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Ribonucleotide incorporation into DNA during DNA replication and its consequences

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Abstract

Ribonucleotides are the most abundant non-canonical nucleotides in the genome. Their vast presence and influence over genome biology is becoming increasingly appreciated. Here we review the recent progress made in understanding their genomic presence, incorporation characteristics and usefulness as biomarkers for polymerase enzymology. We also discuss ribonucleotide processing, the genetic consequences of unrepaired ribonucleotides in DNA and evidence supporting the significance of their transient presence in the nuclear genome.

Keywords

ribonucleotide incorporation; DNA replication; DNA repair; genome stability; genome-wide sequencing

Introduction

The eukaryotic DNA replication machinery is responsible for faithful duplication of genetic information stored in genomic DNA during S phase of each cell cycle. DNA Polymerases (Pols) α, δ and ε conduct the bulk of DNA synthesis by effectively selecting and incorporating correct nucleotides. However, nucleotide selectivity by these replicative polymerases (replicases) is imperfect. They insert roughly one nucleotide with an incorrect base for each hundred thousand correct nucleotides incorporated, resulting in a base-base mispair (Kunkel 2004; McCulloch and Kunkel 2008). These mispairs may be corrected by either the exonucleolytic proofreading activities of Pols δ and ε or by the DNA mismatch repair (MMR) pathway. In addition, imperfect nucleotide selectivity means that replicases also occasionally make errors by incorporating a nucleotide with an incorrect sugar moiety, with the most common type being a ribonucleotide (Nick McElhinny, Watts, et al. 2010; Clausen et al. 2013).

Ribonucleotides embedded in DNA were first identified in mammalian mitochondrial genomes (Grossman et al. 1973), but their biological significance was not appreciated until fairly recently, when their prevalence, mechanism of removal and immediate consequences

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were reported in yeast and mammals (Rydberg and Game 2002; Nick McElhinny, Kumar, et al. 2010; Nick McElhinny, Watts, et al. 2010; Reijns MA et al. 2012). Ribonucleotides are incorporated during genome replication at a remarkably high rate (Rydberg and Game 2002; Nick McElhinny, Watts, et al. 2010). Furthermore, mutations in genes encoding subunits of RNase H2, the main enzyme that initiates ribonucleotide removal, were found in Aicardi-Goutières Syndrome (AGS) patient families (Crow et al. 2006). Replicases incorporate incorrect sugars orders of magnitude more frequently than they do incorrect bases (Nick McElhinny, Watts, et al. 2010; Clausen et al. 2013). Many DNA polymerase active sites select against ribonucleotide incorporation via a steric clash between the 2'-OH on the incoming sugar moiety and a bulky steric gate residue (reviewed in (Joyce 1997; Brown and Suo 2011)). Several studies using archaeal, bacterial and eukaryotic model organisms have demonstrated the importance of such a steric gate residue for ribonucleotide discrimination during DNA replication and repair (DeLucia et al. 2003; McDonald et al. 2012; Vaisman et al. 2012; Donigan et al. 2014; Donigan et al. 2015; Nevin et al. 2015; Diaz-Talavera et al. 2019; Walsh et al. 2019; Zatopek et al. 2020). The majority of incorporated ribonucleotide monophosphates (rNMPs) are removed by the RNase H2-dependent ribonucleotide excision repair (RER) pathway (Rydberg and Game 2002; Nick McElhinny, Kumar, et al. 2010; Hiller et al. 2012; Reijns MA et al. 2012). High cellular concentrations of ribonucleotide triphosphates (rNTPs), relative to deoxyribonucleotide triphosphates (Nick McElhinny, Watts, et al. 2010; Wanrooij et al. 2017; Balachander et al. 2020), guarantee that all transactions involving genomic synthesis incorporate ribose into DNA with some frequency. Individual ribonucleotides dynamically disturb the local duplex, according to simulations (Fu et al. 2019), and can disrupt nucleosome binding (Dunn and Griffith 1980; Hovatter and Martinson 1987). Some critically important questions include where and when ribonucleotides are incorporated and what biological consequences ensue. In this review, we discuss recent developments in pursuit of answers to these questions, with an emphasis on ribonucleotide incorporation in the nuclear genome during DNA replication.

Genome-wide mapping of ribonucleotides

Here we discuss the progress in using these technologies to answer the "where" and "when" questions regarding ribonucleotide incorporation in the nuclear and mitochondrial genome. We also discuss the progress in using the NMP mapping to study DNA polymerase enzymology during DNA replication.

The realization that ribonucleotides are frequently incorporated into the genome during replication sparked interest in developing genome-mapping technologies to define their genomic locations. Major progress was made in 2015 when four groups independently reported sequencing-based ribonucleotide maps of various eukaryotic nuclear and mitochondrial genomes (Clausen et al. 2015; Daigaku et al. 2015; Koh et al. 2015; Reijns MAM et al. 2015).

Using replicase variants that are promiscuous for ribonucleotide incorporation, some of these studies were able to use rNMPs as footprints of replicase usage during normal DNA replication (Clausen et al. 2013; Clausen et al. 2015; Daigaku et al. 2015; Reijns MAM et al. 2015). These studies presented powerful *in vivo* evidence for the division of labor between

Pols ε and δ. They were assigned the majority of leading and lagging strand synthesis, respectively, in confirmation of more limited mutational studies in yeast (Pursell et al. 2007; Nick McElhinny et al. 2008; Larrea et al. 2010; Miyabe et al. 2011). This demonstrated the power of rNMP mapping to study polymerase enzymology during DNA synthesis.

Ribonucleotide incorporation into the nuclear genome

These studies simultaneously describing independent methods for genome-wide rNMP mapping were all based on high-throughput sequencing platforms. Three groups using Illumina sequencing (Clausen et al. 2015; Daigaku et al. 2015; Koh et al. 2015) and one group used Ion Torrent sequencing (Reijns MAM et al. 2015). The unifying principle was incision at genomic rNMPs, either by rNMP-recognizing RNase H2 or by alkaline hydrolysis. Nicked DNA ends were then tagged with strand-specific DNA adaptors (Figure 1).

All four studies confirmed widespread rNMP incorporation with rNMPs detected universally from early to late replicating regions. Two studies in the budding yeast *Saccharomyces cerevisiae* (Clausen et al. 2015; Reijns MAM et al. 2015) and one in the fission yeast *Schizosaccharomyces pombe* (Daigaku et al. 2015) also used their respective rNMP mapping technologies to show the division of labor among replicases.

Pol α-primase synthesizes an RNA-DNA primer required for initiation of DNA synthesis. The short DNA portion synthesized by Pol α was thought to be largely removed by Pol δ-dependent strand displacement during Okazaki fragment maturation (Maga et al. 2001). However, the Pol α variants incorporates significant rNMPs, suggesting that at least some Pol α-synthesized DNA is retained after Okazaki fragment maturation (Clausen et al. 2013; Clausen et al. 2015; Reijns MAM et al. 2015). Rejins et al. estimated that Pol α synthesizes at ~1.5% of the yeast genome. Because Pol α lacks exonucleolytic proofreading activity and is the least accurate eukaryotic replicase (McCulloch and Kunkel 2008), these results indicate that Pol α contributes significantly to the spontaneous mutagenesis across the genome, especially during the lagging strand synthesis (Lujan et al. 2014). Rejins, et al. proposed that retention of DNA synthesized by Pol α may be related to inhibition of Pol δ strand displacement by nucleosome occupancy or other DNA binding proteins such as transcription factors (Reijns MAM et al. 2015). Nucleosomes appear to load before Okazaki fragment maturation (Smith and Whitehouse 2012) and to inhibit other transactions, such as MMR (Lujan et al. 2014). Thus, these protein-binding sites tend to have higher spontaneous mutation rates in both yeast and human cells (Reijns MAM et al. 2015).

These studies also revealed that rNMP incorporation by the replicases is not uniform. Ribose-seq showed that rC and rG are preferred substrates for incorporation over rA and rU in RNase H2-deficient *S. cerevisiae* cells (Koh et al. 2015). HydEn-seq revealed distinctive rNMP preferences for the Pol α, δ and ε variants (Clausen et al. 2013; Clausen et al. 2015). The Pol ε and δ variants favor rC and rG, the Pol α variant prefers rA, and all three disfavor rU. The Storici group recently expanded these initial observations by conducting an expansive analysis of rNMP identity and sequence context in both budding and fission yeast strains using an improved version of Ribose-seq (Balachander et al. 2020). Confirming the imbalanced rNMP incorporation observed previously, they show that rC incorporation is

highly preferred followed by rG in RNase H2-deficient *S. cerevisiae* and *S. paradoxus*. rU is the least abundant ribonucleotide in the genome. This preference only partially reflects the imbalanced cellular rNTP and dNTP pools, with the most notable discrepancy being rATP relative to dATP. In *S. cerevisiae*, rATP is the most abundant ribonucleotide and the rATP/dATP ratio is the highest (Nick McElhinny, Watts, et al. 2010; Balachander et al. 2020). The low rate of rA and rU incorporation is likely due to the high discrimination against rATP by the replicases (Nick McElhinny, Watts, et al. 2010). In contrast, rU is the least frequently incorporated ribonucleotide in *S. pombe*, with rA being the most abundant, perhaps due to the extreme rATP/dATP ratio (Balachander et al. 2020). The Storici group further showed that rNMP incorporation is influenced by the immediate upstream DNA sequence, suggesting certain sequences help to stabilize the incoming ribonucleotide (Balachander et al. 2020). Because a ribonucleotide at the 3'-end of a DNA primer is an impediment to DNA polymerase extension (Watt et al. 2011; Goksenin et al. 2012), it is possible some base-pairing combinations are favored for extension. It is worth noting that different yeast strain backgrounds also impact the ribonucleotide identity and distribution in the genomes (Balachander et al. 2020). The Storici group has developed a computational toolkit named Ribose-Map that streamlined upstream read alignment and certain downstream analyses including ribonucleotide identity, genomic distribution and sequence contexts (Gombolay et al. 2019). This toolkit can be adapted for different rNMP mapping technologies.

rNMP incorporation in the mitochondrial genome

The first genome-wide ribonucleotide mapping studies also confirmed that rNMPs are stably incorporated into mitochondrial genomes (Clausen et al. 2015; Koh et al. 2015). Similar to the nuclear genome, rNMP incorporation in the mitochondrial genome is nonuniform (Clausen et al. 2015; Koh et al. 2015; Berglund et al. 2017; Wanrooij et al. 2017; Balachander et al. 2020). These studies in budding yeast disagree upon which rNMP is the most frequently incorporated (rC or rG) but agree that rU is strongly selected against. This is similar to human mitochondria and seems to be driven by imbalances in nucleotide pools (Berglund et al. 2017). The ribonucleotide spectrum in mitochondrial DNA from solid human tissues is mostly explainable by the nucleotide pools and ribonucleotide discrimination by Pol γ (Moss et al. 2017). The mitochondrial genome rNMP distribution is also nonuniform with regard to location and DNA sequence context (Clausen et al. 2015; Koh et al. 2015; Berglund et al. 2017; Balachander et al. 2020). DNA replication by Pol γ and remnants of unremoved RNA primers could both contribute to rNMP incorporation (Cerritelli Susana M. et al. 2003; Holmes et al. 2015). rNMP peaks corresponding to sites of replication initiation were observed in both yeast and human mitochondrial genomes (Clausen et al. 2015; Berglund et al. 2017). Since RNase H1-dependent removal of an RNA primer leaves two consecutive rNMPs behind (Cerritelli Susana M. et al. 2003), it is possible some of the observed peaks are from residual RNA primers. So far, mitochondria seem to lack the ability to remove single embedded ribonucleotides (Wanrooij et al. 2017). It remains unclear what consequences embedded ribonucleotides may have on mitochondrial biology.

Using rNMPs as a biomarker to map polymerase enzymology

During DNA replication, rNMPs are incorporated at a remarkably high rate. Over a million rNMPs are incorporated into the mouse genome each cell cycle (Reijns MA et al. 2012;

Reijns MAM et al. 2015)). The Pol α, δ and ε active site mutants (e.g. *pol1-Y869A*, *pol3-L612G and pol2-M644G* in budding yeast) incorporate rNMPs even more frequently than their wild-type counterparts, but can still support relatively normal DNA replication (Nick McElhinny, Kumar, et al. 2010; Clausen et al. 2015). In RNase H2-deficient yeast cells, most of the embedded rNMPs are retained, leaving footprints by which the DNA synthesized by each replicase may be tracked. Replication profiles deduced from rNMP mapping in both budding and fission yeasts provided powerful support for the currently accepted model that Pol ε is the main leading strand replicase, and that Pol δ conducts the majority of lagging strand synthesis (Clausen et al. 2015; Daigaku et al. 2015; Reijns MAM et al. 2015). The profiles also yield tremendous information regarding sites of replication initiation, termination and replication timing. However, localized excursions from the canonical division of labor are occasionally observed (Clausen 2015; Daigaku et al. 2015).

Reductions in background noise in subsequent allowed opened windows on local variations in the division of labor among the replicases (Garbacz et al. 2018; Zhou et al. 2019). Restriction digestion of genomic DNA prior to HydEn-seq sample library preparation provided consistent DNA ends to act as internal standards (Garbacz et al. 2018). This improved normalization allowed better background subtraction and quantitation. Meta-analysis of *S. cerevisiae* replication origins, which further reduces the effect of background noise, showed that Pol δ synthesizes both DNA strands at replication origins, suggesting that both leading and lagging strand synthesis are initiated by the first Okazaki fragment (Figure 2A) (Garbacz et al. 2018). Evidence for this model was later supported by an *in vitro* reconstituted system of replication (Aria and Yeeles 2018). Continued modification of the HydEn-seq protocol and the analysis pipeline, including a switch from alkaline hydrolysis to RNase H2 cleavage and measures that reduces background noise, further increased specificity and decreased noise (Zhou et al. 2019). The RHII-HydEn-seq method allowed discernment of polymerase usage down in individual regions as small as 50 bp. Thus, it was shown that nearly all active *S. cerevisiae* origins show an increase in the Pol δ footprint proportional to their efficiency. This provides strong support for nearly all replication initiation events in *S. cerevisiae* involving the mechanism where the first Okazaki fragment primes both leading and lagging strand synthesis. Similar patterns were found in *S. pombe*, which has more diffusive origin activities across the genome, similar to metazoans, including in human cells (Vashee et al. 2003; Dai et al. 2005; Xu et al. 2012; Tubbs et al. 2018). The RHII-HydEn-seq study also provided credible evidence suggesting that Pol δ synthesizes both strand during replication termination (Figure 2A) (Zhou et al. 2019).

Some specialized DNA polymerases such the translesion synthesis (TLS) polymerases assist canonical DNA replication when faced with a damaged template or difficult-to-replicate region (Yang and Gao 2018). The potential role and bias of TLS polymerases in leading versus lagging strand synthesis can also be examined by rNMP mapping. HydEn-seq with an rNMP-permissive Pol η variant, showed that Pol η is primarily engaged in PCNA-dependent lagging strand synthesis (Kreisel et al. 2019). Given that Pol η can help bypass lesions on both strands *in vitro* (Guilliam and Yeeles 2020), the HydEn-seq *in vivo* data suggests that our understanding of Pol η is incomplete. Lagging strand lesions may be more accessible to Pol η, or Pol η has additional roles in lagging strand synthesis.

Most DNA repair processes require DNA synthesis as one of the last steps to fully "heal" the DNA. The roles of the major polymerases in many repair pathways are still unclear. Theoretically, rNMP mapping may help answer these questions, at least for DNA repair processes that require substantial DNA synthesis. One such process is the recombination-dependent break-induced replication (BIR), a specialized DNA double-strand break (DSB) repair pathway. BIR occurs when, following 5'-to-3' end resection, the 3' protruding end of a DSB invades a homologous template and primes extensive DNA synthesis (Sakofsky and Malkova 2017). Such events are studied in yeast using experimental systems wherein the centromere-proximal end of an induced DSB is homologous to one locus on another, donor chromosome. This compels DNA synthesis to proceed to the end of the donor chromosome (Donnianni and Symington 2013). HydEn-seq of purified repair products showed that Pol δ is unequivocally the main polymerase conducting DNA synthesis during BIR, with only minimal Pol α contribution (Figure 2C) (Donnianni et al. 2019). Likewise, in a recent BioRxiv manuscript, the Pu-seq approach was used to show that Pol δ is predominantly used to synthesizing both strands following homologous recombination-dependent replication restart (Figure 2C) (Naiman et al. 2020, under review, available on bioRxiv). These studies demonstrate that rNMP mapping can be used to track polymerase usage during DNA repair. It remains to be tested whether rNMP mapping has the resolution to discern polymerase usage during the small patch repair synthesis that occurs during processes such as nucleotide excision repair or base excision repair.

The biological impacts of ribonucleotides incorporated into genomic DNA during eukaryotic replication—The initial demonstration that RNase H2 can incise a single ribonucleotide in DNA (Rydberg and Game 2002) launched numerous studies of Ribonucleotide Excision Repair (RER) and the biological impacts of unrepaired genomic ribonucleotides. These include both negative and positive outcomes, and additional pathways of genomic ribonucleotide removal. The latter include exonucleolytic proofreading by the 3'−5' exonuclease activities of Pols δ and ε, albeit inefficiently, and, in the absence of RER, incision by topoisomerase 1 (Top1) (Sekiguchi and Shuman 1997; Kim et al. 2011; Williams J. S. et al. 2013).

Negative biological consequences

Several genome instability phenotypes can result from the failure to properly remove genomic ribonucleotides. These include direct impacts on the stability of DNA, as well as the release of problematic intermediates that can be formed during ribonucleotide-processing and removal. These effects have been the subject of several recent reviews (Cerritelli S. M. and Crouch 2016; Williams J. S. et al. 2016; Klein 2017; Kellner and Luke 2020).

Structural effects on DNA.—The presence of a single ribonucleotide in DNA has multiple effects on its structure. These effects have been studied in detail using a variety of approaches, including molecular dynamics simulation (Fu et al. 2019), atomic force microscopy (AFM) (Chiu et al. 2014), nuclear magnetic resonance (NMR) (DeRose et al. 2012) and X-ray crystallography (Egli et al. 1993). These investigations revealed that DNA electrostatic potential, elasticity, deoxyribose pucker, minor groove width, Watson-Crick

pairing, and duplex unwinding are all impacted by the presence of a single ribonucleotide. These structural perturbations change the character of DNA and have the potential to impact DNA transactions that include replication, transcription and DNA repair. One example of this is during bypass of template ribonucleotides by DNA polymerases, a process that becomes increasingly difficult as the number of consecutive ribonucleotides increases from one to four (Watt et al. 2011; Goksenin et al. 2012; Clausen et al. 2013). Finally, at physiological pH, RNA is 100,000 times more susceptible to spontaneous hydrolysis when compared to DNA (Li Y and Breaker 1999) and therefore the presence of ribonucleotides in DNA increases the rate of formation of single-strand DNA breaks (Figure 3A).

Ribonucleotide-dependent mutagenesis.—Much of what is currently known regarding mutations that arise during processing of ribonucleotides in DNA comes from studies performed in budding yeast. Qiu et al. first demonstrated a moderate increase in spontaneous mutation rate in a strain deleted for the gene encoding the catalytic subunit of RNase H2 (*RNH201*) (Qiu et al. 1999). Later frameshift reversion assays found that these mutations were primarily 4 bp deletions (Chen et al. 2000). Reversion and forward mutation reporter assays show that the primary mutations associated with loss of RNase H2 activity in yeast are short deletions (2–5 bp) in perfect or imperfect repeat sequences (Nick McElhinny, Kumar, et al. 2010; Clark et al. 2011; Kim et al. 2011), and the rate of these mutations increases in a yeast strain expressing a ribonucleotide permissive variant of Pol ε (*pol2-M644G*) (Nick McElhinny, Kumar, et al. 2010). These mutations are generated when Top1 cleaves at an unrepaired ribonucleotide (Sekiguchi and Shuman 1997; Kim et al. 2011). Extensive genetic and biochemical analyses demonstrate that these deletions likely result from two cleavage events by Top1, followed by strand realignment and Top1-dependent ligation across the gap (Figure 3B) (Cho et al. 2015; Huang SY et al. 2015; Sparks and Burgers 2015). In the absence of RNase H2 activity, the 2–5 bp deletion rate also depends on polymerase status. It is moderately elevated in strains with wild type polymerases or lagging strand mutator variants (*pol3-L612M* and *pol1-L868M*), but greatly elevated with a leading strand mutator polymerase (*pol2-M644G*) (Williams J. S. et al. 2013; Cho et al. 2015; Williams J. S. et al. 2015). This suggests that unrepaired leading strand ribonucleotides are frequent targets for Top1, possibly due to a requirement for relief of topological stress via Top1 cleavage. This Top1-induced 2–5 bp deletion mutational specificity occurs across the yeast genome (Williams Jessica S. et al. 2019).

In the absence of RNase H2 activity, Top1 cleavage at an unrepaired ribonucleotide generates aberrant 5’-OH and 2’,3’-cyclic phosphate DNA ends (Figure 3A) (Sekiguchi and Shuman 1997; Kim et al. 2011) that can be nucleolytically processed. The Srs2 helicase can unwind the 5’ end to create a flap for processing by the Exo1 nuclease (Potenski and Klein 2014). This generates a gap that can then be filled in by a DNA polymerase. The 2’,3’-cyclic phosphate and its hydrolyzed products can be removed by the Apn2 nuclease (Li F et al. 2019).

RNase H2 is also responsible for removing R-loops that arise when a nascent RNA transcript anneals with its complementary DNA template. This creates a bulky DNA structure that impedes both replication and transcription and can cause DSBs if not properly removed. RNase H2 can cleave at stretches of ribonucleotides and thereby provides a critical

means of R-loop removal. In order to distinguish between mutagenic pathways, a separation-of-function variant of the gene encoding the RNase H2 catalytic subunit (*RNH201* in *S. cerevisiae*) was engineered. This variant, Rnh201-RED (ribonucleotide excision defective), retains activity on stretches of ribonucleotides but is unable to incise at a single genomic ribonucleotide (Chon et al. 2009). The Rnh201-RED mutant has now been used in multiple model systems to demonstrate that both single unrepaired ribonucleotides and R-loops promote genome instability (reviewed in (Cerritelli S. M. and Crouch 2019)). However, it is Top1-cleavage at single ribonucleotides that initiates short deletion mutagenesis in yeast (Chon et al. 2013; Williams J. S. et al. 2017).

Replicative stress and checkpoint activation.—As discussed above, replicative stress arises during DNA polymerase bypass of ribonucleotides in template DNA (Watt et al. 2011; Goksenin et al. 2012; Clausen et al. 2013). Yeast lacking one of the RNase H2 subunits (Rnh201, Rnh202, and Rnh203 in *S. cerevisiae*) cannot repair genomic ribonucleotides, resulting in replication stress in a ribonucleotide permissive background (i.e. *pol2-M644G*). The *pol2-M644G rnh201*Δ strain has elevated dNTP pools (Nick McElhinny, Kumar, et al. 2010; Williams J. S. et al. 2013) and immunoblotting shows Rad53 phosphorylation (Lazzaro et al. 2012) and Rnr3 protein level increases (Williams J. S. et al. 2013), all indicators of S-phase checkpoint activation. Furthermore, the *pol2-M644G rnh201*Δ cells accumulate in S and G_2 phases of the cell cycle (Nick McElhinny, Kumar, et al. 2010), highlighting the fact that ribonucleotides left unrepaired can impede replication and cause cellular stress. In human cells, RNase H2 silencing also causes replication stress and the accumulation of cells in S and G_2, resulting in chronic activation of the post-replication repair pathway (Pizzi et al. 2015).

Chromosomal instability due to processing of single ribonucleotides in DNA.—The presence of unrepaired ribonucleotides in DNA can generate multiple types of instability, including recombination, chromosome rearrangements and chromosome loss. The first demonstration that loss of RNase H2 was associated with increased mitotic recombination in yeast came from a study of hyper-recombination (hyper-rec) mutants (Aguilera and Klein 1988). Since this initial demonstration, loss of RNase H2 activity has been shown to cause elevated gross chromosomal rearrangements (Allen-Soltero et al. 2014) and non-allelic homologous recombination (NAHR) (Conover et al. 2015). RNase H2 deficiency causes increased loss-of-heterozygosity in diploid yeast strains (Conover et al. 2015; O'Connell et al. 2015), suggesting that DSBs are formed when genomic ribonucleotides are not properly removed. DSB formation can be initiated by failure to repair R-loops or single unrepaired ribonucleotides incorporated during replication, both of which contribute to increased chromosomal instability in RNase H2-deficient strains (Cornelio et al. 2017). Unrepaired single ribonucleotides may trigger DSB formation in multiple ways. Spontaneous hydrolysis or Top1 cleavage would generate a DNA single strand break (Figure 3A) that could then be converted into a DSB. In addition, a DSB can be generated directly by a second Top1 cleavage event proximal to the initial Top1-incised ribonucleotide but on the opposite DNA strand (Figure 3C) (Huang SN et al. 2017). Consistent with Top1-dependent DSB formation in RNase H2-deficient strains, the homologous recombination (HR) repair proteins Rad51 and Rad52 are critical for cell

viability in RER-deficient yeast cells (Huang SN et al. 2017). Furthermore, recombination rates are reduced upon deletion of *TOP1* in RNase H2-deficient yeast strains (Potenski and Klein 2014; Conover et al. 2015).

Chromosomal instability associated with RER failure has also been observed in mouse and human cells. RNase H2-null mouse embryonic fibroblasts have increased γ-H2AX foci, substantial levels of micronuclei, and chromosomal rearrangements (Hiller et al. 2012; Reijns MA et al. 2012). Micronuclei and the DNA damage response are also observed in human cells depleted of RNase H2 (Pizzi et al. 2015).

Ribonucleotides in vertebrates: connections to human disease.—RNase H2 is essential for viability in mice (Hiller et al. 2012; Reijns MA et al. 2012). The RNase H2-RED variant also confers lethality, suggesting that removal of single ribonucleotides from genomic DNA is essential (Uehara et al. 2018). Early embryonic arrest in both variants was traced to activation of the p53-dependent DNA damage response (Reijns MA et al. 2012; Uehara et al. 2018).

There are now several lines of evidence demonstrating a connection between RNase H2 deficiency and human disease. The first was the discovery that mutations in any of the three subunits of RNase H2 can cause Aicardi-Goutieres Syndrome (AGS), a severe auto-inflammatory disorder (Crow et al. 2006). Potenski et al. (2019) modelled AGS-associated mutations in the yeast RNase H2 enzyme and found that the yeast AGS mutants have a variety of phenotypes, including a strongly reduced genome stability. Those mutant strains with the most instability had elevated levels of unrepaired ribonucleotides in their DNA (Potenski et al. 2019). Depletion of RNase H2 in human cells causes checkpoint activation and genome instability (Pizzi et al. 2015). Genomes in cells derived from AGS patients accumulate ribonucleotides and damage, suggesting that ribonucleotide removal by RNase H2 contributes to AGS etiology and is critically important for genome maintenance (Figure 3D) (Pizzi et al. 2015).

RNase H2 mutations have also been associated with Systemic Lupus Erythrematosis, another autoimmune disorder (Ramantani et al. 2010; Gunther et al. 2015). The precise link between loss of RNase H2 activity and autoimmune disorders has not been identified. However, mutations in other DNA-processing enzymes such as TREX1 and ADAR1 have also been linked to AGS (reviewed in (Crow 2015)), suggesting that it may involve improper nucleic acid processing. In addition, CRISPR screens of chemotherapy-treated human cells have identified and characterized and a molecular connection between unrepaired genomic ribonucleotides and cancer. All three RNase H2 genes were identified in a targeted screen for mutations that cause sensitivity to inhibitors of poly (ADP-ribose) polymerase (PARP) (Zimmermann et al. 2018). Top1 processing of unrepaired single ribonucleotides causes DNA lesions that trap PARP (Figure 3E). Thus, RNase H2 inactivation could harbor therapeutic potential for some cancers. In addition, a genome-wide CRISPR screen in cells treated with an inhibitor of the ATR checkpoint kinase showed that RNase H2-deficiency was synthetic lethal with ATR inhibition (Wang et al. 2019). Wang et al. also found reduced levels of RNASE H2 in prostate adenocarcinoma patient-derived samples. Together, these observations suggest that the use of ATR inhibition as a chemotherapeutic may be beneficial

in cancer patients with RNase H2-deficiency. Recently, ribonucleotide removal by RNase H2 was shown to be critical for prevention of intestinal tumorigenesis in mice and as a colorectal tumor suppressor in human tumor specimens (Aden et al. 2019). RER inactivation in the mouse epidermis promotes squamous cell carcinoma (Hiller et al. 2018), thereby further highlighting the connection between ribonucleotide removal, genome instability and cancer (Figure 3D).

Positive biological consequences

In addition to the negative effects that ribonucleotides incorporated into DNA have on genome stability, there are several lines of evidence in support of positive signaling roles for ribonucleotides in DNA that include promoting DNA repair and providing important cellular signals. For example, in the active site of Pol ε, a strictly conserved methionine buttresses the steric gate tyrosine that acts to exclude ribonucleotides from polymerization. This buttressing hydrophobic amino acid is always a leucine in Pols α and δ (Lujan et al. 2013). Substitution of a leucine for the methionine in Pol ε confers higher discrimination against ribonucleotide incorporation without increasing mismatch incorporation (Nick McElhinny, Watts, et al. 2010). This suggests that ribonucleotide incorporation into leading strand DNA by Pol ε serves one or more critical cellular functions.

Signaling for strand discrimination during DNA Mismatch Repair.

One potential positive signaling function provided by leading strand ribonucleotides is during mismatch repair (MMR) of errors introduced by Pol ε during replication (Ghodgaonkar et al. 2013; Lujan et al. 2013). Nicks generated when RNase H2 cleaves at a ribonucleotide may allow an entry point for the MMR machinery to repair base-base mismatches or insertion/deletion mutations (Figure 4A). In support of this, a single ribonucleotide can initiate MMR of an adjacent mismatch in human cell extracts in a RNase H2-dependent manner (Ghodgaonkar et al. 2013). RNase H2-deficient (*rnh201Δ*) yeast strain shows moderate increase of mutation rates (Nick McElhinny, Kumar, et al. 2010; Ghodgaonkar et al. 2013; Lujan et al. 2013). Moreover, there is an elevated rate of MMR-dependent single base pair deletions in the *pol2-M644G rnh201*Δ strain containing a high density of unrepaired genomic ribonucleotides (Lujan et al. 2013).

Although this review is focused on ribonucleotides incorporated by the replicases, they are also frequently incorporated by the DNA repair polymerases (reviewed in (Vaisman and Woodgate 2018)). For example, during bypass of DNA lesions, the specialized TLS polymerases can replicate damaged DNA with reduced base and sugar selectivity. Also, during DNA DSB repair by non-homologous end joining (NHEJ), both Pol μ and TdT efficiently incorporate ribonucleotides into DNA to promote efficient ligation by DNA ligase IV (Nick McElhinny and Ramsden 2003; Ruiz et al. 2003; Brown et al. 2010; Martin et al. 2013; Moon et al. 2017; Pryor et al. 2018). This suggests that ribonucleotides incorporated by Pol μ and TdT may be preferred over deoxyribonucleotides for NHEJ, especially when dNTP concentrations are low, e.g., in non-replicating cells.

Additional cellular signaling roles for ribonucleotides.—One example of a putative positive signaling role for unrepaired genomic ribonucleotides involves the epigenetic

imprint formed during mating type switching in *S. pombe*. During this process, an imprint is formed by two ribonucleotides left by incomplete Okazaki fragment processing at a defined location in the lagging strand (Figure 4C) (Vengrova and Dalgaard 2006). These adjacent ribonucleotides cause Pol ε to stall during the next round of replication, thereby triggering recombination between two genomic loci to facilitate a mating type switch. The possibility also exists that ribonucleotides in DNA may play a signaling role during embryonic development in higher eukaryotes.

Ribonucleotides in DNA may also signal for the relief of torsional stress in leading strand DNA (Cerritelli S. M. and Crouch 2016). The discontinuously synthesized lagging strand has free DNA ends that allow for rotation, relieving superhelical stress. In contrast, superhelical stress builds during continuous leading strand synthesis. Ribonucleotides are more abundant in the leading strand than they are in the lagging strand thanks to wild type Pol ε (Clausen et al. 2015; Daigaku et al. 2015). One reason for this may be to promote nicking by RNase H2 to relieve supercoiling (Figure 4E).

Ribonucleotide incorporation beyond animals and fungi.: Ribonucleotides are incorporated into DNA across all three kingdoms of cellular life. Like opisthokonts (animals, fungi and their close relatives), other eukaryotic lineages possess RNase H2 and synthesize their nuclear genomes with B-family replicases Pols α, δ and ε. For example, RNase H2 deficiency causes nuclear genome ribonucleotide accumulation and instability in the plant *Arabidopsis thaliana* (Kalhorzadeh et al. 2014). Unlike opisthokonts, most other eukaryotes do not use A-family Pol γ for mitochondrial or plastid genome synthesis. They instead they use A-family plant organellar polymerase (POP), which is more similar to bacterial DNA Polymerase I (Moriyama et al. 2011). Apicomplexans are an exception, in that their plastid genomes are replicated by a primase/helicase-fused A-family polymerase, the plastidic DNA replication/repair enzyme complex (PREX) (Seow et al. 2005). The first reported ribonucleotide mapping in a plastid-bearing eukaryote, the unicellular green alga *Chlamydomonas reinhardtii*, found disproportionately abundant ribonucleotides in the mitochondrial and chloroplast genomes (El-Sayed et al. 2020, under review, available on bioRxiv). Ribonucleotide ratios in these organelles implicate replication by POPs in the presence of highly imbalanced NTP pools.

Genomic ribonucleotide biology is less well defined in prokaryotes. In bacteria, different combinations of C-family polymerases are responsible for the bulk of genome synthesis (Timinskas et al. 2014), assisted by A-family Pol I and a host of repair and TLS polymerases. The C-family replicative polymerases (Yao et al. 2013) and Y-family TLS polymerases (McDonald et al. 2012; Ordonez et al. 2014) are known to incorporate ribonucleotides. In *Escherichia coli*, ribonucleotides are primarily removed via RER with help from Pol I (Vaisman et al. 2014), with NER as an efficient and apparently accurate backup mechanism (Vaisman et al. 2013). Pol I can incorporate ribonucleotides *in vitro* (Ide et al. 1993; Astatke et al. 1998). In *Bacillus subtilis*, loss of RER is mutagenic (Yao et al. 2013), with a spectrum that implicates error-prone re-synthesis by the C-family DnaE replicative polymerase after NER (Schroeder et al. 2017). In Archaea, DNA replication systems vary widely, using different combinations of D and B-family polymerases (Makarova et al. 2014). Examples from both groups can incorporate ribonucleotides at rates

similar to the eukaryotic replicases (Gardner et al. 2004; Schermerhorn and Gardner 2015), which can be removed by RER (Heider et al. 2017). Though the details differ between kingdoms, ribonucleotide incorporation and RER appear to be conserved across cellular life.

Conclusions and perspectives

Tremendous progress has been made regarding the when, where and how of genomic ribonucleotide incorporation. It is now safe to state that rNMPs can be incorporated by any DNA polymerase during any DNA synthesis. The rate at which rNMPs are incorporated is partly determined by the nucleotide pool imbalance, the extent of which is regulated by cell cycle and DNA damage and replication checkpoint status via ribonucleotide reductase. DNA polymerases are also vastly different in their ability to discriminate against ribonucleotide incorporation. Pols ε and δ incorporate slightly less than one ribonucleotide per kilobase. Some specialized repair polymerases, such as Pols η and μ, incorporate ribonucleotides into DNA at extremely high rates. In fact, Pol μ behaves almost like an RNA polymerase. Thus, the question of why ribonucleotides are so abundantly incorporated into DNA remains. In this review, we discussed evidence of positive roles for ribonucleotides in mating type switching in *S. pombe*, mismatch repair, and non-homologous end joining. Widespread genomic ribonucleotides may influence nucleic acid transactions such as gene expression, nucleosome and histone marks, DNA repair, chromosome architecture and DNA mutagenesis. In contrast to the transient majority of rNMPs in the nuclear genome, embedded rNMPs are not likely removed from mitochondrial DNA (Wanrooij et al. 2017). This is particularly interesting, as the 2'-hydroxyl group makes ribonucleotides naturally less stable. It is tempting to speculate that the lack of selection against ribonucleotides in the mitochondrial genome suggests a positive role for their presence. In both nuclear and mitochondrial genomes, rNMP incorporation is not uniform. There are biases in the identity of the embedded rNMP, locus of incorporation, and in the surrounding sequence context. The biological significance of these biases remains to be determined. The use of embedded rNMPs as biomarkers for DNA polymerase action has been fruitful. Their abundance enables high resolution mapping and provides a means of examining polymerase roles in any processes that involves DNA synthesis. It remains to be seen if current techniques have sufficient resolution to detect rare events like short DNA synthesis tracts from repair events in individual cells. Genetic tools for studying the vast number of specialized polymerases will also be needed.

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Declaration of Interest

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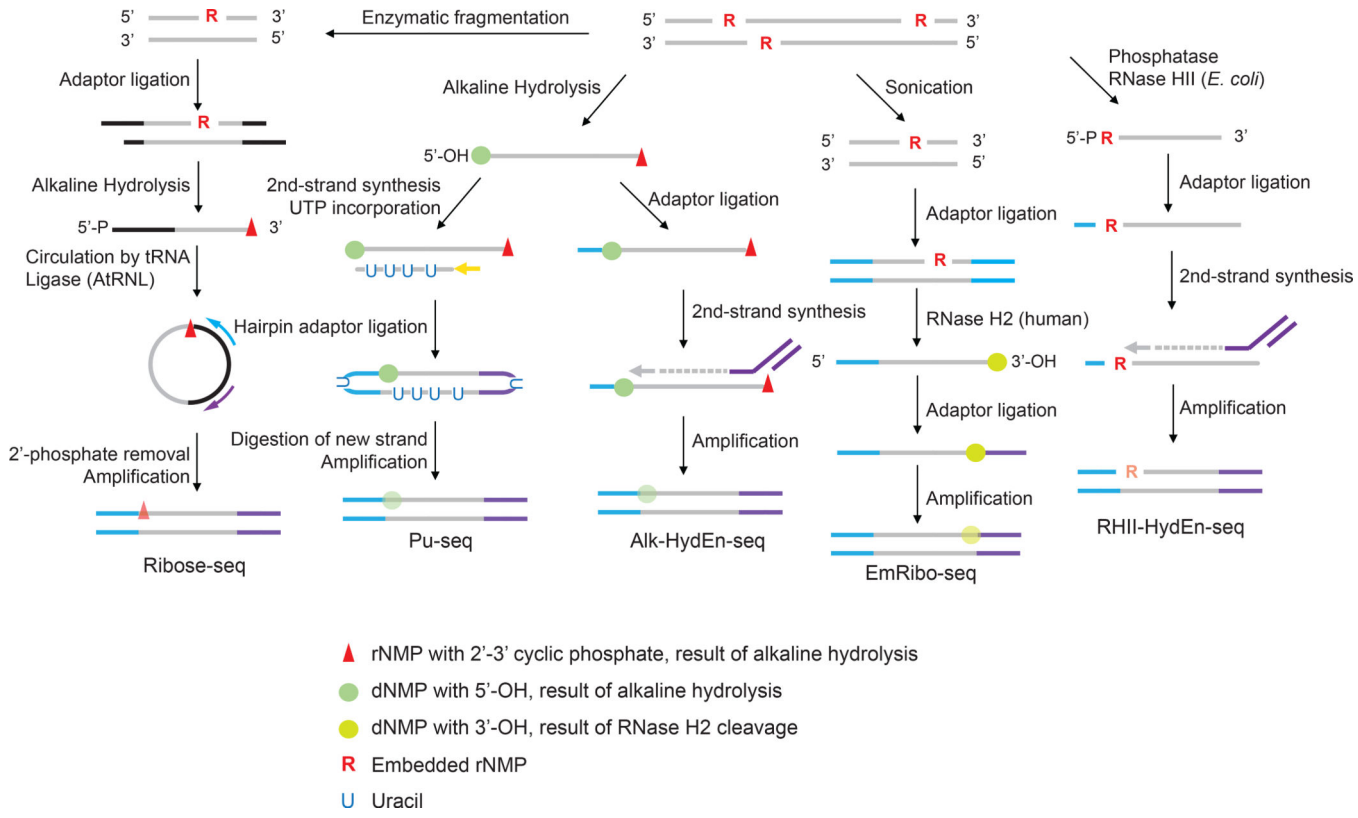

Figure 1.

The current iterations of ribonucleotide mapping technologies. These strategies can be classified by the methods used to incise at embedded ribonucleotides. Ribose-seq, Pu-seq and Alk-HydEn-seq use alkaline hydrolysis which catalyzes hydrolysis on the 3' side of the rNMP, resulting in 2',3'-cyclic phosphate and a 5'-hydroxyl DNA ends. EmRibo-seq uses recombinant human RNase H2 and RHII-HydEn-seq uses *E. coli* type II RNase H (RNase HII), both of which cleave on the 5' side of the rNMP, resulting in 5'-phosphate and 3'-OH ends. These technologies also differ in the location of ribonucleotide with respect to the sequencing read. Ribose-seq maps the rNMP site to the first position of the mapped read but on the opposite strand. Pu-seq and Alk-HydEn-seq identify the rNMP as located one nucleotide upstream of the mapped the read. In EmRibo-seq, the rNMP is similarly positioned one base upstream of the mapped read but on the opposite strand. For RHI-HydEn-seq, the rNMP is the first nucleotide of the mapped read. The symbols denoting the DNA/RNA ends resulting from alkaline hydrolysis or RNase cleavage are kept throughout the steps to help track their locations in the sequencing reads. It is worth noting that after the DNA amplification step during the library preparation, these symbols no longer represent the original form of the nucleotides but rather their base identities. Their opacity to emphasize this difference.

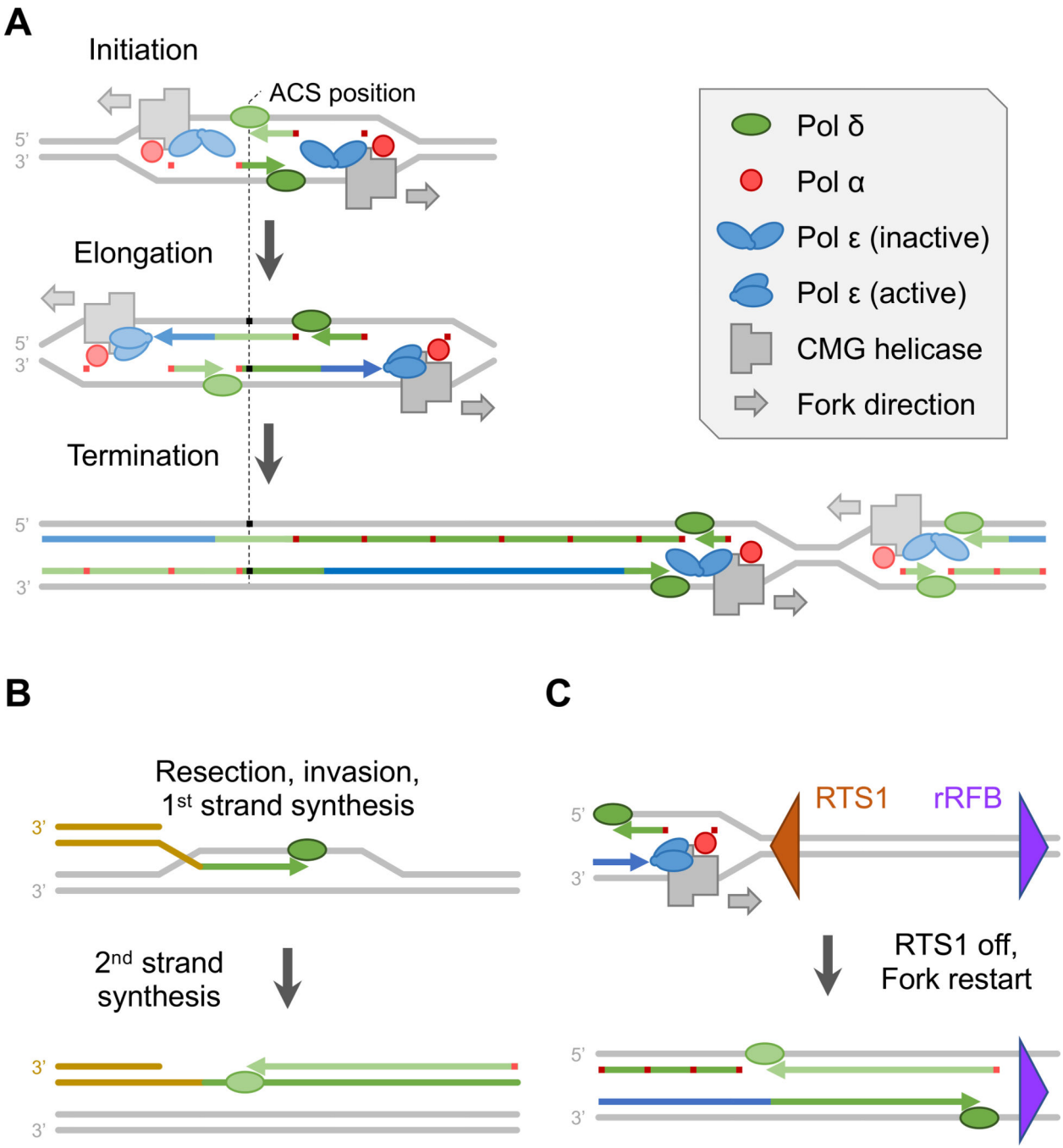

Figure 2.

Polymerase usage during normal and stressed conditions. (A) Polymerase usage differs during replication initiation, elongation and termination. The first Okazaki fragments on both strands initiate leading strand synthesis. Their overlap means that Pol δ synthesize the short stretches of DNA on both strands at replication initiation sites. Pol ε and Pol δ follows the canonical division of labor for the majority of DNA replication. During termination, leading strand replication switches from Pol ε to Pol δ for the last a few Kbps. (B) Polymerase usage after homologous recombination-dependent replication restart. In *S.*

pombe, when the replication fork is forced to stall at the RTS1 site and restart following RTS1 removal, the restarted replication predominantly uses Pol δ for synthesis of both strands. (C) Polymerase using during break-induced replication (BIR). The 3' end of a single-ended DSB can invade the homologous sequence on the donor chromosome. DNA synthesis is forced to copy the rest of the donor chromosome. In contrast to normal DNA replication, the nascent first strand serves as the template for the second strand synthesis. Both strands are predominantly synthesized by Pol δ.

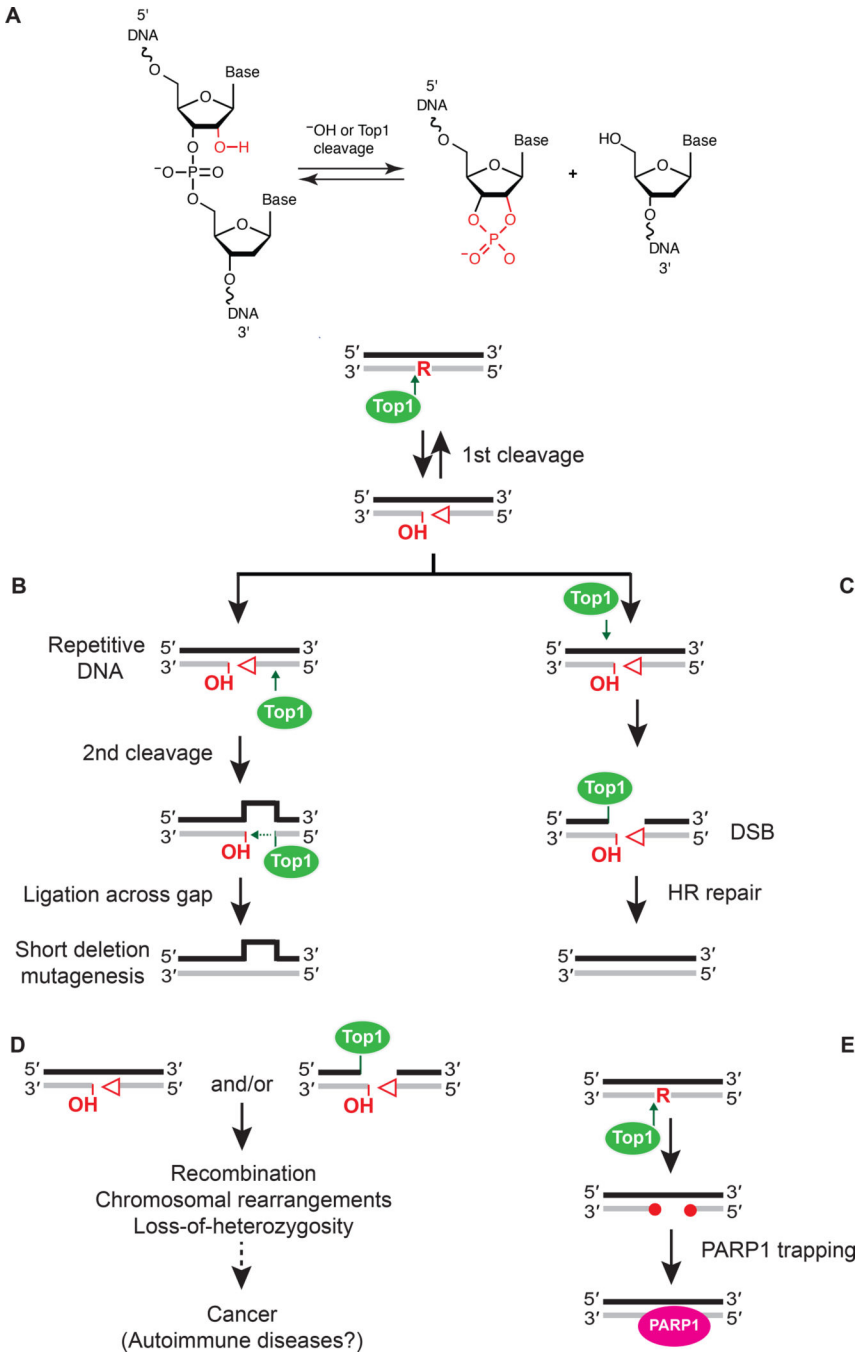

Figure 3.

The negative physiological consequences associated with processing of ribonucleotides in DNA. (A) A single ribonucleotide in DNA can lead to formation of a single strand break (SSB) containing unligatable 5'-OH and 2',3'-cyclic phosphate DNA ends (open triangle). This can occur following spontaneous hydrolysis or Top1 cleavage. (B) Short deletions in repeat DNA sequences are likely generated by two consecutive Top1 cleavage events, the first at the site of a ribonucleotide and the second 5' to this initial cleavage site. This allows for strand realignment and Top1-mediated ligation across the short gap, resulting in loss of a

repeat DNA sequence. (C) A DNA DSB can be directly generated following two Top1 cleavage events. If the initial Top1 cleavage event at an unrepaired ribonucleotide occurs in close proximity to a Top1 cleavage event on the opposite DNA strand, this generates a DSB that is repaired by Rad51- and Rad52-mediated HR. (D) Processing of genomic ribonucleotides results in SSBs and DSBs with unligatable DNA ends that promote various types of genome instability and may contribute to diseases such as cancer and autoimmune disorders. (E) Aberrant DNA ends produced following Top1-cleavage at unrepaired genomic ribonucleotides are recognized and bound by PARP1 to initiate DNA repair.

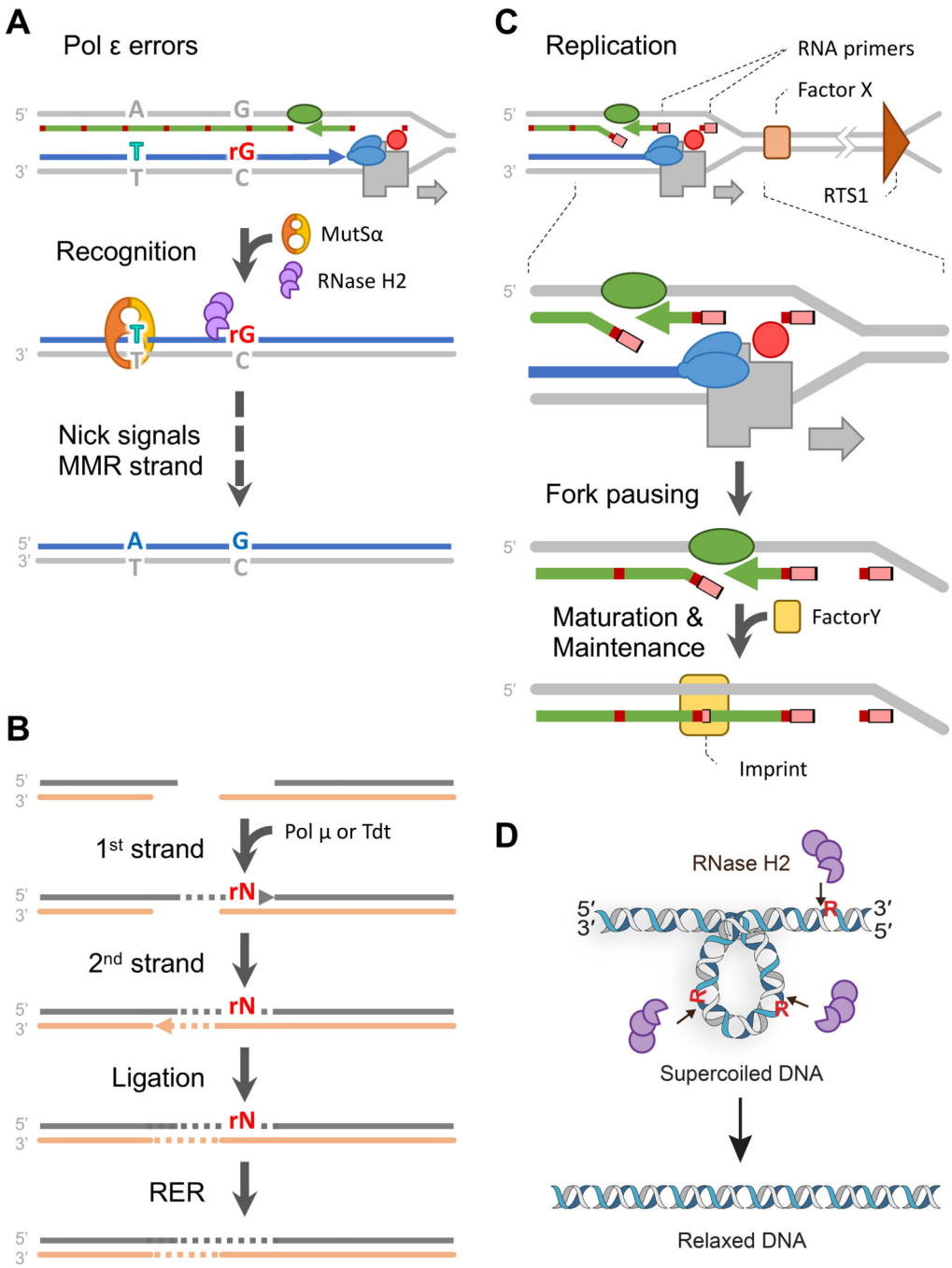

Figure 4.

The positive consequences of ribonucleotide incorporation into DNA. (A) RNase H2 nicking at unrepaired nascent strand ribonucleotides acts as a strand-discrimination signal for MMR. (B) During NHEJ of DNA DSBs, ribonucleotides are frequently incorporated by Pol μ or TdT to promote efficient ligation by DNA Ligase 4. These incorporated ribonucleotides can later be removed during RER. (C) In *S. pombe*, a di-nucleotide imprint in the lagging strand is required for initiation of the mating-type switch. During replication of the mating-type locus, the imprint is suggested to consist of two consecutive ribonucleotides that are

preserved after Okazaki fragment maturation. Imprint formation and preservation requires the concerted effort of several factors. The incoming fork is paused by the unknown factor X. The unknown DNA-binding factor Y is thought to protect the imprint from processing. RTS1 is a replication terminator that blocks the incoming fork from opposite direction. The imprint stalls Pol ε during leading strand synthesis in the next round of replication to promote recombination and allow a mating type switch. (D) RNase H2 nicking at ribonucleotides incorporated by Pol ε on the leading strands results in rotational freedom of the newly synthesized DNA strand and may provide relief of torsional stress.