

Increased *Rrm2* gene dosage reduces fragile site breakage and prolongs survival of ATR mutant mice

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In *Saccharomyces cerevisiae*, absence of the checkpoint kinase Mec1 (ATR) is viable upon mutations that increase the activity of the ribonucleotide reductase (RNR) complex. Whether this pathway is conserved in mammals remains unknown. Here we show that cells from mice carrying extra alleles of the RNR regulatory subunit RRM2 (*Rrm2*^{TG}) present supraphysiological RNR activity and reduced chromosomal breakage at fragile sites. Moreover, increased *Rrm2* gene dosage significantly extends the life span of ATR mutant mice. Our study reveals the first genetic condition in mammals that reduces fragile site expression and alleviates the severity of a progeroid disease by increasing RNR activity.

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Replication stress (RS) has emerged as a source of genome instability in human diseases, including cancer and premature aging (Lecona and Fernandez-Capetillo 2014; Zeman and Cimprich 2014). In brief, RS is defined by the accumulation of abnormal amounts of ssDNA at

stalled replication forks that, due to its recombinogenic nature, can initiate genomic rearrangements. In mammals, RS is sensed and suppressed by a signaling cascade initiated by the ATR kinase, which, together with its target kinase, CHK1, suppresses RS through the phosphorylation of multiple targets (Cimprich and Cortez 2008; Lopez-Contreras and Fernandez-Capetillo 2010). ATR and CHK1 are essential for embryonic development in mice (Brown and Baltimore 2000; de Klein et al. 2000; Liu et al. 2000), which is due to the role of the RS response (RSR) in preventing replication-born chromosome breakage. Whether the RSR protects all forks or a subset of them during replication is unclear. On the one hand, proteomic studies of the human replisome in unchallenged conditions have failed to detect ATR or CHK1 in the vicinity of replication forks (Lopez-Contreras et al. 2013; Sirbu et al. 2013), suggesting that their activity might be particularly necessary for only a subset of forks, such as damaged ones. Accordingly, chromosomal breaks that arise upon ATR inactivation locate preferentially at specific loci named common fragile sites (CFSs) (Casper et al. 2002) and early replicating fragile sites (ERFSs) (Barlow et al. 2013). Regardless of whether ATR works at all forks or only some of them, how it suppresses RS and why it is essential are still not fully understood.

Ribonucleotide reductase (RNR) is a tetrameric enzyme composed of two catalytic (RRM1, Rnr1 in yeast) and two regulatory (RRM2, Rnr2 in yeast) subunits (Jordan and Reichard 1998). It reduces NDPs into dNDPs, which is a rate-limiting step for the production of dNTPs. In yeast, the lethality of *mec1Δ* strains can be bypassed by mutations that increase RNR activity. The first evidence of a connection between ATR and RNR came from the discovery of Crt1 (a transcriptional repressor of RNR subunits) as a suppressor of Mec1 lethality in *Saccharomyces cerevisiae* (Huang et al. 1998). Furthermore, overproduction of Rnr1 was shown to be sufficient to rescue *mec1Δ* viability (Desany et al. 1998; Vallen and Cross 1999). In addition to the transcriptional regulation provided by Crt1, Mec1 and its downstream kinase, Dun1, also increase RNR activity by phosphorylating the RNR inhibitor Sml1, leading to its degradation (Zhao et al. 2001; Zhao and Rothstein 2002). As in the case of Crt1, *sml1* deletion also rescued *mec1Δ* viability (Zhao et al. 1998). Finally, the activity of yeast RNR is also regulated through the control of Rnr2 localization. *S. cerevisiae* Dif1 or *Schizosaccharomyces pombe* Spd1 retain Rnr2 in the nucleus, preventing its interaction with cytoplasmic Rnr1. Like Crt1 or Sml1, deletion of *spd1* or *dif1* increases RNR activity and suppresses the lethality of checkpoint mutants (Liu et al. 2003; Lee et al. 2008; Wu and Huang 2008). Even though no clear orthologs for yeast RNR inhibitory proteins have been found in mammals, evidence suggests that the connection between ATR and RNR is conserved to some extent.

First, the addition of nucleosides has been shown to reduce RS in various instances, such as in response to

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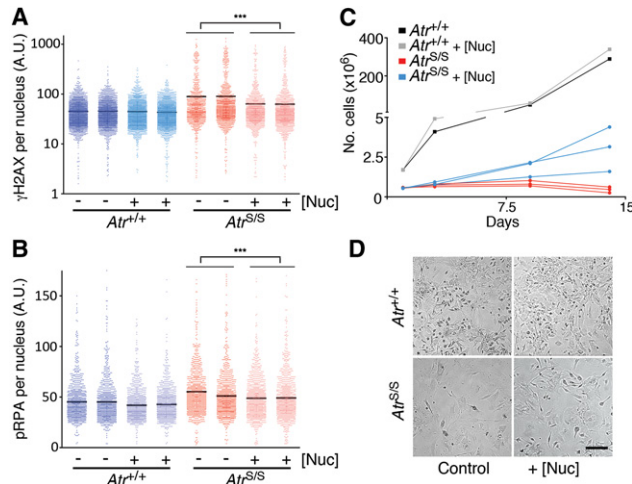


Figure 1. Nucleoside supplementation reduces RS and improves growth of ATR-Seckel MEFs. (A) HTM-mediated quantification of the intensity of γ H2AX per individual nucleus on $Atr^{+/+}$ and $Atr^{S/S}$ MEFs treated or not with 60 μ M nucleosides for 24 h. Data are representative of two independent cell lines. (B) HTM-mediated quantification of the intensity of phosphorylated RPA per individual nucleus on $Atr^{+/+}$ and $Atr^{S/S}$ MEFs treated or not with 60 μ M nucleosides for 24 h. Data are representative of two independent cell lines. (C) Proliferation curves of $Atr^{+/+}$ and $Atr^{S/S}$ MEFs grown in the presence or absence of 60 μ M nucleoside supplementation. Data are representative of two independent analyses. (D) Representative pictures of wild-type and ATR-Seckel MEFs at the end point of the proliferation assay shown in C. Note that while all ATR mutant MEFs show the flattened morphology of senescent cells at this point, those treated with a nucleoside supplement still present cells with the elongated morphology of proliferating fibroblasts. (***) $P < 0.001$.

oncogenes or during zebrafish development (Bester et al. 2011; Danilova et al. 2014). Furthermore, CHK1 activation by topoisomerase inhibitors induces the expression of RRM2 through E2F-dependent transcription (Zhang et al. 2009). In addition, p53 induces the expression of an alternative regulatory subunit (RRM2B or p53R2). However, both of these activities occur only after a prolonged exposure to DNA damage and cannot account for the more immediate role that ATR plays during DNA replication. Regarding Sml1, a recent study reported that IRBIT, a protein known to be involved in ion transport, shares some distant homology with Sml1 in a small fragment of its sequence (Arnaoutov and Dasso 2014). This fragment can bind and inhibit the RNR complex, but its activity seems to be restricted to mitosis and RRM1/RRM2B complexes. In summary, while these experiments suggest that RNR regulation might suppress RS in mammals, whether and how these functions are linked to ATR activity are not understood.

Here we sought to explore the impact of the RNR in the ATR response in mammals. To this end, we generated a mouse model with increased levels of the regulatory subunit RRM2 ($Rrm2^{TG}$). We show that $Rrm2$ transgenic mice present supraphysiological RNR activity, which becomes protective in the context of insufficient dNTP levels. Importantly, we show that increased levels of RRM2 can limit chromosome breakage in response to ATR inhibitors and extend the life span of mice with reduced ATR levels. Altogether, our findings provide strong genetic support for the concept that regulating nucleotide pools is one of the key functions of ATR in mammals.

Results and Discussion

Nucleosides limit RS and improve growth of ATR-Seckel mouse embryonic fibroblasts (MEFs)

To explore whether increased nucleotides could suppress phenotypes related to ATR deficiency in mammals, we used MEFs from a model of the ATR-Seckel syndrome (Murga et al. 2009). ATR-Seckel MEFs present severely reduced ATR levels and undergo premature senescence due to the accumulation of RS (Murga et al. 2009; Monasor et al. 2013). While nucleotides are not cell-permeable, a recent report showed that the addition of nucleosides, the nucleotide precursors, to the culture medium alleviates oncogene-induced RS (Bester et al. 2011). In the case of ATR-Seckel MEFs, the addition of nucleosides significantly reduced the levels of RS, as quantified by measuring the phosphorylation status of histone H2AX (γ H2AX) or the ssDNA-binding protein RPA by high-throughput microscopy (HTM) (Fig. 1A,B). In addition, nucleoside supplementation to the culture medium partially rescued the growth defect in three independent lines of ATR-Seckel MEFs (Fig. 1C,D). These results suggest that increased levels of nucleotides can suppress the RS that derives from reduced ATR activity.

Generation of a mouse model with increased RRM2 levels

To investigate the consequences of increased RNR activity in a mammalian organism, we generated mice carrying extra alleles of the regulatory subunit RRM2. We chose to focus on RRM2 because of the following considerations. First, knockdown of RRM2 increased RS in response to the RNR inhibitor hydroxyurea (HU) in human U2OS cells, whereas overexpression had the opposite effect (Fig. 2A,B; Supplemental Fig. S1). Second, available microarray analyses revealed reduced levels of RRM2 expression on ATR-Seckel embryos (Murga et al. 2009), suggesting that a defective RNR activity might contribute to the phenotypes of these mice. Third, ATR-dependent degradation of cyclin F has been shown to increase RRM2 levels in response to DNA damage (D'Angiolella et al. 2012). Finally, classic reports described HU-resistant hamster cell lines that were associated with genomic amplification of $Rrm2$, but not $Rrm1$, which led to increased RNR activity in these cells (Lewis and Wright 1974; Tonin et al. 1987). Hence, we hypothesized that mice carrying additional copies of the $Rrm2$ gene would similarly have increased RNR activity. To preserve the endogenous regulation of $Rrm2$ transcription, we subcloned a 26-kb region encompassing the $Rrm2$ gene and 10 kb of flanking sequences (Fig. 2C). This strategy, in contrast to classic transgenesis using plasmids harboring cDNA, has been proven successful for generating animals with increased activity of genes that are otherwise deleterious when aberrantly overexpressed, such as $Chk1$ or $Tp53$ (Garcia-Cao et al. 2002; Lopez-Contreras et al. 2012).

Transgenic lines were identified by Southern blotting, and the integration site was subsequently mapped by inverse PCR. We selected one line ($Rrm2^{TG}$) carrying several alleles integrated in tandem (Fig. 2D) on the basis of having the insertion of the transgene at a locus where it did not disrupt any other gene (chr12: 118,863,235). Mapping the insertion also allowed for genotyping of homozygous or heterozygous mice (Fig. 2E). $Rrm2$ transgenic

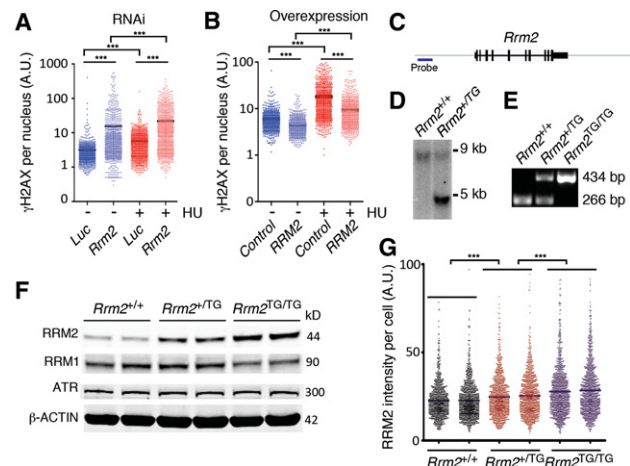


Figure 2. Generation of *Rrm2*^{TG} mice. (A) HTM-mediated quantification of the intensity of γ H2AX per individual nucleus on human U2OS cells 48 h after being transfected with endogenous siRNAs (esiRNAs) targeting either firefly luciferase (*Luc*) or *Rrm2*. Where indicated, cells were also exposed to 2 mM HU for 3 h. Data are representative of two independent experiments. (B) HTM-mediated quantification of the intensity of γ H2AX per individual nucleus on human U2OS cells 48 h after being transfected with a RRM2-expressing plasmid or the empty vector (control). Where indicated, cells were also exposed to 2 mM HU for 3 h. Data are representative of two independent experiments. (C) Scheme of the genomic locus (including the *Rrm2* gene and flanking regions) that was used for the generation of *Rrm2*^{TG} mice. (D) Southern blot illustrating the presence of a single transgene integration site (5 kb) on the *Rrm2*^{TG} strain. The 9-kb band corresponds to the endogenous *Rrm2* gene. (E) Genotyping PCR illustrating the three genotypes that can be obtained at Mendelian ratios on the *Rrm2*^{TG} strain. (F) Western blot illustrating the levels of RRM2, RRM1, and ATR on *Rrm2*^{+/+}, *Rrm2*^{+/TG}, and *Rrm2*^{TG/TG} MEFs. β -Actin was used as a loading control. (G) HTM-mediated quantification of the intensity of RRM2 per individual cell on *Rrm2*^{+/+}, *Rrm2*^{+/TG}, and *Rrm2*^{TG/TG} MEFs. RRM2 Western blot of tissues from wild-type and *Rrm2*^{TG} mice. (***) $P < 0.001$.

MEFs exhibited increased RRM2 protein levels in a transgene dosage-dependent manner, as detected by Western blotting and immunofluorescence (Fig. 2F,G). In contrast, no effect on the levels of RRM1 or ATR was observed. In tissues, RRM2 expression was analyzed by immunohistochemistry (IHC). RRM2 expression was highest in tissues with active turnover, such as spleen or testes, and increased on *Rrm2* transgenic tissues (Supplemental Fig. S2). Interestingly, RRM2 was cytoplasmic in all tissues except testes, where it showed a nuclear distribution consistent with nuclear RNR activity being particularly relevant for DNA repair (D'Angiolella et al. 2012). Regardless of its increased expression, the intracellular localization of RRM2 or its relative abundance between different tissues was not altered on *Rrm2*^{TG} mice. Thus, mice carrying extra alleles of *Rrm2* provide a physiological model to investigate the consequences of increased RRM2 levels.

Increased RNR activity in *Rrm2*^{TG} MEFs

Two independent methods for quantifying dNTP levels failed to detect significant changes on *Rrm2* transgenic MEFs (Supplemental Fig. S3). The absence of an increase in steady-state dNTP levels is in agreement with the existence of feedback inhibitory loops, which inhibit RNR activity when dATP levels increase above physio-

logical levels (Jordan and Reichard 1998). Nevertheless, our finding is in agreement with previous studies. For instance, HU-resistant mouse mammary tumor cells with a 40-fold overexpression of RRM2 have normal dNTP levels (Eriksson et al. 1984). In addition, mouse 3T6 cells made HU-resistant upon a gradual increase in HU concentrations have normal levels of dNTPs despite having a threefold to 15-fold increase in RNR activity (Akerblom et al. 1981). Hence, while steady-state dNTP levels are kept at normal levels on *Rrm2* transgenic cells, they could harbor increased RNR activity that would be particularly relevant in conditions of limited nucleotide availability.

To examine whether *Rrm2* transgenic cells had increased RNR activity, we tested how they responded to the RNR inhibitor HU. A number of independent experiments validated that *Rrm2* transgenic cells had increased RNR activity. First, HTM analyses revealed that *Rrm2*^{TG} MEFs exhibited lower levels of γ H2AX in response to HU (Fig. 3A). While *Rrm2*^{TG/TG} MEFs express more RRM2 than heterozygous cells, this did not translate into a higher resistance. This could indicate that the strategy of increasing RRM2 might reach a limit if not done together with a concomitant increase in RRM1. Besides H2AX, *Rrm2* transgenic MEFs also presented lower levels of RPA phosphorylation in response to HU (Fig. 3B). In

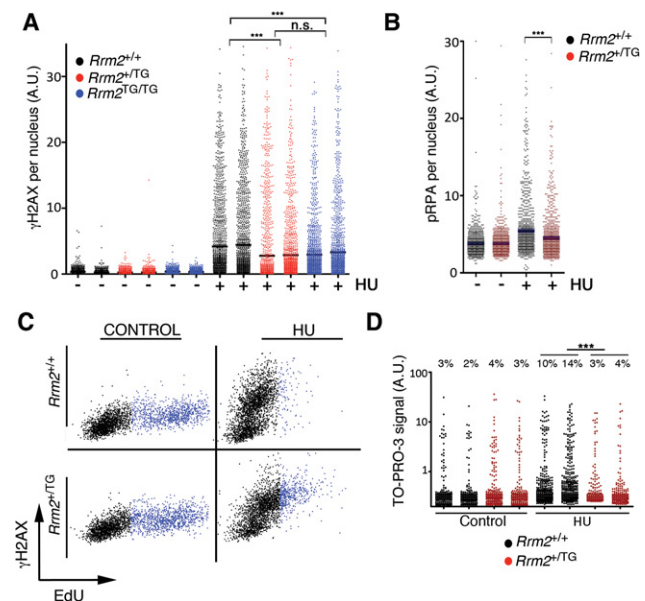


Figure 3. Increased RNR activity on *Rrm2*^{TG} cells. (A) HTM-mediated quantification of the intensity of γ H2AX per individual nucleus on *Rrm2*^{+/+}, *Rrm2*^{+/TG}, and *Rrm2*^{TG/TG} MEFs. Data are representative of three independent experiments. Where indicated, cells were also exposed to 0.5 mM HU for 4 h. (B) HTM-mediated quantification of the intensity of phosphorylated RPA per individual nucleus on *Rrm2*^{+/+} and *Rrm2*^{+/TG} MEFs. Data are representative of three independent experiments. Where indicated, cells were also exposed to 0.5 mM HU for 4 h. Data are representative of three independent analyses in four different MEF pairs. (C) HTM-mediated quantification of γ H2AX and EdU intensities per individual nucleus in wild-type and *Rrm2* transgenic MEFs treated or not with 0.3 mM HU for 4 h. EdU was added to the cultures for the last 30 min of the experiment. Blue dots represent EdU-incorporating cells. (D) HTM-mediated quantification of cell death by analysis of the incorporation of the TO-PRO-3 dye in *Rrm2*^{+/+} and *Rrm2*^{+/TG} treated or not with 0.3 mM HU for 24 h (see the Materials and Methods). The experiment was done in triplicate with three independent MEF pairs. (***) $P < 0.001$.

addition to RS markers, *Rrm2* transgenic cells were able to incorporate EdU at doses of HU that fully prevented replication in wild-type cells (Fig. 3C). Interestingly, EdU incorporation in *Rrm2*^{+TG} MEFs could even be detected in cells that presented H2AX phosphorylation. Of note, the actual degree of HU-resistant replication might be higher than observed with this assay, since higher dNTP levels on *Rrm2* transgenic cells exposed to HU would compete with EdU during replication. Finally, we tested actual HU resistance by looking at HU-induced cytotoxicity upon prolonged exposure to the drug. Once again, increased levels of RRM2 correlated with lower levels of HU-induced cell death in transgenic cells (Fig. 3D). In contrast, *Rrm2* transgenic cells were not protected against other RS-inducing agents, such as aphidicolin or camptothecin, suggesting that increased RRM2 levels become particularly relevant in the context of conditions that limit dNTP levels, such as HU or ATR inhibitors (see below). Of note, neither the *Rrm2* transgene nor the supplementation of nucleosides shown above affected cell cycle progression. Altogether, these experiments demonstrate that *Rrm2*^{TG} cells present increased RNR activity, which protects them from HU-induced RS and might be beneficial in the context of conditions that reduce dNTP availability.

Rrm2^{TG} protects from reduced ATR activity *in vitro* and *in vivo*

As mentioned, evidence from yeast suggests that stimulating RNR activity is one of the essential activities of Mec1 (ATR). To determine whether a similar interaction might be conserved in mammals, we first tested whether *Rrm2*^{TG} cells were resistant to the replicative damage induced by ATR inhibitors previously developed in our group (Toledo et al. 2011). To do this, we looked at the presence of chromosomal abnormalities at CFS and ERFS loci, both of which are sites of preferential chromosomal fragility upon ATR inhibition (Casper et al. 2002; Barlow et al. 2013). Proliferating B lymphocytes from *Rrm2*^{TG} cells presented an overall reduction in the number of chromosome breaks that were induced by ATR inhibitors (Fig. 4A), a difference that was pronounced at CFS and ERFS fragile loci (Fig. 4B,C). Remarkably, ATR inhibitors lead to a reduction in cellular dNTP levels (Supplemental Fig S4), which might contribute to the toxicity of these compounds. Supporting this view, increased levels of RRM2 limit the chromosomal breakage that arises upon ATR inhibition *in vitro*.

Finally, to determine whether increased RNR activity could also rescue the phenotypes associated with deficient ATR signaling in a mammalian organism, we crossed *Rrm2*^{TG} mice into the ATR-Seckel strain. The Seckel syndrome was originally named “bird-headed dwarfism” because of the overall reduced size and the craniofacial anomalies present in these patients (Seckel 1960). Both of these clinical manifestations, which are recapitulated on ATR-Seckel mice, were significantly alleviated by the *Rrm2* transgene (Fig. 4D,E). In addition to the overall appearance, Seckel mice succumb prematurely due to accelerated aging (Murga et al. 2009). Importantly, the presence of the *Rrm2* transgene significantly increased the survival of ATR-Seckel mice (Fig. 4F). *Atr*^{S/S}; *Rrm2*^{TG/+} animals doubled the median life span of *Atr*^{S/S}; *Rrm2*^{+/+} mice (50 wk vs. 24 wk). Moreover, the presence of

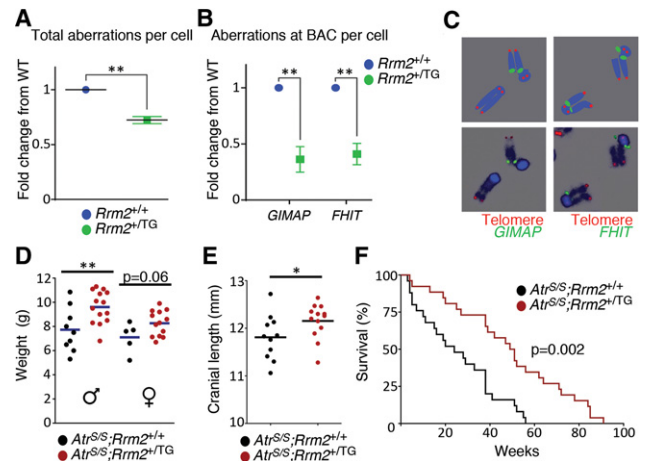


Figure 4. Increased RNR activity limits chromosome breakage in response to ATR inhibition and prolongs survival of ATR-Seckel mice. (A) DNA aberrations per metaphase were counted from *Rrm2*^{+/+} and *Rrm2*^{+TG} B lymphocytes treated with 10 μ M ATR inhibitor for 16 h. Data were normalized as fold change from wild-type levels to account for fluctuations between three independent experimental runs. (B) Aberrations at the *GIMAP* ERFS and the *FHIT* (FRA14A2) per metaphase normalized as fold change from wild-type levels from the metaphases used in A. Aberrations included chromatid and chromosome breaks, radials, and dicentric chromosomes. At least 84 metaphases were scored per replicate. (C, top panel) Diagram of FISH probes, with the DNA in blue, telomeres in red, and BAC probes in green. The bottom panel shows representative rearrangements at *GIMAP* and *FHIT*. (D) The weight of *Atr*^{S/S}; *Rrm2*^{+/+} and *Atr*^{S/S}; *Rrm2*^{+TG} male (left) and female (right) mice at 10 wk of age. (E) The cranial length of *Atr*^{S/S}; *Rrm2*^{+/+} and *Atr*^{S/S}; *Rrm2*^{+TG} mice at 10 wk of age analyzed by computerized tomography (CT). (F) Kaplan-Meier curves of *Atr*^{S/S}; *Rrm2*^{+/+} (n = 25) and *Atr*^{S/S}; *Rrm2*^{+TG} (n = 26) mice. The P-value was calculated with the Mantel-Cox log rank test.

the transgene also increased the maximum life span of ATR hypomorphic mice from 54 wk to 91 wk. Collectively, these results demonstrate that increased RNR activity limits the consequences derived from reduced ATR activity in mammals.

Here we show that RNR activity can be enhanced in mammals by overexpression of the RRM2 subunit. A previous report that generated cDNA-based RNR transgenic mice revealed that broad overexpression of RRM2 increases the incidence of lung neoplasms (Xu et al. 2008). So far, we failed to detect a higher incidence of tumors on *Rrm2*^{TG} mice. It is possible that the difference between these studies relies on the fact that our transgenic RRM2 is expressed under its endogenous promoter so that we are not overexpressing highly increased levels in tissues where RNR expression is normally low (such as the lungs). To further clarify this issue, we forced the development of lung tumors on *Rrm2*^{TG} mice by crossing them with a genetic model of lung carcinogenesis induced by the *K-Ras* oncogene (Guerra et al. 2003), where we also failed to see significant differences (Supplemental Fig. S5). Hence, the increased RNR activity of *Rrm2*^{TG} mice does not promote cancer, which is consistent with the fact that dNTPs are kept within normal levels in RRM2-overexpressing cells. In contrast, our work reveals that increased RRM2 levels can diminish the severity of the pathologies that lead to the premature aging and death of ATR mutant mice. Work in yeast has recently shown that increased RNR activity reduces genomic instability in several DNA repair

mutants besides *mec1Δ* (Poli et al. 2012). To what extent increased RNR activity can also alleviate the phenotypes of additional genomic instability-driven diseases in mammals demands further investigation. Interestingly, folates, the nucleotide precursors, are routinely used in medicine for the prevention or treatment of a wide range of diseases, including various age-related pathologies. Our work suggests that strategies directed to increase RNR activity might also have beneficial effects in mammalian disease. Collectively, our data validate *Rrm2^{TG}* mice as a valuable mammalian model of increased RNR activity and demonstrate that ATR-dependent regulation of nucleotide pools contributes to the phenotypes of Seckel mice.

Materials and methods

Mouse models

For the generation of *Rrm2^{TG}* mice, a 26-kb region from the mouse genome encompassing the *Rrm2* gene was cloned from a BAC (RP23-460K8) into a minimal vector by recombineering (Gene Bridges) and was subsequently used for the generation of transgenic mice by microinjection into fertilized oocytes. ATR-Seckel (Murga et al. 2009) mice have been described.

Nucleoside supplementation

Where indicated, a nucleoside solution commercialized as a supplement for improving the growth of embryonic stem cells was used at the suggested dose (EmbryoMax ES Cell Qualified Nucleosides, Millipore).

Cell viability assays

The assay for measuring cell viability by HTM using TO-PRO-3 and Hoechst 33342 has been described before (Eguren et al. 2014).

Extended methods are in the Supplemental Material.

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