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Effect of the loss of Ribonuclease H activity on genome instability: from DNA damage tolerance to retrotransposition

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Part I

Abstract

Preserving the entire set of hereditary instructions necessary for life of every organism, genome integrity and identity maintenance is a serious concern for cells. Genomic DNA is continuously endangered by exogenous and endogenous factors and one of the most common source of genome instability was recently detected to be the misincorporation of ribonucleotides (rNTPs) during DNA synthesis. RNA is more susceptible than DNA to spontaneous hydrolysis, so the presence of rNMPs in the genome may render chromosomes more unstable. Moreover, embedded rNMPs induce replication forks stalling and alter the B-conformation of a dsDNA, resulting in replication stress. Normally, rNMPs in DNA are processed by Ribonuclease H (RNase H) enzymes, which cleave the RNA of RNA:DNA hybrids allowing the reconstruction of a dsDNA molecule. Cells lacking RNase H activity can survive thanks to the Post-Replication Repair mechanism, which includes an error free pathway, the Template Switch, and a more mutagenic pathway that relies on the ability of special enzymes to replicate across damaged DNA, the Translesion Synthesis (TLS). Here, we focus on the contribution of the three TLS Polymerases (Rev1, Pol ζ and Pol η) to genomic-rNMPs tolerance and incorporation. We previously demonstrated that Pol ζ efficiently replicates rNMPs-containing DNA and that Rev1 plays a non-catalytic role in supporting this function (Lazzaro et al. 2012). In this study we

observed that Rev1 has also a non-catalytic role in preventing Pol η function. Surprisingly, the polymerase activity of Pol η appeared to be toxic for cells where dNTP pools, necessary for replication, are downregulated by hydroxyurea (HU), and lead to cell death when the RNase H is missing. Furthermore, we provide evidence that, in our sperimental conditions, Pol η toxicity is due to its tendency to introduce rNMPs during the TLS when the dNTP levels are low. Our findings describe an unexpected mechanism for TLS that could be relevant to replication stress in cells defective in RNase H, including humans stricken from diseases associated with RNase H defects like the Aicardi-Goutieres Syndrome (AGS). AGS is an autoimmune disease characterized by high levels of interferon α in the serum and cerebrospinal fluid. Due to the fact that identified mutations fall in genes implicated in nucleic acid metabolism or signalling, and that an emerging source of immunostimulatory nucleic acids are fragments derived from endogenous retroelements, a recent hypothesis states that the pathological immune response could be driven by aberrant accumulation of retroelements intermediates. We then decide to use *S.cerevisiae* as model system to investigate the role of RNase H2 in retroelements mobility and try to link it to the possible molecular origin of AGS. We found that yeast RNase H can block the retrotransposition process and, once replaced yeast enzymes with wild type or AGS mutated forms of human RNase H2, we noticed that one of the three tested mutations causes the loss of the RNase H protective function against the mutagenic potential of retroelements. Even if preliminary, these findings could guide the way to better understand the molecular causes of AGS.

State of the art

1. DNA damage and Genome Instability

Since the discovery of the DNA as the molecule that encodes the genetic information more than a half century ago, the mechanisms that protect it, then ensuring its faithful transmission across the generations, have been the subject of extensive investigations.

In all living cells DNA molecules are continuously attacked by numerous factors, endogenous and environmental, that threaten their stability. The chemistry and the biology of DNA damage is very complex and the variety of DNA lesions is enormous, but fortunately nature evolved a multitude of strategies for repairing most of damage or, at least, mitigating its potentially lethal effect caused by interference with normal DNA metabolism (Fig. 1).

The failure of a cell to maintain the integrity of its genomic DNA is reflected in the concept of “genome instability”, a condition characterized by the quick accumulation of different genetic alterations, ranging from point mutations to gross chromosomal rearrangements (Aguilera and Gómez-González 2008).

Genome instability is present in almost all human cancers and is considered a key driving force in tumorigenesis (Loeb 1991) because, in order to develop, a cancer needs a strong accumulation of mutations (Hanahan and Weinberg 2011).

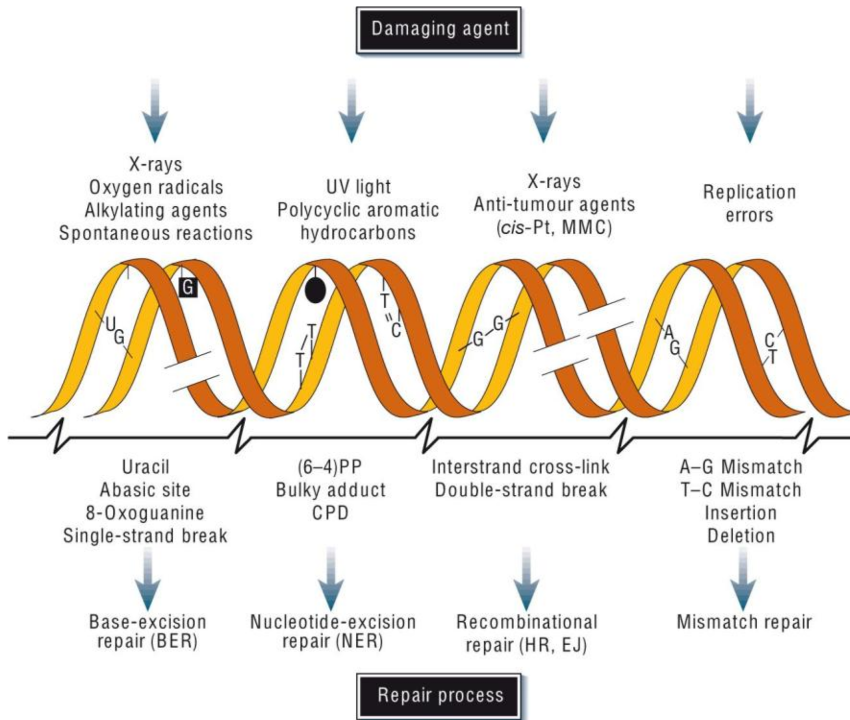

Figure 1: DNA damaging agents and repair systems
The most common source of DNA damaging agents are illustrated at the top, in the middle are reported examples of induced DNA lesions and on the bottom the most relevant DNA repair mechanisms involved in their removal (*from Hoeijmakers 2001*).

Surprisingly, it has been estimated that DNA damage associated with endogenous elements is more extensive than damage caused by environmental factors (Purohit and Basu 2000). In fact, the primary source of genomic damage is the chemical ability of DNA to react with molecules present in the surrounding cellular environment, like water and oxygen, resulting in multiple "spontaneous" lesions. For example, the deamination of four of the five bases that assemble the DNA polymer (cytosine, adenine, guanine and 5-methylcytosine) occurs inherently in pH- and temperature-dependent reactions of DNA and

results in the conversion of the stricken bases to uracil, hypoxanthine, xanthine and thymine, respectively (Friedberg et al. 2006). Among the endogenous species that injure cellular DNA there are reactive oxygen species (ROS) and reactive nitrogen species (RNS), continuously produced as a consequence of normal metabolic activities like mitochondrial respiration.

Other types of endogenous DNA damage are depurination (and to a lesser extent depyrimidination) that originate from the hydrolysis of the glycosidic bonds that hold together the nucleobase and deoxyribose sugars, thus leading to the formation of apurinic (or apyrimidinic) sites.

On the other side, among the external causes of DNA damage there are solar UV radiation and ionizing radiation. Sunlight is considered the most powerful and ubiquitous physical carcinogen in our natural environment (D'Orazio et al. 2013); indeed, the UV portion of the solar spectrum in the 100- to 320-nm region can be directly absorbed by DNA, producing covalent links between two adjacent pyrimidine bases and leading to the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts. Ionizing radiation is routinely used in medical diagnostic and chemotherapeutic applications. There are different forms of radiation that generate a variety of DNA lesions that include double- (DSB) and single-strand breaks (SSB), as well as oxidatively modified nucleobases and deoxyribose moieties.

Moreover, apart from endogenous or environmental sources of DNA damage, a very serious threat to genome integrity comes from the DNA metabolism itself: the two main cellular processes involving DNA, namely transcription and replication, represent a potential

source of chromosome breakage. The so-called replication stress and subsequent replication errors or replication failures appear to be the main origins of genome instability (Kunkel 2004, Gorgoulis et al. 2005, Aguilera and Gómez-González 2008, Halazonetis et al. 2008).

1.1. DNA Replication Stress and Fork Stability

In eukaryotes, DNA replication starts at thousands of individual replication origins that form bidirectional replication forks. Once origins fire and replication begins, the cell has to monitor several parameters, like its speed, the accuracy, the consumption and distribution of different required resources such as nucleotides and replication factors, to complete the process in an efficient manner.

When a replication fork encounters a physiological or environmentally created obstacle on the template strand, its movement is temporarily compromised and this situation is termed “fork stalling” (Carr et al. 2011). Once the obstacle is removed, DNA synthesis can restart. Since the DNA replication fork is asymmetric, it is known that injuries into lagging-strand template represent trivial obstacles to replication fork progression because DNA synthesis is already discontinuous and would leave a gap with a 5’ primer end. On the contrary, injuries into the leading-strand template chain induces a more noteworthy interruption of DNA synthesis and requires the additional step of repriming to resume the process.

The direct consequence of a stalled fork formation is the unhooking of the replicative helicases from the polymerases, which leads to the

accumulation of highly vulnerable single-stranded DNA stretches and then to replication stress (Carr et al. 2011).
The stabilization of stalled forks is an important factor in the maintenance of the genome stability and a pivotal role is played by the DNA damage checkpoint protein kinases Mec1/Rad3/ATR, Rad53/Cds1 and Chk1 (Zegerman et al. 2009, Branzei et al. 2009). The pathways that result from these regulators prevent the worsening of the situation to the so-called "fork collapse" (Lopes et al. 2001). This phenomenon arises when the replisome is no longer associated with the site of DNA synthesis. The replisome contains the full range of replication proteins: the three replicative polymerases (eukaryotic Pol α, Pol δ and Pol ε), the sliding clamp PCNA, the MCM replicative helicase and a range of accessory factors (Yao et al. 2010). Once a fork collapses, rebuilding and restarting a replisome can lead to rearrangements and cell death with mechanisms involving a variety of DNA transactions, engaging exonucleases, endonucleases and helicases that process newly replicated strands. Processing can form any of several structures, including large regions of ssDNA, a "chicken-foot" structure that resembles a Holliday Junction potentially cleavable to form a DSB (Postow et al. 2001), a structure called hemicatenane formed by a template-switch mechanism, and a structure recently termed "closed-fork" (Fig. 2) (Carr et al. 2011).

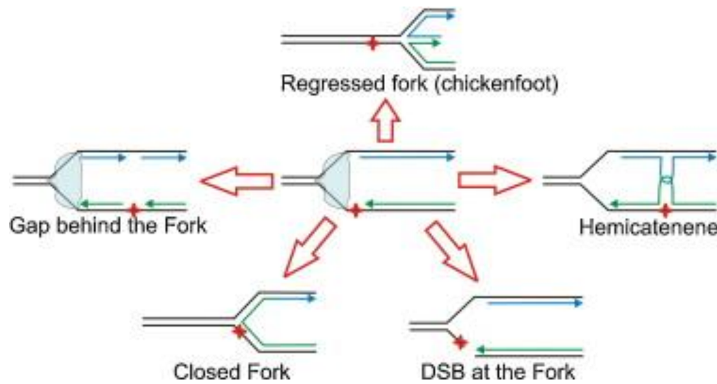

Figure 2: Structure of replication forks.
A fork that is stalled by a barrier such as DNA damage (red X) undergoes one of several fates: restart beyond the fork to form a gap; regression to form a chicken foot; template switch to form a hemicatenane; DNA breakage to form a DSB; or a template switch to form a "closed fork" (*Adapted from Carr 2011)*.

1.2. DNA polymerases fidelity and replication errors

The accuracy of DNA replication is a key aspect required for the correct transmission of an intact genetic information. Errors in DNA synthesis, although important for evolution, represent a source of DNA replication stress and may become major causes for cancer predisposition and genetic diseases.

There are several families of DNA polymerases, grouped depending on the percentage of homology shared: A, B, C, D, X, Y and RT. In bacteria, C-family polymerases carry out the bulk of genomic DNA synthesis, while in eukaryotes B-family polymerases acquire this task (Pavlov et al. 2006, Kunkel 2009). These principal replicative DNA polymerases necessitate both speed and accuracy to replicate large genomes in an efficient and faithful manner.

Many different biochemical mechanisms safeguard the fidelity of replicative polymerases: selectivity for the insertion of the correct nucleotide provided by base-base hydrogen bonding, water exclusion from the catalytic site, and above all a steric selection on base pair shape and size within the active site (Kunkel 2004). Moreover, DNA replicative polymerases usually have the ability to proofread the insertion and detect mistakes. This editing (proofreading) function uses an exonuclease activity to immediately remove most mistakes. The measure of how accurately a DNA polymerase selects and proofreads the correct nucleotide is termed its fidelity.
However, the stable insertion of incorrect nucleotide during DNA synthesis can happen and it results in nucleotide mismatches. It was estimated that the base substitution error rate of replicative polymerases *in vivo* is in the range of 10^{-7} to 10^{-8} (Schaaper 1993, Loeb 1991).
Nonetheless, other specialized DNA polymerases exist: they copy only short segments of DNA during DNA repair processes or Translesion Synthesis (TLS) and thus do not require the same level of speed and accuracy as the replicative enzymes. TLS polymerases of the error-prone Y-family, for example, are deployed to bypass replication-blocking lesions (Pavlov et al. 2006, Kunkel 2009), meaning that bypass of the damage takes place at a potentially mutagenic cost; another example is represented by reverse transcriptases (RT), which catalyze both RNA- and DNA-dependent DNA synthesis of retroviral genomes (Sarafianos et al. 2009).

1.3. Misincorporation of ribonucleotides in DNA

The insertion of an incorrect nucleotide into the genome depends not only on the failure of polymerases to comply with the rule of complementarity between the bases of the two DNA strands. An error can arise even when these enzymes do not succeed in the discrimination of the identity of its sugar component (Joyce 1997).
All cells contain two types of nucleotides, which are the essential building blocks of nucleic acids, DNA and RNA: deoxyribonucleotides (dNTPs) and ribonucleotides (rNTPs) respectively. dNTPs derives from rNTPs and this conversion is catalyzed by Ribonucleotide Reductase (RNR), whose enzymatic activity corresponds to the protonation/elimination of the substrate's 2'-hydroxyl group (Cerqueira et al. 2006) (Fig. 3).

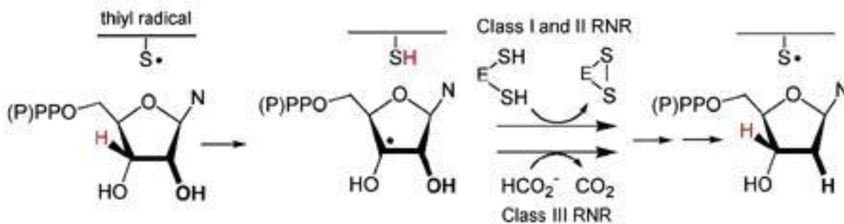

Figure 3: General reaction catalyzed by ribonucleotide reductases.
Hydrogen atom removal from the sugar by the enzyme's thiyl radical and the subsequent radical rearrangements result in the loss of the 2'-OH group in the form of water. The reducing equivalent for the reaction differs depending on RNR class. (*Adapted from Sintchak et al. 2002*).

The two classes of nucleotides have very similar structures, differing only by a little chemical group at the second carbon in the ribose ring, an hydroxyl group in rNTPs and a hydrogen atom in dNTPs. It is thus

reasonable to think that the selection of the correct substrate represents a challenge for polymerases.

Indeed, several studies have shown that the overall discrimination against rNTPs insertion is not 100% efficient and that they can be frequently misincorporated in genomic DNA during DNA synthesis; it has been estimated that more than 10.000 or more than 1.000.000 rNTPs are incorporated, respectively, into yeast and mouse genomes during each cell cycle. So, ribose contamination appears to be the most frequent source of cellular DNA damage in eukaryotic cells (Reijins et al. 2012, Nick McElhinny et al. 2010).

Moreover, just as the probability of base misincorporation and misalignment depend on the absolute and relative cellular concentrations of the four dNTPs (Kumar et al. 2010), the probability of rNTP insertion and stable incorporation in DNA also varies with the cellular concentrations of rNTPs and dNTPs. Measurements in yeast (Nick McElhinny et al. 2010) and mammalian cells (Traut et al. 1994, Ferraro et al. 2010) indicate that rNTPs concentrations are much higher than dNTPs concentrations (36- to 190-fold in *S. cerevisiae* depending on the nucleotide). This greatly increases the chances that rNTPs will be incorporated into DNA, despite the strong polymerase discrimination against this substrate. This point is nicely illustrated in a study (Nick McElhinny et al. 2003) regarding the human DNA polymerase μ, an enzyme that fills 1–2 nucleotide gaps during non-homologous end joining of double strand breaks (DSBs). Increasing the rNTP:dNTP ratio to that estimated in mammalian cells resulted in a several-fold increase in rNTPs incorporation *in vitro*. This observation led also to the suggestion that rNTPs may be frequently incorporated

during gap-filling synthesis associated with DNA repair, especially in non-proliferating cells where dNTPs concentrations are low and rNTP:dNTP ratios are high (Nick McElhinny et al. 2003).

Supported by these facts, other experiments in *S. cerevisiae* suggested that rNMPs misincorporation occurs randomly throughout the entire genome, even if some differences were observed mainly depending on the sequence context *in vitro* (Nick McElhinny et al. 2010a, Nick McElhinny et al. 2010b). However, it has only recently been appreciated in yeast that rNMPs in both genomic and mitochondrial DNA are not randomly distributed and that hotspots of rNMP incorporation are positioned in specific sequences present in multiple copies, such as ribosomal DNA repeats and the yeast retrotransposon (Ty) (Clausen et al. 2015; Reijns et al. 2015; Koh et al. 2015; Daigaku et al. 2015). Moreover, through different rNMP-mapping methods, these studies revealed that more extensive rNMP incorporation occurs during leading strand synthesis by Pol ε and confirmed that sites of incorporation are influenced by sequence context. As a consequence, despite rNMPs are typically viewed as mutations, their presence in DNA could have also a non deleterious role.

A possibile physiological function for genomic rNMPs have been proposed in the fission yeast *Schizosaccharomyces pombe*, where the incorporation of rNTPs can occur via a site-specific mechanism: at the *mat1* locus an imprint consisting of two rNMPs is incorporated into DNA during replication, in order to direct recombination events that eventually result in mating-type switching (Vengrova et al. 2006).

Additional processes are known in which rNTPs play an important role when incorporated into the genome. They are intentionally introduced

into DNA by RNA primase-dependent synthesis of RNA primers that initiate Okazaki fragments during lagging strand replication. These rNMPs are removed during Okazaki fragments maturation (Rossi et al. 2006, Burgers et al. 2009). Moreover, recent findings suggest that the presence of rNMPs may help metabolic processes that need to recognise the newly synthesized DNA strand; in fact, a single rNMP located near a mismatch can act as an initiation site for the Mismatch Repair system in human cell extracts, and Ribonuclease H2 (RNase H2) is crucial for efficient MMR on the leading strand *in vivo* (Ghodgaonkar et al. 2013).

In any case, the main feature that renders the DNA a good template for storing the genetic information is its stability; so it is supposed that rNMPs can stay within the DNA molecule only temporarily and have to be removed before the DNA synthesis restarts. When accumulated at excessive levels, in fact, they might become a danger for a cell for several reasons.

First of all, the chemical nature of rNTPs represents *per se* a challenge to genome stability, because the reactive hydroxyl group at the 2' position of the ribose ring sensitizes the DNA backbone to cleavage, making it more vulnerable to spontaneous hydrolysis (Li and Breaker 1999).

In addition, many structural studies (Egli et al. 1993, Jaishree et al. 1993, Ban et al. 1994, Ban et al. 1994, Wahl et al. 2000) indicate that RNA contamination of a double-stranded DNA causes alterations in the DNA helix shape, promoting a switch from the B- to the A-form and possibly interfering with the functionality of molecular mechanisms based on protein-DNA interactions. Also the proper

assembly of nucleosomes is negatively impacted by rNMPs in the genome, where having 5% or greater rNMPs content in DNA abolishes nucleosome formation (Hovatter et al. 1987). Stunningly, it has recently been observed that even a single rNMP embedded in duplex DNA can result in a helix perturbation at the RNA:DNA base stack which can modify protein recognition and binding (Gao et al. 2014, Tumbale et al. 2014) (Fig. 4).

Finally, genomic rNMPs pose a serious threat to the replication process, acting as a physical obstacle to replication fork progression, when embedded into the template strand. *In vitro* assays demonstrated that replicative polymerases stall when rNMPs are present in template DNA strand, and in budding yeast and mammalian cells it was observed that irreparable genomic rNMPs cause replication stress (Nick McElhinny et al. 2010, Lazzaro et al. 2012, Pizzi et al. 2015).

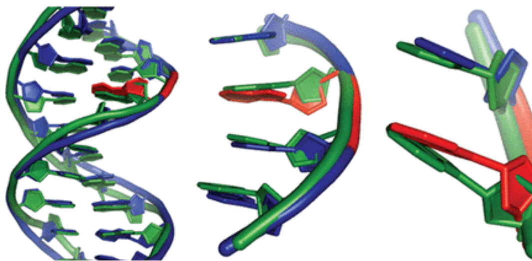

Figure 4: Structure of DNA with an embedded rNMP. Aligned structures of 12nt DNA molecule (green) and a DNA molecule (blue) with an embedded ribonucleotide (red) (*Adapted from Potenski et al. 2014*).

Normally cells try to limit genomic accumulation of rNTPs first preventing their stable insertion. This is possible because of a clash between the 2'-oxygen atom of the rNTP and the "steric gate" residues of the replicative polymerases (Brown et al. 2011); most DNA polymerases in fact strongly reject nucleotides with modifications of the 2'-deoxyribose sugar via a simple steric exclusion model: the small

hydrophobic pocket in the nucleotide-binding site of these enzymes does not accommodate the 2'-hydroxyl group of rNTPs (Joyce 1997). Moreover, most high fidelity polymerases possess 3'-5' exonucleolytic proofreading activity to excise nucleotides carrying the wrong sugar, as well as the wrong base (Williams and Kunkel 2014).

To cope with the addition of rNMPs by replicative polymerases, cells can use a specific repair system designed to detect and remove rNTPs from dsDNA, the Ribonucleotide Excision Repair (RER) (examined in depth below). This mechanism relies on a rNMP-specific endonuclease that can recognise even a single rNMPs embedded in dsDNA, called Ribonuclease H2 in eukaryotic cells, and results in the correct resynthesis of the rNMP/rNMPs-containing DNA patch (Williams et al. 2014).

In addition to this pathway, a backup mechanism based on topoisomerases I ability to target erroneous rNMPs has been recently identified in yeast, even if its activation can compromise genome integrity, causing short deletions in repeated sequences (Nick McElhinny et al. 2010, Clark et al. 2011, Kim et al. 2011). Topoisomerase I (Top1), whose primary role is to regulate DNA supercoiling through the production of transient SSBs, can recognize and cleave misincorporated rNMPs (Williams et al. 2013), but after the hydrolysis of the 3'-bond at an internal rNMP, the 2'-OH group causes the intramolecular hydrolysis of the enzyme bond forming a 2'-3' cyclic phosphate at the nick site instead of maintaining a covalent bond between Top1 and the DNA. The subsequent removal of the nucleotide carrying this cyclic phosphate would then cause slippage and consequent short deletions (Kim et al. 2011, Williams et al. 2013).

However, new evidences shows that Srs2 helicase can repair Top1-induced mutations originating from misinsertion of rNTPs in DNA. According to the model, Srs2 can unwind DNA from the 5'-OH end of the nick produced by Top1, interact with Exo1 and stimulate its exonuclease activity; the resulting gap is then filled by a DNA polymerase in an error-free manner (Potenski et al. 2014).

2. Cellular response to DNA damage

Damaged DNA must be removed for the DNA code to be read properly. During evolution cells have developed a complex network of coordinated mechanisms that uninterruptedly detect the lesions, signal their presence and improve their repair. In eukaryotic cells, the activation of DNA repair mechanisms, specific for different types of lesions, is most of the time combined with the activation of surveillance mechanisms named checkpoints; they respond to genomic perturbations by arresting the cell cycle and also by further promoting DNA repair (Hustedt et al. 2013; Lim et al. 2000).

Collectively, these two types of systems are commonly defined as components of the cellular DNA damage response (DDR) (Fig. 5). The importance of DDR in maintaining genome integrity is underlined by the observation that defects in either DNA repair pathways or DNA damage checkpoints cause cell sensitivity to DNA-damaging agents and genome instability, and inherited mutations in many human DDR components are associated with cancer-predisposing syndromes

(Hoeijmakers 2001, Kennedy and D'Andrea 2006, Kerzendorfer and O'Driscoll 2009, Jackson and Bartek 2009).

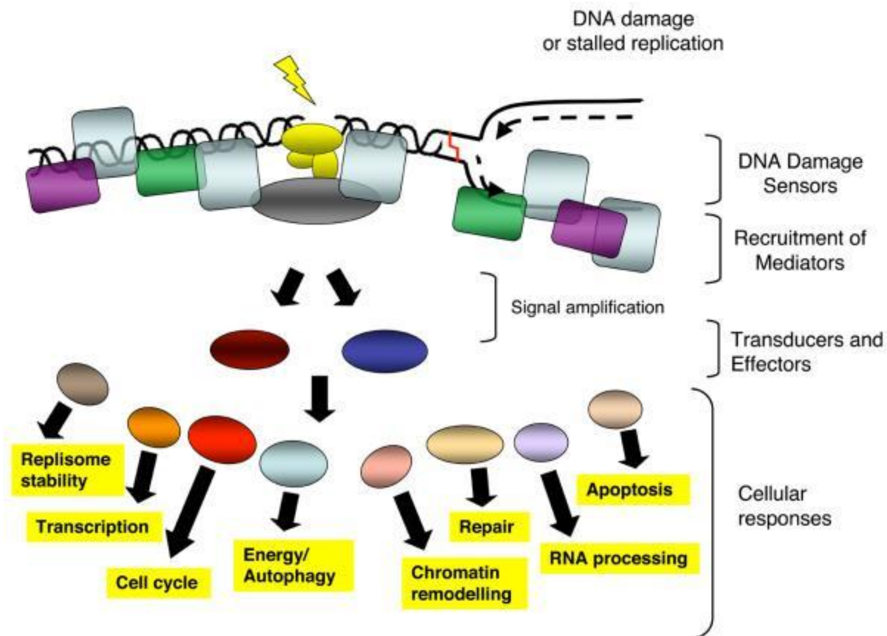

Figure 5: Overview of the DNA damage response. (*from Jackson and Bartek 2009).*

2.1 DNA damage checkpoints

DNA damage checkpoint responses are responsible for the tight feedback regulation of cell cycle progression and frequently referred to as G_1/S, S-phase and G_2/M checkpoints. DNA damage leading to structural changes is initially detected by sensor molecules that are largely operative in all phases of the cell cycle. After sensing the damage, with the help of mediator proteins, the signal is passed on to

transducer proteins. At this point, the transducers modify direct or indirect mediators of cell cycle progression as their effector molecules by phosphorylation, possibly resulting in proteolytic degradation. If phosphorylated or absent, effector molecules cannot promote the transition through cell cycle phases, thereby establishing a cell cycle phase-specific delay or arrest.
Following DNA repair, when the damage has been removed, the checkpoint pathway is inactivated in a process termed recovery that allows cell cycle progression. A related but genetically distinct process, the adaptation, controls cell cycle re-entry in the face of unrepairable damage.

2.1.1. The recognition of the damage: signals and sensors

Because many different kind of injuries can damage the DNA molecule, cells evolved a specific mechanism by which the checkpoint cascade can be activated independently of the lesion type. The current model suggests that lesion processing by DNA repair mechanisms produces a common DNA intermediate that can be recognized by the apical checkpoint factors. Several evidences suggest that this common intermediate comes from stretches of ssDNA generated either by the functional uncoupling of replicative helicases and polymerases during fork stalling or by nucleolytic processing of DNA lesions (Aparicio et al. 1999, Paulsen et al. 2007, Giannattasio et al. 2004, Giannattasio et al. 2010). Indeed, the regions of ssDNA are quickly coated by replication protein A (RPA) that is itself a target of budding yeast

Mec1, the central phosphoinositide 3-kinase (PI3K)-related protein kinase acting as a DNA damage sensor. RPA is able to recruit Mec1 also via its co-factor Ddc2; indeed, Ddc2 foci demonstrate the accumulation of the Mec1-Ddc2 complex at sites of repair (Melo et al. 2001, Rouse et al. 2002).

Another factor recruited on DNA is needed for checkpoint cascade activation: the Rad17-Mec3-Ddc1 complex. This ring-shaped heterotrimer is loaded at the 5' ssDNA-dsDNA junctions adjacent to RPA-coated ssDNA by Rad24 and Rfc2-5 proteins (Majka et al. 2006a). It was demonstrated that the Ddc1 subunit can directly activate Mec1 (Majka et al. 2006b) and also recruit another protein, Dpb11, which further stimulates Mec1 activity (Puddu et al. 2008).

Colocalization of Mec1-Ddc2 and Rad17-Mec3-Ddc1 complexes is a critical step for the checkpoint activation (Bonilla et al. 2008). Interestingly, both in yeast and mammalian cells it was shown that forced tethering of sensor proteins to chromatin is sufficient to elicit the DDR cascade even in absence of DNA damage (Bonilla et al. 2008; Soutoglou and Misteli 2008).

2.1.2. The transduction of the signal: adapters and mediators

Once apical kinases are activated, the signal is transduced and amplified by phosphorylation and activation of numerous targets globally called mediators. In particular, in budding yeast two serine/threonine kinases are thought to be key regulators of the DNA

damage checkpoint response: Rad53 and Chk1. The activation of these mediators requires the presence of adaptor proteins, the most important of which is Rad9 (Schwartz et al. 2002, Naiki et al. 2004, Blankley and Lydall 2004). Activated Rad53 and Chk1 can phosphorylate their downstream targets, then modulating their activity; moreover, two or more molecules of Rad53 can autophosphorylate in-trans, resulting in full kinase activation (Pellicioli and Foiani 2005). Therefore, since Rad53 phosphorylation level correlates with its kinase activity, damage dependent Rad53 phosphorylation is generally used as a marker of the signal transduction cascade activation (Pellicioli et al. 1999).

Changes to chromatin structure, including covalent modification of histone proteins, are known to occur during DNA-damage responses. In particular, active Mec1 can phosphorylate the S129 residue of histone H2A, demarcating large chromatin domains around the site of DNA lesion. This modification is thought to create a specific platform for recruitment and/or retention of DNA damage repair and signaling factors (Redon et al. 2003), including for example Rad9 (Granata et al. 2010; Javaheri et al. 2006).

2.1.3. The cell response: transducers and effectors

Checkpoint responses are multifaceted and integrate cell cycle arrest with DNA repair regulation. In yeast three checkpoint pathways were identified, which elicit different responses according to the cell cycle phase in which the cell experiences DNA damage (Nyberg et al. 2002): G_1 checkpoint, S-phase checkpoint and G_2/M checkpoint.

The first one slows down the entry in S phase, thus preventing the replication of a damaged DNA: the main effector molecule, phosphorylated by Rad53, is Swi6 transcription factor, whose modification inactivates the Swi6/Swi4 complex responsible for the transcription of *CLN1* and *CLN2* (Sidorova and Breeden, 1997), preventing the G_1/S transition.

The S-phase checkpoint slows down DNA synthesis, stabilizes the replication fork and promotes alternative replication systems (Paulovich and Hartwell 1995). Probably, the best example of the S-phase checkpoint is when cells experience DNA damage, or nucleotide starvation, when entering S-phase: in these cases, replication fork stalling activates the checkpoint thanks to proteins associated to the fork itself, among which Sgs1 helicase, Pol2 subunit of DNA polymerase, Dpb11 and Drc1 proteins, and RFC subunits 2, 3, 4 and 5 (Nyberg et al. 2002); moreover the S-phase checkpoint inhibits additional origin firing (mainly late replication origins) and recombination activities at the fork (Branzei and Foiani 2006). In yeast, indeed, it was shown that Rad53 phosphorylates Sld3 and Dbf4 with the consequence to block further origin firing (Zegerman et al. 2010).

Lastly, the G_2/M checkpoint halts the cell cycle before mitosis, preventing the segregation of damaged chromosomes (Weinert et al. 1998): Chk1 phosphorylates Pds1, preventing its degradation, a critical step to achieve chromosome segregation (Sanchez et al. 1999); Rad53 also promotes Pds1 stabilization by preventing its interaction with Cdc20 and its subsequent ubiquitylation by the APC/C^{Cdc20} complex (Agarwal et al. 2003). Moreover, Rad53 inhibits mitotic exit

phosphorylating also Cdc5, thus preventing APC/C^{Cdc20} activation and the degradation of mitotic cyclins (Sanchez et al. 1999).

2.2. DNA repair pathways

Due to the great diversity of the DNA injuries, several specialized repair pathways have evolved and it is increasingly clear that there is a remarkable overlap between them in terms of lesions that each can deal with. This functional redundancy underlines the importance of these pathways in the maintenance of genome integrity.

The S-phase checkpoint has a central role in stimulating the repair processes and, in general, these two mechanisms are closely connected.

The simplest repair mechanism is the so called Direct Reversal of DNA Damage: it consists of a single-step reaction like the photoreactivation of a cyclobutane pyrimidine dimer, which is the major product of UV radiations, by a specific DNA photolyase in a light-dependent manner (Thoma 1999). In addition, in eukaryotes it is possible to identify five main repair pathways: Homologous Recombination (HR) and Non-Homologous End Joining (NHEJ), involved in the resolution of double strand breaks, Mismatch Repair (MMR), Base Excision Repair (BER) and Nucleotide Excision Repair (NER), responsible of the correction of lesions derived from cellular metabolism, strand misalignments and non-Watson-Crick base pairs.

Moreover, recently an additional repair mechanism, called Ribonucleotide Excision Repair (RER), has been identified (Sparks et

al. 2012) to effectively cope with genomic rNMPs that represent potentially the most abundant noncanonical lesion into DNA (Nick McElhinny et al. 2010a).

2.2.1. The Homologous Recombination Pathway

HR is used for the error-free repairing of double-strand breaks (DSBs) during S or G_2 phase of the cell cycle. In order to occur, the system needs a sequence-homologous partner to be copied during repair, so cells must have already replicated the DNA. The key step is represented by the exonucleolytic processing, also called resection, at the 5' strand of the DSB ends, with the subsequent formation of ssDNA tails at the 3' strand (Krogh and Symington 2004). This ssDNA tract allows the loading of different recombination proteins, primarily Rad51, required for the recognition of the homologous chromosome and the subsequent strand invasion. Extension of the invading strand on the sister template then replaces the sequence across the DSB with that of the sister chromatid, after which the extended strand returns to the original chromosome, in a process named resolution (Li and Heyer, 2008).

2.2.2. Non-Homologous End Joining

In NHEJ the broken ends are directly rejoined. This mechanism can occur at any time during the cell cycle, even if it occurs predominantly

during G_1 or stationary phase, when a sister chromatid is not present to provide a template for homologous repair. In yeast it requires three protein complexes: MRX (Mre11-Rad50-Xrs2), Ku (Ku70-Ku80) and DNA ligase IV (Dnl4-Lif1-Nej1). They recognise, bridge and ligate the broken DNA strands (Jackson 2002). Additional processing factors are also used if the DNA termini require resection or polymerization to become ligatable (Ma et al. 2002, Wilson and Lieber 1999). Indeed, repair by this pathway can often be inexact, because of the frequent loss of few nucleotides before the rejoining.

2.2.3. Mismatch Repair

MMR system detects and removes mispaired nucleotides polymerised during DNA synthesis and insertion/deletion loops (IDLs) that can form by slippage during replication of repetitive sequences (Hoeijmakers 2001). It acts by catalyzing the excision of the mispair-containing tract of nascent DNA and by promoting its error-free re-synthesis. In eukaryotes, MutSα (Msh2-Msh6) and Mutβ (Msh2-Msh3) complexes have the role of recognition and verification of the mismatch and of recruitment of MutLα (Mlh1-Pms2) and MutLβ (Mlh1-Pms1) complexes. At this point MutLα, translocating along DNA, recognises the damaged (neo-synthesized) strand thanks to the presence of a nick. Such nick is likely provided by the removal of Okazaki fragments on lagging strand, while it is still unclear how it is generated in leading strand (Jiricny 2006). A recent work demonstrated that it could be produced by the activity of RNase H2 at rNMPs

incorporated by the replicative polymerase ε (Ghodgaonkar et al. 2013). Removal of the mismatch containing strand can be then accomplished by Exo1 and the resulting gap is finally filled by DNA polymerase (Li 2008).

2.2.4. Base Excision Repair

BER system corrects base alterations derived from cellular metabolism like oxidative damages, deaminations, methylations or hydroxylations. It is based on the activity of several dedicated DNA N-glycosylases, specialized in the recognition of a specific base modification. Once one of these glycosylases recognizes the lesion, the erroneous base is released through cleavage of the glycosidic bond that holds together the base and the deoxyribose, leaving thus an abasic site within the DNA molecule. Subsequently, an apurinic/apyrimidinic (AP) endonuclease nicks the DNA backbone immediately 5' of the lesion. Yeast has two AP endonucleases, Apn1 and Apn2. In the short-pach BER (that is the main pathway) one-nucleotide gap filling is performed by DNA pol β that, removing the 5'-terminal baseless sugar, leaves a nick to be sealed by DNA ligase 3. In the long-patch BER, Pol δ/Pol ε re-synthesize a 2-10 nucleotides gap, the endonuclease Rad27 removes the flap generated by strand displacement, and DNA ligase 1 seals the nick (Hoeijmakers 2001).

2.2.5. Nucleotide Excision Repair

NER system corrects a large variety of helix-distorting lesions that interfere with base pairing and impair nuclear functions such as replication and transcription. It can also provide an alternative mechanism to repair AP sites and oxidized bases (Gellon et al. 2001) but, in general, damages faced by NER mainly come from external sources. Eukaryotes possess two different subpathways of NER: the first one, named global genome NER (GG-NER), removes lesions from non-transcribed DNA strands; the second, transcription-coupled NER (TC-NER), takes care of lesions occurring on the transcribed strand which prevent the proper gene transcription (Tornaletti et al. 1999; Hanawalt 2002).

GG-NER is initiated when, with the help of the Rad7-Rad16 complex, the trimeric complex composed of Rad4-Rad23-Rad33 senses the distortion of the DNA structure and unwinds a ~10bp region around the lesion (Tapias et al. 2004). This DNA structure allows the positioning of TFIIH transcription factor, Rad14 and RPA in order to arrange the so called pre-incision complex and further open the DNA double helix. Subsequently, the Rad2 and Rad1-Rad10 endonucleases are recruited: Rad2 nicks 2-8 nt from the damage on the 3'side, while Rad1-Rad10 nicks 15-24 nt from the damage on the 5' side (Evans et al. 1997). The lesion-containing fragment thus generated is released together with other NER factors. Lastly, repair synthesis fills the gap and DNA ligase 1 seals the nick (Thoma 1999; Lindahl and Wood 1999).

On the contrary, TC-NER is triggered because the RNA polymerase itself signals the presence of a lesion. Indeed, it was demonstrated in yeast that Rad26 may be associated with RNA Pol II during transcriptional elongation; accordingly, its positioning to the site of the damage would result from stalling of the enzyme in an elongation mode (Malik et al. 2010). At this step, Rad26 and Rad28 displace RNA polymerase and recruit the other NER factors. Hereafter the pathway is identical to GG-NER (Hoeijmakers 2001).

2.2.6. Repair of ribonucleotides embedded in the genome: Ribonucleotide Excision Repair

The main enzyme of this pathway is the Ribonuclease H2 (RNase H2), an endoribonuclease able to cleave the phosphodiester bond between dNMP and rNMP, or two adjacent rNMPs, into a DNA duplex substrate. This enzymatic activity is present in all kingdoms of life, from retroviruses to humans (Crouch and Cerritielli 1998) and two types of RNases H exist: RNase H1 and RNase H2 (named RNase HI and HII in bacteria). In yeast, RNase H1 is a monomeric enzyme, while RNase H2 has three different subunits (Rnh2Ap, Rnh2Bp and Rnh2Cp). Of these subunits, only the 2A shows catalytic activity, while 2B and 2C have the role of providing a platform for the assembly of the complex, ensuring processivity and ability to interact with PCNA, an essential protein for DNA replication and repair (Chon et al. 2009). Mutations in RNase H2 are associated with the human Aicardi-Goutières syndrome, an autosomal recessive disorder that

could result from mishandling nucleic acids (see below). In mice with complete RNase H2 deficiency, accumulation of rNMPs in genomic DNA causes embryonic lethality due to the activation of the DNA damage response (Reijns et al. 2012, Hiller et al. 2012). On the contrary, yeast RNase H2 null mutant are viable (Lazzaro et al. 2012). RNase H1 and H2 have partially overlapping substrates, since RNase H2 shows an increased activity in yeast *rnh1Δ* deletion strains, possibly in order to compensate for loss of RNase H1 activity (Arudchandran et al. 2000). Indeed, RNase H1 hydrolyzes the RNA moiety when present in a tract of at least four rNMPs, while RNase H2 is the only one able to incise even a single rNMP incorporated within a DNA molecule (Cerritelli and Crouch 2009) (Fig. 6).

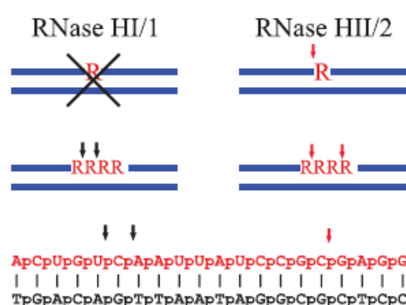

Figure 6: cleavage patterns of RNase H enzymes.
Three types of substrates are shown: a single ribonucleotides in a duplex DNA (top), four consecutive ribonucleotides residues (middle) and an RNA/DNA hybrid (bottom) that are cleaved differently by the two classes of enzymes (*Adapted from Cerritelli and Crouch, 2009*).

The RER pathway begins when RNase H2 recognizes rNMPs within DNA strands and cuts the backbone 5' to an rNMP, creating a nick whose ends have a 3'-OH and a 5'-RNA-DNA junction. RNase H1

fails to substitute for RNase H2 in this RER reaction. Subsequently, strand displacement synthesis by Pol δ occurs, with the help of PCNA and the RFC clamp loader. In this case, Pol ε is able to substitute for Pol δ, but the reaction is slightly less efficient. FEN1 then excises the resulting flap containing the RNA-DNA junction, nicking 3' to the rNMP containing segment (Rydberg and Game 2002). Exo1 can substitute for FEN1, even if less efficiently, but Dna2, able to only act on longer flaps, cannot substitute for FEN1. In order to complete the mechanism, the resulting nick is sealed by DNA ligase 1 (Sparks et al. 2012) (Fig. 7).

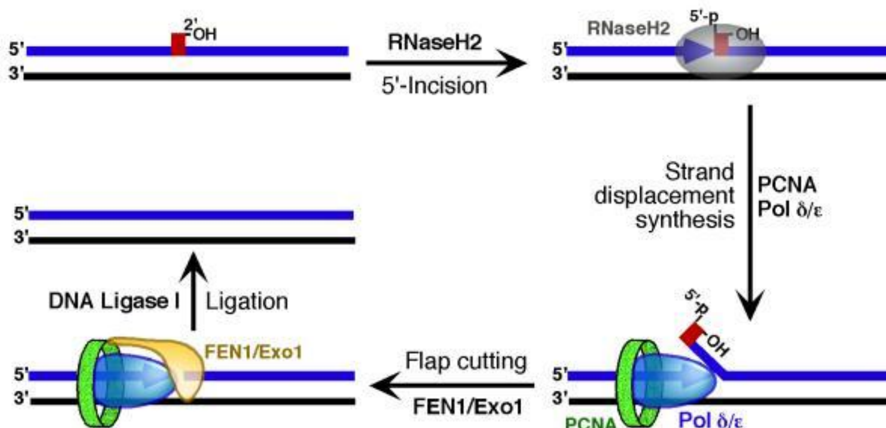

Figure 7: Ribonucleotide Excision Repair. (*from Sparks et al. 2012*).

2.3. Tolerance of DNA damage

Albeit cells have many systems to restore their genome to its original sequence and structure, sometimes another kind of mechanisms are required. Indeed, when the repair pathways are saturated or incapable of replication-blocking lesions removal before the S-phase beginning, cell death could result.

In emergency situations these mechanisms, collectively referred to as Post-Replication Repair (PRR) or DNA Damage Tolerance (DDT) (Friedberg 2005), facilitate cell survival by promoting the completion of the replication process, without mediating lesions repair, according to the "better safe than sorry" philosophy. DNA damage tolerance processes therefore also actively promote the generation of mutations and their transmission to the next generation (Andersen et al. 2008), but their task is not to protect the accuracy of the genetic information.

In eukaryotes, PRR is accomplished by an error free pathway and a parallel, more mutagenic pathway: the Template Switching (TS) and the Translesion DNA Synthesis (TLS), respectively (Fig. 8).

It is still poorly understood what determines the choice between TS or TLS, but decisive factors include the type of lesions, the extent of DNA damage and the phase of the cell cycle (Diamant et al. 2012; Huang et al. 2013). A recent study reveals that also topological changes, particularly the Hmo1-dependent DNA banding, can influence on the mode of tolerance (Gonzalez Huici et al. 2014).

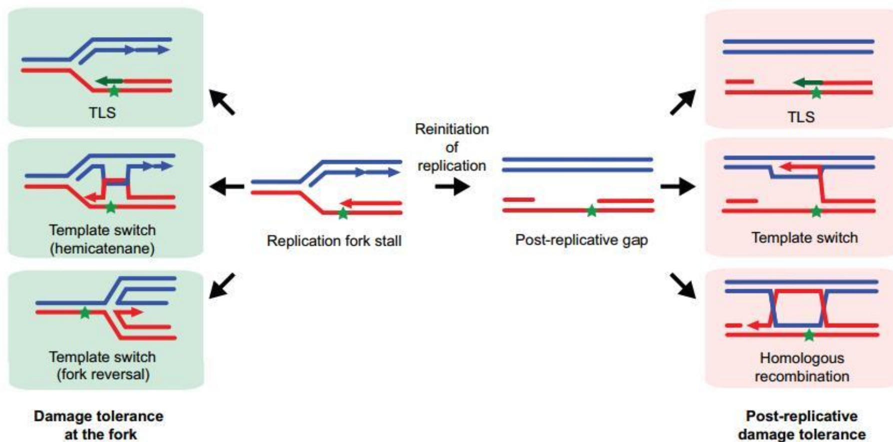

Figure 8: DNA damage tolerance. A simplified scheme of the mechanisms of TLS and template switching. Following replication fork stalling at a DNA lesion (green star) the damage can be overcome using an alternative DNA template, that is the newly synthesised strand on the sister chromatid, or specialised DNA polymerases can directly replicate the lesion by TLS. On the left is represented what happens at the fork; on the right is shown what happens at post-replicative gaps, generated far from the replicative fork because of the recovery of DNA synthesis downstream of the block (*from Sale 2012*).

2.3.1. Activation of the Post Replication Repair

The RPA-coated ssDNA tracts, arranged as a consequence of fork stalling during DNA replication, act as a signal for the recruitment of the E3-ubiquitin ligase Rad18 to chromatin (Davies et al. 2008). Subsequently, also the E2-conjugating enzyme Rad6 is recruited and, together with Rad18, forms a stable heterodimer able to monoubiquitylate PCNA at the conserved residue Lys164 (Hoege et al. 2002).

PCNA is a key regulator of the tolerance pathway selection; its post-translational modification directs bypass into the alternative

subpathways (Fig. 9) (Ulrich and Walden 2010). Monoubiquitylated PCNA activates TLS system (Stelter and Ulrich 2003), but the single Ub mojety can be further extended by another E3-ubiquitin ligase, Rad5, and the E3-conjugating complex Ubc13-Mms2, allowing the formation of a polyubiquitin chain at Lys164 (Hoege et al 2002). Polyubiquitylation of PCNA triggers then the downstream steps of TS (Fig. 9). Importantly, PCNA ubiquitylation is a reversible process, allowing the PRR to be switched off once the lesions have been bypassed. In *S. cerevisiae* the role of Ub molecules removal is carried out by the ubiquitin protease Ubp10 (Gallego Sanchez et al. 2012).

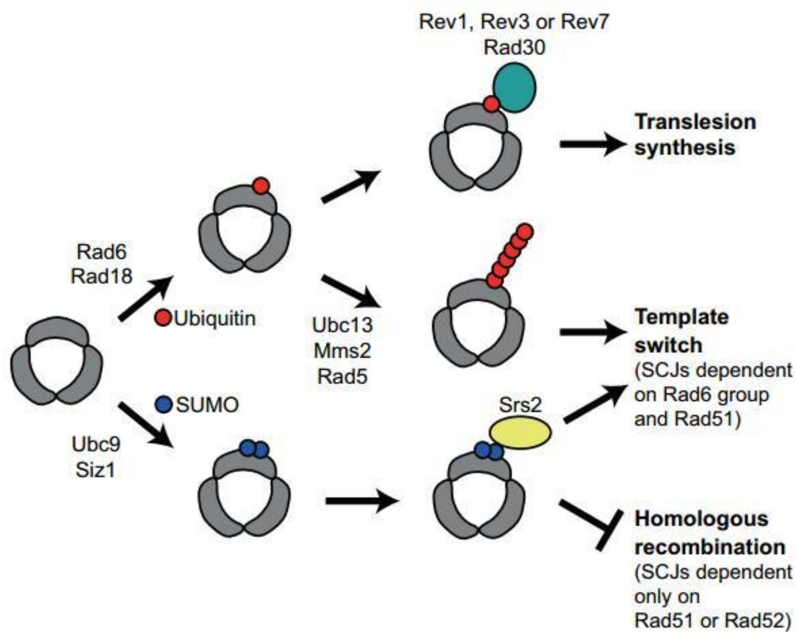

Figure 9: Post-translational modification of PCNA. PCNA can be modified by monoubiquitylation, polyubiquytilation or sumoylation, determining an error free or error prone outcome (*from Sale 2012*).

Moreover, during normal S-phase the replication sliding clamp can be modified at Lys164, and with low frequency at Lys127, by another molecule: small ubiquitin-related modifier (SUMO). In budding yeast this event is dependent on the activity of the E2 and E3 SUMO ligases Ubc9 and Siz1, respectively (Ulrich 2009), and has been associated with the activation of the DNA damage tolerance mechanisms (Hoege et al. 2002; Stelter and Ulrich 2003), supporting Rad18-mediated ubiquitylation in the presence of DNA damage (Parker and Ulrich 2012). As a matter of fact, sumoylated PCNA attracts the antirecombinogenic helicase Srs2, which is able to displace the ricombinase Rad51 in order to avoid unwilled recombination and make room for other proteins like Rad18 (Papouli et al. 2005; Pfander et al. 2005).

2.3.2. Template Switching

How polyubiquitylated PCNA promotes template switching is still mostly speculative. Reputedly, the polyubiquitin chain prevents the access of the TLS polymerases to the site of damage or even throw them out through direct interaction with the ubiquitin-binding motifs in the polymerases. This could allow access to the DNA of other proteins, thereby allowing template switching to occur. Another hypothesis is that polyubiquitylated PCNA acts as a scaffold by recruiting the enzymes that carry out template switching process.
Anyway, this type of error-free bypass is mediated by a transient "template switching" in which the stalled nascent DNA strand takes

advantage of the newly synthesized, undamaged strand of the sister chromatid, using it as a template for replication across the DNA lesion.
Two alternative template switching strategies exists: the Replication Fork Regression, in which after replication fork stalling, the parental DNA partially reanneals while the two newly synthesized strands do the same to form a structural rearrangement defined “chicken foot” (Sogo et al. 2002) (Fig. 8); and the Post-Replication Recombinational Repair, that is an homologous recombination-based lesion bypass triggered by sister chromatin invasion and branch migration across the damaged portion of the template.
Both methods need the association of the two nascent DNA strands, followed by resolution of the intermediary structure via reverse branch migration in the first case, or cleavage of the Holliday junction, here specifically called sister chromatid junction (SCJ), in the second case.
Even if these processes are not yet completely understood, it is becoming clear that a crosstalk exists between classical homologous recombination and template switching (Fig. 8). In effect, recent evidence has shown that the formation of SCJs depends on Rad5 and Rad18 proteins as well as on homologous recombination factors, like Rad51, and requires Pol δ-dependent DNA synthesis but not TLS polymerases (Branzei et al. 2008; Minca and Kowalski 2010; Vanoli et al. 2010). The processing of SCJs is eventually performed by the RecQ helicase complex Sgs1-Top3-Rmi1 (Cejka et al. 2012; Branzei et al. 2008; Karras and Jentsch 2010).

2.3.3. Translesion DNA Synthesis

TLS is a direct mechanism of bypassing unrepaired lesions, which includes several specific DNA polymerases capable of incorporating a nucleotide opposite to the lesions despite the conformational constraints that many of them may impose (Friedberg 2005; Prakash et al. 2005). These particular enzymes are present in organisms throughout all three domains of life. In contrast to the replicative DNA polymerases, they have a more spacious active site that allows the accommodation of large bulky adducts (Ling et al. 2001; Friedberg 2005) which are unable to fit into the active site of their cousins; moreover, they are non-processive low-fidelity enzymes and have lost the proofreading exonuclease activity (Prakash et al. 2005; Friedberg et al. 2002).

Although TLS polymerases are widely referred to as “error-prone” because all are notoriously mistaken on undamaged DNA, the fidelity of each of them relative to replicases is lesion specific. Some TLS polymerases are optimized to bypass one or a few types of lesions in a relatively error-free manner. This feature is strikingly clear in the case of Pol η, which can accurately insert two adenines opposite a thymine dimer caused by UV irradiation, thus reducing UV-induced mutagenesis (Johnson et al. 1999; Kozmin et al. 2003; McCulloch et al. 2004).

Yeast possesses three TLS polymerases: Pol ζ, which is a member of the B family of DNA polymerases, the same including also replicative DNA polymerases; Rev1 and Pol η, which belong to the Y family (Ohmori et al. 2001).

Polymerase	Gene in:				
	S. cerevisiae	*S. pombe*	*D. melanogaster*	Mouse	Human
Pol ζ	*REV3*	*rev3*$^+$	*mus205/dmREV3*	*Rev3*	*REV3L*
Rev1	*REV1*	*rev1*$^+$	*rev1*	*Rev1*	*REV1*
Pol κ		*dinB*$^+$*/mug40*$^+$		*Polκ/DinB1*	*DINB1*
Pol η	*RAD30*	*eso1*$^+$	*DNApol-η*	*Polη*	*RAD30A/XPV*
Pol ι			*DNApol-ι*	*Polι*	*RAD30B*

Figure 10: Types of eukaryotic TLS polymerases and genes encoding their catalytic subunits.
Other non-replicative polymerases exists, as Pol β, λ and μ, members of the X family, and Pol θ, an A family member. They are capable of TLS in certain situations but they have other primary physiological functions (*from Lauren et al. 2009*).

DNA Pol ζ is a heterodimer composed of the Rev3 catalytic subunit and the Rev7 accessory subunit, which enhances the polymerase activity of Rev3 by about 200-fold (Nelson et al. 1996b). Although it can sometimes insert nucleotides across damaged bases, it has a unique architecture to extend DNA synthesis from distorted DNA structures, such as mismatches that may result from inaccurate base insertion by another polymerase (Zhong et al. 2006; Lawrence 2004), methylated bases (Johnson et al. 2007), AP sites (Haracska et al. 2001b), oxidative damages (Johnson et al. 2003; Abdulovic and Jinks Robertson 2006) and DNA-protein crosslinks (De Graaf et al. 2009; Grogan and Jinks Robertson 2012). Moreover, in budding yeast Pol ζ actively replicates rNMPs-containing DNA template (Lazzaro et al. 2012).

Despite the lack of conserved PCNA interaction motifs, Pol ζ exhibits increased lesion bypass activity in the presence of PCNA (Garg et al.

2005), probably because it generally works together with Rev1 (Lazzaro et al. 2012). As a matter of fact, Rev3 subunit interacts with Rev1 *in vitro* and this interaction stimulates the ability of Pol ζ to extend mismatches and bypass specific lesions (Acharya et al. 2006; Guo et al. 2004). Also Rev7 is capable to mediate the interaction with Rev1 (D'Souza and Walker 2006, Acharya et al. 2005); moreover, it can bind to the 9-1-1 alternative processivity clamp, which participates in DNA damage signalling and checkpoint, and this interaction may facilitate the positioning of Pol ζ to the site of damage (Sabbioneda et al.2005).

In contrast with the versatility of Pol ζ, Rev1 has a polymerase activity that is restricted mainly to inserting a dCMP nucleotide across an undamaged template G and across a variety of DNA lesions, like abasic sites and adducted G residues (Lawrence 2004; Nelson et al. 1996a); for this reason Rev1 is also defined as a deoxycytidyl transferase. In spite of this "shortcoming", its catalytic activity is not necessary for the overcoming of many DNA alterations for which Rev1 function is required *in vivo* (Haracska et al. 2001b). Instead, the contribution of Rev1 to yeast cell's resistance to DNA damaging agents and to mutagenesis mainly derives from its interactions with other proteins, as PCNA (Guo et al. 2006) and particularly other TLS polymerases: Pol ζ (D'Souza and Walker 2006) and Pol η (Acharya et al. 2007). Therefore, from these observations comes a model in which Rev1 acts as a scaffold for the recruitment and the coordination of various DNA damage tolerance factors at the site of lesion, rather than directly catalyze DNA synthesis across the lesion (Guo et al. 2003).

Finally, Pol η (also defined Rad30 in yeast) as formerly mentioned is endowed with the ability of accurately bypass particular types of DNA lesion, as *cis-syn* cyclobutane pyrimidine dimers resulting from UV radiation (Johnson et al. 1999; Kozmin et al. 2003; McCulloch et al. 2004). In addition, it is also involved in the bypass of a broad spectrum of other lesions *in vitro*, such as 7,8-dihydro-8-oxoguanine (Haracska et al. 2000b), which is a common form of oxidative base damage, acetylaminofluorene-adducted guanine (Yuan et al. 2000), O^6-methylguanine (Haracska et al. 2000a), thymine glycol (Kusumoto et al. 2002) and adducts derived from cisplatin and oxaliplatin (Vaisman et al. 2000). Unexpectedly, on undamaged template this enzyme shows *in vitro* among the lowest fidelity of any DNA polymerase (Prakash et al. 2005; Matsuda et al. 2000).

As the other TLS polymerases, the catalytic activity of Pol η is regulated through protein-protein interactions; indeed, an important role is played by PCNA, which stimulates its activity *in vitro* (Haracska et al. 2001a). This interaction can be enhanced by the monoubiquitylation of PCNA; mutants disrupting its ubiquitin-binding domain in either budding yeast or mammalian Pol η, cannot complement the UV sensitivity of cells lacking this enzyme (Bienko et al. 2005; Parker et al. 2007). Another binding partner of Pol η is Rev1, but little is known about how this interaction affects its activity, except that the DNA synthesis activity of Rev1, but not of Pol η, seems to be enhanced in the complex *in vitro* (Acharya et al. 2007).

3. Molecular consequences of Ribonuclease H failure

As previously anticipated, RNase H are the major enzymes able to hydrolyze the RNA mojety in RNA:DNA hybrids and are highly conserved during evolution.

The *in vivo* roles of RNase H in eukaryotic cells are still not fully understood, and some differences exist among organisms. For example, in mammals, RNase H1 has been implicated in mitochondrial DNA replication while this role is not conserved in yeast (Cerritielli et al. 2003). Moreover, it is noteworthy that while in yeast the deletion of both types of RNase H is compatible with life, in mammalian cells their functionality is essential (Reijns et al. 2012, Cerritielli et al. 2003, Hiller et al. 2012).

Apart from the involvement of RNase H2 in Ribonucleotide Excision Repair mechanism (described in detail in 2.2.6), which allows the removal of even single rNMPs embedded in dsDNA molecules, RNase H enzymes participate in many other cellular processes.

It is known the involvement of RNase H2 in the processing of Okazaki fragments redundantly, at least in yeast, with Fen1 and the nuclease/helicase Dna2 (Rydberg and Game 2002, Ayyagari et al. 2003). Furthermore, over-expression of RNases H1 has been associated with removal of R-loops (Huertas and Aguilera 2003). These are three-strand nucleic acid structures that form as a consequence of the annealing of the transcribed RNA on its template, thus generating an RNA:DNA hybrid and a displaced ssDNA strand (Aguilera and Garcìa Muse 2012). Moreover, a high-throughput RNAi

screening identified the RNase H2A, containing the catalytic centre, as a factor that increase HIV replication in host cells (Genovesio et al. 2011), thus supporting the hypothesis of a role of RNase H2 also in the regulation of endogenous retroelements metabolism (Rabe 2013).
Finally, mutations in any of the human genes encoding for RNase H2 subunits causes Aicardi-Goutières syndrome (Crow et al. 2006b).

3.1. Aicardi-Goutières Syndrome

Aicardi-Goutières syndrome (AGS) is a chronic inflammatory genetic disorder that typically affects newborns and infants and results in severe mental and physical handicap (Rice et al. 2007). It was first described in 1984 by Jean Aicardi and Françoise Goutières (Aicardi and Goutières 1984) as an early onset encephalopathy characterized by basal ganglia calcifications, white matter abnormalities and chronic cerebrospinal fluid (CSF) lymphocytosis. Few years later, Lebon (Lebon et al. 1998) added a further typical feature to the diagnostic profile of the syndrome: the presence of raised levels of interferon α (INF α) in the CSF and serum in the absence of demonstrable infections of the central nervous system (CNS) (Rice et al. 2007).
To date, only few hundreds of affected individuals worldwide have been classified since the genes responsible of the disease have been identified only recently and because AGS is likely to be misdiagnosed, as it mimics the symptoms caused by congenitally acquired viral infection. The severity of the neurological phenotype, although consistent among patients, presents a high level of variation. Patients

mostly present peripheral spasticity, dystonic posturing, truncal hypotonia and, almost all, are severely intellectually and physically impaired (Rice et al. 2007).

AGS is genetically heterogeneous and up to now, seven genes have been involved in its pathogenesis: TREX1, encoding a DNA exonuclease, RNASEH2A, RNASEH2B and RNASEH2C, encoding the three subunits of the RNase H2, SAMHD1, encoding a dNTP triphosphohydrolase, ADAR1, encoding an adenosine deaminase and IFIH1, which encodes the cytosolic dsRNA receptor also called MDA5 (Crow et al. 2006a, Crow et al. 2006b, Rice et al. 2009, Rice et al. 2012, Rice et al. 2014, Oda et al. 2014). All these proteins are implicated in nucleic acid metabolism or signalling but the molecular origins of AGS are still unknown. However, the relationship between the mutations in the above cited genes with increased interferon α activity in CSF and serum and the increased expression of interferon α-stimulated gene transcripts in peripheral blood, strongly suggests that the interferon α-mediated innate immune response is driven by aberrant accumulation of endogenous nucleic acids (Rabe et al. 2013, Crow and Rehwinkel 2009).

Approximately, 60% of the AGS patients have mutations in the three genes encoding the RNase H2 complex subunits. Among the RNase H2 genes, the RNase H2B is the most frequently mutated, with the recurrent c.529G>A A177T substitution considered a mutation hotspot. This mutation is recurrently present in hetero compound with c.488C>T T163I mutation. RNase H2B mutations has been associated with a significantly later onset, lower mortality and relatively preserved intellectual function (Rice et al. 2007).

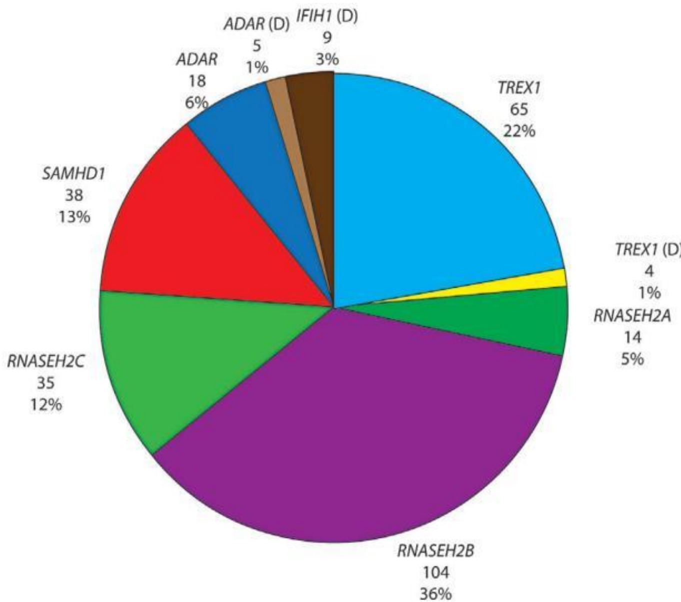

Figure 11: Number and percentage of families with AGS with the seven identified mutations. Data collected in the recent study conducted by Crow group. D: dominant mutation. (*from Crow et al. 2015).*

Conversely, the earliest appearance and most severely affected AGS patients carry mutations in the C and A subunits (Chon et al. 2009, Rice et al. 2007); in particular, missense mutation c.139G>A G37S in subunit A was demonstrated to severely impairs enzymatic activity (Crow et al. 2006b).

3.2. Involvement of RNase H in autoimmunity

In mammals, the detection of non-self nucleic acids, as that ones belonging to pathogens as viruses, by pathogen recognition receptors leads to the production of type I interferons (IFNs) (Akira et al. 2006), including IFN α.

However, although these molecules are a very important factor in cellular response to viral infection, uncontrolled production of IFNs can also induce pathological consequences, most notably the development of autoimmune diseases, as in the case of AGS. The discrimination of viral from self nucleic acids is not always perfect; indeed, different studies have ascertained that the defective processing of self-derived nucleic acids can cause serious IFN-dependent autoimmunity. For example, the deoxyribonuclease I (DNase I) deficiency in mouse causes a syndrome resembling the human systemic lupus erythematosus (SLE), characterized by multisystem inflammation commonly including the skin, kidneys, and joints (Napirei et al. 2000) and DNase I mutations in humans inducing decreased enzymatic activity are associated with SLE (Yasutomo et al. 2001). Moreover, mutations in IFIH1, inducing the over-activity of the anti-viral helicase MDA5, has been recently associated to increased sensitivity to IFNs and increased IFN-induced gene expression in circulating blood cells from SLE patients (Robinson et al. 2011).

In recent years, the IFN-stimulatory DNA (ISD) response has been discovered: it is a antiviral signalling pathway activated by cytosolic DNA, which can activate potent IFNs production through the activation of TANK-binding kinase 1 (TBK1) and IFN regulatory factor 3 (IRF3) (Ishii et al. 2006, Stetson and Medzhitov 2006).

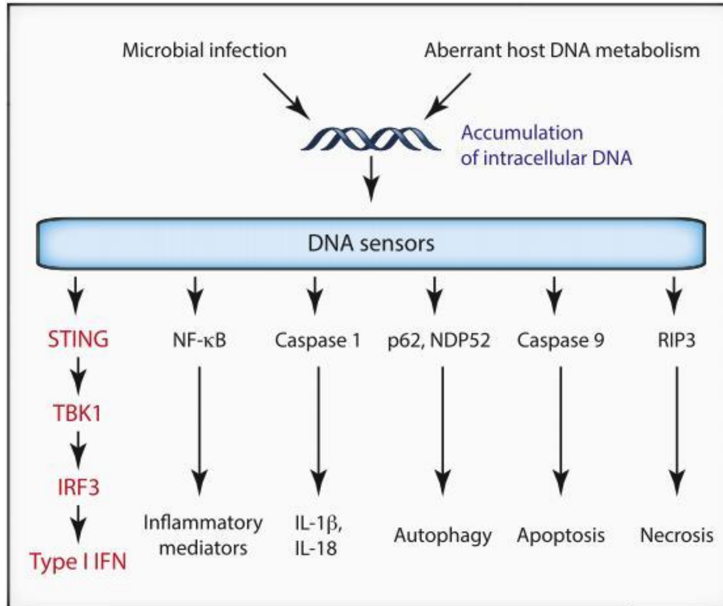

Figure 12: Cellular pathways stimulated by DNA molecules.
The best characterized DNA-stimulated pathway is the one leading to IFNs production. Other pathways are the inflammatory NF-kB and inflammasome pathways, which stimulate expression of inflammatory genes and cleavage of pro-IL-1β and IL-18, respectively. Intracellular DNA also stimulates autophagy, apoptosis and necrosis. (*from Paludan et al. 2013*).

In 2008, Stetson et al. investigated the mechanism underlying the autoimmune disease caused by Trex1 deficiency and observed, in mouse, that this protein is a negative regulator of the ISD response, because mice lacking both IRF3 and TREX1 relentlessly died; moreover, the study showed that fragments of endogenous retroelement DNA are potential Trex1 substrates, since they are accumulated in the hearts of Trex1-deficient mice; lastly, the sequence analysis of cytosolic DNA purified from cells of mice lacking Trex1 revealed that fragments derived from endogenous retroelements were

abundantly represented, suggesting that the reverse-transcribed DNA of these elements can be metabolized by Trex1 (Stetson et al. 2008).
Given that mutations in Trex1 and RNase H2 lead to very similar autoimmune diseases, and both are involved in AGS pathogenesis, a reasonable hypothesis is that they function in the same pathway, maybe sharing a common substrate: the DNA:RNA hybrid probably derived from endogenous retroelements.

3.2.1. Transposition and retrotransposons

The transposition is a particular type of genetic recombination which allows the integration, through at least two molecular mechanisms, of particular DNA sequences, called transposable elements (TEs), into the genome at a new position within the cell of their origin.
TEs are divided in two major classes: DNA transposons and retrotransposons. DNA transposons can be excised and inserted into new genomic site; retrotransposons, on the contrary, have to replicate by forming an RNA intermediate, which is reverse-transcribed in order to obtain a cDNA molecule that can be inserted into a new genomic site (Ayarpadikannan and Kim 2014) (Fig. 13).
Based on the presence of long terminal direct repeats (LTRs), retrotransposons are further divided in LTR and non-LTR transposons. The internal domain of retrotransposons encodes genes that are equivalent to retroviral *gag* and *pol* genes (Fig 13). Indeed, the purpose of the transcription phase is also to synthesize a template for the translation of the reverse transcriptase and nucleocapsid proteins, both

important for the transposition process (Curcio and Garfinkel 1991a). Retrotransposons resemble eukaryotic retroviruses in their structure, as well as in the way of replication: they form cytoplasmic virus-like particles (VLPs), which package the RNA transcript and where the reverse transcription process is circumscribed.

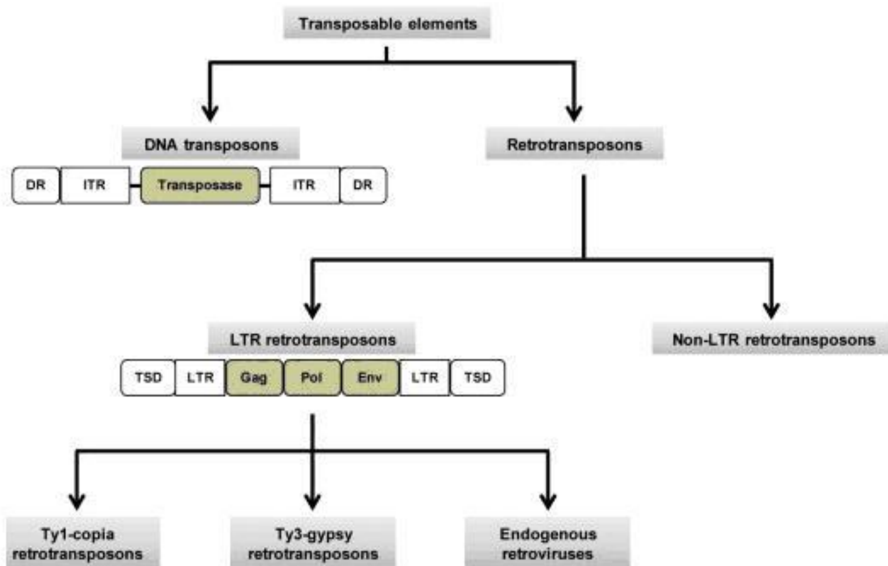

Figure 13: Types and structure of transposable elements.
DR: direct repeats; ITR: inverted terminal repeat; TSD: tandem site duplication; LTR: long terminal repeats. (*from Ayarpadikannan and Kim 2014*).

Among the best characterized retrotransposons are the Ty elements of *S. cerevisiae* and, in particular, Ty1 has been long studied and then genetically modified in order to provide a tool to better understand its retrotransposition (Boeke et al. 1985, Garfinkel et al. 1988, Curcio and Garfinkel 1991a). Ty1 transcript, synthesized by cellular RNA polymerase II, contains two open reading frames (ORFs), *TYA* and

TYB, encoding for nucleocapsid proteins of the VLPs and for protease, integrase, reverse transcriptase and a RNase H, respectively (Curcio and Garfinkel 1991a).

There are about 30 Ty1 elements in the haploid yeast genome (Curcio and Garfinkel 1994) and Ty1 RNA is one of the most abundant mRNA species in yeast (Curcio et al. 1990). Despite this, in normal conditions these elements exhibit transpositional dormancy (Curcio and Garfinkel 1991b) and this probably results from inhibition of one or more post-transcriptional steps in the Ty1 replication cycle. The main characteristic of all TEs, that is their ability to move, can in fact cause genomic instability in many ways, either by insertion or by rearrangements in the genome that may have a large number of different effects on a cell, ranging from silent mutation to alternative splicing of any transcript. The site of insertion along the genome, indeed, appears to be random, even if the integration is particularly frequent in the region upstream of genes transcribed by RNA polymerase III (Ji et al. 1993). Cells have then evolved mechanisms to regulate transposition in order to reduce their mutagenic potential and several host factors can inhibit Ty1 transposition (Rattray et al. 2000, Picologlou et al. 1990). Among them, in 2001 Sholes et al. characterized the *RTT* genes, regulators of Ty1 transposition, which products block the post-transcriptional steps of the process. Many of these proteins are involved in genome maintenance, as those constituting the DNA damage response (Sholes et al. 2001). This observation suggests that Ty1 transposition levels can be modulated in response to alterations of genome integrity and, generalizing, that mutations in genes implicated in genome integrity maintenance, as

RNase H1 and H2, could strongly alter the control mechanisms of transposons mobility with consequences, for different species, yet to be explored.

Aim of the projects

Project 1: Investigating the role of TLS polymerases during genomic-rNMPs tolerance in *S. cerevisiae*

Both repair and tolerance mechanisms represent important cellular defensive weapons against the copious different attacks coming from different sources.

Among the factors that can be considered as a DNA damage, there are ribonucleotides; recent evidence proves a massive ribonucleotides incorporation in genomic DNA, which was shown to promote genome instability (Nick McElhinny et al. 2010a, b).

RNases H are the principal enzymes able to repair this type of damage removing, independently from the sequence, RNA associated with DNA. Indeed, our group described a crucial function for RNase H enzymes in overcoming ribonucleotides wrongly incorporated during DNA replication (Lazzaro et al. 2012).

Moreover, the same study demonstrated a significant contribution of Translesion Synthesis pathway to the survival of cells whose genome is contaminated by ribonucleotides.

The work described in this thesis is aimed at a deeper exploration of the interplay of the three different TLS Polymerases (Rev1, Pol ζ and

Pol η) during genomic-rNMPs tolerance, in absence of RNase H activity and its possible role in rNMPs incorporation.

Project 2: Investigating the role of RNase H in *S. cerevisiae* retrotransposon metabolism

Mutations of the genes coding for RNase H2 subunits cause Aicardi-Goutiéres syndrome in humans, a genetic neurodegenerative disease associated with a perturbation of interferon α metabolism. At the molecular level, the pathogenesis of this syndrome has been attributed to an alteration of the metabolism of intracellular nucleic acids, because of the fact that the mutations identified in the genome of AGS patients include several genes encoding enzymes involved in the processing of nucleic acids: TREX1, SAMHD1 and ADAR1, other than RNase H2 genes.

Moreover, during last years in AGS patients have also been found mutations in IFIH1, a gene coding for a receptor able to recognize dsRNA and activate the antiviral responses.

Not surprisingly, the typical symptoms of the disease are very reminiscent of those of congenital viral infections, although in the blood and in the cerebrospinal fluid of AGS patients are not found viruses.

The immune response caused by RNA:DNA hybrids derived from endogenous retroelements would be the same that is activated in the presence of hybrids belonging to retroviruses; consequently, an

interesting hypothesis states that, in absence of the RNase H2, RNA:DNA hybrids resulting from the metabolism of endogenous retroelements accumulate in cell cytoplasm in an uncontrolled way, thereby triggering the abnormal production of IFN α and the resulting immune response.

Then, we decided to better investigate the role of RNase H enzymes in the metabolism of retrotransposons using an engineered version of *S. cerevisiae* Ty1 retrotransposon. Furthermore, *S. cerevisiae* cells have proven to be a good model organism for the study of AGS because genes coding for the human RNase H2 (RNASEH2A, RNASEH2B, RNASEH2C) are able to complement the lack of yeast genes (RNH201, RNH202, RNH203) in removing rNMPs incorporated during DNA replication (unpublished data, see Figure 16); given that, we started studying also the effects of some AGS mutations on the mobility of retroelements in yeast.

Materials and Methods

Yeast strains and media

All the strains used in the project 2 of my PhD thesis are derivatives of SY2080 strain (W303 *MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100 RAD5*) and are listed in the table below.

Specifying that for solid media 2% Agar was added to the liquid media composition, the media used in this study for budding yeast growth are:

1. Rich media (YPD), composed of 1% yeast extract, 2% peptone and 2% glucose in bidistilled water;
2. Minimal media (YNB + 2% glucose), containing 6.7% yeast nitrogen base (YNB), 1.25 g/l of threonine, 0.625 g/l each of phenylalanine, tyrosine, lysine, isoleucine, arginine, methionine and adenine and 2% glucose in bidistilled water;
3. Synthetic complete media (YNB complete) containing minimal media (YNB + 2% glucose) supplemented with 25 mg/l each of uracil, tryptophan, adenine, histidine and leucine in bidistilled water;
4. Selective media (YNB + 2% glucose, -selective amino acid) containing synthetic complete media with the selected amino acid omitted from the mixture for selective growth.

After preparation, all the solutions are autoclaved to ensure their sterility.

Strain Name	**Genotype**	**Source/ Reference**
YFL2208#82	SY2080 *MATa Ty1H3mHIS3AI his3::*	Available in Lab.
YFL2233/2d	SY2080 *MATa rtt101::KANMX6 Ty1H3mHIS3AI#82 his3::*	This Thesis
YFL2246/6b	SY2080 *MATa rnh1::HPH Ty1H3mHIS3AI#82 his3::*	This Thesis
YFL2230/13a	SY2080 *MATa rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::*	This Thesis
YFL2227/1b	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::*	This Thesis
YFL2229/5c	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::*	This Thesis
YFL2234	SY2080 *MATa Ty1H3mHIS3AI#82 his3::* + pRS316, yCp-lac111, pRS314+pADH (obtained from YFL2208#82)	This Thesis
YFL2236	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::* + pRS316, yCp-lac111, pRS314+pADH (obtained from YFL2227/1b)	This Thesis
YFL2238	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::* + pFL93.1, pFL103.4, pFL105.2 (obtained from YFL2227/1b)	This Thesis
YFL2240	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::* + pFL112, pFL103.4, pFL105.2 (obtained from YFL2227/1b)	This Thesis
YFL2242	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::* + pFL93.1, pFL113, pFL105.2 (obtained from YFL2227/1b)	This Thesis

YFL2244	SY2080 *MATa rnh1::HPH rnh201::KANMX6 Ty1H3mHIS3AI#82 his3::* + pFL93.1, pFL116, pFL105.2 (obtained from YFL2227/1b)	This Thesis

Plasmids

1. pRS316: yeast centromere vector with a *URA3* and a *AMP*^{R} markers;
2. pRS314+pADH: yeast centromere vector pRS314, consisting of *TRP* and *AMP*^{R} markers, in which was added *S. cerevisiae ADH1* promoter;
3. yCp-lac111: yeast centromere vector with *LEU2* and *AMP*^{R} markers;
4. pFL93.1: yeast centromere vector (derived from pRS314) with *RNASEH2A* gene, coding for the subunit A of human RNase H2, under *S. cerevisiae ADH1* promoter control;
5. pFL103.4: yeast centromere vector (derived from pRS316) with *RNASEH2B* gene, coding for the subunit B of human RNase H2, under *S. cerevisiae ADH1* promoter control;
6. pFL105.2: yeast centromere vector (derived from yCp-lac111) with *RNASEH2C*, gene coding for the subunit C of human RNase H2, under *S. cerevisiae ADH1* promoter control;
7. pFL112: yeast centromere vector (derived from pRS314) with a mutated *RNASEH2A* gene, coding for the subunit A of human RNase H2 carrying G37S mutation, under *S. cerevisiae ADH1* promoter control;

8. pFL113: yeast centromere vector (derived from pRS316) with a mutated *RNASEH2B* gene, coding for the subunit B of human RNase H2 carrying T163I mutation, under *S. cerevisiae ADH1* promoter control;
9. pFL116: yeast centromere vector (derived from pRS316) with a mutated *RNASEH2B* gene, coding for the subunit B of human RNase H2 carrying A177T mutation, under *S. cerevisiae ADH1* promoter control.

S. cerevisiae transformation

10 ml of cell culture in exponential phase of growth for each transformation reaction (~ 5×10^6 cells/ml) is centrifuged at 4000 rpm for 2 minutes at room temperature. The cell pellet is washed with 25 ml of sterile water and centrifuged again at 4000 rpm for 2 minutes. It is then resuspended in 1 ml of 0.1 M lithium acetate (LiAc) and the suspension is transferred to a new microcentrifuge tube.
Cells are centrifuged at 14000 rpm for 30 seconds and resuspended in 100 μl of sterile water for each transformation reaction. Aliquots of 100 μl are thus transferred to a new sterile microcentrifuge tube, cells are centrifuged and the pellet is resuspended in 360 μl of freshly prepared T-Mix, composed of 240 μl of 50% weight/volume Polyethylene glycol (PEG), 36 μl of 1M LiAc, 10 μl of 10 mg/ml carrier DNA and 74 μl of sterile water. A suitable amount of plasmid DNA to be transformed is then added to the solution, cells are gently

resuspended and incubated at 42 ° C for 20 minutes, to allow the DNA to enter the cells. The pellet obtained by centrifuging again at 2000 rpm for 2 minutes is washed with 1 ml of sterile water. Finally, cells are plated on the appropriate medium to select for transformants containing the introduced plasmid/plasmids.

Ty1 mobility assay: the fluctuation analysis and the Lea-Coulson median estimator

The purpose of the assay is to estimate the frequency of retrotransposition of the genomic *Ty1H3mHIS3AI* element in cultures of yeast strains having different genetic background.

The fluctuation analysis begins when each yeast strain is streaked on solid medium in order to allow single colonies to grow, and incubated at 20 °C for four days. At this temperature, the frequency of transposition increases about 10 times compared to frequency at 28°C. At least nine colonies for each strain, chosen to represent the particular yeast population, are separately inoculated in 5 ml of liquid medium in the absence of selective pressure and incubated at 20 °C for four days. In this way cultures are allowed to grow to saturation and each of them would not contain preexisting mutants. Then, a culture aliquot is plated on medium lacking histidine (His^-) and incubated at 28 °C for three days, so that only the His^+ mutant cells, in which Ty1 has retrotransposed, generate colonies. In parallel, an appropriate dilution of the initial culture is plated onto medium containing histidine (His^+), as a control of the total number of plated cells. Finally, the number of

grown colonies is recorded and the *Ty1H3mHIS3AI* retrotransposition rates are calculated through the Lea-Coulson method of the median.
The main parameters which must be considered to apply this method are: *m*, the number of mutations (retrotransposition events in this case) per culture, and *r*, the number of mutants per culture. The distribution of the numbers of mutant among the parallel cultures, derived from *r*, is used to calculate *m* thanks to the following equation:

$$\frac{\tilde{r}}{m} - \ln(m) - 1.24 = 0$$

Finally, the estimated value of *m* can be divided by the total number of cell divisions (number of cells-1) to give the mutation rate. The Lea-Coulson method is valid only if the value of *m* is between 1.5 and 15 and if the median number of retrotransposition events in a culture is between 2.5 and 60 (Foster 2006; Pope et al. 2008).

Main Results

Project 2:

1. Analysis of the role of RNase H in controlling the mobility of retroelements in yeast

Retrotransposons replicate through a "copy and paste" mechanism, inserting new copies of themselves into unique genomic positions. However, this is a rare phenomenon, typically occurring approximately one time per 10000 cells (Risler et al. 2012), mainly because retrotransposons are powerful insertional mutagens and their movements are controlled. For this reason, in order to facilitate the monitoring of transposition events in our study, we used the retrotransposition indicator gene *his3AI*, a phenotypic marker that helped us to understand how the transposition of the Ty1 element is controlled (Curcio and Garfinkel 1991). *Ty1H3mHIS3AI* construct consists of the genomic Ty1 element gene, the yeast *HIS3* gene, which is in the opposite transcriptional orientation relative to Ty1, and an artificial intron (AI) that disrupts the *HIS3* coding sequence and is in an antisense orientation relative to the *HIS3* gene. In this way, the intron can be removed by splicing only when Ty1 is transcribed, so the

mobility of *Ty1H3mHIS3AI* element is detected phenotypically by the formation of His^+ prototrophs (Fig. 14).

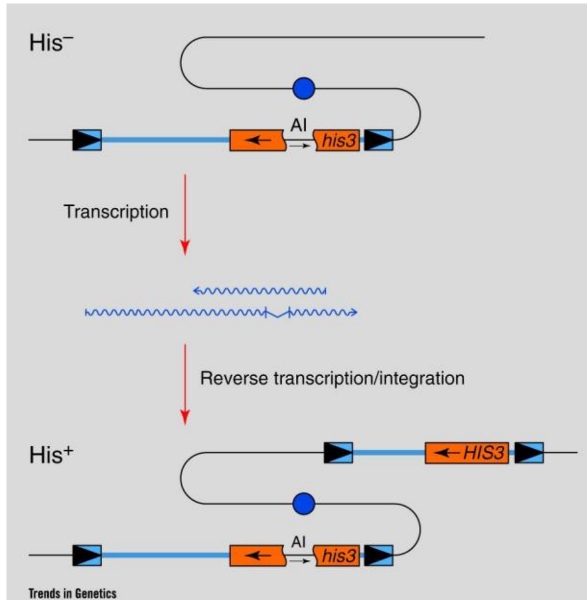

Figure 14: How to detect the retrotransposition of a Ty element marked with the indicator gene *his3AI*.
Due to the antisense orientation of the artificial intron (AI) relative to *HIS3*, a cell hosting the Ty1 element (blue line) marked with *his3AI* within its genome is phenotypically His^-. When the splicing of the transcripts occurs, the AI sequence is removed from the Ty1 RNA and this one can be used as a template for reverse transcription. In this way, a new DNA copy of the element will be synthesized and will contain a functional *HIS3* gene, which would render the cell phenotypically His^+. (*from Curcio and Garfinkel 1991)*.

1.1. Loss of RNase H1 and H2 genes results in a significant increase in Ty1 mobility

Yeast strains containing a single intact copy of the *Ty1H3mHIS3AI* element (meaning that it retains the intron sequence) integrated in a random location of their genomic DNA were used in an assay to measure the retrotransposition frequency. Given that our hypothesis is that the RNase H enzymes could have a role in the degradation of the RNA component of the RNA:DNA hybrids, thus supporting or blocking the movement of transposable elements throughout the genome, retrotransposition frequency was measured for strains lacking RNase H1 or H2 activity or both. As a positive control was used a strain bearing the deletion of *RTT101* gene, encoding the cullin-component of an E3 ubiquitin ligase (Fujii et al. 2009), identified as one of the "regulation of Ty1 transposition" genes of *S. cerevisiae*. The retrotransposition frequency of this mutant is higher then wt (Scholes et al. 2001).

The lack of both RNase H enzymes seems to considerably affect the mobility of the *Ty1H3mHIS3AI* retroelement: the retrotransposition frequency in these cells is higher than that observed in the wild type strain (Fig. 15). From this experiment it seems that host cell RNase H have a negative role in the control of retroelements mobility.

Genotype	Median r	Retrotransposition Frequency	Fold increase relative to wt
wild type	3	1.26×10^{-8} (6.55×10^{-9} - 2.68×10^{-8})	1
rtt101Δ	118	5.12×10^{-7} (4.49×10^{-7} - 6.33×10^{-7})	40.5
rnh1Δ	5	1.49×10^{-8} (1.07×10^{-8} - 2.27×10^{-8})	1.2
rnh201Δ	4	1.59×10^{-8} (1.31×10^{-8} - 2.78×10^{-8})	1.3
rnh1Δ rnh201Δ	67	1.92×10^{-7} (1.84×10^{-7} - 2.83×10^{-7})	15.2
rnh1Δ rnh201Δ	56	1.27×10^{-7} (1.05×10^{-7} - 1.46×10^{-7})	10.1

Figure 15: Loss of RNase H1 and H2 genes results in a significant increase in Ty1 mobility.
To detect spontaneous Ty1 retrotransposition events in strains bearing the *Ty1H3mHIS3AI* element, cells were streaked for single colonies on YPD plates and incubated at 20 °C for four days. Nine colonies for each strain were separately inoculated in YPD and incubated at 20°C for four days. Then, a colture aliquot was plated on medium lacking histidine and incubated at 28°C for three days. Quantitative *Ty1H3mHIS3AI* retrotransposition rates and confidence levels were calculated through the Lea-Coulson median test. Two strains with the same genotype (*rnh1*Δ *rnh201*Δ) were analyzed. Numbers in parentheses represent 95% confidence levels.

On the other hand, the two strains devoid of a single RNase H show retrotransposition frequencies very similar to those observed in the wt strain, indicating that in the control of the mobility of retroelements, as well as for hydroxyurea sensitivity (Lazzaro et al. 2012), the role of the two yeast RNase H is redundant. As expected, the *rtt101*Δ positive control shows a very high frequency of retrotransposition compared to wt strain, confirming the validity of the test.
Future analysis will be conducted to analyze the effect of RNase H enzymes on the moving of *Ty1H3mHIS3AI* element integrated in different positions of the genome in order to assess whether a different site of integration may influence how these enzymes play their role.

2. Investigating the effect of AGS mutations on human RNase H2 during the control of retrotrasposon mobility in yeast

As explained in the introduction, mutations in the genes coding for the RNase H2 cause Aicardi-Goutères (AGS) in humans. Almost all the mutations identified until now in AGS patients are found into genes coding for enzymes involved in the metabolism of nucleic acids. For this reason, the Aicardi-Goutières syndrome is thought to be caused by an incorrect intracellular nucleic acid metabolism. If not properly processed, intracellular nucleic acids may result in a continuous and high production of IFN α and in the consequent chronic activation of the immune system (Chahwan and Chahwan 2012).

Since the previous experimental data suggest that functional RNase H enzymes may reduce the number of retrotransposition events, probably facilitating the degradation of a metabolic intermediate necessary for the pathway, we hypothesized that a family of incorrectly processed nucleic acids in AGS patients cells could just be endogenous retroelements. This could explain the immune response outbreak without any virus has been detected in patients and than provide an answer to the open questions about the pathogenesis.

2.1. G37S mutation in human RNase H2A subunit causes the increasing of Ty1 element mobility

Recent experiments performed in our laboratory show that the three genes coding for the subunits of human RNase H2, introduced into yeast cells lacking the respective endogenous genes other than RNase H1 gene, produce a protein complex able to complement the absence of the yeast RNase H2. Transformed yeast RNase H deficient cells with genes encoding for human RNase H subunits, in fact, recover the sensitivity to a replication stress-inducing agent, hydroxyurea (HU) (Fig. 16), typical of cells lacking RNase H enzymatic activity (Lazzaro et al. 2012).

We then decided to check the accuracy of our previous deductions and possibly confirm, through this assay, that the human proteins are able to functionally replace those of budding yeast. At the same time, the goal of this experiment was to assess whether the mutations responsible for the onset of AGS can alter the retrotransposition control mechanisms.

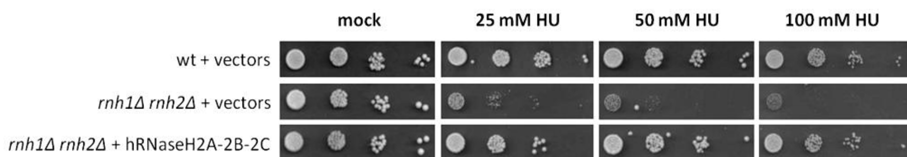

Figure 16: The human heterotrimeric complex fully complement the HU sensitivity due to the deletion of yeast RNase H2.
Sensitivity to sublethal doses of HU of the indicated strains was assayed plating 10-fold serial dilutions on selective medium plates lacking leucine, tryptophan and uracil (selective markers for human RNase H2 subunits genes) and incubated at 28 °C for two days. *rnh2Δ* comprises *rnh201Δ rnh202Δ rnh203Δ*.

We then transformed yeast strains having the *Ty1H3mHIS3AI* element integrated in the genomic DNA, analyzed in the previous experiment, with vectors carrying the human genes of the RNase H2 subunits either wild type or mutated. In particular, tested mutations are: RNASEH2A-G37S (G37S), RNASEH2B-T163I (T163I) and RNASEH2B-A177T (A177T), typical of AGS. The obtained strains underwent a retrotransposition assay to derive the frequency of retrotransposition of endogenous *Ty1H3mHIS3AI* and, also in this case, the resulting data were analyzed with the median test of Lea-Coulson.
As shown in the figure 17, the genes coding for the human RNase H2 are not able to fully complement the phenotype due to the absence of the corresponding yeast genes. Indeed, the frequency of retrotransposition of *rnh1*Δ *rnh201*Δ cells expressing the wild type form of the hRNase H2 is higher than that of wild type cells. Despite this, the catalytic G37S mutant shows a frequency of retrotransposition comparable to the control strain devoid RNase H activity (Fig. 17). Since from previous *in vitro* experiments we know that this mutation affects almost completely the catalytic activity of the enzyme (Shaban et al. 2010), we expected the observed phenotype.
The T163I and A177T mutations within the B subunit, conversely, induce within the cell a frequency of retrotransposition very similar to that observed in the strain *rnh1*Δ *rnh201*Δ expressing the wild type version of the hRNase H2. This reveals that these mutations, in this assay, do not seem to alter the activity of RNase H2 (Fig. 17).

Genotype	Median r	Retrotransposition Frequency	Fold increase relative to wt
wild type + vector	11	1.19×10^{-7} (6.94×10^{-8} - 1.45×10^{-7})	1
***rnh1Δ rnh201Δ* + vector**	72	8×10^{-7} (6.16×10^{-7} - 9.12×10^{-7})	6.07
***rnh1Δ rnh201Δ* + hRNase H2 wt**	31	3.12×10^{-7} (2.42×10^{-7} - 3.81×10^{-7})	2.06
***rnh1Δ rnh201Δ* + hRNase H2 G37S**	61	8.71×10^{-7} (6.52×10^{-7} - 1.04×10^{-6})	7.03
***rnh1Δ rnh201Δ* + hRNase H2 T163I**	24	3.32×10^{-7} (2.56×10^{-7} - 3.95×10^{-7})	2.07
***rnh1Δ rnh201Δ* + hRNase H2 A177T**	33	2.87×10^{-7} (1.88×10^{-7} - 3.46×10^{-7})	2.04

Figure 17: G37S mutation of the human RNase H2 subunit A induces an increase in Ty1 mobility comparable to the one due to the loss of the enzyme. Because the three different human genes, coding for the three subunits of the RNase H2, are localized on three centromeric plasmids into the yeast cells, the strains were streaked on selective medium plates lacking leucine, tryptophan and uracil and incubated at 20 °C for four days. Fifteen colonies for each strain were separately inoculated in the same type of medium and incubated at 20 °C for four days. Then, a colture aliquot was plated on medium lacking also histidine and incubated at 28 °C for three days. Quantitative *Ty1H3mHIS3AI* retrotransposition rates and confidence levels were calculated through the Lea-Coulson median test. Numbers in parentheses represent 95% confidence levels.

Conclusions and future perspectives

The functioning of many molecular processes of a cell can not be fully understood without a deep understanding of RNA metabolism. An important and highly conserved component of RNA metabolism is represented by RNA degradation. This biological system is responsible for monitoring and adapting to the cell needs the levels of different types of RNA, including above all messenger RNAs (mRNA), which amounts is necessary for the ordinary post-transcriptional control of gene expression (Arraiano et al. 2010). Aside from the global regulatory network, RNA degradation is crucial also for some genome integrity surveillance processes or defense mechanisms against viral infections; indeed, the accumulation of misincorporated ribonucleotides during DNA synthesis has been suggested as one of the most common types of DNA damage (Dalgaard et al. 2012) and the importance of cytoplasmic viral nucleic acids destruction has long been known (Goubau et al. 2013; Akira et al. 2006).

Ribonucleases are the main actors involved in RNA degradation and processing activities and they have been extensively studied; however, the complete repertory of their RNA substrates is still misconceived, even for well known Ribonucleases as RNase H. Eukaryotes and prokaryotes are endowed with either type I or type II RNase H, able to process RNA molecules belonging to RNA/DNA hybrids (Cerritielli and Crouch 2009). However, it is very recent the discovery of an *ad*

hoc pathway for the processing of double stranded genomic DNA tracts contaminated with even a single ribonucleotide (Sparks et al. 2012). In parallel, little is known about the role of endogenous RNase H in retroviral replication inside a host cell (Broecker et al. 2012) and which pathway could be affected by RNase H malfunctioning in human cells that then fires up rare genetic diseases, like the autoimmune Aicardi Goutieres Syndrome (AGS).

The purpose of this thesis was to explore the molecular pathways and consequences arising in absence of RNase H enzymes or in presence of different mutated RNase H2 versions, responsible for AGS pathogenesis in human, using *S. cerevisiae* as experimental model.

Cells lacking RNase H activity accumulate ribonucleotides in the genome, thus suffering of replication stress and genome instability; these cells can survive thanks to the Post-Replication Repair (Lazzaro et al. 2012), including a mutagenic pathway that relies on the ability of particular polymerases to replicate across damaged DNA, the Translesion Synthesis (TLS). From a previous study we knew that Pol ζ efficiently replicates rNMPs-containing DNA and that Rev1 plays a non-catalytic role in enhancing this function (Lazzaro et al. 2012). In this study we surprisingly observed that Rev1 has also a non-catalytic role in preventing Pol η activity, which in stressing condition due to the absence of RNase H and to hydroxyurea (HU) treatment, induce cell lethality. Furthermore, we provide evidence that Pol η toxicity observed in absence of RNase H is dependent on its polymerase activity and that is caused by its tendency to introduce an high number of rNMPs during the translesion synthesis when the dNTPs levels are downregulated by HU. It would be very interesting understand why

Pol η introduces ribonucleotides into the genome, if this effect is due only to the low concentration of available dNTPs or if it is driven by unusual structures in the DNA template, as previously fixed ribonucleotides. This last hypothesis would entail the formation of a toxic intermediate, maybe composed by ribonucleotides facing each other, responsible for multiple fractures in the DNA chains, which in turn would be responsible for cell death.

In addition, more work is required to further investigate how the TLS polymerases compete during translesion synthesis in response to dNTP pool size shrinkage and if is there a competition between Rev1 and Pol η for Ub-PCNA binding.

Our findings describe an unexpected mechanism for TLS that, resulting in increased genomic DNA damage, could be relevant to understand the replication stress cause in cells defective of RNase H, including humans affected from AGS; indeed, in AGS patients cells a chronic activation of the DNA damage response has been observed (Yang et al. 2007; Pizzi et al. 2015).

On the other hand, since recent studies showed a 100% correlation between AGS associated mutations in RNase H2 genes and the presence of interferon α-dependent inflammation (Rice et al. 2013), an emerging hypothesis envisage a possible involvement of RNase H2 in the same pathway that provide protection from viruses like HIV (Lepelley et al. 2011). Because of the absence of detectable viruses in AGS patients tissues, and given that another source of immunostimulatory nucleic acids is a class of viruses housing in the human genome, endogenous retroelements, we tried to better examine the role of RNase H in retroelement mobility control. We provide

evidence that in yeast both RNase H1 and RNase H2 are engaged in the retrotrasposition control pathway and that their role is clearly inhibitory. Probably, in the presence of functional RNase H the reverse transcription, a determinant step for retroelement transposition, can not be completed due to the degradation of RNA/DNA hybrids, as already suggested by the results of *in vitro* and *in vivo* experiments on *Escherichia coli* RNase HI (Ma and Crouch 1996). Conversely, loss of RNase H activity could result in the high accumulation of retroelement intermediates, which could then activate the innate immune response.
Moreover, our results on cells transformed with mutated RNase H2 genes confirm that G37S mutation significantly affects the catalytic activity of the complex (Shaban et al. 2010), that in our case is manifested in a loss of the ability to prevent retrotransposition events.
Nevertheless, many open questions remains. For example, which of the nucleic acid intermediates forming during retrotransposition could have the crucial role in triggering the aberrant immune response? Why, in AGS patients, also RNase H2 mutations not compromising the catalytic activity of the enzyme can induce the interferon α-mediated inflammation?
Hopefully, continued investigations will shine lights on these questions and on the complete biological relevance of RNase H activity.

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Part II

Manuscript in preparation

Project 1

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Conflicting roles of Translesion Synthesis Polymerases in cellular response to rNMPs incorporated in the genome.

Manuscript in preparation

Conflicting roles of Translesion Synthesis Polymerases in the cellular response to rNMPs incorporated in the yeast genome

Running head: Pol η, Rev1 and ribonucleotides misincorporation in DNA.

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Abstract

Accumulation of rNMPs in genomic DNA causes replication stress and has toxic consequences for the cells, particularly in the absence of RNases H enzymes required to remove them. In this situation DNA damage tolerance mechanisms are required for cell survival. Here, we investigated the contribution of three Translesion Synthesis (TLS) Polymerases (Rev1, Pol ζ and Pol η) to rNMPs incorporation in the budding yeast genome and in rNMPs tolerance mechanisms. We

previously demonstrated that Pol ζ efficiently replicates rNMPs-containing DNA and that Rev1 plays a non-catalytic role in supporting this function. Here we found that Rev1 has a non-catalytic role also in preventing a toxic effect of Pol η. Indeed, the polymerase activity of Pol η is toxic for cells in which dNTPs pool expansion is prevented, inducing cell death when RNases H are missing. Furthermore, we provide evidence that Pol η toxicity is due to its tendency to introduce higher levels of rNMPs during the TLS process when the dNTPs level is low. These findings highligth an unexpected mechanism for TLS that can cause replication stress in cells defective in RNases H, a situation which is present in the majority of patients suffering the Aicardi-Goutieres Syndrome.

Author Summary

Genomic DNA is continuously damaged by misinsertion of ribonucleotides. Normally, inserted rNMPs are removed by Ribonucleases H (RNases H), able to cleave the RNA component of RNA:DNA hybrid molecules. Cells lacking RNase H activity can survive thanks to tolerance mechanisms that permit the completion of DNA synthesis despite the presence of DNA lesions in an error-free or -prone manner, depending on the type of damage and the pathway involved. We show that accumulation of rNMPs in the yeast genome cause the recruiting of polymerase η (Pol η), a translesion synthesis (TLS) polymerase, which is able to introduce ribonucleotides in the newly synthesized DNA strand and induce cell death. We also

demonstrate that Rev1 can prevent this Pol η toxic effect. These findings are important for a better understanding of the TLS mechanisms and explain how replication stress is arising in the absence of RNase H activities.

Introduction

The accuracy of DNA replication is a key factor required for the correct transmission of an intact genetic information to daughter cells. Errors during DNA synthesis, although required for evolution, represent a powerful source of detrimental mutations and may become major causes for genome instability, cancer predisposition and genetic diseases.

Several biochemical processes exist to guarantee replicative DNA polymerases fidelity, such as their proofreading activity and the evolutionary conserved structural characteristics of the catalytic site which are responsible for water molecules insulation of the catalytic pocket, selectivity of correct nucleotide incorporation and the stringent steric selection on base pair shape and size within the active site [1-3]

Despite this, the misinsertion of ribonucleotides (rNMPs) in place of deoxyribonucleotides (dNTPs) in genomic DNA during DNA synthesis was recently detected to occur quite frequently in normal cells [4,5]. The pool of rNTPs significantly exceeds that of dNTPs both in prokaryotes [6] and in eukaryotes [5] and often replicative polymerases fail in discriminating between ribose and deoxyribose sugars [4]. Human Pol δ can incorporate one rNTP every ~2.000

dNTPs [5] and it has been calculated that an amount of rNMPs exceeding ten thousand/ yeast cell, and a million/mouse cell is inserted into genomic DNA during each cell cycle. Therefore, incorporation of rNMPS into DNA genomes can be considered as the most frequent source of cellular DNA damage in eukaryotic cells [4,7]. Replicative polymerases can potentially correct this mistakes using their 3' to 5' exonuclease activity [5], but if this repair process fails rNMPs would remain permanently incorporated in the genome.

rNMPs embedded in DNA molecules are mutagenic [8] and lead to genome instability [4]. The presence of rNMPs in DNA can also alter the B-conformation of a dsDNA helix [9-11] and negatively affect the proper assembly of nucleosomes [12]. Finally the presence of rNMPs may render chromosomes unstable since RNA is more susceptible than DNA to spontaneous hydrolysis [13] and can sensitize the DNA backbone to enzymatic nicking, as in the case of topoisomerase I-dependent rNMPs processing [14,15].

Unrepaired rNMPs in genomic DNA would impact on normal cell-cycle progression when a RNA-containing DNA template must be duplicated in the subsequent S-phase. In fact, replicative polymerases are not able to replicate rNMPs containing DNA [16,17], resulting in fork stalling, fork collapse and then replication stress. Previous work in several laboratories indicates that replication errors or replication failures are the main origins of genome instability [2,18,19].

The enzymes responsible for rNMPs removal from DNA, are Ribonucleases H (RNases H) which hydrolyze the RNA component of DNA:RNA hybrids molecules and these enzymes are conserved in all kingdoms of life [20,21]. They are involved in many cellular

processes, such as Okazaki fragments maturation [22] or R-loops removal [23]. There are two main types of RNases H: RNase H1 and RNase H2 [24]: RNase H2 can recognize a single rNMP within a DNA duplex substrate and cleave its 5'-phosphodiester bond [21,25,26], while RNase H1 requires the recognition of at least four consecutive rNMPs for nicking [27].

Recently, the Ribonucleotide Excision Repair mechanism (RER) has been characterized as a complete repair mechanism to remove rNMPs embedded in DNA and the main enzyme involved in this pathway is RNase H2. The mechanistic details of toxic rNMPs removal have been reconstituted *in vitro*: RNase H2 is required for the incision of the duplex, Fen1 or Exo1 endonucleases for flap ejection, Pol ε or Pol δ polymerases together with the PCNA clamp and RFC complexes for DNA re-synthesis, and DNA ligase I for nick sealing [28,29].

RNase H activity is crucial for the repair of rNTPs incorporated in DNA in *S. cerevisiae* [17,30] and its role in the preservation of genome integrity through rNTPs removal is conserved in mammals [7,31,32].

Concomitant loss of RNase H1 and RNase H2 in yeast demonstrate that RNase H1 cooperates with RNase H2 in eliminating rNMPs from the chromosomes and suggests that DNA polymerases can incorporate in some cases more then 4 consecutive rNMPs or clustered regions of rNMPs that can be processed by RNase H1 [17].

Loss of RNase H activity may have devastating consequences; indeed, mutations in the three human RNase H2 genes coding for the RNase H2 protein complex are found in ~ 60% of the Aicardi-Goutieres (AGS) patients. AGS is an autoimmunity-linked neurological disorder whose clinical characteristics mimic those of congenital viral

infections. Several studies indicate that AGS pathogenetic mechanisms may be linked to the formation of nucleic acid derivatives arising from altered repair of RNA-contaminated DNA [33-35].[32]

When the repair pathways are saturated or incapable of replication-blocking lesions removal before the S-phase beginning, Post-Replication Repair (PRR) facilitate cell survival by promoting the completion of the replication process, without mediating lesions repair, according to the "better safe than sorry" philosophy [36,37]. Damage tolerance mechanism is composed of two parallel sub-pathways, Template Switching (TS) and Translesion Synthesis (TLS). PCNA is the key regulator of pathway selection: in fact, its post-translational modifications direct damage bypass into one of the alternative sub-pathways [38-40]. TLS includes different specialized DNA polymerases capable of incorporating a nucleotide opposite to a lesion despite the conformational obstacles that most of the DNA lesions may impose [36,41]. In contrast to the replicative DNA polymerases, they have a more flexible active site that allows the accommodation of large bulky adducts [36,42] unable to fit into the active site of replicative polymerases. Moreover, they are non-processive and low-fidelity enzymes having lost the proofreading exonuclease activity [41,43].

Budding yeast has three TLS polymerases: Pol ζ, Rev1 and Pol η [44]. DNA Pol ζ is a heterodimer consisting of the Rev3 catalytic subunit and the Rev7 accessory subunit, which is able to boost the polymerase activity of Rev3 by about 200-fold [45]. Yeast cells lacking RNase H1 and H2 can use Pol ζ to bypass embedded rNMPs, as it can efficiently replicates over 1 to 4 consecutive ribonucleotides [17]. Moreover, the same study demonstrated that Rev1 plays a non-catalytic role

supporting Pol ζ function. Conversely, the contribution of Pol η to the tolerance of rNTPs has not yet been clarified, but in a previous study we observed that loss of this enzyme confers an unforeseen growth advantage when genomic DNA contains rNMPs [17].

This work was aimed to explore more deeply the interplay of these three TLS Polymerases (Rev1, Pol ζ and Pol η) during genomic-rNMPs tolerance, in absence of RNase H activity in *S. cerevisiae*. Here we reveal an unexpected role of Rev1 in preventing Pol η activity, which in low dNTPs concentration and in the absence of RNase H enzymes, is toxic for cells. Furthermore, we provide evidence that, in our experimental conditions, Pol η toxicity is due to its tendency to introduce an high number of rNMPs during the TLS process.

Results

1. Rev1 counteracts a putative toxic effect of Polη at low dNTPs concentration

As previously mentioned, ribonucleotides (rNTPs) are incorporated into the genome at high levels by replicative polymerases [46] potentially generating endogenous replication stress and genome instability[4]. Unremoved rNMPs in genomic DNA would impact on cell-cycle progression since, at the next round of DNA replication, a RNA-containing DNA template must be duplicated, and replicative polymerases are not able to fulfill this task [16].

Recently has been shown that budding yeast *Saccharomyces cerevisiae* cells devoid of both RNase H1 and H2 (*rnh1Δ rnh201Δ)* can use TLS

and Template Switch pathways to completely replicate their rNMPs-containing genome and survive. Moreover, in the absence of a functional Template Switch pathway, rNMPs-containing DNA can only be replicated by the action of TLS Pol ζ [17].
Surprisingly, we observed that the simultaneous deletion of all TLS polymerases suppresses the HU sensitivity of *rnh1*Δ *rnh201*Δ cells; in addition, we found that this suppression is fully recapitulated by deletion of the single *RAD30* gene encoding TLS Pol η, suggesting that such polymerase may have a toxic effect on cell cycle progression in the presence of HU [17] and Fig. 1.
We also observed that deletion of the single *REV1* gene in double mutant *rnh1*Δ *rnh201*Δ cells causes increased HU sensitivity although the Template Switch sub-pathway is fully functional. This result does not seem not to be related to the Rev1 role in supporting TLS Pol ζ activity, since deletion of *REV3* and *REV7*, encoding the two Pol ζ subunits, has no detectable effect on HU sensitivity of *rnh1*Δ *rnh201*Δ mutant cells (Fig. 1a).
On the contrary, the strong HU sensitivity of *rnh1*Δ *rnh201*Δ *rev1*Δ cells can be fully attributed to the toxicity of Pol η; indeed, it is almost completely suppressed by the further deletion of *RAD30* gene (Fig. 1). This observation strongly suggests that Rev1 plays an additional role in preventing a Pol η-dependent toxic function.
Although cells lacking RNases H activities are sensitive to replication stress agents [17], our genetic analysis suggests that Pol η toxicity is only visible in response to HU treatment. The exposure of *rnh1*Δ *rnh201*Δ *rad30*Δ cells to another agent capable of inducing replicative stress, such as methyl methane sulfonate (MMS), does not produce the

same effect. Indeed, while *rnh1*Δ *rnh201*Δ cells are sensitive to MMS, removal of *RAD30* does not suppress, but aggravates, this sensitivity (S1 Fig.).

Figure 1. Absence of TLS Rev1 polymerase increases HU sensitivity of *rnh1*Δ *rnh201*Δ mutant cells, and this effect is abrogated when TLS Pol η is non functional.

A. To test sensitivity to 25mM HU 10-fold serial dilutions of the indicated strains were plated on YPD or YPD + 25 mM HU. **B.** Quantification of cell survival in the presence of HU was calculated by distributing about 100 G_1-synchronized cells on solid medium with or without 25mM HU. Colonies were grown at 28 °C for three days and counted. The histogram is representative of three independent experiments. Error bars describe mean ± s.e.m.

2. The Rev1 catalytic activity is not required to prevent the toxic effect of Pol η

We investigated whether the role of Rev1 in controlling Pol η toxicity in the presence of HU was linked to its enzymatic activity. As shown in Fig. 2, by using a catalytically dead mutant strain (*rev1D467A-E468A*) (ref) we show that Rev1 polymerase activity is not required to prevent Pol η toxicity. In fact, the HU sensitivity of *rnh1*Δ *rnh201*Δ *rev1*Δ cells is rescued at the same level by addition of a wt version of *REV1* or by the catalytically dead mutant of *REV1* (Fig. 2).

This finding indicates that to suppress Pol η toxicity in HU is required the physical presence of Rev1 and not its enzymatic activity. An interpretation of this result might imply a physical competition

between Rev1 and Pol η for the same substrate or, alternatively, Rev1 interaction may negatively regulate Pol η.

Figure 2. Rev1 suppresssion of HU sensitivity in RNases H-deleted cells does not require its catalytic activity.

A. Wild-type *REV1* or catalytically dead *rev1D467A-E468A* are able to recover the HU sensitivity observed in a *rnh1*Δ *rnh201*Δ *rev1*Δ strain. HU sensitivity was tested as described in Fig. 1A.

B. Quantification of cell survival was calculated as explained in Fig. 1B on the basis of three independent experiments.

3. The catalytic activity of Pol η causes cell lethality when genomic DNA is rNMPs-enriched

To analyze the contribution of Pol η in HU sensitivity when RNases H genes are deleted, we have overexpressed wild-type or a catalytic mutant form of *RAD30* in a wt or *rnh1*Δ *rnh201*Δ genetic backgrounds. Serial cellular dilutions have been plated in absence or presence of 25 mM HU and cell growth was measured by drop test (Fig. 3A) and cell survival was quantitatively evaluated (Fig. 3B). Differently from what observed when we analyzed Rev1 contribution to translesion synthesis in *rnh1*Δ *rnh201*Δ double mutant cells in the presence of HU, the sensitivity to the drug is clearly increased when the wt form of Pol η is overproduced, while this is not the case when a catalytically dead form of of Rad30 is overexpressed (Fig. 3B and 3C). This finding indicates that the toxic Pol η effect observed in the absence of RNase H enzymes in HU-containing media requires its catalytic activity.

Figure 3. Pol η is responsible in producing toxic intermediates causing cell lethality when rNMPs-containing DNA cannot be repaired.

A. The overexpression of the wild type form of Rad30 in cells lacking RNase H activities induces cell death in the presence of HU. The sensitivity is suppressed by overexpression of a catalytically-dead Rad30 mutant. The sensitivity to HU was measured as described in Fig. 1A.

B. Quantitative data were assayed as described in Fig. 1B.

C. The levels of overproduced wt or mutant Rad30 forms were monitored by western blotting with anti-His-Tag Ab.

4. The activity of Pol η increases the rNMPs levels in genomic DNA in the presence of HU

It is well established that HU treatment causes a reduced concentration of dNTPs in the cells. Therefore, we hypothesized that in a *rnh1Δ rnh201Δ* double mutant background and in the presence of low dNTPs levels an aberrant replicative mechanism catalyzed by Pol η may introduce high rNMPs levels into the genome that, above a certain threshold, may be toxic for the cells.

To verify this hypothesis, we compared the amount of ribonucleotides incorporated in genomic DNA during a single cell cycle in unperturbed conditions or in the presence of HU. As shown in Fig. 4, we observed that HU treatment significantly increases the levels of rNMPs incorporated when RNase H activities are absent. However, the highest level of rNMPs incorporation is observed in cells lacking both RNase H and Rev1 activities and in such a genetic background the

simultaneous deletion of *RAD30* reduces the level of incorporated rNMPs. We interpret all these data by assuming that, in the absence of Rev1, Pol η gains access to DNA regions in which the replication process is impaired and Pol η activity is responsible for the increased level of rNTPs incorporation.

Figure 4. Pol η toxicity is caused by its capacity to incorporate ribonucleotides into DNA in the presence of HU.
HU-dependent incorporation of rNTPs by Rad30 observed in *rnh1*Δ*rnh201*Δ cells was assayed quantifying the amount of embedded rNMPs in DNA. The indicated strains were arrested in G1 with alpha factor and then released in fresh medium with or without 50mM HU. When cells were in S-phase, nocodazole was added thus permitting a single round of replication. Genomic DNA was extracted and rNMPs incorporation in the genome was measured as described in Material and Methods.

Discussion

Recently has emerged that replicative polymerases can introduce rNTPs instead of dNTPs during physiological DNA replication [46] and that these misincorporated nucleotides represent the most abundant "mistake" of replicative DNA polymerases. To cope with these events, prokaryotic and eukaryotic cells have evolved a specialized repair system, called RER (Ribonucleotide Excision Repair), that removes rNMPs from the genome [29]. The initial step of RER requires the

action of a RNase H enzyme and if RER fails the cells suffer replication problems [4,17,32].

Previously we have shown that yeast and human cells can tolerate rNMPs-containing chromosomes through the action of a tolerance mechanism called Post-Replication Repair (PRR) [17]. PRR comprises two sub-pathways: Translesion DNA synthesis (TLS) acting through the action of several TLS polymerases and Template Switch (TS) acting through recombinational mechanisms [38]. Here, we investigated the role of TLS polymerase in bypassing unremoved rNMPs from the genome and their direct involvement in rNMPs incorporation.

When ribonucleotides accumulate in genomic DNA, cells become sensitive to additional replication stress caused by external agents, such as MMS and HU, and this sensitivity dramatically increases if both branches of PRR are impaired [17]. Surprisingly, the single deletion of *RAD30,* the gene coding for TLS Pol η, recovers almost completely the HU sensitivity of yeast cells defective in both RNase H1 and RNase H2 (*rnh1*Δ *rnh201*Δ cells) (Fig. 1) and [17]. HU inhibits the ribonucelotide reductase, thus causing the depletion of the dNTP pools essential for DNA replication. On the contrary, the sensitivity to MMS, another DNA damaging agent that does not alter dNTP pools, is not altered by the further *RAD30* deletion in RNase H mutant cells (S1). These data suggest that, when cells replicate DNA in conditions of low dNTPs concentration, Pol η plays a negative role that affects the replication process.

Moreover, when Pol η is overexpressed in cells lacking RNase H activity, the HU sensitivity severely increases, while remains unaltered

in a wild-type genetic background. Conversely, the overexpression of a catalytic mutant form of Pol η causes an opposite effect, resulting in a decrease of the HU sensitivity observed in *rnh1*Δ *rnh201*Δ cells (Fig. 3). We also noticed that the overexpression of the catalytic mutant form of Pol η can recover the HU sensitivity of *rnh1*Δ *rnh201*Δ cells also when a wild-type copy of the *RAD30* gene is present, suggesting a dominant negative function of the catalytic mutant allele. Altogether, these genetic data indicate that the polymerase activity of Pol η generates some toxic intermediates when cells defective in any RNase H activity replicate their genomes in the presence of low dNTPs levels. To explain the phenotypes above described, we could suppose that, in the absence of functional RNases H, rNMPs accumulate in the genome and TLS polymerases are recruited to allow the completion of DNA replication. However, when dNTP levels are decreased as a consequence of HU treatment, Pol η assumes a toxic role. In a different, but not mutually exclusive interpretation, we can speculate that replicative polymerases stall in conditions of low dNTPs levels and are substituted by TLS polymerase and, in these conditions, Pol η can incorporate rNMPs more efficiently. In the absence of RNase H activity, this high level of Pol η-dependent rNMPs incorporation induces cell lethality. This hypothesis is supported by the identification of a specific role of Pol η and TLS in genomic DNA replication in presence of HU in human [47] and *E. coli* [48]. Moreover, the monoubiquitylation of PCNA, a crucial step for activation of the TLS sub-pathway in PPR mechanisms, is strongly induced by HU treatment [49].

We observed that deletion of *REV1* gene, encoding a peculiar TLS polymerase with dCMP transferase activity [50], dramatically increases the sensitivity to HU of *rnh1*Δ *rnh201*Δ cells, and that this increased sensitivity is fully abolished by additional deletion of *RAD30* gene (Fig. 1). This function seems to be independent on the additional role of Rev1 in regulating TLS Pol ζ activity, because deletion of *REV3* and *REV7*, encoding the catalytic and the accessory subunits of Pol ζ, does not alter the HU sensitivity of *rnh1*Δ *rnh201*Δ cells (Fig.1). Additionally, this unexpected Rev1 function is independent on its catalytic activity (Fig. 2). From these observations we hypothesize that Rev1 and Pol η likely compete for the same substrate and that Rev1 interferes with Pol η function when the dNTP pools are low. A good candidate for this substrate competition is the ubiquitylated form of PCNA because both Rev1 and Pol η can bind the PCNA complex using specific domains [51,52]. Alternatively, it has been shown in yeast, human and mouse that Rev1 and Pol η stably interact [53-55]; this interaction could inhibit Pol η-dependent toxic function in low dNTPs concentrations.

We hypothesized (Fig. 5) that, during replication in the presence of low dNTP pools, TLS polymerases are recruited on DNA as a consequence of replicative polymerases stalling. Rev1 can control the recruitment of both Pol ζ and Pol η, promoting the activity of the first while inhibiting the latter. In the absence of Rev1, Pol η can be more easily recruited on DNA and introduce additional rNMPs into the genome: this may allow completion of replication in the presence of reduced dNTPs pool. In RNase H-proficient cells, the incorporated rNMPs may be subsequently removed but, if RNase H activity is

impaired, the high amount of rNMPs into the genome becomes toxic for the cells, finally preventing cell cycle progression.

Consistently with this model, we demonstrated that during replication in HU, Pol η introduces higher rNMPs amounts especially in the absence of Rev1 (Fig. 4).

In conclusion, we also identified a novel protective role for Rev1 in preventing genome instability, as a consequence of increased rNMPs misincorporation by Pol η at low levels of available dNTPs. These discoveries can lead to important breakthroughs in the field of replication stress tolerance, even if other efforts are required to better understand TLS mechanisms in response to dNTP pool size changes and RNase H deficiency. Simultaneously, our finding in *S. cerevisiae* could serve as a paradigm for tolerance processes in higher eukaryotes, as humans suffering from genetic disorders which plague the normal replication process, or Aicardi-Goutieres Syndrome, caused by mutations in genes coding for RNase H2 subunits.

Figure 5. Hypothesized Model.

The stalling of replicative polymerases, due to accumulated genomic rNMPs in absence of RNases H or to reduced dNTP pools at replication sites in presence of HU, allow the recruitment of TLS polymerases in order to complete genomic DNA sythesis. In this context, Pol η catalyzes the frequent incorporation of rNTPs in chromosomal DNA, allowing the DNA synthesis to continue. On the other hand, Rev1 prevents Pol η intervention, possibly by direct inhibition or by competition for monoubiquitylated PCNA, simultaneously promoting Pol ζ activity. Functional RNases H would

finally remove rNMPs inserted by Pol η and thus prevent cell lethality.

Materials and methods

Yeast strains, plasmids, media and growth conditions:

All the strains in Table S1 are derivative of W303 *RAD5+*. Standard genetic procedures for cell transformation and tetrad analysis were used to generate them [56]. Deletions were made by the one-step PCR system [57]. pEGUh6-*RAD30* and pEGUh6-*rad30D155A,E156A* plasmids have been kindly provided by T.A. Kunkel and are described in [58]. For the indicated experiments, cell cultures were grown in YPD medium containing 2% glucose, raffinose 3%, raffinose 3% and galactose 2% and enriched or not by addition of the drugs indicated in the Figure legends. All the experiments were performed at 28°C.

DNA estraction

Genomic DNA was extracted with the Teeny Prep method: briefly, yeast spheroplasts were prepared with sorbitol 1 M, EDTA 0.1 M, 2-mercaptoethanol 14 mM and zymoliase 1 mg/ml (US Biological) at 37 °C, followed by cell lysis at 65 °C for 40 min with EDTA 55 mM, Tris-HCl 89 mM pH 7.5, SDS 0.44% and DNA precipitation at -20 °C in ethanol. After DNA resuspension, samples were incubated with RNase A 0.05 mg/ml at 37 °C for 30 min and purified through three washes with equal volume of phenol. Precipitation with isopropanol

was performed at -20 °C, pellets were air-dried and then DNA was resuspended in bidistilled water.

Sensitivity Assay

To evaluate the viability of each yeast strain after HU and MMS treatment, cell cultures in exponential phase of growth were diluted to 1×10^6 cfu/ml, and 10-fold serial dilutions were spotted on solid medium enriched or not with HU or MMS at the indicated concentrations. Pictures of the plates were taken after 2 or 3 days of incubation. In parallel, in order to obtain quantitative data, exponentially growing cells were synchronized in the G_1 phase by adding α-factor to 6 μg /ml. After appropriate dilutions, 100 cfu of each strain were plated on YPD with or without the indicated drugs. After 3 days of incubation the number of grown colonies was recorded. Experimental results were calculated from the ratio between the number of colonies treated or not with the drugs. The standard error of mean (s.e.m.) was calculated on three independent experiments.

Western Blot

TCA protein extracts were prepared and an equal amount for each sample was separated by SDS-PAGE using standard technique [59]. Western blotting was performed with anti-HIS tag Ab (70796-3) Novagen.

Ribonucleotides Incorporation Assay

Yeast cell cultures in exponential phase of growth were normalized at 6×10^6 cells/ml and blocked in G_1 by adding α-factor to 6 µg /ml. Cells were then simultaneously collected by centrifugation and each strain was resuspended in fresh YPD or fresh YPD enriched with 50 mM HU. 45 min after release from the G1 block, 20 µg /ml nocodazole was added to the cultures to arrest the cell cycle in M phase thus allowing the completion of a single cell cycle. Genomic DNA purified with the teeny prep protocol, digested with 0.5 U *Escherichia coli* RNase HII (New England Biolabs) in 50 µl at 37 °C for 2.5 h and precipitated O/N in 0.3 M sodium acetate pH 7 and ethanol at -20 °C. The day after, DNA was resuspended in TE 0.1%. For the subsequent steps we followed a previously described protocol [32].

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FIGURE 1

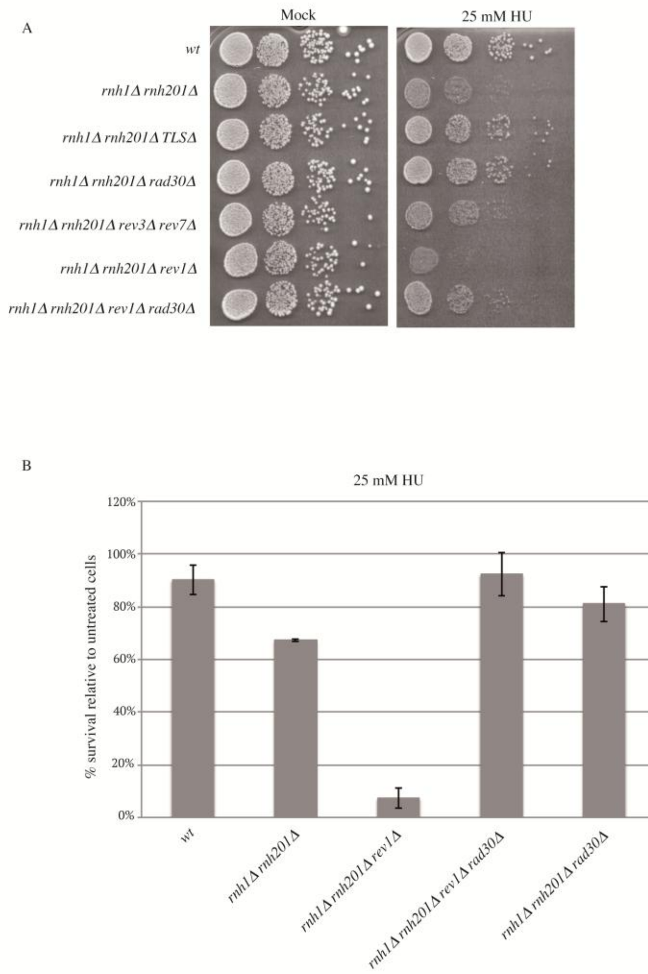


FIGURE 2

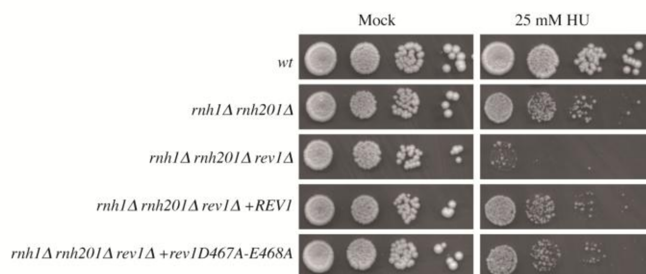


FIGURE 3

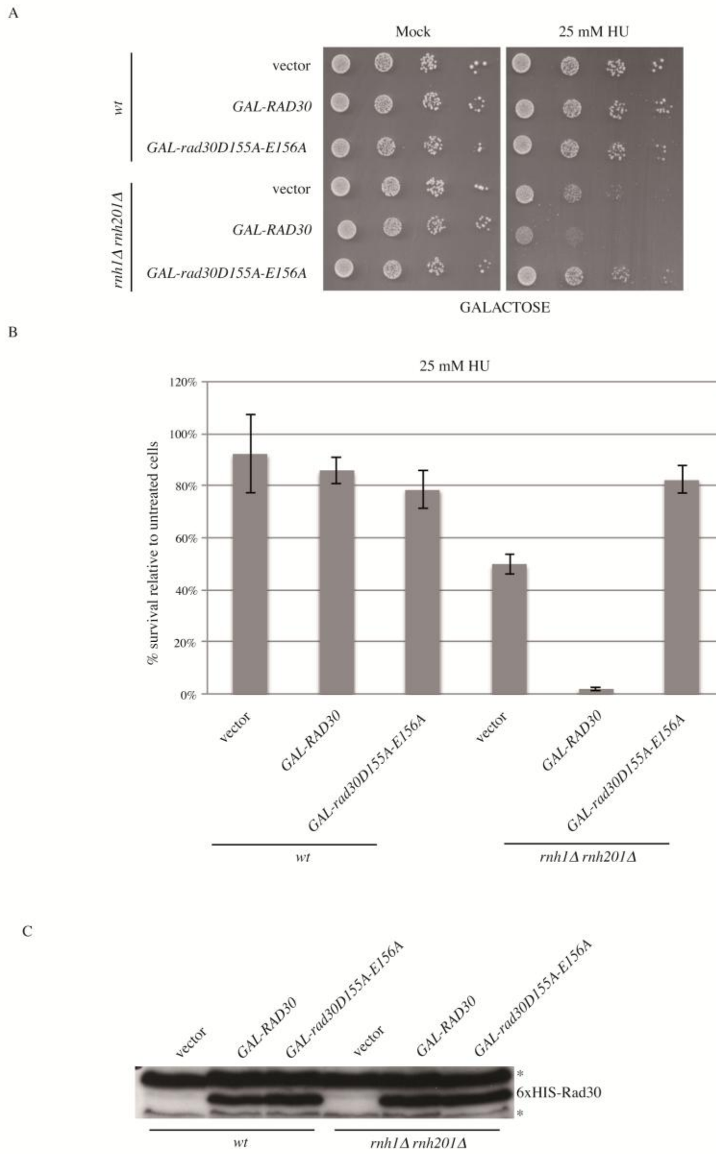


FIGURE 4

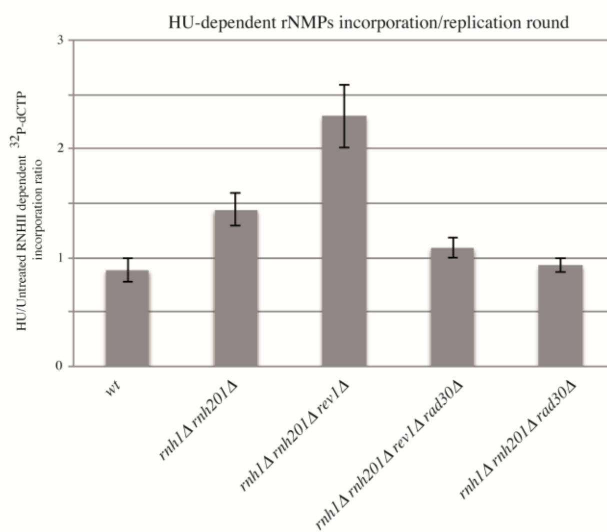
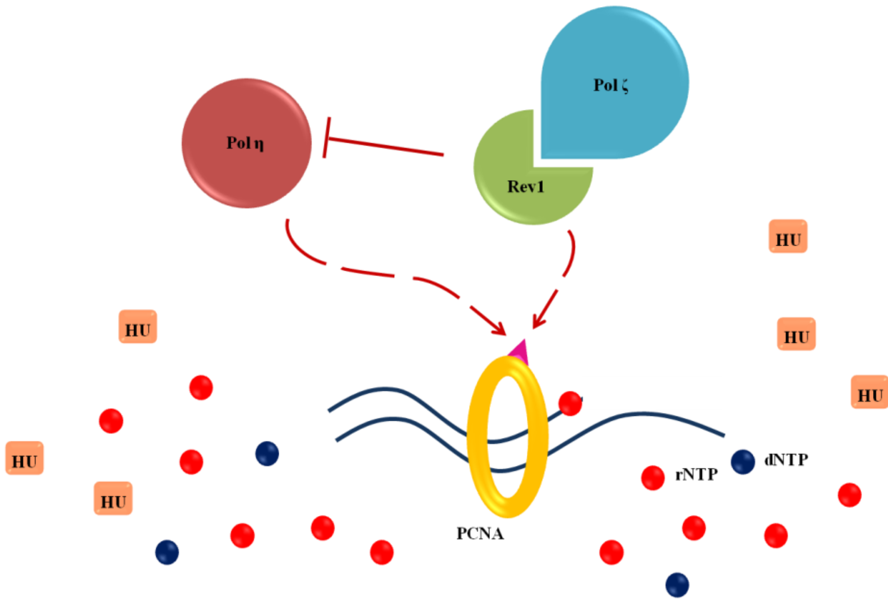


FIGURE 5

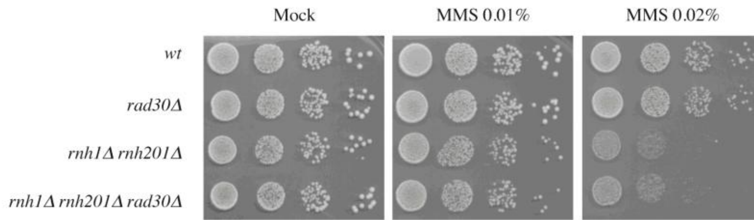


Supporting Information

S1 Figure. Pol η is not toxic for cells devoid of RNases H activities in the presence of MMS.

The figure shows that the sensitivity of *rnh1*Δ *rnh201*Δ cells to MMS is not dependent on the presence of a functional *RAD30* gene. To test sensitivity to 0.01% or 0.02% MMS 10-fold serial dilutions of the indicated strains were plated on YPD or YPD + MMS. The photograph was taken after 3 days of growth.

FIGURE S1



S1 Table. Strains used in this study

Strain name	Genotype	Source/ Reference
SY2080	W303 *MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1 can1-100 RAD5*	M. Foiani
YFL1213	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6*	[17]
YFL1409	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rev1::KANMX*	This study
YFL1775	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rev1::KANMX rad30::TRP1*	This study
YFL1773	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rad30::TRP1*	This study
YFL1559	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rev1::KANMX ura3::REV1:URA3*	This study
YFL1577	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rev1::KANMX ura3::REV1-DE467-468AA:URA3*	This study
YFL1389	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rev3::TRP1 rev7::HIS3*	This study
YFL1271	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 rev3::TRP1 rev7::HIS3 rad30::KANMX6 rev1::KANMX6*	[17]
YFL1419	(SY2080) *MATa* + pRS426	This study
YFL1420	(SY2080) *MATa* + pEGUh6-*RAD30*	This study
YFL1421	(SY2080) *MATa* + pEGUh6-*rad30D155A,E156A*	This study
YFL1422	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6* +pRS426	This study
YFL1423	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 +* pEGUh6-*RAD30*	This study
YFL1424	(SY2080) *MATa rnh1::HIS3 rnh201::KANMX6 +* pEGUh6-*rad30D155A,E156A*	This study
YSS21	(SY2080) *MATa rad30::KANMX6*	[60]