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Time for remodeling: SNF2-family DNA translocases in replication fork metabolism and human disease

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Abstract

Over the course of DNA replication, DNA lesions, transcriptional intermediates and protein-DNA complexes can impair the progression of replication forks, thus resulting in replication stress. Failure to maintain replication fork integrity in response to replication stress leads to genomic instability and predisposes to the development of cancer and other genetic disorders. Multiple DNA damage and repair pathways have evolved to allow completion of DNA replication following replication stress, thus preserving genomic integrity. One of the processes commonly induced in response to replication stress is fork reversal, which consists in the remodeling of stalled replication forks into four-way DNA junctions. In normal conditions, fork reversal slows down replication fork progression to ensure accurate repair of DNA lesions and facilitates replication fork restart once the DNA lesions have been removed. However, in certain pathological situations, such as the deficiency of DNA repair factors that protect regressed forks from nuclease-mediated degradation, fork reversal can cause genomic instability. In this review, we describe the complex molecular mechanisms regulating fork reversal, with a focus on the role of the SNF2-family fork remodelers SMARCA1, ZRANB3 and HLTF, and highlight the implications of fork reversal for tumorigenesis and cancer therapy.

1. Introduction

DNA replication is the process by which genomic DNA is duplicated during cellular proliferation. Over the course of DNA replication, obstacles to replication fork progression cause replication stress, thus resulting in genomic instability, an enabling characteristic for cancer development [1–4]. Replication stress is induced by a variety of endogenous sources, including unbalanced nucleotide pools, abasic sites, modified DNA bases (*e.g.*, oxidized or methylated bases, DNA crosslinks), DNA breaks, protein-DNA adducts, non-canonical DNA structures (*e.g.*, hairpins, G-quadruplexes, and trinucleotide repeats), RNA-DNA

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Conflict of interest

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hybrids (R-loops), replication slow zones (AT-rich regions or replication initiation poor sites), and collisions with transcription or non-histone DNA-bound proteins [5–7]. Replication stress can be further exacerbated by exposure to exogenous DNA damaging agents, including ultraviolet (UV) light, ionizing radiation (IR) and chemical compounds, such as DNA crosslinking and methylating agents (*e.g.*, cisplatin, mitomycin C, methyl methanesulfonate), topoisomerase inhibitors (*e.g.*, camptothecin) and inhibitors of nucleotide synthesis and DNA polymerase activity (*e.g.*, hydroxyurea, aphidicolin) [8].

While replication obstacles on the lagging strand are bypassed by the synthesis of a new Okazaki fragment, impediments on the leading strand may result in the arrest of replication fork progression [9–11] (Figure 1, step I). DNA lesions that block the leading strand polymerase can induce the formation of RPA-coated ssDNA regions [11–13], which in turn results in the recruitment of the ATR kinase through its interacting partner ATRIP [14]. Following recruitment and activation, ATR phosphorylates a multitude of factors involved in cell cycle regulation, DNA replication and DNA repair in order to arrest cell cycle progression, suppress global origin firing, activate local dormant origins, slow replication fork movement, and promote replication fork stability [15–17]. Subsequently, several pathways become engaged to restore DNA synthesis and ensure successful completion of DNA replication by: 1) allowing the direct bypass of DNA lesions (Figure 1, steps II–III); 2) pausing and eventually resuming fork progression once the obstacles have been cleared (steps IV–V); or 3) repairing forks that have collapsed (steps VI–VIII) [18–20]. Additionally, completion of DNA synthesis at sites of replication fork stalling can also be achieved through convergence of replication forks originating from previously activated or newly fired origins [21]. The coordinated action of these pathways depends on the type of DNA lesion, cell cycle regulation, and protein post-translational modifications (*e.g.*, PCNA ubiquitination) [17, 22]. Given the relevance of genome stability for human disease, understanding the crosstalk between the pathways that maintain replication fork integrity in physiological and pathological conditions is of utmost therapeutic relevance.

1.1. Lesion bypass

Certain DNA lesions, such as modified bases and DNA adducts, can be directly bypassed by translesion DNA synthesis (TLS) (Figure 1, step II). TLS is promoted by specialized DNA polymerases capable of DNA synthesis across DNA lesions. TLS polymerases are engaged at DNA damage sites through binding of monoubiquitinated PCNA mediated by their ubiquitin-binding motifs [23–26]. TLS requires the sequential action of two groups of DNA polymerases that insert the nucleotide across the lesion (Pol κ , Pol η , Pol ι , or REV1) and then extend the nascent DNA strand (Pol κ or Pol ζ) [19, 27–34]. Once the lesion has been bypassed, replicative polymerases can then resume DNA synthesis [35]. Distinct from the replicative polymerases, TLS polymerases are characterized by low processivity, efficiency and accuracy [32]. Lesion bypass can also occur by repriming of DNA synthesis downstream of DNA lesions (Figure 1, step III). Repriming is catalyzed by the DNA polymerase-primase PRIMPOL on templates containing DNA lesions (*e.g.*, cyclobutane pyrimidine dimers and (6–4) T-T photoproducts), chain-terminating nucleotide analogs, and non-canonical DNA structures (*e.g.*, G-quadruplexes) [36–43]. PRIMPOL presents weak affinity for DNA, thereby limiting its potentially error-prone repriming activity [39, 44].

Bypass of complex DNA lesions, such as DNA-protein crosslinks (DPCs) and interstrand crosslinks (ICLs), requires specialized machineries. DPCs and ICLs can be induced by exogenous sources, such as UV radiation and chemical agents (*e.g.*, cisplatin, mitomycin C), or result from endogenous sources, like formaldehyde [45–48]. Bulky DPCs impair replication fork progression by delaying CDC45-MCM-GINS (CMG) helicase movement, while ICLs prevent the unwinding of the DNA double helix [49, 50]. RTTEL1 facilitates the bypass of DPCs by the CMG helicase and is required for efficient DPC processing by the metalloprotease SPRTN [49]. MCM helicase complexes can also bypass ICLs at a single blocked replication fork through the actions of FANCD2, the BLM and FANCM helicases, and FANCM's interacting partners MHF1 and MHF2 [51–54]. Subsequently, when two converging forks are arrested by an ICL, ubiquitination of the CMG helicase by the TRAP1 E3 ligase controls whether the lesion is bypassed by TLS after ICL unhooking by the NEIL3 glycosylase [55–57] or is repaired by the Fanconi anemia pathway through ICL incision, DSB formation and homologous recombination (HR) [58–60].

1.2. Repair of collapsed forks

Forks irreversibly arrested by DNA lesions undergo MUS81/SLX4-dependent fork cleavage, which leads to DSB formation and fork collapse (Figure 1, step VI) [61, 62]. DSBs can be formed at single stalled forks or converging forks arrested by DNA lesions (*e.g.*, ICLs) [59, 63]. HR then promotes DSB repair and fork restart through RAD51-dependent strand invasion, followed by DNA synthesis aided by the MCM8–9 helicase in complex with HROB/MCM8IP [64–67] (Figure 1, steps VI–VIII). If unrepaired DSBs persist into late G2 and mitosis, they can be resolved by RAD52-dependent mitotic DNA synthesis (MiDAS) (Figure 1, step VII) [68]. RAD52 facilitates MiDAS by promoting microhomology-mediated strand annealing, followed by DNA synthesis dependent on the POLD3 subunit of Pol δ (Figure 1, steps VI–VIII) [68–70]. Interestingly, in yeast a Rad51- and Rad52-dependent pathway called break-induced replication (BIR) proceeds through the invasion of the broken end into a homologous template, followed by conservative DNA synthesis until the end of the chromosome promoted by the Pif1 helicase [71–76]. While MiDAS and the repair of DSBs resulting from oncogene-induced replication stress, or induced during alternative lengthening of telomeres (ALT), have been suggested to be similar to the BIR pathway described in yeast [68, 69, 77, 78], the molecular mechanisms of BIR-like processes in mammals remain to be fully defined.

1.3. Fork reversal

Fork reversal occurs when the parental fork DNA strands re-anneal, thereby extruding the nascent DNA strands to form a four-way structure, also known as “chicken foot” [18] (Figure 1, step IV). Fork reversal has been proposed to limit excessive ssDNA accumulation at stalled forks, reposition DNA lesions into a dsDNA context to enable their repair, and prevent synthesis across DNA single-strand breaks (SSBs) to avoid their conversion into deleterious DSBs [18]. In addition, fork reversal could enable error-free bypass of DNA lesions by allowing the arrested leading strand to utilize the lagging strand as a template for DNA synthesis through template switching, a pathway suggested to depend on PCNA polyubiquitination [79]. While lesion bypass mediated by fork reversal has been demonstrated *in vitro*, evidence of the occurrence of such events in mammalian cells

remains to be reported [20, 80, 81]. Nonetheless, recent studies using electron microscopy (EM) have enabled the visualization and quantification of reversed fork structures in replicating mammalian cells, demonstrating that exposure to endogenous and exogenous genotoxic stresses leads to an increase in fork reversal events [82–88]. Genetic and biochemical studies have shown that fork reversal is primarily catalyzed by the SNF2-family DNA translocases SMARCAL1, ZRANB3, and HLTF, along with the FBH1 helicase [86, 87, 89–96] (Figure 1, step IV). The RAD51 recombinase has also been proposed to have an HR-independent function in promoting fork reversal through mechanisms that are still unclear [83, 97]. In this review, we describe the functions of SNF2-family fork remodelers in the response to replication stress during physiological and pathological conditions.

2. SNF2-family DNA translocases and replication fork dynamics

The SNF2 family of proteins is structurally characterized by a SWI/SNF2 (switch/sucrose non-fermenting) translocase domain with a bi-lobed RecA-type ATPase configuration, which is present in the yeast protein Snf2 [98]. Functionally, SNF2-family members have been primarily involved in chromatin remodeling. The SNF2 family includes SNF2-like, INO80-like, SSO1653-like, RAD54-like, RAD5/16-like and SMARCAL1-like subfamilies [98, 99] (Figure 2). Below we describe the activities exhibited at the replication fork by the SMARCAL1-like and RAD5/16-like subfamily members SMARCAL1, ZRANB3 and HLTF.

2.1. SMARCAL1

SMARCAL1 (SWI/SNF-related, matrix-associated, actin-dependent, regulator of chromatin, subfamily A-like 1) is a distant member of the SNF2 family [98]. SMARCAL1 associates with the replication fork under unperturbed conditions, but becomes enriched at stalled forks upon replication stress through its direct binding to RPA mediated by an N-terminal RPA2 interaction motif [93, 100–105] (Figure 2). In addition to its RPA2-binding motif and SWI/SNF2 DNA translocase domain, SMARCAL1 contains two HepA-related protein (HARP) domains, which promote binding to synthetic fork-like structures [106]. Biochemical studies have shown that the HARP and DNA translocase domain of SMARCAL1 cooperate to promote ATP-dependent regression of fork-like structures through coordinated re-annealing of complementary strands [89, 91–93, 106] (Table 1). The fork remodeling activity of SMARCAL1 is stimulated by RPA, which targets SMARCAL1 to forks containing gaps on the leading strand [92, 107]. Besides remodeling fork-like structures, SMARCAL1 was also shown to promote Holliday junction branch migration and dissociation of D-loop recombination intermediates [91, 93]. The fork remodeling activity of SMARCAL1 has been confirmed *in vivo* by EM studies showing a 50% reduction in the number of reversed forks in SMARCAL1-deficient human cells [89]. In addition, loss of SMARCAL1 was shown to cause defective restart of stalled forks and delayed cell cycle progression following replication stress [100].

2.2. ZRANB3

ZRANB3 is related to SMARCAL1 and shares with it extensive similarity (44%) in the DNA translocase domain [91] (Figure 2). Distinct from SMARCAL1, ZRANB3 associates

with PCNA via a PIP box and an APIM motif, which are required for ZRANB3's recruitment to stalled replication forks [91, 108–110]. Moreover, ZRANB3 contains a NPL4 zinc-finger (NZF) motif that binds K63-linked polyubiquitin chains and cooperates with the PIP box and APIM motif to promote the association of ZRANB3 with polyubiquitinated PCNA [91, 108], suggesting a possible role for ZRANB3 in the template switching pathway. Biochemical studies have shown that ZRANB3 possesses ATP-dependent DNA translocase activity that promotes remodeling of synthetic fork-like structures and dissociation of D-loop intermediates [89, 91, 92, 108] (Table 1). In addition, ZRANB3 exhibits structure-specific endonuclease activity mediated by its HNH nuclease domain, which promotes ATP- and PCNA-dependent cleavage of splayed DNA duplex structures, generating 5' overhangs *in vitro* [108, 110]. These observations have suggested the possibility that ZRANB3 could utilize its fork reversal activity to facilitate the excision of DNA lesions at stalled forks [108]. ZRANB3's binding to DNA substrates relies on its sequence recognition domain (SRD), which is required for both fork remodeling and endonuclease activities [111]. Recent EM studies have shown that ZRANB3 requires its DNA translocase activity and its ability to associate with polyubiquitinated PCNA, but not its endonuclease activity, to catalyze fork reversal *in vivo* [86]. In line with its fork processing activities, ZRANB3 deficiency causes impaired fork slowing and defective fork restart in mammalian cells in response to replication stress [86, 91, 109].

2.3. HLTF

HLTF is a member of the RAD5/16-like subfamily (Figure 2). Analogous to the yeast Rad5 protein, HLTF promotes polyubiquitination of PCNA via its RING domain, suggesting a potential function in template switching [112, 113]. Similar to SMARCAL1 and ZRANB3, HLTF can also catalyze fork regression *in vitro* and *in vivo* through its DNA translocase activity [90, 94, 114–116] (Table 1). Besides HLTF's ATPase domain, its HIP116/HLTF Rad5 N-terminus (HIRAN) domain plays an important role in fork reversal by recognizing 3'-ssDNA ends formed at stalled replication forks and properly orienting the motor activity of HLTF [116–120]. HLTF has also been shown to promote strand invasion and D-loop formation *in vitro* in an ATP-independent manner, a mechanism that could act as an alternative to fork remodeling for promoting fork restart [121]. In line with these findings, loss of HLTF results in impaired fork restart and defective fork slowing upon replication stress [90, 94, 116], as observed in the case of ZRANB3 deficiency [86, 91, 109].

2.4. Interplay between SMARCAL1, ZRANB3 and HLTF at the replication fork

Although SMARCAL1, ZRANB3 and HLTF share the same ability to reverse stalled forks, they play non-redundant functions during replication stress [122]. For instance, SMARCAL1, but not ZRANB3 and HLTF, protects telomeres from replication stress and is required for efficient telomere replication [123–125]. At a molecular level, the above DNA translocases exhibit differences in their mode of action (Table 1). As discussed above, SMARCAL1 is an RPA-associated factor and is recruited to stalled forks by RPA [100, 101, 103–105], while ZRANB3 associates to sites of replication stress in a manner dependent on PCNA and its polyubiquitination promoted by HLTF and UBC13 [91, 108, 112, 113]. In agreement with these observations, RPA stimulates the fork reversal activity of SMARCAL1, while it inhibits ZRANB3-mediated fork remodeling [92]. Furthermore, the

fork reversal activity of ZRANB3 and HLTf, but not that of SMARCAL1, is thought to depend on PCNA ubiquitination [86, 126]. In addition to their different modes of activation, the above fork remodelers display distinct substrate specificities (Table 1). SMARCAL1 primarily acts on fork substrates containing leading strand gaps [92, 93]. Instead, ZRANB3 and HLTf remodel equally efficiently fork substrates with leading or lagging strand gaps, and HLTf has a strong preference for available 3'-OH groups near the fork junction [92, 116, 117, 119]. ZRANB3 may preferentially act upon forks stalled by damaged DNA bases that could be excised through its endonuclease activity [108]. These observations raise the possibility that SMARCAL1, ZRANB3 and HLTf might operate on distinct types of stalled forks induced in response to replication stress. These structures could arise independently from each other on distinct stalled forks or originate on the same fork during multiple remodeling attempts, especially under conditions where extensive fork remodeling is thought to occur (*e.g.*, BRCA1/2-deficient cells). It has been proposed that, under those circumstances, SMARCAL1, ZRANB3, and HLTf may act cooperatively on their preferred substrates generated on the same fork, and that failure at any point in these sequential steps could impair fork reversal [89, 122]. SMARCAL1 could, for example, generate fork substrates amenable to HLTf-mediated fork remodeling by pulling the leading strand closer to the fork junction, and HLTf could control ZRANB3-dependent remodeling by regulating the levels of PCNA polyubiquitination. However, these models remain to be proven and the dynamics between fork remodelers elucidated. Understanding these processes will likely require the development of novel approaches to study replication fork dynamics that overcome the limitations of currently available methodologies, as discussed below (Section 4.1).

3. Roles of SNF2-family DNA translocases in maintaining genomic stability and preventing the development of cancer and genetic syndromes

3.1. Fork remodeling and genomic instability

Fork reversal is a double-edged sword, given that both failure to reverse stalled forks and excessive fork reversal can result in genomic instability due to enhanced nucleolytic processing of stalled forks [18, 81, 87–90, 96, 103, 127–131]. Therefore, fork remodelers need tight regulation to prevent unscheduled fork reversal activity. One such regulatory mechanism is provided by ATR, which phosphorylates SMARCAL1 on serine 652 to restrict its fork remodeling activity [129]. Moreover, RAD52 has recently been suggested to inhibit SMARCAL1-mediated fork reversal by directly blocking SMARCAL1 loading on stalled forks and possibly competing with SMARCAL1 for the same RPA2-binding site [132, 133]. In addition, the nuclease EXD2 has been suggested to counteract SMARCAL1-mediated fork reversal through mechanisms that need to be elucidated [134]. As previously mentioned, RPA impairs ZRANB3 fork remodeling activity *in vitro*, presumably by sterically hindering ZRANB3's binding to DNA [92]. Furthermore, the helicase FANCF restricts HLTf activity at the fork [135]. In addition to the above negative regulators of fork remodelers, the RECQ1 helicase counteracts fork reversal by resetting regressed forks once lesion repair or fork pausing has been completed, thus allowing fork restart (Figure 1, step V) [88]. RECQ1-mediated fork restoration is inhibited by PARP1, which promotes fork reversal [84, 88, 136].

An alternative mechanism restricts genomic instability caused by excessive fork reversal, namely fork protection. When a fork is reversed, the nascent DNA extruded from the “chicken foot” structure is susceptible to degradation by nucleases, such as MRE11, DNA2 and EXO1 [87, 89, 96, 131, 137–148]. EXO1-mediated processing of stalled forks could be aided by the SNF2-family member SMARCAD1, a chromatin remodeler that promotes DSB resection [149–152]. Interestingly, EM studies have shown that regressed forks contain nucleosomes, indicating that chromatin remodeling may play an important role in fork resection [153]. While limited fork resection by the above nucleases can assist in the removal of the regressed arm of the reversed fork and/or promote HR-mediated fork restart, uncontrolled fork degradation can lead to chromosomal instability [63, 81, 138]. To prevent fork degradation, RAD51 nucleofilaments are assembled on the regressed arm of reversed forks [87, 96, 97, 128, 141, 142, 154–156]. RAD51 nucleofilaments protect stalled forks in an HR-independent manner, as indicated by the observation that a RAD51 mutant deficient for strand invasion is capable of fork protection [97]. Several DDR factors regulate the assembly and stability of RAD51 nucleofilaments to protect stalled forks from degradation. Among those, BRCA1-BARD1 and BRCA2 play an HR-independent role in fork protection by assembling and stabilizing RAD51 nucleofilaments (Figure 1, step IV) [87, 89, 96, 141, 142, 144]. Recent studies have suggested that PIN1-dependent isomerization of the BRCA1-BARD1 complex allows enhanced binding of RAD51 and fork protection [157]. Interestingly, the BRCT domain of BARD1 exhibits an exclusive role in fork protection by recruiting the BRCA1-BARD1 heterodimer to stalled forks in a poly(ADP-ribose) (PAR)-dependent manner [144]. RPA phosphorylation, on the other hand, is thought to favor the recruitment of PALB2 and BRCA2 to stalled forks [158]. BRCA2 mediates fork protection through a RAD51 interaction site that, when mutated, renders BRCA2 unable to prevent fork degradation, despite being still competent for HR [142, 159]. Additionally, BRCA2 requires its DNA binding domain for fork protection and suppresses both MRE11- and DNA2-mediated fork degradation [142, 160]. Other DDR factors, such as FANCA, FANCD2, the RAD51 paralogs (XRCC2 and RAD51C), PDS5B, WRNIP1, ABRO1, and the SETD1A-BOD1L complex, also contribute to protecting stalled forks from nuclease-mediated degradation [141, 143, 145, 146, 161–164]. Distinct from these factors, the recently identified RADX protein has been shown to promote MRE11- and DNA2-mediated fork degradation by antagonizing RAD51 at stalled forks [127, 128].

Fork protection can also be achieved by nuclease inhibition or exclusion from nascent DNA. For instance, the SNF2-family member ATRX has been suggested to protect stalled forks from MRE11-dependent degradation through MRE11 sequestration, similar to its role at telomeres [165, 166]. Moreover, the phosphatase complex RIF1-PP1 negatively regulates the WRN-DNA2 complex to limit over-resection of stalled forks [139, 140]. Failure to protect forks from extensive degradation can lead to fork collapse and DSB formation induced by MUS81/SLX4-dependent fork cleavage [89, 131] (Figure 1, step VI). This observation is supported by the fact that MUS81-depletion prevents DSB formation after fork stalling similarly to deficiency of either MRE11 or EXO1, suggesting nucleolytic processing of reversed forks prior to cleavage [131]. MUS81-dependent fork processing has been shown to be promoted by the histone methyl-transferase EZH2 specifically in BRCA2-deficient cells, implying different roles for BRCA1- and BRCA2-mediated fork protection [167]. In

addition, WRNIP1 protects stalled forks from SLX4-mediated processing to prevent unnecessary fork collapse and DSB formation [143, 168]. In the absence of fork protection mechanisms, as in BRCA1/2-deficient tumors, limitation of fork reversal becomes crucial to prevent extensive fork degradation and collapse [87, 89, 96], and consequent accumulation of DNA damage and chromosomal instability (Figure 3) [87, 89, 134, 141–143].

Failure to resume DNA synthesis after fork stalling/collapse leads to the accumulation of under-replicated regions and DNA breakage [169, 170] (Figure 3). Accordingly, difficult to replicate regions, such as common fragile sites, telomeres, and centromeres, are prone to the formation of sister chromatid bridges induced by replication stress [170]. The SNF2-family ERCC6L (PICH) DNA translocase is involved in the resolution of sister chromatid bridges due to under-replication, and its loss leads to micronuclei, chromosomal aberrations and cell death [171–173]. Overall, these findings indicate that proper processing of stalled/collapsed forks is critical for preventing genomic instability.

3.2. The role of fork remodeling in genetic disorders and cancer

Mutations in SNF2-family fork remodelers have been associated with human disease (Table 1). In particular, biallelic mutations in *SMARCAL1* cause the autosomal recessive disorder Schimke immuno-osseous dysplasia (SIOD), which is characterized predominantly by spondyloepiphyseal dysplasia, growth retardation, T-cell immunodeficiency and renal failure [174–178]. More recently, mutations in *ZRANB3* have been associated with an African-specific type-2 diabetes, implicating *ZRANB3* in glucose response and insulin secretion in pancreatic β -cells [179]. It remains, however, to be determined whether these disorders result from deficiencies in fork remodeling or alterations of other biological processes (*e.g.*, transcription) controlled by *SMARCAL1* and *ZRANB3* (Table 1).

Consistent with the role for DDR factors in suppressing tumor development [17, 180], loss-of-function mutations and down-regulation of SNF2-family members have been described in several human cancers (Table 1). *HLTF* is frequently silenced by promoter methylation in human colorectal and gastric cancers [181, 182]. Interestingly, while *Hltf* loss is insufficient to induce cancer development in mice, it cooperates with *Apc* deficiency to enhance colon cancer formation [183]. Paradoxically, *HLTF* is also commonly amplified and overexpressed in several cancer types, including esophageal, uterine and various types of squamous cell carcinoma [184]. *HLTF* overexpression is also associated with increased cancer metastatic potential and poor prognosis in non-small cell lung cancer patients [185]. While the precise function of *HLTF* amplification in cancer has not been fully investigated, studies conducted in yeast have shown that overexpression of Rad5, the *HLTF* yeast ortholog, induces replication fork instability [184], in line with the observation that alterations in the levels of fork remodelers cause DNA damage [103, 122]. Additional studies have reported a role for *ZRANB3* as a putative tumor suppressor in endometrial cancer [186], whereas *SMARCAL1* mutations have been described in a subtype of glioblastoma carrying wildtype *TERT* promoter and *IDH1/2* genes and exhibiting alternative lengthening of telomeres (ALT) [187]. *ATR*X has also been found mutated in ALT-positive cancers, including gliomas, neuroendocrine tumors and various sarcomas [188–192]. Furthermore, *CHD4* mutations are

associated with serous endometrial carcinomas and *ERCC6L* is significantly mutated in breast and kidney cancer [193–195].

In agreement with their role in maintaining fork stability in response to replication stress, SMARCAL1 and ZRANB3 promote resistance to chemotherapeutic agents that induce replication stress (*e.g.*, DNA crosslinking and methylating agents, topoisomerase inhibitors) in human and mouse cancer cell lines [91, 100, 101, 103, 108, 109] (Table 1). Distinct from SMARCAL1 or ZRANB3 loss, HLTf deficiency in the osteosarcoma U2OS cell line causes resistance to replication stress-inducing agents, such as HU and mitomycin C [90]. Accordingly, while loss of SMARCAL1 or ZRANB3 induces the accumulation of DNA damage and formation of DSBs after replication stress, HLTf deficiency results in decreased DSB induction and reduced DNA damage checkpoint signaling upon HU treatment, possibly contributing to the observed proliferative advantage of HLTf-deficient cells under replication stress [86, 90, 93, 100, 103, 109]. Based on these considerations, small-molecule inhibitors of SMARCAL1 and/or ZRANB3 could provide novel anti-cancer therapies. Inhibition of SMARCAL1 and/or ZRANB3 could be particularly effective in tumors with elevated replication stress induced by oncogenes. It was indeed shown that loss of *Smarcal1* or *Zranb3* increases replication stress and cell death induced by c-Myc overexpression and that *Zranb3* deficiency reduces Myc-driven B-cell lymphomagenesis [196] (Table 1). In addition, loss of *Smarcal1* was shown to reduce lymphomagenesis induced by ionizing radiation [197].

Distinct from the above observations, loss of SMARCAL1 or ZRANB3 in *BRCA1/2*-deficient mammary epithelial cells restores fork protection and reduces DNA damage and DNA break formation without re-establishing HR [89, 159] (Table 1). Similar findings were also obtained for HLTf depletion in *BRCA1/2*-deficient cells [89]. However, loss of SMARCAL1 does not restore cellular viability in *BRCA1/2*-deficient mammary epithelial cells upon treatment with replication stress-inducing agents [89], consistent with the observation that re-establishment of fork protection is not sufficient to suppress the growth defect exhibited by *BRCA2*-deficient mammary epithelial cells [159]. Notably, loss of fork protection is not sufficient to cause *BRCA1/BARD1*-deficient tumors [144]. It remains, however, to be investigated whether loss of fork protection is necessary for *BRCA1/2*-mutant tumor development. In such a scenario, re-establishment of fork protection through inhibition of SNF2-family fork remodelers might lead to a reduction of *BRCA1/2*-mutant tumor formation.

In contrast with the above findings in mammary epithelial cells, re-establishment of fork protection in *BRCA1/2*-deficient tumors has been associated with resistance to chemotherapeutic agents [89, 127, 167, 198]. In particular, loss of SMARCAL1 has been shown to restore chemoresistance in *BRCA1*-deficient breast cancer cells [89] (Table 1). Similar observations have been reported for CHD4, whose lower expression levels significantly correlate with poorer prognosis in patients with *BRCA2*-mutant tumors [198, 199]. Chemoresistance in *BRCA1/2*-deficient tumors can also occur as a result of the restoration of *BRCA1/2* functionality, the re-establishment of HR-mediated DSB repair or the activation of compensatory DNA repair pathways in response to replication stress [200]. Recent studies have shown that the restart of stalled forks by PRIMPOL-mediated repriming

is an alternative to SMARCAL1- or HLF-dependent fork reversal [90, 201]. Remarkably, *BRCA1*-mutant ovarian cancer cells adapt to recurrent cisplatin treatments by activating fork repriming to avoid excessive nascent DNA degradation due to fork remodeling at the expense of ssDNA gap formation [201]. Besides fork reversal and repriming, TLS provides an additional mechanism to restart stalled forks. Notably, REV1-mediated TLS was recently shown to operate as an alternative pathway to HLF-dependent fork reversal [90]. Furthermore, aberrant TLS activation induced by FANCD1 deficiency was reported to reduce fork reversal and facilitate continuous DNA synthesis in the presence of HU, thus avoiding ssDNA gap formation due to repriming events [202]. ssDNA gaps formed following fork repriming can undergo resection and RAD51-mediated HR for post-replicative repair [203]. TLS can function alternatively to HR for post-replicative repair of ssDNA gaps generated during replication stress [156], and may therefore become essential in HR-deficient cells. Interestingly, genomic sequencing studies and analysis of mutational signatures upon treatment with genotoxic agents suggest that TLS is upregulated in *BRCA1/2*-deficient cells [204]. Understanding the complex interplay between fork reversal, repriming, TLS and HR in response to replication stress will be of paramount importance for developing novel cancer therapies.

3.3. Fork instability, innate immunity activation, and cancer immunotherapy

Innate immunity is an evolutionary conserved cell-intrinsic reaction to microbial pathogens, which involves multiple mechanisms, and sensor molecules. Cytosolic DNA sensing recognizes and reacts to foreign DNA found in the cytoplasm (*e.g.*, viral DNA), leading to immune responses. The cGAMP synthase (cGAS)–stimulator of interferon genes (STING) pathway has been identified as a critical component of DNA sensing [205]. Briefly, cytosolic DNA sensed by cGAS activates STING, which triggers the expression of interferon-stimulated genes (ISGs) and induces the inflammatory response [205, 206] (Figure 3).

Recent studies have shown that unresolved DNA damage derived from replication fork instability, telomere fragility, common fragile site expression and chromosome mis-segregation often results in the formation of micronuclei upon mitotic progression, which can be detected by cGAS, thus leading to STING activation and the induction of innate immune signaling [207–209]. Accordingly, micronuclei formed as a result of deficiency in *BRCA1/2* or *RAD51* activate innate immune signaling, and this response is exacerbated by PARP inhibitor treatment [210–214]. Notably, loss of the SNF2-family members *SMARCAL1* and *ERCC6L* has been associated with an increase of micronuclei formation [172, 215]. However, it remains to be determined whether deficiency of these factors leads to innate immunity induction.

In addition to micronuclei, short species of genomic DNA released into the cytoplasm during DNA replication and repair can also be recognized by cGAS, thereby triggering the activation of innate immune signaling. In particular, it was shown that cytoplasmic ssDNA derived from replication fork intermediates following HU treatment is degraded by TREX1 [216], a nuclease that suppresses innate immune signaling and maintains immune tolerance [217, 218], thus providing direct evidence that processing of stalled forks can generate cytoplasmic DNA and activate innate immunity. Additional studies have shown that the

innate immune response is activated also by cytoplasmic ssDNA generated upon treatment with other replication stress-inducing agents, such as mitomycin C and cisplatin, or in response to IR [219]. Of note, depletion of nucleases such as DNA2 and EXO1, but not MRE11 and CtIP, restrains innate immune signaling, suggesting that ssDNA molecules that induce innate immunity require extensive DNA-end resection activities [219]. In addition, loss of SAMHD1, a factor that stimulates the exonuclease activity of MRE11 at stalled forks, was shown to prevent nascent DNA degradation and stimulate the helicase and endonuclease activities of RECQ1 and MRE11, respectively, thus resulting in the accumulation of cytosolic ssDNA fragments and the subsequent activation of the cGAS-STING pathway [220]. Cytoplasmic ssDNA fragments deriving from stalled replication intermediates and cGAS-STING-dependent innate immune signaling have also been observed in *BRCA1*-deficient breast cancer cells, although it remains unclear whether this phenotype is dependent on defective fork protection, as in the case of RAD51-depleted cells [212, 221]. Interestingly, it was reported that prostate cancer cells accumulate cytoplasmic dsDNA derived from nascent DNA in a manner dependent on MUS81, suggesting that it may originate from the cleavage of stalled forks [222]. Further studies are required to understand the relationship of fork remodeling with cytosolic DNA sensing and inflammation and evaluate whether inhibition of SMARCA1, ZRANB3 and HLTF may stimulate the cGAS-STING pathway. Overall, the above findings demonstrate that replication fork instability leads to aberrant inflammatory response in both malignant and non-malignant cells (Figure 3).

cGAS-STING-dependent innate immune signaling enhances tumor immunogenicity, and consequently the response of tumors to immunotherapies, such as immune checkpoint blockade (ICB) treatments that use antibodies inhibiting the immune checkpoint factors PD-1, PD-L1 and CTLA-4 [223–228]. During oncogenesis, PD-L1 expressed on tumor cells restricts T cell-mediated anti-tumor immunity by binding to the PD-1 receptor on tumor-infiltrating T cells, thus promoting tumor development [229–231]. In the context of ICB therapy, higher levels of PD-L1 on tumor cells correlate with better response to PD-1/PD-L1 antibody-mediated immunotherapy [232]. Notably, the expression of PD-L1 depends upon STING activation and is induced in response to replication stress [221]. STING activation following replication stress can also result in the expression of CXCL10, a chemo-attractive cytokine that correlates with high tumor-infiltrating cytotoxic T cells [210], as well as type-I interferon signaling, which promotes anti-tumor immunity [233–235]. However, recent studies have also shown that activation of the cGAS-STING pathway in chromosomally unstable tumors drives metastasis [236]. Understanding the connection between replication fork instability and the cGAS-STING pathway will therefore be critical for defining underlying mechanisms of anti-tumor immunity and metastasis, as well as predicting the response to ICB therapy in cancer patients (Figure 3).

4. Technologies to define the function of SNF2-family members in replication fork metabolism, genome integrity and human disease

4.1. Methodologies to study replication fork transactions: current limitations and novel approaches

Our ability to study replication fork transactions is challenged by technological limitations. The current gold-standard assay used to study replication fork dynamics in response to replication stress relies on DNA fiber and combing techniques [237–244] (Figure 4). These techniques utilize thymidine analogs to label the progression of replication forks, followed by the stretching of the DNA onto a glass coverslip to obtain DNA fibers with a resolution limit of 1 μm , corresponding to 2–4 kb [238, 239, 243–245]. In addition to having resolution constraints that do not enable the visualization of fork structures, DNA fiber/combing techniques are unable to discriminate between leading and lagging strand-dependent DNA synthesis. Unlike DNA fiber techniques, EM enables the visualization and quantification of replication fork structures with a resolution of ~30–50 base pairs, thereby permitting the discrimination of ssDNA gaps, reversed forks and other fork intermediates [83, 155, 246–248]. Despite its high resolution, EM only provides a snapshot of replication intermediates captured at a single time point. Moreover, both EM-based approaches and DNA fiber/combing techniques lack the ability to visualize proteins associated with distinct replication fork intermediates. Current approaches to detect proteins associated with replication forks rely primarily on the iPOND technology, which enables the identification of proteins bound to nascent DNA by western blotting or mass spectrometry [102, 249] (Figure 4). Although highly sensitive for identifying fork-associated proteins, iPOND technologies do not provide spatial information on the location of the identified factors. Recent studies led to the development of proximity ligation assay (PLA)-based approaches that allow the detection of proteins on nascent DNA in response to replication stress using standard microscopy [89, 250, 251]. To enhance signal detection, PLA-based applications can be combined with super-resolution imaging technologies, which have been shown to enable the mapping of chromatin-associated proteins around a replicating area [136, 252]. Future developments of super-resolution imaging may allow the direct visualization of reversed forks and other replication fork transactions, thus enabling the study of replication fork dynamics through live imaging-based analyses. To gain additional mechanistic insights into replication fork transactions, cell-based assays can be coupled to biochemical assays that employ *Xenopus* egg extracts [50, 253–255] or purified replication factors [9, 256, 257] to study replication fork progression *in vitro* with and without the presence of fork barriers. Alternative biochemical assays can be utilized to monitor the enzymatic activities of recombinant DNA replication or repair factors on fork-like substrates [91, 93, 94] and define DNA transactions using single-molecule technologies, such as DNA curtains [258–260] or optical tweezers [261–263] (Figure 4). These approaches will contribute to further define the function of DNA replication and repair factors in replication fork metabolism.

Standard DNA fiber/combing assays can discern global changes in fork progression, but lack the ability to detect alterations caused by specific genomic contexts. To overcome this limitation, DNA combing techniques have been combined with fluorescence *in situ* hybridization to monitor replication fork progression at single loci using SMARD (single

molecule analysis of the replicated DNA) [264–266] (Figure 4). SMARD allows the study of a limited number of genomic loci at a time. More recently, the use of optical mapping technologies have enabled the mapping of fluorescently-labeled replication tracts to genomic sites in human cells and *Xenopus* egg extracts [267, 268]. In addition, nanopore sequencing-based approaches, such as D-NAscent or FORK-seq, have been shown to discriminate thymidine analogue incorporation and identify DNA replication sites in yeast and mouse pluripotent stem cells [269–271]. We expect that further developments of optical mapping and nanopore sequencing technologies will enable genome-wide detection of site-specific replication events under normal or replication stress conditions in human cells.

Classical DNA fiber/combing assays are unable to discern the presence of DNA lesions on replication tracts, thus hindering the study of DNA lesion bypass and repair events. Recent studies have utilized digoxigenin-labeled psoralen to visualize DNA crosslinks on DNA fibers and study the bypass of those lesions [51] (Figure 4). Similar approaches might be possible using antibodies that detect DNA lesions (*e.g.*, antibodies that recognize cisplatin-induced DNA lesions). Alternative methods employing the *E.coli* Tus-*Ter* system have been utilized in yeast and mammalian cells to study DNA repair events occurring at forks stalled by a single, site-specific replication fork barrier [272, 273]. More recently, a nuclease-dead Cas9 has been demonstrated to be a programmable barrier for replication fork progression [274]. Combining site-specific fork barriers with PLA and next-generation sequencing may enable the discrimination of proteins recruited to a single stalled fork and the detection of repair events induced by fork blockage. Ultimately, the development of new techniques to study replication dynamics will provide important insights into the mechanisms that control replication fork reversal and other fork transactions.

4.2. CRISPR-based technologies to investigate the contribution of SNF2-family members to genome stability and human disease

The mapping of genetic interactors of SNF2-family members can provide novel insight into their contribution to genome stability and other biological processes, as well as identify potential therapeutic options to treat SNF2-associated diseases. Epistatic relationships identified by genome-wide genetic interaction screens in SMARCA1- or ZRANB3-deficient cells may potentially uncover new factors that promote fork reversal and/or identify biological pathways relevant to the suppression of multisystem developmental diseases, such as SIOD and diabetes. Furthermore, these same screens may also identify buffering interactions from which we could infer potential therapies to ameliorate disease symptoms, such as T-cell immunodeficiency in SIOD [275]. The identification of synthetic lethal partners of SNF2-family members may also provide supporting rationale for precision medicine in cancer treatment. For example, identifying synthetic lethal partners of ZRANB3 and HLTF may offer novel targets to treat endometrial and colorectal cancers, respectively [181, 186]. Moreover, identifying novel domains, separation-of-function mutations, and regulatory elements of the above SNF2-family fork remodelers may facilitate the classification of pathogenic mutations that contribute to human disease etiology.

The development of CRISPR-based technologies [276] has produced an expansive toolbox of genome editing reagents to define genetic interactions. Mapping of the genetic

interactions of the above SNF2-family members can be achieved through combinatorial CRISPR-based screening in a pooled format [277–282]. Beyond cellular viability in response to replication stress-inducing agents as a readout of genomic stability, recent advances in the application of single-cell analyses to pooled CRISPR-Cas9-based screens have enabled the acquisition of richer content for deeper phenotypic characterization of DNA replication and repair processes and more refined definition of genetic interactions. For example, *in situ* sequencing of single guide RNAs (sgRNAs) or their corresponding barcodes in intact cells [283, 284] enables the use of cellular markers of replication stress and genome instability in pooled CRISPR screens. These markers can include replication stress-associated γ H2AX and RPA foci, 53BP1 nuclear bodies, micronuclei, and DNA synthesis events in response to replication stress. Further advancements to CRISPR screening technologies may also allow the coupling of genetic screens with methods that monitor the association of proteins with nascent DNA, such as PLA-based assays [89, 250, 251]. As an alternative to these approaches, characterization of genetic interactions established by SNF2-family members can also be obtained by analyzing the transcriptional and chromatin state resulting from the deletion of SNF2-family members and their potential genetic interactors under different cellular conditions [285, 286]. These relationships can be elucidated by coupling CRISPR screening with single-cell RNA-seq (Perturb-seq, CROP-seq) and chromatin accessibility analyses (Perturb-ATAC) [285–288].

CRISPR-based technologies can also identify novel structure-function relationships of SNF2-family members beyond known domains that mediate nucleic acid-binding, protein-protein interactions or catalytic activity. To that end, the use of high-throughput saturation mutagenesis screens carries the potential to uncover novel functional domains, regulatory elements and separation-of-function mutations, as well as classify the pathogenicity of variants that occur in the human population. CRISPR-dependent base editing, and the recently developed prime editing are new technologies to study single nucleotide variants that have yet to be applied to systematic screening in human cells [289–291], but a saturation mutagenesis screen has already been conducted for *BRCA1* using CRISPR-mediated HDR to introduce 4000 variants into 13 coding exons [292]. This proof-of-concept study identified hundreds of novel non-functional variants of BRCA1 and allowed for the potential to predict pathogenicity of variants that were previously of unknown significance. Application of these technologies to the SNF2-family members is similarly expected to produce a rich resource of novel structure-function relationships.

5. Conclusions and future perspectives

Members of the SNF2-family have been implicated in maintaining genomic stability in response to replication stress and regulating the development of human diseases. Future studies will better define the interplay among SNF2-family members at the replication fork and the interconnection between fork reversal and chromatin remodeling. The development of new techniques to study replication fork dynamics will help refine our understanding of these and other aspects of replication fork stability. Given the multiple roles of SNF2-family members in DNA metabolism, CRISPR screens will play an important role in defining the precise contributions of those factors to genomic stability, as well as their relationship with different DDR factors and pathways. Further studies will elucidate the contribution of SNF2-

family fork remodelers to tumor development and the response to chemotherapy and immunotherapy. Ultimately, the development of small molecules able to efficiently inhibit the activity of specific SNF2-family fork remodelers may provide new cancer therapies and increase the therapeutic opportunities for cancer patients.

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Figure 1. Simplified schematics of the mechanisms of replication fork restart upon replication stress

When the replication machinery encounters a replication block (stop sign), replication fork progression stalls, leading to the formation of single-stranded DNA (ssDNA) that is rapidly coated by the RPA trimer (I). Restart of stalled forks can occur through lesion bypass by translesion synthesis or fork repriming (II-III). Translesion synthesis allows the direct bypass of the DNA lesion (II), while fork repriming promotes the restart of DNA synthesis downstream of the lesion (III). Alternative to lesion bypass, stalled forks can undergo reversal mediated by fork remodelers, including the SNF2-family members SMARCAL1, ZRANB3 and HLTF (IV). Fork reversal slows down replication fork progression, thus allowing sufficient time for repair of the lesion. Reset of reversed forks by the RECQ1 helicase can then promote the restart of DNA synthesis once the lesion has been removed (V). Fork reversal could also directly promote the bypass of the DNA lesion by enabling DNA synthesis on the nascent DNA strand of the sister chromatid (green) through template switching (not shown). Following fork reversal, the exposed ends of the regressed fork are stabilized by RAD51 to limit fork resection by the nucleases MRE11, DNA2 and EXO1 and protect the integrity of the fork (IV). Limited fork resection can facilitate fork restart through the reset of reversed forks or HR-dependent strand invasion catalyzed by the 3'-end of the resected regressed arm (not shown). BRCA1/2, RAD51 paralogs and Fanconi anemia proteins cooperate with RAD51 to control fork resection and maintain fork protection. Defective fork protection causes extensive fork resection and processing of the regressed fork by the endonucleases SLX4 and MUS81, leading to fork collapse (VI). Fork collapse can also result from MUS81-mediated cleavage of persistently arrested replication forks or from forks encountering single-strand DNA breaks. Collapsed forks are repaired by HR-

mediated fork restart pathways dependent on RAD51 and/or RAD52 (VI-VIII). Key factors involved in each pathway and described in the main text are indicated.

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Figure 2. The SNF2 family of proteins

Depiction of SNF2-family group relationships, based on alignments of the DNA translocase domain (adapted from Flaus *et al.* [98]). Schematic representations (not to scale) of protein domains characteristic of each subfamily in humans are shown. All SNF2-family members contain a DNA translocase domain consisting of DEXDc and HELICc domains. The SNF2-like subfamily consists of ISWI-like, CHD-like and SWI/SNF-like groups. The ISWI-like group includes SMARCA1 (1054 aa) and SMARCA5 (1052 aa). The CHD-like group includes CHD1 (1710 aa), CHD2 (1828 aa), CHD3 (2000 aa), CHD4 (1912 aa), CHD5 (1954 aa), CHD6 (2715 aa), CHD7 (2997 aa), CHD8 (2581 aa), CHD9 (2897 aa) and CHD1L (897 aa). CHD3, CHD4 and CHD5 have N-terminal plant homeodomain (PHD) fingers not represented in the schematic, while CHD1L lacks the N-terminal chromodomains and contains a C-terminal macrodomain (not shown). The SWI/SNF-like group includes SMARCA2 (1590 aa) and SMARCA4 (1647 aa). HELLS (838 aa) is closely related to the SNF2-like subfamily, but it does not contain domains typical of the members of this subfamily (not shown). The INO80-like subfamily includes INO80 (1556 aa), SRCAP (3230 aa) and EP400 (3159 aa), which contain additional A/T hook and SANT domains, respectively, and SMARCAD1 (1026 aa), which lacks the HSA domain and contains two N-terminal coupling of ubiquitin conjugation to ER degradation (CUE) motifs not represented in the schematic. The SSO1653-like subfamily includes BTAF1 (1849 aa), which contains multiple HEAT/ARM repeats, and ERCC6L (1250 aa), which harbors two tetratricopeptide repeat (TPR) motifs and a PICH family domain (PFD) of unclear function not represented in

the schematic. The RAD54-like subfamily includes RAD54 (747 aa) and ATRX (2492 aa), which contains an N-terminal ATRX-DNMT1-DNMT1L (ADD) domain not shown. The RAD5/16-like subfamily includes SHPRH (1683 aa), which contains a linker histone H1/H5 (H15) domain in addition to the domains represented in the figure, and HLTf (1009 aa). Finally, the SMARCAL1-like subfamily includes SMARCAL1 (954 aa) and ZRANB3 (1079 aa). SMARCAL1, ZRANB3 and HLTf (in red) are the only SNF2-family members that have been currently shown to regress stalled replication forks *in vivo*. HLTf contains a RING domain that mediates the interaction between HLTf and substrates to ubiquitinate. SMARCAL1 harbors an RPA2-binding motif to directly interact with RPA. ZRANB3 contains a PCNA-interacting protein (PIP) motif, an AlkB homolog 2 PCNA interacting motif (APIM) and a NPL4 zinc-finger (NZF) motif that cooperate to bind polyubiquitinated PCNA. The HIRAN, HARP and SRD (substrate recognition) domains of HLTf, SMARCAL1, and ZRANB3, respectively, enable structure specific DNA binding. The HNH motif of ZRANB3 is a nuclease domain.

Figure 3. Cellular effects induced by replication stress

Failure to complete DNA replication due to replication stress results in under-replication and sister-chromatid bridge formation. If the bridges are not resolved during anaphase, the sister chromatids remain attached to each other, resulting in chromosome mis-segregation, lagging chromosome formation, or chromosome breakage. Chromosomal breakage and mis-segregation results in the formation of chromosomal aberrations. Chromosomal fragments or lagging chromosomes that fail to be incorporated into the daughter cell nucleus form micronuclei. Errors during DNA synthesis and DNA repair processes can result in the inaccurate duplication of the genome and mutagenesis. Cytoplasmic DNA fragments and micronuclei originating upon replication stress may be recognized by the cGAS-STING pathway, leading to the induction of interferons, immune stimulated genes (ISGs), and immune checkpoint factors (*e.g.*, PD-L1). PD-L1 expression inhibits immune cell surveillance, leading to immune evasion and immunosuppression.

Figure 4. Methods for studying replication fork transactions

Overview of the current techniques used to (1) monitor replication fork dynamics and visualize replication fork structures, (2) identify proteins associated with and localized on nascent DNA, (3) analyze locus-specific replication fork progression, (4) monitor genomic replication at fork barriers, and (5) reconstitute *in vitro* replication fork transactions with and without the presence of barriers. DNA fiber and DNA combing techniques enable the analysis of the progression of individual replication forks, while electron microscopy allows high-resolution detection of DNA intermediates and replication fork structures, such as reversed forks and ssDNA gaps. Protein identification and localization on newly synthesized DNA can be obtained by iPOND, PLA-based approaches, and super-resolution microscopy methods, such as STORM. Techniques, such as SMARD, nanopore-based sequencing methods (D-Nascent, Fork-seq), and optical mapping enable the study of replication fork dynamics at genomic loci of interest. DNA lesions labeled with chemically modified adducts and/or antibodies or site-specific replication fork barriers, such as the *E. coli* Tus-*Ter* system, allow the analysis of events occurring when replication forks encounter obstacles. *In vitro* studies of replication fork transactions can be conducted using *Xenopus* egg extracts, which enable the study of replication fork progression, with and without fork barriers. Similar studies can be performed using purified replication factors that reconstitute DNA synthesis *in vitro*. Purified proteins can also be utilized to detect enzymatic activities on fork-like substrates or define DNA transactions at single-molecule level using DNA curtains or optical tweezers technologies combined with fluorescence microscopy.

Table 1.
List of functions of SNF2-family fork remodelers exhibited in biochemical assays, cellular and animal models, and human disease.

Citations of papers describing the indicated functions of SMARCAL1, ZRANB3 and HLTf are included in the table.

	HLTf	SMARCAL1	ZRANB3
Biochemical Activities	Activities <ul style="list-style-type: none"> • Fork reversal and restoration [90,94,114,116,117,120] • D-loop formation [121] • Ubiquitin ligase activity [112,113] Substrate preference <ul style="list-style-type: none"> • <i>DNA binding</i>: ssDNA and fork substrates with 3'-OH overhang [116–119] • <i>Fork reversal</i>: fork substrate with 3'-OH available in the vicinity of the fork branch point [116,117]; fork with gap on leading or lagging strand [117] • <i>Fork restoration</i>: fork with lagging strand gap (final product) [117] Inhibition by <ul style="list-style-type: none"> • FANCI [135] 	Activities <ul style="list-style-type: none"> • Fork reversal and restoration [87,89,91–93,106,107,129] • D-loop dissolution [91] Substrate preference <ul style="list-style-type: none"> • <i>DNA binding</i>: ssDNA/dsDNA junction with 3' or 5' ssDNA overhangs, fork structures [93,293] • <i>Fork reversal</i>: fork substrate with a leading strand gap bound by RPA [92,107] • <i>Fork restoration</i>: fork with lagging strand gap bound by RPA (final product) [92] Association with <ul style="list-style-type: none"> • RPA2 [100,103,104] Stimulation by <ul style="list-style-type: none"> • RPA [92,107] Inhibition by <ul style="list-style-type: none"> • ATR [129] • MCM10 [294] • RAD52 [133] 	Activities <ul style="list-style-type: none"> • Fork reversal and restoration [89,91,92,108,111] • D-loop dissolution [91] • Inhibition of D-loop formation [91] • ATP-dependent endonuclease activity [108,110,111] Substrate preference <ul style="list-style-type: none"> • <i>DNA binding</i>: fork structures [108,110] • <i>Fork reversal</i>: fork with gap on leading or lagging strand [92] • <i>Fork restoration</i>: fork with lagging strand gap (final product) [92] • <i>Endonuclease activity</i>: splayed duplex [108] Association with <ul style="list-style-type: none"> • PCNA and poly-ubiquitinated PCNA [91,108,109] Stimulation by <ul style="list-style-type: none"> • PCNA [110] (endonuclease activity) Inhibition by <ul style="list-style-type: none"> • RPA [92] (fork remodeling activity)
Cellular Functions	Functions <ul style="list-style-type: none"> • Fork reversal in response to replication stress [90] • Restart of stalled forks [94] • Suppression of PRIMPOL- and REV1-mediated restart of stalled forks [90] • Induction of PCNA polyubiquitination upon replication stress [112,113] • Stimulation of TLS [295,296] • Suppression of cellular resistance to HU [90,135] and MMC [90], promotion of cellular resistance to UV and MMS [112,113] • Suppression of replication stress-induced DSB formation [90] • Gene expression regulation [297,298] <i>BRCA1/2-deficient cells</i> <ul style="list-style-type: none"> • Promotion of replication stress-induced fork degradation and DSB formation [89] <i>FANCI-deficient cells</i> <ul style="list-style-type: none"> • Promotion of replication stress-induced fork degradation and suppression of DSB formation [135] • Promotion of MMC sensitivity and HU resistance [135] 	Functions <ul style="list-style-type: none"> • Fork reversal in response to replication stress [87,89,93] • Restart of stalled forks [100] • Telomere maintenance in ALT [123,125,215] and non-ALT cells [124] and ALT suppression [124,187] • Stimulation of NHEJ [299] • Promotion of cellular resistance to HU, aphidicolin, MMC, IR and camptothecin [100,101,103,197,299] • Suppression of DNA damage formation [100,101,103,124,300] • Gene expression regulation in response to replication stress and heat shock [300–305] <i>BRCA1/2-deficient cells</i> <ul style="list-style-type: none"> • Promotion of replication stress-induced fork degradation [89] <ul style="list-style-type: none"> • Induction of DNA damage [159], replication stress-induced DSB formation and genomic rearrangements [89] • Suppression of cellular resistance to replication stress-inducing agents in breast cancer cells, but not in mammary epithelial cells [89] • Suppression of PRIMPOL-mediated restart of stalled forks [201] <i>Myc-overexpressing cells</i> <ul style="list-style-type: none"> • Promotion of replication fork progression and suppression of fork collapse [196] 	Functions <ul style="list-style-type: none"> • Fork reversal in response to replication stress [86] • Restart of stalled forks [91,109] • Recognition of poly-ubiquitinated PCNA [91,108] • Suppression of hyper-recombination [91] • Promotion of cellular resistance to camptothecin, HU, cisplatin, MMC [91,109] and MMS [108] <i>BRCA1/2-deficient cells</i> <ul style="list-style-type: none"> • Promotion of replication stress-induced fork degradation [89,96] • Induction of replication stress-induced DSBs and genomic rearrangements in mammary epithelial cells [89]; suppression of genomic rearrangements in U2OS cells [96] <i>Myc-overexpressing cells</i> <ul style="list-style-type: none"> • Promotion of replication fork progression and suppression of fork collapse [196] <i>β-cells</i> <ul style="list-style-type: none"> • Promotion of insulin secretion in response to glucose [179]
Mouse Models	<i>HLTf^{del/del}</i> <ul style="list-style-type: none"> • Semi-lethal / neonatal lethal (deletion of exons 11–12) [297,298,306] or exhibiting 	<i>Smarcal1^{del/del}</i> <ul style="list-style-type: none"> • No developmental, growth, or physical abnormalities [305] 	<i>Zranb3^{-/-}</i> <ul style="list-style-type: none"> • No abnormalities [196] <i>Ep-myc transgenic mice</i>

	HLTF	SMARCA1	ZRANB3
	<p>normal development, fertile, and normal life span (deletion of exons 1–5) [183]</p> <ul style="list-style-type: none"> • Formation of carcinogen-induced colorectal cancer [306] <p><i>Apc^{min/+}</i></p> <ul style="list-style-type: none"> • <i>Hlhf</i> deletion increases intestinal adenocarcinoma invasion and malignancy [183] 	<ul style="list-style-type: none"> • Reduced B-cell count [196,305] • Reduced growth and weight and albuminuria upon RNA pol II inhibition [305] • Hypersensitivity to camptothecin, etoposide, and HU [301] • Increased survival, delayed T-cell lymphomagenesis, impaired T-lymphocyte reconstitution after IR [197] <p><i>Ep-myc transgenic mice</i></p> <ul style="list-style-type: none"> • <i>Smarca1^{del/+}</i>, but not <i>Smarca1^{del/del}</i>, mice exhibit increased myc-induced B-cell lymphomagenesis and decreased survival [196] 	<ul style="list-style-type: none"> • Loss of one or both <i>Zranb3</i> alleles inhibits myc-induced B-cell lymphomagenesis [196]
Human Disease	<p>Cancer</p> <ul style="list-style-type: none"> • Frequently silenced in colorectal and gastric cancers [181,182] • Overexpressed in esophageal, uterine, and squamous cell carcinoma [184] • Overexpression is associated with increased metastasis and poorer prognosis in non-small cell lung cancer [185] 	<p>Genetic syndrome</p> <ul style="list-style-type: none"> • Biallelic mutations cause Schimke immuno-osseous dysplasia (SIOD) [174,175,178,305,307] <p>Cancer</p> <ul style="list-style-type: none"> • Mutations identified in glioblastoma carrying wildtype <i>TERT</i> promoter and <i>IDH1/2</i> genes, and ALT-positive [187] 	<p>Genetic syndrome</p> <ul style="list-style-type: none"> • Mutations associated with African-specific type-2 diabetes [179] <p>Cancer</p> <ul style="list-style-type: none"> • Candidate tumor suppressor in endometrial cancer [186] • Mutations associated with carcinosarcoma [308]