*UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO*

**DIPARTIMENTO DISCIENZE ECOLOGICHE E BIOLOGICHE**

**Corso di Dottorato di Ricerca in**

GENETICA E BIOLOGIA CELLULARE – XXVII Ciclo.

# "Investigating the role of Werner syndrome protein in the activation of the ATR-dependent checkpoint in response to mild replication stress"

(**s.s.d. BIO/11**)

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**Data della discussione**

**08/05/2015**

*Dedicated to*

*my boyfriend,*

*my family,*

*my closest friends,*

*my pets and*

*those who will always be*

*in my heart*

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## SUMMARY

Werner syndrome (WS) is a human chromosomal instability and cancer-prone disease caused by mutations in the WRN gene, encoding for the Werner syndrome protein (WRN) that is a member of the RecQ helicases.

It has been previously proposed that WRN helicase activity is a key regulator of common fragile site (CFS) stability, which are the preferential targets of genome instability in precancerous lesions. Moreover, it is known that, under mild replication stress inducing by low dose of Aphidicolin (Aph), WRN and ATR act in a common pathway preventing accumulation of DNA breaks at CFS. Despite WS cells exhibit an ATR-like instability at CFS, and that WRN has been found phosphorylated by ATR under robust replication stress caused by treatments with hydroxyurea (HU), there is no evidence of a functional requirement of WRN in the establishment of the replication checkpoint response to mild replication stress, like that inducing CFS expression.

The aim of this study was to analyze the functional requirement of WRN in the ATR-dependent checkpoint activation under mild replication stress using low doses of aphidicolin treatments.

Our data establish that WRN plays a role in mediating CHK1 activation, a principal target of the ATR kinase activity under replication stress. Moreover, our results demonstrate that WRN and the ATR-mediated WRN phosphorylation are required to phosphorylate CHK1, as they are important for chromatin loading of checkpoint mediators under untreated as well as Aph-treatment conditions. In contrast, although WRN helicase activity is not required for CHK1 phosphorylation, it results essential in supporting replication fork recovery, possibly by the resolution of DNA secondary structures, thus promoting CFS stability.

Analysis of replication fork dynamics shows that loss of WRN checkpoint mediator function, as well as of WRN helicase activity, hamper replication fork progression, and lead to new origin activation to allow recovery from replication slowing upon mild replication stress. Furthermore, bypass of WRN checkpoint mediator function through over-expression of a phospho-mimic form of CHK1 restores fork progression and chromosome stability to the wild-type levels.

Loss of WRN also greatly hampers the phosphorylation of histone H2AX (γ-H2AX), which is the earlier target of ATR kinase following replication stress. Indeed, although, upon mild replication stress, in wild-type cells H2AX is activated by ATR in a time-dependent

manner, its phosphorylation is reduced in WS cells, further confirming defects in the ATR signalling.

Furthermore in this study, others cellular consequences of WRN loss have been explored in response to mild replication stress. Evidences demonstrate that the absence of WRN leads to an ATM pathway activation, which is harmful to the cells, as confirmed by positive effects obtained on cellular survival and chromosomal damage by ATM inhibition in the last part of the treatment with Aph. One way by which ATM inhibition could protect genome stability in WS cells is the recovery of CHK1 defective activation. Noteworthy, in cells expressing the unphosphorylable form of WRN, WSWRN$^{6A}$, ATM inhibition is not able to rescue CHK1 activation, possibly because the presence of the protein, although mutated, could prevent the activation of such alternative pathway. Furthermore, WRN deficiency leads the formation of 53BP1 foci in S phase, after prolonged mild replication stress. The increase of 53BP1 foci in all cell cycle phases has been previously associated with CHK1 depletion, and so it is consistent with defective CHK1 activation observed in WRN-deficient cells following Aph exposure. These findings suggest that, in S phase, in absence of the ATR checkpoint activation, 53BP1 recruitment in foci is instrumental in attracting other proteins implicated in the response to mild replication stress damage.

Therefore, our results suggest a novel role of WRN as checkpoint mediator in response to moderate replication stress and give strong mechanistic support to the notion that defective fork repair/recovery undermines integrity of chromosomes at CFS. This study also unveils a complicated network in which several proteins work tightly linked together. Loss of one protein means altering this network and changing the interaction among proteins.

Moreover, our findings may contribute to shed light into the origin of chromosome instability in WS and more in general to clarify how genome instability accumulates in pre-neoplastic lesions, thus promoting cancer development.

# INTRODUCTION

# INTRODUCTION

## *DNA replication and genome stability*

Genome instability is a common feature of cancer cells. Most of the chromosomal abnormalities arising in tumors come from defective DNA replication (Myung and Kolodner, 2002). Thus, in eukaryotic cells DNA replication process is tightly monitored to ensure that genome is replicated just once per cell cycle, and that DNA duplication is complete before mitosis begins (Branzei and Foiani, 2010).

Given to the complexity of the replication process, it is not surprising that defects in DNA replication or in its regulation may give rise to several human diseases. Therefore, investigating the DNA replication process and the pathways that are involved in preventing genome instability is fundamental to understand the mechanisms by which cancers and others pathological disorders arise.

DNA replication represents a crucial moment in the life of a cell, as chromosomal integrity can be seriously threatened by replication stress, that is the slowing and/or stalling of replication fork progression (Zeman and Cimprich, 2014). In fact, replication stress interferes with fork stability and can be caused by endogenous side-products of cellular metabolism, exogenous agents capable to interfere with DNA replication, as well as intrinsic structural features of specific genomic regions, such as the common fragile sites (CFS). Fork stalling is a very frequent event occurring during S-phase. To guarantee genome integrity , replication forks are endowed with an extraordinary potential to coordinate fork stalling with fork resumption processes. When protection of stalled forks or their processing and replication restart fail, mutations and aberrations accumulate in the genome. Mutations in genes that protect the genome integrity during replication characterize a variety of human genetic syndromes, which lead to cancer predisposition (Branzei and Foiani, 2005).Among these human disease, there is Werner syndrome, that shows defects in resolving DNA replication stress.

To minimize the risk of chromosomal rearrangement accumulation and deal with problems encountered during S-phase, cells have evolved a sophisticated apparatus deputed to the resolution of problems arising at replication forks: the replication checkpoint.

## *The replication checkpoint response*

The link between replication defects, human diseases and cancer underscores the requirement of an efficient and accurate monitoring of genome integrity during DNA replication, and the presence of multiple checkpoint activities in the S-phase may be explained with the complexity of the DNA duplication process.

The replication checkpoint is a complex and coordinated network under the control of the ATR kinase (Abraham, 2001; Zou and Elledge, 2003). These biochemical network contains a class of protein, named mediators or adaptors, which promote functional interactions between sensor and effector proteins. In the case of replication stress, replication checkpoint activation leads to inhibition of origin firing, cell cycle arrest, stabilization, and then restart of stalled forks, and prevention of the entry into mitosis until the DNA has been completely replicated (Budzowska and Kanaar, 2009).

ATR was discovered in the human genome database as a gene with sequence homology to ATM and SpRad3, hence the name ATR (Cimprich et al., 1996). The gene encodes a protein of 303 kDa with a C-terminal kinase domain and regions of homology to other PIKK family members. ATR deficiency in mice results in early embryonic death (Brown and Baltimore, 2000), and mutations causing a partial loss of its activity have been reported to be associated with the human autosomal recessive disorder, Seckel syndrome (O'Driscoll et al., 2003). ATR is capable of specifically phosphorylating Serine or Threonine residues in SQ/TQ sequences (Abraham, 2001). In human cells, ATR exists in a stable complex with ATR-interacting protein (ATRIP), a potential regulatory partner (Cortez et al., 2001; Sancar et al., 2004). ATR is essential for embryonic cell viability and, for this reason, it probably has an important function during cell cycle progression.

Although ATR is activated in response to many different types of DNA damage, including double strand breaks (DSBs), base adducts, crosslinks, it is thought to be mainly responsible for the replication stress response.

The first step of the cellular response to stalled replication forks requires the recognition of a such event. In eukaryotes, replication stress usually results in the formation of stretches of single-stranded DNA (ssDNA) that plays crucial roles in its cellular recognition. When forks are stalled, for example, by hydroxyurea or by Aphidicolin, uncoupling of replicative helicase and DNA polymerases takes place, generating a ssDNA of sufficient length (Byun et al., 2005; Sogo et al., 2002). In fact, often replicative helicases continue to

unwind the parental DNA after the polymerase has stalled. RPA binds to these ssDNA, and protects DNA from erosion. ATRIP brings the sensor/master kinase ATR to the site of the fork stall (Zou, 2007). However, the ssDNA-RPA complex is not sufficient for checkpoint activation (Masai et al., 2010). RPA-coated ssDNA recruits ATR–ATRIP and facilitates the loading of 9–1–1 clamp to ds/ssDNA junctions by the Rad17 complex. Rad17 and Rad9 are phosphorylated by ATR, and the phosphorylated Rad17 and Rad9 recruit Claspin and TopBP1, respectively, allowing them to be efficiently phosphorylated by ATR (Zou, 2007). The binding of TopBP1 with RAD9 localizes TOBP1-ATR-activating domain near ATR, further stimulating the kinase activity of ATR (Kumagai et al., 2006). Once ATR is fully activated at assembled stalled forks, signaling to coordinate cell cycle, repair and replication can begin.

The list of ATR substrates is rapidly expanding thanks to the use of large-scale proteomic profiling methodologies (Matsuoka et al., 2007; Mishmar et al., 1998; Mu et al., 2007; Stokes et al., 2007). However, the best studied is the Ser/Thr kinase checkpoint kinase-1 (CHK1). The phosphorylation of Claspin by ATR may promote its interaction with CHK1, a serine/threonine-protein kinase. Claspin binds to phosphorylated RAD17 (a component of the 9-1-1 clamp loader) and this interaction is important for sustaining CHK1 phosphorylation (Kumagai and Dunphy, 2000; Wang et al., 2006). Claspin interacts with CHK1 in a damage-dependent manner, and this interaction requires the phosphorylation of Claspin on at least two sites (Ser864 and Ser895 in X. laevis) (Kumagai and Dunphy, 2003). In addition to Claspin, a second replication-fork-associated complex that is composed of timeless, and timeless-interacting protein (tipin) might also mediate the activation of CHK1 by ATR (Errico et al., 2007; Leman et al., 2010; Unsal-Kaçmaz et al., 2007).

CHK1 activation requires phosphorylation by ATR on Ser317 and Ser345, which seems to be a reliable indicator of CHK1 activation (Liu et al., 2000; Lopez-Girona et al., 2001; Walworth and Bernards, 1996) and these post-translational modifications are used to amplify the signal. Once phosphorylated, CHK1 is released from chromatin to phosphorylate its substrates(Zhao and Piwnica-Worms, 2001) and signal DNA damage to the rest of the nucleus. Reduced CHK1 activity has been associated with accumulation of ssDNA (Syljuåsen et al., 2005), impaired replication fork progression (Maya-Mendoza et al., 2007), and increased fork stalling (Maya-Mendoza et al., 2007; Petermann and Caldecott, 2006). Once phosphorylated, CHK1 plays a critical role in suppressing late replication origin firing and maintaining fork integrity (Lopes et al., 2001; Maya-Mendoza et al., 2007; Petermann and

Caldecott, 2006).

CHK1 activation inhibits the entry into G2 or M phase by targeting CDC25 phosphatases (Boutros et al., 2006). Human cells have three CDC25 proteins that regulate cell-cycle transitions by removing the inhibitory phosphorylation of cyclin-dependent kinases (CDKs). CHK1 phosphorylation of the CDC25 proteins inhibits their activity and prevents CDK activation (Furnari et al., 1999; Peng et al., 1997; Sanchez et al., 1997). This is a major checkpoint mechanism that prevents entry into mitosis.

ATR signalling through CHK1 is also crucial for regulating replication. In eukaryotes, DNA replication originates on multiple chromosomes from multiple origins that form bidirectional replication forks. The ability to replicate the genome from multiple origins was probably a crucial event in the evolution of eukaryotes, nevertheless, the presence of multiple origins presents challenges to ensure that all parts of the genome are fully replicated in each S-phase and no origin initiates for a second time in one cell cycle. For these reasons cells developed a two-step mechanism that consists in "origin licensing" and "origin firing" that are processes separated temporally and tightly coupled to distinct phases of the cell cycle. Before S-phase, each origin is 'licensed' by the loading of the replicative helicases and a combination of replication initiation proteins to prepare the chromatin for replication at future origins (Masai et al., 2010). Origin firing involves the subsequent activation of the replicative helicases(Masai et al., 2010).Replication origins fire according to a cell-type-specific temporal program, which is established in the G1 phase of each cell cycle. In an unperturbed S-phase only ∼10% of replication origins licensed are normally used in the firing process, while the majority remaining dormant. In response to conditions causing the slowing or stalling of DNA replication forks, the program of origin firing is altered in two contrasting ways, depending on chromosomal context: first, inactive or 'dormant' replication origins in the vicinity of the stalled replication fork become activated and, second, the checkpoint induces a global shutdown of further origin firing throughout the genome. In this way, when DNA replication fork progression is slowed or stalled, nearby dormant origins initiate (Ge et al., 2007; McIntosh and Blow, 2012; Woodward et al., 2006) to ensure the completion of DNA replication at stalled replication forks (Zeman and Cimprich, 2014). ATR signalling globally slows down DNA replication at least in part by inhibiting origin firing , that is important even in the absence of added exogenous replication stress agents (Maya-Mendoza et al., 2007; Shechter et al.). ATR-dependent inhibition of origin firing is crucial in reducing the rate of DNA synthesis under different DNA-damaging conditions (Alvino et al., 2007;

Baynton et al., 2003a; Feijoo et al., 2001; Heffernan et al., 2002; Merrick, 2004; Mickle, 2007; Otterlei et al., 2006; Pichierri et al., 2003; Sakamoto et al., 2001; Shechter et al.; Shirahige et al., 1998; Tercero and Diffley, 2001).

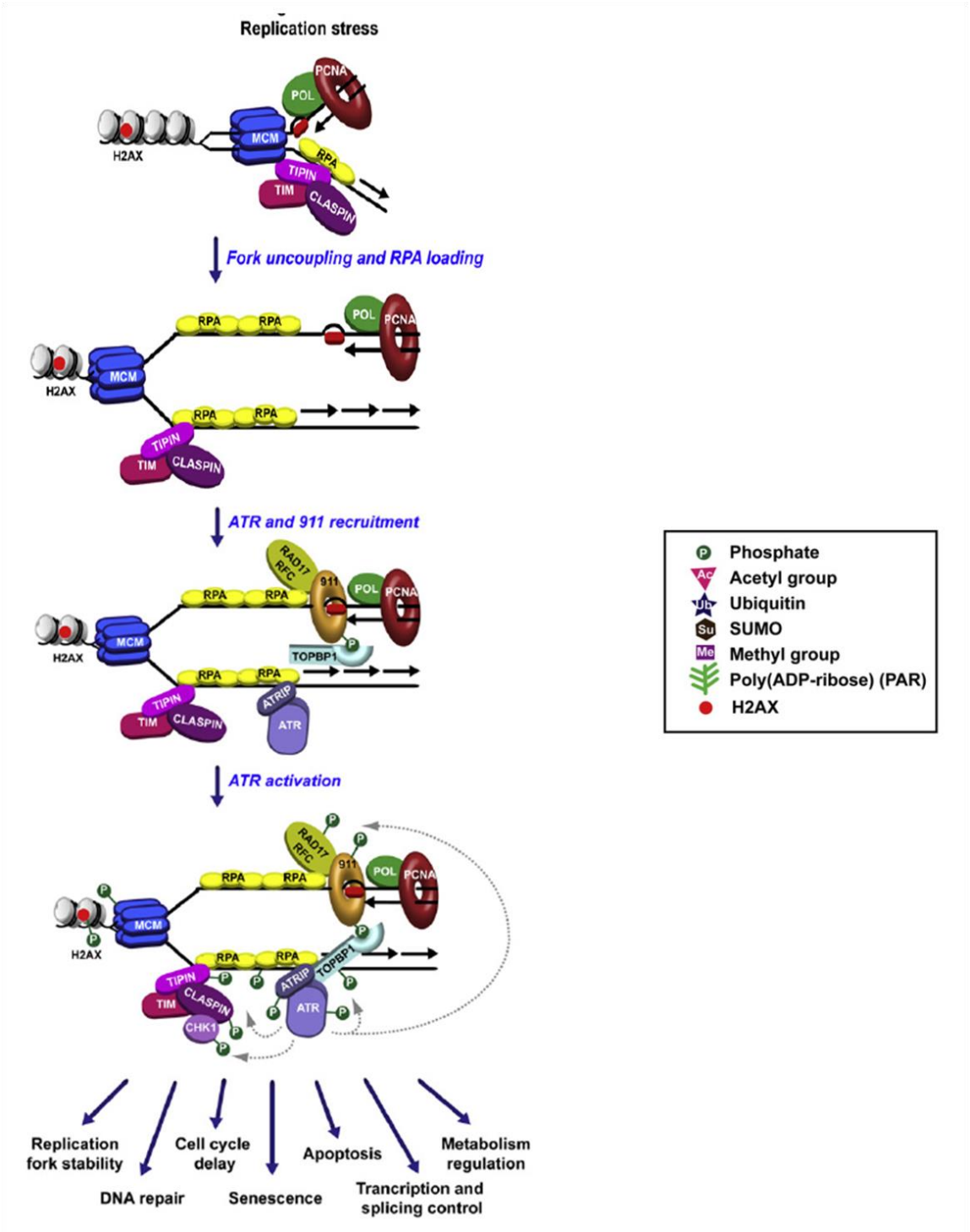



**Figure 1** Replication stress leads to replication fork stalling and accumulation of RPA-coated ssDNA regions, which recruit the ATR/ATRIP and the RAD17/RFC2-5 complexes. Loading of the 9-1-1 complex by RAD17/RFC2-5 and stimulation of the ATR kinase activity by the 9-1-1-associated protein TOPBP1 result in the activation of the ATR signaling cascade and CHK1 phosphorylation. Posttranslational modifications of the DDR factors depicted here are represented by different colored shapes, as indicated by the legend. (Ciccia et al., 2010).

Defective ATR-dependent signaling in the replication regulation might represent one of the majority cause that leads to genome instability. In the human genome there are regions, the common fragile sites, which had been found to be the preferential targets for genome instability in the early stages of tumorigenesis. Interestingly, the ATR-dependent checkpoint together with several proteins involved in response to replication fork stalling have been implicated in maintaining common fragile site stability.

## *Common fragile sites*

Common fragile sites (CFS) are loci that preferentially exhibit chromosome instability visible as gaps and breaks on metaphase chromosomes following partial inhibition of DNA synthesis (Durkin and Glover, 2007). Unlike rare fragile sites, CFS represent a component of normal chromosome structure and are not the result of nucleotide repeat expansion mutations. It was determinatedthat the great majority of CFS are also specifically and reproducibly induced by low doses of aphidicolin (APH), an inhibitor of DNA polymerase α, δ, and (Cheng and Kuchta, 1993; Durkin and Glover, 2007; Ikegami et al., 1978). At low concentrations APH does not greatly affect mitotic index, and slows replication fork movement causing mild replication stress. CFS are considered not only themselves as a source of replication stress but also, DNA breakage at these sites is considered a symptom of replication stress (Zeman and Cimprich, 2014) even at mild levels (Bartkova et al., 2005; Gorgoulis et al., 2005). Until today, there is a consensus considering that moderate slowing of replication fork movement delays completion of CFS replication more than the rest of the genome, and that breaks occur at under-replicated sequences upon chromosome condensation at mitotic onset. In addition to gaps and breaks, CFS display a number of characteristics of DNA instability in cultured cells: following induction, they are ‘hotspots’ for increased sister chromatid exchange (SCE)and show high frequency of translocations and deletions in somatic cell hybrid systems.

In vivo, CFS correlate with chromosomal breakpoints in tumors (Hecht and Glover, 1984; Ma et al., 2012a), and were found to be involved in deletions of tumor suppressor genes and genomic amplification of oncogenes (Hellman et al., 2002; Ozeri-Galai et al., 2012). CFS are hotspots of genome instability since early stages of tumorigenesis, in fact chromosomal instability at these loci precedes the instability in other genomic regions and it is thought to be a driving force in cancer progression (Ma et al., 2012b).

Rassool and colleagues (Rassool et al., 1991) demonstrated that fragile sites are preferred sites of recombination or integration with pSV2neo-plasmid DNA transfected into cells pre-treated with aphidicolin. Perhaps related to this characteristic are reports of the coincidence of viral integration sites in tumors or tumor cell lines and fragile sites(de Braekeleer et al., 1992).

Fragile sites have also been implicated in intrachromosomal gene amplification events in cultured Chinese hamster ovary (CHO) cells and in cancer cells by leading to DNA strand breaks that trigger breakage–fusion–bridge cycles (Coquelle et al., 1997). Despite their inherent stability, CFS have been observed in several other mammalian species(Coquelle et al., 1997; McAllister and Greenbaum, 1997; Ruiz-Herrera et al., 2004; Smeets and van de Klundert, 1990; Soulie and De Grouchy, 1981; Stone et al., 1991, 1993; Yang and Long, 1993), thus suggesting a conserved function. Of those species, CFS are currently best characterized in the laboratory mouse.

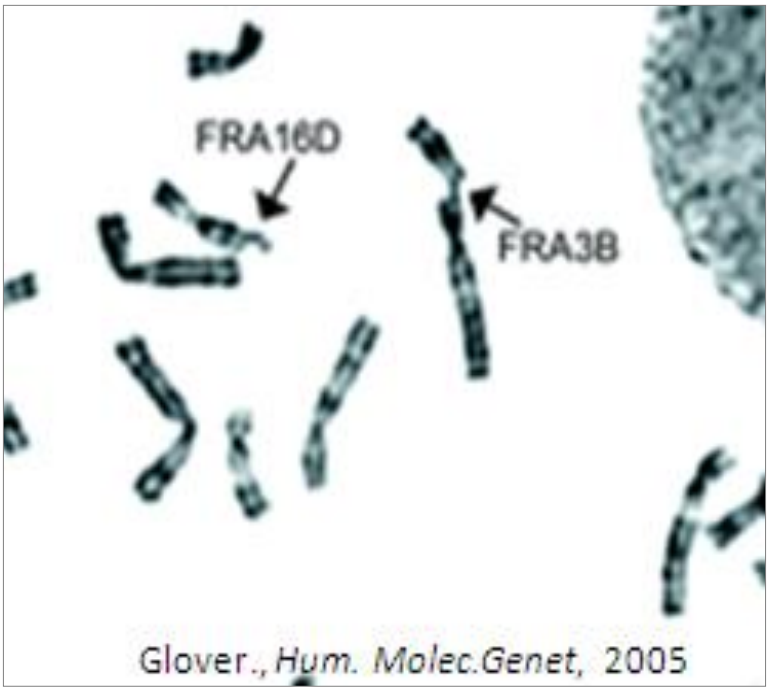



**Figure 2**Examples of common fragile sites. Human G-banded metaphase chromosomes with breaks at fragile sites FRA3B and FRA16D (arrows). (Glover et al. 2005).

Sequence analyses of cloned fragile sites did not clarify why these sites are unstable. The molecular basis of their fragility, indeed, are not fully understood yet. All fragile sites cloned to date are relatively AT-rich (Arlt et al., 2002; Boldog et al., 1997; Ried et al., 2000; Shiraishi et al., 2001), and have no expanded di- or trinucleotide repeats.

Mishmar and colleagues (Mishmar et al., 1998) designed the FlexStab program to measure local variation in the twist angle between bases, and they found that the FRA7H region contained more areas of high flexibility, termed 'flexibility peaks', than the non-fragile regions. These flexible sequences are composed of interrupted runs of AT- dinucleotides (Zlotorynski et al., 2003a), showing similarity to the AT-rich minisatellite repeats that underlie the fragility of the rare fragile sites, FRA16B and FRA10B. Such sequences have the potential to form secondary structures and, hence, may affect replication at fragile sites (Zlotorynski et al., 2003a).

Despite their biological and medical relevance, the molecular basis of CFS fragility in vivo has not been fully elucidated. At present, different models have been proposed to explain how instability at CFS. Mounting evidence suggests that instability at CFS depends on multiple factors, but all the proposed models imply that replication fork progression along these loci is perturbed, and that protection of their integrity relies on an accurate response to replication stress (Glover et al., 2005; Lukusa and Fryns, 2008; Mishmar et al., 1998). Hence, it is reasonable that proteins involved in the stabilization and safe recovery of replication forks could play a crucial role in preserving CFS integrity (Mishmar et al., 1998; Zlotorynski et al., 2003a).

### *Multiple factors underlying CFS instability*

CFS are large genomic regions, spanning hundreds to thousands kilobases, which possess common features but show often different chromosome localizations in different cell types or tissues (Debatisse et al., 2012). About 80 CFS have been identified so far, but not all are expressed at the same frequency and may present different cell-type-specific sensitivity (Letessier et al., 2011; Le Tallec et al., 2011).

While the molecular basis of CFS instability still remains elusive, several factors may contribute to the fragility of these regions. Computational studies proposed that AT-rich sequences of CFS may perturb DNA replication because of their ability to adopt complex secondary structures and their tendency of fork stalling or replication elongation perturbation (Mishmar et al., 1998; Zlotorynski et al., 2003b). An indirect evidence that such sequences may perturb DNA replication because of their potential ability to adopt complex structures has been provided by in vivo studies in a yeast system (Mishmar et al., 1998; Zlotorynski et al., 2003b). In that study, an AT-rich region within the fragile site, FRA16D was predicted to have high flexibility and to form cruciform DNA. At this site replication fork frequently stall,

and increased chromosome breakage was registred, mimicking what might happen at human CFS, also independently from replication stress. This is the first demonstration that links a sequence element within a CFS with replication fork arrest and chromosome breakage. However, the most convincing, even if yet indirect proof is provided by the observation that a stable ectopic integration of FRA3B into non-fragile loci recapitulates the CFS-like phenotype (Zhang and Freudenreich, 2007).

Interestingly, the hypothesis that that intrinsic features of a CFS sequence are associated with breakage at fragile regions has been proven by electron microscopy analysis in human cells transfected with FRA16B-containing constructs. That study revealed a propensity of the FRA16B replication fork template to promote spontaneous fork reversal and DNA polymerase pausing at specific sites within the FRA16B region, suggesting that the secondary structure-forming ability of FRA16B contributes to its fragility by stalling DNA replication (Zhang and Freudenreich, 2007). Overall, these observations confirm that generation of stable secondary structures may be a general mechanism accounting for the fragility of CFS during DNA replication.

Apart from an involvement of DNA secondary structures in determining CFS instability, a role for replication origin density has been described, and the idea that CFS expression is epigenetically defined has been proposed (Letessier et al., 2011). According to that study, fragility of the human FRA3B fragile site in lymphoblastoid cells, but not in fibroblasts, is due to a paucity of initiation events, which forces forks coming from flanking regions to cover long distances to finish replication. Treatment of the cells with aphidicolin leads to reduction of replication fork velocity. Consequently, replication along CFS risks remaining partial, resulting in unreplicated regions which show a remarkable propensity to breakage respect to the rest of the genome (Letessier et al., 2011).

Moreover, it has also been proposed that commitment to fragile site instability in different cell types depends on the same paucity of origins, but different chromosomal regions are committed (Le Tallec et al., 2011). Thus, the scarcity of origins within FRA3B in combination with it being a late-replicating region could be responsible for the incomplete replication of the site at G2/M, leading to its elevated susceptibility to breakage.

A direct demonstration of fork stalling along an endogenous human fragile site has been provided (Ozeri-Galai et al., 2012). Indeed, along the human FRA16C region, high levels of fork stalling close to the AT-rich sequences are observed, clearly indicating that replication is intrinsically perturbed. Moreover, although replication stress further enhances fork stalling, most of the origins are already activated under unperturbed conditions, thus CFS are not able to compensate for replication stress resulting in wide unreplicated regions more sensitive to breakage (Ozeri-Galai et al., 2012). Consistent with that study, the analysis of the replication dynamics of FRA6E, that contains long AT-rich sequences, also showed a slower replication rate along the site, a shorter inter-origins distance, and a higher frequency of replication fork arrest with respect to the rest of the genome (Palumbo et al., 2010). These studies suggest that both paucity of replication origins and fork arrest can contribute to the destabilization of the FRA16C and FRA6E regions.

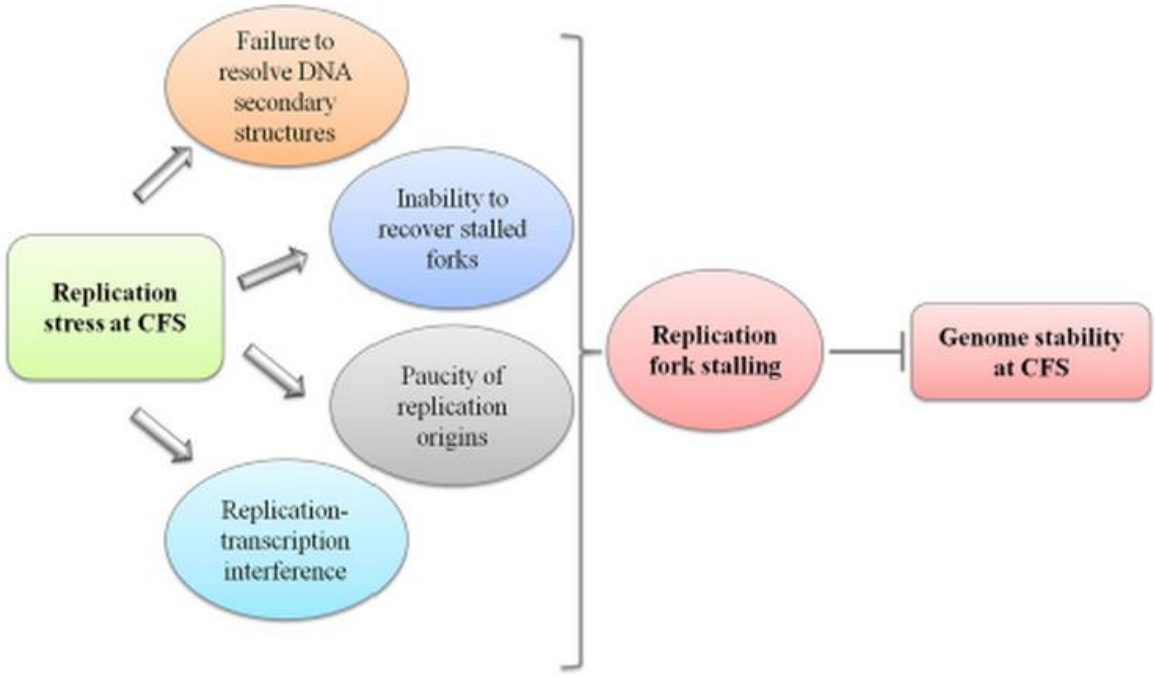



**Figure 3** General scheme of the potential sources of replication stress at CFS. Multiple factors can threaten DNA replication contributing to replication perturbation, and all the proposed causes implicate the requirement of a replication recovery mechanism to avoid CFS instability. (Franchitto and Pichierri 2014).

More recently, collision between replication and transcription complexes has also been considered a potential source of CFS instability, due to the ability of stable R-loops to impede

replication fork progression (Helmrich et al., 2011). Since not all the fragile sites co-localize within very large genes, this mechanism can only explain the fragility of some CFS.

Collectively, all these findings clearly indicate that, although distinct replication features may explain the instability of different fragile sites, replication fork pro-gression along these loci is perturbed, and raise the possibility that maintenance of genome stability depends on an accurate response to replication stress.

## *Replication Checkpoint Is Actively Involved in the Maintenance of CFS Integrity*

The ATR-dependent checkpoint together with several proteins involved in response to replication fork stalling have been implicated in maintaining common fragile site stability. A number of targets or modifiers of the ATR pathway have now been shown to influence fragile site stability, including CHK1, the 9-1-1 complex, the Fanconi anemia (FA) pathway proteins, Claspin, SMC1, BRCA1 and WRN protein (Dillon et al., 2010; Franchitto and Pichierri, 2011).The dependency of CFS stability on checkpoint activity supports the hypothesis that their instability derives from stalled forks or incomplete replication.A first correlation between the replication checkpoint and CFS has been provided by the discovery of the critical role of the replication checkpoint kinase ATR in maintenance of fragile site integrity. Under conditions of mild replication stress ATR protein preferentially binds (directly or through complexes) to fragile site FRA3B as compared to non-fragile site regions (Wan et al., 2010).Moreover, ATR disruption or hypomorphic mutation dramatically and specifically results in CFS expression, even without addition of Aphidicolin (Casper et al., 2002, 2004). In the same way, inactivation of Mec1, the yeast ATR homolog, elicits persistent fork stalling at the replication slow zones, an example of fragile sites in yeast, leading to chromosome breaks at these loci (Cha and Kleckner, 2002).Interestingly, the fact that ATR deficiency alone results in CFS expressionsuggests, once again, that replication fork stalling may occur spontaneously at these regions even during the normal replication. Altogether, these findings demonstrate that ATR plays an important function in recognizingand responding to stalled or incomplete replication at these sites. The model that explains how ATR prevents instability at CFS proposes that in normal cells fragile sites are single-stranded (ssDNA), unreplicated regions, derived from stalled or collapsed forks upon replication stress. When some of the ssDNA regions escape checkpoint, CFS are expressed (Casper et al., 2002).

Several other factors of the ATR-pathway and ATR substrates have been shown to contribute to CFS stability (Dillon et al., 2010; Franchitto and Pichierri, 2011).Among them CHK1, the apical kinase, deputed to the ATR- pathway activation, plays a crucial role in the maintenance of CFS stability. Interestingly, upon replication perturbation at CFS, both CHK1 and CHK2 were activated, but only depletion of CHK1 induces CFS expression(Durkin et al., 2006a). The elevated chromosome instability observed in the absence of CHK1 might be explained by its proposed role in maintaining replication fork integrity upon replication stress, and the high CFS expression may be due to loss of replication checkpoint function after fork stalling.

Downregulation of two other upstream regulators of CHK1 activation in response to replication stress significantly affects CFS stability: Claspin, an adaptor protein in the ATR pathway; and HUS1, member of the 9.1.1 complex a of the RAD9/RAD1/HUS1 (9.1.1) complex (Focarelli et al., 2009; Zhu and Weiss, 2007); SMC1 Component of the cohesion complex, contributes to the replication checkpoint activation.

After exposure to low doses of aphidicolin, down regulation of Claspin or HUS1 significantly affects CFS stability (Zhu and Weiss, 2007; Focarelli et al., 2009).

Studies from the yeast model suggest that the Claspin homolog Mrc1 may be involved in the stabilization of the replisome, by counteracting fork stalling at DNA secondary structures (Katou et al., 2003). Loss of Mrc1 causes fork collapse, accumulation of ssDNA, and then Rad9 activation to trigger checkpoint signaling and allow efficient restart of DNA synthesis (Katou et al., 2003). In human cells Claspin is involved in the maintaining of stalled fork stability and could contribute to dealing with the potential DNA secondary structures formed at CFS, which would hinder replication fork progression leading to fork collapse. The inhibition of the CLSPN gene leads to both genome instability and fragile site expression. Following aphidicolin treatment, Claspin synthesis transiently increase due to its requirement in checkpoint activation. However, Claspin synthesis decreased after a prolonged aphidicolin treatment. It has been proposed that, CLSPN modulation, following an extreme replication block, allows rare cells to escape checkpoint mechanisms and enter mitosis with a defect in genome assembly (Focarelli et al., 2009)

Furthermore, since, in response to replication arrest, RAD9 regulates the S-phase checkpoint activation by mediating CHK1 phosphorylation (Dang et al., 2005), promotes phosphorylation of ATR- substrates, loss of HUS1, which leads to the disruption of the whole

complex, might result in the loss of the checkpoint signal and then of the correct restart of DNA synthesis.

Besides its role in sister chromatid cohesion, the structural maintenance of chromosomes 1 (SMC1) is phosphorylated in an ATR-dependent manner under conditions of replication stress, and has been implicated in the maintenance of CFS integrity (Musio et al., 2005). Notably, SMC1 shows a preferential binding affinity for DNA secondary structures and a strong preference for AT-rich sequences (Akhmedov et al., 1998). Thus, SMC1 might contribute to the activation of the ATR-checkpoint upon replication perturbation at CFS, probably allowing error-free recovery of DNA replication (Dang et al., 2005; Musio et al., 2005).

| Protein | General known function | Suggested function in CFS stability under replication stress conditions | Experimental method | Effect on CFS expression (increase + vs decrease–) | References |
|---|---|---|---|---|---|
| ATR | DNA damage transducer | Replication stress response and maintenance of stalled fork stability | Downregulation, knockout, kinase-dead ATR, Seckel syndrome cells | + | 1) Casper et al. Cell (2002)<br>2) Casper *et al.*, J. Hum. Genet (2004) |
| CHK1 | DNA damage checkpoint | Replication stress response | Downregulation | + | 1) Durkin *et al*, Oncogene (2006) |
| Claspin | S-phase checkpoint | Maintaining stalled fork stability | Downregulation | + | 1) Focarelli *et al.*, Genes Chromosomes Cancer, (2009) |
| HUS1 | Cell cycle checkpoint | Intra S-phase checkpoint and DNA repair | Knockout | + | 1) Zhu, R.S. Weiss, Mol. Biol. Cell, (2007) |
| WRN | Helicase | Resolving DNA secondary structures | Gene mutation, downregulation | + | 1) Pirzio *et al.*, J. Cell Biol., (2008) |

**Figure 4**Proteins of the ATR-pathway involved in the maintenance of CFS stability. Adapted from: Ozeri-Galai et al., 2012.

Interestingly, maintenance of CFS stability requires the collaboration of ATR and another of their targets, the Werner syndrome protein, WRN (Ammazzalorso et al., 2010; Otterlei et al., 2006; Pichierri et al., 2003). Indeed, WRN, a member of the RecQ family of DNA helicases, appears to be essential for fruitful rescue from replication fork arrest (Baynton et al., 2003b; Pichierri et al., 2001; Sakamoto et al., 2001), and it is the first protein involved in this process to be correlated with instability at CFS (Pirzio et al., 2008).

Therefore, if replication checkpoint functions are somewhat impaired, then the entire pathway is probably inactivated and recovery of stalled forks compromised. As a consequence, fork collapse and the inability to accurately replicate through fragile sites might occur.

Although ATR is considered to be the major kinase mediating the response to replication stress because of its ability to activatethe intra-S phase checkpoint, same evidences support a role forprotein kinase ataxia-telangiectasia mutated (ATM) in the activation of a response to replication stress(Mazouzi et al., 2014). This protein is best known for its role as an apical activator of the DNA damage response in the face of DNA double-strand breaks (DSBs). One aspect of ATM function under replication stress conditions could be the activation of the homologous recombination repair pathway, which is important for restart of collapsed replication forks and recovery of replication (Petermann and Helleday, 2010). ATM can also influence replication fork restart by directly regulating the DNA helicases WRN and BLM, both required for resolution of replication intermediates (Ammazzalorso et al., 2010; Davalos et al., 2004).

ATM plays another important role upon mild replication stress, like that caused by low aphidicolin doses. In these conditions the frequency of chromosomal lesions that are transmitted to daughter cells increases (Lukas et al., 2011). Unresolved replication intermediates can occur during S/G2 phases of the cell cycle and can be converted into DNA lesions in M phase, for example into DSBs. It has been shown that a protein that binds p53, 53BP1, forms nuclear bodies at such sites of unrepaired DNA lesions in the subsequentG1 phase, to shield these regions against erosion in an ATM-dependent manner (Lukas et al., 2011).

## *Repair of lesions at CFS*

Little is known about how lesions at fragile sites are repaired. Most studies of repair responses have focused on DNA double-strand break (DSBs), whereas little is known about

the repair of stalled replication forks or lesions resulting from replication stress that occur at fragile sites. As CFS are late replicating region, induced with inhibitors of DNA replication, the major hypothesis on the instability of these regions is that CFS sequences present difficulties during replication process and that the breakage can results from an extreme delayed or incomplete replication, leading to single-stranded gaps on newly replicated DNA strands.

It has been demonstrated that DSBs are formed at CFS as a result of replication perturbation and that the repair of these breaks by both homologous recombination (HR) and non- homologous end- joining pathways NHEJ is essential for chromosomal stability at these sites (Schwartz et al., 2005). Replication stress, in fact, leads to focus formation of RAD51 and phosphorylated DNA-PKcs, key components of the homologous recombination (HR) and nonhomologous end-joining (NHEJ), DSB repair pathways, respectively. Down-regulation of RAD51, DNA-PKcs, or Ligase IV, an additional component of the NHEJ repair pathway, leads to a significant increase in fragile site expression under replication stress (Schwartz et al., 2005).

HR plays the major role in responding to DSBs and stalled or collapsed replication forks during S and G2, when the sister chromatid is present. Interestingly, Glover and Stein(Glover and Stein, 1987) reported that, on average, 70% of all gaps and breaks at FRA3B after aphidicolin treatment had an SCE at that site. The molecular basis for formation of SCEs in mammalian cells is not well-understood, but it has been hypothesized that SCEs are formed by the action of HR during replication repair. It has been shown, moreover, that other proteins, such as the Fanconi anemia proteins (FA), which are involved in the HR-dependent replication recovery, can be required for the regulation of CFS stability (Howlett et al., 2005). These findings suggest that, even under conditions that slow DNA replication, DSBs are formed at fragile sites and that stability at these genomic regions is dependent on the DSB repair pathways.

## *Werner syndrome protein*

WRN, the gene defective in WS, encodes a protein that is homologous to the E. coli RECQ helicase, which plays an important role in the maintenance of genome stability.

In addition to WRN, four other RECQ-like proteins have been identified in humans, including BLM (which is defective in Bloom syndrome), RECQL4 (whichis defective in Rothmund–Thomson syndrome), RecQ1 andRecQ4. These five distinct RecQ helicases possess all a hallmark RecQ helicase conserved domain. RecQ family also includes Sgs1 in

Saccharomyces cerevisiae, Rqh1 in Schizosaccharomyces pombe,and homologs in Caenorhabditis elegans, Xenopus laevis, and Drosophila melanogaster.Indirect immunofluorescence using polyclonal anti-human WRN shows a predominant nucleolar localization in human cells (Marciniak et al., 1998).

WRN gene encodes a large protein of 1432 amino acid (~162kDa). WRN possesses amino terminal exonuclease domain conserved in proteins of the DnaQ family(Huang et al., 1998), and a central helicase domain characteristic of the RecQ family(Gray et al., 1997). In addition, DNA binding (RQC) and protein interaction (HRDC) domains exist distal to the helicase domain.

WRN is a nuclear protein with both NLS and NoLS sequences situated at the C terminus (residues 949-1092 ) (von Kobbe and Bohr, 2002). WRNappears to be mainly located in the nucleoli, except during S phase or upon DNA damage, when it is redistributed

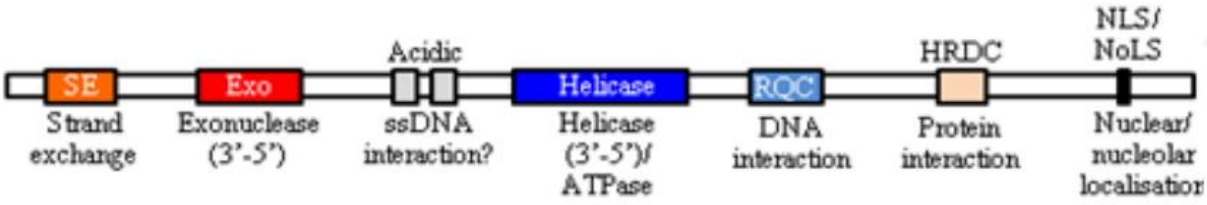



**Figure 5 Werner syndrome protein domain**

to sites of DNA replication or repair, and visible by immunofluorescence as nuclear foci (Baynton et al., 2003a; Otterlei et al., 2006; Pichierri et al., 2003; Sakamoto et al., 2001).Electron microscopy data indicates that WRN is found as a dimer in solution, yet as a tetramer in complex with DNA (Compton et al., 2008). Still, while unwinding DNA, WRN acts as a monomer (Choudhary et al., 2004). Together these results suggest that WRN's oligomeric state may be dependent on its catalytical activity and its interacting with DNA (Rossi et al., 2010).

Loss of function mutations of the WRN gene give rise to a severe human disease: the Werner syndrome (WS)(Oshima, 2000; Salk et al., 1985). Individuals with Werner syndrome (WS) prematurely develop an aged appearance with many common features associated with normal aging and cancer predisposition. Individuals with Werner syndrome develop normally until the end of the first decade. The first sign of the disease is the lack of a growth spurt during the early teen years (Belmaaza and Chartrand, 1994; Epstein et al., 1966; Goto, 1997;

Tollefsbol and Cohen, 1984). Early findingsgenerally occurs in the fifth decade of life beginning in early adulthood (usually observed in the 20s) include loss and graying of hair, hoarseness, and scleroderma-like skin changes, followed by bilateral ocular cataracts, type 2 diabetes mellitus, hypogonadism, skin ulcers, and osteoporosis in the 30s. Myocardial infarction and cancer are the most common causes of death; the mean age of death in individuals with Werner syndrome is 54 years (Oshima et al., 2014).

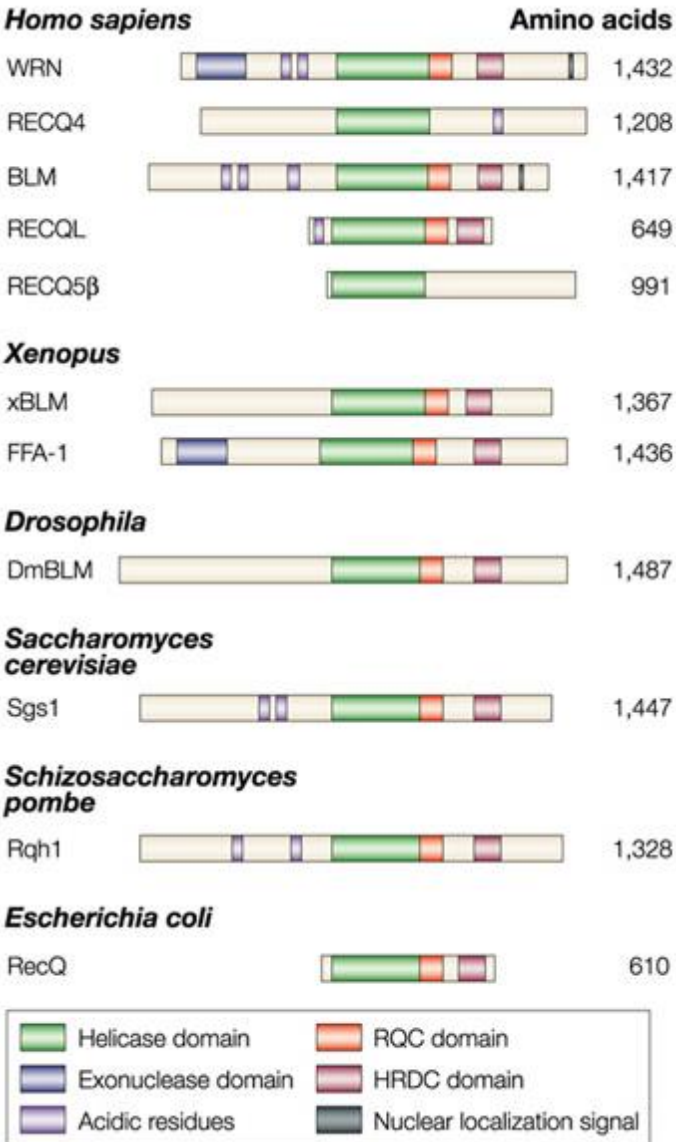



**Figure 6** Schematic representation of selected members of the RecQ family of DNA helicases. Family members have been identified in bacteria (RecQ), fission yeast (Rqh1), budding yeast (Sgs1), flies (DmBLM), amphibians (xBLM, FFA-1) and humans (WRN, RECQ4, BLM, RECQL, RECQ5), as indicated on the left. Proteins are aligned by their conserved helicase domain, which is shown as a green box. The conserved RQC and HRDC domains are shown as orange and purple boxes, respectively. The exonuclease domain in the amino-terminal region of WRN and its orthologues is shown as a blue box. Regions containing patches of acidic residues are shown as violet boxes. The nuclear localization signal sequences identified at the extreme carboxyl terminus of certain family members is shown as a black bar. The remaining pale yellow portions of each protein represent regions that are poorly conserved. At least three splice variants of the human RECQ5 protein are expressed, only one of which is shown. The size of each protein (in amino acids) is indicated on the right.

### *Cellular phenotype caused by Werner syndrome protein loss*

Cells from WS individuals have a short replicative lifespan in culture WS, in fact cells undergo highly premature replicative senescence, failing to proliferate after only 9-11 population doublings, compared with the 50-60 doublings characteristic of wild type fibroblasts (Hayflick, 1979). Transcriptomic studies have demonstrated that gene expression profiling in Werner syndrome closely resembles those of normal aging, with >90% gene expression changes associated with normal ageing seen in young WS cells (Kyng et al., 2003).Moreover, WS cells exhibit genomic instability characterized by chromosomal variegated translocation mosaicism (Salk et al., 1985), and more in general spontaneous chromosomal abnormalities and large deletions in many genes(Fukuchi et al., 1989; Gowans et al., 2005), which may represent an important determinant of the increased risk of cancer and of the aging phenotype (van Brabant et al., 2000; Goto, 1997).WS cells show phenotypes such as non-homologous chromosome exchanges and large chromosomal deletions, caused by deficiency of DSBR (Singh et al., 2009). WRN can also catalyse branch migration of Holliday junctions and melting of D-loops, which represent recombination intermediates. Moreover, it has been established that WRN participates in a multi-protein complex including ATR and the recombination proteins RAD51, RAD52, RAD54 and RAD54B, supporting a role for WRN in the later steps of the HR process (Otterlei et al., 2006). WS cells are sensitive to hydrogen peroxide (Von Kobbe et al., 2004), supporting, together with biochemical evidence (Harrigan et al., 2006), the involvement of WRN in base excision repair BER, that is one of the major DNA repair pathways next nucleotide excision repair (NER), double strand break repair (DSBR), and mismatch repair (MMR).WS cells are very sensitive to a well known DSB generating agent. Rapid accumulation of WRN at laser-induced DSBs has been shown, and it remains at the DSB site for at least for 4 h (Singh et al., 2009). DSBs are repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) processes. WRN is known to physically and functionally interact with two key proteins involved in NHEJ, Ku and DNA-PKcs(Chen et al., 2003; Cooper et al., 2000; Karmakar, 2002).

Notably, not only WS patients are more susceptible to cancer on WRN loss. Epigenetic transcriptional silencing associated with CpG island-promoter hypermethylation of the WRN gene promoter has been reported both in epithelial and in mesenchymal cancers with value in prognosis in colorectal cancer.. Moreover specific WRN

SNPs have been correlated with breast cancer incidence, suggesting that breast cancer can be driven by the aging associated with variant WRN, even though such genetic changes do not alter the helicase or exonuclease activities of the protein or modulate the levels expressed(Ding et al., 2007).

WRN is therefore of interest not only to those attempting to understand the molecular basis of human ageing, but also to cancer biologists. In fact, WRN knockdown is likely to promote cancer cell death and hypersensitise cells to current chemotherapeutic agents. WRN hypermethylation in colorectal tumors is a predictor of good clinical response to the camptothecin analogue irinotecan, a topoisomerase inhibitor commonly used in the clinical setting for the treatment of this tumor type (Agrelo et al., 2006). Therefore, small molecules that specifically inhibit or modulates WRN have attracted great interest for their therapeutic potential (Aggarwal et al., 2011).

## *Roles of Werner Syndrome Protein during DNA replication*

The multiplicity of interactions make very difficult to determine the prominent biological function of WRN protein. Moreover, the characteristics of WS syndrome are not correlable with the loss of a specific activity of WRN protein, due to the pleiotropic nature of the protein. Based on its in vitro substrate preferences, it is thought that in vivo WRN may participate in several DNA metabolic pathways, such as replication, recombination and repair ,telomere maintenance, but also in transcription. Studies both in vitro and in vivo indicate that the roles of WRN in a variety of DNA processes are mediated by post-translational modifications, as well as several important protein-protein interactions (Rossi et al., 2010).WRN is primarily a multifunctional nuclease widely involved in genome stability maintenance. The nuclease activities of WRN are critical for these functions, but WRN plays also nonenzymatic roles, for example in preserving nascent DNA strands from exonuclease activity of MRE11 following replication stress(Su et al., 2014).

Firstly, cellular analyses reveal a role of WRN in DNA replication, because of the observed delay in S-phase in WS cells. The delay has been attributed to either decreased rates of DNA extension(Hanaoka et al., 1985)and replication fork propagation(Rodríguez-López et al., 2002)(Kamath-Loeb et al., 2012) or to disruptions in replication initiation or origin firing(Fujiwara et al., 1985; Hanaoka et al., 1983, 1985; Takeuchi et al., 1982). DNA combing studies have demonstrated a problem with replication fork progression in WS cells, resulting in marked asymmetry of bidirectional forks (Rodríguez-López et al., 2002). Such studies led

to the proposal that replication forks stall at high frequency in cells lacking WRN protein, that WRN could act in normal DNA replication to prevent collapse of replication forks or to resolve DNA junctions at stalled replication forks, and that the loss of this capacity may be a contributory factor in premature aging (Rodríguez-López et al., 2002).

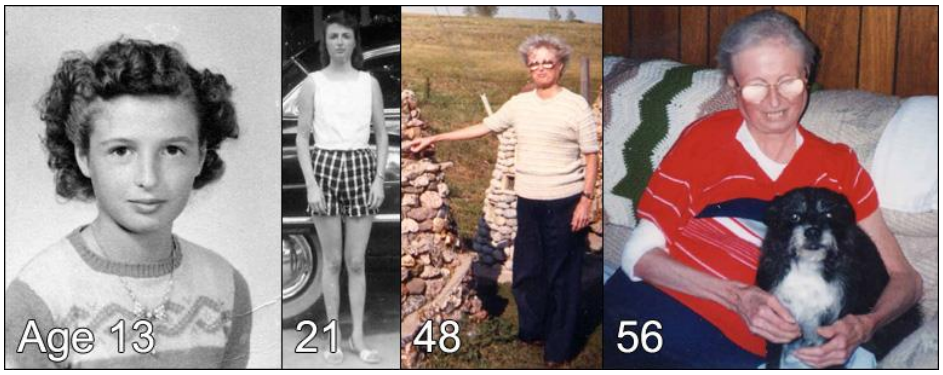



**Figure 7Werner syndrome patient**

Moreover, Pol δ synthesizes the lagging strand during replication of genomic DNA and also functions in the synthesis steps of DNA repair and recombination. It has been shown that WRN assists pol δ (possibly on the lagging strand during Okazaki fragment synthesis) by removing 3' mismatches, thus allowing the polymerase to extend primers (Kamath-Loeb et al., 2012). This supports a direct role for WRN in Okazaki fragment synthesis and in DNA editing. Indeed, WRN could play a role in editing DNA, either during DNA synthesis or in processing free ends, in collaboration with and stimulated by the end-binding protein Ku (Perry et al., 2006). Structural and biochemical similarities have been established between WRN functional exonuclease domain (WRN-exo) and DnaQ-family replicative proofreading exonucleases. Hence, WRN-exo is a human DnaQ family member and supports DnaQ-like proofreading activities stimulated by Ku70/80 (Perry et al., 2006). With regard to WRN role in Okazaki fragment processing, this has not been fully explored. RNA-primed Okazaki fragments must be matured into a single covalent DNA strand, that requires PolB1, Fen1 and Lig1 catalytic activities, coordinated by DNA sliding clamp, proliferating cell nuclear antigen (PCNA) (Beattie and Bell, 2012). WRN binds to and stimulates the nuclease activity of Fen1, which may contribute to efficiency of Okazaki fragment processing (Brosh et al., 2001). Functional interaction is mediated by a 144 amino acid domain of WRN, that shares homology with RecQ DNA helicases. As WRN binds to Fen1 immediately adjacent to its

PCNA binding site, it is likely that there is some interplay between the three proteins (Sharma et al., 2005), that may be important in Okazaki fragment processing (Mason, 2013).

The most efficient mode of replication involves the removal of barriers to fork progression before they lead to fork stalling. In vitro studies demonstrate that the WRN helicase activity can unwind G4-tetraplex structures of the Fragile X syndrome repeat sequence d(CGG)n and other DNA secondary structures such as hairpins or forked DNA, more efficiently than double-stranded duplex DNA. WRN has been shown to be required by DNA pol δ to unwind G4 DNA (Kamath-Loeb et al., 2001a), bubbles and D loops (Kamath-Loeb et al., 2012) to allow pol δ-mediated synthesis over such template sequences without leading to fork stalling.

In vitro and in vivo data demonstrate functional interaction between WRN and the translesion DNA polymerases Pols, Polη, Polκ, and Polι (human cells have four TLS Pols, REV1, Polη, Polκ, and Polι, that belong to the Y family, and a family B Pol, Polζ), specialized Pols whose primary function is to insert nucleotides across DNA lesions that block progression of replicative Pols(Kamath-Loeb et al., 2007). Some lesions such as those caused by MMS or 4NQO present an insurmountable barrier to templating for the high fidelity B family DNA polymerases, but error-prone replication through these small lesions is often less costly for the cell than replication pausing and recruitment of repair complexes. WRN has been found to promote the processivity of Y-family TLS pols on a wide range of substrates including oxidized bases, abasic sites, and thymine dimmers. The functional interaction between WRN and TLS Pols may promote replication fork progression, at the expense of increased mutagenesis, and obviate the need to resolve stalled/collapsed forks by processes involving chromosomal rearrangements.

## *Recovery from replication fork stalling*

WRN has been extensively linked to replication fork recovery (Pichierri et al., 2011). Several groups have shown that WRN cells are sensitive to treatment with replication inhibitors and DNA damaging agents that cause replication fork stalling (Pichierri et al., 2001; Sidorova et al., 2013a).The focus of many current investigations has largely been on the response of WRN deficient cells to these replication disruptions, and the role of WRN in recovery from replication-dependent DNA damage. Upon replication fork stalling, WRN acts to limit fork collapse and/or to promote repair of DSBs.

It is a target of ATR/ATM and interacts with several checkpoint factors, such as the 9.1.1 complex (Ammazzalorso et al., 2010; Pichierri et al., 2012), which are recruited at stalled forks .Even though WRN has also been shown to carry out a function during recombinational repair of DSBs (Prince et al., 2001), the primary function at perturbed forks seems to be unrelated to recombination (Pichierri et al., 2001) and is probably more linked to fork remodeling. WRN is involved in replication resumption after fork arrest induced by DNA damage or nucleotide depletion by HU (Ammazzalorso et al., 2010; Sidorova et al., 2013b), and supports replication at CFS. WRN could act in preventing the replisome disassembly or in the removal of DNA secondary structures that impede fork progression by its helicase activity, and its function could be regulated by the replication checkpoint. This is in agreement with the proposed coordinated action of WRN and DNA polymerase delta in the replication of DNA substrates containing G4-tetraplex structures (Kamath-Loeb et al., 2001b; Shah et al., 2010a). Moreover WRN binds and/or functionally interacts with several proteins at the replication fork, telomere ad proteins involved in fork recovery after stalling. For instance, RPA physically interacts with WRN in vitro, stimulates its helicase activity, and, following HU exposure co-localizes with WRN at replication fork stalling sites and assists WRN in the resolution of replication arrest. Coimmunoprecipitation experiments suggest that WRN and RPA association is enhanced in response to fork blockage inducing-treatments and this interaction is instrumental for the WRN-mediated displacement of RPA from DNA that contributes to fork recovery (Machwe et al., 2011).

During replication, topoisomerases relieve supercoiling in the DNA that occurs as a result of strand separation. Incomplete topoisomerase release from DNA, such as occurs upon treatment with topoisomerase inhibitors, leads to formation of covalent topoisomerase-DNA complexes that can pose a barrier to replication and can result in the formation of strand breaks(Leppard and Champoux, 2005). When exposed to topoisomerase I inhibitor topotecan (TPT), cells with a knockdown of WRN have a greater arrest in S-phase and inhibition of replication compared to control cells. This effect is specific for topoisomerase I inhibitors since the effects are not seen when cells depleted of WRN are treated with the topoisomerase II inhibitor etoposide (ETO).

In WRN knockdown versus wild-type cells, there is an increased propensity for conversion of TPT-induced single strand breaks (SSBs) into double strand breaks (DSBs), suggesting that WRN prevents SSBs at replication forks from being converted into DSBs (Christmann et al., 2008). DSBs accumulate in WRN deficient cells in response to HU

treatment, which induces replication fork stalling. These DSBs form as a result of collapsed replication forks, as indicated by proliferating cell nuclear antigen (PCNA) release from chromatin during S-phase. In the absence of WRN, stalled replication forks are processed via a compensatory pathway, which can be dependent on MUS81 endonuclease, causing DSB formation (Franchitto et al., 2008a). Together the results indicate that WRN functions in protecting cells from DSB formation that can occur as a result of replication fork stalling and collapse.

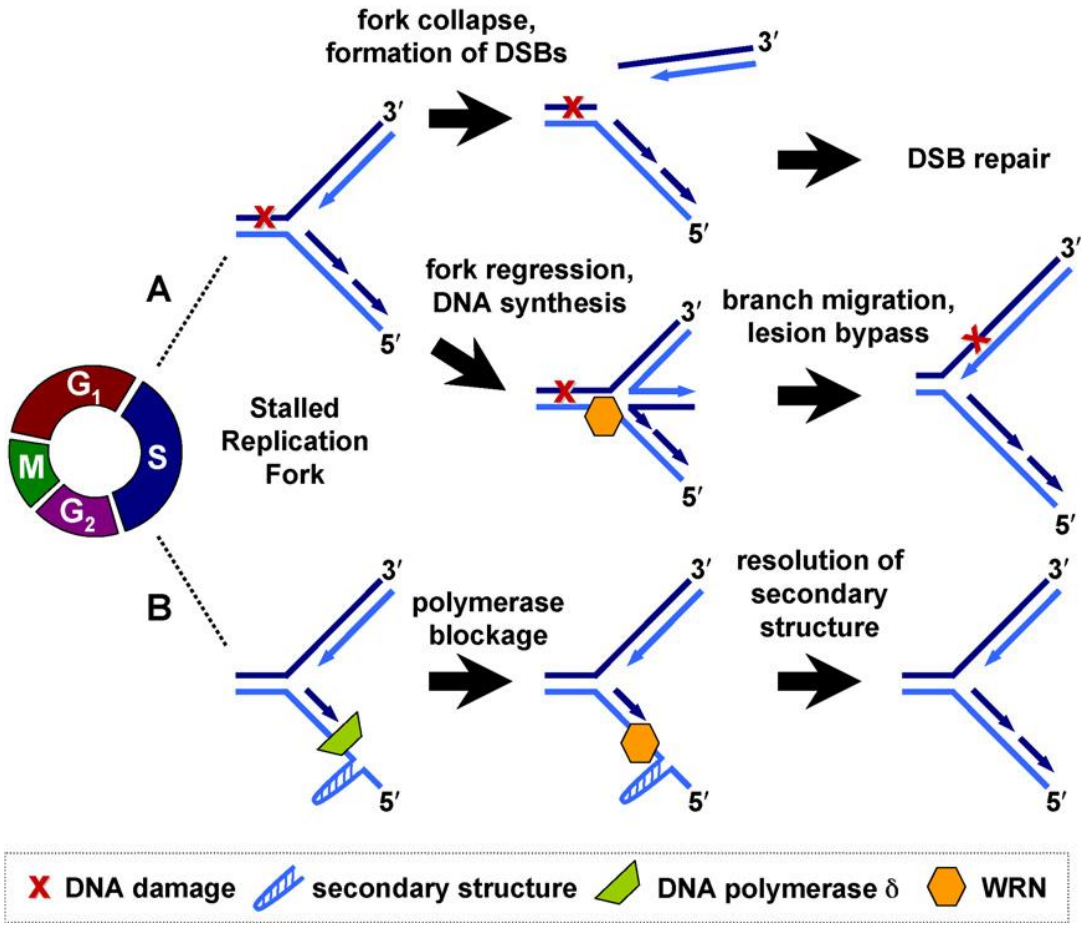



**Figure 8Roles of WRN in S-phase at stalled replication forks.** (A) WRN may participate in repair of double-strand breaks (DSBs) following DNA damage-induced replication fork collapse. Alternatively, WRN may function to regress the fork and allow for synthesis bypass of DNA damage. (B) Secondary structures which block DNA polymerases may be resolved by WRN (see text for details), (Rossi et al., 2010).

Moreover, WRN is involved in the response to replication fork stalling induced by agents that generate crosslinks within the DNA. Chromium is an environmental genotoxin known to affect DNA replication through formation of interstrand crosslinks that inhibit polymerase elongation of the DNA and also through creation of DSBs during S-phase that can lead to replication fork collapse(Bridgewater et al., 1998). Cells depleted of WRN are hypersensitive to chromium exposure, showing increased cell cycle arrest and cell death compared to wild-type cells. In WRN deficient cells exposed to chromium, WRN colocalizes with damage sites, as detected by phosphorylated histone H2AX (γ-H2AX) foci. These cells display a longer recovery time for stalled replication forks and repair of DSBs than control cells. Altogether the results indicate that WRN is involved in the recovery and/or repair of chromium-induced replication stress and DNA damage during replication (Liu et al., 2009). One potential avenue of WRN participation in the restart of damage-induced stalled replication forks is through processing of regressed forks. When damage is encountered at the fork, replication halts and the fork regresses into a chicken foot structure. In this structure, the lagging strand serves as a template for leading strand synthesis. Subsequently, WRN can mediate reverse branch migration of the chicken foot to bypass the damage, which can then be repaired by alternate pathways (Sharma et al., 2004).

### *WRN and the replication checkpoint*

In the last years, several studies on model organisms implicated RecQ helicases in the S-phase checkpoint response. For instance, the budding yeast RecQ, Sgs1, has been found to directly participate in the replication checkpoint response downstream to Mec1 (ATR) and alongside Rad24 (RAD17) (Cobb et al., 2003), and even the bacterial RecQ might be necessary, at least under certain conditions, for the induction of the SOS response(Hishida et al., 2004).In vertebrates, BLM is phosphorylated by ATR and seems to cooperate with checkpoint proteins, such as the MRE11 complex, BRCA1 and 53BP1, after DNA damage induced at traveling forks or replication inhibition by HU (Davalos et al., 2004; Franchitto and Pichierri, 2002). Thus, a connection between WRN and the replication checkpoint is likely to occur and WRN may have a role in the recovery of stalled forks independently from recombination. The first evidence supporting this cross-talk derives from observations, in vitro and in vivo, that WRN can be phosphorylated by ATR after replication fork arrest induced by HU or aphidicolin treatment (Pichierri et al., 2003). Additional data emerged from in silico analysis of the potential ATR/ATM phosphorylation sites in the WRN sequence (Kim et al., 1999; Traven and Heierhorst, 2005), and more recent findings demonstrate that in

response to replication stress, WRN undergoes phosphorylation in an ATR/ATM-dependent manner and co-localizes with ATR at nuclear foci (Ammazzalorso et al., 2010). Moreover, WRN interacts or co-localizes with proteins involved either in the intra-S or replication checkpoint, such as ATR or the MRE11 complex (Ammazzalorso et al., 2010; Cheng et al., 2004; Franchitto and Pichierri, 2004). Of particular interest is that WRN helicase activity and ATR-mediated checkpoint response collaborate in a common pathway to maintain CFS stability.

Interestingly, upon replication arrest, WRN re-localization is completely abrogated in cells depleted of the 9.1.1 complex (Pichierri et al., 2011), suggesting that the replication checkpoint controls WRN function at stalled forks acting at multiple levels. Further supporting the possibility that ATR-dependent phosphorylation may be required to fasten WRN at stalled forks and that phosphorylation and ability to form nuclear foci are two separable events. Indeed, while 9.1.1 complex down-regulation prevents both assembly of WRN nuclear foci and phosphorylation, depletion of TopBP1 reduces WRN phosphorylation without affecting its localization in nuclear foci(Pichierri et al., 2011). Altogether, it seems likely that phosphorylation of WRN follows its recruitment at sites of stalled forks, probably to “activate” fork processing. It is tempting to speculate that phosphorylation of WRN might affect separately helicase or exonuclease activity.

Altogether, these findings reinforce the hypothesis that WRN plays an essential role in the maintenance of genome stability by repairing damaged forks, whenever they stall, most likely in collaboration with ATR-dependent checkpoint.

### *Werner syndrome helicase activity is essential in maintaining CFS stability*

WRN is a key regulator of particular genome regions, called Common Fragile Sites (CFS), that are naturally occurring replication fork stalling sites. WRN is required to limit the formation of single stranded DNA regions and gaps during replication of common fragile sites (CFS) (Ammazzalorso et al., 2010; Murfuni et al., 2012) and enhances processivity of DNA pol δ on fragile site FRA16D over hairpins and microsatellite regions, requiring either the helicase or DNA binding activities of WRN (Shah et al., 2010b).

Notably, the safeguard role of WRN at CFS requires the helicase activity of the protein and its cooperation in an ATR-pathway (Pirzio et al., 2008). Since WRN deficiency

recapitulates ATR defects in terms of CFS instability, it is likely that the ATR-mediated stabilization of stalled forks may be basically carried out through phosphorylation and regulation of WRN by ATR. WRN is mainly located in the nucleoli and relocalizes to nuclear foci after DNA damage or replication fork arrest. It is recruited to sites of DNA synthesis, possibly through association with the sliding clamp PCNA, and to sites of stalled/collapsed forks probably by RPA in concert with the S phase checkpoint kinase ATR and its downstream effectors and mediators Chk1, Rad53, Mec1 and Mrc1. In response to replication perturbation induced at CFS, WRN deficient cells display an increase CFS instability and breaks compared to wild-type even in the absence of treatment (Pirzio et al., 2008). The expression in WS cells of missense mutant forms of WRN protein, that inactivate the exonuclease (WRN-E84A) or helicase (WRN-K577M) activity (Chen et al., 2003; Gray et al., 1997; Huang et al., 1998), led to a significant increase in chromosomal damage after aphidicolin exposure compared to cells with a wild type WRN (WS*WRN*) (Pirzio et al., 2008). However, FISH analyses performed on metaphases after 24 h of treatment indicated that the induction of FRA3B, FRA7H, and FRA16D was enhanced in a statistically significant manner only in WS and WRN-K577M cells (Pirzio et al., 2008). Given the specific requirement of the WRN helicase activity for regulating CFS stability and the high propensity of CFS to adopt DNA secondary structures during DNA replication (Mishmar et al., 1998; Pirzio et al., 2008; Zlotorynski et al., 2003b), it is possible that the helicase activity of WRN is necessary to the unwinding of these structures in order to facilitate replication fork progression or support fork restart. Moreover, WRN could act in preventing the replisome disassembly or in the removal of DNA secondary structures that impede fork progression at these sites by its helicase activity, and its function could be regulated by the replication checkpoint. This is in agreement with the proposed coordinated action of WRN and DNA polymerase delta in the replication of DNA substrates containing G4-tetraplex structures (Kamath-Loeb et al., 2012; Shah et al., 2010b). Since WRN deficiency recapitulates ATR defects in terms of CFS instability, it is likely that the ATR-mediated stabilization of stalled forks may be basically carried out through phosphorylation and regulation of WRN by ATR (Casper et al., 2002).

Instability at CFS can be considered as a hallmark of early precancerous lesions

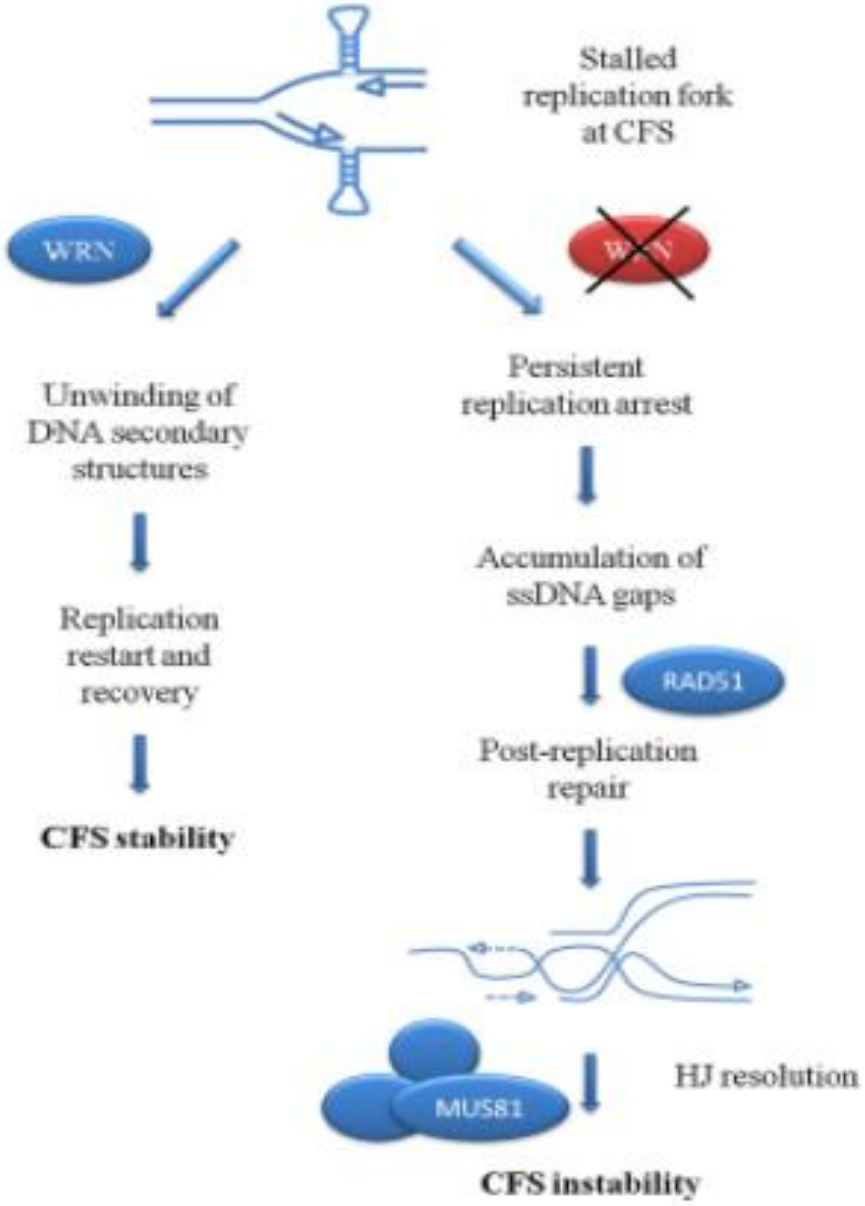



**Figure 9Proposed model of the WRN RecQ helicase function for the replication restart at CFS.** Formation of DNA secondary structures (depicted as hairpins) at a subset of CFS may hinder the progression of the replisome inducing a transient replication arrest. The WRN RecQ helicase, in cooperation with the replication checkpoint, is recruited at the fork stalling site to help in removal of the roadblock and possibly in the restoration of an active replisome, either directly or after fork regression. In the absence of WRN, the fork stalling becomes permanent because the DNA secondary structures cannot be unwound, resulting in regions of unreplicated DNA. In late S or G2, the unreplicated regions are targeted by RAD51-mediated recombi-nation to be replicated. Extensive requirement of this backup mechanism, as probably occurs in the absence of WRN or other factors involved in replication restart at CFS, leads to accumulation of recombination intermediates that have to be resolved before mitosis. Resolution of these intermediates by resolvases such as MUS81 contributes to generate chromosome breaks and gaps at CFS, which are commonly observed in metaphase cells. (Franchitto and Pichierri 2014)

(Gorgoulis et al., 2005) and it is widely accepted that most gross chromosomal rearrangements accumulating in solid tumors originate from fragile sites (Arlt et al., 2006). WS is a cancer-prone and chromosome fragility syndrome characterized by gross chromosomal rearrangements (Martin and Oshima, 2000; Oshima, 2000). Because instability of CFS is readily detected in cells depleted of WRN even under normal division, it is possible that chromosomal instability observed in WS cells could correlate with breaks accumulating at these sites. However, a recent study suggests that most of the chromosomal abnormalities arising in WS cells could be related to erosion of telomeric sequences (Crabbe et al., 2007). These hypotheses are not necessarily incompatible: both the common fragile site and telomere stabilities might require the helicase activity of WRN to clear the way for the replisome, and chromosomal rearrangements observed in WS are most likely derived from a common protective mechanism at telomeric and nontelomeric sequences. Consistently, instability at CFS was also observed in Epstein-Barr virus–transformed lymphoblasts derived from WS patients, which are telomerase proficient and thus protected from telomere erosion (Pirzio et al., 2008).

# AIM OF THE WORK

## AIM

The checkpoints are surveillance mechanisms of genomic integrity, that is fundamental to ensure genetic identity of cells in a multicellular organism. Failure of a checkpoint often causes mutations and genomic arrangements, resulting in genetic instability, which appears to be a leading cause in the development of many diseases, especially cancer. Therefore, studies focusing on checkpoints are very important for understanding mechanisms of genome maintenance, also because resulting data have direct impact on cancer biology.

The link between replication defects, human diseases and cancer, underscores the requirement of an efficient and accurate monitoring of genome integrity during DNA replication, which is provided by a complex and coordinated protein network, under the control of the ATR kinase (Abraham, 2001; Zou and Elledge, 2003). In fact, DNA replication represents a crucial moment in the life of a cell, as chromosomal integrity can be seriously threatened by replication stress, that is the slowing and/or stalling of replication fork progression (Zeman and Cimprich, 2014). Replication stress interferes with fork stability and can be caused by endogenous side-products of cellular metabolism, exogenous agents capable to interfere with DNA replication, as well as intrinsic structural features of specific genomic regions, such as the common fragile sites (CFS).

CFS are difficult-to-replicate regions of the genome, especially prone to fork stalling (Durkin and Glover, 2007). It was determined that the great majority of CFS are specifically and reproducibly induced by low doses of Aphidicolin (Aph), an inhibitor of replicative DNA polymerases,(Cheng and Kuchta, 1993; Durkin and Glover, 2007; Ikegami et al., 1978), which do not greatly affect mitotic index, but slow replication fork progression.

Although they are considered as “hotspots” of genome instability, a recognized causative factor in tumor development, very little is known about the molecular mechanisms of CFS expression. As CFS are normally occurring replication fork stalling sites, they can be considered as useful means to understand how replication fork stalling can be recovered *in vivo.*

It has been previously proposed that WRN, mutated in the cancer-prone disease Werner syndrome, is a key regulator of CFS stability, even under unperturbed conditions through its helicaseactivity (Pirzio et al., 2008).These data support a role of WRN in facilitating replication fork progression of regions affected by replication stress, and suggest

that CFS may represent the physiological substrates of this protein (Pirzio et al., 2008).Moreover, it is known that, under mild replication stress inducing by low doses of Aph, WRN and ATR act in a common pathway preventing accumulation of DNA breaks at CFS. Despite WS cells exhibit an ATR-like instability at CFS, and that WRN has been found phosphorylated by ATR under replication stressupon HU-induced replication stress (Pichierri *et al.*, 2003; Ammazzalorso et al.,2010), there is no evidence of a functional requirement of WRN in the establishment of the replication checkpoint upon mild replication stress, such as the one that causes breaks at CFS.

The aim of this study was to analyze the functional requirement of WRN in the ATR-dependent checkpoint activation under mild replication stress, like that inducing CFS expression.

# RESULTS PART 1

Nucleic Acids Res. 2014 Nov 10;42(20):12628-39. doi: 10.1093/nar/gku1022. Epub 2014 Oct 28.

# RESULTS (part 1)

## *WRN deficiency results in defective ATR-dependent checkpoint activation under mild replication stress*

In order to assess the role for WRN in ATR pathway activation in response to mild replication stress, phosphorylation status of the main target of ATR, CHK1, was examined.

To compare isogenic cell lines, we first generated HEK293T cells stably expressing scrambled (WRN-wt) or WRN-targeting shRNA (WRN-kd).WRN-kd cells showed about 80% depletion of WRN protein under the experimental conditions used in this study (Figure 1A).

A time course analysis was performed treating WRN-wt and WRN-kd cells with low dose (0,4 μM) of Aphidicolin (Aph), and the phosphorylation of CHK1 was measured in cell lysates by Western blot using an antibody that specifically recognizes phospho-CHK1 on Ser345.Treatment with low dose of Aph induced a time-dependent phosphorylation of CHK1 in WRN-wt cells, already noticeable after 1 h and peaking at 24 h (Figure 1A), suggesting that also a modest replication perturbation can trigger a quick checkpoint response.

In contrast, in WRN-kd cells CHK1 phosphorylation was not detectable, or very weak, and it did not rich the wild-type levels even at the late time-points (Figure 1A).

In contrast, treating the cells for 4h with 1 mM HU, a specific inhibitor of DNA synthesis which leads to a strong genome-wide replication arrest, comparable CHK1 phosphorylation levels in both WRN-wt and WRN-kd cells was observed (Figure 10B, lanes 4 and 8).Although CHK1 phosphorylation is hampered in WRN-deficient cells, similar amounts of Cyclin A were detected after treatments in both cell lines, suggesting that defective CHK1 phosphorylation was not attributable to a smaller S-phase population in WRN-kd cells (Figure 10B).

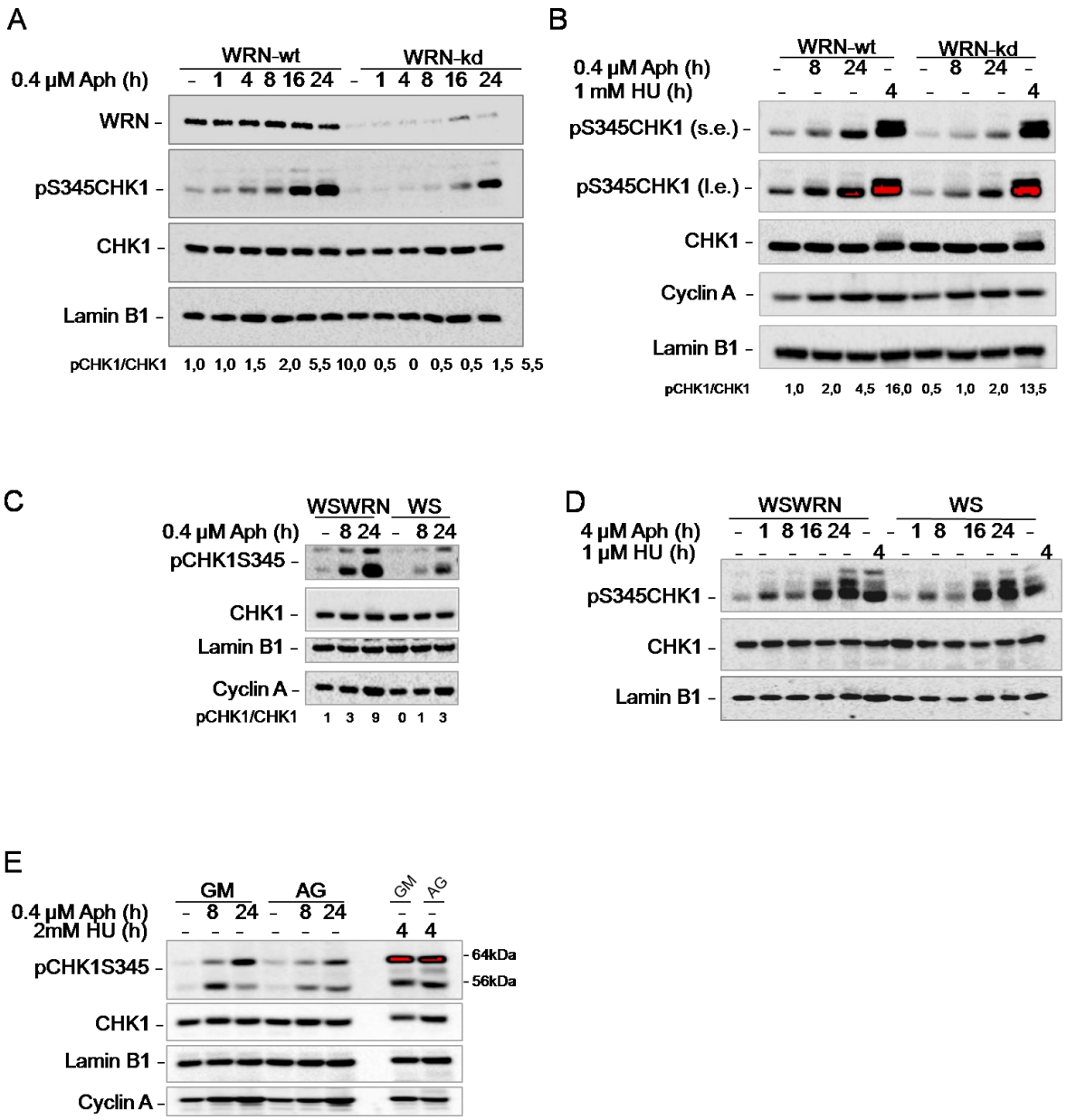



**Figure 10 WRN is required for CHK1 activation following mild replication stress.** Western blot detection of CHK1 phosphorylation: (A) in total extracts of WRN-wt or WRN-kd cells untreated (-) or treated with Aph, as indicated; (B) in WRN-wt and WRN-kd cells treated with Aph or HU (s.e., short-exposure; l.e., long-exposure); ((E) in WS and WSWRN treated with 4 µM Aph for the indicated time points; D) in total extracts of hTERT-immortalized primary fibroblasts, both WRN-proficient (GM01604) and WRN-deficient (AG12975), untreated (-) or treated with 0.4 µM Aph at the indicated times. (A-B-C-D-E) In WRN-kd cells, down-regulation of the WRN protein was verified using a specific anti-WRN antibody. The presence of activated, i.e. phosphorylated, CHK1 was assessed using S345 phospho-specific antibody (pS345). Total amount of CHK1 was determined with an anti-CHK1 antibody. Equal loading was confirmed probing with an anti-Lamin B1 antibody. (B) Cyclin A was used to quantify S-phase cells.

As shown in Figure 10C, Aph treatment induced CHK1 phosphorylation in WSWRN cells, in a manner similar to that seen in WRN-wt cells, whereas, in WS cells resulted in no or minimal activation of CHK1. Nonetheless, treatment of cells with high dose of Aph or 1 mM HU, an inhibitor of DNA synthesis able to induce a complete replication arrest, led to comparable CHK1 phosphorylation in both cell lines (Figure 10D).

Interestingly, a defective phosphorylation of CHK1 after a low dose of Aph was consistently observed in WS-derived hTERT-immortalized primary fibroblasts (Figure 10E), suggesting that the phenotype is unlikely due to cell transformation, but rather is specifically related to the absence of WRN.

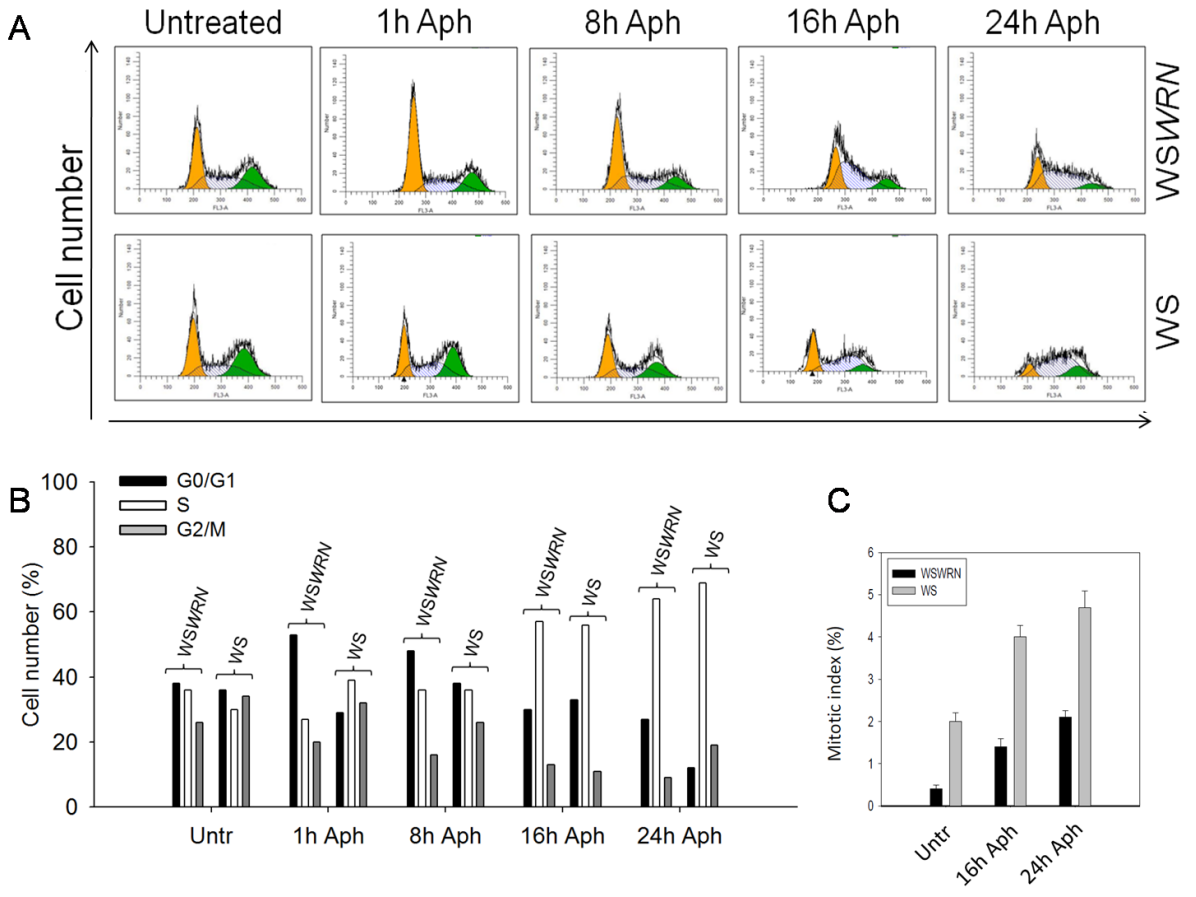



**Figure 11** Analysis of cell cycle progression upon moderate replication stress. (A) Wild-type (WSWRN) and WS cells were treated with 0.4 μM Aph at various periods as indicated, then harvested and stained with PI prior to FACS analysis. Upper panel represents DNA content profiles of cells. Graph shows the percentage of cells distributed into G0/G1, S or G2/M phases. (B) Evaluation of mitotic cells in WSWRN and WS cells treated with 0.4 μM Aph for the indicated times, estimated following 16 or 24 h in the presence of 0.5 μg/ml nocodazole for 6 h before harvesting.. Mitotic index was determined as the number of histone H3-pSer10 positive cells over the total cell population by immunofluorescence.

ATR-checkpoint response in WRN-deficient cells was also investigated by flow-cytometric analysis. As expected, Aph slowed down cell cycle progression of WSWRN cells, and delayed S-G2 phase transition (Figure 11A). In contrast, WS cells exhibited a higher proportion of G2/M phase cells accumulated starting from 8 h of treatment (Figure 11A) with a more pronounced accumulation of cells in the M phase, as evaluated by immunostaining for the mitosis-specific marker phospho-histone H3 (Figure 11C).

Overall, these findings imply that WRN plays a critical function in response to mild replication stress, and support a possible role for WRN as specific mediator of CHK1 activation.

### *WRN is phosphorylated in an ATR-dependent manner after Aph-treatment*

Often checkpoint mediators are themselves targets of apical kinases, and WRN is indeed phosphorylated by ATR after both HU and CPT treatments (Pirzio et al., 2008; Shah et al., 2010).

To reinforce the existence of a functional relationship between WRN and the ATR-dependent checkpoint after mild replication stress, we asked whether WRN was targeted by ATR kinase under Aph treatment.

HEK293T cells were treated with low dose of Aph and cell lysates were subjected to WRN IP. Using phospho-S/TQ antibodies, which specifically recognize phosphorylated ATM/ATR substrates, phosphorylation of WRN was barely detectable in untreated conditions but strongly increased after treatments (Figure 12B), suggesting that moderate stress is able to induce WRN modification.

In an independent experiment, we transfected HEK293T cells with plasmids expressing the Flag-tagged full-length wild-type WRN (wt) or a mutant Flag-WRN6A, which makes WRN unphosphorylable by ATR after replication stress (Pirzio et al., 2008).Following Aph treatment, cell extracts were prepared and subjected to IP using anti-Flag tag antibodies. The expression of wild-type WRN resulted in the expected phosphorylation at S/TQ sites, whereas mutant $WRN^{6A}$ protein abrogated pS/TQ immunoreactivity (Figure 12C), suggesting that, even after low dose of Aph, WRN is mainly phosphorylated at C-terminus residues.

Collectively, these results demonstrate that mild replication stress, like that inducing breaks at CFS, may trigger ATR dependent pathway that requires the presence of WRN.

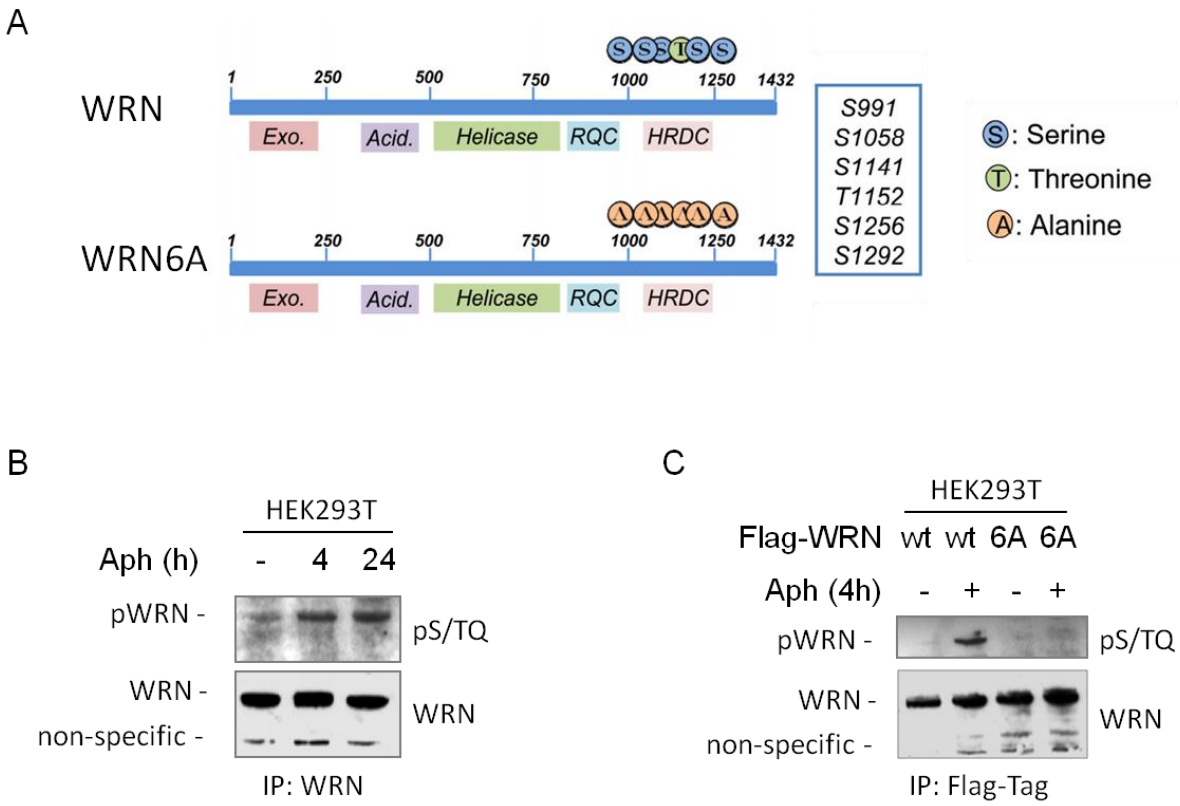



**Figure 12ATR-dependent WRN phosphorylation upon Aph-treatment.** (A) Western blot detection of WRN phosphorylation in HEK293T cells treated or not with 0.4 μM Aph for the indicated time points. Cell extracts were immunoprecipitated using anti-WRN antibody followed by immunoblotting with an anti-S/TQ antibody. Total WRN was used to assess the amount of WRN immunoprecipitated. The ratio of phosphorylated protein to total protein is reported below each lane. (B) Analysis of WRN status in HEK293T cells transfected with plasmids expressing the Flag-tagged full-length wild-type WRN (wt) or a full-length carrying Ala substitutions at all the six S/TQ sites (6A) and treated or not with Aph as indicated. Cell extracts were prepared 48 h post-transfection and used to immunoprecipitate ectopic WRN with anti-Flag tag antibody, followed by immunoblotting with an anti-S/TQ antibody. Total WRN was used to assess the amount of wild-type or mutant form of WRN immunoprecipitated. All experiments are representative images of at least two replicates.

## *WRN phosphorylation by ATR, but not WRN helicase activity, is required for checkpoint activation upon mild replication stress*

WRN is indeed phosphorylated by ATR after both HU and CPT treatments(Ammazzalorso et al., 2010; Pirzio et al., 2008).To verify whether WRN phosphorylation by ATR is a prerequisite for checkpoint activation after replication stress induced by Aph, we studied the ability of WS cells stably expressing the mutant form of WRN unphosphorylable by ATR (WSWRN$^{6A}$) (Pirzio et al., 2008) to phosphorylateCHK1. WSWRN, WS and WSWRN$^{6A}$ cells were used and CHK1 phosphorylation evaluated.

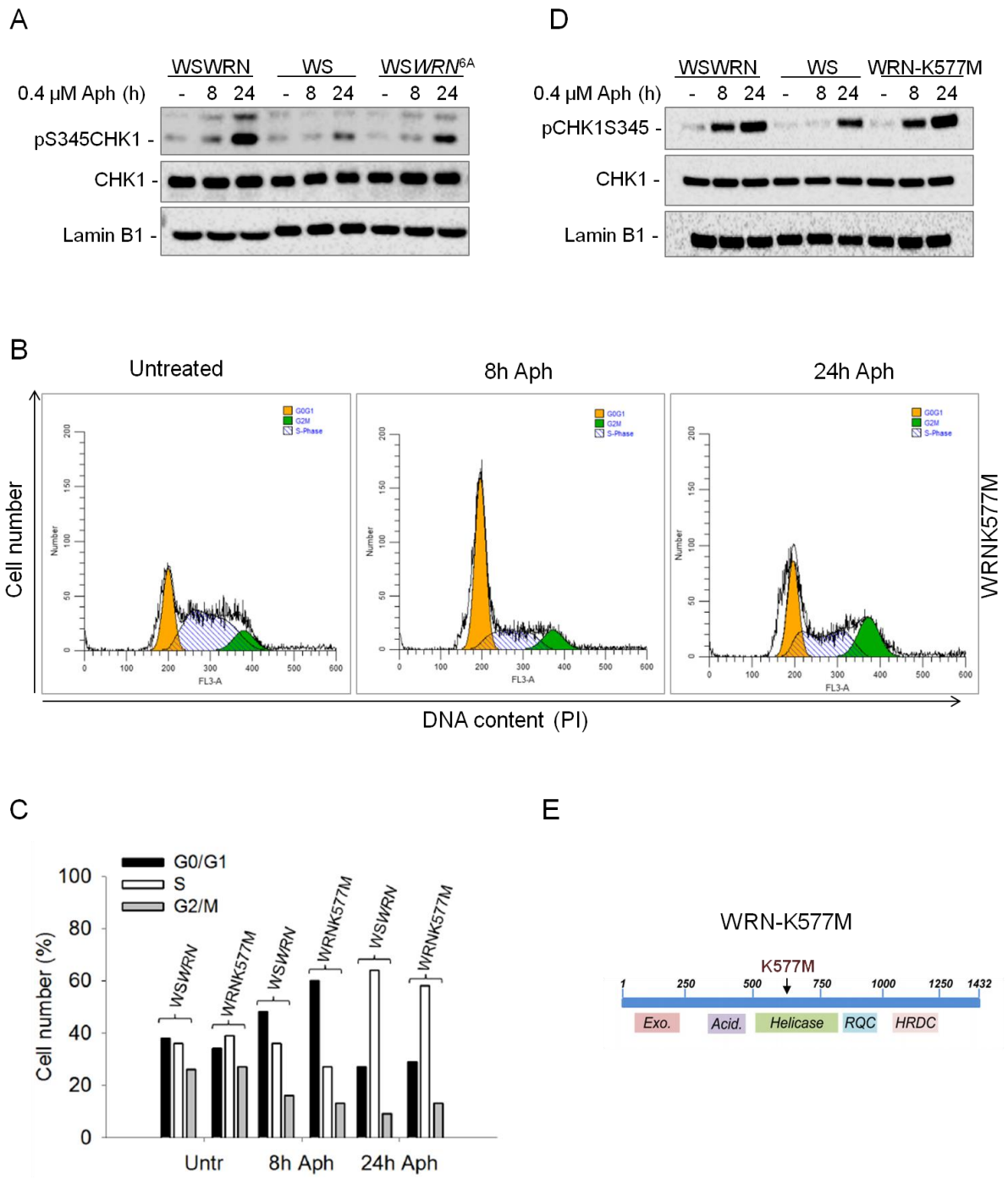



**Figure 13ATR-dependent WRN phosphorylation, but not defective WRN helicase activity, fails to activate CHK1 upon moderate replication stress.** (A) WS cells, WSWRN or expressing an unphosphorylable mutant form of WRN (WSWRN$^{6A}$) were treated or not with Aph for the indicated times. CHK1 phosphorylation was analysed by Western blot using phospho-specific antibodies (pS345). (B) WS cells, WSWRN or expressing a mutant form of WRN affecting helicase (WRN-K577M) were treated or not with Aph for the indicated times. (A-B) CHK1 phosphorylation was analysed by Western blot using phospho-specific antibodies (pS345). Total amount of CHK1 was determined with an anti-CHK1 antibody. Lamin B1 was used as loading control. The ratio of phosphorylated protein to total protein and then normalized to the untreated wild type is listed below each lane. (B) DNA content profiles of WRN-K577M cells treated or not with Aph 0.4 µM for the indicated times and stained with PI prior to FACS analysis. Graph shows the percentage of cells distributed into G0/G1, S or G2/M phases.

As expected, WSWRN cells were proficient in activation of CHK1 after treatment (Figure 13A). In contrast, WSWRN$^{6A}$ cells showed defective CHK1 phosphorylation as WS cells. Therefore, these data suggest that phosphorylation of WRN may play a role in the ATR-checkpoint activation in response to moderate replication stress.

WRN helicase activity, which is crucial in maintaining CFS stability (Sakamoto et al., 2001),could facilitate formation of the single-strand DNA (ssDNA) leading to checkpoint activation (Franchitto et al., 2008). Thus, to verify whether suppression of helicase function hinders CHK1 phosphorylation, WS fibroblasts and WS cells stably expressing the wild-type WRN (WSWRN) or its helicase-dead form (WRN-K577M) were treated with Aph and cell lysates subjected to Western blot (WB). The data confirmed that, in WSWRN cells, CHK1 was properly phosphorylated after Aph treatment, and comparable levels of CHK1 phosphorylation were observed in WRN-K577M cells, indicating that expression of the helicase-dead WRN protein is sufficient to recover from the defective phenotype (to restore checkpoint activity) (Figure 4B). Consistently, flow-cytometric experiments indicated that loss of the WRN helicase activity had no effect on the delay of cell cycle progression induced by Aph (Figure 13C).

We therefore can conclude that the WRN helicase activity is not implicated in the activation of the ATR-dependent pathway in response to low-levels of replication stress, whereas the ATR-dependent phosphorylation of WRN results essential for CHK1 activation.

## *Analysis of the ATR-signaling pathway in WRN-deficient and mutant cells after low-levels of replication perturbation*

Activation of the ATR-pathway depends on RPA binding to ssDNA (Franchitto et al., 2008). To determine whether impaired RPA-coated ssDNA accumulation contributes to defective checkpoint activation observed in the absence of WRN, the amount of chromatin-bound RPA was measured in Aph-treated WSWRN and WS cells. Biochemical analysis of fractionated cell extracts showed that RPA32, a component of the RPA heterotrimer, was only slightly more chromatin-associated in WS cells, either exposed to Aph or left untreated, respect to WSWRN cells (Figure 14A). Indeed, even though WS cells showed more chromatin-bound RPA32, they also seemed to contain more RPA32 in total extracts (Figure 14A, bottom). Consistently with previous results, Western blot analysis showed that the level of Ser33-phosphorylated RPA32, which represents a direct read-out of replication stress (Liu et al., 2000), was greater in WRN-kd cells in comparison with WRN-wt cells (Figure 14C).

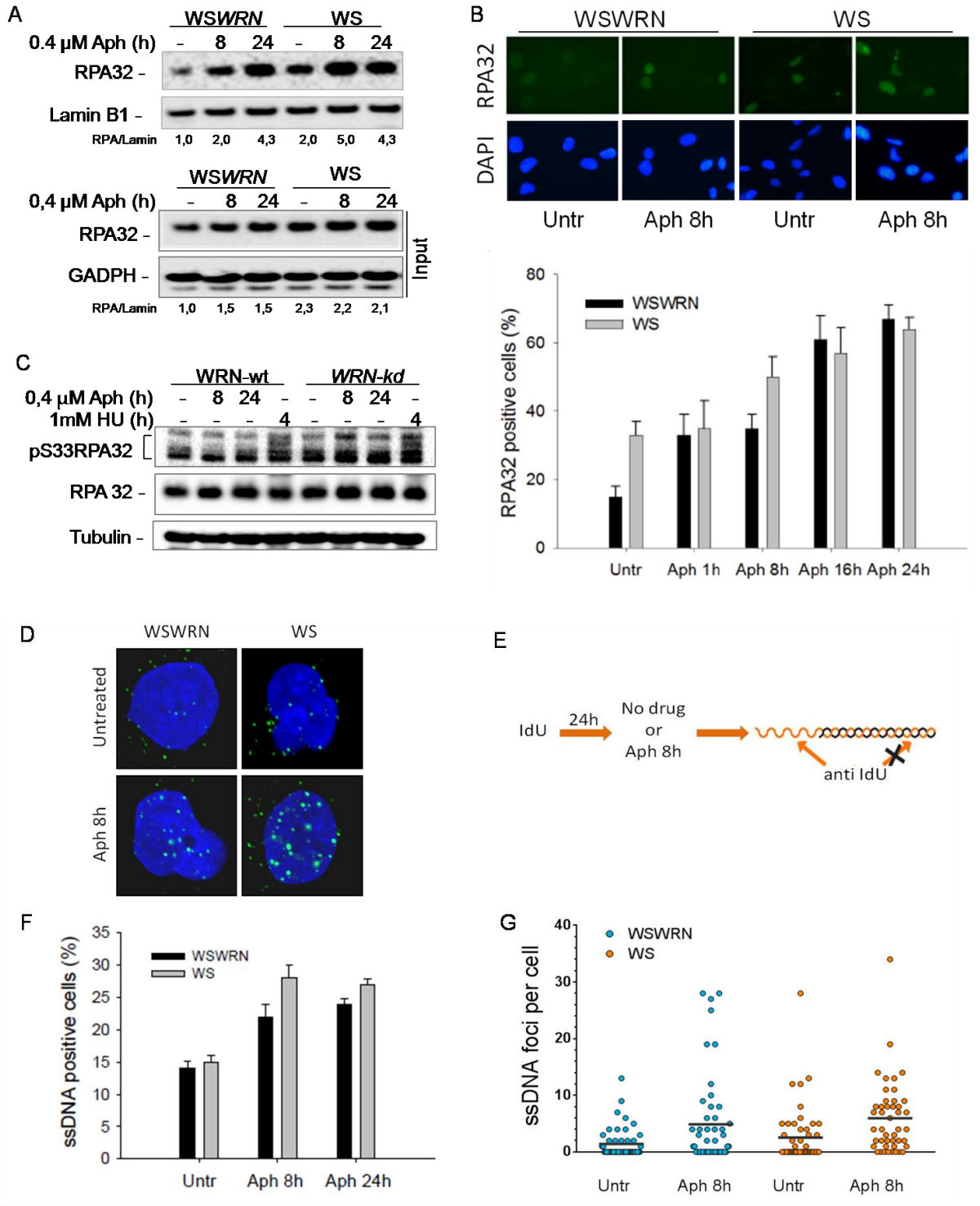



**Figure 14 Analysis of RPA32 accumulation and ssDNA formation in cells upon mild replication perturbation.** (A) WB chromatin recruitment ofRPA32 in WSWRN and WS cells. Total amount of RPA32 was determined with an anti-RPA32 antibody. Lamin B1 was used as loading control. WB of whole cell extracts (Input) (bottom) . (B) Immunostaining with RPA32 of cells untreated or treated with 0.4 µM Aph, representative images. Graph shows the percentage of RPA32-positive cells (bottom). (C) Western blot detection of RPA32 phosphorylation in total extracts of WRN-wt and WRN-kd cells untreated (-) or treated with 0.4 µM Aph or 1mM HU for 4 h. Phosphorylated RPA32 was assessed using phospho-specific antibodies (pS33), total amount of RPA32 was determined with an anti-RPA antibody. Equal loading was confirmed with anti-Tubulin antibody. (D-E) Detection of BrdU incorporation under non-denaturing conditions in the parental DNA of WSWRN and WS cells treated as indicated in the scheme. (F) Graph shows the percentage of BrdU-positive nuclei. (G) Dot plot of the number of ssDNA foci per cell for a representative experiment.

In agreement with this result, a time course analysis of non-extractable RPA32 foci by immunofluorescence revealed that, in the absence of WRN, a higher number of RPA32-positive nuclei accumulated under unperturbed conditions, while only a slight increase at 8 h Aph treatment was detected, even if the RPA32 fluorescence appeared to be more intense than in the control cells (Figure 14B).

Next, to support the evidence that loss of WRN does not affect the generation of ssDNA after exposure to low dose of Aph, the extent of ssDNA formation was quantified through native BrdU staining (Figure 14D-E-F-G). The analysis revealed that the percentage of ssDNA-positive nuclei was roughly similar in both WSWRN and WS cells (Figure 14F), but the number of foci per cell was higher in WS cells (Figure 14G).

Together, these results indicate that, in WS cells, CHK1 activation is not defective because of the inability to form the RPA-coated ssDNA after moderate replication perturbation.

It is known that the TopBP1 mediator protein is required for efficient CHK1 phosphorylation, and that it is recruited to stalled forks by the chromatin-bound RAD1-RAD9-HUS1 (9.1.1) complex (Jackson and Pombo, 1998; Zou and Elledge, 2003). In addition, WRN and the 9.1.1 complex physically interact (Delacroix et al., 2007). To test the possibility that WRN could stabilize 9.1.1/TopBP1 association following mild replication stress, we analyzed chromatin loading of TopBP1 and RAD9 by WB after cellular fractionation in WSWRN and WS cells treated or not with Aph at the indicated times. As shown in Figure15A, in WSWRN cells the amount of chromatin-bound TopBP1 started to increase at 1 h after treatment, and remained high at 8 and 24 h, even if it decreased at 16 h in agreement with other reports (Kumagai et al., 2006).

Similarly, RAD9 was loaded on the chromatin at all times of Aph exposure except at 16 h (Figure 15A). In contrast, in WS cells low-levels of RAD9 loaded to chromatin was detectable under untreated and low dose Aph-treated conditions (Figure 15B). Interestingly, the expression of the unphosphorylable form of WRN, but not, as expected, loss of WRN helicase activity, compromised chromatin association of checkpoint mediators, and a clear reduction of TopBP1 and RAD9 recruitment was found in WSWRN$^{6A}$ cells at 24 h of Aph (Figure 15B). Therefore, defective CHK1 activation upon replication perturbation induced by Aph, in WS and WSWRN$^{6A}$ cells, appears related to the levels of chromatin-bound checkpoint mediators.

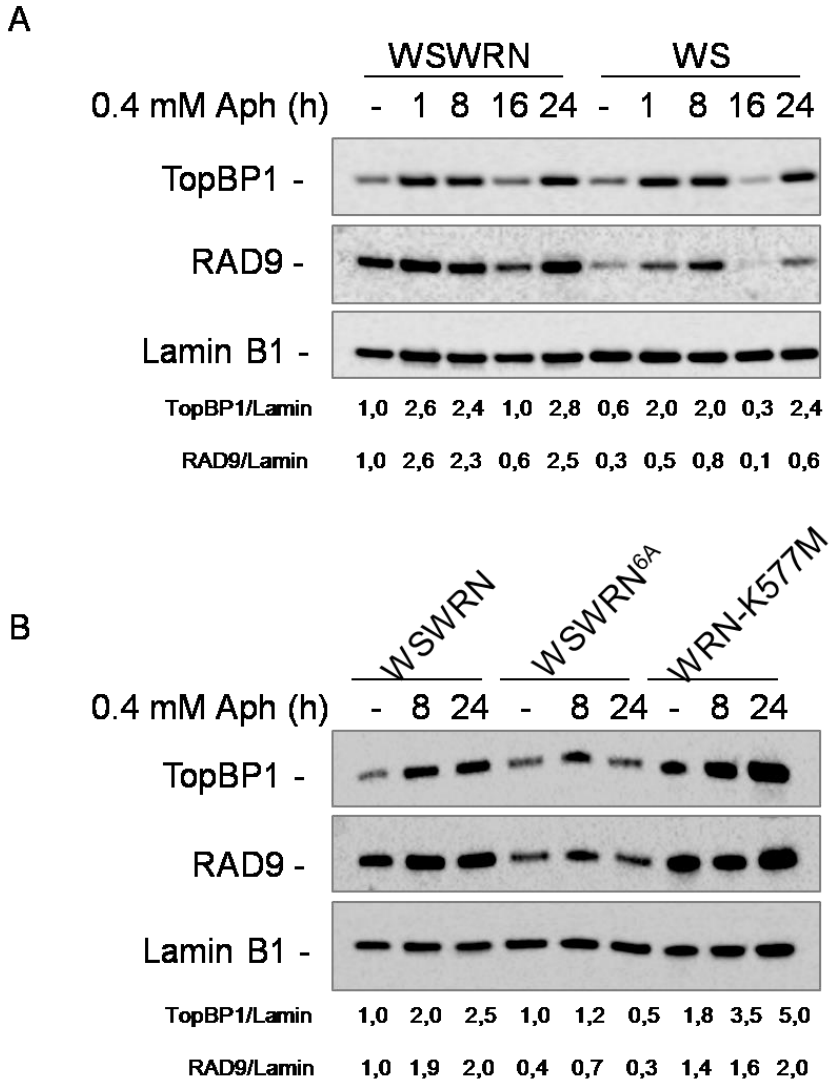



**Figure 15Analysis of chromatin loading of ATR-pathway sensors and mediators in cells upon mild replication stress.** (A) WSWRN and WS cells were treated with Aph for various times or left untreated as indicated. Total amount of TopBP1 and RAD9 were determined with an anti-TopBP1 or anti-RAD9 antibody respectively. Lamin B1 was used as loading control. (B) WSWRN cells and WS cells expressing an ATR-unphosphorylable form of WRN (WSWRN$^{6A}$) or mutant form of WRN helicase (WRN-K577M) were treated or not with Aph for the indicated times. Total amount of TopBP1 and RAD9 and equal loading of total proteins were determined as in (B). Quantitative immunoblotting was used and the ratio of TopBP1 or RAD9 protein to Lamin B1 then normalised to wild-type is reported below each lane.

### *Replication fork dynamics are altered in WRN-deficient and mutant cells*

Having demonstrated that correct CHK1 activation relies on both the presence of WRN and its phosphorylation by ATR, and since CHK1 has been implicated in maintaining fork integrity and proper cell cycle progression under conditions of replicative stress (Koundrioukoff et al., 2013; Pichierri et al., 2012), we examined how the absence of WRN or expression of mutant forms could influence replication dynamics.

We first monitored replication in WSWRN, WS, WSWRN$^{6A}$ and WRN-K577M cells under unperturbed conditions as indicated in the scheme (Figure 16A). Under these conditions, WSWRN showed an average fork progression rate of 1.1 kb/min (Figure 16B). By contrast, WRN-deficient cells or cells expressing the mutant forms of WRN displayed a significant reduction of fork speed (about two-fold) as compared to control cells (Figure 16B). A logical expectation from this result might be that replication stress exacerbated the effect, however, all the cell lines reached identical values of fork velocity upon Aph treatment (Figure 16C). This implies that fork progression is mainly affected by WRN deficiency or expression of WRN mutated forms, and that Aph is able to further delay forks only in control cells.

It has been previously proposed that cells lacking CHK1 activity show reduced rate of replication fork progression and increased origin firing (Feijoo et al., 2001). Thus, we verified whether the inability of Aph to reduce further fork speed in WRN-deficient and WRN-mutant cells could be the end-result of new origins activated to compensate for replication problems.

We used a modified double labeling protocol that enables us to study fork recovery from Aph (Figure 17A). Under such conditions, all the cell lines showed an analogous ability to replicate after removal of Aph, evaluated as length of green tracks (Figure 8B and C). Interestingly, using roscovitine, a CDK inhibitor that prevents origin firing, a reduced length of green tracks in WS, WSWRN$^{6A}$ and WRN-K577M cells was detected, but no effect was found in WSWRN cells (Figure 17B). Such a finding could indicate that DNA is partly replicated from ongoing forks and partly from a downstream fork fired during roscovitine treatment. Consistent with more new origin firing in WS, WSWRN$^{6A}$ and WRN-K577M cells, roscovitine also decreased the number of isolated green tracks detected during the recovery from Aph (Figure 17D). Therefore, we conclude that the increased replication elongation observed in WRN-deficient or WRN-mutant cells is due to increased origin firing of

neighboring dormant origins, and that this back-up mechanism obscures a more severe reduction of fork progression occurring in these cells.

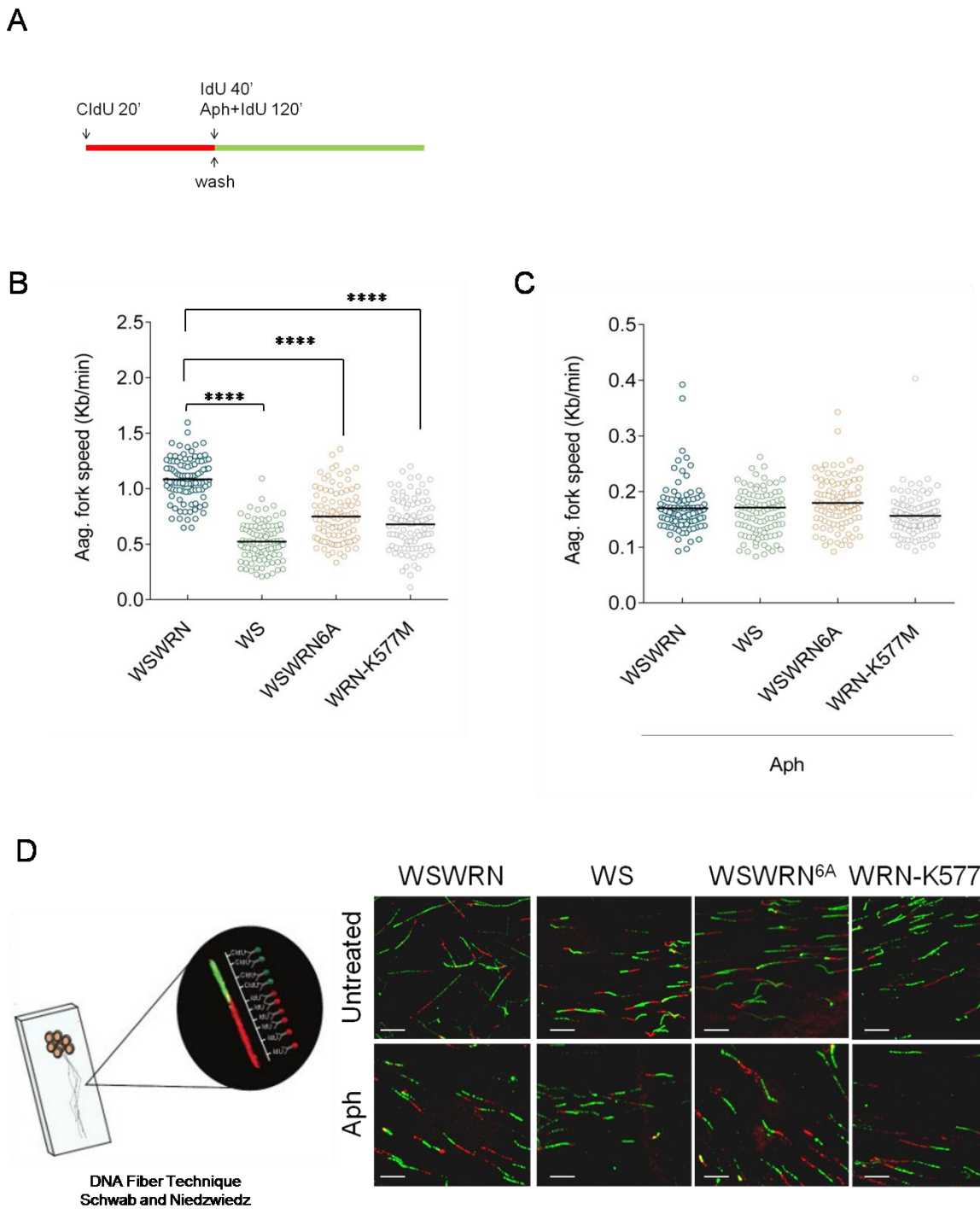



**Figure 16Replication fork progression is impaired in WRN-deficient and mutant cells.** (A) Experimental scheme of dual labelling of DNA fibres. (B) Analysis of replication fork velocity in WSWRN (wild-type), WRN-deficient (WS) and mutant cells (WSWRN$^{6A}$ and WRN-K577M) under unperturbed conditions or (C) exposed to 0.4 µM Aph as indicated in (A). The length of the green tracks were measured. Median values are represented as horizontal black lines. (D) Representative images of actual DNA fibres from WSWRN, WRN-deficient and mutant cells. Scale bars, 10 µm. **** = Statistically significant P < 0.0001 (Student's t-test).

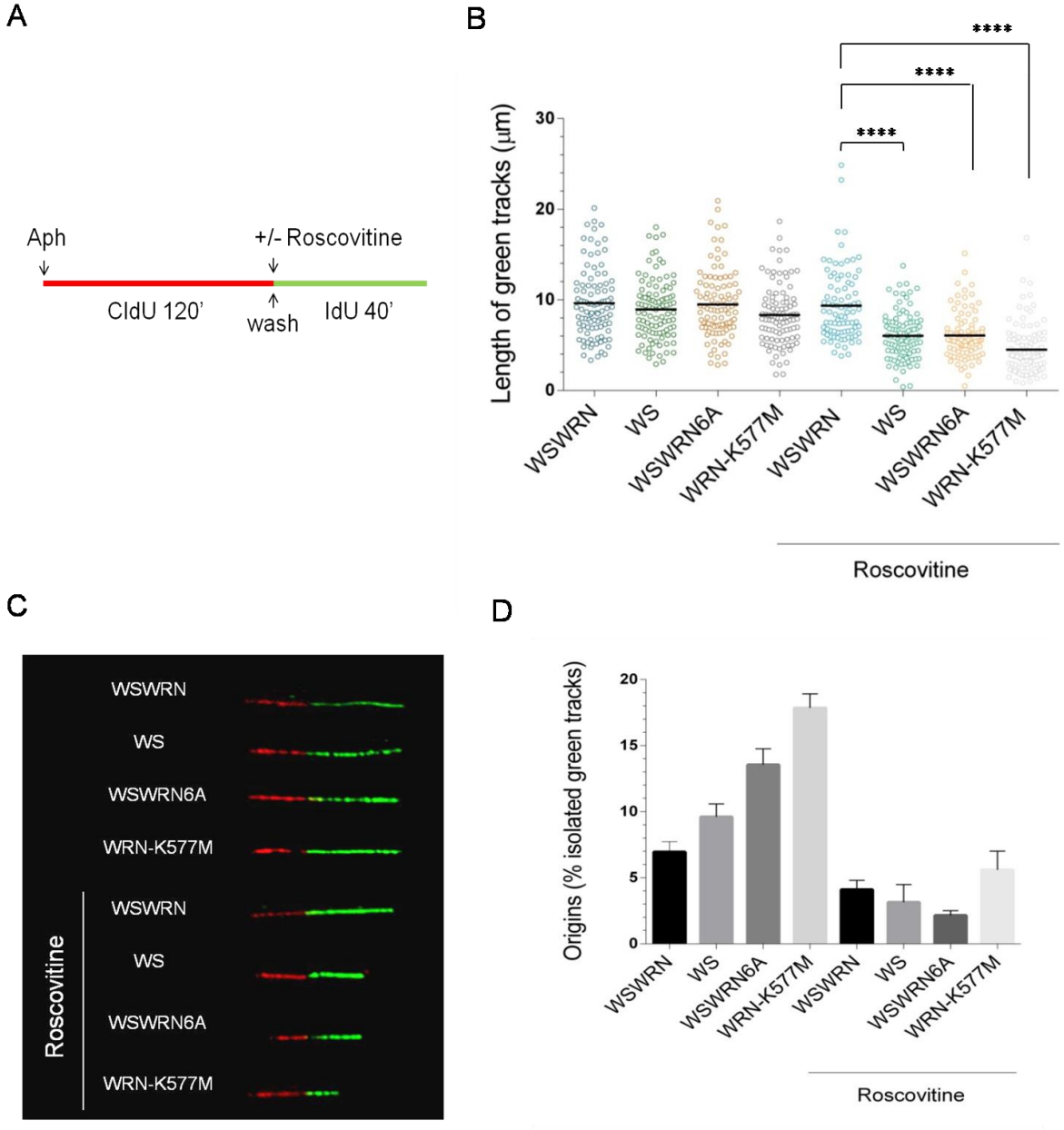



**Figure 17Replication fork restart is affected by roscovitine in WRN-deficient and mutant cells.** (A) Experimental scheme of dual labelling of DNA fibres in presence or absence of roscovitine. (B) Analysis of replication fork recovery in WSWRN (wild-type), WRN-deficient (WS) and mutant cells (WSWRN$^{6A}$ and WRN-K577M) treated as indicated in (A). Aph (0.4 µM) and roscovitine (100 µM) were added for the indicated time. Median values are represented as horizontal black lines. (C) Representative images of replication tracks from WSWRN, WRN-deficient and mutant cells with or without roscovitine treatment. (D) Percentage of new origin firing in cells treated as in (B). Data are reported as mean from three independent experiments. Error bars represent standard error. **** = Statistically significant $P < 0.0001$ (Student's t-test).

## *Expression of phospho-mimic CHK1 mutant recovers cells from sensitivity to Aph treatment and restores fork elongation*

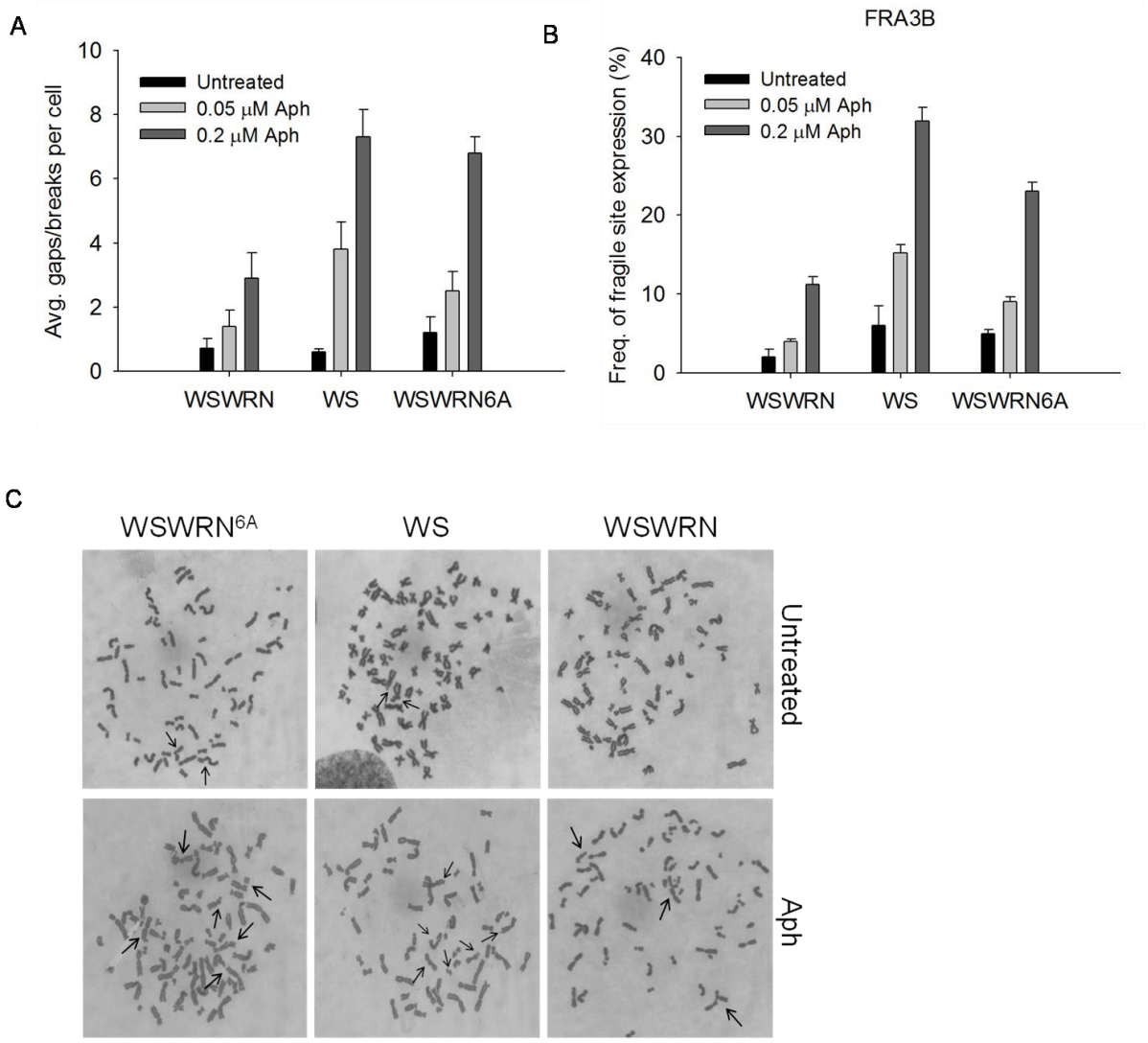



**Figure 18Enhanced CFS induction in cells expressing an ATR-unphosphorylable form of WRN.** (A) Average overall chromosome gaps and breaks in WS cells (WS), WS cells expressing an ATR-unphosphorylable form of WRN (WSWRN$^{6A}$) and in WS cells in which wild-type WRN was reintroduced (WSWRN). Cells were treated with two different doses of Aph for 24 h before harvesting. Data are presented as means of three independent experiments. Error bars represent standard error. (B) Frequency of gaps and breaks at CFS by FISH analysis. Cells were treated as described in (A). (A) Representative Giemsa-stained metaphases of wild-type (WSWRN*)*, WS and WSWRN$^{6A}$ fibroblasts untreated or treated with 0.2 μM Aph. Arrows indicate chromosomal aberrations. FISH was performed using BACs probes corresponding to the FRA3B region. Frequency of fragile site induction is presented as the percentage of chromosome 3 homolog with gaps and breaks at FRA3B. Data are presented as means of three independent experiments. Error bars represent standard error on the mean.

Since low-levels of replication stress induce CFS expression, we asked whether defective checkpoint activation found in WS and WSWRN$^{6A}$ cells could be related to CFS

instability. We first investigated the sensitivity of WSWRN$^{6A}$ to Aph. A dose-dependent enhancement of chromosomal aberrations, similarly to that previously seen in WS cells (Sakamoto et al., 2001), was observed in WSWRN$^{6A}$,which showed approximately two-fold more aberrations than control cells at the higher dose (Figure 18A). These results suggest that WSWRN$^{6A}$ cells are sensitive to Aph treatment, and that loss of ATR-mediated WRN phosphorylation is responsible for chromosome instability.

Next, we examined if increased chromosomal damage induced by Aph in WSWRN$^{6A}$ took place at CFS. As shown in Figure 9C, instability at CFS FRA3B was induced in a dose-dependent manner in WSWRN$^{6A}$ cells, with a number of gaps and breaks higher than in control cells, and at levels comparable to those of WS cells. Interestingly, enhanced FRA3B induction was detected even in the absence of Aph in both WSWRN6A and WS cells (Figure 18C). Similar results were observed testing other CFS (Figure 19).

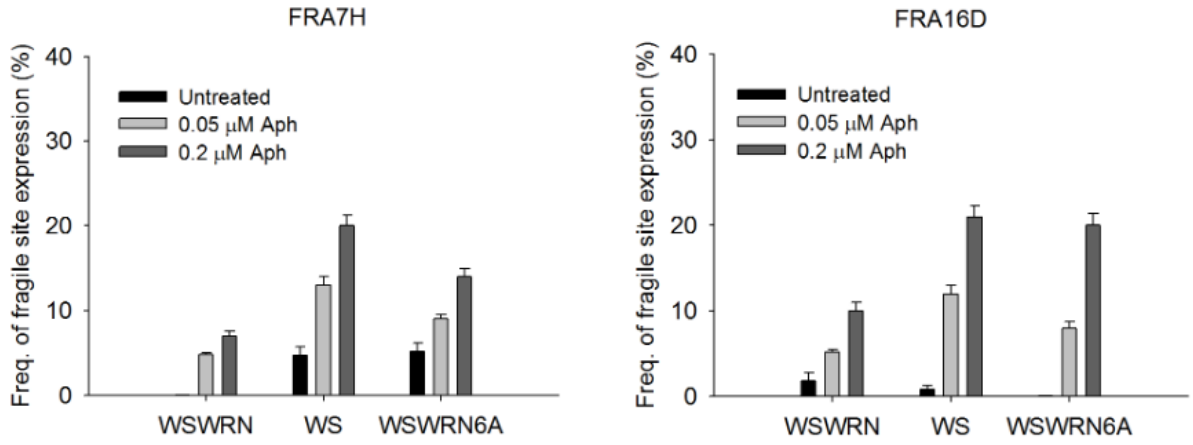



**Figure 19 Analysis of CFS expression in cells expressing an ATR-unphosphorylable form of WRN.** Frequency of fragile site FRA7H and FRA16D expression in WS cells (WS), WS cells expressing an ATR-unphosphorylable form of WRN (WSWRN6A) and in WS cells in which wild-type WRN was reintroduced (WSWRN). Cells were treated the indicated doses of Aph and harvested 24 h later. Frequency of fragile site induction is presented as the percentage of chromosome 7 or 16 homologues with gaps and breaks at FRA7H and FRA16D,R espectively. Data are presented as means of three independent experiments. Error bars represent standard error.

These findings demonstrate that phosphorylation of WRN by ATR is essential not only for correct CHK1 phosphorylation but also, and most notably, for CFS stability. Thus, we wondered whether forced activation of CHK1 could overcome sensitivity to Aph in WS and WSWRN$^{6A}$cells, that is in cells where CHK1 is not phosphorylated.

Cells were transfected with a construct expressing a mutant form of CHK1 in which Ser317 and Ser345 were changed into Asp ($CHK1^{317/345D}$), mimicking the phosphorylated status of the protein (15). WB analysis confirmed that the Flag-tagged phospho-mimic CHK1 mutant (Flag-$CHK1^{317/345D}$) was expressed at comparable levels in both cell lines (Figure 20A). After transfection, cells were treated with Aph and metaphase chromosomes were collected and scored for total gaps and breaks. As expected, Aph treatment resulted in enhanced chromosomal damage in WS and $WSWRN^{6A}$cells (Figure 20B).

Interestingly, expression of the $CHK1^{317/345D}$protein into both WS and $WSWRN^{6A}$ cells resulted in the rescue of the chromosomal fragility induced by low dose of Aph and also determined a slight reduction of the spontaneous number of aberrations per cell in $WSWRN^{6A}$ cells (Figure 20B). These results indicate that DNA breakage observed in both WS and $WSWRN^{6A}$ cells upon mild replication perturbation actually correlates with incorrect CHK1 activation.

Next, we asked whether the introduction of the phospho-mimic CHK1 mutant could allow cells to recover from fork stalling, possible without activating new origins. WS and $WSWRN^{6A}$ cells were transfected or not with the Flag-$CHK1^{317/345D}$plasmid, then treated with Aph and roscovitine prior to analyze their replication recovery (Figure 20C).

Analysis of the length of replicating tracks on DNA fibers showed that the presence of phospho-mimic CHK1 mutant enabled both cell lines to resume replication in a wild-type fashion, overcoming the requirement of new origin firing, as demonstrated by the similar track lengths in cells treated or not with roscovitine (Figure 20C), and the analysis of isolated green tracks, showing that less dormant origins were activated (Figure 20D).

Altogether, these data strongly support the hypothesis that, in WS and $WSWRN^{6A}$ cells, the inability to trigger CHK1 activation leads to replication fork demise and origin firing in the attempt to complete replication.

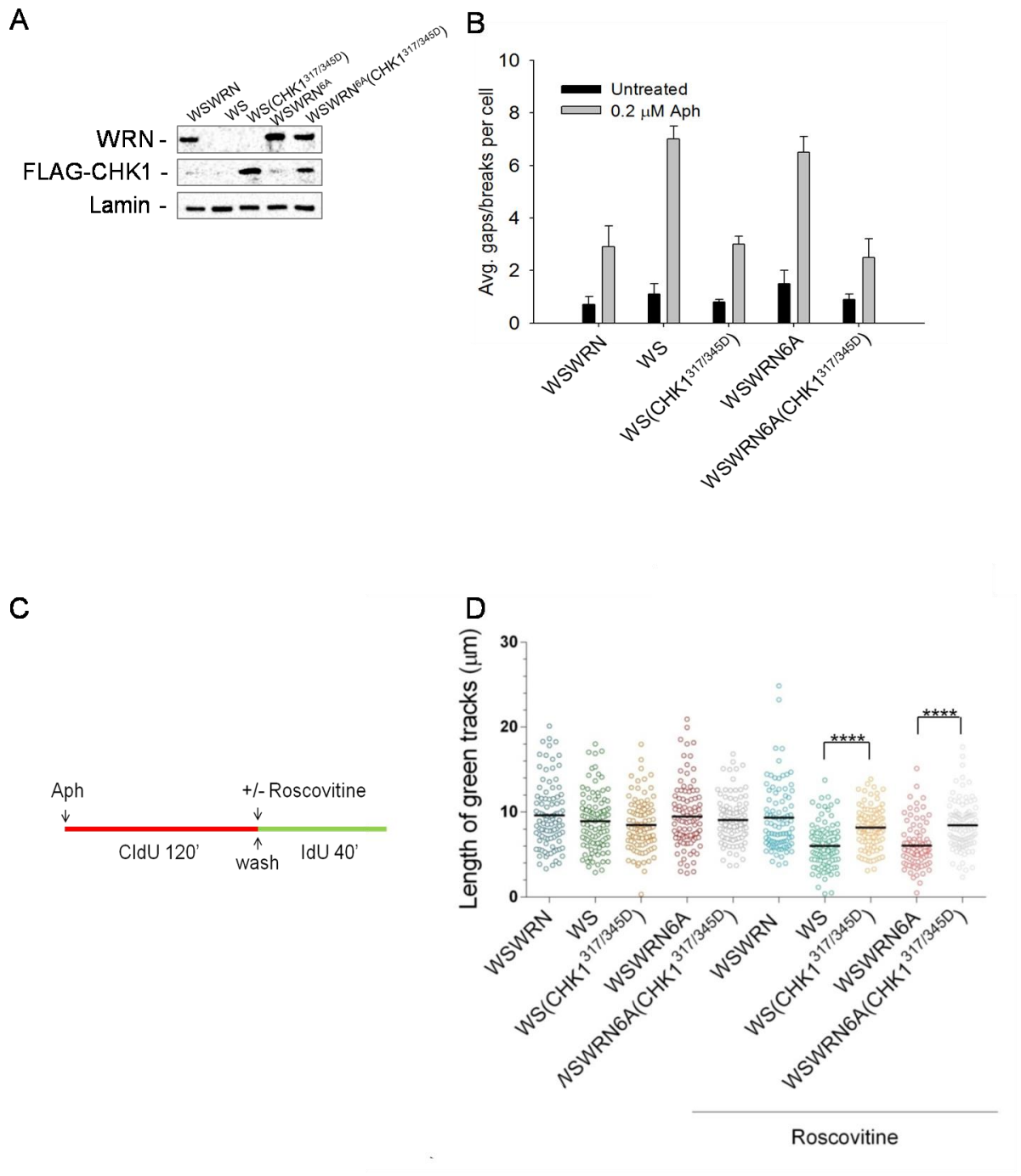



**Figure 20Expression of phospho-mimic CHK1 rescues sensitivity to Aph and replication fork slowing in WS and WSWRN$^{6A}$ cells.** (A) WS and WSWRN$^{6A}$ cells were transfected with Flag-tagged CHK1 S317D/S345D mutant (CHK1$^{317/345D}$). Lysates were collected 48 h thereafter and expression levels of Flag-CHK1$^{317/345D}$ were determined by immunoblotting with anti-Flag antibody. WSWRN cells were used as negative control. Total WRN was used to assess the amount of wild-type or mutant form of WRN. Lamin B1 was used as loading control. (B) Effect of the expression of the Flag-CHK1$^{317/345D}$ plasmid on chromosomal damage. After transfection, cells were treated or not with Aph for 24 h. The graph shows the average overall chromosome gaps and breaks. Data are presented as means of three independent experiments. Error bars represent standard error on the mean. (C) Evaluation of replication fork recovery in WS and WSWRN$^{6A}$ cells transfected with Flag-CHK1$^{317/345D}$. Cells were treated as indicated in the experimental scheme. Aph (0.4 μM) and roscovitine (100 μM) were added for the indicated time. Median values are represented as horizontal black lines. (D) Percentage of new origin firing in cells treated as in (C). Data are reported as mean from three independent experiments. Error bars represent standard error. **** = Statistically significant $P < 0.0001$ (Student's t-test).

# RESULTS PART 2

## RESULTS (part 2)

***H2AX is phosphorylated in an ATR-dependent manner in response to mild replication stress induced by Aph***

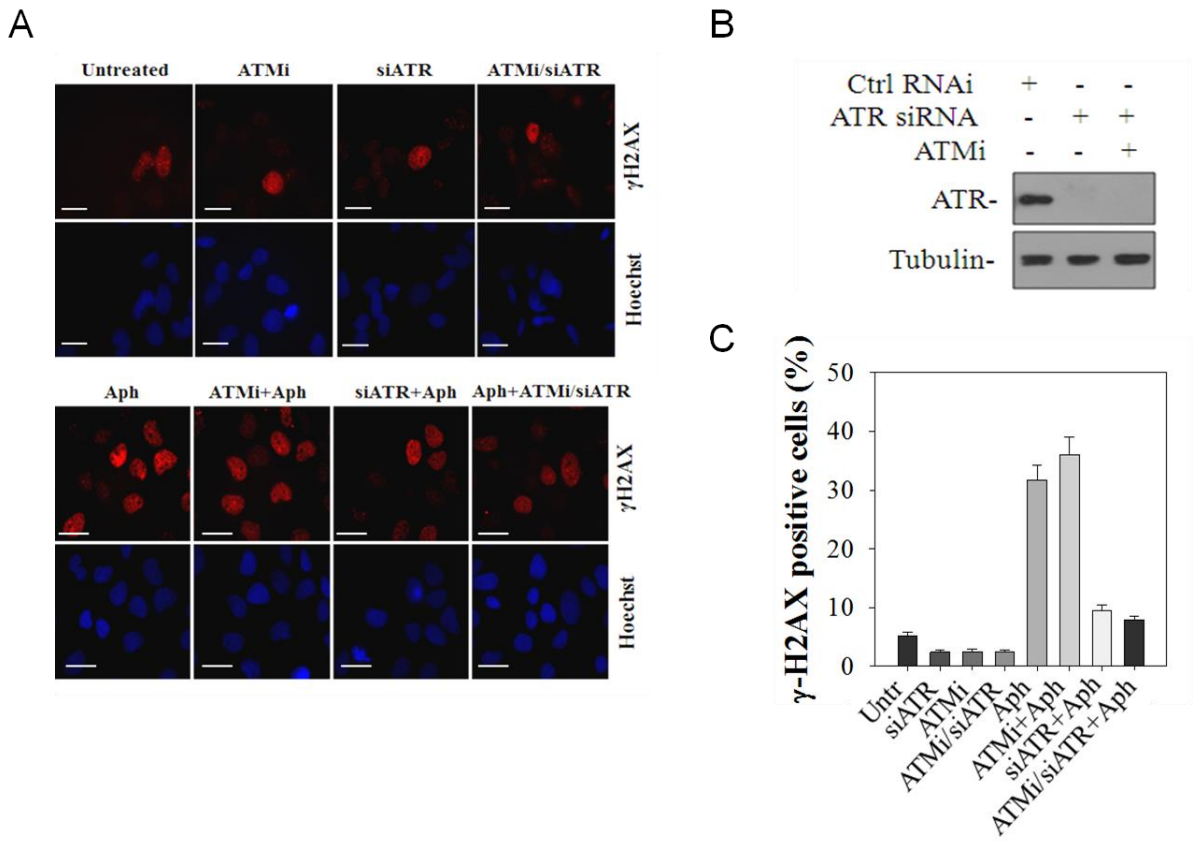



**Figure 21γ-H2AX foci formation in wild-type cells depends on the ATR kinase after low doses of Aph.** (A) In the panel, representative images of each experimental point are shown. Error bars represent standard errors. Scale bars: 10μm. (B) ATR depletion was verified by immunoblotting 48h after transfection using anti-ATR antibody. Tubulin was used as loading control. (C) After depletion of ATR using RNAi and/or inhibition of ATM activity by the KU55933 compound, cells were treated with 0,4μM Aph for 8h and immunostained with anti-γ-H2AX (pSer139) antibody. Graph shows quantification of the number of nuclei with >5 nuclear γ-H2AX foci (γ-H2AX-positive nuclei). Representative images are shown in the panel for each experimental condition. The data are means from triplicate experiments.

To further analyze the cellular response to mild replication stress, we verified whether other targets of the ATR kinase resulted affected by loss of WRN. Among the earlier ATR targets is H2AX, which is phosphorylated in response to single-stranded DNA breaks as well as during genome-wide-induced replication stress, such as replication fork arrest,and whose

activation is easily detected as nuclear foci by specific antibody (anti-γ-H2AX pSer139) for the phosphorylated form of H2AX (Ward and Chen, 2001).

We first verified whether H2AX could be a target of the ATR kinase also under replication perturbation induced by low dose of Aph. To this aim, we performed immunofluorescence experiments in wild-type cells in which ATM (ATMi), ATR (siATR) or both (ATMi/siATR) were depleted.

Down-regulation of ATR was achieved by transfection with siRNA directed against ATR, while ATM activity was inhibited by the use of KU55933 compound. Forty-eight hours after interference, cells were pre-treated with the ATM activity inhibitor (KU55933) for 1h, then Aph was added to a final concentration of 0,4μM for 8h.

The reduction of the ATR level was verified by Western blot (Figure 21B). Our results showed that the percentage of γ -H2AX-positive cells was enhanced by Aph in wild-type cells or cells in which ATM is chemically inhibited, but was absent, or very weak, in cells in which ATR was depleted alone or in combination with ATM (Figure 21A and C).

These data suggest that H2AX phosphorylation induced by Aph-treatment depends mainly on the ATR kinase.

### *Reduced activation of γ-H2AX in WS cells upon Aph-induced replication perturbation*

Next, having confirmed that H2AX is an ATR target upon Aph-induced replication stress, we explored whether H2AX was phosphorylated in WS cells.

To this aim, we examined γ-H2AX foci formation in WSWRN and WS cells,treated with 0,4μM Aph for 8 or 16h, and then immunostained with anti-γ-H2AX antibody.

Our results show that, in wild-type cells, Aph increased the percentage of γ-H2AX-positive nuclei in a time-dependent manner (Figure 2A and B). By contrast, in WS cells, we detected high levels of γ-H2AX foci formation already under unperturbed conditions, but reduced induction of γ-H2AX-positive nuclei after 8h Aph-exposure in comparison to wild-type cells (Figure 22A and B). Moreover, in contrast to wild-type cells, WS cells showed a small increase in the percentage of γ-H2AX foci at later time of treatment (Figure 22A and B).

Consistently with the results obtained by immunofluorescence, Western blot analysis revealed a reduced accumulation of γ-H2AX in WS cells at earlier time of Aph-exposure, and an increased level at later time of treatment (Figure C).

Altogether, these findings suggest that loss of WRN results in reduced activation of γ-H2AX following Aph-induced replication perturbation.

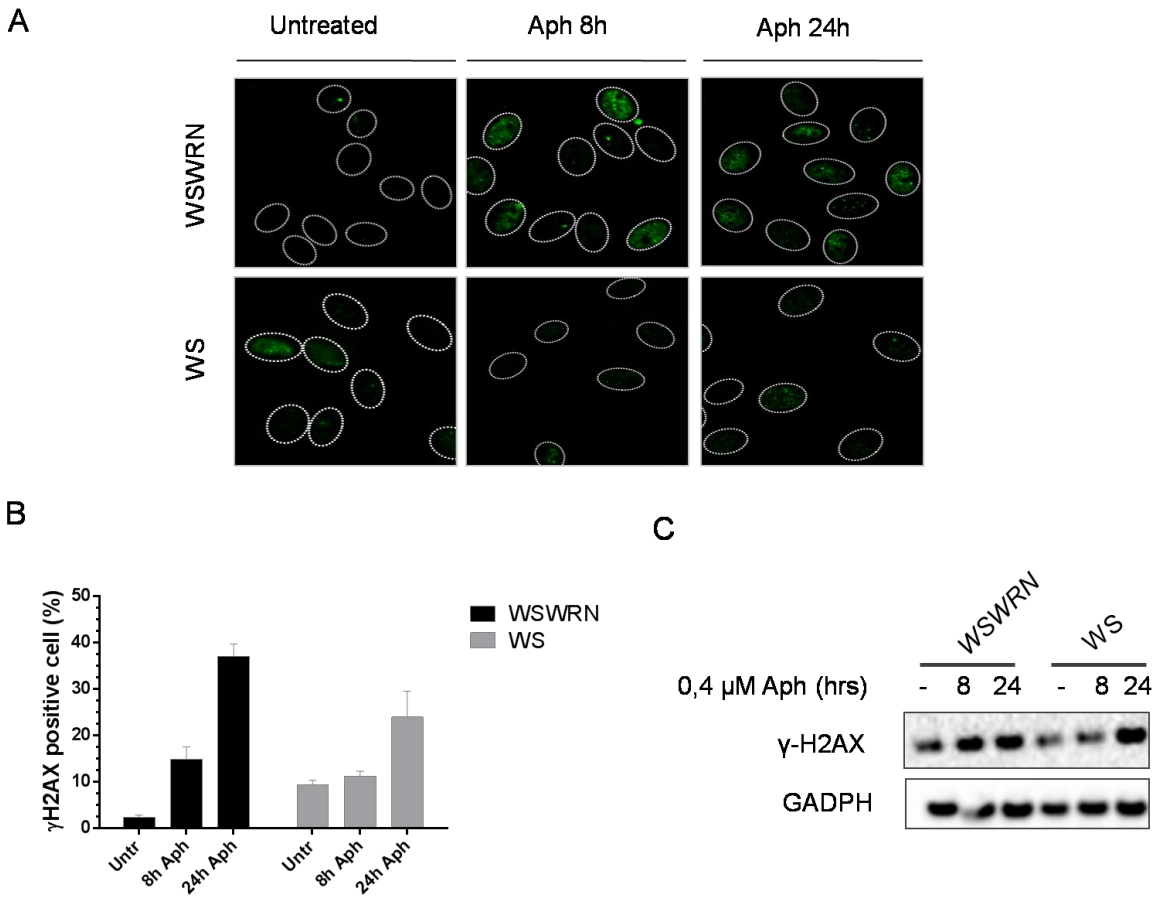



**Figure 22 Loss of WRN results in low levels of γ-H2AX foci formation after Aph-treatment.** A) and B) Immunofluorescence analysis of γ-H2AX. WSWRN and WS cells were left untreated or treated with 0,4μM Aph for the indicated times, and then immunostained with anti-γ-H2AX (pSer139) antibody. Graph shows quantification of the number of nuclei with >5 nuclear γ-H2AX foci (γ-H2AX-positive nuclei). The data are means from triplicate experiments. Error bars represent standard errors. Representative images are shown in the panel for each experimental condition. (C) Western blot detection of γ-H2AX in total extracts from WSWRN or WS cells left untreated or treated with 0,4μM Aph for 8 or 24h using anti-γ-H2AX (pSer139) antibody. Equal loading was confirmed probing with an anti-GAPDH antibody.

### *Loss of WRN leads to the ATM pathway activation after mild replication stress*

In this study, we demonstrate a defect in the ATR-signaling pathway in WRN-deficient cells following mild replication stress (see Results part 1). Since it has been previously reported that phosphorylation of H2AX in ATR-deficiency largely depends on the ATM kinase (Chanoux et al., 2009), and that ATR phosphorylates checkpoint proteins in AT cells (Tomimatsu et al., 2009), we asked whether an ATM-mediated pathway is activated upon Aph-treatment in the absence of WRN.

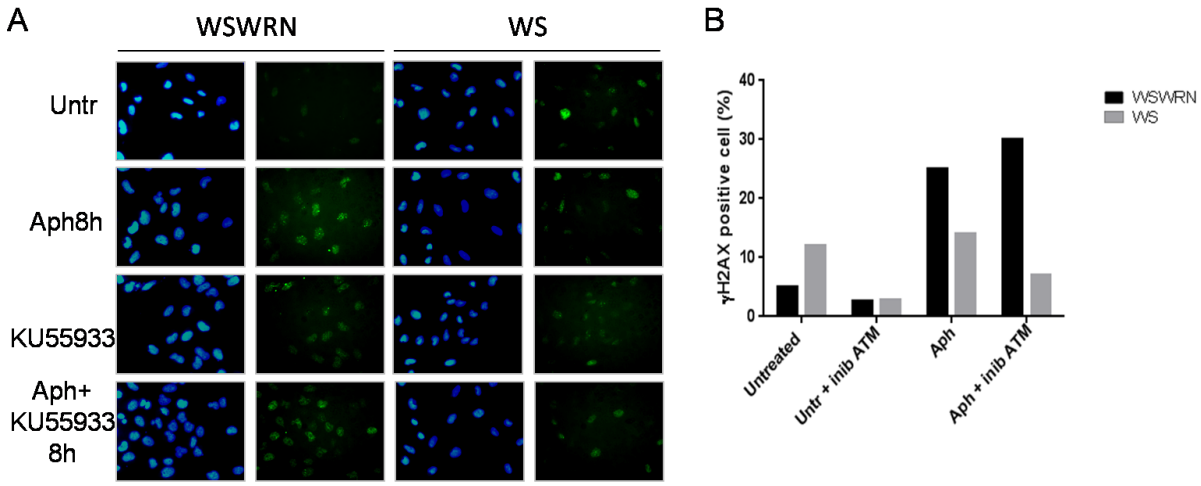



**Figure 23 Evaluation of ATM-pathway inhibition on the H2AX phosphorylation in WS cells.** Immunofluorescence analysis of γ-H2AX in WSWRN and WS cells left untreated or treated with 0,4μM Aph for 8h, with or without addition of 10μM chemical inhibitor of the ATM activity (KU55933). At the end of treatment, cells were immunostained with anti-γ-H2AX (pSer139) antibody. Graph shows quantification of the number of nuclei with >5 nuclear γ-H2AX foci (γ-H2AX-positive nuclei) (B).Representative images are shown in the panel for each experimental condition (A).

To test this hypothesis, WSWRN and WS cells were left untreated or treated with Aph under the same conditions used for the biochemical determination of CHK1 activation (see Result part 1), with or without the concomitant addition of the selective inhibitor of ATM kinase activity (KU55933). After fixation, cells were immunostained with anti-γ-H2AX (pSer139) antibody. As shown in Figure 23, in untreated conditions, chemical inhibition of ATM was able to reduce the percentage of γ-H2AX positive nuclei in both cell lines, but with a greater effect in WS cells. However, after Aph-treatment, KU55933-exposure did not impair γ-H2AX foci formation in wild-type cells, while in WS cells it decreased the percentage of γ-

H2AX positive nuclei, even if at levels not comparable to those observed in untreated conditions (Figure 23A and B).

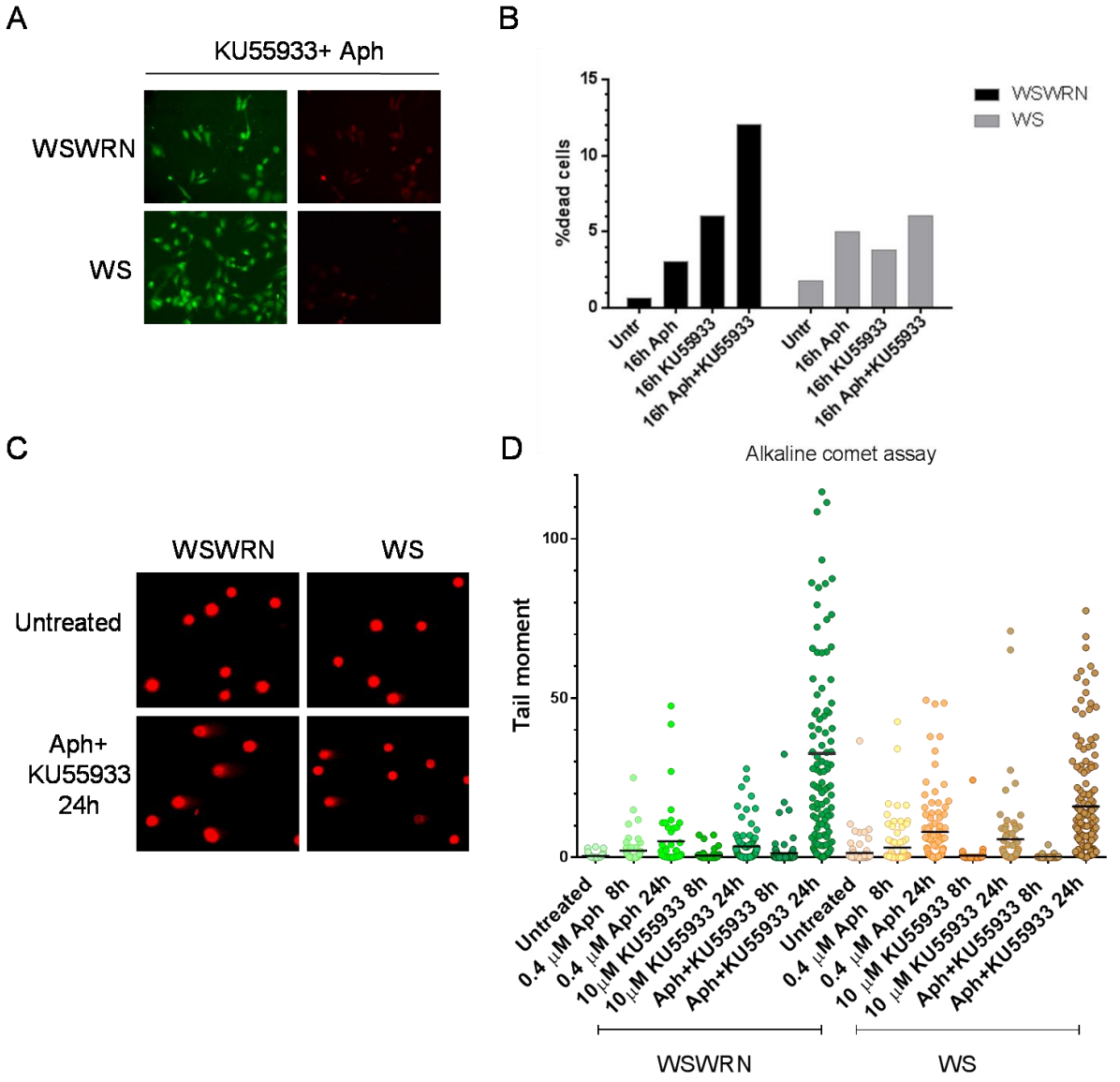
These findings suggest that WS cells likely triggers an ATM-dependent pathway, which remains active in response to mild replication stress, when the ATR-signaling pathway results compromised due to loss of WRN mediator function.

### *Analysis of the consequences of ATM pathway activation in WS cells*

It is well-documented that the checkpoint signaling functions of ATM and ATR are not redundant (Abraham, 2001), and given that we observed that loss of WRN likely elicits an ATM pathway, we investigated the functional consequences of its disruption for WS cells in response to mild replication stress. We first evaluated the effect of inhibition of ATM kinase activity on cell viability. To this purpose, WSWRN and WS cells were treated with Aph for 16h, with or without the addition of the chemical inhibitor KU55933, and subjected to the fluorescence-based LIVE/DEAD assay. As shown in Figure 4A and B, loss of WRN led per se to about two-fold increase in cell death in comparison to wild-type cells. After Aph-exposure, a comparable reduction of cell viability was observed in both WSWRN and WS cells (Figure 24B). Interestingly, inhibition of ATM kinase resulted in a reduction of cell viability in both cell lines, but, surprisingly, at a smaller extent in WRN-deficient cells upon mild replication stress (Figure 24B).

These findings indicate that inhibition of ATM pathway seems to have a little effect on cell survival in WRN-deficient cells following mild replication stress.

To further investigate the consequences of ATM inhibition on WRN-deficient cells, we measured the DNA damage in WRN-proficient or deficient cells by alkaline Comet assay. To do that, we treated WSWRN and WS cells with Aph 0,4μM for 8 or 24h, with or without the addition of the chemical inhibitor KU55933. We performed Comet assay and observed that treatment with low Aph dose led to a time-dependent enhancement of the tail moment both in wild-type and WS cells, with slightly higher values for WS cells (Figure 24C and D). Interestingly, combined adition of Aph and KU55933 reduced DNA damage accumulation in both cell lines at 8h of treatment, conversely ATM inhibition is more tolerated by the cells in the absence of WRN at 24h when the inhibition is performed throughout the whole exposure with low Aph dose (Figure 24D), and it is even advantageous when the inhibitor is added at the end of the treatment (Figure 24E).



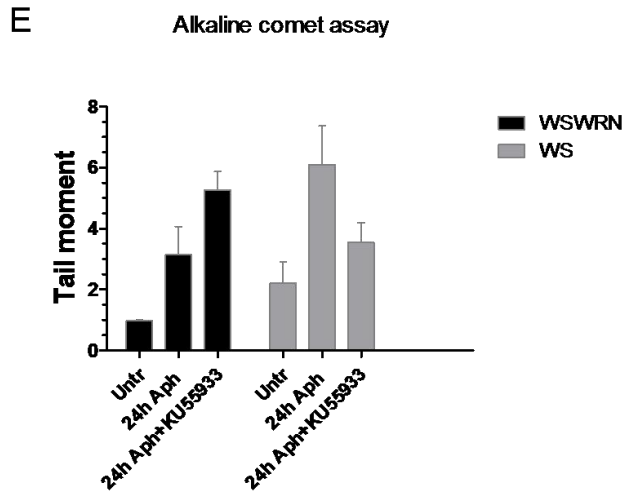




**Figure 24 Evaluation of cellular consequences of ATM inhibition.** (A and B) Evaluation of cell death in wild-type (WSWRN) and WS cells after 0,4μM Aph and/or 10μM KU55933 treatment. Aph was added for 16h and KU55933 1h prior Aph treatment. Cell viability was evaluated by the fluorescence-based LIVE/DEAD assay as described in "Materials and Methods". Data are expressed as percentage of dead cells and are mean values from three independent experiments. Error bars represent standard error. In the panel representative images from selected samples are shown. (C-DE) Analysis of DNA damage accumulation evaluated by alkaline Comet assay. WSWRN and WS cells were treated with 0,4μM Aph and/or 10μM KU55933 as indicated and KU55933 1h prior Aph treatment; (E) KU55933 was added for 3 hours at the end of the treatment .Graph shows data presented as mean tail moment. In the panel representative images from selected samples are shown. Median values are represented as horizontal black lines.

Therefore, although ATM inhibition does not result in a huge difference in cell viability examined with the short-term LIVE/DEAD assay between wild-type and WRN-deficient cells, it diminishes considerably DNA damage accumulation induced by Aph at earlier time of treatment in WRN-deficient cells.

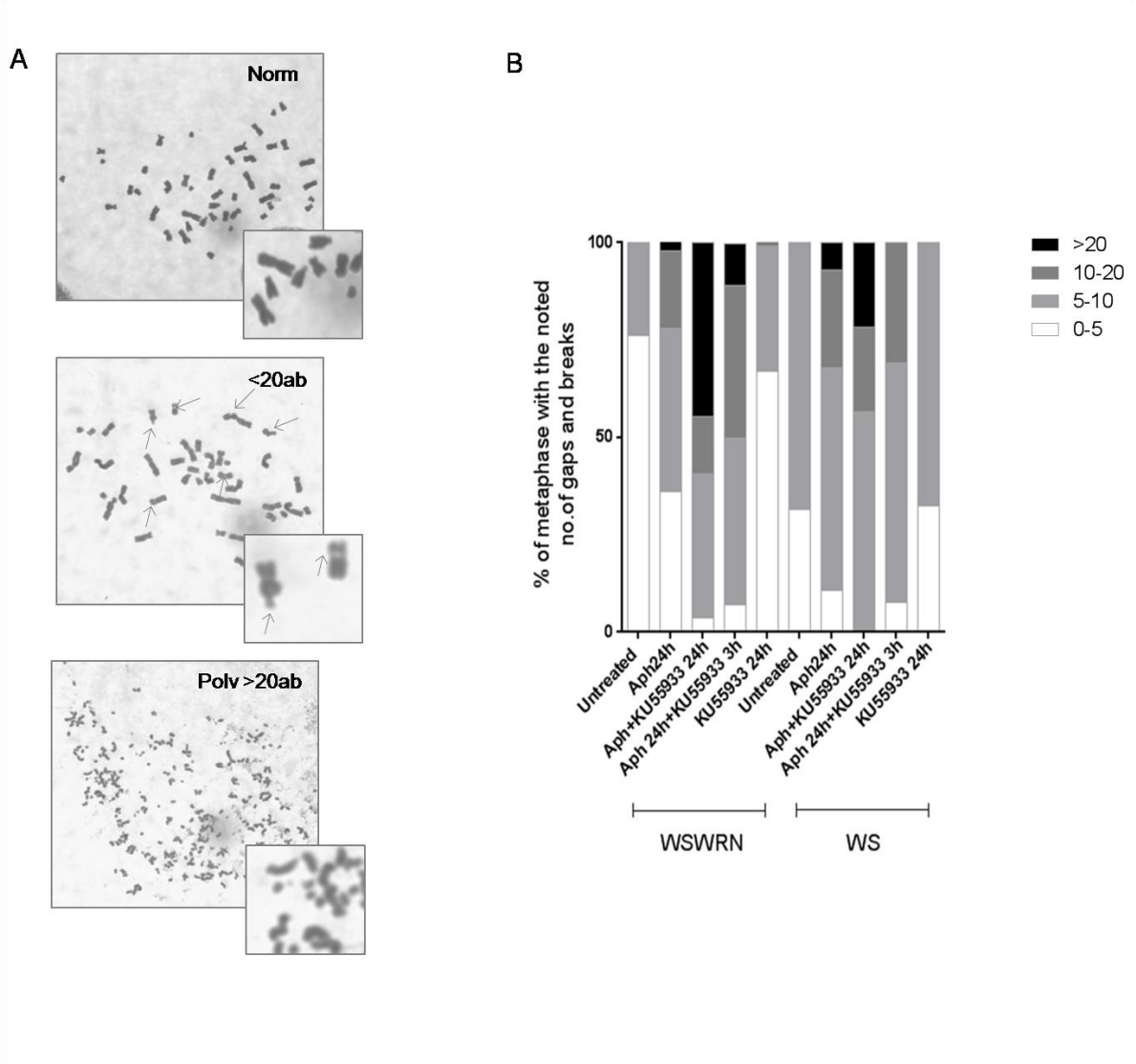



**Figure 25 Analysis of chromosomal damage induced by ATM inhibition in Aph-treated cells.** (A and B) Evaluation of chromosome fragmentation in WSWRN and WS cells treated or not with 0,4µM Aph for 24h, and in which 10µM of ATM inhibitor (KU55933) was added to the medium 3h before harvesting with colchicine. Metaphases were distributed according to the number of gaps and breaks per metaphase in cells. Graph shows the percentage of metaphases with different number of chromosome breakage. Representative Giemsa-stained metaphases of WS cells normal or differently damaged after treatment with Aph and KU55933. Insets show an enlarged portion of metaphases for a better evaluation of chromosome breakage.

It has been previously demonstrated that under conditions of mild replication stress induced by Aph, ATM plays a role in maintaining genome stability at CFS, which is evident only in the absence of ATR (Ozeri-Galai et al., 2012). Thus, we analyzed the frequency of chromosomal damage in WSWRN and WS cells treated with 0,4μM Aph in combination with the ATM inhibitor and, 24h later, metaphases chromosome were collected and scored for total gaps and breaks (Figure 25). Metaphases were divided into three groups, according to the level of chromosome fragmentation: metaphases harboring from 5 to 10 gaps and breaks (5-10), from 10 to 20 (10-20), or metaphases in which chromosomes were fragmented with more than 20 aberrations (>20). Under unperturbed conditions, as expected, WS cells showed higher chromosome fragmentation respect to the wild-type cells (Figure 25). Partial replication inhibition conferred by low dose of Aph, induced chromosomal damage in both cell lines, but, also in this case as expected, with levels of damage higher in WS cells (Figure 25). However, the addition of the ATM inhibitor at the end of the treatment with Aph led to increased DNA breakage in wild-type cells, but reduced levels of chromosomal aberrations in WS cells as compared to the Aph-treated samples (Figure 25).

Together, these results indicate that inhibition of ATM activity seems to reduce the levels of chromosomal instability in WS cells respect to the wild-type cells after mild replication stress.

## *Loss of WRN leads to accumulation of 53BP1 NBs in G1 phase and small foci in S phase upon mild replication stress*

It has been previously reported that mild replication stress, such as low-dose of Aph, induces the formation of 53BP1 nuclear bodies (NBs), which are largely confined to G1 or very early S phase (Lukas et al., 2011). These structures are produced by replication stress and originate from lesions generated at the G2/M transition, when a fraction of "under-replicated" genomic loci enters into mitosis and is converted to DNA/chromatin lesions (Lukas et al., 2011). Moreover, 53BP1 NBs are thought to protect CFS from attack by replication stress, and inhibition of ATM kinase affects their accumulation (Lukas et al., 2011). Thus, we investigated the 53BP1 NBs formation in WSWRN and WS cells, with or without the ATM kinase activity. First, cells were exposed to Aph 0,4μM for 24h, and then fixed and co-immunostained with antibodies against 53BP1 (green fluorescence) and Cyclin A (red fluorescence) to mark individual S phase cells. In wild-type cells,53BP1 NBs were correctly formed in G1 (Cyclin A-negative cells) after 24h Aph (Figure 26A and B). In WSWRN cells the average number of 53BP1 NBs increased from 2,3 to 8,25 after Aph, and

such treatment caused a marked shift towards nuclei with higher numbers of 53BP1 NBs restricted largely to G1 nuclei (Figure 26B). In WS cells, in untreated conditions, the average number of 53BP1 NBs per nucleus is almost two-fold than in control cells, that is about 4,4, and increased to 10,4 after exposure to Aph for 24h (Figure 26B).

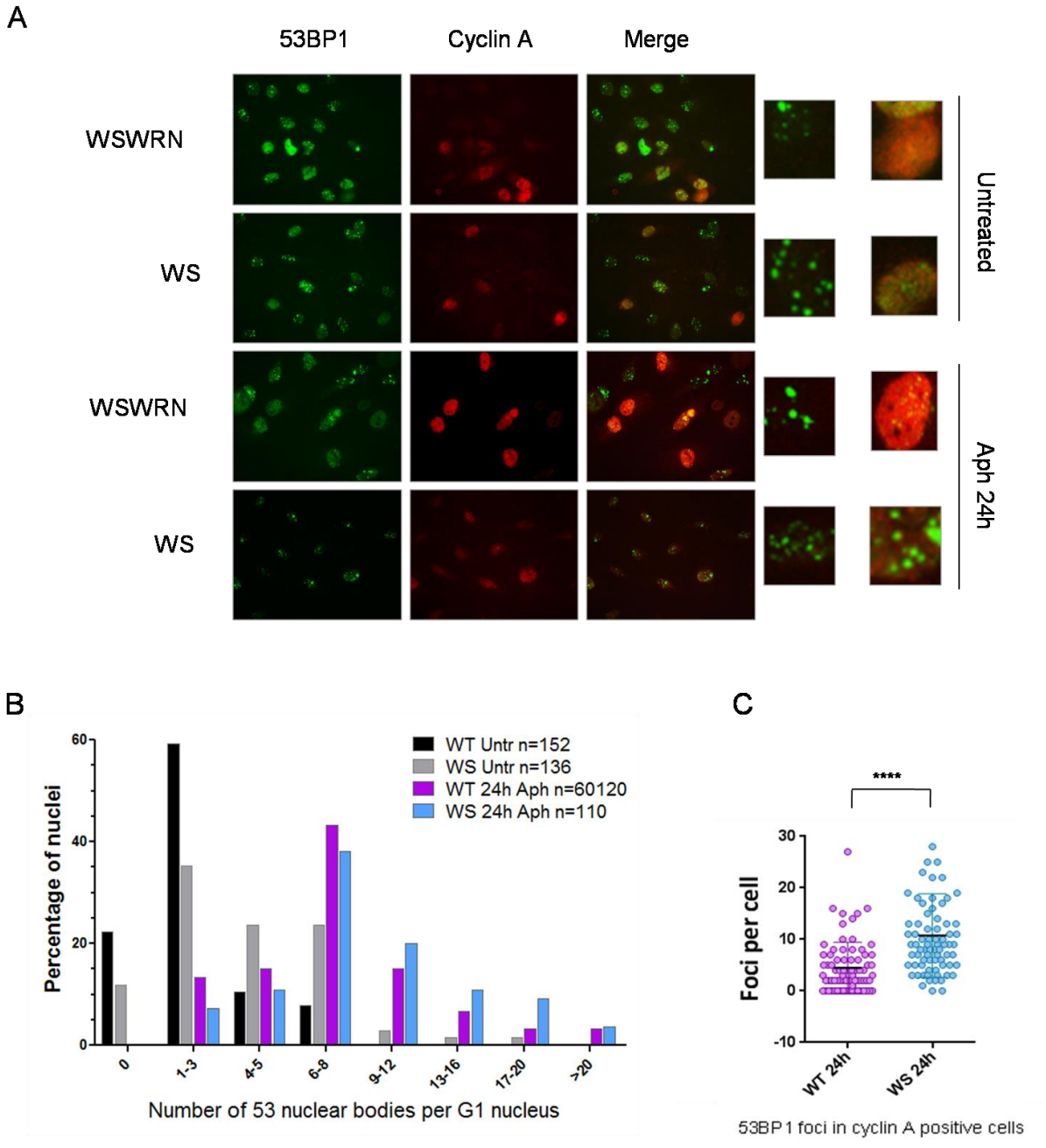



**Figure 26 WRN loss increases 53BP1 relocalisation in all cell cycle phases.** (A) Representative images showing WSWRN and WS cells co-immunostained with antibodies against 53BP1 (green fluorescence) and Cyclin A (red fluorescence) are reported. (B) WSWRN and WS cells were treated or not with Aph 0,4μM for 24h and co-immunostained with antibodies against 53BP1 or Cyclin A. The indicated number of non-overlapping images were acquired and analyzed for the presence of 53BP1 foci in G1 nuclei (Cyclin A-negative cells). (C) Graph shows distribution per cell of the 53BP1 foci in Cyclin A-positive cells in WSWRN and WS cells treated or not with 24h Aph and subjected to dual immunofluorescence with 53BP1 and Cyclin A. Median values are represented as horizontal black lines. Error bars represent standard error.

Notably, small diffuse 53BP1 foci were observed in Cyclin A-positive cells, especially in WS cells, where the 53BP1-positive foci per cell increased from an average number of 3 under untreated conditions to 10,75 after Aph treatment (Figure 26C).

These results indicate that WRN deficiency *per se* promotes 53BP1 NBs accumulation in Cyclin A-negative cells, and a further enhancement was observed after exposure to low dose of Aph. Given that these structures correlate with ATR depletion and are thought to be the end-result of chromosomal stress, occurring during the previous S phase in response to mild replication stress, and taking into account that in WS cells the ATR pathway is compromised, these 53BP1 NBs could represent, also in our case, a system used for protection of DNA lesions formed at CFS.

Furthermore, loss of WRN leads to the formation of small diffuse 53BP1 foci during S phase upon mild replication stress. Since the increase of 53BP1 foci in all cell cycle phases was previously associated with CHK1 depletion (Lukas et al., 2011), we can speculate that the phenotype observed in WS cells could be due to defective CHK1 activation after 8h and 24h of Aph (see Results part 1).

Since it has been reported that ATM kinase is required for efficient accumulation of 53BP1 NBs (Lukas et al., 2011), we asked whether also 53BP1-positive foci formation in S phase are dependent on ATM activity. To test this, WSWRN and WS cells were exposed to Aph 0,4μM for 24h, with or without the addition of ATM inhibitor (KU55933), then fixed and co-immunostained with antibodies against 53BP1 (green fluorescence) and Cyclin A (red fluorescence). First, we confirmed that in our cell lines, ATM inhibition was able to reduce accumulation of 53BP1 NBs (Figure 27A). Then, we examined the relocalisation of 53BP1 into small diffuse foci in S phase (Cyclin A-positive cells), and we found that relocalisation of 53BP1 foci into S phase did not require a functional ATM kinase (Figure 27B).

Next, to verify whether, in WS cells, 53BP1-positive foci in S phase accumulate at CFS after treatment with the low dose of Aph, for 24h, we used a combined immunofluorescence and FISH approach (IF-FISH) to co-localise 53BP1 with CFS. We found that, in WS cells, FISH probes to FRA3B, the most expressed CFS, co-localising with 53BP1 foci in S phase induced by Aph at low frequency (7%) (Figure 27A). These findings suggest that, being fragile sites DNA regions particularly sensitive to replicative stress, 53BP1-S-foci are probably correlated with replication stress response, but not specifically

with common fragile sites processing, actually WRN-deficient cells have enhanced instability at common fragile sites after treatment with Aph (Pirzio et al., 2008).

Altogether, these data allow us to hypothesize that, thus, being fragile sites DNA regions particularly sensitive to replicative stress, 53BP1-S-foci are probably correlated with replication stress response, but not specifically at common fragile sites, actually WRN-deficient cells have enhanced instability at common fragile sites after treatment with Aph (Pirzio et al., 2008).

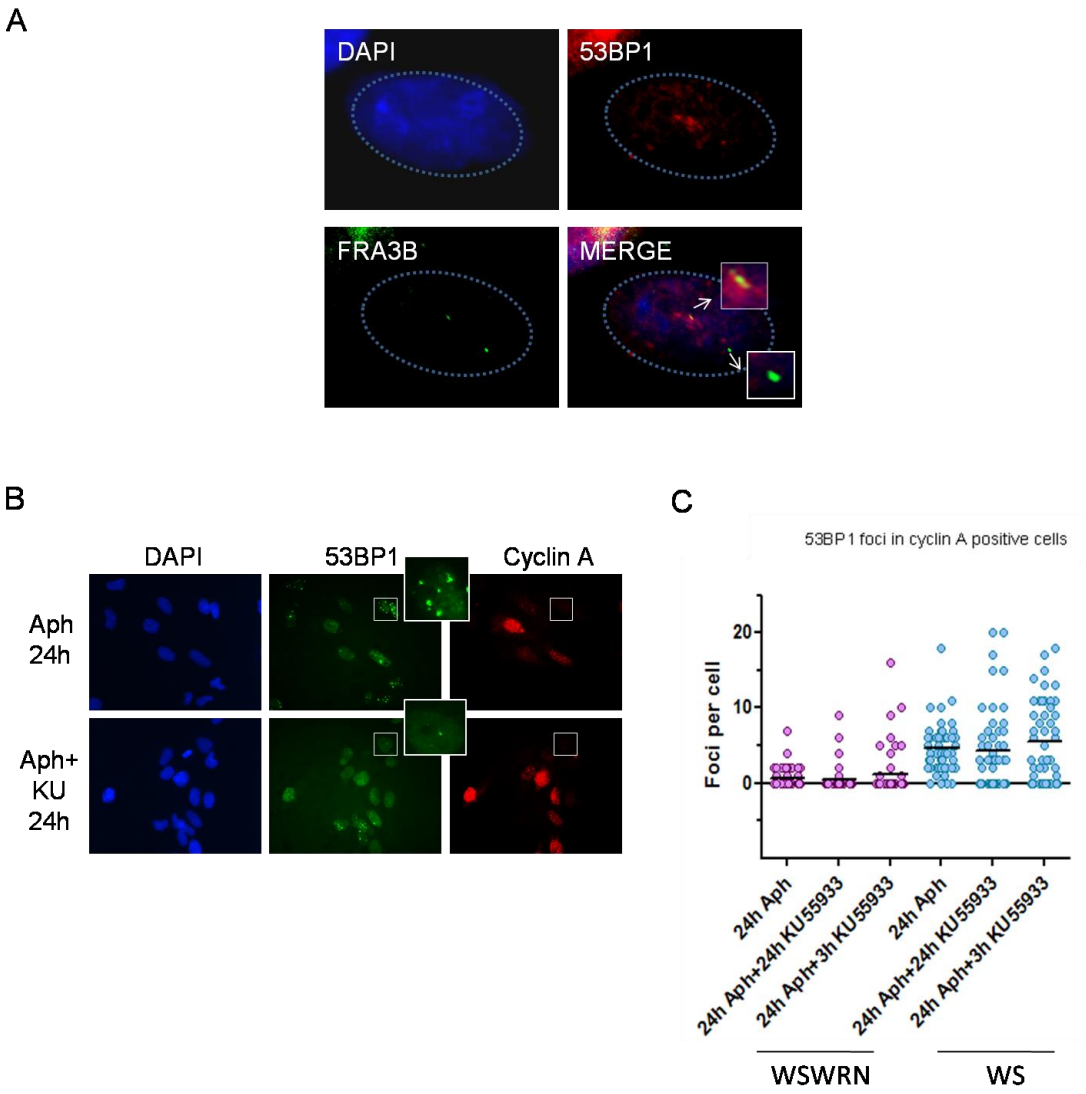



**Figure 27 53BP1 foci formation in Cyclin A-positive cells is not dependent on the ATM activity.** (A) Combined FISH with chromosome-specific probes (green fluorescence) and immunofluorescence staining for53BP1 (red fluorescence) in WS cells after treatment with 0,4μM Aph for 24 h. A mix of BACs (94D19, 149J4 and 48E21) mapping to FRA3B fragile site region was used as probe for FISH analysis.(B) Efficient formation of 53BP1 NBs requires ATM activity. Exponentially growing WSWRN cells were treated or not with 10μM of ATM inhibitor (KU55933) for 24h during 0,4μM Aph treatment and co-immunostained with antibodies to 53BP1 (green fluorescence) and Cyclin A (red fluorescence). In the panel representative images are reported. (B) Graph shows distribution of small diffuse 53BP1 foci in Cyclin A-positive cells using WSWRN and WS cells treated with 0,4μM Aph for 24h, with or without the addition of 10μM of KU55933. Median values are represented as horizontal black lines.

## *ATM inhibition in WRN-deficient cells recovers CHK1 defective activation in response to mild replication stress*

In this study, we reveal that loss of WRN elicits an ATM-dependent pathway in response to mild replication stress, when the ATR-signaling pathway is compromised (see Results part 1). Moreover, it has been previously demonstrated an interplay between ATM and ATR in the regulation of CFS stability (Ozeri-Galai et al., 2008). In particular, it is thought that both ATR and ATM phosphorylate CHK1 under partial replication conferred by Aph (Ozeri-Galai et al., 2008). In the light of these findings, we examined the effect of ATM inhibition on CHK1 activation in WS cells.

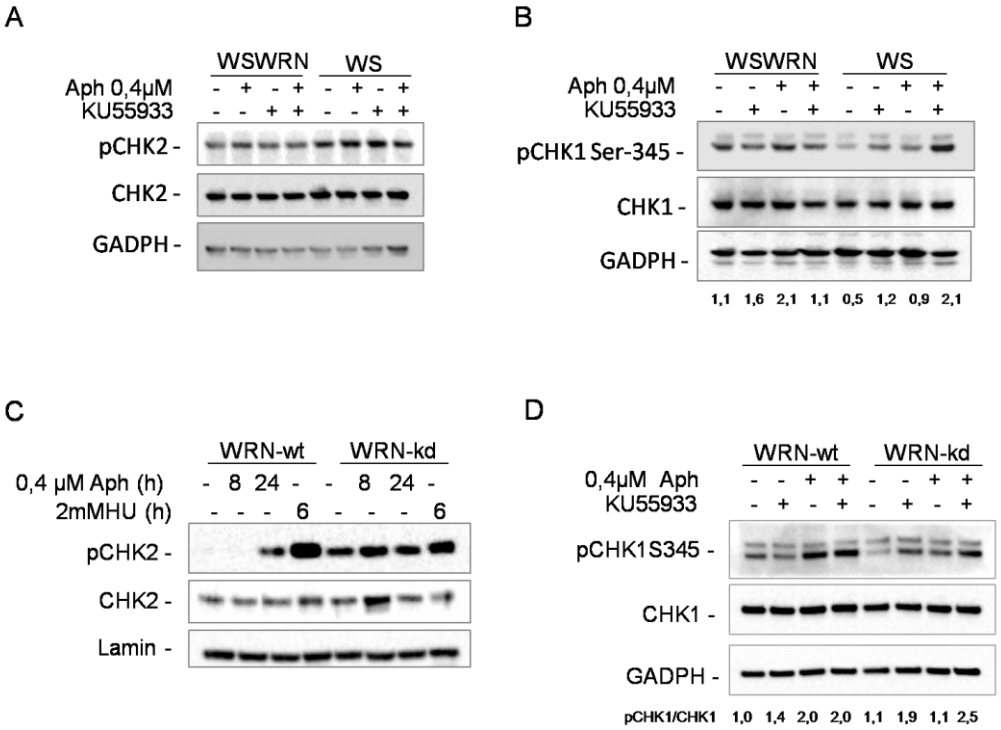



**Figure 28Evaluation of CHK2 and CHK1 activation upon mild replication stress**. (A) Western blot detection of CHK2 in total extracts ofWSWRN and WS cells untreated or treated with 0,4μMAph for the indicated time. Where indicated, cells were treated with 10μMKU55933 added to media 1h prior Aph treatment. The presence of activated, i.e. phosphorylated, CHK2 was assessed using Thr68 phospho-specific antibody (pCHK2). Total amount of CHK2 was evaluated with an anti-CHK2 antibody. Equal loading in WB was confirmed probing with an anti-GAPDH. (B) WRN-wt (wild-type) and WRN-kd (WRN-deficient) cells were treated as in A. Equal loading in WB was confirmed probing with an anti-Lamin B1. (C and D) Western blot detection of CHK1 in WSWRN and WS cells or WRN-wt and WRN-kd cells were treated as in A. The presence of activated, i.e. phosphorylated, CHK2 was assessed using Ser345 phospho-specific antibody (pS345CHK1). Total amount of CHK1 was evaluated with an anti-CHK1 antibody. Equal loading in WB was confirmed probing with an anti-GAPDH. The ratio of phosphorylated protein to total protein normalised to the untreated wild-type is reported below each lane.

First, we investigated whether, under our conditions of mild replication stress, CHK2, a target of ATM kinase, was phosphorylated as a symptom of ATM pathway activation in WS cells.

To carry out our analysis, WSWRN and WS cells were exposed to low dose of Aph for 8h, and phosphorylation status of CHK2 was assessed in cell lysates by Western blot using Thr68 phospho-specific antibody (Figure 28A). Moreover, some parallel samples were treated with ATM inhibitor (KU55933) to verify the dependency of CHK2 phosphorylation on ATM kinase upon Aph (Figure 28A).

In wild-type (WSWRN) cells, a very faint band corresponding to CHK2 phosphorylation was detectable after 8h of Aph, but it was inhibited by KU55933. In contrast, in WS cells CHK2 showed elevated levels of phosphorylation even under untreated conditions. The phospho-CHK2 proportion was sickly intensified by Aph treatment, but it was highly reduced by combined treatment of Aph with ATM inhibitor (Figure 8A).

By using HEK293T cells stably expressing scrambled shRNA (WRN-wt), we observed a CHK2 activation after 24h Aph in wild-type cells (Figure 28C). On the contrary, WRN-deficient (WRN-kd) cells showed increased levels of CHK2 phosphorylation under untreated conditions, and also already at 8h of Aph-exposure (Figure 28C).

For what concerns CHK1 activation, as expected, treatment with Aph for 8h induced CHK1 phosphorylation in WSWRN, which remained unchanged upon ATM inhibition (Figure 28B). Interestingly, in WS cells, although, as expected, CHK1 was not phosphorylated after mild replication stress, however, the addition of KU55933 to the culture medium during Aph treatment recovered from faulty checkpoint activation, leading to CHK1 phosphorylation at levels comparable, if not major, to those of wild-type cells (Figure 28B). Similar results were obtained using HEK293T cells (Figure 28D).

Therefore, our data demonstrate that chemical inhibition of ATM kinase activity confers to WRN-deficient cells the ability to recover CHK1 defective phosphorylation following mild replication stress.

## *Analysis of the consequences of ATM inhibition in WS cells stably expressing the unphosphorylable mutant form of WRN*

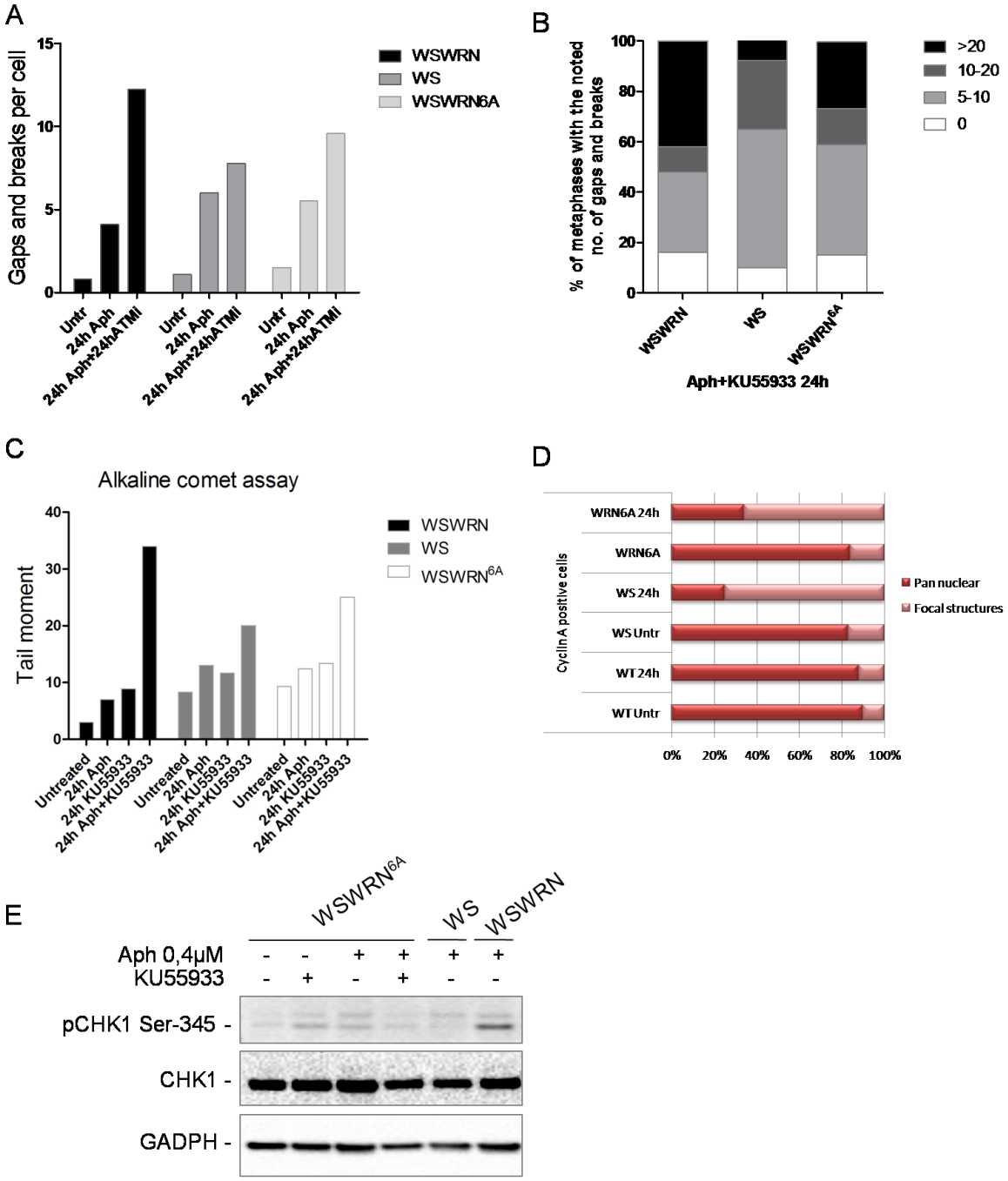



**Figure 29 ATM inhibition consequences in WS cells stably expressing the mutant form of WRN unphosphorylable by ATR** (A) Aberrations per cell in WSWRN, WS and WS cells stably expressing the mutant form of WRN unphosphorylable by ATR (WSWRN6A) (B)Distribution of metaphases according to the number of gaps and constrictions per metaphase in cells following 24 h treatment with 0.4 M aphidicolin and ATM inhibition with KU55933. (B) DNA damage accumulation evalueted by Alkaline Comet assay WSWRN6A.

To further analyse the consequences of ATM inhibition in WS cells we studied the response to ATM inhibition in WS cells stably expressing the unphosphorylable mutant form of WRN (WSWRN$^{6A}$). DNA damage was measured in WSWRN$^{6A}$ cells, compared to WS and WSWRN, by alkaline Comet assay. To do that, WSWRN, WS and WSWRN6A cells were treated with Aph 0,4μM for 24h, with or without the addition of the chemical inhibitor KU55933. Combined addition of Aph and KU55933 is more tolerated in WSWRN6A cells respect to wild type cells, but the damage observer is higher respect to WS cells. ATM inhibition performed throughout the whole exposure with low Aph doses in WSWRN$^{6A}$ leads also to a chromosomal fragmentation in a similar manner than in WSWRN cells, but with minor percentage of metaphases harboring more than 20 aberrations (>20) (Figure 29A-B). Data show that ATM inhibition during mild replication stress increased DNA damage, also at a chromosomal levels, in WSWRN6A at a middle level between WRN-proficient cells and WRN-deficient cells (Figure 29C).

To go deeper inside the causes of this intermediate phenotype, 53BP1 foci in S-phase and CHK1 phosphorylation were evaluated. First, WSWRN$^{6A}$ cells were exposed to Aph 0,4μM for 24h, and then fixed and co-immunostained with antibodies against 53BP1 (green fluorescence) and Cyclin A (red fluorescence) to mark individual S phase cells. By using WS cells stably expressing the unphosphorylable mutant form of WRN, 53BP1 foci in Cyclin A-positive cells are observed, with a similar percentage of cyclin A positive cells with detectable 53BP1 focal structures respect to WS cells. These foci could be a sign of the activation of alternative pathways at later time point, as observed in the absence of WRN, which could partially explain the intermediate phenotype, between that of wild-type cells and that of WS cells, shown by WSWRN6Acells in the comet and metaphase analysis when ATM is inhibited. The analysis of CHK1 phosphorylation point out that after treatment with Aph for 8h in WSWRN6A cells was not phosphorylated after mild replication stress, and the addition of KU55933 to the culture medium does not change CHK1 phosphorylation levels. WSWRN and WS cell treated with Aph for 8 h were used respectly as positive and negative control. In conclusion, data demonstrate that the activation of hypothetic alternative pathways in WSWRN$^{6A}$ cells remain probably incomplete, because, at earlier time, the phosphorylation of CHK1 upon mild replication stress does not change when ATM is inhibited (Figure 29E). The inability of WSWRN6A cells to rescue CHK1 activation could be due to the presence of WRN that, although mutated, could prevent the activation of an alternative pathways. Further

experiments will be needed to explore this issue. Our results show that WRN is tightly regulated by both ATR and ATM to ensure genome stability.

# DISCUSSION

## DISCUSSION

We previously determined that WRN in association with the ATRpathway prevents CFS breakage, but with largely unknown mechanisms(Pirzio et al., 2008). Here, we provide evidence for a crucial role of the WRN protein in mediating CHK1 phosphorylation specifically following low level of replicative stress, such as that causing CFS expression.

Loss of CHK1 has been related to CFS expression, and CHK1 undergoes phosphorylation upon mild replication perturbation(Durkin et al., 2006). Although CHK1 has been widely found activated in response to low-doses of Aph (14,26,27), a recent work suggested that CHK1-dependent protection of CFS might be unrelated to its phosphorylation-dependent activation (Koundrioukoff et al., 2013). Consistently with earlier reports (Durkin et al., 2006; Petermann and Helleday, 2010; Zachos et al., 2005), we detect CHK1 phosphorylation after Aph treatment, albeit at reduced levels if compared to strong replication perturbation as that induced by HU. Since CHK1 phosphorylation was demonstrated from independent studies using different cell lines, it is unlikely that the genetic background of cells could influence this specific phenotype. However, the percentage of S-phase cells might markedly affect the levels of CHK1 phosphorylation, especially in the presence of partial inhibition of DNA synthesis, perhaps accounting for these discrepancies between the data. Noteworthy, in the study where CHK1 phosphorylation has not been detected after low Aph doses, chromatin loading of ATR and its mediators, RAD9 and TopBP1, has been demonstrated (Koundrioukoff et al., 2013). Our findings confirm that mild replication stress determines chromatin accumulation of RAD9 and TopBP1, similarly to robust replication perturbation induced genome-wide.

Interestingly, RAD9 and TopBP1 recruitment to chromatin involves the presence of WRN under unperturbed conditions as well as upon mild replication stress, suggesting that the reported association of WRN with 9.1.1, whose loss increases CFS expression (Pichierri et al., 2012), is important for CHK1 activation. Therefore, our results indicate that the presence of the WRN protein may be required for CHK1 activation, suggesting that WRN acts as a checkpoint mediator protein, specifically when replication is partially inhibited. A similar function of a RecQ helicase has been demonstrated in yeast, where Sgs1 plays a non-catalytic role in correct activation of Rad53 after replication stress (Liu et al., 2000), bridging RPA and Rad53 together (Ozeri-Galai et al., 2008). Consistent with a possible role as mediator protein, WRN is phosphorylated at S/TQ sites by ATR also after Aph-induced replication stress, and this modification of WRN appears to be crucial for promoting efficient CHK1 phosphorylation upon mild replication perturbation, as well as for stabilisation of CFS.

In WS cells expressing the ATR-unphosphorylable mutant form of WRN we detect a decrease of RAD9 and TopBP1 levels respect to the other cell lines especially at the later time. This is not surprisingly since the phospho-WRN mutant is a double mutant that cannot be targeted by ATR and ATM (Pirzio et al., 2008), and we cannot exclude that loading on chromatin of mediators at later times could rely on the ATM-dependent phosphorylation of WRN. Altogether, it is plausible that, upon moderate replication perturbation, WRN may act as a mediator of CHK1 activation, and this function can be regulated by the ATR-dependent phosphorylation. Conversely, WRN helicase mutant does not affect CHK1 activation upon mild replication stress and, consistently, accumulates ATR-mediators properly.

In the absence of ATR or CHK1fork destabilization can occur (Bjergbaek et al., 2005; Koundrioukoff et al., 2013; Maya-Mendoza et al., 2007). The inability of WRN-deficient cells or cells expressing the ATR-unphosphorylable form of WRN to properly activate CHK1 could similarly lead to fork undermining. Indeed, loss of WRN or expression of the ATR-unphosphorylable mutant form affects replication fork progression under unperturbed cell growth, consistently with previous studies (Lopes et al., 2001; Tercero and Diffley, 2001). Also expression of the helicase-dead form of WRN strongly reduces fork speed in untreated cells, suggesting that reduced elongation rates and chromosome instability are connected in cells with absent or dysfunctional WRN protein. In the same cells, mild replication stress further delays fork speed, but in a lesser extent than in control cells. Interestingly, WRN-deficient cells and cells expressing the helicase-dead or unphosphorylable WRN display an apparently normal ability to recover DNA replication after mild replication perturbation, however, their apparently-normal elongation rates are strikingly reduced upon origin firing inhibition by roscovitine. This phenotype possibly indicates that loss of WRN function results in a severe elongation defect or fork stalling, triggering additional local origin firing to complete replication. Consistently, WS cells show more ssDNA formation under unperturbed replication or after Aph. Such increased generation of ssDNA could derive from persistent fork stalling and/or fromregions left unreplicated behind such forks or from additional origin firing. Moreover, the presence of more origin firing in WRN-deficient cells and cells expressing the helicase-dead or the ATR-unphosphorylable WRN mutant is not a mere consequence of deregulated CHK1 activation, which has been reported to induce unscheduled origin firing (Pichierri et al., 2012), as it is even more evident in cells expressing the helicase-dead WRN mutant, which shows wild-type CHK1 phosphorylation. Similarly, since cells expressing the helicase-dead form of WRN are able to support CHK1 activation but also show replication defects, failing to recover forks from Aph-induced replication perturbation, it is

plausible that this reduction of fork speed is linked to a cooperation of CHK1 and WRN. Cells lacking CHK1 reduce fork elongation by half respect to the control under unperturbed conditions (Feijoo et al., 2001; Rodríguez-López et al., 2002), exactly the same extent of reduction associated to loss of WRN function. Expression of a phospho-mimic CHK1 mutant in WS cells or cells expressing the ATR-unphosphorylable form of WRN recovers cells from sensitivity to Aph and restores fork elongation to wild-type level, suggesting that experimentally-induced reactivation of CHK1, bypassing the WRN mediator function, is sufficient to recover perturbed forks and chromosome instability. Thus, stabilisation of perturbed forks by CHK1 can probably overcome loss of WRN, most likely through hyperactivation of compensatory pathways. This result is consistent with our earlier data showing that loss of WRN determines ssDNA gaps accumulation and increased number of RAD51 foci after Aph treatment (Sidorova et al., 2013). Indeed, RAD51 is involved in the maintenance of CFS stability (Petermann and Caldecott, 2006) and in the repair of gaps left behind the stalled fork, and most importantly is activated by CHK1 (Murfuni et al., 2012; Schwartz et al., 2005). One possibility to explain the effect of enhanced recovery in WS cells expressing a constitutively-active CHK1 may be related to a more efficient RAD51-dependent post-replication gap repair to stabilise unreplicated regions. On the other hand, in cells expressing the ATR-unphosphorylable WRN the introduction of the constitutively-active CHK1 might be sufficient to prevent fork destabilisation, thus allowing fork recovery through the helicase activity of WRN, which is likely fully functional in these cells.

Altogether, our data allow to propose a model, which explains how WRN can function to guarantee CFS stability, through a checkpoint-dependent and independent way. The ATR-dependent WRN phosphorylation is required to stimulate CHK1 activation that is instrumental for stabilization of stalled forks, whereas, the helicase activity of WRN might be necessary to support replication restart, possibly by the resolution of DNA secondary structures, to promote CFS stability. When WRN protein is absent or cannot be phosphorylated by ATR, replication checkpoint is not elicited and forks are destabilised, consequently increased CFS expression can be observed (Figure 1). Similarly, the inability to rescue stalled forks, due to loss of WRN helicase activity, results in enhanced CFS instability (Sakamoto et al., 2001).

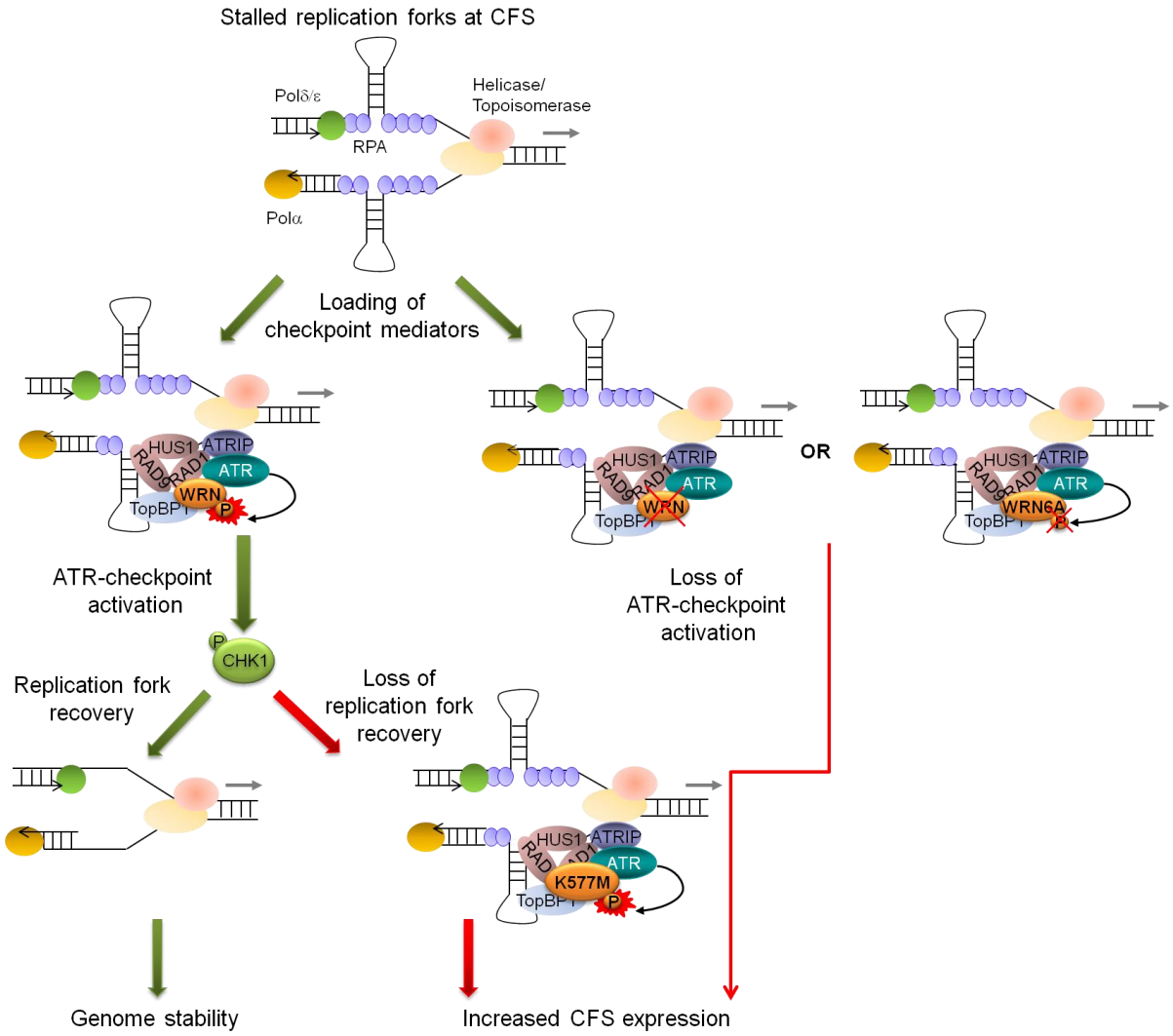



**Figure 30Model of action(s) of WRN upon replication fork stalling at CFS to prevent chromosome breakage.** The ATR-dependent WRN phosphorylation is required to stimulate CHK1 activation that is instrumental for stabilization of stalled forks, whereas, the helicase activity of WRN might be necessary to support replication restart, possibly by the resolution of DNA secondary structures, to promote CFS stability. When WRN protein is absent or cannot be phosphorylated by ATR, replication checkpoint is not elicited and forks are destabilised, consequently increased CFS expression can be observed (Figure 1). Similarly, the inability to rescue stalled forks, due to loss of WRN helicase activity, results in enhanced CFS instability

Therefore, findings of the first part of our study suggest a novel role of WRN as checkpoint mediator in response to moderate replication stress.

In the second part of the study, we explored other cellular consequences of WRN loss in response to mild replication stress. We provide evidence that the absence of WRN leads to an ATM pathway activation, which is harmful to the cells, as confirmed by positive effects obtained by ATM inhibition on cellular survival and chromosomal damage during the last part of the treatment with Aph.

We discovered that, besides reducing the activation of CHK1, loss of WRN also greatly hampers the phosphorylation of histone H2AX, which is the earlier target of ATR kinase activity following replication stress. In fact, although, upon mild replicative stress, H2AX is phosphorylated by ATR in a time-dependent manner in wild-type cells, however, its activation is reduced in WS cells. Interestingly, WS cells showed high levels of histone H2AX phosphorylation (γ-H2AX) in untreated conditions. Phosphorylation of H2AX is a common marker used to detect replication stress (Zeman and Cimprich, 2014). Consistent with high levels of spontaneous DNA damage in WRN-deficient cells, we observed also a high degree of Ser33-phosphorylated RPA32, which represents a direct read-out of replication stress, as well as of RPA-coated ssDNA production (see Results part 1), (Maréchal and Zou, 2013; Nam and Cortez, 2013; Zeman and Cimprich, 2014).

The higher percentage of γ-H2AX positive cells detected in WS cells under untreated conditions may reflect the activation of the ATM pathway due to WRN loss. It has been previously reported that, in response to replication stress, H2AX is phosphorylated by the ATM kinase when ATR action is defective (Chanoux et al., 2009). Notably, in untreated conditions, WS cells showed even elevated levels of CHK2 phosphorylation. Thus, it is likely that WS cells display an endogenously activated γ-H2AX-ATM-CHK2 checkpoint response similarly to that of Bloom syndrome-deficient cells (Rao et al., 2007), and that lack of WRN engages the cells in a similar situation to precancerous tissues with replication stress. Aphidicolin treatment could exacerbate this endogenous replication stress. Even if in WS cells, where the ATR signalling in response to mild replication stress is defective, the γ-H2AX-ATM-CHK2 pathway activation could represent a positive event, however, it results detrimental for the cells. In fact, ATM inhibition is more tolerated by the cells in the absence of WRN when the inhibition is performed throughout the whole exposure with low Aph dose, and it is even advantageous when the inhibitor is added at the end of the treatment. The fact that prolonged ATM inhibition in combination with Aph leads to increased DNA damage than treatment alone, could be due to lack of some function that ATM carries out in the cells

to protect genome stability upon mild replication stress. It has been previously reported that mild replication stress, such as low-dose of Aph, induces the formation of 53BP1 nuclear bodies (NBs), which are largely confined to G1 or very early S phase (Lukas et al., 2011). These structures are produced by replication stress and originate from lesions generated at the G2/M transition, when a fraction of "under-replicated" genomic loci enters into mitosis and is converted to DNA/chromatin lesions (Lukas et al., 2011). Moreover, 53BP1 NBs are thought to require ATM kinase for their accumulation and represent nuclear compartments in which DNA lesions are protected from attack by replication stress (Lukas et al., 2011). Thus, it is plausible that inhibition of ATM function could impede 53BP1 NBs formation, leading to excessive DNA/chromatin lesions during G, particularly when the inhibition is prolonged. As these compartments are considered structures deputed for the protection of CFS from degradation, and given that WRN deficiency results in CFS instability (Lukas et al., 2011), it is possible that 53BP1 NBs is a symptom of problems arising during previous S phase likely along CFS regions.

Interestingly, WRN deficiency leads also to the formation of 53BP1 foci during S phase, which are independent from ATM. These 53BP1 foci in Cyclin A-positive cells could have a role in protecting DNA lesions caused by mild replication stress when ATM is inhibited, and could be the cause of the beneficial effect of ATM inhibition during the final part of the treatment with Aph. Activation of such pathway in S phase could compensate in WS cells in which ATM is inhibited for the loss of 53BP1 NBs formation in the subsequent G1 phase. Noteworthy, the increase of 53BP1 foci in all cell cycle phases was previously associated with CHK1 depletion (Lukas et al., 2011), and it is consistent with to defective CHK1 activation observed in WRN-deficient cells following Aph exposure. We can speculate that, in S phase, 53BP1 recruitment in foci could be instrumental in attracting other proteins implicated in the response to mild replication stress. For example, 53BP1 regulates the accumulation of BLM at sites of DNA damage during replication stress (Sengupta et al., 2004). This pathway could be correctly activated in response to mild replication stress in WS cells, but it is not able to efficiently protect CFS, and more in general to guarantee genome stability. However, it could play a protective role, in WS cells, when ATM function is inhibited, as demonstrated by the fact that ATM inhibition is more tolerated by the cells in the absence of WRN when the inhibition is performed throughout the whole exposure with low Aph dose, and that it is even advantageous when the inhibitor is added at the end of the treatment.

Another way by which ATM inhibition could protect genome stability in WS cells is the recovery of CHK1 defective activation in response to mild replication stress. In the absence of ATM, ATR could be recruited to chromatin as reported following replication arrest by HU (Cuadrado et al., 2006). Increased loading of ATR to chromatin could overcome the WRN mediator function to activate the ATR-mediated checkpoint. Alternatively, in absence of ATM, Aph treatment could produce degeneration of stalled forks into breakage, so that the ATR pathway is activated bypassing the mediator function of WRN protein. Hence, phosphorylation of CHK1 could stabilize perturbed forks through the activation of alternative repair pathways at early time after induced-replication stress, which could cooperate with 53BP1-mediated pathway at later time for DNA damage repair or prevention. In the same way that the expression of a phospho-mimic CHK1 mutant does, the forced activation of CHK1, by ATM inhibition, could lead to the engagement of the RAD51-dependent post-replication gap repair, which could act in stabilizing unreplicated regions.

Our results show that WRN is tightly regulated by both ATR and ATM to ensure genome stability. By using WS cells stably expressing the mutant form of WRN unphosphorylable by ATR after Aph ($WSWRN^{6A}$), 53BP1 foci in Cyclin A-positive cells are observed, as seen in WS cells. These foci could be a sign of the activation of alternative pathways at later times, as observed in the absence of WRN, which could explain the intermediate phenotype, between that of wild-type cells and that of WS cells ,in which ATM is inhibited, shown by $WSWRN^{6A}$cells. The activation of alternative pathways in $WSWRN^{6A}$cells remains probably incomplete, because, at earlier time, the phosphorylation of CHK1 upon mild replication stress does not change when ATM is inhibited. The inability of $WSWRN^{6A}$cells to rescue CHK1 activation could be due to the presence of WRN that,although mutated,could prevent the activation of an alternative pathway. Further experiments will be needed to explore this issue.

Therefore, all our findings together suggest that WRN is a mediator of ATR-checkpoint activation in response to mild replication stress, and this function could be regulated by the ATR-dependent phosphorylation, and give strong mechanistic support to the notion that defective fork repair/recovery undermines integrity of chromosome sat CFS. Since both WRN helicase activity and WRN phosphorylation by ATR are required for guarantee CFS stability, it is possible that the WRN helicase activity is required to support replication restart, whereas WRN phosphorylation by ATR is required for the activation of

ATR-mediated checkpoint, and so indirectly for stabilization of stalled forks. Moreover, our findings suggest that loss of WRN leads to ATM-pathway activation, and ATM inhibition in WS cells recovers CHK1 defective activation.

Collectively, our results may contribute to shed light into the origin of chromosome instability in WS and more in general to clarify how genome instability accumulates in pre-neoplastic lesions, thus promoting cancer development.

Moreover, this study unveils a complicated network in which several proteins work tightly linked together. Loss of one protein means altering this network and changing the interaction among proteins. Playing a role in the game against diseases resulting from alterations of this network requires a deeply understanding of all delicate interactions between the different actors acting in the network.

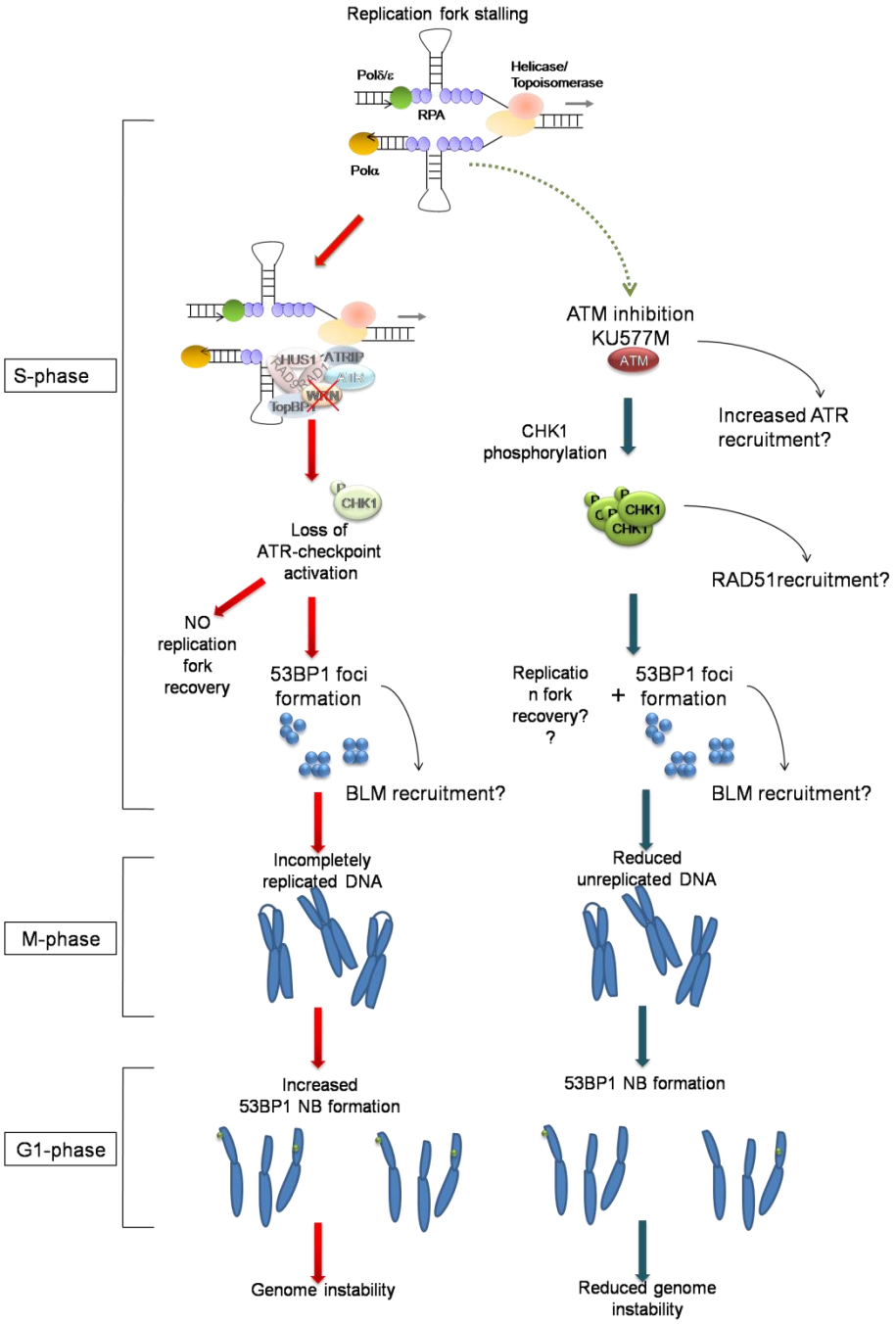



**Figure 31 Hypothesis of consequences of mild replication stressduring the cell cycle in WS cells with and without ATM inhibition.** Replication stress induced by low doses of Aph results in the generation of single strand DNA bound by RPA protein. ATM inhibition stimulates, in different ways, CHK1 activation. In the absence of ATM, ATR could be much more recruited to chromatin. Increased loading of ATR to chromatin could overcome the WRN mediator function to activate the ATR-mediated checkpoint. Alternatively, in absence of ATM, Aph treatment could produce degeneration of stalled forks into breakage, so that the ATR pathway is activated bypassing the mediatorfunction of WRN protein. Hence, phosphorylation of CHK1 could stabilize perturbed forks.Moreover, WRN deficiency leads to the formation of 53BP1 foci during S phase, which are independent from ATM.53BP1 recruitment in S-phase foci could be instrumental in attracting other proteins implicated in the response to mildreplication stress, for example BLM. During mitosis genomic loci, that remain 'under-replicated', are converted into condensation-induced DNA rupture. Throughout G1, these lesions are sequestered in nuclear compartments marked by 53BP1 and other chromatin-associated genome caretakers. CHK1 activation and/or activation of alternative repair pathways during S-phase, could cooperate with 53BP1-NB mediated protection in G1, and these mechanisms could act together for stabilizing unreplicated regions.

# MATERIALS AND METHODS

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## Cell cultures

HEK293T cells were obtained from ATCC. HEK293T cells proficient and deficient for WRN weregenerated by stably expressing, respectively, a scrambled shRNA (shCTRL) or shRNA againstWRN (shWRN) (OriGene). Cells were cultured in the presence of hygromycin (200 µg/ml) (EMDChemicals Inc.) to maintain selective pressure for shRNA expression. The SV40-transformed WSfibroblast cell line (AG11395) was obtained from Coriell Cell Repositories (Camden, NJ, USA).WRN mutation in AG11395 cells (c.1336C>T) is located in exon 9 of the WRN gene resulting inan amino acid change at codon 369 from arginine to a stop codon (p.Arg369Stop) that gives rise toa truncated protein as described (Friedrich et al., 2010). AG11395 fibroblasts retrovirally-transduced with full length cDNA encoding wild-type WRN (WSWRN) and missense-mutant form of WRN with inactive helicase (WRN-K577M) were previously described (Pirzio et al., 2008). WSWRN6A cell line was generated by transfection of the AG11395 (WS) with plasmid expressing a Flag-tagged full-length WRN carrying Ala substitutions at all the six S/TQ sites (6A) as previously described (Ammazzalorso et al., 2010). The hTERT-immortalized human fibroblasts GM01604 (normal) and AG12975 (Werner syndrome) were obtained from Coriell Cell Repositories (Camden, NJ, USA).). All the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; LifeTechnologies) supplemented with 10% FBS (Boehringer Mannheim). The hTERT fibroblasts were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) with 15% FBS (Boehringer, Mannheim, Germany and incubated at 37°C in ahumidified 5% $CO_2$ atmosphere.

## Chemicals and treatments

Aphidicolin (Aph, Sigma-Aldrich), hydroxyurea (HU, Sigma-Aldrich) or roscovitine (Selleck) wasadded to culture medium at the indicated concentrations from stock solutions prepared in DMSO(Aph, roscovitine) or in PBS (HU).Nocodazole (Sigma-Aldrich) was used at final concentration of 0.5 µg/ml to accumulate cells in M-phase. Calyculin A (Abcam) was added for 3 h at the final concentration of 1 nM.

## Plasmids and transfection

Plasmids expressing the Flag-tagged $WRN_{wt}$ or the Flag-tagged $WRN_{6A}$ were generated as earlierreported (Ammazzalorso et al., 2010). The phospho-mimic (Flag-$CHK1_{317/345D}$) mutant form of CHK1 was constructed aspreviously described (Gatei et al.,

2003), and it was a kind gift from Prof. K.K. Kanna (Queensland Institute of Medical Research, Australia). To express the plasmids, cells were transfected using the Neon™Transfection System Kit (Invitrogen), according to the manufacturer's instructions. Forty-eight hours afterwards, cells were appropriately treated and then collected for biochemical analysis, DNA fibre analysis or chromosomal damage scoring.

### Immunofluorescence

Immunofluorescence microscopy was performed on cells grown on coverslips. Cells were treatedwith aphidicolin and harvested at the indicated times. Immunofluorescence was performed aspreviously described (Franchitto et al., 2008a). Staining with anti-RPA32 (Cabiochem) was performed for 1 h at RT 1%BSA/PBS. Specie-specific fluorescein-conjugated secondary antibodies (Jackson ImmunoResearchLaboratories) were applied for 1 h at RT followed by counterstaining with 0.5 μg/ml DAPI. Slideswere analyzed with a microscope (Leica) equipped with a charge-coupled device camera(Photometrics). Images were acquired as greyscale files using Metaview software (MDS AnalyticalTechnologies) and processed using Adobe Photoshop CS3 (Adobe). For each time point, at least100 nuclei were examined by two independent investigators and foci were scored at 60×. Onlynuclei showing more than five bright foci were counted as positive. Parallel samples eitherincubated with the appropriate normal serum or only with the secondary antibody confirmed thatthe observed fluorescence pattern was not attributable to artifacts.To detect parental-strand ssDNA, cells were prelabeled for 24 h with 10 μM BrdU (Sigma-Aldrich),then 0.4 μM Aph was added for 8 h. Next, cells were washed with PBS, permeabilized with 0.5%Triton X-100 for 10 min at 4°C and fixed as previously described (Couch et al., 2013). Fixed cellswere then incubated with mouse anti-BrdU antibody (BD Pharmingen) for 1 h at RT 1% BSA/PBS,followed by specie-specific fluorescein-conjugated secondary antibodies (Jackson ImmunoResearchLaboratories), and counterstained with 0.5 μg/ml DAPI. Images were acquired as reported above.

### Cell lysatesand Western blotting

Cell lysates were prepared as previously reported (Franchitto et al., 2008b). Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose (PROTRAN). Incubation with antibodies was performed for 2 h at RT. Proteins were visualized using ECL+ according to the manufacturer's instructions (GE Healthcare).

**Antibodies** used for Western blotting were commercially obtained for WRN (1:300, rabbit polyclonal, Santa Cruz), ATR (1:1000; rabbit polyclonal, Calbiochem), γH2AX (1:1000; mouse polyclonal, Millipore), PCNA (1:2000; mouse monoclona, Santa Cruz), RPA32 (1:100; mouse monoclonal, Calbiochem), Flag-Tag (Sigma-Aldrich), phospho-S/TQ (Cell SignalingTechnologies), phospho-CHK1-Ser345 (Cell SignalingTechnologies), CHK1 (Santa Cruz Biotechnology, Inc.), Lamin B1(Abcam), GADPH (Millipore), Cyclin A (Santa Cruz Biotechnology, Inc.), RPA32 (Calbiochem),TopBP1 (Bethyl Laboratories), RAD9 (Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-conjugated-goat specie-specific secondary antibodies (Santa Cruz Biotechnology, Inc.) were used.

**Quantification** was performed on scanned images of blots using ImageJ software, and values shown on the graphs represent a percentage compared with the matched untreated control normalized against the protein content evaluated through Lamin B1, or CHK1 (pCHK1/CHK1) immunoblotting.

### Immunoprecipitation

For IP experiments, $2.5X10^6$ cells were used. Lysates were prepared using Co-IP buffer (1% Triton X-100, 0.5% Na-dehoxycolate, 150mM NaCl, 2.5mM MgCl2, 1mM EGTA, 1mM EDTA, 20mM Tris/Cl pH 8) supplemented with phosphatase and protease inhibitors. One-milligram of lysate was incubated overnight at 41C with 20ml of Dynabeads M-280- Tosylactivated (Invitrogen) conjugated with 5mg of anti-WRN antibody according to the manufacturer. After extensive washing in RIPA buffer (PBS, 1% NP-40, 0.5% sodium dehoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml PMSF, 1 mM sodium orthovanadate, and 1 mM NaF), proteins were eluted in 2X electrophoresis buffer and subjected to SDS–PAGE and western blotting. For the analysis of whole protein content, cells were washed with PBS and lysed in standard RIPA buffer.

### Chromatin fractionation

Chromatin fractionation experiments were performed as previously described (Muftuoglu et al., 2008; Pichierri et al., 2001). To isolate chromatin, cells were resuspended in bufferA (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M Sucrose, 10 % Glycerol, 1 mM DTT, protease inhibitor cocktail). Triton X-100 (0.1%) was added, and the cells were incubated for 5 min on ice. Nuclei were collected inpellet 1(P1) by low-speed centrifugation (4min,1,300g, 4°C). The supernatant (S1) was further clarified by high-speed centrifugation (15 min,20,000g, 4°C) to remove cell debris and insoluble aggregates. Nuclei

were washed once in buffer A, and then lysed in for 30 min in 100 µl buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitor cocktail). Insoluble chromatin was collected by centrifugation (4 min, 1,700 g , 4°C), washed once in buffer B,And centrifuged again under the same conditions. The final chromatin pellet(P3) was resuspended in Laemmli buffer, sonicated for and then boil for 5 min at 95°C. Chromatin associated proteins were analysed by SDS PAGE/Western Blot.

### ssDNA immunofluorescent assays

Detection of parental-strand ssDNA was performed as previously described (Couch et al., 2013). Cells were labeled for 24 h with 10 mM BrdU and then treated with Aph 0,4µM for indicated time. Next, the cells were washed once with PBS, permeabilized with 0.5% Triton X-100 for 10 min at 4°C, fixed with 3% paraformaldehyde/2% sucrose solution for 10 min, and blocked for 15 min in 3% BSA-PBS. Fixed cells were then incubated with mouse anti-BrdU antibody (Becton Dickinson)for 60 min at 37°C followed by Alexa-488 goat anti-mouse (Invitrogen) secondary antibody. Images were collected using a Nikon Fluor microscopy at a constant exposure time

### Cell cycle analysis by flow cytometry

Cells were treated with Aph 0.4 µM and harvested at the indicated times. After harvesting, cells were fixed in 70% ethanol at 4°C overnight. The day after, cells were washed three times in PBS, resuspended in PBS/phosphate-citrate buffer 1:1 ($NaHPO_4$ 0,2M, citric acid 0,1M) and incubated 5 minutes at room temperature. Phosphate/citrate buffer is used to improve DNA extraction and to better separate G0/G1 cells from apoptotic cells. After centrifugation at 2000 rpm for 5 minutes, cells were resuspended in 1 ml of propide iodure (50 µg/µl) plus RNasi and incubated 30 minutes in the dark prior cytofluorimetric analysis. Data were analysed with CellQuest software.

### Evaluation of mitotic index by phospho-H3 immunostaining

For evaluation of mitotic index, $3\times10^5$ cells were plated in 35-mm dishes and exposed to 0.4 µM Aph for 16, 24 h before fixation in 2% PFA. After permeabilization in 0.5% Triton X-100, cells were blocked in 10% FBS and incubated with anti-pH3 antibody (Santa Cruz Biotechnology, Inc.) for 1 h at RT, followed by three washes in PBS and incubation with an Alexa 488-conjugated secondary antibody before DAPI counterstaining and evaluation of mitotic index by fluorescence microscopy.

### Fragile site induction and slide preparation

Fragile sites were induced by treating cells with aphidicolin ( 0.4 μM)(Sigma-Aldrich). Cell cultures were incubated with 0.2 μg/ml colcemid at 37°C for 3 h until harvesting. Cells formetaphase preparations were collected and prepared as previously reported (Pirzio et al., 2008). Cells for metaphase preparations were collected according to standard procedure. In brief, the cellular pellet was resuspended in pre-warmed hypotonic solution (0.075 M KCl in distilled water) and incubated at 37°C for 18 min followed by multiple changes of fixative solution (3:1 methanol/acetic acid). Cell suspension was dropped onto cold, wet slides to make chromosome preparations. The slides were air dried overnight and stored at −20°C until analysis. For each condition of treatment, the number of breaks and gaps was observed on Giemsa-stained metaphases.

### FISH analysis

Bacterial artificial chromosomes (BACs) mapping to fragile site regions (provided by D. Toniolo, Dibit-HSR, Milan, Italy; and M. Rocchi, University of Bari, Bari, Italy) were used as probes for FISH analyses. A mix of the BACs 94D19, 149J4, and 48E21 was used for FRA3B; a mix of bacterial artificial chromosomes BAC36B6 (RP-11) or BAC264L1 (RP-11)were used as probes for FISH analyses respectively for FRA7Hor FRA16Dfragile site regions. Probes were labeled with a digoxigenin-11-dUTP nick translation kit (Roche) according to the manufacturer's instructions. FISH experiments were performed according to standard protocols previously reported (Pirzio et al., 2008).Two hundred nano grams of labeled DNA were precipitated with 10μg human Cot-1 DNA , 3μg salmon sperm DNA, 1/10 vol. 3M Na acetate and 3 vol. cold (-20°C) ethanol at -80°C for 15 minutes, then centrifugated for 15' (14,000 rpm) at 4°C and finally denatured at 80°C for 8 min in 10 μl of 2× SSC, 10% dextran sulfate, 50% formamide, 1% tween 20. This hybridization mix was preannealed at 37° C for at least 1 h prior to application to denatured slides.Slides were denatured at 80 ° C for 2 min in 70% formamide, 2× SSC, pH 7.0 and put through a standard dehydrating ethanol series. Hybridization was carried out overnight at 37°C. The hybridated slides were so washed 3 times for 5 min in pre-warmed 0.1xSSC at 60°C, then incubated with blocking solution (3% BSA/4xSSC/0.1 Tween 20) for 30' at 37°C. FISH signals were detected by incubation with anti–digoxigenin-rhodamine Fab fragments (Roche) diluted in detection buffer (1% BSA/1xSSC/0.1 % Tween 20) at 37°C for 30 min. Slides were then rinsed 3 times in pre-warmed washing solution (4xSSC / 0.1 Tween 20) in water bath at 42°C and finally counterstained with DAPI (200ng/ml in 2xSSC). Hybridized metaphases were analyzed with

an epifluorescence microscope equipped with a charge-coiled device camera. Images were acquired as grayscale files using Metaview software and processed using Photoshop. For each time point, at least 100 chromosomes were examined by two independent investigators and chromosomal damage was scored at 100×.

### IF-FISH

Cells were grown in 35mm coverslips, treated with Aph and harvested at the indicated times aftertreatments. Immunofluorescence was performed as described above. Staining with anti-53BP1 antibody (Calbiochem) was performed for 1 h at RT 1% BSA/PBS. After secondary antibodiesincubation, cells were washed 2 times in PBS, fixed again with 4% PFA at RT for 10 min andsubsequently permeabilized with Triton X-100 0.5% at 37°C for 10 min, just before performingFISH analysis as described above, to detect FRA3B.

### DNA fibre analysis

Cells were pulse-labelled with 25 μM chlorodeoxyuridine (CldU) and then labeled with 250 μMiododeoxyuridine (IdU) at the times specified, with or without treatment as reported in theexperimental schemes. DNA fibres were prepared and spread out as previously described (Ammazzalorso et al., 2010). Forimmunodetection of labeled tracks the following primary antibodies were used: rat anti-CldU/BrdU20(Abcam) and mouse anti-IdU/BrdU (Becton Dickinson). Images were acquired randomly fromfields with untangled fibres using Eclipse 80i Nikon Fluorescence Microscope, equipped with aVideoConfocal (ViCo) system. The length of labeled tracks were measured using the Image-Pro-Plus 6.0 software, and values were converted into kilobase using the conversion factor 1μm = 2.59kb as reported (Jackson and Pombo, 1998). A minimum of 100 individual fibres were analysed for each experiment and themean of at least three independent experiments presented.

### Comet assay

DNA breakage induction was evaluated by Comet assay (single cell gel electrophoresis) in non-denaturing conditions. Cell lines were cultured on 12-multiwell coverslips and treated or not with Aph 0.4 μM for indicated times. Dust-free frosted-end microscope slides were kept in methanol ON to remove fatty residues. Slides were then dipped into molten Low Melting Point (LMP) agarose at 0.5% and left to dry. Cells were suspended in PBS and kept on ice to inhibit DNA repair. Cell suspensions were rapidly mixed with LMP agarose at 0.5% kept at 37°C and an aliquot was pipetted onto agarose-covered surface of the slide. Agarose was allowed to gel for 10 min at RT. Agarose-embedded cells

were lysed by submerging slides in lysis solution pH 10 (2.5 M NaCl; 0.1 M EDTA; 0.01 M TRIS) and incubated at 4°C for 20h in the dark for the removal of histone proteins as well as for the breakage of the cell membrane. After lysis, slides were equilibrated in fresh prepared running buffer pH 13.0 (300mM NaOH; 1mM EDTA) for 20 min. Electrophoresis was performed for 20 min in running buffer at 0,6 V/cm. Slides were subsequently rinsed and neutralized with neutralization buffer (0,4 M Tris-HCl). Slides were then washed with distillate water and finally fixed and dehydrated in ice-cold methanol.Nuclei were stained with gel red (EtBr; 1:10000) and visualized with a fluorescence microscope (Zeiss), using a 20X objective, connected to a CCD camera for image acquisition. A minimum of fifty comets per cell line were analyzed using Comet Assay IV software (Perceptive instruments) and data from tail moments processed using Sigma Plot 10 software. Apoptotic cells (smaller comet head and extremely larger comet tail) were excluded from the analysis to avoid artificial enhancement of the tail moment.

### LIVE/DEAD staining

To evaluate cell viability LIVE/DEAD assay (Sigma-Aldrich) was used according to the manufacturer. Cells were seeded in 35mm coverslip. Cells were treated or not with Aph or CPT for 16h, collected and washed once with PBS and incubated in the dark with a solution of 2 µM calcein AM and 4 µM EthD-1 in PBS for 30 min at RT. After removal of the LIVE/DEAD solution, cells are washed once in PBS and fixed in 4% paraformaldehyde (PFA). Live and dead cells were counted using a fluorescence microscope (Zeiss) connected to CCD camera for image acquisition. Cell number was counted in randomly chosen fields and expressed as percent of dead cells (number of red nuclear stained cells/total cell number). For each time point, at least 200 cells were counted.

### Statistical analysis

All the data are presented as means of at least three independent experiments. Statistical comparisons of WS or WRN-mutant cells to their relevant control were analyzed by Student's *t* test. $P < 0.0001$ was considered significant.

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# ACKNOWLEDGEMENTS

# ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my group leaders, Dott.ssa Annapaola Franchitto and Dott. Pietro Pichierri, for their guidance and for having given me the possibility to spent my PhD time in their laboratories. Particularly, I would like to thank my tutor for fruitful discussions in these years and critical reading of my thesis.

I sincerely desire to thank all my colleagues and friends of the Genome Stability Group for their support and friendship: Giuseppe Leuzzi, who also contributed to some experiments of the first part of the results, Veronica Marabitti, who helps me particularly in the last part of my Phd work, and Ivana Murfuni, Eva Malacaria, Chiara Iannascoli, Valentina Palermo, Anita Palma, Monia Pugliese, Francesca Grillini, Francesca Cremona. The group has been a source of friendships as well as good advice and collaboration.

I express my grateful thanks to Professor K.K. Khanna for sharing research material (Queensland Institute of Medical Research, Australia). A special thought to the people who started this work before my arrival in the lab.

I am also grateful to Dott.ssa Eugenia Dogliotti, for welcoming me into the whole group of the Molecular Epidemiology lab. Especially thanks to Marco Crescenzi, Margherita Bignami, Riccardo Crebelli for their hospitality in their labs for my experiments. I gratefully acknowledge the AIRC funding sources that made my Ph.D. work possible. Especially thanks to Giorgio Prantera for his helpfulness during all these years of my PhD. I would also like to express my gratitude Cristina Foggetti for her valuable help for English.

Lastly, I would like to thank my family for all their love and encouragement. For my parents who raised me with the love of science and encouragement and support me in all my pursuits, even though being so neglected. A special thank goes to my loving, supportive, encouraging, and patient boyfriend, Carlo, for taking extra care of me in such stressful study times.

Moreover, I am indebted to all my friends who have supported me over the last few years, expecially Teresa Ardovini and Loredana Guglielmi.

Thank you all.