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Review

Cellular regulation of ribonucleotide reductase in eukaryotes

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ABSTRACT

Synthesis of deoxynucleoside triphosphates (dNTPs) is essential for both DNA replication and repair and a key step in this process is catalyzed by ribonucleotide reductases (RNRs), which reduce ribonucleotides (rNDPs) to their deoxy forms. Tight regulation of RNR is crucial for maintaining the correct levels of all four dNTPs, which is important for minimizing the mutation rate and avoiding genome instability. Although allosteric control of RNR was the first discovered mechanism involved in regulation of the enzyme, other controls have emerged in recent years. These include regulation of expression of RNR genes, proteolysis of RNR subunits, control of the cellular localization of the small RNR subunit, and regulation of RNR activity by small protein inhibitors. This review will focus on these additional mechanisms of control responsible for providing a balanced supply of dNTPs.

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1. Introduction

Ribonucleotide reductases (RNR) are key enzymes in all organisms essential for the *de novo* synthesis pathway of deoxyribonucleoside triphosphates (dNTPs), required for DNA replication and repair. They are of particular interest as their activity largely determines the concentrations and ratios of dNTPs and these factors are critical in ensuring high-fidelity DNA synthesis [1–6]. High concentrations of dNTPs reduce the efficiency of polymerase proofreading, but may also serve to facilitate repair by promoting the ability of polymerases to copy damaged template. Imbalances in dNTP levels reduce the fidelity of the initial polymerization step and even subtle defects can be highly mutagenic [7]. Inhibition of RNR slows DNA replication and activates the intra-S phase checkpoint, which helps to preserve limiting dNTPs [8,9]. If the S phase checkpoint is inactive, DNA synthesis is not restrained by limiting dNTPs and ongoing replication leads to DNA damage and cell death [10]. Failure to upregulate dNTP levels during cell proliferation has been shown to promote oncogene-induced transformation, emphasizing the importance of RNR regulation for genome stability [11]. The key role that RNR has in cell proliferation is exploited in chemotherapy of several types of cancer, using inhibitors such as hydroxyurea, clofarabine and gemcitabine [12,13].

The cellular pool of dNTPs is sufficient for replication of just a fraction of the genome, so upregulation of RNR activity is necessary

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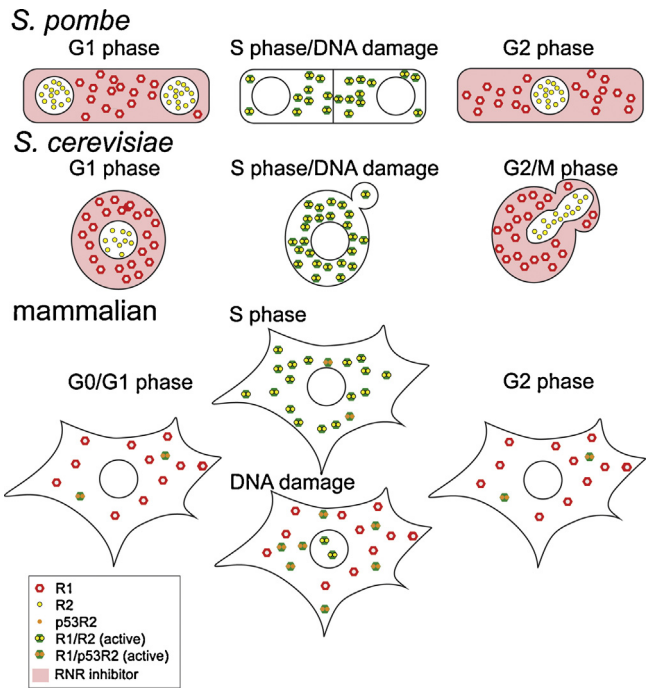

Fig. 1. Overview of cell cycle and DNA damage induced changes in RNR regulation in yeasts and mammalian cells. No attempt is made to depict accurately the cellular localization of small protein RNR inhibitors. Note that in *S. pombe*, S phase normally occurs before cytokinesis is complete, so G1 and S phase cells are shown binucleated.

as cells enter S phase. Allosteric mechanisms regulate the activity and specificity of RNR but a wider range of cellular mechanism impinge on the enzyme. These include altering the expression or proteolysis of RNR subunits, changing the cellular localization of individual subunits, or altering the levels of small protein inhibitors to control the enzyme activity (Fig. 1). In *Saccharomyces cerevisiae*, a combination of regulatory mechanisms serve to elevate the dNTP levels several fold in S phase or after DNA damage [2], although in *Schizosaccharomyces pombe* a much more modest increase is seen [14] (Table 1). Mammalian cells show an even more dramatic elevation of dNTP levels in S phase, although curiously little change after DNA damage ([15] reviewed in [16]). Allosteric regulation of RNR has been the subject of recent reviews [17,18] and will only be summarized here, and the purpose of this review is to consider recent developments in cellular regulatory mechanisms.

2. Outline of RNR structure and biochemistry

RNRs are classified into three main classes depending on the mechanism of free radical generation, which is essential for catalysis (reviewed in [19]). Class I RNRs are aerobic enzymes composed of two subunits, and are further subdivided depending on the metallocofactor used. Eukaryotes predominantly use Class 1a RNRs, which have a Fe(III) metallocofactor in the smaller subunit (R2, β) and a catalytic site in the larger subunit (R1, α). The R2 subunit generates and stabilizes a tyrosyl radical, which creates a reactive cysteine thyil radical in the active site necessary for initiating catalysis (reviewed in [18,20]). During a reaction cycle, a disulphide bond is generated in the R1 subunit which must be reduced by thioredoxin or glutaredoxin to reactivate the enzyme. This does not occur directly, but *via* an intermolecular reaction with the C-terminus of another R1 subunit, where a CX_2C motif functions as an intermediate in reducing the active site disulphide bond (reviewed in [21]). Thioredoxin and glutaredoxin can then reduce the C-terminal disulphide bond. In contrast to Class I enzymes, Class II RNRs (NrdJ) function independently of oxygen and have single subunit which requires 5′-deoxyadenoysylcobalamin for radical generation. Class III enzymes (NrdD) are only active under anaerobic conditions, and use a stable glycine radical for catalysis which is generated with the aid of a second protein NrdG.

RNRs show a particularly elaborate mechanism of allosteric regulation which serves to regulate levels and relative amounts of dNTPs (reviewed in [17]). This involves binding of dATP or ATP to an activity site in the R1 subunit, which respectively inhibits or stimulates the enzyme. A second 'specificity' allosteric site affects the types of nucleotides reduced; thus binding of ATP or dATP stimulates the reduction of pyrimidine nucleotides, while TTP and dGTP stimulate GDP and ADP reduction respectively. The exact stoichiometry of the enzyme has been somewhat unclear, but recent findings suggest a $R1_6R2_2$ ring complex for the inactive and possibly also the active form ([22] reviewed in [23]).

Table 1
Changes in of dNTP levels in S phase or after DNA damage compared to G1 or G0 levels.

	S phase	DNA damage	Reference
S. cerevisiae	x3-6	x6-8	[2]
S. pombe	x2	x2	[14]
Mammalian	x18	<x2	[15,20]

3. Regulation of RNR levels during the cell cycle and after DNA damage

One conserved theme with eukaryotic RNR genes is transcriptional activation during S phase and after DNA damage (reviewed in [18]). In yeasts, two transcriptional pathways are involved, one of which is responsible for cell cycle dependent changes in transcription and another which is activated by DNA damage. *S. cerevisiae* has two R1 genes (*RNR1*, *RNR3*) and two R2 genes (*RNR2*, *RNR4*). *RNR1*/R1 is differentially regulated by the MBF transcription factor, which regulates many other G1/S genes, while *RNR2*/R2, *RNR3*/R1 and *RNR4*/R2 show little variation in expression during the cell cycle [24–29]. In response to DNA damage, the Dun1 kinase is activated by the Mec1-Rad53 pathway and phosphorylates Crt1, thus relieving repression of *RNR2-4* genes. Activation of *RNR1*/R1 upon DNA damage involves the HMG-transcription factor Ixr1, which binds to the *RNR1* promoter and also promotes transcription under basal conditions [30]. The TOR pathway has also been implicated in RNR activation after DNA damage, as inhibition of TORC1 with rapamycin interferes with activation of *RNR1*/R1 and *RNR3*/R1 expression after DNA damage, leading to increased sensitivity to DNA damaging agents [31]. The DNA-binding factor Rap1, which has multiple roles at telomeres, promoters and silencers and, is also required for the activation of *RNR2-4* genes after DNA damage, thus changes in the level of Rap1 could potentially modulate RNR responses [32]. A recent study examined RNR mRNA and protein induction after DNA damage in single cells, and showed that elevation of both mRNA and protein was cell cycle dependent, being striking in S/G2 cells but little affected in G1 cells [33].

In *S. pombe*, the story is similar in that the *cdc22*$^+$/R1 gene is cell cycle regulated by the MBF regulator of G1/S transcription [34]. The *suc22*$^+$/R2 gene generates a smaller, constitutively expressed transcript, and a larger one which is MBF-regulated and induced by DNA damage and heat-shock [35]. The Ino80 nucleosome-remodelling complex appears to be necessary for correct *cdc22*$^+$/R1 expression under basal conditions [36]. After DNA damage, the checkpoint kinase Cds1(Chk2) phosphorylates the Yox1 inhibitor of MBF, allowing reactivation of MBF and transcription of targets such as *cdc22*$^+$/*R1* [37]. For Suc22/R2, regulation of expression may also occur post-transcriptionally since a cytoplasmic poly(A)

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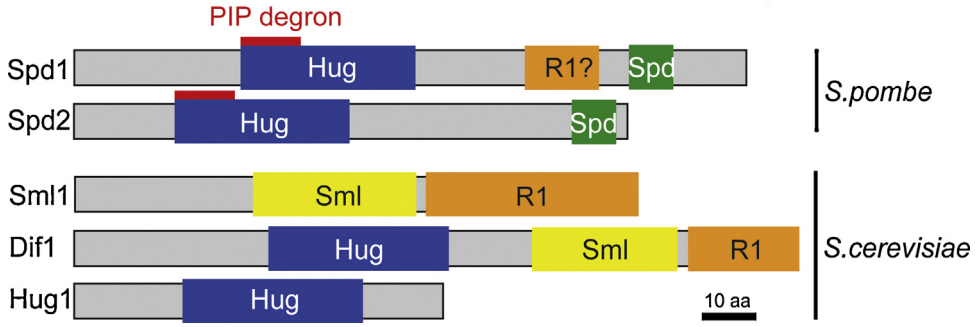

Fig. 2. Comparison of small protein inhibitors of yeast RNRs, showing the location of Hug, Sml, R1 binding, Spd and PIP (PCNA-interacting protein) degron motifs. Figure is adapted from [57,70].

polymerase targets and stabilizes *suc22* mRNA thus enhancing R2 expression [38].

In mammalian cells, expression of R1 and R2 is also cell cycle regulated [39,40], and R2 is also transcriptionally activated after DNA damage in a pathway involving ATR/ATM, Chk1 and E2F1 [41]. In an unperturbed cell cycle, R1 levels are constant, while R2 varies dramatically due to proteolysis from G2 through to and G1, and R2 is not present in quiescent cells. In G2, proteolysis of R2 is initiated by its phosphorylation by CDK, which promotes interaction and ubiquitylation by $SCF^{cyclin\ F}$ ubiquitin ligase [42]. Blocking this degradation leads to an imbalance in dNTP levels and consequent genetic instability. After DNA damage, an ATR-dependent process leads to proteolysis of cyclin F, and this is necessary for the nuclear accumulation of R2 and efficient DNA repair. In G1, proteolysis of R2 is maintained by the APC/Cdh1 ubiquitin ligase and thus R2 levels remain low until APC/Cdh1 is inactivated near the start of S phase [43].

In addition to effects on R2, DNA damage increases, *via* p53 transcriptional activation, the expression of a distinct small RNR subunit p53R2, capable of forming a functional RNR complex with R1 [44–47]. p53R2 is very similar to R2 but lacks an amino-terminal region required for APC-Cdh1 mediated proteolysis. p53R2 is a stable protein, normally expressed at low levels in both proliferating and non proliferating cells and it plays an important role in providing dNTPs for mtDNA synthesis (see below). p53R2 is translocated to the nucleus [45] and quiescent cells with nonfunctional p53R2 are defective in DNA repair [48], implying that the dNTPs supplied *via* R1/p53R2 are important to allow DNA synthesis associated with repair.

4. Regulation of RNR by small protein inhibitors

Yeasts employ a distinct mode of RNR regulation involving small intrinsically disordered proteins that either bind to RNR and inhibit the enzyme or alter the cellular localization of RNR subunits. *S. cerevisiae* possesses three related genes, *SML1*, *DIF1* and *HUG1*, all of which have been implicated in different aspects of RNR regulation (Fig. 2). *S. pombe* has two genes *spd1*$^+$ and *spd2*$^+$ which show limited sequence similarity to the *S. cerevisiae* group.

S. cerevisiae Sml1 inhibits RNR by binding to the R1 subunit in a 1:1 stoichiometry [49–51]. It has been proposed that Sml1 prevents the CX_2C motif in the C-terminus from accessing the active site, thus preventing cysteine reduction needed for enzyme reactivation [21]. During S phase or after DNA damage, activation of the Mec1-Rad53-Dun1 checkpoint kinase pathway leads to Sml1 phosphorylation by Dun1 and its subsequent proteolysis following ubiquitylation by Rad6-Ubr2-Mub1 [52,53]. Failure to degrade Sml1 is the reason why Rad53 inactivation is lethal in *S. cerevisiae* [51]. Sml1 forms a dimer, but the dissociation constant for this is relatively high and the biological significance of dimer formation is unclear [54,55].

Dif1 regulates RNR by promoting the nuclear localization of R2. R1 is constitutively cytoplasmic, and localization of R2 to the nucleus for most of the cell cycle downregulates RNR activity [56]. Dif1 binds to and promotes the nuclear import of R2 [57,58] and following import the subunit is anchored there by interaction with Wtm1 and Kap122 [59,60]. In response to DNA damage or DNA replication, Dif1 is down regulated and, similar to Sml1, is phosphorylated after DNA damage by the Mec1-Rad53-Dun1 cascade, leading to its proteolysis. In addition, genotoxic stress weakens the interaction between Wtm1 and R2, and together this leads to the relocalization of R2 to the cytoplasm and RNR activation.

Relatively little is known about *S. cerevisiae* Hug1. Since deletion of the *HUG1* gene suppresses the lethality of Mec1 inactivation, the protein presumably inhibits RNR, and a recent study suggested that its co-compartmentalization with cytoplasmic Rnr2/Rnr4 may reflect RNR inhibition *via* R2 interaction [61].

In fission yeast, Spd1 seems to combine properties of *S. cerevisiae* Sml1 and Dif1, affecting both the activity and localization of RNR, and as such may be more representative of an ancestral protein. As in *S. cerevisiae*, the active form of RNR appears to be cytoplasmic in fission yeast. The R1 subunit is pancellular, while the R2 subunit is nuclear for much of the cell cycle, but is relocalized to the cytoplasm in S phase and after DNA damage to activate dNTP production. Spd1 plays a role in this localization, since deletion of the *spd1* gene results in constitutive cytoplasmic localization of R2 [62]. However, in addition to *in vivo* effects on the R2 subunit, *in vitro* analysis showed an interaction between Spd1 and R1, but not R2, and inhibition of RNR activity depends on interaction with the R1 subunit [14]. A detailed mutagenesis study of Spd1 identified separation-of-function mutations which affected either nuclear localization of R2 or RNR activity [63]. Interestingly, a mutant defective for R2 nuclear import is fully able to inhibit RNR activity, and conversely, a mutant competent for R2 nuclear localization cannot restrain RNR activity, implying that Spd1 mainly inhibits RNR by directly binding to the enzyme, rather than through effects on R2 localization. The same study used *in vivo* fluorescence techniques to analyze the interaction between RNR subunits and Spd1 and concluded that Spd1 can interact with both subunits, and that Spd1 can promote an R1-R2 interaction in a manner that does not correlate with RNR activity.

Spd1 is degraded in S phase and after DNA damage *via* ubiquitylation by the $Cul4^{Cdt2}$ ubiquitin ligase [64]. The Cdt2 subunit of the ubiquitin ligase is itself cell cycle regulated, and is expressed *via* MBF-mediated transcription [65], but Cdt2 is not sufficient for Spd1 degradation and the additional requirement is Spd1's interaction with DNA-associated PCNA. In this context, Spd1 is ubiquitylated by $Cul4^{Cdt2}$ [66,67]. For reasons that are not clear, free PCNA is unable to promote Spd1 ubiquitylation by $Cul4^{Cdt2}$, and since PCNA is assembled onto DNA specifically during S phase and DNA repair, this provides a switch to synchronize RNR activation with DNA synthesis. This regulatory mechanism is consistent with

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the predominantly nuclear localization of Spd1, but it is less clear how Spd1 regulates the holoenzyme, which is supposedly cytoplasmic. Possibly, Spd1 shuttles between nucleus and cytoplasm and maintains a higher concentration in the nucleus. Incidentally, since Spd1 interacts with both PCNA and RNR, it could localize RNR at sites of DNA synthesis, although direct evidence for this is lacking.

Defects in Spd1 proteolysis result in depressed dNTP pools, an increased mutation rate [4], defective DNA repair by homologous recombination [6], and activation of DNA damage checkpoints [62,68]. Pre-meiotic DNA replication appears to be especially sensitive to defects in Spd1 proteolysis [4]. Previously, these effects were interpreted as directly due to dNTP deficiency, but a recent study showed that an RNR mutant resistant to allosteric feedback inhibition does not suppress Spd1 stabilization defects, even though the dNTP concentration is higher than in wild-type cells [69]. Thus excess Spd1 might lead to replication stress in other ways, for instance by interacting with PCNA and interfering with the binding of other replication or repair factors.

A second Spd1-related protein, Spd2, has been recently described in *S. pombe* [70]. Different phenotypes result from Spd2 inactivation compared to Spd1, in that there is no effect on R2/Suc22 nuclear localization and dNTP levels are not affected. However, Spd2 modulates RNR subunit interactions, is targeted for ubiquitylation by $CRL4^{Cdt2}$ and can delay S phase when in excess, suggesting that it shares some properties with Spd1.

Proteins related to the Sml1 family are found in other fungi, but have not been identified in other eukaryotic groups. However, these proteins are very poorly conserved at the level of primary sequence making their detection by bioinformatics difficult, so it remains to be seen whether this mode of regulation is found in higher eukaryotes.

5. RNR and organelle DNA synthesis

RNR is crucial for maintenance of organelle DNA. Although salvage pathways within the mitochondria provide one source of precursors for mtDNA replication [71], it is clear that RNR is also important. This is dramatically highlighted by the discovery that some human mtDNA depletion syndromes are caused by mutations in p53R2, a phenotype reproduced in mice deficient in p53R2 function ([72], reviewed in [73]). Thus the low level of RNR activity provided by constitutively expressed p53R2/R1 in quiescent cells, which lack normal R2, provides an essential supply of reduced nucleotides for mtDNA synthesis. It is most likely that reduced nucleotides generated by cytosolic RNR are imported into the matrix of mitochondrial as dNTPs, but import of dTMP and possibly other types of reduced nucleotides may occur (reviewed in [74]). The R1 subunit has been reported to be associated with mammalian mitochondria [75], raising the possibility that nucleotides may also be reduced by endogenous RNR.

Mutations affecting plant RNR cause defects in chloroplast maintenance and lead to leaf variegation [76,77]. Partial inhibition of RNR leads to chlorotic leaves without growth retardation, suggesting that chloroplast replication is especially sensitive to defects in dNTP supply [78].

6. Cellular localization of RNR and provision of dNTPs to replication and repair sites

As discussed in Section 4, the active form *of S. cerevisiae* and *S. pombe* RNR is cytoplasmic. A study in mammalian cells also concluded that both subunits are constitutively cytoplasmic, both during the cell cycle and after DNA damage [79]. A simple model to explain provision of reduced nucleotides for DNA synthesis would involve free diffusion across nuclear pores. However the situation after DNA damage is more controversial, since problems in visualizing the R2 subunit in the nucleus may be linked to its rapid turnover there. Furthermore, RNR recruitment has been reported in G1 cells at DNA damage site in a process that requires interaction between R1 and the Tip60 histone acetyltransferase [80]. Impairing this accumulation by blocking the R1-Tip60 interaction reduces DNA repair and sensitizes cells to DNA damaging agents. Localization of RNR in mammalian cells might be especially important since dNTP levels drop outside of S phase and are not significantly increased by DNA damage. The concept of localizing RNR at sites where dNTPs are consumed to improve replication efficiency goes back many years, and the *Escherichia coli* replisome appears to have a hyperstructure which includes RNR [81–84].

A recent study showed that doubling dNTP levels in *S. cerevisiae* increases the rate of DNA synthesis in S phase, implying that under normal conditions the rate of DNA synthesis by replicative polymerases is limited by dNTP concentration [85]. Increased dNTP levels also led to a higher DNA synthesis rate on damaged templates and prevented activation of the DNA replication checkpoint, possibly by promoting lesion bypass. These observations imply that minor deviations in dNTP supply might have significant effects on genome stability. In this connection, it has been suggested that replication of simple repeats with low sequence complexity could transiently reduce the local concentration of specific dNTP precursors and slow replication [86]. Transient fork inhibition could promote the formation of secondary structures that could promote repeat instability.

7. Novel modes of RNR regulation

A recent report shows that iron deficiency also regulates R2 cellular localization in *S. cerevisiae* (reviewed in [29]). Iron is essential for RNR function as it provides the metallocofactor in the R2 subunit (see Section 2). Iron deprivation results in the movement of R2 from nucleus to cytosol to activate RNR, but this movement is independent of Mec1 or Rad53, suggesting the existence of a regulatory mechanism that does not work through the DNA damage checkpoint pathway [29,87]. Iron deficiency results in the expression of Cth1 and Cth2, which bind to the 3′ UTRs of many mRNAs, causing their destabilization [88]. One of these mRNAs is *WTM1* mRNA, thus downregulation of Wtm1 allows release of R2 from the nucleus (see Section 4). Cth1 and Cth2 also interact with Rnr2 and Rnr4-encoding transcripts in response to iron deficiency, and promoting their degradation may allow cells to cope with low iron levels [87].

RNR has also been shown to be responsible for a type of cytoplasmic incompatibility in *Neurospora crassa*, such that co-expression of two allelic forms of RNR results in lethality due to RNR inhibition. This property relies on a region near the C-terminus of the R1 subunits, which is different comparing incompatible alleles and is not conserved in other eukaryotes [89]. Co-expression of incompatible RNR alleles results in the formation of high molecular weight RNR complexes, apparently mediated by disulphide bond formation [90]. It is suggested that with incompatible forms of RNR, an intermolecular disulphide bond is formed between R1 subunits, involving an active site cysteine and one in the C-terminal region. Surprisingly, expression of a small C-terminal region of *N. crassa* R1 in *S. cerevisiae* triggers an incompatibility reaction, showing that it can function in *trans* [91], hinting at new ways in which RNR could be regulated.

Type 1a RNR is also found in *E. coli*, and studies of the inactive form of the holoenzyme, which is composed of $R1_4R2_4$ rings, have shown these rings can interlock to form protein catenes [92]. It remains to be seen whether this “knotted” RNR is relevant to regulation, but it has been suggested that sequestration of the inactive

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form of the enzyme as a catenated complex might modulate the conversion to an active enzyme form. Since eukaryotic RNR forms $R1_6$ rings it will be interesting to determine if these bacterial observations have a more general relevance.

8. Cellular consequences of deregulated RNR and elevated dNTP levels

It is clear that dNTP concentration is a critical factor in ensuring accurate DNA replication, but it is intriguing that cells downregulate RNR outside of S phase in order to depress dNTP levels. One suggestion is that this is a strategy to restrict the replication of viruses [93], and evidence for this comes from a study of mammalian SAMHD1, which encodes a deoxynucleoside triphosphohydrolase and downregulates dNTP pools by hydrolysis outside of S phase [94]. The ability of SAMHD1 to lower dNTP levels blocks HIV-1 replication, and inactivation of SAMHD1 alleviates restriction of viral replication [95–97]. Defects in SAMHD1 are found in Aicardi-Goutières syndrome [98], which is characterized by innate immunity defects and neurological degeneration, but the link between these phenotypes and dNTP levels is unclear.

High dNTP levels outside of S phase could have other deleterious consequences. Mitochondrial nucleotide pools may be distorted by the transient increase in dNTP concentration during S phase, and it is possible that the fidelity of mtDNA replication would be adversely affected by constitutively high dNTP levels. It has also been suggested that low dNTP levels contribute to the block to DNA replication outside of S phase [43], so as to limit replication occurring from an unscheduled initiation. High dNTP levels also disturb cell cycle progression. In *S. cerevisiae*, high dNTP levels resulting from an RNR mutation that deregulates the enzyme for feedback inhibition, inhibit cell cycle progression by delaying initiation of DNA replication [99]. Similarly, in mammalian cells, elevation of dNTPs levels by down-regulating SAMHD1 inhibits S phase entry by an unknown mechanism [94].

Finally, in the light of these recent findings, it is interesting to reconsider the significance of RNR localization at sites of damage in mammalian cells. This has been interpreted as a mechanism to provide an adequate concentration of nucleotides at repair sites to facilitate the activity of repair polymerases, but an alternative interpretation is that this is a compromise mechanism that allows repair while avoiding the deleterious consequences of a pancellular elevation in dNTP levels.

9. Conclusions and perspectives

It is clear that RNR is subject to a wide range of regulatory mechanisms which, together with other factors such as SAMHD1, serve to maintain dNTP concentrations at optimal levels during S phase, while reducing levels in other phases of the cell cycle or in nonproliferating cells. Some control mechanisms are conserved between yeasts and mammalian cells, but others are specific and there appears to be considerable evolutionary plasticity in mechanisms affecting RNR activity. Mammalian cells go to extremes in reducing dNTP concentrations in nonproliferating cells to levels that are compatible with maintenance of mtDNA while preventing adverse consequences of constitutively high levels. This may in part be an adaptation reflecting the high proportion of nonproliferating cells in the adult body and the long lifespan of some nondividing cell types. Mammalian cells show localization of RNR at sites of DNA damage [80] and is intriguing why a nuclear sublocalization mechanism has evolved for repair and not for bulk DNA replication in S phase. RNR localization could potentially occur in other circumstances, such as to support DNA synthesis associated with homologous recombination in meiosis, since inhibition of RNR has adverse effects on homologous recombination in yeast [6]. Yeast cells also have multiple overlapping mechanisms to regulate RNR, including the use of small inhibitory proteins. Defective downregulation of these protein regulators can have serious effects on cell viability or efficiency of DNA replication or repair, but inactivation of these proteins has relatively mild phenotypes. As yet it is unclear whether this mode of regulation has so far escaped detection in mammalian cells, or is a peculiarity of fungi that has been supplanted by different regulatory mechanism in other organisms.

The interaction between RNR activity, dNTP levels and cell cycle progression requires further clarification. High dNTP levels delay cell cycle progression in both yeasts and mammalian cells but it is unclear how these high concentrations of dNTPs are sensed and how this is transduced to delay S phase entry. Low levels of dNTPs activate checkpoint mechanisms but again, what is the sensor for this? A plausible mechanism could involve a mechanism that detects an abnormally slow rate of DNA synthesis, perhaps involving the leading strand polymerase ε in conjunction with checkpoint proteins associated with the fork.

RNR and more generally dNTP regulation is of considerable clinical interest, in part for the use of RNR inhibitors in chemotherapy. Studying novel models of RNR inhibition, such as by small protein inhibitors, may provide new avenues for inhibitor development, and exploiting RNR structural differences between organisms might expand the utility of RNR drugs in combatting parasitic, bacterial or viral infections. The realization that elevated or depressed dNTPs levels are responsible for certain genetic diseases also has more general implications. It will be particularly interesting to work out how the clinical phenotypes caused by SAMHD1 defects are linked to elevation of dNTP levels, and whether defects in RNR *per se* causing elevated dNTP levels are associated with any genetic diseases. Mutations reducing RNR activity have so far only been linked to mtDNA depletion syndromes, but could also affect nuclear DNA synthesis, promoting fork stalling or replication infidelity, which could lead to genomic instability [100]. Relevant here is the recent finding that riboNMP incorporation by replicative polymerases is common and mutagenic [101,102]; this would be predicted to be enhanced under conditions where RNR activity is not adequate. It will be interesting to determine whether aberrant RNR regulation, due to germline or somatically acquired mutations, has any relevance to the development of cancers in highly proliferative tissues.

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