The Mre11-CDK2 Interaction in the DNA Damage Response

by

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ABSTRACT

The MRN (Mre11, Rad50, and NBS1) complex consists of highly conserved proteins integral to DNA double-strand break (DSB) signaling and repair. The unique MRN complex architecture allows direct binding to damaged DNA ends, DSB detection, and activation of cellular repair systems. Defects in the MRN complex lead to cancer predisposition, neurodegeneration, and immunodeficiency.

MRN is required to initiate homologous recombination, the predominant repair pathway during the S and G2 phases of the cell cycle. Mre11 provides the nuclease activity needed for resection, while the NBS1 subunit interacts with ATM kinase, the master regulator of the DNA damage response (DDR) signaling cascade. The nuclease activity of Mre11 are required but alone are insufficient for resection; tumor suppressor BRCA1 and CtIP protein are also needed. CtIP is responsible for cell-cycle regulation of resection, while the major cyclin-dependent kinase (CDK) in S-phase is CDK2 bound to Cyclin A. Phosphorylation of CtIP by CDK2 allows the MRN-CtIP-BRCA1 resection complex to assemble, providing maximum resection capacity for homologous recombination. Mre11 controls these events through direct interaction with CDK2 which is required for CtIP phosphorylation and interaction with BRCA in normal cells. This observation demonstrates the important functions of MRN in both the DDR and in normal cell cycle regulation.

The DDR has been extensively studied, but the role of CDK2 within the DDR remains unclear. The work presented here includes the following investigations: 1) the impact of DNA damage on the Mre11-CDK2/Cyclin A interaction, 2) whether change in the Mre11-CDK2/Cyclin A interaction is ATM-dependent, and 3) whether alterations in the activity of

CDK2 indicate DDR participation. To examine the effect of DNA damage on the Mre11-CDK2/Cyclin A complex, I exposed mammalian cells to ionizing radiation and evaluated the interaction of endogenous Mre11 and CDK2/Cyclin A. These data indicate that DNA damage induces rapid dissociation of the Mre11-CDK2/Cyclin A complex dependent on ATM kinase activity and correlative to a reduction in CDK2 activity. This suggests that the Mre11-CDK2/Cyclin A complex dissociation causes a reduction in CDK2 activity, thereby contributing to the delay of cell cycle progression in S-phase, allowing for DNA repair proteins to restore DNA integrity.

CHAPTER I

Introduction

Function of the MRN complex

Disease: Human syndromes resulting from failure to detect and repair DNA double strand breaks have diverse sequelae including genomic instability, increased cancer incidence, and immunodeficiency (D'amours and Jackson, 2002). A well-studied mutation in Meiotic recombination 11 (Mre11)(Figure I.1) leads to a cellular loss of immediate early response to DNA damage and corresponding downstream biological repair network signal amplification linked to Mre11 (Shan *et al*., 2009). Catastrophic disease disorders are associated with genetic lesions in genes encoding Mre11-Rad50-Nbs1 components in humans. These disorders resulting from genetic mutation demonstrate that Mre11 is an integral component of the MRN complex (Figure I.2), involved in detecting and repairing DNA double strand breaks (Buis *et al.*, 2008). As part of the MRN complex, Mre11 binds to DNA double strand breaks and participates in DNA repair and checkpoint signaling pathways governed by Ataxia Telangiectasia Mutated (ATM), a 350kDa multifunctional serine/ threonine kinase, (Stracker et al. 2004, Stewart et al. 1999)(Figure I.2 and I.3.). This has been demonstrated by the failure of human or murine cells deficient in ATM to arrest at the intra S and G2/M check points after DNA damage (Stewart et al. 1999, Stracker et al. 2004)(Figure I.4). Failure of the S phase checkpoint is defined by a cells hypersensitivity to ionizing radiation, measured chromosomally and by cell survival, radioresistant DNA synthesis (RDS) and an insufficiency to induced stress activated protein kinases following

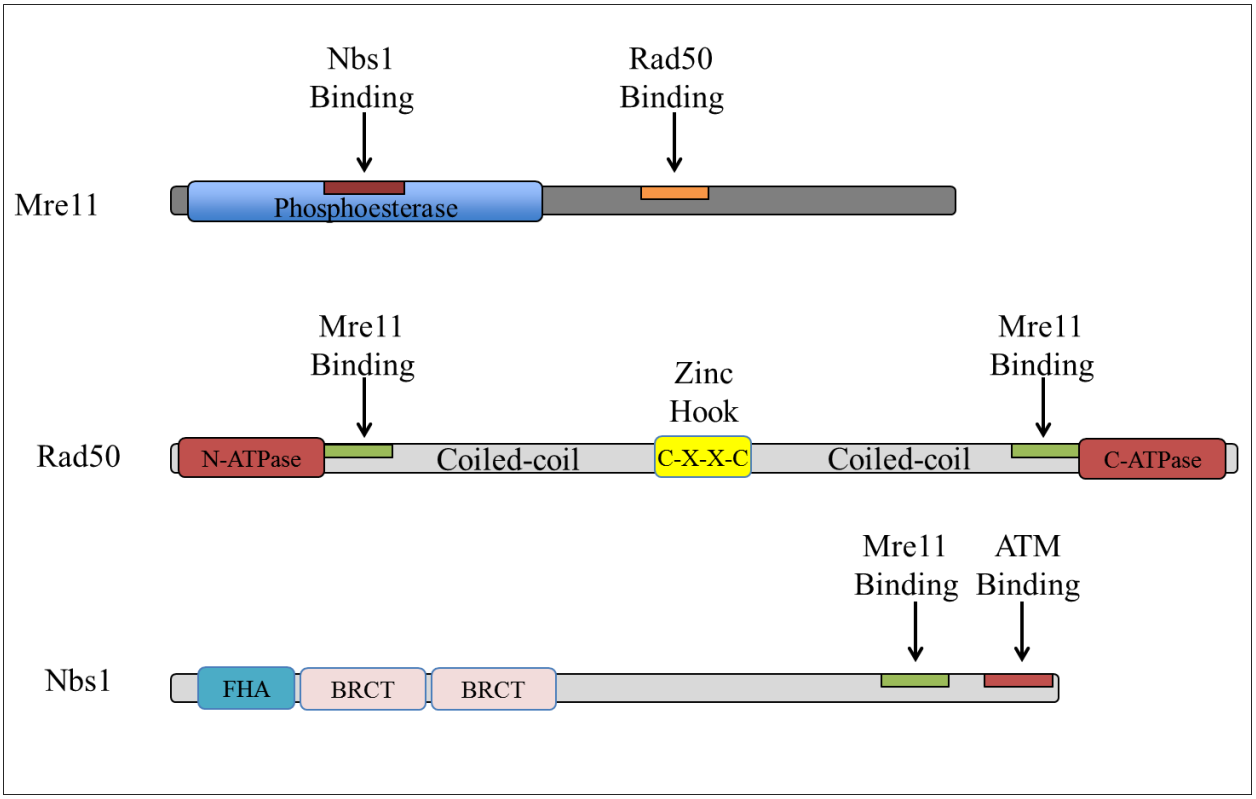

Figure I.1. Components of the MRN complex. Modified from Lamarche et al 2010.

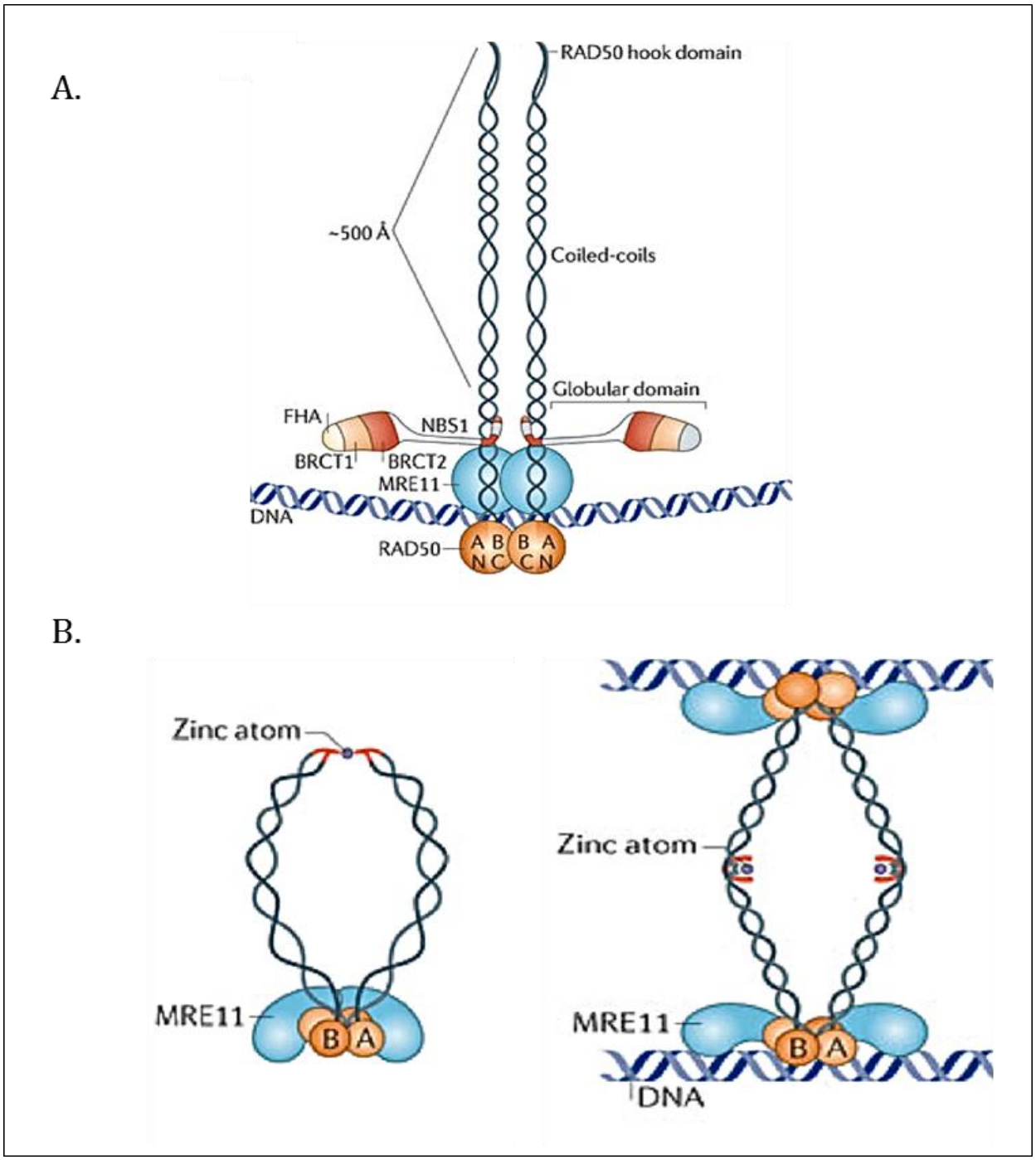

Figure I.2. Representative model of Mre11-Rad50-Nbs1 (MRN) complex. A. The MRN complex consists of MRE11, NBS1, and RAD50. B. Schematic representation of the MRN complex in two possible configurations. Rad50 can dimerize both through its head domain and through a zinc-binding dimerization motif at the opposite end of the coiled-coil known as the "zinc-hook". Adapted from Stracker, Petrini(Stracker, Petrini 2011a)

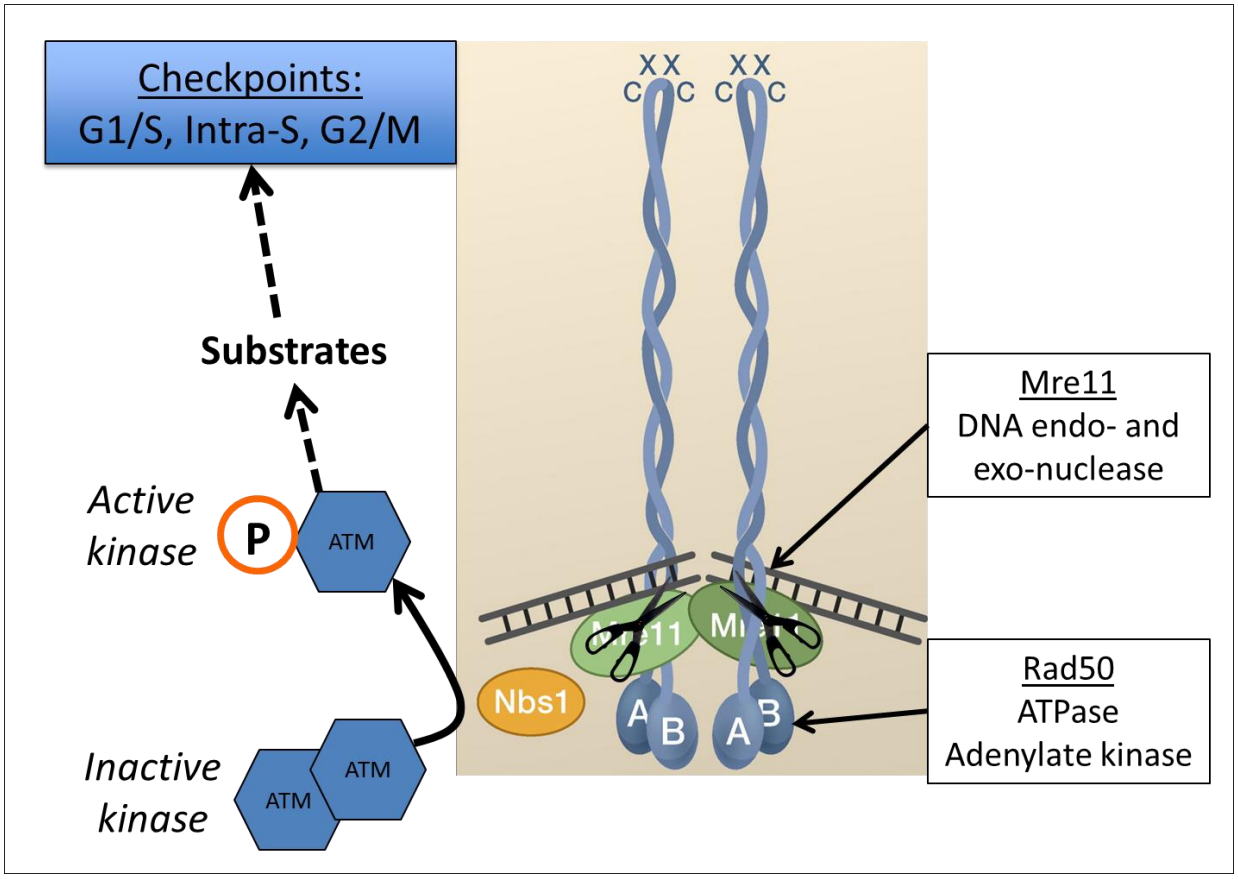

Figure I.3. MRN complex at a DNA double strand break. Mre11 protein has 3'to 5' exonuclease activity and endonuclease activity for early processing of broken DNA ends. Rad50 ATPase and adenylate kinase activities modulate dramatic movement of two large flexible coiled arms. NBS1 protein is critical for recruitment of ATM and activation of ATM in response to DSBs.

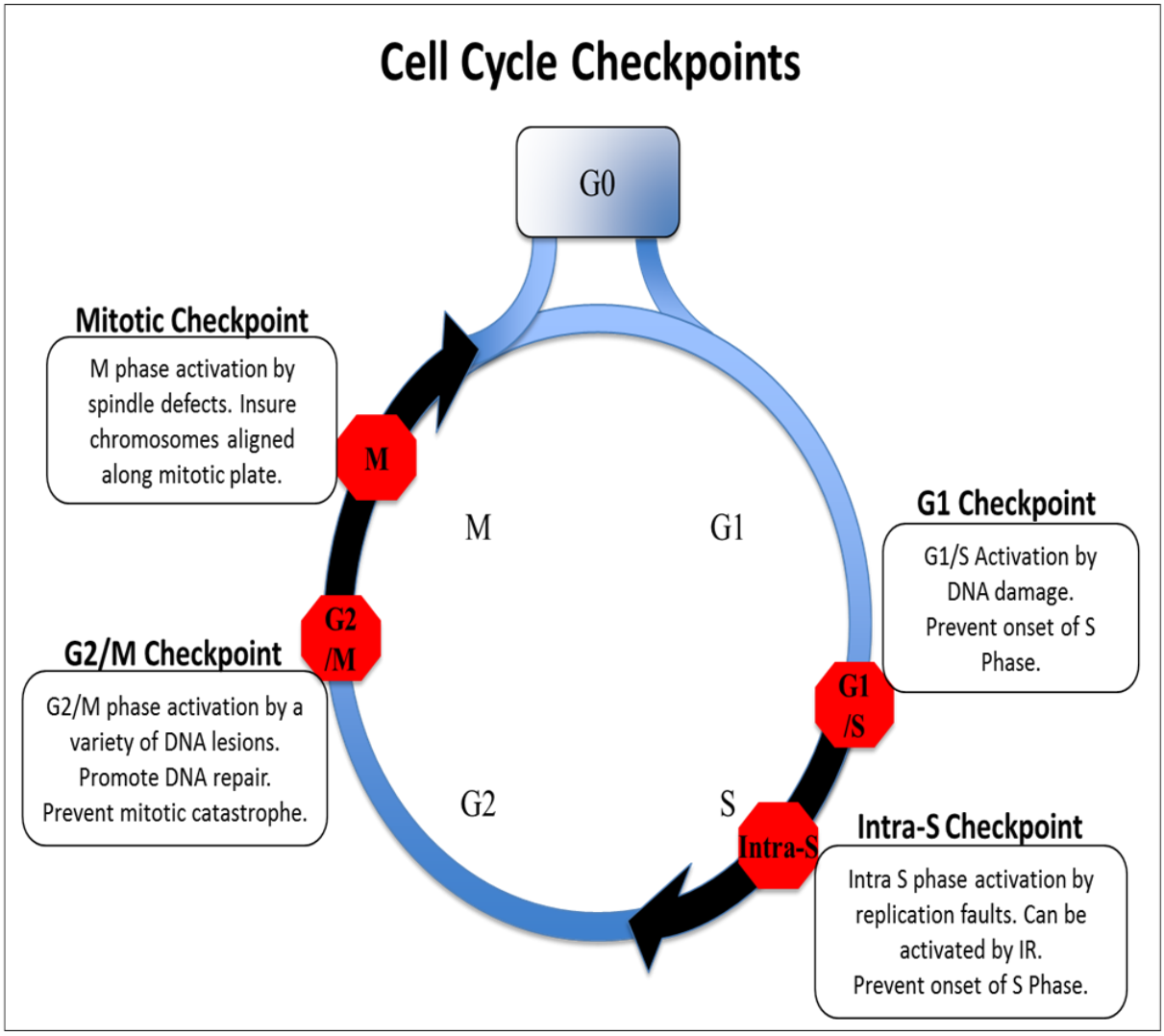

Figure I.4. Cell cycle DNA damage checkpoints. DNA lesions activate distinct cell cycle checkpoints. G1/S checkpoint prevents cells from entering S phase by inhibiting entry into S phase. Intra-S phase is activated by damage encountered during the S phase or by unrepaired damage that escapes the G1/S checkpoint and leads to a block in replication. G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. Pathway is activated to arrest the cell cycle following DNA damage in G2 and inhibit entry into mitosis.

exposure to IR. This checkpoint deficiency in the DNA double strand break is phenotypic of ataxia telangiectasia (AT), ataxia telangiectasia like disorder (ATLD), and Nijmegen breakage syndrome (NBS) (Varon et al. 1998, Stewart et al. 1999). Mutations that alter proteins involved in the DNA repair response are responsible for these conditions.

Patients with mutations in *Mre11* suffer from ATLD and consequently experience extreme sensitivity to ionizing radiation, develop cerebellar degeneration, and have chromosomal translocations in circulating lymphocytes (Stewart et al. 1999b). In addition, somatic mutations of *Mre11* have been found in multiple tumors (Fukuda et al. 2001, Giannini et al. 2002), and *Mre11* is one of two genes linked to DNA repair found in the consensus coding sequence of human breast and colorectal cancers (Sjoblom et al. 2006). Total loss of function or mutation of *Mre11* causes a range of phenotypic variation dependent on the organism and degree of disruption. These outcomes can include reduced growth rate, development, and cellular proliferation, as well as sterility and lethality (Johzuka and Ogawa, 1995) (Xioa and Weaver, 1997)(Yamaguchi-Iwai *et al.*, 1999) (Chin and Villeneuve, 2001) (Bundock and Hooykaas, 2002)(Buis *et al.*, 2008) . Comparatively, total loss of Rad50 in post-mitotic and quiescent cells of mice has no effect on long term viability, while deletion of *Rad50* from rapidly growing cultured fibroblasts and hematopoietic cells causes extensive DNA damage and death (Adelman *et al.*, 2009).

Biology: The MRN complex functions as a heterotetramer comprised of two Mre11 and two Rad50 molecules together with a single Nbs1 monomer (Figure I.2). The binding of the MRN complex to DSB initiates the recruitment and activation of the apical DNA damage response kinase ataxia telangiectasia-mutated protein. Evidence of double strand breaks is communicated through the DNA damage response signal transduction pathway,

cell cycle regulators, the apoptotic machinery, and DNA repair proteins (Figure I.5). (Harrison, Haber 2006, Harper, Elledge 2007, Lazzaro et al. 2009, Lazzaro et al. 2009, Harper, Elledge 2007).

Mre11 is a modular protein with an N-terminus containing four highly conserved DNA nuclease motifs (Stracker et al. 2004b). Mre11 homologues are found in eukaryotes ranging from yeast to humans (Sharples, Leach 1995). These proteins consist of approximately 700 amino acids and share approximately 50% homology in the N-terminus, while the C-terminus is more heterogeneous. All of the proteins include four conserved phosphodiesterase (nuclease) motifs in the N-terminus bearing a marked similarity to *E. coli* nuclease SbcD (Petrini et al. 1995).

Rad50, like Mre11, is conserved from archaea to humans and together form the core of the MRN complex. This highly conserved heterotetramer functions as the DNA binding and processing core of the MRN complex (D'Amours, Jackson 2002, de Jager et al. 2004, Bhaskara et al. 2007). Similar to the structural maintenance of chromosome (SMC) family, the MRN complex retains the ability to bind DNA, and contains ATPase and adenylate kinase activities to modulate dramatic movement of two large flexible coiled arms (Figure I.1 and I.2.) (Hopfner et al. 2002, Williams, Tainer 2005, Williams, Tainer 2007, Bhaskara et al. 2007). To encompass recognition and bridging, Rad50 contains a highly conserved Zn^{2+} hook domain that mediates dimerization and the expansive configurations allowing for nuclease-independent function of the Mre11 complex in DNA recombination and repair (Hopfner et al. 2002, Bhaskara et al. 2007, Hohl et al. 2011).

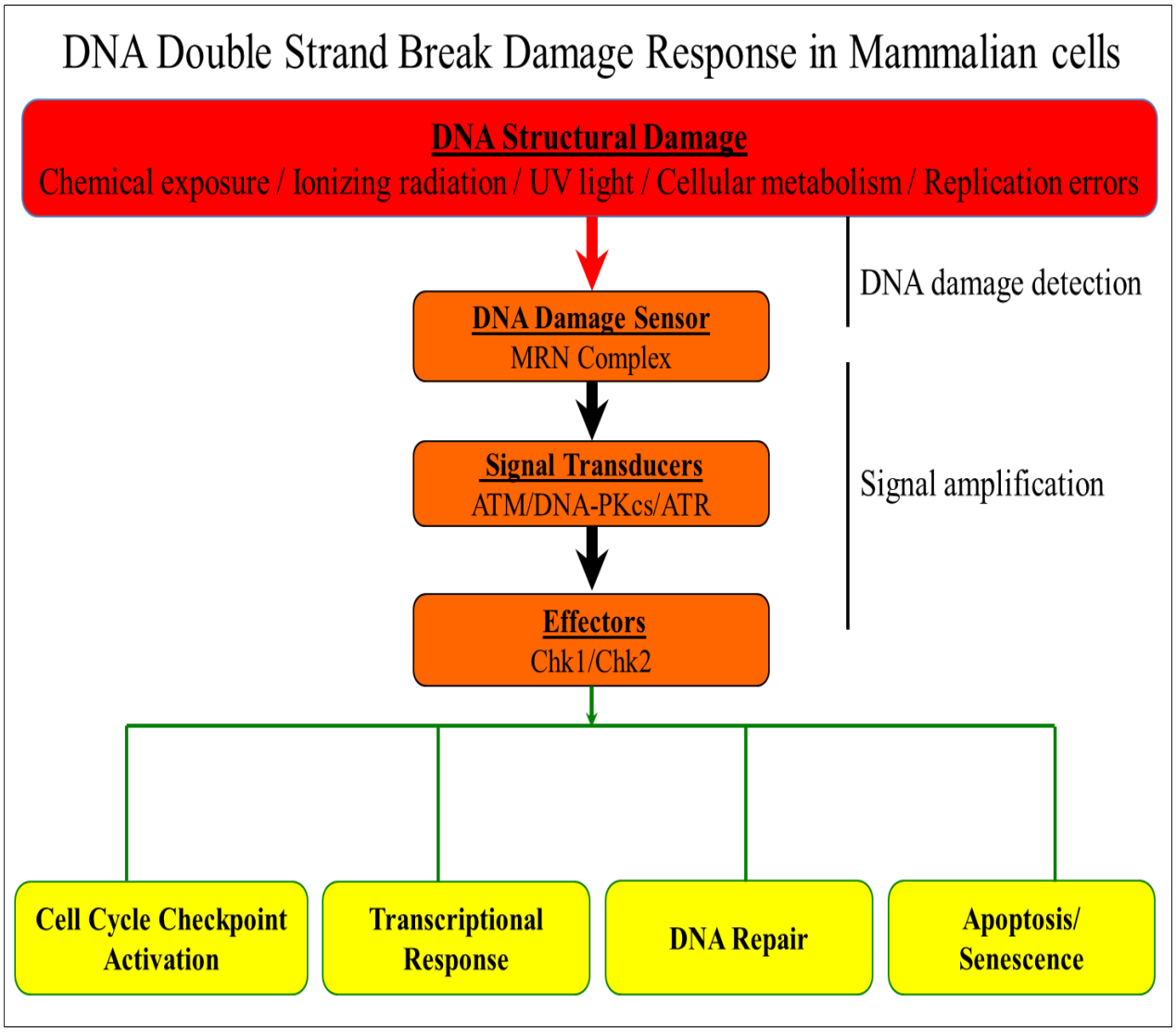

Figure I.5. DNA damage response cascade.

The MRN complex plays a key role in promoting both HR and NHEJ forms of repair (Figure I.5). Investigational data has shown that Rad50 furthers HR by structural conformations centered on the hook and coiled-coil domains and NHEJ repair suggested to be mediated by the globular domains DNA binding sites (Williams et al. 2008, Hohl et al. 2011, Roset et al. 2014). Further investigations into the Rad50 hook domain revealed that the domain is required for MRN complex function in mammals and mutation affecting hook domain residues are deleterious to survival on a cellular or organismal level (Roset et al. 2014).

NBS1 contains forkhead-associated domain (FHA), BRCA1 C terminus (BRCT) domains, and ATM phosphorylation sites. Communication to cell cycle checkpoints is initiated through interaction of Nbs1 with ATM kinase (Lee, Paull 2005a, Berkovich, Monnat & Kastan 2007) (Figure I.3), in combination with other less well understood aspects of the MRN complex (D'Amours, Jackson 2002, Stracker et al. 2004b, Lee, Paull 2005). The interactions with the kinase ATM are dynamic and inadequately understood (Lee, Paull 2005, You et al. 2005, Pellegrini et al. 2006, Williams, Williams & Tainer 2007). The activation and recruitment of the kinase ATM to DSB appears to be mediated by Nbs1, however, studies have shown that ATM makes multiple contacts with the MRN complex (Lee, Paull 2004). Once recruited by the MRN complex ATM homodimers disengage leading to active monomers which are free to migrate and phosphorylate substrate targets such as those in the G1, S, and G2-phase checkpoint signal transduction pathways (Kitagawa, Kastan 2005, Lee et al. 2013). The role of ATM phosphorylation in regulating Mre11 complex function remains to be fully elucidated and can be exemplified by studies showing

the dispensability of NBS1 for ATM activation in vitro (purified human MRE11 and RAD50) and in mouse studies (Stracker, Petrini 2011b).

Mre11 has multiple nuclease activities, including 3'-5' exonuclease, endonuclease activities and DNA hairpin cutting (Furuse et al. 1998, Paull, Gellert 1999, Paull, Gellert 1998). The nucleolytic reactions catalyzed by Mre11 can be abrogated *in vitro* without altering protein structure through mutation of an invariant histidine within the third nuclease motif (Arthur et al. 2004). Mutation of this histidine (H125N) residue in *S. cerevisiae* has been shown to cause minimal phenotypes relative to Mre11 null, suggesting that Mre11 nuclease activities serve little function during general DSB DNA repair and are primarily required during meiosis (Moreau, Ferguson & Symington 1999). However, introduction of the analogous Mre11 mutation into mice (H129N) demonstrated that the nuclease activities of Mre11 are essential for viability and play a fundamental role in DSB repair, particularly in homologous recombination (Buis et al. 2008). Additional recent work on Mre11 in *P. furiosus* and *S. pombe* has shown that Mre11 exonuclease activities can be abrogated without significantly affecting endonuclease activities, and that Mre11 exonuclease deficiency has minimal impact on cell growth and radiation sensitivity compared to loss of all nuclease activities (Williams et al. 2008). Further *in vitro* work on Mre11 suggests that Mre11 nuclease activities are required to facilitate 5' end resection prior to homologous recombination along with HerA and NurA in *P. furiosus* (Hopkins, Paull 2008). Of particular relevance, studies in *S. cerevisiae* have proposed that Mre11 catalyzes the initial 5' end resection while other nucleases, such as Exo1, further resect the single strand DNA prior to strand invasion, resulting in overhangs (Mimitou, Symington 2008). The generation of these overhangs is critical for both checkpoint activation and

subsequent homology directed repair. Current investigations have elucidated a two-step model for DNA-end resection following Mre11 complex end capture (Mimitou, Symington 2008, Zhu et al. 2008). In the initial step, the Mre11 complex and associated C-terminal binding protein (CtBP)-interacting protein (CtIP) initiate limited 5'-3' resection of the break ends. Mre11 endonuclease activity nicks the DNA; this is followed by exonucleolytic resection toward the end in a 3'-5' direction consistent with the polarity of the Mre11 activity (Neale, Pan & Keeney 2005). In tandem, exonuclease 1 (Exo 1) acts on the same strand in the opposite direction, consistent with its respective 5' -3' resection polarity. This effective bidirectional resection model is applicable to DSB resection, as Mre11 nuclease mutants have mild defects in resection in yeast and normal G2/M checkpoint in murine cells (Mimitou, Symington 2008, Buis et al. 2008). In the second step, nucleases such as Exo1 or DNA2 perform progressive 5'-3' resection, resulting in longer ssDNA structures. Providing initial detection and facilitating repair of damaged DNA ends are part of a diverse functionality MRN possesses. MRN initiates damage and checkpoint signaling responses through collaborations with ATM kinase DNA double strand break ends, 2-ended chromatin break, and collapsed replication forks, 1-ended break, through ATR and replication protein A (RPA) interactions. Replication protein A bound structures serve as the foundation to recruit the proteins that jointly facilitate the activation of the ATR, Chk1, and Chk2 kinases (replication factor C (RFC) 2-5, the 9-1-1 complex (Rad9, Hus1, Rad1), TOPBP1, and ATRIP). The activation of Chk1 and Chk2 by ATR and ATM respectively, reduce cyclin-dependent kinase activity (Kastan, Bartek 2004, Riley et al. 2008, Bartek, Lukas 2007). The ATM-Chk2-Cdc25A pathway, followed by the p53-mediated maintenance of the G1/S arrest, initiates the initial rapid arrest. These inhibitory biochemical processes

slow down or arrest cell cycle progression at the G1-S, intra-S and G2-M cell-cycle checkpoints (Figure I.4). Cell signaling of DNA double strand breaks, due to the genotoxic nature of these lesions, is of singular importance for the cell. Arrest prevents or inhibits duplication and segregation of damaged DNA, and is postulated to increase the time available for DNA repair before replication or mitosis ensues.

DNA double strand break response

DNA damage: Integrity of genetic material is essential to the health and viability of organisms and their future generations. DNA incurs a broad spectrum of damage; one of the most potent of these is the DNA double strand breaks. DNA damage occurs in all cells and the genotoxic damage is both endogenous and exogenous. Genotoxic damage from exogenous sources occurs when cells are exposed to physical damage (e.g. ionizing radiation, chemotherapeutic agents, and X-rays) or chemical agents (e.g. naturally occurring Arsenic).These genotoxicological events derived from endogenous sources is caused by multiple internal cellular pathways, including apoptosis, excision repair, oxidative damage, and depurination. A single unrepaired DSB can be sufficient to induce apoptosis (van Gent, Hoeijmakers & Kanaar 2001, Rich, Allen & Wyllie 2000). Reactive oxygen species, ionizing radiation, and chemicals that generate reactive oxygen species all produce DSBs. Double strand breaks are also a result of normal cell function in the case of Variable, Diverse, and Joining (VDJ) recombination and immunoglobulin class switching processes. In unperturbed cells, the consequences of replication fork arrest and collapse may lead to formation of DSBs.

Chromosomal aberrations induced by DSB are linked to cell death, dysfunction, uncontrolled growth, and cancer; consequently, cells expend considerable resources to rapidly and accurately repair DSBs (Shrivastav, De Haro & Nickoloff 2008). These repair processes operate through tightly regulated, complex, multistep responses resulting in modifications to large chromatin domains via ubiquitination, phosphorylation, and the interaction of numerous repair factors, folding mediators, and posttranslational modifiers (Stracker et al. 2004a).

Repair pathways: When incorrectly repaired, DNA DSBs may cause developmental disorders or cancer. To survive these breaks, eukaryotes employ an elaborate set of responses including cell cycle checkpoints, apoptosis, and pathways of DNA repair (Figure I.6) (Rupnik, Lowndes & Grenon 2010). The ability of the cell to control cell cycle progression is an important evolutionary survival mechanism and is a major factor in the success of the repair mechanism. (Sonoda et al. 2006). The primary pathways of DSB repair are non-homologous end joining (NHEJ), which entails direct ligation of DNA ends, and homologous recombination (HR) in which sequences homologous to the broken region are used as a template for repair (Shrivastav, De Haro & Nickoloff 2008). Appropriate repair pathway choice during the different stages of the cell cycle is of critical importance for the preservation of genetic fidelity.

Based on the need for sister chromatids to be present, homologus recombination has a defined role during S and G2 phases of the cell cycle (Figure I.7). Conversely, non-homologous end joining does not require the presence of sister chromatid to join broken ends of DNA, but ligates the ends together regardless of the nucleotide loss and independent of cell cycle phase. These repair pathways typically function in unique cellular

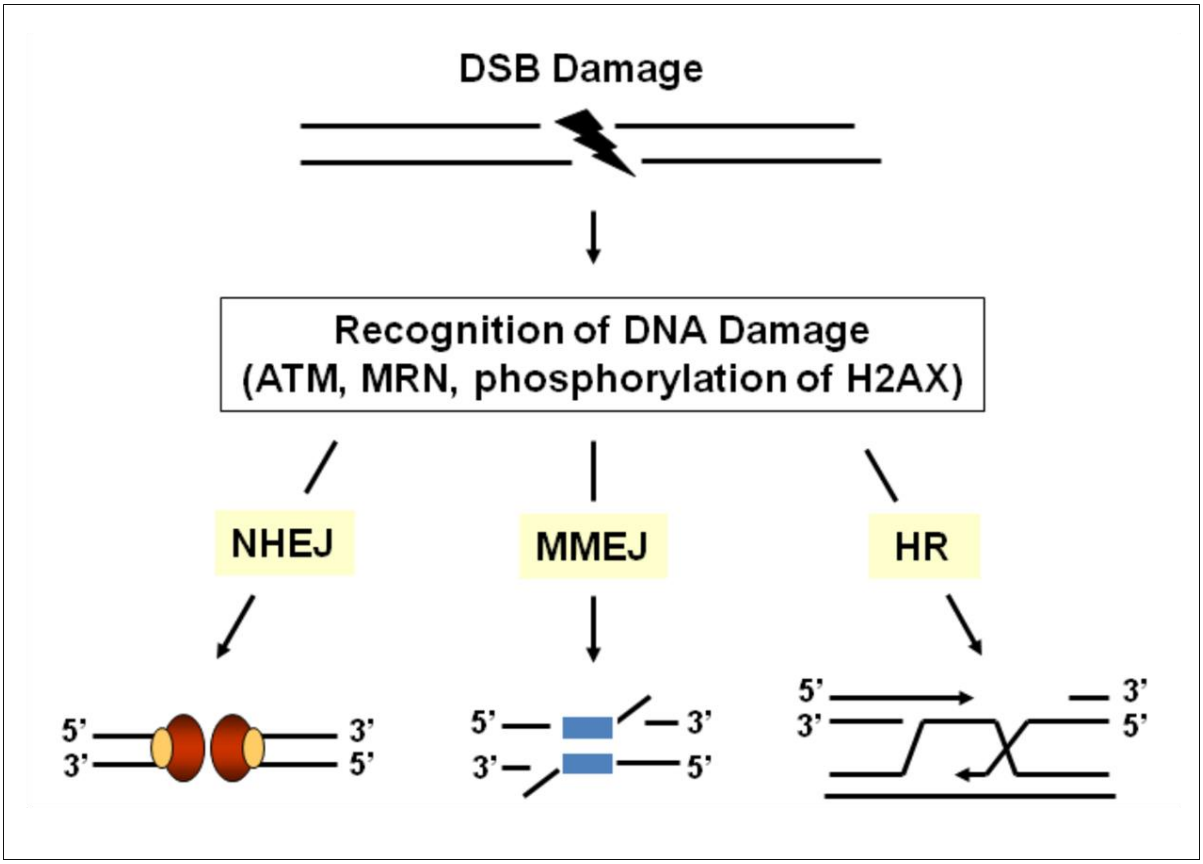

Figure I.6. Pathways involved in the maintenance of genetic integrity after exposure to ionizing radiation. Non-homologous end joining (NHEJ), an error prone pathway, entails direct ligation of broken DNA ends. Microhomology mediated end joining (MMEJ) an error-prone method of repair distinguished by use of 5-25 base pair microhomologous sequences to align the broken strands before joining. Homologous recombination (HR) a high fidelity method of repair occurring in S and G2 phases of cell cycle based on presence of sister chromatids for template DNA repair. Adapted from Kim et al, New Research Directions in DNA Repair, 2013.

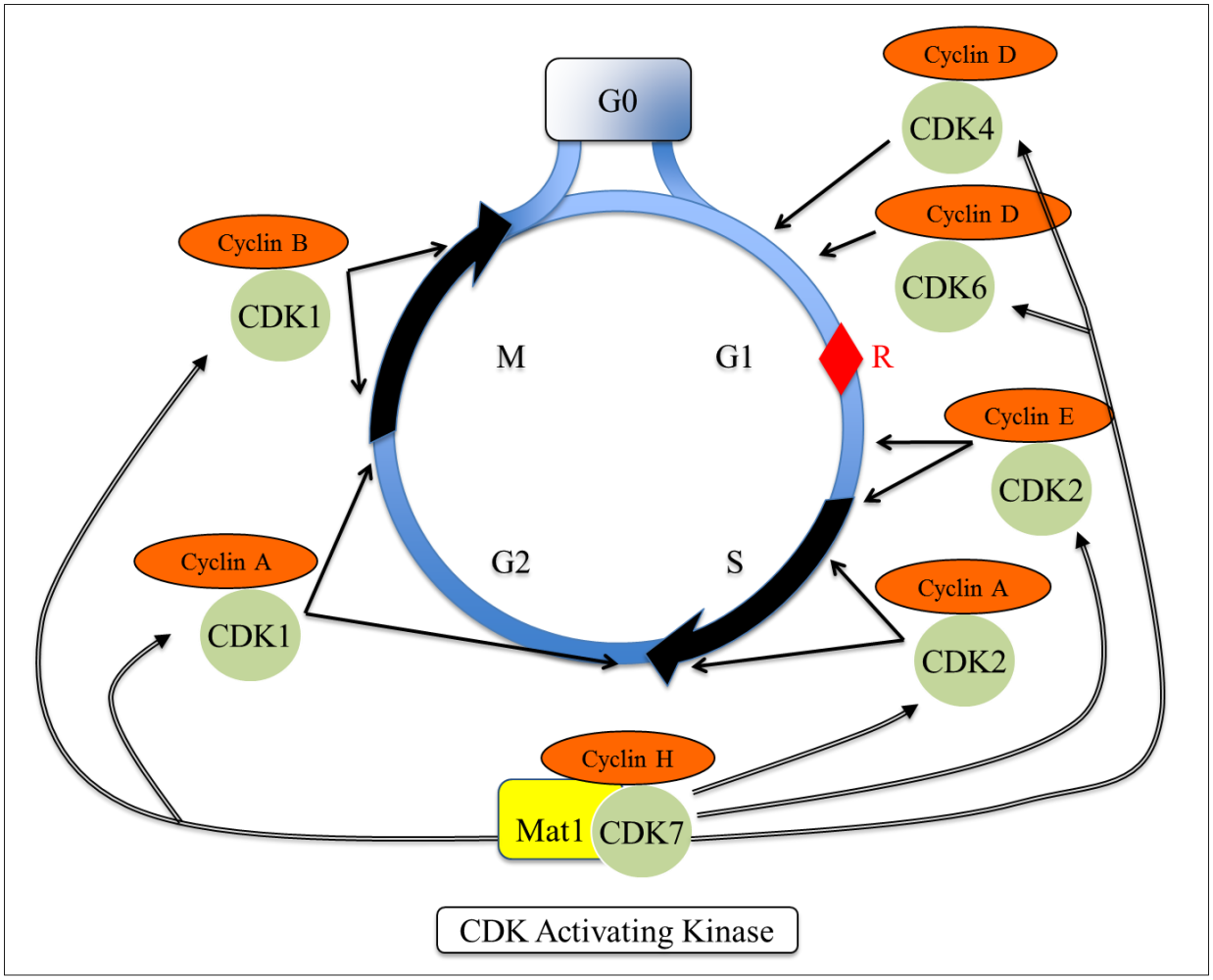

Figure I.7. Roles of CDK-cyclin complexes in mammalian cell cycle. CDK4-CyclinD, CDK6-cyclinD regulate quiescent cells (G0) - Growth phase 1 (G1) transition, and early G1 phase, in proliferating cells. Cyclin E associates with CDK2 forming a complex to regulate progression from G1 into synthesis phase (S). Cyclin A binds with CDK2 and this complex is required for S Phase progression. In late G2 and early M, cyclin A complexes with CDK1 to promote entry into mitosis (M) phase. CDK-activating kinase (CAK) phosphorylates a conserved threonine residue (Thr-160/161) in the activation loop (T-Loop). Restriction point (R) represents the cells independence from growth factors for progression through the remainder of the cell cycle.

situations, although there are instances in which both processes may be appropriate. The requirement of a homologous duplex for this template repair limits the HR biochemical repair pathway to S and G2 phases of the cell cycle. HR complexes such as BRAC1 and CtIP are specific for S and G2 phases, and require cyclin-dependent kinase 2 (CDK2) and corresponding regulatory cyclin A subunit for function (Yu, Baer 2000, Germani et al. 2003). This evidence is suggestive that CDK2/cyclin A participates in regulation of HR-mediated DSB repair (Ruffner et al. 1999, Sonoda et al. 2006b, Sartori et al. 2007, Buis et al. 2012). Mammalian cells deficient in CDK2 have mildly defective HR repair, consistent with CDK2 influencing CtIP (Deans et al. 2006).

Recent studies have elucidated that homologous recombination capacity is controlled by the ability of the cell to catalyze resection throughout the cell cycle. This control begins at the nucleolytic resection of the double strand break ends to generate single stranded DNA with 3' termini (Mimitou, Symington 2011). In mammals, initiation of homologous recombination DNA end resection is dependent on the MRN complex (Figure I.3.) While required, the nuclease activity of Mre11 is insufficient for the resection of double strand break DNA ends. Therefore, interaction of the tumor suppressor BRCA1 and CtIP are required to enhance Mre11 nuclease activity, resulting in effective and efficient resection (Sartori et al. 2007, You et al. 2009).

The generation of ssDNA as a substrate for Rad51 binding allows HR protein machinery to execute strand invasion of a homologous donor chromosome. Strand invasion forms a displacement loop (D-loop) structure (Krogh, Symington 2004, San Filippo, Sung & Klein 2008). The formation of the nascent D-loop structure allows DNA synthesis to proceed. Within the HR repair pathway, DNA double strand breaks is repaired by various

methods, including synthesis-dependent strand annealing (SDSA) and double-strand break repair. In the SDSA pathway, the D-loop is unwound and the liberated ssDNA strand anneals to the complementary ssDNA strand that is associated with the other double-strand break end. The remaining portion of the DSB is repaired by gap filling DNA synthesis and ligation, resulting in double-strand break repair wherein participant chromosome arms are not exchanged during repair (i.e. non-crossover). The double-strand break repair model begins with strand invasion into a homologous sequence, forming a D-loop intermediate, followed by polymerase extension from the 3' termini of the invading strand. Progression of the double-strand break repair model includes capture of second double strand break by annealing to the extended D-loop and formation of two crossed Holliday junctions (a mobile junction between four strands of DNA (Stahl 1994)), leading to resolution and producing crossover and non-crossover products(San Filippo, Sung & Klein 2008, Mimitou, Symington 2009, Mimitou, Symington 2011).

Capacity for Homologous Recombination regulated by Mre11 in unperturbed cell: Homologous recombination is a high fidelity repair mechanism requiring sister chromatid templates for repair, corresponding to S and G2 phases of the cell cycle. The formation of the tripartite complex, MRN-CtIP-BRAC1, during S-phase and subsequent dissolution during G2 defines the resection capacity (i.e. homologous recombination capacity) of the cell. MRN interacts in the tripartite complex through the NBS1 subunit of the complex (Chen et al. 2008, Yun, Hiom 2009). A recent investigation by our laboratory revealed, Mre11, in addition to NBS1, participates in the important formation of the tripartite complex which is important for facilitating double strand break resection to generate ssDNA that is needed for homologous recombination-mediated double strand

break repair (Buis et al. 2012). These data demonstrated Mre11 contribution to the stability of CtIP, wherein, the absence of Mre11 leads to deficient levels of the CtIP protein (Buis et al. 2008). Furthermore, altered or abnormal Mre11, C-terminal truncation (e.g. ATLD1 78 amino acid truncation) or addition (e.g. Mre11 fusion protein with 54 amino acids added), resulted in CtIP deficiency with in the cell (Buis et al. 2008). These data support a role for Mre11 providing stability to CtIP. In order for CtIP to interact with the tumor suppressor BRCA1, the S-phase CDK is required to phosphorylate CtIP (Ruffner et al. 1999, Yun, Hiom 2009, Buis et al. 2012) . Therefore, an investigation of CDK2 influence on CtIP protein stability was conducted. These studies, by chemical or genetic means, perturbed CDK2 resulting in reduced biological activity or the absence of protein, respectively. Collaborating evidence reveals CtIP levels are influenced by CDK2 (Wu-Baer, Baer 2001, Chen et al. 2008). With these data in mind, an association or interaction between Mre11 with CDK2 was hypothesized as a means to stabilize CtIP for the purpose of tripartite complex interaction and therefore resection capabilities for HR. The experiment in mammalian cells competent in CDK2 and through molecular biology techniques manipulated expression levels of Mre11 (reducing endogenous Mre11, over expressing ATLD1 truncated), demonstrated CtIP phosphorylation occurred only when unaltered Mre11 was present in the experimental conditions; suggestive of CtIP reliance on an intact Mre11 C-terminus (Buis et al. 2012). To determine the extent of the association between Mre11 and CDK2 a yeast 2-hybrid assay (Y2H) (established biological method for ascertaining/evaluating protein-protein interaction) was conducted. The assay demonstrated unaltered Mre11 was interacting with CDK2. In follow up co-immunoprecipitation studies conducted in mammalian cells displayed Mre11 interaction

specific to CDK2. Additionally, these mammalian cell based co-immunoprecipitation experiments with CDK2 exhibited an impaired ability to interact with the C-terminal truncated ATLD1 (Mre11 C-Terminal truncation of 78 amino acids) protein, furthermore, correlative to mammalian co-immunoprecipitation data, the yeast 2-hybrid assay revealed C-terminal Mre11 truncations significantly compromised CDK2 interaction(Buis et al. 2012). Interestingly, Y2H experiments conducted using the ATLD1 truncated portion (last 78AA of the C-terminus) demonstrated interaction competence (Buis et al. 2012). Therefore, the modulation of resection capacity is directly correlated with the presence of sister chromatin template material in S-phase. The increase in resection capacity regulated by Mre11-CDK2 interaction results in cell cycle specific phosphorylation of CtIP thereby enabling high fidelity homologous recombination of DNA double strand breaks to become the predominate repair method in S and G2 phases in the unperturbed cell. The impact of genotoxic stress on the Mre11-CDK2 interaction has not been investigated. In addition to HR repair, NHEJ is the other possible mechanism for DSB repair. (Kanaar, Wyman & Rothstein 2008).

While NHEJ is highly efficient, it is imprecise in nature, making it prone to introducing mutations. NHEJ is active throughout the entire cell cycle for repairing non-replication associated breaks and is the preferred mode of repair and predominantes in the G0, G1 and early S phase of the cell cycle, while HR occurs during S-G2 phase (Kanaar, Wyman & Rothstein 2008). The simplest breaks are those with a chemical composition allowing direct ligation. In these circumstances, joining requires the highly conserved core components of NHEJ: the Ku70/80 heterodimer, the DNA-dependent protein kinase catalytic subunit (DNA-Pkcs), $XRCC_4$, XLF, and DNA ligase IV (Lees-Miller, Meek 2003,

Lieber et al. 2003). The NHEJ biochemical pathway does not require a 3' overhang and accepts a variety of end products as substrates for ligation. A DSB is detected by the Ku80-Ku70 heterodimer, which in turn recruits the DNA-Pkcs complex and activates its kinase activity (Dynan, Yoo 1998, Featherstone, Jackson 1999). Increasing evidence suggests that DNA-Pkcs functions as a regulatory component of NHEJ, potentially facilitating and regulating the processing of DNA ends (Misteli, Soutoglou 2009). DNA-Pkcs also recruits and forms a complex with the broad-specificity nuclease Artemis to process DNA ends, an important step for NHEJ (Moshous et al. 2001). The formation of the Artemis/DNA-Pkcs complex provides 5' and 3' endonucleolytic activity in addition to the ability to open secondary hairpin structures (Moshous et al. 2001).

The core biochemical process of NHEJ involves the DNA ligase/IV-XRCC$_4$-XLF complex binding to broken DNA ends (Lees-Miller, Meek 2003, Meek et al. 2004, Yano et al. 2008). This complex covalently links the ends together through the XRCC$_4$-XLF and DNA ligase IV-mediated phosphodiester bonds. In addition to nucleases, DNA polymerases provide increased diversity in the types of DNA ends that can be joined. DNA polymerases pol-mu and pol-lambda are trans-lesion polymerases. Pol-mu DNA polymerase can add nucleotides to the ends of DNA in a template-independent method; pol-lambda readily uses template primers with limited base pair homology, adding nucleotides to one end of the break, using the template strand before it is covalently sealed (Dominguez et al. 2000, Bebenek et al. 2003, Ma et al. 2004). Rejoining of DNA breaks by NHEJ does not require accuracy at the genomic sequence level to avoid deleterious chromosomal translocations; thus NHEJ is highly efficient, but imprecise by nature, making it prone to mutations.

Cell cycle regulation

The cell cycle is the process by which a cell ensures correct replication, and is central to the understanding of all life. For a cell to survive, it is essential that at division it receive full complement of all the components necessary for its survival. Of particular importance is the hereditary material DNA, which needs to be replicated and segregated in each cell cycle. Transitions between cell cycle phases are controlled by a group of protein kinases, termed cyclin dependent kinases (CDK) and their regulatory cyclin subunits (Nurse 1997, Doree, Hunt 2002). The mammalian cell cycle consists of four stages: S phase (CDK2) where DNA synthesis occurs, mitosis (CDK1) during which the actual cell division takes place, and two gap phases G1 and G2 (CDK4, CDK6 and CDK1) during which required cell components are synthesized and assembled (Figure I.7).

In animal cells, CDK2 is considered to be the key protein in the actual onset of DNA replication. Cyclin E and cyclin A sequentially activate CDK2 to initiate S phase. Of particular importance is the hereditary material DNA, which needs to be replicated and segregated in each cell cycle. In the normal cell cycle, S phase is always preceded by M phase and M phase does not begin until the previous S phase is completed (Nurse 1997). These two phases are critical and rigorously ordered in a cyclic process, common to all eukaryotes; cell cycle phases S and M ensure that cellular components are properly duplicated and segregated. Progression of eukaryotic cells through these major transitions is mediated by sequential activation and inactivation of CDKs, a highly conserved family of protein serine/threonine kinases (Morgan 1995). CDK protein levels remain stable while cyclin levels rise and fall during the cell cycle, and in this manner CDK apoenzymes are partially activated by heterodimer complex formation with regulatory cyclin subunits (Evans et al. 1983, Pines 1991)

Cell cycle functions of cyclins and CDKs are outlined in Figure I.7. Three D-type cyclins (cyclin D1, D2, D3) bind to CDK4 and to CDK6, and CDK-cyclin D complexes are essential for entry in G1 (Sherr 1994). Another G1 cyclin is cyclin E, which associates with CDK2 to regulate progression from G1 to S phase (Sherr 1993, Morgan 1995, Ohtsubo et al. 1995). Cyclin A binds with CDK2 and this complex is required during S phase (Girard et al. 1991, Walker, Maller 1991, Morgan 1995). Cellular CDK levels remain at relatively constant levels throughout the normal cell cycle; most of the regulatory controls on catalytic activity are post-translational. Cyclin binding is essential for complete activation of the CDK due to the conformational changes that occur in the heterodimer complex allowing phosphorylation at a conserved threonine residue (Thr-160/161) in the activation loop (T-Loop). The enzyme responsible for this activating phosphorylation is itself a CDK and has been designated CAK for CDK-activating kinase. CAK is a multi-subunit enzyme composed of a catalytic subunit (CDK7) and a regulatory subunit (cyclin H) (Desai et al. 1995, Morgan 1995). CAK is capable of activating all of the major CDK-cyclin complexes involved in vertebrate cell cycle control. During a normal cell cycle, activation of phosphorylation at the threonine residue by CAK tends to oscillate in parallel and enhance the binding of cyclins (Gu, Rosenblatt & Morgan 1992, Jeffrey et al. 1995). Furthermore, CDK activity is regulated by phosphorylation and dephosphorylation on a threonine and a tyrosine residue (Thr14, Tyr15 in CDK1 and CDK2; Tyr 24 in CDK6) near the N-terminus. Phosphorylation of Thr-14 and Tyr-15 parallels the rise in cyclin levels (CDK2/ A, CDK1/ B) that occurs as cells approach transition from S to G2 and G2 to M, respectively. Inhibitory phosphorylation by Wee1 and Myt1 kinases maintain the inhibitory state of the CDK-cyclin complex until dephosphorylation at the transitional point in cell cycle. For example,

CDK2/cyclin A heterodimer activation requires the phosphatase enzyme Cdc25 to dephosphorylate CDK2 at two sites, activating the heterodimer and promoting further progression through the cell cycle (Lew, Kornbluth 1996, Morris et al. 2002, Shreeram, Hee & Bulavin 2008). The kinase activity of the CDKs appears only when the phosphatase, encoded by the gene *Cdc25,* dephosphorylates these two amino acids, thereby, increasing the activity of the CDK. There are three Cdc25 isoforms: A, B and C. Cdc25A is involved in transition G1/S; Cdc25B undergoes activation during S-phase, and Cdc25C activates CDK1-cyclin B during entry into mitosis.

CDK activity can be counteracted by negative regulation of CDK proteins, called CDK inhibitors (CKI). Two families of CDK inhibitor proteins antagonize CDKs by binding to and inhibiting their kinase activity (Sherr 1994, Sherr, Roberts 1995). CKIs may bind to the CDK alone or to the CDK-cyclin complex in order to regulate CDK kinase activity. One of these families is INK4 ($p16^{INK4a}$, $p15^{INK4b}$, $P18^{INK4b}$, $p19^{INK4d}$), which specifically inactivate G1 CDK4 and CDK6. The INK4 family of CKI forms stable complexes with the CDK enzyme before cyclin binding preventing association with cyclin D (Sherr, Roberts 1995, Canepa et al. 2007). The second family of regulators includes Cip and Kip ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$), and in contrast to INK4 binds to CDK-cyclin complexes (Polyak et al. 1994, Lee, Reynisdottir & Massague 1995, Harper et al. 1995, Russo et al. 1996). The Cip/Kip proteins act as negative regulators of CDK2-cyclin E/A and CDK1-cyclin B holoenzymes in a 1:1 stoichiometry, and also act as positive regulators of CDK4/6-cyclin D by allowing assembly of the complex in early G1 (Hannon, Beach 1994, Cheng et al. 1999). In a second step, when the number of p21 and p27 molecules attaching to nascent CDK-cyclin D complexes progressively increases, the effect of inhibition is brought into play on CDK-cyclin D,

thereby relieving CDK2-cyclin E from its main inhibitory function. Interestingly, CDK2-cyclin E is capable of phosphorylating the p27 bound to the complex, thus facilitating the degradation of this inhibitor by the proteasome and triggering its own activation, resulting in cell cycle progression. This unique biochemical mechanism creates a situation where p27 is both inhibitor and substrate of CDK-cyclin E. The factors that determine which of these functions p27 perform remains unclear. Upon the degradation of p27, INK4 proteins compete with D-type cyclins for CDK4/6 binding. Therefore, in cycling cells, a gradient of Cip/Kip proteins between CDK4/6 and CDK2 is established as cells progress through G1 with p21 and p27 alternately acting as positive or negative regulators of CDK activity (Carnero, Hannon 1998, Cheng et al. 1999, Trimarchi, Lees 2002, Massague 2004).

CDK2 function in cell cycle and DNA damage response

Assigning functionality to individual CDKs remains a challenge. A majority of the information derived on CDK structure and function is based largely on studies of the prototypical CDKs of *S. pombe* (Cdc2), *S. cerevisiae* (CDC28), and vertebrates (CDK1 and CDK2). The crystal structure of metazoan CDK1 remains undefined and research has relied on the crystal structure of CDK2 as proxy. Various biochemical pathways of cell cycle progression have been called into question based on studies revealing the existence of functional redundancies between CDK members. These redundant activities may mask essential CDK activities required for Restriction-point passage, G1/S, or G2/M progression in either normal or cancer cells (Ortega et al. 2003, Berthet et al. 2003, Tetsu, McCormick 2003).

Studies have demonstrated the dispensability of the interphase CDKs (iCDK) (CDK4, CDK6, and CDK2), and evidence suggestively points to CDK1 as the absolute essential CDK in

the group. However, whether CDK1 is the dominant regulator of the entire cell cycle in normal cells remains to be elucidated (Santamaria et al. 2007, Hochegger, Takeda & Hunt 2008). Conversely, mice with proficient iCDKs and deficient in the gene *CDK1* die during early embryogenesis, suggesting no other CDK (CDK4, CDK6, or CDK2) can compensate for the loss (Satyanarayana et al. 2008). These data suggest that CDK1 is the essential cell cycle regulator. However, studies in normal cells indicate that the responsibility of CDK1 remains as a regulator of mitosis as demonstrated by appreciable binding with cyclin A in mid S-phase, and investigations resulting in the elimination of iCDKs allows compensatory biochemical interactions resulting in atypical association with cyclins D, E, and A (Doree, Hunt 2002, Aleem, Kiyokawa & Kaldis 2005, Maude, Enders 2005, Merrick et al. 2011). Therefore, CDK1 function as the universal regulator of cell cycle remains to be further studied. Research to define CDK roles using small molecule inhibitors to delineate CDK1 from CDK2 in unperturbed cells has found limited utility due to the lack of specificity with current chemical entities. A recent study using a chemical-genetic approach in human cells revealed a requirement for CDK2 activity in cell proliferation that was until then undetected in gene knockout and RNAi-based investigations (Merrick et al. 2011). The study discovered that CDK2 is a non-redundant, rate-limiting regulator of cell cycle progression in eukaryotic cells. The study also recapitulated findings that CDK2 influences Restriction point passage through catalytic activity. However, the mechanism by which CDK2 promotes Restriction point passage and S phase entry remain (Merrick et al. 2011). Examinations demonstrating the importance of CDKs in cell cycle transitions have been conducted using expression of dominant negative mutants, introduction of inhibitory antibodies, or small molecule inhibitors caused phase-specific cell cycle arrest (Hochegger, Takeda & Hunt 2008, Harashima, Dissmeyer & Schnittger 2013). A limitation

of this type of study is the perturbation of the biochemical pathway in the cell leading to a reallocation of resources. This adaptation to the new cellular environment can cause ectopic cyclin-CDK complexes being generated to compensate for the missing enzyme. Compensatory activities between CDKs and cyclins were discovered during these analyses and have impeded the definition of precise regulatory mechanisms and identification of exclusive functions of the CDKs. Compensatory activities have additionally obstructed the precise identification of non-redundant cell cycle governing mechanisms.

A critical part of cell cycle progression is the assurance that governance over the progression is intact, preventing replication or segregation of a damaged or otherwise compromised genome. To accomplish the task of regulation, the cell monitors DNA fidelity throughout the cell cycle to minimize the accrual of damage. In addition to arresting cells with compromised DNA, these checkpoints are activated in response to incomplete DNA replication or DSB to mediate repair of the damage (Hartwell, Weinert 1989, Yata, Esashi 2009). The aforementioned investigations revealing dispensability of iCDKs (CDK2) along with studies in *CDK2*$^{-/-}$ null mice revealed that the absence of CDK2 from the cell cycle regulatory system was not deleterious to cellular viability. In addition, the absence of CDK2 was not essential for the G1/s checkpoint, leaving CDK2's role in the checkpoint control pathways in question.

The role for CDK2 in the DNA damage repair pathway remains unclear, as current data suggest that CDK2 is dispensable for viability. A majority of the data supports a non-redundant role for CDK2 in the DNA damage repair checkpoint response. However, evidence exists against requirement for CDK2 in DNA damage repair, based on a study in *CDK2* null cells where the G1/S and G2/M checkpoints appear to remain fully functional (Martin et al. 2005, Satyanarayana, Hilton & Kaldis 2008). Current data from the literature does support cell cycle

G1 and S-phase checkpoint enforcement of an inhibitory signal on CDK2 (Abraham 2001, Neganova et al. 2011, Merrick et al. 2011). Data further demonstrates CDK2 involvement in G2/M transitions (Gu, Rosenblatt & Morgan 1992, Aleem, Kiyokawa & Kaldis 2005, Chung, Bunz 2010a, Harashima, Dissmeyer & Schnittger 2013). Literature remains conflicted on how functions of different CDKs are coordinated during unperturbed cell division or after genotoxic stress in mammalian cells. The view of the cell cycle mechanisms has been repeatedly altered by extensive study of the subject.

Research evaluating CDK2 in the DNA damage repair and DNA repair is increasing, but remain limited considering the involvement in control of transcription, cell cycle progression, and capacity to phosphorylate various substrate. CDK2 is a relevant therapeutic target in cancer, a disease of sustained cellular proliferation, based on unperturbed cell function as well as new data revealing roles in DDR and indirect DNA repair functions.

Recent published data supports an emerging role for CDK2 in DNA damage repair and assurance that DNA repair can occur. Studies investigating the G1 to S checkpoint activation in mammalian cells reveal that CDK2 plays an important role in this DNA damage repair (Deans et al. 2006, Satyanarayana, Hilton & Kaldis 2008, Neganova et al. 2011). Investigations into the S-phase checkpoint have revealed CDK2 checkpoint participation in the intra-S-phase checkpoint (Zhu et al. 2004a). In the final CDK2-related cell cycle checkpoint, G2/M, CDK2 is implicated in two separate pathways for checkpoint control by the activation of the ATR-CHK1-Cdc25 biochemical pathway. Furthermore, recently published investigation on this pathway has revealed that CDK2 activates the G2/M checkpoint in a p53-independent manner. Loss of CDK2 alters regulation of several proteins known to regulate S-phase progression, Cdc25A, Chk1, Cdc6, and ATRIP, which allow the cell to effectively control the G2/M checkpoint through the

ATR-Chk1 pathway. This report demonstrated that CDK2 facilitates a DNA damage promotion of the pathways by the formation of an active ATR complex through ATRIP and CDC6 phosphorylation. These data suggest that after IR treatment, the inherently unstable CDC6, a replication licensing protein, is stabilized by CDK2, which leads to a genotoxic stress induced Chk1 phosphorylation by ATR, resulting G2/M checkpoint activation (Chung, Bunz 2010b). Taken together, these studies illustrate that CDK2 is a target for ATR-dependent checkpoint response in addition to functioning as a direct regulator of the ATR-ATRIP checkpoint kinase complex.

Efforts to resolve questions regarding the nature of cellular checkpoints in mammals and the integration of checkpoint pathways with cell proliferation controls will facilitate the understanding of how cells sense and respond to DNA damage in order to maintain genome integrity and stability. The CDKs regulate cell cycle and play roles in tumorigenesis. Despite persistent research, only a limited number of CDK substrates have been identified (Chi et al. 2008). To acquire comprehensive knowledge of CDK function, substrate identification is essential. Of the known and implicated CDK2 substrates, CtIP is one of the best characterized (Huertas et al. 2008, You et al. 2009a, Mimitou, Symington 2011). Seminal experiments in BRCA1 interacting proteins discovered and confirmed a BRAC1-CtIP interaction (Yu et al. 1998). Continuing investigations led to the discovery that CtIP is expressed in a cell cycle-specific pattern. During G1, protein levels are low and increase dramatically as cell cycle progression traverses the G1/S boundary. Additionally, the interaction was shown to be stable after genotoxic stress, Furthermore, the reappearance of BRCA1 foci after genotoxic stress suggests an alteration of the function of BRCA1 to maintain genomic integrity of replicating DNA repair and/or cell cycle checkpoint control (Yu, Baer 2000).

Recent inquiries have identified CDK2 as the CDK responsible for the cell cycle-dependent stability of CtIP. Studies have also revealed the dependence on NBS1 of the DNA sensor complex MRN and BRCA1 for maximum resection capacity during HR (You et al. 2009, Yun, Hiom 2009). Essentially, CDK2 phosphorylation of CtIP associated with the formation MRN-CtIP-BRAC1 complex defines the resection capacity during S-G2 cell cycle phases, and results in the modulation of double strand break repair from non-homologous end joining to homologous recombination. These findings demonstrate a biochemical pathway for DNA resection capacity and thus homologous recombination capacity, directed by a cell cycle regulator (Huertas et al. 2008, Cerqueira et al. 2009).

An important question in the DNA damage repair research field remains concerning the underlying biochemical mechanism for the transition from DSB sensing to resection. Providing more information on these complex biochemical pathways in mammalian cells, the Ferguson laboratory conducted studies to further understand the MRN-CtIP relationship using the ATLD genomic instability syndrome as a starting point of the investigation. This investigation revealed a finding that Mre11 directly interacts with CDK2 in an unperturbed cell. The interaction was further queried to reveal that Mre11 is responsible for the regulation of CtIP phosphorylation through the direct interaction with CDK2. Mre11 is therefore responsible for the capacity of resection required for homologous recombination in unperturbed mammalian cells, which is ATM independent (Wu, Lee 2006, You et al. 2009b, Buis et al. 2012).

An ATM dependent pathway describes the phosphorylation of CtIP by ATM kinase in response to DSB damage (Li et al. 2000). This investigation shows that in response to DSBs, CtIP receives a signal from ATM after MRN-dependent DSB sensing and signaling. The ATM activation results in CtIP binding to damaged DNA through its DNA-binding motif. Once bound,

CtIP cooperates with MRN to promote resection at DSBs, which leads to ssDNA that is then stabilized by RPA proteins. The results surrounding ATM phosphorylation will require further study to define functional relationships of CtIP, CDK, and BRCA1. This is in part due to the inconsistencies between functions in different cell types. For example, ATM phosphorylation sites identified in human CtIP appear to be dispensable (You et al. 2005, You et al. 2009b). The differing reports are consistent in the phosphorylation of CtIP leading to transcription of cell cycle inhibitors and DNA damage response proteins, p21 and Gadd45, respectively (Li et al. 2000, Wu-Baer, Baer 2001).

DNA damage checkpoints

Function of checkpoints: A perplexing diversity of lesions arises in DNA from three main causes. First, environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous genotoxic chemicals cause alterations in DNA structure, which, if left unrepaired may lead to mutations that increases cancer risk. For example, Fukushima and Chernobyl nuclear reactor failures released high levels of radioactive genotoxic compounds into the environment. Compounds such as $Iodine^{131}$, $Cesium^{137}$, and $Plutonum^{239}$ having been released are capable of causing genotoxic stress to a cell or organism through internal or external exposure. Second, normal cellular metabolism constitutes a permanent insult to DNA integrity from within the cell. Metabolites include reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation.

The outcome of DNA double strand break damage is diverse and predictably adverse to the cell. Acute effects arise from disturbed DNA integrity, triggering cell-cycle

arrest or cell death. Long-term effects (i.e. genotoxic exposure) result from irreversible mutations contributing to oncogenesis. Since the amount of undamaged DNA vastly exceeds that of damaged DNA, DNA damage sensors devote far more time associated with undamaged DNA versus damaged DNA. Discrimination between undamaged and damaged DNA is not absolute. Rather, the biochemical processes and signal transduction pathways for recognition of DNA integrity and the DNA damage checkpoints are operative at all times and operate based on the magnitude of the DNA damage. These checkpoints provide for a controlled, temporary arrest at specific stages of the cell cycle to correct possible defects. Genotoxic stress, such as ionizing radiation, has the potential to induce arrest in the G1, S, and G2 phases of the cell cycle. There are multiple pathways involved in the maintenance of genetic integrity after exposure to ionizing radiation (Figure I.5), most of which are related to the cell cycle (Hartwell, Kastan 1994).

Each checkpoint provides a specific function for the cell to maintain integrity of genetic material. The G1 checkpoint prevents the replication of damaged DNA before entry into S phase, and the G2 checkpoint prevents the segregation of aberrant chromosomes during M phase. Therefore, checkpoint reactions may be greatly amplified, halting cell cycle progression or conversely, may have a diminished response with no outward effects on cell cycle progression (Figure I.6).

Molecular components of the DNA damage checkpoints: Key DNA damage response signaling components in mammalian cells are the protein kinases ATM and ATR, which are recruited to and activated by DSBs and replication protein A RPA-coated ssDNA, respectively (Shiloh 2003, Bartek, Lukas 2007). The structurally dynamic MRN complex allows for versatile roles within the DDR, including the initiation of signaling cascades

(Williams, Lees-Miller & Tainer 2010). Targets of ATM/ATR in the DNA damage response and known as the effectors of the signal transduction cascade are CHK1 and CHK2, act to reduce cyclin-dependent kinase activity through various post-translational mechanisms to arrest cell cycle progression (Morgan 1995, Bartek, Lukas 2001, Bartek, Lukas 2007). Inhibition of cell cycle progression at transition points G1-S, intra-S and G2-M cell cycle boundaries increase time available for DNA repair.

Summary of novel findings

Our laboratory recently elucidated a role for Mre11 to facilitate DSB repair by interaction with CDK2 in an unperturbed cell. The interaction of CDK2 with Mre11 allows for the mediation of the capacity for resection as the cell progresses through S-phase. Our laboratory has uncovered the connections between normal cell cycle processes (CDK2) and DDR machinery (Mre11), allowing further exploration of this relationship after genotoxic stress. We present in chapter II our examination of the Mre11 - CDK2 complex response to IR induced genotoxic stress.

There is evidence in the literature suggesting that CDK2 plays an indirect role in DNA repair by activation of several proteins involved in repair pathways, and this is supported by a number of studies in cancer cell lines showing that CDK knockdown or inhibition delays the normal activation of DNA damage signaling, slows the rate of DNA repair, and prolongs the persistence of DSBs (Gu, Rosenblatt & Morgan 1992, Yu, Chen 2004, Zhu et al. 2004b, Esashi et al. 2005, Chen et al. 2008, Chung, Bunz 2010a, Soffar et al. 2013). Therefore, we investigated whether CDK2 in response to genotoxic stress may undergo changes in phosphorylation state as well as kinase activity level. The apical kinase plays an

important role in the signal transduction of DSBs and interacts with the NBS1 component of the MRN complex.

Based on the recent evidence from our lab and others, the Mre11 complex is associated with chromatin during S and G2 phases at sites of DNA replication (Maser et al. 2001, Mirzoeva, Petrini 2003, Buis et al. 2012). The biological activity conveyed by Nbs1 phosphorylation and Mre11 complex functions at sites of DNA replication are not fully understood. Therefore, we investigated the role of ATM in the Mre11-CDK2 complex interaction after IR-induced DSB. It is known that the MRN-CDK2 complex exists in an unperturbed cell; we assessed whether the recruitment and activation of ATM in response to DSB formation alter the physical interaction of these two proteins. A role for CDK2 is emerging in the literature as a DNA repair and response protein (Nyberg et al. 2002, Esashi et al. 2005, Falck et al. 2002, Chung, Bunz 2010a, Lovejoy et al. 2009). From this, we know that CDK2 in an unperturbed cell undergoes post-translational modifications such as cyclin association and phosphorylation at thr160 on the activation T-loop to promote cell cycle, progression. This prompted us to query whether S-phase CDK2 is modified by the induction of DNA double strand break inducing genotoxic stress i.e. ionizing radiation from cesium source. Further, we assessed whether the master double strand break kinase, ATM, influences or modifies CDK2 biological activity and phosphorylation status.

From this investigation we concluded that in response to genotoxic stress a rapid disruption of the Mre11-CDK2 complex occurs in an ATM dependent manner. In a complimentary manner, CDK2 activity (as measured by phosphorylation of thr160 on the T-loop and decrease in kinase activity) was rapidly reduced, which correlates to the dissociation of the Mre11-CDK2 complex. Furthermore, a rapid reduction in CDK2 activity

occurred correlative to this dissociation. These data suggest that a genotoxic dose of ionizing radiation dissociates the Mre11-CDK2 complex rapidly in a process dependent on the ATM kinase. Correlative to the Mre11-CDK2 dissociation, a reduction in activity occurs in the S phase kinase, CDK2, suggestive of a S phase checkpoint activation. The activation of the checkpoint would thereby enable cell cycle halt or reversible arrest for the purpose of DNA damage repair.

Additionally, I collaborated with a fellow researcher in the Ferguson laboratory where my experimentation was published as part of a larger body of work (Regal et al. 2013). The investigation lead by Joshua Regal examined *MRE11A* mutations (ATLD) effect on the MRN complex DNA damage signaling in relation to an associated disease state. My contribution to this endeavor was the assessment of ATLD subcomponent ($MRE11^{ATLD}$-RAD50-NBS1) ability to interact with one another in the presence of a MRE11A disease mutant by co-immunoprecipitation. Through the conduct of these studies the effect of $MRE11^{ATLD17}$ and $MRE11^{ASM}$ N-terminal mutation revealed a reduction in ability to co-immunoprecipitate subunit components. $MRE11^{ASM}$ lysates demonstrated reduced MRE11-RAD50 interaction and $MRE11^{ATLD17}$ lysates had reduced MRE11-RAD50 as well as MRE11-NBS1 interaction. These data contributed to the weight of evidence suggesting subtle differences in multi-protein complex stability can result in different disease states in ATLD patients.

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CHAPTER II

Mre11-CDK2 interaction in the DNA double strand break response

Abstract

The Mre11-Rad50-NBS1 (MRN) is a structurally dynamic multiprotein complex, capable of recognizing damage and initiating signal transduction cascades in response to DNA double strand breaks (DSB) through the activation of the ATM kinase, the master regulator of the DNA damage response (DDR). Functions of Mre11 were recently discovered outside of the DDR during normal mammalian cell cycling. Mre11 directly interacts with cyclin depending kinase 2 (CDK2)/ cyclin A, enabling the phosphorylation and stabilization of CtIP, the protein primarily responsible for the regulation of DNA resection. The tumor suppressor BRCA1 subsequently interacts with CtIP and the MRN complex. The Mre11-CDK2 interaction initializes the assembly of the MRN-CtIP-BRAC1 complex, providing maximum resection capacity for normally dividing cells in S and G2 phase. This recent research demonstrates that the MRN complex functions outside of the DDR signal transduction cascade, functioning in an unperturbed cell and regulating the normal cell cycle.

My current investigation explores the Mre11-CDK2 interaction and potential role of CDK2 as part of the DDR in mammalian cells. Analysis of the Mre11-CDK2/cyclin A interaction in mammalian cells after genotoxic stress has revealed a rapid dissociation of the Mre11-CDK2 complex requiring ATM kinase activity, correlating to a reduction in CDK2 kinase and inhibitory dephosphorylation at thr160 on the activation T-loop. A reduction in

CDK2/cyclin A activity results in arrest of the cell cycle in S-phase, allowing for cellular DNA repair mechanisms to restore DNA integrity prior to restoration of cell cycle progression.

Introduction

Among the types of potentially lethal DNA damage, DNA DSBs pose the greatest threat to cell survival. DNA DSBs are common and arise from both exogenous and endogenous sources. Exogenous lesions are induced through ionizing radiation (IR), chemotherapeutic drugs, and environmental chemicals. The majority of DNA DSB lesions occur endogenously as byproducts of normal cellular metabolism, for instance in the generation of reactive oxygen species. Programmed DNA lesions also form as intermediates during developmentally regulated genome rearrangements in lymphocytes and germ cells (Jackson, Bartek 2009, Stracker, Petrini 2011a) . Additionally, DSBs occur when DNA replication forks encounter single strand DNA breaks or other DNA lesions (Aguilera, Gomez-Gonzalez 2008).

For a cell to survive deleterious DSBs, cellular mechanisms monitor DNA to maintain genome integrity. A DNA DSB involves both stands of the double helix, and erroneous rejoining of broken DNA ends may result in changes to tumor suppressor or oncogenes, leading to the loss or gain of gene function. If DNA damage is left unmitigated, a DSB can give rise to a plethora of chromosomal aberrations, often resulting in cell death or mutations that can lead to cancer phenotypes (Langerak, Russell 2011). A single DNA DSB is sufficient to lead to cell death if inactivation of an essential gene occurs (Lee et al. 1998). Cellular survival is dependent on the coordination of sophisticated biochemical networks

to detect and appropriately respond to deleterious DNA lesions in order to prevent genomic instability and promote faithful genome propagation. When damage is too significant, a cell may initiate apoptosis. The proteins that share a common feature of mobilization in reaction to a genotoxic stress are collectively termed the DDR (Rouse, Jackson 2002, Harrison, Haber 2006, Harper, Elledge 2007).

The MRN complex is a central component of the DDR, involved DSB signaling cascades. This initial and earliest response in the DDR is mediated through ATM and NBS1, a less conserved member of the MRN complex (Lee, Paull 2005, Falck, Coates & Jackson 2005, You et al. 2005) . ATM is quickly activated by intermolecular phosphorylation, which corresponds to the dissociation of the ATM homodimer (Pellegrini et al. 2006). The activated ATM is then free to phosphorylate downstream targets involved in cell-cycle arrest, DNA repair, and stress response (Canman et al. 1998, Matsuoka et al. 2000, Williams, Lees-Miller & Tainer 2010) . The highly conserved core of the MRN complex, the Mre11-Rad50 heterotetramer (two Mre11 and two Rad50 protomers) is capable of binding directly to DNA ends on one or both sides of the broken DNA helix (Hopfner et al. 2001, Roset et al. 2014). Once bound, the coiled-coil arms of Rad50 stabilize the break over long distances (Williams et al. 2008, Roset et al. 2014). Mre11 provides endo- and exonuclease activities that initiate resection of DNA ends (Paull, Gellert 1998, Paull, Gellert 1999, Williams et al. 2008). ATR is recruited by ATR-interacting protein (ATRIP) to replication protein A (RPA)-coated ssDNA. This ssDNA is created in part by Mre11 nuclease activity through the initial resection of DNA damaged ends (Zou, Elledge 2003, Cuadrado et al. 2006). The apical kinases, ATM and ATR, are recruited to sites of DNA damage by related biochemical mechanisms. Once recruited, the response at the sites of damage centers on

the phosphorylation of a number of substrates, including the protein kinases Chk1 and Chk2, which then target downstream proteins to induce cell cycle arrest and facilitate DNA repair (Falck et al. 2001, Bourke et al. 2010, Langerak, Russell 2011).

DSBs constitute one of the most genotoxic forms of DNA damage and pose a substantial threat to cell viability. DSBs can promote chromosomal rearrangements and potentially deleterious mutations that promote tumorigenesis (Rich, Allen & Wyllie 2000, van Gent, Hoeijmakers & Kanaar 2001) . Deregulation of components involved in DDR biochemical processes contribute to genomic instability, displaying heightened sensitivity to DNA-damaging agents, which in turn can lead to tumorigenesis. The human genetic disorders ataxia-telangiectasia (AT), Nijmegen breakage syndrome (NBS), and ataxia-telangiectasia-like disorder (ATLD) exemplify a fundamental loss of DDR function. These are categorized as chromosome instability disorders, and are associated with the defective genes *ATM,* nibrin *(NBS1),* and *Mre11* (Featherstone, Jackson 1998, Stewart et al. 1999, Gumy-Pause, Wacker & Sappino 2004) . Patients diagnosed with AT, Nijmegen breakage syndrome, and ATLD share characteristics that include neurodegeneration, radiosensitivity, immunodeficiency, and cancer predisposition (Matsuoka et al. 2007) . Cells derived from these patients are extremely sensitive to levels of IR and fail to activate specific cell cycle checkpoints in response to DNA damage. These data substantiate fundamental roles linked to signal transduction pathways required to activate ATM-dependent cell cycle checkpoints (Shiloh 1997, Stewart et al. 1999, Shiloh 2003) . A checkpoint is a biochemical pathway that ensures dependence of one process upon another process that is otherwise biochemically unrelated. A null allele in a checkpoint gene results in a loss of this dependency and, thus checkpoints are inhibitory pathways (Elledge 1996).

The loss of functionality in these highly conserved DDR proteins, ATM and members of the MRN protein complex, compromise cellular survivability against genotoxic stresses. Furthermore, phenotypic outcomes indicate that a significant component of S phase DNA damage recognition and checkpoint activation is absent. A distinctive mechanistic defect to these syndromes is S phase checkpoint deregulation, which allows for characteristic radioresistant DNA synthesis, compromising prevention of deleterious genetic material replication. Of particular importance to survivability of a cell or organism is the hereditary DNA, which needs to be replicated and segregated in each cycle. Two events common to all eukaryotic cell cycles ensure this event: S-phase, when chromosome replicate, and M-phase (mitosis) when the replicated chromosomes segregate into the two newly divided daughter cells. Reproduction of heritable genetic misinformation or deleterious information to daughter cells may result in a failure to thrive. Another crucial part of cell cycle control is checkpoint control, which ensure that a cell will not undergo a phase transition with incorrect information.

In mammals, progression through the cell division cycle is governed by cyclin-dependent kinases (CDKs). CDKs belong to a family of serine/threonine kinases that represent, together with particular cyclins, the core of the cell cycle machinery. These kinases phosphorylate numerous substrates that play pivotal roles in major cell cycle events, including DNA replication and mitosis. Genomic integrity is monitored through the precise activation of CDKs and the correctly timed coordination of DNA synthesis (Pines 1991, Morgan 1995, Satyanarayana, Hilton & Kaldis 2008, Enserink, Kolodner 2010) . CDK2 and CDK1 together direct S and G2 phase transit, whereas CDK1 governs the G2/M transition and mitotic progression (Pines 1991, Morgan 1995, Hochegger, Takeda & Hunt

2008) . The collective cell cycle paradigm in eukaryotes begins with G1, followed by an unalterable commitment to division made at the restriction (R) point in mammalian cells, which is governed by a mitogen-dependent CDK signal (Pardee 1974, Johnston, Pringle & Hartwell 1977, Skotheim et al. 2008) . Cyclin D activates CDK4/CDK6 and subsequently initiates phosphorylation and inhibition of the Retinoblastoma protein (Rb)(Sherr 1994). Deactivation of Rb through phosphorylation releases the E2F transcription factor, which is required for enabling cell cycle gene transcription and cell cycle progression (Dyson 1998). In the next cell cycle phase, G1, cyclin E binds with CDK2 for activation of the kinase and completion of Rb phosphorylation for G1/S phase transition through the Rb restriction point (Sherr, Roberts 1999, Sherr, Roberts 2004). The gradual rise of CDK activity during S-phase initiates the onset of DNA replication and appropriate timing of origin firing while preventing untimely licensing of origins throughout S and G2 phases (Diffley 2004). As S-phase progresses, the CDK2/cyclin A complex is established and genome replication is completed. Late G2 transition to M phase includes cyclin A binding to CDK1, and eventually cyclin B complexes with CDK1 to promote entry into M phase. Regulation of CDKs, specifically CDK2, requires the catalytically inactive monomeric form to be activated in a two-step mechanism. In the first step, partial activation of a CDK2 is accomplished through the binding of a cyclin A in early S-phase to form a heterodimer which increases the intrinsic activity of CDK2. With the formation of the heterodimer, structural changes occur allowing the T-loop to reposition, making threonine 160 available for recognition by CDK-activation kinase (CAK). Phosphorylation of threonine 160 by CAK leads to a 100-fold increase in activation (Harper, Adams 2001, Jeffrey et al. 1995). Thus, both cyclin binding and T-loop phosphorylation are required for full activation and biological activity of

CDK/cyclin complexes. Positional subcellular changes have been associated with the regulation of specific CDKs such as CDK7 and CDK4 (Cheng et al. 1999, Miller, Cross 2001, Chen et al. 2003) .

Our knowledge of the complex regulator mechanisms governing DNA damage response and checkpoint activation has advanced significantly over recent years, as demonstrated by the aforementioned study reports. Critical questions remain as to the nature of checkpoints in mammals and the integration of checkpoint pathways with cell proliferation control and DNA repair. Checkpoints figure prominently in chemotherapeutic strategies to eliminate cancer cells. Most current therapeutic strategies rely on checkpoint-mediated apoptosis pathways or target chemical sensitivities due to loss of checkpoint function. CDK2 is known to be a key regulator of cell cycle progression, and is emerging as a checkpoint control contributor in the DDR. A comprehensive understanding of CDK2 functions in these respective fields will aid in development of cell biology and therapeutic adjuvants.

I have expanded upon on my previous studies, performed in unperturbed cells where Mre11 interacted with CDK2 enabling CtIP resection capacity, thereby initiating/promoting HR repair. My current work investigates the Mre11-CDK2/cyclin A interaction in the context of DSB damage response. Assays were designed to investigate the integrity of the Mre11-CDK2 complex and effects on CDK2 activity. The novel interaction of Mre11 and CDK2 has not been studied in the DDR. However, the role of CDK2 in the DDR continues to emerge. Based on current knowledge of CDK2 in the DDR, assays were designed to detect changes in activity based on the phosphorylation of thr160 and the activation T-loop and kinase activity after genotoxic stress. (Harper, Adams 2001, Gu,

Rosenblatt & Morgan 1992). To this end, I investigated the endogenous Mre11 and CDK2 interaction in murine cells carrying a Cre/LoxP allele ($Mre11^{cond/-}$) developed in our laboratory, as well as primary mouse and human fibroblasts (Buis et al. 2008a). I discovered that Mre11 rapidly dissociates from CDK2/cyclin A after a genotoxic dose of ionizing radiation (IR). Correlative to this rapid dissociation of Mre11 from CDK2/cyclin A, the thr160 site on the activation T-loop of CDK2/cyclin A becomes dephosphorylated. Furthermore, I provide evidence that this dissociation and reduction in CDK2/cyclin A occurs in an ATM-dependent manner. These data suggest that in response to genotoxic stress, the cell rapidly reduces CDK2/cyclin A activity, slowing or reversibly halting cell cycle progression.

Methods

MEF engineering and culture: *$Mre11^{cond/\Delta}$* murine embryonic cell lines (Buis et al. 2008b) were maintained using standard tissue culture techniques. To create $Mre11^{-/\Delta}$ lines, cells were treated with replication-defective adeno-cre (University of Michigan Vector Core), and allowed to recover for 48-72 hours before experiments were conducted. *$ATM^{-/-}$* MEFs were described previously (Buis et al. 2012). Primary mouse fibroblast cells and primary human foreskin fibroblasts (Varini laboratory) were used and maintained under standard tissue culture techniques. 10 Grey IR treatment consisted of cells exposed to a $Cesium^{137}$ source.

Drug treatments: Roscovitine (Calbiochem) and KU55933 (Tocris) were dissolved in DMSO at 1000x final concentration. Roscovitine drug treatment *in vivo* lasted for 72

hours, *in vitro* treatment duration of one hour. KU-55933 treatment regimen was one hour prior to IR treatment.

Immunoblots: Cells were lysed in Laemmli Sample Buffer 2x (BioRad Cat# 161-0737) and heated at 95˚C for 10 minutes. Protein concentrations were determined using BCA assay (Thermo Pierce Cat#23225). Proteins resolved by SDS-PAGE, transferred to PVDF membrane (Millipore Immobilon Cat#IPFL00010) and blocked in 5% goat serum (Invitrogen Cat#PCN5000). Primary antibodies included Mre11 (Cell Signaling Technology Cat#4895S), NBS1 (Novus Cat#NB100-143), CDK2 (Cell Signaling Technology Cat#2546S), CDK2 (M2) (Santa Cruz Cat#SC-163), pKAP1 (Bethyl cat#A300-767A), Cyclin A (Santa Cruz Cat#SC-596), p27 (Santa Cruz Cat#A-10), GAPDH (Abcam Cat#9484), CDK2 P-thr160 (Cells signaling Cat#2561), and FLAG (Sigma Cat#7425). Primary antibody concentrations followed manufacturer specified concentrations/ratios and times (typically overnight at 4˚C) Secondary antibodies included fluorophore (LiCor 700, 800, anti-mouse or anti-rabbit) or peroxidase-conjugated Heavy +Light chain Goat anti-Rabbit, or Heavy + Light chain Goat anti-mouse (Jackson Immunolabs) one hour at room temperature. Pierce Enhanced ChemiLuminescence (ECL) West Pico (cat#34080) substrate used for peroxidase-conjugated secondary antibody.

Co-Immunoprecipitation: Between 1 x 10^6 and 2 x 10^6 cells were plated on to 10 cm dishes (Corning Cat#CLS430165) and allowed to grow for 24 hours. Cells were then treated with 10 Grey of IR and harvested at specific time points post-treatment. Cells were washed 3x with 10mL DPBS 1x (Invitrogen Cat# 141-90144) and harvested using ice cold lysis solution (50mM Tris, 150mM NaCl, 10% Glycerol, 1% NP-40, 10mM PIPES, 0.1% Triton X-100, and 1mM MgCl). Protein lysates were pre-cleared with protein A beads (Roche

Cat#11134515001); lysate protein concentrations were measured by BCA assay (Thermo Pierce Cat #23225); 500ug – 2000ug of protein was added to beads; beads were incubated with either anti-Mre11 (Cell Signaling Technology) or anti-CDK2 (Cell Signaling Technology) antibody; HALT phosphatase and protease inhibitor (Pierce Cat#78420, 87786) added to protein Co-IP solution. Duration of incubation is overnight to 24 hours at 4˚C. At the conclusion of the incubation, beads were washed 3x with lysis/wash buffer under low- and high-salt conditions (2x-150mM NaCl, 1x-300mM NaCl). Proteins were eluted from beads with 2x Laemmli sample buffer (BioRad Cat# 161-0737), and sample buffer extracts were heated at 95˚C for 10 minutes prior to resolving by SDS-PAGE.

Kinase Assay. Between 1 x 10^6 and 2 x 10^6 cells were plated on to 10 cm dishes (Corning Cat#CLS430165) and allowed to grow for 24 hours. Cells were then treated with 10 Grey of IR and harvested at pre-determined time points post treatment. Cells were washed 3x with 10 mL of DPBS 1x (Invitrogen Cat# 141-90144) and harvested using ice-cold lysis solution (50mM Tris, 150mM NaCl, 10% Glycerol, 1% NP-40, 10mM PIPES, 0.1% Triton X-100, and 1mM MgCl). Protein lysates were pre-cleared with protein A beads (Roche Cat#11134515001); lysate protein concentrations were measured by BCA assay (Thermo Pierce Cat #23225); 500ug – 2000ug of protein was added to beads; beads were incubated with anti-CDK2 (Cell Signaling Technology) antibody; HALT phosphatase and protease inhibitor (Pierce Cat#78420, 87786) added to protein IP solution. Duration of incubation was overnight to 24 hours at 4˚C. At conclusion of incubation, beads were washed 3x with lysis/wash buffer under low salt conditions (150mM NaCl). The experimental controls included bead control (BC); no antibody for CDK2 conjugated to beads; histone substrate control (H1) designed to detect incorporation of γATP-P^{32} on an artificial non-specific substrate by omitting histone

H1 in kinase reaction buffer. Roscovitine, a selective inhibitor of CDK2, was administered at 25uM *in vivo* and 2.5uM *in vitro*, to reduce CDK2 kinase activity. $CDK2^{-/-}$ cells were used to confirm antibody specificity to CDK2. Beads were rinsed 2x with 1mL kinase buffer solution (Kinase Buffer 10mL total volume; 250µL 1M Tris-HCL, 300uL 5M NaCl (150mM final), 100µL 1M MgCl, 10µL 1M DTT solution, ~pH 7.2). In the following order reagents were added to 100µL Kinase Buffer; Artificial histone H1 substrate (Roche Cat#10223549001) 10µL (1µg/µL stock); 40µM, 100mM ATP^{33} (Roche cat#11140965001) 4µL; gamma P^{32} ATP 3000µCi/mM (Perkin-Elmer C#BLU002A500UC) 1µL. Reaction was allowed to run for 45 min at RT. Reaction was quenched and proteins were eluted from beads with 50µL 2x Laemmli sample buffer (BioRad Cat# 161-0737), and sample buffer extracts were heated at 95˚C for 10 minutes prior to resolving by 4-20% SDS-PAGE. Histone substrate transferred to PDVF using BioRad semi-dry system. Kinase activity evaluated using by autoradiography evaluated using film and/or 9400 Typhoon Scanner (GE Healthcare).

Results

CDK2 kinase activity and dephosphorylation of threonine 160 site on the activation T-loop after genotoxic stress: CDK2 achieves maximum biologic activity by binding to cyclin A, which enables structural conformational changes resulting in the access to thr160 on the activation T-loop for phosphorylation and full activation. Intrinsic activity of CDK2 is increased 100-fold when thr160 becomes phosphorylated (Desai et al. 1995). A dose of 10 Gy was used to experimentally introduce DSB to cells and induce the DDR to reversibly arrest cell cycle progression (Langerak, Russell 2011) .

As a proxy for cell cycle arrest or reversible halt, I assayed biological kinase activity and the thr160 phosphorylation state of the S-phase cell cycle regulator CDK2. The supposition being the reduction of CDK2 kinase activity and inhibitory dephosphorylation at thr160 is indicative of a cell no longer capable of progression through S-phase. In order to evaluate the biological activity of CDK2 I conducted a kinase assay. The kinase assay is commonly used to determine the biologic activity of a CDK through the accumulation of γATP-P^{32} on an artificial substrate, histone H1, measured over a period of time. My target protein kinase, endogenous CDK2, was immunoprecipitated from treated and control cell lysates of equal protein concentrations using anti-CDK2 antibody bound to agarose-A beads. After immunoprecipitation, the beads (having acquired CDK2 protein from both treated and control samples) were combined with kinase assay reaction buffer containing the H1 substrate and γATP-P^{32}. The γATP-P^{32} incorporated onto the artificial substrate served as a mechanism to measure the CDK2 activity, which was visualized by autoradiography. A reduction in signal from the radio labeled H1 substrate relative to controls would be suggestive of an inhibitory dephosphorylation at site thr160 on the activation T-loop. In my seminal study I revealed a rapid decrease in γATP-P^{32} incorporated on H1 substrate after IR as compared to the non-irradiated (0Gy) control dose group (Figure II.1A). In the experiment, cells were treated with 10 Grey (Gy) and allowed to recover for 15, 30, 60, and 120 minutes. Each IR-treated sample (15, 30, 60, and 120 minute recovery time points) showed a marked decrease in γATP-P^{32} signal as compared to corresponding untreated (0Gy IR) control, determined by film radiography. Controls for this experiment included a bead control (BC), which contained cell lysate and beads only, providing evidence no other kinases were adhering to the beads. The second

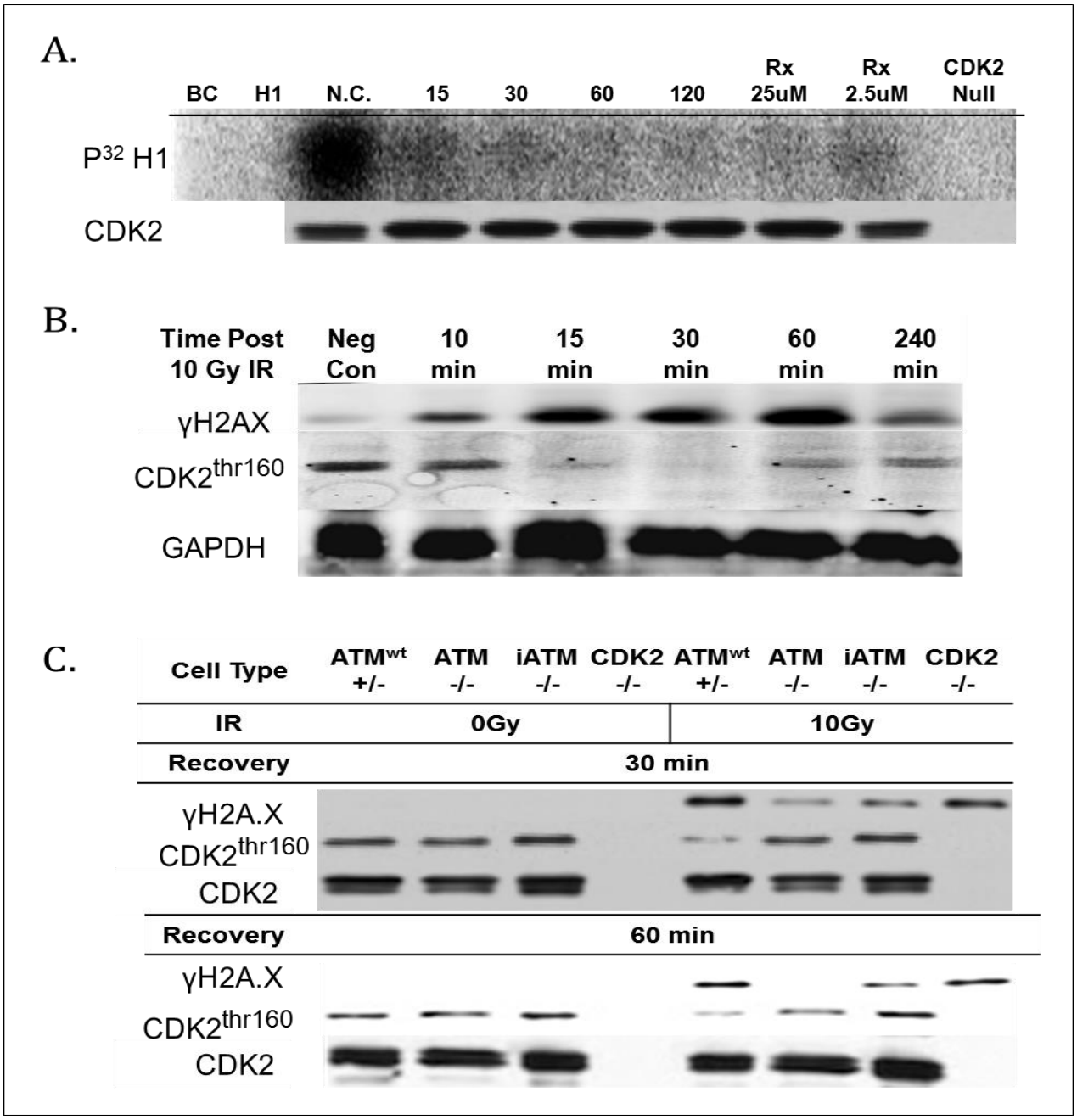

Figure II.1. Cyclin dependent kinase 2 Threonine160 phosphorylation status after genotoxic stress. Immunoblot analysis of unsynchronized Mre11cond/- mouse embryo fibroblast cells with primary antibodies indicated at left. A. Immunoprecipitation of CDK2 for temporal evaluation of activity based on incorporation of radiolabeled substrate on to Histone H1 using in vitro kinase assay. CDK2 protein levels used as a loading control for assay. B. Comparative temporal analysis of phosphorylated threonine160 levels after ionizing radiation. Immunoblot of γH2AX for the assessment of genotoxic stress with GAPDH levels for protein loading control. C. Comparison of threonine 160 phosphorylation status of CDK2 in ATM proficient and ATM null and small molecule inhibited (10uM Ku55933) cells after 30 and 60 minutes recovery. CDK2 used for protein loading control.

control (H1) served to detect any other artificial substrate present in the lysate by removing histone H1 from the reaction mixture. The H1 control contained beads, lysate, and anti-CDK2 antibody only. Roscovitine, a known CDK inhibitor, was used *in vitro* and *in vivo*, at Rx2.5μM and Rx25μM, respectively. These treatments were used to assess the inhibition of CDK2 activity when artificial substrate for kinase was present (in vitro; CDK2 bound beads, kinase reaction solution, H1 substrate) as well as inhibition of CDK2 during cell growth in culture (in vivo) prior to harvest and subsequent anti-CDK2 immunoprecipitation. CDK2 null cells were also used to determine kinase assay performance and antibody specificity (Figure II.1A). Radiolabeled substrate in this lane would have proven the assay invalid due to a non-specific CDK being introduced into the reaction. Protein samples were determined by BCA for the immunoprecipitation protein input, a confirmatory immunoblot of CDK2 total protein was performed at the time of the kinase activity assessment. Based on data from the controls the results of the kinase assay are valid and reflective of a reduced CDK2 level of activity after IR.

CDK2 kinase activity is based on the activation level of CDK2 (Gu, Rosenblatt & Morgan 1992, Harper, Adams 2001) . Therefore, I investigated the phosphorylation status of $CDK2^{thr160}$ in asynchronous $Mre11^{cond/-}$ MEF cells. Cells were treated with 0 and 10Gy of IR and allowed to recover for 10, 15, 30, 60, and 240 minutes post-IR treatment (Figure II.1B). Immunoblotting for $CDK2^{thr160P}$ showed a rapid decrease in the level of phosphorylated $CDK2^{thr160P}$ by 15 minutes, at 30 minutes levels were below the limit of detection. At 60 minutes $CDK2^{thr160P}$ begin to rise and by 240 minutes $CDK2^{thr160P}$ levels are again slightly increased, however, as compared to negative control the $CDK2^{thr160P}$ levels remain decreased (Figure II.1B, C). MRN recruits ATM to DSBs and facilitates kinase

activation through direct interaction of ATM with NBS1 (Falck, Coates & Jackson 2005) . ATM activation and activity can be monitored by the DSB biomarker, γH2A.X, which is directly phosphorylated by ATM at sites of DSB, and thus is commonly used as a marker of DNA damage. (McManus, Hendzel 2005, Kuo, Yang 2008). Genotoxic stress induces DSBs resulting in levels of γH2A.X to rise rapidly and peak at 60 minutes, by 240 minutes the levels have decreased significantly and begin to resemble untreated control, indicating that the damage has been repaired (Figure II.1B, C).

Finally, I conducted an experiment to determine if the rapid dephosphorylation event on $CDK2^{thr160}$ is ATM dependent. ATM null cells and the ATM small molecule inhibitor KU-55933 was used to determine dependency on ATM (Hickson et al. 2004). Cells deficient in ATM, or $Mre11^{-/\Delta}$ cells treated with KU-55933, have reduced or below the level detection of γH2A.X consistent with other reports (McManus, Hendzel 2005, You et al. 2005, Kuo, Yang 2008). Cells treated with 10 Gy IR the phosphorylation of $CDK2^{thr160}$ is again rapidly reduced in $Mre11^{-/\Delta}$ MEF cells as compared to untreated control cells (Figure II.1C). Cells treated with KU-55933 (an ATM inhibitor) or those devoid of ATM protein have undergone no visible change in level of phosphorylation at $CDK2^{thr160}$ as compared to controls (Figure II.1C). CDK2 null cells show no presence of CDK2 at either time point confirming antibody specificity for CDK2.

Mre11-CDK2 complex dissociates after genotoxic stress: Induction of DBS by genotoxic stress initiates cell cycle checkpoints to arrest or reversibly halt progression (Hartwell, Weinert 1989, Paulovich, Hartwell 1995, Langerak, Russell 2011). A recent investigation revealed that Mre11 and CDK2 interacted in an unperturbed cell to influence the capacity for HR resection (Buis et al. 2012). Therefore, I assessed the Mre11-CDK2

complex interaction after genotoxic stress. Cells were treated with 0 or 10 Gy IR to induce DSB, then were allowed to recover for 15, 30, and 60 minutes at which point samples were interrogated for Mre11-CDK2 interaction using co-immunoprecipitation. Endogenous CDK2 was immunoprecipitated from $Mre11^{cond/\Delta}$ cells to determine whether Mre11 associated with CDK2 after genotoxic stress. Mre11 co-immunoprecipitated with CDK2 in the untreated controls as previously discovered. However, after 10Gy IR, Mre11 was not detected at any time point (Figure II.2A). Cyclin A, the S-phase binding partner of CDK2, was consistently immunoprecipitated at each time point (Figure II.2A). By reconfiguring the immunoprecipitation, endogenous Mre11 was immunoprecipitated to investigate CDK2 association. Cyclin A was used as proxy for CDK2 in this experiment. In the control group, Mre11 remained associated with cyclin A/CDK2 at the 30-minute time point (Figure II.2B). The 10Gy treatment group correspondingly disassociated in the CDK2 immunoprecipitation (Figure II.2A, B). NBS1, a member of the MRN complex, remained associated with Mre11 under both treatment conditions (Figure II.2B)

In the final experiment of this set, primary mouse and human fibroblasts were used to investigate the Mre11-CDK2 complex interaction after genotoxic stress. Mre11 was immunoprecipitated from mouse and human primary cells. 10Gy IR was again used to induce DSB and activate the DDR. After 60 minutes, samples were harvested and examined for complex interaction. CDK2 remained associated in both control (0Gy) treatment groups after a 60 minute recovery in mouse and human primary cells. In the 10Gy mouse and human treatment groups, CDK2 dissociated from Mre11 (Figure II.2C, D). This result corresponds to SV40 transformed $Mre11^{cond/\Delta}$ cells at the 60 minute time point (Figure II.2).

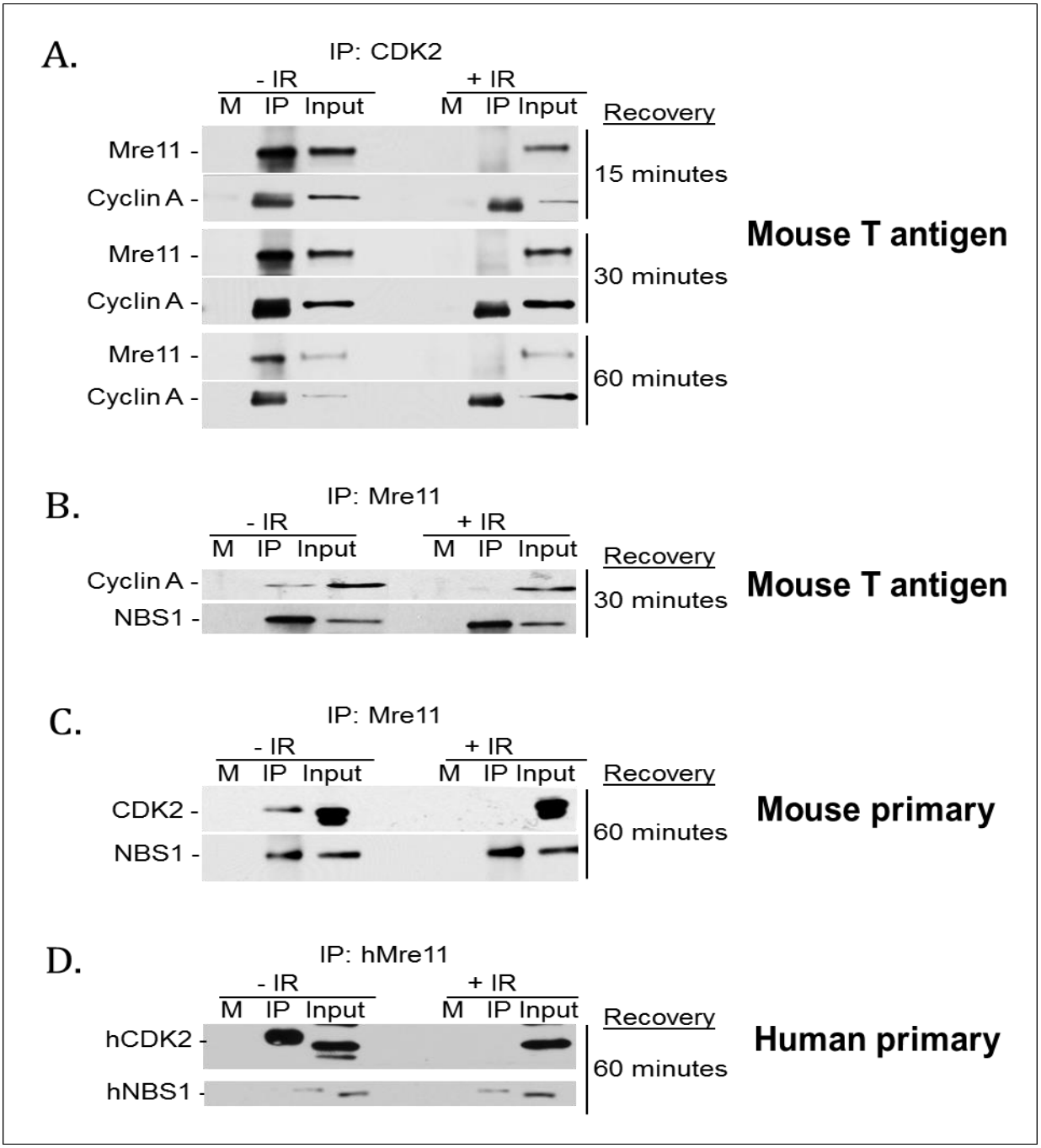

Figure II.2. Mre11-CDK2/Cyclin A complex in primary and transformed mammalian cells before and after genotoxic stress. Immunoblot analysis with primary antibodies indicated at left and recovery times after IR noted at right. A. Temporal evaluations of Mre11 – CDK2/Cyclin A complex in Mre11cond/- MEF cells using anti-CDK2 antibody (CoIP) or beads only mock (M) of complex. Cells were treated with 0 Grey (-IR) and 10 Grey (+IR). B. Reverse CoIP using anti-Mre11 antibody in Mre11cond/- MEF cell lysate. C. Mouse primary cell lysate CoIP using anti-Mre11 antibody. D. Human foreskin fibroblast cell lysate CoIP using anti-Mre11 antibody.

Mre11-CDK2/cyclin A dissociation is dependent on ATM: Upon DNA damage, sensor complex MRN recruits apical kinase ATM to DSB, enabling cell cycle checkpoints and mobilizing repair proteins through signal transduction pathways. I therefore assessed the role ATM plays in the dissociation of Mre11-CDK2/cyclin A complex. To determine MRN recruitment and subsequent activity of ATM, the phosphorylation of KAP1 at S824 was measured (Ziv et al. 2006). ATM null cells and ATM competent $Mre11^{cond/\Delta}$ cells were exposed to 0 and 10Gy IR to cause DSB, and then allowed 60 minutes for recovery. Phosphorylation of pKAP was present in $Mre11^{cond/\Delta}$ -ATM proficient cells. However in ATM null cells, pKAP was substantially abrogated (Figure II.3A). Endogenous CDK2 was immunoprecipitated from ATM null and $Mre11^{cond/\Delta}$ cells to determine Mre11 association. Consistent with previous experiments, $Mre11^{cond/\Delta}$ cells treated with 10Gy IR and allowed to recover showed a substantial decrease in complex formation at 30 and 60 minutes. Contrary to this finding, ATM null cells treated with 10Gy demonstrated an association of Mre11 and CDK2 at 30 and 60 minutes, similar to untreated controls (Figure II.3A). Cyclin A was associated with CDK2 in all samples.

Finally, I analyzed the effect of the ATM inhibitor KU-55933 on $Mre11^{cond/\Delta}$ cells, which express ATM at normal levels. As previously seen, pKAP1 induction occurred in the IR-treated $Mre11^{cond/\Delta}$ cells and was not detected in the KU-55933 treated cells after a 60 minute recovery, suggesting inhibition of ATM activity. Endogenous Mre11 and CDK2 were immunoprecipitated in the presence and absence of IR and KU-55933 to assess the association of CDK2 and Mre11, respectively. In the non-IR group, cells were treated with and without KU-55933 in both the CDK2 IP and Mre11 IP. In each of these groups, the Mre11-CDK2/cyclin A complex is present in the test samples (Figure II.3B). For the IR-

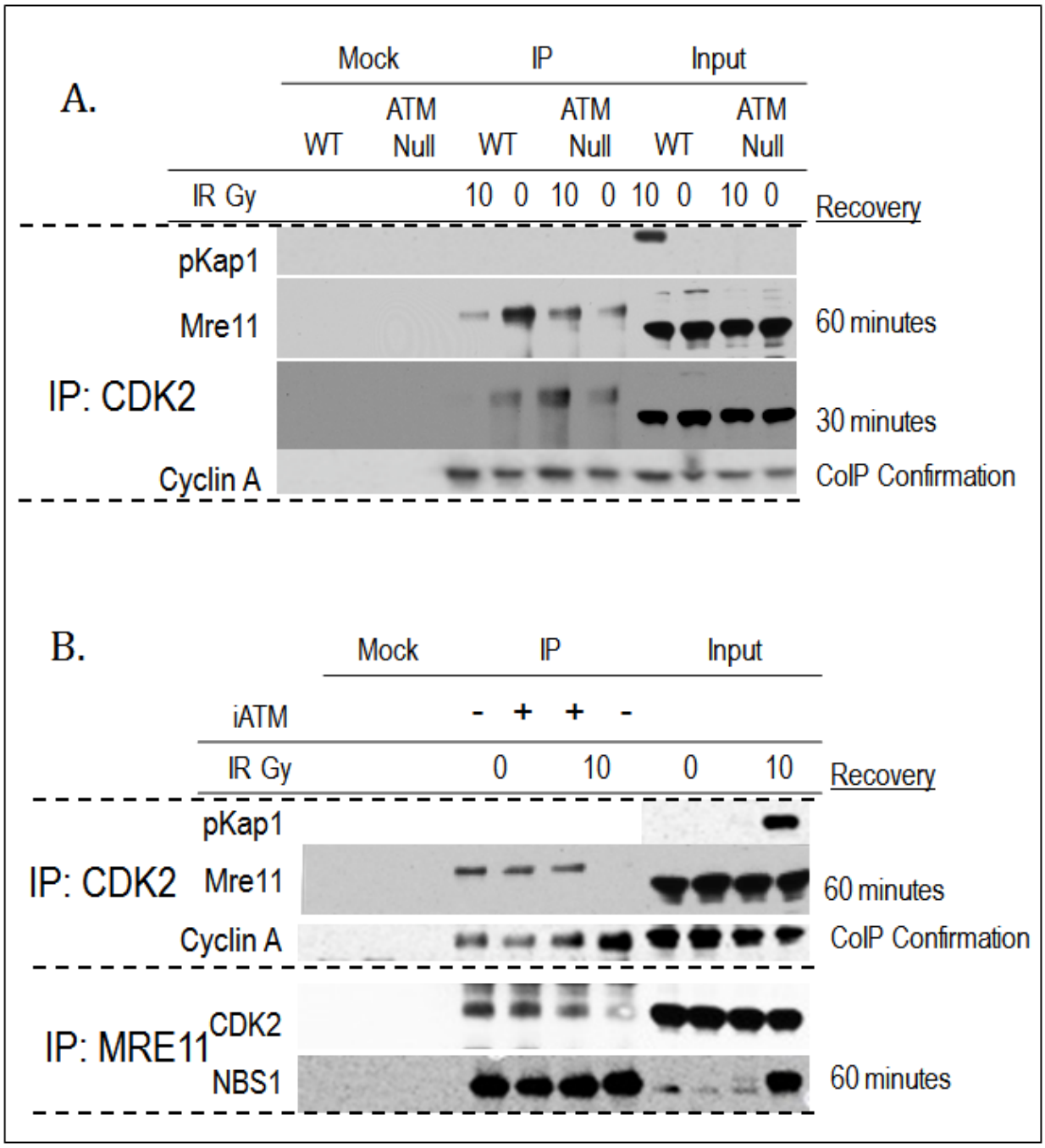

Figure II.3. Mre11- CDK2/Cyclin A complex and ATM activity after genotoxic stress. Immunoblot analysis with primary antibodies indicated at left and recovery times after IR noted at right. ATM activity indicated by presence of pKap1. Mre11-CDK2/CyclinA complex analysis was conducted by co-immunoprecipitation (CoIP) using anti-CDK2 antibody or anti-Mre11 antibody. A. Mre11cond/- and ATM-/- treated with 0 Grey (-IR) and 10 Grey (+IR) and allowed recovery of 30 and 60 minutes. B. Mre11cond/- MEF cells were treated with 0 Grey (-IR) and 10 Grey (+IR). MEFs were additionally treated with Ku-55933 an ATM inhibitor (iATM). Mre11 and CDK2 were co-immunoprecipitated using anti-CDK2 or anti-mre11 antibodies, respectively.

treated cells, with and without KU-55933, in both the CDK2 and Mre11 IP groups, the KU-55933 treatment prevented the dissociation of Mre11 and CDK2. Immunoprecipitation controls (cyclin A or NBS1) were present in all samples, confirming that the CDK and MRN complexes were not disturbed during IR or drug treatment.

Discussion

My investigation reveals that after DNA damage, Mre11 dissociates from CDK2 in an ATM dependent manner. This conformational change in Mre11 and CDK2 correlates temporally to the rapid CDK2 thr160 dephosphorylation, suggestive of a cell cycle halt or arrest for the purpose of repair. Contrary to my findings, another group determined that after genotoxic stress, DT40 cells activated CDK2 phosphorylation of thr160 in a Chk1 dependent manner (Bourke et al. 2010).

The work in DT40 primarily consisted of post-IR recovery time points of 2, 4, 6, 8, 10, and 12 hours, as compared to my post-IR experimental time points of 15, 30, and 60 minutes. My experiment evaluating CDK2 biological activity did include a 2-hour time point and observed a significant decrease in kinase activity as compared to the untreated control. My evaluation of CDK2 thr160 in SV40 large T antigen transformed MEF cells demonstrated a rapid and sustained reduction of thr160 protein levels up to and including the 4 hour post IR mark as compared to control sample. Contrary to my findings in mammalian cells, the research conducted in avian DT40 cells demonstrates a significant increase in CDK2 biological activity and phosphorylation of thr160, treated versus control. However, in the 2-hour post-IR sample, the thr160 levels remain equivalent to or slightly increased, relative to control. The 4-hour time point clearly demonstrates an increase in

CDK2 thr160 levels, relative to control. My research focused on recovery time points of 1 hour or less based on the rapid responses I found in my work using MEF SV40 large T antigen transformed cells, which determined that the critical time points to measure CDK2 phosphorylation status and biological activity occur before 60 minutes. The difference in cell line species and cell type could also contribute to the different results. Nevertheless, one possible hypothesis based the data contrary to mine is that earlier time points may have a decrease in CDK2 phosphorylation and biological activity. If this were the case, evaluation at the later time points (2 and 4 hours) would in fact, be a measure of damaged cell recovery, rather than the inhibitory effects of the DDR.

Using a variety of controls, small molecule inhibitors, and procedures to measure CDK2 activity, I have shown a consistent correlation between inhibition of CDK2 activity at thr160 and Mre11-CDK2 association. The inhibition of CDK2 activity and Mre11-CDK2 interaction both rely on ATM kinase. Previous studies have shown that in response to genotoxic insult, a cell will halt or reversibly arrest cell cycle progression in order to allow time for repair of the damage, partially through CDK inhibition (Chow et al. 2003, Zhu 2004, Maude, Enders 2005, Hughes et al. 2013) . This arrest can be accomplished through the ATM/ATR-Chk1/Chk2-Cdc25 signal transduction pathways. ATM and ATR are activated after genotoxic stress by responding to the formation of DSB and RPA coated ssDNA that is generated by DSB, respectively (You et al. 2009) . Checkpoint signaling kinases 1 and 2 are activated by the apical kinases to phosphorylate Cdc25A, resulting in proteasome-mediated degradation (Bartek, Lukas 2001, Falck et al. 2001, Molinari et al. 2000, Reinhardt, Yaffe 2009) . This prevents Cdc25A from activating CDK2, which is required for initiation of DNA synthesis. The action of Cdc25 does not include the

dephosphorylation of thr160 on the T-loop of CDK2 (Gu, Rosenblatt & Morgan 1992) . Published works have shown kinase associated phosphatase (KAP) is capable of dephosphorylating thr160 on the activation T-loop. However, CDK2/cyclin A complex is resistant to KAP dephosphorylation in a cyclin A-dependent manner (Gyuris et al. 1993, Poon, Hunter 1995).

Based on previous studies and my current investigational data, a supposition can be made to assess the functional significance of the Mre11-CDK2 complex. It is feasible that Mre11 senses genotoxic-induced DSBs, and ATM is recruited and subsequently activated through the MRN complex. Once activated, ATM acts upon the MRN complex, leading to the separation and consequential inhibition of CDK2. Altered by Mre11 physical absence, inhibited CDK2 enables a cell division arrest in S-phase. A hypothetic and simple model for the resolution of the arrest and cell cycle progression begins with the Mre11 binding to CDK2/cyclin A, enabling T-loop thr160 phosphorylation (Desai et al. 1995, Larochelle et al. 2007) . This is an oversimplification of the biochemical activities conceivably involved in the reestablishment of the Mre11 and CDK2/cyclin A association and subsequent progression of the cell cycle. An intricate and multifaceted system for cell cycle progression resumption would provide more control over a system start. Perhaps as the double helix becomes repaired, dynamic conformational change in the MRN complex occurs; it is feasible that when bound to a replication fork collapse, Mre11 an orientation specific to that type of DNA damage and otherwise has an entirely different architectural orientation (Williams, Lees-Miller & Tainer 2010) . Furthermore, the interaction maintained between Nbs1 and Mre11 provides orientation sensing of Mre11 symmetry and consequently, the ability to modulate the magnitude of ATM activation after IR (Williams, Lees-Miller &

Tainer 2010, Stracker, Petrini 2011b) . I hypothesize that resolution of the S-phase checkpoint arrest occurs in the following manner: the DNA double helix is stabilized as lesions are repaired and Mre11 architecture subsequently resumes a non-DNA binding state. This MRE11 conformational modification is sensed by Nbs1, causing a reduction in the magnitude of ATM activation, whereby MRN conformational state is capable of interaction with CDK2/cyclin A, supporting cell cycle progression.

Based on my experiments and observations in the absence or inhibition of ATM, another intriguing supposition can be made. I observed that the inhibition or absence of ATM allowed the Mre11-CDK2 complex to remain stable after genotoxic stress. With an intact Mre11-CDK2 complex I did not observe a loss in phosphorylation at site thr160 on the CDK2 T-loop. The thr160 site remained at levels equivalent to the non-treated control, suggesting normal cell cycle progression despite the damage induced by 10Gy. However, in ATM-competent cells, this resulted in dissociation of Mre11 and CDK2, correlative loss of phosphorylation, and subsequent CDK2 activity, suggesting arrest or reversible halt.

It is possible that my work using ATM null cells and small molecules mimicked the genomic instability syndrome AT, characteristic S-phase checkpoint failure resulting in DNA replication after IR exposure, and subsequent radio-resistant DNA synthesis (RDS) (Carney et al. 1998, Stewart et al. 1999, Petrini 2000) . This is based on the failure of intra-S-Phase checkpoint inhibition and subsequent RDS as one of the characteristic cellular phenotypes shared by individuals affected with inherited genomic instability syndromes.

It is further conceivable that a loss of function related to inhibition of CDK2 could lead to an ability to re-replicate through complete inhibition of CDK2, or alternatively lead to a continuation of replication despite genomic material that is compromised and may

contain deleterious errors. Several studies have illustrated the importance of CDK inhibition in the prohibition of the cell from replication of genetic material (Zhu 2004, Lopez-Mosqueda et al. 2010, Zegerman, Diffley 2010). It is tempting to surmise that my observation may provide insight into the mechanism of RDS and the continuation of replication despite the presence of genotoxic damage. My data provide intriguing formative evidence that the Mre11-CDK2 interaction is a method to govern CDK2 activity through complex association and dissociation due to DSB sensing activity by Mre11-complex in S-phase as a mechanism to maintain genomic stability.

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CHAPTER III

Disease-associated MRE11 mutants impact ATM/ATR DNA damage signaling by distinct mechanisms

Preface

In this chapter, I collaborated with my colleague, Joshua Regal, to investigate the effect of *MRE11A* mutations (ATLD) on the MRN complex DNA damage signaling in relation to an associated disease state (Regal et al. 2013). The investigation centered around four alleles of interest $MRE11A^{ATLD17}$, $MRE11A^{GRM}$, $MRE11A^{ASM}$, and $MRE11A^{ATLD1}$ (Figure. III.1.A). Alleles were chosen to provide a spectrum of severity in respective disease-associated patient outcomes. My contribution to this endeavor was the assessment of ATLD subcomponent ($MRE11^{ATLD}$-RAD50-NBS1) stability and interactions in the presence of an MRE11A disease mutant. Subcomponent interaction capabilities were tested by co-immunoprecipitation of wild type control, $MRE11A^{ATLD17}$, $MRE11A^{GRM}$, and $MRE11A^{ASM}$ cell lysates. Normal and mutant sample MRN subcomponents were immunoprecipitated (IP:MRE11, RAD50, NBS1) followed by immunoblotting for the respective elements in order to assess protein interaction (Figure III.6). Through this work, I was able to demonstrate that the MRE11-RAD50 interaction was reduced in $MRE11^{ASM}$ cell lysates compared to control, and furthermore, that there was a reduction in both MRE11-RAD50 interaction and MRE11-NBS1 interaction in $MRE11^{ATLD17}$ cell lysates. These data contributed to a weight of evidence suggesting that subtle differences in multi-protein complex stability can result in different disease states in ATLD patients.

Abstract

DNA double-strand breaks (DSBs) can lead to instability of the genome if not repaired correctly. The MRE11/RAD50/NBS1 (MRN) complex binds DSBs and initiates damage-induced signaling cascades via activation of the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and rad3-related (ATR) kinases. Mutations throughout MRE11 cause ataxia-telangiectasia-like disorder (ATLD) featuring cerebellar degeneration, and cancer-predisposition in certain kindreds. Here, we have examined the impact on DNA damage signaling of several disease-associated *MRE11A* alleles to gain greater understanding of the mechanisms underlying the diverse disease sequelae of ATLD. To this end, we have designed a system whereby endogenous wild-type *Mre11a* is conditionally deleted and disease-associated MRE11 mutants are stably expressed at physiologic levels. We find that mutations in the highly conserved N-terminal domain impact ATM signaling by perturbing both MRE11 interaction with NBS1 and MRE11 homodimerization. In contrast, an inherited allele in the MRE11 C-terminus maintains MRN interactions and ATM/ATR kinase activation. These findings reveal that ATLD patients have reduced ATM activation resulting from at least two distinct mechanisms: (i) N-terminal mutations destabilize MRN interactions, and (ii) mutation of the extreme C-terminus maintains interactions but leads to low levels of the complex. The N-terminal mutations were found in ATLD patients with childhood cancer; thus, our studies suggest a clinically relevant dichotomy in *MRE11A* alleles. More broadly, these studies underscore the importance of understanding specific effects of hypomorphic disease-associated mutations to achieve accurate prognosis and appropriate long-term medical surveillance.

Introduction

DNA damage is commonplace and results from a variety of endogenous and exogenous sources(Jackson, Bartek 2009). DNA double-strand breaks (DSBs) are a highly toxic form of damage and arise from replication fork collapse, ionizing radiation (IR) or as intermediates in programmed rearrangements during meiosis and lymphocyte development. If not properly repaired, these lesions may result in deletion, duplication or translocation of genomic material leading to cellular dysfunction and potentially neoplastic transformation. Hence, maintenance of genomic integrity is essential for cellular and organismal viability. To deal with the constant threat of DNA damage, organisms have evolved elaborate DNA damage response (DDR) machinery to attempt DNA repair, and if unsuccessful, remove cells from the proliferative pool.

The MRE11/RAD50/NBS1 (MRN) complex is central to the detection and repair of DSBs, as well as the restart of stalled replication forks (Stracker, Petrini 2011). This complex serves as a hub for regulation of kinases involved in damage responses. When DSBs arise, MRN directly binds DNA ends and facilitates activation of ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia- and rad3-related (ATR). These are the apical kinases of the DDR that are responsible for cell cycle arrest, initiation of DNA repair and, if necessary, apoptosis. DNA ends are bound directly and stabilized in proximity by a heterotetramer composed of two MRE11 and two RAD50 protomers. When MRN is engaged at DSBs, it recruits and activates ATM through direct interaction with the NBS1 subunit as well as other possible contacts (Lee, Paull 2004, Falck, Coates & Jackson 2005, Lee, Paull 2005). MRE11 possesses endo- and exo-nuclease activities that initiate resection of DNA ends (Paull, Gellert 1998, Paull, Gellert 1999). This end processing facilitates

generation of single-stranded DNA upon which ATR can be subsequently loaded and activated (Zou, Elledge 2003, Falck, Coates & Jackson 2005). MRN-dependent resection is promoted by ATM; thus, DSB-induced ATR activation is also downstream of ATM activity (Wang et al. 2013, Cuadrado et al. 2006).

Inherited deficiencies in DNA damage signaling are responsible for a spectrum of disorders with diverse sequelae involving the central nervous system, the immune system and musculoskeletal development (Jackson, Bartek 2009). Biallelic mutations in *ATM* or *NBN (NBS1)* result in ataxia-telangiectasia (AT; MIM 208900) or Nijmegen breakage syndrome (NBS; MIM 251260), respectively (Savitsky et al. 1995, Carney et al. 1998, Varon et al. 1998). These syndromes are marked by neurologic phenotypes (cerebellar ataxia and microcephaly, respectively), immunodeficiency, cellular genomic instability and IR hypersensitivity (McKinnon 2004, Demuth, Digweed 2007). As anticipated from the important role of DNA repair in maintaining genomic stability, AT and NBS patients display strong predisposition to malignancy. In fact, a single hypomorphic *ATM* or *NBS1* allele can be sufficient to increase cancer risk(Athma, Rappaport & Swift 1996, Cybulski et al. 2013, Swift et al. 1987, Thompson et al. 2005, Renwick et al. 2006, Seemanova et al. 2007, Ciara et al. 2010, Fletcher et al. 2010, Zuhlke et al. 2012). This cancer predisposition is also apparent in mouse models. Though *Nbs1*-null mice are not viable(Zhu et al. 2001), mice with *Nbs1* heterozygosity or hypomorphism are predisposed to oncogenesis(Kang, Bronson & Xu 2002, Dumon-Jones et al. 2003). Furthermore, *Atm*-null mice are predisposed to lymphoma(Xu et al. 1996), and mice carrying one hypomorphic *Atm* allele are cancer predisposed (Spring et al. 2002). Other components of the DSB repair

machinery are also tumor suppressors—for example, *BRCA1* and *BRCA2* (Miki et al. 1994, Wooster et al. 1995).

Given that MRE11 functions in a complex with NBS1, it would be anticipated that inherited MRE11 deficiencies would phenocopy NBS. However, this is not commonly observed. Germline biallelic *MRE11A (MRE11)* mutation results in a syndrome more similar to AT than NBS and has thus been termed ataxia-telangiectasia-like disorder (ATLD; MIM 604391) (32–37). Like AT, ATLD is characterized by cerebellar ataxia which develops after birth through neurodegeneration rather than the microcephaly at or shortly after birth associated with NBS. In addition, the earliest identified ATLD patients appear to differ from both AT and NBS in their cancer predisposition. ATLD patients homozygous for the first identified ATLD allele, $MRE11A^{ATLD1}$, have not been reported to develop early cancer, nor are $Mre11a^{ATLD1/ATLD1}$ mice predisposed to the development of lymphoma—the malignancy to which AT and NBS patients are most markedly predisposed (Stewart et al. 1999, Theunissen et al. 2003). However, $MRE11A^{ATLD1}$ has been associated with a familial cancer syndrome inherited in an autosomal dominant manner (Bartkova et al. 2008). Also, more recently identified ATLD patients harboring novel *MRE11A* mutations do seem cancer prone. Two ATLD-afflicted brothers both died of lung cancer (pulmonary adenocarcinoma) at the remarkably young ages of 9 and 16 (Uchisaka et al. 2009). These two individuals had cerebellar degeneration but additionally had developmental defects suggestive of NBS.

Comparison of these DDR disorders makes clear that while there are some common features, there are also critical differences among specific patient groups. Complete absence of any MRN component is early embryonic lethal in mice; thus, the disease alleles are partial loss-of-function mutations that must preserve essential functions of the complex

(Luo et al. 1999, Zhu et al. 2001, Buis et al. 2012). It is likely that the differing impact of each of the mutations is largely responsible for the varied spectrum of sequelae in the resulting disorders. Understanding and predicting these differences has important implications for prognosis and treatment for these disorders. Given that mutations in AT and MRN components can affect the wider population through haplo-insufficiency and somatic mutation, greater understanding of these factors has important health implications.

We endeavored to define the specific defects in DNA damage signaling caused by several *MRE11A* alleles associated with diverse clinical outcomes. To this end, we designed a structure–function system to express mutant alleles at physiologic levels in murine cells harboring an endogenous Mre11a conditional Cre/LoxP allele (*Mre11a*^{cond}) engineered in our laboratory (Buis et al. 2008). We find that the MRE11 mutants have distinct consequences on stability of the MRN complex and DNA damage signaling, which appear to correlate with differences in cancer predisposition. Furthermore, these studies provide evidence that MRE11 dimer formation and MRE11–NBS1 interaction are mechanistically linked in mammals thus providing insight into how certain *MRE11A* mutations abrogate DNA damage signaling.

Methods

DNA construct creation: Select *Mre11a* mutations were introduced into pEF6-Mm*Mre11a* using site-directed mutagenesis (Stratagene) or—for the alternative splice mutant—PCR amplification followed by ligation. These mutants were shuttled into pGBKT7

and pGADT7 (Clontech). Mm*Nbn* (GeneCopoeia) were TOPO PCR subcloned (Life Technologies) and shuttled into the Y2H vectors.

MEF engineering and culture: *Mre11a*$^{cond/\Delta}$ murine embryonic cell lines (41) were maintained using standard culture conditions. To create clones, cells were transfected (Lipofectamine 2000, Life Technologies) with empty vectors or wild-type MRE11- or mutant MRE11-expressing constructs and clones were isolated and grown under blasticidin (Life Technologies) selection. Prior to each experiment, cells were grown under blasticidin selection for 3 days, split, allowed to recover for a day, treated with replication-defective adeno-cre (University of Michigan Vector Core), allowed to recover for 2 days, split and allowed to recover for 2–3 days after which time experiments were performed. *ATM*$^{-/-}$ MEFs were as described previously (Buis et al. 2012). Where IR treatment is indicated, cells were exposed to a 137Cs source.

Mre11a RNA typing: 5e^5 cells per sample were pelleted, RNA was isolated (AllPrep DNA/RNA Mini Kit, Qiagen), RT-PCR was performed (forward primer: GCAATCTCAACATTT CCATTCC, reverse primer: GTTTCTTCTTGGGCAACTACTG) and amplicons were subjected to Sanger sequencing (sequencing primers: CAGTATTTAGTATCCACGGCAAC, CATCGTCAT CATCCTCATCTG, GGAGAAGAGATCAACTTTGGG and CTCTTCCTTGTCCACAAACTC).

Immunoblots: Cells were lysed in Laemmli buffer (BioRad) and heated at 100°C for 10 min. Protein concentrations were ascertained by BCA assay (Thermo Scientific). Proteins were resolved by SDS–PAGE, transferred to PVDF membranes (Immobilon) and blocked in 5% milk TBST. Primary antibodies used were as follows: MRE11 (Cell Signaling), RAD50 (Bethyl), NBS1 (Novus), pATM S1987 (Rockland), ATM (Cell Signaling), pKAP1 (Bethyl), KAP1 (Cell Signaling), pCHK1 S345 (Cell Signaling), CHK1 (Cell Signaling), GAPDH

(Abcam), HA-tag (Cell Signaling), MYC-tag (Cell Signaling) and tubulin (Pierce). Either fluorophore (Li-Cor)- or peroxidase (Jackson Immunolabs)-conjugated secondary antibodies were used. Quantitation was performed using a Li-Cor Odyssey infrared imaging system.

G_2/M checkpoint: $7.5e^5$ cells were plated per 10 cm dish, grown for 48 h, treated with 10 Gy IR from a 137Cs source or mock treated, allowed to recover for an hour and fixed. Cells were probed for the mitotic marker p-histone H3 S10 (56) using Cell Signaling primary antibody and FITC conjugated secondary antibody (BD Pharmingen). Flow cytometry (Accuri C6, BD Biosciences) was performed as described previously (Theunissen, Petrini 2006).

Immunoprecipitation: Ten to twenty million cells were lysed with 50 mm Tris-Cl, 300 mm NaCl, 10% glycerol and 1% NP-40. Lysates were pre-cleared with protein A agarose beads (Roche); lysate protein concentrations were measured by BCA assay (Thermo Scientific); 20 μg/μl protein solutions were made; beads were incubated with either anti-MRE11 antibody (Cell Signaling), anti-RAD50 antibody (Bethyl) or anti-NBS1 antibody (Novus); and 0.5 mg protein was added along with phosphatase and protease inhibitors (Roche). After an overnight incubation, beads were washed four times. Proteins were eluted from the beads with Laemmli buffer (BioRad), and extracts were heated at 95°C for 10 min.

Yeast two-hybrid: Y2HGold (Clontech) were co=transformed using the Yeastmaker Yeast Transformation system (Clontech) per the manufacturer's protocol. To select for cotransformed cells, transformation reactions were plated onto SD-L-W agar (Clontech) plates, and colonies were picked and streaked onto SD-L-W agar plates. To test for

interaction by colorimetric assay, the restreaked yeast were picked, grown in SD-L-W (Clontech) overnight, assessed for their density (by OD600nm) and briefly centrifuged. α-Galactosidase activity was visualized as conversion of p-nitrophenyl-α-d-galactopyranoside (colorless) to p-nitrophenoxide (yellow, λmax = 410 nm). Sixteen microliters of supernatant was aliquoted per reaction, 48 µl assay buffer (2 volumes 0.5 m NaOAc, pH 4.5 (aq) and 1 volume 100 mm p-nitrophenyl-α-d-galactopyranoside (Sigma-Aldrich) (aq)) was added and the reactions were incubated at least overnight. Each reaction was quenched with 136 µl 1 Na2CO3 (aq), and OD410 nm readings were taken. Colony growth was assessed by culturing yeast overnight, normalizing yeast density by OD600 nm and plating five 10X serial dilutions onto SD-L-W (loading control) and SD-L-W-H-ade (interaction test) agar plates.

Results

Selection of four distinct disease-associated MRE11A mutant alleles: We sought to determine if disease-associated *MRE11A* mutations impact DNA damage signaling in differing ways and if unique functional defects relate to variability in disease sequelae (Fukuda et al. 2001, Bartkova et al. 2008, Uchisaka et al. 2009). Unfortunately, most ATLD-associated *MRE11A* mutations cause low levels of the entire MRN complex, preventing structure–function analyses through study of patient cell lines (Stewart et al. 1999, Delia et al. 2004, Uchisaka et al. 2009, Chaki et al. 2012, Pitts et al. 2001). To circumvent this limitation, we engineered cell lines to express MRE11 mutants of interest at approximately physiologic levels and in which endogenous wild-type *Mre11a* could be inactivated through Cre/LoxP-mediated deletion (Buis et al. 2008).

Inherited alleles of interest were chosen based on maximum differences in patient outcome (Figure III. 1A). To this end, we compared the impact of the first reported ATLD allele, *MRE11A*^{ATLD1}, to that of recently identified alleles in compound heterozygotes, *MRE11A*^{ATLD17} and *MRE11A*^{ASM} (Figure III.1B). *MRE11A*^{ATLD1} contains a nonsense mutation that truncates 76 amino acid residues from the MRE11 C-terminus (Stewart et al. 1999). Although *MRE11A*$^{ATLD1/ATLD1}$ patients and *Mre11A*$^{ATLD1/ATLD1}$ mice exhibited cellular hallmarks of cancer, they do not appear to be cancer predisposed (Stewart et al. 1999, Theunissen et al. 2003). In contrast, two brothers harboring both *MRE11A*^{ATLD17} and *MRE11A*^{ASM} died of pulmonary adenocarcinoma at the ages of 9 and 16 years (Uchisaka et al. 2009). While this patient cohort is small, the early age of lung cancer is striking given that it is normally a disease of old age (Siegel, Naishadham & Jemal 2013). The brothers' wild-type *MRE11A* heterozygous parents and sibling did not have ATLD or lung cancer at last report (Oba et al. 2010).

The *MRE11A*^{ATLD17} mutation (c.727T>C) results in substitution at a highly conserved residue (p.W243R) near motif IV of the N-terminal phosphodiesterase domain (Figure III.1B). *MRE11A*^{ASM} possesses an intronic mutation, c.1098+5G>A, near a splice donor site associated with skipping of the 81-nucleotide exon 10. The resulting mRNA maintains the open reading frame, thus encoding a predicted protein lacking 27 amino acid residues (p.Δ340–366). The deleted residues reside in the capping domain, a structural feature unique to MRE11 compared with other known phosphoesterases (Hopfner et al. 2001, Park et al. 2011).

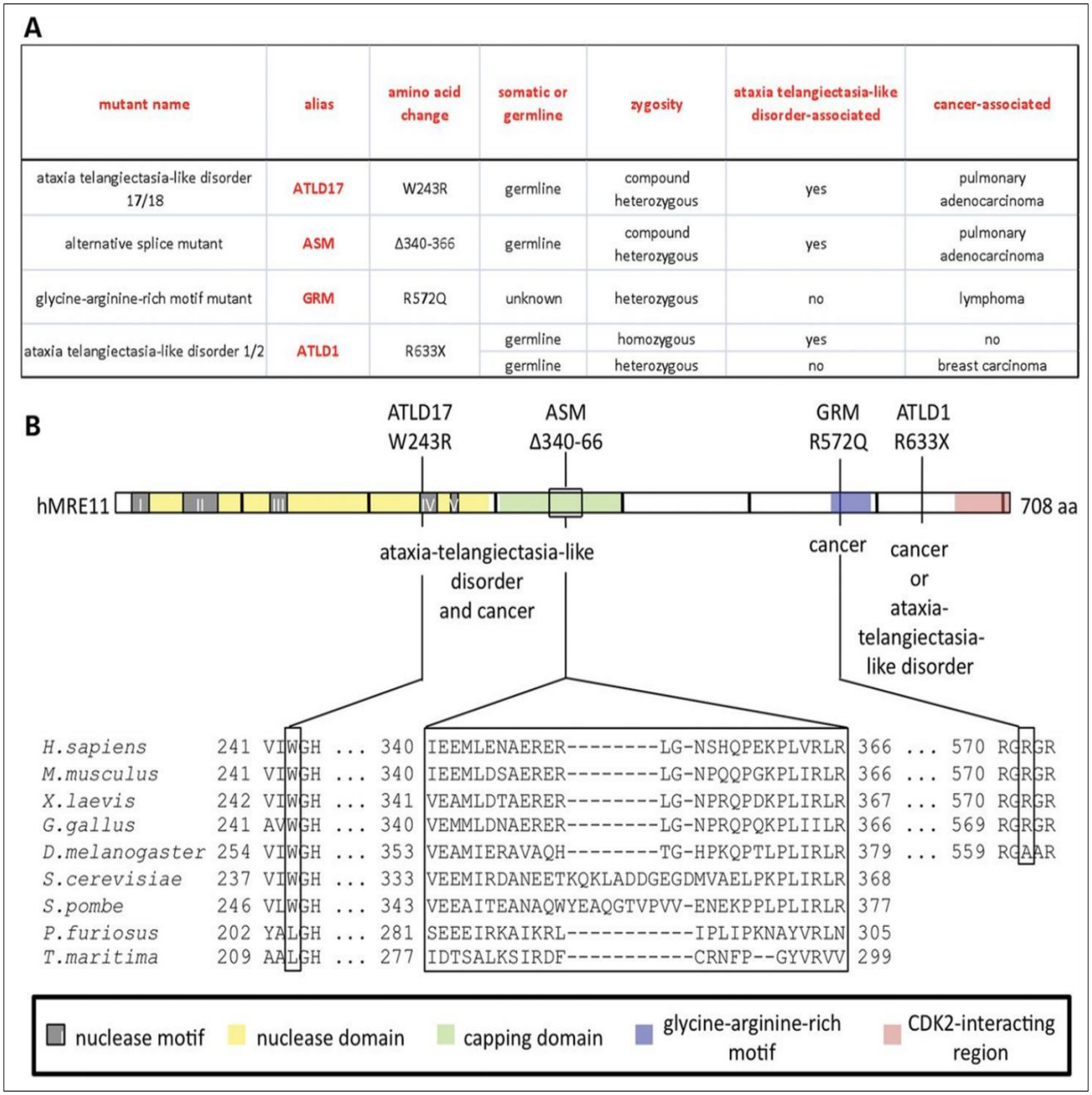

Figure III.1. Disease-associated MRE11 mutants in this study. A. Summary of the name, nickname (alias), amino acid change and clinical context for each mutant. B. *Hs*MRE11 stick diagram with pertinent domains and motifs. Note that the phosphodiesterase domain includes both the nuclease (catalytic) domain and the capping domain. Amino acid changes are labelled along with mutant aliases and associated human diseases (top). Alignments of the residues surrounding the mutant sites are shown (bottom).

In addition to inherited mutations causing ATLD, *MRE11A* has been found mutated in the context of familial and sporadic cancers. Mutational analysis of *MRE11A* in unselected primary tumors revealed a heterozygous mutation (c.1715G>A) encoding MRE11 R572Q in a lymphoma (Fukuda et al. 2001). While it is not known whether this allele was inherited or if it played a causal role in this lymphoma, the mutation nonetheless resides in an interesting region of mammalian MRE11. R572 is in a glycine-arginine-rich (GAR) motif, portions of which are conserved among metazoans. Methylation of GAR motif arginines has been shown to be important for MRE11 exonuclease activity and activation of the ATR kinase (Boisvert et al. 2005a, Boisvert et al. 2005b, Dery et al. 2008, Yu et al. 2012). On the organismal level, alteration of the GAR motif arginines caused radiation hypersensitivity, a trait common among cancer-prone genomic instability syndromes. However, similar to most studied ATLD alleles, the murine MRE11 protein lacking GAR motif arginines was present at low levels relative to wild-type MRE11 (Yu et al. 2012). Thus, we engineered the lymphoma-associated MRE11 GAR motif mutant ($MRE11^{GRM}$) into our expression system to facilitate study of this mutant with the protein at physiologic levels.

Generation of cell lines expressing Mre11a mutant alleles: Immortalized murine embryonic fibroblasts (MEFs) harboring one *Mre11a* allele rendered null in the germline (*Mre11a*–) and one floxed conditional allele ($Mre11a^{cond}$) (Buis et al. 2008) were stably transfected with pEF6-*Mre11a* constructs expressing the mutants of interest from cDNA. Clones were isolated and exposed to adenovirus expressing Cre recombinase to convert the endogenous $Mre11a^{cond}$ allele to null ($Mre11a^{\Delta}$). Clones expressing at least physiologic levels of mutant MRE11 protein were used for experimentation, and protein

levels were confirmed before and after each experimental time period (Figure III.2A). Untagged proteins were expressed to avoid disruption of MRE11 interactions as previously reported (Buis et al. 2012). For *MRE11*^{ATLD1} and *MRE11*^{ASM}, gel mobility distinguished these from wild-type MRE11 (Figure III.2B). For the missense mutants *MRE11*^{ATLD17} and *MRE11*^{GRM}, expression was assessed by an RT-PCR-sequencing strategy (Figure III. 2C) combined with confirmation of endogenous allele deletion by genomic PCR (data not shown) and by comparison with *Mre11a*$^{cond/-}$ cells treated in parallel during each experiment.

Activation of ATM and ATR kinases: MRN recruits ATM to DSBs and facilitates kinase activation through direct interaction of ATM with NBS1 (Lee, Paull 2004, Falck, Coates & Jackson 2005, Lee, Paull 2005). ATM activation and activity can be monitored by measuring ATM autophosphorylation at S1987 (Bakkenist, Kastan 2003) and ATM phosphorylation of KRAB associated protein (KAP1, also called TIF1β, TRIM28 and KRIP-1) (Ziv et al. 2006). Also, ATM kinase and MRE11 nucleolytic activities together mediate DNA resection (Cuadrado et al. 2006, Buis et al. 2008, Wang et al. 2013). The resulting replication protein A-coated ssDNA can be loaded with ATRIP-ATR at which time ATR phosphorylates substrates such as the CHK1 kinase (Zou, Elledge 2003, Falck, Coates & Jackson 2005, Cuadrado et al. 2006). To determine the ability of each MRE11 mutant to activate ATM and ATR in the presence of DSBs, pATM S1987, pKAP1 S824 and pCHK1 S345 induction was measured.

We used IR to experimentally induce DSBs, and observed robust KAP1 and CHK1 phosphorylation in the presence of wild-type MRE11, but only weak induction in the

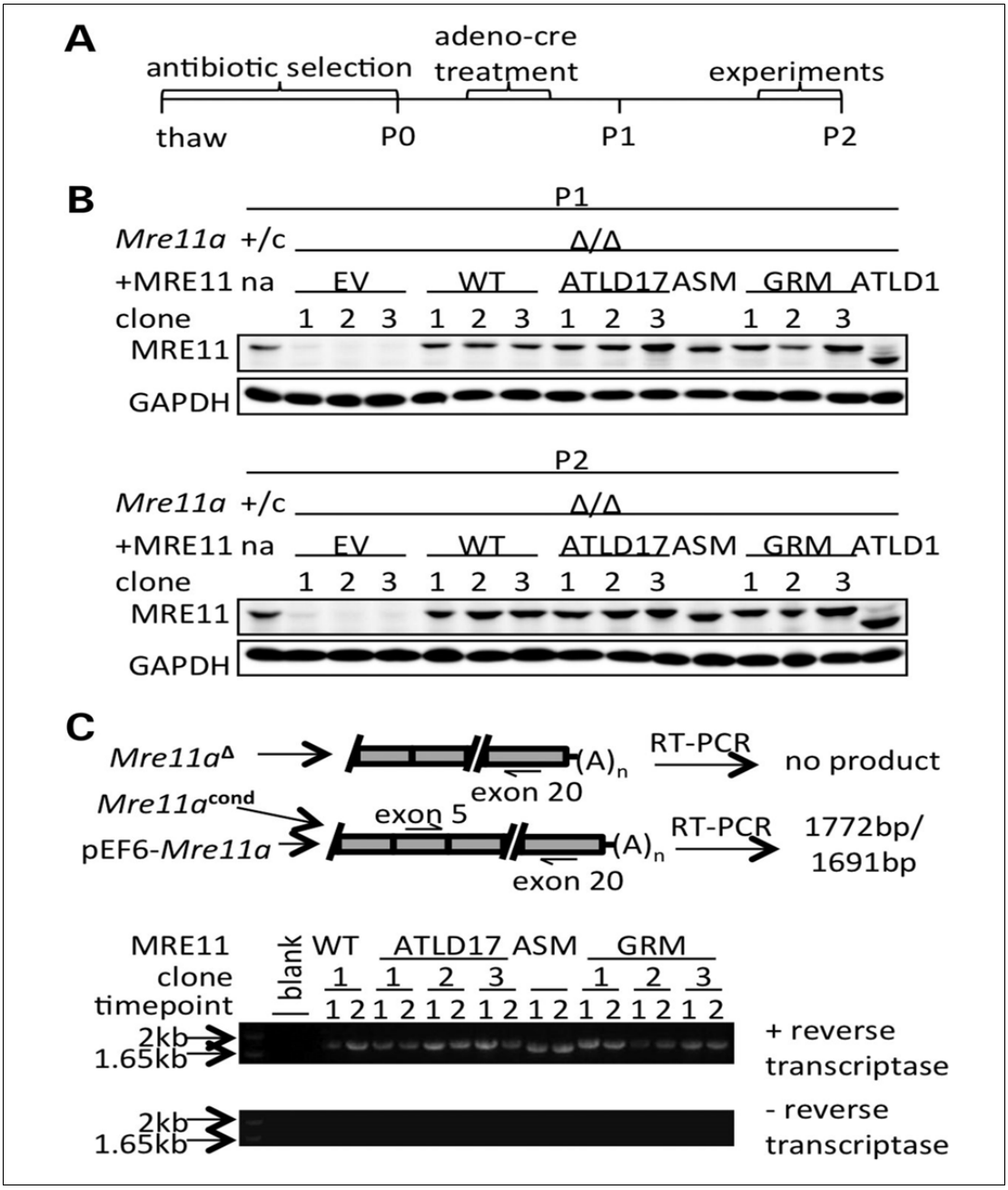

Figure III.2. Generation of MRE11 mutant-expressing cell lines. A. The peri-experimental timeline. Cells were passaged (P) as indicated. B. Western blots demonstrating approximately physiologic levels of MRE11 protein expressed from stably integrated plasmid constructs. Endogenous *Mre11a* was inactivated in these lines through cre-loxP mediated deletion (*Mre11a*$^{\Delta/\Delta}$). Physiologic MRE11 levels encoded by endogenous alleles are shown at the far left (*Mre11a*$^{+/c}$ harbors one wild-type and one floxed conditional allele). EV denotes empty vector controls. C. RT-PCR strategy to type *Mre11a* mRNA of MRE11-expressing clones is shown (above) along with RT-PCR results (below). Bands of the expected sizes were detected for each clone (upper panel). No bands were detected in the absence of reverse transcriptase (lower panel).

absence of MRE11 or ATM (Figure III. 3A and B). We next assessed the impact of MRE11A alleles found in the ATLD siblings who succumbed to pulmonary adenocarcinoma. Cells expressing MRE11^{ATLD17} showed deficiencies in pATM and pKAP1 induction but not pCHK1 induction (Figure III. 3C and D). Decreased pKAP1 induction was observed following IR doses ranging from 0.5 to 10 Gy and following recovery times from 6 min to 8 h. Induction of pKAP1 was also substantially abrogated in cells expressing MRE11^{ASM} (Figure III. 3D). In addition, MRE11^{ASM}-expressing cells showed reduced levels of IR-induced pCHK1, suggesting this mutant was defective in facilitating ATR activity. Hence, whereas both alleles in the ATLD patients with pulmonary adenocarcinoma had a negative impact on ATM activity, only MRE11^{ASM}-expressing cells had deficient ATR activity.

The MRE11^{GRM} mutant identified in a lymphoma supported approximately wild-type levels of ATM activation (Figure III. 3E and F). A modest 10% reduction in KAP1 phosphorylation was noted (Figure III. 3G) but is likely not biologically significant. In contrast, pCHK1 induction was reduced to 50% of wild-type (Figure III. 3E and F), indicating reduced ATR activation. This implies that even a subtle disruption of the MRE11 GAR motif is sufficient to compromise ATR activity.

Finally, we analyzed cells expressing MRE11^{ATLD1}, a mutant from ATLD patients who do not appear to be cancer prone. In stark contrast to the mutants described above, MRE11^{ATLD1} expression was able to complement pATM, pKAP1 and pCHK1 induction (Figure III. 3F). Therefore, this disease-associated mutant retains the ability to activate both ATM and ATR kinases. Quantitation of pKAP1 and pCHK1 induction is shown in Figure III. 3G and H. ATM/ATR activation results are summarized in Table III. 1.

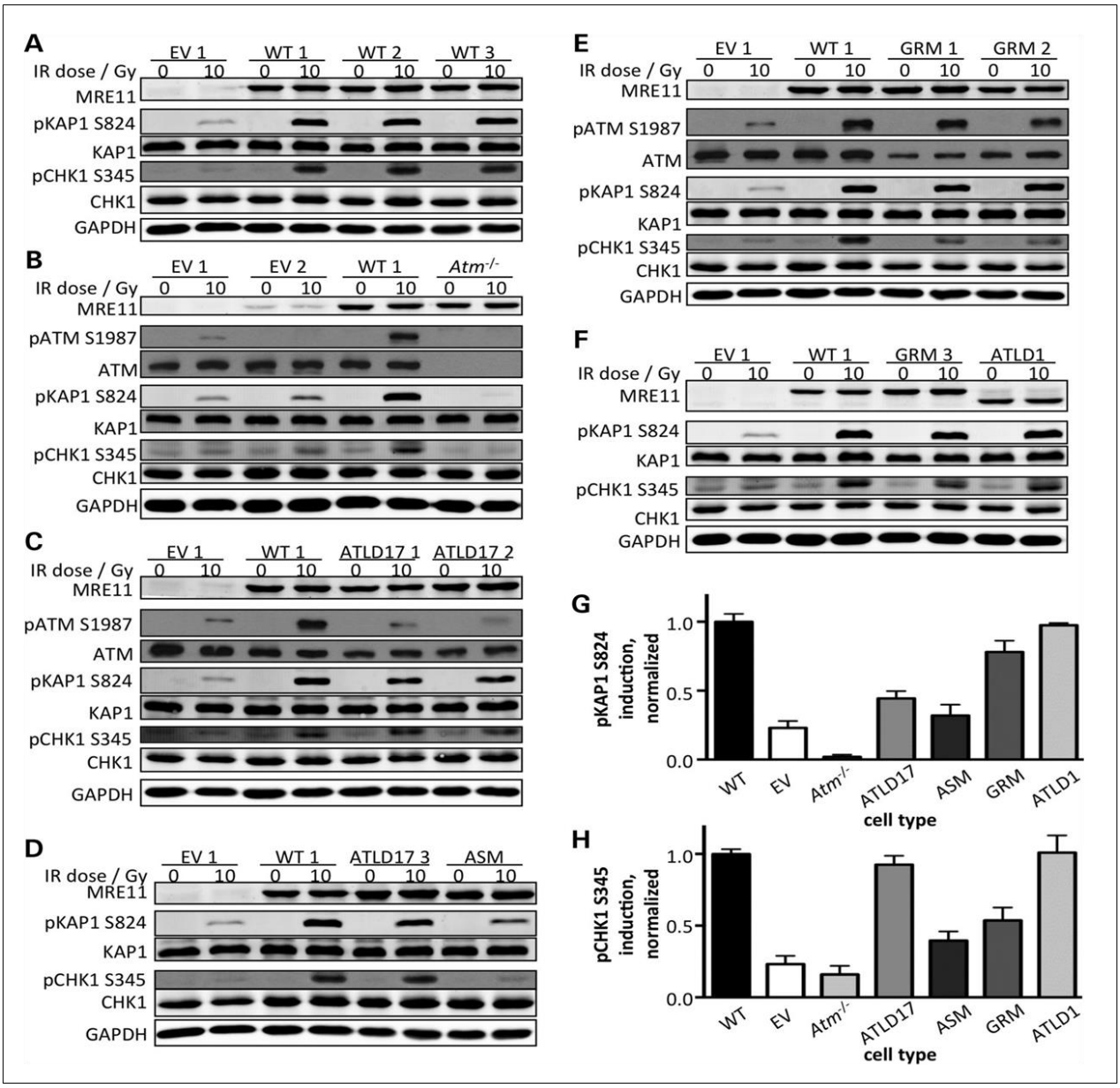

Figure III.3. Assessment of DNA damage-induced ATM/ATR kinase activation. A–F. Western blot analyses for representative substrates of the ATM and ATR kinases. The indicated cell lines (top) were either untreated or treated with 10 Gy IR and allowed to recover for 30 min. Whole cell lysates were immunoblotted for the proteins indicated (left). GAPDH is a loading control. Samples were loaded in various combinations to allow accurate comparison of phospho-protein levels across multiple gels. Immunoblots are representative of at least three independent experiments. EV, empty vector controls. G, H. Quantitation of pKAP S824 (G) and pCHK S345 (H) induction from immunoblots. After accounting for protein loading, induction was normalized to the weighted mean induction of the wild-type lines. Each bar represents at least three independent experiments. Error bars represent standard error of the mean.

Assessment of the G_2/M cell cycle checkpoint: Upon DNA damage, ATM triggers cell cycle arrest at the G_2/M transition (Beamish, Lavin 1994, Liu et al. 2000). We therefore assessed the competency of the early G_2/M checkpoint in our mutant MRE11-expressing lines. Cells were mock-treated or treated with 10 Gy IR, and the mitotic index was calculated based on the percentage of cells positive for the mitosis-specific phospho-histone H3 S10 marker (Van Hooser et al. 1998). Activation of the G_2/M checkpoint is reflected by a significant drop in mitotic index 60 min post-IR. A defective checkpoint is identified by a significantly higher mitotic index post-IR compared with wild-type control. Consistent with previous reports, we found that cells lacking MRN or ATM had a higher mitotic index post-IR compared with wild-type, reflecting the defective G2/M checkpoint (Figure III. 4 and Table III. 1) (Beamish, Lavin 1994, Theunissen et al. 2003).

$MRE11^{ASM}$, which conferred defects in both ATM and ATR kinase activation, caused a defective G2/M checkpoint (Figure III. 4 and Table III. 1). In fact, the impact was similar to that in ATM-deficient cells. $MRE11^{ATLD17}$ did not have a measurable impact on the G2/M checkpoint, despite the reduced activation of ATM. This mutant did permit greater ATM activity than $MRE11^{ASM}$; thus it is likely that the observed difference in checkpoint proficiencies is due to differences in ATM activation. $MRE11^{ATLD1}$, which supported ATM and ATR activation, and $MRE11^{GRM}$, which supported normal ATM activation, both appeared capable of maintaining the G_2/M checkpoint (summarized in Table III. 1).

MRE11/RAD50/NBS1 complex stability: The findings above support the notion that unique *MRE11A* mutations can impact ATM and ATR functions in differing ways.

	Assay	ATLD17	ASM	GRM	ATLD1
ATM activity	pKAP1 induction	+	<+	+++	++++
	pATM induction	+	ND	++++	ND
	G2/M checkpoint	++++	++	++++	++++
ATR activity	pCHK1 induction	++++	+	+	++++

Symbol	+	++	+++	++++	ND
% of wild-type	12.5, 37.5%	37.5, 62.5%	62.5, 87.5%	87.5, 112.5%	Not determined

Table III.1. Comparison of ATM/ATR activation among the four disease associated Mre11 alleles in this study

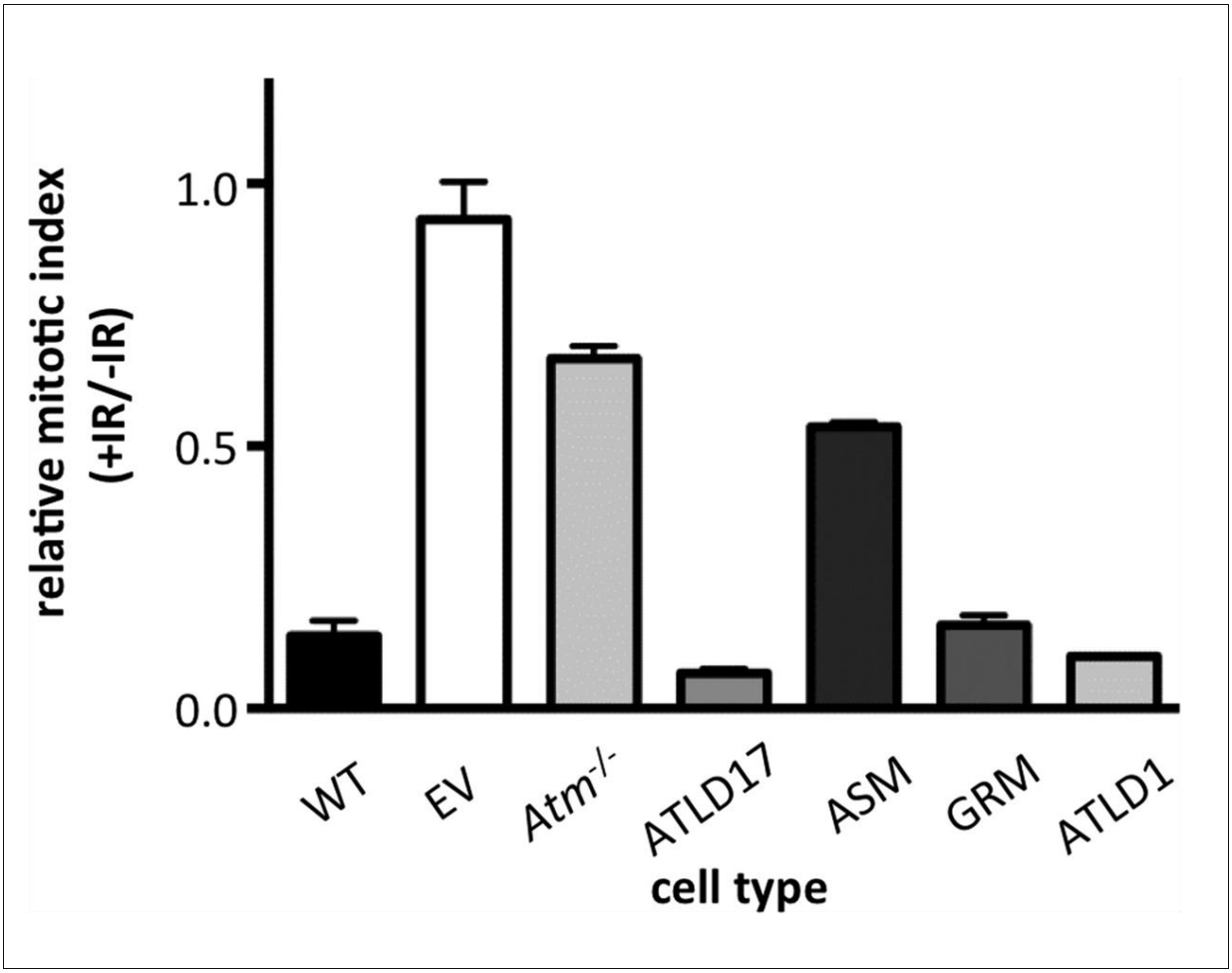

Figure III.4. Assessment of the DNA damage-induced G2/M checkpoint. Cells were either mock treated or treated with 10 Gy IR, allowed to recover for 60 min and then stained for the mitosis-specific p-histone $H3^{S10}$ modification. p-$H3^{S10}$ was detected by flow cytometry, and mitotic index was determined by the percentage of p-$H3^{S10}$ positive cells. Relative mitotic index (*Y*-axis) reflects the ratio of the mitotic index of IR-treated cells to untreated cells for a given line. MRE11 wild-type cells (WT) show a significant reduction in mitotic index after IR, reflecting activation of the G2/M checkpoint. Absence of MRE11 (EV, empty vector) or ATM ($Atm^{-/-}$) shows significantly higher relative mitotic indices compared with WT controls, reflecting a defective G2/M checkpoint. A minimum of three independent experiments were performed for each cell line. Error bars represent standard error of the mean.

Kinase activation by MRN involves conformational changes to all three complex components upon recognition of a DSB (Williams et al. 2008, Hohl et al. 2011, Lammens et al. 2011, Mockel et al. 2012, Schiller et al. 2012, Lee et al. 2013). To gain understanding of the mechanism by which the mutants impact signaling, we compared the stabilities of MRN complexes containing each mutant. Depletion of MRE11 also resulted in decreased NBS1 levels (Figure III.5A and C). In contrast to what was seen for RAD50:MRE11, cells expressing either MRE11^{ATLD17} or MRE11^{ASM} had reduced NBS1:MRE11 ratios suggestive of compromised interaction with NBS1. The least squares fit for wild-type cell lines had a slope appreciably different from the best fit for MRE11^{ATLD17}- or MRE11^{ASM}-expressing lines (Figure III.5C). Cells expressing MRE11^{ATLD1} or MRE11^{GRM} had wild-type-like NBS1:MRE11 ratios, implying normal interactions. In support of our observations, ATLD17/18 patient cells were found to have reduced NBS1:MRE11 ratios (Fernet et al. 2005), whereas ATLD1/2 patient cells had normal NBS1:MRE11 ratios (Stewart et al. 1999) despite both lines having reduced overall levels of MRN. These findings are summarized in Table III. 2.

Previous studies have demonstrated that disrupted MRN complex formation can cause abnormal ratios of the complex components; patients with *MRE11A* mutations that compromise MRE11–NBS1 interaction have reduced NBS1:MRE11 molar ratios (Pitts et al. 2001, Fernet et al. 2005, Uchisaka et al. 2009)). We assessed cellular RAD50:MRE11 and NBS1:MRE11 ratios to determine if they suggested defects in MRN complex stability.

Mre11a deletion resulted in proportionally decreased RAD50 levels (Figure III.5A and B). Expression of wild-type MRE11, or each of the four MRE11 mutants, resulted in a proportional increase in RAD50 levels. A least-squares best fit for all *Mre11a* alleles yielded

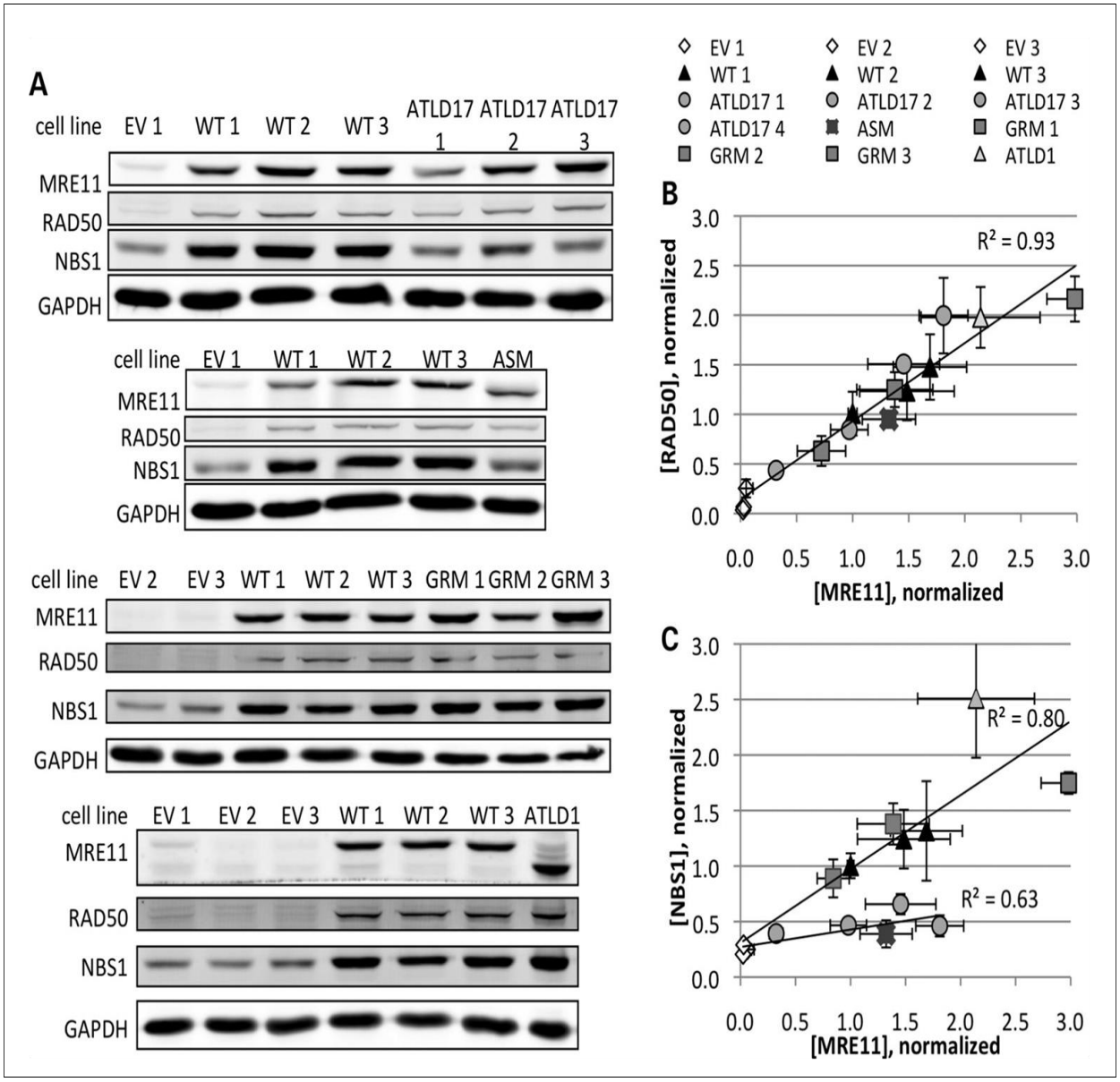

Figure III.5. Comparison of MRE11/RAD50/NBS1 molar ratios. A Western blots comparing protein levels of the MRN components (left). Cell lines (top) are: EV (empty vector controls), WT (exogenously-expressed wild-type MRE11) and the four *Mre11a* mutant alleles as described in the text. Endogenous wild-type *Mre11a* has undergone cre/loxP-mediated deletion in EV and the four mutant lines. B, C. Quantitation of cellular MRN complex component levels from immunoblots as shown in (A). MRE11, RAD50 (B), and NBS1 (C) levels were normalized to those in wild-type-expressing clone 1 (WT 1) whole cell lysate after accounting for protein loading. Each data point represents a minimum of three measurements (range: 3–15, median: 5), and error bars represent standard error of the mean. The trend lines shown with R^2 values represent the least-squares best fits. Note the reduced NBS1:MRE11 ratios in $MRE11^{ATLD17}$- and $MRE11^{ASM}$-expressing lines (C).

a line with an R2 = 0.93 (Figure III.5B). RAD50:MRE11^{ATLD17} and RAD50:MRE11^{ATLD1} ratios were consistent with those found in ATLD17 and ATLD1 patient cells (Stewart et al. 1999, Fernet et al. 2005). Thus, each mutant can maintain RAD50 levels to a similar extent as wild-type MRE11.

Depletion of MRE11 also resulted in decreased NBS1 levels (Figure III.5A and C). In contrast to what was seen for RAD50:MRE11, cells expressing either MRE11^{ATLD17} or MRE11^{ASM} had reduced NBS1:MRE11 ratios suggestive of compromised interaction with NBS1. The least squares fit for wild-type cell lines had a slope appreciably different from the best fit for MRE11^{ATLD17}- or MRE11^{ASM}-expressing lines (Figure III.5C). Cells expressing MRE11^{ATLD1} or MRE11^{GRM} had wild-type-like NBS1:MRE11 ratios, implying normal interactions. In support of our observations, ATLD17/18 patient cells were found to have reduced NBS1:MRE11 ratios (Uchisaka et al. 2009), whereas ATLD1/2 patient cells had normal NBS1:MRE11 ratios (Stewart et al. 1999) despite both lines having reduced overall levels of MRN. These findings are summarized in Table III.2.

We further probed the stability of MRN complexes by co-immunoprecipitation (co-IP) of MRN components. Co-IP of NBS1 with MRE11^{ATLD17} or MRE11^{ASM} was substantially reduced compared with wild-type MRE11 (Figure III.6). In the case of MRE11^{ATLD17}, we also performed co-IPs with NBS1 12 and 30 min after a dose of 10 Gy IR, and we found a defect similar to that seen in unirradiated cells. MRE11^{ATLD17} co-immunoprecipitated with RAD50 to a slightly lesser degree than wild-type MRE11. However, MRE11^{ASM} displayed dramatically reduced co-IP with RAD50. In contrast, MRE11^{GRM} appeared just as capable as wild-type MRE11 in pulling down and being pulled down by RAD50 and NBS1. Previous

	Method	ATLD17	ASM	GRM	ATLD1
Homodimerization	Y2H	++	<+	++++	++++
RAD50 interaction	Molar ratios	++++	++++	++++	++++
	Co-IP	+++	++	++++	++++
NBS1 interaction	Molar ratios	++	++	++++	++++
	Y2H	++	<+	++++	++++
	Co-IP	≥++	≥++	++++	++++

Symbol	+	++	+++	++++	ND
% of wild-type	12.5%, 37.5%	37.5%, 62.5%	62.5%, 87.5%	87.5%, 112.5%	Not determined

Table III.2. Comparison of MRN complex stability among the four disease associated Mre11 alleles in this study

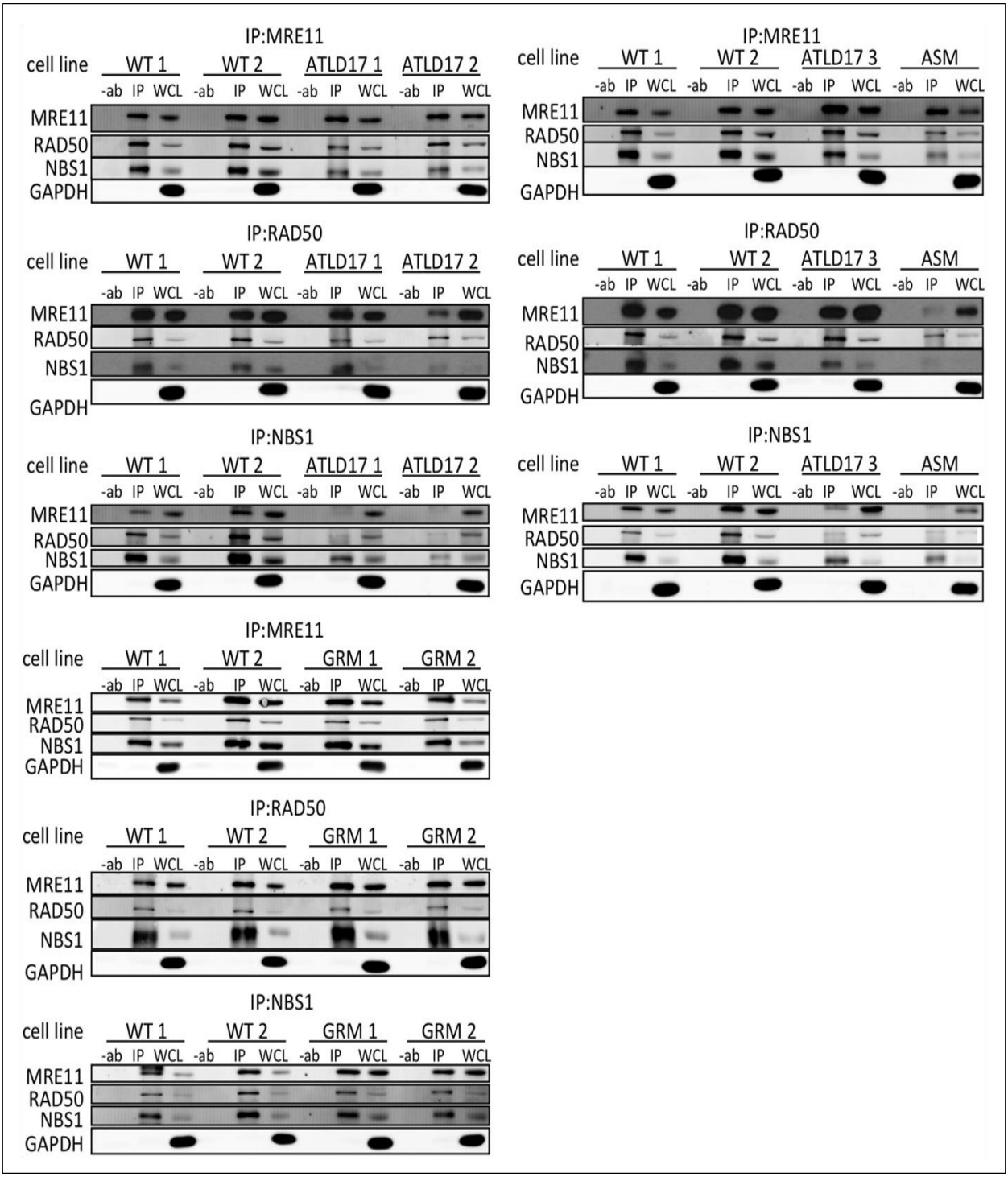

Figure III.6. MRE11/RAD50/NBS1 co-IP. MRE11, RAD50 or NBS1 were immunoprecipitated from whole cell lysates as indicated above each blot. Immunoblots were performed for each MRN complex component as indicated (left). GAPDH was used as a whole cell lysate loading control. Results shown are representative of a minimum of three co-IPs. Reduced MRE11-NBS1 co-IP is observed in lysates from MRE11^{ATLD}- and MRE11^{ASM}-expressing lines. Reduced MRE11-RAD50 co-IP is also observed in MRE11^{ASM} lysates.

work has shown that MRE11^{ATLD1} does not impact the MRN complex as assessed by co-IP (Theunissen et al. 2003, Buis et al. 2012). See Table III. 2 for summary.

These studies imply that MRE11^{ATLD17} and MRE11^{ASM} confer a defect in MRE11–NBS1 binding and, in the case of MRE11^{ASM}, a further impact on RAD50 binding. In contrast, MRE11^{ATLD1} and MRE11^{GRM} appear not to impact MRN complex stability, at least in a manner which can be revealed by co-IP.

MRE11–NBS1 interaction and MRE11 homodimerization: To directly determine whether the four MRE11 mutants in our study impact NBS1 binding, we utilized the yeast two-hybrid system. Wild-type and mutant genes were cloned into pGBK (bait) and pGAD (prey) plasmids, and binding was quantitatively assessed using α-galactosidase expression and qualitatively using colony growth in double selection (-His, -adenine). Both MRE11^{ATLD17} and MRE11^{ASM} displayed substantial reductions in NBS1 binding (Figure III.7A, B). However, MRE11^{ATLD1} and MRE11^{GRM} interacted with NBS1 to similar extents as wild-type MRE11.

Within the MRN complex, MRE11 forms a dimer which bridges DNA ends in close proximity and positions DNA termini within the nuclease domain (Williams et al. 2008, Park et al. 2011). Using the yeast two-hybrid approach, we determined whether the four mutants in our study impact the ability of MRE11 to homodimerize. Compared with wild-type MRE11, MRE11^{ATLD17} homodimerization was reduced (Figure III.7C, D). Strikingly, homodimerization was dramatically reduced for MRE11^{ASM} and MRE11^{ASM} with MRE11^{ATLD17}, appearing similar to the results obtained using empty pGBK and pGAD vectors. The MRE11^{ASM} fusion proteins were confirmed to be present in the yeast strains through immunoblotting.

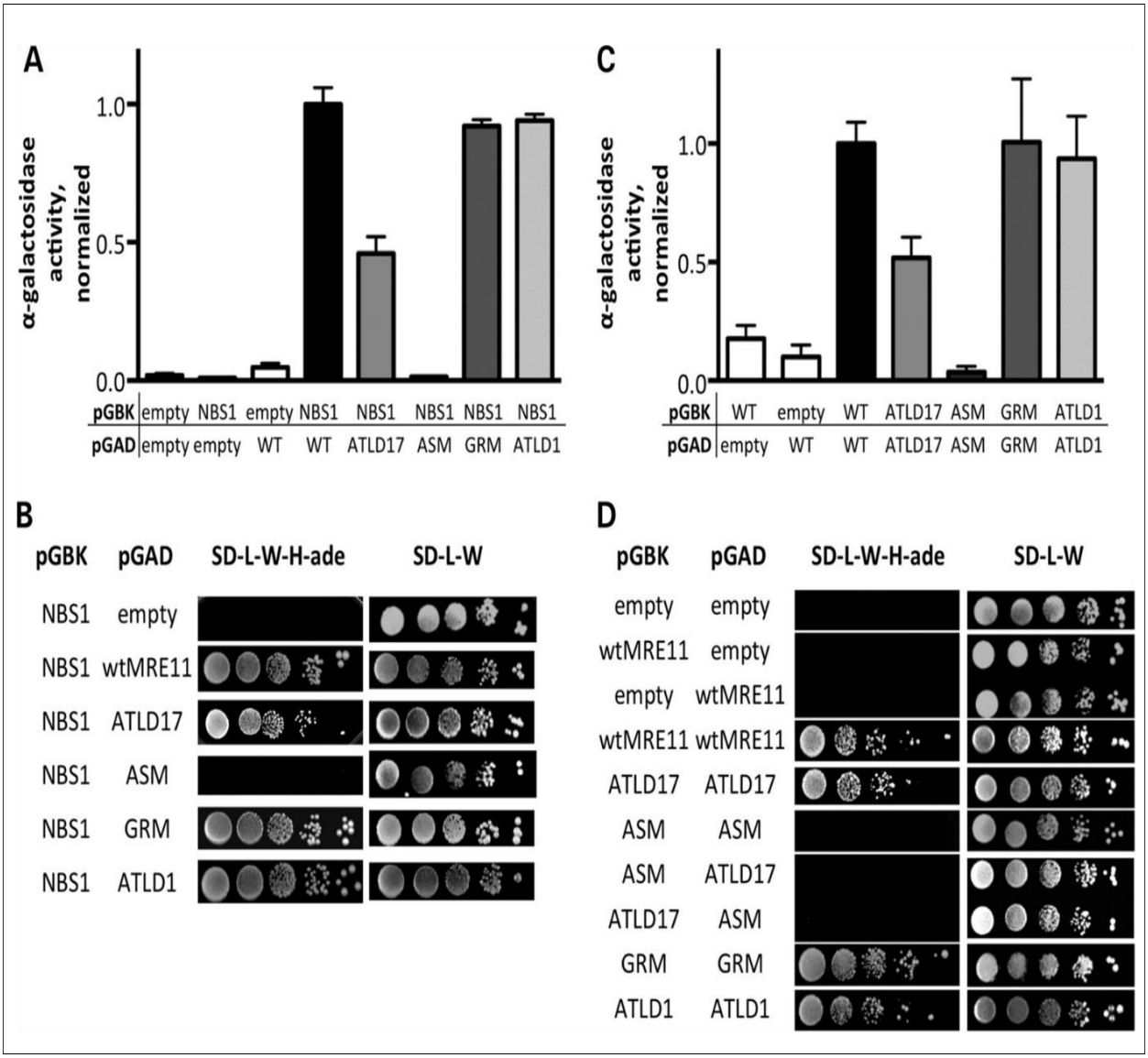

Figure III.7. Direct MRE11–NBS1 interaction and MRE11 homodimerization. Yeast two-hybrid analysis was performed using pGBK and pGAD plasmids—encoding the bait and prey, respectively. Empty vectors are negative controls. Plasmids were selected for by culturing in the absence of leucine and tryptophan (SD-L-W). A. MRE11–NBS1 interaction quantitated by colorimetric detection of α-galactosidase expression. Bait–prey combinations are shown below each bar, which represents at least three clones per combination with at least three measurements per clone. Error bars represent standard error of the mean. B. MRE11–NBS1 interaction by Y2H colony growth assay. Bait and prey proteins are indicated on the left. Ten-fold serial dilution series are shown on interaction test plates (SD-L-W-H-ade, center) and plating efficiency control plates (SD-L-W, right). Results are representative of those for at least three clones per bait–prey combination. C. MRE11 homodimerization by yeast two-hybrid colorimetric assay. D. Homodimerization assessed by colony growth.

No defect in MRE11^{ATLD1} or MRE11^{GRM} homodimerization was apparent. See Table III. 2 for summary.

A mammalian MRE11 homodimerization mutant: Structural studies of proteins from the single-celled eukaryote *Schizosaccharomyces pombe* have suggested a mechanistic link between Mre11 dimer stability and binding of Nbs1 to Mre11 (Schiller et al. 2012). The findings from examination of the four mutants in this study suggest that this relationship may hold true in mammals and represent an important cause of variable disease sequelae for syndromes associated with MRN dysfunction. The two mutants that impacted MRE11–NBS1 interaction, MRE11^{ATLD17} and MRE11^{ASM}, also reduced MRE11 dimer stability, whereas the two remaining mutants, MRE11^{GRM} and MRE11^{ATLD1}, preserved both functions. We therefore wished to determine the extent to which mammalian MRE11 dimer stability and NBS1 interaction are functionally linked. To this end, we sought to design an *Mre11a* mutation that would specifically disrupt dimer formation, using structural and biochemical studies from the archeal species *Pyrococcus furiosus* as a guide (Williams et al. 2008). Archea lack an NBS1 homologue as well as eukaryotic-specific structural motifs of MRE11 involved in NBS1 binding (Park et al. 2011, Schiller et al. 2012). Therefore, residues in mammalian MRE11 that are conserved with those required for dimerization in *P. furiosus* Mre11 likely represent the core features for this function.

We identified murine and human L72 as being homologous to *P. furiosus* Mre11 L61, the latter of which is necessary for Mre11 dimer stability (Figure III.8A) (Williams et al. 2008). L72 is an aliphatic hydrophobic residue that participates in formation of a hydrophobic pocket at the homodimer interface (Figure III.8B). We mutated this residue to aspartate and utilized the yeast two-hybrid system to assess MRE11 L72D homodimerization and direct interaction with NBS1. We observed that homodimeration

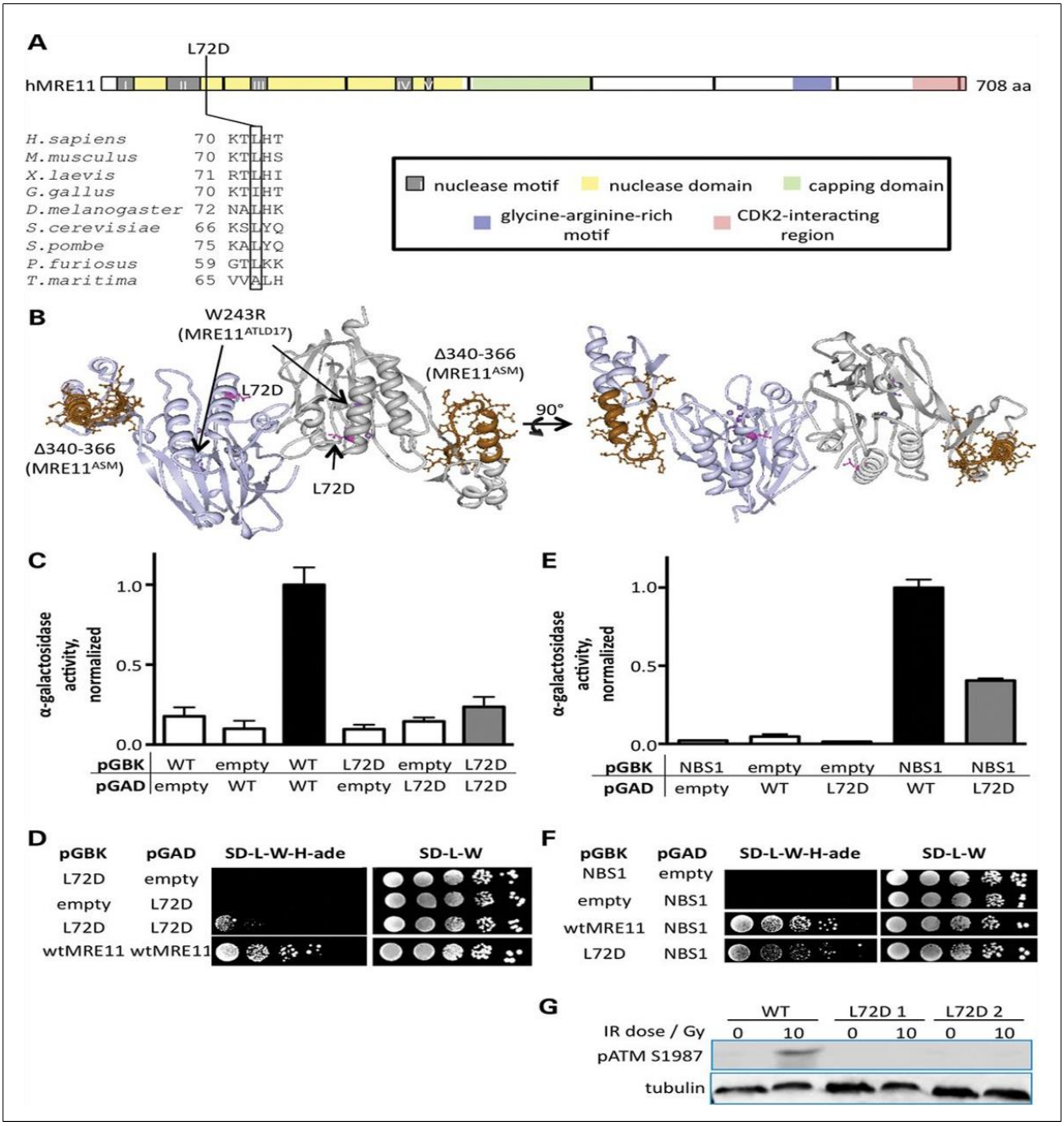

Figure III.8. Engineered MRE11 homodimerization mutant. A. MRE11 diagram and alignment indicating amino acid residue L72. B. Crystal structure of the *Hs*MRE11 N-terminal domain homodimer with mutation sites indicated as follows: L72D (dimerization mutant, pink), W243R (MRE11^{ATLD17}, violet) and Δ340–366 (MRE11^{ASM}, mocha) (46). C. Homodimerization assessed by Y2H colorimetric assay. D. MRE11 homodimerization assessed by Y2H colony growth assay. E. MRE11 L72D interaction with NBS1 assessed by Y2H colorimetric assay. F. MRE11 L72D–NBS1 interaction assessed by Y2H colony growth assay. G. Cells stably expressing either wild-type MRE11 or MRE11 L72D were treated with 10 Gy IR, allowed to recover for 30 min and lysed. MRE11 L72D failed to complement ATM activation as measured by ATM autophosphorylation. Loading control is tubulin.

of MRE11 L72D was impaired, using both α-galactosidase levels and colony growth (Figure III.8C and D). Thus, the function of this portion of mammalian MRE11 is conserved despite significant divergence in nearby structures involved in interaction with eukaryote-specific NBS1 (Park et al. 2011, Schiller et al. 2012). Importantly, direct interaction with NBS1 was also abrogated by the MRE11 L72D dimerization mutant (Figure III.8E and F).

An impact on MRE11–NBS1 interaction would be predicted to affect ATM activation. We tested this by stably expressing MRE11 L72D from cDNA in *Mre11a*$^{\Delta/-}$ cells (data not shown). ATM activation was measured by autophosphorylation 30 min after exposure to IR. Indeed, ATM activation was significantly reduced compared with that in cells expressing wild-type MRE11 (Figure III.8G). Thus, our studies have revealed an important functional link between MRE11 homodimerization, MRE11–NBS1 interaction and ATM signaling in mammals.

Discussion

Here, we have shown that unique inherited alleles of *MRE11A* can have varying impact on control of cellular signaling upon DNA damage. A common theme among the ATLD alleles is reduced activation of the ATM kinase, likely accounting for similar disease sequelae such as cerebellar degeneration. However, the mechanisms differ greatly which may explain clinical variation, such as cancer predisposition. Reduced signaling in *MRE11A*$^{ATLD1/ATLD1}$ individuals likely results primarily from low levels of the MRN complex because we have shown that expression of MRE11^{ATLD1} to normal levels supports ATM activity. On the contrary, in *MRE11A*$^{ATLD17/ASM}$ patients, both mutant alleles impact crucial N-terminal structural features required for MRE11–NBS1 interaction and—therefore—

ATM activation. The specific defects caused by these two N-terminal mutations suggested a mechanistic link between MRE11 homodimer formation and interaction with NBS1, which we demonstrated through generation of a single-amino acid substitution that abrogates both functions.

The N-terminal half of MRE11 is highly conserved throughout nature. Recent co-crystallization of Mre11 and Nbs1 fragments from the single-celled eukaryote *Schizosaccharomyces pombe* shows that *Sp*Mre11 forms a dimer that interacts with two *Sp*Nbs1 fragments (Schiller et al. 2012). Nbs1 binds in a highly asymmetric manner. Although each Nbs1 fragment binds one Mre11 promoter on a peripheral surface, only one Nbs1 fragment binds across the Mre11 dimer interface and makes contact with both Mre11 protomers. This contact occurs at a eukaryote-specific portion of Mre11 recently dubbed 'latching loops' (Schiller et al. 2012). The mutant residue in MRE11^{ATLD17} (*Hs*MRE11 W243, *Sp*Mre11 W248) does not lie within the latching loops. Instead, it forms the hydrophobic core of the structural region encompassing the peripheral binding sites. Hence, its substitution could disrupt folding within the N-terminal domain, thereby effecting binding by Nbs1 at the peripheral sites as well as stability of the Mre11 dimer.

Our studies on the MRE11 L72D mutant have illuminated the mechanistic connection between the homodimer interface helices and NBS1 binding. These helices show high conservation, and studies of Mre11 from prokaryotes and *S. pombe* implicate them in dimer stability independent of the eukaryote-specific latching loops (Hopfner et al. 2001, Das et al. 2010, Lim et al. 2011, Schiller et al. 2012). MRE11 L72 lies within these helices, and the impact of MRE11 L72D on homodimerization provides strong evidence that they function in dimer stability in mammals. The influence of this mutant on NBS1

binding indicates that during evolution eukaryotes have built upon the core dimer structure to provide scaffolding for NBS1-dependent functions such as initiation of DNA damage signaling.

The severe impact of the *MRE11A*^{ATLD17} mutation could in part explain unique aspects of the disease features of the affected patients. *MRE11A*^{ATLD17} was an allele in the two ATLD17/18 children who succumbed to lung cancer, an outcome not reported in other ATLD patients (Stewart et al. 1999, Pitts et al. 2001, Delia et al. 2003, Fernet et al. 2005, Uchisaka et al. 2009). However, the second allele in these individuals must also be considered. *MRE11A*^{ASM} represents a loss of 27-amino acid residues from a feature unique to MRE11, the capping domain (Hopfner et al. 2001). In unicellular organism MRE11 orthologues, the capping domain has DNA- and RAD50-binding sites (Williams et al. 2008, Lammens et al. 2011, Lim et al. 2011, Williams et al. 2011). Therefore, the capping domain is likely to be crucial for folding of the greater N-terminal phosphodiesterase domain and thus required for stability of the MRE11 protein as a whole. In support of this notion, although *MRE11A*^{ASM} mRNA was found in ATLD17/18 patient cells, no evidence of ASM protein was apparent (Uchisaka et al. 2009, Oba et al. 2010). Collectively, the available evidence suggests that *MRE11A*^{ASM} may functionally mimic a null allele in the patient cells, leaving the *MRE11*^{ATLD17} protein as the disease-causing entity.

In contrast to the well-conserved N-terminus, the C-terminus of MRE11 is divergent in several respects. Prokaryotic Mre11 has little sequence beyond the capping domain, whereas eukaryotes have ~300 additional residues. The primary sequence of these residues is poorly conserved when comparing different evolutionary groups of eukaryotes, and no structural information is yet available. The *Mre11a*^{ATLD1} nonsense mutation lies near

the 3-prime end of the *Mre11a* coding sequence and encodes a protein lacking the final 76-amino acid residues (Stewart et al. 1999, Theunissen et al. 2003). This truncated protein maintains interactions among MRN components and supports ATM activation. We conclude from this that ATLD1/2 cells have reduced ATM activity and patients have cerebellar degeneration due to low levels of the MRN complex rather than a specific functional defect in the $MRE11^{ATLD1}$ protein *per se*. This could provide a rational explanation for the differences in disease course of ATLD1/2 patients compared with syndromes resulting from mutations that cause significant structural alterations in the MRN complex. Examples of the latter would include ATLD17/18—as demonstrated in this study—and NBS resulting from early termination and alterative translation initiation of mutant NBS1 (Maser, Zinkel & Petrini 2001)(67). Some ATLD mutations are likely to have intermediate effects compared with those in this study. For example, ATLD3/4 patients harbor a mutation in the N-terminus of MRE11 (N117S), but have not been reported to develop cancer. Interestingly, the ratio of NBS1 to Mre11 proteins in ATLD3/4 is impacted far less than in ATLD17/18, suggesting a lesser impact on MRN structural integrity in ATLD3/4 (Stewart et al. 1999, Uchisaka et al. 2009). Therefore, we postulate that cancer predisposition may arise in the context of major structural abnormalities in the MRN complex and be less probable when mutations have subtle effects on complex stability or primarily effect MRN protein levels.

How the $MRE11A^{ATLD1}$ nonsense mutation causes low MRE11 levels is not understood. The mammalian MRE11 C-terminus has recently been shown to interact with cyclin-dependent kinase 2 (CDK2) and to be required for CDK2-dependent phosphorylation of the DNA repair factor CTIP (RBBP8) (Buis et al. 2012). In principle, loss

of this interaction could potentially destabilize MRE11 in a manner similar to disrupted interactions with NBS1. However, CDK2 levels are not reduced in MRE11 deficiency, and MRE11 is present at normal levels in CDK2 knockout mouse fibroblasts (data not shown and (Buis et al. 2012)). It is possible that additional protein interactions occur at the eukaryote-specific extended MRE11 C-terminus that would explain why levels are low in ATLD1/2. In addition, because mutation of the GAR motif in mice resulted in reduced MRE11 levels, it is formally possible that loss of the C-terminal 76-amino acid residues causes low MRE11 levels by impacting the GAR motif at a distance. Understanding this portion of mammalian MRE11 represents an important future goal for insight into DDRs as well as disease mechanisms of MRN deficiencies.

Although the N-terminal domain of MRE11 is highly conserved, there may be important differences in downstream events under its control. For example, the *S. pombe* mutation analogous to MRE11^{ATLD17} (*Sp*Mre11 W248R) was not defective in activation of Tel1 (the *S. pombe* ATM orthologue) despite causing a significant disruption of MRN complex stability (Limbo et al. 2012). Our studies support the notion that mammalian MRE11 dimer formation and NBS1 interaction are mechanistically linked and that this linkage plays an important role in activation of ATM. While a similar linkage may occur between *S. pombe* Mre11 and Nbs1, communication to the respective DNA damage kinases (ATM versus Tel1) might differ. This makes teleologic sense since simple organisms need only activate checkpoints to provide sufficient time for repair, whereas metazoan cells must decide whether to risk attempting repair, versus initiating senescent or apoptotic programs.

Our studies highlight the notion that crucial differences in disease sequelae among related disorders can result from subtle differences in multi-protein complex stability. In the case of MRN, all inherited disease alleles are hypomorphic owing to embryonic lethality of null alleles. Therefore, this spectrum of disorders provides a superb platform for our greater understanding of inherited syndromes of this sort. Defining the relationship between disease outcomes and specific protein structures and functions has important diagnostic and prognostic implications. With sufficient knowledge, informed decisions can be made regarding long-term medical surveillance and care catered to the specific mutations present. This type of information will also aid in the emerging strategy of developing therapeutics that manipulate protein–protein interactions rather than directly targeting a catalytic site. In the near future, it may be possible to develop compounds that compensate for structural mutations, restoring interactions and catalytic activity.

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CHAPTER IV

Mre11-Cyclin Dependent Kinase 2 complex-associated findings

Summary

This chapter is a compilation of relevant but tangential experiments associated with the main body of my work. Discussed herein are encouraging findings that are additive to my investigational knowledge of the Mre11-CDK2 complex.

Assays were conducted primarily in mouse embryo fibroblast (MEF) cells conditional for *Mre11*. Additional experiments were conducted using the HeLa S3 cervical cancer cell line. The investigations were performed to better understand functional characteristics of the Mre11-CDK2 complex. During these inquires, I characterized CDK2 levels in MEF SV40 large T antigen transformed cell lines, confirmed CDK2 interactions, and revealed unanticipated Mre11-CDK2 interaction properties that are cell type dependent. These findings precipitated further interesting theories and questions that fell outside the scope of my work, but would be worthwhile pursuits for future research on the cellular DNA damage response.

p27 binding to CDK2 after IR in MEF cells

My preliminary experiments established a dephosphorylation event on thr160 of CDK2 that correlated with the dissociation of Mre11-CDK2. I therefore focused my investigation on CDKi p27 as a possible inhibitor of CDK2 activity during normal cell cycle after DNA damage (phosphorylation of thr160 on activation T-loop) based on data showing

that p27 directly inhibits CDK2 (Cheng et al. 1999, Tetsu, McCormick 2003, Martin et al. 2005). Following DNA damage, p27 binds to thr160 phosphorylated CDK2/cyclin A complexes and directly impairs catalytic activity and induces cell cycle arrest. (Morgan 1995, Cheng et al. 1999). The mechanism of action is accomplished through the physical impairment of the catalytic cleft of the CDK2 subunit, thereby blocking ATP loading. Conformational changes further lock the catalytic cleft into an inactive form; however, dissociation of cyclin A from CDK2 does not occur (Sherr, Roberts 1995, Cheng et al. 1999, Sherr, Roberts 1999).

Using the co-immunoprecipitation (Co-IP) protocol described in chapter II, I assessed inhibitory subunit p27 as a regulator mechanism for the reduction in CDK2 after DNA damage. Time points of 10min, 30min, 60min, and 2.5 hours post-IR were tested and analyzed by immunoblot (IB) for p27 (Santa Cruz, C-19, Cat # sc-528, ratio 1:100). My investigation discovered no discernable difference in p27 interaction with CDK2 when comparing Co-IP protein levels of matched controls to treated groups across experimental test points (Figure IV.1).

These data suggest that p27 binding, and therefore inhibition of CDK2, does not increase after IR as demonstrated by treated and control group relative protein levels. Cell cycle inhibitors such as p27 regulate the functions of CDKs to ensure the proper timing and coordination of cell cycle events. While these data infer that p27 has an inconsequential role in CDK2 inhibition, the influence on some portion of CDK2 is clearly evident. Based on the multifaceted regulation of the cell cycle, it is possible that my observed subset of inhibited CDK2 may contribute to the mechanism of CDK2/cyclin A inhibition after DNA damage. An additional inhibitor, p21, is induced by p53, a transcriptional regulator that

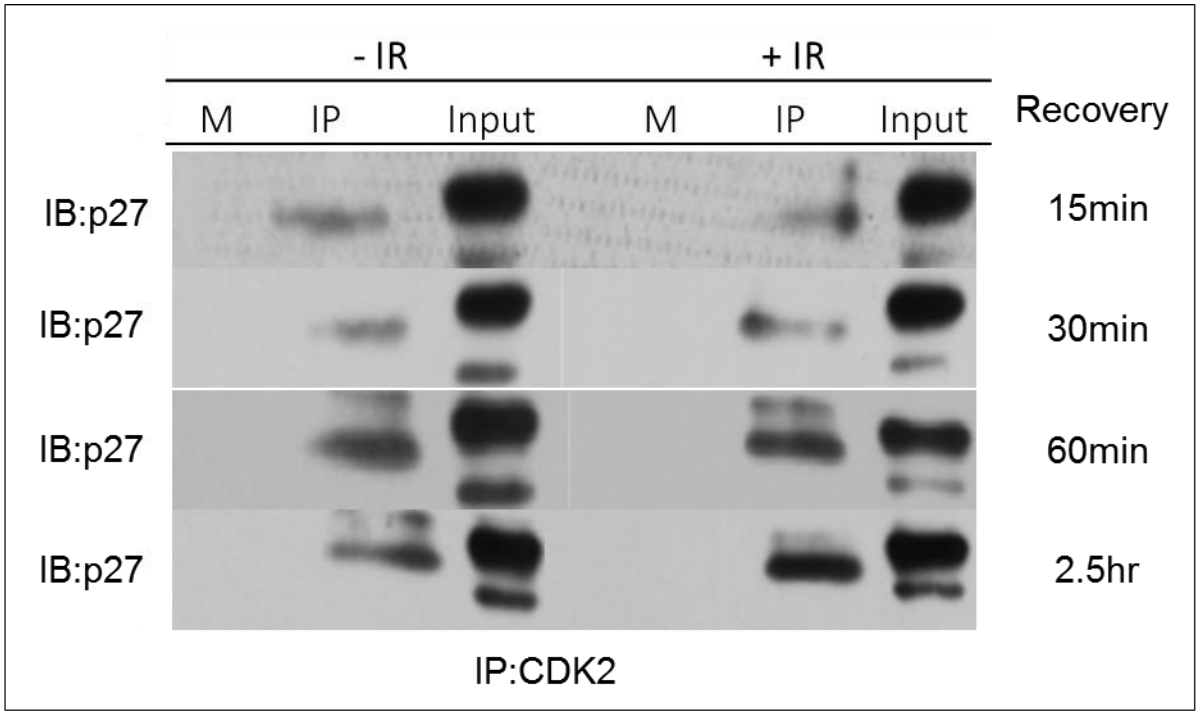

Figure IV.1. Levels of p27 after genotoxic stress in MEF cells. Co-IP: CDK2, IB: p27, IR Rx: 10Gy. Temporal evaluation of p27 interacting with CDK2 at 15 min, 30 min, 60 min, and 2.5 hours.

mediates cell cycle arrest following DNA damage (Dulic et al. 1994, Harper et al. 1995, Canman et al. 1998, Bartek, Lukas 2001, Delia et al. 2003). Further investigations including p21 would be merited based on the scientific evidence available for a role in the dissociation of Mre11-CDK2/cyclin A complex.

My testing required an important modification to the previously effective Co-IP and immunoblot methods, which I maintained for the remainder of testing. I enacted protocol changes when I discovered that the p27 protein had an affinity for the agarose A beads used in the immunoprecipitation. To prevent binding of p27 to the beads, I incorporated a step to block the beads in a fashion similar to the immunoblot membrane blocking procedure. This new step involved pre-incubating the beads with wash/lysis buffer containing 3% serum from the host secondary for 60 minutes at 4° C. Similarly, in an effort to improve immunoblotting methods, secondary antibody host animal serum was incorporated to the protocol to prevent non-specific binding and maintain low background signal. These modifications prevented further non-specific binding of p27 to beads and improved immunoblot performance.

In summary, consistent level of p27-CDK2/cyclin A interaction may be suggestive of a subtle role or one part of a multiplexed mechanism to inhibit CDK2/cyclin A activity in the cell. Further exploration of a sister inhibitor, p21, and CDK2-cyclin A phosphorylation state as determined by inhibitory phosphorylation at thr(T)14 and try(Y)15 require more investigation to fully understand the mechanism providing CDK2 inhibition correlative to Mre11-CDK2/cyclin A dissociation.

CDK2 protein levels in mouse embryo fibroblast cell lines

During the characterization of the Mre11-CDK2 complex, I encountered multiple technical challenges in visualizing the Mre11-CDK2 complex. In this particular case I were unable to detect CDK2 protein by immunoblot after conducting the Mre11 immunoprecipitation, making it impossible to determine Mre11 interaction in treated cells or controls. While the input lane revealed CDK2 protein after immunoblotting, the immunoprecipitation lane did not show CDK2 protein. Upon review of this unexpected result, I determined that my failure was primarily due to the choice of cell line, 513-2, based on the concurrence of method failure and elevating cell line passage number. From another line of investigation, I selected cell line 553-6 for my work, as it was created in our lab, had an analogous genotype, was successfully used in previous work, and had sufficient frozen stocks for continuation without delay. Upon reinitiating experimentation with 553-6, I identified, though immunoblotting, CDK2 protein levels were significantly decreased as compared to 513-2 cells (Figure IV.2 B, C). Based on this evidence, I investigated anti-CDK2 antibodies in an effort to confirm antibody specificity for the target protein. Using cell lysates from previous successful experiments, ID:513-2 as a normal CDK2 level control, the comparison revealed significant differences in CDK2 protein levels in these genetically similar cell lines (Figure IV.2 A). I found evidence supporting this CDK2 protein reduction phenomenon in published data investigating CDKs, clearly demonstrating an association between reduction of CDK2 protein and successive passage of primary $CDK2^{+/+}$ MEFs (Ortega et al. 2003). Therefore, I hypothesized that the maximum level of CDK2 protein is established during the initial isolation of the primary cells. Subsequent

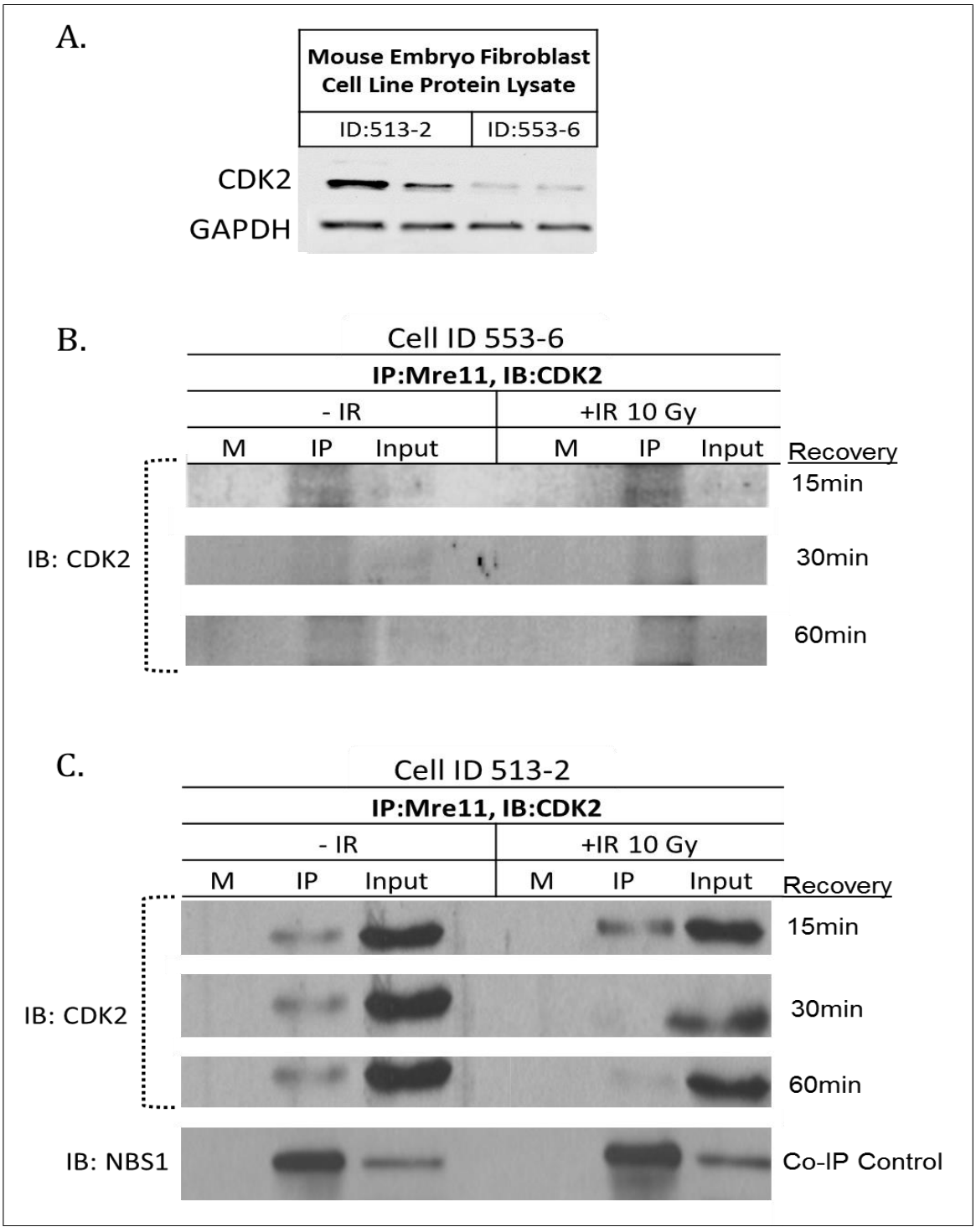

Figure IV.2. CDK2 protein levels in SV40 transformed primary MEF cells with equivalent genetic background. A. Analogous genetic MEF SV40 transformed cell lines created in the Ferguson Laboratory show different levels of CDK2 protein. GAPDH protein loading control B. Co-IP for Mre11-CDK2 interaction using SV40 transformed 553-6 cells. Rx: 10 Grey IR, IP: Mre11/ IB: CDK2. C. Functional Co-IP experiment using SV40 transformed MEF 512-3 cells for Mre11-CDK2 interaction. IP: Mre11/IB: CDK2.

SV40 large T-antigen passages required to attain immortalized cells further reduced the cellular CDK2 protein levels. This resulted in production of immortalized cell lines with variable levels of specific proteins, leading to variability in experimental results.

Technical challenges are inevitable during research and are a consistent part of the scientific endeavor. My unexpected results highlight the importance of maintaining documentation for the purpose of reproducibility and integrity of the experiment. Cell lines often come into question due to frequent manipulation in study conduct and constant passaging for maintenance of stocks. Prior to this intriguing outcome, I had not recognized the possibility of variable CDK2 protein levels between passage numbers and cell lines. After this discovery, I returned to our original cell line stocks with documented, consistent, and acceptable CDK2 protein levels for the remainder of my research.

Mre11-CDK2 interaction in the cervical cancer cell line HeLa S3

During my investigation of the Mre11-CDK2 complex I used HeLa S3 cervical cancer cells because protein levels of the MRN complex are maintained at relatively higher levels in these cells as compared to other cell lines. My initial data revealed the Mre11-CDK2 complex did not dissociate after genotoxic stress in HeLa S3 cells at 8 and 15 min recovery time points (Figure IV.3 A). The assay was repeated with new time points selected based on previous assay data, revealing no dissociation of the Mre11-CDK2 complex. I hypothesized that due to the aggressive and proliferative nature of HeLa cells, the earliest possible time point should be investigated in addition to the known dissociation time point of 30 minutes. To achieve the zero minute recovery, cells were

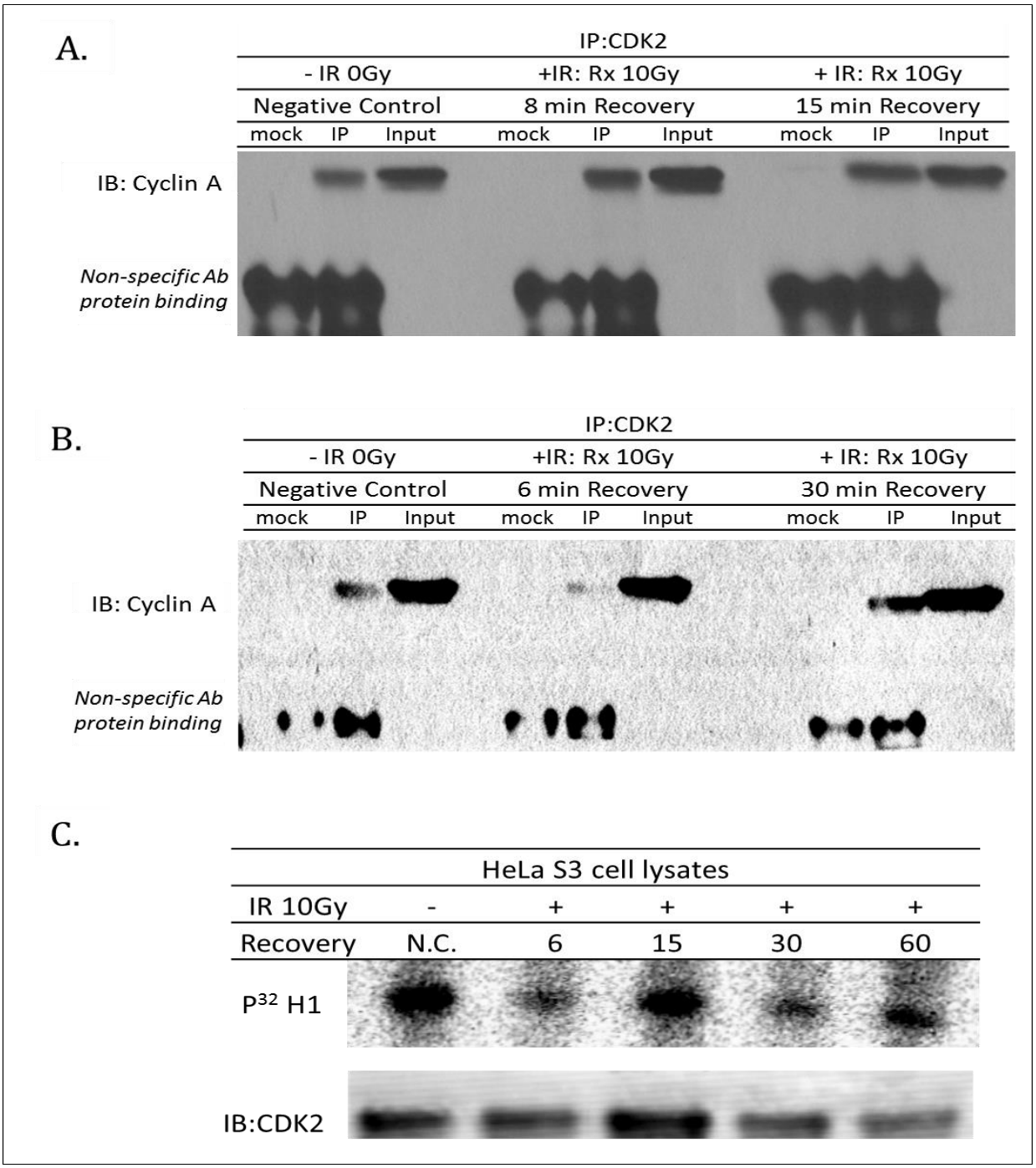

Figure IV.3. Interaction of Mre11-CDK2 and CDK2 kinase activity levels in HeLa S3 cells. A. Mre11-CDK2 complex interaction visualized using IB: Cyclin A as proxy for CDK2. Temporal evaluation of interaction in untreated, 8 minute, and 15 minute recovery groups. Positive control Rx: 10Gy IR. B. Mre11-CDK2 complex second experiment. Temporal evaluation of interaction in untreated, 6 minute, and 30 minute recovery groups. Positive control Rx: 10 Gy IR. C. CDk2 activity in HeLa S3 untreated and treated 6 minute, 15 minute, 30 minute, and 60 minute recovery groups. Protein loading control CDK2.

processed immediately after exposure to the cesium^{137} source. The experimental dose of 10Gy was achieved with an exposure of approximately 6 minutes in duration. These data suggest that in HeLa S3 cells, Mre11 and CDK2 are possibly dissociated during the approximately six minutes (zero recovery time point) the cells were exposed to the cesium source in order to accumulate a dose of 10Gy. The faint band of cyclin A in the immunoprecipitation lane is observable at zero minute recovery; however, is not as definitive as the 8-minute time point. At eight minutes recovery, the complex has assembled and remains intact for the extent of the study design. These temporal data are suggestive of a complex capable of maintaining the interaction between Mre11 and CDK2 during and after 10Gy of IR, and further suggests a deficit of cell cycle checkpoint control that arrests or reversibly halts cellular replication. This finding is significantly different from my outcomes in transformed MEF cells, primary MEF cells, and human primary foreskin fibroblast cells.

These provocative data may allow a supposition based on the characteristics of HeLa S3 cells and my Mre11-CDK2 studies. HeLa S3 cells are used extensively in research based on their characteristic deregulation of important genomic stability mechanisms that create the environment for DNA synthesis and cell division in the presence of DNA damage. My observations of CDK2 and the Mre11-CDK2 complex interaction in HeLa S3 cells suggest a scenario wherein after DNA damage Mre11 and CDK2 are not dissociated, or the disassociation is rapidly reversed, re-establishing Mre11-CDK2 interaction. These actions maintain HeLa S3 characteristic aggressive cell cycle progression (Figure IV.3 A, B). The investigational nature of the initial kinase assay did not include the controls used in later assays. Therefore, the outcome of the assay is not entirely clear, and data interpretation is

limited. Without having direct CDK2 thr160 data (indicator of highest CDK2 activation state) in the study, the kinase assay is representative of broad, non-specific thr160 phosphorylation status.

Data from the kinase assay suggest inhibitory dephosphorylation at thr160 of CDK2 is limited to the six-minute time point, which is reflective of the exposure time needed to cesium^{137} to achieve a 10 Gy dose. The exploratory nature of the HeLa S3 kinase assay, lack of controls, or variability of controls, creates difficulty in extrapolating data from assay results. For example, interpretation of kinase activity is confounded in the presence of variable CDK2 protein levels. Although CDK2 total protein is variable, an evaluation of the CDK2 kinase activity does trend with the dissociation information, suggesting the dephosphorylation of CDK2 in HeLa S3 at site thr160 is constrained during genotoxic stress, and therefore CDK2 maintains a fully active state. I could contend that HeLa S3 cells treated with DNA DSB-inducing IR would continue cycling with minimal or no perceptible alteration in the duration or rate of DNA synthesis, based on my preliminary data. Response to IR in HeLa S3 cells has been examined at different stages of cell division. Those data demonstrate that S-phase is not delayed in HeLa S3 cells upon irradiation with a dose of 10Gy (PAINTER, ROBERTSON 1959, YAMADA, PUCK 1961, TERASIMA, TOLMACH 1963). My exploratory study requires additional and appropriate controls to properly analyze CDK2 thr160 phosphorylation state to make a full evaluation of the influence Mre11-CDK2 complex dissociation has on CDK2 activity.

In hyperploid HeLa S3 cells, interaction between Mre11-CDK2 after IR revealed a distinct dissociation and interaction profile from normal cells derived from our mouse colony (SV40 transformed and primary cells) as well as human primary foreskin

fibroblasts. The extraordinarily aggressive proliferation of these highly malignant cervical cancer cells makes analysis of complex dissociation intriguing. Investigations using HeLa S3 cells may provide further insight into prospective Mre11-CDK2 complex functions as well as the role of CDK2 in the DDR.

CDK2 kinase activity in MEF cells lacking *Mre11*

During the investigation that led to the finding that Mre11 regulates CtIP resection by interaction with CDK2, the Ferguson laboratory made a second significant observation. Data had clearly revealed that Mre11, specifically the C-terminus, is essential for the interaction with CDK2 (Buis et al. 2012) . This stimulated the hypothesis that Mre11 may influence CDK2 biological activity as well as enable CtIP phosphorylation. Therefore, the experiment was conducted in cells engineered in our lab having a *loxP*-flanked conditional allele for *Mre11, Mre11*$^{cond/\Delta}$, in which Mre11 can be rendered deficient (*Mre11*$^{-/\Delta}$) (Figure IV.4). Additional *CDK2*$^{-/-}$ cells were used as a control for the evaluation of non-specific activity in the absence of CDK2 protein. These findings revealed a significant reduction in CDK2 biological activity and dephosphorylation of thr160 correlative to the deletion subsequent diminution of Mre11 (Figure IV.4). These *in vitro* data are suggestive of reliance on the MRN complex for CDK2 activity in the normal cell cycle. The observations made during my investigation of the DDR have interesting comparisons to this *in vitro* assay, specifically the loss of protein-protein interaction leading to dramatic change in CDK2 kinase activity and dephosphorylation at thr160 (Figure II.1A, B and Figure II.2A).

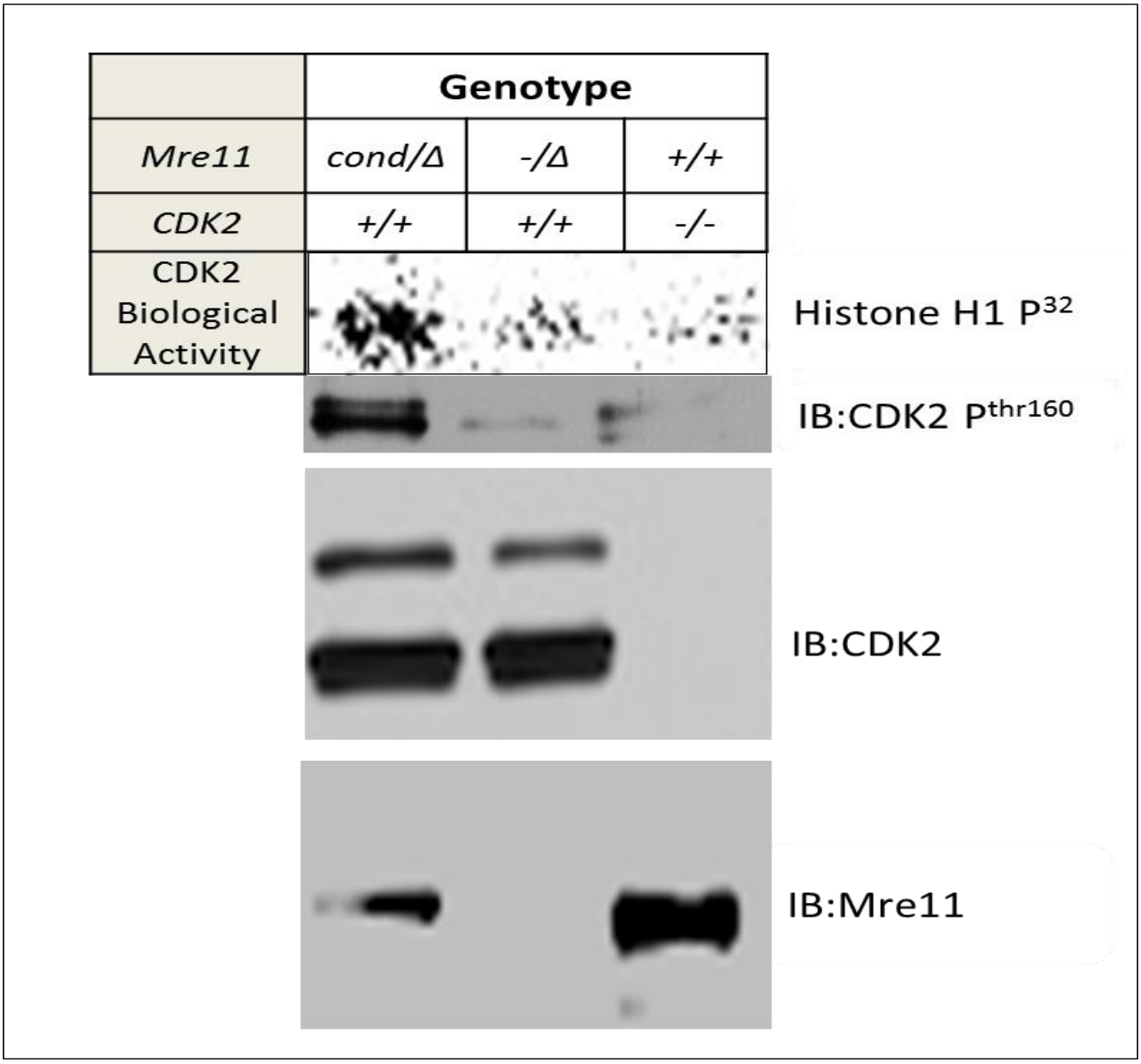

Figure IV.4. Mre11 protein and CDK2 kinase activity *in vitro*. Lane 1. Mre11 / CDK2 proficient cells visualize baseline CDK2 kinase activity. Lane 2. *Mre11* deficient cells, proficient in CDK2. Lane 3. CDK2 deficient cells (*CDK2*$^{-/-}$) proficient in Mre11 (*Mre11*$^{-/\Delta}$).

Mre11 C-terminus immunoprecipitation of endogenous CDK2

A recent study on our lab demonstrated that the CDK2 interaction with Mre11 occurs at the C-terminus (Buis et al. 2012) . I conducted a series of transient transfections in our MEF cell line using P3XFlag-CMV plasmid (N-terminal flag, Sigma, St. Louis, MO) in combination with my co-immunoprecipitation protocol, using Clontech FLAG conjugated beads (anti-DYKDDDDK beads, Clonetech, CA, Cat#635686). The co-immunoprecipitation/transient transfection integrated protocol is listed in Chapter 2. In triplicate repeat experiments, I successfully pulled down CDK2 using the N-terminal FLAG-tagged Mre11 C-terminus fusion protein (Figure IV.5). This data is suggestive that the C-terminus contains critical interaction points. Investigation of the Mre11 C-terminus is inhibited by lack of structural data for eukaryotic Mre11. Furthermore, the C-terminus sequence does not contain any recognizable domains (Buis et al. 2012) . These data clearly demonstrate efficacious binding of a N-terminus DYKDDDDK tagged Mre11 C-terminus fusion protein to endogenous CDK2. These results complement earlier findings wherein a construct of the $MRE11^{ATLD1}$ protein absent of the 76 AA C-terminus was ineffective for binding a HA-tagged CDK2 protein in a co-immunoprecipitation experiment (Buis et al. 2012) . This demonstration of interaction between the Mre11 C-terminus fusion protein and endogenous CDK2 in unperturbed cells reinforces our earlier $Mre11^{ATLD1}$ findings (Buis et al. 2012) . This demonstration of CDK2/cyclin A binding specificity at the C-terminus of Mre11 provides insight towards a possible dissociation mechanism for the Mre11-CDK2/cyclin A complex after IR. I demonstrated that ATM is required for the Mre11-CDK2 complex dissociation after IR. Of particular interest at the Mre11 C-terminus are the SQSQ residues residing within the final 76AA of this protein. The SQSQ motif is a known

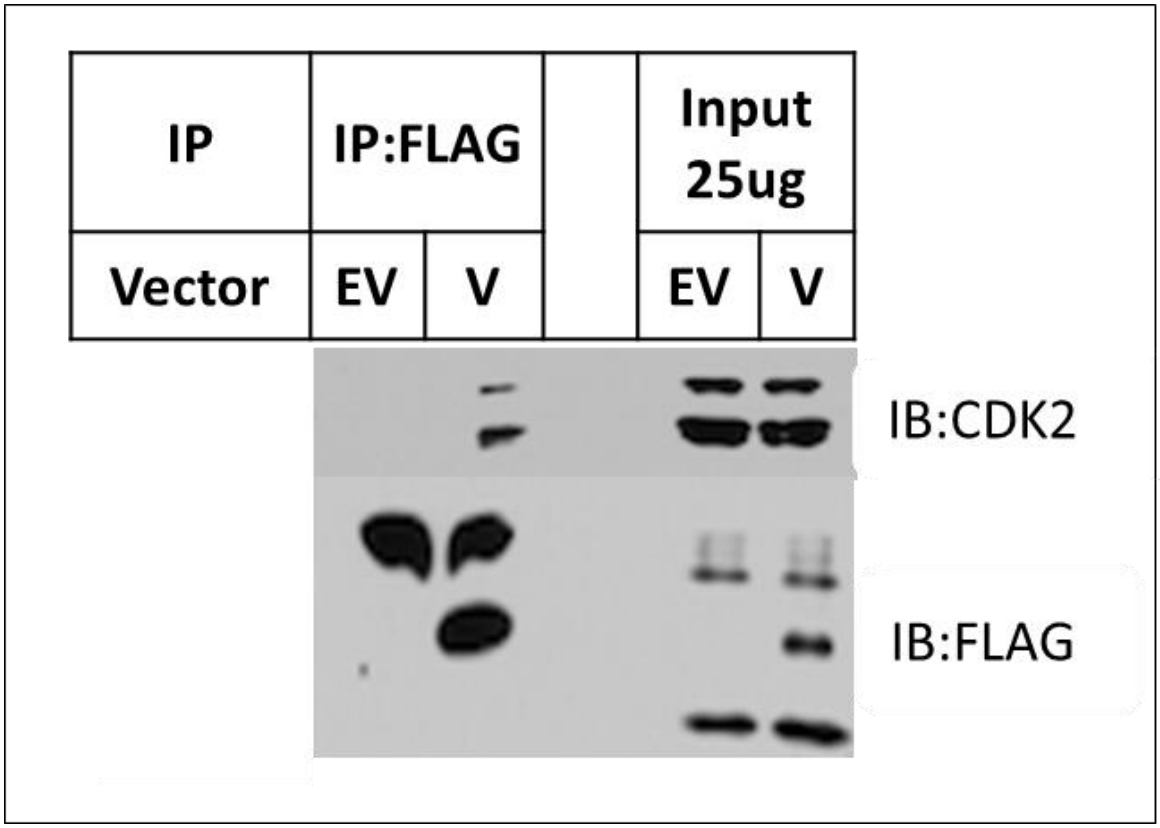

Figure IV.5. FLAG-tagged Mre11 C-terminus fusion protein co-immunoprecipitation of CDK2 endogenous protein in SV40 transformed MEF cells. Empty Vector (EV) cell lysate, Vector (V) cell lysate containing FLAG tagged fusion protein.

phosphorylation target for the ATM and ATR kinases (Kim et al. 1999, Abraham 2001, Matsuoka et al. 2007). The presence of a C-terminus phosphorylation motif, in conjunction with CDK2 binding sites, may be indicative of a possible biochemical method for Mre11 dissociation from CDK2. In this model, the ATM kinase phosphorylates the SQSQ site resulting in modification of the Mre11 C-terminus, resulting in dissociation and a correlative reduction in CDK2 biologic activity (Abraham 2001, Matsuoka et al. 2007). Further investigation is required to determine if the SQ residues become phosphorylated after IR in connection with dissociation of Mre11 from CDK2.

Effects of small molecule inhibitor binding on CDK2-Mre11

This work is part of an ongoing collaboration with the Cierpicki Laboratory, through which I was provided NMR spectra confirming the Mre11-CDK2 interaction. The Cierpicki laboratory is developing and screening new chemical entities (NCE) for the purpose of evaluating efficiency of disrupting protein-protein interactions. The Cierpicki laboratory generously provided two NCE from a library of compounds for testing. Both of the compounds target the RxL hydrophobic motif on cyclin A without disturbing the cyclin A/CDK2 interaction, allowing us to continue using cyclin A as a surrogate for the presence of CDK2 (Figure IV.6). Besides the cyclin A binding motif, residues on the T-loop adjacent to the thr160 site on CDK2 provide multiple points for interaction. The RxL hydrophobic patch has been established as a common binding point for substrates that interact with cyclin A (Schulman, Lindstrom & Harlow 1998) , and studies have revealed that inhibitory proteins such as p21 and p27 bind at this motif (Dynlacht et al. 1997, Vlach, Hennecke & Amati 1997, Schulman, Lindstrom & Harlow 1998) .

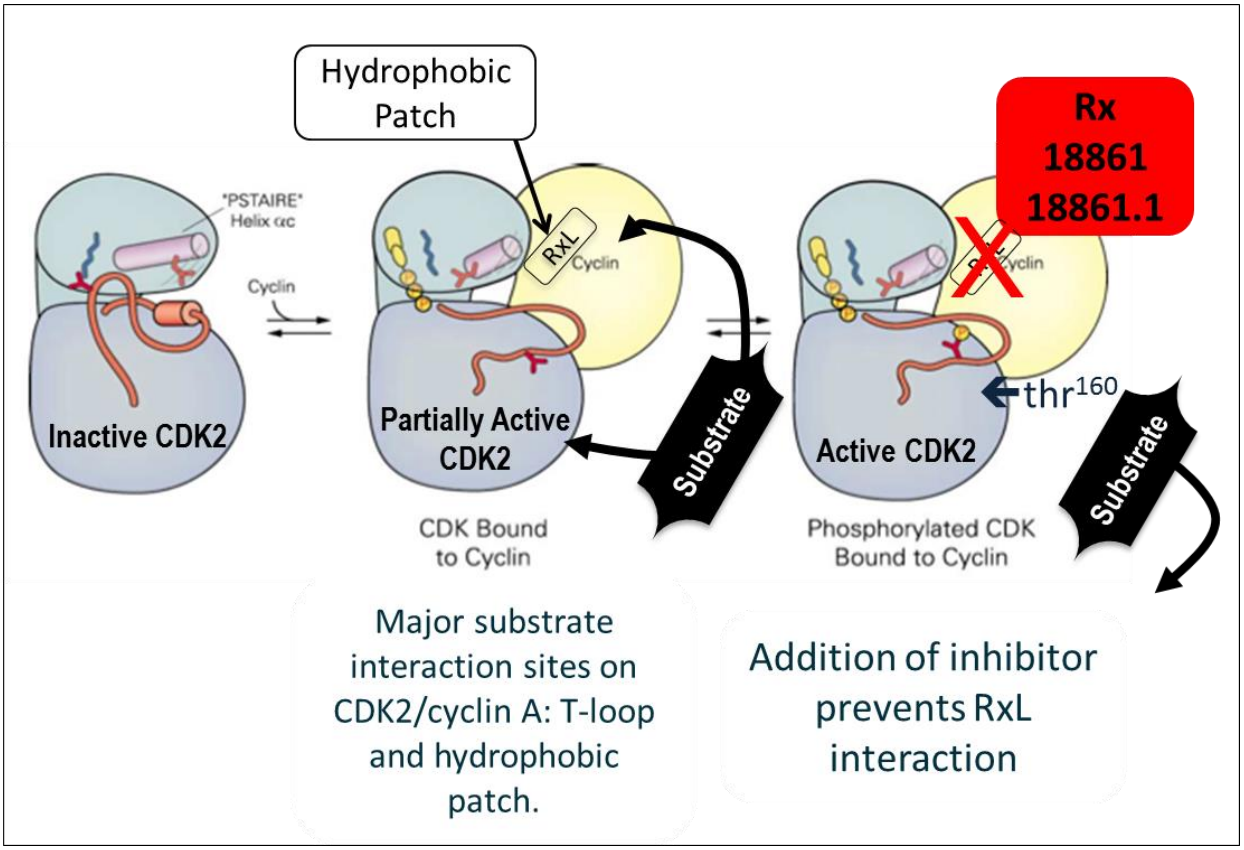

Figure IV.6. CDK/cyclin binding positions for small molecules 18861 and 1881.1 competitively binding RxL hydrophobic patch on cyclin. Adapted from Molecular Biology of the cell, 2008

Using my co-immunoprecipitation procedure, I investigated binding of Mre11 to the CDK2/cyclin A complex in the presence and absence of the NCE18861 and NCE18861.1 compounds. In the first experiment, NCE18861 alone was studied at 20μM and 40μM. Testing conditions included negative control cells treated with DMSO and a positive control group treated 10Gy IR for confirmation of dissociation. The results are shown in Figure IV.7A. Assay controls indicated assay validity; vehicle control showing no dissociation and positive control showing a significant dissociation of Mre11 from CDK2/cyclin A after 10Gy IR. The drug treatment group data was suggestive of a dose-related response wherein 20μM showed negative control values of association and the high dose of 40μM displayed a dissociation of CDK2 from Mre11 at levels equivalent to positive control.

The second experiment was performed with both NCE18861.1 and NCE18861 (40μM as advised the Cierpicki laboratory). The results showed that negative and positive controls performed as expected with respective association and dissociation of Mre11-CDK2 complex (Figure IV.7B). Cells treated with NCE18861 again demonstrated a reduction in binding of Mre11 and CDK2, confirming the initial result. Cells treated with NCE18661.1 demonstrated an absence of interaction between Mre11 and CDK2. The small molecule NCE18861 data suggest a role for cyclin A in providing a docking site for Mre11 *in vitro* and in my endogenous tissue culture based testing. My data shows that 40μM NCE18661.1 can prevent Mre11 from binding CDK2, which was inconsistent with the Cierpicki laboratory's evaluation of NCE18861.1. The Ceirpicki laboratory assessment determined, *in vitro,* that 40μM NCE18861.1 was ineffective in blocking protein-protein binding. Possible explanations for this discrepancy may be attributed to the nature of the NCE testing assay. The Cierpicki laboratory *in vitro* screening assays determine compound

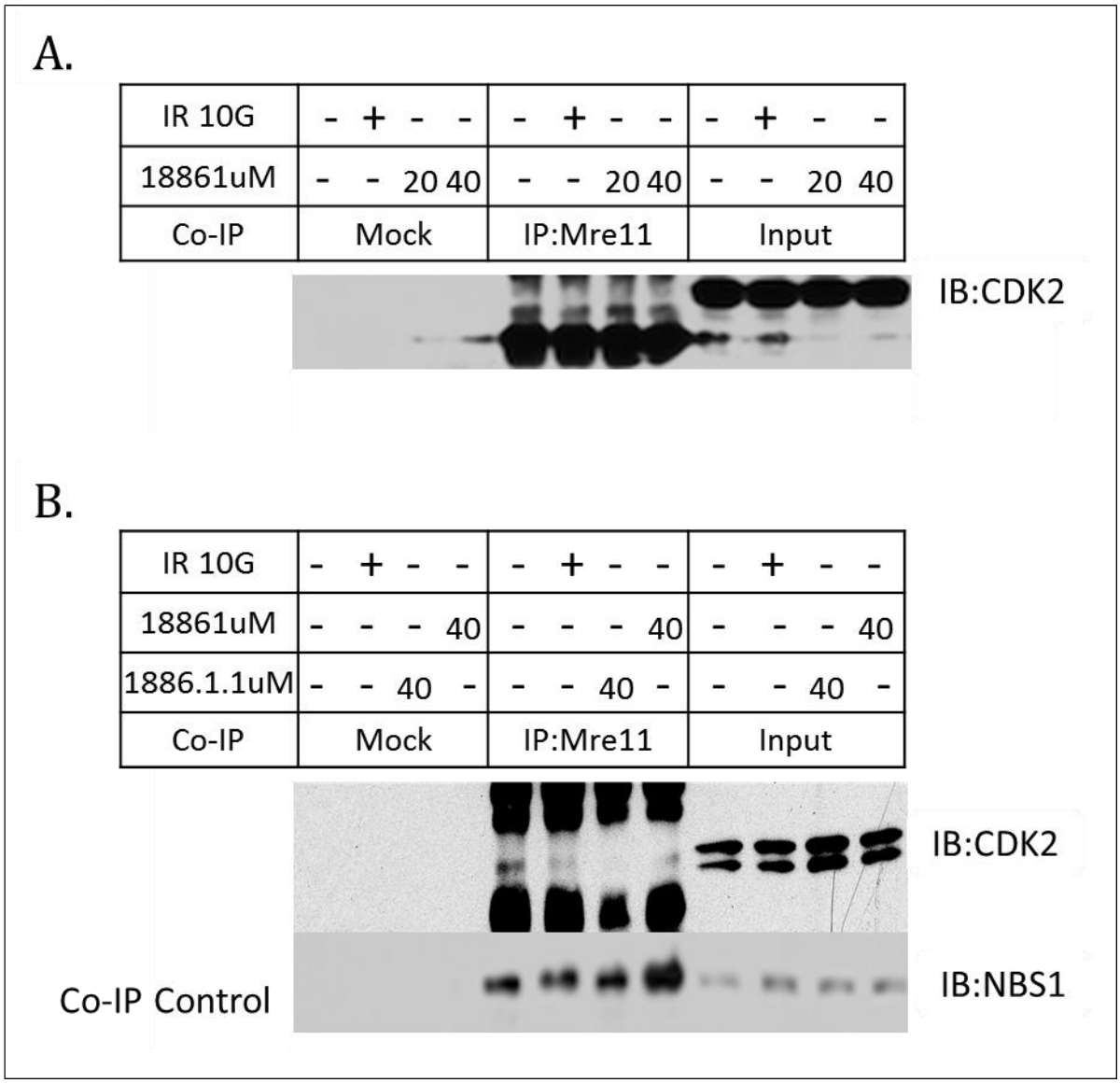

Figure IV.7. Binding of small molecule inhibitors specific for RxL motif on cyclin A. A. SV40 transformed MEF cells dosed with NCE18861 at concentrations of 0, 20, 40μM and allowed to recover for 1 hour. 10 Gy IR positive control. B. SV40 transformed MEF cells dosed with NCE18861 and NCE18861.1 at concentrations of 0 and 40μM and allowed to recover for 1 hour. 10 Gy IR positive control.

activity in an artificial environment in order to evaluate entire NCE libraries under like or similar conditions. This controlled experimental environment permits suitable candidates to be selected. My assessment of 18861.1 was conducted under conditions more reflective of a normal biological system in SV40 transformed MEF cells. In this cellular environment, it is possible that the chemical structure of NCE18861.1 could contribute to non-specific mechanisms lead to the same expected outcome. The NCE18861.1 dose concentration may also responsible for dissociation of Mre11 from CDK2 and not the binding of NCE18661.1 to the RxL motif on cyclin A. Additionally, the possibility of dissociation due to cytotoxicity remains plausible in my testing system; my primary experiments did not include a corollary assessment of toxicity at each respective dose. Nevertheless, these data from NCE18861 remain compelling and merit continued experimentation with addition appropriate controls. With the appropriate controls and dose regime, an accurate result reflective of the mechanism of action can be ascertained. Despite the design limitations of these preliminary experiments, the results from NCE18861 permit speculation of the RxL motif on cyclin A interacting with Mre11. Furthermore, based on my *in vivo* data, the possibility of NBS1 or RAD50 interacting with cyclin A may be entertained, as one member of the complex cannot exist without the others. Of the remaining MRN members, evidence would support NBS1 as a prime binding partner candidate, based on the broad hydrophobic interfaces in the FHA-BRCT domain of NBS1 (Williams et al. 2009). The question of how NBS1 physically integrates DNA end sensing and processing with cell-cycle checkpoint activation remains to be elucidated. Conceivably, a spatial and temporal interaction with a single substrate by one or more members of the MRN complex is plausible based on the dynamic structural characteristics of the MRN complex.

Interaction of CtIP with BRCA1 depends on CtIP-Thr847 phosphorylation by CDK2. Allowing NBS1 to interact with CtIP directly, this interaction is significantly increased in the presence of Mre11 and RAD50 *in vivo* in an unperturbed cell (Chen et al. 2008). The Ferguson laboratory recently published work on CtIP regulation by Mre11 interacting with CDK2; in this body of work it was revealed that Mre11 is capable of immunoprecipitating CtIP, CDK2, and cyclin A in a single co-immunoprecipitation assay (Buis et al. 2012) . Mre11 presence in the immunoprecipitation would be highly suggestive that NBS1 is also present in this complex in unperturbed cells. Moreover, recent data investigating the role of CDK2 in the DDR has shown that CDK2 identifies and phosphorylates NBS1 in response to genotoxic stress in order to promote damage repair and checkpoint signaling (Wohlbold et al. 2012).

My evidence-based hypothesis centered on available data and my observations that MRN forms a complex with CDK2/cyclin A (specifically Mre11 bound to CDK2/cyclin A), upholding CDK2 activity in S-phase and regulating CtIP resection capacity. Control of CtIP resection capacity secures homologus recombination as the primary method of DNA DSB repair in S-phase. These choreographed efforts result in synchronizing high fidelity repair systems with sister chromatids in an unperturbed cell.

In the event of genotoxic stress, the hypothetical MRN-CDK2/cyclin A complex dissociates, resulting in NBS1 phosphorylation by an independent CDK2/cyclin A complex (Wohlbold et al. 2012). The phosphorylation of NBS1 would promote radio-protective effects such as DNA damage sensing, MRN repair capabilities, and initiation of cell cycle checkpoints (Wohlbold et al. 2012). In this postulate NBS1, as an essential part of the MRN

complex, participates in the activities of DNA end sensing and processing in addition to cell-cycle checkpoint activation through MRN complex dissociation from CDK2/cyclin A.

The existence of a small molecule that, based on my data, mimics IR-induced dissociation of the Mre11-CDK2 complex is of immense benefit for future Mre11-CDK2/cyclin A studies, and will allow for a more diverse investigation of the interaction. However, limitations do exist with any method; novel NCEs may not be fully characterized or may have unknown off-target effects. Proper precautions taken during the conduct of the assay, such as appropriate controls, will maintain confidence and validity of the assay. In doing so, accurate data interpretation and results can be reported.

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CHAPTER V

Discussion and Summary

Introduction

The objective of our research included three main biologic questions: The first examined the nature of the interaction of the Mre11- CDK2/cyclin A complex in a genotoxic environment and how the relationship changes between Mre11 and CDK2 in MEF SV40 large T antigen transformed cells, primary cells, and human foreskin fibroblast cells? Our second query asked how CDK2/cyclin A responds to genotoxic stress, specifically whether direct modifications to CDK2/cyclin A play a role in the DDR. Our final research aim was to answer whether the Mre11-CDK2 complex and CDK2 was ATM-dependent.

Summary of Chapters

Chapter II research was based on the recently discovered interaction between Mre11 and CDK2 (Buis et al. 2012). Those studies revealed that during normal cell cycle progression, the temporal and spatial integration of these complexes enables information exchange for the regulation of HR repair (Buis et al. 2012). This novel observation changed the functional paradigm of the MRN complex, repositioning it as a fundamental element of the mammalian cell cycle. The MRN tripartite complex responds to DSB by rapidly binding to DNA ends, stabilizing chromatin structure, and transmitting information of DNA damage to the ATM kinase through the actions of Mre11, Rad50, and NBS1 respectively.

Based on MRN participation as an early responder to DNA DSB and the role of CDK2 in cell cycle checkpoint control, we sought to examine their contributory roles in the DDR. Our studies investigating the Mre11-CDK2 complex provide biological evidence of a dissociation induced by DNA damage. The abrogation of Mre11-CDK2 interaction results in changes to each respective member, resulting in potential structural changes and subsequent protein phosphorylation. In our research we adhered to specific post-IR time points so that the three-dimensional re-orientation between Mre11 and CDK2 occurring after induced damage could be compared to other investigations. We found that the timing of CDK2 thr160 dephosphorylation corresponds to dissociation of the interaction. Evaluation of dissociation timing with the inhibitory dephosphorylation of CDK2 at thr160 on the activation T-loop revealed a temporal correlation, suggestive of dissociation-enabled cell cycle cessation or reversible arrest upon DNA damage (Figure V.1).

Like MRN, the ATM kinase is a key mediator of cellular response to DNA damage (Durocher, Jackson 2001, You et al. 2005). Therefore, we examined the Mre11-CDK2 complex interaction in the absence of ATM kinase activity by studying the interaction in *ATM*$^{-/-}$ cells and using an ATM inhibitor, KU-55933. We discovered the Mre11-CDK2 complex remains bound in the absence of ATM protein or activity, indicating that ATM is required to enable the dissociation of Mre11 from CDK2. This evidence is again suggestive of an intricate spatial and temporal system that detects, propagates, and elicits a response to genotoxic stress to prevent replication and inheritance of deleterious DNA alterations.

In chapter III, we assessed the consequences of Mre11-associated gene mutations on DDR activation and signaling (Regal et al. 2013). Alterations in Mre11 result in the disorder ataxia-telangiectasia like disorder in humans (ATLD; *Mre11* hypomorphism)

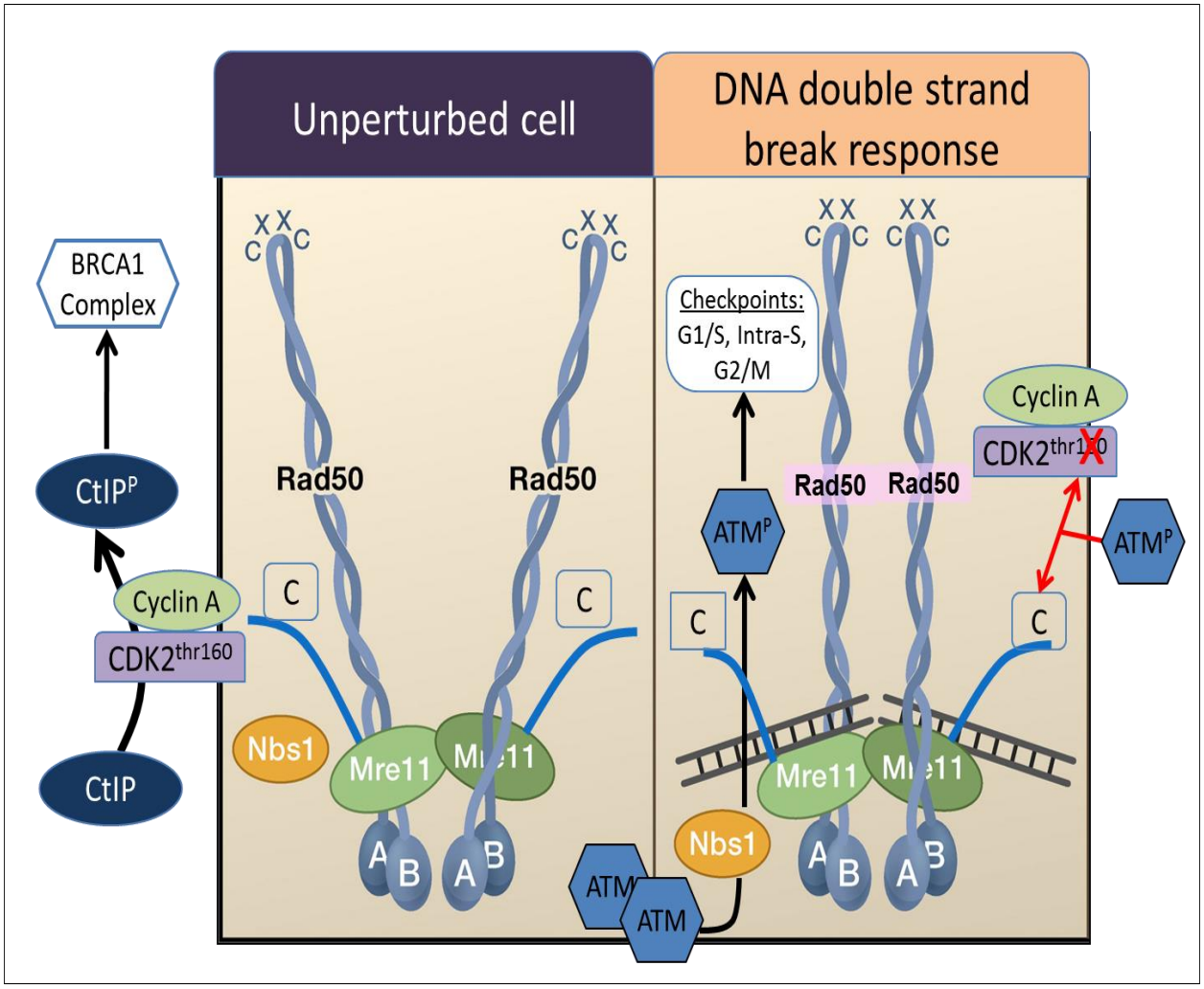

Figure V.1. Schematic model for Mre11 roles in an unperturbed cell and the DNA damage response. In unperturbed cells Mre11 interacts with CDK2 to control the phosphorylation of CtIP thereby regulating resection capacity for HR in S phase. In presence of DNA DSB, the Mre11-CDK2 complex is rapidly dissociated in an ATM dependent manner. Correlative to the dissociation CDK2 is dephosphorylated at thr160 resulting in a reduction of CDK2 biological activity. Suggestive of a cell cycle halt or reversible arrest in S phase for the purpose of DNA damage repair. Modified from Buis et al NSMB 2012.

(Stewart et al. 1999a, Fernet et al. 2005, Uchisaka et al. 2009). Through our research, we created representative cellular systems to study the diverse sequelae of ATLD. This work revealed that the proximity of the alteration to the N-terminus (e.g. MRE11^{ATLD17}, MRE11^{ASM}) or C-terminus (e.g. MRE11^{GRM}, MRE11^{ATLD1}) contributes to the mechanism of reduced ATM/ATR signaling by destabilizing the MRN complex or low MRN complex levels, respectfully (Regal et al. 2013).

Our data on cancer-related N-terminal mutations, *MRE11A^{ATLD17}*and *MRE11A^{ASM}*, demonstrated destabilized interactions between the component members. The destabilization of the MRN complex suggests that dissemination of DNA integrity information to ATM and ATR kinases is reduced or deficient. MRN stability is maintained in C-terminal modified MRE11 (*MRE11A^{ATLD1}*), allowing for informational exchange with PIKK proteins ATM and ATR. Interestingly, C-terminus mutations in MRE11 typically demonstrate reduced levels despite high MRN complex stability. Therefore, the reduction in activation of ATM is based on a decrease in concentration of the MRN complex as compared to a forfeiture of functional performance.

The maintenance of genome stability depends on MRE11 participation with RAD50 and NBS1 to form a three-dimensional complex. Disruption of MRE11 biochemical structure, demonstrated in MRE11^{ATLD17}, MRE11^{ASM}, MRE11^{GRM}, and MRE11^{ATLD1}, results in a change in the protein subunit, loss of protein-protein interactions, and reduction in complex integrity. In chapter III, we propose that the location of the MRE11 alteration and resulting impact on subunit interface and structural stability contribute to differences in ATLD cancer predisposition severity. These studies highlight the intricacy of defining biochemical and biological functions relevant to a cancer predisposition.

In chapter IV, tangential experimental data pertaining to the Mre11-CDK2 interaction as well as CDK2 biological activity in the presence and absence of Mre11 were documented. These experiments added evidence to our research objectives surrounding the role of the Mre11-CDK2 complex interaction in the DDR. Additionally, these experiments brought to light additional valuable information and outcomes meriting further review as stand-alone investigations. By documenting these preliminary experimental findings we hope to provide insight and support into future scientific endeavors carried out in the Ferguson laboratory. Perhaps these data may prove themselves important directly or indirectly to further our understanding of how the MRN complex functions to reduce the rate of DNA synthesis, a role that remains to be completely elucidated.

During the writing and collating of our observations from chapters II, III, and IV, a compelling supposition could be made for a role of the MRN complex in reducing the rate of DNA synthesis in S-phase upon recognizing DNA damage. Our investigation of Mre11 was conducted in the context of normal function in the DDR as well as in disease-associated, functionally compromised Mre11, recapitulating the defective or flawed *MRE11A* present in cell lines generated from affected patients. This approach of understanding normal cellular operation through the study of a defective or complete loss of function can be both effective and worthwhile.

A compelling theme from each component of our research has been the importance of the Mre11 interaction for affecting biological systems in response to DNA damage. For example, our investigation of the Mre11-CDK2/cyclin A complex clearly demonstrated that the inhibition of ATM kinase activity resulted in persistence of the Mre11-CDK2/cyclin A

complex after IR induced DNA damage, suggestive of S-phase checkpoint control deficit by the cellular mechanisms.

Interestingly, ATLD cells lack the S-phase cell cycle checkpoint. Moreover, components of the ATM-NBS1-MRE11 are mutated or deregulated in some cancers (Khanna, Jackson 2001, Shiloh 2003, You et al. 2005, Bartkova et al. 2008, Riley et al. 2008). It is not known how the Nbs1-Mre11-Rad50 complex reduces the rate of DNA synthesis (Falck et al. 2002). Having demonstrated that the Mre11 interaction with CDK2 requires ATM activity (modulated by NBS1) and *in vitro* CDK2 kinase activity is significantly decreased in the absence of Mre11, we continued our investigation of Mre11-CDK2/cyclin A using ATLD hypomorphic *MRE11A* cell lines. These cells contained intra-subunit instability ($MRE11^{ATLD}$-RAD50-NBS1), resulting in reduced MRN complex function. $MRE11^{ATLD}$ instability is inevitable; however, the magnitude of the instability is determined by disruption of specific NBS1 motifs or an absence /truncation of MRE11 that creates constraints on the interaction. Furthermore, modulation of ATM activity necessary for checkpoint control is significantly reduced in the absence of NBS1 protein (Horejsi et al. 2004, Lee, Paull 2005a). As noted in our work on the Mre11-CDK2/cyclin A complex, ATM is necessary to dissociate the complex, which is suggestive of an S-phase arrest.

The Mre11-NBS1 interaction for regulating the degree of ATM activation in association with the Mre11-CDK2/cyclin A complex dissociation allow us to postulate a biological malfunction resulting in radio-resistant DNA synthesis (RDS). The ATLD17 mutation ($MRE11^{ATLD17}$) contains inappropriate N-terminus substitutions and is associated with an aggressive cancer, pulmonary adenocarcinoma. Studies on patients with ATLD observed that $MRE11^{ATLD17}$-NBS1 interaction is reduced as compared to normal MRE11

control groups. Furthermore, the severity of outcomes in *MRE11A* hypomorphs can be differentiated based on the proximity to the N or C terminus. MRE11^{ATLD17} abnormal residues reside in close proximity of the N-terminus, resulting abnormal N-terminal residues result in disruption of the MRN complex.

Our experimental model and collaborative compilation of patient data have shown that the mutational changes to MRE11^{ATLD17} protein structure contravene NBS1 binding, reducing sensing and binding efficiency. Furthermore, interaction with ATM and subsequent activation is constrained by MRE11^{ATLD17} aberrations (Bakkenist, Kastan 2003, Horejsi et al. 2004, Lee, Paull 2005b). An extrapolation could be made that in the absence of NBS1 function, the ability to recruit and activate ATM is repressed, allowing Mre11-CDK2/cyclin A to remain in complex despite DNA damage. In this situation, cell cycle activity is maintained, and as such, DNA synthesis continues, despite the compromised ability to preserve genomic integrity. This is highlighted in cervical cancer-derived HeLa S3 cells, wherein the Mre11-CDK2/cyclin A is rapidly assembled after 10 Gy IR treatment or is maintained during a 10 Gy IR treatment. An alternative pathway to the ATM-Chk2-Cdc25A-CDK2 pathway has been postulated based on studies in ATLD cells (Falck et al. 2002). These studies further suggest a secondary pre-existing genetic defect of the ATM-Nbs1-Mre11 mechanism in ATLD fibroblasts to enable a complete RDS phenotype (Falck et al. 2002). We addressed the mechanism of DNA synthesis reduction by the MRN complex with our cumulative data on the Mre11-CDK2 interaction and additional data demonstrating reduction in CDK2 biological activity in the absence of Mre11.

Our continued examination of the Mre11-CDK2 interaction revealed that CDK2/cyclin A does not immunoprecipitate with the 76 amino acid (76AA) C-terminus

deletion form of Mre11^{ATLD1}(Figure V.1). This finding demonstrated that the last 76AA of the Mre11 C-terminus is required for CDK2 interaction; we therefore focused on this region of the truncated Mre11 protein fragment for CDK2 binding competence. We have demonstrated successful pull down of CDK2 with a FLAG N-terminus Mre11 76AA fusion protein. Furthermore, relevant to our ATM dependent dissociation observation, we noted an ATM target SQSQ motif within this 76AA (Matsuoka et al. 2007). Our evaluation of the Mre11-CDK2/cyclin A interaction demonstrated that CDK2 activity is significantly reduced in the absence of IR. This suggests that when isolated, CDK2/cyclin A activity levels are insufficient for cell cycle progression pending Mre11 binding; once bound, however, cell cycle progression in S-phase may resume.

These research points generate plausibility for dissociation of the Mre11-CDK2 complex based on ATM phosphorylation of SQ sites, thereby reducing C-terminus binding efficiency with CDK2/ cyclin A, which results in dissociation and cell cycle arrest. Evidence supporting this hypothesis demonstrate *in vitro* the varying degrees of ATM and ATR phosphorylation of SQ sites within Mre11 (Kim et al. 1999). Additionally, experimental research has revealed that Mre11 is phosphorylated in an ATM- and NBS1-dependent manner after induction of DSB damage from genotoxic agents (Dong, Zhong & Chen 1999, Yuan et al. 2002b, Yuan et al. 2002a)

Our evaluation of the Mre11-CDK2 interaction clearly demonstrated that the 76AA portion of Mre11 contributes to CDK2/cyclin A binding. Further support of binding interaction between Mre11 and CDK2/cyclin A was provided from our small molecule inhibitor work. These data provided evidence that disruption of the RxL site mimics IR complex disruption, suggesting cyclin A participation in substrate binding.

Intriguingly, the five SQ motifs in Mre11 are within close proximity to the *MRE11A* hypomorphic mutations analyzed in our recent paper (Regal et al. 2013). Our hypothesis supports ATM-dependent phosphorylation of Mre11, such as IR-induced phosphorylation of NBS1 necessary for cell cycle checkpoint control in response to DSB (Rupnik, Lowndes & Grenon 2010). Studies evaluating Mre11 hyperphosphorylation have demonstrated cell cycle arrest after genotoxic treatment to cells (Yuan et al. 2002b). Additional indirect evidence of Mre11 phosphorylation by ATM is demonstrated in AT cell line research. In these studies, normal and AT patient-derived cell lines were exposed to 50Gy of IR; the genotoxic insult resulted in phosphorylation changes in hMre11 and hNBS1 only in normal cells. This research provides data suggesting that ATM phosphorylates SQ motifs within Mre11 in response to DNA damage. The assessment of ATM function, together with patient cell line data, constructs a summary of cellular phenotypes that reveals a trend among AT patient derived cell lines. Specifically, this study showed a correlation between clinically severe patients and low levels of phosphorylated Mre11; conversely, those patients least severely affected retained residual DNA repair function (Stewart et al. 1999b).

Future Directions

Our work has revealed several novel and significant findings involving the roles of the MRN complex, CDK2, and ATM in the DNA damage response. As with all scientific endeavors, new information provokes new questions. There are innumerable pathways for continuation, augmentation, and expansion of the findings presented here; some of the more plausible successions are described herein.

Investigation of reciprocal binding sites on CDK2: These binding sites may provide a better understanding of how the protein-protein interaction is maintained during S-phase in the unperturbed cell. Correspondingly, information on accessibility for ATM dependent DNA damage interruption may be revealed as well.

Alternate new chemical entities: Deregulation of the cell cycle is a common motif for most human malignancies. Our research with HeLa S3 cells is representative of a cancer cell line with an aggressive prolific behavior. Structure-based inhibitory design of chemicals that target specific CDK/cyclin complexes is an important line of research. Designing chemicals that target Mre11 for dissociation resulting in subsequent cell cycle cessation may improve selectivity and reduce common side effects (non-specific inhibition of other CDKs) for this class of therapeutic chemicals.

C-terminal site-directed mutagenesis: Evidence supports the SQ motif as the target phosphorylation site for disruption of the Mre11-CDK2/cyclin A complex. However, there is currently no direct evidence to support the occurrence of phosphorylation. Point mutations that confer constitutive phosphorylation as well as disruptive mutations could provide additional information on the mechanism for complex disruption.

Alternate types and sources of genotoxic damage: Our investigation focused on IR-induced DSB genotoxic lesions in MEF cells. Induction of mutagenesis and DSB by chemical DNA damaging agents may reveal diverse spatial and temporal outcomes as compared to IR-induced damage. This may be of particular interest as a method to assess ATR influence on the Mre11-CDK2 complex, based on the ability of different agents (such as MMS) to induce a replication fork block.

Targeted therapies for ATLD patients: ATLD patients suffer from defective cell cycle checkpoints, among other genomic instability syndrome maladies. Therapeutics directed at Mre11 N- or C-terminal mutations may allow for improved MRN complex function in patients with these disease states. Small molecule therapies targeting NBS1 stability in N-terminal ATLD disease patients may work to improve ATM activation and consequently, cell cycle checkpoint controls. Additionally, therapeutic rehabilitation of CDK2/cyclin A substrate recognition capacity may allow inhibition of structurally altered or low levels of Mre11.

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