability

Iron is an indispensable micronutrient for all eukaryotic organisms because it participates as an essential cofactor in class Ia RNRs, among other reasons. The oxo-diiron center in RNR is responsible for generating and maintaining the stable tyrosyl radical that initiates the electron transfer leading to ribonucleoside diphosphate reduction. The low solubility of ferric iron at physiological pH frequently leads to iron deficiency, which is the most common and widespread nutritional disorder worldwide.[53] Despite this, very little is known about the mechanisms regulating the RNR function in response to iron limitation. Previous studies in mammals have shown that severe iron deficiency conditions can result in decreased RNR activity and dNTP pools.[54,55] A careful analysis of those studies shows that dNTP synthesis slightly increases during early iron deficiency stages,[55] suggesting that cells may possess mechanisms to maintain the RNR function when iron becomes scarce. By using budding yeast, we recently uncovered a novel mechanism to promote the RNR function when iron availability decreases.[56] We observed that in response to low iron, yeast R2 subunits redistribute from the nucleus to the cytoplasm, where R1 subunits permanently reside, in a manner that is independent of the well-established regulatory activation mechanism controlled by checkpoint kinases Mec1 and Rad53. Instead, a novel strategy promotes the Rnr2–Rnr4 subcellular relocalization, and optimizes both the RNR function and dNTP synthesis when iron bioavailability becomes limited. In response to low iron, yeast cells activate the expression of two RNA-binding proteins, denoted as Cth1 and Cth2, which specifically interact with the AU-rich elements within the 3′ untranslated region of many of the mRNAs encoding proteins which either contain iron or participate in metabolic pathways that utilize iron as a cofactor to promote their degradation.[57,58] Specifically, Cth1 and Cth2 mediate the degradation of transcripts that function in the tricarboxylic acid cycle, mitochondrial electron transport chain, lipid and amino acid metabolisms, and heme synthesis, among other

iron-dependent pathways.[57,58] Multiple approaches demonstrate that upon iron deficiency, Cth1 and Cth2 promote the down-regulation of *WTM1* mRNA.[56] The consequent decrease in Wtm1 protein levels facilitates the translocation of Rnr2–Rnr4 to the cytoplasm, leading to dNTP synthesis [Figure 2]. Mutagenesis of either Cth1–Cth2 or *WTM1*-specific binding motifs reduces R2 redistribution and diminishes dNTP pools during iron deficiency.[56] These results suggest that the Sml1-mediated repression of the R1 subunit should be relieved under low iron. However, the mechanisms regulating Sml1 under these conditions have not yet been described. Moreover, Cth1 and Cth2 also interact with *RNR2* and *RNR4* transcripts in response to iron deficiency by promoting their degradation and limiting new R2 subunit synthesis,[56] probably due to the scarce availability of the iron cofactor to incorporate into Rnr2. Interestingly, recent studies in yeast indicate that eukaryotic DNA polymerases rely on iron–sulfur clusters as essential cofactors for the formation of active complexes, thus highlighting the central role of iron in DNA synthesis.[59] Furthermore, a recent study in fission yeast has shown a direct link between DNA synthesis and RNR regulation. Briefly, the interaction of Spd1 with the polymerase processivity factor proliferating cell nuclear antigen (PCNA), complexed onto DNA, is essential for the Spd1 ubiquitylation and degradation that leads to RNR activation.[60] Further studies are required to elucidate how mammalian RNR activity and DNA synthesis are regulated in accordance with iron availability, and how these regulatory mechanisms influence the utilization of RNR as a target for anticancer treatments.

Conclusion

The budding yeast *S. cerevisiae* has outstandingly contributed to our current understanding of how eukaryotic cells function. Yeast cells possess multiple mechanisms to tightly control RNR activity in response to changes during

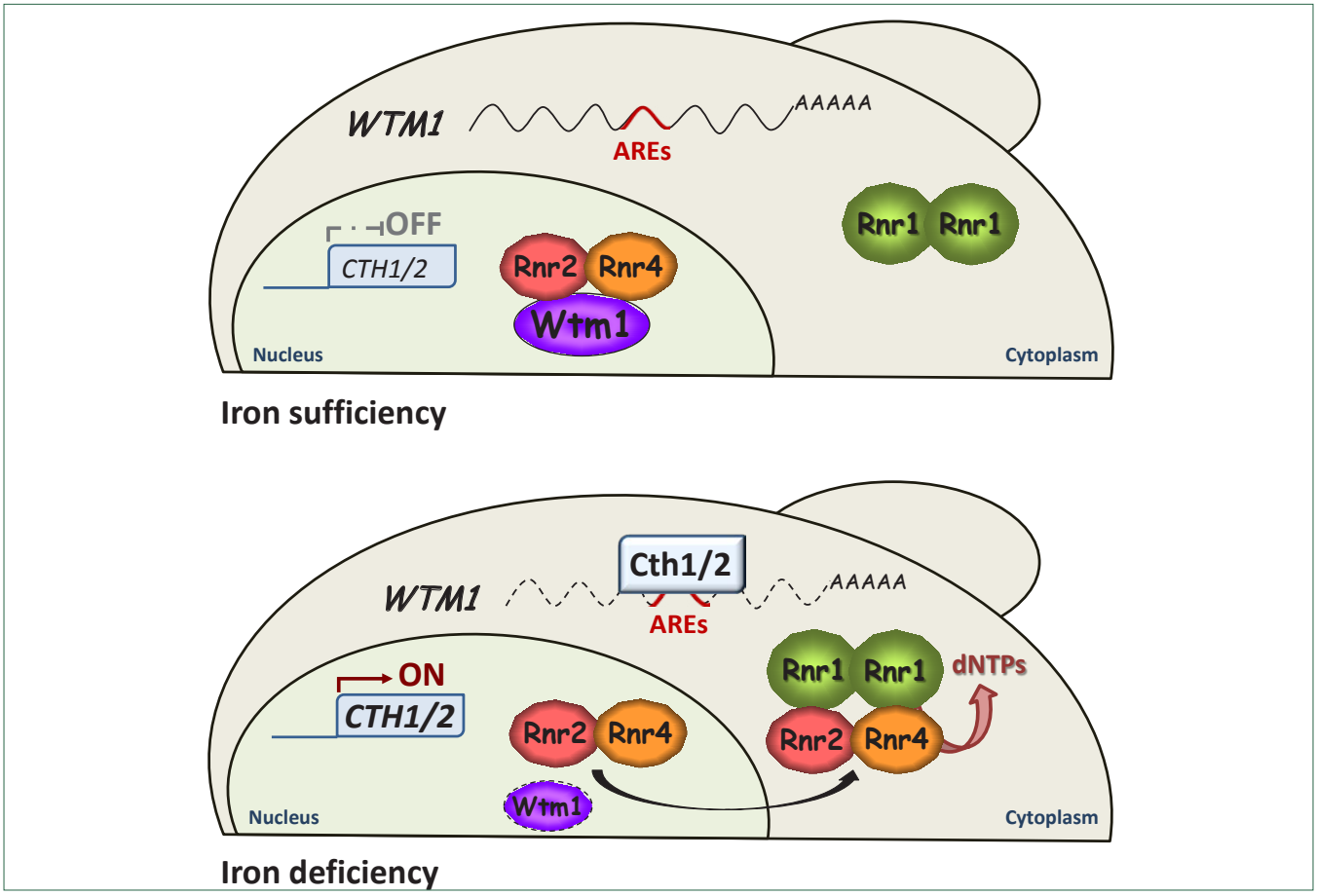

Figure 2: Regulation of RNR activity in response to iron deficiency. RNR activity is partially regulated by controlling the subcellular localization of the Rnr2–Rnr4 heterodimer. Under sufficient iron conditions, the catalytic Rnr1 homodimer resides in the cytoplasm, while iron-containing heterodimer Rnr2–Rnr4 is predominantly localized in the nucleus. Wtm1, a WD40 protein, anchors Rnr2–Rnr4 to the nucleus and limits RNR activity. Iron deficiency activates the expression of Cth1 and Cth2 proteins, which bind to *WTM1* mRNA and promote its degradation. The drop in the Wtm1 protein levels leads to the Rnr2–Rnr4 subunit translocation to the cytoplasm, where it binds to Rnr1 to form an active RNR enzyme

the cell cycle and environmental cues such as genotoxic stress and iron deficiency. The time has now come to decipher how these multilayered strategies are interconnected in order to regulate the yeast enzyme and to what extent they occur in mammalian cells.

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