Regulation of DNA Double Strand Break Response

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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<u>ABSTRACT</u>

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Abstract

To ensure genomic integrity, dividing cells implement multiple checkpoint pathways during the course of the cell cycle. In response to DNA damage, cells may either halt the progression of the cycle (cell cycle arrest) or undergo apoptosis. This choice depends on the extent of damage and the cell's capacity for DNA repair. Cell cycle arrest induced by double-stranded DNA breaks relies on the activation of the ataxia-telangiectasia (ATM) protein kinase, which phosphorylates cell cycle effectors (e.g., Chk2 and p53) to inhibit cell cycle progression. ATM is an S/T-Q directed kinase that is critical for the cellular response to double-stranded DNA breaks. Following DNA damage, ATM is activated and recruited to sites of DNA damage by the MRN protein complex (Mre11-Rad50-Nbs1 proteins) where ATM phosphorylates multiple substrates to trigger a cell cycle arrest. In cancer cells, this regulation may be faulty and cell division may proceed even in the presence of damaged DNA. We show here that the RSK kinase, often elevated in cancers, can suppress DSB-induced ATM activation in both *Xenopus* egg extracts and human tumor cell lines. In analyzing each step in ATM activation, we have found that RSK disrupts the binding of the MRN complex to DSB DNA. RSK can directly phosphorylate the Mre11 protein at Ser676 both *in vitro* and in intact cells and can thereby inhibit loading of Mre11 onto DSB DNA. Accordingly, mutation of Ser676 to Ala can reverse inhibition of the DSB response by RSK.

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Collectively, these data point to Mre11 as an important locus of RSK-mediated

checkpoint inhibition acting upstream of ATM activation.

The phosphorylation of Mre11 on Ser676 is antagonized by phosphatases. Here, we

screened for phosphatases that target this site and identified PP5 as a candidate. This

finding is consistent with the fact that PP5 is required for the ATM-mediated DNA

damage response, indicating that PP5 may promote DSB-induced, ATM-dependent

DNA damage response by targeting Mre11 upstream of ATM.

Dedication

This work is dedicated to my mom for her unconditional support;

and to Lan for her trust, care and love.

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List of Abbreviations

APC   Anaphase promoting complex

ATM   Ataxia telangiectasia-mutated

ATR   ATM and Rad3 related

ATRIP   ATR-interacting protein

BER   Base excision repair

CA   Constitutively active

CAK   CDK-activating kinase

Cdc2   Cell division cycle 2

CDK   Cyclin-dependent kinase

Chk2   Checkpoint kinase 2

MDC1   Mediator of DNA damage checkpoint protein 1

DNA-PK   DNA-dependent protein kinase

DSB   Double strand break

ER   Endoplasmic reticulum

ERK   Extracellular signal-regulated kinases

FL   Full length

FACS	Fluorescence-activated cell sorting
HR	Homologous recombination
HSP90	Heatshock protein 90
IP	Immunoprecipitation
IRIF	Irradiation induced foci
KD	Kinase dead
MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinase kinases
MDC	Mediator of DNA damage checkpoint protein
MEK	MAPK/ERK kinase
MMR	Mismatch repair
Mre11	Meiotic recombination 11
Nbs1	Nijmegen breakage syndrome 1;
NES	Nuclear export signal
NER	Nucleotide Excision Repair
NHEJ	Nonhomologous DNA end joining
NLS	Nuclear localization signal
NTKD	N-terminal kinase domain

OA	Okadaic acid
pCdc2	phospho-Cdc2
PIKK	Phosphoinositide 3 kinase related-kinase
PKA	Protein kinase A
Plk	Polo-like kinase
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP5	Protein phosphatase 5
pRB	Retinoblastoma protein
RSK	Ribosomal protein S6 kinase
Rad50	DNA repair protein RAD50
Raf	Rapidly accelerated fibrosarcoma
SAC	Spindle-asssembly checkpoint
SMC1	Structural maintenance of chromosomes-1
SSB	Single strand break
WT	Wild type
Wip1	Wildtype p53-induced phosphatase

Acknowledgements

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any challenge in life.

Chapter 1: Introduction

1.1 The Cell Cycle and Cell cycle checkpoints

The cell cycle is a process in which cells separate themselves to make two identical daughter cells. This process can be divided into two major phases: interphase, during which the mother cell synthesizes proteins and duplicates its DNA content, and M phase (Mitosis), during which chromosomes segregate and the cell divides. Interphase is further divided into three phases: G1, S and G2. During G1, the cell starts to grow both in size and content. It is critical during G1 that cells monitor their intracellular and extracellular environment to ensure a suitable environment for DNA replication. If conditions are favorable, the cell will enter S phase and start to duplicate their chromosomes and centrosomes. Following this, the cell will enter G2, during which it prepares for M phase by duplicating its organelles and promoting protein synthesis, including the production of microtubules necessary for karyokinesis and cytokinesis (nuclear and cell division, respectively; Fig. 1.1).

M phase can be further divided into five stages: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, chromatin starts to condense, the nucleolus disappears, and the centrosomes move to opposite ends of the cell to prepare for future cell division. During prometaphase, the nuclear membrane breaks down and kinetochores start to form. During metaphase, the chromosomes are attached by spindle microtubules around the middle of the cell to form the metaphase plate,

ensuring that each new daughter cell will receive only one copy of chromosome following separation. In anaphase, the sister chromatids separate at the kinetochores and move along spindle microtubules to the opposite sides of the cell. During telophase, the last stage of mitosis, the chromatids are deconsensed at the opposite poles of cell, spindle apparatus disappears, while at the same time a new nuclear envelope starts to form around chromosomes to form a new nucleus. The cell is subsequently divided into two daughter cells through a process called cytokinesis, marked by the division of cytoplasm and cell organelle membranes.

During the cell cycle, cells can activate several cell cycle checkpoints to ensure the fidelity of cell division. These checkpoints verify whether the processes at each phase of the cell cycle have been accurately completed before progression into the next phase. There are four major checkpoints, G1/S checkpoint, intra-S checkpoint, G2/M checkpoint, and metaphase checkpoint.

The first checkpoint, also termed the restriction point in mammals, is located at the transition between G1 and S phase, during which cells make the key decision of whether they should continue protein and DNA synthesis for division, or enter a resting stage named G0. During the G1/S checkpoint cells evaluate whether the environmental conditions (e.g., extracellular stimuli, nutrients, temperature, pH) favor reproduction.

In eukaryotic organisms, the G2/M DNA damage checkpoint is essential for monitoring DNA for damage, thereby preventing progression into mitosis until cells can

repair damaged DNA. This helps to maintain genomic stability. Cells with abnormal or defective G2/M checkpoints often enter mitosis without completely repairing their DNA. This may result in cell death or accumulation of mutations that may eventually lead to tumorigenesis. In order for the cell to arrest in G2/M, proteins that localize to sites of DNA damage must initiate a signaling cascade that eventually causes a change in the activity of the signaling proteins Wee1 and Cdc25, thereby delaying mitotic entry by inhibition of the Cdc2/cyclin B complex.

Early signaling events in the G2/M DNA damage checkpoint pathway involve activation of members of a family of phosphotidylinositol 3-kinases, ATM, ATR or DNA pK, which are believed to be transducers of the DNA damage response. The details of all G1/S, S and G2/M checkpoints will be further elucidated in the following section of the chapter.

The metaphase checkpoint, which is also known as the spindle assembly checkpoint (SAC), prevents separation of the duplicated chromosomes until each chromosome is properly attached to the spindle apparatus. Compromised SAC often results in aneuploidy, an event that generates cells with fewer or greater number of chromosomes than expected. The SAC stops the cell cycle by negatively regulating Cdc20, thereby preventing the activation of APC (Anaphase Promoting Complex). The proteins responsible for the SAC signal include MCC (Mitotic Checkpoint Complex), which is

consisted of SAC proteins, MAD2/MAD3 (Mitotic Arrest Deficient), BUB3 (Budding Uninhibited by Benzimidazole), and Cdc20.

1.1.1 CDK/Cyclin complexes

Progression of the cell cycle is controlled by the Cyclin-Dependent family of Kinases (CDKs). The expression level of CDKs is usually constant throughout the cell cycle and is cyclically regulated by forming complexes with different Cyclins. At different cell cycle stages, CDKs interact with different Cyclins. During G1, the primary CDK/Cyclin combinations are Cyclin D with CDK4 or CDK6, which promote entry into G1. Cyclin E binds to CDK2 during G1 and S transition while Cyclin A binds to CDK2 to help initiate DNA replication. Cyclin B/CDK1 (Cdc2) forms complexes to promote the progression of mitosis. Each CDK/Cyclin complex has limited substrate specificity, targeting and phosphorylating distinct proteins during the cell cycle. For example, Cyclin D/CDK4 and Cyclin D/CDK6 phosphorylate the tumor suppressor protein pRB during G1, which, in turn, promotes expression of Cyclin E and induces the G1/S transition. At the beginning of mitosis, Cyclin B/Cdc2 triggers an increase in the phosphorylation of proteins that control chromosome condensation, nuclear envelope breakdown and spindle assembly (Fig. 1.2).

1.2 The DNA damage response

Chromosomal DNA constantly encounters potentially harmful assaults from both environmental and endogenous sources. To protect their genomic integrity, cells have evolved a variety of response pathways that stop the cell cycle, initiate repair, and ultimately initiate apoptosis if the damage is beyond the cells' ability to repair. The major DNA repair pathways include direct reversal of lesions, excision of damaged DNA, and rejoining of DNA breaks.

1.2.1 DNA double-stranded breaks (DSBs) and repair mechanisms

DNA double-stranded breaks (DSBs) are widely regarded as the most toxic form of DNA damage due to the difficulty of their repair (Fig. 1.3). These often generate chromosomal aberrations such as translocations, amplifications, or deletions. Excessive DSBs are usually lethal or result in oncogenic transformation if left unrepaired (Zhu 2002). DSBs are generated through several scenarios. Single base lesions that are closely spaced on opposite strands often result in DSBs. These lesions are generated from endogenous base damage or free radicals generated upon exposure of cells to ionizing radiation (IR) (Sutherland 2003 and Wallace 1998). IR can also introduce DSBs directly by depositing energy within the DNA and causing multiple breaks. In addition, DSBs arise frequently during DNA replication when replication forks encounter single-strand breaks and collapse (Thompson and Schild, 2002).

Not all DSBs are harmful, sometimes DSBs can happen during normal cell progression and are important for cellular function. For example, Spo11 transesterase-induced DSBs are required for initiating meiotic recombination (Mahadevaiah 2001 and Sun 1989). V(D)J recombination and class switch recombination (CSR) is part of the normal maturation process of B and T lymphocytes (Honjo 2002).

There are two major pathways responsible for repairing DNA DSBs. Homologous recombination (HR), though highly accurate, requires large regions of homologous sequence from sister chromatids as a template and thereby only occurs during G2 phase. Nonhomologous DNA end joining (NHEJ), which simply joins broken chromosome together, can occur at G1, S, or G2 phase and is an error-prone repair mechanism which often results in deletions, insertions, or base pair substitutions. Most DSBs are repaired by the NHEJ mechanism.

1.2.2 The other types of DNA damage repair mechanisms

Aside from HR and NHEJ, there are three major mechanisms responsible for repairing DNA damage: Nucleotide Excision Repair (NER), Mismatch Repair (MMR) and Base Excision Repair (BER)(Sancar, Lindsey-Boltz et al. 2004). UV-induced photolesions and certain alkylation adducts in humans cells are mostly repaired by the NER pathway, a multistep process including recognition of disrupted base pairing, unwinding of the DNA helix, and subsequent excision of the damaged oligonucleotide

patch. The remaining gap is filled by regular DNA replication using the intact

complementary strand as a template.

The second common excision repair pathway is MMR, which is responsible for

repairing nucleotide insertion, deletions, and mispaired bases as a result of errant DNA

replication. The key proteins responsible for this pathway in eukaryotes are two major

heterodimers: Msh2/Msh6 (MutSα) and Msh2/Msh3 (MutSβ).

The last type of repair pathway, termed BER, targets non-bulky base

modifications, which are generated during the normal cellular metabolism processes

including oxidation, hydrolysis, and non-enzymatic methylation. The intrinsic

molecular instability of the DNA itself sometimes also causes this type of lesion. After

endonuclease removal of the abasic sugar, DNA polymerase removes the 5'-terminal

deoxyribose-phosphate residue and fills the single-nucleotide gap. The remaining nick is

then sealed by a DNA ligase (Fig. 1.4).

1.2.3 The MRN complex and its function

The Mre11/Rad50/Nbs1 (MRN) protein complex has been shown to be essential

for the DSB-induced DNA damage response. All three proteins are important for the

growth of organisms, as it has been shown that hypomorphic mutations of these genes

can result in severe genomic instability and developmental deficiency. During the

process of DNA damage repair, MRN is one of the first factors that are recruited to the

sites of damage. There, it stabilizes broken chromosomes by tethering them together. Besides binding to DNA and stabilizing chromosomal structure, MRN also plays a role in processing and assembling DNA damage foci to facilitate efficient DSB responses. It also activates downstream signaling targets, mainly the transducer ATM. These findings strongly suggest that the MRN complex may function as a sensor of DSBs. Another mediator protein, Mediator of DNA damage checkpoint protein 1 (MDC1), usually co-immunoprecipitates with the MRN complex and participates in the regulation of MRN foci formation.

The Mre11 protein has two distinct DNA-binding motifs that locate to its C-terminus, while Rad50 contains Walker A and B motifs that also participate in nucleotide binding. These Walker domains are separated by two coiled-coil regions that function as linker domains, which are important for intramolecular interactions (de Jager et al., 2001). Through the Cys-X-X-Cys motif, located in the middle of the intramolecular coiled-coil domain of Rad50, the Mre11/Rad50 (MR) complexes can connect the ends of chromosomes and tether them to form the DNA damage foci (Hopfner et al., 2002). The third important components of MRN complex, Nbs1, can interact with Mre11 through its C-terminal region. The N-terminal region of Nbs1 consists of two distinct domains: the FHA domain and the BRCT domain. Both domains can be found in many cell-cycle checkpoint and DNA-damage response proteins, and are responsible for protein-protein interactions (Fig. 1.5) (Soulier & Lowndes, 1999).

1.2.4 Overview of DNA damage DSB response

In order to protect genomic contents from external genotoxic agents, such as IR, chemicals and drugs, or toxic byproducts of internal metabolism, eukaryotic cells have evolved a series of surveillance pathways which are DNA damage response induced cell cycle checkpoints. These cell cycle checkpoint pathways involve three groups of evolutionarily conserved proteins that act in concert to translate the signal of damaged DNA into responses of cell cycle arrest and DNA repair. These sensor proteins recognize damaged DNA directly or indirectly and trigger transducer proteins such as ATM, ATR and DNA-PK to relay and amplify the damage signal. The effector proteins react to these signals by controlling cell cycle progression, chromatin restructuring, and DNA repair (Bakkenist and Kastan 2004).

The most important proteins for DNA damage response are ATM and ATR, which target S/T-Q site on many downstream proteins, including p53, chk1 and chk2. ATM is the central kinase for DSBs, while ATR is mostly activated during single-strand breaks (SSBs). Upon damage, ATM or ATR is recruited to the damaged DNA sites by mediator proteins (MRN complex for ATM, ATRIP for ATR, where they are activated and subsequently phosphorylate multiple substrates. Either Chk1 or Chk2, once activated, can activate Wee1 and inhibit Cdc25 by directly phosphorylating Cdc25 at Ser 287 (Peng, Graves et al. 1997, Donzelli and Draetta 2003, Margolis and Kornbluth 2004,

Uto, Inoue et al. 2004), resulting in the inhibition of CDK2/Cyclin B activity, and stop the cell cycle progression in S or G2 phase to let cells repair damaged DNA content. When damage is too extensive to be repaired, the programmed cell death named apoptosis is initiated to prevent of accumulation of abnormal cells (O'Connell and Cimprich 2005).

1.2.4.1 DSB-induced ATM activation

The activation of ATM upon DSB is well characterized. As mentioned above, the MRN complex is the sensor of the DSB response, binding to the broken ends of DSBs, recruiting ATM and promoting its activation. However, recent studies have found that the MRN complex can directly stimulate the kinase activity of ATM *in vitro*, causing the phosphorylation of p53, Chk2, and H2AX (Lee and Paull 2004, Lee and Paull 2005, Paull and Lee 2005). The C-terminal residues of human Nbs1, which is highly conserved, constitutes an ATM interaction motif and is responsible for recruiting ATM to DSBs. Such interaction is required for ATM-mediated downstream signaling (Gatei, Young et al. 2000, Kastan and Lim 2000, Falck, Coates et al. 2005).

According to experiments done using *Xenopus* egg extracts, the activation of ATM can be divided into two steps. The first step requires binding of MRN complexes to the site of DNA lesions and recruiting inactive ATM dimer through the C-terminal region of Nbs1. Once ATM has interacted with MRN at the DNA lesion, ATM dissociates from dimers to monomers. The next step involves autophosphorylation of

ATM and its direct activation on flanking DNA/chromatin regions (Dupre, Boyer-Chatenet et al. 2006, You, Bailis et al. 2007). This step does not need interaction with MRN complexes. While in human cells, ATM autophosphorylation at the site Ser 1981 has been shown to be essential for its activity (Bakkenist and Kastan 2003, Kozlov, Graham et al. 2006), it has been reported that in mice Ser 1987 (corresponding to human Ser 1981) is dispensable for ATM activation (Pellegrini, Celeste et al. 2006), suggesting that the ATM activation mechanisms may vary from species to species. In my study, I used ATM autophosphorylation on Ser 1981 as indicator of DNA damage response activation in human cells.

As mentioned above, ATR is the essential kinase for SSBs/stalled replication fork-induced DNA damage responses. However, it has been reported that ATR can also be activated indirectly by ATM during DSBs and can play an important role in this signaling event. Upon DSBs, the damaged chromosome ends are processed by the nuclease activity of Mre11 to generate replication protein A (RPA)-coated ssDNA, which forms a platform for the association of ATR via ATRIP (Cuadrado, Martinez-Pastor et al. 2006, Cuadrado, Martinez-Pastor et al. 2006, Jazayeri, Falck et al. 2006). It has also been shown that phosphorylation of TopBP1 on Ser 1131 by ATM strongly promotes the association of TopBP1 with ATR and causes its activation in response to DSBs in *Xenopus* egg extracts (Kumagai, Lee et al. 2006, Yoo, Kumagai et al. 2007). Thus, ATR coordinates

with ATM in response to DSBs to promote the activation of downstream targets and halt the cells at different stages of the cell cycle.

Once ATM is activated, it can activate numerous substrates that are recruited to DSBs by phosphorylating S/T-Q sites, thus amplifying the DNA damage signals. Besides the aforementioned p53 and Chk2, Nbs1 is also a substrate of ATM whose phosphorylation is required for completing the initiation of the DSB response. Another key substrate is MDC1, a signal amplifier of the ATM pathway, the absence of which causes a defect in downstream ATM signaling events (Fig. 1.6) (Lou, Minter-Dykhouse et al. 2006).

1.2.4.2 Post-translational modifications in ATM activation

Besides autophosphorylation of Ser 367, Ser 1897 and Ser 1981, which is required for ATM activation in human cells, ATM can also be regulated by acetylation, another form of post-translational modification. It has been reported that Tip60 histone acetyltransferase can acetylate ATM on Lys 3016 upon DSBs, in parallel with Ser 1981 phosphorylation (Sun, Jiang et al. 2005, Jiang, Sun et al. 2006, Sun, Jiang et al. 2006, Sun, Xu et al. 2007). Another histone acetyltransferase that participates in the ATM activation signaling pathway is hMOF, another histone acetyltransferas (Gupta, Sharma et al. 2005, Taipale, Rea et al. 2005, Rea, Xouri et al. 2007, Gupta, Guerin-Peyrou et al. 2008).

Knocking down hMOF with siRNA resulted in decreased ATM autophosphorylation, as well as ATM kinase activity.

Since a series of phosphorylation events are key in the DSB response, it is natural to assume that phosphatases may also play a role in ATM activation. Indeed, evidence suggests a link between ATM and the phosphatase PP2A. First, inhibition of PP2A by okadaic acid (OA) induced autophosphorylation of ATM on Ser 1981 in non-irradiated cells. (Jazayeri, Falck et al. 2006). In addition, dissociation of the B55 subunit from PP2A is also promoted by IR, which is ATM-dependent (Guo, Brautigan et al. 2002). This suggests that B55 may play an important role in PP2A-mediated ATM Ser 1981 dephosphorylation. The PP2A-interacting protein TIP is a negative regulator of PP2A phosphatase activity in the ATM/ATR signaling pathway (McConnell, Gomez et al. 2007).

One of the most well-characterized phosphatases in the DNA damage response is Wip1 (wildtype p53-induced phosphatase or PPM1D), a PP2C phosphatase that can directly dephosphorylate ATM on pSer 1981 and pSer 367. Overexpression of Wip1 reduces the activation of the ATM-dependent signaling cascade in response to DSBs, while knockdown of Wip1 via siRNA can sensitize cells to DSBs by upregulating ATM activity (Lu, Nguyen et al. 2005, Shreeram, Demidov et al. 2006). In addition to the negatively regulating phosphatases, PP2A and Wip1, another phosphatase, PP5, is required for DSB-induced ATM activation. PP5 has been shown to interact with ATM in

a DNA damage-inducible manner. In addition, reduced expression of PP5 attenuates DSB-induced ATM activation, while overexpression of a catalytically inactivate PP5 mutant inhibits the phosphorylation of ATM substrates and ATM autophosphorylation (Ali, Zhang et al. 2004). The subtle balance between kinase and phosphatase activity during the DNA damage response is critical for proper functioning of the pathway.

1.2.4.3 Detailed mechanisms of DSB-induced cell cycle checkpoints

In order to maintain genomic integrity, cells can be temporarily arrested at G1/S, S, or G2/M in the presence of DNA DSBs by activating a series of protein phosphorylation events that eventually control CDK/Cyclins' activity. These checkpoint pathways stop the cell cycle to give time for the damaged DNA to be repaired. How DNA damage induces the G1/S, intra-S, and G2/M checkpoints are introduced below.

G1/S checkpoint: The key protein for G1/S checkpoint is p53, which can be phosphorylated on Ser 15 or Ser 20 by ATM or Chk2. ATM can also indirectly control the stability of p53 by targeting Mdm2 on Ser 395, an ubiquitin ligase that regulates p53. This interrupts interactions between Mdm2 and p53. Both mechanisms ensure the stabilization as well as activation of p53, which results in the upregulated transcription of p53 targeting genes, such as p21 (Bartek and Lukas 2001, Bartek and Lukas 2001, Kastan and Bartek 2004). p21 can directly inhibit cell cycle progression by binding to CDK4 (or CDK6)/Cyclin D and CDK2/Cyclin E complexes, preventing CDK2 and CDK4

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(or CDK6)-mediated phosphorylation of the Retinoblastoma protein (pRB). The pRB protein, once phosphorylated, can inhibit E2F-dependent transcription and block G1/S progression (Bartek and Lukas 2001, Bartek and Lukas 2001). Another way to activate pRB is through ATM-dependent activated Chk1/Chk2 phosphorylation on Ser 612, which promotes the formation of the complex between pRB and E2F, thereby inhibiting E2F-regulated gene expression and stopping G1/S transition (Inoue, Kitagawa et al. 2007).

Intra S checkpoint: both ATM and ATR mediated pathways can stop S phase progression by targeting Cdc25A. Upon DSB, ATM activates Chk2 by Thr 68 phosphorylation. In addition, ATM-dependent ATR activation can trigger Chk1 activation by phosphorylation on Ser 345 (Cuadrado, Martinez-Pastor et al. 2006). Active Chk1 and Chk2, in turn, can phosphorylate Cdc25A on Ser 123, thereby decreasing the stability of Cdc25A and increasing its binding to the 14-3-3 protein (Falck, Mailand et al. 2001). Both events prevent the Cdc25A phosphatase from activating Cdk2/Cyclin A, whose activity is required for origin firing which initiates DNA replication. Besides the above mentioned pathway, ATM can also trigger the intra-S checkpoint directly by phosphorylating SMC1 (Structural Maintenance of Chromosomes-1) on Ser 957 and 966, subsequently down-regulating DNA replication and impeding S phase progression. Nbs1 is believed to be required for SMC phosphorylation (Yazdi, Wang et al. 2002).

Moreover, direct CDK2 inhibition by p21 can also lead to the intra-S checkpoint through ATM/Chk2 mediated activation of p53 (Zhu, Alvarez et al. 2004).

G2/M checkpoint: The DSB induced G2/M checkpoint pathway is very similar to G1/S, which requires the activation of ATM, Chk1 and Chk2. One of the key proteins for cell cycle progression, Cdc25c, is regulated either by promoting its proteasomal degradation or by sequestration in the cytoplasm via 14-3-3, through phosphorylation of Chk1 and Chk2 (Raleigh and O'Connell 2000, Falck, Mailand et al. 2001). In addition, activated Chk1 and Chk2 can also phosphorylate Wee1 on Ser 549 in *Xenopus* egg extracts to promote its binding with 14-3-3. This leads to nuclear localization of Wee1 and promotes its stability and its ability to inhibit CDK1/Cyclin B (Lee, Kumagai et al. 2001). Both inhibition of Cdc25 and activation of Wee1 kinase results in the inactivation of CDK1/Cyclin B via inhibitory phosphorylation on Thr 14 and Tyr 15 sites (Raleigh and O'Connell 2000). Another mechanism regulating the G2/M checkpoint is the stabilization and activation of p53 by ATM, Chk1 and Chk2, which is similar to the G1/S checkpoint (Fig. 1.7).

1.2.4.4 Checkpoint recovery and adaptation

The major function of the DNA damage checkpoints is to allow cells to repair any damaged DNA before completing either DNA replication or nuclear division. Under normal conditions, cells can only re-enter the cell cycle when DNA repair is

complete, an event termed checkpoint recovery. However, in rare events, cells can resume their cycle even in the presence of damaged DNA, an event termed checkpoint adaptation. Tumor cells sometimes utilize checkpoint adaptation to avoid DNA damage-induced cell cycle arrest and apoptosis. Checkpoint adaptation promotes genomic instability and is often harmful to human cells.

The key proteins underlying the process of checkpoint recovery are Claspin and Wee1. Claspin interacts with Chk1 and plays an important role in Chk1 activation (Kumagai and Dunphy 2000, Kumagai, Kim et al. 2004). Immunodepletion of Claspin from *Xenopus* egg extracts or knockdown of Claspin via siRNA in mammalian cells inhibits DNA damage induced Chk1 activation (Kumagai and Dunphy 2000, Chini and Chen 2003, Lin, Li et al. 2004). During checkpoint recovery, Plk1 can phosphorylate Claspin and Wee1, triggering their recognition by SCFβTCP ubiquitin ligase and subsequent degradation via an ubiquitin-dependent pathway. (Mailand, Bekker-Jensen et al. 2006, Mamely, van Vugt et al. 2006, Peschiaroli, Dorrello et al. 2006). By degrading Claspin and Wee1, Chk1 activity is suppressed and CDK1/Cyclin B is activated due to both Cdc25 activation and elimination of inhibitory phosphorylation on CDK1 by Wee1. The end result is resumption of Cdc2/Cyclin B activity which drives the cell through the G2/M checkpoint to re-enter mitosis. In addition, Chk1 degradation may also play a role in the termination of the DNA damage checkpoint (Zhang, Otterness et al. 2005).

Although Plk1 activity is essential for recovery, it is not sufficient, suggesting

other mechanisms may be involved in this process. Indeed, it has been shown that

several phosphatases, including PP2A and Wip1, may play an important role. As

mentioned above, the PP2C family phosphatase, Wip1, recognizes a p(S/T)Q motif,

allowing it to efficiently dephosphorylate multiple substrates of ATM/ATR in the DNA

damage response, such as Chk1, Chk2, p53, γ-H2AX and ATM itself (Chowdhury,

Keogh et al. 2005, Lu, Nannenga et al. 2005, Lu, Nguyen et al. 2005, Keogh, Kim et al.

2006, Shreeram, Demidov et al. 2006). Particularly, Wip1 can regulate p53 both by direct

dephosphorylation of pSer15, and by activating the ubiquitin E3 ligase Mdm2. PP2A-

B55δ phosphatase can prevent premature mitotic entry by reversing phosphorylations

made by CDK during the interphase (Mochida, Ikeo et al. 2009). In addition, during

G2/M checkpoint recovery, Artemis, a member of the SNM1 gene family, can be

phosphorylated by ATM at Ser 534, Ser 516, Ser 538, and Ser 645. Such phosphorylations

have been shown to be inhibitory, as mutation of both Ser 516 and Ser 645 increases

Wee1-mediated inhibitory phosphorylation on CDK1/Cyclin B, which results in

defective recovery from the G2/M checkpoint (Geng, Zhang et al. 2007).

Checkpoint adaptation was first observed during a study of the yeast DNA

damage response, where certain yeast had the ability to evade the cell cycle arrest even

though DSB damage was sustained (Toczyski, Galgoczy et al. 1997). However, such

adaptation events are very rare in normal human cells, and believed to sometimes occur

in tumor cells following IR treatment. Similar to checkpoint recovery, the most important mechanism underlying checkpoint adaptation involves Plk1-mediated phosphorylation of Claspin, causing release of Claspin from the chromatin and thereby inactivation of Chk1 due to lack of Claspin-mediated Chk1 phosphorylation. This study was done in *Xenopus* egg extracts (Yoo, Kumagai et al. 2004). Plk1 can further promote the degradation of Claspin by triggering the recognition of SCFβTCP ubiquitin ligase and ubiquitin-dependent degradation (Syljuasen, Jensen et al. 2006).

1.2.4.5 DNA damage checkpoint and apoptosis

In the face of severe DNA damage that is beyond the cells' ability to repair, the cell may trigger apoptosis instead of cell cycle arrest. DNA damage-induced apoptosis is centered on the activation of p53. ATM/Chk2-activated p53 upregulates the transcription of many proapoptotic proteins, including Bax, which promotes the release of mitochondrial cytochrome c and Smac into the cytoplasm. BH3-only proteins NOXA, PUMA and Bid promote cytochrome c release by binding to and inhibiting anti-apoptotic proteins Bcl-2 and Bcl-xL. In response to DSBs, p53 can also translocate to the surface of mitochondria, directly inhibit Bcl-2 and Bcl-xL, and at the same time activate pro-apoptotic protein Bak (Essmann, Pohlmann et al. 2005). In addition, the linker histone subtype H1.2 also translocates to the mitochondria in a p53-dependent manner to cause the release of cytochrome c and Smac/DIABLO, which directly couples

damaged chromatin to apoptosis (Konishi, Shimizu et al. 2003, Bree, Neary et al. 2004,

Chen, Shimizu et al. 2005).

1.2.4.6 DNA damage response and cancer

Since the DNA damage response is a key factor for maintaining genomic

stability, any deficiency or misregulation of this pathway may result in genomic

instability, one of the leading causes of tumorigenesis. Also as previously mentioned,

cells can occasionally resume the cell cycle without completion of DNA repair through

checkpoint adaptation. This may result in accumulation of aberrant chromosomes and

gene mutations. The loss of some DNA damage response components also allows cells

to survive DNA damage, which is one of the reasons why several types of cancer are

resistant to radiation therapy. Mutation or deletion of p53, pRB, or BRCA1, each results

in defective DNA damage responses and have been observed in more than half of all

human cancers (Levine 1997). This evidence supports the notion that defects in the

damage-sensing machinery is strongly related to tumorigenesis. Additionally, around

10% of AT (Ataxia Telangiectasia) individuals, whose genomes accumulate mutations in

ATM kinase, are prone to develop many types of cancers (McKinnon 2004).

One of the early events in pre-malignant lesions of the major types of human

tumors are constantly active DNA damage responses. Markers of this activity include

phosphorylated kinases ATM and Chk2 and phosphorylated histone H2AX and p53,

suggesting that the DNA damage response may function as a barrier against early human tumorigenesis (Bartkova, Horejsi et al. 2005), (Gorgoulis, Vassiliou et al. 2005). Defects in the DNA damage induced checkpoint might allow cell proliferation, survival, increased genomic instability and tumor progression. This idea is supported by the finding that the inhibition of ATM abrogated oncogene-induced senescence and promoted cell transformation. In a mouse model, inactivation of the DNA damage response led to increased tumor size and invasiveness. It is possible that oncogene-induced senescence may result as a manifestation of the DNA damage response and, coupled with apoptosis, provides a barrier to malignant progression (Bartkova, Rezaei et al. 2006) (Di Micco, Fumagalli et al. 2006).

Under normal conditions, cells rely on the G1/S, intra S and G2/M checkpoints to protect genomic stability during cell proliferation. However, G1/S or intra S checkpoints are often defective or silenced in many types of cancer cells, rendering cells reliant solely on the G2/M checkpoint for protection. In this case the G2/M checkpoint becomes even more critical, and has been proven to be a powerful therapeutic target in cancer treatment. Combinations of drugs that abolish G2/M checkpoints often render cells more responsive to certain chemotherapeutics. So far, among the many kinases and phosphatases that are involved in the G2-M checkpoint (e.g., ATM, ATR, Chk1, Chk2, Cdc25 and PP2A), Chk1 is the most promising therapeutic target. The selective Chk1 inhibitor, UCN01, is currently undergoing clinical trials as a potential chemotherapeutic

(Yu, La Rose et al. 2002). However, selective inhibitors against ATM and ATR are still yet under development.

1.3 The MAPK signaling cascade and Rsk kinase

1.3.1 The MAPK signaling cascade

As one of the most well-studied signaling cascades, Mitogen-Activated Protein Kinases (MAPKs) are a group of protein Ser/Thr kinases that respond to extracellular stimuli and coordinately regulate gene expression, mitosis, metabolism, motility, survival, apoptosis, and differentiation. MAPKs can be grouped into seven categories: the Extracellular signal-Regulated Kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), p38, ERK5, ERK3/4, ERK7, and Nemo-like kinase (NLK). The first four are termed conventional MAPK while the last three are atypical. Each group of conventional MAPK cascade is composed of a set of three evolutionarily conserved, sequentially acting kinases: MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs) (Cargnello and Roux 2011). For this thesis, I focused mainly on the ERK1/2 signaling cascade.

The Ser/Thr protein kinases MAPKKKs are often activated through phosphorylation or as a result of their interaction with a small GTP-binding protein family. MAPKKKs activation leads to the phosphorylation and activation of MAPKKs,

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which subsequently activate MAPKs through dual phosphorylation on Thr and Tyr residues on a conserved Thr-X-Tyr motif within the activation loop of the kinase domain. Phosphorylation of these residues is essential for enzymatic activities.

ERK1 and ERK2 are the most well-known kinases in the MAPK family, which share more than 80% amino acid identity and are highly expressed in the brain, skeletal muscle, thymus, and heart. Downstream of the Ras-Raf-MEK-ERK signal cascade, the human ERK1/2 can be activated by MEK1/2 through phosporylation at Tyr204/187 and then Thr202/185. More than 30% of all human cancers harbor mutations within the ERK1/2 pathway, which often result in gain of function and consequent ERK hyperactivation (Fig. 1.8) (Hoshino, Chatani et al. 1999).

1.3.2 Rsk kinase family and its activation by sequential phosphorylation

The 90 kDa ribosomal S6 kinases (RSKs), well-known for their two distinct functional kinase domains (N-Terminal Kinase Domain or NTKD, C-Terminal Kinase Domain or CTKD), are a family of Ser/Thr kinases downstream of the MAPK cascade. This family consists of four human isoforms (RSK1–4) and two structurally related homologues, Mitogen- and Stress-activated Kinase-1 (MSK1) and MSK2 (Lara, Seckl et al. 2013). All isoforms share a high degree of sequence homology (more than 70% amino-acid identity).

The spatial and temporal regulation of the RSKs is tightly controlled due to their role in cell motility, gene expression and protein synthesis. Inactive RSKs are mostly localized to the cytoplasm and sometimes to the nucleus along with their upstream activator, ERK1/2. Following growth factor stimulation, RSKs transiently translocate to the plasma membrane allowing them to be fully activated. Fully activated Rsks subsequently relocalize to the nucleus to target their substrates (Richards, Dreisbach et al. 2001). Translocation and activation of RSK at the plasma membrane precede its accumulation in the nucleus. Targeting RSK to membranes by N-terminal myristoylation results in its maximal activation independent of ERK activity.

ERK is recruited in its inactive form to the D-domain of RSKs (Kang, Dong et al. 2007). Phosphorylation of RSK2 on Tyr-529 by receptor tyrosine kinase can initiate the acitvation of Rsk. Following mitogen stimulation, ERK1/2 phosphorylates Thr573 of RSK which locates in the CTKD activation loop, resulting in activation of CTKD. Mutation of Proline-directed ERK consensus sequence in the activation loop or ERK docking site at the C-terminal abolishes this phosphorylation event. ERK1/2 might also phosphorylate Ser363 in the RSK linker region, which is also known as the turning motif. The activated CTKD then phosphorylates the hydrophobic motif in Rsk itself, thus creating a docking site for PDK1, which subsequently phosphorylates Ser221 in the NTKD activation loop, leading to the full activation of Rsk. NTKD is the kinase domain that targets Rsks' substrates (Anjum and Blenis 2008), but similar to CTKD, the NTKD can also

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autophosphorylate Ser 741 located next to the D-domain. This autophosphorylation is believed to be a part of a negative feedback loop, reducing the binding affinity between ERK and RSK (Fig. 1.9).

1.4 Protein phosphatase 5 and its role in the DNA damage response

In eukaryotic organisms, there are two major families of serine/threonine protein phosphatases (PPases), designated as PPM (metal-dependent protein phosphatases) and PPP (phosphoprotein phosphatases). PP5 belongs to the PPP-family, which also contains PP1, PP2A, PP2B, PP4, PP6 and PP7. The phosphatases of the PPP family are among the most highly conserved proteins on earth, whose homology are even greater than that of histones (2A and 2B).

Unlike PP1 and PP2A, purified PP5 has low basal activity, indicating that this phosphatase requires co-factors to be activated. The structure study of purified PP5 revealed that its N-terminal domain functions as an autoinhibitory domain, folding over to block the catalytic site from access to substrates. The currently identified PP5 activation complexes include many proteins known to participate in signaling networks, such as ATM, ATR, the glucocorticoid receptor (GR)- heat shock protein 90 (Hsp-90)-heterocomplex, the CDC16/Cdc27 subunits of the anaphase-promoting complex, cryptochrome 2, Hsp90-dependent heme-regulated eIF2α kinase, apoptosis signal-

regulating kinase 1 (ASK1), DNA-PKcs (DNA-dependent Ser/Thr protein kinase), the A-regulatory subunit of protein phosphatase type 2A, the G12-α/G13-α subunits of heterotrimeric G proteins, Rac and Raf1(Golden, Swingle et al. 2008, Vaughan, Mollapour et al. 2008). However, unlike protein kinases where a binding partner is often a good indication of a substrate, PP5 has no consensus substrate binding site and does not require tight binding to target its substrate. As a result, interaction of these proteins with PP5 in many cases does not indicate an actual substrate, but rather a partner in a complex of proteins that contains a substrate(s) where PP5 sometimes function as a co-chaperone.

1.5 Xenopus oocytes and the egg extract system

Xenopus laevis oocytes and the egg extract system are powerful tools for studying cell cycle regulation, the DNA damage response, and apoptosis. Stage VI *Xenopus* oocytes are arrested at G2 of meiosis I with inactive Cyclin B/Cdc2 (Taieb, Chartrain et al. 1997, Ferrell 1999). By injecting frogs with the steroid hormone progesterone, oocytes can progress through meiosis I and arrest in metaphase of meiosis II, a process named oocyte maturation. One of the major events during maturation is germinal vesicle breakdown (GVBD), marking the breakdown of the oocyte nucleus, which is caused by activation of Cyclin B/Cdc2. Progression of GVBD can be monitored visually, due to the rearrangement of cortical pigment granules at the oocyte's animal pole. This process of

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meiotic maturation provides a useful model for the study of the G2/M transition in isolation. In addition, the rather large size of *Xenopus* oocytes makes them a favorable host for the injection of exogenous mRNA or protein.

Apart from intact oocytes, extracts made from *Xenopus* eggs provide an excellent *in vitro* system for the biochemical dissection of a variety of cellular processes including nuclear transport, DNA replication, apoptosis, mitotic entry, and DNA-responsive checkpoint signaling. These concentrated egg extracts contain around 40 mg/ml of protein and can be made to mimic different cell cycle states, including interphase, mitosis, or meiosis. The egg extracts are an ideal system for studying the DSB response for several reasons. First of all, purified egg extracts are highly synchronized, especially cycling egg extracts (Murray and Kirschner 1989), which oscillate between S and M phases of the cell cycle and can recapitulate DNA damage-responsive checkpoints *in vitro*. Second, egg extracts have a very low amount of endogenous chromosome, but retain the intact DNA damage response machinery. As a result, such a system can recapitulate precisely the DSB response via addition of sperm chromatins or annealed oligonucleotides, which can mimic damaged DNA templates, preventing M phase entry and arresting extracts with fully formed, but inactive, Cyclin B/Cdc2 complexes (Guo and Dunphy 2000, Kumagai and Dunphy 2000, Yoo, Shevchenko et al. 2004). Regardless of the status of the DNA added to these extracts (or even in the absence of added DNA), prolonged incubation of egg extracts on the bench for 4-6 hours results in release of

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cytochrome c from mitochondria and activation of caspases (Newmeyer, Farschon et al. 1994, Kornbluth 1997, Deming and Kornbluth 2006). These cycling and apoptotic properties of the *Xenopus* egg extracts, combined with the ability to directly add and deplete proteins of interest, make them ideal for the analysis of factors implicated genetically in cell cycle progression or apoptosis in other systems (Fig. 1.10).

1.5.1 *Xenopus* egg extracts

In this dissertation, two major egg extracts are utilized:

Interphase extracts: Interphase extracts are prepared by centrifuging dejellied eggs in a prepared egg lysis buffer. During centrifugation, the eggs are crushed and endogenous Ca2+ is released from internal stores, leading to the degradation of Cyclin B and release of extracts into interphase. The addition of cycloheximide to the buffer prior to centrifugation inhibits subsequent Cyclin B translation and functions to arrest the extracts in interphase. When supplemented with non-degradable Cyclin B (◎13), the interphase extracts can be driven into mitosis in a controlled manner, which is useful for the study of the G2/M transition.

CSF extracts: *Xenopus* eggs are arrested at metaphase of meiosis II. However, with the release of Ca2+ upon centrifugation, the APC is activated and the extract releases to interphase. In order to avoid this release, the egg lysis buffer is supplemented

with EGTA, a chelator of Ca2+. This treatment results in an extract that is arrested at the

metaphase of meiosis II, and is referred to as CSF extract.
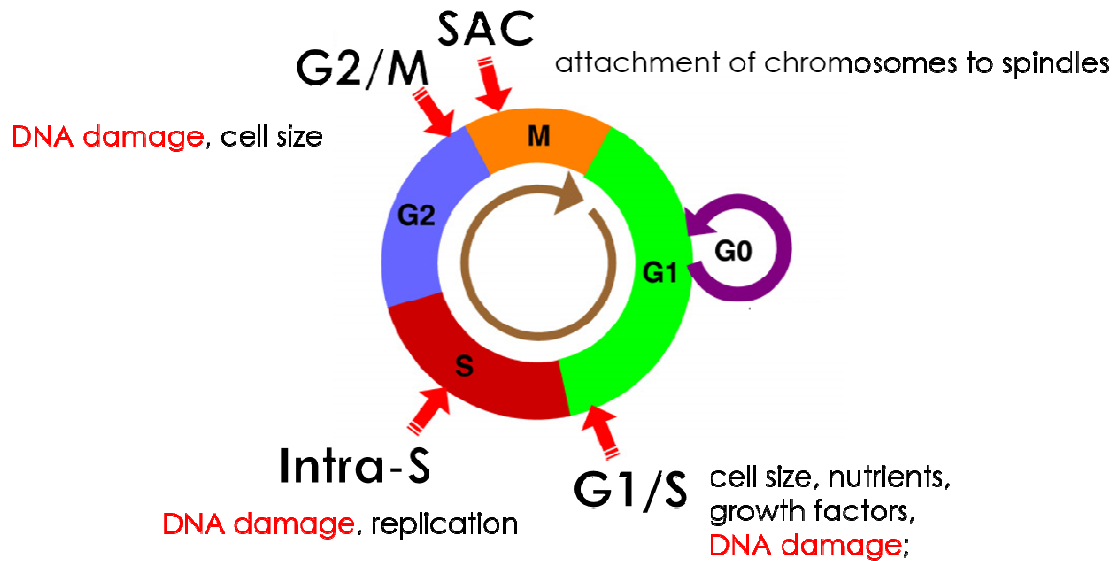
Figure 1.1: The cell cycle and cell cycle checkpoints

Figure 1.1: The cell cycle is separated into two stages: interphase, which can be further divided into G1, S, and G2 phases, and mitosis, during which cells divide to form two identical daughter cells. During cell cycle, cells can be temporarily arrested at G1, S, G2 or M phase by different checkpoints (G1/S, intra-S, G2/M, and spindle checkpoints) if conditions for cell division are not met (e.g. DNA damage, insufficient cell size, un-attached chromosomes). DNA damage usually arrests cells in G1/S, intra-S, or G2/M phase.
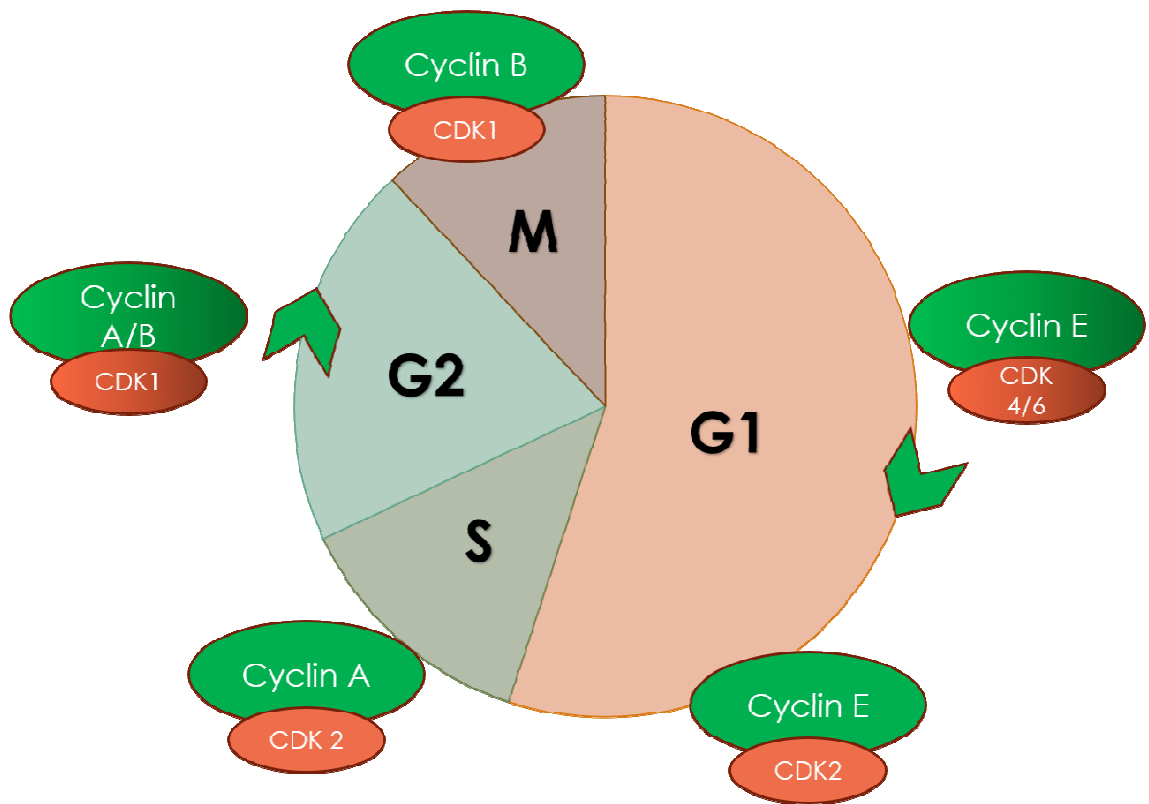
Figure 1.2: Cyclin dependent kinases regulate cell cycle progression

Figure 1.2: The family of cyclin dependent kinases (CDK) and cyclins promotes the progression of cell cycle. CDK4 or CDK6 binds to Cyclin D in order to drive entry into G1 phase. CDK2 binds to Cyclin E at the end of G1 phase which commits the cell to S phase to replicate DNA contents. CDK2 and Cyclin A is the major CDK/Cyclin complex in S phase which initiates DNA replication. CDK1 (Cdc2) binds to Cyclin A and B in G2 phase and the activation of CDK1-Cyclin B complex triggers the entry of mitosis.
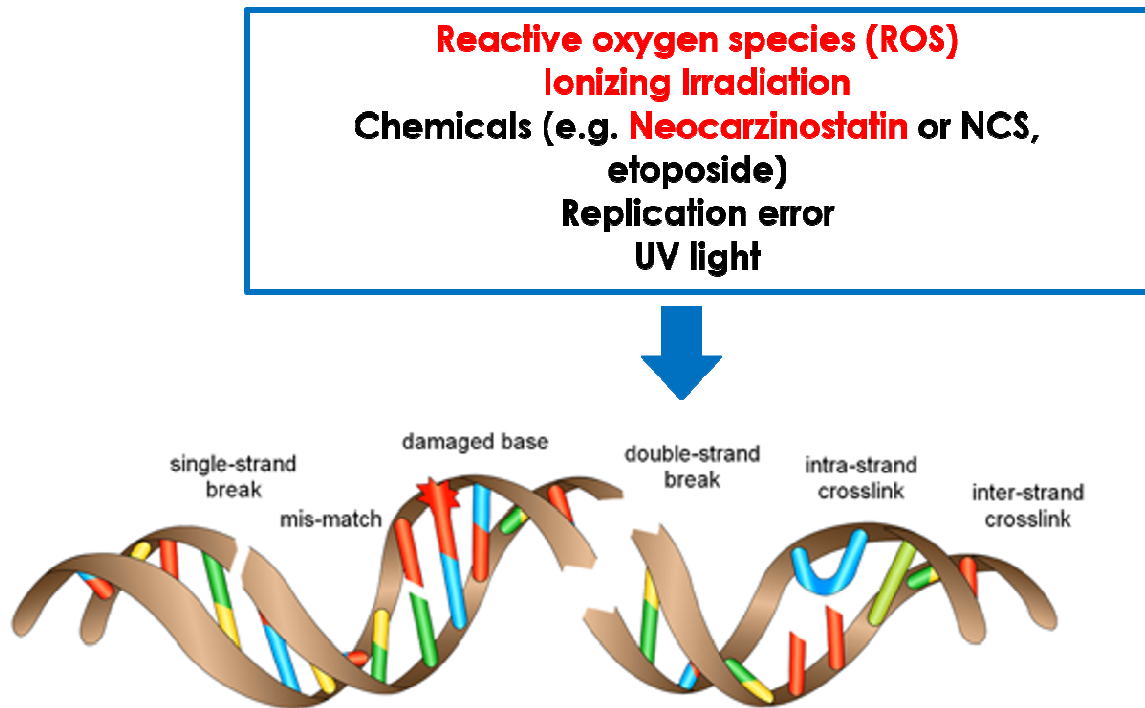
Figure 1.3: The types of DNA damage and causes of DSB

Figure 1.3: The most common types of DNA damage include: base modifications such as methylation and oxidation, mispairs, interstrand/ intrastrand cross-linked nucleotides, single or double strand breaks. Among those, the double strand breaks (DSB) are the most toxic form. Double strand breaks are usually caused by irradiation, chemicals, intense UV light, replication error and ROS (modified from Pears CJ, 2012).
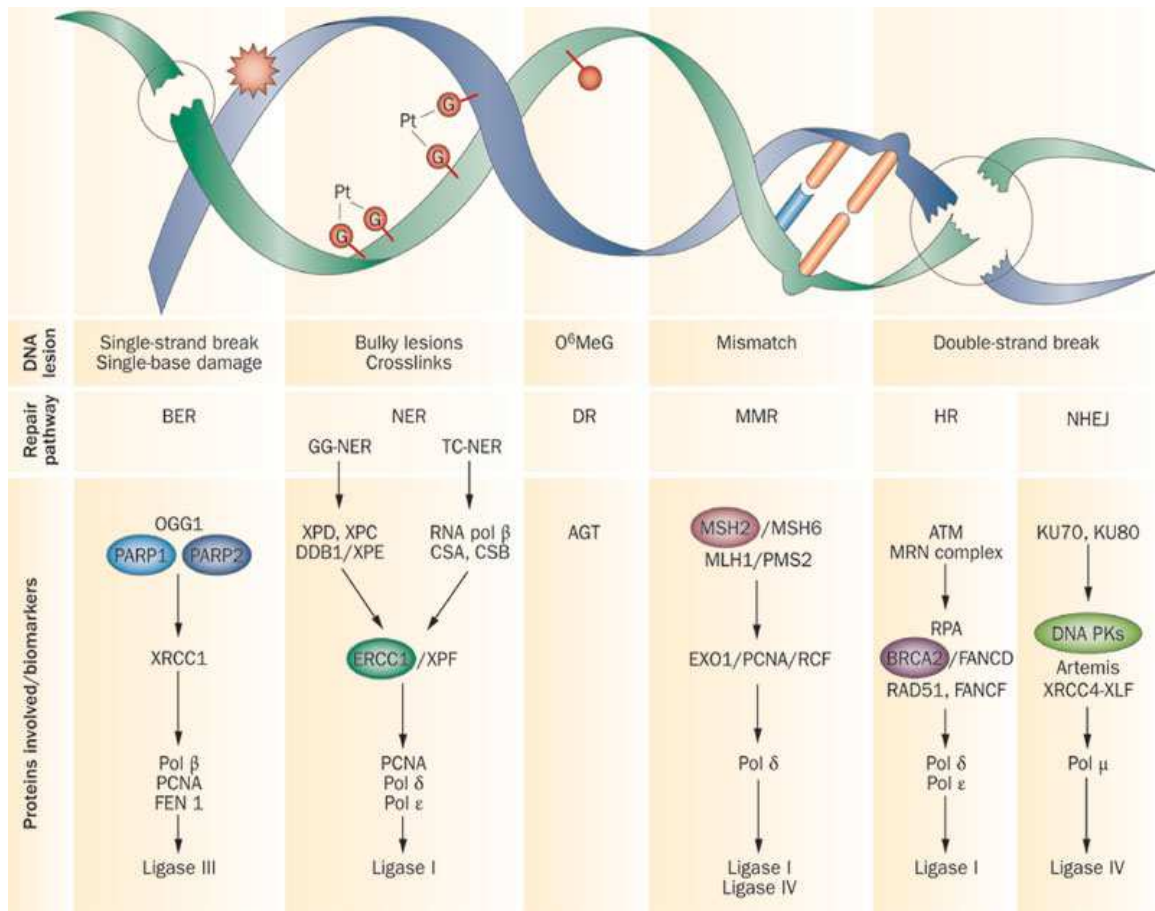
Figure 1.4: Different types of repair fix different types of damage

Figure 1.4: Single strand DNA lesions that does not disrupt the helical structure are generally repaired by base excision repair (BER), while DNA damage such as bulky lesions and crosslinks which significantly distort the DNA helix is repaired by nucleotide excision repair (NER). Direct repair or DR deals with a single base affected small chemical changes whereas mismatch repair or MMR repairs mismatches in the pairing of DNA caused by replication errors. Lastly DSBs are mostly fixed by homologous recombination (HR) and non-homologous end joining (NHEJ), which are both involved in the repair of DNA double-strand breaks. HR allows 'error free' repair of the lesion whereas NHEJ is an 'error-prone' mechanism that repairs DNA but at the cost of introducing mutations into the genome. The selection of HR or NHEJ is primarily based on the phase of the cell cycle and the expression, availability and activation of DNA-repair proteins (adapted from Postel-Vinlay S, et, al. 2012).
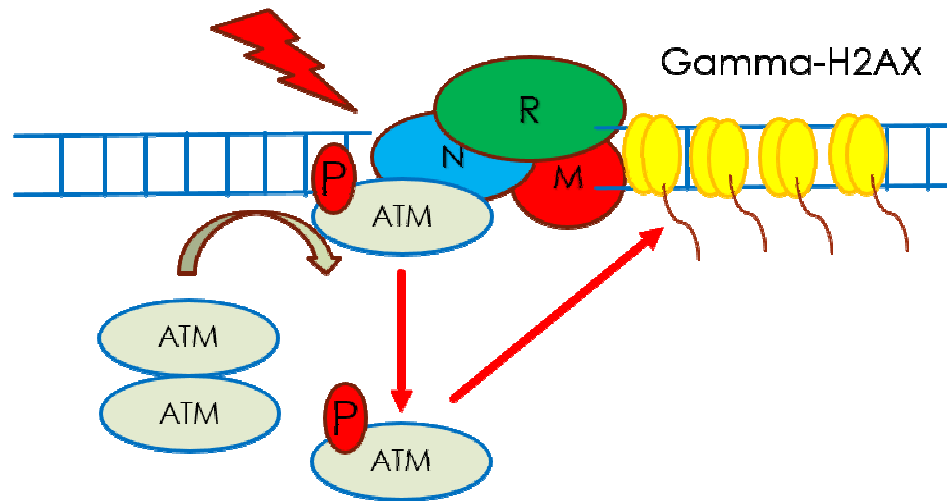
Figure 1.5: MRN complexes are the sensors of DSBs.

Figure 1.5: The Mre11/Rad50/Nbs1 (MRN) protein complex is one of the first factors that are recruited to the sites of damage where it stabilizes broken chromosomes by tethering them together. MRN also plays a role in processing and assembling DNA damage foci to facilitate efficient DSB responses. It also activates downstream signaling targets, mainly the transducer ATM. The inactive ATM exists as dimers or oligomers. After binding to MRN, ATM monomerizes and autophosphorylates itself at several sites including Ser1981. Fully activated ATM subsequently phosphorylates proteins involved in DSB response, which include gamma H2AX, consequently changing the structure of chromosome allowing for recruitment of DNA repair machinery.
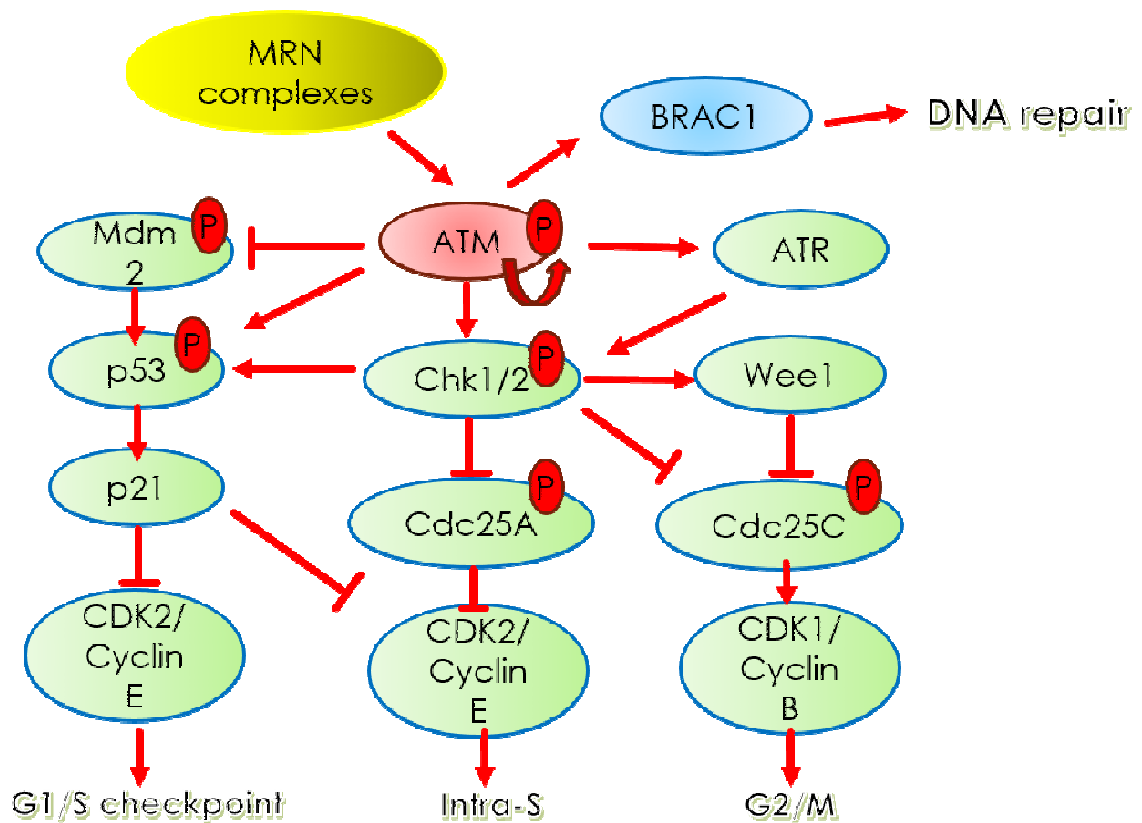
Figure 1.6: ATM signaling pathway

Figure 1.6: Following ionizing radiation, the ATM kinase is activated via intermolecular autophosphorylation of Ser1981 and subsequent dissociation of the ATM homodimer. The ATM kinase is then free to circulate in the cell and subsequently phosphorylate Chk2, p53, and Mdm2 to initiate the G1 arrest; Cdc25A, Cdc25C and Wee1 to initiate intra-S and G2/M arrest; or FANCD2, BRCA1, and SMC1 to initiate DNA damage repair.
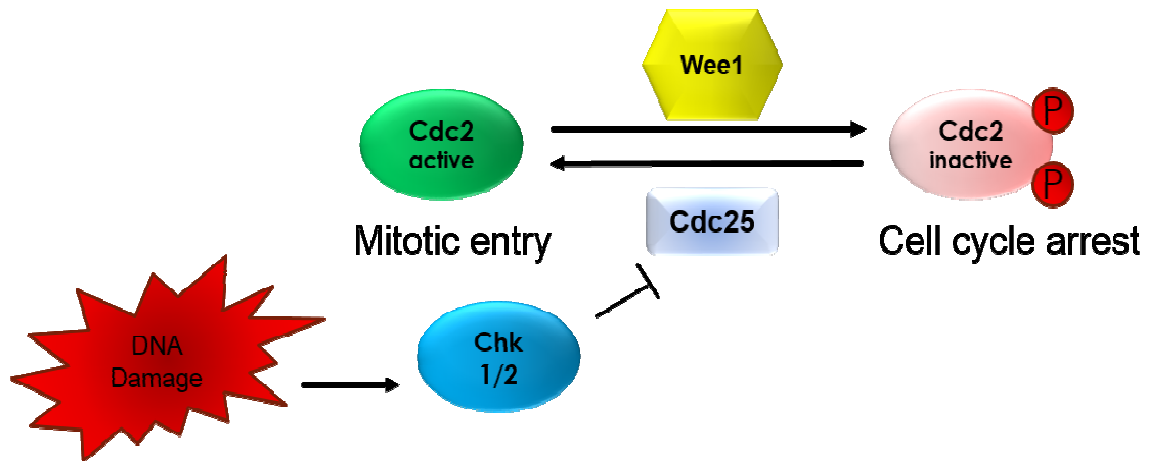
Figure 1.7: Regulation of CDK1/Cyclin B during G2/M transition

Figure 1.7: The entry of mitosis was driven by activation of CDK1/Cyclin B complex, whose activity is regulated by phosphorylation on Thr 14 and Tyr 15. Wee1 and Myt1 kinases target these two sites and keep CDK1 inactive. During the G2/M transition, Cdc25 dephosphorylates CDK1, activating Cdc2 and driving the cell into M phase. DNA damage activated Chk1/2 kinase can trigger Cdc25 degradation by phosphorylation and thus inhibit the G2/M transition
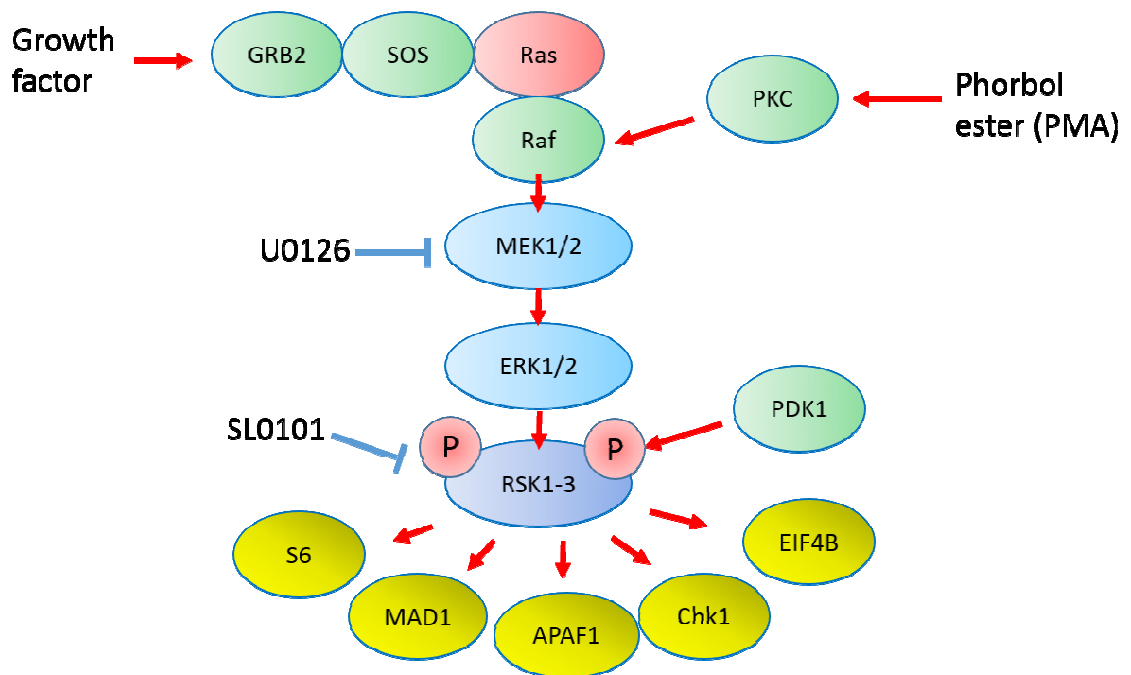
Figure 1.8: MAPK/RSK signaling cascade

Figure 1.8: RSK is activated following the activation of Ras–MAPK cascade upon treatment of growth factors. The GTP-bound form of Ras binds to the Raf protein kinases, which brings them to the plasma membrane to be activated. Subsequently, Raf kinase phosphorylates and activates MEK1 and 2. Activated MEKs then phosphorylate ERK1 and 2 on both a Tyr and a Thr residue. RSK is directly phosphorylated and activated by ERK1/2. In addition, treatment of phorbol esters also activates MAPK/RSK pathway through activation of Raf by PKC. The active Rsk can subsequently phosphorylates many effectors including S6, EIF4B, MAD, Chk1, Apaf1 and so on to regulate apoptosis, EMT, proliferation, DNA damage response, and many other cellular functions. RSK-specific inhibitors SL0101 and MEK inhibitor U0126, have been useful in delineating the activation and biological function of RSK.
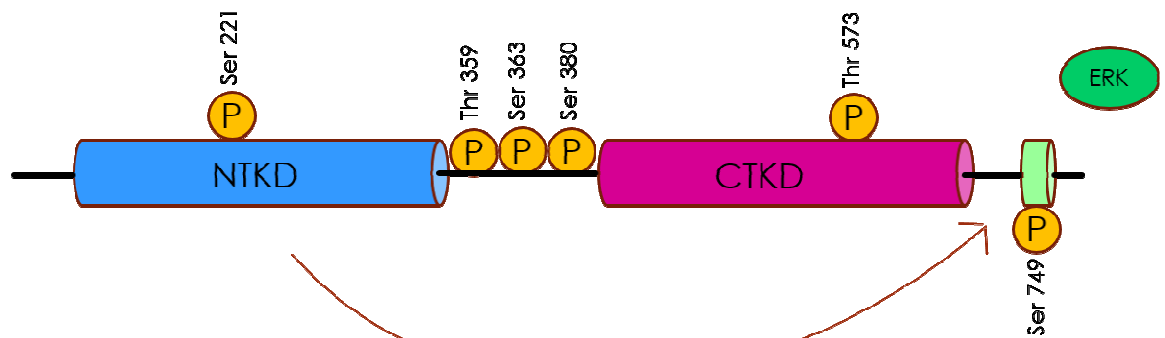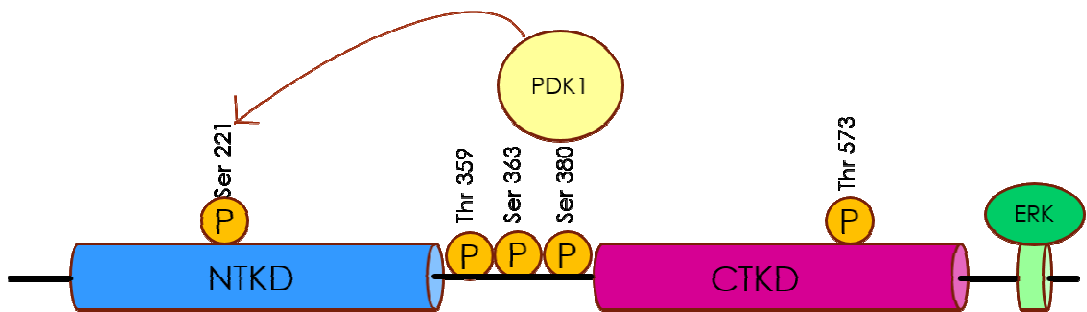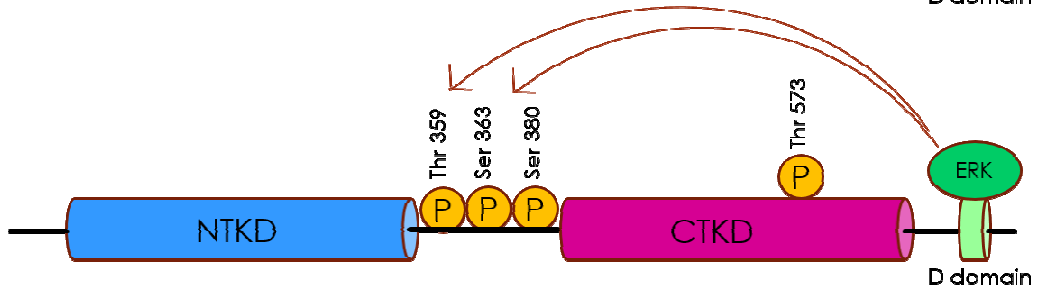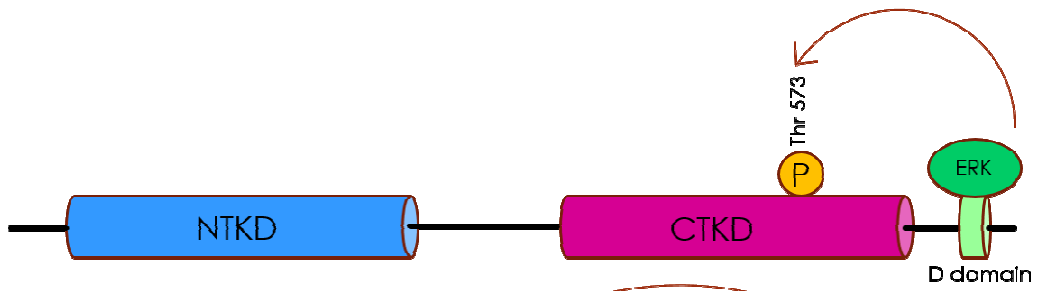
Figure 1.9: RSK is activated by the sequential phosphorylation.

Figure 1.9: Active ERK phosphorylate Thr573 in the C-terminal kinase domain (CTKD) of RSK. This phosphorylation event also requires ERK docking at the D domain of RSK. The activated CTKD subsequently phosphorylates RSK itself at Ser380 in the hydrophobic motif. ERK1/2 might also phosphorylate Thr359 and Ser363. The constitutively active kinase PDK1 docks at the phosphorylated hydrophobic motif and phosphorylates Ser221 in the N-terminal kinase domain (NTKD) to complete RSK activation, followed by the autophosphorylation of Ser749 in the D domain by the NTKD, which in some cases results in dissociation of ERK1/2 from the D domain of RSK.
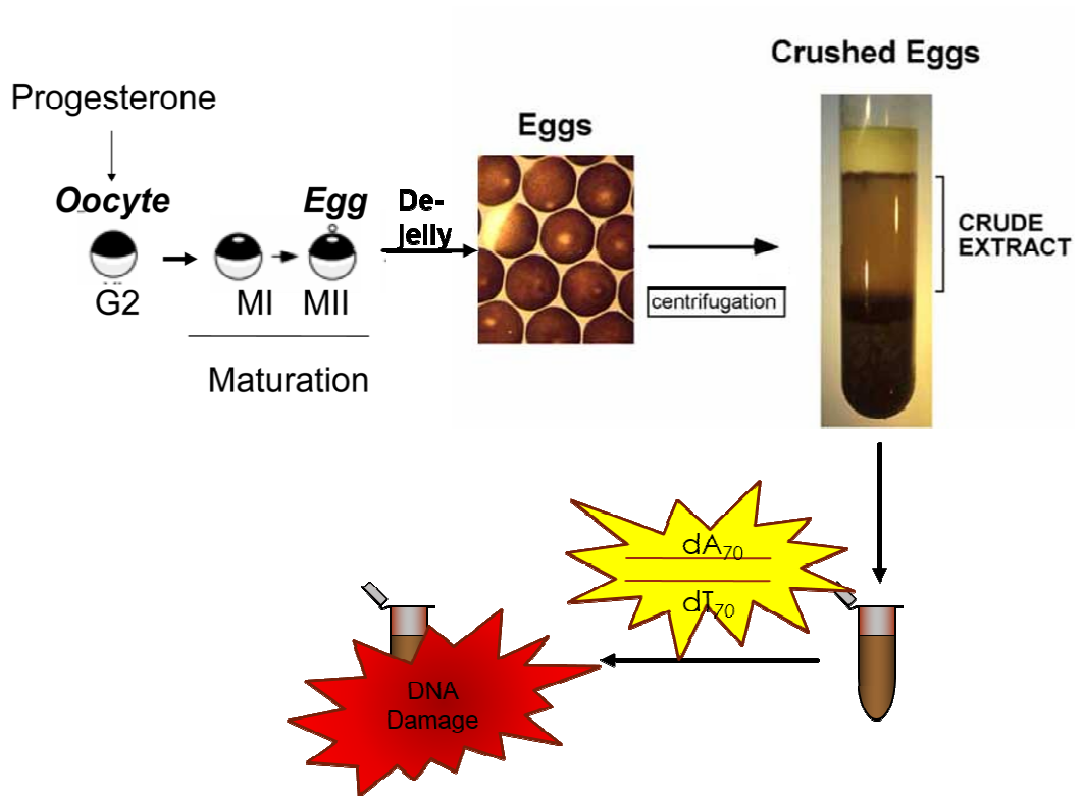
Figure 1.10: Studying DSB response in the *Xenopus* egg extract system

Figure 1.10: Female *Xenopus* were treated with hormones to promote oocyte generation and egg laying. After eggs were laid, they were packed in tubes and crushed by high-speed centrifugation. During centrifugation, endogenous Ca^{2+} is released from internal stores, leading to the degradation of Cyclin B and release of extracts into interphase. The addition of cyclocheximide to the buffer prior to centrifugation inhibits subsequent Cyclin B translation and functions to arrest the extracts in interphase. Such system can recapitulate precisely the DSB response via addition of sperm chromatins or annealed oligonucleotides, making it ideal to study the mechanism of the DSB response.

Chapter 2: Materials and Methods

2.1 Cell culture and drug treatments

293T, HeLa or MCF7 cells were maintained in high glucose DMEM media supplemented with 10% FBS. PC3 cells were maintained in F12K media. Cells were grown to 90% confluence before treating with drugs. For SL0101, 1 µL of 100 mM SL0101 was added into 1mL media for a final concentration of 100uM. Cells were treated with SL0101 for 30min. For PMA, 0.5 µL of 50 ng/µL PMA was added into 1mL media to make final concentration of 25 ng/mL. Cells were treated for 30min. For NCS, 0.2 µL of 500 ng/mL NCS was added into 1mL media to make a final concentration of 200 ng/mL. After treatment for 30min, cells were washed once with PBS.

The following drugs were used in this paper: PMA (Enzo Life Science), NCS (neocarzinastatin, Sigma), SL0101 (Calbiochem), Ku55933 (Calbiochem).

2.2 Transfection and siRNA

FuGENE 6 (Roche) was used to transfect cells with plasmids according to the manufacturer's instructions. Lipofectamine RNAiMAX (Invitrogen) was used to perform siRNA transfections. siRNA targeted against RSK1 and RSK2 were purchased from sigma (Buck M., 2001).

2.3 Plasmid Reagents and antibodies

C-terminal HA tagged Mre11 and N-terminal Flag tagged RSK2 were cloned into pCDNA3 plasmid. Mre11 alanine mutants and the RSK2 707A constitutively active mutant were generated by site-directed mutagenesis. The antibodies against human Mre11 (Abcam), phospho-S6 Ser235/236 (Cell Signaling), phosphor-Histone H3 (Ser10) (Cell Signaling), Actin (Santa Cruz), phosphor-Mre11 Ser676 (Cell Signaling), goat-anti-HA (Abcam), His-probe (Santa Cruz), γH2AX Ser139 (Cell Signaling), human ATM (Abcam), phospho-ATM Ser1981 (epitomics), anti-human/*Xenopus* Mre11 (Calbiochem) were all commercially available.

The antibodies against *Xenopus* Nbs1 and ATM were gifts from William Dunphy.

Biotin-tagged single strand DNA was purchased from integrated DNA technologies. Biotin-tagged double strand DNA was synthesized by annealing tagged single strand DNA with its anti-sense DNA at 70oC and cooling down gradually to room temperature.

2.4 Kinase and phosphatase assay

For kinase assays in mammalian cell lysates, nickel-agarose bound Mre11-His was incubated with 2 μCi γ-32P-ATP in cytosolic lysates from mammalian cells transfected with RSK2 for 1 h at 30°C. Beads were then removed and washed three times in washing buffer (1 mM Hepes-KOH, pH 7.5, 1 mM β-glycerophosphate, 15 mM KCl,

0.1% NP-40, 14.8 mM imidazole, 500 mM NaCl, 1 mM NaVO$_3$ and 5 μg/ml

aprotinin/leupeptin).

For the in-vitro kinase assay, nickel-agarose bound Mre11-His protein was

incubated in kinase buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM ATP, 10 mM MgCl$_2$, 1 mM

DTT, pH 7.2) with 2 μCi γ-32P-ATP and RSK2 kinase (Milipore). After 30 min at 30°C,

the beads were washed with washing buffer. Analysis for all kinase assays was

performed using SDS–PAGE and autoradiography.

For the in-vitro phosphatase assay, Mre11-His protein was pre-treated with

kinase assay in the presence of RSK to be radio-labeled. Prephosphorylated proteins

were then dipped into phosphatase buffer (10 mM Tris-HCl, pH 7.2, 10mM MgCl$_2$, 1mM

DTT, pH7.2) with His-PP2A or PP5. Aliquots were retrieved, washed with PBS plus 300

mM NaCl and 0.1% Triton, eluted by SDS sample buffer and subjected to 10% SDS-

PAGE. The phosphorylation was detected by phosphorimager (Molecular Dynamics).

2.5 Immunoblot analysis

For tissue culture, cells were cultured to 90% confluence before harvesting with

trypsin. Cells were then lysed with extraction buffer (20 mM HEPES pH7.4, 150 mM

NaCl, 12.5 mM M glycerophosphate, 1.5 mM MgCl$_2$, 2 mM EGTA, 10 mM NaF, 2 mM

DTT, 1 mM Na$_3$VO$_4$, 20 μM aprotinin, 1 mM PMSF, 0.5% Triton X-100). The

concentration of cell lysates was then measured. Equal amounts of lysates were loaded

for SDS-PAGE. Proteins were then transferred from gels to PVDF membrane and then

immunoblotted with antibodies. For large molecular weight proteins such as ATM, low

voltage (50V) was used in the overnight transfer.

For *Xenopus* extracts, 5 µL of extract was mixed with 50 µL SDS sample buffer.

Extract samples were then loaded for SDS-PAGE, transferred from gels to PVDF

membrane and processed for immunoblotting.

2.6 FACS

293T cells were treated with different inhibitors based upon experimental

requirement described in detail in the section 3.2 and fixed with 4% paraformaldehyde

for 20 min after 22 hrs. Cells were then permeabilized with 0.2% triton in PBS buffer.

After washing, cells were incubated with primary antibodies at 37 degrees for 2hrs, and

then with secondary antibody for 45 min. Cells were then washed with PBS and treated

with RNase for 15 min and stained with propidium iodium (PI). Cell profiles were

analyzed by flow cytometry. Each experiment was repeated 3 times and Student's t-test

was applied to assess statistical significance. Significance levels employed will be

indicated in results or figure legends of chapter 3.

2.7 Xenopus extracts and DNA IP

CSF extracts and interphase extracts were prepared as previously described (Wu, Hansen et al. 2007). For extracts treated with DSB DNA, 5μL 0.01mM dAdT dsDNA (70 nucleotides) were added into 50μL extracts. Extracts were incubated at room temperature (RT) for at most 1 hr. For DNA precipitations, 1μL biotin-tagged dsDNA (0.25mM) was bound to 25μL avidin beads for each reaction. DNA-bound beads were then incubated with 100μL extracts for 20min at RT. Beads were then retreated from extracts and washed with egg lysis buffer (250mM sucrose, 2.5mM $MgCl_2$, 1mM DTT, 50mM KCl, 10mM Hepes, and pH7.7) 4 times.

2.8 Immunostaining

HeLa cells were grown on cover slips, treated with different drugs as described in section 3.2, and fixed with 4% paraformaldehyde for 20min. Immunostaining was performed as described previously (Zhang, Huang et al. 2012) using anti-γH2AX antibody. Detailed information about microscopy is listed in section 2.9.

2.9 Additional information for microscopy

The following microscope was used: Zeiss Axio Imager widefield fluorescence microscope equipped with Mercury arc lamp (Zeiss HBO100 power supply & lamp housing). Filter cube sets used: DAPI (Chroma 31000v2, excitation wavelength range: 325nm-375nm, beam splitter: 410 nm, emission wavelength range: 435nm-485nm; GFP

(Chroma 41017, excitation wavelength range 450nm-490nm, beam splitter Q495LP, emission wavelength range 500nm-550nm); RFP (Chroma 41004, excitation wavelength range 535nm-585nm, beam splitter 595, emission wavelength range 610nm-680nm). Objectives used: Zeiss 20x/0.50 EC Plan-NeoFluar; Zeiss 40x/0.75 EC Plan-NeoFluar. Camera: Hamamatsu Orca ER monochrome cooled-CCD camera. Acquisition software: MetaMorph Premier Ver 7.7.3.0. Fluorescent intensity in the original raw images was quantified with MetaMorph. Blank areas within images were selected as background during quantification. Images were also adjusted for contrast and pseudo-colored in MetaMorph for presentation in figures.

Chapter 3: Regulation of DNA damage checkpoint signaling by RSK kinase and PP5 phosphatase

3.1 Introduction

Cells have evolved multiple DNA damage response signaling pathways that trigger DNA repair, cell cycle arrest and, in the event of irreparable damage, cell death. Among the various forms of DNA damage, double stranded DNA breaks (DSBs), generated by exposure to ionizing radiation and radiomimetic chemicals (such as neocarzinostatin, NCS) are the most lethal for cells (Agarwal, Tafel et al. 2006).

DSBs are often repaired by homologous recombination during S and G2/M phases of the cell cycle, which involves the Mre11/Rad50/Nbs1 (MRN) complex and the Ataxia Telangiectasia Mutated (ATM) kinase (Abraham 2001, Lee and Paull 2004). The MRN complex first recognizes sites of DNA damage, then promotes binding of ATM to the DSB site. ATM, activated by monomerization and autophosphorylation, phosphorylates downstream proteins including p53, Chk2, and BRCA1 (Hawley and Friend 1996, Kim, Lim et al. 1999, Kastan and Lim 2000, Matsuoka, Rotman et al. 2000). These factors then convey the signal to induce cycle arrest, apoptosis or DNA repair (Jackson 2001). Failure of this process results in genome instability, increasing the risk of cancer, neurodegeneration and other pathologies (Lavin 2007).

3.1.1 MAPK/RSK signaling cascade and DSB induced DNA damage checkpoint

The Ribosomal S6 kinase (RSK), which functions downstream of MEK and ERK, is frequently activated in cancer cells (Dalby, Morrice et al. 1998, Smith, Poteet-Smith et al. 1999). RSK activation can be promoted by multiple signaling pathways in cancer cells, including those triggered by steroids, insulin, EGF, and estrogen (Sturgill, Ray et al. 1988, Zhao, Bjorbaek et al. 1995, Bunone, Briand et al. 1996, De Cesare, Jacquot et al. 1998, Smith, Poteet-Smith et al. 1999). Additionally, RSK activation can be triggered by protein kinase C (PKC) signaling (via the PKC/RAF/MAPK pathway), which is activated by the phorbol ester, PMA (Ryves, Evans et al. 1991, Grove, Price et al. 1993). Previous studies have found that RSK2 is overexpressed in 50% of breast cancers and prostate tumors (Clark, Errington et al. 2005, Smith, Poteet-Smith et al. 2005), and RSK signaling has been implicated in the regulation of survival, anchorage-independent growth, and transformation of breast cancer cells in culture (Xian, Pappas et al. 2009). RSK-specific inhibition (with BI-D1870 or SL0101) significantly reduced proliferation of MCF7, PC3 or LnCaP cancer cells (Clark, Errington et al. 2005, Smith, Poteet-Smith et al. 2005). RSK also inhibits apoptosis in PC3 prostate cancer cells (Kim, Parrish et al. 2012).

A hallmark of cancer cells is their ability to override cell cycle checkpoints, including the DSB checkpoint, which arrests the cell cycle to allow adequate time for damage repair. Previous studies have implicated the MAPK pathway in inhibition of

DNA damage signaling: PKC suppresses DSB-induced G2/M checkpoint signaling following ionizing radiation via activation of ERK1/2 (Lee, Soh et al. 2002); activation of RAF kinase, leading to activation of MEK/ERK/RSK, can also suppress G2/M checkpoint signaling (Fig 1.7) (Warenius, Jones et al. 2000).

Given its prominent role in multiple cancers, the MAPK pathway is an attractive therapeutic target. Indeed, treatment of melanoma using the RAF inhibitor, vermurafenib has shown some clinical success, as has treatment of non-small cell lung carcinoma with MEK inhibitors (Rinehart, Adjei et al. 2004). However, targeting components at the apex of a signaling pathway may induce side effects due to the plethora of downstream effectors (Sebolt-Leopold 2004, Sebolt-Leopold and Herrera 2004). As a terminal kinase in the MAPK pathway, RSK may avoid these complications as a potential target. Thus, there has been interest in targeting RSK for cancers with notable RSK elevation (e.g. prostate cancers) (Eisinger-Mathason, Andrade et al. 2010). Several RSK-specific inhibitors have been described, including SL0101 and BI-D1870 (Cohen, Zhang et al. 2005, Smith, Poteet-Smith et al. 2005, Sapkota, Cummings et al. 2007). It remains unclear whether these, or derivative, drugs will be clinically successful. However, if RSK inhibition can re-instate DSB-induced checkpoint function, then combination therapy of RSK inhibitors with DNA damaging agents may be effective in inducing tumor cell arrest or death.

The precise mechanism underlying checkpoint inhibition downstream of MEK/ERK/RSK signaling is not yet clear. One recent paper has shown that, following doxorubicin-induced DNA damage (both DSB and SSB), RSK can silence the G2/M checkpoint by phosphorylating (and inhibiting) the Chk1 kinase on S280 (the same site that can be targeted by AKT kinase) (Ray-David, Romeo et al. 2013). Yet, another group has reported that phosphorylation of the same site on Chk1 enhanced its ability to enforce the checkpoint by promoting its nuclear translocation (Li, Goto et al. 2012).

We show here that RSK signaling silences DSB-induced G2/M checkpoint by preventing activation of the ATM kinase. Specifically, we have found that RSK targets the Mre11 component of the MRN complex by phosphorylating Ser676, thereby preventing Mre11 binding to DNA. Mutation of Ser676 restored ATM activation, even in the face of high RSK activity, and rendered cells refractory to the checkpoint-inhibitory effects of RSK. This is consistent with a previous study showing that phosphorylation on Mre11 is mostly inhibitory (Di Virgilio, Ying et al. 2009). Taken together, these findings identify a new locus of checkpoint regulation by RSK and lend support to the idea of targeting RSK for therapeutic benefit.

3.1.2 Phosphatases that function in DSB induced DNA damage response

We are further interested in identifying the phosphatase that antagonize RSK's effect on DSB response. As discussed in chapter 1, protein phosphatases play an

important role in the DNA damage response. Both PP2A and Wip 1 can recognize a p(S/T)Q motif and dephosphorylate many proteins that are phosphorylated by ATM/ATR/Chk1/Chk2. Such dephosphorylation may play a role in suppressing intensive checkpoint response as well as in checkpoint recovery.

Another phosphatase, PP5, has also been shown to be involved in the DSB response. Unlike PP2A and Wip 1, whose phosphatase activity is proven to be inhibitory to the activation of ATM or ATR, PP5 is required for both DSBs and SSBs-induced ATM and ATR activation. As mentioned in chapter 1, PP5 forms complexes with several DNA damage activated kinases, such as DNA PK, ATM, ATR, and ASK1. Knockdown of PP5 with siRNA results in attenuation of DSBs-induced ATM activation, while overexpression of a catalytically inactivate PP5 mutant inhibits the phosphorylation of ATM substrates and ATM autophosphorylation (Ali, Zhang et al. 2004). Similarly, it has been reported that PP5 is require for the ATR-dependent phosphorylation of 53BP1 and BRCA1 upon UV-light treatment, and disruption of PP5 expression in mice greatly reduced UV light induced Chk1 phosphorylation(Amable, Grankvist et al. 2011, Kang, Cheong et al. 2011).

Although the importance of PP5 in DNA damage induced cell cycle checkpoint and ATM/ATR activation is well-established, it is still unknown which proteins are targeted by PP5 during this process. Such uncertainty is even more complicated due to the fact that PP5 has no clear consensus targeting sequence and does not always need to

bind to its substrate to function. In addition, the basal activity of PP5 is rather low, indicating some co-factors are also involved in activation of the DNA damage checkpoint by PP5.

As previously described, the phosphorylation of Mre11 on Ser676 by both RSK and ATM inhibits its binding to DNA, reducing the affinity between MRN and dsDNA, and consequently causing RSK-mediated silencing of G2/M checkpoint or ATM-mediated negative feedback loop that might contribute to checkpoint recovery or adaptation. Here we propose that by antagonizing such phosphorylation on Mre Ser676, PP5 may play a role in sensitizing cells for DSBs-induced checkpoints and promoting the recovery from DNA damage response.

3.2 Results

3.2.1 Prostate cancer cells with high levels of endogenous RSK are resistant to DSBs

Rsk is highly expressed in many prostate cancer cell lines, including PC3 and LnCaP cells, which appear to be highly dependent on Rsk activity for growth and survival (Clark, Errington et al. 2005). We hypothesized that Rsk might modulate the response of these cells to DNA damage-induced checkpoint function. To evaluate this, we analyzed cell cycle progression after DSB induction in PC3 cells that endogenously

express twice the level of Rsk protein found in normal prostate tissue (Clark, Errington et al. 2005). Cells were arrested at G1/S using a double thymidine block. Four hours after release by washing away thymidine, they were treated with a Rsk-specific inhibitor SL0101 followed by radiomimetic NCS to induce DSBs during G2 phase. After washing away the drugs, cells were incubated in fresh media for 16 hrs before analyzing DNA content. As shown in Fig. 3.1A co-treatment of cells with SL0101 and NCS resulted in much more robust G2/M arrest than NCS alone. Additionally, following PMA-induced Rsk activation, the DSB-induced G2/M arrest was effectively overridden. We obtained similar results in experiments where Rsk1/2 were knocked-down using siRNA (Fig. 3.1B). Together, these data suggest that Rsk might inhibit the DSB checkpoint in PC3 prostate cancer cells. To examine this further, we stained PC3 cells for γ-H2AX, a downstream target of ATM. As shown in Fig. 3.1C, PC3 cells treated with SL0101 prior to NCS treatment displayed greatly increased γH2AX staining. These data strongly suggest that high Rsk activity can suppress ATM-dependent G2/M checkpoint signaling.

Although it has been reported that inhibiting ATM may enhance G2/M accumulation (Xu, Kim et al. 2002), it should be noted that, in the cell lines we used, inhibiting ATM decreased the G2/M population of cells (Fig. 3.1D). This result is also consistent with other published studies (Zhou, Chaturvedi et al. 2000, Wang, Liu et al. 2012).

To determine if Rsk is sufficient to confer resistance to DSB-induced G2/M arrest, we overexpressed constitutively active Rsk2 in 293T cells, which have much lower endogenous levels of Rsk than PC3 cells. As shown in Fig. 3.1F, Rsk-expressing cells were considerably less sensitive to DNA damage than control cells. To directly demonstrate G2/M checkpoint suppression, we synchronized HeLa cells in G1/S phase by double thymidine block, treated them 4 hrs after washing away thymidine with PMA or SL0101 for 30 min, and then with NCS for an additional 30 min. Three or five hours later, cells were stained with anti-phospho-Histone H3 antibody. As shown in Fig. 3.1F, NCS/DSB significantly reduced pHH3 positive cells by arresting cells in G2 phase. Yet, PMA treatment greatly increased the pHH3 positive population (particularly at 5 hrs), suggesting that the G2/M checkpoint had been silenced. This checkpoint inhibition was reversed by co-treatment with SL0101, confirming a role for Rsk in this silencing.

3.2.2 RSK suppresses the DSB response by inhibiting the activation of ATM

To identify the locus of Rsk action within the DSB signaling pathway, we assessed the status of the ATM kinase following NCS treatment in cells overexpressing constitutively active Rsk2. We found these cells had reduced ATM phosphorylation at SER1981, a hallmark of ATM activation, following treatment with NCS, but no change in ATM protein levels (Fig. 3.2A). Similarly, PMA treatment-markedly decreased ATM autophosphorylation at SER1981. However, ATM phosphorylation was restored when

cells were treated with both PMA and SL0101 (Fig. 3.2B). To further demonstrate down-regulation of ATM, we measured phosphorylation of Nbs1 at S343, a site known to be targeted by ATM. As expected, phosphorylation of Nbs1 was reduced in the presence of PMA, consistent with Rsk-mediated reduction in ATM activity (Fig. 3.2C). Accordingly, siRNA knock-down of Rsk in PC3 cells significantly increased ATM autophosphorylation in response to NCS treatment (Fig. 3.2D). ATM autophosphorylation is a very early event in the DSB response, and these data suggest that Rsk acts either at or upstream of ATM. Consistent with an effect of Rsk signaling on ATM activity, HeLa cells treated with PMA stained more weakly with antibody directed against γH2AX than did untreated cells (Fig. 3.2E), and co-treatment with PMA and SL0101 reversed the diminution in γH2AX staining (Fig. 3.2E). HeLa cells transfected with FLAG-tagged constitutively-activated Rsk2 exhibited significantly less γH2AX staining than did non-transfected cells after NCS treatment (Fig. 3.2F). This effect was reversed by treatment with SL0101 (Fig. 3.2F). Collectively, these data suggest that Rsk signaling suppresses both ATM activation and phosphorylation of its downstream targets, Nbs1 and H2AX.

It is also worth noting that ATM activation is only robustly detected at the early phases of the DSB response (and thus the effects of Rsk could not be detected later, at 24h) (Fig. 3.2G). This is likely due to checkpoint adaptation or recovery from DNA damage, consistent with previous reports (Laval, Kao et al. 2007, Kumareswaran,

Ludkovski et al. 2012). However, cells were still phenotypically arrested at 24h because either 1) the remaining ATM activity/DSB response is sufficient to maintain the checkpoint or 2) checkpoint recovery lags behind DSB response recovery, as cells require time to resume the cell cycle, even though the upstream DNA damage signals have waned.

3.2.3 The Mos/MAPK/RSK pathway inhibits binding of the MRN complex to dsDNA

To identify the direct targets of Rsk responsible for inhibiting the DSB response, we used the *Xenopus* egg extract system, where DSB checkpoint signaling can be reconstituted *in vitro*. This allows for easy manipulation of both DNA templates and Rsk signal components. Interphase extracts prepared from *Xenopus* eggs (crude S extracts) were treated with recombinant MBP (maltose binding protein)-tagged Mos kinase, a strong upstream activator of the MAPK pathway, leading to robust Rsk activation (Wu, Hansen et al. 2007). This treatment markedly inhibited autophosphorylation of ATM induced by dsDNA without altering ATM levels. SL0101 treatment reversed this inhibition (Fig. 3.3A). To rule out the possibility that inhibition of the DSB response might be due to signaling by other Mos-activated kinases, we treated crude S extracts with pre-phosphorylated constitutively-activated Rsk, and observed a similar decrease in ATM autophosphorylation (Fig. 3.3B). Because ATM must be recruited to the site of

DNA break by the MRN complexes prior to autophosphorylation and monomerization, we investigated the binding of ATM to dsDNA; we immobilized biotin-tagged dsDNA on avidin beads to mimic sites of double stranded DNA damage, incubated the beads with crude S extracts, and retrieved DNA bead-associated proteins. We observed a significant decrease in dsDNA-bound ATM after pre-treatment of extracts with Mos, while binding of another DNA damage response protein, Ku70, was unchanged (Fig. 3.3C). To rule out the possibility that decreased ATM binding resulted from reduced ATM activity, we supplemented extracts with the ATM/ATR kinase inhibitor caffeine; this did not reverse the decrease in ATM/DNA binding (Fig. 3.3D). Together, these data suggest that Rsk functions upstream of ATM, affecting its recruitment to sites of DNA damage.

Because ATM recruitment is preceded by recognition of DNA damage sites by the MRN complex, we investigated whether binding of the MRN complex to dsDNA is affected by Rsk. Indeed, less Mre11 protein was associated with dsDNA in the crude S extracts pre-treated with Mos than with control MBP protein. Moreover, SL0101 suppressed the effect of Mos (Fig. 3.3D). The association of Mre11 with the beads was DNA dependent; an excess of free DNA significantly decreased the levels of bead-bound Mre11 (Fig. 3.3E). Moreover, treatment with either the ATM inhibitor KU55933 or ATM/ATR inhibitor caffeine did not reverse Rsk's inhibition of Mre11 binding. This strengthens the suggestion that the observed effect on MRN complex/DSB DNA

assembly was not due to feedback from downstream ATM signaling (Fig. 3.3D and Fig.

3.3E). To confirm these findings in intact cells, we stained HeLa cells with anti-Mre11

antibody. PMA activation of Rsk significantly reduced the formation of Mre11/MRN

foci, suggesting that Rsk was indeed suppressing the binding of the MRN complex to

the DSB DNA (Fig. 3.3F).

To rule out the possibility that Rsk disrupts the interaction between Mre11 and

other MRN complex components, we investigated binding of Mre11 to Nbs1 in egg

extracts without added DNA. We did not observe any effect of Rsk on the physical

interaction between these two proteins (Fig. 3.3G), supporting the hypothesis that Rsk

interferes with Mre11 binding to DNA but not the formation of the MRN complex itself.

To confirm that Rsk mediates suppression of ATM activation through the MRN

complex, we treated cells with H_2O_2, which activates ATM independently of the MRN

complex (Guo, Deshpande et al. 2010). Cells overexpressing Rsk displayed no change in

ATM activation following H_2O_2 treatment, in agreement with our conclusion that Rsk

silences ATM activation through suppression of MRN complex binding to DNA (Fig.

3.3H).

3.2.4 Ser676 on Mre11 is the target of RSK

To determine the specific target(s) of Rsk, we isolated DNA-bound proteins from

crude S egg extracts and incubated the precipitates with Rsk and radio-labeled ATP. The

one major protein band identified by SDS-PAGE and autoradiography was approximately 85 kD, which is the molecular weight of Mre11, suggesting that Mre11 might be a direct target of Rsk (Fig. 3.4A). This was confirmed using His-tagged recombinant Mre11, which could be directly phosphorylated by Rsk2 kinase (Fig. 3.4B). To determine if the decreased MRN/dsDNA binding was due to posphorylation of Mre11, we pre-phosphorylated Mre11 with Rsk2, and then incubated Mre11 with crude S extracts for 15 min (to allow association of other required factors) before precipitating the dsDNA. As shown in Fig. 3.4C, pre-phosphorylation by Rsk2 reduced the binding of Mre11 to dsDNA, suggesting that phosphorylation of Mre11 contributes to Rsk-mediated inhibition of the DSB response.

To identify the relevant sites of phosphorylation on Mre11, we phosphorylated recombinant His-tagged Mre11 with Rsk *in vitro* and analyzed the protein by Mass Spectrometry. We identified four phosphorylated residues: 2, 641, 676 and 688. Although none of those four sites were identical to the Rsk consensus site RXRXXpS/T, Rsk can target non-optimal sites (Buck, Poli et al. 2001). We mutated each of the identified sites and performed *in vitro* Rsk assays using the recombinant mutants. Although several of the sites reduced phosphorylation, the single Ser676ALA mutation almost completely abolished phosphorylation, suggesting that Ser676 was the major site of *in vitro* phosphorylation by Rsk (Fig. 3.4D). Mre11 phosphorylation was also severely compromised by mutation of Ser676 when radiolabeled ATP was added to lysates that

had been prepared from cells transfected with Rsk (Fig. 3.4E). Note that in this experiment, cells were not serum starved before lysate production and thus wildtype or constitutively activated Rsk proteins behaved similarly (due to activation of the transfected WT Rsk). Consistent with our *in vitro* data, we successfully detected endogenous Mre11 Ser676 phosphorylation in the presence of PMA or overexpressed wildtype/constitutively active Rsk, with an antibody that targeted phospho-Ser676 (Fig. 3.4F and G). Phosphorylation induced by PMA was abrogated by co-treatment with the Rsk inhibitor SL0101 (Fig. 4D and E). In addition, we treated Rsk-transfected cells with a DNA PK inhibitor to rule out the possibility that Rsk might cause Mre11 Ser676 phosphorylation indirectly by activating DNA PKc (Fig 3.4H). Although the DNA PK inhibitor was effective against a known DNA PK substrate, Akt, it did not inhibit phosphorylation of Mre11 downstream of Rsk (Fig. 3.4I). Collectively, these data suggested that Rsk can phosphorylate Ser676 of Mre11 both *in vitro* and in intact cells.

3.2.5 Phosphorylation of Mre11 on Ser676 reduces its binding to dsDNA and is responsible for checkpoint inhibition by RSK.

Mre11 has two potential DNA binding domains (DBD) and Ser676 is located within the 2nd of these (Paull and Gellert 2000, Stracker, Theunissen et al. 2004) (Fig. 3.5A). Therefore, we speculate that phosphorylation of Mre11 on Ser676 might directly disrupt its binding to dsDNA. As shown in Fig. 3.5B, Mos treatment of egg extracts

reduced binding of WT Mre11 to DNA beads, but had no effect on the Ser676A mutant.

Moreover, pre-treating recombinant WT Mre11 with Rsk reduced the amount of DNA-

bound Mre11, but not when the Ser676A mutant was used (Fig. 3.5C).

Consistent with a role for Mre11 Ser676 phosphorylation in the control of ATM

by Rsk, we found that the ability of Mos to inhibit DSB-induced ATM activation in

Xenopus egg extracts was significantly dampened by addition of the Ser676A mutant

Mre11 (Fig. 3.5D). Moreover, upon expression in 293T cells, the Ser676A mutant

conferred resistance to Rsk-mediated G2/M checkpoint inhibition, while the control

mutant (mutated at the other sites originally seen by Mass spectrometery,

2A/641A/688A) did not (Fig. 3.5E). We further investigated whether Rsk directly

inhibited Mre11/MRN focus formation in Mre11 mutant cells. We knocked down

endogenous Mre11 in HeLa cells using shRNA and transfected cells with shRNA

resistant WT Mre11 or Ser676A. Importantly, PMA treatment reduced the Mre11 foci in

WT-expressing cells but not in the Ser676A mutant-expressing cells (Fig. 3.5F).

Moreover, SL0101 reversed the effect of PMA in the wild type-expressing cells,

consistent with a role of Rsk in controlling Mre11/MRN focus formation via

phosphorylation of Mre11 Ser676 (Fig. 3.5F).

3.2.6 Identification of potential phosphatases that targets Mre11 Ser676

The importance of the Mre11 Ser676 site suggests that its phosphorylation status

may be tightly controlled by both kinase(s) and phosphatase(s). In order to find out the

potential phosphatase(s) that target Mre11 Ser676, we performed screening experiments

using both drugs and siRNA, where cells were treated with different phosphatase

inhibitors including OA and calyculin A. Via phosphatase inhibitors, we found that the

phosphorylation of Mre11 only increased in the presence of high concentrations of OA

that inhibit PP2A, PP5 or PP4 (Fig. 3.6A). Further experiments using siRNA revealed

that he reduction of PP5 resulted in an increase in Mre11 phosphorylation (Fig. 3.6B).

Together these results suggested that PP5 could be the one of the phosphatases that

targets Mre11 Ser676.

3.2.7 PP5 dephosphorylates Mre11 both *in vivo* and *in vitro*

In order to verify which phosphatase is responsible for dephosphorylation of

Mre11, an *in vitro* phosphatase assay was performed using purified Mre11 that had been

incubated with RSK kinase and radioactive ATP for 30 min, and then with either PP5 or

PP2A for another 2 hrs. Interestingly, PP5 can dephosphorylate Mre11 *in vitro* while

PP2A cannot, suggesting that PP5 is more likely to be the relevant phosphatase (with the

caveat that PP2A-mediated dephosphorylation might require the presence of the correct

targeting subunit) (Fig. 3.6C). This finding was consistent with a previous study, which

suggested that PP2A inhibited the DSB-induced DNA damage checkpoint, while PP5

potentiated the checkpoint. Consistently, knockdown of PP5 in HeLa cells greatly reduced both the number and size of Mre11/MRN complex foci (Fig. 3.6E). Together these data demonstrated a role for PP5 in targeting Ser676 on Mre11.

3.2.8 The negative feedback loop of the DSB response and PP5

As previously described in chapter 1, after being activated, ATM can phosphorylate Mre11 directly or indirectly at multiple S/T-Q sites including Ser676. This inhibits the binding of Mre11, causing its dissociation from dsDNA and contributing to the negative feedback loop within the DSB response. Such a feedback loop may be important for the progression of DNA damage repair and checkpoint recovery. Since PP5 can counteract ATM's phosphorylation on Mre11 by dephosphorylating the same site, it would be interesting to know whether PP5 is involved in the regulation of the negative feedback loop.

If our hypothesis that PP5 is involved in the regulation of the DSB response negative feedback loop is true, it would be interesting to know how cells balance the counter-acting effect of phosphorylation and dephosphorylation between the kinase ATM and the phosphatase PP5. To that end, we studied the interaction between Mre11 and PP5 in mammalian cells before and after treating with NCS. Interestingly, PP5 interacted with Mre11 and such interaction gradually decreased over time upon DSBs (Fig. 3.6 D). We speculated that this gradual dissociation may allow Mre11 to be further

phosphorylated by ATM, tipping the balance towards the completion of the feedback loop.

3.3 Discussion

3.3.1 Mechanism for RSK-induced inhibition of DSB checkpoint function

We have shown here that Rsk signaling impairs cell cycle arrest by the DNA DSB-induced G2/M checkpoint through inhibition of the ATM pathway. This appears to involve a failure to activate ATM through MRN-mediated recruitment to sites of DNA damage. We found that Mre11 protein, a core constituent of the MRN complex, was phosphorylated by Rsk both *in vitro* and in intact cells. Furthermore, through mutagenesis experiments, one of the identified phosphorylation sites, Ser676, was demonstrated to be essential for functionally inhibiting Mre11. Together, these findings provide a mechanism for Rsk-induced inhibition of DSB checkpoint function.

A recent paper investigating the role of Rsk in G2/M arrest following doxorubicin treatment reported that Rsk could phosphorylate Chk1 at Ser 280, the same site that is targeted by Akt kinase to inhibit Chk1 activity (Ray-David, Romeo et al. 2013). However a similar study showed that the same phosphorylation on Chk1 had the opposite effect (Li, Goto et al. 2012). Our findings are distinct from these reports in demonstrating that Rsk-mediated inhibition of ATM activation can be achieved by

targeting steps upstream of ATM activation. In addition, Rsk can suppress G2/M checkpoint function following DSB in cells knocked down for Chk1, suggesting that Rsk phosphorylation of Mre11 contributes to DSB-induced G2 checkpoint silencing independently of Chk1 (Fig. 3.7A).

The finding that phosphorylation on Mre11 at Ser676 inhibited its binding to the DSB is also consistent with a previous report demonstrating that mutation of 8 SQ/TQ sites on Mre11, including Ser676, undermined the negative feedback loop whereby ATM terminates the checkpoint response by inhibiting DNA binding by Mre11 (Di Virgilio, Ying et al. 2009). Our study strongly suggests that Ser676 was, at least in part, responsible for such inhibition and that Ser676 phosphorylation can prevent binding of Mre11 to dsDNA not only in *Xenopus* extracts, but also in mammalian cells. In addition, we implicate Mre11 phosphorylation, as a factor in a critical checkpoint inhibitory pathway that results from high ambient MAPK signaling in cancer cells. Interestingly, Di Virgilio, et al suggested that ATM was not the only kinase responsible for the phosphorylation of the observed 8 SQ/TQ phospho-sites. Indeed, it is possible that Rsk not only participates in a checkpoint inhibitory pathway, but also acts downstream of ATM in the pathway of feedback inhibition. In other words, it may be that ATM can inhibit Mre11 by activating Rsk. We note that the converse cannot be true, i.e., Rsk cannot activate ATM to phosphorylate Mre11, because ATM inhibition did not preclude Rsk-mediated phosphorylation or inhibition of Mre11.

3.3.2 Role of RSK in tumorigenesis

The DNA damage response and its regulation have been shown to play a critical role in tumorigenesis; the DSB response pathway can be turned off early in tumor development in multiple types of cancer. This abrogation of checkpoint function prevents cell cycle arrest, leads to error-prone DNA replication, and promotes accumulation of multiple cellular mutations (that can fuel cancer progression) (Lobrich and Jeggo 2007, Jackson and Bartek 2009). Moreover, as the primary goal of radiation therapy is generation of abundant DSBs to induce permanent cell cycle arrest and/or apoptosis, the override of DSB-induced checkpoint function can undermine the utility of this treatment modality. Indeed as shown in Fig 3.7B, in the cells we tested, up-regulation of Rsk rendered cells resistant to excessive DSB-induced cell death. However, it should be noted that in some radioresistant tumors, the DSB response and DNA repair are up-regulated, rather than inactivated, leading to efficient repair of radiation-induced DNA damage (Sakata, Someya et al. 2007). Also in some situations, loss of the DSB response protein such as ATM/ATM in AT and ATLD patients which result in impaired DSB response signaling, may lead to increased sensitivity to radiation therapy. Consequently, the therapeutic benefit of inhibiting or activating the DSB response is likely to be dependent on tumor type. This has clear implications for Rsk as a target of

tumor therapy. In cancer cells that are inhibiting their checkpoint function through Rsk, it may be that Rsk inhibition would be synergistic with ionizing radiation.

3.3.3 Possibility of targeting RSK in treatment of cancer

The consequences of inhibiting Rsk for cancer therapy extend beyond the impact on cell cycle checkpoints/DNA repair. Recently, our laboratory reported that Rsk can phosphorylate the apoptotic inducer Apaf-1, and thereby reduce its apoptotic activity (Kim, Parrish et al. 2012). Similarly, it has been reported that Rsk can inhibit the action of Bax/Bad complexes, a pivotal mediator of cell death following both chemotherapy and radiation (Hurbin, Coll et al. 2005). Therefore, at least for tumors with high Rsk activity, inhibition of Rsk may restore DNA damage-induced cell cycle arrest, while at the same time lifting the brake on apoptosis. In these tumors, Rsk inhibitors may extend the utility of both IR and DNA-damaging chemotherapeutics.

3.3.4 Possible implication of dephosphorylation on Ser676 of Mre11 by PP5

Currently our results suggest that PP5 may target Mre11 at Ser676. As discussed previously, Mre11 Ser676 can be targeted by both RSK kinase and ATM, though ATM can target multiple sites on Mre11. It is also worth pointing out that other kinases, such as Akt, may also be able to phosphorylate this site, due to the similarity of consensus targeting sequences. The implication of dephosphorylation on Ser676 of Mre11 by PP5 is

two-fold. First of all, PP5 may act against RSK's ability to suppress DNA damage induced G2/M checkpoints, as overexpression of PP5 can override both overexpression of RSK and the effects of PMA treatment in controlling Mre11. . This mechanism may also partially explain why loss of PP5 leads to a hypoactive DSB response, especially in cells overexpressing RSK, such as PC3 cells. We also speculate that RSK may prevent accidental phosphorylation of Mre11 in normal cells, which can be triggered by either basal activity of RSK kinases (or other kinases that can target Ser676), or stimuli from growth factors, thus enabling cells to respond to potential stresses that may threaten genomic stability.

According to previous studies, after activation by MRN complexes, ATM can phosphorylate Mre11 on multiple sites including Ser676, inhibit binding affinity between MRN and DNA, and cause the dissociation of MRN complexes (as discussed in introduction of chapter 3). Such phosphorylation gradually varies over time and may contribute to a negative feedback loop that might be required for the completion of DNA damage repair and checkpoint recovery. Our results suggest that PP5 can counteract ATM's inhibitory phosphorylation. Indeed, Mre11 foci disappear much more quickly in cells depleted of PP5.

3.3.5 A possible co-factor that activates PP5 phosphatase activity towards Mre11: Hsp90

As mentioned in chapter 1, PP5 has very low basal activity due to the N-terminal auto-inhibitory domain that blocks its access to its substrate, i.e., Mre11. This suggests that some other co-factors may play a role in promoting PP5 phosphatase activity towards Mre11.

We have identified Hsp90 as a possible co-factor for PP5. Hsp90 binds to PP5 and has been found in several complexes with both PP5 and its targets. In addition, one proteomic study identified Hsp90 as a potential binding partner of Mre11 (Sharma, Vabulas et al. 2012). Both observations suggests that Hsp90, PP5 and Mre11 may interact with each other. Indeed, it has been reported that Hsp90 is required for both the DNA damage-induced checkpoint and the repair pathway; inhibition of Hsp90 with drugs such DMAG, 17-AAG, and NVP-AUY922 sensitizes cells against radiation-induced DNA damage (Dote, Burgan et al. 2006) (Stecklein, Kumaraswamy et al. 2012, Zaidi, McLaughlin et al. 2012). Hsp90 can interact with ATR, ATM, DNA PK, BRCA1, is phosphorylated and recruited to DNA damage foci upon DSB, and is required for ATM, ATR, and DNA PK mediated DNA damage response (Quanz, Herbette et al. 2012). As a result, an Hsp90 inhibitor (NVP-AUY922) has been introduced in clinical trials in concert with radiation therapy to treat cancer.

In our preliminary experiments, we have found that inhibition of Hsp90 with 17-AAG greatly reduced ATM autophosphorylation, consistent with previous findings. In addition, knockdown of Hsp90 with siRNA reduced the formation of Mre/MRN

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complex foci upon DSB, suggesting Hsp90 may regulate the recruitment of MRN

complexes onto dsDNA, which is upstream of ATM. Indeed, immunoprecipitation

shows that Hsp90 interacts with both Mre11 and PP5. Although our findings are

preliminary, they collectively suggest that in quiescent cells, Hsp90 and PP5 may act in

concert to dephosphorylate Mre11, protecting it from inhibition by RSK or other kinases,

which are occasionally activated due to growth stimuli or normal cell function.

However, the mechanism and consequence of Hsp90's dissociation from PP5/Mre11

upon DSB is largely unknown. We speculate that such dissociation may contribute to the

negative feedback loop of ATM's inhibitory phosphorylation on Mre11, as PP5 may lose

its ability to antagonize such phosphorylation.

A

B

C

71
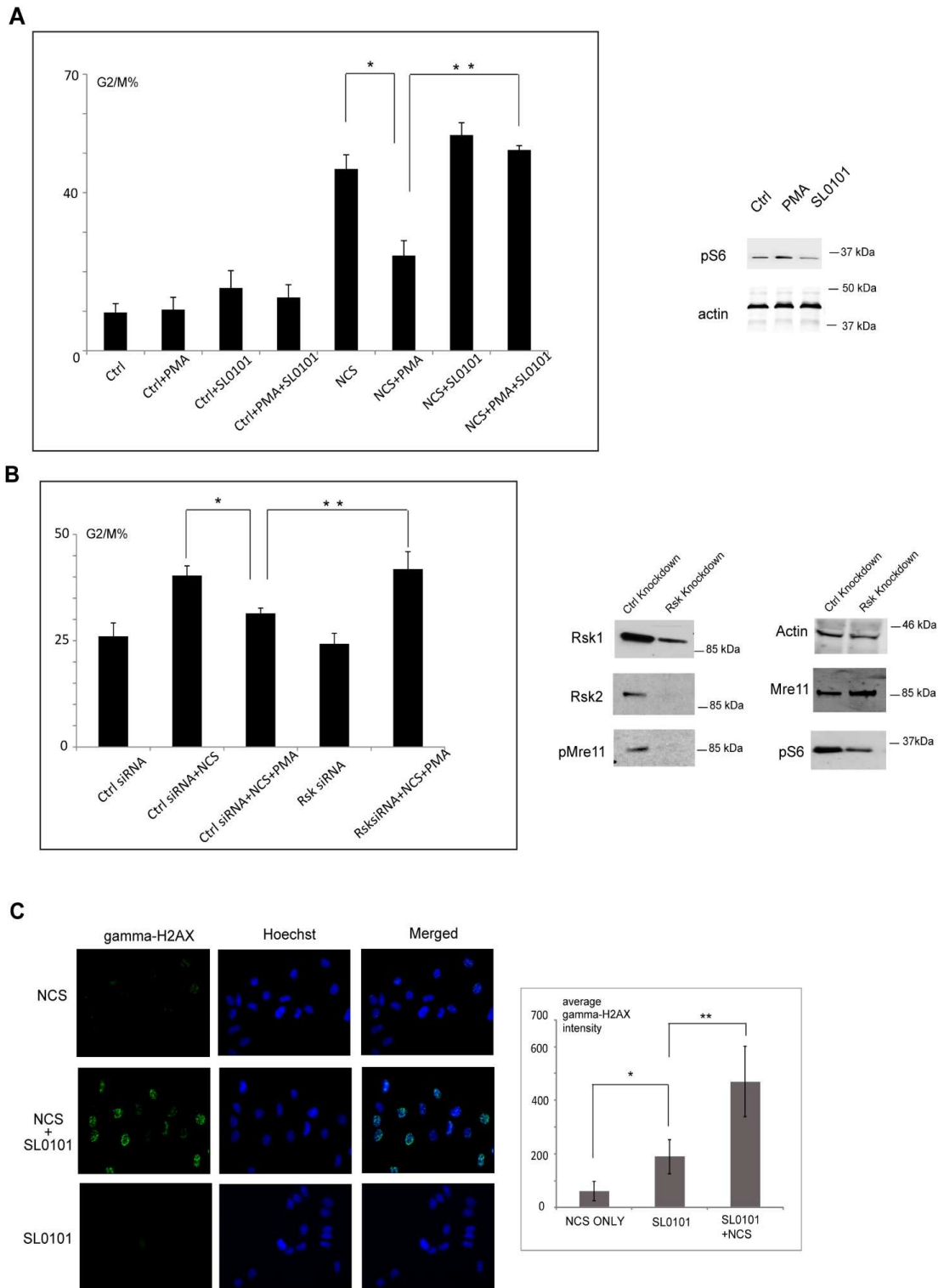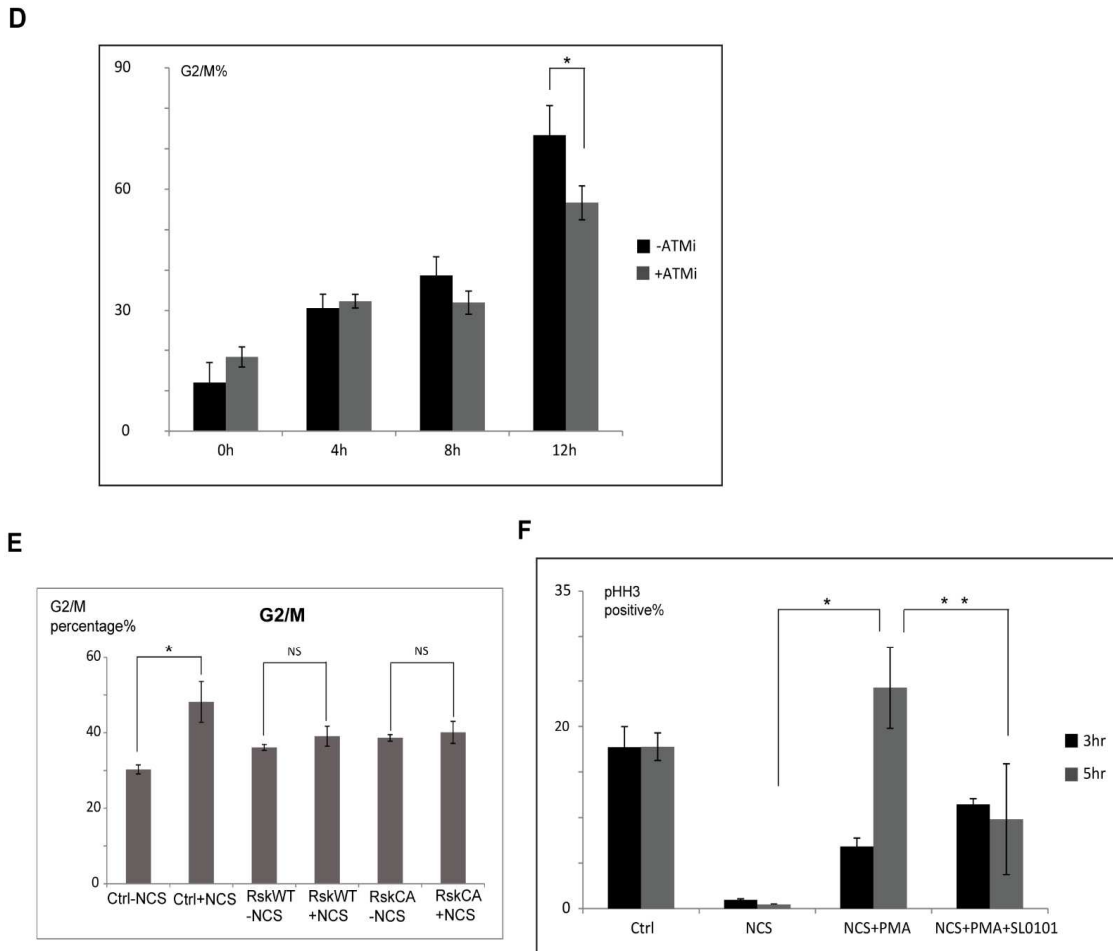
Figure 3.1: Rsk suppresses the DSB response in prostate cancer.

Figure 3.1: (A) PC3 cells were synchronized in G1/S phase by double thymidine block. 4 hrs after release, cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min. 24hrs later cells were collected and DNA profiles were analyzed by flow cytometry. Data from n=3 experiments were combined. Effectiveness of PMA and SL0101 in modulating Rsk activity was shown by immunoblotting for the Rsk substrate, S6. * P<0.01. * * P<0.01. Error bar stands for SD (standard deviation). (**B**) 293T cells were transfected with RSK1/2 siRNA. 48hrs later, cells were treated with PMA for 30 min and then with NCS for another 30 min. Cells were then collected after 16hrs and DNA profiles were analyzed by flow cytometry. The effectiveness of knockdown was analyzed by western blotting. Data from n=3 experiments were combined. * P<0.01.

P<0.01. Error bar stands for SD (standard deviation). (C**) PC3 cells were grown on cover slips. After reaching about 50% confluence, cells were treated with SL0101 or DMSO for 30 min, and then with NCS for another 30 min. Cells were then fixed with 4% formaldehyde and stained with rabbit anti-γH2AX antibody. Cells were examined by fluorescent microscopy for γH2ax staining. Images were taken under 20x objective. The average intensity of γH2ax staining of N>50 cells from 7 random fields were quantified with MetaMorph. Error bars in charts stand for standard deviation unless specified. * P0.01. * * P<0.01. Error bar stands for SD. (**D**) HeLa cells were treated with ATM inhibitor for 30 min and then with NCS for another 30 min. Cells were then collected at the indicated time points and DNA profiles were analyzed by flow cytometry. Data from n=3 experiments were combined. * P<0.01. Error bar stands for SD. (**E**) 293T cells were transfected with FLAG-tagged wildtype or constitutively active RSK2. 20 hrs after treating with NCS, cells were stained with both PI and FITC-conjugated mouse anti-FLAG antibody. FLAG positive cells were gated and their DNA profiles were analyzed to quantify cells in G2/M. Data from n=3 experiments were combined and Student's t-test was applied to assess statistical significance. * P<0.05. Error bar stands for SD. (**F**) HeLa cells were synchronized in G1/S phase by double thymidine block. 4 hrs after release, cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min. 3 or 5 hrs later cells were collected and stained with anti-phospho-Histone H3 antibody. pHH3 positive cells were analyzed by flow cytometry. Data from n=3 experiments were combined. * P<0.001. * * P<0.01. Error bar stands for SD.

A

| | − | − | + | + | Rsk2 |
| | − | + | − | + | NCS |

pATM 1981 — 250 kDa

ATM — 250 kDa

FLAG-Rsk2 — 100 kDa

B

-NCS   +NCS

Control PMA PMA+SL0101 Control PMA PMA+SL0101

ATM 1981P — 250 kDa

pRsk — 85 kDa

Actin — 46 kDa

ATM — 250 kDa

C

	−	+	−	+	+	NCS
	−	−	+	+	+	PMA
	−	−	−	−	+	SL0101

pATM 1981 — 250kDa

pNBS1 343 — 100 kDa

NBS1 — 100 kDa

pS6 — 37kDa

D

Ctr SiRNA   Rsk1/2 siRNA

NCS   − +   − +

ATM 1981P — 250 kDa

ATM — 250 kDa

Actin — 46 kDa

Ctr siRNA Rsk1/2 siRNA

Rsk1 — 85 kDa

Rsk2 — 85 kDa

pS6 — 30 kDa

E

gamma-H2AX   Hoechst   Merged

NCS only

PMA + NCS

PMA +SL0101 +NCS

average gamma-H2AX intensity

* **

Control   PMA   PMA+SL0101

74

F

	gamma-H2AX	Flag-Rsk2	Merged
no NCS			
NCS only			
NCS+SL0101			

gamma-H2AX intensity
relative to non-transfected

G

	No NCS			NCS 30'			NCS 24h			
	−	+	+	−	+	+	−	+	+	PMA
	−	−	+	−	−	+	−	−	+	SL0101
pATM 1981										— 250 kDa
Actin										— 50kDa
ATM										— 250 kDa

Figure 3.2: RSK suppresses the DSB response by inhibiting the activation of ATM.

Figure 3.2: (A) HeLa cells transfected with FLAG-tagged Rsk2 were treated with NCS for 30 min before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981. (**B**) 293T cells were treated with PMA and SL0101 or only PMA for 30 min, and then with NCS for another 30 min before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981. (**C**) HeLa cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min before harvesting. Cell lysates were immunoblotted for phospho-NBS1 Ser 343 as well as phospho-ATM. (**D**) siRNA against RSK1 and RSK2 were used to treat PC3 cells for two days. Cells lysates were immunoblotted for phospho-ATM 1981. RSK1/2 knockdown are cells knocked-down for RSK using siRNA. Control knockdown are cells treated with control siRNA. (**E**) HeLa cells were grown on cover slips and were treated with PMA and SL0101 or only PMA for 30 min, and then with NCS for another 30 min before fixation. Cells were stained with DAPI and rabbit

anti-γH2AX. The pattern of γH2AX staining was examined by fluorescence microscopy. Images were taken under 20x objective and the representative images were shown. The average intensity of γH2ax staining of N>50 cells from 10 random fields were quantified with MetaMorph. * P<0.01. * * P<0.01. Error bar stands for SD. (**F**) HeLa cells were transfected with FLAG-tagged constitutively active RSK2 and co-stained with mouse anti-FLAG and rabbit anti-γH2AX antibody. The pattern of γH2ax staining in FLAG positive and negative cells was examined by fluorescence microscopy. Images were taken under 40x objective lens and the representative images were shown. The average intensity of γH2ax staining of N>40 FLAG-Rsk positive and N>300 FLAG-Rsk negative cells from at least 18 random fields were quantified with MetaMorph. Fold change of intensity of γH2ax staining in FLAG-Rsk positive relative to negative cells were calculated. * P<0.01. Error bars stand for SEM. (**G**) HeLa cells were treated with PMA or SL0101 for 30 min and then with NCS for another 30 min. Cells were then harvested immediately or cultured in regular media for another 24 hrs before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981.

A

(dAdT)$_{70}$

SL0101   MBP   Mos   Mos +SL0101

0'10'20'30' 0'10'20'30' 0'10'20'30' 0'10'20'30'

phospho-ATM   —250 kDa

ATM   —250 kDa

B

-Rsk   +Rsk

0' 10' 20' 30' 0' 10' 20' 30'

phospho-ATM   —250 kDa

ATM   —250 kDa

C

Empty Beads   No Caffeine   +Caffeine

−   −   +   −   +   Mos

ATM   — 250 kDa

KU70   — 75 kDa

D

Empty Beads   No Caffeine   +Caffeine

−   +   +   −   +   Mos
−   −   +   −   −   SL0101

Mre11   — 75 kDa

KU70   — 75 kDa

E

KU55933

Empty beads MBP Mos MBP Mos Free DNA Input

Mre11   — 85 kDa

Loading: Mre11   — 85 kDa

F

MRE11 foci positive%

*

G

IP: IgG   Nbs1

−   −   +   Mos

Mre11   —75 kDa

NBS1   —100 kDa

H

− − + + H_2O_2
− + − +   Rsk2

pATM 1981   —250kDa

ATM   —250kDa

FLAG-Rsk2   —100 kDa
   —75 kDa

Actin   —50kDa

Figure 3.3: The Mos/MAPK/RSK inhibits binding of the MRN complex to dsDNA.

Figure 3.3: (**A**) Xenopus crude interphase extracts (S extracts) were treated with control MBP (Maltose binding protein) or MBP-Mos and then SL0101 or DMSO for 15min. After adding double stranded poly(dA-dT)70, extract samples were then taken at the indicated time points. ATM autophosphorylation was then analyzed by immunoblotting. (**B**) Xenopus crude interphase extracts were incubated with active RSK for 15 min before adding dsDNA. Samples were taken at the indicated timepoints and ATM autophosphorylation was then analyzed by immunoblotting. (**C**) Biotin tagged-dsDNA was bound to avidin beads and incubated with crude interphase extracts that

had been pre-treated with MBP-Mos or MBP alone, and then KU55933 or DMSO. The DNA-bound beads (along with any bound proteins) were then collected and washed. The amount of ATM protein bound to beads was analyzed by immunoblotting. DNA bound Ku70 was immunoblotted as a loading control. (**D**) Biotin tagged dsDNA was bound to avidin beads and added to Xenopus crude S extracts pre-treated with MBP-Mos or MBP and then caffeine or SL0101 and incubated for 20 min. In one of the reactions, free dsDNA was added to compete with the DNA on the beads. The amount of Mre11 protein bound to beads was analyzed by immunoblotting. DNA bound Ku70 was immunoblotted as a loading control. (**E**) Biotin tagged dsDNA was bound to avidin beads and added to Xenopus crude S extracts pre-treated with MBP-Mos or MBP and then KU55933, and incubated for 20 min. In one of the reactions, free dsDNA was added to compete with the DNA on the beads. The amount of Mre11 protein bound to beads was analyzed by immunoblotting. (**F**) HeLa cells were grown on cover slips and were treated with PMA for 30 min, and then with NCS for another 10 min before fixation. Cells were stained with DAPI and rabbit anti-Mre11. The pattern of Mre11 foci staining was examined by fluorescence microscopy. Images were taken under 40x objective. Cells with more than five clear Mre11 foci were counted as Mre11 foci positive. Results from n=3 experiments were combined. N>50 cells from at least 10 random fields was counted from each experiments. * $P<0.001$. Error bar stands for SD. (**G**) Nbs1 protein was immunoprecipitated from Xenopus extract pre-treated with Mos or MBP. Mre11 protein bound to Nbs1 was immunoblotted. (**H**) HeLa cells transfected with FLAG-tagged Rsk2 were treated with 0.9 mM H_2O_2 for 30 min before harvesting. Cell lysates were immunoblotted for phospho-ATM 1981.
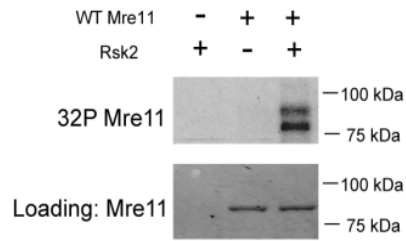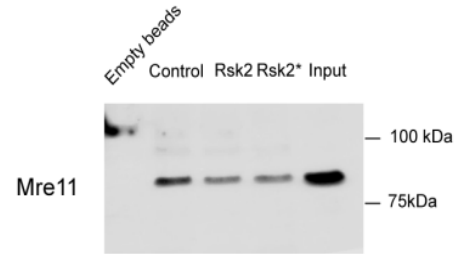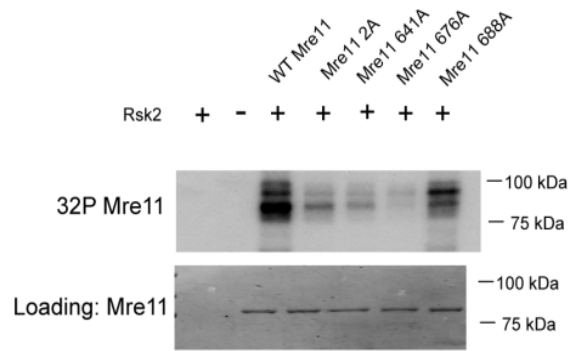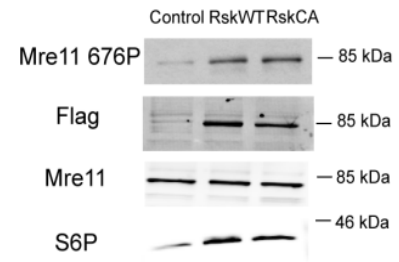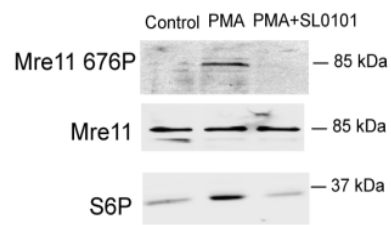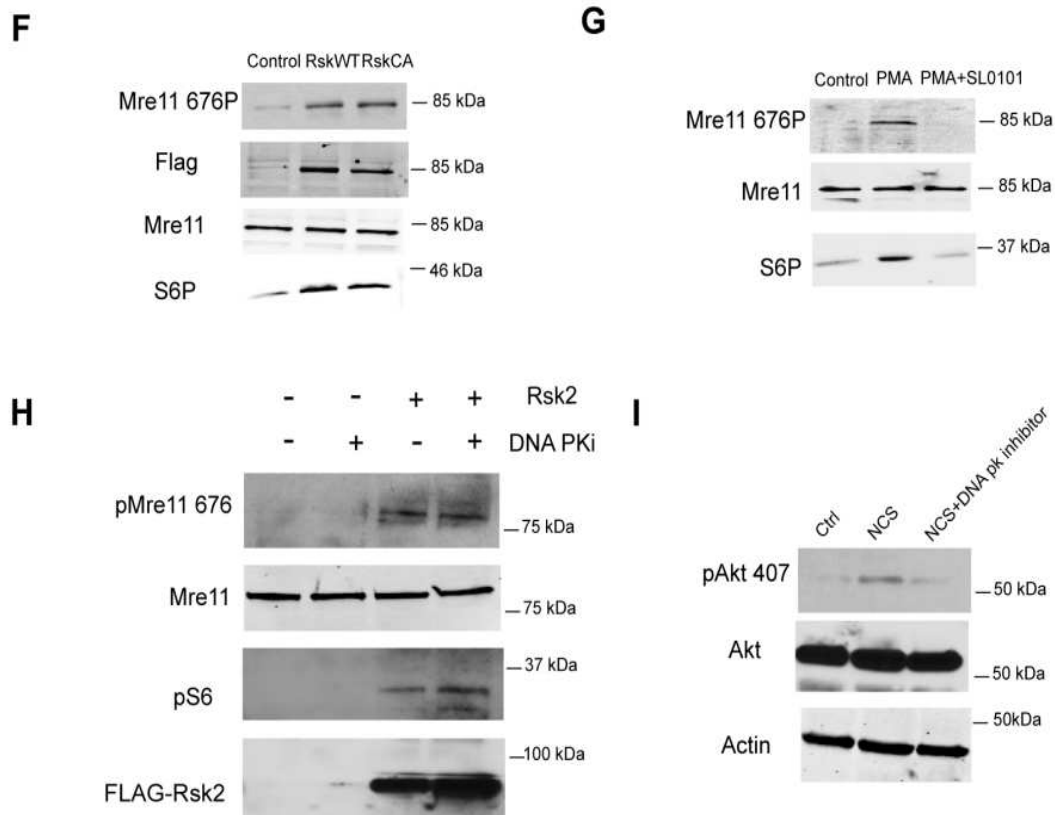
Figure 3.4: RSK phosphorylates Mre11 at Ser676.

Figure 3.4: (**A**) Biotin tagged-dsDNA was bound to avidin beads and incubated with Xenopus crude interphase egg extract for 20 min. After washing with egg lysis buffer (ELB buffer) beads were spilt and treated with RSK2 kinase and γ-32P-ATP or only γ-32P-ATP as a control. Proteins were resolved in SDS sample buffer and analyzed by autoradiography. (**B**) Purified Mre11 protein was treated with RSK kinase and radiolabeled ATP (in kinase buffer) and labeled proteins were subjected to SDS-PAGE and autoradiography. (**C**) His-tagged Mre11 protein was phosphorylated in vitro using RSK kinase and added into Xenopus egg extracts. After incubation for 10 min, biotin tagged dsDNA was added for an additional 15 min. In one sample (RSK*), SL0101 and phosphatase inhibitors were added to extracts to prevent further phosphorylation or dephosphorylation. Beads were then collected by centrifugation and washed with ELB buffer 4 times. Proteins bound to DNA were analyzed by anti-His immunoblotting. (**D**) Equal amounts of His-tagged Mre11 protein and its mutants were treated as in B. (**E**)

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293T cells were transfected with wild type or constitutively active RSK (RSK WT and RSK CA, respectively) and cells were collected 2 days after transfection to make lysates. Equal amounts of wild type His-tagged Mre11 protein and the 676A mutant proteins were mixed with lysates, kinase buffer and γ-32P-ATP at 370C for 45 min. Radio labeled Mre11 protein was analyzed by autoradiography. (**F**) 293T cells were transfected with RSKWT or RSKCA constructs and cells were collected 2 days after transfection to make lysates. Equal amounts of lysates were subjected to SDS-PAGE and endogenous Ser676 phosphorylated Mre11 was detected by immunoblotting with phospho-specific antibody. (**G**) 293T cells were treated with PMA or PMA plus SL0101 for 30min before harvesting to make lysates. Endogenous Ser676 phosphorylated Mre11 was detected by immunoblotting with phospho-specific antibodies. (**H**) HeLa transfected with active Rsk2 kinase were treated with DNA PKc inhibitor for 30 min before harvesting. Lysates were blotted for anti-Mre11 Ser676 antibody. (**I**) HeLa cells were treated with DNA PKc inhibitor for 30 min and then NCS for another 30min before harvesting. Lysates were immunoblotted with anti-Akt Ser407 antibody in order to see whether phosphorylation of Akt by DNA PKc was inhibited.

A

Nuclease Domain   DBD-1   DBD-2

2   641   676   688

B

Empty
Beads   Mre11 WT   Mre11 676A
−   −   +   −   +   Mos

Mre11   —75 kDa

KU70   —75 kDa

Input: Mre11   —75 kDa

C

Empty
beads   WT Mre11   Mre11 676A
Rsk   +   −   +   −   +

IP: DNA
WB: Mre11   — 85 kDa

D

WTMre11
MBP   Mos
Min   0   5   10   20   30   0   5   10   20   30

P-ATM   —250 kDa

ATM   —250 kDa

Mre11 676A
MBP   Mos
Min   0   5   10   20   30   0   5   10   20   30

P-ATM   —250 kDa

—250 kDa

83
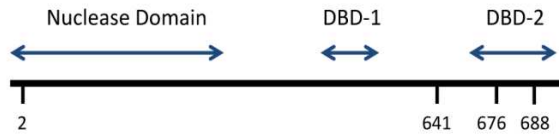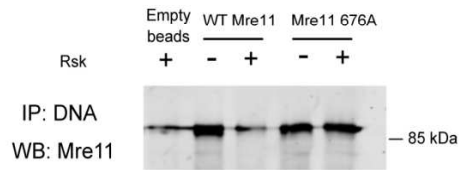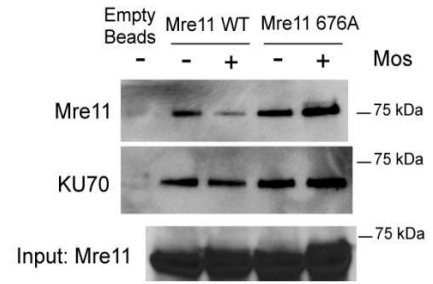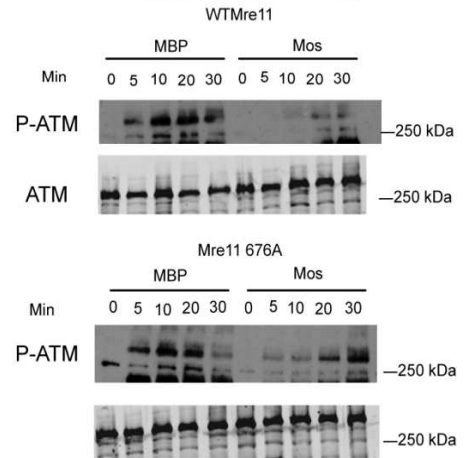
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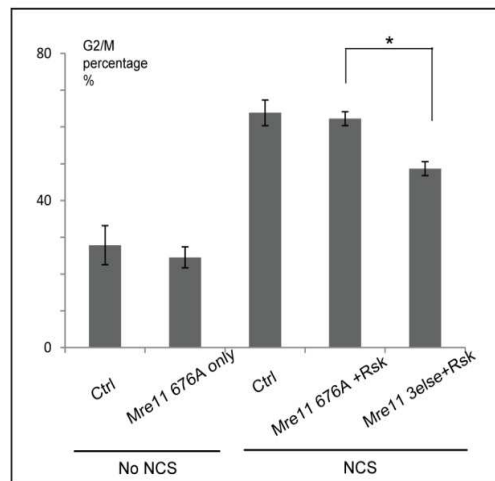
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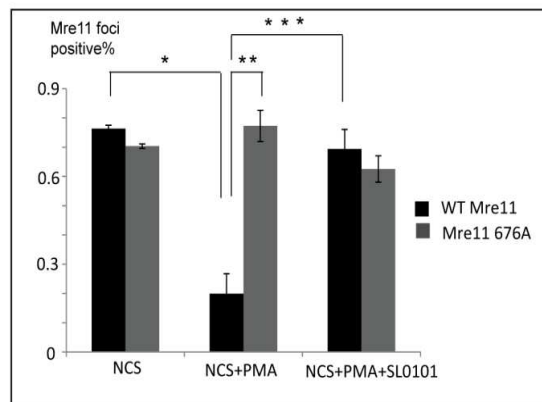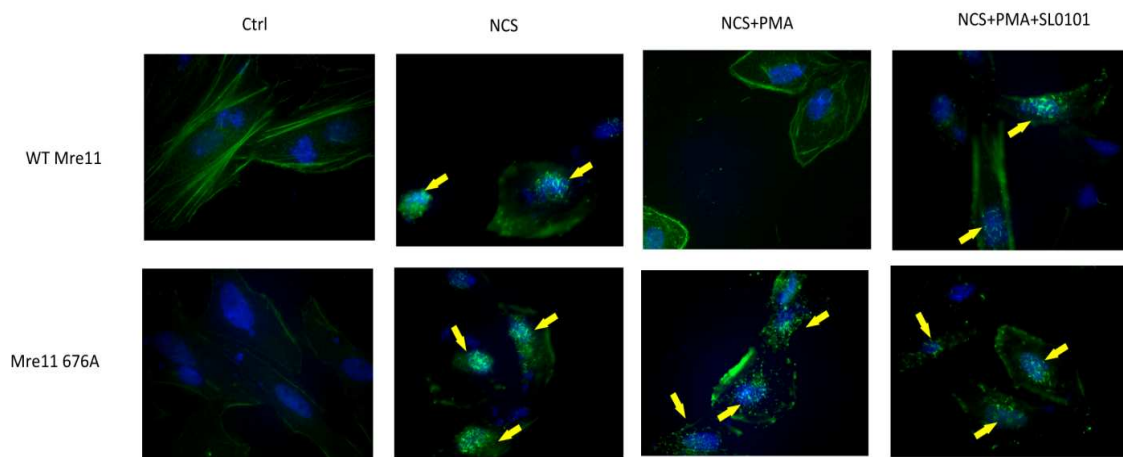
Figure 3.5: Phosphorylation of Mre11 on Ser676 reduces its binding to DNA and reverts checkpoint inhibition.

Figure 3.5: (**A**) Mre11 has two DNA binding domains within its C-terminal half. Both Ser676 and Ser688 are located within the 2nd binding domain. (**B**) Biotin tagged dsDNA was bound to avidin beads and added to Xenopus crude S extracts that were pretreated with MBP-Mos or MBP and supplemented with WT or mutant Mre11 proteins. The amount of Mre11 protein bound to the beads was analyzed by immunoblotting. (**C**) Samples were processed as in B, in the absence of Mos protein, and Mre11 proteins were instead pre-treated with RSK kinase (**D**) Mre11 wildtype or Ser676ALA proteins were added into Xenopus crude S extracts. Extracts were then treated with MBP-Mos or MBP before addition of NCS. Aliquots of extract withdrawn at the indicated time points after NCS addition were immunoblotted for phosphorylated ATM. (**E**) 293T cells were transfected with FLAG-tagged constitutively active RSK2 kinase and either HA tagged Mre11 676A mutant or Mre11 mutated to Ala at Ser/Thr 2, 641, and 688. 20 hrs after treating with NCS, cells were collected and stained with PI, rabbit anti-HA and mouse anti-FLAG antibody. DNA profiles of total population were measured first in order to show NCS' effectiveness. Then HA and FLAG double positive cells were gated and their DNA profiles were analyzed. * $P<0.01$. Error bar stands for SD. (**F**) HeLa cells infected with Mre11 shRNA were grown on cover slips and transfected with shRNA-resistant WT Mre11 or 676A mutant. 48hrs after transfection, cells were treated with PMA and SL0101 for 30 min, and then with NCS for another 10 min before fixation. Cells were stained with DAPI and rabbit anti-Mre11 antibody. The pattern of Mre11 focus staining was examined by fluorescence microscopy. Images were taken under 40x objective and the representative images are shown. Cells with more than five clear Mre11 foci were counted as Mre11 foci positive. Results from n=3 experiments were combined. * $P<0.001$. ** $P<0.001$. *** $P<0.001$. Error bar stands for SD.

A

Mre11 pSer 676 — 85 kDa

DMSO Low OA High OA CalA

Mre11 — 85 kDa

B

Ctrl siRNA   PP5 siRNA

Mre11 pSer 676 — 85 kDa

Mre11 — 85 kDa
          — 58 kDa
PP5

Actin — 46 kDa

C

RSK RSK+PP5 RSK+PP2A

P32 Mre11 — 85 kDa

Mre11 — 85 kDa

D

IP: FLAG   0'   30'   60'   90'

Mre11 — 85 kDa

FLAG-PP5 — 50 kDa

E

Ctrl siRNA No NCS   Ctrl siRNA   PP5 siRNA

Mre11 foci positive %

86
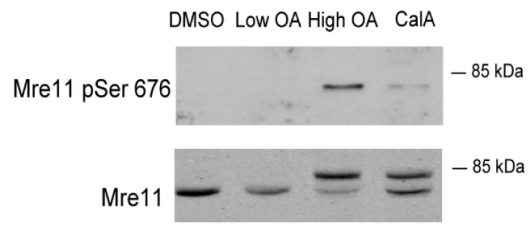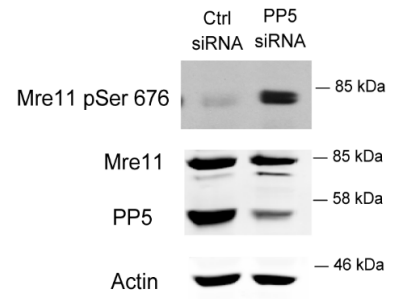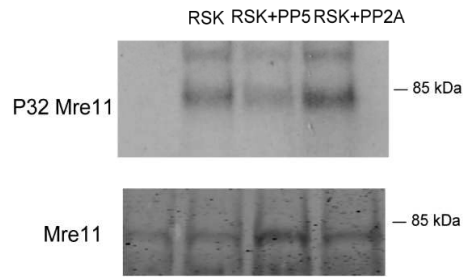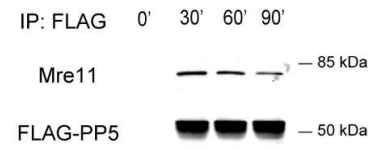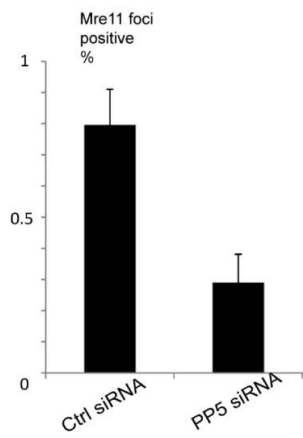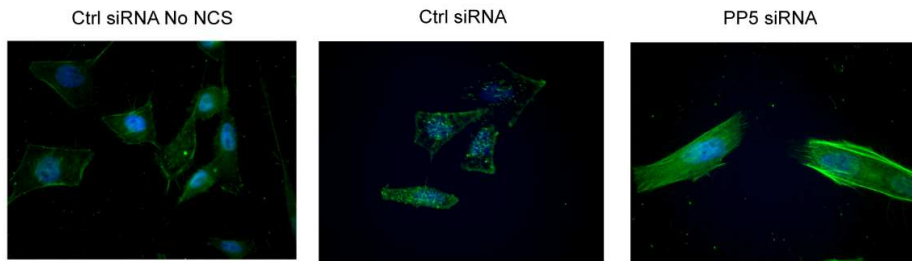
Figure 3.6: PP5 dephosphorylates Mre11 pSer676 both in vivo and in vitro.

Figure 3.6: (**A**) 293T cells were treated with Calyculin A or OA of different concentration (2nM or 10nM) for 30min before harvesting to make lysates. Endogenous Ser676 phosphorylated Mre11 was detected by immunoblotting with phospho-specific antibodies. (**B**) PC3 cells were transfected with siRNA against PP5. Cells were collected to make lysates two days after transfection to make lysates. Endogenous Ser676 phosphorylated Mre11 was detected by immunoblotting with phospho-specific antibodies. (**C**) Purified Mre11 protein was treated with RSK kinase and radiolabeled ATP (in kinase buffer) and labeled proteins were then incubated with purified active PP5 or PP2A in phosphatase buffer for 2hrs. Proteins were resolved in SDS sample buffer and analyzed by autoradiography. (**D**) 293T cells were transfected with FLAG-tagged PP5. Cells were then treated with NCS for different time length and collected to make lysates. FLAG-PP5 was immunoprecipitated from lysates. Mre11 protein bound to PP5 was immunoblotted. (**E**) HeLa cells infected with PP5 siRNA were grown on cover slips. 48hrs after transfection, cells were treated with NCS for 10 min before fixation. Cells were stained with DAPI and rabbit anti-Mre11 antibody. The pattern of Mre11 focus staining was examined by fluorescence microscopy. Images were taken under 40x objective and the representative images are shown. Cells with more than five clear Mre11 foci were counted as Mre11 foci positive. Results from n=3 experiments were combined. Error bar stands for SD.

A

B
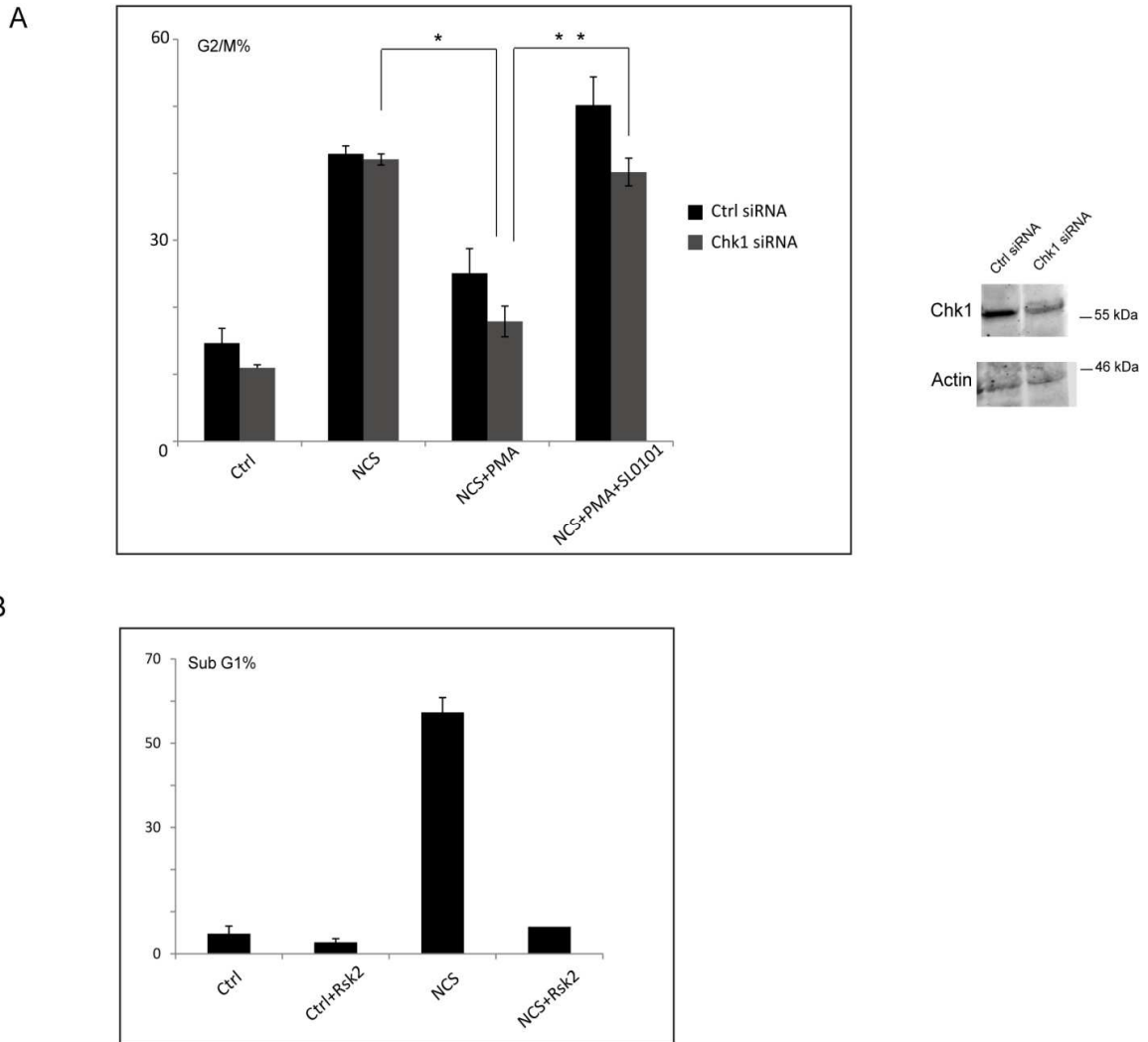
Fig 3.7: RSK inhibits G2/M checkpoints induced by NCS in the absence of Chk1.

Fig 3.7: (**A**) 293T cells were treated with siRNA to knockdown endogenous Chk1. 48hrs later, cells were treated with PMA and/or SL0101 for 30 min and then with NCS for another 30 min. Cells were then grown in DMEM media for 16hrs before harvesting and DNA profiles were analyzed by flow cytometry. Data from n=3 experiments were

combined. * P<0.01. Error bar stands for SD. Note that the knockdown of Chk1 had minimal effect on the response to NCS, possibly due to the fact that ATM/Chk2 pathway, the primary pathway for DSB response, remained intact. (**B**) HeLa cells were transfected with constitutively active Rsk2 kinase. After 36hrs, cells were treated with 200ng/ml NCS for 1 hr. Cells were then grown in DMEM media for another 48hrs before harvesting. DNA profiles were analyzed by flow cytometry and sub G1 population was analyzed. Data from n=3 experiments were combined. * P<0.01. Error bar stands for SD.

Chapter 4: Conclusion and perspectives

Activation of the DNA damage response pathway is critical for cells to maintain genomic stability when stressed by internal or external genotoxic agents. Upon DSB, one of the most toxic forms of DNA damage, cells initiate a series of signaling cascades that center around the formation of MRN complexes and activation of ATM, which halts cell cycle progression and repairs the damaged DNA. MRN complexes are crucial for recognizing and binding to the sites of DNA lesion and initiating the formation of foci to stabilize dsDNA ends. The activation of ATM, the central kinase and signal transducer of DSB response signaling pathway, requires its binding to MRN complexes in the foci, a key step in the initiation of the DSB-induced DNA damage response. As previously discussed, the DSB response is regulated on multiple levels. In this dissertation study, I used two model systems (Xenopus and mammalian cell lines) to show that RSK inhibits DSB-induced ATM activation by targeting binding of the MRN complexes to DSB DNA. Direct phosphorylation the Mre11 protein at Ser676 by RSK inhibits loading of Mre11 onto DSB DNA, while at the same time mutation of Ser676 to Ala can reverse RSK-induced inhibition of the DSB response. Collectively, these data point to Mre11 as an important locus of RSK-mediated checkpoint inhibition acting upstream of ATM activation.

I also expanded my effort to search for the phosphatase that antagonizes Mre11 Ser676 phosphorylation. After screening with both phosphatase inhibitors and siRNA, I

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identified PP5 as a potential candidate. Indeed, PP5 can reverse Mre11 Ser676

phosphorylation by RSK both in intact cells and in vitro. PP5 also interacts with Mre11

in a timely manner after introduction of DSB. Together these findings indicate that PP5

may promote DSB-induced ATM-dependent DNA damage response by targeting Mre11

pSer676.

4.1 RSK suppresses the DSB-induced DNA damage response by targeting MRE Ser676

The activation of ATM is one of the key steps in DSB-induced DNA damage

checkpoints, leading to activation/inhibition of many downstream kinases or

transcription factors, and initiating multiple signaling cascades that profoundly change

cell reproduction, growth, protein synthesis, metabolism, DNA replication, mobility and

apoptosis. Not surprisingly, ATM is one of the focal points of DNA damage-induced

checkpoint regulation, targeted by many kinases, phosphatases, and acetyl-transferases.

As discussed in section 3.1, the Raf/MEK/ERK pathway has long been shown to

suppress G2/M checkpoint induction following irradiation (Lee, Soh et al. 2002).

However, the detailed mechanism how such suppression was accomplished remained

largely unknown. The studies that I have done throughout the course of my graduate

research plan, and as described in this dissertation, may provide the missing link—

identifying RSK as the kinase acting downstream of the MAPK signaling cascade to inhibit ATM activation.

In most situations, ATM is activated by binding to MRN complexes at the sites of DNA lesions. In our study, RSK can suppress ATM activation in both Xenopus egg extracts and mammalian cells, by targeting the MRN complexes upstream of ATM. Indeed, overexpression of RSK or treatment of cells with PMA reduced the formation of MRE/MRN foci. Accordingly, treatment of Xenopus egg extracts with Mos or RSK2 greatly suppressed the affinity between endogenous MRN complexes and dsDNA. Subsequent studies revealed that Mre11 functions as a substrate for RSK, which was further confirmed by in vitro kinase assay, mass-spec, and immuno-blot with antibodies that specifically targeted the Ser676 sites. In fact, mass-spec identified four potential targeting sites on Mre11 but only the mutation of Ser676 completely abolished RSK' phosphorylation, suggesting that Ser676 serves as the primary site for Rsks' inhibition of the DSB response. Indeed, replacing endogenous WT Mre11 with a Ser676 Ala mutant mostly reversed the inhibitory effects of RSK on the DSB-induced DNA damage response, in both Xenopus egg extracts and mammalian cells. However, replacing mutants with exogenous WT Mre11 or Mre11 with mutations at the three other sites did not affect RSK inhibitory activity. Collectively these data support our hypothesis that the ability of RSK to phosphorylate Mre11 on Ser676 confers its function in suppressing the DSB-induced DNA damage response. However, we did not rule out the possibility that

the other components might also be targeted by RSK, mainly Rad50 and Nbs1, nor did

we rule out the possibility that ATM itself may be targeted, either directly or indirectly,

interrupting its binding to MRN complexes. Another possible scenario is that the

formation of MRN complexes was disrupted thanks to RSK activation. This possibility

was ruled out by studying the interaction between Mre11 and other MRN complex

components such as Nbs1 in egg extracts, in which we did not observe any effect of RSK

on the physical interaction between these two proteins (Fig. 3.3G).

However, it should also be noted that ATM can be activated even in the absence

of MRN complexes under certain conditions, such as ROS, and salts (e.g. NaCl) (Guo,

Deshpande et al. 2010). In the case of ROS activated DNA damage response, RSK had no

effect on ATM activation, further lending support to our model that RSK mainly acts on

MRN complexes to suppress DSB-induced DNA damage response (Fig. 3.3H).

RSK also played a role in the SSB- or stalled replication forks-activated

ATR/Chk1 pathways in cells treated with UV lights or chemicals. Two recent papers

both identified Chk1 Ser 280 as a potential target of RSK, the same site that is targeted by

Akt kinase to inhibit Chk1 activity (Ray-David, Romeo et al. 2013). However, the effect

of such phosphorylation was confounded by the fact that their reports contradicted each

other in whether the phosphorylation was inhibitory (Li, Goto et al. 2012). Thus the role

of RSK in the SSB response remains unclear. As mentioned in chapter 1, the ATR/Chk1

pathway lies downstream of ATM following DSBs. To test the possibility that

phosphorylation of Chk1 on Ser 280 might also contribute to G2/M checkpoint silencing upon DSBs, we tested whether RSK can suppress G2/M checkpo0int function following DSB in cells knocked down for Chk1. Our results suggests that phosphorylation of Mre11 by RSK contributes to DSB-induced G2 checkpoint silencing independently of Chk1 (Fig. 3.7A).

In our study we focused primarily on RSK silencing of G2/M checkpoints, because G1/S and intra-S checkpoints are not intact in the cell lines we used. In fact, both G1/S and intra-S checkpoints are compromised in many types of tumors, rendering G2/M as the last resort for keeping genomic fidelity. It should be noted here though that we did not rule out the possibility that G1/S and intra-S checkpoints may also be affected by RSK.

Due to the fact that RSK shares similar consensus targeting sequence with other kinases, RSK may not be the only kinase that can target Mre11 Ser676. In fact, it has already been mentioned that ATM can target the same site, which may be important for checkpoint recovery. It is also entirely possible that some different kinases may utilize the same mechanism to silence the DSB-induced G2/M checkpoint.

4.2 Silencing G2/M checkpoints: the double-edged sword

As mentioned above, the DNA damage response has a complicated effect on cells, as cells usually have to make the choice between arresting the cell cycle to repair

their DNA or initiating programmed cell death. The former choice allows cells to correct

errors in the genome and is usually pro-survival, while the latter option kills cells and

usually happens when the genome is too damaged to be repaired. As a result, the effect

of silencing the checkpoints involved in DSB responses can be either beneficial or

harmful to cells, depending on whether the amount of un-repaired damaged

chromosomes exceeds maximum toleration and whether cells maintain mechanisms to

shut down checkpoint-activated apoptosis. Consequently, the therapeutic benefit of

inhibiting or activating the DSB response is likely to be varied depending on tumor

types.

4.3 Targeting RSK in Cancer therapy

As mentioned in chapters1 and 3, RSK is overexpressed in 50% of breast cancers

and prostate tumors (Clark, Errington et al. 2005, Smith, Poteet-Smith et al. 2005).

Although not an oncogene, the role of RSK in tumorigenesis has been intensively

studied due to its broad range of function in tumor cell proliferation, apoptosis, and

metastasis. It has been reported that RSK promotes the proliferation and metastasis of

tumor cells, and inhibits apoptosis by phosphorylating the apoptotic inducer Apaf-1,

thereby reducing its apoptotic activity (Kim, Parrish et al. 2012). Combined with the fact

that inhibition of RSK may radiosensitize cancer cells (depending on cell types, as

discussed in 3.1.2), we think RSK may have great potential as a therapeutic target for

cancer treatment. However, the development of RSK specific inhibitors is still in an early

stage, and has yet been introduced into clinical trials.

4.4 PP5 as an antagonizer of RSK on Mre11 Ser676 phosphorylation

It is well-established that PP5 is required for both ATM and ATR-mediated DNA

damage responses. However, why PP5 is needed remains unknow

n. It is unlikely that ATM or ATR is the substrate of PP5 because none of the

phosphorylation on ATM or ATR is inhibitory. Here we propose a possible scenario in

which PP5 targets Mre11 on Ser676. Indeed, overexpression of PP5 overrides the effect

of PMA or RSK on Mre11 Ser676 phosphorylation. In addition, PP5 can directly

dephosphorylate Mre11 in vitro. In cancer cells with intrinsically high RSK activity, PP5

may play an important role in keeping Mre11 in the dephosphorylated, functional state.

Unfortunately, little progress has been made in developing PP5 specific inhibitors,

making this phosphatase a very unlikely therapeutic target in cancer treatment. It

should be pointed out here, however, that no evidence suggests PP5 as the only

phosphatase targeting pSer676. There is also no conclusive evidence implying Mre11 as

the ONLY target of PP5 responsible for activation of the DNA damage response.

In cells with very low basal RSK activity, PP5 is still required to activate the DSB

response. We propose that PP5 may function as a "monitor" of Mre11 pho Ser676

phosphorylation status, which keeps Mre11 from occasional phosphorylation due to: 1)

spike activation of MAPK/RSK in response to extracellular stimuli; 2) other active

kinases targeting the same site; or 3) normal activation of RSK during the cell cycle.

Due to very low basal activity, PP5 needs to form complexes with co-factors as

well as its substrates, in this case, Mre11 to perform its function. While not conclusive,

we found some hints suggesting that Hsp90 may be the critical PP5 co-factor. Hsp90 is

located in both the cytosol and the nucleus, and can be found in DNA damage repair

foci (Quanz M et al, 2012). However, to confirm the existence of Hsp90/PP5/Mre11

complexes, immunostaining must be done in the future to study the localization of these

three protein following DSB.

Both PP5 and Hsp90 interact with Mre11 in a timely manner upon DSB, which

may contribute to the negative feedback loop from ATM to Mre11. The function of such

a feedback loop is not yet clear. One possibility is that MRN complexes need to

dissociate from DSB DNA to make room for DNA repair proteins, allowing completion

of the DSB response. Such dissociation may also contribute to the eventual silencing of

ATM activity, which is required for checkpoint recovery.

References

Abraham, R. T. (2001). "*Cell cycle checkpoint signaling through the ATM and ATR kinases.*" Genes Dev **15**(17): 2177-2196.

Ali, A., J. Zhang, S. Bao, I. Liu, D. Otterness, N. M. Dean, R. T. Abraham and X. F. Wang (2004). "*Requirement of protein phosphatase 5 in DNA-damage-induced ATM activation.*" Genes Dev **18**(3): 249-254.

Anjum, R. and J. Blenis (2008). "*The RSK family of kinases: emerging roles in cellular signalling.*" Nat Rev Mol Cell Biol **9**(10): 747-758.

Axton, J. M., V. Dombradi, P. T. Cohen and D. M. Glover (1990). "*One of the protein phosphatase 1 isoenzymes in Drosophila is essential for mitosis.*" Cell **63**(1): 33-46.

Bailis, J. M. and G. S. Roeder (2000). "*Pachytene exit controlled by reversal of Mek1-dependent phosphorylation.*" Cell **101**(2): 211-221.

Bakkenist, C. J. and M. B. Kastan (2003). "*DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation.*" Nature **421**(6922): 499-506.

Bakkenist, C. J. and M. B. Kastan (2004). "Initiating cellular stress responses." Cell **118**(1): 9-17.

Bartek, J. and J. Lukas (2001). "*Mammalian G1- and S-phase checkpoints in response to DNA damage.*" Curr Opin Cell Biol **13**(6): 738-747.

Bartek, J. and J. Lukas (2001). "*Pathways governing G1/S transition and their response to DNA damage.*" FEBS Lett **490**(3): 117-122.

Bartkova, J., Z. Horejsi, K. Koed, A. Kramer, F. Tort, K. Zieger, P. Guldberg, M. Sehested, J. M. Nesland, C. Lukas, T. Orntoft, J. Lukas and J. Bartek (2005). "*DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis.*" Nature **434**(7035): 864-870.

Bartkova, J., N. Rezaei, M. Liontos, P. Karakaidos, D. Kletsas, N. Issaeva, L. V. Vassiliou, E. Kolettas, K. Niforou, V. C. Zoumpourlis, M. Takaoka, H. Nakagawa, F. Tort, K. Fugger, F. Johansson, M. Sehested, C. L. Andersen, L. Dyrskjot, T. Orntoft, J. Lukas, C. Kittas, T. Helleday, T. D. Halazonetis, J. Bartek and V. G. Gorgoulis (2006). "*Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints.*" Nature **444**(7119): 633-637.

Bianchi, M. and E. Villa-Moruzzi (2001). "*Binding of phosphatase-1 delta to the retinoblastoma protein pRb involves domains that include substrate recognition residues and a pRB binding motif.*" Biochem Biophys Res Commun **280**(1): 1-3.

Bree, R. T., C. Neary, A. Samali and N. F. Lowndes (2004). "*The switch from survival responses to apoptosis after chromosomal breaks.*" DNA Repair (Amst) **3**(8-9): 989-995.

Cargnello, M. and P. P. Roux (2011). "*Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases.*" Microbiol Mol Biol Rev **75**(1): 50-83.

Ceulemans, H. and M. Bollen (2004). "*Functional diversity of protein phosphatase-1, a cellular economizer and reset button.*" Physiol Rev **84**(1): 1-39.

Che, S., W. Wu, M. Nelman-Gonzalez, T. Stukenberg, R. Clark and J. Kuang (1998). "*A phosphatase activity in Xenopus oocyte extracts preferentially dephosphorylates the MPM-2 epitope.*" FEBS Lett **424**(3): 225-233.

Chen, C., S. Shimizu, Y. Tsujimoto and N. Motoyama (2005). "*Chk2 regulates transcription-independent p53-mediated apoptosis in response to DNA damage.*" Biochem Biophys Res Commun **333**(2): 427-431.

Chini, C. C. and J. Chen (2003). *"Human claspin is required for replication checkpoint control.*" J Biol Chem **278**(32): 30057-30062.

Chowdhury, D., M. C. Keogh, H. Ishii, C. L. Peterson, S. Buratowski and J. Lieberman (2005). "*gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair.*" Mol Cell **20**(5): 801-809.

Coleman, T. R. and W. G. Dunphy (1994). "*Cdc2 regulatory factors.*" Curr Opin Cell Biol **6**(6): 877-882.

Cuadrado, M., B. Martinez-Pastor and O. Fernandez-Capetillo (2006). ""*ATR activation in response to ionizing radiation: still ATM territory*"." Cell Div **1**(1): 7.

Cuadrado, M., B. Martinez-Pastor, M. Murga, L. I. Toledo, P. Gutierrez-Martinez, E. Lopez and O. Fernandez-Capetillo (2006). "*ATM regulates ATR chromatin loading in response to DNA double-strand breaks.*" J Exp Med **203**(2): 297-303.

Deming, P. and S. Kornbluth (2006). "*Study of apoptosis in vitro using the Xenopus egg extract reconstitution system.*" Methods Mol Biol **322**: 379-393.

Di Micco, R., M. Fumagalli, A. Cicalese, S. Piccinin, P. Gasparini, C. Luise, C. Schurra, M. Garre, P. G. Nuciforo, A. Bensimon, R. Maestro, P. G. Pelicci and F. d'Adda di Fagagna (2006). "*Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication.*" Nature **444**(7119): 638-642.

Dohadwala, M., E. F. da Cruz e Silva, F. L. Hall, R. T. Williams, D. A. Carbonaro-Hall, A. C. Nairn, P. Greengard and N. Berndt (1994). "*Phosphorylation and inactivation of protein phosphatase 1 by cyclin-dependent kinases.*" Proc Natl Acad Sci U S A **91**(14): 6408-6412.

Donzelli, M. and G. F. Draetta (2003). "*Regulating mammalian checkpoints through Cdc25 inactivation.*" EMBO Rep **4**(7): 671-677.

Doonan, J. H. and N. R. Morris (1989). "*The bimG gene of Aspergillus nidulans, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1.*" Cell **57**(6): 987-996.

Dupre, A., L. Boyer-Chatenet and J. Gautier (2006). "*Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex.*" Nat Struct Mol Biol **13**(5): 451-457.

El-Armouche, A., A. Bednorz, T. Pamminger, D. Ditz, M. Didie, D. Dobrev and T. Eschenhagen (2006). "*Role of calcineurin and protein phosphatase-2A in the regulation of phosphatase inhibitor-1 in cardiac myocytes.*" Biochem Biophys Res Commun **346**(3): 700-706.

Essmann, F., S. Pohlmann, B. Gillissen, P. T. Daniel, K. Schulze-Osthoff and R. U. Janicke (2005). "*Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis.*" J Biol Chem **280**(44): 37169-37177.

Falck, J., J. Coates and S. P. Jackson (2005). "*Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage.*" Nature **434**(7033): 605-611.

Falck, J., N. Mailand, R. G. Syljuasen, J. Bartek and J. Lukas (2001). "*The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis.*" Nature **410**(6830): 842-847.

Ferrell, J. E., Jr. (1999). "*Xenopus oocyte maturation: new lessons from a good egg.*" Bioessays **21**(10): 833-842.

Forester, C. M., J. Maddox, J. V. Louis, J. Goris and D. M. Virshup (2007). "*Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1.*" Proc Natl Acad Sci U S A **104**(50): 19867-19872.

Gatei, M., D. Young, K. M. Cerosaletti, A. Desai-Mehta, K. Spring, S. Kozlov, M. F. Lavin, R. A. Gatti, P. Concannon and K. Khanna (2000). "*ATM-dependent phosphorylation of nibrin in response to radiation exposure.*" <u>Nat Genet</u> **25**(1): 115-119.

Geng, L., X. Zhang, S. Zheng and R. J. Legerski (2007). "*Artemis links ATM to G2/M checkpoint recovery via regulation of Cdk1-cyclin B.*" <u>Mol Cell Biol</u> **27**(7): 2625-2635.

Golden, T., M. Swingle and R. E. Honkanen (2008). "*The role of serine/threonine protein phosphatase type 5 (PP5) in the regulation of stress-induced signaling networks and cancer.*" <u>Cancer Metastasis Rev</u> **27**(2): 169-178.

Gorgoulis, V. G., L. V. Vassiliou, P. Karakaidos, P. Zacharatos, A. Kotsinas, T. Liloglou, M. Venere, R. A. Ditullio, Jr., N. G. Kastrinakis, B. Levy, D. Kletsas, A. Yoneta, M. Herlyn, C. Kittas and T. D. Halazonetis (2005). "*Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions.*" <u>Nature</u> **434**(7035): 907-913.

Guo, C. Y., D. L. Brautigan and J. M. Larner (2002). "*ATM-dependent dissociation of B55 regulatory subunit from nuclear PP2A in response to ionizing radiation.*" <u>J Biol Chem</u> **277**(7): 4839-4844.

Guo, C. Y., D. L. Brautigan and J. M. Larner (2002). "*Ionizing radiation activates nuclear protein phosphatase-1 by ATM-dependent dephosphorylation.*" <u>J Biol Chem</u> **277**(44): 41756-41761.

Guo, Z. and W. G. Dunphy (2000). "*Response of Xenopus Cds1 in cell-free extracts to DNA templates with double-stranded ends.*" <u>Mol Biol Cell</u> **11**(5): 1535-1546.

Gupta, A., T. G. Guerin-Peyrou, G. G. Sharma, C. Park, M. Agarwal, R. K. Ganju, S. Pandita, K. Choi, S. Sukumar, R. K. Pandita, T. Ludwig and T. K. Pandita (2008). "*The mammalian ortholog of Drosophila MOF that acetylates histone H4 lysine 16 is essential for embryogenesis and oncogenesis.*" <u>Mol Cell Biol</u> **28**(1): 397-409.

Gupta, A., G. G. Sharma, C. S. Young, M. Agarwal, E. R. Smith, T. T. Paull, J. C. Lucchesi, K. K. Khanna, T. Ludwig and T. K. Pandita (2005). "*Involvement of human MOF in ATM function.*" <u>Mol Cell Biol</u> **25**(12): 5292-5305.

Higuchi, E., A. Nishi, H. Higashi, Y. Ito and H. Kato (2000). "*Phosphorylation of protein phosphatase-1 inhibitors, inhibitor-1 and DARPP-32, in renal medulla.*" <u>Eur J Pharmacol</u> **408**(2): 107-116.

Hoshino, R., Y. Chatani, T. Yamori, T. Tsuruo, H. Oka, O. Yoshida, Y. Shimada, S. Ari-i, H. Wada, J. Fujimoto and M. Kohno (1999). "*Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors.*" <u>Oncogene</u> **18**(3): 813-822.

Inoue, Y., M. Kitagawa and Y. Taya (2007). "*Phosphorylation of pRB at Ser612 by Chk1/2 leads to a complex between pRB and E2F-1 after DNA damage.*" <u>Embo J</u> **26**(8): 2083-2093.

Jazayeri, A., J. Falck, C. Lukas, J. Bartek, G. C. Smith, J. Lukas and S. P. Jackson (2006). "*ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks.*" <u>Nat Cell Biol</u> **8**(1): 37-45.

Jiang, X., Y. Sun, S. Chen, K. Roy and B. D. Price (2006). "*The FATC domains of PIKK proteins are functionally equivalent and participate in the Tip60-dependent activation of DNA-PKcs and ATM.*" <u>J Biol Chem</u> **281**(23): 15741-15746.

Kamer, I., R. Sarig, Y. Zaltsman, H. Niv, G. Oberkovitz, L. Regev, G. Haimovich, Y. Lerenthal, R. C. Marcellus and A. Gross (2005). "*Proapoptotic BID is an ATM effector in the DNA-damage response.*" <u>Cell</u> **122**(4): 593-603.

Kang, S., S. Dong, T. L. Gu, A. Guo, M. S. Cohen, S. Lonial, H. J. Khoury, D. Fabbro, D. G. Gilliland, P. L. Bergsagel, J. Taunton, R. D. Polakiewicz and J. Chen (2007). "*FGFR3 activates RSK2 to mediate hematopoietic transformation through tyrosine phosphorylation of RSK2 and activation of the MEK/ERK pathway.*" <u>Cancer Cell</u> **12**(3): 201-214.

Kastan, M. B. and J. Bartek (2004). "*Cell-cycle checkpoints and cancer.*" <u>Nature</u> **432**(7015): 316-323.

Kastan, M. B. and D. S. Lim (2000). "*The many substrates and functions of ATM.*" <u>Nat Rev Mol Cell Biol</u> **1**(3): 179-186.

Kaufmann, T., L. Tai, P. G. Ekert, D. C. Huang, F. Norris, R. K. Lindemann, R. W. Johnstone, V. M. Dixit and A. Strasser (2007). "*The BH3-only protein bid is dispensable for DNA damage- and replicative stress-induced apoptosis or cell-cycle arrest.*" <u>Cell</u> **129**(2): 423-433.

Keogh, M. C., J. A. Kim, M. Downey, J. Fillingham, D. Chowdhury, J. C. Harrison, M. Onishi, N. Datta, S. Galicia, A. Emili, J. Lieberman, X. Shen, S. Buratowski, J. E. Haber, D. Durocher, J. F. Greenblatt and N. J. Krogan (2006). "*A phosphatase*

complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery." <u>Nature</u> **439**(7075): 497-501.

Konishi, A., S. Shimizu, J. Hirota, T. Takao, Y. Fan, Y. Matsuoka, L. Zhang, Y. Yoneda, Y. Fujii, A. I. Skoultchi and Y. Tsujimoto (2003). *"Involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks.*" <u>Cell</u> **114**(6): 673-688.

Kornbluth, S. (1997). "*Apoptosis in Xenopus egg extracts.*" <u>Methods Enzymol</u> **283**: 600-614.

Kotani, S., S. Tugendreich, M. Fujii, P. M. Jorgensen, N. Watanabe, C. Hoog, P. Hieter and K. Todokoro (1998). *"PKA and MPF-activated polo-like kinase regulate anaphase-promoting complex activity and mitosis progression."* <u>Mol Cell</u> **1**(3): 371-380.

Kozlov, S. V., M. E. Graham, C. Peng, P. Chen, P. J. Robinson and M. F. Lavin (2006). *"Involvement of novel autophosphorylation sites in ATM activation.*" <u>Embo J</u> **25**(15): 3504-3514.

Kumagai, A. and W. G. Dunphy (2000). "*Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts.*" <u>Mol Cell</u> **6**(4): 839-849.

Kumagai, A., S. M. Kim and W. G. Dunphy (2004). "*Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1.*" <u>J Biol Chem</u> **279**(48): 49599-49608.

Kumagai, A., J. Lee, H. Y. Yoo and W. G. Dunphy (2006). "*TopBP1 activates the ATR-ATRIP complex.*" <u>Cell</u> **124**(5): 943-955.

Kwon, Y. G., S. Y. Lee, Y. Choi, P. Greengard and A. C. Nairn (1997). "*Cell cycle-dependent phosphorylation of mammalian protein phosphatase 1 by cdc2 kinase.*" <u>Proc Natl Acad Sci U S A</u> **94**(6): 2168-2173.

Lara, R., M. J. Seckl and O. E. *Pardo* (2013). "*The p90 RSK family members: common functions and isoform specificity.*" <u>Cancer Res</u> **73**(17): 5301-5308.

Lee, J., A. Kumagai and W. G. Dunphy (2001). *"Positive regulation of Wee1 by Chk1 and 14-3-3 proteins."* <u>Mol Biol Cell</u> **12**(3): 551-563.

Lee, J. H. and T. T. Paull (2004). *"Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex."* <u>Science</u> **304**(5667): 93-96.

Lee, J. H. and T. T. Paull (2005). "*ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex."* <u>Science</u> **308**(5721): 551-554.

Levine, A. J. (1997). *"p53, the cellular gatekeeper for growth and division."* Cell **88**(3): 323-331.

Lew, D. J. and S. Kornbluth (1996). *"Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control."* Curr Opin Cell Biol **8**(6): 795-804.

Lin, S. Y., K. Li, G. S. Stewart and S. J. Elledge (2004). *"Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation."* Proc Natl Acad Sci U S A **101**(17): 6484-6489.

Lou, Z., K. Minter-Dykhouse, S. Franco, M. Gostissa, M. A. Rivera, A. Celeste, J. P. Manis, J. van Deursen, A. Nussenzweig, T. T. Paull, F. W. Alt and J. Chen (2006). *"MDC1 maintains genomic stability by participating in the amplification of ATM-dependent DNA damage signals."* Mol Cell **21**(2): 187-200.

Lu, X., B. Nannenga and L. A. Donehower (2005). *"PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints."* Genes Dev **19**(10): 1162-1174.

Lu, X., T. A. Nguyen and L. A. Donehower (2005). *"Reversal of the ATM/ATR-mediated DNA damage response by the oncogenic phosphatase PPM1D."* Cell Cycle **4**(8): 1060-1064.

Mailand, N., S. Bekker-Jensen, J. Bartek and J. Lukas (2006). *"Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress."* Mol Cell **23**(3): 307-318.

Mamely, I., M. A. van Vugt, V. A. Smits, J. I. Semple, B. Lemmens, A. Perrakis, R. H. Medema and R. Freire (2006). *"Polo-like kinase-1 controls proteasome-dependent degradation of Claspin during checkpoint recovery."* Curr Biol **16**(19): 1950-1955.

Margolis, S. S. and S. Kornbluth (2004). *"When the checkpoints have gone: insights into Cdc25 functional activation."* Cell Cycle **3**(4): 425-428.

Margolis, S. S., S. Walsh, D. C. Weiser, M. Yoshida, S. Shenolikar and S. Kornbluth (2003). *"PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation."* Embo J **22**(21): 5734-5745.

McConnell, J. L., R. J. Gomez, L. R. McCorvey, B. K. Law and B. E. Wadzinski (2007). *"Identification of a PP2A-interacting protein that functions as a negative regulator of phosphatase activity in the ATM/ATR signaling pathway."* Oncogene **26**(41): 6021-6030.

McKinnon, P. J. (2004). *"ATM and ataxia telangiectasia."* EMBO Rep **5**(8): 772-776.

Mochida, S. and T. Hunt (2007). *"Calcineurin is required to release Xenopus egg extracts from meiotic M phase."* <u>Nature</u> **449**(7160): 336-340.

Mochida, S., S. Ikeo, J. Gannon and T. Hunt (2009). *"Regulated activity of PP2A-B55 delta is crucial for controlling entry into and exit from mitosis in Xenopus egg extracts."* <u>EMBO J</u> **28**(18): 2777-2785.

Murray, A. W. and M. W. Kirschner (1989). "*Cyclin synthesis drives the early embryonic cell cycle.*" <u>Nature</u> **339**(6222): 275-280.

Newmeyer, D. D., D. M. Farschon and J. C. Reed (1994). *"Cell-free apoptosis in Xenopus egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria.*" <u>Cell</u> **79**(2): 353-364.

Nishiyama, T., N. Yoshizaki, T. Kishimoto and K. Ohsumi (2007). "*Transient activation of calcineurin is essential to initiate embryonic development in Xenopus laevis."* <u>Nature</u> **449**(7160): 341-345.

O'Connell, M. J. and K. A. Cimprich (2005). "*G2 damage checkpoints: what is the turn-on?"* <u>J Cell Sci</u> **118**(Pt 1): 1-6.

Paull, T. T. and J. H. Lee (2005). *"The Mre11/Rad50/Nbs1 complex and its role as a DNA double-strand break sensor for ATM."* <u>Cell Cycle</u> **4**(6): 737-740.

Pellegrini, M., A. Celeste, S. Difilippantonio, R. Guo, W. Wang, L. Feigenbaum and A. Nussenzweig (2006). *"Autophosphorylation at serine 1987 is dispensable for murine Atm activation in vivo.*" <u>Nature</u> **443**(7108): 222-225.

Peng, C. Y., P. R. Graves, R. S. Thoma, Z. Wu, A. S. Shaw and H. Piwnica-Worms (1997). "*Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216.*" <u>Science</u> **277**(5331): 1501-1505.

Peschiaroli, A., N. V. Dorrello, D. Guardavaccaro, M. Venere, T. Halazonetis, N. E. Sherman and M. Pagano (2006). *"SCFbetaTrCP-mediated degradation of Claspin regulates recovery from the DNA replication checkpoint response."* <u>Mol Cell</u> **23**(3): 319-329.

Peters, J. M. (2006). *"The anaphase promoting complex/cyclosome: a machine designed to destroy."* <u>Nat Rev Mol Cell Biol</u> **7**(9): 644-656.

Polit, J. T. and A. Kazmierczak (2007). *"Okadaic acid (1 microM) accelerates S phase and mitosis but inhibits heterochromatin replication and metaphase anaphase transition in Vicia faba meristem cells."* <u>J Exp Bot</u> **58**(11): 2785-2797.

Raleigh, J. M. and M. J. O'Connell (2000). *"The G(2) DNA damage checkpoint targets both Wee1 and Cdc25."* <u>J Cell Sci</u> **113 (Pt 10)**: 1727-1736.

Rea, S., G. Xouri and A. Akhtar (2007). *"Males absent on the first (MOF): from flies to humans."* <u>Oncogene</u> **26**(37): 5385-5394.

Richards, S. A., V. C. Dreisbach, L. O. Murphy and J. Blenis (2001). *"Characterization of regulatory events associated with membrane targeting of p90 ribosomal S6 kinase 1."* <u>Mol Cell Biol</u> **21**(21): 7470-7480.

Sancar, A., L. A. Lindsey-Boltz, K. Unsal-Kacmaz and S. Linn (2004). *"Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints."* <u>Annu Rev Biochem</u> **73**: 39-85.

Shreeram, S., O. N. Demidov, W. K. Hee, H. Yamaguchi, N. Onishi, C. Kek, O. N. Timofeev, C. Dudgeon, A. J. Fornace, C. W. Anderson, Y. Minami, E. Appella and D. V. Bulavin (2006). *"Wip1 phosphatase modulates ATM-dependent signaling pathways."* <u>Mol Cell</u> **23**(5): 757-764.

Skoufias, D. A., R. L. Indorato, F. Lacroix, A. Panopoulos and R. L. Margolis (2007). *"Mitosis persists in the absence of Cdk1 activity when proteolysis or protein phosphatase activity is suppressed."* <u>J Cell Biol</u> **179**(4): 671-685.

Snyder, G. L., J. A. Girault, J. Y. Chen, A. J. Czernik, J. W. Kebabian, J. A. Nathanson and P. Greengard (1992). *"Phosphorylation of DARPP-32 and protein phosphatase inhibitor-1 in rat choroid plexus: regulation by factors other than dopamine."* <u>J Neurosci</u> **12**(8): 3071-3083.

Stegmeier, F. and A. Amon (2004). *"Closing mitosis: the functions of the Cdc14 phosphatase and its regulation."* <u>Annu Rev Genet</u> **38**: 203-232.

Sullivan, M. and D. O. Morgan (2007). *"Finishing mitosis, one step at a time."* <u>Nat Rev Mol Cell Biol</u> **8**(11): 894-903.

Sun, Y., X. Jiang, S. Chen, N. Fernandes and B. D. Price (2005). *"A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM."* <u>Proc Natl Acad Sci U S A</u> **102**(37): 13182-13187.

Sun, Y., X. Jiang, S. Chen and B. D. Price (2006). "*Inhibition of histone acetyltransferase activity by anacardic acid sensitizes tumor cells to ionizing radiation.*" <u>FEBS Lett</u> **580**(18): 4353-4356.

Sun, Y., Y. Xu, K. Roy and B. D. Price (2007). *"DNA damage-induced acetylation of lysine 3016 of ATM activates ATM kinase activity."* <u>Mol Cell Biol</u> **27**(24): 8502-8509.

Syljuasen, R. G., S. Jensen, J. Bartek and J. Lukas (2006). *"Adaptation to the ionizing radiation-induced G2 checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases."* <u>Cancer Res</u> **66**(21): 10253-10257.

Taieb, F., I. Chartrain, S. Chevalier, O. Haccard and C. Jessus (1997). "*Cyclin D2 arrests Xenopus early embryonic cell cycles.*" <u>Exp Cell Res</u> **237**(2): 338-346.

Taipale, M., S. Rea, K. Richter, A. Vilar, P. Lichter, A. Imhof and A. Akhtar (2005). "*hMOF histone acetyltransferase is required for histone H4 lysine 16 acetylation in mammalian cells.*" <u>Mol Cell Biol</u> **25**(15): 6798-6810.

Tang, X., Z. G. Hui, X. L. Cui, R. Garg, M. B. Kastan and B. Xu (2008). "*A novel ATM-dependent pathway regulates protein phosphatase 1 in response to DNA damage.*" <u>Mol Cell Biol</u> **28**(8): 2559-2566.

Toczyski, D. P., D. J. Galgoczy and L. H. Hartwell (1997). *"CDC5 and CKII control adaptation to the yeast DNA damage checkpoint."* <u>Cell</u> **90**(6): 1097-1106.

Trautmann, S. and D. McCollum (2002). *"Cell cycle: new functions for Cdc14 family phosphatases."* <u>Curr Biol</u> **12**(21): R733-735.

Tsai, W. B., Y. M. Chung, Y. Takahashi, Z. Xu and M. C. Hu (2008). *"Functional interaction between FOXO3a and ATM regulates DNA damage response."* <u>Nat Cell Biol</u> **10**(4): 460-467.

Uto, K., D. Inoue, K. Shimuta, N. Nakajo and N. Sagata (2004). *"Chk1, but not Chk2, inhibits Cdc25 phosphatases by a novel common mechanism."* <u>Embo J</u> **23**(16): 3386-3396.

Vandre, D. D. and V. L. Wills (1992). *"Inhibition of mitosis by okadaic acid: possible involvement of a protein phosphatase 2A in the transition from metaphase to anaphase."* <u>J Cell Sci</u> **101 (Pt 1)**: 79-91.

Vaughan, C. K., M. Mollapour, J. R. Smith, A. Truman, B. Hu, V. M. Good, B. Panaretou, L. Neckers, P. A. Clarke, P. Workman, P. W. Piper, C. Prodromou and L. H. Pearl

(2008). *"Hsp90-dependent activation of protein kinases is regulated by chaperone-targeted dephosphorylation of Cdc37."* <u>Mol Cell</u> **31**(6): 886-895.

Wu, J. Q., D. V. Hansen, Y. Guo, M. Z. Wang, W. Tang, C. D. Freel, J. J. Tung, P. K. Jackson and S. Kornbluth (2007). *"Control of Emi2 activity and stability through Mos-mediated recruitment of PP2A."* <u>Proc Natl Acad Sci U S A</u> **104**(42): 16564-16569.

Wu, Q., Y. Guo, A. Yamada, J. A. Perry, M. Z. Wang, M. Araki, C. D. Freel, J. J. Tung, W. Tang, S. S. Margolis, P. K. Jackson, H. Yamano, M. Asano and S. Kornbluth (2007). *"A role for Cdc2- and PP2A-mediated regulation of Emi2 in the maintenance of CSF arrest."* <u>Curr Biol</u> **17**(3): 213-224.

Yazdi, P. T., Y. Wang, S. Zhao, N. Patel, E. Y. Lee and J. Qin (2002). *"SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint."* <u>Genes Dev</u> **16**(5): 571-582.

Yoo, H. Y., A. Kumagai, A. Shevchenko, A. Shevchenko and W. G. Dunphy (2004). *"Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase."* <u>Cell</u> **117**(5): 575-588.

Yoo, H. Y., A. Kumagai, A. Shevchenko, A. Shevchenko and W. G. Dunphy (2007). *"Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM."* <u>J Biol Chem</u> **282**(24): 17501-17506.

Yoo, H. Y., A. Shevchenko, A. Shevchenko and W. G. Dunphy (2004). *"Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses."* <u>J Biol Chem</u> **279**(51): 53353-53364.

You, Z., J. M. Bailis, S. A. Johnson, S. M. Dilworth and T. Hunter (2007). *"Rapid activation of ATM on DNA flanking double-strand breaks."* <u>Nat Cell Biol</u> **9**(11): 1311-1318.

Yu, Q., J. La Rose, H. Zhang, H. Takemura, K. W. Kohn and Y. Pommier (2002). *"UCN-01 inhibits p53 up-regulation and abrogates gamma-radiation-induced G(2)-M checkpoint independently of p53 by targeting both of the checkpoint kinases, Chk2 and Chk1."* <u>Cancer Res</u> **62**(20): 5743-5748.

Zermati, Y., S. Mouhamad, L. Stergiou, B. Besse, L. Galluzzi, S. Boehrer, A. L. Pauleau, F. Rosselli, M. D'Amelio, R. Amendola, M. Castedo, M. Hengartner, J. C. Soria, F. Cecconi and G. Kroemer (2007). *"Nonapoptotic role for Apaf-1 in the DNA damage checkpoint."* <u>Mol Cell</u> **28**(4): 624-637.

Zhang, L., N. J. Huang, C. Chen, W. Tang and S. Kornbluth (2012). "*Ubiquitylation of p53 by the APC/C inhibitor Trim39*." <u>Proc Natl Acad Sci U S A</u> **109**(51): 20931-20936.

Zhang, Y. W., D. M. Otterness, G. G. Chiang, W. Xie, Y. C. Liu, F. Mercurio and R. T. Abraham (2005). "*Genotoxic stress targets human Chk1 for degradation by the ubiquitin-proteasome pathway.*" <u>Mol Cell</u> **19**(5): 607-618.

Zhu, Y., C. Alvarez, R. Doll, H. Kurata, X. M. Schebye, D. Parry and E. Lees (2004). "*Intra-S-phase checkpoint activation by direct CDK2 inhibition.*" <u>Mol Cell Biol</u> **24**(14): 6268-6277.

Zinkel, S. S., K. E. Hurov, C. Ong, F. M. Abtahi, A. Gross and S. J. Korsmeyer (2005). "*A role for proapoptotic BID in the DNA-damage response.*" <u>Cell</u> **122**(4): 579-591.

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Publications

1. **Chen C**, Zhang L, Huang NJ, Huang B, Kornbluth S. Suppression of DNA-damage checkpoint signaling by Rsk-mediated phosphorylation of Mre11. Proc Natl Acad Sci USA. 2013 Dec 17; 110(51):20605-10.

2. Zhang L, Huang NJ, **Chen C**, Tang W, Kornbluth S. Ubiquitylation of p53 by the APC/C inhibitor Trim39. Proc Natl Acad Sci U S A. 2012 Dec 18; 109(51):20931-6.

3. Huang NJ, Zhang L, Tang W, **Chen C**, Yang CS, Kornbluth S. The Trim39 ubiquitin ligase inhibits APC/Cdh1-mediated degradation of the Bax activator MOAP-1. J Cell Biol. 2012 Apr 30; 197(3):361-7.

4. Tang W, Wu JQ, **Chen C**, Yang CS, Guo JY, Freel CD, Kornbluth S. Emi2-mediated inhibition of E2-substrate ubiquitin transfer by the anaphase-promoting complex/cyclosome through a D-box-independent mechanism. Mol Biol Cell. 2010 Aug 1; 21(15):2589-97.

5. Wu JQ, Guo JY, Tang W, Yang CS, Freel CD, **Chen C**, Nairn AC, Kornbluth S. PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation. Nat Cell Biol. 2009 May;11(5):644-51.

6. Huang BF, Huang NJ, Yang ZS, **Chen** C, Kornbluth S. PP2A regulates apoptosis in Xenopus extracts by targeting CamKII. J Chem Bio. submitted

Honors and Awards

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