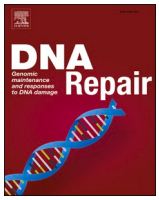
DNA Repair 142 (2024) 103758

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair



Tolerating DNA damage by repriming: Gap filling in the spotlight

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ARTICLE INFO

Keywords:
DNA replication stress
DNA damage tolerance
Repriming
Post-replicative gaps
ssDNA gaps
Gap filling
Post-replication repair

ABSTRACT

Timely and accurate DNA replication is critical for safeguarding genome integrity and ensuring cell viability. Yet, this process is challenged by DNA damage blocking the progression of the replication machinery. To counteract replication fork stalling, evolutionary conserved DNA damage tolerance (DDT) mechanisms promote DNA damage bypass and fork movement. One of these mechanisms involves "skipping" DNA damage through repriming downstream of the lesion, leaving single-stranded DNA (ssDNA) gaps behind the advancing forks (also known as post-replicative gaps). In vertebrates, repriming in damaged leading templates is proposed to be mainly promoted by the primase and polymerase PRIMPOL. In this review, we discuss recent advances towards our understanding of the physiological and pathological conditions leading to repriming activation in human models, revealing a regulatory network of PRIMPOL activity. Upon repriming by PRIMPOL, post-replicative gaps formed can be filled-in by the DDT mechanisms translesion synthesis and template switching. We discuss novel findings on how these mechanisms are regulated and coordinated in time to promote gap filling. Finally, we discuss how defective gap filling and aberrant gap expansion by nucleases underlie the cytotoxicity associated with post-replicative gap accumulation. Our increasing knowledge of this repriming mechanism – from gap formation to gap filling – is revealing that targeting the last step of this pathway is a promising approach to exploit post-replicative gaps in anti-cancer therapeutic strategies.

1. Introduction

Genome duplication is often challenged by obstacles such as DNA damage arising from endogenous or exogenous sources, threatening genome stability and cell survival. DNA damage can lead to replication stress, broadly defined as the slowing or stalling of replication fork progression and interference with DNA replication [1]. One frequent molecular feature of replication stress is the formation of single-stranded DNA (ssDNA) stretches at the replication fork (Fig. 1). This ssDNA can form when the replicative polymerase stalls and becomes uncoupled from the helicase that continues unwinding the DNA double helix [1]. Replication stress can be offset by evolutionary conserved mechanisms of DNA damage tolerance (DDT) that enable replication across damaged DNA (reviewed in [2]). DDT include translesion DNA synthesis (TLS) and template switching (TS). TLS is mediated by specialized low fidelity polymerases whose large catalytic domain can accommodate and replicate damaged DNA, usually at the expense of replication errors [3,4]. During TS, the homologous nascent DNA on the undamaged sister chromatid serves as a template for replication in a mostly error-free reaction [5]. One model of TS is fork reversal in which the two daughter strands anneal and form a four-way junction, thereby avoiding collision with the damage ahead of the replication fork [6]. Alternatively, DNA damage can be "skipped" through *de novo* primer synthesis – or repriming – past the lesion. This lesion-skipping mechanism ensures timely genome duplication by keeping DNA synthesis coupled to replication fork movement, while limiting polymerase-helicase uncoupling and the extent of ssDNA regions at replication forks [7]. Nevertheless repriming comes at the price of generating post-replicative single--stranded DNA (ssDNA) gaps, which are ultimately filled-in separable from genome duplication [8] (Fig. 1).

The first findings on post-replicative gap formation upon DNA damage induction, as well as their repair, were reported more than 50 years ago [9,10] (reviewed in [11]). However, the underlying mechanism in higher order organisms remained poorly understood. Excitingly, the identification of the DNA directed polymerase and primase PRIMPOL in 2013 [12–15] opened new avenues for our understanding of the

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https://doi.org/10.1016/j.dnarep.2024.103758
Received 17 May 2024; Received in revised form 14 August 2024; Accepted 25 August 2024
Available online 30 August 2024

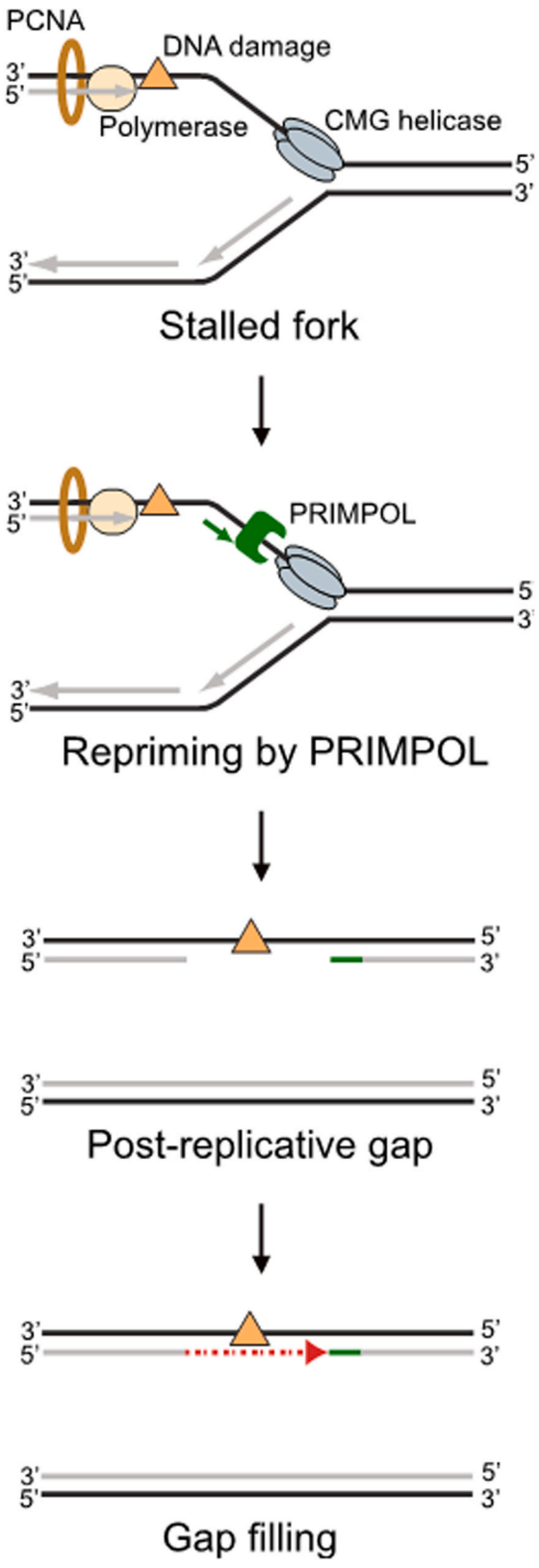

Fig. 1. DNA damage tolerance by repriming. During repriming, replication forks stalled by DNA damage (yellow triangle) restart by "skipping" the blocking DNA damage through synthesis of a short *de novo* primer (green arrow) downstream of the lesion by PRIMPOL (in green). This repriming activity leaves DNA damage at single-stranded DNA (ssDNA) gaps behind advancing forks (post-replicative gaps). Next, gap-filling mechanisms ensure DNA synthesis across DNA damage (red dashed arrow) and post-replication gap repair.

repriming mechanism in vertebrates. After the canonical POLα-primase complex, PRIMPOL is the second primase-polymerase and the first DNA primase characterized in vertebrate cells. Importantly, POLα-primase has limited priming efficiency on leading strand templates [16], supporting the evolutionary need for PRIMPOL in their restart upon DNA damage. The discovery of PRIMPOL was accompanied by the establishment of novel protocols to assess the ensuing post-replicative gaps in human cells, complementing the approaches used in seminal studies such as sucrose gradient sedimentation and transmission electron microscopy (TEM) [9,10,17,18]. These new methods include the accessible and now widely used S1 nuclease modified DNA fiber assay [19–22]. In this genome-wide single-molecule microscopy assay, post-replicative ssDNA gaps, the size of which fall below the resolution limit of the DNA fiber assay, are cleaved by the ssDNA-specific S1 endonuclease, generating shorter fibers that are used as a read-out for the presence of gaps behind ongoing replication forks [19,23].

The past 5–10 years have seen a surge on studies on post-replicative ssDNA gaps, providing novel insights into the repriming mechanism in human models. Moreover, recent studies have implicated TS and TLS in the fill-in of PRIMPOL-mediated gaps and provided insights into factors dictating the choice between them in this reaction. Here, we discuss recent reports on repriming by PRIMPOL in human models, and provide an integrated view of this mechanism, from post-replicative gap formation to gap filling (Fig. 1). We also highlight the biological consequences of activating repriming, and discuss how defects in gap-filling mechanisms underlie gap toxicity.

2. Sources of PRIMPOL-dependent post-replicative gaps

2.1. Replication fork-stalling structures

In wild-type human cells, repriming is activated in response to a broad range of structures perturbing replication fork progression (reviewed in [24]). In particular, studies employing primase-dead PRIMPOL mutants and/or the S1 DNA fiber assay to assess repriming-generated gaps revealed that PRIMPOL can reprime across bulky DNA damage including intra-strand crosslinks induced by ultraviolet radiation C (UV-C) and benzo[*a*]pyrene-diol-epoxide (BPDE) [12,25], as well as alkylation damage caused by methylmethane sulfonate (MMS) [26,27]. In addition to DNA damage, PRIMPOL is able to reprime after chain-terminating nucleoside analogs as well as secondary structures such as G quadruplexes in avian cells [28,29]. Of note, human PRIMPOL contains properties of a TLS polymerase across several replication-fork stalling structures such as G quadruplexes, 8-oxodG and abasic (AP) sites *in vitro*, although the *in vivo* importance of PRIMPOL TLS activity remains to be defined (reviewed in [30]). Interestingly, repriming by PRIMPOL also facilitates the bypass of inter-strand crosslinks (ICL) [31]. ICL is a cytotoxic lesion considered "an absolute block" as it blocks the progression of replicative polymerases and also the CMG helicase. In 2013, this notion was challenged as ICLs were shown to be bypassed by replication traverse [32]. During replication traverse, the DNA translocase FANCM/MHF promotes translocation of the replisome across the ICL in a yet-to-be fully elucidated cascade of molecular events, facilitating restart of DNA synthesis past the ICL [32,33] (reviewed in [34]). More recently, PRIMPOL has been implicated in this process through its repriming activity [31]. Specifically, PRIMPOL primase ensures DNA synthesis restart downstream the ICLs during replication traverse. ICLs are induced by chemicals like mitomycin C and cisplatin as well as by endogenous aldehydes, pointing to a role of PRIMPOL in DNA replication of untreated cells.

Moreover, recent reports have revealed small base modifications as emerging triggers of repriming. For example, expression of APOBEC3A

(A3A) in human cells induces the accumulation of PRIMPOL-dependent gaps [35,36]. A3A is a cytidine deaminase prevalent in human cancers that catalyzes the conversion of cytidine to uracil. Mechanistically, A3A-induced post-replicative gaps are dependent on AP sites generated by UNG, the primary glycosylase removing genomic uracil [35]. In principle, small base lesions are not DNA double helix-distortive enough to stall replication forks, but their processing by DNA glycosylases generates AP sites which are potent blockers for replication [37,38]. The model that emerges is that UNG processing of A3A-triggered uracil gives rise to AP sites that are bypassed at stalled forks by pathways such as repriming [35,37]. Interestingly, in untreated BRCA1-deficient cells, the glycosylase SMUG1 also triggers the accumulation of PRIMPOL-mediated post-replicative gaps [39]. Contrary to UNG, SMUG1 excises uracil derivatives originating from base oxidation such as 5-hydroxymethyl-uracil (5-hmU) or 5-formyluracil [39], raising the possibility that BRCA1-deficient cells accumulate more oxidized damage than wild-type cells. Moreover, as discussed below, cells lacking functional BRCA1 might be unable to fill-in the post-replicative gaps formed upon repriming beyond the AP sites generated by SMUG1 activity, thereby accumulating these gaps. Collectively, these studies unravel that AP sites originating from different base lesions might culminate in the activation of repriming, even in the absence of exogenous stress. Strikingly, loss of UNG also promotes the accumulation of PRIMPOL-dependent gaps [40]. In this context, genomic uracil accumulates to high levels in the absence of UNG, stalls replication forks without being processed into AP sites, and are bypassed by repriming [40]. Hence, both the physiological processing of genomic uracil into AP sites and the pathological accumulation of genomic uracil trigger repriming by PRIMPOL. In summary, the studies discussed in this section point to the primary engagement of repriming by PRIMPOL in the response to replication fork stalling induced by a broad range of DNA modifications, including ones arising endogenously.

2.2. Alterations in the equilibrium between repriming, fork reversal and TLS at stalled replication forks

Recent data point to a remarkable plasticity and adaptation capacity of replication forks during DDT [2,41]. Specifically, a growing body of evidence suggests that repriming is pathologically activated upon dysregulation of its balance with replication fork reversal or "on-the-fly" TLS at stalled replication forks. In this section, we will discuss the scenarios favoring the activation of repriming.

2.2.1. Fork reversal impairment and PRIMPOL upregulation

Replication fork reversal is a protective mechanism activated by different members of the SNF2 translocase family, including SMARCAL1, ZRANB3, and HTLF, that are capable of converting a three-way junction DNA replication fork into a four-way junction reversed fork [42]. Fork reversal is also facilitated by the central recombinase RAD51 [43]. Loss of fork reversal factors RAD51, SMARCAL1 and HLTF promotes PRIMPOL-mediated repriming to deal with stress caused by UV-C, cisplatin and hydroxyurea (HU) [44–46]. Similar results were recently reported for HU-treated cells lacking CSB (Cockayne syndrome B), another member of the SNF2 family with *in vitro* fork reversal activity [47,48]. These findings suggest that loss of one fork reversal factor is enough to trigger repriming by PRIMPOL. Along the same lines, PARP1 was described as a regulator of fork reversal [49] and its inhibition shifts the balance towards PRIMPOL-dependent repriming [45,50,51]. Moreover, PRIMPOL upregulation -either by exogenous over-expression or through an adaptive response to multiple rounds of genotoxic treatments promotes PRIMPOL-dependent post-replicative gap accumulation, while suppressing fork reversal [45,52,53]. Based on these findings, the model that emerges is that repriming and fork reversal are in a competition at stressed replication forks whereby fork reversal is primarily engaged, and repriming is activated when forks cannot reverse [2,41]. Challenging this notion, fork reversal and repriming cooperate in the clearance of re-duplicated DNA [54] and are both active at replication forks upon A3A expression [35]. Of note, on-the-fly TLS was shown to be inhibited by PRIMPOL activity at replication forks stalled by AP sites formed upon A3A-triggered uracil processing [55]. These observations raise the possibility of a collaboration between fork reversal and repriming at stalled forks during the replication stress response. In addition, the nature of the damage, as well as its load might modulate the choice between reversal and repriming at stalled replication forks [2]. For example, in BRCA1-deficient cells, high HU doses promote fork reversal, while low HU doses activate repriming (reviewed in [2]). The underlying mechanisms are unknown and whether this is the case also in wild-type background remains to be tested.

2.2.2. Dysfunctional restriction of PRIMPOL and untimely repriming

The regulators of the balance between replication fork reversal and repriming are currently emerging. Strikingly, the primary role of these regulators is to inhibit PRIMPOL recruitment to damaged replication forks. When these regulators are dysfunctional, PRIMPOL is aberrantly recruited to chromatin and generate post-replicative gaps. For example, BRCA2 interacts with MCM10, a key DNA replication factor, and prevents PRIMPOL recruitment to chromatin upon cell exposure to different genotoxic treatments [56]. These findings provide one of the mechanistic bases underlying post-replicative gap accumulation in BRCA-deficient cells [57,58]. The exact molecular mechanism on how BRCA2-MCM10 inhibits PRIMPOL-dependent repriming remains unclear. Given its large size, BRCA2 could directly block PRIMPOL recruitment by physical competition at stalled forks. Alternatively, BRCA2 could indirectly prevent PRIMPOL loading on chromatin by displacing RPA (replication protein A), the ssDNA-binding protein that facilitates PRIMPOL recruitment to ssDNA at stalled forks by protein-protein interaction [56,59]. Along the same lines, the CST (CTC1-STN1-TEN1) complex restricts PRIMPOL recruitment to replication forks after exposure to high UV-C dose [60]. The CST complex is a RPA-like complex whose canonical function is to promote telomeric maintenance. Loss of CTC1 or STN1 induces PRIMPOL loading to forks triggering post-replicative gaps formation in response to UV-C exposure. Such repriming events are facilitated by the interaction of PRIMPOL with p21, a cyclin-dependent kinase inhibitor which, in the context of CTC1 or STN1 loss, is upregulated independently of p53. Future studies are needed to determine whether PRIMPOL is also regulated by p21 in other contexts. p53 itself also precludes repriming by PRIMPOL and it does so in a complex with POLι [61]. Inhibition of PRIMPOL activity by the POLι-p53 complex depends on POLι interaction with PCNA [61] and on the exonuclease activity of p53 [62,63]. The restriction of PRIMPOL by the POLι-p53 complex is epistatic with ZRANB3 [61], suggesting that the complex promotes fork reversal while suppressing repriming. Moreover, p53 regulates PRIMPOL-associated DNA replication events in manners that are not yet fully understood. While in the absence of p53, PRIMPOL promotes the expected lengthening of nascent DNA tracks, in the presence of high levels of p53, PRIMPOL participation in DNA replication is associated with a shortening of nascent DNA tracks (47). These observations were made in untreated conditions, indicating that PRIMPOL activity is tightly controlled even in the absence of damage from exogenous sources [61,64,65].

The chromatin status also regulates PRIMPOL recruitment, at least in response to replication stress. Specifically, *de novo* heterochromatin assembly at stressed forks precludes PRIMPOL loading in response to HU treatment or oncogene activation [66,67]. Nuclear architecture was also recently implicated in restricting PRIMPOL activity during replication stress response [68]. Indeed, nuclear actin polymerization upon inhibition of topoisomerase I or II by camptothecin (CPT) or etoposide (ETP), respectively, excludes PRIMPOL from the chromatin, while facilitating fork reversal [68]. Impairment of either chromatin compaction or nuclear actin polymerization is sufficient to unleash PRIMPOL and induce post-replicative gaps [66,68]. Collectively these studies point to a complex and multibranched regulatory network for

PRIMPOL recruitment to damaged forks that culminate in limiting its activity.

How are these different pathways regulating PRIMPOL engagement at forks activated upon replication stress induction? The Ataxia telangiectasia and Rad3-related (ATR) is the central kinase in the human replication stress response (reviewed in [69]) and was previously linked to efficient global fork reversal in response to DNA damage induction [70]. It is therefore tempting to speculate that, in certain conditions, ATR activation triggers the regulatory network restricting PRIMPOL recruitment to stressed forks and promotes fork reversal. Supporting this notion, the restriction of PRIMPOL recruitment to forks elicited by chromatin compaction is dependent on ATR activity [66]. Of note, inhibition of ATR does not impact repriming by PRIMPOL upon A3A expression or HU treatment [35,71], but impairs PRIMPOL activity under conditions of impaired fork reversal (as described in section 2.2.1) or high levels of ATR by preventing phosphorylation of its serine 255 [52,67]. These apparent discrepancies could be explained by a multifaceted regulation of PRIMPOL that depends only partially on ATR. Alternatively, the kinetics of PRIMPOL dephosphorylation could be slow, thereby limiting the effect of ATR inhibition on PRIMPOL activation under certain conditions. Instead, ATR could only stimulate repriming under the specific settings described above, raising the question on how repriming is activated otherwise.

2.2.3. *Loss of TLS polymerases and compensation by repriming*

In addition to fork reversal factors, suppression of TLS polymerases also impacts the DDT pathway choice at stalled forks. Specifically, in POLη-depleted cells, repriming acts as a compensatory mechanism to bypass DNA damage induced by UV-C [7,72,73]. In this context, repriming is partially mediated by PRIMPOL and is also promoted by RAD51 through a yet undefined mechanism [73]. Given that RAD51 interacts with POLα as shown in *Xenopus laevis* egg extracts [74], RAD51 could facilitate POLα-mediated repriming in POLη-depleted cells. Supporting this notion, POLα was recently implicated in the accumulation of gaps behind damaged forks, although it remains to be defined whether these gaps are located on the leading and/or the lagging strand [75]. Future studies are needed to understand whether and how PRIMPOL and POLα are coordinated during the replication stress response. Regardless of the potential contribution of POLα to DNA damage tolerance events, POLη-defective cells rely on PRIMPOL for their survival upon UV-C exposure [7,72], underscoring the critical role of PRIMPOL in this context. Interestingly, upon loss of another TLS polymerase, REV1, PRIMPOL activity becomes prominent upon exposure to MMS and cisplatin, but not UV-C [7]. This difference compared to POLη loss could reflect the role of the nature of the DNA damage in defining the TLS polymerase engaged in their bypass (reviewed in [76]). Nevertheless, REV1 appears to have a more general role in restricting post-replicative gaps accumulation. In this regard, REV1 acts during TLS mainly as a scaffold for other TLS polymerases, and disruption of these interactions by a small molecule inhibitor induces gap accumulation in response to HU [77]. Whether these gaps are dependent on PRIMPOL is unknown.

3. Consequences of untimely repriming activation

Here we discuss the consequences of repriming activation under the pathological conditions highlighted in the Section 2.2. In untreated cells, PRIMPOL-dependent repriming triggered by POLι-depletion reduces replication stress-associated signaling and consequently suppresses the S phase checkpoint [61]. These conditions allow faster and apparently unstressed transit through S phase [61]. Nevertheless, the negative impacts of excessive PRIMPOL-mediated replication on DNA replication are apparent during the next M and G1 phases, when mitotic DNA synthesis (MiDAS), mitotic aberrations, micronuclei and 53BP1 bodies accumulate [61]. Hence, unrestricted activity of PRIMPOL in untreated cells leads to genomic instability. Likewise, untimely repriming activation triggers a significant amount of nuclear and chromosomal abnormalities upon DNA damage induction by CPT or *KRAS* oncogene activation associated with elevated ATR expression [67,68]. While unrestrained PRIMPOL activity may induce genome instability, its effect on cell survival remains controversial. For example, PRIMPOL phosphorylation by CHK1 on S255 in response to replication stress stimulates its repriming activity, and overexpression of constitutively active, phosphomimic mutants of PRIMPOL S255 reduces viability of untreated cells [52]. In contrast, unrestricted activity of endogenous PRIMPOL has no effect on the viability of POLι-depleted untreated cells [61]. Results are equally puzzling in the context of DNA damage. While PRIMPOL phosphorylation at S255 promotes cellular resistance to UV-C irradiation and *KRAS* oncogene expression [52,67], untimely participation of PRIMPOL impairs cell viability upon UV-C irradiation when forks cannot reverse due to knockdown of RAD51 [44]. Similarly, disruption of heterochromatin assembly at stressed forks by inhibition of the histone methyltransferase G9a allows local access to PRIMPOL and disfavors cellular viability upon PARP inhibition or cisplatin treatment [66]. Further insights on the cellular impact of untimely repriming activation could be gained from investigating the outcome of the ensuing gaps, as discussed in the next section.

DDT rewiring from fork reversal to repriming also underlies the changes in DNA replication speed required at different stages of the development and organismal homeostasis. For example, we have recently reported that differentiated cells have a faster replication rate than stem cells [65]. In fact, stem cells display a slower DNA elongation led by the POLι-p53 complex involving HTLF and ZRANB3 recruitment to replication forks and frequent fork reversal [65,78]. However, when a burst of proliferation is needed such as during hematopoietic stem - and progenitor cells amplification and bone marrow reconstitution after stress caused for example by viral infection, acceleration of replication fork elongation is achieved by PRIMPOL-dependent replication [79]. The need of changes in DNA synthesis speed can also be imposed by pathogenic signals. In fact, expression of mutagenic $KRAS^{G12V}$ in untransformed cells triggers elevated ATR-mediated participation of PRIMPOL in DNA replication [67]. Hence, DNA replication plasticity at times of sudden physiological or pathological increase in replication speed is achieved by promoting the participation of PRIMPOL in bulk DNA synthesis.

4. Filling in PRIMPOL-mediated post-replicative gaps

Repriming by PRIMPOL ensures overall progression of replication but does not resolve the local challenge to DNA synthesis posed by the DNA damage. Indeed, after PRIMPOL activity, DNA lesions remain unreplicated at ssDNA gaps. Seminal work from bacteria to human cells showed that the fill-in of post-replicative gaps relies on DDT by TLS and homology-dependent mechanisms (reviewed in [5,80,81]) (Fig. 2). This mode of action for DDT was first proposed five decades ago, originating the imprint “post-replication repair” [9]. Here, we discuss recent findings on the fill-in of PRIMPOL-mediated gaps in human models, and how these mechanisms are regulated and coordinated in time. We also discuss the outcome for genome stability of gap filling and the consequences of defective gap filling.

4.1. *Translesion DNA synthesis*

TLS can promote DDT directly at stalled forks (on-the-fly) as discussed above, but also behind replication forks at gaps opposing unrepaired DNA lesions (reviewed in [76]). This labor division depends on several factors including the nature of the damage and the TLS polymerase engaged. TLS is activated upon mono-ubiquitination of the replication clamp PCNA at K164 through the concerted action of the E2 ubiquitin conjugating enzyme RAD6 and the E3 ubiquitin-ligase RAD18 in the *RAD6* pathway [5]. Recent findings support a key role of RAD18-mediated PCNA mono-ubiquitination in promoting filling of post-replicative gaps in human models [7,39,53,82], in agreement with

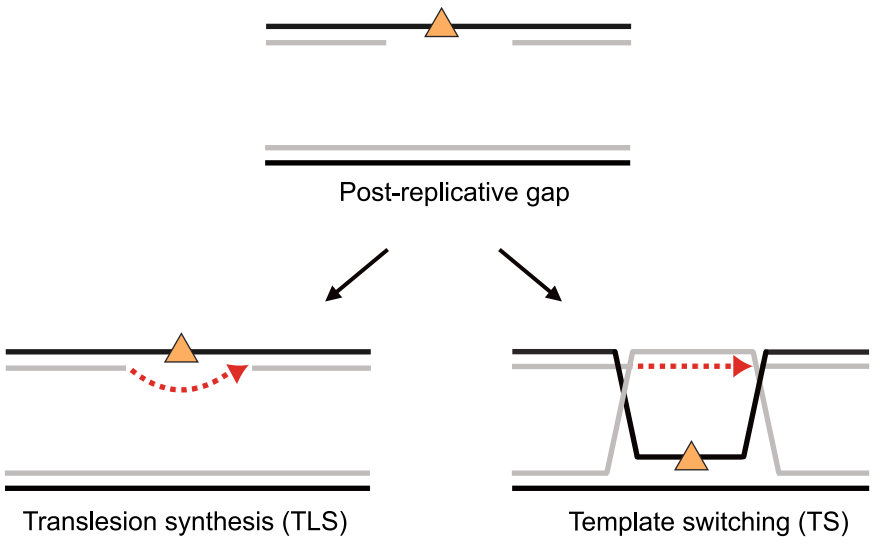

Fig. 2. Gap-filling mechanisms. Schematic representation of a post-replicative gap and gap filling by the DNA damage tolerance mechanisms translesion synthesis (TLS) and template switching (TS). DNA synthesis is represented by the red dashed arrows.

seminal work in budding yeast and DT40 cells [8,83] (Fig. 3A). Loss of function studies in mammalian cells have revealed POLζ as the central gap-filling TLS polymerase in the *RAD6* pathway [19,82,84]. Moreover, inhibition of POLζ interaction with REV1 by the small molecule JH-RE-06 is sufficient to impair gap filling [39,82,85], pointing to a key role of POLζ-REV1 complex in this reaction (Fig. 3A). Of note, as mentioned above, REV1 has a broader function in TLS acting as a scaffold also for other TLS polymerases including POLη, and was shown to also promote TLS on-the-fly [19,83].

4.2. Homology-dependent mechanisms

An alternative strategy to replicate across DNA damage at post-replicative gaps is by template switching (TS). TS is mediated by specific homologous recombination (HR) factors including the central recombinase RAD51 and activated upon polyubiquitination of PCNA at its K164. PCNA is polyubiquitinated in the *RAD6* pathway by the joint activity of an E3 ligase (Rad5 in *S. cerevisiae*), the E2 ubiquitin-conjugating enzyme UBC13 and an E2-like protein (Mms2 in *S. cerevisiae*) (reviewed in [5]). While the role of the human Rad5 homologs HLTF and SHPRH in gap filling remains to be tested, UBC13 was recently implicated in this process [82] (Fig. 3B). In addition, RAD51 operates in the same pathway as UBC13 to fill in post-replicative gaps induced by PRIMPOL [25,35,50,82]. Specifically, upon induction of PRIMPOL-mediated gaps, RAD51 is recruited to chromatin behind ongoing forks, and impairment of RAD51 nucleofilaments formation by the B02 and RI-1 inhibitors blocks gap filling. Mechanistically, TS is stimulated by expansion of post-replicative gaps by the nucleases MRE11 and EXO1 and subsequent docking of RAD51 nucleofilaments [25] (Fig. 3B). The nuclease/helicase DNA2 was also recently implicated in the gap expansion step that precedes TS [27]. Interestingly, RAD51 promotes filling of gaps induced by a range of replication-stress inducing conditions including treatment with BPDE, cisplatin or PARPi, exposure to UV-C and expression of A3A [25,35,50,73,82]. These studies were further validated by direct visualization of RAD51-dependent TS-triggered strand exchange after gap induction by TEM of replication forks and fluorescent microscopy of chromosome spreads [25,73]. Collectively, these observations support a general and key role of RAD51 in filling in PRIMPOL-mediated gaps by TS. Intriguingly, ATR activity was recently implicated in this pathway [35]. One possibility is that ATR cooperates with RAD51 in preventing excessive nucleolytic degradation at post-replicative gaps [35,71,86] (Fig. 4). At the same time, ATR is activated upon expansion of PRIMPOL-mediated gaps by EXO1 and DNA2 [27]. These findings agree with a negative feedback control proposed in yeast whereby nucleolytic expansion of post-replicative gaps triggers ATR activation, which in turn inhibits EXO1 and potentially other nucleases thereafter preventing aberrant gap processing [87].

Besides RAD51, the HR factors BRCA1/2 were recently implicated in gap filling. Interestingly, the role of BRCA1/2 in promoting filling of PRIMPOL-mediated gaps is independent of RAD18 [39], suggesting that BRCA1/2 act separately from the *RAD6* pathway in this scenario. Supporting this notion, BRCA1/2 function at post-replicative gaps is uncoupled from the aforementioned RAD51-mediated TS. Specifically, RAD51 is recruited to nascent DNA containing post-replicative gaps, and promotes filling of gaps induced by A3A expression in a at least partially BRCA2-independent manner [35,88]. These observations open the possibility that BRCA1/2 participate in a HR-mediated mechanism independent from PCNA-ubiquitination to fill in gaps, reminiscent of the "salvage pathway" described in yeast [81]. Alternatively, BRCA1/2 may promote gap filling independently of its HR function. In this sense, BRCA1 facilitates controlled expansion of PRIMPOL-mediated gaps and possibly TS-mediated gap filling [27]. Moreover, loss of BRCA1/2 proteins triggers excessive gap expansion by the nucleases MRE11 and EXO1, thereby impairing their filling [21,82]. These findings point to a protective role of BRCA1/2 at post-replicative gaps from untimely nucleolytic activity, similar to its role at reversed forks (Fig. 4). At the same time, BRCA proteins are involved in Okazaki fragment processing (OFP), and their dysfunction induces gap accumulation associated to defective lagging strand maturation (reviewed in [57]). Collectively, these studies point to a central role for BRCA1/2 proteins in gap filling. Of note, supporting the synergistic role of BRCA1/2 and PARP, PARP trapping impairs filling of PRIMPOL-dependent gaps [35,50] as well as OFP [57], culminating in the accumulation of toxic post-replicative gaps.

4.3. Labor division in gap filling

One long-standing question in the field is how DDT mechanisms are coordinated to promote timely damage bypass. Kinetics experiments of gap filling based on the S1 nuclease modified DNA fiber assay (whereby cells are treated with the S1 nuclease at increasing times after initial gap induction) coupled with cell cycle analyses revealed that gaps are filled in continuously from the first time point in early S phase to complete repair in G2/M [82]. Loss of functions studies showed that the UBC13-RAD51 axis promotes filling of PRIMPOL-mediated gaps in

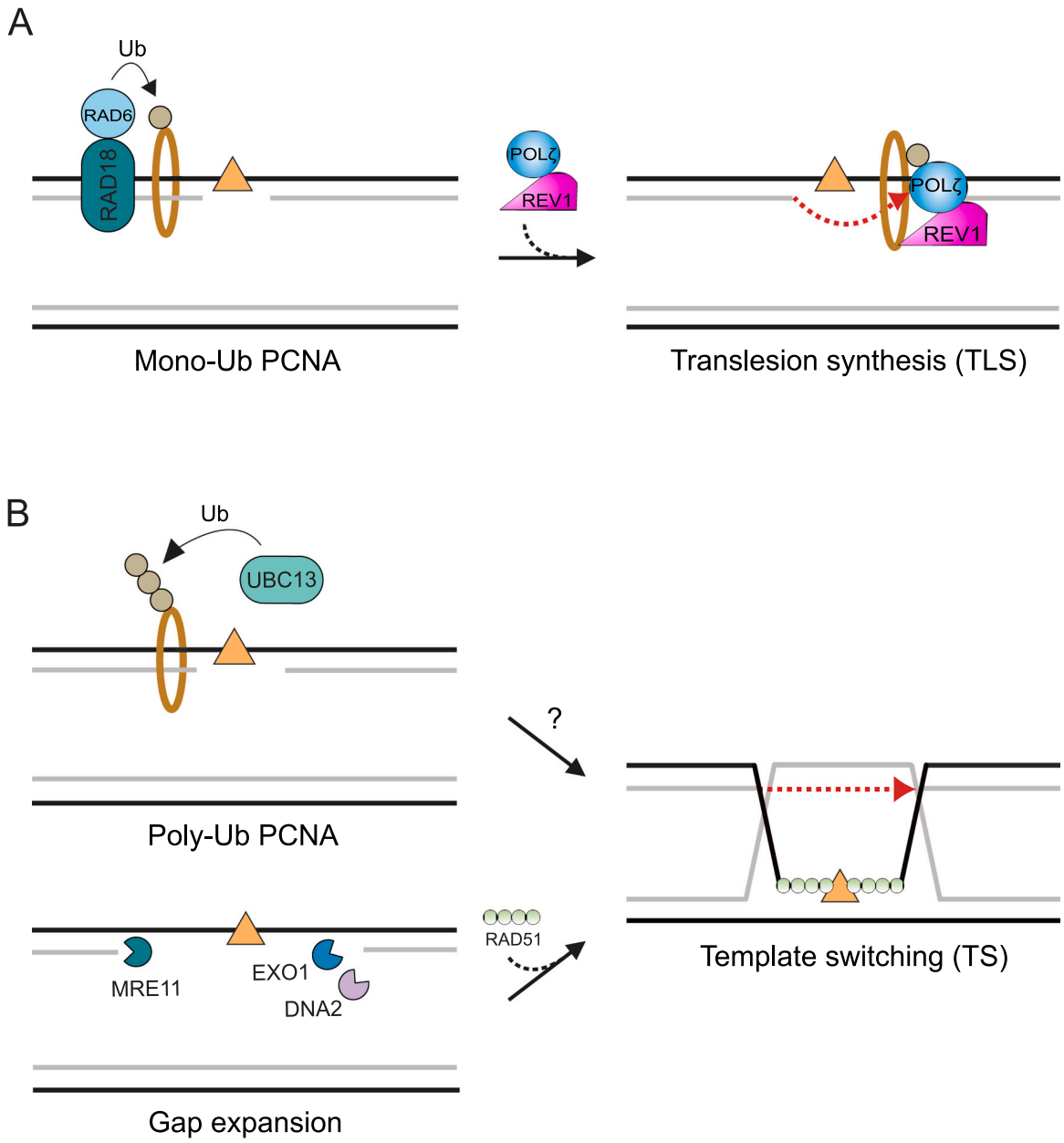

Fig. 3. Regulators of gap filling by translesion synthesis (TLS) and template switching (TS). (A) Mono-ubiquitination of PCNA by RAD6-RAD18 ubiquitin ligase complex promotes the recruitment of POLζ-REV1 complex to fill-in post-replicative gaps *via* TLS. (B) Top: PCNA poly-ubiquitination by UBC13 triggers TS mediated by RAD51 at post-replicative gaps, *via* yet unknown mechanisms. Bottom: Post-replicative gap expansion by the nucleases MRE11, EXO1 and DNA2 generates longer regions of single-stranded DNA surrounding the DNA damage, stimulating RAD51 recruitment and TS.

cisplatin-treated cells in S phase, while POLζ-REV1 complex is essential for filling in these gaps in G2/M [82]. These observations point to a temporal division of labor between TS and TLS during gap filling in human cells, supporting a previously proposed model for DDT at post-replicative gaps based on studies in yeast [80]. As discussed, favoring TS over TLS in the S phase could warrant a timely error-free damage bypass. TLS in G2/M could represent a last resort to fill in gaps and complete genome duplication prior to cell division, avoiding chromosome abnormalities although at the expense of point mutations. Given that POLζ-REV1 was recently implicated in MiDAS [89], our finding of PRIMPOL-induced MiDAS [61] opens the possibility that TLS factors operate also in mitosis to fill in PRIMPOL-induced gaps. Nevertheless, POLζ is also important for gap filling during the S phase [82], thus challenging this model. Further studies are needed to understand whether TLS and TS compete or instead cooperate in the S phase to fill in post-replicative gaps. Interestingly, similar gap filling analysis in human cells expressing A3A showed that PRIMPOL-mediated gap filling at later time points is promoted by RAD51 and to a minor extent by POLζ-REV1 [35]. While the cell cycle phase in this context is unknown, these observations raise the possibility that the nature of the damage modulates how DDT mechanisms cooperate to fill in the induced gaps throughout the cell cycle.

4.4. *Gap-filling plasticity*

The recent efforts in dissecting the mechanisms of gap filling in human models have revealed a surprising plasticity in the *modus operandi*. For example, in the absence of BRCA1/2, human cells increasingly rely on TLS to fill in PRIMPOL-induced gaps [39,90,91]. In this scenario, BRCA-deficient cells display amplified TLS-associated mutagenesis and

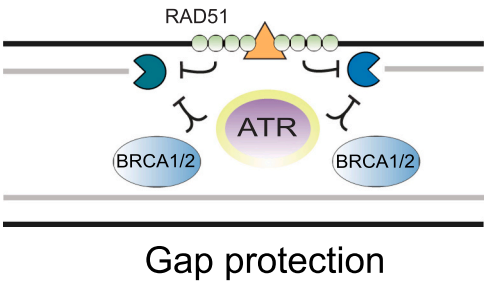

Fig. 4. Gap-protection factors. RAD51 and BRCA1/2 proteins prevent excessive post-replicative gap expansion by limiting the activity of MRE11 and EXO1 nucleases. ATR is activated upon expansion of PRIMPOL-mediated gaps by nucleases, and in turn prevents aberrant gap processing by inhibiting nucleolytic activity and by cooperating with RAD51 in post-replicative gap protection.

depend on TLS activity for their survival. Along the same lines, hypoxia-induced decline of HR proteins is associated with mutagenic TLS filling of post-replicative gaps [26]. Interestingly, low oxygen also triggers MRE11 hyper-activation, and aberrantly processed post-replicative gaps by MRE11 undergo mutagenic TLS [26]. At the same time, cells lacking EXO1 display impaired gap expansion and increased dependency on POLζ-REV1 to survive upon MMS treatment [27]. Collectively, these suggest that an imbalance in the regulation of gap-filling mechanisms can also modulate the DDT mode at post-replicative gaps.

Moreover, recent studies unraveled a novel mechanism of post-replication repair in BRCA-deficient models dependent on the specialized polymerase POLθ [92–95]. Mechanistically, POLθ operates in a mutagenic gap-filling mechanism termed microhomology-mediated gap skipping (MMGS) upon BRCA1/2 loss as well as PARP inhibition [92]. The development of a novel strategy based on TEM to visualize Okazaki fragments revealed that POLθ acts at lagging-strand templates, although a potential activity also on leading strands could not be excluded [93]. These reports laid the ground for future studies on the potential role of POLθ in the fill-in of PRIMPOL-mediated gaps.

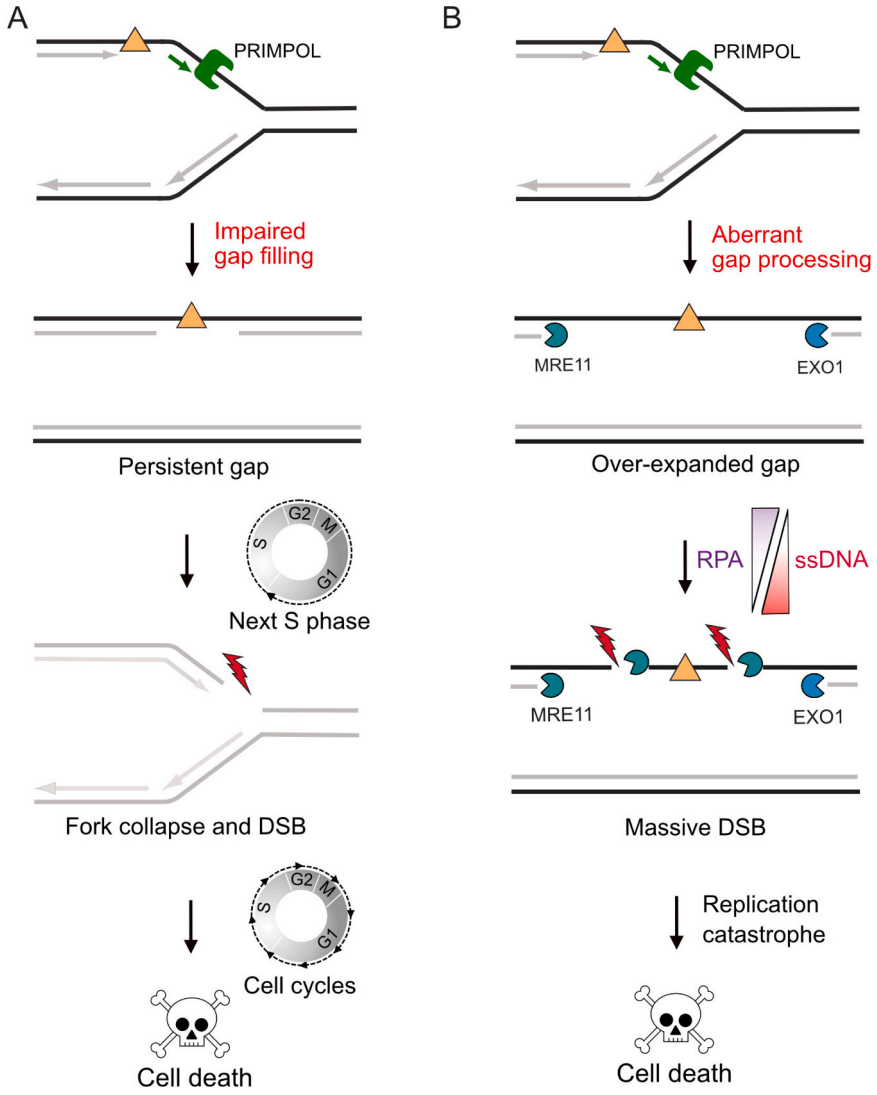

Fig. 5. Proposed mechanisms for post-replicative gap toxicity caused by gap filling defects. (A) When post-replicative gaps are not filled in, they persist until the next S phase where they collide with ongoing forks, leading to fork collapse and double-stranded DNA breaks (DSB) formation. This process may repeat over the next cell cycles, leading to cell death. (B) When post-replicative gaps are unprotected or gap expansion is uncontrolled, gaps become over-expanded. Aberrant gap expansion generates extensive single-stranded DNA (ssDNA) regions, triggering RPA exhaustion and endonucleolytic cleavage of ssDNA by MRE11, culminating in massive formation of DSB associated with replication catastrophe.

5. Defects in gap filling as the underlying mechanism of gap toxicity

The information discussed above highlights that not only the repriming activity of PRIMPOL, but also the processing and fill-in of the ensuing gaps are crucial variables that affect cellular fitness and genome stability. Overall, impaired gap filling – caused either by defects in gap filling mechanisms such as upon loss or inhibition of key TLS or TS factors or by trapping of PARP on DNA by PARP inhibitor – leads to genomic instability, DNA double-stranded breaks (DSB) and cell death [35,39,50,82]. Mechanistically, unrepaired gaps persist into the next S phase where they collide with ongoing replication forks, leading to fork collapse [40,50] (Fig. 5A). Likewise, aberrant gap expansion caused for example by loss of BRCA1/2 proteins or ATR inhibition is emerging as a cytotoxic transaction at post-replicative gaps [21,71,82]. In fact, excessive EXO1 and MRE11-dependent gap expansion triggers endonucleolytic cleavage by MRE11, and formation of toxic DSBs [21] (Fig. 5B). Importantly, the accumulation of aberrantly expanded PRIMPOL-generated gaps leads to excessive formation of ssDNA, triggering exhaustion of the ssDNA-binding protein RPA and cell death in S phase with no build-up of chromosome instability [96] (Fig. 5B).

Can targeting gap filling be exploited in cancer therapy? Certain protumorigenic backgrounds are particularly vulnerable to defective gap filling. For example, cancers with mutational signatures generated by dysregulated activity of A3A employ PRIMPOL-mediated repriming and depend on the repair of the ensuing gaps to survive. In this sense, impairing gap filling by utilizing PARP or ATR inhibitors is proposed as a promising strategy to target these tumors [35]. In fact, PARP and ATR inhibitors act synergistically to selectively kill A3A-expressing cells [35]. The proposed underlying mechanism is that ATR disrupts protection of PRIMPOL-generated gaps that accumulate upon A3A expression and PARP inhibition, thereby promoting excessive gap expansion and consequent cell death [35,71]. Likewise, BRCA-deficient cells sensitivity to PARP inhibitor is exacerbated by ATR inhibition in a PRIMPOL-dependent manner [71]. Of note, EXO1 loss induces killing of BRCA1-deficent tumors [27,97], raising the possibility that impaired gap expansion is also lethal in this genetic background. Moreover, BRCA1/2-deficient cells rely on alternative gap filling mechanisms as discussed above, and inhibiting TLS or POLθ induces synthetic lethality in BRCA-deficient tumors [39,92,93]. Notably, inhibitors of most of the aforementioned factors or pathways, namely TLS, ATR, PARP and POLθ, are under evaluation in cancer therapy and are worth to be considered in other pathological settings in which PRIMPOL-mediated repriming is enhanced. One alternative approach to kill cancer cells could be to simultaneously induce gap formation by PRIMPOL and target gap filling. Supporting this notion, induction of PRIMPOL-dependent gaps by increasing of genomic uracil sensitizes cancer cells with high levels of UNG2 to ATR inhibition [40].

Altogether, these findings indicate that gap filling is a promising target for cancer therapy. On one hand, there is a window of opportunity as the fill-in of gaps is achieved by a network of signals integrated by possibly more than one druggable target. Further studies aiming at unraveling the regulatory network underlying gap filling should reveal new potential therapeutic targets. On the other hand, the cytotoxicity achieved when gaps are excessively expanded may predominantly happen in S phase and such a mechanism may disfavor the accumulation of genomically unstable surviving remnants. In conclusion, targeting the fill-in step of the repriming tolerance pathway is emerging as a novel anti-cancer therapeutic strategy, and new research avenues are worth pursuing to further exploit gap filling as a cancer vulnerability.

CRediT authorship contribution statement

Tiya Jahjah: Writing – review & editing, Writing – original draft, Visualization. **Jenny Kaur Singh:** Writing – review & editing, Writing – original draft, Visualization. **Vanesa Gottifredi:** Writing – review & editing, Writing – original draft. **Annabel Quinet:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work was supported by CFR-CEA (to T.J.), and the Fondation ARC pour la recherche sur le cancer (to J.K.S). Work in the Gottifredi lab is supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT-2019–02201 to VG) and Alexander Von Humboldt Foundation (Research Group Linkage Programme). Work in the Quinet lab is supported by a startup package from UMR008/iRCM/IBFJ, an ANR PIA funding: ANR-20-IDEES-0002, an EDF grant and the ATIP-Avenir program (project #C23002LS).

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