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Mechanisms for stalled replication fork stabilization: new targets for synthetic lethality strategies in cancer treatments

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Abstract

Timely and faithful duplication of the entire genome depends on completion of replication. Replication forks frequently encounter obstacles that may cause genotoxic fork stalling. Nevertheless, failure to complete replication rarely occurs under normal conditions, which is attributed to an intricate network of proteins that serves to stabilize, repair and restart stalled forks. Indeed, many of the components in this network are encoded by tumour suppressor genes, and their loss of function by mutation or deletion generates genomic instability, a hallmark of cancer. Paradoxically, the same fork-protective network also confers resistance of cancer cells to chemotherapeutic drugs that induce high-level replication stress. Here, we review the mechanisms and major pathways rescuing stalled replication forks, with a focus on fork stabilization preventing fork collapse. A coherent understanding of how cells protect their replication forks will not only provide insight into how cells maintain genome stability, but also unravel potential therapeutic targets for cancers refractory to conventional chemotherapies.

Keywords fork stabilization; synthetic lethality; PARP inhibitors
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See the Glossary for abbreviations used in this article.

Introduction

A main task of a cell is to duplicate its genome and pass it on to daughter cells. In human cells, billions of DNA base pairs must be replicated completely and accurately during each cell cycle, which requires proper function of every replication fork travelling along the template DNA. Even under normal conditions, this vulnerable process is often challenged by endogenous DNA lesions [1,2], difficult-to-replicate regions [3–5] and collision with transcription machineries [6,7]. These impediments to replication progression lead to fork slowdown and/or stalling termed replication stress, threatening timely and faithful genome duplication [8]. When the replication stress is prolonged, stalled replication forks can undergo irreversible fork breakage, which eventually results in genome instability [9–12]. However, in the long history of evolution, cells have acquired a multitude of fork protection mechanisms to minimize the genotoxic effects of replication stress by stabilizing, repairing and restarting stalled forks, which represent important barriers to tumorigenesis in nontransformed cells [13,14]. Paradoxically, these mechanisms also act in cancer cells, but only to compromise the cytotoxicity of replication stress-inducing agents such as PARP inhibitors [15–18]. In this consideration, a comprehensive understanding of how cells rescue their stalled forks might lead to new strategies to confront drug resistance challenges in cancer treatment.

A simplified model for the rescue of stalled replication forks consists of two stages—fork stabilization and fork restart (Fig 1). Similar to first aid that preserves life and promotes recovery, stabilization of stalled replication forks prevents them from collapsing into poisonous DSBs, thereby increasing their chance of recovery. In the context of current knowledge, fork stabilization sequentially undergoes RPA-mediated ssDNA protection, RAD51-mediated fork reversal and suppression of nucleolytic fork degradation. Meanwhile, the replication checkpoint serves as a regulator of many cellular events that are required for fork stabilization. When the replication impediments are removed, the rescue mission proceeds to the second stage. According to the types of replication stress, different repair pathways are involved to restart the stalled forks, such that DNA synthesis can be resumed to complete genome duplication (reviewed in references [19,20]). In the following sections, we will focus on the mechanisms underlying stalled fork stabilization and introduce them in more detail from four aspects, which are ssDNA protection, fork reversal, prevention of nucleolytic degradation and checkpoint activation. Though introduced separately, these mechanisms are not mutually independent. In fact, they are rather

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Glossary

9-1-1	Rad9-Hus1-Rad1
ABRO1	abraxas brother 1
ATM	ataxia-telangiectasia-mutated protein kinase
ATR	ataxia-telangiectasia-related protein kinase
ATRIP	ATR interacting protein
BER	base excision repair
BIR	break-induced replication
BLM	bloom syndrome, RecQ helicase-like
BOD1L	biorientation of chromosomes in cell division 1-like
BRCA2	breast cancer-associated 2
CDK1	cyclin-dependent kinase 1
CDK	cyclin-dependent kinase
CHD	chromo-ATPase/helicase/DNA-binding protein
ChIP-PCR	chromatin immunoprecipitation polymerase chain reaction
ChIP-seq	chromatin immunoprecipitation sequencing
CHK1	checkpoint kinase 1
CtIP	CTBP-interacting protein
DDI1/2	DNA-damage-inducible 1 protein
DNA2	DNA replication ATP-dependent helicase/nuclease DNA2
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSBs	double-strand breaks
dsDNA	double-strand DNA
EM	electron microscopy
ETAA1	Ewing's tumour-associated antigen 1
EXO1	exonuclease 1
FAAP24	Fanconi anaemia core complex-associated protein 24
FA	Fanconi anaemia
FANC	FA complementation groups
FBH1	F-box DNA helicase 1
HJ	holliday junction
HLTF	helicase-like transcription factor
HR	homologous recombination
Hus1	HUS1 checkpoint clamp component
ICL	inter-strand crosslink
iPond-MS	isolation of proteins on nascent DNA-mass spectrum
LOH	loss of heterozygosity
mESC	mouse embryonic stem cell
MHF1	MPH1-associated histone-fold protein 1
MHF2	MPH1-associated histone-fold protein 2
MiDAS	mitotic DNA synthesis
MLL3/4	myeloid/lymphoid or mixed-lineage leukaemia protein 3/4
MRE11	meiotic recombination 11
MRN	MRE11-RAD50-NBS1
MUS81	methyl methanesulfonate and ultraviolet-sensitive gene clone 81
Nek1	NIMA-related kinase 1
NHEJ	non-homologous end joining
PALB2	partner and localizer of BRCA2
PARP1	poly(ADP-ribose) polymerase 1
PAR	poly(ADP-ribose)
PARylation	poly(ADP-ribosyl)ation
PCNA	proliferating cell nuclear antigen
PTIP	PAX transcription activation domain interacting protein
RAD51	RAD51 recombinase
RecQ1	ATP-dependent DNA helicase Q1
RFWD3	ring finger and WD repeat domain 3
RNR	ribonucleotide reductase
RPA	replication protein A
RTF2	replication termination factor 2
SMARCAL1	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatinsubfamily A-like 1
SNF2	sucrose non-fermentable 2
SSBs	single-strand breaks
ssDNA	single-strand DNA
TOPBP1	DNA topoisomerase II binding protein 1
WRN	werner syndrome ATP-dependent helicase
XRCC1	X-ray repair cross-complementing 1
ZRANB3	zinc finger RANBP2-type containing 3

coordinated and interweaved. As replication perturbation often underlies genomic instability and chemotherapeutic strategies [13,21–24], this work may expand our knowledge of carcinogenesis and provide new strategies for cancer therapy.

ssDNA protection

Stalled replication forks are characterized by extensive ssDNA, generated by polymerase–helicase uncoupling or nucleolytic processing [25,26], which is very unstable and therefore needs to be protected. The first responder to ssDNA exposure is RPA, which is an ssDNA-binding protein essential for multiple DNA metabolic processes that produce ssDNA intermediates [27–29]. RPA has a higher abundance and ssDNA affinity compared with other ssDNA-binding proteins such as RAD51 and its paralogs; therefore, its assembly on ssDNA occurs earlier than that of other ssDNA-binding proteins [28,30]. The same is true at stalled replication forks, where RPA is quickly loaded onto the ssDNA to prevent formation of secondary structures that may block further fork processing [28,31]. Another major function of RPA on ssDNA is to send out stress signals by activating the replication checkpoint, which involves two parallel pathways that are TOPBP1-dependent and ETAA1-dependent, respectively [32–34]. Furthermore, RPA binding to ssDNA recruits the fork remodelling protein SMARCAL1, which regresses stalled replication forks in the face of impediments to prevent fork collapse [35,36]. Posttranslational modifications of ssDNA-bound RPA also play an important role in fork stabilization. Under replication stress, phosphorylation of RPA by ATR and DNA-PKcs increases its affinity for ssDNA and signals the switch from replicative DNA synthesis to reparative DNA synthesis [29,37]. In addition, site-specific phosphorylation of RPA mediated by ATR and CDK-cyclinB is necessary for targeting PALB2 and BRCA2 to stalled replication forks, which is central to fork stabilization as will be described later [38,39]. Unexpectedly, ssDNA-bound RPA has recently been found to be ubiquitinated by the E3 ligase RFWD3 in reaction to a range of replication-stalling treatments [40]. Interestingly, ubiquitination of RPA does not trigger its degradation by the proteasome, but promotes HR-dependent fork repair and restart [40]. It is still unclear how ubiquitinated RPA escapes from degradation. However, given the role of ubiquitinated RPA in robust fork recovery from replication stalling [40], elucidation of the mechanism that hides it from the degradation machinery may unravel new targets for potentiating the efficacy of replication stress-inducing drugs.

Because the intracellular RPA pool is finite, ssDNA protection also relies on preserving the RPA pool by constraining formation of ssDNA itself (Fig 2). The ATR/CHK1-dependent replication checkpoint is the major pathway fulfilling this task. Under normal

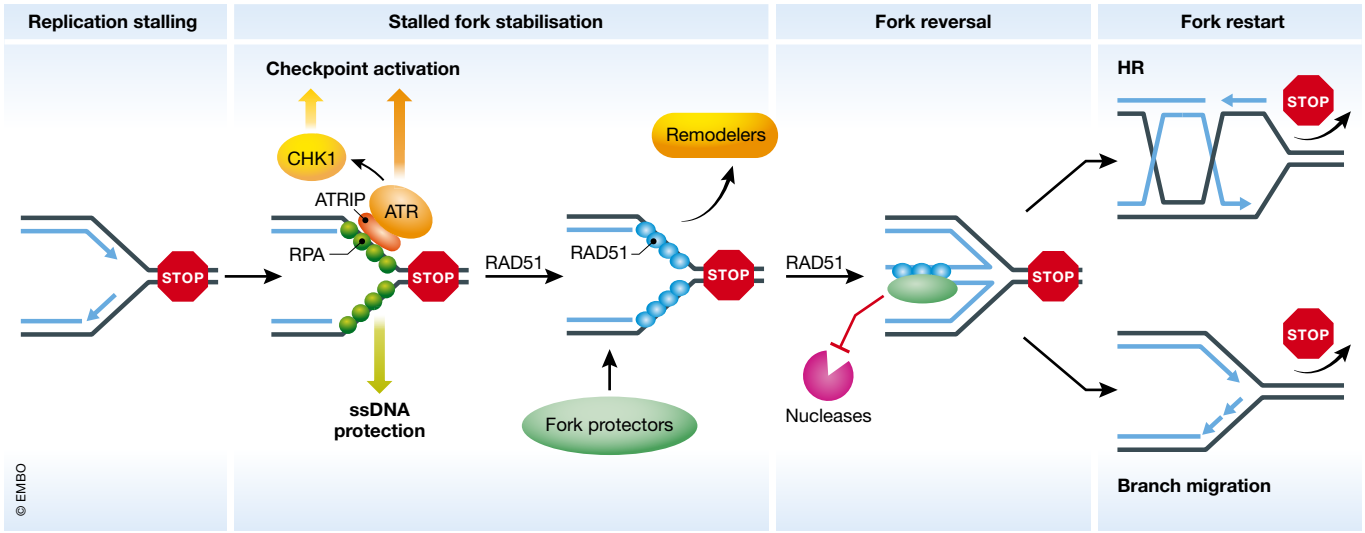

Figure 1. A simplified model for the rescue of stalled replication forks.
After fork stalling, ssDNA generated by polymerase–helicase uncoupling is coated by RPA to prevent secondary structure formation. The ssDNA–RPA complex then induces activation of the replication checkpoint, which will regulate a wide range of cellular events to promote fork recovery. RAD51 soon replaces RPA and mediates replication fork reversal to facilitate fork repair. This process also involves many other replication fork remodelers such as SMARCAL1. The reversed forks are protected by various fork protectors from deleterious fork degradation that can destabilize stalled forks. Finally, after removal of replication stress, stalled replication forks can be restarted in an HR-mediated manner or through branch migration.

conditions, redundant ATR/CHK1 activities play an essential role in regulating replication origin usage. Inhibition of either ATR or CHK1 leads to aberrant origin firing and impedes DNA replication progression [41–43]. In the context of replication stress, ATR inhibition is catastrophic, as unscheduled origin firings produce excessive ssDNA that depletes the intracellular RPA pool, which leaves stalled replication forks unprotected and eventually leads to genome-wide fork collapse [44]. Recently, the DDI1/2–RTF2 pathway was identified as a novel mechanism that prevents accumulation of ssDNA at stalled forks [45]. DDI1/2 is a proteasomal shuttle protein with both ubiquitin and proteasome binding activities. It is responsible for targeting ubiquitinated substrates to the proteasome for degradation [45,46]. Compared with DDI1/2, the role of RTF2 is poorly defined. It was first discovered in *Schizosaccharomyces pombe* as a mediator of site-specific replication termination [47], but since then little progress has been made in characterizing its biological function in human cells. Although a recent proteomic study identified RTF2 as a replisome component on elongating forks [48], its specific role remains enigmatic. However, in the latest study, RTF2 starts to reveal itself as a negative regulator of replication forks. It was shown that under prolonged replication stress, RTF2 must be removed from stalled replication forks by DDI1/2, as it otherwise causes massive ssDNA formation and genome instability [45]. Confined by the poor knowledge of RTF2, it is still mysterious how RTF2 retention at stalled replication forks promotes ssDNA production. It has been speculated that unremoved RTF2 might exacerbate helicase–polymerase uncoupling, which generates an excess of ssDNA [45]. If it is true, inhibiting helicase activities after replication fork stalling should counteract extensive ssDNA formation caused by RTF2 stabilization. Given the shared function of ATR/CHK1 signalling and the DDI1/2–RTF2 axis in restraining ssDNA formation, it is attractive to infer a crosstalk between them. For example, the activity of RTF2 to promote ssDNA formation could facilitate ATR/CHK1 activation at early stages of replication stress, and in turn, ATR/CHK1 activation might promote the posttranslational modifications of RTF2 required for its recognition by DDI1/2. This could represent a balancing mechanism for ssDNA control, which on the one hand ensures sufficient ssDNA generation for full checkpoint activation, while on the other hand prevents ssDNA overloading. More importantly, preventing RTF2 removal from stalled replication forks might potentially be used in combination with ATR inhibitors as a novel therapeutic strategy to kill cancers with high levels of replication stress.

In conclusion, ssDNA protection by RPA is the prerequisite for stabilization of stalled replication forks, which largely depends on checkpoint activation and suppressed origin firing to preserve the RPA pool. However, protection by RPA is not sufficient to rescue stalled forks, as it was shown that a proficient checkpoint or overexpression of RPA only delays but does not prevent fork collapse in cells under prolonged hydroxyurea treatment [44,48]. In fact, the RPA–ssDNA complex is highly dynamic and does not persist long before being displaced by the RAD51 protein [31,49], which mediates replication fork reversal. Replication fork reversal is a pivotal fork remodelling process that bridges fork stabilization and restart, as will be described below.

Fork reversal

Replication fork reversal describes the conversion of a typical three-way junction at the replication fork into a four-way junction in the face of replication blockade [50,51]. During this process, newly synthesized daughter strands anneal to form a new arm that is oriented opposite to the direction of fork progression. It was initially proposed as a mechanism to bypass DNA lesions on the leading strand template, with the lagging strand serving as an alternative template for leading strand DNA synthesis [50–54]. For a long time,

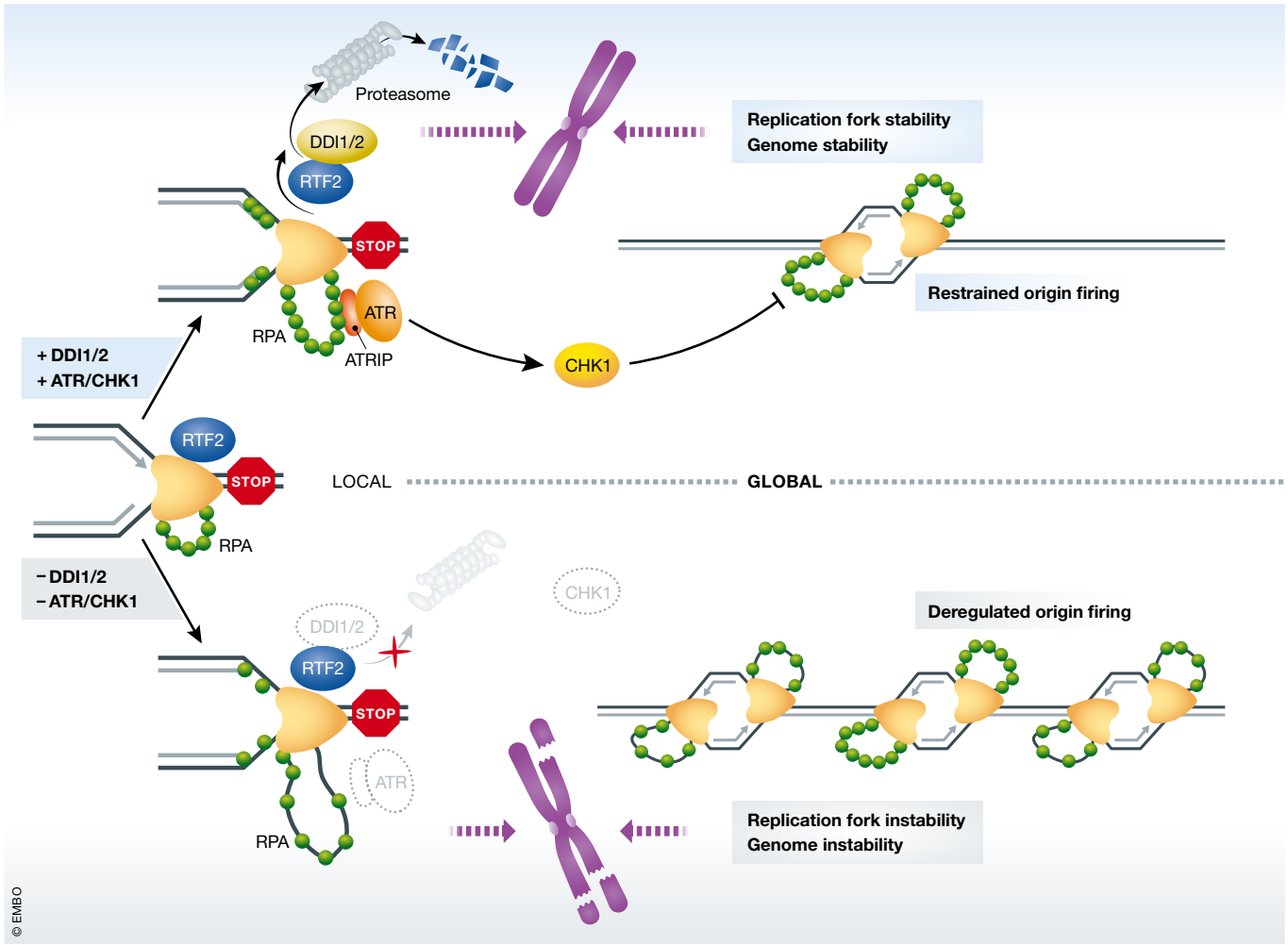

Figure 2. Mechanisms for ssDNA control during stabilization of stalled replication forks.
When replication forks are stalled, the increased ssDNA–RPA level will activate the ATR/CHK1-dependent replication checkpoint, which suppresses origin firing in a cell-wide manner. While ATR/CHK1 signalling prevents ssDNA formation globally, DDI1/2-mediated removal of RTF2 from stalled replication forks is required for limiting local ssDNA generation. These two mechanisms act cooperatively to promote replication fork stability and genome integrity. When the replication checkpoint and DDI1/2 are both absent, excessive ssDNA production will exhaust the intracellular RPA pool and leave stalled forks unprotected, which eventually leads to chromosome breakage and cell death.

most of the evidence for fork reversal was obtained from lower organisms such as prokaryotes or yeasts with deficient checkpoints [11,55,56], raising questions about its evolutionary conservation in higher eukaryotes and about its physiological relevance, albeit in recent years increasing evidence obtained from multiple metazoans including human cells is establishing fork reversal as a conserved response to replication stress to stabilize and promote recovery of stalled replication forks [21,26,57–59].

Protective effects of fork reversal

Replication fork reversal mainly has three protective effects in the context of current knowledge. First, backtracking and annealing of nascent DNA strands prevent replication fork progression across template DNA lesions, thus avoiding replication fork collapse [21,60,61]. For instance, in the face of TOPI inhibition-induced SSBs, PARP1-mediated fork reversal can prevent formation of DSBs by protecting the replication fork from colliding with the SSBs, whereas failure to keep the stalled fork in a regressed state leads to RecQ1-dependent DSB generation [21,62]. Second, replication impediments can be repositioned back onto the double-strand template DNA after fork reversal, allowing extra time and room for the repair machineries to remove those impediments [51,53]. Third, fork reversal generates a Holliday junction with a one-ended DSB, which can be recognized by HJ resolvases such as BLM [63,64], and by DSB repair factors such as BRCA2 [65,66] and DNA-PKcs [1]. Recruitment of these proteins is essential for fork stabilization and restart, though they do not necessarily carry the same functions as they do in DSB repair or HJ resolution. Another potential benefit of HJ formation at the stalled forks is to protect against cleavage by the structure-specific endonuclease MUS81. Compared with replication forks, HJs are cut by MUS81 with lower efficiency [67,68], suggesting that regressed forks should be more resistant to MUS81, thus preventing or at least delaying MUS81-dependent DSB formation. Indeed, this idea is supported by data showing that

SMARCAL1-catalysed fork reversal is required for avoidance of MUS81-induced DSBs [36,69,70]. Most recently, multiple studies have demonstrated that fork reversal provides entry points for different cellular nucleases [71–74]. However, the consequences of the nucleolytic processing of reversed forks depend on the BRCA2 status. In BRCA1/2-proficient cells, reversed forks are resected in a controlled manner, which promotes HR-dependent fork repair and restart [71,75], whereas in cells devoid of BRCA1/2, regressed forks are degraded more extensively and can only be rescued by an alternative pathway called BIR [72–74]. Because of the promiscuous nature of BIR, the contribution of this pathway to the viability of BRCA1/2-deficient cells under replication stress is still controversial. Since BIR is highly mutagenic and closely related to LOH [76,77], its prevalence in BRCA2-deficient cells may in part explain the cancer predisposition of BRCA2 mutation carriers. Overall, replication fork reversal is an active response to replication stress, which may also hold true in clinical settings where replication stress-inducing chemotherapeutics are used. Given the protective effects of fork reversal, it may represent an important mechanism underlying drug resistance. In this consideration, proteins mediating fork reversal could be targeted for manipulating chemosensitivity.

Enzymes that promote fork reversal

Decades of research work have demonstrated that replication fork reversal can be driven by a variety of DNA remodelling enzymes. In human cells, many of them are encoded by genes whose mutations predispose to cancer or developmental defects [35,51,78], which is supportive of a role of replication fork reversal in genome stability maintenance. Early studies mainly used biochemical assays to investigate fork reversal enzymes, with a focus on RecQ helicases due to their disease relevance and intrinsic DNA remodelling activities. Using model replication forks, it was shown that Bloom syndrome protein BLM and Werner syndrome protein WRN are both able to promote stalled fork reversal [78–80]. Since neither of them has been tested for *in vivo* activity to regress stalled replication forks, it remains unclear whether the prominent genome instability in Werner or Bloom syndrome patients is associated with defective fork reversal in response to replication stress. *In vitro* studies have also revealed the ability of two recombinase proteins, RAD51 and RAD54, to regress model stalled replication forks [81,82]. In a recent study, RAD51, for the first time, has been demonstrated as an *in vivo* mediator of fork reversal in response to a range of genotoxic treatments [26]. Although this study does not address the detailed mechanism by which RAD51 mediates replication fork reversal, it presents the advantages of a novel method based on psoralen crosslinking and EM, which allows for the direct visualization and quantification of regressed replication forks extracted from cells. With this method, the fork remodelling activities can be examined under *in vivo* conditions, minimizing artificial effects and reflecting the bona fide function of certain proteins. To date, this methodology has become the standard approach to examine fork reversal, with comprehensive protocols available [83]. Although proving powerful and robust, this methodology has some ingrained caveats. First, EM is barely compatible with other imaging techniques, and it can only provide structural information of regressed forks, while protein localization and DNA–protein interactions cannot be determined. Second, the sample preparation procedures of this method are sophisticated and time-consuming, limiting its applicability.

The SNF2 family proteins, including SMARCAL1, ZRANB3 and HLTF, are also important fork reversal enzymes in human cells and are under active investigation recently. By their DNA translocase activities, they promote replication fork reversal to stabilize stalled replication forks, thus preventing replication-associated genome instability [36,58,84–88]. Although these SNF2 family proteins carry similar functions and can act independently *in vitro*, they do not appear to be redundant or interchangeable *in vivo*. First, depleting any of them in BRCA2-deficient cells is sufficient to disable fork reversal and block fork degradation upon replication stress [18,73]. Second, HLTF has ubiquitin ligase activity and can promote PCNA polyubiquitination, which is required for ZRANB3 recruitment to stalled replication forks [59,86,89,90]. These studies together indicate that SMARCAL1, ZRANB3 and HLTF may act in the same pathway. However, there are also data pointing to the opposite. It was shown that combined loss of SMARCAL1 and ZRANB3 is not epistatic but additive with regard to fork stability and cell viability after hydroxyurea treatment [91], suggesting that they have separate functions independent of each other. Moreover, SMARCAL1 and ZRANB3 are recruited to replication forks by ssDNA-bound RPA and polyubiquitinated PCNA, respectively [35,59,84,86,91], which indicates their substrate specificities. Indeed, *in vitro* biochemistry assays have shown that SMARCAL1 prefers stalled forks with leading strand gaps, while ZRANB3 prefers those with lagging strand gaps [92,93]. Most recently, a study using low-dose camptothecin or mitomycin reveals that under mild replication stress, replication fork reversal is mainly mediated by ZRANB3 [59]. This result can be explained by that mild replication stress does not cause massive dissociation of PCNA from stalled replication forks [26], thus preferring ZRANB3-mediated fork reversal. Because there is no evidence for direct interactions among these SNF2 family fork remodelers, we can only guess whether they act cooperatively or independently and what context specificity they have. It will be very challenging to look into their interplays and individual contributions to replication fork reversal *in vivo*, especially when there are no methods to detect fork reversal both faithfully and efficiently. However, given the emerging role of fork reversal in modulating chemosensitivity and in predicting survival outcomes [18,21,62,94], delineating the interrelationships between fork reversal enzymes will help to translate them into biomarkers of chemosensitivity or into therapeutic targets.

Besides, the FA protein FANCM and DNA helicase FBH1 have also been shown to regress stalled replication forks [57,95,96]. Notably, FBH1 recruitment to stalled forks promotes ATM-dependent checkpoint activation via its helicase activity [57]. Whether ATM signalling is stimulated by FBH1 directly or mediated by FBH1-catalysed fork reversal is still unclear, but it is attractive to speculate that the one-ended DSB of the regressed fork might play a role. FANCM also functions in checkpoint activation, but differently, it promotes ATR signalling by facilitating chromatin retention of the ATR activator TOPBP1 [97,98].

Consequences of replication fork reversal

Although replication fork reversal exerts protective effects on stalled forks, it also carries great risks. As mentioned, regressed forks are the entry points for various cellular nucleases that mediate stalled fork degradation or cleavage [71,73,74]. Limited resection of regressed forks does not have pathological consequences but

promotes HR-dependent fork recovery [71,75]. However, when the controlling mechanisms are compromised, excessive nuclease activities will cause genotoxic consequences leading to chromosome aberrations and cell death [71–73,85]. This is particularly demonstrated in BRCA2- or checkpoint-deficient cancer cells, in which regressed forks are over-processed by MRE11 or converted into DSBs by MUS81, respectively [73,74,85,99]. These genotoxic consequences of fork reversal in BRCA2- or ATR-defective cells could partially underlie their hypersensitivity to replication-stalling agents [18,32,61]. In fact, it was recently demonstrated by multiple studies that escaping from the genotoxic consequences of fork reversal by inactivating fork degradation renders BRCA2-defective cells resistant to PARP inhibitors or hydroxyurea [15,18,73,74]. Hence, in order to provide better strategies to enhance the efficacy of current replication stress-inducing chemotherapeutics, it is imperative to gain mechanistic insight into the pathways cells use to suppress nucleolytic degradation to prevent the bad consequences of replication fork reversal, as will be introduced in the following section.

Protection against nucleolytic degradation

Stalled replication forks are featured by exposed DNA ends in the form of ssDNA or dsDNA, which makes them susceptible to various cellular nucleases including MRE11, CtIP, DNA2 and EXO1 [71–74,100]. It is established that MRE11 and CtIP cooperate to perform short-range resection, while EXO1 and DNA2 act independently in 5′–3′ long-range processing [101–103]. They are all important players in generating HR substrates during DSB repair and are tightly regulated to determine the repair pathway choice between HR and NHEJ [104,105]. Likewise, the nuclease activities at the sites of replication stress are also strictly controlled to prevent excessive fork degradation that will destabilize the stalled/regressed forks. Multiple pathways play in this arena (Fig 3), including the BRCA2–RAD51 axis and the FA pathways, loss of which will cause overt fork breakages and genome instability [65,66,106,107]. In this section, we will summarize the pathways acting in stalled fork stabilization and discuss the interconnections among them.

BRCA2–RAD51 axis

The BRCA2 protein is encoded by the BRCA2 tumour suppressor gene that was identified over two decades ago [108]. The tumour-suppressing function of BRCA2 is largely ascribed to its pivotal role in preventing genome instability, a hallmark of cancer [109]. One major mechanism by which BRCA2 preserves genome stability is to promote HR-mediated DSB repair [110]. During this process, BRCA2 forms a complex with PALB2 and is recruited by BRCA1 to RPA-coated ssDNA overhangs at resected DNA breaks [111]. BRCA2 then, with the help of RAD52, loads RAD51 monomers onto the ssDNA through its BRC repeats, generating ssDNA–RAD51 nucleoprotein complexes termed the presynaptic filament [112]. The presynaptic filament searches and invades into a homologous template to form a heteroduplex intermediate. Afterwards, the RAD51 proteins are removed by RAD54, allowing for subsequent DNA synthesis and junction resolution to complete DSB repair [113,114]. In recent years, a new mechanism for the BRCA2–RAD51 axis to preserve genome stability has been characterized. It was found that under replication stress, BRCA2 relocates to stalled replication forks and promotes the formation of stable RAD51 nucleoprotein filaments, thereby suppressing deleterious fork degradation mediated by the MRE11 nuclease [65,66,73,115]. Centred around the BRCA2–RAD51 axis, BRCA1 also plays a role in fork stabilization. It is widely accepted that BRCA1 and BRCA2 act in the same pathway to suppress the MRE11 nuclease, as it has been seen that loss of BRCA1 mirrors BRCA2 deficiency with regard to fork degradation [18,115,116].

The role of RAD51 in stalled fork stabilization is decisive, as it was observed that overexpression of RAD51 alone can restore stalled fork stability [65,115,117]. In fact, RAD51 serves as a common effector of many pathways that prevent stalled fork degradation, which will recur throughout this section. The mechanism by which RAD51 suppresses nucleolytic fork degradation is not well understood. It may be associated with physical blocking of nucleases, or with inhibitory interactions between nucleases and RAD51. Since RAD51 was shown to mediate replication fork reversal [26], how RAD51 acts to protect against fork degradation becomes even more elusive. In some recent studies, RAD51-mediated fork reversal has been proposed to create an entry point for various exonucleases to initiate fork degradation [71–73]. Supportively, abrogating fork reversal by RAD51 depletion substantially suppresses over-resection of stalled forks [18,71,72]. However, in some other reports, inhibiting RAD51 by BRC4 peptides or the RAD51 inhibitor B02 fails to prevent fork resection [18,65,115]. An explanation to this discrepancy comes from the study of a separation of function mutant of RAD51, RAD51 T131P, which has very low DNA-binding affinity [74,118]. It was shown that RAD51 T131P mediates fork reversal *in vivo* but fails to form stable nucleoprotein filaments, leading to excessive stalled fork degradation and genome instability [73,74]. Since BRC4 and B02 are both designed to inhibit the DNA-binding activities of RAD51 [18,100], as does the RAD51 T131P mutation, it is likely that they will not affect fork reversal, but only disrupt RAD51 filament formation, thus resulting in excessive fork degradation, as observed. These data together suggest that the function of RAD51 to mediate fork reversal is separate from fork protection against degradation. Because of the canonical role of RAD51 in the BRCA2–RAD51 axis and in many other fork stabilization pathways, it is necessary to gain a thorough understanding of how RAD51 is regulated to carry out two distinct functions, such that we can design RAD51 inhibitor-based therapies that maximize the genotoxic risk of fork reversal and minimize the chance of chemoresistance acquired from stalled fork stability. Interestingly, several RAD51 paralogs, including RAD51C, XRCC2 and XRCC3, are also required for preventing MRE11-mediated over-resection of stalled replication forks [119], but whether they act within the BRCA2–RAD51 axis and how they suppress nucleases remain unclear.

Although fork stabilization and HR share the same BRCA2–RAD51 axis, they are inherently different pathways, as evidenced by many studies exclusively manipulating one process without affecting the other [15,17,18,120]. Considering the pivotal functions of BRCA2 in both fork stabilization and HR, an update of the mechanisms by which BRCA2 suppresses tumour occurrence may be well deserved. Because fork stabilization and HR have different RAD51 dynamics [65,118], it is reasonable to speculate a regulatory mechanism that dictates the pathway choice under different contexts. Recently, Nek1 is emerging as part of this regulatory mechanism that tips the balance between fork stabilization and HR. During late

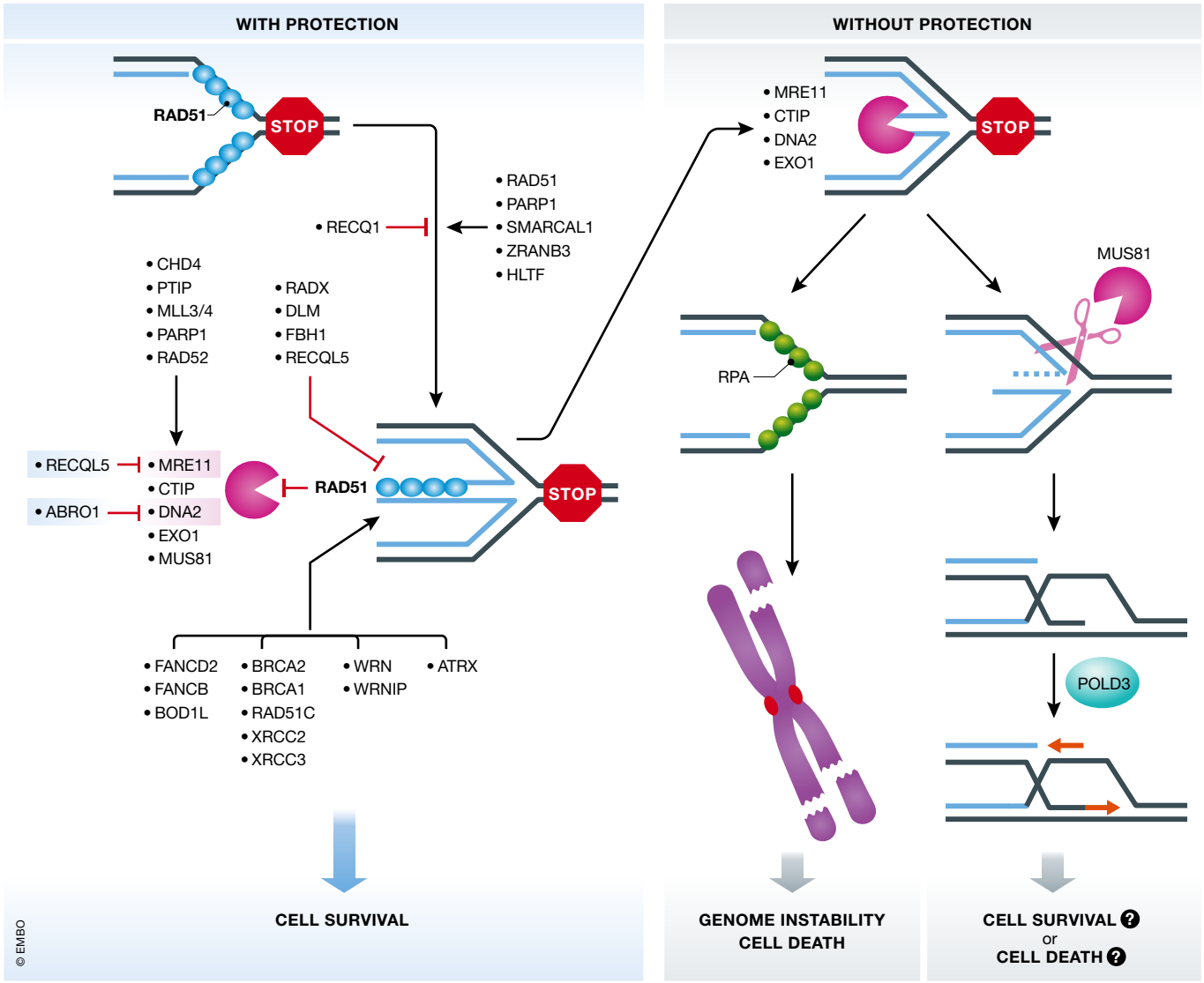

Figure 3. Pathways and proteins involved in preventing or mediating stalled fork degradation.
Regression of stalled replication forks is mediated by RAD51 and other DNA translocases including SMARCAL1, ZRANB3 and HLTF. PARP1 serves to maintain stalled forks in a regressed state by countering RECQ1 helicase. After fork reversal, MRE11 nuclease is recruited to forks in a way dependent on PARP1, RAD52 and PTIP-MLL3/4 and CHD4. MUS81 recruitment depends on EZH2. Other nucleases are also recruited, but the mechanisms are less characterized. These nucleases tend to degrade the fork, which is prevented by different pathways that mainly act through protecting the RAD51 nucleoprotein filaments. Some negative regulators of RAD51 nucleoprotein filaments such as RADX also affect fork stability. When the protective pathways are absent, stalled forks are extensively degraded, leading to genome instability. In some cases, resected forks are cleaved by MUS81 to induce BIR, but how this pathway contributes to cell viability remains a question.

G2 phase, Nek1 phosphorylates RAD54 to enhance its activity to dismantle RAD51 nucleoprotein filaments after strand invasion, thereby promoting completion of HR. But during S phase, RAD54 phosphorylation is inhibited to prevent removal of RAD51 from stalled replication forks. This mechanism is elegant in that it ensures the BRCA2–RAD51 axis is channelled towards fork stabilization when DNA replication is active, but is shunted into mediating HR when DNA damage must be repaired before mitosis onset [113]. Moreover, given that fork stabilization and HR-mediated restart are both involved in stalled fork rescue, there should be some intra-S phase pathways controlling the functional switch in different contexts. Unravelling these pathways will be necessary for understanding how cells coordinate the two fundamental yet antagonistic processes to safeguard their genome.

The FA pathway

Fanconi anaemia is a genome instability-associated disorder characterized by developmental abnormalities, bone marrow failure and cancer predisposition [121]. Up to date, nineteen FANC genes (FANCA-FANCT) have been associated with FA syndromes. The proteins encoded by these genes constitute one of the most important cellular pathways in genome stability maintenance [122]. The major role of the FA pathway is to promote ICL repair. During this process, the FANCM-FAAP24-MHF1-MHF2 anchor complex

relocates to the sites of ICL and then recruits the nine-subunit FA core complex [123]. The FA core complex catalyses the monoubiquitination of the FANCI/FANCD2 heterodimer, which subsequently promotes nucleolytic processing of the ICL and HR-mediated repair [122]. Consistent with the essential role of the FA pathway in ICL repair, the expression of FA proteins is intimately involved in cellular resistance to ICL-inducing agents such as cisplatin and mitomycin [121,124,125]. However, some FA components have also been implied in resistance to non-crosslinking agents including hydroxyurea and PARP inhibitors, which point to ICL repair-independent function of the FA pathway in replication fork stabilization [107,126–128]. Till now, two classical FA proteins, FANCB and FANCD2, have been characterized with a direct role in the stabilization of stalled replication forks. Like the BRCA2–RAD51 axis, FANCB and FANCD2 also suppress MRE11-mediated fork degradation in a manner dependent on RAD51 nucleoprotein filaments [107,115,129]. Recently, a newly identified FA component, namely BOD1L, has been revealed to carry out a similar function. BOD1L stabilizes RAD51 nucleoprotein filaments by counteracting the anti-recombinogenic activities of BLM and FBH1 to displace RAD51 from ssDNA [116]. However, BOD1L does not prevent MRE11-dependent fork degradation; instead, it suppresses DNA2 [116]. Since the functions of BOD1L and FANCD2 are both mediated by RAD51 [115,116], it is counterintuitive that they suppress different nucleases. To understand this, it will be necessary to investigate their additional functions besides stabilizing RAD51 nucleoprotein filaments. Paradoxically, while inhibiting MRE11 and DNA2, the FA pathway facilitates fork resection by the FAN1 nuclease, which is required for the prevention of chromosome abnormalities at stalled replication forks [126]. Taken together, these results suggest that the FA pathway could play a central role in coordinating the activities of different nucleases. It will be interesting to delineate the underlying mechanisms, which might provide new strategies for killing cancer cells by exacerbating genotoxic nucleases activities while inhibiting protective ones.

In contrast to the well-understood interplays between the FA pathway and the BRCA2–RAD51 axis in ICL repair [122], their relationship in fork stabilization remains elusive. It was demonstrated that MRE11 inhibition completely suppresses stalled fork degradation caused by depletion of FANCD2 or BRCA2 individually [65,115]. But recent data show that in cells lacking both FANCD2 and BRCA2, MRE11 inhibition only partially prevents over-resection of stalled forks [107]. It becomes even more complicated when taking BOD1L into consideration. Since BOD1L suppresses DNA2 while BRCA2 dampens MRE11 [65,116], it is expected that combined loss of BOD1L and BRCA2 would confer an additive effect on stalled fork degradation. Nevertheless, an epistatic effect is observed [116]. To reconcile these confounding results, we propose a model in which both BRCA2 and FANCD2 are required to suppress MRE11-mediated fork degradation, but they also act redundantly to suppress some other nucleases. In this scenario, depletion of BRCA2 or FANCD2 alone will only cause MRE11-dependent fork resection; therefore, MRE11 inhibition suffices to prevent stalled fork instability [65,115]. However, when BRCA2 and FANCD2 are both deficient, other nucleases will come into play, exacerbating the uncontrolled fork degradation that can only be partially alleviated by MRE11 inhibition [107,128]. As to the epistatic relationship between BRCA2 and BOD1L with regard to fork instability, we assume there is a negative feedback mechanism that controls ssDNA level under a certain threshold, regardless of the nucleases carrying fork resection. To fully understand these observations, it will be necessary to identify those nucleases responsible for fork degradation under different genetic backgrounds.

Overall, the FA pathway plays an essential role in stabilizing stalled replication forks by suppressing deleterious fork degradation. Its importance in limiting replication stress is especially highlighted after loss of BRCA1/2, as it was seen that FANCD2 expression in BRCA1/2-mutated breast or ovarian cancers is significantly increased [107,128]. In fact, the integrity of the FA pathway is a determinant for the sensitivity of BRCA1/2-deficient cancer cells to mitomycin and olaparib [107,128]. Therefore, therapeutic targeting of the FA pathway might be required to potentiate the PARP inhibitor- or platinum-based treatment for BRCA1/2-mutated tumours.

PARP1 signalling

PARylation is an important posttranslational modification of proteins that regulates their spatial localization and functional activities. In human cells, the bulk of intracellular PAR is synthesized by PARP1, which PARylates numerous proteins in response to cellular stress, including PARP1 itself [130,131]. PARP1-mediated protein PARylation plays a crucial role in genome stability maintenance [132]. Upon detection of DNA damage, PARP1 is activated rapidly and can synthesize long PAR chains within 30 s [133,134]. Notably, the major part of PAR is attached to PARP1 itself, providing a mechanism for PARP1-mediated recruitment of other repair proteins [131,134,135]. During the repair of ssDNA lesions, PARylation of PARP1 itself is required for recruitment of XRCC1, the scaffold protein essential for the assembly and stability of the BER machinery [136,137]. It is also reported that PARP1 facilitates DSB repair by promoting recruitment of HR proteins including MRE11, ATM and BRCA1 [134,138,139].

During the last decade, multiple studies, including our own, have uncovered new roles for PARP1 in stalled replication fork protection, which may hold promise for expanding the therapeutic spectrum of PARP inhibitors. We showed that PARP1 activation at stalled replication forks recruits MRE11 to process the stalled forks, which is required for HR-mediated fork recovery and cell survival [75]. However, this genome maintenance pathway appears to be detrimental in BRCA1/2-deficient cells, as it was shown recently that MRE11 recruitment by PARP1 is responsible for the extensive fork degradation and genome instability in cells lacking BRCA1/2 [15,17]. Most strikingly, PARP1 depletion before BRCA1/2 loss restores stalled fork stability and even confers synthetic viability in mESCs [15,17]. These results seem to be contradictory to our earlier findings that the PARP inhibitor olaparib, when used together with hydroxyurea, exacerbated but did not suppress stalled fork degradation in BRCA2-deficient cells [66]. The reason for this discrepancy is unclear, but since it potentially affects the results of combination therapies involving PARP inhibitors, further studies are urgently required. Moreover, we have found that PARP1 and DNA-PKcs collaborate at stalled replication forks to recruit XRCC1 for fork repair and restart, which implies an involvement of the NHEJ machinery in stalled fork protection [1]. Indeed, two latest studies have reported the functions of 53BP1, a cardinal NHEJ component, to promote fast restart of stalled forks and to restrain stalled fork degradation in checkpoint-deficient cells [140,141]. Since PARP1

and DNA-PKcs bind to stalled forks that are unresected [1], it will be interesting to survey whether they act in the same pathway with 53BP1 to promote a NHEJ-dependent fork recovery that bypasses fork resection. Under topoisomerase I inhibition, PARP1 is required to maintain stalled replication forks in a regressed state, thus preventing DSB formation resulting from replication progression across DNA lesions [21,62]. The mechanism involves inhibitory PARylation on RecQ1 mediated by PARP1 to constrain its branch migration activity, which ensures that stalled forks are restarted only after replication impediments are cleared [62]. Furthermore, a PARylation-independent role for PARP1 to recruit Timeless to stalled replication forks was identified recently, and is proposed to promote HR repair [142]. Collectively, PARP1 presents itself as a multi-functional protector of stalled replication forks. Based on this, PARP inhibitors should confer synthetic lethality not only with DSB repair deficiency, but also with defects in fork stabilization mechanisms, as will be discussed later.

RecQ helicases

RecQ helicases play essential roles in genome stability maintenance. They have substrate specificities for branched DNA structures and can resolve abnormal intermediates occurring during different DNA metabolic processes, including DNA replication, recombination and repair [64,94]. Currently, there are five RecQ helicases that have been identified in human cells (RECQ1, BLM, WRN, RECQL4 and RECQL5), and mutations in three of them (BLM, WRN and RECQL4) cause developmental defects and/or cancer predisposition [64,143], underlining their importance in promoting genome stability. Among these RecQ helicases, BLM and WRN are intimately involved in rescuing stalled replication forks. It was shown that under replication stress, BLM is recruited to stalled replication forks in a manner dependent on the FA proteins, especially on FANCD2 which directly interacts with BLM to protect its stability and mediate its stimulatory phosphorylation [144,145]. Interestingly, it was recently demonstrated that BLM is required for the recruitment and activation of FANCM which acts upstream of the FA pathway [146]. These results suggest that there might be a positive feedback loop between BLM and the FA pathway, which ensures that stalled forks are under sufficient protection. BLM does not affect nuclease activities at stalled forks, as it was observed that BLM depletion has no effects on fork degradation [129]. However, loss of BLM significantly impairs fork recovery from replication stress, suggesting that it mainly acts in later stages of fork rescue [147,148]. Notably, the function of BLM to promote stalled fork restart depends on RAD51, as it was observed that BLM and RAD51 are epistatic with regard to fork recovery efficiency [147]. In addition, the crosstalk between BLM and the FA pathway is also a requirement for suppressing new origin firing [145,148].

Compared to BLM, the role of WRN at stalled replication forks is more complex due to its dual helicase/exonuclease activities. At early stages of fork stalling, WRN is phosphorylated by ATR at multiple sites to prevent MUS81-dependent DSB formation [99,149]. Given that WRN displayed fork regression activities *in vitro* [79,80], it is possible that WRN suppresses DSB formation in manner similar to SMARCAL1. In fact, both helicase and exonuclease activities are necessary for WRN to prevent DSB formation [99], mirroring the requirements for WRN to generate optimal structures for fork regression [80]. Moreover, recruitment of RECQ1 to stalled replication forks is decreased in WRN-deficient cells, as indicated by reduced PARylation by PARP1, suggesting that fork reversal is impaired after WRN loss [150]. To further corroborate whether WRN promotes fork reversal to prevent DSBs, direct examination by EM might be needed. Besides DSB prevention, WRN can protect stalled replication forks from deleterious degradation. It was reported that under mild genotoxic treatment, the exonuclease activity of WRN is required for preventing MRE11/EXO1-dependent over-resection of the stalled replication forks [150]. The mechanism by which WRN exonuclease activity suppresses MRE11 is unclear, but a recent study may help to explain it. It was found that a WRN interacting protein, WRNIP1, prevents MRE11-dependent stalled fork degradation by stabilizing RAD51 filaments [151]. Therefore, it is possible that the WRN exonuclease activity protects against MRE11 by generating substrates for WRNIP recruitment. Importantly, WRN also plays a role in stabilizing and restarting collapsed stalled forks. When replication forks collapse into DSBs under prolonged replication stress, WRN cooperates with RAD51 to counteract uncontrolled resection of the DSB ends by MRE11 [152]. Notably, this function requires neither helicase nor exonuclease activities of WRN, which is consistent with a previous report showing that WRN can play a structural role independent of its enzymatic activities [153]. While antagonizing MRE11 activities, WRN was shown to be phosphorylated by CDK1 to promote DNA2-mediated long-range end resection of the collapsed forks, which is required for HR-dependent stalled fork restart [154]. Collectively, WRN seems to carry out important functions in the rescue of stalled forks, which is in line with its well-established role in countering replication stress induced by oncogene activation of chemotherapeutic intervention [155–157].

In conclusion, RecQ helicases have essential functions in stabilizing and restarting stalled replication forks. Of note, another less known RecQ helicase, RECQL5, also emerges as an important player in fork stabilization during recent years, which prevents MRE11-mediated stalled fork instability and shows promising results when targeted for synthetic lethality with hydroxyurea in JAK2-mutated myeloproliferative neoplasms [129,158]. Although we have made much progress in characterizing the individual roles of RecQ helicases, it is largely unexplored how these RecQ helicases are coordinated during fork stabilization and how they operate under different contexts, as well as what their relationship is with other fork stabilization mechanisms. Answering these questions will help to exploit the RecQ helicases as effective targets to kill cancer cells.

Other pathways

Besides those pathways mentioned above, some less characterized proteins also have important functions in stabilizing stalled replication forks, for example ABRO1 and ATRX. ABRO1 is a paralog of a BRCA1-interacting protein, Abraxas [159]. Though ABRO1 is not involved in HR-mediated DSB repair, its downregulation is frequently seen in human liver, kidney, breast and thyroid gland tumour tissues, indicating its essence in genome stability maintenance [160]. Recently, ABRO1 was found to protect against DNA2/WRN-mediated stalled fork degradation, which may contribute to its tumour suppressor functions [161]. Unlike many other fork stabilization pathways, ABRO1 acts independently of RAD51 filament stabilization, and its depletion has an additive effect to BRCA2 deficiency on stalled fork instability [116]. Moreover, since BOD1L also

stabilizes stalled forks by suppressing DNA2 [116], there might be some genetic interactions between BOD1L and ABRO1. Compared with ABRO1, ATRX operates in a more specific genomic context, that is heterochromatin. ATRX defends against MRE11-dependent degradation of stalled replication forks by promoting BRCA1–RAD51 retention, and its dysfunction leads to rampant fork degradation and genome instability, which could underlie the severe intellectual disability disorder caused by mutations in the Atrx gene [162]. Notably, ATRX-deficient cells display hyperactivation of PARP1 [162], which again reflects the importance of PARP1 in replication stress tolerance.

To conclude, multiple pathways have evolved to protect stalled replication forks by suppressing aberrant nuclease activities. Although their interplays in many biological processes have been firmly established [94,122,163], their interactions in stalled fork stabilization remain largely unknown. However, whether these pathways are redundant, interdependent or complementary can profoundly affect the efficacies of replication stress-inducing agents. For example, the FA pathway impacts on the sensitivity of BRCA1/2-deficient cancer cells to PARP inhibitors [107,128], and PARP1 loss can lead to drug resistance in BRCA1/2-deficient cancer cells [15]. Hence, it is necessary to further unravel the interrelationships among those fork stabilization pathways, which holds great promise for combined therapy design to enhance chemotherapeutic efficacies.

Checkpoint activation

In proliferating cells, various cellular checkpoints play crucial roles in cell cycle control, DNA damage response and replication monitoring. Once activated, the checkpoint kinases phosphorylate hundreds of substrates, causing dramatic alterations in DNA metabolisms, structural biology, enzyme kinetics and so on (reviewed in references [164–167]). Although the numbers and types of checkpoints vary among species, there is one that is highly conserved, the ATR/CHK1-dependent replication checkpoint, which is activated upon replication stress to preserve genome stability at stalled replication forks [30,32,33]. In this section, we will briefly introduce the mechanisms for activation of ATR/CHK1 signalling and describe how checkpoint activation acts to stabilize stalled replication forks.

Pathways involved in ATR/CHK1-dependent checkpoint activation

The mechanisms for activation of the ATR/CHK1-mediated checkpoint are well established. Under replication stress, a pathological amount of ssDNA is generated at stalled replication forks because of helicase–polymerase uncoupling or nuclease activities [25,26], which is recognized and bound by RPA. The ssDNA–RPA then recruits the ATR/ATRIP complex through RPA–ATRIP interaction. Meanwhile, ssDNA–RPA complex also recruits TOPBP1, which then directly activates ATR in a manner dependent on RHINO, and the 9-1-1 and MRN complexes [168–170]. The activated ATR kinase phosphorylates CHK1, and in turn, they phosphorylate a wide range of substrates, leading to full activation of the checkpoint [169]. In recent years, some new mechanisms for ATR/CHK1 activation have been revealed. For instance, CHK1 has been suggested to bind the PAR chain synthesized by PARP1 at stalled replication forks, which facilitates its kinase activity and checkpoint activation independently of ATR [171]. More recently, ETAA1 is identified as a novel checkpoint activator operating independently of TOPBP1. It is also recruited to stalled replication forks by ssDNA–RPA and then interacts with the ATR/ATRIP complex directly to activate ATR [32].

Mechanisms of ATR/CHK1 signalling to stabilize stalled forks

Once activated, the ATR/CHK1-dependent checkpoint modulates both replication and transcription programmes. In mammals, checkpoint activation is well established to promote expression of RNR and a set of G1/S transition genes in response to nucleotide starvation, which mediates replication stress tolerance and cell survival [172,173]. Although transcription regulation is vital for stalled fork stabilization, it takes effect in a rather delayed manner. Therefore, for timely protection of stalled replication forks, posttranslational modifications are also employed by the replication checkpoint.

First, ATR/CHK1 signalling regulates origin firing. In proliferating cells, replication origins are licensed during the G1 phase of the cell cycle [174,175]. During undisturbed S phase, only about 10% of the licensed origins are fired to initiate DNA replication, while the bulk remain dormant throughout S phase and are replicated passively by other travelling forks [176,177]. This tight control imposed on origin firing is mediated by redundant activities of ATR/CHK1 signalling, which promotes replication progression by balancing the number and the velocity of replication forks [41–43]. Under replication stress, the regulation of origin firing by the checkpoint becomes more critical, because deregulated origin firing will generate an excess of ssDNA exhausting the intracellular RPA pool, which will eventually cause genome-wide replication fork collapse [44]. Interestingly, while suppressing global origin firing, the replication checkpoint seems to promote local origin firing in the vicinity of stalled replication forks, which presumably allows the completion of replication by fork convergence [176,178].

Second, ATR/CHK1 signalling controls DNA remodelling. As mentioned earlier, an important configurational change to stalled replication forks is fork reversal, which prevents DSB formation caused by replication runoff or endonuclease cleavage [21,36,99]. However, too much fork remodelling is conversely detrimental to fork stabilization because it will cause aberrant nucleolytic processing that leads to DSBs [85,99]. Many of the enzymes that can catalyse fork reversal *in vitro* or *in vivo* are substrates of ATR kinase, though it is not necessarily their fork reversal activities that are regulated by ATR-dependent phosphorylation. Perhaps the most studied fork remodeler regulated by the replication checkpoint is SMARCAL1, which has been shown to be able to regress stalled forks both *in vitro* and *in vivo* [36,74,179]. At stalled replication forks, ATR fine-tunes the activity of SMARCAL1 by inhibitory phosphorylation on S652 and stimulatory phosphorylation on S889, respectively, which ensures a proper level of fork remodelling [85,180]. In fact, abrogating phosphorylation of either site causes genome instability [85,180]. Phosphorylation of the RecQ helicase WRN by ATR is required for its recruitment to stalled replication forks, and for preventing MUS81-dependent DSB formation [149]. However, unlike SMARCAL1, WRN has not been tested for its fork remodelling activity *in vivo*. Therefore, whether ATR-dependent phosphorylation of WRN prevents DSB formation by regulating its fork reversal activity remains a question. The same is true for BLM, which is phosphorylated by ATR at two residues, Thr99 and

Thr122, to promote stalled fork restart [181]. Recently, the regressed stalled forks are shown to be processed by several nucleases including MRE11, EXO1 and DNA2 in human cells, which affects stalled fork stability and restart [71,72,74]. Because the counterparts of EXO1 and DNA2 in yeasts are targets of the replication checkpoint that increases or decreases their activities [182,183], it may also be the case in human cells. Therefore, the replication checkpoint may stabilize stalled replication forks by modulating fork remodelling and cellular nucleases simultaneously.

Third, ATR/CHK1 signalling maintains replisome stability. Replisome stability describes the stable association of the replisome components with the stalled replication fork. Since the final goal of fork stabilization is to restore replisome integrity and function, it is reasonable to assume that the replication checkpoint plays a role in stabilizing replisome components at the fork. Nevertheless, results from different studies, especially from yeast models or *in vitro* Xenopus systems, are hard to reconcile (summarized in references [8,30]). Some studies report decreased abundance of replisome components at stalled forks when the replication checkpoint is inactive, while others show that replisome stability is not regulated by the checkpoint. The discrepancy is mostly ascribed to the different methodologies that are used to analyse replisome proteins associated with stalled replication forks [8,30]. Earlier studies using ChIP-PCR focused on replication forks that fired early, which might be biased because replisomes at these forks may react to replication stress differently from others [184–186]. Indeed, later studies applying genome-wide ChIP-seq reveal that early firing forks still progress a distance from their origins under replication stress and that they travel further in checkpoint-deficient cells than in checkpoint-proficient ones [187]. This may explain why earlier ChIP-PCR designed for proximal regions of early origins detected reduced replisome components in the absence of checkpoint activity. Albeit the role of the replication checkpoint in replisome stability remains a matter of debate in yeasts, in human cells evidence is favouring that the replication checkpoint does not affect replisome stability at stalled forks, as shown by a recent study using iPond-MS to examine all replisome components simultaneously, and that found no significant change in replisome protein abundance after fork stalling [48].

To conclude, replication checkpoint activation sets an "emergency mode" for cells under replication stress, which promotes stabilization of stalled replication forks to preserve genome stability. Notably, many checkpoint inhibitors targeting ATR and CHK1 are already applied in clinical settings [24]. Therefore, combination of checkpoint inhibition and replication poisons such as PARP inhibitors seems to be a plausible strategy for cancer therapy. In fact, combined treatment with ATR and PARP inhibitors for advanced refractory solid tumours including recurrent ovarian cancer is under active clinical investigations (e.g. NCT03462342, NCT02723864) and represents the rational therapy design based on exaggerating replication fork instability to kill cancers, as will be described below.

Exploiting fork instability in cancer treatment

Stalled fork stabilization is highly important for the cells not only to avoid genome instability but also to promote survival. Hence, compromising fork stabilization mechanisms to confer synthetic lethality with chemotherapy- or oncogene-induced replication fork instability seems to be a promising strategy in cancer treatment. Also, as many cancers have some mutations in proteins mediating fork stability, they may become addicted to alternative pathways that are not required in normal cells. Targeting those alternative pathways also represents a potential strategy for synthetic lethality. Perhaps the most typical anticancer treatment that employs these strategies is PARP inhibitor-based chemotherapies. More than a decade ago, we and another group showed the strong synthetic lethality between PARP inhibitors and BRCA1/2 mutations [60,61]. The original explanation for the potent killing effects of PARP inhibitors on BRCA1/2-deficient cells is that combined loss of PARP and BRCA1/2 will cause severe DNA repair defects allowing for the accumulation of lethal levels of DSBs. However, characterization of a DSB repair-independent role for BRCA2 in fork stabilization leads to revelation of another mechanism underlying the hypersensitivity of BRCA1/2-deficient cells to PARP inhibitors [65,66]. As PARP inhibitors trap PARP on DNA to block DNA replication [22,188], cells will rely on BRCA1/2 to stabilize their stalled replication forks. When BRCA1/2 is defective, those stalled forks will be extensively degraded by MRE11, leading to genome instability and cell death (Fig 4) [65,188].

Although PARP inhibitors have shown great promise with FDA approval for BRCA1/2-mutated breast cancers and ovarian cancers, a major challenge is the acquired drug resistance that leads to cancer relapse (Fig 4) [188,189]. Clinically, patients who acquire resistance to PARP inhibitors frequently harbour secondary mutations in their mutated Brca1 or Brca2 genes, which restore the open reading frame and protein functions [189]. HR reestablishment is usually considered as the underpinning of resistance acquisition [189–191], but since stalled fork instability also contributes to the cytotoxic effects of PARP inhibitors as discussed above, it is likely that fork stabilization is also restored concomitantly in most cases. Interestingly, multiple studies have revealed that restoring fork stabilization alone without restoring HR can drive resistance of BRCA1/2-deficient cells to PARP inhibitors [15,18,120]. It is shown that loss of PTIP, MLL3/4 and CHD, which impairs MRE11 recruitment to stalled forks, restores fork stability and renders BRCA1/2-deficient cells resistant to PARP inhibitors [15]. Notably, cells resistant to PARP inhibitors are also tolerant to cisplatin and topotecan, indicating that stalled fork stabilization confers a general resistance to replication stress-inducing chemotherapeutics [15]. Depletion of the RAD51 antagonist, RADX, also restores stalled fork stability and chemoresistance in BRCA2-deficient cells, which is ascribed to enhanced association of RAD51 with stalled forks [120]. Stalled fork stability and chemoresistance are also induced by inactivating SMARCAL1, which abrogates fork reversal and thus avoids MRE11-dependent fork degradation [18]. Importantly, clinical data show that low expression of PTIP, RADX and SMARCAL1 correlates with poorer survival outcomes of BRCA1/2-mutated cancer patients, underlining the important role of fork stabilization in modulating chemosensitivities [15,18,120]. Since PTIP, RADX and SMARCAL1 are downregulated to mediate drug resistance, they are not easy to target but more suitable to be used as biomarkers for PARP inhibitor sensitivity. In contrast, as mentioned in the former sections, FANCD2 is upregulated in BRCA1/2-mutated cancers to confer PARP inhibitor resistance, which therefore can be targeted to enhance PARP inhibitor efficacies.

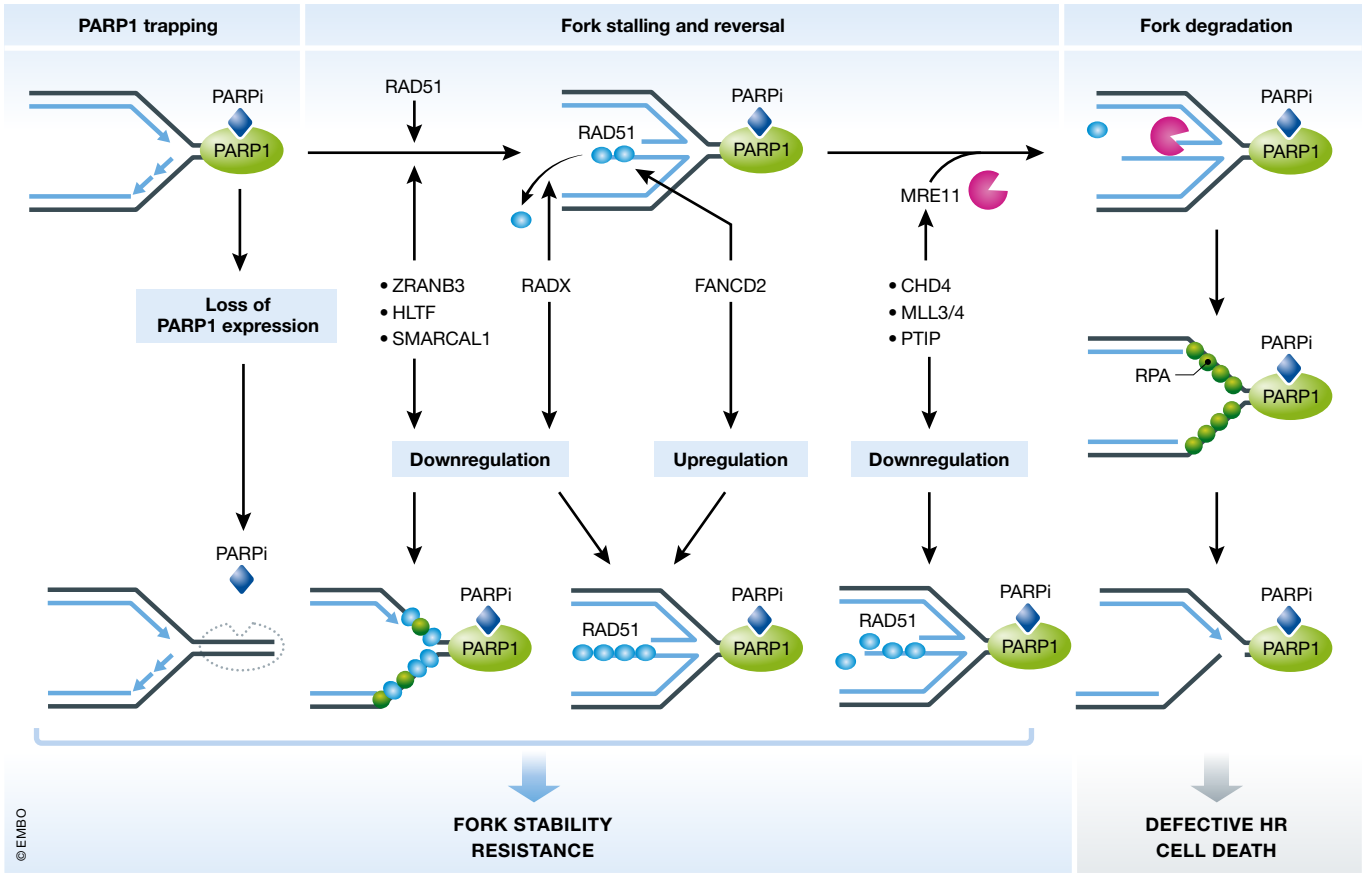

Figure 4. Mechanisms restoring stalled fork stabilization and PARP inhibitor resistance in BRCA1/2-mutated cancers.
PARP inhibitors (PARPi) can trap PARP on DNA, which impedes DNA replication and causes fork stalling. In BRCA2-mutated cancer cells, stalled replication forks are destabilized because of excessive fork degradation, which causes fork collapse and cell death. However, PARP inhibitor sensitivity can be altered by restoring stalled fork stability in some cases. First, through loss of PARP1 expression. As there is no target for PARP inhibitors to trap onto DNA, replication forks are less perturbed. Second, through inactivating SNF2 family fork remodelers. This closes the gate for nucleases by inhibiting fork reversal. Third, through downregulation of RADX. Since RADX promotes RAD51 displacement, loss of RADX results in stabilized RAD51 filaments, which suppresses fork degradation. Fourth, through increased FANCD2 expression. FANCD2 has a role in stabilizing RAD51 nucleoprotein filaments; therefore, its increased expression limits replication stress and promotes fork stability. Lastly, through loss of MRE11 facilitators. In this scenario, MRE11 recruitment to stalled forks is impaired, thus preventing fork degradation and fork destabilization.

Combination therapies involving PARP inhibitors designed to further increase the replication stress burden in BRCA1/2-mutated cancers are also under active clinical studies. However, a paradox about the application of PARP inhibitors in BRCA1/2-deficient cells should be noted. Since PARP1 is required for MRE11 recruitment to stalled forks [75], depletion of PARP1 by gene silencing or using PARP inhibitors prior to replication-stalling treatment restores stalled fork stability and even renders BRCA2-deficient mESCs viable [15,17,73]. In contrast, PARP inhibitors used together with replication-stalling agents increase stalled fork instability and cell death [66]. Though the underlying mechanism is unclear, it is imperative to corroborate whether these observations have implications for the mechanisms clinically driving resistance to PARP inhibitors in BRCA1/2-mutated cancers, such that we can optimize the use of PARP inhibitors within drug combination approaches. Recently, an alternative pathway has been found to fix the unprotected stalled forks in BRCA1/2-deficient cells, which may need to be taken into consideration when using PARP inhibitors. It was shown that resection of the regressed stalled forks assisted by RAD52 triggers MUS81-dependent fork breakage, which is subsequently repaired by the BIR pathway involving POLD3-dependent DNA synthesis [72,73]. The contribution of this pathway to the response of BRCA1/2-deficient cells to PARP inhibitors is currently controversial. Of two recent studies, one reports that MUS81 depletion sensitizes BRCA2-deficient cells to hydroxyurea but has no effects on PARP inhibitor sensitivity [72], while the other one shows that MUS81 inactivation confers resistance to PARP inhibitors in cells lacking BRCA2 [16]. The discrepancy could come from the different cell lines that are used in those two studies, though the latter seems to be more clinically relevant as low MUS81 expression is correlated with poorer survival of BRCA2-mutated ovarian cancer patients [16]. Also, it should be noted that MUS81 depletion does not affect the PARP inhibitor sensitivity of BRCA1-deficient cells [16], which is concordant with a recent finding that BRCA1 and MUS81 act in the same cleavage-coupled BIR pathway at stalled replication forks [140]. It will be necessary to clarify how the MUS81-dependent fork rescue affects the viability of BRCA1/2-deficient cells, such that we can exploit it to further enhance synthetic lethality. Interestingly,

Box 1: In need of answers

(i) How is RAD51 coordinated with other factors like SMARCAL1 to promote fork reversal? What is the specific role of RAD51 in this process?
(ii) How is RAD51 regulated to carry out HR and stalled fork stabilization in different contexts?
(iii) Which nucleases are responsible for fork degradation in different genetic backgrounds?
(iv) What is the aftermath of stalled fork stabilization in BRCA1/2-deficient cancer cells? What pathways are involved in translating fork stabilization into cell viability?
(v) Can fork stabilization alone drive resistance to PARP inhibitors in clinical treatment?
(vi) How will the complex effects of PARP1 on fork stability affect the long-term efficacy of PARP inhibitors for BRCA1/2-deficient cells, especially in combination therapies?

RAD52/MUS81-mediated BIR also operates during mitosis (namely MiDAS) to resolve stalled replication forks that persist into M phase, which promotes faithful disjunction of sister chromatids and cell survival under replication stress [192,193]. Because BRCA2-deficient cells are defective in DNA repair and replication fork protection, they accumulate high-level underreplicated DNA at the G2/M transition point even under unperturbed conditions and are hyperdependent on MiDAS for survival [194,195]. In this context, it is attractive to incorporate MiDAS inhibitors into the PARP inhibitor-based treatments for BRCA2-mutated cancers.

For those cancers without BRCA1/2 mutations, targeting fork stabilization mechanisms holds promise as well. For example, since checkpoint inhibition will cause stalled replication fork instability, it should confer synthetic lethality with PARP inhibitors and other replication stress-inducing agents. Also, it has been shown that PARP inhibitors sensitize cells to topoisomerase poisons, which is underpinned by stalled fork collapse in the face of SSBs [21]. Another fork stabilization mechanism involving RECQL5 has already been targeted in experimental models, which confers synthetic lethality with hydroxyurea for myeloproliferative neoplasms with JAK2 mutations [158].

Concluding remarks

Stalled replication forks are a major source of genome instability in proliferating cells, which need to be stabilized and restarted to promote cell survival. Decades of work have uncovered a multitude of mechanisms that preserve genome stability by protecting stalled replication forks under replication stress. On the one hand, these fork stabilization mechanisms represent important anti-tumour barriers that must be circumvented before a tumour can develop. On the other hand, they are also required by cancer cells to deal with replication stress induced by oncogene activation and/or chemotherapies. Therefore, the integrity of fork stabilization mechanisms plays an important role in modulating chemosensitivities. Based on this, strategies to exacerbate replication stress and/or to compromise fork stabilization mechanisms have been used in cancer treatment, which is represented by PARP inhibitor-based chemotherapies that efficiently kill BRCA1/2-deficient cancer cells. Also, drug resistance has been connected to replication fork stability, underlining the necessity of therapeutic targeting of fork stabilization mechanisms. However, progress in harnessing replication fork instability to improve anticancer efficacies remains slow, largely because of the limited understanding of the interconnections between different pathways and their contributions to cell survival under different genetic contexts (outlined in "In need of answers"). Future studies will need to gain a deeper insight into these questions for better exploitation of fork instability in cancer treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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