Investigating protein-protein interactions of the Bloom syndrome complex

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Abbreviations

BER: Base excision repair

BLM: Bloom syndrome helicase

BrdU: Bromo-deoxyuridine

BS: Bloom Syndrome

BTR: BLM-TOP3A-RMI1-RMI2

CPT: Camptothecin

DHBN: Dimerization helical bundle in N-terminal

DDR: DNA damage response

dHJ: Double Holliday junction

DSBs: Double-strand breaks

HR: Homologous recombination

HRDC: Helicase-RNaseD C-terminal

HU: Hydroxyurea

IP: Immunoprecipitation

MMR: Mismatch repair

MRN: MRE11-RAD50-NBS1

NER: Nucleotide excision repair

NHEJ: Non-homologous End Joining

NLS: Nuclear localisation signal

OB: Oligonucleotide binding

PTMs: Post-translational modifications

RQC: RecQ C-terminal

SCEs: Sister chromatid exchanges

SDM: Site-directed mutagenesis

SDSA: Synthesis-dependent strand annealing

SF2: Superfamily II helicase

SIMs: SUMO interacting motifs

SLIMs: Short linear interaction motifs

ssDNA: single stranded DNA

WRN: Werner Syndrome helicase

WT: Wild type

Abstract

Bloom Syndrome is a rare recessive chromosomal instability disorder, caused by loss-of-function mutations in the BLM helicase tumour suppressor. Patients develop a variety of developmental defects and have an increased predisposition to all cancers.

BLM interacts with TOP3A, RMI1 and RMI2 to form the BTR complex, which functions to promote the restart of stalled forks and the dissolution of double Holliday junctions during homologous recombination repair to the prevent the formation of sister chromatid exchanges.

Since most patient mutations abrogate all of BLM's functions, it is unknown which specific function(s) are important for tumour suppression. Moreover, the functions of the unstructured regions are poorly defined. To address this, I aim to identify and characterize separation-of-function mutations within conserved motifs of the unstructured regions of the BTR complex.

Firstly, I identified several RPA binding motifs in BLM and RMI1 which are important for cellular resistance to HU but are not important the dissolution of dHJs. Secondly, I found that highly conserved FTF residues within the N-terminus of BLM are important for interactions with TOP3A, RMI1 and RMI2, which are partially functional in resistance to hydroxyurea treatment and suppression of sister chromatid exchanges. Thirdly, I found that conserved residues within the N-terminal dimerization helical bundle are important for BLM dimerization and that these residues are partially functional for resistance to HU treatment and suppression of sister chromatid exchanges.

My results demonstrate that there are several conserved unstructured motifs in BLM that mediate specific protein-protein interactions. Furthermore, my data proposes several models of how these motifs and interactions regulate several cellular functions of the BTR complex. This work may provide a future insight into which specific cellular functions of BLM are

important for preventing tumorigenesis in future animal models in which the conserved regions we have studied are mutated.

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1. Introduction and background

1.1 The DNA damage response

1.1.1 Maintenance of genomic stability

Accurate mitotic transmission of genetic information is essential to maintain genomic stability which is required for normal cell function and survival. During DNA replication, the replication fork encounters many obstacles to precisely copy DNA. One consequence of this, is the accumulation of thousands of DNA lesions that occur every day in our genomes. If the damage is not resolved this can change the nucleotide sequence, leading to aberrations in genome replication that can be mutagenic, subsequently leading to genomic instability (Giglia-Mari et al., 2011). If these mutations occur in regions that encode proteins, this can lead to protein dysfunction. Furthermore, mutations in proto-oncogenes or tumour suppressor genes such as DNA repair genes, contribute to tumorigenesis. The nucleotide sequence and chromosomal structure of cancer cells is typically altered compared to that of normal cells, further demonstrating that DNA lesions and the disruption of genetic stability contributes to oncogenesis (Lengauer et al., 1998).

To combat this, cells have evolved a complex series of transduction pathways mediated by protein-protein interactions. This is termed the DNA damage response (DDR), which recognizes DNA lesions to activate signalling pathways to downstream proteins. Activation of the DDR induces checkpoint activation, providing time for specific DNA repair responses to remove the lesions for DNA to continue proper replication. If the number of lesions and the total amount of DNA damage is too great to be repaired, senescence or apoptosis of the cell is initiated (Andrés-León et al., 2016) (Figure 1.1).

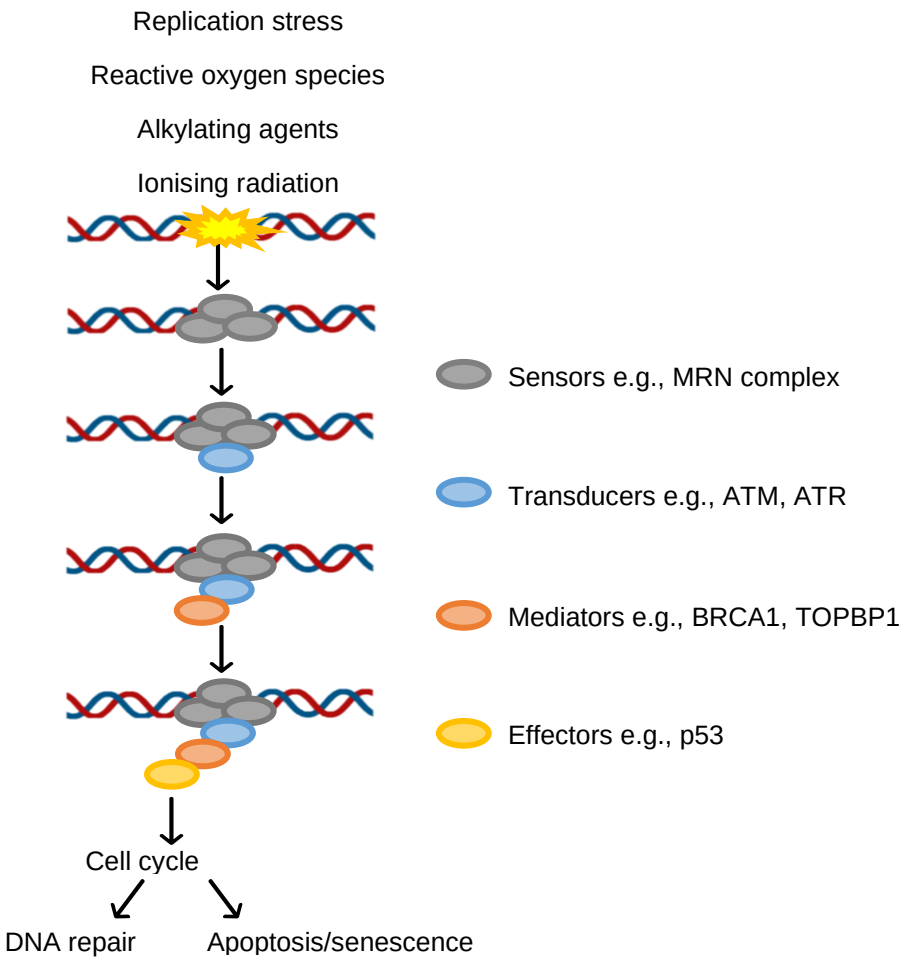


Figure 1.1: The DDR is mediated by protein-protein interactions

The DNA lesion is first detected by sensor proteins, initiating a signalling transduction pathway which involves protein-protein interactions with transducers and mediators to activate effector proteins required for mediating appropriate responses such as cell cycle arrest, DNA repair and apoptosis/senescence.

This intricate network of protein-protein interactions forms several evolutionary conserved repair pathways that are present in cells to resolve specific types of DNA damage (Taylor and Lehmann, 1998). Base excision repair (BER) is a mechanism in which damaged bases e.g. from oxidation, deamination or alkylation are removed by glycosylase enzymes throughout the cell cycle (Krokan and Bjørås, 2013). Mismatch repair (MMR) removes wrongly incorporated bases to repair mismatches. Nucleotide excision repair (NER) removes a region of damaged DNA such as thymidine dimers which is then replaced by a newly synthesised

stand, using the undamaged strand as a template. Double-strand breaks (DSBs) in cells are mainly repaired by one of two pathways, non-homologous end joining (NHEJ) or homologous recombination (HR).

1.1.2 HR repair of DSBs

Unrepaired DSBs are highly genotoxic and present the most lethal form of DNA damage due to their ability to cause deletions, translocations and hence, large-scale chromosomal rearrangements, mutations, and genomic instability. Therefore the NHEJ and HR repair pathways are important to repair spontaneous and programmed DSBs to preserve genomic integrity (Povirk, 2006).

Spontaneous endogenous DSBs are primarily caused by blocks during DNA replication such as single stranded DNA (ssDNA) nicks, thymidine dimers arising from UV radiation, R-loop formation, and other secondary DNA structures. These structures prevent the replication fork from progressing further to copy the DNA, a phenomenon known as replication fork stalling. Replication fork collapse and the formation of a DSB can be a consequence of failure to restart the replication fork (Syeda et al., 2014). Several exogenous agents can lead to the formation of DSBs such as ionizing radiation, topoisomerase inhibitors such as camptothecin (CPT), cross linking agents such as cisplatin, and agents which induce replication fork stalling such as hydroxyurea (HU) (Mehta and Haber, 2014).

Although DSBs are highly genotoxic, the generation of programmed site-specific DSBs have several conserved biological functions. In lymphocytes, DSBs are important for V(D)J and class-switch recombination for the generation of a diverse B and T cell repertoire to recognise a wide variety of antigens. Meiotic recombination presents another example of the importance of programmed DSBs required for generating chiasmata between homologous chromosomes for genetic exchange. These processes are highly regulated and require

appropriate DSB repair mechanisms to balance integral biological functions and genomic stability. NHEJ repair is essential for V(D)J recombination and class switch recombination, whilst HR repair is essential for meiotic recombination (Jackson and Jeggo, 1995, Cortizas et al., 2013, Longhese et al., 2009).

The NHEJ pathway functions to bring the two ends of the DSB for ligation. This pathway often requires the processing of the DSB ends. Hence, although this pathway is relatively accurate in DSB repair, deletions may occur in coding regions that can lead to mutagenesis (Davis and Chen, 2013).

In contrast, the HR pathway requires recombination of the ends of the DSB with an undamaged DNA template with sequence homology found in the sister chromatid, making this pathway error-free and non-mutagenic, thus preserving genome integrity. Since HR requires a sister chromatid, this pathway only occurs during the S and early G2 phases of the cell cycle (Zhao et al., 2017). Loss of HR repair can lead to large chromosomal rearrangements, insertions and deletions, hence defects in HR genes are linked to several cancer predisposition syndromes such as in inherited breast and ovarian cancer syndrome (BOCS).

Following the generation of a DSB, the Ku70/80 heterodimer is initially recruited to the DSB ends. However, at replication forks, Ku is displaced and frees the DSB ends to allow the recruitment of the MRE11-RAD50-NBS1 (MRN) complex contributes to several DNA repair pathways (Shao et al., 2012). In HR, NBS1 acts as a scaffold protein for the recruitment and activation of ATM. One function of ATM is the recruitment and activation of CtIP. MRE11 and CtIP promote short range resection of the DSB ends to generate 3′ ssDNA (Takeda et al., 2007). The ssDNA then undergoes further processing to produce long 3′ ssDNA overhangs, mediated by the DNA2 nuclease or by EXO1 in combination with other helicases. DNA2-

mediated end resection is enhanced by several RecQ helicases such as the Werner Syndrome helicase (WRN) or the Bloom syndrome helicase (BLM) which unwinds the DNA to enhance accessibility of the DNA ends by DNA2 to enlarge the 3'ssDNA resected ends (Mimitou and Symington, 2009, Gravel et al., 2008). As ssDNA is generated by the process of resection, the heterotrimeric RPA complex is loaded on to the ssDNA which protects the DSB ends from nucleases and prevents the formation of secondary DNA structures. (Nimonkar et al., 2011). BRCA2 is recruited to dissociate RPA from ssDNA to produce RAD51 nucleofilaments (Lord and Ashworth, 2007). RAD51 scans for a region of homology within a donor homologous sister chromatid, and once the filament interacts with a homologous region, this results in the formation of a D-loop and undergoes extension by DNA polymerase. There are several pathways in which the D-loop intermediate is processed. In the synthesis-dependent strand annealing (SDSA) pathway of HR, the D-loop can be displaced by branch migration, resulting in displacement of the invading strand from the DNA duplex which then reanneals to the end of the DSB. The gaps are then filled via DNA synthesis (Li et al., 2019).

If the D-loop is not displaced, the invading nucleofilament is synthesised along the region of homology, subsequently leading to second-end capture, forming an intermediate known as a double Holliday junction (dHJ) between sister chromatids (Nimonkar and Kowalczykowski, 2009).

There are two mechanisms by which the dHJ intermediates can be processed to complete HR repair as explained below and in Figure 1.2.

1.1.2.1 Dissolution pathway

The BLM helicase mediates branch migration of the double Holliday junction, resulting in the fusion of the two HJs known as a hemicatenane structure. This intermediate is cleaved

and re-ligated by the topoisomerase TOP3A within a complex that also contains BLM, and the structural subunits RMI1 and RMI2 (Bizard and Hickson, 2014, Swuec and Costa, 2014). This results in no crossovers between the sister chromatids.

1.1.2.2 Resolution pathway

DHJs can also be resolved and cleaved by endonucleases. Depending on how these junctions are cleaved, this can result in a crossover whereby a strand from one chromatid is exchanged with an adjacent stand of its sister chromatid. Crossovers between homologous chromosomes can result in loss of heterozygosity, which is a major driver of cancer. (Matos and West, 2014, West et al., 2015).

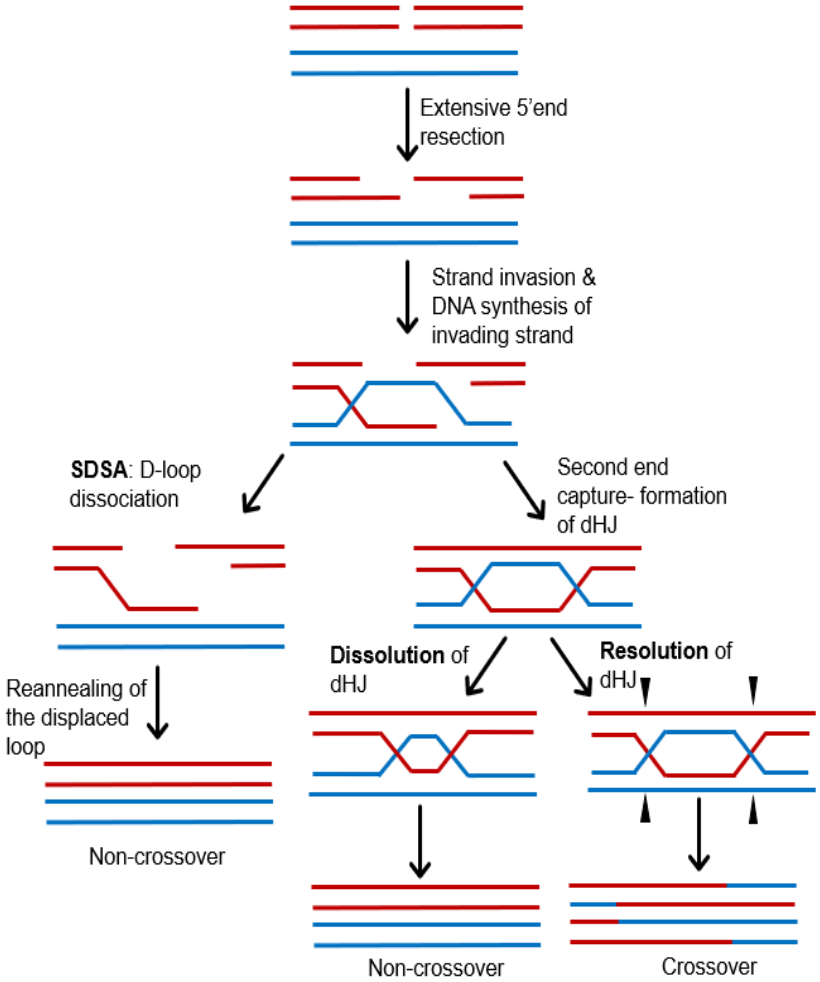


Figure 1.2: HR pathways in response to DSBs

Following RAD51 mediated strand invasion, one of three pathways for HR can take place to complete repair of the DSB. Two of these pathways are anti-recombinogenic, leading to no cross overs occurring between the damaged chromosome and homologous chromosome. On the other hand, the resolution pathway can be pro-recombinogenic whereby depending on how the endonucleases cleave the dHJ, 50% of the repaired products contain cross overs between the homologous chromosomes whilst 50% of the products are non-crossover.

1.1.3 Inherited mutations in DNA repair genes and cancer

Genomic instability is a common driver of tumorigenesis and consequently, inherited mutations in DDR genes are associated with an increased risk of early onset cancers. This is evident in various chromosomal instability syndromes (Jackson and Bartek, 2009, Negrini et al., 2010). For example, inherited mutations in *BRCA1* and *BRCA2* are seen in patients with early onset breast and ovarian cancer (Brody and Biesecker, 1998). Aberrations in *XPA-XPG* result in xeroderma pigmentosum, predisposing individuals to skin cancer due to defects in NER (Gratchev et al., 2003). Inherited defects in MMR genes such as *MSH2*, *MSH3*, *MSH6* and *MLH1* are associated with hereditary non-polyposis colorectal cancer (Fedier and Fink, 2004). Generally, syndromes associated with inherited mutations in DDR genes and defective repair pathways result in increased risk to specific cancer types in one primary site. In contrast, patients with Bloom Syndrome (BS), are deficient in the dissolution pathway of HR repair due to inherited mutations in *BLM*, however these patients have an increased predisposition to a broad range of early onset cancers at multiple primary sites. Therefore, in order to gain a greater understanding of how defects in the DDR confer to genomic instability, it is crucial to investigate BLM and its functions further in this network.

1.2 Bloom syndrome

1.2.1 History

In 1954, BS was first described clinically by Dr David Bloom, a dermatologist in New York who had observed an identical congenital skin rash across the butterfly area of the face in three patients with dwarfism from Ashkenazi Jewish ancestry, suggesting these clinical features could be due to an underlying syndrome (BLOOM, 1954). This was later confirmed as an inherited recessive disease in 1969 through a genetic study in 27 patients who presented similar symptoms (German, 1969). Interestingly in 1974, high levels of sister chromatid

exchanges (SCEs) were observed in lymphocytes from 18 patients, suggesting that BS is associated with high levels of chromosomal rearrangements (Chaganti et al., 1974). There are currently over 200 reported cases in the BS registry (Cunniff et al., 2018).

1.2.2 Causes

1.2.2.1 Mutations in *BLM*

BS is an autosomal recessive disease, predominantly caused by inherited loss-of-function mutations in the *BLM* gene. This means that affected patients have acquired a defective copy of both alleles from each parent. *BLM* is located on chromosome 15q26.1 and encodes the BLM RecQ helicase. BLM is important for several cellular processes required for maintaining genomic stability such as promoting replication fork restart and suppression of inappropriate recombination events during HR.

The BS registry is a database that has documented that most cases of BS are within the Ashkenazi Jewish population. The predominant cause within this population is the BLM^{Ash} founder mutation with a carrier rate of 1 in 100 in this population due to random genetic drift (Ellis et al., 1998). This mutation is a 6bp deletion and 7bp insertion located within exon 10 of the helicase domain of the protein (Cleary et al., 2003). Loss-of-function mutations have also been reported in two other structural domains of the protein; the RecQ and HRDC domains. In addition, several mutations have been reported in unstructured regions of the protein. For example, a Q645X missense mutation has been reported in 19 patients, leading to a premature stop codon (German et al., 2007).

1.2.2.2 Other mutations

Mutations in *TOP3A* have been reported in ten patients displaying BS-like phenotypes such as growth defects, distinct facial features, high levels of SCEs and chromosome instability. There are no records of cancer in these patients, however all were below 15 years of age at

the time of this study and tumorigenesis typically occurs at a later age (Martin et al., 2018). Mutations in *RMI1*, resulting in truncated variants of the RMI1 protein, have been reported in two patients with BS-like phenotypes, similar to patients with *TOP3A* mutations (Martin et al., 2018). Homozygous *RMI2* gene deletions have been reported in two siblings affected by a Bloom-like syndrome as both patients displayed chromosomal abnormalities such as high levels of SCEs and chromatid breaks (Hudson et al., 2016). Interestingly the proteins encoded by these genes (TOP3A, RMI1 and RMI2) form the BTR complex with BLM which has functions in replication restart and non-cross over HR pathways.

1.2.3 Clinical features

Patients with BS display a variety of developmental defects such as a short stature, a high-pitched voice and reduced fertility. Distinct facial features including a long narrow face, small lower jaw and prominent nose and ears are notable. Other characteristics include a skin rash in the shape of a butterfly across the nose and cheeks associated with UV sensitivity following exposure to the sun, increased risk of type 2 diabetes and immune system abnormalities. Patients also have an increased predisposition to all types of cancer, in contrast to other cancer susceptibility syndromes which predispose patients to a specific group of cancers (Kaneko and Kondo, 2004).

1.2.4 Cellular features

The underlying cellular features of BS are important for understanding why the clinical features manifest. Cytogenic studies have identified that BS cells have elevated levels of SCEs and increased somatic mutations which is indicative of loss-of-heterozygosity and genomic instability, providing a possible explanation as to why patients have an increased risk to various types of cancer.

1.2.4.1 Abnormal cytogenic characteristics

Cytogenic analysis of blood lymphocytes and fibroblasts of BS patients in 1974 has shown increased number of chromosomal breaks and rearrangements compared to non-BS cells (Chaganti et al., 1974). A 10- 15-fold increase of SCEs was also observed, a prominent cytogenic hallmark of BS as exposed in Figure 1.3. During mitosis, replicated chromosomes are arranged together as sister chromatids and exchanges between chromatids occurs in the presence of unresolved DNA damage. Deficiency in BLM prevents the dissolution of dHJs during HR, therefore repair is directed through the resolution pathway of HR, consequently leading to crossovers between DNA sequences (Shiraishi, 1990).

1.2.4.2 Increased mutation frequency and cancer predisposition

Several studies have provided evidence that BS cells have an increased incidence of somatic mutations compared to that of normal cells. This was first demonstrated by experiments carried out by Warren et al., showing BS fibroblasts have increased spontaneous mutations at the HPRT locus compared to normal fibroblasts (Warren et al., 1981). Elevated hypermutability has also been reported at the D1Z2 tandem repeat DNA locus in BS cells compared to non-BS cells (Groden and German, 1992). There are several reasons to why this may occur. Firstly, BS cells display shorter resected ends following DSBs compared to normal cells therefore strand invasion and homology search may more likely occur at the wrong sequence of the sister chromatid, leading to chromosomal rearrangements. Secondly, since BS cells elevated levels of SCEs, this may consequently lead to inappropriate crossover events between coding regions of genes and loss-of-heterozygosity, resulting in an increased mutation rate. This may explain why these patients develop such a broad range of early onset cancers.

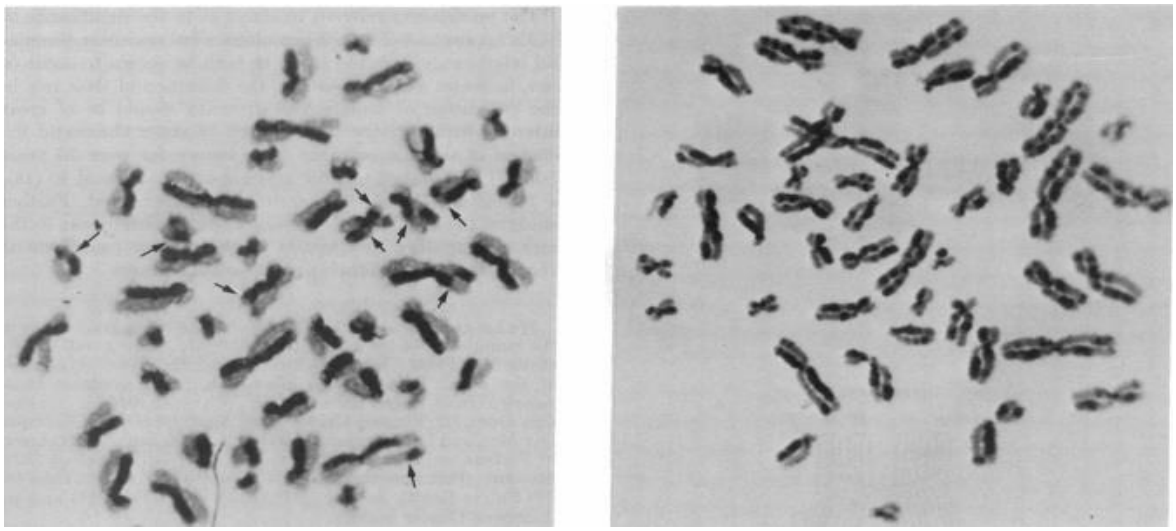


Figure 1.3: First evidence showing elevated SCEs in BS patient samples compared to non-BS cells

These images were taken by James German in 1974, providing the first evidence that BS patients display high levels of SCEs. The image taken on the left represents a metaphase spread derived from lymphocytes of a patients unaffected by BS, these chromosomes display very low levels of SCEs. In contrast, the image on the left has been taken from a patient with BS, it is clearly seen that most chromosomes display cross overs between the sister chromatids, this is a key characteristic of BS.

1.2.5 Diagnosis, management and prognosis

BS diagnosis is first identified through the observation of known clinical features. The diagnosis is then confirmed by cytogenic analysis to detect the presence of high levels of SCEs from cultured somatic cells. Prenatal diagnosis of suspected pregnancies is possible through SCE analysis from obtaining foetal cell samples of the chorionic villus (Howell and Davies, 1994).

There are currently no treatments that can cure BS patients; therefore, the symptoms of the disease are managed to improve quality of life and to prevent the progression of poor prognostic symptoms. Growth hormone therapy is used to improve growth and nutrition defects, however this is maintained with close monitoring of IGF1 levels to prevent malignancy (Brock et al., 1991). Monthly intravenous injections of gamma-globulin are administered to compensate for immune deficiencies, especially as BS patients are more

prone to life threatening infections such as pneumonia (Cunniff et al., 2018). Patients are treated for type 2 diabetes through metformin tablets and the maintenance of a healthy diet to manage blood sugar levels.

Since BS increases risk to a broad range of cancers, there are several screening and prevention recommendations. Daily usage of SPF30 is advised to reduce the risk of skin cancer. Colorectal cancers have been reported with the earliest case in a 16-year-old patient, therefore annual colonoscopies and faecal immunochemical testing are a priority. There has also been an early occurrence of breast cancer, therefore annual breast MRI scans are routine to screen for tumours. Renal ultrasound examinations are standard every 3 months to detect for Wilms tumour. MRI scans are favoured as a screening method over CT scans, since exposure to ionising radiation can further increase the risk of tumorigenesis. Patients and their relatives are made aware of generic symptoms to particular cancer types to seek the appropriate medical attention should they appear (Walsh et al., 2017, Cunniff et al., 2018). Although there are several measures aiming to prevent tumorigenesis, early onset cancer still remains the most common cause of death, with an average lifespan of 27 years.

1.3 The BLM RecQ Helicase

1.3.1 RecQ Helicases

The BLM helicase is a 1417 amino acid protein with a molecular weight of 159 kDa. BLM is a superfamily II (SF2) helicase which are a subfamily of helicases that remodel nucleic acid structures to allow the function of important cellular processes (Beyer et al., 2013). This group of helicases are further characterised into 6 families, and BLM is a member of the evolutionary conserved RecQ helicases. RecQ helicases are important in processes required for the maintenance of normal DNA metabolism such as replication fork protection and homologous recombination (Beyer et al., 2013). There are five RecQ helicase proteins in

humans: BLM, WRN, RECQL4, RECQL1 and RECQ5. RecQ helicases unwind DNA in a 3′ to 5′ direction in an ATP-dependent manner, which is required for accurate DNA replication and DNA repair to maintain genomic integrity (Hickson, 2003). Mutations in WRN and RECQ4 cause the cancer predisposition syndromes Werner's syndrome (WS) and Rothmund-Thomson syndrome (RTS) respectively (Harrigan and Bohr, 2003), suggesting that like BLM, these proteins have important functions in maintaining genomic stability. WS is associated with early ageing and increased risk of early onset cancers of mesenchymal origin (Rincón et al., 2019). RTS is associated with increased risk of osteosarcomas and several types of skin cancer (Zils et al., 2015). Each member of the RecQ helicase family forms specific interactions with other proteins that determine their functions in genome stability.

1.3.2 BLM structure

There are three key structural domains within BLM: the ATPase, the RecQ C-terminal (RQC) and the helicase-RNaseD C-terminal (HRDC) domains (Figure 3).

The ATPase domain is highly conserved among all RecQ helicases and contains a zinc binding subdomain that functions to hydrolyse ATP and is required for 3′-5′ helicase activity (Guo et al., 2005).

The RQC is folded into a winged β-helix motif with an extended C-terminal loop that mediates interactions with the major groove of DNA. This unique RQC structure may allow preferential binding to branched sites such as Holliday junctions, since the β-wing limits interactions with linear base pairs (Kitano, 2014). The RQC β-wing is composed of many polar and acidic residues that mediate DNA strand separation from the 3′ end (Swan et al., 2014, Kim et al., 2013).

Among the RecQ helicases, only BLM and WRN contain an HRDC domain, indicating that the HRDC is not essential for helicase activity, however the function of this is still unclear.

Previous studies have revealed that the isolated HRDC domain of BLM specifically binds to ssDNA with low affinity. Furthermore, there are several regions within the HRDC domain of WRN and BLM that are not structurally conserved, indicating that BLM HRDC may have a role that is specific for the function of the BLM helicase. (Liu et al., 1999) (Kim and Choi, 2010).

BLM forms oligomers via its N terminus, however the function of this in BLM remains to be elucidated. Oligomerisation of proteins generally serves to regulate their functions, however whether oligomerisation of helicases is important for HR functions is controversial. For example the DNA unwinding activity of SF1 and SF2 helicases can be enhanced via self-assembly through oligomerisation but, the helicases can also function in their monomeric forms (Cheng et al., 2001). However, SF3-SF6 helicases requires oligomerisation for their function to unwind DNA. Previous studies have shown that BLM oligomerisation may be important to unwind branched structures of DNA such as dHJs in HR (Gyimesi et al., 2013, Mazina et al., 2012). More recently, a highly conserved dimerization helical bundle in N-terminal domain (DHBN) has been mapped in BLM which induces high order oligomerisation (Shi et al., 2017). However, it still remains unclear as to how oligomerisation is important for BLM's helicase activity in the unwinding of complex structures. Additionally it has been hypothesised that oligomerisation is mediated by N-terminal and C-terminal BLM protein-protein interactions as when these regions are truncated, oligomerisation is diminished (Gyimesi et al., 2013). Perhaps identifying these protein-protein interactions may give a mechanistical insight into how oligomerisation enhances BLM helicase activity.

BLM contains a highly conserved nuclear localisation signal (NLS) in the C-terminus, a short positively charged region of the protein that mediates protein translocation into the nucleus which is important for BLM's functions (Kaneko et al., 1997). Additionally, protein-protein interactions commonly drive the localisation of proteins into the nucleus. In the case of BLM,

a recent study has suggested that direct interactions with importin β1 via the NLS region may be important for mediating BLM nuclear localisation (Duan et al., 2017).

The majority of the BLM protein is composed of unstructured regions with no known function. It is plausible that some of the shorter, conserved sequences of these regions are short linear interaction motifs (SLIMs). In many proteins SLIMs have been found to mediate protein-protein interactions (Davey et al., 2012), but this is yet to be fully investigated in BLM.

1.3.3 The BLM-TOP3A-RMI complex

Many DNA repair proteins are found in complexes and the BLM-TOP3A-RMI1-RMI2 (BTR) complex is an example of this (Figure 1.4). The BTR complex is made of four proteins with different functions, creating a molecular machine that can unwind DNA, cleave regions of torsional stress caused by this unwinding and reseal DNA breaks caused by this cleavage. The function of BTR in the maintenance of genomic stability is further highlighted by congenital loss of any of the proteins within this complex being found in BS patients.

The budding yeast ortholog of TOP3A, Top3, was the first protein demonstrated to interact with the yeast homolog of BLM, Sgs1, via the first 100 amino acids of its N-terminus (Gangloff et al., 1994). Hickson et al., later identified that TOP3A interacts directly with the N-terminus of human BLM (Wu et al., 2000). TOP3A is a type-1 topoisomerase enzyme that is recruited to dHJ structures by BLM. BLM stimulates TOP3A activity that catalyses cleavage of the phosphodiester backbone of the hemicatenane structure to produce non-crossover products (Wu and Hickson, 2002). Patients with biallelic mutations in TOP3A display elevated levels of SCEs, further highlighting the importance of TOP3A in dHJ dissolution (Martin et al., 2018)

A third component of this complex was later distinguished as RMI1 and was found to colocalise with BLM following DNA damage. Loss of RMI1 reduces BLM recruitment to DSB sites and results in high levels of SCEs. RMI1 contains two oligonucleotide binding (OB) fold nucleic acid binding domains, OB1 and OB2. Although OB domains usually function to interact with DNA, the OB domains in RMI1 do not exhibit this function. Instead, OB1 functions to interact with both BLM and TOP3A (Yin et al., 2005). Following cleavage of hemicatenane structures, a break in the DNA backbone is formed and RMI1 functions to allow the released ssDNA to pass through and reseal the break.

Finally, a fourth protein of this complex was recognised as RMI2 which contains an OB domain that interacts directly with OB2 of RMI1 and is important for the stability of this complex. Loss of RMI2 in DT40 cells results in high levels of SCEs and a decrease in TOP3A levels (Singh et al., 2008).

The BTR complex has many versatile functions in replication fork restart to prevent the formation of DSBs by unwinding replication fork blocks, and in promoting non-cross over HR pathways for the repair of DSBs by unwinding HR intermediates (Manthei and Keck, 2013) (Figure 1.5).

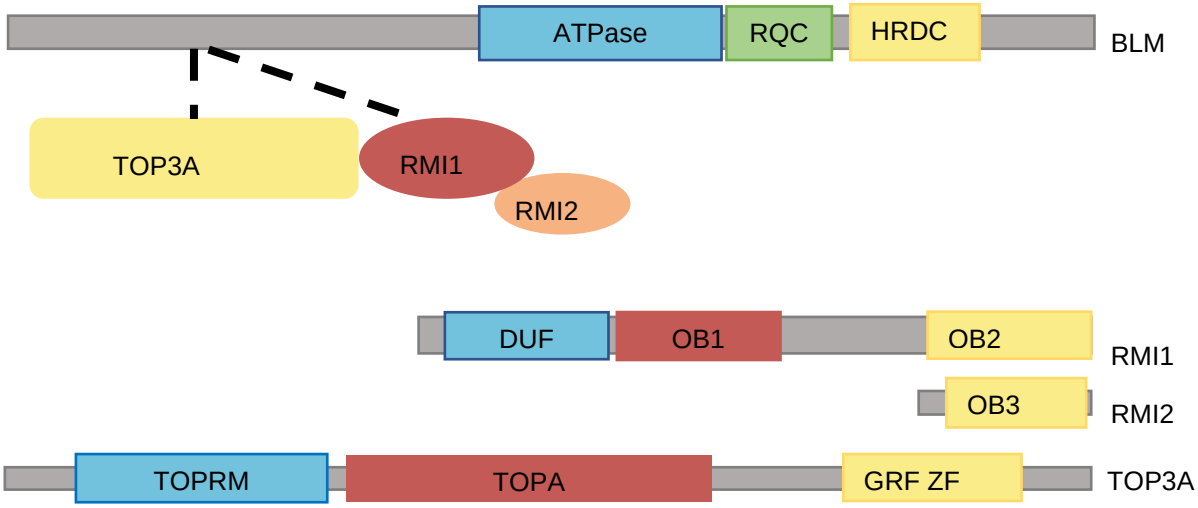


Figure 1.4: Schematic of protein-protein interactions within the BTR complex

This schematic highlights BLM's interactions with TOP3A, RMI1 and RMI2 occur within the unstructured N terminus of the protein.

1.3.3.1 The BTR complex in replication fork restart

Early studies of BS have shown that cells from patients replicate slower compared to normal cells. This is because cell cycle progression is slower due to the accumulation of cells in S-phase causing a delay in replication (Hand and German, 1975). This suggests that BLM functions in replication and several studies have established that the BTR complex functions in the restart of stalled replication forks (Davies et al., 2007, Yang et al., 2012).

During DNA replication, the proteins of the replisome can encounter blocks such as secondary DNA structures which prevents further progression of the replication fork. The BTR complex can function to unwind these structures such as G-quadruplexes, bubbles, and Holliday junctions to remove the block and allow the replication fork to resume.

Additionally, it has been proposed that the BTR complex can promote replication fork reversal (Ralf et al., 2006, Machwe et al., 2006). Fork reversal is the process by which two nascent strands at the site of a DNA lesion peel off their template strands and anneal together. This regresses the replication fork backwards forming a “chicken foot” Holliday junction structure which allows the lesion to relocate downstream of the fork by template switching. The following model of BTR’s roles in fork regression has been proposed, BTR and its interactions with FANCM may be involved in promoting branch migration to push the fork backwards and in unwinding of the chicken foot Holliday junction, thus allowing the replication fork to continue by bypassing the lesion (Ralf et al., 2006) (Lu et al., 2019). It has been suggested that the disruption of the BTR complex and/or disrupting its interactions with FANCM can result in collapse of the replication fork leading to the formation of a DSB (Lu et al., 2019).

1.3.3.2 The BTR complex in HR repair

During HR, RAD51 forms a nucleofilament on ssDNA and mediates strand invasion of the 3′ end of the DSB into a recipient homologous chromosome, thus forming a D-loop structure. Bugreev et al., have shown the BTR complex can interact with RAD51 to unwind and displace this D-loop, resulting in the anti-recombinogenic SDSA pathway of HR of being favoured which produces non-cross over products (Bugreev et al., 2007).

Alternatively, if the D-loop is not unwound, second end capture results in the formation of a dHJ. The BTR complex functions to dissolve this structure whereby BLM promotes branch migration of the dHJ, bringing the two junctions into close proximity. The hemicatenane formed is then cleaved by TOP3A-RMI1 which results in the production of non-cross over products. In contrast, the resolution pathway of HR is dominant in BS cells which display high levels of cross over DNA products.

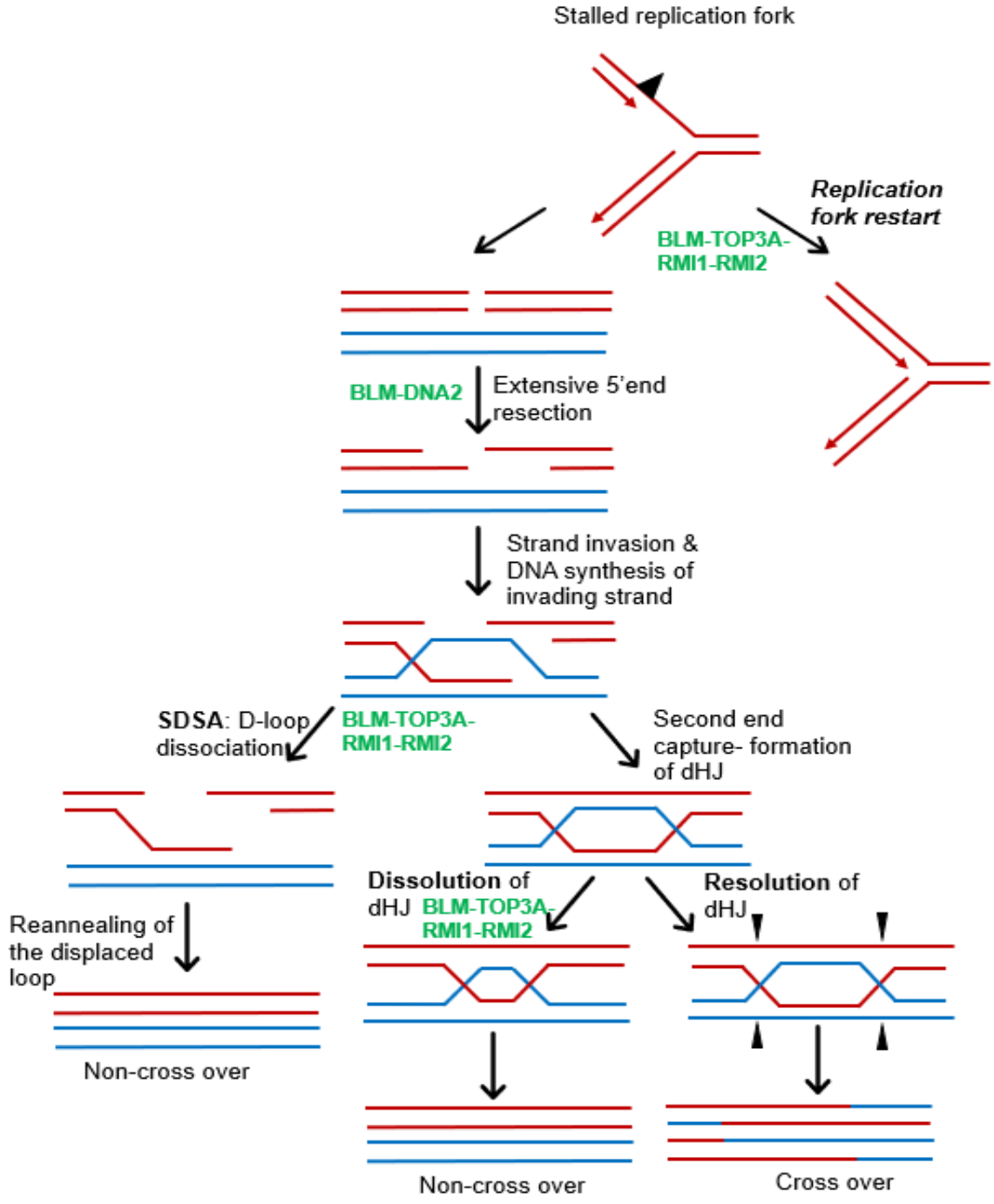


Figure 1.5: BLM protein-protein interactions in replication fork restart and HR

BLM interacts with proteins of the BTR complex to promote replication fork restart to prevent replication fork collapse and the formation of a DSB.

If a DSB occurs, BLM interacts with EXO2/DNA2 to promote extensive end resection of the break and interacts with proteins of the BTR complex to promote the anti-recombinogenic pathways of HR.

1.4 Other BLM protein-protein interactions

As well as interacting with proteins of the BTR complex, BLM also interacts with various other proteins that may be required for promoting or regulating BLM's functions in replication fork restart, ssDNA resection and HR.

1.4.1 BLM-TopBP1

The TopBP1 protein has many important roles in the maintenance of genomic stability by mediating protein-protein interactions via its 9 BRCT domains. For example, these BRCT domains are important for interactions with early responders of the DDR such as MDC1 and 53BP1 (Leimbacher et al., 2019, Yamane et al., 2002). TopBP1 also functions to activate ATR in the early stages of the DDR (Sokka et al., 2010, Kumagai et al., 2006).

In 2015. Blackford et al., showed that within the mostly non-conserved N terminus of BLM, Ser304 is highly conserved. Furthermore, Ser304 to alanine mutation abrogated interactions with TopBP1 in pull-down experiments, confirming that phosphorylation of Ser304 is important for direct interactions with TopBP1 (Blackford et al., 2015). A more recent crystal structure of the TopBP1 BRCT5 domain bound to BLM peptides by Sun et al., provided further evidence that the conserved phosphorylated Ser304 is essential for high affinity interactions with TopBP1 (Sun et al., 2017).

Wang et al., showed that depletion of TopBP1 resulted in high levels of SCEs, indicating that this interaction is important for promoting BLM's functions in supressing cross over events to maintain genomic stability (Wang et al., 2013). This was also confirmed by Blackford et al., whereby S304A mutations in resulted in high levels of SCEs (Blackford et al., 2015). These studies have collectively mapped the BLM-TopBP1 interaction and have provided evidence that this is important for promoting BLM's function in supressing SCE formation during HR to maintain genomic stability.

How the BLM-TopBP1 interaction supresses SCE formation is yet to be fully understood since it remains controversial whether loss of this interaction destabilises BLM levels. Other than BLM's roles in SCE suppression, BLM also functions in replication fork restart and resection, however whether the BLM-TopBP1 interaction is required for this remains to be addressed.

1.4.2 BLM-RPA

RPA is a heterotrimeric protein composed of RPA70, RPA32 and RPA14 subunits. RPA is the main protein in eukaryotes that binds to ssDNA to protect it from degradation and to prevent the formation of secondary structures that could block metabolic reactions such as replication, recombination and repair (Oakley and Patrick, 2010, Wold and Kelly, 1988). RPA is important in regulating DNA damage checkpoints which are activated in response to the DDR. Therefore RPA has important roles in maintaining genomic integrity (Zou et al., 2006). As BLM has poor helicase activity on its own, Brosh et al., suggested that RPA may be a potential binding partner of BLM since RPA functions in replication, recombination, and repair. They confirmed this by incubating human BLM with dsDNA in the presence of elevating RPA70 concentrations, providing evidence that in the absence of RPA, BLM could not unwind the DNA substrate. However, as the RPA concentration increases, the rate at which BLM could unwind DNA also increased. This also occurred in the presence of *S. cerevisiae* RPA, indicating that RPA's function in stimulating BLM helicase activity is evolutionary conserved (Brosh et al., 2000). It was then confirmed that BLM and RPA directly interact via the RPA70 subunit (Brosh et al., 2000).

To identify which region of BLM is important for this interaction, a later study generated various truncated forms of BLM, and using a monoclonal antibody against RPA70, ELISA experiments confirmed that the first 447 amino acids of the BLM N-terminus are required for

direct high affinity interactions with RPA (Doherty et al., 2005). However, the specific residues of the BLM N-terminus that are required for this interaction are yet to be defined.

One function of BLM is to promote DNA end resection with DNA2. A previous study by Nimonkar et al., has shown that in the absence of RPA, DNA2 cleaves both the 5′ and 3′ tails of ssDNA whereas when RPA is present, this limits DNA2- mediated cleavage of the 5′tail only which is important for ensuring that BLM and DNA2 promote resection in a 5′-3′ direction. Likewise RPA stimulates the resection activity of EXO1 by three fold compared to when RPA is absent (Nimonkar et al., 2011). This study presented that RPA is important for promoting the activity of DNA2 and EXO1 in end resection, however, it remains unclear if RPA is important for specifically promoting BLM's functions in resection. Additionally, BLM is not essential for EXO1 mediated resection of DSBs therefore *BLM*$^{-/-}$ cells may still undergo resection via the EXO1 pathway.

Another previous study provided evidence that RPA interacts with an acidic region of RMI1 and mutations in this region abrogated the interaction. Subsequent dHJ dissolution assays demonstrated that inhibition of this interaction diminishes the rate of dHJ dissolution, suggesting that RPA's interaction with RMI1 of the BTR complex is important for the dissolution of dHJs (Xue et al., 2013). As the precise binding sites for the RPA-BLM interaction have not yet been well defined, it remains unclear if RPA's interactions with BLM are important for promoting BLM's functions in dHJ dissolution, as well as its other functions in replication fork restart and resection.

1.4.3 BLM-RAD51

RAD51 has important roles during HR repair whereby it forms a nucleofilament on ssDNA to mediate strand invasion and search homology during the later stages of HR. Loss of RAD51

leads to increased genomic instability and increased mutations in cancers such as renal cell carcinoma highlighting its importance in HR (Liu et al., 2016).

Since RAD51 is essential for HR, Wu et al., hypothesised that BLM and RAD51 may function together during HR and using a yeast-2-hybrid screen, found that the C-terminal amino acid residues 1317-1417 of BLM directly interacts with RAD51. Far western blot analysis also confirmed that the first 212 amino acids of the BLM C-terminus directly interact with RAD51. This interaction is evolutionarily conserved in yeast. Furthermore, fluorescent microscopy has highlighted that BLM and RAD51 colocalise in the nucleus following induced DSBs via ionising radiation, indicating that these two proteins function together in response to DSBs (Wu et al., 2001).

Using a D-loop assay, a study by Bugreev et al., has previously shown that BLM functions to displace ADP-bound RAD51-ssDNA filament to prevent recombination, suggesting this interaction may play a role in promoting BLM's function in mediating D-loop displacement to promote the SDSA non-cross over pathway of HR (Bugreev et al., 2007).

However a later study using a similar biochemical assay revealed BLM functions to promote the strand exchange activity of ATP-bound RAD51-ssDNA filaments, hence highlighting that this interaction may also favour the dissolution or resolution pathways of HR (Bugreev et al., 2009). This suggests that the BLM-RAD51 interaction functions to determine the pathway of HR and this depends on the conformational state of RAD51 which remains controversial. It is also yet to be investigated whether this interaction is important for stimulating BLM's functions in replication fork restart and dHJ dissolution.

1.5 Regulation of BLM via post-translational modifications

Post-translational modifications (PTMs) of proteins are modifications that occur at specific amino acids following translation and often occur in response to a stimulus. PTMs function to

diversify the activity of proteins by regulating protein structure, protein-protein interactions, and protein localisation. PTMs that have been identified in BLM include ubiquitination, phosphorylation and SUMOylation (Böhm and Bernstein, 2014).

1.5.1 Phosphorylation

As discussed previously, phosphorylation of BLM at Ser304 is important for its interaction with TopBP1, therefore this suggests that this PTM is important for mediating BLM protein-protein interactions, although the kinase responsible remains enigmatic.

Following ionising radiation-induced DSBs and HU-induced replication stalling, ATM and ATR phosphorylate BLM at threonine 99 and 122, respectively (Davies et al., 2004, Ababou et al., 2000).

In response to DNA damage, RPA undergoes phosphorylation and this mediates its interactions with several proteins involved in DNA repair such as RAD51 (Shi et al., 2010). More recently, a study has exposed that phosphorylation of RPA inhibits its interaction with BLM, thus abrogating BLM-EXO1/DNA2 mediated end resection (Soniat et al., 2019).

1.5.2 SUMOylation

SUMOylation of proteins involves a three-step enzymatic cascade whereby the SUMO protein is activated by the E1 ligase, followed by conjugation and ligation of SUMO to its target protein via a Lysine-rich SUMO site, this is catalysed by the E2 and E3 ligases (Johnson, 2004). Protein SUMOylation functions in regulating protein-protein interactions, protein translocation, the cellular response to stress and protein stability. More interestingly, SUMOylation has also been reported to be important in regulating HR repair of DSBs (Psakhye and Jentsch, 2012). For example, following induction of DSBs in yeast, PCNA undergoes SUMOylation which mediates recruitment of Srs2 to prevent cross over events

during HR (Papouli et al., 2005). However, the role of SUMOylation in HR remains unclear in human cells.

In 2005, Eladad et al., showed that BLM interacts with UBC9, SUMO-1 and SUMO-2/3. It was then exposed that lysines 317, 331, 334 and 347 are required for the SUMOylation of BLM and that arginine substitution mutations of these residues, that cannot be SUMOylated, inhibits BLM localisation to PML nuclear bodies, highlighting that the SUMOylation of BLM is important for its recruitment to sites of DNA damage. Moreover, BLM defective in SUMOylation showed a 2-fold increase in the formation of SCEs compared to that of WT BLM. Therefore, it is likely that the recruitment of BLM to PML nuclear bodies is required for its anti-recombinogenic roles in HR, such as SCE suppression (Eladad et al., 2005, Zhong et al., 1999).

In 2009, Ouyang et al., showed that following induced replication fork stalling via HU treatment in SUMO-mutant BLM cells, high SCEs were observed indicating that SUMOylation of BLM is required for its functions in HR. Since RAD51 is important for HR, this study also showed that RAD51 interacts with a higher affinity with SUMOylated BLM and that in SUMO mutant BLM cells, RAD51 localisation to sites of DNA damage was impaired, suggesting that SUMOylation of BLM is required for regulating RAD51 (Ouyang et al., 2009).

It appears that SUMOylation of BLM and its functions in HR are evolutionary conserved as studies in yeast have shown that SUMOylation of Sgs1 by Mms1 is required for its functions in dHJ dissolution and resection (Bermúdez-López et al., 2016).

Furthermore, a recent study has revealed that yeast RPA undergoes SUMOylation at two lysine residues in response to DNA damage and this is required for its interactions with Sgs1, functioning to activate Rad53, the yeast homolog of CHK2, and the DNA damage checkpoint

pathway (Dhingra et al., 2019). This indicates that the SUMOylation of Sgs1/BLM's binding partners may be required for mediating protein-protein interactions. Whether SUMOylation of human RPA is required for its interactions with BLM remains to be investigated.

1.6 Aims and objectives

Patients with BS have an increased predisposition to a broad range of early onset cancers, indicating that the BLM helicase protein is a general tumour suppressor (Hickson, 2003). One mechanism in BLM has been proposed to function as a potent tumour suppressor by preventing loss-of-heterozygosity (Groden et al., 1990). However, more recently loss-of-heterozygosity has been indicated to be very rare in patients, suggesting that perhaps another function of BLM is required for tumour suppression (van Wietmarschen et al., 2018). As discussed previously, the BLM helicase protein has many versatile functions such as the restart of stalled replication forks, end resection of double strand breaks during the early stages of HR and double Holliday junction dissolution during the latter stages of HR (Davies et al., 2007, Ralf et al., 2006, Mimitou and Symington, 2009, Gravel et al., 2008).

The majority of studies of the BLM helicase are based on loss-of-function phenotypes whereby loss of the gene or large truncations of the protein are pleiotropic and abrogate all of BLM's functions (German et al., 2007). Although these studies have been fundamental in highlighting the versatile functions of BLM in the DDR, it remains unclear which specific function(s) of BLM is required for its role in tumour suppression.

Additionally, many cellular functions of proteins are mediated by protein-protein interactions from unstructured and intrinsically disordered regions (Tompa et al., 2014). Most studies of the BLM helicase have focused extensively on the structured, folded domains of BLM (the ATPase, RQC and HRDC domains), even though the majority of the BLM protein is made up of these unstructured regions, with little known function.

One approach to delineate BLM's functions in different pathways is to introduce separation-of-function mutations. This can be achieved by identifying important amino acids within the unstructured regions of BLM, which when mutated eliminate and disrupt a specific protein-protein interaction and function whilst maintaining other BLM phenotypes and the structural integrity of the protein (Lubin et al., 2013). This would give an indication as to which specific residues are important for a specific function of BLM, I can then subsequently use this to identify which function(s) of BLM are important for its role in maintaining genome stability and tumour suppression (Figure 1.6).

With this, my aims are to:

1) Identify potential motifs in the unstructured regions of BLM that may constitute novel separation-of-function mutations.
2) Identify if these motifs mediate protein-protein interactions via immunoprecipitation (IP) experiments, and if so the functional significance of these interactions via cell-based assays.

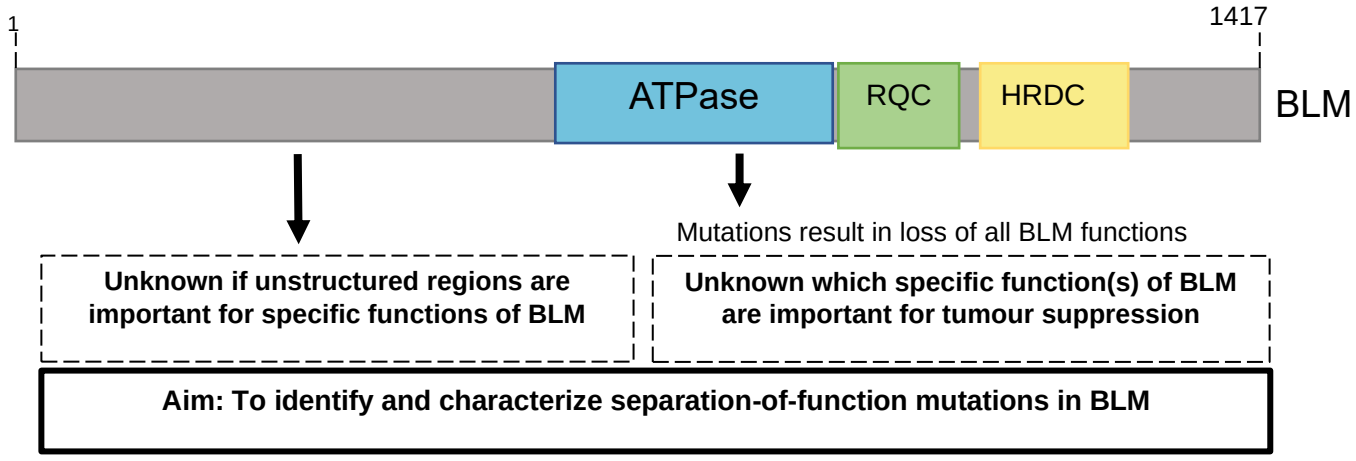


Figure 1.6. Project aims.

The functions of the unstructured regions, which make up the majority of the BLM protein are unknown, I aim to investigate if these regions confer separation-of-function mutations, and whether these phenotypes are required for BLM's role as a tumour suppressor.

2. Materials and methods

2.1 Sequence alignments

Sequences of divergently related BLM orthologs were selected using the NCBI HomoloGene function, and BLM orthologs from the following species were selected to generate sequence alignments: *Homo sapiens* (human), *Pan troglodyte* (chimpanzee), *Macaca mulatta* (Macaque), *Mus musculus* (mouse), *Gallus gallus* (chicken), *Xenopus tropicalis* (frog) and *Danio rerio* (zebrafish). The T-Coffee Multiple Sequence Alignment server (T-COFFEE Multiple Sequence Alignment Server (crg.cat)) was used to generate the alignments, and regions of conservation between aligned sequences were highlighted using the BoxShade server (BoxShade Server (vital-it.ch)).

2.2 Biotin peptide pull-downs

Biotinylated peptides of BLM motif 1 (sequence: DWDDMDDFDTSE), BLM motif 3 (sequence: IDNFDIDDFDD), and RMI1 motif 5 (sequence: LGPSDEELLASLD) were generating using GenScript and bound to streptavidin magnetic Dynabeads M-280 (Thermo Fisher Scientific). Streptavidin bound biotinylated peptides were incubated in HeLa nuclear extracts (Ipracell) for 2 hrs at 4°C, which were then washed and pulled-down with peptide pulldown buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 5mM NaF, 0.2% Igepal CA-630, 1x EDTA free protease inhibitor tablet) at 4°C. Biotinylated peptides were eluted with 2X SDS sample buffer for 5 mins at 100°C.

2.3 Cell culture

RPE-1 FRT cells (Ximbio) and 293FT cells (Thermo Fisher Scientific) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) and supplemented with 10% foetal calf serum (Thermo Fisher Scientific), 500 µL/ml Geneticin (Thermo Fisher Scientific) and 2mM glutamine (Lonza). Cells were incubated with 5% CO_2 at 37°C.

2.4 Western blotting

Western blotting was performed using Tris-Bicine 7% gels (1M Tris-Bicine, 30% Acrylamide, 20% SDS, TEMES, 10% APS) and transferred onto a nitrocellulose membrane overnight. Membranes were blocked in 5% milk and probed using the following primary antibodies: GFP (11814460001, Mouse, 1/10,000, Roche), FLAG (F1804, Rabbit, 1/1000, Sigma-Aldrich), BLM (A300-110A, Rabbit, 1/1000, Bethyl Laboratories), RMI1 (NB100-1720, Rabbit, 1/1000, Novus Biologicals), RMI2 (NBP1-89962, Rabbit, 1/1000, Novus Biologicals), TOP3A (14525-1-1AP, Rabbit, 1/1000, ProteinTech), RPA1 (NA13, Rabbit, 1/1000, Merck), RPA2 (ab10359, Mouse, 1,1000, Abcam), TopBP1 (A300-111A, Rabbit, 1/1000, Bethyl Laboratories). Membranes were then washed in TBST and probed with enzyme conjugated secondary antibodies for chemiluminescence detection. Images of blots were acquired using dark room development.

2.5 Sister chromatid exchange assay

Using the protocol optimised by Stults et al., 2020, I cultured confluent RPE-1 cells with BrdU for two rounds of replication to allow BrdU to become incorporated into one sister chromatid from replicated DNA. Cells were then blocked in metaphase via treatment with 300 ng/ml of colchicine for two hours. Cell cycle arrest was enhanced by prior treatment with CDK1 inhibitor. Cells were then harvested and incubated in 75 mM KCl for 30 mins at 37°C. Cells were then fixed and resuspended in ice-cold 3:1 methanol-acetic acid fixative via 3 rounds of centrifugation at 500g for 5 mins each. Metaphase spreads were carried out by dropping the cells on ice-cold glass slides previously stored in ethanol and cleaned using 0.5% HCl. Before staining, fixed cells were kept in the dark to dry for 2 days. Once dried, cells were treated with 15µg/ml Hoechst for 30 mins at room temperature. Cells were then washed in PBS and were treated with 345 nm UV light for 2 hours to “bleach” the BrdU. Slides were then immersed in 2X saline-sodium citrate (SSC) for 60 mins at 65°C, followed

by staining with Leishman's stain for 90 seconds to allow visualisation of bleached BrdU, hence the observation of SCEs between sister chromatids. Slides were then mounted with glass coverslips using VECTASHIELD. Microscopy of metaphase spreads was carried out using a ZEISS Axio Observer Z1 widefield microscope using the 100x oil objective. Images were acquired using the ZEN pro 2012 software and the number of SCEs per metaphase were counted and analysed using Image J.

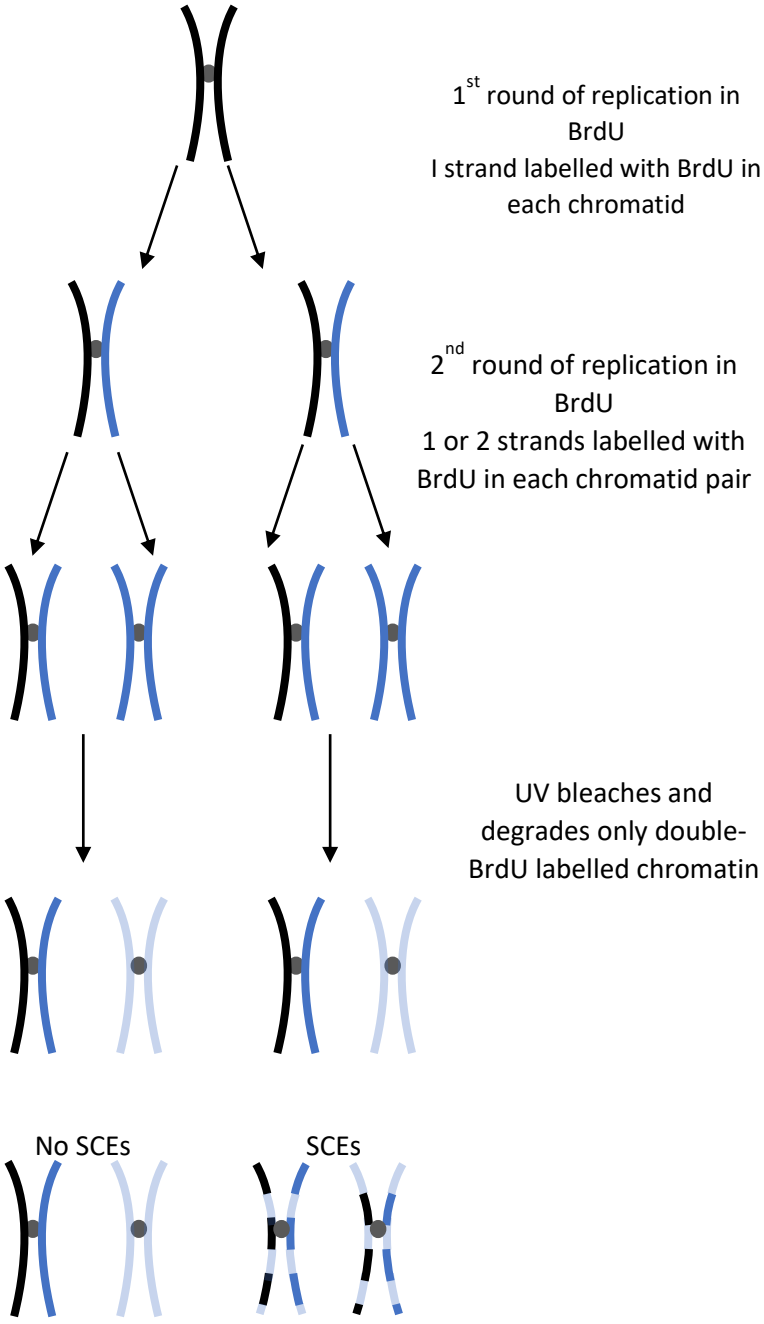


Figure 2.1: Sister chromatid exchange assay: SCEs between sister chromatids of homologous chromosomes occurs during the second round of semi-conservative DNA replication. This procedure allows differential staining between SCEs to be visualised.

2.6 Clonogenic survival assay

RPE-1 cells were plated onto 10 cm dishes at low densities and treated with 0 mM (untreated media), 0.25 mM, 0.5 mM and 1 mM HU concentrations for 24 h. Cells were incubated in a 37°C 5% CO_2 incubator for approximately 2 weeks for colonies to develop. Plates were then washed in PBS and stained using Coomassie stain (0.1% Brilliant Blue R 250, 7% acetic acid, 50% methanol) for 15 mins, rocking at room temperature. Plates were then washed in water and the number of colonies, corresponding to the number of cells that had survived HU treatment, were counted.

Cell densities plated for each RPE-1 cell line used:

Cell line	**No of cells plated for each HU concentration (mM)**			
	0	**0.25**	**0.5**	**1**
BLM$^{+/+}$	300	300	600	1200
BLM$^{-/-}$	600	600	1200	2400
BLM$^{-/-}$ + GFP-BLM^{WT}	300	300	600	1200
BLM$^{-/-}$ +BLM^{min}RMI1^{min}	600	600	1200	2400
BLM$^{-/-}$ + BLM/RMI1^{WT}	300	300	600	1200
BLM$^{-/-}$+BLM/RMI1$^{\Delta RPA}$	600	600	1200	2400
BLM$^{-/-}$+ BLM$^{FTF\text{-}GGG}$	600	600	1200	2400
BLM$^{-/-}$+ BLM^{PATCH8A}	600	600	1200	2400

2.7 PCR site-directed mutagenesis

To generate GFP-tagged and/or FLAG-tagged mutant BLM plasmids, primers for site-directed mutagenesis were designed using QuickChange (Agilent Technologies). PCR using Pfu polymerase was carried out to amplify the plasmids of interest, which were verified via gel electrophoresis. PCR products were then carried out a *Dpn1* digest of the plasmids and transformed in TOP10 competent bacteria in LB media and grown on agar plates treated with antibiotics for selection; kanamycin to select for bacteria transformed with GFP-tagged plasmids and ampicillin to select for bacteria transformed with FLAG-tagged plasmids. Bacteria colonies were then picked and maxi-prepped using Endotoxin Free Plasmid

Maxiprep Kits. Sanger sequencing and ApE software were used to verify and confirm mutagenesis of the plasmids of interest. Figure 2.2 illustrates PCR site directed mutagenesis for the generation of $BLM^{\Delta SIM}$ constructs.

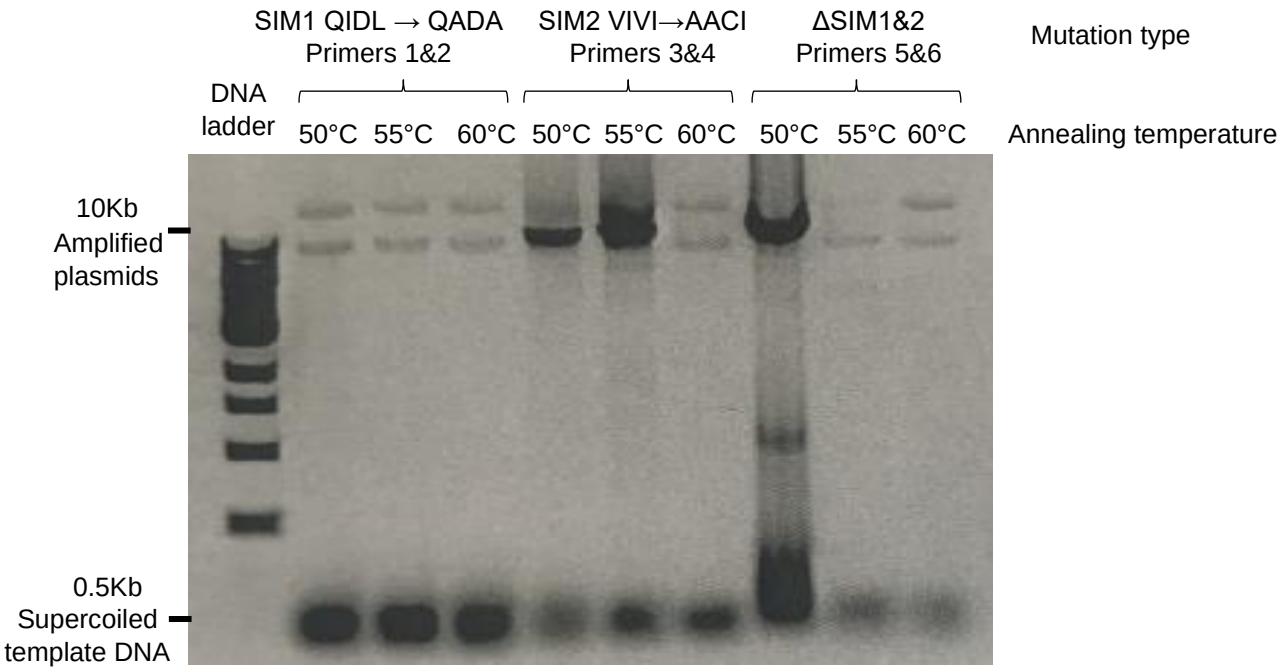


Figure 2.2: SDM of BLM SIMs via PCR amplification.

I carried out several PCR amplifications, using primers we designed, to generate three mutant forms of BLM: SIM1 mutants by substituting QIDL to QADA, SIM2 mutants by substituting VICI to AACI, and simultaneous deletion of both SIM1 and SIM2 (ΔSIM). Our PCR results and subsequent sequencing of our plasmids show that our SDM for mutating SIM2 was successful with an optimal annealing temperature of 50°C and 55°C, and deleting both SIMs was successful with an annealing temperature of 50°C. SDM of SIM1 was not successful. As it was not possible to mutate SIM1, I continued investigating the functions of the SUMO interaction motifs using the FLAG-$BLM^{\Delta SIM}$ plasmid we generated in which both SIMs were deleted.

2.8 Immunoprecipitations

293FT cells were plated into 15cm dishes and were transfected with 18ug of GFP-tagged and/or FLAG-tagged plasmids using Lipofectamine 2000 and Opti-MEM (Thermo Fisher Scientific). Cells were then harvested at 70% confluency in PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 5 mM NaF, 0.2% Igepal CA-630, 1x EDTA free protease inhibitor tablet, Benzonase) at 4°C for 45 mins.

The NaCl concentration of the lysis buffer was then optimised to 200 mM and the EDTA concentration was changed to 2 mM. Cell lysates were then incubated in either GFP or FLAG-Trap magnetic agarose beads, rolling for 2 hours at 4°C and were then washed 5 times in lysis buffer. Peptides attached to the magnetic beads were then eluted using with 2X SDS sample buffer for 5 mins at 100°C.

2.9 Lentiviral infections

Lentiviral constructs of plasmids encoding GFP-BLM$^{FTF\text{-}GGG}$, GFP-BLMP^{ATCH8A} and GFP only were transfected into 293FT cells as well as other lentiviral assembly plasmids (encoding gag, pol, env, vsv) using lipofectamine 2000 and Opti-MEM (Thermo Fisher Scientific). Viral media was then harvested, filtered and incubated in *BLM*$^{-/-}$ RPE-1 cells for 48 hrs lentiviral infection. Cells were then grown in G418 selection media to select for infected cells. To generate stable cell lines expressing GFP-BLM$^{FTF\text{-}GGG}$ and GFP-BLM^{PATCH8A}, GFP-positive cells were selected for by fluorescent-activating cell sort and further verified by western blotting (Figures 3.21 and 3.25).

2.10 siRNA transfections

siRNA constructs (siCtrl and siRMI1, resistant to siRNA) were transfected into *BLM*$^{-/-}$ RPE-1 cell lines using Lipofectamine RNAiMax (Thermo Fisher Scientific). The siRNA plasmid sequences are as follows:

siCtrl: 5′-CGUACGCGGAAUACUUCGA-3′

siRMI1: 5′-AGCCUUCACGAAUGUUGAU-3′.

3. Results

3.1 Unstructured regions of the BLM helicase contain highly conserved residues

Highly conserved regions of proteins often indicate evolutionary functional importance and furthermore, it has been recently recognised that conserved motifs within unstructured and intrinsically disordered regions of proteins often mediate protein-protein interactions (Davey et al., 2012, Van Roey et al., 2014). Therefore, I focused on identifying conserved motifs within the unstructured regions of the BLM helicase and the BTR complex proteins as these would most likely highlight potential separation-of-function mutations. I did this by carrying out a multiple sequence alignment of the BTR complex proteins from different taxonomical classes of large evolutionary distances to identify amino acid sequences that had a high degree of similarity.

Firstly, I found that the BTR complex contains six putative conserved short motifs: four within the unstructured regions of BLM (Figure 3.1), and two within RMI1 (Figure 3.2), leading us to believe that these motifs in BLM and RMI1 may have functional importance, and hence constitute novel separation-of-function mutations. No conserved short motifs were found in TOP3A as the majority of this protein consists of structured regions. Likewise, no short, conserved motifs were identified in RMI2 as this is a small protein containing one OB domain and does not contain unstructured regions.

Secondly, previous studies of BLM have identified that the first 133 amino acids of the N-terminus of BLM mediate protein-protein interactions with the BTR complex proteins TOP3A, RMI1 and RMI2 (Hu et al., 2001) .My sequence alignment of BLM has identified that within the N-terminus of BLM, there is a highly conserved region spanning from amino acids 4-37, leading us to believe that this region of the N-terminus may have functional

importance. Furthermore, unpublished structural analysis from one of our collaborators (the group of Dr Andrew Deans) predicts that three highly conserved FTF residues within the N-terminus may form the interface for protein-protein interactions, and since these residues reside within the N-terminus required for interactions with BTR complex, this lead us to believe that these conserved resides may constitute a novel separation-of-function mutation (Figure 3.3).

The DHBN domain of BLM is important for dimerization (Shi et al., 2017). My sequence alignments illustrate that the DHBN domain is highly conserved and contains many conserved hydrophobic amino acids, named “PATCH”, with unknown function, leading us to believe that residues could be important for interactions and dimerization with BLM, hence may constitute a novel separation-of-function mutation (Figure 3.4).

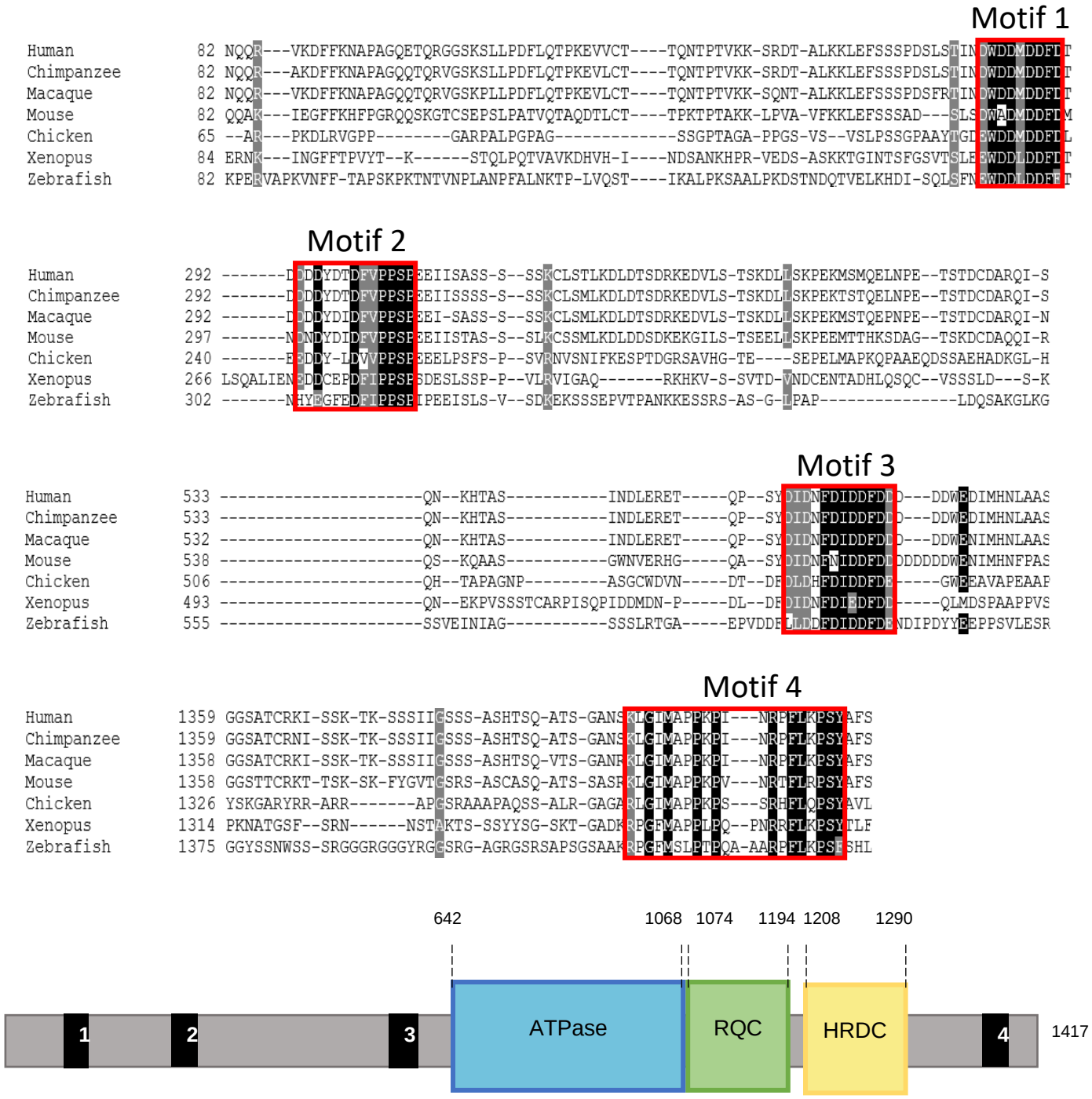


Figure 3.1: Potential conserved short interaction motifs of BLM: Sequence alignment of BLM has identified 4 conserved motifs which have been located at three regions of the N terminus at residues 153-163, 292-306, 552-563 and one region within the C-terminus at residues 1395-1414. We have named these residues as motifs 1-4 for future studies.

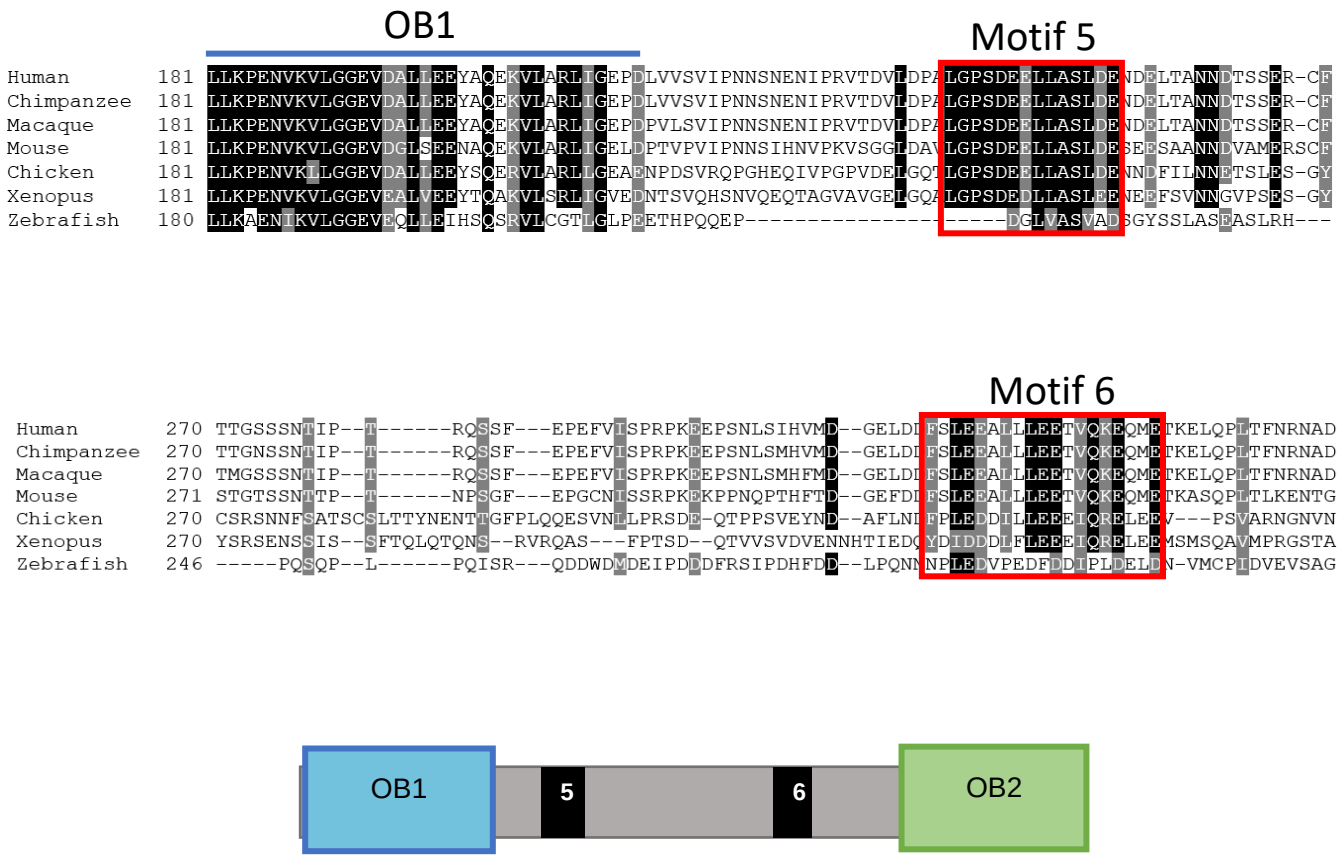


Figure 3.2: Potential conserved short interaction motifs of RMI1: Two conserved motifs were found in RMI1 which we have named motifs 5-6 for future studies.

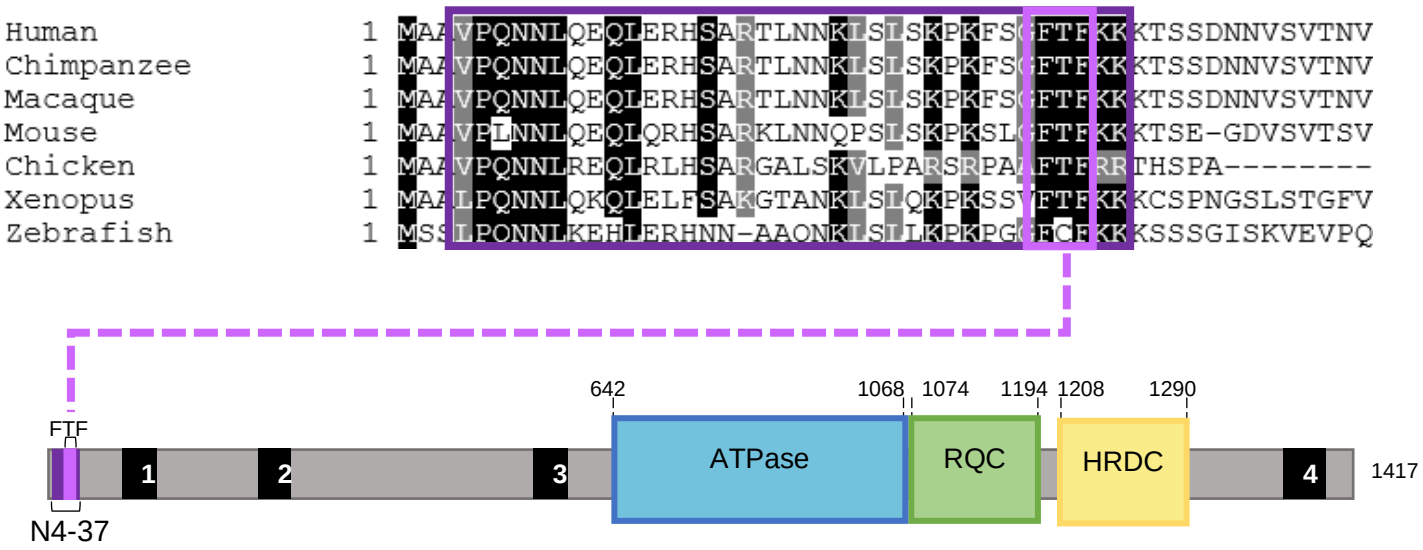


Figure 3.3: The highly conserved BLM N-terminus

The conserved N-terminus contains 3 highly conserved FTF residues located at amino acids 32-34.

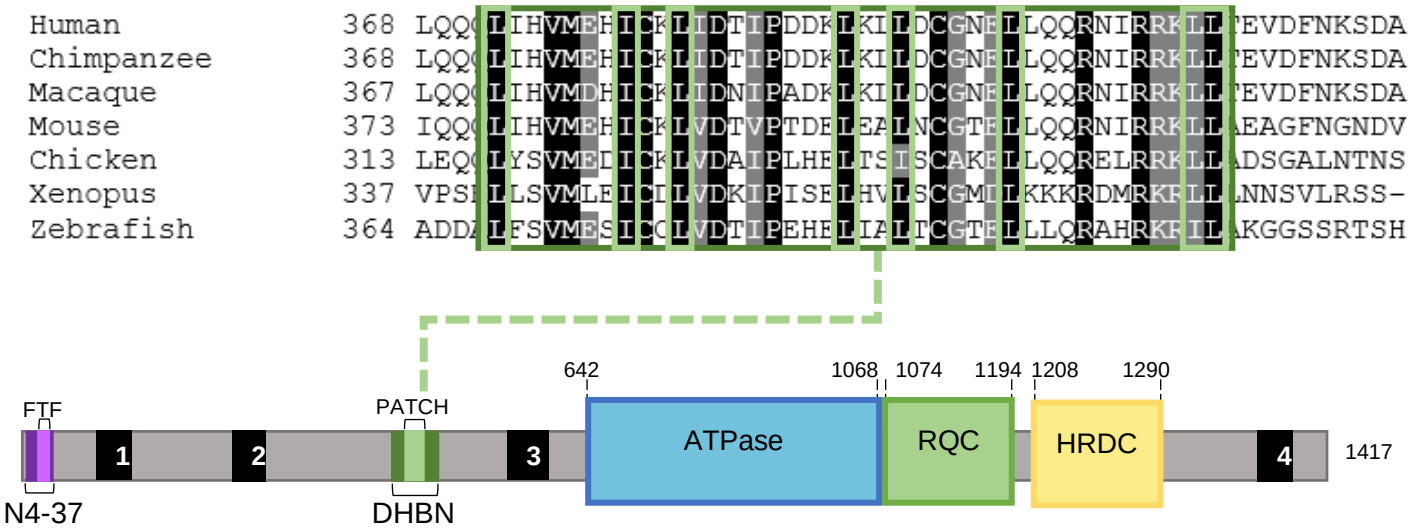


Figure 3.4: The highly conserved DHBN domain of BLM contains 8 highly conserved hydrophobic amino acid residues.

3.2 Functional assays characterising BLM phenotypes

To investigate the functions of the conserved motifs identified in the unstructured regions of BLM, colleagues in the lab generated three *BLM* knockout cell lines using CRISPR-Cas9 gene editing. I then carried out several functional assays in wild type (WT) RPE-1 cells to characterise the phenotype of cells expressing BLM, and in one of *BLM*$^{-/-}$ RPE-1 clones to characterise the phenotype of cells that are deficient in BLM. This was done to determine if these assays and these cell lines would be appropriate to further characterise the functions of the conserved residues of the BLM N-terminus for future experiments.

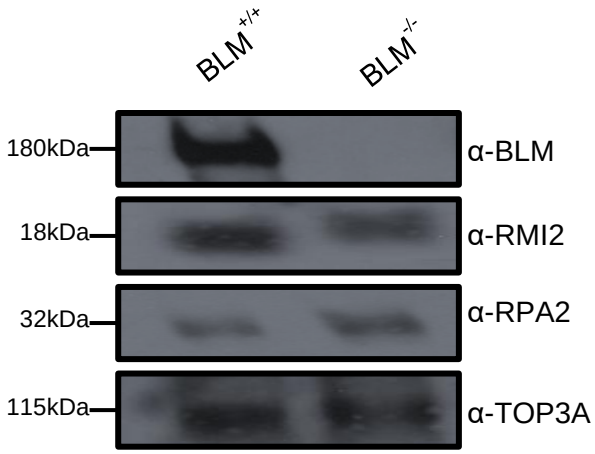


Figure 3.5: CRISPR-Cas9 knock out of BLM from RPE-1 cells. We have been able to knock out BLM from our RPE-1 cells without disrupting the expression of the other BTR complex proteins.

3.2.1 *BLM*$^{-/-}$ cells display high levels of SCEs

Western blot analysis and sequencing data (data not shown) confirms that we have knocked out BLM using CRISPR-Ca9 gene editing (Figure 3.5). I therefore wanted to validate whether our *BLM*$^{-/-}$ cell line display the same phenotypes associated with BS. A well characterised phenotype in BS patients is increased levels of SCEs by approximately 10-fold (Shiraishi, 1990). Therefore, as shown in Figure 3.5, I carried out an SCE assay to validate if our *BLM*$^{-/-}$ cell line mirrors that of a BS cell phenotype. My results in Figure 3.6 present that BLM$^{+/+}$ cells display an average of 4 SCEs per metaphase, while the *BLM*$^{-/-}$ cells display on average 27 SCEs per metaphase. Thus showing that the *BLM*$^{-/-}$ cell line has a 7-fold increase in the number of SCEs compared to that of BLM$^{+/+}$ cells, which is similar to that seen in BS patients. This confirms that BLM has been successfully knocked out in our *BLM*$^{-/-}$ RPE-1 cell line since it is functionally defective. This result also confirms that our SCE assay is appropriate for studying BLM's functions further in future cell-based functional assays.

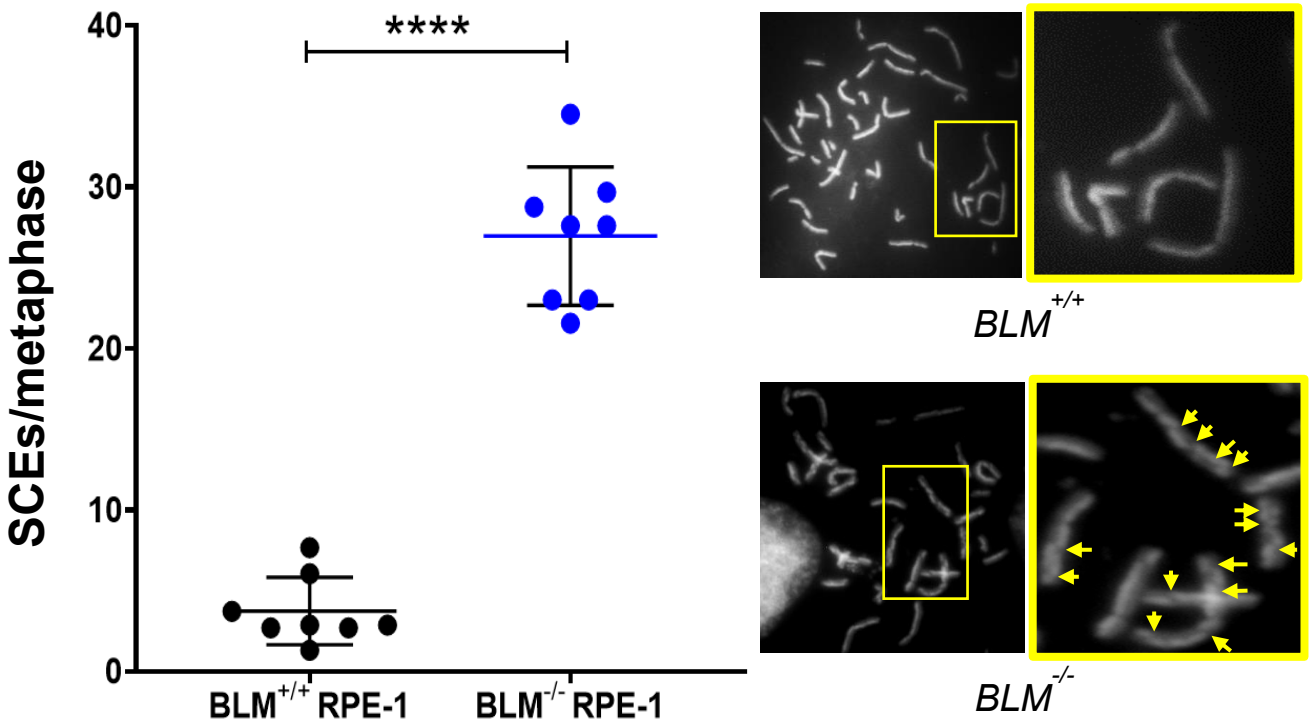


Figure 3.6: SCE assay of WT and BLM$^{-/-}$ RPE-1 cells:

The number of SCEs observed in each metaphase were counted, as depicted by the yellow arrows.

The data displayed as scatter plots derived from the SCE assay are statistically significant with a p-value of <0.0001. Statistical analysis was carried out by a Welch's t test.

3.2.2. *BLM*$^{-/-}$ cells are sensitive to HU

HU is a synthetic analogue of urea and functions to inhibit the ribonucleotide reductase enzyme which is required for production of nucleotides; therefore HU serves to prevent DNA synthesis and induce replication stress, a consequence of this is the formation of stalled replication forks, and can even lead to the formation of double strand breaks (Yarbro, 1992).

A study by Yamagata et al., has shown that yeast cells deficient in Sgs1 are sensitive to HU treatment (Yamagata et al., 1998). Franchitto et al., have demonstrated that that BS lymphocytes are sensitive to HU treatment (Franchitto and Pichierri, 2002). In contrast, others have shown that *BLM*$^{-/-}$ DT40 cells and lymphoblasts derived from BS patients are not sensitive to HU treatment and are resistant to being killed by limited DNA synthesis and fork stalling (Imamura et al., 2001, Ababou et al., 2002). Hence BLM's role in tolerating HU and replication stress is not well defined. However, BS cells do not have an isogenic WT cell line to go with, whereas our *BLM*$^{-/-}$ cell line represents an isogenic model that is more reliable. Therefore, I wanted to characterise BLM's functions in replication stress sensitivity using our cell line.

To do so, I carried out a clonogenic survival assay as presented in Figure 3.7. BLM$^{+/+}$ and *BLM*$^{-/-}$ cells were incubated for 24 hours in the presence of increasing concentrations of HU of 0.25 mM, 0.5 mM and 1 mM. These cells were also incubated in the absence of HU as an untreated control. My results indicate that as the concentration of HU increases, there is reduced survival of the *BLM*$^{-/-}$ cells compared to that of the BLM$^{+/+}$ cells. These results demonstrate our *BLM*$^{-/-}$ cells are hypersensitive to HU treatment, highlighting that BLM functions in tolerating replication stress and limited DNA synthesis caused by HU, thus agreeing with studies by Franchitto et al., and Davies et al.

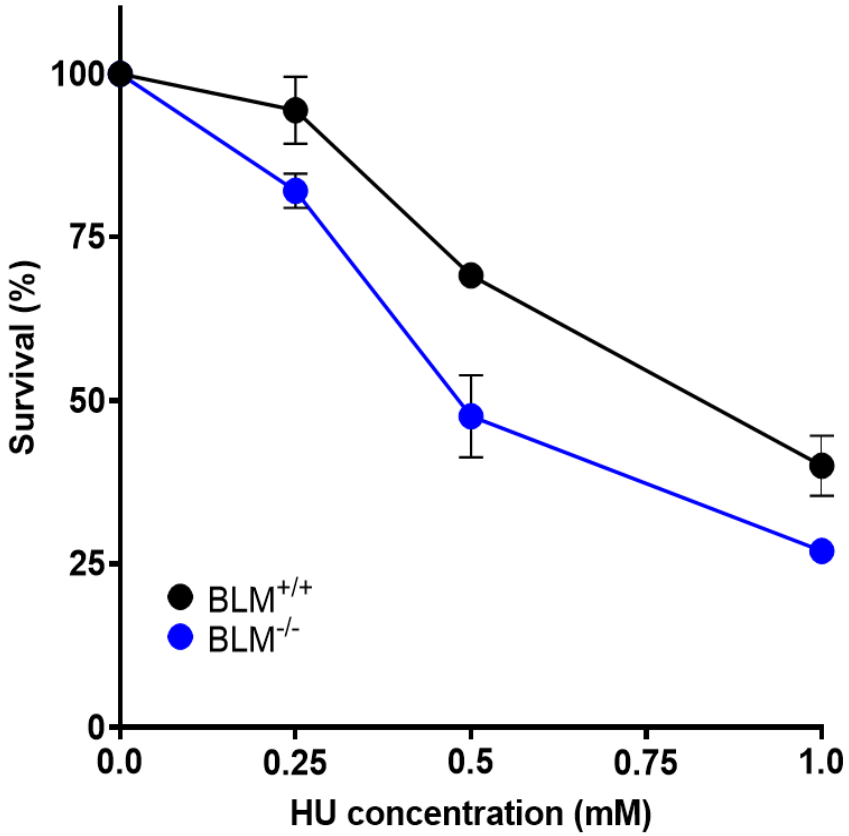


Figure 3.7: Clonogenic survival assay of WT and BLM$^{-/-}$ RPE-1 cells:
The number of colonies counted for each HU concentration were used to calculate the percentage of cells that had survived by automating the number of colonies counted in the untreated conditions as 100% survival. Results were plotted as an XY graph. The mean percentage survival data of the WT RPE-1 cells and BLM$^{-/-}$ RPE-1 cells at 0.25mM are statistically significant with a p-value of 0.016229. Mean percentage survival data at 0.5mM are statistically significant with a p-value of 0.002669. Statistical analysis was carried out via multiple t-tests.

3.3 Conserved short motifs of BLM and RMI1 interact with RPA

To elucidate the binding partners of the motifs identified in Figures 3.1 and 3.2, biotinylated peptides based on these motifs were designed by the lab prior to my arrival. Some motifs have conserved phosphorylation sites such as Ser304 of motif 2, therefore this peptide was designed with and without the phosphorylation PTM (Blackford et al., 2015). These biotin-peptides were then conjugated to magnetic streptavidin beads and incubated in HeLa nuclear extracts and subsequent liquid chromatography-tandem mass spectrometry of the peptides identified potential binding partners. TOPBP1 was found to interact with motif 2 of BLM when Ser304 was phosphorylated, supporting previous studies from Blackford et

al.,(Blackford et al., 2015). In contrast, mass spectrometry data did not show any interactors for motif 4 for BLM and motif 6 of RMI1. Additionally, RPA was found to interact with motifs 1 and 3 of BLM and motif 5 of RMI1. This supports previous studies which showed that the N-terminus of BLM directly interacts with RPA (Doherty et al., 2005). It has been shown that the BLM N-terminus is required for promoting DNA2 mediated resection, however it remains unknown if this interaction also stimulates BLM's activity in end resection (Nimonkar et al., 2011). Other studies have revealed that the central region of RMI1 interacts with RPA and is required for the dissolution of dHJs (Doherty et al., 2005, Yin et al., 2005, Xue et al., 2013). Furthermore, it remains unclear if RPA's interactions with the BTR complex are required for dHJ, resection, fork restart or all of these functions.

To confirm that these motifs are required for interactions with RPA, I conjugated biotinylated peptides 1, 3 and 6 with streptavidin and incubated with HeLa nuclear extracts. I then carried out a peptide pull-down, and then used western blotting for the three subunits of RPA (RPA1, RPA2 and RPA3) to confirm the interaction. My results as displayed in Figure 3.8 confirm that all three subunits of the RPA heterotrimer interact with motifs 1 and 3 of BLM, and motif 5 of RMI1. These results therefore map three specific regions within the BTR complex that mediate interactions with RPA.

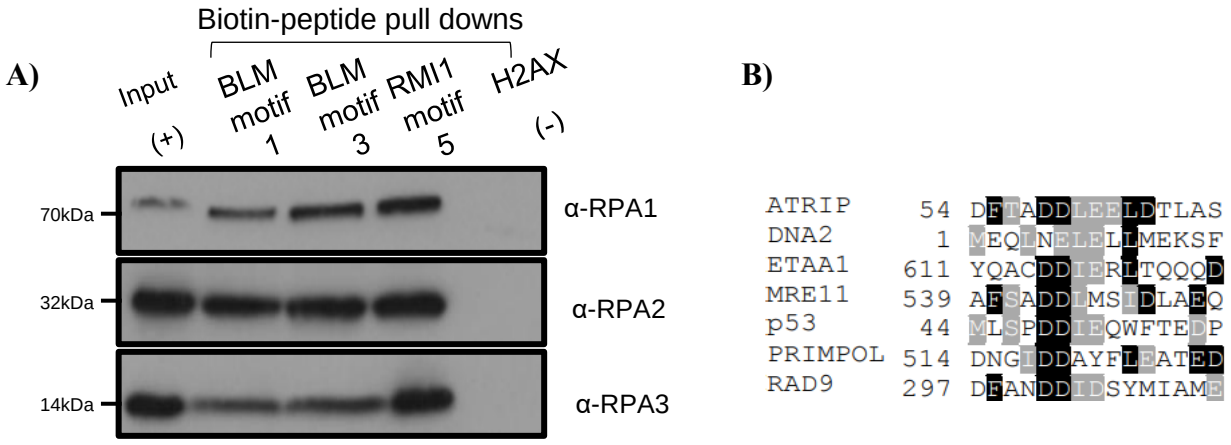


Figure 3.8: Motifs 1 and 3 of BLM and motif 5 of RMI1 interact with the heterotrimeric RPA. A) Western blot analysis of our pull down confirms the BLM/RMI1 interaction with RPA via motifs 1,3 and 5. H2AX was pulled down as a negative control since this protein does not interact with RPA. B) Sequence alignments highlighting sequence similarity of the RPA binding motifs of other DDR proteins.

3.4 Characterisation of the BLM/RMI1-RPA interaction

3.4.1 Validation of a cell line deficient in BLM and RMI1

I next sought to investigate if the BLM/RMI1-RPA interaction is important for the specific functions of BLM and the BTR complex. This requires a cell line whereby both endogenous BLM and RMI1 are depleted so that we can express exogenous mutants of these proteins where the motifs 1, 3 and 5 required for interacting with RPA are deleted. Since I have previously characterised our *BLM*$^{-/-}$ RPE-1 cell line as a successful BLM knock out, we attempted to knock out RMI1 from this cell line using CRISPR-Cas9 editing by targeting the N-terminus region of RMI1, which important for its stability and functions. However, it is worth noting that several previous studies focused on investigating the functions of RMI1 have reported that knocking out RMI1 was embryonically lethal in mice (Chen et al., 2011). Although we were unsure if CRISPR-Cas9 targeting of RMI1 would be viable, we wanted to attempt this to produce a double *BLM*$^{-/-}$*RMI1*$^{-/-}$ cell line for future cell-based assays. Cas9 guides were designed to target the N-terminus of RMI1. Sequencing data shows that this subsequently resulted in mutations in the critical T59 residue in one allele of RMI1 which is important for the folding of the protein, and mutations in a flexible linker region in both alleles of RMI1 (Figure 3.9). Western blot analysis reveals that our CRISPR-Cas9 targeting of RMI1 in *BLM*$^{-/-}$ cells strongly reduced expression of RMI1 but did not completely knock out the protein. Therefore, although RMI1 levels have been strongly reduced, it is not certain if the protein is functional or not in our *BLM*$^{-/-}$*RMI1*^{min} cell line (Figure 3.9).

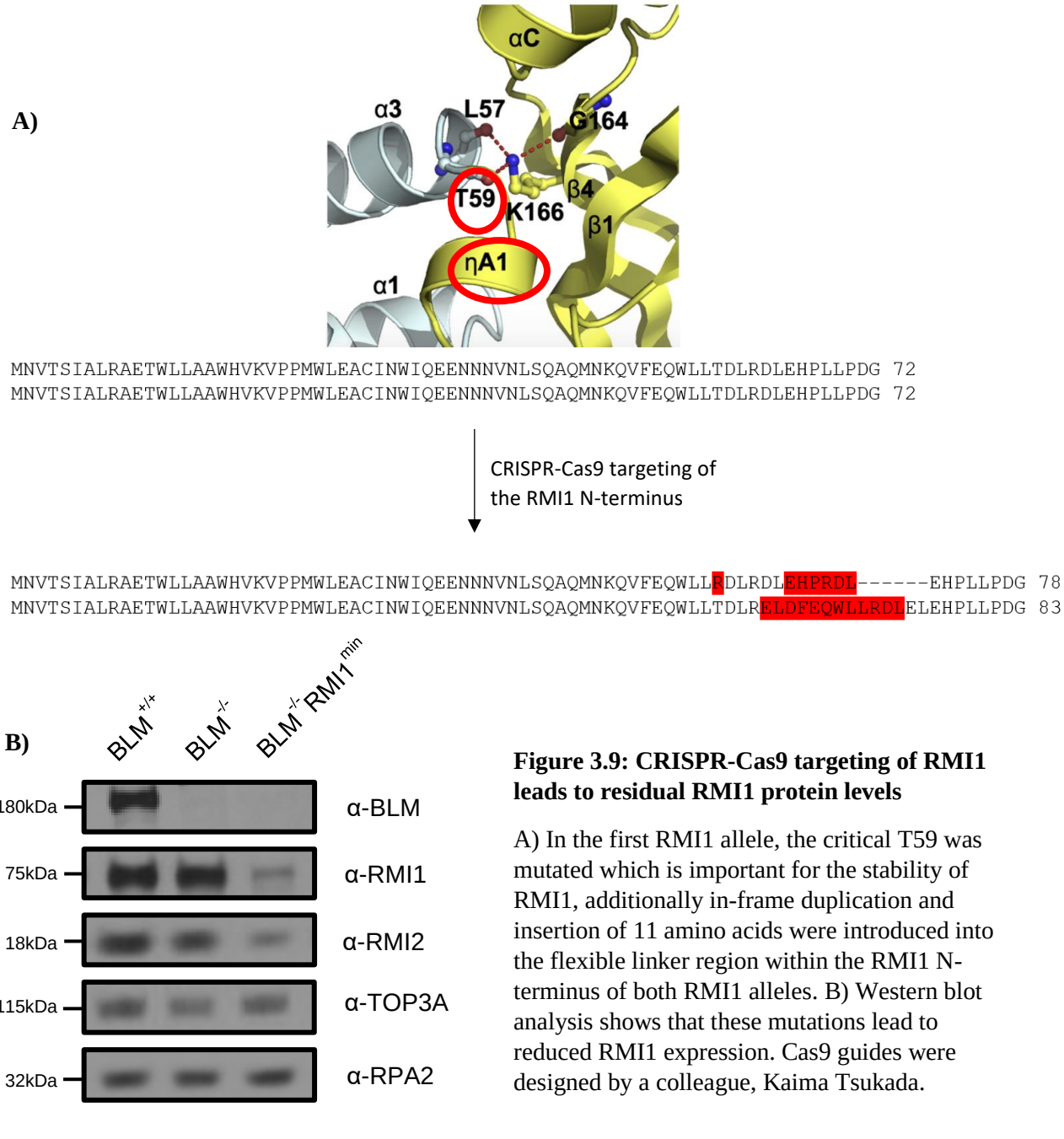


Figure 3.9: CRISPR-Cas9 targeting of RMI1 leads to residual RMI1 protein levels

A) In the first RMI1 allele, the critical T59 was mutated which is important for the stability of RMI1, additionally in-frame duplication and insertion of 11 amino acids were introduced into the flexible linker region within the RMI1 N-terminus of both RMI1 alleles. B) Western blot analysis shows that these mutations lead to reduced RMI1 expression. Cas9 guides were designed by a colleague, Kaima Tsukada.

RMI1 is important for BLM's role in preventing crossovers during HR. It has also been reported that patients with defective RMI1 have BS-like syndrome and display increased levels of SCEs, similar to that seen in patients with BS (Martin et al., 2018). Therefore, to investigate if RMI1 in our *BLM*$^{-/-}$*RMI1*^{min} cell line is functionally defective, we complemented the *BLM*$^{-/-}$ *RMI1*^{min} cell line with GFP-BLM (Figure 3.10). I then carried out an SCE assay to determine if RMI1 is functionally defective in the *BLM*$^{-/-}$*RMI1*^{min} cell line as if RMI1 is functionally defective, reintroducing BLM into these cells should not restore the WT SCE suppression phenotype. My results show that there is no difference in the number of

SCEs of the *BLM*$^{-/-}$*RMI1*^{min} cells compared to that of the *BLM*$^{-/-}$ cells. Importantly, complementation with a GFP-BLM plasmid into the *BLM*$^{-/-}$*RMI1*^{min} resulted in a low number of SCEs similar to that of WT cells (3.11). This indicates that the residual levels of RMI1 present in the *BLM*$^{-/-}$*RMI1*^{min} cells are sufficient to prevent crossover events, restoring the WT phenotype. These data therefore confirm that unfortunately our *BLM*$^{-/-}$*RMI1*^{min} cell line cannot be used to characterise the BLM/RMI1-RPA interaction further. Instead, I silenced RMI1 expression via siRNA in subsequent cell-based assays (Figure 3.12).

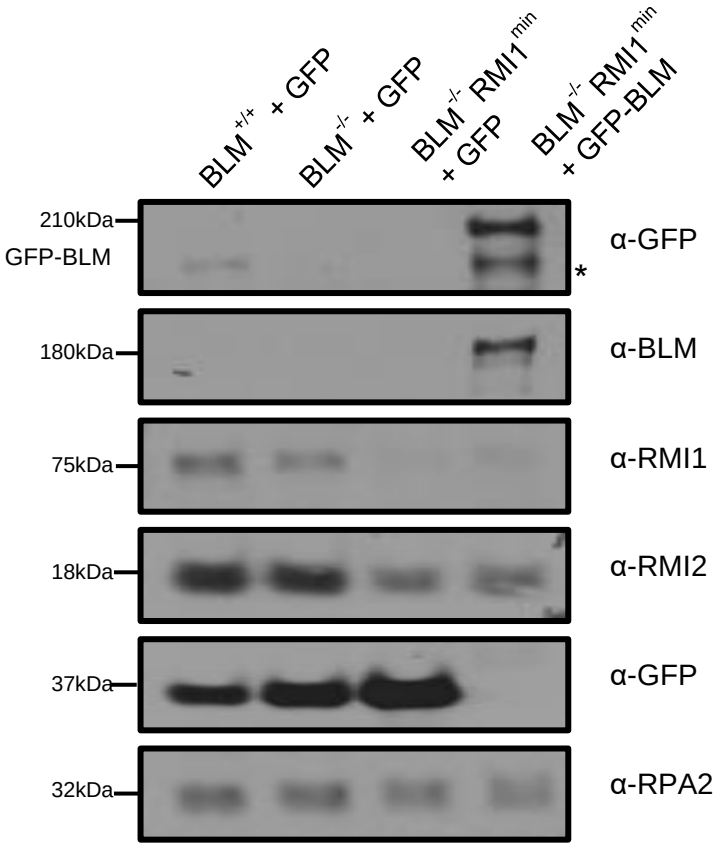


Figure 3.10: Complementation of BLM$^{-/-}$RMI1^{min} cells with GFP-BLM

Western blot analysis confirms successful complementation of exogenous GFP-BLM constructs into our *BLM-/-RMI1*^{min} cell line for functional assays.

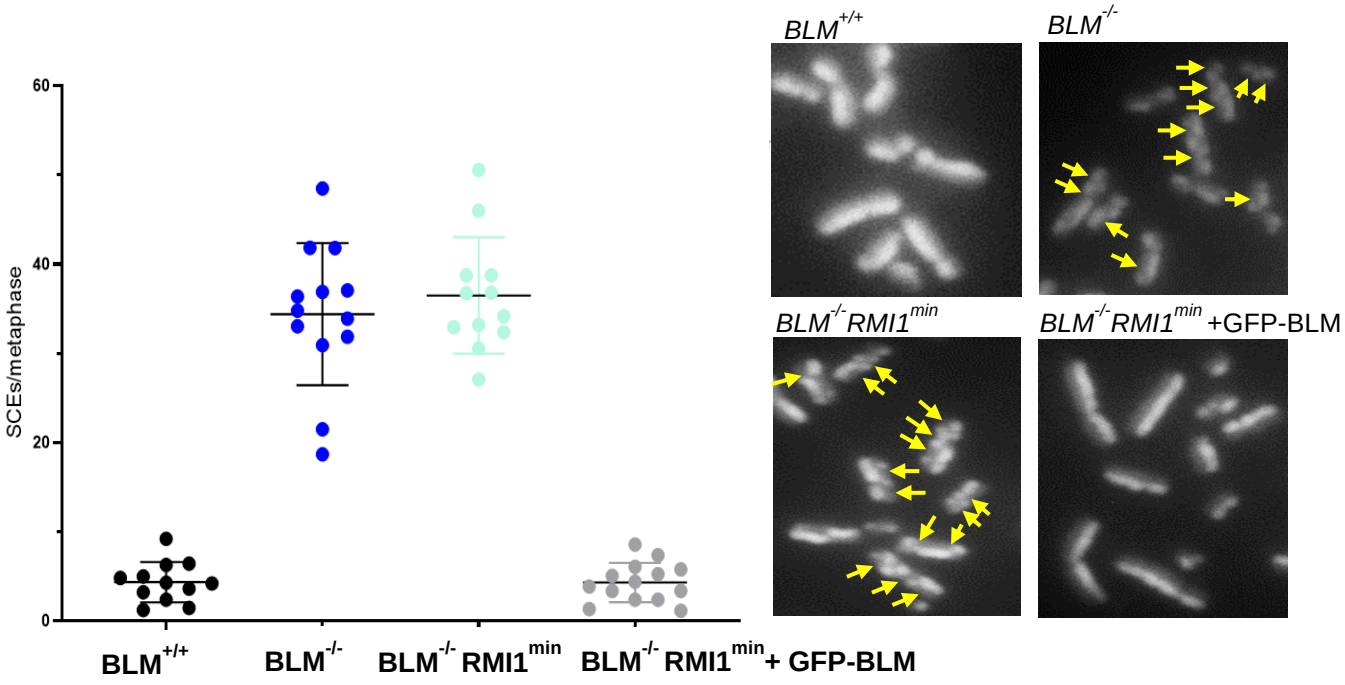


Figure 3.11: RMI1 is not functionally defective in the BLM$^{-/-}$ RMI1^{min} cell line

The scatter plots derived from the SCE assay are statistically significant with a p-value of <0.0001. Statistical analysis comparing the number of SCEs for each cell line was carried out by a Mann-Whitney test, showing that there is no statistical difference between the number of SCEs of the BLM$^{-/-}$ and BLM$^{-/-}$ RMI1^{min} cells, and of that of the WT and BLM$^{-/-}$ RMI1 +GFP-BLM cells.

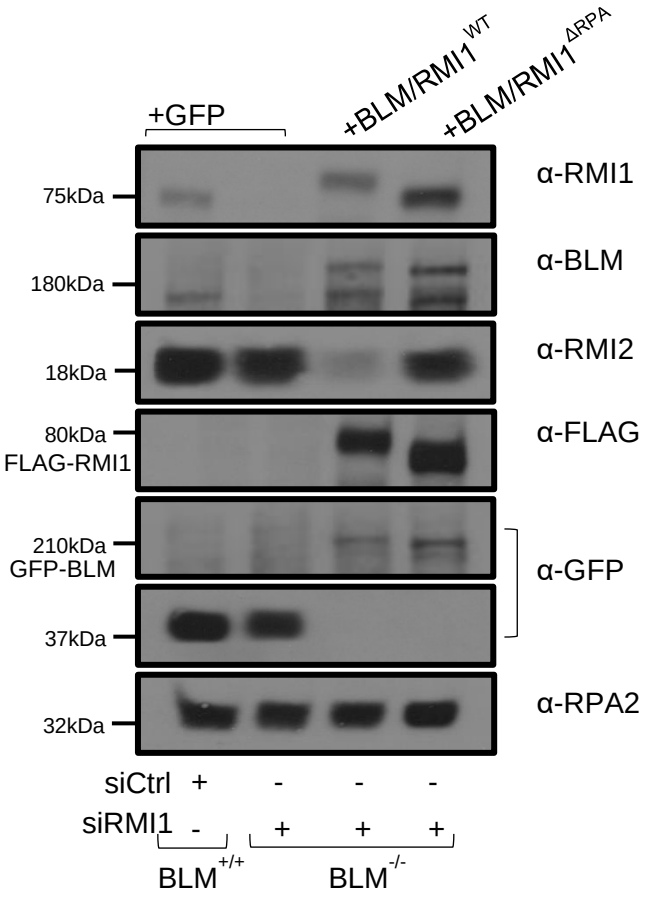


Figure 3.12: siRNA silencing of RMI1

Western blot analysis confirms siRNA mediated endogenous silencing of RMI1 in our GFP-BLM/FLAG-RMI1 expressing cell lines.

3.4.2 The BLM/RMI1-RPA interaction is important for resistance to HU

Sister chromatid exchange assays, which were carried out by my colleague in the lab (Dr Sam Jones), show that cells lacking the RPA binding motifs displayed low levels of SCEs, similar to that seen in $BLM^{+/+}$ cells (Figure 3.13). Thus, suggesting that the RPA binding motifs of BLM and RMI1 are not required for suppression of SCEs via the dissolution pathway of HR.

Previous studies have shown that BLM is important for resistance to genotoxic agents that cause replication fork stalling (Franchitto and Pichierri, 2002). As previously investigated in this study, our $BLM^{-/-}$ cells display increased sensitivity to HU, and one consequence of treating cells with HU is replication fork stalling. I therefore wanted to investigate if the RPA binding motifs of the BTR complex are important for BLM's functions in HU resistance.

To do so, I carried out a clonogenic survival assay by treating cells with increasing concentrations of HU for 24 hours. I found that loss of the RPA binding motifs in BLM and RMI1 rendered cells sensitive to increasing concentrations of HU, and this complemented the sensitivity of $BLM^{-/-}$ cells (Figure 3.14). This suggests that the RPA interaction with the BTR complex via these motifs are important for the resistance of cells to HU-induced replication stress, and perhaps replication fork stalling.

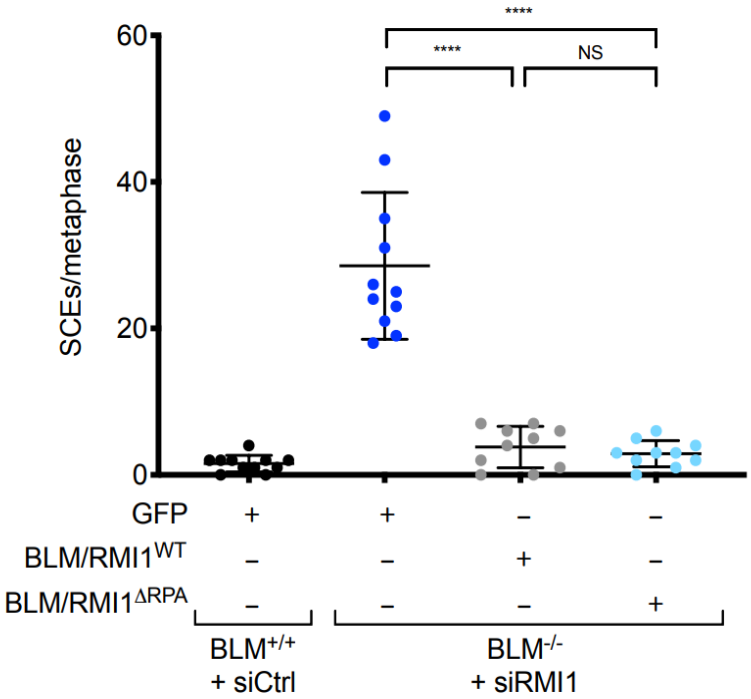


Figure 3.13: RPA binding motifs are not required for the suppression of SCEs.

SCE assay conducted by Dr Sam Jones. Statistical analysis and significance were carried out by a Mann-Whitney test, confirming there is a statistical significance between the number of SCEs of the $BLM^{-/-}$ compared to the WT and ΔRPA cells, with a p-value of <0.0001. There is no statistical significance between the WT and ΔRPA cells.

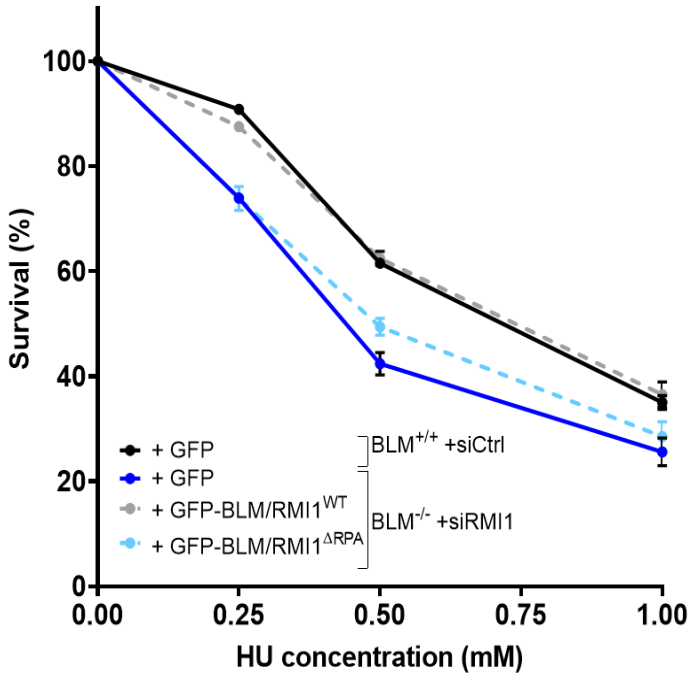


Figure 3.14: RPA binding motifs are important for HU resistance: The number of colonies counted for each HU concentration were used to calculate the percentage of cells that had survived by automating the number of colonies counted in the untreated conditions as 100% survival. Results were plotted as an XY graph. Statistical analysis was carried out via multiple t-tests.

3.4.3 BLM SUMO interacting motifs are not required for interactions with RPA

Previous studies in yeast by Dhingra et al., have reported that SUMOylation of RPA is required for interactions with Sgs1 and this functions to activate the DNA damage checkpoint pathway (Dhingra et al., 2019). Therefore, I wanted to investigate if SUMOylation of RPA is important for interactions with human BLM. Using in vitro SUMO binding assays and sequence analysis, a previous study identified two short amino acid motifs within the N-terminus of BLM, VICI and QIDL, which are required for interactions with SUMO-1 and SUMO-2/3. These motifs are termed SUMO interacting motifs (SIMs) (Zhu et al., 2008). I carried out a sequence alignment and identified that the VICI SIM is conserved, whereas the QIDL SIM is not conserved in human BLM (Figure 3.15).

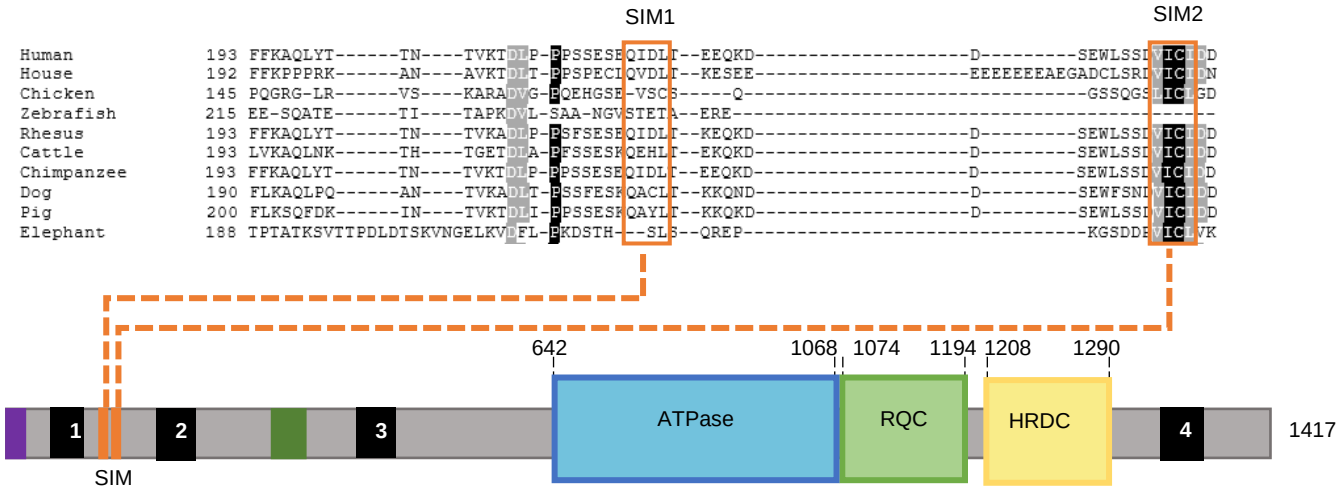


Figure 3.15: There are two SIMs within the N-terminus of BLM. The QIDL SIM is located between amino acid residues 217-220. The conserved VICI SIM is located within amino acid residues 235-238

It has also been reported that simultaneous mutations of the hydrophobic amino acids within these two SIMs abrogates interactions with SUMO-1 and SUMO-2 (Zhu et al., 2008). Therefore, to investigate if these SIMs are important for interactions with SUMOylated RPA, I carried out site-directed mutagenesis (SDM) of ampicillin resistant FLAG-BLM^{WT} plasmids to substitute the hydrophobic residues of these SIMs with alanine residues (Figure 3.16)

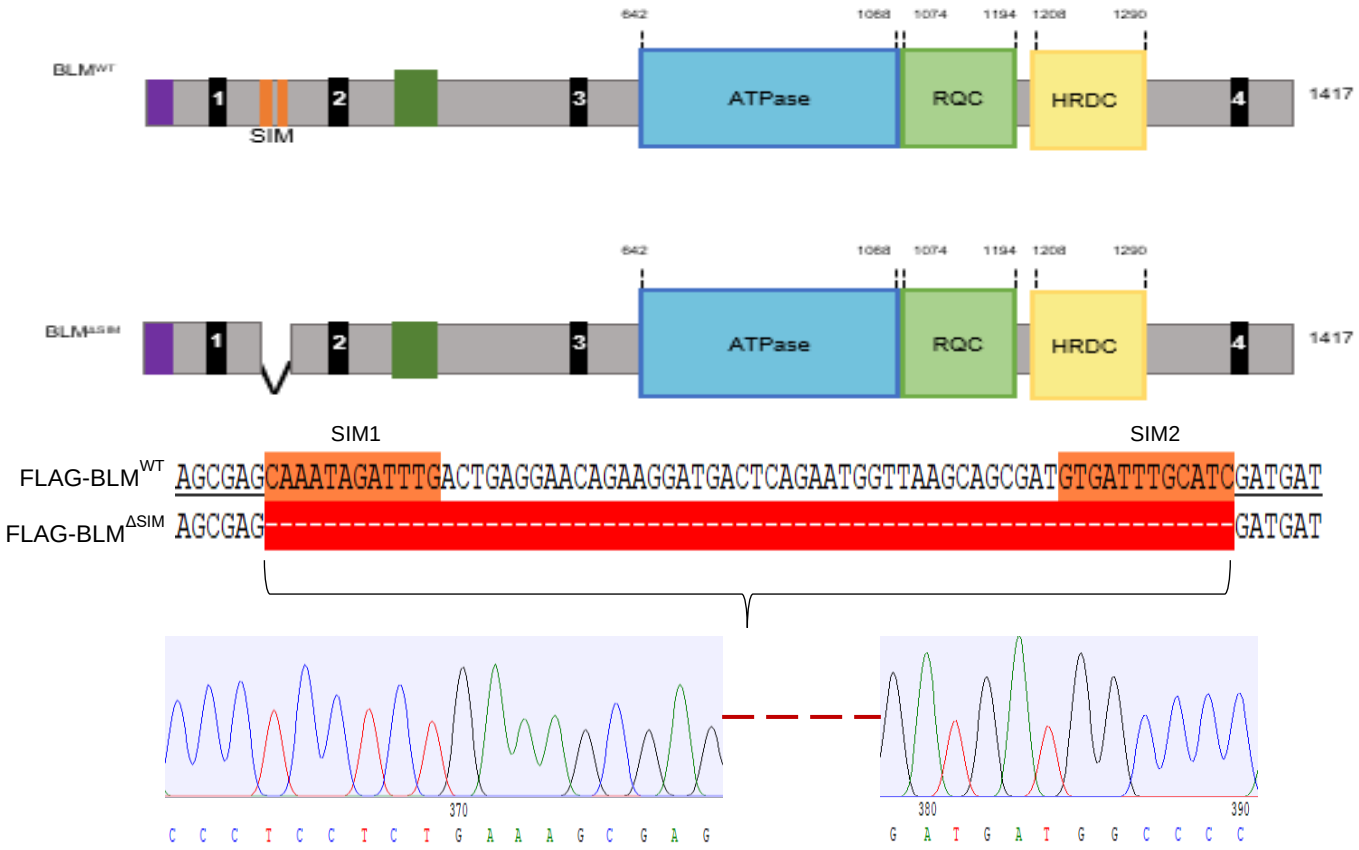


Figure 3.16: SDM to delete the SIMs. Sequence alignment of WT BLM and the SDM PCR product confirms deletion of SIM1 and SIM2. Alignments were carried out by ApE software. The unhighlighted sequence corresponds to the WT plasmid, with the SIMs indicated in yellow. The DNA sequence highlighted in blue corresponds to the SDM DNA product, the deleted SIMs/mismatches are shown in red in comparison to the WT plasmid.

I then extracted the plasmid and transfected three plates of 293FT cells with: FLAG-BLM^{WT} + +GFP-RPA1$^{\Delta BLM}$, FLAG-BLM^{WT}+ GFP-RPA1^{WT}, and GFP-RPA1^{WT}+FLAG$^{\Delta SIM}$. I then carried out a GFP IP to determine if deletion of both SIMs within BLM abrogates or maintains the interaction with RPA. My results in Figure 3.17 show both RPA1 and RPA2 retained interaction with FLAG-BLM$^{\Delta SIM}$ and there is no detectable reduction of this interaction compared to the interaction of FLAG-BLM^{WT} with RPA. This therefore indicates that neither of BLM' s SIMs are necessary for interactions with RPA.

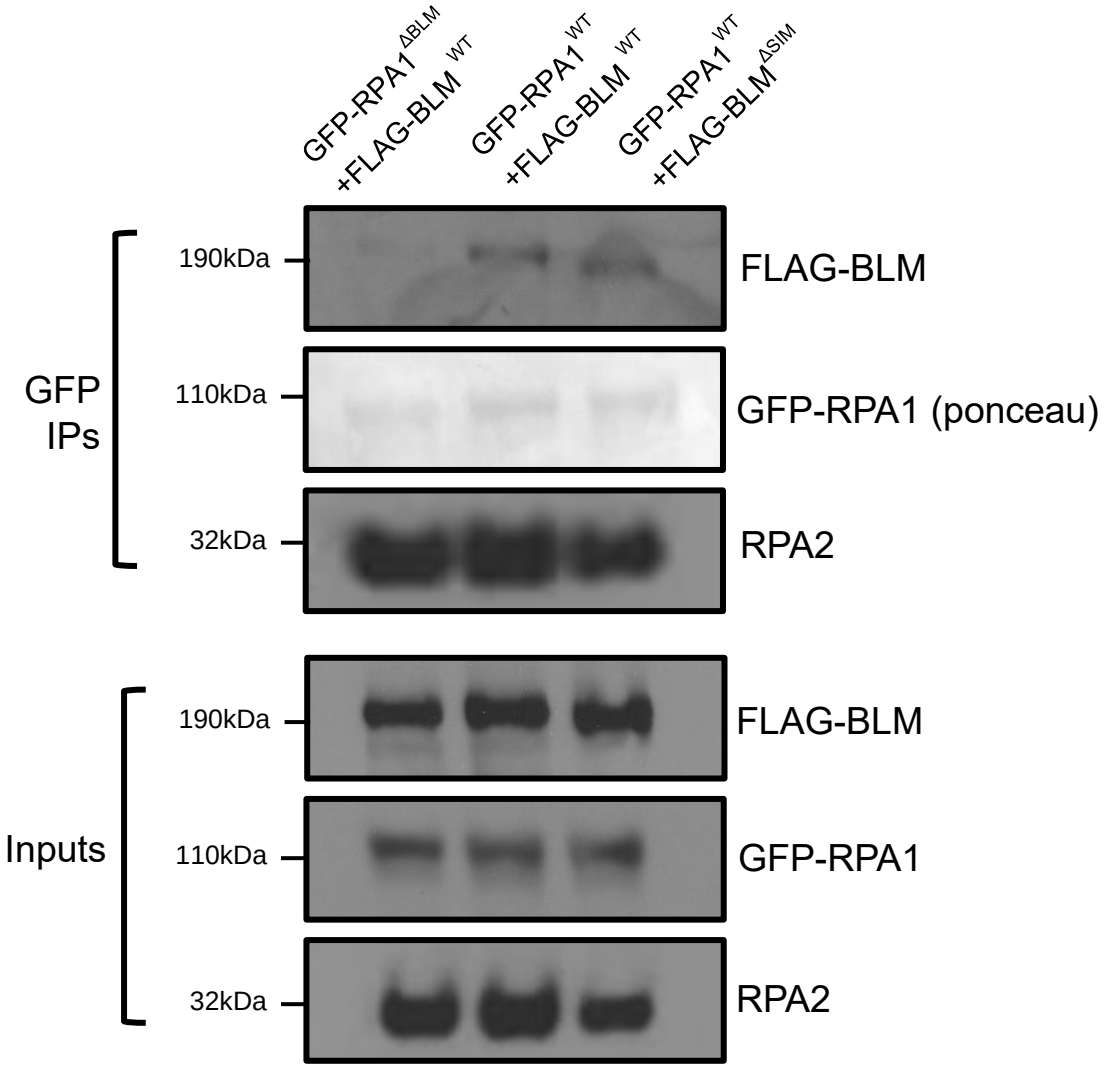


Figure 3.17: ΔSIM BLM interacts with RPA1 and RPA2. GFP-pulldowns from 293FT cells transfected with constructs expressing GFP tagged RPA1 and FLAG tagged BLM.

3.5 Functional significance of the highly conserved residues of the BLM N-terminus

3.5.1 Three highly conserved residues interact with the BTR complex for resistance to HU and suppression of SCEs

As mentioned previously in Figure 3.3, unpublished structural analysis of BLM from one of our collaborators (the group of Dr Andrew Deans) predict that within the highly conserved N-terminus, there are three highly conserved FTF residues that may be important for forming the interface for protein-protein interactions. As these residues are located within the first 133 amino acids of the N-terminus which is required for interactions with the BTR complex proteins, this led us to believe that these three residues may be important for interactions with RMI1, RMI2 and TOP3A, and that this interaction may be important for a specific function of the BTR complex (Hu et al., 2001).

To investigate this, I carried out site directed mutagenesis to mutate the highly conserved FTF residues, generating GFP-BLM$^{FTF\text{-}GGG}$ plasmids and transformed this into competent Top10 *E. coli* which were left to grow overnight in agar plates containing kanamycin. Colonies were then picked and sequenced, confirming that the plasmid of interest was being expressed within the *E. coli* (Figure 3.18). To investigate if the FTF resides are important for interactions with RMI1, TOP3A and RMI2, I carried out a GFP IP from 293FT cell lysates transfected with GFP only, GFP-BLM^{WT}, GFP-BLM$^{FTF\text{-}GGG}$ and GFP-BLM$^{N4\text{-}37}$. I found that loss of the conserved N-terminal residues 4-37 abrogated interactions with RMI1, RMI2 and TOP3A. Furthermore, mutating the FTF residues of the N-terminus abrogated interactions with RMI1, TOP3A and RMI2, similar to that when the N4-37 amino acids were deleted. This indicates that the highly conserved FTF residues within the N-terminus are important for interactions with the BTR complex proteins (Figure 3.19).

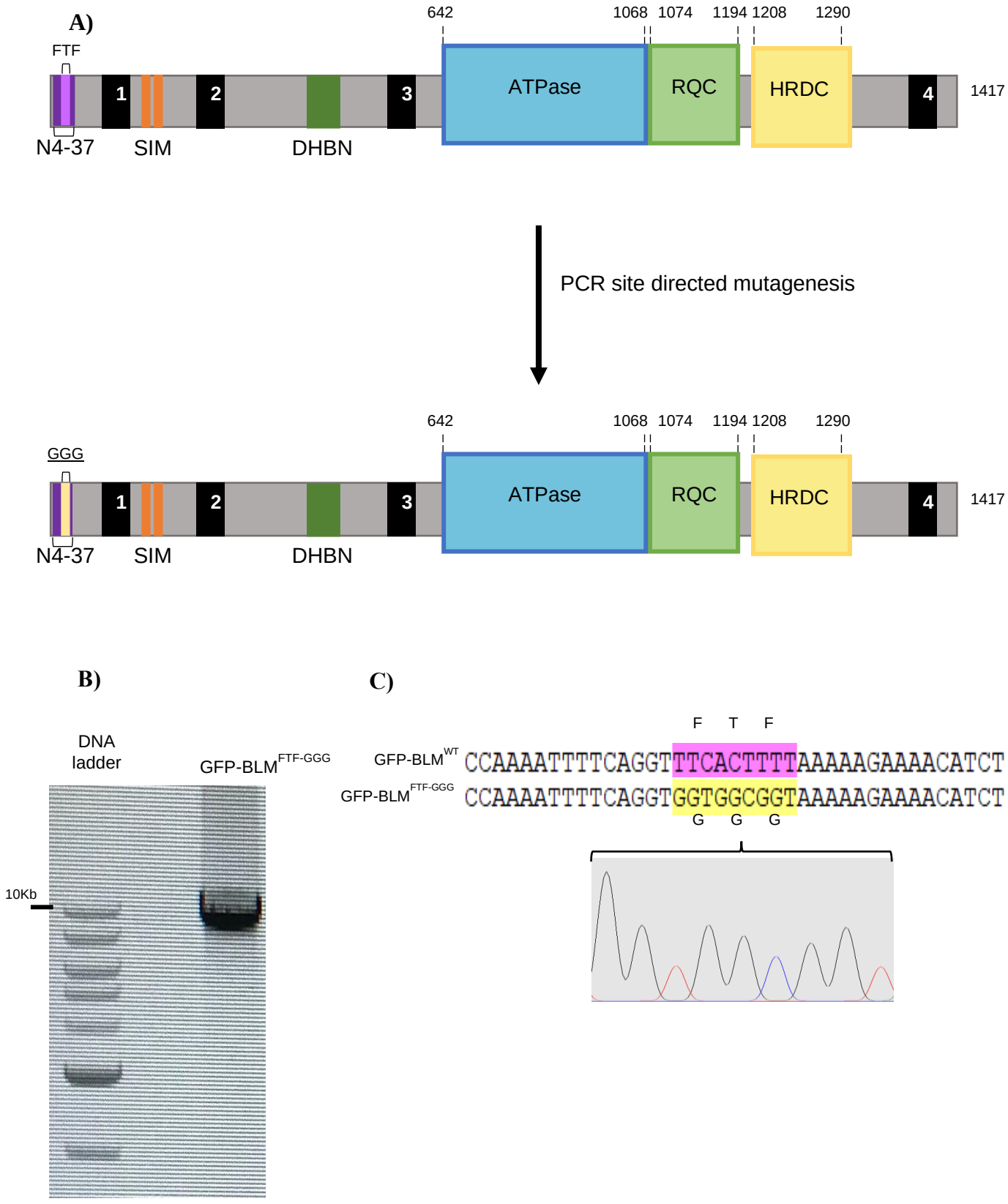


Figure 3.18 SDM to mutate the FTF residues. A) Schematic of SDM to mutate the FTF residues to three Glycine amino acids. B) PCR amplification of the GFP-BLMFTF mutant construct. C) Sequence data and alignments of the GFP BLM^{WT} and GFP-BLM$^{FTF-GGG}$ PCR products confirms mutation of the FTF residues to three Glycine amino acids. Alignments were carried out by ApE software. The highlighted purple sequence corresponds to the WT FTF residues within the WT plasmids. The DNA sequence highlighted in yellow corresponds to the SDM DNA product of the FTF residues to produce three glycine amino acids.

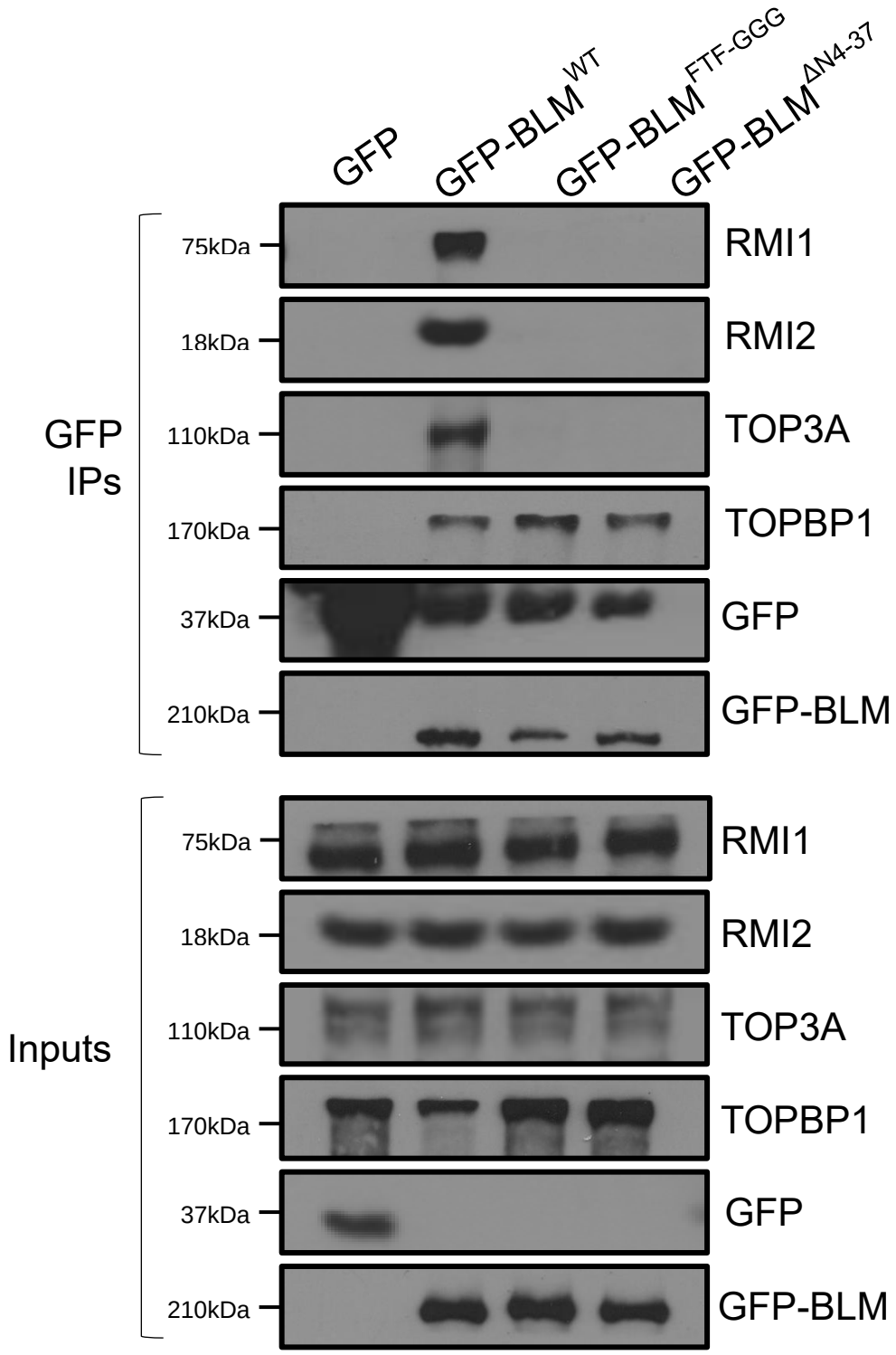


Figure 3.19: GFP-BLM$^{FTF-GGG}$ mutants do not interact with RMI1, RMI2 and TOP3A.

GFP-pulldowns from 293FT cells transfected with constructs expressing GFP tagged BLM showing the effects of BLM interactions with the BTR complex in the absence of the FTF residues.

To carry out cell-based assays to identify the functional importance of BLM protein-protein interactions with the BTR complex via the FTF residues, I generated stable cell lines of *BLM*$^{-/-}$ cells expressing GFP-BLM$^{FTF\text{-}GGG}$ by lentiviral infection methods, I then confirmed that these cell lines were expressing GFP-BLM$^{FTF\text{-}GGG}$ via fluorescent-activating cell sorting and western blotting as shown in Figure 3.20.

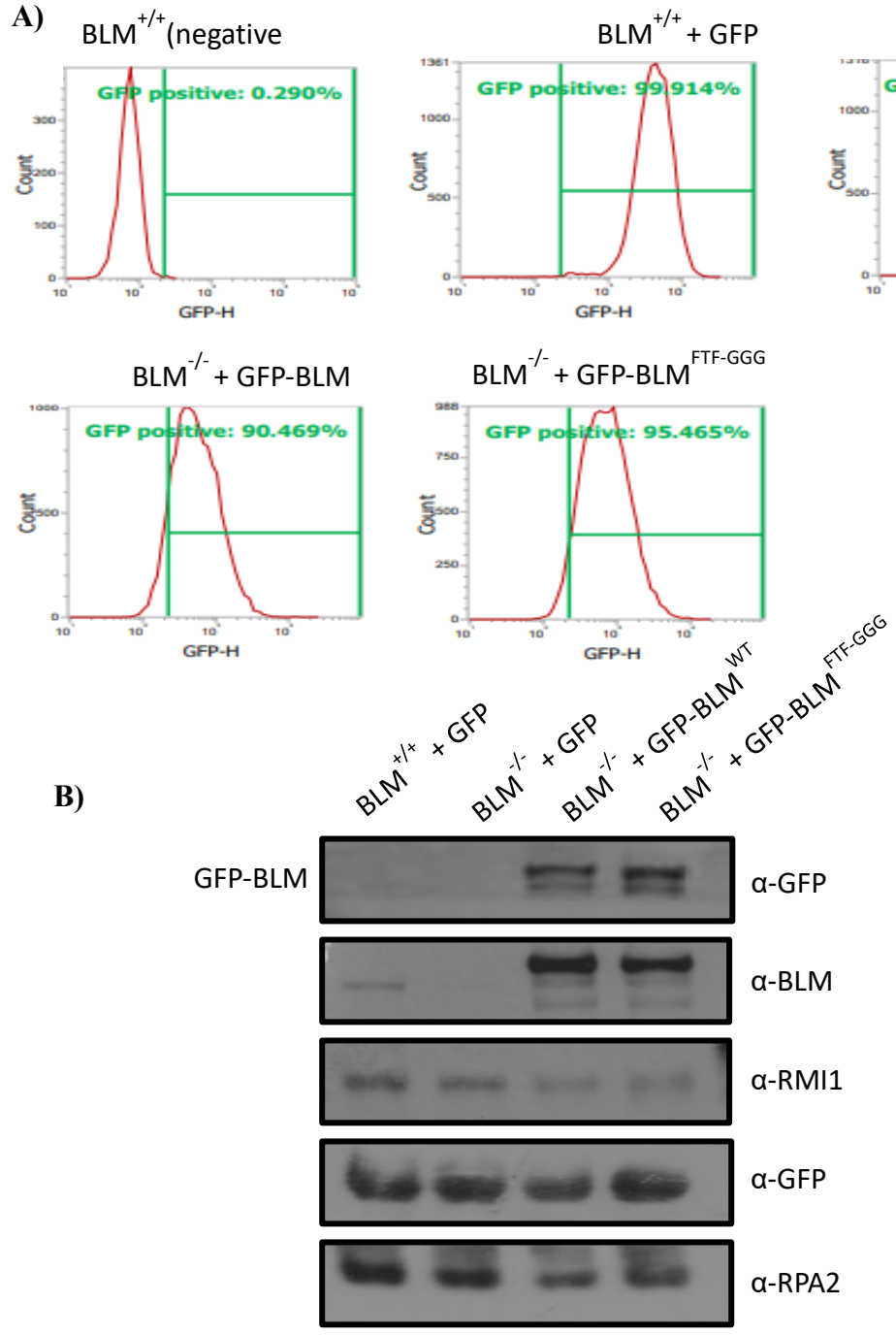


Figure 3.20: Generation and verification of stable cell lines expressing GFP-BLM$^{FTF\text{-}GGG}$

A) Confirmation of GFP-expressing cells *BLM*$^{+/+}$ and *BLM*$^{-/-}$ cells via FACs sort.
B) Western blot analysis confirming expression of GFP in our complemented cell lines.

I carried out a clonogenic survival assay and found that the cells expressing GFP-BLM$^{FTF-GGG}$ displayed hypersensitivity to HU but were not as sensitive in comparison to *BLM*$^{-/-}$ cells (Figure 3.21). Thus, indicating that the FTF residues have a role in cellular resistance to HU induced replication stress, and perhaps fork stalling. Furthermore, SCE assays carried out by my colleague in the lab (Dr Sam Jones) identified that cells expressing GFP-BLM$^{FTF-GGG}$ displayed a significant increase in the level of SCEs compared to that in BLM$^{+/+}$ cells, but lower compared to that observed in *BLM*$^{-/-}$ cells (Figure 3.22). This data suggests that the conserved FTF residues of the BLM N-terminus has a role in the dissolution of dHJs during the later stages of HR repair.

In line with my data and previous studies, I have mapped specific residues within the N-terminus of BLM that are required for protein-protein interactions with TOP3A, RMI1 and RMI2, and that this interaction has a role in cellular resistance to HU, and in the dissolution of double Holliday junctions.

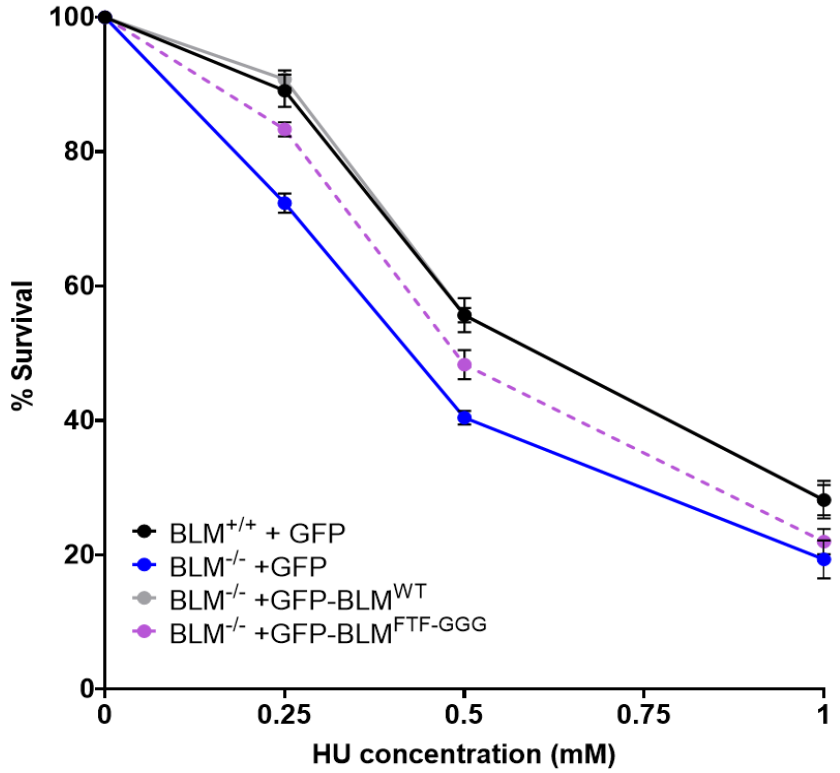


Figure 3.21: Cells expressing GFP-BLM$^{FTF-GGG}$ display sensitivity to HU

Clonogenic survival assay results were plotted as an XY graph. Statistical analysis was carried out via multiple t-tests. P-values comparing GFP-BLM^{FTF} with BLM$^{-/-}$ +GFP and GFP-BLM^{WT} is <0.05.

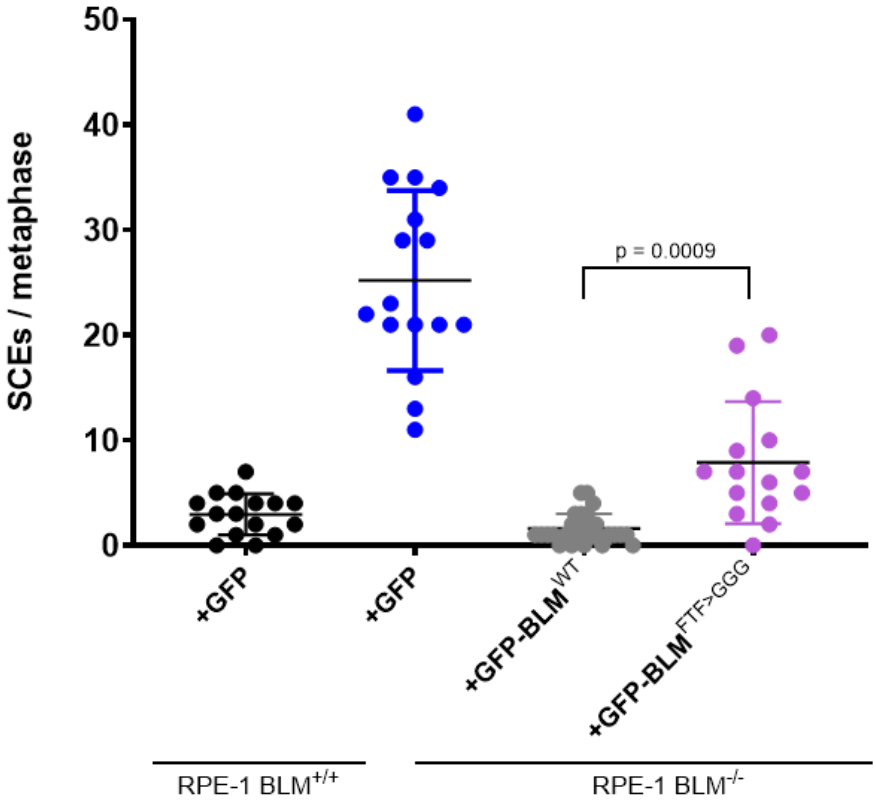


Figure 3.22: FTF residues have a role in supressing SCEs

SCE assay conducted by Dr Sam Jones. Statistical analysis and significance were carried out by a Mann-Whitney test.

3.5.2 Highly conserved residues of the BLM DHBN domain confer resistance to HU and suppression of SCEs

A recent study by Shi et al., has identified the DHBN within BLM which is required for its dimerization. My sequence alignments have identified a highly conserved region within the DHBN which contains hydrophobic residues (Figure 3.4). Furthermore, preliminary data from one of our collaborators (the group of Dr Andrew J Deans) have shown that loss of this conserved region within the DHBN, abrogates interactions with BLM. Therefore, I wanted to investigate the functional significance of these conserved residues within the DHBN.

To do so, I carried SDM to mutate 8 conserved residues into 8 alanine amino acids in GFP tagged BLM plasmids, generating a GFP-BLM^{PATCH8A} construct (Figure 3.23). I then generated stable *BLM*$^{-/-}$ cell lines expressing GFP-tagged BLM^{PATCH8A} via lentiviral infection methods, which were confirmed via fluorescent-activating cell sorting and western blotting (Figure 3.24).

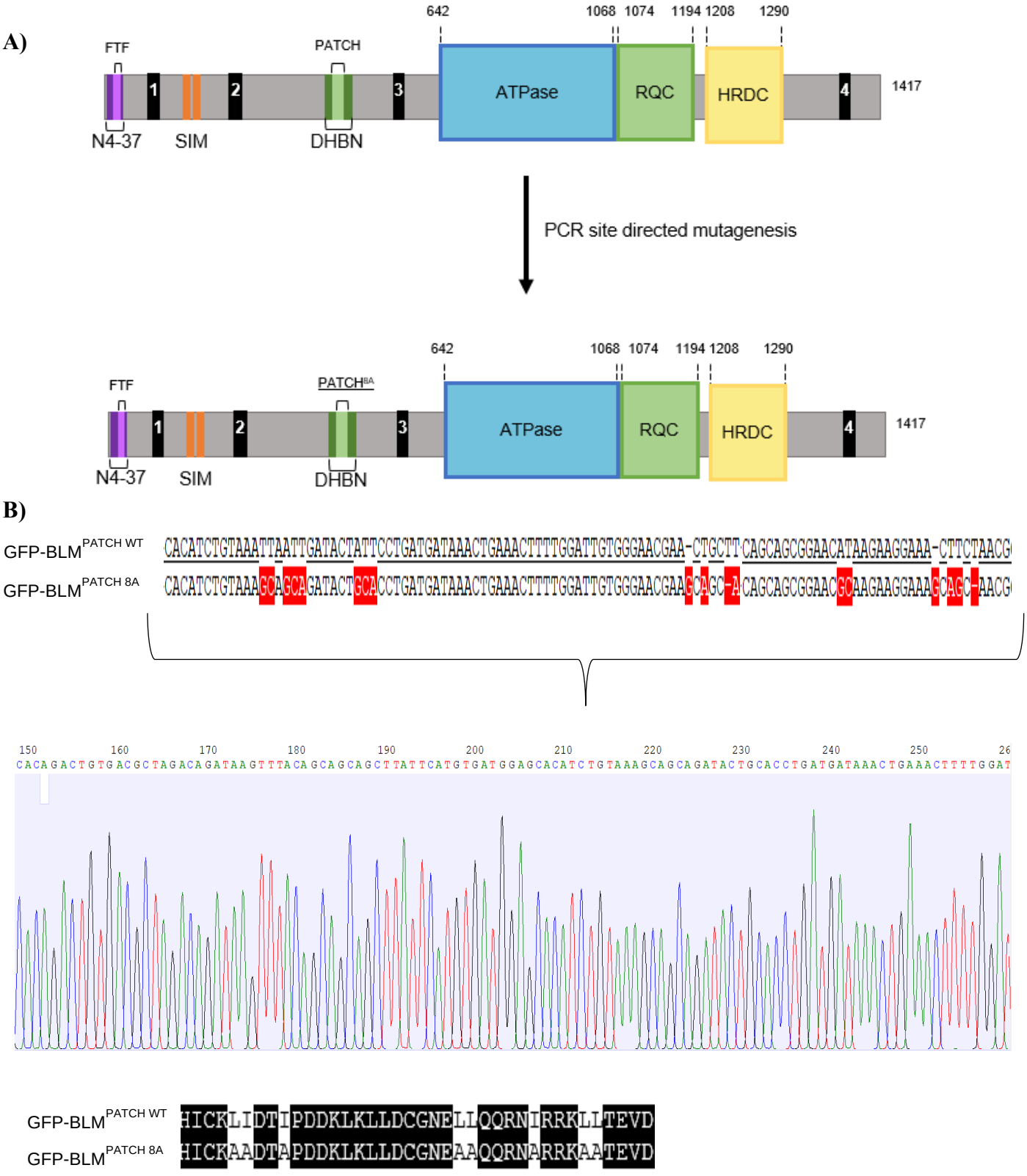


Figure 3.23: SDM to mutate the BLM DHBN residues. A) Schematic illustrating SDM of the BLM dimerization residues to Alanine residues. B)Sequence data and alignments confirms mutation of 8 of the DHBN hydrophobic residues to 8 alanine residues in GFP-BLM and FLAG-BLM plasmid PCR products. Alignments were carried out by ApE software.

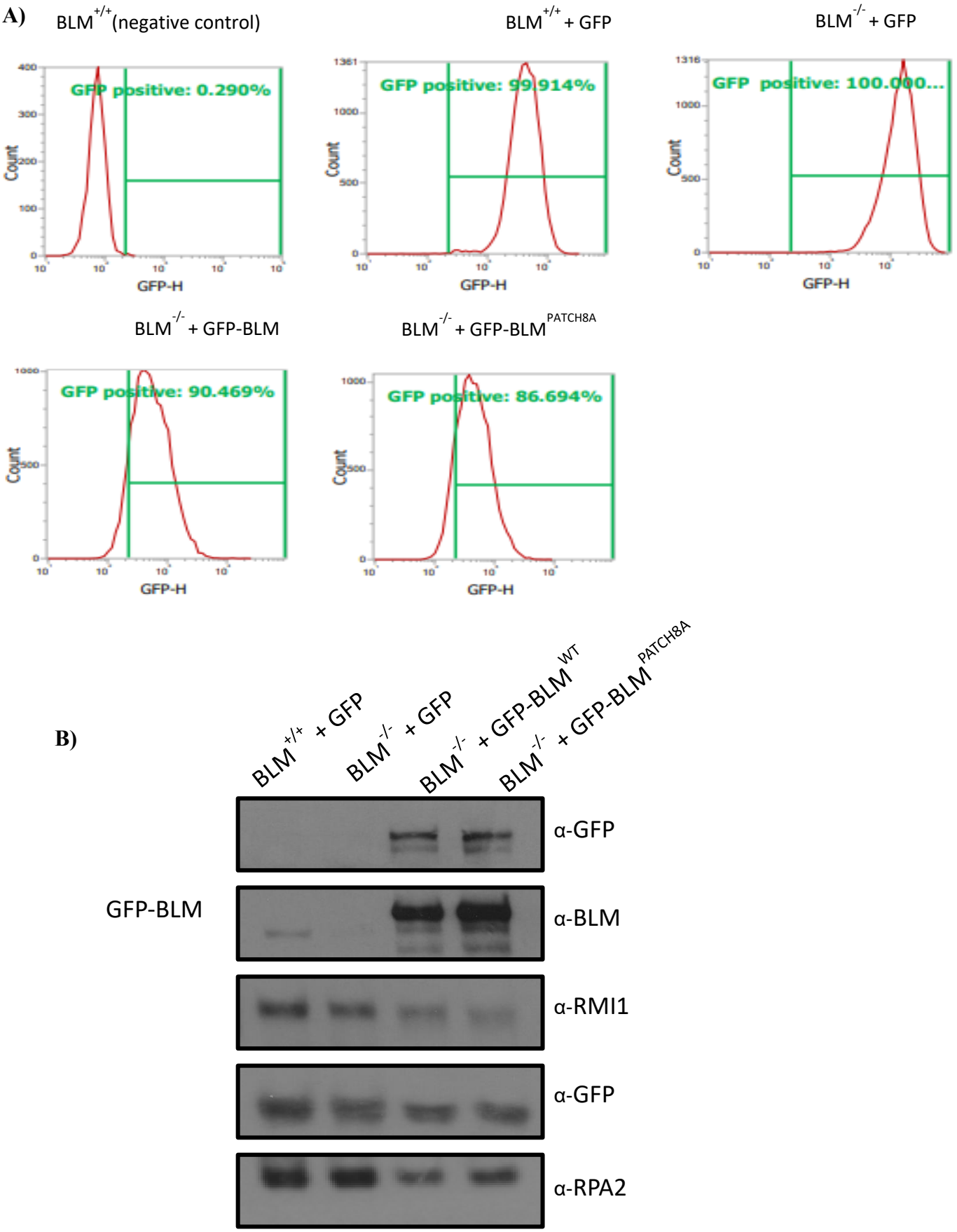


Figure 3.24: Generation and verification of stable cell lines expressing GFP-BLM$^{PATCH\ 8A}$

A) Confirmation of GFP-expressing cells *BLM*$^{+/+}$ and *BLM*$^{-/-}$ cells via FACs sort.
B) Western blot analysis confirming expression of GFP in our complemented cell lines.

Interestingly, my HU clonogenic survival assay shows that cells expressing GFP-BLM^{PATCH8A} are hypersensitive to increasing concentrations of HU compared to WT cells but are not as sensitive to HU compared to *BLM*$^{-/-}$ cells (Figure 3.25). Additionally, to investigate if the conserved residues in the DHBN are important for dHJ dissolution, SCE assays were carried out by my colleague in the lab (Dr Sam Jones). We found that cells expressing GFP-BLM^{PATCH8A} displayed elevated levels of SCEs compared to WT cells, but the levels of SCEs observed in the BLM^{PATCH8A} mutants were lower in comparison to *BLM*$^{-/-}$ cells (Figure 3.26).

This data therefore shows that conserved residues of the DHBN of BLM partially function in both preventing SCE formation and resistance to HU.

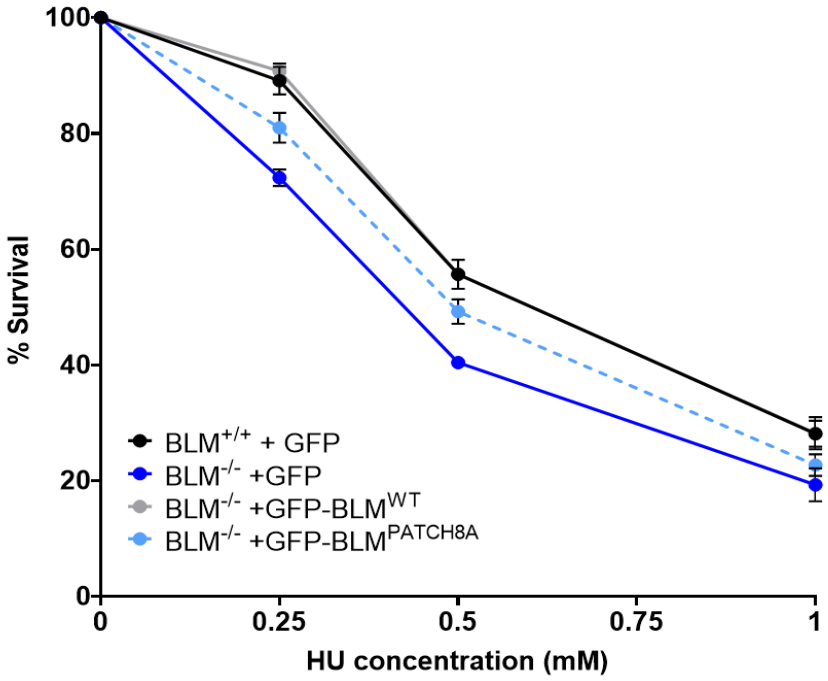


Figure 3.25: Cells expressing GFP-BLM$^{PATCH\ 8A}$ display sensitivity to HU

Clonogenic survival assay results were plotted as an XY graph. Statistical analysis was carried out via multiple t-tests. P-values comparing GFP-BLM^{PATCH8A} with BLM$^{-/-}$ +GFP and GFP-BLM^{WT} is <0.05.

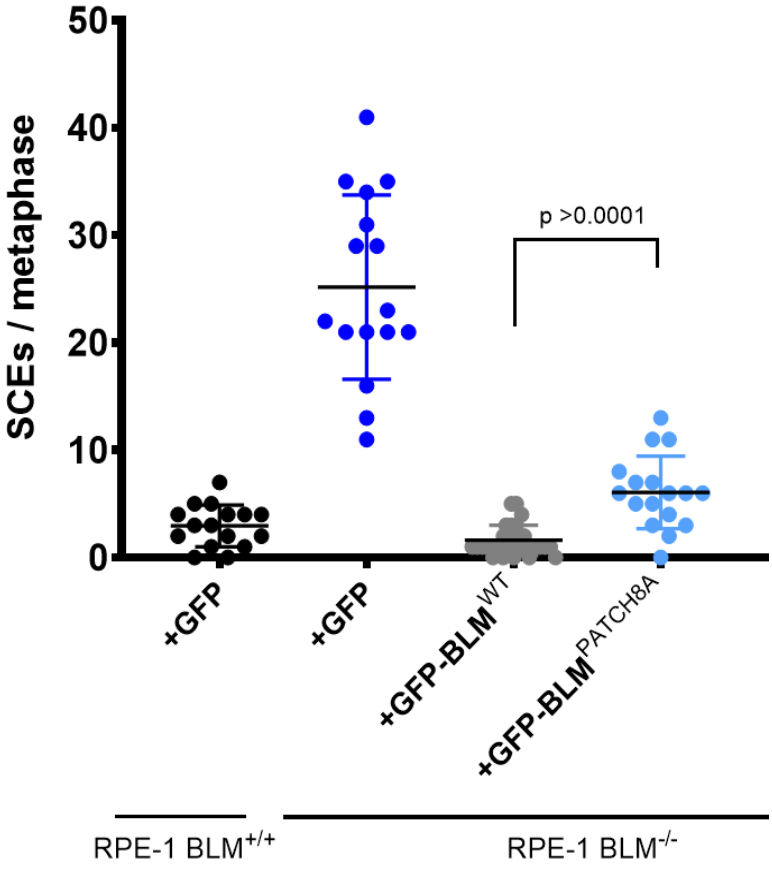


Figure 3.26: Conserved residues of the DHBN have a role in supressing SCEs

SCE assay conducted by Dr Sam Jones. Statistical analysis and significance were carried out by a Mann-Whitney test.

4. Discussion

The aim of my study was to identify separation-of-function mutations within the unstructured regions of BLM. Using sequence alignment analysis of BLM orthologs, I identified several regions within the BTR complex proteins, leading us to speculate that they may have functional importance. Firstly, I identified that there are several conserved motifs in BLM and RMI1 that mediate protein-protein interactions with RPA, and that these interactions are important for resistance to HU but not suppression of SCEs, hence constituting a novel separation-of-function mutation (Figure 4.1). Secondly, I demonstrated that BLM interactions with TOP3A, RMI1 and RMI2 are required for both resistance to HU and suppression of SCEs (Figure 4.1). Thirdly, I found that BLM dimerization is also important for both resistance to HU and suppression of SCEs (Figure 4.1).

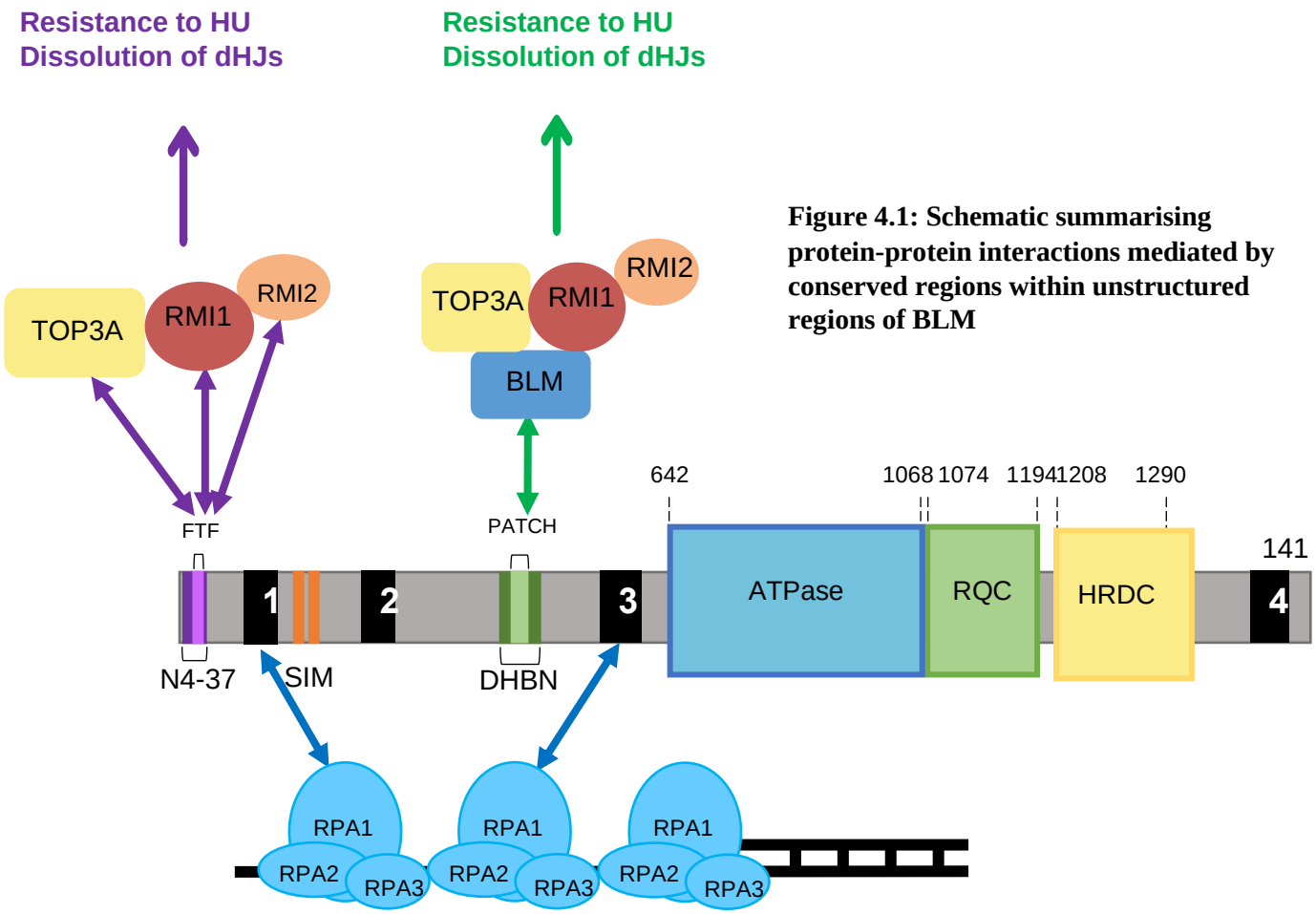


Figure 4.1: Schematic summarising protein-protein interactions mediated by conserved regions within unstructured regions of BLM

4.1 BTR interactions with RPA are required for resistance to replication stress.

Previous studies of the BLM helicase have highlighted that the first 447 amino acids of the BLM N-terminus are important for interactions with RPA (Doherty et al., 2005). Using sequence alignments, have pinpointed the exact regions within BLM and RMI1 which interact with RPA, hence mapping the specific residues within the N-terminus important for the BTR-RPA interaction.

Several previous studies have suggested that RPA is required to stimulate the BTR complex activity in dHJ dissolution. Studies by Xue et al., have demonstrated that RPA may act as a co-factor for BTR's role in dHJ dissolution via interactions with acidic residues 301-334 of RMI1 (Xue et al., 2013). Furthermore, studies by Plank et al., suggest that RPA interactions with BLM-TOP3A are required to stimulate BLM helicase activity at dHJs (Plank et al., 2006). In contrast studies by Cejka et al., have shown that RPA interactions with the BTR complex are not essential for dHJ dissolution (Cejka et al., 2010). Although my sequence alignment has exposed a highly conserved region in RMI1 spanning residues 307-324 we found that these residues are not required for interactions with RPA and instead, RPA interacts with RMI1 specifically at conserved residues 241-254. In addition to this, we found that RPA interacts with BLM via 2 conserved motifs. Interestingly, we found that cells lacking the RPA binding motifs in BLM and RMI1 displayed low levels of SCEs similar to that seen in $BLM^{+/+}$ cells, suggesting that BTR-RPA interactions via these motifs are not required for dHJ dissolution as suggested by previous studies (Xue et al., 2013, Plank et al., 2006), thus agreeing with studies by Cejka et al,. (Cejka et al., 2010). Our proposed explanation for this may be that since dHJ structures do not have regions of ssDNA, RPA is unlikely to be present at these structures during HR and that recruitment of the BTR complex to dHJ structures occurs independently of RPA (Figure 4.3). This led us to believe that RPA

interactions with BTR may be important for a different function in maintaining genome stability.

Several studies have shown that BLM is required for resistance to agents that induce fork stalling such as HU, while others have indicated that BLM is not required for resistance to HU (Yamagata et al., 1998, Franchitto and Pichierri, 2002, Ababou et al., 2002, Imamura et al., 2001). We generated *BLM*$^{-/-}$ cells via CRISPR-Cas9 gene editing that display hypersensitivity to HU. Furthermore, complementation of our *BLM*$^{-/-}$ cells with exogenous BLM/RMI1 that lack the RPA binding motifs were still hypersensitive to HU. My data therefore suggests that the BTR-RPA interactions via these motifs are specifically important for HU resistance, a potential indicator of tolerance to stalled replication forks. This was interesting as stalled forks can have long regions of ssDNA where RPA is recruited to stabilise and prevent degradation and the formation of secondary DNA structures at stalled forks (Zou et al., 2006). We propose that these results may reflect a mechanism by which BTR recruitment to stalled forks is regulated by the availability of RPA coated ssDNA. We suggest that each RPA binding motif in BLM and RMI1 interacts with an individual RPA, hence only one BTR complex is recruited to ssDNA for every three RPA heterotrimers present. This would therefore limit BTR recruitment only to DNA at sites of extensive ssDNA such as at stalled forks and double strand breaks following DNA damage; and prevent recruitment and inappropriate activity of the BTR complex at normal DNA structures with small regions of ssDNA during normal cellular process such as replication (Figure 4.2). Brosh et al., have demonstrated previously that in the absence of RPA, BLM activity in unwinding DNA is reduced (Brosh et al., 2000). Furthermore, Qin et al., have shown that RPA activates BLM for bidirectional unwinding of dsDNA (Qin et al., 2020). Our proposed model that BLM activity is regulated at stalled forks by interactions with RPA may provide a mechanistic explanation as to how BLM's activity in the bidirectional unwinding of DNA is

activated. Future micro irradiation recruitment experiments of BLM/RMI1$^{\Delta RPA}$ mutants to sites of DNA lesions may indicate whether RPA binding is required for recruitment of the BTR complex to stalled forks and DNA lesions (Gaudreau-Lapierre et al., 2018).

This study has identified RPA binding motifs within BLM and RMI1 that constitute a novel separation-of-function mutation which are specifically important for cellular resistance to HU, and perhaps replication fork stalling. Future studies using DNA fibre assays and resection assays may further explain the subsequent role of the BTR-RPA complex at stalled forks in regard to replication fork restart and resection of ssDNA for HR.

4.2 BLM interactions with TOP3A-RMI1-RMI2 are required for resistance to replication stress and dHJ dissolution.

The N-terminus of BLM is important for interactions with the BTR complex proteins TOP3A and RMI1 (Gangloff et al., 1994, Wu et al., 2000, Hu et al., 2001). Moreover, this study has mapped three highly conserved FTF residues that are required for this interaction. This is in line with our collaborator's work (group of Dr Andrew J Deans) who predict that these residues form an interface for protein-protein interactions.

Several studies have presented that BTR recruitment to nuclear foci are dependent on interactions with RMI1, and that this is essential for normal replication fork progression and recovery from replication fork stress (Yang et al., 2012, Yin et al., 2005). I found that mutating the N-terminus FTF residues disrupts interactions of BLM with TOP3A-RMI1-RMI2. Interestingly, although hypersensitivity to HU was observed, this was lower in comparison to the hypersensitivity of the *BLM*$^{-/-}$ cells. We found this surprising since studies by Yang et al., have shown that RMI1 is essential for stalled fork progression (Yang et al., 2012). One possible explanation may be that BLM may still be recruited to stalled forks via interactions with RPA. However, as loss of the FTF residue abrogates interactions with

TOP3A-RMI1-RMI2, this may limit the efficiency of BLM's activity at stalled forks, and that TOP3A, RMI1 and RMI2 act as accessory factors for BLM's activity at stalled forks (Figure 4.2).

There is a multitude of evidence to support that BLM interactions with TOP3A-RMI1-RMI2 are essential for dHJ dissolution. TOP3A-RMI1 stimulates BLM activity to unwind and bring the two HJs in close proximity, forming a hemicatenane structure (Raynard et al., 2006). TOP3A decatenase and strand passage activity is then stimulated by RMI1 and BLM to cleave the hemicatenane structure, which then passes and religates the nicked strand to product non-crossover products (Bussen et al., 2007, Yang et al., 2010). Furthermore, RMI1 is required to stabilise the BTR complex at dHJs (Raynard et al., 2006, Wu et al., 2006).

Interestingly, I found that loss of the FTF residues resulted in only a partial reduction in the formation of SCEs. Since BLM functions with TOP3A and RMI1 to dissolve dHJs, we found this result to be surprising as we would have expected that loss of BLM interactions with TOP3A and RMI1 would have resulted in increased levels of SCEs.

My results show that perhaps BLM is a major BTR complex protein required for resistance to HU and in dHJ dissolution, and that TOP3A-RMI1-RMI2 may enhance BLM's activity at stalled forks and dHJs. However alternatively, in the absence of BLM interactions with RMI1, TOP3A and RMI2, the BTR complex proteins may still be recruited to these DNA structures by another mechanism independently of BLM. Studies by Deans et al., have revealed that FANCM, which is implicated in the genome instability disorder Fanconi Anaemia (FA), recruits the BTR complex at stalled replication forks via direct interactions with RMI1 and RMI2 (Deans and West, 2009). Therefore, in the absence of the FTF residues, it may be possible that FANCM is recruiting RMI1, RMI2 and TOP3A to stalled forks,

however the efficacy of the full BTR complex may be reduced due to loss of direct TOP3A-RMI1-RMI2 interactions with BLM.

FANCM also has functions in supressing SCEs as loss of FANCM results in elevated SCEs as is observed in FA patients (Stoepker et al., 2015). Furthermore, it has been shown that RMI1-RMI2 interactions with FANCM function in supressing SCEs (Hoadley et al., 2012). Therefore, it is plausible that in the absence of interactions with BLM, TOP3A-RMI1-RMI2 may be interacting with FANCM and supressing SCEs via FANCM-RMI activity. It may even be possible that FANCM could be recruiting TOP3A-RMI1-RMI2 to dHJs where BLM is already present to enhance BTR activity at dHJs.

Epistatic experiments by Rosado et al., have highlighted that BLM knockout cells expressing helicase-dead FANCM displayed increased levels of SCEs (Rosado et al., 2009). These experiments have demonstrated that FANCM and BLM cooperate together in preventing SCE formation, indicating that these proteins may function simultaneously in the dHJ dissolution pathway of HR.

I have shown that BLM interactions with TOP3A-RMI1-RMI2 via the FTF residues have a role in resistance to HU, and potentially fork restart. Future DNA fibre experiments may highlight the importance of this interaction in replication fork restart, and DNA resection assays may highlight roles during the early resection stages of HR.

4.3 BLM dimerization mediates resistance to replication stress and dHJ dissolution.

Studies by Shi et al., have shown that BLM contains a DHBN domain, however the function of this region of BLM is unclear (Shi et al., 2017). My sequence alignment shows a highly conserved region within the N-terminus of BLM located at the DHBN region. Unpublished data from Deans et al., have illustrated that loss of this region abrogates BLM dimerization.

Furthermore, I have shown that the conserved residues of the DHBN have a partial role in HU resistance. We suggest that since the DHBN is required for interactions with BLM, that perhaps BLM activity is enhanced by dimerization. Additionally, dimerization may be a mechanism by which multiple BTR complexes are recruited to sites of stalled replication forks to enhance BLM's unwinding activity (Figure 4.2). This supports work by Shi et al., which proposes that BLM dimerization is stabilised by hydrophobic interactions and dimerization enhances BLM helicase activity (Shi et al., 2017). Furthermore, it may be possible that six RPA proteins at ssDNA is required for the recruitment of two BTR complexes for efficient tolerance to HU. Future micro irradiation recruitment experiments of $BLM^{PATCH8A}$ mutants to sites of DNA lesions may give a greater insight to how the conserved residues of the DHBN are implicated in the recruitment of BLM to stalled forks and DNA lesions (Gaudreau-Lapierre et al., 2018).

Interestingly, my results point out that the conserved residues of the DHBN have a partial function in the prevention of SCEs. We propose that the DHBN of the BTR complex is required for the recruitment of two BTR complexes at these dHJ structures to enhance dHJ dissolution, but the presence of one BTR complex is not sufficient to fully dissolve dHJ, but rather this results in reduced availability of BLM for efficient dHJ dissolution.

BLM activity at dHJs is dependent on its interactions with TOP3A, RMI1 and RMI2 (Raynard et al., 2006, Bussen et al., 2007, Wu et al., 2006, Yang et al., 2010). Since we observed an increase in SCEs in cells expressing $BLM^{PATCH8A}$ compared to WT cells, but not as high compared to $BLM^{-/-}$ cells, this indicates there must be some presence of TOP3A-RMI1-RMI2 with BLM at dHJs. This may suggest a mechanism of how BLM dimerization may regulate BTR complex activity at dHJ structures. It is possible that two BLM monomers bind to separate junctions of the dHJ intermediate which then begin to unwind the two junctions forming a hemicatenane, thus also bringing the two BLM monomers in close

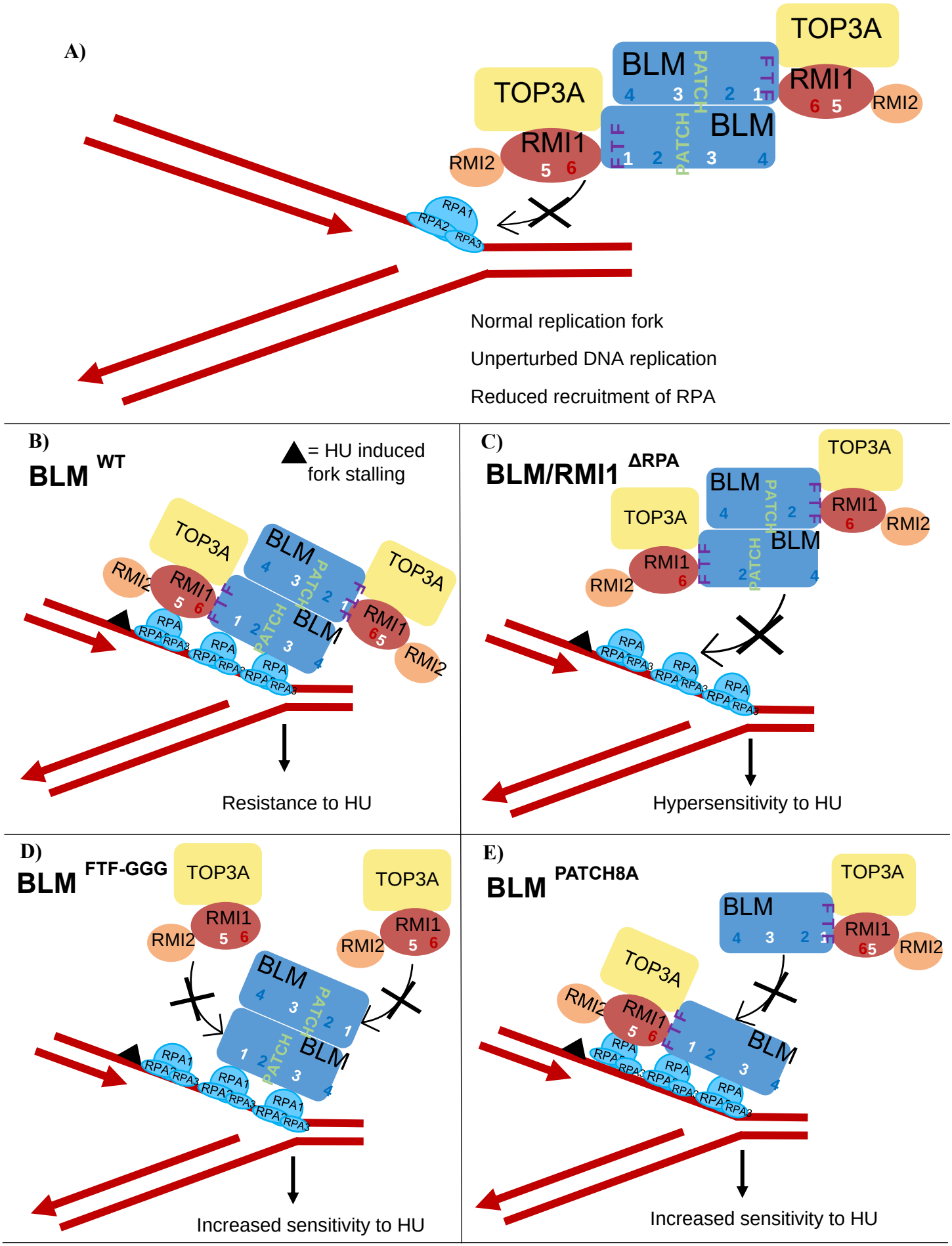
proximity. The two BLM monomers may then act as an interface for interactions with one TOP3A-RMI1-RMI2 complex, via the FTF residues, required to cleave the hemicatenane structure to produce non-crossover products. Therefore, loss of dimerization may limit recruitment of TOP3A-RMI1-RMI2 to dHJs, reducing the efficacy of dHJ dissolution (Figure 4.2).

Figure 4.2: Schematic summarising the functions of conserved unstructured regions of BLM in HU induced fork stalling

- **A)** Recruiment of BLM via the BTR complex to the replication fork during normal DNA replication is limited due to lack of RPA available at normal replication forks.
- **B)** The BTR complex is recruited to stalled replication forks via BLM-RPA interactions to resume DNA replication.
- **C)** Loss of the RPA binding motifs in BLM prevents recruitment of the BTR complex to stalled forks due to inability to interact with RPA.
- **D)** Loss of the FTF residues in BLM limits interactions of BLM with RMI1, RMI2 and TOP3A at stalled forks, hence limiting BTR complex activity at stalled forks.
- **E)** Disruption of the DHBN domain limits BTR complex dimerization at stalled forks, hence limiting BTR complex activity.

Figure 4.3: Schematic summarising the functions of conserved unstructured regions of BLM in dHJ dissolution

- A) Recruitment of BLM via the BTR complex to dHJs, promoting the dissolution pathway of HR repair.
- B) Loss of the RPA binding motifs in BLM does not abrogate recruitment of the BTR complex to dHJs due to absence of RPA at these structures.
- C) Loss of the FTF residues in BLM limits interactions of BLM with RMI1, RMI2 and TOP3A at dHJs, hence limiting dissolution activity of BTR complex.
- D) Disruption of the DHBN domain limits BTR complex dimerization at dHJs, hence limiting dissolution activity of BTR complex.


A)
TOP3A
BLM
PATCH
FTF
RMI1
RMI2
RPA1
RPA2
RPA3
Normal replication fork
Unperturbed DNA replication
Reduced recruitment of RPA
B)
BLM WT
= HU induced fork stalling
Resistance to HU
C)
BLM/RMI1 ΔRPA
Hypersensitivity to HU
D)
BLM FTF-GGG
Increased sensitivity to HU
E)
BLM PATCH8A
Increased sensitivity to HU

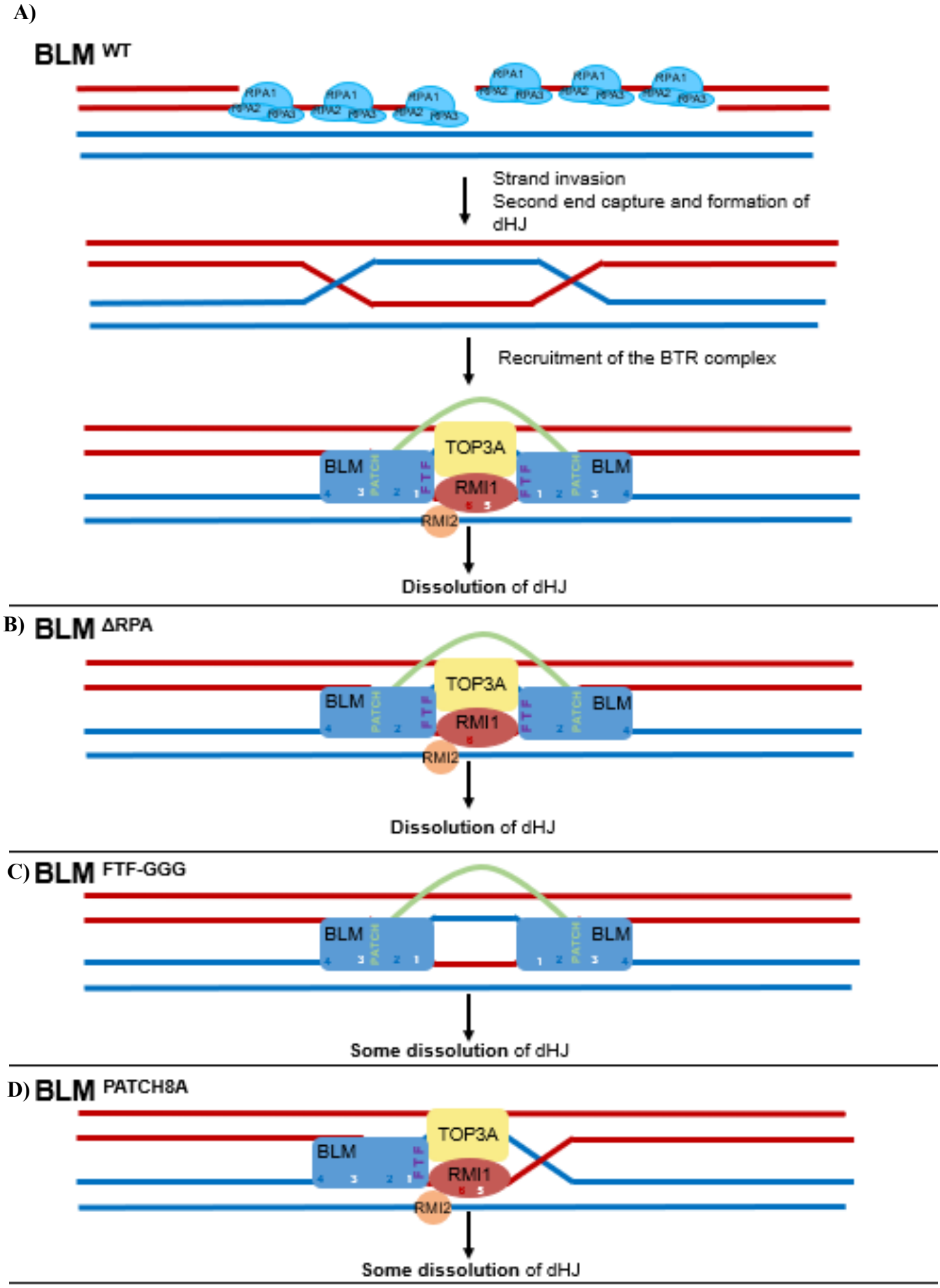


Figure 4.3: Schematic summarising the functions of conserved unstructured regions of BLM in dHJ dissolution

4.4 Clinical relevance and future studies

BLM is a general tumour suppressor and patients with BS have an increased predisposition to a broad range of cancers. BLM has a variety of functions in cells, however the roles that are required for tumour suppression are unclear. In this study, I have extrapolated the specific functions of several conserved regions of BLM. I have found that the RPA binding motifs are important for resistance to HU. I also found that three N-terminal FTF residues are important for interactions with TOP3A-RMI1 and RMI2, whilst conserved residues of the DHBN are important for dimerization. We found that both these regions have a role in cellular resistance to HU and suppression of SCEs. Future studies in animal models which express BLM/RMI1$^{\Delta RPA}$, BLM$^{FTF\text{-}GGG}$ and BLM^{PATCH8A} mutants may determine if these regions are important for BLM's function as a tumour suppressor by observing if these animals develop early onset tumours in comparison to WT animal models.

Previous studies have confirmed that BLM is important for the restart of stalled replication forks, but the mechanism by how this occurs is unclear (Davies et al., 2007, Yang et al., 2012). Since all of the motifs investigated have a function in the cellular resistance to HU, it would be interesting to explore if the RPA binding motifs in BLM/RMI1 are important in stalled fork restart. To study this, we could carry out DNA fibre analysis, an experiment that allows the visualisation of several parameters of DNA replication, including fork restart. This can be achieved by incubating cells with a labelled thymidine analogue (e.g., CldU) which becomes incorporated into synthesising DNA. Cells can then be treated with HU to induce fork stalling, followed by incubation with another labelled thymidine analogue (e.g., IdU). Quantification of the length of CldU/IdU tracts would then determine if replication fork restart has occurred or not (Quinet et al., 2017).

Furthermore, BLM is also important for DNA resection during the early stages of HR. Future cell-based resection assays would determine if these conserved regions of BLM are important for resection (Forment et al., 2012) .

Loss of the FTF residues in BLM abrogates interactions with TOP3A-RMI1-RMI2 and these residues have a partial role in HU resistance and SCE suppression. A proposed reason for these phenotypes is that TOP3A-RMI1-RMI2 may still be recruited to abnormal DNA structures via FANCM. To investigate this, future studies where the MM2 domain of FANCM is mutated, hence abrogating interactions with TOP3A-RMI1-RMI2 would determine if FANCM and BLM are epistatic at stalled forks and dHJ intermediates, and whether these two proteins are dependent or independent of each other in these pathways (Deans and West, 2009, Hoadley et al., 2012).

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