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**hExo1 and hRNaseH: two nucleases required for the maintenance of
genome integrity**

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Part I

Abstract

Genome integrity is continuously jeopardized by environmental insults, such as radiations, UV light and chemicals, as well as by endogenous factors including byproducts of cellular metabolism such as reactive oxygen species. In addition, various DNA transactions necessary for cell survival and proliferation (replication, recombination and transcription) are potential sources for genome instability.

To cope with this situation, cells have evolved mechanisms acting to maintain genome integrity whose failure leads to genome instability, which is the main cause of cancer predisposition and genetic disorders. Nucleases acting in various DNA repair pathways play important roles both in removing altered or mismatched nucleotides and in processing recombination or replication intermediates, thus facilitating subsequent repair steps.

In this thesis we studied the roles of two important nucleases (hExo1 and RNase H2) required for maintaining genome stability in human cells.

We uncovered a role for hExo1 in the cellular response to UV irradiation in human cells by demonstrating its recruitment at sites of UV damage together with NER factors and its role in the activation of the checkpoint signal transduction cascade in response to UV irradiation.

We also gained evidence on the role of RNase H2 in genome stability maintenance by counteracting the misincorporation of ribonucleotides (rNMPs) in the human genome. The presence of rNMPs in DNA molecules has been recently identified as the most common lesion in DNA, and mutations in genes coding for the RNase H2 complex subunits have been reported as the most frequent cause of the Aicardi-Goutières syndrome (AGS), suggesting a possible link between the RNase H2 molecular functions and AGS pathogenesis.

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1. Overview on genome instability: causes and consequences

The survival of all living organisms is based on the correct transmission of the genetic information through generations. Indeed, despite a certain degree of genomic instability caused by mutations or chromosome rearrangement crucial for genome evolution and genetic variation, a decrease in genome stability causes pathological conditions, such as cancer predisposition, premature ageing and inherited diseases. DNA has evolved as the storage molecule of genetic information because of its size and molecular stability; nevertheless, DNA integrity is continuously challenged by exogenous and endogenous insults (Figure 1).

Chemotherapeutic drugs, for example, are often able to induce specific DNA lesions eventually interfering with proper DNA replication at different levels: alkylating agents, such as mitomycin C or cisplatin, introduce covalent links between the two DNA strands or bases of the same strand (interstrand crosslinks or intrastrand crosslink respectively), thus blocking DNA replication and transcription ¹. Antimetabolites, such as pyrimidine or purine analogs, can also interfere with replication by interfering with proper nucleotide incorporation during S-phase². Finally, other drugs can target DNA-protein complexes impeding their proper assembly necessary for DNA processes such as transcription, replication and repair. This is the case of topoisomerase inhibitors, such as camptothecin or etoposide, that induce SSBs or DSBs formation by blocking topoisomerase I or II, respectively³⁻⁶. Other exogenous agents causing alterations of the DNA structure are ionizing radiations (IR) and the ultraviolet component (UV) of sunlight. While IR cause mainly double-strand breaks (DSBs) and single-strand breaks (SSBs), UV generate bulky lesions (cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs)) inducing a distortion of the DNA helix that represents an obstacle for all DNA transactions ⁷.

However, the vast majority of the DNA lesions derive from the normal cellular metabolism. Among the endogenous agents potentially able to cause DNA damage, is the aqueous cellular environment that going through spontaneous hydrolysis can trigger events, such as depurination or deamination, responsible for the formation of abasic sites or miscoding bases, respectively⁸. Byproducts of normal cellular processes such as respiration and lipid peroxidation constitutively threaten DNA integrity. Indeed, reactive oxygen and nitrogen species are responsible for 8-oxo-guanin (8-oxo-G) formation, perhaps the most common oxidative lesion ⁹.

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Finally, DNA replication itself is a source of potential DNA damage. Not only because during replication DNA is particularly vulnerable, but also because high replication fidelity is essential for genome stability.

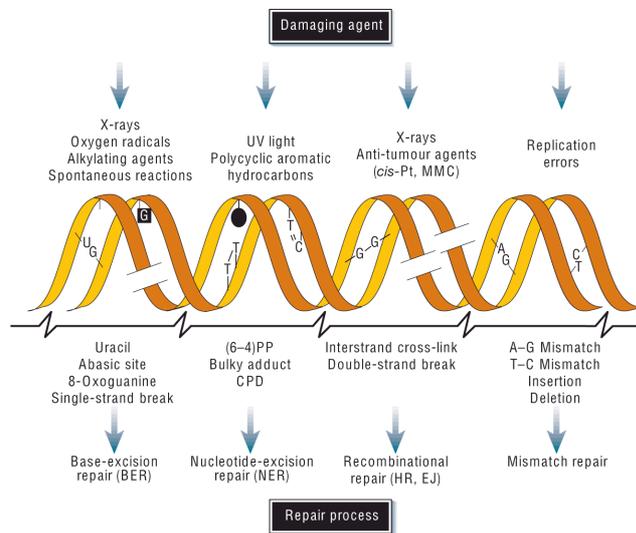


Figure 1: DNA damaging agents and repair systems

The most common source of DNA damaging agents are illustrated at the top, in the middle are reported examples of DNA lesions induced and on the bottom the most relevant DNA repair mechanisms involved in their removal.

(Adapted from Hoeijmakers, *Nature* 2011)

The Replication fork stability

During DNA replication, the replisome machinery must be able to deal with a series of events that may perturb replication fork progression and jeopardize DNA integrity.

Replication-associated DNA breaks can arise in many ways. First of all, replication of a nicked template inevitably results in DSB formation as a consequence of discontinuous synthesis of the lagging strand¹⁰. Second, when the replisome encounters an obstacle on the leading-strand (such as DNA adduct, a DNA secondary structure) or it collides with the transcription machinery a pause and/or an uncoupling between helicases and polymerases may occur. This event leads to the formation of ssDNA tracts, resulting in a situation known as “replication fork stalling”¹¹. Once the obstacle is removed, DNA synthesis restarts. Conversely, if the lesion is not repaired or the replication fork is not properly stabilized, the replisome disassembles and the replication fork “collapses”, generating ssDNA gaps and DSBs^{12,13}. Moreover, after stalling, the fork can reverse forming structures resembling a Holliday junction (HJ), that are often called “chicken foot”^{13,14}; they can revert back to a normal fork, but can also originate a DSB by cleavage¹⁵ or a stretch of ssDNA if processed by nucleases¹⁶.

Alternatively, lesions could block the synthesis of only one strand without impairing replication fork progression. If the lesion blocks the lagging-strand synthesis, a ssDNA gap or a DSB is created between two consecutive Okazaki fragments. If the lesion blocks the leading strand

synthesis, it can be bypassed through activation of a specialized repair pathway^{17,18} (see section *DNA damage tolerance mechanisms*).

Both ssDNA and DSBs are source of genome instability and trigger recombinogenic pathways or repair processes eventually leading to translocations, deletions, inversions or other gross chromosomal rearrangements¹⁹⁻²¹.

DNA Replication fidelity

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

The accuracy of eukaryotic DNA replication *in vivo* is estimated in the range of 10^{-7} to 10^{-8} base substitution error rate²². The fidelity of replicative DNA polymerases is ensured by multiple additive biochemical mechanisms: i) base-base hydrogen bonding; ii) water exclusion and enthalpy-entropy compensation, iii) geometric selection for shape and size, iv) dNTP binding affinity and dNTP-induced conformational changes²³. In addition, the major replicative DNA polymerases have evolved a 3'-5' exonuclease proofreading activity able to excise mispaired bases in the nascent strands²³. Moreover, insertion or deletion of bases can result also from strand misalignment during DNA synthesis, in particular during replication of repetitive sequences.

Misincorporation of ribonucleotides

Recent works provide a new connection between DNA replication fidelity and genome instability. Several studies demonstrated that replicative DNA polymerases could incorporate at unexpected high rates ribonucleotides (rNTPs) instead of deoxyribonucleotides (dNTPs) during DNA synthesis. This process depends on the intrinsic characteristics of the polymerases, but also on the relative concentration of dNTPs and rNTPs in the cell. Indeed, even during S-phase, the concentration of rNTPs exceeds the concentration of dNTPs many-fold (36- to 190-fold in *S. cerevisiae* depending on the nucleotide)²⁴. Normally, cells remove misincorporated rNMPs through the action of specialized nucleases, called Ribonucleases H (see section *Ribonucleases H*), able to recognize and degrade the RNA moiety in RNA:DNA hybrid molecules.

rNMPs misincorporation was estimated by *in vitro* assays using purified DNA polymerases and artificial substrates. Pol α displays the lowest fidelity, as it lacks exonuclease activity. It incorporates on average one rNMPs per 625 nucleotides (nt), while Pol δ and ϵ are estimated to incorporate one rNMPs per 5000 and 1250 nt, respectively²⁴. Considering these numbers, a

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S.cerevisiae cell would incorporate and subsequently remove up to 13.000 rNMPs in its genome per cell cycle; a study in mice lacking RNase H2 activity, estimates a rate of 1.000.000 rNMPs misincorporated per cell²⁵. rNMPs inside the genomes can thus be therefore considered the most frequent type of endogenous DNA lesion²⁴. Studies in budding yeast suggest that rNMPs misincorporation occurs randomly throughout the entire genome²⁶, although some differences are observed mainly depending on the sequence context^{24,26}.

Other processes during which rNMPs are incorporated in genomic DNA exist, such as during the synthesis of Okazaki fragments or during repair of double strand DNA breaks in G1^{27,28}. The latter involves the non-homologous processing of the DSB by two mammalian polymerases, μ and terminal deoxynucleotidyl transferase (TdT), that incorporate rNMPs at high frequency^{27,29}.

This surprisingly high frequency of rNMPs misincorporation by replicative polymerases suggests that a possible physiological role for genomic rNMPs. In the fission yeast *Schizosaccharomyces pombe*, incorporation of rNMPs has been implicated in mating-type switching by site specific incorporation of two ribonucleotides at the *mat1* locus³⁰⁻³².

Furthermore, *in vitro* experiments with human cell extracts, and *in vivo* studies in yeast recently showed that single ribonucleotides in the genome can act as an entry point for the mismatch DNA repair (MMR) machinery especially during leading strand DNA synthesis^{33,34}. In any case, if not removed, rNMPs embedded in DNA represent a problem for cycling cells.

First of all, the chemical nature of ribonucleotides represents *per se* a challenge to genome stability. In fact, ribonucleotides possessing a reactive hydroxyl group at the 2' position render the DNA backbone more vulnerable to spontaneous hydrolysis³⁵. Moreover, structural studies³⁶⁻⁴⁰ indicate that, when embedded in a double-stranded DNA, rNMPs cause alterations in the DNA helix structure, promoting a switch from the B- to the A-form. Finally, changes in the sugar pucker can make a primer terminus more difficult to be extended, thus affecting the efficiency of DNA replication process.

The improper enzymatic processing of genomic rNMPs or their accumulation, can also cause genome instability and increase the rate of short deletions in repeated sequences^{26,34,41}.

It has also been found that in the absence of RNase H2 activity, Topoisomerase I (Top1) can recognize and cleave misincorporated rNMPs, and this alternative processing is associated with an increase of 1-5 bp deletions in yeast⁴¹. Indeed, Top1 can hydrolyze the 3'-bond at an internal ribonucleotide, but the hydroxyl group at the 2' causes the intramolecular hydrolysis of the topoisomerase 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 cause slippage and consequent short deletions^{41,42}. Finally, genomic rNMPs can cause problems during replication acting as an obstacle to replication fork progression. *In vitro* assays demonstrated that replicative polymerases stall when rNMPs are present in template DNA strand, causing replication stress in RNase H-deleted budding yeast strains^{24,43}. The ability of such mutant cells to survive rely on the capacity of Pol ζ to bypass the obstacle, as it can efficiently replicates over 1-4 ribonucleotides stretches⁴³. The deleterious consequences of unrepaired genomic rNMPs summarized above, underline the importance of their correct removal to maintain genome stability.

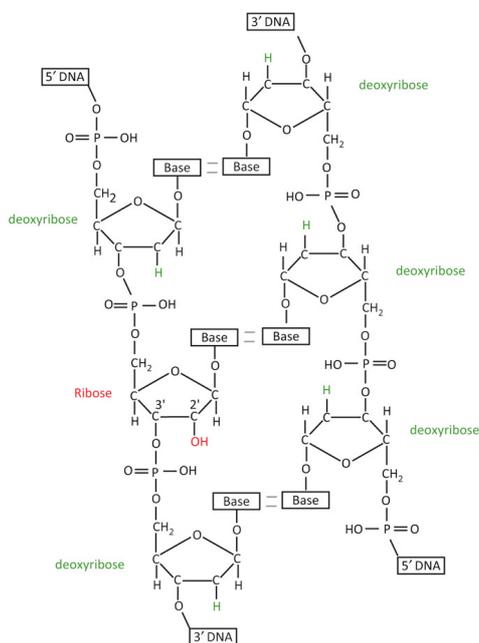


Figure 2: Schematic representation of a single ribonucleotide incorporated into a DNA duplex.

Ribonucleotides and deoxyribonucleotides are shown in red and green, respectively. Bases are outlined as boxes.

(from Dalgaard, *Trends in genetics*, 2012).

2. Genome integrity maintenance: the DNA damage response network

To counteract the number of insults threatening genome integrity, cells developed a complex network of surveillance mechanisms that continuously check the chromosome structure and coordinates repair of the lesions with cell cycle progression. Collectively, these surveillance mechanisms are commonly defined as the cellular DNA damage response (DDR).

DNA damage checkpoints

Checkpoint pathways are evolutionary highly conserved from yeast to humans, and the relevance of their biological functions is further underlined by the fact that several checkpoint genes are relevant for cell survival⁴⁴⁻⁴⁷.

In an unperturbed cell cycle, checkpoints ensure that DNA replication and chromosome segregation occur with the correct order and timing. In addition, in presence of DNA damage, checkpoints respond by inducing, if necessary, a temporary delay or block of cell cycle progression, providing enough time to repair the lesion and/or complete DNA replication.

However, the role of the DNA damage checkpoints reflects much more than a mere pausing. Growing evidence implicates the DNA damage checkpoints in other molecular mechanisms, such as activation and recruitment of DNA repair factors⁴⁸⁻⁵², control of transcriptional programmes⁵³, telomere length^{54,55} and, eventually, induction of apoptotic pathways^{56,57}. Depending on their functions at different cell cycle stages within the checkpoint signal transduction phosphorylation cascade, the various players of the cell cycle checkpoint are often classified as sensors, mediators and effectors.

Step 1: signals and sensors

Because a plethora of different lesions can occur in DNA it is important to understand how the checkpoint machinery can cope with them. Several studies in yeast converged on a model suggesting that all repair mechanisms process the lesions leading to a common intermediate recognized by the apical checkpoint kinases thus initiating the signal transduction cascade. This common intermediate is a tract of ssDNA covered by the Replication Protein A (RPA) complex⁵⁸ that is produced during the excision step in NER⁵⁹, during resection at DSBs⁶⁰ and uncapped telomeres⁶¹ and as a consequence of stalled replication forks⁶².

Two apical kinases, belonging to the phosphatidylinositol-3-related kinase family, are able to activate the checkpoint cascade in response to different types of lesions: ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related). The availability of cell lines derived from ataxia telangiectasia patients has been useful to begin our understanding of the ATM function. ATM is activated in response to DSBs and its recruitment and activation is mediated by the MRN (Mre11-Rad50-Nbs1) complex⁶³. ATM exists as inactive dimers and, once recruited to DSBs by MRN, it became autophosphorylated at Ser1981 and dissociates in active monomers⁶⁴. ATR, instead, is mainly activated in response to stalled replication forks. When a DNA polymerase stalls, a tract of ssDNA is generated by replicative helicases that continue to unwind

DNA helix ahead of the replication fork⁶⁵. The complex formed by ssDNA coated by RPA recruits ATR through ATRIP (ATR-interacting protein) which is the DNA binding subunit of the ATR-ATRIP complex⁵⁸.

Step2: mediators

Mediators are proteins that usually act downstream of the apical kinases, recruiting other substrates or acting as scaffolds for the assembly of complexes. In human cells three main mediator proteins exist: p53-binding protein (53BP1)^{66,67}, the topoisomerase-binding protein (TopBP1)⁶⁸ and the mediator of DNA damage checkpoint 1 (MDC1)⁶⁹⁻⁷¹.

Moreover, at the site of the lesion the histone variant H2AX became phosphorylated at Ser 139 in ATM- and ATR-dependent ways for a tract of 1Mb around the damage site, allowing the recruitment of other repair and checkpoint factors^{72,73} in structures called IR-induced foci (IRIF).

Step3: effectors

With the name of effectors are often indicated other protein kinases able to phosphorylate a large numbers of targets which are often proteins involved in cell cycle regulation or factors playing important roles in DNA replication and repair^{74,75}. Two major serine/threonine effector kinases with overlapping substrate specificity exist in human cells and are called Chk1 and Chk2. Chk2 is mostly activated by phosphorylation of Threonin 68 by ATM in response to DSBs, while Chk1 is activated by phosphorylation of serine 317 and 345 by ATR in response to replication stress and ,in resting cells, in response to UV irradiation⁷⁶.

DNA damage repair systems

Different types of DNA lesions are repaired by different repair processes. In mammals, it is possible to identify five main repair pathways with partially overlapping functions: homologous recombination (HR), non-homologous-end-joining (NHEJ), mismatch repair (MMR), base-excision-repair (BER), nucleotide-excision-repair (NER).

Homologous Recombination and Non Homologous End Joining

HR and NHEJ mostly take care of repairing double strand breaks (DSBs) that alter the integrity of the double helix, thus representing the most dangerous type of DNA lesion. While HR is the prevailing pathway in yeast, in mammals DSBs are mainly repaired by NHEJ; however, the choice of the pathway also depends on the cell cycle phase. HR dominates in S and G2, when the

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sister chromatids are available for the recombination process, while the less-accurate NHEJ acts mainly in G1⁷⁷.

Repair by HR in yeast is mediated by proteins belonging to the Rad52 epistasis group. The main step in this process is carried out by exonucleases that, acting at the 5' end of the DSB in a process called "resection", create a 3'-extended single-stranded overhang. This ssDNA tract allows the loading of recombination proteins required for the recognition of the homologous chromosome and subsequent strand invasion, followed by DNA re-synthesis and crossover resolution⁷⁸.

In mammalian cells, NHEJ is initiated by recruitment of the Ku heterodimer to the DSB site, where it binds the free DNA ends. This step is followed by the recruitment of the DNA protein kinase (DNA-PKcs) that, while bridging the DNA ends through protein-protein interactions, phosphorylates a nuclease required for partial processing of the DSB ends. Finally gap-filling by an X family DNA polymerase together with Ligase IV allows repair and sealing of the break⁷⁹.

Base-excision repair (BER)

BER is the main repair pathway acting in the correction of DNA lesions derived by cellular metabolism (oxidative damage, methylation, deamination, hydroxylation). It involves the action of several glycosylases specialized in the recognition of different base modifications. Once a glycosylase recognizes a lesion, the erroneous base is excised through cleavage of the glycosilic bond between the base and deoxyribose, leaving an abasic site. Subsequently, an apurinic/apyrimidinic (AP) endonuclease cleaves the phosphodiester bond. In the short-patch 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 FEN1/Rad27 removes the flap generated by strand displacement, and DNA ligase 1 seals the nick⁸⁰.

Mismatch repair (MMR)

The mismatch repair system detects and removes non-Watson-Crick base pairs and strand misalignments that take place during DNA replication.

It acts by catalyzing excision of the mispair-containing tract of nascent DNA and by promoting its error-free re-synthesis. It is also involved in removing mispairing caused by slippage during replication at repetitive DNA sequences⁸⁰. Indeed, MMR improves the fidelity of replication by several orders of magnitude⁸¹.

In mammals, the first step of MMR involves MutS α (formed by heterodimers of MSH2/6) that recognizes the mismatches and single-base loops, while hMSH2/3 dimers (MutS β) processes insertion/deletion loops. MutS α and MutS β recruit the heterodimeric complexes MutL α (Mlh1/Pms2) and MutL β (Mlh1/Pms1). It seems that MutL α , based on its ability to translocate along DNA after mismatch recognition, has a key role in the discrimination of the damaged (neo-synthesized) strand, likely due to the presence of a nick. Such nick is likely provided by removal of Okazaki fragments on lagging strand, while it is still unclear how it is generated in leading strand⁸¹. A recent work demonstrated that ribonucleotides incorporated into DNA during could account for this function³³.

Nucleotide-excision-repair (NER)

Eukaryotes possess two different subpathways of NER: global genome NER (GG-NER), which remove damage from non-transcribed chromatin and transcription-coupled NER (TC-NER), which takes care of lesions occurring on the transcribed strand and interfering with the transcription process^{82,83}.

NER is a multistep mechanism consisting of a lesion recognition step followed by excision of a segment of the strand containing the photoproduct, refilling and ligation. GG-NER and TC-NER share several factors, but differ in that employed for the damage recognition step. GG-NER uses XPC-hHR23B and the UV-DDB complexes, while TC-NER is activated by a RNA polymerase block, caused by lesion-dependent helix distortion, and its processing by CSB and TFIIH^{84,85}. The subsequent steps are identical for GG-NER and TC-NER. After the recognition of the lesion the TFIIH complex, that include the two helicases XPB and XPD, unwinds the DNA duplex for a length of ~ 30 nucleotides around the lesion site. At this point RPA stabilizes the generated ssDNA region, while XPA is implicated in the verification of the lesion⁸⁶. In the next step, two endonucleases, ERCC1/XPF and XPG, sequentially cleave near the photoproduct at the opposite extremities. ERCC1/XPF has 5' endonuclease activity, while XPG acts 3' relative to the lesion; the sequential incision of both nucleases generates a 24-32 bp oligonucleotide containing the UV lesion⁸⁷. Polymerases δ , ϵ and κ finally refill the resulting ssDNA gap and DNA ligase seals the nick⁸⁸⁻⁹⁰ (Figure 3).

Defects in NER are associated with three syndromes: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD), all displaying sunlight sensitivity. XP is characterized by severe photosensitivity with increased skin cancer frequency. CS and TTD lead to impaired physical and neurological development and premature ageing^{91,92}.

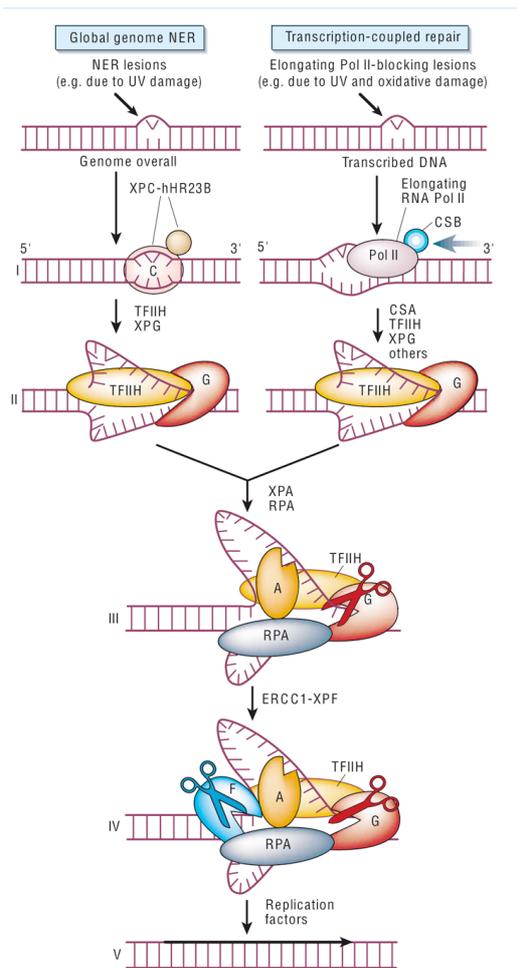


Figure 3: Model for GG-NER and TC-NER

After the initial step of lesion recognition that differs from GG-NER and TC-NER (I), both pathways process the lesion through assembly of preincision complex (II). TFIIH transcription factor contains XPB and XPD helicases that open a ~30bp of DNA around the lesion. XPA might act confirming the presence of the damage. Open intermediate is stabilized by RPA. The nucleases XPF and XPG respectively cleave at 5' and 3' (IV) generating a ssDNA tract that is finally refilled by replication factors (V). (from Hoeijmakers, *Nature*, 2001).

DNA damage tolerance mechanisms

Not all type of lesions can be repaired by the repair mechanisms described above and, remaining in the DNA template, are capable of blocking the replication fork. To avoid the risk of fork collapse, cells have evolved DNA damage tolerance mechanisms (commonly named Post Replication Repair (PRR)), which allow circumventing the block without removing the lesion. The DNA damage tolerance mechanisms include the translesion DNA synthesis (TLS) pathway, that employs non-replicative polymerases to bypass the lesion, and the Template Switch pathway (TS), that uses the undamaged strand to copy DNA thus avoiding DNA replication across the site of damage⁹³.

Translesion Synthesis

Synthesis past DNA lesions blocking replicative DNA polymerases, requires the employment of specialized translesion synthesis polymerases, characterized by different substrate specificities for various types of lesion⁹⁴. Most of TLS polymerases belong to the so called Y-family⁹⁵. Although they function at lower speed, processivity and fidelity compared to replicative polymerases, their active sites can adopt a more open structure that allows accommodation of the altered bases. *S.cerevisiae* possess three different TLS polymerases called Pol η , Pol ζ and Rev1, while in mammals more TLS polymerases exist (Pol θ , ι , κ , λ).

Template Switch pathway

This pathway involves the post-replication recombinational repair (PRRR) and the replication fork regression pathways. Both are error-free strategies, since avoid replicating template DNA at site of the lesion⁹³.

In PRRR, replication restarts 1Mb downstream the lesion, leaving a gap in the newly synthesized daughter DNA duplex that will be filled through recombinational events involving the two nascent DNA daughter molecules.

Replication-fork regression occurs by transient generation of the “chicken foot” structure, formed by the annealing of the two newly synthesized strands, one of which already replicated past the lesion, serving as a new template. Reversal of the “chicken foot” structure restores a normal replication fork, which can carry on replication downstream of the lesion^{13,93}.

Regulation of PRR pathways

Recent studies in yeast and human cells have revealed the direct links between the activation of both PRR pathways and post-translational modifications of proliferating cell nuclear antigen (PCNA). PCNA is a protein acting as scaffold for the binding of replicative DNA polymerases and several other proteins involved in DNA replication, repair and cell cycle regulation⁹⁶⁻⁹⁸.

Studies in *S.cerevisiae* proposed that different ubiquitylation patterns of PCNA would direct the choice between TLS and TS pathways. PCNA mono-ubiquitylation at lysine 164, carried out by the E2 ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin-ligase Rad18, has been linked to the TLS pathway, because it increase the affinity of PCNA for translesion polymerases. Instead, poly-ubiquitylation, resulting from addition of ubiquitin molecules at lysine 63 by the action of the E3 heterodimer Mms2-Ubc13 and the E3 Rad5, would channel the pathway into error-free TS.^{97,99,100} (Figure 4).

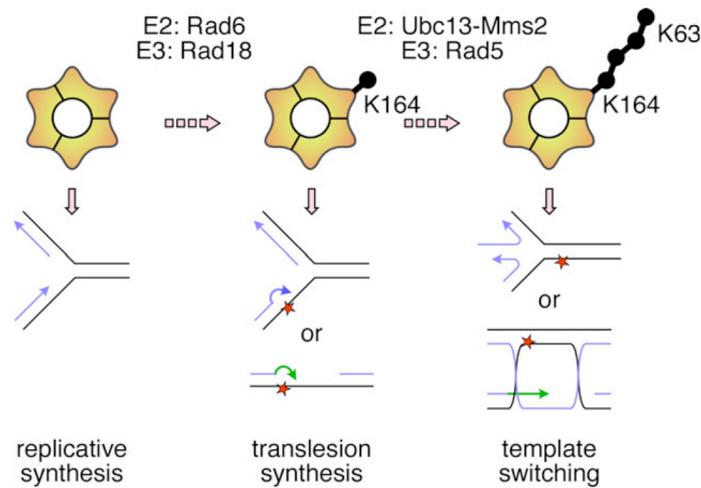


Figure 4: Posttranslational modifications of PCNA during DNA replication in eukaryotes. PCNA (circular symbol) is mono- and polyubiquitylated (black spheres) in response to DNA damage and replication stress. Each modification labels the clamp for alternative downstream pathways. Relevant lysines and conjugation factors are indicated for the individual steps.
(from Ulrich, *FEBS letter*, 2011).

3. Nucleases and genome stability

Nucleases cleave the phosphodiester bonds in a nucleic acid polymer. Nuclease activities are indispensable for life as they are integral parts of several DNA or RNA metabolic processes, including DNA recombination and repair^{101,102}.

In this thesis, I studied the roles of two important human nucleases in genome integrity maintenance: Exonuclease 1 (Exo1) and Ribonucleases H (RNase H1 and RNaseH2).

Exonuclease 1 (Exo1)

Exo1 was first identified in the fission yeast *S.pombe* where it is induced during meiosis¹⁰³.

Exo1 is a member of the Rad2 family of structure-specific nucleases and shows both 5'-3' exonuclease and 5'-flap endonuclease activities¹⁰⁴. Exo1 is present in yeast, fly and mammals and, at least some functions, are conserved since the human Exo1 (hExo1) is able to complement several defects observed in of budding yeast strains in which the *EXO1* gene was deleted (*exo1Δ*)¹⁰⁵.

hExo1 is highly expressed in tissues containing actively proliferating cells, such as fetal liver and thymus or adult bone marrow¹⁰⁶. This pattern of expression suggests that Exo1 has different roles during the development and differentiation processes. In human cells, alternative splicing of the C-terminus, generates two hExo1 isoforms: a shorter form (hExo1a) of 803 aminoacids, and a longer form of 846 aminoacids (hExo1b). So far, functional differences between the two variants has not been described. The nuclease domain is retained in the N-terminal part of the protein, where a domain for interaction with MSH3 (MutS homologue of MMR) is also present. Other protein-interacting domains are located at the C-terminus: a region for interaction with MLH1 and MLH2 (components of the MMR process) and a region for the binding with PCNA¹⁰⁷ (Figure 5). It is therefore evident that hExo1 is mainly involved in MMR, in which plays an essential role by functioning both as 5'-3' and as 3'-5' nuclease, and by contributing to the stability of the MMR complex¹⁰⁸. The activity of hExo1 has also been involved in end resection at telomeres¹⁰⁹⁻¹¹¹. Deletion of the human Exo1 gene reduces the mutation rate in telomerase-deficient cells, thus indicating its contribution in the generation of terminal deletions in cells with dysfunctional telomeres¹¹².

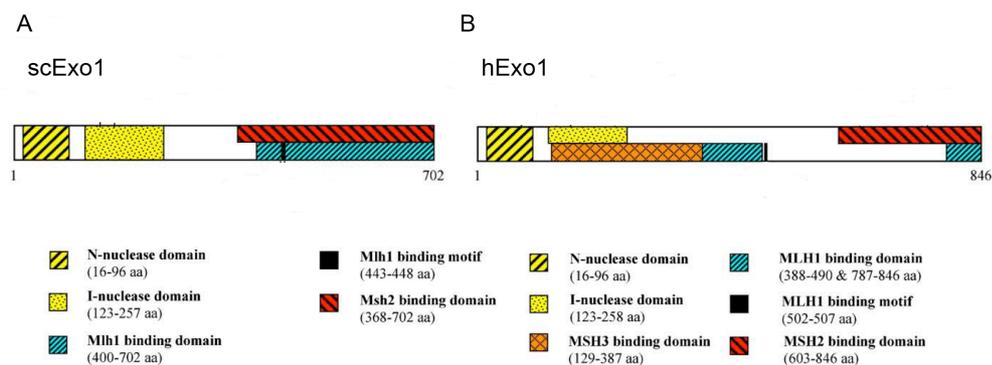


Figure 5: Schematic representation of Exo1

Functional domains and motifs of *S. cerevisiae* Exo1 (A) and human Exo1 (B).

(Adapted from Tran et al, DNA repair, 2004).

Studies in plant also linked Exo1 to the UV damage response, as it resulted up-regulated after UV-B treatment¹¹³. Furthermore, in *S.cerevisiae* Exo1 is strongly induced in response to UV, and cells deleted for the EXO1 gene are sensitive to high UV doses, supporting the involvement of Exo1 in response to UV irradiation¹¹⁴. While this function of Exo1 in yeast was described as NER-independent, a recent work in the same organism demonstrated that Exo1 processes NER intermediates. In yeast, Exo1 acts by generating longer ssDNA gaps allowing the recruitment of Mec1 (the yeast ortholog of ATR) and the activation of the DNA damage checkpoint in response

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to UV irradiation¹¹⁵. Finally, the work presented in this thesis provides evidence for a key role of hExo1 in connecting NER and checkpoint activation after UV damage¹¹⁶.

Mutations in hExo1 have been associated with microsatellite instability cancers (whose hallmark is defective MMR) since certain hExo1 variants could reduce MMR activity¹⁰⁸. Furthermore, the Exo1 mutant mice are characterized by increased susceptibility to lymphomas, higher mutation rate and sterility¹¹⁷. Finally, Exo1^{-/-}KO mice have impaired DNA damage signalling in response to telomere dysfunctions¹¹⁸.

Ribonucleases H (RNase H)

Ribonucleases H (RNases H) are the only enzymes able to cleave the RNA moiety in RNA:DNA hybrid molecules, and these enzymatic activities are present in all kingdoms of life, from retroviruses to humans. Two types of RNases H exist: RNase H1 and RNase H2 (called RNase HI and HII in bacteria) and both are present in the majority of the organisms. Types 1 and 2 have partially overlapping substrates: RNase H1 cleaves 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 molecule¹¹⁹. The *in vivo* roles of RNase H in eukaryotic cells are still not fully understood, and some differences exist among organisms. Indeed, while in yeast deletion of both RNases H is compatible with life, in mammalian cells, both are essential^{25,120,121}. Moreover, in mammals, RNase H1 has been implicated in mitochondrial DNA replication while this role is not conserved in yeast¹²⁰.

RNase H1 is a monomeric enzyme with highly conserved structure among eukaryotes. It consists of a N-terminal region containing the Hybrid Binding Domain (HBD), responsible for the non-sequence specific binding to RNA:DNA hybrids¹²², and a poorly conserved connection domain that links the N-terminus to the C-terminus where the catalytic RNase H domain is located¹²³.

In mammals, RNase H1-mRNAs have two in-frame AUG codons. When translation initiates from the first AUG, a protein with a mitochondrial targeting sequence (MTS) is produced¹²⁴ (Figure 6). Moreover, the levels of mitochondrial RNase H1 are modulated by a uORF that could be differently expressed in various tissues or under different stress conditions¹²⁵.

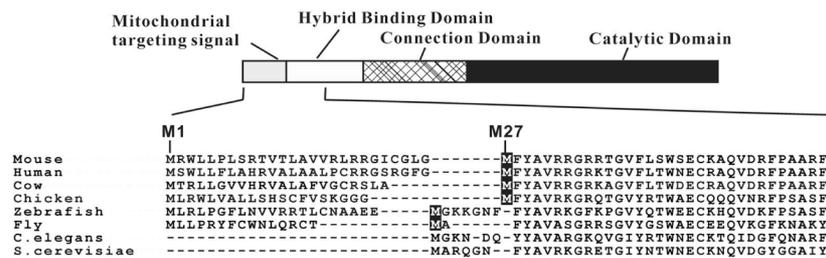


Figure 6: Organization of mouse RNaseH1 mRNA.

Organization of mouse RNase H1 is illustrated as a bar model. Two in-frame AUGs are conserved in RNase H1 from flies to mammals, as illustrated by the alignment of the amino acid sequence of N-terminal part (*Adapted from Suzuky et al, Mol and Cell Biol, 2010*).

The structure of RNase H2 is less conserved. In both archaea and bacteria, the RNase H2 enzyme is present as a monomer, while in eukaryotes it is a complex formed by three subunits, named A, B and C, all of which are required for its activity¹²⁶. The RNase H2A subunit possesses significant sequence homology, also in the catalytic site, with the prokaryotic enzyme, while the subunits B and C have no known prokaryotic equivalents. To date, the role of these auxiliary subunits is not completely understood but they likely act as a platform for assembly of the enzyme complex and for interaction with other proteins¹²⁷. Indeed, RNase H2B possesses a PCNA interacting peptide (PIP) box motif, though the functions of this interaction are not fully understood^{128,129} (Figure 7A).

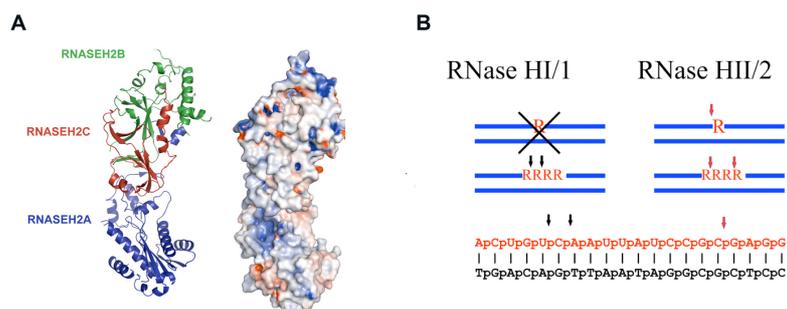


Figure 7: Crystal structure of RNase H2 complex and cleavage patterns of RNaseH enzymes.

A: Representation of human RNase H2 reveals a linear arrangement of the catalytic subunit RNASEH2A (blue) and the auxiliary subunits RNASEH2B (green) and RNASEH2C (red) (*Adapted from Reijns et al, J. Biol Chem, 2011*).

B: 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, FEBS J, 2009*).

State of the art

A recent work proposed a biochemical reconstruction of the Ribonucleotide Excision Repair (RER) pathway, through which RNase H2 would remove ribonucleotides from genomic DNA¹³⁰. Accordingly with the model described, RNase H2 initiates RER providing a 5' incision at rNMP site; subsequent strand displacement synthesis and flap cutting are carried out by Pol δ , in presence of PCNA, and by the nuclease Fen1. Finally Ligase I seals and completes the repair. Both Pol δ and Fen1 could be efficiently replaced by Pol ϵ and Exo1, respectively, conversely, the first incision step is totally RNase H2-dependent as RNase H1 is fully defective in promoting RER¹³⁰.

RNase H2 is also involved in processing of Okazaki fragments redundantly, at least in yeast, with Fen1 and the nuclease/helicase Dna2^{131,132}. Furthermore, over-expression of RNases H has been associated with removal of R-loops¹³³. 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 strand¹³⁴. Moreover, a high-throughput RNAi screening, identified RNase H2A as a factor that increase HIV replication¹³⁵, thus supporting the hypothesis of a role of RNase H2 in the regulation of endogenous retroelements metabolism¹³⁶. Finally, mutations in any genes encoding for RNase H2 subunits causes Aicardi-Goutières syndrome¹³⁷.

4. Aicardi-Goutières Syndrome (AGS)

Aicardi-Goutières syndrome (AGS) is an autosomal recessive inherited disease that typically affects newborns and infants. AGS as first described in 1984 by Jean Aicardi and Françoise Goutières¹³⁸ as an early onset encephalopathy characterized by basal ganglia calcifications, white matter abnormalities and chronic cerebrospinal fluid (CSF) lymphocytosis. A few years later, Lebon¹³⁹ added a further typical feature to the diagnostic profile of the syndrome: the presence of raised levels of interferon-alpha (INF-alpha) in the CSF in the absence of demonstrable infections of the central nervous system (CNS)¹⁴⁰. To date, only few hundreds of cases 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 phenotypes linked to congenital 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 impaired¹⁴¹.

AGS is genetically heterogeneous, characterized by loss of function mutations. Up to now, 6 genes have been involved in AGS: the 3'-exonuclease TREX1 (AGS1)¹⁴², all the 3 genes encoding subunits of RNase H2 (AGS2-4)¹³⁷, the dNTP triphosphatase SAMHD1 (AGS5)¹⁴³ and the RNA-editing enzyme ADAR1 (AGS6)¹⁴⁴.

The molecular origins of AGS are still unknown, but it is considered as an autoimmune disorder caused by aberrant accumulation of nucleic acids. Indeed, all genes involved in AGS are in some way implicated in the breakdown of nucleic acids. It has been suggested that a failure in removing nucleic acids would lead to chronic activation of receptors of the innate immune system, normally implicated in response to viral nucleic acids, thus causing up-regulation of interferon α ^{136,145}.

Approximately, 60% of the AGS patients have mutations in the three genes encoding the RNase H2 complex subunits. Among the RNase H2 genes, RNase H2B (AGS2) 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. Conversely, the earliest appearance and most severely affected AGS patients carry mutations in the C (AGS3) and A (AGS4) subunits^{128,141}, in particular, missense mutation c.139G->A (G37S) in subunit A was demonstrated to severely impairs enzymatic activity¹³⁷.

Aims of the project

AIM 1. Identify the hExo1 function in the NER-dependent DNA damage response after UV irradiation

UV light produces DNA bulky lesions, cyclobutane pyrimidine dimers and 6-4 photoproducts, which are the causes of the pathogenic effects of sunlight. Mammalian cells can cope with such lesions using the nucleotide excision repair (NER) pathway that acts through incision, removal and subsequent refilling and repair of the processed damaged DNA. The clinical relevance of NER is underlined by the existence of genetic syndromes (e.g., xeroderma pigmentosum, Cockayne syndrome, trichothiodystrophy) caused by mutations in genes coding for NER factors¹⁴⁶. Recently, our lab showed that yeast Exo1 is implicated in processing NER intermediates and in activating the G1 checkpoint in response to UV irradiation. Exo1 is a 5'-3' exonuclease and a 5'-flap-endonuclease, conserved from yeast to human, involved in many aspects of DNA metabolism, such as meiotic and mitotic recombination, mismatch repair and telomere processing.

We decided to investigate whether similar hExo1 functions exist in human cells, since it could be helpful for translational medicine to better understand the functional cross-talks between repair of the UV-induced photoproducts and the activation of the checkpoint cascade.

AIM 2. Identify the functional interplay among RNase H functions, post replication repair and causes of the Aicardi Goutières syndrome.

RNases H are the only enzymes able to remove, independently from the sequence, RNA associated with DNA. These RNA:DNA hybrid molecules are generated during different cellular processes such as DNA replication (Okazaki fragments)²⁴, transcription (R-loops)^{133,147} and telomere elongation¹⁴⁸, as well as rNMPs misincorporation during normal DNA replication²⁴. In eukaryotes two principal classes of RNase H exist: RNase H1 and RNase H2. RNase H1 is monomeric enzyme recognizing a stretch of at least 4 rNMPs in DNA, while RNase H2 is a trimeric complex with the unique capacity to remove single ribonucleotides incorporated in DNA. Importantly, mutations in any of the three genes encoding the human RNase H2 subunits cause the Aicardi-Goutières syndrome (AGS). AGS is an early-onset neuroinflammatory condition that mimics congenital viral infection resembling an autoimmune disease characterized by lymphocytosis and raised levels of interferon-alpha (INF-alpha) in the cerebrospinal fluid

Aims of the project

(CSF)¹⁴⁰.

Recently, our laboratory described a crucial function for RNase H enzymes and post replication repair pathways in overcoming ribonucleotides misincorporated during DNA replication⁴³.

We decided to verify whether similar RNase H and PRR functions are conserved in human cells.

In fact, a better understanding of the functions of RNase H2 in human may shed some light on its role in the pathogenesis of AGS.

Main results

AIM 1. Identify the hExo1 function in the NER-dependent DNA damage response after UV irradiation.

During repair of UV-induced lesions in non-cycling budding yeast cells, Exo1-dependent processing of NER intermediates competes with repair DNA synthesis. When the refilling reaction is impeded, Exo1 generates extended ssDNA gaps, which trigger checkpoint activation and will be refilled by long-patch repair synthesis¹¹⁵. We investigated whether hExo1 is involved in the cellular response to UV irradiation also in human cells.

To this aim, we analyzed the cellular localization of hExo1 in human fibroblasts, which have been locally irradiated with UV light. UV irradiation was confined to specific nuclear areas by using filters with 5 μm pores. By immunofluorescence analysis, we found that hExo1 accumulates at UV damage sites where it co-localizes with NER factors (XPA and XPB) (Sertic et al, 2011, Fig. 1A-B). To further understand the mechanisms of hExo1 recruitment at local UV damage (LUD) sites, we tested the physical interactions with components of NER machinery. Co-immunoprecipitation experiments in normal and xeroderma pigmentosum (XP)-derived fibroblasts, demonstrated that hExo1 interacts specifically with XPA in presence or absence of UV treatment, while no co-immunoprecipitation with other NER factors was observed (Sertic et al, 2011, Fig. 1D, Fig. S2).

Furthermore, to exclude the possibility that hExo1 accumulation at LUD was a NER-independent effect, we performed immunofluorescence experiments after UV irradiation in normal MRC5VI fibroblasts and in cells derived from XP patients, defective for different NER factors: XPA, XPG, XPG+XPGE791A (corrected with catalitically dead endonucleases), XPF and XPF+XPFD676A (corrected with catalitically dead endonucleases). The results demonstrated that hExo1 accumulation at LUDs depends on the assembly of the NER pre-incision complex and it requires the 5', but not the 3', incision performed by XPF and XPG, respectively (Sertic et al, 2011, Fig. 2A-B). Indeed, hExo1 was found to accumulate at LUDs in XPG+XPGE791A, since its presence, but not its catalytic activity, is an essential condition for XPF recruitment and activity⁸⁷.

In budding yeast, Exo1 was reported to process NER intermediates, producing ssDNA gaps when the gap-filling reaction is in some way impeded. Exo1 processing will lead to checkpoint activation and repair synthesis¹¹⁵. To verify whether this mechanism is conserved also in human

Main results

cells, we treated human fibroblasts with AraC, an inhibitor of DNA synthesis, thus causing an impediment to repair synthesis. Accordingly with the data obtained in yeast, we observed increased hExo1 accumulation at LUDs after such a treatment (Sertic et al, 2011, Fig. 13A-B). Furthermore, by measuring the incorporation of the nucleoside analog (EdU) in non-S-phase cells, we found that, in the absence of hExo1, repair DNA synthesis after UV irradiation was impaired, similarly to what is happening in NER-deficient XPA cells (Sertic et al, 2011, Fig. 4). Together, these data indicate that hExo1 recruitment at sites of UV damage is enhanced when gap-refilling is impeded and that hExo1 acts by generating larger ssDNA gaps, as it has been measured in yeast by electron microscopy analysis.

It has been shown that in mammalian cells treated with UV light, histone H2A is ubiquitylated by RNF8 and once ubiquitylated it functions as a docking site for DNA damage response factors. Interestingly, we found that H2A ubiquitylation is strongly reduced in cells depleted of hExo1 (Sertic et al, 2011, Fig. 5). Moreover, also the checkpoint signaling activated by UV treatment was impaired as a consequence of hExo1 depletion, as monitored by testing the Chk1 and p53 phosphorylation status (Sertic et al, 2011, Fig. 6).

Altogether, these results suggest that, similarly to what found in yeast cells, hExo1 plays an important role in the proper response to UV irradiation, likely generating ssDNA gaps at a subset of the UV-induced lesions capable to trigger the activation of the DNA damage checkpoint.

AIM 2. Identify the functional interplay among RNase H functions, post replication repair and causes of the Aicardi Goutières syndrome.

As recently published by our group⁴³, the simultaneous deletion of both RNase H1 and RNase H2 in budding yeast causes hypersensitivity to replication stress inducing agents, such as hydroxyurea (HU) and methyl methan sulfonate (MMS), due to the accumulation of ribonucleotides incorporated during DNA replication. Moreover the ability of double mutant cells to survive was dependent on the two post-replication (PRR) pathways, resulting in a constitutively active PRR, as revealed by accumulation of ubiquitylated PCNA.

We decided to verify whether these mechanisms are conserved in human cells, and to further investigate the role of hRNases H on genome stability.

To this aim we generated human cells stably depleted of RNaseH1 or RNase H2 by using lentiviral vectors carrying specific shRNA sequences. Since RNase H1 depletion resulted in high levels of cell mortality, likely due to its role in mitochondrial DNA replication¹²⁰, we focused our work on the phenotypes derived by silencing of RNase H2 alone (Pizzi et al, in preparation, Fig.

Part I

S1).

We obtained an efficient down-regulation of the RNase H2 enzymatic complex and observed that it correlates with a decreased proliferation rate in HeLa cells (Pizzi et al, in preparation, Fig.1A-C). This defect was only partially dependent upon cell mortality, (Pizzi et al, in preparation, Fig.1D-E), suggesting that cell cycle progression was somehow impaired. To confirm this hypothesis, we performed FACS analysis on exponentially growing HeLa cells and we reproducibly detected an accumulation of S and G2-M phase cells in RNase H2 depleted cells, respect to control cells (Pizzi et al, in preparation, Fig.2A-C).

Previous data in yeast suggested that cell cycle defects could be the consequence of replication stress, because, in the absence of RNase H activity, cells cannot counteract the effects caused by misincorporated ribonucleotides⁴³. Thus, we tested the status of PCNA as readout of PRR activation, in control and RNase H2-silenced cells. We found that PRR is indeed activated in cells depleted of RNase H2, as evidenced by the accumulation of ubiquitylated PCNA (Pizzi et al, in preparation, Fig.3A). This result was further supported by the observation that RNase H2 depletion causes hypersensitivity to low doses of the replication stress inducing agent hydroxyurea, respect to control cells (Pizzi et al, in preparation, Fig.3B). Indeed, this treatment is known to be problematic for cells already suffering some form of replication stress.

Although no checkpoint activation was evident by immunoblotting experiments on total cell extracts (Pizzi et al, in preparation, Fig.4A), analysis at single cell levels by immunofluorescence, revealed that depletion of RNase H2 increased the levels of 53BP1 foci formation (Pizzi et al, in preparation, Fig.4B-C), a phenotype linked to double strand breaks repair eventually caused by replication forks collapse¹⁴⁹⁻¹⁵¹. Furthermore, we observed an increased frequency in micronuclei formation in RNase H2-silenced cells (Pizzi et al, in preparation, Fig. 4 D), a phenotype that is indicative of chromosomal breakage¹⁵². These data strongly suggest that the down-regulation of RNase H2 functions is sufficient to trigger a general condition of replication stress and subsequent genome instability in human cells.

Since, AGS is caused by loss of function mutations rather than total loss of RNase H2 activity, our cellular model may mimic the pathological conditions. We thus decided to verify if the phenotypes observed in silenced HeLa cells were also detectable in RNase H2-mutated cells derived from AGS patients.

In agreement with what observed after RNase H2 silencing, we found that AGS cells had increased levels of PCNA ubiquitylation, indicating that also these cells were suffering replication stress (Pizzi et al, in preparation, Fig 5A). Moreover, phosphorylation of p53, a marker of DNA damage, was also detectable (Pizzi et al, in preparation, Fig 5B).

Main results

Finally, we directly verified whether accumulation of misincorporated ribonucleotides, observed both in yeast and mice lacking RNase H2 activity^{24,25,153}, could be a source of replication stress and genome instability also in our cellular model. To test this hypothesis, we used an assay based on the incorporation of radiolabeled nucleotides specifically at sites of rNMPs incorporation¹⁵³. The analysis of genomic DNA extracted from AGS and control cells, actually revealed an accumulation of misincorporated rNMPs in cells carrying RNase H2 mutations, being more evident in those cells carrying the mutation causing the most severe phenotype among the AGS patients (Pizzi et al, in preparation, Fig 6).

Taken together, our results demonstrate human RNase H2 plays a key role in removing ribonucleotides embedded in genomic DNA that, if left unrepaired, cause replication stress and possibly DNA damage and genome instability.

Conclusions and future perspectives

Failure to maintain genome stability is the main cause of cancer predisposition, premature aging and inherited disorders. To avoid such pathological conditions, cells have evolved a complex network of mechanisms, named DNA damage response (DDR), which combines and coordinates DNA damage checkpoints and DNA repair pathways. Among factors that are involved in DDR, nucleases cover important roles in both eliminating damaged bases and in processing intermediates during repair processes. Here, we propose novel roles for two important nucleases involved in genome integrity maintenance in human cells.

Our results on human Exo1, shed light on one of the major questions in this field: namely, how is generated the signal required to activate the DDR checkpoint cascade after UV irradiation and NER-dependent DNA repair. Our work revealed that hExo1 plays a key role in connecting NER processing of UV-induced DNA lesion with checkpoint activation, by producing larger ssDNA gaps at a subset of problematic UV-induced DNA lesions. It will be interesting to further investigate other aspects of the hExo1 mechanisms of action downstream of NER, as for example its involvement in 5'-flap displacement after XPF incision, that could better clarify the cross-talk between NER and checkpoint response. Moreover, it will be interesting to identify the characteristics of the subset of lesions further processed by the hExo1 action.

Recently, accumulation of misincorporated ribonucleotides during normal DNA replication has been proposed as the most common type of DNA damage¹⁵⁴. Therefore, RNase H2, the only enzyme to remove single rNMPs in DNA molecules, has acquired additional relevance in genome stability maintenance. An additional interest derives from the involvement of RNase H2 in the rare autoimmune disease, Aicardi Goutières syndrome (AGS).

In our work, we provide evidences that reduced RNase H2 activity is sufficient to trigger accumulation of ribonucleotides in the genome, thus causing replication stress and genome instability responsible for the activation of the DNA damage response. Since growing evidences link DDR to innate immune response, through induction of stimulatory ligands or expression of IFN genes¹⁵⁵⁻¹⁵⁷, we suggest a possible link between RNase H2 functions *in vivo* and AGS pathogenesis. It will be important to further investigate this aspect, together with other functions of RNase H2, which could be responsible for AGS. In this regard, a possible involvement of RNase H2 in endogenous retroelements metabolism is especially attractive. Indeed, all the other AGS related genes are somehow linked to HIV infection and/or endogenous retroelements pathways¹³⁶; moreover, RNase H2A was identified in a RNAi high-throughput screening as a

Conclusions and future perspectives

factor that facilitate HIV replication¹³⁵.

Beyond the RNase H2 functions and its role in AGS, ribonucleotides misincorporation itself is an intriguing aspect. It will be interesting to understand whether they are randomly incorporated in the genome or whether preferential sites exist. This will be helpful to uncover possible biological roles for rNMPs incorporation as it is suggested by the high frequency of misincorporation observed in various organisms.

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Part II

Published paper I

Sarah Sertic, Sara Pizzi, Ross Cloney, Alan R. Lehmann, Federica Marini, Paolo Plevani and Marco Muzi-Falconi

Human exonuclease 1 connects nucleotide excision repair (NER) processing with checkpoint activation in response to UV irradiation.

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Human exonuclease 1 connects nucleotide excision repair (NER) processing with checkpoint activation in response to UV irradiation

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UV light induces DNA lesions, which are removed by nucleotide excision repair (NER). Exonuclease 1 (EXO1) is highly conserved from yeast to human and is implicated in numerous DNA metabolic pathways, including repair, recombination, replication, and telomere maintenance. Here we show that hEXO1 is involved in the cellular response to UV irradiation in human cells. After local UV irradiation, fluorescent-tagged hEXO1 localizes, together with NER factors, at the sites of damage in nonreplicating cells. hEXO1 accumulation requires XPF-dependent processing of UV-induced lesions and is enhanced by inhibition of DNA repair synthesis. In nonreplicating cells, depletion of hEXO1 reduces unscheduled DNA synthesis after UV irradiation, prevents ubiquitylation of histone H2A, and impairs activation of the checkpoint signal transduction cascade in response to UV damage. These findings reveal a key role for hEXO1 in the UV-induced DNA damage response linking NER to checkpoint activation in human cells.

local UV damage | UV lesion processing | ssDNA | ubiquitin

UV light generates cyclobutane pyrimidine dimers and (6-4) photoproducts in DNA, which are responsible for the pathogenic effects of sunlight. UV-induced photolesions are repaired by nucleotide excision repair (NER), and mutations in genes coding for NER factors are responsible for inherited disorders, such as xeroderma pigmentosum (XP), Cockayne syndrome, and trichothiodystrophy (1). All patients with NER syndrome are photosensitive, but other clinical features vary. This is most likely a consequence of the mutated NER factors involvement not only in the repair of UV lesions, but also in the control of transcription. A specific NER defect can give rise to XP, whereas a transcription defect may cause Cockayne syndrome or trichothiodystrophy (1).

In response to UV irradiation and other genotoxic treatments, eukaryotic cells activate a surveillance system known as the DNA damage checkpoint, which is involved in cancer protection (2). The mechanism underlying the checkpoint response is a phosphorylation-based signal transduction cascade conserved in all eukaryotes, involving the integrated action of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia- and Rad3- related (ATR) PI3-kinases. In noncycling human cells, ATR acts as the apical kinase in the pathway, leading to checkpoint activation in response to UV irradiation, and ATR activation is responsible for the direct or indirect phosphorylation of several substrates (3). Moreover, it was recently established that H2A is ubiquitylated (uH2A) by the RNF8 E3 ubiquitin ligase in response to UV treatment (4), analogous to what has been found after exposure to ionizing radiation (5). Accumulation of uH2A at sites of local UV-induced DNA damage (LUD) was found to be required for MDC1 and 53BP1 recruitment (4). These findings indicate that although UV lesions and double-strand breaks (DSBs) are processed by different repair pathways, they eventually generate the same epigenetic mark.

We and others have shown that in yeast cells and in resting human fibroblasts, activation of the checkpoint induced by UV

irradiation requires a functional NER apparatus (4, 6–9). It is generally assumed that single-stranded (ss) DNA regions represent a common structure required for triggering the DNA damage checkpoint, as the result of damage processing (10, 11). However, NER-mediated excision of UV photoproducts generates short ssDNA gaps (~30 nt long), which might never be exposed, given that cleavage 3' to the UV lesion seems to occur only once the refilling of ssDNA gaps by repair synthesis is well under way (12). According to this scenario, it is difficult to predict the presence of long-lived ssDNA regions during NER.

In *Saccharomyces cerevisiae* cells, Exo1 processes was found to stall NER intermediates, generating longer ssDNA gaps detectable by electron microscopy, the refilling of which by repair synthesis in the presence of BrdU can be monitored by DNA combing (13). Moreover, such processing of NER intermediates is necessary for the recruitment of Mec1, the yeast ortholog of ATR, and for the activation of the DNA damage checkpoint in response to UV irradiation (13).

hEXO1, a member of the Rad2 family of structure-specific nucleases, has 5'-3' exonuclease and 5'-flap endonuclease activities in vitro (14, 15). Two isoforms of the hEXO1 gene, hEXO1a and hEXO1b, differing in a C-terminal extension of 43 amino acids in hEXO1b, have been described; however, no functional difference between the two products has been found (16). Several studies in yeast and multicellular eukaryotes have implicated this enzyme in various DNA metabolic processes, including meiotic and mitotic recombination, DNA repair, telomere maintenance, mismatch repair, and others (reviewed in ref. 17). The importance of EXO1 is underscored by the finding that *Exo1*^{-/-} KO mice have impaired DNA damage signaling in response to telomere dysfunction and increased cancer susceptibility (18, 19).

Our findings regarding the role of Exo1 in yeast led us to investigate whether its function in processing NER intermediates was conserved in human cells. Here we show that endogenous hEXO1 interacts with XPA. hEXO1 is recruited at LUDs in a NER-dependent manner, and this accumulation increases when completion of repair synthesis is inhibited. In UV-irradiated nonreplicating human primary fibroblasts, depletion of hEXO1 causes a noticeable reduction in the level of unscheduled DNA synthesis (UDS), impairs H2A ubiquitylation, and affects the checkpoint-signaling cascade. These findings support a general model in which stalling of repair synthesis at particular chromo-

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somal sites allows hEXO1 to process the repair intermediates, generating ssDNA regions that trigger the checkpoint response.

Results

hEXO1a and hEXO1b Accumulate at LUDs and Interact with the NER Preincision Protein XPA. hEXO1 has been implicated in several DNA transactions (16, 17), but its possible role in processing NER intermediates, generating the signal for checkpoint activation after UV irradiation, has not yet been investigated. To analyze the cellular localization of hEXO1 in response to UV treatment, we tagged both hEXO1a and hEXO1b isoforms with mCherry in MRC5VI-transformed human fibroblasts. We found that in S-phase cells (~15–20% of the cell population), both hEXO1 isoforms accumulate in proliferating cell nuclear antigen-positive foci, as reported previously (20). Application of UV irradiation of confined areas of individual cell nuclei using filters containing pores of a defined size has demonstrated that protein factors involved in the repair of UV lesions localize at LUDs (21). We found that after UV irradiation of cells through 5- μ m Isopore filters (Millipore), both hEXO1a and hEXO1b accumulated similarly at LUDs, colocalizing with NER preincision factors (Fig. 1A and B). Quantification of LUDs in which hEXO1a or hEXO1b colocalized with other NER factors revealed that 30–35% of XPA- and XPB-positive LUDs and 45–50% of RPA-positive LUDs also contained hEXO1 (Fig. 1C). This variation can be related to the reportedly different kinetics of XPA, XPB, and RPA recruitment at UV lesions (21, 22). Cells traversing S-phase and non-S-phase cells can be distinguished after local UV irradiation by staining for ethynyl deoxyuridine (EdU) incorporation. EdU staining is bright and homogeneously distributed in the nucleus in S-phase cells, and is less bright and localized at LUDs in non-S-phase cells (Fig. S1). Interestingly, the accumulation of hEXO1 at LUDs seems to be restricted to non-S-phase cells; no hEXO1 accumulation at LUDs was detected in 100 S-phase nuclei analyzed (Fig. S1).

To gain some insight into the mechanism(s) leading to hEXO1 recruitment at LUDs, we tested whether hEXO1 physically

interacts with known NER factors. hEXO1 is expressed at low levels in human nonproliferating tissues (23–26), and the endogenous protein is barely detectable in total crude extracts from cell lines because of its low abundance and limited affinity to the available Abs (23). However, hEXO1 can be recovered and analyzed by Western blot analysis after immunoprecipitation.

As shown in Fig. 1D, XPA, a protein that acts at an early stage of NER (27), coimmunoprecipitates with hEXO1 in the presence or absence of UV treatment. Attempts to detect interaction of hEXO1 with other NER factors by coimmunoprecipitation have so far been unsuccessful (Fig. S2).

hEXO1b Recruitment at LUDs Depends on Assembly of the NER Preincision Complex and Requires the 5', but Not the 3', Incision of UV-Damaged DNA. Because hEXO1 accumulates at damage sites together with NER factors, we tested the dependency of hEXO1 recruitment at LUDs on NER functions, by analyzing fibroblasts derived from patients with XP. MRC5VI control cells and XPA, XPF, XPF+XPFD676A (corrected with the catalytically dead endonuclease), XPG, and XPG+XPGE791A (corrected with the catalytically dead endonuclease) were transiently transfected with a construct expressing mCherry-tagged hEXO1b and then locally UV-irradiated. Fig. 2A shows that XPB localizes at LUDs in control and XP-mutated cells. Interestingly, hEXO1b is not recruited at LUDs in the absence of the 5' incision to the lesion (in the XPA, XPF, XPF+XPFD676A, and XPG cell lines). hEXO1b recruitment at LUDs was restored in the presence of the 5' incision, even though the 3' cut could not be executed (XPG+XPGE791A) (12). Quantification of the percentage of XPB-positive LUDs that also contain hEXO1b revealed slightly higher recruitment of hEXO1b in XPG cells expressing the catalytically dead nuclease (XPGE791A) than in MRC5VI control cells, with either dramatically reduced or totally abrogated recruitment of hEXO1b in other XP-mutated cell lines.

In conclusion, hEXO1b recruitment at the site of damage requires the NER preincision complex and at least the 5' incision reaction.

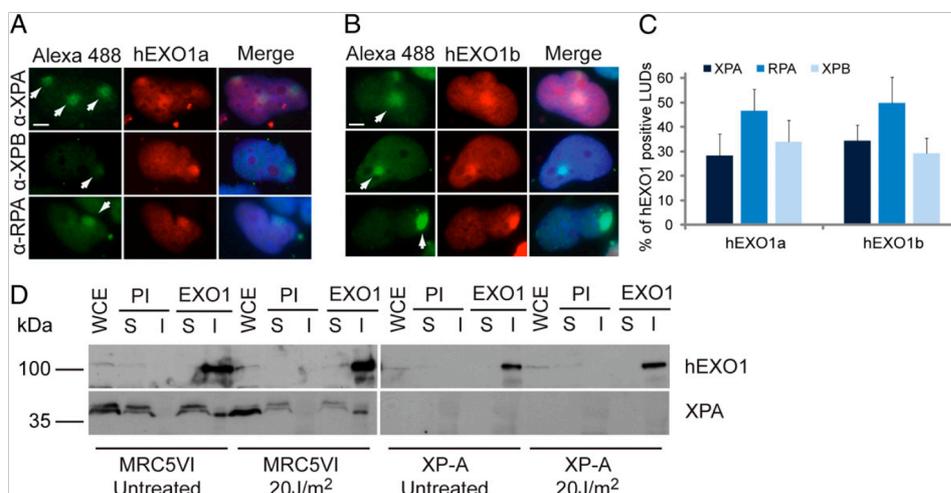


Fig. 1. Accumulation of hEXO1a and hEXO1b at LUDs. (A and B) MRC5VI cells were transfected with hEXO1a-mCherry (A) or hEXO1b-mCherry (B), seeded on coverslips, and locally UV irradiated (40 J/m²) through Isopore filters. After an additional 1-h incubation, cells were fixed and processed for indirect immunofluorescence using Abs against XPA, XPB, and RPA (Alexa Fluor 488), and nuclei were counterstained with DAPI. White arrows indicate LUDs. (Scale bar: 5 μ m.) (C) Quantification of immunofluorescence shown in A and B. Histograms represent the mean \pm SD of three independent experiments. Approximately 40 LUDs were scored for each experiment. (D) MRC5VI cells were mock- or UV-treated and harvested; lysates were prepared and resolved directly on SDS/PAGE (WCE, whole cell extracts, 10%) and incubated with preimmune serum (PI) as a control or anti-hEXO1 Ab (F15). Western blot analysis was performed with hEXO1 Ab (Ab4) and XPA Ab (12F5). XPA runs as a doublet because of incomplete reduction (32). S, immunoprecipitation flow-through; I, immunoprecipitate.

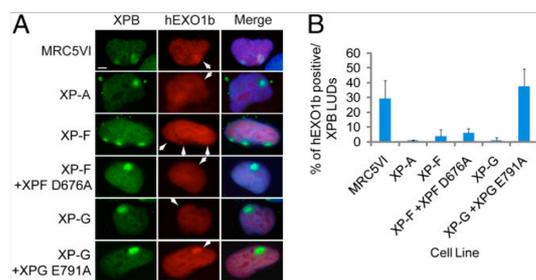


Fig. 2. NER preincision complex and 5' incision are required for hEXO1b accumulation at LUDs. (A) MRC5VI, XP12RO (XPA), XP2YO (XPF), XP2YO (XPF+XPF D676A), XPC51RO (XPG), and XPC51RO (XPG+XPG E791A) cells were transfected with hEXO1b-mCherry, seeded on coverslips, and locally UV-irradiated (40 J/m²) through Isopore filters. After 1 h of incubation, cells were fixed and processed for immunofluorescence against the indicated protein and nuclei were counterstained with DAPI. White arrows indicate the position of LUDs. (Scale bar: 5 μ m.) (B) Quantification of immunofluorescence shown in A. Histograms show the mean \pm SD of three independent experiments. Approximately 40 LUDs were scored for each experiment.

Inhibition of Repair Synthesis Enhances hEXO1 Accumulation at LUDs.

We found that in yeast Exo1 can enlarge NER gaps at a subset of UV-induced lesions when the gap-refilling reaction is somehow slower or impeded. The large ssDNA gaps generated by Exo1 lead to checkpoint activation and are subsequently refilled by repair DNA synthesis (13). If hEXO1 performs a similar function in human cells, then we would expect to see greater recruitment of hEXO1 at LUDs in the presence of 1- β -D-arabinofuranosylcytosine (Ara-C), a DNA synthesis inhibitor. hEXO1 reportedly undergoes degradation after inhibition of replication through hydroxyurea treatment (23), but this did not occur after Ara-C incubation (Fig. S3). Cells locally UV-irradiated and then incubated with Ara-C showed increased accumulation of hEXO1b at XPB-positive LUDs, from 30% to 60% in MRC5VI cells. Such recruitment was lost in XPA cells and was unaffected by Ara-C treatment (Fig. 3). Similar results were obtained for hEXO1a-mCherry (Fig. S4).

Depletion of hEXO1 Reduces UDS and Impairs Checkpoint Signaling.

NER is a multistep process requiring the refilling of ssDNA gaps generated by the processing of the DNA lesions. The UDS DNA resynthesis step requires lesion recognition and processing (27). NER-deficient cells, which are unable to process UV lesions, are UDS-defective. If hEXO1 were involved in extending the NER-induced gaps, similar to what occurs in yeast (13), then we would expect to find lower UDS levels in hEXO1-depleted cells.

A rapid nonradioactive technique for measuring UDS using the nucleoside analog EdU has been reported recently (28). We optimized hEXO1 silencing by using two different siRNA sequences. Human primary fibroblasts were transfected with siRNAs against hEXO1 as a negative control and against luciferase (LUC) and XPA as positive controls. Both XPA and hEXO1 were efficiently down-regulated (Fig. 4B). Mock- or UV-irradiated cells were then incubated with EdU for 3 h, after which EdU incorporation was quantified in nonreplicating cells, which are easily distinguished from S-phase cells (Fig. S5). Fig. 4A shows that EdU incorporation increased 3.6-fold above background after UV irradiation in control LUC-silenced cells, whereas in hEXO1-depleted cells UDS induction was reduced to 1.7-fold, approaching the reduction seen in siXPA cells (1.3-fold). Considering the EdU incorporation measured after UV irradiation in LUC-silenced cells to be 100%, depletion of hEXO1 causes a 52% reduction, and depletion of XPA causes a 65% reduction. These results suggest that hEXO1 activity is indeed involved in the formation of large ssDNA gaps after UV irradiation.

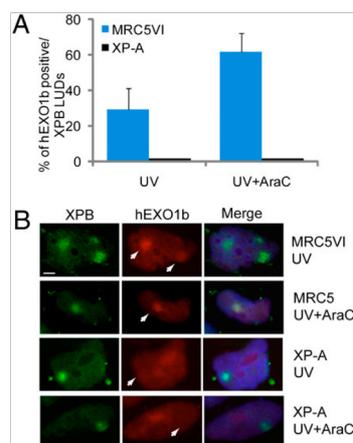


Fig. 3. Accumulation of hEXO1b at LUDs is enhanced when repair synthesis is blocked. MRC5VI and XP12RO (XPA) cells transfected with hEXO1b-mCherry were exposed to local UV irradiation (40 J/m²) and incubated for 1 h at 37 $^{\circ}$ C in the presence or absence of Ara-C. (A) Histograms indicating the percentage of XPB-positive LUDs that also contained hEXO1b-mCherry, with or without Ara-C treatment. Approximately 40 LUDs were scored in each of three independent experiments; values are mean \pm SD. (B) Representative images of cells with hEXO1b-positive LUDs (with or without Ara-C treatment) in the MRC5VI control (Upper) and in the XPA mutated cell lines (Lower). LUDs are indicated by white arrows. (Scale bar: 5 μ m.)

In response to both DSBs and UV irradiation, RNF8 ubiquitylates histone H2A, and this modification is required for recruiting downstream factors in the damage response (4, 5). Strikingly, when hEXO1 was silenced in quiescent primary fibroblasts and cells were locally UV-irradiated, XPA-positive LUDs were clearly detectable, but H2A ubiquitylation (uH2A) at damage sites was strongly reduced (Fig. 5A). Quantification of various experiments indicates that when hEXO1 was down-regulated (Fig. 5C), uH2A was reduced to \sim 40% compared with control (Fig. 5B).

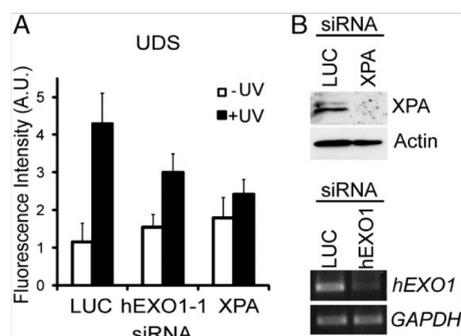


Fig. 4. Down-regulation of hEXO1 impairs UDS after UV irradiation. (A) Primary 48BR cells were depleted of hEXO1, XPA, or control (LUC); cultured on coverslips; and UVC-irradiated (20 J/m²), followed by incubation with 10 μ M EdU for 3 h, fixation, and conjugation of the fluorescent dye to incorporated EdU. The intensity of nuclear fluorescence, which is associated with UDS activity, was measured using a fluorescence microscope and image-processing software. For each sample, at least 100 nuclei were analyzed in three independent experiments; error bars represent SD. (B) Silencing efficiency was monitored by Western blot analysis for XPA (Upper) and by RT-PCR for hEXO1 (Lower).

Formation of ssDNA regions by DNA damage processing activates a signal transduction cascade (10, 11). Previous work demonstrated that recognition and processing of UV-induced lesions by NER factors is required for checkpoint activation in nondividing yeast and human cells (6, 7). Asynchronously growing XPA primary fibroblasts exhibit checkpoint activation after UV irradiation, as detected by Chk1 Ser317 phosphorylation; however, this effect is limited to cells traversing S phase, which do not require functional NER to trigger the checkpoint response (7). Yeast Exo1 also has been found to be required for an efficient and rapid NER-dependent G1 checkpoint response (13). In quiescent human primary fibroblasts, knockdown of hEXO1 expression by siRNA causes a defect in checkpoint activation after UV irradiation. In fact, ATR-dependent phosphorylation of both Chk1-Ser317 and p53-Ser15 was reduced to a level similar to that found in XPA fibroblasts or in cells in which XPA expression was knocked down by siRNA (Fig. 6A) up to 120 min after UV irradiation (Fig. 6B).

Discussion

A long-standing question in the DNA damage response field is how DNA damage signaling initiated by apical kinases is activated in response to various genotoxic agents (11). It is interesting that although DSBs and UV-induced photoproducts are processed by different DNA repair pathways and trigger signaling responses controlled by distinct apical kinases (ATM/ATR in human and Tel1/Mec1 in yeast cells), they eventually generate the same epigenetic mark involving H2A ubiquitylation (4). ssDNA regions covered by RPA are considered the structures

that activate the checkpoint in response to UV irradiation (11). In cycling cells, UV-induced DNA lesions are likely sensed by the DNA replication machinery, whereas in nondividing cells, recognition and processing of UV photoproducts by NER are required to activate the checkpoint (6, 7). However, how the short gaps (~30 nt long) generated by NER (29) mediate activation of damage signaling is unclear.

In *S. cerevisiae* cells, Exo1-mediated processing of a subset of NER intermediates is required for rapid checkpoint response and generates extended ssDNA gaps (11). Here we show that in human cells, both isoforms of hEXO1 localize at the sites of UV-induced DNA lesions, and recruitment of the nuclease is dependent on NER functions. Furthermore, endogenous hEXO1 physically interacts with XPA, whereas no interactions of hEXO1 with other NER factors were detected by coimmunoprecipitation analysis; such interactions may be transient and occur with different kinetics, making the analysis quite difficult.

Down-regulation of human hEXO1 impairs UDS, strongly supporting the idea that hEXO1 activity generates large ssDNA gaps, which are later refilled by repair DNA synthesis. We also found that H2A ubiquitylation is strongly reduced when hEXO1 is silenced, indicating that processing of the UV-induced lesions by the nuclease is required to generate this epigenetic modification. H2A ubiquitylation causes chromatin restructuring and creates docking sites for other DNA damage response factors (4). Down-regulation of hEXO1 also strongly reduces phosphorylation of the downstream checkpoint factors Chk1 and p53. Taken together, these data indicate that, as found in yeast, in human cells hEXO1-dependent processing of at least a subset of UV-dependent NER intermediates is an essential step in the formation of the ssDNA structure that triggers the signaling pathway activated by UV-induced lesions in noncycling human cells. This hEXO1 function in the response to UV irradiation must be added to the long list of DNA transactions in which hEXO1 has been implicated (reviewed in ref. 17).

The available data suggest that hEXO1 processing is triggered when the DNA refilling reaction is somehow impaired; indeed, hEXO1 accumulation at LUDs increases when the gap refilling reaction is inhibited by Ara-C treatment. Moreover, the UDS defect in hEXO1-depleted cells is milder than that seen in XPA siRNA-depleted fibroblasts, consistent with the hypothesis that only a subset of UV-induced DNA lesions are processed by hEXO1 in an NER-dependent manner. Finally, we found that for the accumulation of hEXO1 at LUDs, the 5' incision by XPF is sufficient, even in cases where the 3' incision by XPG is not executed and a flap structure is generated. Formation of a similar structure is predicted if repair synthesis starts but cannot be completed (12). In agreement with previous *in vitro* studies (14), we propose that hEXO1 can process *in vivo* a 5' flap DNA structure that may arise during the processing of some UV-induced lesions.

In human cells, the DNA refilling reaction during NER is complex and involves different DNA polymerases (30, 31). It is possible to speculate that this unexpected complexity might be linked to the chromatin structure around the UV-induced damage, including the number, location, and reciprocal positioning of lesions on the two DNA strands. Gaining a better understanding of these critical issues is a challenge for future studies.

Materials and Methods

Antibodies and Chemicals. The following Abs were used: anti-P-Ser-317-Chk1, anti-P-Ser-15-p53, and RPA70 (Cell Signaling Technology); anti-Chk1 and anti-actin Abs (Santa Cruz Biotechnology); anti-p53 (DO1; GeneSpin); anti-hEXO1 mAb (Ab4; Neomarkers); anti-uH2A (Millipore), rabbit anti-XPA (Sigma-Aldrich); and mouse anti-XPA (12F5; from R. D. Wood, Cancer Center, Smithville, TX), anti-XPB (1B3; from J.-M. Egly, University of Strasbourg), and anti-hEXO1 (F15; from S. Ferrari, University of Zurich). Ara-C (Sigma-Aldrich) was used at a final concentration of 1 mM for 1 h at 37 °C. Secondary Abs were goat anti-mouse or goat anti-rabbit conjugated to HRP (Western blot) or to Alexa Fluor 488 or Alexa Fluor 594 (immunofluorescence).

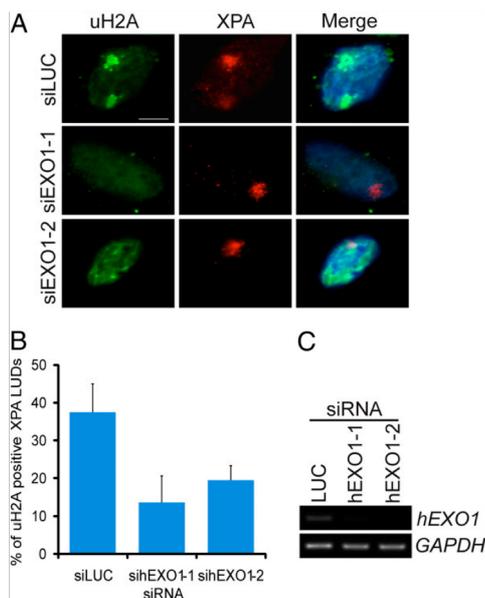


Fig. 5. hEXO1 modulates a crucial step linking UV-induced lesions to histone H2A ubiquitylation. (A) Quiescent 488R cells were transfected with siRNA directed against luciferase or hEXO1 (two different sequences, siEXO1-1 and siEXO1-2, were used) and locally UV-irradiated. At 1 h after UV irradiation, cells were processed to detect uH2A and XPA by immunofluorescence. Images show one representative nucleus for each treatment. (Scale bar: 5 μ m.) (B) Quantification of the percentage (mean \pm SD) of XPA-positive LUDs that are also positive for uH2A in siLUC, siEXO1-1, and siEXO1-2. (C) Silencing efficiency was monitored by RT-PCR.

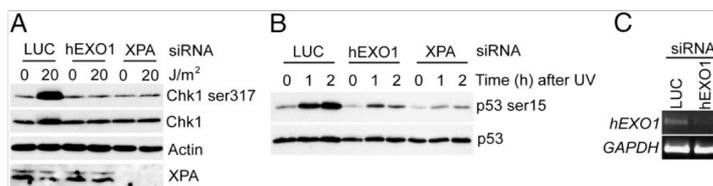


Fig. 6. hEXO1 is required for checkpoint activation after UV irradiation in quiescent human primary fibroblasts. Quiescent human primary fibroblasts transfected with siRNA against hEXO1 (sihEXO1-1 was used) or XPA were held under low serum conditions for 3 d before mock or UV irradiation. (A) Activation of the DNA damage checkpoint was examined at 1 h after UV irradiation by Western blot analysis using anti-P-Ser-317-Chk1. (B) Cells were UV-irradiated (20 J/m²) and harvested at different time points (1 or 2 h) after treatment. The DNA damage checkpoint was monitored by Western blot analysis using anti-P-Ser-15-p53 Ab. (C) hEXO1 knockdown was analyzed by RT-PCR.

Cell Culture and Serum Starvation. MRC5VI, XP12RO, XP2YO, XP2YO+XPF D676A, XPC1RO, and XPC1RO+XPG E791A (all SV40-transformed) were cultured in DMEM containing 10% FCS and kept at 37 °C in a humidified atmosphere with 5% CO₂ (12). The 48BR primary fibroblasts were cultured in DMEM containing 15% FCS. For serum starvation, cells were incubated on plates for 72 h with medium containing 0.5% FCS.

Plasmid and Constructs. hEXO1a and hEXO1b were PCR-amplified from hEXO1b-omnitag [kindly provided by S. Ferrari (23)], and then cloned into pmCherry-N1 at BamHI/HindIII sites.

cDNA Transfection. MRC5VI and XP12RO fibroblasts were transfected using Metafectene Pro (Biontex Labs) following the manufacturer's instructions. XP2YO, XP2YO+XPF D676A, XPC1RO, and XPC1RO+XPG E791A were transfected using Ca₂PO₄ (10 μg DNA each 1.5 × 10⁶ cells) and analyzed at 24–48 h posttransfection.

siRNA Transfection. Cells were seeded and transfected twice with 50 nM siRNA (MWG siLUC: CGU ACG CGG AAU ACU UCG ATT; sihEXO1-1: GCA CGU AAU UCA AGU GAU GTT; sihEXO1-2: CGU AAA UAG AAG AAU AAU UTT; siXPA: ACC UAG AAG AUG ACA UGU ATT) using HiperFect (Qiagen) in DMEM with or without serum depending on the experiment. Cells were harvested and analyzed at 72 h after the first transfection.

UV Irradiation. The medium was removed, and cells were washed once with PBS and then irradiated with a UV lamp (254 nm) at a dose of 20 J/m². The medium was then added back, and cells were returned to culture conditions for different times. LUD was obtained by irradiating cells through a 5-μm Isopore filter (Millipore) with a UV box (254-nm wavelength) at a rate of 0.6 J/m²/s and a final dose of 40 J/m². The same protocol was used for EdU detection at LUDs.

Immunoprecipitation and Western Blot Analysis. MRC5VI and XP12RO cells were either mock-transfected or transfected with hEXO1a-mCherry or hEXO1b-mCherry, cultured up to 80% confluence for 24 h, harvested before and after irradiation (20 J/m² for 1 h) using PBS, and processed as described previously (23). A total of 1.5 mg of protein (as determined by the Bradford method), preimmune serum, and F15 anti-EXO1 Ab were used for the immunoprecipitations, as described previously (23). For total protein extract preparation, cells were lysed in 1% SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, 50 mM DTT, 0.01% wt/vol bromophenol blue], sonicated for 10 s, and heated to 95 °C for 5 min. Equal amounts of proteins were analyzed by SDS/PAGE.

Immunofluorescence. Cells were seeded on a coverslip, transfected, and irradiated as described above. Cells were washed once in PBS, fixed for 20 min with 2% PFA in PBS, and permeabilized for 5 min with ice-cold PBS containing 0.5% Triton X-100. Blocking was performed in 10% BSA in PBS for 30 min, followed by replacement with primary Ab diluted in PBS with 0.1% Tween 20 (PBST 0.1) for 2 h at room temperature. For uH2A staining, permeabilization was performed with PBS-0.2% Triton before fixation, and the Ab was incubated overnight at 4 °C. Coverslips were washed three times in PBST 0.1 for 10 min, after which secondary Ab diluted in PBST 0.1 was added. Nuclei were counterstained with DAPI. Cells were rinsed in PBST 0.1 three times for 10 min and mounted using ProLong Gold (Invitrogen). Images were obtained using a Leica DMRA2 Microscope (with Leica FW4000 software) with a 100× oil immersion objective (1.30 NA). The percentages of hEXO1-positive LUDs were scored, counting RPA, XPA, or XPB accumulation at local damage only in hEXO1-transfected cells. A value of 1 was assigned each time that hEXO1 showed accumulation at local damage and 0 when it did not. Approximately 40 LUDs were scored for each independent experiment.

UDS Measurements. The procedure used was essentially as described previously (28). EdU was purchased from Invitrogen. The 48BR cells were transfected in 24-well plates and then cultured on coverslips. After UV irradiation, cells were incubated with serum-free DMEM supplemented with 10 μM EdU for 3 h. Cells were then washed with PBS, followed by a chase period of 15 min with thymidine. Fixation, permeabilization, and fluorescent labeling were performed using Click-iT (Invitrogen), according to the manufacturer's instructions. Cell images were captured with a Leica DMRA2 fluorescent microscope (40× objective, 0.75 NA) equipped with a Leica DC300F CCD camera. Captured images were processed and analyzed with Adobe Photoshop software. At least 100 non-S-phase cells were selected at random for each experiment.

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Supporting Information

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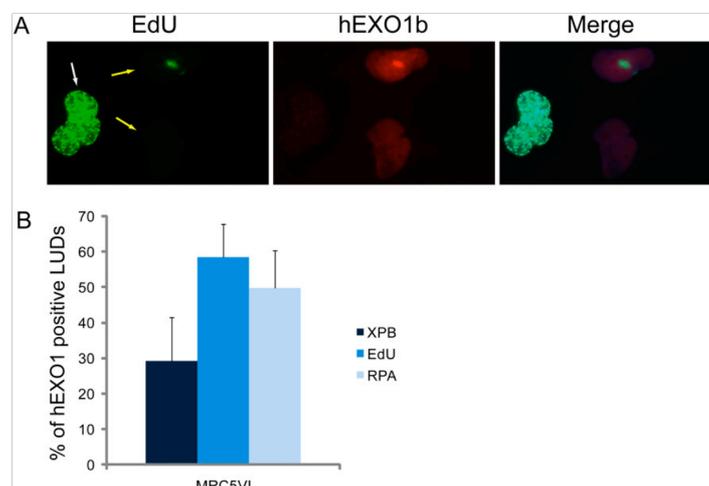


Fig. S1. Accumulation of hEXO1b-mCherry at LUDs occurs in non-S-phase cells. (A) MRC5VI cells transfected with hEXO1b-mCherry were exposed to local UV irradiation (40 J/m^2) and incubated for 1 h at 37°C in the presence of EdU. White arrows indicate S-phase cells; yellow arrows, non-S-phase cells. A representative field is shown. (B) Quantification of non-S-phase cells accumulating hEXO1b at sites of repair synthesis (EdU) compared with percentage of RPA- and XPB-positive LUDs that were also positive for hEXO1b in Fig. 1. The experiment was performed three times. Error bars represent SD.

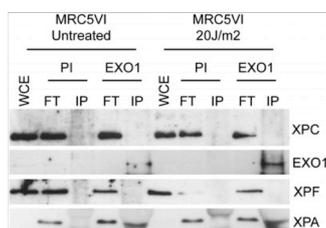


Fig. S2. hEXO1 does not interact with XPC and XPF. MRC5VI cells were mock- or UV-treated and harvested 1 h after UV irradiation. Lysates were prepared and either resolved directly on SDS/PAGE (WCE, whole cell extracts, 10%), or immunoprecipitated with preimmune serum (PI), as control, or anti-hEXO1 Ab (F15). Western blot analysis was performed with Ab against the indicated proteins.

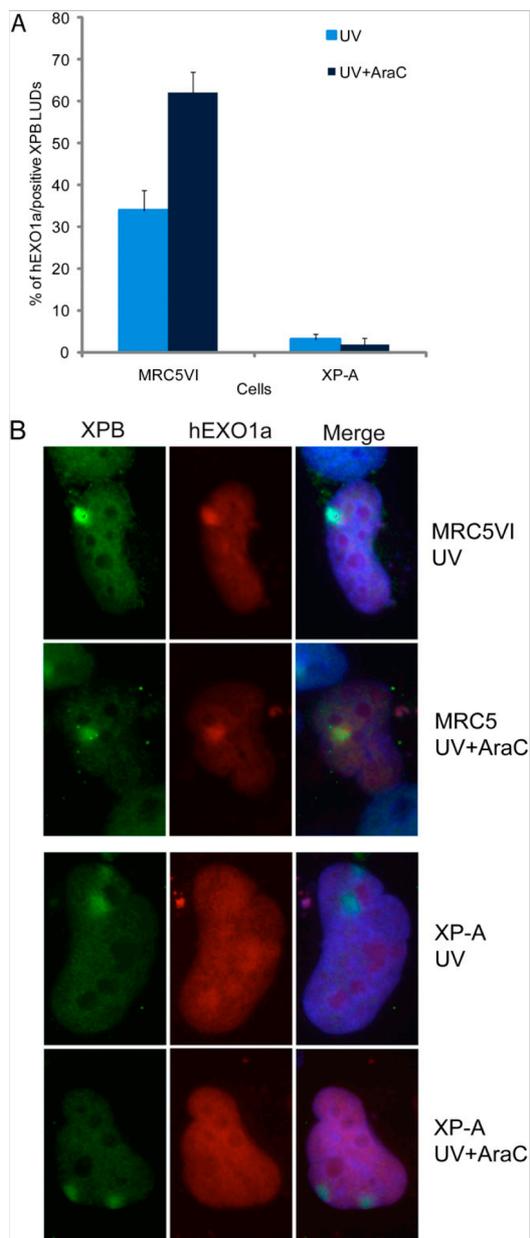


Fig. 54. hEXO1a accumulation at LUDs depends on XPA and is stimulated by inhibition of repair synthesis. MRC5VI and XP12RO (XP-A) cells transfected with hEXO1a-mCherry were exposed to local UV irradiation (40 J/m^2) and incubated for 1 h at 37°C in the presence or absence of Ara-C. (A) Plot of the percentage of XPB-positive LUDs that were also positive for hEXO1a. Approximately 50 local UV damage sites were scored in each of three independent experiments. Error bars represent SD. (B) Representative images of LUD-positive cells in the presence or absence of Ara-C treatment in the control cell line (MRC5VI) and XP complementation group A. (Scale bar: $5 \mu\text{m}$.)

Published paper II

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NER and DDR: Classical music with new instruments

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NER and DDR

Classical music with new instruments

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Genomic insults by endogenous or exogenous sources activate the DNA damage response (DDR). After the recognition of damaged DNA by specific factors, repair mechanisms process the lesions, and a surveillance mechanism, known as DNA damage checkpoint, is triggered by single-stranded (ss) DNA covered by RPA. UV light induces DNA lesions, mainly 6,4 photoproducts (6-4PP) and cyclobutane pyrimidine dimers (CPD), which are removed by nucleotide excision repair (NER). Recent reports shed light onto the mechanism connecting NER and DDR after UV irradiation. How does UV-induced DNA damage activate checkpoint kinases? How is ssDNA generated at UV lesions? In yeast, UV lesions persisting during S phase represent a block for the advancing of replication forks, which temporarily stop and then reinitiate downstream of the damage, leaving a ssDNA region containing the lesion. Non-replicating yeast and human cells with defects in NER are not able to properly activate the checkpoint cascade, indicating that processing of UV lesions is a prerequisite for checkpoint activation. This pathway also requires the function of exonuclease I, which acts on NER intermediates generating long tracts of ssDNA. Here, we review the connections between NER processing of UV-induced lesions and checkpoint activation, discussing the role of recently identified players in this mechanism.

Introduction

In eukaryotic cells, the presence of genomic DNA lesions leads to activation

of the DNA damage response (DDR), which coordinates repair mechanisms and cell cycle events to allow cell survival and preserve genome stability.^{1,2} In the last few years, it has been shown that the DDR modulates senescence and apoptosis, acting as a barrier against tumorigenesis.³⁻⁵ Understanding the molecular mechanisms underlying the cellular response to DNA damage is thus crucial for the comprehension of tumor development and for the definition of specific and effective therapeutic strategies.

ssDNA is a common intermediate in several DNA metabolic processes, ranging from transcription to DNA replication, telomere maintenance and DNA repair. Stable ssDNA regions represent a threat, since they can easily be involved in unscheduled recombination events and are intrinsically fragile. Cells respond to the presence of persistent ssDNA through the activation of the DNA damage checkpoint that helps the cell to cope with this situation. Following the formation of a double-strand break (DSB), a machinery comprising nucleases and helicases processes the broken double-stranded ends, generating 3'-5' ssDNA filaments that are rapidly covered by RPA and activate the apical DDR checkpoint kinase(s).⁶ A long-standing question in the DNA damage response field is how the checkpoint cascade is triggered following genotoxic UV irradiation.

Here, we discuss recent work that addresses this question and traces a connection between NER, translesion DNA synthesis and DNA damage checkpoint in response to UV-induced lesions.

Key words: nucleotide excision repair, DNA damage checkpoint, exonuclease I, translesion DNA polymerases, DNA lesions

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The DNA Damage Response (DDR)

Following genomic insults, the cell activates the DNA damage checkpoint. This surveillance mechanism is based upon a cascade of phosphorylation events that leads to a block or delay of cell cycle progression, regulation of DNA repair mechanisms, and it may eventually trigger the apoptotic pathway.^{2,7} Most of what we have learned about the molecular mechanisms underlying the DNA damage checkpoint derives from studies in yeast and human cells that have been challenged through the formation of DSBs.^{1,2}

Assembly of the NER Machinery and Repair Synthesis Dynamics

UV light generates mostly CPDs and 6-4PPs, which are bulky and helix-distorting lesions and are principally removed by NER. Eukaryotes have two NER sub-pathways: transcription-coupled NER (TC-NER), which removes lesions from the transcribed strand of active genes, and global genome NER (GG-NER), responsible for the removal of damage occurring in non-transcribed chromatin.⁸ The NER and DDR mechanisms have been highly conserved through evolution from yeast to mammals even though a higher level of complexity exists in multicellular organisms. The nomenclature of the different factors changes in various organisms: for the sake of clarity, here we will mainly use the nomenclature used in mammalian or, when specified, *Saccharomyces cerevisiae* cells.

Defects in NER cause severe genetic syndromes, like xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). XP patients suffer from severe photosensitivity and have a high incidence of sunlight-induced skin cancers. CS and TTD are multisystemic diseases characterized by developmental and neurological abnormalities and premature aging. All XP and CS patients show UV sensitivity.⁹

To properly protect cells from damage-induced mutagenesis, DNA repair mechanisms must be able to specifically recognize certain DNA lesions over a large excess of normal DNA. NER is a multistep process

involving a recognition step of the DNA helix distortion leading to partial unwinding of DNA surrounding the lesion, an excision step of the damaged strand and, finally, a refilling and ligation step.¹⁰ The two sub-pathways use many common factors but differ in the DNA damage recognition step: XPC-HR23B and UV-DDB are involved in GG-NER, while a stalled RNA polymerase coordinates with CSB and TFIIH for TC-NER.^{11,12}

Pioneer work on the *in vitro* reconstitution of the core GG-NER reaction^{13,14} led to the identification of ~30 polypeptides whose specific roles and dynamic of recruitment were analyzed in later studies.¹⁵⁻¹⁸ After recognition of the helix distortion and DNA unwinding of a region containing the UV-induced lesion, two endonucleases are involved in the incision step near the photoproducts: ERCC1/XPF and XPG, which are, respectively, acting 5' and 3' relative to the lesion. Recruitment of the ERCC1/XPF and XPG nucleases does not take place simultaneously, but the enzymes participate in a functional complex that requires both XPG and XPF to express its catalytic activities. Indeed, XP-G cells, lacking the 3' endonuclease, fail to cleave on both sides of the lesions, as do XP-F cells, lacking only the 5' endonuclease. If both factors are bound, 5' and 3' incisions are formed, removing a ~30 nt oligonucleotide containing the UV lesion.¹⁹ The resulting ssDNA gap is refilled by different DNA polymerase activities: Pol δ , Pol ϵ and Pol κ in mammalian cells.²⁰⁻²² Endonucleolytic cleavage of the damaged filament is tightly linked to repair DNA synthesis. It was recently demonstrated that the two incision events can be uncoupled and can take place sequentially. Taking advantage of catalytic defective XPG and XPF mutants, it was shown that once the 5' incision is achieved by ERCC1/XPF, DNA repair synthesis can start, and this step allows XPG to cleave 3' to the lesion. Such coordination should prevent the formation of long-lived ssDNA gaps. On the other hand, the refilling reaction stalls if the 3' cut is prevented.¹⁹

NER and Checkpoint Activation

In cells traversing S phase after exposure to UV light, DNA polymerases stall

at the sites of the lesions, and repriming events can occur at DNA lesions, leaving ssDNA gaps behind the replication forks.^{23,24} These structures have been visualized by electron microscopy and are likely responsible for the strong and rapid checkpoint response to UV-induced damage.²⁴ Outside S phase, the scenario is quite different, highlighting connections between NER and checkpoint activation in these conditions. In non-cycling *Saccharomyces cerevisiae* cells, a functional NER is required for the activation of the apical checkpoint kinase Mec1 (homolog of human ATR) and of the major DDR transducer kinase Rad53 (homolog of human Chk2) in response to UV irradiation.^{25,26} Most of differentiated cells in multicellular organisms are post-mitotic, non-dividing cells, a condition that can be compared with non-replicating yeast cells, and similar mechanisms are possibly involved.

Interestingly, while NER was found to be required for checkpoint activation after UV irradiation both in yeast and mammalian non-cycling cells,²⁶⁻²⁹ the relative importance of the two NER sub-pathways was found to be different. In fact, in G₁- or G₂-arrested budding yeast cells, either GG-NER or TC-NER is sufficient to activate the checkpoint in response to UV irradiation, while the signaling is prevented in strains lacking both processes. Conversely, non-cycling human primary fibroblasts derived from XP patients defective in GG-NER but proficient in TC-NER are unable to induce checkpoint activation following UV irradiation, while CS cells specifically defective in the TC-NER sub-pathway are checkpoint-proficient under the same experimental conditions.^{26,29} Therefore, the ability of TC-NER to trigger checkpoint activation following UV irradiation of non-cycling cells is different in yeast and mammals; a diversity that may be related to the lower fraction of the genome that is transcribed in mammals compared with yeast cells.

The Signal Activating the DNA Damage Checkpoint

It is well documented that processing of DSBs by the concomitant action of

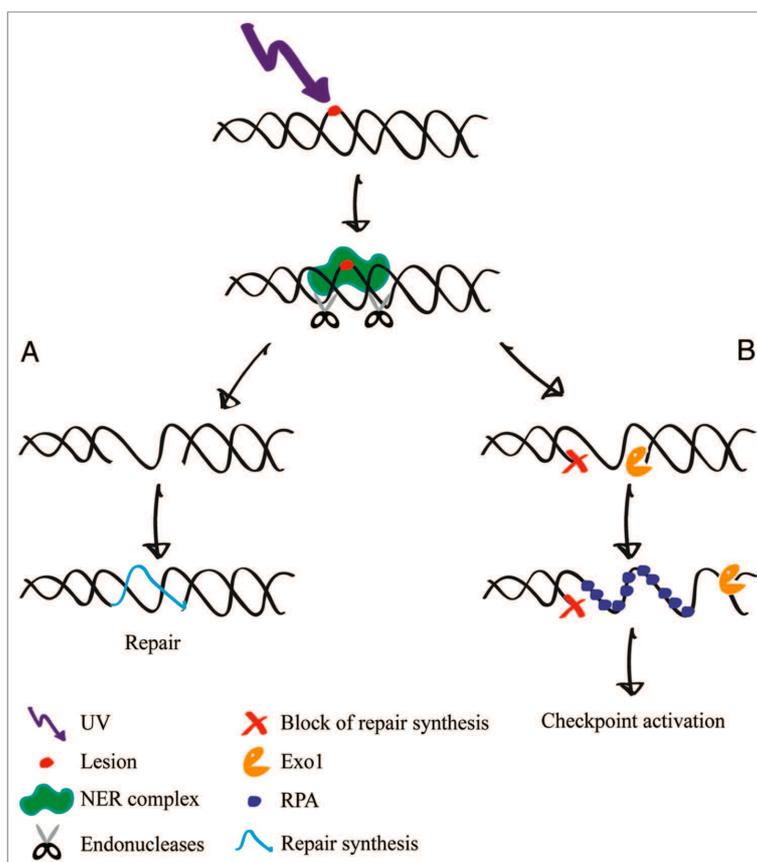


Figure 1. Processing of UV-induced lesions. UV light induces formation of bulky adducts on DNA, which lead to the recruitment of NER pre-incision and excision complexes for their recognition and removal respectively. (A) After the structure specific endonucleases cut, DNA repair synthesis starts and subsequent ligation seals the gap restoring the integrity of the chromosome. (B) If repair synthesis is somehow impaired, the competition between gap refilling and exonucleolytic processing is unbalanced, favoring Exo1 recruitment and formation of larger ssDNA gaps. These structures then trigger checkpoint activation.

nucleases and helicases generates ssDNA covered by replication protein A (RPA), and this nucleoprotein structure is the signal leading to recruitment and activation of the apical kinase in the checkpoint cascade. In mammalian cells, it was shown that UV treatment leads to the formation of RPA foci, where ATR-ATRIP, the main apical kinase responsible for checkpoint activation in response to UV-induced lesions, also colocalizes.^{30,31} This observation suggested that ssDNA coated with RPA may represent the signal required to activate the DDR also in response to UV irradiation. However, the NER

mechanism responsible for the removal of UV-induced DNA photoproducts does not explain the generation of long ssDNA regions. In fact, NER nicks the damaged strand, liberating a tract of approximately 25–30 nt containing the lesion. The corresponding gap on DNA is thus quite short to load the apical checkpoint kinase complex and all the other factors involved in starting the signal transduction cascade. Moreover, the ssDNA region is likely very short lived, being rapidly refilled by a DNA polymerase; indeed, a recent study suggests that during the repair of UV-induced DNA lesions, ssDNA may

never be really exposed, since repair synthesis can start immediately after the first XPF/ERCC1-mediated 5' incision, while removal of the oligo carrying the UV-induced photoproduct requires 3' cleavage by the XPG nuclease, which does not occur unless the refilling reaction is well under way.¹⁹

Another indication of the coupling between initiation of NER and repair synthesis was obtained recently, showing that RPA seems to play a relevant role in controlling NER incision events. The kinetics of repair synthesis and the assembly and disassembly of NER sub-complexes

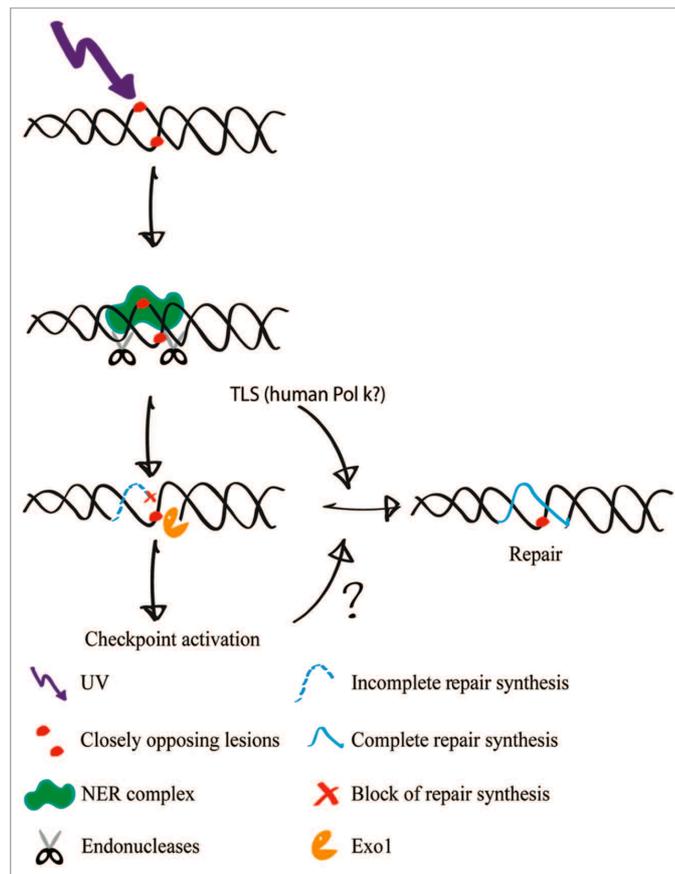


Figure 2. TLS polymerase activity in NER. In particular genomic locations, NER-dependent repair synthesis involves the translesion DNA polymerase pol κ . A possible model that explains the requirement for TLS polymerases invokes closely opposing lesions. NER starts processing one of the two lesions. Sequential coordination of excision steps at one lesion site allows repair synthesis to begin and proceed up to the second lesion on the opposite strand; here the repair DNA polymerase stalls creating an opportunity for Exo1-mediated processing (see Fig. 1). Gap filling and repair completion then require a translesion polymerase to bypass the lesion on the template strand.

after UV irradiation was monitored in normal and XP cells. It was found that when RPA is present at the site of damage, NER-mediated incision can occur and is followed by the recruitment of post-incision factors, with the concomitant release of pre-incision proteins. Under normal DNA repair conditions, RPA is also released and can associate and stabilize newly formed pre-incision complexes. Conversely, if DNA refilling is impaired, RPA remains associated to post-incision

complexes, preventing further incision events and effectively coupling initiation of NER to completion of DNA repair synthesis.³²

Exonuclease 1: A New Player in Checkpoint Activation in Response to UV Irradiation

Exonuclease 1 (Exo1) is a member of the Rad2 family of structure-specific nucleases, and it is endowed with a 5'-3'

exonuclease and a 5'-flap endonuclease activity in vitro.^{33,34} The structure and function of Exo1 is well conserved in eukaryotic cells, where it is implicated in a variety of DNA metabolic processes, such as mismatch repair, DSB repair, telomere maintenance, meiotic and mitotic recombination.³⁵ The relevance of Exo1 is emphasized by the observation that *Exo1*^{-/-}-KO mice are prone to tumor development and impairment of DNA damage signaling in response to telomere dysfunction.^{36,37}

Recently, two reports revealed a new role for Exo1 in the response to UV irradiation in non-cycling yeast and human cells. Deletion of *EXO1* or catalytic *exo1* mutations prevent the activation of the yeast apical checkpoint kinase Mec1 following UV treatment of G₁-arrested cells. Once NER factors start to process the UV-induced lesions and incise the damaged strand, Exo1 generates ssDNA regions that can be much longer in length (> 500 bp, possibly up to ~3 kb) than the canonical ~30 nt ssDNA gap formed after dual incision and unwinding by NER factors.^{38,39} Molecular combing approaches revealed that refilling of these extended gaps during repair synthesis originate long-patch repair tracks.³⁸ The formation and disappearance of these intermediates exposing long ssDNA regions correlates with the activation and inactivation of the checkpoint response after UV irradiation and repair, respectively, suggesting a causal relationship.

A similar mechanism has been proposed to drive UV-induced checkpoint activation in non-cycling mammalian cells. Indeed, hEXO1 is found at the site of local UV damage (LUDs) after UV treatment of non-S-phase human cells. hEXO1 recruitment at LUDs overlaps that of NER pre-incision factors, and a functional NER machinery is needed for hEXO1 accumulation at LUDs. In particular, it was found that hEXO1 recruitment at LUDs was defective when 5' incision by XPF/ERCC1 was impeded. On the other hand, in cells expressing a catalytically inactive XPG protein, where the 5' incision takes place and the 3' incision is prevented, thereby inhibiting completion of gap refilling, hEXO1 accumulated at the sites of damage.⁴⁰ Finally, hEXO1 silencing reduces the extent of repair synthesis

after UV irradiation as well as the activation of the DNA damage response,⁴⁰ analogously to what previously observed in XP patient cells.²⁹

As suggested above, Exo1-dependent extension of the short physiological gaps generated by NER could be a consequence of problematic refilling during NER repair synthesis reaction. Indeed, limiting some essential repair factors or chemically inhibiting repair synthesis with nucleotide analogs led to a stronger Exo1-dependent checkpoint activation even at very low UV doses.³⁸

Altogether, these observations suggest that, under normal conditions, the NER machinery efficiently repairs UV-induced lesions without activating the checkpoint response. However, if repair of the short UV gaps is somehow impaired, a competition is established between the NER refilling machinery and Exo1 that can further extend the gap digesting the 5'-ended filament (Fig. 1).⁴¹

This model is consistent with pioneer work in bacteria, which suggested that in non-replicating cells, the SOS response can originate in a NER-dependent manner from lesion removal and the appearance of DNA gaps.^{10,42}

Mounting evidence indicates the relevance of nucleases in generating the ssDNA that triggers the DNA damage response. As mentioned above, in yeast and mammalian cells, resection of DSBs ends, generating the structure responsible for the recruitment of the apical DDR kinase(s), requires the concerted actions of several nucleases.⁴³ DSB resection takes place in two steps: initially, a short single-stranded oligonucleotide segment is removed from the 5'-ended filament to create an early intermediate with a short 3' overhang, which is then further processed to generate the long-terminal ssDNA region. In budding yeast, the first step is dependent on the highly conserved Mre11-Rad50-Xrs2 complex and Sae2. The second step employs the Exo1 exonuclease and/or the helicase-topoisomerase complex Sgs1-Top3-Rmi1 with the Dna2 endonuclease.⁴³ The findings described above indicate that the DNA damage response following UV irradiation is more similar than previously expected to DDR in response to DSBs. The similarity

between the UV and DSB responses is also suggested by the existence of a common mechanism generating the same epigenetic marks following UV- or DSB-induced DNA lesions. In both cases, the RNF8 ubiquitin ligase and the Ubc13 ubiquitin-conjugating enzyme are recruited at the sites of damage through an interaction with MDC1. The role of these enzymes is to ubiquitylate histone H2A, allowing the recruitment of critical downstream repair factors, such as 53BP1 and Brca1, at the damage sites.⁴⁴⁻⁴⁶ In agreement with the model discussed above, H2A ubiquitylation upon UV irradiation of non-cycling cells requires NER and hEXO1 activities, suggesting an unexpected role for hEXO1 in facilitating repair of UV-induced lesions.^{40,46} Recently, a provocative connection between misregulation of certain epigenetic marks and aging has been also suggested.⁴⁷

Link Between NER Repair Synthesis and Translesion (TLS) DNA Polymerases

NER is generally considered a non-mutagenic process, and it has been assumed that the refilling step of the NER ssDNA gaps is performed by the high-fidelity DNA polymerases δ and ϵ , as indicated by several *in vitro* studies.¹⁰ However, two recent papers reported an unexpected role of the mammalian Y family DNA polymerase κ in NER.^{21,48} siRNA depletion of pol δ , pol ϵ and pol κ diminishes NER-dependent repair synthesis *in vivo*. Moreover, recruitment of these polymerases into repair sites is differentially regulated by the status of PCNA ubiquitylation as well as by usage of distinct clamp loader complexes or the repair scaffold protein XRCC1. Approximately 50% of NER gap refilling is mediated by pol κ recruited through ubiquitylated PCNA and XRCC1, together with pol δ recruited by the classical RFC complex. The remaining 50% is performed by pol ϵ recruited by the alternative CTF18-RFC complex. The authors propose the existence of two modes of repair synthesis dealing with different conformations of the UV-induced lesions or chromatin accessibility of the damaged sites.⁴⁸ About 50% of repair synthesis is in accessible

areas of the genome and is rapidly performed by pol ϵ . The remaining 50% involves chromosomal regions that are more difficult to reach, resulting in the 3' incision being delayed relative to 5' incision, resulting in the formation of a 5' flap, as also suggested by *in vitro* studies.⁴⁹ Because of the steric hindrance, this refilling is more difficult to achieve, and it is performed by pol δ and pol κ . This alternative mode of refilling may correlate with the action of Exonuclease 1,^{38,40} as discussed previously. It is worth noting that in G_1 arrested budding yeast cells, TLS polymerases seem to play a critical role after UV irradiation, possibly in allowing the refilling reaction to take place also where Exo1 has acted. Elimination of TLS increases the accumulation of NER intermediates, resulting in strong checkpoint activation at lower UV doses. It is intriguing that active checkpoint kinase has been reported to support translesion DNA synthesis, suggesting the possible existence of a positive feedback loop.^{50,51} Moreover, cells lacking TLS polymerases exhibit an increased sensitivity to UV light when irradiated in G_1 compared with S-phase irradiation, suggesting a failure to complete NER.³⁸ Mammalian cells contain at least five TLS polymerases likely acting in combined two-polymerase reactions.⁵² A challenge for future studies will be to understand the molecular mechanisms controlling TLS polymerase(s) selection and to explain how an intrinsically mutagenic process may mitigate the mutation load caused by DNA damage.

Concluding Remarks

Processing of NER intermediates by Exo1, linked to impediments in repair synthesis, the involvement of TLS polymerases in G_1 -arrested, UV-irradiated yeast cells and that of pol κ in repairing a subset of NER gaps in human cells raise the problem of identifying the structure(s) and location of the UV-induced lesions that constitute a problem for NER completion. Intriguingly, it has been found that in non-cycling yeast cells, the activation of the checkpoint kinase in response to increasing UV doses suggests that two lesions may concur to generate an efficient checkpoint signal.³⁸ Such events are defined as

“closely opposing lesions” and have been described in different organisms.⁵³

It has been shown that NER cannot remove two closely opposing lesions at the same time.⁵⁴ Therefore, when the lesion on one strand is processed by NER, the refilling polymerase would hit the second lesion on the template and stall; this would tilt the balance between refilling and nucleolytic processing in favor of Exo1, leading to the generation of long ssDNA gaps. Indeed, as discussed above, checkpoint signaling is much stronger in TLS mutant cells. If irradiated in G₁, TLSΔ cells are extremely sensitive to UV. In fact, in the absence of TLS, G₁ haploid cells have no other means to refill a gap containing a lesion in the template strand. A support to these findings rests on the identified role of TLS pol κ in human NER (Fig. 2), at least at a fraction of the lesion sites.²² While budding yeast lacks pol κ, and a similar function for other yeast TLS polymerases has not been reported yet, it is possible that this mechanism will prove to be conserved across evolution and may contribute to the effects described in yeast TLS mutant cells. Particularly intriguing is the finding that human telomeres represent a preferred site for the generation of UV lesions and, at least theoretically, the specific telomeric repeat sequence could favor the formation of closely opposing lesions.⁵⁵ It will be interesting to look for a correlation between TLS polymerase activities and telomeres.

In some NER-defective cells, UV damage has been reported to induce a strong p53Ser15 phosphorylation and to drive cell into apoptosis.⁵⁶ This suggests that a NER-independent response can also be induced by UV, consistent with observations from budding yeast.^{57,58} A recent paper analyzed the molecular details of such response, which, although delayed, does not require NER but seems to rely at least partially on the AP endonuclease Ape1.⁵⁹ The physiological relevance of this pathway is still not understood, but it will be interesting to determine its relationship with the elevated apoptosis observed when NER-defective cells are exposed to UV irradiation.

The findings described in this and in the previous sections reveal an unexpected complexity of the NER repair

synthesis and its functional crosstalk with the action of nucleases that may further process a subset of the NER intermediates at specific locations, where completion of repair is problematic. Moreover, the final outcome appears to be dependent on the cell cycle stage and on the proliferative state of the cell. It will be interesting to identify at the genomic level the location and geometry of these “problematic” sites, which may coincide with slow replicating zones or regions known to be prone to chromosomal breaks. The application of microarray technologies to the analysis on UV light-induced DNA damage allows the determination of the position and frequency of UV-induced lesions⁶⁰ and may be useful to tackle this question.

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Part III

Manuscript in preparation

Down-regulation of hRNase H2 linked replication stress to Aicardi-Goutières syndrome

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ABSTRACT

Ribonucleotides (rNMPs) are incorporated into genomic DNA with relatively high frequency during normal replication making DNA more sensitive to strand breakage. RNase H2 is the only enzyme able to remove single rNMPs embedded in DNA and mutations in any of the genes encoding the three subunits of human RNaseH2 cause the Aicardi-Goutières syndrome (AGS). In budding yeast cell survival in the absence of RNase H activity depends on the two post-replication (PRR) pathways resulting in constitutively activated PRR.

Here we demonstrate that human cells depleted of RNase H2 show impaired cell cycle progression associated with chronic activation of PRR and genome instability. Interestingly, a similar phenotype is observed in lymphoblastoid cells derived from AGS patients, in which rNMPs accumulation in genomic DNA is detectable. Our data indicate that RNase H2 plays a crucial role in overcoming rNMPs misincorporation preventing DNA damage that may induce unscheduled immune response in AGS patients. These findings may be relevant to shed further light on the mechanisms involved in AGS pathogenesis.

INTRODUCTION

Cells have developed various surveillance mechanisms to maintain genome integrity during DNA replication and facilitate repair (Muzi-Falconi et al, 2003; Lazzaro et al, 2009). At the basis of genome stability is the choice of DNA instead of RNA as the storage information molecule. Due to the reactive 2' hydroxyl group in the ribose moiety, RNA is ~100,000-fold more susceptible than DNA to spontaneous hydrolysis under physiological conditions (Li et al, 1999). Incorporation of ribonucleotide triphosphates (rNTPs) in DNA needs to be avoided, as it makes DNA more susceptible to strand breakage and alters DNA helix parameters triggering a switch from B- to A- structure (Nick McElhinny et al, 2010a; Clark et al, 2011, Kim et al, 2011). To prevent this problem DNA polymerases have evolved active sites that distinguish between rNTPs and deoxyribonucleotide triphosphates (dNTPs) (Joyce et al, 1997). However, the fidelity of DNA polymerases is challenged by the higher concentration of rNTPs respect to dNTPs that ranges from 10 to 100 fold in *S. cerevisiae* (Nick McElhinny et al, 2010b) and in mammalian cells (Traut et al, 2003). Indeed, recent evidence revealed that rNMPs are misincorporated into genomic DNA with high frequency during normal replication (Nick McElhinny et al, 2010b; Rejins et al, 2012; Hiller et al, 2012). Moreover, rNMPs may be linked to DNA chain during repair of double strand breaks in G1 (Nick McElhinny et al, 2003; Zhu et al, 2008) and frequent rNMPs incorporation was observed during HIV-1 reverse transcription (Kennedy et al, 2012). It must also be considered that RNA:DNA hybrid molecules are physiological intermediates produced during the synthesis of Okazaki fragments or when a replication fork is colliding with the transcription machinery (Drolet, 2006; El Hage et al, 2010). Altogether, these findings established that incorporation of rNMPs in genomic DNA is the main source of endogenous DNA damage.

In eukaryotes, two classes of RNases H with partially overlapping substrate specificity

remove the RNA moiety from RNA:DNA hybrid molecules (Cerritelli et al, 2009). RNase H1, requires a stretch of at least 4 consecutive ribonucleotides to work: mammalian RNase H1 is essential for mitochondrial DNA replication and there is a nuclear form whose function is still unclear (Suzuki et al, 2010; Cerritelli et al, 2003). RNase H2 is a trimeric complex that besides being able to hydrolyze longer RNA:DNA molecules has the unique property to remove single rNMPs embedded in genomic DNA. Conversely, budding yeast cells carrying combined deletions of RNase H1 and RNase H2 genes are viable, although they show evident cell growth defects (Lazzaro et al, 2012). In *S. cerevisiae* the accumulation of rNMPs into genomic DNA activates the translesion synthesis and template switching PRR sub-pathways that likely act as back up strategies for cell survival (Lazzaro et al, 2012). Conversely, both RNaseH1 or RNaseH2 null mice die during embryogenesis, demonstrating the essential function of these enzymes for cell viability in mammals (Cerritelli et al, 2003; Rejins et al, 2012; Hiller et al, 2012).

In humans the importance of RNase H2 activity is underlined by the fact that mutations in any of the three genes encoding the RNase H2 subunits cause the Aicardi-Goutières syndrome (AGS). This is a rare and under-diagnosed genetic disorder characterized by high levels of type I interferon production and leading to a very severe phenotype characterized by several neuroinflammatory conditions. Interestingly, RNaseH2 mutations that only partially alter the enzymatic activity *in vitro* are responsible for the AGS phenotype (Crow et al, 2006; Rice et al, 2007; Perrino et al, 2009; Shaban et al 2010; Figiel et al 2011; Rejins et al, 2011).

The most significant *in vivo* studies connecting RNase H2 defects with increased genome instability have been carried out in knockout mice and mouse embryonic fibroblast (MEF) cells (Rejins et al, 2012; Hiller et al, 2012). Studies in human cells in which the expression of the RNase H2 genes is modulated by RNA interference could be useful to identify

molecular pathways that are perturbed when RNaseH2 gene expression is down-regulated, likely mimicking what may happen in AGS hypomorphic patients.

We used lentiviral vectors carrying specific shRNA sequences to induce stable RNase H1 and H2 knockdown in human cell lines. Here we report that depletion of RNase H2 causes endogenous replication stress, as evidenced by impaired cell cycle progression and chronic PRR activation. These phenotypes have been tested in lymphoblastoid cell lines derived from AGS patients where an accumulation of rNMPs in genomic DNA linked to the severity of the mutations can be observed. Altogether, our data show that RNase H2 down regulation leads to accumulation of rNMPs in genomic DNA that, in turn, causes replication stress and activation of the DNA damage response (DDR).

RESULTS

1. RNase H2 depletion in human cells impairs normal cell proliferation.

Recent studies on the molecular functions of RNase H2 *in vivo* suggest its role in genome stability maintenance by removing misincorporated rNMPs in genomic DNA (Lazzaro et al, 2011; Reijns et al, 2012; Hiller et al, 2013). However, the link between RNase H2 functions and AGS onset is still poorly understood. It must also be considered that most of the mutations found in AGS patients are hypomorphic rather than loss of function, likely causing decreased RNaseH2 levels or altered protein-substrate or protein-protein interactions.

We thus decided to use HeLa cells in which the level of RNase H enzymes could be modulated by RNA interference techniques. In order to exclude a possible redundant role of RNase H1 we concomitantly silenced both RNase H1 and RNase H2. However, depletion of RNase H1 resulted in high level of cell mortality associated with activation of the Casp3 apoptotic pathway (Fig S1), likely because RNase H1 is essential for mitochondrial

maintenance (Cerritelli et al, 2003; Cerritelli et al, 2009). Thus we focused our work on RNase H2 down-regulation alone.

We generated lentiviral vectors carrying specific shRNA sequences for the catalytic A subunit or the structural B, subunit as they correlate, respectively, with the most severe phenotype or the most common mutations found in AGS patients (Rice et al, 2007).

After lentiviral infection, HeLa cells were analyzed for silencing efficiency (Fig. 1A-B). As shown in Fig. 1A, western blot analysis revealed that down-regulation of one RNase H2 subunit affects the expression levels of the other subunits, likely because the entire heterotrimeric complex is destabilized (Rejins et al, 2012). Upon depletion of RNase H2, HeLa cells began to proliferate slower respect to control cells (Fig. 1C) and this defect increases with the efficiency of silencing (Fig. S2). The decrease in cell number can only partially be ascribed to cell death, as revealed by the modest increase in LDH release respect to control cells; furthermore, silenced cells did not display activation of the Casp3-mediated apoptotic pathway (Fig.1 D-E).

These findings suggest that a decreased level of the RNase H2 complex in human cells affects cell proliferation through mechanisms that cannot be compensated by RNase H1 activity.

2. Depletion of RNase H2 causes replication stress.

Few passages after RNase H2 silencing, the HeLa cell population decreased respect to control cells. Since only a limited level of cell mortality was detectable, we investigated possible defects in cell cycle progression.

Exponentially growing HeLa cells were labeled with BrdU and analyzed by FACS. We observed that RNase H2 silencing slightly affected cell cycle progression respect to control cells, resulting in a limited but reproducible accumulation of cells in S and G2-M phases

(Fig. 2 A-B). Cell cycle was further analyzed in a kinetic experiment in which subpopulations of cells traversing S-phase were labeled with a pulse of BrdU and monitored after release. While control cells progressed with a normal kinetics through S and G2-M phases, silenced cells accumulated in late S/G2 phase and passed into new G1 phase with a significant delay (Fig. 2 C).

Our previous work in budding yeast demonstrated that, in the absence of RNase H activity, misincorporated rNMPs challenge the efficiency of the replication machinery and *rnh1Δ rnh201Δ* cells can cope with such a problem by activating the PRR repair pathway to bypass the lesions (Lazzaro et al, 2012). Because *rnh1Δ rnh201Δ* yeast cells accumulate in the S and G2-M phases of the cell cycle (Lazzaro et al, 2012) and the same phenotype is observed in silenced RNaseH2 HeLa cells, we analyzed in the silenced cells the level of ubiquitylated PCNA, a biochemical readout of PRR activation.

The analysis on total protein extracts from exponentially growing cells, revealed an accumulation of ubiquitylated PCNA in cells depleted of RNase H2, indicating that these cells must activate the post-replication repair pathway to cope with obstacles to replication fork progression (Ulrich et al, 2011; Brown et al, 2009) (Fig. 3A). This hypothesis was strengthened by the observation that, when exposed to low levels of the replication stress-inducing agent hydroxyurea (HU), RNase H2 depleted cells are hypersensitive to the drug compared to control cells (Fig. 3B). Low levels of HU decrease the levels of dNTPs pools and are known to be toxic only for cells suffering replication stress while they are well tolerated by cells normally traversing replications phase (Weinert et al, 1994; Lydall & Weinert, 1997; Lazzaro et al, 2012), supporting the notion that RNase H2 depletion impairs normal replication fork progression.

3. RNase H2 is involved in genome integrity maintenance in human cells

To further investigate the molecular mechanisms linking RNase H2 silencing to cell growth defects, we analyzed the activation of DNA damage response (DDR) pathways.

We failed to detect, in total protein extracts, phosphorylation of Chk1 and Chk2, the two main DDR checkpoint kinases (Fig. 4A). Nevertheless, when RNase H2 silenced cells were analyzed at the single cell level by immunofluorescence, an increase in 53BP1 foci formation was detectable (Fig. 4B, 4C). 53BP1 is a well-known mediator of DNA double strand break (DSB) repair, but it has been recently associated with replication stress (Bekker-Jensen et al, 2005; Doil et al, 2009; Mochan et al, 2004; Lukas et al, 2011). Furthermore, a clear increase in the frequency of micronucleated cells was measured in RNase H2 depleted cells (Fig. 4 D), and this phenotype is indicative of chromosome breakage and increased genome instability (Norppa and Falck, 2003).

Taken together, our data suggest that RNase H2 is involved in maintaining genome integrity and its down-regulation may cause DNA damage and increased genome instability, possibly as consequence of replication fork collapse.

4. Lymphoblastoid cells derived from AGS patients show accumulation of ubPCNA and DNA damage.

The Aicardi-Goutières syndrome is caused by mutations in six genes, but ~ 60% of the AGS patients carry mutations in the three genes encoding the RNase H2 subunits (Rice et al, 2007; Rice et al, 2009; Rice et al, 2012). The most frequent mutations found in patients occur in the gene encoding the structural B subunit, and are associated to a milder phenotype. Conversely, mutation G37S which alters the catalytic site in the A subunit strongly affecting the RNaseH2 activity activity, is less frequent and it is associated to the most severe AGS symptoms. In any case, all mutations in the three RNase H2 genes trigger, to a different extent, a decrease in enzymatic activity and in the stability of the complex

(Crow et al, 2006; Shaban et al, 2010; Perrino et al, 2009; Figiel et al, 2011; Reijns et al, 2012).

We thus hypothesized that down-regulation of RNase H2 by RNA interference could reflect what is happening in AGS patients.

To test this hypothesis, we analyzed: i) the level of the RNase H2 complex subunits; ii) the status of post-replication repair activation, and iii) the presence of markers of DDR activation in EBV-immortalized lymphoblastoid cells derived from AGS patients. We choose cells carrying the G37S mutation in the A subunit or the A177T and the A177T-T1631 heterocompound mutations in the B subunit.

As shown in Fig. 5A, PCNA ubiquitylation was evident in G37S-mutant cells, while it was barely detectable in A177T and A177T T163I- cells, accordingly with the severity of the phenotype observed in patients. Interestingly, phosphorylation of p53 at serine 15, an established marker of DNA damage (Shieh et al, 1997), was detected in all mutant cells at a comparable level, suggesting that impaired RNase H2 function is sufficient to trigger the DNA damage response (Fig. 5B). Furthermore increased 53BP1 foci were detected in G37S-mutated cells (Fig. S3).

Altogether, these data indicate that our shRNA-based cellular model system could be used to mimic molecular pathways linked to AGS pathogenesis.

5. Impaired RNase H2 function triggers rNMPs incorporation in genomic DNA.

Mammalian cells accumulate rNMPs in genomic DNA in the absence of RNase H2 activity (Reijns et al, 2012; Hiller et al, 2012). We hypothesized that such accumulation could be responsible for the phenotypes observed in RNaseH2 silenced HeLa and AGS lymphoblastoid cells. To verify this hypothesis we performed the assay recently developed by Hiller et al. (Hiller et al, 2012), in which genomic cellular DNA is isolated and than

digested or not with bacterial RNaseHII to cause nicks in the position where rNMPs have been introduced in chromosomal DNA. Nicks are then detected by incorporation of a radiolabeled dNTP by DNA polymerase I.

AGS-lymphoblastoid cells were the ideal system to apply the above technique to detect rNMPs in the genome. In fact, we have not been able to produce stable clones of RNase H2-silenced HeLa cells and, on the other hand, a non-homogeneous cellular population goes through clonal selection during the numerous generations likely necessary to accumulate a detectable amount of rNMPs.

Genomic DNA from AGS and control cells was extracted and analyzed as described above, while DNA from the *rnh1Δ rnh201Δ* yeast strain was used as a protocol control (Fig 6B). An increased radioactive signal was reproducibly found in RNHII-treated genomic DNA extracted from G37S-mutated cells. The barely detectable increased signal in A177T and A177T T163I-mutant cells, could be imputed to the high background signal (compare the background in human and yeast DNA) and the subsequent limited sensitivity of the assay (Fig. 6A). The radioactive signal detected in G37S mutant cells was normalized to the DNA amount and quantified with respect to the RNHII-untreated sample. While almost no increase of radioactive signal over the background was detectable in wild-type (WT) cells, it increased more than two fold in G37S AGS-mutant cells (Fig. 6C).

In conclusion, the G37S RNase H2 mutation affecting the enzymatic activity and likely other mutations influencing the heterotrimeric complex stability and/or interactions can cause the accumulation of rNMPs in genomic DNA that, in turn, representing obstacles to replication fork progression will lead to fork stalling and consequent genome instability.

DISCUSSION

Ribonucleotides recently emerged as the main non-canonical nucleotides present in genomic DNA (Nick McElhinny et al, 2010a; Kim et al, 2011; Sparks et al, 2012). Apart from remaining in the genome as a consequence of improper Okazaki fragments processing (Cerritelli et al, 2009), rNMPs can be incorporated into DNA by errors of replicative polymerases with surprisingly high frequency (Nick McElhinny et al, 2010b). Relevant physiological functions for misincorporated rNMPs have been demonstrated (Vengrova et al, 2006; Lujan et al, 2013; Ghodgaonkar et al, 2013), but their excessive accumulation has deleterious consequences (Rejins et al, 2012; Hiller et al, 2012; Lazzaro et al, 2012).

The rising interest toward this recently discovered DNA lesion highlighted the importance of RNase H2 as the only enzyme capable to recognize single rNMPs imbedded in the eukaryotic genomes (Cerritelli et al, 2009; Sparks et al, 2012). The importance of RNase H2 function is further underlined by the fact that mutations in all genes encoding for RNase H2 subunits cause AGS, a rare genetic autoimmune disorder (Rice et al, 2007). Despite the increased interest in this research area, up to now a link between RNase H2 functions and AGS symptoms is still missing.

Knock-in mice carrying the most common AGS mutation in RNase H2 do not display any obvious phenotype, possibly due to differences in the molecular pathways between humans and mice (Rejins et al, 2012). On the other hand, RNase H2 knock-out mice or mice with almost completely abolished RNase H2 activity die early during embryogenesis or perinataly (Rejins et al, 2012; Hiller et al, 2012). Thus, additional cellular mammalian models are necessary to gain more insights on the molecular mechanisms linking RNase H2 defects to AGS pathogenesis.

To overcome the drastic effects caused by total loss of RNase H2 activity and differences linked to the employment of murine models, we used human cells in which RNase H2

expression was modulated by RNA interference. We found that, shortly after infection with lentiviral vectors carrying specific shRNA sequences, HeLa RNase H2-silenced cells showed cell cycle defects leading to accumulation of cells in S and G2/M phases. Moreover, similarly to what we previously observed in budding yeast (Lazzaro et al, 2012), these cell cycle progression defects correlate with activation of the PRR pathways and increased sensitivity to replication stress inducing agents, such defects are not cell line-specific since we obtained similar results with MRC5VI fibroblasts (data not shown). It is plausible that a defective RNase H2 activity leaves unrepaired rNMPs embedded in the template DNA creating obstacles to replication fork progression and requiring PRR activation to complete the replication process.

Genetic analysis in budding yeast demonstrated that replication stress in a strain deprived of both RNases H activities was due to unrepaired rNMPs in genomic DNA rather than to DNA:RNA hybrids derived from R-loops formation or undegraded Okazaki fragments (Lazzaro et al, 2012). Furthermore, a recent biochemical study based on expression of AGS-related mutations in *S. cerevisiae*. It was found that AGS-related mutations did not affect the capacity of RNase H2 to remove R-loops caused by replication fork collapse, while the processing of transcription-dependent R-loops could redundantly be carried out by RNase H1. Conversely, removal of single rNMPs in DNA was totally dependent on RNase H2, and AGS-related mutations had a low but detectable effect (Chon et al, 2013). Nevertheless, it is reasonable to predict that single rNMPs in the genome cannot account for the complex phenotype found in AGS patients. Indeed, the number of generations after RNase H2 depletion are too few to justify a huge rNMPs accumulation in the genome, also taking into account the residual RNase H2 activity associated to most AGS mutations. It is likely that defects in other cellular processes in which RNase H2 is involved will generate the complex genome instability scenario that we observed.

In any case, PRR activation, detectable as the accumulation of ubiquitylated PCNA, was observed in AGS-derived cells, indicating that it is indeed linked to impaired RNase H2 activity. The level of PCNA modification correlates with the severity of the AGS mutation, being particularly evident in G37S-mutated cells. The G37S mutation also causes an evident accumulation of rNMPs in DNA. The most frequent mutations found in the RNase H2 B subunit cause a barely detectable signal related to rNMPs accumulation in genomic DNA, likely related to the limited sensitivity of the assay. It will be necessary to improve the assay itself and/or to evaluate whether differences between wild-type and RNase H2B mutant cells will increase under some stress conditions. Indeed, the AGS phenotype may remain quiescent for a long time and the pathological conditions arise only after some still unknown stress stimuli (Rice et al, 2007).

In agreement with the finding observed in transgenic mice, we observed that RNase H2 silencing in human cell lines or AGS mutations in lymphoblastoid cell lines increased genome instability and activate the DDR response, likely as consequence of replication stress. We thus speculate that DDR *per se* may at least partially account for the clinical symptoms related to RNase H2 mutations in AGS patients. Indeed, growing evidence links DDR to the innate immune response. Activation of the DNA damage response was reported to induce cell surface expression of ligands for NKG2D, the stimulatory receptor expressed by natural killer (NK) and by T cell subpopulations, and more recent data demonstrated that IFN genes are produced in response to DNA damage (Gasser et al, 2005; Coscoy et al, 2007; Brzostek-Racine et al, 2011). It is likely that other molecular pathways are linked to RNaseH2-dependent AGS pathogenesis. Among them it must be considered a possible role of RNaseH2 in retroelement metabolism. Indeed, the TREX1 and SAMHD1 genes, which are mutated in a subset of AGS patients (Crow et al, 2006; Rice et al, 2009) are more or less directly linked to HIV infection and/or endogenous retroelements metabolism (Yan et al,

2010; Yan et al, 2011; Hrecka et al, 2011; Baldauf et al, 2012; Goldstone et al, 2011). Moreover, RNase H2 was identified in a high-throughput screening as a factor that facilitate HIV replication likely by removing ribonucleotides incorporated by errors of HIV reverse transcriptase (Genovesio et al, 2011, Kennedy et al, 2012). It is thus plausible that RNase H2 may play a role in controlling endogenous retroelement spreading, and RNase H2 defects may increase retroelements accumulation leading to a cellular antiviral response. In summary, we provide evidences that reduced RNase H2 activity is sufficient to accumulate rNMPs in genomic DNA, thus triggering replication stress that, in turn, induce genome instability and DNA damage response. Although other pathways likely account for the AGS symptoms, it is possible that increasing IFN levels, a signature of the AGS disorder, are induced by the DDR itself.

MATERIAL AND METHODS

Antibodies and chemicals. The following antibodies were used: anti RNase H1 (Santa Cruz Biotechnology, sc-101114), anti-RNase H2C from (ptglab #16518-AP), anti-RNaseH2A (abcam, ab83943), anti-actin (Santa Cruz Biotechnology, sc-1616); anti-Caspase3 (Cell Signaling, #9662); anti-PCNA was kindly provided by S. Sabbioneda; anti-BrdU FITC-conjugated (BD Biosciences, #347583), anti-Chk1 (Cell Signaling, #2345); anti Chk2 (Cell Signaling, #2662S); anti-P-Chk1-Ser317 (Cell Signaling, #2344S); anti-P-Chk2-Thr68 (Cell Signaling, #2661S); anti p53 (DO1, GeneSpin); anti-P-p53-Ser15 (Cell Signaling, #9284S); anti 53BP1 (Cell Signaling, #4937S); anti Vinculin (Sigma,#V9131); anti-Vimentin (Cell Signaling, #3932). Hydroxyurea (Sigma, #H8627) was used at a final concentration of 0,1 mM. Puromycin (sigma-Aldrich, #P9620) was used to a final concentration of 1 μ M.

Cell Culture. HeLa and HEK293T cells were cultured in DMEM containing 10% FBS, penicillin, streptomycin and L-Glutamine and kept at 37°C in a humidified atmosphere with 5% CO₂. EBV-immortalized lymphoblastoid cells derived from patients and control

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cells were cultured in RPMI containing 20% FBS, penicillin, streptomycin and L-Glutamine and kept at 37°C in a humidified atmosphere with 5% CO₂.

Lentiviral vectors production. shRNA sequences targeting human RNase H1 and subunits A or B of human RNase H2 were cloned using EcoRI and AgeI in pLKO.1-TCR cloning vector (Addgene #10878). As control SCRAMBLE shRNA cloned in pLKO.1 vectors was used (Addgene #1864). Lentiviral vectors were produced by transient co-transfection of pLKO and packaging plasmids psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) into HEK293T. Virus was harvested at 48 post-transfection and infections were carried out in the presence of 10 µg/mL of polybrene (Sigma, #107689). Following transduction, cells were selected with 1 µg/mL puromycin.

shRNA sequences: sense sequences used to silence RNase H1, RNase H2A or RNase H2B are: RNaseH1.1: 5'-GCCGTATGCAAAGCACATGAA-3'; RNaseH1.2: 5'-ACGATAAATGGTATAACTAAC-3'; RNaseH2A: 5'-GAAATGGCAGTTCGTGGAGAA-3'; RNaseH2B.1: 5'-GCTTCTCCACTACCTCATAAA-3'; RNaseH2B.2: 5'-ATCAAACGTGGCAGCATTA-3'

Western Blotting. Cells were lysed in 1% SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% wt/vol SDS, 10% glycerol, 50 mM DTT, 0.01% wt/vol bromophenol blue), sonicated 10 sec, and heated at 95°C for 5 min; equal amounts of total protein extracts were analyzed by SDS-PAGE.

Total RNA extraction and retrotranscription. Total RNA was isolated using High Pure RNA isolation kit (Roche Applied Bioscience) and 0,5-1 µg of RNA was retrotranscribed using EuroScript M-MLV Reverse Transcriptase (RNaseH-) (Euroclone) according to manufacturer's protocol. cDNA was used for RT-PCR using GAPDH as housekeeping control (primers sequences are available upon request).

Proliferation assay. HeLa cells were seeded in 96-well plates at a density of 3,000 to 5,000 cells per well. The viable cells were assessed by MTS assay every 24 h for 4 days at 490nm.

Cell cycle and FACS analysis. Exponentially growing cells were pulse-labeled with 100 μ M BrdU for 30 min and released in BrdU-free medium for different time points or immediately harvested by trypsinization and washed in PBS, fixed in 70% ice cold EtOH and either stained for anti-BrdU and propidium iodide in presence of RNase A. FACS analysis were performed on a BD FACScan and quantified with the Cell Quest software (BD Bioscience).

Colony forming assay. 100 HeLa cells were plated in p60 dishes in triplicate and allowed to grow for 14 days in complete medium in presence of 0,1 mM Hydroxyurea or left untreated. After 14 days cells were fixed in methanol and stained with crystal violet 1%. Colonies of more than 70 cells were scored under microscope.

Immunofluorescence. Cells were plated on glass slides, washed twice in PBS and pre-extracted for 5 min at 4°C in CSK buffer (0.1% Triton X-100 10 mM PIPES [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3mM MgCl₂, 1 mM EGTA, 1 mM EDTA). Cells were then fixed in PFA 4% for 15 min at RT and washed twice in PBS. Cells were permeabilized in PBS/0,5% Triton X-100 for 5 min at 4°C and blocked in PBS/10% BSA for 1h at RT. 53BP1 was diluted 1:100 in PBS/ 0,1% Tween and incubated for 3h at RT. Secondary antibody Goat anti Rabbit 594 Alexa (Invitrogen) diluted 1:1000 in PBS/ 0,1% Tween for 1h at RT.

Foci and micronuclei counting. Cells with more than 5 53BP1 foci per nucleus were counted covering randomly whole coverslip. At least 200 nuclei were scored for each sample. For micronuclei at least 500 cells per sample were counted.

Ribonucleotides incorporation assay. Genomic DNA was purified using Qiagen Genomic-Tip and Qiagen Genomic DNA Buffer set and was digested with 0.5 U E.coli RNase HII (New England Biolabs, Inc.) in 50 μ l of total volume in presence of the recommended buffer at

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37°C for 2.5 h. After precipitation in 0.3 M sodium acetate, pH 5.2, and ethanol, DNA was resuspended in TE 0,1%. Nick translation was performed using 5U of E.coli DNA polymerase I (New England Biolabs, Inc.), 20 µM of unlabeled dA/-T/-GTP (Ambion), 1X buffer 2 (New England Biolabs, Inc.) and 5µC α32PdCTP in a final volume of 20µl. The reaction was incubated at 16°C for 30 min. Labeled DNA was separated from unincorporated nucleotides by electrophoresis on a 1% TAE agarose gel and visualized by autoradiography.

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FIGURE LEGENDS

Figure 1: depletion of RNase H2 complex impairs proliferation of HeLa cells.

HeLa cells were silenced using one shRNA sequence against subunit A and two different sequences against subunit B. As control, non-targeting shSCRAMBLE sequence was used. Efficiency of silencing was monitored by immunoblotting (A) or real-time PCR (B). Silencing of A or B subunits affects protein level of subunit C (A). MTS assay was performed in triplicate to monitor cell proliferation. Cells were plated 7 days after infection and analyzed every 24 hours. MEAN +/- SD (C). Relative LDH release was measured 11 days after infection. MEAN +/- SD of triplicate (D). Immunoblot for Caspase3 was performed on total protein extract 11 day after infection. Protein extract from HeLa cells treated with H₂O₂ were used as positive control (E).

Figure 2: RNase H2-silenced cells accumulate in S and G2/M phase.

Cell cycle distribution of asynchronous HeLa cells. Histograms represent the percentage of cells in G1, S and G2-M phase (A). FACS profiles were analyzed using CellQuest program. Numbers of three independent experiments (MEAN +/- SD) were reported in (B). Cell cycle progression. Representative images of cell kinetic experiment. Asynchronous HeLa cells were pulse labeled with BrdU and released in BrdU-free medium. Cell were harvested and stained with anti-BrdU and Propidium Iodide (PI) at indicated time points (red arrows indicate cells entered in new G1 phase)(C).

Figure 3: depletion of RNase H2 causes replications stress in human cells.

Immunoblot on total protein extract from control and RNase H2A/B silenced HeLa cells against PCNA. Anti-Vimentin was used as loading control (A). Sensitivity to HU was evaluated by colony forming assay. Control and silenced HeLa cells were seeded at low density in p60 dishes. 24 hours after plating medium 0,1 mM HU was added to the medium. Cells were incubated for 14 days and then fixed and stained with crystal violet. Only colonies containing more than 70 cells were scored. The histograms report the percentage of surviving colonies respect to respective untreated sample. Three independent experiments were performed in triplicate. MEAN +/- SD (B).

Figure 4: depletion of RNase H2 induces DNA damage response and causes genome instability in HeLa cells.

Total protein extracted of silenced HeLa cells was blotted with indicated antibodies, HeLa irradiated with UV (20 J/m²) were used as control (A). HeLa cells were immunostained with an

anti-53BP1 antibody and DAPI to stain nuclear DNA (B). The histograms indicate the percentage of cells that have at least 5 53BP1 foci/nucleus. A minimum of 200 cells for shRNA sequence was counted in three independent experiments (C). The histograms in (D) indicate the percentage of micronucleated cells. At least 500 cells were counted in three independent experiments. For each graph MEAN +/- SD is used.

Figure 5: AGS-derived cells have active PRR and DNA damage response.

Immunoblot against PCNA on total protein extract from WT and AGS-mutated lymphoblastoid cells, cells UV irradiated (20 J/m^2) were used as positive control for PCNA ubiquitylation (A). DNA damage response in control and AGS-mutated cells was monitored by western blot analysis using anti-P-Ser-15-p53 antibody (B).

Figure 6: AGS-derived cells show accumulation of genomic ribonucleotides.

DNA extracted from control and AGS-mutated cells were nicked by bacterial RNase HII at ribonucleotide positions. DNA polymerase I-dependent nick translation reaction in the presence of ^{32}P -dCTP was performed at 16°C for 30'. Labeled DNA was run on a 1% agarose gel and visualized by autoradiography. Control samples were not digested with RNase HII to detect background signal (A). DNA from *rnh1rnh201* or wt yeast strains was used as experimental control (B). Radioactive signal from WT and G37S-mutated cells was normalized to respective DNA loading and quantified on relative untreated sample using ImageJ software (C).

Figure.1

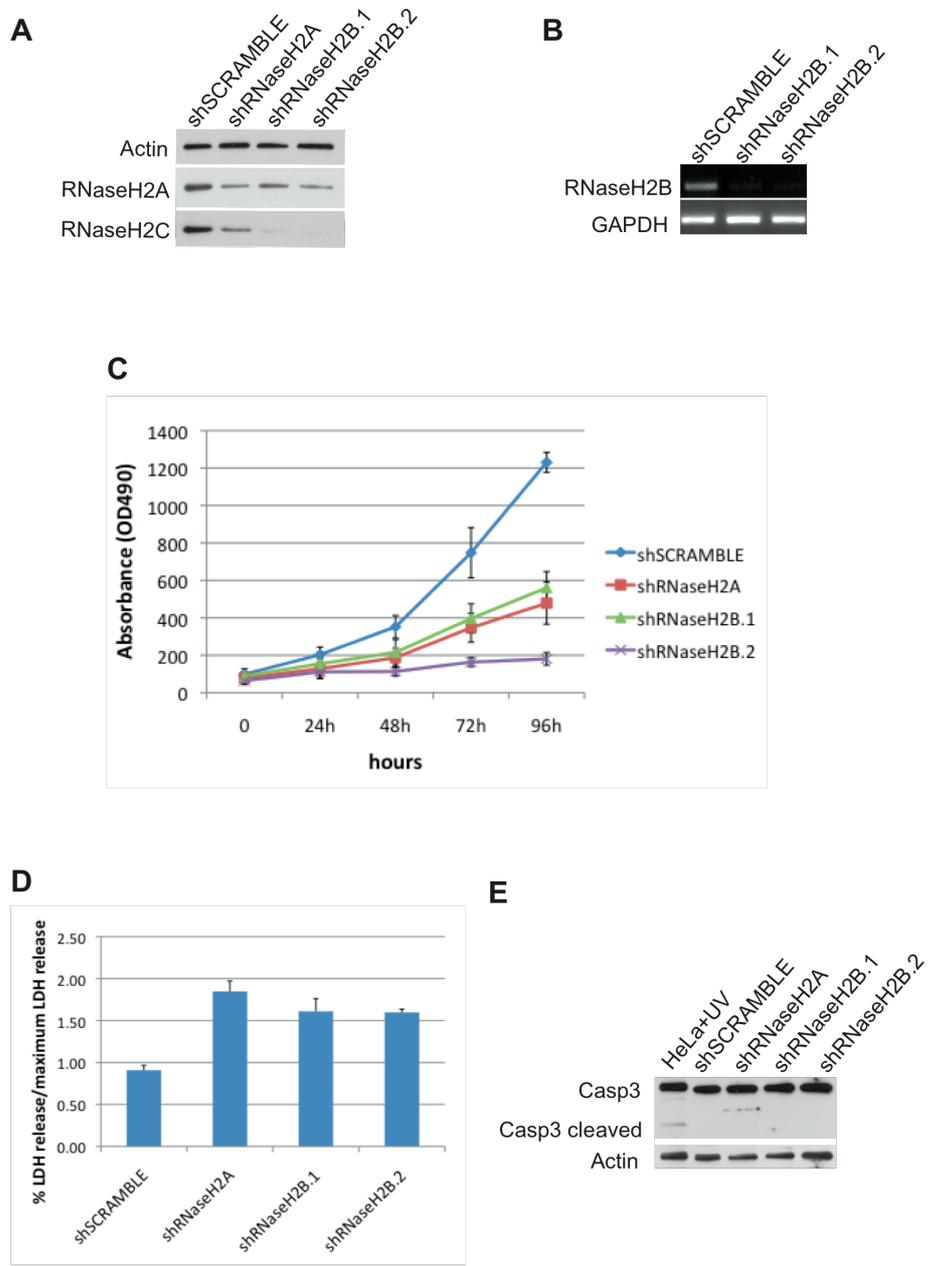


Figure.2

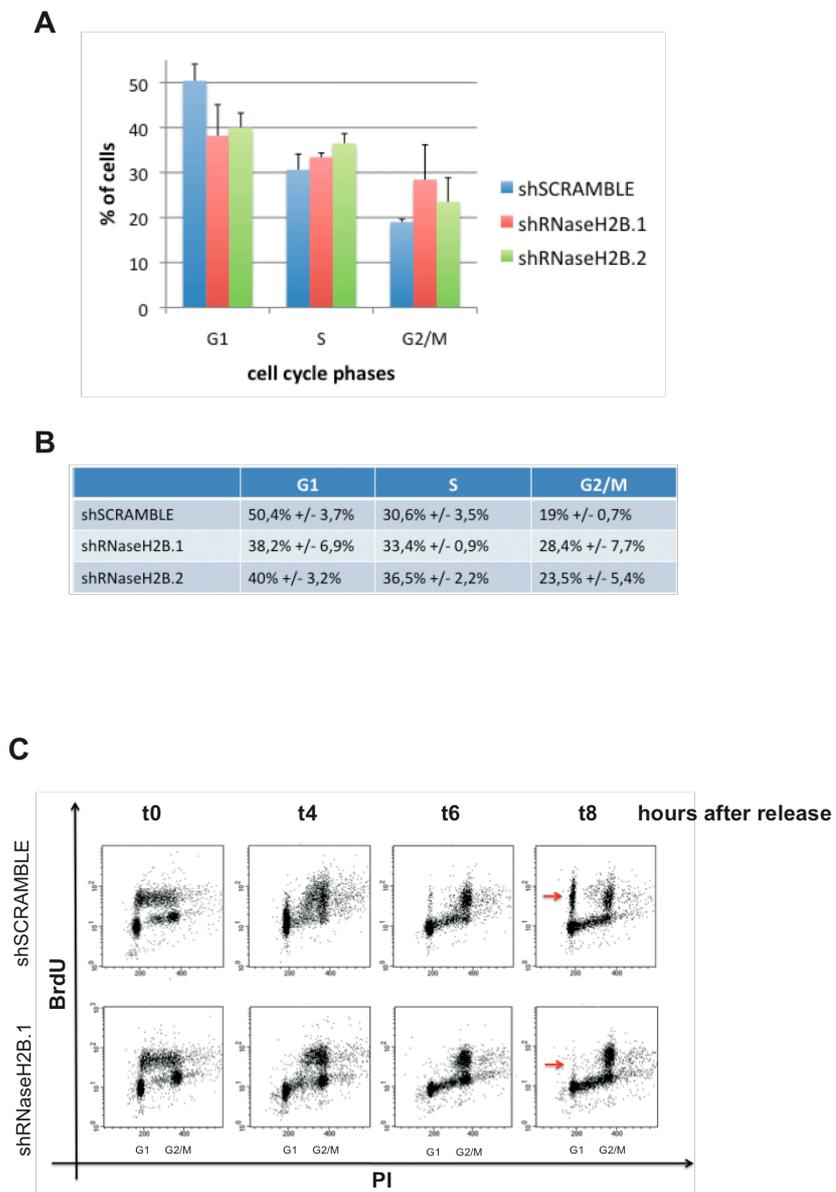


Figure 3

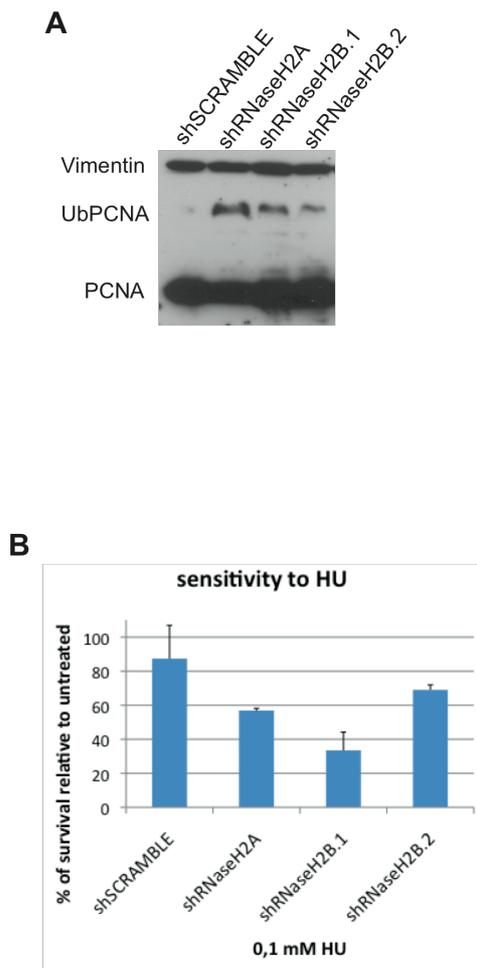


Figure.4

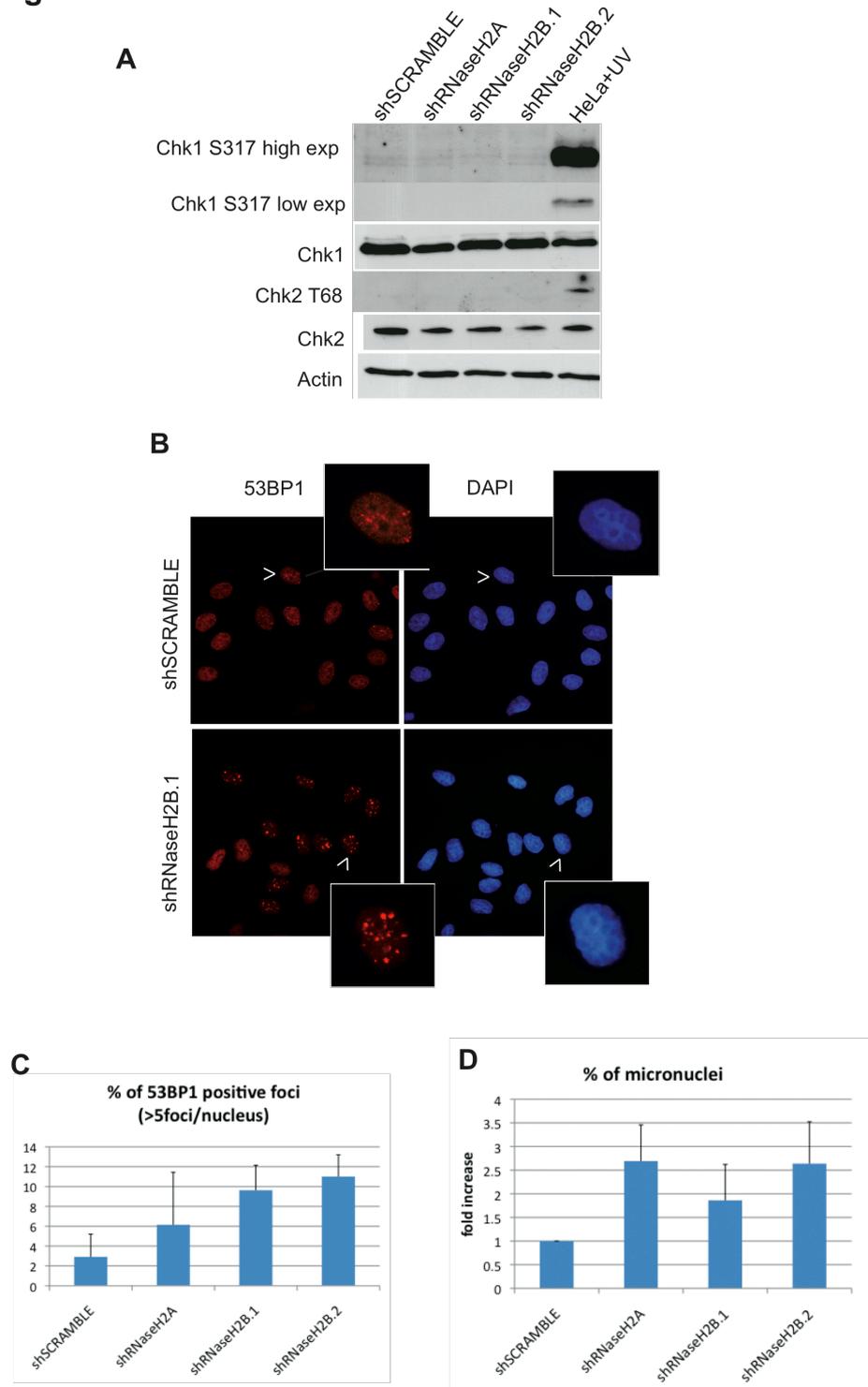


Figure.5

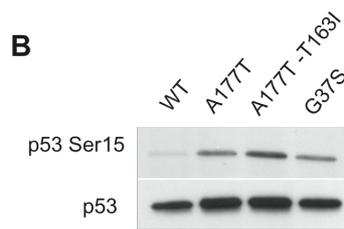
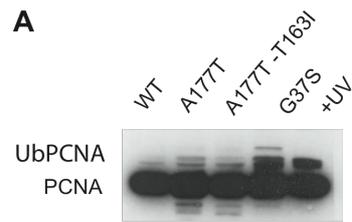
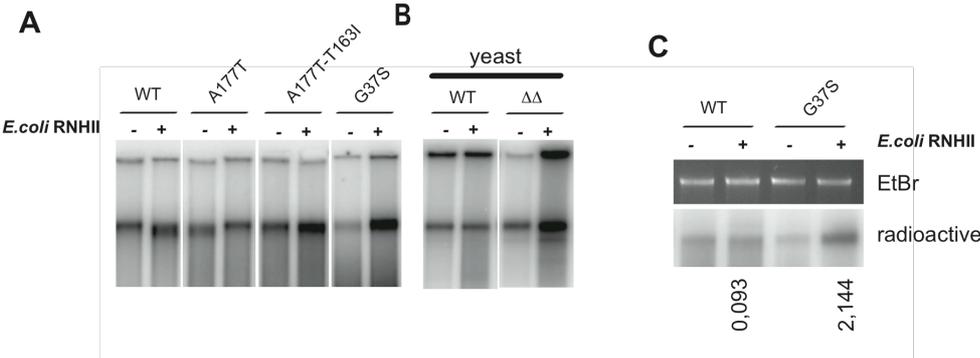


Figure.6



SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Two different shRNA sequences targeting RNase H1 were tested. Efficiency of silencing was evaluated by western blot analysis (A). RNase H1-depleted HeLa cells were defective in colony forming assay in untreated conditions. Control and RNase H1-silenced HeLa cells were plated at low density and fixed and stained with crystal violet 1% after 14 days. The percentage of colonies formed respect to plated cells was scored. Three independent experiment were performed in triplicate. MEAN \pm SD (B). Representative images of colony forming efficiency of RNase H1-depleted cells and control cells (C). Total protein extract of control and RNase H1-depleted HeLa cells was blotted and stained using antibody against Caspase3 (D).

Figure S2: The efficiency of silencing of two different shRNA sequences targeting subunit B of RNase H2 was evaluated. Expression levels of RNase H2B was measured by quantitative RT-PCR (qRT-PCR). Relative expression was normalized against GAPDH. Error bars represent SD

Figure S3: quantification of 53BP1 foci formation in WT and G37S-mutated lymphoblastoid cells. A minimum of 200 cells for sample was counted. This experiment was performed only once.

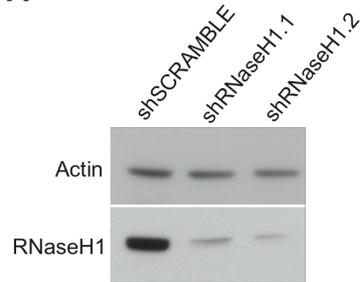
SUPPLEMENTAL INFORMATIONS

Quantitative real-time PCR. The cDNA reaction was diluted 1:20 in TE. PCR primers were designed to amplify 200 bp fragments: sequences are available upon request. GAPDH was used as housekeeping control. Quantitative PCR was performed using SYBR green IQ reagent (Biorad) in the iCycler IQ detection system (Biorad). Reactions were run in triplicates, and the relative sample enrichment was calculated with the following formula: $2^{\Delta\Delta C_t}$, where $\Delta C_t = C_t \text{ input} - C_t \text{ sample}$ and $\Delta C_t = C_t \text{ input} - C_t \text{ control Ab}$.

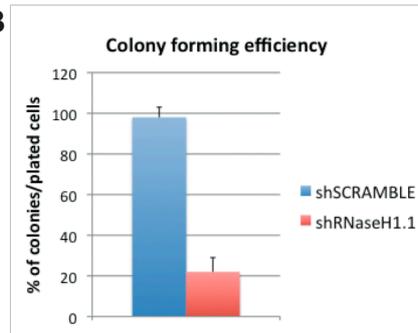
Supplementary figures

S1

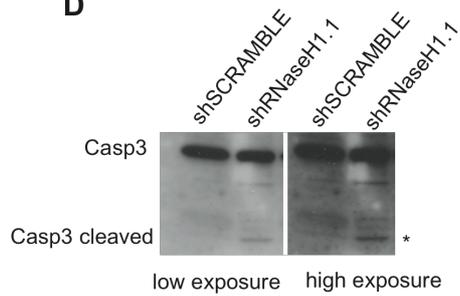
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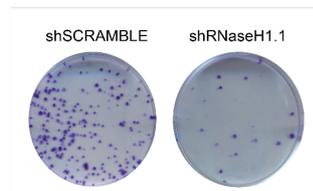
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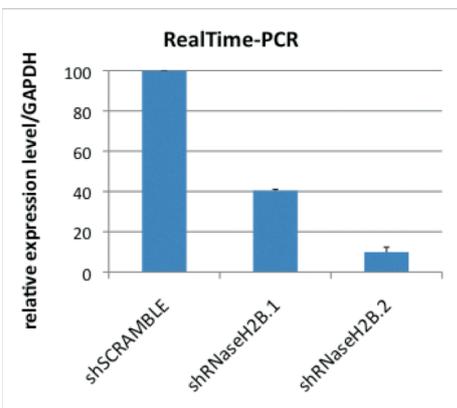
D



C



S2



S3

