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As Published: http://dx.doi.org/10.1073/pnas.1405204111

Publisher: National Academy of Sciences (U.S.)

Version: Final published version


Citable Link: http://hdl.handle.net/1721.1/91532

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Conserved electron donor complex Dre2–Tah18 is required for ribonucleotide reductase metallocofactor assembly and DNA synthesis

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Contributed by JoAnne Stubbe, March 20, 2014 (sent for review January 16, 2014)

Eukaryotic ribonucleotide reductases (RNRs) require a diferric-tyrosyl radical (Fe²⁺–Y•) cofactor to produce deoxynucleotides essential for DNA replication and repair. This metallocofactor is an important target of RNR-based therapeutics, although mechanisms of in vivo cofactor assembly, inactivation, and reactivation are poorly understood. Here, we demonstrate that the conserved Fe-S protein–diffavin reductase complex, Dre2–Tah18, plays a critical role in RNR cofactor biosynthesis. Depletion of Dre2 affects both RNR gene transcription and mRNA turnover through the activation of the DNA-damage checkpoint and the Aft1/Aft2-controlled iron regulon. Under conditions of comparable RNR protein levels, cells with diminishing Dre2 have significantly reduced ability to make deoxynucleotides. Furthermore, the kinetics and levels of in vivo reconstitution of the RNR cofactor are severely impaired in two conditional tah18 mutants. Together, these findings provide insight into RNR cofactor formation and reveal a shared mechanism underlying assembly of the Fe²⁺–Y• cofactor in RNR and the Fe–S clusters in cytosolic and nuclear proteins.

Ribonucleotide reductase (RNR) converts NDPs to dNDPs by using radical-based chemistry and supplies the essential building blocks for DNA replication and repair (1–3). Class Ia RNR, conserved from bacteria to human, is composed of α and β subunits that form active quaternary structure(s) (α₂β₂)(α₃β₃)(m = 1 or 3) in eukaryotes (8–7). The α subunit contains the catalytic and allosteric sites that control overall activity and substrate specificity. The β subunit houses a di-iron center that generates and maintains a tyrosyl radical (Y•), which is essential to initiate nucleotide reduction in the catalytic site of the α subunit via a long-range radical transfer pathway (8, 9). In this study we focus on the mechanism by which the requisite diferric-tyrosyl radical (Fe²⁺–Y•) cofactor is generated in the β subunit of yeast RNR.

The Saccharomyces cerevisiae RNR holoenzyme is proposed to have an (α₂)(ββ)₂ configuration, in which α, β, and β′ are encoded by RNR1, RNR2, and RNR4, respectively. A fourth gene, RNR3, encodes an isoform of subunit α that normally is repressed and is inducible by genotoxic stress. The active form of yeast β₂ is a heterodimer (ββ′) (10, 11). Only β is capable of iron binding and cofactor assembly, and consequently there is a maximum of one Y• per ββ′. However, β′ is essential to maintain β in a conformation competent for iron binding both in vivo and in vitro (12–14).

Eukaryotic cells tightly control their RNR activity to maintain an adequately sized and balanced dNTP pool that ensures high-fidelity DNA synthesis. The levels and activities of S. cerevisiae RNR are regulated by both the cell cycle and environmental signals including genotoxic stress and low iron availability. Cells in S phase have increased expression of the α subunit and redistribution of ββ′ from the nucleus to the cytoplasm, where the α subunit resides (15). In response to DNA damage, an activated Mec1–Rad53–Dun1 checkpoint kinase cascade increases RNR levels by phosphorylation-dependent removal of Ctr1, the transcriptional repressor of RNR2/3/4 (16). Checkpoint kinase-mediated phosphorylation also leads to degradation of two negative regulators of RNR: Smn1 that binds and inhibits subunit α (17, 18) and Dif1 that facilitates nuclear sequestration of ββ′ (19, 20). Another negative regulator of RNR is the nuclear WD40 protein Wtm1, which binds and retains ββ′ in the nucleus (21, 22). Under iron deficiency, mRNAs of RNR2 and RNR4 are decreased to a much greater extent, of WTM1 are degraded in a Cdh1/Cdh2-dependent fashion as part of a metabolic remodeling process to conserve and optimize utilization of iron (23). Cth1/Cdh2 belong to the iron regulon, a group of genes controlled by transcriptional factors Aft1 and Aft2 that are activated upon iron depletion (24).

An additional layer of RNR regulation, given that the level of Y• of the Fe²⁺–Y• cofactor is directly correlated with nucleotide reduction activity, involves the assembly and maintenance of this essential cluster. The cellular machinery required for these processes has been explored only recently (13, 25). The metallocofactor can be generated in vitro by self-assembly from apo-β₂, Fe²⁺, and O₂ with Fe²⁺ supplying the required reducing equivalent (26, 27) (Eq. 1 and Fig. 1A). However, the self-assembly process is inefficient in general, pointing to the importance of a biosynthesis pathway for controlled cofactor assembly (28). The Y• in cells also can be destroyed rapidly by endogenous reductants or exogenous reducing agents such as hydroxyurea (HU) and triapine (29, 30, and thus must be repaired to restore RNR activity (Fig. 1A).
Grx3/Grx4 for iron delivery (13) (Fig. 1B). This role was supported by a synthetic growth defect between dre2 and grx3/4 mutants and by the finding that depletion of Dre2 in yeast cells causes hypersensitivity to the Ye-quenching reagent HU and a decrease in both Ye content and RNR activity (13). However, these studies were complicated by the instability of ββ' in Dre2-deficient cells.

Recently Dre2 has been shown to form a complex with the diflavin reductase Tah18 (33, 34) and to supply reducing equivalents to the early steps of the cytosolic Fe-S assembly (CIA) pathway (33). Thus, it is possible that together Dre2–Tah18 donate the electron for RNR cluster assembly (Fig. 1B). This hypothesis is appealing, because we recently have shown that FeIII•Y maintenance of the Escherichia coli NrdB (β2) is facilitated by a [2Fe-2S]-ferredoxin encoded by yfaE, which resides in the same operon as ndrA (α) and nddB (β) (35). Although the S. cerevisiae ferredoxin-ferredoxin reductase (Fd-Fre) orthologs Yah1-Arh1 are localized exclusively in the mitochondria (36, 37), the Dre2–Tah18 pair has emerged as their cytoplasmic counterparts (33).

In this work, we have characterized the pleiotropic effects of Dre2–Tah18 deficiency on RNR including Cth1/2-mediated RNR2/RNR4 mRNA degradation and activation of the DNA-damage checkpoint leading to RNR induction and activation. Furthermore, using genetic manipulations, we have developed methods of circumventing the variability of ββ' levels to determine the effect of Dre2–Tah18 deficiency on RNR cofactor assembly. We have found that the low ββ' levels in Dre2-depleted cells can be partially suppressed by an increase in intracellular manganese levels. Upon controlling for variability in ββ' levels, depletion of Dre2 causes a significant decrease in Ye content and RNR activity. Moreover, we took advantage of a GalRNR4 Δcrt1 system in which β is constitutively overexpressed because of the removal of transcriptional repression and in which reconstitution of Ye and ββ' activity can be monitored over a time course upon induction of β' by turning on the GAL promoter. Under these conditions, we found that two tah18 conditional mutants exhibit significant defects in both the kinetics and the maximum levels of Ye and ββ' activity reconstitution relative to the WT control. Together, our findings support the model that Dre2–Tah18 functions in RNR cluster assembly and raise the intriguing perspective that the same protein pair functions as a donor of reducing equivalents to different types of cytosolic iron clusters: the Fe-S cluster in CIA and the di-iron cluster in RNR.

Results

GalDRE2 Mutant Has Lower Ye and β Levels Even in the Absence of the RNR2/RNR4 Transcription Repressor Crt1. Because Dre2 is essential for cell viability, the downstream effects of Dre2 deficiency can be investigated by replacing the native DRE2 promoter with the glucose-repressible GAL1 promoter to allow transcriptional shut-off. We have shown previously that Dre2 depletion in GalDRE2 cells led to concurrent decreases in levels of Ye, ββ' activity, and ββ' proteins (13). To determine whether the decrease in ββ' protein levels is mediated transcriptionally or posttranscriptionally, we constructed a Δcrt1GalDRE2 double mutant in which CRT1, the major transcriptional repressor of RNR2 and RNR4, was removed. The protein levels of β and β' in Δcrt1GalDRE2 cells were still threefold lower than in the Δcrt1 single mutant, (Fig. 2A), suggesting that the decrease in ββ' levels in GalDRE2 cells is mediated by a posttranscriptional mechanism. Moreover, the Ye content of Δcrt1 GalDRE2 cells is 5.3-fold lower than that of Δcrt1 (Fig. 2B). Thus, after correction for the difference in ββ' protein levels, Dre2 depletion in Δcrt1 cells resulted in a twofold decrease in Ye/ββ' ratio.

The Decrease of ββ' Levels in GalDRE2 Mutant Is Mediated by CTH2 and CTH1. A recently discovered mechanism of posttranscriptional regulation of RNR2 and RNR4 is targeted mRNA turnover mediated
Dre2 Depletion Activates both the DNA-Damage Checkpoint and Aft1/
Aft2-Dependent CTH2 Transcription and Thereby Exerts Complex
Effects on RNR. We noted that induction of CTH2 in Dre2-depleted
cells is less robust (sixfold) than in the Δatr3Δgxx4 mutant (~10.5-fold), which activates transcription of many genes of the
iron regulon including FET3 (41) (Fig. 3A). CTH2 transcription can be
activated by both Aft1 and Aft2 (41, 42). Moreover, an endogenously
tagged Cth2-GFP fusion protein has been shown to become more abundant in cells under HU-caused replicational stress (43), suggesting that CTH2 expression may be subjected to
other regulation in addition to Aft1/Aft2.

To determine whether the increased CTH2 transcription in
GalDRE2 mutants is mediated by Aft1, Aft2, or the DNA repli-
cation checkpoint, we compared CTH2 mRNA levels by RT-qPCR in
WT cells, GalDRE2 cells, and GalDRE2 cells lacking AFT1, AFT2, or the checkpoint kinase DUN1. The CTH2 mRNA level was
unaffected in Δdun1 and was slightly lower in Δaft1 and Δaft2 cells (Fig. 4A). The increase of CTH2 mRNA in the
GalDRE2 double mutant was independent of DUN1, because the CTH2 level in the Δdun1GalDRE2 mutant cells was comparable to that in GalDRE2 cells. In contrast, CTH2 mRNA levels decreased by 20% in the
Δaft1GalDRE2 cells and 50% in Δaft2GalDRE2 double mutants relative to the GalDRE2 single mutant (Fig. 4A). In keeping with the decrease of CTH2, RNR2 mRNA was restored from 43% in the
GalDRE2 single mutant to ~80% in both Δaft1GalDRE2 and
Δaft2GalDRE2 double mutants relative to the WT strain (Fig. 4A). Together, these data indicate that Aft1/Aft2-mediated induction of
CTH2 is responsible for the decrease of RNR2 mRNA levels in the
GalDRE2 mutant.

We also observed an increase in RNR3 mRNA levels in the
GalDRE2 mutant by RT-qPCR analysis. In contrast to CTH2, RNR3 induction in GalDRE2 cells was clearly DUN1-dependent and Aft1/ Aft2-independent, because it was abolished in the Δdun1GalDRE2
but unchanged in Δaft1GalDRE2 and Δaft2GalDRE2 double mutants relative to the GalDRE2 single mutant (Fig. 4B). Interestingly,
RNR3 also was moderately induced in Δaft1 and Δaft2 single
mutants. Consistent with the increased RNR3 mRNA levels, Rnr3
protein levels also were higher in GalDRE2 and Δaft1 mutant cells
than in the WT control (Fig. 4C). Induction of RNR3 is a signature
of activation of the Mec1–Rad53–Dun1 checkpoint kinase cascade
(16, 44) (Fig. 4D). To determine further whether GalDRE2 cells have a constitutively activated DNA-damage response, we moni-
tored phosphorylation status of Dif1, the DNA-damage–regulated
nuclear import facilitator of ββ (Fig. 4D). The discrepancy between transcript and protein levels of
RNR2 likely reflects decreased stability of apo-β in Dre2-depleted cells.

by Cht1 and Cht2 (23), two homologous proteins that bind to
specific AU-rich elements in the 3′ UTRs of many mRNAs in-
cluding those of RNR2 and RNR4 (38). In response to iron de-
ciciency, cells activate transcription of CTH1 transiently and of
CTH2 persistently, which target specific mRNA degradation lead-
ing to metabolic reprogramming of iron utilization and iron storage
(39, 40). To determine whether CTH2 is induced in Dre2-depleted
cells, we performed reverse transcription and quantitative real-time
PCR (RT-qPCR) to compare CTH2 mRNA levels in WT and
GalDRE2 mutant cells under GAL promoter-off conditions. CTH2
mRNA is ~6.5-fold higher in GalDRE2 cells than in WT cells (Fig.
3A). A similar increase in CTH2 mRNA also was observed in Δcrt1 relative to Δcrt1 cells, suggesting that induction of CTH2 is
caused by deletion of CTH2 instead of by removal of CRT1.
Unlike CTH2, the mRNA level of FET3, another member of the
iron regulon, is induced only slightly in GalDRE2 (Fig. 3A).

Concurrent with an increase in CTH2 levels, we observed a
2.5-fold decrease in RNR2 and RNR4 mRNA levels in GalDRE2
cells (Fig. 3B). The decrease in RNR2 and RNR4 transcripts
appeared to be mediated mainly by CTH1/CTH2 because dele-
tion of both genes in GalDRE2 cells restores RNR2 and RNR4
mRNA levels to ~70% and 100% of those in WT cells (Fig. 3B).
Interestingly, although Rnr4 (β′) protein in GalDRE2 cells was
restored to close to WT levels by removal of CTH1/CTH2, no
significant increase in Rnr2 (β) protein level was observed in
Δcrt1Δcrt2GalDRE2 cells relative to GalDRE2 cells (Fig. 3C).
The discrepancy between transcript and protein levels of RNR2
likely reflects decreased stability of apo-β in Dre2-depleted cells.

Fig. 2. The GalDRE2 mutant has lower Yp and ββ levels even in the absence of the
RNR2/RNR4 transcription repressor Crt1. Cells from a galactose-containing plate
(GAL on) were inoculated into glucose-containing liquid (GAL off) and
grown at 30 °C for 24 h to reach log phase before being harvested for EPR and
Western blotting. (A) Comparison of Rnr2 (β) levels in Δcrt1 and Δcrt1GalDRE2
cells determined by Western blotting. Rnr2/Pgk1 ratios were quantified based on signal intensity. (B) Whole-cell EPR spectra of Δcrt1 (blue, 8.4 × 10^5 cells/mL) and GalDRE2 Δcrt1 (red, 9.3 × 10^5 cells/mL).

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PNAS PLUS
Published online April 14, 2014 | E1697
be measured without concerns for drastically varying ββ' levels. Manganese is known to be able to occupy the iron-binding site of class 1α RNR, resulting in catalytically inactive ββ'. A possible effect of increased cytosolic manganese levels is to form Mn-β that stabilizes the β protein. Because increasing intracellular manganese levels are known to down-regulate manganese uptake in WT cells (46), we chose the Δpmr1 mutant that is defective in manganese transport from the cytosol to Golgi (47) and that apparently is deficient in the negative feedback control of manganese uptake. As a result, Δpmr1 cells have ∼10-fold elevated intracellular manganese levels (48, 49). To assess whether the increased intracellular manganese would affect β protein levels, we constructed the Δpmr1GalDRE2 double mutant. The β protein levels of the Δpmr1GalDRE2 cells remain comparable in GAL-repressed [yeast extract/peptone/dextrose (YPD)] and GAL-induced [yeast extract/peptone/Gal (YPG)] growth conditions (121% and 180%, respectively) but in GalDRE2 single mutant varied by threefold in each strain, and the resulting ratios in WT cells were arbitrarily defined as onefold. Mutant Rnr2/G6PDH ratios were quantified, and the average and SD from triplicates are shown.

**Fig. 3.** The decrease in ββ' levels in the GalDRE2 mutant is mediated by CTH1/CTH2. (A) CTH2 is induced in the GalDRE2 mutant. Levels of CTH2 and FET3 mRNAs in WT, GalDRE2, Δctt1, and Δctt1GalDRE2 cells were determined by reverse transcription and RT-qPCR. Signals of CTH2 and FET3 were normalized against that of ACT1 in each strain, and the resulting ratios in WT cells were arbitrarily defined as onefold. Mutant Δgrx3Δgrx4 was included as a positive control for iron regulon activation (41). (B) Comparison of RNR2 and RNR4 mRNA levels in WT, GalDRE2, Δctt1Δctt2, and GalDRE2Δctt1Δctt2 cells by RT-qPCR as described in A. (C) Comparison of Rnr2 (ββ') and Rnr4 (β') protein levels in WT, GalDRE2, Δctt1Δctt2, and GalDRE2Δctt1Δctt2 cells by Western blot with G6PDH as a loading control. Rnr2/G6PDH ratios were quantified, and the average and SD from triplicates are shown.

**Conditional Mutants of tah18 Exhibit Slow S-Phase Progression and Synthetic Growth Defect with GalRNR4.** The diflavin reductase Tah18 forms a stable complex with Dre2 to transfer electrons from NADPH to Dre2's Fe-S clusters. We inferred that initiation of Tah18 would have effects on RNR similar to that of Dre2 depletion. TAH18 is essential for viability. A previous study has identified two temperature-sensitive (ts) mutant alleles of TAH18, tah18-5H8, and tah18-5I5 (34). We characterized the original tah18 ts strains on HU-containing plates and found that tah18-5I5 was hypersensitive to HU at the permissive temperature 25 °C (Fig. S1). Moreover, both tah18 ts mutants exhibited defects in cell-cycle progression upon release from α factor-mediated G1 arrest: a prolonged S phase for tah18-5I5 and a delay at the G1/S boundary for tah18-5H8 (Fig. S2).

HU sensitivity and defects in G1/S transition or S-phase progression are characteristics of mutants deficient in DNA replication or cellular dNTP pools. To investigate whether the RNR cluster formation is defective in the tah18 ts mutants, we used whole-cell EPR to measure and compare Y• contents in the mutants and their isogenic WT strain. The EPR spectra of these strains had a very high background (Fig. S3) relative to the W303 and S288C strains (11, 13) and thus impeded accurate quantitation. To circumvent this problem, we moved the two tah18 ts mutant alleles into the W303 and S288C backgrounds by crossings with a W303 WT parental strain six times. The outcrossed mutants retained the ts and cell-cycle delay phenotypes but no longer showed obvious HU sensitivity, suggesting that background mutations in the original strains contribute to the growth defect on HU. Interestingly, both the tah18-5I5 and tah18-5H8 mutants showed a delay in S-phase progression after being released from G1 (Fig. 6A). Moreover, both tah18-5I5 and tah18-5H8 enhance HU sensitivity of the GalRNR4 mutant in which the chromosomal RNR4 promoter was replaced by the glucose-repressible GAL1 promoter, on a glucose-containing plate (Fig. 6B), suggesting exacerbation of RNR deficiency when both Rnr4 (ββ') and Tah18 are compro-
mised. The synthetic growth defect between GalRNR4 and tah18 ts mutants is reminiscent of the synthetic defect observed between Δnr4 and GalDRE2 (13).

**Concurrent Decreases in ββ′ Activity and Proteins Levels in the tah18 ts Mutants.** As anticipated, the outcrossed tah18 ts mutants have a much cleaner EPR background and thus allow quantitative comparison of the Y• content in WT and mutants. Both tah18-515 and tah18-5H8 mutants have ∼50% of Y• content seen in the WT strain even at the permissive temperature 25 °C (Fig. 6C). Consistent with the low Y• levels, the activities of ββ′ of the two tah18 ts mutant cells were ∼40% of activities of the WT cells when normalized by cell numbers (Fig. 6D). However, when probing for β protein, we found that the two tah18 ts mutants also had a much lower β levels (Fig. 6E). The protein levels of β in the tah18-5H8 mutant exhibited a further and dramatic decline when the cells were shifted to 25 °C to the nonpermissive temperature 37 °C, dropping to ∼20% of the WT levels after 2 h at 37 °C and becoming undetectable after 4 h (Fig. 6F). Consistent with the decrease in β protein levels, RNR activity of tah18-5H8 dropped to 10% of the WT levels 3 h after being shifted to 37 °C (Fig. 6G).

Thus, as in Dre2-depleted cells, the concurrent decrease in protein levels and activity of ββ′ complicated the assessment of the effect of inactivating Tah18 on RNR.

**Inactivation of Tah18 at Nonpermissive Temperature Impairs Formation of Ye and Reconstitution of ββ′ Activity upon Induction of β′ in GalRNR4 Cells.** To circumvent the effect of Tah18 inactivation on β protein stability, we searched for conditions under which the β protein level is constant and not rate-limiting for measurement of Y• content and ββ′ activity. We have shown previously that induction of β′ in GalRNR4 cells leads to rapid and efficient Fe(III)-Y• formation up to fourfold of the level in WT cells (13). GalRNR4 cells under GAL-repressed conditions accumulate five- to 10-fold more β protein than WT cells because activation of the Mec1-Rad53-Dun1 checkpoint leads to the removal of the transcriptional repressor Crt1. Upon induction of β′, formation of the active ββ′ and replenishment of cellular dNTP pools gradually diminishes the checkpoint signaling, and the levels of both β and Y• eventually return to those of WT cells because of Crt1-mediated negative feedback regulation (13, 16).
We capitalized on the inducible GalRNR4 system and the conditional tah18 ts mutant alleles to investigate whether Tah18 is required for the Ye cofactor formation upon β' induction by generating GalRNR4 tah18 double mutants. To remove Crt1-mediated negative feedback, we deleted Crt1 in GalRNR4 Tah18 (expressing WT Tah18) and GalRNR4 tah18-ts strains so that β is constitutively overexpressed and consequently is not rate-limiting for reconstitution of Ye and β' activity upon induction of β'. Both tah18-5H8 and tah18-5S15 mutants exhibited lower Tah18 protein levels that dwindled quickly when shifted to the nonpermissive temperature 30 °C (Fig. S4). As expected, induction of β' at 30 °C in the GalRNR4 Δcrt1 Tah18 cells led to a time-dependent increase of Ye signal, reaching a plateau that was approximately fourfold higher than that of a WT W303 strain (Fig. 7 A, B, and E). In contrast, formation of Ye signal in GalRNR4 Δcrt1 tah18-5H8 cells (Fig. 7C) and GalRNR4 Δcrt1 tah18-5S15 cells (Fig. 7D) upon β' induction at 30 °C occurred at a much slower pace and reached a plateau that was only 25% of the level in GalRNR4 Δcrt1 Tah18 cells (Fig. 7E).

The β protein of the GalRNR4 Δcrt1 Tah18, GalRNR4 Δcrt1 tah18-5S8, and GalRNR4 Δcrt1 tah18-5S15 cells remained at a constitutive level that is much higher relative to the W303 WT strain over the time course of the experiments as a result of loss of Crt1-mediated transcriptional repression (Fig. 7F). Under such conditions, induced expression of β' protein becomes the rate-limiting step in β' formation and cluster assembly so that the level of Ye formation would be proportional to the level of β' protein induced. The time course and levels of induced β' protein were comparable in GalRNR4 Δcrt1 Tah18 cells and GalRNR4 Δcrt1 tah18-5S8 cells. In both strains, β' levels became detectable at 2 h and plateaued at 5 h (Fig. 7E), as was consistent with the time course of Ye appearance and increase that reached a plateau at 5 h (Fig. 7E). On the other hand, β' induction and Ye formation in GalRNR4 Δcrt1 tah18-5S15 cells occurred at a slower pace, becoming detectable at 3 h and reaching a plateau at 6 h (Fig. 7 E and F). Nevertheless, upon reaching the plateau (6 h for GalRNR4 Δcrt1 Tah18 and GalRNR4 Δcrt1 tah18-5S8 cells and 8 h for GalRNR4 Δcrt1 tah18-5S15 cells), the levels of β and β' proteins were comparable in all three strains, but the Ye levels of GalRNR4 Δcrt1 tah18-5S8 cells and GalRNR4 Δcrt1 tah18-5S15 cells were only ~25% of that of GalRNR4 Δcrt1 Tah18 cells. As we have shown previously (13), there is a good correlation between the Ye signal determined by EPR and the β' activity measured by the permeabilized cell-based RNR activity.

Fig. 5. Depletion of Dre2 causes decrease in β' activity. (A) Comparison of Rnr2 protein levels in GalDRE2 Δmpr1 and GalDRE2 cells under GAL-on (YPG) and GAL-off (YPD) conditions. Total protein extract from an equal number of cells was loaded for each sample. The protein blot was probed with anti-Rnr2 and anti-Rnr4 (Upper) and stained with amino black (Lower) as a control for loading. Relative Rnr2 and Rnr4 signals are shown. (B) Removal of PMR1 in GalDRE2 cells abolished induction of CTH2 and decrease of RNR2 under GAL-off conditions. Relative levels of RNR2 and CTH2 mRNAs were determined by RT-qPCR and normalized against ACT1 mRNA signals; the resulting ratios in WT cells were arbitrarily defined as onefold. (C) Comparison of β' activities of GalDRE2 Δmpr1 and GalDRE2 cells under GAL-on (YPG) and GAL-off (YPD) conditions. The β' activity of each sample was assayed in permeabilized cells in the presence of an excess of α as previously described (13). The β' activities for GalDRE2, YPD, GalDRE2_YPD, GalDRE2/Δmpr1_YPD, and GalDRE2/Δmpr1_YPD are 0.41, 1.46, 0.41, and 2.54 nmol dCDP/min in OD600 cells, respectively. (D) Coimmunoprecipitation of Dre2 and Rnr2 (lanes 1–8). Whole-cell extracts (WCE) of the 3xMycDRE2 (AXY1767) and 3xMycRNR2 (MHY340) strains were incubated with an anti-Myc monoclonal antibody 9E10. The immunoprecipitates (IP) were brought down with Protein A beads, and the protein blots were probed with polyclonal anti-Dre2 (lanes 1–4) and anti-Rnr2 (lanes 5–8) antibodies. The WCE lanes were loaded with lysates of 1.5 × 10^7 cells, and the IP lanes contained immunoprecipitates from lysates of 3 × 10^8 cells.
assay in these strains (e.g., ββ′ activities at 2 h after β′ induction are shown in Fig. S5). Thus these findings strongly support our model that Tah18 is required for de novo formation of the Fe^{III}-Y• cofactor in ββ′.

**Discussion**

In this work, we demonstrated that Dre2–Tah18, a protein complex recently identified as a donor of reducing equivalents to the CIA machinery (33), also plays a critical role in formation of the Fe^{III}-Y• cofactor in RNR. Our efforts to determine the contribution of Dre2–Tah18 to RNR function were complicated by the decrease in ββ′ levels in drc2 and tah18 mutant cells. Our studies to understand the molecular basis for the decrease in ββ′ associated with Dre2–Tah18 inactivation thus have unexpectedly unveiled important regulatory mechanisms linking RNR stability with iron limitation and activation of the DNA-damage checkpoint.

First, we found that depletion of Dre2 induces CTH2 transcription in an Aft1/Aft2-dependent manner. Previous studies have suggested that Aft1/2 sense cellular iron levels by responding to deficiency in the mitochondrial iron-sulfur cluster (ISC) as-
assembly process (50). Because mitochondrial ISC is not affected by Dre2 deficiency (51), it was unclear how Aft1/Aft2 became activated in Dre2-depleted cells. One possible explanation is based on our model that Dre2–Tah18 supplies electrons to Grx3/Grx4 for their function in the delivery of iron for the assembly of all iron-requiring cofactors, including those in the mitochondria (Fig. 1B). This notion is supported by synthetic lethality between grx3/4 and dre2 mutants (13) and by interactions between Dre2 and Grx3 in both yeast and human (52, 53). Moreover, we show that the CTH2 transcript is induced to a much greater extent in the grx3/4 mutant than in GalDRE2 mutant (Fig. 3A). Thus, it is possible that Dre2 depletion may cause deficiency in Grx3/4 activity, which could be sensed directly or indirectly by Aft1/Aft2, leading to transcriptional induction of CTH2. We noted that, unlike CTH2, another...
member of the iron regulon *FET3* is induced in the *gln3*Δ but not much in *GalDRE2* mutant, perhaps reflecting a differential degree of activation of Aft1/Aft2 in these mutants.

Second, we found that Dre2-depleted cells have an activated DNA-damage checkpoint resulting in transcriptional induction of *RNR3* as well as *RNR2/RNR4* through phosphorylation-mediated repressor Crt1 from its target promoters. Thus, the apparent static level of ββ′ would result from the opposing effects of checkpoint-mediated transcriptional induction and Cth1/Cth2-mediated mRNA turnover of *RNR2/RNR4*. Consistent with this notion, Dre2-depleted cells have higher Rnr3 levels but lower ββ′ levels than WT cells. Cth1/Cth2 promote mRNA turnover not only of *RNR2/RNR4* but also of Wtm1, which acts to prevent nuclear release of ββ′ and its colocalization with α (21, 22). The apparent paradoxical down-regulation of both ββ′ and its negative regulator Wtm1 suggests that, in an effort to optimize the use of limited iron, yeast cells prioritize nucleus–cytoplasm redistribution and iron loading of existing ββ′ proteins over the synthesis of more apo proteins.

Third, the findings in *GalDRE2* and *aft1* mutants of *RNR3* induction and DiF1 phosphorylation, two downstream events mediated by checkpoint kinase Dun1, indicate that the Mecl–Rad53–Dun1 checkpoint cascade can be activated in mutants defective in Fe-S cluster synthesis or cellular iron homeostasis. Fe-S cluster–binding domains have been found in an increasing number of nuclear proteins involved in DNA replication and repair including DNA primase, DNA helicases, and DNA polymerases (54). The importance of Dre2–Tah18 in DNA replication also was supported by the synthetic lethality between a mutant of *POL3* encoding the Fe-S cluster containing DNA polymerase δ and dre2 and tah18 mutant alleles (55). Our findings show that the Dre2–Tah18 complex is required for assembly of the di-iron enzyme RNR cluster and for cellular supplies of dNTP. As such, deficiencies in the CIA pathway or proper distribution of intracellular iron utilization would impact many aspects of DNA replication and repair directly, leading to checkpoint activation.

Our finding that removal of Cth1/Cth2 only partially restores the decrease of ββ′ levels in Dre2-depleted cells suggests additional, unidentified regulatory mechanism(s), likely instability of the apo-ββ′ proteins. Therefore, our focus was to identify strains with increased ββ′ levels so that cofactor Fe sulfur–Y• could be made more readily available for Tah18. We achieved this goal by increasing intracellular manganese levels in the conditional strain *GalDRE2* via *apm1* and by keeping β constitutively overexpressed in the *GalRNR4 tah18* ts mutant via *Δctl1*. The results of our studies using these strategies strongly support the requirement of Dre2–Tah18 in RNR cluster assembly, either by delivering the obligatory reducing equivalent for RNR cluster formation in β (Eq. 1) or by being involved indirectly in iron delivery. The finding of coimmunoprecipitation between Dre2 and Rrn2 (β) (Fig. 5D) is consistent with the proposed role of Dre2 in electron delivery. Because Dre2 also has been shown to interact with Grx3/Grx4, we further postulate that Dre2–Tah18 might provide the reducing equivalents to allow Fe sulfur–Y• transfer from the [2Fe2S]–(GSH)2 cluster at the Grx3–dimer interface to apo-ββ′.

The active cluster in both the di-iron– and Fe-S cluster–requiring proteins can form by self-assembly with varying degrees of efficiency in vitro (28, 56). Both require carefully controlled delivery of reducing equivalents. Thus, in both cases biosynthesis and perhaps maintenance pathways may have evolved to ensure highly efficient construction of an essential cofactor. The central role of Dre2–Tah18 in the assembly of the Fe-S cluster in many cytosolic and nuclear proteins, including enzymes involved in DNA replication and repair, complicated experimental designs to obtain evidence for our model (Fig. 1B). Our results together with previous studies by the Lill group (25) suggest that the CIA machinery and RNR cluster assembly share the same sources of iron, in the form of [2Fe2S]–(GSH)2 from Grx3/Grx4, and also the same source of reducing equivalents from Dre2–Tah18. The point of bifurcation of the CIA and the RNR cluster assembly processes remains to be unraveled.

The pathway for Fe sulfur–Y• assembly is likely conserved between *S. cerevisiae* and human despite the differences in structures of the two β2 subunits (heterodimer versus homodimer) (1). The mammalian Grx3, PICOt, recently has been shown to be required for multiple pathways in iron homeostasis, including biogenesis of the Fe-S cluster and hemoglobin maturation (31). The Dre2–Tah18 complex may function as the cytosolic equivalent of the mitochondrial Fd-Fre pair Yah1–Ahr1 to deliver electrons to multiple and divergent pathways of iron cofactor biogenesis. The human counterparts of Dre2 and Tah18, CIAPIN1 and NDOR1, respectively, recently have been shown to function in their place in yeast cells in Fe-S cluster assembly in Leu1, a substrate of the CIA machinery (33, 51). It remains to be determined if CIAPIN1-NDOR1 are involved in the assembly of the Fe sulfur–Y• cluster of RNR and the Fe-S cluster of CIA in mammalian cells. Discovering the cellular machinery required for Fe sulfur–Y• assembly and repair would provide still another tier to the multilayered RNR regulation and would provide new insights into development of RNR-targeted therapeutics.

**Experimental Procedures**

**Yeast Strains, Plasmids, and Growth Conditions.** Yeast strains and plasmids used in this study are listed in Tables S1 and S2, respectively. Growth of yeast strains and genetic manipulations were as described (57). *GalDRE2* and *GalRNR4* strains were constructed by replacing sequences between nucleotides –50 and –1 of each endogenous promoter with the GAL1 promoter (58). AXY1664, AXY1668, and AXY1696 were constructed by integrating an N-terminally Flag-tagged *TAH18* (WT) or tah18–SHR and tah18–SIS mutants into the *tah18::KanMX4* locus. Cell-cycle synchronization and FACS analysis were as described (59).

**Protein Analysis.** Yeast protein extracts were prepared by trichloroacetic acid precipitation (10) or alkaline treatment (60) for Western blotting and by lysis buffer B for immunoprecipitation (20). Antibodies used for immunoprecipitation and Western blotting were anti–RNR3 from Abcam, anti-NDOR1, and monoclonal 9E10 (anti-Myc; Covance). Signals from protein blots were recorded and quantitated using ChemiDoc MP (Bio-Rad).

**RNA Extraction, Reverse Transcription, and RT-qPCR.** Total RNA was extracted from 2 × 106 cells by using a hot-phenol method (62). Total RNA (10 μg) was treated with 10 units of RNase-free DNase I (New England Biolabs) for 30 min at 37 °C to remove contaminating DNA. First-strand cDNA synthesis was carried out by M-MuLV reverse transcriptase (New England Biolabs) on aliquots of 1 μg RNA with a random primer mix. The single-stranded cDNA products were used in qPCR on a Bio-Rad CFX96 real-time PCR detection system based on SYBR Green fluorescence. Sequences of oligo pairs are listed in Table S3.

**Whole-Cell EPR Spectroscopy and ββ′ Activity Assays in Permeabilized Cells.** Whole-cell EPR spectroscopy, preparation of permeabilized yeast cells, and measurement of RNR activity were performed as described previously (13).

**Acknowledgments.** We thank Drs. A. Dancis, S. Puig, R. Lill, and L. Vernis for sharing of yeast strains, antibodies, and plasmids. This work was supported by National Institutes of Health Grants 1GM29595 (to J.S.), R01CA125574 (to M.H.), and R01GM18393 (to J.S. and M.H.).


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