



Role of SAMHD1 in the replication stress response and in the production of type I IFNs

Flavie Coquel

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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Etude du rôle de SAMHD1 dans la réponse au stress réplcatif et la
production d'interférons de type I

Role of SAMHD1 in the replication stress response and in the
production of type I IFNs

Présentée par Flavie COQUEL

Le 19 Septembre 2018

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“The answer to biological problems preexists, it is the question that needs to be discovered”

Jonas Salk

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Abbreviations

9-1-1: RAD9-RAD1-HUS1
ADAR1 Adenosine Deaminase Acting on RNA 1
AGS: Aicardi-Goutières Syndrome
AIM2: Absent In Melanoma 2
APC/C: Anaphase-Promoting Complex/Cyclosome
ATM: Ataxia Telangiectasia Mutated
ATR: ATM and Rad3-related protein
ATRIP: ATR-interacting protein
Bax: BCL-2-like protein 4
Bak: BCL-2 homologous antagonist/killer
BIR: Break-Induced Replication
BLM: Bloom syndrome Mutated protein
BRCA1/2: Breast cancer susceptibility protein
CDC25: cell division cycle 25
CDC45: Cell Division Cycle 45
CDK: Cyclin-Dependent Kinase
CDT1: Chromatin licensing and DNA replication factor 1
CFS: Common Fragile Site
cGAMP: Cyclic GMP-AMP
cGAS: Cyclic GMP-AMP synthase
CHK1/2: Checkpoint Kinase 1/2
CldU: 5-chloro-2'-deoxyuridine
CMG: CDC45-MCM-GINS
CtIP: C-terminal-binding protein
DAPI: 4',6-diamidino-2-phenylindole
DDK: CDC7/Dbf4-Dependent Kinase
DDR: DNA Damage Response
DDX41: DEAD-Box Helicase 41
DNA: deoxyribonucleic acid
DNA2: DNA replication ATP-dependent helicase/nuclease
DNA-PK: DNA-dependent Protein Kinase
dNDP: deoxyribonucleoside diphosphate
dNTP: deoxyribonucleoside triphosphate
DMBA: dimethylbenz[α]anthracene
DSB: Double-Strand Break
dsDNA: double-stranded DNA
EEPD1: endonuclease/exonuclease/phosphatase family domain containing 1 protein
ETAA1: Ewing tumor -associated antigen 1
EXO1: Exonuclease 1
FANCD2: Fanconi anemia complementation group D2
FEN1: Flap structure-specific endonuclease 1
GINS: Go, Ichi, Nii, and San; five, one, two, and three in Japanese
HIN: hematopoietic interferon-inducible nuclear proteins with a 200-amino acid repeat
HU: hydroxyurea

HR: Homologous Recombination
 IdU: Iododeoxyuridine
 ICL: Inter-Strand Crosslinks
 IFI16: IFN-inducible protein 16
 IFN: Interferon
 IKK: I κ B Kinase
 IL: Interleukin
 IRF: IFN Regulatory Factors
 ISGs: Interferon-Stimulated Genes
 ISRE: IFN-Stimulated Response Element
 JAK: Janus kinase
 LGP2: Laboratory of Genetics and Physiology 2
 LINE: Long Interspersed Nuclear Element
 NF- κ B: nuclear factor k-light-chain enhancer of activated B cells
 NK: Natural killer
 NKG2D: natural-killer group 2, member D
 MAVS: mitochondrial antiviral-signaling protein
 MCM: Minichromosome Maintenance Complex
 MDA5: Melanoma Differentiation Associated gene 5
 MEF: Mouse Embryonic Fibroblast
 MHC: Major Histocompatibility Complex
 MLL3/4: myeloid/lymphoid or mixed-lineage leukemia protein 3/4
 MMC: Mitomycin C
 MRE11: Meiotic Recombination protein 11
 mRNA: messenger RNA
 NDP: ribonucleoside diphosphate
 NEMO: NF- κ B essential modulator
 NF- κ B: Nuclear Factor-kappa B
 OAS: 2',5'-oligoadenylate synthetase
 ORC: Origin Recognition Complex
 PAMPs: Pathogens-Associated Molecular Patterns
 PCNA: Proliferating Cell Nuclear Antigen
 PKR: Protein kinase RNA-activated
 PRRs: Pattern Recognition Receptors
 PTIP: Pax Transactivation domain Interacting Protein
 PYHIN: Pyrin and HIN domain
 RFC: Replication Factor C
 RIG-I: Retinoic Acid-Inducible Gene-I
 RLR: RIG-I like Receptor
 RNA: RiboNucleic Acid
 RNR: ribonucleotide reductase
 ROS: Reactive Oxygen Species
 RPA: Replication Protein A
 SAMHD1: SAM And HD Domain Containing Deoxynucleoside Triphosphate
 SINE: Short Interspersed Nuclear Element
 SLX4: Structure-Specific Endonuclease Subunit
 ssDNA: single-stranded DNA

ssRNA: single-stranded RNA

STAT: Signal Transducer and Activator of Transcription

STING: Stimulator of Interferon Genes

TANK: tumor -necrosis-factor-receptor-associated factor (TRAF)-family-member-associated nuclear factor (NF- κ B) activator

TBK1: TANK-binding kinase 1

TLR: Toll-Like Receptor

TLS: Translesion Synthesis

TNF- α : Tumor Necrosis Factor

TopBP1: Topoisomerase II binding protein I

TREX1: Three-prime repair exonuclease 1

WRN: Werner syndrome ATP-dependent helicase

Preamble

Acquired mutations of the deoxynucleotide triphospho-hydrolase SAMHD1 can cause Aicardi-Goutières Syndrome (AGS), a very rare, yet it often fatal disease. In 2015, a study with a cohort of 285 AGS patients revealed that 74% of them had severe deficits of motor and communication abilities. Only 19% of the affected children lived beyond the age of 15. Investigating this disease has proven very powerful. Indeed, studying proteins involved in AGS and other Type I interferonopathies has allowed major discoveries in innate immunity in general and in self vs non/self-discrimination in particular.

In 2014, the identification of an AGS patient with a SAMHD1 mutation that developed chronic lymphocytic leukemia broaden the field of possibilities by showing that SAMHD1 defects may be a founding event in cancer development. From studying a protein involved in a very rare disorder, it was then possible to find implications for cancer, the second leading cause of death worldwide according to the World Health Organization (Ferlay et al., 2015).

During my PhD, I investigated the involvement of SAMHD1 in DNA replication. When I started working on this project, little was known regarding the cause of immune activation triggered by SAMHD1 deficiency. Linked had already been made however between inflammation and DNA damage, a possible consequence of replication stress. Replication stress is considered as one of the hallmarks of cancer, a characteristic that can be used as an Achilles' heel, to specifically eliminate tumor cells. I initially joined the laboratory of Philippe Pasero to find new anti-cancer agents targeting replication stress from Chinese Herbal Medicine, a project that will not be presented in this manuscript for confidentiality reasons. I further studied the response to replication stress and the possible participation of SAMHD1 in this process, a journey which has taken me from DNA replication and cancer to AGS and cell-autonomous innate immunity. These results were published in April 2018.

The introduction of this manuscript first briefly covers the innate immune system and cell-autonomous responses to cytosolic nucleic acids. In the chapter two are presented some transactions taking place during S phase to ensure the faithful duplication of the genome and the impediments that arise during this process. The last chapter describes how these transactions may induce inflammation. The objectives and the questions addressed during my PhD are then presented, followed by a section dedicated to the results. Finally, I discuss these results in light with the literature and suggest ensuing therapeutic outcomes.

| Introduction

Chapter 1: Innate immune responses to cytosolic nucleic acids

Cells have evolved sophisticated pathways to detect pathogens and trigger an appropriate immune response. Cytosolic nucleic acids are part of the molecular patterns recognized by specific immune receptors. DNA and RNA however are not unique to pathogens: host cells also have genomic DNA and produce RNAs. In some circumstances, self-nucleic acids can inappropriately activate the immune system. For instance, several studies have demonstrated that persisting DNA damage leads to the accumulation of DNA in the cytosol, triggering innate immune signaling through the induction of inflammatory genes.

In the next section, I will give a succinct overview about the immune system and the mechanisms by which cytosolic nucleic acids can induce immune signaling. Since responses to endogenous nucleic acids in the cytosol chiefly use the pathways classically induced by pathogens, I will later describe these pathways.

I- The immune system

The immune system is critical for host defense against a variety of exogenous and endogenous threats such as viruses, bacteria, damaged and cancer cells... It is also a major effector of inflammatory diseases. This section is not intended to give an exhaustive view of immunity since the complexity of this field largely exceeds the scope of this manuscript. I will only highlight the main factors involved and how they connect with inflammation, based on Janeway's Immunobiology.

Anatomic and physiological barriers provide a first layer of host defense against exogenous intrusions. The innate and adaptive immune systems constitute the second layer of protection.

At the cellular level, innate immune activity is performed by both hematopoietic and non-hematopoietic effectors. The first category includes, but is not restricted to: macrophages, dendritic cells, and Natural Killer (NK) cells (Figure 1). The second category is made of epithelial and endothelial cells.

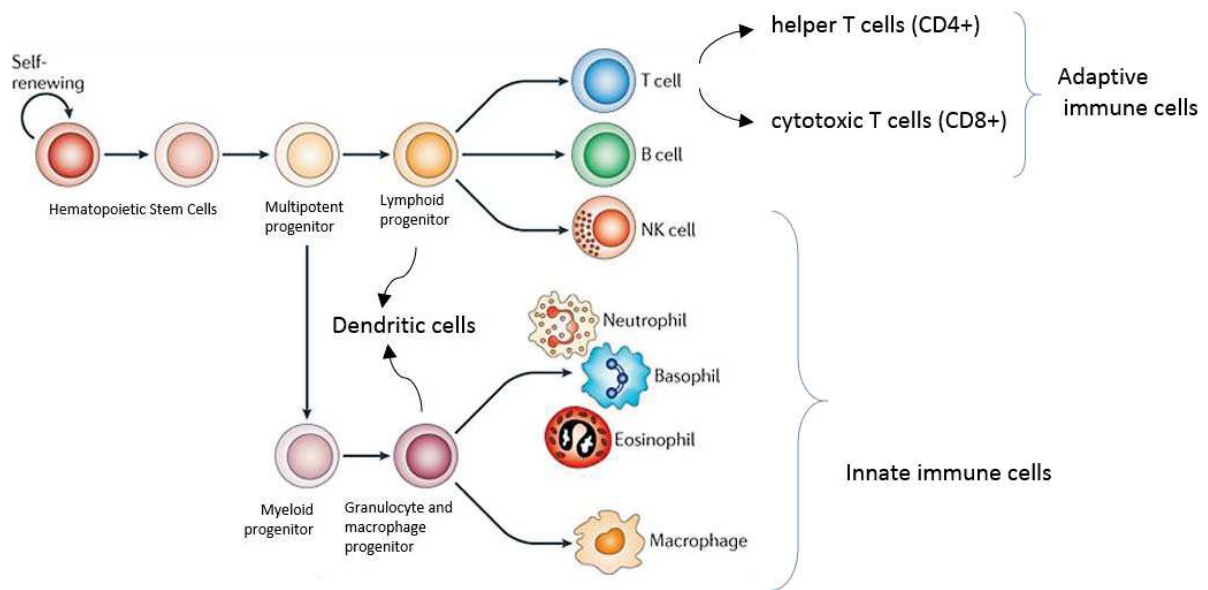


Figure 1 Hematopoietic immune cells: Pluripotent hematopoietic stem cells differentiate into multipotent progenitors, then lymphoid and myeloid stem cells. Lymphoid progenitors generate B and T lymphocytes and NK cells. The myeloid progenitor give rise to the granulocyte lineage (neutrophils, basophils, eosinophils), macrophages. Lastly, dendritic cells may arise from both lymphoid and myeloid progenitors. Simplified model of hematopoiesis modified from King & Goodell, 2011.

Quickly activated after any infection, the innate immune system may be able to eliminate the foreign agent. If not, it can contain the infection while the adaptive response develops.

Monocytes (macrophages and dendritic cells) as well as neutrophils are the first line of the host defense. They harbor Pattern Recognition Receptors (PRRs) allowing them to recognize a number of conserved features of pathogens called Pathogens-Associated Molecular Patterns (PAMPs). PAMPs include for example specific DNA sequences that are much less common in vertebrate DNA than in bacterial DNA. Damage-Associated Molecular Patterns (DAMPs) are also recognized by PRRs. DAMPs are released by damaged and dying cells. Recognition of DAMPs and PAMPs by PRRs triggers multiple signaling cascades, ultimately initiating inflammation.

Activated monocytes and neutrophils secrete the main mediators of inflammation: cytokines. The production of cytokines activates many processes with the final purpose of facilitating migration and activation of immune cells. A special type of cytokines called chemokines attract immune cells to the site of infection.

Monocytes and neutrophils also perform phagocytosis: they are able to internalize pathogens and to digest them into small fragments. This activity allows them to activate lymphocytes of the adaptive immune system by antigen presentation.

Although slower to be implemented, the adaptive response mediated by T and B lymphocytes is highly specific and generates an immune memory allowing for an extremely efficient immune response in the event of a secondary host/pathogen encounter. Rearrangement events are responsible for the generation of an antigen-specific receptors repertoire instrumental for the detection of pathogens and their elimination. T cells can be broadly divided into two categories: helper cells and cytotoxic cells. Both express specific co-receptors helping them to recognize antigens: the CD4 and CD8 receptors respectively. Activated CD4-T cells assist and regulates other immune cells by producing cytokines. CD8-T cells on the other hand can kill infected or cancer cells. The last effectors of this adaptive response, B cells, produce specific antibodies able either to directly neutralize their target or to tag it for attack by other immune cells.

As it is the starting point of a major part of my PhD, the second part of this first introduction chapter covers the immune responses to cytosolic DNA, focusing on the cell-autonomous responses. I also included the response to cytosolic RNA as the two are interconnected and, in my opinion, cannot be dissociated from each other. I will mainly focus on their effects on the innate immune system.

II- Innate immune responses to cytosolic nucleic acids

The reactions to cytosolic nucleic acids take an important part in the immune response against viruses since they replicate inside host cells. As described in the third chapter, we now know that

even self-DNA can be exposed to the cytosol for example after DNA damage (Härtlova et al., 2015) or mitochondrial stress (for review: West & Shadel, 2017).

Whether inflammatory responses are triggered by pathogenic or self-DNA, it roughly engages the main players. This section and the following briefly summarize our current knowledge of the signaling pathways activated in cell-autonomous immunity and gives an overview of the recognition of cytosolic nucleic acids, originating whether from pathogens or self-sources.

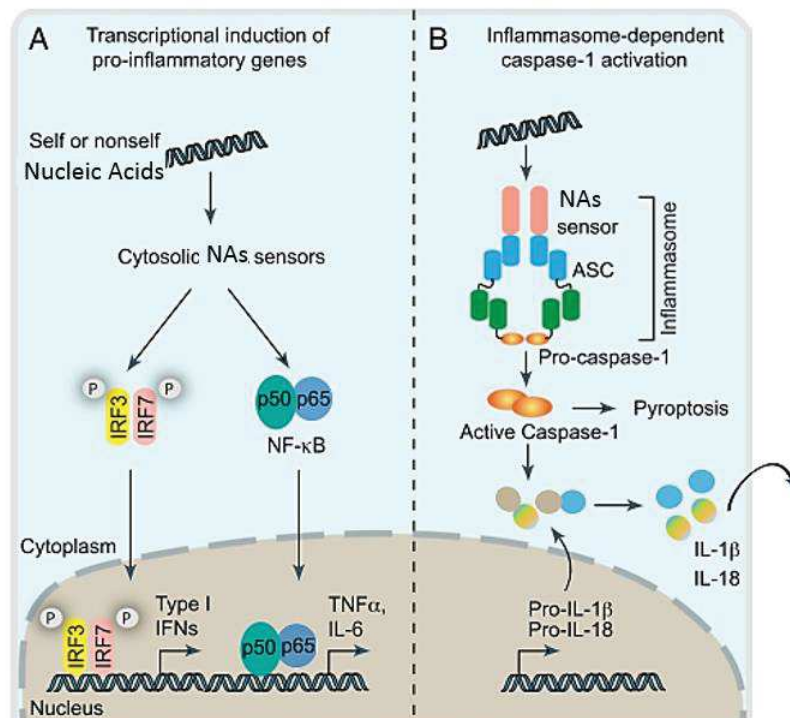
The detection of cytosolic nucleotides triggers multiple signaling cascades, with the finality of either stimulating the formation of inflammasomes or producing pro-inflammatory and antiviral cytokines, as illustrated in the figure 2 (Atianand & Fitzgerald, 2013).

1. Pro-inflammatory and antiviral cytokines

Cytokines are polypeptides secreted by a large range of cells including infected epithelial and immune cells such as macrophages, B and T cells. They are pleiotropic immunomodulators of the innate and adaptive immune responses. Many different cytokines have been described based on their activity. This section addresses two categories of cytokines: pro-inflammatory and antiviral cytokines.

Pro-inflammatory cytokines such as TNF- α (Tumor Necrosis Factor), IL-18 (Interleukin 18), IL-1- β (Interleukin 1- β) are mainly produced by macrophages and dendritic cells. They play pivotal roles in the innate immune response for example by activating macrophages and neutrophils and facilitating their migration to the site of infection (reviewed by Alvine, Knopick, Nilles, & Bradley, 2015; Meager & Wadhwa, 2013).

Antiviral cytokines, on the other hand, can be produced by all nucleated cells. Interferons (IFNs) are the major components of this class of cytokines. Interferons are divided into three groups based on what cell type produces them. While most cells produce type I IFNs (primarily IFN- α and IFN- β), type II (IFN- γ) is produced by T cells and NK cells and type III (IFNs- λ) by fibroblasts, adipocytes, endothelial cells and B cells.



*Figure 2 **Cell-intrinsic responses to cytosolic DNA:** Detection of cytosolic Nucleic Acids (NAs) triggers 2 types of immune responses. On the left, detection of cytosolic NAs by some sensors engages the IRF3/IRF7 or NF-κB pathways of IFN and inflammatory cytokines production. On the right, activation of other sensors triggers the formation of an inflammasome, leading to caspase-1 activation, thereby allowing cleavage of pro-IL-1B and pro-IL-18 into their mature forms. Activated caspase-1 can also initiate pyroptosis, a form of immune cell death. Modified from Atianand & Fitzgerald, 2013.*

Integration of the IFN-induced signaling in the immune response is illustrated in Figure 3.

Three transcription factors regulate the induction of type I IFNs and pro-inflammatory cytokines, through two distinct molecular pathways. These transcription factors are: NF- κ B (Nuclear Factor κ -light-chain enhancer of activated B cells) and the IFN Regulatory Factors IRF3 and IRF7 (for review: Radoshevich & Dussurget, 2016).

Activation of the NF- κ B pathway is generally initiated by phosphorylation of the IKK complex (I κ B Kinase). Activated IKK phosphorylates the NF- κ B negative regulator I κ B, targeting it for proteasomal degradation. NF- κ B can then go to the nucleus where it mediates the transcription of pro-inflammatory genes.

The IRF3/IRF7 pathway is commonly activated following their phosphorylation by TBK1 (TANK-Binding Kinase 1). IRF transcription factors bind to IFN-Stimulated Response Element (ISRE) sequences. These regulatory sequences were first identified in promoters of IFN-inducible genes, but they are also found in the promoters of the genes encoding type I IFNs themselves.

STING (Stimulator of Interferon Genes) is a major mediator of these two pathways (Ishikawa & Barber, 2008; Zhong et al., 2008). Indeed, STING-deficient mice are unable to trigger a robust IFN response upon viral infection and die within 7 days after infection (Ishikawa, Ma, & Barber, 2009; Zhong et al., 2008). STING is expressed in a wide array of tissues and cell types, ranging from immune cells to epithelial cells (Ishikawa & Barber, 2008). Once activated, STING can switch-on both the IRF3/IRF7 and the NF- κ B pathways via TBK1 (T. Abe & Barber, 2014; Tanaka & Chen, 2012).

IFNs have three major functions:

- limiting bacterial and viral spreading
- modulating the innate immune response
- activating the adaptive immune system

They exert these functions by inducing the transcription of key Interferon-Stimulated Genes (ISGs). ISGs are produced through the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathway (for review: Ivashkiv & Donlin, 2014). Upon binding of type I IFNs to their receptor, JAK1 and Tyrosine Kinase 2 are activated, recruiting and activating STAT1, STAT2 and STAT3 proteins.

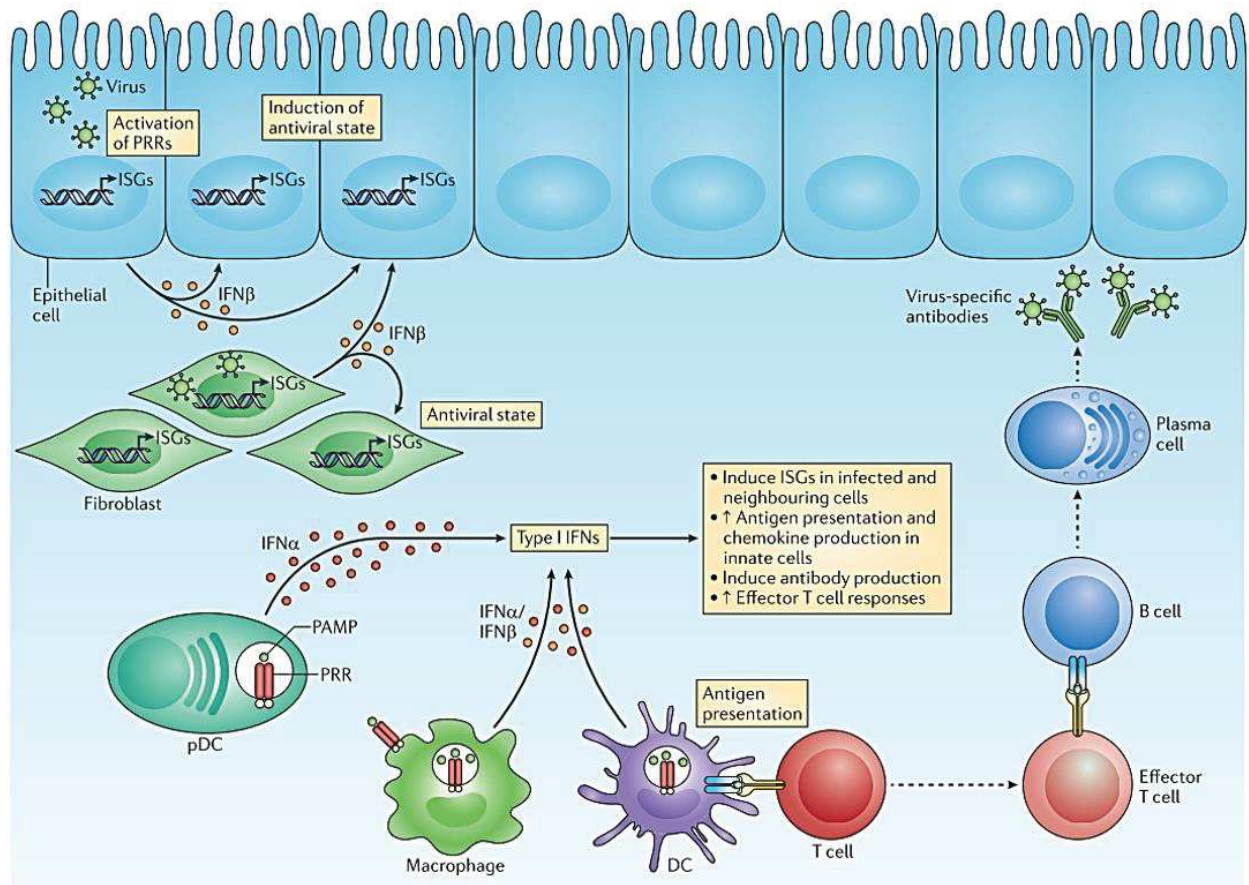


Figure 3 The multiple roles of type I IFNs in innate and adaptive immunity: Upon detection of pathogens, infected fibroblasts and epithelial cells produce IFN- β , inducing the production of IFN-stimulated genes (ISG). Neighboring cells thus switch to an antiviral state. Immune cells such as Dendritic Cells (DC) and macrophages sense Pathogen-Associated Molecular Patterns using their Pattern-Recognition Receptors. They too produce type I IFNs, enhancing antigen presentation and production of chemokines. T and B cells of the adaptive immune system also respond to IFNs: antibody production and T-cell effector functions are increased. Modified from Ivashkiv & Donlin, 2014.

Three different STAT complexes are formed, driving different transcriptional responses (see Figure 4).

The heterodimer STAT1/STAT2 associate with IRF9 to bind ISRE sequences, directing the production of antiviral genes. STAT1 homodimers bind to Gamma-Associated Sequences to induce pro-inflammatory genes. STAT3 homodimers, on the other hand, drives the transcription of negative regulators of inflammatory pathways.

2. The inflammasome

The inflammasome is a large protein complex consisting of three factors: a sensor protein, an adaptor protein and the protease caspase-1. Upon recognition of nonself-nucleic acids by specific sensors, the formation of this multiprotein complex in the cytosol induces the activation of caspase-1 (Martinon, Burns, & Tschopp, 2002). The NLRP3, AIM2 and IFI16 inflammasomes have been linked to immune responses to intracellular DNA (for review: Paludan & Bowie, 2013). For instance, oxidized mitochondrial DNA has been suggested to activate the NLRP3 inflammasome (Shimada et al., 2012). The nuclear DNA sensor IFI16 also forms an inflammasome upon infection of endothelial cells by Kaposi's sarcoma-associated herpesvirus (Kerur et al., 2011).

This complex acts as a molecular scaffold for the proteolytic processing of inactive procaspases into active molecules. In other words, the formation of an inflammasome enables activated caspase-1 to cleave the precursors of Interleukin (IL)-1- β and IL-18 into mature, biologically active cytokines. In turn, IL-1- β and IL-18 promote the activation of distinct immune cells. The former molecule, IL-1- β , is essential for both the innate and adaptive immune responses as it is implicated in the activation of neutrophils, macrophages, dendritic and T cells (Dinarello, 2009). The latter, IL-18, is important for IFN- γ production by NK cells and T cells (Gracie, Robertson, & McInnes, 2003). This post-translational regulation mechanism ensures that the mere formation of an inflammasome triggers a rapid pro-inflammatory response following detection of pathogenic nucleic acids.

Inflammasomes can also trigger pyroptosis, a programmed cell death of immune cells. Pyroptosis has been shown to be important for to eliminate infected immune cells, often used as a "niche" by pathogens (Miao et al., 2010).

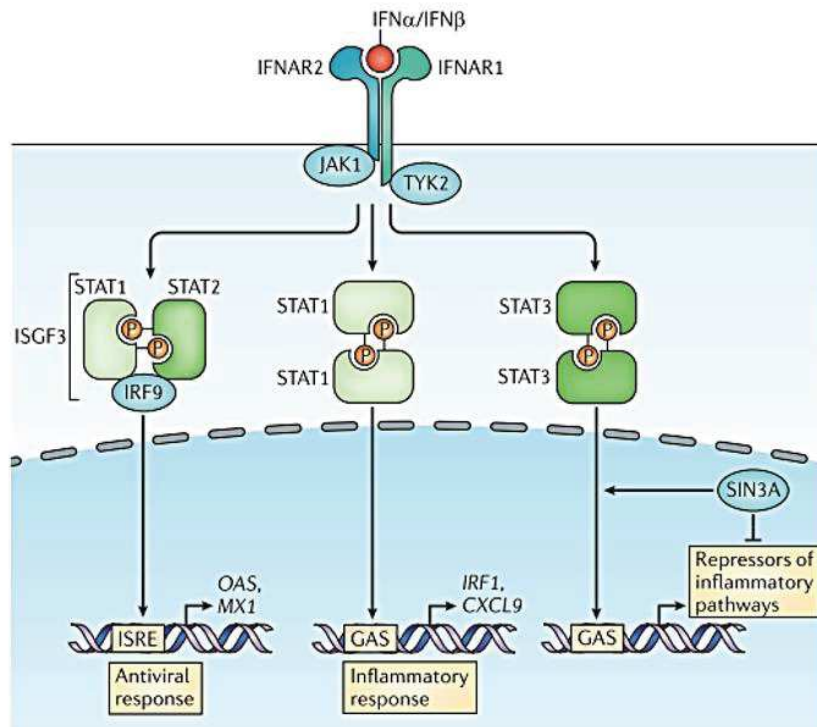


Figure 4 Type I IFN signaling: The IFN receptor is composed of the IFNAR1 and IFNAR2 subunits. Upon IFN binding, it activates the Janus Kinase 1 (Jak1) and Tyrosine Kinase 2 (Tyk2). Activated Jak1 and Tyk2 recruit Signal Transducer and Activator of Transcription (STAT) proteins. 3 different STAT complexes are generated, driving distinct transcriptional programs. The STAT1/STAT2/IRF9 complex activates the antiviral response by, for example, allowing the production of 2'5' oligoadenylate synthase (OAS) and Mx1. The STAT1 and STAT3 homodimers bind to Gamma-Associated Sequences (GAS). The first complex drives the production of pro-inflammatory genes such as the IFN transcription-factor IRF1 and the chemokine CXCL9 while the latter represses inflammatory signaling. Modified from Ivashkiv & Donlin, 2014.

III- Detection of cytosolic nucleic acids

Although we have known for many years that DNA introduced into mammalian cells activates the immune system, only recently have we begun to understand the molecular mechanisms driving this response.

Since my thesis concerned mainly non-pathogenic, self-DNA, an emphasis will be given on the cGAS-STING pathway which is the most reported for the recognition of self-DNA and subsequent inflammatory responses.

As I focus on self-autonomous immunity, I deliberately chose not to describe the family of Toll-Like Receptors (TLRs). Although they have a major role in recognition of pathogen-derived DNAs and RNAs, they are only expressed in specific immune cells where they localize to the cell surface or specialized compartments like endosomes and lysosomes. As such, they mainly sense nucleic acids after pathogen phagocytosis or in the extracellular compartment (Barbalat, Ewald, Mouchess, & Barton, 2011).

1. RNA sensing receptors

As illustrated in figure 5, cytosolic RNA is mainly detected by RNA helicases of the Retinoic Acid-Inducible Gene-I (RIG-I) family (for review: Radoshevich & Dussurget, 2016; Yoneyama, Onomoto, Jogi, Akaboshi, & Fujita, 2015). This family includes 3 proteins: RIG-I, Melanoma Differentiation Associated gene 5 (MDA5) and Laboratory of Genetics and Physiology 2 (LGP2).

MDA5 and RIG-I both have an RNA binding domain and a pair of signaling domains. RIG-I senses dsRNA with a 5' triphosphate end. This structure can be found in RNA viruses such as influenza virus and hepatitis C virus. MDA5 detects long dsRNA known to be generated by picornaviruses. Following RNA recognition, they associate with the adaptor protein MAVS (Mitochondrial Antiviral-Signaling protein). MAVS then activates the IRF3/IRF7 and NF- κ B-dependent pathways of IFN and pro-inflammatory cytokines production. LGP2 lacks the signaling module. Its function in the RNA sensing pathway is not completely understood. Current models implicate LGP2 in helping MDA5-RNA interaction, enhancing antiviral signaling from this pathway and inhibiting RIG-I signaling.

Other cytosolic proteins have been shown to be important for viral RNA detection. For example, Protein kinase RNA-activated (PKR) and 2', 5'-oligoadenylate synthetase (OAS) are important for the response to long dsRNA. Both proteins inhibit viral mRNA translation early in infection, but their role in IFN signaling is elusive (Hartmann, 2017).

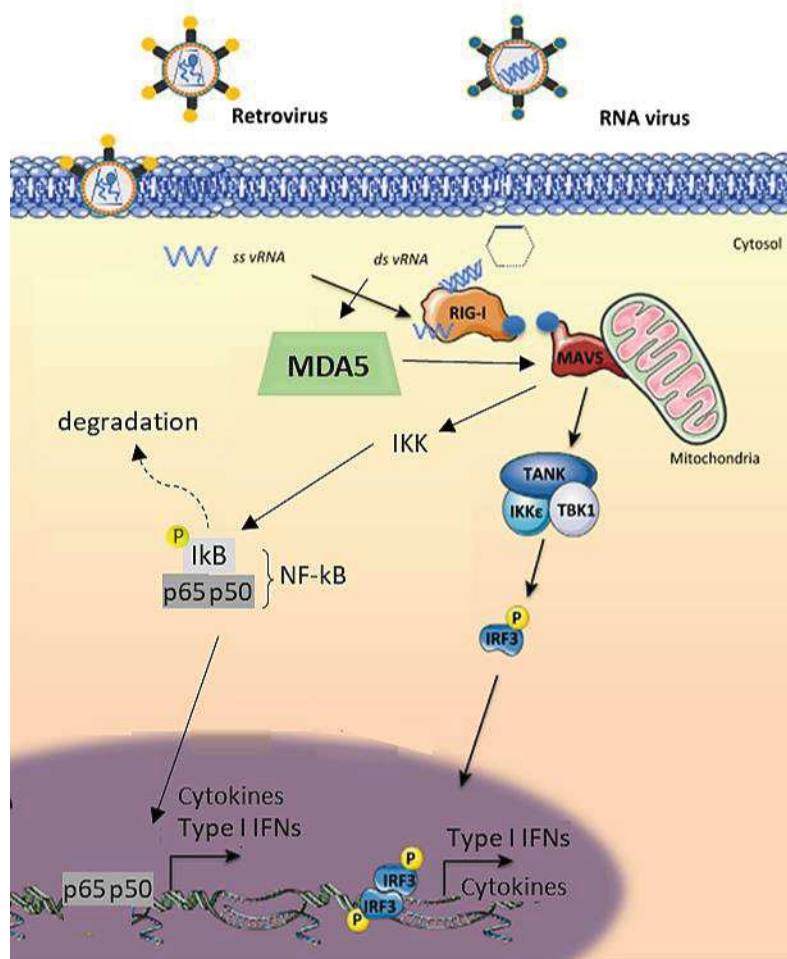


Figure 5 Cytosolic recognition of RNAs: RIG-I and MDA5 recognize dsRNA. Both proteins activate the adaptor protein MAVS. Activated MAVS then induces the production of type I IFN and pro-inflammatory cytokines via the IRF3 and NF-κB signaling pathways. Modified from (Zevini, Olganier, & Hiscott, 2017)

2. DNA sensing receptors

Receptors of cytosolic DNA are illustrated in the figure 6.

DEAD-Box Helicase 41(DDX41)

The RNA helicase DDX41 specifically recognizes cytosolic DNA and colocalizes with STING, inducing IRF3 and NF- κ B-mediated pro-inflammatory responses in dendritic cells and monocytes. DDX41 may directly bind poly(dA:dT), poly(dG:dC) and viral DNA but not poly(I:C) (Z. Zhang et al., 2011).

This result however was not reproduced in fibroblasts (Takayuki Abe et al., 2013; Sun, Wu, Du, Chen, & Chen, 2013), possibly because of cell-type or species-specific involvement of DDX41 in cytosolic DNA recognition.

IFN-inducible protein 16 (IFI16)

IFI16 is member of the Pyrin and HIN (hematopoietic interferon-inducible nuclear proteins with a 200-amino acid repeat) domain (PYHIN) protein family. IFI16 binds dsDNA through its HIN domain, activating the IRF3 and NF- κ B-pro-inflammatory pathways through an interaction with STING (Jin et al., 2012; Unterholzner et al., 2010). IFI16 can additionally signal a pro-inflammatory response via the formation of an inflammasome complex (Kerur et al., 2011).

This sensor also recognizes ssDNA to induce the production of IFNs (Jakobsen et al., 2013).

Absent In Melanoma 2 (AIM2)

As IFI16, AIM2 belongs to the PYHIN protein family. AIM2 binds to dsDNA fragments longer than 50-80 bp, independently of their sequence (Jin et al., 2012). This induces the formation of an AIM2-inflammasome complex resulting in the activation of caspase-1 (Fernandes-Alnemri, Yu, Datta, Wu, & Alnemri, 2009; Hornung et al., 2009).

Cyclic GMP–AMP synthase (cGAS)

cGAS is considered as the major cytosolic DNA sensor (Cai, Chiu, & Chen, 2014). It is predominantly located in the cytosol but can enter the nucleus in proliferating and migrating cells and associates with chromatin during mitosis (Raab et al., 2016; H. Yang, Wang, Ren, Chen, & Chen, 2017).

cGAS is an IFN-inducible nucleotidyl transferase: it catalyzes the synthesis of two phosphodiester bonds between GMP and AMP generating 2'3'-cGAMP (Ablasser et al., 2013; P. Gao et al., 2013; Sun et al., 2013; Xu Zhang et al., 2013).

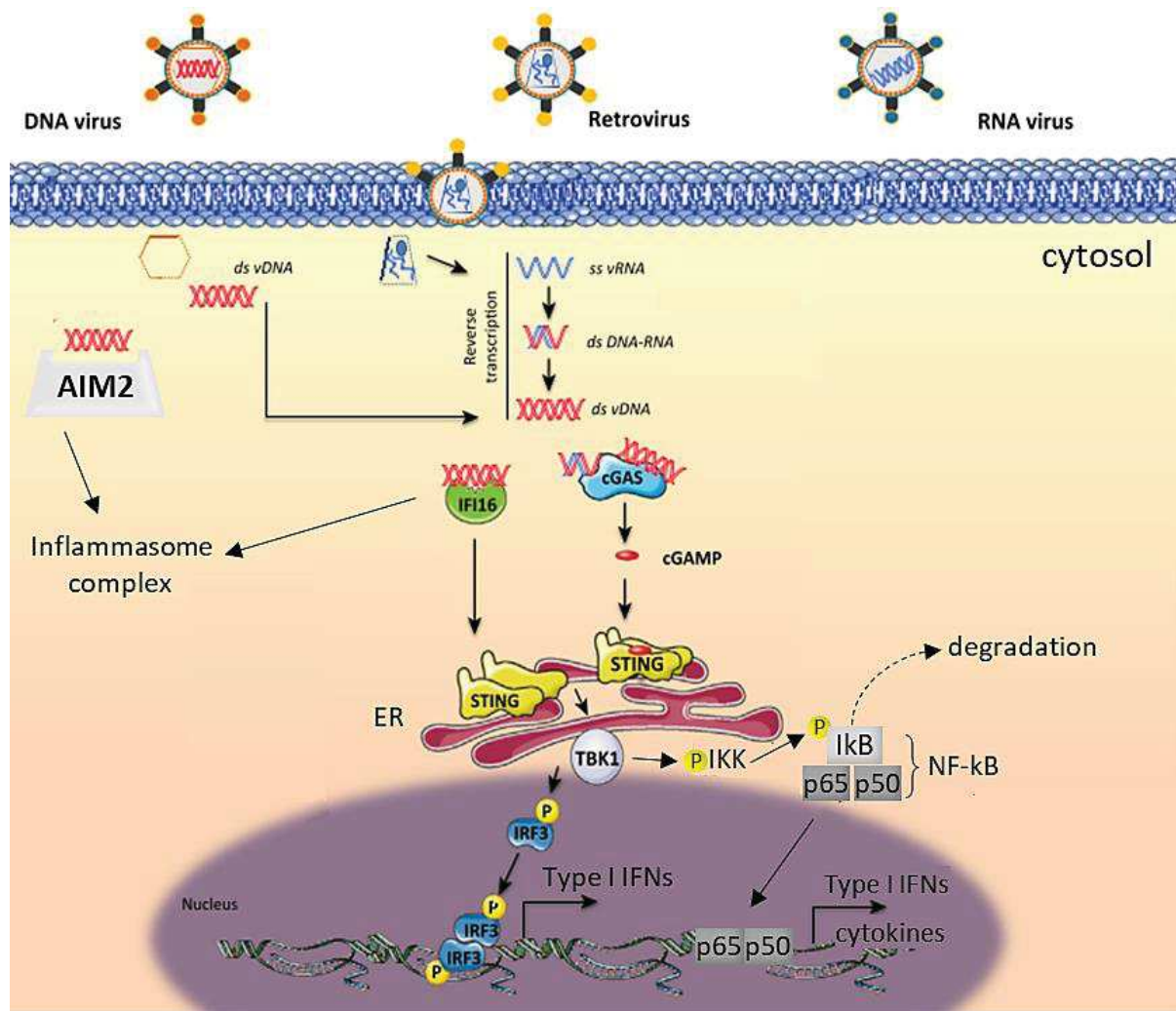


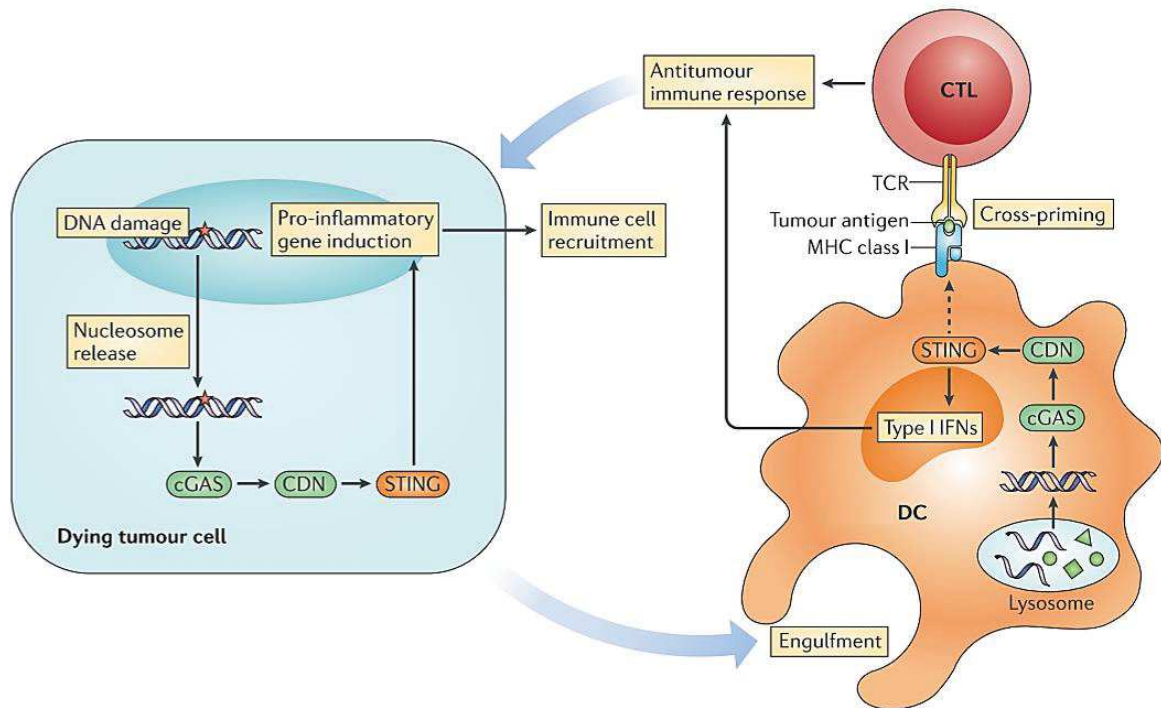
Figure 6 Cytosolic DNA recognition: Following DNA or RNA:DNA recognition, cGAS produces the signaling molecule cGAMP. cGAMP binds to the Endoplasmic Reticulum (ER)-protein STING, inducing TBK1 recruitment. Together they induce the production of type I IFNs and pro-inflammatory cytokines through phosphorylation of IRF3 and IKK. Activated IKK in turn phosphorylates the NFκ-B-negative regulator IκB, unleashing its transcription-factor activity. Other DNA sensors include AIM2 and IFI16. Both can trigger the formation of inflammasome complexes following DNA recognition. IFI16 additionally signals through STING. Modified from Zevini et al., 2017.

Initially, cGAS was shown to bind to the sugar backbone of dsDNA and RNA:DNA hybrids (Civril et al., 2013; P. Gao et al., 2013; X.-D. Li et al., 2013; Xu Zhang et al., 2014), sensing cytosolic DNA independently of its sequence. A recent study, however, has found that cGAS-mediated DNA recognition can be sequence-dependent. Indeed, in HIV-1 detection the minimal sequence necessary for cGAS recognition is Y form DNA containing guanosine (Herzner et al., 2015). ssDNA is also recognized by cGAS, but the structural mechanism is less understood. Lastly, it has recently been proposed that cGAS recognizes micronuclei-forming DNA (MacKenzie et al., 2017).

DNA-bound cGAS undergoes dimerization (Xu Zhang et al., 2014). Activated cGAS dimers then catalyse the secondary messenger cGAMP that next binds to the adaptor protein STING. Of note, cGAMP can also be transferred from one cell to another by viral particles, thus potentially accelerating and broadening immune antiviral responses (Bridgeman et al., 2015; Gentili et al., 2015).

STING is anchored within the endoplasmic reticulum membrane in resting conditions (Ishikawa & Barber, 2008). Upon cGAMP binding, STING undergoes a conformational change and navigates to the Golgi apparatus (Dobbs et al., 2015; Ishikawa et al., 2009; Saitoh et al., 2009). During this process, it recruits TBK1 (Ishikawa et al., 2009; Saitoh et al., 2009). Together, they phosphorylate the transcription factor IRF3 (S. Liu et al., 2015) thereby triggering the production of pro-inflammatory genes. STING also turns on the NF- κ B pathway by activating IKK (T. Abe & Barber, 2014), leading to the expression of pro-inflammatory cytokines, chemokines and interferons including IL-6, IFN- β and CXCL10. STING-mediated NF- κ B activation is required for full production of type I IFNs in MEFs (T. Abe & Barber, 2014). A positive feedback loop may exist between the IRF3 and NF- κ B pathways as it has been recently proposed that activated IKK is required for full TBK1 activation (Fang et al., 2017).

The cGAS-STING pathway mediates immune responses not only against DNA-containing pathogens, but also against self-DNA as well as tumor-derived DNA. This pathway indeed responds to cytosolic DNA, which appearance has been linked to auto-inflammatory diseases such as Aicardi Goutières, or Systemic Lupus Erythematosus. In addition, such metabolites likely affect the host susceptibility to pathogens and contribute to inflammation-associated cancer (for review: Barber, 2015). Importantly, STING signaling is essential both for protecting the cell against a variety of pathogens ranging from bacteria to retroviruses and against the development of cancer by promoting anti-tumor immune responses.



*Figure 7 **STING-mediated anti-tumor response:** STING-dependent type I IFN production is important for Cytotoxic T lymphocyte (CTL) priming and the recruitment of immune cells. Dying tumor cells are engulfed by specialized Dendritic Cells (DC). DNA from the engulfed cell likely triggers STING-dependent cytokine production in the phagocyte, which facilitates cross-presentation and anti-tumor CTL responses. CDN: Cyclic DiNucleotides (eg, cGAMP). Adapted from Barber, 2015.*

Indeed, the STING pathway is essential for a cytotoxic CD8⁺ T cell anti-tumor response, whose activation relies on the production of IFN- β by specialized Dendritic Cells (Figure 7). For instance, STING-deficient mice are unable to mount an efficient adaptive anti-tumor reaction either spontaneously against immunogenic tumors, or in response to radiation (Deng, Liang, Burnette, et al., 2014; S. R. Woo et al., 2014).

Cytosolic DNA recognition by the cGAS-STING pathway is now recognized as a key mediator of the therapeutic efficacy of radiation.

Conversely, STING gain of function mutations results in constitutive type I IFN production, which is associated with an auto-inflammatory vasculopathy disorder. In addition, cytosolic DNA in ATM-deficient cells is recognized by the cGAS-STING pathway, leading to the production of type I IFNs that likely contributes to the inflammatory phenotype of the patients with Ataxia telangiectasia (Härtlova et al., 2015). Aberrant STING signaling is also associated with tumorigenesis.

For instance, the carcinogen polyaromatic hydrocarbon 7,12-dimethylbenz[α]anthracene (DMBA) induces genomic DNA lesions, leading to the leakage of DNA fragments into the cytosol thereby activating STING-mediated inflammatory responses. STING-knock out mice are resistant to DMBA-induced tumor formation, indicating that this signaling pathway indeed plays an important part in tumorigenesis (Ahn et al., 2014).

3. An integrative view of DNA sensing

Individual contribution of these DNA sensors to inflammation is still a matter of debate. cGAS is the only DNA sensor that has been clearly validated using genetics: after infection with herpes simplex virus 1, none of the cGAS ^{-/-} mice survived more than 5 days (X.-D. Li et al., 2013). Engineered mice lacking all AIM2-like receptors including the IFI116 homolog do not have apparent defects in IFN induction following infection with DNA viruses (Gray et al., 2016). However, other results have shown that IFI116 is important for IFN production by cooperating with cGAS, at least in human macrophages (Almine et al., 2017; Jønsson et al., 2017).

Chapter 2: The maintenance of genome stability

During a life time, every dividing cell undergoes around 10^{16} divisions. Each cell synthesizes therefore more than 2×10^{16} meters of DNA, which represents 130,000 times the distance from Earth to the sun. This fundamental process is highly controlled so that DNA, which carries the genetic information required for the functioning, development and survival of all known living organisms, is accurately delivered to each daughter cells. Yet, cells are exposed to tens of thousands of DNA damage events every day, arising from internal and external sources (Hoeijmakers, 2009). Equally important are the natural impediments faced by replication forks every cell cycle, resulting from the nature and organization of the genome itself. Faulty DNA replication likely is a major cause of DNA lesions. If cells continue to proliferate despite a defective repair of these damages, their genetic information can permanently change. Besides, chromosomal aberrations may arise, leading to genomic instability and favoring cancer development (Halazonetis, Gorgoulis, & Bartek, 2008). Persistent DNA damage is also linked with chronic inflammation (Nakad & Schumacher, 2016), as will be described in the next chapter. Accordingly, faithful DNA replication, secured by orderly cell cycle signaling and appropriate responses to fork stalling, is a fundamental process for the maintenance of genome stability and tissue homeostasis.

I- Cell cycle regulation

The cell cycle is constituted of a series of ordered events in which doubled cellular constituents are segregated into daughter cells, before being duplicated again (Figure 8). Genomic DNA is copied during the Synthesis or S phase and chromosome segregation occurs in Mitosis or M phase. Two gap phases, G1 and G2, separate these two steps. During G1, cells integrate growth signals and get ready to duplicate their genomes. Throughout G2, replicated DNA is organized and prepared for chromosome segregation. From G1, cells can also exit the cell cycle and enter a state of cell-cycle arrest termed G0, which can be either transient (quiescence) or permanent (upon terminal differentiation or senescence).

Progression into the cell cycle is highly regulated to avoid unscheduled proliferation, which is known to induce genomic instability and cancer development (for review: Malumbres & Barbacid, 2009).

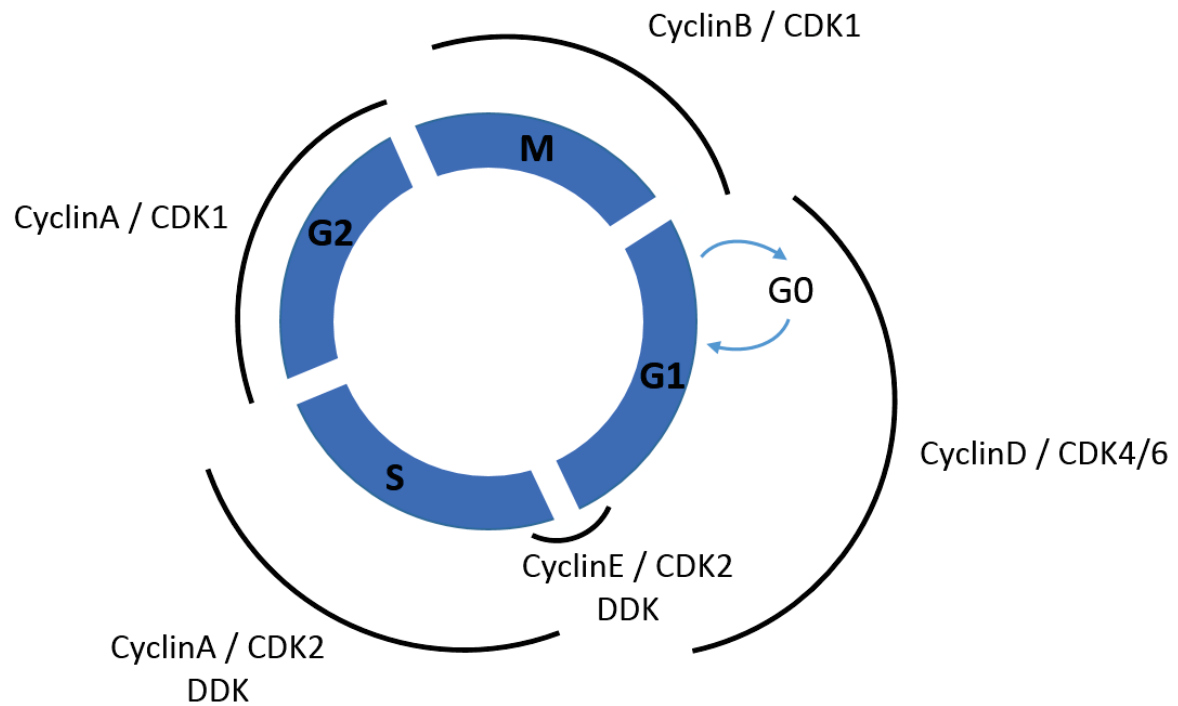


Figure 8 Regulation of the mammalian cell cycle by Cyclin/CDKs. The four steps of the mammalian cell cycle are regulated by distinct Cyclin/CDKs pairs, together with the Cdc7/DBF4 kinase which is required for DNA replication. See text for details.

Cyclin-Dependent Kinases (CDK), a series of serine/threonine kinases, ensure this tight coordination in partnership with cyclin proteins. Progression through each stage of the cell cycle requires the phosphorylation of specific substrates by different cyclin/CDK pairs.

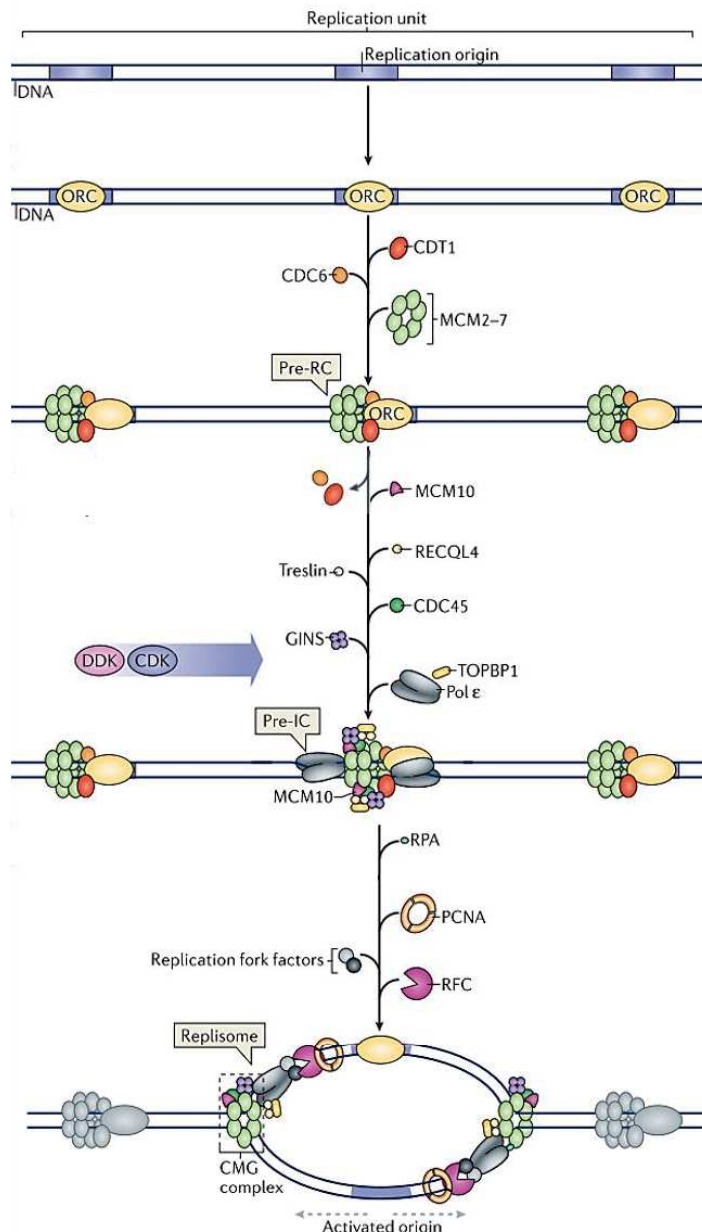
While CDK protein levels remain stable all along the cell cycle, the expression of cyclins fluctuates in a phase-specific manner, thereby allowing for the periodic activation of CDKs. In G1, the activity of Cyclin D/CDKs allow progression into S Phase. Cyclins E and A accumulate at the G1/S boundary where they successively activate CDK2 and CDK1, promoting progression through G2. At this point, cyclins B, chiefly CyclinB1 and CDK1, drives cells to mitosis.

In addition to cyclin binding, CDK activity is controlled by the action of specific kinases and phosphatases. For instance, the phosphatase CDC25 can activate CDK1. On the contrary, the same CDK is turned off by the kinase activity of WEE1. To make things more complex, small inhibitory proteins, the cyclin-dependent kinase inhibitors, also regulate CDKs (for review: Besson, Dowdy, & Roberts, 2008). For instance, the INK4 family member p16 binds to CDK4 and CDK6, blocking their association with cyclins D, thereby impairing their kinase activity. In contrast, the CIP/KIP family member p21 binds to all cyclin-CDK complexes and primarily inhibits the kinase activity of CDK2 and CDK1.

Multiple checkpoints additionally secure the stability of the genome in response to a wide range of internal and external stresses (for review: (Shaltiel, Krenning, Bruinsma, & Medema, 2015). Overall, there are four reversible checkpoints, one in each phase of the cell cycle. The G1/S checkpoint blocks entry into the S phase to prevent replication of damaged genomic material. The S phase checkpoint delays DNA replication to allow the restart and repair of arrested replication forks in case of DNA damage and aberrant DNA replication. In the event of persisting DNA lesions, the G2/M checkpoint delays mitosis thereby preventing cells with damaged DNA to divide. Finally, the spindle assembly checkpoint allows mitotic exit and return to G1 only if chromosomes are properly attached to the mitotic spindle.

II- DNA replication

The duplication of the genome is achieved through a multi-step process (Figure 9). First, replication is initiated at specific sites all along the genome, in a spatial and temporal-regulated manner.



Licensing of origins: G1

The formation of the pre-replication complex occurs through the loading of the initiation factors ORC, CDC6 and CDT1, which recruits the MCM2-7 helicase complex.

Formation of the pre-initiation complex: G1-S transition

The initiation factors CDT1 and CDC6 are released

The loading of additional initiation factors, orchestrated by DDK and S-CDKs, promotes the recruitment of GINS and CDC45 to build a functional replicative helicase.

Origin firing: S phase.

Other proteins are recruited to build the replisome, such as RFC, RPA, PCNA and DNA polymerases.

MCM double hexamers divides into two hexamers that progress bidirectionally.

Two functional replication forks move in opposite directions from the activated origin.

Figure 9 Formation and activation of DNA replication origins in mammalian cells. Modified from Fragkos, Ganier, Coulombe, & Méchali, 2015a.

Second, DNA is synthesized by fired bi-directional replication forks that separate duplex DNA and copy the entire genome. Third, replication is terminated when converging forks meet. These fundamental tasks are described in the following section.

1. The molecular mechanisms

a) The initiation processes

Chromosomal replication initiates from specific regions of the genome, called replication origins. The human genome, ~3 Gb in length, comprises from around 30 000 to 50 000 of them (Leonard & Mechali, 2013).

A detailed molecular mechanism of the helicase loading and activation processes has been thoroughly identified *in vitro* using purified yeast proteins (Devbhandari, Jiang, Kumar, Whitehouse, & Remus, 2017; Ticaú, Friedman, Ivica, Gelles, & Bell, 2015; Yeeles, Deegan, Janska, Early, & Diffley, 2015; Yeeles, Janska, Early, & Diffley, 2017). In eukaryotes, DNA replication starts by the licencing of all replication origins, followed by the formation of the pre-initiation complex and its activation.

-Licencing

This step, beginning in early G1, allows the formation of the pre-replication complex. Two inactive MCM2-7 hexamers are sequentially loaded onto DNA by two ORC proteins (Coster & Diffley, 2017), together with CDC6 and the CDT1 chaperone (for review: Bleichert, Botchan, & Berger, 2017; Deegan & Diffley, 2016). The MCM helicases are loaded as head-to-head double hexamers connected with each other through their N-terminal domains (Evrin et al., 2009; Remus et al., 2009). This oppositely-oriented double MCM helicase complex forms a ring-like structure surrounding dsDNA (Evrin et al., 2009; Remus et al., 2009). After MCM loading, the initiation factors ORC, CDC6 and CDT1 are successively released (Ticaú et al., 2017).

-Formation of the pre-initiation complex and origin firing

Unlike origin licencing, activation of the MCM helicase requires high levels of S-phase CDK activity (for review: (Parker, Botchan, & Berger, 2017) During this step, double MCM hexamers dissociates into two active helicases binding to the leading strand DNA template and moving along in the 3' to 5' direction to unwind double-stranded DNA. A functional replicative helicase is formed following the recruitment of CDC45 and GINS (Ilves, Petojevic, Pesavento, & Botchan, 2010).

At this stage, sequential phosphorylation of MCM subunits carried out by DBF4-dependent kinase (DDK, also known as CDC7-DBF4 kinase) together with S-CDKs is crucial for CDC45 and GINS binding to MCM2-7. Indeed, concerted activity of CDK and DDK recruits key assembly factors such as TopBP1 and Treslin that allows for the sequential recruitment of CDC45 and GINS. Once built,

this CMG (CDC45–MCM–GINS) complex participates in the formation of the pre-initiation complex, constituted of additional proteins such as DNA polymerase ϵ (Pol ϵ), MCM10, RecQL4. The concerted activity of these proteins, regulated by S-CDKs and DDK, is required for the formation of a functional replicative helicase and its activation.

Once the CMG is assembled, many additional proteins are recruited to form the intricate molecular machinery known as the replisome. DNA combing analyses have shown that forks travel approximately at 1–2 kb per min (Conti et al., 2007). *In vitro* reconstitution of replisomes capable of such replication rates requires Mrc1 with Csm3 and Tof1 (Yeeles et al., 2017), which mammalian homologs are Claspin, Tipin and Timeless respectively.

b) DNA elongation

At this point, unwinding of parental strands by the helicase is coordinated with the synthesis of new DNA strands by replicative polymerases.

DNA unwinding by the CMG helicase leads to the generation of ssDNA coated by RPA, a template for the priming of DNA replication by the Pol α primase. This complex synthesizes short RNA–DNA primers through its primase subunit and incorporates a limited number of deoxyribonucleotides triphosphate (dNTPs) after the RNA primers using its DNA polymerase subunit (for review: Pellegrini, 2012). These primers, located both on the leading and the lagging strands, are subsequently elongated by Pol ϵ and Pol δ respectively (Nick McElhinny, Gordenin, Stith, Burgers, & Kunkel, 2008; Pursell, Isoz, Lundstrom, Johansson, & Kunkel, 2007). Primer elongation on the leading strand continuously follows the direction of the CMG complex, with DNA being synthesized on the 5' to 3' direction. On the lagging strand however, DNA is synthesized discontinuously as the polarity of the parental strand is inverted. Consequently, DNA is duplicated through the generation of short Okazaki fragments which are then ligated to form a continuous daughter strand (for review: Balakrishnan & Bambara, 2013).

The Proliferating Cell Nuclear Antigen (PCNA) is also required for this process. Both the Replication Factor C (RFC) and the CTF18-RFC-like complex catalyze its loading onto DNA (Fujisawa, Ohashi, Hirota, & Tsurimoto, 2017; Yao & O'Donnell, 2012). This processivity factor in turn forms a ring-shaped sliding clamp that tethers Pol ϵ and Pol δ to their template to promote high-speed DNA replication (Yeeles et al., 2017).

c) Termination

Termination events are as abundant as initiations, yet they are poorly understood (for review; (Dewar & Walter, 2017).

As replisomes progress along the genome, topological stress accumulates in the form of positive supercoils ahead of the forks. These structures are released by type I and type II DNA

topoisomerases. In *Xenopus* egg extracts, resolution of this topological stress is not rate-limiting for the convergence of two adjacent replication forks (Dewar, Budzowska, & Walter, 2015). In addition, encounter of two converging forks does not induce detectable fork pausing. Completion of DNA replication however requires the disassembly of the replication machinery, including unloading of PCNA and the CMG complex (Kubota, Nishimura, Kanemaki, & Donaldson, 2013; Maric, Maculins, De Piccoli, & Labib, 2014).

2. Local and temporal control of the replicative process

Metazoan Origins of Replication

Unlike in budding yeast, where DNA replication initiates at autonomously replicating sequences containing a consensus element, mammalian origins of replication do not harbor a specific target sequence. Nevertheless, several genetic and epigenetic features are associated with metazoan DNA initiation (for review: Prioleau & Macalpine, 2016). First, ORC preferentially binds to nucleosome-free regions (Karnani, Taylor, Malhotra, & Dutta, 2010; MacAlpine, Gordân, Powell, Hartemink, & MacAlpine, 2010; Sequeira-Mendes et al., 2009). Second, G-rich sequences and CpG islands are also highly enriched at replication initiation sites (Cadoret et al., 2008), likely because they favor the maintenance of a nucleosome-free region (Fenouil et al., 2012). Third, ORC binding is fine-tuned by interactions with nucleosomes and chromatin-associated factors such as histone modifications. For instance, mono-methylation of lysine 20 of histone H4 by the methyltransferase PR-Set7 promotes the loading of pre-RC proteins onto chromatin (Tardat et al., 2010). To make things more complex, replicative helicases may be displaced away from ORC, for instance by transcription machineries (Gros et al., 2015; S. K. Powell et al., 2015).

Regulation of replication initiation

Both origin licencing and firing are highly controlled in G1 and S phases to ensure that origins are fired only once per cell cycle (for review: Siddiqui & Diffley, 2013). These two steps are temporally separated so that new origin licencing is prevented once cells have entered S phase. This is achieved through the concerted action of two enzyme complexes: the CDK and the Anaphase-Promoting Complex/Cyclosome (APC/C) that couples genome duplication with cell-cycle signaling. APC/C is an E3 ubiquitin ligase that polyubiquitinates proteins such as licencing inhibitors to tag them for proteasomal degradation. All pre-RC are assembled before cells enter S phase, so they can be activated by S-phase CDKs and DDK. Origin licensing occurs exclusively during late mitosis and G1, when APC/C activity is high and S-CDK activity is low. On the contrary, CDK phosphorylation events inhibit pre-RC assembly during S, G2, and M phases.

In addition, CDK-independent mechanisms also play a critical role in the regulation of replication initiation. For instance, CDT1 protein levels and activity are thoroughly regulated by degradation during S phase and interaction with geminin, a negative regulator, during S and G2/M phases.

Origin plasticity

Although all replication origins are licenced, not all origins are fired in every cell during each cell cycle (for review: Aladjem & Redon, 2017).

Indeed, replication origins do not fire simultaneously: some are activated early in S phase whereas other begin to replicate later (for review: Rhind & Gilbert, 2013). Replication origins sharing similar replicating timing profiles are often clustered, forming 'replication-timing domains' (see Figure 10). These domains tend to overlap with chromatin compartments known as topologically associated domains (Pope et al., 2014). In fact, early replicating origins associate with euchromatic histone modifications, whereas late replication correlates with repressive chromatin marks (Picard et al., 2014). The first are typically enriched inside the nucleus, the latter at the nuclear periphery. Replication, transcription and chromosome condensation are likely coordinated through the establishment of these domains. In addition, they also may be important to facilitate recovery in case of replication stress (Fragkos et al., 2015). The spatial and temporal regulation of replication is indeed essential to maintain genome stability (for review: Blumenfeld, Ben-Zimra, & Simon, 2017).

III- Causes of replication stress

During each S phase, hundreds of replisomes are susceptible to simultaneously run along chromatin to perform DNA synthesis. Correct execution of this replication program is essential for the maintenance of genome integrity.

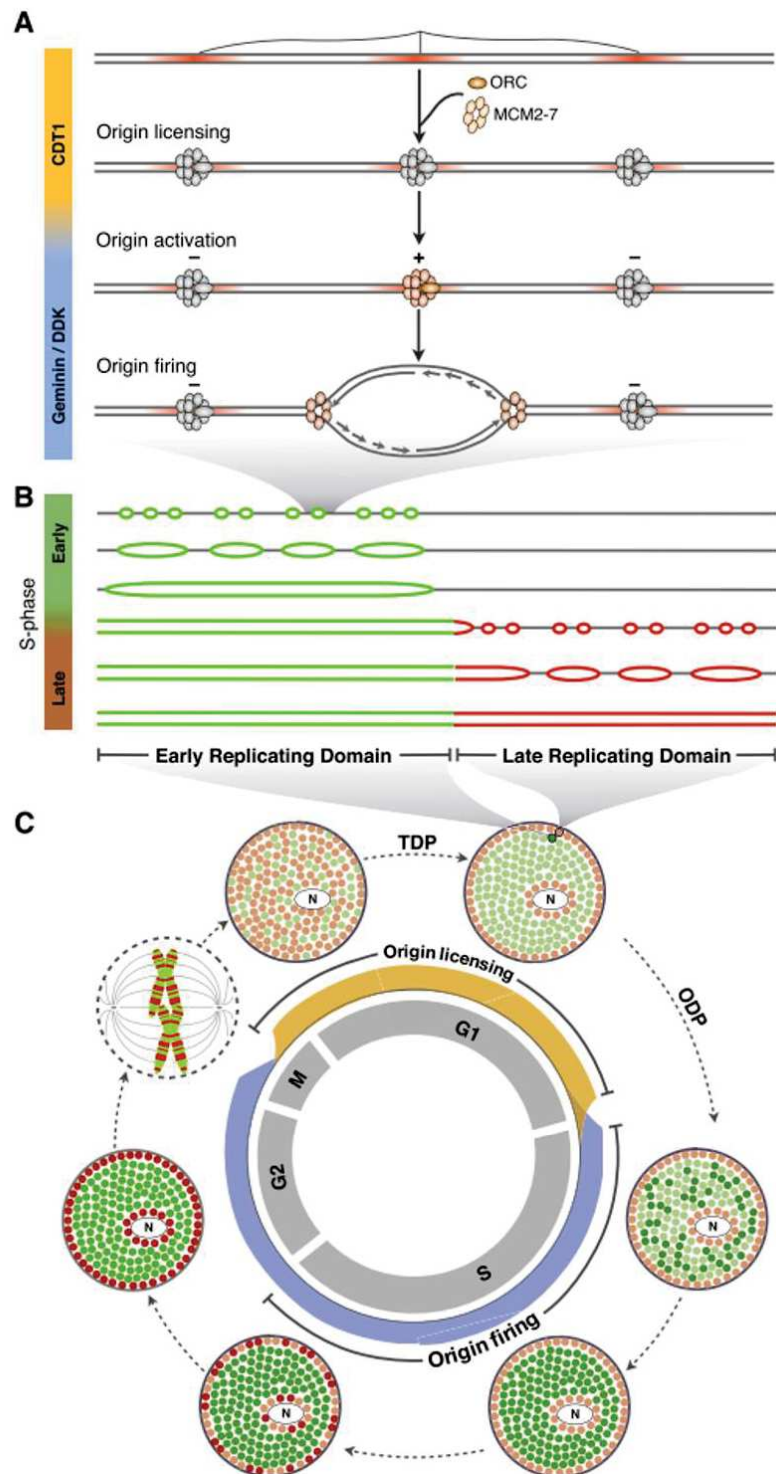


Figure 10 The distinct scales of DNA replication regulation: (A) All potential replication origins are licensed, but only ~10% are fired. (B) Early and late-replicating origins are clustered, partitioning the chromosomes into replicative domains. (C) Replicative domains are themselves segregated in the nucleus such that early replicating domains are located inside the nucleus while late replicating domains are located at the nuclear and nucleolar periphery. Modified from Rivera-Mulia & Gilbert, 2016.

Yet, a large variety of impediments routinely challenge the course of progressing replication forks. Indeed, DNA combing analyses have shown that, in cell lines, 10–20% of the forks display a sign of fork pausing or stalling, as measured by fork asymmetry (Técher et al., 2013). This transient slowing, pausing and stalling of replication forks is known as replication stress.

The progression of replication forks can be altered by diverse blocking agents of endogenous and exogenous origin (Figure 11). Overall, they are distributed in three main categories: physical barriers, shortage in replication factors and transcription-dependent impediments to replication progression. Two additional classes of stresses will be described in this section: oncogene-induced replication stress and common fragile sites. They encompass many causes of fork arrest, some of which are still being investigated.

1. Physical barriers to replication forks

DNA lesions:

Both the nitrogenous bases and the sugar-phosphodiester backbone of DNA can be damaged, causing replication fork slowing. Endogenous sources of base damage can cause up to an estimated ~15 000 lesions per cell per day (Tubbs & Nussenzweig, 2017). These include spontaneous DNA depurination, cytosine deamination and byproducts of cell metabolism such as Reactive Oxygen Species (ROS) (Ciccia & Elledge, 2010). The endogenous production of ROS can generate Inter-Strand Crosslinks (ICL), abasic sites and oxidized DNA bases, all of which are able to block the progression of polymerases (Wallace, 2002). Other endogenous molecules are highly reactive towards DNA such as aldehydes and formaldehyde, byproducts of alcohol metabolism and enzymatic demethylation. These molecules have been extensively studied in the context of Fanconi Anemia, a genetic disease characterized by defects in ICL repair (Langevin, Crossan, Rosado, Arends, & Patel, 2011; Pontel et al., 2015). Cisplatin and Mitomycin C, agents used in cancer therapy, covalently bind the two strands of DNA as well, creating these aberrant DNA structures. Although they unquestionably slow down the progression of replication forks, replisomes are able to traverse these structures with the help of the FANCM translocase (Huang et al., 2013).

Even though they preferentially incorporate deoxyribonucleotides, DNA polymerases can also use ribonucleotides during DNA replication. Indeed, Pol ϵ and Pol δ have high fidelity in base-pairing, yet they are less able to distinguish dNTPs from rNTPs.

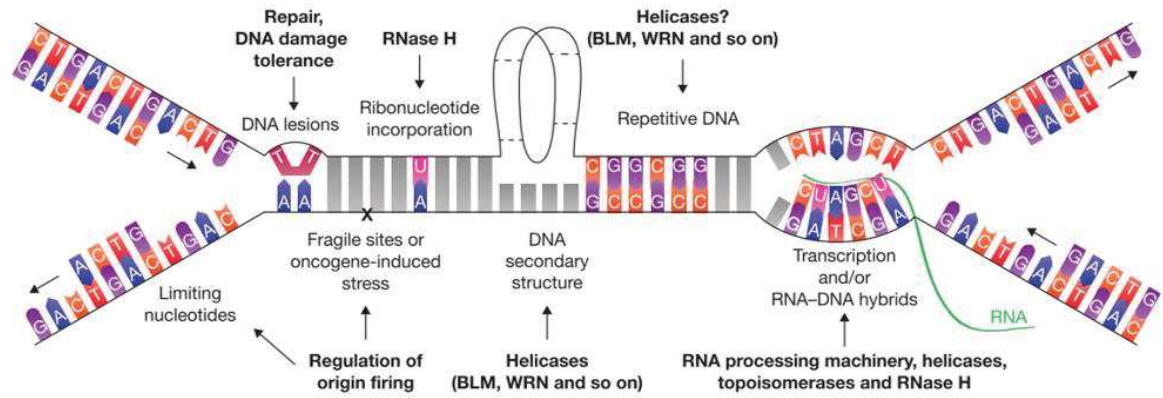


Figure 11 **Sources of replication stress:** The conditions and obstacles that can slow down DNA replication are illustrated, with their respective key resolution pathways in bold. Adapted from (Zeman & Cimprich, 2014)

Misincorporation of rNTPs occurs at high rate during DNA replication, possibly leading to the stalling of the replicative polymerases in the next cell cycle if they are not excised (Clausen, Murray, Passer, Pedersen, & Kunkel, 2013). Single-embedded ribonucleotides are normally removed by a process initiated by the RNaseH2 enzyme (for review: Cerritelli & Crouch, 2016; Dalgaard, 2012).

A plethora of exogenous factors such as environmental elements (ultraviolet radiation from sunlight, ionizing radiation) and chemical genotoxic agents (DNA alkylating agents, topoisomerase inhibitors) can also give rise to DNA lesions (Ciccio & Elledge, 2010).

DNA secondary structures

Non-B DNA structures like hairpins, cruciform, slippage loops, triplex DNA, Z-DNA and G-quadruplexes may also threaten the progression of replication forks (Boyer, Grgurevic, Cazaux, & Hoffmann, 2013; G. Wang & Vasquez, 2017). For instance, hairpins form when two DNA sequences of the same strand fold into a double helix by base-pairing. They act as barriers for the replication machinery as they cause both polymerase and helicase stalling (see Figure 12).

Of particular interest are G-quadruplexes, potentially important for replication initiation in mouse and human cells (Besnard et al., 2012; Cayrou et al., 2011). G-quadruplexes are four-stranded helical structures formed at G-rich sequences (Valton & Prioleau, 2016). They tend to accumulate at promoter regions, in the 5' or 3' untranslated regions of mRNAs and at telomeres. Although they may have a role in normal cell metabolism, G-quadruplexes are potent threats for the replisome, especially in cells already exposed to replication stress such as cancer cells (Subhajyoti & Michor, 2011). Accordingly, in specific backgrounds, fork arrest induced by G-quadruplexes may promote epigenetic instability (Sarkies, Reams, Simpson, & Sale, 2010).

These natural sources of replication stress arise at specific DNA sequences. Among these, repetitive sequences such as trinucleotide repeats, palindromic sequences, microsatellites, minisatellites and satellites are largely found throughout the genome (Lander et al., 2001). In line with this, the accessibility and progression of replisomes can be hindered at heterochromatin regions such as centromeres and telomeres which consist of repetitive sequences and tight DNA-protein complexes.

Repeats and protein-DNA complexes also mediate an additional programmed replicative arrest, located at ribosomal DNA and at tRNA loci.

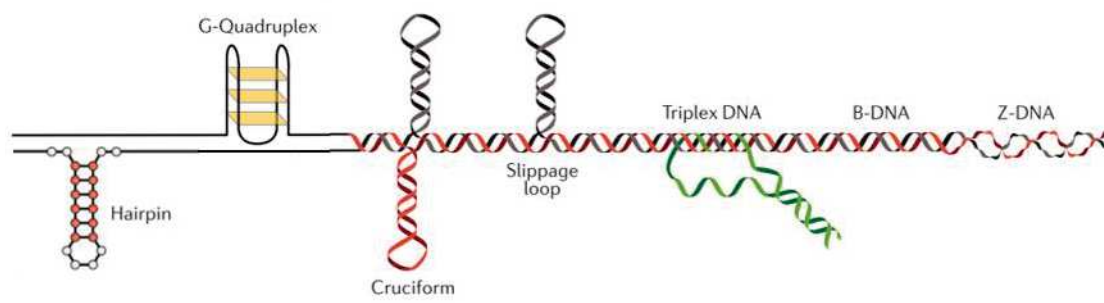


Figure 12 Hard-to-replicate DNA secondary structures: These non-B structures are suspected to induce fork stalling. Adapted from (Técher, Koundrioukoff, Nicolas, & Debatisse, 2017)

At the former set of genes, ribosomal RNAs are massively produced all along the cell cycle. There, replication fork barriers consists of repetitive sequences located downstream of the ribosomal coding region, bound by proteins such as Fob1 in budding yeast (Kobayashi, 2003). These replication roadblocks likely coordinate replication and transcription in S phase to prevent damage (for review: Gadaleta & Noguchi, 2017).

2. Transcription-dependent impediments to fork progression

The transcription and replication machineries may travel along the same DNA sequence at the same time, for example at genes highly transcribed in S-phase. Distinct types of replication stress are induced by transcription (for review, Aguilera & García-Muse, 2013).

First, replication forks and RNA polymerases can collide. Since the polarity of DNA and RNA synthesis is identical, such encounters can happen co-directionally in the leading strand template or head-on on the lagging strand. Recent evidence suggests that these collisions may not always induce fork stalling in mammalian cells, unless they involve the formation of an R-loop (Hamperl, Bocek, Saldivar, Swigut, & Cimprich, 2017).

Second, as both processes unwind DNA, they create topological stress. In fact, tethering of the transcribed genes to the nuclear pore has been reported to generate topological constraints, thereby leading to replication stress even before the two machineries ever meet (Bermejo et al., 2011). Nevertheless, the simultaneous progression of converging RNA and DNA polymerases along the same template generates positive supercoiling. Although this topological constrain is a source of replication stress, cells harbor helicases and topoisomerases that routinely induce DNA breaks to maintain an appropriate level of torsion (Bermejo et al., 2009; Tuduri et al., 2009).

Finally, the rehybridization of nascent RNA transcript with its template DNA can lead to the formation of R-loops, three-stranded nucleic acid structures composed of an RNA-DNA hybrid, plus a single-stranded DNA sequence. R-loops can be formed all along the genome, with a preference for GC-rich sequences (Gino 2009). They have been implicated in specific physiological processes such immunoglobulin class-switch recombination and the regulation of gene expression (see Santos-Pereira and Aguilera 2015 for review). Beyond these physiological roles, R-loops potentially interfere with the replication machinery, induce DNA breaks (Tuduri et al., 2009), and are associated with instability at common fragile sites (Helmrich, Ballarino, & Tora, 2011).

3. Shortage of replication components

A number of factors are required for DNA replication. Their limitation can impede the progression of replisomes, ultimately resulting in replication stress.

dNTP pools

DNA polymerases rely on the availability of dNTPs to accurately synthesize DNA. More specifically, sufficient and balanced dNTP pools are required in order to avoid fork stalling and maintain the fidelity of DNA polymerases (for review: Pai & Kearsey, 2017; Técher, Koundrioukoff, Nicolas, & Debatisse, 2017). This is obtained by two mechanisms (see Figure 13). First, synthesis of dNTPs by the ribonucleotide reductase (RNR) enzyme is important to supply nucleotides during S phase. Various regulatory pathways allow for a timely biosynthesis of the nucleotides, including the activation and increased expression of the RNR. Second, dNTPs levels must be accurately regulated during the G1 phase. An active dNTP degradation pathway is mediated by the dNTPase SAMHD1 whose activity maintains low levels of dNTPs outside of S-phase. Depletion of SAMHD1 in mammalian cells increases the dNTP pools and perturbs cell cycle progression (Franzolin et al., 2013). Upon loss of SAMHD1, non-transformed cycling fibroblasts accumulate in G1 and do not enter S phase until SAMHD1 protein levels are restored. SAMHD1 regulates not only the size but also the proportions of individual dNTP pools. Indeed, SAMHD1 deficiency primarily affects its preferred substrate, dGTP, which is normally the smallest pool (Franzolin et al., 2013). Consequently, both nucleotides catabolism during G1 and synthesis during the S phase are essential for DNA replication.

dNTP biosynthesis is performed and controlled by several dozen proteins (for review: Aye, Li, Long, & Weiss, 2014; Mathews, 2015). The RNR enzyme catalyzes a rate-limiting step in nucleotide biosynthesis: the reduction of a ribonucleoside diphosphate (NDP) to produce the corresponding deoxy-NDP (dNDP). dNDPs are subsequently phosphorylated by the nucleoside diphosphate kinase to generate dNTPs. Meanwhile, ribonucleotides are produced through two pathways: *de novo* synthesis and a salvage pathway. *De novo* synthesis involves the stepwise assembly of purine and pyrimidine rings, while salvage pathways engages nucleobases and nucleosides arising from nucleic acids degradation.

In mammalian cells, RNR consists of two subunits: either RRM1 working together with RRM2 or RRM1 associated with P53R2. All these RNR subunits are constitutively located in the cytoplasm where they produce dNTPs that diffuse into the nucleus (G. Pontarin et al., 2008).

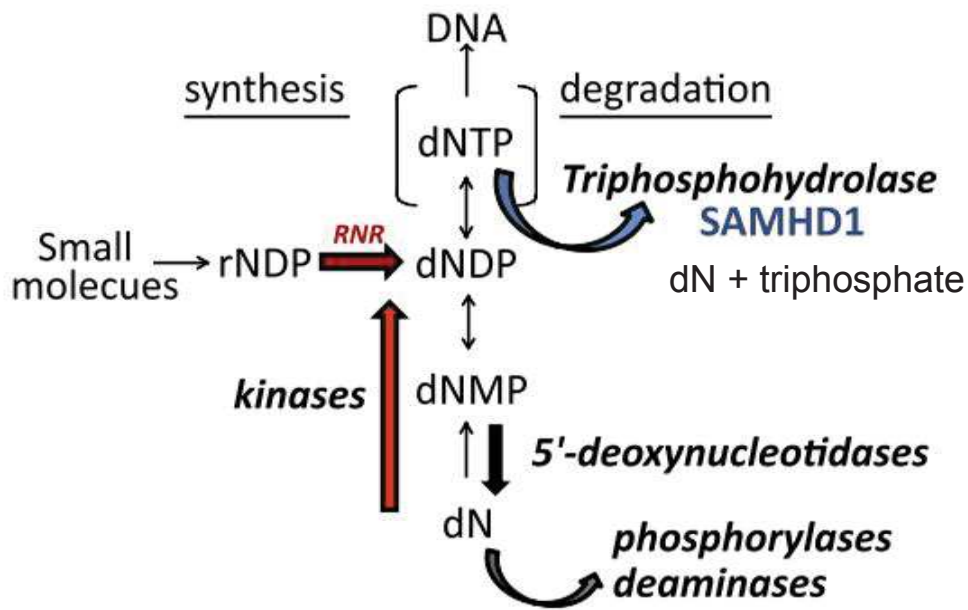


Figure 13 Regulation of dNTP production: Amounts and proportions of the dNTP pools required for DNA replication are regulated both by synthetic and catabolic processes. Synthesis occurs by either *de novo* synthesis or salvage pathways mediated by deoxy-nucleoside kinases. dNTP catabolism is either directly mediated by the nuclear triphospho-hydrolase SAMHD1, or via a stepwise pathway ending with the action of 5'-deoxynucleotidases and phosphorylases/deaminases. Modified from (Franzolin et al., 2013)

RRM1 and RRM2 in part translocate inside the nucleus upon DNA damage (Niida et al., 2010), but it is not known whether dNTP synthesis actually occurs at repair sites. RRM2, together with RRM1, provides dNTPs for genome duplication and repair in proliferating cells. P53R2 is involved in dNTP supply for mitochondrial DNA replication and repair as well as nuclear DNA repair in quiescent cells (Giovanna Pontarin, Ferraro, Bee, Reichard, & Bianchi, 2012).

RNR is tightly regulated along the cell cycle so that dNTPs are synthesized in a balanced manner only during S phase. Its activity, levels and cellular localization are controlled.

For instance, one of the proteins targeted by the APC/C complex during mitosis and the G1 phase is RRM2. Unlike RRM2, RRM1 and P53R2 protein levels are stable throughout the cell cycle.

Increasing the number of active replication forks titrates the number of dNTPs available for each progressing replication forks, thereby slowing down their progression. This is best illustrated by the activation of oncogenes like Cyclin E, Myc, Ras, CDC6 and CDT1 that induces origin hyperactivation and dNTP exhaustion (for review: Hills & Diffley, 2014; Sarni & Kerem, 2017).

Some molecules can also reduce the level and affect the balance of dNTP pools. For example, the reversible ribonucleotide reductase inhibitor hydroxyurea (HU) leads to the depletion of dNTPs by inhibiting the conversion of ribonucleotide diphosphates into deoxyribonucleotides diphosphate. HU induces a dose-dependent depletion of dNTPs and an imbalance between purine and pyrimidine pools thereby slowing down the progression of all active replication forks. Another molecule commonly used to impede DNA replication, aphidicolin, directly inhibits the DNA polymerases α , ϵ and δ . At low doses, aphidicolin selectively block the incorporation of dNTP by pol α by binding to its active site. Unlike HU, polymerase inhibition by aphidicolin results in the formation of long ssDNA stretches as it uncouples the polymerases from the replicative helicase (for review: Vesela, Chroma, Turi, & Mistrik, 2017).

RPA

The DNA binding protein RPA, which protects single-stranded DNA, is essential both for the replication process and for the preservation of fork integrity (for review: Toledo, Neelsen, & Lukas, 2017). Although produced in substantial amounts (according to Toledo et al., human RPA is in 6- to 10-fold excess relative to the amount needed during unstressed replication), RPA becomes limiting if origin firing is mis-regulated, for instance in case of checkpoint inhibition. Under those circumstances, new ssDNA stretches created at each firing origin cannot be protected, making replication forks highly susceptible to collapse and breakage. In other words, arrested replication forks in ATR-inhibited cells undergo massive cleavage, an event that is delayed upon overexpression of RPA (L. I. Toledo et al., 2014). During normal cell metabolism,

RPA titration might be induced by processes generating ssDNA gaps such as nucleolytic degradation of nascent DNA at arrested forks.

The chromatin environment

The accurate duplication of the DNA sequence is not sufficient for genome stability and function. Proper organization of DNA into chromatin is also essential for this process. In fact, DNA replication is coupled with nucleosome disruption ahead of the fork and reassembly on the daughter strands (for review: Prado & Maya, 2017). As a result, histones and histone chaperones are important substrates for DNA replication. Histone availability and supply as well as efficient nucleosome assembly are all required for fork progression (Groth et al., 2007; Mejlvang et al., 2014). For instance, DNA unwinding by the replicative helicase is directly paired with histone supply (Groth et al., 2007). Perturbing the chromatin environment thus contributes to replication stress.

4. Oncogene-induced replication stress

Oncogene-induced replication stress is a major driving force of tumorigenesis (Halazonetis et al., 2008). Several processes underlie oncogene-mediated replication stress, including: replication/transcription conflicts, shortage of replication factors and origin deregulation (Figure 14) (For review: Macheret & Halazonetis, 2015; Sarni & Kerem, 2017). Notably, the group of Thanos Halazonetis recently provided a global mechanism for all these events. They demonstrated that following oncogene activation, the length of G1 phase was reduced leading to an unscheduled firing of intragenic origins (Macheret & Halazonetis, 2018). Activation of these origins is normally suppressed by gene transcription during the G1 phase (S. K. Powell et al., 2015). Conflicts between these unexpected replication forks and transcription machineries thereby induces DNA damage. In other words, hasty entry into S-phase induced by oncogene activation triggers replication stress and DNA damage by increasing conflicts between replication and transcription (Macheret & Halazonetis, 2018).

5. Common fragile sites

As the name indicates, common fragile sites (CFS) are fragile chromosomal regions highly prone to breaking during mitosis, in a cell-type dependent manner.

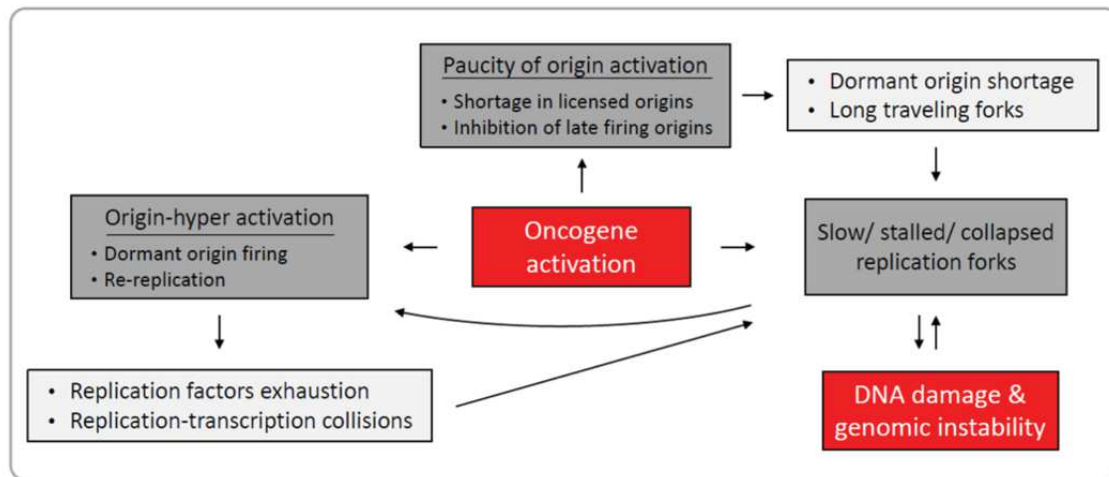


Figure 14 Oncogene activation causes replicative stress and genomic instability: *Oncogene activation can interfere both with replication initiation and elongation, leading to hyper-activation and/or paucity of initiation events as well as fork stalling. Deregulated replication generates DNA damage. Dark grey boxes are manifestations of deregulated replication, light grey boxes are the potential outcomes of origin deregulation. Modified from (Sarni & Kerem, 2017)*

CFS may arise as a result of mitotic entry prior to the completion of replication in late-replicating regions. These regions are highly sensitive to even mild levels of replication stress. Several factors predispose CFS to instability, including delayed replication completion, non-canonical DNA structures, origin shortage and collision between replication and transcription complexes along large genomic regions (for review: Irony-Tur Sinai & Kerem, 2017; Le Tallec et al., 2014). Correspondingly, CFS are located within long genes (from 0.5 Mb to several mega bases in length) in regions replicated very late during S phase. In addition, CFS-containing regions harbor a very low density of replication origins, suggesting that rescue of arrested forks by new initiation events is limited in these regions (Le Tallec et al., 2011; Letessier et al., 2011). This shortage of origin firing implies that neighboring forks have to travel long distances and may not be able to complete replication before mitosis. Moreover, the occurrence of breaks at these loci correlates with gene transcription (Helmrich et al., 2011). This observation indicates that, during their course, replication forks likely encounter transcription machineries operating at large genes located within these regions. The generation of breaks at CFS may be favored both by the low density of replication origins and the presence of large genes at these chromosomal regions.

IV-Stalled fork processing

Initially thought to be detrimental for cell survival (Schlacher et al., 2011), fork remodeling is now acknowledged as essential to warrant resumption of DNA synthesis in case of replication stress (Pasero & Vindigni, 2017). In this manuscript, “remodeling” refers to the three processes that can occur at stalled replication forks, involving DNA strand separation/annealing, degradation, and breakage. These are, respectively, fork reversal, processing and cleavage. If properly controlled, limited degradation of DNA at stalled forks, also known as fork resection or processing, might also be important for checkpoint activation. These processes are presented in the present section.

1. Structures generated

Mammalian cells have evolved intricate mechanisms to respond to replication stress. Measures taken by the cell to allow complete duplication of the genome vary according to the fork structures generated in face of the obstacle. These structures are illustrated in the Figure 15.

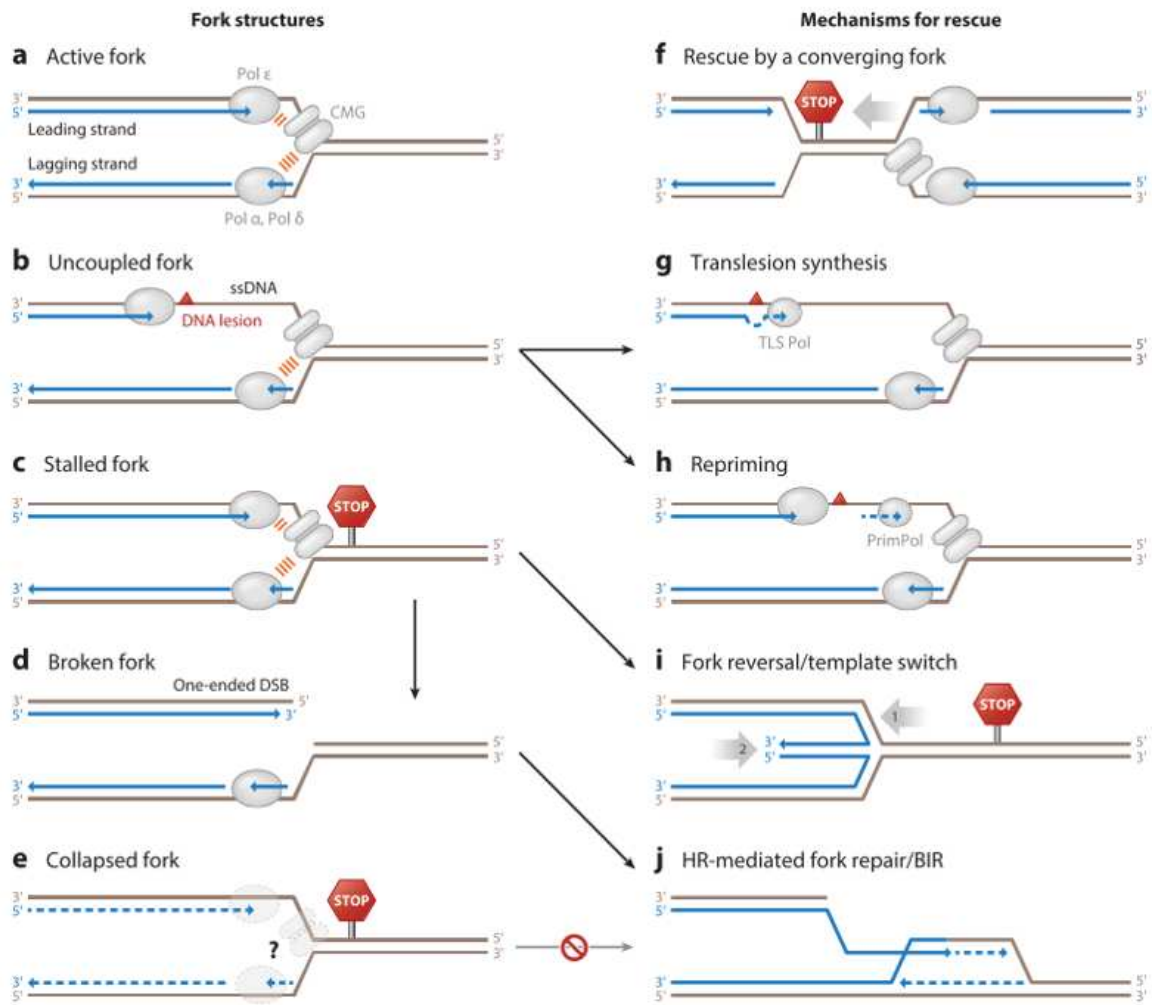


Figure 15 Fork structures generated during replication stress and mechanisms for their recovery: (a) Simplified representation of a functional eukaryotic replication fork. (b) Some lesions that only affect leading-strand DNA polymerases trigger a functional uncoupling between the CMG helicase and the polymerases. Accumulation of ssDNA generates a structure with internal gaps. (c) Other obstacles can affect the replisome entirely, thereby inducing fork stalling. (d) If stalled forks are not properly processed, they may be cleaved by structure-specific endonucleases. Encounter with DNA damage such as ssDNA gaps may also generate such broken forks. (e) Forks unable to restart eventually collapse: they are irreversibly inactivated. (f) Arrested replication fork may be rescued by a converging fork. (g)-(h) gapped fork may bypass the lesion using translesion synthesis (g) or repriming after the lesion (h). (i) Stalled replication forks may be reversed by specific translocases. (j) If they have been cleaved, stalled forks can also be repaired by homologous recombination mechanisms such as BIR. Modified from (Pasero & Vindigni, 2017)

Gaps

It is generally accepted that small DNA lesions can be crossed by the replicative helicase but may impede the progression of DNA polymerases, leading to a physical uncoupling between the two enzymes (Byun, Pacek, Yee, Walter, & Cimprich, 2005). Accordingly, the accumulation of ssDNA at stressed forks may be a passive mechanism in some instances.

Using *Xenopus* egg extracts and budding yeast, the groups of Vincenzo Costanzo and Massimo Lopes showed that, on MMS-damaged DNA templates, single-stranded gaps accumulate behind forks in the absence of RAD51 (Hashimoto, Chaudhuri, Lopes, & Costanzo, 2010).

They managed to roughly quantify the extent of ssDNA generated using the T4 DNA polymerase which has a primer extension and translation synthesis activity but is not able to perform strand displacement. Using electron microscopy as a way to characterize this response more precisely, they additionally showed that MRE11 was responsible for the DNA degradation in RAD51-depleted extracts. They reported one of the first evidence of an active mechanism of DNA degradation at stalled forks. Later, Massimo Lopes reported that mild replication stress induced by treatment with aphidicolin or HU generates ssDNA gaps of ~80 nucleotide-long in human U2OS cells (Zellweger et al., 2015).

Reversed forks

Other DNA lesions such as protein-DNA adducts, or inter-strand crosslinks may frankly halt the progression of the replication machinery. To ensure their stabilization, such stalled forks can be reversed, as illustrated in the figure 16. This operation creates a four-way structure resembling a Holliday junction. Fork reversal or fork regression occurs when the stalled fork reverses its course, leading to the annealing of the newly synthesized DNA strands. This process is considered as an emergency brake, transiently protecting replication forks from DNA damage, and providing space to repair lesions ahead of the fork (Neelsen & Lopes, 2015). If they are not appropriately being taken care of however, reversed forks may be cleaved by structure-specific endonucleases, actively contributing to genome instability (Dehé & Gaillard, 2017).

Fork reversal was first demonstrated at unprotected replication forks in yeast checkpoint mutants in 2002 (Sogo, Lopes, & Foiani, 2002). Its relevance was later recognized in the context of topoisomerase-1 poisoning (Camptothecin) (Ray Chaudhuri et al., 2012).

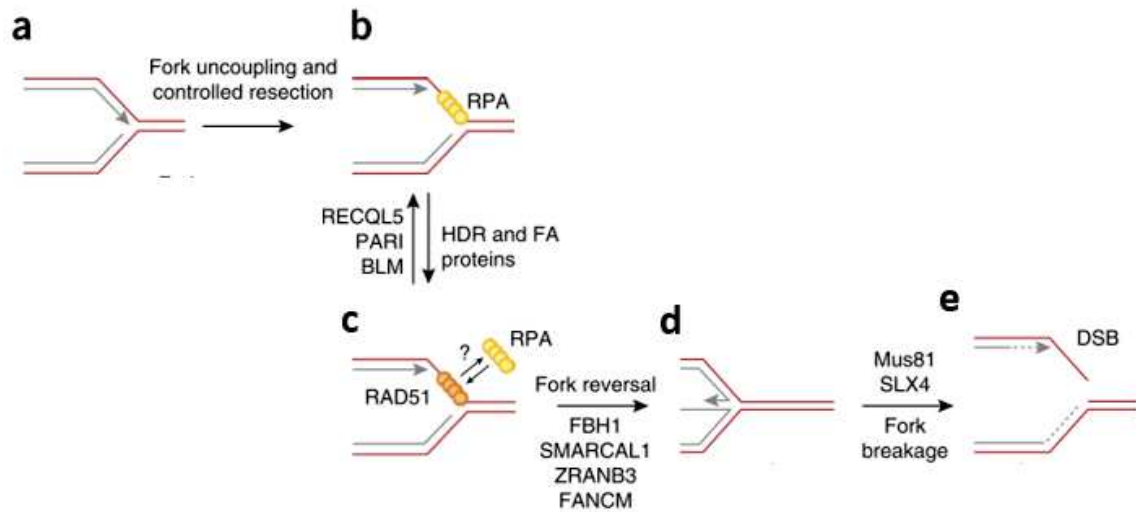


Figure 16 Mechanism of replication fork reversal: (a) Fork uncoupling and active limited degradation of DNA at stalled forks promote the loading of RPA onto ssDNA. (b) BRCA and FANCD2 allow Rad51 to replace RPA at ssDNA. (c) Stalled forks bound by Rad51 are primed for reversal triggered by the FBH1, SMARCAL1, ZRANB3 and FancM translocases. (d) In some instances, prolonged fork reversal are cleaved by structure-specific endonucleases. Modified from (Berti & Vindigni, 2016)

Massimo Lopes and colleagues further revealed that fork reversal is a broad physiological response to replication stress in mammalian cells (Zellweger et al., 2015). Indeed, in U2OS cells, 15 to 30% of all replication forks are reversed upon treatment with various genotoxic agents such as:

- topoisomerase poisons: Camptothecin, etoposide, doxorubicin
- polymerase inhibition: aphidicolin, HU,
- DNA crosslinkers: mitomycin C, cisplatin
- DNA damaging agents: UV irradiation, methyl-methanesulfonate, oxidation.

Moreover, they established that fork reversal is mediated by RAD51, during a process that requires the formation of ssDNA (Ralph Zellweger ; Massimo Lopez, 2015). In fact, RAD51 could be recruited to ssDNA at uncoupled forks to promote the initial step of fork regression by invading the complementary parental strand (Berti & Vindigni, 2016).

Other translocases have been involved in the reversion of stalled forks, including: RAD54 (Bugreev, Rossi, & Mazin, 2011), SMARCAL1 (Bétous et al., 2013), FANCM (Gari, Décaillet, Stasiak, Stasiak, & Constantinou, 2008), ZRANB3 (Ciccio et al., 2012; Yuan, Ghosal, & Chen, 2012) and the Rad5 homolog HLTF (Blastyak, Hajdu, Unk, & Haracska, 2010; Kile et al., 2015).

2. Fork recovery:

Replication forks halted in regions with dormant origins can be rescued by incoming replication forks (Ge & Blow, 2010; Ibarra, Schwob, & Méndez, 2008).

Uncoupled replication forks may be rescued by damage avoidance pathways such as repriming and translesion synthesis (TLS) (Figure 15). The first pathway uses the ability of the polymerase PrimPol to initiate *de novo* DNA synthesis to bypass the lesion. In other words, replisomes skip the damage and restart DNA synthesis past the lesion leaving behind ssDNA gaps that are managed by post-replicative repair pathways (Bianchi et al., 2013; García-Gómez et al., 2013; Mourón et al., 2013). Post-replication repair mechanisms include homologous recombination-mediated template switching and the TLS pathway. The latter makes use of specialized DNA polymerases able to replicate across damaged templates (for review: Sale, Lehmann, & Woodgate, 2012), such as pol η (P. Kannouche et al., 2001; P. L. Kannouche, Wing, & Lehmann, 2004). After bypass of the lesion by the TLS polymerase, a replicative polymerase replaces the latter and continue replication.

Two distinct pathways may be used to restart reversed replication forks (Figure 17).

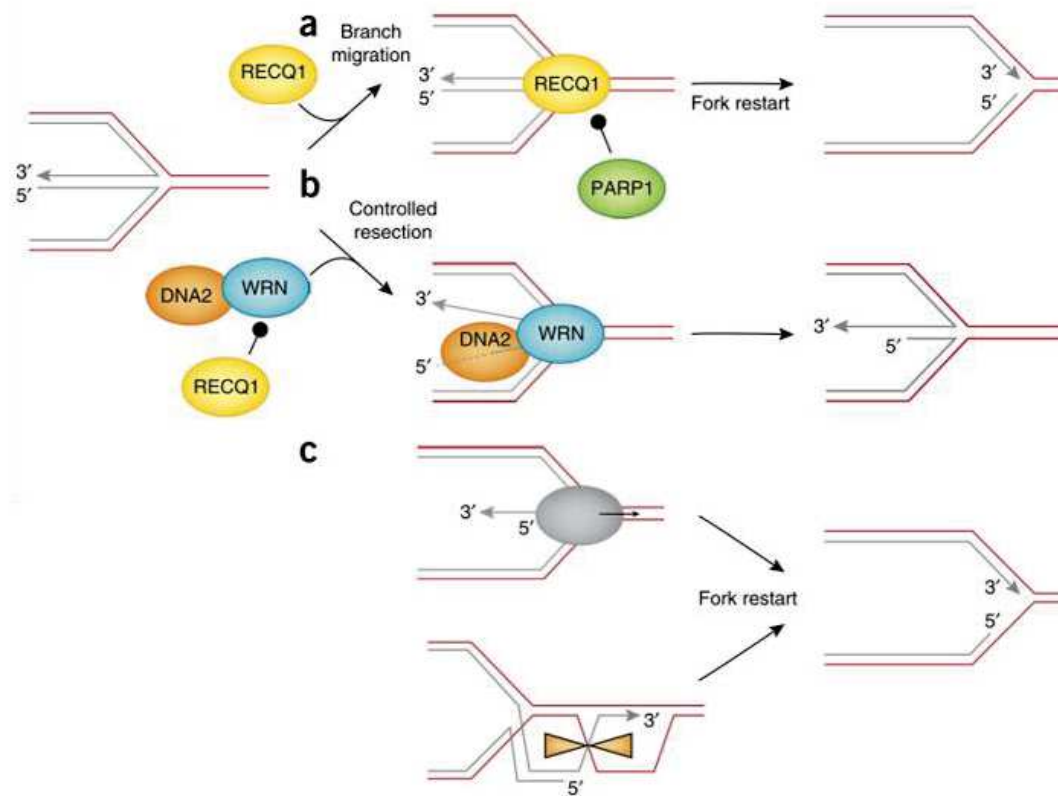


Figure 17 Reversed fork restart: Two mechanisms of reversed fork resolution have been identified, one dependent on the RECQ1 helicase, the other on DNA2/WRN. (a) RecQ1 restarts reversed forks thanks to its ATPase and branch migration activities. (b) Alternatively, limited DNA degradation by DNA2 assisted by the WRN helicase promotes (c) the recognition of the reversed fork by branch-migration factors or the formation of a pseudo Holliday junction subsequently resolved by, for example, Mus81. Modified from (Berti & Vindigni, 2016)

First, replication resumption may be catalyzed by RecQ1, under the control of PARP1 (Berti et al., 2013). This study reported that although RecQ1 is essential for fork restart, unscheduled RecQ1-dependent fork reversion may be a source of DNA damage. In fact, PARP1 transiently inhibits the activity of RecQ1 until the lesion is repaired or the source of replication stress is removed.

Second, stalled forks that have been regressed may instead restart after being processed by nucleases.

For instance, the group of Alessandro Vindigni demonstrated that in U2OS cells DNA2, in cooperation with the RecQ-like helicase WRN, resects reversed replication forks in the 5' to 3' direction. This process was negatively regulated by RecQ1 (Thangavel et al., 2015). Nucleolytic degradation of the nascent regressed strand by DNA2 results in a 3' overhang that mediates the reestablishment of an active fork. Restart of a degraded reversed fork may proceed through two pathways.

An HR-like mechanism where the 3' overhang might be coated by the RAD51 recombinase to invade donor sequences ahead of the reversed fork (Peterman, 2010). This process creates a Holliday junction that needs to be resolved by resolvases or the combined action of the BLM helicase with the type I topoisomerase TOP3. Alternatively, the 3' tail generated by DNA2/WRN-dependent resection of the reversed arm may be recognized by a motor protein, for example, the SMARCAL1 translocase, that drives fork reversion and reestablishment of a functional replication fork (Berti et al., 2013; Bétous et al., 2013).

3. Fork resection

As already mentioned, some replication intermediates need to be processed to ensure fork restart (see also Figure 18). This activity is catalyzed by key nucleases that have been classically described in the repair of double-strand breaks.

Factors involved

Indeed, MRE11 has both endonuclease and exonuclease activities that promotes 5'-3' resection of DNA for the first and 3'-5' trimming of DNA ends for the latter. Biochemical studies have demonstrated that the endonuclease activity, stimulated by CtIP (Anand, Ranjha, Cannavo, & Cejka, 2016), is critical to create an entry point for subsequent DNA resection during the early steps of DSB repair (Nicolette et al., 2010).

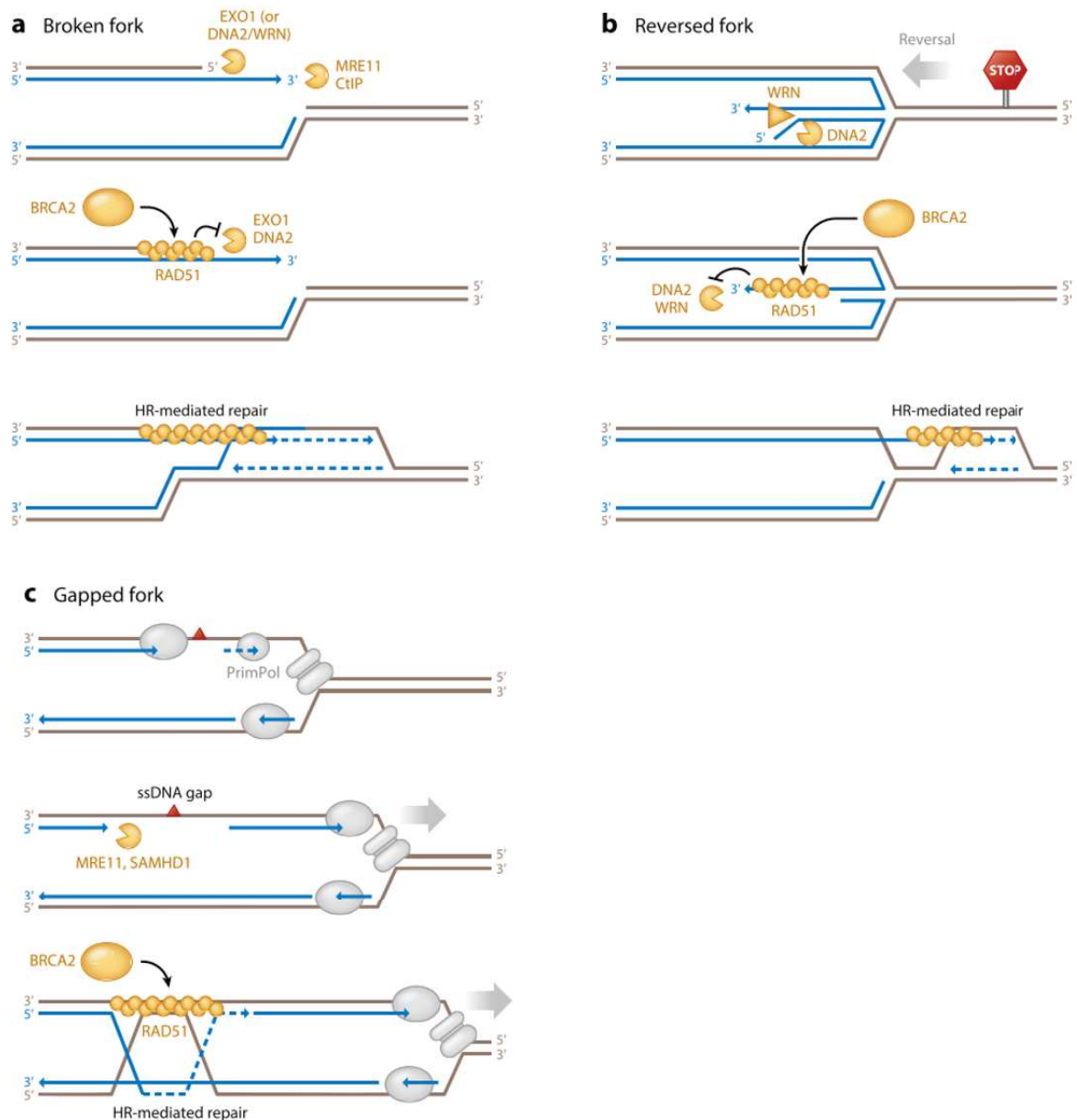


Figure 18 Role of resection in fork restart and repair: The nucleases depicted here are informative as other enzymes can also act at these structures (a) MRE11, CtIP, DNA2 and EXO1 degrade parental and nascent DNA at forks with one-ended DSBs to promote the loading of the recombinase RAD51. (b) At reversed forks, the recruitment of the recombinase is allowed by DNA2-dependent resection assisted by WRN. (c) The MRE11 nuclease acts at gapped forks to generate ssDNA needed for Rad51-mediated repair of the forks. Modified from Pasero & Vindigni, 2017.

The exonucleases EXO1 and DNA2 extend this gap at the 5' ends of DSBs to generate 3' single-stranded overhangs, which are necessary for HR repair (Nimonkar et al., 2011). They are coated by RAD51 filaments that mediate homology search. BRCA2 loads RAD51 filaments onto ssDNA overhangs (Jensen, Carreira, & Kowalczykowski, 2010). While BRCA2 deletion is embryonic lethal (Sharan et al., 1997), BRCA2 is often mutated in cancer, particularly in breast and ovarian cancers (Levy-Lahad & Friedman, 2007).

At gapped forks in *Xenopus* egg extracts, the group of Vincenzo Costanzo provided the first evidence of an MRE11-dependent nucleolytic DNA degradation, counteracted by RAD51 (Hashimoto et al., 2010).

These findings were corroborated by Maria Jasin and colleagues when they reported extensive fork degradation in mammalian cells defective for BRCA2 (Schlacher et al., 2011). Using DNA fiber spreading, they demonstrated that MRE11 processes stalled forks at a rate of 0.7 $\mu\text{m/hr}$, corresponding to $\sim 1.8 \text{ kb/hr}$. Other groups have studied fork resection using this technique, with different labelling strategies. They are illustrated in figure 19.

They used a labelling scheme whereby they first pulse-label cells with IdU, then they induce replication stress by adding HU for 5 hours and they pulse-label cells with CldU. Measuring the IdU track length allowed them to precisely quantify nucleolytic degradation of the replication tracks. They confirmed that RAD51 limits this fork processing using a BRC4 peptide that prevents RAD51 from binding DNA (Hashimoto et al., 2010). In other words, this extensive MRE11-mediated fork degradation event happens only in BRCA2-deficient cells where RAD51 is not properly loaded onto DNA to protect replication forks (Schlacher et al., 2011).

In fact, the group of André Nussenzweig recently proposed that the essential role of BRCA2 is to mediate fork protection (Ray Chaudhuri et al., 2016). They showed that in BRCA2-deficient mouse cells, PTIP and its associated histone methyltransferases MLL3 and MLL4 recruit MRE11 at stalled forks. They also used DNA fiber spreading to evaluate fork resection upon treatment with HU, this time using a different labelling strategy. First, they pulsed-label cells with CldU, then IdU and they add HU for 3 hours. They measured both IdU and CldU tracks to determine an IdU/CldU ratio: if this ratio is below 1, the replication forks were resected. In mice embryonic stem cells and in mice B cells deficient for BRCA2, knocking out PTIP and the methyltransferases MLL3 and MLL4 prevented fork resection without restoring HR at DSBs. Additionally, loss of viability induced by BRCA2 depletion was rescued upon PTIP and MLL3/4 deletion.

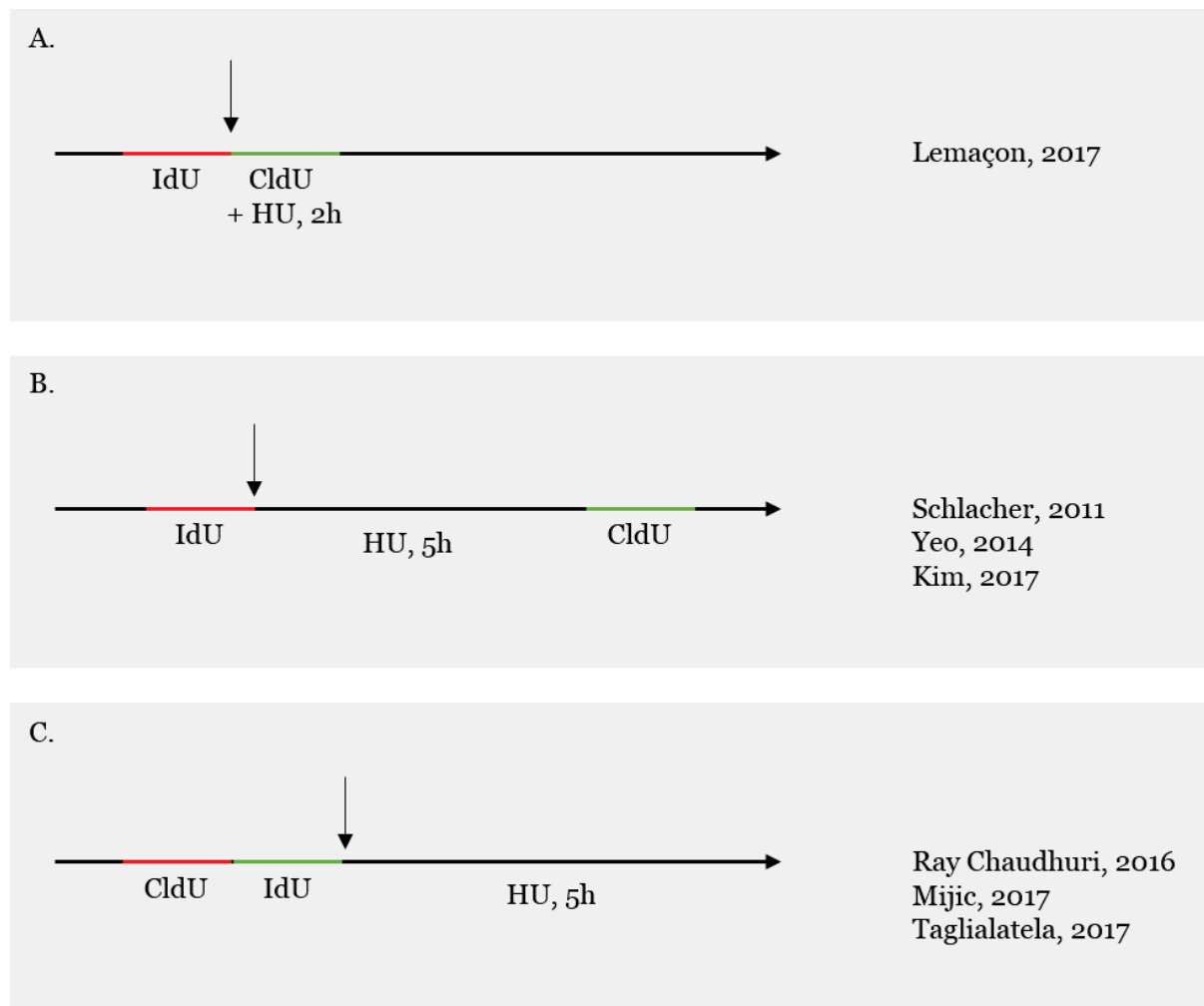


Figure 19 Labelling strategies used to detect stalled fork processing: Ongoing replication forks are labelled with IdU, then cells are treated with the ribonucleotide reductase inhibitor HU together with a pulse of CldU or no labelling (A and B respectively). In B, CldU is added at the end of the HU treatment. Alternatively, DNA synthesis is successively labelled with CldU and IdU (or vice-versa) and HU is added after the labelling (C). HU treatment time may vary between experimental settings. Aphidicolin may also be used instead of HU (for instance Yeo, Lee, Hendrickson, & Sobeck, 2014).

This BRCA2-induced lethality was not rescued by Rif1 depletion that restores HR at DNA damage but has no effect on fork protection. In other words, the essential role of BRCA2 is to prevent MRE11 from excessive fork resection that would induce genomic instability. They also demonstrated that resistance to PARP inhibition in BRCA2-deficient cancer cells could be acquired through the reestablishment of fork stability.

Whether MRE11-dependent degradation of nascent DNA in BRCA2-deficient cells occurs at gapped or stalled forks was recently investigated by three different laboratories (Kolinjivadi et al., 2017; Mijic et al., 2017; Tagliatela et al., 2017). These studies reported that fork reversal mediated by fork remodelers such as ZRAN3B and SMARCAL1 occurs prior to nucleolytic processing. In cells lacking BRCA2, expression of a remodeling-defective ZRAN3B mutant alleviated MRE11-dependent fork resection and the generation of ssDNA (Mijic et al., 2017). Additionally, depletion of ZRAN3B and SMARCAL1 in these cells reduced chromosomal aberrations and DNA damage induced by BRCA2 loss (Tagliatela et al., 2017). These studies demonstrated that fork processing at reversed fork is not restricted to the classical DNA2/WRN pathway.

EXO1, which was first implicated in fork resection in yeast checkpoint mutants (Cotta-Ramusino et al., 2005), has also recently been shown to be involved in reversed-fork processing (Lemaçon et al., 2017). In BRCA2-deficient cancer cells, the combined inhibition of MRE11 using Mirin and depletion of EXO1 further prevented stalled fork degradation, compared to either MRE11 inhibition or EXO1 depletion alone. In other words, MRE11 and EXO1 act in the same pathway to mediate stalled fork resection. Around 25% of fork structures analyzed were reversed forks, indicating that both MRE11 and EXO1 may act at reversed forks. Access of EXO1 to 5' DNA ends may be facilitated by the endonuclease/exonuclease/phosphatase family domain containing 1 protein (EPPD1). EPPD1 is indeed required for EXO1-mediated fork resection (Y. Wu et al., 2015). As EPPD1 is a 5' endonuclease that cleaves replication forks at the junction between the lagging parental strand and the un-replicated DNA parental double strands, its activity may create the structure required by EXO1 for 5'-end resection at reversed forks.

Additional nucleases such as FAN1 and CtIP have been involved in stalled fork processing, under the control of FANCD2, (Chaudhury, Stroik, & Sobeck, 2014; Yeo et al., 2014) but whether they act at reversed forks is unknown. Accordingly, seminal work from the lab of Maria Jasin demonstrated that besides BRCA2, the Fanconi-Anemia protein FANCD2 is able to stabilize RAD51 filaments at stalled forks, thereby protecting nascent DNA strands from extensive nucleolytic degradation (Schlachter, Wu, & Jasin, 2012). FANCD2-mediated fork preservation may not be restricted to RAD51 stabilization, as depletion of FANCD2 in BRCA2-deficient cells results in increased levels of fork degradation compared to FANCD2 or BRCA2 depletion alone (Michl,

Zimmer, & Tarsounas, 2016). Mechanisms of fork protection mediated by BRCA2 and FANCD2 may thus be functionally distinct. The group of Alberto Ciccia however recently showed that fork remodeling was a prerequisite for nascent DNA degradation in FANCD2-deficient cells, as in cells lacking BRCA2 (Tagliatela et al., 2017).

The role of RAD51 in fork protection is ambiguous. RAD51 is necessary for fork reversal (Zellweger et al., 2015), a process now acknowledged to be important for nucleolytic degradation in most fork stalling events (for review: Sidorova, 2017). RAD51 is also stabilized onto regressed arms of replication forks to prevent nucleases from accessing DNA (Kolinjivadi et al., 2017; Mijic et al., 2017; Tagliatela et al., 2017). In other words, RAD51 is both important to create the substrate of nucleases and to restrain their activity. As illustrated in the figure 20 replication forks may actively be reversed and restored in a dynamic cycle during the replication stress response.

Abundance and availability of nucleases and their regulatory factors as well as the nature of the stress causing fork arrest may impact the state of the replication fork and its dynamics. In this pathway, RAD51 may well act as a molecular switch between the different statuses of the fork.

An integrative view of the coordinated action of nucleases at arrested forks together with their regulators is given in the figure 21.

Nucleases recruitment

Some evidence suggests that chromatin plays a role in the recruitment of nucleases at arrested replication forks. First, the histone methyl-transferase activity of MLL3 and MLL4 is necessary for extensive fork degradation in BRCA2- cells (Ray Chaudhuri, 2016). Second, loss of the chromatin remodeler CHD4 which was shown to mediate treatment tolerance of BRCA2-mutated cancer cells (Guillemette et al., 2015) also is involved in the recruitment of MRE11 at stalled forks (Ray Chaudhuri et al., 2016). Third, Metnase was recently shown to recruit EXO1 at stalled replication forks (H. S. Kim, Williamson, Nickoloff, Hromas, & Lee, 2017). Metnase has an endonuclease domain conjugated to a SET histone methyltransferase domain. It methylates histone H3 at lysines 4 and 36, which are associated with 'open' chromatin.

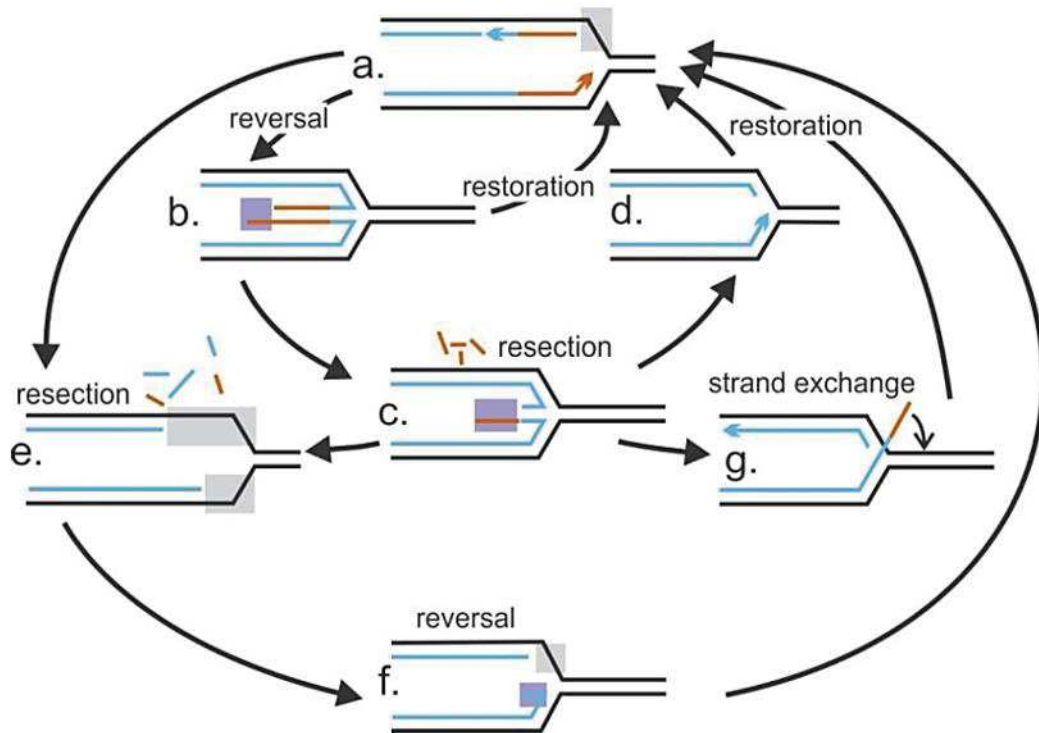


Figure 20 Dynamic cycles of fork reversal and regression during the replication stress response: A stalled fork with limited parental DNA exposure may be directly reversed and restored (a-b-a). After fork reversal, resection of the nascent DNA (c) may also directly (d) or indirectly (e, g) restore a functional replication fork. Upon re-annealing of the parental DNA, reversed forks that have been resected may also experience further reversal (f). Modified from Sidorova, 2017.

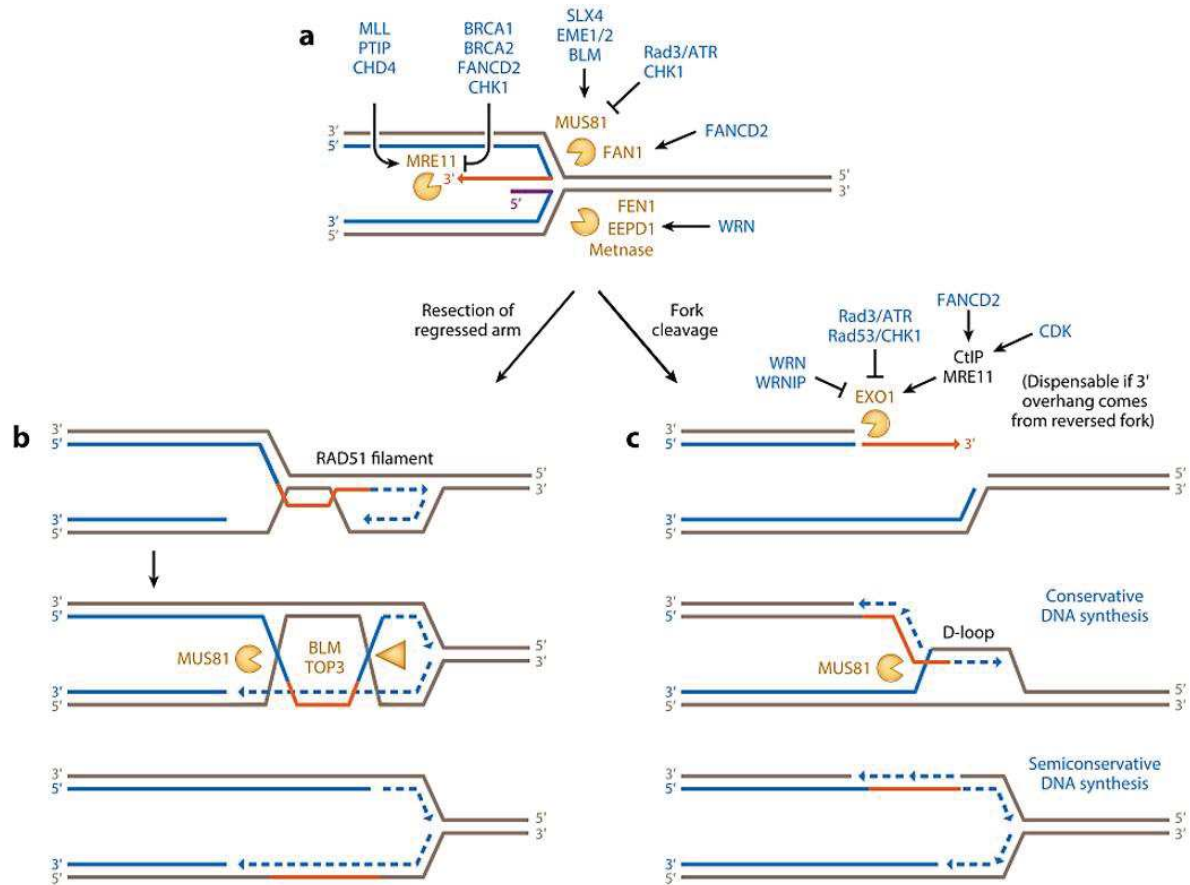


Figure 21 Coordinated action of nucleases at arrested forks: (a) reversed replication forks may be processed to generate 3' overhangs (orange) used to engage homologous recombination-mediated repair (b) or BIR (c). Owing to their polarity, distinct nucleases resect either parental or nascent DNA to generate the overhang. Their regulators are shown in blue. MRE11, counteracted by BRCA1,2 proteins and FANCD2 and recruited by PTIP/MLL3-4 acts on nascent DNA. EXO1 is thought to degrade parental DNA after it has been cleaved by MUS81 or MRE11. Additional cleavage of repair intermediates by Mus81 restores a normal replication fork. Modified from Pasero & Vindigni, 2017.

Metnase facilitates the recruitment of EXO1 at gapped forks *in vitro*, independently of its DNA cleavage activity.

MRE11 is far from being the only nuclease acting at arrested replication forks. In line with the above observations, chromatin modifications may be important not only for its recruitment but also for the engagement of other nucleases involved in fork processing.

Importance of fork processing

Limited fork processing is directly necessary for fork restart in some instances (Figure). For example, regulated DNA resection at reversed forks generates a 3' overhang that may be used for HR-coupled replication restart (Petermann, Orta, Issaeva, Schultz, & Helleday, 2010). Alternatively, this 3' tail may be specifically recognized by motor proteins such as SMARCAL1 that drives branch migration-assisted reestablishment of a functional replication fork (Bétous et al., 2013). At gapped forks, it is possible that fork processing is involved in removing stalled polymerases to allow post-replicative repair. It may also be important to generate ssDNA gaps needed for RAD51 loading during HR-mediated post-replicative repair. The latter mechanism is likewise at play at broken replication forks where the coordinated action of CtIP and MRE11 evict the end-joining factor Ku to allow RAD51 loading (Chanut, Britton, Coates, Jackson, & Calsou, 2016).

If fork stalling is prolonged or if fork restart fails, replication forks become substrates for the structure-specific endonuclease MUS81 (Hanada et al., 2007) or the flap-endonuclease Fen1 (Zheng et al., 2005). Unscheduled cleavage of arrested forks by these nucleases generates single-ended DSBs (Dehé & Gaillard, 2017). These latter may then be repaired by an HR-like mechanism termed Break-Induced-Replication (BIR), a highly error-prone mechanism based on the usage of the sister chromatid as a template for DNA synthesis. BIR is prevalent in human cells upon replication stress as it accounts for the high frequency of genomic duplication after oncogene activation (Costantino et al., 2014).

In BRCA-deficient cells, if the processing of reversed forks is not controlled, forks that have been extensively degraded may also be cleaved by MUS81 to initiate fork rescue via a BIR-like mechanism (Lemaçon et al., 2017). MUS81-mediated cleavage of partially resected reversed forks promotes DNA polymerase δ -dependent DNA synthesis, a process that allow cell survival at the expense of chromosomal aberrations and genomic instability

As described above, limited processing of arrested forks impedes the accumulation of DNA damage by promoting HR-coupled replication repair. DNA resection is not only directly involved in fork protection, but also in the activation of an essential pathway to maintain genome integrity: the replication checkpoint.

V- The DNA replication checkpoint

This protection pathway is activated during S phase to ensure proper duplication of the genome.

It is constituted of a complex network of responses governed by the kinase ATR (ataxia telangiectasia and Rad3-related). Upon replication stress, ATR orchestrates these responses to safeguard replication forks.

Evidence suggests that ATR is activated at every S phase, even when cells are not challenged by any exogenous stress. First, ATR is an essential gene: homozygous deletion is embryonic lethal in mice (Brown & Baltimore, 2000). In line with this observation, a 3-day treatment with 300 nM of a specific ATR inhibitor is sufficient to kill mouse embryonic stem cells (Ruiz et al., 2016). Second, ATR is transiently activated at specific replication forks upon endogenous stress such as at Common Fragile Sites (CFS) (Casper, Nghiem, Arlt, & Glover, 2002). The sole depletion of ATR in human cancer cells is sufficient to activate the expression of CFS. Third, one of the functions of this kinase is to ensure that all the ingredients necessary for DNA synthesis are available in due amounts (for a recent review on the functions of ATR: Saldivar, Cortez, & Cimprich, 2017).

Besides the replication checkpoint, ATR is part of the DNA damage checkpoint as the master kinases ATM (ataxia telangiectasia mutated) and DNA-PK (DNA-dependent protein kinase), with which it shares some sequence and functional homology.

Accordingly, ATR activity is not only crucial for the replication-stress response but is also involved in the response to DNA damage (Maréchal & Zou, 2015 for review), during meiosis (Royo et al., 2013) and at telomeres (for review: (Maciejowski & Lange, 2017).

1. ATR Signaling pathway

In the replication checkpoint, ATR is activated following replication fork slowing and stalling. Fork impediments generate RPA-coated ssDNA, the recruitment signal for ATR. Once recruited to ssDNA, the kinase is stimulated by a group of regulator proteins including RAD17, 9-1-1 and TopBP1. With the help of mediator proteins, activated ATR then recognizes and phosphorylates downstream effectors such as CHK1.

Pioneer *in vitro* work, mainly with purified proteins and in *Xenopus* egg extracts, has allowed for a detailed determination of the molecular steps necessary for ATR activation. For a global view of the factors involved in this process, see Figure 22.

ssDNA fragments generated by fork pausing are coated by RPA, allowing the recruitment of the ATR-interacting protein ATRIP (L. Zou, Liu, & Elledge, 2003). ATRIP is necessary for the recruitment of ATR to stress sites (D. Cortez, Guntuku, Qin, & Elledge, 2001), but is not sufficient

for its activation. The activation of ATR is mediated by TopBP1 (Kumagai, Lee, Yoo, & Dunphy, 2006) and the recently identified regulator protein Ewing tumor-associated antigen 1 (ETAA1) (Bass et al., 2016; Haahr et al., 2016; Y. Lee et al., 2016). The localization of TopBP1 at replication stress sites requires the formation of 5'-ended ssDNA-dsDNA junctions. These junctions support the loading of an RFC-like complex constituted of the clamp loader RFC2-5 with RAD17 that supply the checkpoint clamp complex RAD9-RAD1-HUS1 (9-1-1) onto DNA (L. Zou et al., 2003; Lee Zou, Cortez, & Elledge, 2002). The 9-1-1 complex is necessary for TopBP1 recruitment and subsequent stimulation of ATR kinase activity (Delacroix, Wagner, Kobayashi, Yamamoto, & Karnitz, 2007; J. Lee, Kumagai, & Dunphy, 2007). In other words, after it is bound to the 9-1-1 clamp loaded at ssDNA-dsDNA junctions, TOPBP1 interacts with ATR-ATRIP to stimulate ATR activity.

In addition to its role in TopBP1 loading, RAD17 also plays a key role in establishing interaction between 9-1-1 and TopBP1 (J. Lee & Dunphy, 2010).

ATR signaling is amplified by continued primer synthesis at stalled forks (Van, Yan, Michael, Waga, & Cimprich, 2010). Using *Xenopus* egg extracts incubated with defined DNA structures this study demonstrated that ATR activation, as measured by CHK1 phosphorylation, correlates with the number of ssDNA-dsDNA junctions independently of the length of ssDNA. Stretches of ssDNA necessary for checkpoint activation are generated by distinct mechanisms depending on the nature of the replication stress and whether it affects primarily the leading or the lagging strand (see Figure 23). First, if the leading strand polymerase is slowed down, uncoupling of the helicase and DNA polymerase can occur as the helicase continues to unwind DNA ahead of the stalled polymerase (Byun et al., 2005).

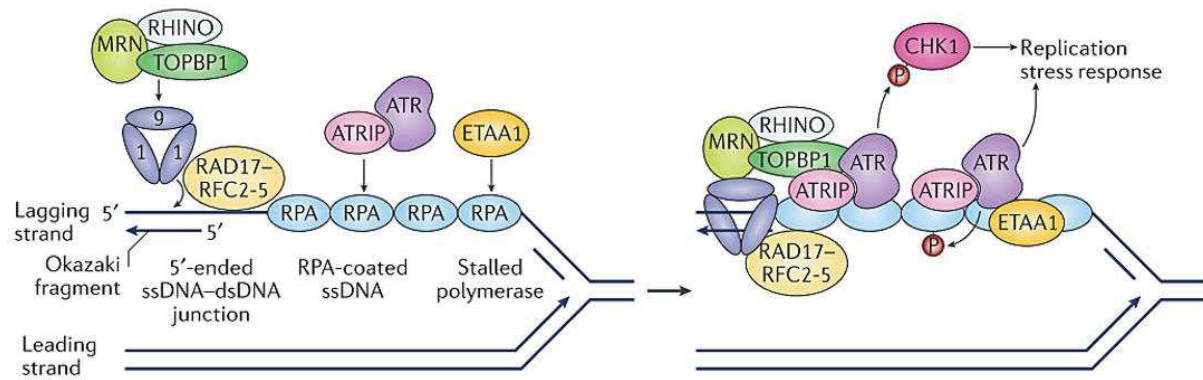


Figure 22 Components of the ATR-activating pathway: In this model, a fork stalling event on the lagging strand generates both structures necessary for ATR recruitment and activation: ssDNA and a 5'-ended ssDNA-dsDNA junction. The first provides a platform for the recruitment of ATRIP-bound ATR. The latter is the loading point for the 9-1-1 clamp complex, loaded onto DNA by the clamp loader Rad17-RFC2-5. The 9-1-1 complex, with assistance from RAD9-HUS1-RAD1-interacting nuclear orphan (RHINO) and the MRE11-RAD50-NBS1 (MRN) complex, recruits the ATR activator topoisomerase II binding protein (TOPBP1), thereby allowing stimulation of ATR and phosphorylation of downstream effectors, including checkpoint kinase 1 (CHK1). Ewing tumor-associated antigen 1 (ETAA1) bound to RPA activates ATR in a parallel pathway. Modified from Saldivar et al., 2017.

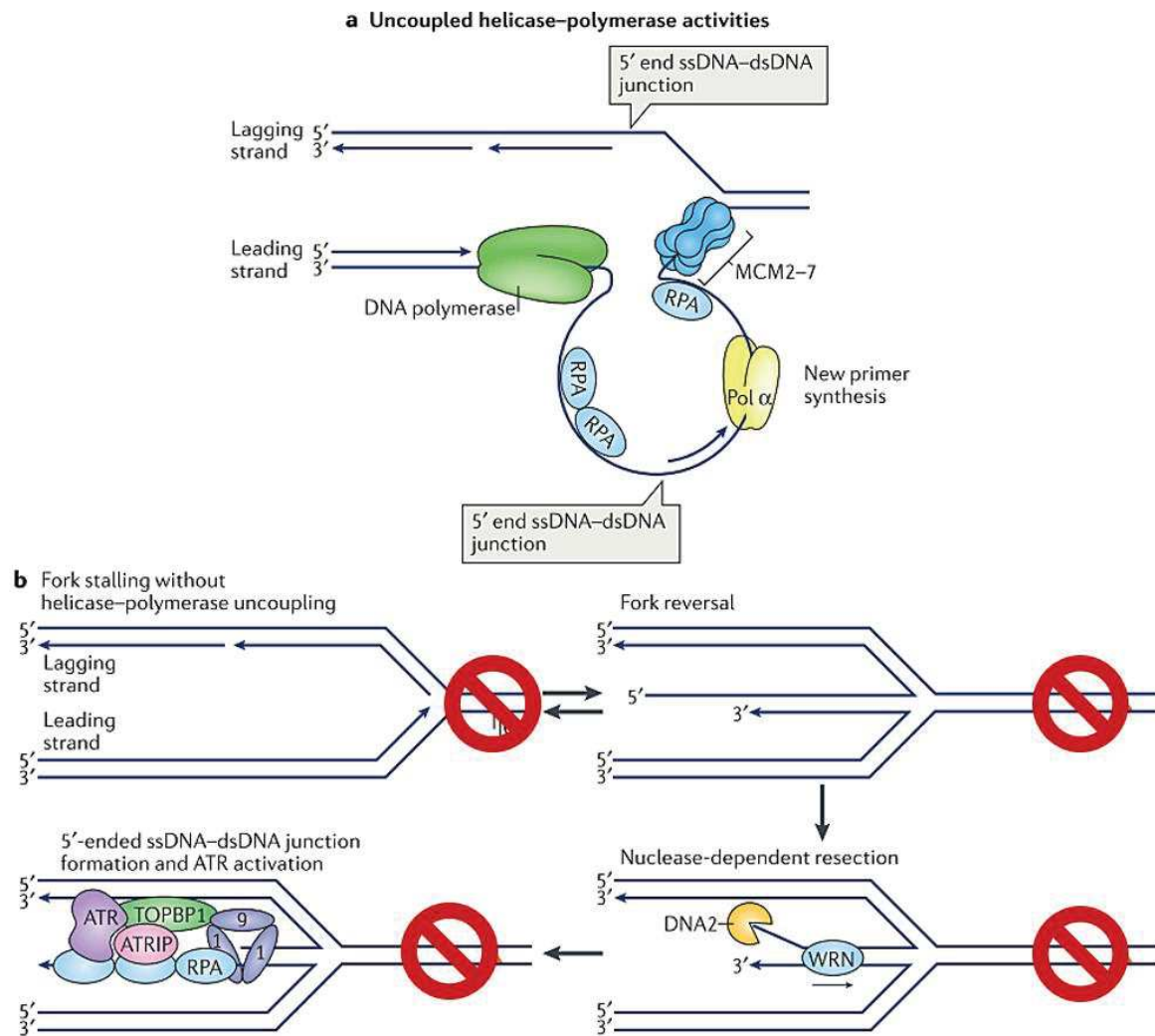


Figure 23 Generation of the ATR activation signal: a) Functional uncoupling between the replicative helicase and the polymerases may generate the ssDNA-dsDNA junctions and the ssDNA gap necessary for full ATR activation. b) If uncoupling does not occur, fork processing may be required to generate these structures. For instance, DNA2 together with WRN degrades nascent DNA allowing the recruitment of ATR-activating proteins. Modified from (Saldivar et al., 2017)

Second, if the lagging strand is affected, repriming should allow the fork to bypass the lesion, leaving behind a ssDNA gap that may be extended by nucleases as it has been described in the past section. Third, if the replication stress blocks both the helicase and polymerases, it is possible that fork processing events are triggered to generate the necessary ATR-activating structures. For instance, the DNA2-dependent resection of reversed fork contributes to ATR activation as depletion of DNA2 reduces ATR-dependent phosphorylation of CHK1 (Thangavel et al., 2015). Genetic depletion of the helicases involved in fork reversal FANCM, FANCD1 and WRN has also been linked to reduced ATR signaling (Basile, Leuzzi, Pichierri, & Franchitto, 2014; Collis et al., 2008; Gong, Kim, Leung, Glover, & Chen, 2010; Schwab, Blackford, & Niedzwiedz, 2010).

2. Functions of the replication checkpoint

Delaying cell cycle transitions, coordinating replication forks and promoting fork stabilization are the essential roles of the replication checkpoint.

While it phosphorylates a complex network of proteins, including the DNA sensor IFI16 (Wagner et al., 2016), the major downstream effector of ATR is CHK1. Itself a kinase, CHK1 is activated by ATR by phosphorylation at S317 and S345 (Q. Liu et al., 2000; H. Zhao & Piwnicka-Worms, 2001).

Cell cycle pausing

Upon replication stress, cell cycle arrest at the G1-S, S-G2 and G2-M transitions are important to prevent cells from entering mitosis with under-replicated DNA.

ATR and CHK1-mediated phosphorylation events cause inhibition of the CDK activators CDC25A, CDC25B and CDC25C (Y. Sanchez et al., 1997) through diverse mechanisms. For instance, phosphorylation of Cdc25A triggers its degradation thereby preventing its association with Cyclin A-Cdk1, Cyclin B-Cdk1, and Cyclin E-Cdk2. This, in turn, results in cell cycle arrest both in G2 and S phases (Mailand et al., 2000; Xiao et al., 2003).

Fork coordination

The replication checkpoint ensures that the reactants necessary for DNA synthesis are available in adequate amounts during S phase by regulating origin firing.

As a matter of fact, replication initiation is increased following inhibition of ATR (Shechter, Costanzo, & Gautier, 2004). Several mechanisms are at play to coordinate origin firing with resolution of the fork arrest, as illustrated in the Figure 24.

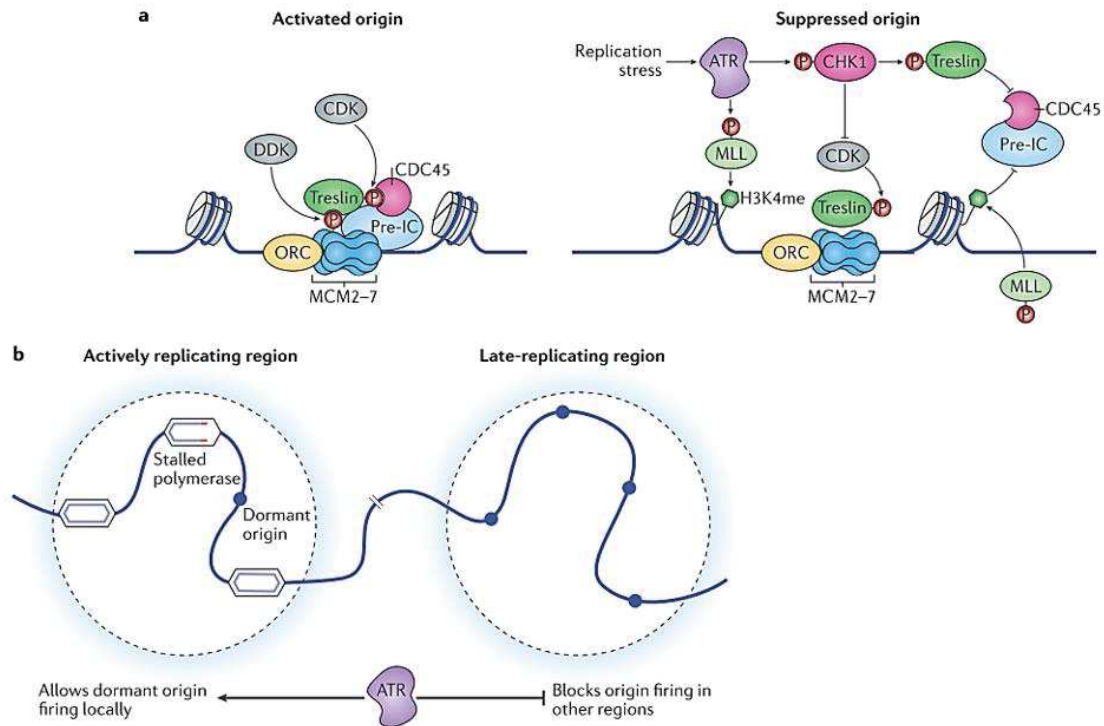


Figure 24 ATR-mediated regulation of origin firing: a) Right: Phosphorylation events catalyzed by DDK and CDK promote the recruitment of replication initiation factors such as CDC45 to activate the replicative helicase. Left: activated ATR regulates origin firing through different pathways. First, phosphorylation of the MLL complex promotes its association with chromatin where it methylates histone H3 Lys4 to block CDC45 loading. Second, activated CHK1 negatively regulates CDK and the initiation factor treslin. b) Upon fork arrest, nearby replication origins fire to help complete DNA synthesis. Simultaneously, the firing of late origins is inhibited to prevent RPA and dNTPs exhaustion. Modified from (Saldivar et al., 2017)

First, CHK1-mediated regulation of the cyclin A–CDK1 complex may be involved in this process as the latter is thought to promote late origin firing (Katsuno et al., 2009). Second, CHK1 directly phosphorylates Treslin to prevent replication initiation by decreasing the loading of CDC45 onto chromatin (Guo et al., 2015). Third, ATR stabilizes the methyltransferase MLL at chromatin, thereby promoting methylation of histone H3 Lys4, a modification shown to prevent CDC45 from binding to chromatin (H. Liu et al., 2010). Consequently, ATR activity regulate origin firing by directly and indirectly preventing the recruitment of initiation factors. In reality, the regulation of origin firing mediated by the ATR-CHK1 axis is complex: replication origins are differentially impacted according to their localization. Upon replication stress, firing of dormant origins is globally prevented but is allowed within an active replication cluster (Ge & Blow, 2010). Global inhibition of firing events in response to fork stalling may be needed to prevent exhaustion of RPA, the leading cause of replication catastrophe (L. I. Toledo et al., 2014). At the same time, supporting replication initiation locally may allow completion of replication within the affected region by allowing rescue of an arrested fork by a converging replisome.

Limiting the amount of simultaneously active replication fork is now acknowledged as a way of regulating the use of limiting factors such as RPA (L. Toledo et al., 2017 for review). The same mechanism may be also true for dNTPs, whose production is indirectly regulated by ATR. Indeed, in the absence of exogenous replication stress, ATR activity is needed for efficient expression of the RNR subunit RRM2 by stabilizing the transcription factor E2F1 (Buisson, Boisvert, Benes, & Zou, 2015).

Accordingly, increased RNR activity can extend the lifespan and decrease chromosomal instability in ATR mutant mice (Lopez-Contreras et al., 2015).

Fork protection

Preserving replication fork stability is the last canonical function of the replication checkpoint upon replication stress. Rather than preventing complete dissociation of the components of the replisome, ATR activity seems to be necessary for DNA synthesis to resume following removal or bypass of the replication stress (Dungrawala et al., 2015). Loss of ATR kinase activity is synthetic lethal with replication inhibition and increases the occurrence of terminally-arrested replication forks (Cliby et al., 1998; Couch et al., 2013). To ensure that replisomes keep their functionality, several pathways are triggered by the checkpoint (Figure 25).

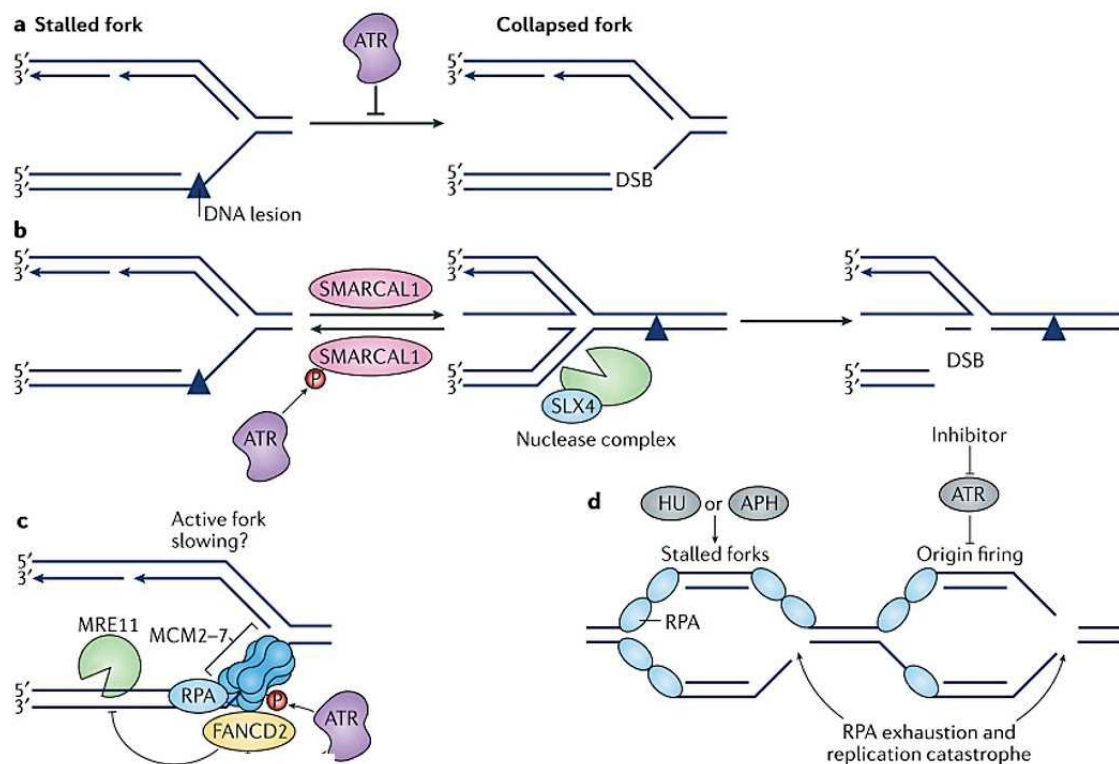


Figure 25 ATR-mediated fork protection mechanisms: a) in the absence an appropriate protection of stalled forks, they may collapse leading to the formation of single-ended DSB. b) ATR phosphorylates SMARCAL1 to limit its fork reversal activity, thereby decreasing the formation of candidate substrates for Mus81/SLX4-mediated cleavage. c) AT-mediated MCM2 phosphorylation may participate in the recruitment of Fancd2 at stressed forks. Fancd2 limits Mre11-dependent DNA degradation. d) ATR also prevents the exhaustion of RPA to prevent replication catastrophe. This has been shown using HU and aphidicolin in wild-type cells as well as in cells where ATR is inhibited. A similar process may be at play, promoting the production of dNTPs to avoid DNA damage. Modified from (Saldivar et al., 2017)

First, ATR directly phosphorylates SMARCAL1 to restrain its fork remodeling activity (Couch et al., 2013). If ATR is inhibited, unleashed SMARCAL1 may cause massive fork reversal. Reversed forks then become substrates for the structure-specific endonuclease SLX4-Mus81 that generates single-ended DSBs. ATR also phosphorylates helicases involved in fork restart such as BLM and WRN (Ammazzalorso, Pirzio, Bignami, Franchitto, & Pichierri, 2010; Davies et al., 2004). At least for WRN, this phosphorylation event promotes its localization at stalled replication forks, precluding the formation of DNA breaks (Ammazzalorso et al., 2010).

Second, ATR signaling mediates the recruitment of FANCD2 at replication forks upon replication stress (Lossaint et al., 2013). The role of FANCD2 in this fork protection pathway is two-fold. First, FANCD2 restrains replisome activity when replication forks are challenged with HU: while FANCD2 depletion had no impact on the progression of replication forks in normal conditions, after HU replisomes were faster in FANCD2-deficient cells compared to control cells. Second, the absence of FANCD2 induced an accumulation of ssDNA in condition of replication stress. Given the known protective role of FANCD2 against fork resection, it is conceivable that ATR indirectly prevents unscheduled fork resection by recruiting FANCD2 at stalled forks. The exact mechanism of ATR-mediated FANCD2 recruitment is unclear. ATR phosphorylates MCM2 at its Ser 108 (David Cortez, Glick, & Elledge, 2004). Loss of this phosphorylation only partially reduced the association of FANCD2 with MCM proteins (Lossaint et al., 2013), indicating that additional mechanisms are required.

Cleavage of the fork by structure specific endonucleases is important to resume DNA synthesis after prolonged fork arrest (Hanada et al., 2007) but drives cell lethality if uncontrolled (Forment, Blasius, Guerini, & Jackson, 2011).

The effector kinase CHK1 might be directly involved in this fork regulatory pathway since its inhibition leads to Mus81-mediated cleavage of stalled forks (Forment et al., 2011; Técher et al., 2017).

As the activity of structure-specific endonucleases is managed by CDKs, the loss of their regulation upon ATR and CHK1 inhibition may be responsible for the unscheduled breakage of stalled replication forks mediated by SLX4 and Mus81 (Dehé & Gaillard, 2017).

Once DNA synthesis has resumed, the replication checkpoint is turned off to allow cell cycle restart. Checkpoint attenuation is mediated through the activity of protein phosphatases and ubiquitin-ligases that remove activation signals and induce proteasomal degradation of key checkpoint proteins (for review: Chaudhury & Koepp, 2016; Hustedt, Gasser, & Shimada, 2013).

Chapter 3: Self-DNA-induced inflammation

Our understanding of the detection and the generation of cytosolic DNA has come a long way since the initial description of viral cytosolic DNA sensing by Stetson and Medzhitov in 2006. As a matter of fact, much of our knowledge comes from the study of inflammatory disorders such as the Aicardi-Goutières Syndrome (AGS). Consequently, this section is dedicated to the description of this disorder, with a special focus on one of the protein involved in its etiology which is also the central player of my PhD: SAMHD1.

I- AGS, a model to study autoimmunity

1. Clinical characteristics

AGS is a rare monogenic disease, with around 500 affected families known worldwide (Crowl, 2017). It is mainly caused by inherited autosomal recessive mutations. So far, seven genes have been shown to be mutated in AGS: TREX1 (Crow, Hayward, et al., 2006), the three subunits of RNASEH2 (Crow, Leitch, et al., 2006), SAMHD1 (G. I. Rice et al., 2009), ADAR (G. I. Rice et al., 2012) and IFIH1 (G. I. Rice et al., 2014). All of them are involved in cytosolic nucleic acids metabolism and sensing.

Most affected patients progressively develop a severe encephalopathy after the first weeks of life, resulting in severe intellectual and physical disabilities (Goutières, Aicardi, Barth, & Lebon, 1998). Some patients however already present systemic inflammation and neurological defects at birth (Goutières et al., 1998), making this disease similar to a congenital viral infection. Regardless of the onset of the disease, the inflammatory response is profoundly activated in all patients. Indeed, prolonged IFN- α production in the cerebrospinal fluid and sera, and constitutive upregulation of ISGs in circulating blood, as measured by RT-PCR, are hallmarks of the disease (first described by Lebon and colleagues in 1988 and confirmed by Rice, 2013). Other classical features of the disease include severe psychomotor retardation owing to the loss of motor neurons and to leukocyte infiltration in the central nervous system, white matter abnormalities and skin lesions (chilblains). Although the main phenotypes are associated with neurological defects, other symptoms such as cardiomyopathy have been linked with AGS in specific mutation backgrounds (Crow, 2015). For a detailed review of the main clinical characteristics of AGS, see Figure 26.

Disease manifestations	Affected gene associated with type I interferonopathy				
	TREX1	RNASEH2A, RNASEH2B and RNASEH2C	SAMHD1	ADAR	IFIH1
Neurological phenotypes					
Developmental delay	•	•	•	•	•
Intracranial calcification	•	•	•	•	•
White matter disease	•	•	•	•	•
Cerebral atrophy	•	•	•	•	•
Spastic paraparesis		•		•	•
Large vessel disease (stenosis, moyamoya or aneurysms)	•		•		
Bilateral striatal necrosis				•	
Dermatological phenotypes					
Digital vasculitis and/or necrosis (chilblains)	•	•	•	•	•
Livedo and/or skin mottling	•	•	•	•	•
Panniculitis			•		
Necrotic cheek lesions					
Lipoatrophy					
Other phenotypes					
Recurrent (sterile) fevers	•	•	•	•	•
Autoimmune features	•	•	•	•	•
Glaucoma	•	•	•		•
Neonatal thrombocytopenia and/or bone marrow suppression	•	•	•	•	•
Hypertrophic cardiomyopathy	•	•			•
Myositis					
Enchondromata					
Lung involvement					
Chronic lymphocytic leukaemia			•		
Premature dental loss					•
Aortic calcification					•
Joint contractures	•		•		•

Figure 26 **Major clinical features associated with AGS**: circles indicate the presence of the phenotype. Since only a small number of patients have been reported so far for some mutations, an absence of a clinical feature does not mean it cannot occur. Modified from Crow & Manel, 2015.

AGS is an extremely serious disease: in 19% of cases, death occurs during childhood, mainly before 5 years, and most of the remaining patients are profoundly disabled (Crow, 2015).

2. AGS-related genes and proteins

The cellular functions of all above proteins are depicted in the Figure 27.

TREX1 (Three-prime repair exonuclease 1):

TREX1 is a 3' – 5' ssDNA exonuclease widely expressed in mammalian cells (Mazur & Perrino, 2001). It is primarily located in cytosol, more precisely at the endoplasmic reticulum, but can translocate to the nucleus on some instances (Chowdhury et al., 2006; Stetson, Ko, Heidmann, & Medzhitov, 2008; Y. G. Yang, Lindahl, & Barnes, 2007).

Two studies have greatly contributed to the understanding of the molecular function of this enzyme. Yang et al. have shown that Trex1 is recruited to the nucleus, more precisely to replication foci, after γ -Irradiation or hydroxyurea treatment. TREX1^{-/-} MEFs massively accumulated 60-65 nucleotide-long DNA fragments in their cytosol, in the absence of any treatment. These DNA species originated from replication forks, as shown by BrdU labelling. This study provided the first hint that DNA replication might be a source of cytosolic DNA. The authors concluded that chronic checkpoint activation and defective DNA repair are the source of the inflammatory response arising in TREX1-deficient cells (Y. G. Yang et al., 2007).

In a completely independent study, Stetson and colleagues again involved TREX1 in cytosolic DNA-mediated inflammation (Stetson et al., 2008). First, they showed that knocking out IRF3, the IFN receptor IFN α R2 and the nuclease RAG2 (essential for B and T cell maturation) completely rescued lethality induced by TREX1 deficiency. This result confirmed that inflammation and more precisely type I IFN production is the leading cause of mortality in this background. Second, they managed to extract cytosolic DNA generated in TREX1-deficient cells, to clone it and sequence it. They found an enrichment for endogenous retro-elements in these cells, implicating TREX1 as a negative regulator of endogenous retro-transposition. Notably, a combination of reverse-transcriptase inhibitors strongly reduces heart inflammation of TREX1-knock out mice and improved mice survival (Beck-Engeser, Eilat, & Wabl, 2011). Correspondingly, the implication of TREX1 as a negative regulator of retro-transposition was recently reported in Trex1-deficient human neural cells (Thomas et al., 2017). How Trex1 prevents the generation of cytosolic retroelements is still not entirely understood.

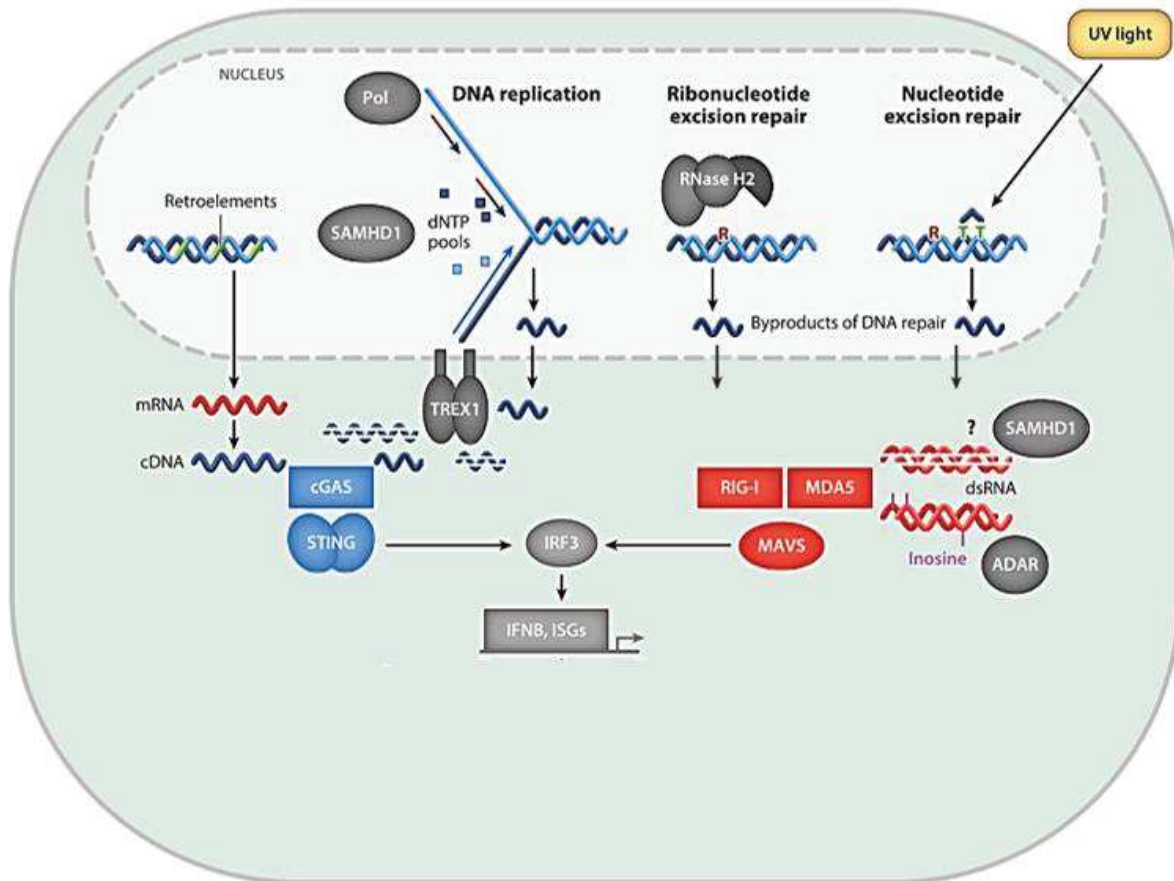


Figure 27 Cellular functions of the proteins associated with AGS: DNA damage, endogenous retroelements and DNA replication are potential sources of cytosolic DNA. Trex1 is a cytosolic DNase degrading ssDNA derived from endogenous retroelements or byproducts of DNA repair. Cytosolic ssDNA not degraded by Trex1 are recognized by cGAS. RNase H2 removes ribonucleotides from genomic DNA and degrades RNA in RNA:DNA hybrids, preventing DNA damage. Besides, ribonucleotides in DNA enhances photodimerization of adjacent pyrimidines in close proximity, increasing nucleotide excision repair. SAMHD1 ensures appropriate dNTP pools levels required for DNA replication. ADAR edits dsRNA by deamination of adenosine to inosine, preventing recognition of dsRNA by MDA5. Modified from Lee-Kirsch, 2017.

Evidence suggests that it may not entirely be due to its exonuclease activity as some AGS-causing Trex1 mutants were shown to be defective in LINE-1 inhibition despite their nucleolytic proficiency (P. Li et al., 2017).

This model however was recently questioned by another report (Achleitner et al., 2017) implying that endogenous retro-elements may not be the sole activators of the immune system in this setting.

Nonetheless, these studies together provide evidence for a second source of self-derived cytosolic DNA: endogenous viral elements.

RNase H2 (Ribonuclease H 2):

RNase H2 is the major RNaseH in mammalian cells (Frank, BraunshoferReiter, Poltl, & Holzmann, 1998). RNase H2 degrades RNA in RNA-DNA hybrids; it can also cleave a single ribonucleotide inserted into DNA (Eder, Walder, & Walder, 1993).

Complete loss of RNASEH2 is embryonic lethal in mice but is not associated with type I IFN induction (Hiller et al., 2012; Reijns et al., 2012). Consistently, most RNASEH2 mutations identified so far in AGS are hypomorphic (G. Rice et al., 2007). Study of RnaseH2 ^{-/-} MEFs has shown that this enzyme is essential to protect genome integrity by removing mis-incorporated ribonucleotides (Hiller et al., 2012; Reijns et al., 2012; Sparks et al., 2012).

RNase H2 contains three subunits, RNase H2A, 2B and 2C, mutations in any of which causes AGS (Crow, Hayward, et al., 2006). Significantly, RNaseH2 loss-of-function mutations account for over half of the AGS cases (Crow & Manel, 2015). RNaseH2 mutations are not associated with a specific clinical phenotype.

Cells of SLE (Systemic lupus erythematosus) and AGS patients with RNase H2 mutations produce increased levels of pro-inflammatory cytokines, particularly after irradiation with UV light (Günther et al., 2015); This study demonstrated that RNaseH2 mutation in AGS cells increases basal DNA damage signaling. Besides, the authors proposed that ribonucleotides embedded into genomic DNA favor the generation of pyrimidine dimers upon UV light exposure, explaining the photosensitivity of these patients.

A mouse model of the most common missense mutation found in AGS patients was generated by Jackson and colleagues (Mackenzie et al., 2016). They demonstrated that RNASEH2 mutation activates a STING-modulated pro-inflammatory response (Mackenzie et al., 2016). In particular, this effect was associated with increased DNA damage and the loss of RNase H2-specific activity in ribonucleotide excision repair (Mackenzie et al., 2016). The exact ligand activating the STING pathway was later identified: RNaseH2 deficiency, similarly to DNA damage, leads to the

formation of micronuclei recognized by cGAS, triggering the production of ISGs (Harding et al., 2017; MacKenzie et al., 2017).

Interestingly, R-loops have not been so far involved in the inflammatory phenotype of RNaseH2-deficient cells, despite one report of large accumulation of R loops across all AGS subtypes (Lim, Sanz, Xu, Hartono, & Chédin, 2015) and the demonstration that RNA:DNA hybrids can be recognized by cGAS (Mankan et al., 2014).

ADAR1 (Adenosine Deaminases Acting on RNA)

ADAR1 is an RNA editing enzyme: it catalyzes the conversion of adenosine to inosine in dsRNA (Hogg, Paro, Keegan, & O'Connell, 2011). Two protein isoforms are expressed in mammalian cells, one of them is an IFN-inducible gene (George, Samuel, & Sinsheimer, 1999). Deletion of this gene is embryonic lethal in mice (Hartner et al., 2004; Q. Wang et al., 2004), as is mutating the RNA editing catalytic site (Liddicoat et al., 2015). Both absence of ADAR1 and mutation of its editing activity results in upregulation of ISGs in mice embryos. Deletion of the cytosolic RNA sensor MDA5 or its adaptor protein MAVS rescue the IFN signature and this embryonic lethality (Liddicoat et al., 2015; Mannion et al., 2014). These results indicate that RNA editing by ADAR1 is essential to prevent endogenous RNA recognition by the RLR pathway.

Evaluation of the ADAR1-editome in human cells has revealed thousands of possible editing sites, most of which mapped to endogenous retro-elements, particularly Alu repeats (Levanon et al., 2004). In mice, genes edited by ADAR1 have the potential to form long perfect dsRNA fragments, legitimate ligands of the RLRs, through duplexing of repetitive regions (Liddicoat et al., 2015). ADAR1 A-to-I editing is hence thought to destabilize host-derived dsRNA structures, chiefly Alu repeats, to prevent their detection by the innate immune system (Ahmad et al., 2018). Lastly, it was recently demonstrated that, in human cells, ADAR1 not only regulates innate immune activation, but also the response to IFNs by preventing the global transcription shutdown induced by inflammation (Chung et al., 2018).

IFIH1 (Interferon Induced with Helicase C domain 1)

This gene encodes for the cytosolic RNA sensor MDA5. AGS patients exhibit gain-of-functions mutations in these gene, leading to increased signaling activities and IFN production either in basal conditions or upon stimulation with dsRNA (G. I. Rice et al., 2014). Using a cell-free system, it was recently shown that gain-of-function MDA5 mutants are constitutively activated by endogenous RNAs (Ahmad et al., 2018). The authors went on to identify the endogenous MDA5 ligand in 293T cells, revealing that Alu retro-elements are recognized by MDA5. Alus in inverted repeat configuration in particular strongly activate MDA5 gain-of-function mutants and not wild-type MDA5 because it is sensitive to dsRNA structure modifications (generated, for example, by A-to-I editing).

3. Disease management

Treatment of patients affected with AGS is currently primarily based on alleviating symptoms rather than reversing the course of the disease. A phase II clinical trial is ongoing in Paris (NCT02363452) to test the use of reverse-transcriptase inhibitors, based on the hypothesis that retro-element activity is a major source of self-induced-immunity. Another clinical trial (NCT03304717) was recently opened in Philadelphia to test the same hypothesis.

The results of another trial were recently published (G. A. M. Sanchez, Macias, & Goldbach-Mansky, 2018), testing the use of the Jak inhibitor baricitinib in auto-inflammatory disorders, including AGS. Only one patient diagnosed with AGS was enrolled in the study. Following treatment, her inflammatory signature decreased, her auto-antibody production was reduced and her clinical symptoms improved but she experienced serious adverse events such as infections and sepsis.

To my knowledge, no other clinical investigation is open.

II- SAMHD1

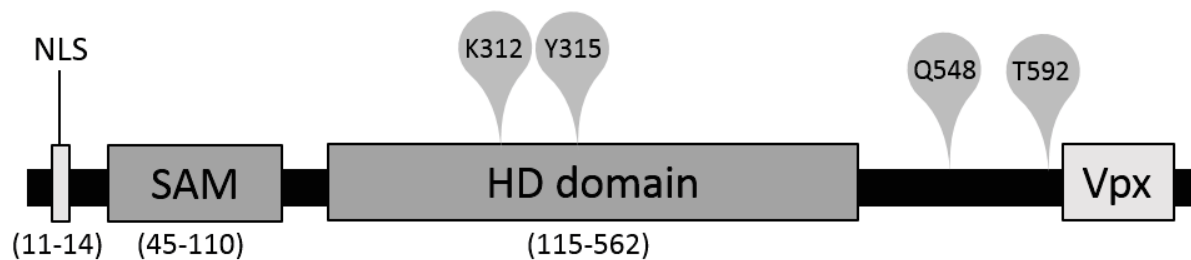
According to a recent survey of 374 AGS patients, SAMHD1 is mutated in 13% of AGS cases (Crow, 2015). AGS caused by SAMHD1 mutation is not only associated with classical features of the disease such as neurological dysfunctions, congenital infection-like syndrome and developmental delay (Crow, 2015) but also with cerebral vasculopathy (Ramesh et al., 2010). SAMHD1 is produced in a wide array of tissues, with a particularly high expression profile in Dendritic Cells and other cells of the myeloid lineage (Su et al., 2004).

1. Structure

SAMHD1 has 2 characteristic domains (see Figure 28):

- An N-terminal sterile alpha motif (SAM) domain, known to mediate protein-protein interactions and potentially protein-nucleic acids interactions (C. A. Kim & Bowie, 2003). This domain is preceded by a Nuclear Localisation Signal (NLS).

	mutation:			
activity:	K312A	Y315A	Q548X	T592A/E
dNTPase:	-	+	+	+/?
HIV restriction:	-	-	-	+/-



*Figure 28 **Structural and functional domains of SAMHD1:** SAMHD1 consists of 4 main domains. First, the N-terminal domain with a nuclear localization sequence. Second, the SAM domain known to mediate protein-protein interactions. Third, the catalytic HD domain. Lastly, a C-terminal domain with a Vpx-binding sequence. Study-relevant amino-acid positions with the corresponding mutations and their effect on SAMHD1 dNTPase and HIV restriction activities are indicated.*

- A catalytic histidine–aspartate (HD) domain typical of a class of enzymes with phosphohydrolase activity (Aravind & Koonin, 1998), encompassing a nucleic-acid binding region (Goncalves et al., 2012).

Indeed, SAMHD1 possesses 2 known enzymatic activities: it is a dNTP triphospho-hydrolase (Goldstone et al., 2011; R. D. Powell, Holland, Hollis, & Perrino, 2011) and a putative nuclease (Beloglazova et al., 2013).

2. Enzymatic activities

SAMHD1 is a dNTP hydrolase

SAMHD1 converts dNTPs to the corresponding deoxynucleoside and inorganic triphosphate. It is not active against nucleosides triphosphates and deoxynucleotides mono- or di-phosphates (R. D. Powell et al., 2011).

Biochemical analysis has shown that GTP and dGTP-induced tetramerization of the protein is necessary for its dNTPase activity (Hansen, Seamon, Cravens, & Stivers, 2014; R. D. Powell et al., 2011; Yan et al., 2013). Further structural analysis has revealed that tetramerization is sequentially regulated by allosteric GTP, dGTP and dNTP binding to different regulation sites (Figure 29). First, allosteric binding of dGTP or GTP to a primary regulation site induces dimerization of the protein. Subsequent recognition of any dNTP by each monomer of dimerized SAMHD1 at a secondary allosteric regulation site allows the formation of tetramers, thereby generating an active protein complex (Hansen et al., 2014; Ji, Tang, Zhao, Wang, & Xiong, 2014; Koharudin et al., 2014; Miazzi et al., 2014). This tetramer is able to cleave any dNTP binding to the catalytic site, albeit its affinity for dGTP is higher than its affinity for any other dNTPs (R. D. Powell et al., 2011).

Is SAMHD1 a nuclease?

SAMHD1 has an *in vitro* 3' – 5' exonuclease activity against ssRNA and, potentially, against ssDNA and RNA in DNA/RNA hybrids (Beloglazova et al., 2013; Ryoo et al., 2014). Accordingly, SAMHD1 was proposed to mediate HIV restriction by the degradation of viral genomic RNA.

This feature is controversial: in 2015, Seamon et al suggested that this exonuclease activity may be due to contaminants (Seamon, Sun, Shlyakhtenko, Lyubchenko, & Stivers, 2015). They used three purification steps to isolate SAMHD1 proteins. They indeed found a nuclease activity in the protein preparations, but the DNase activity was lost upon protein purification. A weak RNase activity persisted, although not dependent on the HD-catalytic site.

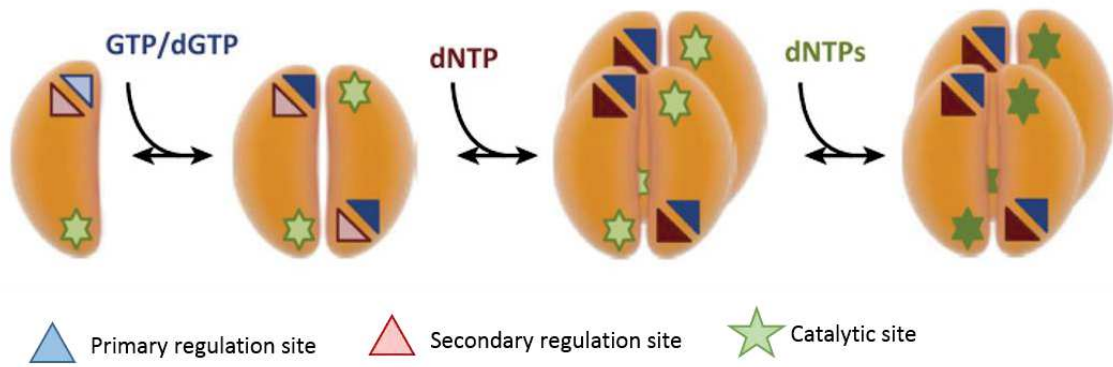


Figure 29 **The dNTPase activity of SAMHD1 is activated by tetramerization:** First, allosteric binding of dGTP or GTP to a primary allosteric regulation site induces dimerization of the protein. Additional binding of any dNTP to a secondary allosteric regulation site on dimerized SAMHD1 proteins induces additional conformational changes, generating active SAMHD1 tetramers enable to accommodate any dNTP in its catalytic site. Modified from Ballana & Esté, 2015.

The authors additionally demonstrated that SAMHD1 binds to ssRNA, ssDNA, RNA:DNA hybrids and dsDNA, with a preference for ssRNA.

The same group further reported that SAMHD1 binds ssDNA and ssRNA through its HD and its C-ter domains in its monomeric and dimeric forms. Single-stranded nucleic acid binding to SAMHD1 prevented its tetramerization. In other words, upon binding to nucleic acid ligands, SAMHD1 is not active as a dNTPase. No RNase activity however was detected in this study (Seamon, Bumpus, & Stivers, 2016).

Up to now, it is still not clear whether SAMHD1 has a nuclease activity altogether.

3. Functions

Viral restriction

The existence of a cell-intrinsic factor counteracted by Vpx and mediating resistance to HIV-1 through the activation of an inflammatory response was known in dendritic cells (Manel et al., 2010). This restriction factor was later identified as being the SAMHD1 protein. Its capacity to inhibit HIV-1 replication in macrophages and dendritic cells was its first cellular function discovered (Hrecka et al., 2011; Laguette et al., 2011). It was later found to also prevent HIV infection of resting CD4 T Cells (Baldauf et al., 2012; Descours et al., 2012). SAMHD1 also blocks infection by other retroviruses such as the Equine Infectious Anemia Virus or the Murine Leukemia Virus (Gramberg et al., 2013; T. E. White et al., 2013). This activity is not restricted to retroviruses as infection by some DNA viruses such as the vaccinia virus and the herpes simplex virus are also counteracted by SAMHD1 (Hollenbaugh et al., 2013). Lentiviruses escaping SAMHD1 restriction express the accessory protein Vpx that target it for ubiquitin-dependent degradation (Hofmann et al., 2012).

How SAMHD1 restricts viral replication is still an open question as there is some controversy regarding which of its enzymatic activity is involved in this process.

Genome maintenance

Fibroblasts from AGS patients with SAMHD1 mutations have higher endogenous DNA damage compared to normal fibroblasts as seen by comet assay (Kretschmer et al., 2015). Accordingly, Clifford et al. demonstrated that SAMHD1 localizes to DNA damage sites (Clifford et al., 2014). In fact, SAMHD1 is involved both in the prevention and in the response to DNA damage.

As already mentioned in chapter 2, SAMHD1 reduces dNTP pools in G1 (Franzolin et al., 2013). Tight regulation of dNTP pools outside of S phase is of primary importance to sustain accurate DNA repair and to allow mitochondrial replication (Håkansson, Hofer, & TheLander, 2006). SAMHD1 also has a more direct role in DSB repair (Daddacha et al., 2017). Indeed, SAMHD1

promotes HR-mediated repair of the breaks by recruiting CtIP, thereby allowing DSB resection (Daddacha et al., 2017). As HR is a major pathway of break repair, especially in S phase when many genotoxic drugs are active, this study lead to the provocative idea that targeting SAMHD1 with Vpx could sensitize cancer cells to DNA-damaging agents. In addition, reducing SAMHD1 levels may also sensitize cells to nucleoside analogues such as Ara-C. Beyond endogenous dNTPs, SAMHD1 indeed hydrolyzes these antimetabolites, thereby preventing their incorporation into genomic DNA and reducing their therapeutic efficacy (Herold et al., 2017; Rudd et al., 2017; Schneider et al., 2017).

SAMHD1 mutation is conversely associated with tumor development in chronic lymphocytic leukemia (Clifford et al., 2014), colorectal cancer (Rentoft et al., 2016) and in T-cell prolymphocytic leukemia (Johansson et al., 2018). Interestingly, in colon adeno-carcinomas, SAMHD1 mutations affecting its dNTPase activity were all found in hyper-mutated tumors associated with defects in Mismatch Repair proteins (Rentoft et al., 2016). In this study, modest dNTP pool imbalance was also linked to elevated mutation rates in Mismatch Repair-deficient cells. Given that dNTP pools homeostasis is critical both for DNA replication and DNA repair, it is likely that the key role of SAMHD1 in genome integrity maintenance results from its dNTPase activity.

Restriction of endogenous retroelements

Retrotransposons constitute over 40% of the base content of the human genome (Lander et al., 2001). Three main families of retroelements are found in humans:

- long terminal repeat (LTR)-like endogenous retroviruses,
- LINEs (long interspersed nuclear element) and
- SINEs (short interspersed nuclear elements) (see Figure 30).

The first two groups are autonomous in that they encode all the necessary proteins for replication, while SINEs hijack the enzymes produced by LINEs to mediate their retrotransposition. LINE-1 retrotransposons, which make up round 17% of genomic DNA, are the only actively replicating retroelements in the human genome. They code for a reverse-transcriptase with an additional endonuclease activity that mediates the “copy and paste” mechanism of reverse transcription. LINE-1 allows the replication of Alu retrotransposons, the main components of human SINEs. 248 known copies of functional LINE-1 reverse-transcriptase can mediate LINE-1 and Alu expansion.

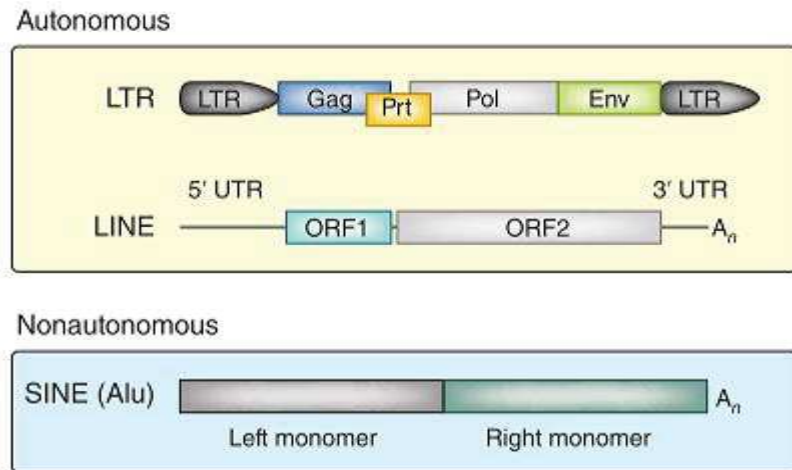


Figure 30 The 3 main types of human retroelements: The autonomous retroelements encode all the necessary proteins for their replication. This category includes LTR-type retroviruses and LINEs. Non-autonomous retroelements such as SINEs require LINEs for their mobility. They mainly consist of Alu elements. Modified from Volkman & Stetson, 2014.

Over 10^6 Alu repeats are inserted in the human genome, which means there is 1 Alu copy every $\sim 3 \cdot 10^3$ base pairs. Both LINE-1 and Alu retro-transposition events can destabilize genome integrity and cause disease, therefore their activity is highly regulated (for a review of endogenous retroelements in autoimmune disease: Volkman & Stetson, 2014).

Although SAMHD1 restrict retroviruses in resting cells only, several groups have implicated SAMHD1 in the inhibition of endogenous retro-elements in proliferating cells (Herrmann et al., 2018; Hu et al., 2015; K. Zhao et al., 2013). They all used variation of a reporter assay whereby a GFP or a neomycin-resistance cassette, inserted in reverse orientation in the 3' UTR of LINE-1 and interrupted by an antisense intron, is expressed only after the L1 transcript is spliced, reversed-transcribed and inserted into the genome.

The first study showed that overexpressing SAMHD1 in 293T cells prevents LINE-1 retro-transposition by inhibiting its reverse transcription (K. Zhao et al., 2013). They also reported that AGS-related SAMHD1 mutants do not sustain this activity, unlike a dNTPase-defective mutant.

On the other hand, Hu and Herrmann found that a functional dNTPase activity is necessary for LINE-1 restriction. The first group implicate SAMHD1 in stress-granule formation where LINE-1 RNA-protein complexes are sequestered, limiting its retro-transposition (Hu et al., 2015). They did not discuss this model in light with the requirement for SAMHD1 dNTPase activity. Herrmann and colleagues analyzed LINE-1 retro-transposition in 293T cells (Herrmann et al., 2018). They show that SAMHD1 overexpression only modestly reduced L1 reverse-transcription but a non-phosphorylatable mutant (T592A) did strongly impairs LINE-1 activity. They compared many mutants to identify which enzymatic activity of SAMHD1 is involved. They found that allosteric GTP binding and an intact HD domain were both important for L1 inhibition mediated by SAMHD1. A Q548A mutant, defective in the so-called RNase activity, did not reduce this activity. Altogether, they favor a model where local dNTP pool alterations mediated by SAMHD1 prevent LINE-1 reverse-transcription. In contrast to Hu and colleagues, they did not detect stress granule formation upon overexpression of LINE-1 and SAMHD1.

Overall, this activity remains controversial, at least in the context of AGS, as transposon reactivation has not been detected in AGS-patient fibroblasts mutated for SAMHD1 (Lim et al., 2015).

4. Post-translational regulation

Cycling cells are permissive to HIV-1 infection, suggesting that an additional layer of protein regulation exists to modulate the restriction ability of SAMHD1. Three groups showed that indeed SAMHD1 is phosphorylated on its threonine 592 (T592) specifically in proliferating cells (Cribier, Descours, Valadão, Laguet, & Benkirane, 2013; Welbourn, Dutta, Semmes, & Strebel, 2013; T. E.

White et al., 2013). They also reported that exogenous expression of a non-phosphorylatable SAMHD1 mutant in permissive U937 monocytic cells is sufficient to make them resistant to HIV-1 infection. In other words, SAMHD1 phosphorylation at T592 negatively regulates its HIV-1 restriction ability. On the other hand, this modification does not reduce its dNTPase activity, its sensitivity to Vpx, its nuclear localization and its capacity to bind nucleic-acids. The effect on the oligomerization abilities is more controversial : using tagged-SAMHD1 proteins expressed in 293T cells, White and colleagues did not find any defect in oligomerization induced by mutation of the T592 residue, but other laboratories disputed this finding using *in vitro* assays (Arnold et al., 2015; A. Bhattacharya et al., 2016; T. E. White et al., 2013). Because the ability of SAMHD1 to oligomerize is primarily regulated by dNTP binding to regulatory sites, phosphorylation may only reduce the stability of the tetramers when dNTP levels are limiting.

Other phosphorylation sites were discovered (S6, T21, S33), none of them affected SAMHD1 restriction activity in U937 cells (Welbourn et al., 2013; T. E. White et al., 2013). T592 phosphorylation is removed by the protein phosphatase 2A at the M/G1 transition, which correlates with an activation of its HIV restriction activity (Schott et al., 2018).

Several Cyclin/CDK complexes seem to catalyze SAMHD1 phosphorylation on T592. Both CDK1 and CDK2 phosphorylates this residue *in vitro*, in partnership with Cyclin A2 and Cyclin B (Cribier et al., 2013; St. Gelais et al., 2014; T. E. White et al., 2013). The relative *in vivo* contribution of the CDK1 and CDK2 kinases might be cell-type dependent. Indeed, CDK1 was implicated by groups using mainly cell lines (Cribier et al., 2013; T. E. White et al., 2013), while the involvement of CDK2 was confirmed using primary cells (Pauls et al., 2014).

Regardless of the kinase involved, regulation of SAMHD1 is tightly linked to cell-cycle progression as the expression and activity of CDKs is highly controlled along the cell cycle.

All the aforementioned studies found a perfect correlation between the phosphorylation status of SAMHD1 and the loss of its viral restriction activity. This observation suggests that this activity is only observed in non-dividing cells because they do not express the CDK proteins phosphorylating the T592. In other words, although SAMHD1 is expressed in proliferating cells, its phosphorylation precludes its HIV restriction function. There is however an exception to this rule: expressing a non-phosphorylatable mutant in 293T cells did not make them resistant to HIV (St. Gelais et al., 2014). This result indicates that phosphorylation on T592 may not be the only regulatory modification mediating the restriction capacity of SAMHD1. Other post-translational modification, yet to be defined, may be involved in the regulation of SAMHD1 in these cells. One such candidate modification has recently been identified. It has indeed lately been shown that SAMHD1 is acetylated in G1, upregulating its dNTPase activity (Ji Lee et al., 2017). To add another layer of complexity, this enzymatic activity seems to be also regulated by oxidation in response

to growth stimuli (Mauney et al., 2017). Whether these regulatory signals are important for the HIV restriction ability of SAMHD1 is at present unknown.

The interaction between SAMHD1 and Cyclin A/CDK is mediated through a cyclin-binding motif localized at the C-terminus of the protein sequence, right next to the threonine 592 (St. Gelais et al., 2018). Studying this motif, Li Wu and colleagues have found that endogenous SAMHD1 has a half-life of 6.5 hours in THP1 cells (St. Gelais et al., 2018). Accordingly, SAMHD1 protein levels seem to be actively regulated in cycling cells but the mechanisms involved are still unknown. Both autophagy and proteasomal degradation may be involved in this process (St. Gelais et al., 2018).

5. Animal models

Early after the discovery of the role of SAMHD1 as an HIV restriction factor, SAMHD1 knock-out mice were generated to study its *in vivo* relevance in HIV infection and AGS etiology. Two groups generated SAMHD1 mice models using similar mice backgrounds (Behrendt et al., 2013; Rehwinkel et al., 2013). In each case, mice were born in Mendelian ratios, without obvious phenotypical defects. One group showed that SAMHD1-deficient mice were more susceptible to HIV-1 infection than control mice (Behrendt et al., 2013), the other modulated this conclusion relatively to the high dNTP levels of the mice strain they used (Rehwinkel et al., 2013). Notably, they reported that an HIV mutant strain having lower affinity for dNTPs infected SAMHD1-null mice more potently than control mice. Both study confirmed the implication of SAMHD1 in HIV restriction *in vivo*.

Regarding AGS development, the results were more disappointing as neither study found an association between SAMHD1 loss and an autoimmune pathogenesis despite an increase in IFN signaling in some tissues (Behrendt et al., 2013; Rehwinkel et al., 2013).

Subsequent development of a Zebrafish model was more successful as it was associated with phenotypes reminiscent of AGS, such as the IFN-associated cerebral vasculopathy (Kasher et al., 2015). Interestingly, the involvement of SAMHD1 in innate immunity in the fish order was recently confirmed (M. Li et al., 2018).

III- Crosslinks between the DDR and self-induced innate immunity

In 2005 Gasser and colleagues showed that the DNA damage response can directly activate the immune system (Gasser, Orsulic, Brown, & Raulet, 2005). Some innate immune cells such as NK cells and CD8⁺ cells express stimulatory receptors such as the NKG2D (natural-killer group 2, member D) receptor. This receptor is activated by molecules resembling major histocompatibility complex (MHC) class I proteins, including MICA and MICB (respectively MHC class I chain-related

protein A and B). Ionizing radiation, 5-FU, cisplatin, aphidicolin all induced the expression of NKG2D ligands in primary mice fibroblasts as detected with a specific NKG2D tetramer antibody. This response was inhibited when ATM, ATR and CHK1 were depleted or inhibited, providing one of the first hints of the involvement of the DNA damage response in innate immunity.

The study of inflammatory disorders such as AGS has provided other clues about the contribution of endogenous DNA in the activation of innate immunity. As stated earlier, endogenous retroelements, ribonucleotides embedded into genomic DNA, replication intermediates and DNA damage all have been proposed to fuel cytosolic DNA recognition. The list of potential activators of cell-autonomous immunity does not stop here as almost all aspects of endogenous DNA metabolism may be sources of immune agonists.

1. Stress-induced inflammation

DNA damage

Several studies have demonstrated that persisting DNA damage promotes inflammation. Indeed, cell treatment with etoposide (but also camptothecin, mitomycin C, and adriamycin) induces the production of ISGs, IFN- α and IFN- λ genes in an NF- κ B-dependent manner (Brzostek-Racine, Gordon, Van Scoy, & Reich, 2011). Cisplatin and MMC were also found to activate the cGAS-STING pathway of type-1 IFN signaling (Erdal, Haider, Rehwinkel, Harris, & McHugh, 2017). As for irradiation, X-ray (Erdal et al., 2017), γ -irradiation (Härtlova et al., 2015), high LET (Linear Energy Transfer) iron irradiation (S. Bhattacharya et al., 2017) were all found to activate pro-inflammatory pathways.

In particular, a direct link between loss of ATM and inflammation was demonstrated by Nelson Gekara and colleagues in 2015. Ataxia-Telangiectasia (AT) is a neurodegenerative disease, displaying a variety of autoinflammatory and autoimmune symptoms, caused by mutations in the ATM gene. Bacterial infection is the major cause of mortality of AT patients. In cells from AT patients and in mice, loss of ATM and DNA damage (γ -irradiation, etoposide) induce the production of cytosolic DNA. The latter is recognized by IFI16 and cGAS, triggering STING-mediated type I IFN signaling (Härtlova et al., 2015). This study proposed that DNA damage induced by the loss of ATM primes the immune system for type I IFN production. Moreover, ATM activity was subsequently implicated in inflammasome activation (Erttmann et al., 2016). ATM deficiency indeed directly impaired the production of IL-1 β in mice. As inflammasome activity is essential for anti-bacterial defense, this study provided an explanation for the high susceptibility of AT patients to bacterial infection.

The molecular mechanisms of DNA damage-induced inflammation are beginning to be elucidated. It was found recently that in breast cancer cells, long-range resection pathways mediated by BLM

and EXO1 generate cytoplasmic ssDNA fragments upon X-ray irradiation (Erdal et al., 2017). Cytosolic RNA signaling has in turn been implicated in the radiation-induced type I IFN response (Ranoa et al., 2016). Mechanistically, the authors of this study proposed that irradiation upregulates the transcription of non-coding RNAs that are exported to the cytosol where they are recognized by RIG-I.

Retrotransposition

Analysis of the cell-cycle arrest at the G2/M transition induced by the HIV accessory protein Vpr has implicated the SLX4 repair complex as a negative regulator type I IFN production (Laguette et al., 2014). Indeed, in the absence of viral infection, silencing of SLX4 and its partner nuclease Mus81 induces an overproduction of IFN- α , IFN- β and the ISG MxA. The activation signal for this pro-inflammatory activity was subsequently identified (Br  gnard et al., 2016). It was found that SLX4-deficient cells have increased cytosolic DNA levels caused by an upregulation of retrotransposition activity. Accordingly, this study reported that SLX4 inhibits LINE-1 retrotransposition. Treatment with a reverse-transcriptase inhibitor (Tenofovir) prevented the production of pro-inflammatory cytokines induced by SLX4 deficiency.

Mitochondrial stress

Mitochondrial stress is also a source of cytosolic DNA. Seminal work indeed demonstrated that injury triggers a sepsis-like inflammatory response induced by the release of mitochondrial DNA in the circulation that is recognized by innate immune sensors, starting the production of pro-inflammatory cytokines (Q. Zhang et al., 2010). How mitochondrial DNA could be released in the bloodstream was not thoroughly addressed in this study and still is a matter of study.

Two groups have simultaneously demonstrated that the pro-apoptotic proteins Bax (BCL-2-like protein 4) and Bak (BCL-2 homologous antagonist/killer) trigger the release of mitochondrial DNA in the cytosol, activating the cGAS/STING pathway to induce the production of IFN- β (Rongvaux et al., 2014; M. J. White et al., 2014). This activity was counteracted by the apoptotic caspases caspase 9, or both caspase 3 and caspase 7. In other words, these two studies report that, *in vivo*, proapoptotic caspase cascades are essential to suppress a pro-inflammatory response induced by programmed cell death. They also suggest that mitochondrial outer membrane permeabilization by Bax and Bak induced mitochondrial DNA release.

Disruption of mitochondrial chromatin organization may be another cause of mitochondrial DNA export. In fact, mitochondrial stress induced by disruption of mitochondrial chromatin organization also activates a type I IFN response in MEFs (West et al., 2015). In mammalian cells, mitochondrial DNA is packed in higher-order chromatin structures names nucleoids. Heterozygous knock-out of TFAM (Transcription Factor A, Mitochondrial), the mitochondrial

DNA-binding protein necessary for nucleoid formation, induced the production of the ISGs Cxcl10, Ifit3 and Isg15. Depletion of cGAS, STING, Tbk1 and IRF3 abrogated this response. Consistently, mitochondrial DNA was enriched in the cytosol of TFAM heterozygous MEFs compared to WT cells.

Interestingly, mitochondria is also known to be an endogenous agonist of inflammasomes. This result was reported studying autophagy-mediated inflammation. In fact, mitochondrial DNA may be released into the cytosol of macrophages where it involved in caspase-1 activation and, therefore, in inflammasome-mediated IL-1- β and IL-18 production (Nakahira et al., 2011). The authors of this study demonstrated that suppressing this release of mitochondrial DNA is one of the key role of autophagy proteins.

This mitochondrial DNA-induced inflammation has been involved in several pathologies such as atherosclerosis, age-related macular degeneration, mevalonate kinase deficiency and certain bacterial infections (West & Shadel, 2017). As pro-inflammatory cytokines such as TNF- α and IL-1- β conversely promote mitochondria damage (López-Armada, Riveiro-Naveira, Vaamonde-García, & Valcárcel-Ares, 2013), a vicious circle of increasing inflammation may entangle the assessment of the physiological relevance of mitochondrial DNA release.

2. Counteracting self-DNA induced inflammation

Mutation or deficiency in proteins involved in the DDR and in DNA repair have been reported to induce inflammation. In particular, it was shown that loss of the repair helicase WRN upregulates inflammatory pathways at the transcription level (Turaga et al., 2009), as does loss of ERCC1 (Karakasilioti et al., 2013) and the loss of BRCA2 (Xu et al., 2014). Fen1 deficiency has been shown to trigger auto-immunity, possibly through the accumulation of apoptotic DNA (Zheng et al., 2007). These early studies however did not fully explain how these proteins protects the cell from inflammatory responses.

One study has demonstrated the involvement of other DDR proteins, namely RPA and RAD51, in an active protection mechanism against self-induced inflammation (Wolf et al., 2016). In fact, the authors demonstrated that RPA and RAD51 prevent nuclear DNA from leaking to the cytoplasm (Wolf et al., 2016). They reported that short DNA fragments microinjected into living cells move between the nucleus and the cytoplasm, their nuclear retention mediated by RPA and RAD51. In Trex1-depleted cells, RAD51 and RPA depletion increases the constitutive type I IFN expression. They also showed that in HeLa cells, RPA and RAD51 depletion is sufficient to induce a spontaneous STING-mediated I IFN production, indicating that RPA and RAD51 may also bind genome-derived DNA fragments to prevent inflammatory signaling.

Recently, another role was proposed for RAD51 in preventing the generation of DNA fragments from stalled replication forks (S. Bhattacharya et al., 2017). The authors showed that RAD51 depletion upregulates STING-mediated production of pro-inflammatory genes such as IL-6 or TNF- α upon irradiation. In the absence of RAD51, IR-stalled replication forks were aberrantly processed by MRE11, generating cytosolic ssDNA and dsDNA fragments that triggered pro-inflammatory signaling (S. Bhattacharya et al., 2017). This study suggests that, in the absence of RAD51, an aberrant processing of stalled replication forks by MRE11 turns on inflammatory responses. As BRCA2 is responsible for the loading of RAD51 at stalled replication forks (Schlachter et al., 2011), it is now tempting to speculate that the same mechanism is in play in BRCA2-deficient cells (Xu et al., 2014).

3. DDR proteins as cytosolic DNA sensors

MRE11/RAD50 (Kondo et al., 2013; Roth et al., 2014) and DNA-PK (Ferguson, Mansur, Peters, Ren, & Smith, 2012; Xing Zhang et al., 2011) were found to be involved in cytosolic DNA recognition, completing the list of DNA sensors (Figure 31).

MRE11 indeed recognizes cytosolic dsDNA (Kondo et al., 2013). This observation was initially made using a non-AT-rich sequence dsDNA ligand, resembling DSB, injected in the cytosol of cells treated with the MRE11 inhibitor mirin or transfected with a siRNA against the nuclease. The recognition of other DNA ligands such as *E. coli* DNA and plasmid DNA was also demonstrated to be dependent on MRE11, indicating a broader role for this protein in the immune response. Supplementary analysis demonstrated that this interaction is important for STING-mediated IFN- β production, placing MRE11 upstream of STING in this DNA sensing pathway. Unlike NBS1, RAD50 was also implicated in this response.

The group of Jürgen Ruland further showed that RAD50 interacts with the adaptor protein CARD9 (Caspase recruitment domain-containing protein 9) to induce the production of IL-1- β in response to multiple dsDNA ligands such as linear synthetic poly(dA:dT), poly(dG: dC), purified genomic calf thymus DNA or circular bacterial plasmid DNA. CARD9 is solely expressed in myeloid cells and is important for pro-IL-1 β production induced by NF- κ B signaling. This study demonstrated that formation of a dsDNA-RAD50-CARD9 complex activates NF- κ B in response to cytosolic dsDNA or during infection with a DNA virus (Roth et al., 2014).

As for DNA-PK, two studies implicated this DNA-binding protein as an innate immune sensor. First, Tomozumi Imamichi and collaborators demonstrated that Ku70 is specifically important for type III IFN production in response to linear plasmid DNA in HEK293T cells (Xing Zhang et al., 2011). One year later, DNA-PK was found to mediate IRF3-induced IFN signaling in response to

exogenous DNA transfection and infection with the DNA virus vaccinia in primary mice fibroblasts and in mice (Ferguson et al., 2012).

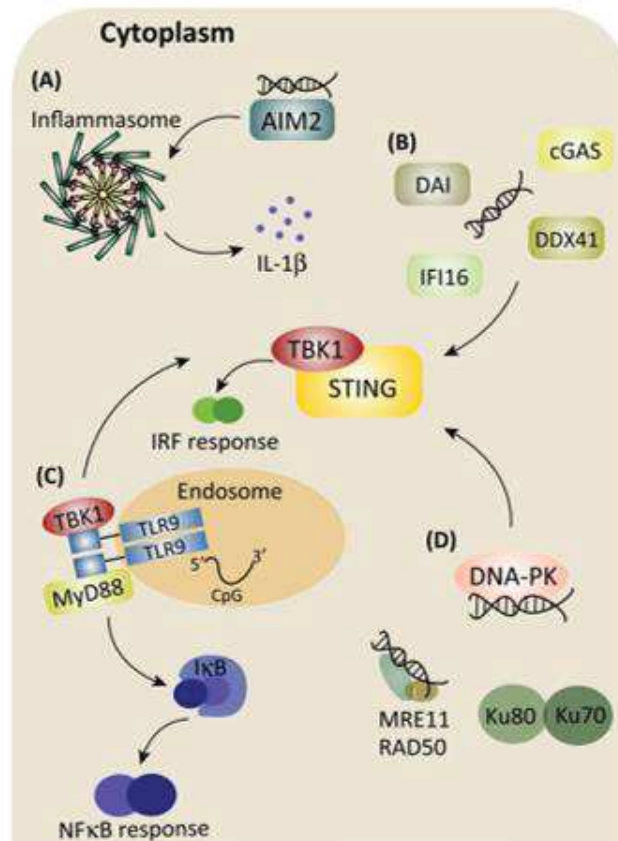


Figure 31 Cytosolic DNA sensors and innate immune signaling: Multiple DNA sensors recognize DNA in the cytosol. A) AIM2 binds dsDNA to generate an inflammasome, allowing for the production of mature IL-1 β . B) cGAS, DDX41, IFI16 also binds cytosolic DNA, activating the IFN response through STING. C) In endosomes, DNA is recognized by TLR9 that activate NF κ B-dependent inflammatory responses. D) DNA-PK and MRE11-RAD50 also signal the presence of DNA in the cytosol via STING. Modified from Chatzinikolaou, Karakasilioti, & Garinis, 2014.

The involvement of DNA-PK in immune sensing seems to be specific to DNA as these findings could not be extended to exposure with poly (I:C) RNA or infection with an RNA virus (influenza). It was later found that vaccinia viruses express a protein that reduces the ability of Ku to bind DNA, highlighting the importance of DNA-PK as a DNA sensor upon vaccinia virus infection (Peters et al., 2013).

It would be interesting to study how these DNA sensors fit in the context of the cGAS-STING DNA sensing pathway. Recently, the group of Thomas Schulz reported that the Kaposi Sarcoma herpesvirus latency-associated nuclear antigen promotes viral reactivation by counteracting cGAS (G. Zhang et al., 2016). They further demonstrated that in the absence of cGAS, this viral antigen now antagonizes MRE11-RAD50-dependent NF- κ B signaling (Mariggiò et al., 2017). I was not able to find a study reporting a comprehensive view of all these sensors in cytosolic surveillance mechanisms, but these two reports outline the fact that they are probably interconnected and controlled to allow an appropriate response to pathogens and protect against cytosolic self-nucleic acids.

All these studies highlight the complex interplay between the DNA damage response and activation of the innate immune system. Self-nucleic acids, arising for instance from genomic DNA or mitochondrial DNA following the induction of damages potentially activates innate immune sensors. What about other transactions taking place inside the nucleus? Aberrant intermediates of DNA replication was proposed to be immune-stimulatory (Y. G. Yang et al., 2007) but this was, to my knowledge, never studied more thoroughly.

| Objectives

Upon replication stress, a complex network of responses is triggered to safeguard the integrity of the genome, providing anti-tumor protection through pathways that coordinate control of the cell cycle, fork restart, apoptosis and senescence. Even so, replication stress is admittedly a significant inducer of genome instability. Besides, unrepaired damaged DNA may be released from the nucleus to the cytosol, where it is usually degraded by the cytosolic nuclease Trex1. In other words, persisting DNA damage or defects in this protection pathway can activate innate immune signaling.

Beyond DNA damage, retro-transposition, mitochondrial stress and micronuclei are all potent immunogenic signals. Replication has also been suggested to be a direct source of cytosolic DNA. How genomic DNA could be exported in the cytosol during DNA replication however was not reported. Stalled replication forks are processed by nucleases and helicases, so they can restart properly. In conditions of replication stress, activation of the interferon response could signal an abnormal processing of stalled replication forks.

Although mainly known as an HIV-restriction factor, SAMHD1 is also important for the regulation of the dNTP pools in G1 and in the repair of DNA damage. Notably, while SAMHD1 mutations in somatic cells are associated with cancer development, germline mutations cause the inflammatory disorder Aicardi-Goutières Syndrome. During the past few years, various studies have unraveled the central role of cytosolic nucleic acids metabolism in the development of AGS. The mechanism of chronic inflammation induced by SAMHD1 loss however remains unclear despite a recent report indicating that it relies on the cGAS/STING pathway of type I IFN production (Maelfait, 2016). Accordingly, immunogenic DNA might be located in the cytosol of SAMHD1-depleted cells. More work was needed to elucidate how this DNA was generated.

The nuclease SAMHD1 has important functions both in the DNA damage response and in the control of the interferon pathway. If SAMHD1 acts at arrested replication forks, its deficiency would result in an aberrant fork metabolism thereby generating DNA fragments able to leak to the cytosol.

The main objectives of my thesis were to:

1. To characterize the involvement of SAMHD1 in response to replication stress;
2. To assess the direct link between replication stress and the interferon response.

| Results

SAMHD1 acts at stalled replication forks to prevent induction of interferon

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Summary

SAMHD1 was previously characterized as a dNTPase that protects cells from viral infections. Mutations in SAMHD1 are implicated in cancer development and in a severe congenital inflammatory disease called Aicardi-Goutières syndrome. The mechanism by which SAMHD1 protects against cancer and chronic inflammation is unknown. Here, we show that SAMHD1 promotes degradation of nascent DNA at stalled replication forks by stimulating the exonuclease activity of MRE11. This function activates the ATR–CHK1 checkpoint and promotes fork restart. In SAMHD1-depleted cells, single-stranded DNA fragments are released from stalled forks and accumulate in the cytosol where they activate the cGAS–STING pathway to induce expression of pro-inflammatory type I interferons. SAMHD1 is thus an important player in the replication stress response, which prevents chronic inflammation by limiting the release of ssDNA from stalled replication forks.

Keywords: DNA replication forks, S-phase checkpoint, replication stress, chronic inflammation, interferons, cytosolic DNA, Aicardi-Goutières syndrome, cancer.

Introduction

SAMHD1 (sterile alpha motif and HD domain-containing protein 1) is a deoxyribonucleoside triphosphohydrolase (dNTPase) that, in quiescent cells, restricts infection by the human immunodeficiency virus-1 and other viruses¹⁻³. In cycling cells, this dNTPase activity is inhibited by phosphorylation of SAMHD1 by cyclin-dependent kinases^{4,5}. Germline mutations in SAMHD1 cause Aicardi-Goutières syndrome (AGS), a rare inflammatory encephalopathy characterized by overproduction of type I interferons (IFNs)⁶. Other genes mutated in AGS include TREX1, a 3'–5' exonuclease that degrades nucleic acids in the cytoplasm^{6,7}. Cytosolic DNA species that are not degraded by TREX1 trigger the production of type I IFNs and other cytokines through the cGAS-STING cytosolic DNA-sensing pathway⁸. The mechanism by which SAMHD1 inhibits this pathway is currently unclear⁹⁻¹¹ but it has been proposed to involve an elusive 3'–5' exonuclease activity¹²⁻¹⁴.

Besides AGS, SAMHD1 is frequently mutated in solid tumors and in chronic lymphocytic leukemia^{15,16}. Since SAMHD1 regulates intracellular dNTP pools, imbalanced dNTP levels in SAMHD1-deficient cells might perturb the progress of replication forks and thus increase spontaneous mutagenesis^{16,17}. Moreover, SAMHD1 colocalizes with DNA repair foci in cells exposed to genotoxic agents¹⁵, suggesting that it may play a more direct role at DNA lesions or at stalled replication forks. Fork stalling occurs when cells are exposed to genotoxic agents such as hydroxyurea (HU) and camptothecin (CPT) or when they encounter sequences that are intrinsically difficult to replicate¹⁸. Single-stranded DNA (ssDNA) accumulates at stalled forks and recruits the checkpoint kinase ATR, which in turn activates CHK1 to promote fork restart and prevent premature entry into mitosis^{18,19}. Fork restart depends on the degradation of nascent DNA strands by MRE11 through a process regulated by BRCA2 and called fork resection²⁰⁻²². Defective fork processing leads to fork collapse, increased genomic instability and cancer development^{18,19}.

Results

SAMHD1 prevents release of ssDNA

To determine whether SAMHD1 has a role at stalled forks that could be important to prevent the accumulation of cytosolic DNA, we first constructed stable HEK293T cell lines expressing either a shRNA against SAMHD1 (sh-SAM) or a scrambled shRNA control (sh-Scr; [Extended Data Fig. 1a](#)). Confocal immunofluorescence microscopy confirmed the presence of cytosolic

ssDNA in sh-SAM cells, but at a much lower level than in TREX1-depleted HEK293T cells, used here as positive control (Fig. 1a, b and Extended Data Fig. 1b). SAMHD1 depletion also induced type I IFN and other pro-inflammatory cytokine mRNAs in HEK293T (Fig. 1c), HL116 cells (Extended Data Fig. 1c) and HeLa cells (Extended Data Fig. 1d). Importantly, both the accumulation of cytosolic ssDNA and induction of IFN genes were substantially increased when sh-SAM cells were exposed to HU to induce replication fork stalling (Fig. 1a, b, d and Extended Data Fig. 1e). Moreover, when newly replicated DNA was labeled with BrdU two hours before addition of HU, this cytosolic DNA contained BrdU (Fig. 1e and Extended Data Fig. 1f), indicating that it comes from arrested forks and not, for example, from damaged mitochondria – another possible source of cytosolic DNA (Extended Data Fig. 1g, h). This induction of IFN genes by the cGAS–STING pathway in HeLa, HEK293 and THP1 cells^{23,24} is inhibited by depletion of SAMHD1 (Fig. 1f and Extended Data Fig. 1i, j, n, o), as it is the case in *Samhd1*^{-/-} mice⁹. It also depends on the interferon regulatory factor IRF3 (Extended Data Fig. 1k–n), both in the presence or the absence of HU. Together, these data indicate that SAMHD1 prevents the release of ssDNA from stalled forks and aberrant activation of the cGAS–STING pathway.

SAMHD1 is involved in DNA replication

To investigate the role of SAMHD1 in DNA replication, we first monitored its subcellular localization by immunofluorescence microscopy. SAMHD1 forms foci in HeLa cell nuclei which colocalize with replication sites (Fig. 2a and Extended Data Fig. 2a). By using iPOND (isolation of proteins on nascent DNA) method²⁵, we confirmed that SAMHD1 is present at replication forks (Fig. 2b and Extended Data Fig. 2b). However, SAMHD1 persisted on newly replicated chromatin after a thymidine chase, unlike PCNA (Fig. 2b). SAMHD1 also interacted with nascent DNA in *Xenopus* egg extracts and was recruited to chromatin in response to replication inhibition and DNA double strand breaks (DSBs; Extended Data Fig. 2d–f).

SAMHD1-depleted cells grew more slowly than control cells (Extended Data Fig. 3a, b) and had a longer S phase (Extended Data Fig. 3c). To determine whether this was due to slower DNA synthesis, we labeled control and sh-SAM cells with IdU and CldU and monitored the progression of individual forks by DNA fiber spreading. CldU tracks were much shorter in both HEK293T and HeLa cells depleted of SAMHD1 than they were in control cells (Fig. 2c and Extended Data Fig. 3d–f), indicating slowed DNA synthesis.

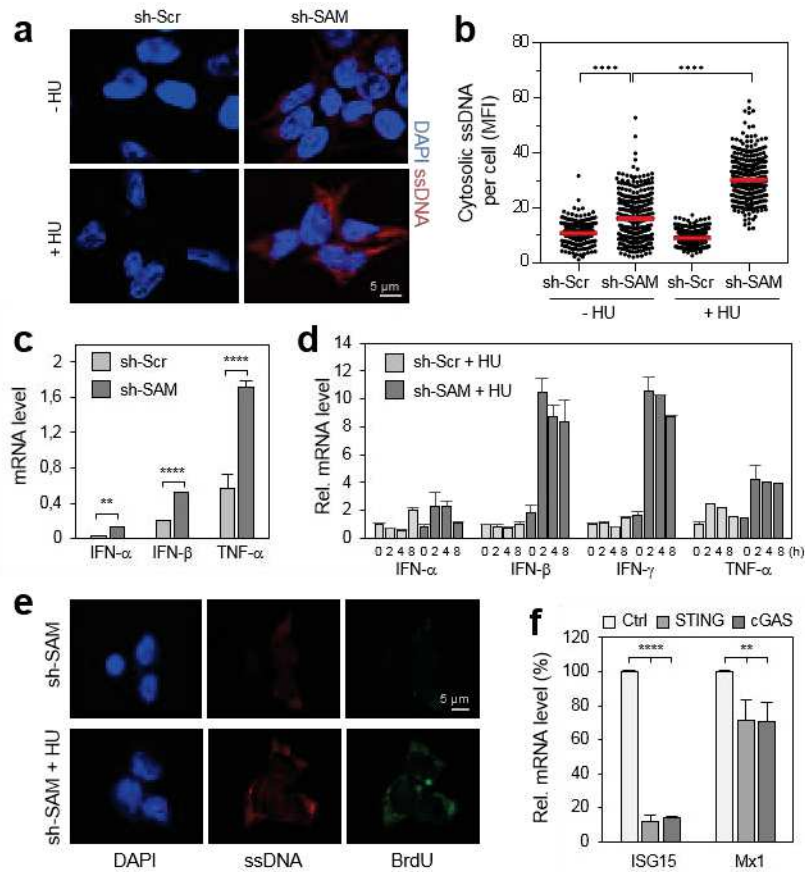


Figure 1 *SAMHD1 prevents accumulation of cytosolic ssDNA and induction of type I IFNs in response to replication stress.* a, Cytosolic DNA (red) in control (sh-Scr) and SAMHD1-depleted (sh-SAM) HEK293T cells exposed for 2 hours to 4 mM HU. b, Mean fluorescence intensity (MFI) of cytosolic ssDNA per cell (n=300). c, Expression of IFN and TNF- α mRNAs in sh-Scr and sh-SAM HEK293T cells (mean and SD of three independent experiments). d, Induction of IFN and TNF- α mRNAs in sh-Scr and sh-SAM HEK293T cells exposed to HU as in part (a). Mean and SD correspond to technical triplicates, representative of three independent experiments. e, Cytosolic DNA and BrdU-labeled DNA in sh-SAM HEK293T cells incubated with BrdU for 2 hours then without BrdU and in the presence of 4 mM HU for 2 hours (n=3). f, Expression of interferon-induced genes ISG15 and Mx1 in cGAS-KO (cGAS), STING-KO (STING) and control (Ctrl) SAMHD1-depleted HeLa cells. Mean and SD of three independent experiments are shown. In parts (b-d) and (f), **** P<0.0001, ** P<0.01, two-sided Mann-Whitney test.

Moreover, increased fork stalling was detected in sh-SAM cells by monitoring the asymmetry of sister forks (Extended Data Fig. 3g), even though the density of active forks was unchanged ($P=0.33$, Extended Data Fig. 3h). Together, these data indicate that SAMHD1 promotes normal fork progression.

To determine whether the replication defects observed in sh-SAM cells were due to imbalanced dNTP pools, we measured dNTP levels in sh-SAM HEK293T cells by HPLC (Fig. 2d). In SAMHD1-depleted cells, dGTP levels were 2.5-fold higher than in controls, but dATP levels were only ~30% higher and dCTP and dTTP levels were unchanged. Moreover, addition of a balanced mix of nucleosides to sh-SAM cells only partially rescued fork speed (Fig. 2e), suggesting that the role of SAMHD1 in S phase is not limited to the regulation of dNTP pools.

SAMHD1 is phosphorylated on T592 by cyclin-dependent kinases (CDKs) during S and G₂/M phases of the cell cycle^{4,5} (Extended Data Fig. 4a). To determine how this phosphorylation affects DNA replication, sh-SAM cells were complemented with phosphomimetic (T592E) or non-phosphorylatable (T592A) SAMHD1 mutants (Extended Data Fig. 4a). Unlike T592A, T592E fully rescued slow forks in sh-SAM cells (Fig. 2f). Since both cell types have similar dNTP levels (Extended Data Fig. 4b), these data indicate that phosphorylation of SAMHD1 on T592 promotes fork progression independently of dNTP pools. Interestingly, normal fork progression was also restored to sh-SAM cells by expression of the dNTPase-deficient K312A mutant but not by the dNTPase-proficient Y315A mutant¹³ (Extended Data Fig. 4a, c). Fork progression was also impaired in immortalized B cells from an AGS patient (AGS5^{-/-}) with a Q548X mutation (Extended Data Fig. 4d), which does not affect dNTP regulation^{12,14}. Together, these data indicate that phosphorylation of SAMHD1 on T592 by CDK is required for normal fork progression.

SAMHD1 promotes fork resection

Since SAMHD1 was proposed to have potential 3'–5' exonuclease activity^{13,14} similar to MRE11, we next asked whether it could be involved in fork resection. We labeled newly synthesized DNA in control and sh-SAM cells with IdU for 15 minutes then exposed them to HU for 120 minutes in the presence of CldU. In control cells, a significant degradation of IdU tracks was observed after HU addition (Extended Data Fig. 5a), indicating resection. This was prevented by SAMHD1 depletion and/or by addition of the MRE11 inhibitor Mirin^{20,21} (Fig. 3a).

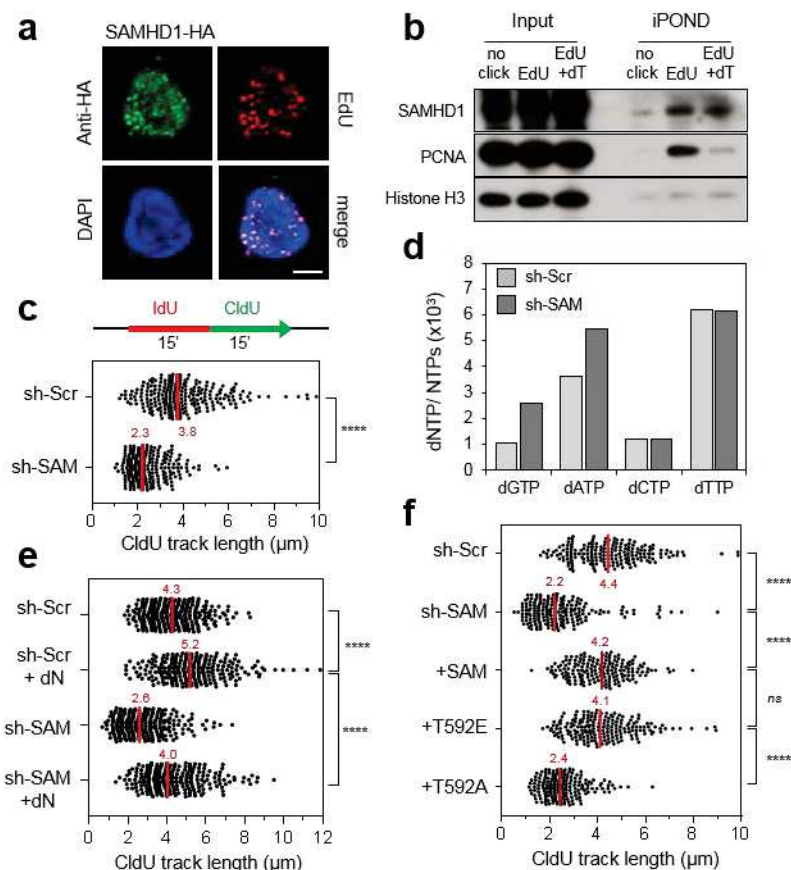


Figure 2 *SAMHD1* colocalizes with replication foci and regulates fork progression. **a**, Immunolocalization of HA-tagged SAMHD1 (green) and EdU-labeled replication foci (red) in HeLa cells, showing their colocalization (merge) in a DAPI-stained nucleus (blue; $n=2$). **b**, iPOND analysis of SAMHD1 and PCNA in HEK293T cells. **c**, DNA fiber analysis of CldU track lengths ($n=250$) in sh-Scr and sh-SAM HEK293T cells. **d**, Intracellular dNTP pools in sh-Scr and sh-SAM HEK293T cells ($n=2$). **e**, Lengths of CldU tracks ($n=250$) after addition of a balanced mix of nucleosides (+dN) 2 hours prior to IdU and CldU labeling. **f**, Fork progression in sh-SAM HEK293T cells complemented with wild-type SAMHD1 or with phosphomimetic (+T592E) or non-phosphorylatable (+T592A) mutants ($n=200$). In parts (c, e) and (f), median track lengths are indicated in red. **** $P<0.0001$; ns not significant, two-sided Mann-Whitney test.

Similar results were obtained by using a different labeling strategy (Extended Data Fig. 5b) and aphidicolin (a drug that inhibits DNA polymerases without affecting dNTP pool) instead of HU to stall replication forks (Extended Data Fig. 5c). Moreover, addition of exogenous nucleosides did not restore fork resection in sh-SAM cells (Extended Data Fig. 5d) and dNTP pools were equally affected by HU exposure in control and sh-SAM cells (Extended Data Fig. 5e). Fork resection was also inhibited by the depletion of the CtIP, a cofactor of MRE11 (Extended Data Fig. 5f) and by depleting SMARCAL1 (Extended Data Fig. 5g). This suggests that resection occurs partly on reversed forks, which form at stalled forks upon reannealing of nascent DNA strands²⁶⁻²⁸.

To investigate whether SAMHD1 is also required to resect DNA ends during DSB repair, we used the single molecule analysis of resection tracks (SMART) method²⁹ and an *in vivo* DNA repair assay³⁰ in sh-SAM and control cells. These approaches demonstrated that SAMHD1 is indeed required to resect DNA ends and repair DSBs (Extended Data Fig. 6a-e), confirming a recent report³¹. Also, we showed that *Xenopus* SAMHD1 is recruited to chromatin after *Eco*RI digestion (Extended Data Fig. 6f). Altogether, these data indicate that SAMHD1 acts with MRE11 and CtIP to promote resection at forks and DSBs, independently of dNTP pools.

Fork resection in sh-SAM cells was fully restored by the phosphomimetic T592E mutant of SAMHD1, but not by the non-phosphorylatable T592A mutant (Fig. 3b) and it was restored by the dNTPase-defective mutant K312A, but not by the dNTPase-proficient mutant Y315A (Extended Data Fig. 5h), suggesting the resection function of SAMHD1 requires phosphorylation but not the dNTPase activity. Also, fork resection in immortalized B cells was impaired by the Q548X mutation found in AGS patients (Extended Data Fig. 5i). The resection-proficient mutants, T592E and K31A, prevented accumulation of cytosolic DNA whereas the resection-deficient mutants, T592A and Y315A, did not (Fig. 3c, d). Altogether, these data indicate that mutations in SAMHD1 affecting fork progression and nascent DNA resection are also associated with the release of ssDNA fragments into the cytoplasm.

SAMHD1 activates the MRE11 exonuclease

To characterize further the function of SAMHD1 at the biochemical level, we assayed the binding of human SAMHD1 to various DNA substrates (Extended Data Fig. 7a, b) and found a high affinity for ssDNA and different fork structures. Remarkably, SAMHD1 stimulated three-fold the exonuclease activity of MRE11 (Fig. 3e, f), whereas it did not increase the activity of DNA2, FEN1 and bacterial ExoIII (Extended Data Fig. 7c).

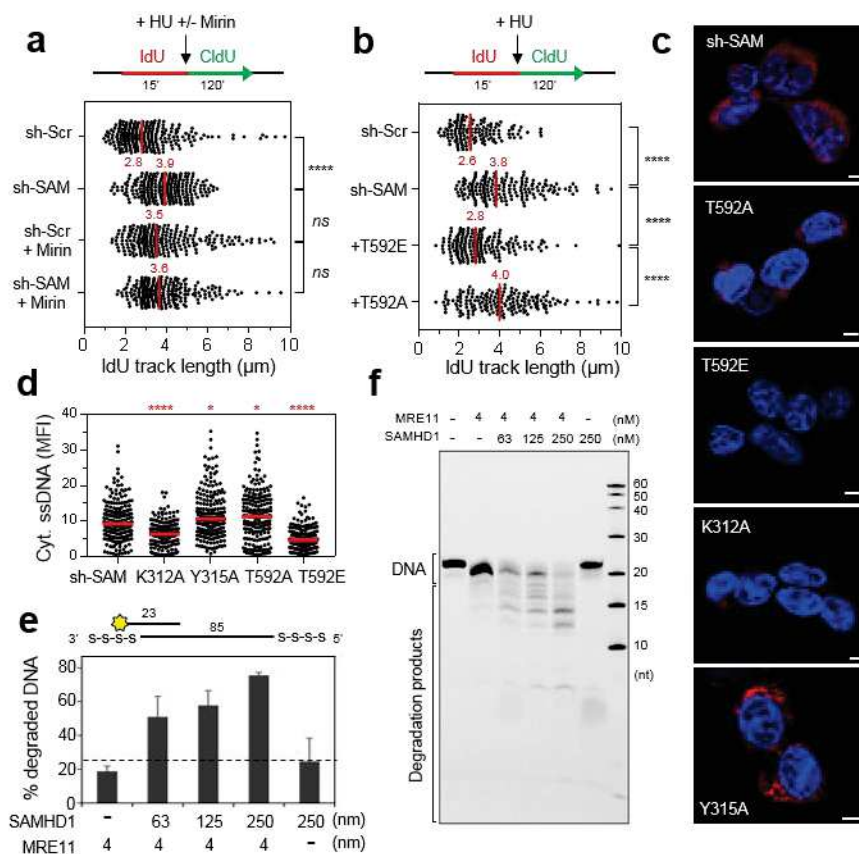


Figure 3 *SAMHD1 promotes the MRE11-dependent degradation of nascent DNA at stalled forks.* a, sh-Scr and sh-SAM HEK293T cells were labeled with IdU and CldU in the presence of 4 mM HU and in the presence or absence of 50 μM Mirin. Median IdU track lengths (n=240) are indicated in red. **** P<0.0001, Mann-Whitney test. b, Analysis as in part (a) of IdU track lengths (n=180) in sh-SAM HEK293T cells expressing the T592A and T592E mutants of SAMHD1. c, Cytosolic ssDNA (red) in sh-SAM HEK293T cells (n=200) complemented with K312A, Y315A, T592A and T592E mutants and exposed for 2 hours to 4 mM HU. Bar, 5 μm. d, Mean fluorescence intensity (MFI) of cytosolic ssDNA per cell (n=200). Relative difference to sh-SAM: **** P<0.0001; * P<0.05, Mann-Whitney test e, In vitro nuclease assays of MRE11 in the absence and presence of increasing concentrations of SAMHD1. Mean and SD are from three independent experiments. f, Representative gel as in part (f).

We assayed direct binding of SAMHD1 to labeled MRE11 by using microscale thermophoresis and measured a dissociation constant (K_d) of 977 ± 176 nM, suggesting that stimulation of the exonuclease activity of MRE11 by SAMHD1 is due to a direct interaction (Extended Data Fig. 7d). In addition to binding to MRE11, we found that SAMHD1 directly interacts with replication protein A (RPA) with a $K_d = 312 \pm 57$ nM as determined by microscale thermophoresis (Extended Data Fig. 7e) and by co-immunoprecipitation in *Xenopus* egg extracts (Extended Data Fig. 7f). *Xenopus* SAMHD1 also interacted with the resection protein CtIP (Extended Data Fig. 7g). Together, these data suggest that SAMHD1 binds with high affinity to RPA and fork structures, where it interacts directly with MRE11 to selectively stimulate its exonuclease activity.

SAMHD1 activates CHK1 at stalled forks

Since SAMHD1 binds to forks, MRE11 and RPA *in vitro* and MRE11 is reported to play a role in CHK1 activation at stalled forks³², we asked whether the recruitment of MRE11 and the formation of RPA-coated ssDNA at stalled forks depends on SAMHD1. To address this possibility, we first examined the colocalization of MRE11 and EdU-labeled replication sites in HU-treated sh-SAM and control cells and found that their colocalization requires SAMHD1 (Extended Data Fig. 8a). To test whether formation of RPA-coated ssDNA also depends on SAMHD1, we looked for ssDNA in HU-treated sh-SAM and control cells by detecting BrdU incorporation under non-denaturing conditions. Both ssDNA formation foci (Extended Data Fig. 8b) and the formation of RPA foci (Extended Data Fig. 8c) depended on the presence of SAMHD1. Incidentally, we also observed a four-fold increase in BrdU-labeled cytosolic ssDNA in sh-SAM cells (Extended Data Fig. 8b, asterisks), consistent with the data shown in Figure 1a, b.

Since RPA recruits ATR to stalled forks, we next monitored the phosphorylation of the ATR targets CHK1 and H2AX in cells exposed either to HU or to CPT (Extended Data Fig. 8d, e). Phosphorylation of both proteins was much reduced in sh-SAM cells when compared to control cells, indicating that activation of the ATR–CHK1 pathway depends on SAMHD1. CHK1 phosphorylation was restored upon complementation of sh-SAM cells with wild-type SAMHD1 (Extended Data Fig. 8d) or with the T592E and K312A mutants, but not with T592A and Y315A mutants, which are defective in fork resection (Extended Data Fig. 8e, f). Moreover, the activation of CHK1 in *Xenopus* egg extracts treated with aphidicolin or DSB-inducing agents was also inhibited in the absence of SAMHD1 or MRE11 activity (Fig. 4d and Extended Data Fig. 8i-k).

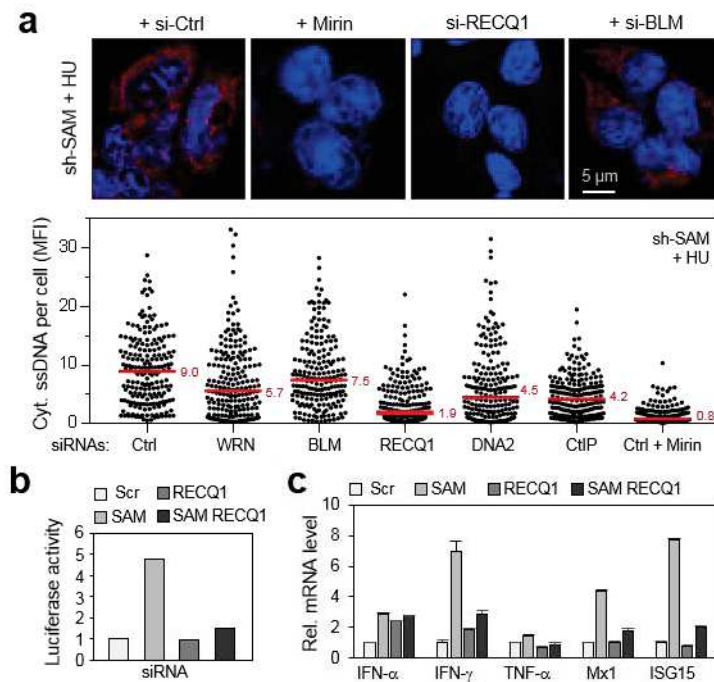


Figure 4 Depletion of RECQ1 prevents the IFN response in SAMHD1-depleted cells. a, Cytosolic ssDNA in HU-treated sh-SAM HEK293T cells transfected for 48 hours with siRNAs against WRN, BLM, RECQ1, DNA2 and CtIP or treated with Mirin (n=200; two experiments). b, Induction of IFNs (luciferase assay) in HL116 cells transfected with siRNAs against SAMHD1 (SAM), RECQ1 (RECQ1) or both (SAM + RECQ1), or with a control scrambled siRNA (Scr; n=3). Data are representative of three experiments. c, Total mRNA was extracted from HL116 cells after siRNA transfection and the expression of IFN- α , IFN- γ , TNF- α , Mx1 and ISG15 was quantified by qRT-PCR (n=3). Error bars: SD of triplicates from representative experiment

We also found that SAMHD1 acts together with MRE11 for the restart of CPT-arrested forks (Extended data Fig. 8l). Together, these findings indicate a novel role for SAMHD1 in the activation of the ATR–CHK1 pathway and in fork restart.

RECQ1 induces IFNs in sh-SAM cells

We hypothesized that the cytosolic ssDNA observed in sh-SAM cells might result from the displacement of nascent DNA by a helicase and cleavage by a flap endonuclease. To test this hypothesis, we depleted sh-SAM cells of BLM, WRN, RECQ1, DNA2 and CtIP (Extended Data Fig. 9a) and exposed them to HU. Depletion of RECQ1 and, to a lesser extent, DNA2 and CtIP resulted in a significant reduction in the cytosolic ssDNA seen in both HU-treated and untreated sh-SAM cells (Fig. 4a and Extended Data Fig. 9b–e). RECQ1 depletion also reduced the expression of IFN genes in HL116 (Fig. 4b, c), HEK293T and HeLa cells (Extended Data Fig. 9f, g) and the degradation of IdU tracks (Extended Data Fig. 9h), suggesting that RECQ1 contributes to fork resection and is responsible for the observed cytosolic ssDNA. Levels of cytosolic ssDNA were also reduced by Mirin (Fig. 4a), suggesting that the endonuclease activity of MRE11 cleaves these displaced strands, releasing short ssDNA fragments, as it does at DSBs³³.

To investigate further how cytosolic ssDNA is produced, sh-SAM cells were labeled with IdU before the addition of HU and with CldU during the HU arrest, and then released from HU in the presence of thymidine to chase CldU. Remarkably, both IdU- and CldU-labeled ssDNA were detected in the cytoplasm (Extended Data Fig. 10a, b), indicating that DNA synthesized both before and after replication fork stalling contributes to the cytosolic ssDNA seen in HU-treated cells (Extended Data Fig. 10c).

We conclude from our data that SAMHD1 functions during S phase at stalled DNA replication forks to activate the ATR–CHK1 pathway and to promote the resection of gapped or reversed forks by binding to and activating the MRE11 exonuclease. This function is independent of its previously described role in maintaining balanced dNTP pools, which is also important for faithful DNA replication^{16,17}. This novel role of SAMHD1 depends on its phosphorylation by S phase CDKs, which act as a switch to control its dNTPase-dependent and -independent functions.

In the absence of SAMHD1, ssDNA molecules are released from stalled replication forks and accumulate in the cytoplasm, where they activate the cGAS–STING pathway inducing expression of type I IFNs and other pro-inflammatory cytokine genes (Extended Data Fig. 10d).

This is consistent with recent reports showing that DNA repair byproducts can also induce the production of type I IFNs ³⁴⁻³⁷. Mutations in SAMHD1 that prevent the degradation of nascent DNA at stalled forks increase cytosolic DNA, whereas mutations that affect the dNTPase activity of SAMHD1 do not, confirming thereby that defective fork processing and aberrant induction of the interferon pathway are directly linked. This mechanism is distinct from the induction of cGAS–STING by micronuclei recently reported in cells deficient for RNase H2, another gene frequently mutated in AGS ^{38,39}. Since replication stress contributes to cancer development¹⁸, our findings also shed new light on the mechanism by which SAMHD1 mutations might promote tumorigenesis.

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Author Contributions

YLL and PP conceived and planned the study. YLL, FC, MJS, ALS and AP designed, performed experiments on replication stress. SS and ACh analyzed dNTP pools. CM and ACr constructed SAMHD1 mutants. YLL, AB and CM performed RT-qPCR experiments. JN and WN performed iPOND experiments. ED and BL performed SSA assay. AS helped with immunofluorescence experiments. VC designed and HT performed experiments with *Xenopus* SAMHD1. KZ and LK designed, performed and analyzed nuclease assays. YLL, MB and PP wrote the manuscript and all authors reviewed it.

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Methods

Cell lines and cell culture

Human embryonic kidney (HEK) HEK293T cells (ATCC CRL-3216) and SAMHD1-overexpressing HeLa cells¹⁵ were cultured at 37 °C under 5% CO₂ in DMEM supplemented with 10% fetal calf serum (FCS). Epstein–Barr virus-immortalized B cells from an AGS5-/- patient were cultured in RPMI-1640 supplemented with 10% FCS. Reporter HL116 cells were cultured in DMEM supplemented with 10% FCS and ultra-glutamine under HAT selection⁴⁰.

Construction of SAMHD1 mutants

A SAMHD1 wild-type clone from Invitrogen (clone ID: IOH55544, sequence NM 015474.3) with a C-terminal FLAG and an HA tag was modified to produce a shRNA-resistant coding sequence. The DNA was amplified by PCR with the appropriate primers to create a SAMHD1 protein-coding sequence with an HA-tag fused to the second amino acid residue from the N-terminus and to restore the normal C-terminus. The PCR reaction also created a *Mlu*I site after the sequence encoding the protein C-terminus and an *Age*I site followed by a Kozac sequence before the sequence encoding the protein N-terminus. The amplified DNA was cloned in p-TripZ (Thermo Scientific Open Biosystems) between *Age*I and *Mlu*I. All mutations were created by using the QuickChange IIXL kit from Agilent. The complete SAMHD1-coding sequence was checked by sequencing for each mutant.

K312A: AAA to GCA

Y315A: TAT to GCT

T592A: ACA to GCA

T592E: ACA to GAA

HIV-1-derived lentiviral vectors were produced in HEK293T cells as previously described⁴¹.

Detection of cytosolic ssDNA

Cells were treated with 4 mM HU for 2 hours, or untreated, as appropriate, and then incubated in fresh medium for 2 hours and fixed for 20 minutes with 80% methanol at -20°C. Coverslips were incubated with a mouse anti-ssDNA antibody (see Supplemental Information) overnight at 4 °C and with a secondary antibody conjugated to an Alexa Fluor dye for 1 hour at room temperature, followed by DAPI staining. Images were acquired by using a Zeiss ApoTome microscope and a LSM780 confocal microscope. For the detection of BrdU-labeled cytosolic DNA, cells were labeled for 2 hours with 10 μM BrdU prior to HU exposure and BrdU was

detected with a rat anti-BrdU antibody without denaturation. The mean fluorescence intensity (MFI) of cytosolic ssDNA and BrdU was quantified with the CellProfiler software. Nuclei were segmented using DAPI signal and masked to quantify ssDNA and BrdU signals.

Quantification of cytosolic mitochondrial DNA

Cells growing on 100 mm coverslips were collected into 200 μ l fractionation buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and left on ice for 15 min. They were then lysed by passing cell suspensions 10 times through a 27 gauge needle attached to a 1 ml syringe and left on ice for a further 20 min. Cell lysates were centrifuged at 720 g for 5 min at 4°C. The supernatant was further centrifuged at 10,000 g for 5 min at 4°C to remove mitochondria. Cytosolic DNA was purified from the supernatant by using DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions. The presence in the cytosolic fraction of the mitochondrial gene MT-CO1 encoding the Cytochrome C oxidase 1 (COX1) was quantified by qPCR using specific primers.

Analysis of interferon production

Cell culture media were collected 48 hours after transfection with various siRNAs. Reporter HL116 cells harboring a type-I IFN-inducible 6-16 promoter fused to the firefly luciferase gene⁴⁰ were cultured for 48 hours in the presence of the collected culture media. Luciferase activity was quantified according to the manufacturer's instructions (Luciferase assay system, Promega). The expression of various IFNs and IFNs-Stimulated Genes (ISGs) was quantified by qRT-PCR (see Supplemental information) and normalized to GAPDH. Reverse transcription was performed by using the Superscript III First Synthesis System for RT-PCR (ref. 18080-051, Invitrogen). A LightCycler 480 SYBR Green I Master Mix (ref. 04887352001, Invitrogen) was used to perform quantitative PCR.

Cell sorting

HEK293T cells expressing the Scramble-control shRNA (sh-Scr) were labeled with propidium iodide. Populations of G₁, S and G₂/M phase cells were sorted in a FACS Aria sorter (Becton Dickinson, MRI facility).

Flow cytometry analysis of S phase progression

HEK293T cells transduced with lentiviral vectors expressing sh-Scr or sh-SAMHD1 (sh-SAM) RNAs were pulse labeled with 10 μ M EdU for 15 min and then chased with 100 μ M thymidine for the indicated periods of time. After fixation with 1% formaldehyde for 30 min at room

temperature, EdU incorporation was detected by using Click chemistry according to the manufacturer's instructions (Click-iT EdU Flow Cytometry Cell Proliferation Assay, Invitrogen). The cells were resuspended in PBS containing 1% (w/v) BSA, 2 μ g/ml DAPI and 0.5 mg/ml RNase A for 30 min at room temperature and were analyzed in a MACSQuant flow cytometer (Miltenyi Biotec). The percentages of cells in G₁, S and G₂/M phases were quantified by using FlowJo single-cell analysis software (FlowJo, LLC).

Confocal microscopy of DNA replication foci

HeLa cells overexpressing HA-tagged SAMHD1 and GFP proteins were grown on coverslips. They were labeled for 1 h with 10 μ M EdU then fixed with 1% paraformaldehyde (PFA) and permeabilized with 0.1% saponin. Coverslips were then incubated overnight at 4°C with an anti-HA antibody. Replication foci were detected by using Click chemistry, as for flow cytometry analysis. The cell nuclei were stained with DAPI and the coverslips were then mounted on glass slides and visualized by using a Zeiss LSM780 confocal microscope. The percentage of HA-EdU foci colocalization was quantified by ImageJ-JACoP (imagej.nih.gov/ij/plugins/track/jacop.html).

iPOND

Logarithmically growing HeLa S3 cells (1×10^6 per ml) or HEK293FT cells were incubated with 10 μ M EdU for 10 min. Following EdU labeling, cells were fixed in 1% formaldehyde, quenched by adding glycine to a final concentration of 0.125 M and washed three times in PBS. Collected cell pellets were frozen at -80°C and cells were permeabilized by resuspending 1.0 – 1.5×10^7 cells per ml in ice-cold 0.25 % Triton X-100 in PBS and incubating for 30 min. Before the Click reaction, samples were washed once in PBS containing 0.5% BSA and once in PBS. Cells were incubated for 1 h at room temperature in Click reaction buffer containing 10 μ M azide-PEG(3+3)-S-S-biotin conjugate (Click ChemistryTools, cat. no AZ112-25), 10 mM sodium ascorbate, and 1.5 mM CuSO₄ in PBS. The 'no Click' reaction contained DMSO instead of biotin-azide. Following the Click reaction, cells were washed once in PBS containing 0.5% BSA and once in PBS. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 1% SDS) containing protease inhibitor cocktail (Sigma) and sonicated with a Diagenode Bioruptor Plus for 40 cycles (30 sec on/30 sec off). Samples were centrifuged at $14,500 \times g$ at 4°C for 30 min and the supernatant was diluted 1:3 with TNT buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.3% Triton X-100) containing protease inhibitors. An aliquot was taken as an input sample. Streptavidin-agarose beads (Novagen) were washed three times in TNT buffer

containing protease inhibitor cocktail. Two hundred microliters of bead slurry were used per 1×10^8 cells. The streptavidin–agarose beads were resuspended 1:1 in TNT buffer containing protease inhibitors and added to the samples, which were then incubated at 4°C for 16 h in the dark. Following binding, the beads were washed twice with 1 ml TNT buffer, twice with TNT buffer containing 1M NaCl and twice with TNT buffer. Protein–DNA complexes were eluted by incubating with 5 mM DTT in TNT buffer. Cross-links were reversed by incubating samples in SDS sample buffer at 95°C for 20 min. Proteins were resolved on SDS–PAGE and detected by immunoblotting using specific antibodies.

DNA fiber spreading

DNA fiber spreading was performed as described previously⁴². Briefly, subconfluent cells were sequentially labeled first with 10 μ M 5-iodo-2'-deoxyuridine (IdU) and then with 100 μ M 5-chloro-2'-deoxyuridine (CldU) for the indicated times. One thousand cells were loaded onto a glass slide (StarFrost) and lysed with spreading buffer (200 mM Tris-HCl pH 7.5, 50 mM EDTA, 0.5% SDS) by gently stirring with a pipette tip. The slides were tilted slightly and the surface tension of the drops was disrupted with a pipette tip. The drops were allowed to run down the slides slowly, then air dried, fixed in methanol/acetic acid 3:1 for 10 minutes, and allowed to dry. Glass slides were processed for immunostaining with mouse anti-BrdU to detect IdU, rat anti-BrdU to detect CldU, mouse anti-ssDNA antibodies (see Supplemental Information for details) and corresponding secondary antibodies conjugated to various Alexa Fluor dyes. Nascent DNA fibers were visualized by using immunofluorescence microscopy (Leica DM6000 or Zeiss ApoTome). The acquired DNA fiber images were analyzed by using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) and statistical analysis was performed with GraphPad Prism (GraphPad Software). The lengths of at least 150 IdU and/or CldU tracks were measured per sample.

Single-molecule analysis of resection tracks (SMART)

Control (sh-Scr) and SAMHD1-depleted (sh-SAM) HEK293T cells were labeled with 10 μ M BrdU for 24 h. They were then treated with 5 μ M bleocin (Calbiochem) for 1 h and harvested at the indicated time points. They were processed for DNA fiber spreading as described. BrdU tracks were stained with anti-BrdU antibody without DNA denaturation and visualized by fluorescence microscopy (Zeiss ApoTome). The acquired DNA fiber images were analyzed by using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices)

and statistical analysis was performed with GraphPad Prism (GraphPad Software). The lengths of at least 200 BrdU tracks were measured per sample.

Single-strand annealing (SSA) assay

Seven hours after plating in six-well plates (1×10^5 cells/well), U2OS-SSA cells were transfected with siRNAs against SAMHD1 or CtIP, or with control siRNAs (see Supplemental Information for sequences) by using Interferin reagent (Polyplus, Ozyme). Forty-eight hours after siRNA transfection with siRNAs, HA-tagged I-SceI was expressed by transient transfection (JetPEI, Polyplus, Ozyme) with 1.5 μ g of the expression plasmid pCMV-I-SceI⁴³ for DSB induction. Expression was verified by immunoblotting with an anti-HA antibody. Forty-eight hours after transfection with the construct expressing I-SceI, cells were fixed in PBS containing 2% formaldehyde for 15 min at room temperature. Cells expressing GFP were monitored by flow cytometry in a BD Accuri C6 flow cytometer.

Collection and processing of HEK293T cells for nucleotide pool measurements

Three 150 x 20 mm cell culture dishes were seeded with (8×10^6) HEK293T cells and grown to 80% confluency. After removal of the medium, the plates were washed twice with cold NaCl (9 g/l). The cells were scraped with a cell scraper after addition of 250 μ l of cold 15% TCA containing 30 mM MgCl₂ to each of the three plates and they were pooled together. The pooled cells were collected in an Eppendorf tube and snap-frozen in liquid nitrogen. They were stored at -80°C until processed. For extraction of dNTPs and NTPs, cells were thawed on ice and vortex-mixed for 10 min in a cold room. The supernatant was then collected by centrifugation at $14,000 \times g$ for 5 min at 4°C and processed as described⁴⁴.

Detection of ssDNA, RPA and MRE11 foci

Cells growing on coverslips were labeled with 10 μ M BrdU for 24 h. They were treated with 1 mM HU for 2 h after the removal of BrdU and fixed with ice-cold methanol for 1 h at -20°C. The coverslips were incubated with an anti-BrdU monoclonal antibody (without DNA denaturation) overnight at 4°C and then with a secondary antibody conjugated to an Alexa Fluor dye for 1 h at room temperature, followed by DAPI staining. Images were acquired by using a wide-field Leica DM6000 microscope and a Zeiss LSM780 confocal microscope. The number of subnuclear ssDNA foci was quantified by using CellProfiler image analysis software. For detection of chromatin-bound RPA foci during S phase, cells growing on

coverslips were pulse labeled with 10 μ M EdU for 10 min and treated with 4 mM HU for 2 h. They were fixed with 4% PFA in PBS for 15 min and then incubated for 3 min at 4°C with CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 300 mM sucrose, 0.5 mM DTT) containing 0.25% Triton X-100 and phosphatase inhibitor cocktail (SIGMA-ADRIK, # P0044). EdU incorporation was detected by using Click chemistry and then blocked with 3% BSA in PBS for 1 h at room temperature. The coverslips were incubated with an anti-RPA antibody (overnight at 4°C) and then with a secondary antibody conjugated to an Alexa Fluor dye for 1 h at 37°C, followed by DAPI staining. Images were acquired by using a Zeiss ApoTome microscope. The mean fluorescence intensity (MFI) in EdU-positive cells was quantified by using CellProfiler (www.cellprofiler.org). For the detection of MRE11 foci, cells seeded on coverslips were labeled with EdU and treated with or without HU as described above. They were incubated in cold extraction buffer (20 mM HEPES pH7.5, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 0.5% Triton X-100) for 5 min at 4°C and then fixed in fixation buffer (3.7% PFA, 2% sucrose, 0.5% Triton X-100) for 20 min at room temperature. The coverslips were incubated with an anti-MRE11 antibody overnight at 4°C after blocking in PBS containing 1% BSA in PBS for 1 h at room temperature. After incubation with a secondary antibody conjugated to Alexa Fluor dye, EdU incorporation was detected by using Click chemistry, followed by DAPI staining. Images were acquired by using a Zeiss LSM780 confocal microscope. The percentage of colocalized MRE11 and EdU foci was quantified by ImageJ-JACoP.

Cloning and purification of recombinant *Xenopus* SAMHD1

The reference sequence of *Xenopus* SAMHD1 (xSAMHD1), available from Uniprot, Q6INN8 (<http://www.uniprot.org>), is of 632 amino acids. Briefly, the cDNA encoding *Xenopus laevis* SAMHD1 (XGC SAMHD1, Clone ID 3402629, from Thermo Fisher Scientific) was cloned into the pET43 plasmid following standard methods. Clones were validated by PCR and DNA sequencing.

Purification of recombinant xSAMHD1 (His- or His-FLAG-tagged) was performed in two steps after IPTG (0.5 mM) induction of the bacteria overnight at 23°C. The bacterial pellet was lysed in a buffer containing 500 mM NaCl, 50 mM Hepes pH 7.5, 2 mM beta-mercaptoethanol, 10% glycerol and protease inhibitors cocktail, then sonicated and clarified by centrifugation. The first purification step was performed by binding the recombinant xSAMHD1 to Ni-NTA resin (HisPur, Thermo Fisher Scientific) for 2 h at 4°C and eluting with 100 mM and 250 mM imidazole. After dialysis, the recombinant protein was further purified by size exclusion

chromatography (Superdex 200 10/300 GL, GE healthcare). For use in *Xenopus* egg extracts, both His- and His-FLAG-xSAMHD1 were purified in buffer containing 150 mM NaCl, 25 mM Tris-HCl pH 7.6, 1 mM DTT, 10% glycerol.

Production of anti-xSAMHD1 antibody

For antibody production, the recombinant His-xSAMHD1 was purified, as described above, but size exclusion chromatography was performed in phosphate-buffered saline. This preparation was supplied to BioGenes GmbH who immunised rabbits and prepared the antiserum. The antibody was affinity purified from the antiserum by binding to recombinant His-xSAMHD1 by the Cogentech service of the IFOM protein facility.

Preparation of interphase *Xenopus* egg extracts and treatments

Xenopus egg extracts were prepared as described⁴⁵. The DNA damage response and DNA replication were analyzed as previously described^{45,46}. To induce DNA double-strand breaks, we added 0.05 U/ μ l EcoRI (New England Biolabs) to the extracts. To slow or stall replication forks, we added aphidicolin (Sigma) to the extracts at the concentrations indicated in the main text.

Immunodepletion of xSAMHD1 in *Xenopus* egg extracts

To immuno-deplete xSAMHD1 from *Xenopus* egg extracts, 0.3–0.5 ml of egg extract were incubated with affinity-purified IgGs (30 mg) and with 120 μ l of Dynabeads–ProteinA (Thermo Fisher, 10002D). The immunodepletion was performed in three rounds of 30–45 minutes at 4°C.

Immunoprecipitation of xSAMHD1 in *Xenopus* egg extract

For coimmunoprecipitation of xSAMHD1 with RPA70, 200 μ l of Dynabeads–Protein A slurry were coupled with 60 μ g of anti-xSAMHD1 purified serum or 50 μ g of rabbit pre-immune serum (IgG control). The beads were then incubated with 500 μ l of clarified *Xenopus* egg extract brought to a final volume of 1.5 ml with NIB buffer (50 mM KCl, 50 mM Hepes pH 7.0, 5 mM MgCl₂, 2 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 20% sucrose, 0.05% Triton X-100). After 1 h at 4°C, a fraction of the unbound material, called ‘flow-through’ (FT), was recovered and boiled in Laemmli sample buffer (Biorad) for further analysis. Beads were washed 5 times in NIB buffer and boiled in Laemmli sample buffer before analysis by western blotting. For coimmunoprecipitation of xSAMHD1 with xCtIP, 100 μ l Dynabeads–Protein A slurry were coupled to 30 μ g of anti-xSAMHD1 (affinity purified serum) or 50 μ g of pre-

immune serum (IgG control). The beads were then incubated with 300 μ l of clarified *Xenopus* egg extract brought to a final volume of 1 ml with a buffer containing 100 mM KCl, 50 mM HEPES pH 7.0, 5 mM MgCl₂, 2 mM DTT, 20 % sucrose, 0.05 % Triton X-100. After overnight incubation at 4°C, a fraction of the FT was recovered and boiled in Laemmli sample buffer for further analysis. Beads were washed 5 times in the IP buffer and boiled in Laemmli sample buffer before analysis by western blotting.

Pulldown of recombinant His-FLAG-xSAMHD1 from *Xenopus* egg extracts

Xenopus egg extracts were first clarified by centrifugation at 15 000 rpm at 4°C for 20 min. Then, 300 μ l of clarified extract and 100 μ l anti-FLAG M2 resin slurry (Sigma) were incubated in the presence, or not, of recombinant His-FLAG-xSAMHD1 at 0.1 mg/ml. The final reaction volume was adjusted to 1.5 ml with buffer containing 100 mM KCl, 50 mM HEPES pH 7.0, 5 mM MgCl₂, 2 mM DTT, 20 % sucrose, 0.05 % Triton X-100 and incubated overnight at 4°C. The resin was washed 5 times in the same buffer and boiled in Laemmli buffer (Biorad). Samples were loaded on SDS-PAGE and analysed by WB.

Immunoprecipitation of xSAMHD1 from nuclear lysates

For each experimental condition, 400 μ l *Xenopus* egg extract were incubated with 4000 nuclei/ μ l of demembranated *Xenopus* sperm nuclei and treated with or without 0.05 U/ μ l EcoRI or 20 μ M aphidicolin. After 70 min of incubation at 23°C, the nuclei were recovered by centrifugation through a sucrose cushion and resuspended in EB (100 mM KCl; 50 mM Hepes pH 7.4, 2.5 mM MgCl₂) containing 0.25% NP40. The chromatin was solubilized by digestion with benzonase nuclease (Sigma) for 20 min at room temperature, and then sonicated for 10 min (Bioruptor, Diagenode). The resulting nuclear fraction was immunoprecipitated by incubating for 3 h at 4°C with anti-xSAMHD1 or an IgG control (pre-immune serum) and Dynabeads–Protein A in a final volume of 1 ml adjusted with NIB buffer. The beads were then prepared for western blotting as described above.

Biotin–dUTP pulldown of nascent chromatin

Biotin–dUTP pulldown was performed as previously described²⁸. Briefly, *Xenopus* sperm nuclei were added to 100 μ l *Xenopus* egg extracts at a final concentration of 4000 nuclei/ μ l. Forty-five minutes after sperm nuclei addition, newly synthesized DNA in the extracts was labeled with 40 μ M Biotin-16-dUTP (Roche) in the presence of either 5 μ M aphidicolin or

DMSO as control for 10 min. DNA replication was stopped by the addition of 200 μ l cold EB–EDTA buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 1 mM EDTA). Samples were homogenized by using a cut Eppendorf p1000 pipette tip and overlaid on 600 μ l EB–EDTA buffer containing 30% w/v sucrose. Nuclei were collected by centrifugation at 8 300 g at 4°C, the nuclear pellets were resuspended in 400 μ l EB–NP40 buffer (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 0.25% NP40) and then subjected twice to 10 min sonication with a Bioruptor device set to maximum power. After the sonication step, 20 μ l from each sample were set aside (5% Input). Biotinylated DNA fragments were then pulled-down by incubation with 40 μ l Dynabeads M-280 Streptavidin for 1 h at 4°C. The beads were then washed three times in EB–EDTA buffer and boiled with 30 μ l of 1x denaturing loading buffer, and the entire volume was separated by SDS-PAGE and analyzed by western blotting.

Protein purification

10xHis-SAMHD1 was overexpressed from pET19b as previously described⁴⁷. All purification steps were carried out at 4°C. The cell pellet was resuspended and lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 1 mM β -mercaptoethanol, 1 mM EDTA, protease inhibitors) and centrifuged for 1 h at 35 000 g. Soluble extract was incubated with pre-equilibrated Ni-NTA agarose resin (Qiagen) for 2 h with continuous mixing. The resin was washed with buffer T (20 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM EDTA) and bound protein was eluted with a gradient of 100–500 mM imidazole in buffer T. The eluted fractions were diluted in buffer T, loaded onto a 1 ml SourceS column and the protein was eluted with a 10 ml gradient of 100–700 mM KCl in buffer T. The peak fractions were stored in small aliquots at -80°C.

MRE11-6xHis and yMRE11 were purified from Sf9 insect cells or yeast cells, respectively, as described previously⁴⁸. DNA2 was purified from Sf9 cells as described previously⁴⁹ and FEN1 was expressed and purified by the same protocol as described for yFEN1⁵⁰. Bacterial ExoIII was purchased from Thermo Fisher Scientific (#EN0191).

Nuclease assays

Fluorescently labeled DNA substrates used for nuclease assays were prepared by annealing as described elsewhere⁵¹. The concentrations of SAMHD1 indicated in the main text were pre-incubated for 5 min at 37°C with MRE11 (4 nM) or yMRE11 (4 nM) followed by addition of a DNA substrate and incubation in buffer P (40 mM KCl, 10 mM TRIS-HCl (pH 7.5), 1 mM DTT, 10 μ g/ml BSA) in the presence of 2 mM dGTPs, 2 mM MnCl₂ and 2 mM MgCl₂ for 50 min at 37°C. The reaction mixtures were then incubated with 0.1% SDS and 500 μ g/ml

proteinase K at 37°C for 20 min, heat-denatured, the DNA was resolved on 20% denaturing PAGE gels (acrylamide:bisacrylamide, 19:1) and scanned by using a Fuji FLA 9000 imager. Where indicated, gels were quantified using Multi Gauge V3.2 software (Fuji).

For nuclease assays with DNA2, reactions were carried out as for MRE11 and yMRE11 but using 0.4 nM DNA2 in buffer P containing 2 mM ATP, 2 mM MgCl₂ and 2 mM dGTPs. Assays of FEN1 (1 nM) and ExoIII (20 μU/μl) were carried out in buffer P containing 2 mM MgCl₂ and 2 mM dGTPs.

Electrophoretic mobility shift assays

Fluorescently labeled DNA substrates were prepared as for the nuclease assays⁵¹. SAMHD1 was incubated with fluorescently labeled DNA substrate for 10 min at 37°C in the presence of 30 mM KCl, 10 mM TRIS-HCl pH 7.5, 1 mM DTT, 2 mM MgCl₂. Protein–DNA complexes were crosslinked by incubation with 0.01% glutaraldehyde for 5 min at 37°C and resolved on 0.8% agarose gels. The gels were scanned by using a Fuji FLA 9000 imager and quantified using Multi Gauge V3.2 software (Fuji).

Microscale thermophoresis

For MRE11–SAMHD1 interaction measurements, MRE11 was fluorescently labeled by using Monolith Protein Labeling Kit RED-MALEIMIDE (Cysteine Reactive) according to the manufacturer's protocol. Measurements were performed with 8 nM labeled MRE11 and the indicated concentration of SAMHD1 in PBS containing 0.05% Tween. Thermophoresis was performed on a Monolith NT.115 (NanoTemper Technologies GmbH) set at 50% LED and 80% MST power at 25°C and with 5 s and 30 s laser off and on times, respectively. For RPA–SAMHD1 interaction measurements, 10xHis–SAMHD1 was labeled by using the Monolith His-Tag Labeling Kit RED-tris-NTA according to the manufacturer's protocol. The thermophoresis measurements were performed with 50 nM SAMHD1 and increasing concentration of RPA in PBS containing 0.05% Tween and 2 mM MgCl₂ a Monolith NT.115 set at 40% LED and 80% MST power at 25°C and with 5 s and 30 s laser off and on times, respectively. Data were analyzed by using MO.Affinity Analysis Software version 2.2.4 (NanoTemper Technologies GmbH).

Supplemental information

Antibodies used in human cells

Antibody	Catalog No.	Dilution
Mouse anti-BrdU clone B44	347580, BD Biosciences	1/100
Rat anti-BrdU clone BU1/75	ABC117-7513, Eurobio Abcys	1/100
anti-BrdU for ssDNA foci	347580, BD Biosciences	1/20
anti-ssDNA	MAB3868, Millipore	1/250
anti-pCHK1 (S345)	2348, Cell signaling	1/1000
anti-pCHK2 (T68)	2661, Cell signaling	1/1000
anti- γ -H2AX (S139)	05-636, Millipore	1/1000
anti-SAMHD1	A303-691A, Bethyl	1/5000
anti-pSAMHD1 (T592)	Cribier et.al. 2013	1/500
anti-HA	Clone3F10, Roche	1/300
anti-actin	A4700, Sigma	1/500
anti-RPA1	Ab79398	1/300
anti-RECQ1	A300-450A, Bethyl	1/5000
anti-cGAS	15102, Cell Signaling Technology	1/1000
anti-IRF3	4302, Cell Signaling Technology	1/1000
anti-TBP	8515, Cell Signaling Technology	1/1000

Antibodies used in *Xenopus* extracts

Antibody	Source	Identifier	Dilution
Anti-Xenopus SAMHD1 (affinity purified Rabbit serum)	This study (Biogenes)	BioGenes Serum #25130	1/500
Anti-Xenopus Pol a p180 (Mouse monoclonal)	Described in ²⁸	Abmart: clone 13026-1-3/C199	1/1000
Anti-Orc1 (Mouse monoclonal)	Described in ⁵²	-	1/5000
Anti-xMre11 (Rabbit polyclonal)	Kindly provided by Jean Gautier, Columbia University, described in ⁵³	-	1/3000
Anti-xRPA70 (Rabbit polyclonal)	Kindly provided by Jean Gautier, Columbia University, described in ⁵³	-	1/5000
Anti-xCtIP (Mouse monoclonal)	Kindly provided by Jean Gautier, Columbia University, described in ⁵⁴	-	1/30
Anti-xATR	Described in ⁵²	-	1/1000
Anti-xATM	Described in ⁵²	-	1/1000
Anti-ATM pSerine 1981	Rockland Immunochemicals	200-301-400	1/1000
Anti-Phospho-CBK1 (Ser345) (133D3)	Cell Signaling	mAb #2348	1/500
Anti-CBK1 (G-4)	Santa Cruz	sc-8408	1/500
Anti-Histone H2B	Millipore	clone 07-371; RRID: AB_310561	1/5000
Anti-Mcm7	Santa Cruz	sc-9966	1/5000
Anti-Rad51 (mouse monoclonal)	Abcam	clone ab123	1/1000
Anti-phospho-Histone H2A.X (Ser139)	Millipore	clone JBW301	1/1000

siRNAs

siRNA name	orientation	siRNA sequences 5' to 3' orientation
si-Ctrl	sense	UUCUCCGAACGUGUCACGUTT
	antisense	ACGUGACACGUUCGGAGAATT
si-RAD51	sense	GAAGCUAUGUUCGCCAUUA
	antisense	UAAUGGCGAACAUAGCUUCTT
si-MRE11	sense	GAUGCCAUUGAGGAAUAAG
	antisense	CUUAUUCCUCA AUGGCAUCTT
si-Non-target	sense	AUGAACGUGAAUUGCUCAA
	antisense	UUGAGCAAUUCACGUUCAUTT
si-CtIP	sense	GCUAAAACAGGAACGAAUC
	antisense	GAUUCGUUCCUGUUUUAGCTT
si-RECQ1	siRNA-SMART pool	#4390824, ID 5965,
		ThermoFisher Scientific
Si-TREX1	TREX-1 siRNA pool	sc-63157, Santa Cruz
si-SAMHD1	SI04243673	Qiagen
si-STING	sense	GGUCAUAUUACAUCGGACAtt
	antisense	UAUCCGAUGUAAUAUGACCat
si-cGAS	sense	CCUUCUCUCACAUCGAAAAtt
	antisense	UUUUCGAUGUGAGAGAAGGat
si-IFR3	sense	AACUCAUCCAGAAUGUCUCCUGGG
	antisense	CCCAGGAAGACAUUCUGGAUGAGUU

Primer sequences

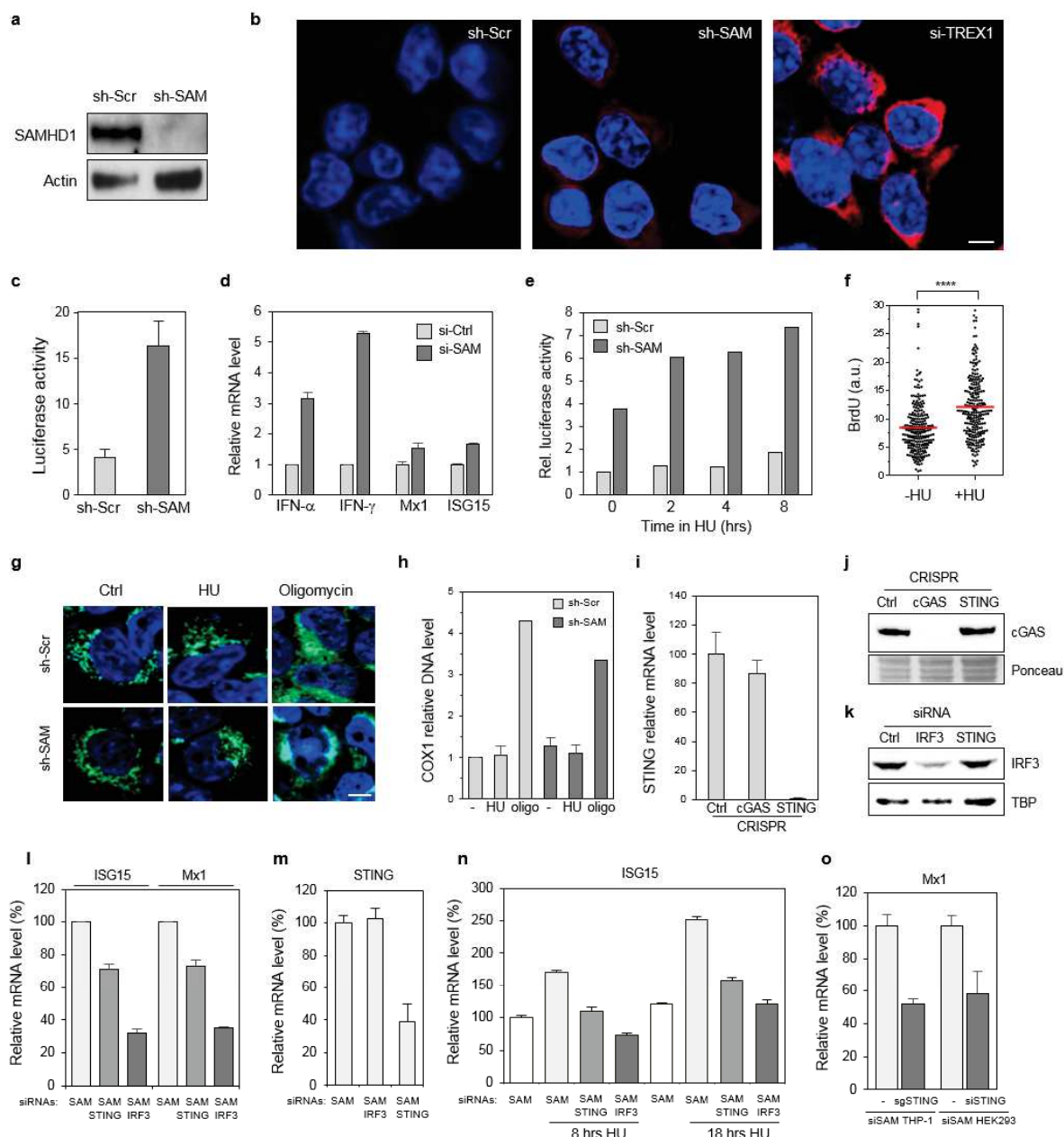
primer name	orientation	Primer sequences 5' to 3' orientation
GAPDH	Forward	TGCACCACCAACTGCTTAGC
	Reverse	GGCATGGACTGTGGTCATGAG
IFN- α	Forward	GTGAGGAAATACTTCCAAAGAATCAC
	Reverse	TTCATGATTTCTGCTCTGACAA
IFN- β	Forward	AGCTGAAGCAGTTCCAGAAG
	Reverse	AGTCTCATTCCAGCCAGTGC
IFN- γ	Forward	GTGGTGCTGGTGACTTTGG
	Reverse	CTCCTGTGGTGACAGAGATTG
TNF- α	Forward	CCTCTCTCTAATCAGCCCTCTG
	Reverse	GAGGACCTGGGAGTAGATGAG
Mx1	Forward	ATGAGCTAATCACCTGGAG
	Reverse	ATACCCAATGTCAGCAGGC
ISG15	Forward	CGCAGATCACCCAGAAGATCG
	Reverse	TTCGTCGCATTTGTCCACCA
STING	Forward	CATGGGCTGGCATGGTCATA
	Reverse	ATATACAGCCGCTGGCTCAC
MT-CO1 (COX1)	Forward	CCCCGATATGGCGTTTCC
	Reverse	GTTCAACCTGTTCTGCTCC

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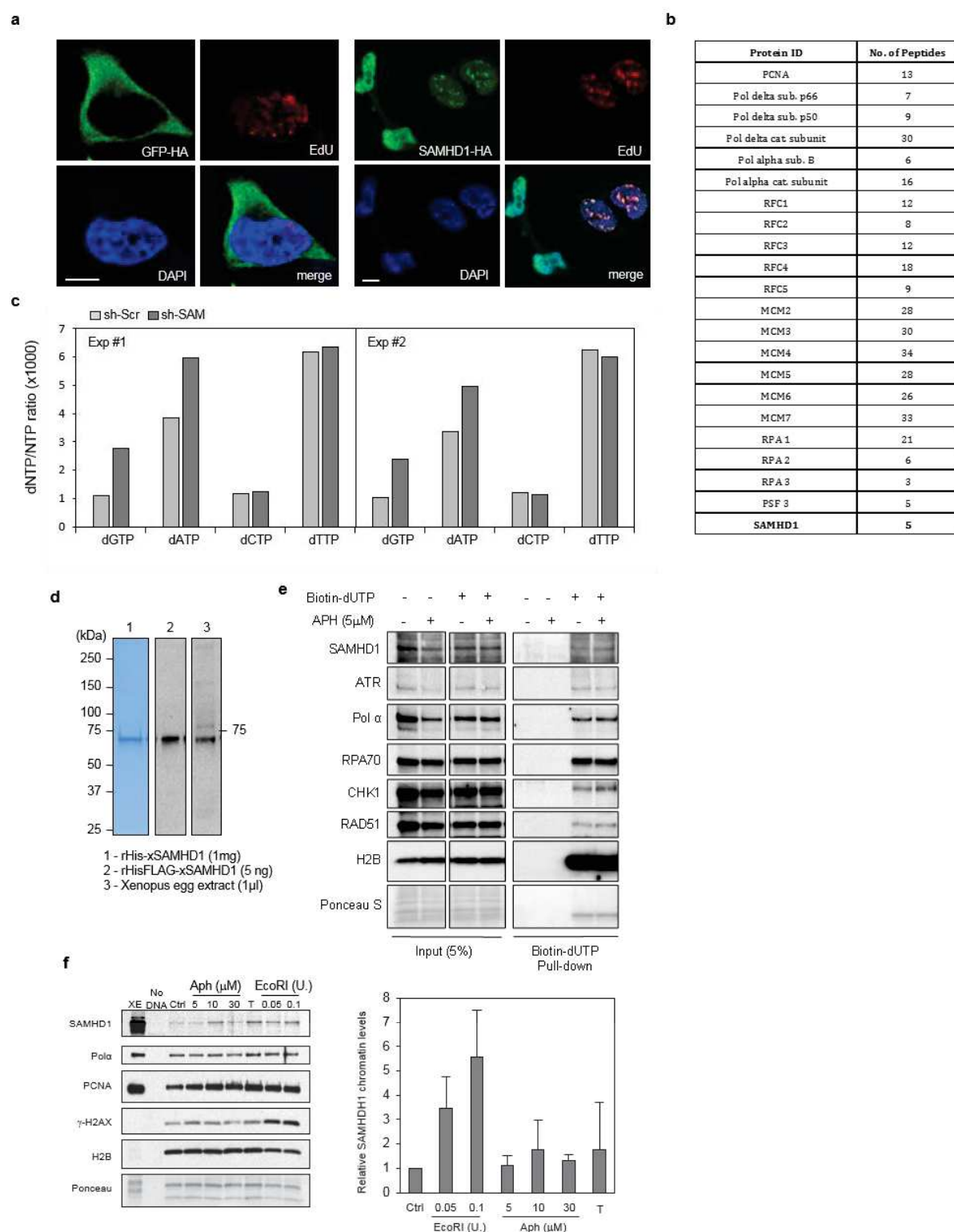
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Extended data



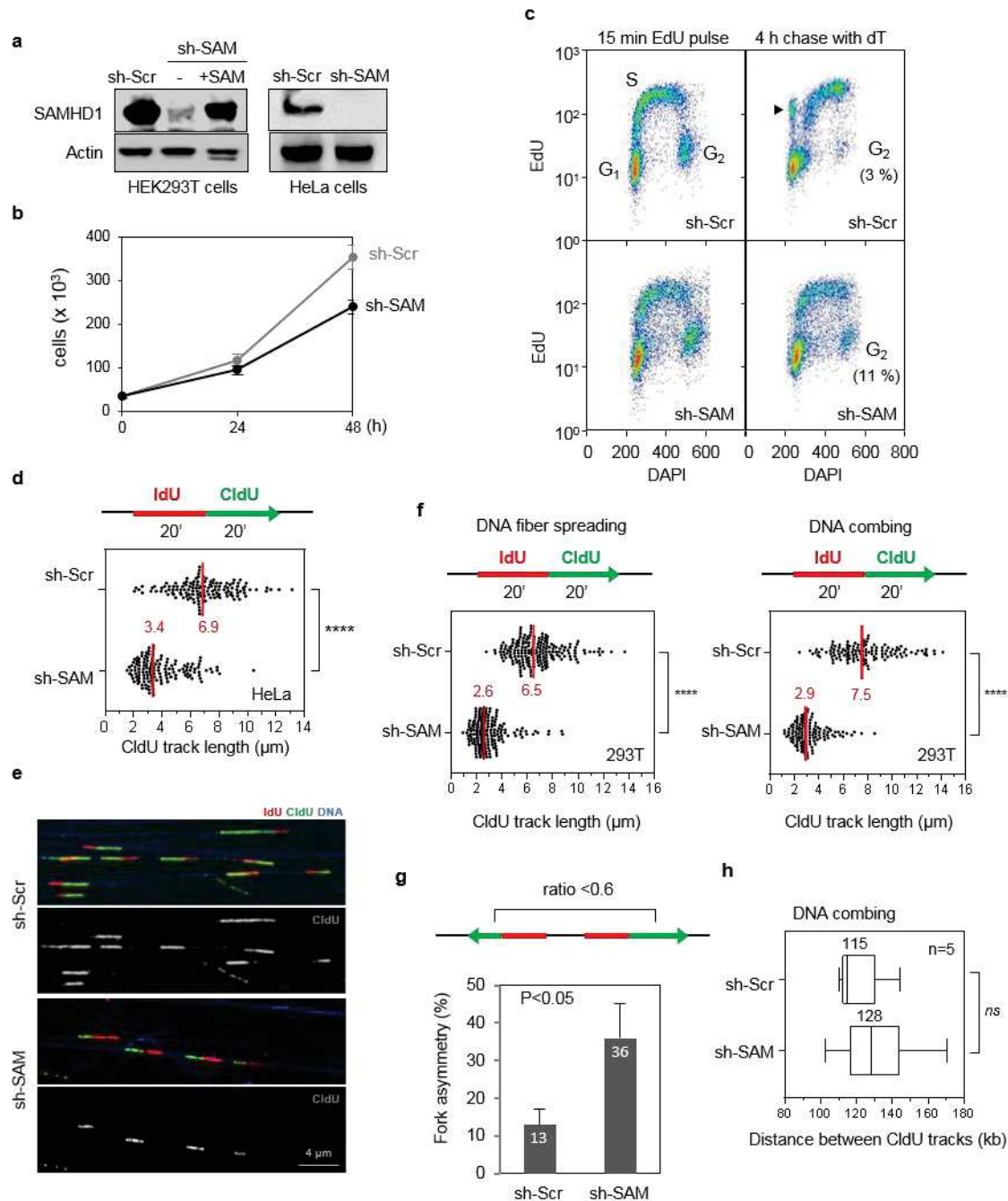
Extended Data Figure 1 SAMHD1-depleted cells secrete IFNs. a, Western blot analysis of SAMHD1 in HEK293T cells expressing a shRNA against SAMHD1 (sh-SAM) or a scrambled control (sh-Scr). b, Cytosolic ssDNA (red) in sh-Scr and sh-SAM HEK293T cells and in HEK293T cells transfected with an si-RNA against TREX1 (si-TREX1). Bar, 5 μ m, n=5. c, HL116 cells containing an interferon-stimulated response element-luciferase reporter gene were incubated with culture medium from sh-Scr or sh-SAM HEK293T cells for 48 h. Mean values of luciferase activity and standard deviation from four independent experiments are shown. d, HeLa cells were transfected for 48 h with control siRNA (si-Ctrl) or SAMHD1 siRNA (si-SAM). Expression of IFN- α , IFN- γ and the interferon-stimulated genes Mx1 and ISG15 was quantified by qRT-PCR. Data are representative of three independent experiments. Error bars correspond to SD for a representative triplicate experiment. e, sh-Scr or sh-SAM HEK293T cells were treated with 4 mM HU for the indicated

times and then transferred to fresh medium for a total of 20 h. Culture media were then collected and incubated with HL116 cells for 48 h prior to luciferase assay. The data shown are representative of three independent experiments. f, Quantification of the mean fluorescence intensity of cytosolic BrdU in the experiment shown in Fig. 1e. Quantification was performed on 250 cells by using CellProfiler. Median BrdU intensity is indicated in red. **** $P < 0.0001$, Mann-Whitney rank sum test. g, sh-Scr and sh-SAM HEK293T cells were treated for 2 h with 4 mM HU or for 6 h with 40 μ M oligomycin, used here to damage mitochondria. The cells were labelled with the mitochondria-selective dye MitoTracker (Invitrogen). The integrity of mitochondria was assessed by confocal microscopy. Representative images are shown. Bar, 5 μ m. h, The abundance of mitochondrial COX1 DNA in cytosolic DNA isolated from cells treated as in part (g) was quantified by qPCR and normalized to GAPDH. Mean values and standard deviations for three independent experiments are shown. i, Levels of STING mRNA in cGAS-KO (cGAS), STING-KO (STING) and control (Ctrl) HeLa cells transfected with siRNA against SAMHD1 were measured by qRT-PCR 48 h post-transfection. Mean values and SD for three independent experiments are shown. j, Levels of cGAS protein in cGAS-KO, STING-KO and control HeLa cells transfected with siRNA against SAMHD1 were monitored by western blotting 48 h post-transfection (n=3). k, Levels of IRF3 protein in SAMHD1-depleted HeLa cells co-transfected with siRNAs against STING (STING) or IRF3 (IRF3; n=3). l, HeLa cells were co-transfected for 48 h with siRNAs against SAMHD1 (SAM) and against STING (STING) or IRF3 (IRF3). Levels of ISG15 and Mx1 mRNAs were analysed by qRT-PCR. Mean values and SD for three independent experiments are shown. m, Expression levels of STING mRNA in HeLa cells co-transfected with siRNAs against SAMHD1 (SAM) and against STING (STING) or IRF3 (IRF3). Mean values and SD for three independent experiments are shown. n, HeLa cells were co-transfected for 48 h with siRNAs against SAMHD1 (SAM) and against STING (STING) or IRF3 (IRF3). They were then treated with 4 mM HU for 8 or 18 h, washed with PBS and further cultured in fresh medium for 18 h. Expression of ISG15 mRNA was quantified by qRT-PCR. Data are representative of three independent experiments. Mean values and SD correspond to triplicates of a representative experiment. o, HEK293 cells (+/- STING siRNA) and CRISPR-Cas9-mediated STING knock-out THP-1 cells were transfected with siRNA against SAMHD1. Expression of Mx1 mRNA was quantified by qRT-PCR. Mean values and SD for three independent experiments are shown.



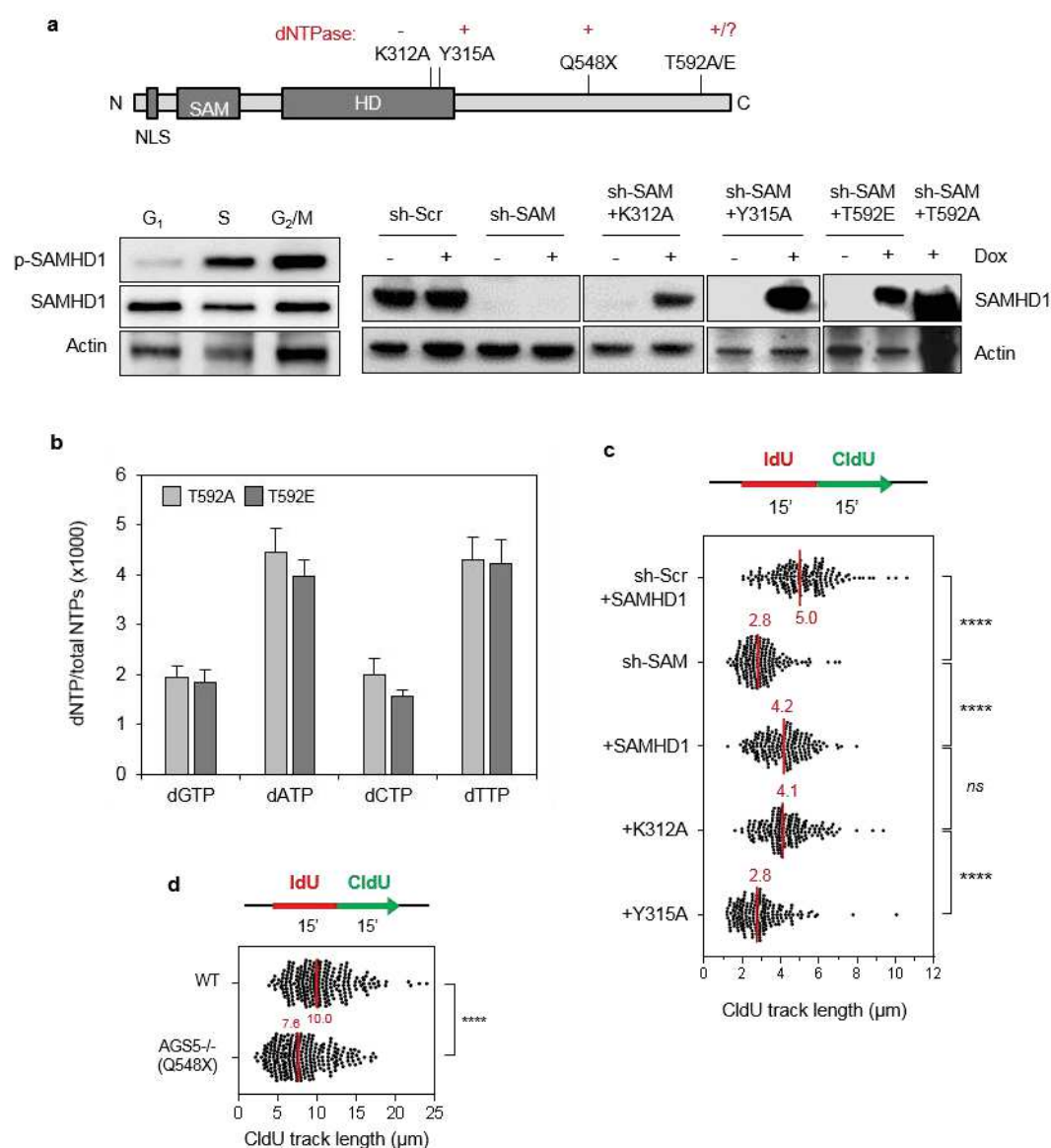
Extended Data Figure 2 SAMHD1 localises to replication foci and binds nascent DNA. a, EdU (red) and SAMHD1 (green) foci in HeLa cells expressing HA-tagged SAMHD1 (SAMHD1-HA) or GFP-HA. Immunofluorescence microscopy was performed as indicated in Fig. 2a. Bar, 5 μ m. n=2. b, HeLa cells were labelled with 10 μ M EdU for 10 min then processed for iPOND analysis. Proteins associated with nascent DNA were analysed by mass spectrometry. The number of

peptides from SAMHD1 and other factors found associated with nascent DNA is indicated. The table summarizes the data from two independent experiments. c, Measurement of intracellular dNTP pools in sh-Scr and sh-SAM HEK293T cells (two independent experiments). d, Production of recombinant His-xSAMHD1 (see Supplementary Information) and characterization of the antibody raised against this protein. e, xSAMHD1 associates with nascent DNA. *Xenopus* sperm DNA was incubated in *Xenopus* egg extract for 45 min then nascent DNA was labelled for 10 min with 40 μ M biotin-16-dUTP in the absence or presence of 5 μ M aphidicolin (APH). Nascent chromatin was isolated on streptavidin beads and analysed by western blotting for the proteins indicated (see extended methods). n=3. f, SAMHD1 binds chromatin in response to DSBs and aphidicolin. Left panel. *Xenopus* egg extracts were treated with the indicated doses of aphidicolin (Aph, μ M), Topotecan (T, 100 μ M) or EcoRI (U/ μ l). Chromatin-bound proteins were then analysed by western blotting (left panel). Histograms (right) show the mean relative signal intensity of SAMHD1 on chromatin and standard deviation for three independent experiments



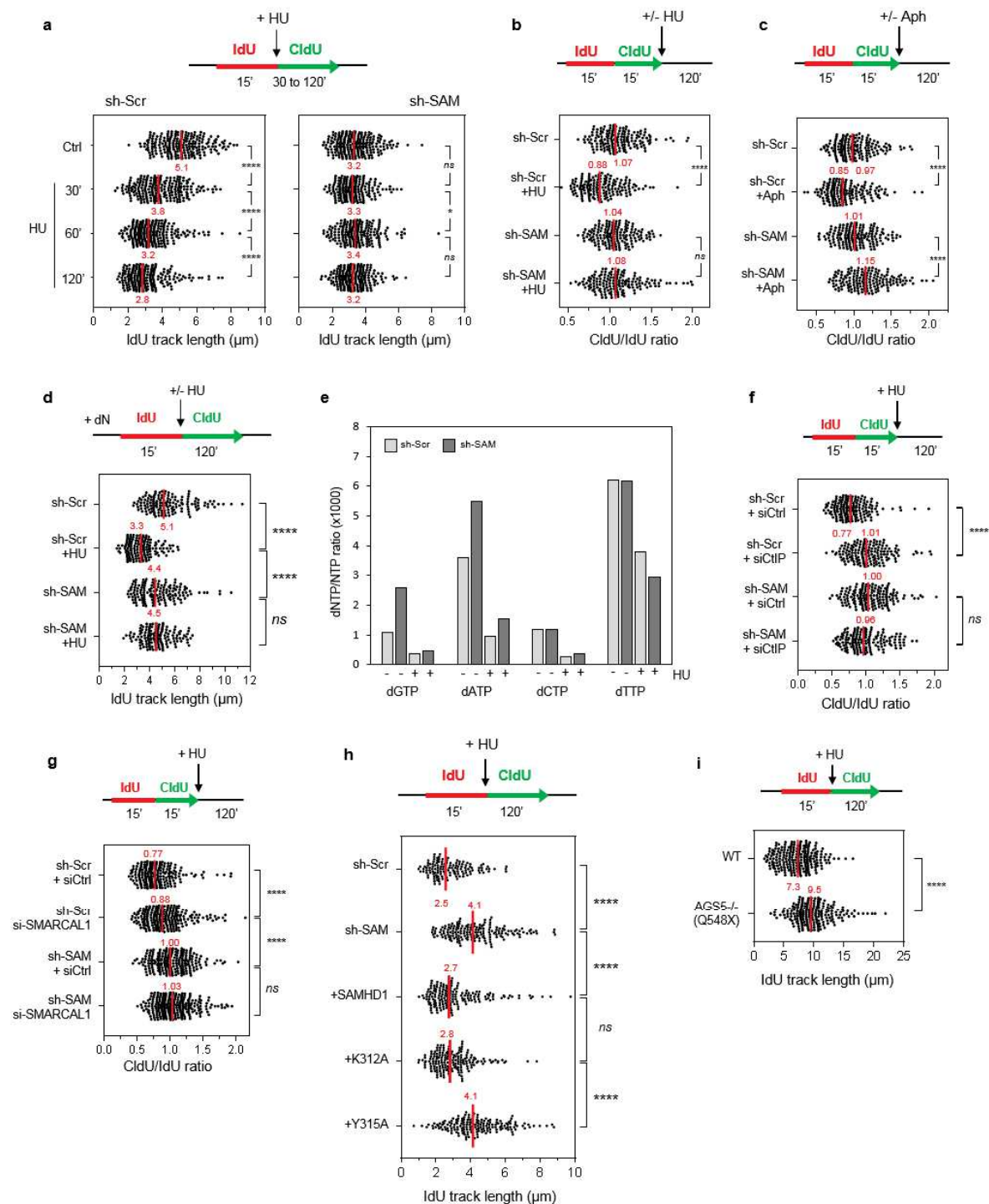
Extended Data Figure 3 Fork progression is altered in the absence of SAMHD1. a, Western blot analysis of SAMHD1 protein in HeLa cells depleted of SAMHD1 (sh-SAM) by using an shRNA and in SAMHD1-depleted HEK293T cells expressing an shRNA-resistant, full-length SAMHD1 (SAM) under the control of a doxycycline-inducible promoter. Expression of SAMHD1 was analysed 72 h after induction with doxycycline. b, Control (sh-Scr) and SAMHD1-depleted (sh-SAM) HEK293T cells were seeded in 24-well plates. Cell number was determined by using trypan blue exclusion and hemocytometry. Mean values and SD are shown for three independent experiments. c, SAMHD1 is required for normal S-phase progression. sh-Scr and sh-SAM HEK293T cells were pulse-labelled with EdU for 20 min and chased with thymidine for 4 h prior

to flow cytometry analysis. The arrowhead indicates the EdU-labelled cell population that completed DNA replication and came back to G1 phase. The percentage of cells in G2/M phase is indicated. N=2. d, sh-Scr and sh-SAM HeLa cells were labelled sequentially for 20 min with IdU and CldU and the length of CldU tracks (n=150) was analysed by DNA fibre spreading. Median track lengths are indicated in red. e, Representative images of stretched DNA fibres. Red, IdU; green, CldU; blue, DNA. The green channel is shown separately for clarity. Bar, 4 μ m. f, DNA fibres from sh-Scr and sh-SAM HEK293T cells sequentially labelled for 20 minutes with IdU and CldU were either stretched on glass slides (DNA fiber spreading; n=190) or combed on silanised coverslips (DNA combing; n=130) and then analysed by immunofluorescence microscopy. The length distribution of CldU tracks is shown. Median track lengths are indicated in red. g, DNA fibres from cells treated as in part (f) were stretched by DNA combing and the distance between CldU tracks, which is indicative of the density of active origins, was determined for five independent experiment. Median distances are indicated. Whiskers correspond to 10-90 percentiles. In parts (d-g), **** P<0.0001; ns not significant, Mann-Whitney rank sum test. h, SAMHD1-depleted cells show increased spontaneous fork arrest. The ratio of the shortest to the longest CldU track from cells treated as in part (d) was calculated for each pair of divergent sister replication forks and the percentage of sister forks showing a ratio of less than 0.6 is shown (n=75). Error bars indicate the standard deviation calculated for three independent experiments. P<0.05, Mann-Whitney rank sum test



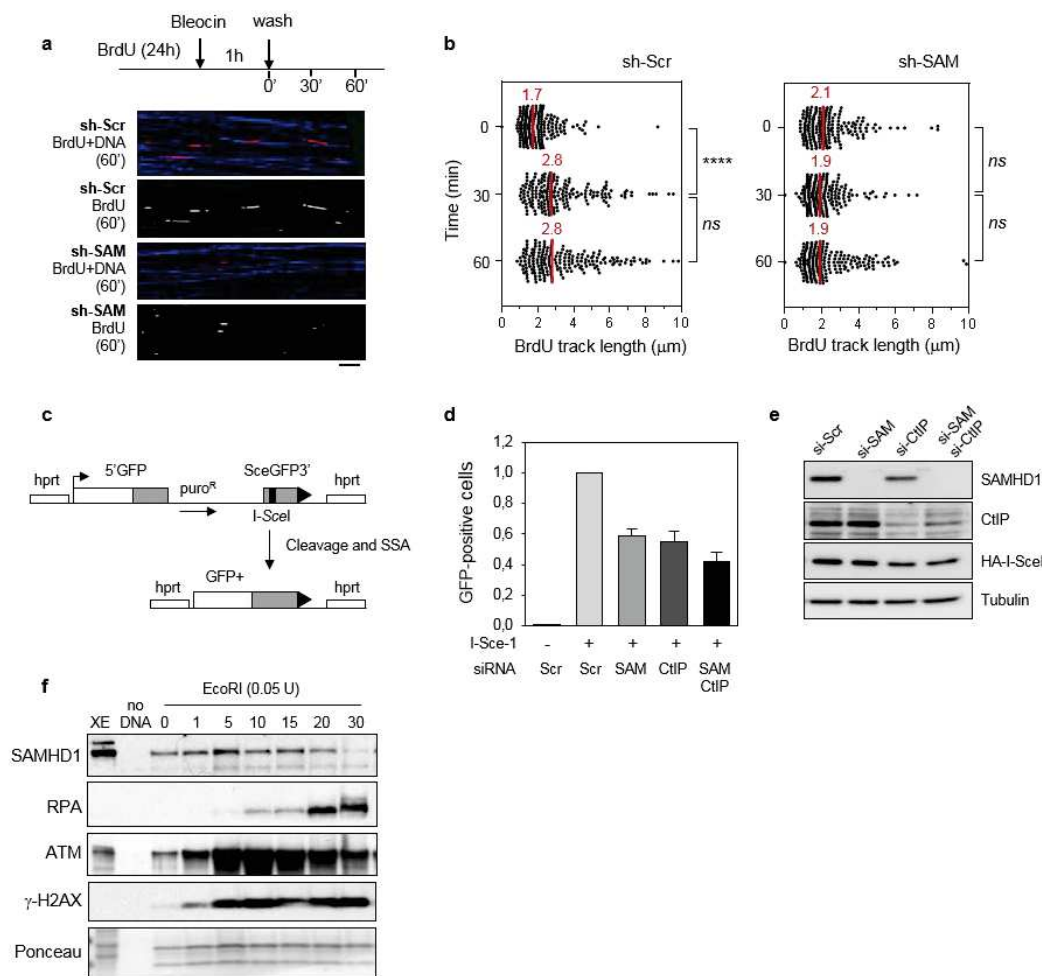
Extended Data Figure 4 SAMHD1 promotes fork progression independently of dNTP pools. a, Map of SAMHD1 protein domains indicating the positions of the mutations analysed in this study. The level of phospho-SAMHD1 (T592) in HEK293T cells collected by FACS in G1, S and G2/M phases was determined with a phospho-specific antibody. Levels wild type and mutant SAMHD1 were also analysed by western blotting after induction of the genes with doxycycline (Dox) for 72 h. n=4. b, SAMHD1-depleted HEK293T cells were complemented with the phosphomimetic (T592E) or non-phosphorylatable (T592A) mutants of SAMHD1. Intracellular dNTP pools were quantified as described in Methods. Median values and SD are shown for 5 independent experiments. c, Wild-type SAMHD1 or the K312A and Y315A mutant forms were expressed in SAMHD1-depleted (sh-SAM) HEK293T cells and the cells were labelled sequentially with IdU and CldU for 15 min each. The lengths of the CldU tracks (n=180) were measured on spread DNA fibres. d, Immortalized B cells from an AGS patient with a homozygous Q548X mutation (AGS5-/-

) or a healthy donor (WT) were labelled with IdU and CldU and the lengths of CldU tracks (n=240) were measured as in part (c). **** $P < 0.0001$; ns not significant, Mann-Whitney rank sum test



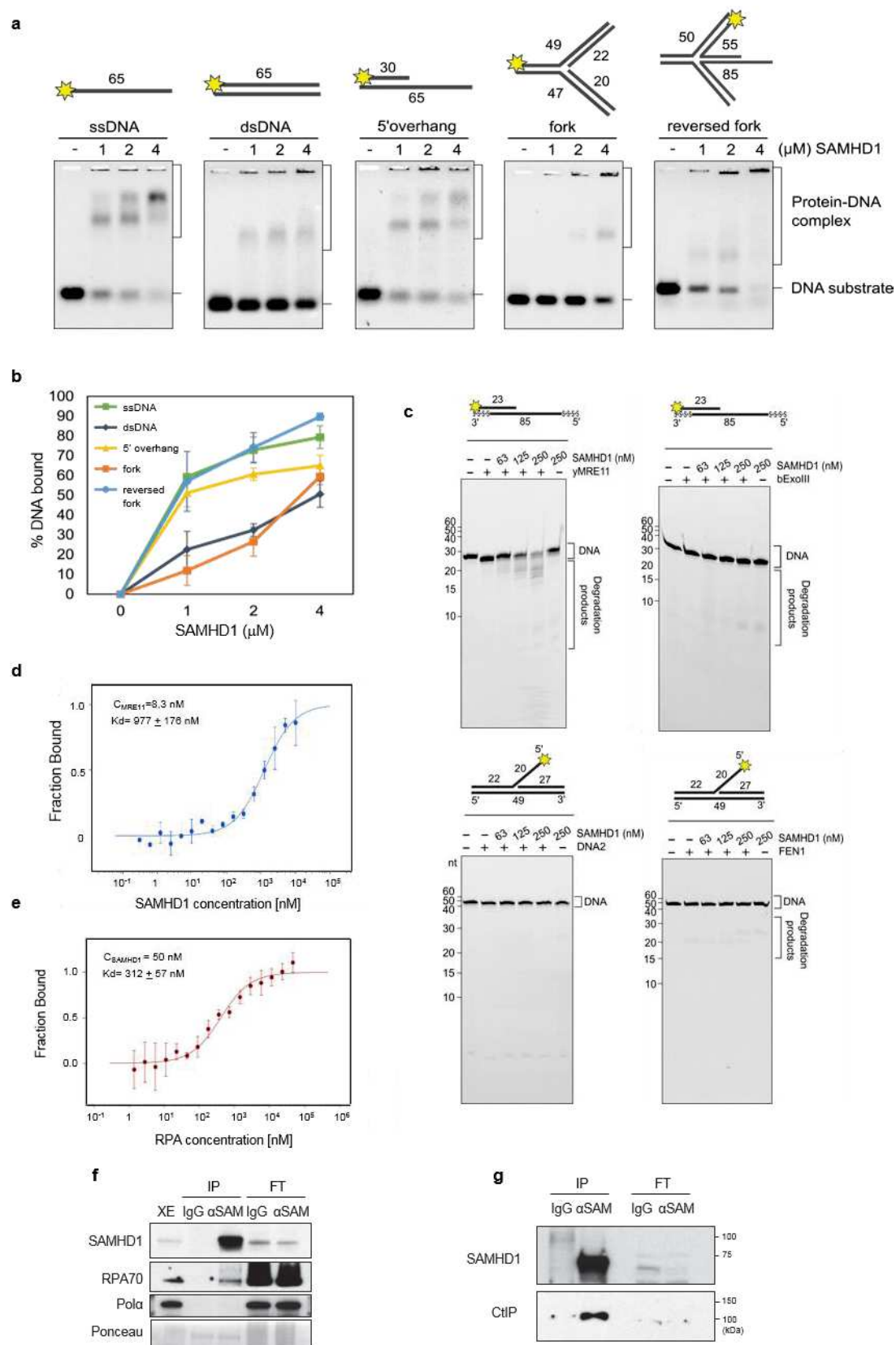
Extended Data Figure 5 Role of SAMHD1 in the degradation of nascent DNA at HU-arrested forks. a, Control (sh-Scr) and SAMHD1-depleted (sh-SAM) HEK293T cells were labelled with IdU for 15 min and were then exposed to 4 mM HU for 30, 60 or 120 min in the presence of CldU. The lengths of the IdU tracks (n=230) were measured on spread DNA fibres. b, sh-Scr and sh-SAM HEK293T cells were sequentially labelled for 15 min with IdU and for 15 min with CldU. Then, they were either collected immediately or treated for 2 h with 4 mM HU before DNA fibre analysis. The lengths of the IdU and CldU tracks (n=160) were plotted as the ratio of CldU to IdU. c, sh-Scr

and sh-SAM HEK293T cells were treated as described in part (b), except that HU was replaced with 1 μ M aphidicolin (+ Aph; n=160). d, sh-Scr and sh-SAM HEK293T cells were incubated for 120 min with a balanced mix of nucleosides (+ dN) and then labelled with IdU and CldU in the presence of 4 mM HU, as indicated. The lengths of IdU tracks (n=160) were measured on spread DNA fibres. e, sh-Scr and sh-SAM HEK293T cells were treated for 2 h with 4 mM HU, or not treated, and intracellular dNTP pools were measured and expressed relative to intracellular rNTP pools in two independent experiments. f, sh-Scr and sh-SAM HEK293T cells were transfected for 48 h with an siRNA against CtIP (siCtIP) or with a control (siCtrl) and then labelled with IdU and CldU in the presence of 4 mM HU, as indicated. The lengths of the CldU and IdU tracks (n=160) were measured on spread DNA fibres and plotted as the ratio of CldU to IdU track lengths. g, sh-Scr and sh-SAM HEK293T cells were transfected with siRNA against SMARCA1 or with a si-Ctrl for 48 h and then labelled with IdU and CldU in the presence of 4 mM HU, as indicated. The lengths of the CldU and IdU tracks (n=200) were measured on spread DNA fibres and plotted as the ratio of CldU to IdU track lengths. h, Wild-type SAMHD1, the dNTPase-deficient mutant K312A or dNTPase-proficient mutant Y315A were expressed in sh-SAM HEK293T cells and the cells were then labelled with IdU for 15 min and then with CldU in the presence of 4 mM HU for 2 h, as indicated. The lengths of the IdU tracks (n=140) were measured on spread DNA fibres. i, Immortalized B cells from an AGS patient with a homozygous Q548X mutation (AGS5-/-) or a healthy donor (WT) were labelled with IdU and CldU in the presence of HU, as in part (h). The lengths of the IdU tracks (n=200) were measured on spread DNA fibres. For parts (a) to (i), median track lengths are indicated in red. ****, $P < 0.0001$; *, $P < 0.05$; ns, not significant, Mann-Whitney rank sum test.



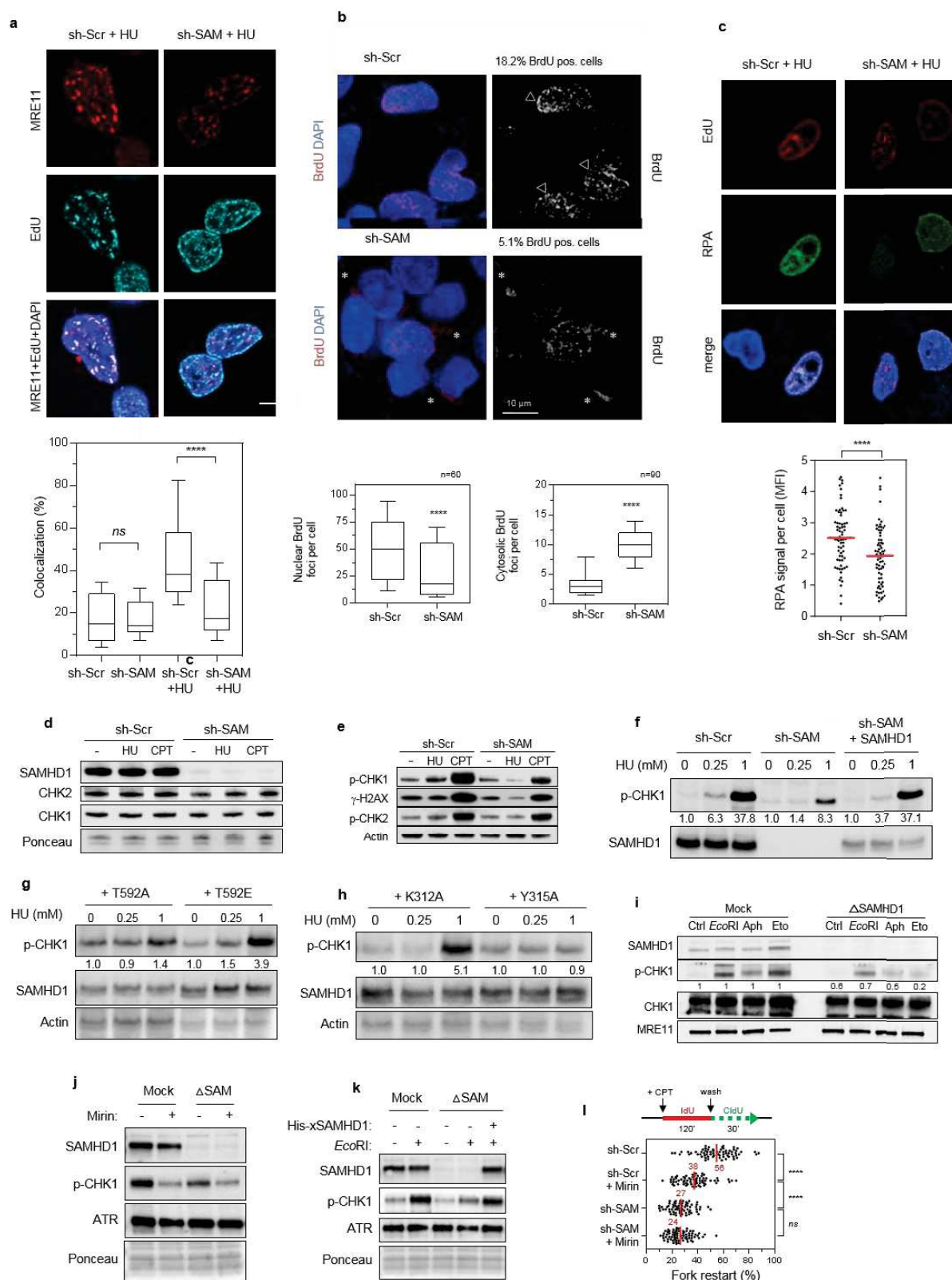
Extended Data Figure 6 *SAMHD1 promotes the resection of DNA DSB ends.* a, Analysis of DNA end resection at the level of individual DNA fibres with the SMART assay. Control (sh-Scr) and SAMHD1-depleted (sh-SAM) HEK293T cells were grown for 24 h in the presence of BrdU to label genomic DNA and DSBs were induced with 5 μg/ml bleocin for 1 hour. Cells were then washed and collected at the indicated times. DNA fibres were spread on glass slides and BrdU was detected without DNA denaturation. Representative images (three independent experiments) of BrdU tracks (red) 1 h after bleocin removal are shown. Bar, 5 μm. b, Quantification of BrdU track lengths (n=200) in sh-Scr and sh-SAM HEK293T cells treated with bleocin as in part (a). Median track lengths are indicated in red. ****, P<0.0001; ns, not significant, Mann–Whitney rank sum test. c, Schematic of the U2OS–SSA cell assay for DNA DSB repair by single-strand annealing (SSA). These cells carry a reporter vector in which an I-SceI site has been incorporated into a GFP gene, such that SSA-mediated repair events result in GFP fluorescence. d, U2OS–SSA cells were transfected with siRNAs against SAMHD1 (SAM), CtIP (CtIP), or both (SAM + CtIP), or with a control scrambled siRNA (Scr). They were then transfected with a plasmid expressing HA-tagged I-SceI under the control of a CMV promoter. Percentages of GFP-positive cells were quantified by flow cytometry and were normalized to the control (Scr) cells. Error bars correspond to SD of

three independent experiments. e, Expression of SAMHD1, CtIP and HA-tagged I-SceI in the experiment shown in part (d) were monitored by western blotting (n=3). f, *Xenopus* sperm DNA was incubated in *Xenopus* egg extract in the presence of 0.05 U/ μ l of EcoRI for the indicated times then the chromatin was purified and analysed by western blotting for the indicated proteins (n=3)



Extended Data Figure 7 SAMHD1 binds MRE11 and stimulates its nuclease activity. a, Various fluorescently labelled DNA substrates (ssDNA, dsDNA, dsDNA with a 5' overhang, forked DNA and reversed fork DNA of the nucleotide lengths indicated) were incubated with a range of

concentrations of purified SAMHD1 and the formation of protein–DNA complexes was determined by electrophoretic mobility shift assay (EMSA) as described in Supplementary Information. Representative gel shift images are shown (n=3). b, Quantification of EMSA in part (a). c, SAMHD1 stimulates the nuclease activity of yeast MRE11, but not that of bacterial ExoIII and human FEN1 and DNA2. Error bars correspond to the SD of three independent experiments. d, e, SAMHD1 binds MRE11 and RPA as monitored by microscale thermophoresis assay, respectively. Error bars correspond to the SD of three independent experiments. f, Co-immunoprecipitation of SAMHD1 and RPA from *Xenopus* egg extracts. The panel shows western blots of the input egg extract (XE), of the proteins immunoprecipitated (IP) by a control antibody (IgG) and an antibody against SAMHD1 (anti-SAM) and the proteins that remained unbound (flow through, FT; n=3) g, Co-immunoprecipitation of SAMHD1 and CtIP from *Xenopus* egg extracts (n=3), as in part (f) and described in Supplementary Information



Extended Data Figure 8 SAMHD1 is required to recruit MRE11 and RPA to stalled forks and to activate the replication checkpoint. a, Top, control (shScr) and SAMHD1-depleted (shSAM) HEK293T cells were labelled with EdU for 10 min then grown without EdU for a further 2 h in the presence of 4 mM HU. Chromatin-bound MRE11 foci were detected by confocal microscopy and

compared to EdU foci. Representative confocal images are shown. Scale bar, 5 μ m. Bottom, the co-localization of EdU and MRE11 foci was quantified by using the JACoP plugin of ImageJ (n = 55). Whiskers indicate the 10th and 90th percentiles. ****P < 0.0001, Mann–Whitney rank sum test.

b, Top, foci (arrowheads) of ssDNA (red) in the nuclei (blue) of HU-treated shScr and shSAM HEK293T cells labelled for 24 h with 10 μ M BrdU. Representative images from two independent experiments are shown. Asterisks indicate BrdU-labelled cytosolic ssDNA. Bottom, quantification of nuclear and cytosolic BrdU foci per cell as in a.

c, Top, shScr and shSAM HEK293T cells were incubated with 10 μ M EdU for 10 min and then treated for 2 h with 4 mM HU. RPA foci (green) were detected by using an anti-RPA1 antibody after CSK extraction. Bottom, mean fluorescence intensity of chromatin-bound RPA1 was quantified from 70 EdU-positive cells by using CellProfiler. ****P < 0.0001, Mann–Whitney rank sum test. Scale bar, 5 μ m.

d, Immunoblots of CHK1 phosphorylated on S345 (p-CHK1), γ -H2AX phosphorylated on S139, and CHK2 phosphorylated on T68 (p-CHK2) in shScr and shSAM HEK293T cells treated for 60 min with 0.25 mM HU or 1 μ M CPT (n = 3).

e, Expression of CHK1 and CHK2 proteins after HU or CPT treatment as indicated in d.

f, shScr and shSAM HEK293T cells, and shSAM HEK293T cells expressing full-length SAMHD1 (shSAM + SAMHD1) were tested for their ability to phosphorylate CHK1 on S345 upon exposure to 0.25 or 1 mM HU for 2 h. The fold of induction was normalized to untreated cells by quantifying the bands on the blots and calculating the ratios of p-CHK1 to SAMHD1 (n = 3).

g, shSAM HEK293T cells expressing the non-phosphorylatable mutant T592A or the phosphomimetic mutant T592E of SAMHD1 were treated with 0.25 mM or 1 mM HU for 2 h. The amounts of p-CHK1 and SAMHD1 were analysed by immunoblotting and quantified as described in f; n = 2.

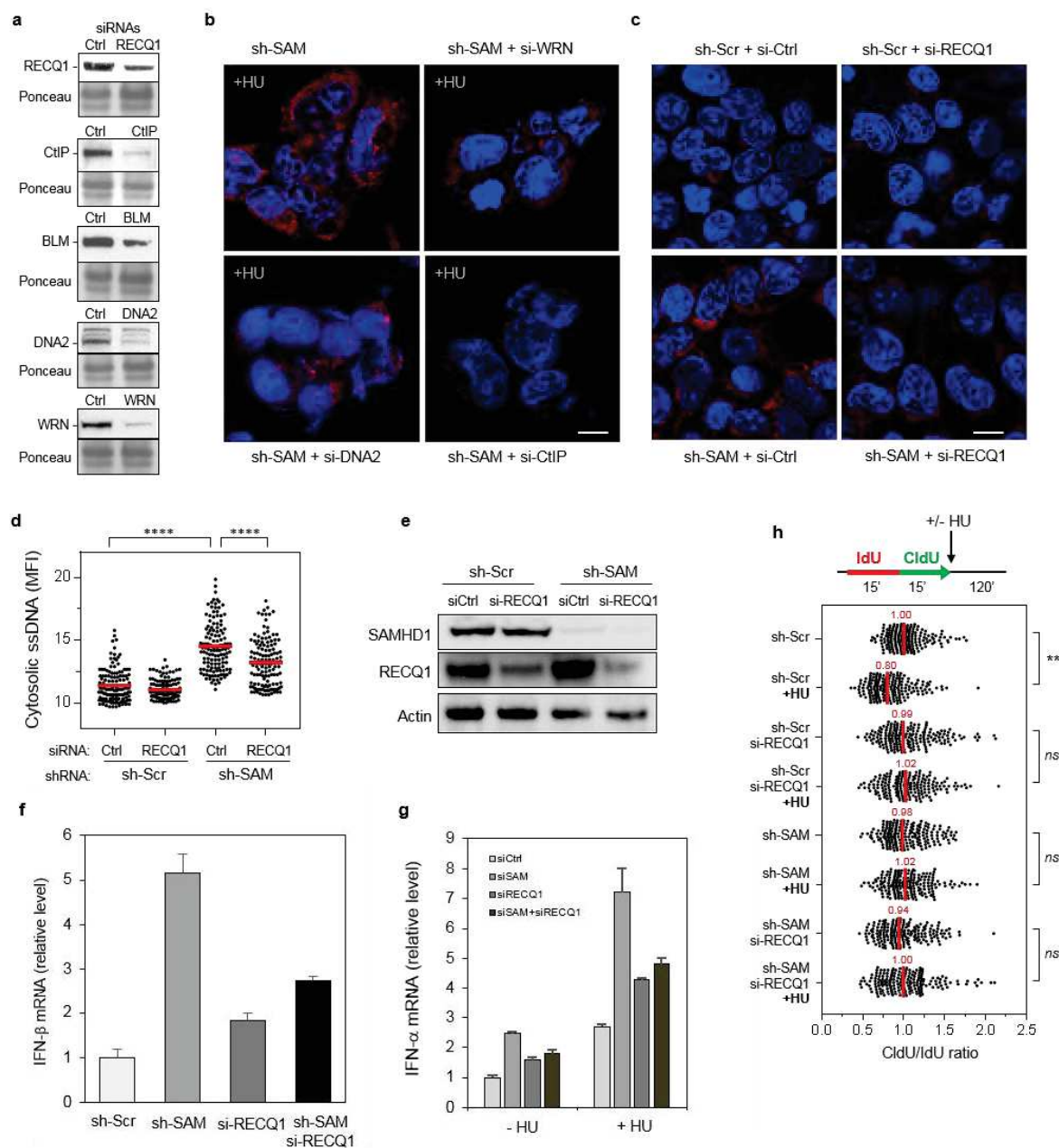
h, shSAM HEK293T cells expressing the exonuclease positive (K312A) or negative (Y315A) mutants of SAMHD1 were treated with the indicated doses of HU for 2 h. The amounts of p-CHK1 and SAMHD1 were analysed by immunoblotting and quantified as described in f; n = 2.

i, Depletion of Samhd1 from *Xenopus* egg extracts impairs the phosphorylation of CHK1 in response to EcoRI (0.05 U μ l⁻¹), aphidicolin (20 μ M) or etoposide (Eto, 30 μ M; n = 3).

j, Samhd1 and Mre11 are required to activate CHK1 in Samhd1-depleted or mock-depleted *Xenopus* egg extracts treated with 0.05 U μ l⁻¹ of EcoRI. Mre11 was inhibited with 100 μ M mirin; this experiment was performed once.

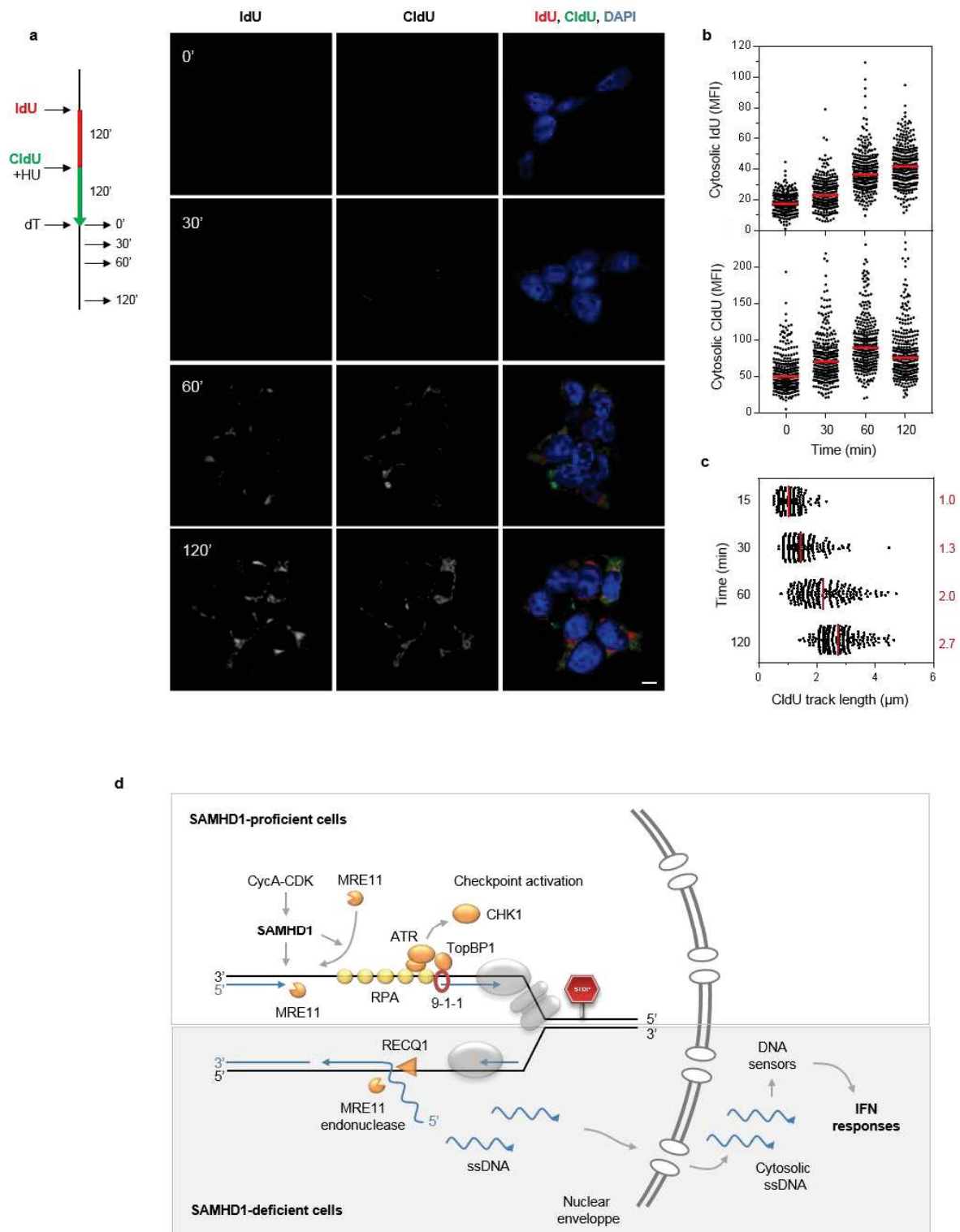
k, CHK1 activation in Samhd1-depleted *Xenopus* egg extracts upon formation of DSBs by addition of 0.05 U μ l⁻¹ EcoRI can be restored by the addition of approximately 25 nM of recombinant His-xSamhd1; a representative experiment is shown (n = 2).

l, DNA fibre analysis of fork restart in shScr and shSAM HEK293T cells treated for 18 h with 10 μ M mirin and for 2 h with 1 μ M CPT. Fork restart (that is, formation of red and green tracks) was monitored 30 min after CPT removal (n = 70; three independent experiments). ****P < 0.0001, Mann–Whitney test.



Extended Data Figure 9 Depletion of RECQ1 prevents accumulation of cytosolic ssDNA in SAMHD1-depleted cells. a, RECQ1, CtIP, BLM, DNA2 and WRN proteins remaining 48 h after transfection with the corresponding siRNAs (n=2). b, SAMHD1-depleted (sh-SAM) HEK293T cells were transfected with the indicated siRNAs for 48 h were then treated with 4 mM HU for 2 h. Cytosolic ssDNA was visualized by immunofluorescence microscopy. Representative confocal microscopy images are shown (n=2). Bar, 5 μm. c, Depletion of RecQ1 prevents the accumulation of cytosolic ssDNA in untreated sh-SAM cells. Cytosolic ssDNA was detected by immunofluorescence microscopy. Representative images are shown (n=3). Bar, 5 μm. d, Mean fluorescence intensity (MFI) of cytosolic ssDNA in the cells in the experiment shown in part (c) was quantified by using CellProfiler (n=130). ****: P<0.0001, Mann-Whitney rank sum test. e,

RECQ1 and SAMHD1 proteins remaining after siRNA transfection, as analysed by western blotting (n=3). f, sh-Scr and sh-SAM HEK293T cells were transfected with siRNA against RECQ1 (si-RECQ1) for 48 h. Expression of IFN- γ mRNA was quantified by qRT-PCR. The data shown are representative of three independent experiments. Error bars represent SD of triplicates. g, HeLa cells were transfected with siRNAs as indicated. They were cultured in the absence or presence of 4 mM HU for 8 h and were then in without HU for a total of 20 h before mRNA extraction. Expression of IFN- α mRNA was analysed by qRT-PCR and normalized to the amount of GAPDH mRNA. The data shown are representative of three independent experiments. Error bars represent SD of triplicates. h, sh-Scr and sh-SAM HEK293T cells were transfected with siRNA against RECQ1 for 48 h. The cells were sequentially labelled for 15 min with IdU and for 15 min with CldU. Then, they were either collected immediately or treated for 2 h with 4 mM HU before DNA fiber analysis. The lengths of the IdU and CldU tracks (n=200) were plotted as the ratio of CldU to IdU. Median values are indicated in red. ****, $P < 0.0001$, ns, not significant, Mann-Whitney rank sum test



Extended Data Figure 10 SAMHD1 depletion promotes accumulation of cytosolic nascent DNA under replication stress. a, SAMHD1-depleted HEK293T cells were labelled for 2 h with IdU and then for 2 h with CldU in the presence of 4 mM HU. They were then chased with thymidine for the indicated times and cytosolic IdU (red) and CldU (green) were visualised by confocal immunofluorescence microscopy. Representative images are shown. Bar, 5 μ m. b, Quantification of the cytosolic IdU and CldU signals in part (a) by using CellProfiler (n=300). c, DNA fibre analysis

showing the increasing length of CldU tracks in HU-treated SAMHD1-depleted HEK293T cells. Median track lengths (n=190) are indicated in red. d, Model of the role of SAMHD1 at stalled replication forks. In SAMHD1-proficient cells (top), phosphorylation of SAMHD1 by CycA-CDK contributes to the MRE11-dependent resection of stalled replication forks and activates the ATR-CHK1 pathway at RPA-coated ssDNA, together with TopBP1 and 9-1-1 complex. In SAMHD1-deficient cells (bottom), nascent DNA is displaced by the RECQ1 helicase and cleaved by an endonuclease, such as MRE11. The resulting ssDNA fragments accumulate in the cytosol and activate the type I interferon response

| Conclusion and discussion

Article conclusion

The absence of SAMHD1 triggers an increase in cell-autonomous type I IFNs production, together with the generation of cytosolic ssDNA. Importantly, this effect is exacerbated when cells are treated with DNA replication inhibitors such as HU. We demonstrated that cytosolic DNA generated in the absence of SAMHD1 directly originates from stalled replication forks. SAMHD1 indeed localizes to replication sites where it promotes DNA synthesis in part via its dNTPase activity. In addition, SAMHD1 promotes the processing of arrested forks and the activation of the replication checkpoint. *In vitro*, SAMHD1 binds reversed fork structures as well as ssDNA and stimulates the exonuclease activity of MRE11. In the absence of SAMHD1, stalled forks are aberrantly processed by the helicase RecQ1, which generates flaps that are cleaved by the endonucleolytic activity of MRE11 or DNA2. This event generates DNA fragments that accumulates in the cytosol and are detected by the cGAS-STING pathway, thereby triggering the production of type I IFNs.

Discussion

I. SAMHD1 is required for stalled fork processing

Multiple nucleases and helicases are involved in the processing of stalled replication forks in mammalian cells (Pasero & Vindigni, 2017). We found that SAMHD1, which possesses a putative nuclease activity (Beloglazova et al., 2013; Ryoo et al., 2014), is required for fork restart under treatment with camptothecin. Correspondingly, SAMHD1 promotes nascent DNA degradation at arrested replication forks and the activation of the replication checkpoint. As discussed below, the exact role of SAMHD1 in this utterly complex process is still obscure.

1. Is fork resection mediated by the nuclease activity of SAMHD1?

To begin with, I wanted to point out that we were able to detect the processing of stalled forks in HRD-proficient cells, which can be surprising at first considering that nascent DNA degradation has mainly been reported in cells deficient for BRCA2 or FANCD2. I can see two main reasons for this seemingly different result. First, we used a labelling strategy whereby we label nascent DNA for a short time (15 minutes), following by a short treatment with HU (15 to 120 min), which may allow us to quantify small differences, something that might be more difficult to obtain with longer treatment times. Off note, it was recently demonstrated that fork reversal is a key prerequisite of nascent DNA degradation in BRCA2-defective cells (Kolinjivadi et al., 2017; Mijic et al., 2017; Taglialatela et al., 2017). Long exposures to HU may provide time for fork reversal, allowing extensive MRE11-dependent fork resection. In our experimental setting with shorter HU exposure, we may actually mainly detect DNA degradation at gapped forks which could eventually be converted into reversed structures if the ssDNA gap persists (Kolinjivadi et al., 2017). Second, this could be caused by differences in cell systems. Indeed, we used human cancer cell lines and human transformed cells (mainly HeLa and HEK293T, respectively), which may respond differently to replication stress from the hamster cells used by Schlacher et al. and the primary murine cells used by Chaudhury et al. (Chaudhuri et al., 2016; Schlacher et al., 2011). When assessing fork protection in non-transformed MCF10-A breast cells, we could not detect nascent DNA degradation (Figure 34 in Appendix). Notably, the laboratory of Maria Jasin reported that MCF7 breast cancer cells (which are not BRCA mutated) are defective in fork protection (Schlacher et al., 2012), which is consistent with our findings (Figure 34 in Appendix).

We used the DNA fiber spreading technique with variations on the labeling scheme to evaluate stalled fork processing. With this method, we were not able to directly measure both degradation of nascent DNA and the appearance of single-stranded DNA at arrested forks. We therefore tried to set-up a modified SMART experiment whereby we could quantify the amount of ssDNA

appearing at stalled replication forks. To do so, we labelled cells with BrdU for one cell cycle and included a short EdU pulse before arresting forks with HU. After the fiber experiment, we performed the immunostaining without the denaturation step to label BrdU tracks embedded into ssDNA. The EdU tracks allowed us to visualize the localization of replications forks. Unfortunately, we observed a lot of background noise associated with BrdU staining and BrdU tracks adjacent to the EdU were too small to be measured. DNA stretching with the fiber technique may not be enough to allow a precise measurement of ssDNA at stalled forks. Alternatively, these structures may be generated very transiently and would be difficult to analyze with this resolution. Electron microscopy (Zellweger et al., 2015) would allow us to visualize the structures of ssDNA and reversed forks, however, this laborious and time-consuming technique is not adequate for the quantification of DNA track length. As of today, a technique allowing sensitive, robust and easy quantitation of ssDNA at stalled forks is still not available.

SAMHD1 mediates DNA resection at arrested forks, which could be through its putative 3'-5' exonuclease activity. For instance, nascent DNA degradation is restored by expressing a nuclease-proficient K312 mutant in SAMHD1-depleted cells, but not by a nuclease-deficient Y315A mutant (Extended data Figure 5h). These observations, however, rely on controverted results (Beloglazova et al., 2013). Besides, we found that SAMHD1 is epistatic to MRE11 in fork restart (Extended data Figure 8-l). We also consistently detect MRE11 activity at arrested forks (Figure 3-a), as previously published (Chaudhuri et al., 2016; Schlacher et al., 2011), and the recruitment of MRE11 at replication foci in HU was lost upon SAMHD1 depletion (Extended data Figure 8-a). Overall, our data indicates that MRE11 is involved in SAMHD1-dependent fork processing.

In parallel, our collaborators found that SAMHD1 interacts with MRE11 and stimulates its exonuclease activity *in vitro* (Figure 3-e, f; Extended data Figure 7-d). Using their *in vitro* nuclease assays, they could not detect any nuclease activity of SAMHD1 by itself, either on gapped or on flapped DNA substrates (Extended data Figure 7-c). We therefore propose that SAMHD1 supports fork processing by recruiting and activating MRE11 at arrested forks. We have not been able to directly show that the nuclease activity of SAMHD1 itself was involved in this process. It is now tempting to speculate that the contaminating DNA exonuclease that co-purify with SAMHD1 (Seamon et al., 2015) is MRE11, which would suggest that the Y315 amino-acid might be important for the interaction between SAMHD1 and MRE11. To test this hypothesis, it is possible to check if the nuclease activity observed upon purification of SAMHD1 is reduced upon addition of the MRE11 inhibitor mirin. In addition, it would be interesting to analyse the interaction of SAMHD1 and MRE11 using the Y315A mutant. Of note, a SAMHD1 AGS variant (G209S) was recently described, preserving all the known enzymatic and biochemical properties of SAMHD1 while being defective in the regulation of interferon production (T. E. White et al., 2017). It would

be interesting to test whether the interaction between SAMHD1 and MRE11 is lost using this variant. If so, it would confirm the importance of appropriately degrading nascent DNA at arrested forks to prevent the generation of pro-inflammatory DNA fragments. And it would exclude any involvement of SAMHD1 intrinsic nuclease activity in this process.

2. SAMHD1 orchestrates the concerted action of multiple factors involved in stalled fork processing

While instigating the factors involved in fork processing, we found that this process was moderately reduced upon loss of the fork remodelling helicase SMARCAL1 (Extended data Figure 5-g), indicating that a subset of arrested forks are reversed so they can be processed. SAMHD1 depletion, in comparison, completely abolished nascent DNA degradation. It is possible, then, that SAMHD1 activity promotes the recruitment and activity of MRE11 both at gapped and reversed replication forks.

Besides MRE11, multiple nucleases have been involved in the degradation of nascent DNA at arrested forks. SAMHD1 may well regulate fork processing by operating as a recruitment platform and an activator for such enzymes (FAN1 and DNA2, for instance). *In vitro*, SAMHD1 does not stimulate the nuclease activity of DNA2 on flapped DNA structures (extended data Figure 7-c), but this was not tested on other fork-related structures such as 4-way junctions or gapped structures. As SAMHD1 probably promotes nascent DNA degradation at reversed forks, it would be relevant to study whether it stimulates the activity of DNA2 at 4-way junctions, which are known substrates of this nuclease (Thangavel et al., 2015). It would be interesting to assess the effect of SAMHD1 on the localization of the aforementioned nucleases in conditions of replication stress by proximity ligation assay and *in situ* protein interactions at nascent and stalled replication forks, techniques that enable measuring the association of proteins to nascent DNA (Roy, Luzwick, & Schlacher, 2018; Söderberg et al., 2006). Another possibility would be that SAMHD1 affects the remodelling of stalled replication forks. One way to evaluate this hypothesis would be to perform electron microscopy experiments to measure fork reversal in SAMHD1-depleted cells.

At double-strand breaks, the recently reported role for SAMHD1 in the resection of DNA ends seems to be dependent on its interaction with CtIP (Daddacha et al., 2017). Our collaborators likewise demonstrated that SAMHD1 promotes the resection of EcoRI-induced DNA DSBs and interacts with CtIP in *Xenopus* egg extracts (Extended data Figure 6-f and 7-g, respectively). It is tempting to speculate that this interaction is important for the DSB resection activity of SAMHD1 in *Xenopus*, as in human cells. Our experiments do not exclude the possibility that CtIP is also recruited at stalled replication forks by SAMHD1. In fact, CtIP and MRE11 remove the end-binding protein Ku from one-ended double-strand breaks (Chanut et al., 2016), a structure also existing

at broken and reversed forks. Accordingly, CtIP is involved in the processing of stalled replication forks, under the control of FANCD2 (Yeo et al., 2014). More specifically, CtIP operates at the regressed arm of reversed forks, allowing MRE11-dependent DNA degradation (Lemaçon et al., 2017). The exact role of CtIP in resection (both at DSBs and at forks) is not entirely understood. It is thought to primarily act as a cofactor for MRE11, stimulating its endonuclease activity (Anand et al., 2016), but it also has an intrinsic endonuclease activity (Makharashvili et al., 2014; H. Wang et al., 2014). During the course of this study, we nonetheless found that fork resection was strongly impaired upon CtIP depletion (Extended data Figure 5-f), supporting its importance in nascent DNA degradation.

3. Functions of fork processing

Fork restart

Nascent DNA degradation at arrested replication forks prevents the accumulation of replication-associated DSBs by promoting the repair of the forks by poorly understood mechanisms such as replication-coupled-Homologous Recombination (Colosio, Frattini, Pellicanò, Villa-Hernández, & Bermejo, 2016; Petermann et al., 2010). At reversed forks, controlled DNA resection mediated by DNA2 together with the helicase WRN is important for fork restart (Thangavel et al., 2015). At gapped forks, limited MRE11-dependent degradation of nascent DNA may remove obstacles, such as stalled polymerases, thereby allowing repriming past the lesion.

Fork restart is defective in SAMHD1-depleted cells treated with camptothecin (Extended data Figure 8-l), indicating that SAMHD1-mediated fork processing allows such pathways to resume DNA synthesis. SAMHD1 might therefore be important to support both fork restart by recombination-mediated processes and to allow fork repriming, possibly through the activation of the replication checkpoint (Trenz, Smith, Smith, & Costanzo, 2006).

Indeed, activation of the ATR effector kinase CHK1, but not the DNA damage kinase CHK2, is decreased upon loss of SAMHD1, both in mammalian cells and in *Xenopus*, in response to HU and aphidicolin, respectively (Extended data Figure 8d, f, i). In other words, the SAMHD1-dependent generation of RPA-coated ssDNA is important to trigger the replication checkpoint in response to replication stress, as it was proposed for DNA2 (Thangavel et al., 2015). This function likely is important during a normal S phase when forks encounter natural obstacles such as tightly bound protein-DNA complexes or DNA secondary structures. Indeed, without addition of any exogenous stress, replication forks are slower in SAMHD1-depleted cells (Figure 2c) and they do not activate compensatory replication origins (Extended data Figure 3h), presumably because of the defective checkpoint activation. This combination of slow replication forks and the lack of compensation by origin activation might explain why S phase is longer when SAMHD1 is depleted.

Replication fork progression

Global progression of replication forks and progression through S phase are strongly impaired by SAMHD1 depletion (Figure 2c; Extended data Figure 3c). Supplementing SAMHD1-depleted cells, which have impaired dATP and dGTP pools (Figure 2d), with a balanced mixture of dNTPs partially rescued fork progression (Figure 2e), thereby indicating that imbalanced dNTP pools are in part responsible for this phenotype. Importantly, SAMHD1 primarily regulates dNTP pools in quiescent cells and in the G1 phase of cycling cells (Franzolin et al., 2013). In our experimental setup, upregulation of dATP and dGTP is partially involved in the reduction of replication fork progression. Accordingly, our results suggest that imbalanced dNTP pools in G1 directly affect the processivity of the replisome.

The progression of replication forks was further analysed in immortalized B cells from an AGS patient carrying a Q548X SAMHD1 mutation, which does not affect dNTP regulation (Ryoo et al., 2014). Interestingly, replication fork progression was reduced in cells from the AGS patient compared to that of cells from a healthy donor (Extended data Figure 4d). Conversely, replication defects observed in SAMHD1-depleted cells are fully rescued by the expression of K312A mutant, defective for dNTP hydrolysis (Extended data Figure 4c). All these observations support the notion that SAMHD1 promotes fork progression somewhat independently of dNTP pools. In other words, an additional SAMHD1-mediated process must also support the activity of the replisome.

Accordingly, while inter-origin distances are not affected in SAMHD1-deficient cells (as measured by DNA combing), fork asymmetry significantly increased (Extended data Figure 3g, h). The occurrence of asymmetric replication forks indicates that fork stalling events either increase or are incorrectly handled when SAMHD1 is depleted. To put it differently, appropriate fork processing mediated by SAMHD1 may participate in the accurate progression of replication forks, possibly by helping their timely restart and by supporting CHK1 activation, which has been shown to be important for normal replication rates (Petermann et al., 2006). Although we do not know whether this mechanism effectively occurs in physiological conditions, fork restart and checkpoint activation indeed drop in SAMHD1-deficient cells upon treatment with the Topoisomerase I inhibitor camptothecin (Extended data Figure 8d, e). In DNA fibre spreading experiments, replication tracks are globally smaller in SAMHD1-depleted cells compared to control cells. Yet, it is highly unlikely that all said forks have been arrested during the experiment. It is possible then that the combined effect of dNTPs dysregulation, fork restart failure and checkpoint deficiency is responsible for the global slow-down of DNA replication and the delay in cell cycle progression. Conversely, replication tracks formed during treatment with HU, a drug that reduces dNTP pools, are longer in SAMHD1-depleted cells than in control cells (see Figure

35 in the Appendix). At first one may hypothesize that increased dNTP pools in SAMHD1-depleted may allow forks to progress further. But replication tracks are still longer in SAMHD1-depleted cells complemented with the dNTPase-proficient Y315A mutant compared to control cells and SAMHD1-depleted cells complemented with the dNTPase-deficient K312A. Since the Y315A mutant is defective for stalled fork resection, this observation suggests that this process participates in restraining fork progression under replication stress. We have shown that dNTP supplementation did not restore stalled fork processing in SAMHD1-depleted cells (Extended data Figure 5d). It would be informative to assess whether it does affect fork asymmetry in normal conditions to separate the two functions of SAMHD1.

It is interesting here to take a look at what happens in *Xenopus* egg extracts. Indeed, if SAMHD1 also associates with nascent chromatin in in this model organism (Extended data Figure 2e), its depletion does not affect DNA synthesis. Our collaborators have not measured dNTP pools before and after SAMHD1 depletion, but in this system replication substrates such as nucleotides are notoriously very abundant. In egg extracts activated for DNA replication, the dNTPase activity of SAMHD1 may either be inactive, or not sufficient to reduce dNTP pools to a level that affects DNA synthesis. In fact, addition of an excess of recombinant SAMHD1 to these extracts does reduce DNA replication (Hervé Técher, unpublished data), indicating that in basal conditions dNTPs are not limiting in this system. Besides, in *Xenopus*, SAMHD1 chiefly associates with nascent DNA upon treatment with aphidicolin. In conditions of replication stress, DNA synthesis is further inhibited following SAMHD1 depletion (Hervé Técher, unpublished data). Because dNTP pools do not seem to be limiting in this model organism, if SAMHD1 is involved in stalled fork resection in *Xenopus* as it is in human cells, this observation would confirm the importance of this process for fork progression.

II. SAMHD1 prevents cell-autonomous type I IFN production

While the involvement of SAMHD1 in the regulation of inflammation was inferred from its role in AGS, the ligand activating type I IFN production in SAMHD1-mutated cells was unknown. A previous study reported that chronic DNA damage signalling, induced by increased and imbalanced dNTP pools, was responsible for type I IFN activation (Kretschmer et al., 2015). How genome instability would activate the IFN response was, however, unclear. Given the primary role of chronic inflammation in AGS and tumor development, identifying this causative agent was important. We found that SAMHD1 prevents the generation of cytosolic DNA (Figure 1a, b). In its absence, cytosolic DNA fragments activate the cGAS-STING pathway, thereby inducing the production of Type I IFNs (Figure 1c, d, f). Importantly, these fragments are released from

arrested replication forks, particularly when cells are treated with the replication stress inducer HU (Figure 1e).

1. Mechanism of DNA fragments release

An intriguing aspect of our results was the generation of DNA fragments in the absence of SAMHD1, which we found was dependent on MRE11 (Figure 4-a). Indeed, if SAMHD1 stimulates MRE11 to promote fork processing, how is it possible that the generation of cytosolic DNA in the absence of SAMHD1 relies on the very same nuclease? Our collaborators found that SAMHD1 stimulates the exonuclease activity of MRE11 *in vitro*. SAMHD1 may act as a molecular switch, guiding MRE11 toward an exonucleolytic activity rather than its endonuclease function. It would be important to address the effect of SAMHD1 on the endonuclease activity of MRE11 which could be done using for instance a flapped DNA substrate in an *in vitro* nuclease assay. To evaluate the involvement of the endonuclease activity of MRE11 in the generation of DNA fragments, specific inhibitors such as PFM03 could be used in cytosolic DNA experiments (Shibata, Moiani, Arvai, & Perry, 2014). Our results indicate that DNA2, whose endonuclease activity is not stimulated by SAMHD1 (Extended data Figure 7c), and CtIP are also implicated in this process, albeit to a lesser extent than MRE11 (Figure 4a). Incidentally, these enzymes are all known to function at stalled replication forks (Lemaçon et al., 2017; Thangavel et al., 2015). It is possible then that in the absence of SAMHD1, the endonuclease activity of MRE11, DNA2 and CtIP is unleashed. Other flap-endonucleases may as well play a role in this process, including FAN1, MUS81/EME1, METNASE, EEPD1. Interestingly, they have all been involved, directly or not, in the processing of stalled replication forks. It would be important to assess their involvement in the generation of DNA fragments in SAMHD1-depleted cells and to further delineate how they coordinate.

Although we do not see nascent DNA degradation in SAMHD1-depleted cells in classic DNA spreading experiments, DNA fragments arising from replication forks are exported in the cytosol upon replication stress. The timing of both experiments is different. When we analyse fork processing by DNA spreading, nascent DNA is labelled with IdU then we treat cells 2 hours with HU together with a pulse of CldU and we directly measure IdU track length. Conversely, when we assess the generation of cytosolic DNA, cells are treated with HU for two hours, then incubated for an additional two hours in fresh media. During this extent of time, forks may have restarted, and DNA fragments cleaved from further away, behind forks, as illustrated in the figure 32.

To test this hypothesis, we designed a specific labelling protocol whereby nascent DNA is first labelled with IdU, then we treat cells with HU together with a pulse of CldU and we chase this labelling with an excess of thymidine.

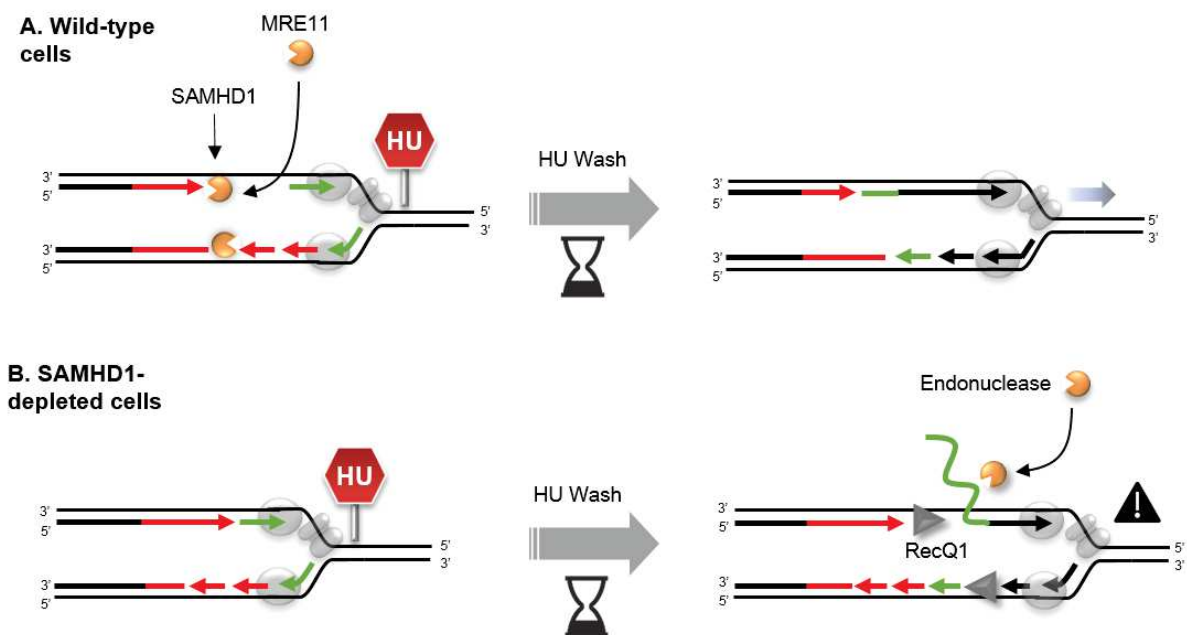


Figure 32 A model for the generation of DNA fragments in SAMHD1-depleted cells. Nascent DNA is successively labelled with IdU (red) and CldU (green). During the CldU labelling, cells are treated with HU, triggering fork arrest. (A) In wild-type cells, nascent DNA is degraded by MRE11-dependent processes, allowing for a timely restart of the replication forks upon HU removal. (B) When SAMHD1 is depleted, arrested forks are not properly resected by MRE11. Upon HU removal, forks may slowly restart and DNA fragments been cleaved behind forks by the concerted activity of RecQ1 and endonucleases.

We used this labelling scheme for immunofluorescence and DNA spreading experiments: the former, to analyse cytosolic DNA; the latter to measure fork processing. In accordance with our model, Cytosolic IdU and CldU levels both start to rise 30 minutes into the thymidine chase, with CldU peaking 60 minutes into the chase and IdU levels still increasing after 2 hours (Extended data Figure 10a, b). Regarding DNA fibres, CldU tracks length start to decrease after 30 minutes of thymidine chase, indicating that DNA is degraded at that moment. If RecQ1 is depleted in SAMHD1-deficient cells, this effect is abrogated (see Figure 36 in the Appendix), thereby confirming the importance of RecQ1 in the generation of DNA fragments.

Forks do not restart as efficiently in SAMHD1-depleted cells as in control cells under camptothecin treatment. It may also be true when cells are exposed to HU. If so, and considering the results described above, it would mean that arrested forks are processed in SAMHD1-depleted cells, albeit in a slow process implicating RECQ1-dependent generation of DNA fragments.

How these pieces of DNA diffuse to the cytosol is currently unknown. RPA and RAD51 prevents the release of nuclear oligonucleotides to the cytoplasm (Wolf et al., 2016). Because these proteins also are involved in DNA replication, it was difficult to study their implication in the release of DNA fragments generated at arrested forks. To assess their involvement in this process, it would be interesting to see if over-expressing RPA and RAD51 prevents the generation of cytosolic DNA in SAMHD1-depleted cells.

2. Involvement of the cGAS-STING pathway of type I IFN production

a) Technical limitations

To evaluate type I IFN production, we used two different kind of assays: the measurement of IFNs, TNF and ISGs mRNA production by qRT-PCR and a type I IFN-induced luciferase assay with HL116 reporter cells that contain the firefly luciferase gene under the control of a type I IFN-inducible promoter (known as a 6- 16 promoter). Both methods have limitations: gene expression analysis by qRT-PCR only reflects the quantity of a given mRNA at a given time-point, and the HL116 reporter assay only indirectly evaluates the presence of type I IFN in given culture media. These two methods did not tell the exact quantity at protein levels induced by SAMHD1 depletion.

To go further and assess if this transcriptional response translates into more IFN and ISG at the protein level, we could for example evaluate ISG15 protein expression by western-blotting as done by many laboratories (see for instance Erdal, Haider, Rehwinkel, Harris, & McHugh, 2017; Maelfait, Bridgeman, Benlahrech, Cursi, & Rehwinkel, 2016). We could also directly measure IFN titres in the culture media of SAMHD1-deficient cells using ELISA kits, providing that the assay is sensitive enough. Indeed, we do not know the absolute amount of IFNs overexpressed upon

SAMHD1 depletion as we always compared SAMHD1-depleted cells relative to control cells. This issue may easily be addressed using defined amounts of IFN- α to draw a calibration curve in the HL116 reporter assay, which would allow us to absolutely quantify the amount of IFN in each culture media. Interestingly, it was recently demonstrated that SAMHD1 knock-out in human monocytic THP-1 cells does not affect the response to type I IFNs and that spontaneous production of ISGs elicited by SAMHD1 deficiency is caused by overproduction of type I IFNs (Martinez-Lopez et al., 2018).

This work mainly focuses on the proof of concept that defective stalled fork processing can induce interferon production. Our findings could surely be further extended to the biological impacts of the IFNs production due to SAMHD1 depletion-induced forks processing defect. For instance, we could perform an IFN antiviral assay using peripheral blood mononuclear cells incubated with culture media from SAMHD1-depleted cells treated or not with HU and infected with HIV-1. In addition, while SAMHD1-knock out mice have little or no inflammatory phenotype (Behrendt et al., 2013; Rehwinkel et al., 2013), in light with our study it would be interesting to assess if a mild replication stress does induce an IFN response in this model.

b) Contribution of a STING-independent pathway?

Some of the experiments performed to study the pro-inflammatory response triggered by SAMHD1 deficiency were done in HEK293T and HeLa cells, which may be surprising since the cGAS-STING IFN pathway may be altered in these cell lines. Indeed, viral oncogenes expressed in HEK293 (and, accordingly, in HEK293T) and HeLa cells inhibit STING (Lau, Gray, Brunette, & Stetson, 2015). Besides, STING expression itself is very low in HEK293T cells (Burdette et al., 2011; J. Wu et al., 2013). Many reports however now show that the cGAS-STING pathway is active in HeLa cells. For instance, the production of IFN- β induced by TREX1 depletion in HeLa cells is dependent on STING (Wolf et al., 2016).

We used STING and cGAS-knock out HeLa cells (Langereis, Rabouw, Holwerda, Visser, & van Kuppeveld, 2015) to evaluate the dependency on this pathway for the SAMHD1-mediated pro-inflammatory response. Transcription of pro-inflammatory genes decreased from 40% to 90% upon cGAS and STING knock out, depending on the gene considered. Overexpression of some pro-inflammatory genes, like ISG15, was largely dependent on cGAS and STING, while others like Mx1 was not. In HeLa cells, ISG15 transcription induced by SAMHD1 depletion actually correlated with STING proteins levels (compare Fig. 1f with Extended data Fig 1l where STING expression was almost fully ablated by CRISPR-Cas9 or incompletely by siRNA). The dependency on STING could easily be confirmed using small molecules inhibitors (Haag et al., 2018).

The partial loss of the IFN response upon STING depletion in the various models tested suggests the additional involvement of a STING-independent pathway in this phenotype. We can

hypothesize for instance that Mx1 gene transcription might be controlled by this pathway, which would explain why STING depletion does not fully abrogate its overexpression in SAMHD1-depleted cells. Interestingly, it has recently been demonstrated that ionizing radiation induces a type I IFN response not only dependent on STING but also on an independent pathway regulated by IRF1 (Purbey et al., 2017). As IRF3-depletion does not fully abrogate the basal IFN response induced by SAMHD1 deficiency (Extended data Fig 1l), it would be interesting to assay if depleting both IRF1 and IRF3 does. Further, if IRF1 does regulate this pro-inflammatory response, it would mean that a STING-independent but IRF1-dependent pathway of type I IFN production contributing greatly to the inflammatory phenotype of SAMHD1-depleted cells is yet to be discovered.

That cGAS depletion alone is not sufficient to abrogate pro-inflammatory signaling is not surprising: other DNA sensors such as DNA-PK for instance might compensate for its absence.

In this work, we demonstrated that cGAS is involved in the induction of IFN following SAMHD1 depletion. However, we could not distinguish whether the involvement of cGAS is direct or indirect. We could verify if SAMHD1 depletion can directly activate cGAS, leading to type I IFN production, through the quantification of cGAMP abundance in SAMHD1-depleted cells (J. Wu et al., 2013).

3. Implications in disease

a) Aicardi-Goutières syndrome

Pathogenesis

In this study, we reported using various assays that depletion of SAMHD1 triggers the production of pro-inflammatory molecules, such as type I IFNs and ISGs, in conditions of replication stress.

As previously mentioned, most of the experiments presented in this manuscript were performed in HEK293T and HeLa cell lines, both transformed by viral oncogenes. One could argue that these cell lines may not be relevant in the context of AGS since transformation may modify the DNA replication process. In addition, viral transformation may also perturb IFN signaling (Lau et al., 2015). This choice was motivated by our interest in the role of SAMHD1 in fork resection, a process that we were not able to detect in non-transformed MCF10A cells (see Appendix Figure 37 A.). It also made sense considering that we were interested in the role of SAMHD1 in cancer. We nonetheless did show that SAMHD1 contributes to fork resection in immortalized B cells from an AGS patient with a SAMHD1 Q548X mutation (Extended data Fig 5i). To assess the physiological relevance of our results, we could go further and test if MRE11 or RecQ1 inhibition reduces the pro-inflammatory phenotype of *samhd1* morphant zebrafish (Kasher et al., 2015).

In addition, we highlight the role of DNA replication stress in the release of type I IFNs in SAMHD1-deficient cells. In the context of AGS however, some patients, including patients with SAMHD1 mutations, display features of the disease already at birth, meaning that chronic exposure to type I IFNs occurred *in utero*. In other words, our results imply that SAMHD1-deficient cells are exposed to replication stress early-on during development, something that actually occurs in rapidly dividing embryonic stem cells (Ahuja et al., 2016), at least in mice.

HU was used to exacerbate replication stress, so that we were able to analyse the responses to fork arrest. Although we could detect interferon production and cytosolic DNA in SAMHD1-depleted cells in basal conditions, it was not possible to directly study the processing of arrested replication forks without using HU. Yet, as reported in the second chapter of the introduction, replication stress is thought to occur during every cell cycle. If SAMHD1 is not active, some replication forks thus potentially arrest during every S phase, becoming substrates for RECQ1-mediated aberrant processing. It is difficult to study the responses to endogenous replication stress, but the analysis of cytosolic DNA would be an indirect way of showing it. It would be interesting to look at the spontaneous generation of cytosolic DNA in stem cells derived from SAMHD1-null mouse that overexpress type I IFNs (Behrendt et al., 2013) or, better, in cells derived from SAMHD1-depleted zebrafish (Kasher et al., 2015). Pulse-labelling ongoing DNA synthesis with thymidine analogues as we have done in this study would indicate if these DNA fragments originate from replication forks.

These experiments, if performed without the addition of exogenous inducers of replication stress, would validate this hypothesis *in vivo* and give insights into replication stress in stem cells. A thorough study of endogenous replication stress, however, may be difficult to perform in SAMHD1-deficient cells because loss of SAMHD1 by itself slows-down the progression of replication forks.

Therapy

Our study not only improves our knowledge of the replication stress response and the development of AGS caused by inherited SAMDH1 mutations, but it also has therapeutic implications for this subset of AGS patients. Indeed, as mentioned in the introduction, their options are for now very limited. 20% of SAMHD1-mutated AGS patients present with symptoms of the disease already at birth. Most of them therefore develop the disease during the first year of life or after, thereby leaving a window of opportunity for therapeutic intervention. With the knowledge generated from our study, it is now possible to envision targeting not merely the effects (inflammation) but also the cause of the disease (release of DNA in the cytosol).

Indeed, if cytosolic DNA increases in the aforementioned models compared to their wild-type counterparts, depleting or inhibiting RECQ1 with specific inhibitors should reduce their release

and the subsequent inflammatory response. This assumption may easily be tested in SAMHD1-depleted zebrafish for instance, since they display phenotypic features of the disease.

Another approach currently being investigated makes use of specific STING and cGAS inhibitors (Haag et al., 2018; Vincent et al., 2017). This strategy is more broadly usable in different subtypes of AGS, and even in other auto-immune disorders dependent on cGAS activity such as SLE (D. Gao et al., 2015). Both methods also have drawbacks: inhibiting RECQ1 may induce chromosomal instability (Sharma et al., 2007), while preventing STING and cGAS activity may render cells more susceptible to viral infections. Nonetheless, a better understanding of the processes leading to chronic inflammation in AGS and related interferonopathies should pave the way for the development of therapies.

b) Cancer

Tumorigenesis

Although replication stress favours loss of cells that are in the process of transformation following oncogene activation, it can also trigger genome instability and promote tumorigenesis (Bartkova et al., 2006; Macheret & Halazonetis, 2018). Much like replication stress, cytosolic DNA-induced inflammation is a double-edged sword in tissue homeostasis. On one hand, cytosolic nucleic acids act as danger signal, inducing immune surveillance of malignancies by promoting senescence upon diverse stimuli like oxidative stress, DNA damage and the activation of oncogenes such as Ras (Dou et al., 2017; Glück et al., 2017; H. Yang et al., 2017). On the other hand, chronic inflammation creates tissue damage and promotes carcinogenesis as well as metastasis (Ahn et al., 2014; Bakhoun et al., 2018). Of note, we can envision here the inflammatory response as an extension of the replication checkpoint, which would signal to the neighbouring cells aberrant transactions taking place at replication forks in any given cell.

In particular, a large body of evidence now shows that the DNA damage response and the immune signaling network cooperate to maintain homeostasis (for review: Nakad & Schumacher, 2016). Persistent DNA damage induces chronic inflammation (Brzostek-Racine et al., 2011; Erdal et al., 2017; Härtlova et al., 2015; Karakasilioti et al., 2013; Turaga et al., 2009). Conversely, inflammatory signaling can in turn damage genomic DNA. This is best illustrated by virus-induced cancers: some viruses, like Hepatitis B/C viruses and Epstein-Barr virus are considered as carcinogenic to humans by the International Agency for Research on Cancer. For instance, the most common liver cancer is associated with chronic liver inflammation induced by either Hepatitis B or Hepatitis C viruses (Perz, Armstrong, Farrington, Hutin, & Bell, 2006). Inflammation generates DNA damage through distinct mechanisms including the upregulation of cell proliferation as well as the production of mutagenic ROS and reactive nitrogen species (for

review: Kawanishi, Ohnishi, Ma, Hiraku, & Murata, 2017). Such oxidative stress directly damages genomic DNA and may also inhibit DNA repair, thereby promoting genome instability.

We showed that loss of SAMHD1 induces cell-autonomous production of type I interferons and other pro-inflammatory cytokines. SAMHD1 mutation is associated with tumor development, including different types of leukemia (Clifford et al., 2014; Johansson et al., 2018), colon cancer (Rentoft et al., 2016), lung cancer (J. L. Wang, Lu, Shen, Wu, & Zhao, 2014). Because cancer-associated SAMHD1 mutations reduce its dNTPase activity, dNTP pools may be altered in pre-cancerous lesions in which SAMHD1 is mutated. Imbalanced pools increase mutation rates, particularly in combination with deficiencies in the mismatch repair pathway (Rentoft et al., 2016). Alternatively, loss of SAMHD1 may also induce genome instability owing to its role in DNA repair (Daddacha et al., 2017). Our results provide additional mechanisms by which SAMHD1 mutations promotes oncogenesis. First, loss of SAMHD1 function induces DNA replication stress, which leads to genome instability and favors the loss of anticancer genes (Macheret & Halazonetis, 2015). Second, it triggers the production of cytokines that fuel inflammation.

Therapy

Cancer cells are immunogenic: upon expression of DAMPs or specific tumor -associated antigens, they may be recognized and eliminated by the immune system (for review: Woo, Corrales, & Gajewski, 2015). Among the mechanisms involved, tumor antigens are presented by dendritic cells in the tumor -draining lymph nodes to prime and stimulate cytotoxic T cells, which then infiltrate the tumor mass to mediate tumor rejection. The production of Type I IFNs by dendritic cells is necessary to activate this adaptive immune response (Diamond et al., 2011; Fuertes et al., 2011). In mice, spontaneous tumor rejection occurring via the priming of cytotoxic T cells requires a functional STING pathway in antigen-presenting cells, which is activated following uptake of tumor -derived DNA (S. R. Woo et al., 2014).

As a matter of fact, evading immune destruction is one of the hallmarks of cancer that allows tumor development through the bypass of immune surveillance (Hanahan & Weinberg, 2011). In some instances, spontaneous T-cell priming, immune infiltration of effector CD8+ T cells and type I IFNs are detected in the tumor microenvironment. Distinct mechanisms allow such tumor s to escape immune surveillance, including the expression of the T-cell negative regulators PD-(L)1 and CTLA-4 (Dong et al., 2002; Leach, Krummel, & Allison, 1996). Based on these observation, antibodies that target the PD-(L)1 and CTLA4 T-cell negative regulators have been designed and efficiently driven from the bench to bedside (for review, Ribas & Wolchok, 2018). They show promising efficacy in various cancers including melanoma and lung cancer, but all patients do not respond, often because their tumors are not immunogenic. Indeed, some tumor s evolve in a non-inflamed microenvironment devoid of T cells, caused by different factors, possibly including a

denser stroma (Hugo et al., 2016). Immune checkpoint antibodies are effective only in a fraction of patients, likely because their immune system is alert. It is therefore useful to stimulate anti-tumor T cell responses to enhance the efficacy of such therapies. In other words, cancer immunotherapy today is faced with the challenge of turning tumors from immune inert to immunogenic.

Radiotherapy and chemotherapeutic agents including doxorubicin, paclitaxel and oxaliplatin indeed potentiate checkpoint blockage in pre-clinical mice models (Deng, Liang, Xu, et al., 2014; Pfirschke et al., 2016; Twyman-Saint Victor et al., 2015). For instance, radiotherapy promotes T-cell priming by Dendritic Cells, a process also known as cross-presentation, through a mechanism dependent on the production of type I IFNs (Burnette et al., 2011). This process requires the detection of DNA from irradiated-tumor cells by cGAS in host dendritic cells, and the subsequent activation of the STING pathway (Deng, Liang, Xu, et al., 2014).

Although type I IFNs are thought to be primarily produced by innate immune cells, evidence now suggests that malignant cells themselves can also be a relevant source of inflammatory signals (Linsley, Speake, Whalen, & Chaussabel, 2014; Sistigu et al., 2014). In the context of radiotherapy, it has recently been demonstrated that the generation of cytosolic DNA in tumor cells and the ensuing cell-intrinsic production of type I IFNs mediates the immune response to irradiation (Vanpouille-Box et al., 2017).

Pioneer work from the laboratory of Stephan Gasser suggested that replication stress may induce immune tumor rejection (Ho et al., 2016). They demonstrated that type I and II IFN production induced by MUS81-dependent generation of cytosolic DNA was critical for tumor immune rejection mediated by macrophages and T cells. More specifically, MUS81 activity enhanced the ability of Dendritic Cells to activate specialised T cells, much like what was observed for radiotherapy (Burnette et al., 2011).

Since we demonstrated that an aberrant processing of stalled replication forks triggers a cell-autonomous production of type I IFNs, replication stress may be envisioned as a way to potentiate the effects of a targeted immune therapy such as checkpoint antibodies (Figure 33). Indeed, we can hypothesize that the type I IFN response triggered by the release of DNA fragments from arrested forks may enhance cancer-antigen cross presentation, as does MUS81 activity (Ho et al., 2016). According to the current extent of our knowledge, we could think that this strategy may only be useful in tumor cells defective in the fork resection pathway such as SAMHD1-mutated tumors. In fact, this therapeutic strategy may be amenable to a broader range of tumors. MEFs shed replication-derived DNA fragments into the cytosol, that are degraded by Trex1 (Y. G. Yang et al., 2007). It is possible that this process occurs in some tumors as well, such as what has been demonstrated for prostate cancer cells (Ho et al., 2016). In the future, a deeper understanding of

this process may allow the development of specific inhibitors which could mimic the lack of fork processing and may allow for the identification of tumors devoid of this pathway.

Using a replication-stress-dependent response rather than agents that directly generate DNA damage as a way to trigger interferon production would be beneficial for the patient. Indeed, not only malignant cells, but also highly proliferative tissues like the bone marrow are vulnerable to damaging agents such as radiotherapy, thereby inducing toxicities. Targeting the replication stress response, in contrast, may offer a larger window of opportunity by specifically targeting replication-addicted cancer cells (For review: Forment & O'Connor, 2018).

An acute boost of interferon production triggered by the induction of replication stress in fork processing-deficient cells may be a new way to enhance immune responses and potentiate the effects of anti-immune checkpoint antibodies. Activation of the inflammatory response would have to be transient however as chronic inflammation, including chronic Type I IFNs production, may promote tumour escape from immune surveillance. For instance, chronic IFN-I induced as a consequence of combination radiation therapy with anti-CTLA4 drive tumor cell resistance to adaptive immunity, at least in part, by increasing PD-L1 expression (Benci et al., 2016). Accordingly, increasing inflammation in the tumor microenvironment to favour immunogenicity may support the development of checkpoint-antibodies-resistant malignant cells. Associating multiple antibodies may provide a better tumour response.

Altogether, our study uncovered a new interplay between DNA replication and the inflammatory response, which have broad implications for AGS and for cancer therapy considering the wide range of drugs inducing replication stress that used today in the clinic.

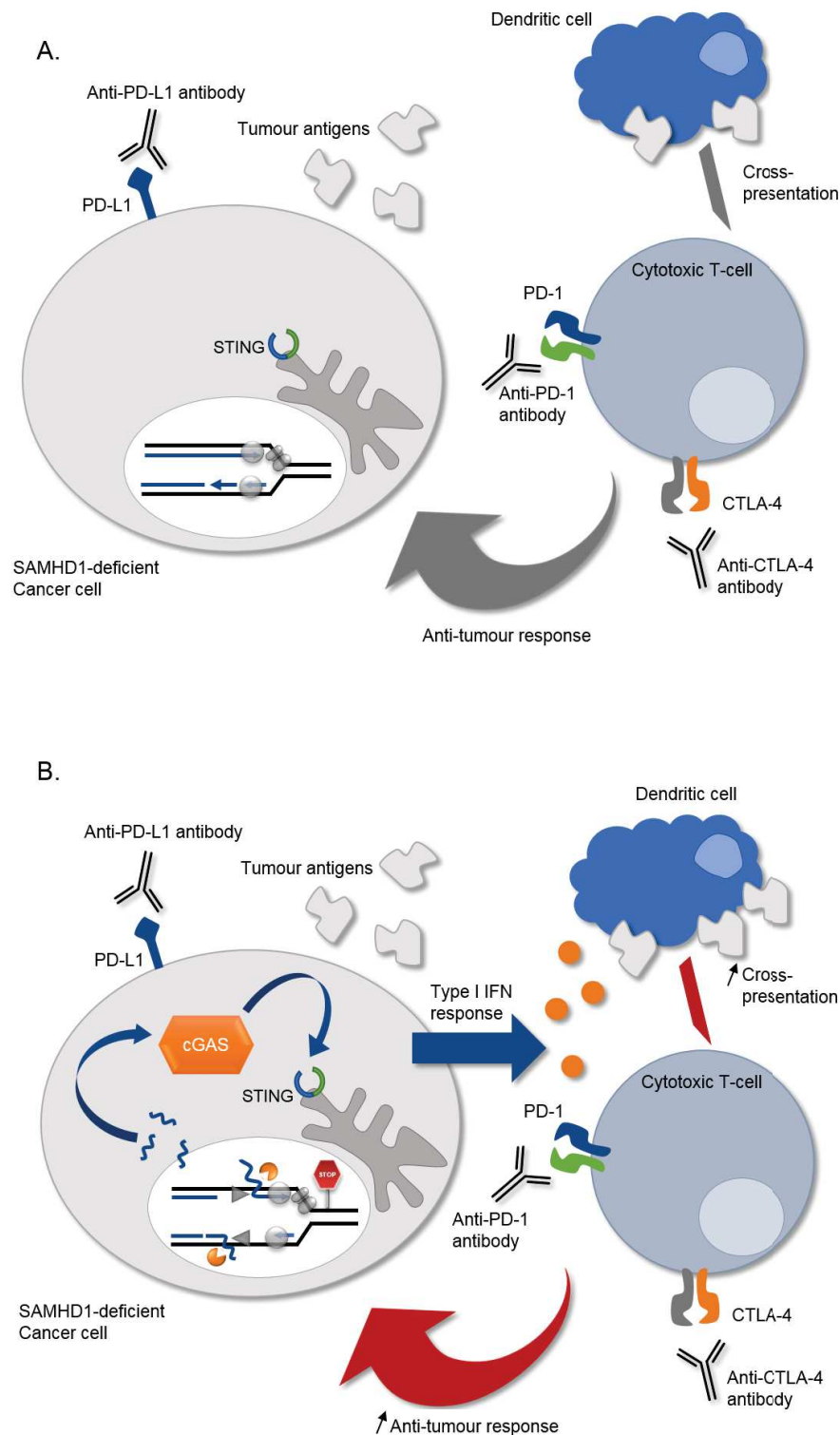


Figure 33 Replication-stress-induced production of type I IFN potentiates immune checkpoint therapy. (A) Specific antibodies are used to block negative regulators of immune responses to cancer cells, namely CTLA-4, PD-1 and its ligand PDL-1. (B) Type I IFNs potentiate immune anti-tumor responses through different mechanisms including increasing cross presentation of tumor antigens by dendritic cells. Inducing replication stress in cancer cells with defects in stalled fork processing may increase cell-autonomous production of type I IFNs and anti-tumor responses.

| Appendix

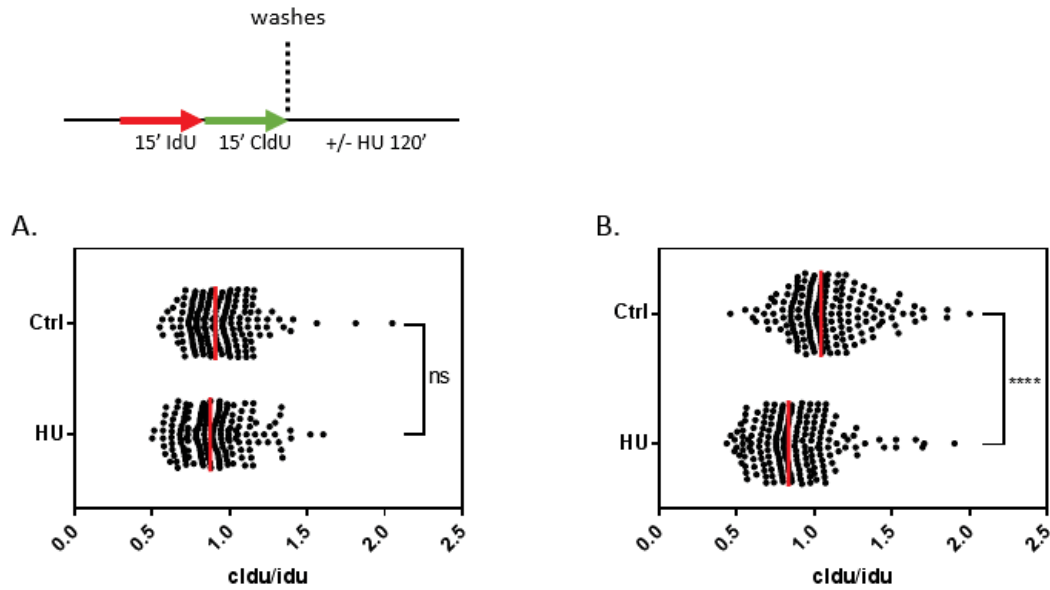


Figure 34 Stalled fork resection in non-transformed MCF10-A cells and in breast MCF-7 cancer cells. A. MCF10-A cells were labelled with IdU and CldU and subsequently treated with 4 mM HU for 120 minutes as indicated in the above scheme. B. MCF-7 cells were labelled with IdU and CldU and subsequently treated with 4 mM HU for 120 minutes as indicated in the above scheme. Median CldU to IdU track lengths ratios ($n \geq 170$) are indicated in red. **** $P < 0.0001$, Mann-Whitney test.

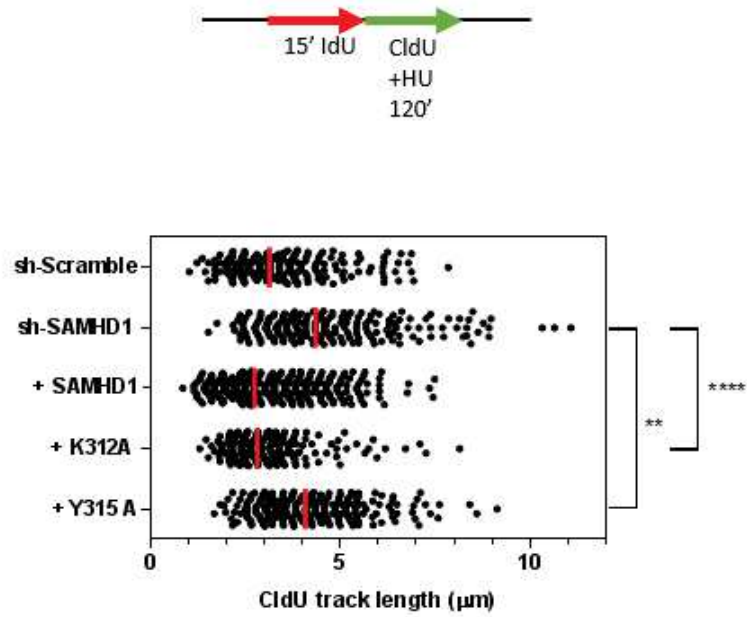


Figure 35 Fork progression during nucleotide depletion. *Sh-Scramble cells, sh-SAMHD1 cells and sh-SAMHD1 cells expressing wild-type, K312A or Y315A SAMHD1 were labelled with IdU and CldU in the presence of 4 mM HU as indicated in the above scheme. Median CldU track lengths ($n \geq 200$) are indicated in red. **** $P < 0.0001$, Mann-Whitney test.*

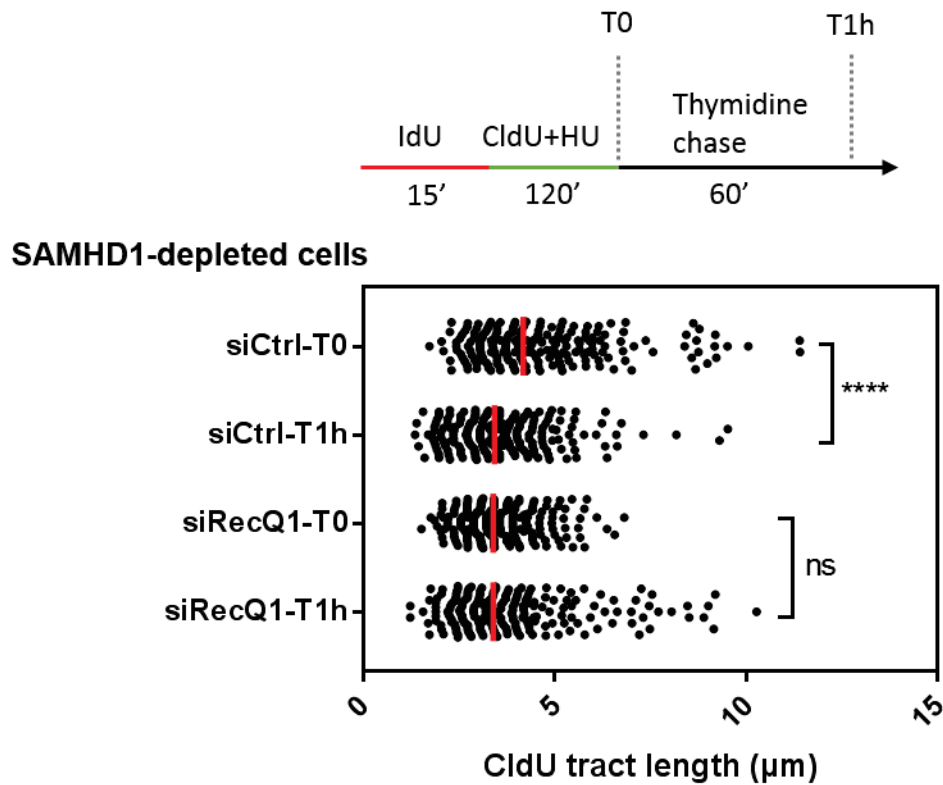


Figure 36 Fork processing in SAMHD1-depleted cells. SAMHD1-depleted HEK293T cells were transfected with siRNAs against RecQ1 or with a siCtrl for 48h. They were then labelled with IdU and CldU in the presence of 4 mM HU for 120 minutes, before a thymidine chase for 60 minutes as indicated in the above scheme. Median CldU track lengths measured at the end of the CldU+HU treatment or after the thymidine chase ($n \geq 210$) are indicated in red. **** $P < 0.0001$, Mann-Whitney test.

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