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Dissecting the role of BRCA2, Rad51 and SMARCAL1 in vertebrate chromosomal DNA replication

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Abstract

DNA replication is a fundamental macromolecular event that is essential for cell division. During each cell cycle the entire genome has to be precisely duplicated to ensure genome integrity and stability. In the process of DNA replication, replication forks encounter endogenous and exogenous lesions and these lesions have to be rectified to transfer stable genetic material to daughter cells. Emerging evidences connected a role for Homologous Recombination (HR) proteins to replication fork protection during unperturbed DNA replication. Since HR protein, BRCA2 and Rad51 are essential for cell survival we used *Xenopus laevis* cell-free extract to dissect the function of BRCA2 and Rad51 during chromosomal DNA replication. Using Electron Microscopy (EM) we show that BRCA2 and Rad51 function together to prevent single-stranded DNA (ssDNA) accumulation at and behind the forks during unperturbed DNA replication. We further discovered that BRCA2 mediates interaction between Rad51, Polymerase alpha (α) and Polymerase delta (δ) and this interaction likely promote efficient re-priming and polymerising activity at stalled replication forks to prevent ssDNA accumulation. Moreover we show that inhibition of replicative polymerases in the absence of Rad51 results in increased frequency of replication fork reversal activity. We further found that replication fork reversal is predominantly induced by an annealing helicase SMARCAL1 in the absence of Rad51. Collectively our findings indicate that, to prevent ssDNA accumulation and aberrant replication fork architecture, timely re-priming of Polymerase alpha mediated by Rad51 is essential. Hence, loss of Rad51 impact replication fork architecture, eventually resulting in chromosomal abnormalities.

Chapter 1. Introduction

1.1 Eukaryotic Chromosomal DNA Replication

DNA replication is the biological process by which cells duplicate their genome before cell division. Duplication of the whole genome has to happen once and only once per cell cycle to give rise to two daughter cells having the same set of chromosomes. DNA replication is spatially and temporally organised in different steps that involve the assembly and coordination of numerous replication factors. The DNA replication process can be divided in to three different steps: initiation, elongation and termination. The initiation step comprises the opening of the DNA double-strand at specific sites of the genome named replication origins and the loading of the replication machinery that starts DNA synthesis. During the elongation step bulk DNA synthesis is performed by specialized multi-protein complex named replisomes. Finally the termination step merges the chromosome regions replicated by different replisomes and complete the DNA replication process.

Although basic DNA replication dynamics as well as some DNA replication factors are conserved between prokaryotes and eukaryotes, the mode of replication factors assembly and activation is more complex in eukaryotes. One reason for this complexity in higher eukaryotes is the large size of their genomes[1] that are divided in multiple linear chromosomes. In the large eukaryotic genome, DNA replication is initiated at thousand different replication origins scattered throughout the genome. Origin activation is carefully regulated to ensure precise chromosomal replication[2]. Another important feature of multicellular organisms is the property of “plasticity”[3].

Plasticity could be better explained by taking metazoan's fertilized egg as a reference. For example in *Xenopus laevis* the fertilized eggs complete one single round of DNA replication very rapidly within ~30 minutes, whereas replication of the whole genome in somatic cells takes 8 – 10 hours[4]. This massive transition in replication timing (kinetics) between embryonic and somatic cells shows that DNA replication is tightly and temporally regulated in higher eukaryotes in different type of cells. Replication programs are defined in part by the DNA sequence, but they are mostly dependent on chromatin structure, in other words, they are epigenetically controlled[5].

Although it is essential to transfer error-free genetic material to daughter cells, the presence of exogenous or endogenous sources of DNA damage challenge the accuracy of DNA replication[6]. In order to protect genome integrity different repair and protection mechanisms are at play during and after DNA replication. Deregulation in DNA replication or repair mechanisms result in deleterious consequences for cells leading to genome instability that is a hallmark of cancer cells.

1.1.1 Origin selection: the binding of the Origin Recognition Complex

Replication origins are specific DNA regions where replication initiates. To ensure that the whole genome is completely duplicated, eukaryotes use a large number of replication origins (typically ~ 10^3 to 10^5) that are scattered throughout the genome[7]. Replication origin activation happens stochastically at different times during the synthesis (S) phase of the cell cycle[8-10]. The first step for origin selection is the binding of hetero -

hexameric Origin Recognition Complex (ORC) to replication origins. ORC directs the binding of accessory factors Cdc6 and Cdt1 to trigger the loading of hetero-hexameric replicative DNA helicase MCM2-7. The assembly of multi-protein complex on chromatin such as ORC, Cdc6, Cdt1 and MCM2-7 helicase named as pre-Replicative Complex (pre-RC) assembly or replication licensing. In yeast *Saccharomyces cerevisiae,* chromatin association of ORC is sequence-specific. Sequences of 17 base pairs (bp), known as Autonomous Replicating Sequence (ARS), act as a landing pad for pre-RC assembly[11-13]. However in metazoans no specific motifs for ORC association have been clearly identified so far[14]. Although the ORC complex is highly conserved from yeast to humans the selection of specific DNA sequences for its binding is not conserved across species[15]. In humans it has been shown that 50% of the active origins are found near or within CpG islands and most origins are strongly associated with transcriptional regulatory elements[16-19]. In agreement, chromatin immunoprecipitation (ChIP) experiments carried out with human cells show that ORC's are enriched at CpG islands and DNaseI hypersensitive regions such as open chromatin and nucleosome free regions[20,21]. In *Drosophila melanogaster* ORC binding is consistently favoured at nucleosome free regions[22]. This suggests that in higher eukaryotes ORC chromatin association is regulated in part by higher order chromatin structure and epigenetic marks.

1.1.2 Pre-Replicative Complex (Pre-RC) Assembly and its regulation

During late-M / early-G1 phase of the cell cycle hexameric ORC, MCM2-7 double hexamer, Cdt1 and Cdc6 bind onto chromatin to complete pre-RC

assembly (origin licensing) (Fig.1.1). In order to duplicate the genome once and only once per cell cycle, regulation of replication licensing reaction is crucial. During unperturbed DNA replication, sufficient origins organised in clusters are activated to complete whole genome duplication. Insufficient licensing or origin activation leads to under replication of the genome. Under replication describes a situation in which a specific segment of genome is left unreplicated[23]. Unreplicated segments of the genome tend to face chromosome breaks during the last steps of mitosis leading to genome instability. Hence precise regulation of replication origins firing is required to prevent chromosome instability. For instance, in cells, partial depletion of MCM proteins has no detectable consequences under normal conditions. However cells partially depleted for MCM proteins are hypersensitive to replication stress inducers, such as Aphidicolin (a DNA polymerases inhibitor) or Hydroxyurea (which deprivate dNTP pools)[24]. During unperturbed DNA replication, 10 to 20 times more MCM2-7 complexes are loaded onto chromatin with respect to MCM2-7 complexes that are being used. Only a few origins are fired and used during unperturbed S phase to complete DNA replication[25]. Thus many more potential origins are formed respect to what are actually used every round of replication. The potential origins that remain inactive are called “dormant origins”[4]. The pools of dormant origins contribute to the plasticity of the replication program, notably in the presence of DNA damage. In the presence of replication stress a specifically chosen pool of dormant origins are activated to enable the completion of DNA replication[26]. Inability to activate or insufficient dormant origins becomes a problem when two converging replication forks stall irreversibly and there

is no MCM helicase between them. In such cases replication of the intervening DNA is challenged.

Similarly, it is equally important that origins do not fire twice on the same replicated segment of the chromosome. When this happens this phenomenon is known as re-replication and it leads to irreversible genetic modifications[23]. Hence replication-licensing reaction functions only during late M – early G1 phases. MCM2-7 moves along the DNA together with the replisome and once a segment of the genome is replicated it dissociates from the chromatin leaving the replicated region in an unlicensed state. Furthermore, cells prevent relicensing and regulate replication initiation using multiple approaches. In metazoans re-licensing, and so re-replication, are predominantly prevented through the regulation of Cdt1 by its inhibiting interactor geminin or by proteolytic degradation[27]. Proteolytic degradation of Cdt1 takes place either by its interaction with Proliferating Cell Nuclear Antigen (PCNA) or by CDK dependent ubiquitination[28]. During the cell cycle, Cdt1 initially accumulates in G1, gets degraded in S phase and again peaks at late mitosis to early G1 transition to facilitate replication licensing[29]. In contrast, geminin is degraded during late mitosis to G1 phase by Anaphase Promoting Complex (APC) and peaks in S, G2 and early M phase of the cell cycle[30]. Collectively, the balance between geminin and Cdt1 levels are critical to ensure that origins fire only once per cell cycle.

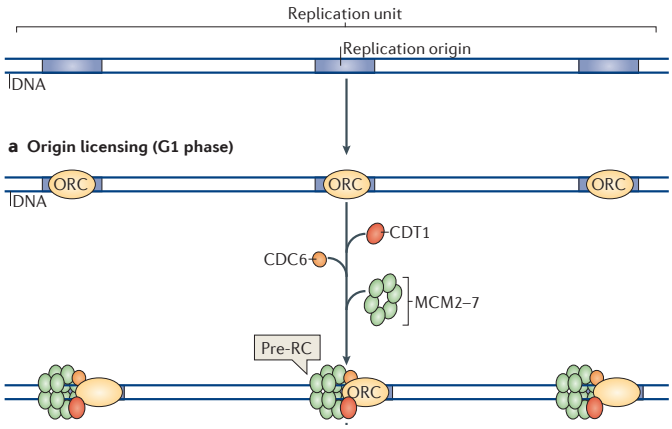


Figure 1.1 Origin placement and pre-replication complex assembly

This figure shows an example of three replication origins. Pre-replication complex assembly or licensing is restricted to late M – G1 phase of the cell cycle. Sequential loading of ORC, CDT1, CDC6 and finally MCM2-7 hetero hexamer completes the licensing reaction or Pre-replication complex assembly. (Picture taken from Fragkos M, 2015). Ref.[31]

1.1.3 Replication Fork Assembly

Once pre-RC is formed, the next step is the DNA helicase activation and replication initiation. At the beginning of S phase, licensed origins initiated by S phase promoting factor (SPF). SPF activity is constituted by two conserved kinases Dbf4-Dependent Cdc7 kinase (DDK) and S-phase Cyclin-Dependent kinase (S-CDK), which are involved in the activation of the replicative helicase MCM2-7 complex[32,33]. The major substrate for Cdc7 appears to be MCM2-7 and specifically the N-terminal tail region of MCM2, 4 and 6 are phosphorylated[34]. These modifications might induce conformational changes on licensing complex and allow the binding of Cdc45, a helicase co-factor[35]. Parallel to DDK, in yeast it has been shown that S phase CDK phosphorylates at least two proteins Sld2 and Sld3.

Phosphorylation of Sld2 and Sld3 facilitates their binding with another replication factor Dpb11 and formation of this complex allows the recruitment of the hetero-tetrameric GINS complex (composed of Sld5, Psf1, Psf2 and Psf3)[36,37]. Dpb11 contains four BRCT domains that are conserved across species. In general, BRCT domains have the tendency to bind to phosphorylated peptides[38]. TopBP1 is a metazoan homolog of Dpb11 that contain conserved BRCT domains[39]. RecQ4, GemC1 and Treslin all appear to be the functional metazoans homologues of yeast Sld2 and Sld3, whose function is critical for replication initiation[40,41]. RecQ4, GemC1 and Treslin's ability to interact with TopBP1, explains why these proteins are essential for DNA replication in higher eukaryotes.

In summary, the series of phosphorylations performed by CDK and DDK activates the MCM2-7 complex through the association with Cdc45 and GINS to form CMG complex (Cdc45, MCM2-7 and GINS), the actual replicative DNA helicase (Fig.1.2). These factors are collectively referred as pre-initiation complex (pre-IC). The CMG complex opens the double helix and enables DNA synthesis by DNA polymerases. Polymerase α (alpha) prime the synthesis, and Polymerase ε (epsilon) and δ (delta) are in charge of chain elongation on the leading and lagging strands, respectively (Fig.1.2 & 1.3) (See section.1.1.4). Initiation of replication or unwinding of DNA in co-ordination with several proteins results in replication fork structure assembly. A series of events enable chromatin association of Cdc45 and the GINS complex to activate the MCM2-7 helicase and initiate DNA replication. This process is referred as "origin firing".

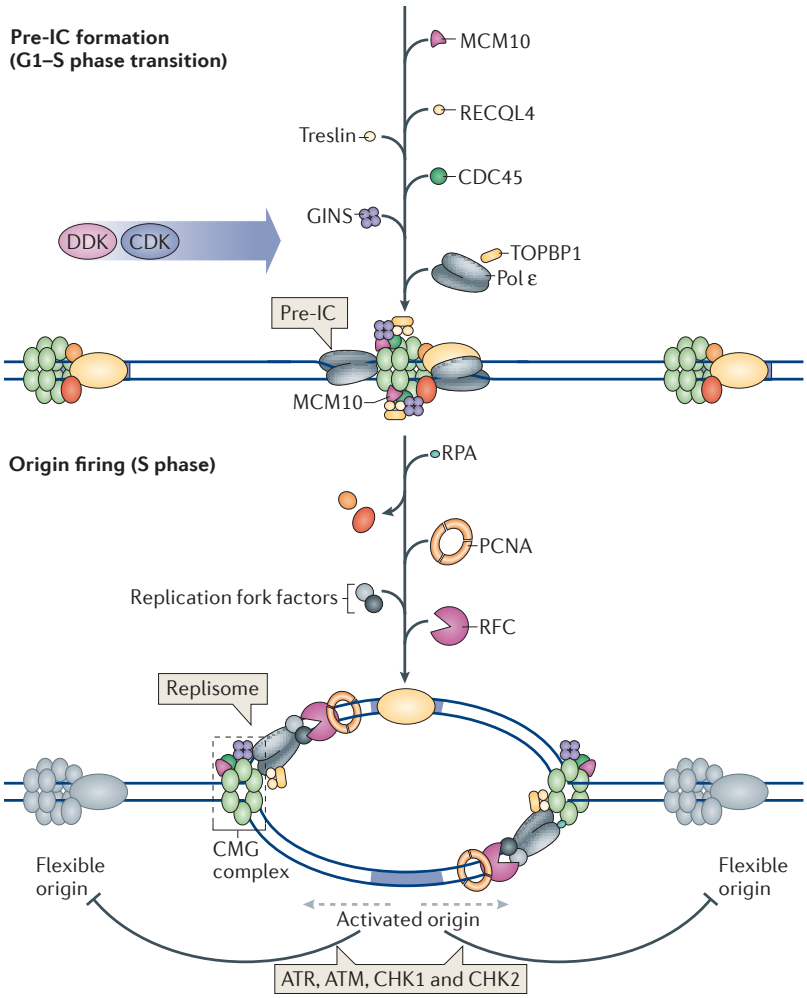


Figure 1.2 Replication Fork Assembly.

Replication fork assembly occurs when pre-initiation complex is formed by the activation MCM2-7 helicase. DBF4-dependent kinase (DDK) and Cyclin-Dependent Kinase (CDKs) phosphorylate several proteins to provide origin firing. In co-ordination with RPA, PCNA and with the action of replicative polymerases α, ε & δ replication fork assembly is established. (Picture taken from Fragkos M, 2015). Ref[31].

1.1.4 Replicative Polymerases in Chromosomal DNA replication

Following replication fork assembly, chromosomal DNA replication takes place by the synthesis of the nascent daughter strands using parental strands as templates. Nascent strand synthesis during unperturbed DNA replication in eukaryotes is mainly carried out by three DNA polymerases named Polymerase α, Polymerase ε and Polymerase δ. Upon unwinding of parental strand by MCM2-7 helicase, primarily the Polymerase α / Primase complex synthesises a short RNA primer of 7 - 14 nucleotides in length[42] and elongates them to a maximum of 20 deoxyribonucleotides[43] (dNTP's). Subsequently elongation from these primers is carried out by Polymerase ε and Polymerase δ in coordination with several proteins and nucleases to complete DNA synthesis of a given segment of the genome (Fig.1.3). DNA polymerising activity of Polymerase ε is carried out in a continuous fashion and hence the nascent strand synthesised by Polymerase ε is called as "leading strand". In contrast on the opposite template strand Polymerase δ synthesises DNA in a discontinuous manner hence named as "lagging strand"[44]. On the lagging strand the discontinuous synthesis is divided into Okazaki fragments of 100 – 200 nucleotides in length[44]. The directionality of polymerising activity of Polymerase δ and ε is 5' to 3'. If the leading strand is synthesized co-directionally with the movement of the fork, the lagging strand synthesise in opposite direction. Besides 5' to 3' polymerising activity, Polymerase δ also possess proof reading 3' to 5' exonuclease activity to minimize the incorporation of incorrect nucleotides being incorporated[45]. Due to discontinuous nature of DNA replication at the lagging strand, together with its proof reading activity polymerase δ displaces RNA primer to create

a 5' single-stranded flap structure[42]. DNA – RNA single stranded flaps have to be removed to allow intact Okazaki fragment maturation[42]. Removal of most of the RNA primer from the RNA – DNA fragment is performed by RNaseH except the last few ribonucleotides[42]. At this point another enzyme Fen1 removes the last ribonucleotide flap by its 5' exonuclease activity and subsequently DNA ligase I ligates two Okazaki fragments resulting in an intact double strand[46]. Recently it has been elegantly shown at the millisecond timescale that reconstitution of the Fen1/Polymeraseδ/PCNA/DNA complex is sufficient to remove completely RNA primer from the DNA – RNA template to promote Okazaki fragment maturation[46]. However, in the absence of Fen1, a protein called Dna2 can function in RNA primer removal. Dna2 is a helicase and a nuclease that localises onto Okazaki fragments and processes long flaps that are generated during normal replication or under stress conditions[47]. This compensatory mechanism protects the integrity of lagging strand synthesis during DNA replication.

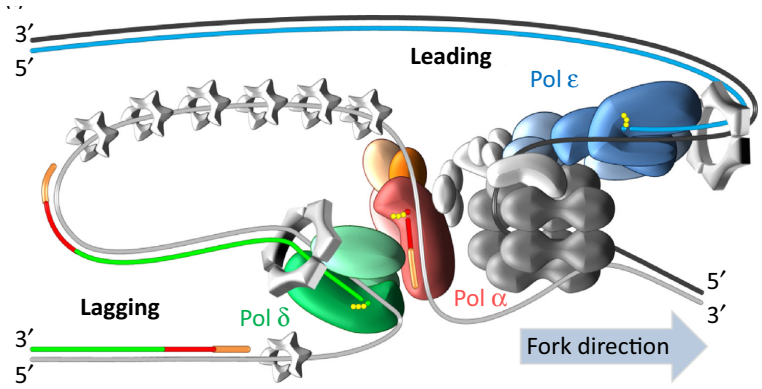


Figure 1.3 Replicative polymerases in chromosomal DNA replication

This cartoon shows the leading and lagging strand synthesis mode of the replication fork. The function of Polymerase α – primase complex that creates a short RNA primer (in yellow), extends ~ 20 deoxyribonucleotides by the Polymerising activity of Pol α (red). Further the lagging strand is extended by Polymerase δ and leading strand by Polymerase ε. As shown the directionality of both the Polymerases are 5' to 3'. (Picture taken from Lujan SA, 2016). Ref[48].

1.1.5 Role of Replication Fork Protection Complex in unperturbed and perturbed DNA replication

In recent years several accessory proteins such as Tipin[49], TIM1[49], Claspin[50], AND1[51], and MCM10[51,52] were discovered to be part of the replisome machinery (Fig.1.4). It has been shown previously that all these factors are required for efficient DNA replication and their function is conserved across species from yeast to humans[49-59]. Recent studies from yeast and *Xenopus* egg extracts showed that Tipin/Tim complex is required for precise duplication of difficult to replicate regions such as centromeric and telomeric segments[59-61]. Owing to the importance of these accessory factors in DNA replication they were named as

Replication Fork **P**rotection **C**omplex (RPC). RPC becomes indispensable when ongoing replication forks are stalled. During unperturbed DNA replication, functional coupling of MCM2-7 helicase and polymerases are essential to prevent ssDNA accumulation. Replication fork stalling refers to the inhibition of the progression of replicative polymerases eventually compromising the completion of DNA replication. Inhibition of replicative polymerases without blocking MCM2-7 helicase progression results in uncoupling of MCM2-7 helicase and polymerases[62]. Hence continuous unwinding of MCM2-7 helicase results in ssDNA accumulation. ssDNA regions are rapidly coated by the ssDNA binding protein RPA[63]. It has been shown that hyper-accumulation of RPA and continued priming activity is indispensable for Chk1 activation, when replication forks are stalled[64,65]. Activation of ATR - Chk1 induces the intra-S-phase checkpoint that will inhibit the late origin firing[66].

Tipin, Tim, Claspin, AND1 and MCM10 are implicated in ATR mediated Chk1 activation during fork stalling conditions. Especially it has been shown that Tipin is indispensable for DNA replication under “Minimal Licensing” condition, a condition when a minimal amount of MCM2-7 complex is recruited on chromatin[49]. Under conditions of minimal licensing, efficiency of DNA replication is same as under maximal (normal) licensing[67] during unperturbed DNA replication. In the presence of DNA damaging agents minimally licensed chromatin lack dormant origins to rescue stalled replication forks[68]. Impairment in DNA replication under minimal licensing in the absence of Tipin is notably due to the inability to recruit Polymerase α on chromatin to support DNA replication in *Xenopus* egg extracts[49,61]. Upon fork stalling, together with Tipin, Tim and AND1,

altogether collaborate to recruit and stabilise Polymerase alpha on chromatin to promote checkpoint activation[51,69]. Intra-S checkpoint is also promoted by factors such as Claspin[50] and MCM10[70]. All these factors collaborate directly or indirectly with RPA and Pol α primarily to stabilise ongoing or stalled replication forks and later to promote efficient checkpoint activation when required[51,69]. These factors are also involved in the cohesion of the replisome, by promoting the coupling of polymerases with helicases.

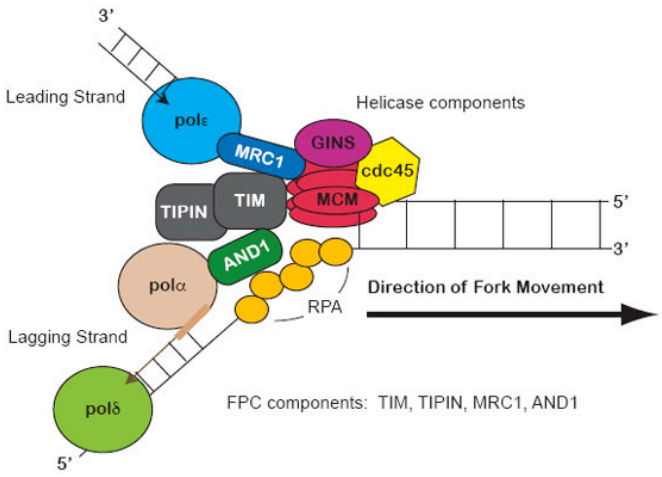


Figure 1.4 Replication Fork Protection Complex as a part of replisome.

This figure shows that Tipin, MRC1/Claspin, AND1, TIM are integral part of the replisome components. These proteins act in response to replication stress to protect replication fork stability. (Picture taken from Sabatinos SA, 2010).

1.1.6 Role of Homologous Recombination (HR) Factors in Replication Fork Protection.

Cells constantly face endogenous and exogenous DNA damages through out cell cycle. These damages induce several kinds of lesions such as a nick on the DNA template, a physical obstacle for replisome progression or single strand gaps or Double Strand Breaks (DSBs). DSBs are the most deleterious kind of DNA damage; inability to repair DSB leads to aneuploidy, chromosomal aberrations or even cell death[71]. Any kind of unrepaired damage present on the template might lead to DSBs formation at the moment it encounters replication fork. During S and G2 phases of cell cycle, DSBs are safely repaired by an error-free mechanism called Homology Directed Repair (HDR). This repair mechanism exploits the presence of a homologous DNA sequence on the sister chromatid to carry out homology search and strand invasion to complete Double Strand Break (DSB) repair.

1.1.6.1 Mechanism of Homology Directed Repair of DSBs

Years of extensive study in DSB repair by Homologous Recombination (HR) resulted in the discovery of several key proteins and in the dissection of different steps of the homologous recombination process (Fig.1.5). The first step in HR after DSBs formation is the nucleolytic resection of DSB ends to provide single-strand tails with 3'-OH protruding ends. The DNA end resection mechanism to promote HDR is conserved between prokaryotes and eukaryotes. In prokaryotes, a heterotrimeric complex consisting of RecB, RecC and RecD initiates the end resection from 5' to 3' direction to generate 3' ssDNA tail overhang[72]. In RecBCD complex, RecB

and D subunits possess ATP hydrolysis dependent DNA helicase activity[72]. RecB also harbors 5' to 3' nuclease activity[72]. Similar to prokaryotes, in eukaryotes the resection is mediated by several helicase and nucleases. In eukaryotes, double strand break end resection is initiated by MRE11, Rad50, Nbs1 (MRN) complex and then CtIP and Exo1 join to resect extensively the 5' to 3' ends to produce 3' ssDNA overhang[71]. The second step involves rapid binding of RPA, a single strand binding protein towards the 3' ssDNA. In order to start homologous recombination a protein called BRCA2 displaces RPA and recruits Rad51 onto 3' ssDNA overhang tail to form Rad51 nucleo-filament and this process is called pre-synapsis[71]. In humans Rad51 recruitment upon DSB is also contributed by Rad51 paralogs such as Rad51B, Rad51C, Rad51D, XRCC2 and XRCC3. Rad51 paralogs contain ~30% sequence homology in Walker ATP motif (which posses ATPase activity) with Rad51[73]. Recently it has been shown *in vitro* that the purified protein named RFS-1/RIP-1, *C.elegans* homologue of Rad51 paralogs, interacts with already formed rigid Rad51 nucleofilaments and remodels it to an open flexible state to allow homology search[74]. In the next step, Rad54, a translocase/motor protein, facilitates a connection between pre-synaptic Rad51 filament and the homologous template to form a D-loop intermediate. Establishment of D-loop is otherwise termed as synapsis[71]. After the establishment of the D-loop, postsynaptic DNA synthesis begins from the 3' end of the invaded strand to complete DNA synthesis. Once DNA synthesis begins there is the possibility of three pathways to be triggered. These are DSBR, Double Strand Break Repair (DSBR), Synthesis Dependent Strand Annealing mode (SDSA) and Break Induced DNA replication (BIR). In DSBR, D-loop DNA synthesis begins

and capture with the second double strand break end by annealing with the synthesised D-loop[71]. Reconstitution of recombination associated DNA synthesis revealed that synthesis at Rad51 mediated recombination intermediates was dependent on PCNA, RPA and Polymerase δ[75,76]. Besides polymerase δ, polymerase η also functions in the synthesis of the D-loop but less efficiently compared to polymerase δ[75]. At this step the formed Holliday Junctions (HJs) or two crossed strands are resolved to generate crossover or non-crossover products[77]. Optionally when DNA synthesis is directed to SDSA mode, the extended nascent strand is displaced and paired with the other 3' tail and further DNA synthesis completes the repair[77]. BIR is triggered when nascent strand in D-loop extends but the second end is not available for capture. At this point D-loop intermediates turns into a replication fork capable of leading and lagging strand synthesis[77].

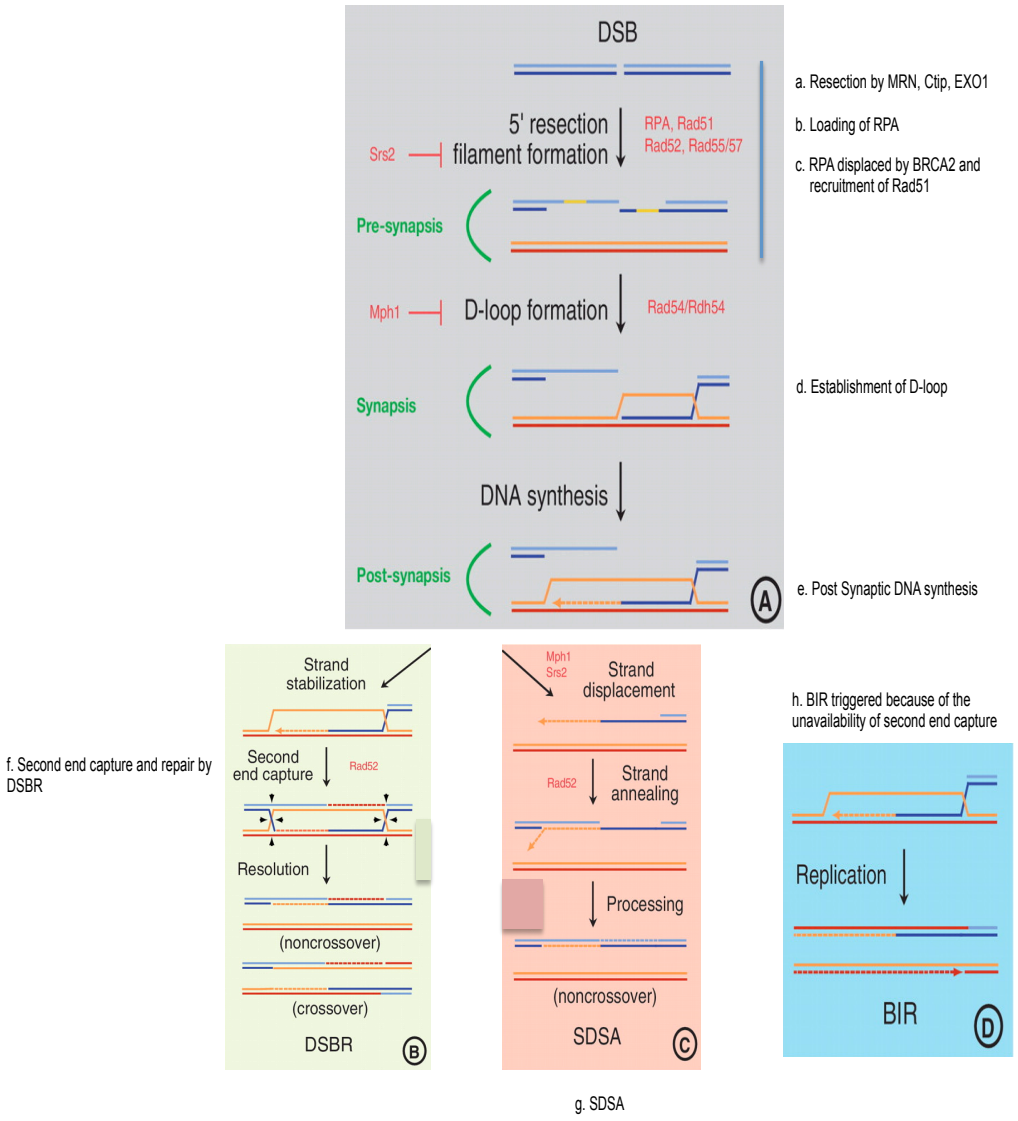


Figure 1.5 Steps involved in Homologous Recombination.

A. Upon DSB induction a. nuclease mediated resection, b. loading of loading of RPA, c. RPA displacement and loading of Rad51 by BRCA2, d. Establishment of D-loop and e. DNA synthesis is initiated. **B.** DNA synthesis in D-loop invokes three pathways **B.** f. DSBR (Double Strand Break Repair), g. SDSA (Strand Displacement induced Strand Annealing) and h. Break Induce Repair. (See text 1.1.6.1 for explanation). Picture taken from Krejci L, 2012. Ref[77].

1.1.6.2 Functional domains of BRCA2 in Homology Directed Repair

As discussed earlier, BRCA2 functions in recruiting Rad51 to promote HR mediated Double Strand Break (DSB) repair. BRCA2 retains single-stranded and double-stranded DNA binding activity through its C-terminal DNA binding domain (Fig.1.6). The DNA binding domain of BRCA2 contains five main modules, a 190 amino acids α-helical domain, three ssDNA associating oligonucleotide binding (OB) domains, and a tower domain within the OB2 domain binds to dsDNA[78]. OB1, OB2 and helical domain interact with a protein called DSS1[78] (deleted in split-hand/split-foot syndrome). It has been reported that DSS1 stabilises BRCA2 on the chromatin[78]. BRCA2 also contains eight BRC repeats that bind to Rad51 to promote HR. Point mutations in the BRC repeats have been reported to compromise its interaction with Rad51 and HR mediated repair[78]. BRC repeats together with ATP bound Rad51 facilitate Rad51 nucleofilament formation on ssDNA, but not onto dsDNA[78]. It has been shown that ssDNA – dependent ATPase activity of Rad51 induces dissociation of Rad51 from ssDNA[78]. BRC repeats of BRCA2 binds Rad51 monomers and inhibits its ssDNA – dependent ATPase activity and thereby allowing Rad51 stably bound onto ssDNA to form nucleofilament. It has been recently defined by means of cryo-electron microscopy that a BRCA2 dimer can accommodate 8-10 monomers of Rad51[79] to recruit it on to RPA bound 3' ssDNA tail and thereby displacing RPA. In addition to these domains, near the Nuclear Localisation Signal (NLS), BRCA2 also contains a Rad51 interacting domain towards the end of the C-terminal region. Binding of Rad51 through its C-terminal domain has been implicated in stabilisation of Rad51 filament on chromatin during DNA replication and it has been

shown that the phosphorylation of C-terminal domain of BRCA2 by CDKs is required for proper regulation of HDR mechanism[78].

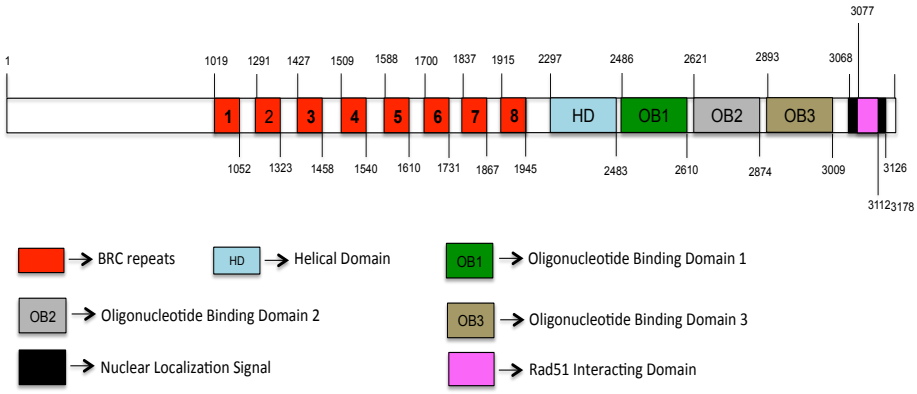


Figure 1.6 Representation of different domains within BRCA2.

Function of each domain is detailed in the text section 1.1.6.2.

1.1.6.3 Homologous Recombination and DNA Replication cross-talk

Remarkably even in unperturbed conditions HR pathway proteins are indispensable to maintain normal karyotype of a cell. Besides the classical role of HR factors in DSBs repair, several studies reported that Rad51, BRCA2 and RPA form nuclear foci and co-localise during S phase upon replication fork stalling[80-84]. In addition to Rad51, BRCA1 and BRCA2 associate together and form damage-induced foci together with PCNA[85,86]. The link between HR factors and the DNA replication is evolutionarily conserved from lower to higher eukaryotes. In budding yeast it was shown that the HR factor Rad52 forms spontaneous S phase foci and these foci tend to increase several folds in a polymerase alpha-mutant background[87] during on-going DNA replication. Similarly Rad22, the fission yeast homologue of Rad52 is required to restart replication fork after releasing from replication fork blockage[88]. Moreover it has been shown that Rad51 knockout vertebrate B-lymphocyte cell lines undergo apoptosis following severe chromosome breaks and gaps[89]. These results were obtained from

cells cultured without any exogenous source of damage, indicating that Rad51 is required to maintain stable chromosomes during unperturbed conditions. Similarly HR factors Rad51, BRCA1 or BRCA2 knock out embryonic cells are incompatible for survival and these cells accumulate different kinds of chromosome breaks prior to cell death[90]. However, HR factors, neither Rad51 nor Rad52 deletion in yeast compromises cell viability[91]. Overall the current literature suggests that the link between HR factors and DNA replication is conserved during evolution and that at least in higher eukaryotes HR factors are essential for life.

One important clue about HR proteins role in DNA replication was suggested by their requirement to restart collapsed fork or stalled replicative forks through the Homologous Recombination mechanism. In higher eukaryotes a first line of evidence for that was shown in 2003 using mouse embryonic fibroblasts deficient for BRCA2. The results of this study suggested that BRCA2 is required to stabilise stalled forks[92]. In the absence of BRCA2, induction of fork stalling through Hydroxyurea (HU) treatment lead to the accumulation of double strand breaks. Hence suggesting a function for BRCA2 in protecting replication forks from break[92]. However at that point authors did not provided any mechanistic insights about the role of BRCA2 in fork protection. Later the same group obtained interesting results working with another key recombination factor Rad51. Owing to the difficulty of immediate cell death phenotype in Rad51 knockout cells[89], they set up a degron system to monitor the effect of Rad51 knockout in different phases of the cell cycle[93]. They showed that Rad51 depletion did not affect bulk DNA synthesis in S phase, but Rad51 knockout cells arrested in G2 with increased RPA foci[93]. Hence suggesting

that Rad51 knockout may result to ssDNA gap accumulation and eventually chromosome breaks. This information also relates to the data of an independent study that showed that Rad51 associates to post-replicative chromatin (Fig.1.7b)[94]. It has been shown that HR pathway is active during S and G2 phases of cell cycle[95]. From these studies it is apparent that HR factors, Rad51 and BRCA2, function to prevent chromosome breaks through a protection function played during DNA replication and post-replication in late S - G2 (Fig.1.7). Rad51 and BRCA2 are probably promoting HR-coupled DNA synthesis after the bulk replication is completed leaving no segment of DNA unreplicated (Fig.1.5).

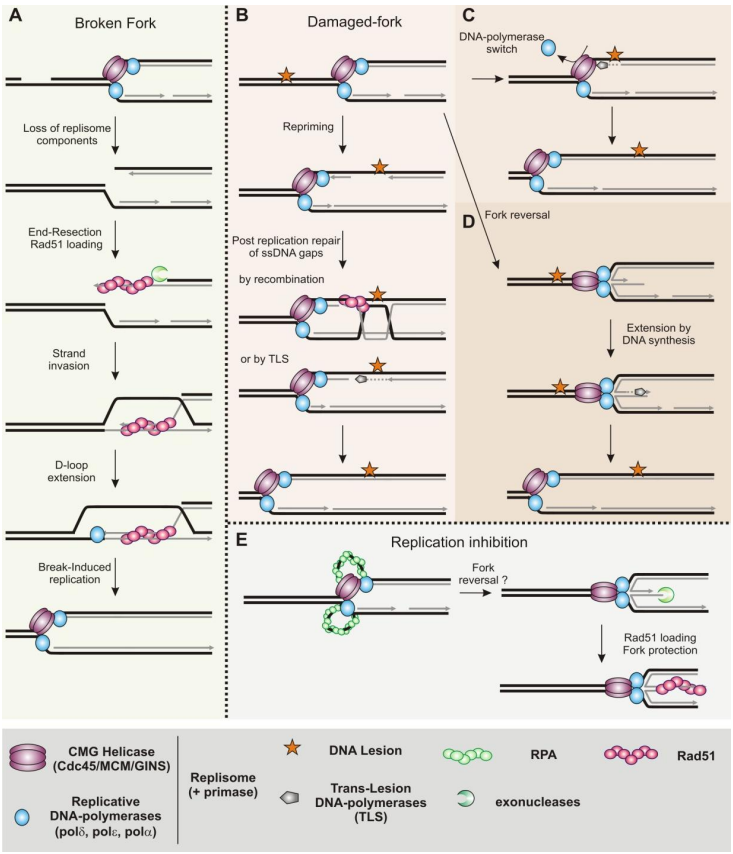


Figure 1.7 Recombination associated function at replication forks in eukaryotes.

(A) Model showing break induced repair. Broken fork with one sister chromatin can be rescued by Homologous Recombination machinery at the fork. (B) Model for post-replicative repair. Error free template switch mechanism using recombination machinery or TLS (Translesion Synthesis Polymerases) pathway is actiated when ssDNA gaps left behind ongoing replication forks. (C) Model for recombination dependent polymerase-switch (D) Model of replication fork reversal during damage (see next sections) (E) Model for replication fork stabilisation by Rad51 (see next section text for details). (Picture Taken from Costes A, 2012). Ref[96].

1.1.7 HR proteins protect replication fork from MRE11 mediated degradation

Recent technical advances in the field of DNA replication such as molecular combing, Electron Microscopy mediated visualisation of replication intermediates[97], isolation of Proteins enriched in Nascent DNA[98] (**iPOND**) have allowed us to better understand the function of several replication fork protection, chromatin remodelling and repair proteins in chromosomal DNA replication. In particular, a direct link between HR and DNA replication has been established using molecular combing to analyse DNA replication dynamics in cells deficient of HR factors such as BRCA2, Rad51 and XRCC2. These cells show reduced rate of replication fork movement and increased density of replication origin firing[99]. Using *Xenopus* egg extracts our group has shown that the inhibition of Rad51 chromatin association during unperturbed DNA replication resulted in accumulation of ssDNA gaps in daughter strands[68]. This direct evidence hinted that Rad51 has a role to perform at the fork during normal DNA replication. Similarly another study using molecular combing approach showed that in the absence of BRCA2, HU treated mammalian cells resulted in MRE11 nuclease mediated degradation of newly synthesised DNA strands (of ~1.8 kb/60min)[100]. The extensive degradation generated in BRCA2 deficient cells is because of reduced chromatin recruitment of Rad51, when replication forks are stalled by HU[100]. Furthermore the C-terminal - DNA binding domain, the Rad51-interacting and stabilising domain and the Nuclear Localisation Signal of BRCA2 have shown to be functionally involved in the stabilisation of Rad51 onto chromatin. Collectively it is apparent that BRCA2 protect

replication fork from nuclease-mediated degradation by recruiting Rad51 at stalled replication forks[100]. Interestingly a recent study showed that Fanconi Anemia (FA) pathway proteins FANCD2 and FANCA are both involved in stabilizing Rad51 at the fork to prevent nascent strand degradation[101]. This study suggests that together with BRCA2 a portion of the FA pathway proteins protect nascent DNA strands by stabilising Rad51 onto chromatin.

1.1.7.1 MRN complex function in DNA replication and Double Strand Break Repair

MRN protein complex is composed of MRE11, Rad50 and NBS1. MRE11 (Meiotic Recombination Enzyme 11) as a part of the MRN complex contains 3' to 5' exonuclease and 5' to 3' endonuclease activities. MRE11 plays a critical role in DSB repair by beginning short resection of the broken DNA ends and later CtIP and Exo1 continue the resection with a 5' to 3' direction to generate 3' ssDNA tail[102]. As discussed earlier (Section 1.1.6.1), the single-strand overhang generated by this resection mechanism is crucial for RPA and subsequent loading of Rad51 to promote Homologous Directed Repair. Besides the function of MRE11 in HR, first line of evidence that MRE11 is required for the integrity of DNA during replication[103] came from the depletion of MRN complex (MRE11, NBS1 and Rad50) using *Xenopus* egg extract. In this experimental system depletion of MRN complex resulted in chromosome breaks during unperturbed DNA replication, suggesting a role for MRE11 nuclease in DNA replication[103]. As discussed earlier, cells deficient of HR factors showed enhanced degradation of nascent DNA strands that are dependent on MRE11 (Fig.1.8). Using *Xenopus laevis* egg extract and

advanced electron microscopy techniques our laboratory has elegantly shown that when Rad51 chromatin association was inhibited in the presence of BRC4 recombinant polypeptides (BRCT repeats-containing peptides from BRCA2 protein), ssDNA gaps are formed in daughter strands due to uncontrolled MRE11 nuclease activity[68]. During unperturbed DNA replication, in the absence of Rad51 chromatin association, DNA replication intermediates showed two different kinds of ssDNA gaps in daughter strands - ssDNA gaps near the junction of the replication fork called as ssDNA gaps “at the fork” and ssDNA gaps far from the junction of replication fork called as ssDNA gaps “behind the fork”. The addition of Mirin, a specific inhibitor of 3’ to 5’ exonuclease activity of MRE11 limited the degradation of ssDNA gaps observed “behind the fork”, when Rad51 chromatin association is inhibited[68]. Interestingly ssDNA gaps “at the fork” persisted even in the presence of mirin to comparable levels and length respect to the untreated controls (in the presence of Rad51). Thus the degradation of nascent strands observed in human cells, in the absence of BRCA2, is possibly due to MRE11 mediated degradation - “behind the fork” but not “at the fork”. Although molecular combing is an appropriate tool to study the replication fork progression, the discrimination on the specificity of nucleases “at” and “behind the fork” can only be achieved with the direct visualisation of the DNA replication intermediates by electron microscopy.

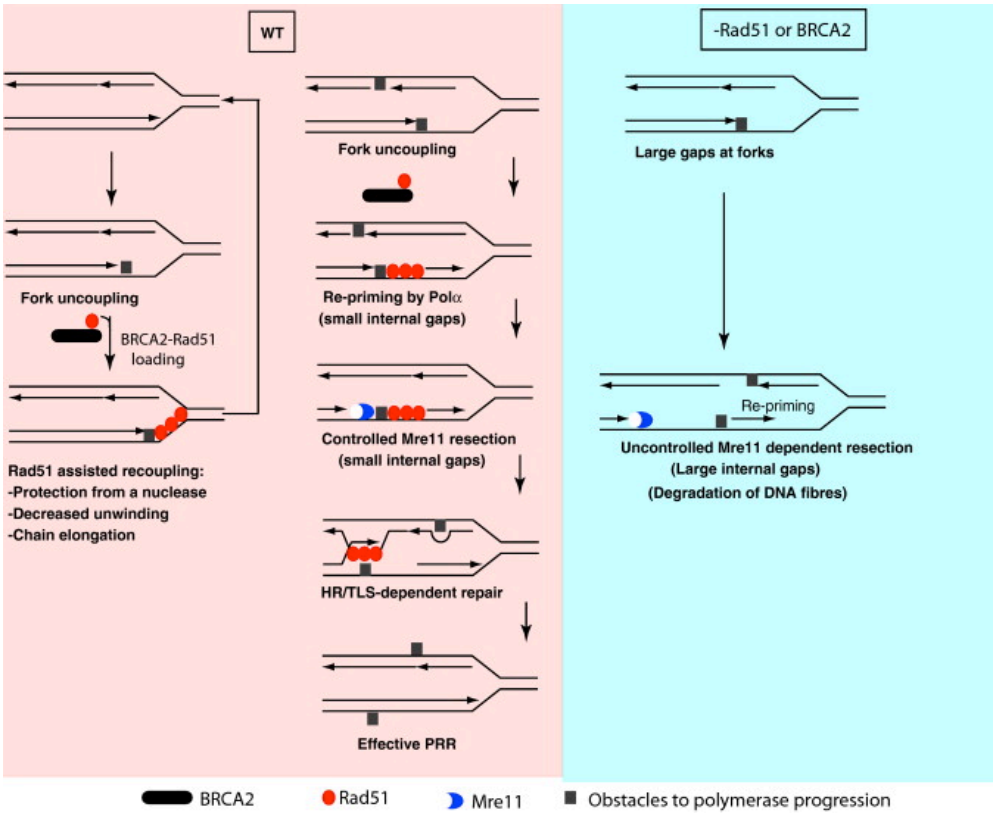


Figure 1.8 Acting in balance: BRCA2/Rad51 and MRE11 at forks.

This model shows the replication fork association of Rad51 recruited by BRCA2 in the presence of obstacles to polymerase progression. In the absence of Rad51 or BRCA2 long stretch of internal gaps accumulate due to uncontrolled resection by MRE11. (Picture taken from Costanzo V, 2011). Ref[104].

1.1.8 Effect of MRE11 inhibition on BRCA1/2 deficient cells viability

In this part of the thesis I discuss about two recent discoveries suggesting that inhibition of MRE11 nuclease activity at forks rescue cell viability in BRCA1/BRCA2 knockout cells[105,106]. As mentioned earlier BRCA1/BRCA2 knockout Embryonic Stem Cells (ESC's) are incompatible for survival[107]. Two main pathways repair DSB's, one is Homologous Recombination (HR) and other is Non-Homologous End Joining (NHEJ). DSB in S phase is predominantly repaired by HR pathway and DSB in G1 phase is repaired by NHEJ pathway. It should be noted that DSB repair by NHEJ pathway in S phase results in toxic chromosomal aberrations. During S phase of cell cycle, in the presence of Double Strand Break's BRCA1 recruit CtIP and MRE11 to promote HR[108]. It has been shown that factors such as 53BP1, RIF1 and PTIP functions in promoting NHEJ by blocking the 5' resection mediated by BRCA1-CtIP, which is a key step to initiate HR[109-116]. Hence, during S phase in the absence of functional BRCA1 the DSB repair is inappropriately routed to error-prone NHEJ instead of error free HR, resulting in accumulation of radial chromosomes and other lethal chromosomal aberrations. Interestingly, in ES cells, it has been found that loss of NHEJ promoting factor 53BP1 results in synthetic viability in $BRCA1^{-/-}$ (knock out) cells[117]. Moreover 53BP1 loss can rescue proliferation defect by restoring HR in BRCA1 mutant cells[117]. Mechanistic evidences suggest that 53BP1 loss allows functional HR mediated repair by restoring end resection by CtIP and ATM in BRCA1 mutant cells[117].

In the context of replication fork stalling, similar to BRCA2, BRCA1 prevents MRE11 mediated degradation of replication forks[118] whereas in case of Double Strand Break's BRCA1 and BRCA2 function to promote

CtIP and MRE11 mediated resection for HDR. This suggests a separation of function for BRCA1 and BRCA2 in replication fork stalling and in DSB repair pathway. On one hand, BRCA1 and BRCA2 prevent the action of nuclease-mediated degradation of stalled replication fork to inhibit DSB formation and on the other hand, when there is DSB's BRCA1 and BRCA2 promote MRE11 mediated resection to initiate error free HR mediated repair. Recently, Dr.Nussenzweig group showed that loss of the MLL3/4 complex protein, PTIP rescues cellular lethality of BRCA1 or BRCA2 knockout cells in the presence of DNA damage[105]. Authors demonstrated that the cell viability of BRCA1 or BRCA2 knockout cells in the absence of PTIP is due to limited degradation of nascent strands by MRE11. Moreover, they showed that inhibition of MRE11 by mirin or siRNA mediated knockdown of MRE11, both led to cellular viability of BRCA1 or BRCA2 knockout cells in the presence of DNA damaging agent HU[105]. However in the presence of HU, loss of neither 53BP1 nor RIF1 limited the degradation of nascent strand and induced survival in BRCA1 knockout cells[105]. Collectively, the authors suggest that in the presence of HU, replication fork protection by inactivating PTIP or inhibiting directly MRE11 mediated degradation can render cell viability to BRCA1 or BRCA2 knockout cells.

1.1.8.1 PARP1 – A FRENEMY (Friend and an enemy)

Poly-ADP-Ribose Polymerase 1 (PARP1) is an enzyme responsible for Poly-ADP ribosylation (PARylation) of several proteins involved in DNA damage response. It also undergoes auto-parylation in response to DNA damage. Recently, inhibitors of PARP1 were discovered and employed for

cancer therapies on BRCA mutation carrier patients. One of the commercially available PARP1 inhibitor Olaparib is frequently employed both in basic and applied cancer research. In normal cells, inhibition of PARP1 activity with Olaparib induces formation of ssDNA breaks on replicating DNA. ssDNA breaks induced by Olaparib is mainly repaired by HDR pathway[119]. Due to inefficient HDR in BRCA2 mutant cells Olaparib treatment results in apoptosis[119]. It has been reported that BRCA2 mutant cells showed 1000-fold sensitivity to PARP1 inhibitor Olaparib[120]. This was the rationale behind using PARP1 inhibitor as a drug in BRCA2 mutated cells. Clinical Phase I and Phase II trials showed a promising response to Olaparib in BRCA mutation carriers with breast, prostate and ovarian cancers[121]. In one phase II[122] study it has been shown that 40% of the germline BRCA mutation carriers with ovarian and breast cancer responded well to Olaparib. In a different phase II[123] trial patients who have undergone chemotherapy did not respond to Olaparib treatment. These latter results indicated that chemotherapy likely led to acquired resistance because of Olaparib treatment.

A recent study showed that mouse embryonic stem cells (mESC's) pre-treated with Olaparib or PARP1 knockdown followed by BRCA2 conditional knock out, those cells are surprisingly viable[106]. Authors demonstrated that this synthetic viability between PARP1 deficiency and BRCA2 conditional knockout cells is due to limited replication fork association and degradation of nascent DNA strands by MRE11[106]. This is in line with studies suggesting that PARP1 activates MRE11 at the stalled forks to mediate resection and promote HDR at stalled replication forks[124,125]. However synthetically viable cells in PARP1 and BRCA2

deficient background, showed different chromosomal aberrations and were assessed to be potentially tumorigenic[106]. Authors suggest that synthetic viability or lethality with respect to BRCA2 and PARP1 deficiency is associated with different order of events. For instance, in the absence of BRCA2, replication fork protection is lost because PARP1 activates MRE11 at forks resulting in hyper-resection of nascent DNA strands. In this condition DNA damage checkpoints are activated and cells are destined to apoptosis. Similarly when BRCA2 loss is followed by PARP1 inhibition, cells die due to synthetic lethality. In contrast when cells are pre-treated with PARP1 inhibitor, MRE11 activation is blocked. Pre-treatment with PARP1 inhibitor, followed by BRCA2 loss limits the resection of MRE11 because MRE11 is inactive. In this scenario, limiting excessive MRE11 resection in BRCA2 knock out cells results in cellular viability. Considering these observations, PARP1 inhibition besides inducing apoptosis in BRCA2 deficient cancer cells might facilitate survival in normal BRCA2 heterozygous cells that would go to loss of heterozygosity. According to this scenario these cells could either undergo apoptosis because of continuous DNA breaks or become potentially resistant cancer cells.

1.1.9 Replication Fork Re-priming

Transient stalling of replication forks is unavoidable during chromosomal DNA replication and such stalling events not necessarily bring to the uncoupling between the replicative helicase and DNA polymerases[126]. To ensure continuous DNA synthesis, lesion or base modifications on the template during replication can be repaired during replication by

specialised polymerases like Translesion Synthesis (TLS) Polymerases such as POLK, POLH, REV1, POLQ, POLN, and REV3L-REV7[127]. The signal for the recruitment of TLS polymerases is mediated by the PCNA replication factor. Once replication fork is stalled PCNA is poly- or mono-ubiquitinated at lysine K164 residue, primarily monoubiquitination is responsible for recruiting TLS polymerases in response to fork stalling[128]. Mono-ubiquitination of PCNA is mediated by Rad18 in response to HU treatment and UV irradiation. It has been shown that Rad18 is also controlling the poly-ubiquitination of PCNA[128]. Base modifications or lesions can happen both on leading and lagging strand. Because of the discontinuous nature of lagging strand synthesis, continuous re-priming using translesion polymerases efficiently repairs lesion on the DNA template. This situation can be much different on the leading strand because of the continuous nature of the leading strand synthesis. In bacteria it has been described that re-priming activity is also carried out when there is a lesion on the leading strand template[129]. Similar to prokaryotes, evidence in yeast model suggests that there are discontinuities in both the leading and lagging strand synthesis when DNA replication is challenged by UV treatment[130]. Cells likely require re-priming mechanisms to resume replisome activity and complete DNA synthesis during normal and challenged DNA replication. DNA gaps left unreplicated are then repaired by means of post replicative repair using specialised TLS polymerases or error-free Homologous recombination mechanism.

1.1.10 Connecting Fanconi Anemia (FA), Homologous Recombination (HR) and Translesion Synthesis pathway in response to replication fork stalling

Eukaryotes evolved with two modes of DNA damage tolerance pathway (DDT) during cell cycle. In one mode lesion on template during replication can be repaired by specialised polymerases called Translesion Synthesis Polymerases (TLS). Other mode is through template switch using the sister chromatid. TLS polymerases mediated repair is not accurate and it likely increases the DNA mutation rate; hence the TLS pathway is considered as error-prone repair mechanism. A recent study in *Saccharomyces Cerevisiae* showed that template switch is the preferred way of repairing template lesions likely because of its error free nature of repair[131].

The template switch mechanism requires strand invasion of the sister chromatid and it is mediated by Homologous Recombination protein Rad51. It has been reported a cross talks between TLS pathway and Homologous Recombination proteins exists in prokaryotes. Bacterial RecA the prokaryotic orthologue of Rad51, is required to promote leading and lagging strand synthesis in cooperation with translesion polymerases PolII and PolIV[132]. However RecA showed inhibitory activity on another translesion polymerase, named PolIII[132]. In mammalian cells E3 ubiquitin ligase Rad18 besides its function in recruiting TLS polymerases, it is also involved in orchestrating homologous recombination reactions by binding to Rad51C and recruiting Rad51 onto chromatin during DSB[133]. This suggests that Rad18 signaling acts as a platform to regulate the DNA repair mechanism both for the TLS and HDR mediated repair.

1.1.10.1 Targeting Fanconi Anemia and TLS, pathways as a possible new strategy to kill BRCA mutant cells.

Fanconi Anemia is a genomic instability disorder caused by mutations in genes that are involved in DNA inter-strand crosslink repair. The Fanconi Anemia (FA) multisubunit core complex is composed by FANCA, B, C, E, F, and G. This complex catalyses the ubiquitination of FANCD2 in response to a variety of DNA damaging agents[134]. Firstly FANCD2 was observed to form nuclear foci in response to DNA damage. Moreover ubiquitinated FANCD2 was found to co-localise with BRCA2, BRCA1, Rad51, REV1, and PCNA[135]. Also Rad18 signaling and mono ubiquitination of PCNA was shown to be required for the ubiquitination of FANCD2 at Lys561[135].

FA proteins are also implicated in protecting replication forks during stress. It has been shown recently that cells mutated in BRCA1/2 are mainly dependent on FANCD2 for their survival[136]. Hence loss of FANCD2 results in synthetic lethality for BRCA1/2 mutated cells[136]. FANCD2 has been shown to be a part of replisome component using iPOND experiment and also FANCD2 interacts with MCM2-7 complex to suppress new origin firing in the presence of HU[137]. Similar to BRCA1/2, FANCD2 functions in protecting the replication fork by recruiting Rad51 to prevent MRE11 mediated nascent DNA degradation[101]. Gene expression profile from HR deficient BRCA1/2 tumour subsets revealed that the expression level of FANCD2 is increased and FANCD2 is mono-ubiquitinated to a greater extent[138]. Mono-ubiquitinated FANCD2 is thus engaged at the fork to protect nascent strands from nuclease mediated degradation[138].

Besides FANCD2 over expression it has been shown that translesion polymerase, Polymerase θ is highly expressed in BRCA1/2 knock out cells[139] and in BRCA1/2 deficient epithelial ovarian cancers[140]. Polymerase θ has a conserved function in error-prone alternative–end joining to repair DSBs[140]. In BRCA1/2 deficient cancer cells HR mediated repair of DSB is compromised, therefore to repair DSB, Pol θ directs to error-prone alternative-end joining leading to chromosomal aberrations. It is worth mentioning that Pol θ knockout is synthetic lethal with BRCA1/2 mutant epithelial ovarian cancer cells[139]. Interestingly a recent study suggested that in the absence of BRCA1/2, FANCD2 induces alternative–end joining pathway by recruiting Polymerase θ[138] at stalled forks. Hence FANCD2 deficient cells lack the capacity for alternative – end joining. Link between FANCD2 and Pol θ is interesting, this suggest a dual role for FANCD2 in the presence or absence of BRCA1/2. In the presence of BRCA1/2, FANCD2 co-operate with BRCA1/2 complex to promote HR by recruiting Rad51[101]. In the absence of functional BRCA1/2, although FANCD2 is involved in protecting replication fork from nascent strand degradation, it is also involved in activating Pol θ mediated NHEJ[138]. Considering the synthetic lethality between FANCD2 and BRCA1/2 deficient cells. FANCD2 appears to be an interesting target for cancer treatment in BRCA1/2 mutation carriers. Inhibiting its mono-ubiquitination by targeting E3 ligase activity of FANC-A core complex would be one of the strategies. To this aim further understanding of the function of FA core complex is necessary. Because in a BRCA1/2 mutation background inhibiting E3 ligase activity of FA core complex impairs Rad51 recruitment to stalled forks and allow excessive MRE11 mediated degradation of

nascent strands[101]. Enhanced resection of nascent strands at stalled forks results in ssDNA accumulation resulting in checkpoint activation. Similarly inhibition of E3 ubiquitin ligase activity of FANC-A core complex in parallel inhibit Pol θ-mediated alternative–end joining in BRCA1/2 deficient cancer cells, this would possibly result in cell death for cancer cells (Fig.1.9). Although conceptually relevant, this strategy may also induce chromosomal aberrations in normal cells because FANCD2 deficient cells behave similar to BRCA2 deficient cells leading to enhanced degradation of nascent strands even in a BRCA2 wild type background resulting in a FA phenotype[101]. However developing a drug against Pol θ will be another option that is of current under consideration by scientific community to treat BRCA1/2 mutation carriers (Fig.1.9).

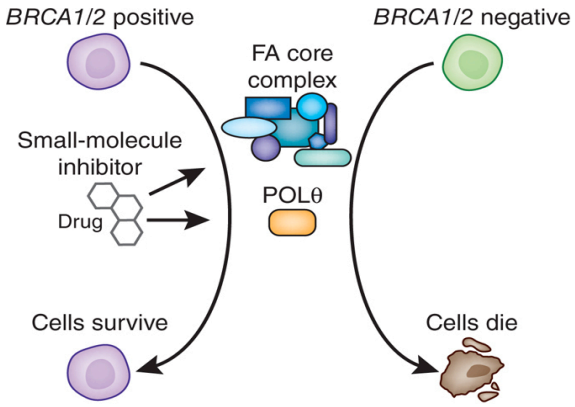


Figure 1.9 Strategy to target BRCA-mutated cancer cells by FA pathway interference.

The model shows inhibition of Polymerase θ and E3 ubiqutin ligase activity of Fanconi Anemia (FA) core complex as a better strategy to target BRCA1/2 mutated cancer cells. (Picture taken from Lachaud C, 2016). Ref[141].

1.1.11 Replication Fork Reversal: Causes and Consequences

Replication fork reversal is a four-way junction structure induced upon perturbation of replication fork progression. Fork reversal is formed by the annealing of the two newly-synthesised nascent strands together with the concomitant re-annealing of the parental strands, thus creating a four-way junction, also known as chicken foot (Fig.1.10). Replication fork reversal is also called as replication fork regression. The concept and experimental evidence of replication fork reversal ware reported in seminal studies using *E.coli* as model system back in 1976[142]. In eukaryotes, *in vitro*, several proteins are involved in inducing replication fork reversal. A list of proteins involved in replication fork reversal *in vitro* is shown in the table

(Table.1.1). It has been shown in yeast that fork regression induced genome instability on rDNA loci or on RNA polymerase II actively transcribed regions upon replication fork stalling induced by HU or UV[142]. In yeast using Electron Microscopy (EM) to visualise DNA replication intermediates it has been shown *in vivo* that in the absence of the checkpoint kinase Rad53, HU treatment induces extensive fork reversal coupled with ssDNA accumulation suggesting that the checkpoint machinery prevent replication fork reversal[143]. Recently a study from yeast has shown that in the presence of MMS, in primase mutant background, the frequency of replication fork reversal is increased several folds[144]. In the same study authors also observed that primase mutant displayed extensive ssDNA gaps accumulation at and behind the replication fork[144]. Similarly mutating Ctf4, a protein that tethers polymerase alpha and the MCM2-7 helicase resulted in ssDNA gaps accumulation and enhanced reverse fork formation[144]. This suggests a strong correlation between ssDNA gaps at the fork and reverse fork formation.

A recent study in mammalian cells has shown that, in the presence of different DNA damaging agents (treated at sub-lethal concentration) resulted in increased frequency of replication fork reversal as visualised by EM[145]. Authors concluded that reverse forks are induced in response to mild impediment to fork progression[145]. Interestingly, in the same study authors reported that the HR factor Rad51 is required to promote replication fork reversal activity likely because of its strand exchange activity[145]. The extent of Rad51 mediated fork reversal was addressed *in vitro* in the presence of the chromatin remodeller Rad54. It has been shown that Rad51 and Rad54 co-operate together for both fork regression

and fork restoration[146]. In this context the role of Rad51 regulators, such as BRCA1, BRCA2, Rad51 paralogs in replication fork reversal *in vivo* is still unknown. Apart from Rad51, in the presence of nucleotide deprivation FBH1, a helicase, *in vivo* reported to be involved in replication fork reversal. The role of FBH1 is mainly attributed to its function in replication fork stability and post replication repair[147]. In addition, PARP1 is required to stabilise the reversed replication forks in the presence of topoisomerase inhibitor[148]. Hence it is becoming clear from literature that many factors are involved in inducing and stabilising replication fork reversal activity when replication fork progression is challenged.

It is still unclear whether replication fork reversal is a physiological or a pathological condition. In a positive outlook, when DNA replication is challenged transient replication fork reversal could be a way to prevent deleterious DSB's[145]. Fork reversal could act as a brake by transiently reversing the fork and gives some time to rectify the damage and resume DNA replication[147]. For instance in the presence of DNA damaging agents such as DNA lesions, discontinuities in template, nucleotide shortage, all these damaging agents induce replication fork reversal. Under these circumstances, fork reversal limits genome instability by minimising toxic single-stranded DNA (ssDNA) accumulation, thereby favouring DNA damage tolerance during replication (for example: template switch). It is tempting to speculate that endogenous replication stress could contribute to replication fork reversal. However, it has been shown by Massimo Lopes group that the frequency of replication fork reversal in somatic cells are about 8% where as undifferentiated embryonic stem cells showed higher fork reversal rate (~30%)[149]. Study conducted by same group

indicates that increased fork reversal in embryonic stem cells was due to increased ssDNA gaps and higher expression level and chromatin association of RPA and Rad51 in embryonic stem cells compared to somatic cells[149]. Collectively, observations from Lopes group suggest that replication fork reversal do happen during the course of DNA replication during endogenous replication fork stalling as a way to protect genome integrity albeit at a low frequency. Further studies are required in different model system and cell lines to clarify about the frequency of fork reversal. In contrast there is no report showing replication fork reversal during unperturbed DNA replication in yeast. From another point of view possible pathological consequences of unresolved reversed forks have to be taken into account. It could be envisaged that unresolved reverse forks might lead to chromosomal abnormalities. Recently some nucleases and helicases have been shown to have a role in restarting reversed fork. The nuclease/helicase DNA2 and the Werner syndrome ATP-dependent helicase (WRN) have been shown to be involved in restarting reversed forks by resecting regressed arms in a controlled manner[150]. Failure to resolve reversed forks might lead to chromosomal abnormalities at the end of mitosis.

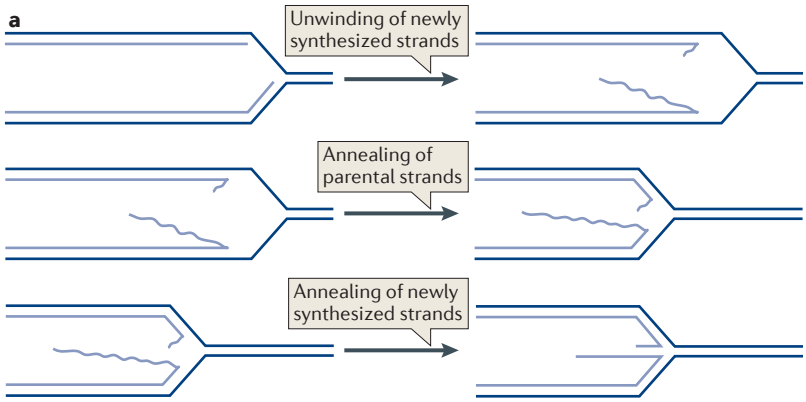


Figure 1.10 Model for replication fork reversal.

Fork reversal is formed by annealing of the two nascent strands together with the concomitant re-annealing of parental strands, thus creating a four-way junction, also known as chicken foot. (Picture taken from Neelsen KJ, 2015). Ref[147].

Protein	Fork reversal activity (plasmid or oligonucleotide substrate)*	Fork restoration activity (oligonucleotide substrate)*	Impact of ssDNA-binding proteins on protein activity *in vitro*
RECQ1	No (plasmid)[57]	Yes (oligonucleotide)[29,57]	RPA inhibits strand annealing[59]
RECQ5	Yes (oligonucleotide)[83]	ND	RPA partially inhibits strand annealing[83]
BLM	Yes (oligonucleotide[84,87] and plasmid[85])	Yes (oligonucleotide)[86]	The role of RPA is controversial[57,83,86]; no effect of RAD51 (REF. 102)
WRN	Yes (oligonucleotide)[83,84,90]	Yes (oligonucleotide)[29,86]	The role of RPA is controversial[83,87]
HLTF	Yes (oligonucleotide and plasmid)[96]	ND	HLTF displaces RPA from the DNA[98]
RAD54	Yes (oligonucleotide)[102]	Yes (oligonucleotide)[102]	Fork reversal activity requires RAD51 (REF. 102)
SMARCAL1	Yes (oligonucleotide and plasmid)[94,106]	Yes (oligonucleotide)[94]	RPA promotes fork reversal on leading-strand gap substrates and inhibits reversal of lagging-strand gap substrates[94]
ZRANB3	Yes (plasmid)[113]	ND	RPA inhibits fork reversal on leading-strand gap substrates[94]
FANCM	Yes (oligonucleotide[117,119] and plasmid[118])	ND	No effect of RPA on fork reversal[118]

BLM, Bloom syndrome helicase; FANCM, Fanconi anaemia complementation group M; HLTF, helicase-like transcription factor; ND, not determined; RECQ, ATP-dependent DNA helicase Q; RPA, replication protein A; SMARCAL1, SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1; ssDNA, single-stranded DNA; WRN, Werner syndrome ATP-dependent helicase; ZRANB3, zinc finger RAN-binding domain-containing protein 3. *Holliday junction migration activity has been shown for all enzymes in the table (see the main text for further details and references). 'Plasmid' and 'oligonucleotide' indicate the substrate in the respective *in vitro* assay.

Table.1.1. List of proteins reported to possess fork reversal activity *invitro* has been shown. (Picture taken from Neelsen, KJ, 2015). Ref[147]

1.1.12 *Xenopus* egg extract as a model system to study DNA replication and repair

Xenopus laevis egg extracts serves as a good model system for studying DNA replication and DNA repair mechanisms. Due to high concentration of proteins and RNA molecules, the egg extract system is capable of recapitulating several aspects of cell biology such as cell cycle progression, regulation of DNA replication and repair, chromatin structure, mitotic spindle dynamics and system chromatid cohesion[151]. Similar to other vertebrate eggs, *Xenopus* eggs are arrested in meiosis II metaphase. Fertilized eggs as well as egg extracts supplemented with sperm nuclei are able to trigger DNA replication and undergo 12 rounds of cell cycle with no significant transcription[152]. Hence this system becomes a valuable tool to study protein complexes and to biochemically dissect protein–DNA transactions in a temporally controlled manner.

Owing to the ability of this embryonic system to complete DNA replication in a very short time, studying DNA replication and repair using *Xenopus laevis* egg extract is extremely useful. After the addition of sperm nuclei to interphase egg extract, DNA replication can be monitored over time and the efficiency of replication can be assessed quantitatively and qualitatively by evaluating the incorporation of radiolabelled nucleotides by means of TCA precipitation assays and agarose gel electrophoresis[153]. A unique tool to study protein function in egg extract is antibody mediated protein depletion strategy. Specific antibodies directed against the protein of interest are used to deplete proteins or protein complexes from the extract by a method called immunodepletion. High affinity antibodies are capable of depleting near ~100% of proteins from egg extract. Phenotypes

observed could then be confirmed by rescuing the *wild* type conditions by means of recombinant proteins added back to the depleted extract.

Furthermore combining immunoprecipitation from egg extract or from chromatin fractions and coupling with mass spectrometry offers an advantage to find new interacting partners for the protein of interest. Also due to enriched pool of proteins and the ability to fire many more replication origins with respect to the non-embryonic systems, *Xenopus* egg extracts become a valuable tool for visualising replication intermediates using Electron Microscopy[68]. All of these techniques have been employed during my PhD study using *Xenopus* egg extract as model system.

Chapter 2. Materials and Methods

2.1 Preparation of interphase *Xenopus laevis* egg extracts

Japanese interphase egg extracts were prepared as described[154]. *Xenopus* eggs are laid naturally arrested in metaphase of Meiosis II. High quality inactivated or metaphase arrested eggs are crucial for the preparation of best Low Speed Supernatant (LSS) extracts. Initially, eggs were rinsed several times in 1X Marc's Modified Ringer (MMR) (20X MMR: 100 mM HEPES, pH7.5, 2 M NaCl, 10 mM KCl, 5 mM $MgSO_4$, 10 mM $CaCl_2$, 0.5 mM EDTA) to remove excessive skin shed or food regurgitated by the frogs from the laying buffer. Then activated or apoptosed eggs were removed carefully with a pastuer pipette. The eggs were dejellied in a dejellying buffer (20 mM Tris, pH 8.5, 110 mM NaCl, 5 mM DTT) for 10 min. Then eggs were washed again with 1X MMR three times and activated with 1 µg/ml calcium ionophore A23187 in MMR for 5 min. The activated eggs were then washed with 1X MMR and thrice with S buffer (0.25 M sucrose, 50 mM KCl at pH 7.5). The eggs were spin crushed at 13,000 rpm for 12 min. The cytoplasmic fraction were retrieved and supplemented with cytochalasin B (40 µg/ml) and centrifuged at 70,000 rpm for 15 min. The cytoplasmic and membrane fractions are collected and supplemented with cyclohexamide and snap frozen with 3% glycerol.

2.2 Antibodies

Rabbit polyclonal antibodies against *Xenopus laevis* BRCA2 were raised against immunogenic peptide sequence of N-terminal – KPHIKEDQNEPESNSEYC – C-terminal and coupled with Keyhole Limpet Hemocyanin (by Biogenes, Germany). Rabbit polyclonal against Rad51 were raised against a 320 to 336 amino acids (CAEAMFAINADGVGDAKD) of *Xenopus laevis* Rad51. *Xenopus laevis* SMARCAL1 antibodies were gift from Dr. Anna De Antoni and Guilia Rota (From Dr. Vincenzo Costanzo's group). Polyclonal rabbit *Xenopus laevis* Anti-SMARCAL1 antibodies were raised against His-tagged SMARCAL1 recombinant protein (by Biogenes, Germany). Antibodies against *Xenopus* ORC1, MCM7, AND1, Polymerase α and δ Cdc45, Psf3, H2B, γH2AX has been described previously[68,69,154].

2.3 Western Blotting

Unless otherwise specified throughout the study 4 - 15% Bis – Tris Poly Acrylamide gels (From Biorad) were used. All gels were run at 100 volts to allow proper migration of different molecular weight proteins until the dye front reached the bottom of the gel. Proteins from gel were transferred on methanol activated PVDF membrane in buffer containing 20% methanol and 80% 1X transfer buffer (10X Transfer Buffer composition: 25 mM Tris, 192 mM glycine) for 40 volts overnight. Transferred membrane was washed twice with Milli-Q water and was blocked for 1 hour with 5% milk dissolved in PBST (1X Phosphate Saline Buffer was supplemented with

0.1% Tween-20). Further the membranes were incubated for 2 hours with primary antibody. Again the membranes were rinsed thrice for 10 min with PBST and incubated with an HRP conjugated secondary antibody in blocking solution. Afterwards membranes were rinsed and developed using ECL pico or 10% femto detection buffer.

2.4 Chromatin binding experiment

Chromatin was isolated as described previously with little modifications. Forty µl of extract was incubated with 4,000 sperm nuclei/µl for required time points. To isolate the chromatin, the extracts were diluted with 400 µl of EB buffer (50 mM KCl, 50 mM HEPES, 2.5 mM $MgCl_2$) supplemented with 0.25% NP-40 and layered onto 200 µl of a sucrose cushion made with EB buffer. The chromatin was spun in swinging bucket rotor at 10,000 rpm for 5 min at 4^{o}C. The resulting pellet was washed with 300 µl EB buffer and spun in fixed bucket rotor centrifuge at 10,000 rpm for 5 min. Final pellet was resuspended in 3X laemelli buffer and analysed by western blotting.

2.5 Immunoprecipitation

Clarified extract were obtained by diluting interphase egg extract five times with IP buffer (40 mM HEPES-KOH pH 7.5, 10% sucrose, 50 mM KCl and protease inhibitor cocktail) and spun in centrifuge at 13,000 rpm for 30 min. Fifty µl of the clarified extract was incubated with 3 µg of affinity purified anti-BRCA2 or anti-Rad51 antibody or non-specific IgG one hour on ice. After an hour of incubation on ice the samples were mixed with 30 µl

Protein A Dynabeads (Invitrogen) and incubated for 2 hours at 4^{o}C in a gentle rotation mode. Then beads were washed thrice with IP buffer and twice with IP buffer containing 0.1% triton X-100. Later the proteins were eluted from the beads by boiling with 3X laemelli buffer (4% SDS, 20% glycerol, 120 mMTris-HCl pH 6.8) supplemented with fresh 10% β-mercaptoethanol.

2.5.1 Chromatin Immunoprecipitation

Two hundred µl of egg extract was incubated with 4000 sperm nuclei/ µl for 90 min, optionally supplemented with 20 µM aphidicolin or 0.05 U EcoRI. Later chromatin pellet was isolated as described in section 2.4. Chromatin pellet was resuspended in 300 µl IP buffer and incubated with 2 units/ µl final concentration DNaseI for 10 min. Then the samples were sonicated in (Bioruptor, Diagenode) with medium setting 10 s sonication and 45 s brake. Later the sample was centrifuged 10,000 g for 5 min and supernatant was considered as the solubilised chromatin. The solubilised chromatin sample was incubated with either non-specific IgG or 3-µg/ µl anti-Rad51 antibodies for one hour on ice. From them standard immunoprecipitation protocol is followed as described in section 2.5.

2.6 Immunodepletion

2.6.1 BRCA2

Eighty µg of affinity purified BRCA2 antibody was incubated with 150 µl of Protein A Dynabeads overnight. 150 µl dynabeads were divided into three tubes and unbound antibodies were removed by placing the dyna beads in

a magnetic rack. To the antibody bound dyna beads 300 µl of extract was added and incubated rotating at 4^{o}C cold room. Three rounds of depletion were carried out with timing of 60 min, 45 min and 30 min. The resulting supernatant extract was considered as immunodepleted extract and 1 µl of extract was immunoblotted to check the efficiency of depletion.

2.6.2 Rad51

Two microgram of affinity-purified anti-Rad51 or equal amount of non-specific IgG was incubated with 50 µl of protein A dynabeads for overnight. Unbound antibodies were removed from the beads and 420 µl of extract was added to antibody bound beads and one round of immunodepletion was carried out for one hour. The supernatant was considered as immunodepleted extract and 1 µl of extract was immunoblotted to check the efficiency of depletion.

2.7 Isolation of Proteins enriched on Nascent Chromatin (iPOND).

iPOND experiment was performed as described previously with slight modifications[98]. Hundred µl of egg extract was incubated with 4000 sperm nuclei/µl for different time points as required. DNA replication in egg extract usually begins within 20 – 30 min after incubation with sperm nuclei. At 50 min chromatin reaction was optionally supplemented with 40 µM biotin-dUTP and incubated for further 10 min to allow replication forks to incorporate biotin-dUTP at the nascent strands. After 10 min the reaction diluted by the addition 10 fold EB buffer and chromatin fraction was pelleted as described above (section 3.4). The isolated chromatin was

sonicated (bioruptor) 30 sec on and 40 sec off for 60 cycles in medium setting. Sonicated samples were bound to 30 µl of streptavidin-coated dynabeads for 1 hour. Bound fractions were washed and eluted by boiling with laemelli buffer and immunoblotted.

2.8 Replication Assay

2.8.1 Agarose Neutral Gel Electrophoresis

To analyse the bulk replication activity in egg extract neutral agarose gel electrophoresis is used to separate the ^{32}P-labelled replication products. 20 µl of depleted or mock depleted egg extract was supplemented with 2000 sperm nuclei/µl and replicated in the presence of α-^{32}P-dCTP for 120 min. At 120 min the reaction was terminated by the addition of stop buffer (1% SDS, 80 mM Tris pH.8.0, 8 mM EDTA) supplemented together with 1 mg/ml proteinase K and incubated for 2 hours. Later the reaction was extracted with phenol/chloroform/isoamylalcohol. Finally ethanol precipitated and separated on 0.8% agarose gel. Then the agarose gel was fixed with 30% TCA for 30 min, dried and exposed for autoradiography. The signal was obtained from Phosphoimager and quantified using ImageJ software.

2.8.2 TCA Replication Assay

TCA replication assay is used to quantify accurately the amount of DNA synthesised based on the incorporation of radiolabelled nucleotide to replicating DNA[152]. Twenty µl BRCA2 or Rad51 or mock depleted egg extract was incubated with 100 nCi/µl α^{32}P-dCTP at 23^{o}C for different time

points to follow the kinetics of DNA replication. Reactions were terminated by the addition of 160 µl stop buffer (as mentioned in section.3.8.1) supplemented with freshly added proteinase K and the reaction were incubated at room temperature for one hour. The samples were precipitated with 4 ml of 10% TCA (5 % w:v TCA, 2% w:v $Na_4P_2O_7$ $10H_2O$) for 60 min at 4°C. Then 40 µl of the TCA sample was spotted onto the paper filter to measure total ^{32}P-dCTP. Remaining TCA sample is filtered under vacuum and the filter was washed twice with 8 ml of 5% TCA (5 % w:v TCA, 0.5% w:v $Na_4P_2O_7$ $10H_2O$) and finally with 8 ml of ethanol. The amount of α^{32}P-dCTP on filter is quantified using scintillation counter. By dividing the α^{32}P-dCTP incorporated into the DNA by the total α^{32}P-dCTP on the paper filter gives the percentage of total ^{32}P incorporated.

2.9 Electron Microscopy sample preparation and analysis of replication intermediates

Electron microscopy sample preparation including isolation genomic DNA, enrichment of replication intermediates from *Xenopus laevis* egg extracts was performed as described previously[68]. Two hundred µl mock or depleted extract was incubated with 4000 sperm nuclei/ µl approximately for 60 min. As an internal control we assessed the incorporation of Cy3-dCTP in a separate aliquot of extract. Nuclear assembly and strong incorporation Cy3-dCTP as visualised by fluorescence microscope suggests that replication is ongoing. At this time point the extract was three fold diluted in EB-EDTA buffer (50mM HEPES,pH 7.5, 100 mM KCl,

2.5 mM $MgCl_2$, 1 mM EDTA) and layed in EB-EDTA-sucrose buffer (EB-EDTA buffer + 30% sucrose). Then the sample was spun at 10,000 rpm at 4°C for 5 min and the supernatant was removed gently without disturbing the pellet. Hundred µl nuclei was then cross-linked by the addition of 10 µl 4,5',8-trimethylpsoralen (TMP) (200 µg/ml) and followed by irradiation with 366 nm UV light (Stratalinker UV2400). Crosslinking step was performed three more times. To 400 µl of nuclei suspension 10 µl of 10 mg/ml RNase and 10% SDS were added. After an hour of incubation at 37°C, 29 µl of 18 mg/ml Proteinase K was added and incubated at 50°C for 2 hours. Then the sample was processed for chloroform/iso-amyl alcohol and ethanol method of DNA extraction. Purified DNA is digested with 3–5 hours with NdeI restriction enzyme at 37°C. BND cellulose chromatography columns were prepared. Chromatographic columns were washed six times with 1 ml of 10 mM Tris-HCl pH 8.0,1M NaCl. Then the columns were again washed six times with 1 ml of 10 mM Tris-HCl pH 8.0, 300 mM NaCl. Then the bound replication intermediates were eluted in buffer containing 10 mM Tris-HCl pH 8.0, 1M NaCl with 1.8% caffeine. The eluted sample containing enriched replication intermediates was further concentrated with Amicon size-exclusion filters (EMD Millipore). Quantity of the final replication intermediates was checked using agarose gel electrophoresis. Then the DNA samples were spread on carbon-coated grids and visualised by transmission electron microscope and the images were analysed by ImageJ software. All the experiments related to electron microscopy were performed in collaboration with Dr. Vincenzo Sannino. Dr. Vincenzo Sannino captured all the Electron Micrograph images. Dr.

Giorgio Baldi analysed EM pictures generated from BRCA2 depleted egg extract (Fig.3.7b).

Chapter 3. BRCA2 and Rad51 maintains replication fork integrity during unperturbed DNA replication.

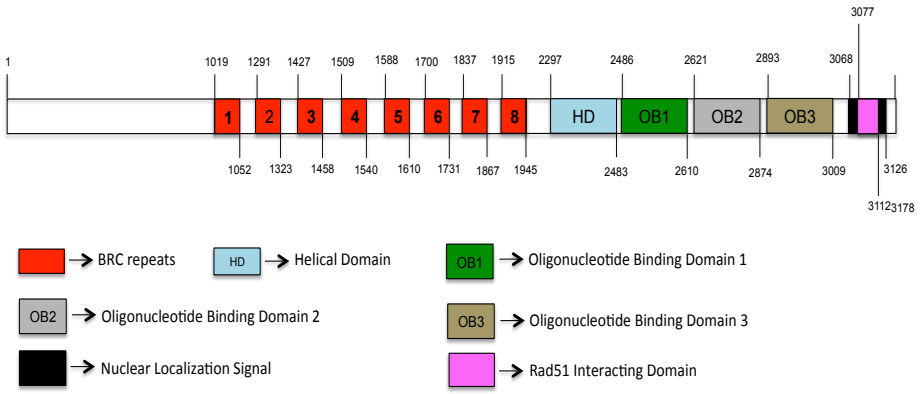
To examine the role of BRCA2 in unperturbed DNA replication.

In this part of the results, I will present characterisation of Xenopus laevis BRCA2 and discuss the function of BRCA2 and Rad51 in unperturbed DNA replication.

3.1 Protein sequence comparison between *Xenopus laevis* and *Homo sapiens* BRCA2

Xenopus laevis genome is not completely sequenced. Hence BRCA2 protein sequence is not yet available in NCBI database. In an effort to sequence *Xenopus* whole genome, *Xenopus* Genome Project was initiated from Wallingford and Marcotte labs from Texas Institute for Drug and Diagnostic Development. The sequencing of the *X.laevis* genome was performed at ~20x coverage using ABI SOLiD next-generation sequencing. To be useful for the *Xenopus* community unpublished intermediate datasets obtained were made online by Wallingford and Marcotte labs, in their laboratory website (http://www.marcottelab.org/index.php/Xenopus_Genome_Project). We retrieved the BRCA2 protein sequence from this database and analysed the sequence conservation with respect to *Homo sapiens*. Figure.3.1. show different domains present in *Homo sapiens* BRCA2. Fig.3.1 b shows the percentage identity and similarity between *Homo sapiens* and *Xenopus laevis* BRCA2.

a



b

BLAST analysis – Individual Domains of BRCA2 with *Homosapiens Vs Xenopus laevis*

Domains	%Identity	% Similarity	% Query Coverage
BRC-1	56	65	93
BRC-2	47	62	100
BRC-3	41	59	100
BRC-4	52	72	90
BRC-5	----	-----	-----
BRC-6	35	70	96
BRC-7	73	84	83
BRC-8	62	80	83
Helical Domain	66	80	99
OB1	66	76	100
OB2	60	76	99
OB3	42	62	99
Rad51 Interacting Domain	69	83	100

Figure 3.1 Conservation of BRCA2 domains in *X. laevis*

a. Shows different domains of BRCA2 (See Introduction section for details about known function of individual domains of BRCA2). **b.** Table shows the percentage sequence similarity and identity between *Xenopus* and *Homo sapiens* BRCA2.

3.2 Antibody production and characterisation for *Xenopus laevis* BRCA2.

Xenopus laevis BRCA2 protein consists of 3177 amino acids with a predicted molecular weight of ~353 kDa. Highly specific antibodies represent a fundamental tool in order to investigate the function of DNA replication factors with the *Xenopus* system (see section 1.1.12 *Xenopus* system in the introduction). They can be used to specifically deplete the protein from the *Xenopus* extracts in order to carry out functional analyses. Also commercially available antibodies were not found to be useful for the detection of *Xenopus laevis* BRCA2 in western blot analyses. Hence, we raised peptide based rabbit polyclonal antibodies against *Xenopus laevis* BRCA2. Antigenicity Predication Software was used to predict the highest immunogenic sequence for antibody production. Finally, N-terminal KPHIKEDQNEPESNSEYC –C-terminal sequence coupled with Keyhole Limpet Hemocyanin was chosen to immunise the Rabbits (By Biogenes, Germany).

Xenopus egg extract contains ~60 mg of total protein per ml of extract[155]. Generally, we use 0.5 µl or 1 µl of extract to detect proteins by immunoblotting. Hence an immunoblot was performed using 1 µl interphase egg extract or MBP tagged recombinant BRCA2 and probed with Anti-BRCA2 antibody (Fig.3.2). MBP-BRCA2 was produced in baculovirus system and the lysate was used for Western Blotting as positive control with respect to extract. As expected the antibody

recognised a clear band in extract and also the recombinant MBP-BRCA2 confirming that the serum is specific for *X.l*.BRCA2 (Fig.3.2).

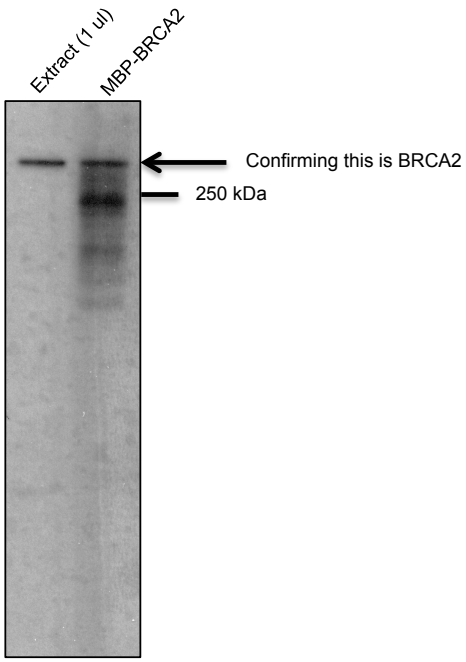


Figure 3.2 *Xenopus laevis* BRCA2 antibody characterisation.

1µl of interphase egg extract or MBP-BRCA2 is loaded on to 4-15 % SDS-PAGE Gel and immunoblotted with peptide raised anti-BRCA2 antibody. (MBP – BRCA2 was cloned and expressed by Dr. Anna De Antoni, Dr. Vincenzo Costanzo Laboratory).

3.3 BRCA2 associates with replicating chromatin

Xenopus laevis egg extracts represent a powerful tool to characterise the function of proteins involved in DNA replication and repair[151]. It has been shown that cells defective in homologous recombination machinery have impaired replication fork progression[99]. When on-going replication forks are slowed down or stalled, BRCA2 and Rad51 are recruited onto DNA in order to stabilize replication forks and protect newly synthesised DNA from nuclease-mediated degradation[68,100,156]. Since BRCA2 or Rad51 depleted cells accumulate chromosome breaks prior to cell death[93], it has been long speculated that BRCA2 and Rad51 function at the fork during unperturbed DNA replication to protect their spontaneous collapse and prevent chromosomal breakage[92]. In order to verify whether BRCA2 associates with chromatin, we monitored the chromatin association of BRCA2 during unperturbed and perturbed DNA replication. As shown in Fig.3.3, BRCA2 associates with replicating chromatin with a binding pattern similar to MCM7 and Polymerase alpha suggesting that like Rad51, BRCA2 might travel together with active replication forks during DNA replication. We also monitored the chromatin association of BRCA2 in the presence of DNA damaging agents namely Aphidicolin, S1 nuclease + Aphidicolin and EcoRI (Fig.3.3). These treatments led to increase in BRCA2 loading onto chromatin, consistent with the role of BRCA2 in resolving stalled replication fork and in repairing DSB's. Aphidicolin (APH) is an inhibitor of replicative polymerases that block fork progression. Addition of S1 nuclease in the presence of low APH induces breaks on DNA at the

level of stalled fork, a situation mimicking fork collapse[157]. The restriction enzyme EcoRI generates DSBs. All these treatments led to increase BRCA2 levels onto chromatin, suggesting that, in egg extract as in mammalian cells, BRCA2 binds to stalled forks and damaged DNA template. We next asked whether BRCA2 chromatin association was dependent on replication origin assembly. Therefore, egg extracts were supplemented with recombinant geminin, which inhibits the loading of MCM2-7 helicase[49]. Similar to Rad51, BRCA2 chromatin association was impaired when origin assembly was inhibited (Fig.3.4). Hence these data confirm that BRCA2 and Rad51 are involved in unperturbed DNA replication, likely at the level of replication forks.

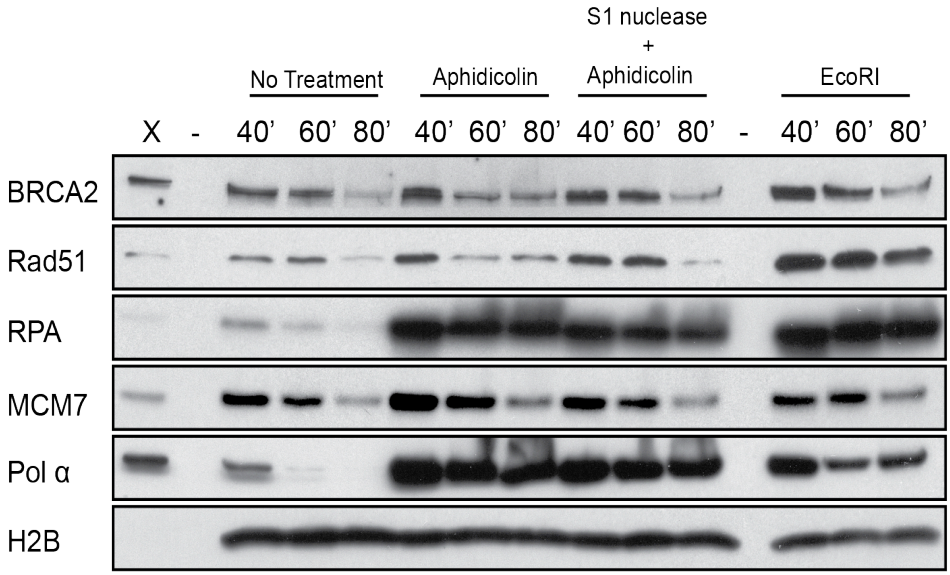
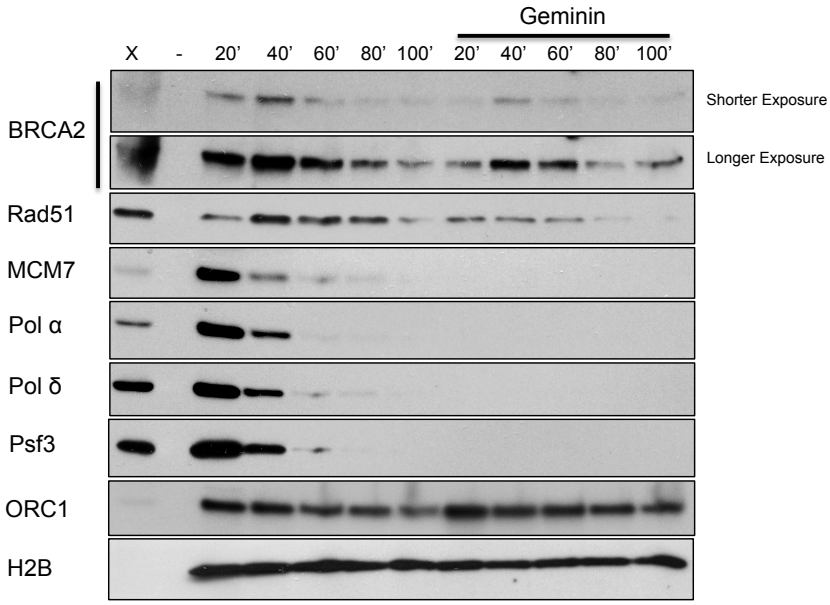


Figure 3.3 BRCA2 associates with replicating chromatin.

Time course of chromatin association of BRCA2, Rad51 and indicated replication factors were assessed in the presence or absence of different DNA damaging agents (Aphidicolin 20 μM, S1 nuclease 0.32 U/μl + aphidicolin 1 μM, EcoRI 0.05 U/μl). Western blotting was carried out with the chromatin fraction from 40 μl of extract incubated with 4000 nuclei/μl for the indicated times. X and – indicate extract (X) and empty lane (-), respectively. Similar results were obtained at least in three independent experiments.

a



b

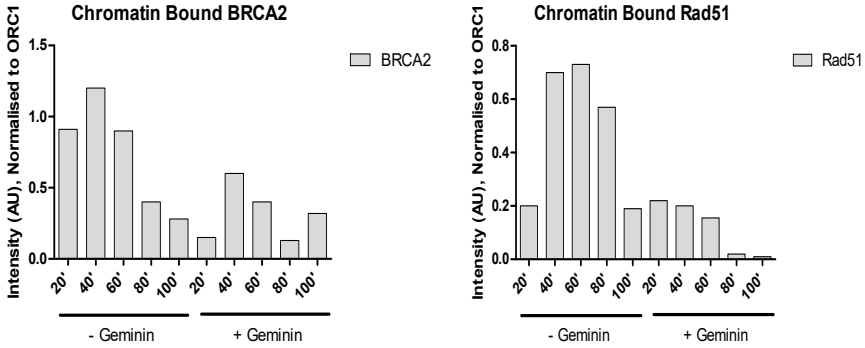


Figure 3.4 BRCA2 chromatin association is dependent on replication origin assembly.

a. Sperm nuclei (4000 nuclei/µl) were incubated with 30 µl of egg extract in presence and absence of ~ 200nM geminin for different time points as indicated and immunoblotted against indicated proteins **b.** Histograms represents the quantification of chromatin bound Rad51 and BRCA2 (Quantified by Image J software). Similar results were obtained at least in two independent experiments.

3.4 BRCA2 depletion does not affect bulk DNA synthesis in *Xenopus laevis* egg extract

To verify the role of BRCA2 in DNA replication, we assessed the replication potential of the *X.laevis* extracts depleted of BRCA2 in comparison with Mock-depleted extracts (pre-immune antibodies). As shown in Figure.3.5, BRCA2 was immunodepleted > 90% from *X.laevis* egg extract. These depleted extracts were subjected to DNA replication assays in which the level of DNA synthesis is measured based on the amount of radioactive dCTP incorporation. Mock and BRCA2 depleted extracts were optionally treated with 50 μM Topotecan an inhibitor of Topoisomerase I. Topoisomerase I is an essential enzyme that relaxes topological stress induced by supercoiling during replication and transcription. Topoisomerase I perform this function by creating ssDNA breaks to release the topological stress and re-ligate to reestablish intact dsDNA. Addition of Topotecan, a Topoisomerase I inhibitor, stabilises the cleavable complex and prevent re-ligation, therefore inducing lethal Double Strand Break's (DSB's)[158]. Figure.3.5 shows the mean efficiency of DNA replication from three different experiments carried out with three independently depleted extracts. The assessment from non – denaturing agarose gel electrophoresis suggests that the BRCA2 depletion does not affect the overall rate of DNA replication in *Xenopus* egg extracts, challenged or not with topoisomerase I inhibitor. Since non – denaturing Agarose gel gives only a rough estimate of DNA synthesis, we next performed TCA replication assay at different time points (Fig.3.6a). Binding of replisome components to chromatin was

also assessed in parallel experiments (Fig.3.6b). These experiments confirmed that BRCA2 depletion does not affect bulk DNA synthesis.

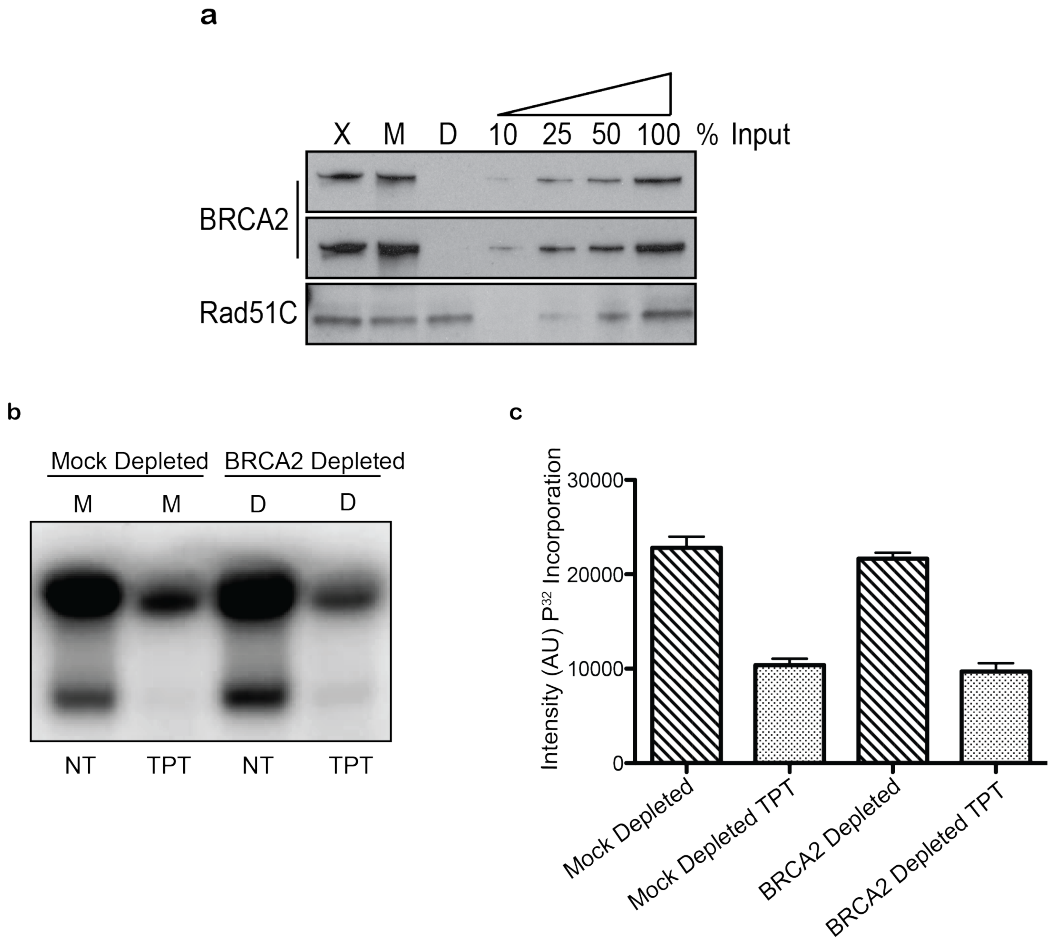


Figure 3.5 BRCA2 depletion does not affect bulk DNA replication in *Xenopus* egg extract.

a. 1 µl of egg extract (X), Mock depleted extract (M), BRCA2 depleted extract (D) are loaded in SDS-PAGE gel and immunoblotted to test the level of depletion. Different percentage of extract was loaded as input to semi - quantitatively compare the efficiency of depletion. **b.** Interphase extract was supplemented with sperm nuclei (2000 nuclei/ µl) and α^{32}P - dCTP in the presence or absence of 50 µM Topotecan (TPT) for 120 minutes and DNA synthesis was monitored by Neutral Agarose Gel Electrophoresis. **c.** Intensity of two bands from neutral agarose gel

electrophoresis qualitatively represents the degree of incorporation of $\alpha^{32}P$ - dCTP to DNA template. Bar graph shows the mean intensity of $\alpha^{32}P$ – dCTP at 120 min. Error bars indicate standard deviation of the mean (from three independent experiments).

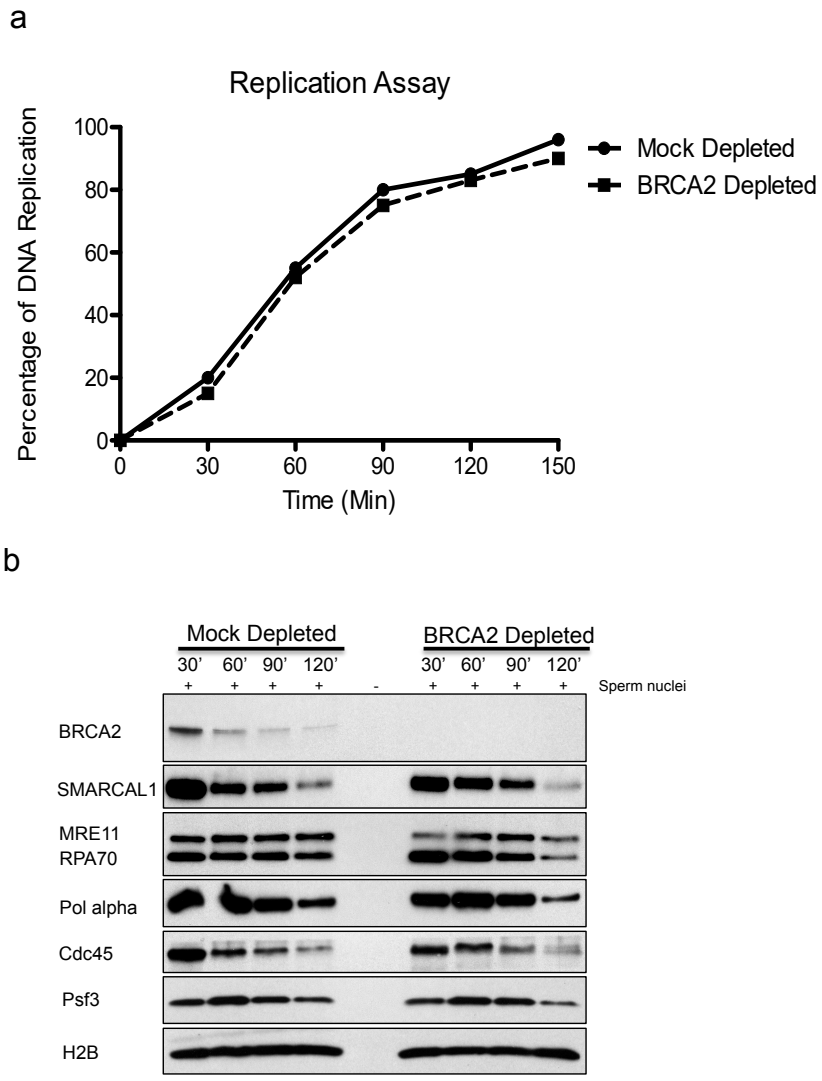


Figure 3.6 BRCA2 depletion results in a slight delay in replication timing.

a. Mock or BRCA2 depleted extract was incubated in the presence of sperm nuclei 2000 nulcei/µl with $\alpha^{32}P$ - dCTP and the replication was quantified with TCA precipitation assay[159]. **b.** Mock or BRCA2 depleted

extract was analysed for the indicated proteins enriched on chromatin, by chromatin fractionation and immunoblotting.

3.5 BRCA2 depletion results in ssDNA gaps accumulation at the fork.

Chromosome breakage or gaps are hallmarks of BRCA2 or Rad51 depleted cells[104,160,161]. Of note, formation of chromosome breaks during mitosis is commonly viewed as the consequence of incomplete DNA replication known as under-replication[162]. Recently, evidence from my host laboratory showed by Electron Microscopy (EM) analyses of DNA replication intermediates that Rad51 chromatin association is required to prevent single strand DNA gaps accumulation in daughter strands during unperturbed DNA replication[68]. Failure to replicate a segment of the genome may lead to chromosome gaps or breakage at the end of mitosis[23,163-165]. Hence it can be envisaged that although inhibition of Rad51 chromatin association does not affect overall DNA replication, it is required to prevent the formation of gaps that arise during DNA replication likely due to endogenous DNA replication stress sources.

Thus we analysed by EM DNA replication intermediates isolated during unperturbed DNA replication in extracts depleted of BRCA2. Similar to Rad51[68], BRCA2 depletion also resulted in ssDNA gaps accumulating at the fork with an average length of ~ 80nm that corresponds to ~ 0.2kb (Fig.3.7 and 3.8). Overall these data clearly suggest that BRCA2 and Rad51 are required to perform a peculiar protective function during

unperturbed DNA replication to prevent ssDNA gaps accumulation at the fork.

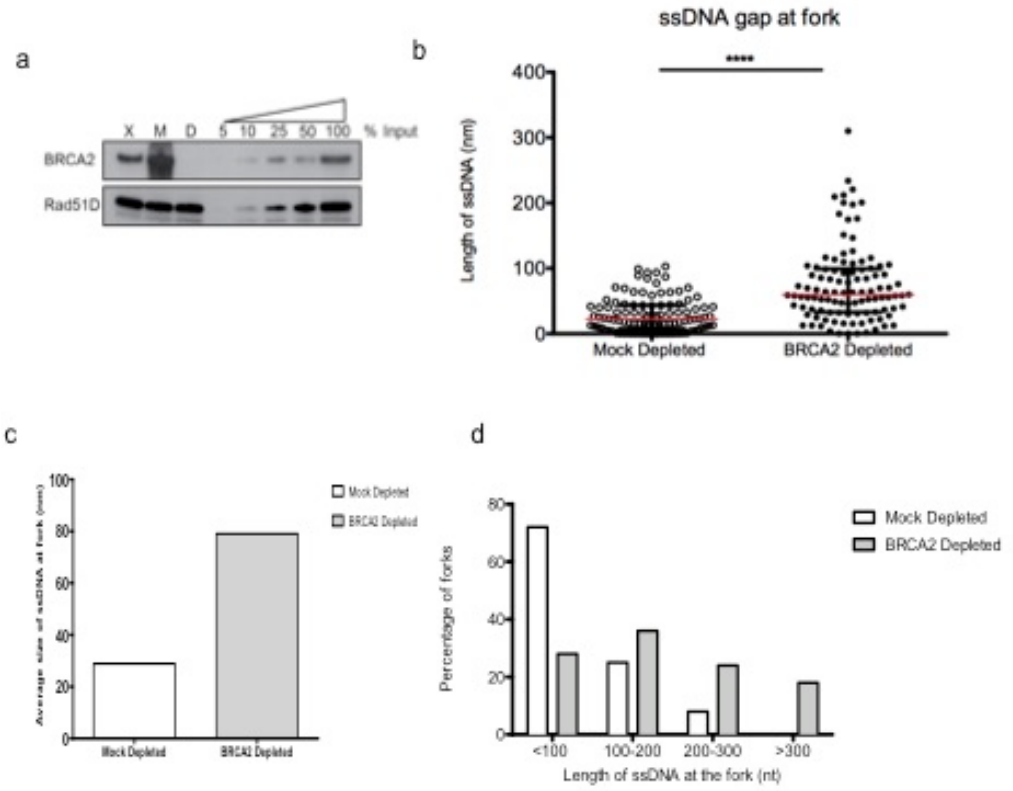


Figure 3.7 BRCA2 depletion results in ssDNA gaps accumulation at the fork.

a. Interphase extract immunodepleted with Pre-Immune IgG (M) or BRCA2 antibodies (D). 1 µl of Mock or depleted extract was loaded on SDS-PAGE gel and immunoblotted against BRCA2 proteins to check the efficiency of depletion. X – Untouched extract, M – Mock depleted, D – BRCA2 depleted. Different percentage of extract was loaded as input. **b.** Scattered distribution of ssDNA gap length in nanometre (nm), obtained from DNA replication intermediates. ****P<0.0001 (Mann-Whiteney test). **c.** Average size of ssDNA at the fork (nm) is represented in histogram**. d.** Histogram shows the distribution of length of ssDNA at fork, measured at nucleotides level (nt). Total number of

molecules analysed in Mock or BRCA2 depleted extract is 100. Results obtained in b, c, d represents one independent experiment.

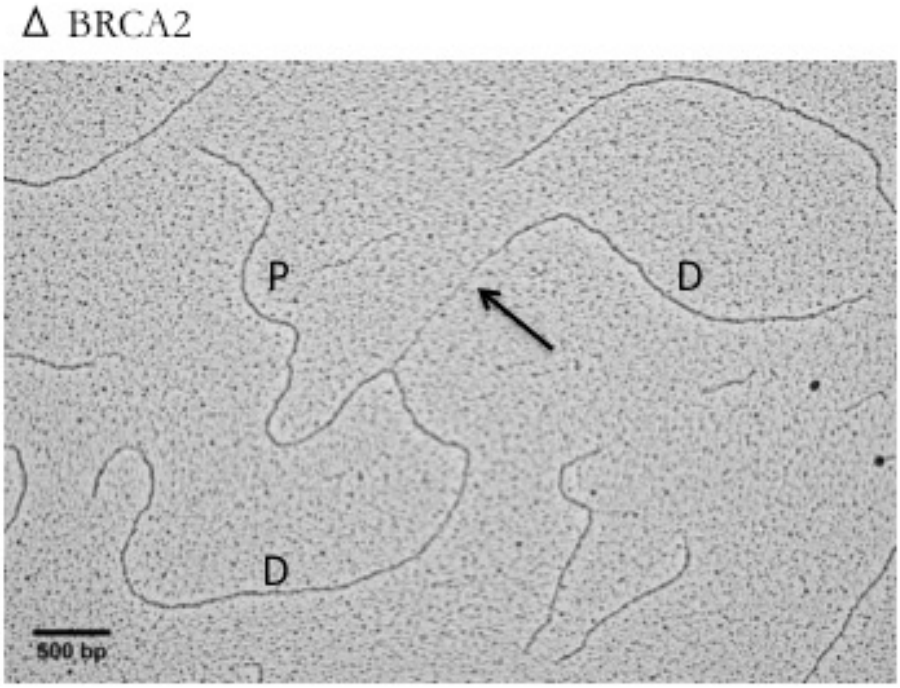


Figure 3.8 Electron Microscopic visualisation of ssDNA gaps accumulation.

Representative replication intermediate isolated from *Xenopus* egg extracts from BRCA2 depleted extracts. Letter P indicate the parental strand, D indicates the daughter strands. Arrow indicates ssDNA gap accumulation at one of the daughter strands.

3.6 The length of ssDNA gap accumulation at the fork in the absence of Rad51 mirrors BRCA2 depletion.

BRCA2 deficient cells suffer from chronic genome instability with different kind of chromosomal abnormalities[100,166]. A previous study from my host laboratory showed that inhibiting Rad51 chromatin binding by GST-BRC4 peptide resulted in accumulation of ssDNA gaps at and behind the replication fork[68]. During my PhD study I took advantage of Rad51 depletion strategy rather than using GST-BRC4 peptide to abolish Rad51 chromatin binding. Consistent with the published results[68,157], we found that Rad51 depletion does not impact overall rate of replication (Fig.3.9 a, b) but we observed a marked increase in the level of ssDNA gaps at the fork of ~100nm that corresponds to 0.28 kb in the absence of Rad51 (Fig.3.10). Interestingly the ssDNA gaps in the absence of BRCA2 or Rad51 resulted in similar gap length at the fork, indicating that during unperturbed DNA replication BRCA2 and Rad51 likely operates in the same pathway to protect replication forks.

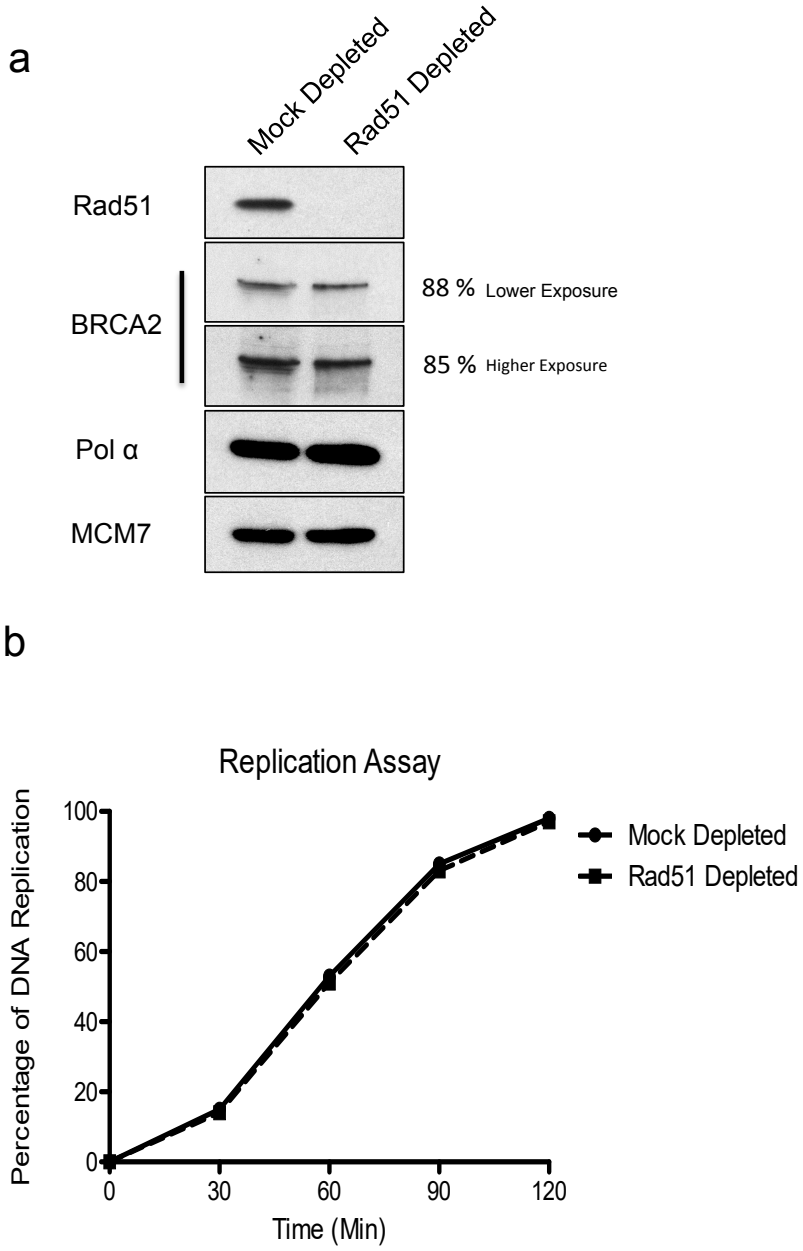


Figure 3.9 Rad51 depletion does not affect DNA replication in *Xenopus laevis* egg extract.

a. 1 µl of Mock or Rad51 depleted extract was loaded on 4-15% SDS-PAGE gel and immunoblotted for indicated proteins. **b**. TCA replication assay was carried out in Mock or Rad51 depleted extracts incubated with 2000 sperm nuclei/ µl with α^{32}P - dCTP.

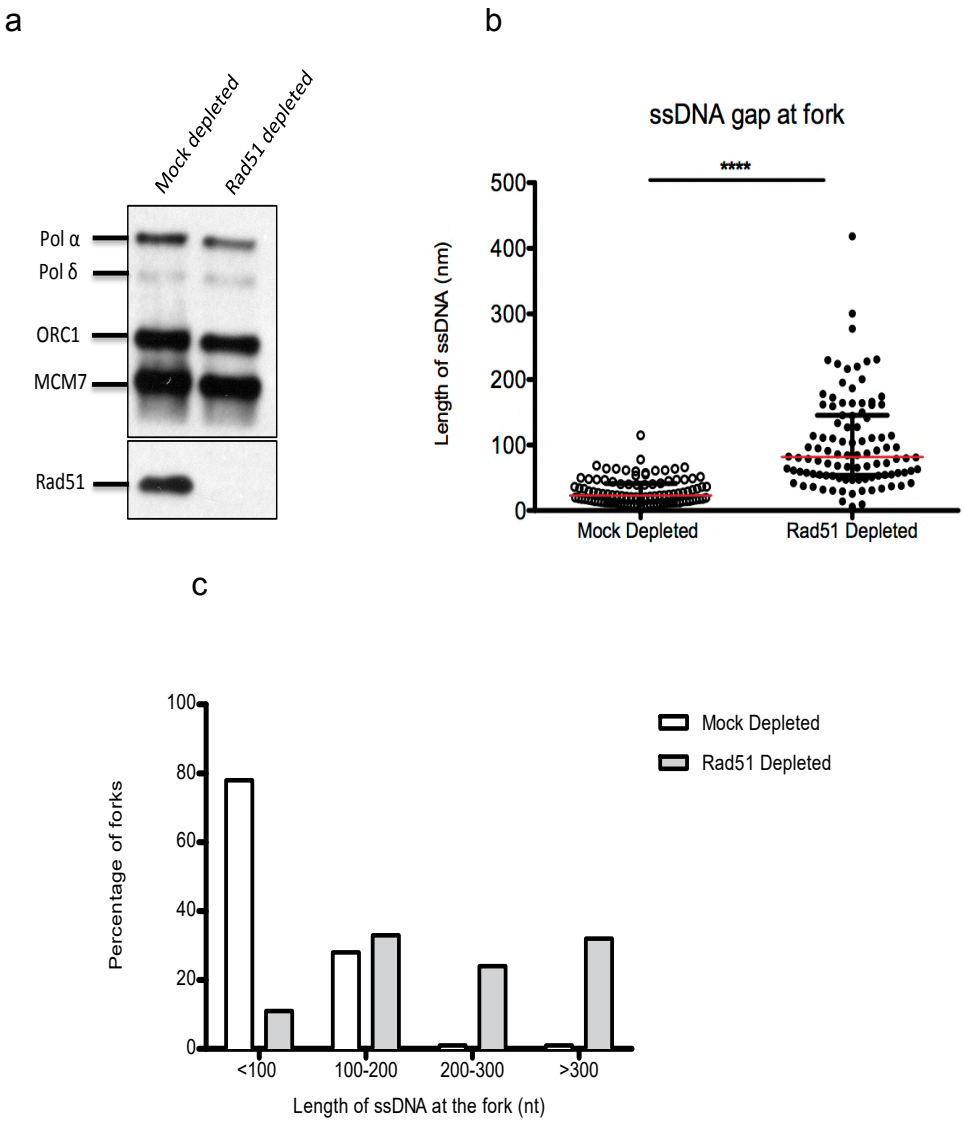


Figure 3.10 Rad51 depletion results in ssDNA gaps at the fork.

a. 1 µl of Mock or Rad51 depleted extract was loaded on 4-15% SDS-PAGE gel and immunoblotted for indicated proteins. **b**. Scattered plot shows the distribution of ssDNA size (in nm) from DNA replication intermediates. 105 molecules were analysed for Mock and Rad51 depleted extracts. ****P<0.0001 (Mann-Whiteney test). **c.** Histogram shows the percentage of molecules distributed in terms of different ssDNA size (at the level of nucleotides, nt). Results obtained in a, b, and c are one representative image obtained from at least three independent experiments.

3.7 Bio-chemical characterisation of BRCA2 and Rad51 from *Xenopus laevis* egg extract

3.7.1 BRCA2 depletion do not co-deplete Rad51 from *Xenopus* egg extract

As shown earlier, BRCA2 or Rad51 depletion resulted in ssDNA gap accumulation at the fork with similar length. Vast body of literature suggests that BRCA2 strongly interact with Rad51 both *in vitro* and *in vivo* and that these proteins are present in the same complex[167-169]. Considering this, we next questioned the level of Rad51 in BRCA2 depleted egg extracts. If BRCA2 and Rad51 exist in a stable complex in egg extracts, one would expect depletion of BRCA2 might co-deplete Rad51. Contrary to our expectation, BRCA2 depletion did not co-deplete Rad51 from *Xenopus laevis* egg extracts (Fig.3.11a). However approximately 20% reduction in the level of Rad51 was observed upon BRCA2 depletion from egg extract (Fig.3.11a).

3.7.1.1 Rad51 depletion do not co-deplete BRCA2 from Xenopus egg extract

Since BRCA2 depletion did not affect the level of Rad51 in egg extract, we next asked whether Rad51 depletion affects BRCA2 quantitatively in egg extract. As shown in immunoblot Fig.3.9, Rad51 was depleted near to 100 % and its depletion did not significantly affect the level of BRCA2 in egg extract. Overall these data hints that most of Rad51 in the cytoplasmic extract is not in complex with BRCA2. This is also suggested by the fact that cytoplasmic BRCA2 and Rad51 do not co-

elute in the same complex in size exclusion fractions (Fig.3.11b). To preserve interactions between protein complexes we used 50mM KCl as salt concentration in the buffer in which the extract was diluted and processed for gel filtration. As shown in Fig.3.11b majority of BRCA2 is eluted in void volume ~2000 kDa indicating that BRCA2 is a part of a multimeric complex and major proportion of Rad51 eluted near to its monomeric form ~ 37 kDa. These data further suggest that the majority of BRCA2 and Rad51 are not in a same complex in interphase *Xenopus laevis* egg extract.

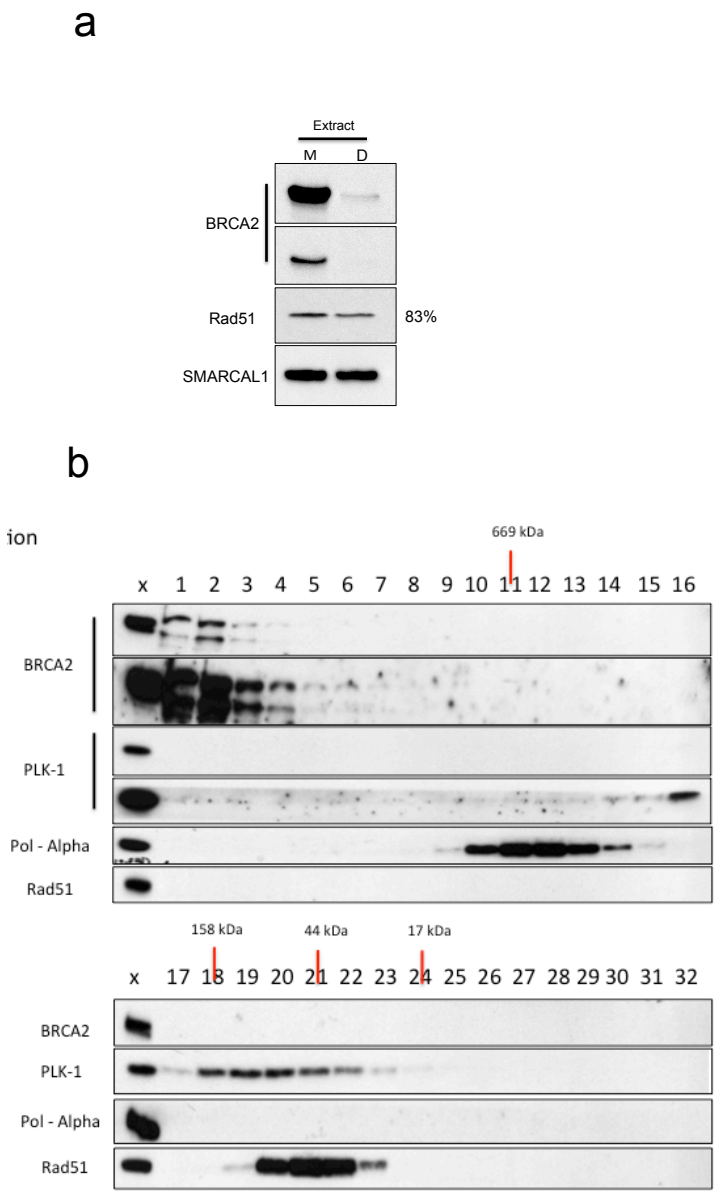


Figure 3.11 BRCA2 depletion does not co-deplete Rad51 from *Xenopus* laevis egg extract.

a. Interphase egg extract was immunodepleted with either non-immune IgG or anti-BRCA2 antibody and then 1 µl of extract was loaded on gel to test the efficiency of depletion. b. 500 µl Interphase egg extract was diluted five fold in IP buffer and loaded onto Superose6 gel filtration column. 15 µl fractions were immunoblotted against indicated proteins.

3.7.2 Rad51 interacts with BRCA2 in interphase *Xenopus laevis* egg extract

Although size exclusion chromatography is a good technique to separate proteins based on complex sizes, the clarified extract loaded onto the column gets diluted several folds and proteins might eventually escape to western blot detection. We therefore sought to immunoprecipitate Rad51 from *Xenopus* egg extract and probed for BRCA2 to check whether it co-immunoprecipited with Rad51. Consistent to previously published *in vivo* and *in vitro* data[167-169] from human cells, we observed an interaction between Rad51 and BRCA2 in *Xenopus* egg extracts (Fig.3.12).

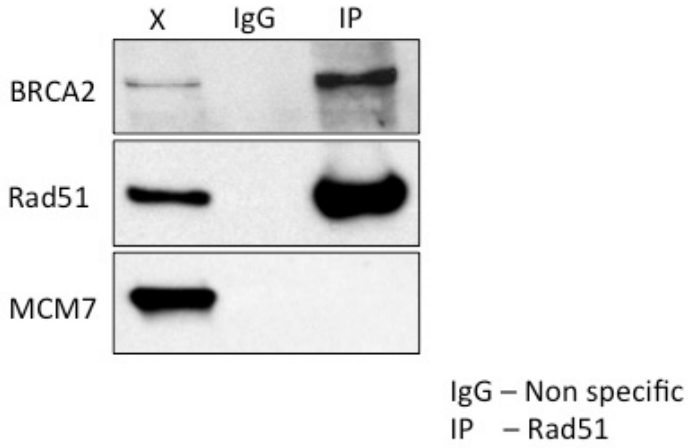


Figure 3.12 Rad51 interacts with BRCA2 in interphase *Xenopus laevis* egg extract.

Interphase *Xenopus laevis* egg extract was immunoprecipitated with non-immune IgG as Mock and Rad51 antibody. Precipitated proteins were immunoblotted with antibodies to BRCA2, Rad51 and MCM7.

3.8 BRCA2 and Rad51 are interdependent for chromatin binding during chromosomal DNA replication

Even if BRCA2 or Rad51 depletion did not significantly co-deplete each of these factors, it is possible that BRCA2 and Rad51 assemble onto DNA. We next questioned the interdependency of these factors for chromatin recruitment during DNA replication. To this end, we depleted BRCA2 from egg extract and monitored the ability of Rad51 chromatin binding during normal DNA replication. Interestingly, BRCA2 depletion impaired Rad51 chromatin association during unperturbed DNA replication (Figure.3.13). This result indicates that BRCA2 is required for stable Rad51 binding onto replicating DNA. However, residual Rad51 binding was noticed at higher exposure, suggesting that Rad51 can also bind chromatin without BRCA2, although less efficiently. Notably, BRCA2 depletion also mildly delayed the kinetics of chromatin association of replication factors Polymerase alpha, Psf3, Cdc45. Delay in the kinetics of replication factors loading reflects the slowing down of replication progression obtained by TCA precipitation assay (Fig.3.6a). Similarly, Rad51 depletion impaired BRCA2 binding to chromatin. At longer exposure (Fig.3.14) we observed that there is a fraction of BRCA2 still bound onto chromatin. Overall these data suggest that majority of the chromatin bound Rad51 and BRCA2 are inter-dependent on each other but a small fraction of Rad51 and BRCA2 binds to the chromatin independently.

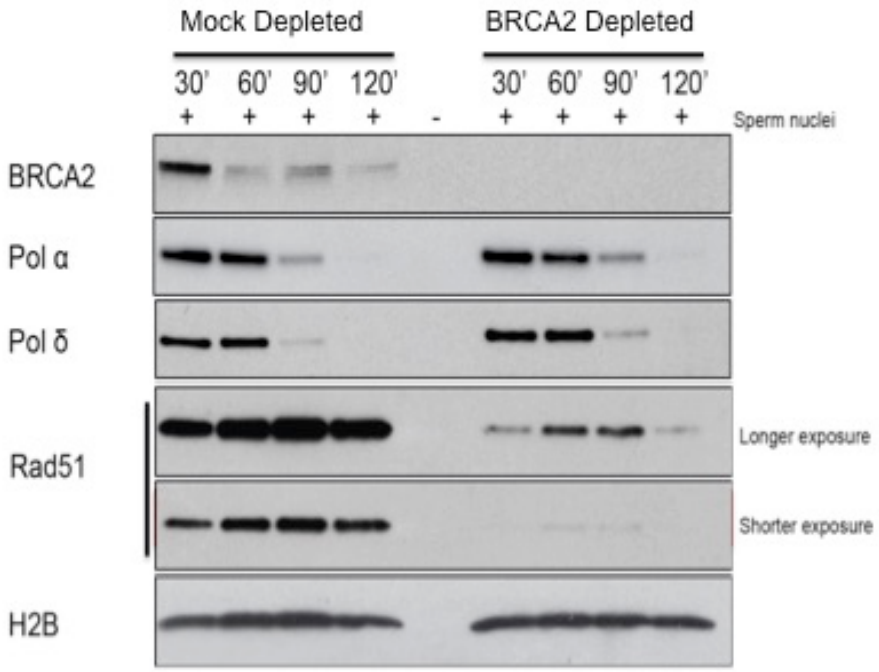


Figure 3.13 BRCA2 depletion impairs chromatin recruitment of Rad51 during chromosomal DNA replication.

Mock or BRCA2 depleted egg extract was incubated with 4000 sperm nuclei/μl for indicated times then chromatin fraction was immunoblotted against BRCA2, Rad51, Polymerase alpha and Polymerase delta as indicated. Similar results were obtained at least in two independent experiments.

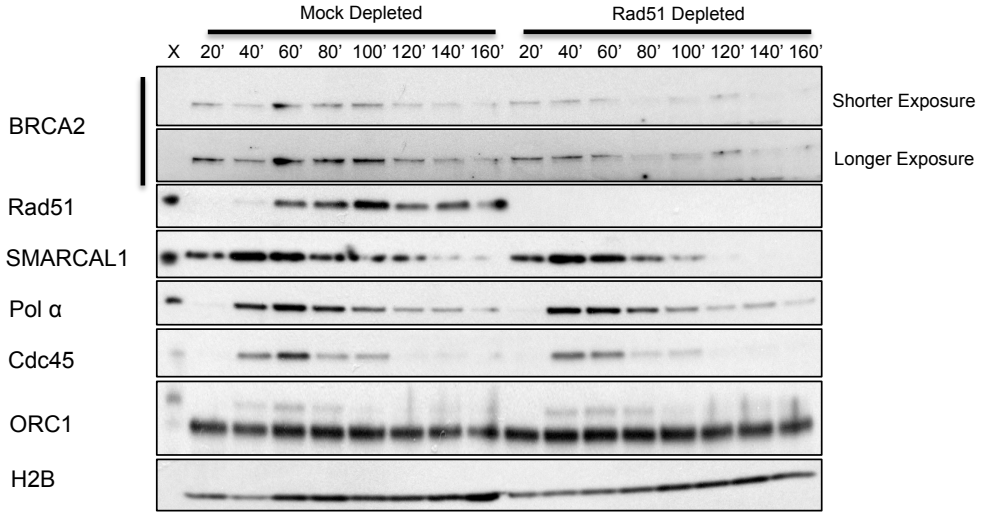


Figure 3.14 Rad51 depletion impairs chromatin recruitment of BRCA2 during unperturbed DNA replication.

30 µl of Mock or Rad51 depleted egg extract was incubated with 4000 sperm nuclei/µl for indicated times. Then chromatin fraction was purified and immunoblotted against indicated proteins.

3.9 Conclusions

In summary, these results indicate that BRCA2 is a replication dependent chromatin-associated protein required for replication fork integrity during unperturbed DNA replication. Electron Microscopy (EM) analysis of DNA replication intermediates in BRCA2 depleted egg extracts revealed striking accumulation of ssDNA gaps in daughter strands. Interestingly, ssDNA gap length observed in BRCA2 depleted extract was similar to that of Rad51 depleted extracts. Furthermore, during unperturbed DNA replication Rad51 is predominantly recruited onto chromatin by BRCA2. Overall observations from first part of the results section indicate that BRCA2 and Rad51 chromatin association is required to limit the size of ssDNA gaps at replication forks during unperturbed DNA replication.

Chapter 4. Rad51 interacts with Polymerase alpha in the presence of DNA damage

To further investigate replication fork associated function of Rad51 during perturbed and unperturbed DNA replication.

The observed ssDNA gaps accumulation in the absence of BRCA2 or Rad51 suggests a replication fork associated function for these proteins during unperturbed DNA replication. In this chapter the link between Rad51 and replisome components will be discussed. Results obtained in this section led to the identification of an interaction between Rad51 and Polymerase alpha in the presence of DNA damaging agents.

4.1 Rad51 is an integral part of the replisome machinery in *Xenopus laevis* egg extract

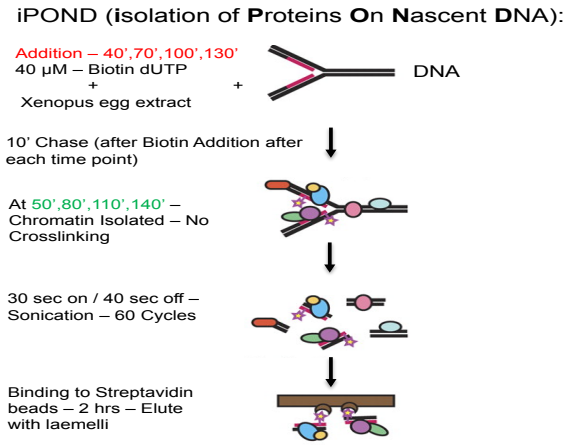
To ask whether BRCA2 and Rad51 travel with replication fork during unperturbed DNA replication we performed **i**solation of **P**roteins enriched **O**n **N**ascent **D**NA (iPOND) experiments, a recently developed technique, routinely used in mammalian cells to test proteins enriched at replication fork[98]. The schematic representation of iPOND experiment performed from *Xenopus* egg extract is shown in Figure.4.1a.

In early *Xenopus* embryos replication origins are activated in clusters[170]. Origins within each clusters are activated stochastically every 10 – 15 kb to complete fast duplication of the genome[14,31,170,171]. The inter-origin distance in the *Xenopus* embryonic system is much shorter compared to somatic mammalian cells. In embryonic cells, origins are spaced every 10 to 15 kb whereas in somatic cells origins are placed every 50

to 150 kb hence the overall timing of replication is slowed down in somatic cells[31]. However, the replication fork rate in embryonic cell is ~ 1.2 kb/min compared to ~ 2 kb/min in somatic cell[98,170,172].

Considering the similar rate of fork progression in embryonic and mammalian system we adapted protocol for **iPOND** used in mammalian cells[98]. As shown in Figure.4.1b at 50', 80', 110', and 140' time points we could detect Pol alpha, Pol delta and Cdc45 in the biotin-dUTP pull-down indicating the presence of *bonafide* replication fork components. Interestingly we observed a reproducible binding of Rad51 at the fork together with replisome components. As shown earlier in Figure.3.13 and 3.14 that Rad51 and BRCA2 are interdependent for chromatin binding during normal replication. Therefore it is possible that BRCA2 travels with the fork together with Rad51 in embryonic DNA replication. Our data is consistent with published results obtained from mammalian cells using Nascent Chromatin Capture approach and CIdU Immunoprecipitation[173,174] during unperturbed DNA replication. Hence preventing ssDNA gap accumulation at the fork could actually be BRCA2 and Rad51 function while working in co-ordination with the replisome machinery.

a



Taken From Sirbu BM , Nature Protocol, 2012

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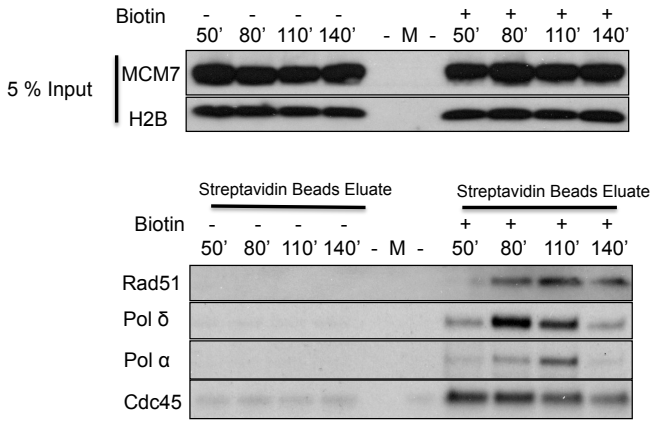


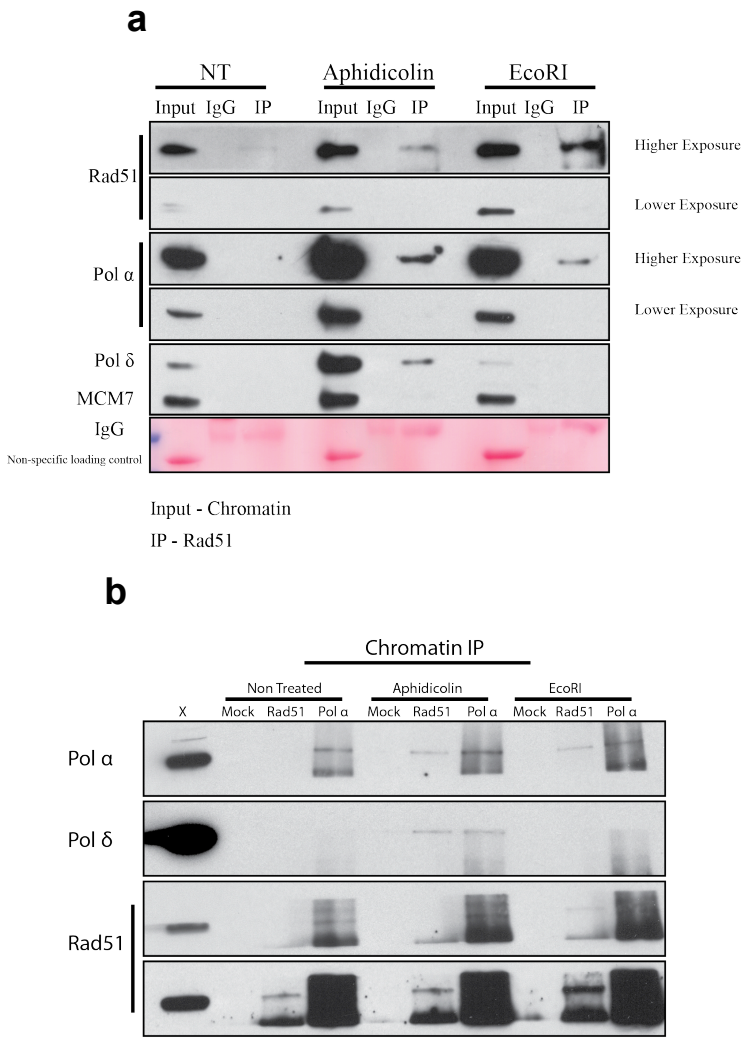
Figure 4.1 Rad51 travels with replication fork components.

a. Schematic representation of iPOND experiment performed using egg extracts. 100 µl of interphase egg extract was incubated with 4000 sperm nuclei/µl for 40', 70', 100', 130' in different reaction tubes. At 40', 70', 100', 130' time points extracts were optionally supplemented with 40 µM biotin-dUTP was added and incubated for further 10 min to allow incorporation of biotin-dUTP. At 50', 80', 110' and 140' min chromatin was fractionated; sonicated and nascent chromatin was pull-down with streptavidin beads. The eluted samples were immunoblotted against Rad51, Polymerase δ, α and Cdc45.

4.2 Rad51 interacts with Polymerase alpha on chromatin during replication stress and DSB

Since Rad51 or BRCA2 depletion results in ssDNA gap accumulation at the fork and these factors also travel with the fork, we asked whether Rad51 interact with replisome components on chromatin assembled in *Xenopus laevis* egg extract. Considering the literature and known interacting partners, we immunoprecipitated Rad51 from chromatin and probed for several factors involved in DNA replication. Surprisingly in the presence of replication stress and double strand break, Rad51 interacts with Polymerase alpha on the chromatin (Fig.4.2a). Reciprocal immunoprecipitation of Polymerase alpha also showed an interaction with Rad51 in the presence of replication stress and double strand breaks formation (Fig.4.2b).

Having discovered an interaction between Rad51 and Polymerase alpha on chromatin, we next monitored the level of Polymerase alpha on the damaged DNA template in the presence or absence of Rad51. Of interest, treatment of egg extract with Aphidicolin or Topotecan (Topoisomerase I inhibitor) damaging agents reduced the level of Polymerase alpha on the chromatin in the absence of Rad51 (Fig.4.3). Overall these data hint that in the presence of replication stress, BRCA2/Rad51 complex might promote efficient repriming through polymerase alpha against nuclease-mediated degradation. At this point we hypothesise that the single strand DNA gap observed in the absence of BRCA2/Rad51 is due to impaired repriming activity of Polymerase alpha at stalled replication fork.



Mock – Non-specific IgG, Rad51 – IP with anti-Rad51 antibody, Pol α – IP with anti-Polymerase α antibody.

Figure 4.2 Rad51 interacts with Polymerase alpha on chromatin.

a. Interphase egg extract was incubated with sperm nuclei and optionally treated with 20 μM aphidcolin or 0.05 U/μl EcoRI for 90 min. Then chromatin fraction was purified, DNaseI digested and sonicated. Proteins released from the chromatin were immunoprecipitated with Rad51 antibody and immunoblotted against Rad51, Polymerase α, δ, MCM7. **b.** Reciprocal immunoprecipitation from chromatin was performed using Polymerase α and δ antibodies. Different exposures are shown. Result obtained in “a” has been validated at least in two

independent experiments. Result obtained in “b” represents one independent experiment.

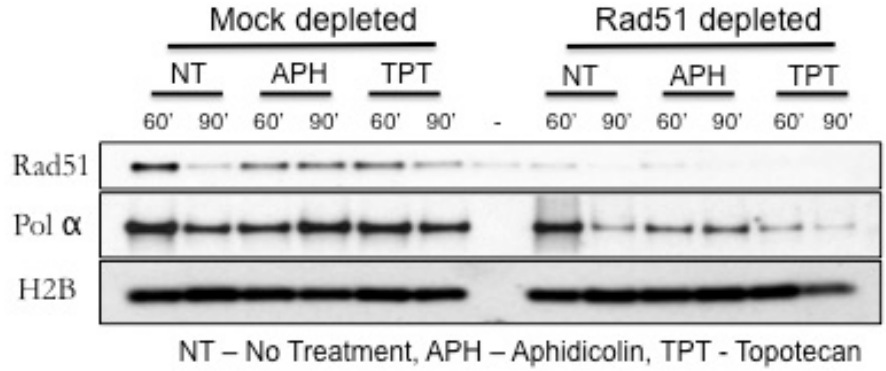


Figure 4.3 Rad51 depletion impairs Polymerase α chromatin recruitment in the presence of DNA damage.

Mock Depleted or Rad51 depleted extract was incubated with sperm nuclei optionally treated with 20 μM aphidicolin or 50 μM Topotecan. Then the chromatin fraction is purified and immunoblotted against Rad51, Polymerase α and H2B. Similar results were obtained at least in two independent experiments. Dr.Hérve Techer (Vincenzo Costanzo’s laboratory) performed the experiment shown above.

4.3 Conclusions.

In conclusion, these results indicate that the Homologous Recombination (HR) protein Rad51 is an integral part of replisome component. Isolation of Proteins enriched On Nascent DNA (iPOND) experiment revealed that Rad51 associates with replication fork during unperturbed DNA replication. In addition, we found that Rad51 interacts with Polymerase alpha when replication forks are stalled by aphidicolin. Taken together, our results indicate a possible role for Rad51 in Polymerase alpha mediated re-priming to promote continuous DNA synthesis in the presence of replication stress.

Chapter 5. SMARCAL1 induces replication fork reversal when replication fork progression is challenged.

To investigate and identify factors that contributes to replication fork reversal when replication fork progression is challenged.

Results described in chapter 3 and 4 indicate that Rad51 and BRCA2 functions during unperturbed and perturbed DNA replication to maintain replication fork integrity. In this chapter, we show that in the presence of replication fork stalling, replication forks remodel into a four-way junction called replication fork reversal. We discuss about a factor called SMARCAL1, an annealing helicase that is required for fork reversal activity, when replication fork progression is challenged.

5.1 Aphidicolin induced massive ssDNA accumulation and replication fork reversal in *Xenopus laevis* egg extracts.

Rad51 interaction with Polymerase alpha on chromatin hinted the possibility that ssDNA accumulation at the fork may be due to inefficient priming and polymerising activity of polymerase alpha. Similar to Rad51 or BRCA2 knock out cells, aphidicolin treated cells also show massive chromosomal aberrations[175]. Aphidicolin is a highly specific inhibitor of Polymerase α[176] but it inhibits Polymerase δ and ε weakly without affecting the exonuclease activity of Polymerase ε on ssDNA[177,178]. Two different studies reported that aphidicolin has no inhibitory activity against DNA repair enzymes Polymerase β and γ[179,180]. Considering the

stronger specificity of aphidicolin to Polymerase α, we intended to know the length of ssDNA gap generated when Polymerase α activity is inhibited by aphidicolin using Electron Microscopy mediated visualisation of DNA replication intermediates. Knowing the minimal length of ssDNA gap at the fork in the presence of aphidicolin will allow us to directly compare the severity of Rad51 or BRCA2 knock out cells. We first titrated the concentration of aphidicolin and monitored the concentration at which maximum RPA or Polymerase α bound to chromatin. As shown in Figure.5.1, RPA and Polymerase α saturates on chromatin from 10 to 40 μM. However, addition of aphidicolin at such concentrations to *Xenopus* egg extract together with sperm nuclei abolishes DNA replication completely[67].

In order to stall replication fork from on-going replication and visualise the replication intermediates, we used a different protocol as shown in Figure.5.2. We allowed replication to happen in *Xenopus* egg extract in the presence of sperm nuclei for 60 min where usually more than 50 to 60% of the genome is replicated (Fig.3.9) and then aphidicolin was added. This strategy allow proteins involved in replication fork protection and Homologous Recombination proteins to hyper accumulate on the chromatin to choose HR mediated structure formation (presumably reversed forks) or processing of replication forks by nucleases resulting in ssDNA gap accumulation. At the concentration of aphidicolin that we used replication forks are stalled but the nuclear assembly is not affected. As shown in Figure.5.3a aphidicolin mediated fork stalling induced average ssDNA gap length of ~0.5 to 0.6 kb in length at the fork. Nearly 15% of forks showed

extensive ssDNA gap length of 1 to 2 kb (Fig.5.3b). On the other hand, it should be note that Rad51 or BRCA2 depletion alone induced average ssDNA gap length of ~ 0.28 kb or ~ 0.20 kb, respectively (Fig.3.9 and 3.7). These results indicate the severity of Rad51 or BRCA2 depletion in the absence of replication fork stalling agents.

Aphidicolin treatment also induced reverse fork formation. Reverse forks are four-way junction DNA replication intermediates caused by re-annealing of the parental duplex and the consequent re-annealing of the two nascent DNA strands (see introduction, section. 1.1.11). We have finely evaluated the percentage of fork reversal in *Xenopus* egg extract in the presence of aphidicolin. As shown in Figure.5.3c, we observed that fork stalling by aphidicolin led to the formation of ~15% reversed forks. A representative reverse fork has been shown in Figure.5.4.

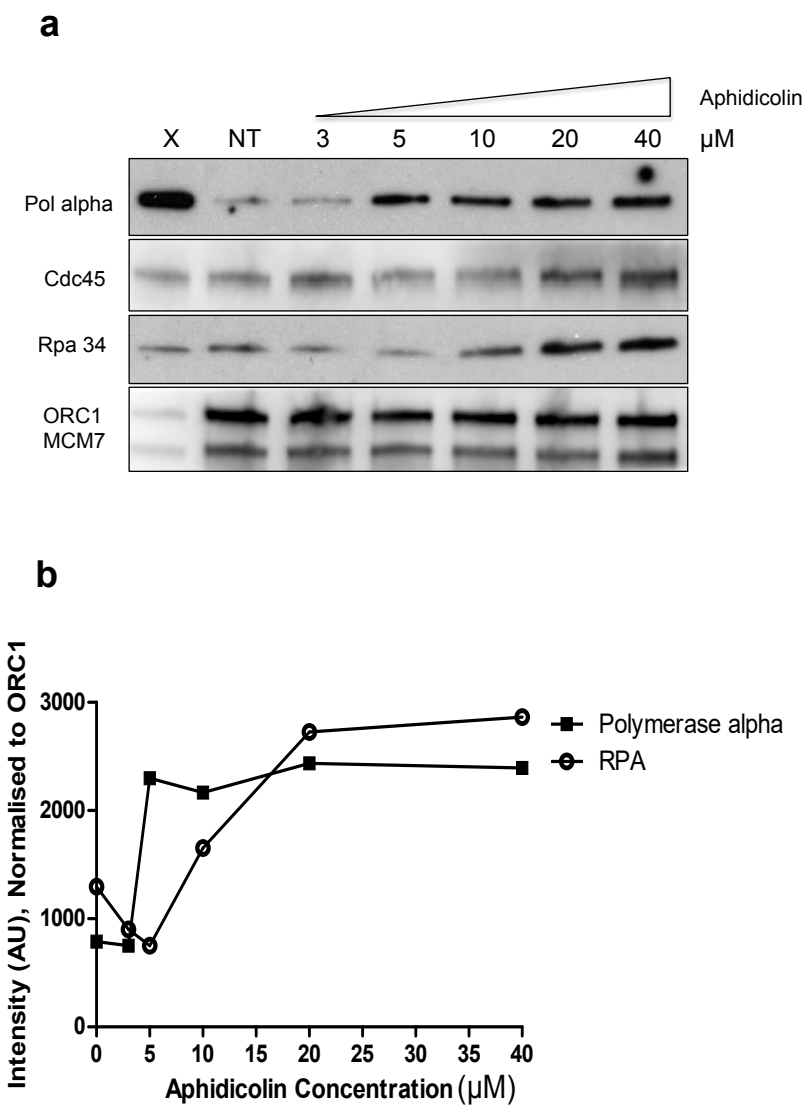


Figure 5.1 10μM Aphidicolin is enough to saturate chromatin bound RPA and Polymerase α.

a. Interphase egg extract was incubated with sperm nuclei and optionally supplemented with 3, 5, 10, 20, 40 μM aphidicolin. NT indicates non-treated. Chromatin bound fractions at 90' were then immunblotted against Polymerase α, Cdc45, RPA, ORC1/MCM7. **b**. Quantification of chromatin bound Polymerase α and RPA.

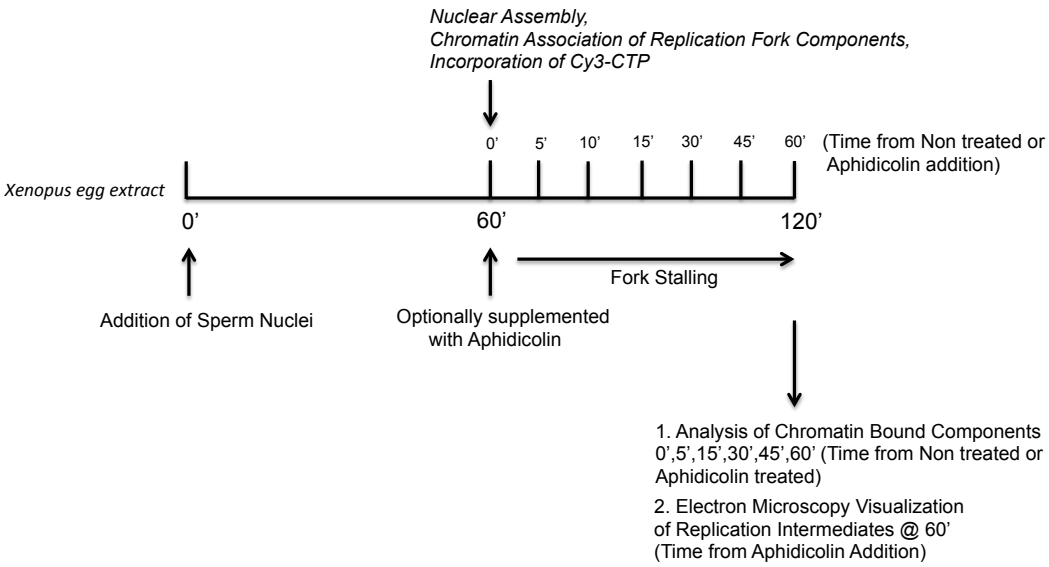


Figure 5.2 Aphidicolin treatment procedure for EM analysis.

Scheme detailing the procedure of aphidicolin treatment and time points isolated for chromatin binding and electron microscopic visualisation of replication intermediates.

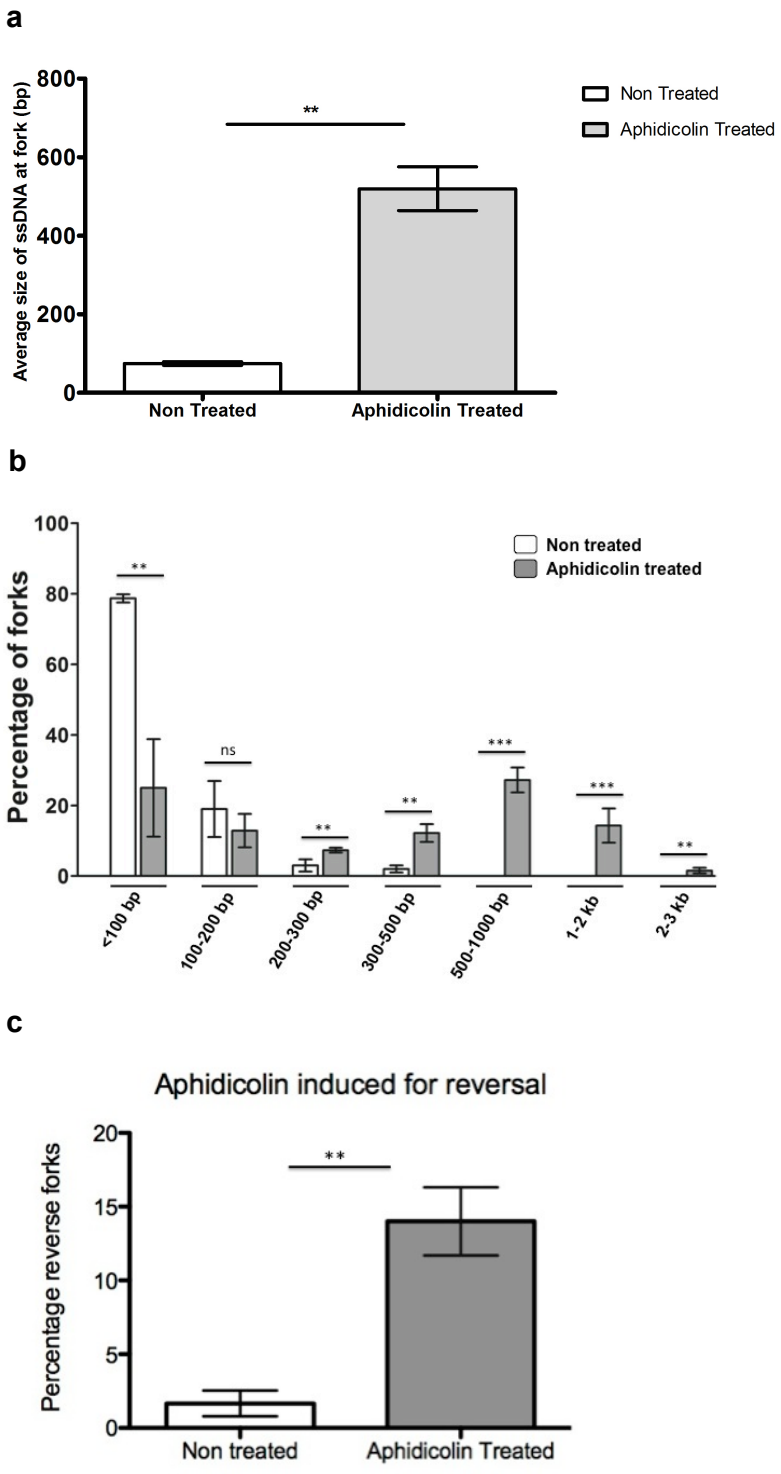


Figure 5.3 Aphidicolin induces ssDNA gap accumulation at the fork of ~ 0.5 – 0.6 kb and replication fork reversal in *Xenopus laevis* egg extracts.

a. Histogram shows the average size of ssDNA gap accumulated at the fork in nm. Error bars represent standard deviation. *n* = 3

independent experiments; *P* < 0.0014 when comparing non treated and aphidicolin treated samples; unpaired two-tailed *t*-test **b.** Statistical distribution of ssDNA length (bp and kb's) at the fork in the population of analysed molecules (Nontreated – 105 or Aphidicolin treated – 105 molecules analysed). Error bars represent standard deviation of the mean. *n* = 3 independent experiments; *P* < 0.0052 (<100 bp), *ns* (100-200 bp), *P* < 0.010 (200-300 bp), *P* < 0.0039 (300-500 bp), *P* < 0.0008 (500-1000 bp), *P* < 0.0009 (1-2 kb), *P* < 0.0031 (2-3 kb) when comparing with non treated and aphidicolin treated samples ; unpaired two-tailed *t*-test **c.** Histogram represents the mean percentage of reverse fork formation in the presence and absence of aphidicolin treatment. Error bars indicate the standard deviation of the mean. *n = 3* independent experiments. *P* < 0.075; when comparing with non treated and aphidicolin treated samples.

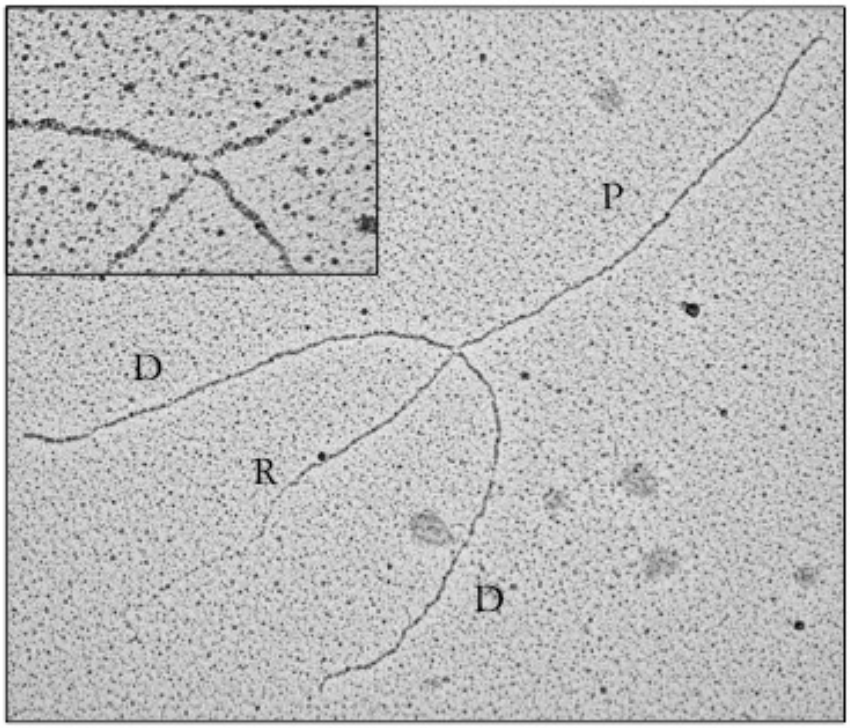


Figure 5.4. Electron microscopic visualisation of reverse fork formation.

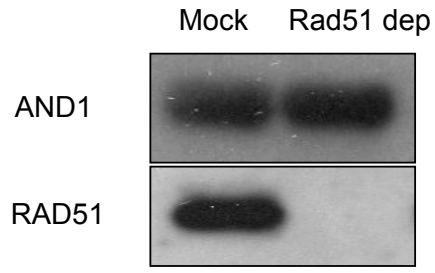
Representative replication intermediate isolated from *Xenopus* egg extracts treated with aphidicolin. P indicates the parental strand; D indicates the daughter strands (of equal length) and R indicates the reversed fork. Picture in the small box is the zoomed-in picture of four-way junction of the reverse fork.

5.2 Rad51 depletion does not compromise aphidicolin induced replication fork reversal

Using a validated electron microscopy based technique to visualise DNA replication intermediates we established the average size of ssDNA gap generated when replication forks are stalled by aphidicolin (Fig.5.3). Insights on replication fork reversal in the presence of aphidicolin were interesting because those were observed for the first time using *Xenopus laevis* egg extracts. During these years several reports have been published regarding fork reversal and proteins involved in these transactions in the presence of different DNA damaging agents[147]. With regard to my PhD thesis, a recent report suggested that fork reversal could be mainly dependent on the key homologous recombination factor Rad51[145], when cells are treated with sub-lethal concentrations of a variety of DNA damaging agents including aphidicolin. Considering these data we next questioned whether Rad51 was required for aphidicolin induced replication fork reversal in *Xenopus laevis* egg extracts. As shown in Figure.5.5a Rad51 was depleted near to 100%. To our surprise Rad51 depletion did not negatively affect replication fork reversal in *Xenopus laevis* egg extracts (Fig.5.5b). To be sure about the observed data we reproduced the experiment thrice and carried out a statistical analysis by assessing at least 100 DNA replication intermediates per sample for each experiment. Rad51 depletion showed an increase in the level of replication fork reversal. As shown in Fig.3.14, since Rad51 depletion impairs BRCA2 chromatin recruitment, at this point we hypothesize that

BRCA2 depletion also should have similar effect on fork reversal as Rad51 depletion. However, our hypothesis has to be tested and investigated directly in future.

a



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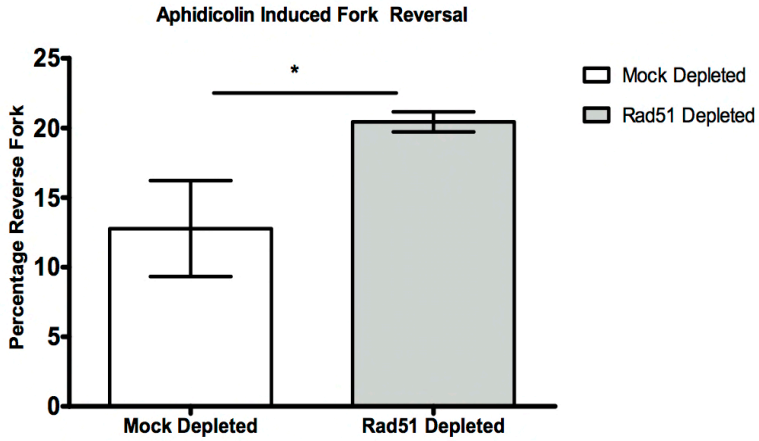


Figure 5.5 Aphidicolin induced fork reversal is not dependent on Rad51.

a. To check the efficiency of depletion, Mock or Rad51 depleted extract was loaded onto 4–15% SDS – PAGE gel immunoblotted against AND1 and Rad51. **b**. Histograms represent the mean percentage of reverse forks in extract treated with aphidicolin, in the presence and absence of Rad51. Error bars represent standard deviation of the mean. *n* = 3

independent experiments; $P < 0.0467$ when comparing Mock depleted and Rad51 depleted samples ; unpaired two-tailed *t*-test.

5.3 SMARCAL1 induces replication fork reversal in the absence of Rad51.

Having ruled out that Rad51 is required for replication fork reversal after aphidicolin treatment we further investigated about the proteins that could be involved in replication fork reversal during aphidicolin treatment. SMARCAL1 is a DNA dependent - ATPase in the SNF2 family of proteins. Biochemically SMARCAL1 is an annealing helicase that has been reported to induce replication fork reversal *in vitro*[181]. As shown previously SMARCAL1 depletion did not compromise DNA replication[182] (Data not shown). We next asked whether SMARCAL1 accumulate onto chromatin in the presence of DNA damaging agents. As shown in Figure.5.6, SMARCAL1 associates with replicating chromatin and its chromatin association increases progressively in the presence of fork stalling agents (aphidicolin) and break induced repair (S1 nuclease + aphidicolin) and SMARCAL1 binding peaks in the presence of double strand breaks (EcoRI). We next monitored the status of reverse forks formed in the absence of SMARCAL1 when extract was treated with aphidicolin. As shown in Figure.5.7, SMARCAL1 depletion reduced the frequency of replication fork reversal. Co-depletion of SMARCAL1 with Rad51 further reduced replication fork reversal (Fig.5.7). Hence the data suggest that the fork reversal is predominantly mediated by SMARCAL1 in the presence of aphidicolin. In the absence of SMARCAL1, Rad51 could be involved in inducing or

stabilising replication fork reversal to a minor extent. Absence of SMARCAL1 or Rad51 leads to impairment in fork reversal possibly leading to fork collapse.

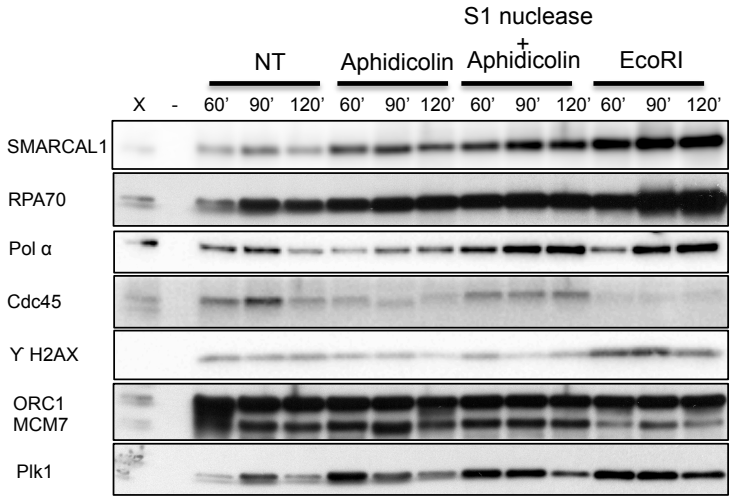
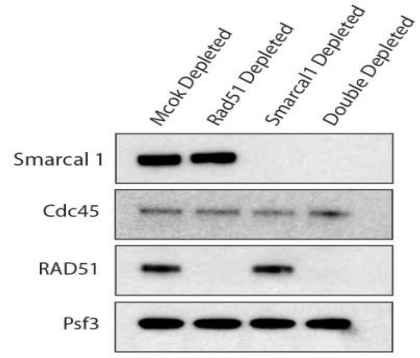


Figure 5.6 Chromatin dynamics of SMARCAL1.

a. Time course of chromatin association of SMARCAL1 and indicated replication factors were assessed in the presence or absence of different DNA damaging agents (Aphidicolin 20 μM, S1 nuclease 0.32 U + aphidicolin 1 μM, EcoRI 0.05U). Western blotting was carried out with the chromatin fraction from 40 μl of extract incubated with 4000 nuclei/ μl for indicated times. Similar results were obtained at least in three independent experiments.

a



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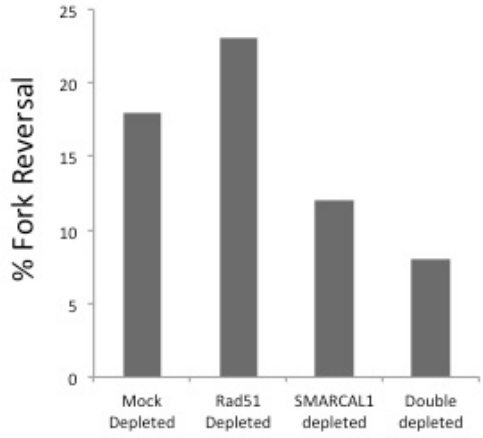

Figure 5.7 SMARCAL1 induces replication fork reversal in the absence of Rad51.

a. Interphase egg extract was immune depleted with non-immune IgG, anti-Rad51, anti-SMARCAL1 and co – depleted with anti-Rad51 and SMARCAL1. 1 µl of immunodepleted extract was loaded on SDS-PAGE and immunoblotted to check the efficiency of depletion. **b.** Mock, Rad51, SMARCAL1, Rad51 + SMARCAL1 depleted extract was treated with aphidicolin according to the scheme (Fig.5.2) and subjected to electron microscopic visualisation of replication intermediates. The percentage of double stranded (ds) and single stranded (ss) reversed forks has been calculated and represented in the graph. 150 molecules counted for statistical analysis for each condition.

5.4 Conclusions:

In summary, results suggest that in Xenopus egg extracts, aphidicolin induced replication fork reversal is predominantly induced by a DNA – dependent ATPase called SMARCAL1. However, in the absence of SMARCAL1, possibly fork reversal activity is mediated by Homologous Recombination (HR) factor Rad51 to a minor extent. Loss of both SMARCAL1 and Rad51 further reduced the frequency of replication fork reversal. Finally these results reveal a function for SMARCAL1, which protect the stalled replication forks from collapse by inducing replication fork reversal.

Chapter 6. Dynamic behaviour of replication and repair factors in the presence of Double Strand Break's (DSB).

To monitor the kinetics of chromatin association of replication and repair factors in the presence of Double Strand Break's (DSB's).

In this chapter, the kinetics of recruitment of DNA repair and replication factors to Double Strand Break- containing chromatin in interphase egg extract will be addressed.

6.1 Double Strand Break induces dynamic behaviour of replication and repair factors on chromatin

DNA double-strand breaks (DSBs) are the most toxic lesions generated by replication fork collapse, exogenous damage, nucleases or topoisomerases. Upon double strand the decision to choose HR or NHEJ is partly governed by resection of DSB ends[183]. Double strand break ends are converted into 3' ssDNA overhang; an intermediate for HR and this 3' over hang acts as an inhibitor for NHEJ[183]. In eukaryotes resection is mediated by MRN complex and CtIP. MRN complex mediates a shorter resection from 5' to 3' together with CtIP and then Exo1 and Dna2 can act for an extensive resection independent of MRN and CtIP[183]. Resection is followed by accumulation of ssDNA binding proteins and subsequent repair. In this scenario of double strand break repair we monitored the chromatin association of replication factors. As shown in Figure.6.1, surprisingly we found dissociation of replication factors like Cdc45, Psf3, Polymerase δ, but chromatin bound

Polymerase α, MCM7 and ORC1 stayed on the chromatin unchanged. As a positive control there was several fold increase of Rad51 and γ – H2AX indicating that the DSB response induced by EcoRI worked. Having this information about the status of replication factors upon DSB at single time point, we then monitored the chromatin association of BRCA2, Rad51, Polymerase α, MRE11 (nuclease), Cdc45 (Replisome component), AND1 (a protein that tethers Polymerase α to MCM2-7) and γ – H2AX. We assessed their chromatin association at different time points to understand the relationship between BRCA2, MRE11, Rad51 and other components of replisome machinery. Interestingly upon DSB induction, BRCA2 and MRE11 showed a very early recruitment to the chromatin within 10 min and later Rad51 was hyper loaded on the chromatin followed by Polymerase alpha and AND1 (Fig.6.2 a, b). Upon DSB resection by MRE11, RPA coated ssDNA regions become the signal for BRCA2 association and thus aids Rad51 recruitment subsequently. These data adds even more precise detail about the sequence of action of BRCA2, MRE11, Rad51 and Polymerase alpha in double strand break repair in *Xenopus* egg extracts. From these results (Fig.6.1 and 6.2) it is clear that DSB although induced dissociation of Cdc45, Psf3, Polymerase δ but not Polymerase α. As discussed earlier similar to aphidicolin treatment we did observe an interaction of Rad51 and Polymerase alpha in DSB's (Fig.6.3). Overall these data propose a model that Rad51 might be required for Polymerase alpha mediated repair of DSB's in *Xenopus laevis* egg extracts.

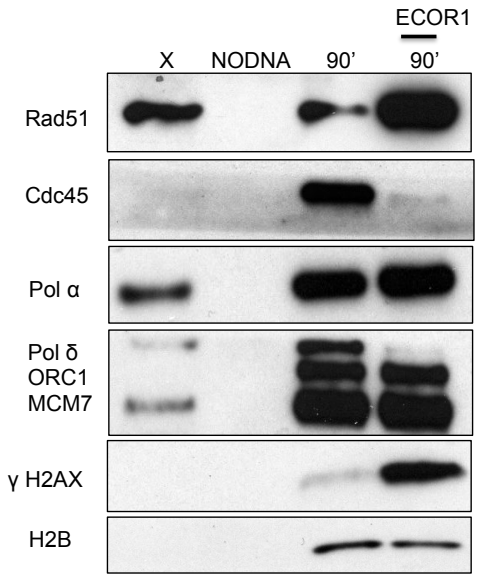

Figure 6.1 Double Strand Break induces dissociation of Polymerase δ, Cdc45 but not Polymerase α from the chromatin.

Interphase egg extract was incubated with sperm nuclei in the presence and absence of (0.05U) EcoRI. At 90 min chromatin samples were subjected to western blotting and probed for the indicated proteins.

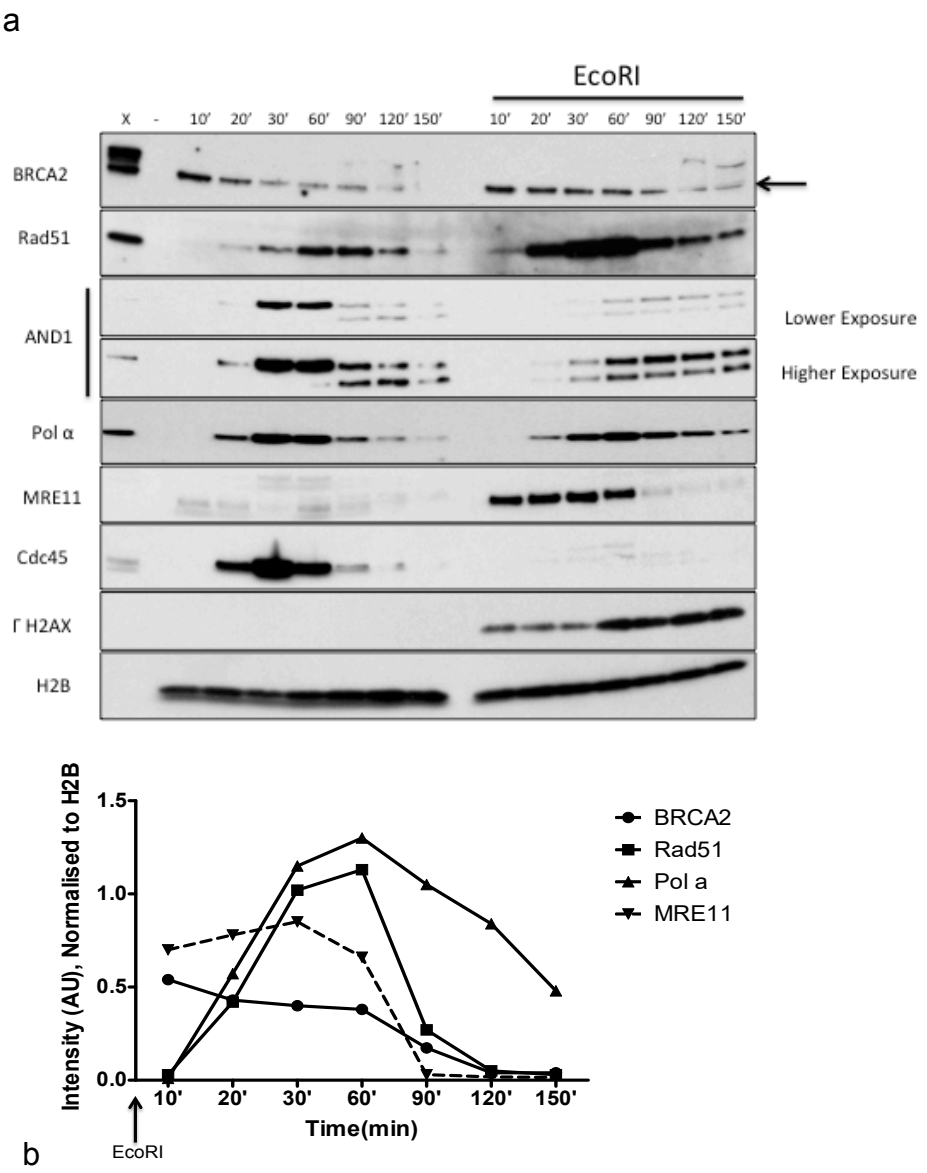

Figure 6.2 Double Strand Break dynamics of BRCA2, Rad51, MRE11 and other replication factors.

a. 30 µl egg extract was incubated with sperm nuclei (4000 nuclei/µl) in the presence or absence of 0.05U EcoRI and chromatin fraction was purified at different time points and probed for indicated proteins. **b.** Intensity of chromatin bound BRCA2, Rad51, Polymerase α and MRE11 were quantified at different time points for sample treated with EcoRI. Similar results were obtained in at least three independent experiments.

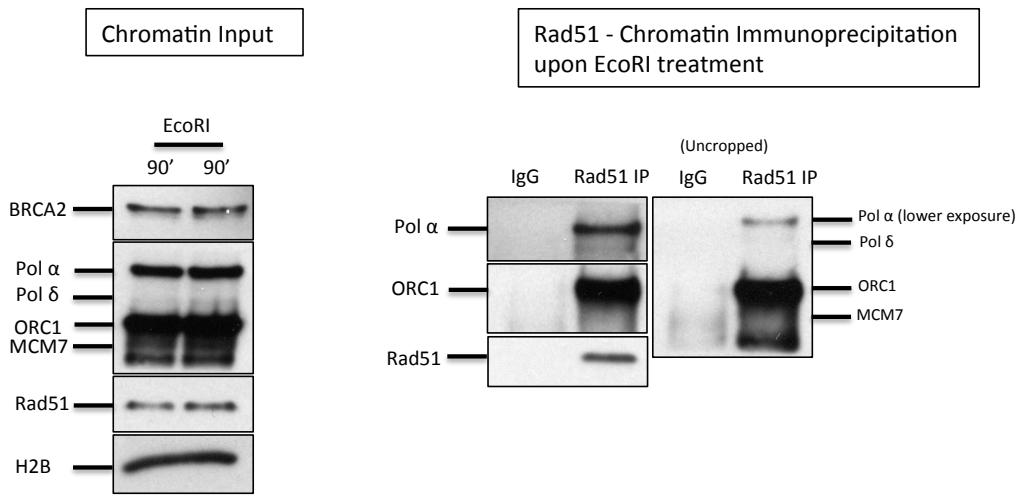

Figure 6.3 Rad51 interacts with Polymerase alpha upon DSB.

a. Chromatin input upon treatment with EcoRI treatment and immunoblotted for proteins mentioned above. **b.** Rad51 was immunoprecipitated and immunoblotted for proteins mentioned above.

6.2 Conclusions.

In summary, results from this section indicate that BRCA2 associate with the chromatin earlier in response to EcoRI induced Double Strand Break's (DSB's) and then directs the recruitment of Rad51 to initiate DSB repair. Finally our preliminary observations suggest a role for Polymerase alpha in response to DSB's. The interaction observed between Rad51 and Polymerase alpha in the presence of DSB's further indicate a potential role for Rad51 – Polymerase alpha complex DSB repair.

Chapter 7. Discussion

7.1 BRCA2/Rad51 act together to protect replication forks during unperturbed DNA replication

During perturbed DNA replication HDR is required to repair collapsed forks. In this regard BRCA2 mediated Rad51 recruitment is crucial to promote HR[100]. In human cells it has been shown that BRCA2 functions in preventing nascent strand degradation in the presence of HU[100]. To dissect the function of BRCA2 and Rad51 in fork protection we studied the role of these two proteins in *Xenopus* egg extract. Using this system we showed that BRCA2 and Rad51 are chromatin-associated proteins (Fig.3.4). Importantly I showed for the first time that BRCA2 loading onto chromatin is largely dependent on pre-replication complex assembly (Fig.3.4). This is similar to Rad51 chromatin binding pattern[68]. Monitoring chromatin association of BRCA2 and Rad51 in short time points during replication also revealed a sequential order of action. During unperturbed DNA replication BRCA2 binds early to chromatin within 10 minutes and then follows Rad51 association together with replication fork components (Fig.6.2).

We further showed majority of chromatin association of Rad51 is strictly dependent on BRCA2 during normal DNA replication (Fig.3.13). Similarly BRCA2 chromatin association is dependent on Rad51 during unperturbed DNA replication. Hence from our data we conclude that BRCA2 and Rad51 are interdependent for chromatin association especially at replication forks. A recent study using iPOND, authors showed that cells expressing BRCA2 Y3308X showed reduced Rad51

replication fork association with respect to cells expressing wild type BRCA2[184]. Of note BRCA2 Y3308X cells and BRCA2 knock out cells are incapable of DSB induced HR but are able to maintain basal level of sister chromatid exchange under spontaneous replication stress[184]. These observations suggest that BRCA2 is dispensable for spontaneous HR at the fork and it is partially required for Rad51 loading during normal replication and HU induced stress conditions in mammalian cells. In *Xenopus laevis* egg extract BRCA2 is required for most of the Rad51 chromatin association during unperturbed DNA replication. However, similar to mammalian cells residual Rad51 recruitment can take place in the absence of BRCA2 (Fig.3.13). However we still need to confirm BRCA2 involvement in Rad51 chromatin association in the presence of replication fork stalling.

To further confirm that BRCA2/Rad51 operates in same pathway to protect replication fork during normal replication, we used electron microscopy to reveal the structure of replication intermediates isolated from BRCA2 and or Rad51 depleted extracts. Using this approach we showed ssDNA gap accumulation at the fork in the absence of Rad51 or BRCA2. The analysis of the length of ssDNA gaps at the fork suggests approximately similar length of ssDNA gaps in Rad51 and BRCA2 depleted extracts. In BRCA2 depleted extract the average length of ssDNA gaps accumulation at the fork was ~ 80 nm that is ~ 0.2kb (Fig.3.7 b, c, d) where as in Rad51 depleted extracts the average ssDNA gap at the fork was ~100 nm that corresponds to ~ 0.28 kb (Fig.3.10 b, c). Due to discontinuous nature of DNA replication, usually the average ssDNA gaps at the fork during normal replication is ~10 to

30 nm that corresponds to 40 to 80 bp[68]. The effect we observe in the absence of BRCA2 is more than two folds compared to mock depleted extracts and similarly for Rad51. In addition to that 20 – 30 % of the molecules in BRCA2 and Rad51 depleted extracts showed ssDNA gaps upto > 300 bp (Fig. 3.7 d and 3.10 c). Hence from these results it appears that BRCA2/Rad51 likely operates in the same pathway to protect replication fork during unperturbed DNA replication.

7.2 Distinct replication fork protection at and behind the fork by BRCA2/Rad51

Previous study from my host laboratory has shown that Rad51 depletion induces ssDNA gaps also behind the replication fork. In the presence of mirin the ssDNA gaps “behind the fork” are suppressed in Rad51 depleted extracts whereas ssDNA gaps “at the forks” persist in same length as Rad51 depleted extract that has not been treated with Mirin (See Fig.7.1c to differentiate gaps at the fork and behind the fork). Since BRCA2 controls the recruitment of Rad51 during normal replication it is possible that BRCA2/Rad51 protect the replication fork from MRE11 mediated degradation that is occurring behind the fork. In human cells majority of the degradation that has been observed by molecular combing is probably due to MRE11 degradation behind the fork in Rad51 or BRCA2 deficient cells[100].

During unperturbed DNA replication, it is not clear why ssDNA gap length of ~ 200 - 300 bp occurs in the absence of Rad51 or BRCA2 ‘at the fork’. We cannot exclude that a nuclease different from MRE11 is generating these gaps in the absence of BRCA2 or Rad51. In this study

we have shown (Figure.4.2 a and b) that in the presence of replication stress Rad51 interacts with Polymerase alpha and Polymerase delta on chromatin. Although the interaction has to be validated in future with recombinant Rad51 and polymerases at this point this interaction appears to be a possible explanation for ssDNA gaps observed at the fork in the absence of Rad51 or BRCA2, in the presence of replication. Owing to the discontinuous nature of lagging strand, lesion in lagging strand could be re-primed efficiently by polymerase alpha and extended by polymerase delta. In contrast, impediment in the leading strand might result in large ssDNA gaps 'at the fork' because of continuous unwinding of MCM2-7 helicase, if left unrepaired. In this scenario we hypothesize (Fig.7.1) that BRCA2 recruits Rad51 at the blocked leading strand and further re-prime and polymerise the leading strand by directly loading polymerase alpha and polymerase delta to prevent long ssDNA stretches. Although several studies from the past suggest that during unperturbed DNA replication polymerase epsilon polymerises the leading strand and polymerase delta polymerises the lagging strand[48], recently it has been proposed that in the presence of replication fork stalling the re-priming and polymerising activity at the leading strand could be taken care of Polymerase alpha and delta instead polymerase epsilon[48]. When replication fork encounter endogenous or exogenous impediments in replication fork progression, these impediments may result in replication fork stalling and uncoupling leading to ssDNA accumulation. In such cases, possibly, BRCA2/Rad51 promote efficient re-priming and polymerising activity of Polymerase alpha and

Polymerase delta at the leading strand to prevent ssDNA accumulation and eventually chromosome breaks at the end of mitosis.

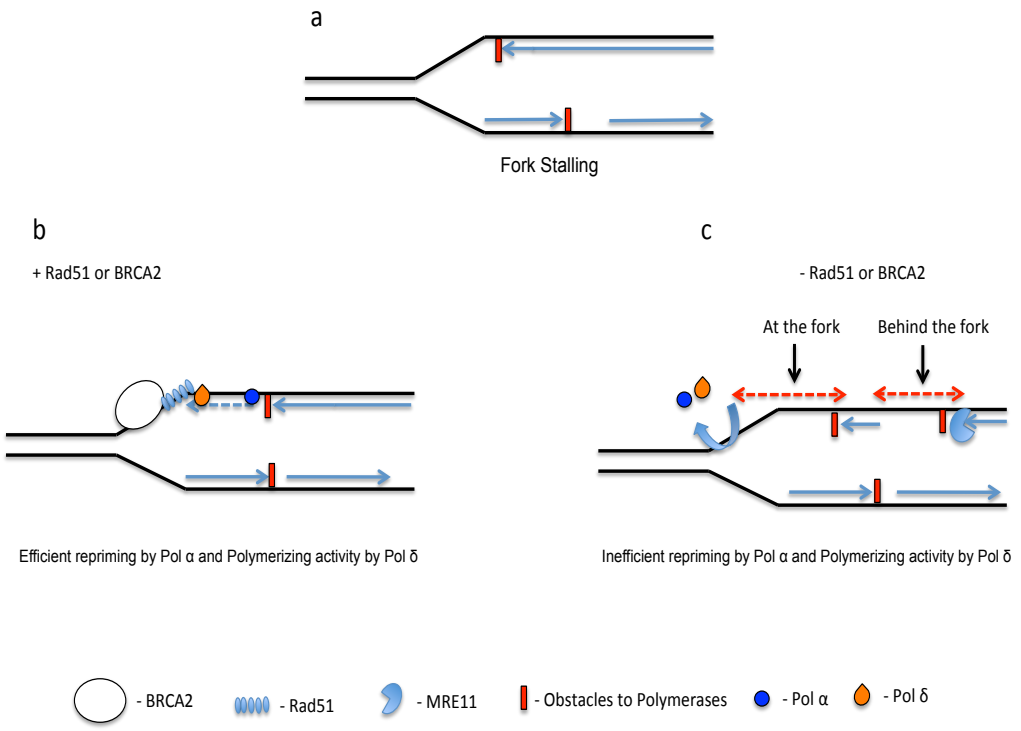

Figure 7.1 Model showing the requirement of Rad51 or BRCA2 for efficient priming and polymerising activity of Pol α and Pol δ.

a. Cartoon of fork stalling induced by obstacles to replicative polymerases. **b.** Reloading of a new of Pol α and Pol δ in the presence of Rad51/BRCA2. **c**. Rad51/BRCA2 depletion results in dissociation of Pol α and Pol δ at forks, possibly reducing the priming and polymerising activity of Pol α and Pol δ leading to large ssDNA gaps at the fork.

7.3 Annealing helicase SMARCAL1 induces replication fork reversal

A recent report from mammalian cells showed that inhibition of replication fork progression with wide range of replication inhibitors results in replication fork reversal, a four-way junction generated by annealing of two nascent strands and re-annealing of parental strands[145]. The same study suggested that in response to sub-lethal concentration of replication stress mainly Rad51 mediates reverse forks[145].

In this project having concluded that Rad51/BRCA2 depletion results in ssDNA gaps accumulation, we next asked the requirement of Rad51 in replication fork reversal in the presence of aphidicolin, a polymerase alpha inhibitor. As shown in Figure.5.5 aphidicolin treatment in the absence of Rad51 resulted in an increase in replication fork reversal. Hence at this point we concluded that Rad51 is not required for replication fork reversal in the presence aphidicolin in this embryonic system. These data is in shear contrast with data published using mammalian cells[145]. Given these observations we tested whether other factors possibly implicated in replication fork reversal *in vitro* played a role in *Xenopus* (Table.1.1). Among these we tested SMARCAL1 to verify its requirement for fork reversal in the presence of aphidicolin. The reason to choose SMARCAL1 was because of its potent fork reversal activity *in vitro*[185] and also for the availability of specific anti-SMARCAL1 antibody in the laboratory for depletion and biochemical experiments. As shown in Figure.5.7, SMARCAL1 depletion reduced

the frequency of replication fork reversal. However we observed further reduction in replication fork reversal when Rad51 and SMARCAL1 are co-depleted from egg extracts. Based on these data we propose a model as shown below (Fig.7.2). Inhibition of replicative polymerases by aphidicolin induces fork reversal in *Xenopus laevis* egg extracts. Increased fork reversal observed in the absence of Rad51 might be caused by the hyper action of SMARCAL1 at ssDNA gaps generated in the absence of Rad51. It has been shown *in vitro* that the branch migration and annealing activities of SMARCAL1 is directed by RPA[186] (the single strand binding protein). Of note, *in vitro*, in co-operation with RPA, SMARCAL1 selectively reverse the stalled replication forks caused by lesion at the leading strand[186]. Hence, in the presence of replication fork-stalling ssDNA gaps generated at the fork in the absence of Rad51 or BRCA2 becomes the substrate for fork reversal activity of SMARCAL1 directed by hyper-accumulation of RPA (Fig.7.2.). In the presence of persistent replication block caused by aphidicolin or hydroxyurea the reversed forks are susceptible for nuclease-mediated degradation. Alternatively, reversed fork degradation might set the stage for replication fork restart. This hypothesis needs to be tested in the future.

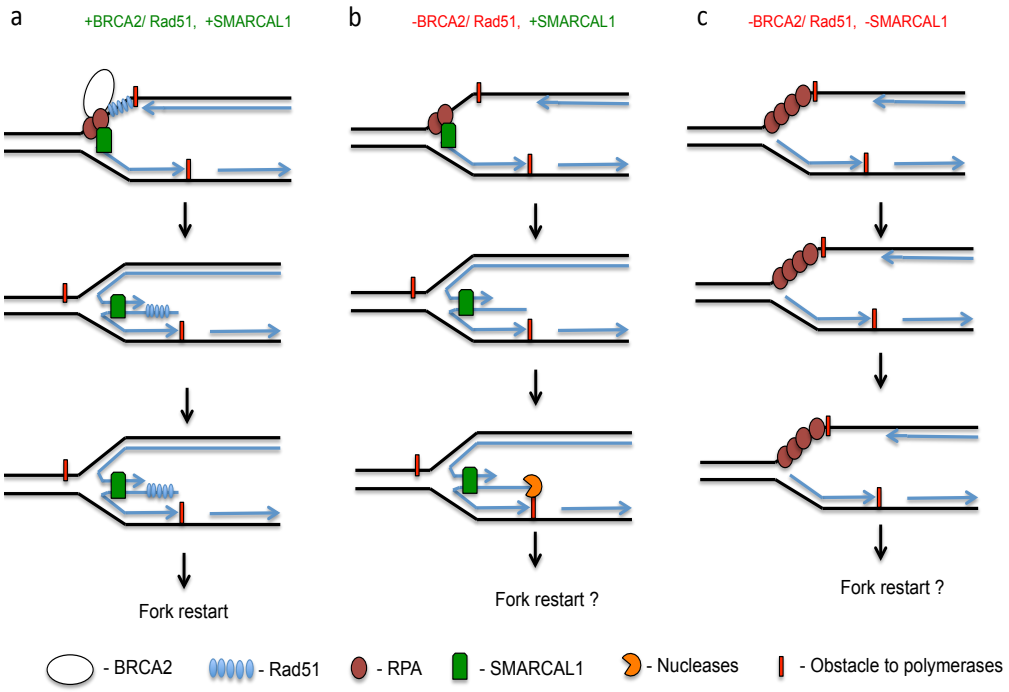

Figure 7.2 Model showing requirement of SMARCAL1 in replication fork reversal.

a. In the presence of BRCA2 and SMARCAL1 inhibition of replicative polymerases results in replication fork reversal. **b**. Loss of BRCA2/Rad51 shows an increase in replication fork reversal due increased ssDNA gap accumulated at the fork. **c.** Depletion of Rad51/BRCA2 and SMARCAL1 impairs fork reversal and probably fork collapse.

Other important questions that require further investigations include

1. In results section 3.5, I have shown that BRCA2 depletion results in ssDNA gaps accumulation in daughter strands. To confirm that the effect we observe is specific to BRCA2 depletion, in future we will add-back purified recombinant MBP-BRCA2 (that is currently available in lab, produced by Dr. Anna De Antoni) and monitor by Electron Microscopy whether we can rescue the ssDNA gaps in BRCA2 depleted extract. Further, to confirm that BRCA2 and Rad51 operates in the same pathway during unperturbed DNA replication, co-depletion of BRCA2 and Rad51 will be performed and DNA replication intermediates will be analysed by EM.

2. We have shown in Figure.3.13 that Rad51 chromatin recruitment during normal replication is predominantly dependent on BRCA2. To further confirm this observation we will next add-back recombinant MBP-BRCA2 in BRCA2 depleted extract and monitor whether recombinant MBP-BRCA2 is able to recruit Rad51 during unperturbed DNA replication.

3. In results section 4.2, I have shown that Rad51 and Polymerase alpha and delta interact with each other in the presence of DNA damage. In future we will reconstitute recombinant Rad51, Polymerase alpha and delta to test whether these factors interact directly *in vitro*.

4. We have shown in Figure.5.7 that SMARCAL1 is required for replication fork reversal in the presence of aphidicolin. To validate that the observation is specific for SMARCAL1 depletion, in future we will add-back recombinant SMARCAL1 in SMARCAL1 depleted extract and monitor the frequency of replication fork reversal. If our hypothesis is

true we expect increased replication fork reversal in the presence recombinant SMARCAL1 compared to SMARCAL1 depleted extract. This will further confirm that in the presence of aphidicolin SMARCAL1 predominantly induces replication fork reversal. Together information obtained from the present and future results will give a better insight to target cancer cells by specifically inhibiting factors that induce fork reversal.

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