

Targeting DNA Repair Mechanisms in MYC-Driven Cancer

by

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Dedication

To my loving and supportive parents, Tom and Lynn Nelson

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List of Abbreviations

4-OHT	4-hydroxytamoxifen
AID	Activation induced cytidine deaminase
Alt-NHEJ	Alternative non-homologous end joining
APH	Aphidicolin
ATR	Ataxia telangiectasia and Rad3-related
ATM	Ataxia telangiectasia mutated
ATLD	Ataxia telangiectasia-like disorder
ATRIP	ATR-interacting protein
BCR	B cell receptor
B-ALL	B-cell acute lymphoblastic leukemia
BER	Base excision repair
BLM	Bloom helicase
BM	Bone marrow
BFB	Breakage-fusion-bridge
BRCA1	Breast cancer 1
BrdU	bromodeoxyuridine
CHK1	Checkpoint kinase 1
CML	Chronic myeloid leukemia

CSR	Class-switch recombination
C-NHEJ	Classical non-homologous end joining
CtIP	CtBP-interacting protein
CDK	Cyclin-dependent kinase
DNA	Deoxyribonucleic acid
DR-GFP	Direct repeat GFP
DDR	DNA damage response
LIG3	DNA Ligase 3
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	Double-strand break
dsDNA	Double-stranded DNA
ER	Estrogen receptor
EXO1	Exonuclease 1
FA	Fanconi anemia
FANCD2	Fanconi anemia complementation group D2
HR	Homologous recombination
HU	hydroxyurea
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IRES	Internal ribosome entry site
IR	Ionizing radiation
MRE11	Meiotic recombination 11

MMEJ	Microhomology-mediated end joining
MEF	Mouse embryonic fibroblast
MRN	MRE11/RAD50/NBS1
NBS	Nijmegen breakage syndrome
NBS1	Nijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
53BP1	p53-binding protein
PARP	Poly (ADP-ribose) polymerase
PI	Propidium iodide
RAD51	Rad51 recombinase
RS-SCID	Radiosensitive severe combined immunodeficiency
ROS	Reactive oxygen species
RAG1	Recombination activating gene 1
RAG2	Recombination activating gene 2
RSS	Recombination signal sequence
12RSS	Recombination signal sequence, 12 nucleotide spacer
23RSS	Recombination signal sequence, 23 nucleotide spacer
RPA	Replication protein A
RPA	Replication protein A
ssDNA	Single-stranded DNA
TCR	T cell receptor
TOPBP1	topoisomerase-binding protein 1

XRCC1	X-ray repair cross-complementing protein 1
XRCC4	X-ray repair cross-complementing protein 4
XLFB	X-ray repair cross-complementing protein 4-like factor

Abstract

Genetic instability is a hallmark of cancer and contributes to tumorigenesis, since it can lead to acquisition of the remaining cancer hallmarks. Consistent with this, DNA damage is detected at early stages of tumor development, specifically in some premalignant human tumors. The source of this DNA damage is, in part, due to oncogene-induced replication stress. Replication stress is defined as slowing of DNA synthesis or stalling of the replication fork, and can lead to accumulation of genomic damage if not resolved or repaired efficiently. Oncogene activation has been identified as one source of replication stress due to the influence these proteins have on the cell cycle. For example overexpression of *C-MYC*, one of the most highly amplified oncogenes in human cancer, leads to deregulated cyclin dependent kinase (CDK) activity, a shortened G1, and premature entry into S-phase of the cell cycle. These effects can lead to replication stress, and it has previously been shown that the DNA damage response (DDR) is activated upon *C-MYC* overexpression. With the growing knowledge of the cellular consequences of oncogene overexpression, there is great interest in identifying the proteins required for repair of oncogene-induced DNA damage. The Mre11/Rad50/Nbs1 (MRN) DNA repair complex has roles in all known DNA DSB repair pathways. Previous studies have shown MRN plays important roles in resolving stalled replication forks, and promoting fork

restart. This thesis is focused on investigating the role of the MRE11 DNA nuclease in tumorigenesis and specifically in cells that overexpress the MYC oncogene.

The studies in my thesis utilize a mouse model of spontaneous lymphoma associated *IgH:Mye* translocations leading to C-MYC or N-MYC overexpression. These mice harbor gene targeted mutations in *Artemis*, the V(D)J recombination DNA nuclease, and *p53*, and have a strong predisposition to early onset pro-B lymphoma. Interestingly, we found B cell specific deletion of MRE11 or inactivation of MRE11 nuclease activity completely suppressed pro-B lymphomagenesis. Although MRE11 has been implicated in the DSB repair pathway proposed to generate some translocations, we provide evidence that shows MRE11 is not required for the generation of specific *IgH:Mye* translocations. Based on these data, we hypothesize that MRE11 is required during repair of oncogene-induced DNA damage and inactivation of MRE11 leads to catastrophic genomic instability and cell death.

To address these questions, the impact of pharmacologic inhibition of MRE11 nuclease activities in cells that overexpress C-MYC was examined. I observed MRE11 exonuclease activity, and not endonuclease activity, is critical for survival in these cells. I demonstrate that pharmacological inhibition of MRE11 exonuclease activity results in increased DNA damage, decreased cellular survival, and increased apoptosis specifically in cells that overexpress C-MYC. The findings presented in my thesis provide promising mechanistic preclinical

evidence in support of inhibiting MRE11 exonuclease activity to therapeutically target MYC-driven and replication stress-associated cancers.

Chapter I

Introduction

Summary

Cancer is a disease driven by genetic mutation. Recurrent chromosomal translocations are one type of genomic alteration that is observed in many hematological malignancies and some solid tumors. Frequently, lymphoid malignancies develop from and are characterized by recurrent translocations involving genetic regions on two separate chromosomes: the immunoglobulin heavy chain (IgH) locus and an oncogene such as *C-MYC*, resulting in oncogene dysregulation. The DNA double strand breaks (DSBs) generated within the IgH locus that participate in chromosomal translocations are a result of misrepaired programmed DNA DSBs that occur during lymphocyte development. Programmed DNA DSBs generated during V(D)J recombination, a lymphocyte-specific process that gives rise to the diverse set of antigen receptors necessary for proper immune function, require the ARTEMIS DNA nuclease for proper ligation. My studies utilize *Artemis/p53* deficient mice that spontaneously develop pro-B lymphomas characterized by *IgH:Myc* translocations and either *C-MYC* or *N-MYC* overexpression. Overexpression of oncogenes, including *MYC*, leads to

DNA damage, which can result in genomic instability and tumorigenesis. Therefore, determining the mechanisms involved in generating oncogenic chromosomal translocations and the pathways involved in repairing oncogene-induced DNA damage is critical for better understanding cancer initiation and progression. In this chapter, I will provide a focused overview of the current knowledge in the field in addition to the gaps that my thesis has aimed to fill.

Genomic Alterations and Cancer

The transformation of a normal cell into a cancer cell is a multi-step process, driven by an accumulation of genetic changes. Normal cells possess highly evolved mechanisms to ensure genome integrity and to limit the number of spontaneous deoxyribonucleic acid (DNA) mutations. In contrast, cancer cells often exhibit a rapid mutation rate, referred to as the mutator phenotype [1-3]. The mutator phenotype is thought to be adopted at an early step in tumorigenesis, and gives rise to genomic instability, a hallmark of cancer [4, 5]. Genomic instability plays a role in both cancer initiation and progression by allowing accumulation of additional oncogenic mutations required for acquisition of the other cancer hallmarks. Such genetic mutations can transpire at the simple nucleotide level, or at the chromosomal level.

Genomic alterations in cancer come in the form of insertions, deletions, duplications, amplifications, inversions, and translocations [6, 7]. Recurrent translocations are most frequently identified in lymphoid malignancies, but have

more recently been observed in some solid tumors, such as in lung, thyroid, prostate, and pediatric bone cancer [8-11]. The generation of a translocation involves two DNA double strand breaks (DSBs) that have occurred within the genome of a cell at the same time, close proximity of these broken ends, and ligation of the heterologous ends [12]. The outcome of ligation can result in two different types of translocations: reciprocal or nonreciprocal (Figure 1.1). Reciprocal translocations result in a simple exchange of genetic material, usually from two separate chromosomes, and can be classified as balanced or unbalanced [7]. Balanced translocations result in an even exchange of genetic material, whereas unbalanced translocations result in deletions or duplications in genes located near the breakpoint [7]. Nonreciprocal translocations are a one-way transfer of genetic material from one chromosome to the other. A Robertsonian translocation is a type of reciprocal translocation that involves two chromosomes with centromeres at or near the end of the chromosome (telocentric or acrocentric) with fusion occurring at the centromere, resulting in a metacentric chromosome [13, 14]. When acrocentric chromosomes are involved, the two long q-arms of each chromosome fuse, and although the short p-arms also fuse at the centromere, this rearrangement is typically lost within a few cell divisions [15].

Translocations can have a role in the initial steps of tumorigenesis, and can also contribute to clonal evolution and tumor progression. In order for these rare translocation events to become tumorigenic, they must provide a

proliferative advantage to the cell, usually by oncogene activation. There are three common mechanisms by which chromosomal translocations can lead to dysregulation of oncogenes. First, chromosomal rearrangement can lead to the juxtaposition of an oncogene with a strong promoter or enhancer, placing the oncogene under the control of these regulatory elements, resulting in aberrant activation. Second, fusion of two genes that are normally located on separate chromosomes can lead to an aberrant transcript and aberrant protein with potential oncogenic activity. Finally, a translocation that results in a dicentric chromosome can lead to oncogene deregulation through amplification, likely through breakage-fusion-bridge (BFB) cycles [16]. BFB cycles can be initiated during anaphase when the two centromeres are pulled in opposite directions, causing a break in the chromosome between the centromeres [17]. During the next cell cycle, the sister chromatids are available to fuse, and break again during anaphase. This process can quickly lead to amplification of genes located near the breakpoints, and if an oncogene is involved, can lead to oncogene overexpression (Figure 1.2).

Lymphoid Malignancies

The most common genomic alterations found in hematological malignancies are translocations [18]. According to the World Health Organization, lymphoid neoplasms are the fourth most common cancer type, and are the sixth leading cause of death by cancer in the United States [19]. Approximately 95% of

lymphomas are of B cell origin and harbor recurrent chromosomal translocations that involve the immunoglobulin (Ig) loci and various oncogenes, leading to their dysregulation by multiple mechanisms [18]. For example, in the t(8;14) translocation observed in Burkitt's B-cell lymphoma, c-myc (on chromosome 14) is placed under the control of a strong IgH (on chromosome 8) enhancer leading to deregulated c-myc expression. In the chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL) t(9;22) translocation, a portion of BCR from chromosome 22 is fused to a portion of ABL1 on chromosome 9 (referred to as the Philadelphia chromosome), leading to expression of a constitutively active oncogenic BCR-ABL fusion kinase. In other human B cell malignancies, more complex dicentric chromosomal translocations can lead to amplification of oncogenes through BFB cycles [20, 21]. While we have knowledge of the translocations that arise, the mechanisms underlying the susceptibility of loci to translocation formation and the mechanisms underlying the generation of translocations remain outstanding questions.

Factors that Give Rise to Double Strand Breaks

One factor that plays a role in translocation frequency is the number of DSBs in a cell at a given time [22]. DSBs can be generated by many cell-intrinsic mechanisms including errors made during replication or transcription and oxidative stress from byproducts formed during cellular metabolism [22-24]. In human cells, 10-50 DSBs are estimated to occur per cell cycle as a result of

replication [25, 26]. Some regions in the genome are more susceptible to DNA DSBs such as fragile sites, repetitive structures, or several non-B DNA structures (structures other than the conventional right-handed Watson-Crick structure) [23, 27-29]. Cell extrinsic mechanisms that lead to DNA DSBs include ionizing radiation, x-rays, and topoisomerase inhibitor treatment [24].

Furthermore, programmed DNA DSBs occur during lymphocyte development. Briefly, recombination activating genes 1 and 2 (RAG1 and RAG2) generate DSBs during V(D)J recombination, a lymphocyte-specific process that is responsible for producing the highly diverse antibodies and T cell receptors (TCR) expressed on B and T lymphocytes, respectively. V(D)J recombination is a main subject of this thesis and will be discussed in detail below. At later stages of B cell development, activation induced cytidine deaminase (AID) generates DSBs during class switch recombination (CSR), a process that increases antibody diversity by changing the effector properties of the antibody, and thereby influencing antibody function. Misrepair of these DSBs has been shown to give rise to translocations involving the immunoglobulin loci, although the mechanism for breaks generated in the translocation partner genes are not always well understood.

The MRE11/RAD50/NBS1 Complex

To preserve genome integrity, the cell has evolved sophisticated mechanisms to sense and repair all types of DNA damage, collectively known as

the DNA Damage Response (DDR). The DDR encompasses proteins that function to sense the break (sensors), transduce the damage signals (transducers), and initiate cellular responses including induction of cell-cycle-checkpoints, repair pathways, apoptosis, or senescence (effectors).

The MRE11/RAD50/NBS1 (MRN) complex is comprised of meiotic recombination 11 (MRE11), RAD50, and Nijmegen breakage syndrome 1 (NBS1), and has many roles in the DDR including detecting, signaling, and repairing DNA DSBs. Homozygous mutations in *Mre11*, *Rad50*, or *Nbs1* result in embryonic lethality in mice, revealing a critical role for the complex during development [30-32]. The MRN complex has been implicated in all known pathways of DNA DSB repair. It has roles in sensing DSBs, tethering ends, DNA end processing, and activating DNA damage checkpoint signaling cascades [33-35]. Importantly, in response to DSBs, MRN is required for the activation of ataxia telangiectasia mutated (ATM), a serine/threonine kinase. ATM signaling can result in cell cycle checkpoint activation, DNA repair, and apoptosis. MRE11 has single-stranded DNA (ssDNA) endonuclease activity as well as 3'-to-5' double-stranded DNA (dsDNA) exonuclease activity [36, 37]. Together, MRE11 and RAD50 form a heterotetramer that bridges DNA ends over both short and long distances (Figure 1.3) [38, 39]. NBS1 is responsible for the ATM-activation functions of the complex [40]. Specific roles for the MRN complex in DNA DSB repair pathways will be described in the sections below.

Human patients with hypomorphic mutations in MRE11, NBS1, or RAD50 present with ataxia telangiectasia-like disorder (ATLD), Nijmegen breakage syndrome (NBS) and NBS-like diseases, respectively [41-45]. ATLD is characterized by cerebellar ataxia, immunodeficiency, and a subset of patients is predisposed to cancer [44, 46]. Patients with NBS exhibit microcephaly, immunodeficiency, and a strong cancer predisposition [41, 42]. Therefore, there is interest in understanding the roles of MRN in tumor biology.

DNA Double-Strand Break Repair Pathways

To preserve genome integrity, highly conserved DNA DSB repair pathways have evolved to efficiently repair DSBs, including homologous recombination (HR) and non-homologous end joining (NHEJ). HR precisely joins the ends in an error-free manner by utilizing a sister chromatid template, restoring the original genomic sequence. Thus, HR is limited to S and G2 phases of the cell cycle, when a sister chromatid is available. In contrast, NHEJ is active in all phases of the cell cycle, but is most important in G1, when repair through HR is not available. NHEJ promotes the potentially inaccurate ligation of ends, and can be subdivided into classical-NHEJ (C-NHEJ) and alternative-NHEJ (alt-NHEJ).

Homologous Recombination and Roles of MRN

HR is initiated by 5' resection of a DSB end, an important regulatory step in DSB repair pathway choice [47]. Paradoxically, the generation of the 3'-ssDNA overhangs requires MRE11 3'-to-5' exonuclease activity [48]. Thus, a proposed two-step mechanism from MRE11 nuclease activity has been proposed [49-51]. First, end resection is initiated by an MRE11 endonucleolytic DNA nick upstream of the DSB, followed by bidirectional resection via MRE11 3'-to-5' exonuclease activity and Exonuclease 1 (EXO1) and DNA2/Bloom helicase (BLM) 5'-to-3' exonuclease activities [49, 50, 52-54]. Phosphorylated CtBP-interacting protein (CtIP) promotes the initial MRE11 endonuclease activity [55-58]. Once resection occurs, the cell is committed to HR [49, 59]. The resulting 3' ssDNA overhangs are rapidly coated by replication protein A (RPA), which protects against nuclease cleavage and formation of hairpins [60, 61]. RPA is replaced by RAD51 recombinase (RAD51), forming a nucleoprotein filament that catalyzes a search for homologous sequence, invasion, and strand exchange [62, 63].

In addition to essential roles during the initial steps of end resection, the MRN complex plays crucial roles in sensing and tethering the DNA DSB. MRN binds to DNA first, acting as a sensor to DNA damage, leading to activation of the DDR transducing kinases, ATM and ATR [64-66]. MRN has two important roles for activating ATM. First, MRN tethers the DSB ends, thereby increasing the local concentration of damaged DNA, and thus, promoting recruitment and dissociation of dimeric ATM into monomers [40]. The RAD50 zinc-hook domain is

responsible for this tethering function, which facilitates MRN intercomplex interactions [38]. Second, the NBS1 component of the MRN complex interacts with ATM, leading to ATM autophosphorylation and activation [40]. Once ATM is activated, it has critical roles in DSB signaling.

Non-Homologous End Joining and Roles of MRN

Non-homologous end joining (NHEJ) can be subdivided into classical (C-NHEJ) and alternative-NHEJ (alt-NHEJ). C-NHEJ is required for repairing programmed breaks generated during lymphocyte development, but is also utilized for the repair of general DSB repair throughout the cell cycle. Within seconds after DSB formation, the Ku70/80 dimer binds to DNA ends and then recruits the serine/threonine kinase DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to the ends which activates downstream signaling and repair pathways [67, 68]. DNA-PKcs helps to tether the DNA ends, which may need to be processed by nucleases or polymerases, an essential step involving the ARTEMIS DNA nuclease during C-NHEJ repair of programmed breaks during lymphocyte development. The ends are ligated by DNA ligase 4(LIG4)/X-ray repair cross-complementing protein 4 (XRCC4) and this process is enhanced by X-ray repair cross-complementing protein 4-like factor (XLF) [69, 70].

C-NHEJ deficient cells are still capable of NHEJ, and therefore reveal a much less characterized NHEJ pathway, termed alternative-NHEJ (alt-NHEJ),

also known as microhomology-mediated end joining (MMEJ) [71-73]. Although the details of alt-NHEJ are not well understood, studies have implicated alt-NHEJ to depend on NBS1, MRE11, CtIP, Poly ADP ribose polymerase 1 (PARP1), X-ray repair cross-complementing protein 1 (XRCC1), and DNA ligase 3 (LIG3) [74-80]. Sequence analysis of alt-NHEJ joins indicates the use of microhomologies (5-25 base pairs) between broken DNA ends for repair. Alt-NHEJ has been implicated in the generation of oncogenic chromosomal translocations, largely based on the presence of microhomologies at translocation junctions [81, 82]. Alt-NHEJ repair can take place in the presence of functioning HR and C-NHEJ, although at lower levels [83]. Further, Alt-NHEJ has also been implicated in repair of DSBs resulting from hydroxyurea (HU)-induced collapsed replication forks [83].

Initially, a major role for MRN in C-NHEJ was proposed to be ATM activation. However, ATM-independent functions of MRN, and specifically MRE11 nuclease activity, have now been implicated in having a role in both C-NHEJ and alt-NHEJ. In both wild type and XRCC4^{-/-}, depletion of MRE11 or inhibition of MRE11 exonuclease activity by mirin decreased end-joining efficiency of I-SceI nuclease-induced DSBs, indicating a role for MRE11 and MRE11 nuclease activity in both C-NHEJ and alt-NHEJ repair pathways [77, 80]. However, repair of I-SceI-induced DSBs requires MRE11 nuclease activity only in alt-NHEJ, and is not required for C-NHEJ [84]. It has recently been shown that alt-NHEJ and HR share the initial end resection step, requiring MRE11 nuclease activity to promote repair [83, 85]. Further, roles for MRN in C-NHEJ and alt-

NHEJ have been identified during lymphocyte-specific class-switch recombination (CSR) [76].

V(D)J Recombination and Lymphocyte Development

The ability of the adaptive immune system to recognize antigens from a wide variety of pathogens depends on the large repertoire and specificity of the antigen receptors. The antigen receptors contain constant regions and variable regions. The constant region determines the effector function of the antibody, while the variable region is responsible for the specificity of antigen binding. The variable regions of the antigen receptor are encoded by a single exon that is assembled through somatic recombination of germline variable (V), diversity (D), and joining (J) gene segments in a process referred to as V(D)J recombination (Figure 1.5) [86]. This process is restricted to developing B and T lymphocytes and results in the diverse B cell receptors or immunoglobulins (BCR or Ig) and T cell receptors (TCR) that are expressed on the B and T lymphocyte cell surfaces, respectively. Each lymphocyte expresses multiple receptors with the same antigen recognition and specificity.

Specifically, B cell receptors are comprised of two identical immunoglobulin heavy (IgH) chains covalently attached to two identical light (IgL) chains (Figure 1.4A). Both the heavy and light chains contain a constant region (C_H and C_L) and a variable region (V_H and V_L). V(D)J recombination begins in a lymphoid progenitor cell (pre-pro-B) in the bone marrow. During B cell

development, rearrangement of the immunoglobulin loci is carefully orchestrated in a specific sequence of events. First, the heavy chain (IgH) locus on both chromosomes rearranges a D gene segment to a J_H gene segment and transitions to an early progenitor B (pro-B) cell (Figure 1.4B). Successful rearrangement initiates V_H to DJ_H gene rearrangement on one chromosome, and further rearrangement on the other chromosome only occurs if rearrangement on the first chromosome was unproductive (Figure 1.4B). If neither heavy chain rearrangement is productive, the cell undergoes apoptosis. Successful V_H to DJ_H gene rearrangement completes the late pro-B cell stage. At this point, the pre-B-cell receptor (pre-BCR) is expressed on the cell surface, and includes a rearranged heavy chain and a surrogate light chain. Expression of the pre-BCR stimulates these cells to divide, and are considered large pre-B cells. The cell enters the small pre-B cell stage after downregulation of the B cell marker CD43 and the cell is no longer cycling.

Next, only if one chromosome successfully rearranged the heavy chain, the light chain attempts rearrangement in a similar manner, except the light chain lacks the D gene segment. The rearranged light chain of the final BCR will be comprised of either the kappa (κ) chain or the lambda (λ) chain. Whether the light chain of the BCR is comprised of a rearranged kappa chain or a rearranged lambda chain depends on the first successful rearrangement in a specific order of events. First, in the small pre-B cell, rearrangement of the κ gene on the first chromosome occurs. If this is a productive rearrangement, the cell becomes an

immature B cell expressing the B-cell receptor (BCR) comprised of a rearranged heavy chain, and either a rearranged kappa or lambda light chain. If, however, the rearrangement is unproductive, the κ gene on the second chromosome, the λ gene on the first chromosome, and the λ gene on the second chromosome will rearrange in this order until there is a productive rearrangement. If there are no productive light chain rearrangements, the cell undergoes apoptosis. A process called allelic exclusion prevents both chromosomes from completing V(D)J recombination, so that only one chromosome undergoes complete rearrangement. This ensures that in an individual B cell, only one type of immunoglobulin is expressed on the cell surface.

Similar to rearrangements in the immunoglobulin loci, the TCR chains in developing thymocytes undergo rearrangements to produce a functional TCR. There are two categories of TCRs classified by the type of receptor on their cell surface. TCRs either have an α -chain and β -chain or a γ -chain and δ -chain.

V(D)J Recombination: Cleavage Stage

V(D)J recombination initiation requires the products of recombination activating genes 1 and 2 (RAG1 and RAG2) [87, 88], which make up the lymphocyte-specific Rag endonuclease [87, 89, 90]. RAG recognizes specific noncoding DNA sequences that are adjacent to each of the many V, D, and J segments [91]. The sequences consist of a conserved heptamer—5'CACAGTG3'—followed by a conserved length of either 12 or 23 nucleotides

referred to as the spacer. This is followed by a conserved sequence of nucleotides called the nonamer—5'ACAAAAACC3'. Although the sequence of the spacer is nonconserved, the conserved length of the spacer, either 12 or 23 nucleotides, corresponds to one or two turns of the DNA double helix, respectively, allowing for the heptamer and nonamer to be in close proximity so they can be bound by the protein complex that carries out recombination [91, 92]. The heptamer-spacer-nonamer region is collectively referred to as the recombination signal sequence (RSS), and more specifically 12RSS or 23RSS, depending on the length of the spacer.

Generally, a gene segment flanked by a 12RSS will only recombine with a gene segment flanked by a 23RSS, ensuring that productive DNA rearrangements occur. This restriction is known as the 12/23 rule [86]. Further, regulation of recombination events between V, D, and J gene segments relies on the strategic placement of 12RSS and 23RSS, typically with V and J segments in a given locus being flanked by the same type of RSS. For example, in the IgH locus, V_H and J_H gene segments are flanked by 23RSS, whereas the D gene segments are flanked by 12RSS, thereby allowing only V_H-to-D and D-to-J_H recombination events [91].

RAG initiates V(D)J recombination by first recognizing and binding to a 12RSS or 23RSS, followed by nicking one strand of the double stranded DNA precisely between the heptamer and the coding segment, leaving a 3' hydroxyl (OH) group (Figure 1.5) [93-95]. Next, this RAG-RSS complex captures a second

23RSS or 12RSS (referred to as synapsis), and nicks between the second RSS and coding segment, generating another 3' hydroxyl (OH) group (Figure 1.5) [96]. Once the 12RSS and 23RSS pair is in a synaptic complex with RAG, the 3' hydroxyl (OH) groups mediate nucleophilic attack on the phosphodiester bonds between the coding segments and the RSSs on the opposite strands via a transesterification reaction (Figure 1.5) [97]. This reaction results in two DNA double-strand breaks, each consisting of a hairpin coding end adjacent to the coding segment, and a blunt end adjacent to the RSS, referred to as the signal end (Figure 1.5) [98, 99]. The requirement for both RSSs to be in complex with Rag before generation of the DSB is an important mechanism of control to prevent aberrant rearrangements [94, 95, 100-103]. Additionally, to provide another mechanism of regulation and to ensure proper joining, the four DNA ends are held together by RAG in a DNA-protein post-cleavage complex (PCC) [104]. This complex functions to protect DNA ends from degradation or inappropriate ligation, to prevent the ends from inducing p53-mediated apoptosis, and to recruit important factors necessary for ligation of the DNA ends [105]. The hairpin coding ends must be opened, allowing for the two ends to be joined, generating a coding join (CJ) (Figure 1.5). The signal ends are blunt, and therefore, do not require additional processing before being ligated to generate a signal join (SJ) (Figure 1.5).

V(D)J Recombination: Joining Stage

These RAG-induced DNA DSBs are joined by the ubiquitously expressed canonical non-homologous end joining (C-NHEJ) machinery [106]. Specifically, the KU70/KU80 heterodimer binds to the Rag-induced DSB DNA ends by forming a hollow ring around the DNA ends, and is required ultimately for both coding and signal end joining [107, 108]. KU70/KU80 recruits DNA-protein kinase catalytic subunit (DNA-PKcs) to form the complex DNA-protein kinase (DNA-PK), which bridges the coding ends, and is required for coding end processing [109-112]. Autophosphorylation of DNA-PKcs is important for activating the DNA nuclease, ARTEMIS, and when in complex with one another, ARTEMIS gains endonuclease activity required for nicking open the hairpins at the coding ends (Figure 1.5) [113, 114]. Finally, the processed coding ends and the signal ends are both ligated by a complex comprised of Ligase IV (LIG4), X-Ray Repair Cross Complementing 4 (XRCC4), and XRCC4-like factor (XLF) [115-118].

Roles of MRN during V(D)J Recombination

Roles for the MRN complex have been implicated in V(D)J recombination. Although mice with homozygous-null mutations in *Mre11*, *Rad50*, or *Nbs1*, exhibit embryonic lethality, human patients and mice with hypomorphic mutations in *Mre11* or *Nbs1* present with mild immunodeficiency, demonstrating a possible role for the MRN complex in the repair of DSBs generated during V(D)J

recombination [30, 31, 119-122]. Further, thymocytes with a hypomorphic mutation in *Mre11* have increased trans-rearrangements, suggesting that mutations in MRN can lead to aberrant V(D)J recombination [121]. Studies have also shown an increase of unrepaired coding ends associated with hypomorphic *Mre11* or *Nbs1* mutations in developing lymphocytes [123].

Human V(D)J Defects and Mouse Models

Defects in C-NHEJ generate unrepaired RAG-induced DSBs in pro-B cells. Under normal conditions, the G1 cell cycle checkpoint would lead to elimination of these cells; however, in the context of p53 deficiency, these cells survive, carrying with them unrepaired DSB substrates for translocation formation [124, 125]. Indeed, mice with C-NHEJ mutation combined with p53 deficiency have a strong predisposition to pro-B lymphomas associated with oncogenic chromosomal translocations involving the antigen receptor loci [12].

Human patients with inactivating mutations in *RAG1* or *RAG2* present with severe combined immunodeficiency (SCID), a term that refers to a group of disorders generally characterized by a combined block in both T lymphocyte and B lymphocyte development, and therefore, an absence of T and B lymphocytes with high susceptibility to infection [126]. Hypomorphic mutations in *RAG1* or *RAG2* result in Omenn Syndrome, a less severe combined immunodeficiency disorder [126, 127]. Mouse models lacking either RAG1 or RAG2 recapitulate the

SCID phenotype observed in human patients, and a hypomorphic *Rag1* mutant is predisposed to thymic lymphoma [90, 128, 129].

Human patients with mutations in proteins required for proper repair of RAG-induced breaks, specifically the C-NHEJ pathway of repair, typically have radiosensitive severe combined immunodeficiency (RS-SCID). Mutations in *DNA-PKcs*, *LIG4*, and *XLF* all cause RS-SCID in humans, while hypomorphic mutations in *ARTEMIS* or *LIG4* result in radiosensitive combined immunodeficiency or Omenn syndrome with a subset of patients predisposed to lymphoid malignancies [130-133]. Mouse models of *Ku70*, *Ku80*, *DNA-PKcs*, or *Artemis* deficiency are SCID or leaky-SCID in the case of *Ku70* and *Artemis* [134, 135]. A mouse model with a hypomorphic *Artemis* mutation caused by deletion of the C-terminus results in partial immunodeficiency and a predisposition to lymphoma [136]. Knockout of *Lig4* or *Xrcc4* in mouse models are embryonic lethal [137-139]. *p53* deficiency rescues embryonic lethality in *Lig4* or *Xrcc4* deficient mouse models, and these mice are associated with a predisposition to pro-B lymphoma with oncogenic translocations, similar to *Ku80*, or *Artemis* null mice with a *p53* deficiency [135, 140-143]. These phenotypes highlight the importance of C-NHEJ factors in mammalian cells.

V(D)J Errors that Lead to Lymphoid Malignancies

While the mechanism for generating the vast diversity of antigen receptors is highly regulated, it is accompanied with the risk of genomic rearrangements

due to the essential involvement of DSBs. A role for V(D)J recombination defects, as well as errors during CSR, in lymphomagenesis was first suspected when translocation breakpoints in certain tumors were found to have an antibody receptor locus fused to other chromosomal regions, frequently placing a highly expressing antigen receptor promoter next to an oncogene [144, 145]. Since then, a large number of translocations associated with the IgH locus have been identified (Figure 1.6).

Aberrant V(D)J rearrangements leading to lymphoid malignancies arise from two major types of errors—those in target recognition, and those in joining [146]. Errors in the ability of RAG to recognize an authentic RSS are one cause of illegitimate DNA DSB formation. In random DNA sequence, cryptic RSS (cRSS) sites exist once per kb, and there are estimated to be more than ten million cRSS sites within the genome [147]. These sites can occur in non-antigen receptor loci, and resemble an authentic RSS [147]. There is evidence of translocations between the Ig or TCR loci and either cRSS or non-B DNA structures [147-150]. V(D)J-mediated translocations involving an authentic antigen receptor RSS and a cRSS bordering a proto-oncogene were first identified in human lymphoid malignancies by cytogenetic analysis [151, 152]. Additionally, RAG can misidentify and cleave at non-B DNA structures, implicated by RAG-mediated cleavage at the major breakpoint region (Mbr) in *BCL2*, a common translocation partner observed in human follicular lymphoma [149].

Errors in joining occur when a seemingly normal RAG-mediated DSB at an authentic RSS is joined to a DSB generated by other mechanisms elsewhere in the genome. While C-NHEJ may be involved in translocation generation in some circumstances, evidence implicates that the error-prone alternative NHEJ (alt-NHEJ) pathway of repair is favored in the generation of translocations [153]. In fact, analysis of breakpoint sequences in hematological translocations reveals microhomologies (1-6 base pairs) at the junction, a characteristic of joining by alt-NHEJ [16, 154].

MYC Family

C-MYC belongs to the MYC family of transcription factors, including N-MYC and L-MYC [155, 156]. They share similar structures and functions, but have distinct targets and differential expression patterns. *C-Myc* and *N-Myc* knock out mice are embryonic lethal, implicating an important role for normal development and embryogenesis [157, 158]. However, when C-MYC is replaced with N-MYC, mice survive, and N-MYC can function similarly to C-MYC in murine development, growth, and differentiation [159].

In normal cells, MYC is under strict control to ensure the delicate balance between normal and tumorigenic conditions leans toward normalcy. MYC expression and transcription are tightly regulated on transcriptional, translational, and post-translational levels [160-163]. Additionally, MYC is autoregulated by a negative-feedback loop demonstrated by exogenous expression of MYC resulting

in a down-regulation of endogenous MYC levels [164]. MYC is involved in the tight regulation of replication by interaction with the pre-replication complex (pre-RC), cell cycle control by regulation of cyclin-dependent kinases (CDKs), and nucleotide synthesis.

MYC is one of the most highly amplified oncogenes in human cancer [165]. Interestingly, the expression patterns in normal cells and overexpression of each oncogene is cancer specific, with C-MYC broadly overexpressed but most frequently in blood-borne and solid tumors, N-MYC most frequently overexpressed in solid tumors that originate from the neural crest, and L-MYC most often expressed in small cell lung cancer [7]. Amplification and overexpression of MYC family transcription factors have been observed in a variety of human cancers including breast, colorectal, gastric, lung, medulloblastoma, neuroblastoma, and prostate [166]. C-MYC/N-MYC deregulation has also been implicated in bladder, cervical, endocrine, gastric, glioblastoma, liver, melanoma, osteosarcoma, and ovarian cancers [167]. It has been estimated that MYC expression is deregulated in up to 70% of human cancers, and is correlated with genome instability in many cancers [160, 168].

The large variety of cancers that activate MYC has made MYC an attractive potential target for cancer therapies. However, successful pharmacologic targeting of MYC is scarce. MYC is a transcription factor, and thus, located in the nucleus where it is largely inaccessible to antibody-based therapies. MYC lacks intrinsic enzymatic functions and a classic active site,

making it difficult for small molecule pharmacologic inhibition to be successful. Further, MYC is essential in normal cells, making toxicity of drugs targeting MYC a potential issue in human patients. For these reasons, MYC has been deemed “undruggable”, a frustrating restriction since it is such a well-known driver of cancer. Thus, it is important to identify strategies to target MYC overexpressing tumors.

Oncogene-Induced Replication Stress

Faithful replication in dividing cells is essential for transmitting an exact copy of the genome from a parental cell to daughter cells, ensuring genome stability. Threats to DNA replication that can impede this process arise from both endogenous and exogenous sources including limited nucleotides, DNA lesions, fragile sites, ribonucleotide incorporation, DNA secondary structures, repetitive DNA, collisions with the transcription machinery, and oncogene overexpression (Figure 1.7) [169]. These conditions or obstacles can lead to slowing of DNA synthesis or replication fork stalling, referred to as replication stress. The details of how oncogene overexpression leads to replication stress are not simple, as studies have shown different oncogenes induce replication stress through distinct mechanisms, and an individual oncogene may act through a number of mechanisms. Many oncogenes induce replication stress due to their influence on the cell cycle as well as the regulation of DNA replication initiation. For example,

overexpression of oncogenes MYC, RAS, and C-FOS lead to premature S-phase entry and hyper-proliferation [170-172].

Under normal replication conditions, the pre-replication complexes (pre-RCs) assemble at origins of replication during late mitosis and G1, licensing these sites as origins (Figure 1.8 A). CDC45 is loaded onto the pre-RC, DNA is unwound, and DNA replication is initiated in S-phase [173]. This process is controlled by cyclin-dependent kinases (CDKs). Interestingly, oncogenes regulate all of these initial replication steps. For example, C-MYC directly interacts with CDC45, stabilizing components of the pre-replication complex [174]. A decreased inter-origin distance has been observed in MYC, cyclin E, RAS, and HPV E6 and E7 overexpressing cells, consistent with increased origin firing [170, 175-177]. Increased origin firing can lead to depletion of nucleotide pools, and/or collisions with the transcription machinery [177]. In fact, replication stress induced by a number of oncogenes has been shown to be rescued by an exogenous supply of nucleotides [177]. On the contrary, MYC regulates genes that are involved in pyrimidine and purine synthesis, having a direct role in nucleotide biosynthesis and likely counters this effect [178, 179].

Replication and transcription both utilize and function on DNA. Therefore, increased origin firing or increased transcription could lead to collisions of the replication and transcription machinery. Collisions between the replication and transcription machinery can lead to the formation of R-loops, RNA:DNA hybrids formed between one strand of DNA and the nascent RNA transcript [169, 180].

This can lead to stalling of the replication fork, and DSBs following transcription-coupled nucleotide excision repair [181]. Cyclin E enhances replication initiation and causes collisions between the replication and transcription machinery [176]. Overexpression of other oncogenes, such as HRAS^{V12}, results in increased transcription levels, which can lead to an increase in the frequency of collisions [182].

Overexpression of Cyclin E and CDC25A also slows replication fork progression and induces reversal of the replication fork, which could be a result of increased topological stress from increased origin firing [183]. Further, MYC overexpression deregulates CDK activity, a key protein that controls cell cycle progression [184, 185]. All of these effects of oncogene dysregulation can lead to impaired replication fork progression and fork stalling (Figure 1.8 B).

Upon fork stalling, it is thought that the replicative helicase continues to unwind DNA, exposing single-stranded DNA (ssDNA) [186]. Replication protein A (RPA) coats ssDNA leading to activation of ataxia-telangiectasia related (ATR) and the ATR-dependent signaling cascade resulting in checkpoint kinase 1 (CHK1) activation, fork stabilization and restart, reduced origin firing, and cell cycle arrest. If the fork is unable to restart, it may collapse, resulting in a DSB, and activation of the DNA damage response (DDR). Interestingly, the DDR is activated early on in tumor development, specifically in premalignant lesions in human bladder, lung, and skin hyperplasias [187, 188]. The DDR is absent in normal tissues, is activated in premalignant lesions, and is decreased in more

advanced carcinomas, indicating the DDR acts as a barrier to tumorigenesis. Many questions still remain regarding the mechanisms that are involved in repairing oncogene-induced DNA damage.

Targeting the DNA Damage Response in Cancer

Genomic instability is a hallmark of cancer [5]. It is an early event in tumorigenesis, and can lead to the acquisition of the other cancer hallmarks, promoting tumorigenesis [5, 187, 188]. Therefore, it may seem counterintuitive to inhibit the very proteins responsible for maintaining genomic stability as a treatment for cancer, however strategies to inhibit the DDR in cancer are gaining tremendous amounts of interest. The rationale for targeting the DDR relies on key differences between cancer cells and normal cells [189]. First, nearly all cancers evolve to become deficient in one or more DDR pathways, and therefore become dependent on the remaining pathways (Figure 1.9). Second, as discussed above, many cancers have activated oncogenes, and thus increased levels of oncogene-induced replication stress. Third, cancer cells have an increased level of endogenous DNA damage. These three characteristics provide potential therapeutic windows for inhibitors to target cancer cells. The intent of targeting the DDR is to exacerbate the endogenous levels of damage inherent to cancer cells.

One strategy takes a synthetic lethal approach. For example, inhibitors of poly (ADP-ribose) polymerase (PARP), a sensor protein that has roles in DNA

base excision repair (BER), single-strand break (SSB) repair, and alt-NHEJ have been developed [190, 191]. PARP inhibitors such as olaparib (Lynparza; AstraZeneca) suppress SSB repair, BER, and cause PARP to be trapped on SSBs, resulting in replication fork stalling, fork collapse, and DSBs [192, 193]. In normal cells, these breaks would be repaired via HR, however, cells mutant for HR protein BRCA1/2 repair the break through error-prone pathways, resulting in catastrophic damage and cell death [194]. Olaparib has recently been clinically approved for use in patients with *BRCA1/2* mutations.

Another strategy, relevant to the studies presented in this thesis, takes advantage of the increased levels of replication stress observed in cancer cells. The cellular response to replication stress is dependent on the ATR/CHK1 signaling pathway. ATR and CHK1 inhibitors were originally utilized in combination to enhance the effect of other chemotherapeutic agents. However, now ATR and CHK1 inhibitors are being considered for potential as a monotherapy. Not surprisingly, use as a monotherapy was not effective in a variety of tumor models; however, CHK1 inhibitors have proved to be effective in MYC-driven lymphoma cell lines and mouse models, N-MYC-driven neuroblastoma, and melanoma cells that display high levels of replication stress [195-199]. Importantly, K-RAS^{G12V}-induced pancreatic adenocarcinomas do not respond to CHK1 inhibitor treatment, which do not have increased levels of replication stress [195]. A number of ATR and CHK1 inhibitors are currently being tested in clinical trials, and provide an exciting new strategy to selectively target

cancer cells (Figure 1.10). Thus, there is great interest in pursuing this type of therapeutic strategy for targeting cancer cells.

Thesis Summary

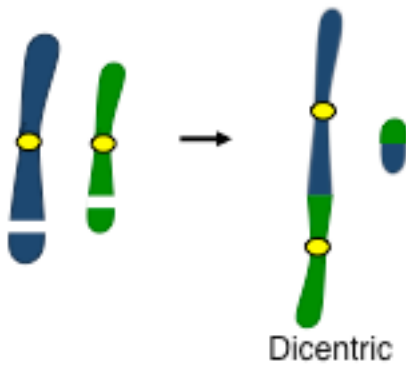
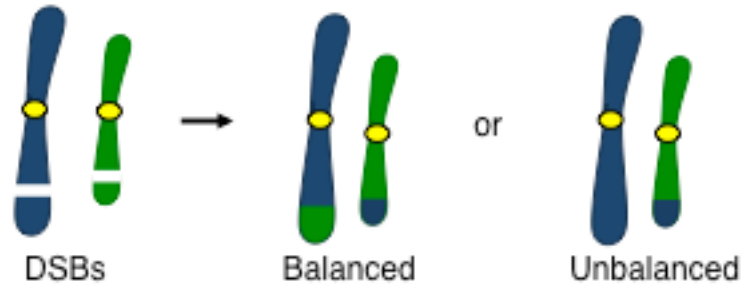
The MRN complex is involved in all pathways of DNA DSB repair, including pathways known to generate chromosomal translocations and pathways known to repair replication stress. The studies presented in this dissertation aim to investigate two potential cancer-promoting functions of MRE11. First, the studies in Chapter II aim to address the role of MRE11 and MRE11 nuclease activity in tumorigenesis. I utilize an *Artemis*^{-/-}*p53*^{-/-} mouse model that spontaneously develops pro-B lymphomas associated with *IgH:MyC* translocations leading to MYC overexpression. Experiments in this chapter test the hypothesis that MRE11 is involved in facilitating oncogenic chromosomal translocations, and thus, the initiating genetic events that can lead to cancer. Second, the experiments in Chapter III test the hypothesis that MRE11 nuclease activity is required for cellular survival in C-MYC overexpressing cells, and inactivation of nuclease activity leads to catastrophic amounts of damage and cell death.

The findings in this dissertation demonstrate that pharmacological inhibition of MRE11 exonuclease activity results in increased DNA damage, decreased cellular survival, and increased apoptosis specifically in cells that overexpress C-MYC. I hypothesize MRE11 exonuclease activity could be an

effective target for MYC-driven tumors and cancers with increased replication stress. The studies in my dissertation provide promising mechanistic preclinical evidence, in support of MRE11 exonuclease activity as a novel therapeutic target. This dissertation makes important contributions to the fields of DNA repair and cancer biology, and has provided a foundation of data from which future studies can build to better understand and treat MYC-driven cancer.

Figures

Reciprocal



Nonreciprocal

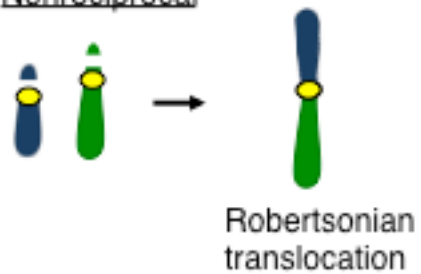


Figure 1.1. Types of chromosomal translocations. Misrepair of DNA DSBs can result in reciprocal (balanced or unbalanced) or nonreciprocal translocations. Centromeres, yellow dots.

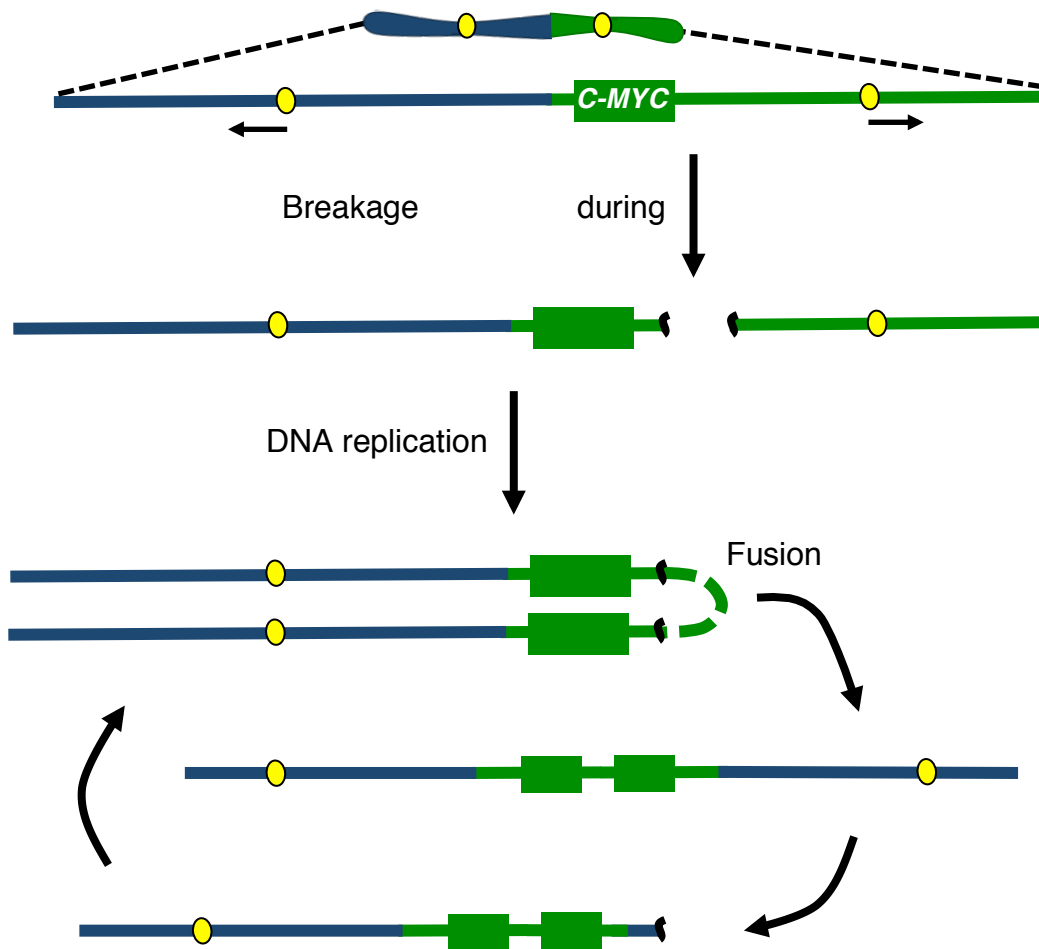


Figure 1.2. Gene amplification through breakage-fusion-bridge (BFB) cycles. BFB cycles can be initiated during anaphase when the two centromeres are pulled in opposite directions, causing a break in the chromosome between the centromeres. During the next cell cycle, the sister chromatids are available to fuse, and break again during anaphase. This process can quickly lead to amplification of genes located near the breakpoints, and if an oncogene is involved, can lead oncogene overexpression [200].

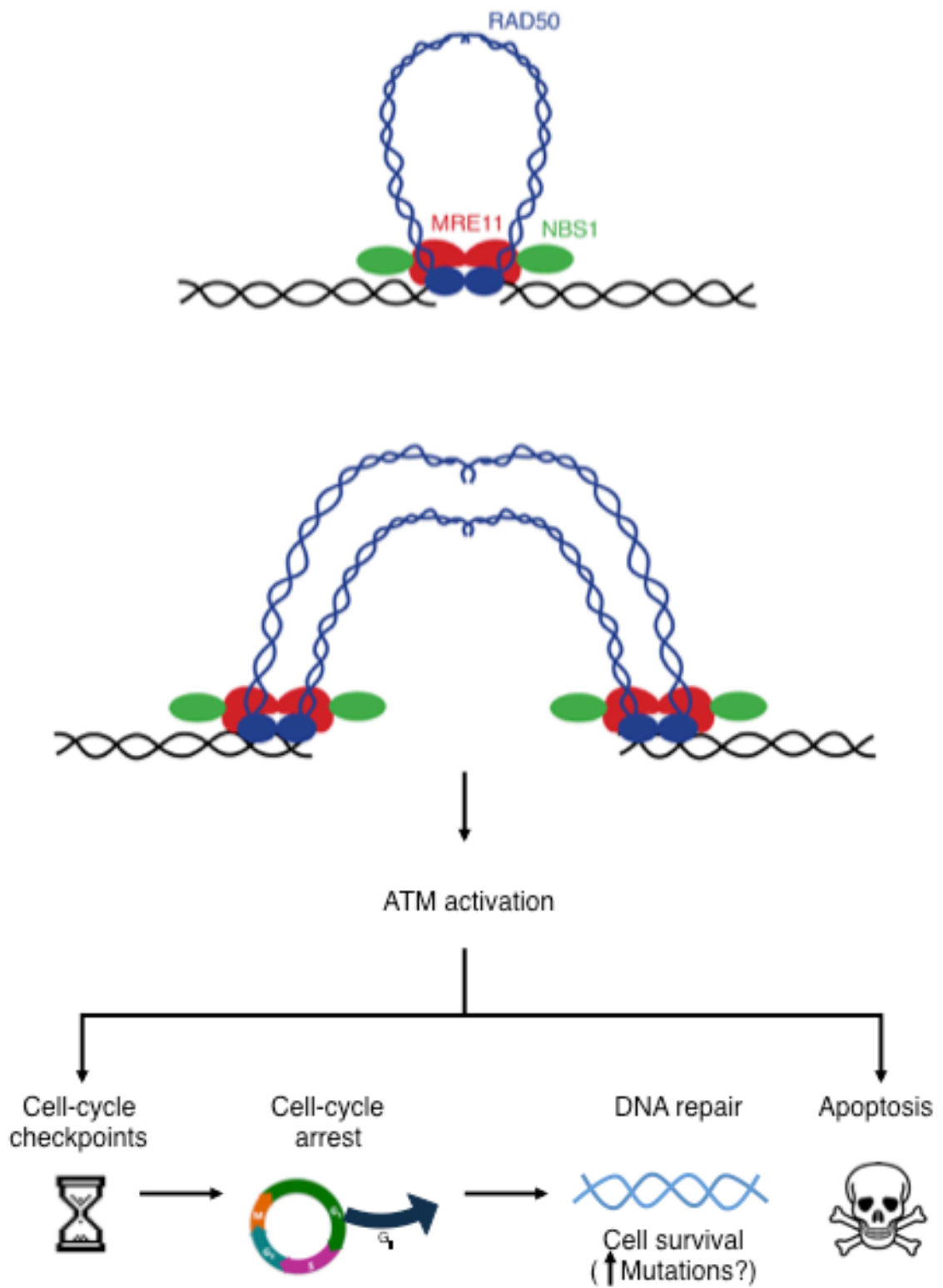


Figure 1.3. The MRE11/RAD50/NBS1 (MRN) complex. Together, MRE11 and RAD50 form a heterotetramer that bridges DNA ends over both short and long distances [38, 39]. NBS1 is responsible for the ATM-activation functions of the complex. ATM signaling leads to cell cycle, checkpoints and cell cycle arrest, allowing the cell time to repair DNA damage. This can restore the genome, but may also increase mutation frequency which could drive tumorigenesis. If the damage to the DNA is too severe, the cell will undergo apoptosis.

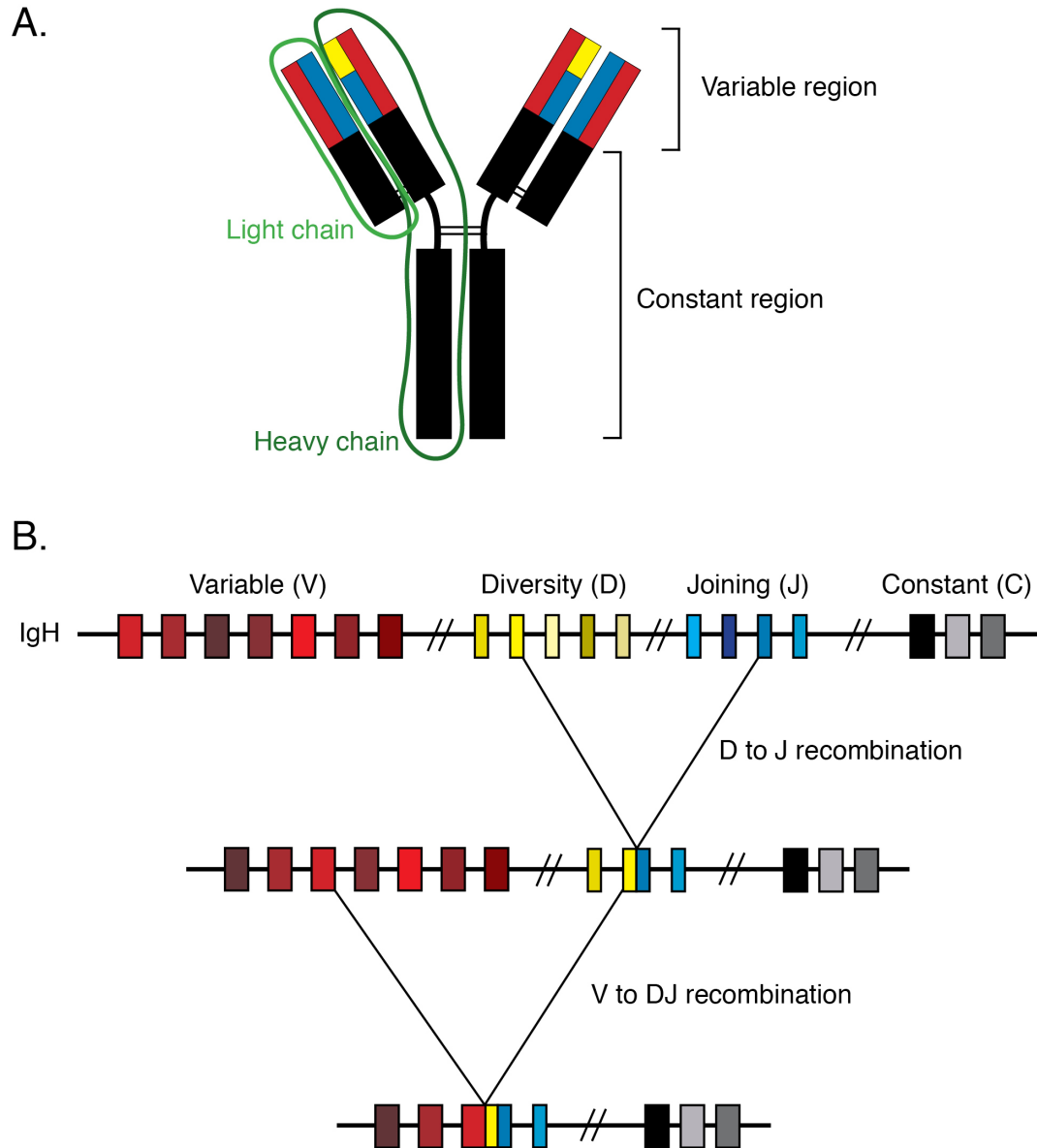


Figure 1.4. The B cell receptor (BCR) and V(D)J recombination. (A) The B cell receptor is comprised of two identical immunoglobulin heavy chains covalently attached to two identical light chains. Both the heavy and light chains contain a constant region and a variable region. (B) During V(D)J recombination, variable (V), diversity (D), and joining (J) segments are rearranged to generate the antigen receptor. First, D and J segments are joined followed by V to DJ rearrangements.

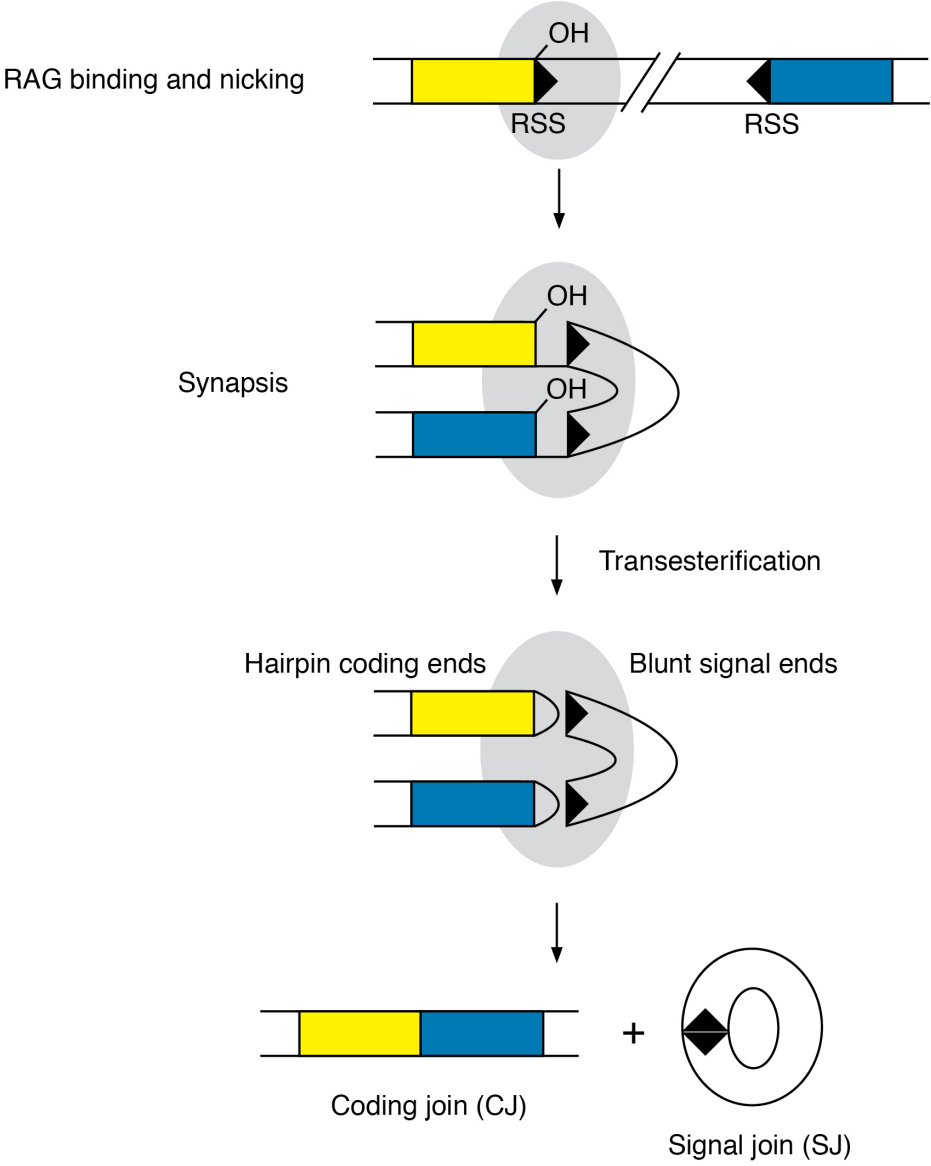


Figure 1.5. RAG initiates V(D)J recombination and generate hairpin ends.

The RAG endonuclease (Gray ellipse) initiates V(D)J recombination by first recognizing and binding recombination signal sequences (RSS) (black triangles) followed by nicking one strand of the double stranded DNA between the RSS and the coding gene segment (yellow rectangle) leaving a 3' hydroxyl (OH) group [93-95]. Next, this RAG-RSS complex captures a second RSS (referred to as synapsis), and nicks between the second RSS and the coding gene segment (blue rectangle), generating another 3' hydroxyl (OH) group [96]. Once the RSS pair is in a synaptic complex with RAG, the 3' hydroxyl (OH) groups mediate nucleophilic attack on the phosphodiester bonds between the coding segments and the RSSs on the opposite strands via a transesterification reaction [97]. This reaction results in two DNA double-strand breaks, each consisting of a hairpin coding end adjacent to the coding segment, and a blunt end adjacent to the RSS, referred to as the signal end. The hairpin coding ends must be opened, allowing for the two ends to be joined, generating a coding join (CJ). The signal ends are blunt, and therefore, do not require additional processing before being ligated to generate a signal join (SJ).

<u>Type of translocation</u>	<u>Gene relocated to IgH locus</u>	<u>Type of cancer</u>
t(8;14)(q24;q32)	c-MYC(8q24)	BL, B-ALL
t(2;8)(p12;q24)	c-MYC(8q24)	B-ALL
t(8;22)(q24;q11)	c-MYC(8q24)	B-ALL
t(8;12)(q24;q22)	c-MYC(8q24) BTG(12q22)	B-CLL, ALL
t(3;14)(q27;q32)	BCL6(3q27)	DLCL, FL
t(14;18)(q32;q21)	BCL2(18q21)	FL
t(11;14)(q13;q32)	BCL1(11q13)	B-CLL
t(10;14)(q24;q32)	LYT10(10q24)	B lymphoma
t(14;19)(q32;q13.1)	BCL3(19q13.1)	B-CLL
t(5;14)(q31;q32)	IL-3(5q31)	pre-B-ALL
t(1;14)(q21;q32)	BCL-9(1q21)	B-ALL and MALT lymphoma

Figure 1.6. Chromosomal translocations involving the IgH locus in haematopoietic tumors leading to dysregulated gene expression. Figure adapted from Nambiar et al., 2008 [201].

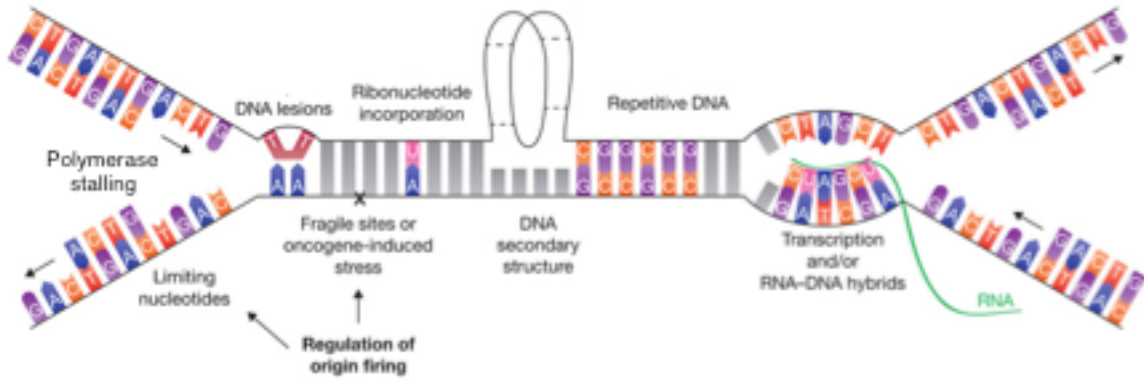
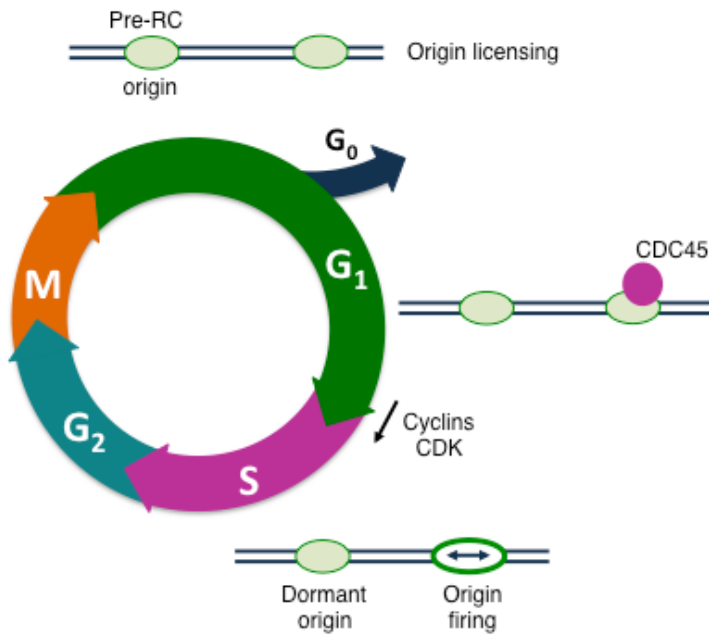


Figure 1.7. Sources of DNA replication stress. Limited nucleotides, DNA lesions, fragile sites, ribonucleotide incorporation, DNA secondary structures, repetitive DNA, collisions with the transcription machinery, and oncogene overexpression can lead to a stalled replication fork. Figure adapted from Zeman and Cimprich, 2014 [169].

A. Normal replication conditions



B. C-MYC overexpression

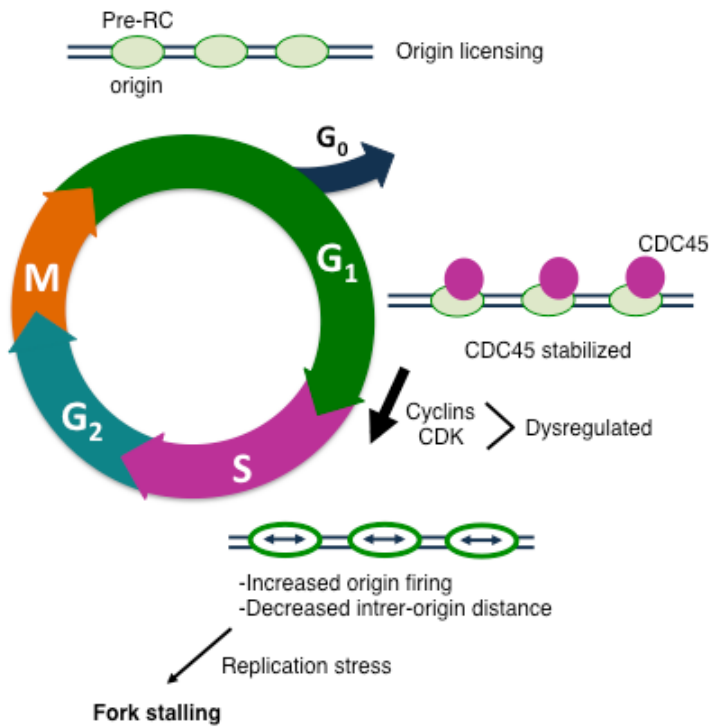


Figure 1.8. The consequences of C-MYC overexpression on cell cycle progression. (A) Normal replication conditions. The pre-replication complexes (pre-RCs) assemble at origins of replication during late mitosis and G1, licensing these sites as origins. CDC45 is loaded onto the pre-RC, DNA is unwound, and DNA replication is initiated in S-phase. This process is controlled by cyclin-dependent kinases (CDKs). (B) Consequences of C-MYC overexpression on cell cycle progression. MYC directly interacts with CDC45, stabilizing components of the pre-replication complex, decreases inter-origin distance and leads to increased origin firing. Cyclin and CDK activities are dysregulated, leading to inappropriate S-phase entry.

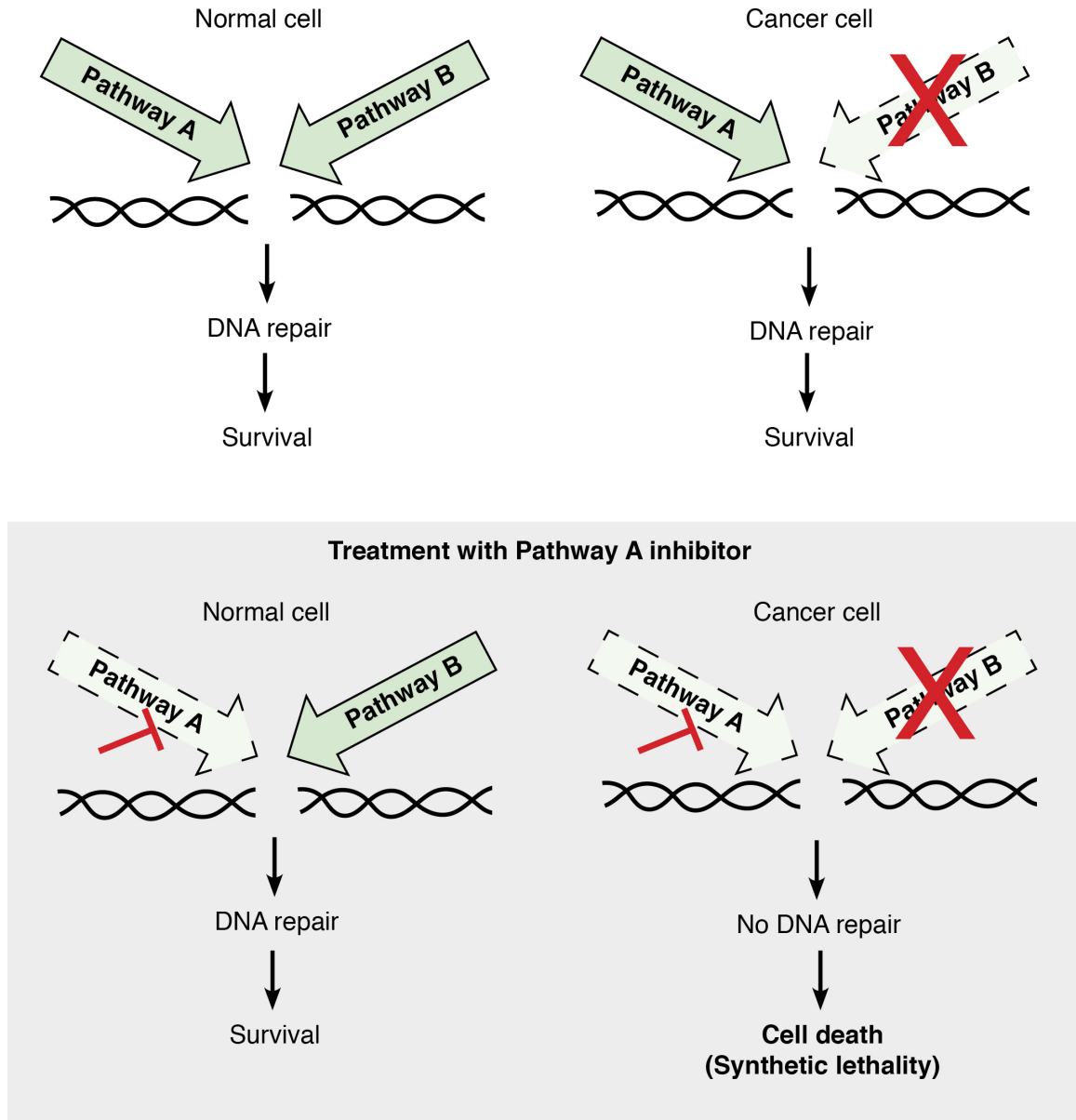


Figure 1.9. Synthetic lethal approach to selectively target cancer cells. Cancer cells frequently become deficient in one or more DNA repair pathways, and therefore can become vulnerable to inhibition of the remaining DNA repair pathways. Normal cells are able to compensate for the loss of one DNA repair pathway, and thus cellular death is specific to cancer cells.

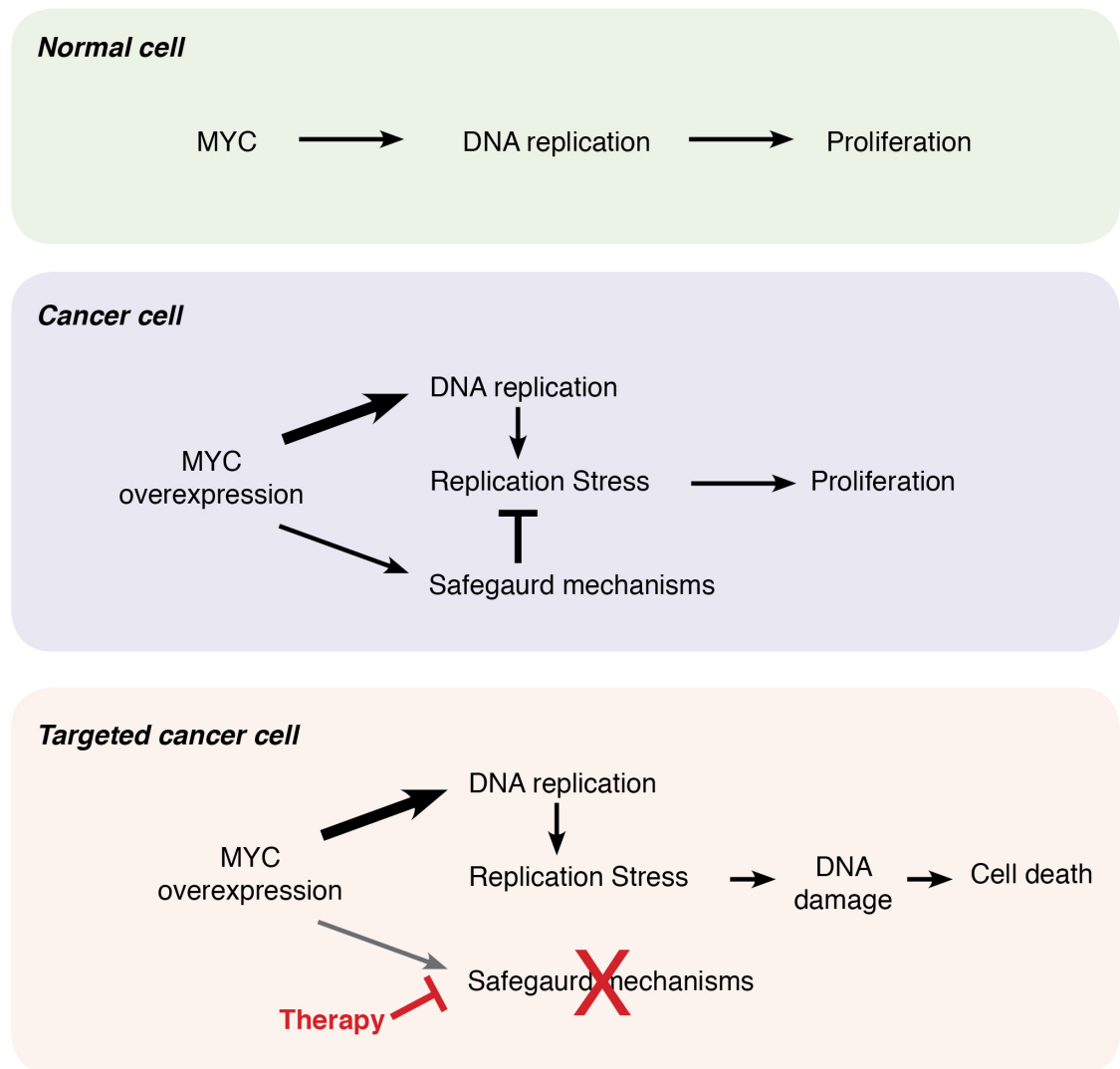


Figure 1.10. Targeting the pathways required to repair replication stress-associated damage is a promising therapeutic strategy. In normal cells, MYC functions to regulate DNA replication. Dysregulated MYC expression leads to replication stress, but the replication response pathways act as a safeguard mechanism, allowing survival of cancer cells. Targeting the proteins required for damage associated with replication stress is one way to specifically target MYC-driven cancer. Figure adapted from Rohban, 2015 [202].

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Chapter II

***Mre11* deletion suppresses tumorigenesis in a lymphoma prone mouse model**

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Abstract

Recurrent chromosomal translocations are the underlying cause of many lymphoid malignancies and have been identified in a growing number of solid tumors. Translocations in lymphomas and leukemias frequently involve the immunoglobulin heavy chain locus (IgH)—the site of V(D)J recombination and class-switch recombination (CSR). These lymphocyte-specific DNA rearrangements involve the generation of site-specific programmed DNA double strand breaks (DSBs) and subsequent joining by the classical non-homologous end joining DNA repair pathway. Mutations in the classical non-homologous end joining (C-NHEJ) genes can lead to misrepair of DSBs, thereby resulting in translocations that often involve oncogenes. Juxtaposition of an oncogene and a strong immunoglobulin enhancer or promoter results in oncogene overexpression. Indeed, mice deficient in C-NHEJ proteins, including the Artemis DNA nuclease, in a p53 deficient background have a strong predisposition to pro-B lymphomas associated with *IgH:Mye* translocations and C-MYC (and N-MYC, in the case of Artemis) overexpression.

The Mre11/Rad50/Nbs1 (MRN) DNA repair complex has roles in all known DNA DSB repair pathways, and evidence suggests that MRN participates in V(D)J recombination. MRE11, a DNA nuclease, is also involved in the repair of DNA replication associated damage.

In this study, we examine the roles of the MRE11 nuclease and the MRN complex on the tumorigenesis phenotypes observed in *Artemis/p53* mutant mice.

We demonstrate that B cell specific deletion of MRE11 or inactivation of MRE11 nuclease activity completely suppressed pro-B lymphomagenesis. We provide evidence that MRE11 is not required for the generation of specific *IgH:Myc* translocations. We find that C-MYC or N-MYC overexpression elicits cellular DNA damage responses likely due to oncogene-induced replication stress. We also observe that upon overexpression of C-MYC or N-MYC, cells expressing MRE11 are given a selective advantage over those that have *Mre11* deleted. Together, these findings in this chapter indicate a critical role for MRE11, and specifically MRE11 nuclease activity in tumorigenesis.

Introduction

Recurrent chromosomal translocations are a cause of many hematological malignancies including lymphoma and leukemia, as well as some solid tumors. Aside from the recurrent translocations associated with prostate cancer, the molecular mechanisms underlying formation of recurrent translocations in solid tumors are not well understood [9, 203, 204]. There is, however, significant insight into the mechanisms involved in generating DNA double strand breaks (DSBs) that are involved in recurrent translocations in many hematological malignancies. Many translocations in lymphomas and leukemias involve the immunoglobulin heavy chain locus (IgH)—the site of V(D)J recombination. V(D)J recombination is a programmed DNA rearrangement that occurs during lymphocyte development to assemble the genes that encode the variable regions

of antigen receptors from component V, D, and J exons. It is initiated by the RAG1/2 endonuclease, which generates site-specific DNA double strand breaks (DSBs) that are subsequently processed and joined by the classical non-homologous end joining (c-NHEJ) pathway. Defects during end processing and joining can lead to misrepair of RAG1/2 induced DSBs, thereby resulting in chromosomal anomalies, including oncogenic translocations. A subset of human lymphoid malignancies are associated with recurrent translocations involving the V(D)J loci and cellular oncogenes.

Our lab has developed mouse models of C-NHEJ deficiencies to study the consequences of defective V(D)J recombination on tumor predisposition. Studies of mice harboring gene targeted mutations in *Artemis*, the V(D)J recombination DNA nuclease, and *p53* revealed a strong predisposition to early onset pro-B lymphomas. The tumors are characterized by translocations and co-amplification involving the rearranging immunoglobulin heavy chain locus and either the *C-myc* or *N-myc* loci, leading to increased oncogene expression. The translocation breakpoints are characterized by short regions of homology (microhomology) of 2-8 nucleotides, which are also observed at breakpoints in recurrent translocations associated with human lymphoid malignancies and other human cancers [143, 205-208]. Therefore, DNA DSB repair pathways, such as alternative non-homologous end joining (alt-NHEJ), that utilize microhomologies between the two DNA strands to be joined have been proposed to catalyze translocation formation.

Aside from roles in homologous recombination (HR), previous studies demonstrated that the Mre11/Rad50/Nbs1 (MRN) DNA repair complex facilitates both classical and alternative non-homologous end joining [76, 77, 80, 143]. Upon generation of a DSB, the MRN complex is responsible for activating the DDR serine/threonine ATM kinase. ATM activation phosphorylates a number of proteins, resulting in the activation of cell cycle checkpoints, DNA repair pathways, and apoptotic pathways. In mouse B lymphocytes, loss of MRE11 results in both C-NHEJ and alt-NHEJ deficiencies during immunoglobulin class switch recombination (CSR), and is independent of the ATM activating functions of MRE11 [76]. Thus, the DNA tethering and nuclease functions of MRE11 may be important for joining via NHEJ. Evidence also suggests that MRN participates in V(D)J recombination. Human patients and mice with hypomorphic mutations in *Mre11* or *Nbs1* present with mild immunodeficiency, revealing a potential role for the MRN complex in the repair of DSBs generated during V(D)J recombination [30, 31, 119-122]. Furthermore, a *Mre11* hypomorphic mutation leads to increased trans-rearrangements in thymocytes, implicating that mutations in MRN can lead to aberrant V(D)J recombination [121]. Other studies have shown an increase of unrepaired coding ends associated with hypomorphic *Mre11* or *Nbs1* mutations in developing lymphocytes [123]. Based on these observations, we hypothesized that the MRN complex facilitates end joining during defective V(D)J recombination and may be involved in generating translocations.

The pro-B tumors in Artemis and p53 double-deficient mice are associated with either C-MYC or N-MYC oncogene overexpression. Oncogene activation can result in replication stress, leading to an induction of the DNA damage response (DDR), which acts as an anti-cancer barrier [187, 209]. Studies have shown MRN involvement in repairing DNA damage associated with replication stress. MRN is involved in activation of ataxia-telangiectasia and Rad3-related protein (ATR) upon recruitment to sites of replication stress [210-212]. MRN has been implicated in restarting replication forks, and is important for resolution of replication intermediates [213-215]. Upon DNA damage, MRN is responsible for activating ATM, a key step in activating cell-cycle checkpoints [216]. Based on these experiments, we hypothesized that the MRN complex is required for cell survival in cells overexpressing oncogenes.

Thus, we predicted that inactivation of MRN, in particular the MRE11 DNA nuclease, may suppress tumorigenesis via two non-mutually exclusive mechanisms: (1) MRE11 facilitates the formation of oncogenic translocations, and (2) MRE11 is required for cellular survival of cells overexpressing either the C-MYC or N-MYC oncogene. We examined the impact of Mre11 mutation on the tumorigenesis phenotypes observed in *Artemis/p53* double mutant mice. Interestingly, B cell specific deletion of *MRE11* or inactivation of MRE11 nuclease activity suppressed pro-B lymphomagenesis.

Results

Establishing a potential B cell lymphoma system using conditional MRE11 deficiency

The existence of programmed DNA rearrangements in B lymphocytes makes this lineage ideal to study roles of errant DSB repair in cancer initiation and progression. Such mistakes in repair are directly responsible for oncogenic translocations involving the immunoglobulin heavy chain (IgH) locus and oncogenes such as *C-MYC* (*MYC*), *BCL2* and *BCL6* [217]. Similarly in mice, B lineage tumors harbor IgH:Myb translocations. Therefore, we generated mice that lack MRE11 entirely, or express an *Mre11* nuclease-deficient allele, specifically in the B lymphocyte lineage to determine the impact on the initiation or progression of lymphomagenesis [32, 76]. The single amino acid change in the active site of MRE11, a conserved histidine to asparagine, results in loss of both endo- and exonuclease activities. In contrast to deletion of *Mre11*, the H129N mutation does not destabilize the MRN complex, it maintains ATM activation and signaling, but, similar to *Mre11* deletion, it is deficient in DNA DSB repair [32]. These studies highlight a role for MRE11 nuclease activity in DNA DSB repair.

Homozygous mutations that result in complete loss of MRE11 (*Mre11*^{-/-}) or defective nuclease activity (*Mre11*^{H129N/-}) are embryonic lethal, so we utilized mice harboring a conditional *Mre11* allele (*Mre11*^c) that can be inactivated through Cre/Lox-P-mediated deletion of a region that includes exon 5, resulting in no full-length or truncated MRE11 protein (Figure 2.1A) [32]. Through breeding,

we generated $Mre11^{+/cond}$, $Mre11^{-/cond}$, and $Mre11^{H129N/cond}$ mice harboring one copy of the CD19-Cre transgene. The B-lineage specific CD19 promoter initiates Cre recombinase expression in the pro-B cell lineage at the same time RAG1/2 is expressed and V(D)J recombination begins, and continues to be expressed throughout B-cell development [218, 219]. Thus, deletion of endogenous *Mre11* in our system is specific to the B-cells. This breeding scheme results in mice with B lymphocytes of the following genotypes: $Mre11^{+/-}$, $Mre11^{-/-}$, and $Mre11^{H129N/-}$. We previously demonstrated complete loss of MRE11 or inactivation of MRE11 nuclease activity in the B cell lineage supports B-cell survival and normal spleen development [32]. In this study we generated mice with the *Mre11* genotypes described above that are also deficient for the p53 tumor suppressor by breeding in a germline knockout null allele ($p53^{-/-}$) [220].

Lifespans and tumorigenesis in mice with combinations of p53 and B cell-specific *Mre11* deficiencies

p53 wild type and deficient mice with B cell genotypes $Mre11^{+/-}$ (control), $Mre11^{-/-}$, and $Mre11^{H129N/-}$ were aged to evaluate survival and tumor development. p53 wild type mice with B cell specific MRE11 deficiencies were observed to have a normal life span with a median survival of 27.5 months (compared to 29 months for the controls) (Figure 2.1B). Mice with $p53^{-/-}Mre11^{-/-}$ and $p53^{-/-}Mre11^{H129N/-}$ B cell genotypes became moribund at about 16-18 weeks of age (Figure 2.1C). However, the control $p53^{-/-}Mre11^{+/-}$ mice also became moribund

around 16-18 weeks of age (Figure 2.1C). Upon necropsy, the majority of p53^{-/-} mice presented with an enlarged thymus that was negative for the B cell marker B220. Further flow cytometric analyses of these tumors indicated the masses were thymic lymphomas of T cell origin, a common outcome of murine p53 deficiency (Figure 2.1D) [129, 220-222]. A smaller percentage of mice succumbed to non-lymphoid tumors (Figure 2.1D). Therefore, it appears that B lymphocyte specific deficiency of MRE11, or of MRE11 nuclease activity, does not impact the survival of mice, and does not appear to predispose to B cell malignancy.

Mre11 deletion suppresses pro-B lymphomagenesis in Art/p53 deficient mice

In order to examine the role of MRE11 in tumorigenesis, we examined the impact of MRE11 deletion in a mouse model of spontaneous pro-B lymphomagenesis [143]. We crossed mice harboring the targeted *Mre11* mutant alleles and the CD19-Cre transgene with pro-B lymphoma-prone *Artemis/p53* double null mice to obtain mice with B cell genotype *Mre11*^{-/-}*Artemis*^{-/-}*p53*^{-/-}. Compared to *Artemis*^{-/-}*p53*^{-/-} mice (average survival 14 weeks), *Mre11*^{-/-}*Artemis*^{-/-}*p53*^{-/-} mice (average survival 17 weeks) survived significantly longer (Log-rank Mantel-Cox test, p=0.003) (Figure 2.2A). Once moribund, mice were dissected, and lymphoid tissues were dissociated into single cells for flow cytometric analysis. Using markers expressed on the surface of B cells, we determined 12

out of 19 tumors (63%) that arose in *Artemis*^{-/-}*p53*^{-/-} mice were pro-B lymphomas, staining B220⁺CD43⁺IgM⁻, consistent with previous studies [143].

Upon analysis of tumors that arose in *Mre11*^{-/-}*Art*^{-/-}*p53*^{-/-} mice, we remarkably did not observe any pro-B lymphomas (Figure 2.2B). Instead, we observed 11 out of 11 mice (100%) succumbed to thymic lymphoma of a T cell origin, staining negative for B cell markers (B220⁻CD43⁻IgM⁻) and instead, staining positive for T cell markers (CD4⁺CD8⁺TCRβ⁻). Since Cre expression is driven by the CD19 promoter and is specific to the B cell lineage, T cells do not express Cre, and therefore, maintain MRE11 expression. These results suggest that B cell specific deletion of MRE11 suppresses pro-B lymphoma.

Inactivation of Mre11 nuclease activities suppresses pro-B lymphomagenesis in *Art/p53* deficient mice

We next wanted to determine the impact of loss of MRE11 nuclease activities by H129N mutation in *Artemis/p53* double null mice. We crossed mice harboring these *Mre11* mutant alleles and the CD19-Cre transgene with *Artemis/p53* double null mice to obtain mice with B cell genotype *Mre11*^{H129N/-}*Artemis*^{-/-}*p53*^{-/-}. Compared to *Artemis*^{-/-}*p53*^{-/-} mice (average survival 14 weeks), *Mre11*^{H129N/-}*Artemis*^{-/-}*p53*^{-/-} mice (average survival 19 weeks) survived significantly longer (Log-rank Mantel-Cox test, p=0.0006) (Figure 2.2A).

Flow cytometric analysis of tumors arising in *Mre11*^{H129N/-}*Artemis*^{-/-}*p53*^{-/-} mice revealed 9 out of 11 tumors stained negative for B cell markers (B220⁻

CD43⁻IgM⁻), and thus, were not pro-B lymphomas (Figure 2.2C). Upon further analysis, these tumors were classified as thymic lymphomas, staining positive for T cell markers (CD4⁺CD8⁺TCRβ⁻) (Figure 2.2B-C). One *Mre11*^{H129N⁻}*Artemis*^{-/-}*p53*^{-/-} mouse became moribund from unknown causes, with no observable cause of death, and one tumor was classified as a pre-B cell tumor. Importantly, like the thymic lymphomas arising in this cohort, the *Mre11* conditional allele was retained and MRE11 protein was expressed. Taken together, these results indicate that deleting MRE11 or inactivating MRE11 nuclease activity suppresses pro-B lymphomagenesis in a lymphoma-prone mouse model.

IgH:Myc translocations can occur in the absence of Mre11

Our findings that B cell specific deletion of MRE11 or inactivation of MRE11 nuclease activity suppress pro-B lymphomas in *Artemis/p53* double null mice suggests that MRE11 may be required for tumorigenesis. Programmed DNA DSBs generated in the IgH locus are known substrates for oncogenic translocation formation [7]. DSB repair deficiencies often increase the frequency of translocation formation by mis-repairing breaks through alternative DSB repair mechanisms. Because the MRN complex has been implicated in having roles in all known DSB repair pathways, we hypothesized that translocations cannot be generated in the absence of MRE11.

To this end, we determined if specific IgH:Myc translocations can be generated in *Mre11*^{-/-}*p53*^{-/-} B cells by taking an established nested PCR-based

approach [223]. This assay utilizes primers that can amplify translocations occurring between J_H4 and C_μ of IgH (chromosome 12) and exon 1 of *c-myc* (chromosome 15) (Figure 2.3A). Indeed, translocations were detected in *Mre11*^{-/-} *p53*^{-/-} and *Mre11*^{H129N/-} *p53*^{-/-} B cells as well as *Mre11*^{+/-} *p53*^{-/-} B cells, and were confirmed by sequencing (Figure 2.3B). These data indicate that *Mre11* is dispensable for generating IgH:Myc translocations.

Chromosomal anomalies in MRE11 and MRE11/ARTEMIS deficient MEFs

To examine the levels and types of chromosomal anomalies that arise in cells lacking MRE11 and ARTEMIS, I took advantage of *Mre11*^{c/-} and *Mre11*^{c/-} *Artemis*^{-/-} mouse embryonic fibroblasts (MEFs). Cells were treated with control adenovirus (adeno-empty) or adenovirus expressing the Cre recombinase to delete the *Mre11* conditional allele to generate *Mre11*^{-/-} or *Mre11*^{-/-} *Artemis*^{-/-} cells (Figure 2.3C). Upon *Mre11* deletion, an increase in chromosomal anomalies compared to control cells was observed, which is consistent with previous studies (Figures 2.3D and 2.3F) [32]. *Artemis* deletion alone did not have a significant impact on spontaneous chromosomal anomalies; however, *Mre11/Artemis* deficiency led to a moderate increase in spontaneous chromosomal anomalies (Figures 2.3D and 2.3F). Importantly, in the absence of both MRE11 and ARTEMIS, dicentrics, ring chromosomes, and Robertsonian translocations were observed at elevated levels, indicating that spontaneous

translocations can be generated in cells lacking MRE11, even in the absence of ARTEMIS (Figure 2.3G).

The tumors observed in *Artemis*^{-/-}*p53*^{-/-} tumors are associated with either C-MYC or N-MYC oncogene overexpression, and oncogene overexpression can lead to replication stress. Thus, we were interested in examining chromosomal anomalies in cells lacking MRE11 and ARTEMIS in cells treated with the DNA polymerase inhibitor, aphidicolin (APH), which leads to replication stress. APH treatment in *Mre11*^{-/-} MEFs resulted in increased chromosomal anomalies, as previously observed [32]. Following adeno-Cre deletion of *Mre11* and a 24 hour treatment with APH, *Artemis* deletion has an additive effect on chromosomal anomalies in the presence of *Mre11* deletion (Figures 2.3E-F). These results suggest that Artemis and Mre11 may have independent functions in repairing APH-induced damage. Furthermore, MRE11 and ARTEMIS deficient MEFs treated with APH can still generate dicentrics, ring chromosomes, and Robertsonian translocations (Figure 2.3G).

C-MYC leads to increased MRE11 and NBS1 protein levels

Protein expression levels of the MRN complex and most DNA repair proteins are generally believed to remain constant even in the presence of damage, and post-translational modifications are what lead to rapid cellular responses observed. However, previous studies demonstrate C-MYC directly regulates transcription of MRN complex member Nbs1, and members of the

MRN complex are transcriptionally regulated by MYCN during mouse cerebellar development [224, 225]. Thus, it is not surprising that increased MRE11 expression is observed upon C-MYC and N-MYC overexpression. To examine the levels of NBS1 protein levels upon C-MYC overexpression, MEFs were transduced with either control retrovirus or retrovirus expressing *C-myc*, and then sorted on the GFP positive population of cells. Upon western blot analysis of these cells, 5-fold and 2.5-fold increases of MRE11 and NBS1, respectively, were observed upon C-MYC overexpression (Figure 2.4A and 2.4B). These results demonstrate that levels of the MRN complex are increased upon MYC overexpression.

C-MYC or N-MYC overexpression leads to increased DNA damage

Our observations that translocations can be generated in the absence of MRE11 led us to hypothesize that rather than having a role in generating oncogenic translocations, MRE11 may promote survival of oncogene overexpressing cells resulting from oncogenic translocations. Overexpression of a number of oncogenes has been identified as a source of replication stress due to their influence on the cell cycle as well as the regulation of DNA replication initiation [170, 226, 227]. Inappropriate cell cycle progression can lead to replication stress in the form of stalled replication forks that can collapse, and result in DSBs and induction of the DDR. In fact, the DDR is activated in some preneoplastic lesions and early human tumors, and acts as an anti-cancer barrier

[187, 188]. This response has the purpose of maintaining genome stability, but may also enhance the probability of malignant transformation and promote tumorigenesis by allowing cancerous cells to survive.

Both *c-myc* and *N-myc* are transcription factors that are key regulators of development and cell growth and have overlapping functions. Interestingly, the expression patterns in normal cells and overexpression of each oncogene is typically cancer specific, with *c-myc* most frequently overexpressed in blood-borne and solid tumors, and *N-myc* most frequently overexpressed in solid tumors that originate from the neural crest. While *C-MYC* regulates a large number of genes required for proliferation, DNA repair, and metabolism, it is unclear whether DNA repair processes are altered in cells overexpressing *N-MYC*. Likewise, until recently, little was known about the impact of *N-myc* overexpression on induction of DNA damage. Although both transcription factors regulate similar cellular processes, they have distinct targets, and thus, it is unknown whether overexpression of *N-MYC* will elicit similar phenotypes as observed for *C-MYC*.

In order to determine if the DDR is activated, in response to *C-MYC* or potentially *N-MYC* oncogene-induced replication stress, I generated retroviral constructs containing murine *c-myc* or *N-myc* to overexpress the oncogenes. These retroviral constructs contain the internal ribosome entry site (IRES), which links either *c-myc* or *N-myc* and GFP transcription from a single promoter, and allows for the simultaneous expression of oncogene and GFP separately but

from the same RNA transcript [228]. Therefore, GFP expression can be used as a readout for transduction and transfection efficiency using flow cytometry (Figure 2.5A). First, 293T cells were transfected with these constructs, or a control IRES-GFP construct and then the virus-containing supernatant was used to transduce primary wild type and *Artemis* null MEFs to overexpress either C-MYC or N-MYC.

One key protein in the DNA damage response is the variant histone, H2AX, which is rapidly phosphorylated to γ H2AX. Phosphorylation of H2AX occurs upon DNA DSB formation, as well as in response to replication stress at sites of stalled forks in response to replication stress[229]. Phosphorylated H2AX forms nuclear foci at sites of DNA DSBs and at sites of stalled replication forks, which can be visualized as punctate dots, referred to as foci, using immunofluorescent microscopy. Upon C-MYC overexpression in primary wild type MEFs, increased γ H2AX were observed compared to controls, as expected (Figure 2.5B and 2.5D). N-MYC overexpressing in these cells led to similar levels of γ H2AX foci seen in C-MYC overexpressing cells, indicating that N-MYC overexpression also induces the DDR in these cells (Figures 2.5B and 2.5D). In order to determine if *Artemis* mutation has an impact on activating the DDR, I examined γ H2AX foci in ARTEMIS deficient primary MEFs with overexpressed C-MYC or N-MYC. Unsurprisingly, *Artemis* deficient MEFs have an increased baseline level of damage (Figure 2.5C-D). *Artemis* deletion does not further increase γ H2AX foci upon C-MYC or N-MYC overexpression (Figure 2.5C-D).

This suggests ARTEMIS does not play a major role in repair of oncogene-induced damage.

C-MYC overexpression induces the DNA damage response in primary pre-B lymphocytes

The cellular response to DNA damage can be variable between different cell types. For example, MEFs can tolerate DNA damage induced by irradiation, leading to cellular arrest. In contrast, B cells are very sensitive to DNA damage induced by irradiation and will undergo apoptosis. Thus, it is important to determine if the cellular response to C-MYC overexpression in B-lymphocytes, as MYC-overexpressing tumors in *Artemis/p53* deficient mice originate from the B cell lineage. To address this question, bone marrow (BM) was harvested from wild type mice, and pre-B cells were purified using B220 BD-IMag magnetic particles. These are nanoparticles that have a monoclonal antibody conjugated to their surfaces, allowing for the positive selection of B220 positive cells. Pre-B cells in the purified sample were confirmed by B220⁺CD43⁻ staining by flow cytometric analysis (Figure 2.6A). These pre-B cells were transduced with retrovirus expressing C-MYC. Upon C-MYC overexpression, increased γ H2AX foci were observed in pre-B cells that overexpress C-MYC compared to controls (Figure 2.6B-C). This result suggests C-MYC overexpression in B lymphocytes also leads to increased amounts of DNA damage.

MRE11 expression is selected for in cells overexpressing C-MYC or N-MYC

In order to evaluate the impact of *Mre11* deletion on survival in cells overexpressing C-MYC or N-MYC, I generated both a primary and SV40 transformed MEF line from an embryo with genotype $Mre11^{c/-}Art^{-/-}p53^{-/-}$. $Mre11^{c/\Delta}Art^{-/-}p53^{-/-}$ SV40 MEFs were transduced with retrovirus expressing *c-myc* or *N-myc* and subsequently treated with adenovirus expressing *Cre* recombinase to delete *Mre11*. If MRE11 is required for survival in C-MYC or N-MYC overexpressing cells, a decrease in proliferation would be expected compared to controls. The proliferation rate of C-MYC or N-MYC overexpressing cells with MRE11 deletion was significantly increased compared to controls (Figure 2.7A). However, upon western blot analysis comparing day 3 and day 8 post-*Mre11* deletion, I observed *MRE11* protein levels were no longer diminished from *Cre*-deletion by day 8 (Figure 2.7B). This suggests that MRE11 expression is selected for in cells overexpressing oncogenes C-MYC or N-MYC. Furthermore, these studies were performed in primary $Mre11^{c/-}Art^{-/-}p53^{-/-}$ MEFs and similar results were observed, suggesting the selection is independent of SV40 transformation (Figure 2.7C).

Discussion

In this study, we sought to understand the roles of MRE11 in promoting tumorigenesis. MRE11 deficiency or inactivation of MRE11 nuclease activity in the B lymphocyte lineage did not promote B cell lymphomagenesis, even in the absence of p53. Previously, partial loss-of-function mutations in *MRE11* have been observed in human patients that present with a predisposition to cancer, suggesting preserved functions of the protein are necessary for tumor formation [46]. Deficiency of the entire MRE11 protein does not support MRN complex formation, ATM signaling, or the ability to sense DNA breaks through the DNA tethering and end-bridging [32]. However, the single amino acid H129N mutation retains MRN complex stability, ATM signaling, and DNA tethering and end-bridging. Both mutations are deficient in DNA repair. Thus, these mouse studies indicate that these mutant *Mre11* alleles do not promote tumorigenesis, even though the cells are deficient for DNA repair.

Artemis/p53 deficient mice are strongly predisposed to pro-B lymphomagenesis and harbor oncogenic translocations involving immunoglobulin *IgH* and either *C-myc* or *N-myc* leading to overexpression of the oncogenes. If ATM activation or DNA tethering/end-binding functions of MRE11 were responsible for pro-B lymphomagenesis in *Artemis*^{-/-}*p53*^{-/-} mice, I would expect pro-B lymphomas to arise in mice deficient in MRE11 nuclease activity. However, if MRE11 nuclease activity were responsible for promoting pro-B

lymphomagenesis, I would not expect to observe pro-B tumors in mice deficient in MRE11 nuclease activity.

In this study, we found that B-cell specific deletion of *Mre11* in an *Artemis/p53* deficient background suppressed pro-B lymphomas (Figure 2.2B). Additionally, B-cell specific inactivation of MRE11 nuclease activity by the H129N mutation suppressed pro-B lymphomas (Figure 2.2B). Instead, mice with B cell genotypes *Mre11*^{-/-}*Artemis*^{-/-}*p53*^{-/-} and *Mre11*^{H129N/-}*Artemis*^{-/-}*p53*^{-/-} predominantly succumbed to thymic lymphomas. Based on these observations, and the identified functions of MRE11 in all known pathways of DNA DSB repair, we hypothesized that MRE11 may be involved in facilitating oncogenic translocations, or not mutually exclusively, depended upon for survival in tumor cells overexpressing oncogenes C-MYC or N-MYC.

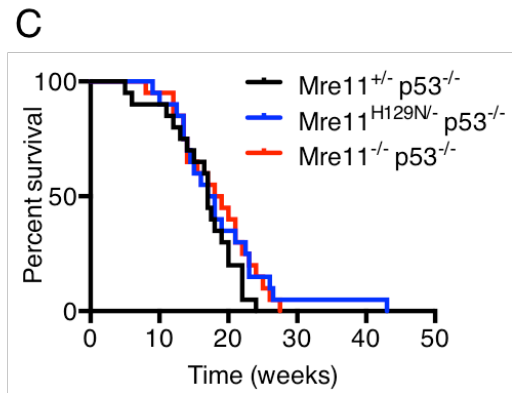
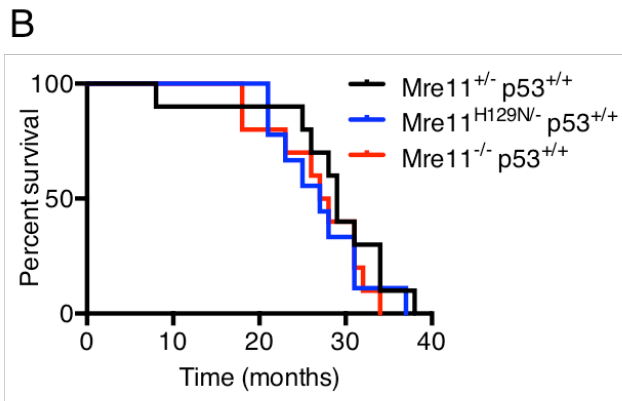
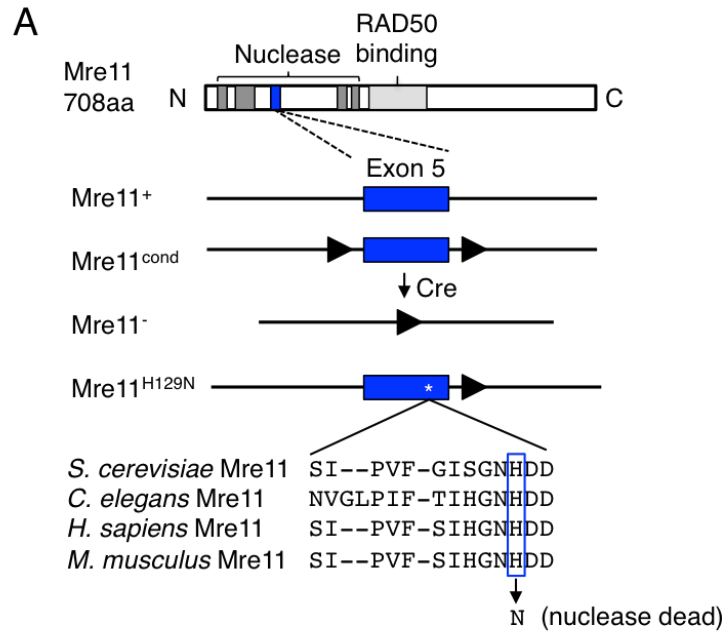
In order to determine if MRE11 can facilitate translocations, we observed whether translocations could be generated in the absence of MRE11 or specifically without MRE11 nuclease activity. We observed that specific *IgH:c-Myc* translocations could still be generated in the absence of MRE11 (Figure 2.3B). Furthermore, upon analysis of spontaneous chromosomal anomalies, we observed ring chromosomes, dicentrics, and Robertsonian translocations in MRE11 deficient MEFs, indicating that translocations can indeed be generated, even in the absence of the DNA repair nuclease ARTEMIS (Figure 2.3D). Upon replication stress induced by APH, *Mre11* and *Artemis* deletion displayed an additive effect on the number of chromosomal anomalies (Figure 2.3F). Previous

studies have shown ATR phosphorylation of Artemis is important for repairing replication fork stalling induced by APH, suggesting that ARTEMIS may be required to process the ends of difficult-to-repair breaks in a way that MRE11 cannot compensate [230].

One hypothesis for why we do not observe pro-B lymphomas in *Mre11* mutant *Artemis/p53* deficient mice is that translocation-mediated oncogene overexpression may lead to replication stress reliant on MRE11 for survival. Previous studies have demonstrated C-MYC overexpression can lead to replication stress by deregulating S-phase entry into the cell cycle, resulting in increased genome instability [170, 226]. Here, we show increased DNA damage by examining γ H2AX foci formation in both MEFs and pre-B cells upon overexpression of C-MYC or N-MYC (Figures 2.5B-C, and 2.6B). Interestingly, increased levels of MRE11 and NBS1 were observed upon C-MYC overexpression, suggesting that the MRN complex is regulated by MYC (Figure 2.4A-B). Future studies should address the mechanism of *Mre11* regulation by C-MYC. Previous studies have shown *Mre11* is transcriptionally regulated by N-MYC, and NBS1 is transcriptionally regulated by C-MYC, so it would not be surprising if *Mre11* is also regulated transcriptionally by C-MYC. This is an interesting question because if MRE11 is induced by C-MYC overexpression to restrict replication stress, this would give rationale for targeting MRE11 in MYC-driven cancer.

Together, these results demonstrate that *Mre11* deletion or inactivation of MRE11 nuclease activity suppresses pro-B lymphomas in *Artemis/p53* deficient mice that normally develop these tumors associated with *c-Myc* or *N-myc* overexpression. It would therefore be interesting to delete *Mre11* or inactivate MRE11 nuclease activity in other spontaneously arising MYC-driven tumor models such as the E mu-myc transgenic mouse, which more closely resembles human lymphomas [231]. In our mouse model, CD19-*Cre* is expressed at the beginning of RAG1/2-initiated V(D)J recombination, so *Mre11* is deleted as V(D)J recombination begins. Thus, it would be interesting to delete *Mre11* at earlier and later time points, before and after translocations are generated.

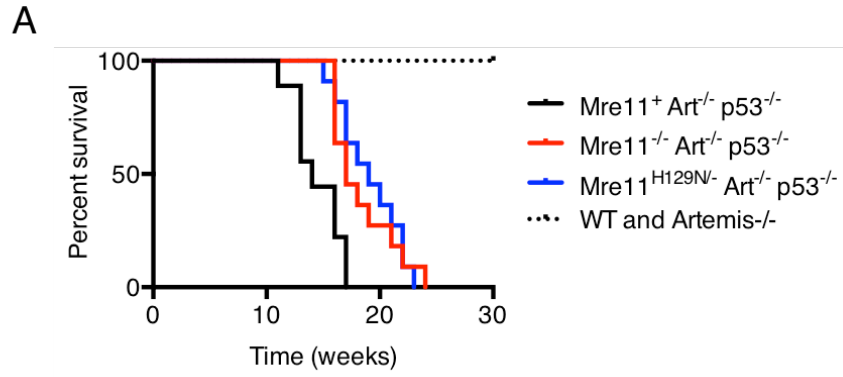
Figures



D

Cause of death	B cell genotype		
	Mre11 ^{+/-} p53 ^{-/-}	Mre11 ^{H129N/-} p53 ^{-/-}	Mre11 ^{-/-} p53 ^{-/-}
B cell lymphoma	0 (0%)	0 (0%)	0 (0%)
Thymic lymphoma	14 (70%)	13 (65%)	15 (75%)
Non-lymph tumor	2 (10%)	3 (15%)	1 (5%)
Non-tumor	4 (20%)	4 (20%)	4 (20%)
<i>n</i>	20	20	20

Figure 2.1. Survival and tumor spectrum in mice with combined p53 and B cell specific Mre11 deficiencies. (A) Mammalian MRE11 domains and location of the invariant histidine required for nuclease activity. The four murine germline *Mre11* alleles used in this study and described previously [32]. Blue rectangle, histidine exon 5 (grey box) essential for nuclease activities; line, introns; triangles, LoxP sites; asterisk, H129N nuclease dead mutation. (B) Kaplan-Meier plots representing the survival percentages of mice with B cell genotypes of *Mre11*^{-/-} (median survival, 27.5 months, p=0.72) and *Mre11*^{H129N/-} (median survival, 29 months, p=0.50) in a p53 wild-type background. p values from Mantel-Cox, log rank test. (C) Plots as in (B) of mice with p53^{-/-}*Mre11*^{-/-} and p53^{-/-}*Mre11*^{H129N/-} B cell genotypes and p53^{-/-}*Mre11*^{+/-} controls with median survival of 17.5wks (p=0.36), 18.5wks (p=0.25). (D) Causes of death for mice of the indicated B cell genotypes. No mice developed pro-B cell lymphoma.



B

B cell genotype

Cause of death	$Mre11^+ Art^{-/-} p53^{-/-}$	$Mre11^{H129N/-} Art^{-/-} p53^{-/-}$	$Mre11^{-/-} Art^{-/-} p53^{-/-}$
ProB lymphoma	12 (63%)	0 (0%)	0 (0%)
TCR β - T cell lymphoma	6 (32%)	9 (82%)	11 (100%)
PreB lymphoma	1 (5%)	1 (9%)	0 (0%)
Non-tumor	0 (0%)	1 (9%)	0 (0%)
<i>n</i>	19	11	11

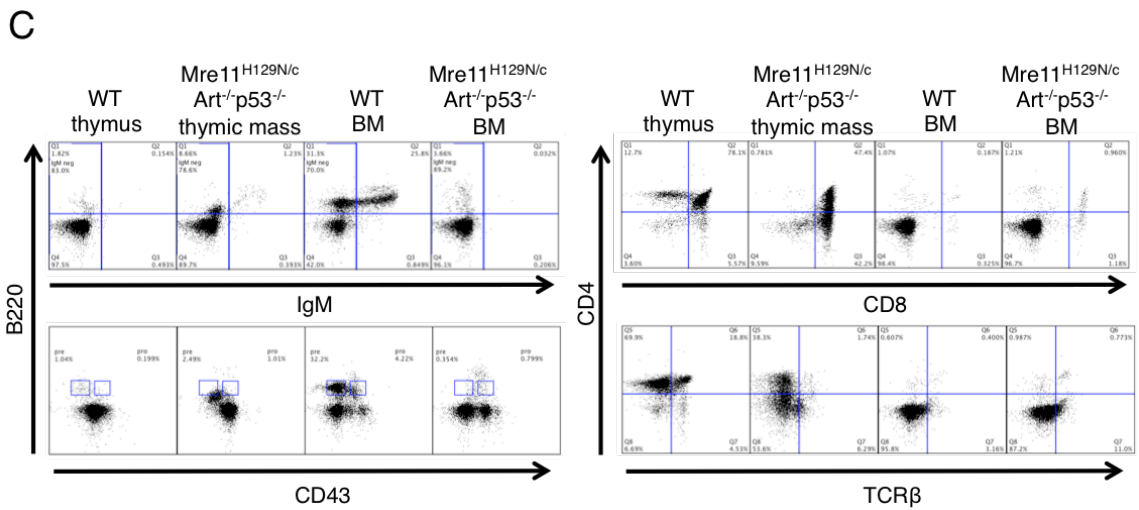
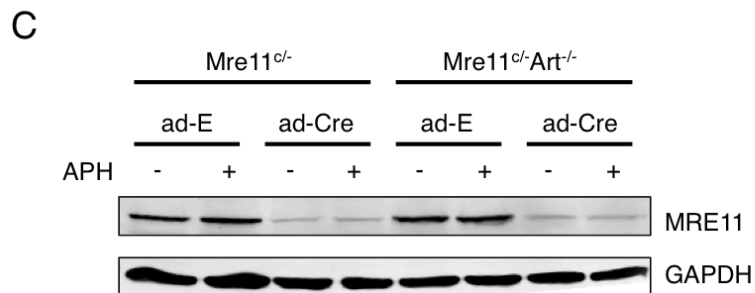
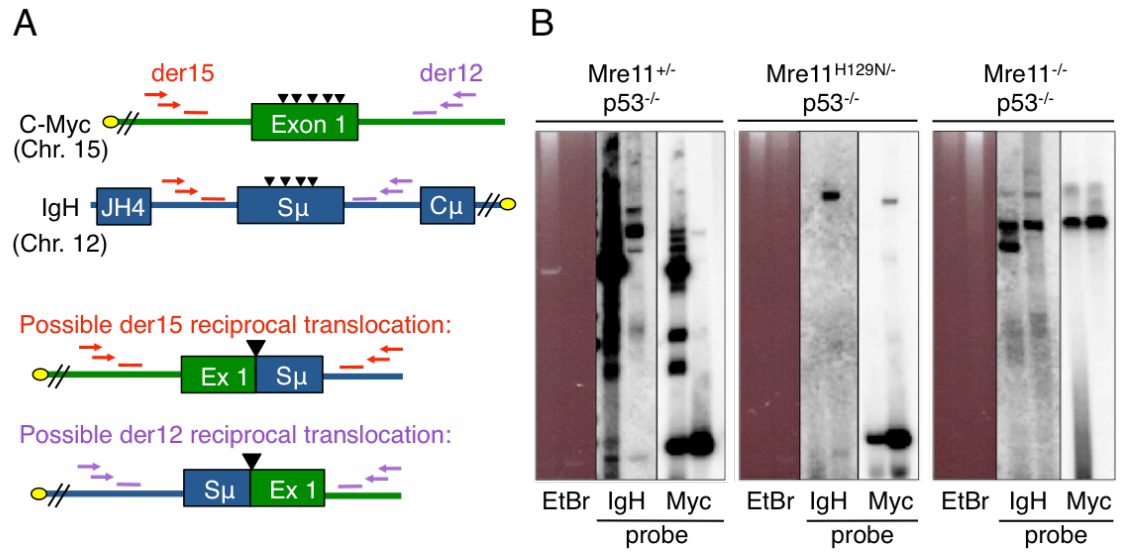


FIGURE 2.2. Survival and tumor spectrum in Artemis/p53 double null mice with Mre11 mutation. (A) Kaplan Meier survival curve showing the percent survival of mice with the indicated B cell genotypes is plotted as a function of time (weeks). All mice harbor the CD19-Cre recombinase transgene. Mice with B cell genotypes $Mre11^{-/-}Art^{-/-}p53^{-/-}$ and $Mre11^{-/H129N}Art^{-/-}p53^{-/-}$ survive longer than $Art^{-/-}p53^{-/-}$ mice ($p < 0.01$, Mantel-Cox, log rank test). (B) *Mre11* mutation suppresses pro B lymphomagenesis in *Art/p53* double null mice. Summary table of lymphoid tumor spectrum and incidence in mice with the indicated B cell genotypes. No pro-B lymphomas were observed in mice harboring B cell specific *Mre11* mutations in $Art^{-/-}p53^{-/-}$ double null mice ($p < 0.0001$, Fisher's exact, two tailed test). (C) Representative flow cytometric analysis of primary tumor with B cell genotype $Mre11^{-/H129N}Art^{-/-}p53^{-/-}$.



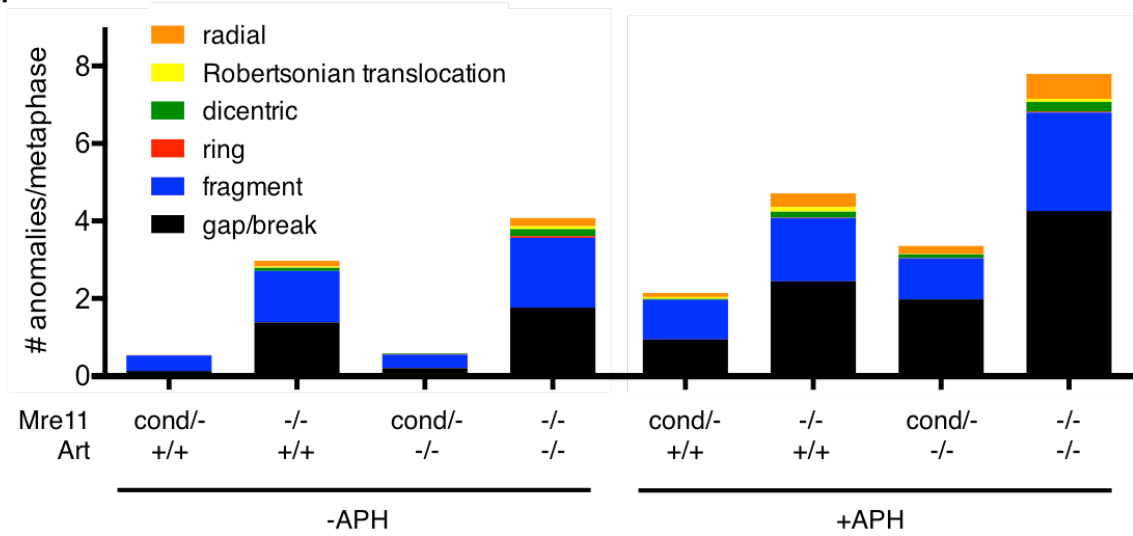
D Spontaneous chromosomal anomalies in Mre11/Artemis deficient MEFs

Genotype	Mre11 ^{cond/-}	Mre11 ^{-/-}	Mre11 ^{cond/-} Art ^{-/-}	Mre11 ^{-/-} Art ^{-/-}
Total Metaphases	140	141	166	151
Gaps/Breaks	18 (0.13)	195 (1.38)	34 (0.20)	67 (1.77)
Fragments	56 (0.40)	187 (1.33)	58 (0.35)	271 (1.79)
Ring	0 (0)	0 (0)	1 (0.01)	7 (0.05)
Dicentric	1 (0.01)	12 (0.09)	3 (0.02)	27 (0.18)
Radial	1 (0.01)	21 (0.15)	1 (0.01)	32 (0.21)
Robertsonian translocations	0 (0)	4 (0.03)	1 (0.01)	12 (0.08)
Total anomalies	76	419	98	616
Anomalies per metaphase	0.54	2.97	0.59	4.08

E

Chromosomal anomalies in Mre11/Artemis deficient MEFs (+0.5μM Aphidicolin)				
Genotype	Mre11 ^{cond/-}	Mre11 ^{-/-}	Mre11 ^{cond/-} Art ^{-/-}	Mre11 ^{-/-} Art ^{-/-}
Total Metaphases	117	124	159	142
Gaps/Breaks	111 (0.95)	304 (2.45)	315 (0.20)	605 (4.26)
Fragments	119 (1.02)	202 (1.68)	168 (0.35)	361 (2.54)
Ring	0 (0)	1 (0.01)	2 (0.01)	3 (0.02)
Dicentric	4 (0.03)	20 (0.16)	15 (0.02)	37 (0.26)
Radial	12 (0.12)	43 (0.35)	34 (0.01)	91 (0.64)
Robertsonian translocations	5 (0.04)	15 (0.12)	1 (0.01)	11 (0.08)
Total anomalies	251	584	535	1110
Anomalies per metaphase	2.15	4.71	3.36	7.82

F



G

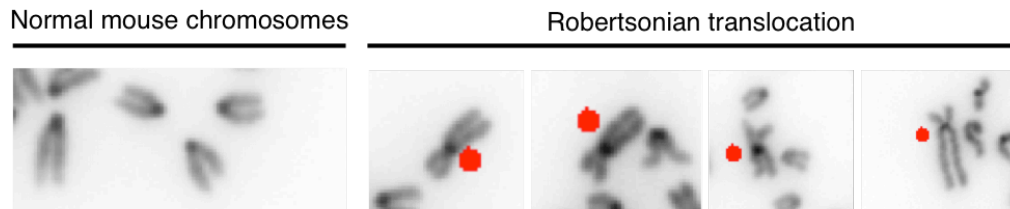


FIGURE 2.3. Translocations can be generated in the absence of MRE11.

(A) Schematic of IgH:Myc translocation PCR strategy performed in B cells. Nested primers (arrows) flanking exon 1 of Myc and S μ of the IgH locus detect translocations between the two loci [223]. Southern blot probes are represented as horizontal lines. Possible translocation products are shown. (B) B cells were stimulated to undergo class switch recombination (CSR) in culture. Nested PCR products were run on ethidium bromide (EtBr) stained agarose gel and then analyzed by Southern blotting using chr. 12 (IgH) and chr. 15 (Myc) probes. Translocations were confirmed by sequencing the PCR products. (C) Mre11^{c/-} and Mre11^{c/-}Art^{-/-} SV40 MEFs were treated with adeno-empty (ad-E) or adeno-Cre (ad-Cre) to delete endogenous MRE11. Cells were treated with DMSO or 0.5 μ M aphidicolin (APH) for 24 hours. Cells were harvested for Western blot analysis. Spontaneous chromosomal anomalies (D) and chromosomal anomalies after APH treatment (E) from cells as treated in (C). Metaphases were stained with DAPI and scored in a blinded manner for chromosomal anomalies. (F) Bar graph shows the number of anomalies per metaphase with type of anomalies indicated in the color scheme. Data are from 3 independent experiments. (G) Representative images of normal mouse chromosomes (left panel) or Robertsonian translocations (right panel) observed in cells lacking MRE11.

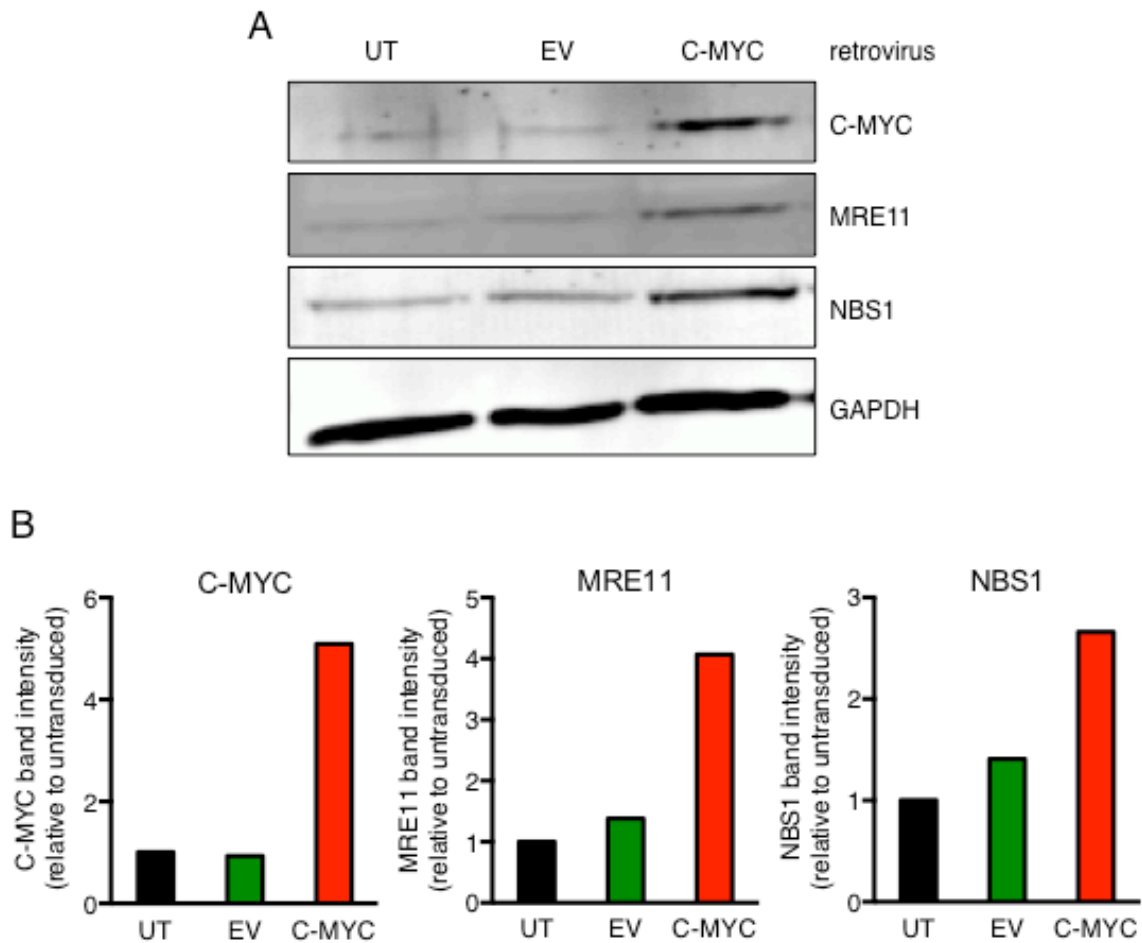


FIGURE 2.4. C-MYC overexpression leads to increased MRE11 and NBS1 protein levels. Primary WT MEFs were untransduced (UT), transduced with control retrovirus (empty vector, EV), or retrovirus expressing *C-myc*. After 72 hours, cells were sorted for GFP positive cells, and harvested to make protein for western blot analysis. (A) Western blot analysis. GAPDH, loading control. (B) Quantitated protein levels for C-MYC, MRE11, and NBS1 relative to untransduced cells.

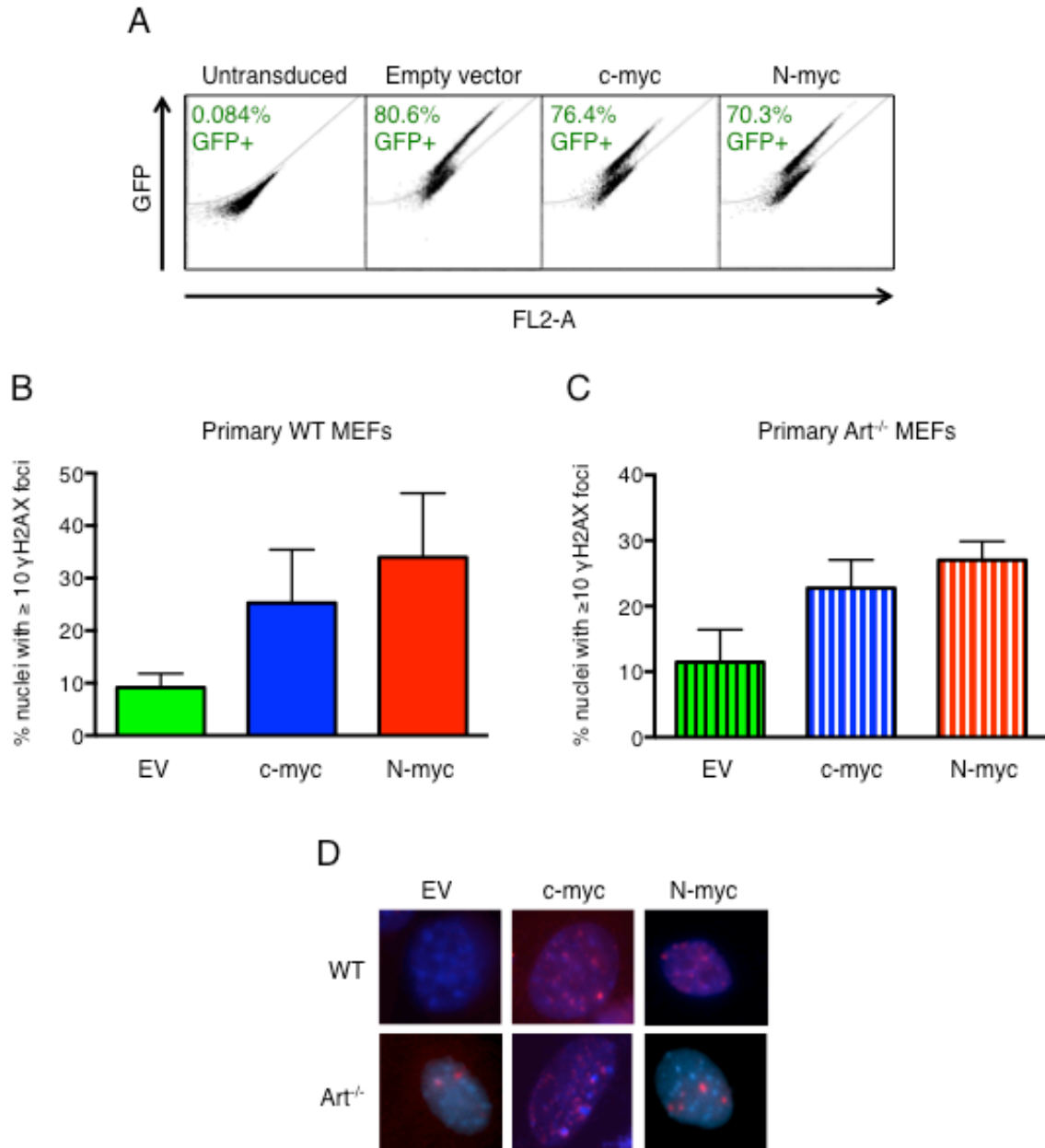


FIGURE 2.5. γ H2AX foci increase upon C-MYC or N-MYC oncogene overexpression in MEFs. Primary WT MEFs or *Art*^{-/-} MEFs were transduced with control empty vector (EV) retrovirus or retrovirus expressing *C-myc*, or *N-myc*. (A) Retroviral transduction efficiency measured by GFP percentage using flow cytometry. γ H2AX foci were detected by immunofluorescence microscopy. Bar graphs show percentage of nuclei with ≥ 10 γ H2AX foci in primary WT MEFs (B) or *Art*^{-/-} MEFs (C). (D) Representative images of γ H2AX foci (red); DAPI (blue).

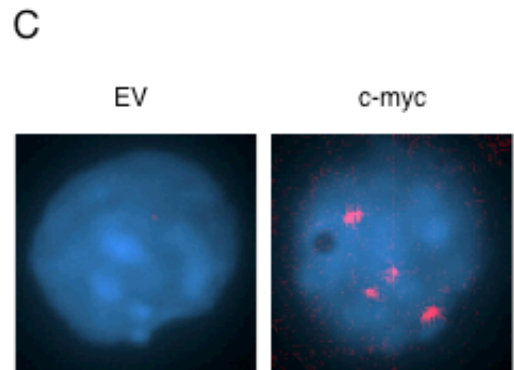
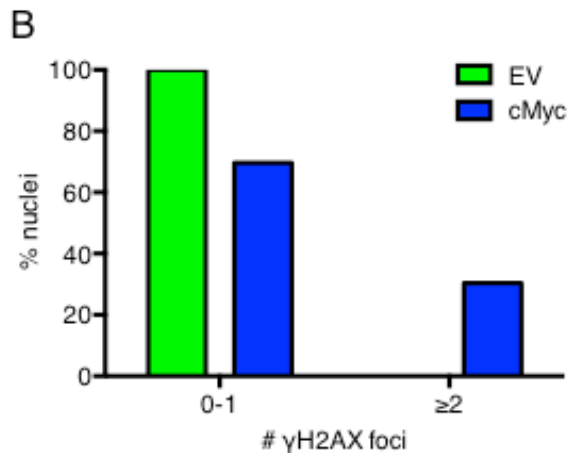
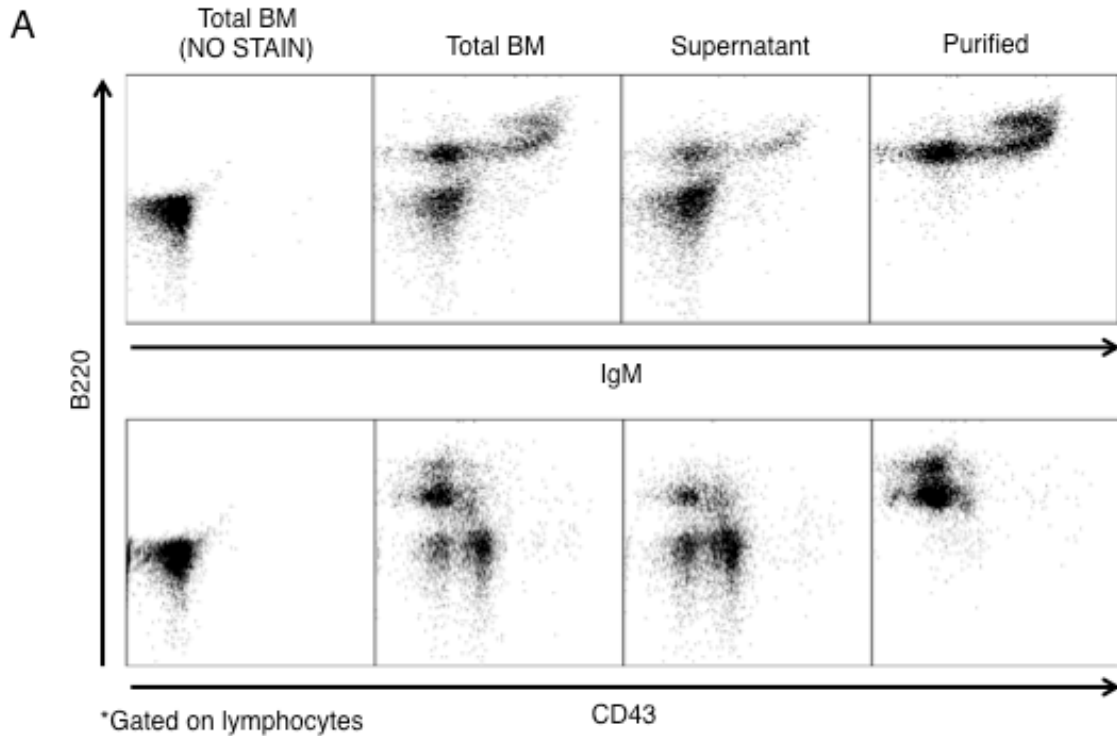


FIGURE 2.6. γ H2AX foci increase upon C-MYC or N-MYC oncogene overexpression in primary pre-B cells. (A) Primary WT pre-B cells were purified from mouse bone marrow (BM), and confirmed by analysis of B cell markers using flow cytometry. Purified pre-B cells stain B220⁺/CD43⁻. (B) Purified primary pre-B cells in (A) were transduced with control retrovirus (empty vector, EV) or retrovirus overexpressing *C-myc*, and sorted for GFPdf positive cells. γ H2AX foci was detected by immunofluorescence microscopy. Bar graphs show percentage of nuclei with indicated number of γ H2AX foci. (C) Representative images of γ H2AX foci (red); DAPI (blue).

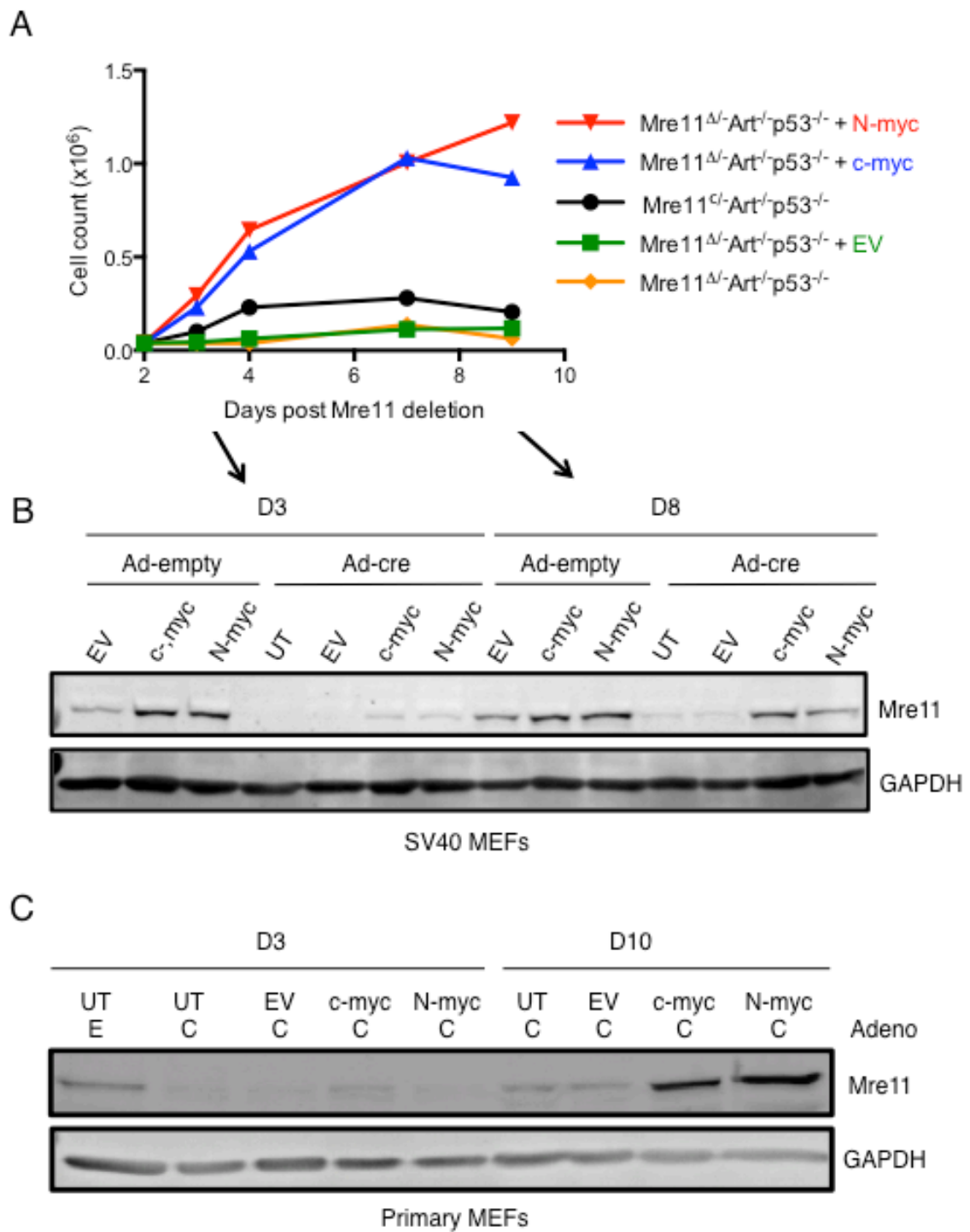


FIGURE 2.7. MRE11 expression is selected for in cells overexpressing oncogenes c-myc or N-myc. *Mre11*^{C/-}*Art*^{-/-}*p53*^{-/-} MEFs were untransduced (UT), transduced with control retrovirus (empty vector, EV), or retrovirus expressing *C-myc* or *N-myc* and then treated with control adenovirus (Ad-empty) or adenovirus expressing *Cre* recombinase to delete endogenous *Mre11*. (A) Proliferation curve of *Mre11*^{C/-}*Art*^{-/-}*p53*^{-/-} SV40 MEFs following MYC-overexpression and *Mre11* deletion. (B) Western blot analysis of cells in (A) on day 3 (D3) and day 8 (D8) post *Mre11* deletion. On day 8, MRE11 protein levels are increased in cells that overexpress C-MYC or N-MYC. (C) Western blot analysis of primary *Mre11*^{C/-}*Art*^{-/-}*p53*^{-/-}MEFs on day 3 (D3) and day 10 (D10) post *Mre11* deletion. On day 10, MRE11 protein levels are increased in cells that overexpress C-MYC or N-MYC.

Materials and Methods

Characterization of Tumors

Mouse lymphoid tumors were stained with antibodies against B-cell markers (B220, CD43, IgM), and T-cell markers (CD4, CD8, TCR β , CD3, CD25, CD44) and analyzed by flow cytometry.

Metaphase spread analysis—Mre11^{cl}-Artemis^{-/-} SV40 MEFs +/- Aphidicolin

MEFs were infected with control Adeno-Empty or Adeno-Cre at an MOI of 500. Cells were grown for 3 days after infection, split, and doubly infected before plating for the experiment. Cells were treated with 0.5 μ M aphidicolin (APH) or DMSO for 24 hours, followed by incubation with colcemid (KaryoMAX) for 8 hours. Cells were harvested and incubated with 0.4% KCl for 15 minutes at 37°C, followed by a series of fixations in ice cold fixative (3:1 methanol:glacial acetic acid). Fixed cells were dropped onto glass slides and stained with DAPI (Invitrogen) according to manufacture's instructions. Images were acquired on an Olympus BX61 microscope using 60X objective, and viewed with SKYview software (Applied Spectral Imaging).

Western blot analysis

Cells were harvested and lysed in lysis buffer (200mM KCL, 0.1% NP40, protease inhibitors, phosphatase inhibitors, 1mM DTT, 25mM Hepes, pH 7.4, glycerol) on ice for 5 minutes and then spun for 30 minutes at high speed at 4°C.

Concentration of supernatant protein was calculated by Bradford assay per manufacturers instructions. The following primary antibodies were used: Mre11 (Cell Signaling 4895S), C-MYC (Santa Cruz), Nbs1 (Novus NB100-143), and GAPDH (Santa Cruz).

Transfection and immunofluorescent analysis of MEFs

293T cells (5.3×10^6 cells) were plated in T-75 flasks 16 hours before transfection in DMEM media supplemented with 10%FBS, 20mM HEPES (Invitrogen 15630-080), non-essential amino acids (Invitrogen 11140-050), 1mM sodium pyruvate (Invitrogen 11360-070), 1% pen/strep (Invitrogen 15140-122), 2mM L-glutamine (Invitrogen 25030-081) and β -mercaptoethanol). Cells were transfected with pCL-Eco and either MSCV-IRES-GFP, MCSV-Nmyc-IRES-GFP, or MSCV-cMyc-IRES-GFP by the calcium chloride method and at 72 hours the virus containing supernatant was collected and filtered through a 0.45mm PVDF filter (Millex-HC Cat#SLHV033RS). MEFs (4.0×10^4 cells) were plated onto coverslips in 12-well dishes then transduced with 1ml viral supernatant (empty vector, c-Myc or Nmyc), 1ml culture media, and 2ml 4mg/ml polybrene (final concentration 4mg/ml) for 72 hours. Cells were fixed with ice cold 3.7% paraformaldehyde, 2% sucrose for 20 m and washed 3x with PBS then incubated with ice cold buffer (20mM HEPES, 50mM NaCl, 3mM $MgCl_2$, 0.5% TX-100, 300mM sucrose) for 4 minutes. After incubation, cells were washed 3x in PBS and blocked with PBSBT (0.5% BSA, 0.05% Tween-20 in PBS) for 1 hour. Cells were incubated 45

minutes in primary γ H2AX antibody diluted in PBSBT and then stained with secondary antibody Alexa Fluor 488 diluted in PBSBT for 45 minutes. Coverslips were mounted on slides using DAPI (Invitrogen), and cells were visualized using Olympus BX61 microscope using 100X objective.

Pre-B purification and immunofluorescence

Bone marrow was isolated from mice, lysed with RBC lysis buffer, and washed with 1X BD IMag buffer (PBS with 0.5% BSA, 2mM EDTA). 5 μ l BD IMag anti-mouse CD45R/B220 (BD 551513) was added for every 1.0×10^7 cells and incubated 30 minutes at 4°C. Next, volume was brought up to 1ml with 1X IMag buffer, and then placed on the BD IMagnet for 30 minutes at room temperature. Supernatant was removed while still on IMagnet. Magnet steps were repeated two more times with 8 minute room temperature incubations. After final wash, the positive purified fraction was resuspended in FACS buffer (5% FBS in PBS) and analyzed by flow cytometry. Purified cells were plated in pre-B cell media (optimem with 15% Hy-clone FBS, pen/strep, L-glutamine, β -ME, and 5ng/ml IL-7) at a concentration of at least 2.0×10^6 cells/ml. For infection, control empty vector and c-myc retrovirus was prepared as described above, and pre-B cells were spininfected with virus for 30 minutes. After 72 hours in culture, cells were cytospun on to slides, fixed with ice cold 3.7% paraformaldehyde, 2% sucrose for 20 minutes, and then incubated with ice cold buffer (20mM HEPES, 50mM NaCl, 3mM MgCl₂, 0.5% TX-100, 300mM sucrose) for 4 minutes. After incubation, cells

were washed 3x in PBS and blocked with PBSBT (0.5% BSA, 0.05% Tween-20 in PBS) for 1 hour. Cells were incubated 45 minutes in primary α - γ H2AX antibody (Millipore 05-636) diluted in PBSBT and then stained with secondary antibody Alexa Fluor 594 for 45 minutes. Slides were mounted using DAPI (Invitrogen), and visualized using Olympus BX61 microscope using 100X objective.

MEF proliferation assay

293T cells were transfected as described above to generate retrovirus. MEFs (2.0×10^5 cells) were plated in 6-well dishes then transduced with 1ml viral supernatant (empty vector, c-Myc or Nmyc), 1ml culture media, and 2ml 4mg/ml polybrene (final concentration 4mg/ml). After 48 hours, MEFs were plated (5.0×10^5 cells) into 10cm dishes, and 24 hours later, MEFs were infected with Adeno-empty or Adeno-Cre at an MOI of 500 to delete endogenous *Mre11*. MEFs were replated (4.0×10^4 cells) in 6-well plates for the experiment 48 hours after Adeno infection. At the indicated timepoint, cells were harvested, and cell count was determined by trypan blue exclusion and a hemocytometer.

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Chapter III

MYC overexpressing cells require DNA repair protein MRE11 for cellular survival

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Abstract

Oncogenes are activated in a significant number of human cancers. A consequence of oncogene overexpression is dysregulated DNA synthesis, which can lead to replication-associated DNA damage and elicit cellular DNA damage responses and repair. Some cancer cells exhibit high levels of replication stress, and can become dependent on the repair proteins that respond to oncogene-induced replication stress for survival. Therefore, there is great interest in identifying these proteins so they may be explored for therapeutic purposes.

Previous studies have shown the Mre11/Rad50/Nbs1 (MRN) DNA repair complex has a role in resolving stalled replication forks and promoting replication fork restart. We previously observed that deletion of *Mre11* or inactivation of MRE11 nuclease activity suppressed pro-B lymphomagenesis in a mouse model with a strong predisposition to pro-B lymphomas associated with *IgH:MyC* translocation and C-MYC or N-MYC overexpression. Therefore, I hypothesized that MRE11, and specifically MRE11 nuclease activity, is required during repair of oncogene-induced DNA damage and inactivation of Mre11 leads to catastrophic genomic instability and cell death.

To address these questions, I utilized small molecule inhibitors of MRE11 endo- or exonuclease activity in C-MYC overexpressing cells. I observed Mre11 exonuclease activity, and not endonuclease activity, is critical for survival in these cells. I demonstrate that pharmacological inhibition of Mre11 exonuclease activity results in increased DNA damage, decreased cellular survival, and increased

apoptosis specifically in cells that overexpress C-MYC. These findings provide promising mechanistic preclinical evidence, in support of MRE11 exonuclease activity as a novel therapeutic target for use in MYC-driven and replication-stress associated cancers.

Introduction

Genetic instability is a hallmark of cancer. Genomic changes that lead to activated growth signaling contribute to sporadic cancer initiation [5]. For example, translocations involving the immunoglobulin heavy chain locus (IgH) and the potent MYC oncogene are responsible for Burkitt's lymphoma. The accumulation of additional genetic alterations such as point mutations, translocations, amplifications, deletions, and aneuploidy allow for changes that lead to the acquisition of the other cancer hallmarks. The selection of these mutations leads to further cancer progression, evolution, and the development of drug resistance after therapeutic intervention. Given the potentially harmful effects of genomic instability, cells have evolved an efficient DNA damage repair system to maintain genome stability and protect against cancer. This repair system, collectively termed the DNA damage response (DDR), involves recognition of DNA damage, transduction of signals, and activation of cellular responses to induce cell cycle arrest, promote repair, or induce cell death if the damage is too severe. In this regard, the DDR acts as a double-edged sword, as it both prevents genetic instability that might lead to cancer, and aids in resisting

current chemotherapies and radiotherapies. In the last decade, researchers have discovered the DDR is activated early on in cancer development, specifically in some premalignant human tumors [187, 188]. The DDR can be activated in response to oncogene-induced replication stress [187, 232]. Replication stress is defined as DNA synthesis slowing or replication fork stalling.

Overexpression of a number of oncogenes has been identified as a source of replication stress due to their influence on the cell cycle as well as the regulation of DNA replication initiation. For example, the oncogene Cyclin E enhances replication initiation and causes collisions between the replication and transcription machinery [176]. Mutant oncogenic Ras (H-RasV12) produces high levels of reactive oxygen species (ROS), which can generate abasic sites and oxidized bases, requiring additional steps in order for proper replication to continue [233]. Overexpression of the oncogene Myc leads to deregulated Cyclin-dependent kinase (CDK) activity, a key protein in controlling cell cycle progression. MYC overexpression also leads to a shortened G1, premature entry into S-phase of the cell cycle, and hyper-proliferation [170]. MYC directly interacts with and stabilizes components of the pre-replication complex, which can stimulate origin firing [174]. Increased origin firing can lead to depletion of nucleotide pools, and/or collisions with the transcription machinery [177]. All of these effects of oncogene dysregulation can lead to impaired replication fork progression and fork stalling.

A stalled replication fork can be resolved by restarting the fork. Often, the stalled fork cannot be restarted, resulting in fork collapse, a double-stranded break (DSB), and activation of the DDR. The DSB is sensed by the ataxia-telangiectasia mutated (ATM) kinase which promotes recruitment of the MRE11, RAD50, and NBS1 (MRN) complex to the collapsed fork. During S and G2 phases of the cell cycle, sister chromatids are present for use by the error-free homologous recombination (HR) repair pathway. MRE11 endonuclease and exonuclease activities facilitate end resection, which is an essential step in HR. One strand on each side of the DSB is resected 5'—3' to expose ssDNA to initiate repair through HR. The knowledge of the consequences of oncogene overexpression on accumulation of DNA replication associated DNA damage is increasing. Therefore, there is great interest in identifying the proteins required for repair of oncogene-induced DNA damage, as abrogation of these specific proteins might prove to be a promising strategy in the development of targeted cancer therapies.

Previous studies have shown the MRN complex has a role in resolving stalled replication forks. MRE11 tethers DNA ends through short-range interactions, and has endonuclease and 3'—5' exonuclease activity. MRE11 nucleolytic processing initiates HR. MRN has been implicated in promoting fork restart after the replication fork has been blocked with mitomycin C or camptothecin [213]. The MRN complex is important for preventing replication intermediates from accumulating and is required for the resolution of these

intermediates [214]. Additionally, the recruitment of MRN to stalled forks following replication stress contributes to activation of the kinase ataxia-telangiectasia and Rad3-related protein (ATR) [210-212]. Upon DNA damage, MRN is important in the activation of ATM, a key step in activating cell-cycle checkpoints [216]. While MRN has clear roles in resolving replication-associated DNA damage, it is not well understood if these functions overlap with repairing oncogene-induced replication stress.

In Chapter II, we discovered B cell specific deletion of *Mre11* or inactivation of MRE11 nuclease activity suppressed tumorigenesis in a mouse model predisposed to early onset pro-B lymphomas associated with amplification and overexpression of oncogenes C-MYC or N-MYC. Here, I provide a molecular mechanism for tumor suppression observed upon *Mre11* mutation. The MRN complex localizes to sites of oncogene-induced damage. Inhibition of MRE11 exonuclease activity selectively kills cells overexpressing C-MYC, thereby suggesting that the tumor suppression phenotypes results from elimination of cells harboring IgH:MYC translocations. Taken together, our data reveal a promising therapeutic strategy for treating MYC-driven tumors.

Results

MYC overexpression leads to DNA damage and MRN localization to sites of damage

Previous work has provided evidence for C-MYC-induced replication stress, and recently, the pathways involved in repair of oncogene-induced DNA damage [174, 175, 185, 234]. As C-MYC regulates a large number of genes required for proliferation, DNA repair, and metabolism, it is unclear how C-MYC overexpression affects DNA repair processes [235-238]. Based on our previous observations that *Mre11* mutation prevents pro-B lymphomas associated with C-MYC or N-MYC overexpression, we hypothesize that MRE11 is required during repair of oncogene-induced DNA damage. In order to determine the impact of oncogene overexpression on the DNA damage response, I first examined the localization of key repair proteins to sites of C-MYC-induced DNA damage.

To do so, human *C-MYC* cDNA fused to the hormone-binding domain of the estrogen receptor (ER) was introduced into human osteosarcoma U2OS cells using the retroviral expression vector pBabehc-MycER to generate U2OS-cMyc-ER cells [239]. To avoid ER activation by endogenous estrogen or estrogen in serum, the estrogen receptor used contains a mutation, G525R, in the ligand-binding domain that does not allow it to bind the ligand 17 β -oestradiol. Additionally, the mutant ER no longer possesses ligand-dependent transactivation activity, but can still be activated by the synthetic steroid 4-hydroxytamoxifen (4-OHT) [240]. In the absence of 4-OHT, ER is associated with

an inhibitory complex. Increased C-MYC-ER protein expression in U2OS-cMyc-ER cells can be visualized by western blot analysis in the presence or absence of 4-OHT (Figure 3.1A). Upon 4-OHT binding, ER is released from the complex, and can translocate to the nucleus where it is active. An increase of nuclear C-MYC in U2OS-cMyc-ER cells treated with 4-OHT can be visualized by immunofluorescent staining, and a 2 to 3-fold increase of nuclear C-MYC fluorescence intensity is observed in these cells (Figures 3.1B-C). Consistent with the previously reported functions of C-MYC that cause accelerated entry into S-phase of the cell cycle, an increased accumulation of cells in S-phase is observed upon c-myc overexpression (Figure 3.1D) [170, 237].

One key protein in the DNA damage response is the variant histone, H2AX, which is rapidly phosphorylated to γ H2AX. Phosphorylation of H2AX occurs upon DNA DSB formation, as well as in response to replication stress at sites of stalled forks in response to replication arrest mediated by hydroxyurea (HU), a deoxynucleotide synthesis-blocking agent [229]. Phosphorylated H2AX forms nuclear foci at sites of DNA DSBs and at sites of stalled replication forks, which can be visualized as punctate dots, referred to as foci, using immunofluorescent microscopy. Following 4-OHT treatment in U2OS-cMyc-ER cells, a two-fold increase in the percentage of nuclei with γ H2AX foci compared to controls was observed, consistent with observations in primary mouse embryonic fibroblasts (MEFs) (Figures 3.2A, 3.2G).

H2AX phosphorylation can lead to recruitment of p53-binding protein (53BP1), which, in turn, is important for recruiting other repair factors. 53BP1 responds to replication stress, and is involved in DNA DSB repair pathway choice [241, 242]. Upon C-MYC overexpression, a significant increase in 53BP1 nuclear foci formation was observed (Figures 3.2B, 3.2G).

Frequently, when a replication fork has stalled, the replicative helicase becomes uncoupled from the polymerase, and continues to unwind the DNA, resulting in long stretches of single-stranded DNA (ssDNA) [173, 186, 243]. Replication Protein A (RPA) binds to ssDNA during the early stages of repair and acts as a major signal to downstream events such as checkpoint activation and fork restart and/or repair [244-246]. RPA bound to ssDNA recruits the ataxia-telangiectasia and Rad3 related (ATR) kinase through an ATR-interacting protein (ATRIP)-RPA interaction. The RAD9-HUS1-RAD1 complex (9-1-1 complex) is loaded onto RPA-bound ssDNA through a RAD9-RPA interaction and recruits topoisomerase-binding protein 1 (TOPBP1), which leads to ATR activation [247-251]. ATR phosphorylates and activates checkpoint kinase 1 (CHK1), which phosphorylates effector proteins, leading to initiation of the intra-S phase checkpoint, activation of the Fanconi anemia (FA) pathway, recruitment of DNA repair proteins, stabilization of stalled forks and, if necessary, repair of collapsed forks [61, 252-256].

Upon 4-OHT treatment in U2OS-cMyc-ER cells, an increase in the percentage of nuclei with RPA foci compared to controls was observed (Figure

3.2C, 3.2G). Mutations in members of the FA family as well as BRCA1 are associated with an increased cancer incidence. FA pathway member Fanconi anemia complementation group D2 (FANCD2) and homologous recombination protein breast cancer 1 (BRCA1) are important proteins for resolving replication stress. During the later stages of repair, both proteins localize to sites of stalled replication forks formed upon replication stress. We sought to determine if FANCD2 and/or BRCA1 localize to sites of oncogene-induced DNA damage. Indeed, we observed a two-fold increase in both FANCD2 foci and BRCA1 foci upon C-MYC overexpression (Figures 3.2D-E, 3.2G).

Although the MRN complex has also been implicated in promoting fork restart after the replication fork has stalled, and has recently been shown to be important for preventing replication intermediates from accumulating, we wanted to determine if the MRN complex is involved in the DNA damage response to oncogene-induced DNA damage [214]. We examined the localization of the MRN complex by visualizing NBS1 foci upon C-MYC overexpression. Following 4-OHT treatment, we observed an increase in the percentage of nuclei with NBS1 foci in U2OS-cMyc-ER cells compared to controls (Figure 3.2F-G). These findings indicate that proteins involved in the restart and/or repair of stalled replication forks also localize to C-MYC-induced damage, and importantly, the MRN complex is recruited to sites of damage.

MRE11 nuclease activity is important for survival of cells that overexpress C-MYC

The observation that the MRN complex localizes to MYC-induced damage led me to hypothesize that MRE11 may have a role in repairing damage associated with oncogene-induced replication stress. I was especially interested in utilizing a small molecule inhibitor of MRE11 due to the increasing attention DNA repair proteins have received with regard to making improvements to current chemotherapies for the treatment of cancer [257-260].

Mirin is a small molecule inhibitor of MRE11, identified in a 2008 forward genetic screen of 10,000 compounds [59]. Mirin specifically inhibits MRE11 exonuclease activity, and does not cause dissociation of the MRN complex, still allowing for ATM activation, consistent with reports of the H129N nuclease dead *Mre11* mutation [32, 49, 59]. Mirin inhibits phosphate rotation necessary for dsDNA exonuclease activity, and it is worth noting that off-target effects of this drug have not been fully explored [49]. In order to analyze the effect of mirin on homologous recombination levels in MEFs, *Mre11*^{o/-} MEFs containing a single chromosomally integrated copy of the DR-GFP reporter (direct repeat GFP) was utilized. This system contains two tandem mutant genes encoding green fluorescent protein (GFP) in a direct repeat. The 18-basepair I-SceI recognition sequence is inserted within one copy of full length GFP causing it to be inactive [261, 262]. The second mutant GFP copy is truncated at the 3' end and homologous sequences are positioned 3.7 kilobases apart. A single site-specific

DSB can be introduced by expressing I-SceI in cells by infecting cells with adeno-I-SceI. When a sister chromatid is present, during S or G2 phases of the cell cycle, homologous recombination can occur whereby the truncated GFP gene acts as a donor of sequence information for the I-SceI-induced broken GFP gene, restoring functional GFP expression (Figure 3.3A). The recombination event can easily be detected by flow cytometry, scoring the percentage of GFP positive cells as the level of homologous recombination. Consistent with previous studies, decreased levels of homologous recombination with increasing concentrations of mirin in *Mre11^{cl-}* DR-GFP MEFs was observed (Figure 3.3B) [49, 59].

To specifically address the role of MRE11 nuclease activity on cellular survival of cells overexpressing oncogenes, I generated retroviral constructs containing murine *C-myc* or *N-myc* to overexpress the oncogenes. These retroviral constructs contain the internal ribosome entry site (IRES), which links either *C-myc* or *N-Myc* and GFP transcription from a single promoter, and allows for the simultaneous expression of oncogene and GFP separately but from the same RNA transcript [228]. Therefore, GFP can be used as a readout for transduction and transfection efficiency using flow cytometry. Control (IRES-GFP), C-MYC, and N-MYC expressing retroviruses were generated and used to transduce *Mre11^{cl-}* SV40 MEFs to overexpress either C-MYC or N-MYC. After treatment with DMSO or MRE11 inhibitor, mirin, cellular survival was analyzed. A significant decrease in relative cellular survival in cells overexpressing

oncogenes c-myc or N-myc was observed upon mirin treatment in Mre11^{cl-} SV40 MEFs (Figure 3.3C).

In Chapter II, I observed Mre11 deletion suppressed lymphomagenesis in mice with Artemis/p53 deficiency. Therefore I wanted to determine if *Artemis* deletion had an additive effect in combination with MRE11 inhibition on cell survival in cells overexpressing oncogenes C-MYC or N-MYC. Cells with and without ARTEMIS showed similar levels of sensitivity to mirin treatment in C-MYC or N-MYC overexpressing cells (Figure 3.3C). These findings indicate *Artemis* deficiency does not further sensitize cells to Mre11 inhibition in cells that overexpress C-MYC or N-MYC.

Furthermore, I examined the dose response of mirin in human U2OS-cMyc-ER cells and pBABE empty vector control cells that I generated, using concentrations of the drug that have an impact on homologous recombination (Figure 3.4A). Upon 4-OHT treatment, U2OS-cMycER cells treated with mirin exhibit significantly decreased cell viability compared to U2OS-pBABE empty vector controls in a dose-dependent manner (Figure 3.4B). Even at the highest dose of mirin treatment, at concentrations that lead to significant cell death in C-MYC overexpressing cells, there was minimal impact on cellular survival in control cells.

Next, to specifically address the role of MRE11 exo- or endonuclease activity on cellular survival, I utilized newly developed small molecule MRE11 inhibitors that have specificity for MRE11 endonuclease activity [49]. Based on the structure of mirin bound to *Thermotoga maritime* MRE11, mirin derivatives

that block the hypothesized ssDNA-binding groove were developed to specifically inhibit MRE11 endonuclease activity. The endonuclease inhibitors, PFM01 and PFM03, are named for the researchers who made the drugs. Similar to the exonuclease inhibitor mirin, these endonuclease inhibitors do not cause defects in the recruitment of MRN to sites of DNA damage, and do not disrupt ATM activation [49]. As previously reported, homologous recombination levels decrease with MRE11 endonuclease inhibitor treatment (Figure 3.4C) [49]. At concentrations that have an effect on homologous recombination levels, MRE11 endonuclease inhibitors (PFM03 or PFM01) do not have an effect on C-MYC overexpressing U2OS cells compared to controls (Figures 3.4D-E). The results of these studies suggest that MRE11 exonuclease activity, but not endonuclease activity, is required for cellular survival in cells overexpressing C-MYC.

Inhibiting Mre11 nuclease activity in c-myc overexpressing cells leads to cell death through apoptosis

In order to understand the cause of reduced cellular viability induced by mirin in C-MYC overexpressing cells, the extent of mirin-induced apoptosis was examined by FITC-Annexin V/Propidium Iodide (PI) double staining, followed by flow cytometry analysis. One of the earliest characteristics of apoptosis is loss of plasma membrane symmetry, and the normally internally localized membrane phospholipid phosphatidylserine residues become externalized. Annexin V binds with high affinity to PS, and allows for detection of apoptotic cells through flow

cytometry [263-265]. During later stages of apoptosis, the membrane loses integrity. Cells with permeable membranes will stain with the intercalating dye, propidium iodide (PI), whereas cells with intact membranes exclude PI [265-267]. Therefore, viable cells are Annexin V/PI negative, early apoptotic cells stain Annexin V positive and PI negative, and late apoptotic cells are Annexin V/PI positive. It is important to note that cells dying via the necrotic pathway will also stain positive for both Annexin V and PI [268, 269].

In U2OS cells, mirin induced apoptosis in a dose-dependent manner and was statistically significant compared to controls (Figure 3.5). At either 0 hours (Figure 3.5B) or 48 hours (Figure 3.5C) post mirin treatment in C-MYC-overexpressing cells, there was at least a 2-fold increase in the percent of Annexin V positive cells compared to controls. Compared to 0 hours post mirin treatment, there was a further increase of myc-overexpressing cells undergoing apoptosis 48 hours post mirin treatment. These findings suggest that while survival of control cells is relatively unaffected by mirin treatment, cells overexpressing MYC continue to apoptose even 48 hours after mirin is removed. These results reveal that decreased cell viability observed upon mirin treatment in cells overexpressing c-myc occurs through mechanisms associated with apoptosis.

Chromosomal anomalies in MYC-overexpressing MEFs treated with mirin

I hypothesized that MRE11 inhibition in myc-overexpressing cells leads to catastrophic genome instability and cell death by apoptosis. To test this hypothesis, I generated Mre11^{cl-} MYC-ER MEFs, overexpressed C-MYC by treating with 4-OHT, and treated with mirin for 24 hours. In order to examine chromosomal anomalies, cells were treated with colcemid, a drug that prevents spindle formation and leads to arrest in mitosis. This allows for separation of chromosomes in mitotic cells, and visualization of chromosomal anomalies by examining metaphase spreads from these cells. While increasing amounts of chromosomal anomalies per metaphase were detected with increasing doses of mirin, and increased anomalies were consistently observed upon 4-OHT treatment, increased chromosomal anomalies in C-MYC-overexpressing cells that were treated with mirin were not detected (Figures 3.6A-C). Together with the previous results demonstrating that apoptosis occurs as early as 24 hours in mirin at all doses used in these metaphase spread experiments (25 μ M, 50 μ M, and 75 μ M), it is possible that I am not able to detect an increase in chromosomal anomalies in C-MYC-overexpressing cells treated with mirin because these cells have already undergone apoptosis.

Compared to controls, mirin treatment in C-MYC overexpressing cells leads to increased DNA damage

Based on my previous findings that mirin treatment in C-MYC-overexpressing cells leads to no detectable increase in chromosomal anomalies compared to controls, I hypothesized that damage induced by mirin in C-MYC-overexpressing cells led to apoptosis before cells reach metaphase. Immediately after mirin treatment, 50% of myc-overexpressing cells are undergoing apoptosis, and by 48 hours post-mirin treatment, 70% of cells are undergoing apoptosis (Figures 3.5B-C). Therefore, to examine an earlier measure of chromosomal damage, 53BP1 foci were examined immediately after mirin treatment. Strikingly, compared to controls, a significant increase in 53BP1 foci in C-MYC-overexpressing cells treated with mirin was observed (Figure 3.7A-B). These results support my hypothesis that Mre11 inhibition in cells overexpressing C-MYC leads to increased DNA damage, and cell death.

Inhibiting MRE11 nuclease activity in c-myc overexpressing cells leads to decreased cell proliferation.

Next, I wanted to determine if C-MYC-overexpressing cells that have been treated with mirin continue to proliferate. Therefore, cells were treated with mirin following C-MYC overexpression in U2OS-cMycER cells. Immediately after removing mirin from the media, bromodeoxyuridine (BrdU), a synthetic analog of the nucleoside thymidine, was added for the indicated times. Replicating cells will

incorporate BrdU into the DNA during S phase. After fixing, in order for labeled cells to be detected by anti-BrdU antibodies, the DNA must be denatured, and can then be analyzed by flow cytometry analysis. Upon mirin treatment, I observed a very low percentage of BrdU positive cells in myc-overexpressing cells compared to control cells treated with mirin (Figure 3.8B). These results indicate that mirin treatment slows or halts DNA synthesis in cells that overexpress C-MYC.

Discussion

In this chapter, I examined the impact of inactivating MRE11 nuclease activity in cells overexpressing the C-MYC oncogene. Previously, we observed *Mre11* deletion or inactivation of Mre11 nuclease activity in a lymphoma prone mouse model suppresses tumorigenesis. We hypothesized that oncogene overexpression driven by translocations between IgH and either *C-myc* or *N-myc* led to replication stress, and the DNA repair protein MRE11 is important for the repair of this oncogene-induced damage. To test this hypothesis, in this study, I examine the role of MRE11 and MRE11 nuclease activity in cells overexpressing C-MYC. To this end, I analyzed the DNA damage response to oncogene overexpression, and visualized foci formation of DNA repair proteins previously known to localize to sites of replication stress (γ H2AX, 53BP1, RPA, FANCD2, and BRCA1). Strikingly, I observed localization of the MRN complex to sites of c-myc-induced damage, suggesting that the MRN complex is important for repairing damage induced by C-MYC overexpression. MRE11 exonuclease activity is required for resolving replication intermediates formed upon replication stress to initiate fork restart. If a replication fork collapses, MRE11 exonuclease activity is required for resection to initiate repair by HR.

Furthermore, MYC-overexpressing mouse and human cells were significantly more sensitive to MRE11 inhibition compared to non-overexpressing cells. Interestingly, I observed C-MYC-overexpressing cells had increased sensitivity MRE11 exonuclease inhibitors only, and not MRE11 endonuclease inhibitors.

These findings indicate that myc-overexpressing cells rely on MRE11 exonuclease activity for survival. Therefore, it is possible that MRE11 endonuclease activity is dispensable for repair of oncogene-induced DNA damage. After MRE11 exonuclease inhibition, myc-overexpressing cells exhibit increased DNA damage, halt proliferation, and begin to undergo apoptosis, while control cells are largely unaffected.

My findings provide a molecular basis for tumor suppression upon *Mre11* deletion in our MYC-driven tumor mice. It is possible that inactivation of MRE11, and specifically MRE11 exonuclease activity, strips myc-overexpressing cells of the ability to maintain enough genome stability required for cellular survival. Instead, these cells accumulate large amounts of DNA damage, and are targeted for cellular death through apoptosis.

Many cancers are associated with MYC overexpression, which is associated with replication stress. Replication stress-associated damage relies on DNA damage proteins for repair and survival. Small amounts of damage are beneficial to cancer cells, as this allows for the accumulation of mutations that may lead to chemotherapeutic resistance. On the other hand, too much damage leads to cell death. In this regard, our results have high clinical and therapeutic relevance. If patient tumors that overexpress C-MYC or N-MYC are reliant on MRE11 activity for survival, using an MRE11 inhibitor could be an effective therapeutic strategy for treating these MYC-driven tumors. Specifically inhibiting MRE11 exonuclease activity has implications for further investigating MRE11

inhibitors for cancer therapeutics. Genetic inactivation or pharmacological inhibition of MRE11 nuclease activity does not impair ATM signaling functions and checkpoint responses [32, 49]. Therefore, normal cells would still have an intact checkpoint, while cancer cells would be more sensitive to exonuclease inhibition.

Future studies will need to focus on developing more potent MRE11 inhibitors, as the concentrations of inhibitors used in this study do not meet standards for use in the clinic. Additionally, future studies will need to assess the effectiveness and potential toxicities of MRE11 targeted therapies *in vivo*. To begin to address this, studies could be done using MRE11 inhibitors in our *Artemis/p53* deficient mouse model of lymphoma or in the E mu-myc transgenic mouse [231]. As with almost all cancer drugs entering the clinic, combination therapies should be considered and tested, although use as a monotherapy may be possible in MYC-driven cancer and cancers with increased replication stress.

Figures

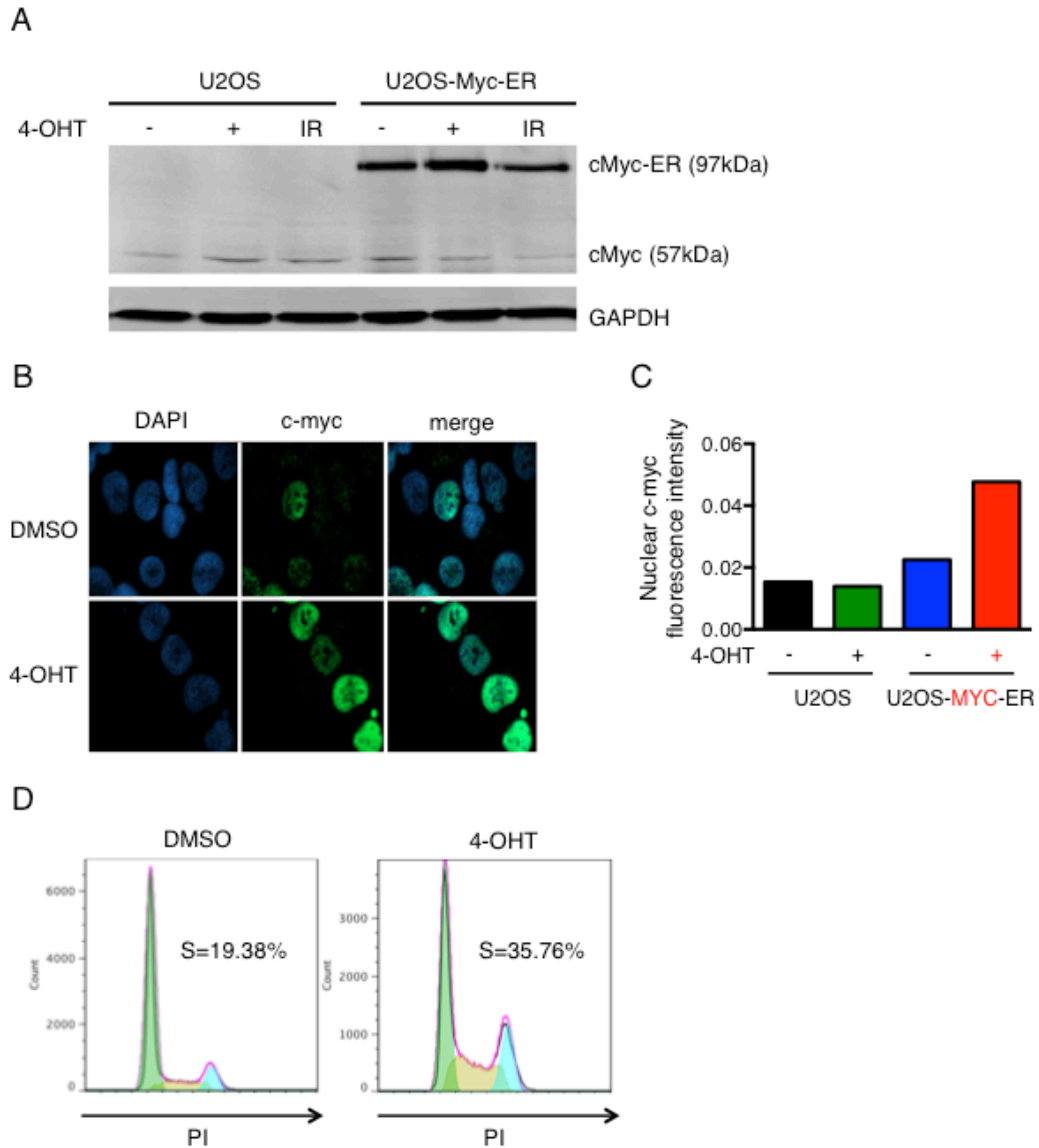
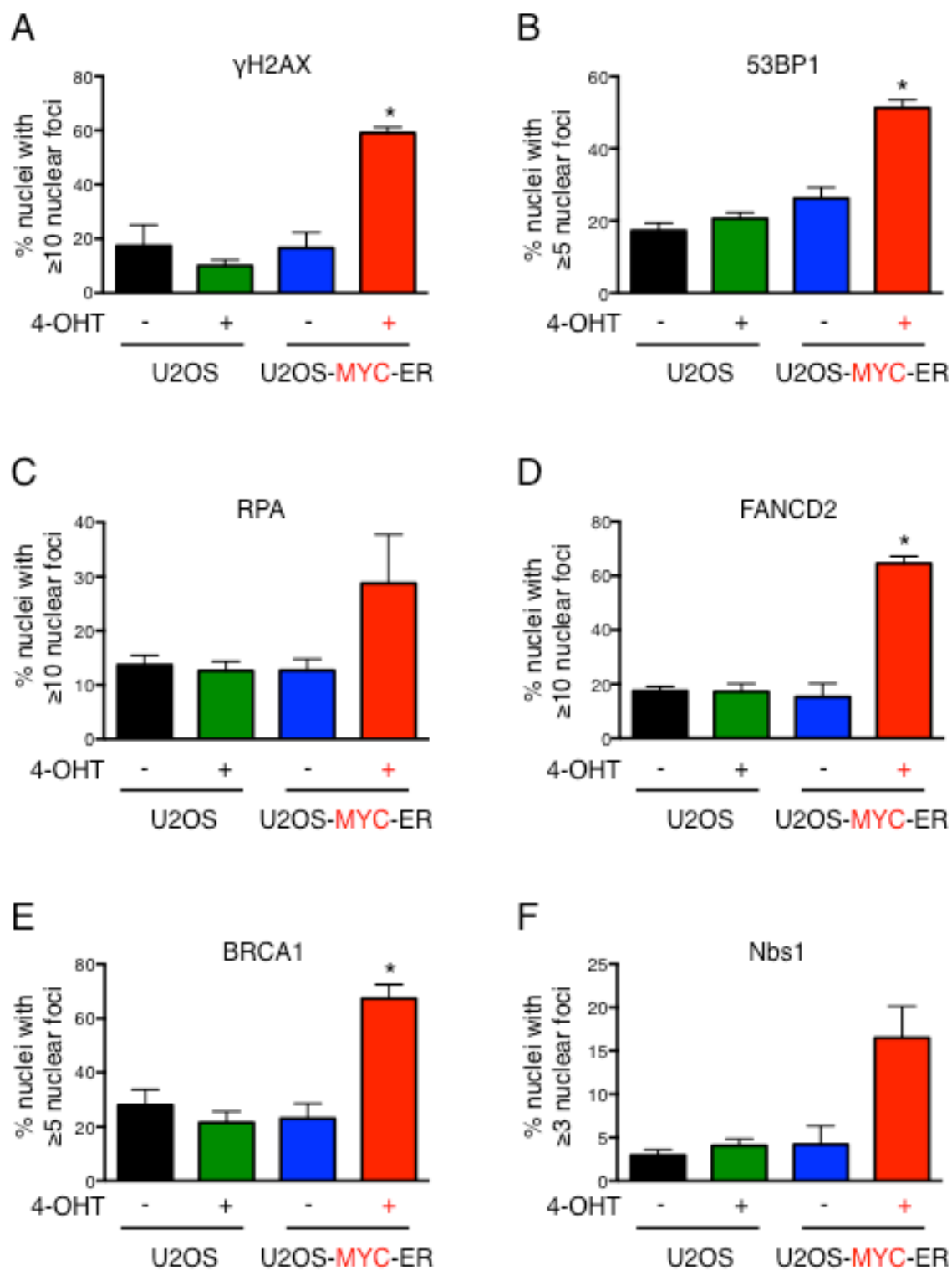


FIGURE 3.1. Inducible MYC overexpression assay system. U2OS and U2OS-MYC-ER cells were treated with DMSO or 200nm 4-OHT for 72 hours to induce MYC overexpression in the nucleus. (A) Immunofluorescent images of nuclear c-myc. (B) Fluorescence intensity of nuclear c-myc was quantitated using Applied Spectral Imaging software. (C) Western blot analysis of MYC-ER. Cell lysates from untreated and 4-OHT treated cells were analyzed by western blotting using an α -MYC antibody. The MYC-ER fusion protein (97kDa) is overexpressed compared to low levels of the endogenous MYC protein (57kDa). GAPDH, loading control. (D) Flow cytometry analysis of U2OS-MYC-ER cells. DNA content was analyzed by fixing cells and staining with propidium iodide (PI). Percent indicates cells in S-phase of the cell cycle.



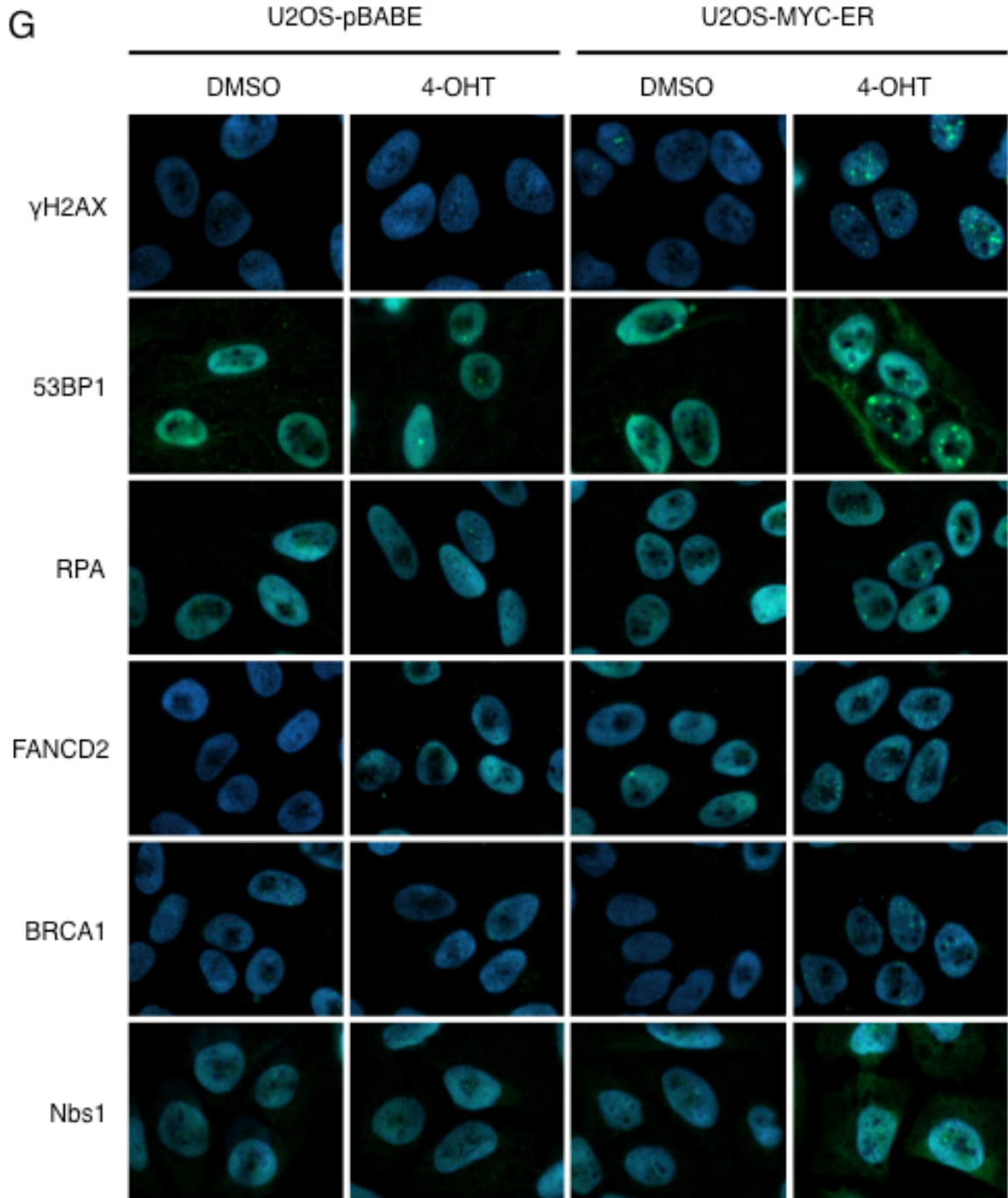


FIGURE 3.2. DNA repair foci increase upon c-myc overexpression and the MRN complex is localized to sites of damage. U2OS-pBABE and U2OS-cMyc-ER cells were treated with DMSO or 200nm 4-OHT to induce c-myc overexpression in the nucleus. Localization of DNA repair proteins at sites of DNA damage was detected by immunofluorescence microscopy. Percentage of nuclei with γ H2AX (A), 53BP1 (B), RPA (C), FANCD2 (D), BRCA1 (E) and Nbs1 (F) foci is plotted. Mean + SEM of 3 or more independent experiments. *, $p < 0.05$. G. Representative images of nuclear foci; DAPI (blue), indicated protein (green).

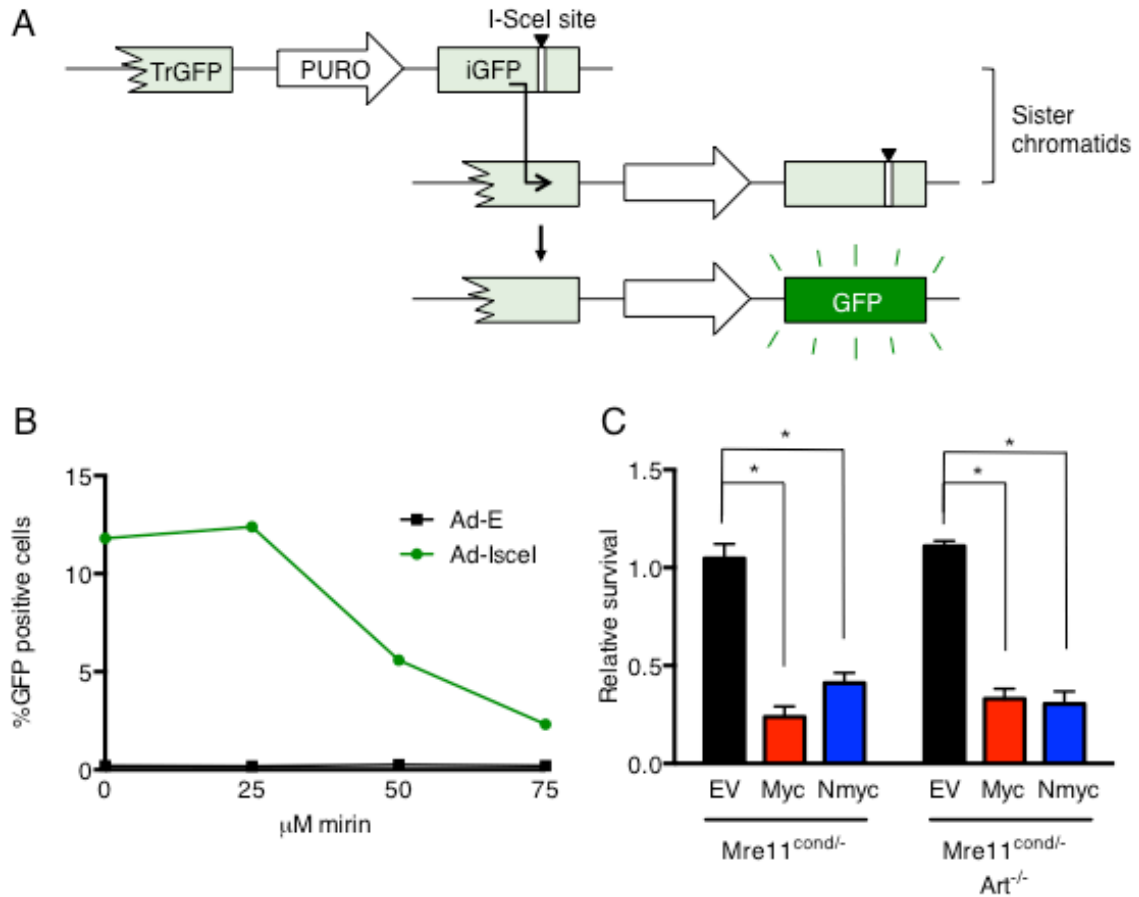


FIGURE 3.3. Inhibition of Mre11 nuclease activity by the small molecule inhibitor, mirin, reduces survival of cells overexpressing oncogenes. (A) Schematic of DR-GFP assay depicting two tandem mutant genes (light green) encoding green fluorescent protein (GFP) in a direct repeat. TrGFP, truncated GFP; iGFP, full length GFP interrupted by I-SceI cut site, black triangle. A single site-specific DSB can be introduced by expressing I-SceI in cells by infecting cells with adeno-I-SceI. When a sister chromatid is present, homologous recombination can occur whereby the truncated GFP gene acts as a donor of sequence information for the I-SceI-induced broken GFP gene, restoring functional GFP expression (dark green). (B) Mirin inhibits homologous recombination in a dose-dependent manner in *Mre11^{cond/-}* DR-GFP MEFs. (C) *Mre11^{cond/-}* and *Mre11^{cond/-} Artemis^{-/-}* MEFs were transduced with retroviruses expressing Myc or N-myc, then treated with the Mre11 inhibitor, mirin, or vehicle for 24hr. Live cells were counted and survival (mirin/DMSO) relative to untransduced cells is plotted. Mean \pm SEM of 3 or more independent experiments. *, $p \leq 0.05$.

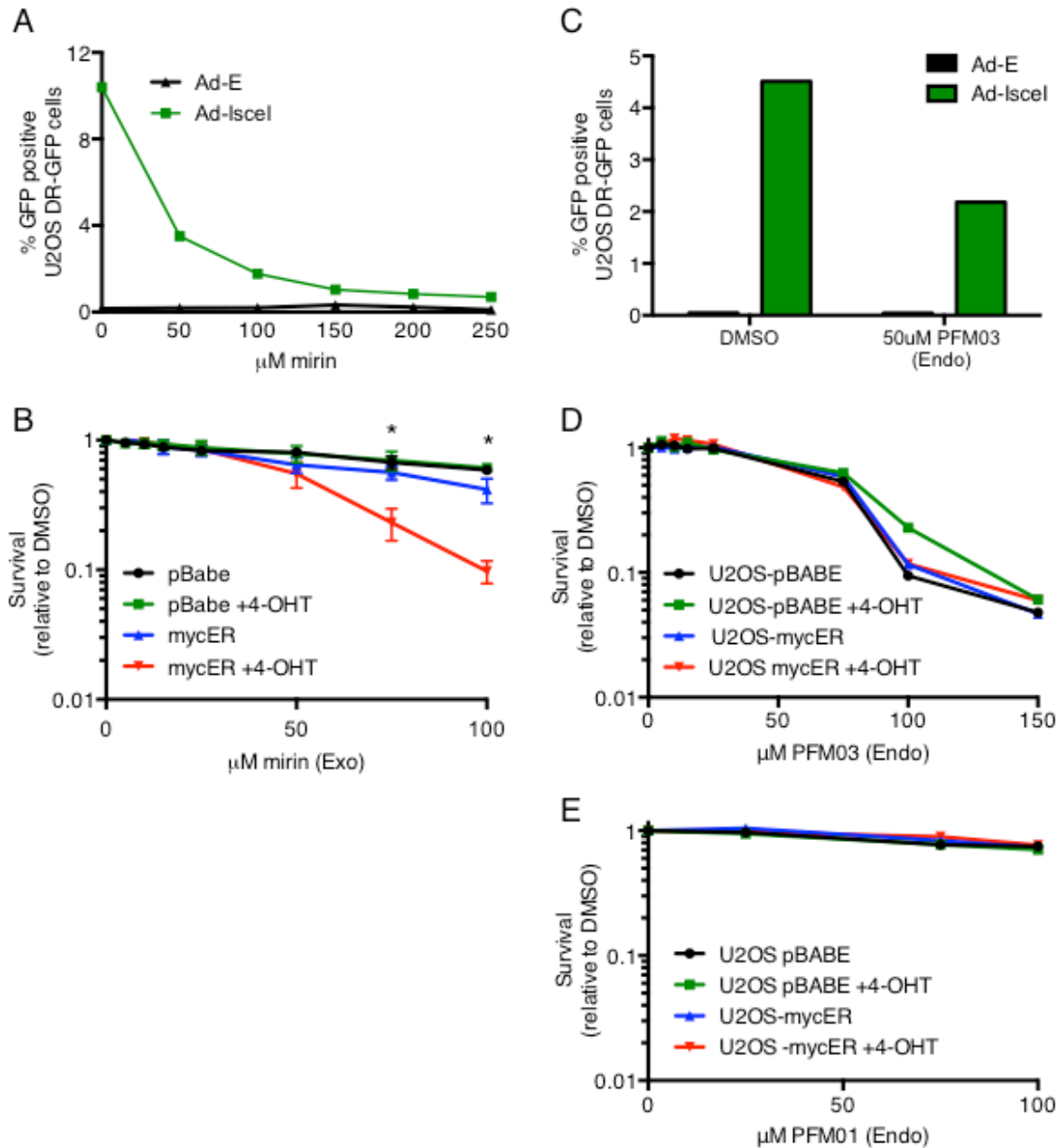


FIGURE 3.4. c-Myc overexpressing cells have increased sensitivity to Mre11 exonuclease inhibitor, mirin. (A, C) U2OS-DR-GFP cells were infected with Adeno-Isce-I or Adneo-empty, and subsequently treated with indicated doses of mirin (A) or PFM03 (C). Percent GFP positive cells was measured by flow cytometry. (B, D, E) U2OS-pBABE and U2OS-cMyc-ER cells were treated with DMSO or 200nm 4-OHT for 72 hours, then treated with increasing concentrations of mirin (B) or indicated Mre11 endonuclease inhibitor (D, E) for 24 hours, and recovered for 48 hours. Values represent crystal violet absorbance relative to vehicle DMSO control. Graph represents the average of three independent experiments. Error bars; SEM. (* $p \leq 0.05$).

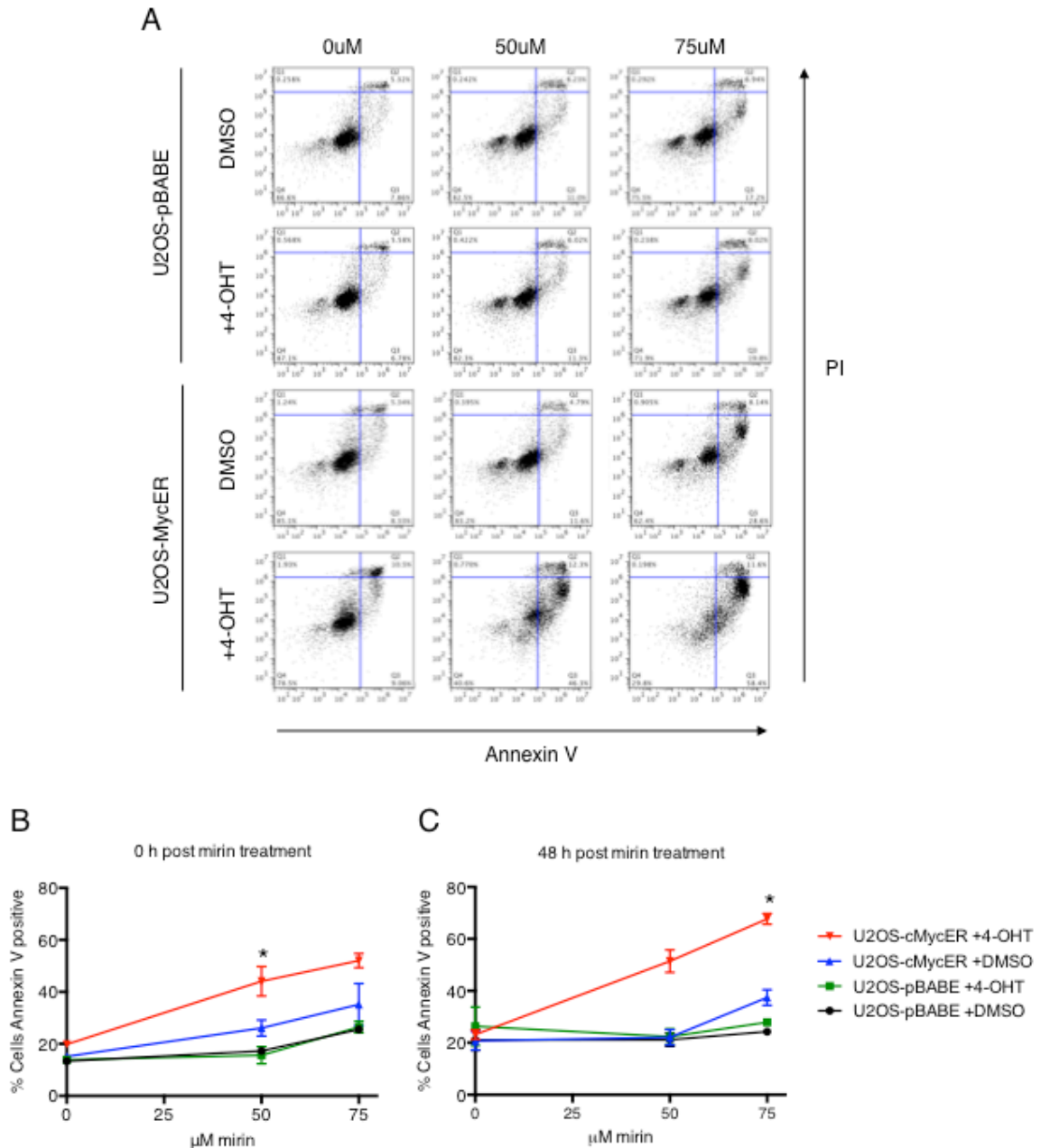


FIGURE 3.5. Mre11 inhibition in C-MYC overexpressing cells leads to apoptosis. U2OS-pBABA and U2OS-cMycER cells were treated with the indicated doses of mirin for 24 hours after 72 hours 200nM 4-OHT treatment. Cells were allowed to recover for 0 hours (B) or 48 hours (A, C), stained with FITC-Annexin V/PI, and the percent Annexin V positive cells was determined by flow cytometry. (A) Representative flow analysis of Annexin V/PI stained cells 48 hours post mirin treatment. (B, C) Graph represents the averages of three independent experiments. Error bars; SEM. (* $p \leq 0.05$).

A

Chromosomal anomalies in SV40 Mre11c ^{-/-} MYC-ER-MEFs (+/- 25 μM mirin)				
Treatment	DMSO	25 μM mirin	4-OHT	25 μM mirin/4-OHT
Total Metaphases	50	50	50	50
Gaps/Breaks	2(0.04)	2(0.04)	1(0.02)	3(0.06)
Fragments	19(0.38)	39(0.78)	32(0.64)	37(0.74)
Ring	0(0.00)	1(0.02)	1(0.02)	1(0.02)
Dicentric	2(0.04)	1(0.02)	0(0.00)	3(0.06)
Radial	1(0.02)	0(0.00)	2(0.04)	1(0.02)
Robertsonian translocations	0(0.00)	0(0.00)	4(0.08)	8(0.16)
Total anomalies	24	43	40	53
Anomalies per metaphase	0.48	0.86	0.80	1.06

B

Chromosomal anomalies in SV40 Mre11c ^{-/-} MYC-ER-MEFs (+/- 50 μM mirin)				
Treatment	DMSO	50 μM mirin	4-OHT	50 μM mirin/4-OHT
Total Metaphases	51	49	52	51
Gaps/Breaks	6(0.12)	14(0.29)	26(0.50)	20(0.39)
Fragments	31(0.61)	32(0.65)	70(1.35)	55(1.08)
Ring	0(0.00)	1(0.02)	0(0.00)	3(0.06)
Dicentric	4(0.08)	0(0.00)	0(0.00)	3(0.06)
Radial	0(0.00)	1(0.02)	1(0.02)	0(0.00)
Robertsonian translocations	1(0.02)	3(0.06)	4(0.08)	1(0.02)
Total anomalies	42	51	101	82
Anomalies per metaphase	0.82	1.04	1.94	1.61

C

Chromosomal anomalies in SV40 Mre11c ^{-/-} MYC-ER-MEFs (+/- 75 μM mirin)				
Treatment	DMSO	75 μM mirin	4-OHT	75 μM mirin/4-OHT
Total Metaphases	51	49	50	49
Gaps/Breaks	3(0.06)	31(0.63)	17(0.34)	26(0.53)
Fragments	23(0.45)	72(1.47)	44(0.88)	85(1.73)
Ring	1(0.02)	5(0.10)	0(0.00)	0(0.00)
Dicentric	0(0.00)	2(0.04)	0(0.00)	3(0.06)
Radial	0(0.00)	2(0.04)	2(0.04)	2(0.04)
Robertsonian translocations	1(0.02)	1(0.02)	1(0.02)	2(0.04)
Total anomalies	28	113	64	118
Anomalies per metaphase	0.55	2.31	1.28	2.41

FIGURE 3.6. Chromosomal anomalies in SV40 Mre11^{c/-} MYC-ER MEFs after mirin treatment. SV40 Mre11^{c/-} MYC-ER MEFs were treated with 4-OHT for 72 hours, followed by 25μM (A), 50μM (B), or 75μM (C) mirin treatment for 24 hours. Metaphases were stained with DAPI and scored in a blinded manner for chromosomal anomalies.

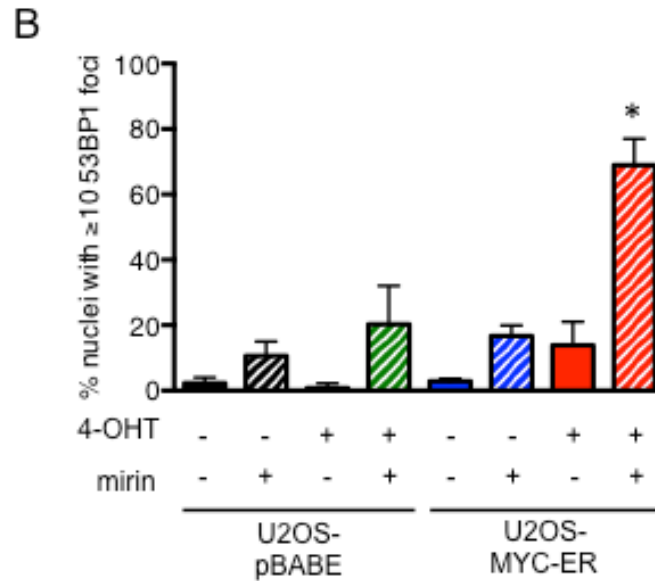
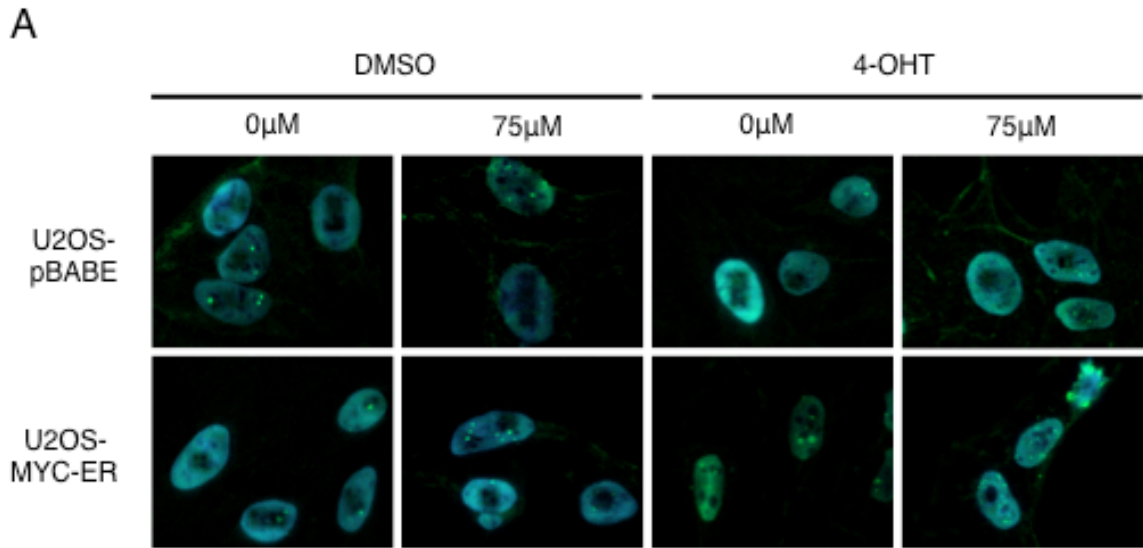
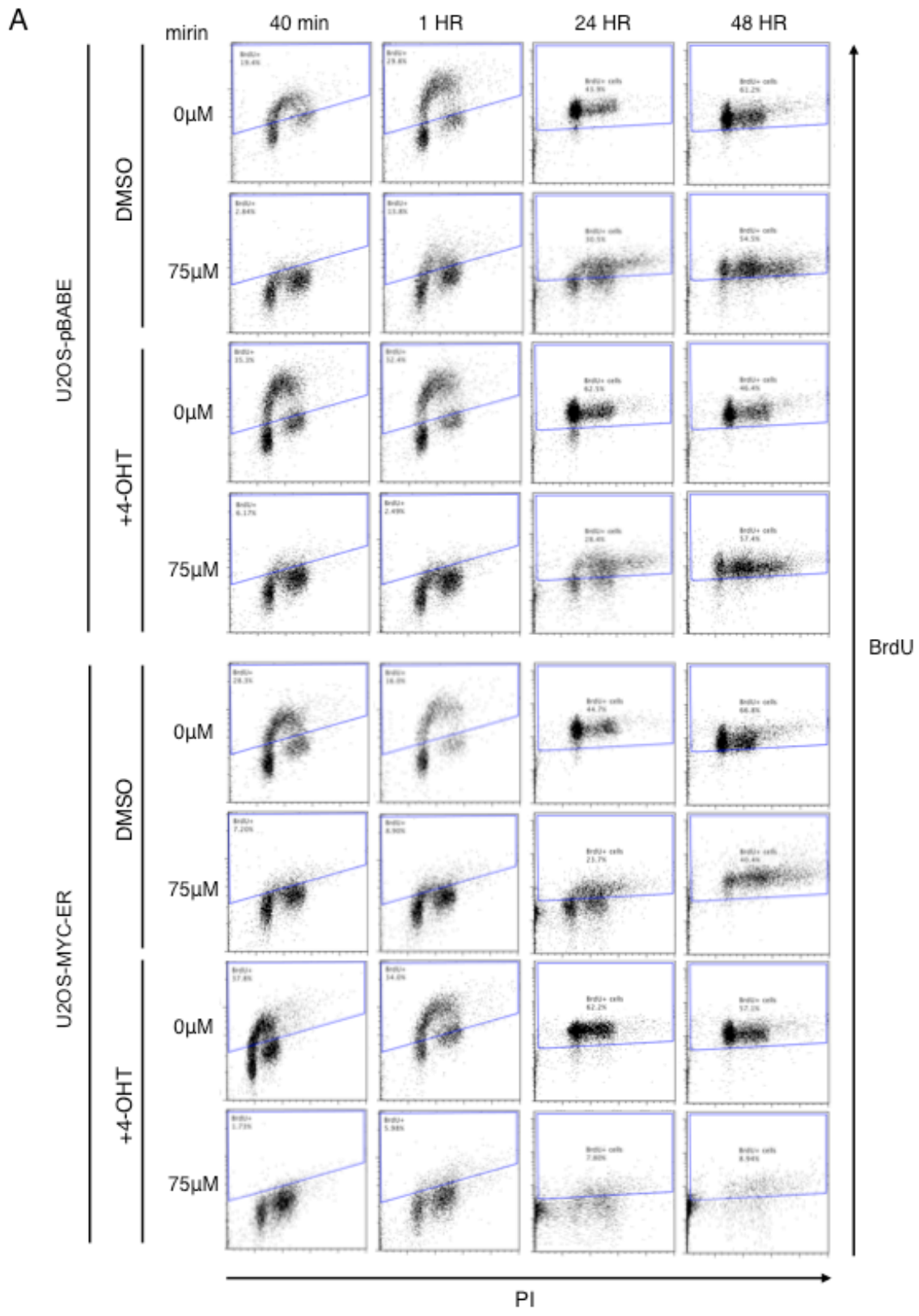


FIGURE 3.7. MYC-overexpressing cells treated with mirin have increased damage compared to controls. U2OS-pBABE and U2OS-cMycER cells were treated with DMSO or 75 μ M mirin for 24 hours after 72 hours 200nM 4-OHT treatment, and 53BP1 foci was analyzed by immunofluorescent microscopy. (A) Representative images of nuclear 53BP1 foci (green); DAPI (blue). (B) Percentage of nuclei with 53BP1 foci is plotted. Mean \pm SEM of 3 independent experiments. *, $p < 0.05$.



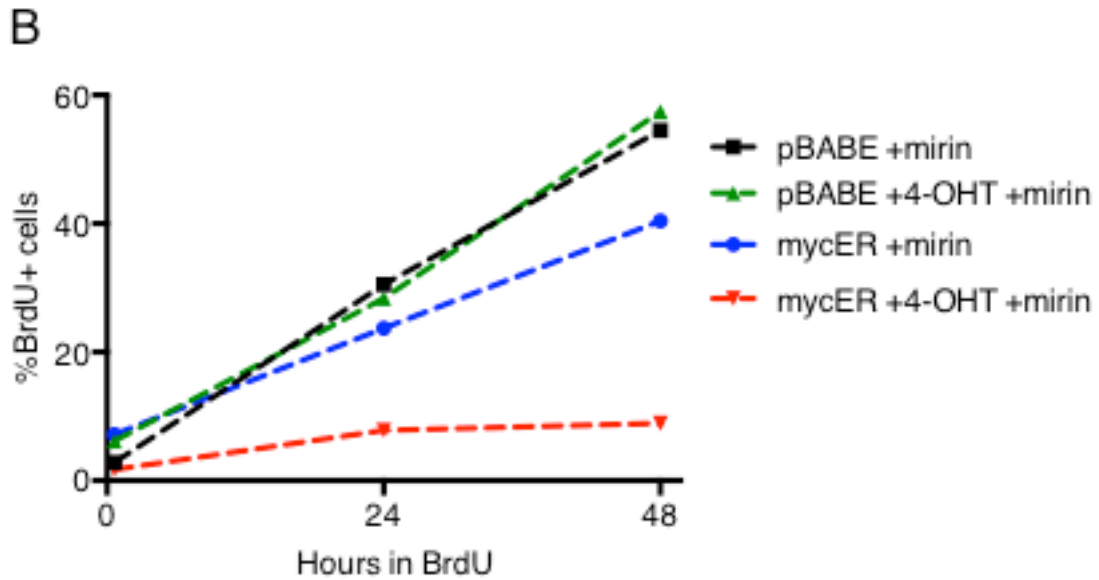


FIGURE 3.8. MYC-overexpressing cells treated with mirin have decreased BrdU incorporation, compared to controls. U2OS-pBABE and U2OS-cMycER cells were treated with DMSO or 75 μ M mirin for 24 hours after 72 hours 200nM 4-OHT treatment. Cells were then incubated with BrdU for the indicated times, fixed, and stained with anti-BrdU and PI and analyzed by flow cytometry (A). Graph shows percent of BrdU positive cells treated with mirin for the indicated times (B).

Materials and Methods

Cell cycle profile

U2OS-pBABE and U2OS-cMYC-ER cells were treated 72 hours with 200nM 4-OHT. Cells were harvested, fixed with 70% ethanol, and stored at -20°C overnight. Cells were stained with 50ug/ml propidium iodide (PI), 50ug/ml RNase A, 0.1% Triton-X solution for 20 minutes at room temperature and then analyzed by FACS analysis on an Accuri C6 flow cytometer. FlowJo software was used to analyze cell cycle profiles.

Homologous recombination DR-GFP assay

Mre11^{cl-} DR-GFP MEFs or U2OS-DR-GFP cells were plated at 1.0×10^5 cells/well in 6-well plates. 24 hours later, cells were treated with the Mre11 inhibitor indicated at the concentrations indicated for 4 hours. Next, cells were infected with Adeno-Isce-I (AdNGUS24:#9) or Adeno-empty (AdCMVpLpA.d/E3#1) and incubated for 72 hours. GFP% was analyzed by flow cytometry.

Transfection and Mre11 inhibitor treatment

293T cells (5.3×10^6 cells) were plated in in T-75 flasks 16 hours before transfection in DMEM media supplemented with 10%FBS, 20mM HEPES (Invitrogen 15630-080), Non-essential amino acids (Invitrogen 11140-050), 1mM sodium pyruvate (Invitrogen 11360-070), 1% pen/strep (Invitrogen 15140-122), 2mM L-glutamine (Invitrogen 25030-081) and β -mercaptoethanol). Cells were

transfected with pCL-Eco and either MSCV-IRES-GFP, MCSV-Nmyc-IRES-GFP, or MSCV-cMyc-IRES-GFP by the calcium chloride method and at 72 hours, the virus containing supernatant was collected and filtered through a 0.45mm PVDF filter (Millex-HC Cat#SLHV033RS). MEFs (2.0×10^5 cells) were plated in 6-well dishes then transduced with 1ml viral supernatant (empty vector, c-Myc or Nmyc), 1ml culture media, and 2ml 4mg/ml polybrene (final concentration 4mg/ml) for 72 hours. At 24 hours after transduction, MEFs were replated at 2.0×10^5 cells/well in 6-well plates. 24 hours later, cells were treated with given concentrations of Mre11 inhibitor mirin, or DMSO. Cells were harvested after 24 hours, and cellular survival was determined by cell counting using a hemacytometer and trypan blue exclusion. The results represent the mean of cellular survival relative to untransduced cells \pm standard error from 3 or more independent experiments.

Immunofluorescence microscopy

U2OS-pBABE and U2OS-cMycER cells were plated on coverslips (4×10^4) in a 12-well dish 24 hours before treatment with 200nm 4-OHT. After 72 hours in 4-OHT, cells were fixed with ice cold 3.7% paraformaldehyde, 2% sucrose (γ H2AX, Nbs1), 3.7% paraformaldehyde, 2% sucrose, 0.5% Triton-X (FANCD2, BRCA1, RPA, 53Bp1), or methanol (c-myc) for 20 minutes and washed 3x with PBS. For γ H2AX, FANCD2, and BRCA1 foci, cells were then incubated with ice cold buffer (20mM HEPES, 50mM NaCl, 3mM $MgCl_2$, 0.5% TX-100, 300mM sucrose) for 4

minutes. For RPA and 53BP1 foci, cells were incubated with ice cold buffer (10mM HEPES, 50mM NaCl, 3mM MgCl₂, 0.5% TX-100, 200mM sucrose) for 5 minutes. For Nbs1 foci, cells were incubated with 0.2% Triton-X for 10 minutes. After incubation, cells were washed 3x in PBS and blocked with PBSBT (0.5% BSA, 0.05% Tween-20 in PBS) for 1 hour. Cells were incubated 45 minutes in primary antibody diluted in PBSBT and then stained with secondary antibody Alexa Fluor 488 or 594 for 45 minutes. Coverslips were mounted on slides using DAPI (Invitrogen), and cells were visualized using Olympus BX61 microscope using 100X objective. For immunofluorescent analysis after mirin treatment, cells were plated as described above. After 72 hours 200nM 4-OHT treatment, cells were incubated with 75 μ M mirin for 24 hours. Cells were then fixed and stained for 53BP1 as described above.

Antibodies

α - γ H2AX was from Millipore (05-636). α -Nbs1, α -FANCD2, and α -53BP1 were from Novus (NB100-143, NB100-182, NB100-304). α -BRCA1 and α -RPA were from Calbiochem (OP92, NA19). α -cMyc was from cell signaling (D84C12).

Crystal Violet colorimetric analysis

U2OS-pBABE and U2OS-cMycER cell lines were generated by infecting the U2OS cell line with the retroviral vector pBABE-PURO or pBabepuro-myc-ER (Addgene plasmid #19128 [239]) expressing human c-myc cDNA fused to the

hormone-binding domain (HBD) of the estrogen receptor (ER). Briefly, 293T cells were transfected with PEQ/PAM, VSVG (retro), and either pBABE-PURO or pBabepuro-myc-ER by the calcium chloride method. After 72 hours, the virus containing supernatant was collected, filtered through a 0.45mm PVDF filter (Millex-HC Cat#SLHV033RS), and frozen overnight at -80°C. U2OS cells were infected with viral supernatant, and after 48 hours, treated with 2.5mg/ml puromycin. U2OS-pBABE and U2OS-cMycER cells were plated in triplicate in 96-well plates 24 hours before treatment with 200nm 4-OHT (Sigma 6892-35-8). After 72 hours in 4-OHT, cells were treated with increasing concentrations of mirin (or DMSO) for 24 hours and then fixed for 10 minutes with 10% methanol/10% glacial acetic acid 48 hours after removing the drug. Cells were stained with 1% crystal violet in methanol for 10 minutes, washed and dried overnight. The crystal violet was solubilized using 0.1% SDS in methanol and absorbance was measured (595nm). Survival (%) was calculated relative to vehicle-treated controls. Three or more independent experiments were performed.

Annexin V/PI apoptosis assay

U2OS-pBABE and U2OS-cMycER cells were plated in 6-well plates 24 hours before treatment with 200nm 4-OHT. After 72 hours in 4-OHT, cells were treated with mirin for 24 hours. After 0 hours recovery or 48 hours recovery, cells were harvested, washed, and resuspended in 1X binding buffer (10 mM HEPES (pH

7.4), 140 mM NaCl, 2.5 mM CaCl₂). Cells were stained with FITC Annexin V antibody (BD Pharmingen 556419) and propidium iodide (PI) then analyzed by flow cytometry.

Metaphase spread analysis in Mre11^{cl-} MYC-ER SV40 MEFs after mirin treatment

MEFs were treated with 200nM 4-OHT for 72 hours, then treated with mirin at the indicated concentration for 24 hours in the presence of 4-OHT. Cells were treated with colcemid (KaryoMAX) for 8 hours, harvested, and incubated with 0.4% KCl for 15 minutes at 37°C. Cells were fixed in ice cold fixative (3:1 methanol:glacial acetic acid), dropped onto glass slides, and stained with DAPI (Invitrogen) according to manufacturer's instructions. Images were acquired on an Olympus BX61 microscope using 60X objective, and viewed with SKYview software (Applied Spectral Imaging).

BrdU analysis

U2OS-pBABE and U2OS-cMycER cells were plated in 6-well dishes 24 hours before treatment with 200nm 4-OHT. After 72 hours in 4-OHT, cells were treated with DMSO or 75µM mirin for 24 hours. Mirin was removed and cells were incubated with 20µM BrdU for the indicated times, and then harvested, and fixed in 70% ethanol overnight. To denature DNA, 2N HCl/0.5% Tx-100 was added slowly to cells while vortexing, and then incubated for 30 minutes at room

temperature. Cells were resuspended in sodium tetraborate decahydrate (0.1M $\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{H}_2\text{O}$ pH8.5) to neutralize the acid. Next, cells were blocked with 0.5% Tween-20/1% BSA in PBS (PBST), and then stained with primary anti-BrdU antibody (B44 BD 347580) for 30 minutes at room temperature. Cells were washed, and stained with secondary antibody Alexa Fluor 488 goat anti-Mouse IgG for 30 minutes at room temperature. Next, cells were washed, resuspended in PBS containing 5 $\mu\text{g}/\text{ml}$ propidium iodide (PI) and 10 $\mu\text{g}/\text{ml}$ RNaseA, then analyzed by flow cytometry.

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Chapter IV

Discussion

Summary and Insights

MYC expression is estimated to be deregulated in up to 70% of human cancers [1]. Recurrent human lymphoid malignancies are often associated with MYC overexpression resulting from oncogenic translocations involving the immunoglobulin *IgH* locus and the *C-MYC* locus. These lymphoid translocations have been extensively studied and well characterized. The oncogenic translocations in these malignancies are products of aberrant repair of lymphocyte-specific programmed DSBs generated during V(D)J recombination or class-switch recombination. It is not clear, however, what proteins facilitate the generation of these oncogenic translocations, and what proteins are required for survival in cells harboring oncogenic translocations. In this thesis, I investigated the molecular mechanisms underlying pro-B lymphomagenesis in a lymphoma prone mouse model.

Human patients with mutations in proteins required for proper repair of programmed DSBs during V(D)J recombination, specifically the C-NHEJ pathway of repair, are immunocompromised (or present with radiosensitive severe

combined immunodeficiency (RS-SCID) in the case of *Artemis* deficiency), and a subset of these patients are predisposed to lymphoid malignancies [2-5]. Mice with C-NHEJ deficiencies are immunocompromised and have a strong predisposition to pro-B lymphomas. Roles for the MRN complex have been implicated during C-NHEJ. Using a reporter substrate in wild type and *XRCC4* deficient cells, siRNA knockdown of *MRE11* or inhibition of MRE11 exonuclease activity by mirin decreased end-joining efficiency of I-SceI-induced DSBs [6, 7]. Additionally, MRE11 deficiencies in B cells led to decreased levels of CSR [8]. These findings indicate a role for MRE11 and MRE11 nuclease activity in both C-NHEJ and alt-NHEJ repair pathways.

In this study, we observed an absence of tumor formation in mice with B cell specific *Mre11* deficiencies in a $p53^{-/-}$ background. This is in stark contrast to mouse models with partial loss-of-function mutations in *Mre11*, which present with ataxia telangiectasia-like disorder (ATLD) and an increased predisposition to cancer in a *p53* deficient background [9]. Our results are also distinct from two human patients with germline *MRE11* mutations presenting with ATLD and predisposition to lung adenocarcinoma, and other patients with *Mre11* mutations associated with lymphoma or breast cancer [10-12]. Taken together, these data suggest hypomorphic mutations in *MRE11* must retain some MRE11 functions that can promote tumorigenesis, likely MRE11 nuclease activity.

The V(D)J recombination DNA nuclease, ARTEMIS, is required during C-NHEJ to nick open hairpins at the ends of the generated DSBs. Our lab has

developed a mouse model with gene targeted mutations in *Artemis* and *p53* that is predisposed to early onset pro-B lymphomagenesis. The tumors in these mice were previously characterized by oncogenic translocations involving the *IgH* locus and either the *C-myc* or *N-Myc* loci, leading to oncogene overexpression. In order to better understand the roles of MRE11 and MRE11 nuclease activity in tumorigenesis, we crossed *Mre11*^{-/-} or *Mre11*^{H129N/c} mice with B-cell specific CD19-cre recombinase to pro-B lymphoma prone *Artemis*^{-/-}*p53*^{-/-} mice.

In this study, we observed B cell specific *MRE11* deletion or inactivation of MRE11 nuclease activity suppressed pro-B tumorigenesis in *Artemis*^{-/-}*p53*^{-/-} mice, suggesting that MRE11 has tumor promoting functions. Instead, these mice succumbed to thymic lymphomas, which still express wild type MRE11. It is possible that thymic lymphomas arise before pro-B lymphomas in this context. However no microscopic B cell lesions are detectable by histology in *Mre11*^{-/-}*p53*^{-/-} and *Mre11*^{H129N/-}*p53*^{-/-} mice collected upon necropsy (Spehalski and Ferguson, personal communication). These findings provide additional supporting evidence that B cell tumors, even at the pre-neoplastic microscopic level, are not developing in these mice.

Roles of the MRN complex have been implicated in V(D)J recombination, possibly functioning to tether coding ends and stabilize the postcleavage complex [13, 14]. Humans and mice with MRE11 deficiencies exhibit phenotypes that would suggest defects during V(D)J recombination, including immunodeficiency [15-20]. Further, MRN deficiency leads to an increase of unrepaired coding ends

and increased trans-rearrangements, suggesting that MRE11 deficiency can lead to aberrant V(D)J recombination [13, 19]. Importantly, a role for MRE11 has been implicated in alt-NHEJ. The alt-NHEJ pathway of DSB repair has been implicated in the generation of oncogenic chromosomal translocations [21, 22]. Thus, we hypothesized that MRE11 may be involved in facilitating the formation of translocations during aberrant repair of Rag-initiated DSBs during V(D)J recombination. Our results demonstrate, however, that in the absence of MRE11, or MRE11 nuclease activity, specific *IgH:Myc* translocations in *Mre11^{-/-}p53^{-/-}* and *Mre11^{H129N/-}p53^{-/-}* B cells can be detected. Further, I observed dicentric, ring chromosomes, and Robertsonian translocations in *Mre11^{-/-}Artemis^{-/-}* MEFs, indicating that spontaneous translocations can be generated in cells lacking MRE11, even in the absence of ARTEMIS.

A separate, not mutually exclusive, hypothesis for why *Artemis^{-/-}p53^{-/-}* mice no longer develop pro-B lymphomas in the absence of MRE11 or MRE11 nuclease activity, could be due to a requirement of MRE11 for tumor cell survival. The pro-B tumors that arise in *Artemis^{-/-}p53^{-/-}* mice harbor translocations involving the *IgH* and either the *C-Myc* or *N-Myc* oncogenes, leading to their amplification and overexpression. Oncogene overexpression, and specifically C-MYC overexpression, has been shown to induce replication stress through a number of mechanisms. MYC overexpression deregulates CDK activity, a key protein in controlling cell cycle progression. MYC also stabilizes components of the pre-replication complex, leading to increased origin firing [23]. Further, MYC

overexpression leads to a shortened G1, inappropriate S-phase entry, and asymmetric replication forks, and hyper-proliferation [23, 24]. All of these effects can ultimately result in fork stalling and replication stress and activation of the DDR. Importantly, the MRN complex has been implicated in replication fork restart and the resolution of replication intermediates [25-27]. Thus, MRE11 could be involved in the repair of oncogene-induced replication stress, and thus, maintaining a level of genome stability necessary for tumor cell survival.

In this thesis, I present evidence for DDR activation upon C-MYC overexpression. Key DNA repair proteins localize to sites of C-MYC-induced damage. Importantly, as visualized by Nbs1 foci, the MRN complex localizes to sites of MYC-induced damage, indicating that the MRN complex may be involved in the repair of this type of damage. While an increase in γ H2AX foci is visualized in MEFs, mouse primary B cells, and human U2OS cells upon C-MYC overexpression, the rest of the foci experiments are performed in human U2OS cells. It is not clear if the cellular response to MYC overexpression is the same in different cell types, as they may have different responses. For example, MEFs are more tolerant to IR-induced DNA damage, while B cells are very sensitive and will undergo apoptosis. It is possible that the cellular response to oncogene-induced replication stress in B cells might differ from the response in U2OS cells. Thus it will be important to determine the cellular response to oncogene-induced damage in other cell types, specifically in B lymphocytes, as MYC-

overexpressing tumors in *Artemis/p53* deficient mice originate from the B cell lineage.

A recent cancer therapeutic approach that is currently gaining recognition takes advantage of increased replication stress associated with certain types of cancer [28-30]. The reliance on proteins involved in repairing damage associated with replication stress provides potential attractive molecular targets to selectively eliminate cancer cells. I showed that MYC overexpression induces the DDR, and MRN localizes to site of damage. Together with our observations that simply inactivation of MRE11 nuclease activity in *Artemis*^{-/-}*p53*^{-/-} mice suppresses pro-B lymphoma led us to utilize small molecule inhibitors of MRE11 in order to determine the role of MRE11 nuclease activity on the survival of C-MYC overexpressing cells [31, 32]. The MRE11 inhibitors available allowed me to discern the function of MRE11 endo- and exonuclease activities in cells overexpressing C-MYC [31, 32]. Interestingly, MRE11 exonuclease inhibitors, and not endonuclease inhibitors, selectively led to decreased survival and increased apoptosis in C-MYC overexpressing cells, while control cells were largely unaffected at the same dose. While the results I observed were striking, it is clear that the necessary concentrations used in this study do not lend themselves for use in a therapeutic setting. Thus, Mre11 inhibitors of exonuclease activity must be further developed to be more potent in order to decrease the concentrations of the drug required for cancer cell specificity. An MRE11 inhibitor with clinical promise would exhibit selectivity for the exonulcease

activity in the low nanomolar range. It would be valuable to increase solubility and determine bioavailability of the drug if introduced orally. Developing a small molecule that can be administered orally would be ideal for patients (less frequent clinic visits), however intravenous administration would increase bioavailability and may be more effective.

Studies by Shibata et al. use selective MRE11 inhibitors and show that following exposure to ionizing radiation (IR), MRE11 exonuclease inhibition prevents end resection, decreases RPA and RAD51 loading, and decreases levels of repair via HR without increasing levels of NHEJ [32]. This resulted in increased γ H2AX foci and increased chromosomal breaks. In contrast, MRE11 endonuclease inhibitor treatment led to decreased levels of HR, but increased levels of NHEJ, suggesting that cells were able to shuttle repair from HR to NHEJ without the increased level of DSBs observed with MRE11 exonuclease inhibitor treatment. The results of these and other studies have led to a two-step mechanism for MRE11 nuclease activities during HR [32-34]. First, MRE11 endonuclease activity makes a nick upstream of the DSB, committing repair through HR. Next, bidirectional resection via MRE11 3'-to-5' exonuclease activity coupled with EXO1/BLM 5'-to-3' exonuclease activity.

The studies performed by Shibata et al. examined the functions of MRE11 nuclease activity at two-ended DSBs, and not those associated with replication complications (one-ended DSBs resulting from fork collapse) [32]. Oncogene-induced replication stress can lead to fork stalling, and if unable to restart, the

replication fork can collapse, generating a one-ended DSB. If the functions of MRE11 nuclease activities function in a similar way at both one- and two-ended DSBs, I would hypothesize that MRE11 exonuclease inhibitor treatment would allow for MRE11 endonuclease activity to create the commitment nick for HR, but inhibit resection, decrease RPA and RAD51 foci, and lead to increased amounts of DNA damage resulting in cell death. Indeed, I observed increased 53BP1 foci, demonstrating increased amounts of DNA damage, and increased apoptosis upon treatment with mirin in cells that overexpress C-MYC. I did not observe decreased survival in C-MYC overexpressing cells upon treatment with MRE11 endonuclease inhibitors, potentially because damage could be repaired through alternative pathways before a commitment to HR. To address this question experimentally, I could take advantage of copy number variants (CNVs) that are observed upon C-MYC overexpression. CNVs are proposed to arise when a broken replication fork is repaired by NHEJ rather than error-free HR during high stress conditions [35]. Therefore, I would expect to see higher CNVs with Mre11 endonuclease inhibitor treatment, as HR would be defective and more error-prone pathways would be responsible for repair. Alternatively, recent studies in *Xenopus* egg extracts reveal a requirement of Mre11 endonuclease activity only at ends with 5' bulky adducts, and other studies in mitotic yeast cells reveal that Mre11 endonuclease activity is not required for resection of HO-endonuclease induced DSBs, indicating the structure of DNA ends is important for the requirement of MRE11 endonuclease activity [36, 37]. Therefore, it is possible

that the types of ends at oncogene-induced DSBs may not require MRE11 endonuclease activity for repair.

Future Directions

Although $Artemis^{-/-}p53^{-/-}$ mice develop tumors associated with translocations involving the C-MYC or N-MYC oncogenes, the studies in this thesis largely focus on C-MYC overexpression. While I did observe increased DNA damage by γ H2AX foci in cells overexpressing N-MYC at comparable levels to C-MYC overexpression, I did not determine if the cellular response to N-MYC overexpression is similar to what I observe in cells that overexpress C-MYC. It would be interesting to see if the MRN complex also localizes to sites of N-MYC-induced DNA damage, and if N-MYC overexpressing cells also rely on MRE11 nuclease activity for survival. Previous studies have shown a role for the MRN complex in controlling N-MYC-induced replication stress during normal neural cell proliferation, but it is not clear if the role of MRE11 in different cell types or in the context of cancer is similar to what we observe in C-MYC overexpressing cells [38, 39]. Utilizing neuroblastoma cell lines that express varying levels of N-MYC expression and treating with mirin would be one way to test this hypothesis.

$Mre11^{+/c}Artemis^{-/-}p53^{-/-}$ and $Mre11^{H129N/c}Artemis^{-/-}p53^{-/-}$ mice that harbor the B-cell specific CD19-cre transgene do not develop pro-B tumors, but rather succumb to thymic lymphomas. The thymic tumors that arise are not associated

with C-MYC or N-MYC overexpression. Therefore, it could be informative to delete MRE11 in the T cell lineage. If these mice still develop thymic tumors, this would provide stronger evidence that MRE11 functions specifically in MYC-driven tumorigenesis. However, if these mice do not develop thymic tumors, it could reveal a broader requirement for MRE11 in cancers with inherent genomic instability, such as the thymic tumors. If this is the case, it is possible MRE11 inhibitors could be utilized more broadly than only for use in MYC-driven cancers, as genomic instability is a hallmark of cancer [40, 41].

Further, to take a genetic approach to determine the role of MRE11 nuclease activity in a developed tumor *in vivo*, the development of a “tet-on” inducible B-cell-specific Cre mouse would be informative. Inducing Cre expression, and thus deleting MRE11 in a B-cell tumor, by administration of doxycycline once $Mre11^{H129N/c} Art^{-/-} p53^{-/-}$ mice develop tumors could reveal interesting results. Based on the findings in Chapter III, I would hypothesize that pro-B tumors would shrink due to increased damage leading to apoptosis.

Use of mirin in tumors *in vivo* is an appealing experiment. Utilizing a mouse model of myc-driven cancer, such as the E mu-Myc model of spontaneous lymphoma would test the effectiveness of MRE11 nuclease inhibitors in MYC-driven tumors [42]. The E mu-Myc mouse has an inherited IgH-Myc fusion transgene that drives spontaneous B lymphomas [42]. Importantly, comparing treatment of mirin, or other newly developed MRE11 exonuclease inhibitors, with the standard of care, or in combination would be the goal [32].

Interestingly, ATR and CHK1 inhibitors were originally utilized in combination to enhance the effect of other chemotherapeutic agents. However, ATR and CHK1 inhibitors are being considered for potential as a monotherapy, as the repair of damage induced by replication stress is dependent on these proteins. Not surprisingly, use of CHK1 inhibitors as a monotherapy was not effective in a variety of tumor models; however, CHK1 inhibitors have proved to be effective in MYC-driven lymphoma cell lines and mouse models, N-MYC-driven neuroblastoma, and melanoma cells that display high levels of replication stress [30, 43-46]. Importantly, K-RAS^{G12V}-induced pancreatic adenocarcinomas did not respond to CHK1 inhibitor treatment, and do not have increased levels of replication stress [30]. Comparing MRE11 nuclease inhibition to CHK1 and ATR inhibitors will be important. The ATR-CHK1 pathway is activated by a large range of cellular and DNA abnormalities including stalled replication forks, interstrand DNA crosslinks, and virus infection [47-50]. MRE11 exonuclease inhibitors keep the MRN complex and ATM signaling intact, therefore I hypothesize MRE11 exonuclease inhibitors would have decreased cytotoxicity on normal cells compared to CHK1 inhibitors. To test this hypothesis, non-MYC-overexpressing cells could be treated with MRE11 exonuclease inhibitors or CHK1 inhibitors and cellular survival, levels of apoptosis, and senescence could be assayed. Further, these drugs could be compared in MYC-overexpressing cells to determine any differences in sensitivity.

In summary, the findings in this thesis provide insight into the mechanism of MYC-driven lymphoma tumor cell survival, and provide evidence for a role of MRE11 exonuclease activity in this context (Figure 4.1). Taken together, the data reveal a new target, MRE11 exonuclease activity, for replication stress associated cancers. I hope that the findings in this thesis will contribute to the advancement of personalized therapies for cancer patients.

Figures

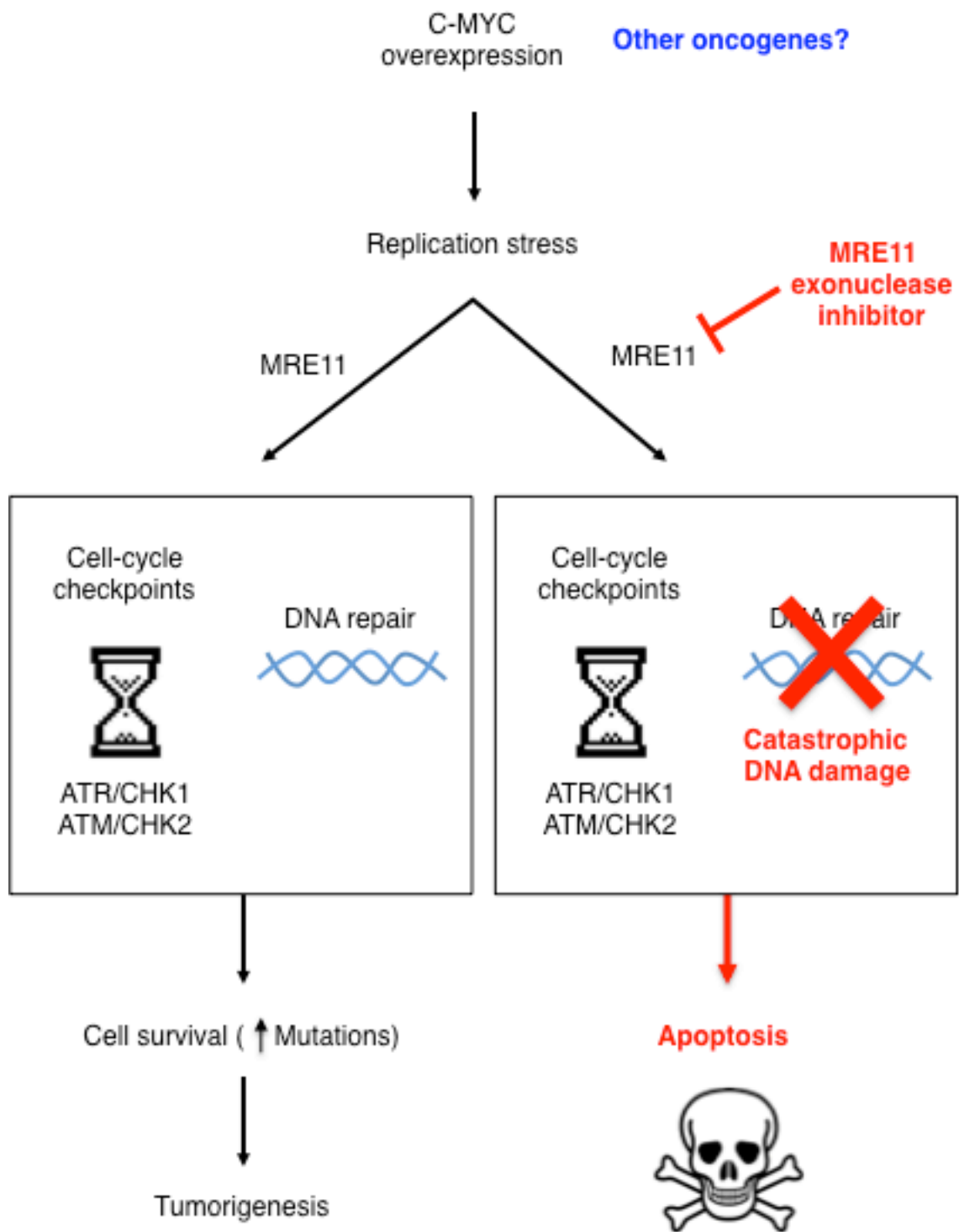


Figure 4.1. Inhibition of MRE11 exonuclease activity in C-MYC overexpressing cells leads to apoptosis. C-MYC overexpression leads to replication stress. MRE11 is involved in the DNA damage response, which leads to cell cycle checkpoints and allows the cells time to repair DNA damage. This may restore the genome, but may also increase mutation frequency, which could drive tumorigenesis. Treatment with an MRE11 exonuclease inhibitor in cells that overexpress C-MYC retains ATM signaling, but inhibits the DNA repair functions of MRE11, resulting in catastrophic amounts of DNA damage and cell death through apoptosis.

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