

# **Investigating the role of RAD51 phosphorylation during the cell cycle**



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## **Abstract**

The RAD51 recombinase safeguards genomic integrity by promoting double-strand break repair and replication fork protection. PLK1-dependent RAD51 phosphorylation contributes to RAD51 recruitment at DNA double strand breaks and stalled replication forks. During an unperturbed cell cycle, RAD51 phosphorylation peaks in late G2 phase and mitosis, suggesting a role for (phosphorylated) RAD51 during the later stages of the cell cycle.

In this thesis, CRISPR/Cas9 RAD51 phospho-mutants were generated to assess the importance of RAD51 phosphorylation during the cell cycle. Consistent with previous research, I present data which suggest that RAD51 phosphorylation contributes to RAD51 recruitment at stressed DNA. I further demonstrate that RAD51 phosphorylation protects against the detrimental consequences of replication stress and that RAD51 phosphorylation may influence replication rate and mitotic duration in unperturbed conditions.

I present evidence for the role of RAD51 in mitotic DNA synthesis (MiDAS), as well as the contribution of RAD51 phosphorylation in this process. Based on the data presented here, I hypothesize that RAD51 phosphorylation enables the recruitment of RAD51 to mitotic chromatin to promote MiDAS. Break-dependent and break-independent mechanisms of MiDAS are proposed and the potential roles of RAD51 are discussed in each. The interplay between MiDAS and mitotic duration is explored. Anaphase onset is found to be delayed upon RAD51 inhibition, while polymerase inhibition decreases mitotic duration. A model is proposed in which the presence of mitotic DNA breaks and active replication intermediates in mitosis prevent SAC silencing, thereby ensuring enough time for the completion of replication prior to anaphase.



# Table of content

## Chapter 1 Reviewing the role of RAD51 beyond classical double-strand break repair

|      |                                                                                                           |    |
|------|-----------------------------------------------------------------------------------------------------------|----|
| 1.1  | Genomic instability and DNA double-strand break repair                                                    | 11 |
| 1.2  | ATM- and ATR- mediated DNA damage signalling                                                              | 13 |
| 1.3  | DNA damage signalling is attenuated during mitosis                                                        | 14 |
| 1.4  | RAD51 recruitment and loading at DSBs catalyses HR                                                        | 15 |
| 1.5  | PLK1-dependent RAD51 phosphorylation: an alternative pathway for RAD51 recruitment                        | 17 |
| 1.6  | RAD51 is essential in higher eukaryotes                                                                   | 18 |
| 1.7  | RAD51: Fork reversal, fork protection and fork restart                                                    | 20 |
| 1.8  | Proposed mechanisms of RAD51-dependent fork restart                                                       | 22 |
| 1.9  | Break-induced replication mediates fork restart and alternative lengthening of telomeres (ALT) in mitosis | 25 |
| 1.10 | A potential role for phosphorylated RAD51 in later stages of the cell cycle                               | 27 |
| 1.11 | References                                                                                                | 28 |

## Chapter 2 Materials and Methods

|        |                                                 |    |
|--------|-------------------------------------------------|----|
| 1.     | Materials                                       | 37 |
| 2.     | Methods                                         | 48 |
| 2.1    | Bacterial techniques                            | 48 |
| 2.1.1  | Transformation                                  | 48 |
| 2.1.2  | Growth and storage of bacterial strains         | 48 |
| 2.1.3  | Plasmid DNA preparation                         | 48 |
| 2.2    | DNA analysis techniques                         | 49 |
| 2.2.1  | Quantification of nucleic acids                 | 49 |
| 2.2.2  | Isolating mammalian genomic DNA                 | 49 |
| 2.2.3  | Polymerase chain reaction (PCR)                 | 49 |
| 2.2.3a | PCR from genomic DNA template                   | 49 |
| 2.2.3b | Colony PCR                                      | 51 |
| 2.2.3c | Site-directed mutagenesis                       | 51 |
| 2.2.4  | Agarose gel electrophoresis                     | 52 |
| 2.2.5  | Sanger sequencing                               | 52 |
| 2.2.6  | Screening of CRISPR-targeted locus by PCR       | 52 |
| 2.2.7  | Allele-separation through cloning               | 52 |
| 2.2.7a | Insert preparation                              | 52 |
| 2.2.7b | Vector preparation                              | 52 |
| 2.2.7c | Ligation                                        | 53 |
| 2.2.8  | Next-generation sequencing (NGS)                | 53 |
| 2.3    | Protein analysis techniques                     | 54 |
| 2.3.1  | Cell lysate preparation and Western Blotting    | 54 |
| 2.3.1a | Whole cell lysate preparation                   | 54 |
| 2.3.1b | Western blotting                                | 54 |
| 2.3.2  | Protein enrichment by immunoprecipitation       | 54 |
| 2.3.2a | Antibody crosslinking to Affiprep Protein beads | 54 |
| 2.3.2b | Immunoprecipitation                             | 55 |
| 2.3.3  | Chromatin fractionation                         | 55 |
| 2.4    | Mammalian cell culture techniques               | 56 |
| 2.4.1  | Cell culture propagation and storage            | 56 |
| 2.4.2  | Ionizing irradiation                            | 56 |
| 2.4.3  | Transfection                                    | 56 |
| 2.4.4  | Generation of stable cell lines                 | 57 |

|        |                                                                |    |
|--------|----------------------------------------------------------------|----|
| 2.4.5  | Generating CRISPR cell lines                                   | 57 |
| 2.4.5a | Transfection                                                   | 57 |
| 2.4.5b | Clonal expansion and storage                                   | 57 |
| 2.4.6  | Cell synchronization                                           | 58 |
| 2.4.6a | Double thymidine block-and-release                             | 58 |
| 2.4.6b | Cell synchronization for immunofluorescence experiments        | 58 |
| 2.4.6c | Cell synchronization for chromatin fractionation experiments   | 59 |
| 2.4.6d | Cell synchronization for live microscopy experiments           | 60 |
| 2.5    | Cell analysis techniques                                       | 62 |
| 2.5.1  | Immunofluorescence microscopy                                  | 62 |
| 2.5.2  | Click-iT fluorescent-labeling                                  | 62 |
| 2.5.3  | Proximity ligase assay (PLA)                                   | 62 |
| 2.5.4  | Flow cytometry                                                 | 62 |
| 2.5.4a | Analysing cell cycle distribution by propidium iodide staining | 62 |
| 2.5.4b | Analysing mitotic index                                        | 63 |
| 2.5.4c | Analysing EdU incorporation                                    | 63 |
| 2.5.5  | Analysing mitotic progression by live microscopy               | 63 |
| 2.6    | Cell survival assays                                           | 64 |
| 2.6.1  | WST1-assay                                                     | 64 |
| 2.6.2  | Clonogenic assay                                               | 64 |
| 2.7    | Data analysis techniques                                       | 65 |
| 3      | References                                                     | 65 |

## **Chapter 3** CRISPR/Cas9-generation of RAD51 phospho-mutants

|       |                                          |    |
|-------|------------------------------------------|----|
|       | Summary                                  | 69 |
| 3.1   | Introduction                             | 70 |
| 3.2   | Approach                                 | 71 |
| 3.2.1 | CRISPR/Cas9-mediated gene editing        | 71 |
| 3.2.2 | Design and selecting of CRISPR guide RNA | 71 |
| 3.2.3 | High resolution melt analysis (HRMA)     | 73 |
| 3.2.4 | Screening strategy                       | 76 |
| 3.3   | Results                                  | 78 |
| 3.4   | Discussion                               | 86 |
| 3.5   | References                               | 89 |

## **Chapter 4** Characterization of RAD51 phospho-mutants in the context of DNA repair and replication

|     |              |     |
|-----|--------------|-----|
|     | Summary      | 93  |
| 4.1 | Introduction | 94  |
| 4.2 | Results      | 95  |
| 4.3 | Discussion   | 106 |
| 4.4 | References   | 112 |

## **Chapter 5** Investigating the role of RAD51 phosphorylation in mitotic DNA synthesis

|     |              |     |
|-----|--------------|-----|
|     | Summary      | 117 |
| 5.1 | Introduction | 118 |
| 5.2 | Results      | 120 |
| 5.3 | Discussion   | 136 |
| 5.4 | References   | 148 |

## **Chapter 6** Final conclusions and future work

|     |                                     |     |
|-----|-------------------------------------|-----|
| 6.1 | Summary of experimental findings    | 153 |
| 6.2 | Remaining questions and future work | 155 |
| 6.3 | Final remarks                       | 158 |
| 6.4 | References                          | 160 |

# **Chapter 1**

## **Reviewing the role of RAD51 beyond classical double-strand break repair**



## **1.1 Genomic instability and DNA double-strand break repair**

Despite encoding the hereditary information that underlies all cellular processes, DNA is an inherently unstable molecule that readily decomposes in physiological solvent conditions. It was this realisation which led to the discovery of DNA repair mechanisms which counteract the accumulation of DNA damage and hence ensure the genetic stability that is essential to life.<sup>1</sup> While the reactivity of the DNA molecule mainly involves the modification of its bases, the phospho-diester backbone is not exempt from breakage. DNA double-stranded breaks (DSBs), in which the backbone of both complementary DNA strands is broken such that the DNA helix is entirely cleaved in two, are particularly lethal if unrepaired.<sup>2</sup> Moreover, DSBs are liable to be repaired incorrectly and give rise to chromosomal translocations and genomic rearrangements that contribute to malignant transformation.<sup>3,4</sup> Several exogenous DNA damaging agents, such as ionizing radiation, can generate DNA DSBs.<sup>4</sup> However, DNA metabolism itself can give rise to DSBs, specifically during the process of DNA replication in which the stable double-stranded helix is temporarily opened up and vulnerable to nucleolytic attack. Conditions of replication stress, in which replication slows down or stalls, maintains DNA in this vulnerable state and hence represents a major endogenous source of DNA DSBs. Indeed, oncogene-induced replication stress is thought to be the driver for tumour development in sporadic cancers.<sup>5</sup>

DSBs can be repaired via several different mechanisms that can be subdivided into two main categories: homology-directed repair (HDR) and non-homologous end-joining (NHEJ). During NHEJ the two broken DNA ends are re-ligated to seal the break. As DNA end processing is often required to create suitable blunt ends for ligation, some loss of genetic information may occur during this process. HDR utilizes a homologous sequence to template DNA synthesis of the broken DNA, which, in case the identical sister chromatid is used, can lead to the error-free repair of the break.

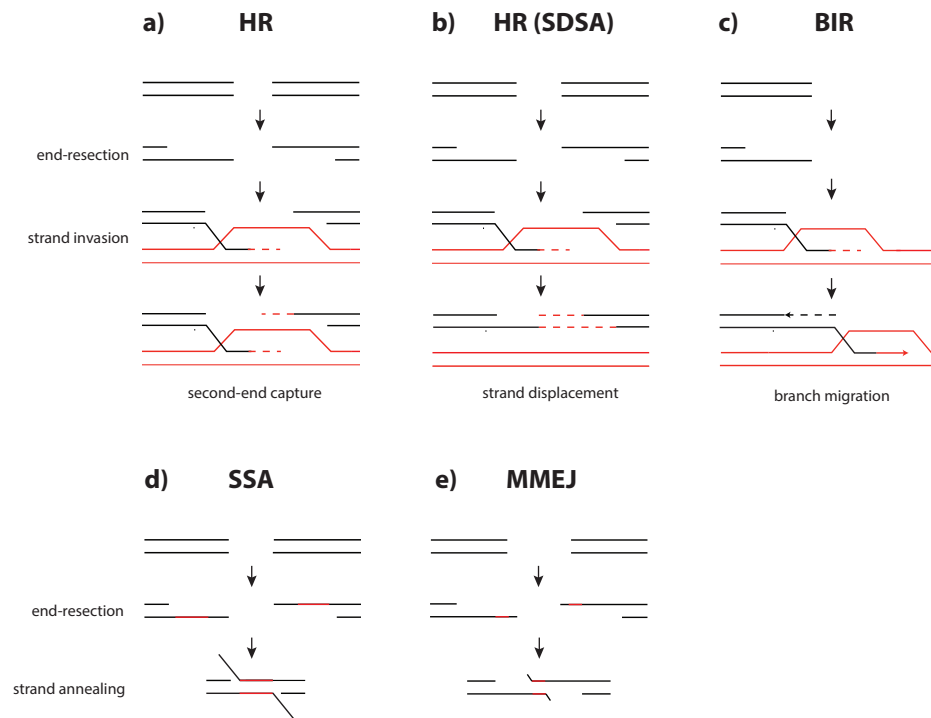
HDR is initiated by 5' resection of the broken DNA ends, a process which has been identified as a key step in repair pathway choice.<sup>6</sup> A bidirectional mechanism for end-resection is proposed in which Mre11 endonuclease activity provides the



necessary entry point for subsequent exonuclease activity of the 3'-5' Mre11 exonuclease and 5'-3' Exo1 exonuclease.<sup>7,8</sup> Alternatively, end-resection can be achieved via the combined activity of RECQ helicases and the DNA2 nuclease.<sup>9,10</sup> During homologous recombination (HR) the resultant 3' single-stranded DNA (ssDNA) overhang invades the repair template in search of optimal homology. Once this has been established, DNA synthesis of the invading strand ultimately bridges the gap across the DSB. A displacement loop (D-loop) structure is formed during this process due to the disruption of the double-stranded repair template upon strand invasion. The displaced DNA strand can anneal with the other resected end of the DSB and serve as a repair template through the process of second-end capture (Figure 1 a). This leads to the formation of a double Holliday junction, which can be resolved to generate crossover (CO) or non-crossover (NCO) products. Alternatively, the invading DNA strand is released from the repair template after DNA synthesis and reanneals the other end of the DNA break (Figure 1 b), resulting in exclusively NCO products. This pathway is known as synthesis-dependent strand annealing (SDSA). A similar mechanism underlies break-induced replication (BIR). BIR occurs at one-ended DSBs arising from broken replication fork or at uncapped telomeres. In this pathway, the resected DNA end invades the repair template to re-establish leading and lagging strand DNA synthesis. Studies in yeast demonstrate that, contrary to bulk DNA replication, BIR proceeds via conservative DNA synthesis, as the invading DNA strand is displaced from the D-loop via branch migration to serve as a replication template for the lagging strand (Figure 1 c).<sup>11,12,13</sup> In mammalian cells, conservative DNA synthesis is observed during alternative lengthening of telomeres (ALT) as well as mitotic DNA synthesis (MiDAS), two related processes which are proposed to be mediated via a BIR mechanism, as discussed further below<sup>14,15,16</sup>

Lastly, homology between the resected DNA ends can be utilized to reanneal the broken DNA ends without the need of strand-invasion into a repair template. Protruding non-homologous tails are clipped off, such that these repair processes are inherently error prone. Single-strand annealing (SSA) involves extended end resection to uncover long stretches of homology, while micro-homology mediated end-joining (MMEJ) acts between very short stretches of homology (Figure 1 d-e). While these pathways appear to be upregulated in NHEJ- or HDR- deficient backgrounds, growing evidence suggests they can occur even when both NHEJ- and HDR-repair pathways

are available.<sup>17,18,19,20,21,22</sup> Which of the above pathways is used for repair is thought to be largely dependent on the extent of DNA end-resection, which is in turn cell cycle-regulated.<sup>23,24,25,26</sup>



**Figure 1.1 – Pathways of homology-directed repair.**

## 1.2 ATM- and ATR- mediated DNA damage signalling

The ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinase pathways constitute two major signalling pathways activated in response to DNA damage. This DNA damage response not only initiates repair of the detected DNA lesion but also triggers checkpoint activation, thus preventing cell cycle progression in the presence of DNA damage. Both ATR and ATM initiate a cascade of protein phosphorylation, which is relayed through the activation of effector kinases CHK1 and CHK2 respectively. However, ATM- and ATR- pathways cross-talk on various levels, including the activation of CHK1 and CHK2. Additionally, CHK1 and CHK2 target a wide array of overlapping substrates, such that the relative contribution of each pathway is difficult to distinguish.<sup>27,28</sup> Recruitment and activation of ATM at the chromatin is mediated by the MRN (MRE11-RAD50-NBS1) complex, which, by

binding to broken DNA ends, acts as one of the earliest DSB sensors.<sup>29</sup> Conversely, ATR is recruited and activated at ssDNA in an RPA-, ATRIP- and TOPBP1-dependent manner.<sup>30,31,32</sup> Large stretches of RPA-bound ssDNA are generated upon replication fork uncoupling, such that ATR acts as a sensor to replication fork stalling.<sup>33</sup> Although activated separately, ATM and ATR signalling pathways converge to induce checkpoint activation via the downregulation of CDK activity. Depending on the cyclin with which it is associated, CDK substrate specificity may range from the replication machinery to the mitotic apparatus, such that DNA damage checkpoint activation can mediate G1/S, intra-S or G2/M arrest.<sup>34,35,36,27,37,38</sup>

Contrary to ATM, ATR knock-out mice are embryonically lethal, suggesting that the role of ATR is integral to genomic stability during normal proliferation.<sup>39</sup> Indeed, a major function of ATR is its regulation of origin firing.<sup>40</sup> Although dormant origin firing is proposed to allow for the completion of replication in the presence of replication stress, excessive origin firing upon ATR inhibition leads to RPA exhaustion and is proposed to be a major cause of replication fork breakage.<sup>41,42</sup> Given that ATR inhibition results in fork breakage even in the absence of exogenously-induced replication stress, ATR-dependent inhibition of origin firing is likely important during normal S phase progression.<sup>43,44,45</sup> Another source of replicative stress upon ATR inhibition is introduced due to the CDK-mediated degradation of ribonucleotide reductase subunit RRM2, which leads to dNTP depletion.<sup>46</sup>

Apart from this, Saldivar et al. recently demonstrated that ATR is continually activated during S phase to prevent the CDK-dependent activation of a promitotic transcription programme, such that a basal level of ATR activation ensures the completion of replication prior to mitosis.<sup>47</sup> Apart from their role in checkpoint activation, ATM- and ATR- mediating signalling is also responsible for the recruitment of proteins involved in DNA repair or fork remodelling.<sup>48,49,50,51,52</sup>

### **1.3 DNA damage signalling is attenuated during mitosis**

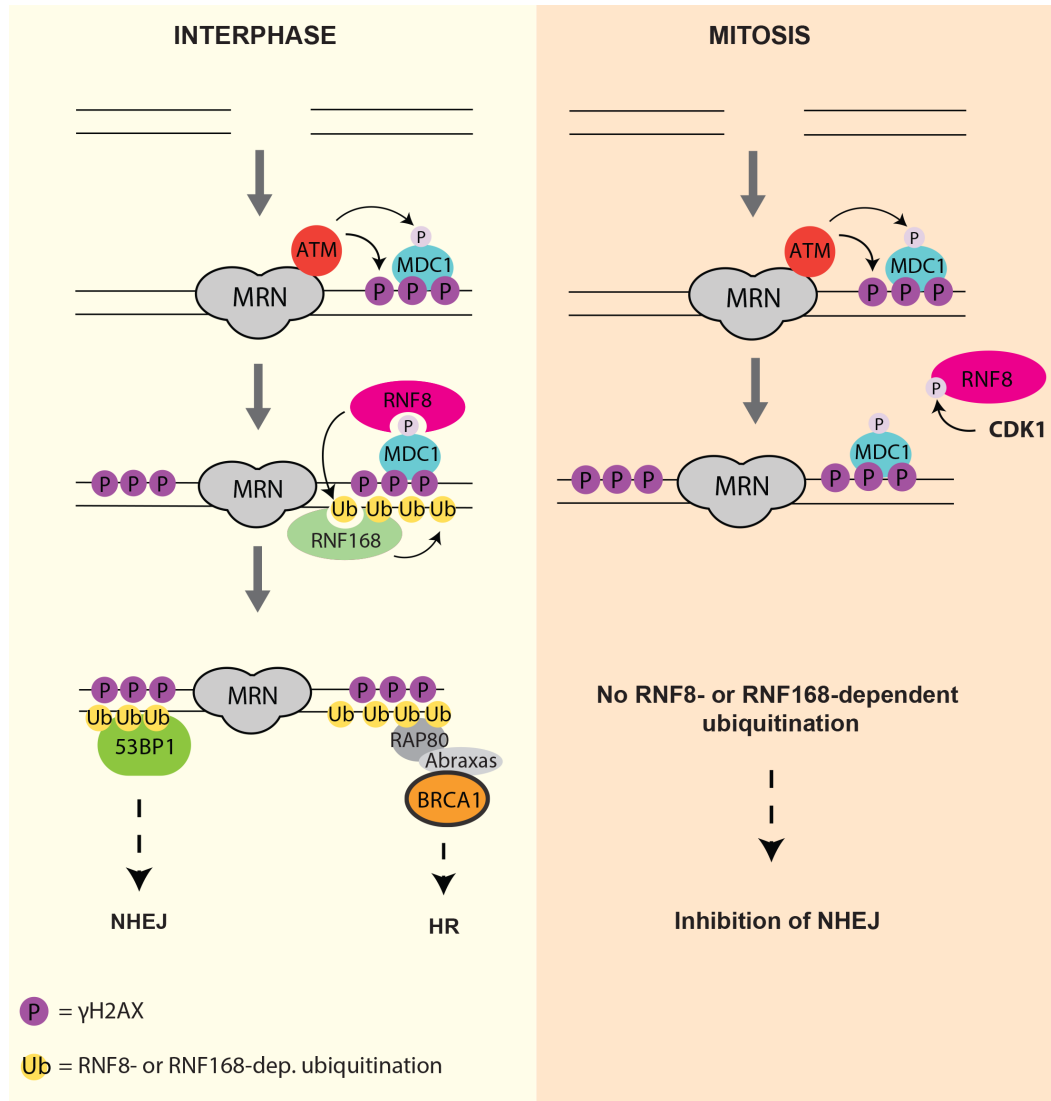
The major role of ATM activation is to propagate the DNA damage signal at the chromatin level, ultimately leading to the mobilization of the downstream DSB repair machinery. Upon activation, the ATM kinase initiates a wave of chromatin modifications. The DNA damage signal is firstly reinforced via direct phosphorylation

of histone variant H2AX (known as  $\gamma$ H2AX in its phosphorylated form).  $\gamma$ H2AX recruits MDC1, a key conductor of the DNA damage response, which acts as a platform for the recruitment of E3 ubiquitin ligase RNF8. RNF8, in turn, promotes the recruitment of E3 ubiquitin ligase RNF168. In this way, the DNA damage signal is propagated from an initial burst of chromatin phosphorylation to a subsequent wave of protein ubiquitination at the damaged chromatin.<sup>53</sup> This allows for the ubiquitin-dependent recruitment of various DNA repair proteins, including 53BP1 and BRCA1, which direct DSB repair via NHEJ or HR respectively (Figure 1.2).<sup>54</sup>

Interestingly, ATM-mediated recruitment of DNA repair proteins seems to be inhibited during mitosis.<sup>55,56</sup> This occurs via the inhibitory CDK-dependent phosphorylation of RNF8 during mitosis, which prevents RNF8- and RNF168-mediated ubiquitination of its downstream targets. 53BP1 accumulation at mitotic chromatin is additionally inhibited through its phosphorylation by mitotic kinases CDK1 and PLK1.<sup>54,57,58</sup> Indeed, restoration of RNF8 and 53BP1 recruitment to mitotic chromatin results in telomere fusions and reduced cell survival upon irradiation, indicating that the activation of NHEJ must be inhibited in during mitosis. However, there is no evidence that the recruitment of HR-mediating proteins is similarly deleterious. Indeed, recent studies suggest stalled replication forks and telomeres are processed via a BIR-like mechanism during mitosis, such that mitosis is not necessarily a DNA repair-devoid phase of the cell cycle.<sup>59,60,16</sup>

#### **1.4 RAD51 recruitment and loading at DSBs catalyses HR**

The RAD51 recombinase is the main catalyst of HR, as it promotes strand-invasion of the broken DNA end into the homologous repair template through its strand-displacement and homology-pairing activities.<sup>61</sup> Upon DNA end resection, the ssDNA overhang is rapidly bound by the RPA protein, thereby inhibiting RAD51 binding. In a key step towards homologous recombination, RPA must be displaced from the resected DNA end to load RAD51 in its place. This process requires the action of a mediator protein, which facilitates the release of RPA from the ssDNA to promote the establishment of the RAD51 nucleoprotein filament and subsequent strand-exchange reactions.



**Figure 1.2 – RNF8- and RNF168- dependent ubiquitination at damaged chromatin is inhibited in mitosis.**

Rad52 fulfils this function in *S. cerevisiae*, as it binds ssDNA, RPA and Rad51 to facilitate the displacement of RPA and subsequent loading of Rad51.<sup>62,63</sup> Importantly, Rad52 also exhibits DNA strand annealing activity and can mediate DSB repair in the absence of Rad51.<sup>64,65</sup>

While RAD52 similarly binds ssDNA, RPA and RAD51 in higher eukaryotes, it does not appear to be an important mediator of RAD51 strand-exchange activity. Instead, breast cancer susceptibility gene BRCA2 represents the key mediator of RAD51 recruitment and loading at DSBs.<sup>66</sup> Indeed, based on the discovery that RAD52 depletion is synthetically lethal with BRCA2 deficiency RAD52 is proposed

to act as a back-up mediator of HR.<sup>67</sup> Nevertheless, similarly to its homolog in *S. cerevisiae*, RAD52 retains its DNA strand annealing activity and can promote DSB repair in its own right. RAD52 single-strand annealing activity promotes DSB repair via the SSA pathway, while mounting evidence implicates RAD52 in break-induced replication, as discussed further below.<sup>23,14,68,69,</sup>

BRCA2 directly binds RAD51 either through its centrally located BRC-repeats or through the C-terminal TR2 domain. The BRC motif binds RAD51 in its monomeric form and is important for the recruitment of Rad51 to the break site.<sup>70</sup> RAD51 interaction with the TR2 domain of BRCA2 has been shown to both stabilize the Rad51 nucleoprotein filament and promote its extension at the resected DNA end.<sup>71,72,73</sup> Therefore, through the concerted action of these binding domains, BRCA2 facilitates RAD51 recruitment and establishment of the RAD51 nucleoprotein filament. BRCA2 recruitment to the DSB itself is mediated through the formation of a BRCA1-PALB2-BRCA2 complex at the break site.<sup>74,64</sup> Interestingly, RAD51 binding at the TR2 domain was shown to be disrupted by CDK-dependent phosphorylation of BRCA2.<sup>75</sup> This phosphorylation peaks in mitosis, suggesting that the TR2-RAD51 interaction (and TR2-dependent RAD51 filament formation) is actively prevented as the cell goes into mitosis. Indeed, *in vivo* studies have suggested that the disruption of TR2-RAD51 interaction allows for quicker disassembly of RAD51 foci and concomitant faster entry into mitosis.<sup>76</sup> These findings point towards the inhibition of BRCA2-dependent RAD51 loading in mitosis, which is in line with the reported attenuation of the DNA damage response during mitosis.<sup>53,54,56</sup>

## **1.5 PLK1-dependent RAD51 phosphorylation: an alternative pathway for RAD51 recruitment**

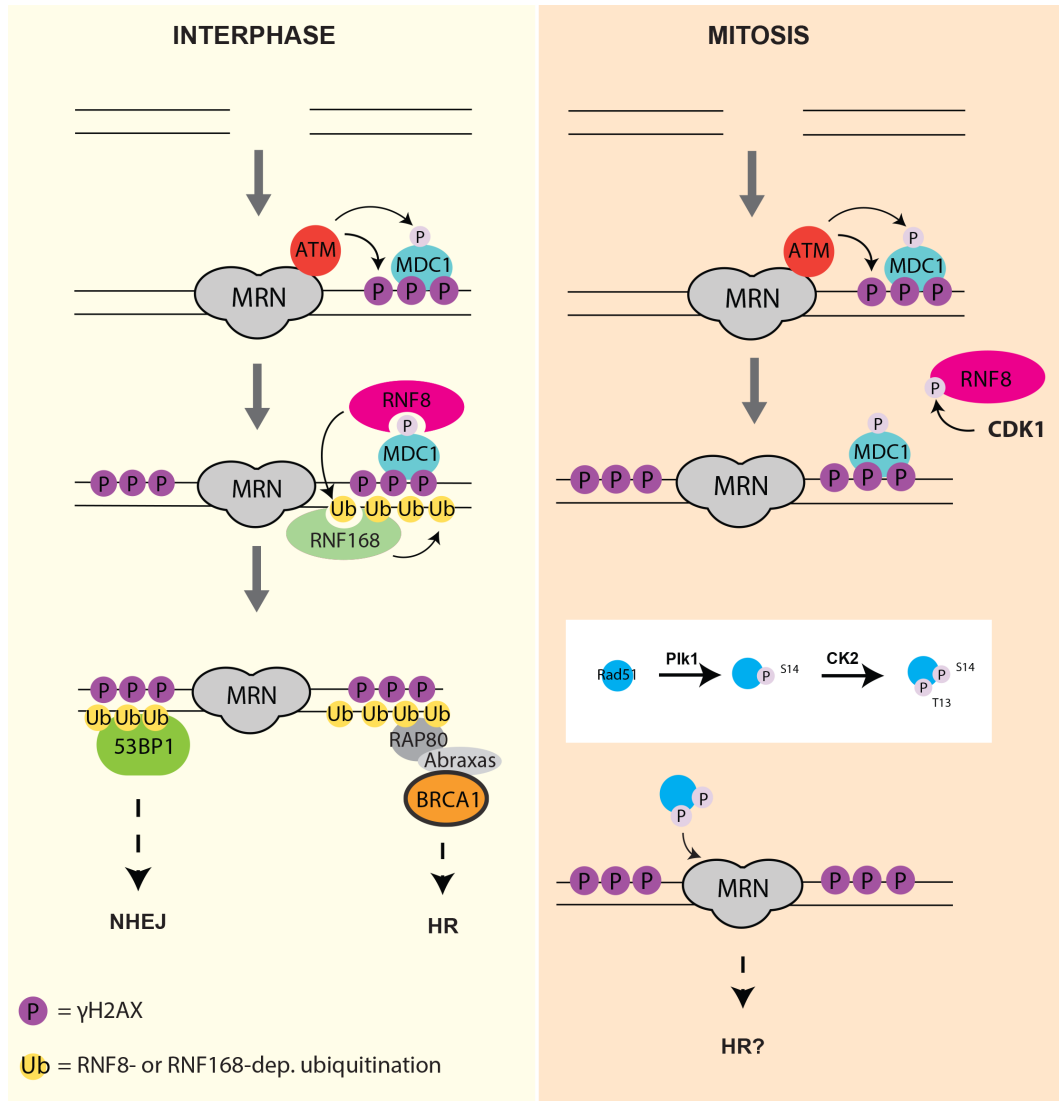
Importantly, RAD51 can be recruited to the chromatin in a pathway that does not depend on BRCA1-PALB2-BRCA2 complex formation.<sup>77,78</sup> This alternative pathway for RAD51 recruitment depends on RAD51 phosphorylation by the mitotic kinase Polo-like kinase 1 (PLK1), which phosphorylates RAD51 at serine 14 (S14). The phosphorylated residue creates a recognition site for the acidophilic casein kinase 2 (CK2), which subsequently phosphorylates RAD51 at threonine 13 (T13). T13 phosphorylation mediates a direct interaction between RAD51 and the NBS1

component of the MRN complex, which is sufficient to load RAD51 at the site of DNA damage and promote HR in a BRCA2 deficient background.<sup>76</sup> Nevertheless, BRCA2 was shown to act as a binding platform for both PLK1 and RAD51, hence promoting PLK1-dependent RAD51 phosphorylation.<sup>79</sup> It should be noted that this interaction does not require BRCA2 recruitment to chromatin, as it seems to be detected primarily in the nuclear soluble fraction. A similar role has been reported for TOPBP1, such that different binding platforms likely support PLK1-dependent RAD51 phosphorylation even in the absence of BRCA2.<sup>80</sup>

Importantly, RAD51 phosphorylation peaks during late G2 phase and mitosis in unperturbed cells, suggesting that phosphorylated RAD51 may also target endogenous DNA damage or stress during mitosis.<sup>76,77</sup> Indeed, given that the MRN complex is actively recruited at mitotic DSBs, NBS1-RAD51 interaction may be sufficient to support RAD51 recruitment at mitotic chromatin in a phosphorylation-dependent manner (Figure 1.3).

## **1.6 RAD51 is essential in higher eukaryotes**

Interestingly, Rad51 deletion confers high sensitivity to  $\gamma$ -ray radiation in yeast, but is not detrimental to viability, while the loss of RAD51 is fatal in vertebrate cells.<sup>81,82</sup> However, the contribution of HR in the repair of DSBs is drastically reduced with increasing complexity of the organism<sup>83</sup> – while *S. cerevisiae* mostly depends on HR for DSB repair, NHEJ repairs at least 75% of enzymatically induced breaks in immortalized human fibroblasts, and up to 85% of X-ray induced DSB in G2 in primary human fibroblasts.<sup>84,85,86,87</sup> NHEJ has been characterized as a fast-acting repair mechanism, initially targeting all DSBs in G2, while HR is observed at those breaks where NHEJ cannot progress efficiently due to the presence of clustered DNA damage or surrounding heterochromatin.<sup>88</sup> Moreover, the NHEJ pathway can compensate for the loss of HR in irradiated cells upon inhibition of end-resection.<sup>8,86</sup> Given the decreased dependency on HR-mediated DSB repair in higher eukaryotes, the role of RAD51 in classical two-ended DSB repair does not provide a satisfying explanation for the lethality of RAD51 deletion in vertebrates. RAD51 must play an important role beyond the classical DSB repair pathway.



**Figure 1.3 – PLK1-dependent RAD51 phosphorylation potentially mediates its recruitment in mitosis.**

Indeed, the essentiality of RAD51 in higher eukaryotes is thought to stem from the increased size and complexity of the genome. Compared to yeast, the mammalian genome is larger and more repetitive.<sup>89</sup> Repetitive DNA is thought to be inherently more difficult to replicate due to its propensity to generate secondary structures that impede replication fork progression.<sup>90,91,92,93,94</sup> Additionally, the elevated number and length of introns in mammalian genes introduce another source of replication stress, as long genes are associated with increased replication-transcription collisions.<sup>95,96,97</sup> All together, the increased complexity of the mammalian genome increases the amount of replication stress the cell must deal with. Importantly, several studies demonstrate that



RAD51 function at the stalled replication fork contributes to genome stability, suggesting that this may account for the essentiality of RAD51 in higher eukaryotes.<sup>98,99,100,101</sup>

## **1.7 RAD51: Fork reversal, fork protection and fork restart**

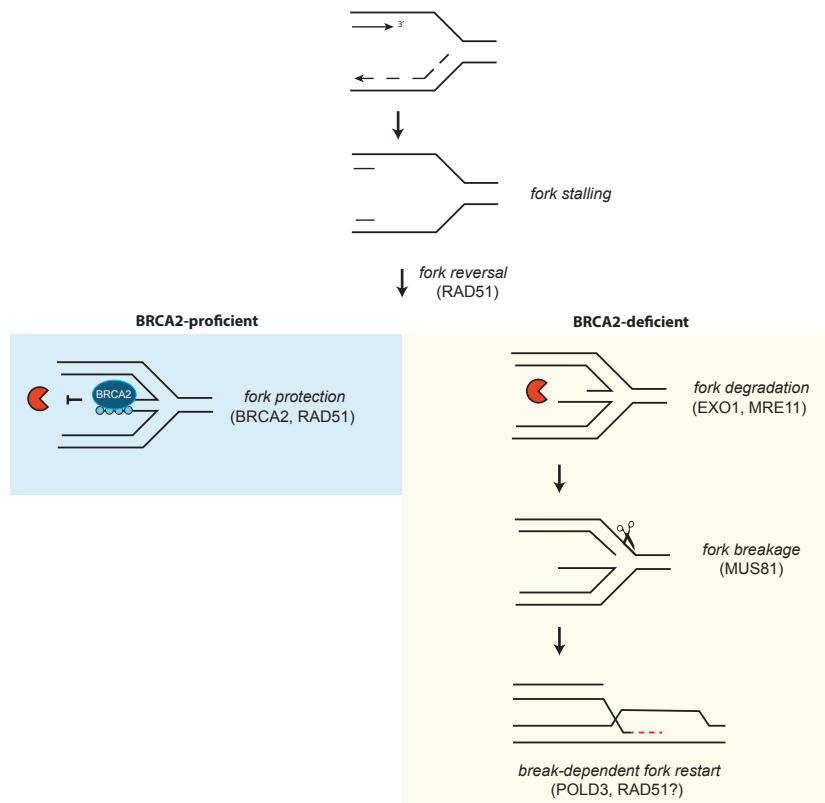
Replication fork remodelling into a reversed ‘chicken-foot’ structure has been identified as a global response to replication-stress induced fork uncoupling.<sup>102,103</sup> Several factors mediating fork reversal have been identified, including the SWI/SNF family translocases ZRANB3, SMARCAL1 and HLTf, as well as PARP1 and RAD51.<sup>100,101,104,105,106</sup> Importantly, RAD51 depletion severely reduces fork reversal and increases DSB formation under conditions of replication stress, suggesting that RAD51-mediated fork reversal contributes to genomic stability.<sup>101</sup>

RAD51 is also proposed to preserve genome stability through the stabilization of stalled replication forks. Schlacher et al. first reported that BRCA2 prevents MRE11-dependent degradation of nascent DNA at stalled replication forks.<sup>96,97,98,107,108</sup> Interestingly, this protective function of BRCA2 depends on its stabilization of the RAD51 nucleofilament. Indeed, BRCA2-mediated fork protection depends on RAD51 interaction at the BRCA2 TR2 domain, which stabilizes RAD51 nucleofilaments.<sup>69,96</sup> FANCD2-mediated RAD51 stabilization was similarly shown to prevent nascent DNA degradation at stalled forks.<sup>97,109</sup> RAD51 K133R mutation disrupts RAD51 ATPase activity and impairs its dissociation from the DNA, such that the RAD51 K133R mutant forms hyper-stable RAD51 nucleofilaments that do not support HR.<sup>110,111</sup> Interestingly, expression of the HR-defective RAD51 K133R mutant is sufficient to maintain fork protection in BRCA2- or FANCD2- deficient cells, indicating that the stability of the RAD51 nucleofilament, not its function in HR, is essential for fork protection.<sup>96,97</sup> The separate function of RAD51 in fork protection versus HR is further indicated by the Fanconi anaemia-associated dominant-negative RAD51 T131P mutant. Contrary to K133R mutants, heterozygous RAD51 T131P patient cells are HR proficient but display increased MRE11-dependent degradation of nascent DNA strands upon fork stalling.<sup>112,98</sup>

Several recent studies further suggest that MRE11- or EXO1-dependent DNA degradation at stalled forks specifically occurs at the reversed ‘chicken-foot’ structure.

Indeed, the inhibition of fork reversal upon RAD51-, ZRANB3- or SMARCAL1-depletion, as well as PARP1 inhibition, rescues the enhanced DNA degradation observed at stalled forks in a BRCA2-deficient background.<sup>98,105,106</sup> This suggests that fork reversal provides the entry-point for nucleolytic DNA degradation at stalled forks (Figure 1.4). Lemaçon et al. show that MRE11- and EXO1-dependent degradation at reversed forks promotes MUS81-dependent fork breakage and subsequent fork restart.<sup>105</sup> The authors further find that this break-dependent fork restart is reduced upon POLD3 or RAD51 depletion. POLD3 is associated with BIR in mammalian cells<sup>113</sup> such that break-dependent fork restart likely proceeds via BIR. The observed involvement of RAD51 in break-dependent fork restart likely reflects its role in fork reversal, although a downstream role for RAD51 after fork breakage cannot be excluded (Figure 1.4).

Expression of the HR-defective RAD51 K133R mutant rescues cell viability in RAD51-null DT40 cells, suggesting that HR is dispensable when fork protection is sustained. Conversely, viability of the T131P heterozygous mutant demonstrates that fork protection is dispensable in HR-proficient cells. It is therefore tempting to speculate that the importance of HR in the absence of fork protection depends on its ability to mediate BIR-dependent fork restart. Nevertheless, BIR is highly mutagenic and has been shown to increase copy-number alterations in human U2OS cells, as well as chromosomal rearrangements in yeast.<sup>111,114,115,116</sup> Indeed, the loss of BRCA2 - mediated fork protection was shown to underlie the lethality of BRCA2-deficiency in embryonic stem cells, suggesting that BIR-mediated fork restart is not sufficient to sustain viability in these cells.<sup>117</sup>



**Figure 1.4 – Nascent DNA degradation at reversed forks triggers break-dependent replication fork restart in BRCA2-deficient cells.**

Considering the mutagenic consequences of BIR, break-independent mechanisms of fork restart are likely important to maintain genomic stability.

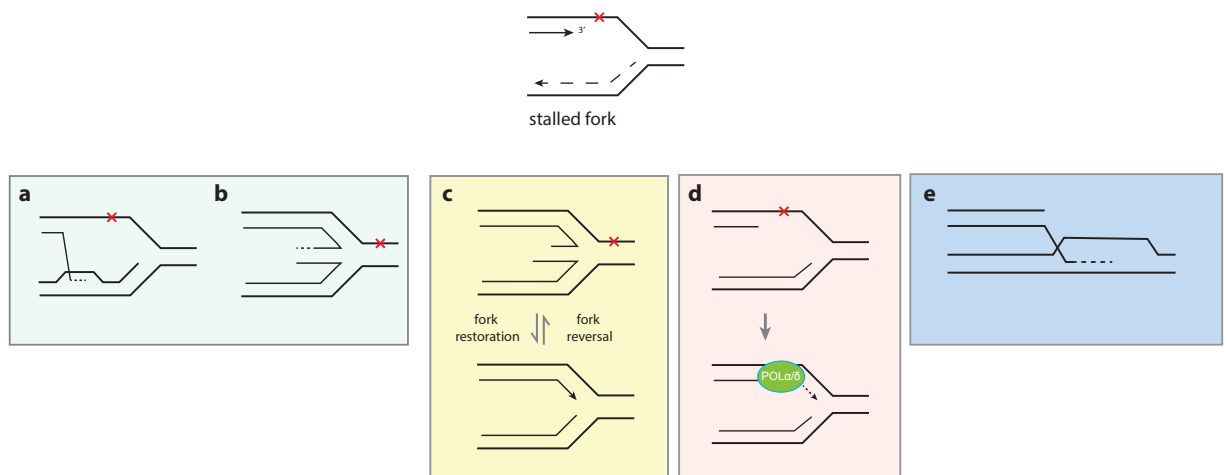
## 1.8 Proposed mechanisms of RAD51-dependent fork restart

Petermann et al. demonstrated that RAD51 mediates replication fork restart after hydroxyurea (HU)-induced fork stalling.<sup>118</sup> Importantly, this was observed under conditions that did not appear to induce massive DSB accumulation, suggesting that RAD51 mediates fork restart from intact stalled forks structures. Several mechanisms can be envisioned for RAD51-mediated restart of stalled forks (Figure 1.5). Template switching refers to the temporary pairing of the stalled DNA strand with the sister chromatid (or any other homologous sequence), hence providing an undamaged replication template to bypass the replication-obstructing DNA lesion (Figure 1.5 a-b). Template switching conceivably involves RAD51-mediated strand invasion into the

homologous template. Alternatively, RAD51-mediated fork reversal potentially promotes template switching and subsequent lesion bypass (Figure 1.5b).<sup>119</sup> Indeed, replication restart by template switching was observed to occur via a regressed fork intermediate in the T4 bacteriophage replication system.<sup>120</sup>

Fork reversal may also simply provide a stable replication fork intermediate from which replication can be continued once the cause of replication stalling has been resolved (Figure 1.5c). Indeed, RECQ1-mediated branch migration was shown to restore the reversed fork to an active replication fork structure to promote replication restart upon repair of the obstructing DNA lesion.<sup>121</sup> Controlled resection of the reversed replication fork has also been shown to mediate replication restart, potentially by generating a suitable substrate for fork restoration.<sup>122,104</sup>

The accumulation of ssDNA gaps behind the replication fork suggest that re-priming is a common mechanism to continue replication ahead of an obstructing DNA lesion.<sup>123,124</sup> Interestingly, a recent study in *Xenopus laevis* egg extract shows that RAD51 interacts with polymerase Pol  $\alpha$  and mediates Pol  $\alpha$  and Pol  $\delta$  chromatin association, hence introducing the intriguing possibility that RAD51 recruits the primase complex at stalled replication forks to mediate replication restart (Figure 1.5d).<sup>106</sup> Lastly, RAD51 can promote the restart of broken replication forks via BIR (Figure 1.5e).<sup>125,126,111</sup>



**Figure 1.5 – Proposed models for RAD51-mediated fork restart**

So far, RAD51 has been implicated in three replication-associated functions: replication fork reversal; replication fork protection against excessive nucleolytic

degradation; and replication fork restart. Interestingly, a recent study investigates the importance of RAD51 strand-exchange activity in each of these functions by analysing a RAD51 mutant that binds ssDNA but cannot catalyse *in vitro* D-loop formation or *in vivo* DSB repair. Consistent with previous studies, RAD51-mediated fork protection does not rely on its strand-exchange activity. Surprisingly, RAD51 strand-exchange activity was also found to be dispensable for fork reversal.<sup>127</sup> It is not yet understood how RAD51 can mediate fork reversal in the absence of strand-exchange activity, although it has been proposed that RAD51 may stabilize the reversed fork structure after initial regression by capturing the ssDNA end protruding from the chicken-foot structure.<sup>128</sup> Intriguingly, restart of the majority of stalled replication forks does not require RAD51 strand-exchange activity after 5 hours HU incubation, whereas fork restart after 8 hours HU incubation exhibits a much greater requirement for RAD51 strand-exchange activity. This suggests that replication restart from a reversed fork structure initially does not necessarily require RAD51 strand-exchange activity (such as in Figure 1.5 b-d), while, after prolonged replication block, the fork structure changes such that replication restart requires RAD51 strand-exchange activity. It is tempting to speculate that replication fork breakage is increased after the longer replication block, such that RAD51 strand-exchange activity is required for BIR-mediated restart.

All together, it appears that both replication fork reversal and replication fork protection can occur in the absence of RAD51-mediated strand-exchange.<sup>96,126</sup> These findings may be interpreted to indicate that HR does not play a very important role at the replication fork. However, Feng and Jasin recently found that the role of BRCA2 in fork protection is dispensable for the viability of untransformed mammalian cells.<sup>129</sup> Furthermore, BRCA2 was found to prevent under-replication in a way that depends on RAD51, but not on its role in fork protection. This study therefore suggests that BRCA2 and RAD51 facilitate efficient replication in untransformed cells, likely through a role in HR, although a RAD51-mediated role independent from HR and fork protection cannot be excluded. Evidently, the full contribution and importance of RAD51 at the replication fork is not yet understood completely.

## **1.9 Break-induced replication mediates fork restart and alternative lengthening of telomeres (ALT) in mitosis**

Replication stress has long been associated with chromosomal breakage or ‘fragility’.<sup>130,131</sup> Genomic loci which are inherently difficult to replicate and exhibit chromosomal gaps upon exposure to mild replication stress are termed common fragile sites (CFSs).<sup>129</sup> CFSs are generally found in long genes that exhibit late replication. Several hypotheses for their fragility have been proposed, including sparsely distributed initiation events; lack of dormant origin firing upon replication stalling, as well as impaired replication progression.<sup>132,133,134,135,95</sup> The latter may be explained by a propensity for the underlying DNA sequence to form secondary structures that impede efficient replication.<sup>133,134</sup> Additionally, long genes take a very long time to be transcribed. This increases the likelihood of replication-transcription collisions and hence introduces a transcription-dependent source of chromosomal fragility and genetic instability.<sup>95,136</sup>

Replication stress promotes the formation of FANCD2 foci which persist into mitosis and associate to the base of ultra-fine DNA bridges (UFBs) formed between sister chromatids. Indeed, these characteristic mitotic FANCD2 ‘twin-foci’ serve as a marker for under-replication at CFSs.<sup>137</sup> This indicates that this level of DNA under-replication are checkpoint blind, as cells can enter into mitosis before having completed replication completely. The persistence of under-replicated DNA leads to impaired chromosome disjunction, observed as anaphase bridges, UFBs and micronuclei, as well as 53BP1 nuclear bodies which represent unresolved DNA damage that passes into the next cell cycle.<sup>135,138,139,140</sup> Several proteins have been found to act at CFSs to alleviate these phenotypes– including FANCD2, ERCC1, MUS81, TOPBP1 and TLS polymerases Pol  $\eta$  and Pol  $\zeta$  - thereby indicating a role for these proteins to facilitate replication at CFS.<sup>133,135,137,138,141,142</sup> This could either stem from the facilitation of replication during S phase or from the resolution of under-replicated regions at later stages of the cell cycle. MUS81 and TOPBP1 associate with fragile sites specifically upon mitotic entry, suggesting a role beyond facilitating replication in S phase.<sup>137,139</sup> Careful cell synchronization prior to EdU incubation confirmed that DNA synthesis occurs during early mitosis and that this process is specifically initiated in mitosis.<sup>57</sup> The observed mitotic EdU incorporation was shown

to be a MUS81- and POLD3-dependent process, suggesting that MUS81-dependent fork breakage acts at stalled replication intermediates to trigger BIR-mediated mitotic DNA synthesis (MiDAS).

A similar mechanism is proposed to enable replication restart from stalled replication forks in BRCA2-deficient cells, as described above (Figure 1.4). Importantly, both MUS81-dependent fork restart and chromosomal aberrations are reduced upon MRE11 inhibition in BRCA2-deficient cells, indicating that break-induced fork restart is a significant source of genetic instability.<sup>96,105</sup> Lemaçon et al. find that, while fork restart in BRCA2-deficient cells is largely POLD3-dependent, this is not the case in BRCA2-proficient cells. This suggests that BIR-mediated fork restart acts as a back-up mechanism to support replication restart in the absence of adequate fork protection.<sup>105</sup> Similarly, MiDAS is proposed to act as a ‘last resort’ mechanism to complete DNA replication at persistently under-replicated sequences prior to anaphase.

While several reports of BIR in higher eukaryotes suggest that RAD51 catalyses this process, MiDAS at FANCD2 twin-foci appears to be primarily RAD52-dependent.<sup>14,111,125</sup> Indeed, the importance of RAD52 in oncogenic-induced BIR was recently demonstrated.<sup>66</sup> Alternative lengthening of telomeres (ALT), a homology-directed pathway for the maintenance of telomere length, is similarly proposed to proceed via a BIR mechanism. Analysis of ALT telomeres revealed the importance of a POLD3-dependent conservative DNA replication process in the maintenance of telomere length, thereby demonstrating that ALT exhibits characteristic features of BIR.<sup>15</sup> Indeed, Dilley et al. show that the induction of telomere DSBs triggers telomere synthesis.<sup>143</sup> Interestingly, this process was shown to be RAD51 independent. MiDAS at telomeres was similarly shown to be RAD51-independent, and instead relies on RAD52 activity.<sup>16,58</sup>

The idea that a BIR-related processes can occur independently of RAD51 is not new – indeed, Rad51-independent (Type II) ALT has been described in yeast and relies on the strand annealing activity of Rad52. Since MiDAS occurs at regions of stalled replication, it is conceivable that these areas are associated with non-B DNA structures which might allow for strand annealing without requiring RAD51-mediated strand invasion. Furthermore, repetitive sequences might be particularly amenable to strand-pairing without requiring the homology-search capacity of RAD51. Further

research will be necessary to elucidate under which conditions BIR and MiDAS are RAD52- or RAD51-dependent, and to what extent they might cooperate in these processes.

## **1.10 A potential role for phosphorylated RAD51 in later stages of the cell cycle**

As reviewed in this chapter, RAD51 appears to have several functions at the (stalled) replication fork which may have a bigger impact on cell viability and genetic integrity than its well-described function in classical DSB repair. A PLK1-dependent recruitment mechanism of RAD51 has been identified, which does not necessarily depend on BRCA2. Indeed, the impact of RAD51 depletion on fork reversal and fork restart in BRCA2 deficient cells, as described previously, demonstrates that RAD51 recruitment at stalled replication forks does not necessarily depend on BRCA2.<sup>98,105</sup> Previous research has shown that PLK1-dependent RAD51 phosphorylation enables RAD51 recruitment at both irradiation-induced DSBs and HU-stalled replication forks after BRCA2 depletion.<sup>76,77</sup> The PLK1-dependent RAD51 phosphorylation pathway appears to sustain RAD51 recruitment in BRCA2-deficient backgrounds. This BRCA2-independent RAD51 functionality may explain why BRCA2 null cells can be viable (albeit genetically unstable), whereas RAD51 nullzygosity appears to be lethal no matter the genetic background.

Interestingly, RAD51 T13/S14 phosphorylation is observed during an unperturbed cell cycle, suggesting that PLK-dependent RAD51 recruitment may also be important during normal proliferation. Given that RAD51 T13/S14 phosphorylation peaks during late G2 phase and mitosis, phosphorylated RAD51 may have an important role during the later phases of the cell cycle. In this thesis I investigate the function of PLK1-dependent RAD51 phosphorylation during the cell cycle, focusing on a role during replication and mitotic DNA synthesis.



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## **Chapter 2**

### **Materials and Methods**





## **1. Materials**

All materials used during this study are listed in tables 2.1-2.10.

### **1.1 Mammalian cell lines**

Mammalian cell lines used and generated during this study are summarized in table 2.1-2.3.

The HeLa mCh-H2B EGF-tub cell lines was a generous gift from U. Gruneberg.

### **1.2 Oligonucleotides and plasmids**

All RNA and DNA oligonucleotides used during this study are summarized in table 2.4 and 2.5 respectively. All plasmids used during this study are summarized in table 2.6.

pcDNA3.1/GFP-LaminB1 was a kind gift from David Vaux.

### **1.3 Buffers and solutions**

Buffers and other solutions used during this study are summarized in table 2.7.

### **1.4 Antibodies**

All antibodies and the dilutions used during this study are summarized in table 2.8-2.9.

All remaining materials used during this study are listed in table 2.10.

**Table 2.1 – Mammalian cell lines used**

| <b>Cell line</b>                               | <b>Abbreviation</b>      | <b>Parental</b> | <b>Disease</b>           | <b>Further characteristics</b>                                           | <b>Medium</b> | <b>Additional antibiotics</b>                       |
|------------------------------------------------|--------------------------|-----------------|--------------------------|--------------------------------------------------------------------------|---------------|-----------------------------------------------------|
| U2OS                                           | -                        | -               | Osteosarcoma             | Hyperploid                                                               | DMEM          | -                                                   |
| hTERT RPE-1                                    | RPE1                     | -               | Normal                   | hTERT immortalized, near-diploid                                         | DMEM/F12      | -                                                   |
| Engineered HAPloid                             | eHAP                     | HAP1            | Chronic myeloid leukemia | Fully haploid (CRISPR/Cas9-engineered)                                   | IMDM          | -                                                   |
| HeLa mCherry-Histone H2B<br>EGFP-Alpha Tubulin | HeLa mCh-H2B<br>EGFP-tub | HeLa S3         | Adenocarcinoma           | Constitutively expresses mCherry-Histone H2B and EGFP- $\alpha$ -tubulin | DMEM          | 2 $\mu$ g/ml blasticidin;<br>1 $\mu$ g/ml puromycin |

**Table 2.2 – Stable cell lines generated<sup>1</sup>**

| <b>Cell line</b>    | <b>Parental cell line</b> | <b>Exogenous gene expressed</b> | <b>Plasmid</b>           | <b>System</b>           | <b>Additional antibiotics</b> |
|---------------------|---------------------------|---------------------------------|--------------------------|-------------------------|-------------------------------|
| U2OS mCh-RAD52      | U2OS                      | mCherry-RAD52                   | pcDNA/DEST/mCherry-RAD52 | Constitutive expression | 400 $\mu$ g/ml G418           |
| U2OS S14A + WT      | U2OS S14A (CRISPR)        | RAD51 (wild-type)               | pcDNA/FRT/RAD51          | Constitutive expression | 400 $\mu$ g/ml G418           |
| U2OS S14D + WT      | U2OS S14D (CRISPR)        | RAD51 (wild-type)               | pcDNA/FRT/RAD51          | Constitutive expression | 400 $\mu$ g/ml G418           |
| U2OS WT GFP-Lamin   | U2OS                      | GFP-Lamin B1                    | pcDNA3.1/GFP-LaminB1     | Constitutive expression | 400 $\mu$ g/ml G418           |
| U2OS S14A GFP-Lamin | U2OS S14A (CRISPR)        | GFP-Lamin B1                    | pcDNA3.1/GFP-LaminB1     | Constitutive expression | 400 $\mu$ g/ml G418           |
| U2OS S14D GFP-Lamin | U2OS S14D (CRISPR)        | GFP-Lamin B1                    | pcDNA3.1/GFP-LaminB1     | Constitutive expression | 400 $\mu$ g/ml G418           |

<sup>1</sup> All cells lines were generated by I. Wassing

**Table 2.3 – Cell lines generated by CRISPR/Cas9-mediated gene editing**

| Parental cell line | Mutation                               | Medium   | Antibiotics |
|--------------------|----------------------------------------|----------|-------------|
| U2OS               | Knock-in, RAD51 S14A <sup>a</sup>      | DMEM     | -           |
|                    | Knock-in, RAD51 T13A S14A <sup>a</sup> | DMEM     | -           |
|                    | Knock-in, RAD51 S14D <sup>b</sup>      | DMEM     | -           |
| RPE1               | Knock-in, RAD51 S14A <sup>a</sup>      | DMEM/F12 | -           |
|                    | Knock-in, RAD51 T13A S14A <sup>a</sup> | DMEM/F12 | -           |
|                    | Knock-in, RAD51 S14D <sup>a</sup>      | DMEM/F12 | -           |
| eHAP               | Knock-in, RAD51 S14A <sup>a</sup>      | IMDM     | -           |
|                    | Knock-in, RAD51 T13A S14A <sup>a</sup> | IMDM     | -           |
|                    | Knock-in, RAD51 S14D <sup>a</sup>      | IMDM     | -           |

**Table 2.4 – RNA oligonucleotides used**

| Name            | Sequence (5'-3')        |
|-----------------|-------------------------|
| guide RNA       | agcaaatgcagatacttcagtgg |
| siRNA RAD51 (1) | gacugccaggauaaagcuu     |
| siRNA RAD51 (2) | gugcugcagccuaaugaga     |

<sup>a</sup> Generated by I. Wassing and L. Rampazzo<sup>b</sup> Generated by C. Ralf

**Table 2.5 – DNA oligonucleotides used**

| Name                           | Sequence (5'-3')                                                                                                                                            | Purpose                                                                                                             |
|--------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------|
| ssDNA repair template S14D     | ccaaaattagcaactaaccacatacctctaaccgtgaaatgggtgtgggccc<br>agctttcttccacatcagtatctgcatttgcttcaagctgcatctgcattgccat<br>tactgaaaaatacaaatgcttatcagtataaacacta* g | CRISPR/Cas9-mediated knock-in<br>(Methods 2.5.5)                                                                    |
| ssDNA repair template S14A     | ccaaaattagcaactaaccacatacctctaaccgtgaaatgggtgtgggccc<br>agctttcttccacggcagtatctgcatttgcttcaagctgcatctgcattgccat<br>tactgaaaaatacaaatgcttatcagtataaacacta* g | CRISPR/Cas9-mediated knock-in<br>(Methods 2.5.5)                                                                    |
| ssDNA repair template T13AS14A | ccaaaattagcaactaaccacatacctctaaccgtgaaatgggtgtgggccc<br>agctttcttccacggcgcatctgcatttgcttcaagctgcatctgcattgccat<br>ttactgaaaaatacaaatgcttatcagtataaacacta* g | CRISPR/Cas9-mediated knock-in<br>(Methods 2.5.5)                                                                    |
| HRM forward                    | ggggagagagatgcaccta                                                                                                                                         | PCR-mediated amplification of<br>CRISPR-targeted locus                                                              |
| HRM reverse                    | aaaggttgtaatacctccactagg                                                                                                                                    |                                                                                                                     |
| Sticky-HRM forward             | agagagggatccggggagagagatgcaccta                                                                                                                             | PCR-mediated amplification of CRISPR-targeted<br>locus / contains <i>Bam</i> HI restriction site<br>(Methods 2.3.8) |
| Sticky-HRM reverse             | agagaggtcgacaaaggttgtaatacctccactagg                                                                                                                        |                                                                                                                     |
| WT forward                     | cttgaagcaaatgcagatacttca                                                                                                                                    | Screening of CRISPR-targeted locus by PCR<br>(Methods 2.3.6)                                                        |
| S14D forward                   | cttgaagcaaatgcagatactgat                                                                                                                                    | Screening of CRISPR-targeted locus by PCR<br>(Methods 2.3.6)                                                        |
| S14A forward                   | cttgaagcaaatgcagatactgcc                                                                                                                                    | Screening of CRISPR-targeted locus by PCR<br>(Methods 2.3.6)                                                        |
| T13A/S14A forward              | gcaaatgcagatactgccgcc                                                                                                                                       | Screening of CRISPR-targeted locus by PCR<br>(Methods 2.3.6)                                                        |
| Reverse                        | tgcagcctttccacaggatt                                                                                                                                        | Screening of CRISPR-targeted locus by PCR<br>(Methods 2.3.6)                                                        |
| MiSeq forward                  | acactcttcctacacgacgctctccgatcttactgataagcatttgatt                                                                                                           | PCR primers compatible with barcoded Illumina<br>adaptors (Methods 2.3.9)                                           |
| MiSeq reverse                  | tggcattcctgctgaaccgctctccgatctaaaggttgtaatacctccactag                                                                                                       |                                                                                                                     |

\* Denotes position of phosphorothioate bond

**Table 2.6 – Plasmid DNA used**

| <b>Name</b>                            | <b>Vector backbone</b>      |
|----------------------------------------|-----------------------------|
| pBluescriptKS                          | Standard cloning vector     |
| pgRNA- Cas9-GFP 93                     | pSpCas9 (BB)-2A-GFP (PX458) |
| pcDNA/DEST/mCherry-RAD52               | pcDNA/DEST/mCherry          |
| pcDNA/FRT/RAD51                        | pcDNA/FRT                   |
| pcDNA3.1/GFP-LaminB1                   | pcDNA3.1                    |
| pcDNA5/FRT/TO/RAD51                    | pcDNA5/FRT/TO               |
| pcDNA5/FRT/TO/RAD51 S14A               | pcDNA5/FRT/TO               |
| pcDNA5/FRT/TO/RAD51 S14D               | pcDNA5/FRT/TO               |
| pcDNA5/FRT/RAD51 T13T_S14S_V15V (55.2) | pcDNA5/FRT/TO               |
| pcDNA5/FRT/RAD51 S14A_V15V (42.2)      | pcDNA5/FRT/TO               |
| pOG44                                  | -                           |

**Table 2.7 – Buffers and other solutions used**

| <b>Name</b>                               | <b>Composition</b>                                                                                                                                                                                        |
|-------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1x Phosphate Buffered Saline (PBS) pH 7.4 | 136.9 mM NaCl, 2.7 mM KCl, 17.2 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.76 mM KH <sub>2</sub> PO <sub>4</sub>                                                                                             |
| NETN150 lysis buffer                      | 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8, 0.5% NP40, 1x Sigma protease inhibitors, 10 mM Benzamide Hydrochloride, 20 mM NaF, 1 mM Na <sub>3</sub> VO <sub>4</sub> , 20 mM Na-β-Glycerophosphate |
| 1x MOPS running buffer                    | 0.2 mM MOPS, 0.2 mM Tris, 0.1% SDS, 4 mM EDTA                                                                                                                                                             |
| 1x Transfer buffer                        | 100 mM Tris, 767 mM Glycine                                                                                                                                                                               |
| Ponceau staining solution                 | 0.1% Ponceau in 5% acetic acid                                                                                                                                                                            |
| Coupling buffer (pH 9)                    | 22 mM Boric Acid, 22 mM Sodium tetraborate                                                                                                                                                                |
| DIM-PIM buffer (pH 8.5-9)                 | 0.063 g Dimethyl pimelimidate dihydrochloride in 0.1M Sodium tetraborate                                                                                                                                  |
| Genomic DNA extraction buffer             | 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8, 25 mM NaCl, 200 µg/ml Proteinase K                                                                                                                                 |
| TAE buffer                                | 1.25 mM EDTA, 40 mM Tris, 5.7% acetic acid                                                                                                                                                                |

**Table 2.7 (continued) – Buffers and other solutions used**

| <b>Name</b>                 | <b>Composition</b>                                                                                |
|-----------------------------|---------------------------------------------------------------------------------------------------|
| PTEMF buffer                | 20 mM PIPES (pH 6.8), 10 mM EGTA, 0.2% Triton X-100, 1 mM MgCl <sub>2</sub> , 4% paraformaldehyde |
| Coomassie staining solution | 0.1 % Brilliant blue R-250, 7% acetic acid, 50% Methanol                                          |

**Table 2.8 – Primary antibodies used**

| <b>Antibody</b>              | <b>Supplier</b>                 | <b>Purpose</b>     | <b>Dilution</b> |
|------------------------------|---------------------------------|--------------------|-----------------|
| anti-RAD51<br>(7946)         | Biogenes                        | Western blotting   | 1:5000          |
|                              |                                 | Immunofluorescence | 1:1000          |
| anti-pS14                    | Biogenes                        | Western blotting   | 1:1000          |
| anti-Lamin A                 | Sigma Aldrich (L1293)           | Western blotting   | 1:10 000        |
| anti-Histone H3              | Bethyl Laboratories (A300-822A) | Western blotting   | 1:1000          |
| anti-RAD52                   | Santa Cruz (sc-365341)          | Western blotting   | 1:200           |
| anti-PLK1                    | Bethyl Laboratories (A300-251A) | Western blotting   | 1:1000          |
| anti-Nbs1                    | BD Biosciences (34/NBS1)        | Western blotting   | 1:1000          |
| anti-BRCA2                   | Calbiochem (OP95)               | Western blotting   | 1:1000          |
| anti-γH2AX<br>(clone JBW301) | Merck Millipore                 | Western blotting   | 1:1000          |
|                              |                                 | Immunofluorescence | 1:1000          |
| anti-mCherry                 | GeneTex (GTX59788)              | Immunofluorescence | 1:1000          |
| anti-MUS81                   | Santa Cruz (sc-47692)           | Immunofluorescence | 1:100           |
| anti-RPA                     | Bethyl Laboratories (A300-244A) | Immunofluorescence | 1:5000          |

**Table 2.9 – Secondary antibodies used**

| <b>Antibody</b>      | <b>Supplier</b>                                            | <b>Purpose</b>     | <b>Dilution</b> |
|----------------------|------------------------------------------------------------|--------------------|-----------------|
| Goat anti-rabbit HRP | Dako (PO448)                                               | Western blotting   | 1:2000          |
| Goat anti-mouse HRP  | Dako (PO447)                                               | Western blotting   | 1:1000          |
| Alexa-Fluor 647      | Thermo Scientific (A-21235)<br>Thermo Scientific (A-21244) | Immunofluorescence | 1:500           |
| Alexa-Fluor 555      | Thermo Scientific (A-21422)<br>Thermo Scientific (A-21428) | Immunofluorescence | 1:500           |
| Alexa-Fluor 488      | Thermo Scientific (A-11001)                                | Immunofluorescence | 1:500           |
|                      | Thermo Scientific (A-11008)                                | Flow cytometry     | 1:100           |



**Table 2.10 – List of other materials used**

| <b>Item</b>                                                                               | <b>Supplier</b>                |
|-------------------------------------------------------------------------------------------|--------------------------------|
| Dulbecco's Modified Eagle Medium (DMEM)                                                   | GIBCO                          |
| Dulbecco's Modified Eagle Medium with F-12<br>Nutrient mixture and L-Glutamine (DMEM/F12) | GIBCO                          |
| Iscove's Modified Dulbecco's Medium with<br>L- Glutamine (IMEM)                           | GIBCO                          |
| Heat Inactivated FBS Fetal Bovine Serum<br>(EU approved)                                  | GIBCO                          |
| Trypsin (Tryple Express Enzyme)                                                           | GIBCO                          |
| Penicillin-Streptomycin                                                                   | GIBCO                          |
| Blasticidin                                                                               | Invivogen                      |
| Zeocin                                                                                    | Invivogen                      |
| Puromycin                                                                                 | Invivogen                      |
| G418 disulfate salt                                                                       | Sigma                          |
| JetPrime transfection reagent                                                             | Polyplus-transfection          |
| Lipofectamin LTX with Plus Reagent                                                        | ThermoScientific               |
| Opti-MEM I (1X) Reduced Serum Medium                                                      | GIBCO                          |
| Turbofectin 8.0                                                                           | OriGene                        |
| Lipofectamine RNAiMAX reagent                                                             | ThermoScientific               |
| One Shot™ TOP10                                                                           | ThermoScientific               |
| 10% NP-40 detergent solution                                                              | ThermoScientific               |
| Benzamidine Hydrochloride hydrate                                                         | Sigma Aldrich                  |
| Benzonase Nuclease                                                                        | EMD Millipore                  |
| Protease inhibitor cocktail                                                               | Sigma Aldrich                  |
| 0.2 µm Nitrocellulose membrane<br>(Amersham Protan)                                       | GE Healthcare Life<br>Sciences |
| Dried skimmed milk                                                                        | Marvel                         |
| Tween 20                                                                                  | Sigma Aldrich                  |
| PBS (BioWhittaker)                                                                        | Lonza                          |
| NuPAGE 4-12% Bis-Tris protein gels                                                        | ThermoScientific               |
| BlueEasy Pre-stained protein ladder                                                       | Geneflow                       |
| DNA Loading dye Purple (6x)                                                               | New England Biolabs            |
| NuPAGE LDS sample buffer                                                                  | ThermoScientific               |
| ECL Western Blotting detection reagents                                                   | GE Healthcare                  |

**Table 2.10 (continued)– List of other materials used**

| <b>Item</b>                                | <b>Supplier</b>       |
|--------------------------------------------|-----------------------|
| Bradford protein assay                     | Bio-Rad               |
| DTT                                        | Sigma Aldrich         |
| Ponceau                                    | Sigma Aldrich         |
| Re-Blot Plus Mild Solution                 | EMD Millipore         |
| Bovine Serum Albumine                      | Sigma Aldrich         |
| Affiprep Protein-A beads                   | Bio-Rad               |
| BamBanker Direct                           | NIPPON Genetic EUROPE |
| Proteinase K                               | Sigma Aldrich         |
| SOB broth                                  | Fisher BioReagents    |
| LB agar                                    | Merck                 |
| LB broth                                   | Merck                 |
| Ampicillin                                 | Melford               |
| GeneJET Plasmid Miniprep Kit               | ThermoScientific      |
| GeneJET genomic DNA purification kit       | ThermoScientific      |
| PCRBio HIFI Polymerase                     | PCR Biosystems        |
| PCRBio HIFI buffer                         | PCR Biosystems        |
| SensiFAST SYBR No-ROX kit.                 | Bioline               |
| PfuTurbo polymerase                        | Agilent               |
| PfuTurbo buffer with MgSO <sub>4</sub>     | Agilent               |
| DpnI                                       | New England Biolabs   |
| Ethidium bromide                           | Sigma Aldrich         |
| Ultra Pure Agarose                         | Invitrogen            |
| Qiaquick PCR purification kit              | Qiagen                |
| CutSMART buffer                            | New England Biolabs   |
| BtgI                                       | New England Biolabs   |
| Sall                                       | New England Biolabs   |
| BamHI                                      | New England Biolabs   |
| Calf Intestinal Alkaline Phosphatase (CIP) | New England Biolabs   |
| Qiaquick Gel extraction Kit 50             | Qiagen                |
| DNA Ligation kit, Mighty Mix               | Takara                |
| Triton X-100 (10%)                         | Sigma Aldrich         |
| Thymidine                                  | Sigma Aldrich         |

|             |            |
|-------------|------------|
| Aphidicolin | Santa Cruz |
|-------------|------------|

**Table 2.10 (continued) – List of other materials used**

| Item                                     | Supplier            |
|------------------------------------------|---------------------|
| RO3306                                   | Axon Medchem        |
| Poly-L-lysine                            | Sigma Aldrich       |
| ProLong Gold antifade reagent            | Life Technologies   |
| Nocodazole                               | Calbiochem          |
| 5-ethynyl-2'-deoxyuridine (EdU)          | Life Technologies   |
| WST-1 Cell Proliferation Reagent         | Roche               |
| DMSO                                     | Sigma Aldrich       |
| DNA ladders (100 bp; 1 kB)               | New England Biolabs |
| RNAse A                                  | Sigma Aldrich       |
| Propidium iodide                         | Sigma Aldrich       |
| B02 (Rad51 inhibitor)                    | Calbiochem          |
| MK1775 (Wee1 inhibitor)                  | Selleckchem         |
| AZ3146 (MPS1 inhibitor)                  | Santa Cruz          |
| AICAR (RAD52 inhibitor)                  | Sigma Aldrich       |
| MISSION siRNA universal negative control | Sigma Aldrich       |

## **2. Methods**

### **2.1 Bacterial techniques**

#### **2.1.1 Transformation**

For amplification of plasmid DNA, chemically competent Oneshot™ TOP10 *E. coli* cells were transformed by heat shock. 50 ng of plasmid DNA was gently added to 50 µl of thawed bacterial stock and incubated for 30 minutes on ice. The mixture was subsequently incubated for 30 seconds at 42 °C and returned on ice for 2 min. 500 µl of pre-warmed SOB medium supplemented with 20 mM glucose was added to the heat-shocked bacteria and incubated for 1 h at 37 °C with shaking (225 rpm). Transformed cells were next plated on LB agar plates containing the appropriate antibiotic for selection and grown overnight at 37 °C. Ampicillin was used at 100 µg/ml.

#### **2.1.2 Growth and storage of bacterial strains**

Bacterial cultures were started from a small aliquot of bacteria picked either from LB agar plates or from frozen glycerol stock. Bacteria were grown up overnight at 37 °C in 5 ml of LB broth containing the appropriate antibiotic. Ampicillin was used at 100 µg/ml. For storage, 500 µl of the overnight-grown culture was added to 500 µl of 50% glycerol and frozen at -80 °C.

#### **2.1.3 Plasmid DNA preparation**

For small scale preparations of plasmid DNA (5 ml overnight cultures), GeneJet Plasmid Miniprep Kit was used following manufacturer's instructions.

## 2.2 DNA analysis techniques

### 2.2.1 Quantification of nucleic acids

The concentration of DNA nucleic acids was quantified from the absorbance at 260 nm by Nanodrop 1000 (Thermo Scientific).

### 2.2.2 Isolating mammalian genomic DNA

A GeneJET genomic DNA purification kit (Thermo Scientific) was used following manufacturer's instructions.

#### *From low cell numbers*

When working with many different mammalian cell lines in parallel laborious cell culture was avoided by extracting genomic DNA from low cell numbers. Genomic DNA was extracted from a confluent 96-well cell culture by scraping and resuspending adherent cells in 50  $\mu$ l Genomic DNA extraction buffer. Resuspended samples were subsequently incubated for 30 min at 65 °C, followed by a 2 min incubation at 95 °C. The resultant DNA extract was used as genomic DNA template in subsequent PCR strategies (2.2.3).

### 2.2.3 Polymerase chain reaction (PCR)

During this study the polymerase chain reaction (PCR) was used (i) to amplify DNA for sequencing or cloning; (ii) to screen CRISPR clones for the presence of a mutation (see 2.2.6 and 2.2.7 for more details) or (iii) for site-directed mutagenesis of plasmid DNA (2.2.3c). For each PCR primer set used during this study, table 2.5 lists the purpose of the PCR. To assess the success of a PCR reaction, 1/10<sup>th</sup> of the reaction volume was resolved on a 1% agarose gel (as described in 2.2.4).

#### 2.2.3a PCR from genomic DNA template

DNA regions of interest were amplified from genomic DNA template using PCRBIO HiFi Polymerase or SensiFAST SYBR No-ROX kit as described below. To amplify the CRISPR-targeted DNA locus, primers flanking the mutation site were used (listed in table 2.5 as 'HRM forward' and 'HRM reverse').

### *Using PCRBIO HiFi polymerase*

PCR was performed using 400 ng of genomic DNA template in a 50  $\mu$ l reaction volume, containing 10  $\mu$ M of the corresponding forward and reverse primers and 1U PCRBIO HiFi Polymerase in 1x PCRBIO HiFi buffer. PCR was performed using a T100 Thermal Cycler (BioRad), using the PCR programme summarized in table 2.11.

**Table 2.11 – PCR programme used with PCRBIO HiFi Polymerase**

| Step                    | Temperature (°C)                         | Duration                             |
|-------------------------|------------------------------------------|--------------------------------------|
| 1) Initial denaturation | 95                                       | 3 min                                |
| 2) Denaturation         | 95                                       | 30 s                                 |
| 3) Primer annealing     | Varies per primer set<br>(between 60-65) | 30 s                                 |
| 4) Extension            | 72                                       | 30 s<br><b>Repeat steps 2-4, 35x</b> |
| 5) Final extension      | 72                                       | 5 min                                |

### *Using SensiFAST SYBR No-ROX*

When encountering difficulties in amplifying DNA from certain template DNA samples, PCR was alternatively performed using SensiFAST SYBR No-ROX.\* PCR was performed using 100 ng of genomic DNA in a 10  $\mu$ l reaction volume, containing 10  $\mu$ M of the corresponding forward and reverse primers in 1x SensiFAST SYBR No-ROX mix. PCR was performed in a RotorGene Q (Qiagen), using the PCR programme summarized in table 2.12.

**Table 2.12 – PCR programme used with SensiFAST SYBR No-ROX mix**

| Step                       | Temperature (°C)                         | Duration                             |
|----------------------------|------------------------------------------|--------------------------------------|
| Initial denaturation       | 95                                       | 2 min                                |
| Denaturation               | 95                                       | 5 s                                  |
| Primer annealing/extension | Varies per primer set<br>(between 60-65) | 30 s<br><b>repeat steps 2-3, 40x</b> |

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\* Although this reagent allows real-time quantitative PCR (qPCR), the sole aim here was to successfully obtain the PCR product - no quantification of the amplified DNA was performed.)

### 2.2.3b Colony PCR

Instead of using genomic DNA template, PCR was performed as described in 2.2.3a using a small amount of bacterial colony picked from a LB-agar plate as template. This method allows for the screening of DNA plasmids from bacterial colonies without requiring prior plasmid DNA preparation.

### 2.2.3c Site-directed mutagenesis

Site-directed mutagenesis was used to insertion of a desired mutation into plasmid DNA, following a protocol based on the QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies). PCR was performed in a 50  $\mu$ l reaction containing 400 ng of the corresponding forward and reverse primers, 100 ng of the plasmid DNA template (pcDNA/FRT/RAD51), 0.5 mM dNTPs, 3.75 U PfuTurbo DNA polymerase in 1x PfuTurbo reaction buffer with MgSO<sub>4</sub>. Table 2.5 lists the primer sets used. PCR was performed in a T100 Thermal Cycler (BioRad), using the PCR programme summarized in table 2.13. The duration of the extension step required depends on the size of the template plasmid and was determined assuming extension occurs at a rate of 0.5 kb/min. After PCR-mediated amplification, 20 U of Dpn I restriction enzyme was added directly to the reaction mixture and incubated for 2 h at 37 °C to digest the parental (non-mutated) DNA. The Dpn I-treated sample was subsequently transformed into Oneshot™ TOP10 *E. coli* cells and plated on LB-agar plates (as described in 2.1.1). Colonies were picked, grown and plasmid DNA extracted (as described in 2.1.2; 2.1.3). Plasmid DNA was screened for the desired mutation by sequencing (as described in 2.2.9)

**Table 2.13 – PCR programme used for mutagenesis**

| Step                    | Temperature (°C)                         | Duration                               |
|-------------------------|------------------------------------------|----------------------------------------|
| 1) Initial denaturation | 95                                       | 3 min                                  |
| 2) Denaturation         | 95                                       | 30 s                                   |
| 3) Primer annealing     | Varies per primer set<br>(between 60-65) | 1 min                                  |
| 4) Extension            | 68                                       | 12 min<br><b>Repeat steps 2-4, 35x</b> |

#### 2.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to screen for the presence and size of expected DNA products. DNA samples were prepared in 1x DNA loading dye and resolved at 100V in 1-2% agarose gels containing 1x TAE buffer and 0.25 µg/ml Ethidium bromide. 100 bp and 1 kb DNA ladders were used to estimate the size of DNA products. Ethidium bromide-bound DNA was visualized under UV light.

#### 2.2.5 Sanger sequencing

Unless stated otherwise, all DNA sequencing of DNA plasmids or PCR products was performed by Source BioScience UK Ltd, in compliance with corresponding sample requirements.

#### 2.2.6 Screening of CRISPR-targeted locus by PCR

PCR was performed from genomic DNA as described in 2.2.3a using primer pairs that are perfectly complementary to a specific RAD51 T13S14 variants (listed in table 2.5 as 'WT forward' and 'WT reverse'; 'S14D forward' and 'S14D reverse'; 'S14A forward' and 'S14A reverse' or 'T13AS14A forward' and 'T13AS14A reverse').

#### 2.2.7 Allele-separation through cloning

##### 2.2.7a Insert preparation

PCR was performed from genomic DNA using primers that amplify the CRISPR-targeted DNA locus and contain either a *SalI* or *BamHI* restriction site (listed in table 2.5 as 'sticky-HRM forward' and 'sticky-HRM reverse'). The resultant PCR product was purified using the Qiaquick PCR purification kit and approximately 500 ng was subsequently digested for 1 h at 37 °C in a 50 µl reaction volume containing 20 U *BamHI* and 100 U *SalI* in 1x CutSMART Buffer. The *SalI*- *BamHI*- digested PCR product was again purified using a Qiaquick PCR purification kit and used for subsequent ligation (2.2.8c).

##### 2.2.7b Vector preparation

Approximately 1 µg of pBluescriptKS plasmid was digested for 75 min at 37 °C in a 50 µl reaction volume containing 40 U *BamHI*; 200 U *SalI*; 4 U Calf Intestinal Alkaline



Phosphatase (CIP) and 5  $\mu$ g BSA in 1x CutSMART Buffer. The *SalI*- *BamHI*- digested vector was subsequently resolved on a 1% agarose gel to allow for the extraction of the major linearized fragment using a Qiaquick Gel extraction kit according to manufacturer's instructions. The purified, linearized vector fragment was subsequently used for ligation (2.2.8c).

### 2.2.7c Ligation

Ligation was performed with 1:3 molar ratio of vector to insert. 50 ng of *SalI*- *BamHI*-linearized pBluescriptKS (2.2.8b) and 12 ng of *SalI*- *BamHI*- digested insert (2.2.8a) were ligated overnight at 16 °C in a 10  $\mu$ l reaction volume of 1x DNA ligation mix (Takara). The ligated mixture was subsequently transformed into OneShot TOP10 *E. coli* (2.1.1), after which the CRISPR-targeted DNA locus was amplified from the cloned construct by colony PCR (2.2.3b) using primers listed in table 2.5.

### 2.2.8 Next-generation sequencing (NGS)

Next-generation sequencing was used to conclusively confirm the sequence at the CRISPR-targeted locus at every allele for heterozygous clones. PCR was performed from genomic DNA as described in 2.3.3a using primers that amplify the CRISPR-targeted DNA locus (listed in table 2.5 as 'HRM forward' and 'HRM reverse'). The resultant PCR product was purified using a Qiaquick PCR purification kit and served as the DNA template in a subsequent PCR reaction using 'MiSeq' primers listed in table 2.5. The resultant PCR product was purified using Qiaquick PCR purification kit and sequenced by our collaborator (A. Bassett) at the Wellcome Trust Sanger Institute, using an Illumina MiSeq Next Generation Sequencer.

## 2.3 Protein analysis techniques

### 2.3.1 Cell lysate preparation and Western Blotting

#### 2.3.1a Whole cell lysate preparation

Cells were collected by trypsinization, washed with ice-cold in PBS and incubated on ice in NETN150 buffer supplemented with 5 mM MgCl<sub>2</sub> and 125 U/ml Benzonase for at least 30 min. Cell lysate was separated from cell debris by centrifugation (16000 g, 30 min, 4 °C) and collected. Protein concentration was determined by the Bradford protein assay (Bio-Rad), according to manufacturer's standard procedure. Samples were prepared for polyacrylamide gel electrophoresis (PAGE) in 1x NuPAGE LDS sample buffer and 25 mM DTT.

#### 2.3.1b Western blotting

Subsequent protein separation was performed in 1x MOPS running buffer using NuPAGE 4-12% Bis-Tris gels at 100-120V. Electrophoretic protein transfer onto membrane was performed in 10% methanol Transfer buffer using Mini Trans-Blot system, at 70-90V. Transfer efficiency was assessed by staining the membrane in Ponceau staining solution. De-stained membranes were subsequently blocked in PBS with 5% Milk and 0.05% Tween for at least 30 minutes. Primary antibodies were applied at the corresponding dilutions (listed in table 2.8) for 2-3 hours or overnight. The membrane was washed in PBS with 0.05% Tween (3x 5 min) and secondary antibodies (listed in table 2.9) were applied for 1 hour. Finally, after washing the blotted membrane in PBS with 0.05% Tween, immune complexes were detected by chemiluminescence using ECL Western Blotting detection reagents according to manufacturer's protocol. Where indicated, the membrane was treated with Re-Blot Plus Mild Solution according to manufacturer's instructions to allow for re-blotting with another antibody.

### 2.3.2 Protein enrichment by immunoprecipitation

#### 2.3.2a Antibody crosslinking to Affiprep Protein beads

Affiprep Protein-A beads were crosslinked to the appropriate antibody for subsequent use in immunoprecipitation. 20  $\mu$ l of beads were incubated with 1  $\mu$ l of the appropriate

antibody in 1 ml PBS and rotated for 2-3 hours at room temperature. The beads were successively washed in 1 ml PBS (3x, 5 min); 1 ml coupling buffer (2x, 5 min) and 1 ml DIM-PIM buffer (1x, 5 min) before being incubated in 1 ml DIM-PIM for at least 6 hours. The beads were subsequently washed in 1 ml of 1M Tris-HCl buffer (pH 9.0) (1x, 10 min). Prior to immunoprecipitation, the antibody-crosslinked beads were extensively washed in NETN150 buffer and blocked in NETN150 buffer with 5 mg/ml BSA for at least 1h at 4 °C. Unless stated otherwise, all incubation and wash steps were performed using a tube rotator at 4 °C.

### 2.3.2b Immunoprecipitation

Starting from an 80% confluent 10-cm dish, cell lysate was prepared as described in 2.3.1a. The obtained cell lysates were diluted to 1 mg/ml in NETN150 buffer with 5 mg/ml BSA. To pre-clear the cell lysate, the extract was incubated with uncrosslinked Affiprep Protein-A beads for at least 1 hour at 4 °C. For subsequent immunoprecipitation, the pre-cleared lysate was collected by centrifugation (3000 rpm, 3 min) and incubated with the antibody-crosslinked Affiprep Protein-A beads overnight at 4 °C. After extensive washing of the beads in NETN150 buffer, the cross-linked immune complexes were eluted from the beads in 4x NuPAGE LDS sample buffer and 12 mM DTT by heating the sample at 85 °C for 5 min. The eluted sample was subsequently used for PAGE and Western blot analysis as described in 2.3.1.

### 2.3.3 Chromatin fractionation

Starting from an 80% confluent 15-cm dish, cells were collected by trypsinization and washed with ice-cold PBS. Approximately 1/8th of the collected cells were prepared as whole cell lysate as described in 2.3.1a. The remaining cells were resuspended in 70  $\mu$ l NETN150 buffer and incubated on ice for 30 min. The supernatant was separated from the pellet by centrifugation (1000 g, 5 min, 4 °C) and collected as the soluble fraction. Next, the remaining chromatin-containing pellet was washed once in 150  $\mu$ l ice-cold NETN150 buffer, and re-pelleted by centrifugation (1000 g, 5 min, 4 °C). The pellet was next resuspended in 20  $\mu$ l NETN150 buffer supplemented with 5 mM MgCl<sub>2</sub> and 125 U/ml Benzonase and incubated on ice for at least 30 min. Both the resultant insoluble fraction and the previously collected soluble fraction were centrifuged (16100

g, 30 min, 4 °C) to pellet any cell debris. Supernatants were collected as the insoluble (chromatin) fraction and soluble fraction respectively. Protein concentration was determined by the Bradford protein assay (Bio-Rad), according to manufacturer's standard procedure. Samples were prepared for polyacrylamide gel electrophoresis (PAGE) in 1x protein NuPAGE LDS sample buffer and 25 mM DTT. Western blotting was performed as described in 2.3.1b.

## 2.4 Mammalian cell culture techniques

### 2.4.1 Cell culture propagation and storage

All cell lines were cultured at 37 °C with 5% CO<sub>2</sub> in the corresponding cell culture medium supplemented with 10% FBS, streptomycin (0.1 mg/ml) and penicillin (100 units/ml) and any additional antibiotics as listed in table 2.1-2.3. Cells were passaged every 3 days (or upon reaching approximately 80% confluency) at 1:10 or 1:20 dilution by trypsinization. For cell line storage, pre-confluent cells were trypsinized and frozen down at -80 °C in 10% DMSO in FBS in a cryo freezing container, unless stated otherwise. Frozen stocks were revived by gradual warming at 37 °C and replacement into complete cell culture medium. Any centrifugation of mammalian cell culture was performed at no more than 5000 g.

### 2.4.2 Ionizing irradiation

Wherever indicated, a GRAVITRON RX 30/55 (Gravatom) was used to irradiate the cells at the stated dose.

### 2.4.3 Transfection

Unless stated otherwise, DNA transfection was performed using jetPRIME transfection reagent according to manufacturer's instruction when cells are approximately 80% confluent. siRNA transfection was performed using Lipofectamine RNAiMAX reagent according to manufacturer's instruction. siRNA oligos used are listed in table 2.4. A 1:1 mix of RAD51 siRNA (1) and (2) was used to knock down RAD51, a 1:1 mix of the corresponding scrambled control siRNA (1) and (2) was used as a negative control of RAD51 knockdown.

#### 2.4.4 Generation of stable cell lines

Stable cell lines generated during this study are listed in table 2. Stable cell lines for constitutive expression of the exogenous gene were generated by co-transfecting the plasmid of interest and pcDNA-DEST26, followed by G418 selection at 400  $\mu\text{g}/\text{ml}$ . Transfected cells were plated at dilutions suitable for single colony growth. Colonies were subsequently picked to establish monoclonal cell lines. In case of stable cell lines expressing fluorescently-tagged proteins (U2OS mCh-RAD52), monoclonal cell lines were established by single-cell sorting of fluorescence-positive cells using MoFlo Astrios (Beckman Coulter).

#### 2.4.5 Generating CRISPR cell lines

CRISPR mutant cell lines generated during this study are listed in table 2.3.

##### 2.4.5a Transfection

Cells were transfected at approximately 80% confluency with the corresponding ssDNA repair-template (table 2.5) and pgRNA-Cas9-GFP 93; a Cas9-, GFP- and gRNA-expressing plasmid (as listed in table 2.6; see table 2.4 for gRNA sequence). U2OS or RPE1 cells were transfected with 3  $\mu\text{g}$  ssDNA repair-template and 3  $\mu\text{g}$  pgRNA-Cas9-GFP 93 using 9  $\mu\text{l}$  Lipofectamine LTX reagent with 3  $\mu\text{l}$  Plus reagent according to manufacturer's instructions. eHAP cells were transfected with 2.5  $\mu\text{g}$  ssDNA repair-template and 2.5  $\mu\text{g}$  pgRNA-Cas9-GFP 93 using 7.5  $\mu\text{l}$  Turbofectin 8.0 transfection reagent according to manufacturer's instructions.

##### 2.4.5b Clonal expansion and storage

24 hours after transfection cells were collected by trypsinization and 960 GFP-positive cells were sorted as single cells into 96-well plates by MoFlo XDP (Beckman Coulter). Plates were incubated for 2-4 weeks, after which growing clones were expanded and screened for the desired mutation by PCR (see 2.2.6) or western blot (2.3.1b). To avoid unmanageable quantities of clones being expanded in parallel, 96-well plates containing growing clones stored at  $-80\text{ }^{\circ}\text{C}$  in Bmbanker Direct freezing medium according to manufacturer's instructions until further expansion and processing could be

accommodated. Briefly, cells were trypsinized in the 96-well plate and suspended in equal volumes of Bambanker Direct freezing medium. The 96-well plate was subsequently directly stored at -80 °C, thereby avoiding lengthy centrifugation steps and allowing the freezing of low cell numbers. Cells were revived by thawing at 37 °C and re-seeding in 96-well plate in complete medium. As soon as cells attach, complete medium was replenished to remove Bambanker Direct freezing medium.

## 2.4.6 Cell synchronization

### 2.4.6a Double thymidine block-and-release

Thymidine was added to the cell culture at a final concentration of 2 mM. Cells were incubated in the presence of thymidine for 18 hours and subsequently released from the thymidine block by gently washing the dish 2x in PBS. Next, cells were incubated in complete medium for 9 hours, after which thymidine was again added to a final concentration of 2 mM. Cells were incubated in the presence of thymidine for 18 hours and subsequently released from the second thymidine block by gently washing the dish 2x in PBS.

### 2.4.6b Cell synchronization for immunofluorescence experiments

#### *Cell synchronization in Mitosis (without aphidicolin)*

Starting from  $3 \times 10^5$  cells seeded in a 10-cm dish, cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were incubated in complete medium for 8 hours, after which RO3306 was added at a final concentration of 9  $\mu$ M. Cells were incubated for a further 8 hours in the presence of RO3306 and subsequently released from the RO3306-induced G2 block by washing 2x in PBS. Cells were incubated 30 minutes in complete medium containing 10  $\mu$ M EdU. Mitotic cells were subsequently collected by mitotic shake off. The collected cells were resuspended in PBS and seeded onto a poly-L-lysine treated coverslips and incubated for 5 min at 4 °C to allow time for cell attachment to the surface. Fixation and sample preparation was subsequently carried out as described in 2.5.1.

### *Cell synchronization in Mitosis (with aphidicolin)*

Starting from  $1 \times 10^5$  cells seeded in a 6-well plate, or  $3 \times 10^5$  cells seeded in a 10-cm dish, cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were returned to complete medium. After 1 hour, aphidicolin was added to the medium at a final concentration of 0.4  $\mu\text{M}$ . After 18 hours, RO3306 was added at a final concentration of 9  $\mu\text{M}$ . Cells were incubated an additional 5 h (such that cells were incubated a total of 23 hours in presence of aphidicolin) before being released from the RO3306-induced G2 block by gently washing the dish 2x in PBS medium. Cells were incubated 30 minutes in complete medium containing 10  $\mu\text{M}$  EdU. Mitotic cells were subsequently collected by mitotic shake off. The collected cells were resuspended in PBS and seeded onto a poly-L-lysine treated coverslips and incubated for 5 min at 4 °C to allow time for cell attachment to the surface. Fixation and sample preparation was subsequently carried out as described in 2.5.1.

### 2.4.6c Cell synchronization for chromatin fractionation experiments

For all chromatin fractionation experiments,  $0.75 \times 10^6$  cells were seeded on a 15-cm dish one day prior to synchronization.

### *Cell synchronization in S phase (with aphidicolin)*

Cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were incubated in complete medium for 30 min, after which aphidicolin was added at a final concentration of 0.4  $\mu\text{M}$ . Cells were incubated for a further 3.5 hours or 10 hours before collecting the cells for chromatin fractionation as described in 2.5.3.

### *Cell synchronization in G2 phase (with aphidicolin)*

Cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were incubated in complete medium for 30 min, after which aphidicolin was added at a final concentration of 0.4  $\mu\text{M}$ . After 18 hours, RO3306 was added at a final concentration of 9  $\mu\text{M}$ . Cells were incubated an

additional 5 hours (such that cells were incubated a total of 23 hours in presence of aphidicolin) before collecting the cells for chromatin fractionation as described in 2.5.3.

#### *Cell synchronization in M phase (with aphidicolin)*

Cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were incubated in complete medium for 30 min, after which aphidicolin was added at a final concentration of 0.4  $\mu$ M. After 18 hours, RO3306 was added at a final concentration of 9  $\mu$ M. Cells were incubated for a further 5 hours (such that cells were incubated a total of 23 hours in presence of aphidicolin) before being released from the RO3306-induced G2 block by gently washing the dish 2x in PBS medium. Cells were incubated 2 hours in complete medium containing 0.33  $\mu$ M Nocodazole, before collecting the cells for chromatin fractionation as described in 2.5.3.

#### 2.4.6d Cell synchronization for live microscopy experiments

##### *Cell synchronization in Mitosis (without aphidicolin)*

Starting from  $1 \times 10^5$  cells seeded in name of dish, cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were incubated in complete medium for 8 hours, after which RO3306 was added at a final concentration of 9  $\mu$ M. Cells were incubated for a further 12 hours and subsequently released from the RO3306-induced G2 block by washing 2x in PBS. Cells were subsequently analysed in complete medium by live cell microscopy as described in 2.5.5.

##### *Cell synchronization in Mitosis (with aphidicolin)*

Starting from  $1 \times 10^5$  cells seeded in name of dish, cells were synchronized in early S by a double-Thymidine block-and-release (2.4.6a). Upon release of the second Thymidine block, cells were incubated in complete medium for 30 minutes, after which aphidicolin was added at a final concentration of 0.4  $\mu$ M. After 18 hours, RO3306 was added at a final concentration of 9  $\mu$ M. Cells were incubated an additional 12 h (such that cells were incubated a total of 30 hours in presence of aphidicolin) before being released from the RO3306-induced G2 block by gently washing the dish 2x in PBS



medium. Cells were subsequently analysed in complete medium by live cell microscopy as described in 2.5.5.

## 2.5 Cell analysis techniques

### 2.5.1 Immunofluorescence microscopy

Cells to be analyzed were grown or seeded on coverslips as described in 2.4.6b and subsequently fixed and permeabilized in PTEMF buffer for 20 minutes. Coverslips were washed in 1x PBS and blocked for 1 hour in PBS containing 3% BSA and 0.5% Triton X-100. Primary antibodies were applied at the dilutions listed in table 2.8 and incubated for 2-3 hours at room temperature. Coverslips were washed in PBS containing 0.5% Triton X-100 (3x 5 min). Fluorophore-conjugated secondary antibodies were applied at the dilutions listed in table 2.9 and incubated for 45 min (in the dark) at room temperature. Coverslips were subsequently washed in PBS containing 0.5% Triton X-100 (3x 5 min) and mounted with ProLong Gold antifade reagent on glass slides. Images were captured using an Olympus FV1000 Laser Scanning Microscope with Becker and Hickel FLIM system and Olympus imaging software.

### 2.5.2 Click-iT fluorescent-labeling

To visualize the incorporation of nucleotide analog EdU in the DNA, the ‘Click-iT’ reaction was performed to fluorescently label incorporated EdU. Cells that were fixed, permeabilized and blocked (as described in 2.5.1 or 2.5.4) were next incubated for 1 hour (in the dark) in PBS containing 2 mM CuSO<sub>4</sub>, 10 mM sodium ascorbate and 10 uM Alexa Fluor 555 or Alexa Fluor 647 Azide.

### 2.5.3 Proximity ligase assay (PLA)

The proximity ligase assay was performed according to the Duolink PLA Fluorescence Protocol (Sigma-Aldrich). Primary antibody incubation was performed as described in 2.5.1.

### 2.5.4 Flow cytometry

#### 2.5.4a Analysing cell cycle distribution by propidium iodide staining

Starting from approximately 10<sup>6</sup> cells per sample, cells were collected by trypsinization and fixed in 70% ethanol. Fixed cells were stained for at least 30 min in PBS with 0.1% BSA, 0.98 mg/ml RNase A and 19.6 µg/ml propidium iodide. Cells were collected by

centrifugation (1000g, 5 min) and resuspended in PBS. Cell cycle distribution was analyzed using a FACSCalibur (Becton Dickinson); software name and FlowJo analysis software. Samples were gated on the single-cell population as determined from fluorescence area vs. width (to exclude doublets) and/or FSC vs. SSC (to exclude debris).

#### 2.5.4b Analysing mitotic index

Cells were collected and fixed as described in 2.5.4a. After centrifugation (1000g, 5 min, 4 °C), cell were resuspended in PBS containing 0.1% Triton X-100 and 1% BSA and incubated for 15 min. Next, the permeabilized cells were incubated for 1 hour with pH3S10 antibody (see table 2.8) in PBS containing 0.1% Tween-20 and 1% BSA. The sample was washed in PBS containing 0.1% Tween 20 and incubated for 30 min with the corresponding secondary antibody (see table 2.9). Propidium iodide staining and subsequent analysis were performed as described in 2.5.4a.

#### 2.5.4c Analysing EdU incorporation

Cells were pulse labeled for 30 min with 10  $\mu$ M EdU before being collected, fixed and permeabilized as described in 2.5.4b. The permeabilized cells were incubated for 1 hour in 100  $\mu$ l of Click-iT reaction described in 2.5.2. Propidium iodide staining and subsequent analysis were performed as described in 2.5.4a.

#### 2.5.5 Analysing mitotic progression by live microscopy

Cells were synchronized as described in 2.4.6d. Upon release from the G2 block, live cell images were captured using a Zeiss 880 Airyscan inverted confocal microscope and name of software. Live cell images were captured every 4-10 minutes for at least 4 hours.

## 2.6 Cell survival assays

### 2.6.1 WST1-assay

Cells were seeded in a 96-well plate at a density of 5000 per well. Once adhered to the plate, cells were incubated with drug-containing medium (prepared by serial dilution) at the indicated concentrations and replenished after 48 hours. After an additional 72 hours of incubation, cell survival was assessed using the WST-1 reagent (Roche), according to manufacturer's instructions. Cells were incubated with medium containing WST-1 reagent (1:10 dilution) for 30 min. Absorbance was measured at 450 nm (650 nm was used as reference wavelength) using the name of microplate reader. Percentage survival was calculated relative to the DMSO-treated control.

### 2.6.2 Clonogenic assay

Cells were seeded in a 6-well plate at a density of 40 cells (untreated samples) or 100 cells (DMSO-treated control samples) per well. Once adhered, the corresponding drug was added to cells at the indicated dose and incubated for 8 days. Cells were then fixed and stained in Coomassie staining solution and colonies of  $> 50$  cells were counted. Percentage survival was calculated relative to the DMSO-treated control.

## 2.7 Data analysis techniques

Unless stated otherwise, foci counting was performed using FIJI (Image J version 2.0.0-rc-65/1.52a) and the GDSC (FindFoci) ImageJ plugin.<sup>1</sup> Prior to foci counting, all images were processed in the same way with Image J ‘Subtract Background’ and ‘Despeckle’ functionalities to reduce noise or uneven background signal. The parameters used for the FindFoci algorithm were optimized for a representative sample of each experimental condition and applied in bulk using the FindFoci Batch plugin.

All statistical analysis was performed using GraphPad PRISM 7 For Mac OS X. Unpaired two-tailed Student’s t test were performed and, where significant differences were found, P-values are depicted according to the GraphPad Prism convention:

P-value  $\leq$  (or equal) 0.05 = \*

P-value  $\leq$  (or equal) 0.01 = \*\*

P-value  $\leq$  (or equal) 0.001 = \*\*\*

P-value  $\leq$  (or equal) 0.0001 = \*\*\*\*

## 3. References

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<sup>1</sup> Herbert, Alex D., Antony M. Carr, and Eva Hoffmann. "FindFoci: a focus detection algorithm with automated parameter training that closely matches human assignments, reduces human inconsistencies and increases speed of analysis." *PloS One* 9.12 (2014): e114749.

## **Chapter 3**

### **CRISPR/Cas9-generation of RAD51 phospho-mutants**



## Summary

CRISPR/Cas9-mediated gene editing was performed to generate non-phosphorylatable RAD51 mutants (S14A or T13A-S14A) and phospho-mimetic RAD51 mutants (S14D) in the U2OS osteosarcoma cell line; the hTERT-immortalized RPE1 cell line and the engineered HAPloid (eHAP) myeloid leukemia cell line. The design, screening and verification of RAD51 CRISPR/Cas9-mediated gene editing is described in this chapter. The importance of RAD51 T13/S14 phosphorylation in cell proliferation is suggested by the relative ease in which the phospho-mimetic mutants were obtained compared to the phospho-null mutants. The U2OS RAD51 S14A mutant appears highly transformed and has likely adapted to the loss of RAD51 T13/S14 phosphorylation by obtaining several secondary mutations.



### 3.1 Introduction

PLK1-dependent RAD51 T13/S14 phosphorylation has been extensively studied in the context of DNA damage and replication stress.<sup>1,2</sup> However, the role of RAD51 T13/S14 phosphorylation has not yet been properly addressed during an unperturbed cell cycle. Given that RAD51 is essential in higher eukaryotes and that the PLK1-dependent phosphorylation of RAD51 contributes to its recruitment at DNA, we hypothesize that RAD51 phosphorylation may also be important for cell proliferation. The function of phosphorylated RAD51 has so far been analysed in clonal stable cell lines exogenously expressing either the non-phosphorylatable RAD51 S14A mutant, or the phospho-mimetic RAD51 S14D mutant. Yata et al.<sup>1</sup> found no significant difference in colony formation between these mutant cell lines under unperturbed conditions - even upon siRNA-mediated depletion of the endogenous wild-type RAD51 - suggesting that this cellular system is not affected by the loss of RAD51 phosphorylation during normal proliferation. However, it is possible that the loss of RAD51 T13/S14 phosphorylation in this system is masked by the constitutive expression of the RAD51 mutant from an exogenous promoter, which is unlikely to approximate the endogenous expression of RAD51. Additionally, incomplete siRNA-mediated knock-down of the endogenous RAD51 gene may further conceal the importance of RAD51 phosphorylation.

I therefore set out to generate CRISPR/Cas9-mediated ‘knock-in’ RAD51 phospho-mutants, such that the function of T13/S14 phosphorylation can be analysed at the level of endogenous RAD51 expression. The importance of RAD51 T13/S14 phosphorylation may additionally differ in cancer cells compared to primary cells. For this reason, CRISPR/Cas9-gene editing was performed both in U2OS osteosarcoma cells and in hTERT-immortalized RPE1 cells, which maintain the physiology of a primary cell line. The engineered HAPloid (eHAP) myeloid leukemia cell line was also included, as gene-editing is facilitated by its haploid karyotype.

## 3.2 Approach

### 3.2.1 CRISPR/Cas9-mediated gene editing

The type II CRISPR system was first identified as a bacterial adaptive immune system in *Streptococcus pyogenes*.<sup>3</sup> Since then it has been modified into a revolutionary gene editing technology, which allows for the induction of a DSB at any target sequence in genome.<sup>4,5</sup> The Cas9 nuclease forms a complex with a ‘guide RNA’ (gRNA), which, through base pairing with a complementary DNA sequence, directs the nuclease to cleave at a specific genomic target site. Cas9 activity is further dictated by the presence of a 5’ PAM sequence (5’-NGG), as it will cleave 3 nucleotides upstream of the PAM site.<sup>6</sup> The Cas9-induced DSB will either be repaired by NHEJ – potentially introducing short insertions or deletions at the break site – or by HR. Mutagenic NHEJ-mediated repair of the break site may lead to a frameshift mutation and premature termination codon (PTC), such that the gene transcript will be targeted for nonsense-mediated mRNA decay (NMD). In this way, a ‘knock-out’ mutant is generated. Alternatively, by providing a homologous DNA repair template containing a desired mutation, HR-mediated repair can be exploited to generate a ‘knock-in’ mutant.

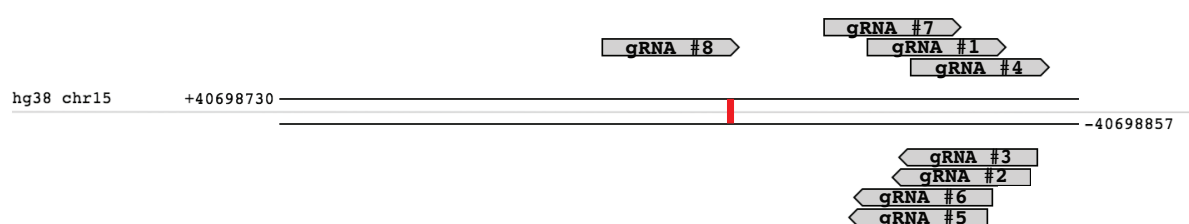
### 3.2.2 Design and selection of CRISPR guide RNA

The CRISPR gRNA used to direct Cas9 to the RAD51 T13/S14 locus was designed using the CRISPR design tool (V1) (<http://crispr.mit.edu>). In the genomic region of interest, the tool computes all possible CRISPR guides (i.e. 20 nucleotides followed by a PAM sequence). Each CRISPR guide is additionally scanned across the target genome to identify any potential off-target matches. For each identified off-target hit, an off-target hit score is calculated which takes into account the number of mismatches between the gRNA and the off-target DNA sequence, as well as the position of these mismatches (cleavage is less likely to take place if the gRNA-DNA mismatch is found close to the PAM site, or if the mismatches are closely distributed). The weighted sum of all off-target hit scores is finally used to calculate a quality score for each gRNA. Quality scores above 50 are deemed suitable for CRISPR-targeted gene editing, provided that none of the high-scoring off-targets fall within genic regions.

Table 3.1 lists the potential guide RNAs in the vicinity of the RAD51 T13/S14 locus with an acceptable quality score (>50). Their position and orientation are shown in Figure 3.1.

**Table 3.1 – List of gRNA candidates**

| Guide # | Quality score | Sequence (5'-3')        | Off-target sites |
|---------|---------------|-------------------------|------------------|
| #1      | 90            | ACAACCCATTTACGGTTAGAGG  | 71 (0 in genes)  |
| #2      | 88            | CATACCTCTAACCGTGAAATGGG | 89 (0 in genes)  |
| #3      | 87            | ACATACCTCTAACCGTGAAATGG | 90 (0 in genes)  |
| #4      | 84            | ATTTACGGTTAGAGGTATGTGG  | 91 (0 in genes)  |
| #5      | 80            | CTAACCGTGAAATGGGTTGTGGG | 70 (0 in genes)  |
| #6      | 79            | TCTAACCGTGAAATGGGTTGTGG | 80 (0 in genes)  |
| #7      | 68            | TTGGCCCACAACCCATTTACCGG | 198 (0 in genes) |
| #8      | 55            | AGCAAATGCAGATACTTCAGTGG | 426 (0 in genes) |



**Figure 3.1 - Position and orientation of gRNA candidates relative to desired mutation.** The position of the mutation site is indicated in red; the arrow head indicates the PAM site within each gRNA. Modified from <http://crispr.mit.edu>.

While the gRNA quality score gives an indication of its specificity for the desired target site, it cannot predict the ultimate activity of the corresponding gRNA-Cas9 complex. Indeed, cleavage efficiency varies greatly when using different gRNAs, as it is likely affected by several factors such as the formation of secondary structures in the gRNA or the accessibility of the DNA target within the chromatin. It is therefore recommended to select at least 3 suitable gRNA candidates and test their respective cleavage efficiencies by high resolution melt analysis (HRMA)<sup>7</sup>.

Cleavage efficiency was assessed by for gRNA #1, #2 and #8. Although not among the top-scoring gRNA candidates, gRNA #8 was selected due to its ideal location with respect to the RAD51 T13/S14 locus, as shown in Figure 3.2. CRISPR-mediated knock-in efficiency was shown to drastically decrease with increasing distance from the Cas9-cleavage site – at a mere 10 bp distance from the cut-site, mutation incorporation drops by 50%.<sup>8</sup> Given that the PAM site of gRNA #8 is located 1 nucleotide downstream of the S14 codon, it is ideally positioned for optimal knock-in efficiency, whereas gRNA #1 and #2 are found 43 and 27 nucleotides downstream respectively. Additionally, successful incorporation of the mutation will decrease gRNA #8 complementarity for the RAD51 T13/S14 locus, hence decreasing the probability of re-editing at the mutated site.



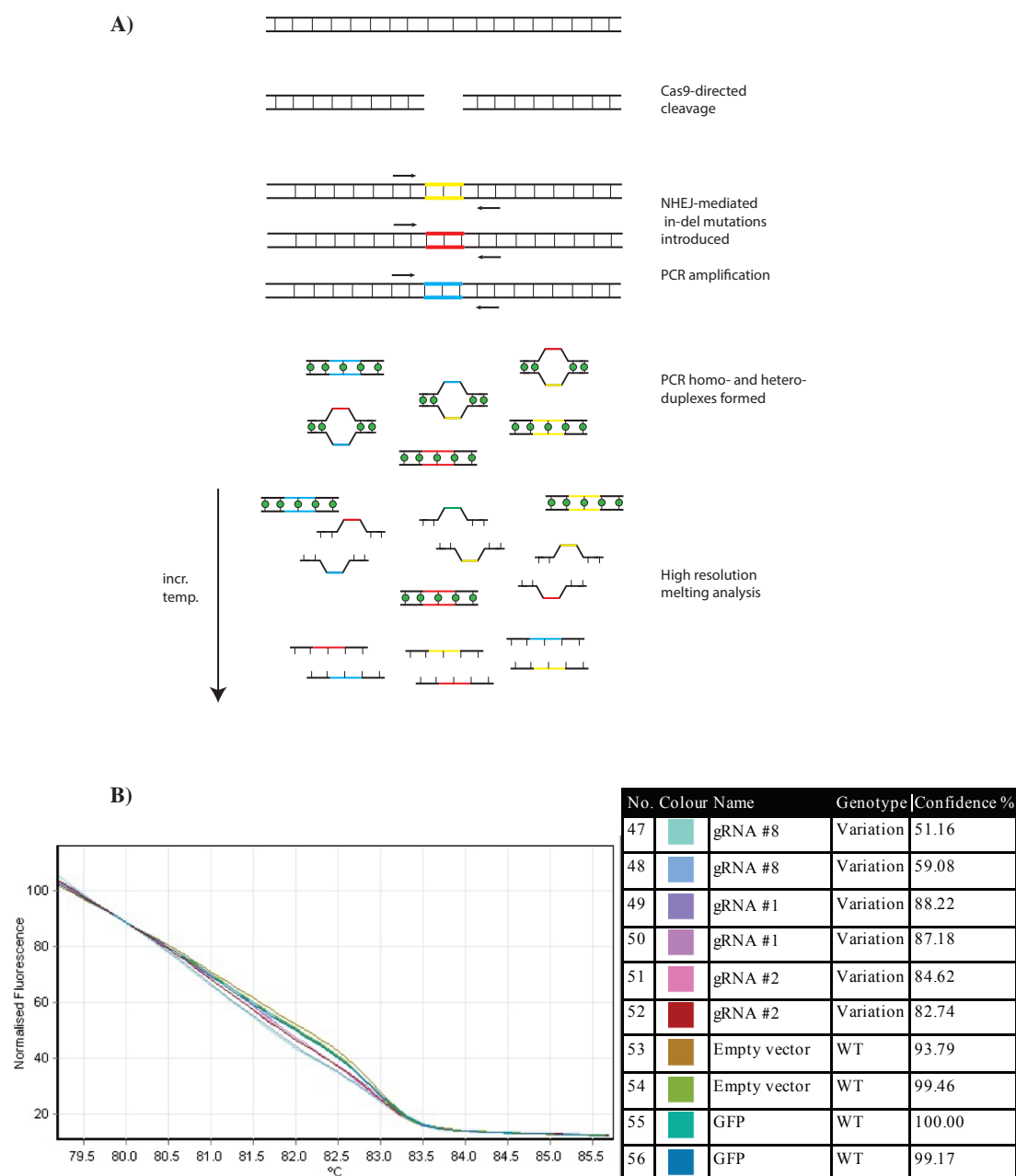
**Figure 3.2 – Position of 3 selected gRNA candidates and the corresponding Cas9 cleave sites.** PAM sites are in bold, the RAD51 T13 and S14 codons are in red. The amino acid sequence is presented in capitals below the nucleotide sequence.

### 3.2.3 High resolution melt analysis (HRMA)

HRMA assesses the efficiency of Cas9-cleavage by detecting genetic variation at the CRISPR-targeted locus. When the gRNA-Cas9 complex cleaves the target locus, subsequent NHEJ-directed break repair will introduce small indel mutations at the break site. Importantly, the mutations introduced may vary from cell to cell, hence introducing genetic variation at the target locus. As depicted in Figure 3.3 a, PCR-amplification of such a genetically variable locus results in heteroduplex PCR products which have a lower melting temperature than homoduplex PCR products. Increased genetic variation at the CRISPR-targeted locus can therefore be detected from precisely measured melting curves of the corresponding PCR product and serve as a read-out for Cas9-cleavage activity (i.e. the larger the shift to a lower melting temperature, the higher the cleavage efficiency). As shown in Figure 3.3 b, samples transfected with gRNA #8

exhibit the largest shift in melting temperature, indicating that gRNA #8 leads to the most efficient induction of DSBs at the RAD51 T13/S14 locus.

Based on the results of the HRM analysis, as well as the convenient location of gRNA #8 with respect to the RAD51 T13/S14 locus, all CRISPR/Cas9 gene-editing in this study was performed using gRNA #8. For more detail on the experimental procedures, see Materials and Methods 2.4.5.



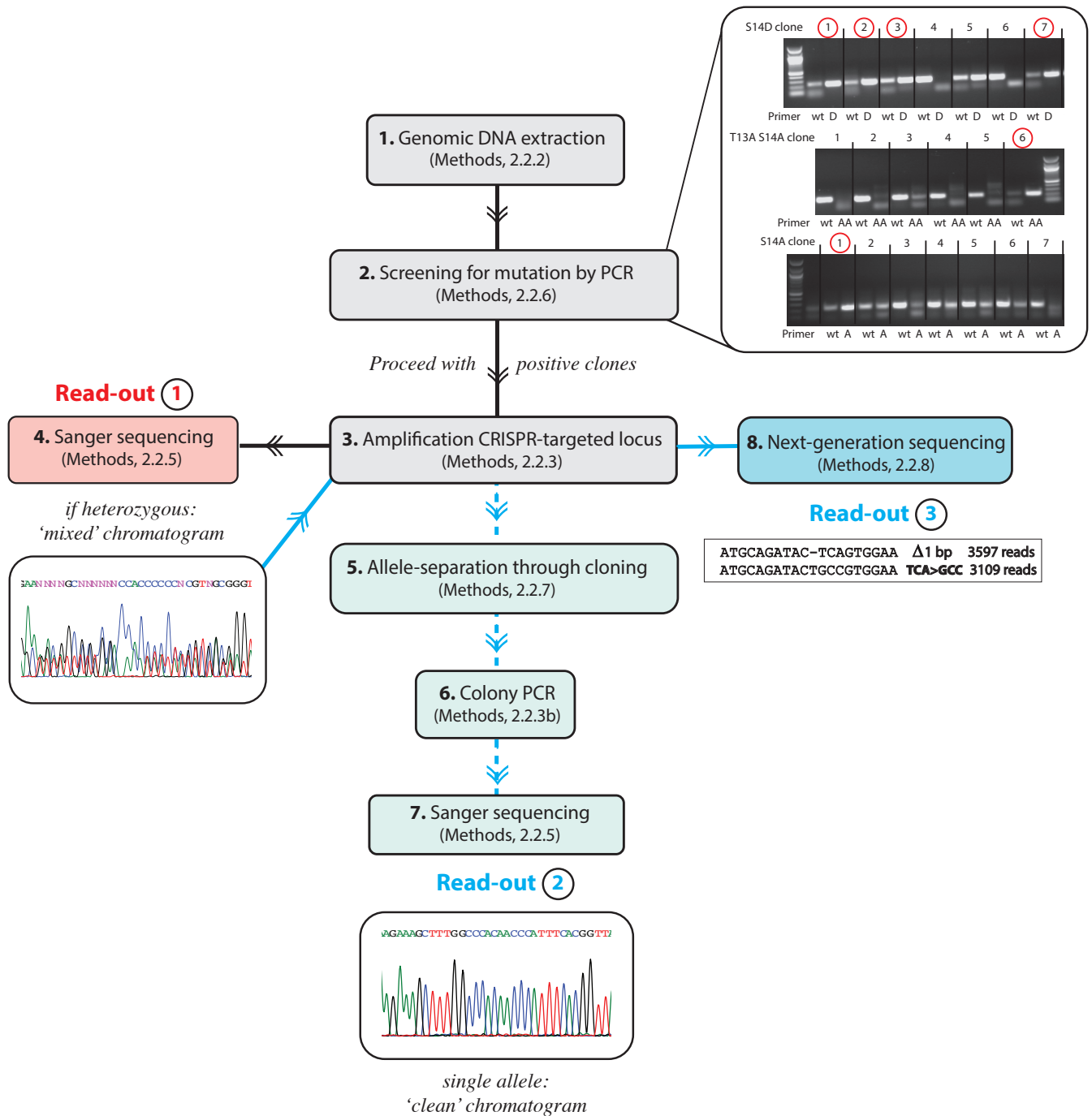
**Figure 3.3 – High resolution melt analysis of the candidate gRNAs.** (A) Efficient Cas9-cleavage introduces DSBs which are repaired by NHEJ, resulting in a variety of indel mutations at the CRISPR/Cas9-target site. PCR amplification of the CRISPR-targeted locus generates a mix of homo- and hetero-duplex PCR products. Hetero-duplex molecules will melt at a lower temperature than homo-duplex molecules. When PCR products dissociate, the intercalating dye (green circles) can no longer bind and fluorescence decreases. Fluorescence is monitored with increasing temperature to generate a melt curve. (B) HRMA was performed by A. Bassett, 24 hours after gRNA-Cas9 transfection. Transfections with an empty vector or GFP vector served as negative controls. Two replicates were included for each transfection. A confidence interval (% confidence) is reported for each transfection, which reflects the similarity of that sample's melt curve compared to those within the negative control (i.e. WT genotype) cluster. The lower the % confidence, the less likely that this particular melt curve represents a WT genotype.

### 3.2.4 Screening strategy

#### For CRISPR ‘knock-in’ clones

The screening and verification strategy for CRISPR knock-in clones is depicted in Figure 3.4, step 1-8. Clones were initially screened for the desired mutation by PCR using forward primers which are perfectly complementary to either the wild-type RAD51 T13/S14 locus or the mutated RAD51 T13/S14 locus (Figure 3.4, step 1-2). Efficient PCR amplification can only occur if the PCR primer encodes the same mutation as is incorporated in the genomic DNA template, such that clones were considered positive only if a bright PCR product was obtained with the mutation-specific primer (e.g. S14D) but *not* with the WT-specific primer (see Figure 3.4, step 2. Positive clones are encircled). The CRISPR-targeted locus of the selected positive clones was next amplified using primers flanking the T13/S14 locus and the resultant PCR product was sent for sequencing (Figure 3.4, step 3-4). In case of a homozygous clone, incorporation of the desired mutation is directly inferred from the sequencing chromatogram. However, in cells containing more than one RAD51 allele, heterozygous mutants may give rise to a ‘mixed’ sequencing chromatogram, impeding the conclusive identification of the RAD51 genotype. In such cases, the mixed-allele PCR product was cloned such that a single PCR molecule is incorporated per vector molecule. The plasmids - now containing separated RAD51 alleles – were subsequently transformed into *E. coli*, resulting in single-allele containing bacterial colonies. Colony PCR was next performed to amplify the T13/S14 locus from a single RAD51 allele. Sequencing of the colony PCR product gives rise to a clear sequencing chromatogram, which identifies the mutation incorporated at the specific RAD51 allele. By picking sufficient bacterial colonies, this approach eventually allows every RAD51 T13/S14 allele to be sequenced (Figure 3.4, step 5-7). The process is laborious and further complicated by the fact that U2OS cells are triploid for chromosome 15 and therefore contain at least three RAD51 alleles. A large number of colonies must be sequenced to ensure every RAD51 allele is recovered. This realisation led to a change in strategy – instead of colony PCR, next generation (deep) sequencing (NGS) was used to determine allele-specific sequences at the S14/T13 locus of RAD51. This high-throughput strategy allows for the quantitative analysis of the sequence composition in a mixed sample, thereby conclusively determining the genotype at every RAD51 allele (Figure 3.4, step

8). Finally, loss of wild-type RAD51 phosphorylation was confirmed by Western blotting.



**Figure 3.4 – Flow chart of screening strategy for CRISPR knock-in mutation.** Each step depicted includes a reference to the corresponding experimental protocol (Chapter 2, Methods). Step 2 (inset) displays a representative agarose gel obtained during PCR-mediated screening. Encircled samples are considered positive.



### 3.3 Results

A first round of CRISPR/Cas9-mediated gene editing was performed in U2OS and RPE1 cell lines in an attempt to generate phospho-mimetic (S14D) or phospho-null (T13A-S14A) mutants. Table 3.1 summarizes the screening and verification of all clones obtained. Clones were confirmed to be knock-in mutants if (i) the mutant is homozygous for the desired mutation or (ii) the mutant is heterozygous, whereby at least one allele encodes the desired mutation and the other allele(s) contain(s) a gene-disrupting frameshift mutation. CRISPR/Cas9 knock-in efficiency (%) was calculated based on the number of correctly mutated clones obtained relative to the total number of clones screened. It should be noted that in some cases not all positively screened clones were confirmed by sequencing, thereby precluding calculation of the precise knock-in efficiency. In this case, a lower estimate of knock-in efficiency was calculated by assuming that only the sequenced clones are correctly mutated. Conversely, an upper estimate of knock-in efficiency was calculated by assuming that all of the unsequenced positive clones are also correctly mutated. For every clone sequenced by NGS, the corresponding indel size distribution as well as the number of reads found for each specific mutation, are displayed in Supplementary Figure 3.1.

#### **Highest knock-in efficiency is achieved for phospho-mimetic (S14D) mutation**

Four homozygous S14D mutants were obtained in U2OS and two heterozygous S14D clones were obtained in the RPE1 cell line. No positive clones were obtained for the T13A-S14A mutation in either the U2OS or RPE1 cell lines. The absence of any T13A-S14A mutants may reflect the importance of T13/S14-phosphorylation in cell viability. Alternatively, the failure to obtain the T13A-S14A mutant may simply stem from (or be exacerbated by) limited efficiency of CRISPR/Cas9-induced HDR. In order to improve the latter, a second round of CRISPR/Cas9-mediated gene editing was performed in which the ssDNA repair template was stabilized against exonuclease degradation by the addition of a 3' phosphorothioate bond.

#### **Phospho-null (S14A) mutant in U2OS appears to overexpress BRCA2 and PLK1**

The second round of CRISPR/Cas9-mediated gene editing aimed to generate either S14A or T13A-S14A phospho-null mutants in U2OS and RPE1 cell lines. Furthermore,

**Table 3.1 – Screening and verification of CRISPR mutants (round 1)**

| Cell line | Desired mutation | # clones screened | # positive clones | # clones sequenced | # clones confirmed | Genotype (per allele) |                                              | knock-in efficiency (%) |
|-----------|------------------|-------------------|-------------------|--------------------|--------------------|-----------------------|----------------------------------------------|-------------------------|
| U2OS      | S14D             | 30                | 7                 | 4                  | 4                  | clone 1 - 4           | S14D (homozygous)                            | 13.3-23.3 *             |
|           | T13A-S14A        | 15                | 0                 | 0                  | 0                  | -                     |                                              | 0                       |
| RPE1      | S14D             | 64                | 4                 | 3                  | 2                  | clone 1 & 2           | 1. 1 nt. insertion<br>2. S14D (heterozygous) | 3.1-4.7 *               |
|           | T13A-S14A        | 64                | 0                 | 0                  | 0                  | -                     |                                              | 0                       |

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\* Not all positive clones were sequenced. In this case, the upper- and lower- estimates of the knock-in efficiency were calculated assuming either all or none of the unsequenced clones contained the desired mutation.

**Table 3.2 - Screening and verification of CRISPR mutants (round 2)**

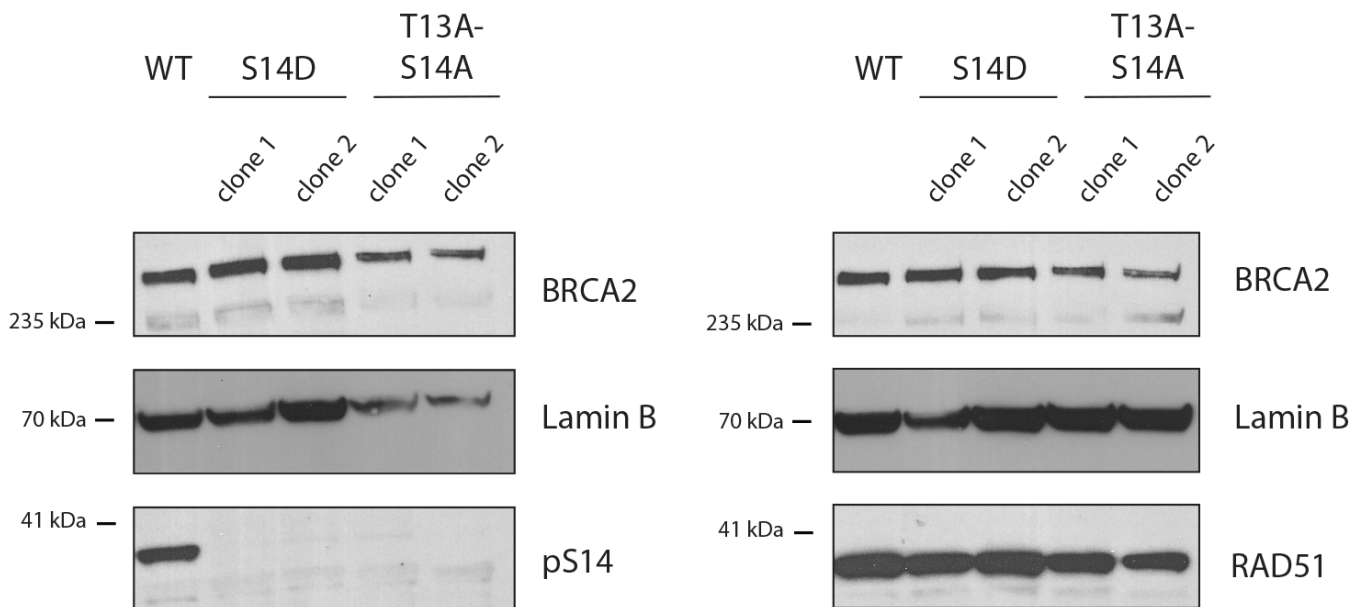
| Cell line | Desired mutation | # clones screened | # positive clones | # clones sequenced | # clones confirmed | Genotype (per allele) |                                                   | knock-in efficiency (%) |
|-----------|------------------|-------------------|-------------------|--------------------|--------------------|-----------------------|---------------------------------------------------|-------------------------|
| U2OS      | S14A             | 27                | 1                 | 1                  | 1                  | clone 1               | 1. 1 nt deletion<br>2. S14A (heterozygous)        | 3.7                     |
|           | T13A-S14A        | 19                | 1                 | 1                  | 0                  | -                     |                                                   | 0                       |
| RPE1      | S14A             | 96                | 1                 | 1                  | 0                  | -                     |                                                   | 0                       |
|           | T13A-S14A        | 167               | 3                 | 3                  | 2                  | clone 1               | T13A-S14A (homozygous)                            | 1.2                     |
|           |                  |                   |                   |                    |                    | clone 2               | 1. T13A-S14A<br>2. 10 nt insertion (heterozygous) |                         |
| eHAP      | S14D             | 13                | 8                 | 3                  | 3                  | clone 1 - 3           | S14D (homozygous)                                 | 23.1-61.5 *             |
|           | S14A             | 41                | 8                 | 4                  | 2                  | clone 1 & 2           | S14A (homozygous)                                 | 4.9-14.6 *              |

\* Not all positive clones were sequenced. In this case, the upper- and lower- estimates of the knock-in efficiency were calculated assuming either all or none of the unsequenced clones contained the desired mutation.

**Table 3.2 (continued) - Screening and verification of CRISPR mutants (round 2)**

| <b>Cell line</b> | <b>Desired mutation</b> | <b># clones screened</b> | <b># positive clones</b> | <b># clones sequenced</b> | <b># clones confirmed</b> | <b>Genotype (per allele)</b> |                        | <b>knock-in efficiency (%)</b> |
|------------------|-------------------------|--------------------------|--------------------------|---------------------------|---------------------------|------------------------------|------------------------|--------------------------------|
| eHAP             | T13A-S14A               | 23                       | 4                        | 4                         | 4                         | clone 1 - 4                  | T13A-S14A (homozygous) | 17.4                           |

the haploid eHAP cell line was used to generate S14D, S14A and T13A-S14A mutants. It should be noted that PLK1- and CK2- phosphorylation of RAD51 is strictly sequential, such that no T13 phosphorylation can be detected *in vivo* in the RAD51 S14A mutant.<sup>1</sup> The T13A-S14A phospho-null mutants are therefore equivalent to the S14A phospho-null mutants. Table 3.2 summarizes the screening and verification of all clones obtained. Compared to the previous round of CRISPR, use of the stabilized ssDNA repair template may indeed have improved CRISPR/Cas9 knock in efficiency. Two separate T13A-S14A mutants (one homozygous, the other heterozygous) were obtained in RPE1, thereby raising the knock-in efficiency from 0% to 1.2%. Western blot analysis confirmed the absence of S14 phosphorylation in the obtained RAD51 phospho-mutants in RPE1 (Figure 3.5).



**Figure 3.5 - Western blot analysis of RPE1 knock-in mutants obtained by CRISPR/Cas9-mediated gene editing.** Cells were treated overnight in 0.33  $\mu$ M Nocodazole, to enhance the S14 phosphorylation signal.

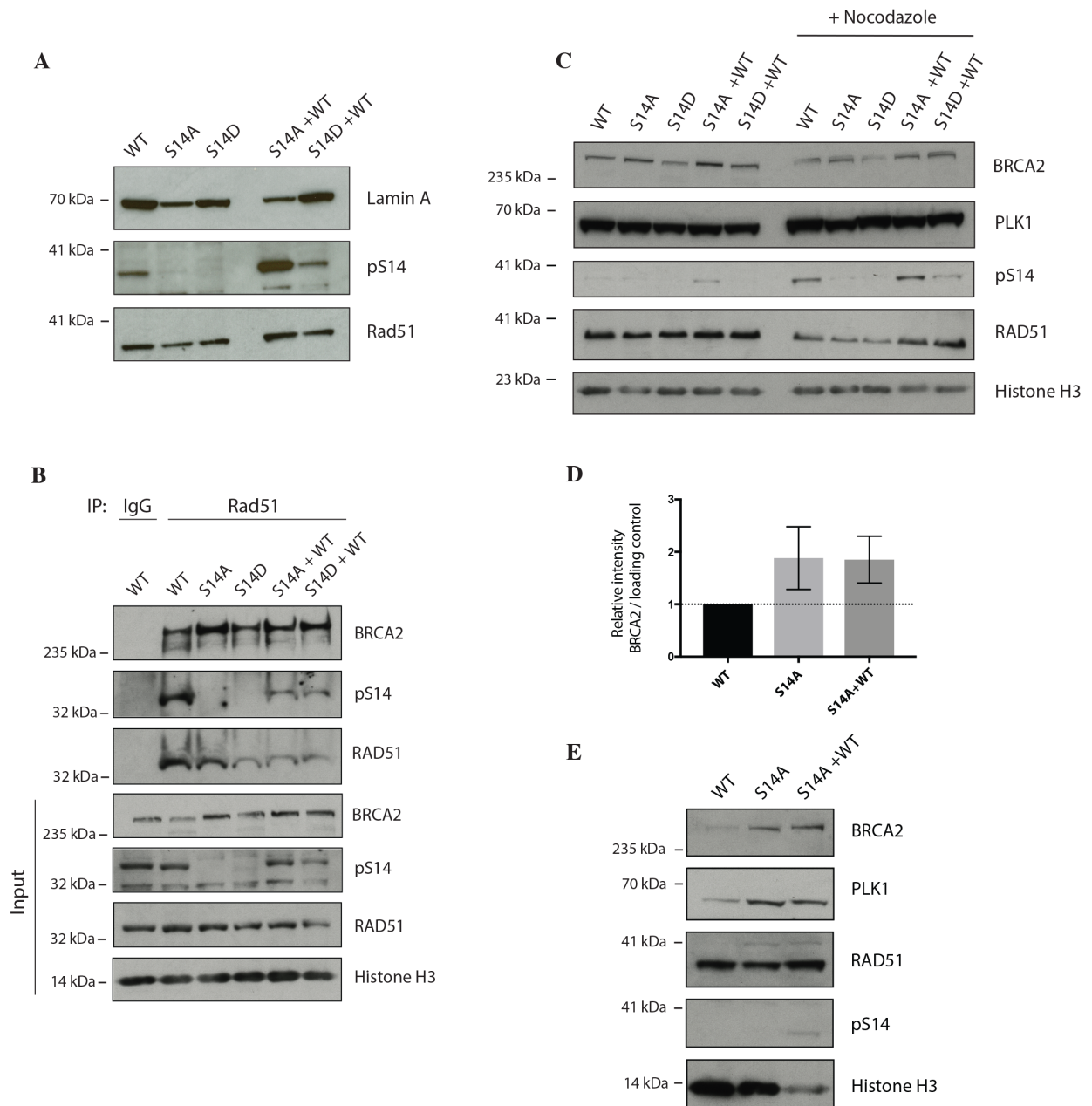
One heterozygous S14A mutant was obtained in U2OS. This mutant has a markedly different morphology compared to the parental U2OS cell line and displays both decreased cell adhesion and decreased contact inhibition in cell culture, as is characteristic for highly transformed cancer cell lines. The U2OS S14A clone has therefore likely obtained secondary mutations, potentially to alleviate detrimental effects of the loss of T13/S14 phosphorylation. Both the U2OS S14A and S14D mutants

were complemented by the stable expression of wild-type RAD51. This is of particular importance in the U2OS S14A cell line, as it allows any mutant phenotype observed in future analysis to be attributed to the phospho-null RAD51 mutation rather than the potential secondary mutations acquired in this cell line. RAD51 analysis by Western blot and immunoprecipitation confirmed the absence of S14 phosphorylation in the U2OS phospho-mutants (Figure 3.6 a-b). Interestingly, the wild-type complemented S14A cell line exhibits strikingly higher S14 phosphorylation compared to the other wild-type RAD51 expressing cell lines, as detected upon nocodazole treatment and in untreated conditions (Figure 3.6 a, c). This suggests that the genetic background of the U2OS S14A cell line promotes enhanced S14 phosphorylation. Indeed, BRCA2 was found to be consistently overexpressed in the U2OS S14A genetic background (Figure 3.6 d) and PLK1 overexpression could be detected in S phase-synchronized U2OS S14A cell lines (Figure 3.6 e). Given that BRCA2 acts as a binding platform for both PLK1 and RAD51 to promote RAD51 T13/S14 phosphorylation, enhanced expression of both BRCA2 and PLK1 can account for the increased level of T13/S14 phosphorylation detected in the U2OS S14A+WT mutant.<sup>2</sup>

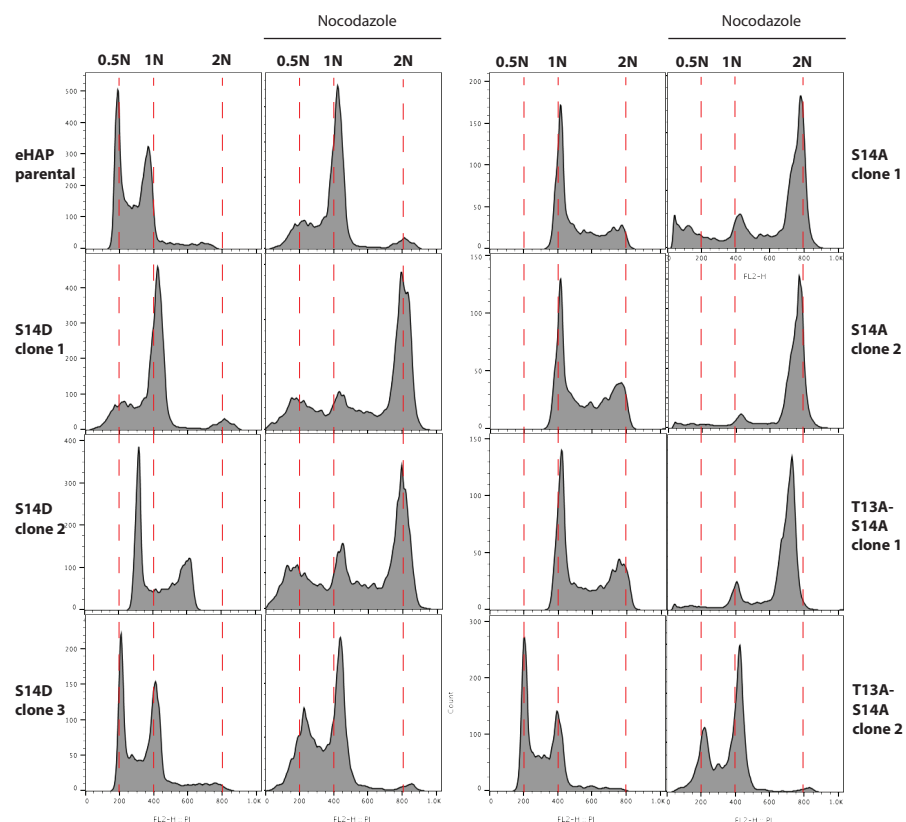
### **Improved CRISPR knock-in efficiency is achieved by targeting a haploid genome**

The CRISPR/Cas9 knock-in efficiency was greatly increased in the eHAP cell line – three homozygous S14D mutants; two homozygous S14A mutants and four homozygous T13A-S14A mutants were obtained (Table 3.2). The percentage of positive clones identified during the PCR-mediated screen was higher for the S14D (61.5%) than for the phospho-null mutations (14.6% and 17.4%), potentially indicating that the loss of T13/S14 phosphorylation also negatively impacts survival in the eHAP cell line. The overall increased knock-in efficiency achieved in the eHAP cell line compared to U2OS and RPE1 is likely due to the haploidy of the target genome, given that just a single allele mutation is required to generate a complete knock-in mutant in eHAP cells. However, flow cytometry of several eHAP mutants revealed that the mutants did not remain haploid (Figure 3.7). While some remaining haploid cells can be detected in eHAP T13A-S14A clone 1 and S14D clone 1-3, all other analyzed mutants appear entirely diploid. Indeed, diploidization of haploid cells is a well-reported phenomenon. While the spontaneous duplication of the genome has been

shown to be a relatively infrequent event, diploid cells gain a significant growth advantage and rapidly outcompete the haploid cells from the population.<sup>9</sup>



**Figure 3.6 - Western blot analysis of U2OS knock-in mutants obtained by CRISPR/Cas9-mediated gene editing.** (A) The level of RAD51 S14 phosphorylation was analyzed with the pS14 antibody. Cells were treated overnight in 0.33  $\mu$ M Nocodazole to enhance the pS14 signal. (B) Immunoprecipitated RAD51 was analyzed with the pS14 antibody. Cells were treated overnight in 0.33  $\mu$ M Nocodazole. (C) Cells were untreated or treated overnight with 0.33  $\mu$ M Nocodazole. The levels of RAD51 S14-phosphorylation; BRCA2 expression and PLK1 expression were assessed. (D) The relative intensity of BRCA2 (in B and C) was quantified relative to the loading control (Lamin or Histone H3) and normalized against WT. (E) Cells were synchronized in S phase by double thymidine block-and-release. Whole cell lysates were prepared 2 hours after release from final thymidine block. The levels of RAD51 S14-phosphorylation; BRCA2 expression and PLK1 expression were assessed.



**Figure 3.7 – FACS analysis of eHAP cell lines with or without Nocodazole treatment. Where indicated, cells were treated overnight with 0.33  $\mu$ M nocodazole.**



### **3.4 Discussion**

#### **Further validation of CRISPR/Cas9-generated mutants is necessary**

The mutants generated during this study have been validated by sequencing and Western blotting. However, only two RAD51 alleles were sequenced in the heterozygous U2OS S14A mutant. U2OS is triploid for chromosome 15 and should therefore contain three RAD51 alleles. The third allele most likely contains a very large deletion which removes one of the primer binding sites, thereby preventing its amplification by PCR and subsequent sequencing. Longer-range PCR should be performed to confirm that this is indeed the case.

Furthermore, recent studies have shown that CRISPR/Cas9-mediated gene knock-out may lead to exon skipping, resulting in a translated gene-product rather than the expected nonsense-mediated decay of the mRNA transcript.<sup>10,11,12</sup> It is therefore possible that any of the mutants containing a frameshift mutation express an N-terminal truncated RAD51 variant. The possibility that an aberrant RAD51 gene product may impact the function of full-length RAD51 cannot be ruled out, as such a gene product may evade detection by the RAD51 antibody. Therefore, RT-PCR must be performed - and the PCR products sequenced- to ensure that only the intended RAD51 variant is expressed in these mutants.

#### **T13/S14 phosphorylation may be important for cell survival**

Phospho-mimetic mutants were more readily obtained than phospho-null mutants across all three cell lines, suggesting that the phospho-null mutation may negatively impact cell survival and therefore decrease the number of positive clones obtained. This is the first indication that RAD51 phosphorylation may be important for proliferation under unperturbed conditions. Previous experiments in cell lines exogenously expressing RAD51 phospho-mutants did not reveal an impact on cell survival, even when endogenous (wild-type) RAD51 was down-regulated.<sup>1</sup> In these cell lines, the constitutive expression of the RAD51 mutant from a viral promoter likely leads to overexpression of the protein, which could compensate for the loss of phosphorylation. The importance of T13/S14 phosphorylation is therefore better analyzed under conditions of endogenous RAD51 expression. However, the observations made in this

study are not based on an exhaustive and systematic screening of all growing clones, thereby preventing any conclusive comparison of knock-in efficiency.

Interestingly, the only phospho-null mutant obtained in the U2OS cell line has likely acquired secondary mutations. The U2OS S14A cell line appears to overexpress BRCA2 and PLK1 – the latter specifically during S phase. Phospho-null RAD51 was previously shown to be impaired in its recruitment to DNA damage-induced foci, as well as stalled replication forks.<sup>1,2</sup> Given that BRCA2 promotes RAD51-nucleofilament formation at broken DNA ends and is known to stabilize stalled replication forks, the overexpression of BRCA2 likely compensates for the loss of RAD51 T13/S14 phosphorylation. PLK1 overexpression may similarly aid survival in the absence of RAD51 T13/S14 phosphorylation. Several studies have indicated a role for PLK1 in the maintenance of replication during a normal cell cycle<sup>13</sup> as well as specifically during conditions of replication stress.<sup>14,15,16</sup> It is conceivable that the loss of RAD51 phosphorylation induces endogenous replication stress to which the cell has adapted by overexpressing PLK1 during S phase. To confirm the importance of these supposed adaptations, it would be interesting to test the sensitivity to BRCA2 knock-down or PLK1 inhibition in the S14A mutant compared to the WT complemented S14A mutant. A last potential indication for the importance of T13 phosphorylation was made during the screening and verification of the RAD51 knock-in mutants. Eleven negative clones were analyzed throughout the process, of which the nine were found to have obtained a 3-nucleotide deletion of the S14 codon. This suggests the loss of the S14 amino acid is not necessarily detrimental to cell survival. However, it should be noted that S14 deletion shifts a downstream glutamic acid (E) patch closer to the T13 amino acid (Figure 3.2). The increased proximity of this negatively charged amino acid-patch may compensate for the loss of S14 phosphorylation and directly recruit the acidophilic CK2 to phosphorylate T13. While the lack of a complete analysis of all obtained mutants again renders these observations qualitative, it is interesting that no mutant phenotype was encountered in which the T13 amino acid was deleted – perhaps indicating the importance of T13 phosphorylation.

To further investigate the effect of T13/S14-mutation on cell viability, a CRISPR/Cas9-mediated screen could be performed in which cells are transfected with both S14A- and S14D- repair templates simultaneously (a WT-repair template harbouring silent mutations could also be included). Exhaustive screening of all clones

obtained would reveal whether a significantly lower number of S14A mutants is obtained. Alternatively, the pooled DNA from the CRISPR/Cas9-treated cells could be analyzed for the frequency of S14A alleles versus S14D or WT by deep sequencing, such that monoclonal growth and analysis of individual CRISPR/Cas9-treated clones is no longer necessary.

### **Is the importance of RAD51 phosphorylation linked to ploidy?**

The fact that RAD51 is essential in higher eukaryotes, but not in yeast, is commonly attributed to the higher levels of DNA damage and replication stress associated with the increased size and complexity of the human genome. Indeed, it has been shown that tetraploidization of budding yeast leads to an increase of replication-associated DNA damage, as well as a dependence on RAD51 for cell viability.<sup>17</sup> It is conceivable that the number of spontaneous DNA lesions is similarly reduced in mammalian haploid cell lines, which could lead to a decreased dependence on RAD51 recruitment – and therefore RAD51 T13/S14 phosphorylation – in human haploid cells. Given that haploidy inherently facilitates the generation of CRISPR/Cas9-mediated knock-in mutants, knock-in efficiency is not an ideal measure by which to assess the importance of RAD51 phosphorylation with varying ploidy. However, the fact that the eHAP cell line is prone to spontaneous diploidization could be exploited to investigate this question. As shown by Olbrich et al.<sup>11</sup>, haploid cells display problems in chromosome segregation and a concurrent decrease in overall fitness compared to their diploid counterpart. A single spontaneous event of genome duplication will therefore give rise to a diploid cell than can readily grow and outcompete haploid cells – hence leading to the ‘diploidization’ of the haploid cell culture. However, if the loss T13/S14 phosphorylation is a bigger disadvantage in diploid cells than in haploid cells, this would reduce the overall gain in fitness upon diploidization. It could therefore be interesting to compare the rate of ‘diploidization’ of haploid S14A mutants compared to the corresponding parental cell line to assess the importance of RAD51 phosphorylation in relation to genome size.

CRISPR/Cas9 was here employed to generate RAD51 phospho-mutants in three different cell lines. The remainder of this thesis will focus on the phenotypical analysis of U2OS RAD51 phospho-mutants. However, further analysis of the RAD51 phospho-

mutants obtained in RPE1 will be important to elucidate the function of RAD51 phosphorylation in non-cancer cell lines.

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## **Chapter 4**

### **Characterization of RAD51 phospho-mutants in the context of DNA repair and replication**



## **Summary**

CRISPR/Cas9-generated U2OS RAD51 phospho-mutants were characterized in the context of DSB repair and replication. Preliminary evidence presented here suggests that the S14A mutation negatively impacts RAD51 recruitment and DNA replication. Loss of RAD51 T13/S14 phosphorylation was further shown to induce increased levels of endogenous DNA damage. While the S14D mutation potentially impairs DSB repair, it was also shown to confer increased cell survival in conditions of replication fork stalling. Lastly, the accumulation of RAD51 in mitotic cells suggests a function of RAD51 during mitosis.

## 4.1 Introduction

Aside from its role in canonical DSB repair, mounting evidence over recent years implicates the involvement of RAD51 in DNA replication. RAD51 has been shown to mediate break-induced replication as well as HR-dependent restart of stalled replication forks.<sup>1,2</sup> Additionally, an HR-independent role for RAD51 at stalled replication forks has been elucidated, in which RAD51 protects nascent DNA at the fork from degradation and thereby stabilizes the stalled fork structure.<sup>3</sup> Importantly, these replication-associated functions of RAD51 may not be confined to situations of exogenously introduced replication stress. Indeed, RAD51 has been shown to prevent the accumulation of unresolved replication intermediates during an unperturbed cell cycle and has further been proposed to play a role in the progression of the replication fork under unperturbed conditions.<sup>4,5</sup> The importance of RAD51 during replication therefore potentially accounts for the essentiality of RAD51 in higher eukaryotes. As discussed in Chapter 3, RAD51 T13/S14 phosphorylation appears to be important for cell proliferation. PLK1-dependent RAD51 phosphorylation contributes to RAD51 recruitment at HU-stalled replication forks and mediates replication restart, such that the potential importance of RAD51 phosphorylation in undamaged conditions may similarly stem from replication-associated functions.<sup>6</sup>

Importantly, as discussed in Chapter 3, all previous analysis of RAD51 T13/S14 phosphorylation have been performed in stable cell lines exogenously expressing RAD51 phospho-mutants. In this chapter, I investigate the effect of RAD51 phospho-mutations in the context of DSB repair and replication using the CRISPR/Cas9-generated endogenous RAD51 phospho-mutants. The loss of RAD51 phosphorylation and its consequences were studied by analysing the non-phosphorylatable RAD51 S14A mutant. The RAD51 S14D mutation mediates T13 phosphorylation without requiring prior PLK1-dependent S14 phosphorylation, as the negatively charged aspartate introduces a binding site for the acidophilic CK2 kinase.<sup>7</sup> The RAD51 S14D mutant was therefore used to study the effects of constitutive RAD51 T13 (hyper)phosphorylation.



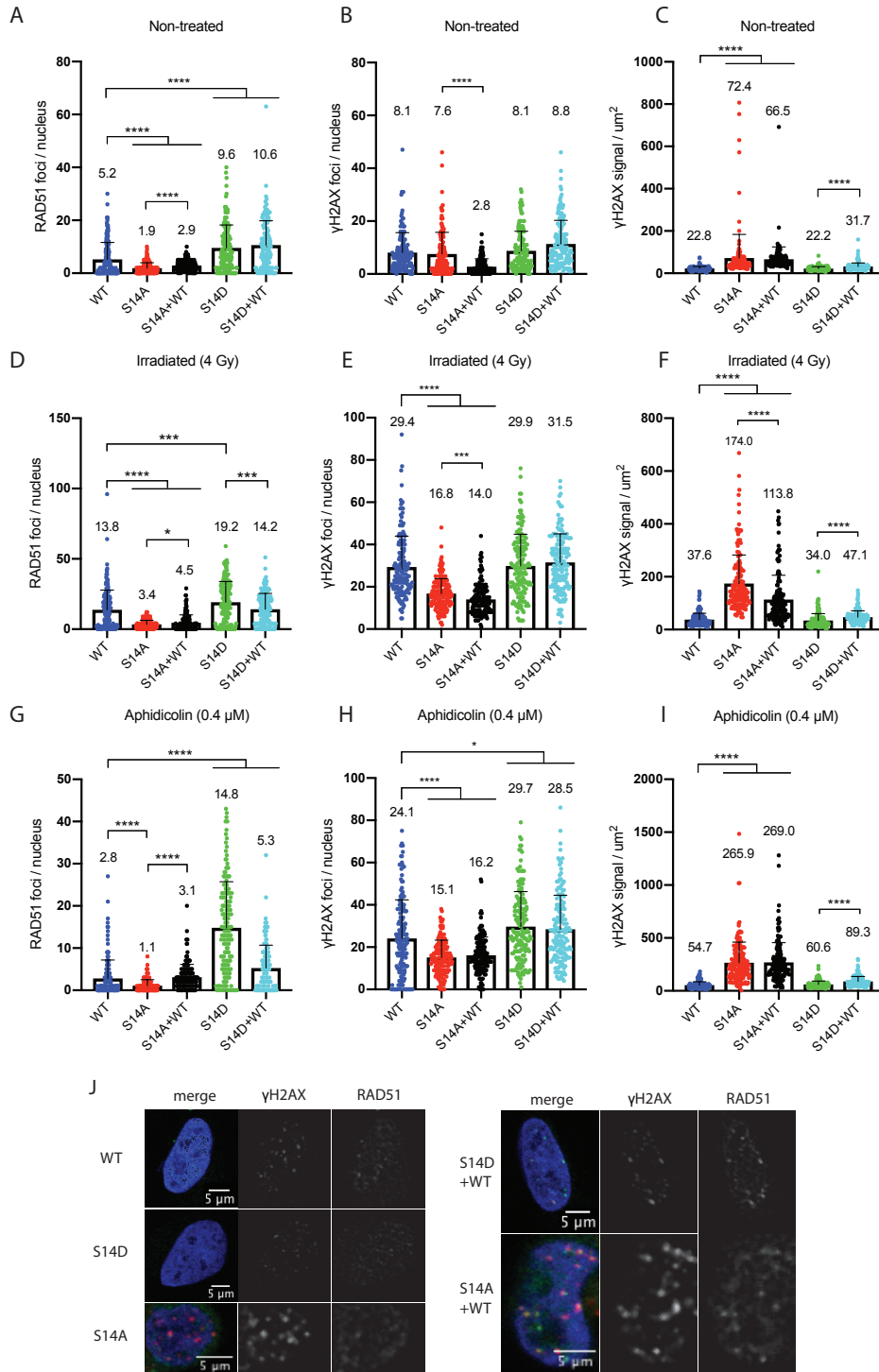
## 4.2 Results

### **RAD51 phosphorylation enhances RAD51 foci formation**

The RAD51 S14D mutation was previously shown to enhance RAD51 T13 phosphorylation and consequentially increase RAD51 recruitment to sites of DNA damage. Conversely, the RAD51 S14A mutation was shown to negatively impact RAD51 recruitment in irradiated cells.<sup>7</sup> RAD51 recruitment was here assessed in CRISPR/Cas9-generated U2OS RAD51 phospho-mutants, either under undamaged (non-treated) conditions or upon the induction of DNA damage by irradiation or aphidicolin treatment (Figure 4.1).

A significant increase in RAD51 foci formation was detected in S14D cells compared to WT cells under all conditions tested (Figure 4.1 a, d, g). Conversely, RAD51 foci formation was significantly decreased in the S14A cell line compared to WT. This decrease was (partially) rescued in the wild-type complemented S14A cell line (S14A+WT). It must be noted that RAD51 foci formation was significantly decreased in the wild-type complemented S14D cell line (S14D+WT) compared to S14D cells upon irradiation (Figure 4.1 d) and aphidicolin treatment (Figure 4.1 g), indicating that the exogenous (over)expression of RAD51 itself does not enhance RAD51 foci formation, but may even pose a disadvantage to RAD51 foci formation. Therefore, the rescue in RAD51 foci formation observed in the S14A+WT cell line is likely attributed to the complementation of the RAD51 phospho-null genotype. All together, these results suggest that S14 phosphorylation indeed enhances RAD51 recruitment and foci formation, in line with previous analyses.<sup>6,7</sup>

Unexpectedly, the number of DNA damage-induced  $\gamma$ H2AX foci was decreased in the S14A cell line compared to WT (Figure 4.1 e, h). However, as shown in Figure 4.1j, the  $\gamma$ H2AX foci in both the S14A and S14A+WT cell lines tend to be bigger and brighter than in the WT or S14D cell lines, such that the overall intensity of the  $\gamma$ H2AX signal may be a better read-out for DNA damage experienced in these cell lines than the number of  $\gamma$ H2AX foci. The  $\gamma$ H2AX signal intensity per nuclear area (for the same raw data as in Figure 4.1 b, e and h is displayed in Figure 4.1 c, f and i, respectively. Indeed, the highest levels  $\gamma$ H2AX signal intensity are consistently observed in the S14A and S14A+WT cell lines in all conditions tested. While the levels of  $\gamma$ H2AX signal intensity are significantly reduced in the S14A+WT cell line compared to S14A upon irradiation (Figure 4.1 f), this is not the case in untreated conditions (Figure 4.1 c) or upon aphidicolin treatment (Figure 4.1 i). Given that replication stress is thought to be a major source of endogenous DNA damage, this suggests that the S14A cell line is inherently



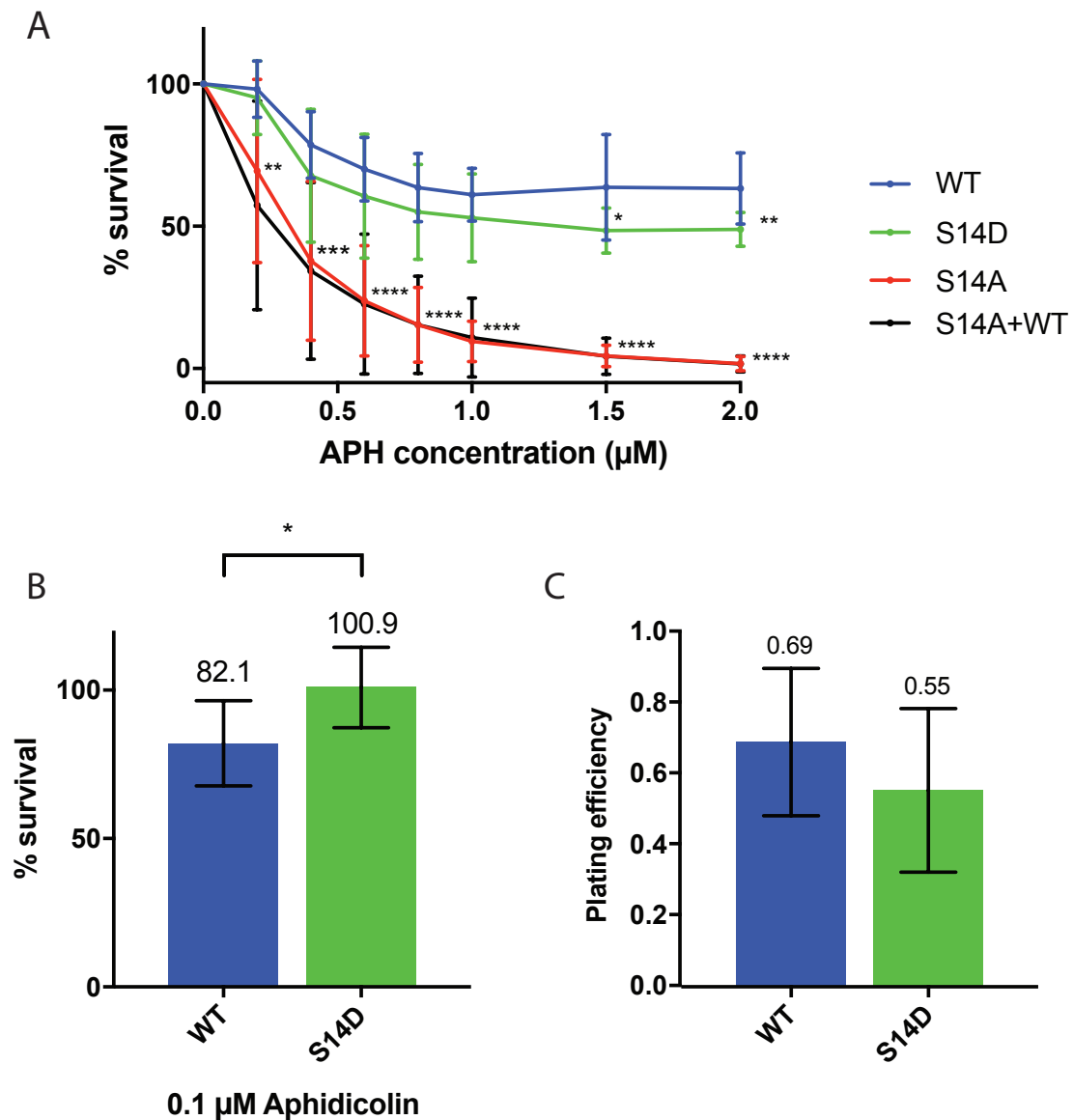
**Figure 4.1 – Analysis of RAD51 and  $\gamma$ H2AX signal in U2OS RAD51 S14 mutants.** (A) RAD51 foci per nucleus in untreated cells. (B)  $\gamma$ H2AX foci per nucleus in untreated cells. (C) Pixel value of  $\gamma$ H2AX signal per nuclear area, for the same experimental data as in (B). (D-F) Same as (A-C) in irradiated cells. (G-I) Same as (A-C) in cells treated with 0.4  $\mu$ M for 23 hours. (J) Representative images of RAD51 and  $\gamma$ H2AX staining per cell line upon irradiation. (F-G) Where indicated, cells were irradiated at 4 Gy and fixed after 3h. 150 cells were analyzed per sample. The mean value is indicated above each column. Error bars (SD),  $n=150$ . Images were obtained with 60x magnification.

vulnerable to replication stress (whether endogenous or aphidicolin-induced) independently of the RAD51 phosphorylation status. The level of  $\gamma$ H2AX signal intensity is significantly increased in the S14D+WT compared to S14D for all conditions tested, suggesting that the exogenous expression of wild-type RAD51 does not protect against DNA damage. Therefore, the complementation of the phospho-null genotype likely accounts for the decrease in  $\gamma$ H2AX signal intensity upon irradiation observed in the S14A+WT cell line compared to S14A. Taken together, these results suggest that RAD51 T13 phosphorylation enhances RAD51 recruitment.

### **RAD51 phospho-mimetic mutation protects cell against mild replication stress**

A past study has shown that S14 phosphorylation contributes to RAD51 recruitment at stalled replication forks and aids replication restart.<sup>6</sup> This suggests that RAD51 T13/S14 phosphorylation may be important to protect the cell against the detrimental effects of replication stress. To test whether this hypothesis holds true in the CRISPR/Cas9-generated cell lines, I analyzed cell viability by metabolic activity (WST-1 assay) (Figure 4.2 a) and clonogenic survival (Figure 4.2 b) in the presence of aphidicolin, which introduces replication stress through the inhibition of B-family DNA polymerases. Cell survival is significantly reduced in both of the S14A cell lines compared to the WT, even at low concentrations of aphidicolin (0.2  $\mu$ M) (Figure 4.2 a). Given that the wild-type complemented S14A cell line does not rescue this decrease in cell survival, the sensitivity to aphidicolin-induced replication stress of the S14A cell line cannot be attributed to the loss of RAD51 S14 phosphorylation. Considering that the S14A mutant appears to have obtained several secondary mutations (as discussed in Chapter 3), it is likely that the S14A genetic background has introduced an inherent vulnerability to replication stress.

The S14D cell line also exhibits a significant decrease in cell survival compared to the WT, but only at higher concentrations of aphidicolin (1.5-2  $\mu$ M). Conversely, the clonogenic survival of the S14D mutant is significantly increased relative to the WT in the presence of 0.1  $\mu$ M aphidicolin (Figure 4.2 b). Interestingly, the plating efficiency of the S14D mutant tends to be lower than that of the wild-type (Figure 4.2 c). These results suggest that RAD51 S14D mutation protects against the consequences of mild replication stress but may confer an overall disadvantage to cell proliferation in unperturbed conditions.



**Figure 4.2 – Analysis of cell viability in the presence of aphidicolin.** (A) Cell survival of RAD51 phospho-mutants was analyzed in the presence of aphidicolin by WST-1 assay at indicated concentrations. Error bars (SD),  $n=3$ . (B) Cell survival of RAD51 phospho-mutants was analyzed in the presence of aphidicolin by clonogenic assay in the presence of  $0.1 \mu\text{M}$  aphidicolin. Error bars (SD),  $n=3$ . (C) Plating efficiency of WT and S14D cells in untreated conditions. Error bars (SD),  $n=3$ .

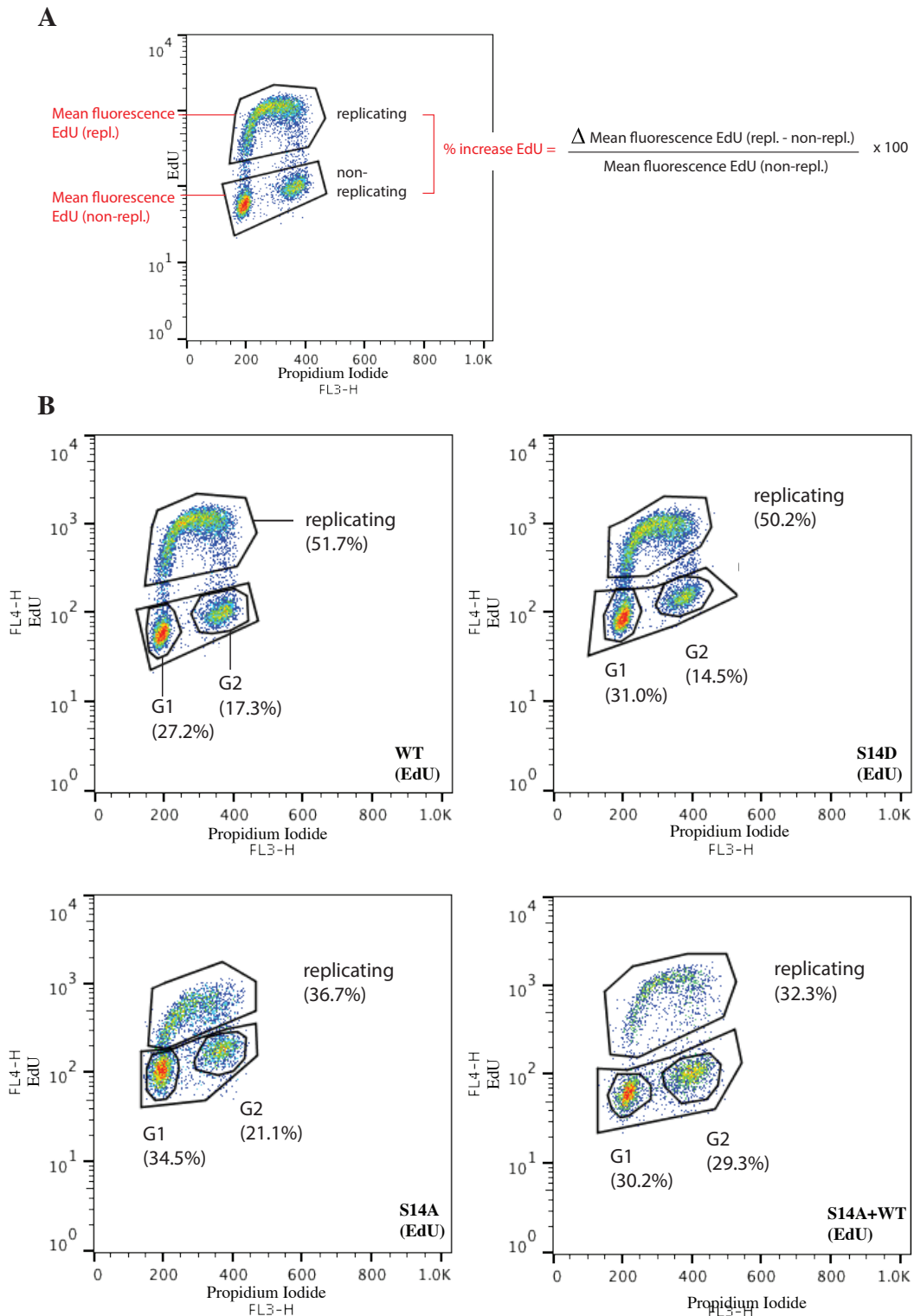
### RAD51 phospho-null mutation may impact replication rate and mitotic duration

The RAD51 phospho-mutants exhibited significantly different sensitivities to aphidicolin-induced replication stress (Figure 4.2), suggesting that T13/S14 phosphorylation status in these cell lines likely impacts replication. To test this more directly, replication was next assessed by EdU incorporation. After a 30-min EdU pulse, cells were fixed and incorporated EdU was fluorescently labelled by click chemistry to distinguish replicating from non-replicating cells

(i.e. G1 and G2 populations) (Figure 4.3). Propidium iodide staining of all DNA further allows to separate G1 and G2 cell populations. The percentage increase in mean fluorescence (EdU) intensity of replicating cells compared to non-replicating cells was calculated for each cell line as depicted in Figure 4.3 a and summarized in table 4.1.

A strikingly smaller percentage increase in EdU signal upon replication was detected for S14A cells compared to WT, suggesting that the S14A cell line exhibits a reduced replication rate (Figure 4.3 b, table 4.1). This apparent decrease in replication rate was rescued by complementation with wild-type RAD51 (S14A+WT). Interestingly, S14D also exhibited a slight reduction in EdU signal upon replication relative to WT. This data suggests that the loss of dynamic PLK1-regulated RAD51 S14/T13 phosphorylation negatively impacts overall replication rate. S14A cells also have a noticeably smaller population of replicating cells compared to WT (Figure 4.3). However, this is not rescued in the S14A+WT cells such that this change in cell cycle distribution cannot be attributed to the RAD51 phospho-null mutation.

Cell cycle distribution was further analyzed in the absence and presence of aphidicolin, whereby histone H3 phosphorylation at serine-10 serves as a mitotic marker to determine the mitotic index (Figure 4.4). A noticeable increase in mitotic index was detected in the S14A and S14A+WT cell lines compared to WT in untreated conditions indicating that both these cell lines may take longer to complete mitosis (Figure 4.4 a). A significant difference in cell cycle distribution was observed between S14A and S14A+WT cells when treated with aphidicolin (Figure 4.4 b). S14A displays a markedly high cell population in mid-S phase. This mid-S phase arrest appears to be rescued in the S14A+WT cell line. However, relative to WT, S14A+WT appears to accumulate in late S and G2 phase. Interestingly, the S14D cell line also displays a subtle shift in cell cycle distribution towards G2 phase. Taken together, these data suggest that the S14A cell line is impaired in replication progression, leading to S phase arrest. While both the decreased replication rate and mid-S phase arrest appear to be rescued by complementation with phosphorylatable RAD51, the S14A+WT cell line displays aphidicolin-induced G2 arrest, which may account for the persistent sensitivity to aphidicolin-induced replication stress as detected by the cell viability assay.

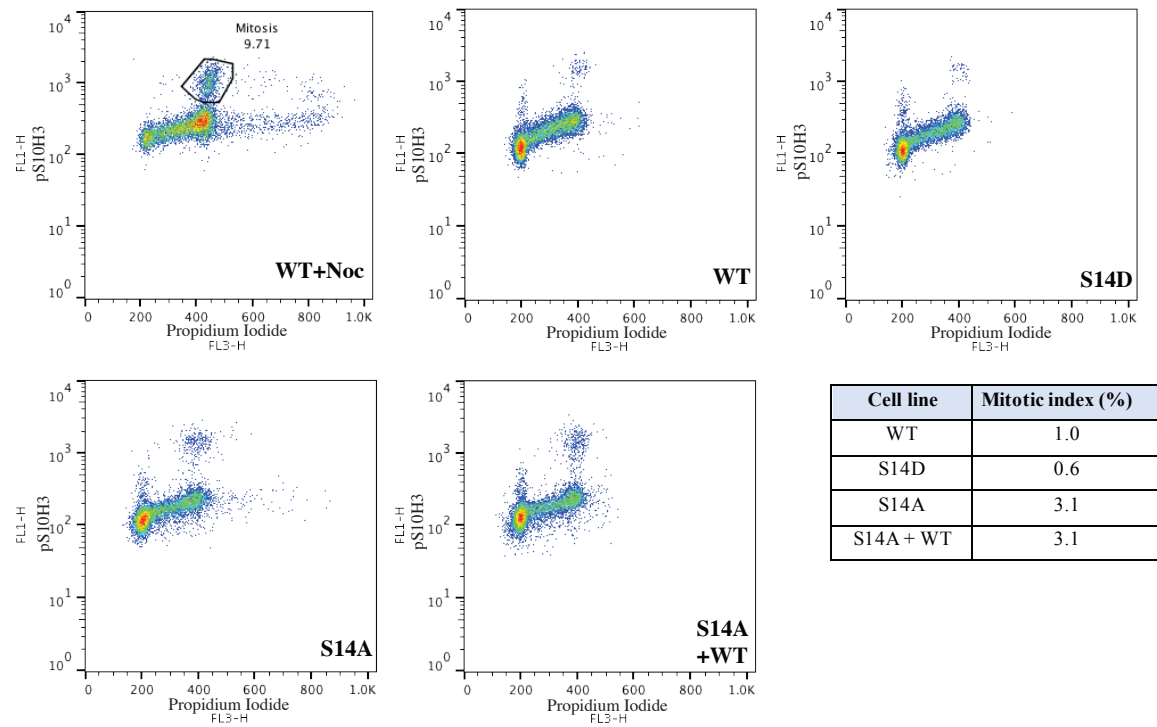


**Figure 4.3 – EdU incorporation in RAD51 phospho-mutants.** Cells were incubated with 10  $\mu$ M EdU for 30 min prior to fixation. (A) Graphical representation of how % EdU increase values listed in table 4.I are calculated. (B) Flow cytometry was used to detect EdU incorporation. Percentage of total cells in each subpopulation are displayed.

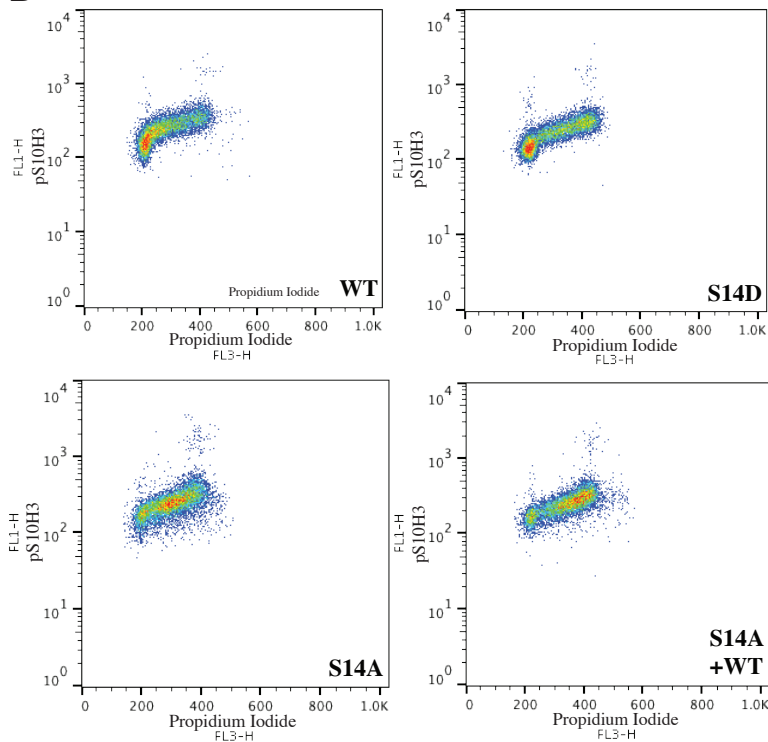
**Table 4.1 – Mean EdU signal in RAD51 phospho-mutants**

| <b>Cell line</b> | <b>Cell population</b> | <b>Mean fluorescence intensity (EdU)</b> |
|------------------|------------------------|------------------------------------------|
| WT               | Non replicating        | 77.3                                     |
|                  | Replicating            | 892                                      |
|                  | Percentage increase    | <b>1054 (%)</b>                          |
| S14D             | Non replicating        | 112                                      |
|                  | Replicating            | 794                                      |
|                  | Percentage increase    | <b>609 (%)</b>                           |
| S14A             | Non replicating        | 133                                      |
|                  | Replicating            | 497                                      |
|                  | Percentage increase    | <b>274 (%)</b>                           |
| S14A +<br>WT     | Non replicating        | 82                                       |
|                  | Replicating            | 840                                      |
|                  | Percentage increase    | <b>924 (%)</b>                           |

**A**



**B**

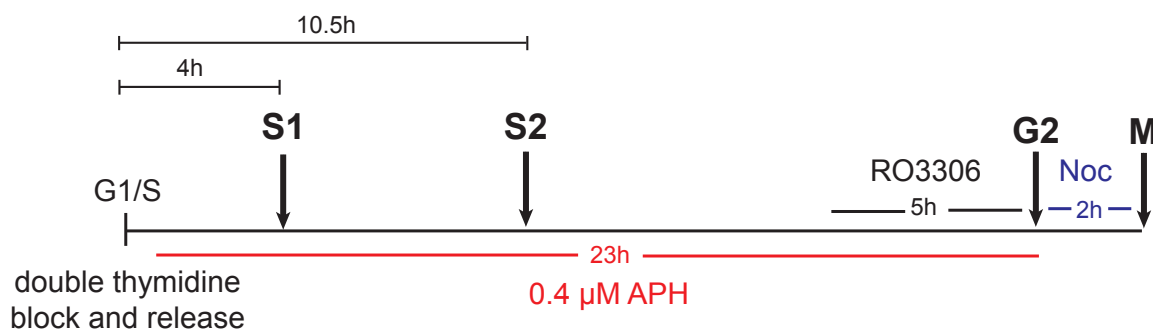


**Figure 4.4 – Cell cycle distribution for untreated and aphidicolin-treated RAD51 phospho-mutants. (A)** Where indicated, cells were treated with 0.33  $\mu$ M Nocodazole for 16 hours. **(B)** Cells were treated with 0.4  $\mu$ M aphidicolin for 23 hours prior to FACS analysis.



### Analysis of RAD51 phospho-mutant recruitment during the cell cycle

The results discussed thus far suggest the importance of RAD51 T13/S14 phosphorylation during replication, which is in line with previously reported evidence for its role at stalled replication forks. However, given that PLK1-dependent RAD51 phosphorylation peaks in G2 phase and mitosis, the phosphorylation-dependent recruitment of RAD51 may be specifically important during the later stages of the cell cycle. To test this, the recruitment of RAD51 was analyzed at the insoluble chromatin fraction for cells enriched in early S phase (S1); late S phase (S2); G2 phase (G2) and mitosis (M) (Figure 4.6). Cells were synchronized and released in the presence of low-dose aphidicolin such that a mild replicative stress is induced in interphase, as summarized in Figure 4.5 and validated by flow cytometry (Figure 4.6 d-e). Unfortunately, synchronization of the S14A and S14A+WT cell line proved problematic, such that only WT and S14D have been analyzed thus far.



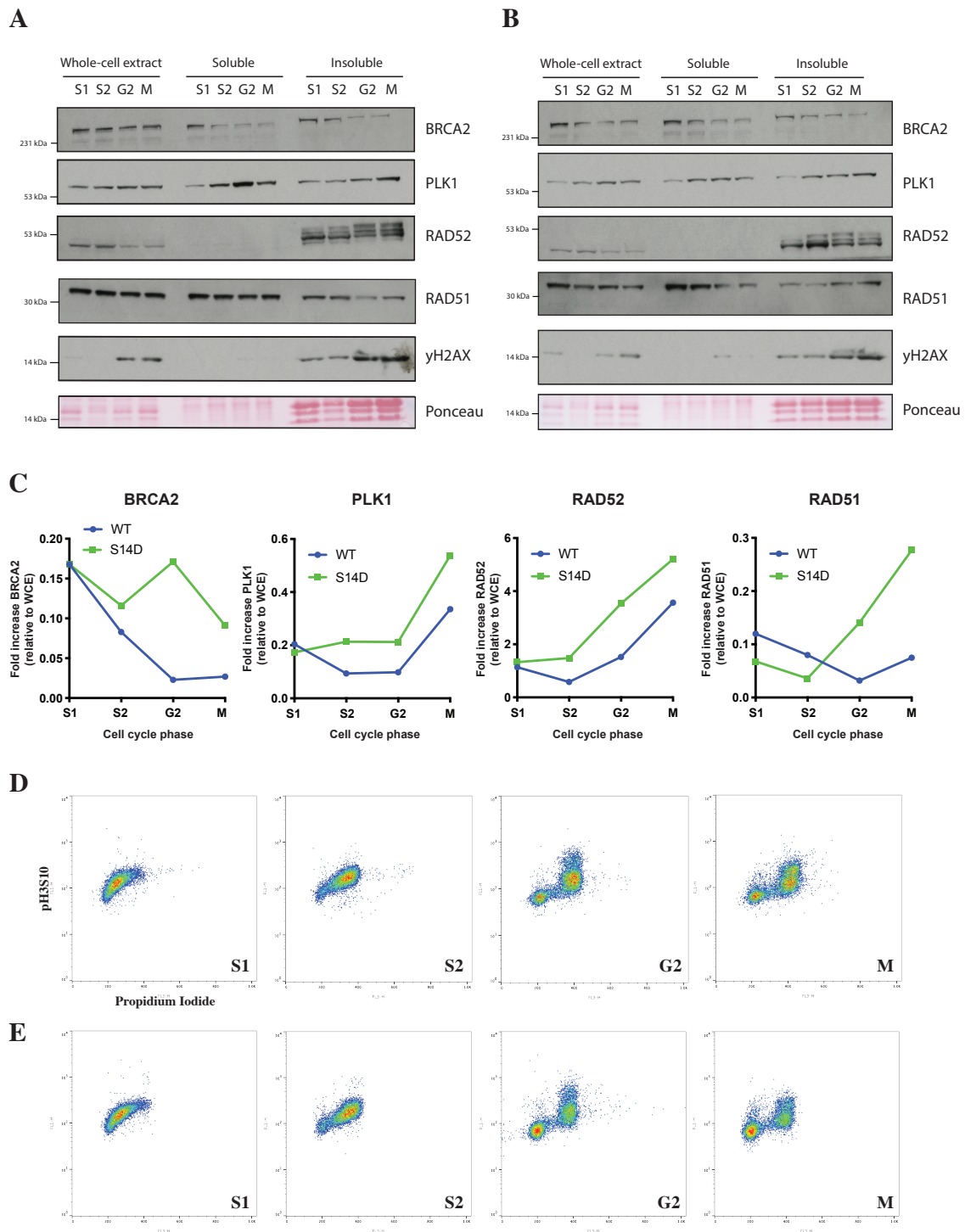
**Figure 4.5 – Graphical summary of synchronization process.** For more details see Methods 2.4.6c.

$\gamma$ H2AX signal was increased in G2 and M fractions, coinciding with the accumulation of mitotic cells as observed by flow cytometry (Figure 4.6 d-e). This suggests that the mild replication stress experienced during S phase leads to DNA damage in mitosis. The recruitment of RAD51 and several other proteins to the chromatin was analyzed in WT and S14D cells (Figure 4.6 a-c). BRCA2 association at chromatin is generally decreased as both WT or S14D cells progress into mitosis, consistent with the previously reported inhibition of BRCA1 recruitment in mitosis<sup>11</sup>. Conversely, RAD52 recruitment at chromatin is increased in the mitotic fractions, consistent with the previously reported role of RAD52 in mitotic DNA synthesis.<sup>12</sup>

Reduced levels of wild-type RAD51 were found in the S2 and G2 insoluble chromatin enriched fractions compared to the S1. RAD51 levels are recovered in the M insoluble fraction,

potentially indicating a mitosis-specific recruitment of RAD51. Relative to the S1 insoluble fraction, RAD51 S14D is decreased in the S2 insoluble fraction, but increases in both the G2 and M insoluble fractions, when mitotic S14D cells are accumulated (Figure 4.6 e). Higher levels of chromatin-bound RAD51 S14D are observed in both the G2 and M fraction compared to wild-type RAD51, which could be indicative of enhanced mitotic RAD51 recruitment conferred by the phospho-mimetic mutation. Unexpectedly, lower levels of chromatin-bound RAD51 S14D are observed in the S1 and S2 fractions compared to wildtype RAD51. Repeated analysis will be necessary to determine whether these trends are reproducible.

All together, these results suggest RAD51 is recruited to mitotic chromatin. Improved synchronization of the RAD51 phospho-mutants will be necessary to determine the importance of RAD51 phosphorylation in this process.



**Figure 4.6 – Protein recruitment to the chromatin fraction in different phases of the cell cycle.** Western blot of the whole cell extract; the soluble fraction and insoluble fraction of (A) WT and (B) S14D cells synchronized in early S phase (S1); late S phase (S2); G2 phase (G2) and mitosis (M). (C) Quantification of (A) and (B). Protein signal was normalized against ponceau loading control and overall protein expression in the corresponding whole cell extract. (D) Flow cytometry control of WT cell synchronization. (E) Flow cytometry control of S14D cell synchronization. .

## 4.3 Discussion

### Impact of RAD51 phospho-mutations on RAD51 recruitment

RAD51 recruitment was analysed in untreated, irradiated and aphidicolin-treated cells. Fewer RAD51 foci were detected in the S14A mutant compared to WT cells under all conditions tested, while RAD51 recruitment was found to be significantly increased in the wildtype complemented S14A cell line (S14A+WT) compared to S14A. It should be noted that this rescue of RAD51 foci formation was only partial - the number of RAD51 foci detected in S14A+WT cells remains significantly lower compared to WT in untreated and irradiated cells. Indeed, given the high level of DNA damage observed in the S14A+WT cell line, RAD51 foci formation may be expected to be increased compared to WT cells. It is therefore likely that RAD51 foci formation is somewhat deficient in the S14A+WT cell line. Indeed, RAD51 foci formation was significantly decreased in the wildtype complemented S14D (S14D+WT) cell line compared to the parental S14D cells, suggesting that exogenous (over)expression of RAD51 may impede efficient RAD51 foci formation. Nevertheless, wildtype RAD51 complementation in the S14A cell line leads to enhanced RAD51 foci formation under all tested conditions, as well as decreased  $\gamma$ H2AX signal intensity upon irradiation. This suggests that, while the exogenous expression of wildtype RAD51 is unlikely to emulate endogenous RAD51 function, it can still partially compensate the lack of RAD51 recruitment in the phospho-null mutant. These results therefore indicate that the loss of S14 phosphorylation negatively impacts RAD51 foci formation. Conversely, RAD51 foci formation was found to be significantly increased in the S14D cell line compared to WT under all conditions tested. The enhanced recruitment of RAD51 in the CRISPR/Cas9-generated S14D mutant is further suggested by the increased survival of this mutant in the presence of replication stress, as discussed below.

All together, these results corroborate previously reported observations indicating the importance of T13/S14 phosphorylation in RAD51 recruitment upon DNA damage.<sup>6,7</sup> However, the observed trends in RAD51 recruitment as described here must be confirmed through biological replicates. It would be interesting to analyse RAD51 recruitment in the RAD51 phospho-mutants upon BRCA2 knock-down, which may make the impact of RAD51 S14 mutation on RAD51 recruitment more pronounced.

### **The RAD51 phospho-mimetic mutation protects against mild replication stress**

The S14A cell line displayed a marked vulnerability to aphidicolin-induced replication stress, even at low doses of aphidicolin. However, this is not rescued by wild-type RAD51 complementation (S14A+WT) such that the S14A genetic background likely imparts an inherent vulnerability to replication stress in these cell lines. Indeed, markedly high levels of  $\gamma$ H2AX signal were observed in both the S14A and S14A+WT cell lines upon aphidicolin treatment. It is interesting to note that the S14A cell lines appear to overexpress PLK1 (as discussed in Chapter 3), which has been implicated in the activation of dormant origins upon replication stress<sup>16</sup>. If so, increased origin firing may lead to RPA exhaustion, which has recently been proposed as a mechanism for replication catastrophe in the absence of checkpoint activation.<sup>17</sup> Further analysis origin firing and checkpoint activation in the S14A mutant could be performed to test this hypothesis.

Exogenous expression of the RAD51 S14D mutant (as analyzed by Yata *et al.*<sup>7</sup>) increases DNA damage-induced RAD51 foci formation and confers increased cell survival upon irradiation and olaparib treatment. Indeed, RAD51 S14D mutation confers enhanced cell survival and decreased  $\gamma$ H2AX signal in the presence of low dose aphidicolin (0.1 and 0.4  $\mu$ M aphidicolin respectively). Conversely, at higher aphidicolin concentrations (1.5-2  $\mu$ M), cell survival appears to be negatively impacted by the S14D mutations. High dose aphidicolin treatment (3  $\mu$ M) has been shown to introduce DSBs even after short (10 min) incubation.<sup>18</sup> Therefore, impaired survival of S14D compared to WT cells occurs under conditions that are presumably more likely to result in replication fork breakage, while enhanced survival of S14D over WT cells is observed at low concentrations of aphidicolin that generally do not induce fork breakage. If this is indeed the case, the opposing impact of RAD51 S14D mutation on cell survival at low- and high- doses of aphidicolin suggests that the RAD51 S14D mutant is proficient in protection of stalled replication forks but impaired in DSB repair (HR-deficient). Indeed, several RAD51 separation-of-function mutants demonstrate that RAD51-mediated fork protection does not require HR-activity and vice versa.<sup>19,20,21,22</sup>

It is interesting to note that the CRISPR/Cas9-generated RAD51 S14D displays somewhat decreased plating efficiency compared to WT cells, perhaps indicating reduced cellular proliferation. Furthermore, Yata *et al.* observed decreased DSB-induced gene targeting in S14D-expressing cells compared to the WT control, suggesting that RAD51 S14D may indeed be impaired in HR.<sup>7</sup> Future experiments should focus on validating the above hypothesis. Pulsed field gel electrophoresis (PFGE) could be used to assess DSB formation and repair in

S14D cells to investigate the proposed S14D-dependent HR-deficiency. Further analysis of S14D cell survival in response to other genotoxic stresses could also be informative. Finally, the mechanism by which S14D potentially impairs homologous recombination should be investigated. Can the inhibitory effect of the S14D mutation be reconstituted in an *in vitro* biochemical reaction? Given that the S14 residue is located in the unstructured N-terminal region of RAD51 (far from the RAD51 ATPase or oligomerization domains) S14D mutation is not expected to directly affect RAD51 filament formation, although this has not yet been tested. However, it is interesting to note that the RAD51 K133R mutation forms hyper-stable RAD51 filaments which impair RAD51 HR-activity but not RAD51-mediated protection at the stalled fork.<sup>24,25</sup> It would be interesting to investigate whether the S14D mutant similarly increases the stability of the RAD51 nucleoprotein filament. Perhaps S14D mutation enhances RAD51 accumulation at the DNA to such an extent that it impacts RAD51 filament stability or impedes the efficient removal of RAD51 from recombination intermediates formed downstream of RAD51-dependent strand invasion. A time-course analysis of RAD51 foci formation and disassembly after DNA damage may reveal whether S14D mutation impacts the persistence of RAD51 foci.

### **Impact of RAD51 phospho-mutations on replication**

Given that replication stress is a major endogenous source of DNA damage, the high levels of  $\gamma$ H2AX signal detected in the S14A mutant may indicate replicative problems. Indeed, preliminary evidence points towards a reduced replication rate in the S14A mutant, as detected by EdU incorporation, which is rescued in the S14A+WT mutant. Interestingly, a slight reduction in EdU incorporation was also detected for the S14D mutant, which potentially accounts for the decreased cell proliferation observed in the S14D mutant, as described above.

Importantly, while the role of RAD51 at stalled or collapsed replication forks has been well-described, the role for RAD51 during unperturbed replication is more contentious. Indeed, RAD51 appears to be recruited to replication forks even in the absence of exogenously-induced replication stress (as detected by iPOND analysis)<sup>6,23,24</sup>. Since several endogenous replication impediments can lead to fork stalling, the accumulation of RAD51 at nascent DNA in unperturbed conditions could simply represent RAD51 recruitment to the (few) forks stalled at inherently difficult-to-replicate regions. However, depletion of BRCA2 or RAD51 from *Xenopus laevis* egg extract was found to generate long (300-400 nt) ssDNA gaps at the replication fork, potentially indicating a role for RAD51 in the maintenance of continuous DNA replication in unperturbed conditions.<sup>4,25</sup> Conversely, no evidence for the involvement of

RAD51 in replication progression was found upon acute degron-mediated depletion of RAD51 in chicken DT40 cells, as neither S phase progression nor replication rate appeared to be affected.<sup>5</sup> In short, the function and importance of RAD51 during unperturbed replication remains unclear and further experiments will be required to elucidate whether a role of RAD51 in fork progression may be responsible for the decreased EdU incorporation observed in the S14A mutant. It will also be interesting to investigate whether a similar phenotype is observed in the RPE1 RAD51 phospho-null mutant. Fibre analysis should be performed to investigate the impact of RAD51 phospho-mutations on replication progression. If the involvement of RAD51 during unperturbed replication is confirmed, it would be interesting to investigate whether the HR- or 'fork protection' activity of RAD51 is specifically important for replication fork progression.

### **Effect of RAD51 phospho-mutation on cell cycle distribution**

Interestingly, no S phase arrest was observed in either S14A or S14D cells under unperturbed conditions, suggesting that whatever the effect of RAD51 phospho-mutation during replication, it does not elicit a full-strength checkpoint response. However, upon low dose aphidicolin treatment, a striking accumulation of S14A cells in S phase is observed, possibly indicative of replication stress-induced checkpoint activation. Surprisingly, while the S14A+WT appears equally vulnerable to aphidicolin-induced replication stress, it does not exhibit the same S phase arrest as observed in S14A cells. Given that both the reduced EdU incorporation as well as the aphidicolin-induced S phase arrest in S14A cells are rescued in the S14A+WT cell line, this suggests that T13/S14-phosphorylated RAD51 supports replication fork progression and that this role is crucial for the completion of replication in the presence of mild replicative stress. Considering that RAD51 recruitment is impaired in S14A cells, replication intermediates may be particularly vulnerable to collapse in this cell line. Indeed, if we consider the possibility that aphidicolin treatment in the S14A genetic background leads to replication catastrophe due to excessive origin firing, as outline above, it is likely that the S14A and S14A+WT cell lines must deal with extremely unstable replication intermediates. The S phase arrest detected in the S14A cell line may indicate a failure to complete replication due to frequent replication fork collapse.

Although increased RAD51 recruitment in the S14A+WT cell line apparently allows for the replication of bulk DNA, the S14A+WT cell line displays a prominent accumulation of cells in G2 phase, indicative of DNA damage-induced checkpoint activation. Perhaps the replication stress encountered in the S14A genetic background is not completely rescued by

wild-type RAD51 complementation, and (while an earlier S phase arrest is avoided) results in a G2/M arrest. It is interesting to note that the S14D mutant also exhibits a slight accumulation of cells in G2 phase upon aphidicolin treatment, perhaps indicative of persistent DNA damage generated upon replication stress. Given that RAD51 was shown to be hyperphosphorylated in the S14A+WT cell line, any HR-deficiency associated with the S14D mutant potentially also applies to the S14A+WT mutant and may explain the G2/M arrest detected in this cell line.

### **Potential role of RAD51 in mitosis**

Preliminary analysis of protein recruitment to the insoluble chromatin fraction of synchronized aphidicolin-treated cells suggests that PLK1, RAD52 and RAD51 are actively recruited to mitotic chromatin. Whether or not T13/S14 phosphorylation is important for RAD51 recruitment in the mitotic chromatin fraction is as-yet inconclusive. Whereas RAD52 was recently implicated in mitotic DNA synthesis at common fragile sites and telomeres, RAD51 function at the mitotic chromatin has never before been demonstrated. Indeed, RAD51 foci disassembly was shown to occur prior to mitotic entry such that RAD51 foci are absent from mitosis, even upon suppression of the G2/M checkpoint in irradiated cells.<sup>26</sup> Various attempts were made during this study to visualize RAD51 foci formation in aphidicolin-treated mitotic cells under different conditions (i.e. different fixation methods, RAD51 antibodies and time points in mitosis). No convincing RAD51 foci formation was observed, even by proximity ligase assay (data not included in thesis). However, it is important to note that not all RAD51 functions correlate with RAD51 foci formation such that the effect of RAD51 depletion, rather than RAD51 foci formation, may be a better indication for RAD51 activity.<sup>2</sup> The absence of RAD51 foci formation in mitosis therefore does not necessarily exclude a possible RAD51 function at mitotic chromatin. Indeed, the impact of RAD51 inhibition during mitosis suggests a function for RAD51 in mitotic DNA synthesis, as will be discussed in Chapter 5. Nevertheless, improved cell synchronization of RAD51 phospho-mutants may allow for further analysis and confirmation of RAD51 recruitment to mitotic chromatin by fractionation. It will also be interesting to investigate whether DNA damage-induced recruitment of PLK1 occurs at the mitotic chromatin and how this impacts RAD51 function in mitosis.

Many results discussed in this chapter are based on a single experiment. Biological replicates will be crucial to conclusively characterize the impact of RAD51 phospho-mutation during the cell cycle. The fact that the S14A cell line is heavily transformed and likely contains secondary mutations further complicated interpretation of the obtained results. Future work should therefore focus on confirmation the described cellular phenotypes during unperturbed



or stressed conditions in other S14A mutants, such as those obtained by CRISPR/Cas9-generated knock-in in eHAP or RPE1 cell lines.

## 4.4 References

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## **Chapter 5**

### **Investigating the role of RAD51 phosphorylation in mitotic DNA synthesis**

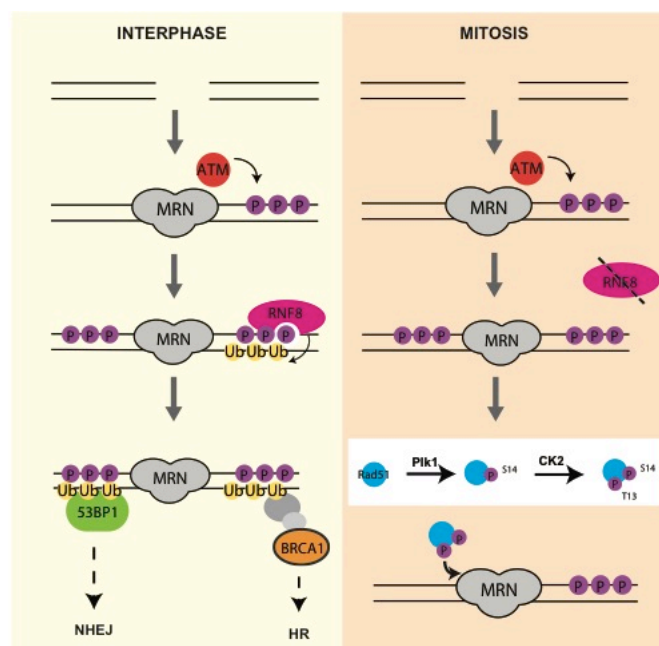


## Summary

The involvement of RAD51 in mitotic DNA synthesis and mitotic progression is investigated in this chapter. Results presented here suggest that RAD51 phosphorylation recruits RAD51 to mitotic chromatin and promotes mitotic DNA synthesis. MUS81 recruitment in mitosis is assessed upon mitotic aphidicolin treatment, as well as the inhibition of RAD51, RAD52 or MRE11 during mitosis. A molecular mechanism is proposed in which RAD52 and MRE11 act upstream to MUS81-mediated fork cleavage. Increased cell survival in S14D cells compared to WT is observed in the presence of MPS1 kinase inhibitor. Considering that S14D confers increased protection against replication stress during S phase, this suggests that the spindle assembly checkpoint (SAC) is important to resolve mitotic DNA damage prior to anaphase. Mitotic duration was found to be increased in the S14A cell line and decreased in the S14D cell line compared to WT, while RAD51 inhibition delays anaphase onset. Conversely, mitotic duration was decreased upon the inhibition of mitotic DNA synthesis by aphidicolin treatment. A model is discussed in which the presence of mitotic DNA breaks and active replication intermediates in mitosis signals to the SAC.

## 5.1 – Introduction

RAD51 T13/S14 phosphorylation peaks in G2 phase and mitosis, suggesting that phosphorylation-dependent recruitment of RAD51 may be especially important during the later phases of the cell cycle.<sup>1,2</sup> Interestingly, phosphorylation of the BRCA2 TR2 domain at residue S3921, which is thought to prevent the formation of stable RAD51 nucleofilaments, similarly peaks in G2 and mitosis.<sup>3</sup> A cell cycle-dependent switch in the mode of RAD51 recruitment can therefore be envisioned, in which RAD51 recruitment is primarily mediated via BRCA2-dependent RAD51 loading during S phase, while phosphorylation-mediated RAD51 recruitment takes over as the cell progresses into mitosis. Importantly, BRCA1 recruitment to DSBs is impaired during mitosis, while recruitment of the MRN complex to mitotic DSBs remains intact.<sup>4</sup> Therefore, RAD51 recruitment during mitosis may heavily rely on the phosphorylation-dependent pathway, rather than via the BRCA1-PALB2-BRCA2 complex formation (Figure 5.1).



**Figure 5.1 – Model for RAD51 recruitment in mitosis.** MRN recruitment and PLK1-dependent RAD51 phosphorylation are proposed to mediate RAD51 recruitment to mitotic DNA damage. See Chapter 1 for more details.

Preliminary evidence discussed in Chapter 4 suggests that RAD51 is actively recruited to mitotic chromatin. Although a mitotic role for RAD51 has not yet been identified, a possible target for mitotic RAD51 recruitment is the induction of DSBs from the cleavage of replication intermediates in mitosis. This has been shown to occur at under-replicated DNA, which is

proposed to undergo a break-induced replication (BIR)-like mechanism to complete replication in early mitosis.<sup>5,6</sup> This process is also termed mitotic DNA synthesis (MiDAS). Several proteins have been shown to mediate MiDAS, including MUS81, RAD52 and POLD3.<sup>6,7,8,9</sup> Interestingly, PLK1 and TOPBP1 have also been found to promote MiDAS. PLK1 inhibition is proposed to affect MiDAS through the altered progression of mitosis, while TOPBP1 was shown to mediate mitotic SLX4 recruitment and is thereby proposed to mediate the resolution of persistent under-replication or DNA recombination intermediates in mitosis.<sup>6,10</sup> However, it is interesting to note that both PLK1 and TOPBP1 promote RAD51 T13/S14 phosphorylation, such that their involvement in MiDAS may also implicate the function of phosphorylated RAD51 at under-replicated DNA in mitosis.<sup>11</sup> I hypothesize that RAD51 phosphorylation is important to recruit RAD51 to under-replicated DNA in mitosis, where RAD51 promotes MiDAS by catalysing strand-invasion and homology search. To test this hypothesis, the formation of mitotic EdU foci was assessed in the CRISPR/Cas9-generated RAD51 phospho-mutants.

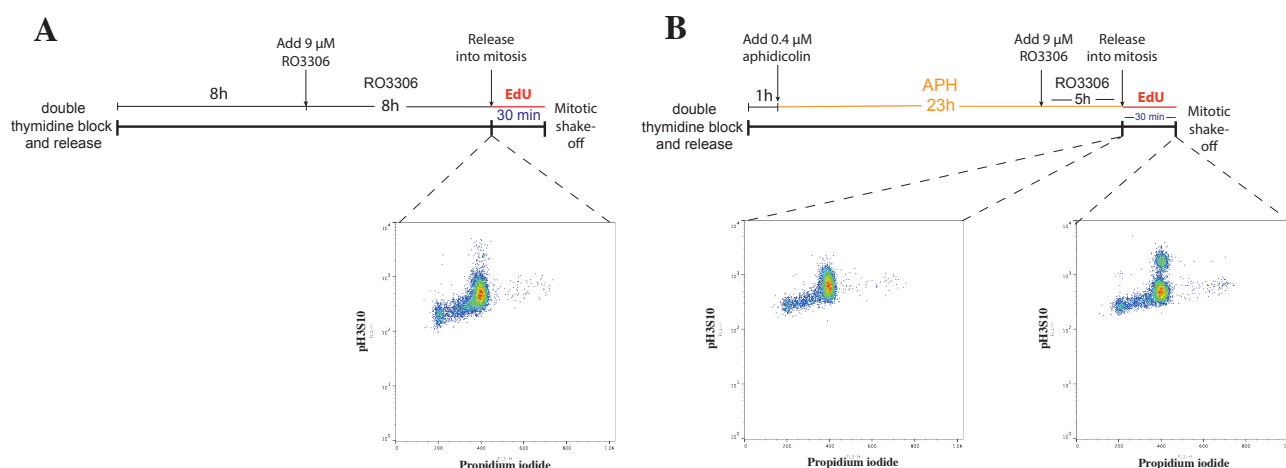
Studies so far have analyzed MiDAS by assessing mitotic EdU incorporation at common fragile sites (CFSs) and telomeres. In this context, CFSs are defined by FANCD2 twin-foci. In this chapter I analyse mitotic DNA synthesis based on the total number of EdU foci detected in the mitotic nucleus. However, it is important to consider the possibility that different molecular mechanisms underlie MiDAS at different genomic loci. In the context of this thesis, MiDAS refers to any mitotic process which results in DNA synthesis during mitosis, regardless of the molecular mechanism or at which genomic loci this occurs.



## 5.2 Results

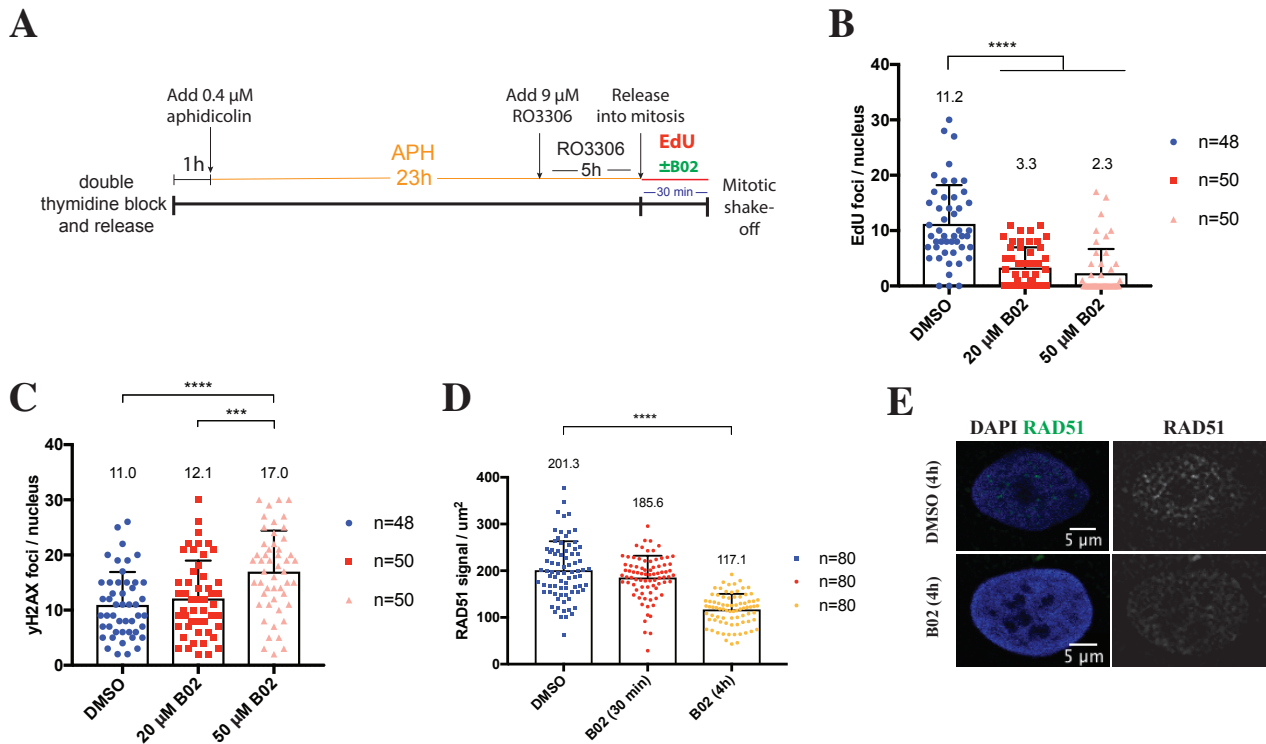
### RAD51 promotes mitotic DNA synthesis

Immunofluorescent visualization of EdU incorporation during mitosis was used as a read-out for MiDAS in this study. Cells were synchronized at G1/S by double thymidine block and released into S phase in medium with or without low dose (0.4  $\mu$ M) aphidicolin. CDK1 inhibitor RO-3306 was added as cells enter G2 phase to further accumulate cells at G2/M phase border, before releasing the cells into mitosis in medium containing EdU. Mitotic cells were collected 30 minutes upon release by mitotic shake off and EdU incorporation was analyzed using Click chemistry. Synchronization was optimized for wild-type U2OS cells in conditions with or without interphase aphidicolin treatment, as shown in Figure 5.2.



**Figure 5.2 – Graphical representation of cell synchronization for analysis of mitotic EdU incorporation.** (A) No aphidicolin added in S phase. FACS analysis of cell synchronization prior to release from RO3306-block is shown. (B) 0.4  $\mu$ M aphidicolin added in S phase. FACS analyses of cell synchronization prior and after 30-min release from RO3306-block are shown. See Chapter 2, methods 2.4.6b for further details.

To assess whether RAD51 is involved in MiDAS, a small molecule inhibitor of RAD51 (B02)<sup>12</sup> was added to the medium when aphidicolin-treated cells were released from RO3306-induced G2 arrest (Figure 5.3 a). Biochemical analysis has shown that B02 inhibits both RAD51 binding to ssDNA during nucleoprotein filament formation, as well as dsDNA binding to a pre-formed RAD51-ssDNA filament (i.e. synaptic complex formation).<sup>13</sup> The use of this small molecule inhibitor allows for the rapid inhibition of RAD51 during mitosis specifically, without affecting RAD51 functions during the previous S phase. The number of EdU foci counted per mitotic nucleus was significantly decreased at 20  $\mu$ M or 50  $\mu$ M concentration of the inhibitor, indicating that RAD51 promotes MiDAS (Figure 5.3 b). A corresponding increase in  $\gamma$ H2AX foci was observed upon mitotic RAD51 inhibition, suggesting that the lack of mitotic RAD51 function increases mitotic DNA damage (Figure 5.3 c).



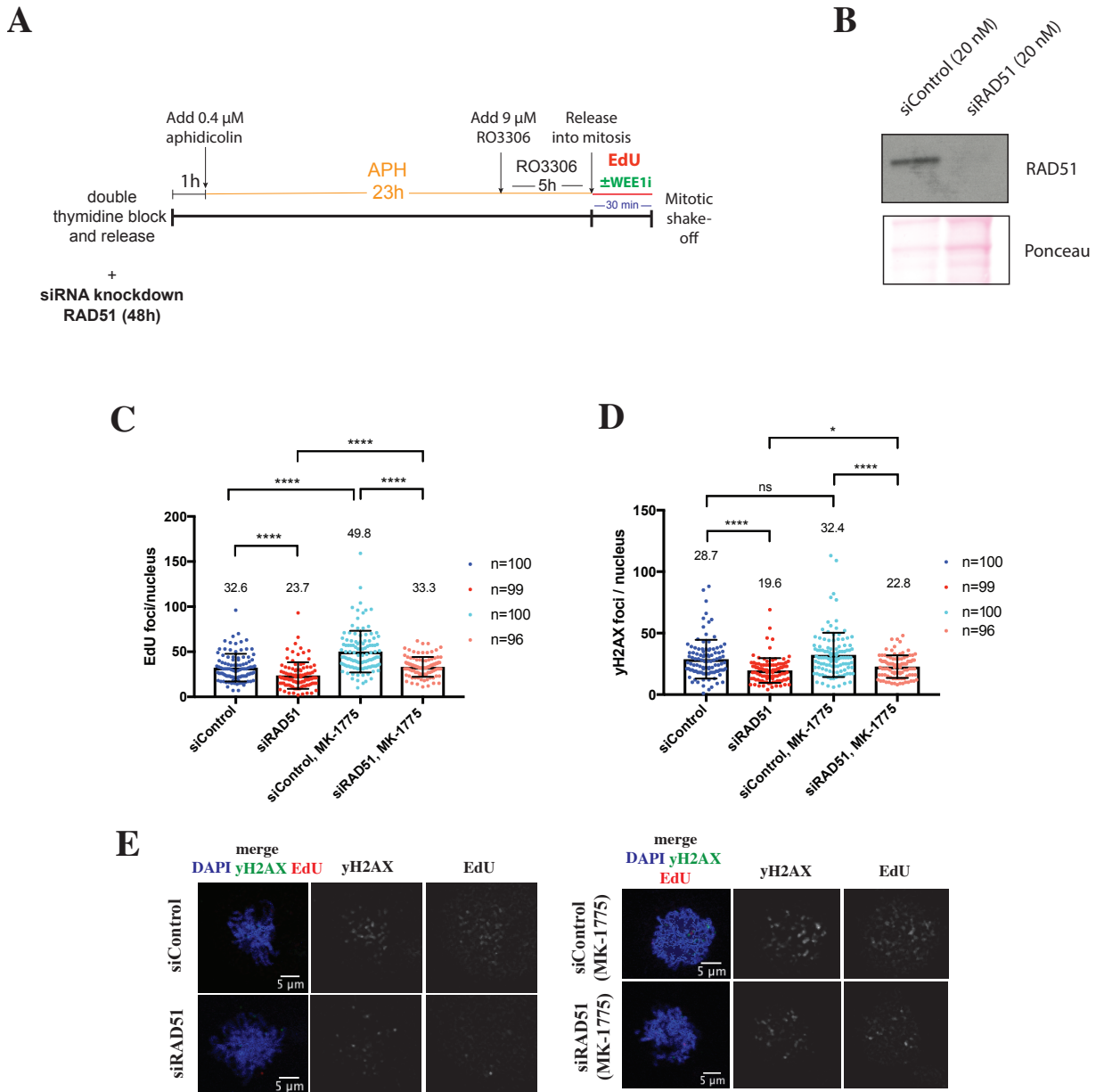
**Figure 5.3 – RAD51 inhibition reduces mitotic EdU incorporation.** (A) Graphical representation of cell synchronization and drug treatment for the analysis of EdU incorporation and  $\gamma$ H2AX foci in (B) and (C). (B) EdU foci or (C)  $\gamma$ H2AX foci per mitotic nucleus were quantified in a DMSO-treated control sample, or upon B02 treatment at 20  $\mu$ M or 50  $\mu$ M concentration. Foci were counted by eye. (D) All cells were incubated for 24 hours with 2 mM HU and treated with DMSO for 4 hours, or with 50  $\mu$ M B02 for 30 min or 4 h prior to fixation and immunofluorescence analysis. RAD51 signal intensity per nucleus was quantified. (E) Representative images of RAD51 signal in DMSO-treated control cells or B02-treated cells, as quantified in (D).

RAD51 inhibition by B02 treatment was validated by assessing RAD51 signal intensity in hydroxyurea (HU)-treated cells (Figure 5.3 d). While 30 minutes of B02 treatment is sufficient to drastically decrease mitotic EdU incorporation (Figure 5.3 b), a significant decrease in RAD51 signal intensity is only observed after 4 hours of B02 treatment (Figure 5.3 d-e). The time frame at which RAD51 inhibition is detected likely depends on whether B02 is added before or after RAD51 recruitment to the DNA break. In HU-treated cells, RAD51 nucleoprotein filaments were likely already established such that the effect of B02 treatment relies on the RAD51 turnover at the break. Since EdU incorporation decreases within 30 min, this suggests that B02 was added before RAD51 recruitment to under-replicated DNA (i.e. *de novo* RAD51 recruitment occurs in mitosis to promote MiDAS).

To further investigate the involvement of RAD51 in MiDAS, siRNA knockdown of RAD51 was performed during the cell synchronization process. EdU incorporation and  $\gamma$ H2AX foci formation were assessed in RAD51-depleted mitotic cells (Figure 5.4 c-d). Contrary to

using a small molecule inhibitor, siRNA-mediated RAD51 knockdown is not immediate and cannot be limited to mitosis. To achieve efficient RAD51 knockdown, siRNA transfection was performed 48 hours prior to mitotic shake-off, such that RAD51 depletion likely occurs throughout the cell cycle (Figure 5.4 b). Mitotic EdU incorporation was significantly reduced in the siRAD51 treated cells compared to the siControl treated sample (Figure 5.4 c). However, mitotic  $\gamma$ H2AX signal was similarly decreased upon RAD51 knockdown (Figure 5.4 d, Supplementary Figure 5.1). The reduced EdU incorporation observed upon RAD51 knockdown may therefore simply reflect a reduction in mitotic DNA damage. Given that RAD51 depletion induces G2 arrest, cells that experience efficient RAD51 knockdown may not be collected by mitotic shake off.<sup>14,15</sup> Perhaps this bias in the population of mitotic cells collected partially accounts for the differences observed in mitotic EdU incorporation and mitotic DNA damage – in the absence of RAD51-mediated fork stabilization, fork breakage and subsequent G2/M arrest likely occurs in those cells experiencing replication stress, such that overall ‘healthier’ cells may be collected through mitotic shake-off in the RAD51 depleted sample. In order to circumvent this potential bias, WEE1 inhibitor was used to override the DNA damage-induced G2/M checkpoint in RAD51 depleted cells and presumably enhance the collection of mitotic cells with efficient RAD51 knockdown. WEE1 inhibitor was added upon release from the RO3306-induced G2 arrest (Figure 5.4 a). In RAD51 depleted cells,  $\gamma$ H2AX was significantly increased upon WEE1 inhibition, potentially indicating the accumulation of damaged cells that are otherwise arrested by the G2/M checkpoint (Figure 5.4 d). However, mitotic DNA damage remained significantly lower in RAD51 depleted cells compared to control cells, perhaps indicating that 30 minutes of WEE1 inhibitor treatment is not sufficient to abrogate the proposed bias in mitotic cells collected. Nevertheless, WEE1 inhibition does enhance the overall population of cells entering mitosis, both in siControl and siRAD51 treated cells, thereby indicating that the G2/M checkpoint is at least partially suppressed (Supplementary Figure 5.2).

Unexpectedly, mitotic EdU incorporation was significantly increased upon WEE1 inhibition in both siControl and siRAD51 treated cells. Given that WEE1 inhibition increases the population of cells entering mitosis in both samples, this increase in mitotic EdU incorporation may simply reflect the entry of severely under-replicated cells into mitosis, which would normally arrest in G2. However, WEE1 inhibition did not increase mitotic DNA damage in the siControl treated cells (Figure 5.4 d). This introduces the intriguing possibility that WEE1 normally inhibits MiDAS during mitosis. Perhaps WEE1 inhibition leads to hyper-activation of CDK and origin firing in mitosis (similar to what has been reported in S phase<sup>16</sup>)



**Figure 5.4 – Analysis of mitotic EdU incorporation in RAD51 depleted cells.** (A) Graphical representation of cell synchronization and drug treatment for the analysis of EdU incorporation and  $\gamma$ H2AX foci in RAD51 depleted cells, 48 hours after siRNA transfection, as quantified in (D) and (E). (B) Western blot analysis of RAD51 knockdown, 48h after transfection. (C) Cells were treated with the indicated siRNA. Where indicated cells were treated with 100 nM MK-1775 (WEE1 inhibitor) when released from G2 arrest. EdU foci were quantified per mitotic nucleus. (D) Quantification of  $\gamma$ H2AX foci per mitotic nucleus for the same experimental conditions as in (C). (E) Representative images of EdU and  $\gamma$ H2AX foci as quantified in (C) and (D).

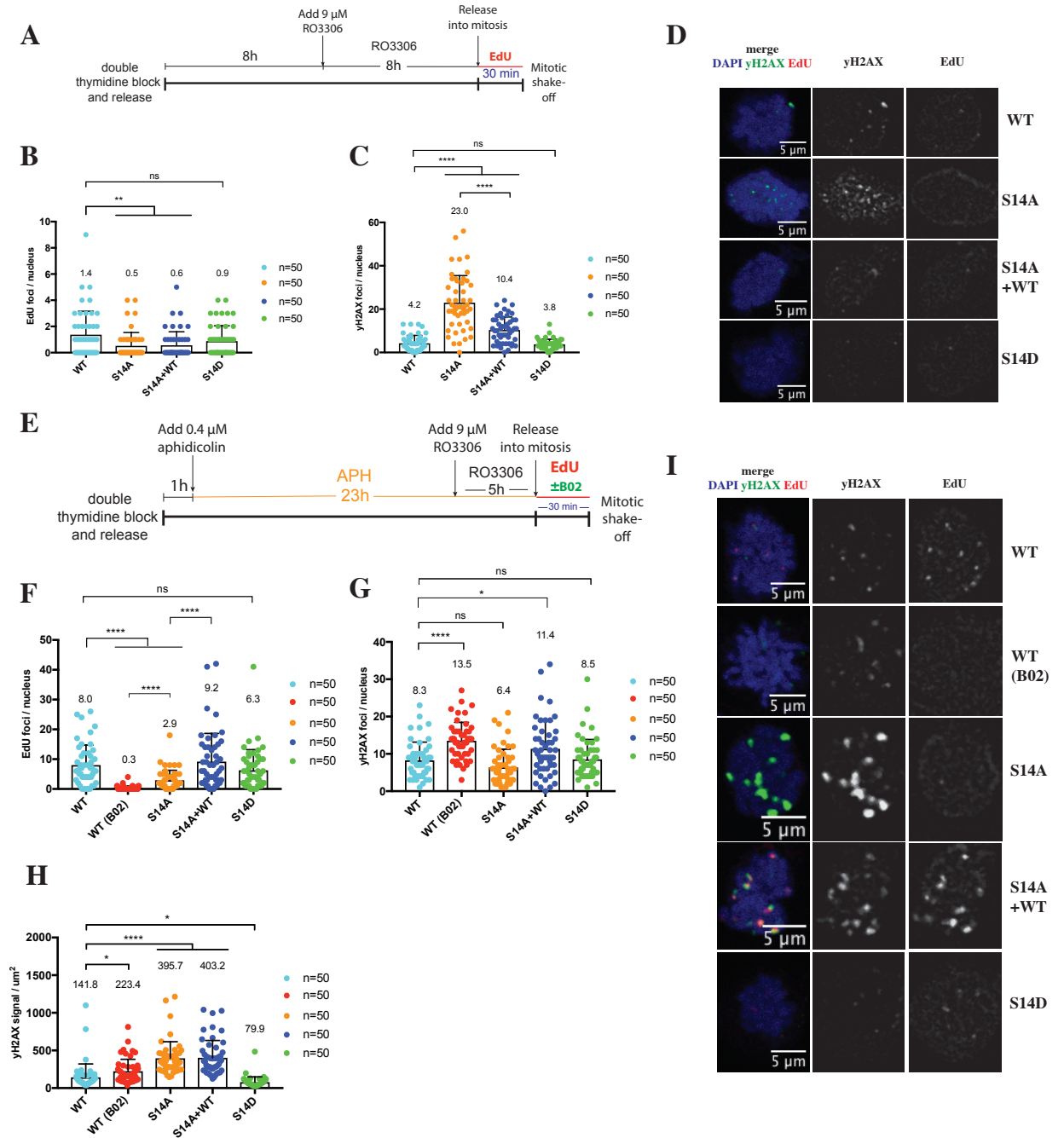
thereby promoting increased mitotic EdU incorporation in a break-independent manner. Future research should focus on validating the possibility of CDK-dependent origin firing during mitosis.

## **RAD51 phosphorylation promotes mitotic DNA synthesis**

RAD51 recruitment is predicted to depend heavily on RAD51 T13/S14 phosphorylation during mitosis. As discussed in Chapter 4, RAD51 foci formation was not observed during mitosis. However, given that RAD51 promotes EdU incorporation in mitosis, this provides an alternative read-out for the recruitment of RAD51 during mitosis. Mitotic EdU incorporation was therefore analyzed in the CRISPR/Cas9-generated U2OS RAD51 phospho-mutants.

Few EdU foci were detected in mitotic cells synchronized in the absence of low dose aphidicolin in S phase (Figure 5.5 a, b). A significant decrease in EdU foci was observed in the S14A cell line compared to WT, which was not rescued in the wild-type complemented S14A cell line (S14A+WT). However,  $\gamma$ H2AX foci were significantly increased in mitotic S14A cells compared to WT, while this increase in mitotic DNA damage was partially rescued in the S14A+WT cell line (Figure 5.5 c-d). This suggests that RAD51 T13/S14 phosphorylation prevents the accumulation of mitotic DNA damage, likely due to the enhanced (RAD51-mediated) protection against replication stress during S phase in the WT complemented cell line. The fact that S14A cells do not display increased mitotic EdU incorporation despite exhibiting increased levels of mitotic DNA damage may indicate a decreased ability to repair DNA breaks during mitosis in this cell line.

Mitotic EdU incorporation was further analyzed upon cell synchronization in the presence of low dose aphidicolin in S phase (Figure 5.5 e). The addition of low dose aphidicolin in S phase further impairs the timely completion of DNA replication prior to mitotic entry, thereby enhancing MiDAS, as reflected by the increase in mitotic EdU foci detected in all cell lines (Figure 5.5 f). Under these conditions, mitotic EdU incorporation was significantly decreased in the S14A cell line compared to WT. However, the level of mitotic EdU incorporation was rescued in the S14A+WT cell line, indicating that MiDAS is impaired in the absence of phosphorylated RAD51. Consistent with earlier data, mitotic EdU incorporation is also significantly decreased upon RAD51 inhibition (B02 treatment) during mitosis. Although a significant increase in  $\gamma$ H2AX foci is observed upon mitotic RAD51 inhibition, no significant difference in  $\gamma$ H2AX foci is observed in S14A cells compared to WT (Figure 5.5 g). However, as shown in Figure 5.5 j, the  $\gamma$ H2AX foci in both the S14A and S14A+WT cell lines are bigger and brighter than in the WT or S14D cell lines. Indeed, when analysing the overall intensity of  $\gamma$ H2AX signal in the different cell lines, a drastic increase is observed in the S14A and S14A+WT cell lines compared to the WT, suggesting that these cell lines experience higher replication stress and concomitant mitotic DNA damage (Figure 5.5 h). A significant decrease in the intensity of  $\gamma$ H2AX signal was detected in the S14D mutant compared to WT, consistent with



**Figure 5.5- Mitotic EdU incorporation in U2OS RAD51 phospho-mutants.** (A) Graphical representation of cell synchronization for the analysis of mitotic EdU incorporation in the absence of aphidicolin. (B) EdU foci per mitotic nucleus were quantified in the indicated cell lines. (C)  $\gamma$ H2AX foci per nucleus were quantified for the same experimental conditions as in (B). (D) Representative images of data analysed in (A-B). (E) Graphical representation of cell synchronization and drug treatment for the analysis of mitotic EdU incorporation in the presence of low dose aphidicolin. (F) EdU foci per mitotic nucleus were quantified in the indicated cell lines. Where indicated cells were treated with 50  $\mu$ M B02 when released from G2 arrest. (G)  $\gamma$ H2AX foci per nucleus were quantified for the same experimental conditions as in (F). (H) The pixel value of  $\gamma$ H2AX signal per nuclear area, for the same experimental data as displayed in (G). (I) Representative images of data analysed in (F-H).

the idea that S14D mutation protects against the consequences of replication stress and decreases the extent of DNA under-replication upon mitotic entry. Interestingly, mitotic EdU incorporation was found to be significantly lower in B02-treated cells compared to S14A cells (Figure 5.5 f), perhaps suggesting that some mitotic RAD51 recruitment can still be achieved in the absence of T13/S14 phosphorylation. Nevertheless, the reduced mitotic EdU incorporation in the S14A cell line is rescued in the S14A+WT cell lines, suggesting that RAD T13/S14 phosphorylation is important to recruit RAD51 to under-replicated DNA in mitosis and promote MiDAS.

### **RAD52 and MRE11 inhibition reduces mitotic MUS81 foci formation in WT cells**

Previous studies have identified several key players in MiDAS, as recently reviewed by Özer and Hickson.<sup>9</sup> RAD52 and MUS81 depletion was shown to significantly reduce MiDAS at FANCD2 twin-foci, while inhibition of RAD52 or MRE11 activity has been reported to significantly reduce MiDAS at telomeres.<sup>7,8</sup> MiDAS is proposed to occur via a BIR-like mechanism at the collapsed replication fork, in which RAD52 mediates strand-annealing of the broken DNA end to the repair template. Interestingly, RAD52 was shown to promote MUS81 and POLD3 recruitment, thereby placing RAD52 upstream of MUS81 and POLD3 activities in the MiDAS pathway.<sup>7</sup> Given that MUS81 depletion leads to a reduction in mitotic EdU incorporation, MUS81-mediated fork collapse likely generates the DSB substrate for subsequent BIR-mediated MiDAS.<sup>6,17</sup> Taken together, this suggests RAD52 promotes MiDAS by acting upstream to replication fork collapse. However, given that MUS81 has also been implicated in the resolution of Holliday junction (HJ)-containing recombination intermediates,<sup>18</sup> the decrease in MUS81 recruitment may also stem from reduced mitotic recombination in the absence of RAD52.

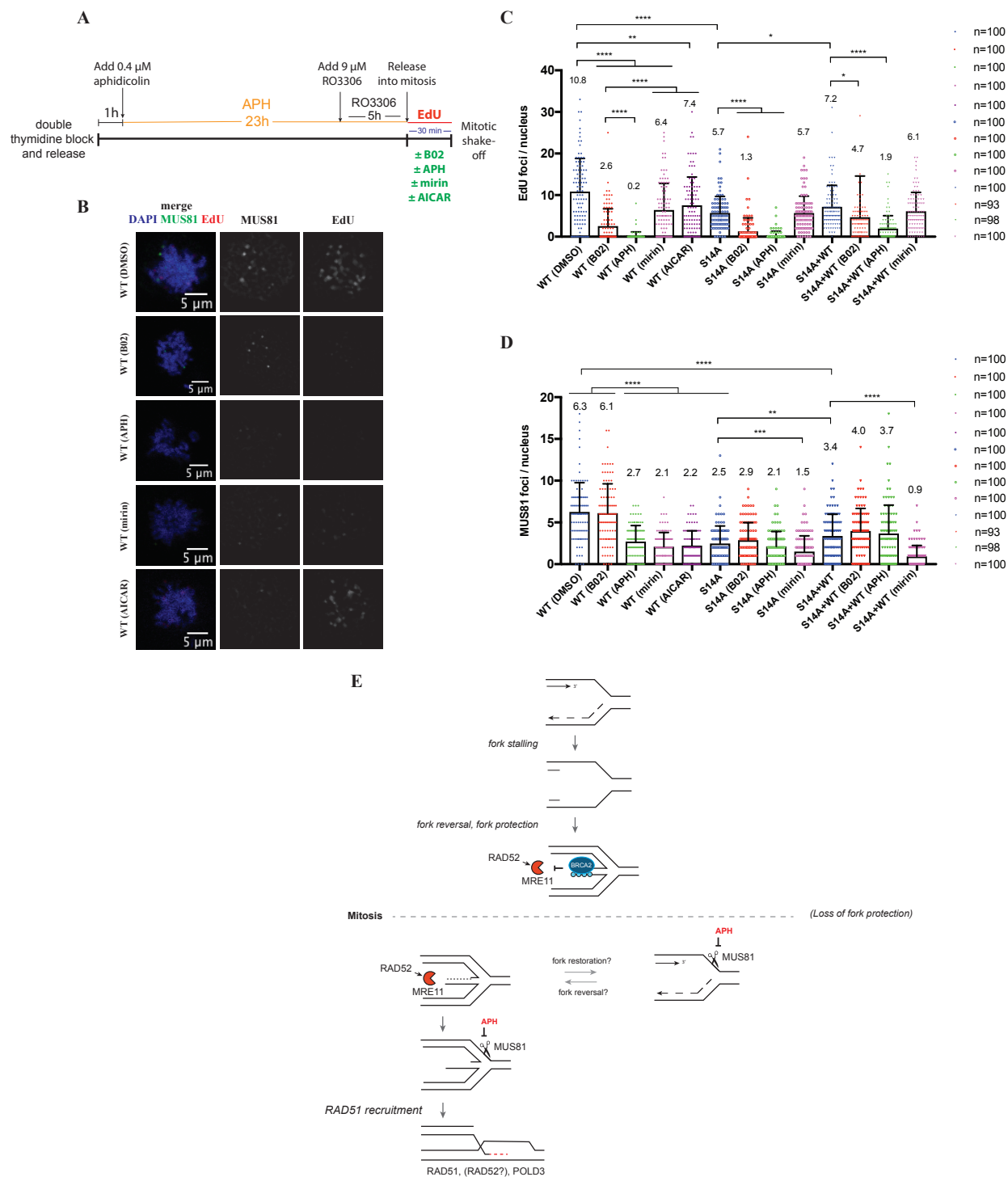
To further elucidate at which stage RAD52, RAD51, MRE11 and MUS81 might act in the MiDAS pathway, mitotic EdU incorporation and MUS81 recruitment were analyzed upon the inhibition of RAD51, RAD52, MRE11 and replicative polymerases in mitosis by the addition of B02, AICAR, mirin and aphidicolin respectively (Figure 5.6). Mitotic EdU incorporation is significantly reduced upon all the above drug treatments in WT cells (Figure 5.6 b, c). Indeed, MiDAS was most efficiently inhibited by the addition of high dose (2  $\mu$ M) aphidicolin in mitosis. Consistent with previous experiments, mitotic RAD51 inhibition lead to a drastic reduction in mitotic EdU incorporation. Mitotic MRE11 or RAD52 inhibition also significantly decreased mitotic EdU incorporation in WT cells, albeit to a lesser extent compared to RAD51

inhibition or mitotic aphidicolin treatment. The concomitant effect on MUS81 recruitment is quantified in Figure 5.6 d. As mentioned above, MUS81 recruitment may represent MUS81 accumulation at (i) stalled replication forks or (ii) recombination intermediates. Interestingly, while RAD51 inhibition clearly impairs mitotic EdU incorporation, it did not affect MUS81 recruitment compared to the DMSO-treated control. Assuming that RAD51 acts to promote MiDAS via a mitotic recombination process, the decrease of RAD51-mediated recombination in mitosis did not impact MUS81 recruitment. The MUS81 foci analyzed here are therefore unlikely to represent the downstream accumulation of the protein at recombination intermediates, and must therefore indicate MUS81 accumulation at stalled fork structures. MUS81 recruitment was significantly reduced upon mitotic MRE11 or RAD52 inhibition, as well as mitotic aphidicolin treatment. This suggests that MRE11, RAD52 and DNA synthesis may play a role in the recruitment of MUS81 to stalled replication forks in mitosis. Interestingly, a recent study suggests a cooperative role for RAD52 and MRE11 in the degradation of reversed replication forks in the absence of BRCA2-mediated fork stabilization, which leads to increased chromosome breakage.<sup>19</sup>

Considering that BRCA2 recruitment to mitotic chromatin is thought to be inhibited during mitosis, perhaps RAD52- and MRE11-dependent degradation at the reversed fork promotes MUS81 recruitment. Indeed, Lemaçon et al. propose that MRE11-dependent DNA degradation at the reversed fork generates a MUS81-cleavable DNA substrate.<sup>20</sup> A possible model for the role of RAD52 and MRE11 in MiDAS can therefore be envisioned in which their combined action at reversed replication forks promotes MUS81-mediated fork collapse and subsequent BIR-mediated repair (Figure 5.6 e). Since fork reversal is thought to be a reversible process, it is possible that the reversed replication fork is ‘restored’ to a normal fork structure.<sup>21</sup> Fork restoration may provide an alternative substrate for MUS81 cleavage which does not depend on MRE11- and RAD52-mediated degradation of nascent DNA and may explain why the inhibition of MRE11 and RAD52 only leads to a mild reduction of MiDAS. Alternatively, perhaps only a subset of stalled forks is reversed when the cell enters mitosis. Unexpectedly, mitotic aphidicolin treatment also significantly decreases MUS81 recruitment, suggesting that DNA polymerases do not exclusively act downstream of collapsed replication forks. It is interesting to note that, under conditions of aphidicolin-induced replication stress, POLD1 and POLD3 remain associated to chromatin in G2 and mitosis.<sup>6</sup> This suggests that the replisome may remain associated to at least a subset of stalled replication forks upon mitotic entry. Perhaps mitotic aphidicolin treatment maintains replisome association at stalled replication forks in such a way that MUS81 is prevented from accessing and cleaving reversed replication



forks. Restored replication forks possibly attempt to resume replication in mitosis without requiring prior fork collapse. However, active replication forks are readily cleaved by MUS81 and are specifically vulnerable to breakage in mitosis, given the mitosis-specific stimulation of MUS81 and other resolvases.<sup>22,23</sup> In general, I propose that polymerase stalling during mitosis prevents MUS81 from accessing and cleaving the replication fork, such that mitotic aphidicolin treatment inhibits MUS81 recruitment, potentially at either reversed or ‘restored’ replication forks (Figure 5.6 e). Interestingly, the addition of B02, aphidicolin or mirin during mitosis affect EdU incorporation and MUS81 recruitment differently in the S14A genetic background. It is important to note that the number of MUS81 foci are significantly reduced in both the S14A and S14A+WT cell lines compared to WT, perhaps indicating that fork collapse is less dependent on MUS81 activity in these cell lines. As described in Chapter 4, these cell lines exhibit extreme vulnerability to aphidicolin treatment (potentially indicative of replication catastrophe). It is not unlikely that some fork breakage occurs prior to mitotic entry. This may also account for the high level of  $\gamma$ H2AX signal observed in mitotic S14A and S14A+WT cells. MUS81 recruitment in S14A and S14A+WT cells appears to be further reduced upon MRE11 inhibition, but not mitotic aphidicolin treatment. The latter observation possibly suggests that the replisome does not remain associated to those replication intermediates accumulated in the S14A genetic background, such that aphidicolin treatment does not protect against MUS81-dependent fork cleavage. I propose that the majority of stalled replication forks in S14A and S14A+WT cells are collapsed, such that any replication restart must proceed via a BIR-like mechanism. I therefore hypothesize that the reduction in mitotic EdU incorporation in the S14A cell line compared to the WT stems from a deficiency in BIR-mediated MiDAS, which is rescued upon complementation with wildtype RAD51. It must be emphasized that the above models are based on the assumption that RAD51 mediates MiDAS through a role in BIR. However, perhaps a break-independent manner of replication restart (or initiation) can take place in mitosis. It cannot be ruled out that RAD51 promotes this hypothetical break-independent form of MiDAS. To better understand the requirement of MUS81 activity for mitotic EdU incorporation, it will be interesting to investigate how MUS81 depletion affects MiDAS in the different CRISPR/Cas9-generated RAD51 mutants.

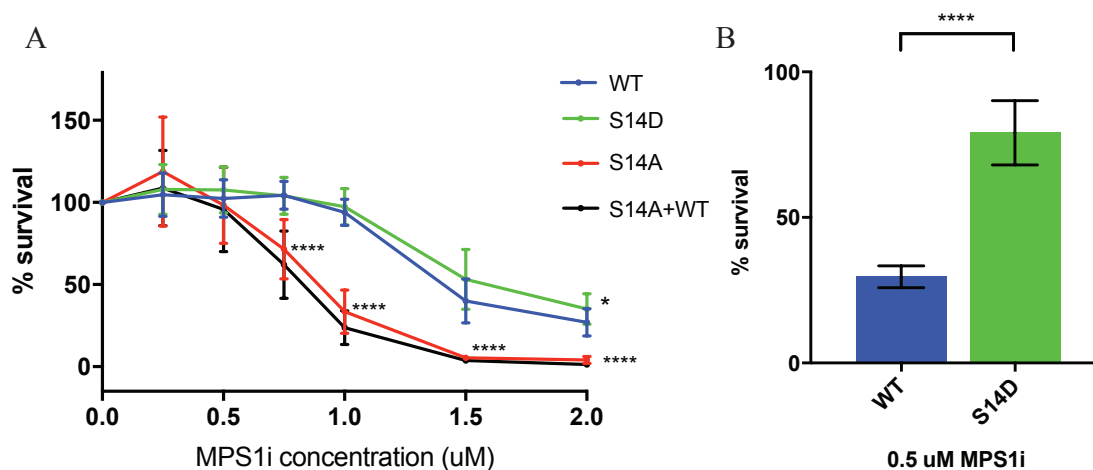


**Figure 5.6 – MUS81 recruitment in U2OS RAD51 phospho-mutants.** (A) Graphical representation of cell synchronization for the analysis quantified in (C) and (D). (B) Representative images of data analysed in (C) and (D). (C) EdU foci and (D) MUS81 foci per mitotic nucleus were quantified in the indicated cell lines. Where indicated cells were treated with 50  $\mu$ M B02; 2  $\mu$ M APH; 50  $\mu$ M mirin or 50 M AICAR. (E) Proposed MiDAS mechanism.

## RAD51 S14D mutant is less sensitive to spindle assembly checkpoint inhibition

Upon mitotic entry, the spindle assembly checkpoint (SAC) is activated and prevents metaphase-anaphase transition until the mitotic spindle (composed of microtubules) is correctly anchored at the centromere of each sister chromatid. This is achieved by microtubule attachments to the kinetochore, a large protein complex that is formed at the centromere. Bioriented kinetochore-microtubule attachments are required for the successful separation of sister chromatids during anaphase. Once properly established, kinetochore-microtubule attachments silence the SAC to initiate chromosome segregation. In this way, the SAC ensures that chromosomes are properly aligned and attached to the mitotic spindle prior to anaphase onset.

The inhibition of MiDAS has been shown to decrease cell survival and increase chromosome mis-segregation and non-disjunction, suggesting that the inability to resolve under-replication prior to anaphase is detrimental to cell survival.<sup>6</sup> If so, premature anaphase onset upon SAC inhibition is expected to aggravate the negative consequences of under-replication. Cell survival of the RAD51 phospho-mutants was assessed in the presence of an MPS1 kinase inhibitor, which inactivates the SAC. The S14A cell line exhibited an extreme sensitivity to MPS1 inhibition, which was not rescued by complementation of wild-type RAD51 (S14A+WT) (Figure 5.7 a).



**Figure 5.7 – Analysis of cell viability in the presence of MPS1 inhibitor.** (A) Cell survival of RAD51 phospho-mutants was analyzed in the presence of MPS1 inhibitor (AZ3146) by WST-1 assay at indicated concentrations. Error bars (SD),  $n=3$ . (B) Cell survival of RAD51 phospho-mutants was analyzed in the presence of 0.5  $\mu$ M MPS1 inhibitor by clonogenic assay. Error bars (SD),  $n=2$ .

Conversely, a significant increase in cell survival was observed for the S14D cell line relative to WT at high concentrations of the MPS1 inhibitor (2  $\mu$ M). Clonogenic survival upon

MPS1 inhibition is also significantly increased in the S14D mutant relative to WT (Figure 5.7 b). Indeed, the S14A and S14A+WT cell lines were previously found to exhibit the highest level of mitotic DNA damage (Figure 5.5 c), presumably due to the increased replication stress experienced in the S14A genetic background. The increased cell survival observed for the S14D cell line upon MPS1 inhibition supports the notion that S14D mutation protect against the consequences of replication stress, thereby reducing the amount of under-replication remaining upon mitotic entry.

Indeed, the trend in cell survival observed upon SAC inhibition follows the trends previously observed upon aphidicolin treatment (as shown in Chapter 4), suggesting that the importance of SAC activity may be directly related to the cell's ability to cope with replication stress.

### **RAD51 inhibition delays anaphase onset**

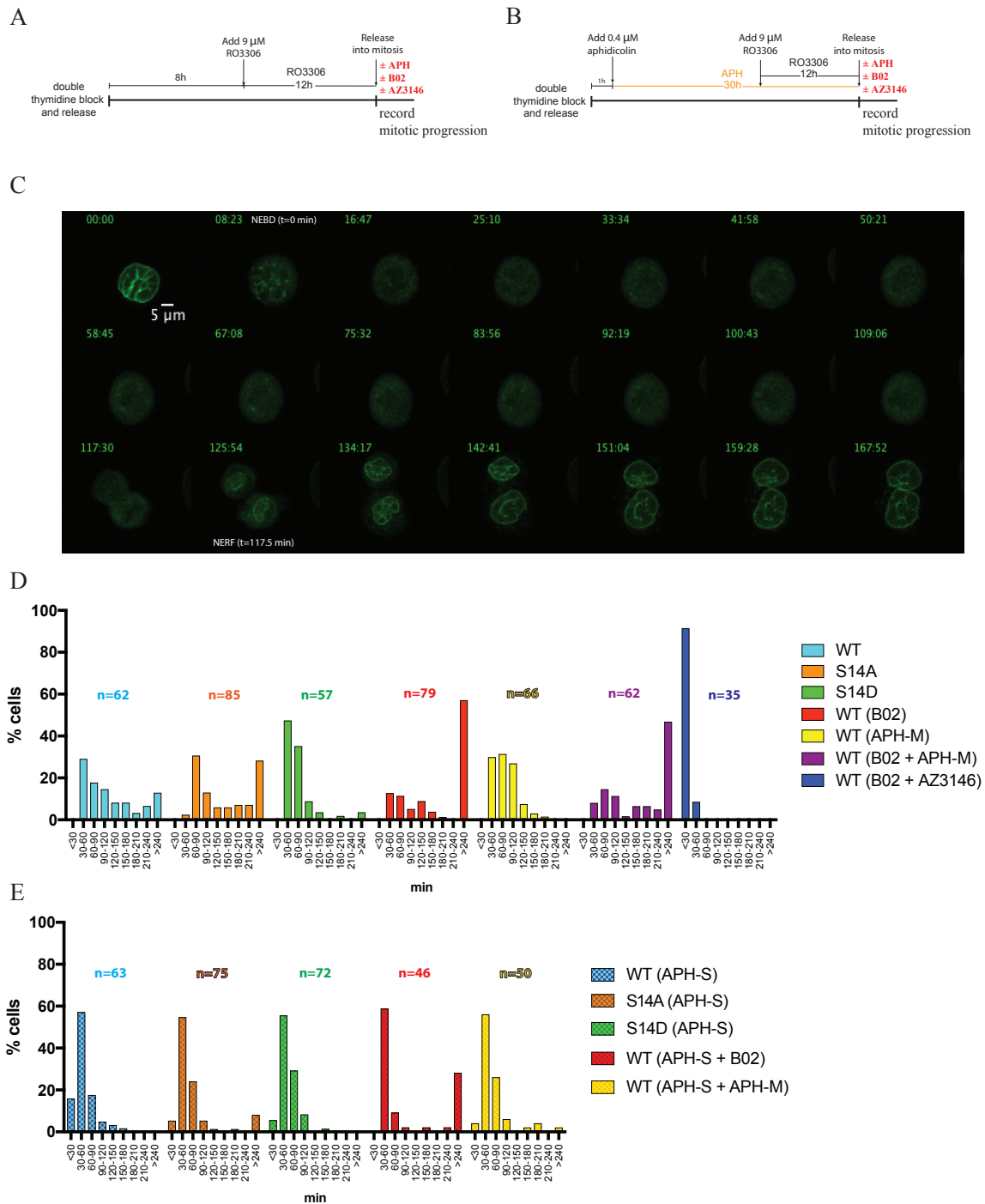
Interestingly, an increased mitotic index was observed for the S14A and S14A+WT cell lines compared to WT (as described in Chapter 4), suggesting that these cells take longer to complete mitosis, while the S14D cell line displays a reduced mitotic index compared to WT. RAD51 phospho-mutation apparently impacts mitotic duration, possibly via altered SAC activity. One intriguing possibility is that the SAC may 'sense' mitotic DNA damage or under-replication, thereby preventing SAC silencing until MiDAS has been completed.

To assess mitotic duration more directly, CRISPR/Cas9-generated RAD51 phospho-mutants were stably transfected with GFP-Lamin and analyzed by live microscopy. This allows for the detection of nuclear envelope breakdown (NEBD) and nuclear envelope reformation (NERF), which serve as markers for mitotic duration. Cells were synchronized by double thymidine block-and-release combined with RO3306-induced G2 arrest (Figure 5.8 a). Upon release from G2 arrest, cells were followed during mitosis to monitor the time taken from NEBD to NERF (Figure 5.8 c). As previously suggested by FACS analysis, mitotic duration indeed appears to be increased in a subset of S14A cells compared to WT. Conversely, mitotic duration appears to be decreased in the S14D cell line compared to WT. Given that S14A cells exhibit higher levels of endogenous replication stress than WT cells - and that the S14D mutation appears to protect against the consequences of replication stress - the trends in mitotic progression observed in the RAD51 phospho-mutants potentially arise from the level of replication stress experienced in each cell line. Alternatively, mitotic duration is directly impacted by the effect of S14 mutation on RAD51 recruitment during mitosis.

B02 treatment was used to investigate the effect of impaired mitotic RAD51 recruitment on mitotic duration. Interestingly, RAD51 inhibition drastically increases mitotic duration for the majority of cells. This delay in mitotic progression is abolished upon combined treatment with the MPS1 inhibitor. Given that RAD51 inhibition prevents efficient MiDAS, these results support the hypothesis that remaining under-replication in mitosis results in the persistent activation of the SAC. To further corroborate this, MiDAS was inhibited by the addition of 2  $\mu$ M aphidicolin in mitosis and mitotic duration was analyzed (APH-M). Surprisingly, contrary to the effect of B02 treatment, mitotic aphidicolin treatment decreases mitotic duration. Combined treatment of B02 and aphidicolin in mitosis reinstates delayed mitotic progression (> 240 min) in the majority of cells, indicating that the effect of RAD51 inhibition in mitosis dominates that of aphidicolin. If the model for MiDAS as depicted in Figure 5.6 e is considered, the inhibition of RAD51 would lead to an accumulation of mitotic DNA breaks. Mitotic aphidicolin treatment either inhibits MUS81-dependent fork cleavage or DNA synthesis downstream of strand invasion leading to the accumulation of repair intermediates rather than exposed DSBs. It is therefore possible that the presence of DNA breaks in B02-treated cells is signalled to the SAC, culminating in delayed anaphase onset.

Addition of aphidicolin during S phase of the synchronization process (APH-S) increases the level of under-replication remaining in mitosis. However, aphidicolin treatment during S phase drastically decreases mitotic duration compared to the corresponding non-aphidicolin treated control. This was consistently observed in all cell lines and mitotic drug treatments analyzed (Figure 5.8 d-e). Why the addition of aphidicolin in S phase decreases mitotic duration is unclear and merits further investigation. It is interesting to note that, even in the presence of aphidicolin in S phase, the absence of RAD51 recruitment in mitosis appears to delay mitotic progression in some cells. Indeed, B02 treatment drastically increases mitotic duration in a subset of cells compared to the WT (S-APH) control (Figure 5.8 e). Similarly, mitotic duration after S-phase aphidicolin treatment is increased for a subset of S14A cells compared to WT.

No significant difference in mitotic duration is observed in S14D (S-APH) cells compared to the WT (S-APH) control (Supplementary Figure 5.3). However, an interesting observation was made when monitoring the fate of daughter cells after cell division. In cells that were filmed for at least 500 min after NERF, a striking number of S14D daughter cells appeared to undergo cell death (table 5.1). In all cases, cell death occurred in just one daughter cell, suggestive of problematic chromosome segregation (Supplementary Figure 5.4). If so, this implies that the RAD51 S14D mutant may not properly resolve replication intermediates in



**Figure 5.8 – Monitoring mitotic progression in GFP-Lamin expressing cells.** (A) Graphical representation of cell synchronization for the analysis quantified in (D). (B) Graphical representation of cell synchronization for the analysis quantified in (E). (C) Example of cell completing mitosis. Nuclear envelope breakdown (NEBD) is used as a marker for mitotic entry, nuclear envelope reformation (NERF) is used to mark the end of mitosis. (D) Mitotic duration as detected in the indicated cell lines, upon the indicated drug treatments. (E) Cells were synchronized in the presence of aphidicolin in S phase as depicted in (B). Mitotic duration (NEBD-NERF) as detected in the indicated cell lines, upon the indicated drug treatments.

mitosis. More detailed analysis of the cell fate after mitotic division will be required to confirm these observations.

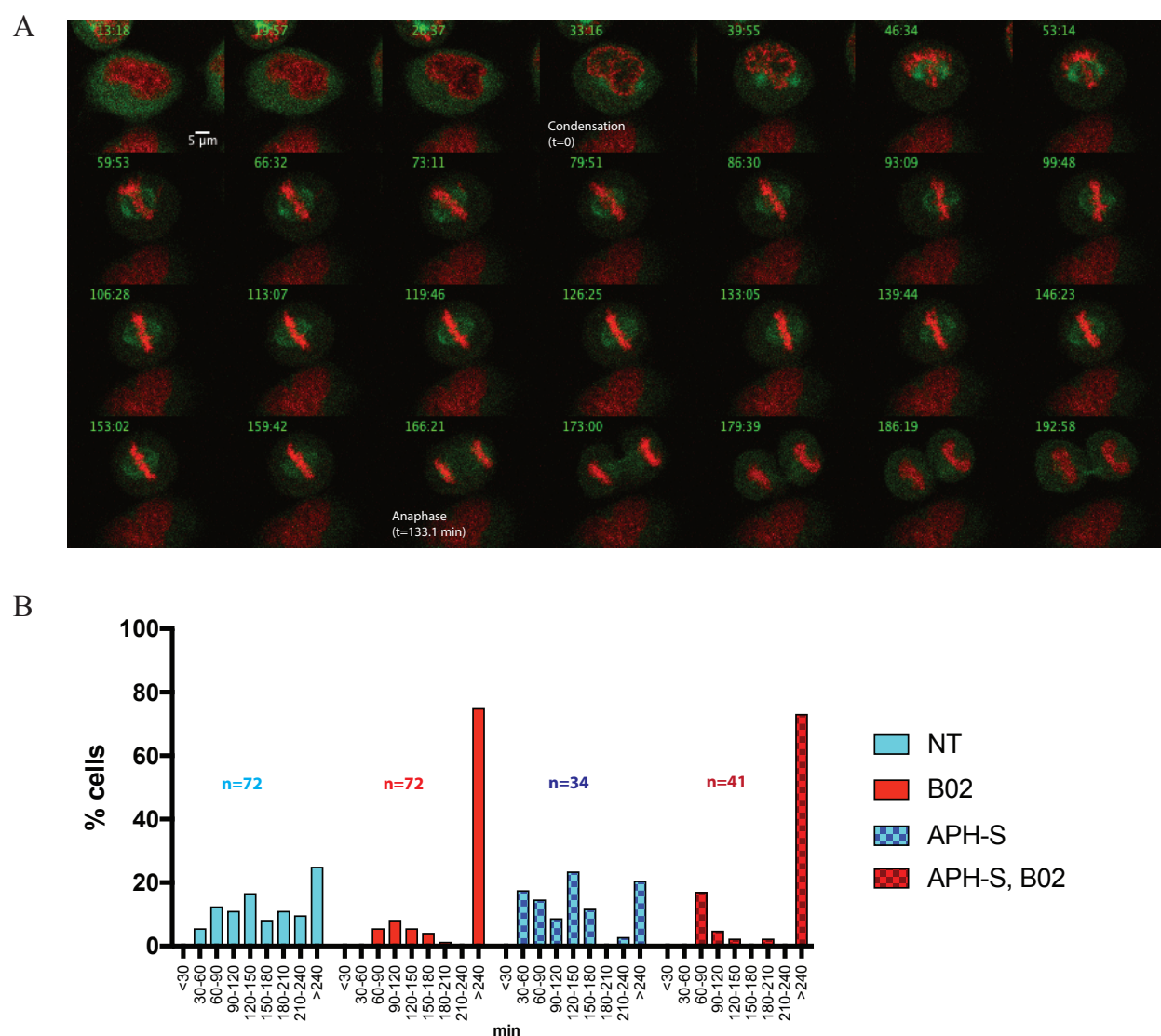
Mitotic progression was also monitored in HeLa cells expressing mCherry-Histone 2B and GFP-tubulin, allowing for the visualization of DNA during cell division. Mitotic duration was here measured from the onset of chromatin condensation to the first frame of anaphase captured (Figure 5.9 a). RAD51 inhibition in these cell lines drastically delays the onset of anaphase (Figure 5.9 b). Aphidicolin treatment in S-phase seems to decrease anaphase onset in a subset of cells, although the effect is not as striking as the decrease in mitotic duration observed in Figure 5.8 e. Repeat analysis will be required to confirm these observations.

**Table 5.1 – Cell death occurring within 500 minutes after NERF**

| Cell line         | Total cells recorded<br>(for > 500 min after NERF) | Total cell deaths recorded<br>after NERF |
|-------------------|----------------------------------------------------|------------------------------------------|
| WT (S-APH)        | 31                                                 | 0                                        |
| WT (S-APH, B02)   | 28                                                 | 1 *                                      |
| WT (S-APH, M-APH) | 35                                                 | 2 *                                      |
| S14A (S-APH)      | 43                                                 | 0                                        |
| S14D (S-APH)      | 40                                                 | 8 *                                      |

\* All cell deaths recorded occurred in only one of two daughter cells

Taken together, the analysis of mitotic duration presented here suggests that RAD51 recruitment and mitotic polymerase function impact activation of the SAC. Whether or not RAD51 impacts mitotic duration due to its role in MiDAS is not yet clear and requires further investigation



**Figure 5.9 - Monitoring mitotic progression in mCherry-H2B and GFP-tubulin expressing HeLa cells. (A)** Example of cell division. Chromatin condensation is used as a marker for mitotic entry; mitotic duration is quantified until anaphase. **(B)** Mitotic duration (condensation-anaphase) as detected upon the indicated drug treatments.



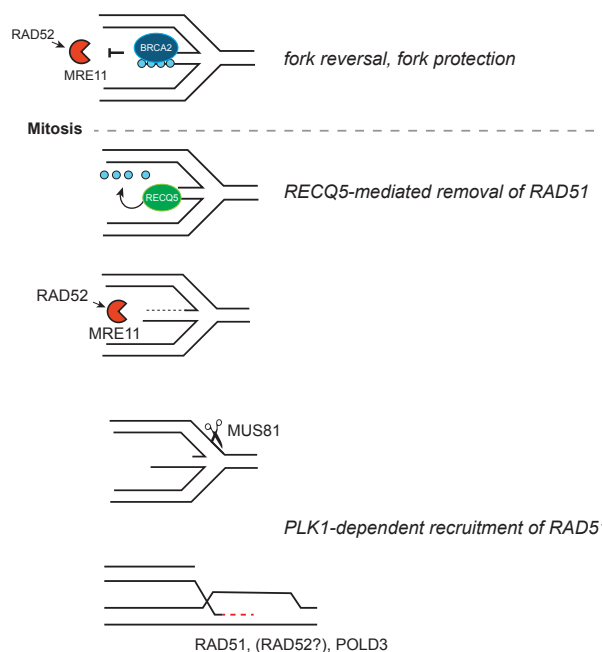
## 5.3 Discussion

### Identification of RAD51 as a new player in mitotic DNA synthesis

The involvement of RAD51 in MiDAS was assessed via three different approaches.

Firstly, a small molecule inhibitor (B02) was used to inhibit RAD51 recruitment specifically in mitosis. Mitotic B02 treatment consistently leads to a significant reduction in mitotic EdU incorporation, indicating that RAD51 promotes MiDAS. RAD51 inhibition was also shown to increase the level of mitotic DNA damage as assessed by  $\gamma$ H2AX foci formation and signal intensity. This potentially reflects the persistence of mitotic DNA damage in the absence of RAD51-mediated MiDAS. However, it is important to note that the increase of  $\gamma$ H2AX foci is detected 30 minutes after mitotic entry. The decline in irradiation-induced  $\gamma$ H2AX foci is reported after at least 1-2 hours recovery time, while reduction of irradiation-induced DNA breaks can be detected within 30 minutes.<sup>24,25</sup> Repair-associated decline in  $\gamma$ H2AX foci may therefore not be observable 30 minutes after mitotic fork cleavage. It is therefore possible that the increase of mitotic  $\gamma$ H2AX foci observed in B02 treated cells represents an increase in the induction of mitotic DSBs, rather than a deficiency in mitotic DNA repair. In other words, RAD51 inhibition potentially promotes the induction of DNA breaks in mitosis, while also preventing their repair via a MiDAS-dependent process. Indeed, RECQ5-mediated removal of RAD51 has been proposed to facilitate MUS81-dependent cleavage of stalled replication forks at CFSs.<sup>26</sup> RAD51 signal intensity in HU-treated cells was not significantly reduced upon 30 minutes of B02 treatment. This is likely explained by the fact that RAD51 nucleofilaments are already established prior to the addition of B02 in this experiment. Indeed, *in vitro* inhibition of RAD51 was found to be less efficient when B02 is added after RAD51-ssDNA filament formation.<sup>13</sup> The fact that B02 treatment impacts mitotic EdU incorporation within 30 minutes therefore suggests that MiDAS is mediated by newly recruited RAD51 in mitosis, consistent with the idea that RAD51 is removed before being recruited to under-replicated regions in mitosis (Figure 5.10).

Secondly, mitotic EdU incorporation was assessed in the RAD51 phospho-null mutant (S14A), in which RAD51 recruitment is proposed to be impaired particularly during late stages of the cell cycle. Indeed, mitotic EdU incorporation is significantly reduced in the S14A cell line compared to WT after low dose aphidicolin treatment in S phase. The decrease in mitotic EdU foci was rescued upon complementation with wild-type RAD51 (S14A+WT), suggesting that RAD51 phosphorylation promotes MiDAS via the recruitment of RAD51 to under-replicated DNA in mitosis (Figure 5.10). However, it cannot yet be completely excluded that



**Figure 5.10 – Model for removal and recruitment of RAD51 during mitosis**

the difference in MiDAS observed between the S14A and S14A+WT cell line are due to the absence of RAD51 phosphorylation during S phase – perhaps the lack of RAD51 recruitment during S phase in the S14A cell line impacts the nature of replication intermediates remaining in mitosis. This could in turn affect the rate at which replication restart takes place in mitosis. For example, it is conceivable that break-induced MiDAS occurs with lower processivity than a hypothetical break-independent mechanism of MiDAS. If S14A cells accumulate more DNA breaks in mitosis than S14A+WT cells (due to decreased replication protection during S phase), this could then contribute to the decrease in EdU incorporation during mitosis. However, the fact that S14A and S14A+WT cells exhibit comparable levels of mitotic  $\gamma$ H2AX signal suggest that a similar level of mitotic DNA damage and DSBs accumulate in both cell lines, such that the observed rescue of mitotic EdU incorporation in the S14A+WT likely reflects a rescue in RAD51-mediated BIR. Future research should focus on confirming this.

Indeed, the fact that mitotic  $\gamma$ H2AX signal is not reduced in the S14A+WT cell line compared to S14A supports the notion that repair-mediated decline of  $\gamma$ H2AX signal does not occur within a 30-minute time frame.  $\gamma$ H2AX signal was significantly reduced in S14D cells compared to WT, suggesting that the S14D mutation protects against mitotic DNA damage. This is consistent with the observation that S14D protects against the consequences of replication stress, as determined in Chapter 4. However, it cannot be excluded that the protection conferred by the S14D mutation arises during mitosis itself - if the S14D mutation

increases the stability of the RAD51 nucleoprotein filament (as proposed in Chapter 4) this may in turn affect mitotic fork cleavage. Interestingly, B02 treatment further decreases mitotic EdU incorporation in S14A cell lines, suggesting that some mitotic RAD51 recruitment is sustained in the absence of RAD51 phosphorylation.

Lastly, mitotic EdU incorporation was assessed upon siRNA-mediated RAD51 depletion. Mitotic EdU incorporation is significantly decreased in siRAD51 treated cells compared to siControl treated cells. Unexpectedly, mitotic  $\gamma$ H2AX foci formation is reduced in RAD51 depleted cells, suggesting that these cells collected in this sample experience less mitotic DNA stress and potentially have a lower requirement for MiDAS. RAD51 depletion is known to generate DNA DSBs, even in unperturbed conditions<sup>27</sup>, such that RAD51 depletion is not expected to mitigate DNA damage. I therefore hypothesized that the activation of a DNA damage-induced G2/M arrest upon RAD51 depletion prevents the collection of damaged mitotic cells (i.e. those in which RAD51 is efficiently knocked-down) by mitotic shake-off. However, addition of WEE1 inhibitor did not increase the amount  $\gamma$ H2AX in RAD51 depleted cells compared to control cells. This either suggests that the WEE1 inhibitor treatment was not sufficient to enrich the mitotic shake-off with RAD51 depleted cells, or that the above hypothesis is incorrect. Does RAD51 depletion actually decrease  $\gamma$ H2AX formation? It must be noted that a similar observation was recently made by Sotiriou et al., who find that the  $\gamma$ H2AX signal resulting from hydroxyurea (HU) treatment is significantly lowered in RAD51 depleted cells compared to control cells.<sup>28</sup> It is therefore worth investigating whether the reduction in  $\gamma$ H2AX foci formation upon RAD51 depletion observed here is specific to cells collected by mitotic shake-off, or whether it is generally observed during S phase.

While the addition of WEE1 does not facilitate the interpretation of the above described experiment, it did reveal an interesting effect on mitotic EdU incorporation. In both siControl and siRAD51 treated cells, WEE1 inhibition increased the number of mitotic EdU foci. WEE1 normally inhibits CDK activity – could the hyperactivation of CDKs in early mitosis stimulate MiDAS? WEE1 inhibition and concomitant CDK hyperactivity has been reported to enhance SLX4-MUS81 interaction and thereby MUS81 activity. The increased mitotic EdU incorporation in MK-1775 treated cells could therefore simply represent more efficient fork cleavage. However, since  $\gamma$ H2AX foci are not increased upon WEE1 inhibition in siControl cells, a CDK-dependent but break-independent mechanism for mitotic EdU incorporation should be considered. One intriguing possibility is that elevated CDK activity may enhance origin firing in mitosis. Further research will be necessary to confirm that this is indeed the case.

All together, these results support the hypothesis that RAD51 promotes MiDAS. Importantly, previous reports suggest RAD51 is not involved in MiDAS at CFSs marked by FANCD2 twin-foci or at telomeres.<sup>6,8</sup> These discrepancies may partially be explained by the loci-specific analysis of MiDAS in these studies, contrary to the global mitotic incorporation of EdU as assessed here. It is likely that MiDAS proceeds via different mechanisms depending on the site and nature of the mitotic DNA damage which arises. How many of these mechanisms rely on RAD51 activity? Indeed, although B02 treatment drastically impairs MiDAS, it does not abolish it completely, suggesting that some RAD51-independent MiDAS may occur. Further investigation will be required to analyse the site-specificity of RAD51-mediated MiDAS. It will be important to determine whether RAD51 activity is required for a break-dependent mechanism of MiDAS (as proposed in Figures 5.6 and 5.10) or whether RAD51 acts at different replication intermediates.

### **Proposed mechanism for MUS81 recruitment at stalled replication forks**

Minocherhomji et al. show that MUS81, EME1 or SLX4 depletion reduces mitotic EdU incorporation at FANCD2 twin-loci, suggesting that MUS81-mediated fork cleavage triggers MiDAS at CFSs.<sup>6</sup> Whether MUS81 activity similarly mediates MiDAS observed under the experimental conditions used in this chapter has yet to be confirmed. However, total mitotic EdU incorporation was shown to significantly decrease upon MUS81 depletion in a previous study.<sup>17</sup> Since MiDAS therefore largely appears to be a MUS81-dependent process, mitotic MUS81 foci likely represent MUS81 recruitment to stalled replication forks in mitosis. However, MUS81 is also involved in the resolution of recombination intermediates, such that MUS81 foci may also reflect its recruitment downstream of MiDAS. Interestingly, while RAD51 inhibition drastically reduces mitotic EdU incorporation, it does not significantly impact MUS81 recruitment. This suggest that the MUS81 foci detected within 30-minute after mitotic entry primarily represent MUS81 recruitment to stalled replication forks (upstream of a RAD51-mediated mitotic recombination process). Analysis of the colocalization of MUS81 foci and EdU foci may be useful to identify whether the observed MUS81 accumulation occurs downstream or upstream of mitotic EdU incorporation.

If the observed MUS81 foci indeed represent the protein's accumulation at stalled forks, the fact that either RAD52 inhibition or MRE11 inhibition significantly reduce mitotic MUS81 foci suggests that these players act upstream of MUS81-mediated fork cleavage. Mijic et al. propose that RAD52 recruits MRE11 to reversed replication forks, thereby promoting MRE11-

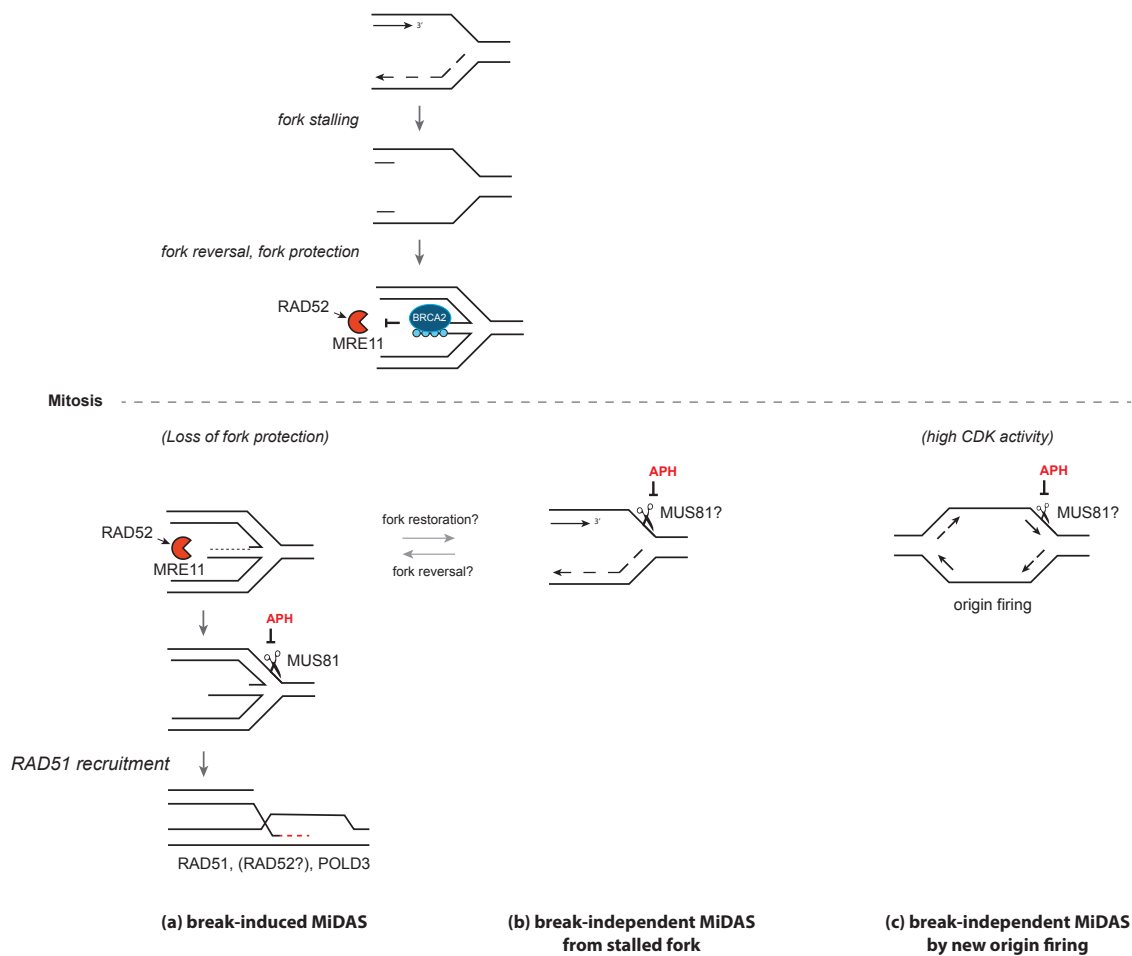
dependent degradation of nascent DNA at stalled forks in the absence of BRCA2.<sup>19</sup> Given that BRCA1 recruitment is impaired in mitosis and reduced chromatin association of BRCA2 was observed as cells progress into mitosis (Chapter 4), BRCA2 is likely absent from the mitotic chromatin.<sup>4</sup> Therefore, mitotic entry may promote RAD52- and MRE11-dependent degradation at reversed replication forks. Another recent study suggests that MUS81-mediated fork cleavage is facilitated at partially resected forks in a BRCA2 deficient background.<sup>20</sup> Based on these findings, I propose a model in which RAD52 and MRE11 mediate DNA degradation at stalled replication forks in mitosis, thereby promoting MUS81-dependent fork collapse (Figure 5.6 e). To further test this hypothesis, the effect of RAD52 and MRE11 inhibition on the accumulation of mitotic DNA damage should be assessed. Does the amount of mitotic DNA breaks decrease upon RAD52 or MRE11 inhibition? It will also be interesting to determine whether the importance of RAD52 and MRE11 is negated in MUS81-depleted cells, as might be expected if these proteins primarily act to recruit MUS81 to stalled forks.

The addition of 2  $\mu$ M aphidicolin in mitosis almost completely abolishes total mitotic EdU incorporation, suggesting that MiDAS is exclusively mediated by replicative polymerases. Surprisingly, mitotic aphidicolin treatment also significantly decreases MUS81 foci in mitosis. If we accept that MUS81 foci formation represents its recruitment to stalled replication forks, this suggests that mitotic replication promotes fork cleavage. Does the inhibition of DNA polymerase by aphidicolin treatment protect replication forks from MUS81-dependent cleavage in mitosis? Indeed, resolvases are thought to be particularly active in mitosis, such that active replication forks must be especially vulnerable to breakage during mitosis.<sup>22,23,29</sup> A model is proposed in which the replisome remains associated to stalled replication forks into mitosis and protects forks from MUS81-dependent cleavage (Figure 5.6 e). Analysis of mitotic DNA breaks upon mitotic aphidicolin treatment will be necessary to test this hypothesis and exclude the possibility that the observed reduction in MUS81 foci simply reflects a decrease in recombination intermediates.

Analysis of S14A and S14A+WT cell lines suggests that MiDAS may occur via a different mechanism in these cell lines compared to WT cells. MUS81 recruitment is significantly reduced in both S14A and S14A+WT cells compared to WT, suggesting that MiDAS may be less MUS81-dependent in the S14A genetic background. Given the fact that both S14A and S14A+WT cell lines experience high levels of mitotic DNA damage and are particularly vulnerable to replication stress (as determined in Chapter 4), perhaps replication stalling is more frequently accompanied by fork breakage in these cell lines. In this case DSBs might pass into mitosis from S phase, preventing the need for MUS81-mediated fork cleavage

in mitosis. The TUNEL assay could be used to elucidate whether the increased mitotic DNA damage observed in the S14A genetic background indeed correlates with an accumulation of mitotic DNA breaks, and whether or not the number of mitotic DNA breaks decreases upon MUS81 depletion. It will also be interesting to assess the proficiency of the G2/M DNA damage checkpoint in S14A and S14A+WT cells. Assuming that both cell lines primarily rely on break-induced MiDAS for mitotic EdU incorporation, (i.e. the same number of broken forks accumulate from S phase into mitosis in S14A and S14A+WT cells) the fact that wildtype RAD51 complementation rescues MiDAS likely suggests that RAD51 can mediate break-dependent MiDAS. The increase in MiDAS observed in S14A+WT cells is not accompanied by a decrease in  $\gamma$ H2AX signal. However, this is not unexpected if we consider that the decline in  $\gamma$ H2AX foci is significantly delayed to DSB repair, as introduced earlier. Perhaps it would be interesting to monitor the persistence of DNA breaks in S14A and S14A+WT cells to confirm that MiDAS in S14A+WT indeed reflects a break-repair process, rather than a break-independent process. As discussed previously, it must further be considered that a different mechanism for MiDAS might occur in S14A cells compared to S14A+WT (i.e. break-induced vs. break-independent), such that the presence of phosphorylated RAD51 is not necessarily responsible for the increased mitotic EdU incorporation in S14A+WT cells.

So far, a model for break-induced MiDAS has been proposed in Figure 5.6. Perhaps this should be expanded to include potential mechanisms of break-independent MiDAS (Figure 5.11). Mitotic entry induces the removal of fork protection by inhibiting BRCA2 association at mitotic chromatin. As proposed earlier, RECQ5-mediated removal of established RAD51 nucleofilaments may contribute to this loss of fork protection. This allows for mitotic-specific nucleolytic degradation of reversed forks and subsequent MUS81-dependent fork cleavage. MiDAS is proposed to occur via a BIR-like mechanism (Figure 5.11 a). Alternatively, a subset of stalled forks may retain an active replisome that continues to replicate once cells are released into aphidicolin-free medium (Figure 5.11 b). Lastly, given that mitotic entry is associated with increased CDK activity, perhaps this triggers late origin firing during mitosis (Figure 5.11 c). A fundamental question arises with each of the above proposed mechanisms. How is MiDAS sustained when resolvase activity is at its highest during mitosis? While MUS81-dependent cleavage is proposed to contribute to the BIR mechanism proposed in (a), it should also be able to cleave the recombination intermediates formed during BIR. Indeed, MUS81 activity is proposed to limit the length of POLD3-dependent BIR during S phase.<sup>30</sup> Similarly, MUS81 activity may be expected to cleave active replication forks, such that both break-independent mechanisms proposed (Figure 5.11 b, c) should be targeted.



**Figure 5.11 – Hypothetical break-dependent and break-independent forms of MiDAS.**

Indeed, the inhibition of replication by mitotic aphidicolin treatment reduced MUS81 recruitment. Perhaps these replication intermediates are protected from MUS81 activity during MiDAS. In fact, this introduces another possible function for RAD51 – could RAD51 act to stabilize such intermediates against nucleolytic cleavage, even in the absence of BRCA2 stabilization? Indeed, mitotic RAD51 inhibition is associated with an increase in mitotic DNA damage. Initial experiments should focus on determining how much of the observed mitotic EdU incorporation occurs via a break-dependent or break-independent MiDAS pathway. It may be specifically interesting to assess the effect of the different drug treatments on mitotic EdU incorporation in MUS81-depleted cells, when break-independent MiDAS may be more pronounced.

In general, the nature of mitotic DNA damage and, consequentially, the mechanism of mitotic DNA repair likely varies depending on the cell lines and specific genomic loci analyzed, as well as the experimental conditions used during cell synchronization. It may

therefore be difficult to define a single molecular mechanism of MiDAS from the analysis of global mitotic EdU incorporation. It will be important to assess whether the observations made in this chapter are consistent upon analysis of loci-specific mitotic EdU incorporation, such as at FANCD2 twin-foci or at telomeres.

### **S14D mutation protects against replication stress and SAC inhibition**

Since the SAC prevents the premature onset of anaphase, SAC activation is logically required for the completion of MiDAS prior to chromosome segregation. Given that the inability to complete replication in mitosis negatively impacts cell survival, SAC inactivation will be more detrimental to cells coping with increased mitotic DNA under-replication. In Chapter 4, RAD51 S14D mutation is proposed to confer replication fork protection under conditions of mild replication stress. S14D cells also demonstrated increased cell survival compared to WT cells in the presence of MPS1 kinase inhibition. The S14A and S14A+WT cell lines are extremely vulnerable to either replication stress or SAC inhibition. All together, these results suggest that the importance of SAC activation is directly related to amount of under-replicated DNA remaining in mitosis and therefore the level of replication stress experienced in S phase. A comparison of the sensitivity to MPS1 inhibition in non-cancerous RPE1 cells compared to U2OS cells may further support this hypothesis, as U2OS cells must deal with oncogenic-induced replication stress and exhibit higher levels of MiDAS than untransformed cells.<sup>6</sup>

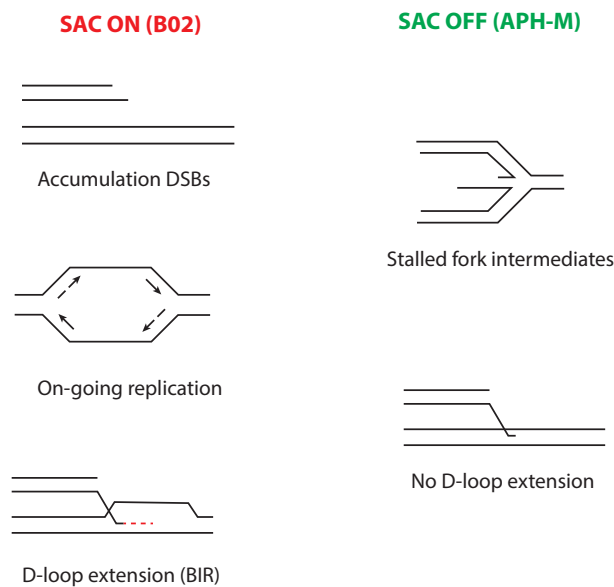
### **Spindle assembly checkpoint (SAC) potentially senses DSBs in mitosis**

Assuming that the appropriate duration of SAC activity is important to enable sufficient time for the completion of MiDAS in early mitosis, it is conceivable that the duration of the active SAC depends on the level of DNA under-replication remaining in mitosis. In other words, is SAC activation regulated independently of MiDAS or does the presence of under-replicated DNA prevent SAC silencing? To further investigate this, I assessed whether mitotic duration was affected in conditions where MiDAS is inhibited. Interestingly, mitotic RAD51 recruitment greatly influences mitotic duration. RAD51 inhibition by B02 treatment drastically increases mitotic duration in WT U2OS cells and delays anaphase onset in HeLa cells. Increased mitotic duration is also observed in the S14A cell line, while S14D cells appear to progress through mitosis more quickly. Analysis of mitotic duration in the S14A+WT cell line will be important to see whether the increased MiDAS observed in S14A+WT cells correlates



with a decrease in mitotic duration and to exclude the involvement of any secondary mutations in the S14A genetic background. Alternatively, the effect of RAD51 S14 mutation on mitotic duration may not result from a mitotic role of RAD51, but simply stem from the difference in replication stress experienced in these cell lines.

Importantly, the addition of 2  $\mu$ M aphidicolin in mitosis (which inhibits total mitotic EdU incorporation more effectively than RAD51 inhibition) significantly decreases mitotic duration compared to the untreated WT cells, suggesting that mitotic replication prevents SAC silencing. Considering the proposed role of RAD51 in MiDAS (either through mediating strand-invasion, or a possible fork-protection related function), RAD51 inhibition is predicted to lead to the accumulation of mitotic DNA breaks. Mitotic aphidicolin treatment potentially inhibits MUS81-dependent fork cleavage, thereby avoiding mitotic DSBs. However, perhaps the most important effect of mitotic aphidicolin treatment is the absolute abrogation of mitotic replication, thereby preventing either D-loop extension after strand-invasion (Figure 5.11 a); replication restart from intact forks (Figure 5.11 b) or origin firing (Figure 5.11 c). I propose a model in which active mitotic DNA synthesis or the presence of mitotic DNA breaks impedes silencing of the SAC, such that B02 treatment leads to prolonged SAC activation (Figure 5.12).



**Figure 5.12 – Mitotic DNA breaks and active replication intermediates are proposed to induce DNA damage signalling which integrates with the spindle assembly checkpoint to delay the onset of anaphase. Stalled replication forks and D-loops are proposed to be checkpoint blind.**

Conversely, the DNA intermediates accumulated during mitotic aphidicolin treatment are likely checkpoint blind so that SAC activity is reduced compared to untreated cells. According

to this model, DNA under-replication can be monitored from the presence of persistent mitotic DNA breaks and/or active replication intermediates, such that the SAC is silenced only once all DNA has been replicated and no DSBs remain.

However, it must be noted that a previous study found no significant change in mitotic duration upon acute TOPBP1 depletion in mitosis.<sup>10</sup> TOPBP1 was shown to promote SLX4 foci formation in mitosis, and either TOPBP1 or SLX4 depletion reduces mitotic EdU incorporation.<sup>6,10,17</sup> This data is consistent with a role for TOPBP1 in MiDAS upstream of fork collapse, thereby predicting a decrease in accumulated DNA breaks upon TOPBP1 depletion. However, a subsequent decrease in mitotic duration was not observed. The analysis of TOPBP1 depletion on mitotic progression was performed in the absence of any induced replication stress. Perhaps under these conditions the level of under-replicated DNA in mitosis is negligible. Conversely, the experiments performed here consistently introduce at least some level of replication stress during thymidine block-and-release, potentially accounting for the above described discrepancy. Further analysis should focus on determining whether mitotic DNA breaks are indeed increased under all conditions which exhibit delayed anaphase onset (i.e. S14A; B02; M-APH+B02). It will be interesting to see how mitotic inhibition of RAD52 or MRE11 (which similarly reduce MUS81 recruitment in mitosis) affect mitotic duration, as well as the effect of siRNA-mediated MUS81 depletion. It is also important to confirm that the observed increase in mitotic duration indeed stems from a delay in anaphase onset and persistent SAC activation. The latter is perhaps best addressed by analysing the persistence of MAD2 binding at the kinetochore by immunofluorescence, as MAD2 serves as a marker for unattached kinetochores.

If the presence of mitotic DNA damage or under-replicated DNA indeed results in persistent SAC activation, how is this achieved? It is conceivable that the activation of a DNA damage signalling pathway in mitosis cross-talks with the SAC signalling network at the kinetochore. Indeed, activation of ATM during mitosis was shown to promote SAC activity, although this does not seem to be related to a DNA damage response.<sup>31</sup> Kabeche et al. recently showed that ATR recruitment and activation at the centromere promotes Aurora B activation and prevents whole chromosome mis-segregation.<sup>32</sup> Aurora B destabilizes erroneous kinetochore-microtubule attachments to prevent premature silencing of the SAC, such that persistent activation of the reported ATR/Chk1/Aurora B signalling axis likely results in a delayed anaphase onset.<sup>33</sup> It would be interesting to confirm that mitotic ATR activity indeed delays anaphase onset and to further investigate whether ATR activation at under-replication regions can contribute to this potential delay in mitotic progression. The role of ATR in MiDAS

is contentious - while MiDAS at CFSs appears to be ATR independent, conflicting observations on ATR involvement in telomeric MiDAS are reported.<sup>8,17</sup> Dilley et al. find that break-induced DNA synthesis is increased at telomeres upon ATR knock down. Similarly, Özer et al. observe an increase in mitotic EdU incorporation at telomeres upon ATR inhibition. Given that ATR inhibition induces replication stress, these results likely reflect the exacerbation of under-replication and/or fork collapse at telomeres which leads to an increase in break-induced telomeric DNA synthesis. However, Min et al. find that mitotic EdU incorporation at telomeres is significantly decreased upon mitosis-specific ATR inhibition. Importantly, ATR inhibitor was here added exclusively after cells were released from a G2 arrest, thereby largely preventing ATR inhibition (and consequential replication stress) during interphase. This may account for the discrepancies in the above-described findings – when ATR is inhibited during interphase, the concomitant increase in replication stress (and thereby telomeric MiDAS) potentially masks the decrease of a subset of ATR-dependent telomeric MiDAS events. Indeed, the impact of ATR activity, as well as other DNA damage signalling pathways, on MiDAS should be analyzed under the experimental conditions used in this thesis.

If DNA damage signalling at under-replicated or broken DNA integrate with the SAC signalling network, it will be interesting to determine whether this requires the mitotic DNA damage to occur in the vicinity of the kinetochore complex. In other words, can a DNA damage response generated at under-replicated DNA distant to the centromere diffuse through the cells and impact SAC signalling at the kinetochore? This is perhaps best addressed by the induction of DNA breaks proximal or distant to the kinetochore, specifically during mitosis. Expression of a Cas9 nuclease which lack a nuclear localization signal (NLS) would possibly enable the induction of gRNA-targeted DNA breaks exclusively in mitosis, as the Cas9 nuclease can only access the chromatin upon nuclear envelope breakdown.

The notion that under-replicated DNA or mitotic DNA breaks signal to the SAC suggests that mitotic duration should be increased under conditions of enhanced replication stress. As described previously, the increased mitotic duration observed in the S14A mutant correlates with the higher levels of replication stress experienced in this cell line. Furthermore, the average mitotic duration of U2OS cells is reported to be approximately 30 minutes, while the average mitotic duration of U2OS cells analyzed here is greater than 100 minutes.<sup>34</sup> This presumably reflects the introduction of replication stress during the cell synchronization process by thymidine block-and-release. It is also noteworthy that RPE1 cells reportedly progress through mitosis more quickly (18 min +/- 3) than either U2OS cells (30 min +/- 8) or HeLa cells (44 min +/- 13). Indeed, RPE1 cells experience lower levels of endogenous

replication stress and exhibit very low levels of MiDAS (data not included in thesis). Additionally, low levels of HU were found to delay metaphase-anaphase transition in hamster cells.<sup>35</sup> Overall, these findings suggest that increased under-replicated DNA in mitosis does delay mitotic progression. It is therefore very surprising that aphidicolin treatment in S phase was consistently found to decrease mitotic duration in all cell lines and conditions analyzed. This suggests that an aphidicolin-specific characteristic of replication stress introduced in S phase can lead to a decrease in mitotic duration. Alternatively, perhaps an excessive amount of DNA under-replication in mitosis can overwhelm the SAC signalling network. It may be interesting to assess how different doses and types of replication inhibitors affect mitotic duration.

### **RAD51 S14D mutants potentially experience problematic chromosome segregation**

Lastly, preliminary observations suggest that S14D cells exhibit enhanced cell death after mitosis, as observed during live cell imaging. This cell death is limited to only one of two daughter cells suggesting that unequal chromosome segregation may occur S14D cells. More detailed analysis of chromosome segregation and the fate of cells beyond mitosis will be necessary to confirm these preliminary observations. If S14D cells indeed experience problematic chromosome segregation, how can this be reconciled with the enhanced cell survival conferred by the S14D mutation in the presence of MPS1 inhibition? Perhaps the problems in chromosome segregation arising in S14D cells are not exacerbated by premature anaphase onset, such that the relative cell survival of the S14D mutant is not drastically decreased upon SAC inhibition. As discussed in Chapter 4, while the S14D mutant exhibits enhanced protection against mild replication stress, it appears to be somewhat defective in HR-mediated repair. It is tempting to speculate that the S14D mutation protects against under-replication and the associated accumulation of mitotic DNA breaks (resulting in decreased mitotic duration and reduced sensitivity to SAC inhibition), but that the few stalled forks that do persist into mitosis are not resolved properly (resulting in repair intermediates that are blind to the SAC and give rise to chromosome non-disjunction).

Overall, the intriguing effect of RAD51 inhibition and replication stress on mitotic duration merits further investigation. Based on the current evidence, the involvement of a MiDAS-independent RAD51 function in SAC signalling cannot be excluded.

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## **Chapter 6**

### **Final conclusions and future work**





## 6.1 Summary of experimental findings

To analyse the impact of PLK1-dependent RAD51 T13/S14 phosphorylation during the cell cycle, CRISPR/Cas9 RAD51 phospho-null (S14A or T13AS14A) and RAD51 phospho-mimetic (S14D) mutants were generated in U2OS, RPE1 and eHAP cell lines, as described in Chapter 3. Given the relative ease with which the phospho-mimetic mutants were obtained compared to the phospho-null mutants, it is tempting to suggest that RAD51 T13/S14 phosphorylation is important for cell proliferation. A single phospho-null (S14A) mutant was obtained in the U2OS cell line, which appears to have adapted to the loss of RAD51 T13/S14 phosphorylation and exhibits characteristics of highly transformed cells. I hypothesize that this cell line has obtained several secondary mutations to suppress the detrimental consequences of the loss of RAD51 phosphorylation.

The RAD51 phospho-mutants generated in U2OS were analysed in the context of DSB repair and replication in Chapter 4. RAD51 recruitment appears to be impaired in the S14A cell line upon irradiation, as well as in unperturbed conditions. Conversely, RAD51 foci formation was significantly increased in the S14D cell line in unperturbed conditions. These results corroborate previous research indicating the importance of RAD51 T13/S14 phosphorylation in RAD51 recruitment.<sup>1,2</sup>

S14A cells exhibit a decreased replication rate as detected by EdU incorporation. Given that S14A cells accumulate in S phase in the presence of 0.4  $\mu$ M aphidicolin, this suggests that RAD51 phosphorylation supports replication fork progression and is crucial for the completion of replication under conditions of mild replication stress in the S14A genetic background. Indeed, S14A cells display extreme vulnerability to low dose aphidicolin as assessed by cell survival assay. While complementation with wildtype RAD51 is sufficient to rescue both the decreased replication rate as well as the observed S phase arrest in S14A cells, it does not rescue the decreased cell survival in the presence of low dose aphidicolin. I hypothesize that the S14A genetic background confers impaired checkpoint activation and de-regulates origin firing when exposed to mild replication stress, which leads to replication catastrophe. I propose that the expression of phosphorylatable RAD51 in the S14A+WT cell line increases the protection of vulnerable replication intermediates under these conditions, thereby rescuing bulk replication, but that this is insufficient to fully rescue replication fork breakage. Indeed, the S14A+WT cell line displays a prominent accumulation of cells in G2/M in the presence of low dose aphidicolin.

The enhanced recruitment of RAD51 in the S14D mutant appears to protect against the consequences of replication stress, as indicated by the increased cell survival of S14D cells relative to the WT control in the presence of low dose aphidicolin (0.1  $\mu$ M). However, at higher doses of aphidicolin (1.5-2  $\mu$ M), cell survival is significantly decreased in S14D cells compared to WT cells. I propose that RAD51 S14D is proficient in fork protection but impaired in HR, such that enhanced survival of S14D cells is only observed under conditions of replication stress that do not induce fork breakage.

Lastly, the role of RAD51 in mitotic DNA synthesis (MiDAS) was analysed in Chapter 5. I find that RAD51 inhibition by a small molecule inhibitor (B02) during mitosis significantly reduces mitotic EdU incorporation. Mitotic EdU incorporation is also significantly decreased in the S14A cell line compared to WT. This decrease in mitotic EdU incorporation is rescued in the wildtype complemented S14A+WT cell line, suggesting that RAD51 phosphorylation promotes MiDAS. Based on these findings, I hypothesize that RAD51 phosphorylation enables RAD51 recruitment to under-replicated DNA in mitosis, where RAD51 promotes MiDAS.

I further investigated the effect of RAD52 and MRE11 inhibition on mitotic EdU incorporation and MUS81 recruitment. Consistent with previous research, I find that RAD52 significantly decreases mitotic EdU incorporation and MUS81 recruitment<sup>3</sup>, and that MRE11 inhibition follows the same trends. I propose a model in which RAD52 and MRE11 act at reversed replication forks to promote MUS81 recruitment and subsequent fork breakage, which triggers a break-induced mechanism of MiDAS. I further suggest that MiDAS may also proceed via break-independent mechanisms – indeed, a substantial amount of mitotic EdU incorporation occurs even with RAD52 or MRE11 inhibition. I hypothesize that stalled replication forks potentially continue to replicate in mitosis and/or that dormant origins may fire in mitosis. Indeed, WEE1 inhibition (which increases CDK activity) was shown to increase mitotic EdU incorporation.

Previous research has shown that the inability to resolve under-replication prior to anaphase induces chromosome non-disjunction and is detrimental to cell survival.<sup>4</sup> Inhibition of the spindle assembly checkpoint gives rise to premature anaphase onset and is here shown to drastically reduce cell survival in the S14A and S14A+WT cell lines, while cell survival is significantly increased in the S14D cell line compared to the WT. Since the S14D mutation protects against mild replication stress, it is likely that this cell line exhibits less DNA under-replication in mitosis so that the consequences of premature anaphase are less severe.

Since an active SAC allows for the completion of MiDAS prior to anaphase, it is possible that SAC activity is regulated according to the presence of under-replicated DNA. To investigate this, mitotic duration was analysed under conditions that effect MiDAS. The inhibition of RAD51 in mitosis increases mitotic duration and delays the onset of anaphase. Conversely, mitotic aphidicolin treatment decreases mitotic duration. Based on these preliminary results, I hypothesize that a signalling pathway which is activated at mitotic DNA breaks and active replication intermediates cross-talks with the SAC to maintain its activation. In this way, SAC silencing and subsequent anaphase onset are delayed until any under-replicated DNA remaining in mitosis has been fully replicated. I propose that the inhibition of RAD51 leads to persistent mitotic DNA breaks and thus increases mitotic duration. Upon mitotic aphidicolin treatment, mitotic DNA synthesis is completely abrogated. I propose that, under these conditions, under-replicated DNA remains as stalled fork intermediates which are checkpoint blind, thus resulting in decreased mitotic duration.

## **6.2 Remaining questions and future work**

Future work should focus on confirming several observations made in this thesis. As discussed in Chapter 3, the validity of the CRISPR/Cas9-generated mutants should be confirmed by RT-PCR and sequencing of the PCR product to ensure that only the intended RAD51 variant is expressed in each cell line. This is specifically relevant for those CRISPR mutants which contain a knock-out frameshift mutation at one of the RAD51 alleles, as exon-skipping may occur. The presence of secondary mutations in the U2OS RAD51 S14A mutant should be further investigated. Preliminary evidence presented in Chapter 3 suggests that BRCA2 and PLK1 are overexpressed in the S14A and S14A+WT cell lines. This could be confirmed by RT-qPCR. Furthermore, the extreme vulnerability of these cell lines to replication stress is potentially indicative of replication catastrophe due to impaired checkpoint activity. Proficiency of the replication and DNA damage checkpoints should be assessed in all cell lines. DNA fibre experiments may be useful to demonstrate the potential deregulated origin firing in the S14A genetic background under conditions of replication stress. In general, it will be interesting to see whether the phospho-null mutants obtained in the RPE1 or eHAP cell lines similarly adapted to the loss of RAD51 phosphorylation.

As proposed in Chapter 3, a CRISPR/Cas9-screen could be designed to conclusively determine the knock-in efficiency of the RAD51 phospho-mutants. This would confirm whether or not the loss of RAD51 phosphorylation is truly disadvantageous in U2OS, RPE1

and eHAP cells, as is currently suggested by the estimated knock-in efficiencies in Chapter 3. If so, it is particularly interesting to consider the importance of RAD51 phosphorylation in the non-cancerous RPE1 cell line. Indeed, RPE1 cells experience much lower levels of endogenous replication stress than U2OS and exhibit little mitotic EdU incorporation. Is the importance of RAD51 phosphorylation related to replication stress and/or MiDAS in this cell line or is the importance of RAD51 phosphorylation unrelated to a function in mitosis?

In this thesis, a novel mitotic function for RAD51 is demonstrated which promotes mitotic DNA synthesis. However, the exact role of RAD51 in MiDAS is yet unclear. As discussed in Chapter 5, it will be important to determine how much of mitotic EdU incorporation is achieved via a nuclease-dependent BIR mechanism, and how much proceeds via a break-independent mechanism. Analysis of MiDAS in MUS81 depleted cells, as well as detailed analysis of the colocalization of EdU foci and  $\gamma$ H2AX may be useful. Based on the surprising increase in mitotic EdU incorporation upon WEE1 inhibition, the firing of dormant origins in mitosis is proposed as a potential mechanism of MiDAS. However, many of the factors involved in origin initiation appear to be required for BIR, which may complicate the identification of replication initiation events in mitosis.<sup>5</sup> Perhaps the most straight forward way to identify origin firing is through DNA fibre analysis, although this may be technically difficult to perform with mitotic chromatin.

Based on the results presented in Chapter 5, I suggest that RAD52 promotes MUS81-dependent cleavage of the stalled replication fork. This should be further confirmed by analysing the presence of mitotic DSBs, which would be expected to decrease upon RAD52 or MRE11 inhibition. However, it must be noted that RAD52 may also be involved in MiDAS beyond promoting MUS81-dependent fork breakage. Indeed, the results presented in this thesis do not necessarily contradict the previously proposed model in which RAD52 mediates MiDAS by promoting strand annealing of the broken DNA end to a homologous repair template.<sup>3</sup>

Mitotic EdU incorporation is more drastically decreased upon RAD51 inhibition compared to RAD52 inhibition, which may indicate that RAD51 promotes MiDAS via multiple mechanisms. As proposed in Chapter 5, it is conceivable that RAD51 plays a role in the protection of active replication forks or newly fired origins. Indeed, considering the hyper-activation of resolvases in mitosis, the protection of replication or recombination intermediates is likely important for efficient MiDAS. It may be interesting to analyse separation-of-function mutants of RAD51, such as the RAD51 T131P mutant, to find out whether a role of RAD51 in

fork protection is integral to its function in MiDAS. Indeed, the role of RAD51 at stalled forks is not associated with RAD51 foci formation, such that the lack of RAD51 foci formation in mitosis may be more consistent with a role for RAD51 in replication fork protection than in break repair.<sup>6</sup>

Interestingly, while mitotic EdU incorporation is significantly reduced in the RAD51 S14A cell line compared to WT, it is even further reduced upon inhibition of RAD51. How is RAD51 recruited to mitotic chromatin in the absence of T13/S14 phosphorylation? An interesting possibility is that RAD52 may act as a mediator for RAD51 recruitment to under-replicated DNA in mitosis.

Another question which is yet unanswered concerns the nature of the RAD51 S14D nucleofilament. The results presented in Chapter 4 suggest that RAD51 S14D is defective in HR, but it is not clear why. Given that the hyperstable RAD51 K133R nucleofilaments are also proficient in fork protection but deficient in HR, it is conceivable that the RAD51 S14D nucleofilament is similarly resistant to disruption. Indeed, the removal of RAD51 from post-synaptic filaments is likely important for the proper resolution of HR intermediates. Perhaps the hyperstability of the RAD51 S14D nucleofilament accounts for an intriguing observation made during live cell imaging of the S14D cell line: S14D cells often undergo cell death after mitosis, specifically in one of the two daughter cells. I interpret this as an indication of problematic chromosome segregation, although further research should focus on confirming this. Interestingly, this characteristic cell death was observed more frequently in S14D cells than in WT cells, even when the latter were analysed under conditions at which MiDAS is completely abrogated, i.e. mitotic aphidicolin treatment. Furthermore, the S14D mutant does not appear to be impaired in MiDAS, as assessed by mitotic EdU incorporation. Could the increased cell death after mitosis be indicative of problems arising after mitotic EdU incorporation, perhaps due to problematic resolution of recombination intermediates? In general, it will be important to assess the effects of impaired MiDAS under the different conditions assessed in this thesis. Chromatin bridges, ultra-fine anaphase bridges (UFBs) and 53BP1 nuclear bodies in G1 are commonly associated with DNA under-replication and may help to interpret some of the observations made in this thesis. For example, is the proposed protection against replication stress in the S14D cell line reflected in a decrease of these hallmarks of DNA under-replication?

Finally, the proposed model for the signalling of ongoing mitotic replication and mitotic DSBs to the SAC should be further tested. I have analyzed mitotic duration in the CRISPR/Cas9-generated mutants based on nuclear envelope breakdown and reformation.

Visualization of DNA will be important to confirm that the observed increase in mitotic duration indeed stems from delayed metaphase-anaphase transition and that the observed decrease in mitotic duration results from the earlier onset of anaphase. It will also be interesting to follow the fate of those cells that appear to be arrested in mitosis. When anaphase onset is delayed upon RAD51 inhibition, does this culminate in cell death or are cells able to eventually resume cell cycle progression?

If the SAC does indeed ‘sense’ the presence of ongoing MiDAS, how is the presence of MiDAS intermediates integrated into the SAC signalling network? The potential activation of DNA damage signalling pathways at sites of MiDAS should be analyzed. Lastly, the surprising decrease in mitotic duration observed in cells treated with low dose aphidicolin during S phase should be further addressed. Is this effect specific to replication stress introduced by aphidicolin? It will be interesting to see whether aphidicolin treatment in S phase is associated with increased markers of DNA under-replication and impaired MiDAS, as would be expected upon premature anaphase onset. Indeed, an intriguing possibility is that aphidicolin treatment during S phase enables more efficient completion of MiDAS in mitosis, hence resulting in faster mitotic progression which is not necessarily ‘premature’. Interestingly, aphidicolin has been shown to lead to helicase-polymerase uncoupling at origins.<sup>7</sup> It is tempting to speculate that the partial unwinding of dormant origins in S phase allow for the rapid progression of MiDAS once these origins are fired in mitosis.

### **6.3 Final remarks**

Cancer cells generally cope with increased levels of oncogene-induced replication stress, such that MiDAS is expected to be particularly important for their survival. The identification of players in the MiDAS pathway may therefore reveal promising targets for cancer therapy. Current research focuses on elucidating the molecular mechanisms behind MiDAS. The work presented in this thesis suggests that several different mechanisms for MiDAS likely exist and that RAD51 may play a major role in promoting several of these pathways. Furthermore, RAD51 phosphorylation appears to mediate RAD51 recruitment in mitosis. Further research into understanding exactly how PLK1-dependent RAD51 phosphorylation is regulated upon DNA damage or replication stress may reveal druggable targets to abrogate RAD51 recruitment specifically in mitosis, and thereby inhibit MiDAS. Lastly, inhibition of SAC activity was shown to severely decrease cell survival in cells that exhibit high levels of replication stress. This suggests that therapies aimed at increasing cellular

replication stress may be particularly effective when combined with chemotherapeutic treatments targeting the spindle checkpoint.

## 6.3 References

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