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RIF1: A novel regulatory factor for DNA replication and DNA damage response signaling

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ABSTRACT

DNA double strand breaks (DSBs) are highly toxic to the cells and accumulation of DSBs results in several detrimental effects in various cellular processes which can lead to neurological, immunological and developmental disorders. Failure of the repair of DSBs spurs mutagenesis and is a driver of tumorigenesis, thus underscoring the importance of the accurate repair of DSBs. Two major canonical DSB repair pathways are the non-homologous end joining (NHEJ) and homologous recombination (HR) pathways. 53BP1 and BRCA1 are the key mediator proteins which coordinate with other components of the DNA repair machinery in the NHEJ and HR pathways respectively, and their exclusive recruitment to DNA breaks/ends potentially decides the choice of repair by either NHEJ or HR. Recently, Rap1 interacting factor 1 has been identified as an important component of the DNA repair pathway which acts downstream of the ATM/53BP1 to inhibit the 5′–3′ end resection of broken DNA ends, in-turn facilitating NHEJ repair and inhibiting homology directed repair. Rif1 is conserved from yeast to humans but its function has evolved from telomere length regulation in yeast to the maintenance of genome integrity in mammalian cells. Recently its role in the maintenance of genomic integrity has been expanded to include the regulation of chromatin structure, replication timing and intra-S phase checkpoint. We present a summary of these important findings highlighting the various aspects of Rif1 functions and discuss the key implications for genomic integrity.

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1. Introduction

1.1. Rif1 evolved from yeast to humans

Rap1 interacting factor 1 (Rif1) was identified in *Saccharomyces cerevisiae* as being important for the maintenance of telomeric length [1]. The repressor/activator protein 1 (Rap1) binds to telomeric repeat tract and maintain telomeric length by a negative feedback loop. Rap1 recruits Rif1 and Rif2 via its C-terminus, and the same domain facilitates the recruitment of the silencing protein Sir3p and Sir4p [1–3]. Later its orthologue was identified in *Schizosaccharomyces pombe.* Unlike budding yeast Rif1 (herein *ScRif1*), the fission yeast Rif1 (herein *SpRif1*) does not bind to Rap1 and is recruited to telomeres through a different telomeric protein Taz1, where it promotes telomere length homeostasis [2,4]. Rif1 is a part of the telomeric complex and its involvement in the inhibition of telomeric end resection has only recently been established [5,6]. Presence of Rif1 orthologues in vertebrates suggests important functions of Rif1 which has evolved in complex eukaryotic organisms [7].

High expression of mouse Rif1 was detected in totipotent and pluripotent cells, as well as in the testes [7]. Bioinformatic analysis reveals a characteristic HEAT repeat domain in Rif1 homologues found in yeast, invertebrates and vertebrates [8]. X-ray structural study of the yeast Rif1 has only recently been reported. This study reveals that Rif1 and Rif2 bind to Rap1 C-terminal domain via two independent Rap1 binding epitopes. Specifically, the C-terminal domain of Rif1 serves as Rap1 binding and tetramerisation sites [9]. However, a C-terminal protein interaction domain unique to the mammalian Rif1 is required for its interaction with BLM and the chromatin recruitment of BLM [10]. Unlike mammalian Rif1, drosophila Rif1 do not localize to sites of DNA damage, suggesting

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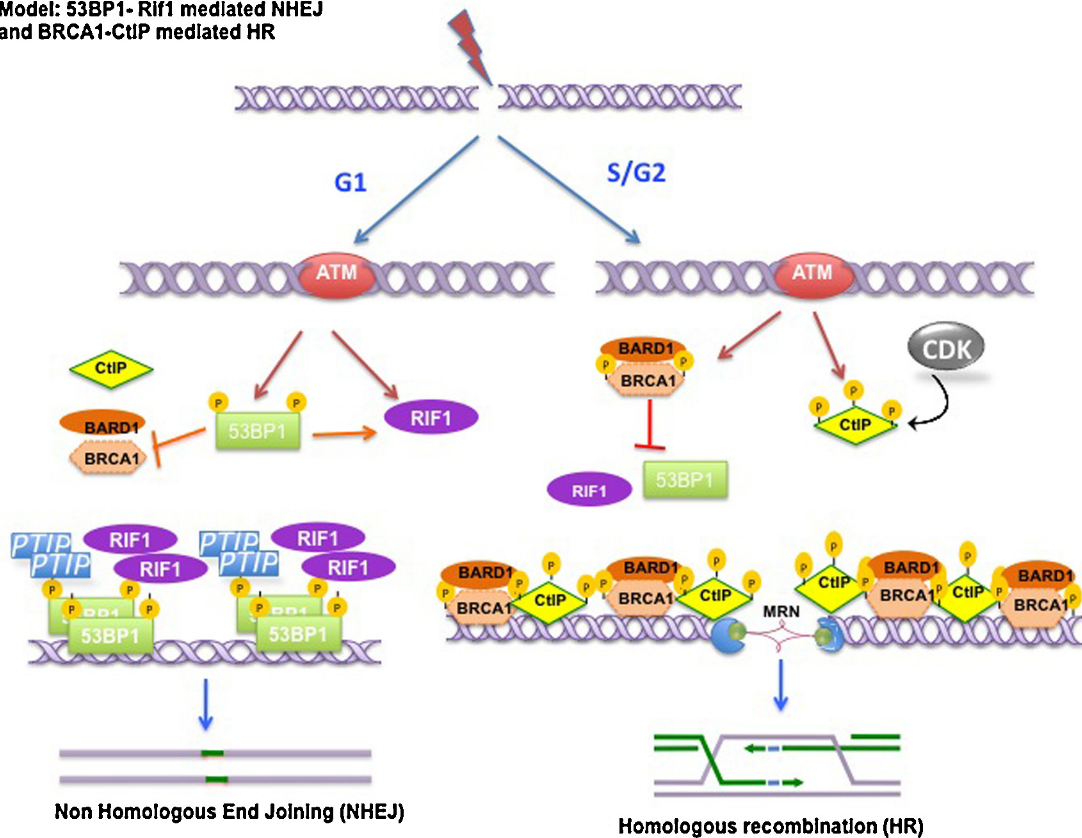

Fig. 1. 53BP1/Rif1 mediated NHEJ and BRCA1-CtIP mediated HR facilitates the DSBs repair during different phages of the cell cycle. In response to DNA double strand break, ATM dependent phosphorylation of checkpoint signaling molecules facilitate DNA double strand break repair by two main processes, NHEJ (favored in G1 cells) and HR (favored in S/G2 cells). Due to lack of a sister chromatid, 5′ end resection is suppressed and HR is inhibited in G1. RIF1 has been identified as a suppressor of BRCA1, a protein that facilitates the break resection. ATM phosphorylated 53BP1 recruits RIF1 which in turn inhibits the 5′–3′ end resection. ATM phosphorylated 53BP1 also binds to the RIF1 and PTIP via its C and N-terminus respectively. In BRCA1 deficient cells, in contrast to Rif1 depletion, PTIP ablation supports the continuous resection required to rescue the HR. During S/G2 phase of the cell cycle, ATM phosphorylated BRCA1/BARD1 complex is recruited at the sites of DNA DSBs, which in turn negatively regulates the RIF1 functions. CDK phosphorylation of CtIP promotes its interaction with BRCA1 and it also binds to the MRE11 complex to facilitate the nucleolytic resection of the 5′ end to generate the homology ends required for HR-mediated DNA DSBs repair.

a functional diversification of Rif1 in vertebrates and invertebrate [8]. Human Rif1 was identified by blast search where a significant similarity of RIF1 sequence was observed with *SpRif1* and *ScRif1* [1,4; Rif Gene Bank accession no. AY585745]. To understand the functional conservancy between *ScRif1* and human Rif1, Xu and Blackburn overexpressed human Rif1 in wild type and *rif2Δ* yeast cells which resulted in significant telomere elongation suggesting that the telomeric function of Rif1 is somewhat conserved from yeast to humans [11].

2. Rif1, an important component of DDR signaling

Unlike *ScRif1*, human Rif1 protein only binds aberrant telomeres. In human cells, uncapped telomeric ends are recognized as DNA damage site which promotes the recruitment of DDR factors including NBS1, ATM, 53BP1, Rad17 and γ-H2AX [12,13]. The pattern of foci generated at the aberrant telomeric ends is quite similar to DNA damage-induced foci and are referred to as telomere dysfunction-induced foci or TIFs [14]. Indeed, DNA damage-induced RIF1 foci formation was detected in cells treated with various DNA damaging agents including etoposide, hydroxyurea (HU) and ultraviolet light (UV), suggesting a potential role of human Rif1 in DDR signaling. Rif1 foci colocalise with 53BP1 foci but was completely abolished in 53BP1 depleted cells, attributing a significant role of 53BP1, a key player in the NHEJ pathway, to the regulation of Rif1 function [14]. Given the role of Rif1 in DDR signaling, recently many research groups are trying to understand the molecular mechanism of Rif1 function and so far their findings are quite encouraging.

2.1. Rif1 specifically acts in the ATM/53BP1 signaling pathway

Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) protein kinase signaling are central to the DNA damage response and repair pathways. DNA damage-induced Rif1 foci was reduced in ATM signaling defective cells, as demonstrated in two independent patient-derived AT cell lines. Moreover, Rif1 foci formation was severely reduced in cells treated with PIKKs (PI3 kinase-related family of kinases) inhibitors. Likewise, in the absence of 53BP1, IR induced Rif1 foci formation was reduced. However, in response to UV treatment, Rif1 foci formation was unaffected in ATRIP (an essential component of the ATR signaling) depleted cells suggesting an ATM dependent but ATR independent regulation of Rif1 function [14]. These findings clearly indicate that Rif1 DDR function is regulated by ATM and 53BP1 [14]. Rif1 depleted HeLa cells displayed normal levels of IR-induced ATM, Nbs1, Chk1, BRCA1, and p53 phosphorylation and IR-induced 53BP1 foci formation, however, these cells display increased radiosensitivity similar to ATM or 53BP1 deficient cells [15,16], suggesting that Rif1 do not directly affect the checkpoint signaling functions of the key DDR checkpoint proteins but may have a more direct impact on the repair processes. Fanconi anemia (FA) and ataxia telangiectasia (AT) share some common characteristics

of DNA damage sensitivity. In response to IR treatment, ATM dependent phosphorylation of the key FA protein "FANCD2" is essential for the activation of S phase checkpoint [17,18]. The observed radiosensitivity phenotype in the absence of Rif1 questions if Rif1 plays a role in Fanconi Anemia (FA) pathway. Rif1 depletion do not enhance sensitivity to the cross-linking agent mitomycin C (MMC), unlike FANCD2-deficient cells, hinting that Rif1 probably do not participate in the FA pathway [14]. Given that Rif1 depleted cells are deficient in intra-S phase checkpoint, it appears that Rif1 deficiency phenocopies the lack of ATM or 53BP1 proteins [14]. Interestingly, in xenopus egg extracts, Rif1 interacts with TopBP1, ATM and NBS1. [19]. However, the significance of this finding remains to be further clarified. Overall, these data concludes that Rif1 is an important component of ATM/53BP1 driven signaling cascade which specifically acts in intra-S phase checkpoint, contributing to the inhibition of DNA replication associated with ATM activation.

2.2. ATM/53BP1 mediated Rif1 function promotes the NHEJ mediated repair process in G1 cells

In response to DNA Double strand breaks, components of DDR signaling drives two main repair pathways NHEJ and HR. NHEJ is an efficient DSB repair mechanism that do not require excessive processing of the broken DNA ends and homology-directed base pairing with a DNA template and, is functional in all phases of cell cycle despite the risk for deleterious consequences. In contrast, HR is limited to S/G2 phase of the cell cycle, as HR requires an undamaged template DNA strand for base pairing and repair synthesis [20]. DNA repair is tightly coupled to cell cycle control partly via the interplay between cyclin-dependent kinases (CDKs) and DDR proteins [21–23]. In budding yeast CDK activity is specifically required to generate accurate processing and repair of DSBs during S/G2 phase, suggesting that prior to checkpoint activation, some DDR factors are activated by CDK phosphorylation [23,24]. Indeed recent study in budding yeast has shown that, regulation of the key DDR component Rad9 (human 53BP1) is coupled to cell cycle control. CDK phosphorylation of Rad9 promotes the DSBs processing [25]. CDK activity regulates various steps of the homologous recombination process [21]. In human cells, CDK2 inhibition leads to defective HR and delay in DSBs signaling [26]. In G1 cells, in the absence of sister chromatid and inadequate CDK activity, nucleolytic resection of 5′ end is inhibited which promotes the 53BP1-mediated NHEJ break processing [24]. However, in S and G2 phases, CDK phosphorylation of BRCA1/CtIP promotes the 5′–3′ DNA end resection which facilitates the HR process to repair the DNA DSBs [27]. For more detail please see Fig. 1.

Growing evidence suggests that 53BP1 binds to DSB and promotes NHEJ by suppressing the 5′ end resection of DSBs required for HR. Recent studies by several laboratories has elucidated the role of Rif1 in promoting NHEJ and inhibiting HR pathway, primarily through its recruitment by phosphorylated 53BP1 [28–30]. ATM phosphorylation of 53BP1 is required for the recruitment of Rif1 at DSBs. In this context, Rif1 acts as an effecter molecule of 53BP1 and directs the repair of DSBs toward the NHEJ pathway. Recent studies also suggested that like 53BP1, Rif1 is also required for joining DSB during Class Switch Recombination [29,31,35]. Damage-induced Rif1 foci is increased in G1 cells [30]. Upon cell cycle progression, CDK phosphorylation of CtIP facilitates its interaction with BRCA1 and activated BRCA1-CtIP complex inhibits Rif1 binding in S/G2 cells. CtIP-BRCA-1 complex is required to inhibit the recruitment of Rif1 at the sites of DNA lesion in S/G2, facilitating the initiation of DNA end resection for HR [30,32]. In BRCA1 deficient avian cells (DT40) and human cells, IR-induced Rad51 foci was increased in Rif1 depleted cells, to the same extent to that in 53BP1 depleted cells suggesting that Rif1 and 53BP1 may both suppress HR, in an epistatic manner. Notably, loss of ATM, Chk2 and p53 in BRCA1 deficient mice has been shown to reduce embryonic lethality [33]. Indeed, the sensitivity of BRCA1 deficient cells to PARP1 inhibitor was significantly rescued by the deletion of 53BP1 or Rif1 [29,34]. Loss of 53BP1 rescues the inviability of BRCA1 deficient mice by preventing the occurrence of deleterious toxic end products arising from the NHEJ/Ligase IV pathway [36,37]. These data indicate that BRCA1 prevent abnormal NHEJ by inhibiting 53BP1-RIF1 activities at the sites of DSBs.

To further provide mechanistic insights into the regulation of 53BP1/RIF1-mediated NHEJ and BRCA1 driven HR pathway recently several laboratories attempted to identify new components of DDR signaling pathways. Depletion of either Rif1 or 53BP1 resulted in significant increase in BRCA1 complexes at DSBs and no change in the foci level was observed in the double mutant. Consistently BARD1 foci accumulation at the aberrant telomeric end was also increased in these mutants [35]. PTIP (Pax Transactivation Domain-Interacting Protein) has been identified as an additional factor which is recruited at DSBs and is regulated by ATM/53BP1 signaling. PTIP and RIF1 are recruited independently by distinct 53BP1 phophorylation events. Despite recruitment by distinct phosphorylation of 53BP1, both PTIP and RIF1 recruitment are essential for NHEJ [38,39]. In the absence of BRCA1, PTIP is required for 53BP1-mediated inhibition of HR and largely dispensable for NHEJ during class switch recombination (CSR). In contrast Rif1 is absolutely required for CSR and only partially contributes to the HR defect [28,29,35]. Indeed Rif1 primarily appeared to play inhibitory role in the initiation DNA end resection and its activity is not sufficient to block the sustained resection [34]. This finding explains the fact why Rif1 depletion only partially rescues the HR in the absence of BRCA1. In contrast to Rif1, PTIP depletion promotes the extensive resection and rescues HR in the BRCA1 deficient cells. The relationship between 53BP1, Rif1 and PTIP is probably complex and requires more investigation to ascertain how the recruitment of Rif1 and PTIP is coordinated at DSB and how they may modulate or compete with each other to execute NHEJ [38].

2.3. Rif1 plays a vital role in the replication stress response

While fully viable ATM or 53BP1 knockout mice were born at expected mendalian ratio [16,40–42], however, Rif1 knockout mice resulted in embryonic lethality (Buonomo et al., 2009 unpublished data). This finding suggests that in addition to ATM/53BP1-dependent functions, Rif1 likely possess additional function(s) specifically needed in embryonic development. Mouse embryonic fibroblast (MEF) depleted of Rif1 display a partial sensitivity with IR and high sensitivity with HU treatment, suggesting a likely role of Rif1 in S phase regulation/replication stress response [14]. Investigation of the ATR signaling cascade reveals that Chk1 phosphorylation is elevated in Rif1 depleted cells in response to aphidicolin, indicating that Chk1 activation do not promote the inhibition of DNA end resection [43]. Importantly, end resection during HR generates ssDNA and ssDNA–dsDNA junctions that led to the conclusion that end resection is needed for sustained ATR/CHK1 activation. In the light of the data discussed earlier that Rif1 inhibits resection directly, it is foreseeable that loss of Rif1 results in an increase in resection activity, which could account for the increase in ATR/Chk1 signaling.

Increased phosphorylation of the Chk1 is a surrogate marker for increased replication stress and ATR kinase activity. Alternatively, the elevated Chk1 phosphorylation observed in Rif1 depleted cells may suggests the involvement of Rif1 in the sensing and/or repair of DNA damage occurring during replication stress. In line with that, Rif1 localizes at the site of stalled replication fork mostly at pericentromeric heterochromatin region. The colocalisation of Rif1 and BrdU foci at pericentromeric heterochromatin, well known to display increased frequency of replication fork stalling, suggests a role

for Rif1 at sites of fork stalling [43]. Both ATM and 53BP1 signaling is required to drive RIF1 recruitment at the IR-induced DSBs [14]. Rif1 localization at aphidicolin-induced ssDNA was intact in ATM defective MEFs but was compromised in cells lacking 53BP1 or ATR function, suggesting that both ATR and 53BP1 is required for the binding of Rif1 at the stalled replication fork. In MEFs, Rif1 depletion greatly reduces homology directed repair. Given that Rad51 is the key protein involved in strand invasion during homology directed repair, Rif1 deficient MEF cells were analyzed for endogenous Rad51 and 53BP1 by Co-IF. This data revealed that 15% of the RAD51 positive cells accumulated an aberrant Rad51 aggregates in Rif1 deficient cells [43]. In fission yeast it has been shown that Rad51 function is essential to suppress gross chromosomal rearrangement at centromeres [44]. So it is possible that Rif1 regulates Rad51 dependent HR at stalled replication fork in the genome.

2.4. Rif1 promotes BLM functions to maintain the genomic integrity

The BLM helicase is a part of multiprotein complex that maintains genomic integrity. 3′–5′ DNA unwinding activity of the BLM resolves a wide range of DNA structures including Holiday Junctions (HJs), replication forks, D loops and G4 DNA [45–49]. Mass-spectrophotometry analysis on the BLM protein complex revealed the 250 kDa Rif1 as an important component. BLM binding of Rif1 is absolutely dependent on its conserved C-terminal domain (which also harbor DNA binding activity) and is independent of the N-terminal Heat repeat region of Rif1 [10]. In response to replication stress, both $Rif1^{-/-}$ and $BLM^{-/-}$ DT40 cells display an increase in collapsed forks, to the same extent observed in $Rif1^{-/-}$ $BLM^{-/-}$ cells, suggesting that BLM and Rif1 may work in the same pathway to promote the recovery of stalled replication fork [10]. Other than NHEJ and HR, an alternative end joining (A-EJ) process has also been implicated in the repair of DNA DSBs. Indeed BLM, RIF1 and 53BP1 work in an epistatic manner for protection against long range deletions mediated by alternative end joining process [50]. BLM and Rif1 interact physically and recruited at the stalled replication fork with similar kinetics. DNA binding activity of the C-Terminal Domain (CTD) of Rif1 facilitates its recruitment at the stalled replication forks, where it promotes the efficient recovery of these forks resulting in cellular resistance to DNA damage [10]. BLM forms nuclear foci and its co-localization with RAD51 increased significantly in response to DSBs [51]. Rif also promotes the chromatin recruitment of the BLM, suggesting that in addition to the inhibition of BRCA1 mediated end resection, Rif1 has an important role in promoting BLM function in DNA DSB repair [34].

3. Rif1 – key regulator of replication timings

Evidences from both yeast and mammalian systems suggest that Rif1 functions go beyond telomere regulation, DDR signaling and determination of DSB recombination pathway. Indeed, recent reports demonstrated a global change in replication timings in Rif1-deleted cells, implicating Rif1 as a major regulator of the replication process. Rif1 colocalises with other DNA replication proteins during mid-S phase [52]. Replication foci pattern analysis in Rif1 deleted cells indicate a specific loss of mid-S replication foci. Strikingly, genome wide replication profiling in mammalian cells show that loss of Rif1 led to a change in replication patterns, with a shift in replication timing in over 40% of the replication segments, resulting in early-to-late and late-to-early replication timing changes. While overall S phase progression is not affected in Rif1 deleted cells, the replication domains appear to be fragmented when Rif1 is lost.

Chromatin is organized into chromosome segments known as "replication domains," which are highly regulated and the timing of early, mid- and late-replication is determined at a discrete point during early G1 phase ("timing decision point" TDP), where major chromatin repositioning and anchoring takes place. This creates chromatin loops which allow for the spatial and temporal organization of replication. Rif1 depletion causes increase in size of chromatin loops indicating a role in the regulation of higher order chromatin architecture. The influence of chromatin looping on gene transcription is recognized as a regulatory mechanism in developmentally regulated transcriptional programs for the fine-tuning of transcriptional regulation. Transcription changes brought about by chromatin looping which brings promoters and enhancers in close proximity are reported to result in massive global chromatin reorganization that occurs during differentiation of ES cells. Not surprisingly, transcriptomic analysis revealed a significant change in the transcription profile in the Rif1 depleted cells. More than 600 genes are either up regulated or down regulated indicating perhaps that the regulation of chromatin structures by Rif1 significantly alters gene transcription [52]. In fission yeast, deletion of Rif1 alters replication timing, resulting in suppression of early-firing origins and activation of dormant origins [53]. It is clear that DNA replication is initiated at pre-RC (replication complex) binding sites during S phase and the sequence and timing of origin firing from various pre-RCs are determined in M-G1 phase. In yeast phosphorylation of MCM proteins by CDK and Cdc7-Dbf4 activates pre-RC, followed by the loading of replisome factors including Cdc45, PCNA and DNA polymerases [54]. The level of Cdc7 dependent MCM protein phosphorylation was increased in Rif1 depleted cells while the binding of Mcm4 to origins is not affected. Interestingly, chromatin binding of Cdc45 and PCNA (Proliferating Cell Nuclear Antigen) was increased in Rif1 depleted cells, which is consistent with the enhanced action of Cdc7. Rif1 binds to chromosome arm segments during late M-G1 phase, coincident with TDP, when chromatin repositioning takes place in early G1 phase, thus raising the intriguing possibility that Rif1 may play a role during TDP in the temporal regulation of replication timing. ChIP-chip analyses show that 41% of the Rif1 binding sites overlap with the pre-RC sites. Together these findings propose that Rif1 influences both replication and transcription perhaps through its role in modulating the global nuclear architecture of chromatin [52,55]. For more detail information on role of Rif1 on replication timings, please read the recently published excellent review by Yamazaki and colleagues [56].

4. Concluding remarks

Rif1 is a highly conserved protein whose functions have diverged during the course of evolution, from its primary role in telomere length maintenance to a broader role in DNA replication, DNA repair and the maintenance of genomic integrity. Recent studies have highlighted Rif1 as an important factor in the regulation of replication timings in the mammalian genome. Replication timings might be a very crucial factor to coordinate between the number of the fired origins and the level of available dNTPs. The exact mechanism by which Rif1 achieve this is a topic of great interest. Indeed questions about how Rif1 regulates chromatin structure and if this function of Rif1 directly impacts upon replication timings requires more work to clarify. The role of Rif1 in DNA damage response has been implicated long time ago but unlike other DDR signaling components the molecular basis of Rif1 functions has only been recently deciphered by different research groups. ATM/53BP1 signaling is absolutely required for Rif1 localization at IR-induced DNA DSBs sites while ATR signaling is required for Rif1 binding to ssDNA at sites of stalled replication [43]. DNA Damage induced Rif1 foci is observed in G1 cells and upon cell cycle progression its abundance is inhibited by BRCA1/CtIP complex. Rif1 inhibits the

DNA end resection required for HR and Rad51 loading in BRCA1 deficient cells through a mechanism that involves a direct recruitment by phosphorylated 53BP1. In contrast, in S/G2 cells, CDK phosphorylation of CtIP promote BRCA1-CtIP mediated DNA end resection and prevent NHEJ through the removal of 53BP1-RIF1 from DSBs. Overall, Rif1 is a novel DDR protein that has diverse roles in G1 and S/G2 phases of the cell cycle in the repair of DSBs and the precise cell cycle dependent regulation of Rif1 functions remain to be investigated. Of note, the Rif1 protein expression level is significantly higher in cancerous lesions than those in benign lesions [57]. This observation indicates a potential link between Rif1 expression level and breast cancer and highlights a possibility of using Rif1 as a diagnostic marker for detecting tumor cells.

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Conflict of interest statement

The authors state no conflict of interest.

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