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Ribonucleotide reductase is not limiting for mitochondrial DNA copy number in mice

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ABSTRACT

Ribonucleotide reductase (RNR) is the rate-limiting enzyme in deoxyribonucleoside triphosphate (dNTP) biosynthesis, with important roles in nuclear genome maintenance. RNR is also essential for maintenance of mitochondrial DNA (mtDNA) in mammals. The mechanisms regulating mtDNA copy number in mammals are only being discovered. In budding yeast, RNR overexpression resulted in increased mtDNA levels, and rescued the disease phenotypes caused by a mutant mtDNA polymerase. This raised the question of whether mtDNA copy number increase by RNR induction could be a strategy for treating diseases with mtDNA mutations. We show here that high-level overexpression of RNR subunits (Rrm1, Rrm2 and p53R2; separately or in different combinations) in mice does not result in mtDNA copy number elevation. Instead, simultaneous expression of two RNR subunits leads to imbalanced dNTP pools and progressive mtDNA depletion in the skeletal muscle, without mtDNA mutagenesis. We also show that endogenous RNR transcripts are downregulated in response to large increases of mtDNA in mice, which is indicative of nuclear-mitochondrial cross-talk with regard to mtDNA copy number. Our results establish that RNR is not limiting for mtDNA copy number in mice, and provide new evidence for the importance of balanced dNTP pools in mtDNA maintenance in postmitotic tissues.

INTRODUCTION

Ribonucleotide reductase (RNR) catalyzes the rate-limiting step in *de novo* synthesis of deoxyribonucleoside triphosphates (dNTPs) [reviewed in (1)]. The enzyme is known to contribute to malignant transformation (2,3). During S-phase, RNR is abundant as a tetramer composed of homodimers of the large Rrm1 and small Rrm2 subunits. In non-cycling cells, Rrm2 is replaced by the alternative small subunit p53R2 (4). The latter is induced by the tumor suppressor p53 and was initially considered to contribute to nuclear DNA damage responses (5,6).

Mutation of p53R2 has recently been found to be an important cause of human inherited diseases. Inactivating mutations of *RRM2B*, the gene encoding p53R2, were not associated with neoplasms, but caused early onset fatal depletion of mitochondrial DNA (mtDNA) (MIM #612075) (7). Similarly, knock-out mice lacking p53R2 exhibited near-total loss of mtDNA and died shortly after weaning (7,8). We recently reported that a dominant *RRM2B* mutation led to truncated p53R2 and caused adult-onset progressive external ophthalmoplegia (PEO) with multiple mtDNA deletions (9). Certain compound heterozygote *RRM2B* mutations have also been shown to result in mitochondrial neurogastrointestinal encephalopathy (MNGIE; MIM #603041) (10). Defects in p53R2 can therefore cause diseases of differing severity, ranging from fatal multisystem disorders with mtDNA depletion in children, to an adult-onset muscle disorder with multiple mtDNA deletions. These results highlight the essential role of RNR in mtDNA maintenance in postmitotic cells.

Increasing mtDNA copy number has emerged as an attractive target of intervention for mtDNA diseases.

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Even a minimal amount of wild-type mtDNA can compensate a functionally recessive mutant (11). In the budding yeast *Saccharomyces cerevisiae*, RNR overexpression led to elevation of mtDNA copy number (12) and to the rescue of the 'petite' phenotype of mtDNA polymerase mutants that carried mutations equivalent to those in autosomal dominant PEO (13). This suggested that RNR levels, by affecting dNTP pools, might be rate limiting for mtDNA synthesis, and that increasing the availability of the enzyme might be a tool for increasing mtDNA abundance. Whether RNR induction could increase mtDNA in mammals, as it does in yeast, has so far not been investigated.

In mice, considerable increase of mtDNA copy number has only been achieved in two mouse models, overexpressing the mitochondrial transcription factor A (TFAM) or the mtDNA helicase Twinkle (14,15). TFAM is required for mtDNA transcription, but it also binds DNA with low specificity and packages mtDNA in a histone-like manner (16–18). Its levels closely follow mtDNA levels. Twinkle helicase is known to increase mtDNA copy number by affecting the replication initiation rate (19). In this study, we set out to determine whether overexpression of RNR subunits, alone or in combination with each other, influence mtDNA copy number in mammalian tissues.

MATERIALS AND METHODS

Generation of transgenic mice

The Rrm1^{Tg}, Rrm2^{Tg} and p53R2^{Tg} mice have been previously described (3). Briefly, the mice were maintained on a pure *FVB/N* strain background and expressed their transgene under the control of chicken β-actin promoter and cytomegalovirus enhancer regulatory sequences. The transgenic mice seemed grossly normal and were fertile. The presence of the transgene was verified by PCR analysis as described earlier (3). Rrm1^{Tg} mice showed restricted overexpression of Rrm1 protein primarily in the skeletal muscle by immunoblotting whereas Rrm2^{Tg} and p53R2^{Tg} mice had widespread, high-level overexpression of the transgene in all tested tissues. For this study, we created mice overexpressing Rrm1 together with either Rrm2 or p53R2 by cross-breeding mice that were hemizygous for each transgene. Because the PCR assay used to detect Rrm1^{Tg} and p53R2^{Tg} is not transgene-specific (3), the presence of Rrm1^{Tg} or p53R2^{Tg} in offspring from these crosses was determined by Southern blotting. Briefly, tail DNA obtained from offspring was digested overnight with BamHI (Rrm1^{Tg}) or EcoRV (p53R2^{Tg}). After DNA was immobilized on a nylon membrane (GeneScreen Plus, Perkin Elmer), transgene bands were detected by hybridization with radio-labeled probes derived from previously described pCaggs RNR expression constructs (3). Pathological examination of lung neoplasms was performed as before (3).

The Twinkle^{Tg} (15) and TFAM^{Tg} mice (20) used here were also described earlier. Both were in *C57BL/6* background, and expressed the transgene under a ubiquitous β-actin promoter. The Twinkle-mice were backcrossed to *C57BL/6* from *FVB/N* for more than 12 generations, and the congeneity was confirmed with the Mouse Medium Density SNP Panel (Illumina).

Quantification of RNR transgene overexpression

Western blotting for RNR subunits was performed as described earlier (3). Chemiluminescent signal was detected on a VersaDoc Imaging system and quantified using Quantity One software (Bio-Rad Laboratories). Band intensity was determined for each dilution series sample and plotted following subtraction of background signal. The measured intensity for each undiluted wild-type band was fitted to the generated line for the corresponding RNR^{Tg} dilution series. Fold overexpression values were corrected for loading by standardization based on α-tubulin signal.

Southern blotting for mtDNA copy number determination

Total DNA was isolated from tissues by proteinase K digestion and standard phenol–chloroform extraction. Southern blotting was performed essentially as previously described (21). Briefly, 3 μg total DNA was digested with SacI overnight at 37°C, samples were then separated by electrophoresis in an agarose gel and blotted by alkaline transfer onto a Hybond N+ membrane (Amersham Biosciences). The membrane was hybridized overnight at 68°C in a roller hybridizer using 5 μCi/ml ^{32}P-dCTP labeled (PCR-generated) mouse mtDNA probe, and 18S rDNA probe in pBR322 plasmid. Phosphoimager analysis was done with Typhoon 9400 (Amersham Biosciences) and mtDNA was quantified against the 18S rDNA signal using ImageQuant v5.0 software (Amersham Biosciences).

Real-time PCR

For mtDNA quantification, the quantitative real-time (q)PCR reactions were done with 25 ng total DNA used as template and normalizing the *mt-Cytb* gene amplification level (primer sequences: 5′-GCTTTCCACTTCATCT TACCATTTA-3′ and 5′-TGTTGGGTTGTTTGATCC TG-3′) against the nuclear β-actin gene (primer sequences: 5′-GGAAAAGAGCCTCAGGGCAT-3′ and 5′-GAAGA GCTATGAGCTGCCTGA-3′). Samples were run on an Abi Prism SDS 7000 machine (Applied Biosystem). Amplification conditions were: 95°C for 7 min followed by 35 cycles of 95°C for 10 s and 60°C for 30 s. Dissociation curves were checked to ensure the existence of a single PCR product. Each sample was run in duplicate, and samples with significant variation between duplicates were excluded. qPCR data were analyzed using 7000 System Sequence Detection Software version 1.2.3 (Applied Biosystems).

For gene expression analysis, 1000 ng total RNA was DNase digested using the Amplification Grade DNase I kit (Invitrogen) according to the manufacturer's instructions. Reverse transcription was done using M-MLV reverse transcriptase (Promega). Taqman gene expression assay for *Rrm2b* (assay ID Mm01165706_m1) and the *Gapdh* endogenous control were purchased from Applied

Biosystems. The PCR reactions for *Rrm2b* and *Gapdh* were done using TaqMan Universal PCR Master Mix (Applied Biosystems), and run on an Abi Prism SDS 7000 machine (Applied Biosystem) according to the manufacturer's protocol. The PCR for *Rrm1* was done using the DyNAmo™ Flash SYBR® Green QPCR Kit (Finnzymes) as above (primer sequences 5′-TGGACTCA ACATGGACTTTG-3′ and 5′-GGCCTTGGATTACTTT CATG-3′). QPCR data were analyzed using 7000 System Sequence Detection Software version 1.2.3 (Applied Biosystems). The amplification level of *Rrm2b* or *Rrm1* was normalized by dividing by the *Gapdh* amplification level.

MtDNA point mutation analysis

For mtDNA point mutation analysis we used primers that specifically amplified the *mt-Cytb* gene (nucleotide pair 14 073–14 906) and non-coding control region (15 357–138) of mouse mtDNA. In the PCR, we utilized the high-fidelity Phusion polymerase (Finnzymes) according to the manufacturer's instructions, with 25 ng total DNA from quadriceps femoris muscle as template. The PCR program was 98°C for 30 s followed by 30 cycles of 98°C for 10 s, 61°C for 10 s and 72°C for 30 s. PCR products were cloned into pCR®2.1 plasmid using the TA cloning kit (Invitrogen) according to the manufacturer's instructions. Multiple PCR clones were sequenced to generate ~30 000 bp of sequence for each region.

Long PCR

Long PCR to amplify the entire mitochondrial genome or selectively deleted mtDNA molecules was done using the Expand Long Template PCR System (Roche). Fifty-nanogram total DNA was used as template. Cycling conditions were: 92°C for 2 min followed by 30 cycles of 92°C for 10 s and 68°C for 12 min. PCR products were separated by electrophoresis on 1% agarose gels and visualized with a Typhoon 9400 scanner (Amersham Biosciences). Primers hybridized to the control region of mtDNA located at nucleotide positions 1953–1924 and 2473–2505.

Measurement of nucleotide pools in skeletal muscle

Nucleotides were extracted from skeletal muscle of aged mice using previously described methods with modifications (22,23). Briefly, skeletal muscle was excised following euthanasia by CO_2 asphyxiation and snap-frozen in liquid nitrogen. When sufficient tissue was available, samples were divided into two equal portions, processed separately, and compared to assess measurement consistency. Tissues were weighed and immersed in ~5 µl/mg 10% trichloroacetic acid, 10 mM $MgCl_2$. Tissues were homogenized in a Qiagen Tissue Lyser by six cycles of 30 s at 30 Hz followed by 30 s on ice. Tissue homogenates were incubated on ice for 20 min, then spun at 13 000 g for 1 min. The supernatant was taken to a fresh tube and re-spun. An approximately equal volume of 0.5N trioctylamine in fluorotrichloromethane was added to the supernatant. The samples were vortexed briefly and spun for 2 min at 16 000 *g*. The volume of the upper phase was estimated by pipetting and aliquots were snap-frozen in liquid nitrogen and stored at −80°C until nucleotide measurements were performed. dNTPs were measured using an indirect enzymatic assay as reported by Ferraro *et al.* (24). Standard curves were prepared from concentrated stocks of pure individual dNTPs (Fermentas). Reactions in 50 µl total volume were incubated for 1 h and then 30 µl was spotted to Whatman DE81 paper discs and dried. Discs were washed three times in 5% Na_2HPO_4, and once each in dH_2O and 95% EtOH. Discs were dried and counted on a Beckman Coulter LS6500 scintillation counter. Measurement of ribonucleosides was performed as in Kochanowski *et al.* (25) with modifications. Analyses were carried out on a Shimadzu UHPLC system. Samples were separated on a Supelco LC-18T column in 30 mM KH_2PO_4 and 10 mM tetrabutylammonium hydrogen sulfate, pH 6.5: methanol (A = 91.7:8.3, B = 71.6:28.4) over a 40-min time period. The program ran 0–100%B from 0–24 min, 1 min at 100% B and 100–0% B from 25–30 min at a flow rate of 1 ml/min. Identities of analytes were confirmed both by comparison of elution time to known standards and by wavelength of maximum absorbance (λ_{max}). Sample extracts in which ATP comprised <65% of the total adenine nucleotides were excluded from further analysis.

RESULTS

Generation of RNR transgenic mice

Transgenic mice that express recombinant Rrm1, Rrm2 or p53R2 (referred to as 'Rrm1^{Tg}', 'Rrm2^{Tg}' and 'p53R2^{Tg}' mice hereafter) were generated previously and showed broad, high-level RNR overexpression (3). Since active RNR requires the presence of the large and the small subunit, simultaneous overexpression of both subunits could cause a greater increase in RNR activity. We therefore interbred Rrm1^{Tg} mice with Rrm2^{Tg} or p53R2^{Tg} mice to generate bitransgenic mice. The Rrm1^{Tg} + Rrm2^{Tg} and Rrm1^{Tg} + p53R2^{Tg} mice were born at expected frequencies and showed no gross abnormalities (Supplementary Table S1). The endogenous RNR subunits were barely or not at all detectable by western blotting of 25 µg protein for each muscle tissue extract, whereas significant overexpression was observed in RNR^{Tg} samples. We therefore made serial dilutions from each RNR^{Tg} sample, and plotted the band intensities against the dilution level. Fitting the corresponding wild-type samples onto this plot allowed us to estimate the fold overexpression for each transgene-encoded protein (Figure 1). The calculated threshold dilutions for bitransgenic mice are shown in Table 1. The level of transgene overexpression was comparable between single RNR transgenic and bitransgenic mice (Supplementary Figure S1).

Rrm2^{Tg} and p53R2^{Tg} mice were previously found to have increased lung tumor prevalence at ~17 months of age (3), Rrm2 being a more potent tumor inducer than p53R2. In bitransgenic mice, the lung tumor formation occurred with similar frequency as in mice overexpressing

either of the small RNR subunits alone (Supplementary Table S2). The bitransgenic mice were followed up to ~17-month age and, excluding lung tumor formation, they appeared grossly normal during this time.

RNR overexpression does not elevate mtDNA copy number in mouse tissues

We used Southern blotting to investigate the effect of RNR expression on mtDNA copy number in the skeletal muscle (Figure 2A), heart (Figure 2B), liver and kidney (Supplementary Figure S2) of the various RNR^{Tg} mice. No significant increases in mtDNA copy number were observed in any of the tissues from 9–12-weeks-old mice. In the skeletal muscle of the bitransgenic mice, there was actually a modest reduction in mtDNA abundance: $Rrm1^{Tg}+Rrm2^{Tg}$ mice had on average 71% ($P = 0.026$) and $Rrm1^{Tg}+p53R2^{Tg}$ mice 62% ($P = 0.0075$) residual mtDNA amounts compared to their wild-type littermates (Figure 2A). The mtDNA depletion in skeletal muscle was progressive; at 11–15 months of age, the residual mtDNA copy number was 42% in $Rrm1^{Tg}+Rrm2^{Tg}$ mice ($P = 0.030$) and 34% in $Rrm1^{Tg}+p53R2^{Tg}$ mice ($P = 0.0011$) (Figure 2A). The mtDNA levels in the other examined tissues remained unchanged also at the older age. Therefore, overexpression of RNR did not elevate mtDNA copy number in differentiated mouse tissues and was associated with progressive mtDNA depletion in bitransgenic animals.

RNR overexpression does not affect mtDNA integrity or mutagenesis

Overexpression of RNR causes nuclear genome mutagenesis and promotes cancer (3). We therefore asked whether RNR overexpression leads to mtDNA instability as well. We examined the presence of mtDNA point mutations in the skeletal muscle of a 16-month-old $Rrm1^{Tg}+Rrm2^{Tg}$ mouse by amplifying and cloning mtDNA regions from the control region (1080 bp) and *mt-Cytb* gene (833 bp), which encodes cytochrome b, followed by sequencing of ~30 kb of DNA from each region. We previously reported the point mutation rate in wild-type *FVB/N* mice to be ~0.5 mutations per 10 kb in the control region and ~0.25 mutations per 10 kb in the *mt-Cytb* gene (15). The 16-month-old $Rrm1^{Tg}+Rrm2^{Tg}$ showed similar or lower point mutation loads compared to a wild-type control (Table 2). In a long-range PCR assay, full-length

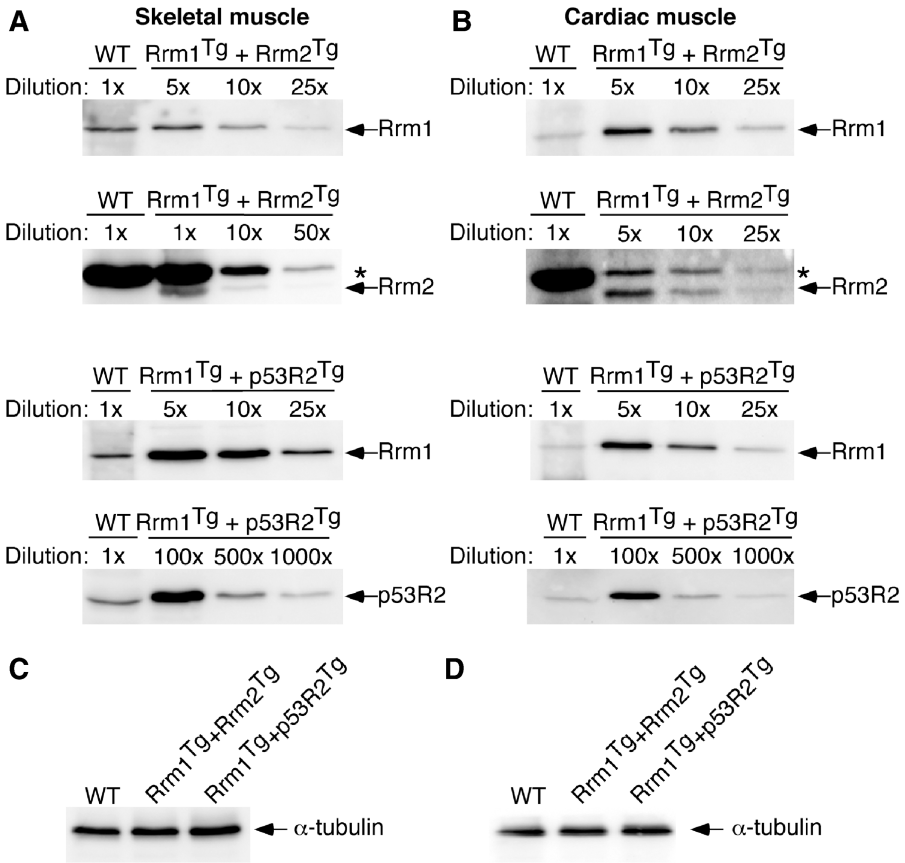

Figure 1. RNR overexpression in skeletal muscle and cardiac muscle from $Rrm1^{Tg}+Rrm2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ bitransgenic mice. Western-blot analysis of RNR protein expression was performed on total protein extracts prepared from skeletal muscle (**A** and **C**) or cardiac muscle (**B** and **D**) from wild-type (WT) as well as $Rrm1^{Tg}+Rrm2^{Tg}$ or $Rrm1^{Tg}+p53R2^{Tg}$ bitransgenic mice. Extracts were left undiluted (1×) or diluted as indicated and then subjected to immunoblotting with antibodies specific to Rrm1, Rrm2 or p53R2. The asterisk indicates a non-specific band in the anti-Rrm2 immunoblot. α-Tubulin signal in undiluted skeletal (C) and cardiac (D) muscle samples was used to normalize for protein loading. The analysis was performed on three mice of each genotype, and representative bands are shown for clarity of comparison. The calculated fold overexpression values are shown in Table 1.

mtDNA was readily amplified in the skeletal muscle of aged bitransgenic mice, with no additional small amplifying products. This ruled out the presence of large mtDNA deletions (Figure 3). These analyses showed that bitransgenic RNR overexpression did not increase deletion formation or point mutagenesis of mtDNA, but only caused mtDNA depletion.

Table 1. Transgene–encoded protein levels

Genotype	Protein	Skeletal muscle	Cardiac muscle
$Rrm1^{Tg}+Rrm2^{Tg}$	Rrm1	3.3 (±2.1)	33.7 (±7.6)
	Rrm2	37.0 (±7.2)	>23.7 (±3.8)
$Rrm1^{Tg}+p53R2^{Tg}$	Rrm1	11.7 (±6.7)	34.7 (±24.5)
	p53R2	355.3 (±25)	975.3 (±726.3)

The relative levels of transgene encoded proteins were determined by western blotting serial dilutions of skeletal and cardiac muscle tissue lysates, as shown in Figure 1. The table shows the average (±standard deviation) calculated threshold dilutions from three mice of each genotype, for each of the proteins in $Rrm1^{Tg}+Rrm2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ bitransgenic mice.

RNR overexpression leads to dNTP pool imbalance in skeletal muscle

In many human diseases and mouse models, mtDNA depletion and instability have been suggested to occur as the result of perturbed dNTP pools (26–28). To understand the mechanism of mtDNA depletion in RNR overexpressors, we measured the relative levels of dNTP pools in muscle extracts. Nucleotides are known to easily undergo quick dephosphorylation during the extraction procedure (29–31). Under conditions when dNTPs are dephosphorylated during sample preparation, ATP is similarly degraded to ADP and AMP. Therefore, the level of ATP in the extract closely mirrors that of the dNTPs, and can be used as an internal marker of unwanted dNTP destruction (30). We measured dNTP levels in skeletal muscle extracts from 12-month-old mice by a primer extension assay, using 2–3 mice of each

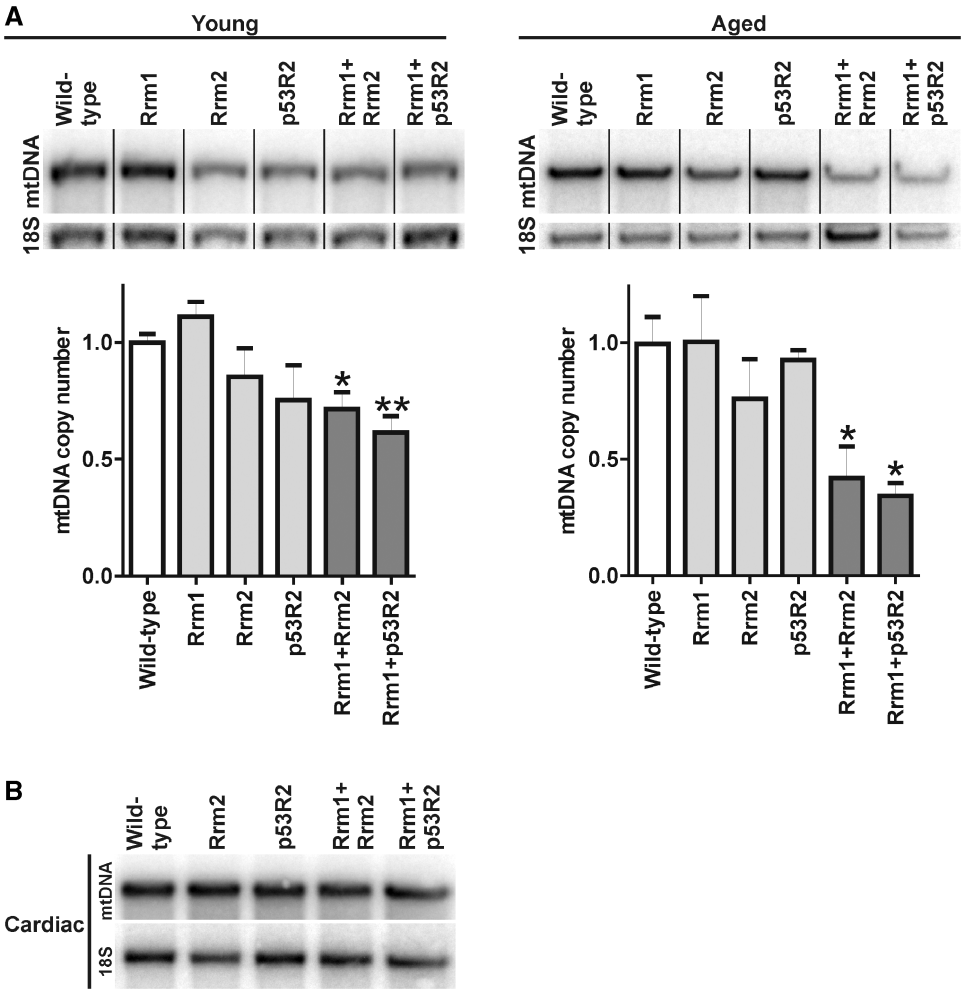

Figure 2. mtDNA quantification in young (9–12 weeks) and aged (11–15 months) mice. Southern blots of digested total DNA from skeletal (**A**) and cardiac and (**B**) muscle were probed for full-length mtDNA and the nuclear 18S rDNA gene to control for loading. (A) mtDNA to nuclear DNA ratios were determined by densitometric quantification of Southern blots and by QPCR, both methods yielding similar results. mtDNA depletion was found in young and aged bitransgenic mice. (B) No change in mtDNA copy number was observed in the cardiac muscle of RNR^{Tg} mice. Error bars indicate SEM. $*P<0.05$, $**P<0.01$, Student's *t*-test compared to wild-type where $N \geq 3$ individual animals.

genotype. The levels of ATP, ADP and AMP were measured from the same extracts by HPLC.

The average ATP levels were similar across the genotypes, the levels of ADP were >10-fold lower than that of ATP, and AMP amounted to <1% of the total adenine nucleotide pools (Figure 4). These ATP/ADP ratios corresponded well with previously reported numbers from mouse liver (30), and suggested that general dNTP dephosphorylation did not have a major effect on the dNTP level determination. The only exceptions were the $Rrm1^{Tg}+Rrm2^{Tg}$ mice, which did exhibit a somewhat lower average ATP/ADP ratio than the other genotypes (Figure 4), suggesting that dNTP measurements may have been underestimated in those mice. To ensure that the high level of RNR overexpression did not lead to substrate depletion, we also measured the levels of CDP, UDP and GDP, and found no significant differences in their levels between the genotypes (Supplementary Figure S3).

The amounts of the individual dNTPs are shown in Figure 5. Data are presented according to the respective crosses, with the $Rrm1^{Tg}$ X $p53R2^{Tg}$ crosses in panels A–D and the $Rrm1^{Tg}$ X $Rrm2^{Tg}$ crosses in panels E–H. We observed increases in the levels of dATP and dCTP in all of the transgenic lines, as compared to wild-type control samples. The mice expressing either small subunit showed greater relative increases in dATP and

Table 2. Effect of RNR overexpression upon mtDNA point mutagenesis

Mouse (genotype)	*mt-Cytb* gene		Control region	
	Sequenced (base pairs)	Mutation rate (per 10 kb)	Sequenced (base pairs)	Mutation rate (per 10 kb)
Wild-type	30 844	0.648	27 332	0.366
$Rrm1^{Tg}+Rrm2^{Tg}$	39 438	0.000	28 034	0.356

Fragments from the control region (1080 bp) and *mt-Cytb* gene (833 bp) of mtDNA, were amplified by PCR, cloned and multiple clones were sequenced. Data are from the skeletal muscle of a 16-month-old $Rrm1^{Tg}+Rrm2^{Tg}$ mouse and a 14.5-month old wild-type mouse. The mtDNA point mutation load was not increased in the bitransgenic mouse.

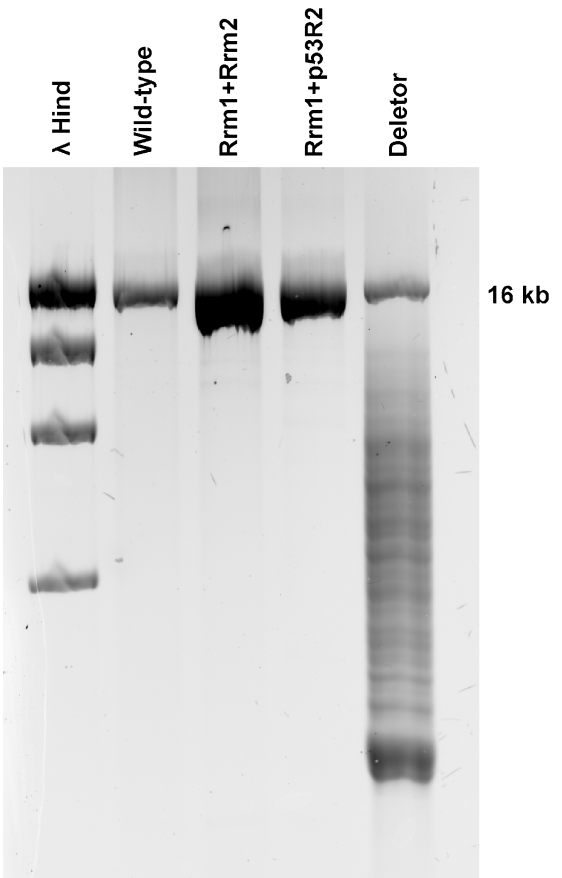

Figure 3. Analysis of mtDNA integrity in RNR^{Tg} mice by mtDNA deletion assay. To look for mtDNA deletions, a long-PCR assay was used to amplify the entire mtDNA genome from the skeletal muscle of 12-month old wild-type, $Rrm1^{Tg}+Rrm2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ mice. The full-length mtDNA (16 kb) was readily amplified from all samples. A Deletor mouse sample was included as a positive control; this sample shows multiple mtDNA fragments of smaller size. The DNA ladder λ Hind was used as a size marker.

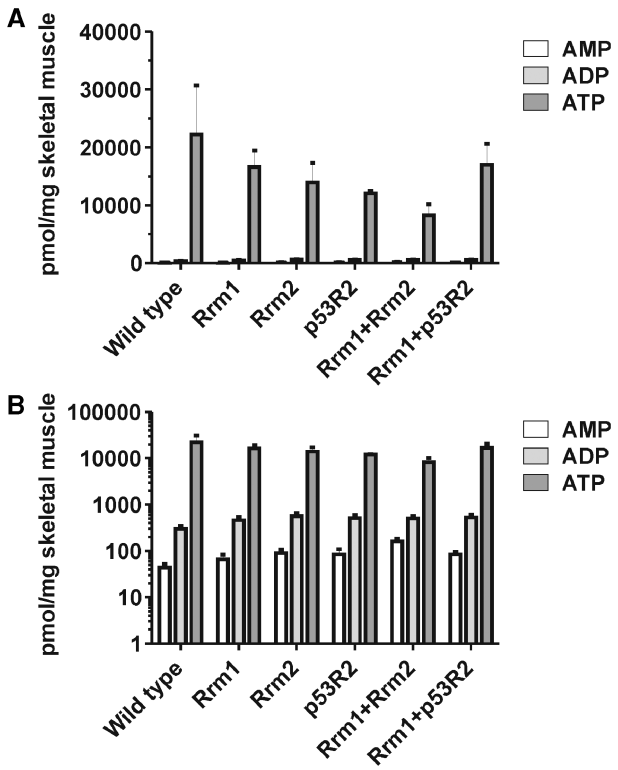

Figure 4. Measurement of AMP, ADP, and ATP levels in skeletal muscle extracts from RNR^{Tg} mice. The pools of ATP, ADP and AMP were measured from skeletal muscle extracts by an HPLC assay and quantified relative to standard curves. Results are presented as the means of results from two (for wild-type) or three (for all other genotypes) mice. The average levels of the adenine nucleotides were plotted on linear (**A**) and logarithmic (**B**) scales, and found to be similar across genotypes, which suggested comparable extraction efficiencies. The only exception was with the $Rrm1^{Tg}+Rrm2^{Tg}$ mice, which had lower average ATP levels and ATP/ADP ratios compared to the other genotypes. This may cause dNTP pool levels in these mice to be underestimated. Error bars indicate SEM.

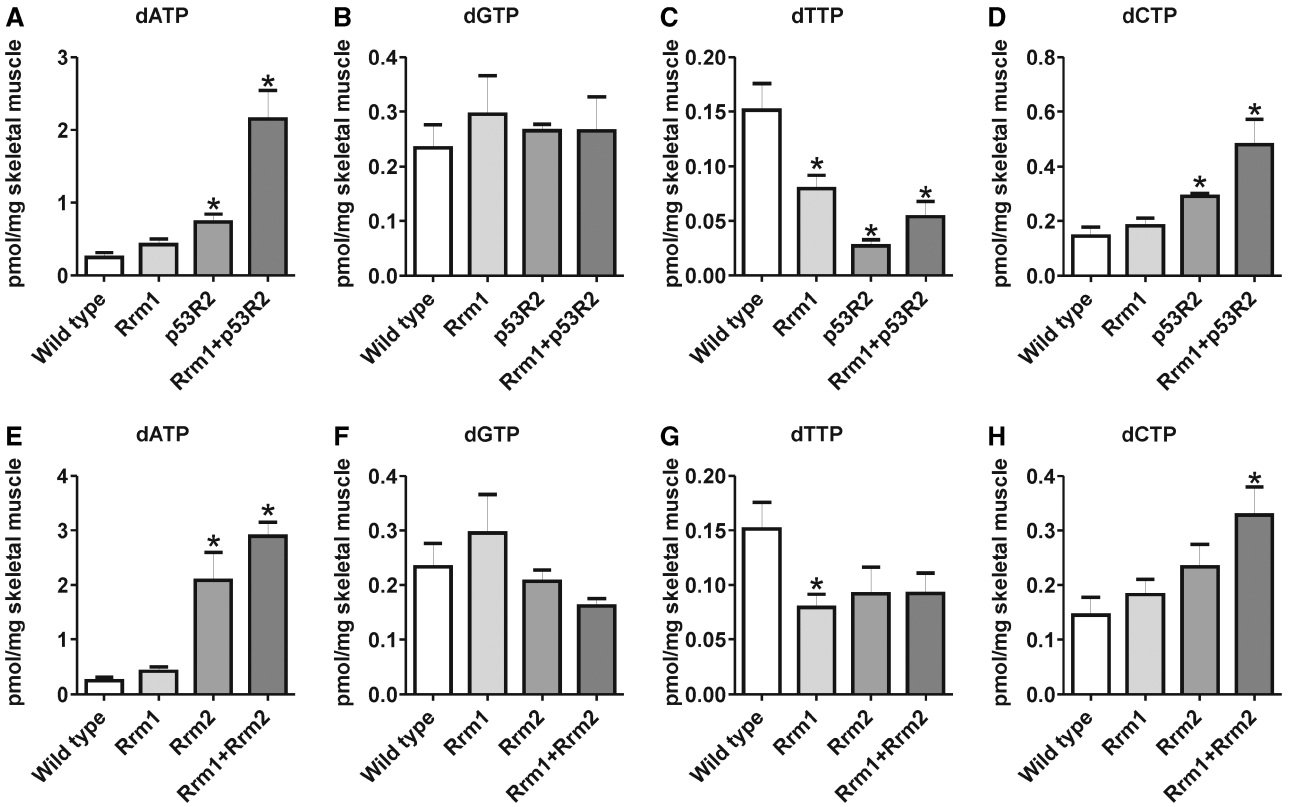

Figure 5. RNR overexpression alters dNTP pools in skeletal muscle. The levels of the four dNTPs were measured by polymerase assay and expressed per unit weight of skeletal muscle. Pool sizes for the indicated dNTPs were compared between wild-type control mice and mice from $Rrm1^{Tg} \times p53R2^{Tg}$ (**A–D**) or $Rrm1^{Tg} \times Rrm2^{Tg}$ (**E–H**) breedings. Results are presented as the means of results from two (for wild-type) or three (for all other genotypes) mice. Results for the same wild-type and $Rrm1^{Tg}$ mice are presented in (A–D) and (E–H) for comparative purposes. The levels of dATP (A and E) were significantly increased in $p53R2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ mice as well as in $Rrm2^{Tg}$ and $Rrm1^{Tg}+Rrm2^{Tg}$ mice. The dGTP levels (B and F) were unchanged in all mice. dTTP (C and G) was decreased in $p53R2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ mice and to a lesser degree also in $Rrm1^{Tg}$, $Rrm2^{Tg}$, and $Rrm1^{Tg}+Rrm2^{Tg}$ mice. dCTP (D and H) showed a similar pattern as dATP, with increases in $p53R2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ mice as well as in $Rrm2^{Tg}$ and $Rrm1^{Tg}+Rrm2^{Tg}$ mice. In each case, the $Rrm1^{Tg}$ mice showed similar but less pronounced dNTP changes as the mice overexpressing the small subunits. Error bars indicate SEM, $^*P < 0.05$, Student's *t*-test as compared to wild-type.

dCTP than the $Rrm1^{Tg}$ mice. There was also a clear tendency towards higher dATP and dCTP values in the bitransgenic $Rrm1^{Tg}+p53R2^{Tg}$ and $Rrm1^{Tg}+Rrm2^{Tg}$ mice as compared to the $p53R2^{Tg}$ (Figure 5A and D) and $Rrm2^{Tg}$ (Figure 5E and H) single transgenic mice, respectively. In contrast to the elevated levels of dATP and dCTP, the levels of dGTP were unchanged in all of the transgenic mice (Figure 5B and F), and the levels of dTTP were actually decreased significantly in the $p53R2^{Tg}$ and $Rrm1^{Tg}+p53R2^{Tg}$ mice (Figure 5C), and, to a lesser degree, in the $Rrm1^{Tg}$, $Rrm2^{Tg}$ and $Rrm1^{Tg}+Rrm2^{Tg}$ mice (Figure 5G). Taken together, these data suggested that RNR overexpression led to significant changes in dNTP pools and considerable imbalances between the different dNTPs, with the most pronounced changes in bitransgenic RNR^{Tg} mice.

Increased mtDNA copy number is associated with decreased RNR expression

RNR is involved in a signalling pathway that regulates mtDNA copy number in yeast (12), and regulation of RNR expression could be part of a homeostatic mechanism to control mtDNA copy number in mammals. We therefore utilized mouse models that express recombinant wild-type mouse Twinkle (15) or human TFAM (20), with ~2- and ~3-fold respective increases of mtDNA copy numbers in their skeletal muscle at the age of ten weeks. We measured the expression levels of *Rrm1* and *Rrm2b*, the genes encoding Rrm1 and p53R2, respectively, in skeletal muscle of $Twinkle^{Tg}$ and $TFAM^{Tg}$ mice. *Rrm1* mRNA was decreased to 76% ($P = 0.011$) and 70% ($P = 0.010$) in the $Twinkle^{Tg}$ and $TFAM^{Tg}$ mice, respectively (Figure 6A). *Rrm2b* mRNA was similarly decreased to 78% ($P = 0.045$) and 77% ($P = 0.0093$) of normal in the $Twinkle^{Tg}$ and $TFAM^{Tg}$ mice, respectively (Figure 6B). Thus the expression of RNR genes was found to correlate inversely with mtDNA copy number in two mouse models.

DISCUSSION

RNR is essential for both nuclear and mtDNA replication. The enzyme is limiting for mtDNA copy number in yeast, but whether the same is true in mammals has not been studied earlier.

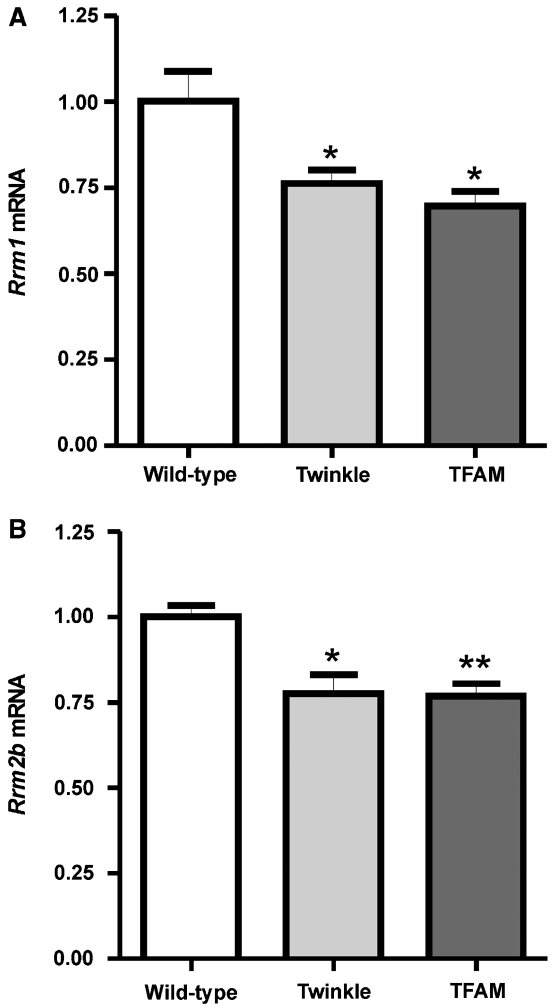

Figure 6. RNR expression levels in mice with high mtDNA copy number. A QPCR assay was used to measure the expression levels of *Rrm1* and *Rrm2b*, the genes encoding Rrm1 and p53R2, respectively, in the skeletal muscle of mice overexpressing Twinkle or TFAM. These mice have increased mtDNA copy number. (**A**) *Rrm1* expression was downregulated in the Twinkle-mice and TFAM-mice compared to controls. (**B**) *Rrm2b* was similarly downregulated. Error bars indicate SEM, $*P < 0.05$, $**P < 0.01$, Student's *t*-test compared to wild-type where $N \geq 3$ individual animals.

dNTP pool regulation has emerged as a potential tool to increase mtDNA levels, and thereby to slow down progression of mtDNA disease. *In vitro* supplementation with two deoxyribonucleoside monophosphates (dNMPs) rescued mtDNA depletion in cultured patient myotubes with mutations in the mitochondrial deoxyribonucleoside salvage pathway enzyme deoxyguanosine kinase (dGK) (32). However, mtDNA depletion due to patient mutations in the mtDNA polymerase gamma (Pol γ) was not restored through nucleotide supplementation in the same study. Likewise, nucleotide supplementation to healthy myotubes did not increase mtDNA levels significantly above normal (32). These results suggested that although dNTP pool expansion may be beneficial in cases of dNTP deficiency, other factors than the size of the mitochondrial dNTP pool are limiting for mtDNA copy number in normal cells or in mtDNA replication defects.

RNR overexpression in yeast resulted in elevated mtDNA levels (12), and complementation of the respiratory chain deficient phenotype of Pol γ disease mutations (13). The results presented here suggest that RNR overexpression has partially opposite effects in mammals, which illustrates the marked differences in dNTP pool maintenance between organisms. However, we cannot exclude the possibility that increased RNR activity or dNTP availability *in vivo* could be beneficial in cases with increased mtDNA turnover or mutagenesis. The increased carcinogenesis in RNR^{Tg} mice (3) illustrates the hazards of altering dNTP pool maintenance and further reduces the potential of RNR as a therapeutic tool.

The mechanisms governing mtDNA copy number in tissues are starting to come into focus. In mice, overexpression of the histone-like packaging protein TFAM or of the mtDNA helicase Twinkle increase mtDNA copy number 2- to 3-fold (14,15). The mRNA level of Twinkle and the protein level of TFAM correlate linearly with mtDNA content, suggesting that these may be limiting factors in determining the amount of mtDNA. Defects in mitochondrial dNTP pool maintenance proteins cause loss of mtDNA, indicating that these factors are essential for mtDNA maintenance (7,33–36). However, their contribution to the physiological control of mtDNA copy number is not well characterized. Heart-specific overexpression of the salvage pathway enzyme thymidine kinase 2 (TK2) in mice resulted in a 300-fold increase in enzyme activity and produced a ~30% increase in mtDNA copy number (37). Therefore, a very large increase in TK2 activity modestly influences mtDNA levels, potentially through increases in deoxycytidine and thymidine nucleotide pools. Our results show that although RNR is involved in the synthesis of all four dNTPs, very high levels of the enzyme do not increase mtDNA copy number and instead perturb mtDNA homeostasis. This finding is in line with Twinkle and TFAM being the main regulators of mtDNA level under normal circumstances.

A possible mechanism for nuclear control over mtDNA copy number would be to alter the transcription of mtDNA maintenance genes. In yeast, the first established signaling pathway that regulated mtDNA copy number is activated by the Mec1p/Rad53p kinases and leads to induction of RNR expression (12). The related ATM (ataxia-telangiectasia mutated) kinase in humans was also found to influence RNR expression and mtDNA homeostasis (38). We found the transcription of *Rrm1* and *Rrm2b* to be downregulated in two independent mouse models with increased mtDNA copy number, suggesting an intimate feedback mechanism between transcriptional regulation of RNR subunits and mtDNA levels. RNR could thus contribute to a regulatory mechanism for nuclear control of mtDNA copy number *in vivo*. Such a signaling pathway could involve p53, a tumor suppressor and ATM target, which influences the expression level of p53R2 and is known to localize in small amounts to mitochondria (39).

RNR is rate-limiting for *de novo* dNTP synthesis, so any alteration to RNR activity is expected to induce changes in dNTP pools. These changes are transmitted into

mitochondria, since the mitochondrial and cytosolic dNTP pools are in rapid communication (40). Direct measurement of dNTP pools from animal tissues is complicated by dNTP dephosphorylation caused by the anaerobiosis that immediately follows the death of the animal (30). Our extraction method was optimized to minimize nucleotide degradation during extraction, and the ATP, ADP and AMP levels were used as internal controls to ensure comparability across samples. We documented clear and reproducible differences in total dNTP pools between RNR overexpressors and wild-type mice.

First, there were significant increases in the levels of dATP and dCTP both in the $Rrm2^{Tg}$ and $p53R2^{Tg}$ mice. Rrm2 induced higher dNTP increases than p53R2, which is consistent with Rrm2 being more active than p53R2 *in vitro* (41). Rrm1 overexpression alone was able to induce a detectable, albeit not statistically significant, increase in dATP and dCTP levels, and overexpression of Rrm1 together with either small subunit led to a clear trend towards higher levels of dATP and dCTP. This apparent synergy suggested that co-overexpression of both subunits led to an increase in the abundance of the tetramer, with subsequent effects on nucleotide pools.

The allosteric regulation of RNR promotes a balanced production of all four dNTPs and therefore manipulation of RNR expression might be expected to influence the levels of all four dNTPs equally (1). However, we found remarkable dNTP pool imbalances in the RNR^{Tg} mice. The dGTP pools were unchanged whereas dTTP pools were actually decreased in $p53R2^{Tg}$ as well as $Rrm1^{Tg}+p53R2^{Tg}$ mice. This finding illustrates the need for tight control of the relative activities of the large number of anabolic and catabolic enzymes that determine the final dNTP composition *in vivo* (42). For instance, the synthesis of thymidine phosphates requires—as an additional step—reductive methylation of RNR-generated deoxyuridine monophosphate, which is catalyzed by thymidylate synthase. Hence, induction of RNR could cause a substrate overload for the endogenous thymidylate synthase, which in turn could explain why increased RNR does not increase the dTTP level. Furthermore, the specificity of RNR for GDP reduction is induced by the binding of dTTP to the specificity site of Rrm1 (1). Thus, a relative lack of dTTP could explain why the dGTP pools did not increase in our mice. The dNTP imbalance was not due to depletion of any of the substrates of RNR, since the levels of ADP, CDP, UDP and GDP were similar in all genotypes.

Unbalanced dNTP pools are known to cause mutagenesis in both nuclear (43) and mitochondrial (28) genomes. Given the presence of altered dNTP pools in RNR^{Tg} mice, the likely mechanism of progressive mtDNA depletion in bitransgenic mice is inefficient mtDNA replication caused by perturbed dNTP balance. Initiation of nuclear DNA replication is influenced by the dNTP pool (44,45), and an imbalanced pool could lead to reduced frequency of mtDNA replication initiation. mtDNA depletion was specifically restricted to bitransgenic mice, which was consistent with them displaying the largest dNTP alterations. The fact that $Rrm2^{Tg}$ and $p53R2^{Tg}$ mice also had altered dNTP pools but no mtDNA depletion, suggests a threshold effect for the relative dNTP levels above which mtDNA replication becomes inefficient. Nevertheless, we cannot exclude that co-overexpression of Rrm1 and the small subunit gave rise to additional, unidentified effects, which did not occur upon overexpression of one subunit. Moreover, mtDNA depletion was observed only in the skeletal muscle, although the transgenes were overexpressed in both the skeletal and cardiac muscles, suggesting tissue specific mechanisms in the regulation of nucleotide pools and/or mtDNA maintenance.

Altered dNTP pool balance is a hallmark of MNGIE disease, where deficiency of the catabolic enzyme thymidine phosphorylase (TP) leads to elevated dTTP pools, which cause mtDNA depletion, deletions and point mutations in humans (28). Contrary to MNGIE, however, we found no increase in mtDNA point mutations or deletions in the RNR^{Tg} mice. There are at least two possible explanations for the absence of mtDNA instability, other than depletion, in our mice. First, the dNTP changes were almost opposite to those in MNGIE, i.e. the relative dTTP level decreased instead of increasing. Further studies are needed to elucidate the exact effect of changes in the relative levels of each of the four dNTPs on mtDNA replication frequency and fidelity. Second, the life-span of a mouse may be too short to develop significant amounts of mtDNA deletions or point mutations in the setting of dNTP imbalance. In support of this, the MNGIE mouse model lacking TP and the related uridine phosphorylase (UP) displayed increased dTTP in brain, and, similar to RNR^{Tg} mice, developed progressive mtDNA depletion, but no deletions or point mutations (27). The authors argued that this was at least partly due to the short life-span of mice, emphasizing the differences in dNTP maintenance and disorders between species.

In conclusion, we have established that expression of recombinant RNR in mice leads to dNTP pool imbalance and progressive depletion of mtDNA. This is in contrast to previous findings in yeast, in which RNR is a positive regulator of mtDNA abundance. Furthermore, endogenous RNR expression is responsive to increased mtDNA copy number and a potential modifier of mtDNA homeostasis. The dNTP pool imbalance and mtDNA decrease caused by excess RNR suggests that balanced amounts of RNR are essential for mtDNA maintenance *in vivo*, and that RNR is unlikely to be an optimal target for therapeutic engineering of mtDNA levels in mammals.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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