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# Impact of low replication stress on the replication program of cancer and non-tumor cells

Lilas Courtot

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# THÈSE

En vue de l'obtention du  
**DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE**  
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Présentée et soutenue par  
**Lilas COURTOT**

Le 7 octobre 2020

**Conséquences d'un faible stress réplicatif sur le programme de  
réplication des cellules normales et cancéreuses**

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**CRCT - Centre de Recherche en Cancérologie de Toulouse**

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“Les espèces qui survivent ne sont pas les espèces les plus fortes, ni les plus intelligentes, mais celles qui s'adaptent le mieux aux changements.”

**Charles Darwin**

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## State of the art



Deoxyribonucleic Acid, commonly called DNA, is the first essential molecule of life. It carries genes encoding for RNAs and proteins that are the origin of structure and function of cells, the basic units of a living organism. Complex organism, such as the human body, does count not less than 37.2 trillions of cells (Bianconi et al., 2013), with many distinct roles and structures while harbouring a unique genetic information. Human embryonic and proliferating cells must divide to ensure the development or regeneration of a tissue. To do so, cells need to duplicate its DNA in a semi-conservative and faithful manner to transmit equal genetic information to two daughter cells. The human genome contains a huge amount of DNA, 6 billions of nucleotides distributed among 23 pairs of chromosomes, highly packed in 3D into the nucleus. In light of this and knowing that typical proliferating human cells divide approximately once a day, there is little doubt that DNA replication is a very challenging process.

To ensure DNA replication, cells activate thousands of DNA replication origins distributed along the genome, gathering many proteins with specific functions such as DNA unwrapping and DNA synthesis. Given the large size and complex structure of the human genome, errors in DNA copying process are inevitable. In addition to inserting errors, the replication machinery can encounter some obstacles, from endogenous or exogenous sources, that will slow down the process and create what is called the replication stress. The slowing down of replication fork is a normal event during DNA replication, but it can have deleterious consequences such as un-replicated DNA or DNA breaks that could lead to genome instability. Fortunately, human cells regulate tightly this whole process in order to avoid/repair potential replication errors and counteract replication stress.

It is very well known that oncogene activation and additional mutations lead DNA replication stress, genomic instability and cancer. In addition, cancer cells are immortal and present uncontrolled and excessive proliferation. Targeting DNA replication with genotoxic agents has been and still remains a good chemotherapeutical approach for cancer treatment. However, recurrent disease after chemotherapy is the major cause of cancer-related mortality, suggesting that some cancer cells can escape to this treatment. Thus, we clearly need more understanding about the exact link between replication stress and cancer and to do so, it is essential to better characterize the cellular response to DNA replication stress and to explain how it can lead to cancer and/or resistance to therapies.

Few years ago, several studies described a link between the temporal order of replication origin activation, named replication timing, and cancer. They showed not only correlations between replication timing and mutational landscape (Sima and Gilbert, 2014) but also that the replication timing itself can be deregulated in cancer (Rivera-Mulia et al., 2017; Ryba et al., 2012). A more recent study highlighted a link between specific replication timing alterations of leukemic cells and relapse (Rivera-Mulia et al., 2019).

Replication timing is described as a very robust phenomenon reflecting cellular identity. Shifts in DNA replication timing are very well characterized during human development (Pope et al., 2010; Siefert et al., 2017; Takebayashi et al., 2012), but very little is known about replication timing alterations in cancer. Indeed, the mechanism leading to replication timing modifications is not clear, and we totally ignore how and when these replication timing modifications appear in tumours. Therefore, studying replication timing and deciphering mechanism leading to its alteration could be relevant to better characterize cancer cells and cancer recurrence.

All things considered, the aim of my thesis was to characterize and compare whole genome DNA replication timing in response to mild replication stress induced by aphidicolin in 6 human cell lines from different backgrounds, including 4 cancerous and 2 non-cancerous.

The thesis introduction starts with a non-exhaustive description of DNA replication process and replication origins function in normal condition and under replication stress, integrating the review I wrote during my thesis (Courtot et al., 2018). Then it goes into more details about the DNA replication timing, its regulations and biological significance, to finish with an overview about replication stress, genomic instability and cancer.

# Introduction

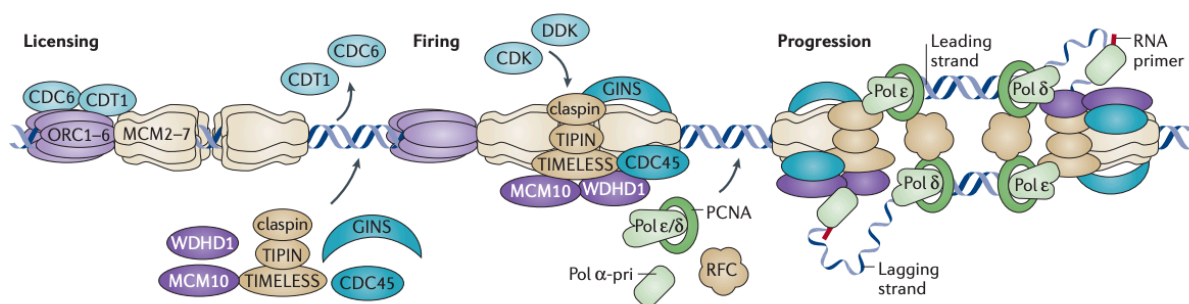
## DNA replication: a crucial step of the cell cycle

All eukaryotic dividing cells are following the very same and specific scheme through well-defined states, called the cell cycle. This latest is first divided into two main parts: the interphase, and the mitosis. During mitosis, the two identical sister chromosomes are segregated to give rise to two genetically identical daughter cells. The interphase is the moment when cells are preparing their future division by duplicating their cellular components, including DNA. The interphase can be subdivided into three main phases: the “gaps” phases of the cell cycle, G1 and G2 phases, and in between the DNA replication, or S phase. DNA replication is the biological process by which an exact copy of the entire DNA is produced, allowing correct genome duplication and biological inheritance through cellular generations. DNA replication process, included in a very well-regulated cell cycle, is very well orchestrated in order to ensure the correct cell division and transmission of the genetic information. This first part aims to give a short introduction to the DNA replication process and the cell cycle.

## a. The DNA replication process

The DNA replication is semi-conservative because each of the two strands of the double helix DNA structure serves as a template for the creation of the complementary strand. DNA replication initiates at specific genomic loci called replication origins that are recognized by specific proteins named origin-recognition complex (ORC). In the beginning of S phase, ORC and MCM proteins are already loaded onto the chromatin at replication origins, together with cell cycle division 6 (CDC6) and the chromatin licensing and DNA replication factor 1 (CDT1) forming the pre-replication complex (pre-RC) (**Figure 1**). The process of DNA replication origin licensing will be further discussed in a following part of the introduction.

Activation of licensed origins is termed origin firing and is triggered by the activity of cyclin dependent kinase-7 (CDK7) and its regulatory subunit (DBF4) allowing the stable association of cell division cycle protein 45 homolog (CDC45) and the DNA replication complex GINS (Boos et al., 2012). This process also requires additional factors including helicase RECQL4, MCM10 and AND1 (or WDHD1), which bind to one of the replicative DNA polymerase Pol  $\alpha$  (Masai et al., 2010).



**FIGURE 1:** DNA REPLICATION AT A GLANCE: FROM LICENSING TO REPLICATION FORK PROGRESSION. (FROM GAILLARD ET AL., 2015)

The first step necessary to start the replication process is the opening of the double stranded DNA into two single strands by mini-chromosome maintenance complex (MCM2-7) helicase proteins and DNA unwinding by topoisomerase enzymes. At each fired origin, two replication forks are established and move on opposite directions, away from the origin thanks to the activity of the holo-helicase complex formed by CDC45-MCM2-7-GINS (CMG). Two CMG helicases translocate in an “N terminus-first” direction, and in doing so, pass each other within

the origin (Douglas et al., 2018). Then, MCM10, together with ATP hydrolysis trigger further DNA untwisting and helicase activation. As the polymerase activity only works in a unique direction, from 5' to 3', depending on the DNA strand template, the replication fork will move continuously (leading strand) or un-continuously, through the production of short Okazaki fragments (lagging strand). On both DNA strands, synthesis of new DNA molecule is initiated by Pol  $\alpha$  complex (Pol  $\alpha$ -pri) which contains DNA primase and DNA polymerase catalytic activities within respective subunits. Leading and lagging strands are then extended by Pol  $\epsilon$  and Pol  $\delta$  respectively, together with the ATP-dependant activities of the Proliferating Cell Nuclear Antigen (PCNA) and the replication factor C (RFC), that anchor DNA polymerases to the DNA template in the form of a sliding clamp and a clamp loader (Goswami et al., 2018; Yeeles et al., 2017). Other key component of the replisome is the claspin-TIMELESS (or TIM) and TIMELESS-interacting protein (TIPIN) complex that coordinates DNA unwinding during DNA synthesis, allowing the replication fork progression and the elongation steps.

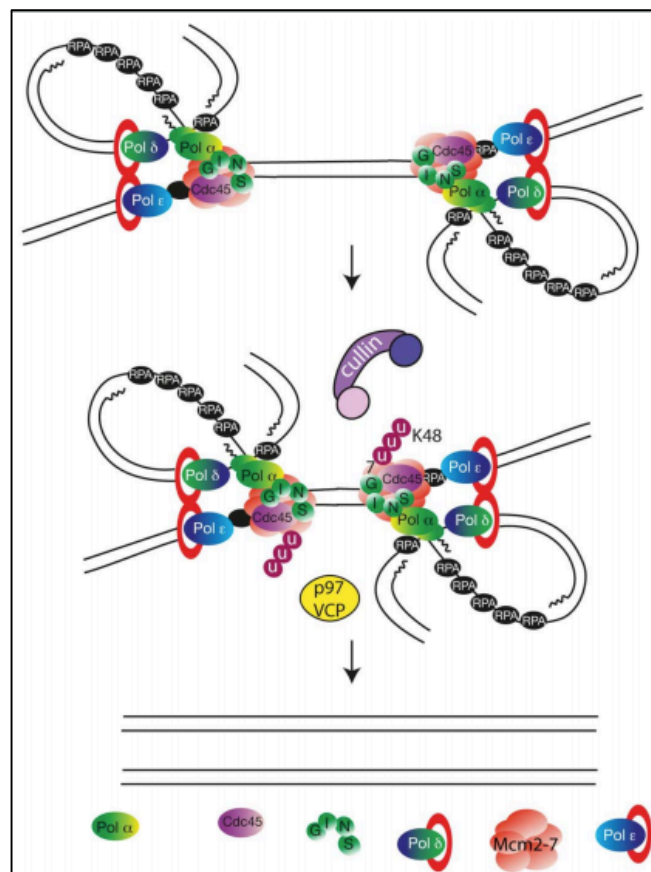


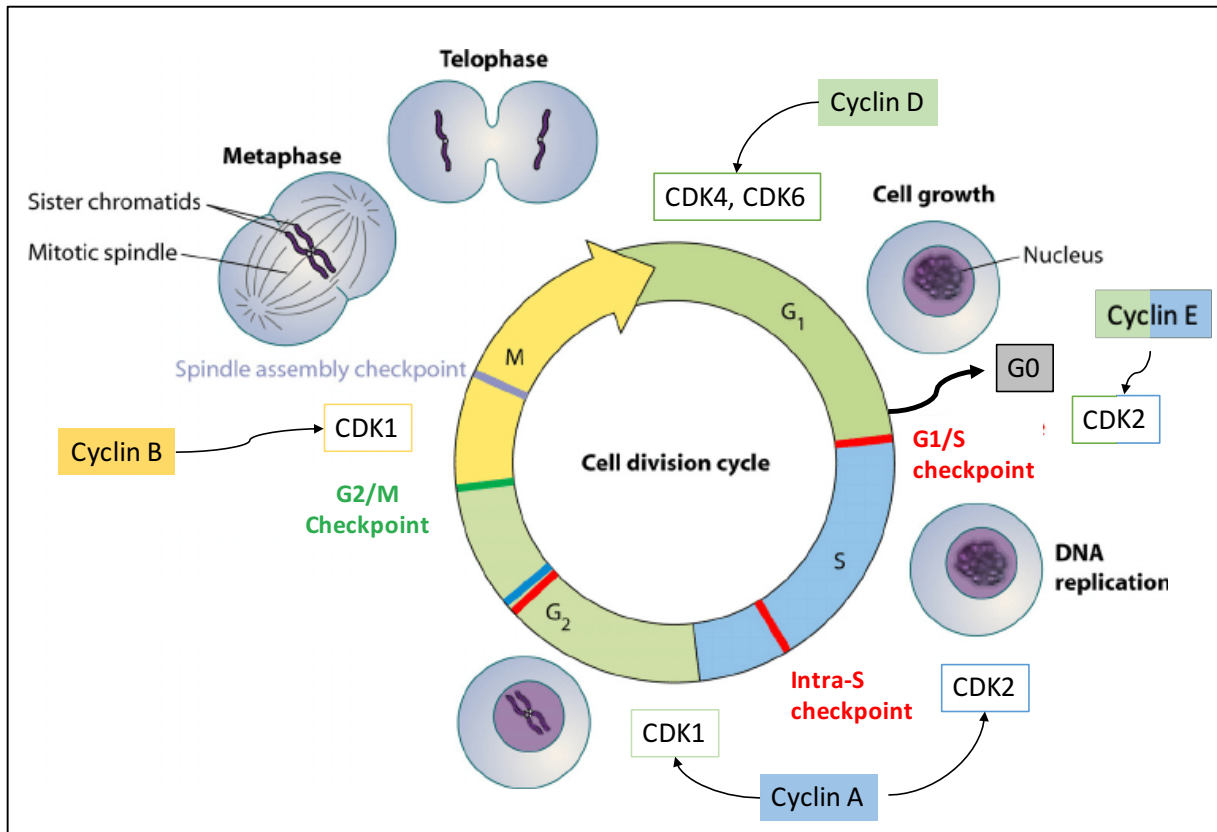
FIGURE 2: A CURRENT VIEW OF THE DNA REPLICATION TERMINATION IN EUKARYOTES. (FROM BAILEY ET AL., 2015)

Following the successful DNA elongation, the replication has to be terminated to end up with two separate copies of the DNA. As the eukaryotic cell have a large number of replication origins, the termination involves merging of two adjacent replication forks that includes different steps. First, the DNA strand between the two adjacent forks is unwound and the approaching CMGs intersect each other and the last Okazaki fragment is processed by DNA pol  $\delta$  and FEN1. Then, the gaps in the new daughter strands are filled in, just like in normal Okazaki fragments, and the two oppositely approaching strands are ligated. After the convergence of the two replication forks, the replisome complex dismantles. This process involves cullin dependant poly-ubiquitylation of MCM7 that in turn is recognized by the p97/VCP/Cdc48 segregase and remodelled causing the helicase to be removed from the DNA (**Figure 2**). Finally, if there are any inter-twinings in the daughter DNA strands or catenanes, they are removed by topoisomerase II, allowing proper segregation of the two strands (Bailey et al., 2015; Dewar and Walter, 2017; Moreno et al., 2014).

As already mentioned, DNA replication is not the only essential process to ensure cell proliferation genome stability. Indeed, after duplicated its DNA, cell will divide to give rise to two daughter cells that will in turn replicate its genetic information and so long. Mitosis is thus the other essential step of the cell cycle, ensuring proper cell division and genomic stability. As the regulation and description of the mitosis is not necessary for the understanding of this thesis, I have decided to not describe this process in detail. The correct cell division and transmission of genomic information are ensured by a tight regulation of the cell cycle, mainly the two gaps phases (G1 and G2), during which checkpoints and cell-cycle regulatory protein (cyclins) are present to impede the passage to another phase if the conditions are not fulfilled.

## b. The cell cycle

The mammalian cell cycle is a ubiquitous and essential cellular process at the origin of the growth and proliferation of cells. This latest can be morphologically divided into two main phases: the interphase and the mitosis. The interphase is subdivided into the G1 (or Gap-1) phase, when cellular components are duplicated, genes are highly transcribed and the cell is getting ready for the DNA replication process. Then, S phase (or replication phase) is the moment when the genome is duplicated. Finally, the G2 phase is when chromosomes are being condensed in order to be correctly segregated during the mitosis (**Figure 3**).



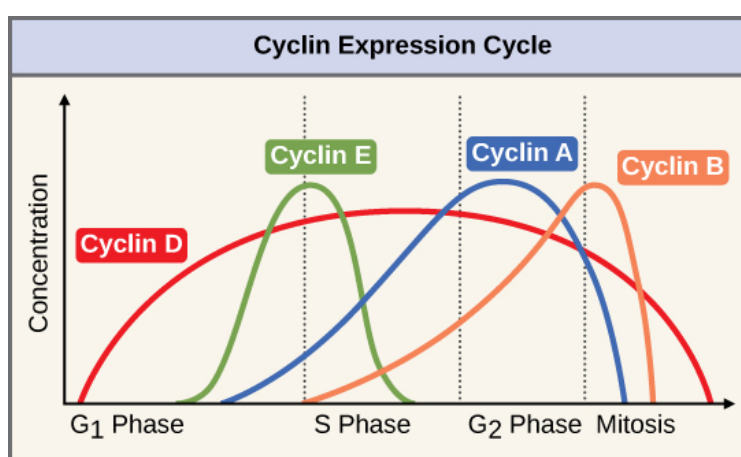
**FIGURE 3:** THE CELL CYCLE. DIVIDED INTO TWO MAIN PARTS: THE MITOSIS (IN YELLOW), THE SPECIFIC MOMENT WHEN CHROMOSOMES ARE SEGREGATED GIVING RISE TO TWO DAUGHTER CELLS. THE INTERPHASE IN WHICH WE CAN DISTINGUISH THE G<sub>1</sub>, S AND G<sub>2</sub> PHASE. MANY CHECKPOINTS ARE PRESENTS THROUGHOUT CELL CYCLE TO ENSURE THE PROPER EXECUTION OF EACH PHASE BEFORE ENTERING INTO THE NEXT PHASE. (ADAPTED FROM CHIN AND YEONG, 2010)

The cell cycle involves numerous regulatory proteins, each having a specific role to ensure the progression of one mother cell until ending up with mitosis and the production of two daughter cells.

At the heart of cell cycle regulation are the cyclin proteins (or cyclins) and cyclin dependent kinases (CDKs), that regulate the progression of cells through cell cycle and help signalling and activating important phenomenon such as gene expression and DNA replication. More specifically, CDKs are serine/threonine protein kinases that are activated at specific points in the cell cycle. There is at least nine different CDKs in mammalian cells, and many have been shown to be critical for cell cycle progression, their inactivation preventing mitosis (Devault et al., 1991; Heuvel and Harlow, 1993; Parker and Piwnica-Worms, 1992; Pines, 1995). The activation of CDK induces downstream processes by the phosphorylation of selected proteins. For example, CDK7 acts together with cyclin H to activate all the other CDKs



(Fisher and Morgan, 1994). CDK protein level remain relatively stable throughout cell cycle while their activating proteins, the cyclins, are differentially present depending on the phase in the way that they specifically activate CDKs (**Figure 4**). Indeed, different cyclins are required depending on the cell cycle phase: cyclins of D type (cyclin D1, D2 and D3) bind to CDK4 and CDK6 to allow the G1 entry (Sherr, 1994). Then cyclin E associates with CDK2 to allow progress from G1 to S phase (Ohtsubo et al., 1995). Cyclin A binds to CDK2 during the S phase and interact with CDK1 in late G2/early M to promote the entry into mitosis (Pagano et al., 1992). The M phase is further regulated by the binding of cyclin B to CDK1 (Arellano and Moreno, 1997; King et al., 1994) (**Figure 3 and 4**).



**FIGURE 4:** THE EXPRESSION OF DIFFERENT CYCLINS DURING CELL CYCLE. (FROM "WIKIMIMA"/WIKIMEDIA COMMONS)

Although CDKs/cyclins are the main regulatory molecules that determine the forward momentum of the cell cycle, there are other important mechanisms that fine-tune the progress of cell cycle with a more counteracting effect. Indeed, among these mechanisms we can mention cell cycle checkpoints, that block the progress of cell into another phase until problematic conditions are not resolved.

At the end of the G<sub>1</sub> phase, the checkpoint determines whether all the conditions are favourable to a proper cell division. The G<sub>1</sub>/S checkpoint, also known as the restriction point in yeast, is the moment when the cell irreversibly enters into the cell division process. External agents, like growth factors, play an important role in favour or against the decision of passing this checkpoint. In addition to good reserves and cell size, there is also a checking of the DNA damage at the end of G<sub>1</sub> phase. The major regulator of the G<sub>1</sub>/S checkpoint is the very well-

known p53 tumour suppressor protein. p53 is a multi-functional protein that has a major role in the genome integrity by acting when there is DNA damage in cells. When damaged, the DNA is detected by the kinase protein ataxia-telangiectasia mutated (ATM) that phosphorylate the MDM2 protein leading to the cell cycle arrest by p53 and the recruitment of DNA repair proteins (Canman et al., 1994; Khosravi et al., 1999). If the DNA is not or cannot be repaired, p53 can trigger the apoptosis to prevent the duplication of damaged chromosomes (Aubrey et al., 2018; Clarke et al., 1993; Fridman and Lowe, 2003; Yonish-Rouach et al., 1991). As the p53 protein level rises, p21 protein production is stimulated and leads to the reinforcement of the cell cycle blockade through the inhibition of the CDK/cyclins complex activity. Finally, if the criteria of this checkpoint are not fulfilled, the cell can halt the cycle in order to attempt to correct the problem or wait for further positive signals when conditions improve entering then into a quiescent state called the G0 phase (Nakamura-Ishizu et al., 2014; Pardee, 1974) (**Figure 3**).

During the DNA replication in the S phase, cells can activate the intra-S checkpoint, or DNA damage response (DDR) to protect genomic integrity and ensure faithful replication. This checkpoint is dependent on the ATR/Chk1 and/or the ATM/Chk2 protein kinases signalling cascades that mainly help the replication fork to restart and/or regulate origin firing (Bartek and Lukas, 2003; Cimprich and Cortez, 2008; Flynn and Zou, 2011; Lukas et al., 2004; Rhind, 2009; Shechter et al., 2004). The intra-S checkpoint activation and DDR process will be further discussed in another part of the introduction.

The G2/M checkpoint stop the entry into the mitosis if conditions are severe. Just like the G1/S checkpoint, cell size, protein reserve and genome integrity are assessed. The most important role of the G2/M checkpoint is to ensure that all chromosome has been entirely and properly duplicated (Löbrich and Jeggo, 2007; Stark and Taylor, 2004). To do so, the cell sensitise the amount of double-strand breaks (DSB) at the end of the G2 thanks to ATR/Chk1/p53 signalling and inhibit CDC2 if the genome is above the tolerance threshold (Stark and Taylor, 2006).

The M checkpoint, also known as spindle checkpoint, occurs near the metaphase stage and assess the correct anchoring of all the sister chromatids to the spindle microtubules. Because separation of sister chromatids during anaphase is irreversible, the cell cycle will not proceed until each pair of sister chromatin kinetochores are firmly attached to at least two

spindle fibers arising from opposite poles of the cell (London and Biggins, 2014; Pinsky and Biggins, 2005).

Finally, an important cell cycle regulator is the Rb protein that influence checkpoints either positively or negatively. Rb is the main safeguard of the cellular size through its interaction with the very well reported transcription factor E2F associated to cellular growth. For example, interaction between Rb and E2F blocks the production of proteins required for the G1/S transition. As the cell increases in size, Rb is more and more phosphorylated until its inactivation and the E2F factor release, which can in turn induces transcription of important genes for the DNA replication process (Giacinti and Giordano, 2006; Hatakeyama and Weinberg, 1995).

## DNA replication origins and their role in response to replication stress

Genome stability requires tight regulation of DNA replication to ensure that the entire genome of the cell is duplicated once and only once per cell cycle. In mammalian cells, origin activation is controlled in space and time by a cell-specific and robust program called replication timing. About 100,000 potential replication origins form on the chromatin in the gap 1 (G1) phase but only 20–30% of them are active during the DNA replication of a given cell in the synthesis (S) phase. When the progress of replication forks is slowed by exogenous or endogenous impediments, the cell must activate some of the inactive or “dormant” origins to complete replication on time. Thus, the many origins that may be activated are probably key to protect the genome against replication stress. This review aims to discuss the role of these dormant origins as safeguards of the human genome during replicative stress.



Review

# The Protective Role of Dormant Origins in Response to Replicative Stress

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**Abstract:** Genome stability requires tight regulation of DNA replication to ensure that the entire genome of the cell is duplicated once and only once per cell cycle. In mammalian cells, origin activation is controlled in space and time by a cell-specific and robust program called replication timing. About 100,000 potential replication origins form on the chromatin in the gap 1 (G1) phase but only 20–30% of them are active during the DNA replication of a given cell in the synthesis (S) phase. When the progress of replication forks is slowed by exogenous or endogenous impediments, the cell must activate some of the inactive or “dormant” origins to complete replication on time. Thus, the many origins that may be activated are probably key to protect the genome against replication stress. This review aims to discuss the role of these dormant origins as safeguards of the human genome during replicative stress.

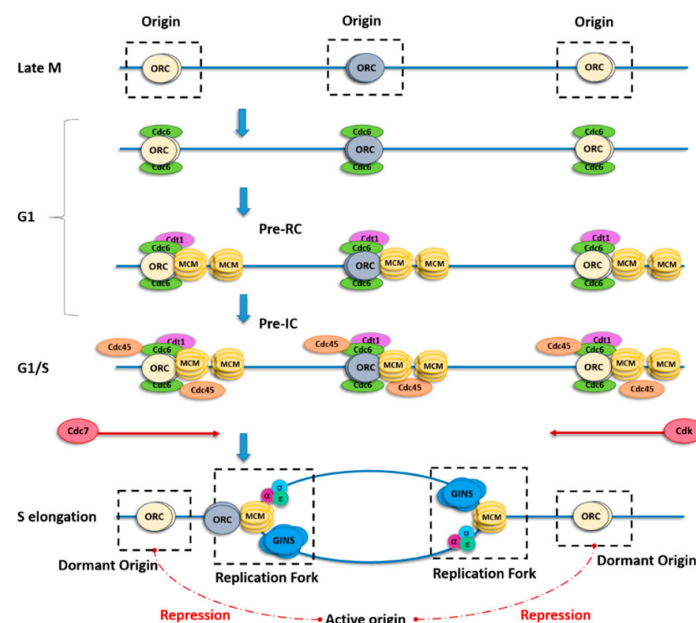
**Keywords:** dormant origins; replicative stress; replication timing; DNA damage; genome instability; cancer

## 1. Introduction: Eukaryotic Origins and the Replication Program

Because of their large genomes, mammalian cells need thousands of replication forks, which initiate from replication origins, to ensure the complete duplication of their DNA within a specific time frame before they can divide. In human cells, the replication process takes about 10 h and involves the activation of roughly 30,000 replication origins. In normal replication conditions, replication origins are spread over about 100 kb of DNA, and only a single origin will be active within an individual DNA unit that we call a replicon. A coordinated group of adjacent replicons, “replicon cluster”, can be visualized as DNA replication foci [1]. Several studies, which compared replication timing (RT) and genome topology, suggested the term “replication domains” for replicons clustered inside large chromatin regions (~1 Mb), close to the size of one replication foci. They are located at discrete territories of the nucleus in the gap 1 (G1) phase and replicate at the same moment during the synthesis (S) phase [2–4]. At any given time of the S phase, about 10% of replicons are activated and replicate simultaneously [5]. In addition, the temporal activation of origins in a specific region of the genome correlates with a distinct pattern of replication foci as cells progress from early to late S phase. The sequential activation of potential origins within replication domains is thought to play a direct role in defining the S phase program or replication program. Temporal and spatial organization of DNA replication was adopted by metazoans cells to finely control the challenging goal of replicating the entire genome in a limited time and to overcome any obstacles that replication forks may encounter.

### 1.1. Origin Licensing and Firing

Complete and robust DNA duplication requires loading of minichromosome maintenance DNA helicase complex (MCM2–7) onto the replication origins. This step, called origin licensing, is restricted to the G1 phase of the cell cycle. A key initial step in origin licensing is the building of pre-recognition complex (Pre-RC) which starts with loading of the origin recognition complex (ORC) onto the chromatin. This ORC complex marks all potential origins providing spatial control of origin position. In higher eukaryotes, ORC binding sites were proven to be unrelated to DNA sequence, in contrast to other organisms such as yeast and bacteria [6,7]. It is currently assumed that multiple factors can characterize an origin, such as cytosine–phosphate–guanine (CpG) islands, G-quadruplexes, epigenetic marks, chromatin accessibility, sites of active transcription, or secondary DNA structures [8–13]. This is the reason why it is so difficult to identify metazoan replication origins. In the budding yeast *Saccharomyces cerevisiae*, a recent structural study [14] showed that two ORC molecules are required to ensure MCM2–7 complex loading onto the chromatin. During late mitosis and the G1 phase, ORCs bind cell division cycle 6 (Cdc6), which then interacts with chromatin licensing and DNA replication factor 1 (Cdt1) to allow loading of the six MCM subunits (MCM2–MCM7) and formation of the Pre-RC. The total amount of MCM complex does not change throughout the cell cycle, but the number of MCM complexes loaded onto DNA increases from telophase to the end of the G1–S phase transition. The final step of licensing requires the loading of Cdc45 and go-ichi-ni-san (GINS) onto the MCM complex to form the pre-initiation complex (Pre-IC). This complex requires the activities of the Dbf4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) for its activation at the G1–S phase transition; then, the polymerases and other replication factors are recruited to allow origin firing (Figure 1).



**Figure 1.** Scheme describing origin licensing and firing. In late mitosis (M), the origin recognition complex (ORC) binds to origins, thus determining where replication forks might initiate, and for the subsequent recruitment of cell division cycle 6 (Cdc6) and chromatin licensing and DNA replication factor 1 (Cdt1) in the gap 1 (G1) phase. Binding of both Cdc6 and Cdt1 is necessary, in turn, for recruitment of the minichromosome maintenance DNA helicase complex (MCM) to form the pre-recognition complex (Pre-RC). Each ORC has two Cdt1-binding sites, which may explain the cooperative loading of two MCM complexes per origin. The MCM pair remains catalytically inactive until the G1–synthesis (S) phase transition, when it is phosphorylated by both cyclin-dependent kinase (CDK) and Cdc7. Once the principal origin is fired, adjacent origins from the same replicon (flexible or dormant) are repressed (red dotted lines) by a yet unclear mechanism.

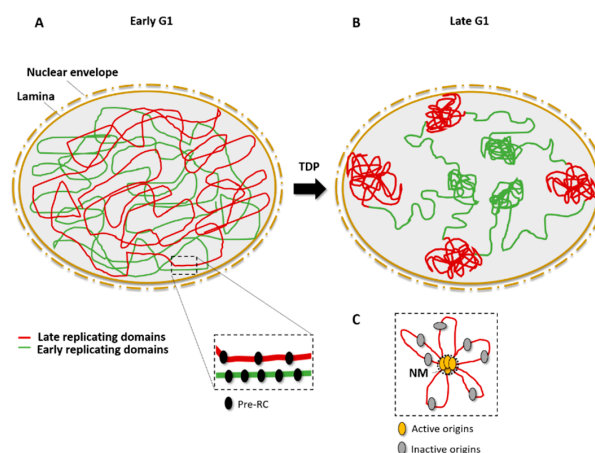
During the first step of origin firing, the MCM pair slides along DNA by encircling the double helix. Recent papers proposed a switch of the MCM double hexamer from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) mediated by N-tier ring movement, allowing the two helicases complexes to pass each other within the origin and permitting lagging-strand extrusion [15,16] (Figure 1). During the elongation step, excess MCMs that are not initiated are removed by the passage of the replication fork [17].

The cell must balance its need for sufficient origins to replicate the entire genome against the risk of re-replication of DNA in the S phase due to an excess of origins. Thus, the control of origin licensing is crucial. Repression of new origin licensing during the S phase is important to avoid re-replication, which can lead to aneuploidy, DNA double-strand breaks, gene amplification, and general genome instability [18–20]. DNA that is not replicated due to an insufficient number of origins or to replication fork stalling, by contrast, can also lead to genome instability and rearrangements if the DNA replication checkpoint is inactive or deficient [21–23].

### 1.2. Spatial and Temporal Organization of Replication Origins

Origin usage in eukaryotes is mainly dependent on two important factors: space and time. Replication origins fire at a defined time that remains the same among cell generations and is closely related to their spatial organization. Early replicating origins are mainly found in replication domains that are enriched in active epigenetic modifications and highly transcribed genes [24–29]. These chromosomal regions have a consequent amount of MCMs, providing potential origins that replicate early in the S phase [7,30]. Conversely, late replication occurs in origin-poor domains with low gene density, and enriched in heterochromatin hallmarks [29,31–33].

Replication clusters are organized in the three-dimensional (3D) nuclear space, where early-replicating domains locate mainly at the center of the nucleus while late-replicating domains are found predominantly at the nuclear periphery (Figure 2B). Chromatin conformation mapping methods such as Hi-C are very powerful for visualizing the spatial organization of early- and late-replicating domains [34,35]. Replication domains are created by topological reorganization of the chromatin in nuclear space. In metazoans, the association of particular replication domains with sub-nuclear compartments determines their replication timing. The set-up of this compartmentalization occurs at a specific time of the G1 phase and is called the timing decision point (TDP) [36,37] (Figure 2).



**Figure 2.** Spatial organization of origins and replication timing. (A) In the early G1 phase, Pre-RCs (black) are assembled on the chromatin and mark potential origins; early-replicating domains (green) and late-replicating domains (red) are disordered in the nuclear space. (B) After the timing decision point (TDP), in the late G1 phase, early-replicating domains are close to center of the nucleus whereas late-replication domains are associated with the lamina, close to the nuclear periphery. (C) Active origins (yellow) cluster in replication domains that are associated to the nuclear matrix (NM), leaving inactive (dormant or flexible) origins in DNA loops (gray).

Accumulating evidence indicates that DNA attachment to the nuclear matrix is important for the initiation of DNA replication [38–42]. The nuclear matrix permits the separation of chromosome territories and allows the formation of replication clusters [39]. The organization of replicon clusters might, thus, reflect chromatin looping to bring the origins from different replicons into a single domain and to exclude the flexible and/or dormant origins from this replication factory (Figure 2C). The cohesin complex may be a key player in chromatin looping because it was found to interact physically with the MCM complex and to be enriched at origin sites [43].

### 1.3. Techniques to Detect and Identify Origins

The first quantitative method for determining origin density in the genomes of bacteria and mammals was DNA fiber autoradiography [44,45]. This time-consuming technique is now replaced by other assays, such as DNA combing or spreading, which label newly replicated DNA with nucleosides analogs, including bromo-, chloro-, and iododeoxyuridine, and visualize the newly replicated DNA by immunofluorescence microscopy using antibodies specific for the analog [46].

The use of next-generation DNA sequencing led to the discovery of tens of thousands of potential replication origins in the human genome. Several independent approaches were used that exploit the direct identification of DNA replication initiation intermediates. The first approach is based on the purification and quantification of short nascent strands (SNS) of DNA [26]. In this method, 1.5–2.5-kb nascent strands specific to replication origins are purified thanks to their resistance to  $\lambda$ -exonuclease digestion due to the incorporation, by the primase, of small RNA primers at their 5' ends [47]. The exonuclease digests the large excess of broken genomic DNA that would generate a background signal if not correctly removed. These genome-wide SNS analyses showed that active origins often co-localize with transcription start sites (TSS) and are located in GC-rich regions, close to CpG islands or G-quadruplexes, confirming previous microarray hybridization results [24,25,48]. A second approach [29] is based on the sequencing of an early intermediate called the DNA replication bubble, which forms when two replication forks diverge from a single origin. The technique consists of fragmenting the replicating DNA via a restriction endonuclease, and then trapping the circular replication bubbles in agarose gel [29]. This so-called “bubble-seq” method led to the mapping of more than 100,000 origins in the human genome. A third genome-wide approach relies on sequencing purified Okazaki fragments (“OK-seq”) to determine replication fork polarity, which allows the identification of initiation and termination sites [49]. With this approach, between 5000 and 10,000 broad initiation zones of up to 150 kb were detected. These sites are mainly non-transcribed but often surrounded by active genes, and they contain a single randomly located initiation event. Finally, a fourth method for identifying metazoan replication origins is called initiation-site sequencing (“ini-seq”) [50]. In this method, initiation events are synchronized biochemically in a cell-free system in which newly replicated DNA, synthesized a few minutes after initiation, is directly labeled and subsequently immuno-precipitated. This original approach has the important advantage of allowing functional genome-wide studies of origin activation. As these approaches become more and more accurate and complementary to each other, they provide an increasingly large, novel dataset on the characteristics of replication origins.

### 1.4. Origin Flexibility, Dormancy, and Efficiency

The replication initiation program of metazoan cells is remarkably flexible, with many origins firing at disparate frequencies depending on the cell lineage. MCM complexes and all the components of the Pre-RC are loaded in excess onto the chromatin in the G1 phase to provide this flexibility. In addition to differences between cell lineages, origin flexibility is also observed within a cell population [42,51].

Very few origins are activated almost all the time; they are called “constitutive” origins [52]. The majority of origins do not initiate replication in all cell cycles; these are called “flexible” origins. Origins that are activated only when replication from adjacent origins is compromised are called



“dormant” origins. Unlike constitutive and flexible origins, dormant origins are not detectable in whole-genome analyses. Inter-origin distances measured by whole-genome sequencing are shorter than those measured by single-fiber analyses. This discrepancy may be explained by the flexibility of origin choice within replicons [53], which might also help coordinate DNA replication with transcription [54,55] and other nuclear processes, such as DNA repair, in order to facilitate recovery when replication is compromised. Given that there is no DNA consensus sequence for metazoan origins and that there exists such a flexibility in establishing which potential origins are activated, one might wonder how initiation ever occurs accurately and at consistent origins [56].

There are currently two theories to explain how origins are selected. One relies on the idea of an origin decision point (ODP)—which occurs in the G1 phase, after the timing decision point—that determines which origins are activated during replication [57]. The second theory postulates increasing origin efficiency based on the random use of replication origins [58], with the idea that the efficiency of origin firing increases throughout the S phase as the replicative DNA polymerases recycle to new origins. Moreover, replication origin efficiency also depends on their location in the nucleus, epigenetic marks, and mainly on the amount of loaded MCM complexes [7,59,60] or nucleosome occupancy [61]. Chromosome architecture also plays an important role in the regulation of DNA replication origin localization and activation [62], although chromosomal loops and loop anchors are still poorly defined biochemically. Further studies using single-cell technologies will be required in the future to better understand the mechanism of origin choice.

## 2. Dormant Origin Activation in Response to Replicative Stress

### 2.1. The Notion of DNA Replication Stress

During DNA replication, the appearance of endogenous or exogenous sources of stress leads to replication forks slowing or stalling. Exogenous sources of stress comprise mainly genotoxic chemicals, and ultraviolet and ionizing radiation. Endogenous sources of stress that are considered to be barriers to replication include repetitive sequences, G-quadruplexes, telomeres, DNA–RNA hybrids, errors in the incorporation of ribonucleotides, collisions between replication and transcription machineries, compaction of chromatin, deregulation of origin activity, and reduction of the deoxyribonucleotide triphosphate (dNTP) pool. Some regions of the genome, such as early-replicating fragile sites (ERFSs) and common fragile sites (CFSs), are more prone than others to replicative stress. Moreover, evidence is emerging that constitutive activation or overexpression of oncogenes, such as Harvey rat sarcoma (HRas) and myelocytomatosis (c-Myc), are a potential source of replication stress [63]. These oncogenes promote replication initiation or origin firing, leading to an elevated risk of nucleotide pool depletion and/or increased collisions with transcription complexes [64,65]. This may explain why supplementing cancer cells with exogenous nucleosides helps decrease chromosomal instability [66].

The first consequence of replication stress is fork collapse, creating DNA single-strand breaks and/or double-strand breaks. These lesions must be resolved before cell division by repair mechanisms such as homologous recombination (HR), non-homologous end-joining (NHEJ), or micro-homology mediated end-joining (MMEJ). In normal cells, the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia Rad3-related (ATR) checkpoint signaling pathways prevent cell division when the genome is damaged. When some proteins of the checkpoint pathway, for example p53, are mutated, the cell can divide despite the presence of DNA lesions (including breaks and unreplicated DNA), which may lead to chromosome fragmentation, rearrangements, and genomic instability [67–70].

### 2.2. The Discovery of Dormant Origins and Their Link to Replicative Stress

In 1977, J. Herbert Taylor [71] first described the firing of new origins in response to replication fork stalling during DNA replication in Chinese hamster ovary (CHO) cells, a finding that later suggested the existence of dormant origins. Moreover, several studies in a range of eukaryotes, including *S. cerevisiae*, *Xenopus laevis*, and human cells, demonstrated that MCM complexes are loaded

onto DNA in a large excess when compared to the number of DNA-bound ORCs and the number of active replication origins [72–77]. It was later shown in *X. laevis* [78] and in human cells that this excess of MCM provides a reservoir of dormant origins, which are activated when replication forks are arrested by agents such as aphidicolin (APH) or hydroxyurea (HU) [79,80]. These studies also showed that depletion of MCM by small interfering RNAs leads to hypersensitivity to replication inhibitors due to the lack of dormant origins [79,80]. Moreover, checkpoint kinase 1 (Chk1) activation is required for firing of dormant origins within active replication clusters, as well as for repression of other replicons that are not yet active [81], suggesting a link between the DNA damage response and dormant origin activation. Indeed, in vertebrates, inactivation or depletion of various proteins involved in genome maintenance, such as ATR [82,83], Chk1 [84–87], Wee1 [88,89], bloom syndrome protein (BLM) [90], Claspin [91,92], breast cancer type 2 susceptibility protein (BRCA2), and Rad51 [93], slows replication forks and also increases the number of initiation events, at least in studies where initiation events were examined. This finding indicates a link between fork speed and the number of active origins, as we examine further below.

### 2.3. The Density of Active Origins Depends on Replication Fork Speed

Under normal conditions, dormant origins do not fire and are passively replicated by the fork coming from adjacent activated origins. Thus, it makes sense to assume that replication fork speed can be a regulator of active origin density. In two complementary studies on CHO cells [62,94], it was demonstrated that replication fork speed has a direct impact on the number of active origins. When the fork is slowed down by HU treatment, the density of active origins increases. In contrast, in conditions that accelerate fork speed (addition of adenine and uridine to the culture medium), fewer origins are active. These studies further showed that the cell starts compensating for the decrease in fork speed within half an hour of treatment by activating dormant origins, which are then able to change their status within the S phase. Regulation of the number of initiation events occurs at the level of individual clusters, consistent with the functional organization of origins into replicon clusters [95]. Another study demonstrated that, in the absence of Cdc7 or ORC1, replication forks progress more rapidly than in control cells and fewer origins fire [96], again suggesting that the number of active origins and the fork rate are interdependent. Similarly, using chemical inhibitors of origin activity (a Cdc7 kinase inhibitor) and of DNA synthesis (APH), a more recent study found that the primary effects of replicative stress on fork rate can be distinguished from those on origin firing [97]. Collectively, these results support the conclusion that the density of origin firing depends on fork speed and, thus, is affected by endogenous or exogenous replicative stress.

### 2.4. CFS Fragility Due to the Lack of Dormant Origins

CFSs play a major role in cancer initiation because of their instability in conditions of replication stress. CFSs were first described as gaps and constrictions in the metaphase chromosomes of human lymphocytes grown under mild replication stress conditions (i.e., a low dose of APH) [98]. These observations were since seen in other organisms and are very likely to be the consequence of under-replication and/or DNA breaks caused by replication stress [99,100].

Although CFSs have been known for over two decades, the cause of their fragility is still controversial [55,101]. CFS fragility was first linked to non-B DNA sequences, such as AT-rich sequences, which are able to adopt secondary structures, constituting barriers to replication forks [102–105]. Deletion of these sequences from some cancer cell lines does not prevent breaks at these loci [106–108], suggesting that DNA sequence is not the sole reason for the instability of CFSs. Genome-wide analysis of replication and DNA combing experiments found a paucity of replication origins within the core of CFSs [109,110] and an incapacity to activate additional origins in response to replicative stress [111]. This suggests that, in order to replicate these regions, the fork must pass through long stretches of DNA containing multiple non-B DNA conformation sequences, and that their fragility correlates with the absence of additional replication origin firing when replication is

slowed down. Most CFSs correspond to long genes (>300 kb), which might increase the risk of collision between the transcription and replication machineries [112]. Although one study showed that the transcription of large genes does not systematically dictate CFS fragility [113], other studies found that replication stress induces locus- and cell-type-specific genomic instability at active, large transcription units corresponding to CFSs [114,115]. Moreover, it is thought that fragility of these sites result from entry into mitosis before their complete replication [116,117]. Taken together, these observations suggest that replication defects at fragile sites may be due to a low density of licensed origins or may reflect inefficient or delayed activation of replication forks under replication stress.

### 3. Regulation of Dormant Origins: A Passive or Active Mechanism?

#### 3.1. Activation of Dormant Origins by a “Passive” Mechanism

It is currently not clear what drives the firing of dormant origins when forks are slowed down or inhibited. One first hypothesis could be that it does not involve an active mechanism, but occurs as a consequence of the stochastic nature of origin firing [18,79]. Dormant origins have a precise lap of time to fire before being passively replicated then inactivated by forks from adjacent origins. When fork progression is impeded, the replication at dormant origins is delayed and, therefore, they have an increased probability to fire. By means of computational modeling, a study showed that the same levels of dormant origin activation seen *in vivo* can be reproduced by a passive mechanism [118]. In this model, the mechanism relies simply on the stochastic nature of origin firing, without any need for additional regulatory pathways.

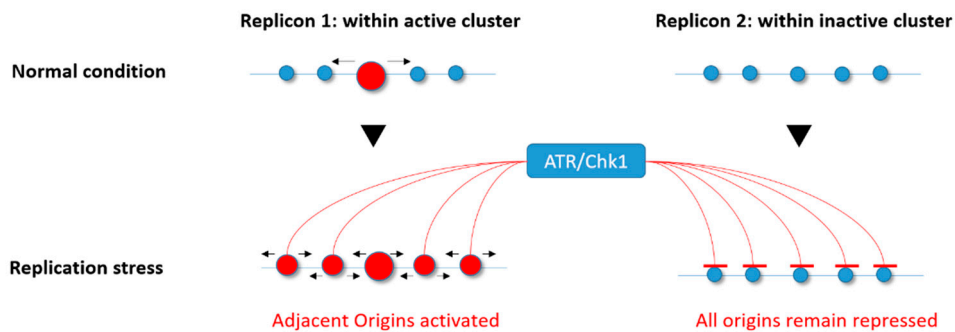
This simple theory can be sufficient to explain the activation of dormant origins in response to replicative stress. Nonetheless, it cannot be ruled out that dormant origins may also be regulated by active mechanisms, involving DNA damage response and other replication-related pathways.

#### 3.2. Regulation of Dormant Origins by “Active” Mechanisms

##### 3.2.1. ATR/Chk1 Kinases as Modulators of Origin Activation

The inhibition of replication forks activates the DNA damage checkpoint kinases ATR–Chk1 and ATM–Chk2, which have many different functions, including stabilizing replication forks, delaying or blocking the progress of the cell cycle, and promoting DNA lesion repair [119–121]. It may seem surprising that, in response to replication stress, the cell can both activate dormant origins and suppress overall origin initiation; however, when replication forks stall, it makes sense that dormant origins should be activated in their vicinity and not elsewhere in the genome.

In the normal S phase, Chk1 affects replication fork speed by inhibiting excess origin firing [23,85,86]. In response to low levels of replication stress induced by APH or HU, ATR and Chk1 impede the activation of new replicon clusters while allowing dormant origins to fire within those already activated and affected by the drug [79,81], thereby avoiding the deleterious impact of replication fork stalling (Figure 3). The mechanism responsible for this phenomenon is not yet elucidated, but one possibility is that ATR and Chk1 mildly reduce CDK levels, resulting in activation of fewer replication clusters [122]. Alternatively, Chk1 might directly inhibit the initiation process through an interaction with Treslin, which is required to stabilize Cdc45, GINS, and the MCM complex together with topoisomerase 2-binding protein 1 (TOPBP1) [123–127]. Moreover, a recent study found that an ATR inhibitor not only induced unscheduled origin firing, but also revealed another mechanism of origin regulation through a Cdc7-dependent phosphorylation of GINS [128]. Finally, a very recent study found that the ATR-activation domain of TOPBP1 is required to suppress origin firing during the S phase [129], further supporting an important role for the ATR–Chk1 pathway in regulating the activation of origins.



**Figure 3.** Ataxia telangiectasia Rad3-related (ATR)/checkpoint kinase 1 (Chk1) involvement in the differential regulation of origin firing under replicative stress. In response to replication stress, the ATR/Chk1 kinases allow the activation of dormant origins within active replicon clusters (active origin(s) in red) while repressing any firing within those that are not yet activated (inactive origins in blue).

### 3.2.2. Mannose Receptor C-Type 1 (Mrc1)/Claspin Is a Central Regulator of Origin Firing under Normal and Stressed Replication

*S. cerevisiae* Mrc1 and its metazoan ortholog Claspin are not only involved in the S phase checkpoint signaling pathway, but are also important components of replication forks. They interact with many factors known to function in or to regulate DNA replication, including MCM4, MCM10, ATR, Chk1, Cdc7, Cdc45, DNA polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$ , and proliferating cell nuclear antigen (PCNA) [130–133]. The presence of Mrc1/Claspin is necessary for normal DNA replication [91,92,134,135], probably by making a connection between the helicase components and replicative polymerases at the replication fork. Also, Claspin plays another role in the initiation of DNA replication in human cells during the normal S phase by recruiting Cdc7 to facilitate phosphorylation of MCM proteins [136]. It was recently discovered in yeast that Mrc1 has two crucial functions in regulating the firing of origins: a checkpoint independent-role to activate early-firing origins during normal replication, and a checkpoint dependent-function to inhibit late/dormant origins in the presence of HU [137].

### 3.2.3. Fanconi Anemia Proteins in the Regulation of Dormant Origins

The role of the Fanconi anemia (FA) pathway in the DNA repair of interstrand cross-links (ICLs) was studied for many years. A clear model emerged describing that FA proteins orchestrate the interplay between multiple DNA repair pathways, including homologous recombination (HR) and translesion synthesis (TLS) [138–140]. However, treatment of cells with a low dose APH robustly activates the FA pathway, indicating a role of the FA proteins during DNA replication [141].

FA complementation group 1 (FANCI) was shown to be involved in dormant origin firing upon low replication stress through a FA pathway-independent mechanism [142]. FANCI associates with MCM3 and MCM5, localizes with replication origins, and acts as a regulator of DDK activity to allow the activation of the MCM2–7 helicase complex in response to mild replicative stress. In contrast, under high replicative stress, FANCI is phosphorylated by ATR. This phosphorylated form of FANCI negatively regulates dormant origin firing and activates replication fork restart/DNA repair that is FA-dependent. In this context, FA complementation group D2 (FANCD2), which is known as a close partner of FANCI, acts as a negative regulator of dormant origin firing [142].

Finally, FANCD2 was shown to facilitate replication of repeat-rich genomic regions such as CFSs by decreasing DNA–RNA hybrid accumulation, thus reducing the need for dormant origin firing [143].

### 3.2.4. Rap1-Interacting Factor 1 (RIF1) Orchestrates Origins and Replication Timing

RIF1 (Rap1-interacting factor 1) was first discovered in budding yeast as a telomeric chromatin-interacting protein required for the regulation of telomere length via its interaction with Rap1 [144,145]. It was then demonstrated in *S. cerevisiae* that RIF1 inhibits activation of the DNA

damage checkpoint close to telomeres [146,147] and affects telomere replication timing [148]. Although the RIF1 protein is evolutionarily conserved, in metazoans, it was described not to play a specific role at telomeres, but rather to orchestrate the DNA double-strand break repair pathway and DNA recombination [149–153].

Further studies implicated RIF1 from the fission yeast *Schizosaccharomyces pombe* and mammalian RIF1 in regulating genome-wide DNA replication. *S. pombe* RIF1 binds selectively not only to telomeres, but also to specific regions of the genome where it may regulate the choice and timing of origin firing in late-replicating regions of chromosomes [154]. In RIF1-deficient cells, activation of dormant or late origins is concomitant with suppression of some active early-firing origins, indicating that RIF1 is a crucial player in the genome-wide origin activation program in *S. pombe*. In human cells, depletion of RIF1 results in increased early-S phase initiation events, loss of mid-S phase replication foci, and global changes in replication timing domain structures. Domains that normally replicate in the early S phase are delayed, whereas those that normally replicate in the late S phase are advanced [155]. Thus, replication timing is completely disturbed in the absence of RIF1. Another study observed that, in the absence of RIF1, the distance between origins is greater than in control cells during the normal S phase, and there are fewer dormant origins upon replication stress [156].

Also, RIF1 binds tightly to insoluble nuclear structures in late mitosis and the early G1 phase, and regulates chromatin-loop size [155]. Interestingly, RIF1 binding to consensus G-quadruplex-like sequences in fission yeast was identified [157]. These sequences tend to be near dormant origins, and the binding of RIF1 on these sites would allow their repression over a great distance. Overall, these findings indicate that RIF1, through its role in organizing higher-order chromatin architecture, is an essential regulator of replication timing.

Thus, the accumulating data suggest that, through its interaction with chromatin and nuclear structures, RIF1 plays an important role in the regulation of dormant origin availability not only in response to replicative stress, but also in normal conditions.

### 3.2.5. Chromatin Loop Size Correlates with Dormant Origin Activation

The fluorescent DNA halo technique was essential for establishing the link between chromatin loops and replicon size [158], and for describing the importance of replicon remodeling events in *Xenopus* embryonic development [159]. Basically, the technique relies on cell permeabilization and soluble protein extraction, allowing supercoiled DNA loops to unroll around an insoluble scaffold, the nuclear matrix. Those structures called DNA “halos” can be visualized by 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining. Active origins are in or near the nuclear matrix, whereas dormant/inactive origins are in the DNA loops [160] (Figure 2C).

Using the fluorescent DNA halo technique, one study [62] observed a strict correlation between dormant origin activation at a given S phase and reduced chromatin loop size in the next G1/S phase. Combining the DNA halo experiment with fluorescent in situ hybridization (FISH) using a probe targeting the highly amplified adenosine monophosphate deaminase 2 (AMPD2)-specific locus in CHO cells, they demonstrated that, in response to replication stress, activation of dormant origins relocates this locus toward the nuclear matrix.

Cohesin also influences the size of interphase chromatin loops since its absence results in longer chromatin loops due to a limited origin usage [43], showing that, independently of the effect of cohesin acetylation on replication fork progression [161], this structural protein is present at origins and impacts their activity. Finally, chromatin loop size increases in RIF1-depleted cells [155], suggesting that the RIF1 protein is required for proper chromatin loop formation, as already mentioned above.

## 4. Dormant Origin Deficiency, Genome Stability, and Pathologies

### 4.1. MCM Mutants and Dormant Origins in Mice

Homozygosity for a null allele of any of the six *Mcm* genes in mice (*Mcm2–7*) causes embryonic lethality [162–164], consistent with the evidence that these *Mcm* genes are essential for DNA replication. Only hypomorphic alleles such as *Mcm4<sup>Chaos3</sup>* and *Mcm2<sup>IRES-CreERT2</sup>* (IRES, internal ribosome entry site; ERT2, estrogen receptor 2) result in mice that are viable into adulthood. The *Mcm2<sup>IRES-CreERT2</sup>* allele expresses a tamoxifen-inducible Cre recombinase (CreERT2) inserted into the 3' untranslated region (UTR) of the endogenous *Mcm2* locus, which reduces the expression of MCM2 by 65% when compared to wild-type cells [165]. The *Mcm4<sup>Chaos3</sup>* allele produces an MCM4 protein with a Phe345Ile mutation, which does not affect the helicase activity of the MCM complex in vitro, but does reduce the efficiency of its assembly [164].

Surprisingly, mouse embryonic fibroblasts (MEFs) from *Mcm4<sup>Chaos3</sup>* mice also have a reduced MCM7 protein level in addition to MCM4 [164]. Moreover, immortalized homozygous *Mcm4<sup>Chaos3</sup>* cells display less stable association of MCM2–7 at replication forks compared to wild-type cells [166]. Finally, *Mcm4<sup>Chaos3/Chaos3</sup>* MEFs exhibit about a half reduction in chromatin bound MCM2–7 that causes a lower ability to activate dormant origins in response to treatment with low doses of APH [162,167].

Mice with only one-third of the normal MCM2 level were shown to develop lymphomas at a very young age, and have diverse stem cell proliferation defects. Similarly to *Mcm4<sup>Chaos3</sup>*, these mice also have 27% less MCM7 protein than wild-type mice. Moreover, *Mcm2<sup>IRES-CreERT2</sup>* cells exhibit decreased replication origin usage due to lower dormant origin availability even in the presence of HU, as demonstrated by DNA combing experiments [165,168].

Hence, these two mouse models are close phenotypically, showing dormant origin deficiency due to reduced levels of loaded MCM onto the chromatin. Even in an unchallenged S phase, the inability to activate dormant origins leads to accumulation of stalled replication forks that reach mitosis and interfere with chromosome segregation. Both phenotypes lead to improper chromosome stability and premature tumorigenesis, with several differences in the latency of disease development [165,166,168,169].

### 4.2. MCM Mutants and Dormant Origins in Stem/Progenitor Cells

The fact that *Mcm2* expression has a global effect on cell proliferation within many tissues might explain why the majority of *Mcm2<sup>IRES-CreERT2</sup>* mice develop tumors and display a range of additional hallmarks of age-related disorders. A study that set out to determine the effect of *Mcm2* deficiency observed an approximately threefold reduction in the level of neurogenesis within the sub-ventricular zone in *Mcm2<sup>IRES-CreERT2</sup>* mouse brains [165], fewer stem cells in intestinal crypts and in skeletal muscle, and a modest increase in DNA damage.

Consistent with the conclusion that *Mcm* mutants affect stem cells, neural stem-cell progenitors in *Mcm4<sup>Chaos3/Chaos3</sup>* mouse embryos display a high level of Chk1 activation, increased phosphorylated H2A histone family X ( $\gamma$ H2AX) and p53-binding protein 1 (53BP1) foci, an accumulation in the G2–M phase, and more apoptosis, resulting in a reduced ability to form neurospheres in vitro [170]. The renewal of stem cells in the brain appears to be normal, but their ability to differentiate into intermediate progenitors is highly reduced due to an increase of apoptotic cells in the sub-ventricular and intermediate zones [170].

These observations suggest that normal expression of MCM complex proteins is essential for stem/progenitor cell function by reducing the risk of replication-associated genome instability, an idea that was supported by two other studies. One demonstrated that human embryonic stem cells, which have a remarkably short G1 phase, load MCM onto chromatin very rapidly when compared to differentiated cells, in order to have a similar total amount of loaded MCM at the G1–S phase transition [171]. In the second study, hypomorphic expression of the origin licensing factor MCM3 in mouse reduced the number of licensed origins and affected the function of hematopoietic stem

cells, as well as the differentiation of highly proliferative erythrocyte precursors, thus demonstrating that the rate of MCM loading is crucial for correct organism development [163]. These observations suggest that hematopoietic progenitors are exceptionally sensitive to replication stress, and that they must license an excess of origins to ensure their correct differentiation and function.

Intriguingly, aging hematopoietic stem cells suffer from replication stress even in wild-type mice. This might be due to the fact that old stem cells have reduced expression of MCM complex proteins, resulting in reduced numbers of dormant origins and, as a consequence, more chromosome instability and cell-cycle defects [172].

#### 4.3. Consequences of Limited Licensing and Firing in Humans

A mutation in the *Mcm4* gene, which results in a truncated form of this protein lacking the N-terminal serine/threonine-rich domain, was identified in a group of patients with a syndrome including growth delay, natural killer cell deficiency, adrenal insufficiency, and genome instability [173–175]. This truncated form of MCM4 does not affect MCM complex loading [174]. Nevertheless, immortalized fibroblasts from these patients have a high level of chromosome breakage and defects in cell-cycle progression, and they are sensitive to low doses of APH [174], suggesting that the N-terminal amino acids of MCM4 protein are involved in the maintenance of genome integrity during replication. Further studies will be necessary to elucidate the mechanism via which normal MCM4 ensures genome maintenance. One possibility is the role of MCM4 phosphorylation in the checkpoint response, where it was shown that the N-terminal domain of MCM4 has a crucial role. In unperturbed replication, this domain exerts an inhibitory effect on replication initiation, and this inhibitory effect is relieved upon its phosphorylation by DDK. However, in the context of replication stress, this N-terminal phosphorylation by DDK becomes a prerequisite for proper checkpoint activation [176].

Another disease that appears to involve defective replication origin licensing is Meier–Gorlin syndrome (MGS), an autosomal recessive primordial dwarfism syndrome characterized by pre- and post-natal impaired growth. Several studies identified marked locus heterogeneity in this syndrome including mutations in five genes encoding components of the Pre-RC: *Orc1*, *Orc4*, *Orc6*, *Cdt1*, and *Cdc6* [177,178]. The molecular and cellular phenotypes include impaired licensing, altered S phase progression, and proliferation defects, which partially overlap with the phenotypes due to MCM mutations, except for chromosomal instability, and an increased predisposition to cancer. Nonetheless, MGS mutations in *Orc1* and *Orc6* can cause quite a significant reduction in MCM loading and replication origin licensing [177,179,180].

Mice and human phenotypes caused by mutations in the licensing system illustrate our limited understanding of what happens to cells when the DNA replication program is compromised. For example, the threshold value for the number of licensed origins needed to activate the licensing checkpoint is still not known, nor whether this value varies between cell types.

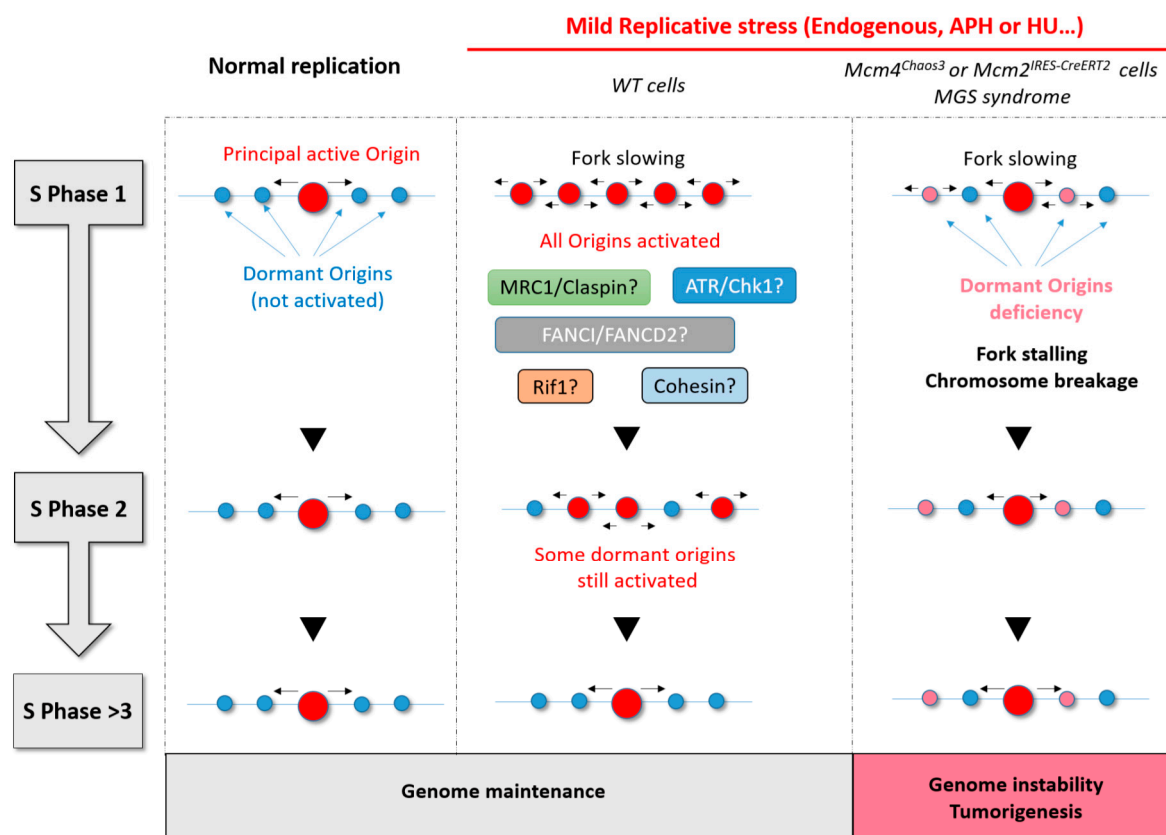
## 5. Conclusion and Prospects

Dormant origins are now recognized as an important safeguard against under-replication of the genome, thus ensuring genome maintenance. Activation of dormant origins plays a central role in the rescue of stalled forks in the context of replicative stress, contributing to the complete replication of the DNA. The interactions between dormant origins and other fork restart mechanisms (such as TLS) are mostly unknown, even though some links with DNA damage checkpoint or FA pathways are becoming evident. What determines whether the cell activates dormant origins or induces these other mechanisms in response to fork stalling still remains to be investigated.

How replicon clusters are activated at the molecular level remains unclear, although we know that origin activation is regulated by both Chk1 and CDKs. The RIF1 protein might be the most interesting factor in this process since it is present both at the replication fork and at replication origins, where it plays a role in the DNA damage response, as well as in replication timing.

The study that found a direct correlation between origin activation and chromatin loop size [62] also reported that origins located near the anchorage sites of chromatin loops are preferentially activated in the S phase of the following cell generation (Figure 4). This suggests that cells respond to changes in fork dynamics by adapting origin usage in the next cell cycle, in addition to their rapid response of origin activation. It appears that cells can adapt to grow under conditions of fork slowing by increasing the efficiency of some origins that are usually dormant in normal growth conditions.

Perhaps most exciting is the prospect that the regulation of dormant origins might be different in cancer cells to that in normal cells. MCM complex proteins are often misregulated at the early stage of cancer [18,181,182], and tumor cells are more sensitive to replicative stress when they have a reduced origin licensing capacity [183]. Mice hypomorphic for *Mcm* gene expression demonstrate the real importance of dormant origins, but any link with spontaneous cancer development remains to be determined to see whether this information can be useful to deal with anti-cancer molecules more accurately.



**Figure 4.** Summary diagram showing the importance of dormant origin activation in response to replicative stress. During normal replication, only the principal origin is activated. If there is no replicative stress, this same principal origin is also activated in the next S phase. Under conditions of mild replicative stress, adjacent or dormant origins fire to compensate for fork slowing and to allow complete replication on time. Many proteins (ATR/Chk1, mannose receptor C-type 1 (Mrc1)/Claspin, Fanconi anemia complementation group 1 (FANCI)/ Fanconi anemia complementation group D2 (FANCD2), and Rap1-interacting factor 1 (RIF1)) are thought to be involved in the regulation of dormant origins under mild replicative stress. RIF1 and Cohesin are two good candidates to explain the persistence of some origin activation in the next S phase. Finally, when cells have few origins or a deficiency in dormant origins, replicative stress leads inevitably to fork stalling, DNA breaks, and genomic instability with a consequent risk of tumorigenesis.



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## Abbreviations

|          |  |
|----------|--|
| 53BP1    | p53-Binding protein 1                            |
| APH      | Aphidicolin                                      |
| ATM      | Ataxia telangiectasia mutated                    |
| ATR      | Ataxia telangiectasia and Rad3-related protein   |
| BLM      | Bloom syndrome RecQ like helicase                |
| BRCA2    | Breast cancer 2                                  |
| Cdc45    | Cell division cycle protein 45                   |
| Cdc6/7   | Cell division cycle 6/7                          |
| CDK      | Cyclin-dependent kinase                          |
| Cdt1     | Chromatin licensing and DNA replication factor 1 |
| CFS      | Common fragile site                              |
| Chk1     | Checkpoint kinase 1                              |
| Chk2     | Checkpoint kinase 2                              |
| CHO      | Chinese hamster ovary                            |
| c-Myc    | Myelocytomatosis                                 |
| DDK      | Dbf4-dependent kinase                            |
| DNA      | Deoxyribonucleic acid                            |
| dNTP     | Deoxyribonucleotides                             |
| ERFS     | Early-replicating fragile site                   |
| FANCI/D2 | Fanconi anemia complementation group I/D2        |
| FISH     | Fluorescence in situ hybridization               |
| GINS     | Go-ichi-ni-san                                   |
| HR       | Homologous recombination                         |
| HRAS     | Harvey rat sarcoma                               |
| HU       | Hydroxyurea                                      |
| ICL      | Interstrand cross-link                           |
| MCM      | Minichromosome maintenance                       |
| MGS      | Meier–Glorin syndrome                            |
| MMEJ     | Micro-homology mediated end-joining              |
| Mrc1     | Mannose receptor C-type 1                        |
| NHEJ     | Non-homologous end-joining                       |
| ODP      | Origin decision point                            |
| ORC      | Origin recognition complex                       |
| PCNA     | Proliferating cell nuclear antigen               |
| Pre-IC   | Pre-initiation complex                           |
| Pre-RC   | Pre-recognition complex                          |
| RIF1     | Rap1-interacting factor 1                        |
| RNA      | Ribonucleic acid                                 |
| RT       | Replication timing                               |
| SNS      | Short nascent strand                             |
| SVZ      | Sub-ventricular zone                             |
| TDP      | Timing decision point                            |
| TOPBP1   | Topoisomerase 2-binding protein 1                |
| TLS      | Translesion synthesis                            |
| TSS      | Transcription start sites                        |

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## DNA replication timing

Given the large size of eukaryotes genome, mechanisms of regulations are needed to orchestrate the challenging process of DNA replication. Among these mechanisms, lies DNA replication timing that consist in regulating the temporal order of replication origins activation. This mechanism is essential to avoid re-replication or under-replication, protein and nucleotide exhaustion or DNA damage while ensuring fast and faithful duplication of the highly complex whole genome.

## a. Replication timing: the temporal regulation of DNA replication

Replication timing phenomenon has not been discovered recently but almost a century ago and its description and characterization evolved together with advances in genomic approaches. This first part aims to present replication timing following the timescale of scientific discoveries regarding this phenomenon.

### i. First evidences of replication timing

Evidences of a DNA replication timing in eukaryotes were described 80 years ago thanks to autoradiography of tritium labelled thymidine incorporation in mammalian cells genome, showing that chromosome replicate asynchronously, and identifying chromosome units, of various size, that replicates at different times during the S phase (**Figure 5**) (Goldman et al., 1984; Lima-De-Faria and Jaworska, 1968; Taylor, 1960; Taylor et al., 1957). This autoradiography technique allowed researchers to study replication fork rate and origin activation, which lead to the conclusion that replication timing is not due to replication fork rate variation but to differential time of origin activation (Rivin and Fangman, 1980). Later, it was shown by immunofluorescence staining of nucleotides analogs that replication timing of a given chromosome loci is always identical and transmitted through cell generations (Ma et al., 1998).

During the replication process, origins fire in many discrete sites that can be visualized by immuno-labelling of thymidine analogues. Different foci pattern exist within S phase nuclei among which we can identify small early replication foci predominantly at the centre of the nucleus and late replicating foci that are bigger and usually found at the nuclear or nucleolar periphery (Nakamura et al., 1986). This three dimensional replication foci pattern is highly conserved in eukaryotes among species, as it has been observed in plants and other animals as well (Alexandrova et al., 2003; Dimitrova and Berezney, 2002; Mayr et al., 2003; Postberg et al., 2005).

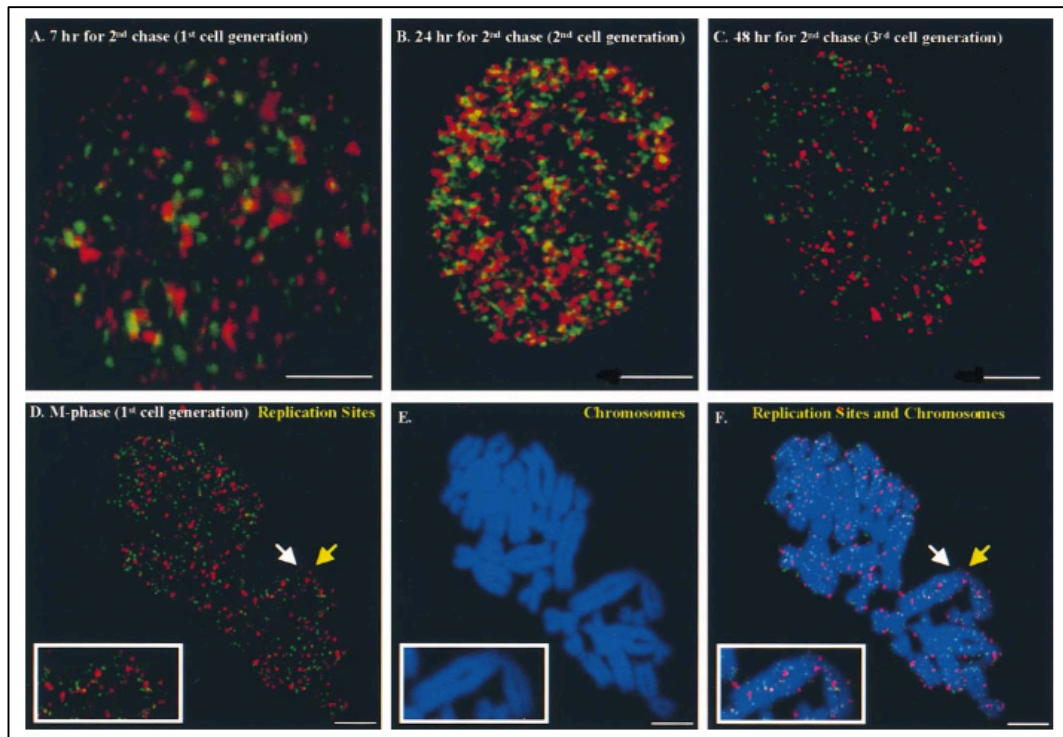
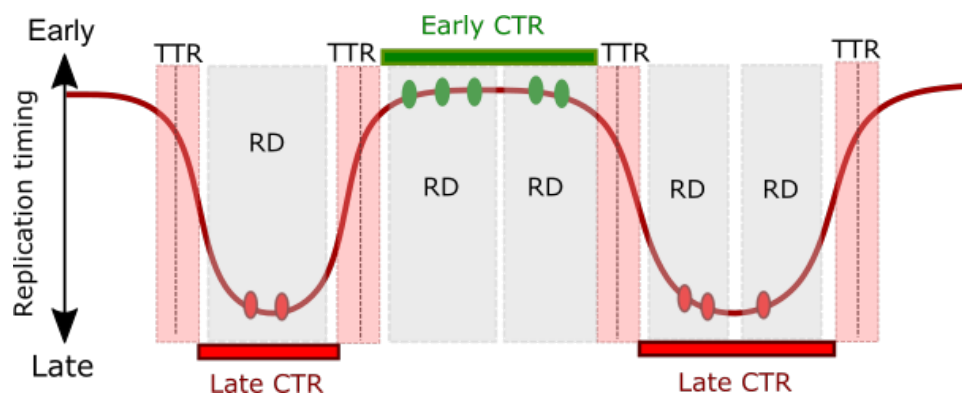


FIGURE 5: VISUALISATION OF REPLICATION TIMING BY DOUBLE PULSE–DOUBLE CHASE EXPERIMENTS. REPLICATION SITES IN SYNCHRONIZED MOUSE 3T3 FIBROBLASTS WERE FIRST LABELLED FOR 2 MIN WITH CLDU (GREEN), CHASED FOR 2 H, AND THEN PULSED FOR 5 MIN WITH IDU (RED). CELL WERE THEN CHASED A SECOND TIME FOR (A) 7 H (LATE S- OR G2-PHASE); (B) 24 H (2<sup>ND</sup> CELL GENERATION); (C) 48 H (3<sup>RD</sup> CELL GENERATION); OR (D–F) 12 H (M-PHASE). (E) CHROMOSOMES WERE COUNTERSTAINED WITH PROPODIUM IODIDE (PI) (BLUE). BARS, 5  $\mu$ M. (FROM MA ET AL., 1998)

## ii. Identification of replication timing domains

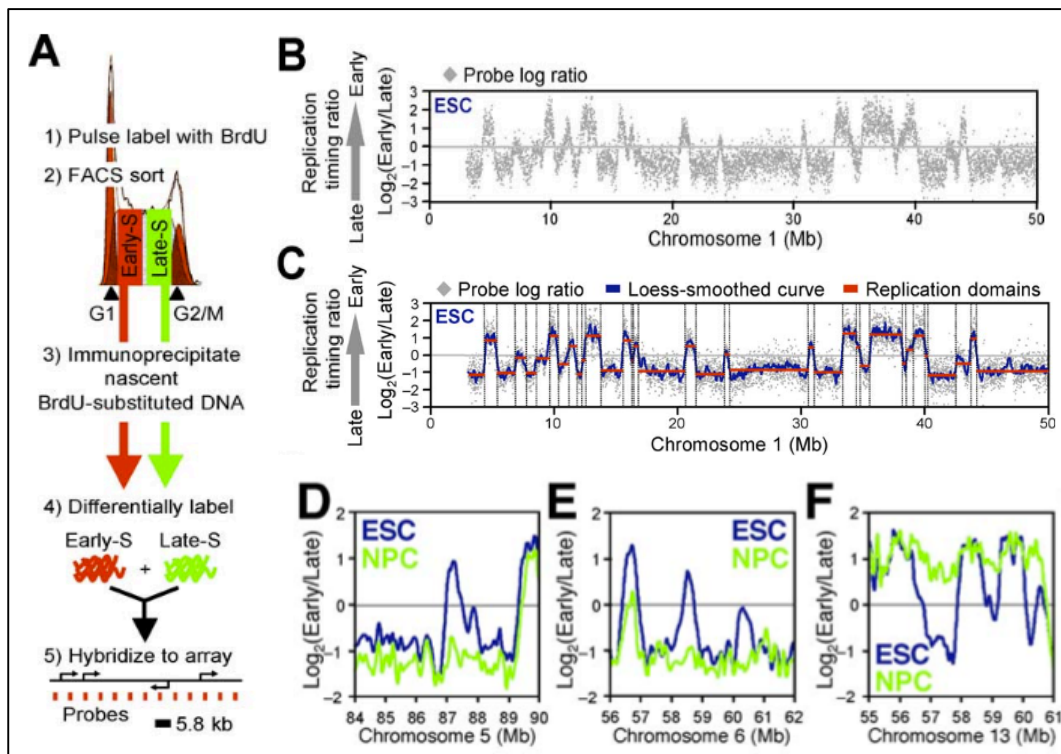
Modern genomic approaches, that map mean replication timing in a population of asynchronous cells, resulting with replication timing profiles, revealed that chromosomes are mosaic structures of early and late replicating domains (1.5–2.5Mb mean size) separated by relatively sharp boundaries (**Figure 6**). Segments that have a relatively uniform replication timing are called constant timing regions (CTR), delimited by temporal transition regions (TTR). CTR replicate in coordination with non-random multiple origins activation in a specific time along the S phase, determined by the replication timing itself (Desprat et al., 2009; Farkash-Amar and Simon, 2010; Farkash-Amar et al., 2008; Hiratani et al., 2008; Woodfine et al., 2004). Thus, even though regulation of origin activation within CTR is not completely understood, it is well established that temporal activation of origin within CTR is tightly and non-randomly regulated by the replication timing (Courtot et al., 2018; Rhind, 2006; Rhind et al., 2010;

Rivera-Mulia and Gilbert, 2016). In human cells, replication timing is characterized by synchronous and robust activation of replicons, creating replication factories of about 400-800kb, called replication domains (RDs) (**Figure 6**).



**FIGURE 6:** DIAGRAM OF REPLICATION TIMING DOMAINS IN HUMAN CELLS. CONSTANT TIMING REGIONS (CTR) REPLICATE IN EARLY S (GREEN, EARLY CTR) OR IN LATE S (RED, LATE CTR) AND ARE DELIMITED BY TRANSITION TIMING REGIONS (TTR). CTR CONTAINS MANY REPLICATION ORIGINS (OVAL SHAPE) THAT ARE NOT ALWAYS ACTIVE IN EACH CELL CYCLE. CLUSTER OF ORIGINS THAT HAVE ALWAYS THE SAME REPLICATION TIMING WITHIN SPECIFIC CELL TYPE ARE CALLED REPLICATION DOMAINS (RDs).

The mapping of whole-genome replication timing by oligonucleotide arrays, after Early-S and Late-S cell sorting, allowed strong advances in replication timing program characterization. Thanks to the ability to compare the replication timing profile from cells that come from different tissue or different stage of differentiation (**Figure 7**), these studies demonstrate that chromosomes can be segmented in replication timing domains and that these RDs are well conserved in the same cell type. Another complementary study in human cells led to the same observations (Desprat et al., 2009; Hiratani et al., 2008). In both studies, they identified RDs where replication timing is modified during development (**Figure 7D and F**), corresponding to 20-45% of the genome. Finally, they defined smaller replication domains and higher density of TTR as specific characteristics of replication timing in pluripotent cells. A recent approach called E/L Repli-seq, very close but more powerful than Repli-ChiP (Ryba et al., 2011b), confirmed replication timing profiles obtained by Repli-ChiP (Brison et al., 2019a; Marchal et al., 2018). However, these approaches are based on bulk cells population, which means that it only represents an average of many cells and not the exact replication timing of a given cell.



**FIGURE 7:** GENOME-WIDE ANALYSIS OF REPLICATION TIMING IN mESCs AND NPCs. (A) PROTOCOL FOR REPLICATION TIMING ANALYSIS USING 5.8KB PROBES OLIGONUCLEOTIDES ARRAY. CELLS ARE PULSE-LABELLED WITH BRdU FOR 2H AND SORTED BY FACS INTO TWO FRACTIONS (EARLY-S AND LATE-S). EACH FRACTION IS DIFFERENTIALLY LABEL (EARLY-S IN RED, LATE-S IN GREEN) THEN HYBRIDIZE INTO A MOUSE WHOLE-GENOME MICROARRAY (NIMBLEGEN SYSTEMS). THE RATIO OF THE ABUNDANCE OF EACH PROBE IN THE EARLY AND LATE FRACTION ( $\text{LOG}_2(\text{EARLY}/\text{LATE})$ ) WAS THEN USED TO GENERATE A REPLICATION-TIMING PROFILE FOR THE ENTIRE GENOME (B) EXAMPLE OF ROW REPLICATION TIMING PROFILE FOR CHROMOSOME 1 IN mESCs. (C) IDENTIFICATION OF REPLICATION DOMAINS (RED LINES) AND THEIR BOUNDARIES (DOTTED LINES) BY A SEGMENTATION ALGORITHM AND REPRESENTATION OF A LOCAL POLYNOMIAL LOESS SMOOTHING CURVE (BLUE LINE); FOR CHROMOSOME 1 IN mESCs. (D-F) EXAMPLES OF REPLICATION TIMING MODIFICATIONS DURING mESCs TRANSFORMATION INTO NPCs. IN (D) AND (E), WE CAN OBSERVE SWITCHES FROM EARLY TO LATE (ETOL) AND IN (F) FROM LATE TO EARLY (LTOE). (ADAPTED FROM HIRATANI ET AL., 2008)

Thus, to assess the question of cell-to-cell heterogeneity in replication timing, single-cell whole genome replication timing have been set-up (Dileep and Gilbert, 2018; Donaldson and Nieduszynski, 2019; Hiratani and Takahashi, 2019; Takahashi et al., 2019). Using scRepli-seq, it has been demonstrated that replication domain organization is very well conserved between individual mouse cells (mESCs and differentiated mESCs) (Takahashi et al., 2019). In another context, single cell versus bulk cell replication timing has been assessed and again it was observed that cell-to-cell replication timing profiles variability was very low (Shaikh et al., 2019); confirming the robustness of replication timing. These discoveries are of a great importance because it helped to define DNA replication timing phenomenon as a robust cellular program, very well conserved among mammalian species and in which changes have

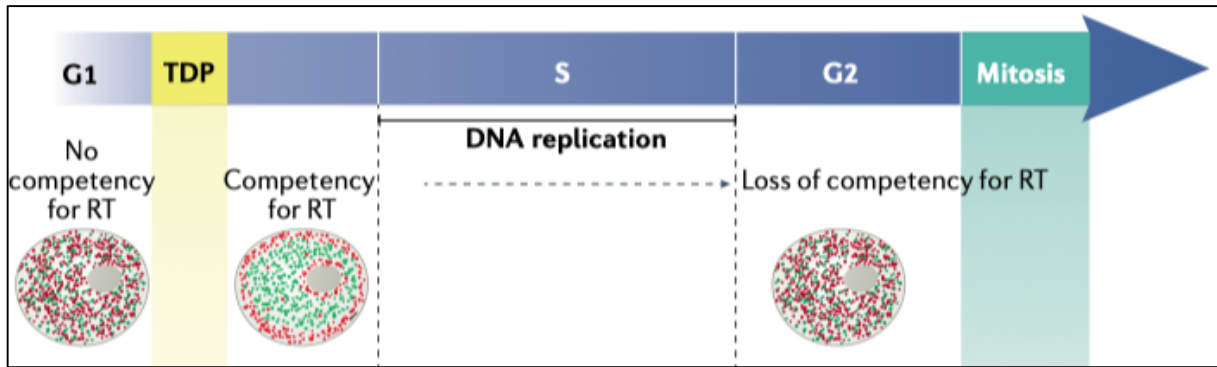
a real developmental significance. Altogether, these observations suggest that DNA replication timing is a very well-regulated phenomenon.

### iii. The replication timing decision point (TDP)

Replication timing reflects the temporal activation of replication origins during the S phase, the moment of the interphase when DNA is actually replicated. Nonetheless, it has been shown that the decision of when a given origin is activated within a specific genomic region, takes place long before the entry into S phase. By monitoring early and late replication domains throughout the cell cycle and introducing pre-labelled nuclei into *Xenopus* eggs extracts, it was demonstrated that the replication program was established in early G1 phase (Dimitrova and Gilbert, 1999a). During a brief period of time in early G1 phase, large scale 3D chromatin remodelling happens to give rise to nuclear arrangement of replication factories, called “timing decision point” (TDP), establishing the replication timing program of the next S phase (**Figure 8**). The TDP is re-established in each cell cycle, giving a window of opportunity for potential replication timing changes during cellular differentiation (Dimitrova and Gilbert, 1999a; Lu et al., 2010).

Up to now, mechanisms under TDP are not well described, but it has been shown that epigenetic modifications, pre-replication complex formation and transcription reestablishment are all upstream events of TDP (Dimitrova and Gilbert, 1999a; Wu et al., 2006). Thus, chromatin remodelling is the most correlated event to TDP because chromatin mobility is very high in early G1 while chromatin motion is locally limited during the rest of interphase (Thomson et al., 2004; Walter et al., 2003). Even though replication timing information is lost during G2 phase (Lu et al., 2010), early and late replication foci detected by replication labelling in the previous S phase are still here at the TDP and persist until the next G2 phase. This demonstrates that spatial organization is not sufficient to dictate replication timing and suggesting that it exists a “memory” of the previous replication timing, probably carried out by epigenetic modifications.





**FIGURE 8:** REPLICATION TIMING DECISION POINT (TDP) IS ESTABLISHED DURING THE G1 PHASE. REPLICATION TIMING AND CHROMATIN ARCHITECTURE ARE BOTH TIMELY COINCIDENT WITH THE TDP IN G1 PHASE. THERE IS EVIDENCE THAT REPLICATION TIMING DETERMINANTS ARE LOST DURING THE G2 PHASE. MAJOR ARCHITECTURAL CHANGES IN GENOME ARCHITECTURE OCCUR DURING ENTRY INTO AND EXIT FROM MITOSIS. (FROM MARCHAL ET AL., 2019)

## b. Structural determinants of replication timing

Thanks to the establishment of replication timing profile of entire genomes, it has been possible to quickly highlight correlations between genome, epigenome features, gene expression, 3D nuclear organization and DNA replication timing. However, the understanding of structural determinant of replication timing is still in progress, and it is very difficult to distinguish a key player among all.

### i. DNA sequence features

In higher eukaryotes, there is a clear correlation between features of primary genomic sequence and DNA replication timing. Indeed, early replication occurs in GC rich isochores, (Marchal et al., 2019) whereas late replicating regions are associated to repetitive elements, AT rich and Long Interspersed Element (LINE) rich (**Figure 9**) (Lander et al., 2001). To have a better idea of the role of DNA sequence features in replication timing and gene expression, people are working with the very well-characterized and conserved  $\beta$ -globin locus. This chromosomal domain is a good tool to study replication timing determinants because it is replicated in early S phase in erythroid cell and late S phase in cell types that do not express  $\beta$ -globin (Epner et al., 1988; Mn et al., 2003). Interestingly, ectopic insertion of the  $\beta$ -globin locus control region (LCR) into a specific isogenic locus normally replicated in mid-late S phase and devoid of replication origins led to local advance of replication timing (Hassan-Zadeh et

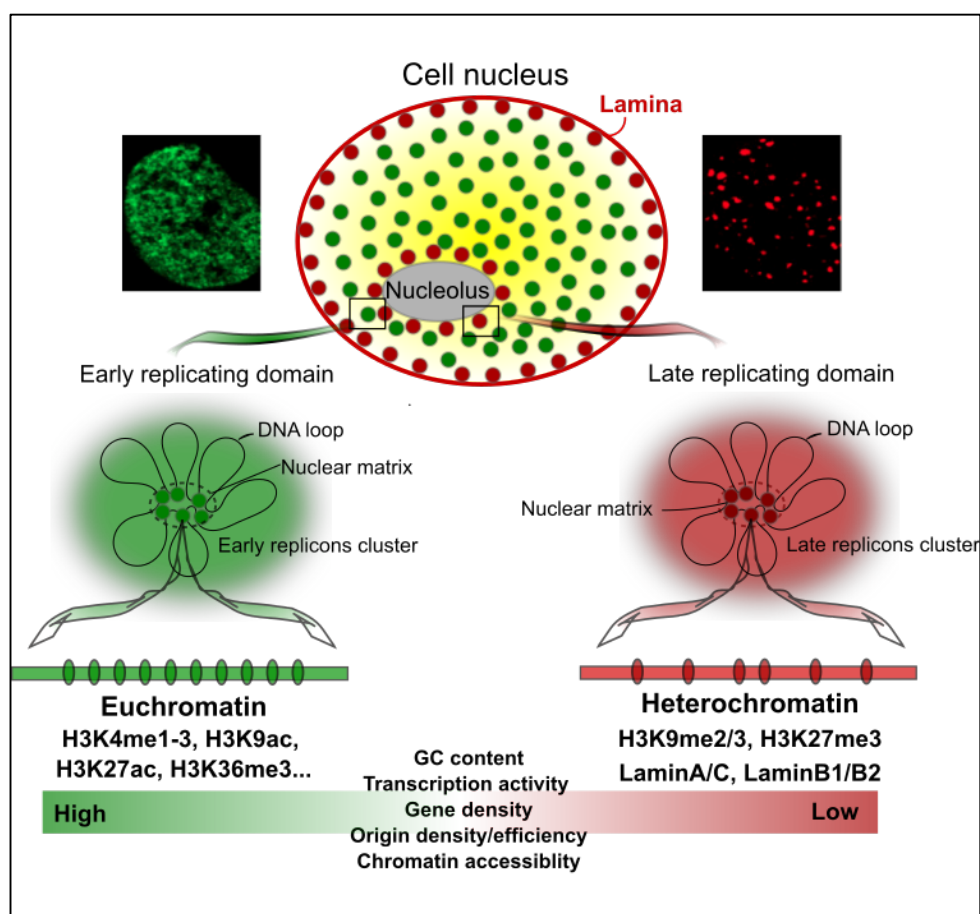
al., 2012; Simon et al., 2001). In the human genome, significant modifications in nucleotide composition coincide with peaks of earlier replication timing at about a thousand sites (Chen et al., 2011; Touchon et al., 2005). These modifications are believed to arise from different mutation frequencies due to fork polarity (leading versus lagging strand), leaving evolutionary fingerprint in the sequence. Homologous regions from the same chromosome can replicate at different times in the same cell (Farkash-Amar et al., 2008; Hansen et al., 2010). By constructing separate replication timing profiles for each homologous chromosome in human erythroblasts, a study identified asynchronously replicating regions enriched in imprinted genes that are linked to large structural variations but not to the DNA sequence (Mukhopadhyay et al., 2014). Altogether, even though sequence composition can be associated to replication timing, it was reported more than forty years ago that RT was more related to chromosome localization than DNA sequence (Calza et al., 1984), proving that this latest is not sufficient to dictate RT.

## ii. Replication origins

As the replication origin matter has been already developed in the first part of the introduction, the main goal of this part is to debate the eventual link between replication origins efficiency and replication timing.

While it is known that replication origins firing during S phase goes hand in hand with replication timing, the relationship between replication timing and replication origins remains unclear. It is well accepted that early replicating domains contains higher origin density and efficiency than late replicating domains (**Figure 9**) (Dellino et al., 2013; Guilbaud et al., 2011; Langley et al., 2016; Mesner et al., 2013; Miotto et al., 2016; Petryk et al., 2016). However, the origin density within replication domains that switch from late to very early replication during development is not significantly different from constitutively late replicating regions (Besnard et al., 2012; Dileep et al., 2015a). Many evidences support the fact that replication timing acts upstream of origin firing (Rivera-Mulia and Gilbert, 2016). In budding yeast, forkhead transcription factors (Fkh1, Fkh2) regulates replication timing upstream and independently of their role in origin activation (Fang et al., 2017; Ostrow et al., 2017). A study in allele-specific replication timing showed that asynchrony could be linked to changes in origin efficiency but not to origin choice, which is consistent with the independency of origin

sites and timing of origin firing (Bartholdy et al., 2015). Studies on dormant origins, that showed their activation in response to low replication stress, do not mention a modification of replication timing in this context (Alver et al., 2014; Blow and Ge, 2008, 2009; Blow et al., 2011; Courbet et al., 2008a). Thus, we can suggest that dormant replication origin activation is a back-up mechanism for cells to respect the replication timing despite replication stress inducing fork slowing. Overall, more studies are needed to have a clear view on the topic but it looks like it is replication timing that will be more determinant for replication origin activation than the opposite.



**FIGURE 9:** GLOBAL OVERVIEW EARLY AND LATE REPLICATING DOMAINS CHARACTERISTICS. EARLY AND LATE REPLICATION FOCI ARE EASILY DISTINCT WITHIN S PHASE NUCLEUS BY THEIR DIFFERENT PATTERNS. EARLY FOCI (IN GREEN) ARE VISUALIZED AS SMALL POINT IN THE CENTRE OF THE NUCLEI AND LATE FOCI (IN RED), BIGGER, AT THE NUCLEAR AND NUCLEOLAR PERIPHERY. FOCI (EARLY OR LATE) ARE ACTUALLY A CLUSTER OF REPLICONS, REPLICATING IN PARALLEL. EARLY REPLICATING DOMAINS ARE EUCHROMATIN (H3K4ME1/2/3, ETC.), WITH HIGH GC CONTENT, ACCESSIBILITY, GENE AND ORIGIN DENSITY AND TRANSCRIPTION ACTIVITY WHEREAS LATE REPLICATING DOMAINS ARE HETEROCHROMATIN (H3K9ME3...) CHARACTERIZED BY POISED TRANSCRIPTION, POOR GC CONTENT, ACCESSIBILITY, GENE AND ORIGIN DENSITIES.

### iii. Transcription activity

In the early 1960s, experiments conducted on frog embryos described “G bands” (Giemsa dark chromosome bands) as genetically inactive heterochromatin, replicating in late S phase. On the contrary, they observed that light Giemsa bands or “R bands”, replicate early in the S phase and are the loci where most transcription takes place (Stambrook and Flickinger, 1970) (**Figure 9**). The link between transcriptional activity and replication timing has been reinforced in many complementary studies on drosophila, mouse and human cells (Desprat et al., 2009; Goldman et al., 1984; Hiratani et al., 2008; Karnani et al., 2007, 2010; MacAlpine et al., 2004; Müller and Nieduszynski, 2017; Schübeler et al., 2002; Simon et al., 2001; Taljanidisz et al., 1989; White et al., 2004; Woodfine et al., 2004). Briefly, these studies showed that genes within RDs that shifted from early to late replication are silenced and conversely, increased gene transcription is observed in RDs whose replication timing switch from late to early replication. In mammals, several studies attempted to establish a causality between these two processes, but all results have been difficult to reconcile.

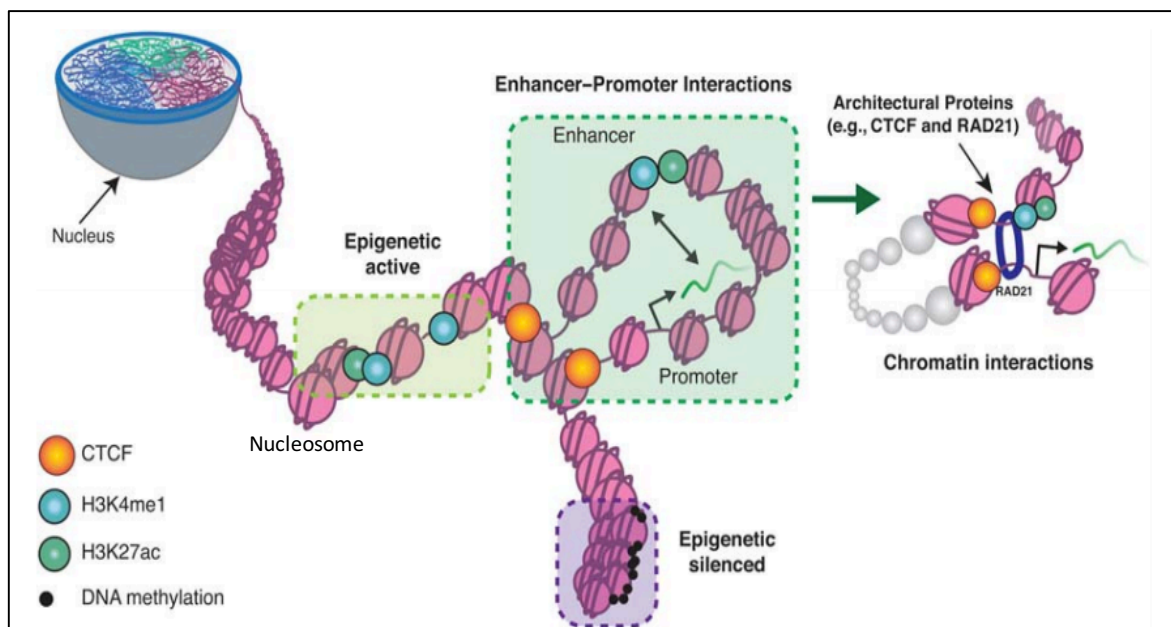
To better understand the relationships between transcription, chromatin condensation, and replication timing, a study in ESCs measured the effect of artificial transcription activation of three late-replicating genes (Ptn, Nrp1 and Sox6). Very interestingly, transcription activation of these genes led to chromatin opening, re-localization to the nuclear interior and earlier replication timing, whereas chromatin opening and nuclear re-localization without transcription activation were not sufficient to induce replication timing modification (Therizols et al., 2014). However, past studies showed that disruption of  $\beta$ -globin gene activation during erythropoiesis does not prevent the switch from late to early replication (Cimbora et al., 2000; Reik et al., 1998; Schübeler et al., 2000). Studies in yeast and humans have also demonstrated that some transcriptionally active genes can be replicated in late S phase (Friedman et al., 1996; Hiratani et al., 2008), and it was later observed that replication timing changes are not necessarily linked to modifications in transcription activity within a given RD (Rivera-Mulia et al., 2015) giving evidences that correlation between replication timing and transcription is not complete. Finally, no specific mechanism has been described to explain the origin of replication timing modifications nor how exactly these modifications impact transcription during development. It is assumed that replication timing and gene activation or repression during development are too synchronous

to be temporally separated (Hiratani et al., 2010). Overall, replication timing may thus function as regulatory mechanisms to facilitate or limit transcriptional activity and other nuclear processes in different segments of the genome, and therefore help orchestrate the complex implementation of the overall genetic program.

#### iv. Chromatin and epigenetic landscape

Between 1928 and 1935, Emil Heitz described for the first time the concept of euchromatin (genetically active) and heterochromatin (genetically inactive or passive) chromosomal regions thanks to cytological staining method in plants (Heitz E (1928) Das heterochromatin der moose. I Jahrb wiss Bot 69: 762–818). His work suggested that DNA can have distinct structures within the same chromosome. DNA double helix structure was first observed by Franklin, Gosling, Watson and Crick in 1953 thanks to their expertise in X-ray crystallography and modelling (Franklin and Gosling, 1953; Watson and Crick, 1953). Since that time, it was discovered that double helix is only the first level for DNA structure. To contain around two meters of genomic information molecule inside the nucleus, cells compact its DNA thanks to a structure called chromatin. This structure consists in wrapping DNA around octamer of four different histones (H3, H4, H2A and H2B) giving rise to a 11nm particle called nucleosome (Richmond and Davey, 2003) (**Figure 10**). An additional histone protein, H1, called the “linker”, binds to the DNA just next to the nucleosome and helps to create further compaction and higher chromatin structure, discussed below.

Histone stabilization occurs via numerous protein-protein interactions, hydrogen bonding and electrostatic forces (Fyodorov et al., 2018; Hashimoto et al., 2010; Hergeth and Schneider, 2015; Woodcock et al., 2006). Histones tails can be modified by post-translational modifications (PTM) such as methylation, acetylation or phosphorylation giving rise to what is commonly called epigenetic code. These modifications play a role in chromatin structure; acetylations tend to help chromatin opening whereas methylations are more linked to chromatin condensation. Thus, what described before as euchromatin, is called the epigenetic active chromatin where active transcription occurs and is marked by specific histone modification such as H4K3me1/2/3 or H3K27ac.



**FIGURE 10:** CHROMATIN ARCHITECTURE REGULATED BY EPIGENETIC LANDSCAPE. THE EPIGENOME IS SEPARATED INTO INACTIVE OR ACTIVE GENOMIC DOMAINS. HETEROCHROMATIN IS MARKED BY HIGH COMPACTION AND REPRESSIVE MODIFICATIONS (I.E. DNA METHYLATION) AND EUCHROMATIN IS MARKED BY OPEN CHROMATIN AND ACTIVE HISTONE MARKS (I.E. H3K4ME1 AND H3K27AC). GENE ACTIVATION IS INDUCED BY PHYSICAL CHROMATIN INTERACTION THAT ENABLE GENOMIC REGIONS TO COME IN CLOSE PROXIMITY IN THREE-DIMENSIONAL SPACE AND RELAY SIGNALS (E.G., VIA ENHANCER–PROMOTER INTERACTIONS). CTCF AND RAD21 ARE TWO ARCHITECTURAL PROTEINS THAT HELP TO MAINTAIN CHROMATIN STRUCTURES. (ACHINGER-KAWECKA ET AL., 2017)

Heterochromatin, is the epigenetically silenced chromatin, with high level of compaction due to DNA and histone methylations (**Figure 10**). Heterochromatin is a specific feature of eukaryotes and can be distinguish into two distinct classes. The first is called constitutive heterochromatin, marked by H3K9me3 (Rea et al., 2000), includes pericentromeric and telomeric regions that are both important for higher order chromatins structures and the maintenance of chromosome stability. It was recently described that H3K9me3 heterochromatin is a barrier to cell reprogramming (Becker et al., 2017). The second is the facultative heterochromatin, marked by mH2A (Gamble et al., 2010) and/or H3K27me3 (Plath et al., 2003), a repressive environment able to rapidly shift to activated state (Huisinga et al., 2006). It is important to empathize that these heterochromatin marks are not restricted to gene inactivation and chromatin silencing as they can also be detected in active and accessible genomic regions.

Epigenetic marks have been associated to replication timing. First, a study on the  $\beta$ -globin domain in human cells pointed out a role for histone acetylation in the control of

replication timing (Schübeler et al., 2000). Another study, observed that the inhibition of histone deacetylation by trichostatin A (TSA) in HeLa cells led to earlier origin firing in this very same locus (Kemp et al., 2005). Then, forcing deacetylation of this locus on a transgene integrated into the genomes of erythroid cells delayed its replication (Goren et al., 2008). Interestingly, H3K9me2 is a histone mark strongly correlated to late replication (Ryba et al., 2010), but removing H3K9me2 by knocking out G9a, the methyltransferase responsible for its deposition, does not affect replication timing (Yokochi et al., 2009). A more recent study demonstrated that the H3K9me3 demethylase Kdm4d facilitates the recruitment of pre-initiation complex by reducing H3K9me3 level and allows proper replication fork initiation and elongation (Wu et al., 2017). It has also been described that epigenetic marks H4K20me1/2/3 are important for the proper replication of some late replicating domains showing that impairing H4K20me2/3 leads to delays in these late replicating (Brustel et al., 2017; Pannetier et al., 2008).

The role of epigenetic as a determinant of replication origins and replication timing is still under debate. However, by an integrative analysis of the genome-wide distributions of many epigenetic marks in the human cell line K562, a recent work described four major groups of chromatin marks with shared features and different replication timing: very early replicated transcriptionally active euchromatin state, an early-mid replicated repressive type of chromatin and associated with polycomb complexes, a silent state not enriched in any available marks that is replicated in mid-late S, and a gene poor HP1-associated heterochromatin state replicated in late S (**Figure 9**) (Julienne et al., 2013). It is well accepted that chromatin environment plays an important role in origin recruitment and activation just like in the replication timing. However, as for replication origins, many evidences suggest that epigenetic state and replication timing can be distinguished and, up to now, no study has described the molecular mechanism linking epigenetic modifications and replication timing.

#### **v. 3D chromatin and nuclear architecture**

For many years, the study of large-scale chromatin architecture was restricted to cytogenetic methods, with the labelling of DNA replication foci, corresponding to coordinated replicons activation. After multiple cell divisions, these foci retained their shape, size and intensity, suggesting the existence of stable chromosome units and tight regulation for their structural organization (Dimitrova and Gilbert, 1999a; Ferreira et al., 1997; Jackson and

Pombo, 1998; Sparvoli et al., 1994). These stable chromosome units can also be visualized by FISH (Fluorescence *In Situ* Hybridization) against chromosome specific probes (Cremer and Cremer, 2001). Over the past decade, development of chromatin conformation capture methods (Hi-C), and mainly high-throughput chromosome conformation capture (capture Hi-C), has allowed precise quantification of intra- and inter-chromosomes interactions genome-wide, leading to the molecular identification of 3D chromatin architecture.

#### 1. Chromatin loops, CTCF, cohesin complex and TADs

It has been shown that CCCTC-binding factor (CTCF) and the ring shaped cohesin complex colocalize onto the chromatin (Wendt et al., 2008). More precisely, CTCF interacts with the STAG1 subunit of cohesin and brings cohesin complex to specific CTCF binding sites on chromosome arms (Parelho et al., 2008; Rubio et al., 2008). These two proteins have been described as regulators of genome folding (Merkenschlager and Nora, 2016), lying at the anchors of chromatin loops (Rao et al., 2014; Splinter et al., 2006) and at the boundaries of contact domains called “topologically associated domains” (TADs) (**Figure 11A**) (Dixon et al., 2012; Lieberman-Aiden et al., 2009; Nora et al., 2012; Rao et al., 2014; Szabo et al., 2019). Although CTCF and cohesin binding at TADs boundaries can restrict enhancer–promoter interactions within a TAD (Rodríguez-Carballo et al., 2017), there is no evidence that the TAD boundaries provide a regulation for coordinated replicon activation within the domain. Furthermore, CTCF sites deletion by CRISPR/Cas9 approach or cohesin depletion impairs chromatin loops and TADs formation (Guo et al., 2015; Rao et al., 2017) but do not seem to have an effect on chromatin compartment (Nora et al., 2017; Rao et al., 2017) nor on replication timing (Oldach and Nieduszynski, 2019; Sima et al., 2019). Interestingly, high-resolution oligopaint tracing of individual chromosome segments revealed a high degree of cell to cell heterogeneity in TAD structures, that was not due to different phases of the cell cycle, and that cohesin depletion in HCT116 cells does not induce changes in TAD boundaries (Bintu et al., 2018). These contradictory results might be due to cell type specific regulation of chromatin architecture and TADs. Finally, the relationship between RD and TAD structure remains unclear. When using high resolution analysis, TADs alignment with RDs is lost and they become further divided into unique chromatin loop or sub-TADs. The current challenge is to understand the biological significance of TADs defined at different scales, and their relationship DNA replication timing and chromosome functions.



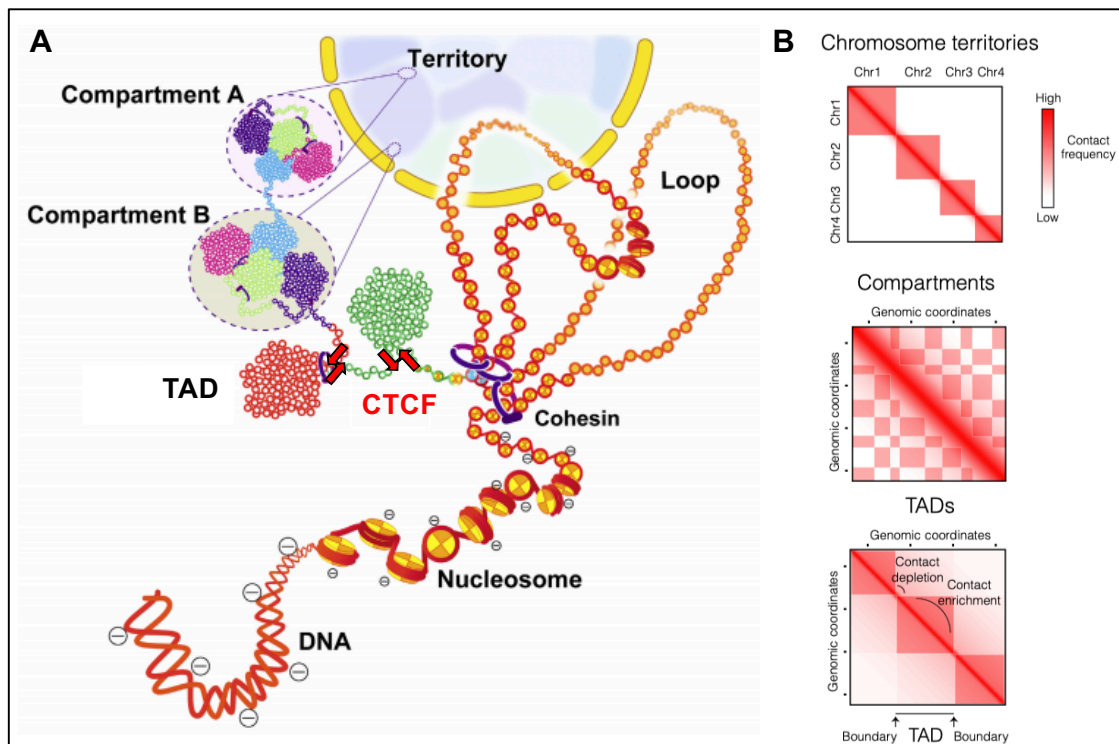
## 2. LADs

The nuclear lamina (NL) is considered to be an essential guardian of the nucleus and is involved in many nuclear activities (Prokocimer et al., 2009). This sub nuclear compartment creates a lattice within the inner nuclear membrane, thanks to intermediate filament proteins (LaminA/C, LaminB1, LaminB2) and coats the nucleoplasm side (Prokocimer et al., 2009; Shevelyov and Nurminsky, 2012; Steensel and Belmont, 2017). It provides anchorage point for chromatin to the nuclear envelope (Farkash-Amar et al., 2012; Peric-Hupkes et al., 2010; Pope et al., 2014; Ragojczy et al., 2014) and to nucleolar periphery or other internal heterochromatic sites (Kind et al., 2013). Thanks to cytogenetic or genomic approaches (i.e. Lamin-Dam-ID), clear interactions between NL and chromatin regions of about (0.1-10Mb) were described; these regions are called “lamina associated domains” (LADs). LADs includes most of the late replicating compartment, enriched in H3K9me2/3 and gene silencing (Kind et al., 2013; Leemans et al., 2019; Towbin et al., 2012) and represent not less than 35-40% of the mammalian genome (Peric-Hupkes et al., 2010; Virgilio et al., 2013). This remarkably large proportion suggests that LADs and NL interaction plays a crucial role in the shape and the positioning of chromosomes. LADs are dynamic genomic regions specific to cellular identity, regulating gene expression, and involved in cellular development and differentiation (Peric-Hupkes et al., 2010).

Almost all mammalian cells contain LaminB while LaminA/C expression is appearing only at later stage of development, in more differentiated cells. Interestingly, hyper-dynamic binding of laminB and other chromatin proteins was described as a hallmark of plasticity in mouse pluripotent embryonic stem cells (ESCs) (Meshorer et al., 2006). Finally, we still ignore if LADs structure is created by the replication timing or if replication timing is regulated by LADs. A very recent and elegant study showed that gene activation within LADs creates NL detachment of the transcription unit associated to a switch from late to early replication timing (Brueckner et al., 2020), proving that replication timing can be modified by modifications of LADs.

### 3. Compartments A/B

Hi-C experiments consist in the cleaving DNA within fixed chromatin and re-ligation of it to create chimeric fragments composed of two or more interacting regions. Chimeric DNA fragments are then analysed by quantitative PCR or sequencing (Lieberman-Aiden et al., 2009). Using either *cis* or *trans* associations scored for TADs from Hi-C data, chromatin has been separated in two main compartments: A and B (**Figure 11**).



**FIGURE 11:** TRAVELLING INTO THE DEPTHS OF THE NUCLEUS: CHROMATIN ARCHITECTURE AT DIFFERENT SCALES. (A) SCHEMATIC VIEW OF CHROMOSOME FOLDING INSIDE THE NUCLEUS. INDIVIDUAL CHROMOSOMES OCCUPANCY WITHIN THE NUCLEUS ARE CALLED CHROMOSOME TERRITORIES (CTs). CHROMATIN IS SEGREGATED INTO ACTIVE “A” AND REPRESSED “B”. CHROMATIN IS PACKED AT DIFFERENT NUCLEOSOME DENSITIES DEPENDING ON GENE REGULATION AND EPIGENETIC FEATURE, FOLDS INTO HIGHER-ORDER DOMAINS OF PREFERENTIAL INTERNAL INTERACTIONS, KNOWN AS TADs. THE SMALLER LAYER OF CHROMATIN FOLDING IS THE NUCLEOSOME, DNA-HISTONE ASSOCIATION. (B) SCHEMATIC REPRESENTATION OF HI-C MAPS REFLECTING THE DIFFERENT LAYERS OF HIGHER-ORDER CHROMOSOME FOLDING. GENOMIC COORDINATES ARE INDICATED ON BOTH AXES, AND THE CONTACT FREQUENCY BETWEEN REGIONS IS REPRESENTED BY THE HEATMAP (HIGH CONTACT IN RED, NO CONTACT IN WHITE). INTRA-CHROMOSOMAL INTERACTIONS ARE OVERREPRESENTED COMPARED TO INTER-CHROMOSOMAL CONTACTS, CONSISTENT WITH THE FORMATION OF INDIVIDUAL CHROMOSOME TERRITORIES. AT THE CHROMOSOMAL SCALE, TWO MUTUALLY EXCLUDED A AND B COMPARTMENTS CAN BE DETECTED BY A CHARACTERISTIC PLAID PATTERN. FINALLY, TADs APPEAR AS SQUARES ALONG THE DIAGONAL ENRICHED IN INTERACTIONS, SEPARATED BY CONTACT DEPLETION ZONES DELIMITED BY TAD BOUNDARIES. (ADAPTED FROM MAESHIMA ET AL., 2020; SZABO ET AL., 2019)

The compartment A is known to be the “active” compartment, enriched in epigenetic marks for transcription, correlated to early replication and euchromatin structure. The compartment B, or “inactive” compartment, on the other hand, has been associated to heterochromatin like structures, with late replicating regions and transcription repressive histone marks (Beagan et al., 2016; Dixon et al., 2012; Lieberman-Aiden et al., 2009; Nora et al., 2012; Rao et al., 2014; Ryba et al., 2010). At very high resolution, Hi-C can resolve these compartments into several sub-compartments that still overlap with active and inactive transcription units but no longer with DNA replication timing (Rowley et al., 2017), indicating that replication timing might be a larger scale regulated phenomenon than transcription, that would be more locally regulated. Finally, it has been described that replication timing and chromatin A/B compartments are less correlated in hESCs than in differentiated cells (Dileep et al., 2019). These results demonstrate that in poorly differentiated cells, changes in one of these properties can be independently regulated and that, during this period, the genome of cells present a high level of plasticity. To better understand mechanisms explaining the close but indirect connection between replication timing and compartment, it will be necessary to independently manipulate these two processes. The identification of *cis* element regulating both replication timing and nuclear compartment could provide a good molecular tool for such experiments (Sima et al., 2019).

#### 4. Nuclear architecture

In the last century, some researchers discovered a network of filaments that was named the nuclear matrix (NM) (Berezney and Coffey, 1974). NM is a protein-rich insoluble network present throughout the nucleoplasm, having a strong resistance to high salt extraction (Georgiev and Chentsov, 1963; Verheijen et al., 1988; Zbarsky and Georgiev, 1959) containing chromatin organized into topologically closed loops with average size of 50-250 kb (Cook et al., 1976; Razin et al., 1996). The chromatin attached to the NM is called S/MARs for scaffold/matric associated regions (Bode et al., 1995). This latest do not contain specific nucleotide sequences but some common characteristics has been observed such as AT enrichment and the ability to be preferentially melted in super coiled DNA (Bode et al., 1992). NM is composed of many proteins playing a structural role (i.e: lamins, actin) but also proteins involved in transcription, replication or RNA processing such like ribonucleoprotein (RNP) particles (Nickerson, 2001; Razin et al., 2014; Skowronska-Krawczyk and Rosenfeld, 2015). NM

has been described as an important factor implied in nucleus organization (Berezney et al., 1995) and a good tool study the organization of DNA replication factories, thanks to DNA Halo approach (Guillou et al., 2010). However, it was demonstrated that NM is an artefact structure arising from attracting forces created under conditions of macromolecular crowding (Hancock, 2000). Even though the techniques to visualize NM were improved over years, it remains unclear to what extent the isolated structure correspond to an *in vivo* existing structure or an artefact. Overall, the existence of a functional platform for nuclear compartmentalization still remain elusive.

Interphase chromosomes are not randomly distributed within the nuclear space and occupy what has been named chromosome territories (CTs) (**Figure 11**). Solid basis suggests that CTs structure is provided by the folding of genomic DNA itself into chromatin (Razin et al., 2013). In interphase nuclei, territorial organization shows evidences of “inter-chromatin domains” that contain many nuclear compartments including Cajal bodies, splicing speckles (or SC35speckles) and PML bodies which contain DNA replication and damage-repair as well as transcriptional and splicing machineries (Cremer and Cremer, 2001; Misteli, 2005; Misteli et al., 1997; Phair and Misteli, 2000). After establishing the basis of CTs organization, investigators wanted to know how individual territories were arranged in the 3D nuclear structure and if this arrangement was random or not. First, it was described that spatial arrangement of chromosomes depends on their size, gene density and GC content (Cremer and Cremer, 2001; Foster and Bridger, 2005; Misteli, 2005). Then, it was demonstrated that individual CT in the nucleus followed a high level of order in the radial positioning; excluding the hypothesis of random distribution (Misteli, 2007; Schneider and Grosschedl, 2007).

This observation has inspired the pursuit in the investigation of how spatial genome organization contributes to functions such as gene expression. Analysis of the location of genes in different cell type and tissues revealed that genomic element occupies preferential position within the nucleus, that vary among tissues and are modified during physiological process like cellular differentiation or pathological situations (Hewitt et al., 2004; Meaburn and Misteli, 2008; Ragoczy et al., 2006; Williams et al., 2006). An interesting and consistent emerging idea is that clustering of genes in transcription hotspots would contribute to efficient regulation of expression (Fraser and Bickmore, 2007) while relative positioning of chromosomes would influence the appearance of specific translocations (Lanctôt et al., 2007). Last, but not least importantly, nuclear positioning has been linked to replication timing about twenty years ago (Gilbert, 2001). More recently, using a large set of DNA replication timing

data on human, non-human and cancer cells, a study has determined the 3D nuclear arrangement of very-early and very late replicating loci by 3D-FISH. Overall, loci topology was well conserved between cell types. As expected, early replicating loci were localized in the nuclear interior and late replicating loci were preferentially found at the nuclear periphery. Interestingly, these data argue in the favour of genomic properties and gene density as decisive parameters of 3D genome organization more than replication timing or translocations (Grasser et al., 2008). Once again, even though chromosome territories and replication timing appears to be correlated, the exact link between those two remains to be elucidated.

Taking together, all these data lead to the conclusion that even though we still ignore the exact determinant of RT, it is a very informative functional readout of large-scale chromatin organization.

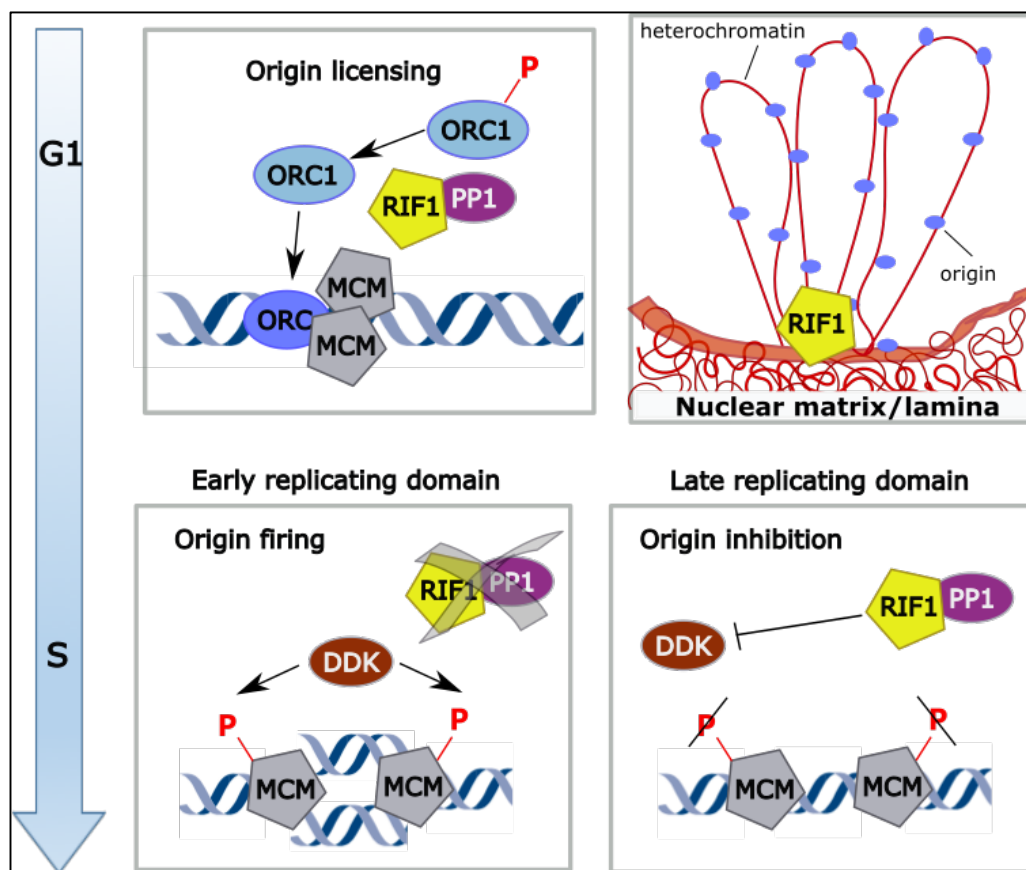
#### **vi. Cis and trans regulation of DNA replication timing**

Identification of replication timing regulators has been a big challenge for the last decade and no major discovery has allowed to fully understand it. Many genes knockdowns have been tested, but only a minority led to replication timing alterations (Marchal et al., 2019, Table 1).

##### 1. RIF1, a trans regulator of replication timing

RIF1 is involved in many nuclear functions such as DNA repair (Noordermeer et al., 2018; Spies et al., 2019), telomere length regulation in yeast (Mattarocci et al., 2016; Ribeyre and Shore, 2012; Shi et al., 2013), epigenetic (Li et al., 2017; Zofall et al., 2016), cytokinesis (Bhowmick et al., 2019) and even in DNA replication factories (Ribeyre et al., 2020). Importantly, up to now, RIF1 is the only protein considered as a whole genome regulator of DNA replication timing. RIF1 depletion in mouse and human cells lead to huge perturbations of DNA replication timing, early to late and late to early replication (Cornacchia et al., 2012; Foti et al., 2016; Yamazaki et al., 2012). A more recent study in human embryonic stem cells (hESC) and HCT116 claimed that, instead of inducing discrete RT alterations, RIF1 KO cells presented a more random replication timing and thus an increased cell-to-cell heterogeneity (Klein et al., 2019). In this unpublished study, they further demonstrate that in RIF1 KO cells, only the H3K9me3 genomic regions maintained the normal RT, especially in the cancerous

cells HCT116. Overall, this study supports the important role of RIF1 in the regulation of RT and also suggests that its role in nuclear architecture might not be direct but through its effect on the epigenetic recycling during DNA replication.



**FIGURE 12:** DISTINCT ROLES OF RIF1 (WITH OR WITHOUT PP1) IN THE REGULATION OF DNA REPLICATION ORIGIN AND CHROMATIN STRUCTURE. IN EARLY G1, RIF-PP1 ACTS TOGETHER AND PLAY A ROLE FOR ORC1 DEPHOSPHORYLATION AND ORIGIN LICENSING. IN PARALLEL, RIF1 HELPS ANCHORING OF LATE REPLICATING REGIONS ONTO THE NUCLEAR LAMINA. IN S PHASE, RIF1-PP1 BEING LESS PRESENT IN EARLY REPLICATING DOMAINS, DDK CAN PHOSPHORYLATES MCMS AND ALLOWS ORIGIN FIRING. RIF1-PP1 COMPETITION WITH DDK IN LATE REPLICATING DOMAINS LIMITING MCMS PHOSPHORYLATION AND FIRING UNTIL THE END OF S PHASE.

Molecular mechanism explaining the role of RIF1 in the regulation of replication timing are not clear yet but several clues suggest that RIF1 acts on replication origin regulation and also in 3D chromatin architecture. In mammals, RIF1 interacts with components of the nuclear lamina (Cornacchia et al., 2012; Foti et al., 2016; Roux et al., 2012; Yamazaki et al., 2013), playing an important role in the insoluble nuclear matrix scaffold and chromatin organization. RIF1 associates with the late replicating genome, forming large domains of mega-bases called RIF1 associated domains (RADs) (Foti et al., 2016). Right after the end of mitosis, RIF1 binds to the chromatin independently but concomitantly to the recruitment of the pre-replication

complex. Thus, RIF1 may play an important role in the TDP through its role in nuclear organization of replication origin in G1 (**Figure 12**) (Aladjem, 2012; Cornacchia et al., 2012; Hayano et al., 2012; Kanoh et al., 2015; Yamazaki et al., 2012).

RIF1 was also described as an important regulator of the nuclear architecture in G1 phase and an essential determinant for the late replication of non-LaminB1 genomic regions (Foti et al., 2016). Several lines of evidence indicate that RIF1 together with protein phosphatase-1 (PP1) counteract DDK activity on the replicative MCM helicase during S phase (Hiraga et al., 2014). Other recent studies showed a dual role of RIF1-PP1 depending on cell cycle: RIF1-PP1 interaction allows origin licensing during G1 by dephosphorylating ORC1 and RIF1 limits origin firing in S phase by counteracting DDK activity (Davé et al., 2014; Hiraga et al., 2017). Finally, a recent unpublished study indicated that just like RIF1, PPI depletion led to replication timing alterations, and that replication timing depends specifically on RIF1-PP1 interaction (Gnan et al., 2020) (**Figure 12**).

## 2. The DNA polymerase Theta

The DNA polymerase Theta (Pol  $\theta$ ) is a unique polymerase-helicase fusion protein that promotes a specific DNA damage repair pathway called micro-homology end-joining (MMEJ) (Black et al., 2019). This interesting polymerase is also involved in translesion synthesis (TLS) (Yoon et al., 2014, 2019). In a study published by my host lab, Pol  $\theta$  was described as another potential molecular actor in the regulation of DNA replication origins and replication timing. In this study, they observed that Pol  $\theta$  binds to G1 chromatin, just like RIF1, and interacts with ORC1 and ORC4. Interestingly, its depletion led to early MCMs recruitment to the G1 chromatin and significant modifications in the replication timing, with both advances and delays switches. Finally, overexpression of this protein exclusively induces replication timing delays (Baldacci et al., 2015; Fernandez-Vidal et al., 2014).

## 3. PREP1

In 2018, another potential regulator of late replicating regions was described: the transcription factor and tumor suppressor, PREP1 (pKnox1) (Palmigiano et al., 2018). PREP1 is essential for development (Fernandez-Diaz et al., 2010; Ferretti et al., 2006) and its depletion

leads to an accumulation of DNA damage *in vitro* (Iotti et al., 2011). In this study, they observed that PREP1 downregulation induces late to early replication timing switches in 25% of the genome and early to late replication timing switches in 5% of the genome in HeLa cells. Moreover, they showed that replication timing switch from late to early happens especially in LADs and TTR and independently of PREP1 role in transcription. Finally, they assumed that these replication timing modifications can be due to the impact of PREP1 downregulation on LaminB1 protein level, that is reduced in this context.

#### 4. Cis regulatory elements

The replication timing is possibly determined at each whole chromosome scale by long non-coding RNAs (lncRNAs). Indeed, three lncRNAs were described as regulators of the replication timing of the very same chromosome from which they are transcribed. As an example, in mice, Xist is important for the late replication of the inactive chromosome X. Xist deletion in human cells leads to a later replication of the inactive X chromosome (Diaz-Perez et al., 2005, 2006). Then, it was observed that the depletion of other lncRNAs namely ASAR6 and ASARA15 for asynchronous replication and autosomal RNAs (ASARs), induces drastic delays in the replication timing of chromosome they are coating and, in some cases, chromosome is being replicated during mitosis which leads to improper condensation (Donley et al., 2013, 2015; Stoffregen et al., 2011). Current hypothesis to explain mechanism by which ASARs regulate replication timing is that different ASARs would be expressed by homologous chromosomes and act together to ensure their synchronous replication (Virgilio et al., 2013).

The replication timing of early replicating domains was shown to be regulated by the presence of multiple specific genomic regions enriched in enhancer epigenetic marks, called early replication control elements (ERCEs). ERCEs interact together in 3D nuclear space and, when deleted, lead to replication delays within the entire domain. Importantly, ERCEs interact with each other independently of CTCF/cohesin and ERCE sites were shown to be like super-enhancers where many transcription factors can bind (Bintu et al., 2018; Sima et al., 2019). Overall, ERCEs are assumed to be necessary for sub-nuclear compartmentalization and 3D architecture of replication domains and they play roles in transcription, further supporting their potential role in the regulation of RT.



### c. Biological relevance of DNA replication timing

Higher eukaryotic cells replicate its DNA in a specific temporal order that is very robust among cells and species. Notably, the main differences observed between the replication timing of cells, has been linked to cellular development, suggesting that replication timing is very important to maintain biological functions of an organism.

#### i. Replication timing is a fingerprint of cellular identity

The study of Ryba and colleagues on 67 whole genome replication timing profiles of human and mouse cells allowed to identify genomic regions as fingerprint of cellular identity. They proved that the analysis of the replication timing of these regions alone, is sufficient to identify the cell type. Genome wide analysis of replication timing profiles in human and mouse by Repli-ChIP (microarrays) provided evidences that overall replication timing profile is very well conserved between mammalian species (Farkash-Amar et al., 2012; Hiratani et al., 2008, 2009, 2010). More importantly, replication timing in a given human cell type is close to the replication timing of the corresponding mouse cell type (Ryba et al., 2010).

#### 1. TDP: a window of opportunity for cellular reprogramming

As 3D nuclear architecture and replication timing are correlated genome-wide, it is assumed that TDP drives the assembly of TADs and nuclear compartments (A/B), which in turn regulate replication timing during the S phase. In 2010, the TDP has been proposed to be a window of opportunity for cellular reprogramming (Gilbert, 2010). In the review, D. Gilbert argue that this is the only moment of the cell cycle where chromatin architecture can be modified by intracellular or extracellular signalling to induce changes of cellular identity (**Figure 13**).

To confirm this theory, experiments of nuclear lamina targeted gene expression showed that re-localization of gene is only established after cell mitosis, in the early G1, presumably during the TDP (Finlan et al., 2008; Kumaran and Spector, 2008; Reddy et al., 2008;

Shachar and Misteli, 2017). Moreover, another study described that contact between LADs and NL is established in early G1 (Virgilio et al., 2013). In addition, transcription factors, that play an important role in chromatin structure, dissociate from chromatin in mitosis, as transcription is repressed, and re-associate after (Egli et al., 2008). It is then reasonable to suggest that transcription factor binding affinities, playing a role in chromatin re-modelling in G1, might be linked to TDP and cell fate. Interestingly, at the end of mitosis, chromatin de-condense, probability of chromatin-chromatin and chromatin-proteins interactions and chromatin mobility increases (Chubb et al., 2002) and this could create an opportunity for cellular factors to induce reprogramming (Deng and Blobel, 2010; Fraser and Bickmore, 2007; Therizols et al., 2014). Finally, Ryba and colleagues identified a cluster of genes encoding for Histone H1 downregulated during differentiation; again suggesting a potential role of chromatin de-compaction in cellular differentiation process (Ryba et al., 2011b).

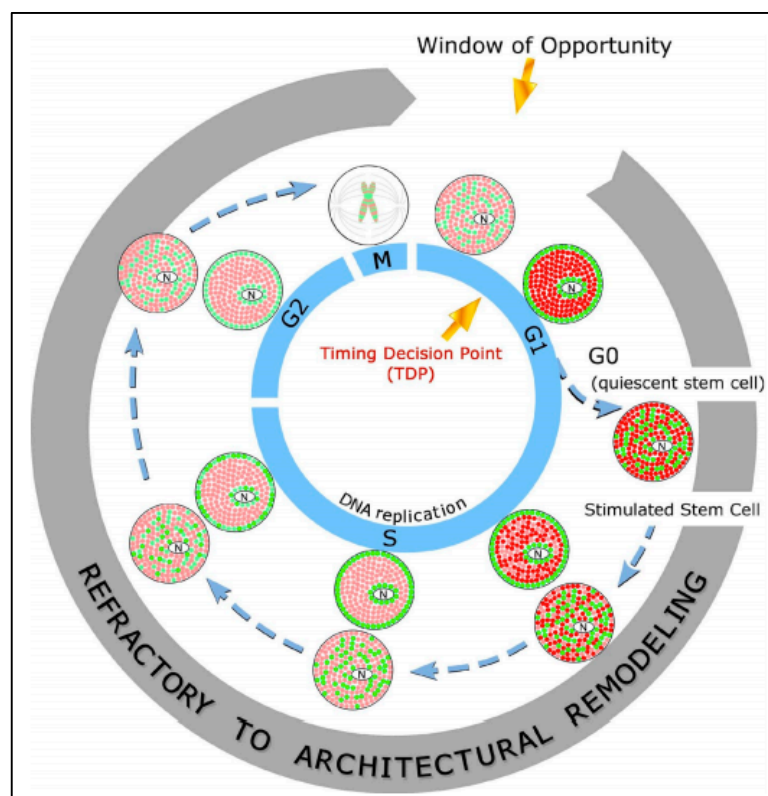


FIGURE 13: The TDP, a small window of opportunity for cellular reprogramming. 4 phases of the cell cycle are represented. TDP happens in early G1 phase, where large scale-chromatin remodelling lead to TADs that will replication in early (red) or late (green) in the next S phase. Architectural remodelling is strongly limited during other phase of cell cycle, leaving TDP window the only opportunity for cellular signalling for reprogramming. (FROM GILBERT, 2010)

## 2. Replication timing switches during organism development and cellular differentiation

Several experiments in *Drosophila melanogaster* embryos and *Xenopus laevis* eggs extracts suggested that replication timing program was not established until the Medula-Blastula Transition (MBT) (Hyrien et al., 1995; Sasaki et al., 1999). However, a more recent study in Zebrafish proved that replication timing is dynamically regulated throughout the organism development (Siefert et al., 2017). More precisely, this study showed that before MBT, chromosome ends are replicated in late S and that RT shift gradually towards earlier replication during development. During MBT, RT is highly dynamic across most of the genome and changes progressively, with specific genomic regions displaying sharp RT switched often associated to enhancer activation. In the same line, it was shown that during human and mouse cellular differentiation, global reorganization of RT occurs and about 20-50% of the genome, depending on cell fate transition (Hiratani et al., 2008, 2010; Rivera-Mulia et al., 2015). Surprisingly, in the work of Rivera-Mulia et al., transcriptome data during cellular differentiation revealed that the well-accepted correlation between RT and gene expression was only respected for constitutive genes. Furthermore, they proved that these genes were frequently restricted to the lineage in which RT was changing but their expression was modified prior to RT. These results first provided new evidences that RT is correlated to cellular identity and it brings new insight about the interplay between RT and transcription during human development.

## 3. Genomic imprinting

Genomic imprinting refers to mono-allelic expression of an autosomal gene due to the inheritance of parental specific methylation of one allele (Delcuve et al., 2009). Until now, these imprinting mechanisms have only been observed in therian mammals, relying on extensive intrauterine foetal-maternal exchange during their early development. According to the parental conflict hypothesis, imprinting would be a way for parental genomes to counteract the effects of each other during foetal development (Moore and Haig, 1991). Imprinted genes present asynchronous replication timing (Delcuve et al., 2009). In most cases the imprinted allele is methylated and transcriptionally silent. The transcriptional state of an

allele seems to be coordinated to replication timing, whereby the expressed allele is early replicated, while the silenced allele is replicated in the late S phase (Zakharova et al., 2009). The imprinted *Igf2-h19* domain represents the most studied imprinted locus. However, many studies led to contradictory results, indicating a lack of consensus regarding allele-specific replication timing of this imprinted locus (Greally et al., 1998; Kawame et al., 1995; Kitsberg et al., 1993; Simon et al., 1999; Virgilio et al., 2013; Windham and Jones, 1997).

#### 4. X chromosome inactivation

To date, sex chromosome inactivation is a remarkable example of chromatin state changes from active to inactive at the whole chromosome level. Indeed, in mammalian females, one of the two X chromosomes is silenced (Xi), heterochromatic and late replicated (Lyon, 1961). X chromosome inactivation is an early event during female development, achieved to ensure equivalent level of gene expression. The key regulator of X chromosome inactivation lies in a unique loci named *Xist* gene, coding for *Xist* RNA that is expressed exclusively from the inactive X chromosome, accumulating on and coating this latest (Brown et al., 1992). Interestingly, it was reported that inactive X chromosome was replicated rapidly *via* random and synchronous DNA synthesis at numerous adjacent regions (Casas-Delucchi et al., 2011; Koren and McCarroll, 2014). This apparently synchronous mode of DNA replication within the inactive X chromosome clearly demonstrate that replication timing is mainly important to properly coordinate replication speed and transcription activity. Finally, it was proposed that RT of the Xi is important for inheritance of its epigenetic state and that RT could be responsible for the transmission of epigenetic information across cell generations, supporting epigenetic maintenance of X chromosome inactivation and other chromatin states elsewhere in the genome (Chadwick and Willard, 2003; Heard and Disteche, 2006).

As a conclusion, the temporal regulation of DNA replication is an essential mechanism to ensure proper genome duplication while maintaining cellular identity. In other word, RT robustness is important for the maintenance of cell-type specific genetic and epigenetic states (Klein et al., 2019) while its plasticity can provide a window of opportunity for chromatin remodelling and gene expression changes during cellular differentiation. Thus, decrypting the relationship between gene expression, chromatin architecture, replication timing and cellular differentiation is an important challenge for the future.

## ii. Replication timing and diseases

The link between DNA replication timing and diseases is an emerging concept that remains poorly explored. However, increasing amount of evidences of a link between RT and cancer have been put forward in the past decade. This part mainly addresses the existing relationships between replication timing and cancer but also intends to prove that RT can also be related to aging and other diseases.

1. The link between replication timing, genetic and 3D chromatin alterations in cancer

It was described a long time ago that some cancer specific replication timing changes appear to be linked to epigenetic and not involved in detectable genetic lesions (Adolph et al., 1992; Eul et al., 1988). In these studies, they were able to restore normal pattern of replication timing by the fusion of mouse lymphoma cells with normal mouse fibroblasts and this reversed the malignant phenotype. This important discovery strongly suggests a role of replication timing in tumour development. In the beginning of the 21<sup>th</sup> century, several studies identified replication timing alterations in cancer, with at least one chromosome being seriously delayed (Amiel et al., 2001; Korenstein-Ilan et al., 2002; Smith et al., 2001; Sun et al., 2001). These studies often associate replication timing delays with chromosome translocations resulting from condensation and mitotic defects. Later, whole genome replication timing analysis described sites of abnormal developmental control of DNA replication in paediatric leukaemia (Ryba et al., 2012) and specific cancer related gene replication timing analysis described alterations in replication timing of these genes in human breast cancer model (Fritz et al., 2013). Interestingly, in these two studies, not only replication timing delays were observed, but also come shifts from late to early replication. Notably, they associated replication timing alterations with chromosome rearrangement and copy number variation (CNV) (Ryba et al., 2012).

Tumour development and progression is associated to genetic and epigenetic alterations. It is well known that mutational rate is associated to replication timing, with a higher rate in late replicating genomic regions (Chen et al., 2010; Cui et al., 2012; Koren et al., 2012; Sima and Gilbert, 2014; Stamatoyannopoulos et al., 2009; Watanabe et al., 2002). In

this same line, a recent study emphasized the fact that the nature of chromosomal rearrangement in cancer depends on both replication timing and the epigenome (Du et al., 2019). In this study, they proposed a model where prostate and breast cancer cells display a higher level of facultative heterochromatin (H3K27me3), in the detriment of constitutive heterochromatin (H3K9me3), giving more susceptibility to chromosomal rearrangement. This observation might reveal an abnormal nuclear compartmentalization, giving rise to more flexibility between active A or repressive B compartment in cancer cell nucleus and thus a higher risk of chromosome rearrangement.

There are experimental and clinical evidences that human malignant cells harbour mutations in chromatin remodellers (Kaur et al., 2019). Absence of functional chromatin remodelling complexes seems to fuel progression of some tumours. For example, about 90% of small ovary cells carcinoma harbour inactivating mutations in Brg1 gene, with concomitant loss of SMARCA4 chromatin remodeller, one of the two mutually exclusive ATPases of the SWI/SNF complex (Witkowski et al., 2014). Moreover, depletion of Snf2L in Hela cells led to enhanced proliferation and increased invasion (Eckey et al., 2012). This phenomenon was explained thanks to transcriptome profiling by the identification of Snf2L as a modulator of the Wnt signalling network. These non-exhaustive list of examples above prove that down-regulation of particular chromatin remodellers expression is one way for cancer cells to proliferate and survive. On the other hand, in some cancer type, overexpression of chromatin remodellers can also be detected. For example, in colon cancer, up-regulation of Brg1 allows tumour progression through concomitant up-regulation of WNT3A expression. Interestingly, knock-down of Brg1 gene expression significantly suppresses proliferation and migration of colon cancer cells (Lin et al., 2016) and the specific inhibition of this very same gene increases the efficacy of chemotherapy in breast cancer (Wu et al., 2016). It is suggested that disruption in chromatin remodellers expression allows cancer cells to re-wire the transcriptional machinery in order to adapt to environmental changes, and thus resist to chemotherapy. The careful knowledge of manipulation of histone modifiers by cancer cells may help scientist to design new epigenetic therapy, such as histone deacetylase inhibitors that are currently in clinical trials for different cancer types (Lakshmaiah et al., 2014; Lu et al., 2020; McClure et al., 2018; Romero, 2019; Suraweera et al., 2018).

Interphase nuclei architecture is more and more considered as a major player in the control of gene expression, epigenetic and replication timing. It is then relevant to analyse chromatin structure in cancer. Indeed, was demonstrated that cancer mutation rate is

influenced by 3D chromatin organization (Schuster-Böckler and Lehner, 2012). Development of high-resolution techniques such as chromatin conformation capture (3C) and sequencing (4C, 5C) and subsequent genome-wide studies with Hi-C and ChIA-PET, allowed the precise analysis of 3D nuclear structure, replication timing and cancer. Assessing this question, it was shown that cancer is associated with spatial chromatin rearrangement and replication timing modifications (Achinger-Kawecka et al., 2017; Grasser et al., 2008; Li et al., 2019; Taberlay et al., 2016). More recently, in a very nice study of whole-genome sequencing data from 2,658 cancers and 38 tumour types and 288,457 somatic structural variations (SVs), Akdemir and colleagues demonstrated that significant changes in chromatin folding and gene expression were due to disruption of TAD boundaries induced by SVs (Akdemir et al., 2020). However, in this study, they did not look at copy number alterations or dysregulation of chromatin regulators, which could also affect the expression of genes. Finally, a work on ER+ breast cancer cells revealed a role for chromatin remodelling in chemo-resistance (Achinger-Kawecka et al., 2020).

To conclude, genomic alterations in cancer cells is clearly associated to 3D nuclear structure and replication timing alterations. One important remaining question is the order of appearance for these alterations during tumorigenesis and/or relapse. If we can prove that RT and/or 3D chromatin alterations occur very early during tumorigenesis, it would be relevant to propose RT or gene positioning as biomarkers or diagnosis tool. Another hypothesis would be that the replication stress induced by chemotherapy lead to RT and chromatin alterations, creating an open window for cellular adaptation and chemo-resistance. An interesting recent study supporting this hypothesis identified specific RT signature associated to relapse in B-lineage acute lymphocytic leukemia (B-ALL) (Rivera-Mulia et al., 2019).

## 2. Replication timing and premature aging

Cancer and aging are closely related genetic diseases. Common markers of genetic instability and chronic inflammation are found in both pathologies (Finkel et al., 2007). However, the important difference between aging and cancer cells is the proliferation state due to the biology of telomeres: cancer cells are immortal and highly proliferative whereas aging is marked by a stochastic progressive failure of telomere and cellular maintenance, known as senescence. It seems that physiological ageing cannot be linked to RT modifications as a recent study in senescent cells does not show particular alterations in RT (Rivera-Mulia et

al., 2018). However, a work from the same author compared RT of fibroblast from normal individuals of different ages and patients with distinct premature aging syndromes (progeria) and revealed a set of common RT alterations associated to the aging diseases (Rivera-Mulia et al., 2017). In addition to this work, tumour-associated genes (RB1, TP53, cMyc and Her2) present large temporal differences in replication timing in tissues of patients suffering from Down's syndrome, in contrast to the high level of synchrony shown in all samples from normal individuals (Amiel et al., 1998).



## Replication stress and genomic instability as hallmarks of cancer

During the duplication of the eukaryote genome, many impediments can lead to the slow-down or arrest of the replication fork (or replisome), defined as DNA replication stress. This latest can be highly deleterious for the cells if the problem is not resolved, which is the case in cancer. This last introduction part aims to explain the causes of DNA replication stress, to show its consequences on genomic instability and its major role in cancer development and finally to describe its relevance as a therapeutic strategy.

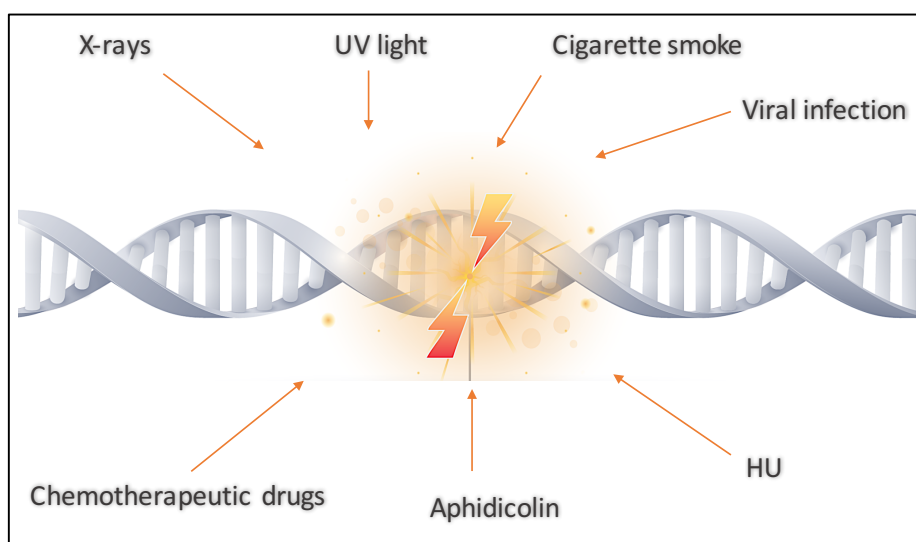
## a. Causes of DNA replication stress

The definition of replication stress is continually evolving and therefore difficult to precisely specify. In the following part, I will give a preview of the main replication stress sources, arising from exogenous or endogenous sources.

### i. Exogenous sources of replication stress

The environment of a living organism is full of potential sources of replication stress (**Figure 14**). First, UV sunlight induces chronic DNA damages by creating adjacent pyrimidines dimerization mainly resulting in cyclobutane pyrimidine dimers (CPDs). Another example could be the 6-4 photoproducts (6-4 TT) that hampered normal replication fork progression (Cadet et al., 2005; Schuch and Menck, 2010) and lead to nicks, gap, single-stranded DNA (ssDNA) or worse unrepaired DNA lesions, which represent a big challenge for replication progression (Ciccia and Elledge, 2010; Zeman and Cimprich, 2014). UV radiation can also indirectly cause DNA damage by generating reactive oxygen species (ROS) mainly in the 7,8-dihydro-8-oxoguanine (8-oxoG) form. Viral infection is another indirect external source of DNA replication stress as the viruses monopolise many essential proteins for the replication and repair processes of the host cell to duplicate its own DNA, allowing its survival and proliferation but leading to genome duplication defects. One of the best example is the papillomavirus (HPV) that has been reported to be a driver of cervical or other cancers (McBride, 2017; Moody, 2019; Sitz et al., 2019). It is now well accepted that chemical agents and product such as benzo-a-pyrene derived from smoke combustion form covalent DNA adduct that are major source of replication stress (Rs et al., 1988). Finally, a large number of chemotherapeutic drugs for cancer treatment are genotoxic agents designed and selected for their strong capacity to create DNA damage and replication stress. Among these drugs, we can distinguish alkylating agents (Nitrogen mustards, Nitrosoureas or Temozolomide), platinum drugs (cisplatin, oxaloplatin or carboplatin), antimetabolites (5-FU) or topoisomerase poisons (etoposide or camptotecin) (Swift and Golsteyn, 2014). Another classical anticancer therapeutic approach is radiotherapy based on the specific X-rays towards

the tumour, directly inducing DNA breaks and Reactive Oxygen Species (ROS), that aim to specifically target cancer cell replication (Baskar et al., 2012; Kim et al., 2019).



**FIGURE 14:** NON-EXHAUSTIVE LIST OF REPLICATION STRESS EXOGENOUS SOURCES. EXOGENOUS SOURCES OF DNA REPLICATION STRESS CAN COME FROM THE NATURAL ENVIRONMENT UV LIGHT OR VIRUSES, THAT ARE ALSO POTENTIAL SOURCES OF DNA LESIONS, OR ARTIFICIAL SOURCES LIKE CHEMICAL DRUGS THAT ARE ALSO ABLE TO TARGET DNA STRUCTURE OR REPLICATION AND GENERATE REPLICATION STRESS.

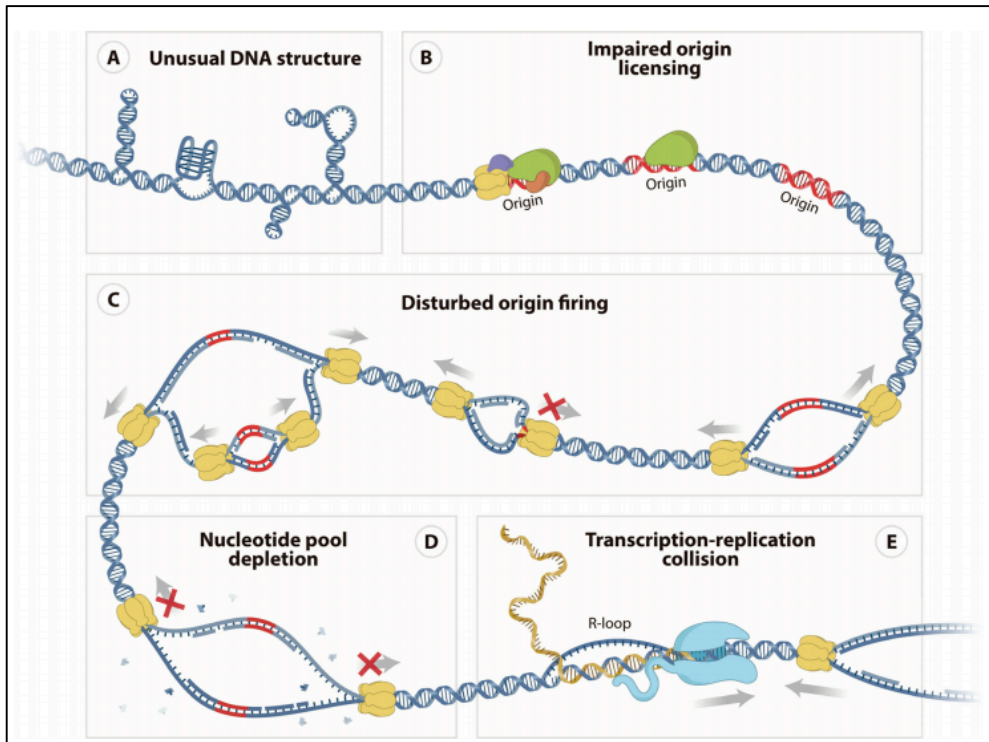
Finally, other replication stress inducers are currently used in research studies such as aphidicolin that directly triggers replication fork speed or hydroxyurea (HU) that affects the nucleotide pool and indirectly target replication fork speed (Koç et al., 2004). Aphidicolin is a tetracyclic diterpenoid antimetabolic and antiviral metabolite secreted by the mold *Cephalosporium aphidicola* (Bucknall et al., 1973). It was described a long time ago as a potent inhibitor of DNA replication in several organisms, including eukaryotes (Huberman, 1981). Aphidicolin affect DNA replication by specifically inhibiting B-family DNA polymerases (Cheng and Kuchta, 1993; Krokan et al., 1981). Because of its specificity and reversibility, aphidicolin is employed at high doses for cell synchronisation in the G1/S border (Matherly et al., 1989). Finally, the treatment with low dose aphidicolin, that do not completely block the cell cycle, allowed the discovery of specific regions of human chromosomes, names fragile sites, that are prone to break (Glover et al., 1984). This concept of fragile sites will be further described in following parts.

## ii. Endogenous sources of replication stress

In normal growth conditions, every duplicating cell of a given organism encounters problems of replication, named endogenous replication stress. This latest is caused by numerous sources such as spontaneous DNA alterations, DNA intrinsic secondary structures (non-B DNA) or collision between replication fork and transcription machinery.

Spontaneous DNA alterations can be due to loss or interconversion between two DNA nitrogenous bases caused by depurination and deamination respectively, modification of DNA bases by alkylation and finally dNTP mis-incorporation during the replication process (Lindahl and Barnes, 2000). Some DNA nucleotide sequences can be highly repetitive (AT rich or GC rich) and form more easily secondary structures like G-quadruplex (Bochman et al., 2012) or hairpins (Kaushal and Freudenreich, 2019) that represent an important obstacle for the helicases of the replisome (**Figure 15A**) (Boyer et al., 2013). Additionally, oxidized DNA bases and DNA breaks can be generated by reactive oxygen species (ROS) derived from normal cellular metabolism. Altogether, a given cell experience up to 10<sup>5</sup> spontaneous DNA lesions per day (Hoeijmakers, 2009). Deoxyribonucleotides (dNTP) are the main substrate essential for DNA replication (Lane and Fan, 2015). Thus, it makes sense to assume that a lack of dNTPs would be a source of replication stress (**Figure 15D**). In fact, the metabolism of nucleotide synthesis must be well regulated in the cell to ensure normal level of dNTPs that regulate replication fork speed and normal replication.

DNA replication and transcription are two essential processes that can take place concomitantly in space and time within the cell nucleus. An important cause of replication stress is the collision between replication fork and transcription machinery (**Figure 15E**). These conflicts have higher risk to occur in genomic regions where large genes (> 400 kb) are actively transcribed during the S phase (Helmrich et al., 2006, 2011) and create particular RNA-DNA hybrid structures, called R-loops, very difficult to resolve (García-Muse and Aguilera, 2019). When not resolved, these structures lead to fork stalling and subsequently DNA breaks or fork reversal (Neelsen and Lopes, 2015).

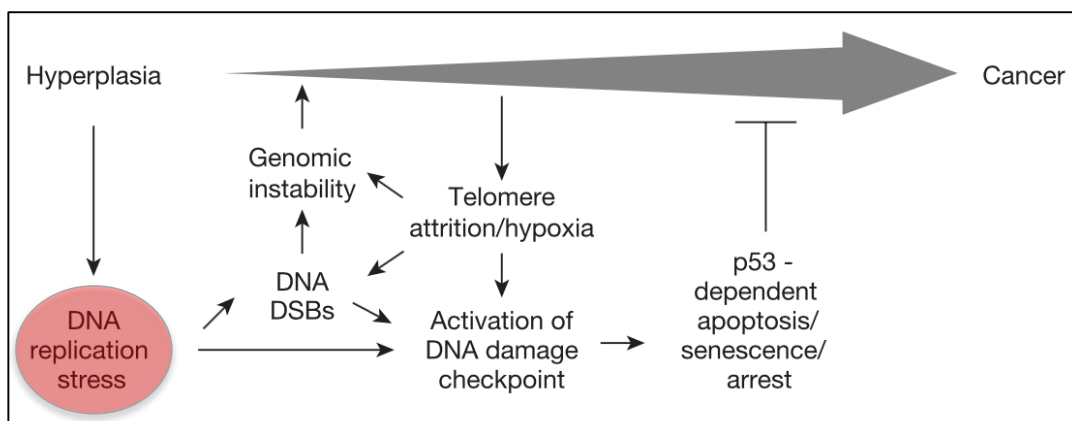


**FIGURE 15:** ENDOGENOUS SOURCES OF DNA REPLICATION STRESS. IN THIS SCHEME, THE DOUBLE-HELIX OF DNA IS REPRESENTED IN BLUE, REPLICATION ORIGINS ARE IN RED, MCM HELICASE COMPLEX IN YELLOW, MESSENGER RNA IN DARK YELLOW AND RNA POLYMERASE IN LIGHT BLUE. (A) SECONDARY/UNUSUAL DNA STRUCTURES SUCH AS G-QUADRUPLEX (SECOND LEFT), STEM-LOOPS OR HAIRPINS (OTHERS) HAS HIGHER SUSCEPTIBILITY TO FORM AT SPECIFIC GENOMIC LOCI SUCH AS TELOMERES, CENTROMERES OR FRAGILE SITES (SEE B). (B) IMPAIRED ORIGIN LICENSING (MIDDLE) OR ABSENCE OF REPLICATION ORIGIN (RIGHT) MAY LEAD TO A DEFICIENCY OF DNA REPLICATION, OR UNDER-REPLICATED DNA. (C) DISTURBED ORIGIN FIRING COULD LEAD TO ASYMMETRIC FORK PROGRESSION (MIDDLE) OR RE-REPLICATION OF DNA (LEFT). (D) NUCLEOTIDE POOL DEPLETION DIRECTLY IMPACT REPLICATION FORK SPEED. (E) TRANSCRIPTION-REPLICATION COLLISION OR R-LOOPS CAN AFFECT REPLICATION FORK SPEED AND EVEN LEAD TO FORK STALLING (RED CROSS). (FROM PRIMO ET AL., 2020)

To finish, a last endogenous source of replication stress is improper origin licensing and firing (**Figure 15B and C**). As previously mentioned in the review (Courtot et al., 2018), replication origin components must be accurately loaded onto chromatin during G1 phase to allow appropriate origin firing in the next S phase (McIntosh and Blow, 2012). The large majority of licensed origins are backup (or dormant) origins, that will not be activated during the replication process but are essential to rescue adjacent stalled replication fork. Coordinated origin firing is also essential to avoid perturbation during DNA replication. For example, it has been shown that reduced or asymmetric origin firing can force replisome to travel very long distances with an increased chance to collapse. On the opposite, an increase in the number of origin or in replication fork speed can also be a source of replication stress (Maya-Mendoza et al., 2018).

### iii. Evidences of RS and oncogene activation in pre-cancerous lesions

Cancer is probably the best described disease characterized by replication stress. Nonetheless, the association between RS and tumorigenesis is not as straightforward. Indeed, we still ignore which is the chicken and the egg in that story; we cannot be sure if the arising of tumour cells comes from RS or if RS is a consequence of tumour stage of the cell. However, replication stress and the presence of DNA damage has been described at early-stages of tumour development (Bartkova et al., 2005; DiTullio et al., 2002; Gorgoulis et al., 2005) (**Figure 16**). As an example, Gorgoulis and colleagues showed that at the very early stages of carcinogenesis, RS arises first and is a driving force for TP53 inactivation, allowing tumour development through (Gorgoulis et al., 2005).



**FIGURE 16:** MODEL FOR INDUCTION OF CANCER WITH RS AS AN EARLY EVENT. DNA REPLICATION STRESS IS AN EARLY EVENT IN TUMORIGENESIS AND IS CAUSED BY ABERRANT STIMULATION OF CELL PROLIFERATION. ACCUMULATION OF RS INDUCES DNA DSBs, ACTIVATION OF THE DNA DAMAGE CHECKPOINT, AND GENOMIC INSTABILITY. THIS CASCADE OF CELLULAR EVENTS PROVIDES A SELECTIVE PRESSURE FOR P53 OR OTHER ONCOGENE INACTIVATION. TELOMERE ATTRITION AND HYPOXIA CAN ALSO CONTRIBUTE TO DNA DSB FORMATION, ACTIVATION OF THE CHECKPOINT AND GENOMIC INSTABILITY. (GORGOLIS ET AL., 2005)

Studies in the early 1980s demonstrated the existence of cellular genes, termed oncogenes, that drive cancer development (Bishop, 1987; Land et al., 1983; Weinberg, 1995). Many oncogenes have been identified regarding to two main criteria: level of expression and/or mutation in human cancer cells and their ability to transform cells when mutated or overexpressed. Oncogene activation leads to an increase in DNA damage and can be defined as an endogenous replication stress. Indeed, it has been shown that oncogene activation

increased all the risk to RS like transcriptional activity and/or replication initiation leading to many transcription-replication conflicts, R-loops formation resulting in a high level of DNA damage (Jones et al., 2013; Kotsantis et al., 2016). Another important effect of oncogene activation is on the regulation of origin licensing and firing. Indeed, the gene CCNE1/2 that encodes for cyclin E1/E2 respectively, is overexpressed in many cancers and induces a reduction of G1 phase length (Ohtsubo and Roberts, 1993), resulting in uncomplete pre-RC assembly (Ekholm-Reed et al., 2004). Moreover, it was observed that Cyclin E1 overexpression results in either decreased (Liberal et al., 2012) or increased origin firing (Jones et al., 2013) in different models. Finally, aberrant activation of the Rb-E2F pathway by HPV-16 E6/E7 or cyclin E oncogenes significantly decreased the cellular nucleotide levels in the newly transformed cells that failed to support normal replication and genome stability (Bester et al., 2011).

Imbalances created by replication stress in pre-cancerous lesions preferentially trigger regions of the genome that are prone to RS and breakage, such as the well described common fragile sites (CFS). An important number of studies have proposed CFSs to not only being susceptible to DNA replication stress induced by oncogenes but also associated to genes that contribute to the neoplastic process. FRA3B contains the famous tumour suppressor gene FHIT, which is involved in nucleotide metabolism and thus in replication stress (Joannes et al., 2010; Karras et al., 2016; Saldivar and Park, 2019; Siprashvili et al., 1997). FRA3B fragility is mainly due to a combination between paucity of origins at the centre of the genomic region and transcription-replication collision due to extended transcription of the large gene FHIT. Similar to FRA3B, FRA16D fragility is associated to large tumour suppressor gene WWOX (Helmrich et al., 2011; Letessier et al., 2011).

Thus, it seems that RS is an early event necessary for tumorigenesis and is the driver for oncogene activation that will in turn create and amplify RS, DNA damage and genome instability, leading to cancerous state (**Figure 16**). A large number of well-described cancer-prone human syndromes like Fanconi anemia, Lurch, Werner or Bloom syndromes have also been associated to replication stress due to mutations in DNA replication or repair proteins (Gaillard et al., 2015).

## b. The replication stress response

Even though replication stress is well recognized as an essential problem for genome stability, there is no single consensus for the characterization nor detection of this phenomenon and it is hard to define a clear set of cellular markers that perfectly characterize this state. This might be due to the fact that cells use many distinct pathways to counteract replication stress and that there is a kinetic for each sensor to act at a specific moment during replication stress response.

### i. Detection and markers of replication stress

However numerous are the ways to induce RS response, it usually results in stretches of single-strand DNA (ssDNA), that are formed when the replicative helicases continue to unwind the parental DNA whereas the polymerases are blocked at the level of the DNA lesion. The persistence of ssDNA coated by a replication protein A (RPA) generates the first signal for replication stress response (Byun et al., 2005) (**Figure 17a**). This structure is a platform to recruit many replication stress response proteins, including the protein kinase Ataxia telangiectasia and Rad3 related (ATR) (Ca et al., 2007; Nam and Cortez, 2011; Zou and Elledge, 2003). ATR is one of the main replication stress response kinases, together with ATM that phosphorylate many substrates which lead to fork rescue or DNA repair that will allow faithful DNA duplication and thus survival of the cell and genomic stability.

The type and intensity of stress challenging the replication fork progression will dictate the level of checkpoint activation and pathways of DNA repair proteins invoked for the cellular response. The wide majority of common markers used to detect RS depend on the activation of ATR pathway, including phosphorylation of the histone variant H2AX ( $\gamma$ H2AX). It is important to notice that ATR is not the unique kinase that can phosphorylate H2AX as it can also be done by the ATM-DNA-PK pathways when ATR signalling is not sufficient (Chanoux et al., 2009; Furuta et al., 2003). Thus,  $\gamma$ H2AX might not be the most appropriate marker to detect replication stress in a sensitive way. ATR-dependant phosphorylation of RPA (on serine 33), Chk1 (on serine 345) or direct detection of ssDNA by native BrdU immunofluorescence are more specific readout of RS (Maréchal and Zou, 2013; Nam and Cortez, 2011). Notably, all

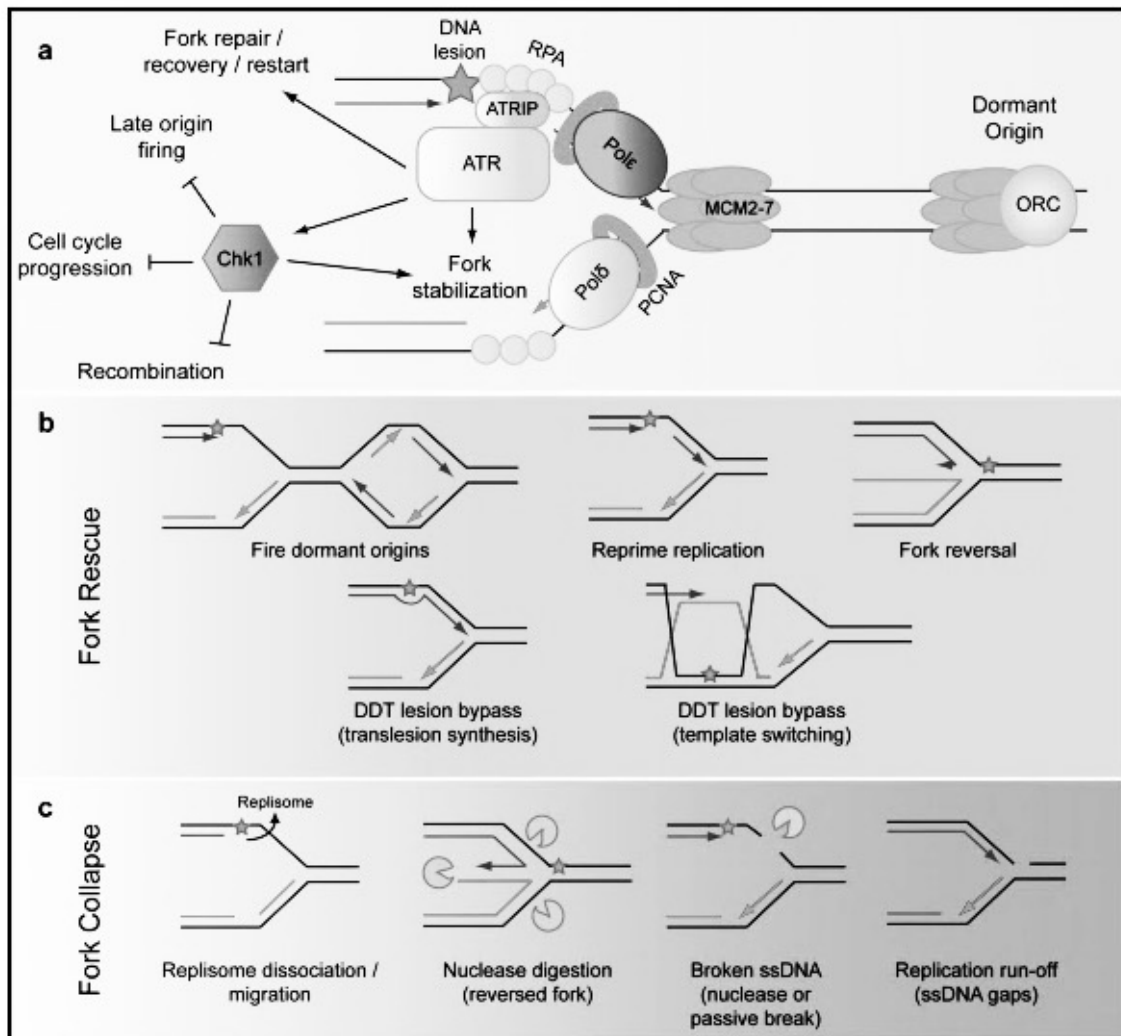


the ATR substrates or ssDNA are good marker of acute replication stress, with a high level of ATR activation inducing wide spreading of phosphorylation of its downstream targets.

However, the level of RS can be low or mild, especially when it comes from endogenous sources or in a non-pathologic context. This mild replication stress does not necessary lead to detectable levels of ATR activation marker. Indeed, when the cell experience replication stress at one or few stalled forks, a local response is set-up and is thus poorly detectable at the cellular level (Koundrioukoff et al., 2013). In this case, the best readout of RS would be the direct measure of replication fork velocity and inter-origin distance by molecular DNA fiber stretching or combing (Bianco et al., 2012; Kaykov et al., 2016).

## ii. Mechanisms to prevent or rescue replication fork stalling

The first response to RS happens at the level of the replication fork by the local activation ATR pathway (Petermann et al., 2010) (**Figure 17a**). Possible ways to rescue replication fork can be dormant origin activation adjacent to stalled fork (Courtot et al., 2018; McIntosh and Blow, 2012; Woodward et al., 2006). Replication machinery can also re-start replication downstream of the lesion by repriming (Elvers et al., 2011; Lopes et al., 2006). These gaps can be bypassed thanks to specialized polymerases from the translesional or Y-family (Bournique et al., 2018; Goodman and Woodgate, 2013) or by the use of sister chromatin as a template called template switching, both mechanisms named DNA damage tolerance (DDT) pathway (Bi, 2015; Ghosal and Chen, 2013). Other mechanisms of replication fork rescue have been described such as fork reversal (Neelsen and Lopes, 2015) (**Figure 17b**). Altogether, these mechanisms allow the rescuing of replication preventing replication fork stalling.



**FIGURE 17:** MECHANISM OF STALLED REPLICATION FORK SIGNALLING, RESCUE AND COLLAPSE. (A) STALLED REPLICATION FORK SIGNALLING BY FIRST ssDNA COATED BY RPA, THEN THE CHECKPOINT SIGNALLING RESPONSE THROUGH ATR MEDIATOR ACTIVATION AND DOWN-STREAM CHK1 PHOSPHORYLATION ALLOWING REPLICATION STRESS RESPONSE. (B) EXAMPLE OF SEVERAL MECHANISMS ALLOWING FORK RESCUE/RESTART AT DNA LESIONS (STAR) LIKE DORMANT ORIGIN FIRING, FORK REVERSAL OR TRANSLATION SYNTHESIS. (C). WHEN STALLED FORK IS NOT PROTECTED OR PERSIST DURING AN EXTENDED PERIOD OF TIME, REPLICATION FORK COLLAPSE AND PREVENT REPLICATION RESTART. FOUR POSSIBLE MECHANISMS OF FORK COLLAPSE ARE REPRESENTED HERE INCLUDING DISSOCIATION OF REPLISOME COMPONENTS, NUCLEASE DIGESTION OF THE REVERSED FORK OR PASSIVE DNA BREAK INDUCTION BY NUCLEASES. (FROM ZEMAN AND CIMPRICH, 2014)

Despite various ways to rescue replication fork, this latest may not restart and may collapse, especially when the RS is acute or when some components of RS response are lost. The exact composition of both stalled or collapsed replication fork is not well characterized and the mechanism of fork stalling is still under investigation. Current potential models are described in the **Figure 17c**. One first proposed model was described in yeast model and suggests that when ATR is not present at the fork or not functional, the replisome is no longer

stabilized and thus the components of the fork dissociate from DNA, resulting in fork collapse (Lopes et al., 2001; Tercero and Diffley, 2001).

Another study based on more recent genome-wide data showed that the replisome is still intact at stall fork but is not functional or properly localized onto the chromatin (Sirbu et al., 2011). Finally, a work in human cells under replication stress (HU) revealed that rapid replication fork breakage (RRFB) achieved by two endonucleases (Artemis and XPF) contributes to fork collapse followed by rapid restart and replication efficiency (Bétous et al., 2018). Once collapsed, the fork is processed into one-ended DSBs, which are repaired by a specific and well described repair pathway named break-induced replication (BIR) (Anand et al., 2013), that may produce tandem genomic duplications when the collapse fork that is repaired is close to another processing fork (Costantino et al., 2014). Overall, even though it is well established that ATR/Chk1 activation is essential to respond to RS and maintain fork stability, the exact function(s) of ATR once activated at stalled replication fork remains a matter of intense investigations (Nam and Cortez, 2011).

### iii. Double strand breaks repair pathways

In a context of acute RS, the management of stalled replication fork is more complicated and may lead to the accumulation of DNA double strand breaks (DSBs). These latest can be repaired by several well described mechanisms.

The first and most faithful DNA repair mechanism, Homologous Recombination pathway (HR), begins with a first step of resection, and then repair by copying the homologous sequence from sister chromosome. This repair pathway is thus restricted to the S and G2 phases of the cell cycle and is inhibited during G1 phase by 53BP1, RIF1 and the polymerase  $\theta$  (POLQ) (Bunting et al., 2010; Heyer et al., 2010; Mateos-Gomez et al., 2015; Ranjha et al., 2018; Yousefzadeh et al., 2014). Principal DNA repair proteins involved in this pathway are MRN, DNA2, BLM, WRN, BRCA1/2 and RAD51 (**Figure 18, right panel**).

The second principal DNA repair mechanism, Canonical-Non-Homologous End-Joining (c-NHEJ), mainly but not exclusively taking place in G1, is based on the direct ligation of the two broken DNA ends, leading inevitably to a less faithful repair (Chapman et al., 2012; Peng and Lin, 2011). This repair mechanism is allowed by the sequential action of Ku70/70

heterodimers that bind to DNA ends, 53BP1, RIF1, DNA-PKcs, Artemis, XRCC4 and DNA ligase IV (Figure 18, left panel).

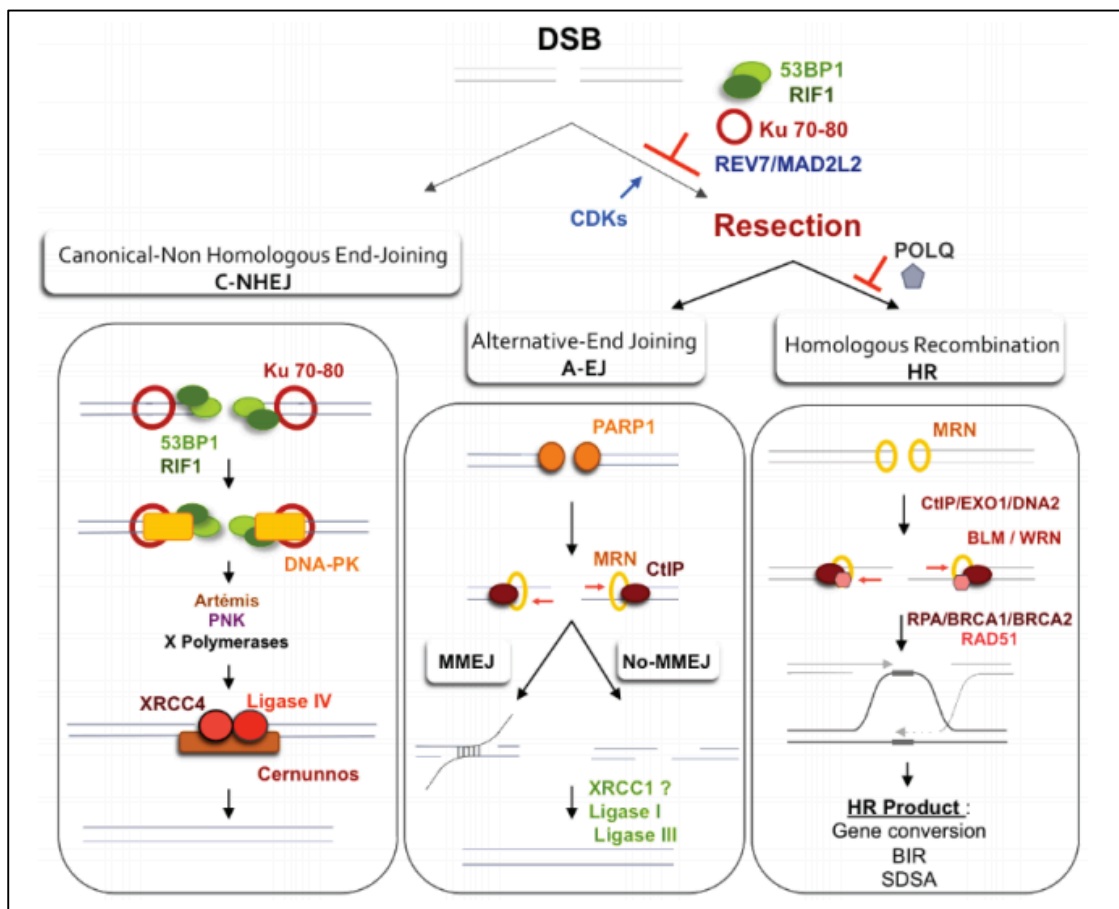


FIGURE 18: The MAJOR DSB repair pathways. ON THE RIGHT PANEL, HOMOLOGOUS RECOMBINATION (HR), MEDIATED BY BRCA1, THAT CAN ONLY BE USED BY THE CELL DURING S AND G2M PHASES BECAUSE IT REQUIRES THE HOMOLOGOUS CHROMOSOME AS TEMPLATE. IN THE MIDDLE, NON HOMOLOGOUS END JOINING (NHEJ), MEDIATED BY THE TP53-BINDING PROTEIN-1 (53BP1) AND IT CONSISTS IN THE RELIGATION OF BROKEN DNA AND, UNLIKE HR, IT CAN OCCUR ANY TIME DURING THE CELL CYCLE BUT IS MORE RESTRICTED TO THE G1 PHASE, WHEN HR CANNOT HAPPEN. (FROM GELOT ET AL., 2015)

Another possible DNA repair pathway is the alternative NHEJ (Alt-NHEJ or A-EJ) that shared the first resection step with HR, while requiring a smaller amount of ssDNA. This repair process is initiated by PARP1, followed by MRN/CtIP activity and the Microhomology-Mediated End-Joining (MMEJ) repair is ensured by the DNA polymerase POL theta (Black et al., 2019; Kent et al., 2015; Sharma et al., 2015). Alt-NHEJ is always associated to deletions at the junction or can involve microhomologies distant from the DSB. The last step of alt-NHEJ process is ensured by XRCC1 and Ligase III (or ligase I).

Overall, c-NHEJ, alt-NHEJ (together EJ) and HR are critical repair pathways that has to be well regulated to ensure genome stability and cell survival. Therefore, the choice between HR and EJ is essential for efficient DNA repair. Interestingly, it has been recently described that the pathway used for the repair depend on the replication timing and gene expression level of a specific genomic loci (Canela et al., 2017; Marnef and Legube, 2017).

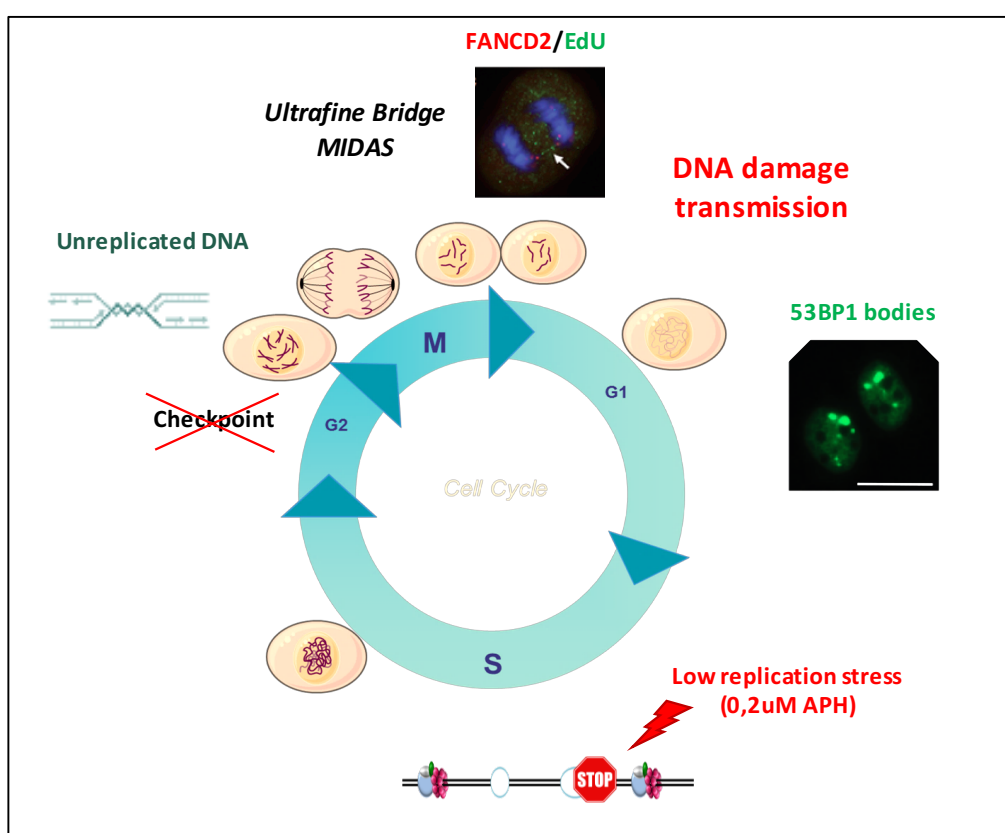
### c. Genomic instability in cancer

Genomic instability is the consequence of chronic replication stress and non-efficient DDR or checkpoint signalling. Thus, it is a hallmark human cancer (Abbas et al., 2013; Negrini et al., 2010). However, the moment during when this event arises in cancer development and the molecular basis under this phenomenon are only beginning to be elucidated.

#### i. **Fragile sites and DNA damage transmission**

In all individual cells, particular regions of the genome are prone to break in mitosis after replication stress. These regions were identified a long time ago, by the use of low doses of aphidicolin, and termed common fragile sites (CFSs) (Glover et al., 1984; Yunis and Soreng, 1984). It is largely suggested that genomic instability (mutations or CNAs) at these particular sites can be at the origin of cancer (Beroukhim et al., 2010; Bignell et al., 2010; Miron et al., 2015; Richards, 2001, 2001, 2001; Tsantoulis et al., 2008). Causes of their fragility are numerous and remain a matter of debate. It is likely due to an accumulation of endogenous sources of replication stress mentioned above, including lack of replication origins, repetitive sequences (AT rich) and replication-transcription conflicts (Brison et al., 2019a; Burrell et al., 2013; Helmrich et al., 2011; Irony-Tur Sinai et al., 2019; Letessier et al., 2011; Sarni et al., 2020; Tamura et al., 2019). It was suggested for a long time that CFSs are replicated in late S phase, explaining the increasing like hood of incomplete replication at the mitotic entry (Le Beau et al., 1998; Letessier et al., 2011). However, recent studies have shown that the replication of CFSs do not necessarily initiate in late S, but more in early/mid S phase, and their fragility lies on the lack of constitutive origin in the centre of the regions, leading to long replication fork travelling with higher susceptibility to collapse, and no dormant origins as a rescue (Brison et al., 2019a; Sarni et al., 2020).

CFSs are particularly difficult to replicate, explaining the high sensitivity of these genomic regions to replication stress. In many cases, these latest are not completely replicated before the onset of mitosis. These "under-replicated" regions will form anaphasic DNA bridges that can break during chromosome segregation. In 2009, two studies demonstrated that low replication stress leads to the formation of DNA breaks detected during mitosis at CFSs (Chan et al., 2009; Naim and Rosselli, 2009). In 2011, studies from Lukas et al. and Harrigan et al. described that replication stress induced by a dose of aphidicolin leads to the transmission of un-replicated DNA to daughter cells (Harrigan et al., 2011; Lukas et al., 2011) (**Figure 19**).



**FIGURE 19:** MIDAS AND DNA DAMAGE TRANSMISSION AT CFSs. LOW REPLICATION STRESS (I.E APHIDICOLIN LOW DOSE) LEADS TO UNREPLICATED DNA AT PARTICULAR FRAGILE SITES (CFSs). THIS REPLICATION STRESS IS NOT STRONG ENOUGH TO ACTIVATION THE G2/M CHECKPOINT AND THUS WILL LEAD TO MIDAS OR EVEN DNA DAMAGE TRANSMISSION DETECTED BY 53BP1 NUCLEAR BODIES IN G1 DAUGHTER CELLS.

This damaged DNA will be protected in the G1 daughter cells by a complexe of multiple signalling and repair proteins, including the 53BP1, giving the name of “53BP1 nuclear bodies”. In these works, they confirmed that the CFSs genomic regions are particularly interacting with

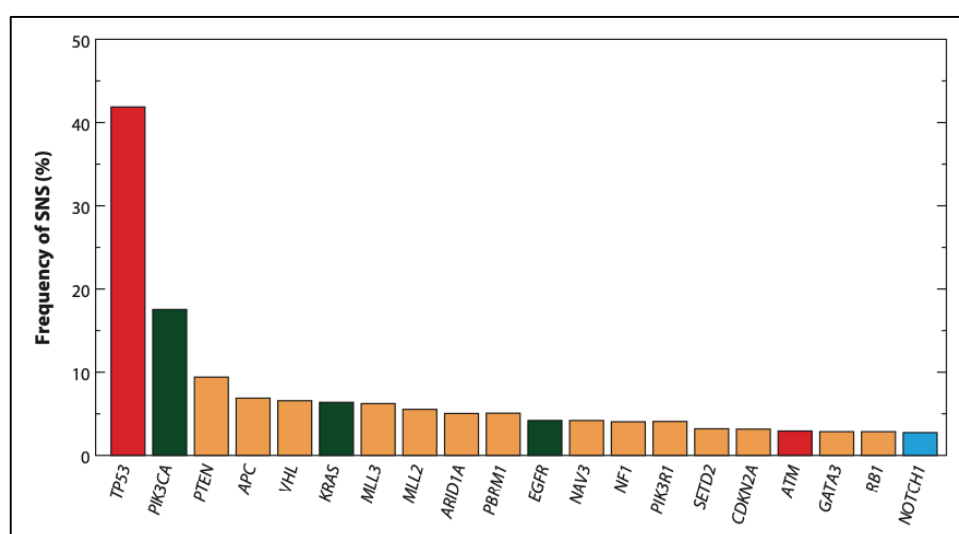
53BP1 in G1 phase and thus they have a high probability to be found within 53BP1 nuclear bodies. It was demonstrated that, to limit this dangerous transmission, cells use a “last chance” mechanism to replicate these under-replicated loci before dividing. This mechanism is based on break induced replication (or BIR) and has been called late-DNA synthesis or mitotic DNA synthesis (MiDAS) (**Figure 19**). MiDAS has been discovered thanks to the detection of EdU and/or FANCD2 foci in mitotic cells, which again specifically colocalized with CFSs. Repair proteins MUS81, SLX4, SMC2, WAPL and POLD3 have been described to play a role in this process (Bergoglio et al., 2013; Minocherhomji et al., 2015).

Unlike CFS, another type of instable sequences named early-replicating fragile sites (ERFS) is associated to short, GC rich, poly dA:dT, increased origin density, highly transcribed, and early replicated genes (Barlow et al., 2013; Mortusewicz et al., 2013; Tubbs et al., 2018). It is then very likely that ERFS instability is also caused by high amount of transcription-replication conflicts. Besides CFS and ERFS, two clear examples of fragile regions are telomeres and centromeres, that are both rich in repeated sequences and highly condensed heterochromatin regions. These regions are particularly prone to formation of DNA secondary structures, which can directly interfere with fork progression (Black and Giunta, 2018; Bloom and Costanzo, 2017; Higa et al., 2017). Finally, member of recently discovered ERFS described in B-cells, frequently coincides with breakpoints and recurrent rearrangements in B-cell lymphoma (Barlow et al., 2013; Le Tallec et al., 2013). Overall, the contribution of CFSs or ERFS to tumorigenesis may be essentially driven by the function of genes contains in these genomic loci.

## **ii. Oncogenes and tumour suppressor genes**

As previously mentioned, oncogenes are associated to tumour progression when being mutated or up-regulated. On the opposite, a second class of cancer-related genes are named tumour suppressor genes, whose wild type form expression counteract cancer development (Weinberg, 1995). These latest are often inactivated in cancers by point mutations, epigenetic silencing or deletions and most of them are antagonist of oncogenes. It was demonstrated that oncogene-induced DNA damage may explain two key features of cancer: genomic instability and the high frequency of p53 mutations (Bartkova et al., 2006; Di Micco et al., 2006; Halazonetis et al., 2008).

There are two main mechanisms by which oncogenes and tumour suppressors can be respectively activated or inactivated. First, point mutations, at the level of a single nucleotide, called single nucleotide substitutions (SNSs) and second, copy number alterations (CNAs) that directly target one or more genes expression level. The genomes of thousands of human cancers have been analysed, providing values of SNSs frequencies at which oncogenes are affected. Sequencing analysis of more than three thousand tumours from 12 cancer types described that the TP53 (p53) tumour suppressor gene is affected in 42% of analysed cancers (Kandoth et al., 2013). Oncogenes like PI3KCA (PI3K) is the second most affected gene in cancers followed by PTEN, APC, VHL and KRAS (**Figure 20**).



**FIGURE 20:** THE TOP 20 MOST FREQUENTLY TARGETED GENES BY SNSs ACROSS 12 HUMAN CANCER TYPES. FOR EACH GENE, THE PERCENTAGE OF CANCERS WITH SNSs (SINGLE NUCLEOTIDE SUBSTITUTION) TARGETING THE SPECIFIC GENE IS INDICATED. ONCOGENES BARS ARE COLOURED IN GREEN, TUMOUR SUPPRESSORS IN ORANGE OR RED FOR THE ONES INVOLVED IN DNA DAMAGE RESPONSE (DDR). NOTCH1 IS COLOURED IN BLUE BECAUSE DEPENDING ON THE CANCER TYPE, IT CAN BE AN ONCOGENE OR A TUMOUR SUPPRESSOR. (FROM MACHERET AND HALAZONETIS, 2015)

Among the 20 most frequently cancer-driver genes the targeted by SNSs, 3 are oncogenes and 17 are tumour suppressors. CNAs analysis in the same cohort of 12 cancer types had led to the identification of peaks for frequently amplified or deleted genomic regions (Zack et al., 2013). Among the 10 most significantly amplified regions, 9 contained established oncogenes among which CCND1, EGFR, MYC and ERBB2. Interestingly, amplified regions that did not harbour known oncogenes were enriched in genes involved in epigenetic



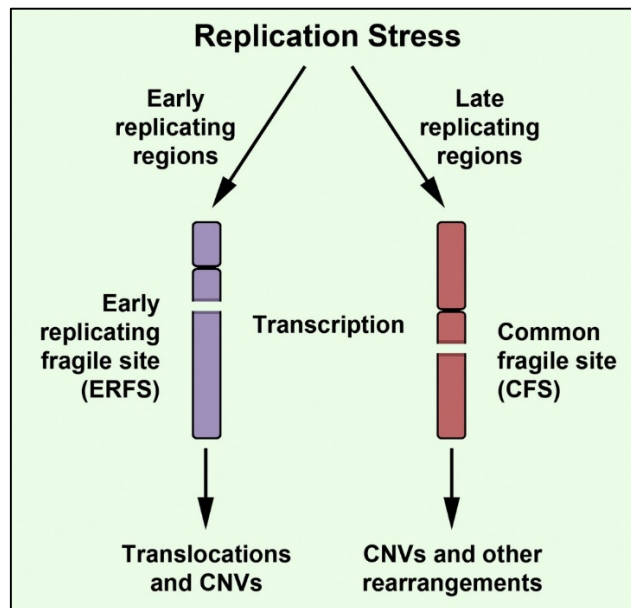
regulations. In contrast to genomic amplifications, only 4 out of the 10 most significantly deleted regions were identified in tumour suppressor genes.

Except for replication stress induction, oncogene activation/overexpression also bring cells capacity to highly proliferate (i.e PI3KCA, KRAS, EGFR). On the other hand, tumour suppressors inactivation allows cells to escape apoptosis (i.e PTEN, APC or RB1 silencing) and/or to accumulate DNA damages and genetic instability (i.e TP53 mutations or silencing).

### iii. Chromosomal instability and aneuploidy in cancer

There are various forms of genomic instability among which the very famous chromosomal instability (CIN), that refers to the high rate of chromosome structure aberrations like copy number variations (CNVs) or translocations. CIN was identified in cancer more than a century ago by Hansemann (Hansemann, 1890). Some of these CIN were visualized in all cancer cells whereas others were only observed in a subpopulation of tumour cells. This results suggested that cancer cells are the progeny of a clonal evolution of genetically unstable cell that acquire more and more abnormalities over time (Nowell, 1976). Many attempts have been made to link CFSs to translocations or other chromosome rearrangements in cancers. FRA3B and FRA16D, two very well described fragile genomic loci, are the most frequently affected CFSs in a panel of many human cancers, including colon, breast, oesophageal, renal and lung carcinomas (Durkin and Glover, 2007). While some positive correlations were found at the chromosomal level, most have not been confirmed by higher resolution inspection.

However, CFSs have been linked to gene amplifications and to a small number of translocations in cancers (Arlt et al., 2011). For example, replication stress induced by Aphidicolin or HU has been shown to induce chromosome instability (CIN), mainly copy number variations (CNVs) and some were found in CFSs (Arlt et al., 2011) (**Figure 21**). Finally, it was recently described that the type of CIN resulting from RS is dependent on the replication timing of the genomic loci (Du et al., 2019) (**Figure 21**). Although CIN is the major form of genomic instability in cancer, others have been described, including microsatellite instability (MSI). MSI is characterized by expansion or contraction of the number of nucleotide repeats present in microsatellite sequences (Fishel et al., 1993; Leach et al., 1993) that increased the frequency of base pair mutations (Al-Tassan et al., 2002).



**FIGURE 21:** DIFFERENT CLASSES OF GENOME INSTABILITY INDUCED BY RS. DEPENDING ON THE REPLICATION TIMING OF GENOMIC REGIONS IMPACTED, REPLICATION STRESS CAN INDUCE DIFFERENT TYPES OF CHROMOSOME INSTABILITY. TRANSLOCATIONS ARE MORE SPECIFIC TO ERFSS WHILE CNVs ARE FOUND IN BOTH ERFSS AND CFS. (FROM GLOVER AND WILSON, 2013)

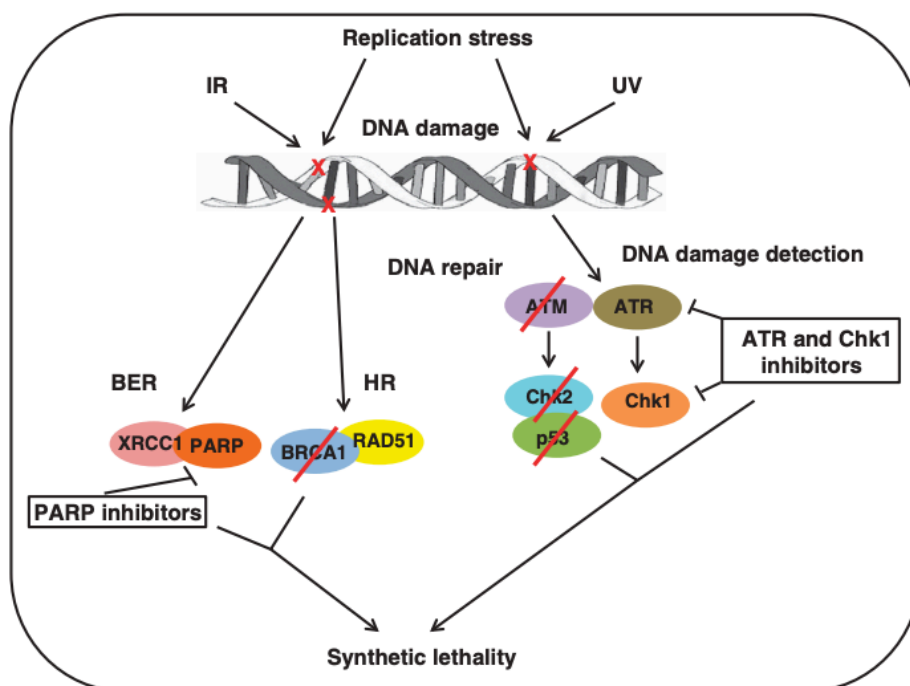
Besides CNV or translocations that are marks of aberrant chromosome structures, another and more severe kind of genome instability frequently found in cancer is aberration in chromosome number, called aneuploidy (Boveri, 2008). Quite surprisingly, even though it can be highly deleterious for the cells, aneuploidy is well tolerated by cancer cells, with about 88% of tumour being aneuploid (Taylor et al., 2018). Unlike some pan-cancer oncogenes or tumour suppressor genes, aneuploidy is not a universal promoter of tumorigenesis. Indeed, several recent studies suggested that aneuploidy is more cancer-type specific and context-dependant (Chunduri and Storchová, 2019; Santaguida and Amon, 2015; Sheltzer and Amon, 2011; Sheltzer et al., 2017; Williams et al., 2008). Hence, aneuploidy is very likely to have a clinical relevance as a prognostic marker and/or as a potential therapeutic target.

#### iv. Targeting replication stress and DDR in cancer

Although conventional DNA-damaging chemotherapies have been used for decades by oncologist to treat cancer, their applicability and efficacy is limited by the toxicity. Indeed, because it targets highly proliferative cells regardless of their state, it affects also normal hematopoietic cells and all cells that regenerate tissues. This leads to undesirable and sometimes intolerable side effects. Moreover, classical treatments with anticancer drugs lead to relapse by the development of highly resistant tumour cells. In light of this, several rationally designed and more specific combination therapies targeting tumour cells are currently emerging. The fact that replication stress is a common feature of pre-cancerous and cancer cells and is rather rarely observed in normal cells opens up new possibilities for new therapeutic approaches. A non-exhaustive list of new therapeutic approaches targeting replication stress and DNA damage response in cancer will be described in the following paragraphs. As it is well described that cancer cells harbour mutations in DDR and cell cycle regulation, a new global approach is based on the inhibition of a pathway that cancer cells are especially dependent on, allowing a specific killing of cancer cells. This approach is named synthetic lethality (Huang et al., 2020; O'Neil et al., 2017; Ryan et al., 2018; Ubhi and Brown, 2019) (**Figure 22**).

The concept of synthetic lethality was very well demonstrated in a famous experiment using poly(ADP-ribose) polymerase (PARP) inhibitors (PARPi), to block DNA repair in breast cancer cells that lost BRCA1/2 proteins and are thus mainly survive thanks to PARP1 activity (Bryant et al., 2005; Farmer et al., 2005). Recently, a growing body of evidences indicated that, rather than only BRCA1/2 mutated patient, a wider population of cancer patients could benefit from PARPi treatment. Numerous biomarkers including HR deficiency and high level of replication pressure can help to predict efficacy of PARPi therapeutic approach (Brown et al., 2017; Lord and Ashworth, 2017; Pilié et al., 2019; Yi et al., 2019). In the same logic, inhibitors of ATR and CHK1 are also being tested in clinical trial for cancer therapies (Hall et al., 2014; Mei et al., 2019; Weber and Ryan, 2015; Williamson et al., 2016). A promising therapeutic approach based on the specific incorporation of damaged dNTPs by cancer thanks to the targeting of a protein that prevents mis-incorporation of oxidized dNTPs, MTH1 (or NUTD1) was described in relevant studies (Gad et al., 2014; Huber et al., 2014; Yin and Chen, 2020). As cancer cells have also deficiency in cell cycle regulators like p53, G2/M checkpoint becomes essential to prevent mitotic catastrophes or DNA damage transmission. Exciting

therapies targeting regulators of mitotic entry, such as Wee1 inhibitors, are currently being explored (Lee et al., 2019; Matheson et al., 2016; Sand et al., 2020).



**FIGURE 22:** EXAMPLE OF SYNTHETIC LETHALITY THROUGH PARP AND ATR/CHK1 INHIBITION. KNOWING THE HIGH LEVEL OF DNA DAMAGE IN CANCER CELLS, SOME DEFICIENCY LIKE BRCA1/2, ATM, CHK2 OR P53 CAN BE TRIGGERED TO SELECTIVELY KILL THESE CELLS. BRCA1/2 TUMOUR CELLS ARE PARTICULARLY SENSITIVE TO PARP INHIBITORS AND ATM, CHK2 OR P53 DEFICIENT CANCER CELLS HAVE AN INCREASE SENSITIVITY TO ATR OR CHK1 INHIBITOR. (FROM KARANIK ET AL., 2015)

Another interesting current approach is to specifically increase the already high replication stress in cancer cells. In this line, it has been described that KRAS positive cells, that are already deficient in licensing factors, are sensitive to CDC6 depletion (Lim and Townsend; Steckel et al., 2012). It has also been demonstrated that different tumour-derived cell lines are hypersensitive to HU and H<sub>2</sub>O<sub>2</sub> when being deficient in origin licensing factor (ORC1 depletion) (Zimmerman et al., 2013). Finally, another opposite strategy is to increase origin firing by geminin inhibition in checkpoint deficient cancer cells, leading to CDT1 stabilization, re-replication, senescence and apoptosis (Lin et al., 2010; Steckel et al., 2012).

In conclusion, synthetic lethality is a simple but powerful genetic concept that plays a major role in the current cancer research and drug discovery. For many years, potential drug

targets were identified through genome sequencing data and direct screen in human cancer cell lines. Recent analysis of a genome-scale CRISPR-based screen in a large panel of tumour cells provided consistent evidences that the number of cancer drug targets that can be used for selective killing is likely more than a hundred, the large majority being context dependant (Behan et al., 2019). Thanks to the continuously evolving CRISPR technology, together with single-cell approaches, it is now easier to address tumour heterogeneity involved in primary drug resistance and genetic pressure that drives relapse and secondary resistance. Nonetheless, the better understanding of cancer resistance and relapse also highlighted the complex mechanism underlying cancer phenotypes that will need to be unravelled to successfully identify specific targets that can be used for the selective killing of cancer cells and to operate the full potential of personalized anticancer therapies.

## Thesis objectives and approach

The understanding of how replication timing can be regulated and/or affected by mild DNA replication stress is still poorly explored in healthy as well as tumour context. The principal aim of this thesis was to investigate the potential modification and regulation of DNA replication timing in the context of mild replication stress and to compare the cellular response of cancer versus healthy cells.

To address this first question, we decided to simulate mild replication stress using low doses of the well-known DNA polymerase inhibitor aphidicolin (APH) and to analyse the whole genome replication timing of cells thanks to CGH arrays approach described in the introduction (**Figure 3**) (Hadjadj et al., 2016; Hiratani et al., 2008). We chose six well characterized (ATCC) cellular models currently used in laboratories, that harbour cellular differences such as tissue of origin, tumorigenicity, differentiation stage and molecular singularities such as oncogene expression, high or low genetic instability and different telomere maintenance mechanism (telomerase, ATL or none). Two of these models are from healthy tissues: RPE-1 cells from retina and MRC5-N from embryonic lung tissue. The four other cellular models were from different tumour tissues: RKO and HCT116 from colon cancer, U2OS from osteosarcoma, and K562 from chronic myeloid leukaemia (Table S1, submitted manuscript).

Before starting the whole-genome replication timing experiment, it was first essential to set-up the experimental strategy and choose appropriate time and dose for aphidicolin treatment in mother cells as well as appropriate time of release to evaluate the replication timing of daughter cells. Indeed, as we wanted to assess the direct response of cells to aphidicolin treatment, we needed to treat the cell a long time enough to have the global population response but not too long to avoid the analysis of cells that have been treated with aphidicolin during two cell cycle in a row. Then, it was essential to verify that the dose of aphidicolin we will use would be optimal to induce mild replication stress without affecting too much the progression of cells through cell cycle. This experimental set-up will be further described in the first part of this thesis results.

Assessing DNA replication timing of the 6 cell lines in normal or aphidicolin-treated condition, we ended-up with the identification of specific genomic regions harbouring replication timing alterations under replication stress, that we named aphidicolin-Replication Timing Impacted Loci (aRTIL).

The second aim of my thesis was to characterize these aRTIL at the genomic and epigenomic level and to try to identify potential actors/causes of replication timing modifications. Genetic and epigenomic characterizations were done by bioinformatics analysis of our data, combined with public available data (i.e: ChiP-seq in HCT116 and K562). To better understand the cellular response to aphidicolin, we performed RNA-ChiP and ATAC-seq experiments.

Finally, for the third objective of this thesis, knowing that DNA damage can be transmitted to the next cellular generation (Harrigan et al., 2011; Lukas et al., 2011), we wondered if a low replication stress undergone by a given cell could have an impact on the next cellular replication program. After setting-up an appropriate time of release for cells that were treated or not with aphidicolin in order to be sure to analyse daughter cells, we performed whole-genome replication timing analysis of the 6 cell lines released from DMSO or aphidicolin treatment.



# Results

## **Experimental set-up of aphidicolin treatment and release in the 6 human cell lines**

As mentioned in the thesis objectives and approach, to properly answer our question, we needed first to set-up the optimal experimental condition for each cell line.

a. Set-up of aphidicolin dose

First, we wanted to verify that the common dose of 0.2 $\mu$ M aphidicolin was adapted for the 6 cell lines. Our criteria were the cell cycle and Chk1 activation (pChk1 on S345). In fact, we did not want cells to be too much impacted by the aphidicolin treatment - to be able to analyse the replication timing in S phase - while inducing a replication stress strong enough to observe a cellular response. In other words, it was important that the aphidicolin dose chosen lead to a mild but not too strong checkpoint activation. Thus, after treating cells with 0.1, 0.2 and 0.3 $\mu$ M aphidicolin for 10 or 24h, we analysed the cell cycle by FACS and pChk1 (S345) by western blot.

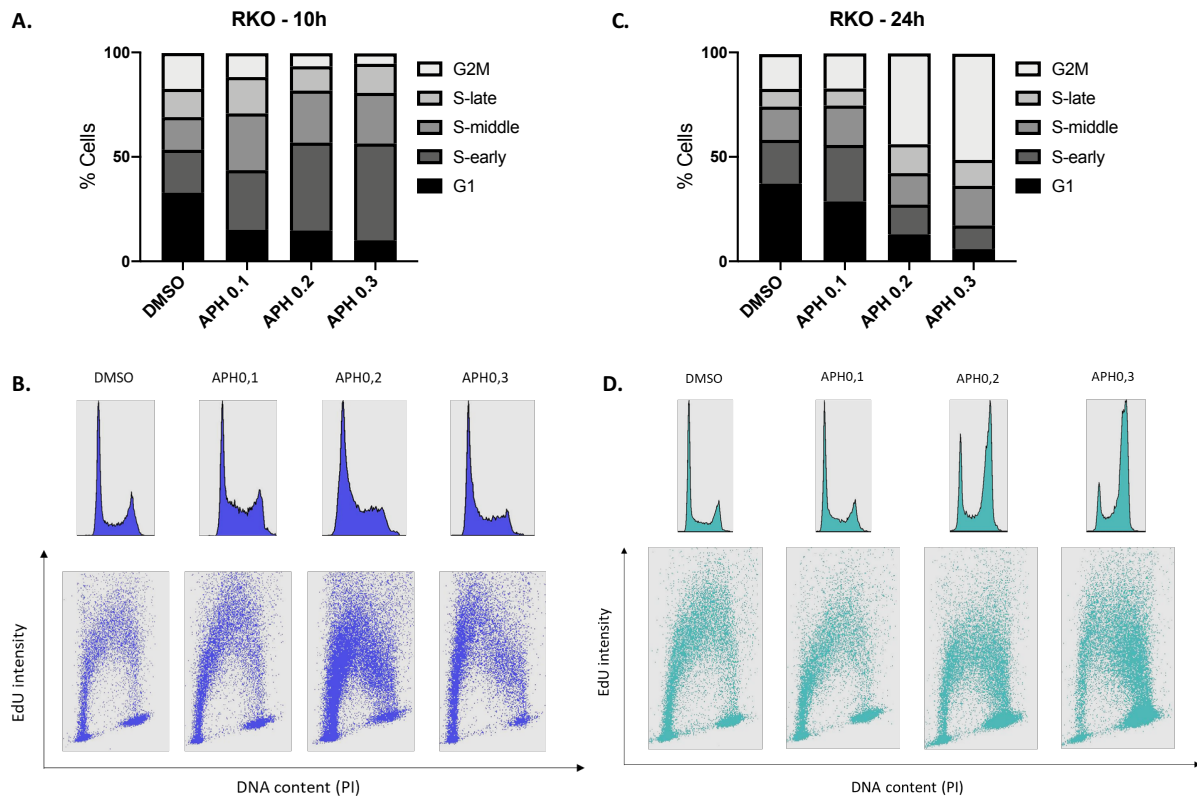
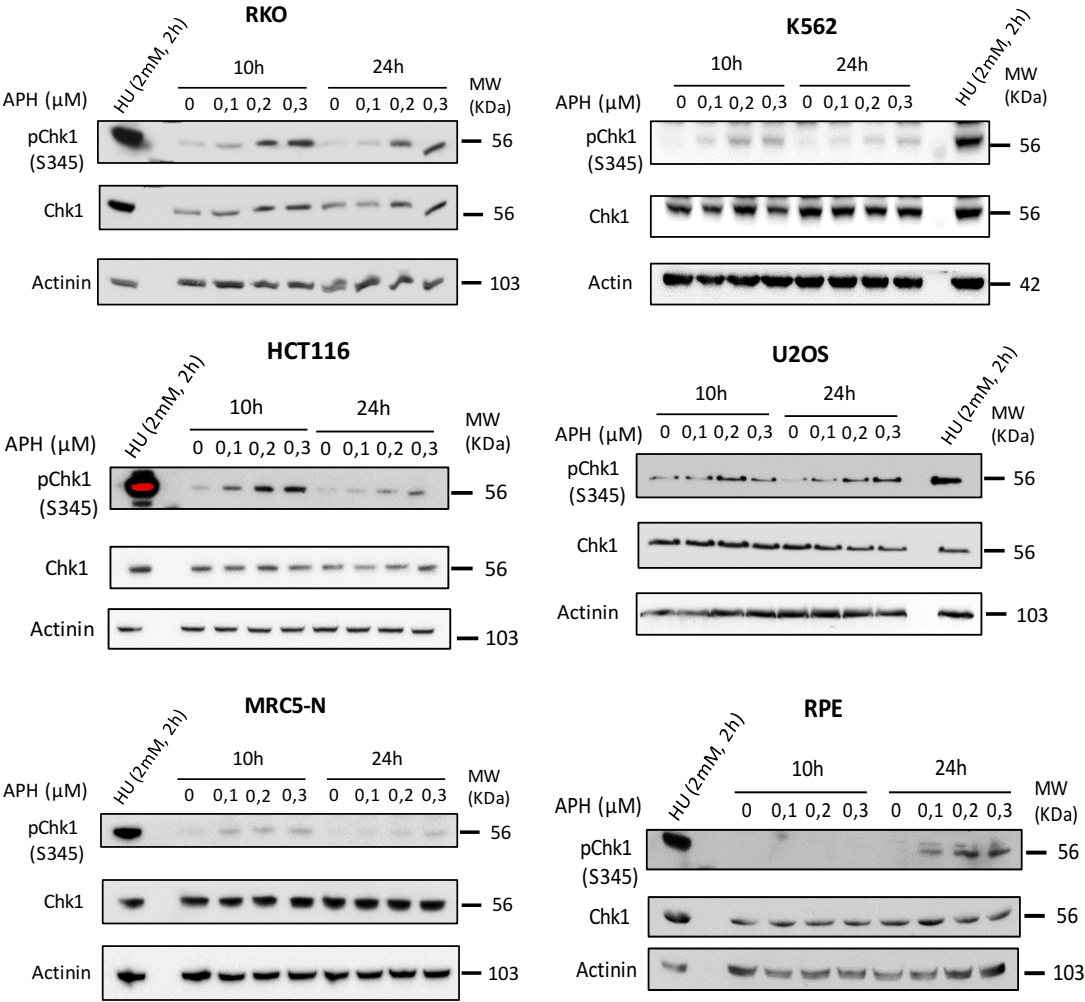


FIGURE 23: CELL CYCLE ANALYSIS IN DIFFERENT CONDITIONS OF APHIDICOLIN TREATMENT IN RKO CELLS. (A) AND (C): HISTOGRAMS REPRESENTING THE PERCENTAGE OF CELL IN THE DIFFERENT PHASES OF THE CELL CYCLE: G1, S-EARLY, S-MIDDLE, S-LATE AND G2M. (B) AND (D): ILLUSTRATIONS OF THE CELL CYCLE ANALYSIS FACS: ON THE TOP THE DENSITY PLOTS ONLY BASED ON IP CONTENT, ON THE BOTTOM CELL CYCLE SCATTERPLOT BASED ON IP CONTENT (X-AXIS) AND EdU INTENSITY (Y-AXIS). THE DATA ON THE LEFT ARE FOR A TREATMENT OF 10H AND ON THE RIGHT 24H. THE SAME ANALYSIS WAS DONE IN THE 5 OTHER CELL LINES (FIGURE S1).

In the 6 cell lines, we observed a slight accumulation in S (10h) or G2 (24h) phases of the cell cycle under aphidicolin treatment (**Figure 23**). Depending on the cell line, the accumulation was more visible at 0.2μM aphidicolin compared to 0.1 μM while in the majority the 0.3μM aphidicolin dose effect was not particularly different from 0.2μM (**Figure 23 and Figure S1**).

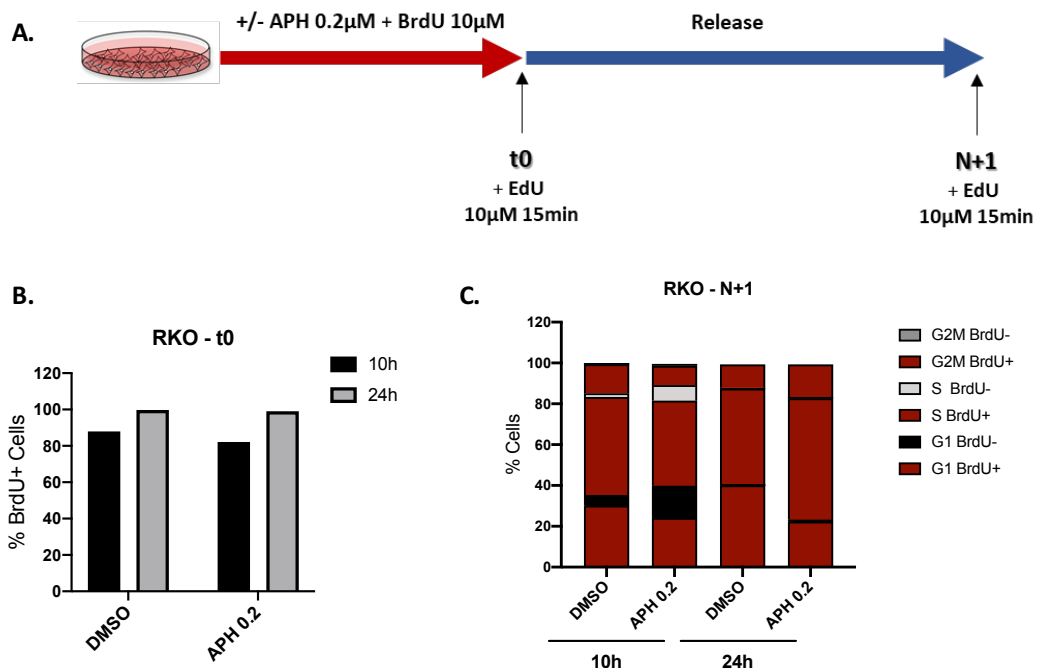
pChk1 analysis allowed us to validate the dose of 0.2μM aphidicolin as 0.1μM was sometime not enough (K562, U2OS, RPE-1) and the 0.3μM too strong in some cells (RKO mainly) (**Figure 24**). We noticed that generally, cancer cells exhibit a stronger pChk1 response to aphidicolin compared to normal cells. With this experiment, it was the first time we notice a difference between normal and cancer cells.



**FIGURE 24:** WESTERN BLOTS TO QUANTIFY THE LEVEL OF PCHK1 IN THE 6 CELL LINES UNDER SEVERAL DOSES OF APHIDICOLIN. MEMBRANES OF WESTERN BLOT USING CHK1, PCHK1 (S345) ANTIBODIES AND ACTIN OR ACTININ AS LOADING CONTROL. DIFFERENT CONDITIONS ARE WRITTEN FOR EACH MEMBRANE AND CELL LINES. 2mM, 2h HU WAS USED AS A POSITIVE CONTROL OF CHK1 ACTIVATION.

## b. Set-up of treatment and release durations

Once the aphidicolin dose validated, we needed to determine the appropriate time of treatment. Indeed, each cell lines having their proper proliferation capacities, it was essential to assess the appropriate time of aphidicolin treatment in order to be sure that cells were treated only during one cell cycle. Then, as we wanted to specifically analyse the replication timing of daughter cells coming from the one that has been treated once with aphidicolin, we needed to set-up a specific time of release for each cell line. This time needed to be long enough in order to let cells pass through mitosis, then G1 and finally enter in the next S phase, but not too long in order to be sure that cells that were considered as daughter cells are the direct descendent of mother cells treated with aphidicolin.



**FIGURE 25:** CELL CYCLE ANALYSIS BY FACS TO SET-UP THE APPROPRIATE TIME OF APHIDICOLIN TREATMENT AND RELEASE IN RKO CELLS. (A) SCHEME REPRESENTING THE EXPERIMENTAL PROTOCOL. BRD U 10MM WAS ADDED TO THE CULTURE MEDIA TOGETHER WITH 0.2MM APHIDICOLIN (APH 0.2) OR DMSO. CELL WERE HARVESTED RIGHT AFTER TREATMENT OR AFTER 15H OF RELEASE IN A FRESH MEDIA. 15MIN BEFORE FIXATION, CELLS WERE LABELLED WITH EDU 10MM. AFTER FIXATION, IMMUNODECTION OF BRDU, EDU AND DAPI WAS ASSESSED BY FACS TO MEASURE THE PERCENTAGE OF BRDU POSITIVE CELLS IN THE DIFFERENT CONDITIONS. (B) HISTOGRAM FOR THE QUANTIFICATION (%) OF BRDU POSITIVE (BRDU+) CELLS RIGHT AFTER 10H (BLACK) OR 24H (GREY) OF TREATMENT (T0). (C) STACKED HISTOGRAM REPRESENTING THE PROPORTION OF BRDU+ CELLS (IN RED) IN THE DIFFERENT PHASES OF THE CELL CYCLE (G1, S OR G2M) AFTER RELEASE (N+1).

To define the proper time of treatment and release, we labelled mother cells thanks to BrdU incorporation during the whole time of treatment (with DMSO or aphidicolin), then release the cells in a fresh media for a selected time of 15h. At this time, cells were labelled in S phase cells thanks to EdU incorporation and we measured the amount of BrdU positive cells in mother ( $t_0$ ) or daughter cells ( $N+1$ ), in different phases of the cell cycle (G1, S or G2M) (**Figure 25A**).

Here we only describe the results for the RKO cell line (**See Figure S2 and S3 for the 5 other cell lines**). We observed in the mother cells that 10h of treatment led to 82% of BrdU positive cells in the Aphidicolin treated condition against 99% BrdU positive cells after 24h of treatment. To be sure that we do not treat cells twice, we decided to select a time of treatment of 16h. Regarding the time of release, after 15h we found some BrdU positive in G2/M and some BrdU negative cells in S phase, meaning that this time is too long. Thus, for RKO cells, we decided to release the cells for a time of 13h. All the appropriate duration of treatment and release chosen for the 6 cell lines are recapitulated in the **Table S2** and FACS analysis is presented in the **Figure S1 of the submitted manuscript** (following part),

# Advances in DNA replication timing highlight genome plasticity of cancer cells in response to low replication stress

*(Submitted manuscript)*

# Low replication stress leads to specific replication timing advances associated to chromatin remodelling in cancer cells

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## ABSTRACT

DNA replication is well orchestrated in mammalian cells through a tight regulation of the temporal order of replication origin activation, named the replication timing, a robust and conserved process in each cell type. Upon low replication stress, the slowing of replication forks induces delayed replication of fragile regions leading to genetic instability. The impact of low replication stress on the replication timing in different cellular backgrounds has not been explored yet. Here we analysed the whole genome replication timing in a panel of 6 human cell lines under low replication stress. We first demonstrated that cancer cells were more impacted than non-tumour cells. Strikingly, we unveiled an enrichment of specific replication domains undergoing a switch from late to early replication in some cancer cells. We found that advances in replication timing correlate with heterochromatin regions poorly sensitive to DNA damage signalling while being subject to an increase of chromatin accessibility. Finally, our data indicate that, following release from replication stress conditions, replication timing advances can be inherited by the next cellular generation, suggesting a new mechanism by which cancer cells would adapt to cellular or environmental stress.

## INTRODUCTION

DNA replication is a highly complex process that ensures the accurate duplication of the genome, hence the faithful transmission of genetic material to the cell progeny. DNA replication occurs during S phase



through the replisome activity, but it requires important upstream regulation during the G1 phase and checkpoints in G2 phase, together with a tight control throughout the process itself. Multicomplex replication machinery performs the coordinated initiation of DNA synthesis at hundreds of replication origins spread throughout the whole length of the genome (1). Adjacent origins that initiate DNA replication at the same time have been called “replicon clusters” (2), giving rise to chromosomal domains replicating synchronously. Each replicon cluster starts replicating at a precise moment during the S-phase, either at the beginning (Early-S), the middle (Mid-S) or the end (Late-S). This coordination of the temporal program of DNA replication is called “replication timing” (RT), allowing a complete and faithful duplication of the entire genome before cell division.

The RT program is modified during organism development and cell differentiation (3,4) and is coupled with gene expression, chromatin epigenome and nuclear 3D compartmentalization (5–8). In somatic cells, the RT pattern is very robust through cell generations (8–11) with early-replicating DNA residing deep within the nucleus within the A compartment containing active chromatin while the later-replicating regions occur at the nuclear periphery or near the nucleolus (9, 12, 13) within the B compartment, containing inactive chromatin. Additional complex associations have been highlighted such as the link between early-replicating regions and GC nucleotides enrichment, enhanced gene expression, and active epigenetic marks corresponding to open or euchromatin. Conversely, late-replicating regions tend to be enriched in AT nucleotides, low gene content, and have heterochromatin repressive epigenetic marks (13, 14).

DNA replication stress is defined as the slowing or stalling of the replication fork resulting in inefficient DNA replication. Many exogenous or endogenous sources of impediment on DNA as well as pathological perturbations such as oncogene activation, conflicts between DNA replication and transcription or shortage of nucleotides affect the progression of replication forks, inducing replication stress (15–19). Experimentally, replication stress can be induced by the specific inhibition of replicative DNA polymerases by treatment with the drug aphidicolin. Notably, low doses of aphidicolin (0.1 to 0.6  $\mu$ M) are well known to cause the induction of common fragile sites (CFS) expression and the generation of under-replicated DNA that leads to DNA damage transmission (20–24). CFS are chromosomal regions harbouring cancer-related genes (25) that are prone to breakage upon replication stress (26) and whose instability is often observed at the early stages of carcinogenesis (27). The fragility of these chromosomal regions has been widely studied, revealing incomplete DNA replication before mitosis (21, 23, 28, 29) mainly due to conflicts with large transcription units (30–32) or/and origin paucity (33, 34).

Evidence of aberrant RT in many different genetic diseases and cancers suggests this cellular process is important for genomic stability (35–37). Interestingly, replication stress inducing CFS expression also affects the RT of these specific chromosomal domains (30, 32). The extent to which these RT changes influence tumour transformation process is still largely unknown.

The major aim of this study was to explore whether low replication stress affects differentially RT of cells from diverse types, and whether a common mechanism for RT change can be found upon low RS.

To do so, we characterized and compared the impact of mild replication stress induced by low doses of aphidicolin on 4 cancer and 2 non-tumour human cell lines (colon, blood, osteoblast, retina, and lung) (**Table S1**). Our experiments revealed that a low dose of aphidicolin have a stronger effect on the RT of cancer cells, promoting RT delays but also unexpected RT advances. We demonstrated that RT advanced loci can be housed in CFS but, contrary to RT delays, they are poorly targeted by DNA damage signalling while being characterized by stronger chromatin accessibility in response to aphidicolin. Finally, we observed the persistence of RT advances in daughter cells released from replication stress which is correlated with modification of chromatin loop size and pre-replication complex (pre-RC) loading in G1 and an increase in the expression of genes contained within these chromosomal regions. Altogether, our results indicate that low replication stress, which leads to RT advances onto flexible heterochromatin regions, can influence the DNA replication program and gene expression of the next generation of cancer cells.

## **MATERIAL AND METHOD**

### **Cell lines, cell culture and drugs**

The 6 human cell lines were purchased from ATCC. Cells were grown in culture medium supplemented with 10% fetal bovine serum (Gibco Life Technologies A31608-02) at 37°C, 5% CO<sub>2</sub> and 5% O<sub>2</sub>. HCT116, U2OS and RKO cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Life Technologies 31966021), MRC5-N cell line was grown in Minimum Essential Medium Eagle (MEM-alpha, Gibco Life Technologies 22561021), RPE-1 cell line was grown in Roswell Park Memorial Institute Media (RPMI, Gibco Life Technologies 61870044) and K562 in Iscove Modified Dulbecco Media (IMDM, Gibco Life Technologies 21980032) supplemented with decomplexed serum. Aphidicolin (Sigma-Aldrich A0781-1MG) stock solution was diluted in DMSO (Sigma-Aldrich D8418-250mL) and kept at -20°C for a maximum of 2 months after first thawing. Cells were synchronized in G1/S with 0.5mM L-Mimosine (Sigma-Aldrich M0253) for 24h.

### **Fluorescence Activated Cell Sorting (FACS)**

Cells were pulse-labelled with 10µM BrdU and/or EdU for indicated times then collected by trypsinization and fixed in 70% ice-cold ethanol overnight at -20°C. For EdU and BrdU immunodetection, we followed the protocol described in Bradford and Clarke 2011 (38). Finally, after washing in PBS-BSA 0.5%, then in PBS, cells were resuspended in PBS with propidium iodide (25 µg/mL, Invitrogen, p3566 ) and RNase A (100 µg/mL, Thermo Fisher Scientific, ENO531) or with DAPI (1/1000, Sigma-Aldrich, D9542). After 20 min of incubation at room temperature, cell cycle analysis was carried out by flow cytometry with a MACSQuant 10 or VYB cytometer (Miltenyi Biotec) and analysed with FlowLogic software.

### **Cell lysis, fractionation and Western blotting**

For whole cell extract, cells were lysed for 30min on ice with classic lysis buffer (0.3M NaCl, 1% triton, 50mM Tris pH7.5, 5mM EDTA, 1mM DTT and 1X Halt protease and phosphatase inhibitor cocktail from Thermo Fisher Scientific 78445). For subcellular fractionation, cells were lysed in Buffer A (Hepes 10mM pH 7.9, KCl 10mM, MgCl<sub>2</sub> 1.5mM, sucrose 0.34M, Glycerol 10%, dithiothreitol (DTT) 1mM, 1X Halt protease and

phosphatase inhibitor cocktail) complemented with Triton X-100 0.1% for 5min on ice. After centrifugation at 1,500rcf, 5min, 4°C, the supernatant was clarified by high-speed centrifugation (18,000rcf, 4°C, 15min) to obtain the cytoplasmic fraction. The pellet was washed once with Buffer A and then incubated in Buffer B (EDTA 3.2mM, DTT 1mM, Halt protease and phosphatase inhibitor cocktail) for 30min on ice. After centrifugation (1,700rcf, 5min, 4°C), the supernatant was collected as the soluble nuclear fraction. The pellet (chromatin fraction) was washed once with Buffer B and resuspended in the same buffer. The whole cell extracts and chromatin-enriched fractions were then sonicated (10 pulses of 1s at 40% amplitude with a Sonics Vibra Cell Ultrasonic processor) and Laemmli buffer was added in order to have a final protein concentration of 2µg/µL and 0.5µg/µL respectively. The detection of pChk1 (S345, Cell signalling 2341 , Rabbit), Chk1 (Santa Cruz sc 8408, Mouse), Actinin (MBL 05-384, Mouse), MCM2 (Abcam ab-4461, Rabbit), p-MCM2 S40 (Abcam ab133243 , Rabbit), ORC2 (MBL M055-3, Mouse) Lamin A/C (Santa cruz sc-7293, Mouse) and Tubulin (Sigma T5168, Mouse) was done by running SDS-page gels, transferring on PVDF membranes, blocking with 5% milk, incubating with primary antibody (in TBS-T, adapted dilutions) followed by secondary antibody (MBL 70765, Mouse or MBL 70745 Rabbit, 1/10 000 in TBS-T) and finally revealing thanks to ECL (Biorad 170-5161) under the ChemiDoc imaging system (BioRad).

### **Replication timing analysis**

10-20 millions of exponentially growing mammalian cells (with DMSO or aphidicolin) were incubated with 0.5mM BrdU (Abcam, #142567), protected from light, at 37°C for 90 minutes. After washing in PBS, cells were fixed in 75% final cold EtOH and stored at -20°C. BrdU labeled cells were incubated with 80µg/mL Propidium Iodide (Invitrogen, P3566) and with 0,4 mg/ml RNaseA (Roche, 10109169001) for 15min at room temperature and 150 000 cells were sorted in early (S1) and late (S2) S phase fractions using a Fluorescence Activated Cell Sorting system (FACS Aria Fusion, Becton Dickinson) in Lysis Buffer (50mM Tris pH=8, 10mM EDTA, 0.5% SDS, 300mM NaCl) and stored at -20°C until the following steps. DNA from S1 and S2 fractions of sorted cells was extracted using Proteinase K treatment (200µg/ml, Thermo Scientific, EO0491) followed by phenol-chloroform extraction and sonicated to a size of 500-1,000 base pair (bp), as previously described (39). Immunoprecipitation was performed using IP star robot at 4°C (indirect 200µl method, SX-8G IP-Star® Compact Automated System, Diagenode) with an anti-BrdU antibody (10µg, purified mouse Anti-BrdU, BD Biosciences, #347580). Denatured DNA was incubated for 5 hours with anti-BrdU antibodies in IP buffer (10mM Tris pH=8, 1mM EDTA, 150mM NaCl, 0.5% Triton X-100, 7mM NaOH) followed by an incubation for 5 hours with Dynabeads Protein G (Invitrogen, 10004D). Beads were then washed with Wash Buffer (20mM Tris pH=8, 2mM EDTA, 250mM NaCl, 1% Triton X-100). Reversion was performed at 37°C for 2 hours with a solution containing 1% SDS and 0.5mg Proteinase K followed, after the beads removal, by an incubation at 65°C for 6 hours in the same solution. Immunoprecipitated BrdU labeled DNA fragments were extracted with phenol-chloroform and precipitated with cold ethanol. Control quantitative PCRs (qPCRs) were performed using oligonucleotides specific of mitochondrial DNA, early (BMP1 gene) or late (DPPA2 gene) replicating regions (10, 39). Whole genome amplification was performed using SeqPlex™ Enhanced DNA Amplification kit as described by the manufacturer (Sigma-Aldrich, SEQXE). Amplified DNA was purified using PCR purification product kit as described by the manufacturer (Macherey-Nagel, 740609.50). DNA

amount was measured using a Nanodrop. Quantitative PCRs using the oligonucleotides described above were performed to check whether the ratio between early and late replication regions was still maintained after amplification. Early and late nascent DNA fractions were labelled with Cy3-ULS and Cy5-ULS, respectively, using the ULS arrayCGH labeling Kit (Kreatech, EA-005). Same amounts of early and late-labeled DNA were loaded on human DNA microarrays (SurePrint G3 Human CGH arrays, Agilent Technologies, G4449A). Hybridization was performed as previously described (39). The following day, microarrays were scanned using an Agilent C-scanner with Feature Extraction 9.1 software (Agilent technologies). To determine the replication domains and do the comparative analysis in different conditions, the online platform specific for replication timing data START-R (40) was used, with biological duplicates for each condition. The output bed file gave the list of significantly impacted genomic regions (ADVANCED or DELAYED) and the report of number and percentage of genomic regions impacted. We also used the output replication timing smooth files to identify the early ( $RT > 1$ ) the mid ( $-1 < RT < 1$ ) and late ( $RT < -1$ ) replicating regions.

### **BrdU ChIP-qPCR**

The same protocol as for replication timing was performed until BrdU immunoprecipitation (IP). For the BrdU-IP, 140 $\mu$ L of IP buffer (Tris pH8 50mM, EDTA 2mM, NaCl 300mM, Triton 1%, H<sub>2</sub>O qsp, 14mM NaOH extemporaneously) and 10 $\mu$ g of the monoclonal anti-BrdU antibody (BD Biosciences, 347580) were added to DNA and incubated on rotating wheel overnight at 4°C. 1.5mg of magnetic beads (Dynabeads™ Protein G, Thermofisher 1004D) previously washed with PBS (15min on wheel at RT) and IP buffer was added to the mix and incubated on wheel 2 hours at 4°C. After washing twice with 800 $\mu$ L of Buffer B (Tris pH8 20mM, EDTA 2mM, NaCl 250mM, Triton 0.2%, H<sub>2</sub>O qsp) and with 800 $\mu$ L of Tris pH8 10mM, beads were resuspended in 100 $\mu$ L of Tris pH8 10mM. Immuno-precipitated DNA was then recovered by a reversion step with a solution containing 1% SDS and 0.5mg Proteinase K for 2h at 37°C while shaking. The supernatant was incubated overnight at 65°C while shaking. A final phenol-chloroform purification was performed and DNA concentration was measured with Nanodrop technology before performing qPCR. qPCR were performed for the specific amplification of 2 early and 2 late replicating control regions, 3 ADV aRTIL and amplicon from neo-synthesized mitochondrial DNA for normalization (**Table S3**). StepOne technology was used to do the qPCR. For each genomic region amplified, we quantified the percentage of S1 and S2 after normalization with mitochondrial DNA (41).

### **Gene expression microarrays**

Exponentially growing cells (with DMSO or aphidicolin) were harvested and RNAs were extracted with RNeasy plus mini kit (Qiagen). RNAs quality and quantity were controlled using Nanodrop ND-1000 and Bioanalyzer 2100 Expert from Agilent. cDNAs were prepared according to the standard ThermoFisher protocol from 100ng total RNA (GeneChip™ WT PLUS Reagent Kit Manual Target Preparation for GeneChip™ Whole Transcript (WT) Expression Arrays User Guide). Following fragmentation, 5.5  $\mu$ g of single

stranded cDNA were hybridized on Human Clariom S Arrays in GeneChip Hybridization Oven 645 for 16 hr at 45°C. The arrays were washed and stained in the Affymetrix Fluidics Station 450. Arrays were scanned using the GeneChip Scanner GC3000 7G and images were analysed using Command Console software to obtain the raw data (values of fluorescent intensity). The data were analysed with TAC (Transcriptome Analysis Console, version 4.0.2.15) from ThermoFisher. Microarrays were normalized with the “Robust Multichip Analysis” (SST-RMA) method. Statistical analysis allowed tagging of genes according to the fold change (FC) and the p-value adjusted together with ANOVA approach.

### **ATAC-seq**

100,000 exponentially growing RKO cells were trypsinized, washed in PBS and treated with 1:100 volume of RNase-free DNase (QIAGEN) and DMEM media for 30min at 37°C in the incubator. Cells were trypsinized, washed in PBS and resuspended in 500µL of ice-cold cryopreservation solution (50% FBS, 40% DMEM, 10% DMSO), transferred into a 2mL cryotubes and frozen in a pre-chilled Mr. Frosty container at -80°C overnight or more before sending to Active Motif to perform ATAC-seq assay. The cells were then thawed in a 37°C water bath, pelleted, washed with cold PBS, and tagmented as previously described (42), with some modifications based on (43). Briefly, cell pellets were resuspended in lysis buffer, pelleted, and tagmented using the enzyme and buffer provided in the Nextera Library Prep Kit (Illumina). Tagmented DNA was then purified using the MinElute PCR purification kit (Qiagen), amplified with 10 cycles of PCR, and purified using Agencourt AMPure SPRI beads (Beckman Coulter). Resulting material was quantified using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems), and sequenced with PE42 sequencing on the NextSeq 500 sequencer (Illumina). Analysis of ATAC-seq data was very similar to the analysis of ChIP-Seq data. Reads were aligned using the BWA algorithm (mem mode; default settings). Duplicate reads were removed, only reads mapping as matched pairs and only uniquely mapped reads (mapping quality  $\geq 1$ ) were used for further analysis. Alignments were extended in silico at their 3'-ends to a length of 200 bp and assigned to 32-nt bins along the genome. The resulting histograms (genomic “signal maps”) were stored in bigWig files. Peaks were identified using the MACS 2.1.0 algorithm at a cutoff of p-value  $10^{-7}$ , without control file, and with the `-nomodel` option. Peaks that were on the ENCODE blacklist of known false ChIP-Seq peaks were removed. Signal maps and peak locations were used as input data to Active Motifs proprietary analysis program, which creates Excel tables containing detailed information on sample comparison, peak metrics, peak locations and gene annotations. To annotate the ATAC-seq peak value and coverage within genomic regions of interest, we used Merge BedGraph and AnnotateBed bedtools functions respectively (Galaxy Version 2.29.2). We then normalized the three biological replicates values across all genomic regions (Early, Mid, Late, ADV and DEL) by a 2way ANOVA Sidak’s multiple comparisons test.

### **Processing ChIP-seq data from public databases**

ChIP-seq, pDamID and RepOri data were download from ENCODE, GEO, 4DN project and Replication domain data base respectively (**Table S4**). The epigenetic marks coverage of each given regions list (Early, Mid, Late, ADV, DEL) was calculated using AnnotatedBed bedtools function (Galaxy Version 2.29.2). The

mean for each epigenetic mark coverage in given regions was calculated to generate clustering tree heatmap based on Pearson correlations with ClustVis software (44).

### **Fluorescent DNA halo**

400,000 cells were harvested after synchronization and treated with nuclei buffer (10 mM Tris at pH 8, 3 mM MgCl<sub>2</sub>, 0.1 M NaCl, 0.3 M sucrose, protease inhibitors) plus 0.5% Nonidet P40 for 5-10 min on ice (depending on cell line). Nuclei were attached to coverslips using cytospin (1500-1800 rpm for 5-10 min, depending on cell line); stained with DAPI (2 mg/mL for 4 min); and immersed in a buffer containing 25 mM Tris (pH 8), 0.5 M NaCl, 0.2 mM MgCl<sub>2</sub>, 1 mM PMSF, and protease inhibitors for 1 min, then in Halo Buffer (10 mM Tris at pH 8, 2 M NaCl, 10 mM ethylene diamine tetra acetic acid [EDTA], 1 mM DTT, protease inhibitors) for 4 min. After two washing steps with wash buffer 1 (25 mM Tris (pH 8), 0.2 M NaCl, and 0.2 mM MgCl<sub>2</sub>) for 1 min, and with buffer 2 (buffer 1 without NaCl) for 1 min extracted nuclei were fixed in 2% formaldehyde for 10 min and processed for immunofluorescence. Images containing about 200 halo per condition acquired with a Nikon Ni-E microscope and a DS-Qi2 camera with 64X objective and MFHR (Maximum Fluorescence halo Radius) was measured in Image J software.

## **RESULTS**

### **Low replication stress differentially impacts cancer and non-tumour cells**

In order to evaluate cellular responses to mild replication stress, we treated cells with 0.2 $\mu$ M aphidicolin and DMSO as a control. We used 6 well characterized cell lines that are common models (HCT116, RKO, U2OS, K562, MRC5-N and RPE-1) and differ in tissue origin, tumorigenicity, differentiation stage and molecular characteristics such as oncogene expression, genetic instability type and telomere maintenance mechanisms (**Table S1**). The duration of aphidicolin treatment was adapted to each cell line in order to treat a maximum number of cells in S phase for a single generation. We selected the longest treatment duration ensuring that no cells were treated during two consecutive cell cycles (**Table S2** and **Figure S1a**). This optimal treatment duration was also determined in order to analyse the effect of replication stress in the daughter cells released from the drug.

Analysis of the S phase checkpoint induction at the end of aphidicolin treatment, by monitoring Chk1 phosphorylation on serine 345, revealed a low level of checkpoint activation compared to acute replication stress (HU, 2mM, 2h), with nevertheless a tendency towards higher p-Chk1 level in cancerous cells (**Figure S1b,c**). Furthermore, we noticed a global accumulation of cells in the S phase under aphidicolin treatment, which again was more pronounced in cancerous cells (**Figure S1d,e**).

To study RT under low replication stress, BrdU was added to the culture medium before cell sorting into Early (S1) and Late S-phase (S2) fractions, and the neo-synthesized DNA was hybridized on human whole genome microarrays, as previously described (39, 45, 46) (**Figure S2a**). RT differential analyses were performed on biological replicate experiments using the START-R suite software (40) and only significant modifications between aphidicolin and control condition were retained.

It has been reported that the RT profile of somatic cells is closely related to the cell type and tissue origin (10). By performing hierarchical clustering of RT for the different cell lines, we found that, in absence of replication stress, non-tumour cells clustered together (cluster 1) and are separated from cancer cells (cluster 2) (**Figure 1a**). In cluster 2, we noticed that the replication timing of RKO is closer to HCT116, consistent with the fact that they are both colon cancer cell lines with microsatellite instability due to mismatch repair deficiency (MMR-) (**Table S1**). Upon aphidicolin treatment, the two distinct clusters of cancer and non-tumour cells remain apart but distances measuring relatedness between cancer cells are altered. Indeed, the RT of RKO cells appears to be closer to that of K562 cells and that of HCT116 to be closer to U2OS (**Figure 1b**). Overall, this observation indicates firstly that without any replication stress, RT itself can discriminate non-tumour from cancer cells, and secondly, that aphidicolin treatment affects differentially the identity of cancer cells since we observed that the RT of the two colon cancer cell lines (RKO and HCT116) are now further apart among the cancer cells cluster 2 (**Figure 1b**).

### **Advance RT signature in cancer cells**

We recapitulated the genome wide percentage of altered RT in the different cell lines in response to aphidicolin treatment (**Figure 1c**). Normal RPE-1 and MRC5-N cells were the least impacted cell lines with 1.54% and 1.94% of the genome undergoing RT alterations respectively (**Figure 1c**) while the RT in cancerous cells was globally more impacted by low replication stress. We noticed that the RKO cell line showed the highest response with 6.54% of the genome impacted (**Figure 1c,e** and large visualization of chromosomes in **Figure S3**).

Analysis of the aphidicolin-RT-impacted loci (aRTIL) led to the identification of regions with significant RT delays (DEL aRTIL), as previously reported (30, 32) (**Figure 1c,d**). In RPE-1 and HCT116, these DEL aRTIL represent the majority of impacted loci with 38/47 and 74/96 loci respectively. Importantly, in all cell lines, we also observed significant RT switches towards earlier RT (RT advances; ADV aRTIL) (**Figure 1c-e**). These ADV aRTIL represent the main type of RT changes in RKO cells, with the largest domain coverage (**Figure 2c** and **Figure S3**) and strongest amplitude (**Figure 1e** and **Figure S3**). By performing BrdU-ChiP-qPCR on three independent domains in the RKO cell line, we confirmed that these newly- identified advanced domains are effectively replicated earlier (relative to BrdU incorporation) upon aphidicolin treatment (**Figure S2b**).

To explore the potential similarities of RT modifications between cell lines, we quantified the number of common DEL and ADV aRTIL (**Figure 1f,g**). We found 9 common large ADV domains in RKO and K562 cells and 4 common ADV aRTIL in U2OS and MRC5-N. The few ADV domains detected in RPE-1 and HCT116 were mainly specific to each cell line. The two non-tumour cell lines (RPE-1 and MRC5-N) shared a majority of DEL aRTIL (14 common) and also had many in common with U2OS (8 and 6 with RPE-1 and MRC5-N respectively) and HCT116 cell lines (11 and 6 with RPE-1 and MRC5-N respectively). In contrast, DEL aRTIL found in RKO and K562 cells were poorly shared with other cell lines.

Collectively, these data demonstrate that RT is significantly modified in cancer cells for a subset of genomic domains in response to mild replication stress. RT of non-tumour cells RPE-1 and MRC5-N was less affected, together with the lower induction of the S-phase checkpoint. Importantly, these data reveal for the first time that, in addition to RT delays, replication stress can also induce RT advances. Finally, the overlapping of aRTIL between cell lines highlights two main distinct RT modification signatures: the first one in RKO and K562 cell lines, characterized by major shared RT advances in specific genomic domains, and the second for U2OS, HCT116, MRC5-N and RPE-1 that are sharing similar RT delayed genomic regions.

#### **ADV aRTIL occur in heterochromatin late replicated chromosomal regions.**

To investigate whether aRTIL are associated to specific epigenomic signatures, we next analysed histone modifications and other epigenomic features such as Lamina-Associated-Domains (LADs) and chromatin accessibility (DNase-seq). Given that the most advanced domains in RKO are shared with K562, we used public epigenomic data on K562 to characterize ADV aRTIL. First, this approach allowed us to validate our experimental RT through the expected enrichment of the typical chromatin marks of Early, Mid and Late replicated regions (**Figure 2a,b**). For instance, Early replicated regions are enriched in euchromatin marks such as H3K27ac and H3K9ac, while Late replicated regions correlate with heterochromatin marks (H3K9me3) and with LaminB1 (LADs) (**Figure 2a,b**). We also confirmed that chromatin accessibility (DNase-seq) decreases from Early to Late replicated regions. Interestingly, chromatin features of the ADV aRTIL are similar to those of Late replicated domains and we noticed that these are even more enriched in H3K9me3 and in LaminB1, while being poorer in H3K27me3, and display very low chromatin accessibility (**Figure 2a,b**). This result suggests that ADV aRTIL domains belong to constitutive heterochromatin while DEL aRTIL are more likely Mid-replicated regions (**Figure 2b**).

We next investigated if genomic features can help distinguish ADV from DEL aRTIL. In accordance with the epigenomic features, we showed that in untreated cells, the large majority of regions converted to ADV aRTIL are replicated in Late S-phase while those changed to DEL aRTIL are mainly replicated in the Early/Mid S-phase (**Figure S4a**). At the genomic sequence level, we observed that ADV aRTIL are large regions (**Figure 2c**) that share similar features with Late replicated regions, such as poor GC content (**Figure 2d**), few constitutive origins (**Figure 2e**) and low gene abundance (**Figure 2f**). In contrast, DEL aRTIL are enriched in GC content, origins and gene coverage that characterize Early and/or Mid replicated regions (**Figure 2d-f** and **Figure S4c-e**).

#### **The ADV aRTIL signature is related to CFS but is not targeted by DNA damage signalling.**

Given that CFS are the most sensitive chromosomal regions to replication stress and that RT delays have been described in these fragile loci, we wondered if aRTIL would also overlap with CFS. To answer this question, we analysed the overlap between aRTIL and the CFS already identified (30). We found a clear enrichment of CFS within aRTIL in all 6-cell lines, with 22-44% of CFS being located within aRTIL (**Figure 3a-c**). The percentage of RT delays within CFS was higher in the HCT116, MRC5-N and RPE-1 cell lines (**Figure 3b**). Quite surprisingly, we also identified ADV aRTIL associated to CFS, notably for the cancerous RKO and



K562 cells. Therefore, besides the well-documented delayed replication dynamics within CFS, replicative stress can also induce RT advances in these particular regions.

It has been recently reported that macroH2A1.2, a variant from the canonical H2A (47), having roles both in replication stress response and in cell fate decisions (48–51), is more abundant at CFS than non-fragile regions of the genome. In response to mild aphidicolin treatment (0.5  $\mu$ M), its enrichment is directly correlated with  $\gamma$ -H2AX peak coverage (52). Using ChIP-seq data from this study, we analysed the coverage of histone variants  $\gamma$ -H2AX and mH2A1.2 in K562 with or without aphidicolin treatment. As we did for epigenomic marks, we compared ADV and DEL aRTIL coverage with Early, Mid and Late S-phase regions. We confirm that, without aphidicolin, Early-S regions are the most enriched in  $\gamma$ -H2AX and mH2A1.2 histone variants (**Figure 3d-e**) (53). As expected, aphidicolin induces a significant increase in  $\gamma$ -H2AX and mH2A1.2 in all control regions. Interestingly, aphidicolin treatment does not modify the coverage of  $\gamma$ -H2AX and mH2A1.2 within ADV aRTIL whereas it induces a strong increase of these two histone modifications coverage within DEL aRTIL (**Figure 3d-e**). This suggests that, while DEL regions are likely prone to DNA damage under replication stress, ADV aRTIL could be protected from DNA damage or more efficiently repaired.

Taken together, these results show that replication stress differently affect aRTIL. In contrast to DEL aRTIL, ADV aRTIL are associated to a low level of DNA damage response (DDR) signalling.

#### **Low replication stress impacts the regulation of genes involved in chromatin organization**

It has been established that RT switches in human cells can be linked to developmental genes expression (3, 13, 54, 55). Nonetheless, the exact correlation between RT and gene expression is not entirely clear. Indeed, several studies have discovered genomic sequences that do not fit the general correlation between gene expression and RT (56–58). Therefore, we checked if, in our experimental condition, aphidicolin has a global impact on gene expression and if this could be related to RT modifications. We performed gene-expression profiling by microarray in RKO cells using the same conditions as for RT analysis. We found that aphidicolin treatment has a mild impact on gene expression, with 14 genes significantly differentially expressed (APH DOWN or APH UP genes) (**Figure 4a and Figure S5a**). We analysed APH DOWN genes by performing a Gene Ontology (GO) enrichment analysis and found these genes were enriched in chromatin and nucleosome organization, gene silencing and cellular differentiation pathways (**Figure S5b**). We did not observe particular enrichment in GO pathways for APH UP genes (FDR > 0.01). Nevertheless, we noticed an up-regulation of the transcription factor gene ZBTB38 which is a biomarker for prostate cancer (59) and that plays an important role in the regulation of DNA replication, cell cycle and cell fate (60, 61). Importantly, the expression of genes that fell inside aRTIL was not affected by low replication stress (**Figure 4b**). Altogether, we concluded that while RT modifications under low replication stress are not related to modification of gene expression within aRTIL, replication stress induces a specific down-regulation of genes involved in chromatin organization.

#### **Aphidicolin modulates chromatin accessibility within ADV aRTIL**

Since we identified APH DOWN genes in RKO cells involved in chromatin and nucleosome organization, we examined the impact of aphidicolin on chromatin structure by performing the Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq), a method for assaying chromatin accessibility genome-wide (62, 63). We observed a global remodelling of chromatin accessibility under aphidicolin treatment demonstrated by a lower strength of ATAC-seq peaks and an increase ATAC-seq peaks coverage in both Early and Late replicated genomic regions (**Figure S5c,f**). More importantly, we observed a significant increase of both ATAC-seq peaks strength and coverage within ADV aRTIL while this was not the case within DEL aRTIL (**Figure 4c-e** and **Figure S5e**). Thus we can conclude that under low replication stress chromatin accessibility is specifically increased within ADV aRTIL.

We also investigated the chromatin accessibility of the UP-regulated genes under aphidicolin treatment. In contrast to ADV aRTIL, we did not find an increase of ATAC-seq peaks value within these specific genes (**Figure S5g**). This result further supports that differential gene expression is not associated to chromatin remodelling and RT modifications induced by aphidicolin treatment.

Overall, this approach enabled us to demonstrate that aphidicolin treatment induces a local increase of chromatin accessibility within ADV aRTIL that can be linked to RT modifications while also inducing whole genome chromatin remodelling.

#### **ADV aRTIL can be transmitted to daughter cells**

RT is faithfully established at the beginning of the G1 phase in each cell cycle, at a precise time named the “timing decision point” or TDP (64, 65). In G1, RT setting up is dependent on 3D nuclear replication domains organisation through chromatin loop formation mediated by the Rif1 protein (66). Moreover, at the G1/S transition, chromatin loops are also maintained by the transient recruitment of pre-replication complex proteins and active origins to the nuclear matrix (NM) (67–70).

We first wondered if RT changes under replication stress in mother cells can be preserved beyond the G1 phase and thus transmitted to daughter cells. To answer this question, we released the 6 cell lines from aphidicolin or DMSO treatment for the appropriate duration (**Table S2**) and analysed the RT of daughter cells in S phase (N+1). Our results indicate that DEL and ADV aRTIL observed in mother cells were no longer detected in the next cell generation of K562, HCT116, U2OS, MRC5-N and RPE1 (**Figure 5a** and **Figure S6a**). Strikingly, in RKO cells, the majority (28 of 49) of the strongest and largest ADV aRTIL were transmitted to the next cell generation and we noticed that the amplitude of these RT advances was less pronounced in daughter cells (**Figure 5a,b** and **Figure S3**). Conversely, DEL aRTIL returned to normal RT in RKO daughter cells, comparable to untreated cells. These results indicate that while the majority of the RT modifications within aRTIL are reversible and tend to be eliminated through the G1 phase, severe ADV aRTIL can be transmitted to the next generation.

To investigate if the persistence of ADV aRTIL in RKO daughter cells could be linked to changes in chromatin loop organisation in G1 phase, we performed a fluorescent DNA halo experiment to evaluate the chromatin loops size in G1/S. We used RKO cells as positive control and RPE-1 cells as negative control. We

measured the maximum fluorescence halo radius (MFHR) formed around the nuclear matrix (NM) and noticed a significant shrinkage of DNA loops in the aphidicolin-released RKO cells (**Figure 5c-d**) while no effect was measured in RPE-1 cells (**Figure S6 b-c**). To test if this reduced halo size correlates to an increase in licensed origins, we quantified the loading of pre-replication complex components onto the chromatin under the same conditions. Our results clearly show an increase of MCM2 and p-MCM2 loading onto the chromatin in G1/S of RKO aphidicolin-released daughter cells, while this was not the case in RPE-1 cells (**Figure S6d**), further supporting the results from the DNA halo experiment (**Figure 5e**). The transmission of ADV aRTIL in RKO daughter cells is therefore associated to a decrease in chromatin loop size and an increase in pre-RC proteins loading in G1 phase, predicting greater activation of replication origin in the next S phase.

Finally, we wondered if chromatin remodelling during the G1 phase and RT advances in RKO daughter cells would modulate gene expression. Even though the expression of genes within ADV aRTIL was not significantly impacted in mother cells, we noticed an up-regulation of genes within ADV aRTIL in daughter cells released from replication stress (**Figure 5f**). Therefore, we performed a gene ontology analysis to determine if these ADV aRTIL genes were involved in a specific molecular pathway(s) and found a strong enrichment for cell-to-cell adhesion and synapse assembly pathways (**Figure 5g**).

Together these data show a correlation between the transmission of RT advances and chromatin structure modification at the time of the G1 phase. In addition, we observed that persistence of RT advances in daughter cells is associated to an increase of gene expression within these specific genomic regions. Overall, we revealed that replication stress in mother cells directly affects chromatin accessibility and replication timing, which will further affect the cell fate of the next generation.

## DISCUSSION

DNA replication is tightly regulated in order to guarantee the accurate transmission of genomic information from mother to daughter cells. Challenging structures of DNA are very often encountered by the replication fork and oncogene expression or metabolism changes can also play a deleterious role in DNA replication program efficiency by inducing replication stress. Thus, regardless of the sources, replication stress will give rise to many cellular responses that will depend on the genetic background of the cell. Importantly, replication stress was identified at the early stage of cell transformation and cancer development (71, 72) and persists in cancer cells after the selection steps (73). As replication stress compromises genome stability, cancer cells have to adapt in order to survive and maintain their proliferation. Due to a high level of genomic instability, cancer cells can lose some of the classical mechanisms involved in DDR, for example through well-known mutations in BRCA1/2 repair genes. However, many compensatory processes take place in cancer cells to allow survival (74–76), also conferring resistance to chemotherapeutic treatments.

In the present work, we demonstrated that one of the components of the replication program, RT, is affected in response to low dose of aphidicolin. Besides the already described DNA replication delays following replication stress induced by the inhibition of replicative DNA polymerases  $\alpha$ ,  $\delta$  and  $\epsilon$ , we revealed here that aphidicolin also promotes RT advances (**Figure 1 and S2, S3**). In order to understand how the

same replication stress induces such opposite effects, we characterized DEL and ADV aRTIL at both the genomic and epigenomic level. DEL aRTIL are normally replicated in the Early/Mid S-phase, they harbour many genes and a high number of constitutive origins of replication (**Figure S4**). These features explain the high probability for replication forks to be affected by aphidicolin treatment and hampered by transcription activity, leading to DNA damage signalling associated with high levels of  $\gamma$ -H2AX and macroH2A1.2 (**Figure 3d,e**).

ADV aRTIL are mainly observed in RKO and K562 cancer cells, even if they are also detected in the other cell lines. These regions correspond to Late replicated heterochromatin with few and poorly expressed genes, low number of constitutive origins and a low coverage of the two DDR histone marks  $\gamma$ -H2AX and mH2A1.2, not significantly increased under low replication stress (**Figure 2, 3**). Interestingly, in RKO cells, we demonstrated higher chromatin accessibility within ADV aRTIL in the mother cells that is not linked to higher gene expression (**Figure 4**). Altogether, ADV aRTIL appear to be heterochromatin flexible regions which might be resistant to replication stress.

Under low dose of aphidicolin, the decrease in DNA replication fork velocity can be compensated by the firing of dormant origins, preventing under-replication (77–82). Interestingly, it was demonstrated that in cancer cells replication origin usage is more flexible (83). Moreover, cancer cells rely on a higher rate of origin licensing protein expression (84, 85), allowing a more efficient usage of dormant origins in response to replication stress (86). Taking our data into consideration, we can imagine that, in cancer cells, the higher chromatin accessibility described within ADV aRTIL favours the access of DNA replication and repair proteins to these specific heterochromatin regions, leading to activation of dormant origins and earlier DNA replication. It was described that activation of replication stress-induced dormant origins within a given S phase can be persistent in the next S phase (78). This result is consistent with our data in RKO cells, in which we still observe RT advances in daughter cells after aphidicolin release accompanied by a reduction in DNA halo size and an increase in MCMs loading to chromatin at the G1/S transition (**Figure 5**). Altogether, we propose that some cancer cells are able to modify their replication program and display a higher flexibility of chromatin organisation and replication origin usage in order to respond to replication stress. In addition, we observed that genes within ADV aRTIL are up-regulated in daughter cells (**Figure 5**). These results would suggest that the chronic replication stress described during the first step of cancer development could be involved in the acquisition of replication timing modifications that subsequently lead to changes in the transcription level of specific genes. Thus, low replication stress would change cell fate of cancer cells whose chromatin is more flexible.

RT modifications are historically associated with organism development and cellular differentiation (3, 4, 8, 87). Furthermore, the pluripotent capacity of mammalian cells has been linked to genome plasticity (88, 89) as well as deficiency in Lamin A protein expression (90, 91). In our study, we observed that replication stress induced higher level of RT modifications in cancer cells. Interestingly, we showed that RT of the cancerous RKO cell line is strongly affected by aphidicolin treatment, inducing major ADV aRTILs (**Figure 1c,d,e and S3**). We linked this particular phenotype to the poorly differentiated status of RKO cells

together with a very low expression of Lamin A/C (**Table S1, Figure S7**). In agreement with this, we noticed that the K562 cell line, which had an aRTIL signature close to the RKO cells, is poorly differentiated and also expressed a low level of Lamin A/C (**Table S1, Figure S7**). Overall, we propose that the less differentiated cancer cells are, the more they will harbour RT advances in response to replication stress, as a sign of their more flexible chromatin organization.

Finally, we unveiled a new mechanism in response to replication stress that may have a strong impact on gene expression and cell identity, mainly for cancer cells. Importantly this work paves the way for future studies investigating the molecular effectors involved in advancing replication timing in cancer cells, with great potential as new targets to prevent cancer cells adaptation to replication stress leading to therapy resistance.

### **ACCESSION NUMBERS**

DNA replication timing, ATAC-seq and microarray gene expression data are available under accession numbers GSE156618, GSE156552 and GSE156521, respectively.

### **SUPPLEMENTAL DATA**

Supplementary Data are available at NAR online.

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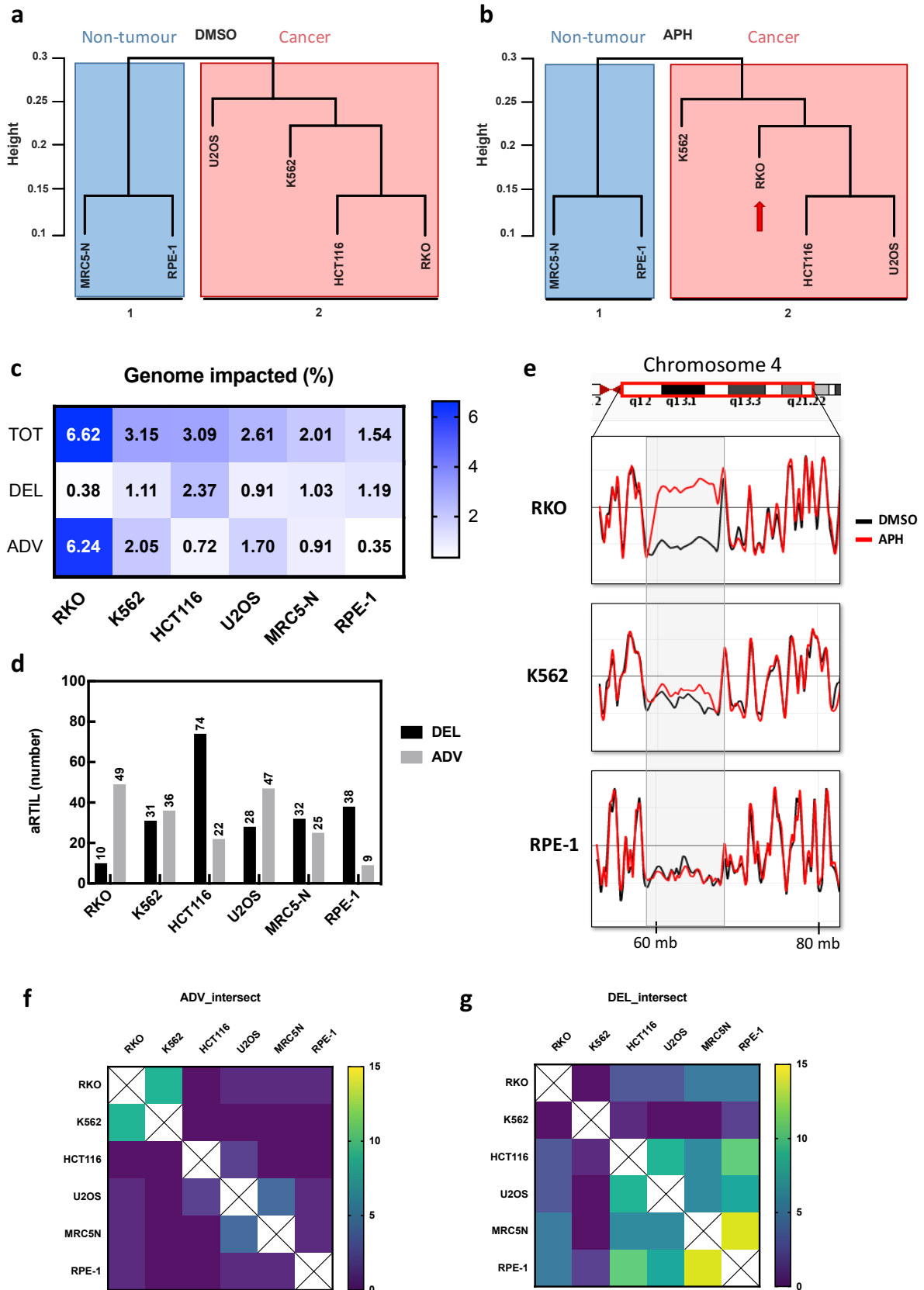
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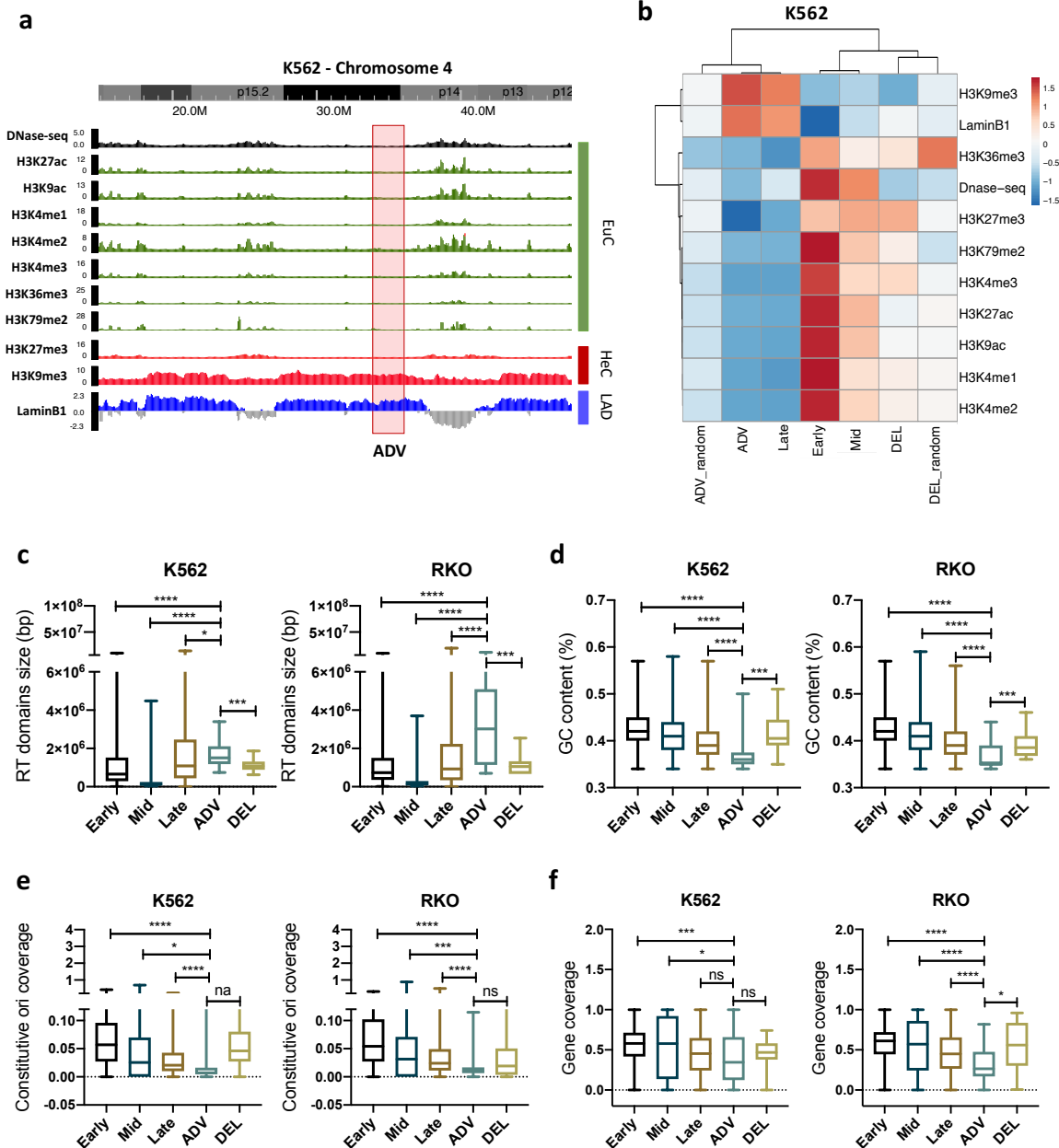
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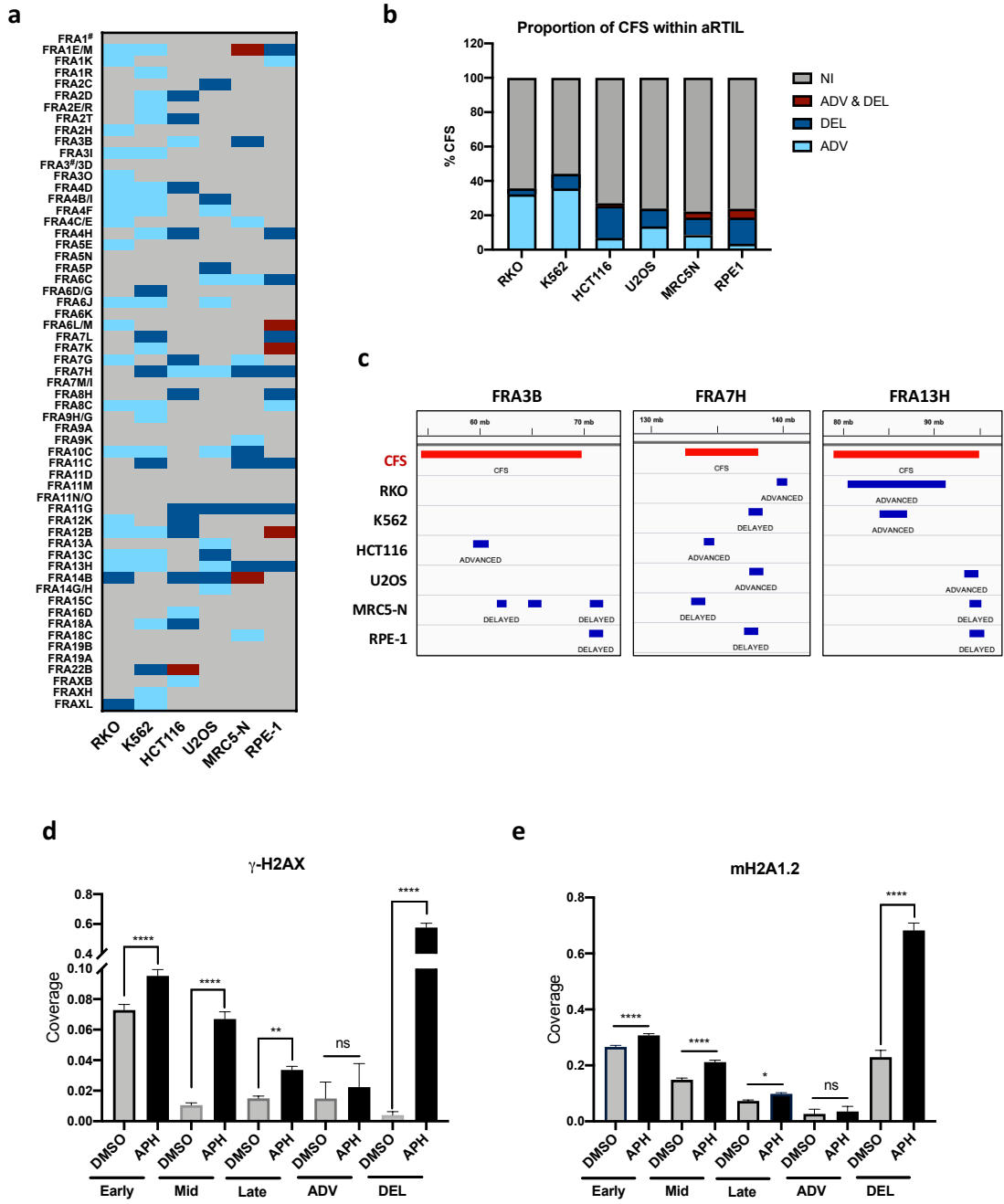
**Figure 1: Low replication stress differentially impacts cancer and normal cells.** **a** Cluster dendrogram based on p-values reflecting RT signatures in DMSO condition of the six cell lines. Distance: correlation and clustering method: average. **b** Cluster dendrogram based on p-values reflecting RT signatures in APH condition of the six cell lines. Distance: correlation and clustering method: average. **c** Heatmap representing the coverage (in % of the genome) of impacted genomic regions: total, delayed or advanced (TOT, DEL and ADV) for each cell line. **d** Quantification of the number of genomic regions significantly impacted (DEL, black

and ADV in grey) by aphidicolin treatment. **e** Screenshot of Loess-smooth RT profiles for the same region in chromosome 4 for RKO, K562 and RPE-1 cells. The dark lines correspond to replication timing of control (DMSO) replication timing (2 independent replicates) and the red lines are replication timing of APH treated cells (2 independent replicates). **f** Heatmap with intersections (the sum of the number of common domains) for ADV genomic regions between cell lines. **g** Heatmap with intersections (the sum of the number of common domains) for DEL genomic regions between cell lines.



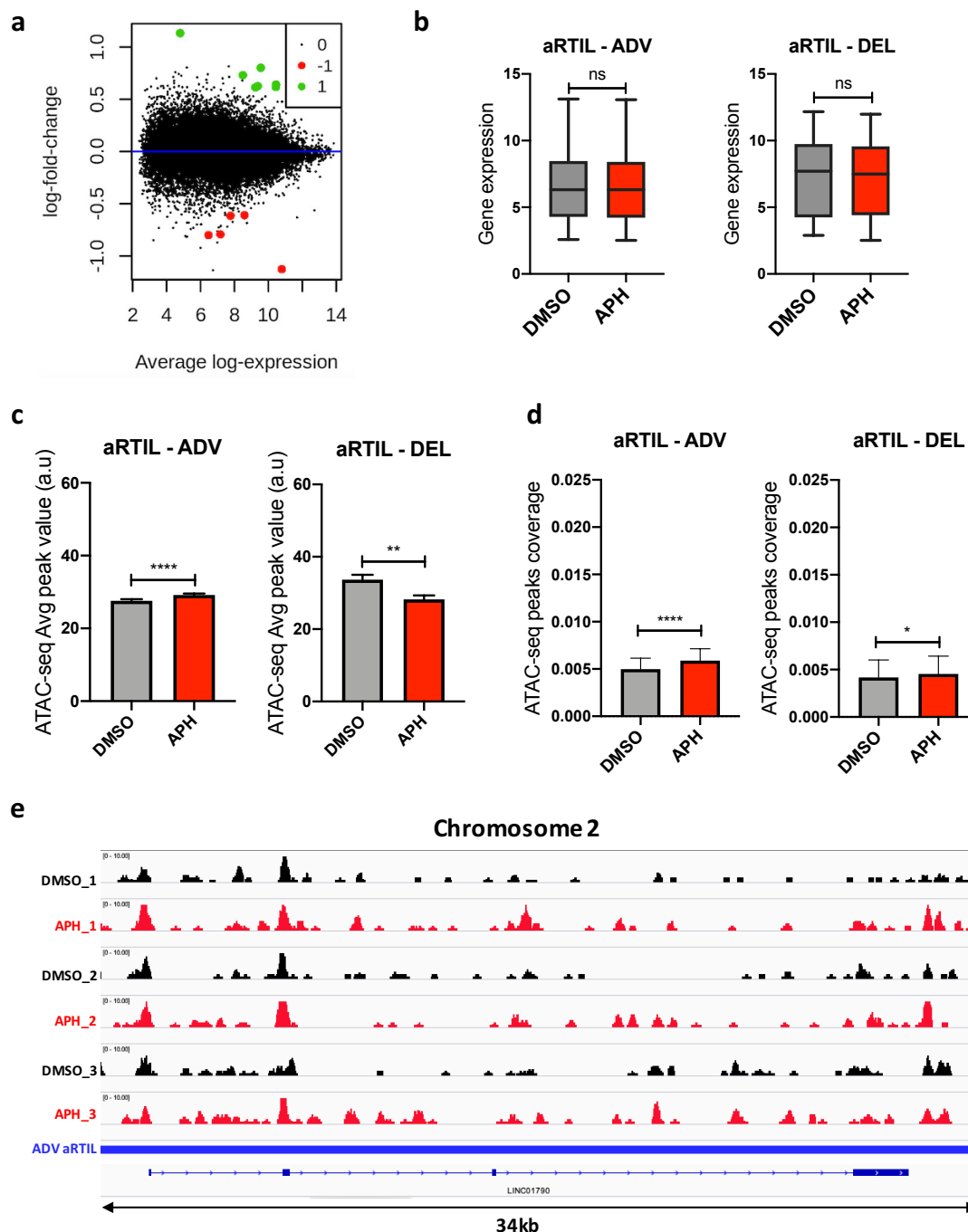
**Figure 2: ADV aRTIL occur in heterochromatin late replicated chromosomal regions.** **a** Screenshot of WashUEpigenome Browser for chromosome 4 in K562 with an example of ADV aRTIL (in red). **b** Heatmap and clustering trees based on Pearson correlations for epigenetic marks coverage in K562 regions (ClusVis Software, ENCODE ChIP-seq data). **c** Boxplots of RT domains size (in kb) for Early, Mid, Late, ADV aRTIL and DEL aRTIL in K562 and RKO cell lines. **d** Boxplots of GC content in Early, Mid, Late, ADV aRTIL and DEL aRTIL genomic regions in K562 and RKO cell lines. **e** Boxplots of constitutive origins coverage in Early, Mid, Late,

ADV aRTIL and DEL aRTIL genomic regions in K562 and RKO cell lines. **f** Boxplots of gene coverage in Early, Mid, Late, ADV aRTIL and DEL aRTIL genomic regions in K562 and HCT116 cell lines. Statistics (for all the boxplots): Wilcoxon rank sum test \*\*\* $p < 0.005$ , \* $p < 0.05$ , ns when  $p > 0.05$ .



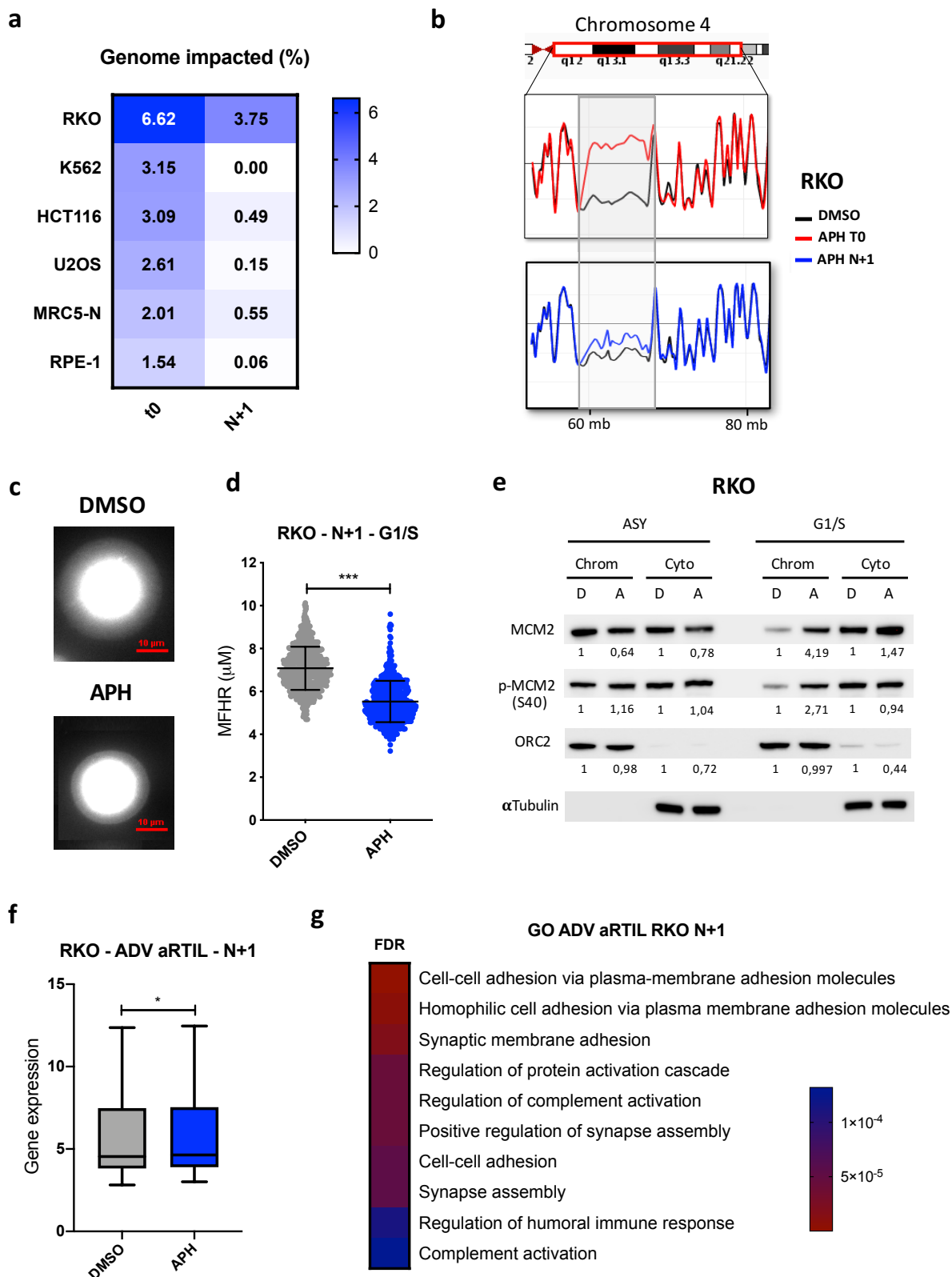
**Figure 3: ADV aRTIL are closely related to CFSs but are not targeted by DNA damage signalling.** **a** Categorical heatmap to visualize impacted CFSs by aRTIL in all cell lines: ADV & DEL in dark red, DEL in dark blue, ADV in light blue and non-impacted (NI) in grey. **b** Histogram representing the proportion (in %) of CFSs within aRTIL. ADV & DEL in dark red, DEL in dark blue, ADV in light blue and non-impacted (NI) in grey. **c** Genome Browser (IGV) snapshots to visualize aRTIL (in blue) in all cell lines for three CFS (in red): FRA3B, FRA7H and FR13H. **d** Histogram representing the coverage of  $\gamma$ -H2AX histone mark (ChIP-seq data) on Early, Mid, Late, ADV aRTIL and DEL aRTIL genomic regions in DMSO- and APH-treated conditions. Statistics: Two-

way ANOVA, Multiple comparison: \*\*\*\* $p < 0.0001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , ns  $p > 0.05$ . **e** Histogram representing the coverage of mH2A1.2 histone mark (ChIP-seq data) on Early, Mid, Late, ADV and DEL aRTIL genomic regions in DMSO- and APH-treated conditions. Statistics: Two-way ANOVA, Multiple comparison: \*\*\*\* $p < 0.0001$ , \*\* $p < 0.01$ , \* $p < 0.05$ , ns  $p > 0.05$ .



**Figure 4: Aphidicolin modulates chromatin accessibility within ADV aRTIL without impacting gene expression.** **a** Scatterplot for RNA-ChIP data in RKO cells representing significantly UP (green) and DOWN (red) -regulated genes in response to aphidicolin. **b** Boxplots measuring the expression of genes within ADV (left) and DEL (right) aRTIL in DMSO (grey) and APH (red) conditions. Statistics: Wilcoxon matched-pairs signed rank test ns  $p > 0.05$ . **c** Comparison of ATAC-seq peak value within ADV and DEL aRTIL regions between DMSO (grey) and APH (red) conditions. **d** Comparison of ATAC-seq peak coverage within ADV and DEL aRTIL between DMSO (grey) and APH (red) conditions. Statistics (N=3): Wilcoxon matched-pairs signed

rank test \*\*\*\* $p < 0.0001$ , \*\* $p < 0.01$ , \* $p < 0.05$ . e Screenshot of integrative genome viewer (IGV) session with ATAC-seq triplicates bigwig files on chromosome 2 within a specific ADV aRTIL (194,728,595-194,763,435kb, LINC01790).





**Figure 5: ADV aRTIL can be transmitted to daughter cells.** **a** Heatmap representing the coverage (in %) of impacted genomic regions in mother cells (t0) or released daughter cells (N+1) for the six cell lines. **b** Screenshots of Loess-smooth replication timing profiles for the same region in chromosome 4 for RKO, in mother (APH T0) and daughter cells (APH N+1). The dark lines correspond to replication timing of control (DMSO) replication timing, the red line is replication timing of T0 APH-treated cells and the blue line is the replication timing of N+1 daughter cells released from APH treatment. **c** Visualization and **d** Quantification of DNA Halo size (MFHR) in RKO G1/S synchronized daughter cells released from DMSO or aphidicolin treatment. Statistics (N=3): Unpaired t test with Welch's correction \*\*\*p<0.001. **e** Western blot on chromatin (Chrom) and cytoplasmic (Cyto) protein fractions to quantify the amount of MCM2, p-MCM2 and ORC2 in asynchronous cells (ASY) and cells synchronized in G1/S with L-mimosine. The fold change between DMSO and APH condition measured in three independent experiments is reported in the figure. **f** Boxplot measuring the expression of genes within ADV aRTIL in DMSO and APH released daughter cells (N+1). Statistics: Wilcoxon matched-pairs signed rank test \*p<0.05. **g** Gene ontology for RKO N+1 ADV aRTIL UP-regulated genes (ShinyGO, P-val cutoff: FDR < 0.05).

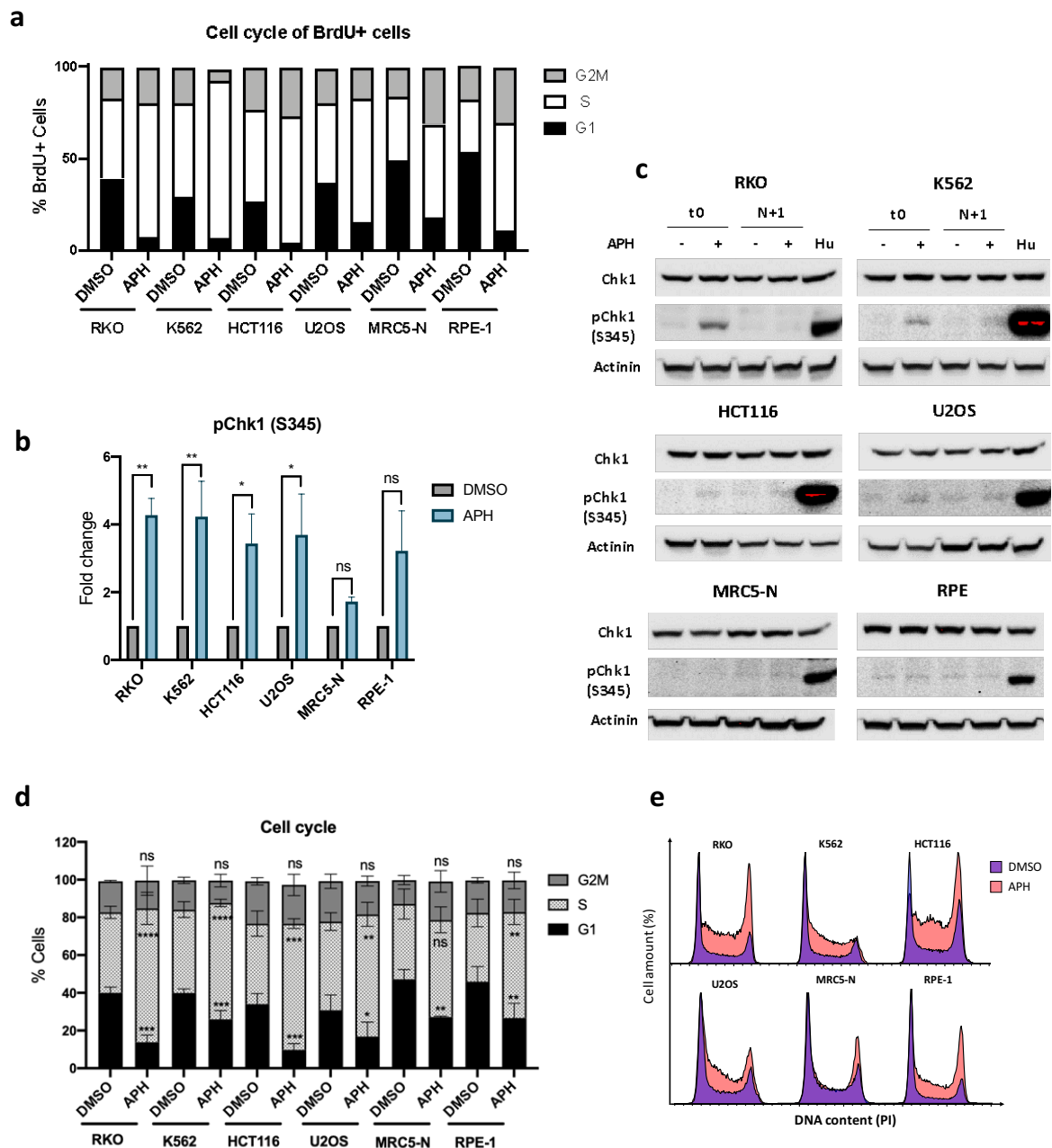
## Supplemental data

| Cell line                 | RKO                                     | HCT116                                  | U2OS                             | K562                               | MRC5-N                              | RPE-1                              |
|---------------------------|---|---|----------------------------------|------------------------------------|-------------------------------------|------------------------------------|
| Gender                    | Female                                  | Male                                    | Female                           | Female                             | Male                                | Female                             |
| Cancer type               | Carcinoma                               | Carcinoma                               | Osteosarcoma                     | Leukemia                           | Non-cancerous                       | Non-cancerous                      |
| Tissue                    | Colon                                   | Colon                                   | Bone                             | Bone marrow                        | Lung                                | Retina                             |
| Differentiation           | Poorly differentiated                   | Poorly Unable to differentiate          | Moderately differentiated        | Undifferentiated progenitor        | Embryonic                           | Terminally differentiated          |
| Morphology                | Epithelial                              | Epithelial                              | Epithelial                       | Hematopoietic                      | Fibroblast                          | Epithelial                         |
| Molecular characteristics | p53+ (WT)<br>MMR-<br>Telomerase<br>CIN- | p53+ (WT)<br>MMR-<br>Telomerase<br>CIN- | p53+ (WT)<br>MMR+<br>ALT<br>CIN+ | p53-<br>MMR+<br>Telomerase<br>CIN+ | p53+ (WT)<br>MMR+<br>Normal<br>CIN- | p53+ (WT)<br>MMR+<br>hTERT<br>CIN- |

**Table S1: Cellular and molecular characteristics of the 6 human cell lines used for the study.** The four cancer cell lines are coloured in red. RKO and HCT116 are two colon cancer cell lines, the U2OS cell lines are from osteosarcoma and the K562 cells lines from haematological cancer chronic myeloid leukaemia. The two human cells from healthy tissues MRC5-N and RPE-1 are coloured in blue and are respectively from lung and retina. Cellular background regarding differentiation state, morphology and finally some molecular characteristics like p53 status or telomere maintenance mechanism are listed in this table. MMR: Mismatch repair, CIN: chromosome instability.

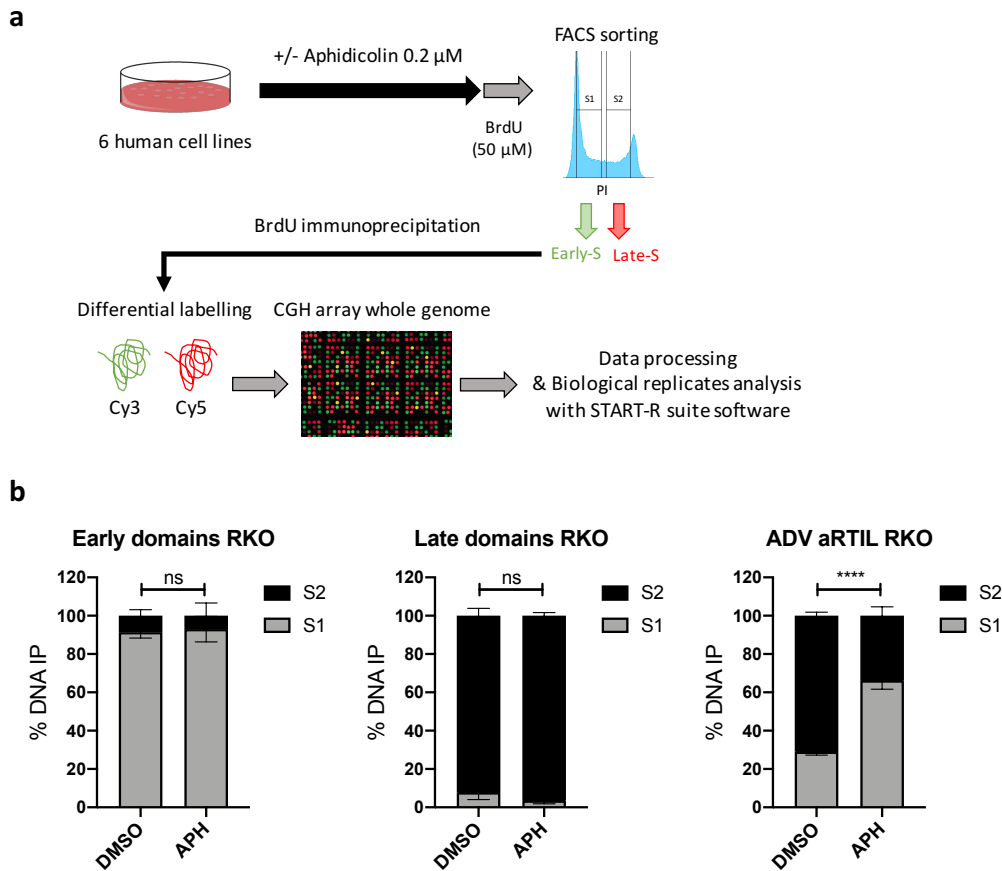
|                        | RKO | K562 | HCT116 | U2OS | MRC5-N | RPE-1 |
|------------------------|-----|------|--------|------|--------|-------|
| Time of treatment (T0) | 16h | 12h  | 15h    | 15h  | 15h    | 12h   |
| Time of release (N+1)  | 13h | 18h  | 14h    | 15h  | 12h    | 13h   |

**Table S2: Optimised times of treatment with aphidicolin and release in a fresh media set-up for the 6 cell lines and used for this study.**



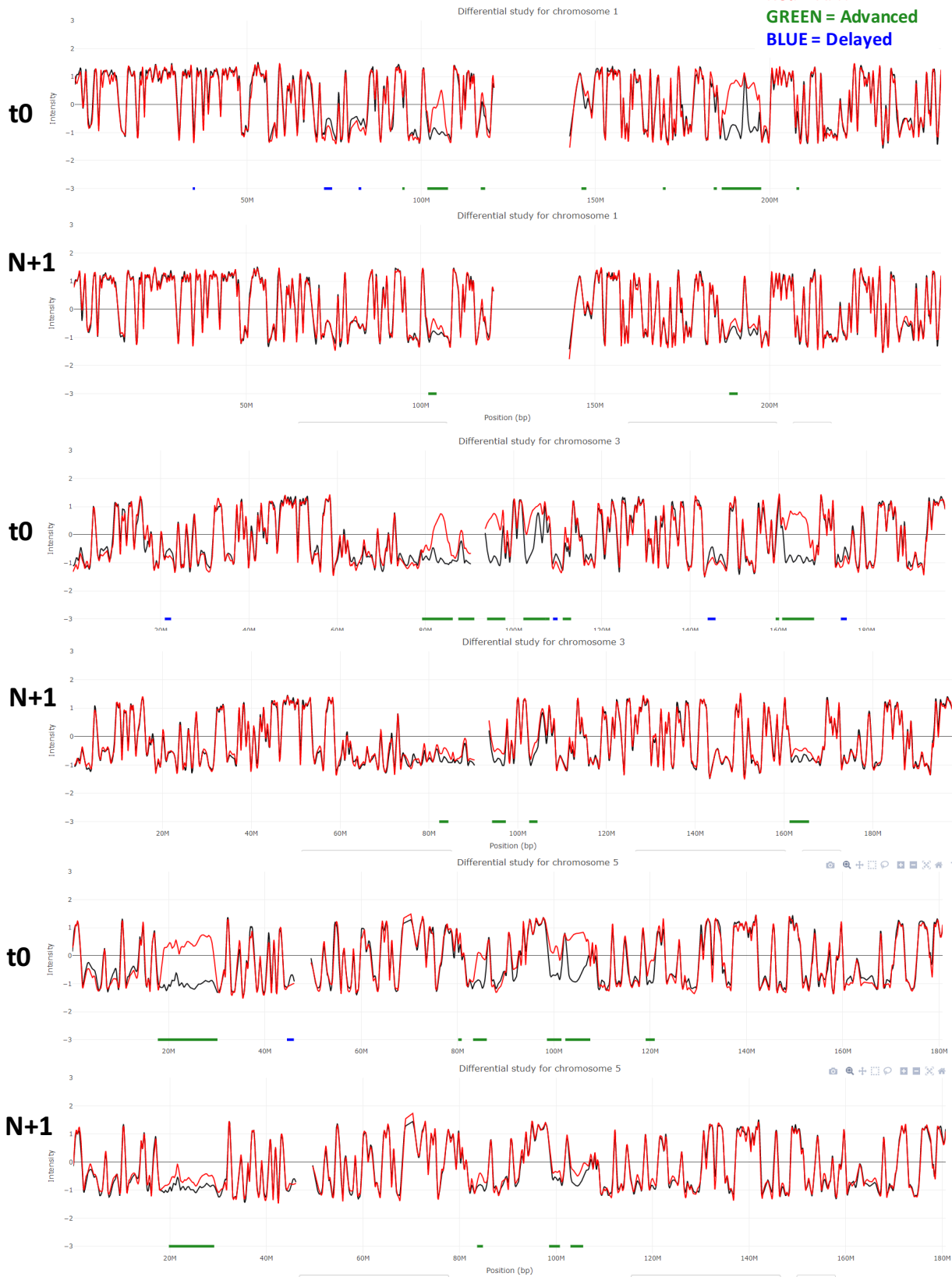
**Figure S1: Low replication stress differentially impacts cancer and normal cells.** **a** Stacked histograms representing the percentage of BrdU positive cells in each cell cycle fraction (G1, S or G2M). Cell cycle analysis was assessed by FACS (DAPI, EdU and BrdU) labeling after BrdU incorporation during the treatment (12-16h) and 15min EdU pulse before cell harvesting and fixation. **b** Quantification of p-Chk1 intensity between DMSO and aphidicolin (APH) treatment detected by western blot. Fold change correspond to normalized APH pChk1 intensity (by Chk1 and Actinin intensities) divided by normalized DMSO pChk1 intensity. Statistics (N>4): Two-way ANOVA, Multiple comparison: \*\*p<0.01, \*p<0.05, ns when p>0.05. **c** Western blot of pChk1 (Serine 345), Chk1 and Actinin with and without APH for the first generation of cells (t0), daughter cells (N+1) or positive control Hu (2mM, 2h). **d** Stacked histograms representing cell cycle distribution (in percentage) between G1, S and G2/M phase. Statistics (N=3): Two-way ANOVA, Classical

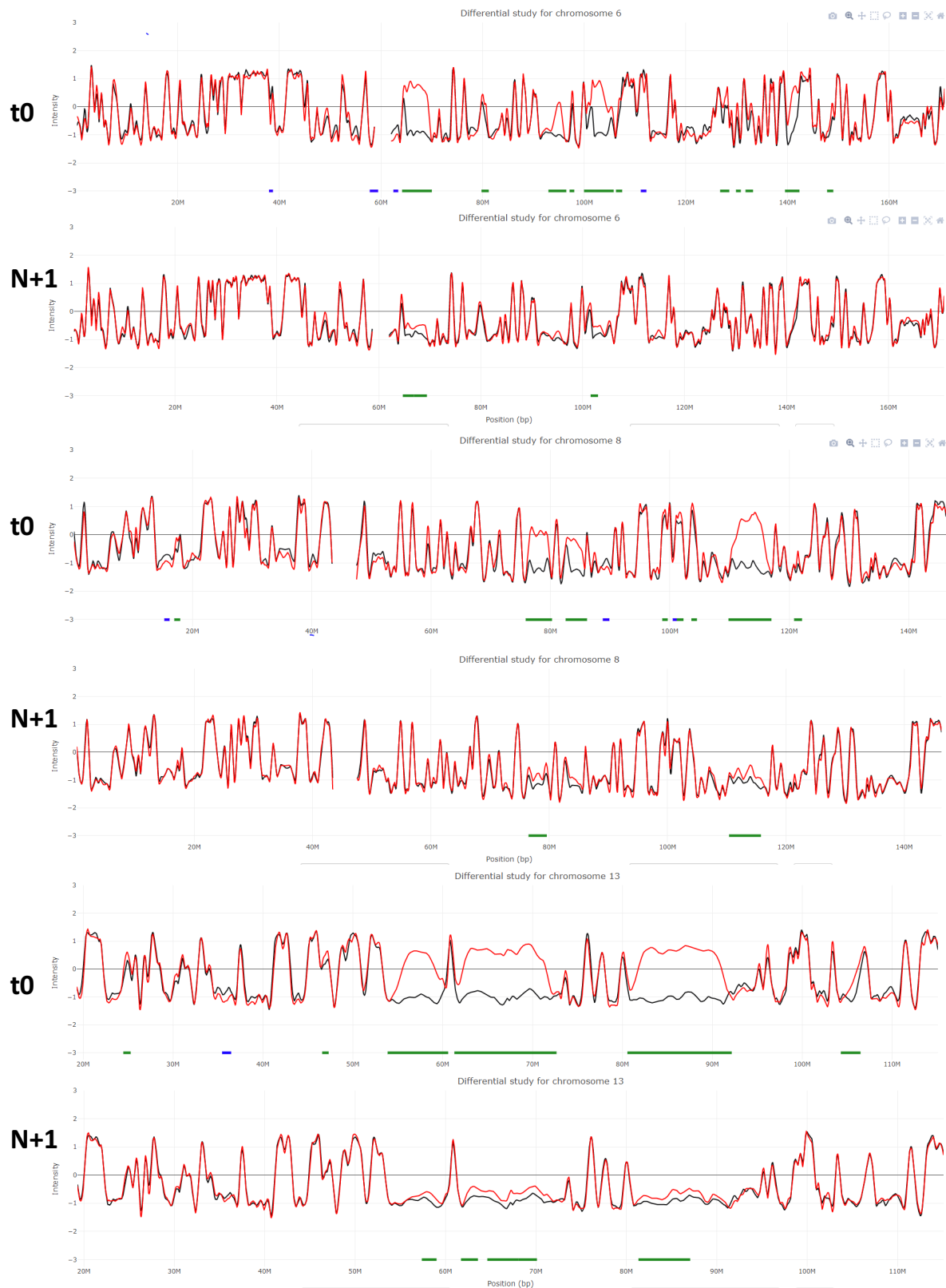
\*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.005$ , \*\* $p < 0.01$ , \* $p < 0.05$ , ns when  $p > 0.05$ . **e** Overlaps of DMSO (purple) and APH (pink) density plot showing cell cycle based on PI content by FACS analysis.



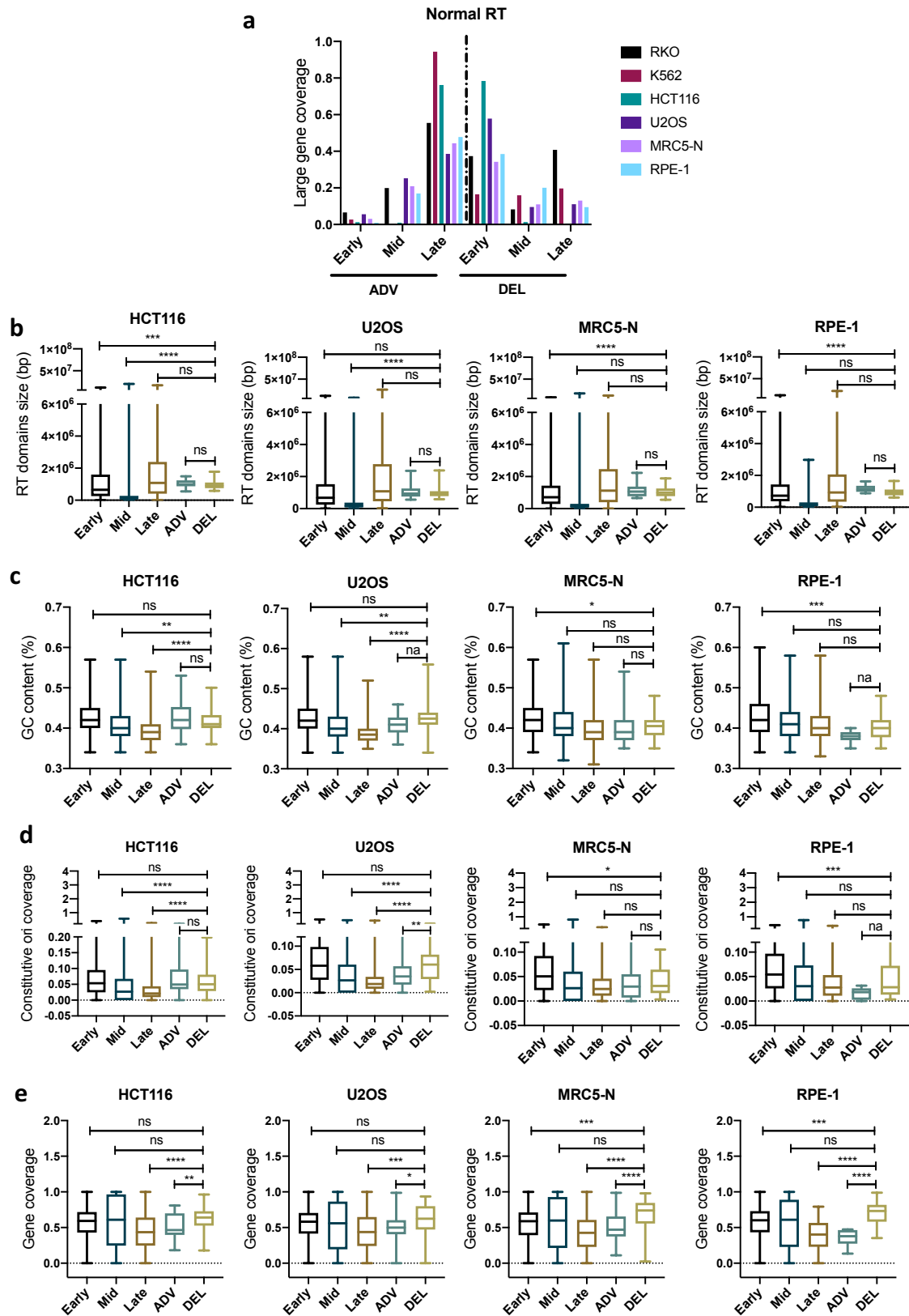
**Figure S2: Design and validation of replication timing experiment.** **a** Experimental protocol to assess whole genome-RT. Briefly, cells were treated with 0.2 $\mu$ M aphidicolin or DMSO during a time adapted for each cell line (10-15h), cells were labelled by BrdU during 1h30 and FACS sorted into two S phase fractions (S1 for early S and S2 for late S). DNA from each fraction is then immunoprecipitated with BrdU antibody, immunolabelled with Cy3 (S1) and Cy5 (S2), and then hybridized on whole human genome arrays. **b** BrdU-ChiP-qPCR after cell cycle sorting between early (S1) and late (S2) S phase. Stacked histograms represent the % of DNA IP for a given genomic domain in the S1 or S2 fraction. The first histogram recapitulates the results for the two early control domains (E1 and E2), the second for the two late control domains (L1, L2) and the last one for the three advanced regions in the aphidicolin condition (ADV aRTIL) (D1, D2, D3). Statistics (three biological replicates, N=3): 2way ANOVA Sidak's multiple comparison test \*\*\*\* $p < 0.0001$ , ns when  $p > 0.05$ .

**Black = DMSO**  
**Red = APH**  
**GREEN = Advanced**  
**BLUE = Delayed**



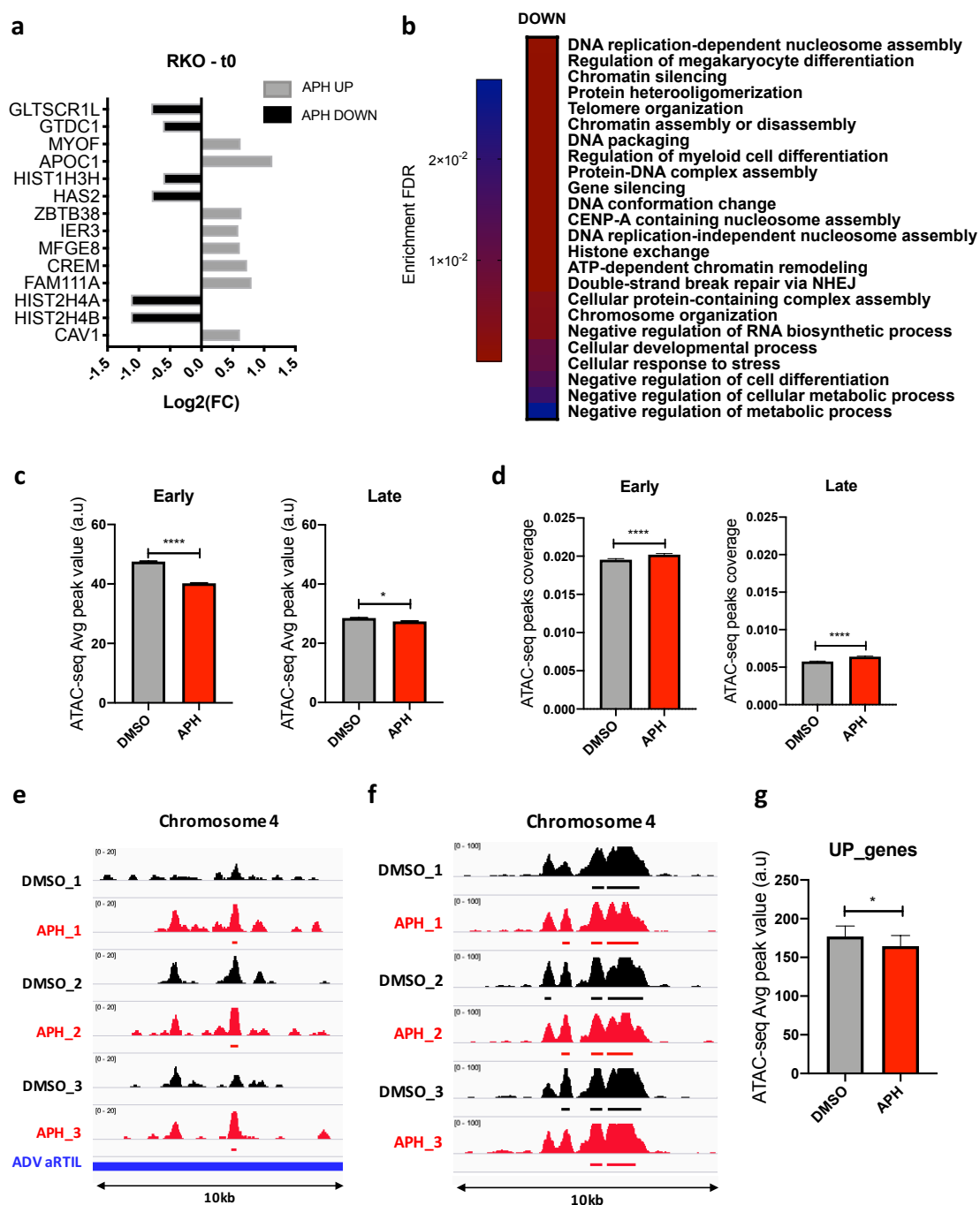


**Figure S3: DNA replication timing visualisation in RKO cells.** Screenshots of the 6 first chromosome visualisation of whole Loess-smooth RT profiles in mother (t0) and daughter cells (N+1). The dark lines correspond to RT of control (DMSO) replication timing and the red lines to RT of APH treated cells (2 independent replicates). ADV aRTIL are underlined in green and DEL aRTIL in blue.



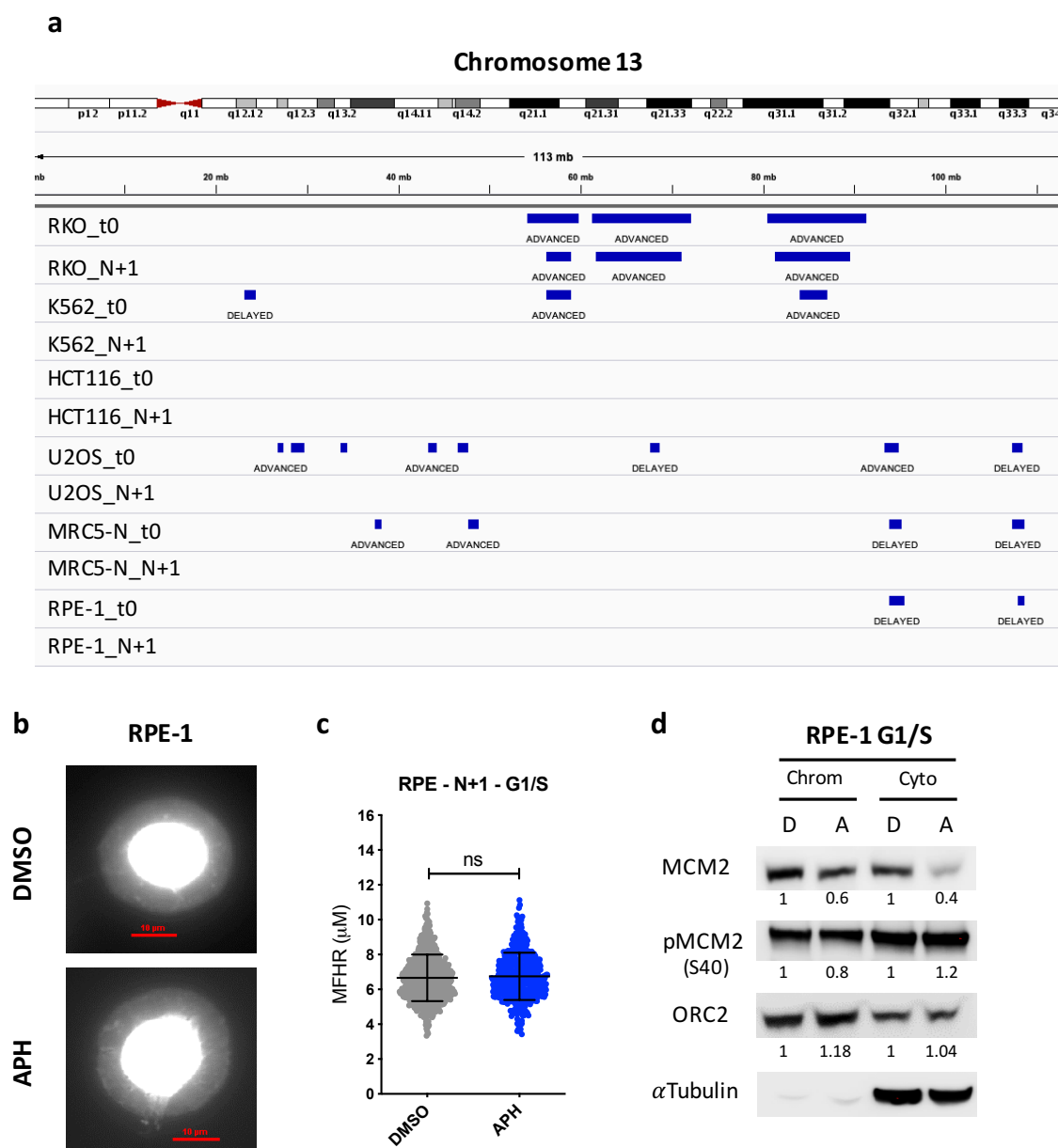
**Figure S4: aRTIL genomic characterisation.** **a** Histograms representing the normal replication timing (DMSO condition) of ADV and DEL aRTIL genomic regions in RKO, K562, HCT116, U2OS, MRC5-N and RPE-1. **b** Boxplots of RT domains size (in kb) of Early, Mid, Late, ADV aRTIL and DEL aRTIL genomic regions in HCT116, U2OS, MRC5-N and RPE-1. **c** Boxplots of GC content (in percentage) in Early, Mid, Late, ADV aRTIL and DEL

aRTIL genomic regions in HCT116, U2OS, MRC5-N and RPE-1. **d** Boxplots of constitutive replication origins coverage in Early, Mid, Late, ADV aRTIL and DEL aRTIL genomic regions in RKO, U2OS, MRC5-N and RPE-1. Statistics: Wilcoxon rank sum test \*\*\*\* $p < 0.0001$ , ns when  $p > 0.05$ . **e** Boxplots of gene coverage in Early, Mid, Late, ADV aRTIL and DEL aRTIL genomic regions in HCT116, U2OS, MRC5-N and RPE-1. Statistics (for all the boxplots): Wilcoxon rank sum test \*\*\* $p < 0.005$ , \* $p < 0.05$ , ns when  $p > 0.05$ .



**Figure S5: Low replication stress impacts the regulation of genes involved in chromatin organization and modulates chromatin accessibility within ADV aRTIL.** **a** Names and values of log2 Fold change gene expression induced by aphidicolin for the most significantly impacted genes ( $\text{Log}_2(\text{FC}) < 0$ , APH DOWN, in black;  $\text{Log}_2(\text{FC}) > 0$ , APH UP, in grey). Statistics: ANOVA test  $p < 0.05$ . **b** Gene ontology for DOWN-regulated genes (ShinyGO, P-val cutoff: FDR < 0.05). **c** Comparison of ATAC-seq peak value within Early and Late

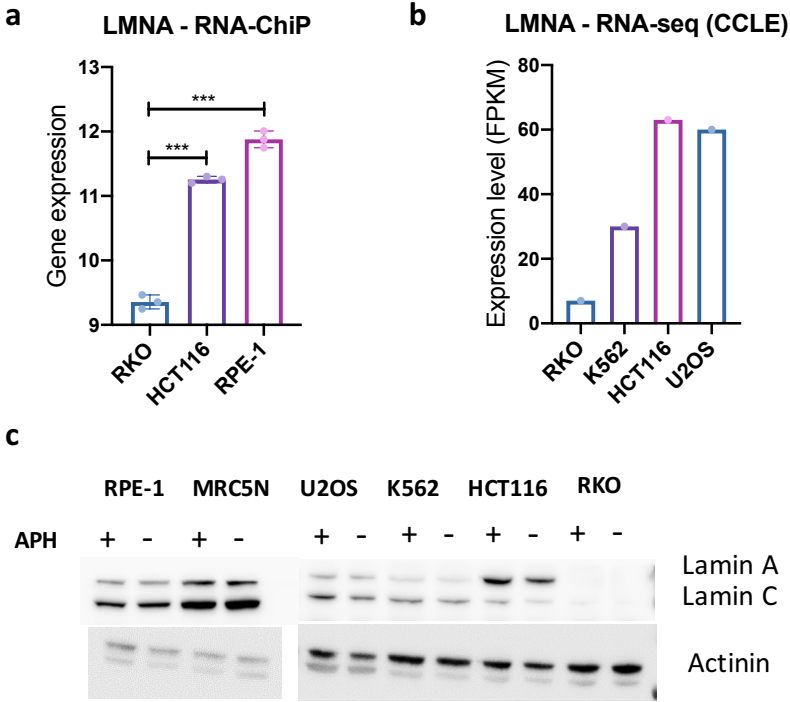
regions between DMSO (grey) and APH (red) conditions. **d** Comparison of ATAC-seq peak coverage within Early and Late replicating regions between DMSO (grey) and APH (red) conditions. Statistics (N=3): Wilcoxon matched-pairs signed rank test \*\*\*\* $p < 0.0001$ , \* $p < 0.05$ . **e** Screenshot of integrative genome viewer (IGV) session with ATAC-seq triplicates bigwig files on chromosome 4 within a specific ADV aRTIL (91,820-91,830kb). **f** Screenshot of integrative genome viewer (IGV) session with ATAC-seq triplicates bigwig files on chromosome 4 within a random non impacted region (151,010-151,020kb). **g** Comparison of ATAC-seq Average peaks value (Avg peak value) between DMSO (black) and APH (grey) conditions within APH UP-regulated genes (UP-genes). Statistics (N=3): Wilcoxon matched-pairs signed rank test \* $p < 0.05$ .



**Figure S6: ADV aRTIL can be transmitted to daughter cells.** **a** Screenshot of integrative genome viewer (IGV) session with aRTIL on chromosome 13 in mother (t0) and released daughter cells (N+1) for the six cell lines. **b** Visualization and **c** Quantification of DNA Halo size (MFHR) in RPE-1 G1/S synchronized daughter cells released from DMSO or aphidicolin treatment. Statistics (N=3): Unpaired t test with Welch's correction



ns when  $p < 0.05$ . **e** Western blot on chromatin (Chrom) and cytoplasmic (Cyto) protein fractions to quantify the amount of MCM2, p-MCM2 and ORC2 in the G1/S cells (synchronized with L-mimosine). The fold change between DMSO and APH conditions is reported on the figure.



**Figure S7: Gene expression and protein level of LaminA/C in cancer and non-tumour cells.** **a** RNA-ChIP expression of LMNA gene in RKO, HCT116 and RPE-1. Statistics: Two-way ANOVA Tukey's multiple comparisons test \*\*\*\* $p < 0.0001$ . **b** Expression level (FPKM) of LMNA gene expression in RKO, K562, HCT116 and U2OS (RNA-seq values from the CCLE data set). **c** Western Blot for LaminA/C and actinin (for loading control) protein in the six cell lines aphidicolin treated (APH+) or not (APH-).

| Name    | Forward sequence       | Reverse sequence      | chr   | Start     | End       |
|---------|------------------------|-----------------------|-------|-----------|-----------|
| L1      | CCCCATCCCCAGTTCTTTCC   | TGGTGGGACTTGTGCTGTTT  | chr17 | 67077058  | 67077088  |
| L1bis   | TGGCACGTTCTTGTCACT     | AAGGTCCTCAGCCATTCAGC  | chr17 | 67129038  | 67129068  |
| L2      | TCACTGCCAGTTCGACACAG   | ACCCAGTCCCACATCACTCT  | chr16 | 75360480  | 75360510  |
| L2bis   | TGTGTCCATGCTGTGCCTAG   | TGATGGAAGCAGCTACGTGG  | chr16 | 75255289  | 75255319  |
| E1      | TGACTCCGCTTCGAACCTC    | GATGCTTGCACTCCCTCTGT  | chr1  | 45058256  | 45058286  |
| E2      | TCCATCCTCAGGTCCTCGAG   | AATGGCACGGTTCAGGAG    | chr12 | 119955736 | 119955766 |
| ADV1    | GTTTCCTGGATGTTGCGCAG   | CCAACAGAGACCCAGCAGAG  | chr13 | 88356053  | 88356083  |
| ADV1bis | CAGCTTCACAGACCTCTCCG   | GCTTGCATCACTGGAGTCGA  | chr13 | 81884982  | 81885012  |
| ADV2    | GCCAAGCTCGACAATGTTT    | TCTTCCACTTGACCTGGG    | chr8  | 114462446 | 114462476 |
| ADV2bis | GTTGCCCATCACGAAAACC    | GCATGACCCTGTATCTGCC   | chr8  | 114378227 | 114378257 |
| ADV3    | ATGGATTAGCCCCGCTACT    | CTCACTGCAATCTGACGGT   | chr1  | 187080568 | 187080598 |
| ADVbis  | GAAGCTTCAGAGGCCGACAT   | GGAGACCATCAATGCCCGTT  | chr1  | 187034792 | 187034822 |
| Mito    | CCTAGGAATCACCTCCCATTCC | GTGTTAAGGGGTTGGCTAGGG | /     | /         | /         |

**Table S3:** Name, genomic sequences and location of BrdU-ChIP-qPCR probes.

|             | <b>K562</b>  |
|-------------|--------------|
| H3K4me1     | ENCFF320NKZ  |
| H3K27ac     | ENCFF770UZZ  |
| H3K36me3    | ENCFF676RW   |
| H3K27me3    | ENCFF369ZKL  |
| H3K9ac      | ENCFF842GQO  |
| H3K4me3     | ENCFF729NQN  |
| H3K9me3     | GSM607494    |
| H3K79me2    | ENCFF520XBR  |
| H3K4me2     | ENCFF298QOP  |
| DNase-seq   | ENCFF433TIR  |
| LaminB1     | 4DNFIHGCLSYW |
| gH2AX_NT    | GSM2808044   |
| gH2AX_APH   | GSM2808042   |
| mH2A1.2_NT  | GSM2808015   |
| mH2A1.2_APH | GSM2808019   |

**Table S4:** Reference of public ChIP-seq data used in this study. Data from the ENCODE project are highlighted in yellow, those from the GEO dataset in blue and those from the 4DN project in green.

## Additional results

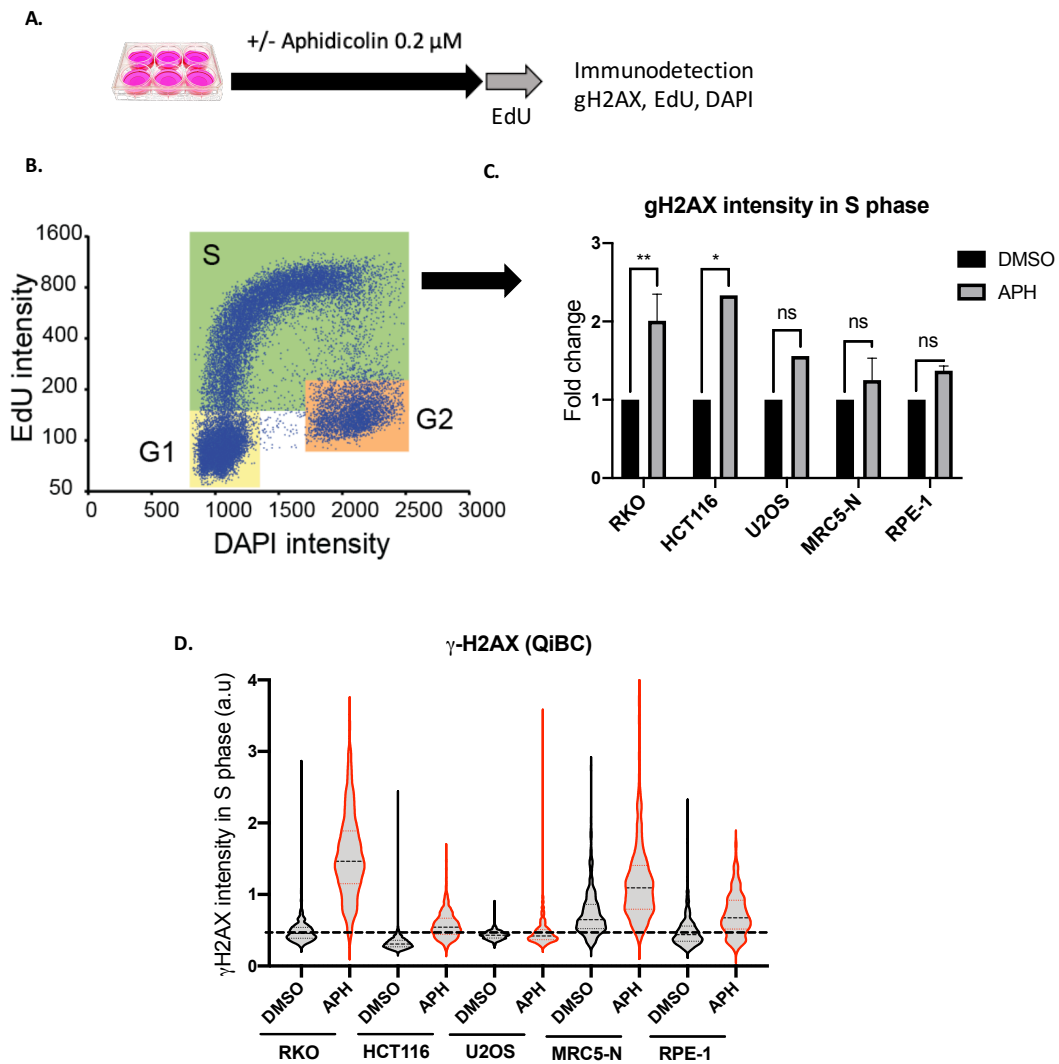
In this part, we will present and describe additional results, not included in the submitted manuscript, that I have performed during my thesis for the same project in the 6 human cell lines, providing additional information about the molecular and cellular response to the low replication stress induced by aphidicolin.

a. Increase of  $\gamma$ -H2AX signal under aphidicolin in cancer and healthy cells

As mentioned earlier in this thesis introduction, there are many different manners to address and measure the replication stress undergone by the cell during S phase. In the submitted manuscript, we described the direct replication stress response through measurement of cell cycle repartition and Chk1 phosphorylation (S345) (**submitted manuscript, Figure 1a and Figure S1b**). To address the impact of aphidicolin on replication during the S phase, we have also performed immunofluorescence of the very well-known  $\gamma$ -H2AX, histone H2AX phosphorylation signal for DNA breaks.

Briefly, cells were treated with the usual dose of aphidicolin during the appropriate time for each cell lines and EdU was added to the media before cell fixation and immunodetection (**Figure 26A**). For each condition, about a thousands of cells were analysed and the  $\gamma$ -H2AX intensity was especially measured in S phase thanks to QiBC technique (**Figure 26B**). For technical reasons, we unfortunately do not have this analysis for the K562 cell line. We observed a global increase of  $\gamma$ -H2AX intensity in S phase under aphidicolin treatment (**Figure 26C and D and Figure S4**). This observation confirms that the dose of aphidicolin used in this study is sufficient to affect the replication process to induce DNA breaks signalling in all the cell lines. Just like it was described for pChk1 and the cell cycle accumulation, we observe a differential response depending on cell lines. Indeed, the RKO cells, followed by MRC5-N present the stronger increase while U2OS and HCT116 only present a very low increase. With this experiment, we can conclude that the aphidicolin treatment at this time and duration does not impact all the cell lines by inducing a global DDR signalling.

At the endogenous level, we could already detect differences of  $\gamma$ -H2AX signal between cell lines. Quite surprisingly, the MRC5-N normal cells present the higher endogenous  $\gamma$ -H2AX level. This might be explained by the fact that these cells are the only non-transformed cells and thus maybe more sensitive to the culture conditions. However, this observation with is not consistent with our observation of p-Chk1 by western blot in which we described a very low basal level in MRC5-N and a very low increase under aphidicolin treatment (**Figure 26D**). These two results are quite difficult to reconcile and maybe it would have been interesting to look at other markers like p-ATM, p-ATR or p-Chk2 to have a clearer idea of the response of each cell line.



**FIGURE 26:** DETECTION OF DSBs BY  $\gamma$ -H2AX IMMUNOFUORESCENCE STAINING WITH AND WITHOUT LOW REPLICATION STRESS. (A) SCHEME OF THE EXPERIMENTAL PROCEDURE. CELLS WERE TREATED WITH DMSO OR APHIDICOLIN, EDU WAS INCORPORATED 15 MIN BEFORE CELL FIXATION AND IMMUNOSTAINING. (B) SCHEMATIC REPRESENTATION OF QIBC THANKS TO MEASUREMENT OF DAPI AND EDU INTENSITIES. (C) HISTOGRAM REPRESENTING THE FOLD CHANGE (APH/DMSO) OF  $\gamma$ -H2AX INTENSITIES IN THE S PHASE OF THE CELL CYCLE BETWEEN DMSO (BLACK) AND APH (GREY) CONDITIONS. STATISTICS (N=2): 2WAY ANOVA \*\* $p < 0.01$ , \* $p < 0.05$ , ns WHEN  $p > 0.05$ . (D) VIOLIN PLOTS OF NORMALIZED  $\gamma$ -H2AX INTENSITY IN S PHASE IN DMSO (BLACK) AND APH (RED) CONDITIONS.

To conclude, if we compare the  $\gamma$ -H2AX signal only between cancer cells, we found that the RKO cell line is the most impacted by the treatment, which is in line with our observation on the replication timing. In addition, regarding  $\gamma$ -H2AX intensity, we found that MRC5-N are also more sensitive to aphidicolin compared to RPE-1, observation that also reflects our replication timing data.

b. Low RS is transmitted to the next cellular generation in cancer cells

In the submitted manuscript, we described the impact of replication stress in mother cells on the replication timing of daughter cells and empathize the fact that RT modifications in mother cells are only transmitted to daughter cells in the particular case of RKO cells. In the paper, we suggest that replication timing transmission happens mainly when the amplitude of the RT modifications in mother cells is strong enough, like it is the case in RKO. However, we still ignore what happens mechanistically in RKO and other cells during the next G1 and S phase after cell division. In this part, we will present some results about the impact of low replication stress on the next cellular generation.

i. **53BP1 bodies in G1**

It has been described several years ago that, upon low replication stress, there is an increased frequency of chromosomal lesions transmitted to daughter cells in G1. These lesions are sequestered in nuclear bodies, containing the p53-binding protein 1 and other chromatin associated caretakers, named 53BP1 bodies (Harrigan et al., 2011; Lukas et al., 2011). This phenomenon is called the DNA damage transmission and it was described in cancer as well as normal cells. Thus, we decided to address the question whether if in our context of low replication stress, we could observe this mark of DNA damage transmission in all the 6 cell lines. To do so, we analysed by immunofluorescence against 53BP1 protein, combined with Quantitative-Image-Based-Cytometry (QiBC), the amount of 53BP1 nuclear bodies in G1 cells. Briefly, we treated cells with or without aphidicolin 0.2 $\mu$ M for appropriate time and released the cell in a fresh media in order to let them divide and enter in the next G1 phase. Cells were labelled with EdU for 15min before fixation and immunofluorescence staining of 53BP1, EdU and DAPI was performed. For each condition, about two hundreds of cells were analysed for each fraction of the cell cycle (G1, S and G2M) by QiBC technique. The mean number of 53BP1 bodies detected in G1 nuclei for each cells and conditions is represented in the **Figure 27** and immunofluorescence images for each cell line in **Figure S5**.

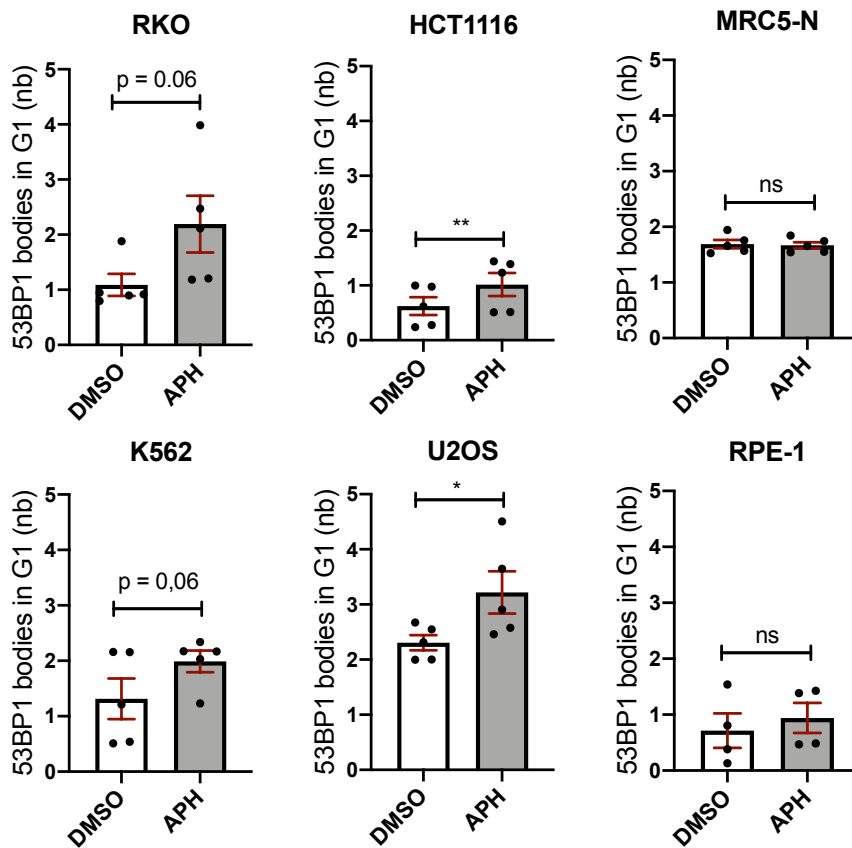


FIGURE 27: ASSESSING DNA DAMAGE TRANSMISSION BY THE QUANTIFICATION OF 53BP1 BODIES IN G1 DAUGHTER CELLS. HISTOGRAMS REPRESENTING THE QUANTIFICATION OF THE MEAN NUMBER OF 53BP1 BODIES IN G1 DAUGHTER CELLS RELEASED FROM THE DMSO OR APH TREATMENT. STATISTICS (N>3): PAIRED WILCOXON TEST \*\*p<0.01, \*p<0.05, NS WHEN P>0.05.

We first observe heterogeneity in the basal level of 53BP1 bodies between the cells, with a high amount in U2OS and particularly low mean number in HCT1116. Then, we can notice a global tendency of increase after aphidicolin treatment in all the cell line except for the MRC5-N. Statistical analysis revealed that cancerous cell lines present a stronger increase in the number 53BP1 bodies than healthy cells. We can imagine that mechanisms of repair and checkpoints are more efficient in normal cells and this would explain why there is less DNA damage transmission. Next, it is interesting to notice that RKO and K562 have a lower DNA damage transmission compared to HCT1116 and U2OS, which is consistent with the fact that they have less replication timing delays and thus, less under-replicated DNA, the major source of DNA breaks in mitosis and induction of 53BP1 nuclear bodies. Finally, as HCT1116 and U2OS has a strong difference in the basal level of 53BP1 bodies whereas they have the closer response in term of RT and DNA damage transmission increase, we can assume that low replication stress induced by aphidicolin is probably not the only explanation for the generation of 53BP1 bodies. Indeed, the cellular background of the cells may better explain

why some cancer cells like U2OS have a high level of 53BP1 bodies in G1 whereas other not. This could be linked to the observation that in U2OS, aphidicolin induces less increase of  $\gamma$ -H2AX intensity in S phase (**Figure 26**), maybe suggesting that these cells have a defect in DSB signalling and thus they enter in mitosis with more DNA damage. Normal cells, mainly MRC5-N, does have endogenous 53BP1 bodies in G1, meaning that the mechanism of DNA damage transmission also exist in these cells, but the low replication stress generated in our context is not sufficient to increase it.

To conclude, this experiment provides a new evidence of a differential response to RS between normal and cancer cells. However, these results are not providing clear evidence of a link between DNA damage transmission and persistence of replication timing modification as RKO cells are clearly not presenting a stronger increase of 53BP1 bodies than other cell lines. As we observed that U2OS and HCT116 were the most impacted cells, we can imagine that 53BP1 bodies shapes the un-replicated DNA coming from strong RT delays, and thanks to this shaping, RT delays are not transmitted to the next cellular generation as it was described in a recent study (Spies et al., 2019).

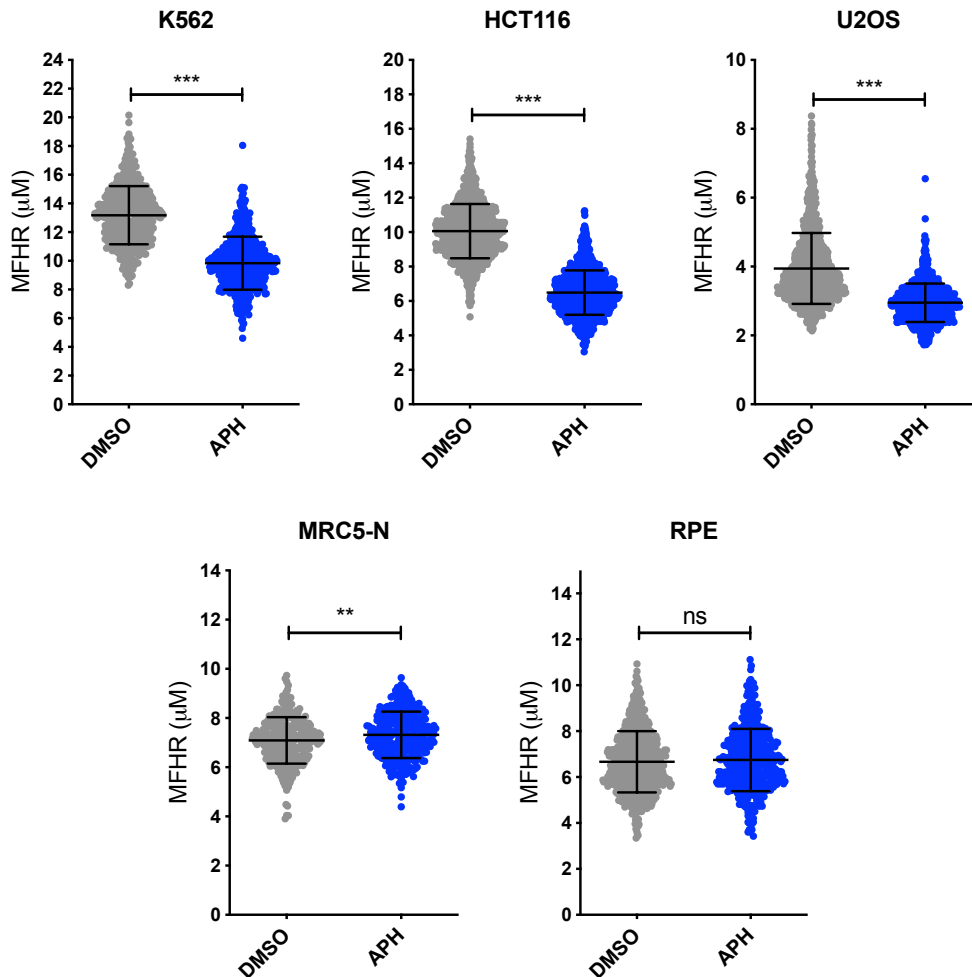
## ii. Origin licensing is impacted in G1/S of cancer cells

As described in the manuscript for the RKO cell line, we also performed DNA Halo experiment in the 5 other cell lines of the study.

Like in RKO cells, we observed a decrease of DNA Halo size in cancerous cell lines released from aphidicolin, compared to the DMSO control. Interestingly, this DNA loop shrinking was not observed in normal cells (**Figure 28** and **Figure S6**). Indeed, no significant modification of the DNA Halo size was detected in RPE-1 cells while we measured an increase in MRC5-N cells.

This last observation may suggest that there is a fewer amount of licensed origin, suggesting that some cell within the population are entering in senescence or quiescence state (Blow and Hodgson, 2002; Kingsbury et al., 2005). Two recent studies in non-tumorigenic cell lines (including RPE-1), have shown that low replication stress in mother cells lead to an increase of senescence entry in the next G1 daughter cells (Arora et al., 2017; Barr et al., 2017), further support this assumption.





**FIGURE 28:** QUANTIFICATION OF DNA HALO SIZE (MFHR) IN G1/S DAUGHTER CELLS. QUANTIFICATION OF DNA HALO SIZE (MFHR) IN G1/S SYNCHRONIZED DAUGHTER CELLS RELEASED FROM DMSO OR APHIDICOLIN TREATMENT. STATISTICS (N=3): UNPAIRED T TEST WITH WELCH'S CORRECTION \*\*\* $p < 0.0001$ , \*\* $p < 0.005$ , NS WHEN  $p > 0.05$ .

Overall, it is interesting to notice that in K562, U2OS and HCT116 cell line, this shrinkage of DNA Halos after aphidicolin stress in mother cells is not sufficient to induce modification of replication timing in the next S phase. Thus, we cannot directly correlate the DNA Halo shrinkage with the persistence of RT alterations in daughter cells. As we mentioned in the paper, the ADV aRTIL in RKO daughter cells (N+1) were less important in amplitude compare to mother cells. As the amplitude of RT modification in other cancer cells was already lower in mother cells, it is consistent to imagine that they were not strong enough to persist or be detected in daughter cells, explaining why we saw very few RT modifications in daughter cells.

### iii. The replication dynamic is still affected in RKO daughter cells

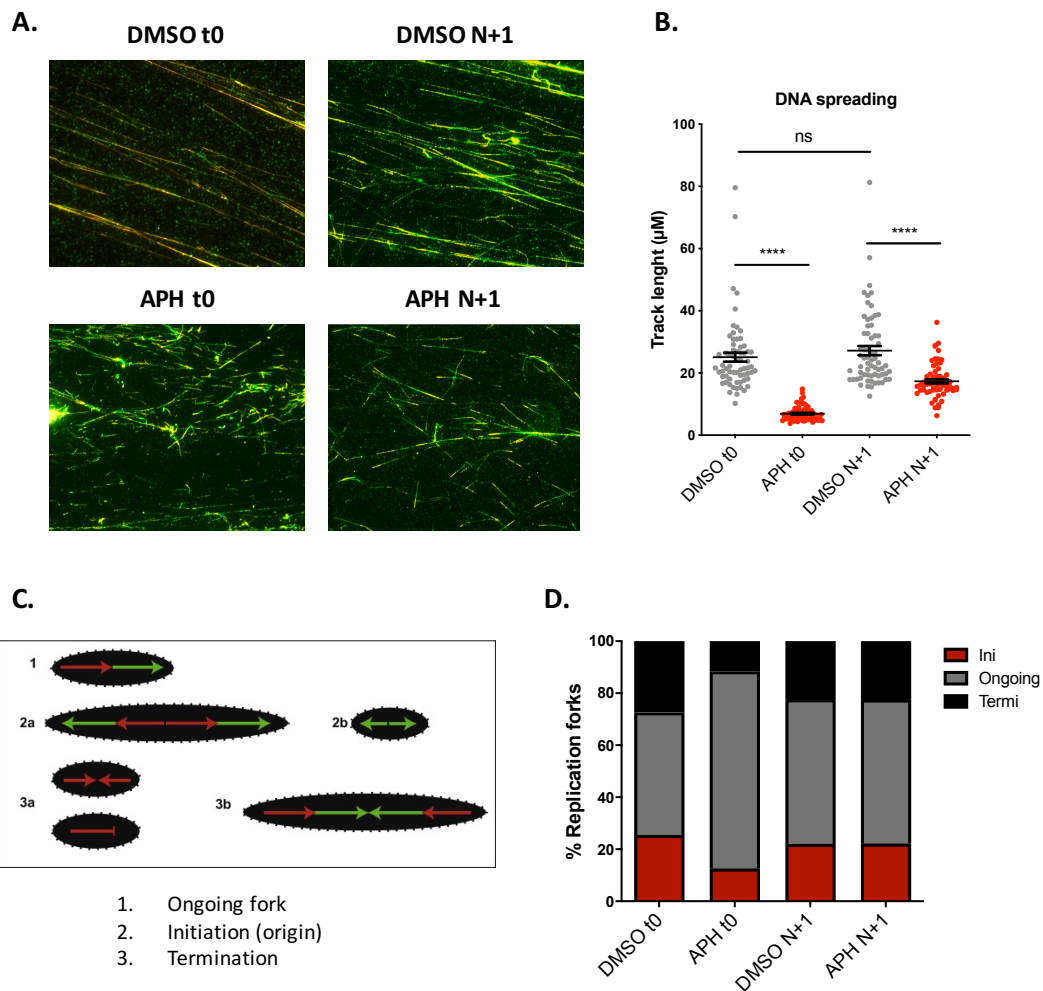
As mentioned in the introduction, as we intended to apply a very low replication stress to answer our question, the best approach to detect this replication stress was to measure replication fork speed at the molecular level. Here we describe the molecular response to RS by directly measure replication dynamic thanks to DNA spreading and DNA combing experiments in RKO mother (t0) and daughter cells (N+1).

We first decided to perform DNA spreading as a control experiment to confirm that replication fork was indeed slow down by aphidicolin treatment (Koundrioukoff et al., 2013; Rodriguez-Acebes et al., 2018) and to see the global impact on replication dynamic. Briefly, cells were treated in the usual conditions, pulse-labelled 30min with CldU followed by 30 min of IdU to then do the DNA spreading on coverslips and then the immunodetection. About 100 DNA tracks were counted to do the statistical analysis. DNA replication fork speed was measured only on ongoing fork, by doing the sum of IdU + CldU length.

As expected, aphidicolin induces a significant diminution of the replication fork track length (reflection the fork speed) in the mother cells (t0) and interestingly, we still noticed a decrease of track length in daughter cells (N+1), suggesting that many replication forks are still slowed down (track length in **Figure 29A and B**). Then, to have a better idea of the global replication dynamic, we quantified the proportion of initiation, ongoing fork and termination (**Figure 29C and D**). Although we observed an increase in the percentage of ongoing forks under aphidicolin treatment in mother cells at the expense of initiations and terminations, we quantified no such differences in daughter cells. Overall, this first DNA spreading approach allowed us not only to validate the effect of aphidicolin on replication fork speed but also to discover that replication dynamic in RKO daughter cells may also be disturbed in after the release from aphidicolin.

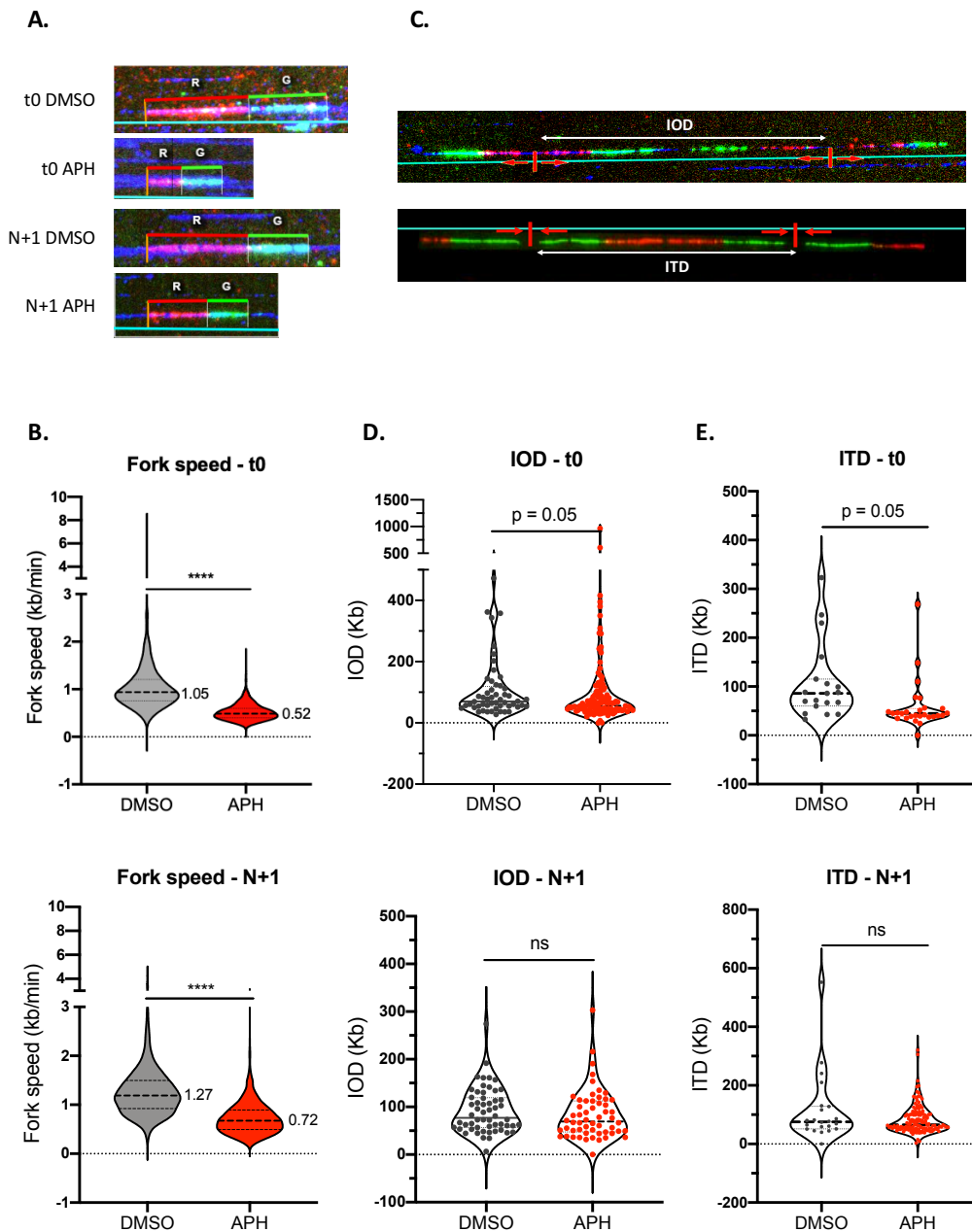
DNA spreading was a good approach to have an idea of the global replication dynamics but it is not precise enough to measure many replication parameters such as inter-origin distance. Indeed, to do such analysis, DNA combing is much more appropriated. We thus performed DNA combing in the same experimental conditions. Our results confirm the replication fork slowing in both mother (t0) and daughter cells (N+1) (**Figure 30A and B**). Thus,

thanks to these two intendant experiment and approach, we confirmed that the persistence of replication fork slowing in RKO daughter cell appears to be real.



**FIGURE 29 :** DNA SPREADING IN RKO MOTHER (t0) AND DAUGHTER (N+1) CELLS. (A) IMAGES OF DNA SPREADING FOR EACH CONDITION. (B) SCATTERPLOT REPRESENTING VALUES OF TRACK LENGTH (IdU + CldU) MEASURED BY DNA SPREADING EXPERIMENT. STATISTICS (N=1): UNPAIRED T-TEST \*\*\*\*p<0.0001, NS WHEN p>0.05. (C) SCHEME EXPLAINING HOW INITIATIONS, ONGOING FORKS AND TERMINATIONS WERE IDENTIFIED. (D) STACKED HISTOGRAM REPRESENTING THE PERCENTAGE OF INITIATION, TERMINATION AND ONGOING FORK IN THE DNA SPREADING EXPERIMENT.

As previously mentioned many time, it is assumed that under low dose of aphidicolin, fork slowing induces an increase of dormant origin activation. To confirm this mechanism in RKO cells, and also to see if we still observed dormant origin activation in daughter cells, we measured the inter-origin and inter-termination distances (IOD and ITD) in the different conditions.



**FIGURE 30:** ANALYSIS OF DNA REPLICATION DYNAMIC BY COMBINING IN RKO CELLS. (A) SNAPSHOT OF DNA COMBINING IMAGES EXAMPLES OF ONGOING REPLICATION FORK FOR EACH CONDITION. (B) VIOLIN PLOTS REPRESENTING VALUES OF REPLICATION FORK SPEED (IN KB/MIN) IN DMSO AND APH IN MOTHER (T0) AND DAUGHTER CELLS (N+1). (C) SNAPSHOT OF DNA COMBINING IMAGES EXAMPLES REPRESENTING INTER-ORIGIN DISTANCE (IOD) AND INTER-TERMINATION DISTANCE (ITD). (D) VIOLIN PLOT REPRESENTING THE VALUES OF INTER-ORIGINS DISTANCE (IOD, IN Kb) IN DMSO AND APH IN MOTHER (T0) AND DAUGHTER CELLS (N+1). (E) VIOLIN PLOT REPRESENTING THE VALUES OF INTER-TERMINATION DISTANCE (ITD, IN Kb) IN DMSO AND APH IN MOTHER (T0) AND DAUGHTER CELLS (N+1). STATISTICS (N=1, FOR DNA SPREADING AND COMBINING): WILCOXON TEST \*\*\*\* $p < 0.0001$ , NS WHEN  $p > 0.05$ .

In RKO mother cells, we saw a tendency to a decrease of IOD and ITD in aphidicolin-treated mother cells (**Figure 30 C and E**), confirming that there must be dormant origin activation in response to the aphidicolin-induced replication stress. However, IOD and ITD

were not anymore different in RKO daughter cells even though we still observed an impact on DNA replication fork speed (**Figure 30 C and E**). This result may be in contradiction with the previous observation with DNA Halo and MCM loading in G1/S, suggesting that potentially more replication origin will be activated in the S phase of daughter cells. We can hypothesize that dormant origin activation in daughter cells will not be throughout the genome and this might explain why we did not detect a significant decrease of IOD and ITD. As we also quantified increased MCM loading onto the chromatin at the global level, we cannot know if this increased loading is happening at the specific locus of constitutive origin or if it is at new/dormant replication origins. As we assessed the IOD and ITD and did not observed significant differences, this may suggest that the increase of MCMs onto the chromatin happens at locus of constitutive origins, to counteract the replication stress sudden by the mother cells in the previous S phase. Finally, if the global fork is slowed down and that the cell has not a significant increase of origin activation in S phase, we may wonder how this cell manage to complete its whole genome replication on time. The ADV aRTIL in these cells may be an explanation, if the replication of these regions started earlier, cells can complete replication on time even with a global decrease of the DNA replication fork.

Overall, even though this DNA combing approach brought some interesting information about the DNA replication process in RKO daughter cells, some mechanism behind this replication fork slowing remains to be explained. First, it would be nice to reproduce this experiment (just one experiment presented here) to confirm our observations. Then, we would also need stronger sensibility and specificity to directly target genomic regions of interest like ADV aRTIL to see if the daughter cells, ADV aRTIL are due to more origin/earlier origin activation. To do so, the best known approach to data would be the OK-seq, that directly map the activation origins of replication.

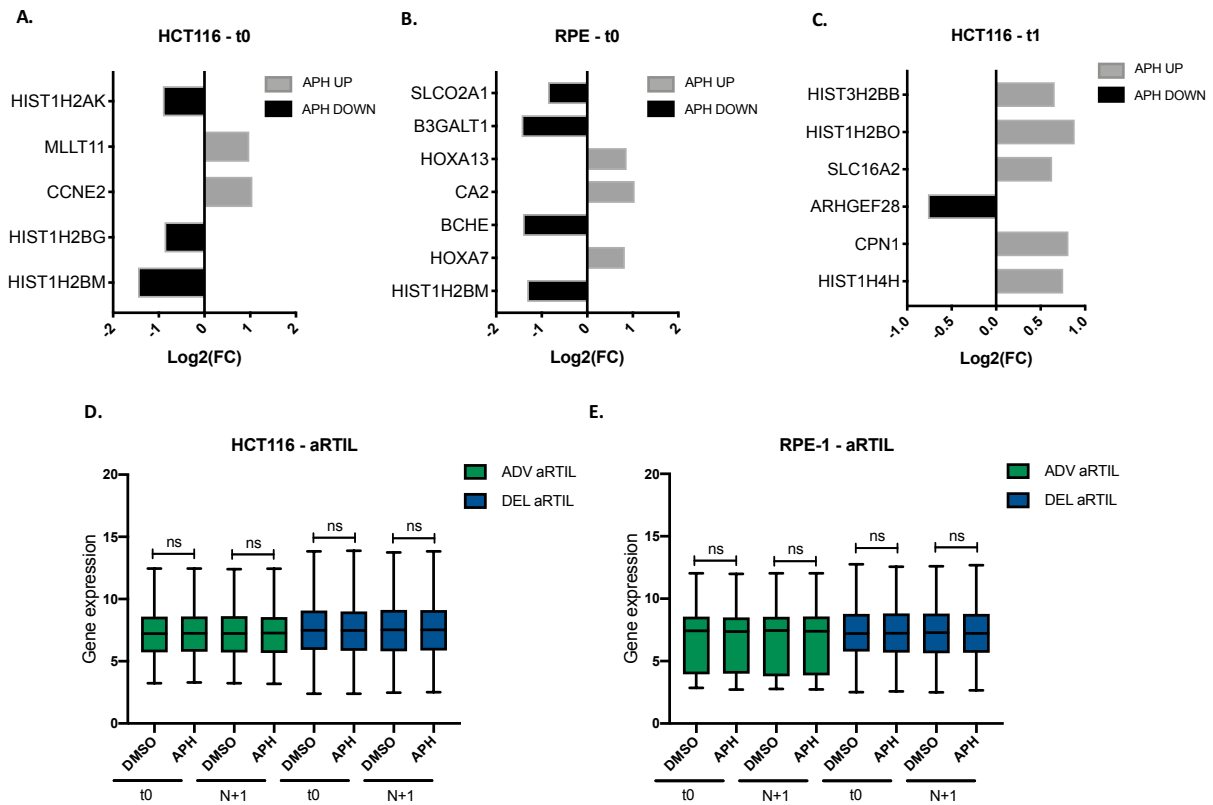
### c. Impact of aphidicolin on gene expression in HCT116 and RPE-1 mother and daughter cells

In the paper, we described that in RKO cells, the gene expression was very poorly impacted by aphidicolin during the treatment (mother cells, t0), and that the few genes impacted were linked to chromatin organization (REF, three histone genes). We also performed RNA-ChIP analysis in HCT116 and RPE-1 cells, to see if the conclusions we made in RKO were also true in another cancerous cell line and in a normal cell line. Globally, we also observed that aphidicolin has a very low impact on gene expression, as we only found respectively 5 and 7 significantly impacted genes in HCT116 and RPE-1 mother cells (**Figure 31A and B**). However, when we looked the list of these impacted genes in more details, we found the same tendency of down-regulation of histone genes (3 in HCT116, 1 in RPE-1).

When we analysed differential gene expression between cells released from DMSO or aphidicolin, we found 6 significantly impacted genes in HCT116 (**Figure 31C**) while no impacted genes were detected in RPE-1. It is interesting to notice here that, on the opposite to RKO daughter cells, the significantly impacted genes in HCT116 daughter cells were mainly histone genes. This up-regulation of histone gene in daughter cells may counteract the down-regulation in mother cell, and thus allow the chromatin to be correctly reorganized allowing the replication timing to be back to normal. Again, with another approach, we confirmed a memory of the replication stress in cancer cell and no significant impact on the next generation of normal cells.

We also wondered if the gene expression within ADV or DEL aRTIL was impacted in mother and/or in daughter cells. Just like we did in RKO cells, we compared level of expression of the genes specifically within aRTIL. As in RKO mother cells, our results did not show significant modification of genes expression within aRTIL in HCT116 nor in RPE-1, in mother (t0) nor in daughter cells (N+1) (**Figure 31D and E**).

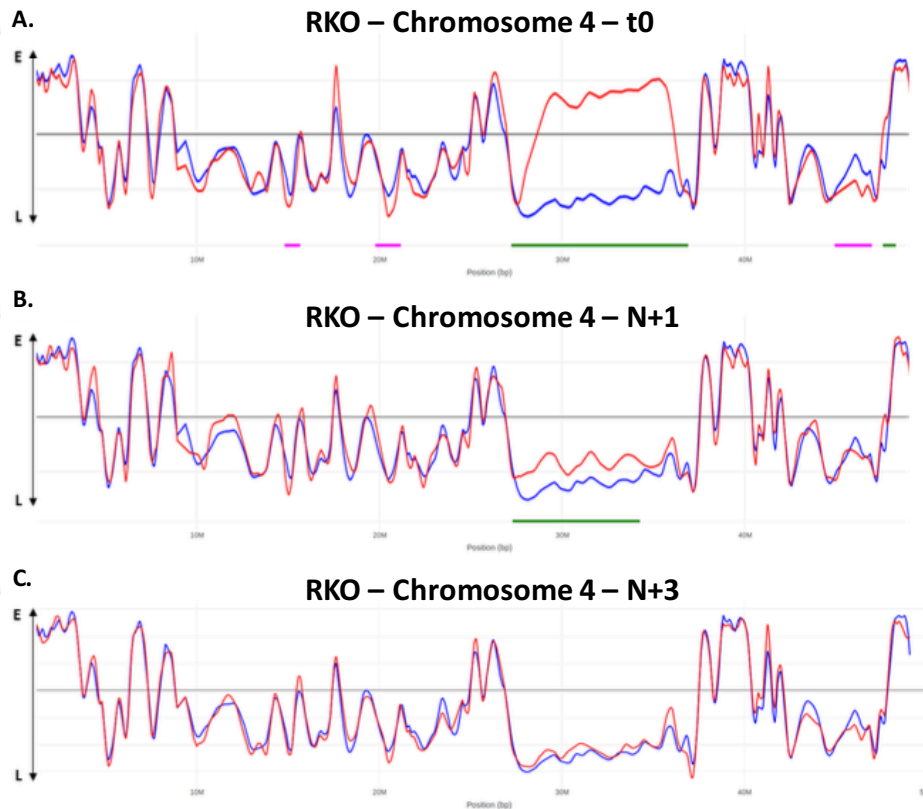
Overall, these data further support the idea that replication timing modifications in mother cells are not associated to modification of gene expression.



**FIGURE 31:** IMPACT OF APHIDICOLIN ON GLOBAL GENE EXPRESSION OR WITHIN ARTIL IN HCT116 AND RPE-1 DAUGHTER CELLS. (A) NAMES AND VALUES OF LOG2 FOLD CHANGE (FC) OF GENE EXPRESSION FOR THE MOST SIGNIFICANTLY IMPACTED GENES BY APHIDICOLIN ( $\text{Log}_2(\text{FC}) < 0$ , APH DOWN, IN BLACK;  $\text{Log}_2(\text{FC}) > 0$ , APH UP, IN GREY), IN HCT116 MOTHER CELLS AND (B) DAUGHTER CELLS (N+1). (C) NAMES AND VALUES OF LOG2 FOLD CHANGE (FC) OF GENE EXPRESSION FOR THE MOST SIGNIFICANTLY IMPACTED GENES BY APHIDICOLIN ( $\text{Log}_2(\text{FC}) < 0$ , APH DOWN, IN BLACK;  $\text{Log}_2(\text{FC}) > 0$ , APH UP, IN GREY), IN RPE-1 MOTHER CELLS. STATISTICS: ANOVA TEST  $\text{FDR} < 0.05$ . (D) GENES EXPRESSION WITHIN HCT116 ARTIL (ADV IN GREEN AND DEL IN BLUE) IN THE MOTHER (t0) AND DAUGHTER (N+1). STATISTICS: WILCOXON MATCHED-PAIRS SIGNED RANK TEST NS WHEN  $\text{P} > 0.05$ . (E) GENES EXPRESSION WITHIN RPE-1 ARTIL (ADV IN GREEN AND DEL IN BLUE) IN THE MOTHER (t0) AND DAUGHTER (N+1). STATISTICS: WILCOXON MATCHED-PAIRS SIGNED RANK TEST NS WHEN  $\text{P} > 0.05$ .

d. The DNA replication timing is no more modified after few cellular generations

The observation of RT persistence in RKO daughter raises the question of the reversibility of this phenomenon. We wondered if in RKO cells, these RT alterations were also persistent in the following cellular generations or if these RT alterations were only transient. To do so, we decided to analyse the RT of cells three generations after the release from the treatment with DMSO or Aphidicolin (N+3).



**FIGURE 32:** SNAPSHOTS OF LOESS-SMOOTH REPLICATION TIMING PROFILES IN RKO CELLS THROUGH 3 GENERATIONS. CHROMOSOME 4 (0-48Mb) RT PROFILES IN THE DMSO (BLUE) AND THE APHIDICOLIN (RED) CONDITIONS. (A) RT PROFILES IN MOTHER (T0) CELLS. (B) RT PROFILES IN DAUGHTER CELLS RELEASED FROM THE STRESS (N+1). (C) RT PROFILE OF CELLS THREE CELLULAR GENERATIONS AFTER THE RS (N+3). SIGNIFICANTLY ADV ARTIL ARE UNDERLINED IN GREEN AND DEL ARTIL IN PINK.

We observe that after few cellular divisions, the RT of RKO cells is the same between cells released from DMSO or Aphidicolin (**Figure 32** and **Figure S7**). Thus, together with what we observed in the daughter cells of the 5 other cell lines, we can conclude that RT alterations is a transient and reversible phenomenon.



## Discussion

The main purpose of this thesis was to explore the direct impact of low replication stress on the replication program of human cells and its consequence on the next cellular generations. This project led to new discoveries as well as consistent observations. As a matter of fact, the major discovery is the replication timing advances in mainly two cancerous cell lines (RKO and K562) and the transmission of these alterations to the next cellular generation in RKO cells. This project brought to light the important fact that the RT of a given cell is sufficient to determine its tumorigenicity as we demonstrate by clustering approach on RT data that cancer cells cluster together regardless of their tissue of origin (**Submitted manuscript Figure 1e,f**). Finally, in line with previous studies, we observed that the expected RT delays could be linked to CFSs, gene expression and/or acute DNA damage signalling. However, this study did not lead to a specific mechanism explaining the RT advances nor their biological significance. In this discussion, I will try to go further in our data interpretations, suggesting potential mechanism under RT modifications to finally bring a point of view regarding the contribution of this study to the field of fundamental and cancer research.

## Connecting ARTIL, CFSs, and genome instability

This study confirmed expected replication timing delays (DEL aRTIL) under low replication stress, as it has been already described in recent studies (Brison et al., 2019a; Sarni et al., 2019). As we observed this effect in the 6 human cell lines, and the two mentioned studies described in two other human normal cells (JEFF and BJ-hTERT), it is reasonable to suggest that this effect on RT is universal. Next, we found some already described CFSs within DEL aRTIL but not exclusively. As Sarni et al. mentioned in their study, analysis of DNA replication timing under low replication stress may be a new way to identify and characterize fragile sites, and provide a specific list for each cell type.

It is well-known that CFSs are enriched in CNVs (Glover and Wilson, 2013). We looked at the enrichment of aRTIL within previously reported CNVs (Zarrei et al., 2015) and observed a tendency for ADV aRTIL to correlate with CNV loss in three cell lines (RKO, K562 and MRC5-N) (**Figures S8 and S9**). Regarding DEL aRTIL, we did not see specific enrichment within CNV gain nor loss. Thus, we could not clearly link CNVs and aRTIL. One possible explanation for this lack of relevance is the fact that we used the same list of CNVs (compilation of published high-quality data on healthy individuals of various ethnicities) whereas to be more accurate it would be preferable to use a cell-type specific list.

## Trying to decipher mechanism(s) under RT modifications

During this thesis, we have made the striking observation that cells are able to advance the replication timing of specific genomic loci while there are harbouring low replication stress and global fork slowing (**Figures 29 and 30**). This mechanism allowing replication timing advances seems to be more pronounced in some cancer cells (RKO and K562), suggesting a stronger response to RS and/or ability to adapt in this context. In our study, we have characterized these ADV aRTIL in term of genomic and epigenomic features and concluded that it recapitulates well all the common features of late replicating heterochromatin and/or LADs domains. Unlike DEL aRTIL for whose mechanism explaining the delays have already been described, the way low replication stress inducing fork slowing leads to replication timing advances has not been explained yet. The aim of this part is to propose potential mechanism(s) that may explain aphidicolin-induced RT modifications, focussing especially on RT advances.

### a. Dormant origins and replication fork speed stochasticity may play a role in RT modifications

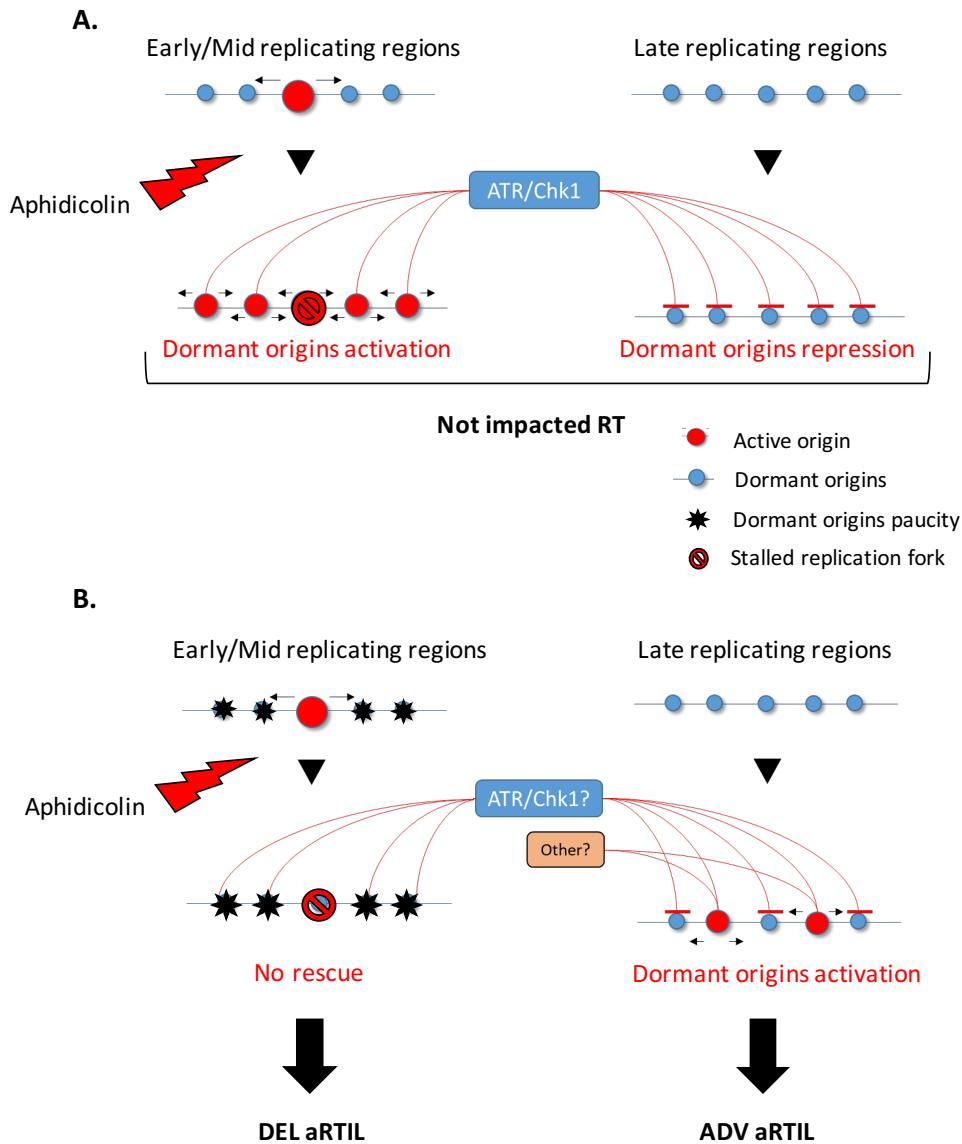
It is well reported that endogenous or low replication stress triggers the activation of dormant origin to complete the DNA replication on time (Alver et al., 2014; Blow and Ge, 2008; Courbet et al., 2008b; Kawabata et al., 2011). Indeed, it has been described that under RS, stalled replication forks within active replicons are rescued by the local dormant origin activation mediated by the ATR/Chk1 kinases while these two proteins inhibit the firing of replication origin in late replicating regions, allowing the RT maintenance (Ge and Blow, 2010; Santocanale and Diffley, 1998) (**Figure 33A**). As we detected RT alterations under aphidicolin treatment in all the cell lines, and especially in cancer cells, we can speculate that RT modifications could be mediated by the absence or excess of dormant origin firing.

First, the most likely scenario to explain RT delays is the presence of many endogenous sources of replication stress that, when combined with aphidicolin treatment, lead to replication fork stalling and an incapacity to rescue these latest by activating dormant origins, due to dormant origin paucity in these specific regions (**Figure 33B**, left panel).

Second, we can imagine that ADV aRTIL are the consequence of dormant origin activation in response to the replication stress. Indeed, we observed a good tendency in our DNA combing results showing differences in the IOD and ITD between untreated and aphidicolin treated conditions (**Figure 30**). However, we can wonder if the mechanism of dormant origin activation would be sufficient to counteract aphidicolin and induce RT advances. Indeed, if replication fork speed is slow down by the aphidicolin drug, it would require a high amount of dormant origin to, at least, complete the replication of a given domain on time and even more dormant origin to finish DNA replication in advance compared to its normal timing. Moreover, dormant origin activation is supposed to happen only when the principal active origin is stalled. This suggesting that, to allow replication timing advances within a given ADV aRTIL the principal origin must have been fired during the early S phase.

Finally, if dormant origin activation was sufficient to induce RT advanced, it would be observed much more frequently in the genome under RS. Thus, it is reasonable to suggest that dormant origin activation may contribute to RT advances but is probably not the only explanation. It would be nice to perform a DNA-combing-FISH on several ADV and DEL aRTIL as well as in control regions like some CFSs and early regions or to map active replication origins in the two conditions (DMSO and APH) genome-wide by SNS-seq or OK-seq approach, to identify dormant origin activation within specific genomic regions and confirm that dormant origin activation contribute to RT advances.

The exact mechanism regulating origin firing is not clearly understood yet (Courtot et al., 2018) and one hypothesis is that this phenomenon is stochastic within a replication domain, having a well-defined replication timing (Rhind, 2006; Rhind et al., 2020; Yousefi and Rowicka, 2019). In the particular case of cancer cells, that have strong endogenous replication stress together with alterations in many checkpoints pathways, we can imagine that, under this additional replication stress (aphidicolin), origin firing is not well regulated and some usually late firing origins are activated earlier in the S phase, resulting in RT advances within heterochromatin regions (**Figure 33B**, right panel).



**FIGURE 33:** THE POTENTIAL ROLES OF DORMANT ORIGIN ACTIVATION OR PAUCITY IN RT ALTERATIONS UNDER LOW RS. (A) AN ALREADY DESCRIBED MECHANISM TO EXPLAIN THE ROLE OF DORMANT ORIGIN ACTIVATION MEDIATED BY ATR/CHK1 PROTEINS IN THE COMPLETION IF DNA REPLICATION AND THE MAINTENANCE OF REPLICATION TIMING UNDER RS. (B) HYPOTHETICAL MECHANISM TO EXPLAIN DEL (ON THE LEFT) AND ADV aRTIL (ON THE RIGHT).

DNA replication fork speed and replication origins activation are two closely related phenomena, very difficult to uncouple. In our experimental context, we directly trigger DNA replication polymerases, it is thus very unlikely that replication timing advances are due to an increase of replication fork speed. In this line, we confirmed by DNA combing that aphidicolin leads to a significant reduction of replication fork velocity (**Figures 29 and 30**). Nevertheless, as our DNA combing analysis were performed genome wide, we cannot exclude the hypothesis of a local replication fork speed increase or a very efficient replication fork restart at the specific RKO ADV aRTIL loci, to compensate the general replication fork decrease and

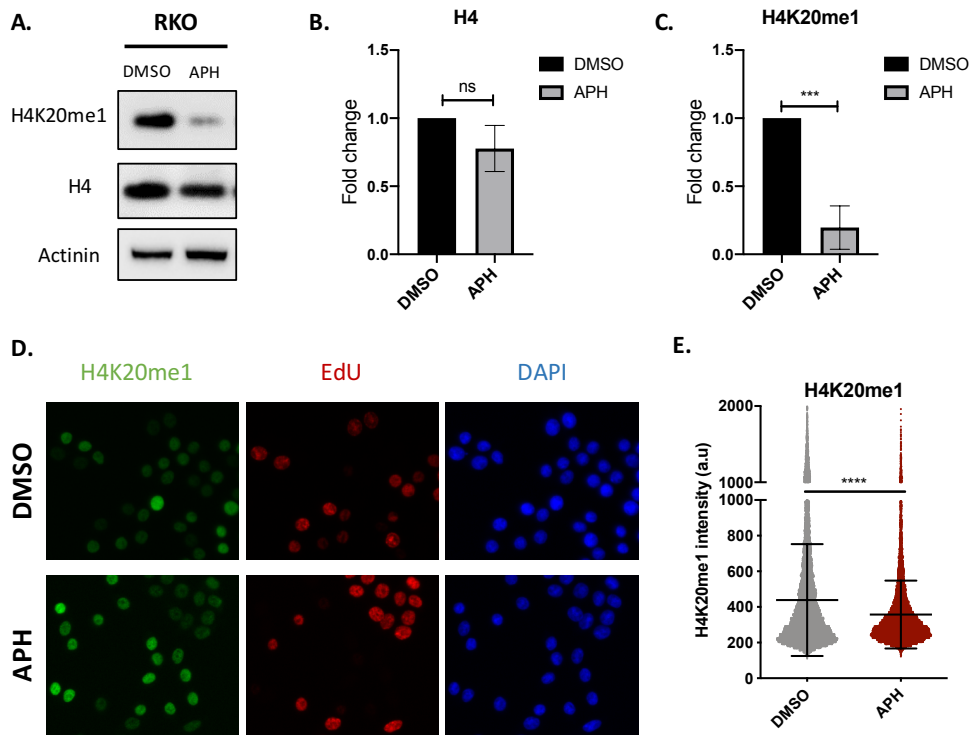
promote timely completion of DNA replication (Berti et al., 2020). To address this question, we can perform a DNA-combing-FISH on several ADV and DEL aRTIL as well as in control regions.

b. DDR, chromatin structures, nuclear organisation and RT modifications

It is now well accepted that replication stress not only target the DNA replication process itself, but also the nucleosome assembly, chromatin structure and to a higher extend, nuclear organisation. Knowing that DNA replication, DNA repair and chromatin remodelling are inter-dependant mechanisms (Demeret et al., 2001; Hauer and Gasser, 2017; Polo and Almouzni, 2015), I will try to discuss about eventual links between the DNA damage response induced by aphidicolin, chromatin structures regulations (TADs, cohesin, RIF1, 53BP1), general nuclear organization (LADs and nuclear compartments) and replication timing alterations described in this study.

i. **The impact of low RS on the nucleosome and chromatin stability**

In the submitted manuscript, thanks to ATAC-seq experiment in RKO cells, we described an increased chromatin compaction (less accessibility) in the surrounding of high accessible genomic regions (**Submitted manuscript, Figure S6c**). This result is quite consistent with the fact that chromatin compaction is known to be increased around fork stalling (Feng et al., 2019; X et al., 2008). On the opposite, we found that low accessible regions have an increased accessibility under aphidicolin treatment.



**FIGURE 34:** IMPACT OF APHIDICOLIN ON HISTONE H4 AND EPIGENETIC MARKS H4K20ME1 IN RKO CELLS. (A) WESTERN BLOT OF WHOLE CELL EXTRACT H4K20ME1 AND H4 HISTONES IN THE DMSO OR APHIDICOLIN (APH) CONDITIONS. (B) HISTOGRAMS OF WESTERN BLOT QUANTIFICATION FOR H4 (FOLD CHANGES). STATISTICS (N=3): UNPAIRED T-TEST, NS WHEN  $p > 0.05$ . (C) HISTOGRAMS OF WESTERN BLOT QUANTIFICATION FOR H4 (FOLD CHANGES). STATISTICS (N=3): UNPAIRED T-TEST, \*\*\* $p < 0.005$ . (D) IMAGES FOR IMMUNOFLUORESCENCE AGAINST H4K20ME1, EdU AND DAPI IN THE DMSO AND APHIDICOLIN CONDITIONS. (E) QUANTIFICATION OF THE H4K20ME1 INTENSITY. STATISTICS (N=1): UNPAIRED T-TEST, \*\*\*\* $p < 0.001$ .

Aphidicolin induces replication fork stalling and can also have an impact on the process of histone recycling (Jasencakova et al., 2010), which is coupled with the replication process. Interestingly, we observed that aphidicolin strongly reduces the amount of histone H4 and its associated modification H4K20me1 (**Figure 34**). This observation may reflect the nucleosome destabilization induced by RS and delays in the epigenome recycling that, however, might be necessary for the increase chromatin mobility to allow the repair of the impacted genomic loci (Krawczyk et al., 2012).

At a broader scale, we described a global effect of the aphidicolin on the chromatin accessibility. This observation was also described in a recent study using CHK1 inhibitor and camptothecin as replication stress agents (Murai et al., 2020) and is also consistent with the numerous reports suggesting that replication stress induce increased chromatin movement to allow DNA repair (Caron and Polo, 2020; Groth et al., 2007; Hauer and Gasser, 2017). Finally, while performing our experiment on the 6 cell lines, we also noticed a global effect of



aphidicolin on the nuclear size of cells (**Figure S10**). We can imagine that a larger nuclear area reflects increased chromatin accessibility and movement necessary for DSB repair and genome stability.

Altogether, our observation and data from the literature suggest that under aphidicolin treatment, histone and epigenetic modification loss can contribute to an increased chromatin mobility together with a loss heterochromatin condensation, that may be useful to relocalize damaged regions (DSB) in order to be properly repaired.

## ii. **The DDR induces TADs and loops reorganization that could trigger RT of specific genomic regions**

Recent advances in sequencing-based methodology designed to investigate chromosome architecture with high resolution helped to tackle and better describe the function of chromatin structure and looping in essential molecular processes such as DNA replication, transcription (Almeida et al., 2018) and even DNA repair processes (Ochs et al., 2019; Raschellà et al., 2017), collectively allowing genome stability. For example, a very recent study revealed the role of TADs in DNA damage repair (DDR), and thus the critical impact of chromatin structure in the maintenance of genome stability (Arnould and Legube, 2020; Arnould et al., 2020). In this work and review, they showed that the establishment of  $\gamma$ -H2AX/53BP1 chromatin domains involved cohesion mediated loop extrusion on both sides of the DSB (**Figure 35**). In addition, chromatin looping has been also linked to the DNA replication timing, by showing that chromatin organization can directly affect the replication origins usage in the subsequent S phase (Blow and Ge, 2008; Courbet et al., 2008b) and chromatin loops anchors have been associated to genome instability in cancer (Kaiser and Semple, 2018). As we observed an increase of chromatin accessibility within APH aRTIL in RKO cells, we can assume that, especially in cancer cells, local reorganisation of chromatin structure induced by RS can lead to a more global chromatin remodelling associated to an increase in chromatin accessibility within heterochromatin regions, an earlier induction of DNA replication and thus RT advances within these specific regions. The large amount of studies describing alterations of the chromatin structure such as relaxation or increased accessibility and of the epigenetic landscape in cancer further support this hypothesis (Achinger-Kawecka et al., 2017; Brock et

al., 2007; Kaur et al.; Luijsterburg and van Attikum, 2011; Morgan and Shilatifard, 2015; Nguyen et al., 2001; Taberlay et al., 2016).

Overall, we can suggest that the direct or indirect effect of aphidicolin on the chromatin accessibility and nuclear shape would facilitate a global chromatin movement and reorganization of TADs leading to RT modifications.

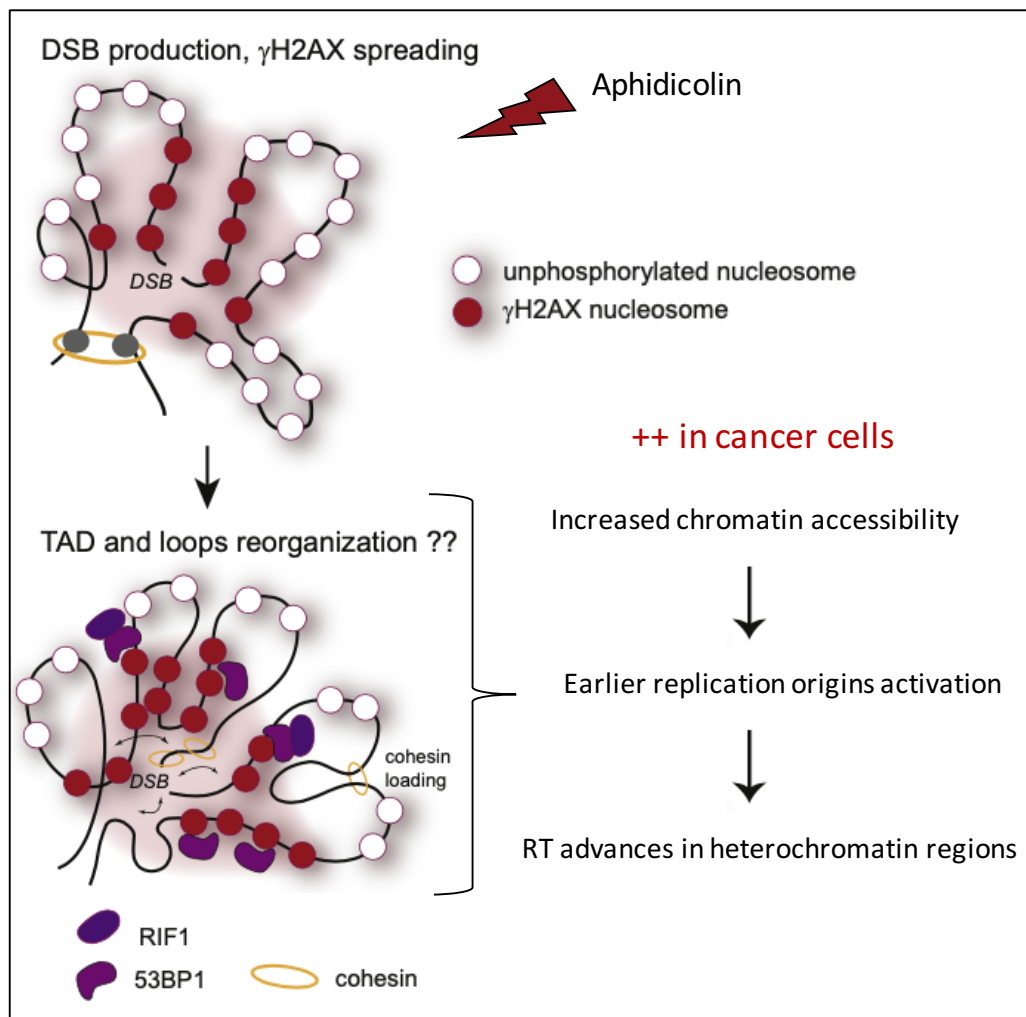


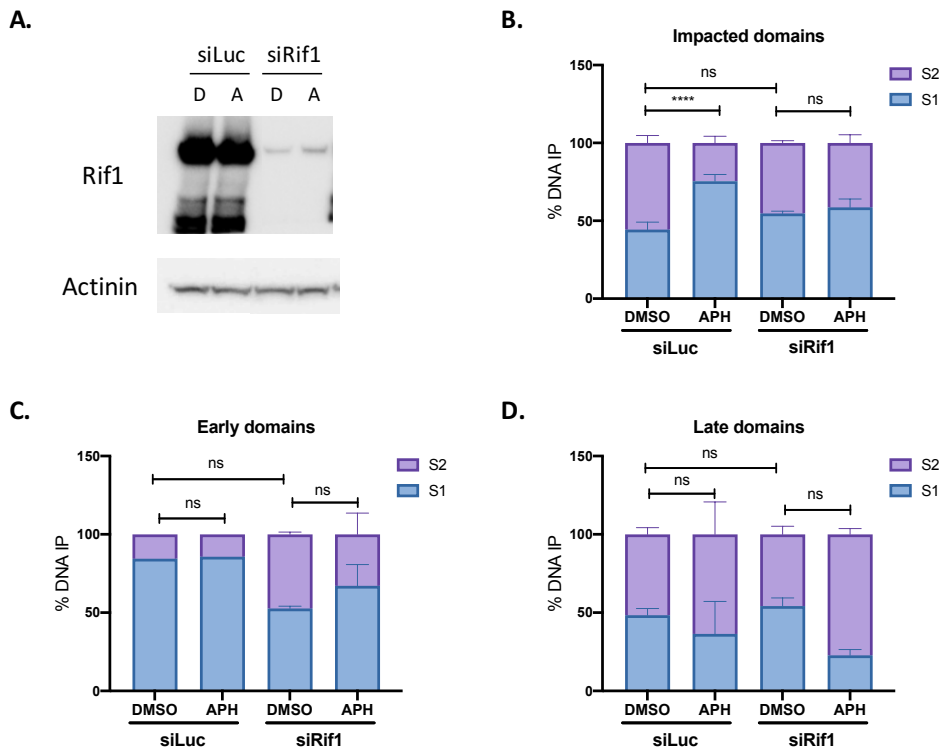
FIGURE 35: A ROLE FOR TADs AND CHROMATIN LOOPS REORGANIZATION IN RT ADVANCES IN CANCER CELLS. DSB INDUCTION LEADS TO LOCAL SPREADING OF  $\gamma$ -H2AX (UP RIGHT) AND MODIFICATIONS OF TADs STRUCTURE DUE TO THE BINDING OF CTCF REGULATING CHROMATIN LOOPS AND REPAIR PROTEIN 53BP1 AND RIF1 THAT MAY PLAY A ROLE IN CHROMATIN ARCHITECTURE (DOWN RIGHT) (OCHS ET AL., 2019). NUCLEOSOME LOSS AND/OR GENERATION OF ssDNA MAY ALSO COLLECTIVELY CHANGE CHROMATIN DYNAMIC WITHIN TADs. ALTOGETHER, ENHANCED CHROMATIN MOBILITY CAN LEAD TO INCREASED CHROMATIN ACCESSIBILITY AND EARLIER ORIGIN ACTIVATION RESULTING IN REPLICATION TIMING ADVANCES WITHIN HETEROCHROMATIN REGIONS. (ADAPTED FROM ARNOULD AND LEGUBE, 2020)

As already discussed in the paper, DEL aRTIL are very sensitive regions to aphidicolin, prone to breakage. We can hypothesize that DEL aRTIL regions are important for the cellular function and thus absolutely need to be repaired. On the opposite, ADV aRTIL contains few, poorly expressed genes and also very few constitutive origin, which make these regions more resistant to aphidicolin. The necessity to relocalize DEL aRTIL to be repaired may be compensated by a movement of ADV aRTIL that harbour very low  $\gamma$ -H2AX signal towards more active genomic regions, leading to an earlier activation of their replication origins (**Figure 35**). To confirm this theory, it would be very nice to perform capture-Hi-C experiment with and without aphidicolin, to first see if indeed we observe modifications in TADs organizations and more specifically if the interactions between the regions of interest (DEL and ADV aRTIL) and the rest of the genome are modified under aphidicolin.

If indeed the hypothesis of a role for chromatin looping in the RT modification is true, we can also imagine that the three key players of TADs structure (cohesin) and chromatin architecture around DSBs (53BP1 and Rif1) may have direct or indirect roles in RT modifications.

### iii. **Rif1 structural and functional role in RT and DDR could explain RT advances**

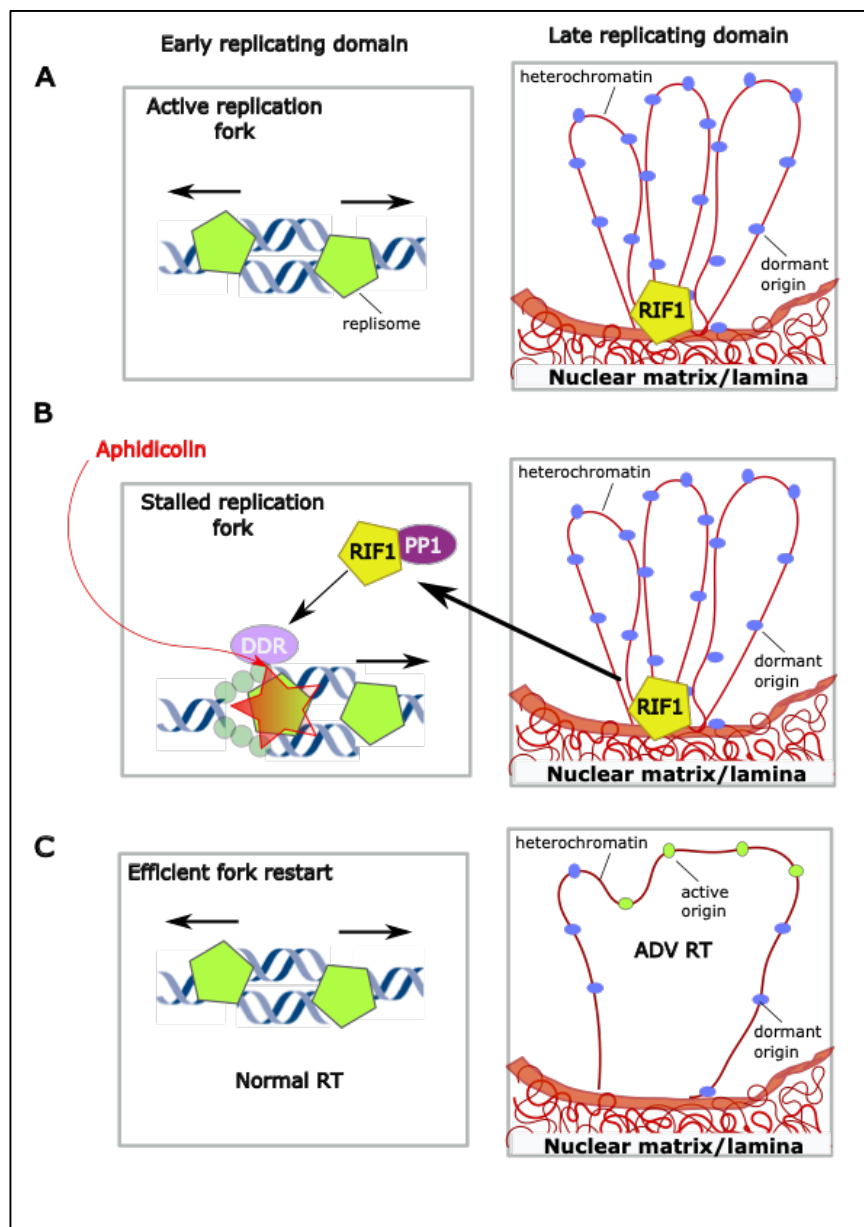
In the literature, it has been described that the replication timing of late replicating regions, especially non-Lamin B1, can be regulated by the Rif protein (Foti et al., 2016). Thus we could imagine that strong RT advances in cancer cells such as RKO, can be linked to the role of Rif1. We have performed the same BrdU-ChiP-qPCR approach, like previously described (**Submitted manuscript, Figure S1d**) in cells depleted for Rif1, with and without aphidicolin treatment. Interestingly, our preliminary data revealed that in absence of the protein Rif1, the impacted domains (ADV aRTIL) under low RS are no longer impacted (**Figure 36**). This observation might suggest a role for Rif1 in the mechanistic of RT advances in RKO cells.



**FIGURE 36:** PRELIMINARY QPCR RESULTS ON RKO CELLS WITH RIF1 EXTINCTION (siRif1). (A) WESTERN BLOT TO VERIFY THE EXTINCTION OF THE RIF1 PROTEIN IN BOTH APHIDICOLIN (A) AND DMSO (D) CONDITION. siLUC IS A CONTROL FOR siRif1 EXTINCTION AND ACTININ IS A LOADING CONTROL. (B) PERCENTAGE OF BRDU-IMMUNO-PRECIPITATED DNA AMPLIFIED IN THE EARLY S FRACTION IN THE DIFFERENT CONDITIONS: WITH OR WITHOUT RS (APH, DMSO) AND WITH OR WITHOUT RIF1 (siLUC, siRif1). HISTOGRAM FOR QPCR-AMPLIFICATION IN THREE (WITH DUPLICATES) ADV ARTIL. (C) HISTOGRAM FOR QPCR-AMPLIFICATION IN TWO EARLY REPLICATED REGIONS. (D) HISTOGRAM FOR QPCR-AMPLIFICATION IN TWO (WITH DUPLICATES) LATE REPLICATED REGIONS. STATISTICS: (BIOLOGICAL N=1, TECHNICAL N>2) 2WAY ANOVA \*\*\*\*p<0.0001, NS WHEN P>0.05.

It was described that, in a context of acute replication stress (HU, 2mM, 4h), Rif1 is recruited to reversed stalled replication forks to protect the DNA and allow replication fork restart (Garzón et al., 2019; Hiraga et al., 2018; Mukherjee et al., 2019; Ribeyre et al., 2020). In normal condition, Rif1 is assumed to localize at late replicating foci (**Figure 37A**). We can imagine that in cancerous cells, as replication stress is already high, the low dose of aphidicolin is sufficient to induce fork stalling and subsequent recruitment of Rif1 to stall/reversed fork (**Figure 37B**). The decrease of Rif1 protein localized at heterochromatin loci may lead to a modification of chromatin anchoring to the nuclear matrix (lamina or other) and to the anticipated activation of replication origins (**Figure 37C**), changing the replication timing of the normally late replicating regions towards earlier replication timing. A ChIP-seq against Rif1 approach will be a good way to prove that Rif1 delocalize from late to early impacted regions

under RS. However, it looks like current Rif1 antibodies are not good enough to perform ChIP-seq as no such experiment has been done yet.



**FIGURE 37:** HYPOTHETIC MECHANISM FOR RT ADV IN LATE HETEROCHROMATIN INVOLVING THE ROLE OF RIF1. (A) IN NORMAL CONTEXT, RIF1 ASSOCIATES TO LATE REPLICATING HETEROCHROMATIN (RIGHT PANEL), PREVENTING REPLICATION ORIGIN ACTIVATION WHEREAS IT IS NOT STRONGLY REPRESENTED AT THE FORK (LEFT PANEL). (B) RIF1 IS RECRUITED TO STALLED REPLICATION FORK IN SOME EARLY REPLICATING REGIONS AFFECTED BY APHIDICOLIN (LEFT PANEL). (C) THE RECRUITMENT OF RIF1 IN ACTIVE FACTORIES MAY LEAD TO A LOSS OF RIF1 IN HETEROCHROMATIN DOMAINS LEADING TO MODIFICATION OF HETEROCHROMATIN INTERACTION WITH LAMINA AND SUBSEQUENT ACTIVATION OF NORMALLY LATE REPLICATING ORIGINS.

As Rif1 is assumed to allow proper replication fork restart, we can imagine that its recruitment to stalled replication fork is efficient enough to not see an impact on RT. This hypothetical model involving Rif1 in RT ADV is mainly for RKO and K562 cells, in which we

observed strong RT ADV in response to low RS. As Rif1 also plays a major role in the NHEJ repair, we can further imagine that in the next RKO G1/S cells, Rif1 is more localized at the damaged DNA (within 53BP1 nuclear bodies for example) and it does not play its role for the TDP, thus leaving the loading and licencing of usually late replication origins. This would explain why we observed reduction of DNA Halo size in daughter cells from Aphidolin treated mother cells and we still observe RT ADV in RKO daughter cells.

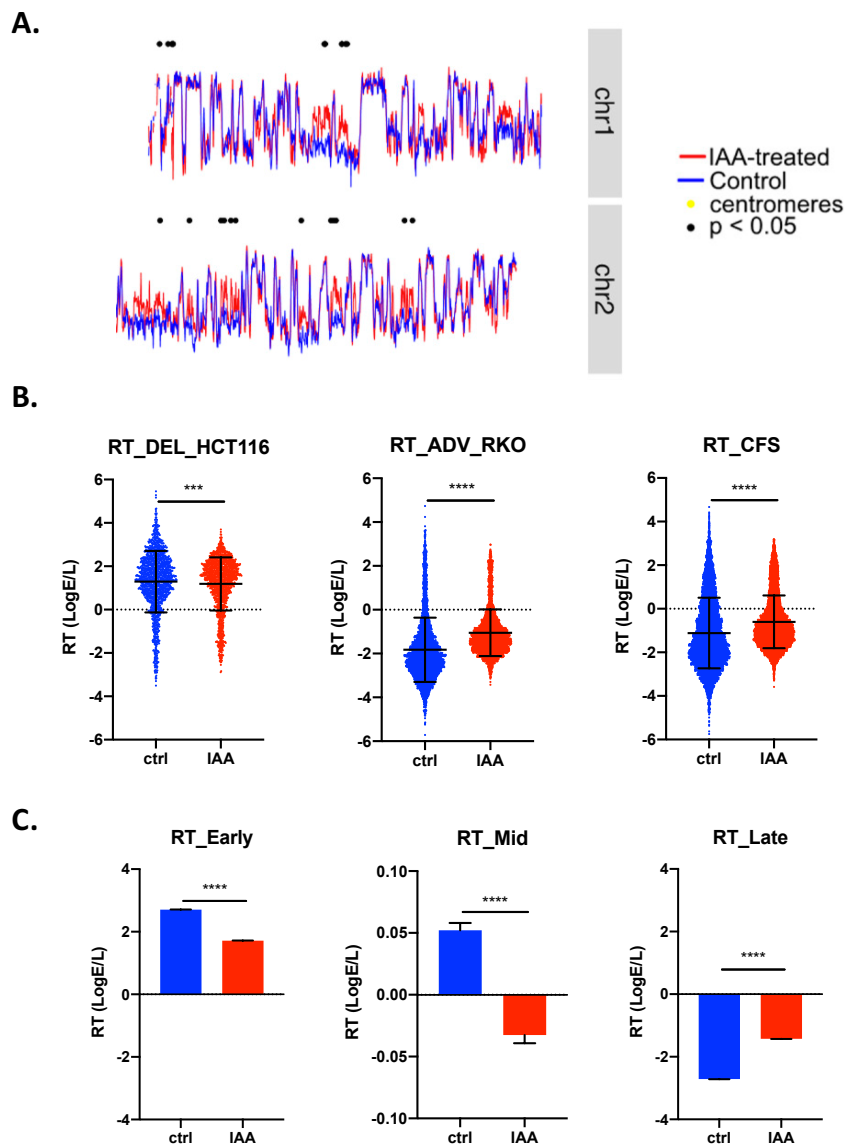
On the other hand, it is known that checkpoint signalling can be deficient in tumour cells. Thus, we can also hypothesize that Rif1 recruitment is not sufficient to maintain normal RT and this would lead to RT delays. This may explain why in Rif1 deficient cells, we observed a tendency for RT delays in early replicating regions (**Figure 36C**) and this might be even more the case for U2OS and HCT116 in which delays RT is the major effect of RS.

#### **iv. Cohesin, TADs and RT modifications**

As mentioned in this thesis introduction, TADs are functional genome units whom boundaries were found to map almost all replication domain boundaries (Pope et al., 2014). In addition, cohesin is enriched at replication origins and TADs boundaries (Guillou et al., 2010) and the link between replication origin, replication timing and DNA loops is further supported by the fact that they are all established concomitantly in G1 (Dileep et al., 2015b; Dimitrova and Gilbert, 1999b). Moreover, an elegant study described that the cohesin loss eliminate all loops domains without strongly impacting the global gene expression but only a down-regulation of genes near super-enhancers (Rao et al., 2017). Thus it would make sense to wonder if cohesin loss would induce local or genome-wide or RT modifications, just like it has been described for the RIF1 structural protein (Aladjem, 2012; Buonomo, 2017; Foti et al., 2016).

A recent study suggested that cohesin-mediated genome architecture is not required for the execution of replication timing patterns in S phase, nor for the establishment of replication timing domains in G1 (Oldach and Nieduszynski, 2019). In this study they performed Repli-seq analysis on HCT116 cells asynchronously growing and treated or not with auxin, that induces acute cohesin loss (Natsume et al., 2016; Rao et al., 2017). Even though they did not observe genome wide replication timing alterations, while reading the paper and watching the figures, we could notice that replication timing profiles in the cohesin loss

condition was similar to the one we observe under aphidicolin treatment, and especially the one in RKO cells.



**FIGURE 38:** THE EFFECT OF COHESIN LOSS ON THE REPLICATION TIMING IN HCT116. (A) ILLUSTRATION OF CHROMOSOME 1 AND 2 REPLICATION TIMING DATA IN HCT116 CELL LINE UNDER CONTROL (BLUE LINE) AND IAA TREATMENT INDUCING COHESIN LOSS (RED LINE). BLACK DOTS ARE HIGHLIGHTING RT SIGNIFICANTLY IMPACTED REGIONS ( $p < 0.05$ ) (FROM OLDACH AND NIEDUSZYNSKI, 2019). (B) COMPARISON OF RT VALUES BETWEEN CONTROL (BLUE, CTRL) AND COHESIN LOSS CONDITION (IAA, RED) IN SPECIFIC REGIONS OF INTEREST (DEL\_ARTIL IN HCT116, ADV ARTIL IN RKO AND CFS). (C) COMPARISON OF RT VALUES BETWEEN CONTROL (BLUE, CTRL) AND COHESIN LOSS CONDITION (IAA, RED) IN EARLY, MID AND LATE REPLICATING REGIONS. STATISTICS: WILCOXON MATCHED PAIRED-SIDE RANK TEST \*\*\*\* $p < 0.0001$ , \*\*\* $p < 0.0005$ .

Indeed, we noticed some switches of late replicating regions towards earlier replication timing in many examples (**Figure 38A**). This observation was intriguing and I thus decided to further analyse these Repli-seq data. To do so, we quantified the RT differences (based on loess-smoothed data) between the control (ctrl) and cohesin loss conditions (IAA) in different genomic regions: DEL aRTIL in HCT116, ADV aRTIL in RKO, CFS (**Figure 38B**) and Early, Mid, Late as control regions (**Figure 38C**). First, we observed that DEL aRTIL in HCT116 tends to be also delayed when cohesin is lost while ADV aRTIL in RKO tends toward RT advances without cohesin (**Figure 38B**).

It was recently reported that the core fragility of CFS may be within TAD boundaries in which DNA replication and repair are improper (Sarni et al., 2020). In this line, the analysis of the RT of CFS (Listed in Brison et al., 2019) in normal or IAA conditions allowed us to observe that the RT of regions tend to be advanced in the absence of cohesin. Thus, cohesin may be an important player in the maintenance of the RT of specific genomic regions. Finally, we also wanted to analyse the impact of cohesin loss at a more global level. Interestingly, cohesin loss has a similar impact than RIF1 on RT: early/mid regions are replicated later while late regions are replicated earlier (**Figure 38C**).

Overall, these observations further support the hypothesis that aphidicolin leads to TADs disruption and thus to RT modifications. Even though it is true that cohesin loss does not strongly disturb the RT program, it is also reasonable to suggest that cohesin may be important to maintain the RT of specific genomic regions (i.e: ADV and DEL aRTIL).

#### **v. LADs and ADV aRTIL**

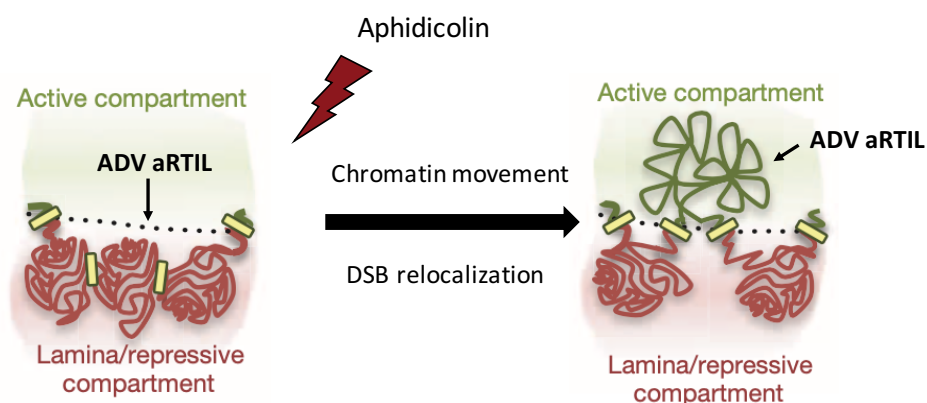
Lamina-associated domains, or LADs, has been described to be reorganized during the process of cellular differentiation in mouse and are involves in the control of gene expression program from lineage commitment to terminal differentiation (Peric-Hupkes et al., 2010). Moreover, alterations of the nuclear lamina are currently emerging as an additional event promoting malignant transformation. The involvement of Lamins in cancer has been principally related to their role in basic nuclear activities such as DNA replication and gene expression (Alvarado-Kristensson and Rosselló, 2019; Irianto et al., 2016; Jia et al., 2019; Lochs et al., 2019). Interestingly, it has been shown that depletion of Lamin A induces an increase of chromatin mobility within the nuclear interior, suggesting that this protein plays a critical role



in the maintenance of genome organization (Bronshtein et al., 2015). A recent study in *Drosophila* further support this assumption by showing that upon NL disruption, LADs become more acetylated and less compact while background transcription is getting activated (Ulianov et al., 2019).

It was recently reported that proximity to NL is correlated with a more controlled late replication (Duriez et al., 2019). As we described that ADV aRTIL are heterochromatin domains enriched in H3K9me3 and LaminB1, we can wonder if, under aphidicolin treatment, that induces DDR, modification of chromatin accessibility may abrogate the interaction between ADV aRTIL and NL which would lead to a subsequent loss of their repression and earlier activation of replication origins within these regions.

As in RKO cells we observe a strong amplitude in the RT modifications and notice a very low expression of LaminA/C in these cells (**Submitted manuscript, Figure S8**), we may further suggest that ADV aRTIL are already poorly interacting with NL in the untreated cells and that aphidicolin treatment completely abolish this low interaction, inducing a shifting of these specific regions from the inactive compartment B to the active compartment A (**Figure 39**). Again, a 3D nuclear structure approach could allow us to confirm this suggestion.



**FIGURE 39:** SCHEME REPRESENTING THE POSSIBLE COMPARTMENT SHIFTING TO EXPLAIN ADV ARTIL IN RKO CELLS. IN NORMAL CONDITION, ADV ARTIL ARE LATE REPLICATING HETEROCHROMATIN/LADs REGIONS (LEFT PANEL). THE APHIDICOLIN TREATMENT, INDUCING DDR REPAIR MECHANISM AND CHROMATIN MOVEMENT, MAY ALSO LEAD TO A SHIFT OF ADV ARTIL FROM INACTIVE/REPRESSIVE COMPARTMENT B (IN RED) TOWARDS ACTIVE COMPARTMENT A (IN GREEN), THUS INDUCING EARLIER REPLICATION OF THESE LOCI.

## A role for epigenetic in the transmission of RT alterations in RKO cells?

Epigenetic modifications are maintained from cell to cell and are known to be the “memory” of cellular identity. The histone deposition and their modifications should be sufficiently stable during interphase to ensure that any epigenetic information carried by the genome is not lost before the next round of replication. As previously mentioned, epigenetic and replication timing program are both closely related and hard to dissociate. In RKO cells, in which we observed a strong impact of aphidicolin on replication timing, we also noticed a global loss of the core histone H4 and epigenetic modification H4K20me1 (**Figure 34**) together with an increase of chromatin accessibility within heterochromatin regions. Interestingly, recent studies reported a role of histone H4K20 methylations and demethylase of the H3K9me3 repressive mark in origin licensing and/or in the regulation of replication timing (Peric-Hupkes et al., 2010). Shoaib et al. observed that, in the absence of H4K20 methylation, there is a genome-wide chromatin decompaction allowing an excessive increase of origin recognition complex (ORC) in G1 cells followed by aberrant origin licensing (Shoaib et al., 2018). Brustel et al. and Wu et al. identified demethylation of H4K20me3 and H3K9me3 as facilitators of pre-initiative complex (pre-IC) formation in late-replicating heterochromatin (Brustel et al., 2017; Wu et al., 2017). Thus, we can speculate that in RKO cells, the epigenetic landscape of ADV aRTIL is strongly disturbed in the S phase of mother cells, not well restored before mitotic entry, leading to aberrant/earlier origin activation within heterochromatic regions of daughter cells.

## What is the biological relevance of these RT modifications?

- a. What is the impact of aRTIL on genomic stability and cell resistance?

It is very likely that strong replication timing delays lead to un-replicated DNA that, if not resolved, breaks during the mitosis creating the phenomenon of DNA damage transmission (Harrigan et al., 2011; Lukas et al., 2011). This is the case for the well-described genomic loci called CFS. As CFS are specifically expressed in tumour cells, they have been widely studied and many correlations between CFS and genomic instability were made. Thus, it makes sense to consider that DEL aRTIL described in our study may also contribute to the appearance of genomic instability in normal cells and its increase in cancer cells.

On the opposite, as the replication timing advances induced by replication stress have never been described before, we completely ignore their impact on genomic stability. It is tempting to speculate that ADV aRTIL would be there to counteract the deleterious effect of DEL aRTIL, and thus would confer a better resistance against genomic instability induced by RS. A first observation supporting this theory is that, in RKO cells, only 10 genomic regions (corresponding to 0.38% of the genome) were significantly delayed under aphidicolin treatment (**Submitted manuscript, Figure 2a,b**).

In order to explore this hypothesis, as a first approach, we decided to measure  $\gamma$ -H2AX intensity in the S phase of daughter cells under a second aphidicolin treatment (t<sub>2</sub>), directly originating from aphidicolin-treated mother cells (t<sub>1</sub>) (**Figure 40A**). In order to be able to compare the effect of DEL or ADV aRTIL on replication stress resistance, we performed the experiment in RPE-1 and RKO cells, as we described that RT replication signature of normal RPE-1 cells was characterized by a majority of DEL aRTIL (38 DEL, 9 ADV) whereas RT signature of RKO cells was associated to a majority of ADV aRTIL (10 DEL, 49 ADV). As expected, RS signal response was stronger in cancer than normal cells (**Figure 40C and D**) (FC  $\approx$  2 in RKO, FC < 1.5 in RPE-1). Quite surprisingly, RKO daughter cells originating from mother treated cells were significantly less sensitive to the second aphidicolin treatment (**Figure 40B and C**).

Furthermore, this effect was not observed in RPE-1 daughter cells originating from mother treated (Figure 40D). This approach provides first evidences that the ADV aRTIL signature may be a real adaptive mechanism to confer cellular resistance to RS.

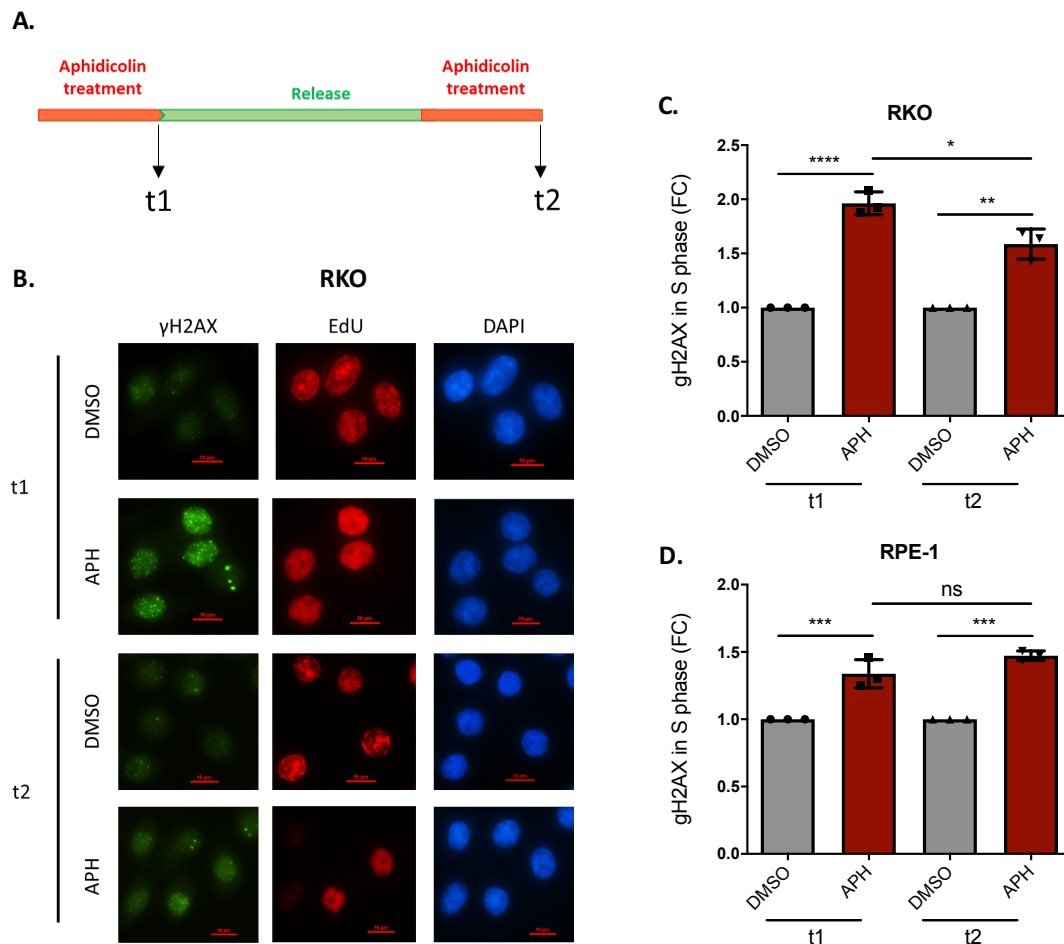


FIGURE 40: THE IMPACT OF A SECOND APHIDICOLIN TREATMENT ON RKO AND RPE-1 CELLS. (A) SCHEME OF THE EXPERIMENTAL PROTOCOL. CELLS WERE TREATED OR NOT WITH APHIDICOLIN (0.2 $\mu$ M, 15H) AND  $\gamma$ -H2AX SIGNAL IN S PHASE WAS MEASURED BY QIBC AT THIS TIME POINT (T1). OTHER CELLS WERE RELEASED FROM THE TREATMENT IN A FRESH MEDIA FOR 12H AND TREATED A SECOND TIME WITH OR WITHOUT APHIDICOLIN (SAME DOSE AND TIME) AND  $\gamma$ -H2AX SIGNAL IN S PHASE WAS MEASURED BY QIBC AT THIS TIME POINT (T2). (B) IMAGES OF IMMUNOFLUORESCENCE FOR  $\gamma$ -H2AX, EDU AND DAPI ARE REPRESENTED FOR EACH CONDITIONS IN RKO CELLS (RPE-1 IMAGES IN FIGURE S11). (C) HISTOGRAM REPRESENTING FOLD CHANGE (FC) OF  $\gamma$ -H2AX INTENSITIES (A.U) IN S PHASE RKO CELLS IN THE FOUR CONDITIONS. (D) HISTOGRAM REPRESENTING FOLD CHANGE (FC) OF  $\gamma$ -H2AX INTENSITIES (A.U) IN S PHASE RPE-1 CELLS IN THE FOUR CONDITIONS. STATISTICS (N=3): 2WAY ANOVA \*\*\*\* $p$ <0.0001, \*\*\* $p$ <0.005, \*\* $p$ <0.01, \* $p$ <0.05, NS WHEN  $p$ >0.05.

Additional experiments need to be done to really demonstrate that ADV aRTIL are protective against genomic instability and this could be one of the perspective of my thesis project. As example, to directly correlate RT modifications (ADV or DEL) with the impact on

the stability of the genomic loci, we can consider doing metaphase chromosome spread combined to locus specific-FISH on previously identified aRTIL and control regions such as famous CFSs and stable regions, and associate the percentage of breakage with the type of RT modification under RS.

On the other hand, even though we do not find increased breakage within ADV aRTIL, we still cannot exclude a deleterious effect of ADV aRTIL. Indeed, it has been described that the replication timing of a given genomic regions would dictate its type of genomic alterations so it is not because their ADV aRTIL are replicated earlier, that these regions are necessarily protected against chromosome rearrangement (Du et al., 2019). To further explore the direct link between RT alterations and genomic instability we could directly assess the correlation between ADV aRTIL and CNAs at the single-cell level, just like it was done in a very recent unpublished study (Shaikh et al., 2019).

#### b. Chronic low RS may induce oncogene activation, EMT and metastasis in tumour cells

This thesis project focused on the direct effect of low RS on the RT, gene expression and chromatin accessibility and its impact on the next cellular generation, that is not triggered by RS. However, an interesting question remains: what would be the impact of a chronic replication stress? It is very likely that during chemotherapeutic treatments, many generations of cells are treated before being released. We can thus wonder to which extent these RT alterations are reversible. Interestingly, some preliminary data from our lab (in RKO and U2OS cells) suggest that, upon 72h of aphidicolin treatment, there a stronger impact on previously described aRTIL (DEL aRTIL and ADV aRTIL), but also an appearance of new aRTIL. Finally, we noticed that the longer aphidicolin treatment is, the more likely RT alterations are persistent in the next cellular generation (even for U2OS cells) (data not shown).

Thus, as it seems like chronic RS could strongly impact the replication timing and as in RKO cells we observed that ADV aRTIL were maintained in daughter cells and, more noteworthy, genes contained within ADV aRTIL tended to be up-regulated, we wondered if these genes are involved in specific cellular pathways. By doing a gene ontology (GO) on aRTIL genes in RKO cells, we found strong enrichments for genes within ADV aRTIL ( $FDR < 5.10^{-4}$ ) with high representation of cell-to-cell adhesion, synapse activation and protein assembly

pathways (**Figure S12**). In the metastatic process, there is a disturbance of cell-to-cell adhesions (mainly in cadherin pathway) as well as cell interaction with extracellular matrix, allowing tumour cells migration and angiogenesis (Friedl and Gilmour, 2009; Janiszewska et al., 2020; Jolly et al., 2019; Yu et al., 2019). Moreover, interactions between cancer cells and cancer-associated fibroblasts (CAFs), that is essential of successful metastasis, is mediated by an accumulation of synapse-like structures at the membrane surface allowing not only a strong adhesion but also a high communication between these cells (Alekseenko et al., 2020). Indeed, synapses are stable adhesive structures between two neighbouring cells playing an important role in information processing and in cell-to-cell communication (Baluška and Mancuso, 2014). Thus, the overexpression of ADV aRTIL genes could drive tumour progression and metastasis.

In our project, we generated microarrays data in RKO, HCT116 and RPE-1 cells treated or not with aphidicolin and did not find a large amount of genes impacted by aphidicolin. In addition to the fold change analysis, we performed a gene set enrichment analysis (GSEA) between DMSO and aphidicolin condition, using the HALLMARK GSEA gene set (Liberzon et al., 2015). For the 3 cell lines, the KRAS signalling pathway was significantly enriched in the aphidicolin condition compared to DMSO (**Figure S13**). In addition, we noticed that the two cancer cell lines present more differences between DMSO and aphidicolin condition than RPE-1, with 13 gene sets significantly enriched in HCT116 and RKO (FDR < 25%) against 5 in RPE-1. Moreover, among the significantly enriched gene sets in cancer cells we found the IL6\_JAK\_STAT3 and IL2\_STAT5 signalling pathways, the inflammatory response and the epithelial mesenchymal transition (EMT) (**Figure 41 and Figure S13**). Then, if we imagine that cells are treated during a longer time with RS inducing agent, just like during chemotherapy, it is very likely that these tendencies will be exacerbated, leading to a true activation of these cellular pathways and finally ending up with cells harbouring a strong metastatic potential.

Overall, through the analysis of genes within ADV aRTIL impacted and by a global overview of transcriptional landscape in cells stimulated by aphidicolin, we could observe a tendency towards specific activation of important pathways related to metastasis in cancer cells. Thus, these observations raise the eventual role of chemotherapy in the tumour progression and the setup of optimal environment driving metastatic process.

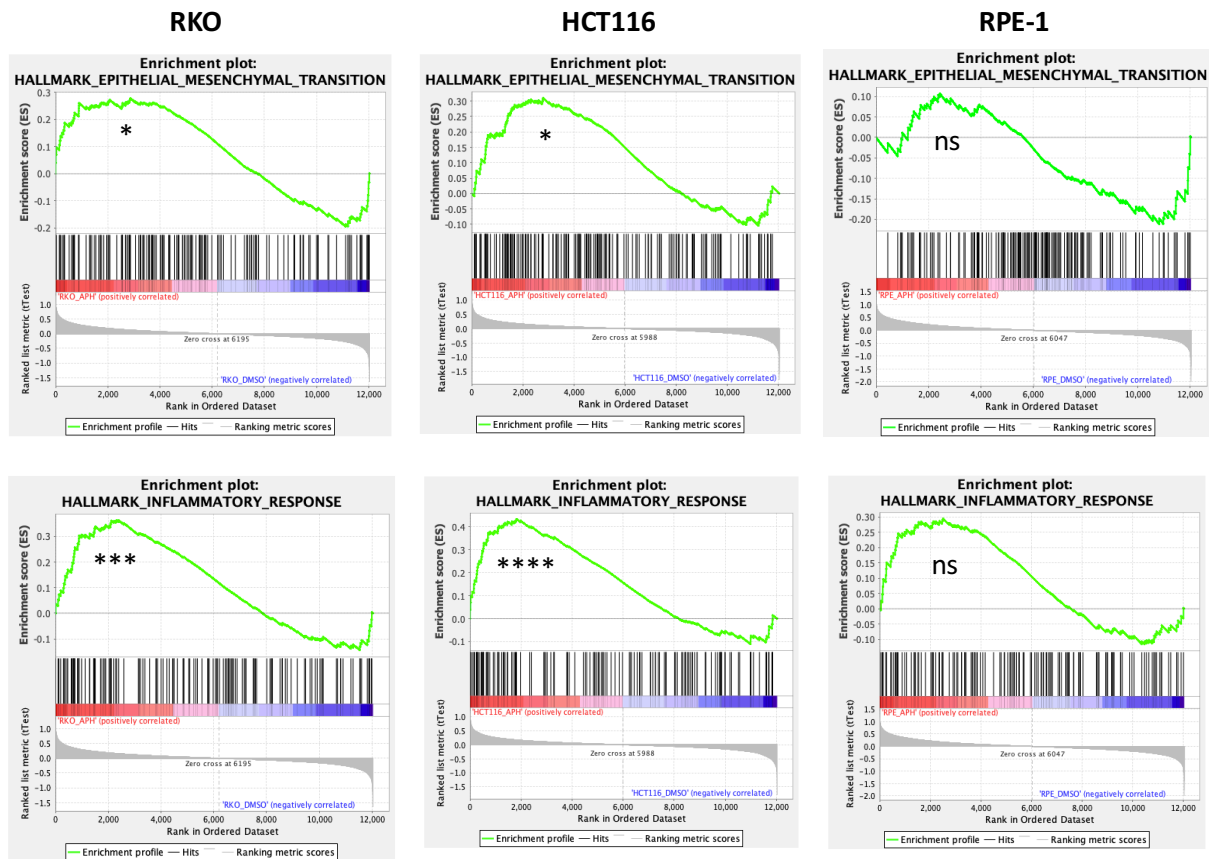


FIGURE 41: GSEA WITH THE HALLMARK DATA SET BETWEEN DMSO AND APH CONDITIONS IN RKO, HCT116 AND RPE-1. SCREENSHOT OF GSEA ENRICHMENT PLOTS FOR EMT AND INFLAMMATORY RESPONSE HALLMARKS IN THE THREE ANALYSED CELL LINES. STATISTICS: NOM P-VAL \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.1$ , NS WHEN  $p > 0.1$ .

An emerging picture in cancer biology is the paradoxical effect of chemotherapy that, while killing the majority of tumour cells, can actively induce the release of inflammatory factors that drives an adaptive response of residual cells towards metastasis (D’Alterio et al., 2020; Karagiannis et al., 2019; Keklikoglou et al., 2019; Middleton et al., 2018; Vyas et al., 2014). A nice recent review points out the fact that “if genetic damage is the match that lights the fire of cancer, some types of inflammation may provide the fuel that feeds de flames” (Güç and Pollard, 2019). In our context, it looks like a low dose of aphidicolin compound, that can be assimilated to chemotherapeutic agent, is sufficient to directly induce an increase in cell-to-cell communication, inflammatory and EMT.

Thus, we can suggest that the combinatory effects of low RS on global gene expression together with its impact on the replication timing of specific genes in RKO cells, may lead to cancer cells adaptation and metastatic capacities. To support this hypothesis, we could imagine an experiment in which we would compare the metastatic by injecting RKO and HCT116 cells in mice, with and without aphidicolin treatment, and see if there are differences

between the two cell lines. This type of experiment could also be carry out by in vitro approach with 3D multicellular tumour spheroids (Pouliot et al., 2013).

c. RT signature: a biomarker for cancer cell plasticity and aggressiveness?

Thanks to a clustering approach with the 6 cell lines RT data in DMSO and aphidicolin conditions, we were able to discriminate cancer to normal cells. This observation was published recently by another team (Du et al., 2019), where they also demonstrated that some RT alterations were conserved in many cancerous cell lines. Finally, two complementary studies from the same research team working on acute lymphoblastic leukaemia revealed clinically relevant genomic domains in which replication timing is specifically altered (Rivera-Mulia et al., 2019; Sasaki et al., 2017). Together with these other studies, our observations suggest that RT profiles can be considered as a signature or a biomarker of cancer cell.

While taking a closer look to the cluster of cancer cells, we distinguished two RT signatures in response to RS. The first one for U2OS and HCT116, associated to RT delays and probably to a more deleterious effect of aphidicolin on genomic stability and the second RT signature with RT advances that we proposed to be associated to cellular plasticity and adaptation to RS. Knowing these two RT signatures, we could imagine that a chemotherapy targeting RS would be more efficient in U2OS and HCT116 cells while it would not be efficient in RKO or K562 and even drive potential aggressiveness to these cells. Thus it would be now very important to discover proteins and/or mechanisms under genomic plasticity, and potential mutation or overexpression/extinction that would bring new biomarker and thus develop more appropriate therapeutic approach depending on the genomic plasticity of the tumour cells.

As we hypothesize that LaminA/C could be involved in RT advances and genome plasticity, we wondered if the expression level of this protein could be associated to good or bad prognosis in cancer. Interestingly, we found that the low level of LaminA/C is associated to a poor prognosis in many cancers (Belt et al., 2011; Bronshtein et al., 2015; Willis et al., 2008; Wu et al., 2009). Finally, as it is known that LaminA/C is very poorly expressed in non-differentiated cells we could also suggest that the cell differentiation state of cancer would predict the RT advances in response to RS.



## Conclusion

To conclude, this thesis aimed to explore the direct impact of low replication stress on the replication program of human normal and cancer cells and its consequence on the next cellular generations. Asking this question, we end up with new discoveries together with already known effect of low replication stress on the replication timing.

A first global approach to address the replication stress response on the 6 human cell lines lead to the conclusion that low replication stress has a stronger impact on cell cycle accumulation, intra-S phase checkpoint and DNA replication timing of cancer cells compared to normal cells. By a clustering approach on RT data, we discovered that cancer cells cluster together regardless of their tissue of origin which brought to light the important fact that the RT of a given cell is sufficient to determine its tumorigenicity.

The analysis of RT differences in the 6 cell lines led to the identification of specific genomic regions in which the RT was delayed under aphidicolin (DEL aRTIL). Interestingly, some of these regions were common between at least two cell lines, while other were not and sometime these regions also correlate with previously identified CFS, which is in line with recently published studies (Brison et al., 2019b; Sarni et al., 2020). Putting together our different analysis to characterize DEL aRTIL, we can conclude that these regions are fragile in the way that they have many genes, including large genes, that tends to be more expressed under aphidicolin, which justify well their high susceptibility to DNA damage (shown by  $\gamma$ -H2AX ChIP-seq data) and the RT delays.

The major discovery of this project was the fact that, mainly in cancer cells, replication timing can be advanced in response to low replication stress (ADV aRTIL) and that these replication timing advances can be transmitted to the next cellular generation. Genomic and epigenomic analysis of ADV aRTIL allowed us to show that they correspond to heterochromatin regions, very poor in gene and constitutive replication origin. Interestingly, we were also able to correlate these ADV aRTIL with some already identified CFS, suggesting that the RT of these fragile regions is not always delayed under replication stress, the reverse is the case. Thanks to ATAC-seq experiment, we could correlate RT advances with higher chromatin accessibility. Finally, our RNA-ChIP data led to the conclusion that the expression of genes within ADV aRTIL is not directly impacted (in mother cells) but when transmitted, ADV aRTIL genes tended to be up-regulated.

Finally, putting all our result together, and regarding to the literature, we can say that our study is a new way to demonstrate the stem cells like phenotype of cancer cells. Indeed, just like it has been described in non-differentiated cells, the chromatin has a higher flexibility allowing quick replication timing modifications in response to any kind of stress that presumably increase variability within cancer cells, leading to selective pressures and resilience to a changing and hostile environment. In other words, to resist to a high endogenous level of replication stress and/or to the exogenous stress from chemotherapeutic agents, cancer cells tend to de-differentiate in order to be more plastic at the level of chromatin and replication timing allowing a faster and better adaptation to any kind of stress.

# Perspectives

As mentioned in the discussion, two points remain elusive in this project. The real mechanistic of replication timing advances and the biological relevance of RT modifications.

To go further in this project, I will first go deeper in the RT experiments with RIF1 and cohesin depleted cells to eventually prove that these proteins are potential actors of RT advances and/or delays under low replication stress. As we strongly suggest that the 3D chromatin structure might play an important role in the RT modifications, it would also be important to do Hi-C experiments with and without aphidicolin and also we want to run already existing 3D networks (from Pol2 ChiA-Pet, CTCF ChiA-Pet and Hi-ChOP) on HCT116 and K562 to eventually show that ADV and DEL aRTIL regions are interacting or not in 3D and/or if these regions are link to promoter/enhancer/polycomb regions that may justify they particular phenotype under RS.

An important point that we did not discuss was the limits of the RT approach we used for our analysis. In fact, as we are working on the RT of bulk cells, we only see a global and cannot know if RT modifications are indeed observed un all cells or if it results from the heterogeneous response within the population. In other words, we cannot exclude the possibility that RT modifications are only occurring in a subpopulation of cells, and that the amplitude of the effect is directly linked to the amount of impacted cells. To elucidate this question, we should perform RT analysis on single-cells and compare to bulk cell population to see if we can generalize the effect or if it is due to a heterogeneous response.

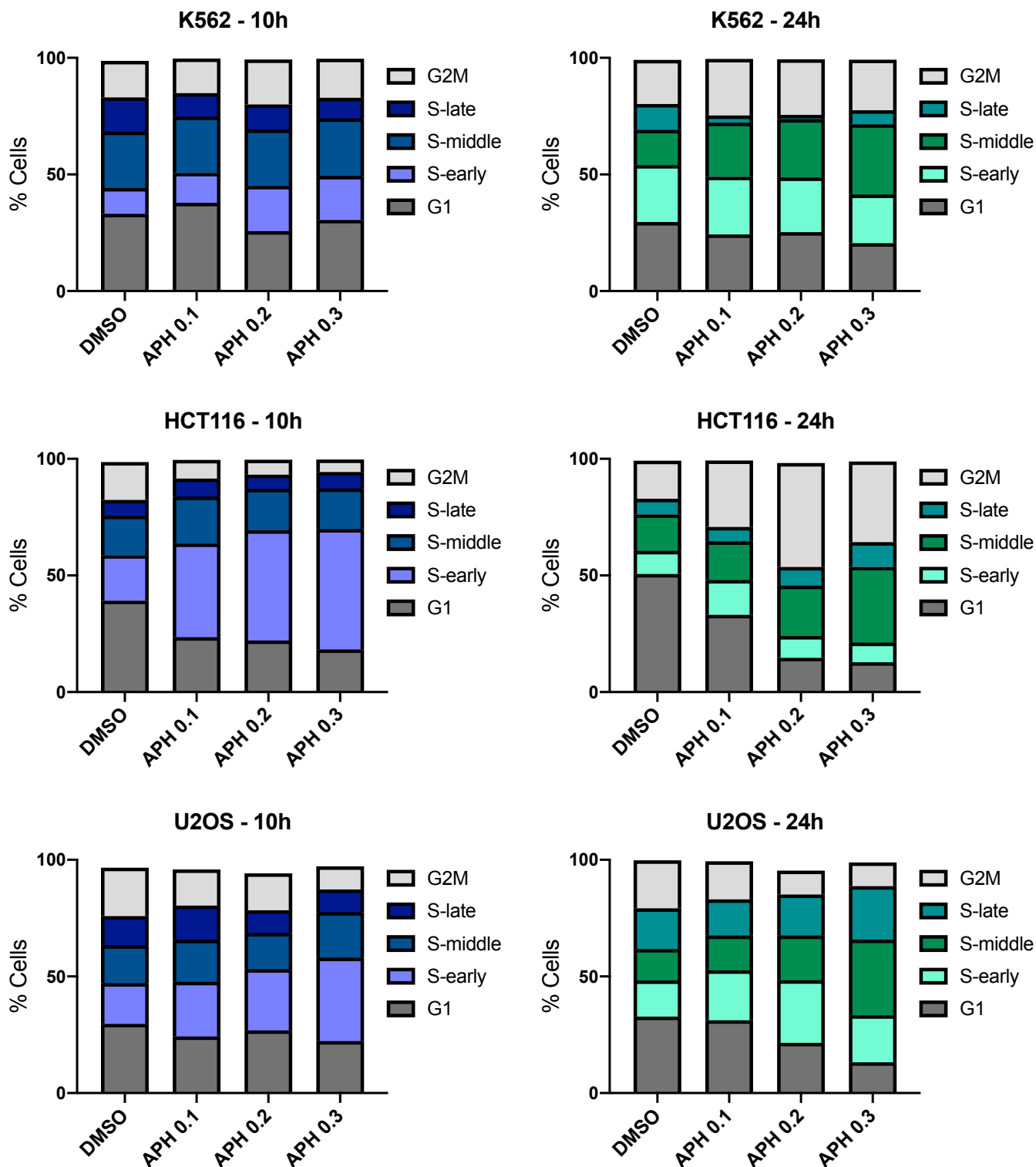
Another limit of our approach is the fact that we only used the aphidicolin drug to induce low replication stress and thus we cannot exclude the fact that the impact we observed on the 6 cell line is aphidicolin-dependant. It would thus be very interesting to test the effect of other genotoxic drugs like HU or cisplatin with the very same approach.

Finally, as mentioned in the discussion, this project only aimed to analyse the direct response of cells to a short replication stress. However, as we observed that this short stress is already sufficient to lead to RT modifications that are inheritable, it would be relevant to now address the effect of chronic replication stress on DNA replication timing of cells, to the if there would be a “non-return point” for RT modifications or if the RT will continuously and randomly change from cell-to-cell generations.

## Supplemental Figures

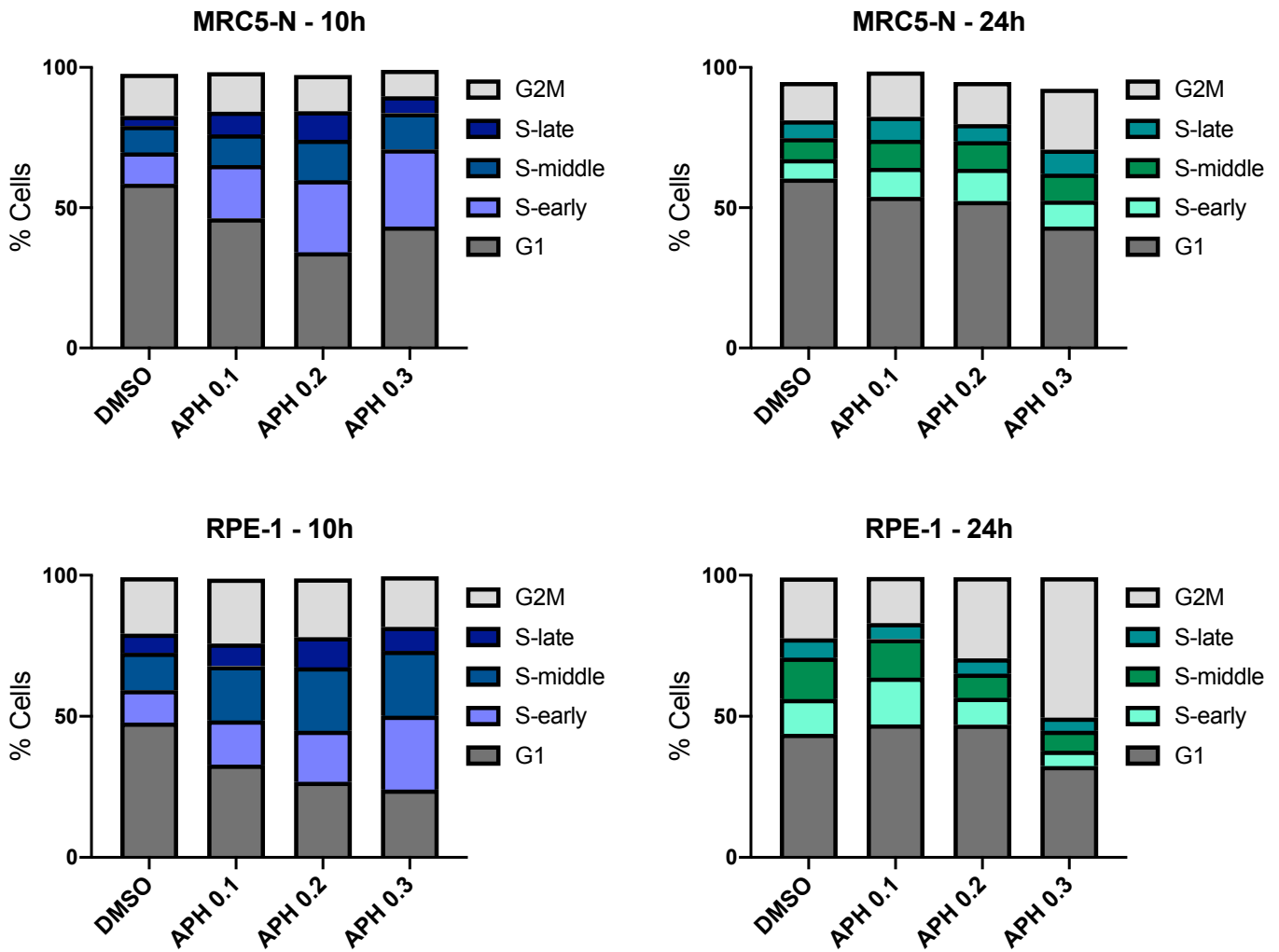
# RESULTS

**Figure S1:** Histograms representing the percentage of K562, HCT116 and U2OS cancer cell lines in the different phases of the cell cycle. Respectively treatments with 10h or 24h, G1 cells are in dark grey, S-early in purple-light green, S-middle in blue-green, S-late in dark blue and G2M in light grey.

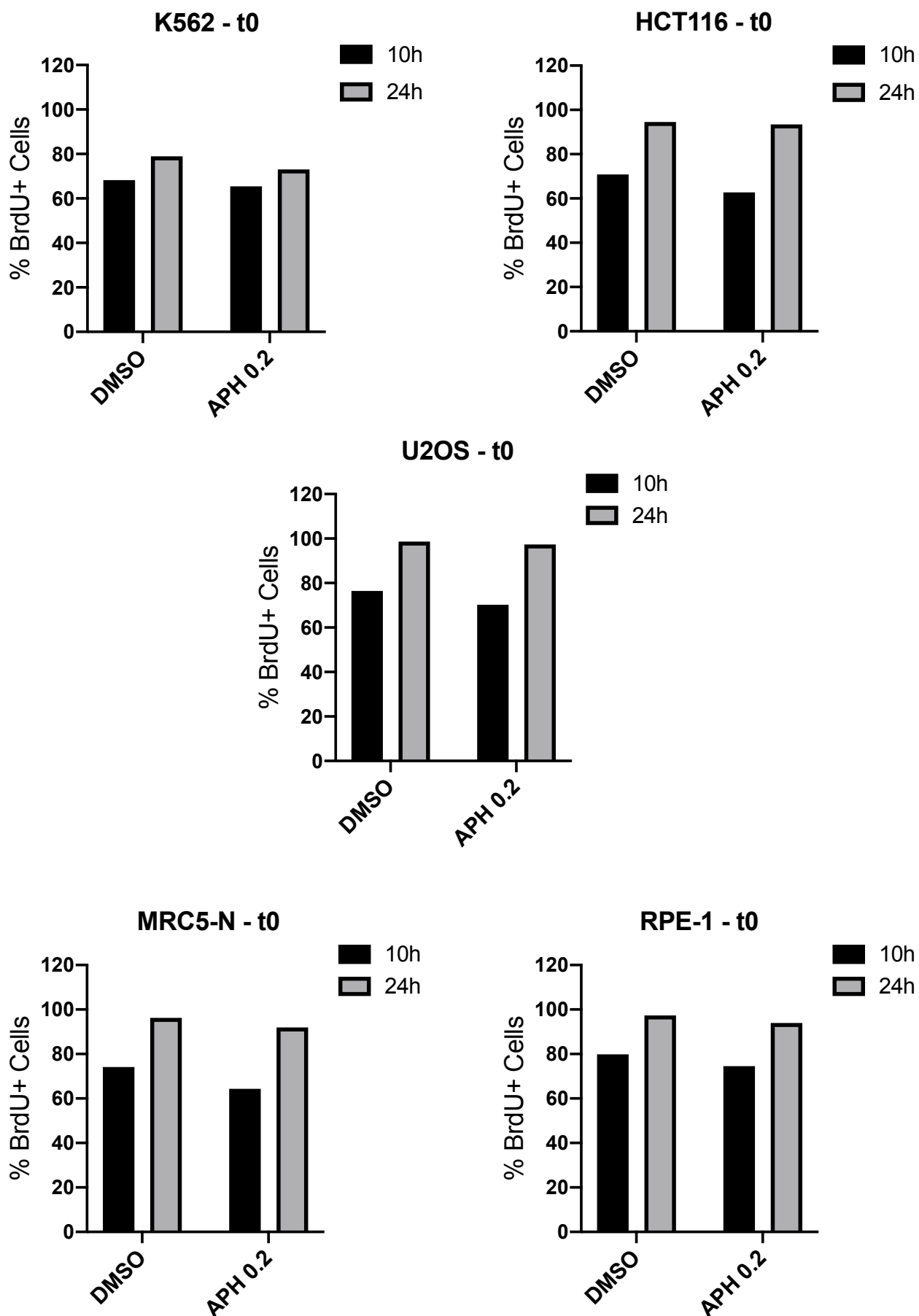




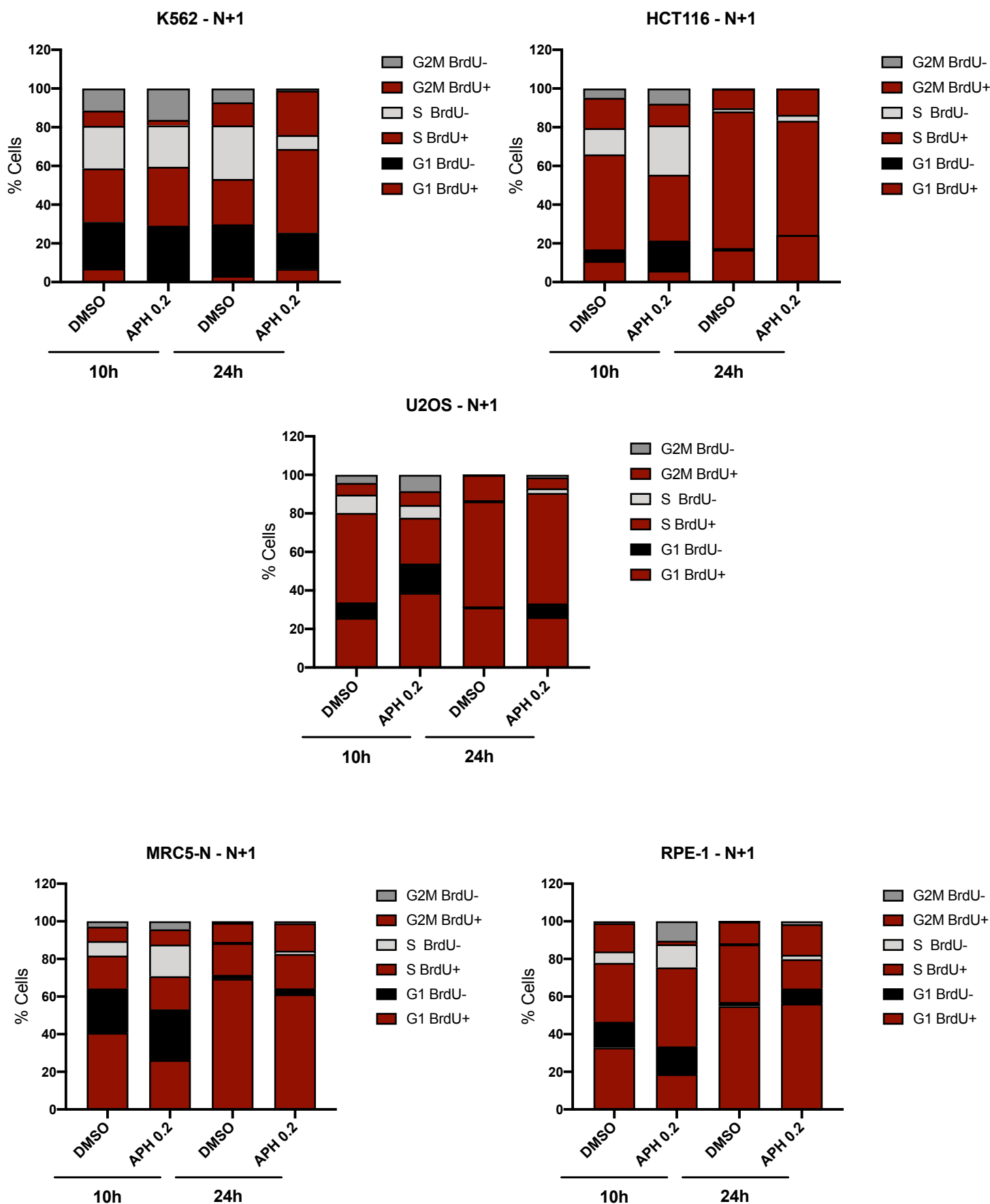
**Figure S1-bis:** Histograms representing the percentage of MRC5-N, and RPE-1 normal cell lines in the different phases of the cell cycle. Respectively treatments with 10h or 24h, G1 cells are in dark grey, S-early in purple-light green, S-middle in blue-green, S-late in dark blue and G2M in light grey.



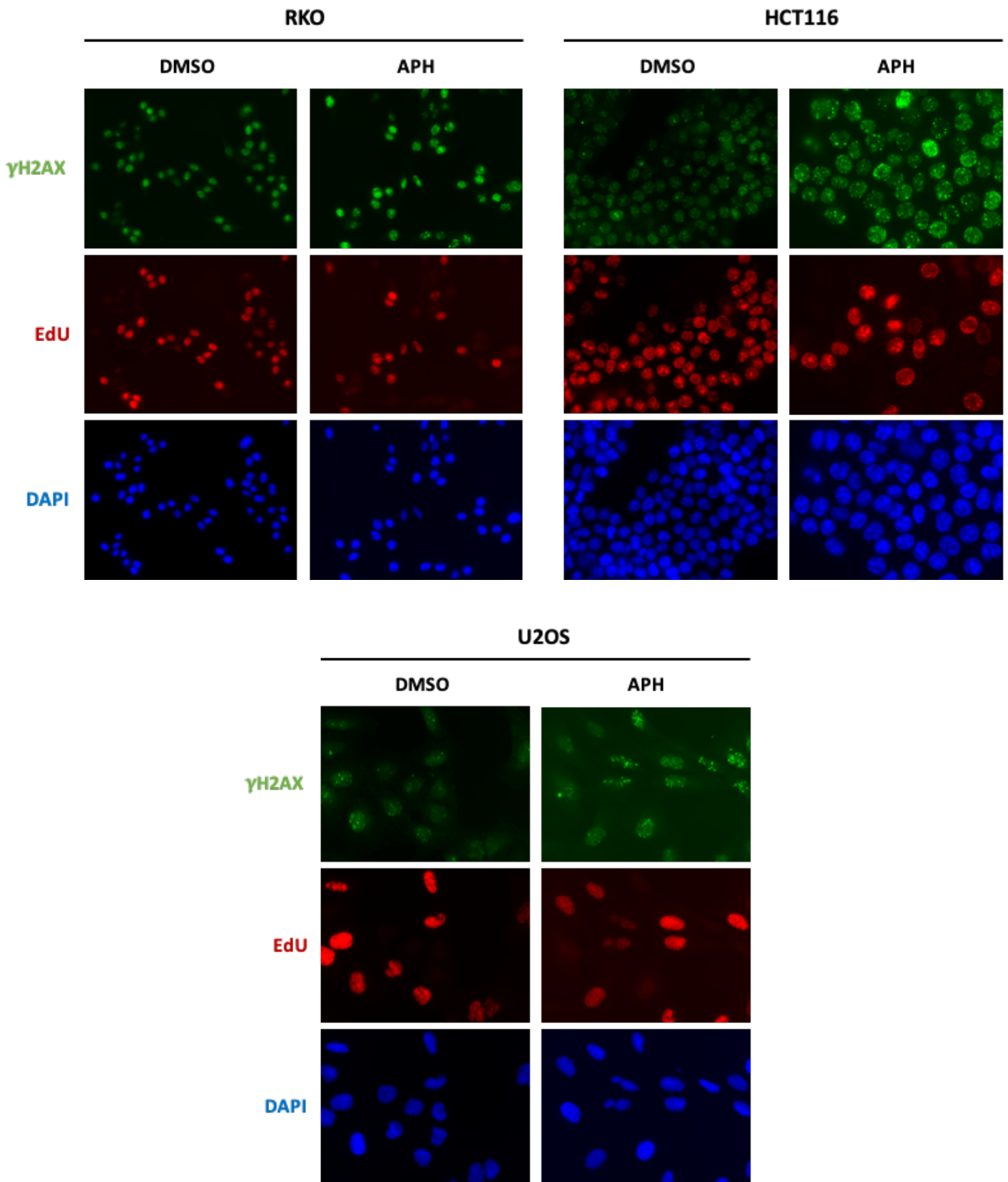
**Figure S2:** Histogram for the quantification (%) of BrdU positive (BrdU+) cells right after 10h (black) or 24h (grey) of treatment (t0) in K562, HCT116, U2OS, MRC5-N and RPE-1.



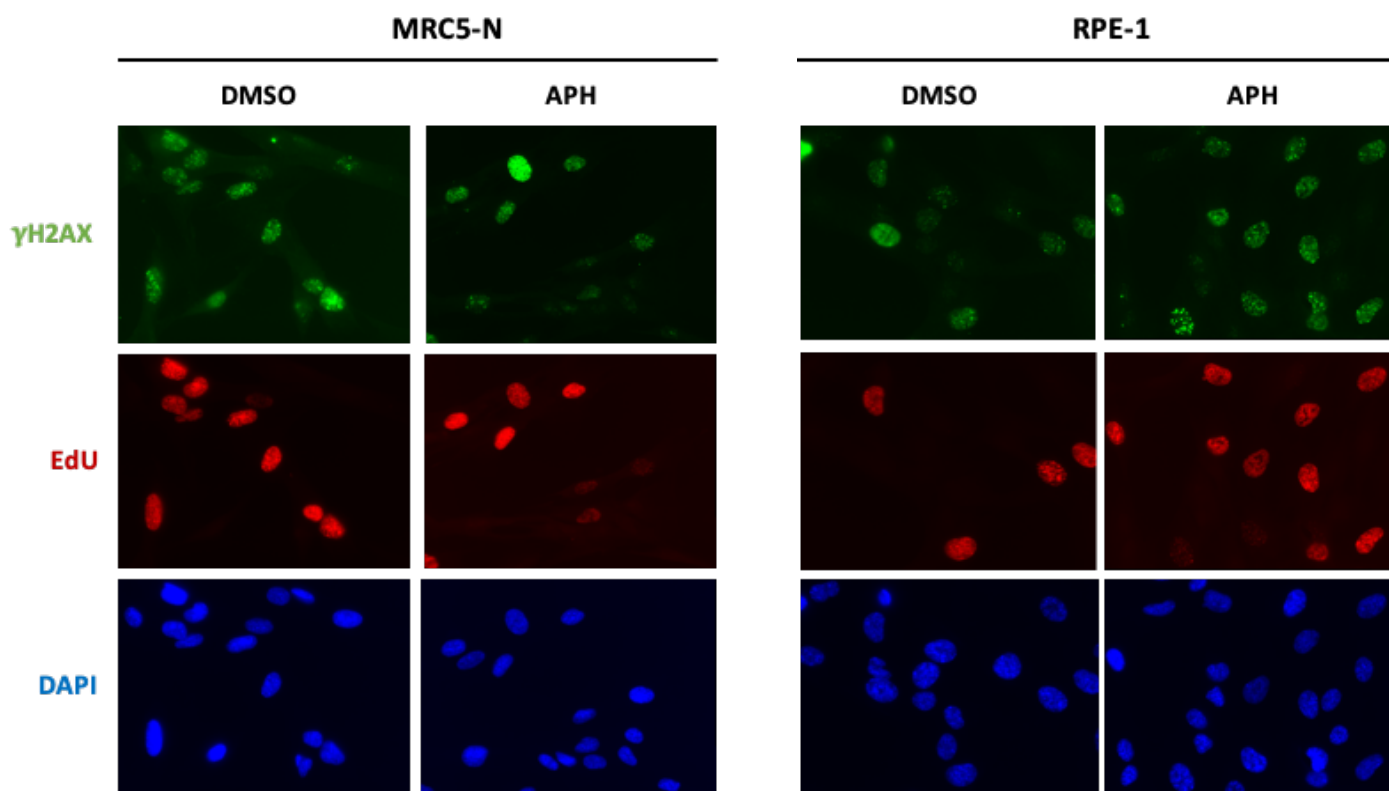
**Figure S3:** Stacked histogram representing the proportion of BrdU+ cells (in red) in the different phases of the cell cycle (G1, S or G2M) after release (N+1) in K562, HCT116, U2OS, MRC5-N and RPE-1.



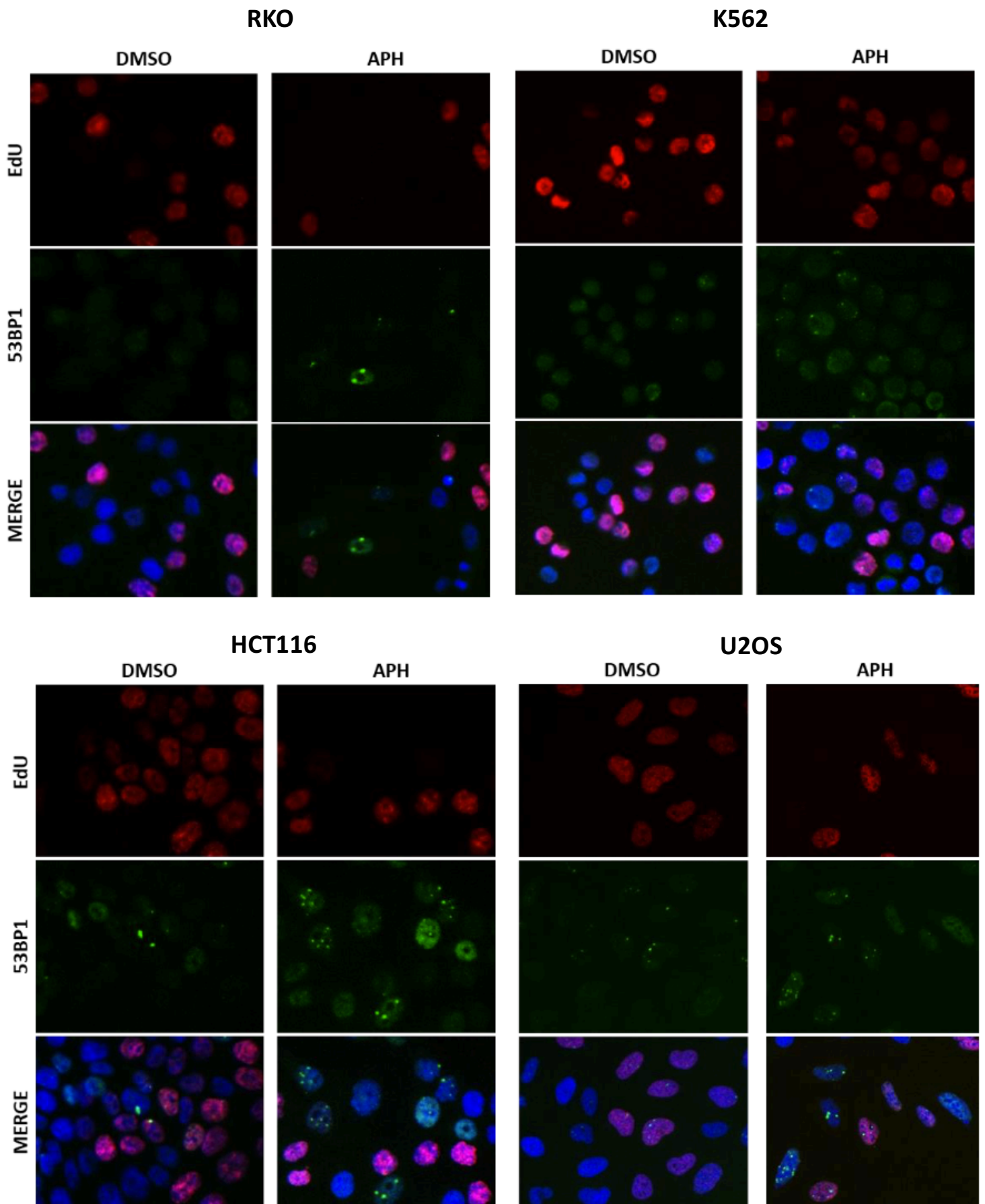
**Figure S4:** Immunofluorescence image for  $\gamma$ H2AX intensity quantifications in the 6 human cells lines. In red, EdU signal, in green  $\gamma$ H2AX antibody signal and in blue DAPI signal.



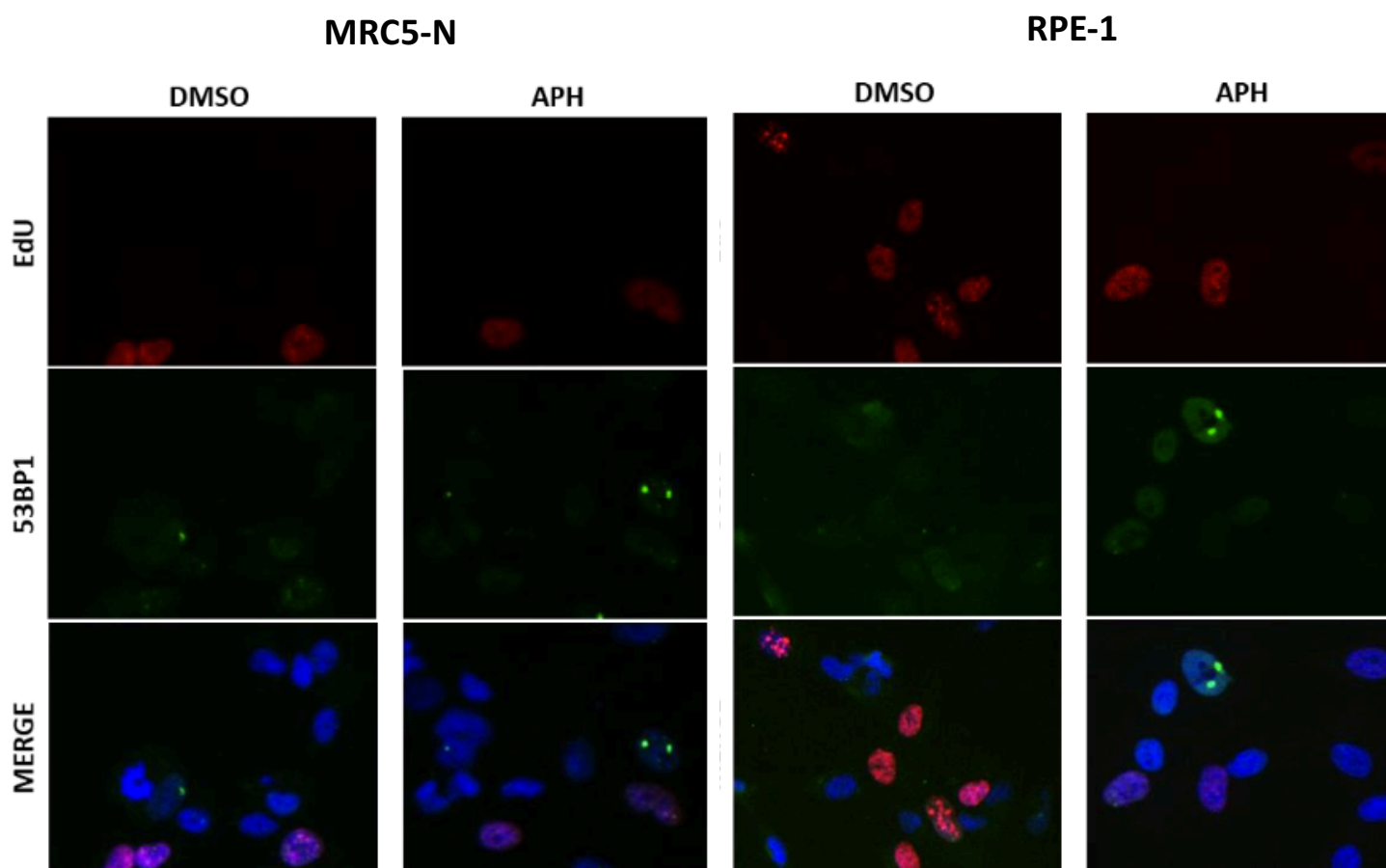
**Figure S4bis:** Immunofluorescence image for  $\gamma$ H2AX intensity quantifications in the 6 human cells lines. In red, EdU signal, in green  $\gamma$ H2AX antibody signal and in blue DAPI signal.



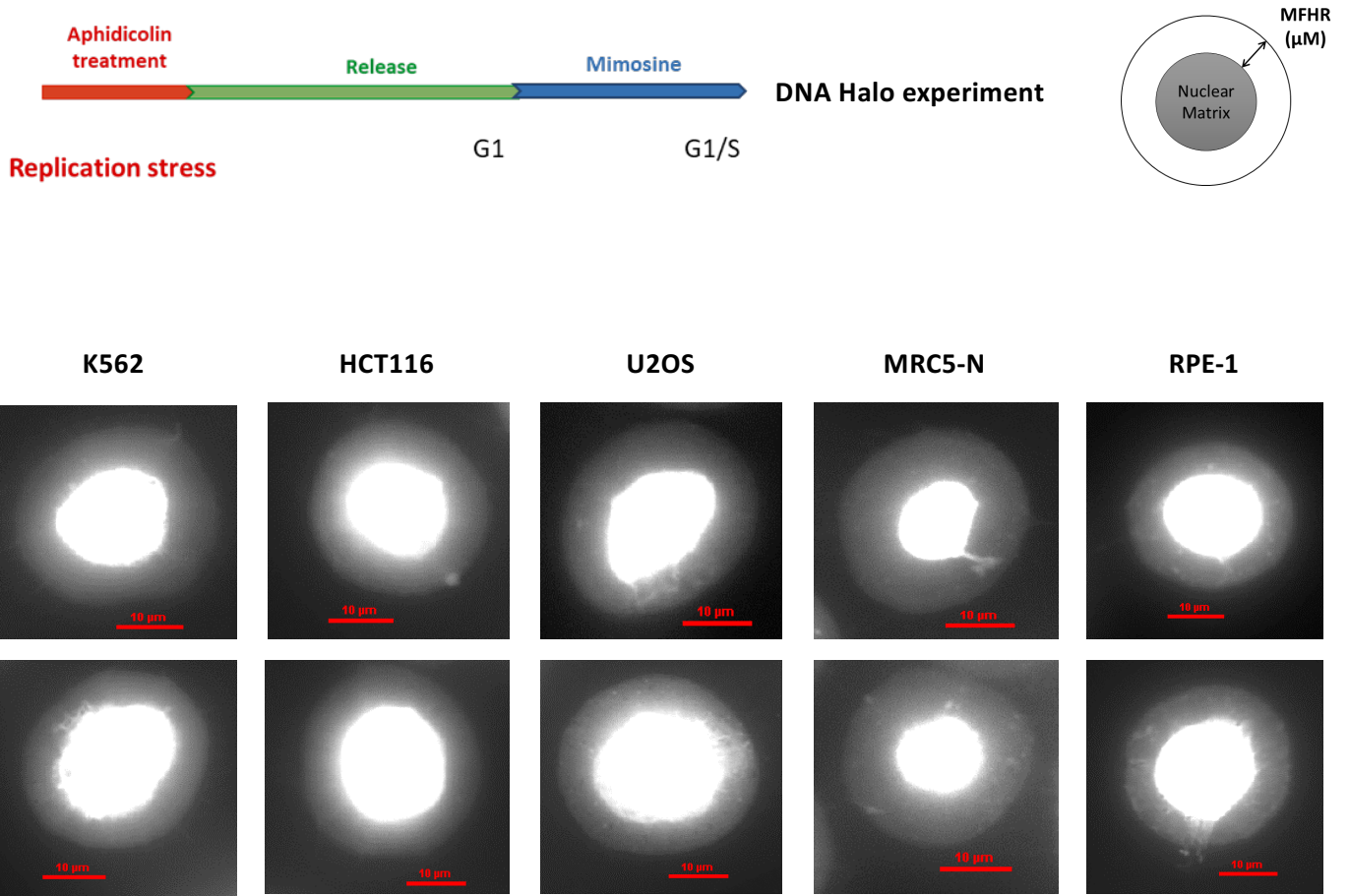
**Figure S5:** Immunofluorescence image for 53BP1 bodies quantifications in the 6 human cells lines. In red, EdU signal, in green 53BP1 antibody signal and in blue DAPI signal.



**Figure S5bis:** Immunofluorescence image for 53BP1 bodies quantifications in the 6 human cells lines. In red, EdU signal, in green 53BP1 antibody signal and in blue DAPI signal.

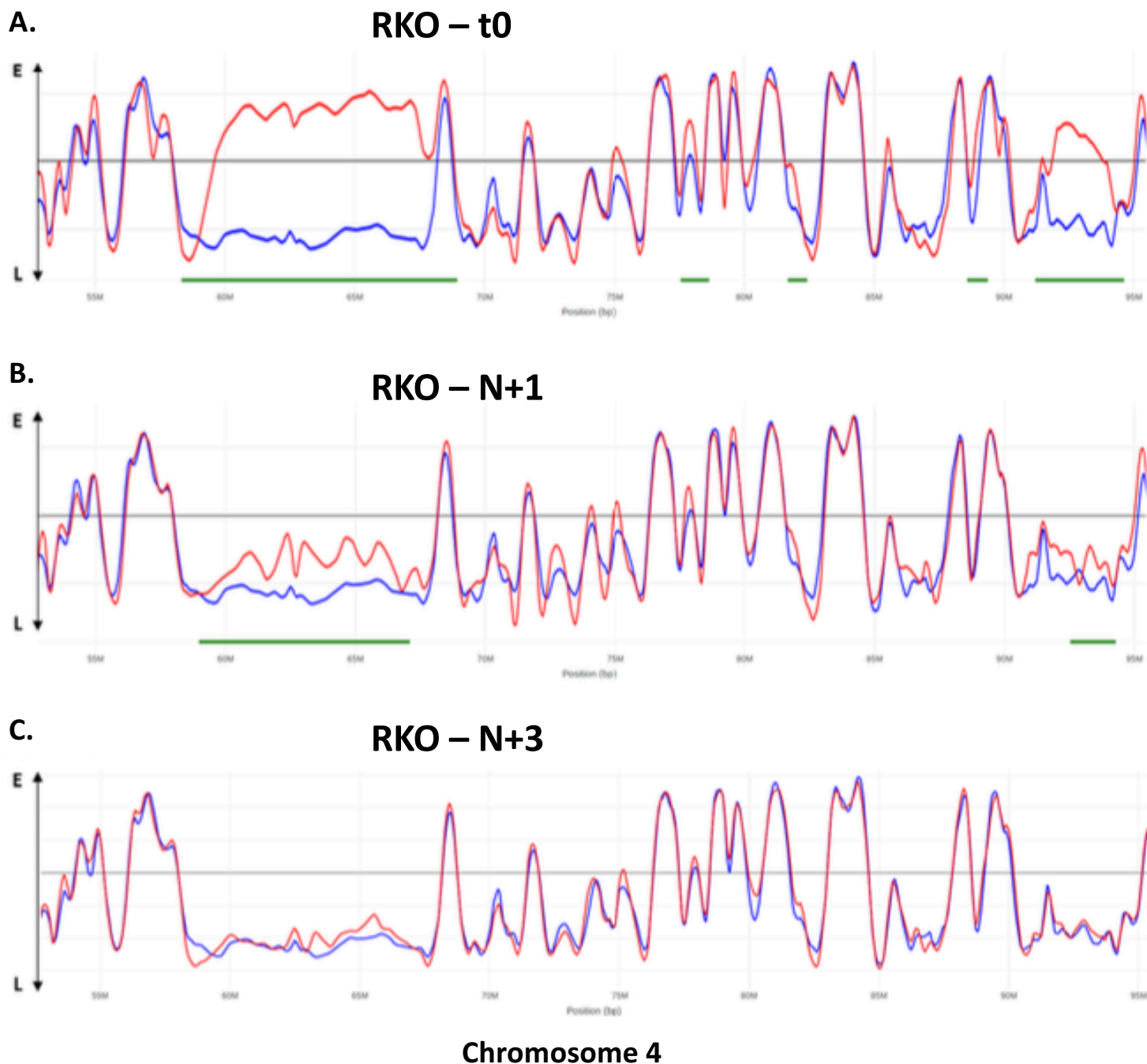


**Figure S6:** Immunofluorescence image for 53BP1 bodies quantifications in the 6 human cells lines. In red, EdU signal, in green 53BP1 antibody signal and in blue DAPI signal.



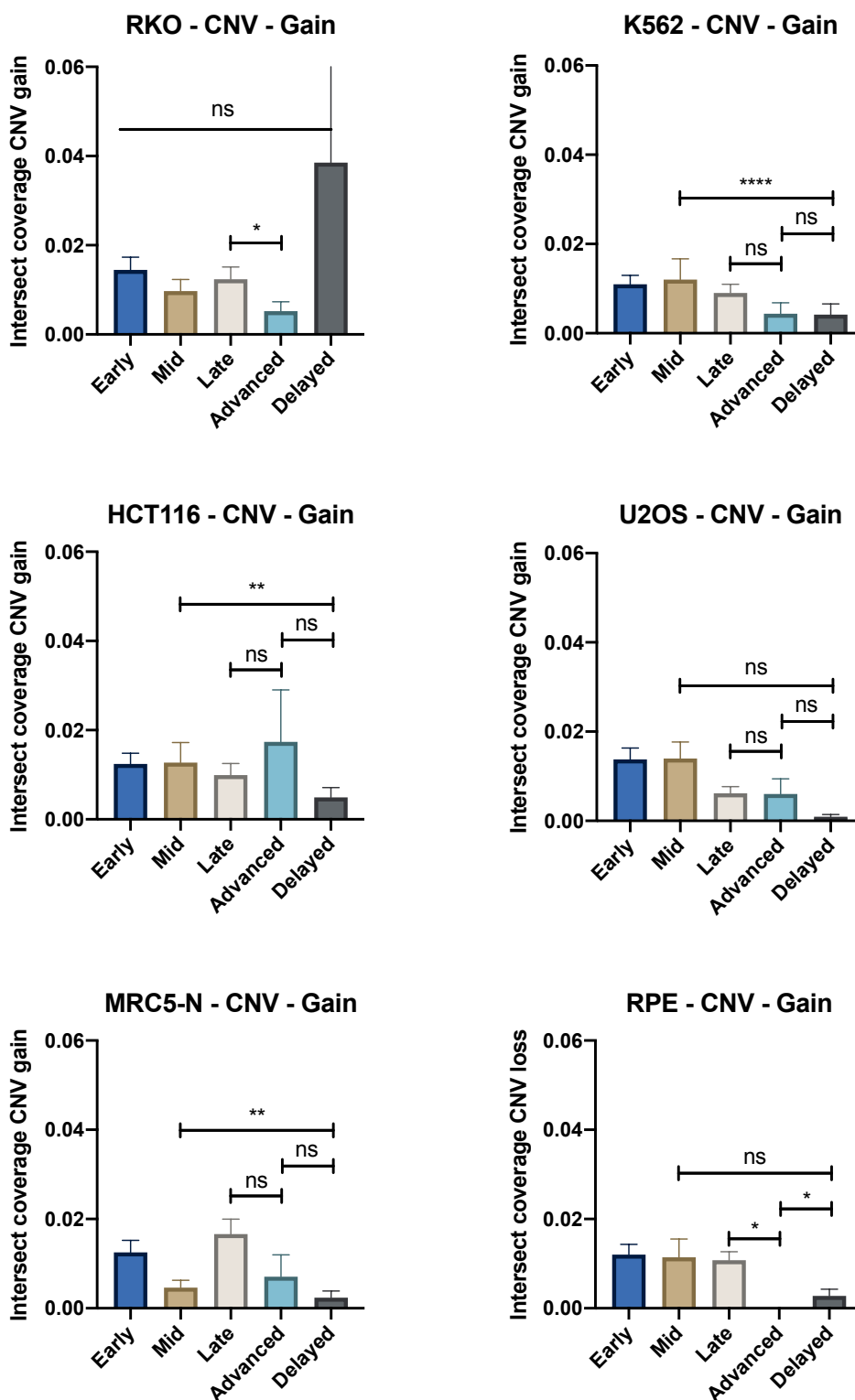


**Figure S7:** Snapshots of Loess-smooth replication timing profiles in RKO cells through 3 generations. Chromosome 4 (52-95Mb) RT profiles in the DMSO (blue) and the Aphidicolin (red) conditions. **(A)** RT profiles in mother ( $t_0$ ) cells. **(B)** RT profiles in daughter cells released from the stress ( $N+1$ ). **(C)** RT profile of cells three cellular generations after the RS ( $N+3$ ). Significantly ADV aRTIL are underlined in green and DEL aRTIL in pink.

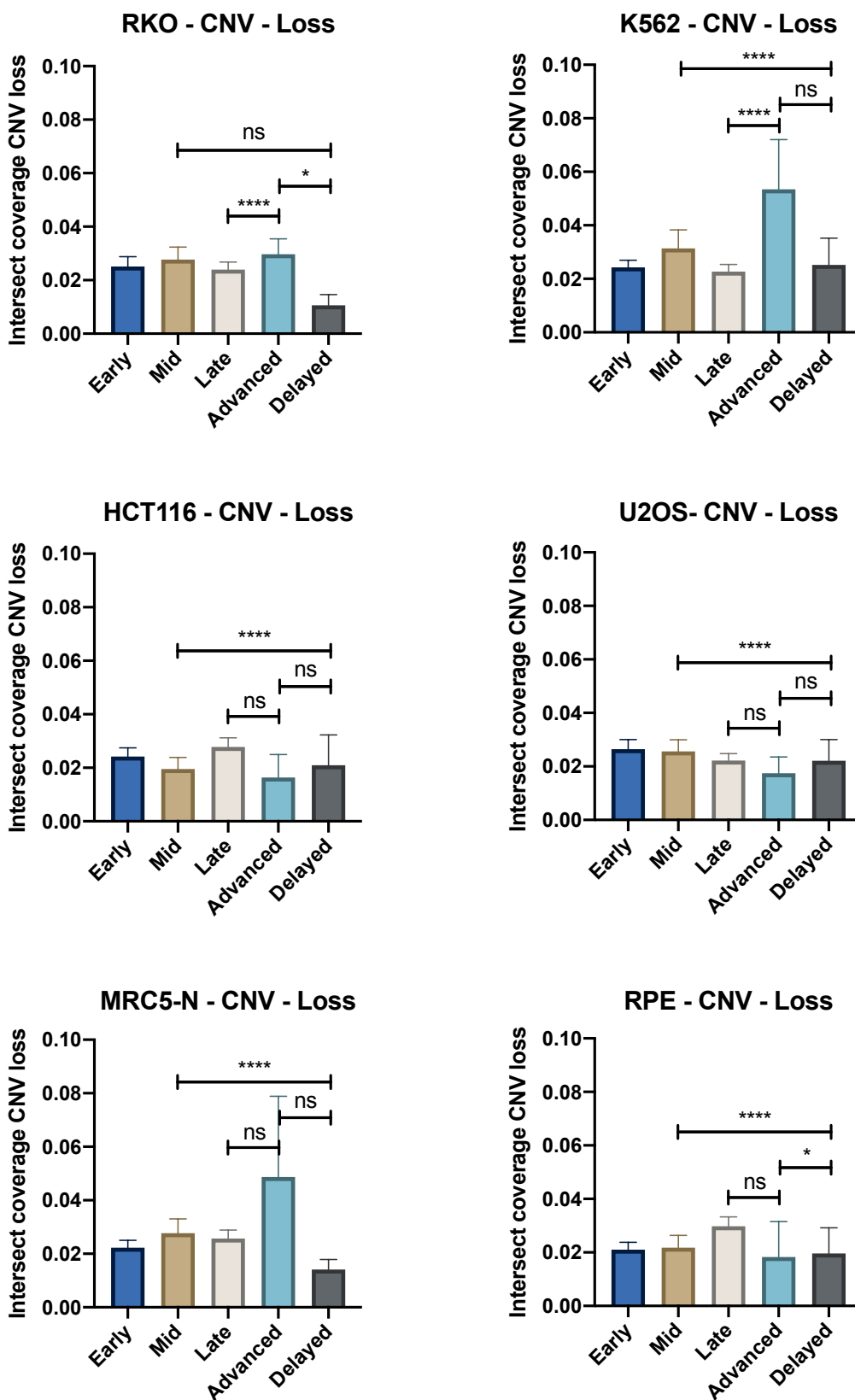


# DISCUSSION

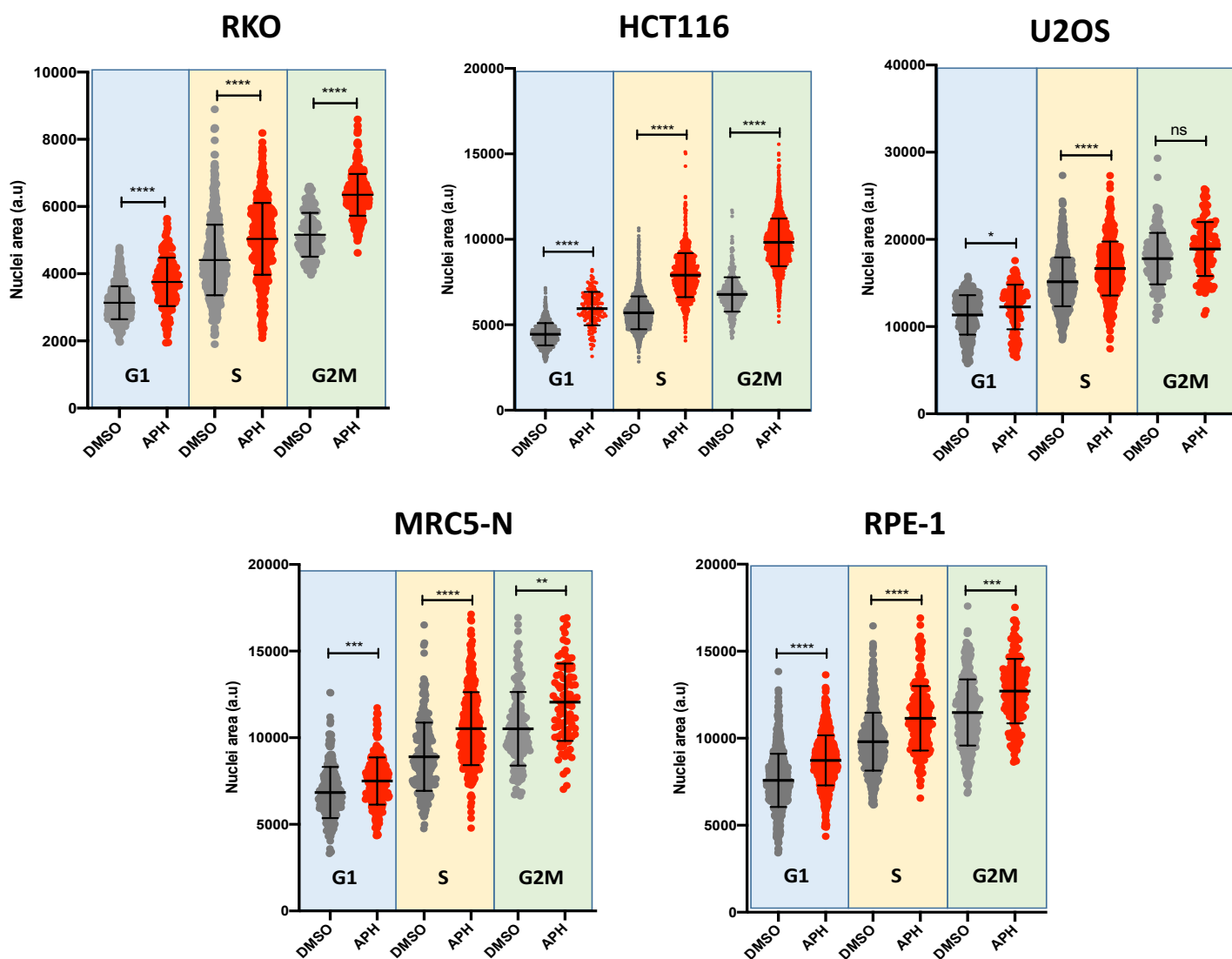
**Figure S8:** Histogram representing the intersect coverage between different genomic regions (Early, Mid, Late, Advanced and Delayed aRTIL) and the CNV gain.



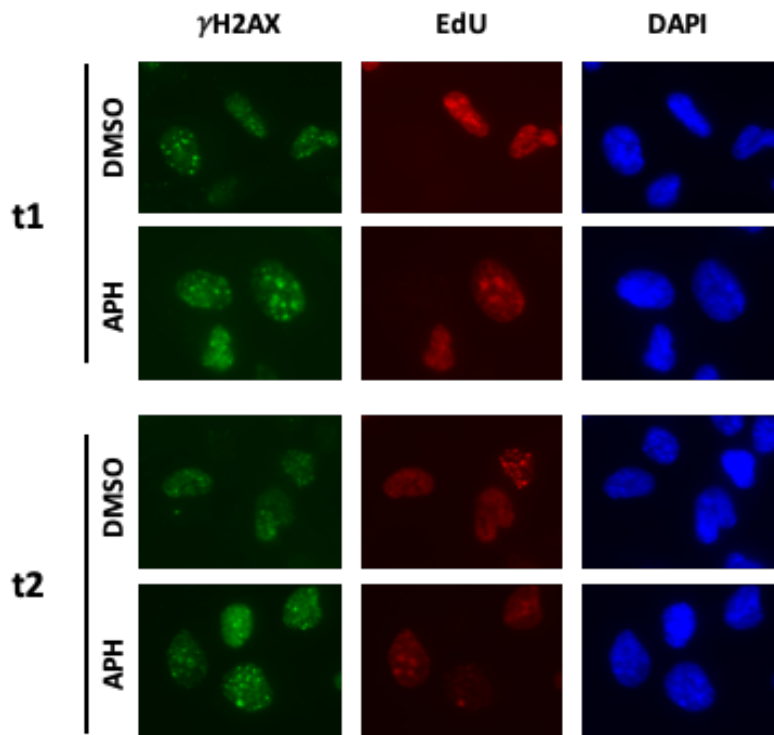
**Figure S9:** Histogram representing the intersect coverage between different genomic regions (Early, Mid, Late, Advanced and Delayed aRTIL) and the CNV loss.



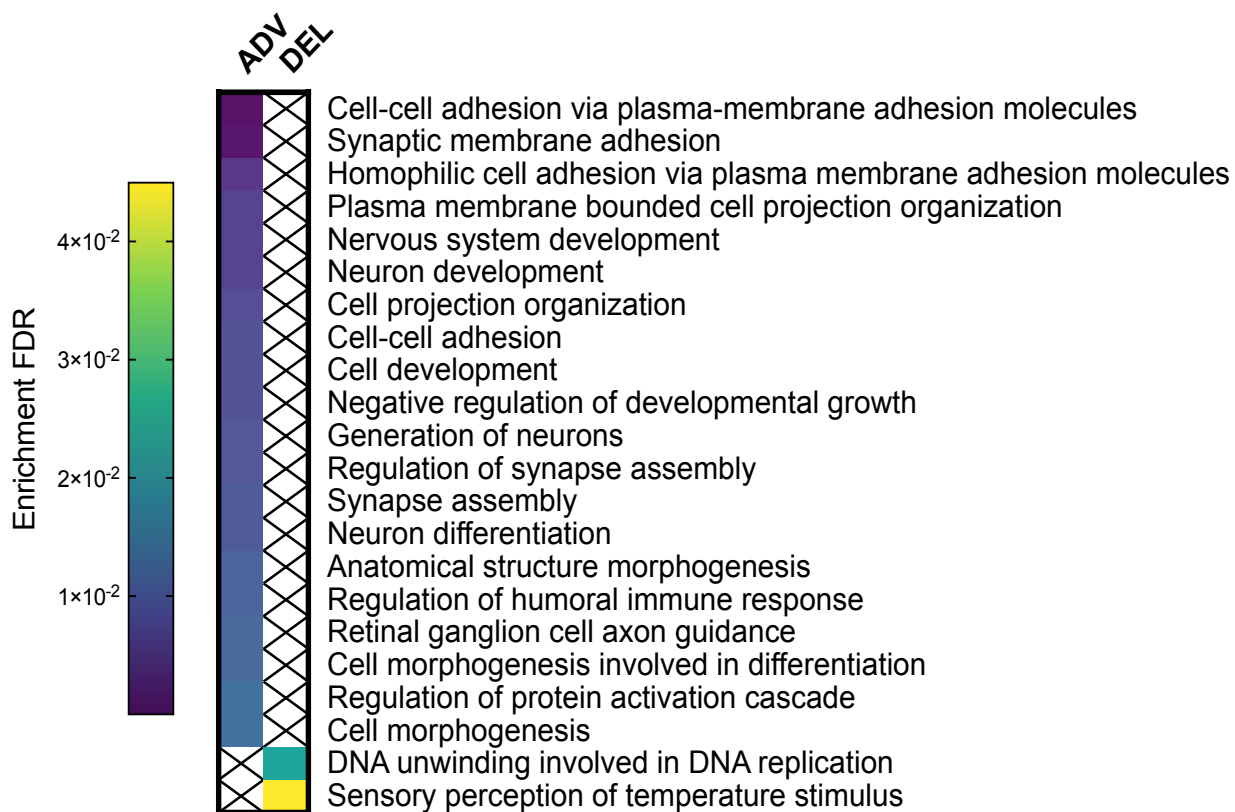
**Figure S10:** Plots of nuclei area measurement by immunofluorescence and QiBC in RKO, HCT116, U2OS, MRC5-N and RPE-1 cells in different phases of the cell cycle (G1, S and G2M).



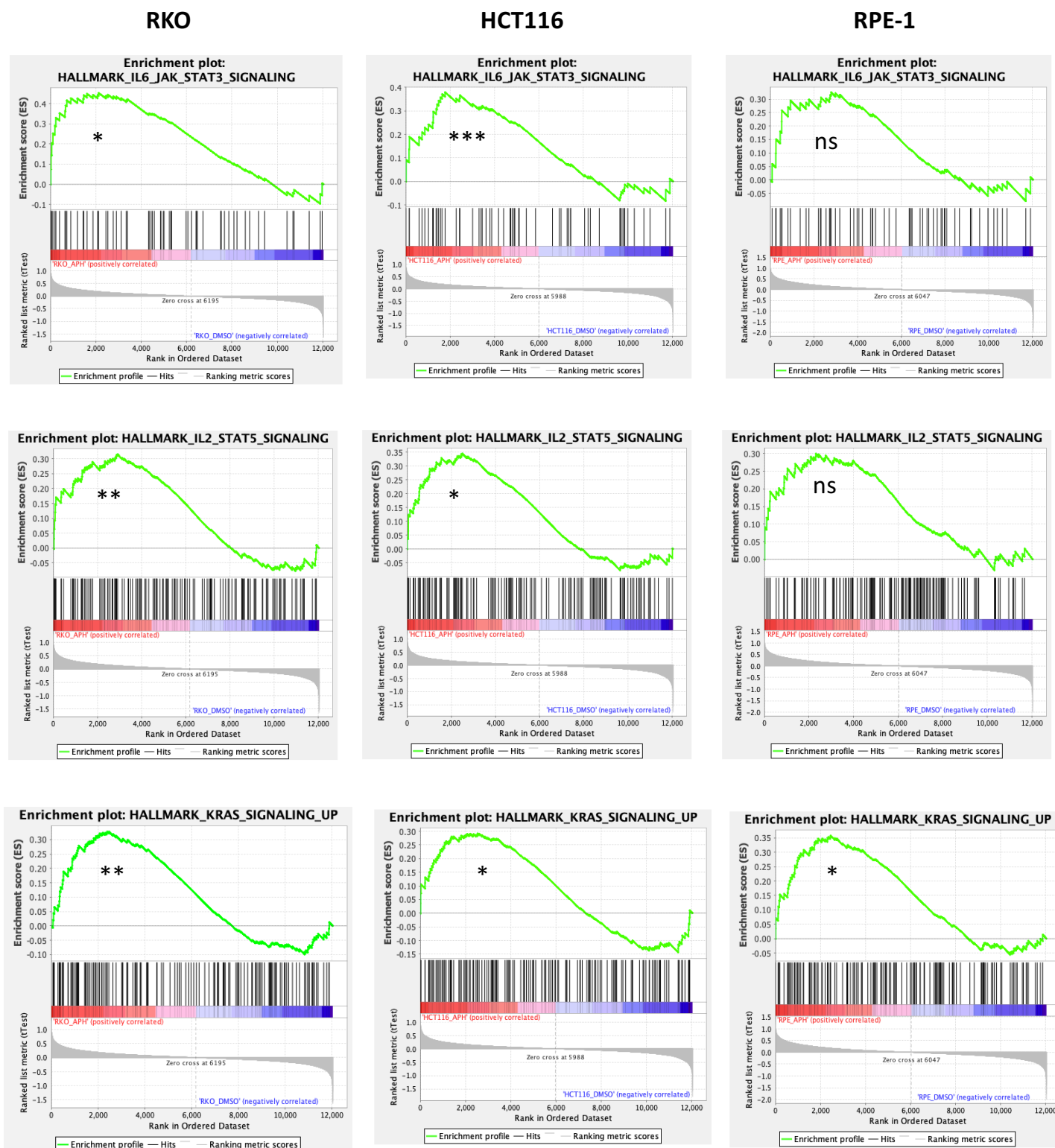
**Figure S11:** Immunofluorescence image for  $\gamma$ H2AX intensity quantifications in RPE-1 cells treated once (t1) or twice (t2) with aphidicolin or DMSO. In red, EdU signal, in green  $\gamma$ H2AX antibody signal and in blue DAPI signal.



**Figure S12:** Gene ontology (GO) enrichment analysis for genes within ADV and DEL aRTIL in RKO cells.



**Figure S13:** GSEA with the HALLMARK data set between DMSO and APH conditions in RKO, HCT116 and RPE-1. Screenshot of GSEA enrichment plots for KRAS, IL6\_JAK\_STAT3 and IL2\_STAT5 signalling hallmarks in the three analysed cell lines. Statistics: NOM p-val, \*\*\*p<0.01, \*\*p<0.05, \*p<0.1, ns when p>0.1.





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## Abstract (Français)

La réplication de l'ADN est un processus finement orchestré dans les cellules eucaryotes grâce à la régulation temporelle de l'activation des origines de réplication, phénomène mieux connu sous le nom de *timing* de réplication. Le *timing* de réplication d'un domaine génomique est très robuste et dépend de l'identité cellulaire. Lors d'un faible stress réplicatif, la fourche de réplication est ralentie et certaines régions fragiles du génome voient leur *timing* de réplication retardé voire ne sont pas du tout dupliquées. Ainsi, le ralentissement du processus de réplication peut avoir pour conséquence des cassures de l'ADN et à fortiori de l'instabilité génomique, un marqueur bien connu des cancers. Hormis ces régions fragiles déjà identifiées, l'impact direct d'un faible stress sur le "timing" de réplication de l'ensemble du génome n'a pas encore été étudié. L'objectif de ma thèse était donc d'analyser et de comparer le *timing* de réplication de 6 lignées cellulaires de différents tissus humains (sain ou tumoral) en réponse à un faible stress réplicatif. J'ai ainsi pu observer une réponse hétérogène entre les différentes lignées et un *timing* de réplication plus impacté dans certaines lignées cancéreuses en réponse au stress. En particulier, certaines lignées cancéreuses présentent de très fortes avancées de *timing* dans certaines régions précises du génome en réponse au stress réplicatif. De surcroît, nous avons observé que ces avancées de *timing* qui sont retrouvées dans la génération cellulaire suivante. Ainsi, lors de ma thèse, j'ai mis en évidence un nouveau mécanisme mis en place surtout par les cellules cancéreuses en réponse au stress réplicatif qui apporte une preuve supplémentaire de la plasticité du génome de ces cellules, leur permettant de répondre et de s'adapter rapidement à des conditions de stress et, éventuellement, de mieux résister aux agents génotoxiques.

## Abstract (English)

DNA replication is very well orchestrated in mammalian cells thanks to a tight regulation of the temporal order of replication origin activation, commonly called replication timing (RT). The replication timing of a given replication domain (RD) is very robust and depends on the cell type. Upon low replication stress, replication forks progress slower and it has been shown that some fragile regions are replicated later or even under-replicated. These replication delay leads to DNA damage and genetic instability, a common marker of cancers. Except for these fragile regions, the direct impact of low replication stress on the RT of the whole genome has not been explored yet. The aim of my thesis was to analyse and compare the replication timing of 6 human cell lines from different tissues (healthy or from tumours) in response to mild replication stress. Assessing this question, I have first observed heterogeneous response in between cell lines, some cancer cells were much more impacted by low replication stress. Strikingly, in some cancer cells, specific RD are undergoing a switch from late to early replication in response to replication stress. Very interestingly, this RT alteration was still detected in daughter cells. These findings disclosed a new mechanism mainly used by cancer cells in response to replication stress that brings another proof of their genome plasticity, allowing a quick response and adaptation to stress that, eventually, gives better resistance to genotoxic agents.