Investigation of in Vivo Diferric Tyrosyl Radical Formation in Saccharomyces cerevisiae Rnr2 Protein

REQUIREMENT OF Rnr4 AND CONTRIBUTION OF Grx3/4 AND Dre2 PROTEINS

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Background: Yeast RNR small subunit is an Rnr2-Rnr4 heterodimer; only Rnr2 contains a cluster.
Results: mnr4 and dre2 mutations are defective in Rnr2 cluster formation and display synthetic growth defects with grx3/4.
Conclusion: Rnr4 stabilizes Rnr2 for cluster assembly via a pathway dependent on monothiol glutaredoxins Grx3/Grx4 and Fe-S cluster protein Dre2.
Significance: Understanding RNR cluster assembly may provide new cancer therapeutic strategy.

The β2 subunit of class Ia ribonucleotide reductase (RNR) contains a diferric tyrosyl radical cofactor (FeII-Tyr*) that is essential for nucleotide reduction. The β2 subunit of Saccharomyces cerevisiae is a heterodimer of Rnr2 (β) and Rnr4 (β′). Although only β is capable of iron binding and Tyr* formation, cells lacking β′ are either dead or exhibit extremely low Tyr* levels and RNR activity depending on genetic backgrounds. Here, we present evidence supporting the model that β′ is required for iron loading and Tyr* formation in β in vivo via a pathway that is likely dependent on the cytosolic monothiol glutaredoxins Grx3/Grx4 and the Fe-S cluster protein Dre2. mnr4 mutants are defective in iron loading into nascent β and are hypersensitive to iron depletion and the Tyr*-reducing agent hydroxyurea. Transient induction of β′ in a GalRNR4 strain leads to a concomitant increase in iron loading and Tyr* levels in β. Tyr* can also be rapidly generated using endogenous iron when permeabilized Δmnr4 spheroplasts are supplemented with recombinant β′ and is inhibited by adding an iron chelator prior to, but not after, β′ supplementation. The growth defects of mnr4 mutants are enhanced by deficiencies in grx3/grx4 and dre2. Moreover, depletion of Dre2 in Gal-DRE2 cells leads to a decrease in both Tyr* levels and ββ′ activity. This result, in combination with previous findings that a low level of Grx3/4 impairs RNR function, strongly suggests that Grx3/4 and Dre2 serve in the assembly of the diferric Tyr* cofactor in RNR.

Ribonucleotide reductases (RNRs) provide the building blocks for DNA replication and repair by catalyzing the reduction of nucleoside 5′-diphosphates to the corresponding deoxy forms using free radical-based chemistry (1, 2). Class Ia RNRs, which are conserved from bacteria to mammals, consist of two homodimeric subunits α2 and β2 that form an active (α2)2β2 complex (n = 1 or 3) (3–7). α2 contains the binding sites for substrates and allosteric effectors. β2 houses a diferric tyrosyl radical cofactor (FeII-Tyr*) that is essential for initiation of nucleotide reduction in α2 (8). A docking model of the x-ray structures of α2 and β2 (9–11) and biochemical studies have led to the proposal that the FeII-Tyr* of β2 plays an essential role in the first step in formation of a reversible and transient thyl radical at the active site in α2 via a proton-coupled electron transfer pathway (12, 13). Efforts to obtain insight into the factors involved in FeII-Tyr* formation in β are reported here.

The active form of the RNR small subunit in budding yeast Saccharomyces cerevisiae is a heterodimer of β and β′, encoded by the RNR2 and RNR4 genes, respectively (14–16). Only β is capable of iron binding and Tyr* formation, and thus there is a maximum of 1Tyr*/β′β′ (17–20). Although β′ lacks three residues required for iron binding and is unable to assemble its own metallo-cofactor (18), deletion of RNR4 causes lethality or a severe growth defect depending on the genetic backgrounds (15, 16). The Δmnr4 mutant is viable but exhibits an enlarged cell morphology (19) and is hypersensitive to the Tyr*-reducing agent hydroxyurea (HU) (21). Whole-cell EPR studies and β′ activity assays indicate that Δmnr4 cells have very low Tyr* content and <1% β′ activity relative to wild-type (WT) cells, despite a 15-fold increase of β levels in the mutant. These results suggest that β′ is essential for FeII-Tyr* cofactor assembly in β in vivo (19). β′ is also critical for efficient cofactor assembly in vitro. β2 and β′ expressed in Escherichia coli (recombinant (r)-β2 and r-β′2) are soluble and folded and predominantly in the apo-form. Efforts to reconstitute the cluster in yeast β2 alone by self-assembly from FeII and O2, which is successful for both mouse (22) and E. coli β2 (23), resulted in very low specific activity (19). However, upon mixing apo-r-β′2 and apo-r-β2, the homodimers undergo rapid monomer

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3 The abbreviations used are: RNR, ribonucleotide reductase; α2, ribonucleotide reductase large subunit; β2, β′/β′′ ribonucleotide reductase small subunit; RNR2, ribonucleotide reductase small subunit; α2, ribonucleotide reductase large subunit; β2, β′/β′′ ribonucleotide reductase small subunit; ISC, iron sulfur cluster; r-β2 (r-β′2), recombinant β2 and β′2/r, recombinant.
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![Diagram of RNR cluster assembly](image)

**FIGURE 1.** Generic RNR diferred-Tyr’ cluster assembly. The in vitro self-assembly of the Fe\(^{II}\)-Tyr’ cluster requires Fe\(^{2+}\), O\(_2\), and an electron (26). Our hypothesis is that these requirements are likely to be the same in vivo but involve specific protein factors.

exchange to form an apo-ββ’ heterodimer. Self-assembly of apo-ββ’ with Fe\(^{2+}\) and O\(_2\) exhibits 200-fold higher specific activity relative to β\(_2\) under the same cluster reconstitution conditions (19).

Despite evidence indicating the critical role of β’ in Fe\(^{II}\)-Tyr’ cofactor assembly in β, its mechanistic function remains unclear. Previous *in vitro* studies have ruled out β’ as a noncatalytic chaperone for iron delivery directly to β (18). Comparison of the structures of the homodimers and heterodimer (with limited metal occupancy) suggests that β’ might stabilize β in a conformation favorable for iron binding (24, 25). However, to date neither mechanisms nor pathways within β for iron loading in any small RNR subunit are understood.

Previous studies have demonstrated that the Fe\(^{II}\)-Tyr’ cofactor of *E. coli* NrdB (β\(_3\)) as well as other eukaryotic β\(_2\) can be generated *in vitro* by self-assembly from apo-β, Fe\(^{3+}\), and O\(_2\) (Fig. 1). However, the inability to control iron loading and reducing equivalent delivery has resulted in sub-stoichiometric amounts of loaded iron and Tyr/β’s (18, 23, 26). It also should be noted that there is a consensus from the study of this process in many organisms that there is only one Tyr’ per β\(_2\) (26). The variable iron loading *in vitro* suggests the existence and importance of a biosynthetic pathway for effective cluster formation *in vivo*. Moreover, rapid Tyr’ loss in crude cell lysates through endogenous reductants or exogenous reducing agents such as HU and triapine (27) also suggests the importance of a regulatory maintenance pathway, for reactivation of the reduced, inactive met-β\(_2\) or ββ’ (28, 29) under different growth conditions.

Recent studies of the *E. coli* NrdB (β\(_3\)) have shown that both biosynthetic and maintenance pathways involve a [2Fe2S] ferredoxin, YfaE (26), and suggest the importance of the flavin-dependent ferredoxin reductase, Fre (29, 30). YfaE is proposed to provide reducing equivalents as well as to facilitate choice of iron over manganese in cluster assembly (26, 31), whereas Fre may reduce YfaE for it to be used catalytically. Our hypothesis is that these pathways will be universal. Nonetheless, the only known *S. cerevisiae* ferredoxin, Yah1, is localized in the mitochondria and, together with the Fre counterpart Arh1, constitutes the electron transfer chain for the mitochondrial iron-sulfur cluster assembly machinery (32–35). The exclusive mitochondrial localization of Yah1-Arh1 makes them unlikely to perform the role in cluster assembly in the cytoplasmically localized ββ’ (20, 32). However, very recently the evolutionarily conserved [2Fe2S]-containing protein Dre2 and diflavin reductase Tah18 have been proposed to act as the cytoplasmic equivalents of the mitochondrial Yah1-Arh1 pair, providing electrons to the cytosolic Fe-S assembly machinery (36, 37). It is unknown whether Dre2-Tah18 can also provide electrons to other iron-utilizing reactions, including RNR cluster assembly.

Another unsolved question is the source of the bioavailable iron for RNR cofactor assembly. A recent study by Mühlenhoff et al. (38) has shown that two conserved cytosolic monothiol glutaredoxins, Grx3 and Grx4, are essential in intracellular trafficking of bioavailable iron for assembly of all iron cofactors in proteins specifically those with Fe-S clusters, hemes, and diiron proteins, including RNR (38). In this study, we investigate the functional role of β’ in iron delivery and cluster assembly in β and the potential roles of Grx3/Grx4 as the source of iron and Dre2 as the source of reducing equivalents in yeast cells. We show that *rnr4* mutant cells exhibit severe growth defects under iron-deficient conditions, which are rescued by increasing iron in the medium. Cells lacking β’ are defective in iron loading into newly synthesized β and have extremely low levels of Tyr’ and RNR activity. Transient induction of β’ in cells lacking β’ leads to increased iron incorporation into β and a concomitant increase in both Tyr’/β’ and RNR activity. We also demonstrate that adding purified β’ to permeabilized Δmrnr spheroplasts results in rapid Tyr’ generation and reconstitution of RNR activity, suggesting that β’ facilitates pre-existing apo-β\(_2\) to form ββ’, to acquire endogenous bioavailable iron, and to assemble its Fe\(^{II}\)-Tyr’ cofactor. Finally, we show genetic interactions between *rnr4*, *grx3*/*grx4*, and dre2 mutant alleles suggesting that these proteins may play important roles in cluster assembly. The impairment of Tyr’ formation and ββ’ activity in cells depleted of Dre2, in conjunction with our previous studies showing defective RNR function in cells depleted of Grx3/4 (38), suggests a pathway consisting of Grx3/Grx4, Dre2-Tah18, and β’ that is involved in iron mobilization, delivery, and reduction in Fe\(^{II}\)-Tyr’ cofactor assembly in β.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Plasmids, Media, and Growth Conditions**—
Yeast strains used in this study are listed in Table 1. *GalRNR2* and *GalRNR4* strains were constructed by replacing the endogenous promoters of *RNR2* and *RNR4* with the *GAL1* promoter as described previously (39). Swapping of the *GAL1* promoter used a PCR-generated cassette with a HIS3 marker. Transformants were selected on complete supplement mixture (CSM)-His raffinose and galactose agar plates and confirmed by Western blot of repressed and induced cell lysates using anti-Rnr2 monoclonal antibody. 

Rich YPD medium contained 1% yeast extract, 2% peptone, and 2% glucose. YP-raffinose contained 2% raffinose instead of
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**TABLE 1**

| Yeast strains used in this study | Genotypes | Parental strain |
|---------------------------------|-----------|-----------------|
| BY4741                          | MATa, his3Δ1, leu2ΔΔ0, met15Δ0, ura3Δ0 | BY4741          |
| GalIRNR2                        | MATa, rnr2::HisMX6::pGAL1-RNR2 | BY4741          |
| GalIRNR4                        | MATa, rnr4::HisMX6::pGAL1-RNR4 | BY4741          |
| Δrnr                           | MATa, rnr4::KanMX4 | BY4741          |
| Δgrx3grx4                       | MATa, grx3::LEU2, RX4::KanMX4 | BY4742          |
| GalIRRNR2Δgrx3grx4             | MATa, rnr4::HisMX6::pGAL1-RNR4, grx3::LEU2, grx4::KanMX4 | BY4742          |
| GalDRE2                        | MATa, dre2::HisMX6::pGAL1-DRE2 | BY4742          |
| ΔrnrGalDRE2                   | MATa, rnr4::KanMX4, dre2::HisMX6::pGAL1-DRE2 | BY4741          |
| YPH499                         | MATa, ura3-52, lys2-801, ade2-101, trplΔ63, leu2Δ1 | YPH499          |
| GalDRE2 (YPH499)              | MATa, dre2::HisMX6::pGAL1-DRE2 | YPH499          |

Glucose. Defined medium contained 6.7 g/liter yeast nitrogen base without amino acids from Difco, 2% glucose, CSM, or CSM with amino acid dropout from MP Biomedical, and 1.5% Difco agar as a solidifying agent for agar plates. Iron-buffered plates consisted of modified defined medium to which buffer (50 mM MES, pH 6.1) and iron chelator (1 mM ferrozine) were added. Different amounts of ferrous iron (0, 20, 50, and 350 μM) were added back to establish windows of available iron as described previously (40).

55Fe Labeling and Rnr2(β) Immunoprecipitation—Radiolabeling of yeast cells with 55FeCl3 and measurement of 55Fe incorporation into proteins by immunoprecipitation were performed as described previously (41). Briefly, yeast cells were grown in iron-free medium overnight (16 h) to an A600 1.0–2.0, and 2 x 106 of cells (wet weight 0.25 g) were harvested for each sample. The cells were washed once with 10 ml of water and resuspended in 10 ml of iron-free medium, and 15 μCi of 55FeCl3 (PerkinElmer Life Sciences, specific activity 76 mCi/mg) diluted in 100 μl of 0.1 M sodium ascorbate was added to the medium (the final concentrations were 1.8 μM Fe3+ and 1 mM ascorbate). Cells were incubated for 2 h at 30 °C before being harvested and lysed by glass bead disruption, followed by protein extraction in TNETG buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol) supplemented with 1 mM PMSF and 1 protease inhibitor mixture (Roche Applied Science). Immunoprecipitation of β was carried out by addition of anti-Rnr2 polyclonal antibodies (18) at 1:100 v/v ratio into 2 mg (~450 μl) of whole-cell extracts, which was incubated at 4 °C overnight followed by incubation with 20 μl of protein A-Sepharose beads (GE Healthcare) for 3 h. The bead-bound immunocomplex was washed with 500 μl of TNETG buffer three times at 4 °C, each time for 30 min, before being subjected to scintillation counting.

Purification of Recombinant r-α, r-β, His6-r-β, His6-r-β Δ8aa—Previous protocols were used to purify E. coli-expressed recombinant yeast RNR subunits (18, 19, 42). The α purification employed a DEAE-Sephadex column and a DAP affinity column (42) and had a specific activity of 102 nmol min⁻¹ mg⁻¹ when assayed in the presence of a 10-fold excess of FLAG-β′ (specific activity ~3000 nmol min⁻¹ mg⁻¹) from yeast Δrcl1 MYH619 cells (43). Untagged β′, N-terminally His6−tagged β′, and N-terminally His6−tagged β′ with the C-terminal 8 amino acids removed (His6-r-β Δ8aa) were purified using anion exchange column and nickel-nitrilotriacetic acid column, respectively (18, 42).

Preparation and Quality Assessment of Permeabilized Yeast Spheroplasts—Logarithmically grown yeast cells in 1 liter of YPD culture were harvested at A600 ~0.8 with a typical yield of 2 g of cell paste. Spheroplasts were prepared and then permeabilized using a protocol previously described with minor modifications (44, 45). Briefly, the cell wall was digested with 0.2 mg of zymolyase 100T (US Biological)/g wet weight cells that were resuspended in a spheroplast buffer containing 20 mM potassium phosphate buffer (KP), pH 7.5, 1.2 m sorbitol for 20 min. Completion of cell wall digestion and spheroplast formation was confirmed by microscope-aided observation of cell lysis in hypotonic buffer. Metabolically active spheroplasts were then recovered in YPD medium containing 0.7 m sorbitol at 30 °C with gentle shaking for 20 min. The recovered spheroplasts were resuspended in a permeabilization buffer containing 20 mM HEPES-KOH, pH 6.8, 0.4 m sorbitol, 150 mM potassium acetate, and 2 mM magnesium acetate and split into 300–400-μl aliquots in 1.7-ml Eppendorf tubes. The permeabilized spheroplast suspensions were slowly frozen for 10–15 min in the vapor of liquid nitrogen before being stored at −80 °C.

To assess the quality of spheroplast permeabilization, an aliquot of suspended permeabilized spheroplasts that were centrifuged at 1400 × g and separated into supernatant and cell pellets were analyzed for protein concentrations. After normalizing each for sample cell number, a Bradford assay revealed that 90–95% of total cellular protein was retained within the cell plasma membrane. Western blotting of β and RNR activity assays of the pellet and supernatant also confirmed minimal (5–10%) protein leakage, while achieving desired permeability to reagents that were used for the RNR activity assay. Previous studies of permeabilized spheroplasts prepared with the same method showed that subcellular organelles were intact under these conditions (44, 45).

Assays of RNR Activity—For a typical assay of the WT strain, 26 μl of permeabilized cell suspension (57 A600/ml) was thawed and assayed for RNR β activity. Typically, a total volume of 180 μl containing 0.1 m KP, pH 7.5, 0.6 m sorbitol, 3 mM ATP, 10 mM NaF, and 50 mM DTT (reduant required for RNR turn-over) with or without 4.4 μM α (specific activity 102 nmol min⁻¹ mg⁻¹) was mixed with permeabilized spheroplasts at 4 °C and warmed at 30 °C for 1 min. The reaction was then started by addition of 1 mM [5-3H]CDP (ViTrax, specific activity 17 Ci/mmol, diluted with cold CDP to 5790 cpm/nmol, 73 mM stock solution) and mixed, and aliquots (58 μl) were removed at 0, 5, and 10 min, respectively. DTT concentrations from 10 to 50 mM and E. coli thioredoxin (100 μM, specific
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activity 40 units/mg) and E. coli thioredoxin reductase (2 μM, specific activity 1600 units/mg) were also examined as reductants in this assay and found to give similar results. The reaction was stopped by placing the sample in a boiling water bath for 2 min. Each sample was then incubated with 10 units of alkaline phosphatase (Roche Applied Science, from calf intestine), and the amount of dCDP produced was analyzed as described previously (46, 47).

The activity assay for active Fe^{II-III}–Tyr’ formation in β in the Δrnr4 mutant strain also used ~1.5 A_{600} permeabilized spheroplasts and was a variation of the protocol described above. Initially, both α and DTT were added as above to the permeabilized spheroplasts at 4 °C. In one set of experiments, His_{α-β’2} (or untagged β’2, the results were similar) was added to this mixture at a final concentration of 7.7 μM, and the reaction was mixed and incubated at 30 °C for 1 or 3 min. Both times were sufficient to assemble the Fe^{II-III}–Tyr’ cofactor. [3H]CDP was then added, and the assay for dCDP formation was carried out as described above. To examine the effect of chelator on this process, chelator (dipyridyl or EDTA, 2 mM final concentrations) was added before and after the 3-min incubation of His_{α-β’2} and incubated for 3 min at 30 °C. The assay for dCDP formation was as described above.

Whole-cell EPR Spectroscopy of GalRNR4 Cells and EPR Spectroscopy Measurements of Tyr’ Reconstitution in Permeabilized Δrnr4 Spheroplasts—The GalRNR4 cells (2 liters) were grown in YP-Raffinose (GAL uninduced) to log phase, and galactose was added to a final concentration of 0.5% (w/v, GAL induced). Cells from 250 ml of culture (A_{600} 0.8–2) were harvested at different time points subsequent to GAL induction, washed with 1 liter of ice-cold PBS buffer, and resuspended in 300 μl of PBS buffer, 30% glycerol at 7–10 × 10^9 cells/ml. The cell suspensions were then packed into EPR tubes and frozen immediately in liquid nitrogen.

For a typical experiment, 60 μl of Δrnr4 permeabilized spheroplast suspension (32 A_{600}/ml) was added to 200 μl of 0.1 M KP i, pH 7.5, 0.6 M sorbitol buffer containing 50 mM DTT, 6.5 μM His_{α-β’2} (1.7 μl from 1 mM stock) and mixed, and the reaction was incubated at 30 °C for 3 min before loading into an EPR tube and rapidly freezing in liquid nitrogen. The effect of chelators on Fe^{II-III}–Tyr’ assembly was studied by addition of 2 mM dipyridyl or EDTA for 30 s before addition of His_{α-β’2}. The effect of Fe^{II} in cluster assembly was studied by adding 20 μM ferrous ammonium sulfate to permeabilized spheroplasts, followed by immediate addition of 6.5 μM His_{α-β’2}. In a control experiment, the chelator, dipyridyl or EDTA, was added after the 3-min incubation of His_{α-β’2} with permeabilized Δrnr4 spheroplasts.

EPR spectra were acquired on a Bruker EMX X-band spectrometer at 30 K using an Oxford Instruments liquid helium cryostat. Acquisition parameters were as described previously (19, 43, 48) at 9.4 GHz, 2 milliwatt power, 2.52 × 10^4 gain, 1.5 G modulation amplitude, and 100 kHz modulation frequency. Spin quantitation was performed by double integration of the signal and comparison with an E. coli NrdB sample in which the Tyr’ content had been determined by the drop line method using UV-visible spectroscopy (49). Analysis was carried out using WinEPR software (Bruker).

RNR Activity Assay and Whole-cell EPR Spectroscopy Measurements of GalDRE2—Single-cell EPR spectroscopy measurements, GalDRE2 (BY4741 background) cells were grown in 50 ml of YP-Raffinose/Galactose overnight before being harvested. Cells were washed with water and then diluted into 1 liter ofYPD or 1 liter ofYP-Raffinose/Galactose liquid media for continued incubation. Cells were harvested at different time points, while the cultures were in log phase, and prepared for EPR analyses as described above.

RESULTS

Cells Lacking β’ Grow Poorly under Iron-deficient Conditions—Previous studies have shown that the Δrnr4 mutant cells have very low Tyr’/β’ content and <1% of WT-β’ activity (19). These observations could result from inefficient iron loading and/or unstable iron binding to β in the absence of β’, which could be further exacerbated by limiting iron in the growth medium. To address this possibility, we compared growth of WT, GalRNR2, GalRNR4, and Δrnr4 mutants on synthetic medium containing 1 mM ferrozine, which has maximum chelating capacity of 333 μM Fe^{II} (40), and varied the concentrations of supplemental ferrous ammonium sulfate. All four strains were from the S288C parental WT strain BY4741. GalRNR2 and GalRNR4 strains contain replacement of the chromosomal RNR2 and RNR4 promoters with the GAL1 promoter that is under the control of glucose repression (50). All strains were grown in YP-Raffinose/Galactose medium to allow expression in the GalRNR2 and GalRNR4 cells, respectively, before being dot-plated onto the ferrozine/ferrous medium that contained glucose, which represses the GAL1 promoter. Under iron deprivation (0, 20, and 50 μM iron), all three mutants grew much more slowly than the WT (Fig. 2) with Δrnr4 exhibiting the worst growth defect. The poor growth phenotype of both GalRNR4 and Δrnr4 mutants is suppressed on plates supplemented with 350 μM Fe^{II} (i.e. iron-replete conditions) (40). In contrast, increasing iron did not rescue the growth defect of the GalRNR2 cells (Fig. 2). These results are consistent with a role of β’ in facilitating iron incorporation into β without which there is little RNR activity to support mitotic growth.

Rapid and Efficient Tyr’ Formation upon Induction of β’ in GalRNR4 Cells—We have previously shown that the Δrnr4 mutant has undetectable Tyr’ content and extremely low RNR activity, despite a 15-fold elevated level of β (19). Similar to Δrnr4, GalRNR4 cells also have undetectable levels of Tyr’ (Fig. 3A, black line) when grown in YP-raffinose (uninduced), suggesting a critical role of β’ for Tyr’ formation. To determine whether induced β’ expression is able to facilitate Tyr’ formation in these cells, we added 0.5% galactose into a log phase culture grown in YP-raffinose and harvested cells at different time points for whole-cell EPR analyses and Western blotting for β and β’. Levels of β’ were undetectable in uninduced Gal-
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RNR4 cells and started to increase by 1 h, reaching a plateau between 2 and 8 h after induction (Fig. 3B). Concomitant with the increasing β' levels, the Tyr' signal from whole-cell EPR spectra became visible at 1 h of β' induction (Fig. 3A, red line) and reached much higher levels at the 2- and 3-h time points (Fig. 3A, green and purple lines, respectively). The concurrent and proportional increases in Tyr' and β' levels suggest a non-catalytic role of β' in facilitating Fe^{III}-Tyr' cluster formation in β. The β protein levels gradually declined during the 8-h period of β' induction (Fig. 3B), which is likely due to Crt1-mediated negative feedback control of the RNR2 promoter (51). Consistent with the decrease in β levels, the Tyr' levels also decreased by the 8-h time point (Fig. 3A, blue line). Fig. 3C shows the changes in Tyr' content normalized by cell number during the 18 h; the Tyr' content is summarized in Table 2. It is worth noting that over 10-fold more Tyr' content per cell was generated within 3 h of β' induction in the GalRNR4 cells relative to WT (378 versus 33 fmol/cell (19)). Thus, the rapid and efficient Tyr' production in GAL-induced GalRNR4 cells provides an excellent tool to investigate protein factors involved in iron delivery and electron donation during active RNR cofactor formation in vivo.

Cells Lacking β' Are Defective in Iron Loading into Newly Translated β—We wanted to directly monitor iron incorporation into newly translated β in vivo and determine the effects of β' on this process. Toward this end, we used the $^{55}$Fe radiolabeling and immunoprecipitation assay developed by Lill and co-workers (41). WT and Δrnr4 cells were grown in iron-free medium overnight before being pulse-labeled with $^{55}$FeCl$_3$ for 2 h. Analysis of $^{55}$Fe incorporation into β from the WT and Δrnr4 cells revealed 5-fold lower $^{55}$Fe-β loading in Δrnr4 (Fig. 4A, bottom panel). However, interpretation of the observed differences in $^{55}$Fe-β loading is complicated by the fact that the Δrnr4 cells contained 10–15-fold higher levels of β relative to the WT cells (Fig. 4A, top panel) (19) and exhibited an ~50% increase in cellular $^{55}$Fe uptake relative to WT cells (data not shown). To circumvent these problems, we performed $^{55}$Fe radiolabeling and β immunoprecipitation assays in the GalRNR4 strain. GalRNR4 cells were grown in iron-free YP medium overnight and pulse-labeled with $^{55}$FeCl$_3$ for 2 h under either uninduced (−Gal) or induced (+Gal) conditions for β' expression. The level of β remained relatively unchanged within the first 2 h of β' induction (Fig. 4B, top panel). Furthermore, no difference in cellular $^{55}$Fe uptake was observed between −Gal and +Gal cells (data not shown). The uninduced cells had an 8-fold lower $^{55}$Fe-β loading compared with GAL-induced cells (Fig. 4B, bottom panel). Together, the data from the Δrnr4 and GalRNR4 strains strongly indicate that β' plays a critical role in iron loading into newly translated β polypeptide. In the absence of β', either iron is not loaded into β at all or iron is so loosely bound that it is easily lost during the process of cell lysis and immunoprecipitation.

Rapid Activation of Pre-existing β and Efficient Reconstitution of the Fe^{III}-Tyr' Cluster in Permeabilized Δrnr4 Spheroplasts by Addition of r-β'$_2$—Considering the difficulties we encountered in the self-assembly of Fe^{III}-Tyr' in ββ' in vitro (19), we decided to investigate this process in an environment that is similar to the intact yeast cells. Toward this end, we adopted a previously described permeabilized spheroplast assay protocol with minor modifications (see “Experimental Procedures”) (44, 45), which increases plasma membrane permeability to macromolecules, while maintaining integrity of intracellular organelles.

To establish the validity of the method, we determined RNR activity in permeabilized WT spheroplasts based on CDP reduction in the presence and absence of an excess of E. coli-expressed α using DTT as reductant. As shown in supplemental Fig. 1A, addition of α, DTT, and $[^3]$H]CDP resulted in a marked increase in the rate of production of dCDP (0.2–4.0 nmol of dCDP/A$_{600}$ at 10 min). This result is consistent with our previous findings that α is the limiting factor for RNR activity in partially purified yeast cell extracts (19). As a second control, the specific activity of β was examined in WT cells pretreated with 100 mM HU for 2 h before spheroplast permeabilization. The specific activity of ββ' in the HU-treated cells is 8-fold lower than the WT cells (supplemental Fig. 1B), consistent with previous results of HU-mediated Tyr' loss measured by whole-cell EPR and in vitro RNR inhibition by HU (43). Taken together, our results demonstrate that RNR activity measurements in permeabilized spheroplasts faithfully reflect changes in Tyr' levels and the activity of ββ' inside the cell.

We then measured RNR activity in permeabilized Δrnr4 spheroplasts using the same approach. As expected, Δrnr4 cells contained very low RNR activity (Fig. 5A). Surprisingly, addition of r-β'$_2$ to the permeabilized Δrnr4 spheroplasts resulted in rapid and significant increase in RNR activity within 5 min
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FIGURE 3. Induction of β′ expression leads to efficient generation of Tyr′ in GalRNR4 cells. GalRNR4 cells were inoculated with a single colony from a CSM-His Raffinose/Galactose plate into 20 ml of YP-Raffinose (uninduced) liquid medium and cultured at 30 °C overnight. Cells were diluted into 2 liters of YP-Raffinose and cultured until A600 = 0.8, followed by addition of galactose to a final concentration of 0.5% and the growth continued. Cells (250 ml) were harvested at different time points (0, 0.5, 1, 2, 3, 8, and 18 h) after galactose addition. Dilution of cultures into fresh media was performed when necessary to maintain all cultures in log phase (A600 = 0.8–2) when cells were harvested. A whole-cell EPR spectra of Tyr′ in GalRNR4 cells at different time points subsequent to induction of β′. Harvested cells were washed thoroughly with 1 liter of cold PBS buffer and resuspended in PBS containing 30% glycerol at a high cell density (7.2 × 10^9–10.4 × 10^9/ml) before being packed into EPR tubes. X-band EPR spectra were acquired at 9.4 GHz, 2 milliwatts power, 2.52 G modulation amplitude, and 100 kHz modulation frequency; spectra taken with 0 h (black), 1 h (red), 2 h (green), 3 h (purple), and 8 h (blue) are shown. B, Western blots were used to monitor changes in β and β′ levels over an 8-h time course subsequent to β′ induction in GalRNR4 cells. Protein extracts from 2 × 10^5 cells were loaded in each lane, and the blot was probed with anti-β, anti-β′, and anti-Pgk1 (as a loading control). C, amount of Tyr′ per cell was calculated by spin quantitation, normalized against cell number at each time point, and plotted over an 18-h time course subsequent to Gal induction.

TABLE 2

Tyr′ content in GalRNR4 cells under different growth conditions

| Hours induced | Cell density (10^9/ml) | Tyr′ (μM) | Tyr′ (pmol/cell) |
|---------------|------------------------|----------|-----------------|
| 0 h           | 8                      | 0        | 0               |
| 0.5 h         | 8.9                    | 0.58     | 76              |
| 1 h           | 7.6                    | 2.35     | 326             |
| 2 h           | 7.2                    | 3.25     | 378             |
| 3 h           | 8.6                    | 1.16     | 145             |
| 8 h           | 8                      | 0.89     | 86              |

*No detectable Tyr′ was found.

The specific activity of the reconstituted β from the Δrnr4 cells is comparable with that from the WT cells (700 nmol/min/mg, see Fig. 5B).

We also performed EPR analyses on permeabilized Δrnr4 spheroplasts that were incubated with exogenous β′ to monitor the kinetics of the Tyr′ formation. The Tyr′ signal was undetectable in the absence of exogenous β′ (Fig. 5C, blue) but appeared within 3 min, upon β′ addition (Fig. 5C, red). Subtraction of the blue spectrum from the red spectrum allowed spin quantitation of the amount of Tyr′, which along with quantitative Western analysis (supplemental Fig. 1) gave 0.5 to 0.6 Tyr′/β′, slightly lower than the Tyr′ levels in WT strains measured by whole-cell EPR (43). Thus, the exogenous β′ introduced into permeabilized Δrnr4 spheroplasts can rapidly and efficiently promote Fe_{32}Ⅲ-Tyr′ cofactor formation in β.

Intriguingly, we found that the production of Tyr′ is dependent on the addition of 50 mM DTT as well as the exogenous β′. Attempts to lower DTT concentration or to replace DTT with other reductants, including glutathione, dithionite, β-mercaptoethanol, and sodium ascorbate, failed to reconstitute Tyr′ signal (data not shown). It is worth noting that no exogenous iron...
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was added in the Tyr' reconstitution assay and thus the iron appears to have come from inside the cell. One possible role of DTT might be to reduce FeIII from iron-containing proteins or small molecules to supply FeII for Tyr' assembly. To test this possibility, permeabilized Δmr4 spheroplasts were supplied with 20 μM freshly prepared ferrous ammonium sulfate in addition to exogenous β' in the absence of DTT for 3–10 min at 30 °C. The EPR spectra of these spheroplasts contained no Tyr' signal (data not shown). Thus, DTT is likely needed for reasons other than generation of FeII.

Pre-existing β in Δmr4 Cells Is in Apo-form, Effect of Metal Chelators—To address whether the pre-existing β is devoid of iron in Δmr4 cells, we performed the RNR activity assay and EPR analyses of permeabilized Δmr4 spheroplasts in the presence of the metal ion chelator EDTA and ferrous iron chelator, 2,2'-dipyridyl. Preincubation with chelators before adding exogenous β' significantly diminished reconstitution of RNR activity (Fig. 5D) and Tyr' production (Fig. 5F, blue) in permeabilized Δmr4 spheroplasts. These results indicate that the source of iron and/or the process of its delivery are chelatable.

Previous studies have shown that the loaded FeIII in RNR is no longer accessible to chelators (52). Consistent with this notion, incubation of permeabilized WT spheroplasts, in which β is loaded with the FeIII-Tyr' cofactor with EDTA, showed no inhibitory effect on RNR activity (Fig. 5E) (43). Moreover, adding chelators 3 min after addition of β' to the permeabilized Δmr4 spheroplasts also showed no diminution of Tyr' (Fig. 5F, red). Taken together, our results strongly suggest that the pre-existing β in the Δmr4 cells is in the apo-form and that iron loading and FeIII-Tyr' assembly occur de novo upon addition of exogenous β'. Although we cannot completely rule out the possibility that iron may be weakly bound to β in the absence of β',

FIGURE 4. Δmr4 mutants are defective in iron loading into nascent β poly-peptide. A congeneric BY4741 (WT) and Δmr4 cells were grown in iron-free medium overnight before being radiolabeled with 15 μCi, 1.8 μM of 55Fe for 2 h. Cell lysates were prepared, and immunoprecipitation of β was performed with anti-Rnr2. Incorporation of 55Fe into β was determined by liquid scintillation counting. Protein levels were assessed by Western blotting with anti-

FIGURE 5. Reconstitution of RNR activity and Tyr' formation in permeabilized Δmr4 spheroplasts by addition of exogenous β'. A and B, measurement of RNR activity in permeabilized Δmr4 spheroplasts. Cells from log phase cultures were subjected to permeabilization, and RNR activity was determined in the presence of an excess of α (4.4 μM) and DTT as reductant, with (open circle) or without (filled circle) addition of His6-r-β' (7.7 μM). RNR activity in permeabilized BY4741 (WT, filled square) spheroplasts was performed under the same conditions as a control. Production of [3H]dCDP at 0-, 5-, and 10-min time points was calculated using the method of Steeper/Steuart (46) and normalized against cell number using A600 (A) or β protein levels (B) as measured by quantitative Western blotting (see supplemental Fig. 1C). C, measurement of Tyr' by EPR analysis. Permeabilized Δmr4 spheroplasts (60 μl, A600 = 32/ml) were diluted into 0.1 M KP buffer, pH 7.5, containing 0.6 M sorbitol and 50 mM DTT, with addition of 6.5 μM of His6-r-β' (final volume 260 μl), and incubated at 30 °C for 3 min before being loaded into an EPR tube (red). Omission of either DTT or His6-r-β' abolished the Tyr' spectrum (blue). D, RNR activities in permeabilized Δmr4 spheroplasts were determined as described in A in the presence (filled square) and absence (open square) 2 mM EDTA or dipyridyl prior to addition of His6-r-β'. E, RNR activities in permeabilized WT spheroplasts were determined as described in A with (filled square) and without (open square) addition 2 mM EDTA. F, preincubation with a metal chelator abolishes Tyr' formation in permeabilized Δmr4 spheroplasts. EPR analyses were performed as described in C except that 2 mM of EDTA was added before (blue) or 3 min after (red) the addition of His6-r-β' and DTT.

A

B

C

D

E

F

FIGURE 4.

FIGURE 5.
previous findings that iron cannot be loaded into β₄ crystals (24, 25) and the inefficient assembly of the active cluster in vitro (18, 19) also argue that β is unable to bind iron in the absence of β'.

β' Is Unlikely to Act as an Iron Chaperone for Cluster Assembly in β—We previously proposed that the C-terminal tail of β' might bind Fe²⁺ and donate it to β upon forming the ββ'-heterodimer in a manner similar to that of copper loading in Sod1 by the chaperone Ccs1 (53, 54). The C-terminal tail of β' is rich in acidic amino acids (Asp and Glu), potential iron-binding ligands (supplemental Fig. 2A). To test the role of the C-terminal tail of β', the full-length β' and a C-terminally truncated β' mutant lacking the last eight amino acids (β' Δ8aa) (17, 18) were introduced into permeabilized Δnr4 spheroplasts, and RNR activity and Tyr' levels were measured. The β' Δ8aa mutant was as efficient as the WT-β' in reconstituting Tyr' signal (supplemental Fig. 2B, red versus green) indicating that the C-terminal tail of β' is not required for Fe³⁺-Tyr' formation. Thus, β' is unlikely to be an iron chaperone of β, similar to previous conclusions from in vitro studies (19).

Synthetic Growth Defects between Mutant Alleles of rnr4, grx3/4, and dre2, Source of Intracellular Iron and Electron for RNR Cofactor Assembly?—Our results indicate that β' acts to facilitate iron loading into β rather than to directly bind and deliver iron. Hence, we have focused our attention on the possible sources of iron and reductants required for Fe³⁺-Tyr' cluster assembly (Fig. 1). Recent studies from Mühlenhoff et al. (38) strongly indicate that the two cytosolic monothiol glutaredoxins Grx3 and Grx4 function in intracellular iron trafficking to provide iron to all cellular iron-requiring proteins. In fact, in collaboration, we demonstrated that RNR was one of the proteins with reduced iron loading and activity upon Grx3/4 depletion (38). To probe genetic interactions between Grx3/4 and RNR, we compared growth phenotypes of congenic BY4741 (WT), Δnr4, GalRNR4, Δgrx3/4, and Δnr4/GalRNR4 strains under GAL-repressed (YPD) and GAL-induced conditions (YP-Raffinose/Galactose) by serial dilution and dot-plating assay. The triple mutant Δnr4/GalRNR4 exhibited a synthetic growth defect that was worse than Δnr4/GalRNR4 strains under GAL-repressed conditions (Fig. 6A). The synthetic sickness phenotype is consistent with the notion that Grx3/4 provides the source of iron used for Fe³⁺-Tyr' cofactor synthesis in ββ', either directly or indirectly. It is worth noting that the Δgrx3/4 mutant strain contains a significantly elevated cytosolic iron pool due to Aft1-dependent activation of the iron regulon (38, 55). Moreover, β is overexpressed in the GalRNR4 cells under uninduced conditions (data not shown). Therefore, the synthetic sickness of the Δgrx3/4/GalRNR4 cells despite the increases in both cellular iron and β levels strongly suggests that the pool of iron in Δgrx3/4 mutant is not bioavailable for use in RNR cofactor assembly and that iron loading into β is very inefficient in the absence of β'.
between Dre2 and Grx3 has been reported in both yeast and human cells (56, 57). To further investigate the interaction between Dre2 and Grx3/4, we constructed a GalDRE2 strain in the BY4741 (WT) strain background by replacing the endogenous Dre2 promoter with the GAL1 promoter, and we crossed the resulting strain with the Δgrx3/4 double deletion mutant (both in BY4741 background in which Δgrx3/4 is viable) to obtain the triple mutant. Growth phenotypes of Δgrx3/4/GalDRE2 were compared with those of Δgrx3/4 and GalDRE2 under GAL-repressed growth conditions (YPD). Both Δgrx3/4 and GalDRE2 were viable on YPD and exhibited a slow growth phenotype relative to WT cells (Fig. 6B). The mild growth defect of the GalDRE2 cells on YPD (GAL-repressed) suggests that a small amount of Dre2 is available even under glucose repression and that the low level of Dre2 is sufficient for mitotic growth. By contrast, the Δgrx3/4/GalDRE2 triple mutant showed a much more severe growth defect than either Δgrx3/4 or GalDRE2 and failed to grow on YPD plates (Fig. 6B). The observed synthetic lethality between Δgrx3/4 and GalDRE2 excludes the notion that Dre2 simply functions downstream of Grx3/4. Hence, Grx3/4 and Dre2 might play essential and non-overlapping roles or function in the same multiprotein complex in cellular iron utilization.

To probe the potential genetic interactions between DRE2 and RNR4, we crossed GalDRE2 with Δrnr4 to obtain the double mutant and compared growth of each single and double mutant under different conditions. The Δrnr4/GalDRE2 double mutant exhibited more severe growth defects on YPD relative to the GalDRE2 and Δrnr4 single mutants, which were further exacerbated in the presence of 5 mM of HU (Fig. 6C). The Δrnr4/GalDRE2 double mutant also showed more severe growth defects on synthetic media with limited iron supply (Fig. 6D). Intriguingly, the double mutant exhibited a worse growth phenotype than either single mutant even on the 350 μM surplus of nonchelatable iron (1 mM ferrozine chelates a maximum of 333 μM iron, which has a minimum of 17 μM surplus of nonchelatable iron (1 mM ferrozine chelates a maximum of 333 μM iron (40)). In contrast, the slow growth defect of GalDRE2 and Δrnr4 cells are both rescued under such iron-replete conditions. The synergistic growth defect of the Δrnr4/GalDRE2 double mutant suggests that Dre2 and β’ act in concert in iron-dependent pathways to provide optimal RNR activity for mitotic growth.

**Depletion of Dre2 Leads to Diminished RNR Activities and Tyr’ Levels**—To determine the role of Dre2 in RNR function, we asked whether RNR activity or its diferric-Tyr’ content was affected by Dre2 deficiency. WT and GalDRE2 cells were grown in YPD (GAL-repressed) to log phase, and β’ content were determined in permeabilized spheroplasts in the presence of an excess of α. The β’ activity in the dre2-deficient cells was severely impaired (~6-fold lower) relative to the WT control (Fig. 7A). To directly measure Tyr’ content, we grew GalDRE2 cells in YP-Raffinose/Galactose (GAL-induced) medium to log phase and split them in half; one was kept in YP-Raffinose/Galactose and the other was switched to YPD (GAL-repressed). The cells were collected at different time points for whole-cell EPR analyses. We observed a time-dependent decrease in Tyr’ levels under Dre2-depleted conditions (Fig. 7B). The protein level of β in GalDRE2 cells also exhibited a gradual decline upon Dre2 depletion based on Western blotting (Fig. 7C). The decrease in β level is likely due to increased instability of its apo-form instead of a general defect in cellular ribosomal activity that may result from defective iron-sulfur assembly upon Dre2 depletion (36) because the overall protein expression patterns revealed by Amido Black staining were similar between GAL-induced and GAL-repressed samples (Fig. 7C). Taken together, these results demonstrate that a Dre2 deficiency causes a severe defect in cofactor formation and activity of β’.

**DISCUSSION**

RNR is an essential enzyme for DNA synthesis in almost all organisms. Although iron is necessary for class Ia RNR activity, much remains to be learned about the biosynthetic machinery required for Fe4H2-Tyr’ and Tyr’ cluster assembly and cluster maintenance in the small subunit (26). In *S. cerevisiae* RNR, this subunit provides an opportune system for investigation of cluster assembly because of its unusual heterodimeric (β’/β) composition in which only β is capable of iron binding and cofactor formation. Results presented in this study establish the critical role of β’ in iron loading and cofactor assembly in β in vivo. Moreover, the observed genetic interactions between *rnr4, grx3/4, and dre2* and the impairment of RNR activity and Tyr’ content in dre2-deficient cells strongly suggest a connection of Grx3/4 to Dre2 and each of these to Fe4H2-Tyr’ cluster formation in β of β’. These observations together have led to the model in Fig. 8 for the biosynthetic pathway of the essential cluster in the *S. cerevisiae* RNR.

In our model, iron is delivered to β in the β’ heterodimer, directly or indirectly, via a Grx3-Grx4 homo- or heterodimer. Grx3/4 contains at its subunit interface an unusual [2Fe2S] cluster with a glutathione molecule bound to each iron in the cluster (58, 59, 60).
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The GalRNR4 allele allows us to manipulate the ratios of $\beta'$ and $\beta$ in vivo by switching on and off the GAL promoter and to monitor de novo assembly of the cluster in real time by whole-cell EPR analysis. The concomitant induction of $\beta'$ and $\beta$ in the induced GalRNR4 cells (Fig. 3) has shown that newly synthesized $\beta'$ can rapidly and efficiently mediate cofactor assembly inside the cell and that the $\beta$ amounts likely respond to changes in dNTP pools. The GalRNR4 allele, when introduced into various mutants such as $\Delta rnr4$, $dre2$, and $tah18$, will allow us to study the specific contribution(s) of each candidate protein to the RNR $\text{Fe}^{\text{III}}$-$\text{Tyr}$ cluster assembly process in vivo.

The rapid and efficient reconstitution of $\text{Fe}^{\text{III}}$-$\text{Tyr}$ formation and $\beta$ activity in permeabilized $\Delta rnr4$ spheroplasts also provides an ideal system to investigate the roles of Grx3/4, Dre2/Tah18, and other candidate proteins in $\text{Tyr}^*$ formation and RNR activity reconstitution. The protein of interest can be depleted from the $\Delta rnr4$ cells by conditional repression of the gene promoter (e.g. GAL-repressed state) to assess the potential effects on $\text{Fe}^{\text{III}}$-$\text{Tyr}^*$ reconstitution. Conversely, purified candidate proteins can be added back into the permeabilized cognate mutant spheroplasts for biochemical reconstitution (36, 37, 59). A challenging task of such experiments will be obtaining the correct redox states of these proteins required for efficient cluster assembly.

Our studies suggest that de novo $\text{Tyr}^*$ biosynthesis involves an endogenous iron source that is chelatable, consistent with the notion of a labile iron pool in the cytosol (38, 60). The inability of iron chelators to inhibit RNR activation once r-$\beta'$ has been introduced into the permeabilized $\Delta rnr4$ spheroplasts also reveals that once the cofactor formation is complete, the iron in the $\text{Fe}^{\text{III}}$ cluster is stable and no longer susceptible to chelation. The stability of the $\text{Fe}^{\text{III}}$ cluster to chelation has been established for the E. coli RNR (52) but is less clear in eukaryotic systems (61). Our serendipitous finding that $\text{Fe}^{\text{III}}$-$\text{Tyr}^*$ cluster assembly in permeabilized $\Delta rnr4$ spheroplasts requires not only $\beta'$ but also high levels of the reductant DTT is intriguing and demands further biochemical investigation. In vitro cluster reconstitution using purified $\beta$ and $\beta'$ in the presence of $\text{Fe}^{\text{III}}$ and $O_2$ (where $\text{Fe}^{\text{III}}$ is the ligand and also the reductant) is inefficient, usually resulting in only 0.25 to 0.3 $\text{Tyr}^*/\beta'$ (43). In contrast, in the permeabilized $\Delta rnr4$ spheroplasts we are able to achieve rapid and efficient cluster formation (0.5–0.6 $\text{Tyr}^*/\beta'$) despite the 15-fold elevated levels of $\beta_2$. Cluster reconstitution is not detected when $\text{Fe}^{\text{III}}$ is provided instead of DTT, suggesting that DTT is unlikely to be required for reducing $\text{Fe}^{\text{III}}$ to the $\text{Fe}^{\text{II}}$. The basis for the high level of DTT required remains unknown.

DTT may function directly to reduce the tryptophan cation radical (Trp$^+$) intermediate involved in one electron transfer essential for $\text{Fe}^{\text{III}}$-$\text{Tyr}^*$ assembly (8) or alternatively to reduce Grx3/4. It is interesting to note that in many recent papers focused on trying to understand Fe-S cluster biogenesis, including transfer of a cluster precursor from a scaffold/chaperone protein to an apo-protein in vitro, moderately high levels of DTT are required to observe efficient transfer (62–64). As more studies are carried out and the factors required for cluster assembly revealed, the DTT requirement will likely become understood.

In summary, our results have led us to propose a working model for the process of $\text{Fe}^{\text{III}}$-$\text{Tyr}^*$ cluster assembly in RNR in vivo. Factors involved in delivery of metals and reducing equivalent into RNR have been a major unsolved problem in RNR biology (26). A mechanistic understanding of this process has important clinical implications in RNR targeting therapeutics such as triapine (27), especially considering the sequence and functional conservations of the major players in the model, including Grx3/4 and Dre2/Tah18 (Fig. 8) between yeast and human (36, 37, 56, 65). With the tools developed in this study, we are now well equipped to test the model using a combination of biochemical and genetic approaches.

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