Caractérisation d'un nouveau rôle de Gnl3 dans la maintenance de la stabilité de génome

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CARACTERISATION D'UN NOUVEAU ROLE DE GNL3 DANS LA MAINTENANCE DE LA STABILITE DE GENOME

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It is my reflection in your eyes that made

me believe nothing is impossible for me to achieve

Abstract

DNA replication requires a plethora of proteins to maintain its accuracy during replicative stress. In order to fully understand how DNA replication is sustained at proper pace, it is important to study the mechanisms that are guarding DNA replication during normal and perturbed conditions. Using the iPOND (isolation of proteins on nascent DNA) based screen, we uncovered a new protein, GNL3 (aka nucleostemin), to be associated with replisome. GNL3 is overexpressed in several cancers and is involved in maintaining genomic integrity in stem and cancer cells. However its precise role(s) is unclear.

One of the key mechanisms that protects the genomic stability during replication stress is the proper regulation of origin firing. In this project, we show that GNL3 limits replicative stress by limiting replication origin firing. We proved that GNL3 is in proximity of nascent DNA using different approaches and that its depletion reduces forks speed but increases forks density and replication origin firing. Conversely, overexpression of GNL3 leads to a decrease in origin firing. When subjected to exogenous replicative stress, cells impaired for GNL3 exhibit an increased MRN-dependent resection and RPA phosphorylation. Interestingly, we found that inhibition of origin firing using CDC7 inhibitor decreased resection in absence of GNL3 but not in absence of BRCA1, suggesting that GNL3 protects the integrity of stalled forks indirectly by regulating origin firing efficiency. In addition, using various approaches (BioID, PLA, coIP), we established that ORC2 and GNL3 interact together in the nucleolus. We propose that GNL3 level is crucial to determine the correct distribution of ORC2 on chromatin to regulate origins licensing. Our data present insights into a new role of GNL3 in the regulation of origin firing that protects genomic stability.

Keywords: GNL3 – ORC2 - DNA Replication - DNA replication origins – Replication stress – iPOND – Genomic Stability

Résumé

La réplication de l'ADN nécessite une pléthore de protéines afin d'assurer sa processivité en particulier en présence de stress réplicatif. Afin de mieux comprendre le processus de réplication de l'ADN, il est important d'étudier les mécanismes qui permettent la réplication dans des conditions normales et en présence de stress réplicatif. A l'aide de la méthode iPOND (*isolation of proteins on nascent DNA*), nous avons découvert une nouvelle protéine associée avec la machinerie de réplication de l'ADN : GNL3 (appelée aussi nucleostemin). GNL3 est surexprimées dans plusieurs cancers et est impliquée dans la réponse aux lésions de l'ADN dans les cellules souches et cancéreuses, néanmoins ses fonctions précises au sein de la cellule ne sont pas connues.

Un des mécanismes majeurs de la protection de l'intégrité du génome durant la réplication en présence de stress est le contrôle précis de l'activation des origines de réplication. Durant ma thèse de Doctorat j'ai montré que GNL3 limite le stress réplicatif en contrôlant l'activation des origines de réplication. J'ai montré que GNL3 est à proximité de l'ADN naissant en utilisant plusieurs approches et que sa déplétion réduit la vitesse de progression des fourches de réplication tout en augmentant la leur densité et l'activation de origines de réplication. Inversement, la surexpression de GNL3 inhibe l'activation des origines de réplication. En présence de sources exogènes de stress réplicatif, l'inactivation de GNL3 conduit à une résection de l'ADN naissant par le complexe MRN et à un la phosphorylation de RPA. J'ai montré que l'inhibition de l'activation des origines de réplication (en utilisant un inhibiteur de CDC7) conduit à une baisse du niveau de résection en absence de GNL3 mais pas en absence de BRCA1. Il apparait donc que GNL3 joue un rôle clé dans la stabilité des fourches de réplication bloquées en régulant l'efficacité d'activation des origines. De plus, à l'aide de plusieurs approches (BioID, PLA, CoIP), j'ai établi que GNL3 interagit avec ORC2 dans le nucléole. Je propose que GNL3 joue un rôle clé dans la distribution d'ORC2 sur la chromatine permettant ainsi la régulation correcte de l'activation des origines. Au final il apparait que le rôle de GNL3 dans la régulation des origines est crucial pour assurer la stabilité du génome.

Mots-clés : GNL3 - ORC2 - réplication de l'ADN - origines de réplication - stress réplicatif – iPOND – stabilité du génome

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List of Abbreviations

ACS: ARS Consensus Sequence

ALT: Alternative Lengthening of Telomeres

AP: Apurinic/Apyrimidinic site

APB: ALT-associated PML Bodies

ATR: Ataxia telangiectasia and Rad3 related

BER: Base Excision Repair

BIR: Break Induced Replication

CDC25: Cell Division Cycle 25

CDK: Cyclin-Dependent Kinase

CFS: Common Fragile Site

ChIP: Chromatin Immunoprecipitation

Chk1: Checkpoint kinase 1

CKI: Cyclin Kinases Inhibitor

CPT: Camptothecin

DDR: DNA Damage Response and Repair

DPC: DNA-Protein Crosslink

DSB: Double Strand Break

DSBR: Double Strand Break Repair

dsDNA: double stranded DNA

EGFR: Epidermal Growth Factor Receptor

ERFS: Early Replicating Fragile Site

ETP: Etoposide

GNL3: Guanine Nucleotide-binding Like 3

HR: Homologous Recombination

ICL: Interstrand Crosslinks

IFN: Interferon

IOD: Inter Origin Distance

IP: Immunoprecipitation

IR: Ionizing Radiation

MCM: Minichromosome Maintenance protein

MiDAS: Mitotic DNA synthesis

MMR: Mismatch Repair

NER: Nucleotide Excision Repair

NHEJ: Non-Homologous End Joining

NS: Nucleostemin

OGRE: Origin G-rich Element

ORC: Origin Recognition Complex

PLA: Proximity Ligation Assay

Pre-RC: pre-Replication Complex

RB: Retinoblastoma Protein

ROS: Reactive Oxygen Species

RPA: Replication Protein A

RTC: Replication-Transcription Collision

SSB: Single Strand Break

SSBR: Single Strand Break Repair

ssDNA: single stranded DNA

TDP: Time Decision Point

TLS: Translesion Synthesis

Top I: Topoisomerase 1

Top II: Topoisomerase 2

UV: Ultraviolet

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Introduction

Chapter 1: Cell Cycle

The cell cycle is a tightly organized and regulated process that directs the cells into a chain of events leading to the duplication of their genetic material and eventually the production of two daughter cells. The cell cycle is divided mainly into two steps: interphase and mitosis. The interphase includes 3 phases: G1, S and G2. During the G1-phase the cell grows in preparation for DNA replication that occurs during S-phase. The G2-phase is the preparation period needed for the cellular growth and protein synthesis prior to mitosis; the cellular division process which is composed of four phases: prophase, metaphase, anaphase and telophase. On the other hand, the cell could exist to a quiescent state known as the G0 phase, where the cell doesn't divide any further. The cell cycle is the essence process for the growth and development of organisms; hence, any error if not well controlled during this process would lead to serious consequences such as development of different types of cancer.

The main regulators of this process are two classes of proteins: (1) Cyclin-dependent kinases (CDKs), a family of Serine/Threonine Kinases, and (2) Cyclins (Rev et al., 1997). These two classes of proteins interact together forming checkpoint complexes that control the progressions of cells between different stages of the cell cycle. CDKs and Cyclins are in turn subjected to regulation by numerous proteins such as p21, p53, and p16, and on the other hand, they regulate several targets such as RB and E2Fs proteins.

1- The Resting Phase- G0

After the end of each cell cycle, the cell might either engage into another round of cell cycle and continue proliferating, or might stop and exit into a non-dividing state, the G0-phase.

The G0-phase is not considered as a part of the cell cycle; however, it is a resting state where cells are still metabolically and transcriptionally active but have stopped to divide temporarily or permanently. Cells enter G0-phase due to several reasons, such as external signals that push the cells to stop dividing and differentiate or due to the lack of mitogens. Moreover, the cells might also exit the cell cycle and enter into another type of resting state known as senescence or cellular aging.

During G0-phase, the genes that are required for entering into the cell cycle are repressed by the DREAM complex (<u>D</u>P,<u>RB</u> like, <u>E</u>2F, and <u>M</u>uvB) (Litovchick et al., 2007). Different components of DREAM complex binds and repress genes required for DNA synthesis and genes required for progression through mitosis (Sadasivam et al., 2012; Schmit et al., 2007). Upon signals that promote cell cycle entry, P130 (an RB like protein) gets phosphorylated by CDKs, which leads to its dissociation from DREAM complex. This will relieve the inhibitory effect of the DREAM complex and eventually allow cells to enter into the cell cycle again (Guiley et al., 2015).

2- G1-Phase

Upon stimulating signals, the expression of cyclin D increases where it forms a complex with CDK4 and CDK6 (Figure 1). The Cyclin D/CDK4-6 complex becomes activated and will phosphorylate P130, thus leading to its dissociation from the DREAM complex as discussed above (Schade et al., 2019). This will allow MuvB to form a complex with B-Myb and FoxM1 which activates late cell cycle genes (Sadasivam et al., 2012). As a response to E2F repression is relieved by CDK4 phosphorylation of RB, the expression of cyclin E is elevated and together with CDK2 will furtherly phosphorylate RB thus completely relieving the inhibition of E2F. At this point, the expression of early cell cycle genes will push the cell to pass through the first cell cycle checkpoint, the G1/S transition point. It is important to note that at this point the expression of cyclin A will increase where it also binds to CDK2 to help Cyclin E/CDK2 complex in crossing the G1/S restriction point (Figure 1).

This stage of the cell cycle is critical and must be well regulated; otherwise, the balance between cell death and cell division could be perturbed, leading to either development of necrotic tissues or malignant ones, respectively. The regulation of CDKs acting in G1 is done by two types of Cyclin Kinases Inhibitors (CKI). The first family is the INK4 family including p16 (Aprelikova et al., 1995) which competes with cyclin D for CDK4 binding, thus inhibiting its activation and phosphorylation. The other family is Cip/Kip. p21 (Cip1) acts on preventing the phosphorylation of CDK2 on Thr160, an activating phosphorylation

(Wade Harper et al., 1993). Whereas p27 (Kip1) binds to the catalytic cleft of CDK2 family, thus inhibiting its action (Toyoshima and Hunter, 1994).

3- S-Phase

During S-phase the cell starts to synthesize a duplicate of its genome. As with other phases of the cell cycle, S-phase should be well regulated to ensure the faithful transmission of the genetic material to the daughter cells during mitosis. After G1/S transition, cyclin E is degraded and cyclin A becomes the main cyclin expressed, forming a complex with CDK2 (Figure 1) (Hengstschläger et al., 1999) . Cyclin A is important for the initiation of S-phase through interacting with MCM7 (Chibazakura et al., 2011), one of the MCMs complex (DNA helicases). CDK2 likewise phosphorylates proteins of the pre-replication complex (Pre-RC) (Hua and Newport, 1998) in order to initiate DNA synthesis. In addition, Cyclin A/CDK2 acts on protecting the genomic integrity by limiting DNA synthesis to one full round where they phosphorylate MCMs during late S phase to prevent their re-loading onto the chromatin, thus inhibiting re-replication (Ishimi et al., 2000) .

4- G2/M-Phase

During late S-phase, Cyclin A/CDK2 down-regulates the level of the checkpoint protein Chk1 (Oakes et al., 2014), relieving its inhibitory effect thus promoting S/G2 transition and facilitating entry into mitosis. It is also been shown that the ATR pathway, a pathway activated in response to the presence of single stranded DNA (ssDNA) detailed elsewhere, plays a role at S/G2 transition (Saldivar et al., 2018a). As replication is ongoing during S phase, ssDNA is generated and coated by RPA which is recognized and bound by ETAA1. The ATR pathway is subsequently activated by ETAA1 until the S-phase ends. When ATR activity drops, FOXM1 is phosphorylated thus promoting S/G2 transition (Saldivar et al., 2018b).

During G2, Cyclin A stimulates the expression of multiple mitotic regulators (Hein and Nilsson, 2016; Laoukili et al., 2008; Lukas et al., 1999a; Oakes et al., 2014). Cyclin A also

contributes in a feedback loop in order to activate CDK1 (Mitra and Enders, 2004) and binds to it forming Cyclin A/CDK1complex. Cyclin B/CDK1 complex, which is responsible for mitotic entry and progression is expressed during G2 (Figure 1); however, it is inhibited by Wee1 kinase (Harvey et al., 2005). As cyclin A stimulates nuclear envelope breakdown (Gong et al., 2007), CDC25 activates Cyclin B/CDK1 which initiates prophase (Timofeev et al., 2010).

As the cell divides into two daughter cells after telophase, the levels of CDKs decrease again leading to dephosphorylation of RB protein, and therefore the repression of its downstream target E2F. This will cause the cell to arrest at G1-phase, where it will either exit to G0-phase or proceed with another round of cell cycle if mitogenic signals are present.
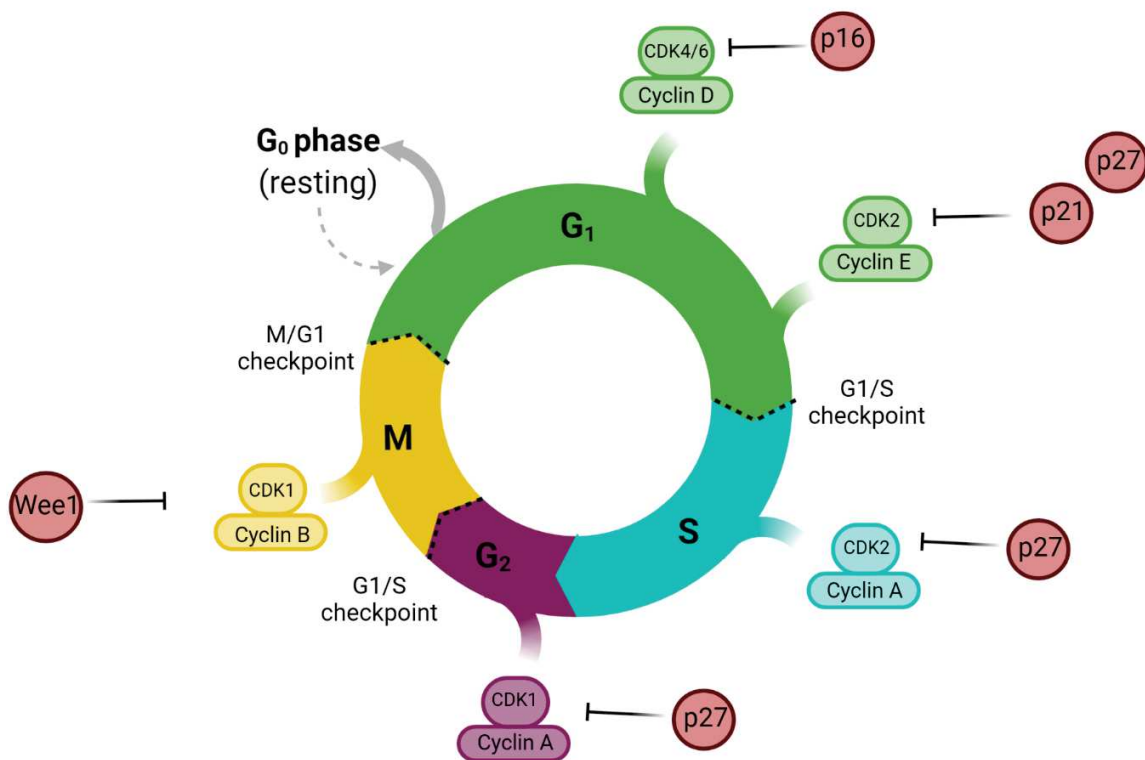
Figure 1. Cell cycle regulation by Cyclins and CDKs. The classical model for Cyclins/CDKs complexes dependent cell cycle regulation.

5- Cell Cycle Deregulation and Cancer

The main trigger for malignant transformation is the loss of control over cellular division, resulting in a non-controllable cellular proliferation. Usually this is due to mutations occurring in two types of proteins: (1) oncogenes: genes that are responsible for inducing cellular division and are usually overexpressed in cancer cells such as EGFR and Myc, and (2) tumor suppressor genes: genes that negatively control the cell cycle and are usually either mutated or deleted in cancer cells such as pRb, p53, and p21. Deregulation of proteins controlling the cell cycle is tightly associated with the development of cancer, since the cells are continuously proliferating with loss of control. The mutations can be in genes encoding Cyclins, CDK, CDKI, CDK activating enzymes, and CDK substrates.

During G1-phase, Cyclin D expression is induced by mitogens to initiate the entry into cell cycle; therefor, if the level of cyclin D is not well-regulated, cells can continuously proliferate independently of mitogens. Cyclin D gene amplification, which results in an increased level of expression, was found to be elevated in different types of cancer such as breast, esophageal, bladder, lung, and squamous cell carcinomas (Hall and Peters, 1996). In addition to Cyclin D, Cyclin A and E, which control the S phase, were found to be overexpressed in lung cancer (Dobashi et al., 1998).

The deregulation might take place at the level of CDKs, which could be at two levels: (1) mutations of CDK, such as mutations in the CKI binding domain that were found in CDK4 and CDK6, leaving them with no negative regulation (Yamamoto, 1998). (2) Overexpression of CDKs such as overexpression of CDK1 and CDK2 in subset of colon adenomas (Yamamoto, 1998).

CDKs and cyclins could be classified as oncogenes, since the over expression or inhibition of their downregulation accelerates malignant transformation. However, CKIs represent tumor suppressor functions since they mainly suppress cellular proliferation through RB activation. Mutations in CKIs are very frequent in human tumors. During G1, p16 binds to Cyclin D/CDK4 to inhibit it from phosphorylating RB, thus maintaining E2F suppression. Therefore, any deregulation of p16 leaves the cells free to proceed through

the G1-phase with no control. The deregulation of p16 is common in a high percentage of cancers where its corresponding genes can be mutated, hypermethylated or even deleted (Lukas et al., 1999b). Deregulation of other CKIs is also common, such as p19, p27 and p21 (Shi et al., 1996; Tan et al., 1997; Wade Harper et al., 1993).

In order to activate CDK during different stages of the cell cycle they must be dephosphorylated by members of Cdc25 phosphatase family, the CDK-activating enzymes. Cdc25A plays an important role during G1/S transition, Cdc25B is activated during S-phase, while Cdc25C activates cyclin B/CDK1 during mitotic entrance. Any deregulation of these enzymes allows an uncontrollable activation of CDKs and could be associated with malignant transformation. Since the expression of Cdc25A and Cdc25B is controlled by c-Myc, one of the most common mutated oncogenes in cancer, these two are considered potential oncogenes (Nilsson and Hoffmann, 2000).

One of the most important substrates of CDKs is the RB protein, and due to its function in inhibiting E2F (subsequently its targets) any mutations targeting this gene which lead to its absence or loss of function will drive the cells into uncontrollable proliferation. Expectedly, RB is frequently deregulated in retinoblastomas, acute lymphoblastic leukemia, and lung cancer (Field et al., 1996; Hall and Peters, 1996; Knudson, 1971).

The discovery of cell cycle regulators and how they might be altered in cancer gave a good target for cancer therapy. Compounds that inhibit CDK have been developed and even some of them are approved by the FDA for cancer treatment. For example, palbociclib, which is a selective inhibitor of CDK4/6, is used as a breast cancer treatment. Hence, palbociclib represents the first successful clinical translation in this field (Fry et al., 2004).

Chapter 2: Origins Licensing,

Firing and Regulation

The main aim of the interphase is first to prepare the cell and prime the DNA for replication and then to ensure the faithful duplication of the genetic material in order to secure its accurate transmission to the daughter cells. DNA is duplicated by a physiological process known as DNA replication, which is monitored strictly to establish the complete replication of the whole genome. The importance of this control has been emphasized by the fact that any defect in the proteins controlling any step of this process may lead to genomic instability, which could be translated into a series of diseases including carcinogenesis.

The outline of DNA replication mechanism is similar between prokaryotes and eukaryotes. However, due to the multiple layers of complexity of the eukaryotic genome in comparison to the prokaryotic one, the modes of recognition and regulation of DNA replication initiation is significantly more sophisticated in eukaryotes and may even differ between their different kingdoms.

1- Definition of Origins

DNA replication starts from genomic sites known as replication origins that are recognized by specific proteins and from which DNA synthesis is carried on in a bidirectional manner. In *Escherichia coli* (*E.coli*), this is limited to a single sequence-specific element known as OriC, from which its relatively simple genome is efficiently replicated within 20 minutes. However, in higher eukaryotes the completeness of genome duplication is particularly complex and requires multiple thousands of origins in order to finish this task within a limited time. For example, the human genome is 700-fold larger than the genome of *E.coli* and it requires 30,000 to 50,000 active origins at each cell cycle to be fully replicated in an average of 8 hrs. (Cvetic and Walter, 2005).

Replication origins are recognized by a family of proteins called origin recognition complex (ORCs) and they are set by three Steps: (1) recognition of origins by ORCs, (2) origin licensing, which constitutes of the assembly of pre-replicative complex (pre-RC) during G1-phase, and (3) Origin firing.

2- Features of DNA Replication Origins

As described above, the sequence of replication origin in *E.coli*, OriC, is well-defined with boxes for DnaA, the homolog of ORC in eukaryotes (Erzberger et al., 2006). Similarly, in the yeast *S.cerevisiae*, ORCs bind to ARS, which has in common a specific 12 bp consensus sequence (ACS) (Xu et al., 2006). However, in other eukaryotic cells there is no defined origin sequences but some common features were reported. In *S.pombe*, ARS elements do not share specific consensus, but they are characterized by AT-rich islands (Dai et al., 2005; Heichinger et al., 2006; Segurado et al., 2003). In multicellular organisms, ARS do not exist and identifying any common elements was unsuccessful. However, several characteristics have been identified at replication origins that are not necessarily present at all origins. These characteristics are found at different levels (Figure 2). (1) At the level of the sequence: AT-rich sequences, asymmetrical purine-pyrimidine sequences and matrix attachment sequences (MAR) were identified (Masai et al., 2010). It had also been reported that half of the replication origins are localized within or near CpG islands (Cadoret et al., 2008). (2) At the level of DNA structure: the topology of DNA has been reported to play a role in DNA origin selection. For example, in *Drosophila Melanogaster*, ORC displayed a preference for supercoiled DNA (Masai et al., 2010). Moreover, other studies have found that topoisomerases are associated with human replication origins (Abdurashidova et al., 2007). (3) At the level of transcription: transcription factors and elements exhibited a possible role in specifying the localization of ORC. Also in humans, ChIP-ChIP assays for mapping replication origins identified 283 origins which largely localized with transcriptional regulatory elements such as c-Jun and c-Fos (Masai et al., 2010). (4) At the level of chromatin: some features such as nucleosome free regions and histone deacetylation site have been described as characteristics of replication origins, however these features could be a consequence of chromatin remodeling in transcriptionally active regions.
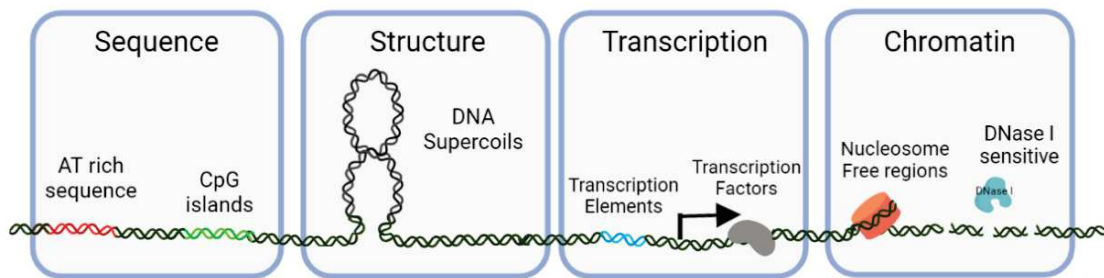
Figure 2. Features of DNA replication origins in eukaryotes. Several characteristics have been described at metazoan replication origins. These features are at the level of sequence, structure, transcription elements and factors, and chromatin. Although they are not present at all origins, they represent different markers that promotes the selection of a given origin.

3- Mechanism of Origin Licensing and Firing

The initiation of DNA replication in eukaryotes is a tightly regulated event that requires the ordered assembly of multiple proteins at the site of replication origins. This process is divided into 2 steps: Origin licensing and origin firing. These two steps are relatively well described in budding yeast, where the essential pre-RC and origin firing factors were identified and characterized for their fundamental roles and regulation. Licensing of origins occurs during late M-phase and in the G1-phase where the CDKs activity is low (Diffley, 2004). It is dependent on ORCs, Cdc6, Cdt1, and DNA helicases.

The first step is the assembly of heterotypic six subunits of ORC (1-6) on the DNA during late mitosis (Weinreich et al., 2001) which is followed by cdc6 recruitment that stabilizes the binding of the ORC complex to the DNA. This allows the recruitment of Cdt1 and eventually the recruitment of the helicase complex which is formed of the six subunit minichromosome maintenance (MCM2-7) thus forming the pre-RC complex (Figure 3A) (Kang et al., 2014). Since each origin produces two bi-directional replication forks after its activation, two helicases are loaded in a head to head dimer that encircles the DNA in opposite directions (Evrin et al., 2009; Remus et al., 2009).

The second step is origin firing, which is the activation of the pre-RC complex (Figure 3B). It requires additional factors: Sld2, Sld7, Sld3, Dpb11, Cdc45, GINS (Sld5, Psf1, Psf2, and Psf3), and DNA polymerase ε. Because this step requires high activity of two kinases

(DDK:Cdc7/Dbf4 and Cdk2) it can only occur at the G1/S transition where these two kinases are active (Gómez-Escoda and Jenny Wu, 2017). The first step of the firing is the activation of the MCM2-7 complex. This is executed by Cdc7, which phosphorylates the N-tail segments of MCM2, MCM4, and MCM6 (Masai et al., 2000). These phosphorylations are recognized by Cdc45, or a complex containing Cdc45 (Sld2, Sld7, and Cdc45) (Masai et al., 2006; Sheu and Stillman, 2006). The other kinase Cdk2, in turn phosphorylates Sld3 and Sld2 (Kamimura et al., 1998; Tanaka et al., 2007) which associates with and recruits DNA polymerase ε and GINS. p-Sld3 and p-Sld7 bind to two different pockets of Dpb11 protein, thus forming the SDS complex. At this step, the pre-initiation complex is formed which is composed of the SDS complex, Cdc45, GINS, DNA polymerase ε and the pre-RC complex all together (Miyazawa-Onami et al., 2017; Zou and Stillman, 1998). Here GINS, Cdc45 and MCM2-7 stably assemble to form the CMG complex, the active replicative helicase where the SDS complex dissociates to form the pre-initiation complex (pre-IC) (Figure 3B). After the formation of the CMG, MCM-10 is recruited where it forms homo-multimers and promotes conversion of the MCM2-7 complex from the double-stranded DNA (dsDNA) binding state into single-strand DNA (ssDNA) one through its interaction with MCMs complex (Figure 3C) (Van Deursen et al., 2012). The exposed ssDNA will recruit replication protein A (RPA), replication factor C (RFC), proliferating cell nuclear antigen (PCNA), DNA polymerase α and DNA polymerase δ to establish the active replisome complex where bi-directional DNA synthesis can start.

The basic principle of origin firing appears to be the same in metazoans. The majority of proteins described above have a homologue in metazoans like Treslin that plays the role of Sld3, TopBP1 which is an orthologue of Dpb11, RecQL4 the vertebrate Sld2, and MTBP which is the homologue of Sld7 (Fragkos et al., 2015).
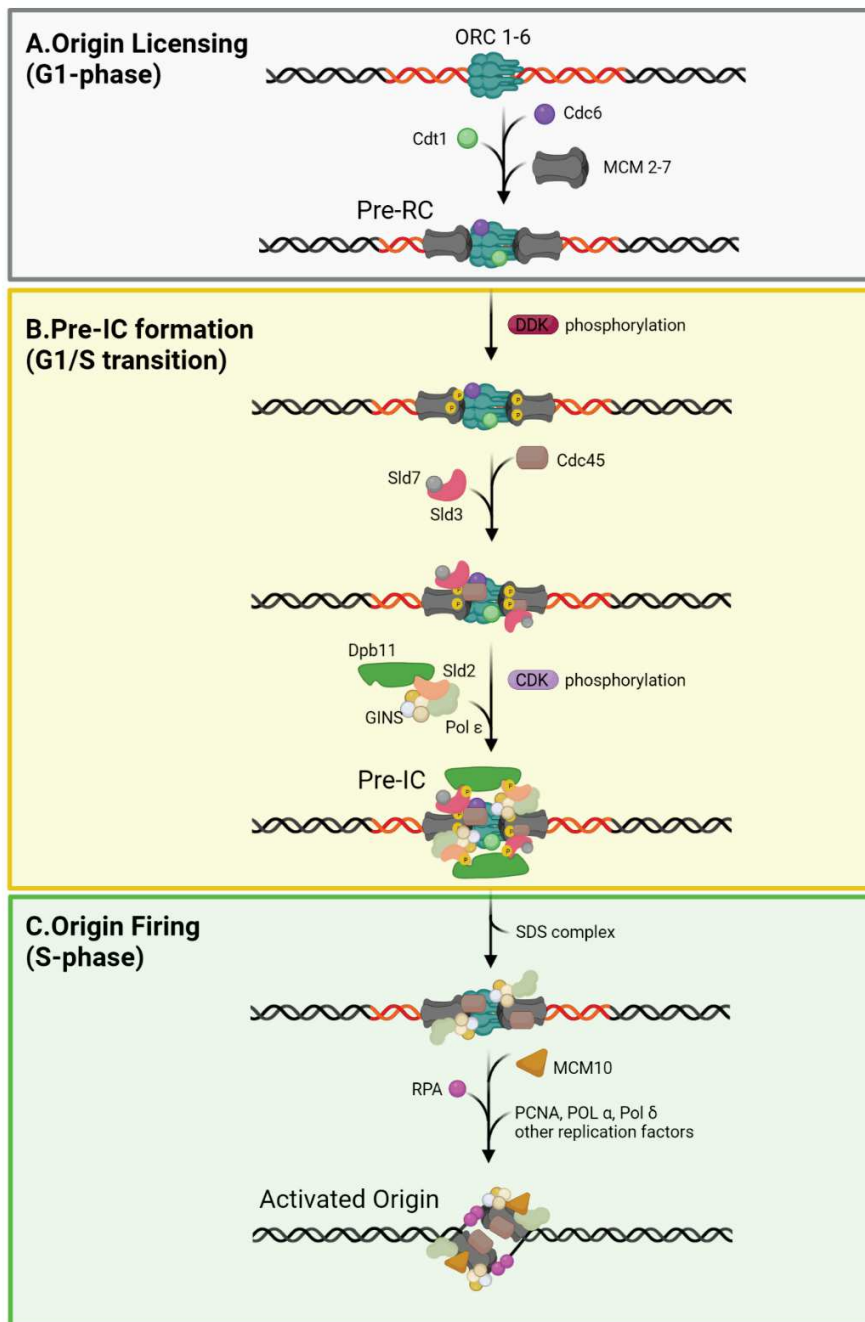
Figure 3. Molecular mechanisms of origin firing. Schematic representation of (A) origin licensing during G1-phase where pre-RC is formed by the sequential loading of ORC, Cdc6, Cdt1, and MCMs on all potential origins in the genome. (B) During G1/S transition, DDK and CDK dependent phosphorylations will recruit the different component of pre-IC. Finally, (C) Origin firing takes place during S-phase resulting in two active bi-directional replisomes.

4- Different Classes of Origins

Completeness of the replication in eukaryotes is a complex issue due to the fact that they have a large genome and limited replication time lasting from several minutes in yeasts to several hours in metazoans. Unlike bacteria, which need only one replication origin to replicate their genome, eukaryotic cells are equipped with multiple or even up to ten thousand replication origins to be able to carry on a faithful duplication of the genome. With multiple origins present comes the risk of large inter-origin distance (IOD) that may leave some un-replicated regions behind if not well monitored. To avoid this problem, origins must be regularly spaced, and the efficiency and the order of the origin firing must be well regulated.

Eukaryotic cells generate much more licensed potential origins than what is actively utilized for DNA replication in S-phase. In a study conducted in human and mouse cells, 30000-50,000 fully active replication origins were detected during S-phase. However, deep sequencing of short nascent DNA strands revealed ten times more replication sites with an average of 11 Kbp IOD (Besnard et al., 2012; Leonard and Mechali, 2013). This shows that DNA replication is carried out by a small subset of the available potential origins.

Replication origins can be categorized into three different classes depending on their use (Figure 4). The first class is the constitutive origins which represent the minority in eukaryotes. These are used all the time in every cell cycle or cell type and are set at the same position according to chromatin or transcriptional constraints. The second class is the flexible origins, which are potential ones that can be used stochastically in different cells. These explain the concept of the initiation zone, where multiple origins are found within the same domain such as the DHFR locus (Mesner et al., 2003). In this zone each cell will fire one of these origins; however, if a whole cellular population is analyzed all origins will be scored as active ones. This elucidates the nature of the stochasticity of origin firing. The flexibility of this origin is affected by different growth conditions, differentiation programs and DNA damage. The third class is the dormant origins. These are origins that are never used in unperturbed S-phase unless needed for facing endogenous replicative stress and are mainly replicated passively by upcoming replication

forks. In case of any genotoxic stress, dormant origin will have enough time to fire, and they replicate region between stalled forks. Thus, preserving the genomic integrity.
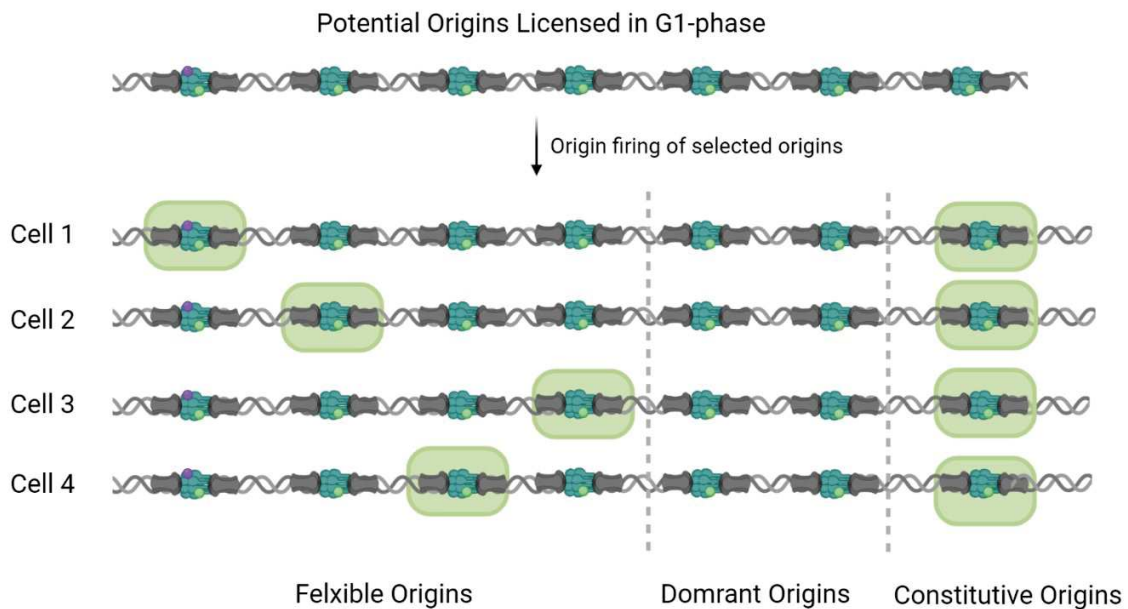
Figure 4. Different types of DNA replication origins. Potential DNA replication origins are licensed during the mitosis–G1 phase by the formation of the pre-RC. The selection of the origins that will be activated at the next S phase occurs during G1 phase according to the spatial and temporal regulations. Origins can be classified into different types. (1) Flexible origins that can be used differently in different cells. (2) Dormant origins that are rarely used except in cases of replication stress. (3) Constitutive origins that are always active, are set at the same position by chromatin or transcriptional constraints.

5- Regulation of Origin Firing

As mentioned above, the number of potential replication origins found in eukaryotic cells is much more than the actual number of origins that are fired during S-phase. What makes flexible origins vary in their activation pattern between different cell cycles and different cells is a notion known as replication origin efficiency. The major challenge is to try to understand how origins are determined, whether to be active or just remain potential. Is it a stochastic event as described in the literature? Or is it based on chromatin features? Although it seems that replication origins are being chosen stochastically, nevertheless

there are accumulating evidences showing that the choice of active origins is spatially and temporally regulated.

5.1- Spatial Regulation of Origin Choice

The selection of replication origins among many of the potential ones depends on the chromatin structure and epigenetics.

5.1.1- Genetic Determines

Several genetic elements were reported to play a role in the activation of potential origins. In metazoans, replication origins are more likely to be localized in CG-rich regions. For example, Origin G-rich repeat element (OGRE) was identified in mammalian cells (Besnard et al., 2012; Cayrou et al., 2011; Delgado et al., 1998). This element can form G-quadruplex (G4) that has the potential to affect the efficiency of replication origins (Valton et al., 2014). Some distal elements can also have an extensive effect on the choice of initiation sites. In humans, replication initiation of β-globin locus depends on the presence of a locus control region which is located upstream of the globin gene cluster. In addition to its role in controlling initiation, it also serves as a control element of β-globin gene expression (Aladjem et al., 1995).

5.1.2- Chromatin Structure

Chromatin structure was reported to be a crucial determinant for origin selection. In general, the presence of efficient replication origins is correlated with an open chromatin structure or euchromatin. It was reported in yeast and multiple metazoans that the presence of active origins overlaps with regions that are nucleosomes free (Eaton et al., 2010; Givens et al., 2012; Lubelsky et al., 2011). In yeast, ARS consensus elements are associated with nucleosome free regions and the positioning of a single nucleosome is sufficient to disturb the firing of this origin (Simpson, 1990).

Chromatin remodeling complexes are also important for the formation and efficiency of replication origins. In *S.cerevisiae*, mutations in the histone deacetylase Sir2 inhibit the activity of replication origins by promoting the position of nucleosomes at these sites (Crampton et al., 2008). On the other hand, it was shown that different acetylations of Histone 3 (H3) and Histone 4 (H4) could enhance replication initiation in a replicating

plasmid (Unnikrishnan et al., 2010). In human cells, histone methyltransferase binding to ORC1 (HBO1) is required for loading of the MCM complex (Iizuka et al., 2006). It also directly interacts with Cdt1 and enhances replication (Miotto and Struhl, 2008). Although the acetylation status of histones seems to be a key feature, it is not a universal feature of replication origins (Cadoret et al., 2008; Dazy et al., 2006; Grégoire et al., 2006). The methylation status of histones seems also important in defining active origins. For example, methylations of H3 were associated with replication origins. This includes H3k56me1, which is involved in recruiting PCNA (Yu et al., 2012), and H3k79me2, which might prevent re-replication events during cell cycle (Fu et al., 2013).

ORCs binding to origins in heterochromatin regions is harder than to origins found in euchromatin regions. For example, ORCs might be recruited via the interaction of ORC1 with heterochromatin protein 1 α (HP1α), which is a specific heterochromatin reader that recognizes H3k9me2 and H3k9me3 that are reported to promote gene silencing (Pak et al., 1997; Sherwood et al., 2010). The significance of this interaction could be explained by the fact that the recruitment of ORCs to less accessible chromatin structure is difficult; hence, the presence of a protein that recruits the ORC complex will facilitate this process.

5.1.3- Nuclear Structure

In eukaryotes, the nucleus is organized into subnuclear compartments, some of which play a role in the activation of replication origins. It was reported that the nuclear envelope is needed for replication origin activation but not for the assembly of the pre-RC (Newport and Spann, 1987; Sheehan et al., 1988). High concentrations of egg extract from *Xenopus laevis* is able to initiate DNA replication without the presence of a nuclear membrane, pointing to the fact that the nuclear membrane role may be to locally concentrate replication factors (Walter et al., 1998).
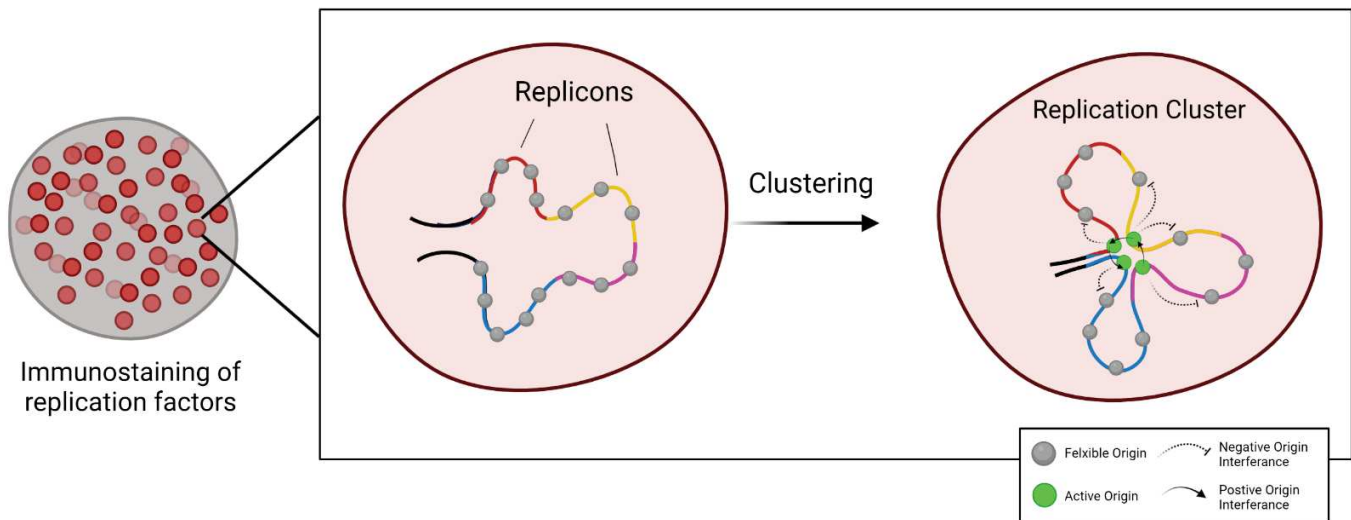
Figure 5. Organization of replication origins. Schematic representation showing a chromatin domain containing four replicon units (shown in different colors). Each replisome contains three to four potential flexible replication origins (gray circles) on average. These replicons are tethered together forming a replication cluster in which the origins that will be activated (one per replicon; green circles) gather within the cluster. In a cluster, DNA replication origins that interact (green circles) fire synchronously by the phenomena of positive origin interference. However, the fired origin within the replicon exerts negative origin interference on the other potential origins, thus inhibiting their firing.

DNA replication is organized in well-defined structures (Figure 5) (Huberman and Riggs, 1968) that are composed of the following: (1) pre-RC that assembles at all potential origins. (2) Replicons which are up to 50-120 kb in metazoan consisting of all potential replication origins in this sequence. In each replicon only one origin is chosen to be activated and the rest are kept dormant by a phenomenon known as negative origin interference (Lebofsky et al., 2006). However, these origins can fire in case of DNA damage or change in the cellular growth conditions. (3) All replicons are associated in a replication cluster/domain consisting of 400 kb to 1 Mb that are tethered by cohesins which were reported to organizes chromatin loops at DNA replication clusters (Guillou et al., 2010). The firing of replication origins in a replication cluster occurs synchronously through a mechanism known as positive origin interference (Marheineke and Hyrien, 2004). These active origins are brought all together forming the core of replication domain, and the rest of the replicons are organized in loops (Figure 5) (Buongiorno-Nardelli et al., 1982; Courbet et al., 2008) that are anchored to the nuclear matrix probably

by the help of Lamins (Moir et al., 1994). These structures form replication foci that can be visualized by immunofluorescence of some replication factors such as PCNA. The organization of replicons into loops could explain why only one origin is active. However, it is still not clear whether the formation of replication foci is what triggers their firing or whether their firing is what allows their clustering into replication foci.

5.1.4- Transcription

Transcription could regulate replication initiation negatively or positively. It affects the choice of replication origins directly or indirectly by changing the topology of chromatin nearby origins. It is reported that transcription events create strong negative supercoils behind the passage of the transcription machinery. In addition, the presence of two transcription bubbles will eventually lead to a strong negative supercoil in the intergenic region where most of origins are present, thus facilitating the opening of the double helix and the recruitment of initiation factors (Hayashi et al., 2007). Moreover, the presence of a transcription promoter in vicinity of replication origins may positively influence its activation (Ghosh et al., 2004; Kalejta et al., 1998). This could be due to the open chromatin status or the crosstalk between transcription factors and proteins involved in DNA replication initiation.

Although many origins are found in intergenic regions, they can also be localized within genes. In this case, it was reported that transcription could silence replication initiation at these origins (Haase et al., 1994; Sasaki et al., 2006). However, the majority of origins are most likely to be found within a non-coding region. And therefore, transcription like other elements, cannot be the only mechanism by which active origins are selected.

5.1.5- Origin Decision Point (ODP)

Nuclei isolated from early G1-phase in mammalian cells exhibit an unspecific pattern of replication when incubated with *xenopus* egg extracts (Dimitrova, 2006). However, after a certain point during the G1-phase, these nuclei showed a site-specific pattern during initiation. This specific time during the G1-phase is known as Origin Decision Point (ODP) at which replication origins are selected for firing (Wu and Gilbert, 1996). Reports have shown a possible crucial role of mitosis in reorganizing the nucleus, a necessary process

for the selection of origins that will be active. For example, after undergoing mitosis, differentiated nuclei showed shorter IOD, which correlated with the size of chromatin loops (Lemaitre et al., 2005). ODP is independent from the Time Decision Point (TDP), the restriction point that controls the timing of origin firing, and it seems to occur 2-3 hours following TDP.

5.2- Temporal Regulation of Origin Firing

Replication origins which were selected to be activated are not fired within the same time interval during S-Phase; however, they follow a temporal program known as the replication timing program. This program is biologically important for several reasons. First, it limits the number of replication forks at a given moment in order to avoid exhaustion of nucleotides, replication factors (Mantiero et al., 2011), and proteins required for replicative stress response that are in limiting amounts (Rivera-Mulia and Gilbert, 2016). Second, this program could be tightly regulated with transcription (Müller and Nieduszynski, 2017). However, replicated genes are subjected to mechanism that induce expression reduction (Padovan-Merhar et al., 2015; Voichek et al., 2016) . This program is executed by the intervention of several factors such as the localization and the topology of the chromosomes in the nucleus, the limiting concentration of replication factors, and proteins that directly control replication timing. Some of the features controlling the spatial regulation of origin firing may also influence the temporal one.

5.2.1- Time Decision Point (TDP)

During time decision point (TDP) chromatin domains move to the final position within the nucleus (Dimitrova and Gilbert, 1999). Single Cell Hi-C technology showed that in early G1-phase during TDP, chromatin interactions are re-established. TDP is a highly deterministic decision that occurs at the level of replication domains or clusters (Dileep and Gilbert, 2018; Hayashi et al., 2007).

5.2.2- Early and Late Replicating Domains

Replication timing domains are divided into early and late ones. Early replication domains are in general observed in accessible, transcriptionally active regions that possess active epigenetic marks, and are enriched with pre-RC complexes (Gineau et al., 2012; Sequeira-Mendes et al., 2009). On the contrary, late replicating domains are associated to origin-poor-domains that are characterized with low gene density and high repressive epigenetic marks of the heterochromatin. Different replication patterns have been described during S-phase which are early, mid, and late (Figure 6). They have been observed by immunostaining of replication factors or dNTP analogs incorporated into the DNA. During early S-phase euchromatin is mainly replicated, while in mid S-phase replication of facultative heterochromatin which constitutes mainly of ribosomal DNA takes place, and finally during late S-phase constitutive heterochromatin is replicated (Dimitrova and Berezney, 2002).
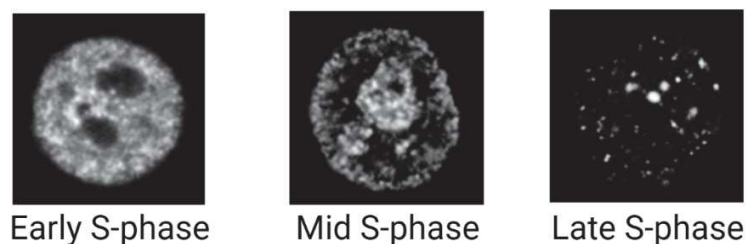
Figure 6. Patterns of DNA replication. Immunofluorescence images of EdU (dNTP analogue) showing the different patterns of DNA replication during S-phase.

5.2.3- Factors Defining Early and Late Replication Domains

i- Nuclear Localization of Replication Domains

Chromatin folding within the nucleus defines that two nuclear components, A and B, which closely correlate with the early and late replicating DNA (Figure 7). Compartment A correlates with actively transcribed chromatin that is diffused in the central regions of the nucleolus. Compartment B, on the other hand, correlates with regions of the chromatin localized to the nuclear periphery which are labeled as Lamina Associated Domains (LADs) (Vogel et al., 2007), and the ones localized near the nucleolus periphery known

as Nucleolar Associated domains (NADs) (Kind et al., 2013; Ragoczy et al., 2014). It was reported that late replication regions are located within LADs and NADs (Demeret et al., 2002; Sansam et al., 2010).
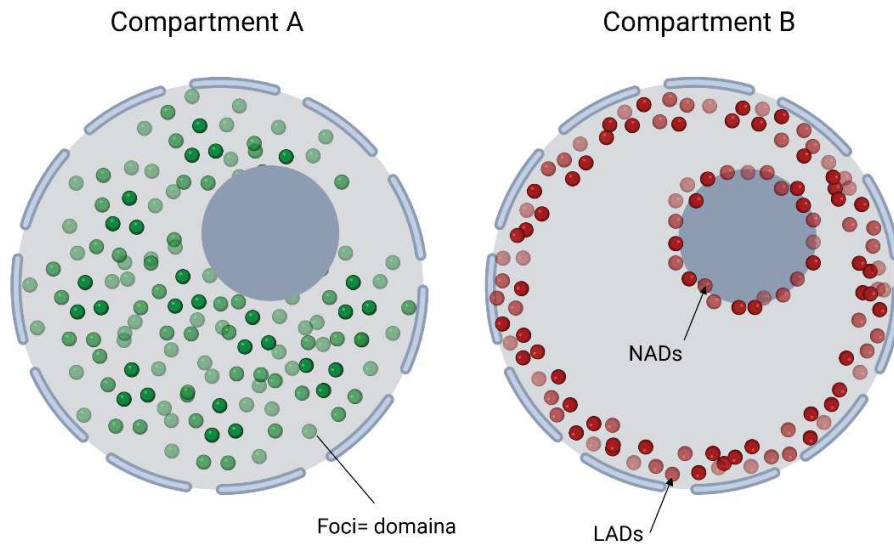
Figure 7. Nuclear localization of replication domains. Schematic Representation showing compartment A and B which corresponds to early and late replicating domains.

ii- Topology

The 3D organization of chromosomes is tightly related to the replication timing domains. Each chromosome is divided into distinct domains that are folded in a specific manner to interact with other domains, but not the ones adjacent. These domains are known as Topology Associated Domains (TADs). TADs were shown to overlap with replication timing domains (Christov et al., 2006); this supports the hypothesis that TADs could be playing a role in the determination of replication timing. However, it was shown that disturbing TADs didn't have an effect on replication timing (Oldach and Nieduszynski, 2019), so although TADs could be playing a role in replication timing, it is not sufficient to execute this alone.

iii- Epigenetic Modification

Epigenetic modification of the chromatin also has an important role in defining the replication timing of different domains. In budding yeast, depletion of the histone deacetylase Rpd3 causes earlier origin firing (Aparicio et al., 2004; Vogelauer et al., 2002), which is accompanied by an advanced binding of Cdc45. Moreover, depletion of Rpd3L, one of the members of Rpd3 complex, induces deregulation of more than 100 late firing origins (Knott et al., 2009). This suggests that histone deacetylation can directly influence the timing of replication initiation. Other studies also showed that Cdc45 loading is affected by the methylation status of H3, where it increases with H3k36me1 and decreases with H3k36me3 (Pryde et al., 2009). These results prove that regulation of replication timing requires multiple histone modifications.

iv- Limiting Factors

As described earlier, Cdc7 and CDK2 are the main kinases activating the initiation of DNA replication. In fission yeast, the increased level of the HSK1 catalytic subunit (homologue of Cdc7) or Dfp1 (homologue of Dbf4/Cdc7) accelerates origin firing efficiency (Patel et al., 2008; Wu and Nurse, 2009a). This indicates that the limited level of these two kinases is critical for controlling the timing of replication. This also applies for Cdc45 protein, where its overexpression led to increased origin efficiency (Patel et al., 2008). This control mechanism is linked to the chromatin accessibility. Histone modifications near origins could change the chromatin status, making it more or less accessible for Cdc7, CDK2, and Cdc45. Because these factors are limited, the firing of origins within less accessible regions is delayed until these factors are available again to induce their firing.

v- Proteins Controlling Timing Decision

In *S. cerevisae*, the forkhead transcription factors Fkh1 and Fkh2 are required for earlier replication of nearly 30% of origins (Knott et al., 2012a). The role of these two proteins is independent from the one in transcription. Fkh1 and Fkh2 bind in the vicinity of origins, where they promote clustering of early origins (Knott et al., 2012b) probably through

interaction with ORCs. This mechanism would help concentrate limiting factors around early replicating clusters (Knott et al., 2012a). In addition, these proteins are also able to recruit DDK kinase to early origins in order to control their firing (Fang et al., 2017). Another positive regulatory mechanism for early firing origins is executed by Ctf19 in budding yeast and Swi6 in fission yeast. Despite the fact that heterochromatin is typically a late replicating domain, pericentromeric heterochromatin is replicated in early S-phase in fission yeast. This depends on DDK recruitment by Ctf19 and Swi6 to the pericentromeric origins of the early replicating centromeres (Hayashi et al., 2009; Natsume et al., 2013).

   Another transacting element is the telomere associated protein RIF1. It was reported that RIF1 regulates the timing of mid S-phase replicating regions by restricting the accessibility of Cdc7, which delays origin firing. Depletion of RIF1 in mouse and human cells showed a strong change in the replication timing profile (Cornacchia et al., 2012; Yamazaki et al., 2012) where the mid S-phase pattern was completely lost and the early replication pattern remains present during the majority of S-phase. There are two possible mechanisms by which RIF1 might regulate the timing of these domains. First, RIF1 prevents the phosphorylation of MCMs by directly binding to PP1 to counteract the activity of DDK. Second, RIF1 also regulates nuclear organization. In the absence of RIF1, chromatin loops are more relaxed (Yamazaki et al., 2012) which could increase the accessibility of initiation factors to these replication domains. This possibility reinforces the concept of connecting replication timing with the nuclear organization. Recent studies had shown that the RIF1-PP1 interaction is required for both replication timing and nuclear organization (Gnan et al. 2021). However, the nuclear organization, but not the replication timing, was sensitive to the level of RIF1 dosage, indicating that these two processes are independent.

vi- ORC Binding during Mitosis

   The ORC complex is the first to recognize and bind to replication origins. The time when ORC binds to replication origins differs between species. In budding yeast, it was found to be in constant association with origins (Diffley et al., 1994). However, in Xenopus egg extracts, ORC binding is low at the beginning of mitosis, then peaks at

anaphase/telophase and is released from chromatin as S-phase is progressing (Romanowski et al., 2000). In humans, ORC1 (the first subunit to bind origins) binds to the DNA between the mitotic exit and G1-phase entry (Li and DePamphilis, 2002) . A strong correlation was found between the timing of ORC binding to replication origins and the origin efficiency (Wu and Nurse, 2009a). A study by Wu and Nurse showed that in fission yeast the periodic binding of the ORC complex during mitosis dictates the timing of origin firing during S-phase (Wu and Nurse, 2009b). Although no study was reported, this might also be dictating the replication timing program in metazoans.

6- Regulation of DNA Replication

During development, proliferating cells must produce only one copy of their genetic material prior to cell division; otherwise they might face genomic instability and aneuploidy. The way to control this is by the tight regulation of origin licensing and firing to prevent both endoreplication and re-replication. Several proteins act in pathways that negatively regulate origin licensing and firing (Ding and Koren, 2020). Moreover, the checkpoint activation during DNA replication is crucial in regulating origin firing and will be addressed in chapter 5.

6.1- Prevention of unscheduled endo-replication

When two consecutive S-phases take place without being followed by mitosis or cytokinesis is termed as endoreplication, which can be scheduled in developmental stages of flowers, amphibians, fish, and rarely in mammals (Zielke et al., 2013). Endoreplication is driven by the inhibition of CDK1 during G2/M and the oscillating levels of Cyclin E/CDK1 which initiate the pre-RC formation while cells have not gone through mitosis. In cases where cells are exposed to DNA damage, CDK1 will be inhibited leading to arrest at G2-phase or mitosis. If this arrest lasts for a long time, cells can either undergo mitotic death or in some cases they can undergo an event known as mitotic slippage where they skip through mitosis and cytokinesis to undergo G1-phase and another round of S-phase, thus unscheduled endoreplication. In normal cells, checkpoints are present to inhibit endoreplication (Greer Card et al., 2010). Furthermore, to prevent endoreplication

sister chromatids must be well separated when DNA replication is completed. This is maintained by protecting sister chromatid cohesion through S-phase and mitosis, untangling of sister chromatids at the end of S-phase and the proper sister chromatid segregation (Zielke et al., 2013).

6.2- Prevention of Re-replication

Re-replication could occur by relicensing or reactivation of an already existing origin or by licensing of a new one in a replicated region of the DNA. Re-replication leads to replicative stress and problems during mitosis. Thus, as cells go through S-phase, origin re-licensing and re-refiring should be completely inhibited until mitosis is completed. This is carried out by different mechanisms (Figure 8) including regulation of ORC binding, also known as the ORC cycle, regulation of Cdt1, and regulation of MCMs.

6.2.1- The ORC Cycle

The variation in behavior and post-translational modifications of the ORC complex during different phases of the cell cycle is known as the ORC Cycle. In yeast, ORC remains intact and stably bound throughout the cell cycle (Diffley et al., 1995; Fujita et al., 1998; Kong and DePamphilis, 2001; Liang and Stillman, 1997). However, ORC subunits undergo cell cycle dependent phosphorylation that inhibits their action just until mitosis. ORC2 and ORC6 are phosphorylated by Cyclin B/CDK1 during G1/S transition and remains hyper-phosphorylated until mitosis, where they get dephosphorylated for pre-RC assembly to take place (Romanowski et al., 1996).

In Xenopus, the ORC complex is also stable; however, its affinity for DNA in egg extracts decreases once pre-RC is assembled. If Xenopus egg extracts are incubated with the sperm chromatin ORC binds to the chromatin to initiate the pre-RC assembly, and it remains stable until it gets phosphorylated by Cyclin A/CDK1 during mitosis, which leads to its dissociation. However, if somatic cell chromatin is added to the extract, the ORC complex loses its affinity directly after the formation of the pre-RC and not during mitosis (DePamphilis, 2003).

In mammalian cells, the events occurring during the ORC cycle are different. With the exception of ORC1, all other ORCs subunits are stable on chromatin throughout the cell cycle. During the G1-phase the level of ORC1 is stable, however it was shown in tumor cells that ORC1 is degraded during S-phase (Kreitz et al., 2001; Méndez et al., 2002; Nguyen et al., 2001; Tatsumi et al., 2003). It was reported otherwise in Chinese hamster ovary (CHO) cells that the level of ORCs remains stable but its affinity to the DNA decreases during S-phase (Li and DePamphilis, 2002; Natale et al., 2000). Therefore, ORC1 is subjected either to post-translational CDK-dependent phosphorylation and/or ubiquitin-dependent degradation (Li et al., 2004; Méndez et al., 2002). Restoration of ORC binding to chromatin during M/G1 transition follows the same time course of cyclin B degradation, suggesting that the mitotic exit is a prerequisite for establishment of ORC1 binding to the DNA. Since ORC1 binding to replication origins is essential for ORC binding to DNA, losing ORC1 means that the other subunits will be destabilized (Lee et al., 2012; Siddiqui and Stillman, 2007). And since Cdc6 binding to DNA is dependent on ORC2-6, destabilizing ORC binding to DNA will therefore destabilize the pre-RC reassembly. Moreover, Cdc6 is also targeted for proteasomal degradation in human cells (Kalfalah et al., 2015). In another study using HeLa cell lines, ORC 2-5 complex was also shown to dissociate from replication origins by Cyclin A/CDK2 dependent phosphorylation of ORC2 (Lee et al., 2012). Therefore, there are multiple mechanisms that control the ORC cycle that would need further investigation.

6.2.2- Cdt1 Cycle

Cdt1 has an essential role in loading the MCMs onto DNA; thus, its regulation is a key control mechanism that inhibits re-replication. In *S. cerevisae*, Cdt1 activity is regulated by CDK-dependent phosphorylation that inhibits its interaction with ORC6 (Chen et al., 2007) and induces its nuclear export during the G1-phase. However, in *S. pombe*, Cdt1 is subjected to degradation upon S-phase entry (Gopalakrishnan et al., 2001; Nishitani et al., 2004; Wohlschlegel et al., 2000; Zhang et al., 2010). During S-phase, Cdt1 interacts with PCNA where it gets ubiquitinated by Cullin ring ligase (CRL4) and is subjected to proteasomal degradation (Arias and Walter, 2005, 2006). Another possible pathway for Cdt1 degradation is CDK dependent phosphorylation which leads to its recognition by

SCF E3 ubiquitin ligase (Kondo et al., 2004). Cdt1 ubiquitination could be reversed by ubiquitin hydrolase USP37 that would stabilize Cdt1 and promote assembly of the pre-RC complex (Hernández-Pérez et al., 2016).

Another mechanism exists where Cdt1 can be bound to Geminin, a protein that is highly expressed during S-phase. This protein interacts with Cdt1 and inhibits its binding to MCM6. Cdt1-Geminin interaction restricts Cdt1 activity to the G1-phase, which prevents the re-replication (Cook et al., 2004; Lutzmann et al., 2006). On the other hand, although Geminin inhibits Cdt1, it preserves a subset of Cdt1 by protecting it from degradation (Ballabeni et al., 2004). Thus, during the G1-phase, Cdt1 could be released from Geminin and directly promotes pre-RC assembly.

6.2.3- Helicase Regulation

Although the main mechanisms to inhibit the re-replication reside in the ORC cycle and Cdt1 regulation, MCM helicases can also be regulated to prevent re-replication. In *S. cerevisiae*, CDK targets MCM2-7 to prevent its interaction with ORC-Cdc6 complex. CDK action occurs through inducing the nuclear export of MCM 2-7 (Labib et al., 1999; Liku et
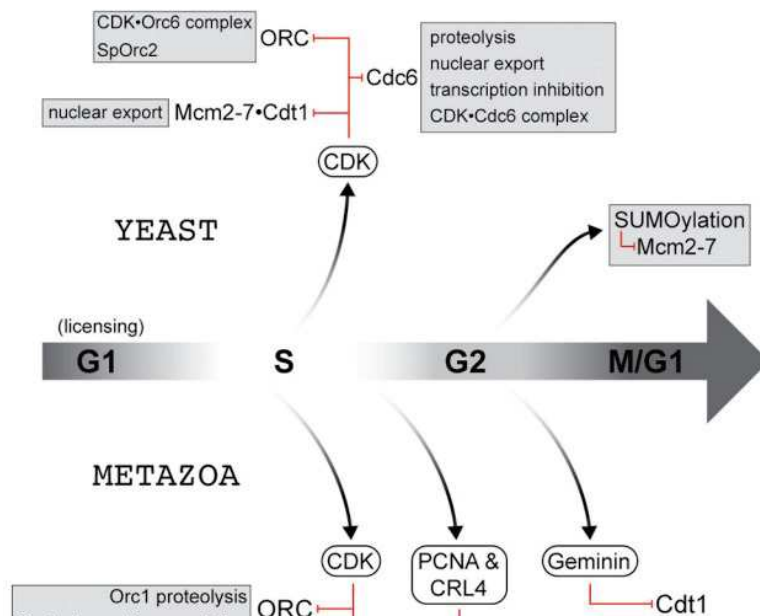
Figure 8. Mechanisms preventing re-Replication. Different mechanisms are applied to prevent re-licensing and re-firing of replication origins after initiation of S-phase in both yeast and metazoan. Adapted from Parker *et al.* 2017

al., 2005; Tanaka and Diffley, 2002). A new mechanism has also been described which involved SUMOylation of the MCM hexamer in the G1-phase. The presence of SUMO inhibits phosphorylation of MCMs that would activate initiation (Wei and Zhao, 2016).

7- Non-Replicative functions of ORC2

Despite the fact that the ORC complex is the first building block of the pre-RC complex formation, several studies have reported the involvement of ORCs in other processes. ORC subunits were shown to be expressed in terminally differentiated mammalian cells that do not undergo any cellular division (Thome et al., 2000), which supports the presence of non-replicative functions of ORCs. These functions include the formation of heterochromatin, chromosomal condensation and segregation, centrosome division, cytokinesis, and gene expression.

One of the aims of this study that will be described elsewhere was to understand the relationship between ORC2 and our protein of interest. ORC2 was reported to be involved in heterochromatin formation. In *S. cerevisiae*, a genetic screen has identified that mutations in ORC2, as well as ORC5, leads\ to defects in establishing silent mating type loci (HMR and HML) (Gineau et al., 2012). Moreover, ORC2 was shown to contribute to the heterochromatin formation in Drosophila. Mutations in ORC2 led to changes in the localization of heterochromatin protein 1 (HP1), a protein involved in the position effect variegation and heterochromatin formation. ORC1 was found to interact with HP1 through its N-terminal domain (Pak et al., 1997). However, ORC1 is degraded after the entry into S-phase (Sun et al., 2002); in contrast, ORC2 is associated with HP1 for the rest of the cell cycle.

HP1 is able to recognize H3K9 methylation and is important for inducing gene silencing and centromere functions. Although ORC2 depletion affects the localization of HP1 to the chromatin, it did not affect these modifications (Prasanth et al., 2004). This suggests that ORC2 recruits and maintains HP1 to these regions and not that HP1 recruitment induces these modifications. The interaction between ORC and HP1 was confirmed also in mammalian cells (Auth et al., 2006; Prasanth et al., 2004, 2010). However, depletion of

each subunit of the ORC complex had a different effect on the localization of HP1 on heterochromatin. with respect to ORC2, its depletion was found to be important for recruiting HP1 to centromeric regions (Prasanth et al., 2010).

Recent studies reported an additional protein which interacts with the ORC complex named as ORC Associated protein (ORCA) and otherwise LRWD1. ORCA associates with ORC and histone methyltransferases (HMT) in one complex that is important in heterochromatin formation through installing repressive histone modifications (Giri and Prasanth, 2015). ORCA levels decrease at G1/S transition by a ubiquitin dependent degradation mechanism. ORCA is polyubiquitinated at the WD40 domain, the same domain required for its interaction with ORC2 (Shen and Prasanth, 2012). Indeed, depletion of ORC2 was reported to induce ORCA degradation, indicating that binding of ORCA to ORC2 protects it from degradation. It was proposed that after G1, ORC2 might be released from chromatin and then ORCA would be subjected to degradation in order to insure a proper program of origin firing. The regulation of ORCA along the cell cycle which leads to less ORCA-ORC2 complex might also contribute to ORC2 function in the replication of specific genomic sites or other unknown functions. Indeed, it was demonstrated in human cells that the downregulation of ORCA leads to changes in the timing of late replicating regions (Wang et al., 2017b) due to a change in the chromosomal organization.

ORC2 was also shown to localize to the centromere during G2/M phase where it is modified by SUMO2 that is important for the recruitment of the histone demethylase KDM5A to the centromeric region (Huang et al., 2016). KDM5A converts H3k4me3 into H3k4me2, a permissive histone mark that allows the transcription of α-satellites at the centromeres. This transcript is crucial for heterochromatin silencing and inhibition of re-replication. Thus, ORC2 is important to maintain the genomic stability of this genomic region.

Another possible function of ORC2 is its role in sister chromatid cohesion. This function is not exclusive only to ORC2 but seemingly for all ORCs. It was reported that depletion of ORC2 during G1 would lead to disruption of sister chromatid linkage in a mechanism independent of the function of cohesins in linking sister chromatids. Loss of chromosome

pairing was observed in specific loci, such as near telomeric and centromeric regions as well as the middle of the chromosome long arm (Shimada and Gasser, 2007). How exactly ORC sustains sister chromatid linkage was not elucidated; however, possible mechanisms have been proposed. It may be involved in forming a special chromatin feature that promotes pairing of sister chromatids, or it might be that the ORC might serve as or recruits bridging factors to link sister chromatids other than cohesins. Although this function is well reported in yeast, there is no proof that it exists in mammalian cells. Therefore, it is clear that ORC has other functions than DNA replication, but these other functions are indirectly affecting DNA replication and genomic stability.

Chapter 3: DNA Replication

As originally hypothesized by Watson and Crick (Watson and Crick, 1953) and proven by Meselson and Stahl (Meselson and Stahl, 1958), DNA replication is carried out in a semi-conservative manner. DNA replication is a process that is composed of three phases: Initiation (previously described in chapter 2), elongation, and termination. The replication fork is composed to two antiparallel replicating strands: (1) the leading strand which is replicated continuously (5' to 3') in the same direction of the unwinding helicase, (2) the lagging strand which is replicated discontinuously in the opposite direction to the movement of the replication fork.

It was reported that about 40-50 proteins are needed to constitute the replisome in eukaryotic cells (Littlechild, 2013). Nonetheless, with new methods to identify replisome components such as iPOND (**i**solation of **P**roteins **O**n **N**ascent **D**NA) (Figure 9) the number of new proteins associated with the replisome is in constant increase.
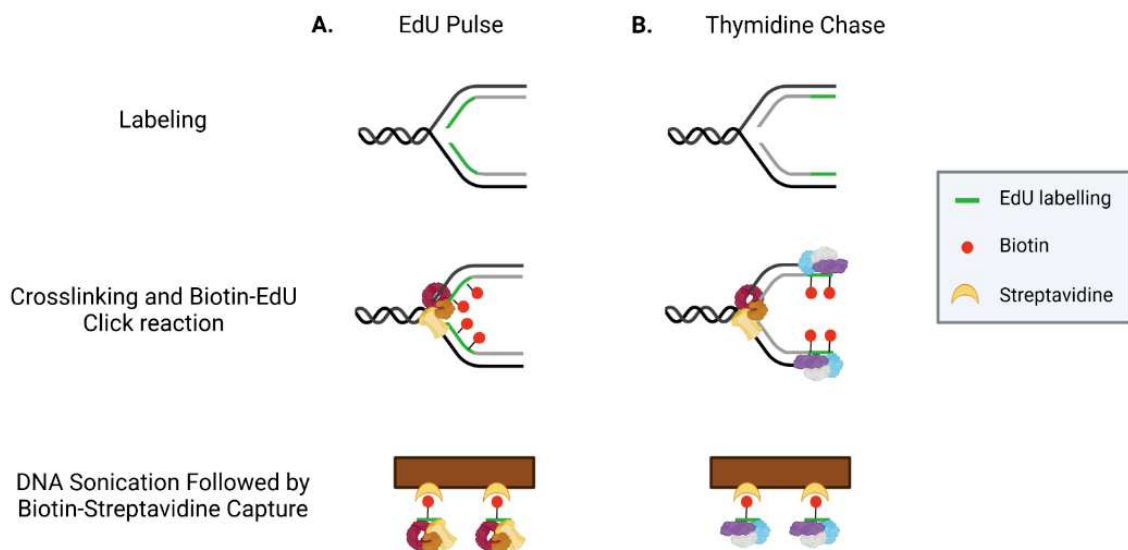
Figure 9. Schematic representation of the iPOND technique. **A.** Pulse condition aims to detect proteins associated with the replication machinery. Newly synthesized DNA is labeled with EdU. This is followed by proteins crosslinking to the DNA and coupling of EdU to biotin using the Click-it reaction. Finally, biotin-labelled DNA-protein mix is captured using streptavidin beads. **B.** Chase condition aims to identify proteins involved in chromatin maturation. Newly synthesized DNA is labeled with EdU followed by a thymidine chase. The rest of the steps are common with the pulse condition. Captured proteins can be analyzed using Western Blot or mass spectrometry.

1- DNA replication Elongation

Initiation of DNA replication is associated with a change in the status of the MCMs from an inactive form encircling dsDNA to an active form where it shifts to encircling ssDNA after unwinding of the dsDNA helix using the energy from ATP hydrolysis. At least three DNA polymerases are associated with the replisome: DNA polymerase (Pol) α, DNA Pol ε, and DNA Pol δ (Figure 10). The unwinding of dsDNA generates ssDNA that will be recognized and bound by RPA, which is a heterotrimeric complex composed of RPA70-RPA32-RPA17. The RPA complex binds ssDNA protecting it from nuclease dependent degradation (Krasikova et al., 2016; Liu and Huang, 2016; Oakley and Patrick, 2010) and recruits DNA Pol α, the only DNA polymerase that can start the process of DNA synthesis. DNA Pol α is a polymerase/ short RNA primers primase complex that synthesizes for the leading and the lagging strands (Littlechild, 2013; Oakley and Patrick, 2010).

After primer synthesis, polymerase switching occurs. In the leading strand, DNA Pol α is replaced by DNA Pol ε, which is recruited via a strong physical interaction with the GINS complex. This interaction tethers the polymerase to the CMG, placing it behind the helicase and giving it the right processivity to duplicate the leading strand (Langston et al., 2014). On the other hand, the lagging strand, which is repeatedly primed and synthesized, DNA Pol α cooperates with DNA Pol δ to carry out the replication process and produce discontinuous DNA fragments known as Okazaki fragments (Burgers and Kunkel, 2017; Lujan et al., 2016). DNA Pol δ is not a part of the replisome; however, it is recruited to the lagging strand primer-template junction after the loading of the clamp protein PCNA. The interaction between DNA Pol δ and PCNA gives the former the processivity to replicate the lagging strand (Georgescu et al., 2015).

The extra events needed for the replication of the lagging strand suggest that the lagging strand polymerases might be faster in order to catch up with the leading strand polymerases. However, it was shown that both polymerases synthesize DNA at the same speed (Graham et al., 2017), and that replication is often disturbed by different barriers which oblige the helicase to slow down so that it will not be uncoupled from the polymerases (Graham et al., 2017).

DNA replication elongation requires other multiple factors:

- Replication Factor C (RFC): the clamp loader which assembles the sliding clamp PCNA (Boehm et al., 2016; Kelly, 2017).

- Topoisomerases I and II: two enzymes critically essential for the relieving of topological stress by resolving the supercoils generated infront of the forks that form due to the unwinding of the double helix (Lodish et al., 2000).

- Flap endonuclease 1 (FEN1): Structure-specific nuclease, which is recruited by PCNA. It is in charge of cleaving the 5' overhangs composed of RNA primers and DNA that are generated by displacement synthesis of the lagging strand. Its actions leave behind a nick that is sealed by Ligase I (LIGI) (Balakrishnan and Bambara, 2011).

- Replication Pause Complex: A complex composed of TIMELESS, Tipin, Claspin, and And1 proteins. This complex coordinates the DNA unwinding by helicases and the activity of DNA polymerases and functions as a fork accelerator (Errico et al., 2009; Kilkenny et al., 2017).

- The Cohesin Complex: A complex composed of SMC1, SMC3, Rad21, and SA1/2 (Remeseiro and Losada, 2013; Sherwood et al., 2010). This complex is responsible for maintaining a physical link between the sister chromatids during DNA replication and therefore ensures their proper segregation during mitosis.

- Mismatch proteins: these proteins are implicated in correcting the occasional mismatches formed by the DNA polymerases during replication. They include MSH2 and MSH6 (Kunkel and Erie, 2015).

- Chromatin remodelers: these include factors such as CAF1, FACT, BAZ1B-SNF2h, EHMT1/2, and DNMT1. These factors play a key role in facilitating and regulating DNA replication through chromatin modification and the propagation of epigenetic information to the newly synthesized DNA during DNA replication (Falbo and Shen, 2006).
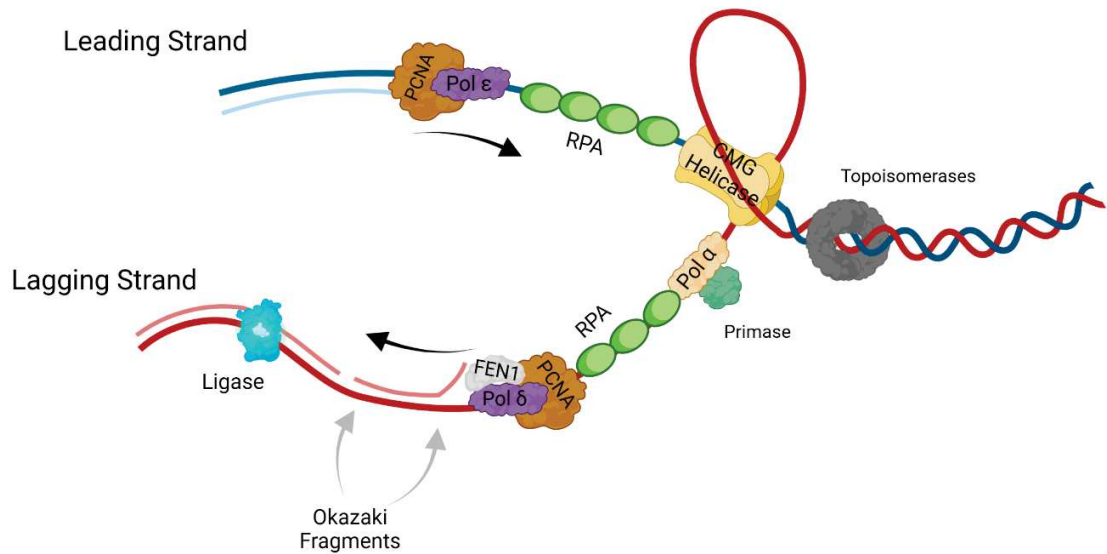
Figure 10. Replication fork structure. The CMG complex is responsible to the unwinding to the double strand helix. Topoisomerases are acting ahead of the CMG complex to relieve the topological stress. DNA Pol ε is synthesizing the leading strand, and DNA pol α together with DNA Pol δ are synthesizing the lagging strand. CMG helicase: CDC45, MCM2/6, and GINS. Pol: DNA polymerase.

2- DNA Replication Termination

Replication termination occurs all through S phase when two converging replication forks meet after finishing the replication of their corresponding DNA fragments with the help of Pif1 and Rrm3 DNA helicases (Deegan et al., 2019). This process occurs in several steps:

1- The topological stress caused by the accumulation of positive supercoils between the two forks must be relieved by the action of Topoisomerase I and II (Pommier et al., 2016).

2- The two CMGs of the opposite strands encounter each other.

3- The replisome will disassemble with the help of SCF/CRL2 that mediates ubiquitination and extraction from the chromatin thanks to p97 ATPases (Dewar and Walter, 2017).

4- Finalization of DNA synthesis is accomplished by completing the gap between the end of the leading strand and the Okazaki fragment from the opposite lagging strand.

Chapter 4: DNA Damage Response

Preserving the genomic sequence from mutations is essential for protecting against cancer development and early cellular ageing. Moreover, it is also crucial in order to avoid transmission of any mutations to the offspring. DNA is composed of nucleotides which in nature are intrinsically reactive molecules, highly prone to chemical modification when exposed to different types of damaging agents. Moreover, the natural process of DNA replication and repair mechanisms may also burden the cell with an excess of mutations. It had been estimated that every cell may experience up to 10^5 lesions per day (Liu et al., 2012). However, cells are able to combat this by a plethora of proteins that take part in pathways of DNA damage signaling, repair, damage tolerance, cell cycle checkpoints, and cell death. All these pathways are collectively functioning to diminish the deleterious consequences of DNA damage.

When the cell is subjected to damaging sources, different pathways of DNA damage repair and response (DDR) are activated to signal and repair the damage taking place. The major DNA repair pathways are: (1) Base Excision Repair (BER), (2) Nucleotide Excision Repair (NER), (3) Mismatch Repair (MMR), (4) DNA-Protein crosslink (DPC) repair (5) Homologous Recombination (HR) and (6) Non-Homologous End Joining (NHEJ). Some types of damage could also be repaired by a simple chemical reversal or by interstrand crosslink (ICL) repair. The pathway of choice is dictated by the type of DNA damage occurring and the stage of the cell cycle. On the other hand, there are subtle cases where the cell decides to endure the damage through the activity of DNA damage tolerance pathways.

When the amount of DNA damage is too high for the cell, programed cell death or apoptosis is activated to eradicate the cells with genomic instability. Expectedly, many cancers are favored by mutations in DDR pathways that would increase the rate of mutations and genomic instability, thus favoring the progression of cancer (Bouwman and Jonkers, 2012; Ghosal and Chen, 2013).

1- Sources and Types of DNA Damage

Based on their origin, DNA damage sources are categorized into two main classes: endogenous and exogenous sources (Figure 11).

1.1- DNA Damage Induced by Endogenous Sources

DNA damage could result from nucleotide base deamination, which occurs when the nucleotides Cytosine (C), Adenine (A), Guanine (5), and 5-methyl Cytosine (5mC) lose their exocyclic amine thus becoming Uracil (U), Hypoxanthine, Xanthine and Thymine (T), respectively. In the case of Cytosine deamination, the native C:G base pair will be altered into U:A base pair and if not repaired before DNA replication will lead to stable sequence mutation CG → TA. In addition to the natural endogenous deamination, environmental sources such as UV and some intercalating agents can enhance the base deamination of the nucleotides (Chen and Shaw, 1993; D'Ischia et al., 2011; Ikehata and Ono, 2011; Moyer et al., 1993).

Abasic site (AP) is another type of DNA damage that can occur as a spontaneous event that is triggered by extreme pH or high temperature, or by the action of DNA glycosylase during the BER pathway (Lindahl, 1993; Wang and Smith, 2008). AP sites arise when the N-glycosylase bond, which links the nitrogenous base and the sugar phosphate is hydrolyzed. AP sites could be transformed into single strand breaks (SSB), a type of DNA damage discussed elsewhere (Bailly and Verly, 1988).

Reactive Oxygen Species (ROS) are natural byproducts of the electron transport chain that occurs during cellular respiration (Henle and Linn, 1997). At low concentrations, ROS are important for normal cellular processes (Friedberg et al., 2005); however, when produced at high concentrations, ROS can lead to ~100 different types of oxidative base lesions such as the formation of 8-oxo guanine (Henle and Linn, 1997).

On the other hand, endogenous DNA damage could also be developmentally programmed. For example, during meiosis, Spo11 triggers the formation of DNA double-strand breaks (DSBs) that initiates a recombination mechanism that promotes new combinations of genes (Yadav and Claeys Bouuaert, 2021). This is essential in maximizing the genetic diversity of the offspring. Another example is the DSB induced by

the action of RAG1 and RAG2, two proteins that are exclusively expressed in lymphocytes during development. These breaks induce the rearrangement and recombination of the genes encoding immunoglobulin and T cell receptor molecules, thus creating the repertoires of the B and T lymphocytes (Nagafuchi et al., 2004). Moreover, telomeres resemble DSBs and also their shortening induces a DNA damage response (Raynaud et al., 2008).

1.2- DNA Damage Induced by Exogenous Sources

Exogenous sources can be divided into physical and chemical ones. Physical genotoxic agents include ionizing radiation (IR). IR is abundant such as ones coming from microwaves from an oven, X rays from an X-ray tube, and can damage DNA either directly by inducing DNA breaks (particularly DSBs) or indirectly by promoting radiolysis of water molecules into highly reactive radicals (•OH) (Desouky et al., 2015; Friedberg et al., 2005). Ultraviolet (UV) radiation is another type of physical genotoxic agent. UV radiation emanates mainly from the sun, and it can damage the DNA by inducing the formation of covalent links between two adjacent pyrimidines including thymidine dimers. Exposure to high levels of UV may lead to diseases such as skin cancer/melanoma in humans (Rastogi et al., 2010).

Chemical exogenous sources include alkylating agents. They are mainly produced from tobacco smoke, biomass burning, industrial processes, and importantly, several chemotherapeutic agents (Grutzen and Andreae, 1990; Lawley, 1966; Pegg, 1990). For example, methyl methanesulfonate (MMS) is an alkylating agent that can methylate the DNA and induces mutations in guanines and adenines that lead eventually to AP sites (Wyatt and Pittman, 2006). Another example is nitrogen mustard, a chemical weapon used during the First World War. This agent induces the formation of intra/intercrosslinks and DNA-protein crosslinks (DPC) that can block the metabolic activity of the DNA (Lawley, 1966; Pegg, 1990). Chemotherapeutic alkylating agents include cisplatin, a platinum compound that is used to treat a variety of cancers (Dasari and Bernard Tchounwou, 2014). Cisplatin can create a crosslink with the urine bases on the DNA. Thus preventing its repair and leading to DNA damage and subsequently apoptosis. Another

type of chemical genotoxic agents are aromatic amines that are produced from cigarette smoke, pesticides, and high temperature cooking (Sugimura, 1986). Aromatic amines can be converted to alkylating agents that attack guanines, leading eventually to base substitution and frameshift mutations (Mah et al., 1989). Natural toxins are also one type of exogenous genotoxic agents, such as ones produced naturally by microorganisms as a defense mechanism (Ames et al., 1990). Aflatoxins are one good example. It is produced by *Aspergillus parasiticus*, a type of fungi, and can attack guanines resulting in its depurination (Essigmann et al., 1977).
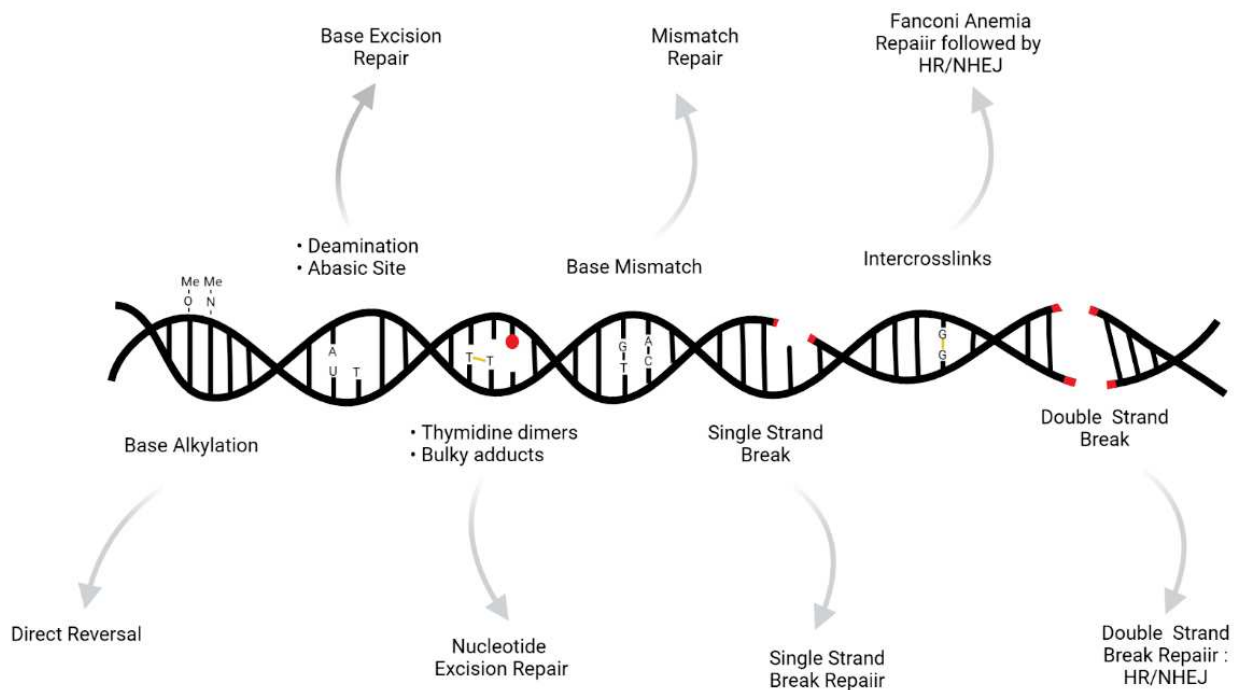
Figure 11. An overview of different types of DNA damage and their corresponding repair pathways. DNA is continuously assaulted by different type of lesions from base alkylation to double strand breaks. The choice of the repair pathway depends mainly on the type of lesions, however, could also be affected by the stage of the cell cycle.

Chemotherapies that induce the formation of DNA-protein crosslinks are important examples of exogenous sources of DNA damage. Examples are camptothecin (CPT) and etoposide (ETP), two natural molecules that specifically inhibit the action of Top1 and Top2, respectively. As discussed previously, Top1 and Top2 are involved in relieving the topological stress resulting from DNA replication and transcription (Baldwin and Osheroff, 2005; Pommier, 2006). During this process, both topoisomerases induce breaks into the DNA helix during which they become covalently linked to the DNA for a short period of time before they re-ligate the break. When the cells are exposed to CPT or ETP, the bond is transformed into a DPC and leaves behind SSB or DSB, respectively.

2- DNA Damage Repair

2.1- Repair of Base DNA Damage

2.1.1- Reversal of DNA Damage

There is a small subset of DNA lesions (alkylated bases and UV photo lesions) that are simply reversed by an error-free process. Two different classes of enzymes are responsible for the reverse of alkylated bases in mammals. The first is the O^6-alkyl guanine DNA alkyl transferase (AGT) enzyme. AGT is able to reverse alkylation in a one-step reaction by transferring the alkyl group from the oxygen molecule of the DNA base to the cytosine residue found in its catalytic pocket (Kaina et al., 2007). The second is the AlkB-related α-ketoglutarate-dependent dioxygenases (AlkB) which oxidize the alkyl group inducing its release as a formaldehyde molecule, thus recovering the original base (Drabløs et al., 2004).

2.1.2- Base Excision Repair (BER)

BER is in charge of repairing base lesions such as oxidation, deamination, alkylation, and AP sites (Figure 12A). Although these lesions are small lesions, they can be highly mutagenic if not well repaired. This repair process is mainly active during G1 phase (Machida et al., 2005), where lesions are first recognized by DNA glycosylases (Odell et al., 2013). There is at least 11 different DNA glycosylases (Huffman et al., 2005; Kovtun

et al., 2007) classified as monofunctional or bifunctional, that remove the damaged base leaving behind an AP site which will be repaired later by short-patch repair or long patch repair, respectively (Machida et al., 2005).

2.2- Repair of multiple and Bulky Base Damage

2.2.1- Nucleotide Excision Repair (NER)

NER is the main pathway to remove bulky adducts created by UV radiation and damaging chemotherapeutic agents (Figure 12C). Any deficiency in this pathway could lead to serious outcomes that are manifested by diseases such as Xeroderma Pigmentosis (XP), a skin cancer predisposition syndrome. The main damage sensor of NER is Xeroderma-Pigmentosis-Complementation C (XPC) which forms a complex with other factors in order to recruit the specific endonucleases XPG and XPF-ERCC that are in charge of cleaving and resecting the damaged strand within a short distance from the 3' and 5' ends of the lesion, respectively (Fagbemi et al., 2011). This is followed by the recruitment of PCNA, RFC, and either of DNA Pol δ, DNA Pol ε, or DNA Pol κ in order to fill the gap left behind by the action of the nucleases. The final step is the ligation of the newly synthesized fragment that is carried by either LIG1 or XRCC1-LIG3 (Moser et al., 2007).

2.2.2- Mismatch Repair (MMR)

MMR is the pathway of choice that ensures replication fidelity (Figure12B) (Kunkel, 2009). The mismatch repair machinery can distinguish between the newly synthesized strand and the template (parental) thus scanning for any mismatches in the newly incorporated bases. MMR repairs mismatches that occur during DNA replication and insertion-deletion loops (IDLs) that result from strand slippage events within repetitive sequences (Friedberg et al., 2005). MutS is responsible for detecting the mismatches that could be at the level of one base, one –to-two nucleotide IDLs, or long IDs. MutS recruits MutL which creates a nick that is recognized by the MCM9 helicase in charge of unwinding the mismatch containing strand that will be subjected to digestion by Exonuclease 1 (EXO1) (Kadyrov et al., 2006). DNA Pol δ, RFC, high mobility group box 1 (HMGB1), and

LIG1 performs the final steps of DNA synthesis and ligation (Genschel and Modrich, 2003).

2.2.3- Intercrosslink (ICL) Repair

ICL occurs when two bases from complementary strands become covalently linked due to exposure to DNA damaging agents such as MMC. ICL, in addition to other similar lesions such as intra-crosslinks and DPCs are recognized and resolved by the Fanconi Anemia (FA) proteins (Figure 12D). To date, 21 Fanconi anemia proteins have been identified known as Fanconi Anemia Complementary Groups (Bluteau et al., 2016). In addition to these 21 proteins four other proteins have been described as a part of this pathway such as Fanconi Anemia Associated Protein (FAAP) and MpH-associated Histone-Fold proteins (MHF) (Ciccia et al., 2007; Yan et al., 2010). Upon recognition of the ICL, FANCM is recruited along with FAAP24 and MPH. This complex remodels the replication fork into a Holliday Junction and creates single stranded DNA (ssDNA) that will activate the ATR pathway and its main effector Chk1. Chk1 will phosphorylate FANCE, FANCD2, FANCI, and the nuclease complex MRN (Mre11-Rad50-NBS1) (Andreassen et al., 2004; Duquette et al., 2012; Smogorzewska et al., 2007; Wang et al., 2007). Next, the core complex will assemble at the damaged site and activate FANCDI/FANCD2 heterodimer through FANCL-dependent monoubiquitination (Smogorzewska et al., 2007). Subsequently, 5'-3' DNA excision will commence by the structure specific endonucleases (Clauson et al., 2013). The final repair step of the ICL could either occur by HR if the cells are in S-phase or by NER and TLS polymerases if the cells are in a non-proliferative state (Clauson et al., 2013)

2.3- Translesion Synthesis (TLS)

Translesion synthesis is a DNA damage tolerance process by which the replisome copies aberrant DNA lesions such as thymidine dimers or AP sites. TLS is carried out by TLS polymerases, specialized polymerases with lower fidelity than the canonical replicative polymerases (Waters et al., 2009). The switching from a replicative polymerase into a TLS polymerase is promoted by the ubiquitination of PCNA by RAD18 (Tian et al.,

2013). A total of eleven TLS polymerases are known so far including Rev1, Pol ζ, Pol κ, Pol η, and Pol ι. Notable features of these polymerases are limited sequence homology and the absence of a 3'-5' exonuclease domain for proof reading (Waters et al., 2009).

Two models have been proposed to explain how TLS bypasses lesions. In the first model, an inserter TLS polymerase promotes the incorporation of nucleotides opposite to the DNA lesion and an extender TLS enzyme extends this primer-template terminus (Washington et al., 2002). In the second model, the gap filling model, replicative polymerases will skip the sequence where the lesion is present, thus leaving behind a gap that will be filled by TLS polymerases such as Pol η (Diamant et al., 2012). Due to the low fidelity of these polymerases, there is a high possibility of nucleotide misincorporation that, if not repaired, will be fixed into a mutation with the next cell cycle.

2.4- DNA-Protein Crosslink (DPC) Repair

DPCs can be resolved by canonical DNA repair pathways such as NER and HR. According to several studies, NER is able to repair DPCs within a size limit, mainly small DPCs or large DPCs that have been processed previously with proteases (DJ et al., 2007). HR has also been shown to resolve DPC lesions as HR deficiency results in hypersensitivity to DPCs-inducing agents in mammalian cells (Nakano et al., 2009). Moreover, a specific type of repair had been discovered recently, which resolves DPCs regardless of the protein identity. This repair is mediated by a protein called Spartan (SPRTN), a homologue of yeast protease wss1. SPRTN was found to protect human proliferative cells from DPC toxicity through association with the replication machinery and by removing DPCs during DNA synthesis (Mórocz et al., 2017). In the presence of DPCs, the stalled DNA helicase and polymerases activate the RAD6-RAD18 complex, which marks the stalled replication fork by PCNA monoubiquitination. The monoubiquitinated PCNA will recruit SPRTN, which through its protease activity will digest the protein forming the DPC. After digestion, a small peptide of the protein will remain covalently attached to the DNA. The latter will be bypassed by TLS (Mórocz et al., 2017).

2.5- Repair of DNA Breaks

2.5.1- Single Strand Break Repair (SSBR)

SSB are lesions often generated directly by IR and ROS, they also could be caused by AP sites that are produced during BER or by errors during the enzymatic activity of TOP1 (Hegde et al., 2008; Wang, 2002). Unresolved SSB can lead to collapse of replication fork thus leading to the formation of DSB and could also stall the ongoing transcriptional machinery (Heeres and Hergenrother, 2007; Zhou and Doetsch, 1993). SSBs activate the PARP family members. PARP1 and PARP2 are the main sensors of SSB and DSB, and their activation leads to the synthesis of poly-ADP-ribose (PAR) chains at the site of the lesion within a short interval of time (Schreiber et al., 2006).

PAR chains which are usually synthesized on proteins such as Histone 1(H1), Histone 2B (H2B), and PARP1 itself. PAR chains are removed rapidly by PAR hydrolyzing enzyme (PARG) (Schreiber et al., 2006). PAR chains act as a platform to recruit protein that are involved in the repair of SSBs. The repair of SSB can occur through different pathways depending on the source of the break. The first pathway is the long patch SSBR. After PARP signaling, the ends of the break are processed by Apuring-Apyrimidic endonuclease 1 (APE1), Polynucleotide Kinase 3' phosphate (PNKP) and aprataxin (APTX) (McKinnon and Caldecott, 2007). FEN1 then removes the damage 5' end flaps leaving behind a ssDNA gape which will be filled by DNA Pol β together with DNA Pol δ/ε and the synthesized fragment will be finally ligated by LIG1 (Mortusewicz et al., 2006). The second pathway is the short patch SSBR which is specific for AP produced by BER. In this pathway APC1 recognizes the lesion and the same process occurs as the one in the long patch SSBR with only DNA Pol β filing the gap and LIG3 performing the DNA ligation instead of LIG1 (McKinnon and Caldecott, 2007). Another pathway exists which is specific for SSB induced by the action of Top1. It is a variant of the long patch SSBR where the end processing in order to remove Top1 is carried by the action of TDP1 (Caldecott, 2008).
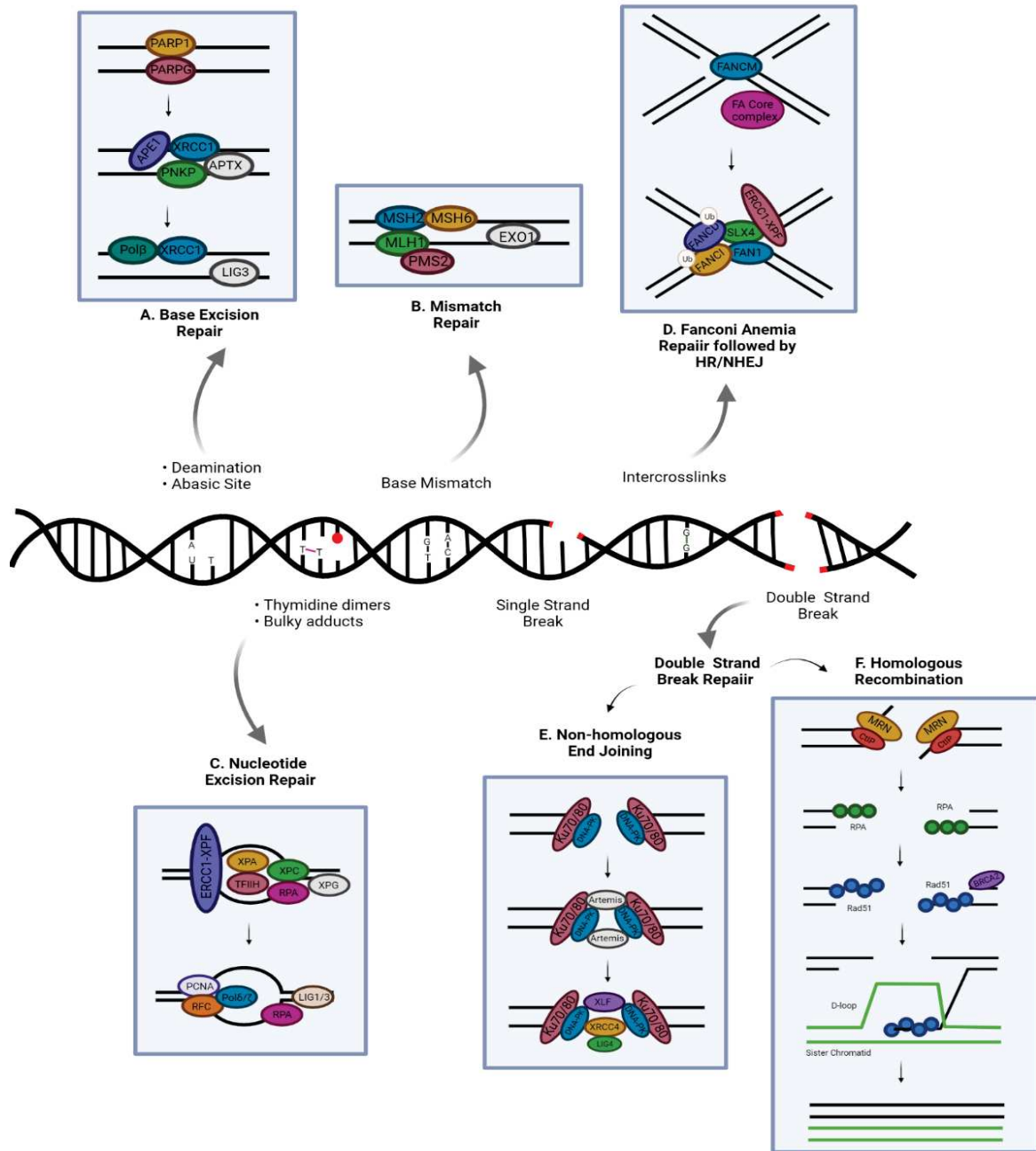
Figure 12. DNA damage repair pathways. Depending on the type of lesions the DNA can be repaired by (A) Base Excision Repair, (B) Mismatch Repair, (C) Nucleotide Excision Repair, (D) Fanconi Anemia, (E) Non-homologous End Joining or (F) Homologous Recombination.

DSBs are highly toxic lesions that can be induced by various physical and chemical DNA damaging agents (Pfeiffer et al., 2000). DSBs are repaired either by Non-Homologous End Joining (NHEJ) or by Homologous Recombination (HR). Other pathways for the repair are alternative NHEJ (Alt-NHEJ) and Single-Strand Annealing (SSA) that are not detailed in this manuscript. The pathway of choice is mainly affected by the cell cycle and also by the extent of DNA resection that has occurred at the site of the break. NHEJ does not require any DNA resection and usually occurs during the G1-phase. However, the HR pathway requires extensive DNA end resection and it usually occurs during S-phase since it utilizes the sister chromatid as a template (Hartlerode and Scully, 2009). DSBs are sensed by at least four proteins: PARP, Ku70/Ku80, MRN, and RPA (in case of DNA resection). Signaling of DSBs is primarily mediated via ataxia-telangiectasia mutated (ATM) and its main effector Chk2, DNA-PK, and the PARP family (Harper and Elledge, 2007; Meek et al., 2008), and the single strand DNA generated by end resection is signaled by the ATR pathway (Cimprich and Cortez, 2008).

2.5.2.1- NHEJ

Since the DSB could be repaired by either NHEJ or HR, two key proteins (BRCA1 and 53BP1) play an important role in determining the pathway of repair. During NHEJ, 53BP1 plays an important regulatory role by recruiting proteins that are implicated in this pathway (Panier and Boulton, 2014). For example, RIF1 is recruited to the N-terminal phosphorylated domain of 53BP1. RIF1 promotes the break repair by NHEJ during G1; however, its action is counteracted by BRCA1 during S-phase (Escribano-Díaz et al., 2013) .DSBs are rapidly recognized and bound by the Ku (Ku70/Ku80) heterodimer that prevents end resection of the break and promote the recruitment of other proteins (Figure 12E) (Doherty and Jackson, 2001; Mari et al., 2006) such as DNA-PK that initiates NHEJ (Mahaney et al., 2009). DNA-PK plays an important role in stabilizing the ends of the DSB (Meek et al., 2008) through a series of phosphorylation events that will recruit XRCC4/LIG4 to the break (Gottlieb and Jackson, 1993; Weterings and Chen, 2008; Yoo and Dynan, 1999)). XRCC4/LIG4 stabilizes the NHEJ complex by bridging and finally

ligating the ends of the breaks. DNA termini that contain lesions preventing the ligation are processed by ARTEMIS, APLF, WRN, ATTX, and KU (Bernstein et al., 2005; I et al., 2006; Li et al., 2011; Ma et al., 2002; Perry et al., 2006; Roberts et al., 2010). Finally, the gaps left behind after the processing are filled by family X DNA polymerases Pol μ in a template dependent manner, or by DNA Pol λ in a template independent manner (Ramadan et al., 2004; Roberts et al., 2010). Eventually, LIG4 will ligate the ends of the break (Grawunder et al., 1997).

2.5.2.2- HR

Repair by HR is following a series of steps: DSB recognition, DNA ends resection, DNA strand invasion, and template-dependent DNA repair synthesis (Figure 12F) (Li and Heyer, 2008). As mentioned previously, DSB can be recognized by the Ku complex; however, they can also be recognized by the MRN (MRE11-RAD50-NBS1) complex, which initiates the HR pathway (Stracker and Petrini, 2011; Sun et al., 2005). RAD50 contains an ATPase domain that interacts and stabilizes the ends of the DSB and recruits MRE11which has an endonuclease/exonuclease activity that initiates DNA ends resection (RS et al., 2007). NBS1 is also recruited to the site of the break where it interacts with MRE11 and promotes its function. NBS1 recruits ATM to the DSB via its C-terminal region. ATM is then activated and phosphorylates the histone variant H2A.X at Ser-139, known as γH2AX, that serves as an anchor for MDC1 (Bhatti et al., 2011). MDC1 is phosphorylated by ATM and functions as a platform to recruit the ubiquitin ligases RNF8 and RNF168 (Altmeyer and Lukas, 2013) that will ubiquitinate H2AX, which will recruit 53BP1 and BRCA1. However, during S/G2 BRCA1 is predominant over 53BP1, thus favoring HR (Escribano-Díaz et al., 2013).

The next step is DNA end resection where the ends are exposed to 5'-3' nucleolytic degradation leaving behind 3' overhangs. This occurs only during S/G2 phase when sister chromatids can be used as a template for the replication of the resected DNA (You and Bailis, 2010). BRCA1 recruits and initiates ubiquitination of CtIP (Huen et al., 2010). CtIP recruitment is also mediated by the MRN complex and ATM kinase activity (You and Bailis, 2010). DNA resection starts by the endonuclease activity of MRE11 with the help

of CtIP, which together cleaves about 15-20 nucleotides (Cannavo et al., 2013). This is followed by extensive resection that is carried either by EXO1 or DNA2 together with BLM (Chen et al., 2008; Nimonkar et al., 2011). The 3' ssDNA overhangs formed due to the DNA resection is coated by RPA in order to protect and stabilize it (Wold, 1997). The RPA-coated filament will activate the ATR pathway. Then RPA will be replaced by RAD51 with the help of recombination mediators including RAD52 and RAD55/57. RAD51-bound DNA will form the nucleoprotein filaments which perform the homology search. BRCA2 and PALB2, two other components of the HR pathway, allow the formation of these nucleoprotein filaments and in the sister chromatid invasion that results in the formation of the D-Loop (Holloman, 2011; Sebesta et al., 2013). Next, RAD51 will be excluded from the DNA by the action of RAD54 and RAD54B, allowing the 3'OH group to be engaged in DNA synthesis by DNA Pol δ, κ, and ν (Mazin et al., 2010; Sebesta et al., 2013). Finally the newly synthesized strand will be annealed to the processed second end of the break (West, 2003) thus forming Holliday Junction (HJ) that is later resolved by the action of BLM/Top3 complex or cleaved by structure specific nucleases SLX1/SLX4, MUS8/EME1, or GEN1 which will either generate crossover products or non-crossover products (Ciccia et al., 2008; Fekairi et al., 2009; Jeong et al., 2008; Rass et al., 2010).

3- Regulation of p53 in Response to DNA Damage

p53 is one of the most important tumor suppressor genes that orchestrates cell cycle and apoptosis. p53 maintains genomic stability and inhibition of tumorigenesis by initiating cell cycle arrest in order to provide the time necessary for DNA to be repaired before DNA replication or DNA segregation during mitosis. Evidently, mutation or loss of p53 is strongly associated with the development of tumors. In support of this, p53 is mutated in half of the tumors (Vogelstein et al., 2000) .

Throughout the unperturbed cell cycle, the activity of p53 is repressed by different mechanisms including the regulation of its transcriptional activity and stability. The main regulator of p53 is the E3 Ubiquitin Ligase MDM2, which regulates it in two ways. First, MDM2 binds to the N-terminal of p53 where it inhibits its ability to function as a transcriptional activator (Momand et al., 1992; Oliner et al., 1993). Second, MDM2

ubiquitinates p53, which targets it to proteasomal degradation, thus controlling its level by modulating its stability (Maki et al., 1996).

p53 activation is induced by several types of cellular stress including nutrient deprivation, hypoxia, ribosomal stress, oncogene activation, and importantly, DNA damage. Levels of p53 increase within minutes of exposure to DNA damaging agents, and this is achieved via post-translational modifications of p53 which include phosphorylation and acetylation. It was reported that phosphorylation of p53 at its N-terminus promotes its dissociation from the MDM2/p53 complex, thereby becoming active and allowing the increase of its half-life (Maki and Howley, 1997; Maltzman and Czyzyk, 1984; Price and Park, 1994). As described previously, the presence of DNA damage activates 3 main kinases: DNA-PK, ATM and ATR. Upon their activation, p53 is phosphorylated at Ser-15 by ATM (Khanna et al., 1998), ATR (Tibbetts et al., 1999) and its main effector Chk1 (Goudelock et al., 2003), and DNA-PK (Shieh et al., 1999) (Figure 13). p53 is also phosphorylated at Ser-20 by Chk2 (Craig et al., 2003), the main effector of ATM. Other phosphorylations on different residues also occur but are not addressed in this manuscript.

The main role of p53 during DNA damage is to induce cell cycle arrest. Upon activation, p53 will transcriptionally induce the expression of p21 which will inhibit both Cyclin E/CDK2 and Cyclin A/CDK2 complexes thus inducing an arrest in G1 (Ko and Prives, 1996; Levine, 1997). p53 activation also induces G2/M arrest thought p21 dependent inhibition of Cyclin B/CDK1 (Martín-Caballero et al., 2001), or by another mechanism that involves the transcriptional inhibition of CDC25C (Hoege et al., 2002a). By arresting the cells, p53 allows time for the repair of DNA breaks that have the potential to be lethal to the cells. In addition, p53 could contribute to the regulation of proteins involved in DNA recombination and repair, such as RAD51 (Gatz and Wiesmüller, 2006). Furthermore, p53 plays a role in regulating genes involved in heterochromatin formation to facilitate the repair of damaged DNA (Zheng et al., 2014).

Upon persisting DNA damage, p53 drives the cells to either senescence or apoptosis (Figure 13). Upon p53-dependent upregulation of p21, cells undergo premature

senescence, which is a unique state of stable cell-cycle arrest (Brown et al., 1997). On the other hand, p53 can also induce a large number of genes that are involved in the apoptosis. These genes include pro-apoptotic proteins (PUMA, Bad, Bax and Bak) and execution factors such as Caspase6 (Chen, 2016).
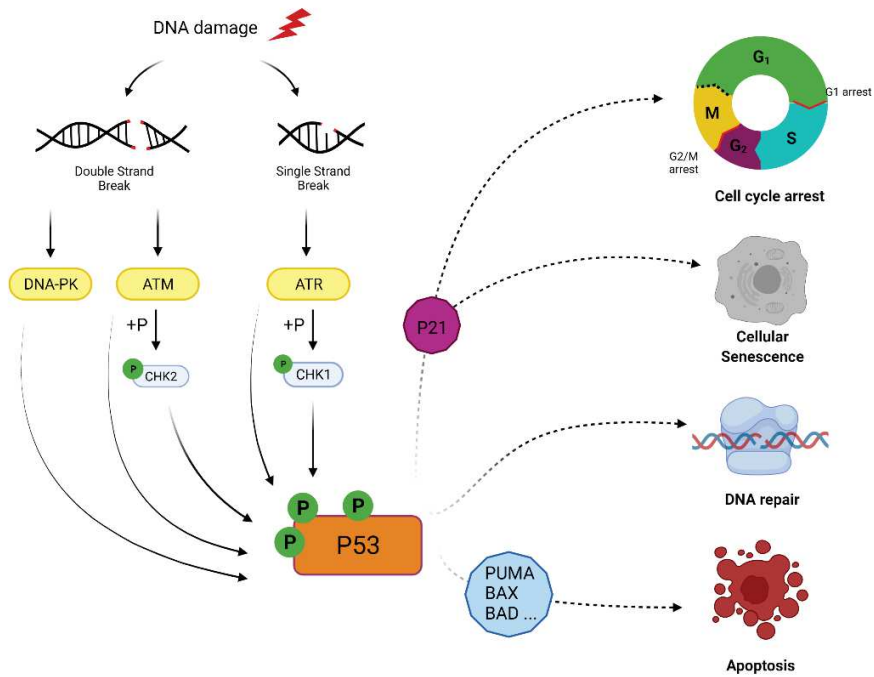
Figure 13. p53 dependent DNA damage signaling. DNA lesions activate different kinases: DNA-PK, ATM and ATR. p53 is activated downstream to the three kinases or their main effectors (Chk2 and Chk1). The activation of p53 will result in cell cycle arrest, cellular senescence, DNA repair, or apoptosis.

Chapter 5: Replicative stress

The DNA replication machinery is constantly assaulted and perturbed by numerous obstacles coming from both intracellular and extracellular origins. These obstacles, if left improperly addressed, will result in replication fork collapse and eventually genomic instability, one of the main drivers of tumorigenesis. DNA Replication stress defines all types of DNA replication deregulation including slowing or stalling of the fork progression as a result of different insults. DNA replication stress can be induced by endogenous or exogenous sources (Figure 14).

1- Sources of Replicative Stress

1.1- DNA Structure

At specific regions of the genome, unusual DNA structures may form during processes that generate ssDNA such as replication, transcription, and different pathways of the DDR (Bochman et al., 2012; Kaushal and Freudenreich, 2019). Formation of secondary structures such as hairpins, triplexes, and cruciform structures are mostly pronounced at tandem repeats and inverted sequences (Leonard and Mechali, 2013). Other alternative DNA structures such as stem loops and G quadruplex (G4) may be formed at AT and CG rich regions and can lead to the increase of topological stress or pose a barrier during replication of the leading strand, and would lead to replication fork stalling (Chambers et al., 2015; Ozeri-Galai et al., 2011; Tubbs et al., 2018). Impeding normal replication fork progression, these structures threaten genomic stability and may contribute to the development of diseases (Ge et al., 2007). Helicases such as Pif1 (Hou et al., 2015; Ribeyre et al., 2009), FANCJ (London et al., 2008) and BLM (Sun et al., 1998) can resolve these structures in vitro and in vivo thus alleviating their effect on replication fork progression.

1.2- Fragile Sites

In the human genome there are certain loci that are particularly complex to replicate, which makes them more prone to breaks and genomic instability during replication stress. These specific regions of the genome are known as fragile sites and can be classified into either Common Fragile Sites (CFS) or Early Replicating Fragile Sites (ERFS). CFS are

usually characterized by having AT-rich sequences and low origin density, and are located in late replicating regions/heterochromatin. Due to the repetitive AT sequences, some CFS are prone to form secondary structures that impose an endogenous obstacle for the progression of the replication fork (Debatisse et al., 2012; Glover et al., 2017; Ozeri-Galai et al., 2012). The low density of replication origins, on the other hand forces two converging forks to travel within a long stretch of DNA in order to finish its replication, and this increases the risk of incomplete replication (Letessier et al., 2011). The probability of incomplete replication along with the fact that CFS replicates during late S-phase might lead to mitotic entry with under-replicated regions due to the short period of time during the end of S-phase (Le Beau et al., 1998). In contrast, ERFS are GC-rich with an open chromatin status. They are rich in replication origins, and they replicate during the early S-phase in proximity to highly transcribed regions. ERFS are prone to replication fork stalling and DNA breaks (Barlow et al., 2013) most probably due to the conflicts occurring between the replication and transcription machinery (to be detailed). CFS are frequently subjected to deletions in a broad spectrum of human tumors (Aird et al., 2013). FRA3B and FRA16D are two of the most affected CFS in human cancers including colon, breast, and lung carcinomas (Durkin and Glover, 2007). For example, FRA3B is located within Fragile Histidine Triad (FHIT), a tumor suppressor gene involved in nucleotide metabolism (S. JC & D, 2019), and this explains why instability of FRA3B participates in tumorigenesis.

1.3- Replication-Transcription Collision (RTC)

An additional source of replicative stress is the collision between the replication and the transcription machineries. In general, both processes are spatially and temporally separated and well-coordinated. It was proved that early replicating genes show increased transcription late in S-phasem whereas late replicating genes are predominantly transcribed early in S-phase (Meryet-Figuiere et al., 2014). However, transcribed genes might lead to RTC. This collision may lead to an increased topological stress caused by anchoring of the newly synthesized mRNA to the nuclear pore complex for further processing, which is known as gene gating (Helmrich et al., 2013). It was reported that the ATR-dependent checkpoint is able to relieve this stress and retain normal fork

progression by releasing the transcribed genes from nuclear pores (Toledo et al., 2011). Another effect of RTC is the formation and accumulation of RNA-DNA hybrids (R-loops). One of the main pathways to avoid the formation of R-loops is the function of RNase H enzyme which act as an endonuclease cleaving the RNA-DNA intermediates (Helmrich et al., 2011).

1.4- Oncogene-Induced Replicative Stress

Malignant transformation is driven mainly by the altered expression of oncogenes, tumor suppressor genes, and microRNAs. A proto-oncogene is a protein involved in the tight regulation of cell growth, differentiation, and apoptosis. When the expression level or the function of a proto-oncogene is deregulated, it results in an activated oncogene. Oncogenes drive the uncontrolled proliferation of cancer cells and cause replicative stress through deregulating the cell cycle, replication initiation program, cellular metabolism, and transcription.

DNA replication initiation, as previously described, is a tightly regulated process. Any deregulation of proteins that monitor this process such as CDKs and RB/E2F leads to the perturbation of either the licensing or the firing. This will eventually result in either a decrease, increase or re-firing of replication origins. The implication of oncogenes in the regulation of origin firing and replication stress will be addressed elsewhere in details.

Oncogenes can induce replicative stress by inducing the production of ROS, one of the main sources of DNA lesions that leads to stalling of replication forks and generation of DSB. It was shown that overexpression of RAS, one of the main oncogenes in cancer development, causes a change in the cellular metabolism leading to an increased production of ROS (Irani et al., 1997; Lee et al., 1999). For example, Myc overexpression was also reported to induce genomic instability by oxidative stress (Vafa et al., 2002). Moreover, oncogenes may target RNR activity or induce increased proliferation that will in both cases reduce the dNTP pool affecting fork progression (Aird et al., 2013).

Oncogene overexpression also leads to an increase in the transcription activity, which results in RTC, and thus replicative stress. For example, RAS proteins were shown to

promote cellular proliferation through upregulation of the level of transcription factors that have the ability to stimulate RNA synthesis by increasing the number of transcription units and R-loops (Pylayeva-Gupta et al., 2011). The same finding was reported with the overexpression of Cyclin E, where it resulted in an increase in transcription and RTC (Jones et al., 2013). The alteration of cellular metabolism caused by oncogenes could also affect the production of dNTPs. In one study, it was reported that RAS interferes with the levels of cellular dNTPs by downregulating ribonucleotide reductase subunit M2 (RRM2). As a consequence, dNTP pools are depleted, forks are stalled, and replication forks undergo premature termination (Aird et al., 2013). Oncogenes mostly cause replication stress indirectly; however, it could also cause replication stress directly by interfering with DDR proteins. For example, it has been shown that RAS causes dissociation of BRCA2 from chromatin and interferes with its ability to repair the damaged DNA (Tu et al., 2011).
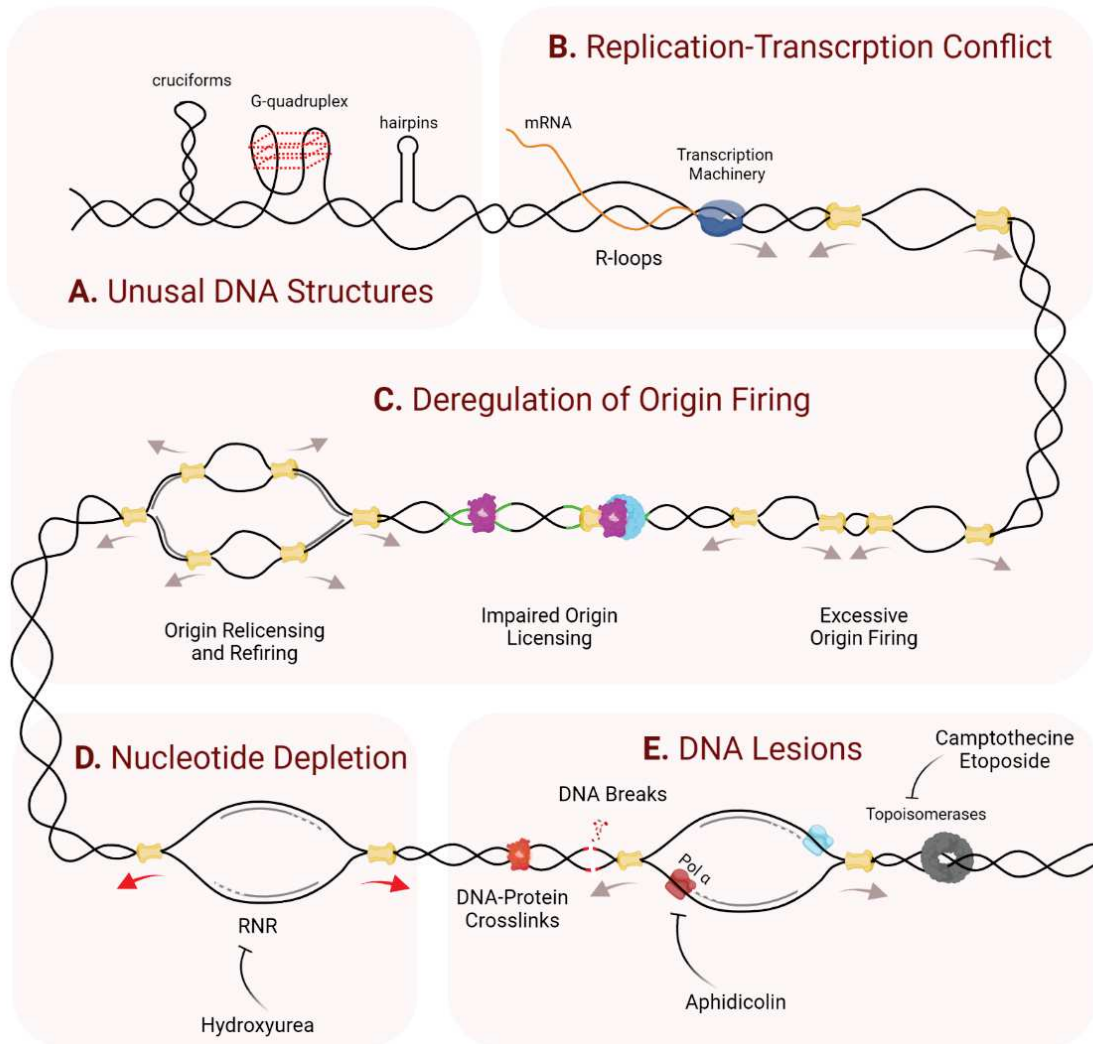
Figure 14. Molecular mechanisms of DNA replication stress caused by different sources. (A) Unusual DNA structures as specific such as cruiciforms, G-quadreplex and hairpins might form at specific genomic sequences. They represent natural obstacles to replication fork progression. (B) Collisions between replication and transcription machineries may also impair DNA replication fork progression through generation of DNA topological stress and formation of persistent R-loops. (C) Deregulation of origin firing can interfere with DNA replication and replication fork progression. The deregulation could be at the level of extra origin firing, impairment of origin licensing, or re-replication. (D) Depletion of nucleotide pool by hyroxyurea for example impairs DNA replication and induce fork stalling. (E) Different DNA lesions including DSB and DPCs may jeopardize the progression of replication fork and induce collapse.

1.5- Exhaustion of Replication Factors

1.5.1- dNTPs

A crucial factor in maintaining replication efficiency and genomic stability is establishing an optimal pool of deoxynucleotide triphosphate (dNTPs). Any shortage in dNTPs would slow down the DNA polymerases compared to the activity of helicases leading to the generation of ssDNA and possible genomic instability (Poli et al., 2012). It was estimated that the pool of dNTPs would cease within minute into S-phase entry if not renewed (Murthy and Reddy, 2006). The regulation of dNTP pools occurs at the level of synthesis and degradation. dNTP synthesis is carried out by the ribonucleotide reductase (RNR) complex, which is composed of two copies of the catalytic unit R1 and two copies of the regulatory unit R2 or p53R2 (Mathews, 2015). R2 expression peaks during S-phase and is degraded during G2-phase by the action of APC/C (Chabes et al., 2003), where it remains at low concentration through mitosis and G1-phase of the next cell cycle (Mathews, 2015). These enzymes are usually localized in the cytoplasm, and once they synthesize dNTP, they shuttle into the nucleus (Niida et al., 2010). The maintenance of the proper dNTP pool levels is also executed at the level of nucleotide degradation. An active pathway involves the action of the dNTP triphosphatase SAMHD1, which degrades dNTPs during the G1-phase (Técher et al., 2017). Any perturbation of the proper level of dNTPs can affect replication initiation program, fork speed, and DNA repair (Pai and Kearsey, 2017).

Depletion of dNTPs by the action of hydroxyurea mediated RNR inhibition for example results in a global replication fork arrest. In general, the generation of ssDNA by the uncoupling of polymerases and helicases activates checkpoints that will stabilize stalled replication forks and induce the firing of backup origins to rescue the stalled forks. However, firing of extra replication origins also contributes to dNTP starvation(Anglana et al., 2003). Eventually, the arrested forks will resume replication once the dNTP pool is restored. However, prolonged dNTP starvation leads to replication fork collapse and DNA damage, especially at specific genomic loci such as fragile sites (Debatisse et al., 2012).

1.5.2- RPA

During the normal course of DNA replication, the generated ssDNA is bound and protected from any assault by the trimeric complex RPA. When the replication fork is challenged with any obstacle, the excess of ssDNA produced would accumulate the available RPA to protect them from nucleases attacks (Toledo et al., 2013). Therefore, any shortage in the RPA pool would subject the cell to higher levels of replicative stress and genomic instability. In normal conditions, RPA is synthesized in 6-10 fold excess than needed, but this supply can be exhausted when excessive stalling occurs and in the case of unscheduled activation of origin firing (to be detailed elsewhere) (Syljuåsen et al., 2005; Toledo et al., 2017).

1.5.3- Histones

During DNA replication, proper DNA organization is as important as the faithful copying of the DNA sequence for ensuring genomic stability. When the cell divides, the chromatin landscape must be reproduced, and this takes place during S-phase. The chromatin structure is disrupted as replication forks progress and is restored behind on the two sister chromatids. Chromatin restoration occurs mainly through nucleosome assembly, which relies on recycling of parental histones along with newly synthesized ones through the AsF1-CAF pathway, since the number of required histones is doubled (Alabert and Groth, 2012; Annunziato, 2012). The high demand on the canonical histones (H1/H2B/H2A/HB.1/H3.2/H4) through S-phase is well coordinated by the expression of new ones (Marzluff et al., 2008). S-phase impairment due to the inhibition of histone biosynthesis was reported in several studies (Barcaroli et al., 2006; Nelson et al., 2002). In detail, it was shown that the inhibition of histone biosynthesis leads to the disturbance of replication fork progression and DNA damage, and impairment of PCNA recruitment due to the lack of nucleosome assembly (Mejlvang et al., 2014).

1.6- Replication Stress Induced by Chemotherapeutic agents

All sorts of DNA damage could lead to replication stress if not well addressed. For example, DPC and DNA breaks caused by either CPT or ETP treatments, and other bulky adducts produced by crosslinking agents such as cisplatin lead to replication fork stalling and collapse if not repaired before the passage of replication forks. In addition, there is a panel of drugs that were designed to perturb the progression of replication forks and induce replicative stress by specifically inhibiting the function of replisome components or checkpoints. A good example is Aphidicoline, a drug that inhibits DNA polymerase α, stalls the replication fork and induces the expression of fragile sites (Debatisse et al., 2012). Inhibiting checkpoint inhibitors such as ATR, Chk1 and Wee1 kinase were also reported to augment the level of replicative stress and induce cancer cell targeting when combined with other chemotherapeutic drugs (Do et al., 2013).

2- Replicative Stress Response

ATR is the key kinase activated in response to replication stress (Figure 15) (Cimprich and Cortez, 2008). After the generation of ssDNA at stalled forks, ATR is recruited physically by RPA loading along with its partner ATR Interacting Protein (ATRIP). RPA on the other hand, also recruits RAD17-RFC and RAD9-RAD1-HUS1 (911) complex. This complex is essential to recruit TopBP1, the activator of the ATR-ATRIP kinase, leading to the phosphorylation of several downstream factors. Moreover, ATR was shown to be activated by ETAA1 (Haahr et al., 2016). ETAA1 accumulates at DNA damage sites and interacts with ATR activating it independently of TopBP1. Fork stability is promoted by TIMELESS and TIPIN complex, which associates with RPA and triggers the accumulation of Chk1 and Claspin to the RPA-ssDNA junction. There, ATR will phosphorylate its main effector Chk1 at Ser317 and Ser345 and RPA at Ser33. ATR also phosphorylates Histone H2AX at Ser319 (γH2A.X), which spreads away from the stalled replication forks to amplify the signal. Moreover, stalled forks could also activate the two other kinases ATM and DNA-PK, depending if there is a lesion associated with the stalling.
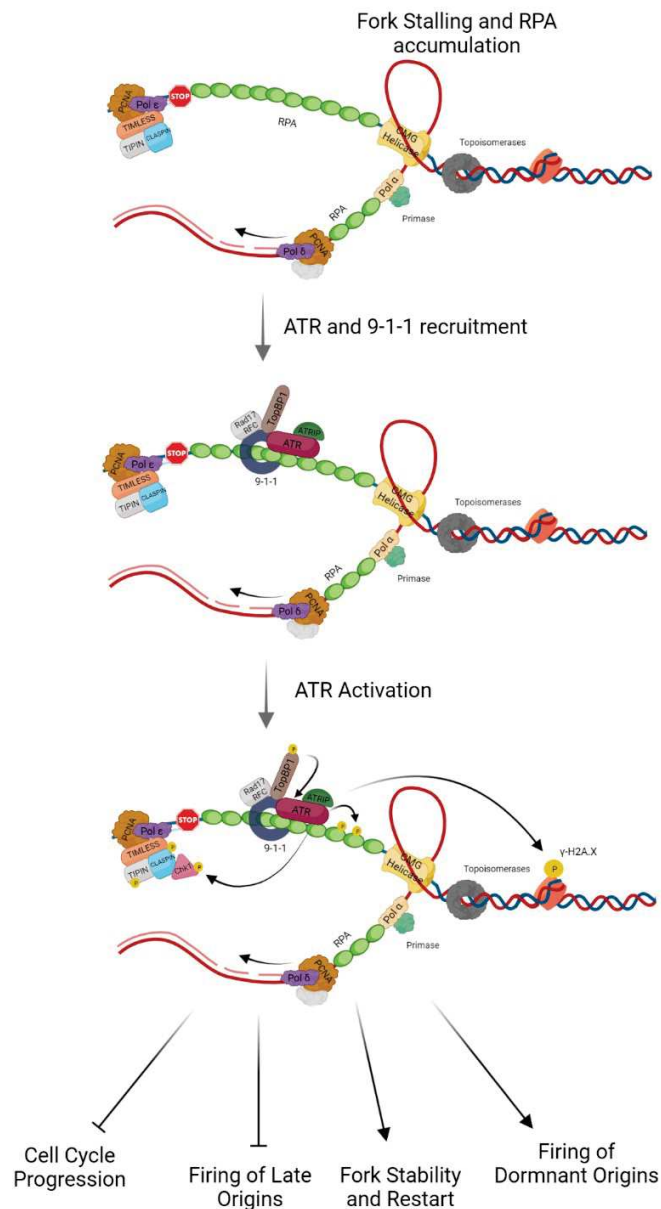
Figure 15. Activation of the ATR/Chk1 pathway. ssDNA is generated as an intermediate structure during DNA repair or DNA replication. RPA binds to ssDNA, which then recruits ATR-ATRIP and RAD17-RFC to load the 9-1-1 (RAD9-RAD1-HUS1). TopBP1 interacts with ATRIP-ATR and activates the kinase activity of ATR. Upon its activation, ATR phosphorylates the effector kinase Chk1, RPA, Rad9 of 9-1-1 complex, claspin and Tipin, and H2AX. The activation of ATR leads to different outcomes including cell cycle arrest, firing of dormant origins and inhibiting late ones, and importantly ensuring the fork stability and restart.

ATR/Chk1 pathway stabilizes stalled forks by two mechanisms. Chk1 organizes cellular response to stalled forks by inducing cell cycle arrest; therefore, providing sufficient time for the cell to restart replication or repair any DNA lesions to prevent premature mitotic entry with under-replicated DNA (Saldivar et al., 2018a). As described previously, activation of ATR and Chk1 leads to the phosphorylation of p53 that induces cell cycle arrest. Moreover, Chk1 degrades the CDK activator CDC25A (Sørensen et al., 2003) . This phosphorylation leads to the degradation and the nuclear export of CDC25, which triggers cellular arrest at S/G2 or G2/M phases. Chk1 also phosphorylates and activates wee1 kinase, the CDK antagonist, thus leading to G2 arrest (Kotsantis et al., 2018).

The second mechanism by which ATR/Chk1 stabilizes stalled forks is by controlling replication origin firing. This mechanism will be described thoroughly elsewhere. Briefly, upon activation, ATR/Chk1 suppress origin firing of new replication clusters and activate firing of dormant origins within active clusters, thus ensuring the rescue of stalled forks and the maintenance of RPA pools (Toledo et al., 2017).

3- Resolving of Stalled forks

Processing of stalled forks can occur by different mechanism including fork reversal, fork repriming, DNA damage tolerance bypass, and break-induced replication (Figure 16).

3.1- Fork Reversal

Stalled replication forks can undergo remodeling into a reversed structure formed by parental DNA strands reannealing and nascent DNA strands annealing, forming a ''regressed arm'' or a ''chicken foot'' structure (Figure 16). Replicating cells show a basal level of reversed forks that increases in response to exogenous replicative stress (Berti et al., 2013; Zellweger et al., 2015). Fork reversal is a tuning mechanism by which the cells undergo rapid proliferation use in order to preserve genomic stability when facing endogenous or exogenous replicative stress (Ahuja et al., 2016). It prevents the generation of excess ssDNA and provides access to DNA repair machinery (Cortez, 2015). However, these structures, if not well protected, could be subjected to nuclease processing and DSB formation (Couch et al.; Schlacher et al., 2011; Ying et al., 2012).

Many factors are involved in the formation of reversed forks. Efficient fork reversal requires the recombinase RAD51 (Scully et al., 2019); however, its function is exclusive from the one in homologous recombination (Bhat and Cortez, 2018; Mijic et al., 2017; Zellweger et al., 2015). RAD51 loading into extended ssDNA regions promotes reannealing of parental DNA strands (García-Rodríguez et al., 2016). This loading is regulated by different factors such as F-box helicase 1 (FBH1), RecQ-like helicase 5 (RECQ5) and RPA1 related-single strand DNA-binding protein X (RADX). It was proposed that these factors modulate RAD51- fork reversal (Chappidi et al., 2020; Fugger et al., 2015).For example, RADX is able to bind to ssDNA and destabilizes RAD51 nucleofilaments and depend ending on the level of replication stress, will either inhibit or promotes fork reversal (Krishnamoorthy et al. 2021). On the other hand, RAD51 nucleofilaments are stabilized and protected by BRCA2 against nucleolytic processing (Bhat and Cortez, 2018; Lemaçon et al., 2017; Mijic et al., 2017). Other proteins such as BRCA1 and FANCD2 were also reported to play a role in stabilizing stalled forks during the process of fork reversal (García-Rodríguez et al., 2016; Guilliam et al., 2017) .

Other remodeling enzymes or translocases are also recruited to stalled forks to mediate fork reversal. (1) SMARCAL1 is recruited via RPA coated ssDNA to stalled replication forks and promote reversal, specifically at forks blocked at the leading strand (Bétous et al., 2012; Couch et al.). (2) HTLF is a protein that promotes ubiquitination of PCNA, binds to the blocked 3' OH of the stalled fork, and mediates fork remodeling (Blastyák et al., 2007; Kile et al., 2015). (3) ZRANB3, dsDNA translocase, is also recruited to ubiquitinated PCNA and mediates fork reversal (Ciccia et al., 2012; Vujanovic et al., 2017; Weston et al., 2012).

Fork reversal could require the activation of the ATR/Chk1 pathway; however, in some cases fork reversal might occur in absence of ATR signaling (Zellweger et al., 2015). Indeed, the activation of ATR was reported to prevent SMRCAL1 mediated fork reversal (Couch and Cortez, 2014; Couch et al.) and promote repriming of stalled forks (described elsewhere) (Quinet et al., 2020).

3.1.2- Resolving of Reversed Forks

In order for reversed forks to be restarted, the normal replication fork structure must be restored. This is performed by mainly two different pathways. The first pathway is mediated via RECQ1, which is a specific human helicase involved in the restart of reversed forks. It was reported that RECQ1 restores normal structure of reversed forks after restoration of nucleotide pools, or repair of Top1 crosslinks or ICL (Berti et al., 2013; Zellweger et al., 2015). RECQ1 is transiently inhibited by PARP1-mediated parylation during persistent replicative stress (Berti et al., 2013). Thus, PARP1 acts as a molecular switch to control the proper timing of a reversed fork restart following replication stress (Zellweger et al., 2015). The second pathway is mediated through two nucleases, DNA2 and WRN. After a prolonged period of nucleotide depletion DNA2 along with WRN assist in the removal of chicken foot structure by resecting the regressed arm and promoting HR-mediated restoration of the typical replication fork architecture (Lorenz et al., 2009). Remarkably, in humans this end processing is exclusively carried by DNA2 and not any other nuclease like MRE11 or EXO1 (Thangavel et al., 2015). A third possible pathway is through structure-specific nucleases SLX4 and MUS81 that have the ability to attack the parental DNA strands causing fork breakage in case of prolonged periods of replicative stress (Fekairi et al., 2009). However, this pathway could lead to deleterious consequences regarding the genomic stability since it includes the formation of DSBs.

Beside the main factors that initiate and resolve reversed forks, there is also a plethora of proteins functioning in order to preserve that integrity of reversed or stalled forks. Several studies reported that depletion of any of these factors would result in extensive DNA resection by MRE11, CtIP, EXO1, and DNA2 (Cotta-Ramusino et al., 2005; Schlacher et al., 2011; Thangavel et al., 2015). These factors function either by promoting stable RAD51 filament formation, limiting the accessibility and activity of nucleases at stalled forks, or by contributing to the complex nuclear organizations (Schlacher et al., 2011; Xu et al., 2017). Table 1 summarizes all the factors that were reported to be important for the protection of stalled forks describing their different mechanisms.

Table 1. Role of Different Factors in Protection of Stalled Replication Forks.
Adapted from (Tye *et al.* 2020)

Factor	In fork protection
ATR	Promote 'rewired' fork protection
BLM	Promote RAD51 dissociation
BRCA1-BARD1	Promote RAD51 recruitment by PIN1
BRCA2	Stabilise RAD51
CtIP	Restrict fork reversal/protect reversed forks and aid MRE11-mediated degradation
DNA2	Resect 'wild-type' and de-protected forks
EXD2	Suppress fork reversal
EXO1	Extend MRE11 resection
FANCD2	Histone mobilisation, increase TLS, direct RAD51 stabilisation
FBH1	Promote fork reversal, promote RAD51 dissociation
MRE11	Resect unprotected forks, promote fork restart
PALB2	BRCA1-independent recruitment of BRCA2
PARP	Inhibit fork restoration, recruit MRE11, inhibits fork restoration, RAD51 stabilisation
PDS5	Promote BRCA2 recruitment
RAD51	Promote fork reversal, protect forks from degradation
RAD51 paralogs	Promote RAD51 recruitment
RAD52	Suppress SMARCAL1 recruitment, promote MRE11 recruitment
RECQL5	Protect against degradation
RIF1-PP1	Inhibit DNA2/WRN
RNF168	Restrict fork reversal
SLX4	Scaffold for nucleases involved in fork cleavage
WRN	Support DNA2 (helicase), prevent MRE11-dependent degradation (exonuclease)

3.2- Repriming of Stalled Forks

Efficient fork restart without remodeling can occur by repriming of the stalled replication forks (Figure 16). Repriming is performed by the human DNA direct primase/polymerase (PrimPol) (Mourón et al., 2013; Wan et al., 2013) which is recruited to stalled forks by RPA where its activity is regulated. PrimPol prevents ssDNA accumulation on the leading strand by repriming the DNA and allowing the resumption of replication leaving behind a ssDNA gap (Mourón et al., 2013). Filling the post-replicative gaps can occur by TLS

polymerases or by a complex template switching mechanism that utilizes the sister chromatid as a template (Denison et al., 2003). The human PrimPol ensures resumption of DNA replication after exposure to UV(Bianchi et al., 2013), oxidative stress, and upon dNTP depletion (Mourón et al., 2013).

3.3- DNA Damage Tolerance (DDT) Lesion Bypass

DDT lesion bypass is another mechanism that enables replication to resume and replicate past the lesions faced by the replisome, leaving it behind to be repaired later on (Figure 16) (Ghosal and Chen, 2013; Sale, 2012). DDT can occur either by translesion synthesis or template switching. As mentioned previously, translesion synthesis is carried out by the specialized TLS polymerases. Upon fork stalling, RAD18 mediated PCNA monoubiquitination recruits the TLS polymerases to the stalled forks where they carry on with the replication (Kannouche et al., 2004; Watanabe et al., 2004; Yang et al., 2013). The other DDT mechanism is strand switching (TS). During TS, the stalled nascent strand switches temporarily to the newly synthesized strand sister in order to replicate over the lesion. Unlike TLS, TS is an error-free process since it utilizes the non-damaged sister strand as a template.

PCNA can act as a molecular switch between TLS and TS. As described, PCNA is first monoubiquitinated by RAD18 which recruits TLS polymerases. However, PCNA could be furtherly ubiquitinated by RAD5/Ubc13/Mms2 E2-3 ubiquitinase (Hoege et al., 2002b) which activates RAD5-dependent TS pathway(Moldovan et al., 2007). Moreover, there is evidence that both mechanisms are separated temporally where TS occurs in Early S-phase while TLS occurs in late S or G2/M phase (Karras et al.; Lang and Murray, 2011; Waters and Walker, 2006). TS requires the unwinding of newly synthesized DNA from the parental strand followed by annealing of the two newly synthesized strands which forms the structure needed to replicate past where the lesion exists on the parental strand. DNA helicases and translocases are required for the branch migration and DNA recombinases and DNA polymerases are required to replicate the nascent DNA (Marians, 2018).

3.4- Break Induced Replication (BIR)

An alternative mechanism for replication fork restart is mediated by several structure-specific endonucleases, especially when fork stalling is prolonged (Dehé et al., 2013). These endonucleases have the ability to target stalled forks with three-way junctions or reversed forks with four-way junctions. Although they enable replication fork restart, they could be a source of genomic instability. Published studies have shown that the nucleases MUS81 and SLX4 are implicated in this process.

MUS81 induces replication-dependent DSB due to nucleotide depletion (Hanada et al., 2007) and due to oncogenes-induced replicative stress (Murfuni et al., 2012; Weston et al., 2012). It is mainly active during mitosis where its activity is linked to mitotic DNA synthesis (MiDAS) at common fragile sites(Kunkel and Erie, 2015) (Constantinou et al., 2002). Its activity is regulated through its partner EME1 (essential meiotic structure-specific endonuclease 1) which is phosphorylated by CDK1 and PLK1 to inhibit its activity outside mitosis. In *S.pombe*, MUS81 is activated by Rad3^{ATR} mediated phosphorylation of EME1 (Dehé et al., 2013), while in human cells MUS81 is activated by alternatively binding to EME2 (Hanada et al., 2006). SLX4, on the other hand, was proposed to cleave SMARCAL1 reversed forks where it interacts with SLX1 and processes branched DNA that results following nucleotide deletion (Couch et al.).

Endonucleolytic cleavage of stalled forks produces one-ended DSB that demand accurate processing to restore the integrity of forks and allow continuation of DNA synthesis. Since this type of DSB is from one end only, canonical HR or NHEJ cannot restore the integrity of the forks. This kind of break is repaired via strand invasion and unusual maintenance of DNA replication through a migrating bubble that could copy many hundreds of kilobases (Malkova, 2018). The break could also be resolved by microhomology-mediated Template switching, where the 3' end of the ssDNA can undergo multiple strand invasions (Lydeard et al., 2007). Another repair mechanism is independent of strand invasion, where the broken ends could be directly ligated by RAD52, DNA LIGASE4 and XRCC4 (Chappidi et al., 2020). In yeast, BIR requires the function of Pif1 helicase and the polymerase accessory factor DNA polymerase delta 3 (POLD3) (Wilson et al., 2013). BIR is highly mutagenic and could lead to genomic

rearrangements, making it an unfavorable repair mechanism and an important source of genomic instability during replication stress (Malkova, 2018).
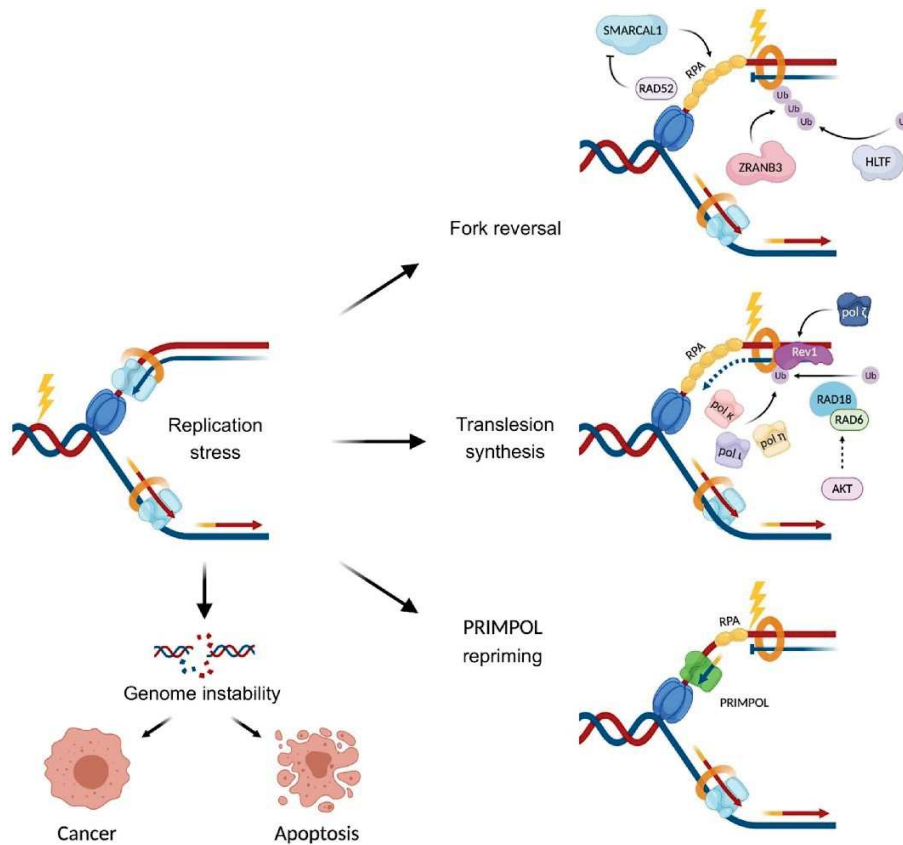
Figure 16. Mechanisms of resolving stalled replication forks. Stalled forks can be resolved by three main pathways including fork reversal, translesion synthesis by the TLS, or repriming by PrimPoL. Prolonged unresolved exposure to replication stress results in fork collapse that leads to genome instability, which is a hallmark of cancer cells. Genomic instability could also signal programmed cell death in certain genetic contexts. Adapted from (Baillie and Stirling, 2020)

4- Origin Firing and Replicative Stress

4.1- Regulation of Dormant Origin Firing

As described previously, the number of origins that are licensed during the G1-phase is much higher than the number actually fired during S-phase. When replication forks are perturbed by any barrier, some of the origins which otherwise would have been inactive and replicated passively are activated. These origins are known as dormant origins and their firing during replicative stress is one of the mechanisms utilized by the cell to rescue stalled forks. During unperturbed replication, the level of MCMs could be lowered to 3-10 folds without affecting the kinetics of S-phase. However, it was shown that during replicative stress, cells with decreased level of MCM will become more susceptible to replicative catastrophes and DNA lesions with decreased level of survival, which is due to the absence of dormant origins (Blow et al., 2011; Woodward et al., 2006).

Dormant origins must be well regulated in order to be activated only when needed; otherwise, instead of rescuing stalled forks, they would cause replication catastrophe when the cells are challenged with replicative stress (Toledo et al., 2017). Dormant origins can be regulated by passive mechanisms such as low concentrations of firing factors or chromatin organization (Lubelsky et al., 2011). They can also be regulated actively by checkpoint pathways. One of the pathways is the ATR/Chk1, which maintains fork stability during stressful conditions by modulating the program of origin firing (Figure 17) (Petermann et al., 2010). In unperturbed S-phase, the basal level of ATR/Chk1 activation limits the number of origins fired by stabilizing RIF1-PP1 interaction through inhibiting CDK-dependent phosphorylation of RIF1 at Ser2205, which releases it from PP1 (Moiseeva et al., 2019). ATR/Chk1 inhibits CDK1 by degrading its positive regulator CDC25 (Moiseeva et al., 2019). RIF1/PP1 complex act on inhibiting CDK2 and CDC7, thus inhibiting origins firing (Moiseeva et al., 2017). Alternatively, Chk1 might also be inhibiting origin firing by interacting with Treslin, a factor that is required for the CMG complex and TopBP1 stability(Guo et al., 2015; Kumagai et al., 2010). However, upon replication stress induced by APH or HU, ATR/Chk1 act on activating dormant origins within active clusters and inhibiting the firing of new ones (Tsantoulis et al., 2008; Wong et al., 2011). For the moment there is no clear mechanism to explain how this is executed.

Active ATR is recruited to stalled forks and is known to phosphorylate MCM2-7 proteins (Chuang et al., 2010). However, there is no clear evidence whether this is the mechanism by which it activates local dormant origins.

Other pathways than ATR/Chk1 may intervene with the firing or inhibition of dormant origins. For example, Claspin facilitates MCM phosphorylation by recruiting CDC7 during normal S-phase (Yang et al., 2016). However, although its yeast homologue Mrc1 does the same function during S-phase, it had a checkpoint dependent function to inhibit late and dormant origin firing in response to HU treatment (Matsumoto et al., 2017). FA proteins also have a role in controlling origin firing independently of their function in ICL repair. Upon mild replicative stress, FANCI associates with MCM3 and MCM5, and acts as a positive regulator of DDK to promote firing of origins. However, if the level of replicative stress is elevated, FANCI is phosphorylated by ATR and, along with its partner FANCD2, acts as a negative regulator of dormant origin firing (Chen et al., 2015).

4.2- Deregulation of Origin Firing and Replicative Stress

DNA initiation, as previously described, is a tightly organized and regulated process. Any deregulation of proteins that monitor this process such as CDKs, RB, or checkpoint pathways leads to perturbation of either licensing or firing. This would result in deregulation of the firing program where there is either increased, decreased, or re-fired replication origins. This is accompanied with replicative stress where cells might enter into mitosis with under/over-replicated DNA, contributing to genomic instability.

4.2.1- Causes and Consequences of Decreased Origin Firing

It is extensively reported that deregulation in replication origin licensing and firing leads to genomic instability and different diseases, including cancer. Studies have showen that these limitations can be due to mutations in the MCM genes that hinder its loading onto chromatin, or mutations in other components of the pre-RC, or oncogene expression.

Three mice models harboring different holomorphic MCM alleles: MCM $^{chaos3/chaos}$, MCM2$^{Ires-CreERT2/ Ires-CreERT2}$, and MCM4 D573H showed limited number of dormant origins due to the defects in MCM loading. Cells having any of these 3 mutations showed an increased level of DNA damage and genomic instability and are prone to malignant

transformation (Alver et al., 2014). In humans, patients with MCM4 mutations that result in a truncated form of this protein present with different syndromes including natural killer deficiency, adrenal insufficient growth retardation and genomic instability (Casey et al., 2012; Gineau et al.; Hughes et al., 2012). The loading of replicative helicases is not affected in cells from these patients; however, they exhibit cell cycle defects and chromosome breakage.

Another syndrome that results from defective origin licensing is Meier-Gorlin Syndrome (MGS), a rare autosomal recessive primordial dwarfism syndrome. The origin of this syndrome is mutations in five non-MCM pre-RC components: ORC1, ORC4, ORC6, Cdt1 and Cdc6 (Bicknell et al., 2011; Karras et al., 2013). The molecular and cellular phenotypes include impaired licensing, altered S-phase progression, chromosomal instability and predisposition to cancer.

During late G1, the cells pass the licensing checkpoint that ensures that a sufficient number of origins are licensed to avoid the risk of having to duplicate the genome with few origins (Machida et al.). This checkpoint must occur before the G1/S transition where high levels of CDK suppress further origin licensing. The precise mechanism of this checkpoint is not well elucidated. However, the tumor suppressor gene RB seems to be involved since the absence of a functional p53 allows the cell to enter S-phase with low number of origins (Nevis et al., 2009), which leads to incomplete S-phase and DNA damage response activation.

Moreover, oncogenes have been proven to play a role in affecting replication origin licensing. An example is the Cyclin E. In one study, it was proven that the overexpression of Cyclin E impaired MCM loading onto the chromatin, thus inducing a decrease in origin licensing(Ekholm-Reed et al., 2004). However, this was controversial because another study showed that overexpression increased origin firing during S-phase (Bester et al., 2011), reflecting the possibility that different cellular models behave differently and that the consequence of Cyclin E overexpression may be affected by other biomarkers.

4.2.2- Increase of Replication Origin Firing and Replication Catastrophe

As the decrease in origin licensing and firing would affect the genomic stability, an increase in origin firing also leads to replication stress and catastrophe. Replication stress could be derived from an increased replication-transcription collision when the number of replication forks increases. Most importantly, the replication stress could be derived from the exhaustion of replication building blocks including dNTPs (Beck et al., 2012; Bester et al., 2011; Poli et al., 2012), RPA (Toledo et al., 2013), or histones (Mejlvang et al., 2014), which worsen when cells start firing excess dormant origins to rescue stalled forks (Toledo et al., 2013).

In X.laevis, the increase of CDK activity results in and increase in origin firing. Consistent with this, deregulation of proteins controlling CDK or CDC7 in humans such as ATR, Chk1 and Wee1 kinase causes extensive origin firing. This was reported to put the cells at risk of replication catastrophe when faced with replicative stress (Beck et al., 2012; Petermann et al., 2010; Shechter et al., 2004).

i- ATR/Chk1

As discussed above, ATR has a basal role in inhibiting excess origin firing during unperturbed S-phase. This function is crucial for the maintenance of genomic stability, especially during replicative stress. It was reported by Toledo *et al.* that using an ATR or Chk1 inhibitor induces an increase in origin firing. When subjected to replicative stress by hydroxyurea, excess dormant origins would fire in an aim to rescue the stalled forks (Figure 17). However, since the pool of available RPA is limited and already used to cover the ssDNA generated by the extra origins fired, the ssDNA generated by dormant origin firing will be left unprotected and are more prone to DSBs (Toledo et al., 2017).

ii- Wee1 Kinase

Wee1 kinase is involved in the regulation of G2/M checkpoint. It inactivates CDK2 bound to Cyclin B through phosphorylation of tyrosine 15 in response to DNA damage and promotes G2 cell cycle arrest (Do et al., 2013). Wee1 also contributes to the proper replication timing through phosphorylation of CDK1 and CDK2 on their tyrosine 15 residues, therefore controlling DNA replication during S-phase and mitotic entry. It was

shown that the use of Wee1 inhibitor not only leads to immature mitotic entry, but also leads to premature G1/S transition with hyperactivity of CDK1 and CDK2 and an increase in the frequency of origin firing (Figure 17). This was also reported to increase the consumption of dNTPs and RPA pools leading to replication stalling and SLX4/MUS81 dependent endonucleolytic DNA breaks in S-phase (Beck et al., 2012). Treatment with hydroxyurea in the presence of Wee1 inhibitor furtherly increases the number of forks and thus leads to replication catastrophe due to RPA exhaustion (Figure 17) (Toledo et al., 2013).Thus, these kinases prove that controlling the proper origin firing during unperturbed S-phase is essential to protect the genomic stability during replicative stress.
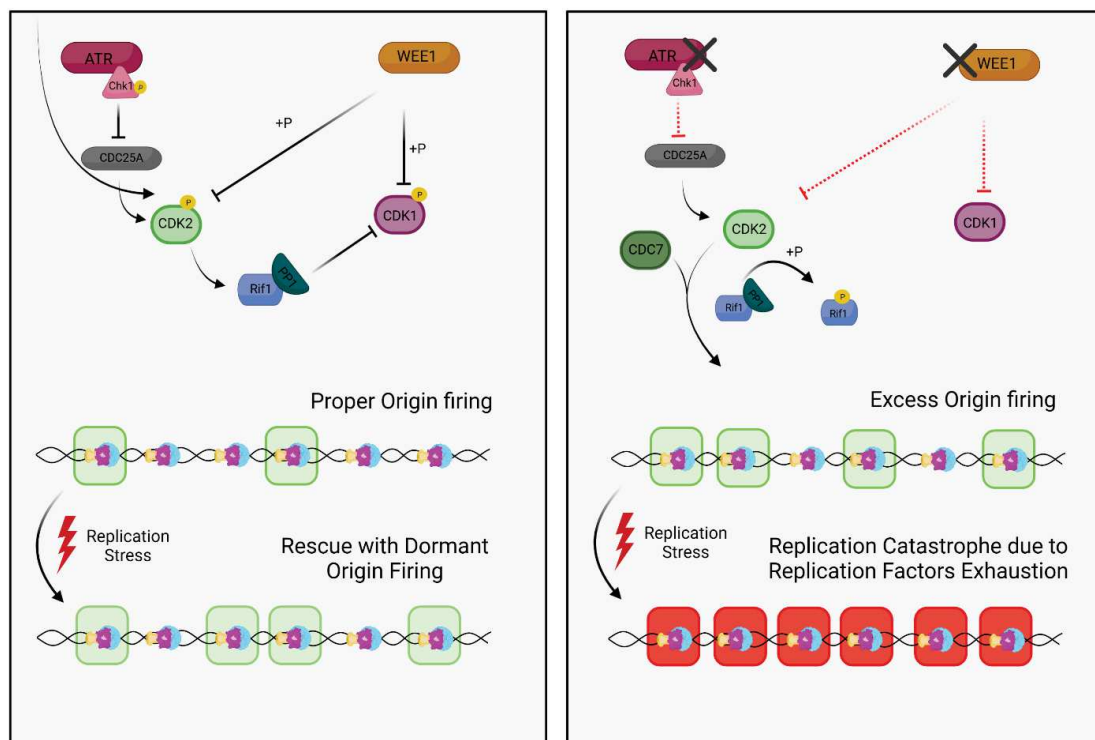
Figure 17. Regulation of origin firing by ATR and Wee1 kinases. In normal conditions (left panel), basal activity of ATR and its main effector Chk1 inhibits CDC25A and subsequently the phosphorylation of CDk2. In non-phosphorylated state CDK2 is not active and the RIF1-PP1 complex is stable where it acts on inhibiting CDK1. Wee1 also inhibits CDK2 and CDK1 by phosphorylating both of them at tyrosine 15. In this case the number of fired origins is regulated during S-phase, and in case of replicative stress dormant origins will fire to rescue the stalled forks. In case of ATR/Chk1 or Wee1 inhibition (right panel), the inhibitory effect on CDK1 and CDK2 will be disturbed, therefore more origins will be fired during S-phase. In case of replicative stress, extra dormant origins will be fired in order to rescue the stalled one. However, due to the exhaustion of replication factors, the forks will be subjected collapse due to nuclease activity for example.

On the other hand, Wee1 was also shown to protect stalled replication forks for DNA end resection by CDK2-dependent regulation of DNA2 (Elbaek et al. 2022).

iii- Oncogenes

Many oncogenes can also disrupt and accelerate the program of origin firing. Oncogene RAS has been described thoroughly for inducing DNA replication stress. One of the mechanisms is by increasing origin firing and generating asymmetrical replication forks (Di Micco et al., 2006). It is possible that it does so by increasing the level of Cdc6 in the cells. Myc oncogene also participates in increasing origin firing. It has been demonstrated that Myc localizes to replication origins and interacts with pre-RC components, and it increases origin firing by recruiting CDC45 to chromatin (Srinivasan et al., 2013).

4.3- Re-firing of Replication Origins

Perturbation in the control of replication origin licensing can lead also to re-licensing and re-firing of replication origins. As discussed earlier, pre-RC components such as ORCs, Cdt1, CDC45 and Sld2/3 are subjected to tight regulations during the cell cycle to inhibit origin re-firing. Any deregulation would lead to replication stress and genomic instability. The main consequence of origin re-firing is the head-to-tail collision that occurs between the unligated Okazaki fragments of the ongoing fork and the leading strand of the re-fired origin. This results in DSB and DNA damage checkpoint activation (Davidson et al., 2006).Overexpression of oncogenes also leads to origin re-firing. For example, RAS upregulates the expression of Cdc6 (Irani et al., 1997) which beside increasing the frequency of origin firing, also leads to re-firing of origins (Mortusewicz et al., 2013). Moreover, the overexpression of Cyclin D1 with a mutation for nuclear localization stabilizes Cdt1 and promotes re-firing of origins (Bartkova et al., 2005).

5- Replication Stress and the Inflammatory Response

The immune system is activated when the cell encounters any infection or tissue damage triggering the inflammatory response. The latter must be well balanced because an insufficient response can result in higher susceptibility to infections or tumor development, while an excessive response could lead to autoimmunity. A possible initial step to activate the inflammatory response is the host cell recognition of pathogens or intrinsically unwanted self-nucleic acid in cytoplasmic spaces (Newton and Dixit, 2012; Paludan and Bowie, 2013; Roers et al., 2016). Many reports have shown an important link between DDR, self-DNA, and the immune response. It was demonstrated that the occurrence of DNA damage could signal the activation of the immune response directly, or indirectly, by the accumulation of nuclear DNA fragments in the cytoplasmic (Gasser et al., 2017; Li and Chen, 2018).

5.1- Cytoplasmic DNA-mediated inflammatory response

The cyclic GMP-AMP synthase (cGAS) and stimulator of interferon gene (STING), cGAS-STING pathway plays a crucial role in triggering the immune response in response to DNA damage (Ablasser and Chen, 2019; Gasser et al., 2017). cGAS acts as a DNA sensor by which it is triggered to produce cyclic guanosine monophosphate- adenosine monophosphate (CGMP-AMP). Human cGAS response depends on the length of DNA, where it was reported that longer DNA (500-4000bp) triggers higher amounts of cGMP compared to DNA of around 20 bp length (Civril et al., 2013; Zhang et al., 2014). STING is a dimeric ER binding protein which is activated by cGMP (Burdette et al., 2011). When cGAS is activated, it produces cGMP which activates and induces the oligomerization of STING (Shang et al., 2019; Zhang et al., 2019) that in turn activates downstream transcriptional response by activating TANK-binding kinase 1 (TBK1) and NFK β pathways (Motwani et al., 2019). TBK1 is recruited and activated by STING. Phosphorylated STING will next recruits Interferon regulatory factor 3 (IRF3), where it also gets phosphorylated and activated by TBK1 (Ishikawa et al., 2009; Shang et al., 2019) a modification that triggers its nuclear translocation where it induces the expression of cytokines and type I interferon (IFN) genes thus starting the inflammatory response.

5.2- Mechanism by Which Replication Stress Induce Inflammatory Response

Defects in proteins involved in DNA repair and DNA damage tolerance are common in tumors and cancer cell lines. These defects promote the accumulation of cytoplasmic ssDNA and dsDNA (Hong et al., 2019; Lam et al., 2014), thus elevating the immune response.

Two main mechanisms have been described to induce activation of the immune response by replication stress (Figure 18). First, upon replication stress, the cells may enter mitosis with DNA that is not fully duplicated or with damaged DNA leading to mitotic defects and formation of micronuclei (Mankouri et al., 2013; Wilhelm et al., 2014). Due to different reasons such as RNase H deficiency, BRCA2 mutations, or γ-irradiation, micronuclei will form and at some point, the envelope will be raptured allowing the release of DNA fragments into the cytosol and the activation of cGAS-STING pathway (Dou et al., 2017; MacKenzie et al., 2017; Reisländer et al., 2019). Second, small DNA fragments could be directly released from DNA processing (Jazayeri et al., 2008) and escape the nucleus (Wolf et al., 2016). The inactivation of proteins involved in repairing DNA and maintaining its integrity leads to accumulation of cytoplasmic DNA.

Stalled and reversed forks are major sources of cytosolic DNA. When both structures are not well maintained they could be targeted to nucleolytic activity such as MUS81 (Ho et al., 2016; Shen et al., 2015) which generates DNA fragments that can escape to the nucleus (Coquel et al., 2018). Deficiencies in proteins regulating nucleases such as SAMHD1, which regulate RECQ1 and MRE11, lead to aberrant fork processing and the release of ssDNA into the cytoplasm (Coquel et al., 2018).

It is well described in the literature that replicative stress triggers the inflammatory response by cGAS-dependent STING activation; however, Dunphy *et al.* reported that etoposide-induced replicative stress can activate STING independently from cGAS. In their report, they showed that ATM and PARP-1 together with the DNA binding protein IFI16 resulted in the assembly of a complex that includes p53 and E3 ubiquitin ligase TRAF6 (Dunphy et al., 2018). This complex activates STING in a non-canonical pathway,

where TRAF6 catalyzes the ubiquitination of STING, thus leading to its activation and its downstream target factor NF-kB, which triggers that inflammatory response.

To avoid abnormal DNA-driven immune or autoimmune reactions, the cell has different types of DNases (DNase I, DNase II, and TREX1) which act on different cellular components to degrade DNA fragments before they activate the inflammatory response (Atianand and Fitzgerald, 2013). TREX, for example, degrades DNA as it enters the cytoplasm and also targets ssDNA coated with RPA and RAD51 in the cytosol and the nucleus (Huffman et al., 2005; Wolf et al., 2016).
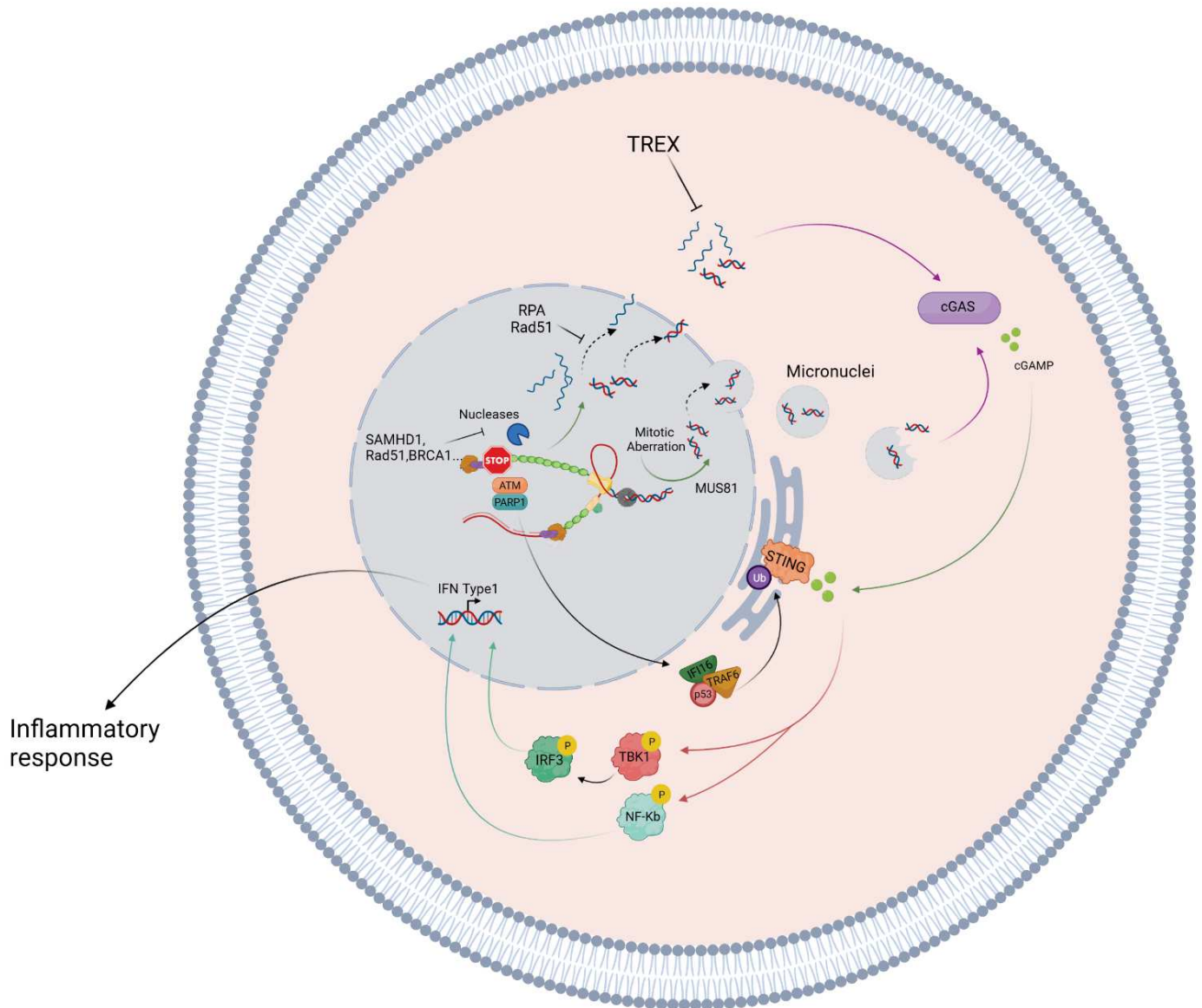
Figure 18. Mechanism of activation of the cell-intrinsic innate immune response by DNA replication stress. During replication stress and in cases of fork instability self DNA can accumulate in the cytoplasm. ssDNA or dsDNA are generated from stalled forks by the action of nucleases such as Mre11 and MUS81. These fragments are released into the cytoplasm, however RAD51 and RPA are known to inhibit this translocation. TREX is the main cytosolic exonuclease that degrade these fragments to inhibit the activation of cGAS-STING. In case of presence of under-replicated DNA, chromosomes will have defects during segregation which will induce the formation of micronuclei. Upon the rupture of the micronuclei membrane, cGAS detects the DNA and activates STING which will induce the transcription of type II interferons through the activation of TBK1 or NF-κb. Eventually the inflammatory response will be activated.

5.3- Impact of Inflammatory Response on Cancer Progression

The effect of activating the immune system by cancer cells is controversial. The increased production of cytokines and interferons by DNA damage could increase immune cell infiltration into tumors and promote its rejection (Erdal et al., 2017; Harding et al., 2017; MacKenzie et al., 2017). For example, treatment of triple negative breast cancer models with PARP inhibitors promoted CD8^{+}T cell tumor infiltration, which was dependent on cGAS-STING activation (Huffman et al., 2005). However, chronic inflammation that is associated with genomic instability of some cancer cells may also boost cancer progression by stimulating metastasis (Bakhoum et al., 2018). Immunotherapy has been used as a therapeutic approach to target tumors and it is very important to understand how replication stress-mediated inflammation could potentiate its effect. Cancer cells may exploit the cGAS-STING mediated immune pathway to promote the formation of a microenvironment favoring the tumor growth. For that reason, immunocheckpoints inhibitors have been used as an approach to exploit the immune rejection of tumors such as targeting TREX or STING pathway. However, immunotherapy cannot be used against 'cold' tumors that have managed to escape the immune system. These 'cold' tumors could be actually turned into 'hot' tumors by strategically targeting the DNA integrity using chemotherapeutic drugs. The combination of immunotherapy with chemotherapy could have synergistic effects, which makes it a promising therapeutic strategy. For example, combining platinum-based chemotherapy with anti-PD-1 therapy successfully increased the survival rate of non-small-cell lung carcinomas (Goto et al., 2012).

6- Replication Stress and Human Diseases

Identification of driver mutations for different genetic syndromes has revealed an implication of proteins functioning in DNA replication and DNA repair pathways (Zeman and Cimprich, 2014). These syndromes share common characteristics such as developmental defects, growth retardation, common neurological disorders and high susceptibility to cancer development. The mutations occurring at the level of the DNA replication process include ones in the pre-RC complex proteins that lead to the

development of Mier-Gorlin syndrome as described previously. Defects in replication stress signaling also lead to several diseases. One prominent example is Seckel syndrome caused by mutations in the ATR Gene. This syndrome is characterized by developmental delay, microcephaly, and mental retardation (Murga et al., 2009; O'Driscoll and Jeggo, 2008). Loss of the MRN complex, which leads to the loss of ATR activation and DSB repair (Stracker and Petrini, 2011), is also correlated with a number of developmental disorders such as ataxia telangiectasia like disease (OMIM 604391) and Hickman breakage syndrome (OMIM 251260). Loss of proteins that recognize or repair lesions also leads to a variety of human diseases. RNaseH2 is one of multiple genes that could lead to a neurological disorder known as Aicardi-Goutières syndrome (Crow et al., 2002). When not expressed, RNaseH2 deficiency leads to the development of Fanconi Anemia that could be caused either by rNTP misincorporation, r-loops accumulation, or both (Kim and D'Andrea, 2012). In addition, mutations in proteins involved in chromatin remodeling during DNA replication have also been associated with human diseases. For example, mutations in the fork reversal enzyme SMARCAL1 lead to the development of Schimke immuno-osseous dysplasia (SIOD) (Boerkoel et al., 2006).

Chapter 6: Guanine Binding Like 3 - GNL3

1- Identification and Structural Characteristics of GNL3

In 2002 Tsai and Mckay first reported the identification of mammalian GNL3, also known as Nucleostemin, as a protein enriched in neural stem cells, embryonic stem cells, and cancer cells (Tsai and McKay, 2002) .

GNL3 belongs to the YlqF/Yaw GTPase family that is involved in ribosomal biogenesis, cell proliferation, and cellular growth. It is characterized by circularly permutated order of GTP binding motifs (Tsai and McKay, 2002). YlqF/Yaw GTPase family is conserved all over Eukarya, Bacteria and Archea and its members are characterized with different compartmental localization suggesting location dependent functions (Mier et al., 2017; Reynaud et al., 2005). In Archea and Bacteria, only one protein of this family is found which are YAG and YlqF, respectively. However, in Eukarya each cellular compartment has its specific protein: Lsg1/GNL1 in the cytoplasm, Mtg1/Noa1 in in mitochondria, CylaF/cYjeQ in chloroplast and GNL2/GNL3/GNL3L in the nucleolus. In vertebrates GNL3, GNL3L and Ngp1 from a distinct subgroup that is localized mainly in the nucleolus, however they have distinct functions.

GNL3 is 77 kDa and is composed of five domains: NH2-terminal - Basic (B) domain, coiled coil (C) domain, two GTP binding motifs (G4: KXDL; GnGXXXXGK[S/T], intermediate (I) domain and COOH terminal acidic domain (A) (Tsai and McKay, 2002) (Figure 19). Thanks to its several domains, GNL3 was shown to interact with several proteins such as p53, MDM2 ARF, TRF1, and RSL1D1 (Dai et al., 2008; Meng et al., 2006; Tsai and McKay, 2002; Zhu et al., 2006). These different interactions reflect the implication of GNL3 in several biological processes.

A. Functional Domains

NH2 **B** **CC** **G1** **G4** **I** **A** COOH

B. Predicted Structure
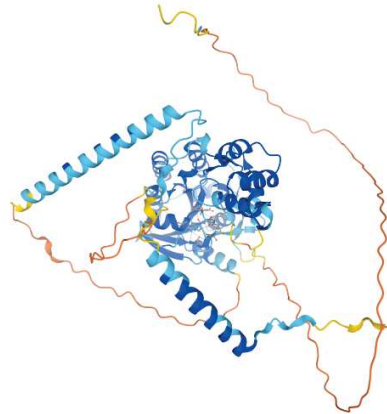
Figure 19. The structure of GNL3 protein. (A) Schematic representation of the functional domains of human GNL3. (B) GNL3 structure prediction by Alphafold tool.

2- Localization and Functional domains

To dissect the molecular functions of GNL3, especially its GTP binding activity, a series of deletion of domains were performed (Tsai and McKay, 2002). It was revealed by immunostaining of GNL3 that its main localization is within the nucleolus; however, it is also diffused in the nucleoplasm, yet at lower concentrations.

Processes controlling cellular growth, such as ribosomal biogenesis take place within the nucleolus. Given that GNL3 is present both in the nucleolus and the nucleoplasm and that it is involved in cellular proliferation (Tsai and McKay, 2002), it was hypothesized that the regulation of GNL3 localization between the two compartments might provide a functional mean to regulate its activity.

The localization of GNL3 in the nucleolus is not static. Actually, FRAP experiments showed that GNL3 is able to shuttle bidirectionally between the nucleolus and the nucleoplasm (Tsai and McKay, 2005). In order to uncover the mechanism that enables

this shuttling, different GNL3 mutants were generated. In the first study done by Tsai and McKay aiming to understand how GNL3 is functioning, they generated different GNL3 sequences with mutation for the basic domain (dB), coiled coil (dCC), G4-GTP binding motif (dG4), G1-GTP binding motif (dG1), acidic domain (dA), or both basic and G1 domains (dB/G1). These mutants were expressed into U2OS cells. Although all of them were localized to the nucleolus, dB mutant was the only one that was diffused into the nucleoplasm, while other mutants localized mainly into the nucleolus (Tsai and McKay, 2002). GTP mutants showed irregular aggregates when they were localized into the nucleolus or into the nucleoplasm with a double mutant dB/G1. These data showed that the basic region is required for GNL3 nucleolar localization and that the GTP-binding motifs are important to the appropriate distribution of GNL3. Moreover, GTP-binding motifs were found to be responsible for the regulation of cell cycle through interacting with p53. In another study using U2OS and CHO cell lines, two mutations of the GTP-binding domain of GNL3 (G265V and G261V) showed a diffusion of GNL3 signal in the nucleoplasm (Tsai and McKay, 2005). This indicated that the basic domain and the GTP binding domain are both responsible for the nucleolar shuttling of GNL3. However, combining the GTP mutation with a deletion of the intermediate domain could restore the nucleolar localization. To conclude (Figure 20A), the basic domain of GNL3 was found to be responsible for its nucleolar localization, but it is inhibited by its intermediate domain that acts as an anchor keeping GNL3 in the nucleoplasm when it is not bound to GTP. Once GNL3 binds to GTP, the conformation of GNL3 changes and the intermediate domain is no longer able to retain GNL3, thus allowing the basic domain to shuttle GNL3 into the nucleolus. Other studies have shown that the nucleolar localization of GNL3 is mediated through the interaction of B domain and G domain with the nucleolar protein RSLD1 (Meng et al., 2006).
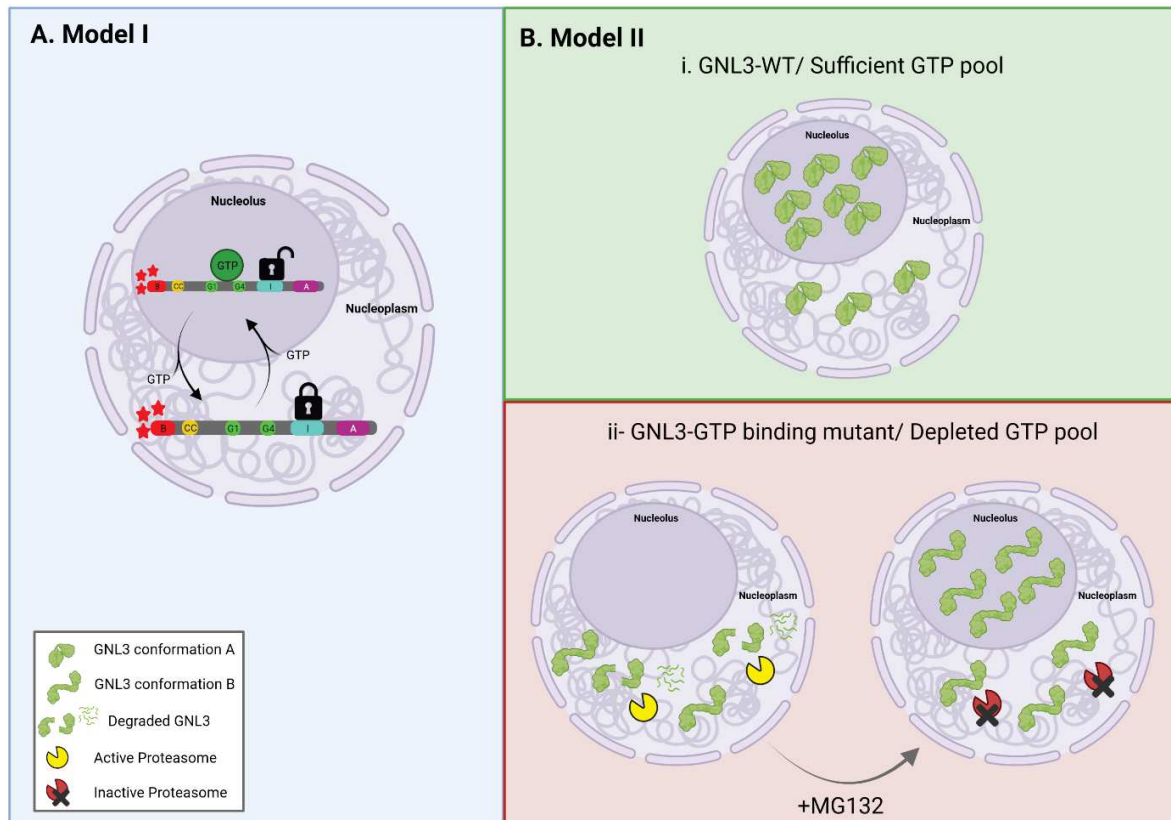
Figure 20. Regulation of GNL3 localization. (A) In the first model, the localization of GNL3 depends on its GTP binding state. In absence of GTP, the intermediate domain will retain GNL3 in the nucleoplasm. When GNL3 is bound to GTP, its conformation will change, and the B domain will shuttle GNL3 into the nucleolus. **(B)** In the second model, the localization of GNL3 depends on its cellular level. When GNL3 is not bound to GTP it has conformation B, which is susceptible to proteasomal degradation, therefore it is not stable. Upon inhibition of the proteasomal activity with MG123, the level of GNL3 will increase in the nucleoplasm, and the excess will shuttle into the nucleolus.

The GTP–driven nucleolar cycle is an event where nucleolar proteins are relocated between the nucleolus and the nucleoplasm. Several nucleolar proteins where shown to change their localization into the nucleoplasm when the GTP pool was downregulated by inhibition of *de novo* synthesis of GTP by the enzyme IMP dehydrogenase (IMPDH) (Huang et al., 2008; Tsai and McKay, 2005). MPA and AV93 are two molecules that inhibit the activity of IMPDH. It was reported that GNL3 showed nucleoplasmic relocalization when cells are treated with either MPA or AV93, resembling the phenotype of the GTP

mutant (Huang et al., 2009; Lo et al., 2012; Tsai and McKay, 2005). It was shown that GNL3 is degraded upon GTP depletion, where its half-life is reduced from more than 9 hrs. to less than 4 hrs.(Lo et al., 2012). Consistent with that, it was reported that GNL3 mutant for GTP binding also shows a decrease in its half-life nearly to 3 hrs.. Studies have reported that the presence of MG132, a proteasomal inhibitor, would not only rescue GNL3 from degradation but also restore its nucleolar localization (Huang et al., 2009). These data suggest that the GTP unbound state of GNL3 is in a specific conformational state that would increase its susceptibility to being degraded by the proteasome. Interestingly, the fact that GNL3 does not require GTP to shuttle into the nucleolus indicates that the shuttling mechanism may be passive (Figure 20B). It might be a storage mechanism that prevents too much GNL3 from residing in the nucleoplasm.

How GNL3 is degraded is still not clear. In one study it was reported that in U2OS cells this degradation is dependent on the E3 ubiquitin ligase activity of MDM2 (Huang et al., 2009). However, in another study using MEFs cells, it was reported that this degradation is independent of ubiquitination and MDM2 (Lo et al., 2012).

3- Role of GNL3 in cell cycle and Apoptosis

As mentioned before, GNL3 was first identified in rat central nervous system (CNS) stem cells and later on it was reported to be expressed in human bone marrow and mouse embryonic stem cells (Kafienah et al., 2006; M et al., 2003). GNL3 expression is high during the early stages of CNS stem cells and it gradually decreases as cells are differentiating. Interestingly, several studies have used GNL3 as a marker for stemness of the cells (Cai et al., 2004; M et al., 2003). In addition, GNL3 is re-expressed as cells are transforming into malignant ones (Liu et al., 2004; Ma and Pederson, 2007; Politz et al., 2005) . From this expression profile, it was expected that GNL3 would be a key factor in controlling cellular proliferation. The first attempt to understand the role of GNL3 was reported by Tsai and Mckay, where their study showed that depletion or overexpression of GNL3 in cortical stem cells and U2OS cell line would lead to a reduction in the rate of cellular proliferation. A lot of studies using different cellular models showed that the depletion of GNL3 result in a reduced proliferation rate and either G1/S or G2/M arrest

(Tsai, 2014). Although the outcome of GNL3 depletion was clear, the biological explanation underlying this outcome was explained either through p53 action or through a p53 independent pathway. It is important to mention that the dependency on p53 is biased by the type of the cellular model and differences between normal stem cells and cancerous ones.

3.1- The p53-dependent model

Several studies have reported the implication of p53 in the biological function of GNL3 regarding cellular proliferation (Figure 21). Physical interaction between GNL3 and p53 was first described after the identification of GNL3 (Tsai and McKay, 2002) In order to explain the implication of this interaction in the function of GNL3, knockdown or overexpression experiments were performed and linked with p53 profile (expression or depletion). Surprisingly, as GNL3 knockdown would lead to reduced cellular proliferation, overexpression of GNL3 had the same outcome. Experiments in several cellular models showed that knockdown of GNL3 elevated the level of p53 (Dai et al., 2008; Huang et al., 2009; Tsai and McKay, 2002). On the other hand, the overexpression of GNL3 would also stabilize the activity of p53 (Dai et al., 2008; Meng et al., 2008).

This controversy was later on explained. It was reported that GNL3 binds directly to the acidic domain of MDM2 (Dai et al., 2008), where it abrogates the ability of MDM2 to mediate ubiquitination-dependent degradation of p53. Thus, overexpression of GNL3 would increase excessively its binding to MDM2 and lead to a steady state elevation of p53, which explains the cell cycle arrest and the decrease in proliferation rate. However, unlike ARF, which inhibits MDM2 by sequestering it in the nucleolus, GNL3 interaction with MDM2 was observed in the nucleoplasm (Meng et al., 2008) which suggests a new regulatory mechanism. Furtherly, if GNL3 would sequester MDM2 in the nucleolus, MDM2 should be released into the nucleoplasm upon GNL3 depletion to degrade p53, yet this is not what was reported.

Previous studies had shown that under cellular stress, large ribosomal proteins L5 and L11 interact with MDM2, inhibit its action, and thus elevate the level of p53 (Dai et al., 2006; Pederson, 1998). It was reported that the depletion of GNL3 might affect the rRNA processing (described elsewhere), thus yielding to less mature rRNAs which cause

ribosomal stress (Romanova et al., 2009a). As a result, unassembled rRNA could be released into the nucleoplasm where they would signal the stress. Consistent with this, depletion of GNL3 increased the interaction between L5, L11 and MDM2, therefore inhibiting MDM2 and elevating the level of p53 which leads to cell cycle arrest.
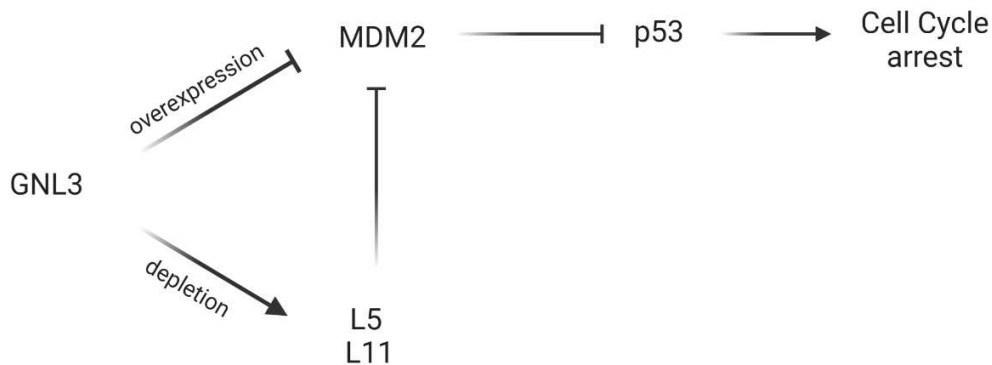
Figure 21. The p53 dependent role of GNL3 in cell-cycle progression.

How p53 guides the response of GNL3 loss in normal and cancer cells?

In order to study the role of p53-GNL3 interplay in normal and cancer cells, a study published by Hung et al reported the different phenotypes of GNL3 depletion in MEFs and HCT116 cells (Huang et al., 2015). They showed that GNL3 depletion in both cell lines resulted in G2/M arrest; however, the mechanism underlying this phenotype was different. In MEFs cells, depletion of GNL3 in the presence of WT p53 increased the expression of reprimo (RPRM), a protein involved in G2 arrest through reduction of CDK1 (Cdc2) expression and cytoplasmic cyclin B export. In p53 knock-out MEFs, depletion of GNL3 led to an increase in the phosphorylation of CDK1 at tyrosine 15, an activating phosphorylation mediated by Wee1/MiK1 kinase, that plays a role in G2/M arrest (Berry and Gould, 1996). Although in the two cases cells underwent G2/M arrest, the outcome of GNL3 depletion was more serious and it was translated by the formation of polyploid giant cell (PGC). Thus, indicating the dependency of normal cells on p53. In HCT116 cancer cells, depletion of GNL3 had a similar phenotype in the absence or presence of

p53. Phosphorylation of CDK1 was elevated upon GNL3 depletion and it mildly increased upon p53 knock-out. RPRM expression was also higher upon depletion of GNL3 in both conditions. However, depletion of GNL3 in HCT116 did not show any PGCs. The p53 independency of HCT116 cells response to GNL3 depletion shows that GNL3 presence is still crucial for the proper cycling of the cells. However, its function is no longer translated via p53, which indicates that the cells may have developed an alternative pathway to regain control.

3.2- The p53-independent model

Although experiments showed a clear regulatory connection between GNL3, MDM2 and p53, several studies have reported that p53 is dispensable for the function of GNL3 regarding cell cycle control and proliferation as reported in Huang *et al.* (Huang et al., 2015)

In 2006, Beekman et al reported the generation of a mouse model with a specific gene trap event that inactivates the GNL3 gene (Beekman et al., 2006). They showed that heterozygous mice had no defects in development; however, GNL3$^{-/-}$ embryos died around the fourth day of embryonic development. Analysis of these blastocysts showed that the cells failed to enter into S-phase. Importantly, they showed that knockout of p53 could not rescue the lethality of these mice. This indicated that GNL3 is a multifunctional factor exerting its role(s) in a p53 dependent and independent manner. Other studies have confirmed this finding where depletion of GNL3 had no effect on p53 or its downstream, and the phenotypes of GNL3 depletion were not rescued by p53 depletion (Liu et al., 2008)

There is no one clear pathway that describes how GNL3 controls the cell cycle independently of p53; however, there are several processes that could be implicated in this control. It was reported that GNL3 depletion leads to upregulation of the INK family genes and downregulation of CyclinD1 and HDAC (Liu et al., 2008). Importantly, as discussed before INK are proteins that control the cell cycle progression during G1 and affect the Cyclin D/CDK4-6 complex. It is unclear how GNL3 would affect the expression of these genes; however, it is consistent with its ability to control the cell cycle. Other studies have shown that GNL3 depletion increases the expression of p27 (Yoshida et al.,

2011). GNL3 interacts with p27 and triggers its nucleolar sequestration and polyubiquitination, thus its activation. Therefore, when GNL3 is depleted, p27 is activated and it binds to Cyclin E/CDK2 complex where it inhibits its action and lead to cell cycle arrest (Hu et al., 2017).

Another mechanism proposed is through modulation of ARF. It was found that overexpression of GNL3 would lead to increased GNL3-ARF interaction that would stabilize ARF/Nucleophosmin complex within the nucleolus. On the other hand, GNL3 is able to bind to ULF, the E3 ligase of ARF, preventing the ubiquitination and proteasomal degradation of ARF. Both mechanisms lead to ARF-dependent G1 cell cycle arrest (Lo et al., 2015).

4- Role of GNL3 in maintaining genomic integrity of cancer and stem cells

The contradictory results concerning the link between GNL3 and p53 concerning cellular proliferation and the fact that GNL3 is an essential gene for embryonic development indicated that GNL3 has a crucial role outside the MDM2-p53 regulation loop. The first clue about the implication of GNL3 in maintaining the genomic integrity (Figure 22) was provided by Hsu et al when they showed that GNL3 is important in protecting telomeric DNA by recruiting PML-IV to SUMOylated TRF1 (Hsu et al., 2012). It was reported by several studies that the depletion of GNL3 increase the level of phosphorylated H2AX at Ser-139 (γH2AX) in different cellular models such as dividing hepatocytes, hematopoietic stem cells, neural stem cells, mammary tumor cells, and hepatocellular carcinomas (Lin et al., 2013, 2019; Meng et al., 2013; Wang et al., 2020; Yamashita et al., 2013). This lesion was described to be replication-dependent since several studies showed that γH2AX-positive cells are in S-phase (Lin et al., 2014; Meng et al., 2013). Other DNA damage markers were also reported to be increased in the absence of GNL3, such as 53BP1, ATR, BRCA1, and RPA (Meng et al., 2013). This was consistent with the fact that GNL3 depletion caused an increase in the DSB incidents (Lin et al., 2019; Wang et al., 2020). Moreover, incubation of GNL3-depleted cells with 2 mM

HU for 24 hrs. increased the levels of γH2AX and phosphorylated ATR (Lin et al., 2019). Therefore, depletion of GNL3 sensitizes cancer and stem cells to replication stress. Thus, it was hypothesized that the overexpression of GNL3 would make these cells more resistant to replicative stress. This hypothesis was validated by several studies showing that overexpressing GNL3 in hepatocellular carcinomas, mammary tumors and neural stem cells led to a decrease in the level of γH2AX when cells were treated with HU. However, it should be noted that GNL3 overexpression was reported to induce G2/M arrest; therefore, the decrease of γH2AX observed could be a consequence a reduced number of cells in S-phase. Consistent with this, an analysis of COSMIC (Catalogue Of Somatic Mutations In Cancer) and CTRP (Cancer Therapeutics Response Portal) databases showed that high level of GNL3 expression is synthetic lethal with DNA damaging drugs and checkpoint inhibitors such as cisplatin, SN38, CPT, ETP-4646 (ATR inhibitor) and PHA-79388 (CDC7 inhibitor) (Wang et al., 2020). Therefore, the controversy in the phenotypes resulting from GNL3 overexpression needs to be furtherly addressed in order to have a clear correlation between the levels of GNL3 and cancer that would predict the prognosis of chemotherapeutic treatments.

While trying to understand the mechanistic role of GNL3 in protecting the genomic integrity, several studies showed a link between GNL3 and RAD51 (Figure 22). The fact that GNL3 depletion increases the level of DSBs and that RAD51 is the core protein in DSBs repair by homologous recombination, looking for a possible link between these two proteins seemed logical. While exploring the role of GNL3 in repairing telomeric DNA damage, Zhu *et al.* reported that GNL3 depletion or overexpression led to decrease in RAD51 foci that colocalize with TRF1 (Zhu et al., 2006) Another study by Lin et al. furtherly reported that depletion of GNL3 decreased the number of RAD51 foci formed in response to HU treatment (Lin et al., 2013). They also reported that RAD51 overexpression, but not overexpression of BRCA2 or RPA70, would slightly rescue the spontaneous γH2AX signal that occurs upon GNL3 depletion (Meng et al., 2013).

To further understand the role of GNL3 in inducing the formation of RAD51 foci, Meng *et al.* utilized DSB-ChIP (Chromatin Immunoprecipitation) assay in U2OS cells to assess if GNL3 is responsible for RAD51 recruitment to DSBs. Their report showed that depletion of GNL3 leads to a decrease in RAD51 recruitment to the site of the DSB (Meng et al., 2013). Another report by Lin *et al.* also reported a similar result. A DSB induced by the endonuclease I-Sce1 can be repaired by different pathways (HR, NHEJ, alt-NHEJ, or SSA). Depletion of GNL3 showed a decrease in repair by HR (Lin et al., 2019). This finding was supported by the co-enrichment of GNL3 with proteins involved in HR. On the other hand, GNL3 depletion increases repair by alt-NHEJ, suggesting that it could be a consequence of HR impairment or a change in the cell cycle distribution.
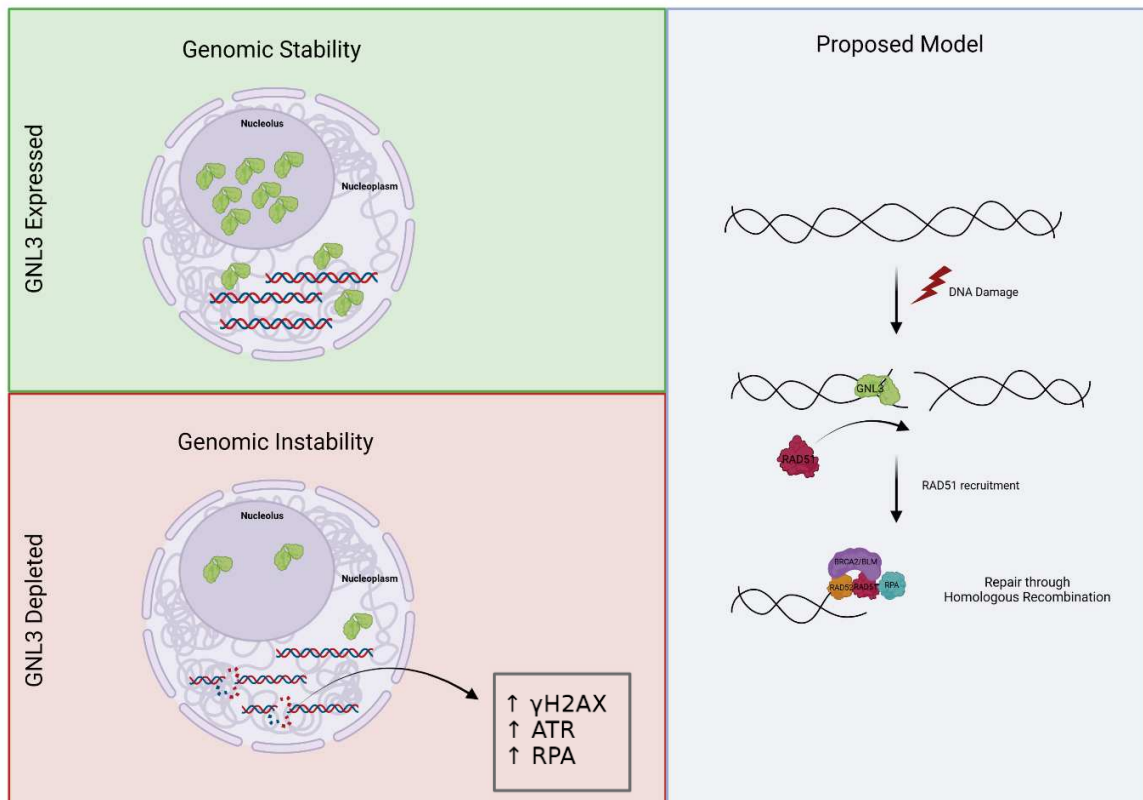
Figure 22. GNL3 is implicated in maintaining the genomic integrity. Upon GNL3 depletion, spontaneous DNA lesions appears and therefore the level of γH2AX, ATR, and RPA increases. It is proposed that GNL3 maintain the genomic integrity by recruiting RAD51 to DSB in order to initiate homologous recombination.

5- Role of GNL3 in the maintenance of telomeric DNA

The role of GNL3 in the maintenance of the proliferative capacity of cancer and stem cells was first linked to its role in regulating the p53/MDM2 loop. However, later studies showed that GNL3 is actually implicated in maintaining genomic stability. This role was first described by an interaction between GNL3 and TRF1 that prevented senescence (Zhu et al., 2006).

During replication, the replisome faces difficulties in replicating the telomeric DNA leading to progressive telomere shortening. To counteract this, a specific enzymatic machinery, the telomerase, composed of a reverse transcriptase (TERT) and RNA component (TERC) is able to lengthen telomeres. Moreover, telomeres resemble a DSB; therefore, it is important to protect them from DNA resection and repair mechanisms that would result in fusing sister chromatids ends by NHEJ, for example. Telomeres are protected by a complex known as Shelterin composed of TRF1, TRF2, RAP, POT1, TPP1 and TIN1. This complex prevents repair and therefore stabilizes the telomeres and controls cellular senescence (De Lange, 2018). The maintenance of telomeric integrity is favored by their length. In 80% of cancer cells, the telomere length is maintained by the telomerase (TA+) (Greider and Blackburn, 1996). In cancer, where telomerase activity is not detected, the telomeres are stabilized by a mechanism named ALT (Alternative Lengthening of Telomeres) that is using HR to maintain telomeres length (Hsu et al., 2012). A unique feature of ALT is the formation of ALT- associated PML bodies (APB), which are composed of SUMOylated TRF1, TRF2, PML-associated proteins, MRN complex, RAD52 and RPA (Hsu et al., 2012). Although APB contains proteins implicated in recombination and repair, their exact role in the ALT mechanism is still not known. TRF1 binds to telomeric repeats (5'TAGGGTT3') and its binding affinity is dependent on the formation of homodimers (De Lange, 2018). Inactivation of TRF1 disrupts the telomeric localization of the Shelterin complex and induces genomic instability. It also negatively regulates telomeres elongation by telomerase (Van Steensel and De Lange, 1997).

Zhu *et al.* showed that GNL3 interacts with TRF1 and positively regulate its degradation but not ubiquitination (Zhu et al., 2006) (Figure 23A). This was described as a mechanism by which GNL3 establishes early embryogenesis and inhibition of senescence in MEFs.
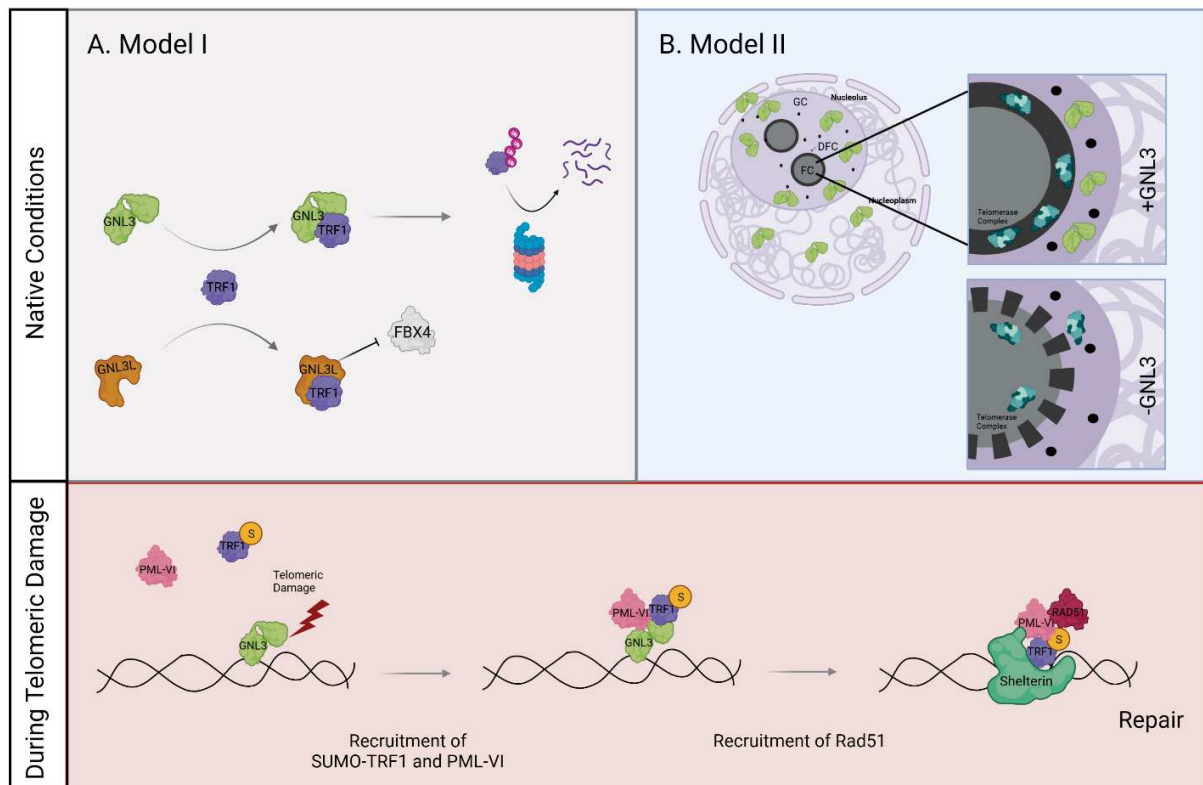
Figure 23. Role of GNL3 in maintenance of telomeric DNA. In native conditions GNL3 was proposed (in the first model) **(A)** to maintain the telomeric integrity by enhancing the degradation of TRF1, which is a negative regulator of the telomerase enzyme. GNL3 and GNL3L bind to TRF1 and exert opposite effects, GNL3 enhances the degradation while GNL3L stabilizes TRF1 through inhibiting the Ubiquitin Ligase FBX4. In the second model, **(B)** GNL3 functions as a structural protein that maintains the DFC structure of the nucleolus which harbors the telomerase complex. In absence of GNL3, the DFC is disorganized which alters the activity of the telomerase complex, thus affecting the telomeric maintenance. During Telomeric damage, GNL3 is responsible form recruitment of SUMOylated TRF1 together with PML-VI to form the APB bodies and initiate repair of the telomeric ends.

In humans, the GNL3 family is constituted of GNL3, GNL3L and Ngp-1. GNL3L and GNL3 are the most closely related. Zhu *et al.* showed in another report that GNL3L is also able to bind to TRF1 (Zhu et al., 2009). Unlike GNL3, GNL3L decreases the degradation of TRF1 by preventing its binding to the E3 ubiquitin ligase FBX4, which allowed its

accumulation during mitosis where it had a role in spindle assembly. Moreover, GNL3 and GNL3L bind to different regions of TRF1 (Hsu et al., 2012). GNL3 inhibits homodimerization of TRF1 and decreases its association with telomeres (Meng et al., 2011). However, GNL3L promotes its homodimerization and reduces the formation of APB. Both GNL3 and GNL3L are localized in the nucleolus, which may act as a hub for regulating telomeres. However, it was reported that their interaction with TRF1 occurs in the nucleoplasm.

It was described that the effect of GNL3 on the dynamic of TRF1 might regulate the access of repair proteins to telomeric DNA (Figure 22). This hypothesis was validated by a report showing that GNL3 promoted telomeric maintenance by allowing the association of PML-IV, a component of the APB bodies, and SUMOylated TRF1 (Hsu et al., 2012). This complex would inhibit telomeric DNA damage and fusions of sister chromatids. It was also responsible for the recruitment of RAD51 to telomeric ends, thus favoring ALT. On the other hand, GNL3L played the opposite role where it inhibited MMS21-dependent SUMOylation of TRF1, thus preventing its association with PML-IV.

The expression pattern of GNL3 and GNL3L is different. GNL3 expression is high in undifferentiated cells, whereas GNL3L levels are high in differentiated ones. This suggests that GNL3 is functioning in extending the proliferative lifespan by providing tolerance to telomeric DNA damage, while GNL3L may play a role in stabilizing telomeres in differentiated cells.

The role of GNL3 in protecting telomeres through SUMO-TRF1 and PML-IV was also proposed to be a similar mechanism in TA+ cells, where they use HeLa cells as a model (Hsu et al., 2012). However, the fact that RAD51 is recruited to maintain telomeric ends is not applicable since TA+ cells do not undergo HR-mediated repair. The implication of GNL3 in protecting telomeric ends in TA+ cells was reported by other groups; however, the mechanism is completely different. In their report, Romanoca *et al.* showed that depletion of GNL3 changed the nucleolar architecture (Figure 22B) (Romanova et al., 2009b). Nucleolus is composed of 3 layers: (1) Granular Component (GC), where late steps of pre-ribosomal assembly take place. (2) Dense Fibrillar Component (DFC), where rRNA transcription takes place. (3) Fibrillar Center (FC), where early steps of pre-

ribosome assembly take place. In their report, they describe GNL3 as a component of the GC, and its depletion disorganizes the nucleolar architecture, especially the DFC where telomerase complex resides. They showed that GNL3 is required for the integrity of the the telomerase complex, which provides another link between GNL3 and the telomerase length, especially since they did not reproduce the interaction between GNL3 and TRF1 in HeLa cells.

6- GNL3 and heterochromatin Maintenance

Okamato *et al*. reported that GNL3 is able to interact with human telomerase TERT within a complex composed of TERT-BRG1- Nucleostemin (GNL3) (TBN) (Okamoto et al., 2011). This complex was identified while they were trying to understand how GNL3 expression would contribute to the maintenance of tumor initating cells. At first, this complex was thought to affect telomere length or telomerase activity; however, this was not the case. This suggested that this complex operates in a telomere-independent function and, on the other hand, may trigger transcriptional programs that might maintain tumor initiation. However, an additional role for this complex has been described. Components of the TBN complex were shown to colocalize with the mitotic spindle during M-phase, and any suppression of either component would lead to mitotic arrest (Maida et al., 2014). The TBN complex is localized to centromeric DNA, where it binds to ssRNA transcribed from α-satellite DNA and human LINE1 elements. TERT produces dsRNA from these ssRNA that will be processed into siRNA by the function of ARGO2. The produced siRNA is targeted to these corresponding heterochromatin centromeric regions during mitosis to maintain the heterochromatin state. Indeed, it was shown that any disruption of the TBN complex leads to increased expression of these regions, troubles during mitotic progression and genomic instability.

7- GNL3 role in pre-RNA processing

GNL3 and GNL3L share high homology in their sequences. They exist as separate genes only in vertebrates (Tsai and Meng, 2009). However, in other species such as

D.melanogaster and *C.elegans*, only one homologue for these two proteins exists. Because of their nucleolar localization, GNL3 and GNL3L have been presumed to have a role in ribosomal biogenesis. Most of the reports showing the implication of this family of proteins have been described in invertebrates (Du et al., 2006; Kudron and Reinke, 2008; Rosby et al., 2009). The first attempt to study the role of GNL3 in ribosomal biogenesis was reported by Romanoca *et al.* (Romanova et al., 2009a). In their study, they showed using a sucrose gradient that GNL3 co-fractionates with a complex containing proteins that are involved in pre-rRNA processing inducing the Pres1, DDX21 and EBP2. Moreover, depletion of GNL3 disrupts the nucleolar retention of DDX21 and EBP2. They also showed that the depletion of GNL3 is delaying the processing of 32S pre-RNA into 28S rRNA. However, it is important to mention that all the phenotypes reported by this study were observed after prolonged depletion of GNL3, (two rounds of depletion over a period of five days). Therefore, one might speculate that these phenotypes could be a side effect of GNL3 depletion and not a direct one. Consistent with this, the direct role of GNL3 in pre-rRNA processing has never been proved. For example, impairment of 32S pre-rRNA in the yeast GRN1 (GNL3 homologue in yeast) mutant was only restored by human GNL3L and not GNL3 (Du et al., 2006). Moreover, human GNL3 failed to rescue the lethality of NST-1 deficient in *C. elegans* (Kudron and Reinke, 2008). As previously mentioned,GNL3 is mainly localized in the GC of the nucleolus (Romanova et al., 2009b). Its depletion did not only affect the integrity of the telomerase complex but also that of small nucleolar ribonucleoproteins (snoRNPs). It is also important to mention that the knockdown conditions are the same as in the report showing the implication of GNL3 in pre-rRNA processing. Another study reported the same observation (Politz et al., 2005). However, they proved that GNL3 is localized in the subnucleolar regions that are deficient in nascent 28S rRNA and nucleolar domains where ribosomes are born.

Trying to answer to the question whether GNL3 is implicated in pre-RNA processing, Lin *et al.* showed that depletion of GNL3 increases the level of DNA damage within 12 hrs., but it had no significant effect on rRNA synthesis nor on the nucleolar structure (Lin et al., 2014). But they could reproduce that upon six days of GNL3 depletion, the level of rRNA decreased as previously discussed. This suggests that the effect of GNL3 on pre-RNA processing is an indirect effect. On the other hand, they showed that GNL3L

depletion disrupts the pre-rRNA processing. This is consistent with the fact that the GNL3L but not GNL3 would rescue the impairment of pre-RNA processing in yeast.

Therefore, this indicates that although GNL3 and GNL3L are closely related, their functions diverge into genome protection and ribosomal biosynthesis, respectively.

8- GNL3 Implication in Cancer Progression

GNL3 is considered as a marker for stemness of the cells (Cai et al., 2004; Schwartz et al., 2003). However, its expression was also found high in several types of cancer, such as gastric, colorectal, liver and others (Liu et al., 2004; Zia-Jahromi et al., 2014). It was described as a bad prognosis factor for the progression of the tumor (Yoshida et al., 2014) and the reoccurrence (Nakajima et al., 2012). How exactly GNL3 maintains the tumorigenicity of the cells is not exactly clear. GNL3 is mainly expressed in normal undifferentiated cells, but what drives its re-expression in cancer cells is not really understood. However, a report by Zwolinska *et al* showed that GNL3 expression could be induced by the oncogene c-Myc, by its ability to bind to a well conserved E-box in the promoter of GNL3 (Zwolinska et al., 2012). Re-expression of GNL3 in cells gives them the characteristics of tumor initiating cells (TIC) (Okamoto et al., 2011). When GNL3 is expressed, the levels of K5, CD114, OCT4, human telomerase and CXC increase (Lin et al., 2010). Moreover, upon overexpression of GNL3, the level of TWIST and phosphorylated STAT3 increased and the cells showed an enhancement to radioresistance (Zhang et al., 2020). Apart from its possible ability to activate the STAT3 pathway, GNL3 overexpression was also reported to activate the Wnt/B-catenin signaling pathway (Bao et al., 2016; Tang et al., 2017). Moreover, GNL3 role in modulating the p53/MDM2 loop might also be one of the pathways where GNL3 is initiating tumorigenesis. However, the effect of GNL3 on p53 is cell type dependent, and this indicates that the role of GNL3 in this process extends to affecting other important parameters, such as its implication in maintaining genomic integrity as well as the telomeric one.

Objectives

The replisome is a large machine composed of a plethora of proteins needed to achieve DNA replication. These include helicases, polymerases, signaling proteins, structural proteins such as cohesins, and proteins involved in the turnover of epigenetic marks. In addition, since the replisome is in constant threat due to DNA lesions or replication forks barriers, some proteins involved in the response to replicative stress can be recruited to the replisome to stabilize, repair and restart stalled replication forks. Several proteins described in the literature are able to accomplish these tasks. However, there is a need to identify new proteins to increase our knowledge and to understand how their activities are coordinated in unperturbed S-phase and in response to replication stress in order to understand how the genomic stability is preserved. Most importantly, it may contribute to the identification of key biomarkers of the resistance to chemotherapeutic treatments in order to target them to enhance the efficacy of anti-cancer therapies.

Nowadays it is possible to isolate newly synthesized DNA along with the proteins that constitute the replisome components by using the iPOND (Isolation of Proteins On Nascent DNA) technique (Sirbu et al., 2011). Previously, my lab used this method and coupled it with mass spectrometry to uncover new proteins recruited at replication forks (Lossaint et al., 2013; Ribeyre et al., 2016). The most promising candidates were validated using a secondary screen based on high-throughput immunofluorescence. GNL3 (also known as nucleostemin) turned out to be the best candidate and therefore the goal of my thesis project was to understand its role in DNA replication.

My project was divided into two parts:

1- Characterization of the role of GNL3 during S-phase in order to understand the reason for its association with the replisome.
2- Determine the role of GNL3 in response to replicative stress.

Results

GNL3/nucleostemin links DNA replication homeostasis with forks stability

 I contributed to 90% of the experimental work done for this manuscript. I was also fully involved in experimental design and the writing of the manuscript that will be submitted by the time I defend my thesis.

GNL3 regulates replication origin firing and protects stalled replication forks

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Summary

DNA replication by the replisome requires specific proteins that protect replication forks and so prevent the formation of DNA lesions that may damage the genome. Here, we show that human GNL3 (also known as nucleostemin), a GTP-binding protein localized in the nucleolus and the nucleoplasm, is a new component of the replisome. Depletion of GNL3 reduces fork speed but increases replication origin firing indirectly by interacting with ORC2, whereas overexpression of GNL3 decreases origin firing. When subjected to replication stress, the nascent DNA in cells depleted of GNL3 undergoes nuclease-dependent resection, a source of DNA lesions. Inhibition of origin firing decreases this resection, indicating that the increased replication origin firing seen upon GNL3 depletion mainly accounts for the observed DNA resection. Our results suggest that GNL3 and possibly other proteins that are required to protect replication forks act indirectly by regulating origin firing.

Keywords

Introduction

In all cells, DNA replication must occur precisely before their division to ensure faithful transmission of the genome. In humans, accurate DNA replication is particularly important for stem cells – which are responsible for renewing organs and tissues – and to prevent premature aging and/or cancer (Macheret and Halazonetis, 2015; Schumacher et al., 2021). Replication must occur correctly in space and time to ensure that the whole genome is copied entirely once per cell cycle with no under-replicated or over-replicated regions. Moreover, the replication forks – the sites at which the replication machinery (the replisome) replicates DNA – must be free of impediments that perturb their progression because collapsed replication forks can result in DNA lesions.

DNA replication initiates from specific sites distributed all over the genome, called replication origins (Fragkos et al., 2015; Mechali, 2010). Initiation of replication is a two-step process. First, the origins are 'licensed' for replication by binding of the origin recognition complex (ORC, composed of six subunits, ORC1–6) and the replicative helicase MCM2–7, which forms the pre-replicative complex. Second, origin firing (the start of DNA synthesis) requires activation of cyclin-dependent kinases and CDC7/DBF4 kinases. One of the ORC subunits, ORC2, also plays structural roles independent of the ORC complex, which may impact DNA replication indirectly (Huang et al., 2016; Prasanth et al., 2004; Shimada and Gasser, 2007).

After DNA replication starts, the progression of the replisome may be perturbed by factors of endogenous and exogenous origin that induce replication stress (Lambert and Carr, 2013). The main pathway activated to prevent fork collapse and genomic instability, the ATR–Chk1 checkpoint, prevents further progress through S phase, thus providing time for stalled forks to be stabilized to avoid formation of DNA lesions (Zeman and Cimprich, 2014). Many other proteins, for example BRCA1, protect stalled forks by preventing the action of specific nucleases like MRE11 or CtIP (Berti et al., 2020; Liao et al., 2018; Rickman and Smogorzewska, 2019). ATR–Chk1 also maintains genomic stability by limiting the firing of replication origins in response to replication stress (Blow et al., 2011; Courtot et al., 2018; Toledo et al., 2013). WEE1, a kinase that limits entry into mitosis by inhibiting CDK1, acts in a similar way (Beck et al., 2012; Moiseeva et al., 2019; Toledo et al., 2013).

We previously used the iPOND (isolation of proteins on nascent DNA) method coupled with mass spectrometry (iPOND-MS) to identify novel factors associated with replication forks (Lossaint et al., 2013; Ribeyre et al., 2021; Ribeyre et al., 2016). Here, we used an siRNA screen to identify those novel factors whose depletion increases the number of DNA lesions in response to replication stress. The protein whose depletion had the greatest effect was GNL3 (also known as nucleostemin), a GTP-binding protein localized

in the nucleoplasm and mainly in the nucleolus, which is highly expressed in stem cells and cancer cell lines (Tsai and McKay, 2002). Previous studies found that GNL3 depletion leads to activation of the DNA damage response during S phase (Lin et al., 2013; Meng et al., 2013; Yamashita et al., 2013). GNL3 is recruited to DNA double-stand breaks (DSBs), and its depletion prevents RAD51 – a key protein for DSBs repair by homologous recombination – from being recruited at DSBs and hydroxyurea (HU)-induced lesions (Lin et al., 2013; Meng et al., 2013). Consistent with this, GNL3-depleted cells are more sensitive to HU (Lin et al., 2014) and are less able to repair DSBs by homologous recombination (Meng et al., 2013). The current model suggests that GNL3 in the nucleoplasm maintains genome stability in S phase by being recruited to DNA lesions in order to stabilize RAD51 (Tsai, 2014). The precise functions of GNL3 in S phase, its role in DNA replication and genome stability, are poorly understood, however.

In this report, we demonstrate that GNL3 is constitutively associated with nascent DNA at replication forks throughout normal DNA replication in human cells. GNL3 depletion decreases fork speed but increases origin firing without affecting replication timing. It interacts with ORC2 in the nucleolus, suggesting an indirect mechanism for the regulation of origin firing. In GNL3-depleted cells subjected to various sources of replication stress, the resection of nascent DNA increases. We show that this increased resection in the absence of GNL3 is a consequence of the increased origin firing; GNL3 does not directly protect replication forks from resection by endonucleases. The same observation was made for inhibition of ATR or WEE1 that also increases origin firing, suggesting that resection of stalled forks depends partially on origin firing.

Results

GNL3 is a new replisome component

We reported previously our use of the iPOND (isolation of proteins on nascent DNA) method coupled with mass spectrometry (iPOND–MS) to identify novel factors associated with replication forks (Ribeyre et al., 2021). Briefly, we pulse-labelled newly synthesized DNA in Hela S3 cells with 5-ethynyl-2'-deoxyuridine (EdU, a nucleoside analogue of thymidine that can be labelled by Click chemistry) or pulsed with EdU then chased for two hours with thymidine, then we purified the proteins associated with EdU (Figure 1A). Those proteins that were significantly enriched in the pulse-labelled samples when compared to the chase were defined as components of the replisome (Ribeyre et al., 2021). These components included many proteins that were not previously known to be associated with nascent DNA. To select candidates for further analysis, we designed an orthogonal approach based on a mini screen using 25 individual endoribonuclease-prepared siRNAs (esiRNAs; against 24 candidates plus a negative control esiRNA against EGFP). We reasoned that if these proteins are important for DNA replication, their depletion should increase the number of DNA lesions upon replication stress. We analyzed DNA lesions by quantifying the amount of γH2A.X phosphorylation after 4 hours of replication stress due to treatment with 1 μM camptothecin (CPT, an inhibitor of DNA topoisomerase 1). Briefly, HCT116 cells growing in 96 well plates were transfected with each of the 25 esiRNAs. Forty-eight hours after transfection, the cells were treated for 4 hours with 1 μM CPT and the amount of γH2A.X in the nucleus (seen by staining with DAPI) was analyzed by immunofluorescence microscopy in a Celigo high-throughput microscope (Figure S1A). We ranked the effects of the 25 esiRNAs based on the amount of γH2A.X and found that GNL3 ranked highest, suggesting that it may be important to tolerate replication stress (Figure S1B).

Using the iPOND–MS data (Ribeyre et al., 2021), we calculated the logRatio of GNL3 in the pulse and the chase samples and found that it was similar to that of the known replisome components PCNA, RFC1 and FEN1 (Figure 1B). Also, by western blotting the iPOND proteins, we observed that GNL3, like PCNA, was associated with EdU only when the Click reaction was performed and was not found in the chase (Figure 1C), further supporting the conclusion that it is a replisome component. To confirm that GNL3 is in the vicinity of replication forks, we performed proximity ligation assays (PLAs) between GNL3 and the EdU-containing nascent DNA Click-labeled with biotin, using antibodies against GNL3 and biotin to identify foci where the two antigens are in close proximity. We found many foci showing the proximity of GNL3 to nascent DNA in the nuclei of normal cells; by contrast, the number of foci was much decreased when GNL3 was depleted (Figure 1D, 1E) or when EdU was not Click-labeled with biotin (Figure 1F). To determine

whether GNL3 is close to replication forks throughout S phase, we synchronized cells in S phase by using a thymidine block and analyzed the proximity of GNL3 to nascent DNA before release (T0) and 2, 4, 6 and 8 hours after release, corresponding to early (T2), mid (T4 and T6) and late S phase (T8; Figure 1G). As expected, no signal was observed at T0 due to the lack of EdU incorporation; by contrast, GNL3 was seen in proximity to EdU-containing DNA at T2, T4, T6 and T8 hours (Figure 1H). GNL3 depletion strongly decreased the signal, thus validating its specificity. The EdU-GNL3 signal mimicked the patterns of S phase (Figure 1G) corresponding to the replication of different regions of the genome (Dimitrova and Berezney, 2002) and also the EdU-PCNA signal (Roy et al., 2018). We conclude from these data that GNL3 is constitutively associated with nascent DNA throughout S phase.

GNL3 depletion increases firing of replication origins

If GNL3 is a component of the replisome, its depletion might be expected to have an impact on the cell cycle. We found no obvious effect of GNL3 depletion, however, either on the distribution of cells in various phases of the cell cycle whether in an unsynchronized population (Figure S2A) or in a population synchronized with a thymidine block (Figure S2B). To confirm this conclusion, we measured the length of S phase by examining the timing of entry into mitosis after a thymidine block, as indicated by phosphorylation of histone H3 on Ser 10 (Prigent and Dimitrov, 2003). Confirming that the length of S phase was unaffected by GNL3 depletion, no sign of early mitotic entry was detected 8 hours after release (Figure S2C). Ten hours after release, however, we noticed a small increase in the percentage of pH3S10-positive cells in GNL3-depleted cells when compared to the control, suggesting the cells accumulate in mitosis in the absence of GNL3, a phenomenon observed also in breast cancer cells lacking GNL3 (Lin et al., 2014). In those cells, loss of GNL3 increased the number of foci containing the DNA damage response protein 53BP1 (Lin et al., 2014; Yamashita et al., 2013), potentially an indicator of incomplete replication due to replication stress (Harrigan et al., 2011). Cells undergoing replication stress have been observed to continue replicating their DNA in early mitosis, a phenomenon known as mitotic DNA synthesis (MiDAS; (Minocherhomji et al., 2015). To test whether GNL3 depletion induces MiDAS, we synchronized cells with thymidine, released them for 8 hours and then labelled nascent DNA for 15 min with EdU (Figure S2D). GNL3 depletion increased the number of mitotic cells with an EdU signal by about two-fold (Figure S2E), indicating that these cells enter mitosis with incompletely replicated DNA, suggesting problems during DNA replication. To determine whether GNL3 depletion has a global impact on DNA synthesis during S phase, we measured incorporation of the thymidine analogue iodo-deoxyuridine (IdU) and found it was increased when compared to control cells (Figure 2A, 2B, Figure S2F). Since the length of S phase is not

affected by GNL3 depletion, this may reflect a change in the number of active replication origins. To test this, we isolated the chromatin from cells depleted of GNL3 and from control cells and analyzed the presence of markers of origin firing by western blotting. We found more CDC45, MCM2 phosphorylated at Ser 40/41 (pMCM2 S40/41) and PCNA in the chromatin fraction of cells depleted of GNL3 than in control cells (Figure 2C, 2D) indicating that more origins are firing in the absence of GNL3. To confirm this finding by using another approach, we used DNA combing (Figure 2E): we labelled the cells with IdU for 20 min and then with another thymidine analogue, chloro-deoxyuridine (CldU), for 20 min and observed that GNL3 depletion reduced fork velocity by about 25% (Figure 2F, Figure S2G). This indicates that the increased IdU incorporation in GNL3-depleted cells is not due to increased fork velocity but that it might reflect more replication forks. To investigate this possibility, we determined the number of forks per megabase of combed DNA by using a highly accurate assay for global instant fork density (Bialic et al., 2015), which reflects the density of origins. An increase in the number of forks per megabase in GNL3-depleted cells indicated that indeed more origins fire in absence of GNL3 than in control cells (Figure 2G). To investigate whether GNL3 affects the firing of replication origins globally or only at specific regions, as does RIF1 (Yamazaki et al., 2012), we analyzed the effect of GNL3 depletion on replication timing. As expected from previous studies (Cornacchia et al., 2012; Yamazaki et al., 2012), depletion of RIF1 had a substantial impact on replication timing; some regions were delayed and others advanced when compared to the control (Figure S2H). GNL3 depletion, by contrast, had little or no effect on replication timing (Figure 2H). We conclude that GNL3 depletion increases the firing of replication origins globally without affecting the replication timing.

GNL3 overexpression inhibits firing of replication origins

Since GNL3 depletion increases origin firing, GNL3 overexpression might decrease origin firing. To test this prediction, we used a Flp-In T-Rex HeLa cell line expressing a doxycycline-inducible GNL3-FLAG fusion protein gene (Figure 3A). GNL3 overexpression had no effect on the cell cycle (Figure S3A), however, it slightly decreased IdU incorporation (Figure 3B and 3C, Figure S3B), suggesting inhibition of replication origin firing. Consistent with this conclusion, GNL3 overexpression decreased the amount of pMCM2 S40/41, CDC45 and PCNA on chromatin (Figure 3D, 3E). We conclude that, contrary to GNL3 depletion, GNL3 overexpression inhibits origin firing, indicating that the amount of GNL3 is important for the regulation of origin firing.

GNL3 interacts with ORC2

To understand how GNL3 might influence replication origin firing, we used proximity-dependent biotinylation identification (BioID; Roux et al., 2012) to identify the proteins in proximity to GNL3 by mass spectrometry. We established a Flp-In T-Rex HEK293 cell line expressing a doxycycline (DOX)-inducible gene encoding GNL3 fused to the biotin ligase BirA and FLAG. Upon induction with DOX for 16 hours, we observed by immunofluorescence microscopy GNL3-BirA-FLAG in the nucleoplasm and the nucleolus (Figure S4A). Moreover, by using streptavidin conjugated to Alexa Fluor 488 to detect exogenous biotin, we observed a strong signal (Figure S4A) demonstrating that GNL3-BirA-FLAG is well localized and can biotinylate proteins in its proximity. In four independent experiments, we induced expression of GNL3-BirA-FLAG with DOX for 16 hours and labelled proteins in its proximity with exogenous biotin for 4 hours. Then we purified the biotinylated proteins on streptavidin beads and analyzed them by mass spectrometry. We calculated the logRatio of the peptides detected upon addition of DOX and biotin compared to the peptides detected in the negative controls (treatment with either DOX or biotin alone) and represented the data in a Volcano plot (Figure 4A). As expected, GNL3 was highly enriched as were several nucleolar proteins that are known to be in proximity (e.g., GNL3L, GNL2, DDX21, Ki67 or NPM1). In addition, consistent with the presence of GNL3 on nascent DNA, several of the enriched proteins are known to be associated with the replisome. Notably, enrichment of ORC2, one of the components of the origin recognition complex, suggested a possible mechanism in the regulation of replication origin firing by GNL3. To confirm the association of ORC2 with GNL3, we immunoprecipitated each of the proteins and analyzed the immunoprecipitates by western blotting; we found GNL3 in immunoprecipitates of ORC2 and *vice versa* (Figure 4B). Mass spectrometry analysis of the proteins that co-immunoprecipitated when using a specific antibody against ORC2 confirmed the presence of GNL3 and most of the ORC subunits, whereas immunoprecipitation with an irrelevant control IgG contained neither GNL3 nor ORC subunits. Moreover, there was a significant overlap between the co-immunoprecipitated proteins and those found by BioID of GNL3: among the 88 proteins significantly enriched by BioID, 35 were found by coimmunoprecipitation with ORC2 (Figure S4B) and most of them (24/35) are proteins localized in the nucleolus. This suggests that at least a subset of ORC2 might be localized in the nucleolus and that the interaction between ORC2 and GNL3 is likely to occur in this compartment. The association of GNL3 with nascent DNA, however, suggests that GNL3 and ORC2 also interact at or near replication origins. To test this, we performed GNL3 chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) and found 3412 binding sites for GNL3. We compared these binding sites with ORC2-binding sites (Miotto et al., 2016) but found no significant overlap (Figure 4C, Figure S4C), indicating that the GNL3–ORC2 interaction occurs in the nucleolus rather than on

vicinity of replication origins. To confirm this, we analyzed the GNL3–ORC2 interaction by using PLA (as above) and found most foci at the border of regions that stained lightly with DAPI and that correspond to nucleoli (Figure 4D), thus supporting our hypothesis. The PLA signal in the nucleoli was strongly decreased upon depletion of GNL3, validating its specificity. If the interaction between ORC2 and GNL3 is important for origin firing, this interaction might be modulated by inhibition of WEE1, CDC7 or ATR, all of which affect origin firing. Inhibitors of all three factors increased the number of GNL3–ORC2 PLA foci (Figure 4E, Figure S4D) although they affect origins firing differently. All three inhibitors caused the cells to accumulate in G2/M phase (Figure S4E), indicating that the interaction between GNL3 and ORC2 may occur preferentially in this phase of the cell cycle. This is consistent with several studies showing that ORC2 may play structural roles in G2/M independent of its function in the ORC complex, possibly at centromeres (Huang et al., 2016; Prasanth et al., 2004; Shimada and Gasser, 2007). Interestingly, centromeres are often localized in the vicinity of the nucleolus (Padeken et al., 2013; Wong et al., 2007). To investigate whether ORC2 recruitment at centromeres depends on GNL3, we performed PLA between ORC2 and the centromere-specific histone H3 variant, CENP-A. As expected, many PLA foci of ORC2 and CENP-A were found in normal cells when compared to controls treated with only the antibody against ORC2 or that against CENP-A. When the cells were depleted of GNL3, however, the average number of PLA foci per cell was reduced by about two-fold (Figure 4F and 4G), indicating that ORC2 recruitment at centromeres depends in part on the availability of GNL3. We propose that GNL3 interacts with ORC2 to facilitate its recruitment to centromeres that in turn impacts the regulation of origin firing globally, thus suggesting a mechanism to explain the role of GNL3 on replication origin firing.

GNL3 prevents DNA resection at stalled replication forks

GNL3 depletion leads to activation of the DNA damage response during S phase and GNL3-depleted cells are more sensitive than control cells to HU, an inducer of replication stress (Lin et al., 2013; Lin et al., 2014; Meng et al., 2013; Yamashita et al., 2013), suggesting a role in the response to replication stress. Indeed, we found more γH2A.X in the nucleus of CPT-treated cells depleted of GNL3 than in control cells (Figure S1B); therefore, we investigated further whether GNL3 regulates replication fork progression in the presence of CPT. To do so, we labelled cells for 30 min with IdU followed by labelling for 30 min with CldU in the presence or absence of 1 μM CPT and measured the length of both tracks to obtain the CldU/IdU ratio (Figure 5A). As expected, addition of CPT strongly reduced the CldU/IdU ratio, however, depletion of GNL3 had no additional impact (Figure 5A, Figure S5A). This indicates that GNL3 has no great influence on replication fork progression during brief treatments with CPT. When the cells were treated with CPT for 1,

2 and 4 hours (Figure S5B), CPT treatment induced rapid phosphorylation of the DNA damage response kinase Chk1 on Ser 345, as expected, however, the kinetics of its phosphorylation was not markedly affected by GNL3 depletion, further supporting our conclusion that GNL3 does not affect fork progression in response to CPT. By contrast, after 4 hours of treatment with CPT the level of phosphorylation of RPA on both Ser 33 and Ser 4/8 was higher in the absence of GNL3 than in the controls (Figure S5B). To determine if this effect was specific to CPT, we performed the same experiment but treated the cells with HU or etoposide (ETP), a topoisomerase 2 inhibitor. Treatment with 5 mM HU or 10 μM ETP induced phosphorylation of Chk1 on serine 345 in control cells but, as with CPT, no obvious difference was seen when GNL3 was depleted (Figure 5B and Figure S5C). Also, as with CPT, we observed stronger phosphorylation of RPA on Ser 33 and Ser 4/8 in the absence of GNL3 than in control cells after 4 hours treatment with HU (Figure 5B) and after 2 hours treatment with ETP (Figure S5C). Thus, we hypothesized that GNL3 depletion may not impact replication stress signaling through Chk1 but, rather, the stability of stalled replication forks, since RPA phosphorylation is a marker of DNA resection (Soniat et al., 2019). Several proteins, including BRCA1, BRCA2 and FANCD2, have been shown to protect nascent DNA from resection in response to replication stress (Rickman and Smogorzewska, 2019). To test if GNL3 protects nascent strand DNA, we sequentially labelled cells with IdU and CldU for 30 min each and then treated the cells with HU for 4 hours (Figure 5C). In the controls, the CldU/IdU ratio was close to 1, indicating that the nascent DNA was protected from extensive degradation, as expected. In cells depleted of GNL3, by contrast, the CldU/IdU ratio was significantly lower (Figure 5C, Figure S5D), indicating resection of the fork DNA by nuclease(s). Likewise, we saw similar effects in response to CPT (Figure S5E) and ETP (Figure S5F), consistent with the increased level of RPA phosphorylation induced by these agents in GNL3-depleted cells.

The resection observed in the absence of fork protectors is most probably initiated by the endonuclease activities of MRE11 and CtIP (Rickman and Smogorzewska, 2019). To test further the function of GNL3 as a fork protector, we depleted GNL3 and MRE11, or GNL3 and CtIP, and found that loss of the nucleases prevented the resection seen upon depletion of GNL3 alone (Figure 5D, Figure S5G, Figure S5H), further supporting our conclusion that GNL3 protects nascent strand degradation by nucleases. To show definitively that GNL3 protects against DNA resection at stalled replication forks, we depleted the endogenous GNL3 with a specific siRNA and complemented its function by expressing an siRNA-resistant, DOX-inducible GNL3-FLAG gene in Flp-In T-Rex HeLa cells. We treated these cells with HU and analyzed the level of resection by IdU and CldU incorporation, as before. Expression of GNL3-FLAG suppressed almost completely the increased resection due to GNL3 depletion (Figure 5F, Figure S5I).

The protection of stalled replication forks allows them to restart DNA synthesis and avoid collapse and conversion into a DSB (Rickman and Smogorzewska, 2019). Consequently, GNL3 depletion should impair the ability of HU-stalled forks to restart. To test this prediction, we labelled cells with IdU, treated them with HU for 4 hours and then removed the HU in the presence of CldU to label the forks that restarted DNA synthesis (Figure S5J). We determined the percentage of permanently stalled forks by counting the number of fibers labelled only by IdU and those labelled by both IdU and CldU and found a greater percentage of stalled forks in the absence of GNL3 (Figure S5J). These data demonstrate that depletion of GNL3 permanently destabilizes stalled replication forks, which may potentially lead to DSBs.

Resection in the absence of GNL3 is a consequence of increasing origin firing

The other proteins known to protect replication forks – BRCA1, RAD51 and FANCD2, for example – accumulate on HU-stalled forks (Dungrawala et al., 2015; Lossaint et al., 2013; Zellweger et al., 2015), suggesting that they may protect them directly from the action of nucleases. To determine whether GNL3 protects stalled replication forks from nucleases in the same way, we again used iPOND to identify the proteins on nascent DNA. Cells were pulse labelled for 15 min with EdU and then chased for 2 hours with thymidine or with HU (Figure 6A). As we showed already (Figure 1C), the replisome components PCNA and GNL3 were enriched on nascent DNA after the pulse but not after the chase and, as expected, treatment with HU induced recruitment of RAD51 (Figure 6B). By contrast, recruitment of GNL3 was strongly decreased in response to HU, as was PCNA (Figure 6B), indicating that GNL3 does not accumulate at stalled forks. This suggests that the ability of GNL3 to protect from resection might be indirect and possibly related to its role in inhibiting origin firing. If so, inhibiting origin firing might suppress the HU-induced resection observed upon GNL3 depletion (Figure 6C). To test this, we sequentially labelled cells with IdU and CldU for 30 min each and then treated them with HU for 4 hours in the presence of an inhibitor of CDC7 to inhibit replication origin firing. Resection was strongly decreased when CDC7 was inhibited, indicating that in the absence of GNL3 an excess of origin firing in response to HU accounts for the increased resection (Figure 6D, Figure S6A). Consistent with the decrease in DNA resection, CDC7 inhibition also decreased the phosphorylation of RPA on Ser4/8 (Figure 6E, Figure S6B).

BRCA1 is recruited to HU-stalled forks (Dungrawala et al., 2015) and BRCA1 depletion increases resection induced by HU (Schlacher et al., 2012), thus this protein is thought to protect stalled forks from resection by directly blocking nucleases. If this is the case, inhibition of CDC7 should have no effect on protection by BRCA1. To test this prediction, we depleted cells of BRCA1 and measured the level of resection in the absence or presence of the CDC7 inhibitor. As expected, depletion of BRCA1 increased resection;

treatment with CDC7 inhibitor, however, did not decrease the level of resection (Figure 6F, Figure S6C, Figure S6D), confirming that the resection observed in the absence of BRCA1 is not a consequence of faulty origin firing. Thus, fork protection by BRCA1 differs mechanistically from fork protection by GNL3.

We saw above that in cells depleted of GNL3, replication stress resulted in both increased replication origin firing and increased resection. If this increased resection is a consequence of increased origin firing, other causes of increased replication origin firing should have a similar effect. Inhibition of ATR or WEE1, for example, increase replication origin firing (Beck et al., 2012; Moiseeva et al., 2017; Moiseeva et al., 2019). We therefore tested the effect of inhibiting ATR or WEE1 on resection in response to HU by sequentially labelling cells with IdU and CldU and then treating them with HU for 4 hours, as before, but in the presence of an inhibitor of ATR or an inhibitor of WEE1 (Figure 6G, Figure S6E). As predicted, inhibition of ATR (Figure 6H, Figure S6F) or inhibition of WEE1 (Figure 6I, Figure S6G) increased resection in response to HU. Moreover, inhibiting the increased origin firing with an inhibitor of CDC7, reversed this effect. This experiment demonstrates that limiting the number of origins that fire is crucial to preventing resection in response to replication stress and supports our conclusion that the enhanced resection observed upon GNL3 depletion is a consequence of increased origin firing.

Discussion

GNL3/nucleostemin was discovered twenty years ago as a nucleolar protein required for cell proliferation (Tsai and McKay, 2002) and several studies have highlighted its role(s) in maintaining genome integrity (Tsai, 2014). Here, we investigate the role of GNL3 during DNA replication. We demonstrate that GNL3 is a new replisome component that limits origin firing and interacts with ORC2. During replication stress, GNL3 protects the DNA at stalled replication forks from resection by endonucleases and this protection depends on the number of replication origins that fire. We propose a model in which GNL3 is required for accurate DNA replication by controlling origin firing through its interaction with ORC2 in the nucleolus (Figure 7A); this explains why GNL3 deficiency increases genomic instability.

GNL3 is a new replisome component

We show that GNL3 is new replisome component that it is present on nascent DNA throughout S phase. This suggests that it is not required at specific domains such as euchromatin or heterochromatin, which are replicated in early S phase and late S phase, respectively. Quantification of iPOND-MS data, however, indicates that GNL3 is not as abundant as the canonical components of replication forks (Ribeyre et al., 2021), suggesting that it may not be associated with every replication fork. One possibility is that GNL3 is associated with forks in regions of chromatin that are difficult to replicate, as, for example, in FANCJ-knockout cells, which exhibit constitutive replication stress and in which GNL3 was found on nascent DNA (Peng et al., 2018).

The association of GNL3 with replication forks might explain why its depletion leads to stalling of S phase, cell cycle arrest at the G2–M phase transition and γH2A.X phosphorylation (Lin et al., 2014), as well as the increased frequency of MiDAS that we observed. Upon GNL3 loss, replication fork speed slows, suggesting GNL3 might act as fork accelerator, as does PRIMPOL (Bianchi et al., 2013; Schiavone et al., 2016). The fact that GNL3 depletion has no effect on the slowing of fork velocity by CPT, however, argues against this hypothesis.

The GTPase activity of GNL3 might provide further clues to its function at the replication fork. In *Escherichia coli*, for example, the GTPase *obgE* is required for correct basal DNA replication and for replication in the presence of replication stress (Foti et al., 2005). GNL3 may play similar roles in vertebrates. GTPases often act as molecular switches through their ability to change conformation upon GTP hydrolysis, thus GNL3 might act as a switch that signals the presence of regions that are difficult to replicate. We attempted to express a GNL3 mutant unable to bind GTP but its instability (Huang et al., 2009; Lo et al., 2012) prevented

us to draw any strong conclusions (data not shown). Studies of other GNL3 mutants should further light on these possible functions at replication forks.

GNL3 regulates the firing of replication origins

The level of GNL3 expression has a profound effect on the number of origins fired: when GNL3 is depleted many origins fire, whereas when it is overexpressed origin firing is inhibited (Figure 7A). Surprisingly, we saw no impact of GNL3 depletion on global replication timing. How might we explain this apparent discrepancy? The measurement of replication timing is an average of thousands of cells and does not represent stochastic variations between individual cells. Recent data suggest that the firing of replication origins is more stochastic than previously thought (Klein et al., 2021; Wang et al., 2021). GNL3 depletion may, therefore, increase the firing of specific origins without impacting replication timing globally.

Loss of ORC2 increases inter-origin distance, indicating a reduced number of origins firing (Shibata et al., 2016). Thus, GNL3 might interact with ORC2, preventing its association with chromatin and limiting origin firing. The interaction between GNL3 and ORC2 is not likely to occur on chromatin, however, as our studies found ORC2 was not in proximity to nascent DNA. Moreover, GNL3 ChIP-seq revealed that the binding sites of GNL3 on chromatin do not overlap with those of ORC2. More likely, our findings indicate it occurs in or near the nucleolus. In *Saccharomyces cerevisiae*, the nucleolar protein Yph1p interacts with the ORC (Du and Stillman, 2002), reinforcing the evidence for a link between the nucleolus and the ORC, at least in this budding yeast.

What might be the relationship between the nucleolus and ORC2? Growing evidence indicates that the nucleolus is involved in the 3D organization of the genome (Iarovaia et al., 2019) and particularly of centromeric DNA (Padeken et al., 2013; Wong et al., 2007). ORC2 also plays roles at centromeric DNA during sister-chromatid cohesion through its interaction with the non-histone heterochromatin protein HP1α and the Lys-specific demethylase KDM5A (Huang et al., 2016; Prasanth et al., 2004; Shimada and Gasser, 2007). Moreover, GNL3 maintains the heterochromatin state of centromeres and transposons in mitotic chromosomes (Maida et al., 2014; Oktar et al., 2011). We propose that GNL3 is required to recruit ORC2 at centromeres by keeping them in proximity to the nucleolus (Figure 7B). This may explain why the level of GNL3 in the nucleolus is tightly regulated by GTP binding (Tsai and McKay, 2005) and that the global level of GNL3 correlates directly with replication origin firing. This function may be important to regulate replication origins firing globally, although we cannot exclude that GNL3 depletion reduces ORC2 recruitment at other regions. More work is required to understand how ORC2 binding at centromeres affects origin firing globally.

GNL3 protects stalled replication forks from resection

GNL3 protects nascent DNA at stalled replication forks from resection by endonucleases. The increased resection seen upon GNL3 depletion, we conclude, is related to the increased replication origin firing because it is suppressed by an inhibitor of CDC7 that decreases origin firing. This conclusion is consistent with data showing that CDC7 inhibition prevents nascent strand resection (Jones et al., 2021; Sasi et al., 2018). We propose that the replication stress induced by HU in GNL3-depleted cells is exacerbated since a proper control of firing is required (inactivation of late origins and activation of dormant origins), thus leading to DNA resection and replisome collapse (Figure 7A). Consistent with this, we found that inhibition of ATR or WEE1, both of which increase origin firing, increases the resection of nascent DNA in a CDC7-dependent manner. Also, inhibition of ATR or WEE1 increases DNA lesions upon exposure to HU due to the exhaustion of the RPA pool (Toledo et al., 2013) and confirms that incorrect control of origin firing leads to DNA resection.

The nascent DNA resection that occurs in the absence of BRCA1, in contrast to that which occurs in the absence of GNL3, was not suppressed by CDC7 inhibition. This indicates a direct role for BRCA1 in protecting nascent DNA but not in origin firing. Thus, we conclude that nascent DNA resection can be promoted either by loss of a protein that protects the DNA directly, like BRCA1, or by loss of proteins such as GNL3 and WEE1 that are not recruited to nascent DNA and therefore must act indirectly. BRCA1, FANCD2 and RAD51 were the first proteins found to act as fork protectors (Hashimoto et al., 2010; Schlacher et al., 2011; Schlacher et al., 2012) by being recruited to nascent DNA (Dungrawala et al., 2015; Lossaint et al., 2013). Since then, several other proteins have been found to protect stalled forks from resection by nucleases (Berti et al., 2020; Liao et al., 2018; Rickman and Smogorzewska, 2019), including the sister chromatid cohesion protein PDS5 (Morales et al., 2020), RIF1 (Mukherjee et al., 2019), the exonuclease EXD2 (Nieminuszczy et al., 2019), the spindle assembly factors TPX2 and Aurora A (Byrum et al., 2019), and the AAA ATPase WRNIP1 (Porebski et al., 2019). Given our findings here, it would be interesting to investigate whether these proteins protect replication forks directly or indirectly.

GNL3 in the nucleoplasm and nucleolus

GNL3 is present both in the nucleoplasm and the nucleolus. We propose that the fraction of GNL3 present in the nucleoplasm affects directly the speed of replication fork progression whereas that in the nucleolus has a structural role that regulates origin firing by interacting with ORC2 (Figure 7B). In this regard, GNL3 resembles Yph1p in *S. cerevisiae* (Du and Stillman, 2002) and may belong to two different protein

complexes. We cannot exclude that the slow replication fork speed observed upon GNL3 depletion may compensate for the increased replication origin firing (Ge et al., 2007; Ibarra et al., 2008).

Although GNL3 is found only in chordates, it belongs to the family of YlqF-related GTPases that is conserved in Eukarya, Bacteria and Archea and has evolved in parallel with the compartmentalization (Mier et al., 2017; Reynaud et al., 2005). GNL3 is the more recent member of the family and seemed to have co-evolved with sub compartments of the nucleolus that are present only in chordates. It is tempting to speculate that compartmentation of the nucleolus is important for the regulation of replication origins firing in metazoans possibly by affecting nuclear organization.

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Author contributions

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Declaration of interests

The authors declare no competing interests.

Methods

Cell lines

HeLa S3 (obtained from ATCC), Flp-In T-Rex 293 (obtained from ThermoFisher) and HeLa Flp-In T-Rex (gift from Jean-Hugues Guervilly and Pierre-Henri Gaillard, Centre de Recherche en Cancérologie de Marseille, France) cells were cultured in Dulbecco's modified Eagle's media (DMEM). HCT116 (obtained from SIRIC Montpellier Cancer) and K562 (authenticated with Eurofins) cells were cultured in Roswell Park Memorial Institute medium (RPMI). Culture media was supplemented with 10% fetal bovine serum (Biowest) and penicillin/streptomycin (Sigma-Aldrich). Cells were incubated in a 5% CO_2 at $37^\circ C$. Selection of integrated clones in Flp-In cells were done using hygromycin and blasticidin.

Inhibitors, drugs and antibiotics

The following reagents were used: etoposide (Sigma-Aldrich E1383), camptothecin (Sigma-Aldrich C9911), hydroxyurea (Sigma-Aldrich H8627), doxycycline (Clontech 631311), hygromycin B Gold (InvioGen), zeocin (Invitrogen 46-0509), blasticidin (InvivoGen), ATR inhibitor VE-821 (TINIB-TOOLS), WEE1 inhibitor AZD1775 (Selleckchem), CDC7 inhibitor PHA-767491 (Selleckchem).

Plasmids construction

GNL3 cDNA cloned in pDONR223 (obtained from Montpellier Genomic Collection) was introduced using Gateway method in pDEST-pcDNA5-FLAG C-term and pDEST-pcDNA5-BirA-FLAG C-term (gifts from Anne-Claude Gingras, Lunenfeld-Tanenbaum Research Institute at Mount Sinai Hospital, Toronto, Canada)

Gene silencing

For GNL3 depletion siGENOME SMARTpool (M-016319-00) and individual siRNA oligonucleotides (D-016319-01-0002, D-016319-02-0002, D-016319-03-0002 and D-016319-04-0002) were purchased from Dharmacon and transfected using INTERFERin (Polypus transfection). siRNAs against MRE11 and CtIP were provided by Yea-Li Lin (Institut de Génétique Humaine, Montpellier) and are described in (Coquel et al., 2018).

Western-blot

Cellular extracts were resuspended in Laemmli buffer (65.8 mM Tris, 26.3% glycerol, 2.1% SDS, and Bromophenol blue) and boiled at 95°C for 5 min. Proteins were separated by SDS-PAGE using home-made or precast gels (Bio-Rad) with suitable percentage then transferred on nitrocellulose membranes (GE

Healthcare or Bio-Rad). Membranes were blocked with 5% non-fat milk in TBS-T (10 mM Tris pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 1 hr then incubated with the primary antibodies overnight. Membranes were washed 3 times with TBS-T then incubated with the corresponding secondary antibody. Finally, membranes were developed with Clarity Western ECL Blotting Substrate (Bio-Rad) and images were acquired using a ChemiDoc System (Bio-Rad). Antibodies against the following proteins were used: Ser345 Phospho-Chk1 (Cell Signaling Technology 2348), Chk1 (Santa Cruz sc-8408), PCNA (Sigma-Aldrich P8825), Ser4/8 Phospho-RPA32 (Bethyl A300-245A), RPA32 (Calbiochem NA18), histone H3 (Abcam ab62642), GNL3 (Bethyl A300-600A and Santa Cruz sc-166460), Ser33 Phospho-RPA32 (Bethyl A300-246), Tubulin (Sigma Aldrich T5168), CDC45 (Santa Cruz sc-20685), Ser40 Phospho-MCM2 (Abcam ab133243), MRE11 (Novus NB100-142), CtIP (Abcam ab70163), RAD51 (Santa Cruz sc-8349).

esiRNA screening

The 25 esiRNA (Sigma-Aldrich) corresponding to 24 candidates plus 1 negative control (EGFP) are described in SupTable1. HCT116 were seeded in 96 wells plates and transfected with esiRNAs using Oligofectamine (ThermoFisher). After 48 hours, transfected cells were subjected to 4 hrs treatment with 1 μM camptothecin then fixed for 15 min using 4% paraformaldehyde (PFA). Cells were permeabilized with 75% EtOH for 30 min on ice. 96 wells plate was incubated with primary antibody against Ser139 Phospho-H2A.X (Millipore 05-636) for 60 min then with secondary antibody anti-mouse coupled with Alexa568 (ThermoFisher A-11011) and finally with DAPI for 30 min. All the washes were performed with PBS-BSA 1%. 96 wells were scanned using a Nexcelom Celigo and images were analyzed using Celigo software. DAPI staining was used to measure the level of Ser139 Phospho-H2A.X in the nucleus for each esiRNA.

Proximity Ligation Assay (PLA)

Cells were grown on coverslips to reach 70-80% confluency then fixed with 2% paraformaldehyde (PFA) and 0.02% sucrose in PBS for 20 min at room temperature. When specified cells were incubated with EdU (5-ethynyl-2'-deoxyuridine) for the indicated times. Cells were permeabilized with 0.5% Triton X100- PBS for 20 min then washed PBS-3% BSA. EdU was conjugated to biotin-TEG-azide (Eurogentec) using Click-it reaction (30 min at room temperature) using indicated concentrations (10 mM sodium Ascorbate, 5 μM biotin-TEG-azide, 3 μM $CuSO_4$). For Click-it negative controls, biotin-TEG-azide was replaced by DMSO. Coverslips were incubated with primary antibodies in PLA blocking solution (Sigma-Aldrich) overnight at 4°C then washed with PBS. PLA probes (anti-mouse minus DUO92004 and anti-rabbit plus DUO92002, Sigma-Aldrich) were incubated together in PLA blocking solution for 20 min then added on the coverslips

for 1 h at 37°C then washed 2 times with buffer A (150 mM NaCl, 10 mM Tris, 0.5 % Tween). PLA kit was used (DUO92014, Sigma-Aldrich) for the following steps. Coverslips were incubated with ligase (1/40 dilution in ligase buffer) for 30 min at 37°C. Coverslips were washed 2 times with buffer A and incubated with polymerase (1/80 dilution in amplification buffer) for 100 min at 37°C. Coverslips were washed 2 times with buffer B (200 mM NaCl, 400 mM Tris-Base), dried and then mounted on glass slides with DAPI containing mounting medium (DUO82040 Sigma-Aldrich). Cells were analyzed by fluorescence microscopy and quantification the number of foci was performed using Fiji software. Antibodies against the following proteins were used: Biotin (Bethyl A150-109 and Jackson Immunoresearch 200-002-211), ORC2 (Bethyl A302-734A), CENP-A (Thermo Fisher MA1-20832) and GNL3 (Bethyl A300-600A and Santa Cruz sc-166460).

Flow Cytometry

When indicated cells were first labeled with 20 µM IdU for 10 min and then fixed with ice-cold 70% ethanol. Then cells were treated with RNase during 60 min and then for 30 minutes with 2M HCl. Next, the cells were incubated with a BrdU/IdU antibody from BD Biosciences (347580) for 60 min or with an anti-pH3S10 (Cell Signaling 9701) overnight, and then with an Alexa 488 conjugated anti-mouse IgG (Invitrogen) at room temperature for 30 min. Finally, the cells were stained with 5 µg/ml of propidium iodide in PBS and analyzed using a MACSquant analyzer (Miltenyi Biotec). Results were analyzed using Flowjo (https://www.flowjo.com).

Replication analysis by DNA Combing

Asynchronous cells were labeled 20 min with IdU, 20 min with CldU and then chased 90 min with thymidine. Purification of HMW gDNA, DNA combing and replication analysis was performed as in (Bialic et al., 2015) with the following modifications. Agarose plugs containing gDNA were washed in TNE50 containing 100 mM NaCl, digested O/N at 42°C with 3U β-agarase (New England Biolabs) and again for 2 hrs with 2U β-agarase. DNA was combed in MES buffer also containing 100 mM NaCl. Briefly, genomic DNA was combed on silanized coverslips, denatured with NaOH, and sites of DNA synthesis revealed using anti-IdU (red), anti-CldU (green), and anti-ssDNA (blue) antibody pairs. Primary antibodies were rat anti-BrdU (clone BU1/75, Abcam ab6326) for CldU, mouse anti-BrdU (clone B44, Becton Dickinson), for IdU and mouse autoanti-ssDNA (from DSHB) for DNA. Washes were performed with PBS-T containing 0.05% Triton X100. Secondary antibodies were Alexa488 Goat anti-rat IgG, Alexa546 Goat anti-mouse IgG, Alexa647 Goat anti-Mouse IgG2a (Life Technologies). Imaging was performed on a Zeiss AxioImager Z1 microscope with YFP, Cy3 and Cy5 filter blocks, equipped with a 40× objective (EC Plan Neofluar 1.3 NA oil) and scMOS

ZYLA 4.2 MP camera (2048*2048 pixels, 6.5μm pixel size). Red-to-green signals show fork direction (yellow arrow). Fork velocity (FV) is calculated by dividing the length of the green tract by the pulse time (in kb/min). Global instant fork density (GIFD) was calculated using the formula that accounts for the doubling of DNA during S phase:

$$\text{GIFD} = \frac{\text{Nf/DNA} \times (G1\% \times 0.66) + S\% + (G2M\% \times 1.33)}{S\%}$$

where Nf is the number of bicolor forks, DNA the total length of DNA measured (in Mb) and G1%, S% and G2M% the fraction of cells in G1, S and G2 or M phases, respectively, calculated from flow cytometry profiles using the same cells as for DNA combing.

Isolation of proteins on Nascent DNA (iPOND)

iPOND was performed largely as previously described (Lossaint et al., 2013; Ribeyre et al., 2016). HeLa S3 cells were pulse labeled with 10 μM EdU for indicated times and chases were performed with 10 μM thymidine. Cells were fixed with 1% formaldehyde for 5 min or 2% for 15 min followed or not by quenching of formaldehyde by 5 min incubation with 0.125 M glycine. Fixed samples were collected by centrifugation at 1000 g for 3 min, washed three times with PBS and stored at -80°C. Cells were permeabilized with 0.5% triton for 30 min and click chemistry was used to conjugate biotin-TEG-azide (Eurogentec) to EdU-labelled DNA in PBS containing 10 mM sodium Ascorbate, 10 μM biotin-TEG-azide, 2 mM $CuSO_4$. Cells were re-suspended in lysis buffer (10 mM Hepes-NaOH; 100 mM NaCl; 2 mM EDTA PH8; 1 mM EGTA; 1 mM PMSF; 0.2% SDS; 0.1% Sarkozyl) and sonication was performed using a Qsonica sonicator with the following settings: 30% power, 20 sec constant pulse and 50 sec pause for a total sonication time of 5 min on ice with water. Lysates were centrifuged at 15,000 g for 10 min at room temperature. Supernatants were normalized by DNA quantification using a nanodrop device. Biotin conjugated DNA-protein complexes were captured using overnight incubation with magnetic beads coated with streptavidin (Ademtech). Captured complexes were washed with lysis buffer and 500 mM NaCl. Proteins associated with nascent DNA were eluted under reducing conditions by boiling into SDS sample buffer for 30 min at 95°C and analyzed by Western-blot or mass spectrometry as indicated in (Kumbhar et al., 2018). Analysis of raw files was performed using MaxQuant (Cox and Mann, 2008) using default settings with label-free quantification option enabled. Raw file spectra were searched against the human UniProt reference database. Protein, peptide, and site false discovery rate (FDR) were adjusted to < 0.01.

DNA fibers labelling

DNA fibers labelling was performed as previously described (Lossaint et al., 2013; Ribeyre et al., 2016). Cells were labeled with 25 μM IdU, washed with warm media and exposed to 50 μM CldU. Cells were lysed and DNA fibers were stretched onto glass slides are left to air dry then are fixed in methanol/acetic acid (3:1) for 10 min. The DNA fibers were denatured with 2.5 M HCl for 60 min, washed with PBS and blocked with 2% BSA in PBS-Tween for 60 min. IdU replication tracks were revealed with a mouse anti-BrdU/IdU antibody from BD Biosciences (347580) and CldU tracks with a rat anti-BrdU/CldU antibody from Eurobio (ABC117-7513). The following secondary antibodies were used: Alexa fluor 488 anti-mouse antibody (Life A21241) and Cy3 anti-rat antibody (Jackson Immunoresearch 712-166-153). Fibers were visualized and imaged by Carl Zeiss Axio Imager Apotome using 40X Plan Apo 1.4 NA oil immersion objective. Replication tracks lengths were analyzed using ImageJ software. Statistical analysis was performed using Graphpad Prism software.

Immunofluorescence

Cells were grown on coverslips to reach 70-80% confluency then fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at room temperature. Cells were permeabilized by with 0.2% Triton X100- PBS for 10 min then transferred into 0.1% Tween-PBS for 5 min. Coverslips were then incubated with primary antibodies in 0.1% Tween-5% BSA-PBS for 1-2 hrs, washed with 0.1% Tween-PBS, then incubated with secondary antibodies (anti-mouse or anti-rabbit coupled with Alexa fluor 488 or Alexa Fluor 546) in Tween 0.1%-BSA 5%-PBS for 1 hr. All the incubations were carried out in darkness in a humidified chamber at room temperature. Finally, coverslips are washed again with 0.1% Tween-PBS, incubated with Hoechst to label DNA for 5 min, and then mounted on glass slides with Prolong (Life). Cells were analyzed by fluorescence microscopy. Antibodies against the following proteins were used: FLAG (Sigma Aldrich F1804), Streptavidin-Alexa Fluor 488 (Life S32354) and GNL3 (Bethyl A300-600A).

Replication timing experiments and microarrays

Cells were incubated with 50 μM of BrdU for 90 min and collected, washed three times with PBS and then fixed in ethanol 75%. Cells were re-suspended in PBS with RNAse (0.5 mg/ml) and then with propidium iodide (50 μg/ml) followed by incubation in the dark at room temperature for 30 min with low agitation. Two fractions of 150,000 cells, S1 and S2 corresponding to Early and Late S-phase fractions respectively, were sorted by flow cytometry using a Becton Dickinson FACS Melody. Whole DNA was extracted with lysis buffer (50 mM Tris pH 8, 10 mM EDTA, 300 mM NaCl, 0.5% SDS) and 0.2 mg/ml of Proteinase K for 2

hrs at 65°C. Neo-synthesized DNA were immunoprecipitated with BrdU antibodies (Anti-BrdU Pure, BD Biosciences, #347580) as previously described (Fernandez-Vidal et al., 2014). To control the quality of enrichment of early and late fractions in S1 and S2, qPCR was performed with BMP1 oligonucleotides (early control) and with Dppa2 oligonucleotides (late control; data not shown, (Hiratani et al., 2008)). Microarray hybridization requires a minimum of 1000 ng of DNA. To obtain sufficient specific immunoprecipitated DNA for this hybridization step, whole genome amplification was conducted (WGA, Sigma) on immunoprecipitated DNA. A post WGA qPCR was performed to preserve specific enrichment in both S1 and S2 fractions. Early and late amplified neo-synthesized DNA were then labeled with Cy3 and Cy5 ULS molecules, respectively (Genomic DNA labeling Kit, Agilent). The hybridization was performed according to the manufacturer instructions on 4×180K mouse microarrays (SurePrint G3 Mouse CGH Microarray Kit, 4x180K, AGILENT Technologies, reference genome: mm9). Microarrays were scanned with an Agilent High-Resolution C Scanner using a resolution of 3 µm and the autofocus option. Feature extraction was performed with the Feature Extraction 9.1 software (Agilent Technologies). For each experiment, the raw data sets were automatically normalized by the Feature extraction software. Analysis was performed using the STAR-R software described in (Hadjadj et al., 2020). The statistical comparison was conducted between early and late domains from both cell lines in order to determine segments where replication timing changes. Graphical representation was generated with START-R suit.

Chromatin immunoprecipitation and deep sequencing (ChIP-seq)

About 20.10^6 of Hela S3 cells per sample were prepared for sonication following the True-ChIP chromatin shearing kit protocol for High Cell concentration from Covaris. Cells were cross-linked in 1% methanol-free formaldehyde during 5 min before cell lysis and nuclei preparation. Washed nuclei were sonicated for 15 min at 6°C to obtain DNA fragments of 100-800pb using the E220evolution Covaris machine following parameters indicated in the provided protocol. After dilution with one volume of immunoprecipitation dilution buffer (Covaris), sonicated samples were pre-cleared with 3 µL/mL of protein G magnetic beads (Ademtech) during 1 hr at 4°C. Each sample was then normalized to an equal amount of protein (associated to pre-cleared chromatin) and input samples were collected after this step. Normalized samples were then incubated with 1 µg of GNL3 antibody (Bethyl A300-600A) overnight at 4°C, before incubation with 20 µL/mL of protein G magnetic beads (previously blocked overnight at 4°C in immunoprecipitation dilution buffer with 1% BSA) during 4 hrs at 4°C. Chromatin bound to beads was then washed 5 min at room temperature in each following buffers: low salt buffer (150 mM NaCl, 20 mM Tris HCl pH=8, 2 mM EDTA, 1% Triton, 0.1% SDS); high salt buffer (500 mM NaCl, 20 mM Tris HCl pH=8, 2 mM EDTA, 1% Triton, 0.1%

SDS); LiCl buffer (0.25 M LiCl, 10 mM Tris-HCl pH=8, 1 mM EDTA, 1% Sodium deoxycholate, 1% NP-40); TE buffer (10 mM Tris-HCl pH=8, 1 mM EDTA). Washed beads were eluted in 200 µL of elution buffer (100 mM NaHCO3, 1% SDS) during 15 min at 30°C with shaking. Eluted chromatin and input samples were reverse-crosslinked overnight at 65°C with 0.2 M NaCl and 0.02 mg/mL of RNAse A and incubated 1 hr with Proteinase K (400 µg/mL final concentration). DNA was purified using the ChIP DNA Prep Adem kit (Ademtech) following the provided protocol. DNA bound to beads was eluted in 50 µL of elution buffer. Quantity of DNA was measured with the Qubit 1X dsDNA HS Assay kit (Invitrogen), using a Qubit 2.0 fluorometer (Thermofisher scientific). GNL3 ChIP was repeated three times and 10 ng of each ChIP and each corresponding input were pooled together and send to the MGX sequencing platform of Montpellier, France (https://www.mgx.cnrs.fr/). DNA banks were sequenced using the Illumina-Novaseq-6000 machine to obtain 150 bp paired-end reads. Sequencing data were processed and analyzed using the online Galaxy platform (https://usegalaxy.org/). Reads were aligned on the February 2009 human reference genome (GRCh37/Hg19) using Bowtie2 tool with default parameters. GNL3 Peaks were discovered using MACS2 callpeak tool using input as control file with a q-value<0.005. ORC2 peaks file was taken from Miotto et al. (Miotto et al., 2016).

MiDAS

Cells were seeded on coverslips and synchronized using 2 mM of thymidine for 18 hrs. After the thymidine block cells were washed twice with pre-heated media and released for 8 hrs after which they were labelled with 10 µM EdU for 15 min and collected by direct fixation of 4% PFA into the media to avoid loss of mitotic cells. Cells were then immunostained with anti-pH3S10 (Cell Signaling 9701) and EdU was clicked with Alexa fluor 555 using Click chemistry.

Chromatin Fractionation

Cells were seeded at 80% confluency and collected by trypsinization followed by centrifugation for 3 min (1200g) at room temperature. The pellets were washed with PBS then resuspended with CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM Sucrose, 1 mM MgCl$_2$, 1 mM EGTA, 0.5 mM DTT, 0.1% Triton X-100, 1 mM ATP, 1X protease inhibitor) and kept for 10 min on ice. Lysed cells were then centrifuged for 3 min (3000g) at 4°C. The resulting supernatant presenting the soluble protein fraction was transferred to another Eppendorf tube and the pellet was washed with CSK buffer for 10 min on ice followed by centrifugation for 3 min (3000g) at 4°C. the resulting pellet which represents the in-soluble fraction of

proteins was then resuspended in 2X Laemmli buffer and incubated at 95°C for 10 min before western blot analysis.

Bio-ID

Flp-In T-Rex 293 cell lines were stably transfected with Flag-BirA-GNL3. Cells seeded at 75% confluency were incubated with 10 μg/ml of doxycycline for 16 hrs and then with 50 μM biotin for 4 hrs. Cells were washed once with PBS and lysed with RIPA/SDS buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.2% SDS, 0.5% Sodium deoxycholate) complemented with 1X complete protease inhibitor and 250U benzonase (Sigma-Aldrich, CE1014). Lysed cells were incubated on a rotating wheel for 1 hr at 4°C followed by sonication on ice with 30% amplitude for 3 cycles of 10 sec (2 sec ON-2sec resting) separated with 10 sec of resting. Sonicated lysate was next centrifuged for 30 min (7750g) at 4°C, the cleared supernatant was transferred to a new tube and protein concentration was quantified using Bradford protein assay. For each condition, 500 μg of proteins were incubated with 30 μl of Streptavidin-Agarose beads (Sigma-Aldrich, CS1638) on a rotating wheel for 3 hrs at 4°C. Beads were next washed sequentially with 1 ml of each buffer starting with lysis buffer, wash buffer 1 (2% SDS in H_2O), wash buffer 2 (0.2% sodium deoxycholate, 1% Triton X-100, 500 mM NaCl, 1mM EDTA, and 50mM Hepes pH 7.5), wash buffer 3 (250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1mM EDTA, 500mM NaCl and 10mM Tris pH 8) and finally wash buffer 4 (50 mM Tris pH 7.5 and 50 mM NaCl). Biotinylated proteins were eluted from the magnetic beads using 40 μl of 2X Laemmli buffer and incubated at 95°C for 10 min.

Proteomics analysis of Bio-ID samples

Biotinylated proteins were migrated on SDS PAGE for a short migration. After reduction (DTT 1 M, 30 min at 60°C) an alkylation (IAA 0.5 M, 30 min RT) proteins were digested using trypsin (Gold, Promega, 1ug / sample, overnight at 37°C). For LC MSMS analysis, samples were loaded onto a 50 cm reversed-phase column (75 mm inner diameter; Acclaim PepMap 100 C18; Thermo Fisher Scientific) and separated with an UltiMate 3000 RSLC system (Thermo Fisher Scientific) coupled to a QExactive HF system (Thermo Fisher Scientific). Separation of the peptides was performed following a gradient from 2 to 25% buffer B (0.1% AF in 80% ACN) for 100 min at a flow rate 300 nl / min, then 25 to 40% in 20 min and finally 40 to 90% in 3 minutes. Tandem mass spectrometry analyses were performed in a data-dependent mode. Full scans (350–1,500 m/z) were acquired in the Orbitrap mass analyzer with a resolution of 60,000 at 200 m/z. For MS scans, 3e6 ions were accumulated within a maximum injection time of 60 ms. The 12 most intense ions with charge states ≥2 were sequentially isolated (1e5) with a maximum injection time of 100 ms and

fragmented by higher-energy collisional dissociation (normalized collision energy of 28) and detected in the Orbitrap analyzer at a resolution of 30,000. Raw spectra were processed with MaxQuant v 1.6.5.0 (Cox and Mann, 2008) using standard parameters with match between runs option. Spectra were matched against the UniProt reference proteome (release 2019_06; http://www.uniprot.org) of Homo sapiens and 250 frequently observed contaminants, as well as reversed sequences of all entries. The maximum false discovery rate for peptides and proteins was set to 0.01. Representative protein ID in each protein group was automatically selected using the in-house developed Leading tool (Raynaud et al., 2018)

Immunoprecipitation

Whole-cell extracts of K562 cells were prepared using lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM EDTA pH8, 0.5% NP40) supplemented with protease inhibitor cocktail (Roche), 1mM PMSF, 1mM MgCl2 and Benzonase Nuclease 250 units/ 10 millions of cells (E1014-25KU, Sigma). Immunoprecipitations were performed overnight at 4°C with protein G Dynabeads (Thermo Fisher Scientific) coupled to either rabbit immunoglobulin G (IgG) (P120-201, Bethyl Laboratories) or rabbit ORC2 antibody (A302-734A, Bethyl Laboratories). Beads were washed 4 times with lysis buffer, then washed 3 times with 50 mM Tris HCl pH8. The immunoprecipitated complexes were eluted in 50 mM Tris HCl pH8 containing 1% SDS for 15 min à 56°C with agitation. IP samples were mixed with 1X Bolt Sample Reducing agent (Thermo Fisher Scientific) and 1X Bolt LDS Sample Buffer (Thermo Fisher Scientific), loaded and resolved on pre-cast Bolt Bis-Tris gels (Thermo Fisher Scientific), then transferred onto nitrocellulose membrane (GE Healthcare). Membranes were blocked in 5% fat-free milk in PBS, incubated overnight at 4°C with primary antibodies directed against ORC2 (A302-734A, Bethyl Laboratories) and GNL3 (sc-166460, Santa Cruz Biotechnology). A cognate secondary antibody coupled to horseradish peroxidase was used and revealed with the Super Signal West Dura Extended Duration Substrate kit (Thermo Fisher Scientific). Acquisition was performed using the Fusion FX (Vilber) and image analysis was performed using ImageJ (https://imagej.nih.gov/ij/).

Proteomics analysis of immunoprecipitation

Sample preparation: Tryptic peptides from the immunoprecipitated complexes (=eluate) were obtained by Strap Micro Spin Column according to the manufacturer's protocol (Protifi, NY, USA). Briefly: proteins from 140 µL of the eluate were diluted 1:1 with 2x reducing-alkylating buffer (20 mM TCEP, 100 mM Chloroacetamide in 400 mM TEAB pH 8.5 and 4% SDS) and left 5 min at 95°C to allow reduction and alkylation in one step. Strap binding buffer was applied to precipitate proteins on quartz and proteolysis took place during 14 hrs at 37°C with 1 µg Trypsin sequencing grade (Promega). After speed-vacuum drying

of eluted peptides, these were solubilized in 0.1% trifluoroacetic acid (TFA) in 10% Acetonitrile (ACN). Liquid Chromatography-coupled Mass spectrometry analysis (LC-MS): LC-MS analyses were performed on a Dionex U3000 HPLC nanoflow system coupled to a TIMS-TOF Pro mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). One µl was loaded, concentrated and washed for 3 min on a C18 reverse phase precolumn (3 µm particle size, 100 Å pore size, 75 µm inner diameter, 2 cm length, from Thermo Fisher Scientific). Peptides were separated on an Aurora C18 reverse phase resin (1.6 µm particle size, 100Å pore size, 75 µm inner diameter, 25 cm length mounted onto the Captive nanoSpray Ionization module, (IonOpticks, Middle Camberwell Australia) with a 60 minutes overall run-time gradient ranging from 99% of solvent A containing 0.1% formic acid in milliQ-grade H2O to 40% of solvent B containing 80% acetonitrile, 0.085% formic acid in mQH2O. The mass spectrometer acquired data throughout the elution process and operated in DDA PASEF mode with a 1.1 second/cycle, with Timed Ion Mobility Spectrometry (TIMS) mode enabled and a data-dependent scheme with full MS scans in PASEF mode. This enabled a recurrent loop analysis of a maximum of the 120 most intense nLC-eluting peptides which were CID-fragmented between each full scan every 1.1 second. Ion accumulation and ramp time in the dual TIMS analyzer were set to 50 ms each and the ion mobility range was set from 1/K0 = 0.6 Vs cm-2 to 1.6 Vs cm-2. Precursor ions for MS/MS analysis were isolated in positive mode with the PASEF mode set to « on » in the 100-1.700 m/z range by synchronizing quadrupole switching events with the precursor elution profile from the TIMS device. The cycle duty time was set to 100%, accommodating as many MSMS in the PASEF frame as possible. Singly charged precursor ions were excluded from the TIMS stage by tuning the TIMS using the otof control software, (Bruker Daltonik GmbH). Precursors for MS/MS were picked from an intensity threshold of 2.500 arbitrary units (a.u.) and resequenced until reaching a 'target value' of 20.000 a.u taking into account a dynamic exclusion of 0.40 s elution gap. Protein quantification and comparison : The mass spectrometry data were analyzed using Mascot version 2.5.1 (http://www.matrixscience.com/). The database used was a concatenation of Homo sapiens sequences from the Swissprot databases (release June 2020 : 563,972 sequences; 203,185,243 residues) and an in-house list of frequently found contaminant protein sequences. The enzyme specificity was trypsin's. The precursor and fragment mass tolerances were set to 20ppm. Oxidation of methionines was set as variable modifications while carbamidomethylation of cysteines was considered complete. False discovery rate (FDR) was kept below 1% on both peptides and proteins. For comparative analysis, peptide count results from Mascot were assembled with the MyPROMS (Poullet et al., 2007) software (version 3.1).

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Figures Legends

Figure 1. GNL3 is a new replisome component. A. Experimental set-up of iPOND-MS experiment. HeLa S3 cells were pulse-labelled with EdU and chased with thymidine for 120 min. **B.** Bar plot showing the LogRatio (pulse/chase) of average peptides intensities corresponding to the indicated proteins from (Ribeyre et al., 2021). Pulse experiments have been repeated 6 times and chase experiments 4 times. **C.** iPOND experiment analyzed by western-blot. HeLa S3 Cells were pulsed for 15 min with EdU and chased for 2 hrs with thymidine. In no click sample, biotin-TEG azide was replaced by DMSO. **D.** Western-blot analysis of HeLa S3 cells depleted with a pool of 4 siRNA targeting GNL3 (siGNL3) or not (siControl). **E.** PLA (proximity ligation assay) analyzing the proximity between EdU (coupled with biotin-TEG azide) and GNL3 in HeLa S3 cells depleted or not for GNL3. **F.** PLA (proximity ligation assay) analyzing the proximity between EdU (coupled or not with biotin-TEG azide) and GNL3 in HeLa S3 cells. **G.** Experimental set-up of the synchronization procedure. HeLa S3 cells were submitted to thymidine block (TB) for 18 hrs and released into S-phase. Cells were collected and fixed just before release (T0) and then 2 hrs (T2), 4 hrs (T4), 6 hrs (T6) and 8 hrs (T8) after release. A 10 min EdU pulse was performed just before fixation. Replication patterns showing the different phases are represented. **H.** PLA (proximity ligation assay) analyzing the proximity between EdU and GNL3 during S-phase in HeLa S3 cells.

Figure 2. GNL3 depletion increases firing of replication origins. A. Flow cytometry experiment of HeLa S3 cells. Nascent DNA was labelled IdU and total DNA stained with propidium iodide. **B.** Quantification of the intensity of IdU signal from flow cytometry analysis. For statistical analysis Mann-Whitney test was used; ****$p < 0.0001$. **C.** Western-blot analysis of the indicated proteins upon chromatin fractionation. **D.** Quantification of chromatin fractionation based on 3 independent experiments. **E.** DNA combing experiment. HeLa S3 cells were subjected to two consecutive 20 min pulses of IdU and CldU and analyzed by DNA combing. A representative microscopy image of combed DNA molecules containing IdU (red) and CldU (green) tracks in presented with arrows indicating the direction of replication. **F.** Analysis of replication forks velocity by DNA combing. For statistical analysis Mann-Whitney test was used; ****$p < 0.0001$. **G.** Analysis of GIFD (Global Instant Fork Density) by DNA combing in HeLa S3 cells, 4 independent experiments are represented. The red line indicates the average of the 4 experiments. **H.** Loss of GNL3 has no effect on replication timing. HeLa S3 cells were pulse-labelled with BrdU for 90 min and sorted by flow cytometry in two fractions, S1 and S2, corresponding to early and late S-phase. Neo-synthesized DNA was immunoprecipitated with BrdU antibodies. Early and late neo-synthesized DNAs were labeled with Cy3 and Cy5 and hybridized on microarrays. After analyzing with the START-R software,

replication-timing profiles can be obtained from two replicates. Shown are the zoomed microarray profiles of the timing of replication on chromosome 1 and chromosome 15 as example. Blue lines represent replication timing from siControl cells and red lines represent siGNL3 cells and grey spots represent the log ratio intensity for each probes of the microarray. Any significantly disturbed regions are detected by START-R software.

Figure 3. GNL3 overexpression inhibits firing of replication origins. A. Immunofluorescence analysis of Flp-in T-Rex HeLa cells expressing GNL3-FLAG. **B.** Flp-in T-Rex HeLa cells expressing (+DOX) or not (-DOX) GNL3-FLAG analyzed by flow cytometry experiment. Total DNA was stained with propidium iodide and nascent DNA labelled with IdU. **C.** Quantification of the intensity of IdU signal from flow cytometry. For statistical analysis Mann-Whitney test was used; ****$p<0.0001$. **D.** Western-blot analysis upon chromatin fractionation. **E.** Quantification of chromatin fractionation based on 3 independent experiments.

Figure 4. GNL3 interacts with ORC2. A. GNL3-BioID experiment analyzed by mass spectrometry. Expression of GNL3-BirA-FLAG in HEK293 Flp-in cells was induced with doxycycline for 16 hrs then biotin was added for 4 hrs. For negative controls cells were treated either 16 hrs with doxycycline either 4 hrs with biotin. Each condition was performed four times and analyzed by mass spectrometry. Label free quantification was performed using MaxQuant (Cox and Mann, 2008) and statistical analysis using Perseus (Tyanova et al., 2016). The volcano plot shows the proteins that are significantly enriched upon induction of GNL3-BirA-FLAG and addition of biotin. **B.** Western-blot analysis of GNL3 and ORC2 immunoprecipitates in K562 cells. **C.** Comparison of the genomic location of GNL3 and ORC2. Chromatin immunoprecipitation of GNL3 followed by deep sequencing was performed in HeLa S3. GNL3 binding sites were compared to ORC2 binding sites obtained from Miotto et al. (Miotto et al., 2016). **D.** PLA (proximity ligation assay) analyzing the proximity between ORC2 and GNL3 in HeLa S3 cells. **E.** Graphic representation of the average number of PLA GNL3-ORC2 foci upon inhibition of WEE1, CDC7 and ATR. The error bars represent the variation between 3 independent experiments. **F.** PLA (proximity ligation assay) analyzing the proximity between ORC2 and CENP-A in HeLa S3 cells using the indicated antibodies. **G.** Graphic representation of the average number of PLA ORC2-CENP-A foci upon inhibition of WEE1 (AZD1775), CDC7 (PHA-767491) and ATR (VE-821). The error bars represent the variation between 3 independent experiments.

Figure 5. GNL3 prevents DNA resection at stalled replication forks. **A.** HeLa S3 cells were sequentially labelled for 30 min with IdU and for 30 min with CldU with or without 1 µM CPT. Ratios between CldU and IdU are plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****$p<0.0001$. ns, not significant. **B.** Western-blot analysis of HeLa S3 cells treated with 5 mM HU during
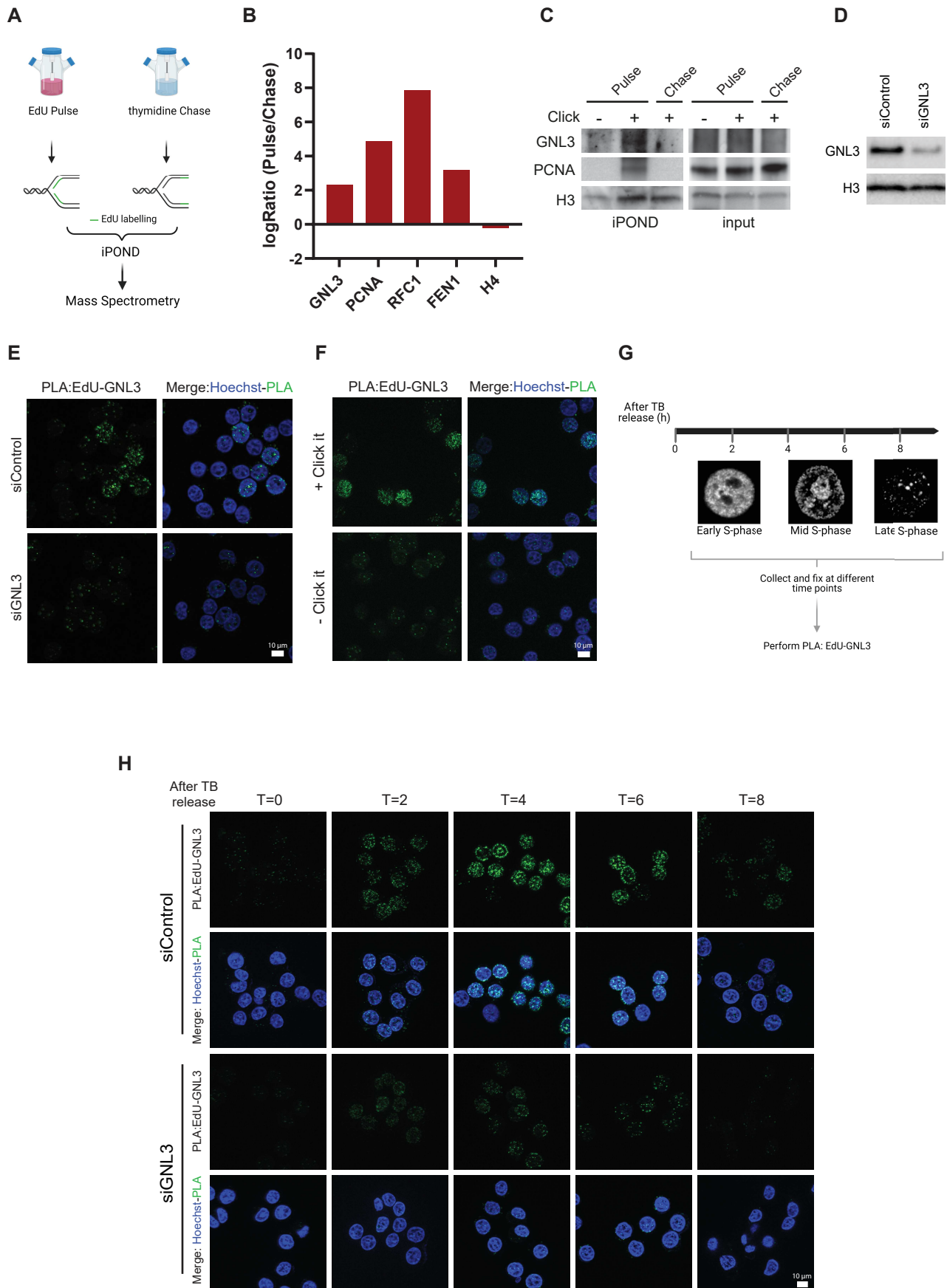
the indicated time. **C.** HeLa cells were sequentially labelled for 30 min with IdU and for 30 min with CldU then treated with 5 mM HU for 240 min. The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **D.** HeLa S3 were sequentially labelled for 30 min with IdU and for 30 min with CldU then treated with 5 mM HU for 240 min. The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **E.** Western-blot analysis of Flp-in T-Rex HeLa cells expressing GNL3-FLAG. Cells were first transfected with siControl or siGNL3 for 48 hrs then expression of GNL3-FLAG (resistant to the siRNA against GNL3) was induced using 10 ng/ml of doxycycline (DOX) for 16 hrs. **F.** Flp-in T-Rex HeLa cells were sequentially labelled for 30 min with IdU and for 30 min with CldU then treated with 5 mM HU for 240 min. The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001.

Figure 6. Resection in the absence of GNL3 is a consequence of its role in origin firing. A. Experimental set-up of iPOND experiment. **B.** iPOND experiment analyzed by Western-blot. Cells were pulsed with 15 min EdU and chased for 2 hrs with 10 µM thymidine or 5 mM HU. In no click sample, biotin-TEG azide was replaced by DMSO. **C.** Scheme to explain how CDC7 inhibition is affecting forks stability. **D.** HeLa S3 were sequentially labelled for 30 min with IdU and for 30 min with CldU then treated with 5 mM HU for 240 min with or without 10 µM of CDC7 inhibitor PHA-767491. The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **E.** Western-blot analysis of the indicated proteins upon treatment with 5 mM HU for 240 min with or without 10 µM of CDC7 inhibitor PHA-767491. **F.** HeLa S3 cells were sequentially labelled for 30 min with IdU and for 30 min with CldU then treated with 5 mM HU for 240 min with or without 10 µM of CDC7 inhibitor PHA-767491. The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001. ns, not significant. **G.** Cells were sequentially labelled for 30 min with IdU and for 30 min with CldU then treated or not with 5 mM HU for 240 min with or without 10 µM of ATR VE-821 inhibitor or 500 nM of WEE1 inhibitor AZD1775. **H.** The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **I.** The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis Mann-Whitney test was used; ****p<0.0001.

Figure 7. Model to explain how GNL3 is affecting origin efficiency. A. GNL3 level is crucial to ensure the correct level of replication origins firing possibly via its ability to interact with ORC2. This is particularly important in presence of exogenous replication stress where the absence of GNL3 leads to replication

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Figure 1

A

EdU Pulse · thymidine Chase

— EdU labelling

iPOND

Mass Spectrometry

B

logRatio (Pulse/Chase)

GNL3 · PCNA · RFC1 · FEN1 · H4

C

Click

GNL3
PCNA
H3

iPOND · input

D

siControl · siGNL3

GNL3
H3

E

PLA:EdU-GNL3 · Merge:Hoechst-PLA

siControl

siGNL3

10 μm

F

PLA:EdU-GNL3 · Merge:Hoechst-PLA

+ Click it

- Click it

10 μm

G

After TB release (h)

0 · 2 · 4 · 6 · 8

Early S-phase · Mid S-phase · Late S-phase

Collect and fix at different time points

Perform PLA: EdU-GNL3

H

After TB release · T=0 · T=2 · T=4 · T=6 · T=8
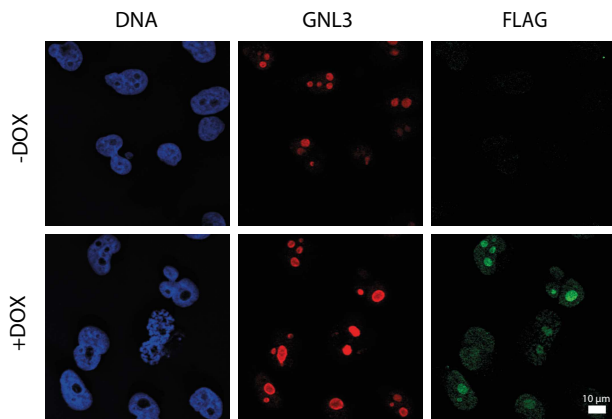
siControl

PLA:EdU-GNL3

Merge: Hoechst-PLA

siGNL3

PLA:EdU-GNL3

Merge: Hoechst-PLA

10 μm

Figure 2

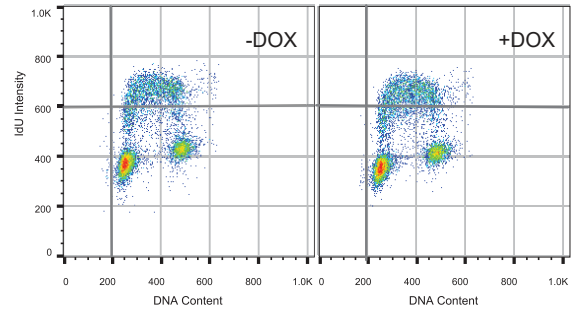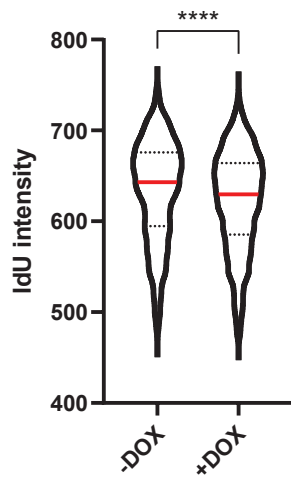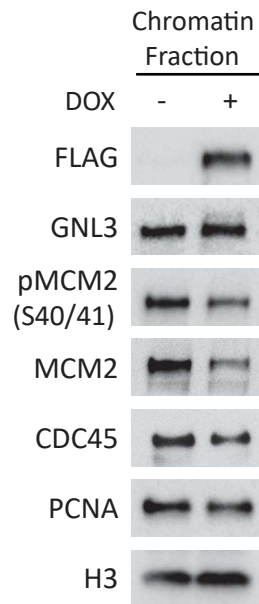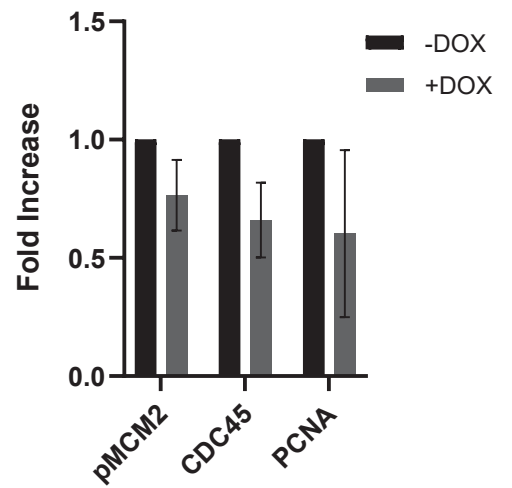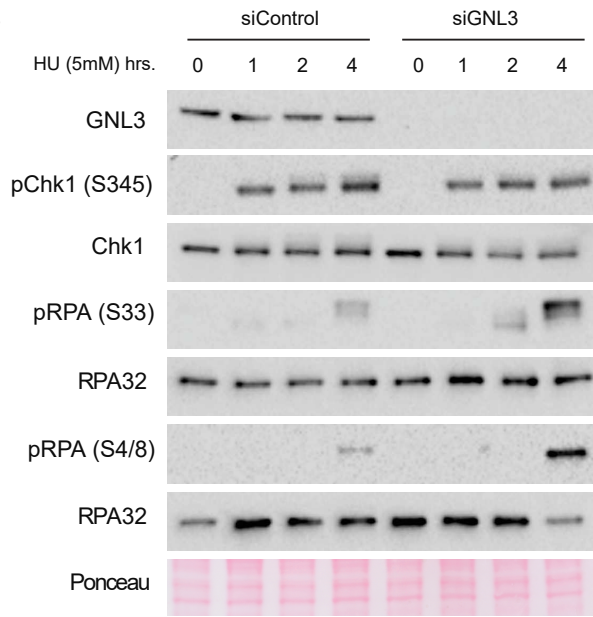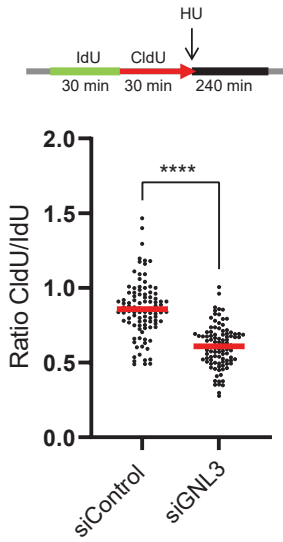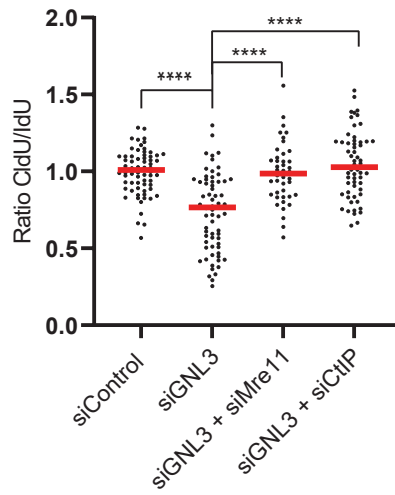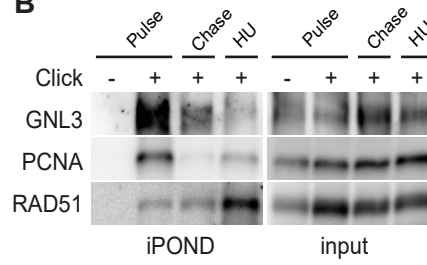# Figure 3

A

B

C

D

E

Figure 4
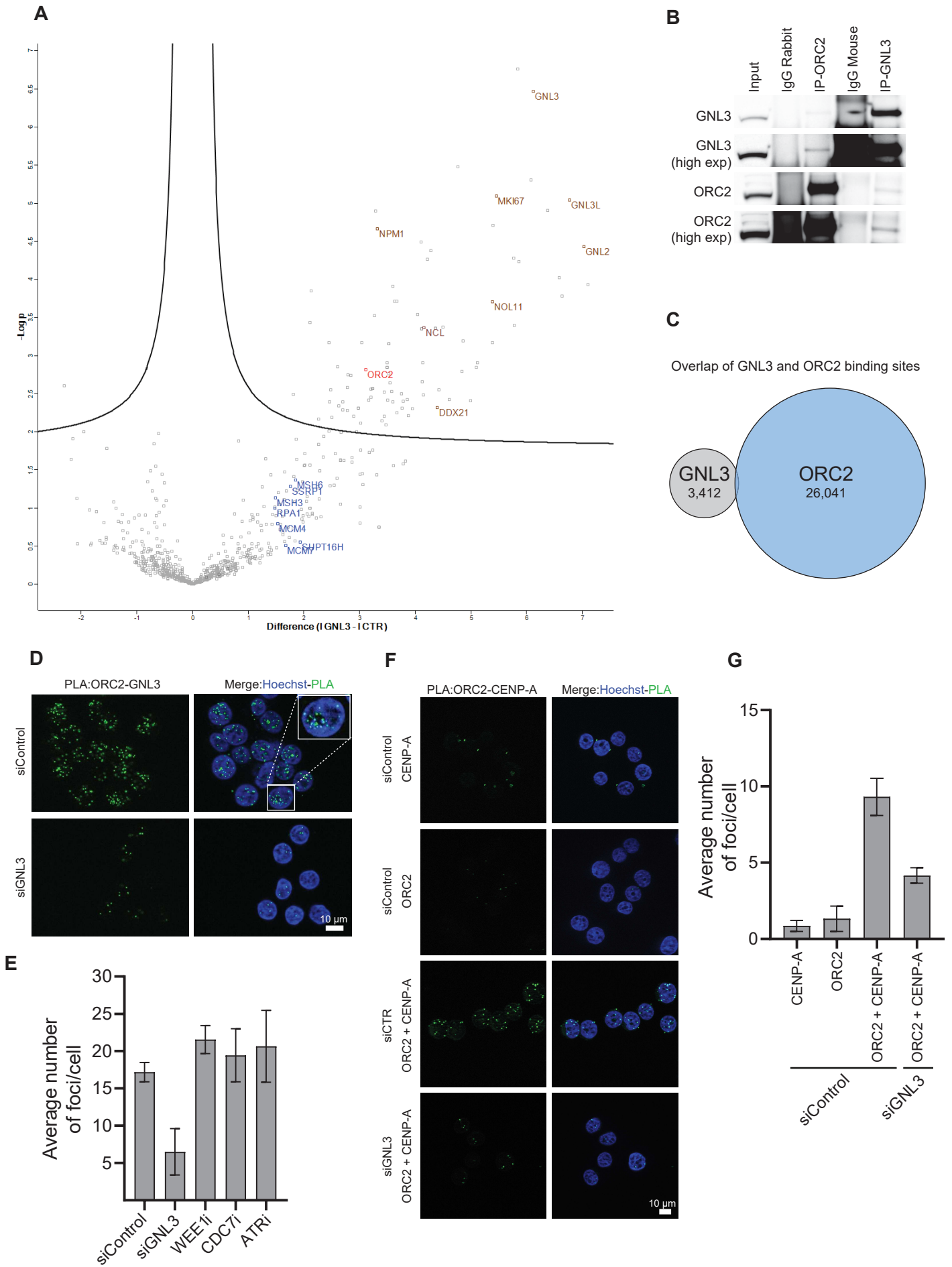
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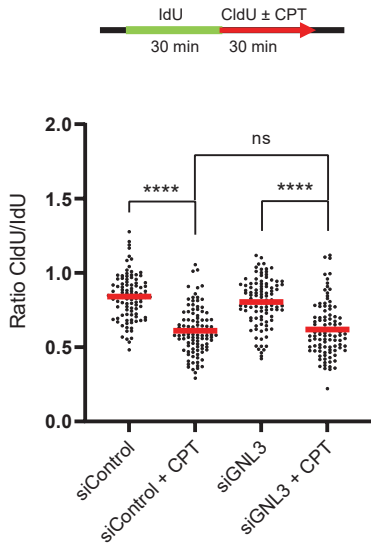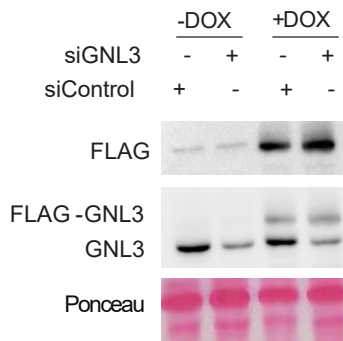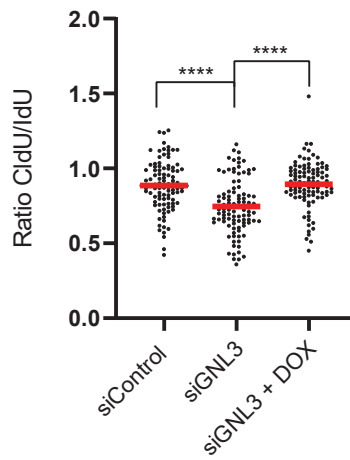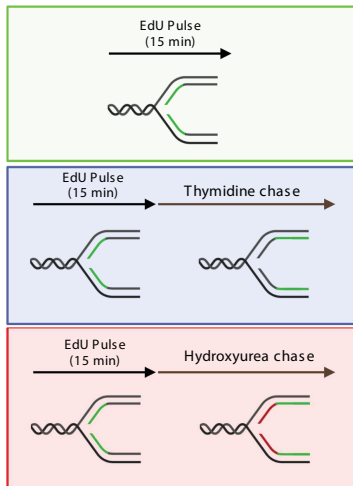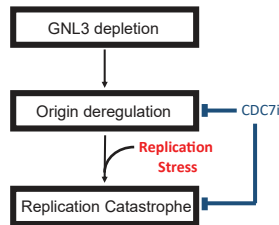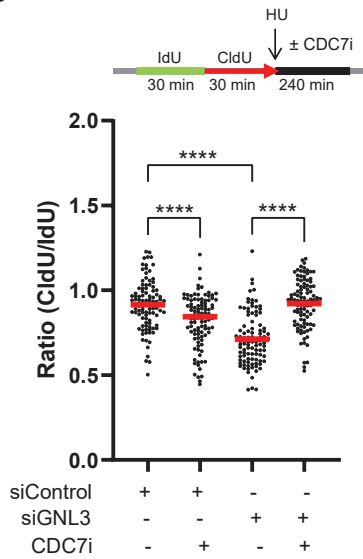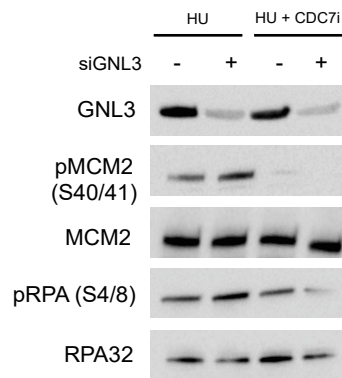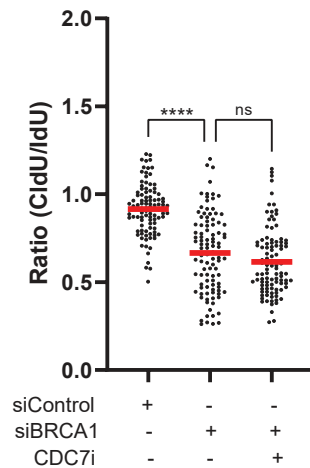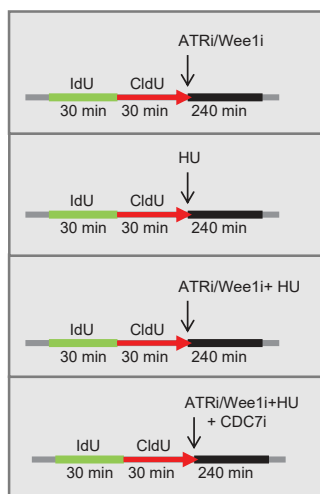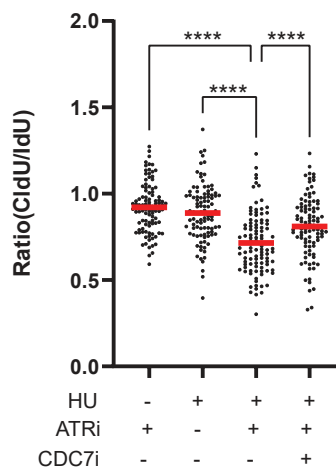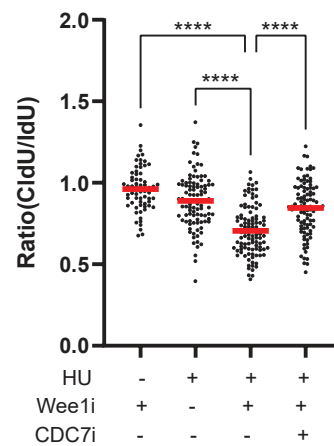
Figure 6

Figure 7

Figures Legends

Figure 1. GNL3 is a new replisome component. A. Experimental set-up of iPOND-MS experiment. HeLa S3 cells were pulse-labelled with EdU and chased with thymidine for 120 min. **B.** Bar plot showing the LogRatio (pulse/chase) of average peptides intensities corresponding to the indicated proteins from (Ribeyre et al., 2021). Pulse experiments have been repeated 6 times and chase experiments 4 times. **C.** iPOND experiment analyzed by western-blot. HeLa S3 Cells were pulsed for 15 min with EdU and chased for 2 hrs with thymidine. In no click sample, biotin-TEG azide was replaced by DMSO. **D.** Western-blot analysis of HeLa S3 cells depleted with a pool of 4 siRNA targeting GNL3 (siGNL3) or not (siControl). **E.** PLA (proximity ligation assay) analyzing the proximity between EdU (coupled with biotin-TEG azide) and GNL3 in HeLa S3 cells depleted or not for GNL3. **F.** PLA (proximity ligation assay) analyzing the proximity between EdU (coupled or not with biotin-TEG azide) and GNL3 in HeLa S3 cells. **G.** Experimental set-up of the synchronization procedure. HeLa S3 cells were submitted to thymidine block (TB) for 18 hrs and released into S-phase. Cells were collected and fixed just before release (T0) and then 2 hrs (T2), 4 hrs (T4), 6 hrs (T6) and 8 hrs (T8) after release. A 10 min EdU pulse was performed just before fixation. Replication patterns showing the different phases are represented. **H.** PLA (proximity ligation assay) analyzing the proximity between EdU and GNL3 during S-phase in HeLa S3 cells.

Figure 2. GNL3 depletion increases firing of replication origins. A. Flow cytometry experiment of HeLa S3 cells. Nascent DNA was labelled IdU and total DNA stained with propidium iodide. **B.** Quantification of the intensity of IdU signal from flow cytometry analysis. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **C.** Western-blot analysis of the indicated proteins upon chromatin fractionation. **D.** Quantification of chromatin fractionation based on 3 independent experiments. **E.** DNA combing experiment. HeLa S3 cells were subjected to two consecutive 20 min pulses of IdU and CldU and analyzed by DNA combing. A representative microscopy image of combed DNA molecules containing IdU (red) and CldU (green) tracks in presented with arrows indicating the direction of replication. **F.** Analysis of replication forks velocity by DNA combing. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **G.** Analysis of GIFD (Global Instant Fork Density) by DNA combing in HeLa S3 cells, 4 independent experiments are represented. The red line indicates the average of the 4 experiments. **H.** Loss of GNL3 has no effect on replication timing. HeLa S3 cells were pulse-labelled with BrdU for 90 min and sorted by flow cytometry in two fractions, S1 and S2, corresponding to early and late S-phase. Neo-synthesized DNA was immunoprecipitated with BrdU antibodies. Early and late neo-synthesized DNAs were labeled with Cy3 and Cy5 and hybridized on microarrays. After analyzing with the START-R software,

replication-timing profiles can be obtained from two replicates. Shown are the zoomed microarray profiles of the timing of replication on chromosome 1 and chromosome 15 as example. Blue lines represent replication timing from siControl cells and red lines represent siGNL3 cells and grey spots represent the log ratio intensity for each probes of the microarray. Any significantly disturbed regions are detected by START-R software.

Figure 3. GNL3 overexpression inhibits firing of replication origins. A. Immunofluorescence analysis of Flp-in T-Rex HeLa cells expressing GNL3-FLAG. **B.** Flp-in T-Rex HeLa cells expressing (+DOX) or not (-DOX) GNL3-FLAG analyzed by flow cytometry experiment. Total DNA was stained with propidium iodide and nascent DNA labelled with IdU. **C.** Quantification of the intensity of IdU signal from flow cytometry. For statistical analysis Mann-Whitney test was used; ****p<0.0001. **D.** Western-blot analysis upon chromatin fractionation. **E.** Quantification of chromatin fractionation based on 3 independent experiments.

Figure 4. GNL3 interacts with ORC2. A. GNL3-BioID experiment analyzed by mass spectrometry. Expression of GNL3-BirA-FLAG in HEK293 Flp-in cells was induced with doxycycline for 16 hrs then biotin was added for 4 hrs. For negative controls cells were treated either 16 hrs with doxycycline either 4 hrs with biotin. Each condition was performed four times and analyzed by mass spectrometry. Label free quantification was performed using MaxQuant (Cox and Mann, 2008) and statistical analysis using Perseus (Tyanova et al., 2016). The volcano plot shows the proteins that are significantly enriched upon induction of GNL3-BirA-FLAG and addition of biotin. **B.** Western-blot analysis of GNL3 and ORC2 immunoprecipitates in K562 cells. **C.** Comparison of the genomic location of GNL3 and ORC2. Chromatin immunoprecipitation of GNL3 followed by deep sequencing was performed in HeLa S3. GNL3 binding sites were compared to ORC2 binding sites obtained from Miotto et al. (Miotto et al., 2016). **D.** PLA (proximity ligation assay) analyzing the proximity between ORC2 and GNL3 in HeLa S3 cells. **E.** Graphic representation of the average number of PLA GNL3-ORC2 foci upon inhibition of WEE1, CDC7 and ATR. The error bars represent the variation between 3 independent experiments. **F.** PLA (proximity ligation assay) analyzing the proximity between ORC2 and CENP-A in HeLa S3 cells using the indicated antibodies. **G.** Graphic representation of the average number of PLA ORC2-CENP-A foci upon inhibition of WEE1 (AZD1775), CDC7 (PHA-767491) and ATR (VE-821). The error bars represent the variation between 3 independent experiments.

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Sup Figure 1

A

Seed HCT116 → Transfection with a mini (25 targets) esiRNA library → 48 hrs incubation → 4 hrs treatment with 1μM camptothecin → Quantify γH2A.X signal in the nucleus using Celigo high-throughput microscope

B

Average rank based on γH2A.X level (n=5)
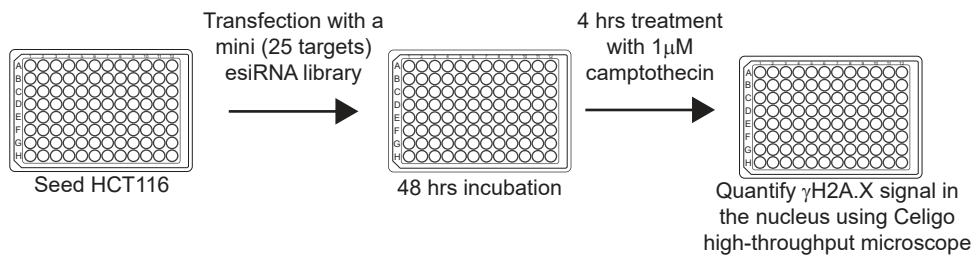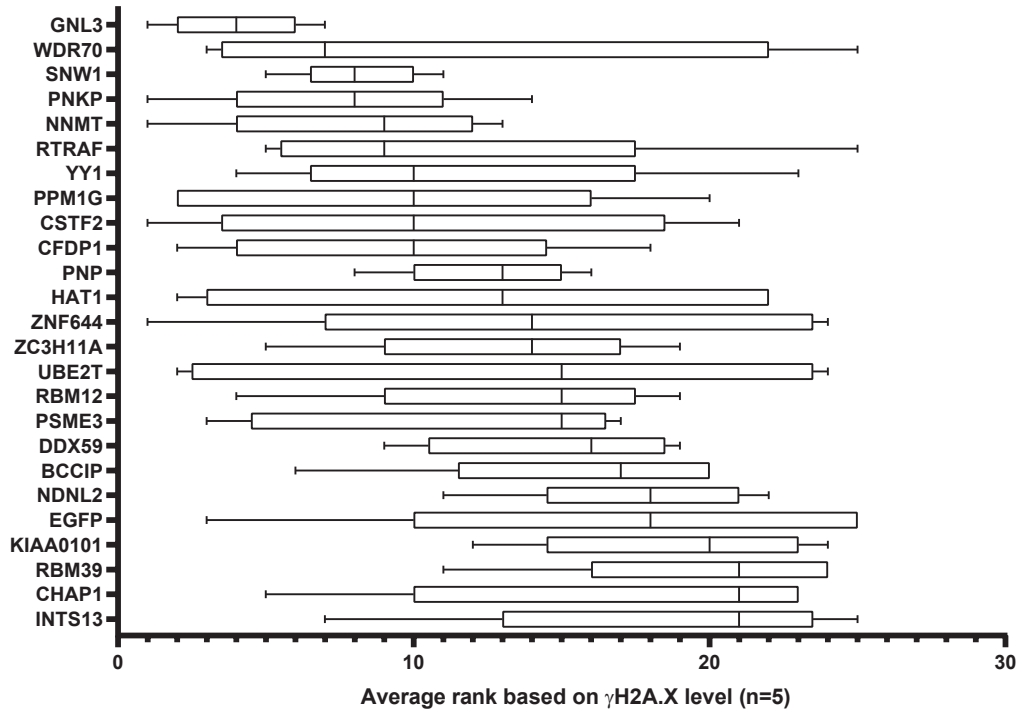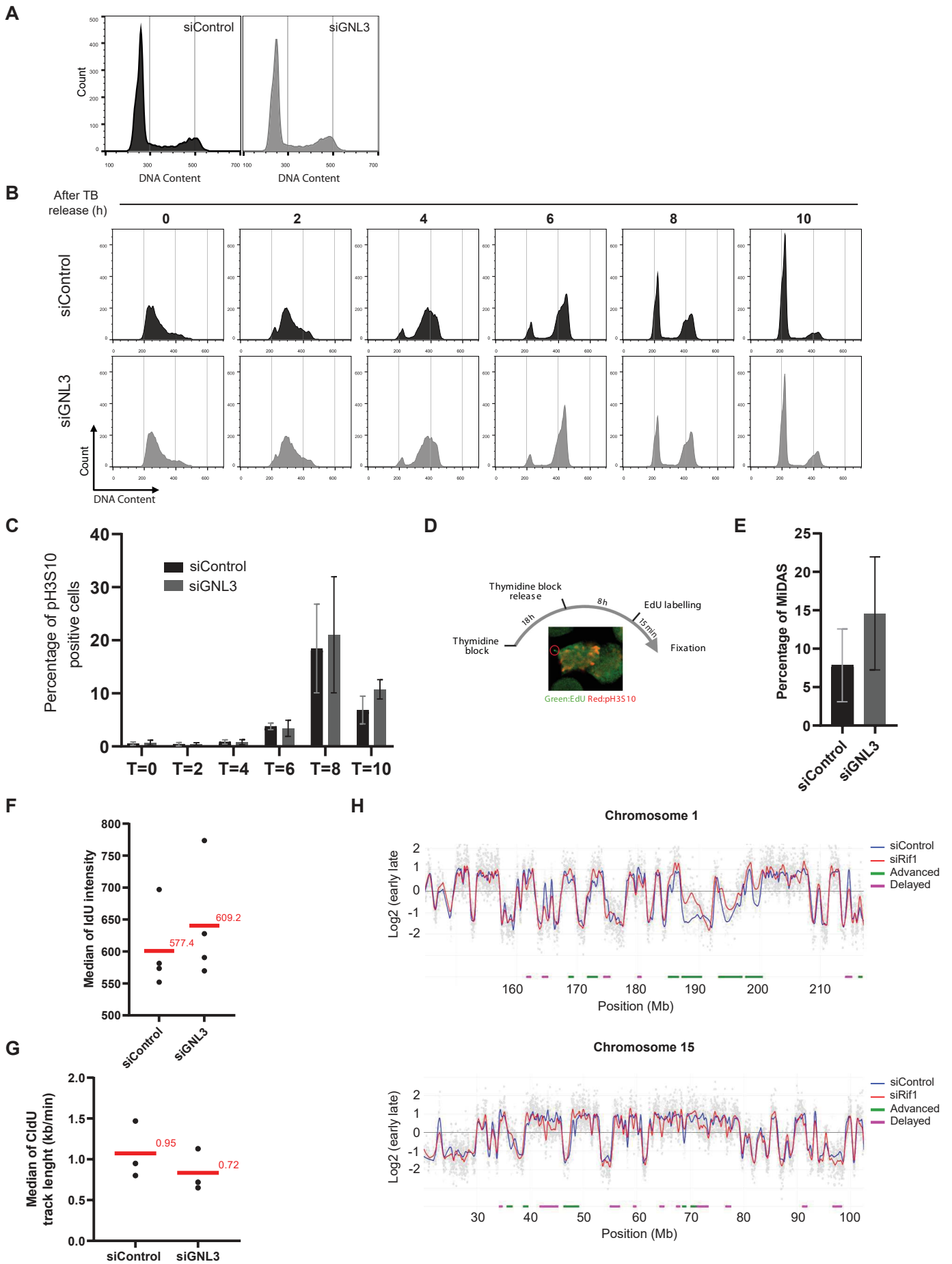
Sup Figure 2

Sup Figure 3

A

B

Sup Figure 4

A

DNA　　FLAG　　Streptavidin

-Biotin

+Biotin

10 µm
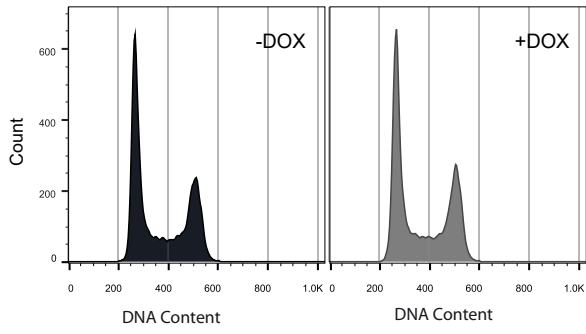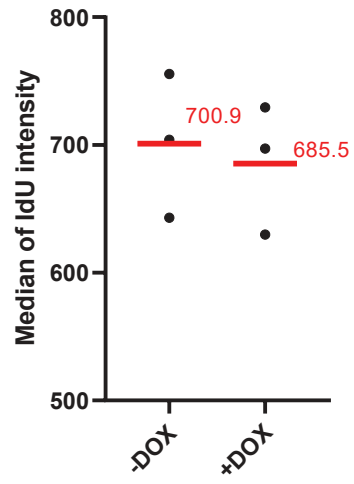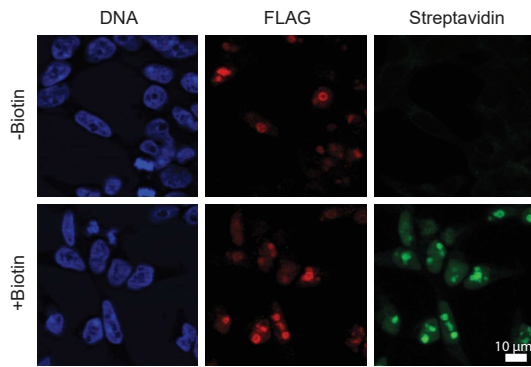
B

Commons hits between ORC2-IP and GNL3 BioID	
Present in the nucleolus	Not detected in the nucleolus
COIL	DDX49
DDX10	JMJD1C
DDX18	MRE11
DDX21	PRPF3
EBNA1BP2	SF3B1
ESF1	SNRPA1
EXOSC10	THRAP3
FTSJ3	TMPO
GNL2	TRAP1
MKI67	XRCC6
MYBBP1A	ZC3H11A
NAT10	
NCL	
NKRF	
NOP53	
NPM1	
PES1	
RPF2	
RPL5	
TCOF1	
TRMT1L	
UTP14A	
WDR36	
XRN2	

C

D

siGNL3　siControl　siControl + Wee1i　siControl + CDC7i　siControl + ATRi

PLA:ORC2-GNL3

Merge:Hoechst-PLA

E

Control			Wee1i		
G1	54.7		G1	43.2	
S	14.3		S	14.0	
G2	29.2		G2	40.9	

CDC7i			ATRi		
G1	44.1		G1	47.8	
S	14.3		S	13.3	
G2	39.3		G2	37.1	

Sup Figure 5

Sup Figure 6

A

B

C

D

E

F

G
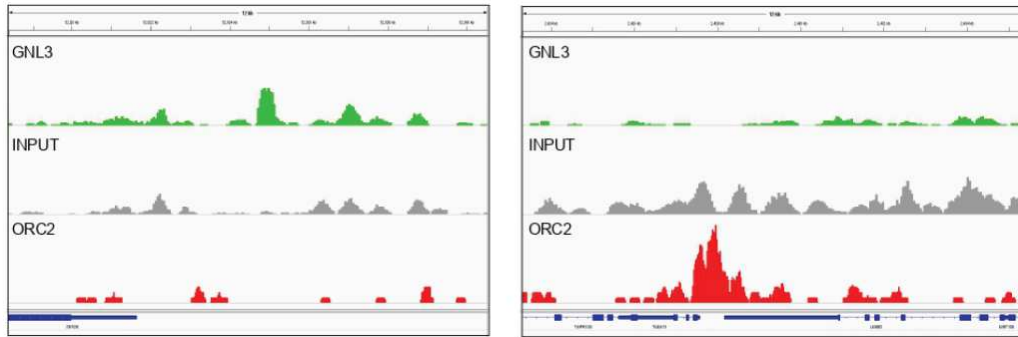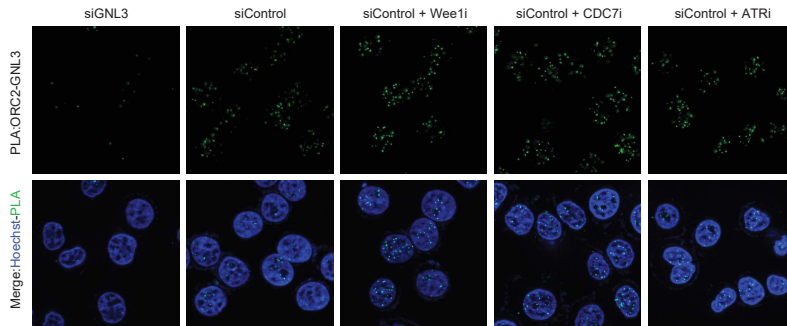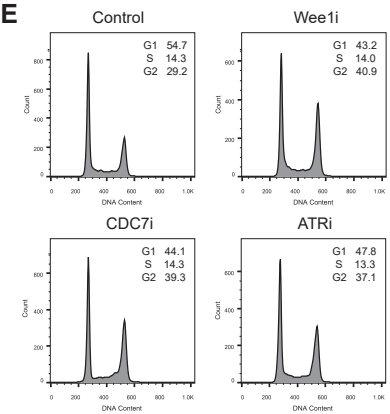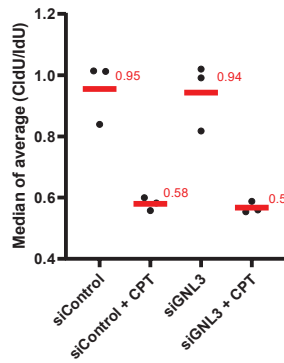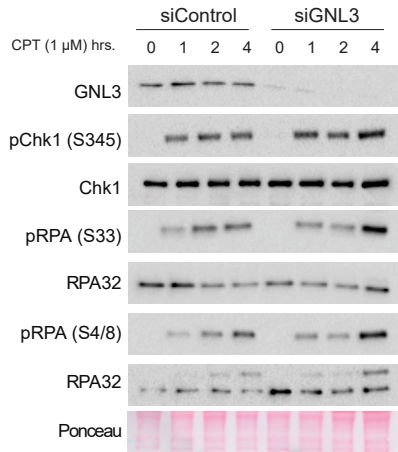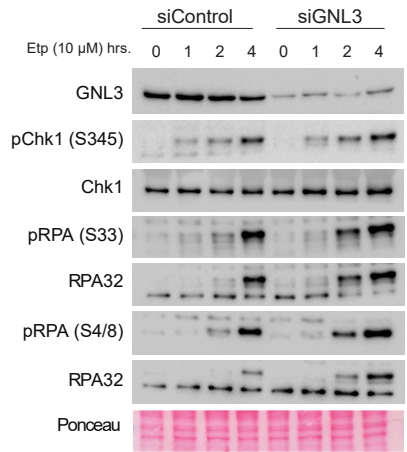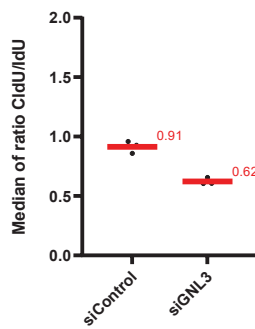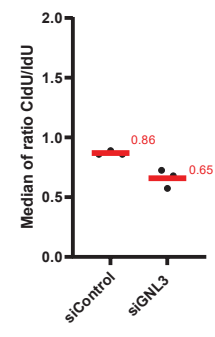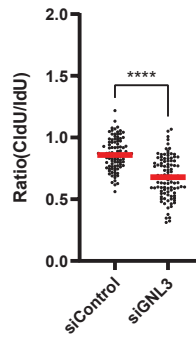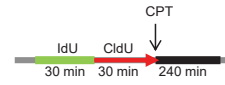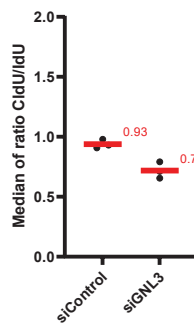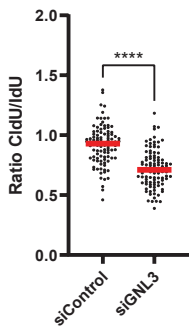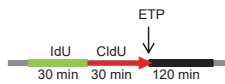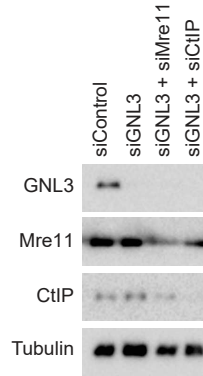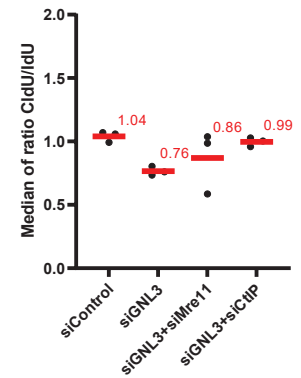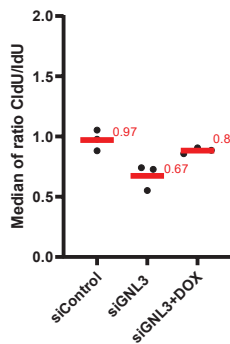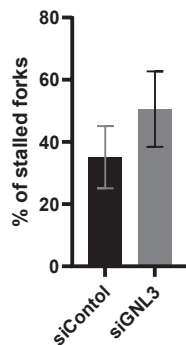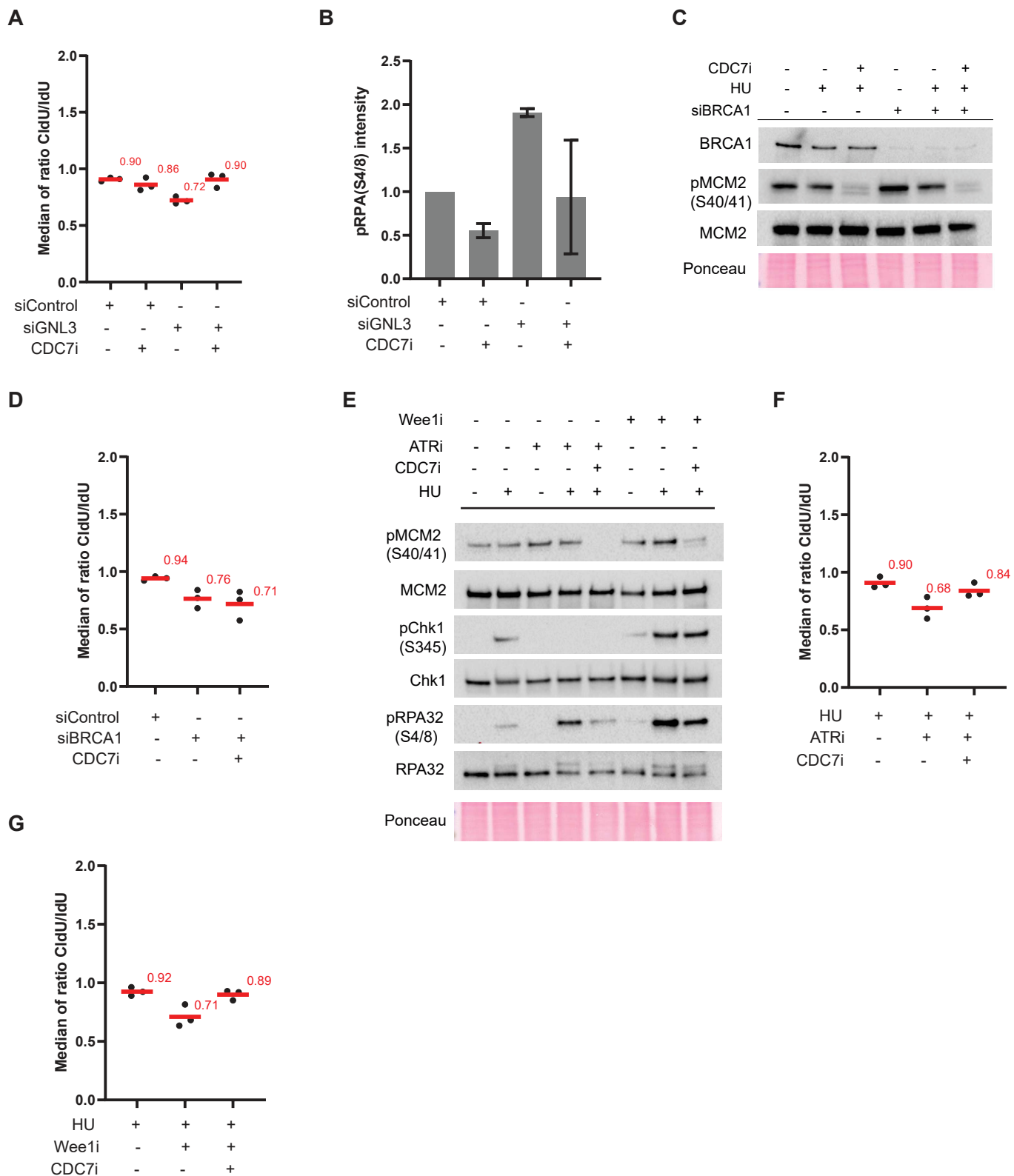
Additional Results

In this part I will present additional results and approaches that were not included in the written manuscript.

1- Depletion of GNL3 Leads to Accumulation of Mid-S Replication Foci

One of the possible ways to understand the role of GNL3 during DNA replication and to explain the increase in the efficiency of origin firing was to study the dynamic of S-phase progression by taking advantage of the existence of different replication patterns corresponding to early, mid and late S-phase (Dimitrova and Berezney, 2002) that can be visualized using EdU staining (Figure 24A). I synchronized cells using thymidine block and measured the percentage of each pattern 2, 4, 6 and 8 hours after release. I clearly observed a faster appearance of the mid-S pattern in absence of GNL3 2 hrs. after the release (Figure 24B). Since synchronization with thymidine block is known to introduce replicative stress (Kurose et al., 2006), I analyzed the percentage of each pattern in
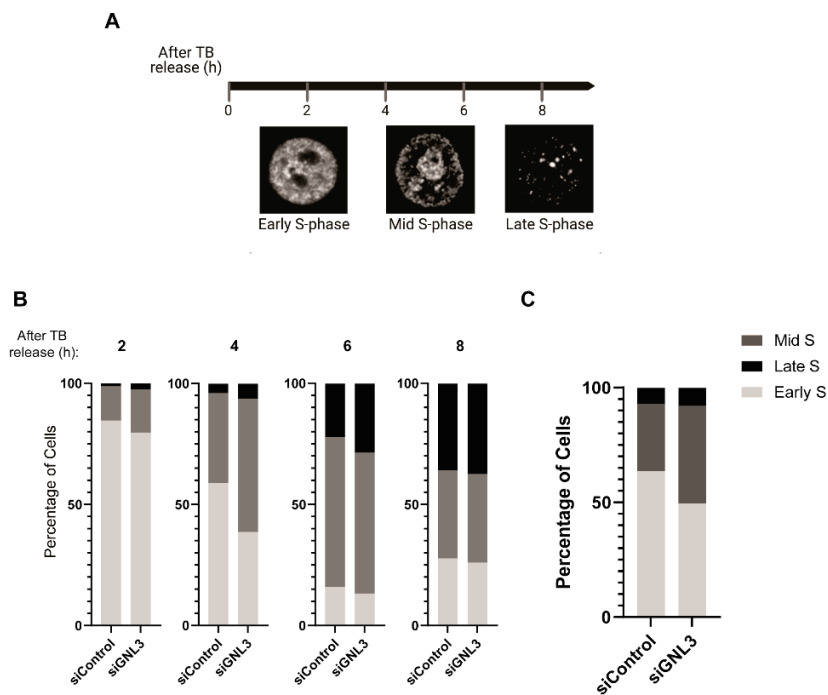
Figure 24. Mid-S-phase replication foci pattern is enriched in GNL3-depleted cells. **A.** Experimental set-up of the synchronization procedure. HeLa S3 cells were synchronized using thymidine block for 18 hours and released into S-phase. Cells were labelled with EdU for 10 mins collected and fixed at 2 hrs. (T2), 4 hrs. (T4), 6 hrs. (T6) and 8 hrs. (T8) after release. Different replication patterns are represented. **B.** Graphical representation of the percentage of each S-phase pattern (early, mid and late) 2, 4, 6 and 8 hours after release from thymidine block in HeLa S3 cells. **C.** Graphical representation of the percentage of each S-phase pattern (early, mid, and late) in non-synchronized HeLa S3 cells. The values correspond to three independent experiments

asynchronous conditions. Similarly, I observed a higher frequency of cells harboring the mid-S pattern in GNL3-depleted cells compared to control (Figure 24C).

Overall, these results indicate that GNL3 depletion might induce a change in the replication timing program, as described in the absence of RIF1, for example (Yamazaki et al., 2012). However, this was not the case since the replication timing experiment we performed showed no significant difference between the control and GNL3 depleted cells. The other explanation could be that GNL3 depleted cells are replicating more rapidly than control cells at the beginning of S-Phase due to the excessive origin firing, and could explain why the mid S-phase pattern appears faster compared to the control cells.

2- GNL3 Depletion Increases the Level of DSBs

It was reported that in the absence of GNL3, the cells accumulate DNA double-strand breaks (DSBs) in mammary and hepatocellular cancer cells (Lin et al., 2019; Wang et al., 2020). I aimed to validate this phenotype in HeLa S3 cells and also to study the level of DSBs induced by HU in absence of GNL3 by performing pulse field gel electrophoresis. Cells were depleted from GNL3 and collected directly or after treatment with HU for 16 or 24 hrs. As previously reported, I confirmed that the depletion of GNL3 increased the level of DNA breaks by two folds (Figure 25). Addition of HU for 16 hrs. Increased the level of DNA breaks in the control cells, and there was a slight increase in GNL3 depleted cells compared to the control. However, although the level of DNA breaks in GNL3 depleted cells were higher than the control upon treatment for 24 hrs., the level of DNA breaks decreased in both compared to the non-treated condition. This could be explained by the fact that cells might have already undergone apoptosis due to the high concentration of HU.
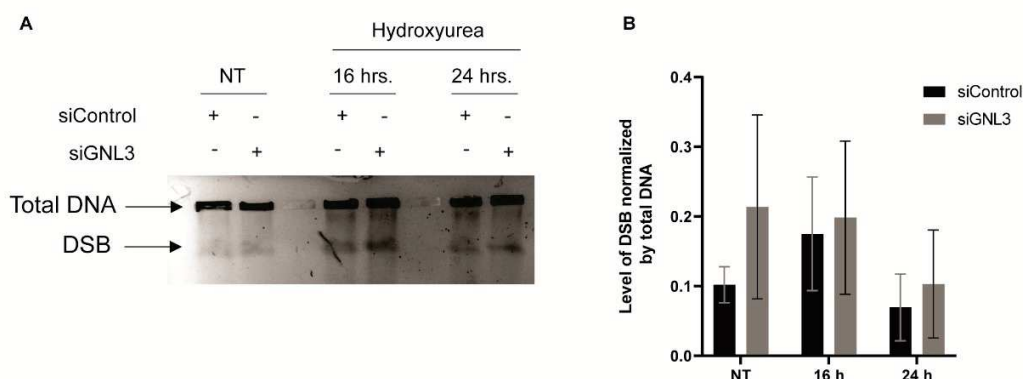
Figure 25. GNL3 depletion increases the level of spontaneous DSB and in response to hydroxyurea treatment. A. Ethidium bromide staining of DNA DSBs visualized with pulsed-field gel electrophoresis. Cells were tested for the level of DSB in untreated conditions or after treatment with HU (5 mM) for 16 or 24 hrs. **B.** Graphical representation of the percentage of the DSB formed in control and GNL3 depleted cells. The values correspond to three independent experiments.

3- Localization of GNL3 is not affected by replication stress

In order to address whether GNL3 might change its location or form DNA damage foci upon replication stress such as BRCA1 and RAD51, I treated HeLa S3 cells with a panel of molecules that induce replicative stress. I could not see any difference in the localization of GNL3 when cells were treated with hydroxyurea or etoposide or when exposed to UV, (Figure 26A,B). Upon treatment with CPT, the signal of GNL3 was different than the control. This observation is due to the fact the CPT is also targeting transcription, which causes nucleolar stress (Figure 26A) (Capranico et al., 2007). Actinomycin D (ActD) is a drug that induces nucleolar stress by inhibiting RNA polymerase I at low concentrations and inhibits both RNA polymerase I and II at high concentrations (Cooper and Braverman 1977). Consistent with the previous observation, inducing nucleolar stress with low concentration of ActD, which changes the structure of the nucleoli, therefore affecting the signal of GNL3 (Figure 26A). Nucleolar stress could be observed by the nucleolar caps that were formed by RNA Pol I. On the other hand, higher concentrations of Act D that completely disrupts the nucleolus, also disrupts the localization of GNL3 (Figure 26B).

Therefore, this suggests that the main localization of GNL3 is not affected by replication stress.
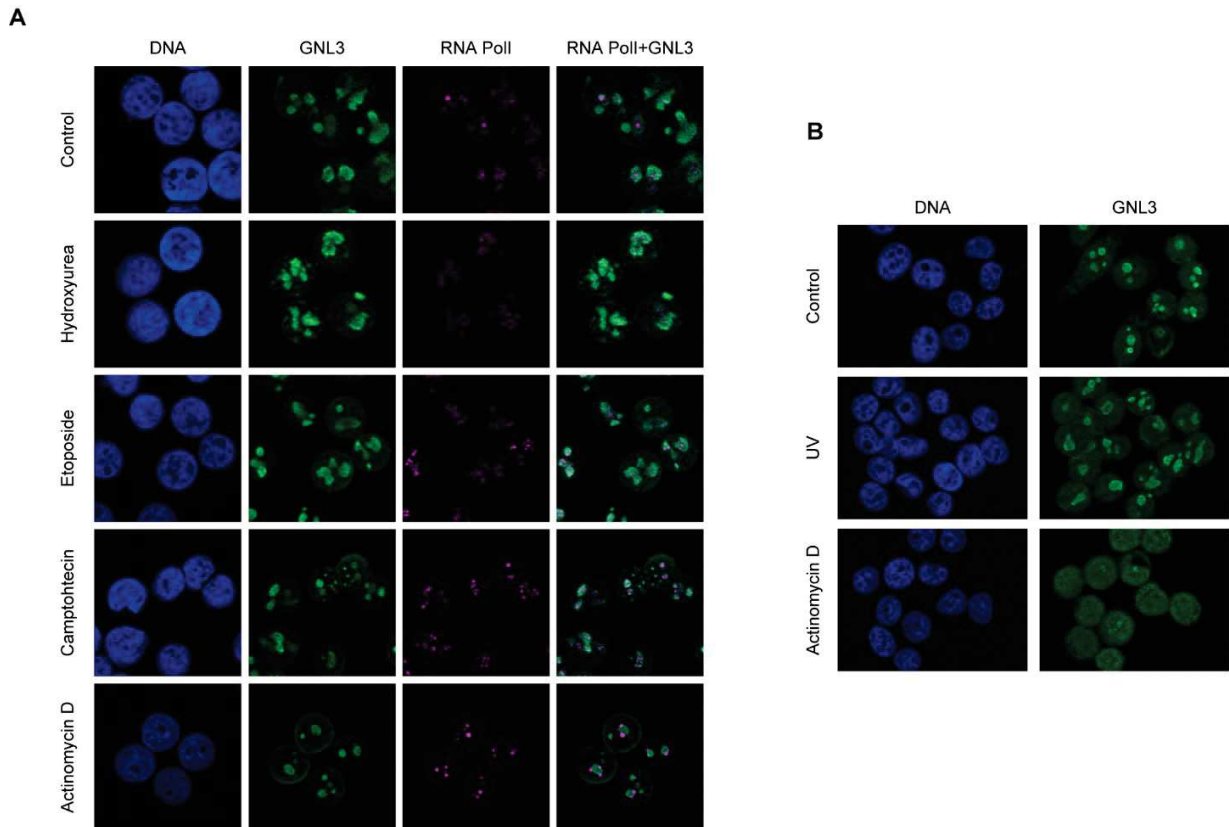
Figure 26. GNL3 localization in response to DNA damage. A. Double immunofluorescence staining of HeLa S3 cells treated with hydroxyurea (5 mM for 4 hrs.), etoposide (10 µM for 2 hrs.), camptothecin (1 µM for 4 hrs.) or actinomycin D (50 nM for 2 hrs.). **B.** Immunofluorescence staining of HeLa S3 cells exposed to 50 KJ of UV light then released for 4 hrs, or treated with actinomycin D (10 µM for 2 hrs.). Antibodies against NS and RNA pol II were used. DNA was counterstained with Hoechst-33342 (blue). Scale bar, 10 µm.

4- Overexpression of GNL3 Leads to DNA Resection in Response to Hydroxyurea

Since GNL3 is required for the recruitment of RAD51 to DSBs (Lin et al., 2013; Meng et al., 2013), I questioned whether the increased resection I observed upon GNL3 depletion could be due to a defect in RAD51 recruitment to stalled forks (Hashimoto et

al., 2010). To test this hypothesis, I performed chromatin fractionation assay to test for the recruitment of RAD51 in response to HU in the absence of GNL3. Interestingly, I did not observe any impact on RAD51 recruitment to chromatin upon GNL3 depletion (Figure 27A). Moreover, if GNL3 was able to protect stalled replication forks directly from DNA resection, its overexpression should not have any impact on DNA resection. Surprisingly, overexpression of GNL3 with DOX induction, using the system previously described, increased the level of resection in response to hydroxyurea like GNL3 depletion (Figure 27B). Therefore, I conclude that GNL3 is not acting directly on forks to protect it from DNA degradation and that maintaining the level of GNL3 within a specific range is essential for the maintenance of fork stability. Moreover, I propose that since the overexpression of GNL3 led to a decrease in the origin efficiency, the DNA resection observed could be due to failure of dormant origin firing.
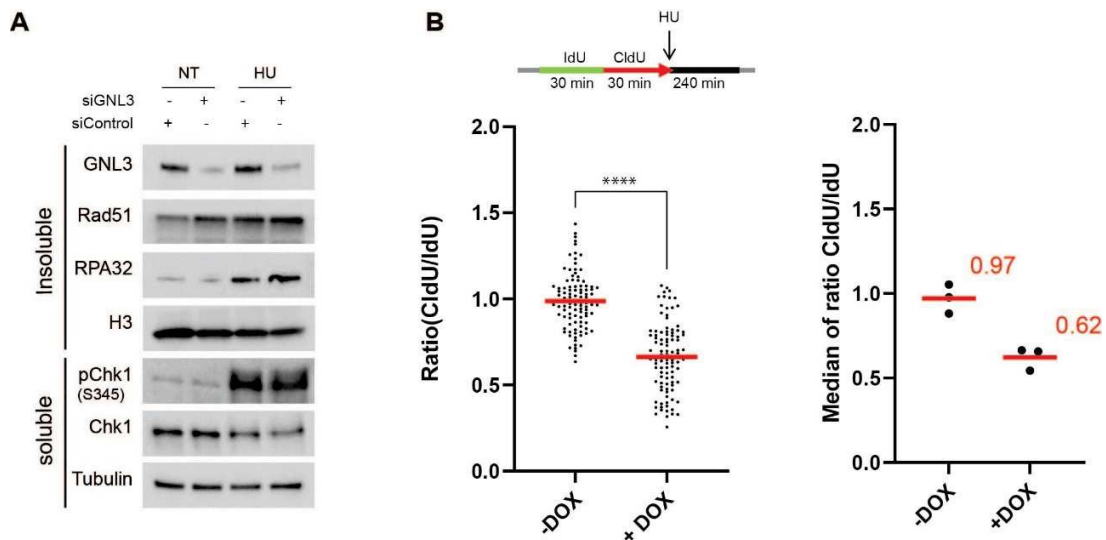
Figure 27. Overexpression of GNL3 leads to DNA resection. A. Chromatin Fractionation of HeLa S3 cells upon treatment with 5 mM HU for 4 hrs.. Western-blot analysis was performed for soluble and insoluble fractions. Flp-in T-Rex HeLa cells expressing (+DOX) or not (-DOX) GNL3-FLAG were sequentially labelled for 30 mins with IdU and for 30 mins with CldU then treated with 5 mM HU for 240 min. The ratio between CldU and IdU is plotted, the red line indicates the median. An average of 3 independent experiemtns is represented. For statistical analysis Mann-Whitney test was used; ****p<0.0001

5- Sensitivity of GNL3 Depleted Cells to Chemotherapeutic Drugs

GNL3 depleted cells are more sensitive to hydroxyurea (Lin et al., 2014). In order to reproduce these results with a variety of other chemotherapeutic drugs and we assessed the survival rate of GNL3 depleted cells using colony forming assay and CellTiter-Glo upon different treatments. Using colony forming assay (Figure 28A) we could confirm that the depletion of GNL3 decreases the ability of cells to form colonies (Figure 28B), indicating either the decrease of the proliferative capacity of these cells or their death by apoptosis.

Control and GNL3 depleted cells were also challenged with increasing concentrations of hydroxyurea (HU), etoposide (ETP), and camptothecin (CPT) for 24 hrs. and then cultured at low concentration and monitored them for their capacity to form colonies. Treatment with CPT had a catastrophic effect on the survival of HeLa S3 cells; therefore, no useful information could be concluded from comparing the control to GNL3 depleted cells. Upon treatment with HU, GNL3 depleted cells showed a slight decrease in the number of colonies formed after treatment with 100 µM for 24 hrs. (87% vs 73%); however, as the concentration increased, the number of colonies formed was similar in both control and GNL3 depleted cells (Figure 28C1). On the other hand, treatment with ETP showed a stronger impact on the ability of cells to form colonies in the absence of GNL3 (Figure 28C2). It is important to keep in mind that the depletion of GNL3 in basal conditions results in less colonies; therefore, the real sensitivity level of GNL3 depleted cells might be masked by the fact that the cells that survived to form colonies are the ones that had low or mild depletion levels.

To answer this possibility, we performed CellTiter-Glo assay exactly after 72 hrs. of GNL3 depletion where the cells were treated for 48 hrs. (Figure 28D1). GNL3 depleted cells showed the same sensitivity to HU and ETP (Figure 28D2,D3), but they were more sensitive to CPT (Figure 28D4). This led me to conclude that although these drugs might induce DNA resection within short period of treatments in GNL3 depleted cells, the real effect on the survival is not translated directly; however, it takes several cellular cycles to really see the effect of GNL3 depletion. This suggestion is consistent with the fact that GNL3 depleted cells harbor higher levels of 53BP1 foci in response to replication stress

(Yamashita et al., 2013), which indicates accumulation of lesions from the previous cell cycles.

Since we have described a role of GNL3 in regulating origin firing, I asked what would be the effect of combining GNL3 depletion with another drug that increases origin firing, such as Wee1 or ATR inhibitors on cellular proliferation. As previously described, Wee1 kinase plays an important role in cell cycle progression, especially at G2/M transition and origin firing regulation during S-phase (Beck et al., 2012). Moreover, Wee1 inhibitor (AZD1775) is in clinical trial for its antitumor effect on cancer cells where it was purported to potentiates chemotherapeutic drugs by modulating DNA damage response (Ha et al., 2020). Interestingly, treating GNL3 depleted cells were more sensitive to Wee1i (Figure 28D5). This could be due to the fact that GNL3 depleted cells may accumulate DNA lesions and possibly under-replicated regions that in the absence of Wee1 would slip through G2/M transition, thus accumulating as the cells are proliferating, leading eventually to apoptosis. However, this could also be explained by the fact that DNA lesions appearing in the absence of GNL3 might not be repaired in the absence of Wee1, therefore leading to increased sensitivity. On the other hand, treating with an ATR inhibitor (ve-821) increased cellular proliferation that would increase furthermore upon GNL3 depletion (Figure 28D6). One of the possible explanations could be the excess of origin firing when both conditions are combined therefore leading to a shorter period of S-phase, and eventually faster cellular proliferation.
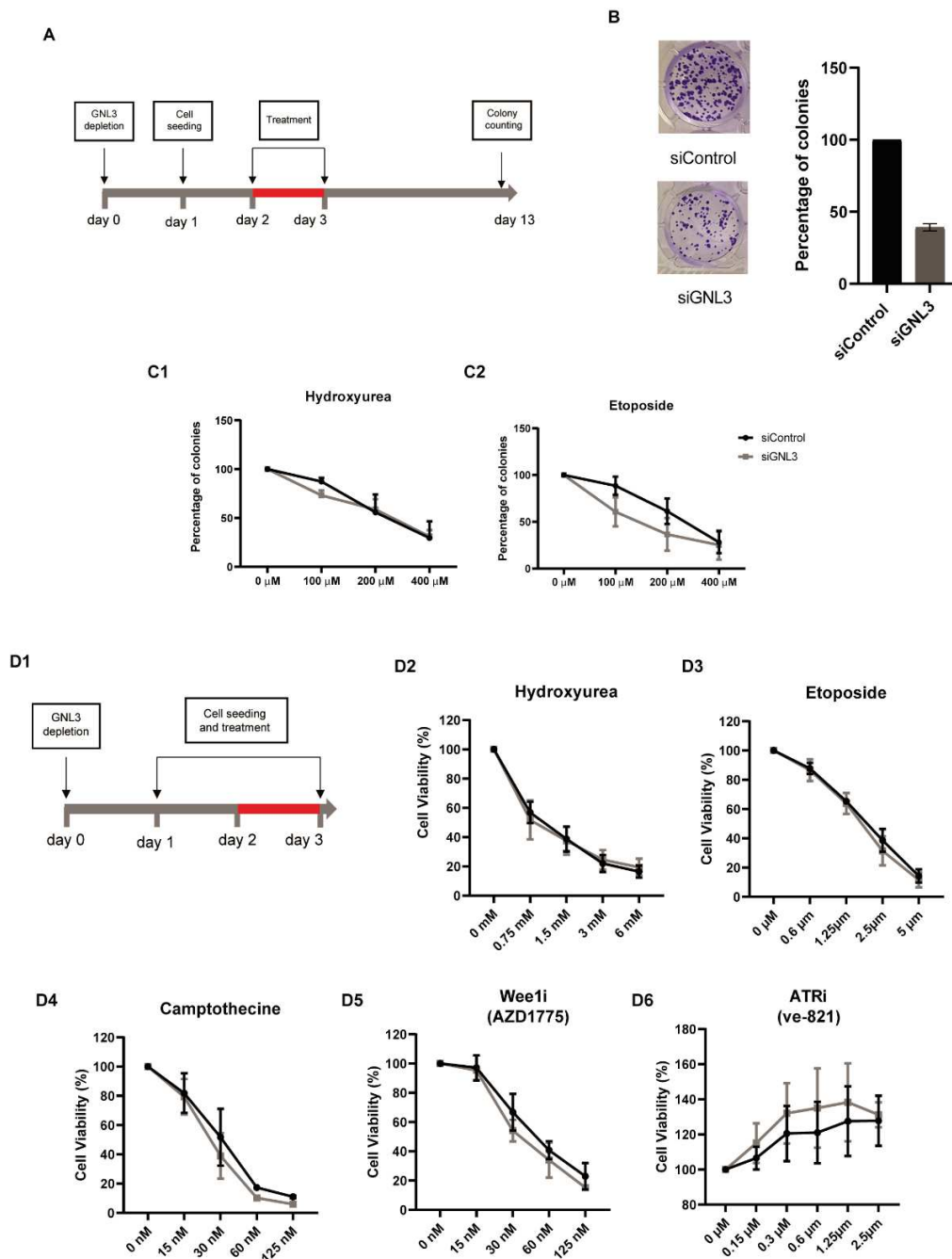
Figure 28. Sensitivity of GNL3-depleted cells to different chemotherapeutic treatments. **A.** Experimental set-up of sensitivity testing using Colony formation Assay. **B.** Graphical representation of the percentage of number of colonies formed in control and GNL3 depleted cells after 13 days of depletion. **C.** Graphical representation of clonogenic survival of control (black) and GNL3 depleted (grey) cells treated with hydroxyurea (**C1**) or etoposide (**C2**) with the indicated concentrations. **D. (D1)** Experimental set-up of cellular viability testing using Cell-Titer Glo Assay. Cellular viability was measured for Control (black) and GNL3 depleted cells (grey) upon increased concentrations of hydroxyurea (**D2**), etoposide (**D3**), camptothecin (D4), Wee1i (**D5**) and ATRi (**D6**). Y-axis shows the relative survival compared with the no-drug**.** All the values correspond to three independent experiments.

6- GNL3 GTP Binding Activity Is a Key Regulator of GNL3 Level

The first attempt to try to understand GNL3 function in accordance with its localization was described by Tsai and Mckoney where they found that the GTP binding activity is essential for GNL3 localization within the nucleolus and in limiting its functions in the nucleoplasm that would perturb DNA replication (Tsai and McKay, 2002). In order to study whether the role of GNL3 in regulating origins and protecting the stalled forks integrity is related to its GTP binding activity, I used the same HeLa Flp-In system described previously to express a FLAG tagged double mutant (G261V and G266V) GNL3 that is not able to bind to GTP. These two mutations where described earlier for disturbing the nucleolar localization of GNL3 (Tsai and McKay, 2005). In this system, the endogenous GNL3 is depleted and an exogenous mutant (GNL3-RGG) which is resistant to the siRNA depletion, is expressed. Upon 16 hrs. of Doxycycline induction, we could detect a very low level of GNL3-RGG when compared to the same condition used to induce the WT (Figure 29A). According to literature, GNL3-RGG is unstable and is subjected to proteasomal degradation resulting in a very low level of GNL3 that would reside in the nucleoplasm (Huang et al., 2009; Lo et al., 2012). However, upon inhibiting the proteasomal activity with MG132, GNL3-RGG is protected from degradation and it restores its nucleolar localization. I followed the same strategy in order to increase the levels of GNL3-RGG in our cellular model. First, I optimized the concentration of MG132 by which GNL3-RGG is stabilized. I found that treatment with 10 µM for 6 hrs. after 16 hrs. of DOX induction was enough to stabilize GNL3-RGG (Figure 29B lower band of FLAG). I validated this by FLAG Immunostaining, and we could detect its signal upon DOX induction that would increase in the presence of MG132 and somehow re-localize into the nucleolus (Figure 29C, arrows). However, I could not detect the signal of GNL3-RGG using an antibody against GNL3, which could be caused by the change in its confirmation. In order to study whether this mutation affects the new functions of GNL3 that have been characterized, I first started by testing if GNL3-RGG is still able to be in proximity of the replisome. To answer this, we performed PLA between GNL3-RGG(FLAG) and EdU and compared it to the binding of GNL3-WT. We saw that the number of PLA foci in both GNL3-WT and GNL3-RGG were approximately the same (Figure 29D), indicating that the mutation did not

abolish the ability of GNL3 to be associated with the replisome. The other aspect I studied was GNL3-RGG ability to rescue the DNA resection phenotype observed in the absence of GNL3 when cells are challenged with replication stress. I performed the same strategy than previously and sequentially labeled the cells with IdU and CldU (30 minutes each) and challenged them with etoposide for 2 hrs.. The CldU/IdU ratio indicated that GNL3-RGG was able to rescue the DNA resection (Figure 29E). Therefore, I conclude that the key point required for GNL3 function is not its GTP binding ability but its cellular level. Other domains are likely to be responsible for these functions that are yet to be discovered.
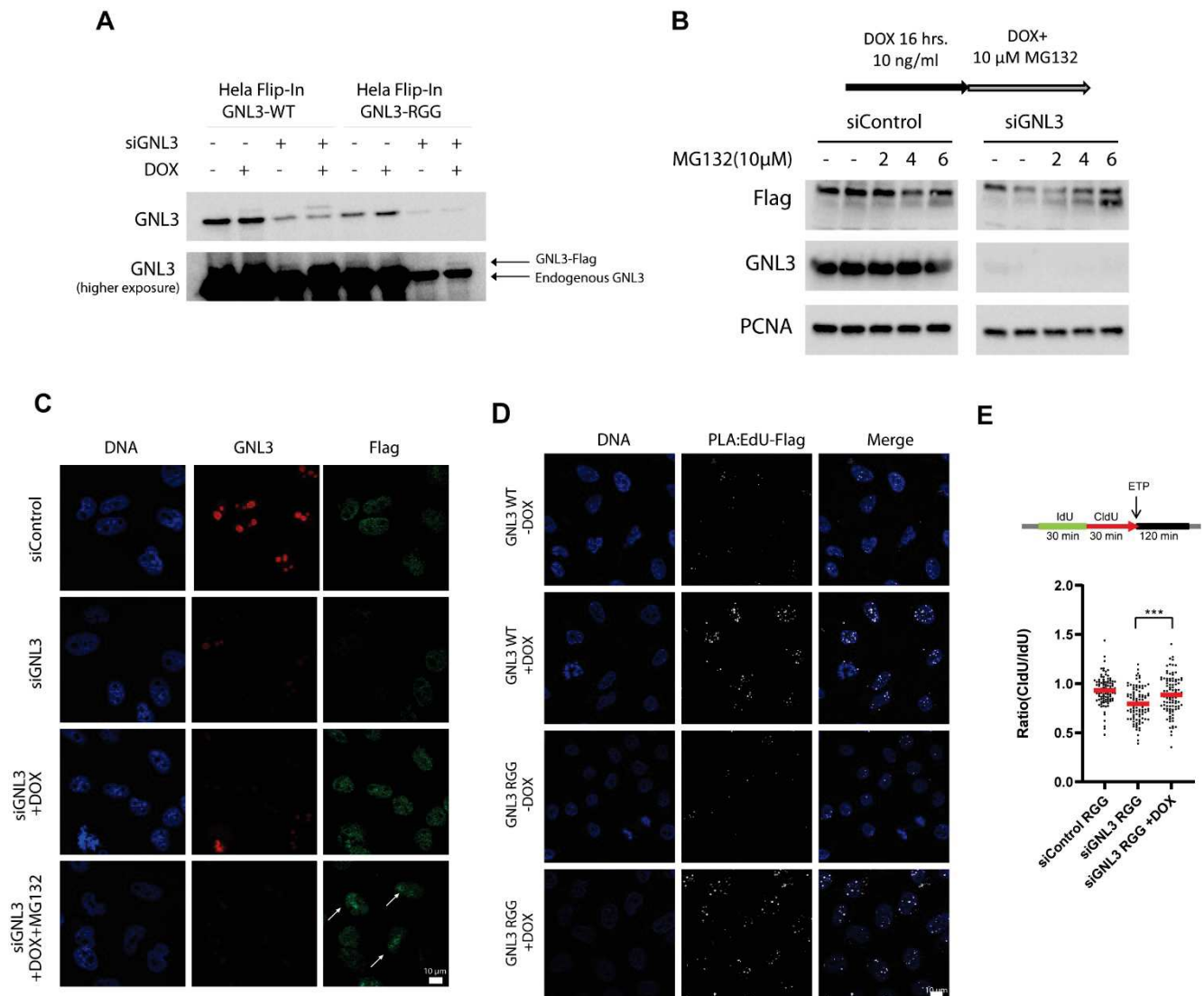
Figure 29. Characterization of GNL3 GTP binding mutant (RGG). A. Western Blot analysis of total cell extracts. Control and GNL3 depleted HeLa Flp-IN cells were induced for GNL3-WT or GNL3-RGG expression by Doxycycline (10 ng/ml for 16 hrs.), collected and analyzed for their levels of endogenous and exogenous level of GNL3 expression. **B.** Western Blot analysis of total cell extracts. Control and GNL3 depleted HeLa Flp-IN cells were induced for GNL3-RGG expression by Doxycycline (10 ng/ml for 16 hrs.) then with increasing time point of MG132 (10μM) treatment, collected and analyzed for their levels of exogenous GNL3 expression using anti-FLAG, endogenous GNL3 using anti-GNL3, and for PCNA. **C.** Double immunofluorescence staining of HeLa Flp-IN cells induced for the expression of GNL3 RGG by Doxycycline with or without MG132 treatment. Antibodies against GNL3 and FLAG were used. DNA was counterstained with Hoechst-33342 (blue). **D.** PLA (proximity ligation assay) analyzing the proximity between EdU and FLAG. Scale bar, 10 μm. **E.** HeLa Flp-In cells were induced for expressing GNL3-RGG, sequentially labeled for 30 mins with IdU and for 30 mins with CldU then treated with 10 μM Etoposide for 120 mins. The ratio between CldU and IdU is plotted, the red line indicates the median. For statistical analysis, Mann-Whitney test was used; ****p<0.0001.

Extra Materials and Methods

This part includes descriptions of experimental procedures that were not included in the submitted manuscript.

1- Pulse Field Gel Electrophoresis

Cells were grown to 40-50% confluency then collected with trypsinzation and washed once with PBS. 10^6 cells were then melted in 0.5% agarose insert. These inserts were then incubated in lysis buffer (100 mM EDTA pH8, 0.2% sodium deoxycholate, 1% sodium 1% sodium lauryl sarcosine, 1 mg/ml Proteinase K) at 37 °C for 48h and then washed 2-4 times with wash buffer (20 mM Tris pH 8, 50 mM EDTA pH 8) before loading onto a 0.9% agarose prepared in 0.25X TBE. Chromosomes were separated by pulsed-field gel electrophoresis for 24 h (Biometra Rotaphor 8 System, 23h; interval: 30-5 s log; angle: 120-110 linear; voltage: 180-120 V log, 13°C). The gel was subsequently stained with ethidium bromide for analysis.

2- Colony Forming Assay

Cells were subjected to siRNA depletion and treated with different DNA damage inducing reagents for 24 hrs. then harvested, counted and seeded at a density 200 cell/well in 12 well plates. Cells were then incubated at 37 °C for 1-2 weeks then fixed with 100% methanol then incubated with crystal violet (0.5% crystal violet in 25% methanol) for 20 mins, washed with water and left to dry. Crystal violet was then solubilized with 10% acetic acid. The obsorbance was finally measured at 570-595.

Discussion and Perspectives

The aim of this study was to discover new proteins associated to the replisome and their role in unperturbed DNA replication and during replication stress. For this aim, an iPOND-based mass spectrometry was performed previously in my lab in order to discover new candidates. In this screen, around 25 new candidates were found to be enriched in the vicinity of replication forks. Using the validation screen described previously in the results section, we found that GNL3 was the most promising candidate.

The most common characteristic between stem cells and cancer cells is their ability to proliferate and expand. A variety of proteins that are essential for the proliferative capacity of stem cells are re-expressed when cells undergo malignant transformation, such as OCT4, SOX2 and NANOG (Zhao et al., 2017). GNL3, a GTP binding protein, is also found to be highly expressed in both stem and cancer cells where it plays a role in inducing the characteristics of tumor initiating cells (Lin et al., 2010; Okamoto et al., 2011). GNL3 was shown to be crucial for the proliferative capacity of cancer and stem cells, and for the maintenance of the genomic integrity (Lin et al., 2013; Meng et al., 2013; Rosby et al., 2009). Several studies have reported the occurrence of spontaneous DNA lesions upon depletion of GNL3. This was demonstrated by the increase of DNA damage markers such as γH2AX and ATR, and also by a higher level of DSBs (Lin et al., 2013, 2019; Meng et al., 2013; Wang et al., 2020; Yamashita et al., 2013). It was established that GNL3 would maintain the genomic integrity by recruiting RAD51 to DSBs and damaged telomeres through its interaction with TRF1 (Hsu et al., 2012; Meng et al., 2013). However, this explained how GNL3 might contribute to repairing DSBs but not how the absence of GNL3 would cause spontaneous lesions in the first place. By revealing that GNL3 is associated with the replisome, I have uncovered the first thread that could explain how exactly GNL3 is implicated in maintaining the genomic integrity of replicating cells.

1- GNL3, a fork accelerator or a regulator of origin firing?

In this report I have demonstrated the first evidence that GNL3 is associated with the replication fork using the iPOND method. This association was validated by PLA EdU-GNL3, a method I also used to show that GNL3 is associated with the replication forks throughout S-phase. Although this finding led me to characterize a novel role of GNL3 in maintaining the genomic stability, I could not address directly the role of GNL3 at replication forks.

The two main phenotypes observed upon GNL3 depletion were the decrease in fork velocity and the increase in origin firing efficiency. While on the other hand, the overexpression of GNL3 led to a decrease in the origin firing efficiency. This finding could be explained by either two hypothesizes.

Hypothesis I: GNL3 a fork accelerator

The first hypothesis suggests that GNL3 is associated with the replisome and functions as a fork accelerator, and that explains why its absence decreases the replication fork velocity. Therefore, in order to compensate the decrease in fork speed, indirect augmentation in the origin firing efficiency would take place. By definition, a fork accelerator can be a protein that overcomes or clears barriers facing replication forks during DNA replication. This hypothesis is very likely to be true, however there are several facts that would argue against it.

First, experiments with short treatments of CPT indicated that depletion of GNL3 does not increase the impact of replicative stress on fork progression. This indicates that GNL3 is not required to remove or overcome the impediments imposed by CPT, otherwise the effect of CPT should have increased the level of fork stalling in absence of GNL3. One good example to compare with is PrimPol, a protein participating in the repriming pathway (replication stress tolerance pathway described previously). PrimPol facilitates the fork progression through endogenous stress such as G-quadruplex (Schiavone et al., 2016) and exogenous ones such as UV (Bianchi et al., 2013). It was shown that depletion of PrimPol would decrease the fork speed and as a consequence would increase the origin firing efficiency (Rodriguez-Acebes et al., 2018). However, unlike GNL3, depletion of

PrimPol increases the effect of replication stress induced by UV on the progression of replication forks.

Second, I have showed that GNL3 overexpression induces DNA resection upon replication stress. If GNL3 was able to accelerate replication forks movement by removing obstacles facing the replisome, one might expect that overexpression of GNL3 would maintain the replication fork intact in presence of replication stress instead of having DNA resection. However, these results could be interpreted differently, where it remains possible that GNL3 is bypassing replication fork impediments that can be processed after the passage of the fork (post-replicative repair).

Third, according to our mass spectrometry data, the number of GNL3 molecules associated with the replisome is less than these of canonical replication proteins such as PCNA and DNA polymerases. This suggests that there is not one GNL3 molecule per replisome, and logically the amount of fork accelerator should be the same than known fundamental components of the replisome.

In conclusion, regardless of these arguments, it remains possible that GNL3 is functioning as a fork accelerator, and this would explain why it is associated with the replication forks. In order to address this question, the group of Juan Méndez previously described an experimental strategy that can be performed which utilizes an inhibitor of origin firing such as CDC7 inhibitor or CDK inhibitor (Figure 30) (Rodriguez-Acebes et al., 2018). In principle, if GNL3 depletion mainly affects the fork speed, the addition of an inhibitor of origin firing should not rescue the defect in fork speed. However if the decrease of fork speed is a consequence of increased origin firing, then the addition of an inhibitor should restore the original fork speed. Performing this experiment would help us to solve the ''chicken and egg'' problem between the origin activation and fork speed.
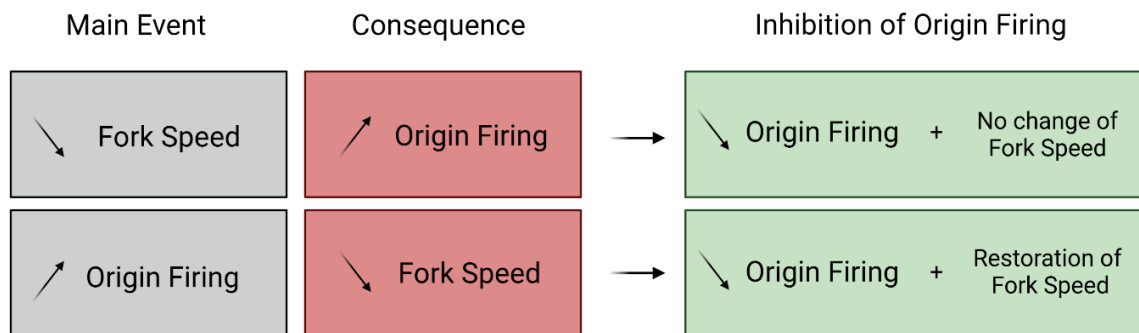
Figure 30. Experimental strategy to explore the role of GNL3 as fork accelerator.

Hypothesis II: GNL3, a protein implicated in the regulation of origin firing

The second hypothesis, which I supported in my project, would be the implication of GNL3 in the regulation of origin efficiency. Here, I would suggest that the first outcome of GNL3 depletion is the increase in origin firing and consequently the velocity of the replication fork is decreased. Such a mechanism was described for Chk1 inhibition or depletion for example where its inhibition leads to firing of dormant origins, and as a compensation mechanism the fork velocity decreases (Petermann et al., 2010). There are several reasons why I would support this hypothesis.

First, and most importantly I proved that GNL3 is in proximity of ORC2, one of the origin recognizing proteins. Although we could not detect other ORCs in proximity of GNL3, the interaction with ORC2 is significant enough since it has a dual role in regulating origin firing and the chromatin state (Huang et al., 2016; Pak et al., 1997; Prasanth et al., 2010) all of which are affecting the efficiency of replication origins directly or indirectly. Second, it was shown that overexpression of GNL3 is synthetically lethal with the Cdc7 inhibitor PHA-767491 (Wang et al., 2020). I have shown that GNL3 overexpression decreases origins firing efficiency, therefore it is possible that inhibition of origins firing using Cdc7 inhibitor induces lethality due to the defect of firing of cells overexpressing GNL3. Moreover, it was reported that GNL3 depletion increases the number of cells with more than 2N DNA content (Wang et al., 2020). Cells with increased DNA content are considered to undergo re-replication such in cases where Geminin (the negative regulator

of Cdt1) is depleted (Melixetian et al., 2004). This could explain why I observed more origin firing in absence of GNL3. However, using HeLa S3 I could not reproduce this finding which could be mainly due to the basal level of re-replication and polyploidy that those cells undergo.

In this report I used HeLa S3 cells to characterize the role of GNL3. Knowing that HeLa cells harbor inactive p53 (Ajay et al., 2012), we have avoided any misleading effects of GNL3 depletion on the p53 pathway. Moreover, it would help to understand the role of GNL3 independently of p53, especially because several reports indicate the presence of p53-independent phenotypes resulting from GNL3 depletion. Inconsistent with previous results, depletion of GNL3 in HeLa S3 did not result in any cell cycle arrest. However, I validated the inability of GNL3 depleted cells to form colonies (Yamashita et al., 2013), which was consistent with the fact that the GNL3 knockouts I tried to generate using CRIPS-Cas9 were not viable. Thus, furtherly validating that GNL3 is important for cellular proliferation.

It was reported that GNL3 depletion increases the level of DSBs, the level of ATR and RPA and γH2AX foci (Lin et al., 2013, 2019; Meng et al., 2013; Wang et al., 2020; Yamashita et al., 2013). In our study we have confirmed that GNL3 depletion increases the level of DSBs. The question that was not fully addressed before, is why GNL3 depletion would lead to DSBs. I propose that in absence of GNL3, the excess of origin firing results in an excess of replication forks that leads to the decrease availability of limiting factors such as RPA and dNTP pool shortage,thus rendering the forks more prone to breaks (Petermann et al., 2010; Toledo et al., 2013). A similar mechanism was described for inhibition of Wee1 that increases the firing of replication origins and leads to an increase in SLX4/MUS81-dependent DSBs formation that could be rescued by addition of dNTPs (Beck et al., 2012).

2- *Possible mechanisms by which GNL3 is regulating origin firing*

RIF1 is a protein implicated in determining the replication timing in human cells (Yamazaki et al., 2012). RIF1 was shown to regulate higher-order chromatin architecture including special organization of chromatin loops by which it limits the accessibility of replication initiation factors. Depletion of RIF1 increases origin firing with a specific loss of

mid S-phase patterns and changes in replication timing. While characterizing the role of GNL3 during unperturbed S-phase, I found that GNL3 depletion leads to an increase in the mid S-phase pattern in non-synchronized conditions and two hours after the release from thymidine block. I hypothesized that this could be due to a change in the replication timing, and therefore would explain why we have deregulation in origin firing, a similar situation than the one described for RIF1 impairment. However, GNL3 depletion did not induce a significant change in the timing of DNA replication. Yet I cannot exclude that there might be a subtle change in the replication timing that is masked by the heterogeneity of replication timing between individual cells. To definitely address if GNL3 depletion has an effect on replication timing (even if it is a subtle one), the measurement of replication at single cell level should be performed. This would help us answer the question of whether GNL3 is implicated in the replication of early or late domains and would explain the change in replication pattern that I observed.

Another possibility would be the implication of GNL3 in origin firing through all S-phase. It is known that the density of licensed origins is much higher in early replicating regions than in late ones (Miotto et al., 2016); thus, one might speculate that the effect of GNL3 depletion on the regulation of origin firing might be stronger in early replicating domains. That might lead to faster replication during early S-phase and might explain the enrichment of mid S-phase patterns. In support of this, the general increase in origin firing reported upon ATR inhibition did not change the timing of replication domains (Moiseeva et al., 2019), but it is unknown whether it affects the S-phase replication patterns. However I could not obtain clear evidence supporting this hypothesis.

Interaction between GNL3 and ORC2

In order to dissect the possible mechanism by which GNL3 may be regulating the efficiency of origin firing, I performed a mass spectrometry screen based on the technique of BioID. In the list of proteins in proximity, there was a great number of nucleolar proteins, reflecting the major localization of GNL3. However, some of the replisome components were recapitulated such as MCMs, RFC and polymerases. Interestingly, ORC2 was found in close proximity with GNL3, but not other components of the ORC complex. I have validated this finding using other approaches (PLA and IP). Since I hypothesized that

GNL3 is implicated in the regulation of origin firing, it was puzzling to find only ORC2 in proximity. However, this was consistent with the fact that we could not detect an enrichment of GNL3 on known replication origins using chromatin-immunoprecipitation (ChIP). It is also known that ORC2 itself has independent functions in maintaining the genomic integrity. I tried to understand the functional meaning of this proximity. One of the interesting observations was the fact that there is no overlap between the GNL3 and ORC2 chromatin binding sites. This tells us that either the interaction is very limited on chromatin or it does not occur on it. It is also possible that we could not detect any overlap since ORC2 ChIP-seq was not performed in HeLa S3 cells (Miotto et al., 2016). We attempted to ChIP-ORC2 in HeLa S3 but we failed to obtain the optimal conditions for this experiment. Interestingly, using PLA, we could detect that the interaction between GNL3 and ORC2 is occurring mainly inside or at the borders of the nucleolus. Moreover, when comparing GNL3 and ORC2 interactors, the proteins I found in common were the ones that resided mainly in the nucleolus. Furthermore, I observed that this interaction is maintained during G1 and S-phase and that it peaked mostly at the G2/M border by using different inhibitors.

My first hypothesis was that GNL3 would be required for sequestering ORC2 in the nucleolus to limit its concentration in the nucleoplasm and therefore regulate the number of origins that are licensed. Indeed, I observed that the level of ORC2 in the nucleolus was lower by 25% when GNL3 was depleted. However, it is not convincing that this would be a sequestration mechanism since the majority of ORC2 is still localized within the nucleoplasm.

It was reported that GNL3 is important for the maintenance of heterochromatin at centromeres and transposons (Maida et al., 2014). For this function, GNL3 interacts with the human TERT (hTERT) and Brahma-related gene 1 (BRG1) forming the TBN complex. This complex produced double-stranded RNAs homologous to centromeric alpha-satellite (alphoid) repeat elements and transposons that were processed into small interfering RNAs targeted to these heterochromatic regions to maintain their silencing. Moreover, CENP-A, a centromere-specific histone H3 variant, showed proximity with GNL3 during DNA replication (Zasadzińska et al., 2018).

On the other hand, a subset of ORC2 also localizes to the centromeric region throughout the entire cell cycle independently from the other ORCs (Prasanth et al., 2004). It was shown that ORC2 recruits and maintains HP1 to centromeric regions through which it participates in the heterochromatin formation. Moreover, it was reported that SUMOylated ORC2 is important for the recruitment of KDM5A that converts H3K4me3 to H3K4me2, which favors α-satellite transcription at the centromere (Huang et al., 2016). The transcript coming from this region is crucial for condensation of pericentric heterochromatin by which DNA re-replication is inhibited and genomic stability is maintained.

Given these findings, I would hypothesize that GNL3 may be recruiting ORC2 into the centromeric regions, where it functions in regulating heterochromatin formation by which it maintains genomic integrity (Figure 31). Several arguments support my hypothesis. First, GNL3 and ORC2 do not interact at replication origins, and both are crucial for maintaining the heterochromatin structure at centromeric and pericentromeric regions. Second, their interaction occurs mostly at the border of the nucleolus, and it was shown that centromeric regions are mostly anchored to the nucleolar regions (NADs). Third, I have proved that the signal of ORC2 within the nucleolus decreases upon GNL3 depletion. And fourth, GNL3 was reported to recruit SUMOylated TRF1 along with PML IV to telomeres. And since GNL3 is predicted to have a strong SUMO-interacting motif at the intermediate domain (328-332), we suspect that GNL3 might interact with SUMOylated ORC2 at centromeric regions.

Moreover, centromeric regions are known to replicate in mid/late S-phase. Therefore, if GNL3 and ORC2 where to maintain the stability of this regions, it would explain why GNL3 depleted cells are enriched in mid S-phase patterns (since they spend more time to replicate this region). It is also possible that the resection detected upon the addition of exogenous replicative stress could occur in cells that are struggling to replicate these regions.
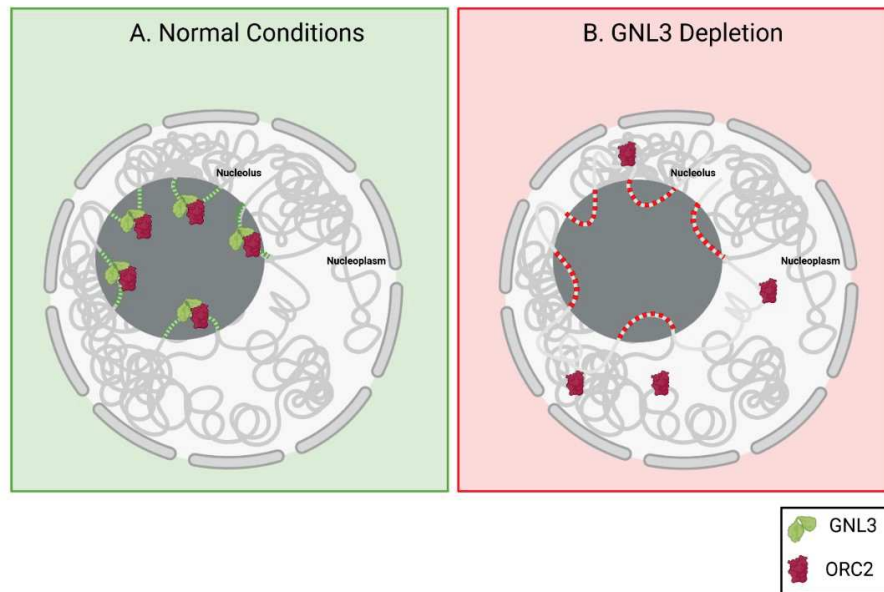
Figure 31. Hypothetic mechanism for GNL3 and ORC2 interaction. (A) In normal conditions, GNL3 is implicated in the recruitment of ORC2 to the centromeric regions, where it functions in maintaining the heterochromatin status by which it limits replication origins and maintains genomic stability. (B) Upon GNL3 depletion, ORC2 is no longer recruited to the centromeric regions thus impairing centromeric heterochromatin silencing, which resultes in re-replication or increased origin firing in heterochromatin DNA leading to genomic instability.

In order to explore this hypothesis, several experimental approaches can be performed. The key experiment that would validate our hypothesis would be to perform ORC2 ChIP-Seq in control and GNL3-depleted cells. Ideally, if my hypothesis is valid, ORC2 recruitment to centromeric regions, or possibly to other regions of heterochromatin, should be impaired upon GNL3 depletion. It was reported that ORC2 is SUMOylated by the SUMO ligase PIAS4 (Wang et al., 2017a) and that a mutation in the K36 and 51R would inhibit its SUMOylation (Huang et al., 2016). Therefore, to test if this interaction is dependent on the SUMOylation of ORC2, we can either deplete PIAS4 or generate an ORC2 mutant to test if GNL3 and ORC2 still interact. This would also answer whether GNL3 is implicated in ORC2 recruitment of KDM5A, by which it maintains the genomic stability.

 If we were able to prove that ORC2 recruitment is impaired by GNL3 depletion, more detailed experiments should be performed such as looking for DNA methylation profiles in absence of GNL3 especially within α-satellite using ChIP-PCR and testing for chromatin

organization using chromosome conformation capture (Hi-C) for example. Finally, we would address the effect of GNL3 depletion on mitosis, such as analyzing the chromosome structures using metaphase spreads and analyzing features of mitotic cells using immunofluorescence to look for the shape of mitotic cells and the centromeres.

However, in this model it remains unknown why GNL3 would be associated with the replication forks throughout S-phase. One possible mechanism that would require intensive exploration is the possible role of GNL3 is the organization of replication factories, since it seems to have a structural role such as in the nucleolar architecture (Romanova et al., 2009b). One possible strategy to answer this hypothesis would be to analyze the proximity of cohesins to replication factories using PLA, for example, or chromosome conformation capture methods such as Hi-C. It is also possible that GNL3 maintains genomic stability by being implicated in two different processes, one that ensures the proper regulation of origin firing and another that signals endogenous or exogenous stress encountered by the replisome. However, these two functions might not be mutually exclusive, similarly to the case of ATR and TIMELESS, for instance. In *E.Coli*, *obgE* which is a GTP binding protein has been implicated for the correct DNA replication in basal conditions and during replication stress (Foti et al., 2005). This suggests that GTP binding protein like GNL3 may play a broader role in the control of DNA replication. Future work using separation of function mutants of GNL3 will be required to validate this possibility.

3- The level of GNL3 is crucial for the genomic integrity

GNL3 is expressed during the early stages of embryonic development; afterwards its expression ceases as cells are undergoing differentiation. During malignant transformation GNL3 expression is resorted probably due to Myc transcriptional activity as it was reported previously (Zwolinska et al., 2012). However, unlike other oncogenes, GNL3 levels should be maintained within a specific range, otherwise very low or very high levels would lead to a decrease in the cellular proliferation (Zhu et al., 2006). Overexpression of GNL3 was previously reported to induce cell cycle arrest and therefore inhibit proliferation by stabilizing the level of p53 (Dai et al., 2008; Meng et al., 2008). In

another study, it was shown that, independently of p53, overexpression of GNL3 leads to an accumulation in late S/G2-phase. One of the conclusions in this study was that GNL3 gain of function phenotypes is achieved only by an optimal level of expression; however, the mechanism of action was not understood.

In this study, I showed that GNL3 overexpression results in a decrease in origin firing. This result could explain the phenotypes described above. I would suggest that due to the low levels of replication origin firing, the cells start to accumulate under-replicated DNA that will cause the cells to pause at G2/M and undergo mitotic DNA synthesis. This eventually will increase the genomic instability and cellular senescence/death, which explains why overexpression of GNL3 is synthetic lethal with Cdc7 inhibition.

Not only the level, but also the localization of GNL3 was described to be important for its proper function (Tsai and McKay, 2005). After discovering the possible role of GNL3 in the regulation origin firing, I asked whether this function requires it's nucleoplasmic or nucleolar localization. For that, I constructed a GNL3 mutant with two mutations (G261V and G266V) in the GTP binding domain that prevents its localization in the nucleolus. However, while trying to express this mutant in our system, I reproduced the other aspect of this mutation which makes GNL3 susceptible to proteasomal degradation (Huang et al., 2008; Tsai and McKay, 2005). To overcome this problem, I inhibited the proteasomal activity using MG132 in order to stabilize the mutant. I found that the mutant was still able to associate with the newly synthesized DNA. Moreover, the mutant was able to protect stalled forks from DNA resection when cells were challenged with etoposide. These observations indicate that the GTP binding activity is protecting GNL3 from degradation andthat it is the key mechanism to regulate the proper level of GNL3 in the cell. In support of this argument, it was reported that the high level of GNL3 is accompanied with high levels of GTP (Uema et al., 2013), which is probably how these levels are stabilized. Therefore, I conclude that the GTP binding domain of GNL3 is responsible for regulating the level but not the molecular activity I have uncovered in this study. Additional deletions or mutations must be performed in order to define which domain is implicated in this regulatory function.

4- GNL3 is crucial for the protection of stalled forks

One of the main aims of this project was to characterize how the newly discovered candidates are implicated in maintaining the genomic integrity in the presence of replication stress. As noted before, we have selected GNL3 based on the high level of γH2AX produced upon CPT treatment when it is depleted from the cell. Therefore, GNL3 must be implicated in the replication stress response. Depletion of GNL3 did not increase the effect of CPT on replication forks and did not result in a change in the level of phosphorylation of neither Chk1 (S345) nor Chk2 (T68). Moreover, upon prolonged periods of replication stress that were induced by either HU, ETP, or CPT, the level of Chk1 phosphorylation did not vary in GNL3 depleted cells. However, I detected an increase in the levels of RPA phosphorylation (S33 and S4/8) in GNL3 depleted cells which reflected the nascent DNA resection occurring at the same conditions. Therefore, I concluded that GNL3 functions as a fork protector.

The real challenge was to understand how GNL3 would be protecting these stalled forks. The first step to understanding how it may protect stalled forks was to test whether it is enriched at stalled forks. I found that GNL3 dissociates completely from HU stalled forks as PCNA does, while RAD51 accumulates. According to literature, GNL3 is required for recruitment of RAD51 to DSBs (Meng et al., 2013). Therefore, one possible explanation could be that GNL3 depletion leads to impairment of RAD51 recruitment, therefore inducing DNA resection. However, if GNL3 is required for RAD51 recruitment to stalled forks, the overexpression of GNL3 should not have led to DNA resection as well. Moreover, I validated with chromatin fractionation that the recruitment of RAD51 to chromatin in the presence of HU is not impaired upon GNL3 loss. Therefore, I conclude that GNL3 is not likely to be required for the recruitment of other fork protectors even though I have not tested all of them.

ATR and Wee1 were both described for maintaining the proper number of origins firing during unperturbed S-phase. If these kinases are inhibited, extra origins are fired and in the presence of replication stress further dormant origins are firing to rescue the stalled forks (Beck et al., 2012; Moiseeva et al., 2019; Toledo et al., 2013). This will result in an increased number of replication forks that exceed the available pools of dNTPs, RPA, and

other possible protectors, leading eventually to replication catastrophe. In this study I have proved that these catastrophic events are linked to DNA resection. Moreover, it was shown that Cdc7 inhibition prevents those catastrophic events (Toledo et al., 2013). I could also prove that Cdc7 inhibition was able to rescue the DNA resection phenotype.

Since I showed that in absence of GNL3 there is an increase in the origin firing efficiency, I hypothesize that the presence of replication stress would increase this number furtherly leading to the same phenomena of the exhaustion of replication factors/protectors and replication catastrophe.

To prove this, I showed that inhibition of Cdc7 during replication stress rescues the DNA resection caused by GNL3 depletion and decreases the levels of RPA phosphorylation. It was reported recently that Cdc7 is implicated in activating Mre1; therefore, Cdc7 inhibition could prevent DNA resection by inhibiting Mre11. To make sure that the results I obtained using Cdc7 inhibitors are not caused by the inhibition of Mre11, I performed the same experiment using BRCA1 depletion as a negative control. It is well demonstrated that BRCA1 is involved in the protection of stalled forks against resection (Chaudhuri et al., 2016), however it has no implication in regulation of origin firing. Therefore, if Cdc7 inhibitor is rescuing DNA resection by the inhibition of origin firing and not Mre11 activity, it should not rescue the DNA resection resulting from BRCA1 depletion in the presence of replication stress. Our experiment showed that Cdc7 inhibition didn't rescue the DNA resection. Therefore, I was able to conclude that inhibition of origin firing is what rescued DNA resection seen in the absence of GNL3, ATR, and WEE1.

On the other hand, I have proved that overexpression of GNL3 leads to DNA resection in response to replicative stress. I hypothesize that this could be due to the fact that there would be less origins to rescue the stalled forks. In support of this hypothesis, it was found that the downregulation of MCMs does not affect the genomic stability unless the cells are subjected to replication stress, which is due to the absence of back-up origins (Ibarra et al., 2008).

In conclusion, I present a new mechanism by which GNL3 is affecting the regulation of the origin firing efficiency. A mechanism that is essential to maintain the genomic integrity during unperturbed replication and during replication stress (Figure 32).
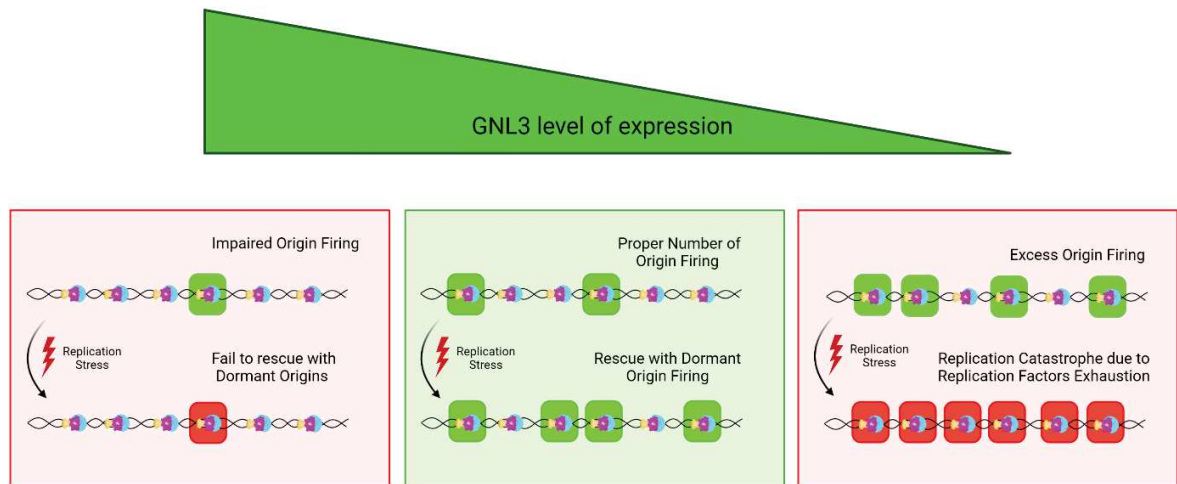
Figure 32. GNL3 maintains the genomic stability during replication stress by fine-tuning the level of replication origin firing. High levels of GNL3 induce a decrease in origins firing efficiency that upon replication stress is leading to replication catastrophe due to the failure to activate dormant origins. On the contrary, low levels of GNL3 lead to an increase in origin firing efficiency, during replication stress extra dormant origins will fire that would eventually lead to replication catastrophe due to exhaustion of replication factors. The level of expression of GNL3 must be maintained within a specific range that would result in the proper number of origins fired that would maintain the genomic stability in case of replication stress.

Conclusion

Maintaining the integrity of replicating DNA is crucial for preserving the genomic stability and the proper functioning of the cells. This study aimed to discover the role of new proteins during DNA replication and to understand their function(s) in the maintenance of genomic stability during normal DNA replication and in response to replicative stress. Using iPOND technique, we have uncovered GNL3, a new protein associated with the replisome. GNL3 is a GTP binding protein that is highly expressed in stem and cancer cells. It was previously described to be essential for the proliferation and maintenance of genomic stability by recruiting RAD51 to DSBs and modulating the binding of TRF1 to telomeres. However, its precise role(s) during DNA replication was not explored.

In this study I have uncovered the implication of GNL3 in the regulation of origin firing. I propose a model where GNL3 interacts with ORC2 in the nucleolus in order to maintain the stability of centromeric DNA, a mechanism by which GNL3 regulates indirectly the origin firing efficiency. It was reported that GNL3 levels should be maintained within a specific window; otherwise, high or low levels would lead to a decrease in the cellular proliferation (Zhu et al., 2006). In this study, I have provided an explanation for these observations. I have shown that low levels of GNL3 expression lead to an increase in the origin firing efficiency, thus affecting the integrity of the genome. And on the other hand, I proved that high levels of GNL3 expression decrease the origin efficiency, explaining why cells overexpressing GNL3 would undergo senescence.

The proper regulation of origin firing is critically linked to the maintenance of genomic stability. Previous studies have shown that ATR and WEE1 play a key role in regulating origin firing through different phosphorylation of CDKs (Beck et al., 2012; Moiseeva et al., 2019; Toledo et al., 2017). Importantly, this role is crucial for protecting the genomic integrity during replication stress. In this study I have provided the first evidence that combining ATR or WEE1 inhibition with replication stress is leading to DNA resection that can be rescued by impeding origin firing using a CDC7 inhibitor. Importantly, I have shown that depletion or overexpression of GNL3 results in DNA resection during replication stress. Interestingly, I showed that DNA resection upon GNL3 loss could be rescued by inhibiting origin firing, similarly to the case of ATR and WEE1. I therefore provided evidence linking DNA resection in the absence of GNL3 to its function in regulating the

origin firing efficiency. Thereby, in this study I described another insight about the importance of maintaining the proper level of origin firing during unperturbed DNA replication and during replication stress. In conclusion, with these findings, I present a mechanism explaining how GNL3 is implicated in the maintenance of genomic stability, a question that was not fully addressed before.

Résumé

Introduction

Avant chaque division cellulaire, le génome est dupliqué par un processus appelé réplication de l'ADN qui doit garantir la transmission fidèle du matériel génétique aux cellules filles. Ceci est crucial pour maintenir un pool sain de cellules souches afin de permettre le renouvellement des organes et éviter le vieillissement cellulaire ainsi que le développement de maladies comme le cancer. Pour assurer cette tâche, le processus de réplication doit être capable de faire face à de multiples difficultés. Par exemple, le contrôle spatio-temporel du processus de réplication de l'ADN est extrêmement important pour assurer que la totalité du matériel génétique soit dupliqué avant la division cellulaire en ne laissant aucune région sous-répliquée ou sur-répliquée. Un autre défi majeur consiste à maintenir la stabilité de la fourche de réplication en réponse au stress réplicatif afin d'éviter son effondrement qui pourrait conduire à des lésions de l'ADN et donc à des mutations ou des réarrangements. Le stress réplicatif provient de sources endogènes (répétitions en tandem, quadruplexes de guanines, collisions avec la machinerie de transcription...) ou exogènes (rayons ultraviolets, rayons ionisants, molécules utilisées en chimiothérapie...). La réplication de l'ADN est initiée à partir de sites spécifiques répartis dans tout le génome appelés origines de réplication. Chez la bactérie *Escherichia coli*, la réplication est initiée à partir d'une seule origine appelée oriC. En revanche, chez la levure *Saccharomyces cerevisiae*, plusieurs centaines d'origines appelées ARS (*autonomously replicating sequence*) possédant une séquence consensus sont nécessaires à la réplication du génome. Dans les cellules de mammifères les origines de réplication n'ont pas de séquences consensus définie. En revanche, elles partagent certaines caractéristiques au niveau de la séquence d'ADN, de l'état chromatinien et de la présence de certains facteurs.

L'initiation de la réplication est un mécanisme en deux étapes : (i) le « *licensing* » : le complexe ORC (*origin recognition complex*) et l'hélicase réplicative MCM2-7 sont chargés sur la chromatine formant ainsi le complexe de pré-réplication (Pré-RC) et (ii) le « *firing* » : le complexe pré-RC est activé par les protéines kinases DDKs et CDKs. Il est important de noter que le nombre d'origines de réplication prêtes à êtres activées est bien plus élevé que le nombre d'origines de réplication réellement utilisées durant la phase S. En effet

seul 10% des origines sont nécessaires à la réplication du génome entier. La régulation spatio-temporelle des origines de réplication conduit à l'existence de régions dites précoces et tardives qui correspondent approximativement à la réplication de l'euchromatine et de l'hétérochromatine respectivement. Le contrôle spatio-temporel de la réplication de l'ADN est extrêmement complexe et varie selon les types cellulaires. En effet, il dépend de plusieurs facteurs comme l'accessibilité et la topologie de la chromatine, l'organisation nucléaire, les marques épigénétiques et de protéines spécifiques comme Ctf19/Swi6 qui favorise la réplication précoce des centromères ou Rif1 qui favorise la réplication des régions tardives.

En plus du contrôle spatio-temporel, le processus de réplication de l'ADN doit également assurer l'intégrité des fourches de réplication en présence de stress réplicatif comme décrit plus haut. Afin d'empêcher l'effondrement des fourches bloquées et leur conversion en cassures double-brins de l'ADN, plusieurs mécanismes existent tels que l'activation des origines dormantes, le redémarrage de la fourche de réplication, la réversion de fourche, la synthèse translésionnelle ou le changement de brin matrice. De nombreuses protéines ont été impliquées dans la stabilisation des fourches bloquées en empêchant l'action de nucléases spécifiques telles que le complexe MRE11-RAD50-NBS1 (MRN). Le point de contrôle ATR/Chk1 est la voie principale empêchant l'effondrement de la fourche de réplication et l'induction d'instabilité génomique. ATR/Chk1 prévient la progression du cycle cellulaire afin de laisser suffisamment de temps à la cellule pour stabiliser et réparer la fourche de réplication bloquée. L'activation d'ATR/Chk1 en réponse au stress réplicatif dans une région inhibe les origines de réplication tardives mais active des origines dormantes au sein de la région en cours de réplication. Ainsi, l'inhibition d'ATR induit des cassures double brins de l'ADN en réponse au stress réplicatif du fait de l'absence de régulation des origines de réplication. Néanmoins un faible niveau d'activité d'ATR est nécessaire pour limiter le déclenchement incontrôlé d'origines durant la réplication normale. En plus d'ATR/Chk1, WEE1 et RIF1 sont également requis pour la stabilité des fourches de réplication et la régulation des origines dormantes. Il apparait donc que la régulation fine des origines de réplication est un élément clé pour maintenir l'intégrité du matériel génétique.

GNL3 (aussi connue sous le nom de *nucleostemin*) a été identifiée à l'origine chez *Rattus norvegicus* comme une protéine de liaison au GTP localisée principalement dans le nucléole et fortement exprimée dans les cellules souches et cancéreuses. GNL3 appartient à la famille YRG (*YlqF related GTPases*) conservée chez les eucaryotes, les procaryotes et les archébactéries. GNL3 peut faire la navette entre le nucléoplasme et le nucléole en raison de sa capacité à se lier au GTP ce qui empêche sa dégradation dans le nucléoplasme, permettant ainsi son accumulation dans le nucléole. GNL3 est principalement impliquée dans la régulation du cycle cellulaire et la stabilité génomique. Par exemple, GNL3 interagit avec MDM2 et régule sa stabilité. Ainsi en l'absence de GNL3, p53 est stabilisé ce qui conduit à l'arrêt du cycle cellulaire. De plus, GNL3 interagit avec la protéine télomérique TRF1 et module sa stabilité prévenant l'instabilité des télomères et la sénescence. L'inactivation de GNL3 conduit à l'activation de la réponse aux dommages de l'ADN pendant la phase S, ceci se traduisant par une augmentation du nombre de de foyers γH2A.X, RPA, ATR et 53BP1. En outre, GNL3 est recrutée ou niveau des cassures double-brins de l'ADN pour faciliter le recrutement de RAD51. Ainsi, les cellules inactivées pour GNL3 sont plus sensibles au stress réplicatif et présentent des défauts de réparation de l'ADN par recombinaison homologue. Le modèle actuel suggère que GNL3 maintiendrait la stabilité du génome en recrutant RAD51 au niveau des lésions de l'ADN afin de les réparer. Cependant, le rôle précis de GNL3 dans la réparation des lésions de l'ADN durant la phase S n'est pas encore connu, son étude fait donc l'objet de cette thèse.

Objectifs

Afin de mieux comprendre le processus de réplication de l'ADN, il est important d'étudier les mécanismes qui permettent la réplication dans des conditions normales et en présence de stress réplicatif. Il est aujourd'hui possible d'étudier systématiquement les protéines associées au réplisome par la technique iPOND (*isolation Of Proteins On Nascent DNA*). Les expériences iPOND réalisées dans des conditions basales ont permis d'isoler des composants connus du réplisome (PCNA, ADN polymérases, MCM2-7…), des protéines impliquées dans la résolution des fourches bloquées (BRCA1/2, RAD51,

ATR…) et des constituants de la chromatine comme les histones. Néanmoins, l'intérêt majeur de la méthode iPOND est la découverte de nouveaux composants du réplisome afin d'avoir une meilleure compréhension du processus de réplication de l'ADN. Dans ce sens ce projet se base sur l'utilisation de cette méthode qui a permis de mettre en évidence la protéine GNL3 comme nouveau composant du réplisome.

Les objectifs de ce projet étaient divisés en deux parties :

1- Caractérisation du rôle de GNL3 pendant la phase S afin de comprendre la raison de son association avec le réplisome.

2- Déterminer le rôle de GNL3 dans la réponse au stress réplicatif afin de comprendre comment elle contribue à préserver l'intégrité génomique.

Résultats et discussion

1- Rôle de GNL3 durant la réplication de l'ADN

Au cours de cette étude j'ai montré que GNL3 est impliquée dans l'activation des origines de réplication. Le niveau cellulaire de GNL3 doit être maintenu dans une fenêtre précise car des niveaux trop élevés ou trop faibles entraînent une diminution de la prolifération cellulaire. Dans cette étude, j'ai fourni une explication à ces observations. J'ai notamment montré que l'inactivation de GNL3 augmente l'efficacité des origines de réplication en utilisant des techniques telles que le peignage de l'ADN et le fractionnement de la chromatine. De plus, cette dérégulation impacte l'intégrité du génome. D'autre part, j'ai prouvé que la surexpression de GNL3 entraîne une diminution de l'efficacité des origines de réplication, expliquant pourquoi les cellules surexprimant GNL3 deviennent sénescentes. De plus, afin d'explorer plus en détail le mécanisme moléculaire de cette nouvelle fonction de GNL3, j'ai recherché des partenaires de GNL3 en couplant la méthode BioID à la spectrométrie de masse. Il apparait que GNL3 est à proximité d'ORC2, une des protéines du complexe ORC. Cette proximité a été validée par d'autres

approches comme la co-immunoprécipitation et le PLA (*proximity ligation assay*). Je propose que GNL3 interagit avec ORC2 au niveau du nucléole afin de maintenir la stabilité de l'ADN centromérique, une région liée spécifiquement par ORC2. Ainsi l'inactivation ou la surexpression de GNL3 conduirait à des défauts de recrutement d'ORC2 pouvant expliquer les défauts d'activation des origines de réplication.

2- Rôle de GNL3 dans le maintien de la stabilité génomique en réponse au stress réplicatif

La régulation des origines de réplication est étroitement liée au maintien de la stabilité génomique. Des études antérieures ont montré qu'ATR et WEE1 jouent des rôles clés dans la régulation des origines à travers les différentes phosphorylations de CDK. Ce rôle est crucial pour protéger l'intégrité du génome pendant le stress réplicatif. Dans cette étude j'ai montré que la combinaison d'inhibiteurs d'ATR ou de WEE1 avec du stress réplicatif conduit à la résection de l'ADN naissant. Ce phénotype peut être supprimé par l'inhibition de CDC7, démontrant ainsi que la résection est une conséquence de la dérégulation des origines de réplication. J'ai montré que l'inactivation de GNL3 entraîne une résection de l'ADN naissant en présence de stress réplicatif. De plus, la surexpression de GNL3 conduit également à une résection de l'ADN en réponse au stress réplicatif. Ces résultats montrent que le niveau de GNL3 est crucial pour maintenir la stabilité du génome en réponse au stress réplicatif. Il apparait, tout comme pour l'inhibition d'ATR et WEE1, que la résection de l'ADN naissant observée en absence de GNL3 est supprimée par l'inhibition de CDC7. Ainsi la résection de l'ADN naissant observée en absence de GNL3 est une conséquence de son rôle dans la régulation des origines. Ces résultats montrent que le contrôle correct de l'activation des origines de réplication en présence de stress réplicatif est essentiel pour prévenir la stabilité des fourches de réplication bloquées. Ces résultats me permettent de proposer pour la première fois un mécanisme expliquant comment GNL3 est impliqué dans le maintien de la stabilité génomique.

Conclusion

Pour conclure, j'ai pu montrer au cours de ma thèse de doctorat que GNL3 est un nouveau composant du réplisome qui régule l'activation des origines de réplication au cours de la

réplication, expliquant ainsi son rôle dans le maintien de l'intégrité du génome. De façon plus générale, mes résultats illustrent l'importance du contrôle de des origines de réplication dans le maintien de la stabilité du génome.

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