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How I Wrote My Prospectus

My advisor was a brand-new faculty member at Yale, and she started her lab with three broad questions that extended from her post-doctoral work. During my rotation, I picked one of these questions, happened to make an interesting observation, and proposed follow-up work in my qualifying exam and prospectus. I came up with the ideas for the aims and the core experiments, and the technical details of the experiments were repeatedly refined by discussions with my labmates, advisor, and senior students. My advisor and committee helped to calibrate the scope of the research to make sure it was achievable during my PhD. Once I had decided on the scope and skeleton of these documents, I was solely responsible for writing them. My department required that the prospectus was one or two double-spaced pages and is therefore very broad. The qualifying exam, in contrast, was formatted like an NIH-style proposal, so it has more experimental detail and consideration of alternative outcomes. We were required to write a proposal in which half of the aims (the extended aims) used experimental approaches that are unfamiliar to the thesis lab.

Advice for Prospectus Writers

Be open to criticism. Writing and defending your prospectus is an opportunity to workshop the project that you will invest years of work in and build important skills in writing, presenting, and experimental design. Your committee is a group of highly experienced and intelligent people dedicated to helping you and your project be in the best possible place to succeed, not a group of people wanting you to fail. Learning to be vulnerable and take feedback (both about writing and scientific content) early in graduate school will help you avoid pitfalls and effectively communicate the importance of your work to funding agencies, editors, and your peers.

Elizabeth Black
April 15, 2021
Prospectus

Investigating DNA damage-independent functions for MRN complex members in mitosis

Background: Faithful genome maintenance and inheritance are essential for proper cell function and require the coordinated efforts of many pathways. Compromised genomic stability is a hallmark and driver of many cancers. One of the major mechanisms by which cells maintain genome stability is the DNA damage response (DDR) pathway. Although once thought to be interphase-specific, novel roles for DDR proteins as critical mitotic regulators have been discovered[1-3]. For example, ATR, a DDR kinase that is canonically active at single-stranded DNA lesions, is active at mitotic centromeres and promotes faithful chromosome segregation in the absence of DNA damage[2]. DDR proteins, therefore, promote genome stability through two discrete mechanisms—canonical DDR in interphase and proper chromosome segregation in mitosis. Despite their important role in maintaining genome stability, we currently understand very little about the functions of the DDR pathway in mitosis. My proposed research will identify novel mitotic functions of the DDR pathway.

Progress: I am working on mitotic functions of the MRE11-RAD50-NBS1 (MRN) complex, which promotes efficient DNA double-stranded break repair by activating the DDR kinase ATM[4]. I have identified a novel and DNA damage-independent localization for two members of the MRN complex, RAD50 and NBS1, at prometaphase kinetochores. I have demonstrated that this localization is not an artifact of nonspecific antibody staining, as it can be recapitulated with multiple NBS1 and RAD50 antibodies, GFP-tagged NBS1, and IF staining is sensitive to siRNA knockdown and in knockout cell lines. In contrast to RAD50 and NBS1, MRE11 does not appear to localize to kinetochores and instead localizes to the mitotic spindle, prompting the hypothesis that members of the MRN complex may have independent functions than in interphase.

My preliminary data suggests that NBS1 and RAD50 are important for promoting proper chromosome segregation independently of their known DDR role. Knockout RAD50 and NBS1 cells have increased micronuclei and chromosome missegregation events. Furthermore, they have a significant increase in lagging chromosomes, a specific chromosome segregation error that is caused by mitotic defects, not unresolved DNA damage. I am currently developing an auxin-inducible degron (AID) system[5] that will allow me to acutely deplete RAD50 and NBS1 in mitosis, which will be important to differentiate the putative mitotic functions of RAD50 and NBS1 from their canonical role in interphase DDR.

One major focus for the last year has been identifying the factors that recruit NBS1 and RAD50 to prometaphase kinetochores. Poly(ADP-ribose) polymerases (PARPs) are enzymes that orchestrate the DDR by catalyzing the formation of poly(ADP-ribose) (PAR) chains to acceptor molecules following DNA damage. Interestingly, PARP-1 and PARP-2 localize to mitotic centromeres and kinetochores and modify important mitotic proteins[6,7]. Given that PARP-1 activity is important for recruiting the MRN complex to sites of DNA damage in interphase[8,9], I hypothesized that PARylation might also promote NBS1 and RAD50 localization to mitotic kinetochores in a DNA damage-independent manner. Indeed, acute treatment with the PARP1/2 inhibitor Olaparib causes a significant reduction in RAD50 and NBS1 localization to the kinetochore. I am currently working to identify the PARylated proteins that interact with NBS1 and RAD50 at mitotic kinetochores using candidate protein screening and mass spectrometry.

Future work and impact: The immediate goals for this project are to 1) determine how PARylation is regulating and promoting NBS1 and RAD50 localization to mitotic kinetochores and 2) determine mitotic functions of NBS1 and RAD50 by developing a high temporal resolution AID system. Together, this work will reveal novel mitotic functions for the MRN complex and DDR pathways in promoting genome stability. I expect that these mitotic and DNA damage-independent functions, together with the well-studied function of the MRN complex in interphase DDR, will demonstrate that the MRN complex has dual function in promoting genome stability.

References

1 Petsalaki, E. & Zachos, G. DNA damage response proteins regulating mitotic cell division: double agents preserving genome stability. *FEBS J* **287**, 1700-1721, doi:10.1111/febs.15240 (2020).

2 Kabeche, L., Nguyen, H. D., Buisson, R. & Zou, L. A mitosis-specific and R loop-driven ATR pathway promotes faithful chromosome segregation. *Science* **359**, 108-114 (2018).

3 Giunta, S., Belotserkovskaya, R. & Jackson, S. P. DNA damage signaling in response to double-strand breaks during mitosis. *J Cell Biol* **190**, 197-207, doi:10.1083/jcb.200911156 (2010).

4 Uziel, T. *et al.* Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J* **22**, 5612-5621, doi:10.1093/emboj/cdg541 (2003).

5 Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat Methods* **6**, 917-922, doi:10.1038/nmeth.1401 (2009).

6 Saxena, A. *et al.* Poly(ADP-ribose) polymerase 2 localizes to mammalian active centromeres and interacts with PARP-1, Cenpa, Cenpb and Bub3, but not Cenpc. *Human Moledular Genetics* **11**, 2319-2329 (2002).

7 Saxena, A., Saffery, R., Wong, L. H., Kalitsis, P. & Choo, K. H. Centromere proteins Cenpa, Cenpb, and Bub3 interact with poly(ADP-ribose) polymerase-1 protein and are poly(ADP-ribosyl)ated. *J Biol Chem* **277**, 26921-26926, doi:10.1074/jbc.M200620200 (2002).

8 Haince, J. F. *et al.* PARP1-dependent kinetics of recruitment of MRE11 and NBS1 proteins to multiple DNA damage sites. *J Biol Chem* **283**, 1197-1208, doi:10.1074/jbc.M706734200 (2008).

9 Li, M., Lu, L. Y., Yang, C. Y., Wang, S. & Yu, X. The FHA and BRCT domains recognize ADP-ribosylation during DNA damage response. *Genes Dev* **27**, 1752-1768, doi:10.1101/gad.226357.113 (2013).

Defining the DNA damage-independent functions of the MRN complex in proper mitotic progression

Elizabeth Black

Qualifying Exam

August 18th, 2020 1:00-3:30 PM

Abstract:

Faithful maintenance and inheritance of the genome is essential for proper cellular function. There are multiple mechanisms in place to promote genome integrity. One such mechanism is the DNA damage repair (DDR) pathway. DDR proteins have noncanonical functions in mitosis that promote faithful chromosome segregation. ATR is a master regulator kinase that is canonically activated by single-stranded DNA lesions and is also activated at mitotic centromeres on RNA-DNA hybrids known as R-loops. This DNA damage-independent ATR activity promotes proper chromosome segregation [1]. The MRE11-RAD50-NBS1 (MRN) complex has a fundamental role in interphase DDR signaling by activating ATR and related kinases. My preliminary data show that NBS1 and RAD50 colocalize with ATR at centromeric R-loops in prometaphase. I hypothesize that the MRN complex has a novel function to promote faithful chromosome segregation and chromosome stability in mitosis.

Aim 1 will determine if the MRN complex activates ATR at prometaphase centromeres. I hypothesize that the MRN complex activates ATR at centromeres in a DNA damage-independent manner. I will test this by using the auxin-inducible degron (AID) system to acutely deplete the MRN complex in mitosis and assay the effects on ATR activity and chromosome segregation by immunofluorescence (Aim 1.1). I will use immunofluorescence of MRN complex members to determine how its centromeric localization is temporally regulated (Aim 1.2), which may uncover a novel mechanism to regulate ATR signaling in mitosis. In addition to the hypothesized ATR-activating function, the MRN complex promotes chromosome alignment and spindle turnover in mitosis [2,3]. **Aim 2** will explore how these mitotic MRN activities are coordinated. I will identify kinase-specific phosphorylation events on the mitotic MRN complex using mass spectrometry (Aim 2.1) and determine their functional significance by creating phosphomimetic and phosphonull mutants at modified residues and identifying their effects on MRN protein interactions (Aim 2.2). These experiments will identify how specific posttranslational modifications regulate MRN localization and function in mitosis.

Extended aim 1 will investigate how the MRN and ATR complexes interact to promote ATR signaling. I will determine the structural mechanism for NBS1-mediated ATR activation by generating and comparing the structures of the active NBS1-ATR complex and inactive ATR using cryo-EM. **Extended aim 2** will explore whether MRN complex function is regulated by phase separation. Based on its physical interactions with known phase-separated domains and predicted disordered regions, I hypothesize that the MRN complex is capable of coacervation. I will test this hypothesis by purifying members of the MRN complex and testing for coacervate formation in vitro (Ext aim 2.1). Additionally, I will test whether the MRN complex forms coacervates in vivo by fluorescence correlation spectroscopy and optogenetic manipulation (Ext aim 2.2). Together, I expect that these experiments will reveal coacervation as a mechanism to regulate MRN activity in mitosis.

Specific aims:

Aim 1: Investigate the function and regulation of the MRN complex at prometaphase centromeres. My preliminary data demonstrate that RAD50 and NBS1, two components of the MRN complex, colocalize with active ATR at prometaphase centromeres in the absence of DNA damage. Given that the MRN complex activates ATR in response to interphase replication stress [4-8], I hypothesize that the MRN complex has a novel and noncanonical function to promote ATR activity at prometaphase centromeres in a DNA damage-independent manner. I will test this hypothesis by depleting the MRN complex in mitotic cells using the auxin-inducible degron (AID) system [9] and assaying ATR kinase activity by immunofluorescent staining with phosphospecific antibodies against known substrates in this pathway (Aim 1.1). My preliminary data also suggest that RAD50 localizes to centromeres specifically in prometaphase. I will test whether cyclin A degradation and kinetochore-microtubule attachment, both features of the prometaphase to metaphase transition [10,11], regulate MRN localization to mitotic centromeres (Aim 1.2). These experiments will determine how the MRN complex is temporally regulated at centromeres, and by extension, how downstream MRN functions, such as ATR signaling, are controlled to promote proper chromosome segregation.

Aim 2: Investigate PLK1-dependent regulation of the MRN complex at spindle poles. Previously published work and my preliminary data suggest that the MRN complex functions with the kinase PLK1 at centrosomes and spindle poles to promote spindle establishment [2]. However, we lack critical information about the mechanisms by which PLK1 and MRN regulate the mitotic spindle. I will identify the PLK1-dependent phosphorylation sites on members of the MRN complex using mass spectrometry. I will directly test whether these phosphorylation events regulate MRN localization and spindle formation by creating phosphomimetic and phosphonull mutations at modified residues (Aim 2.1). I will also identify the mechanism by which MRN functions downstream of PLK1 to promote spindle establishment by comparing protein interactions in MRN phosphomutants at PLK1-dependent residues using proximity biotinylation (Aim 2.2). I expect that these experiments will reveal that the MRN complex is directly phosphorylated by PLK1 and that this phosphorylation is essential for regulating spindle establishment by modulating protein interactions between the MRN complex and microtubule-regulating proteins.

Extended Aim 1: Identify the structural mechanism of ATR activation by NBS1. In vivo and in vitro work demonstrate that NBS1 directly binds to ATR and promotes its kinase activity [4-7], although the structural basis for this activation remains unclear. I will identify the structural mechanism for this activation by using cryo-EM to visualize how NBS1 interaction alters ATR conformation. Characterizing structural changes in ATR will increase our understanding of how ATR activity is regulated by upstream DDR factors and the mechanism by which ATR and its activators promote proper chromosome segregation in mitosis.

Ext aim 2: Investigate the role of phase separation in regulating the MRN complex in mitosis. Phase separation plays an important role in regulating cellular organization and function. I hypothesize that the MRN complex is regulated by coacervation and this biophysical property is important for coordinating its diverse mitotic functions. The MRN complex is a promising candidate for coacervate formation, as it oligomerizes, interacts with other known coacervates, and contains both intrinsically disordered regions as well as scaffolding domains that can promote coacervate formation. I will test whether purified MRN is capable of forming phase-separated coacervates in vitro (Ext aim 2.1). Finally, I will test whether the MRN complex forms coacervates in vivo using optogenetics and fluorescence correlation spectroscopy (FCS) (Ext aim 2.2). I expect that understanding MRN phase behavior could demonstrate another regulatory mechanism for the MRN complex in mitosis.

Background and significance:

Every division cycle, a cell must accurately replicate its genome and faithfully divide this genetic information into two daughter cells. Additionally, cells must repair DNA damage that occurs from endogenous and exogenous sources. There are sophisticated pathways in place to maintain genome integrity by repairing damaged DNA and promoting its accurate segregation in mitosis. If the mutational burden becomes too high, cells may die or, in multicellular organisms, become cancerous. Mutations in some DNA damage repair (DDR) proteins, such as BRCA1, lead to an increased mutation rate and confer a susceptibility to developing early-onset cancer. In addition to increased mutation rates from compromised DDR, some cancer cells are also karyotypically unstable. Aneuploidy, an abnormal number of chromosomes, is found in approximately 90% of solid tumors and arises from faulty chromosome segregation during mitosis [12]. Together, these observations suggest that genome instability resulting from DDR defects and chromosome missegregation are essential to understanding cancer.

The connection between the DDR pathway and mitotic chromosome segregation is currently poorly understood. Cells do not repair DNA damage that occurs during mitosis, prompting the hypothesis that the interphase DDR machinery is inactivate in mitosis [13]. Recent evidence contradicts this hypothesis, as some DDR proteins have mitotic functions that are DNA damage-independent and important for normal mitotic progression [14]. These DDR proteins, therefore, promote genome stability through two discrete mechanisms—canonical DDR in interphase and proper chromosome segregation in mitosis. This dual function for DDR proteins may represent an important capability that can be used to target genomically unstable cancer cells.

One DDR protein that promotes proper chromosome segregation in mitosis is the ataxia telangeistia and Rad3-related (ATR) kinase [1]. ATR is canonically activated at single-stranded DNA lesions in S-phase to protect the genome during replication stress [8,15]. It is additionally activated at RNA-DNA hybrids known as R-loops that form at unperturbed prometaphase centromeres in a manner that is independent of DNA damage or replication [1]. ATR promotes faithful chromosome segregation by phosphorylating and activating its downstream effector kinase, Chk1. Chk1 then promotes Aurora B activity as part of the error correction machinery, a pathway that destabilizes aberrant kinetochore-microtubule attachments and promotes proper chromosome biorientation and segregation [1]. In interphase, ATR functions within an extensive network of DDR proteins to halt cell cycle progression and control DDR signaling [16]. Robust ATR activity requires a number of activating proteins, such as TopBP1, ETAA1, and the MRE11-RAD50-NBS1 (MRN) complex [4-6,8,17-30]. It is currently unclear, however, whether these activators also have DNA damage-independent functions and how they interact with ATR in mitosis.

The MRN complex is a DNA damage sensor important for detecting double-stranded breaks and promoting kinase activity of the apical kinases ataxia telangiectasia-mutated (ATM) and ATR [4-6,8,17-21,31-33]. Once activated, ATR and ATM initiate a signaling cascade to halt cell cycle progression until DNA damage is repaired [34]. The MRN complex is highly conserved in all sampled eukaryotes and essential in vertabrates, as knockouts of any of the genes coding for MRN complex members are lethal in mice [18,35,36]. Homozygous mutation in *NBN,* the gene coding for NBS1, confers Nijimijen breakage syndrome (NBS), which is characterized by defective DDR, susceptibility to early-onset cancer, developmental defects, and chromosomal instability [31,35]. Homozygous mutations in *MRE11A* and *RAD50*, while less frequent, confer similar phenotypes [37-40].

A clinical feature of NBS is chromosome instability, which most often results from chromosome segregation errors. This suggests a mitotic function for NBS1 and the MRN complex. Recent studies have suggested important roles for the MRN complex in mitosis. First, immunodepleting MRE11 or inhibiting its catalytic activity in mitotic cytostatic factor (CSF)-arrested *Xenopus* extracts in prophase leads to defects in spindle establishment and chromosome alignment [3]. The mechanism for this function, as well as its relevance to human cells, is currently unclear. Additionally, another study identified an independent pathway where members of the MRN complex localize to the centrosome and spindle pole and affect spindle turnover [2]. These data, together with our understanding of the DNA damage-independent functions of ATR and the DDR pathway in mitosis, prompt the hypothesis that the MRN complex is working with other DDR proteins and the mitotic machinery to promote genome stability and proper chromosome segregation in mitosis.

Here, I will investigate the DNA damage-independent functions and regulation of the MRN complex in mitosis. These aims will reveal novel and noncanonical functions of the MRN complex in promoting genome stability. I expect that these results will demonstrate a role for the MRN complex in ATR activation at prometaphase centromeres (Aim 1.1) and identify the structural mechanism for ATR activation by the MRN complex (Ext. aim 1). Results from the proposed experiments will also determine how MRN is temporally regulated at centromeres (Aim 1.2). I will also test whether the MRN complex is directly regulated by the essential

mitotic kinase PLK1 at spindle poles to promote its localization and proper spindle establishment (Aim 2.1). I will explore the mechanism by which PLK1-dependent phosphorylation of the MRN complex promotes proper spindle assembly by comparing MRN protein interactions in phosphomimetic and phosphonull mutants (Aim 2.2). Finally, I will determine whether phase separation, an important mechanism to organize subcellular compartments, can regulate the mitotic MRN complex by testing for coacervate formation in vitro (Ext aim 2.1) and in vivo (Ext aim 2.2). Together, these experiments will reveal critical information about the DNA damage-independent functions and regulation of the MRN complex in mitosis. This will identify novel mechanisms by which the MRN complex promotes genome stability throughout the cell cycle. This critical role in maintaining genome stability, which is often dysregulated in cancer, could therefore be a promising therapeutic target.

Innovation:

Members of the DDR pathway do not repair damaged DNA in mitosis, but instead function in a DNA damage-independent manner to promote faithful chromosome segregation. We currently lack a complete understanding of the DNA damage-independent functions of DDR proteins in mitosis. The work proposed here is innovative because it will uncover novel and noncanonical functions of DDR proteins in mitosis and how they interact with pathways that promote faithful chromosome segregation. Specifically, this work will identify both upstream mitotic regulators of MRN activity and downstream targets that affect normal mitotic progression, with emphasis on MRN function in the centromeric R-loop-driven ATR pathway [1] and centrosomal PLK1-dependent spindle establishment [2]. These hypothesized noncanonical and DNA damage-independent mitotic roles for the MRN complex, coupled with its essential function in interphase DNA damage signaling, make it essential for maintaining genome stability throughout the cell cycle. These MRN functions may provide insight into the mechanisms of genomically unstable cancers and offer promising therapeutic targets in cancer treatment.

Dissecting the mitotic DNA damage-independent functions of DDR proteins from unresolved interphase DNA damage has been challenging because traditional protein depletion methods, like siRNA or shRNA, rely on natural protein turnover, often taking more than 24 hours to fully deplete protein expression. These systems, as well as loss-of-function mutations, affect DDR function throughout the cell cycle and lead to an accumulation of DNA damage. Cells depleted of DDR proteins in interphase will enter mitosis with damaged or underreplicated DNA, which may cause mitotic errors independently of any mitosis-specific function [41]. The auxin-inducible degron (AID) tag has emerged as a method to initiate rapid proteasomal degradation of AID-tagged proteins upon addition of the plant hormone auxin [9]. Throughout the course of the experiments described here, I will develop auxin-inducible degron (AID)-tagged cell lines to analyze the activity of DDR proteins in mitosis. My approach will be innovative in that I will be able to deplete DDR proteins in mitosis with high temporal resolution. This will allow us to define the contributions of DDR proteins to mitotic progression and chromosome segregation independently of DNA damage.

Aim 1: Investigate the function and regulation of the MRN complex at prometaphase centromeres

Recent work from our lab and others has demonstrated that the DNA damage repair (DDR) pathway and mitotic machineries are interconnected and play important roles throughout the cell cycle to ensure genome stability. Our lab previously demonstrated that the mitotic R-loop-driven ATR pathway is active at prometaphase centromeres and is vital for proper chromosome segregation [1]. R-loops are three-stranded RNA-DNA hybrids that form when a nascent RNA transcript reanneals to its template DNA strand and displaces single-stranded DNA. The displaced single-stranded DNA is bound by the replication protein A (RPA) complex, which recruits ATR to mitotic centromeres via its interacting partner ATRIP [1,42]. ATR kinase activity at these R-loops promotes proper chromosome segregation [1]. It is currently unclear if other DDR proteins play a role in this pathway.

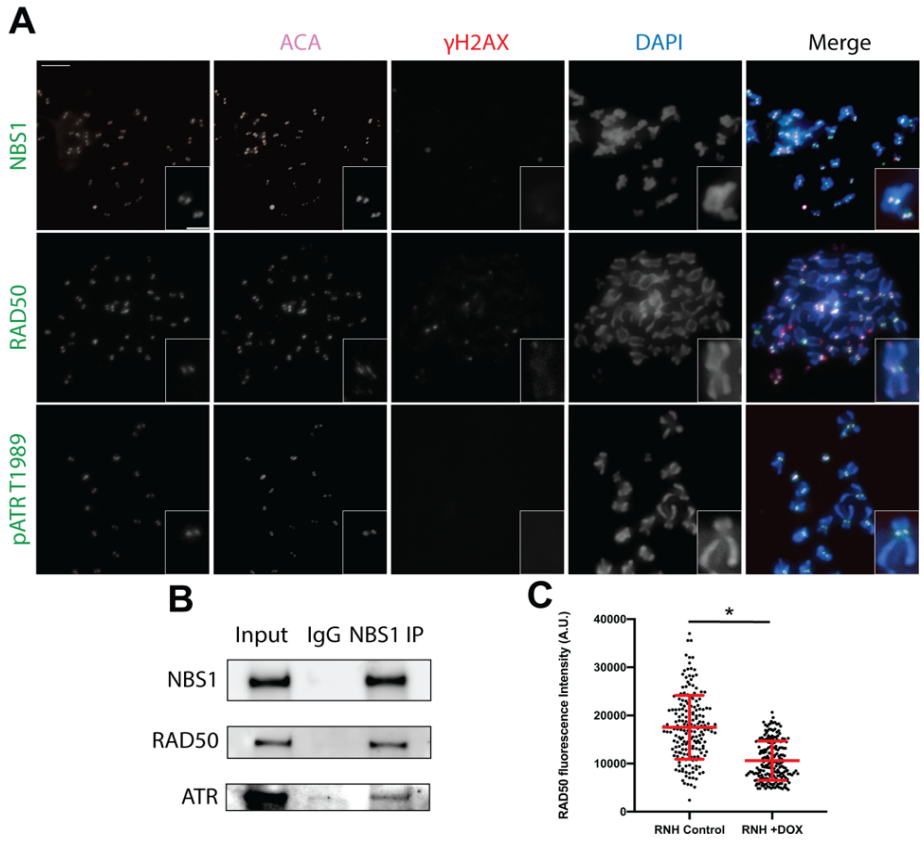

Figure 1. RAD50 and NBS1 are part of the mitotic R-loop pathway. A. NBS1 and RAD50 colocalize with active ATR (pATR T1989) at centromeres by immunofluorescence on unfixed prometaphase chromosome spreads; ACA, anti-centromere antibody. Scale bar: 5 μm large images, 2 μm inset **B.** NBS1 co-immunoprecipitates with ATR in nocodazole-arrested cells. **C.** RAD50 centromeric localization is dependent on R-loops, as RNaseH1 overexpression (RNH +DOX) decreases RAD50 localization to prometaphase centromeres. Dots mark RAD50 centromere intensity (A.U., arbitrary units) on one chromatid. Experiment performed once (n > 190). Error bars represent mean ± SD. *p ≤ 0.05, two-tailed *t* test.

My preliminary data demonstrate that RAD50 and NBS1 colocalize with active autophosphorylated ATR (pATR T1989) at prometaphase centromeres (Figure 1a). Importantly, this localization is independent of detectable DNA damage, which is marked by histone H2AX phosphorylation (γH2AX) (Figure 1a). Additionally, ATR coimmunoprecipitated with NBS1 in prometaphase cells (Figure 1b), suggesting that the MRN complex is interacting with ATR in a DNA damage-independent manner in prometaphase. Moreover, RAD50 localization to centromeres is also R-loop-dependent, as overexpressing doxycycline-inducible wild-type RNase H, which eliminates R-loops [1,43], significantly reduces RAD50 centromere localization as measured by immunofluorescence (Figure 1c). Based on these preliminary data and the MRN complex's role in interphase ATR activation following replication stress [4,6,17,44], I hypothesize that the MRN complex is activating ATR at prometaphase centromeres in a DNA damage-independent manner. In this aim, I will directly test whether the MRN complex functions as an upstream regulator of ATR activity at prometaphase centromeres (Aim 1.1) and how this interaction is temporally regulated (Aim 1.2) (Figure 2).

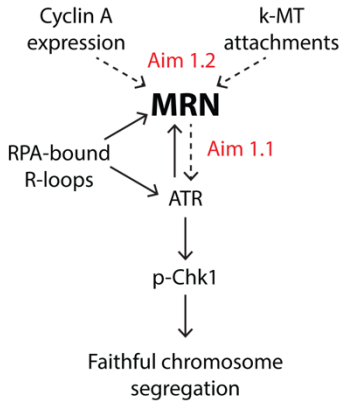

Figure 2. Schematic of Aim 1

Aim 1.1: Determine if the MRN complex is an upstream activator of ATR at prometaphase centromeres

The MRN complex promotes ATR kinase activity both in vitro and in vivo in response to DNA damage and replication stress [4-6,8,17-21]. I hypothesize that the MRN complex promotes ATR activity at prometaphase centromeres in a DNA damage-independent manner. I will test this hypothesis by generating auxin-inducible degron (AID)-tagged cell lines to acutely deplete the MRN complex in mitosis and then assay ATR kinase activity at prometaphase centromeres.

Develop cell lines to acutely deplete the MRN complex in mitosis

I will deplete the MRN complex using the AID system [9] and assay ATR activity at prometaphase centromeres. I will first use the CRISPR/Cas9 system to endogenously tag the members of the MRN complex in HCT116 cells with a tag that includes both a fluorescent protein and an AID. Upon addition of auxin in the presence of the plant-specific adaptor protein (Os)TIR1, AID-tagged proteins are polyubiquitinated and degraded [9]. This depletion method will give us high temporal resolution, as protein degradation in mitosis has been reported in as short as 4 minutes from the addition of auxin [45]. Because proteins will have a combination fluorescent protein and AID tag, live-cell fluorescence imaging will allow us to monitor protein degradation after adding auxin. Given the essential role of the MRN complex in interphase DNA damage repair and DNA replication, it is essential to deplete the complex specifically in mitosis, because mitotic errors can arise from unresolved DNA damage and underreplicated DNA in interphase [41]. Traditional depletion methods used to study MRN function, such as siRNA, shRNA, or hypomorphic mutations, are insufficient to identify the cause of mitotic errors in the absence of the functional MRN complex. However, the AID system will allow us to separate MRN mitotic functions from those related to interphase DNA damage repair.

MRN complex stability depends on the presence of all three proteins, as depletion of one of the proteins leads to codepletion of the others [2,5,18-21,44]. I expect that depleting any of the MRN complex members individually in mitosis will deplete or mislocalize the other complex members from centromeres, similar to what has been shown in interphase. To test this hypothesis, I will deplete the members of the complex individually and measure the centromeric localization of the other members by immunofluorescence. Furthermore, I will compare the effects of single versus combinatorial depletion of MRN complex members on ATR activity. I expect that combinatorial depletion will cause similar phenotypes as individual depletion, supporting the idea that members of the MRN complex are functioning together and are necessary for function. If I find that depleting individual complex members is sufficient to codeplete the complex, I will use the AID-tagged protein with the most rapid degradation as the model system for depleting the entire complex.

Use AID cell lines to determine if the MRN complex promotes ATR activity and faithful chromosome segregation at prometaphase centromeres

I will compare ATR activity at prometaphase centromeres with and without the MRN complex by staining chromosome spreads prepared from MRN-AID HCT116 cells with phosphoantibodies against the well-characterized ATR substrates Chk1 S317, RPA32 S33, and ATR T1989 (trans-autophosphorylation site) after addition of auxin or a vehicle control in nocodazole-arrested prometaphase cells. Nocodazole is a microtubule depolymerizing agent that triggers prometaphase arrest by activating the spindle assembly checkpoint. The phosphorylated species of Chk1 S317, RPA32 S33, and ATR T1989 are present at prometaphase centromeres when ATR is active [1]. I hypothesize that the MRN complex promotes ATR activity. Therefore, I expect to see a reduction in the amount of the phosphorylated ATR substrate at prometaphase centromeres when the MRN complex is depleted. By comparing the relative amounts of phosphorylated substrates in cells with and without auxin-induced depletion of MRN complex members, I will be able to determine if the MRN complex functions as an upstream regulator of ATR kinase activity in mitosis.

Loss of ATR signaling in mitosis increases the rate of lagging chromosomes, a specific mitotic error leading to chromosome missegregation [1]. I will test if depletion of the MRN complex also leads to an increased rate of lagging chromosomes, which will support my hypothesis that the MRN complex is functioning as an upstream regulator of ATR. I will measure the rate of lagging chromosomes with and without depletion of the MRN complex by stably expressing a fluorescently labelled histone (GFP-H2B) in MRN-AID-tagged HCT116 cells to visualize chromosome segregation. Specifically, I will arrest cells in G2 using a CDK1 inhibitor (RO-3306) to allow for complete DNA replication, add either a vehicle control or auxin to deplete the MRN complex, and then wash out the CDK1 inhibitor to allow cells to enter mitosis. I will then count the rate of lagging chromosomes in the subsequent anaphase using live-cell imaging. Although chromosome instability and mitotic defects have already been associated as clinical phenotypes of MRE11, RAD50, and NBS1 deficiency [46], it is currently unclear whether this is due to unresolved DNA damage from interphase or a distinct mitotic function of the MRN complex. I expect that MRN function is promoting faithful chromosome segregation in a DNA damage-independent manner in mitosis as part of the R-loop-driven ATR pathway, and therefore acute depletion of MRN in mitosis will lead to chromosome segregation errors independently of unresolved DNA damage or underreplicated DNA. Together, I expect that these experiments will provide insight into a novel function for the MRN complex in mitosis by promoting ATR activity.

Aim 1.2: Analyze how the MRN complex is temporally regulated at mitotic centromeres

RAD50 colocalizes with prometaphase centromeres but is not detected at metaphase centromeres by immunofluorescent staining (Figure 3), suggesting that RAD50 localization is temporally regulated in mitosis. ATR activity is also temporally regulated in mitosis, as it is essential for downstream Aurora B activity in prometaphase but not in metaphase [1]. The prometaphase-specific RAD50 localization to centromeres may provide a mechanism for differential ATR activity throughout mitosis. In this aim, I will investigate the mechanism for temporal localization of the MRN complex at centromeres. Understanding how the MRN complex is temporally regulated at centromeres will partially reveal how MRN acts within larger mitotic regulatory pathways to promote proper chromosome segregation.

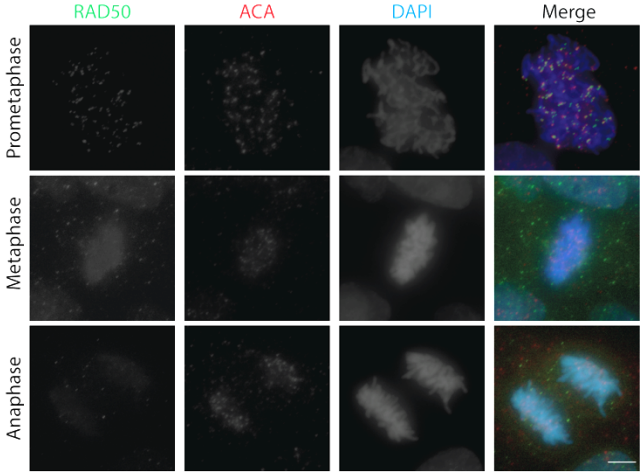

Figure 3. RAD50 centromere localization is prometaphase-specific. ACA, anti-centromere antibody. Scale bar, 5 μm.

The prometaphase to metaphase transition is characterized by the alignment of condensed chromosomes to the metaphase midzone. Additionally, cyclin A, which is highly expressed in early prometaphase, is rapidly degraded and is reduced to 40% of its prometaphase levels in metaphase, distinguishing prometaphase and metaphase as distinct biochemical states [10,11,47]. High expression of cyclin A in prometaphase destabilizes kinetochore-microtubule (k-MT) attachments. In contrast, k-MT attachments are stabilized in metaphase as cyclin A is degraded below a threshold value [11]. Thus, there are two key events that occur in the prometaphase to metaphase transition that I expect may influence MRN centromere localization: 1) degradation of cyclin A and 2) k-MT capture and biorientation. I hypothesize that these two events may be directing the temporal regulation of the MRN complex at centromeres.

<u>Determine if MRN centromere localization is cyclin A-dependent</u>

Prometaphase and metaphase are biochemically distinct states that can be distinguished by the expression of cyclin A. Given the observation that RAD50 localizes to centromeres in prometaphase, where cyclin A levels are high, and not in metaphase, where they are low [11](Figure 3), I hypothesize that MRN centromere localization may be positively regulated by cyclin A expression and cyclin-dependent kinase (CDK1) activity. The MRN complex is regulated by cyclin-CDK activity in interphase, as phosphorylation of NBS1 by CDK2 modulates downstream repair [48]. I will test whether cyclin A expression regulates MRN centromere localization by measuring NBS1 and RAD50 localization to centromeres during the course of nocodazole arrest. Although nocodazole-arrested cells do not progress through prometaphase to metaphase, cyclin A is degraded during prolonged SAC arrest [11,49]. I will synchronize cells in G2 using a CDK1 inhibitor (RO-3306) and then release them into nocodazole-containing media. I will isolate mitotic cells after 1, 4, 8, and 24 hours of nocodazole arrest and measure MRN localization to centromeres by immunofluorescent staining on chromosome spreads. If cyclin A is positively regulating MRN localization to centromeres, then I will see an inverse relationship between the time following nocodazole arrest and MRN intensity at centromeres. MG132 is a small molecule inhibitor of the proteasome that will prevent cyclin A degradation during nocodazole arrest. As a control, I will add MG132 to cells 1 hour after CDKi release into nocodazole to allow cells to enter mitosis but not degrade cyclin A, and then measure how MG132 treatment modulates MRN localization compared to untreated nocodazole-arrested cells, which I expect that this will increase MRN centromere localization.

I will also test the effect of cyclin A expression on MRN centromere localization by ectopically expressing a nondegradable variant of cyclin A that lacks the degradation box necessary for targeting by the proteasome or overexpressing the wild-type protein, both of which have been shown to be sufficient to induce a prometaphase-like biochemical state even after chromosome alignment [11]. By comparing MRN centromere localization in nocodazole-arrested chromosome spreads between these nondegradable cyclin A constructs and the endogenous cyclin A, I will further confirm the effect of cyclin A expression on MRN centromere localization. Inversely, I will endogenously tag cyclin A with an AID tag and compare MRN prometaphase centromere localization between addition of auxin or a vehicle control. If I observe more localization of the MRN complex to centromeres with auxin and cyclin A degradation, this would support the hypothesis that MRN localization to the centromere is regulated by cyclin A expression.

Determine if MRN localization to centromeres is responsive to k-MT attachments

During prometaphase, kinetochores form unstable attachments with spindle microtubules that are important for aligning the chromosomes along the midzone in metaphase. My preliminary analysis of MRN centromeric localization suggests that k-MT attachments are not necessary for MRN centromere localization, as immunofluorescent staining of chromosomes from nocodazole-arrested cells demonstrates robust RAD50 and NBS1 localization (Figure 1a). This observation prompts the hypothesis that MRN colocalization with centromeres is negatively regulated by k-MT attachments, as most kinetochores are largely unattached in prometaphase but are attached in metaphase.

I will test this hypothesis by comparing MRN centromere localization in nocodazole- and taxol-arrested cells. Whereas nocodazole depolymerizes microtubules, taxol stabilizes microtubules. By comparing MRN localization to centromeres in these two conditions, I will determine how microtubule binding to kinetochores influences MRN localization. If MRN centromere localization is negatively regulated by k-MT attachments, I will observe less MRN localization in taxol-arrested cells compared to nocodazole-arrested cells.

Additionally, I will validate these findings by perturbing the machinery that links the kinetochore to spindle microtubules. NDC80 is a flexible linker protein that is responsible for bridging the kinetochore and spindle microtubules. I will endogenously tag NDC80 with an AID tag, arrest cells in G2, deplete NDC80 by addition of auxin, and then release cells into mitosis. NDC80 depletion will eliminate k-MT attachments, which I predict will mimic nocodazole arrest, as kinetochores will remain unattached to microtubules. I expect to observe that NDC80 depletion enhances MRN localization to centromeres, supporting the hypothesis that MRN centromere localization is negatively regulated by k-MT attachments.

Together, these experiments will determine whether cyclin A and k-MT attachments regulate MRN localization to the centromere specifically in prometaphase. Identifying the mechanism by which the MRN complex is temporally regulated to centromeres is essential for understanding how MRN and ATR activity is regulated in mitosis.

Potential pitfalls and alternative approaches: It is possible that I will not be able to make endogenously tagged homozygous AID cell lines with CRISPR. If this occurs, I will make stable knock-down cell lines with lentiviral infections of shRNA and then express a plasmid containing the ORF for a shRNA-resistant AID-tagged protein, which, similarly to the endogenously tagged protein, will be depleted with addition of auxin.

Additionally, members of the MRN complex may have mitotic functions independent of one another. This is highly unlikely, as protein stability for each of the complex members is dependent on the presence of the others. Other studies have indicated that overexpressing NBS1 when MRE11 is depleted is sufficient to rescue NBS1 expression and restore NBS1-specific function in DT-40 cells [7]. If I suspect that members of the MRN complex have functions independent of one another, I will deplete the complex with AID and systematically overexpress other complex members to test for restoration of function. If the entire complex is not necessary for centromere function, then I expect a rescue after overexpressing individual complex members.

Aim 2. Investigate how noncanoncial functions of the MRN complex are coordinated in mitosis

In addition to MRN's hypothesized role in the mitotic ATR pathway, previously published work suggests that the MRN complex regulates the mitotic spindle through its association with the kinase PLK1 [2,3]. However, it remains unclear how MRN activity and localization to mitotic spindles is regulated. Here, I will explore the function of the MRN complex at spindle poles. I will determine whether PLK1 phosphorylates members of the MRN complex and how this phosphorylation affects MRN spindle localization and the consequences for proper spindle establishment (Aim 2.1). I will determine the mechanism by which PLK1 phosphorylation of the MRN complex affects spindle formation by identifying how phosphorylation at these sites on the MRN complex affect its interactions with other proteins (Aim 2.2). Together, these experiments will reveal the functional consequences of MRN phosphorylation by PLK1 and will provide a mechanistic understanding of how the MRN complex promotes spindle assembly.

Aim 2.1. Identify the PLK1-dependent phosphorylations sites on MRN and whether phosphorylation regulates MRN localization to spindle poles

My preliminary data and previously published work demonstrate that components of the MRN complex localize to spindle poles and interact with PLK1, an essential kinase necessary for proper and efficient spindle formation [2,50](Figure 4). Specifically, PLK1 phosphorylates MRE11 in vitro, and this phosphorylation is important

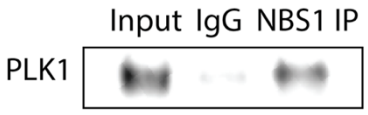

Figure 4. PLK1 and NBS1 interact in prometaphase-arrested cells by coimmunoprecipitation.

for regulating spindle assembly in vivo [2]. PLK1 also extensively phosphorylates the C-terminus of MMAP, a newly identified protein that interacts with the MRN complex in mitosis and is essential for prometaphase spindle formation [2]. Moreover, a whole cell mass-spectometry screen demonstrated a decrease in NBS1 phosphorylation at (T485, S486, S488) in mitotic cells treated with PLK1 inhibitor [51]. These data demonstrate a connection between the MRN complex and PLK1 in mitosis, although we currently lack a comprehensive understanding of how PLK1 phosphorylates the MRN complex in vivo. Furthermore, the functional significance of these phosphorylation events are unknown. I hypothesize that PLK1 directly phosphorylates members of the MRN complex to regulate its localization to, and function at, spindle poles.

To test this hypothesis, I will determine the phosphorylation status of the cytoplasmic MRN complex in the presence and absence of the PLK1 inhibitor BI2536 (PLK1i). I will use subcellular fractionation to isolate the cytoplasmic protein population and then use immunoprecipitation followed by mass spectrometry to identify their phosphorylation status. This approach will focus mass spectrometry sequencing depth on members of the MRN complex to more clearly identify peptide modifications. By isolating the cytoplasmic fraction, I expect to isolate the spindle pole-associated, rather than the centromeric, MRN complex, which is likely regulated by other mitotic kinases and may introduce heterogeneous signal in the mass spectrometry reading. These data will allow me to identify PLK1-dependent phosphorylation sites on the MRN complex that may have functional consequences for regulating spindle dynamics.

I will confirm the mass spectrometry findings using immunofluorescence and western blotting with phospho-specific antibodies against phosphorylated residues where high-quality commercial antibodies are available. Once confirmed, I will test whether these modifications are necessary and sufficient to drive MRN localization to spindle poles. Although PLK1i treatment has already been shown to affect MRE11 spindle localization [2], how individual phosphosites affect spindle localization is currently unknown. I will clone the open reading frame of MRN complex members with phosphomimetic or phosphonull mutations at identified phosphosites into a doxycycline-inducible and fluorescently tagged vector and test whether these modifications are necessary (phosphonull) or sufficient (phosphomimetic) to drive protein localization by expressing these proteins in mitosis and tracking their localization with live-cell imaging. I expect that phosphomimetic, but not phosphonull, mutants will localize to the spindle.

Proper spindle establishment and morphology are essential for proper chromosome segregation. I will use live-cell imaging of MRN phosphomutants to visualize spindle establishment and determine how MRN phosphomutants affect chromosome segregation. I will arrest cells expressing GFP-H2B and mCherry-alpha tubulin in G2 using a CDK1 inhibitor, add doxycycline to induce expression of phosphomutants or a wild-type control, and then wash out CDK1 inhibitor to allow cells to enter mitosis. Consistent with the data from MMAP phosphonull mutants, I expect that expressing MRN phosphonull mutants will compromise spindle establishment and lead to asymmetric metaphase spindles [2]. PLK1 is also important for regulating the time to complete mitosis. I expect that MRN phosphonull mutants will phenocopy PLK1 inhibition and have a prolonged mitosis, consistent with phenotypes of MRN deficiency [3,52]. I will also assess chromosome segregation defects associated with phosphomutants by counting the rate of lagging chromosomes in the subsequent anaphase using live-cell imaging. I expect that phosphonull, but not phosphomimetic, mutants will have spindle establishment and chromosome segregation defects.

Finally, I will test whether the phosphorylated MRN complex is sufficient to rescue defects associated with PLK1i by comparing spindle establishment in PLK1i cells when MRN phosphomimetic mutants are expressed. If PLK1 regulates spindle establishment through the MRN complex, I expect to that expressing phosphomimetic mutants in PLK1i-treated mitotic cells will rescue the spindle establishment defect associated with PLK1i [50]. Given that PLK1 has many centrosomal targets that may be independent of the MRN complex, I expect that MRN phosphomutants will partially rescue PLK1i spindle establishment defects. These experiments will demonstrate that MRN phosphorylation by PLK1 in mitosis is important for regulating spindle establishment.

Aim 2.2 Identify how PLK1-dependent MRN phosphorylation affects MRN protein interactions.

I expect that PLK1-dependent phosphorylation of the MRN complex is important for MRN localization to spindle poles and proper spindle establishment (Aim 2.1). However, we do not currently understand how the MRN complex acts downstream of PLK1 to regulate proper spindle establishment. I hypothesize that PLK1-dependent phosphorylation of the MRN complex increases its affinity for microtubule regulators, such as KIF2A, that are important for regulating the spindle.

I will identify NBS1-interacting proteins by proximity ligation and mass spectrometry to increase our understaning of how the MRN complex functions with PLK1 to promote spindle establishment. I will first transiently express a construct in which doxycycline-inducible NBS1 is tagged with ascorbate peroxidase (APEX), which, upon addition of hydrogen peroxide, leads to the covalent addition of a biotin molecule on proteins within 20 nm [53]. I will tag NBS1 rather than RAD50 or MRE11 because NBS1 serves as the scaffolding component of the MRN complex and is therefore more likely to capture biologically meaningful interactions between the complex and other proteins. APEX labelling is preferable to other biotin labelling methods, such as BioID, because the labelling reaction is performed within minutes, allowing high temporal resolution. To identify molecules that interact with NBS1 in mitosis, I will arrest cells in G2 with a CDK1 inhibitor, release them into mitosis, isolate mitotic cells by shake off, and then add hydrogen peroxide to mitotic cells to biotinylate proteins within 10 nm of NBS1-APEX. Biotinylated proteins can be isolated and identified by streptavidin pull down followed by mass spectrometry. As a negative control, I will perform the same experiment in untagged cells. I expect that this approach will identify known NBS1-interacting proteins, such as RAD50, MRE11, and PLK1, as well as novel interacting partners. It is also likely that I will identify both centromeric and centrosomal proteins, which will further support the MRN localization data from Aim 1 and 2.1 [2](Figures 1a, 3) in an antibody-independent manner.

To determine how PLK1-dependent phosphorylation of the MRN complex affects its protein interactions, I will make NBS1 constructs with an APEX tag and phosphonull and phosphomimetic mutations at the sites identified in Aim 2.1. Following the experimental approach described above, I will identify what proteins the phosphomutant NBS1 interacts with in mitosis. I expect that the phosphomimetic mutant will resemble the wild-type NBS1 and interact with centrosomal proteins. I expect that the phosphonull mutant, however, will have relatively fewer interactions with centrosomal proteins.

I will confirm candidate protein association with NBS1 by performing co-immunoprecipitation against wild-type NBS1 followed by western blotting for the candidate protein. Reciprocal immunoprecipitations will also be performed. From the list of confirmed NBS1-associated proteins, I will prioritize hits for functional studies by performing gene ontology (GO) and STRING network analysis to identify proteins that are known to regulate microtubule dynamics and mitotic spindle establishment.

Once confirmed, I will test how NBS1 phosphorylation affects protein binding by immunoprecipitating against the fluorescent tag on wild-type or phosphomutant NBS1 and blotting for the candidate protein. Associated proteins that are bona fide interactors and sensitive to NBS1 phosphorylation will immunoprecipitate with wild-type and phosphomimetic NBS1, but to a lesser extent with phosphonull NBS1. These experiments will not only identify NBS1-interacting proteins in mitosis, but will also allow us to form detailed hypotheses about the mechanism by which PLK1 and NBS1 promote proper spindle assembly in mitosis.

Potential pitfalls and alternative approaches: PLK1 regulates other mitotic kinases that may also phosphorylate the MRN complex. Thus, our PLK1i-based identification of PLK1 phosphorylation sites may also identify phosphorylations installed by other kinases. I will prioritize studying PLK1-dependent sites on the MRN complex that contain the canonical PLK1 motif, D/E-X-S/T [54]. To confirm that PLK1-dependent phosphorylation sites are bona fide PLK1 targets, I can perform an in vitro PLK1 kinase assay with MRN peptides as substrates.

I do not expect difficulty identifying MRN-associated proteins with APEX labelling; however, this method may fail to capture all MRN-associated proteins due to the small radius around which APEX acts (<20 nm). RAD50 can extend 50 nm away from the site that associates with MRE11 and NBS1, so distal interactions with RAD50 will not be identified by APEX. Given that most MRN-interacting partners associate with NBS1 and not the distal ends of RAD50, I do not expect that NBS1-APEX labelling will cause us to miss biologically relevant protein interactions. If I wish to identify proteins that interact with RAD50, I can directly immunoprecipitate RAD50 and identify associated proteins by mass spectrometry. This approach will generate a higher background signal and may make it more difficult to identify direct and relevant MRN-interacting proteins, but I expect that it will also generate a more comprehensive list of MRN-interacting proteins.

Ext Aim 1. Investigate the structural basis for NBS1-mediated ATR activation

ATR must interact with other DDR proteins for full activation. The MRN complex interacts with ATR through NBS1 and has been shown to promote ATR activity. In vitro, NBS1 is sufficient to significantly increase ATR kinase activity towards the ATR substrate Chk1 [7]. Here, I will investigate the structural mechanism for how NBS1 promotes ATR activity.

Determine the structural basis for ATR activation by NBS1

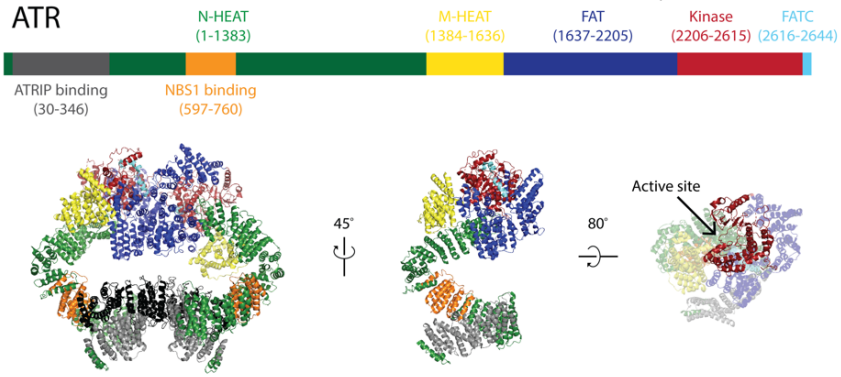

Figure 5. Structure of the ATR-ATRIP complex. Top) Domain map of human ATR. Bottom) Ribbon diagram of ATR-ATRIP complex (PDBID:DYZ0) [55] colored according to ATR domain map with monomers in different orientations. ATRIP dimer shown in black.

Two independent studies have identified non-overlapping fragments within the N-terminal half of NBS1 that can bind ATR in vivo, indicating that there are several regions within the N-terminus of NBS1 that are sufficient, but not necessary, to bind ATR [5,7]. NBS1 binds to ATR at residues 597-760, which fall in the largely uncharacterized N-HEAT repeat domain, an alpha-helix-rich region of the protein that connects the N-terminal ATRIP-interacting region and kinase domain (Figure 5)[7]. Given these data, as well as a spatial understanding of where on ATR NBS1 interacts, I hypothesize that NBS1 promotes ATR activation via an allosteric mechanism.

The overall structure of the heterotetrameric (2:2) ATR-ATRIP complex has been solved by cryo-EM at 4.7 Å resolution, and the C-terminal region of ATR (residues 1521-2644) containing the kinase domain has been determined at 3.9 Å resolution [55]. This structural analysis, however, was performed without ATR activators, conditions in which ATR lacks robust kinase activity [23]. Because I am interested in how NBS1 promotes ATR activity, I will compare ATR conformations, especially of the kinase domain, in the presence or absence of NBS1 to determine the mechanism by which NBS1 promotes ATR activity. I hypothesize that NBS1 binding promotes ATR activity by inducing a structural change to the active site of ATR. I will test this hypothesis by determining the structure of the ATR-ATRIP complex in the presence or absence of the NBS1 peptide using cryo-EM.

I will first purify full-length ATR and ATRIP from human cells using the protocol described previously [55]. I will separately express and purify the N-terminal half (FHA and BRCT domains, residues 1-325) of NBS1 in 293T cells. I will use the N-terminal half of NBS1 because this fragment has been shown to be sufficient for ATR interaction and activation in vitro [5,7] and because the middle and C-terminus of NBS1 contains intrinsically disordered regions (Figure 6) that will likely challenge structural analysis.

Before structural analysis, I will confirm that I have isolated a functional ATR-ATRIP complex by performing an in vitro kinase assay with purified NBS1, ATR-ATRIP, and a synthesized Chk1 peptide as a substrate. If protein purification has successfully isolated functional proteins, ATR will be able to phosphorylate the Chk1 peptide, and ATR kinase activity will increase in the presence of NBS1, determined by the relative amount of phosphorylated Chk1. Size-exclusion chromatography will be used to isolate stoichiometric ATR-ATRIP-NBS1 or ATR-ATRIP complexes from unbound proteins. Before imaging the protein complexes to generate their structures, I will use negative-stain electron microscopy to confirm that our protein complexes are of sufficient quality for cryo-EM analysis. Finally, I will determine the structure of active (ATR-ATRIP-NBS1) and inactive (ATR-ATRIP) complexes by electron microscopy imaging and generating a comparative homology model to the recently solved structure of the ATR-ATRIP complex [55].

I expect that the ATR kinase domain and active site structures will be different when NBS1 is bound to ATR. NBS1 binding may promote ATR activity by inducing a structural change to increase the thermodynamic favorability of the kinase reaction. This may occur if NBS1 binding changes the affinity between ATR and its substrate or the ATP/ADP nucleotide. Alternatively, NBS1 may cause a change in a regulatory structure within the kinase to allow substrate binding in a more favorable conformation for the phosphorylation to occur. If I do not observe changes to active site conformation in the presence of NBS1, this would suggest that NBS1 mediates ATR activation by a more indirect method, such as by increasing the affinity between ATR and other ATR activators, for example. As a negative control, I will perform structural analysis of ATR-ATRIP along with a NBS1 mutant that cannot bind ATR (NBS1 R28A Y176A E309A) [5]. If I observe changes in te ATR active site conformation in the presence of wild-type NBS1, but not ATR-binding deficient mutants, that would support the hypothesis that NBS1 binding promotes ATR activation.

I will assess the quality of these structures by performing Fourier Shell Correlation (FSC) analysis on our structures to compare the consistency within the data, with a more consistent dataset reflecting a higher quality structure. I expect to determine the structural basis for ATR activation by NBS1 using this method. In total, these experiments will provide a mechanistic understanding of how NBS1 promotes ATR activity.

Potential pitfalls and alternative approaches: I do not expect that RAD50 and MRE11 are directly contributing to ATR activation, as they have not been demonstrated to directly bind to or activate ATR in vitro. However, the MRN complex is most stable when all members are present [2,5,18-21,44]. If I experience difficulty determining the structure of NBS1-bound ATR-ATRIP due to NBS1 instability, I can include purified full-length NBS1, MRE11, and RAD50 to promote stability of the entire complex.

In addition to being an activator of ATR, NBS1 is also a substrate [5,6]. Therefore, our structural analysis of the NBS1/ATR interaction might be challenged if the NBS1 peptide has a stable interaction with the ATR active site. I do not expect this to be a challenge because an ATR fragment containing only the kinase domain does not interact strongly enough with NBS1 to confer a positive result in a yeast two-hybrid assay [7]. If I do encounter problems with NBS1-ATR interactions in the active site, I can mutate the ATR consensus sequence ([phospho]S/T-Q) to preclude recognition by ATR.

If I am unable to generate a structure with sufficient resolution to identify changes in ATR kinase domain conformation, I will chemically crosslink purified ATR-ATRIP complex in the presence and absence of N-terminal NBS1 and use mass spectrometry to identify how intramolecular interactions in ATR change with NBS1 binding. To better understand if NBS1 affects binding kinetics between ATR and its substrates or ADP/ATP, I can use isothermal titration calorimetry (ITC) to compare the thermodynamics and kinetics of these binding reactions in the presence and absence of NBS1. These data will determine if NBS1-mediated ATR activation is due to conformational changes in ATR and/or a more thermodynamically favorable catalytic reaction.

Ext Aim 2. Determine the contribution of phase separation to coordinated MRN function in mitosis

Liquid-liquid phase separation (LLPS) is a biophysical phenomenon that drives the organization of membraneless subcellular compartments [56,57]. Well-known examples of this organization include the nucleolus and stress-induced p-granules. LLPS occurs when the homotypic interactions between solute or solvent molecules are more energetically favorable than heterotypic interactions between solute and solvent [56]. This typically occurs in poor solvents, such as the concentrated cytoplasm [58,59]. LLPS is a rapid, reversible, and energy-efficient way to regulate biochemical processes [60]. It can be highly selective for proteins and nucleic acids, and thus can rapidly increase the kinetics of biochemical reactions [57].

Work in recent years has identified a role for LLPS in regulating essential mitotic functions, such as microtubule spindle dynamics and activation of Aurora A and B [61-64], two regulatory kinases important for proper mitotic progression. There is strong evidence that two mitotic structures, the centrosome and inner centromere, behave as phase separated domains both in vivo and in vitro [61-65]. Members of the MRN complex localize to centrosomes and centromeres in mitosis [2](Figure 1a), prompting the hypothesis that the MRN complex might also be regulated by phase separation. These data, along with primary sequence features of MRN complex members, prompt the hypothesis that the MRN complex can phase separate under physiological conditions. I will test this hypothesis by exploring MRN phase separation in vitro (Ext aim 2.1) and in vivo (Ext aim 2.2). Together, I expect that these experiments will demonstrate that phase separation is an important regulator of MRN in mitosis.

Aim 2.1 Determine if MRN demonstrates phase separation in vitro and how different protein regions affect coacervate formation

There are two main types of inter- and intra-molecular forces thought to determine a macromolecule's ability to phase separate: the strong, specific interactions typically found in folded domains, such as between a ligand and its receptor, and weak, nonspecific interactions generally found in intrinsically disordered regions (IDRs) [60,66-72]. The MRN complex has many features that make it a promising candidate for phase separation and coacervate formation. All three members of the complex contain intrinsically disordered regions as predicted by IUPRED [73,74](Figure 6), are capable of oligomerization [75], and have a known spatial and functional relationship to bona fide phase-separated domains (PSDs). Additionally, NBS1 contains two breast cancer-associated C terminal (BRCT) domains, which in another DDR protein 53BP1, were shown to promote phase separation [76]. Here, I will test whether the MRN complex can coacervate in vitro and identify the protein regions that promote this biophysical behavior.

Determine whether purified MRN complex can coacervate in vitro

Analysis of MRN primary sequence and understanding of its relationship to other phase separated domains strongly suggests that MRN can act as a phase-separated domain. However, this has yet to be tested experimentally. I will first test whether MRN is able to phase separate in a minimal in vitro reconstitution system.

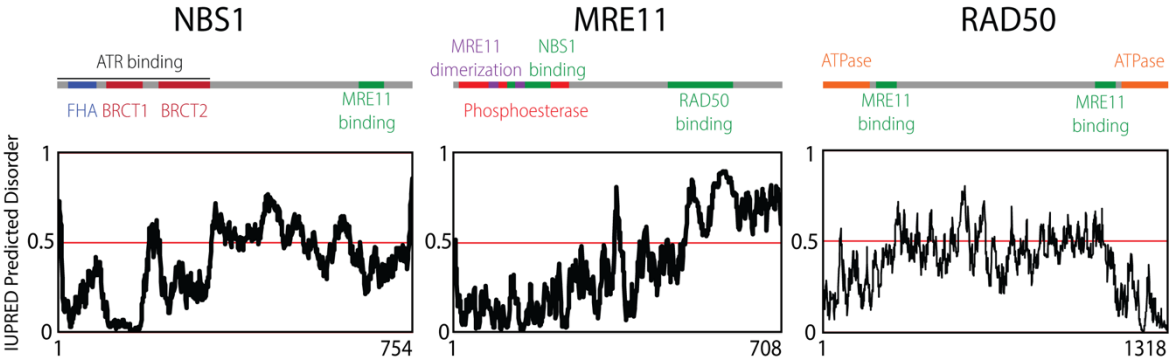

Figure 6. Intrinsic disorder prediction for MRN complex proteins. Top, domain maps of each member of the MRN complex. Bottom, disorder score for 30 residue sliding window calculated by IUPRed2A [73, 74]. FHA, fork head-associated. BRCT, breast cancer-associated C-terminal.

I will fluorescently tag each member of the MRN complex with a unique fluorescent protein (such as GFP, RFP, and BFP) and purify the complex from mitotic human 293T cells using protocols described previously [77]. To test if MRN is capable of forming physiologically relevant PSDs, I will increasingly concentrate the complex and determine if there is a threshold at which MRN becomes an inhomogeneous mixture. It is important to maintain physiological conditions that mimic the biochemical environment of the cytoplasm. If I observe demixing, evident by the formation of characteristic droplets within the mixture, the concentration of the MRN complex in the dense phase (protein droplets) relative to the light phase (buffer) will be assessed by measuring fluorescence from the tagged proteins. Additionally, I will purify the fluorescent tags alone as a negative control, as they are not able to form phase separated domains in vitro and therefore should not form condensates. Another indication that condensates are bona fide phase separated domains is their ability to deform, fuse, and fission, all properties of liquids. I will observe this behavior with time-lapse imaging of purified fluorescently tagged proteins. I expect that under physiological conditions, MRN will form condensates that display these liquid-like behaviors.

Additionally, I will test whether condensates are sensitive to 1, 6-hexanediol treatment. Hexanediol is frequently used as a chemical method to acutely disrupt liquid condensates, as it interferes with the weak hydrophobic interactions, particularly between aromatic phenylalanine residues, that often promote LLPS [64,76,78-80]. Reversible disruption of condensate formation in vitro with hexanediol treatment will support the hypothesis that condensates form via LLPS and that hydrophobic interactions are important for this property. A negative result from this assay, however, does not preclude LLPS as a driving force for coacervate formation, as other types of interactions, such as pi-cation and electrostatic interactions, are not disrupted by hexanediol.

Identify the regions of the MRN complex that are necessary to drive coacervate formation

If I discover that the MRN complex coacervates in vitro, I will next identify the minimal protein regions that are sufficient to drive coacervation. I will first purify individual members of the complex and test whether all components are necessary for coacervate formation, which would suggest that phase separation is driven by intermolecular forces. If the entire complex is necessary for phase separation, I will demonstrate that intermolecular interactions are driving coacervation by disrupting the MRN intracomplex interaction regions, which have already been identified (Figure 6). I will then purify these truncated proteins and test for coacervation using the approach described above. If these intracomplex interactions are necessary for coacervate formation, then truncated mutants should no longer be able to coacervate.

I will also identify whether IDRs are necessary for coacervate formation. IDRs are common in phase separating proteins [66,68-71] and form interactions mainly through hydrogen bonding between polar amino acids [81]. For example, mutating serine residues to alanine in the IDR of the protein BuGZ, a mitotic microtubule-binding protein, disrupts coacervate formation without compromising its microtubule-binding function [61-63]. I will disrupt IDRs in MRE11, NBS1, and RAD50 by mutating serine residues to alanine, purifying the protein, and analyzing coacervate formation. I expect that mutating these residues will disrupt the protein's ability to form phase separated domains. Together, these experiments will identify how different protein regions contribute to the MRN complex's biophysical properties and the types of interactions driving coacervation.

Ext Aim 2.2 Determine if MRN coacervates in vivo and its relevance for mitotic function

To understand the functional consequences of MRN phase separation, I will first demonstrate that the MRN complex forms coacervates in vivo. Analysis from our lab and others have identified that members of the MRN complex form foci at mitotic centromeres (Figure 1) and centrosomes [2]. I will test whether these foci are sensitive to disassembly by treatment with 1,6-hexanediol, which disrupts weak hydrophobic interactions and has been shown to reversibly disassemble some PSDs [64,76,78-80]. I will arrest cells in prometaphase with

nocadazole and treat with hexanediol to observe whether the focal accumulation observed in unperturbed cells at spindle poles and centromeres dissipates with chemical treatment. Furthermore, I will test whether the disassembly of PSDs is reversible by washing out hexanediol and observing whether the MRN complex can resume focal accumulation using live-cell fluorescence microscopy. I hypothesize that MRN focal accumulation at centromeres and centrosomes is driven by coacervation. Therefore, I expect that treatment with hexanediol will reversibly disassemble these foci, providing support for the observation that these foci act as PSDs.

An emerging method to test coacervation in vivo has been with optogenetic tools, such as optoDroplet [82]. In this system, the protein or fragment of interest is cloned with fused mCherry and partial Cry2. Cry2 is a protein originally identified in *Arabidopsis thaliana* that dimerizes upon exposure to blue light. Proteins that are capable of coacervation will form condensates in vivo when fused to Cry2 and exposed to blue light, whereas the mCherry protein alone is incapable of forming condensates under these conditions. This system, which is both reversible and tunable, can determine if individual proteins of the MRN complex are capable of forming condensates within the biochemical cellular environment. If I observe that full length MRN proteins are form condensates following blue light exposure, this will not only support the hypothesis that members of the MRN complex are capable of coacervation in vivo, but will also allow us to reversibly manipulate the timing and degree of condensate formation in vivo for future functional studies. A caveat of this experiment is that blue light dimerization can introduce extra interactions between peptide molecules that do not occur in physiological conditions and may artifactually induce phase separation.

Although LLPS domains are biophysically heterogeneous and difficult to define, one feature common to all LLPS domains is the ability to change volume (through fission and fusion) while maintaining a constant concentration. I will test if putative MRN complex coacervates have this property by cloning fluorescently tagged MRN complex members behind a doxycycline-titratable promoter and using live-cell imaging and fluorescence correlation spectroscopy (FCS) to measure and calculate how the volume and concentration of fluorescent concentrates changes over time. I expect that as the MRN concentration increases above a critical threshold it will form coacervates that are able to fuse and fission. If I observe that members of the MRN complex form coacervates that maintain a constant concentration while changing volume, this will strongly support the hypothesis that MRN forms bona fide coacervates in the cellular environment. Together, these experiments will determine whether the MRN complex is capable of coacervation in vivo. This will help us understand how the MRN complex is coordinated to promote proper chromosome segregation in mitosis.

Potential pitfalls and alternative approaches: It is possible I will not be able to concentrate the MRN complex enough in vitro to observe coacervation. One common method to induce phase separation in vitro is treatment with a crowding agent such as polyethylene glycol (PEG), which facilitates phase separation by increasing the effective protein concentration [64,67,83,84]. However, I am wary of this approach because crowding agents may increase the concentration beyond the cellular concentration [85]. Thus, I will first test MRN phase separation in vitro without crowding agents, but if we fail to observe coacervates, will use low concentrations of PEG.

MRN coacervation might be dependent on phosphorylation. Phosphorylation has been shown to directly modulate LLPS properties, such as by adjusting the concentration threshold at which proteins phase separate [64] or disassembling coacervates in mitosis [65]. I will use MRN proteins purified from mitotic human cells, so proteins will likely be phosphorylated. To promote coacervate formation, I can express and purify mutants with phosphomimetic mutations at residues identified to be phosphorylated in mitosis.

Conclusions and impact

The work proposed here will reveal novel functions of the MRN complex in promoting genome stability in mitosis. I expect that Aim 1 will identify a role for the MRN complex in ATR activation at prometaphase centromeres (Aim 1.1) and how the centromere localization of the MRN complex is temporally regulated (Aim 1.2). Aim 2 will explore how PLK1 regulates the MRN complex at spindle poles and centrosomes (Aim 2.1) and a mechanistic basis for how PLK1 phosphorylation affects MRN function in spindle establishment (Aim 2.2). Work from extended aim 1 will reveal the structural mechanism for ATR activation by the MRN complex. Finally, extended aim 2 will test whether the MRN complex is capable of coacervation in vitro (Ext aim 2.1) and in vivo (Ext aim 2.2), which I expect will reveal a novel regulatory mechanism for the localization and function of the MRN complex in mitosis. These mitotic and DNA damage-independent functions, together with the well-studied function of the MRN complex in interphase DDR, will demonstrate that the MRN complex has dual function in promoting genome stability, making it a promising candidate to target genomically unstable cancers.

References:

1 Kabeche, L., Nguyen, H. D., Buisson, R. & Zou, L. A mitosis-specific and R loop-driven ATR pathway promotes faithful chromosome segregation. *Science* **359**, 108-114 (2018).

2 Xu, R., Xu, Y., Huo, W., Lv, Z., Yuan, J., Ning, S., Wang, Q., Hou, M., Gao, G., Ji, J., Chen, J., Guo, R. & Xu, D. Mitosis-specific MRN complex promotes a mitotic signaling cascade to regulate spindle dynamics and chromosome segregation. *Proc Natl Acad Sci U S A* **115**, E10079-E10088, doi:10.1073/pnas.1806665115 (2018).

3 Rozier, L., Guo, Y., Peterson, S., Sato, M., Baer, M., Gautier, J. & Mao, Y. The MRN-CtIP Pathway is Required for Metaphase Chromosome Alignment. *Molecular Cell* **49**, 1097-1107 (2013).

4 Stiff, T., Reis, C., Alderton, G. K., Woodbine, L., O'Driscoll, M. & Jeggo, P. A. Nbs1 is required for ATR-dependent phosphorylation events. *EMBO J* **24**, 199-208, doi:10.1038/sj.emboj.7600504 (2005).

5 Olson, E., Nievera, C. J., Lee, A. Y., Chen, L. & Wu, X. The Mre11-Rad50-Nbs1 complex acts both upstream and downstream of ataxia telangiectasia mutated and Rad3-related protein (ATR) to regulate the S-phase checkpoint following UV treatment. *J Biol Chem* **282**, 22939-22952, doi:10.1074/jbc.M702162200 (2007).

6 Manthey, K. C., Opiyo, S., Glanzer, J. G., Dimitrova, D., Elliott, J. & Oakley, G. G. NBS1 mediates ATR-dependent RPA hyperphosphorylation following replication-fork stall and collapse. *J Cell Sci* **120**, 4221-4229, doi:10.1242/jcs.004580 (2007).

7 Kobayashi, M., Hayashi, N., Takata, M. & Yamamoto, K. NBS1 directly activates ATR independently of MRE11 and TOPBP1. *Genes Cells* **18**, 238-246, doi:10.1111/gtc.12031 (2013).

8 Shiotani, B., Nguyen, H. D., Hakansson, P., Marechal, A., Tse, A., Tahara, H. & Zou, L. Two distinct modes of ATR activation orchestrated by Rad17 and Nbs1. *Cell Rep* **3**, 1651-1662, doi:10.1016/j.celrep.2013.04.018 (2013).

9 Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nat Methods* **6**, 917-922, doi:10.1038/nmeth.1401 (2009).

10 den Elzen, N. & Pines, J. Cyclin A is destroyed in prometaphase and can delay chromosome alignment and anaphase. *J Cell Biol* **153**, 121-136, doi:10.1083/jcb.153.1.121 (2001).

11 Kabeche, L. & Compton, D. A. Cyclin A regulates kinetochore microtubules to promote faithful chromosome segregation. *Nature* **502**, 110-113, doi:10.1038/nature12507 (2013).

12 Taylor, A. M., Shih, J., Ha, G., Gao, G. F., Zhang, X., Berger, A. C., Schumacher, S. E., Wang, C., Hu, H., Liu, J., Lazar, A. J., Cancer Genome Atlas Research, N., Cherniack, A. D., Beroukhim, R. & Meyerson, M. Genomic and Functional Approaches to Understanding Cancer Aneuploidy. *Cancer Cell* **33**, 676-689 e673, doi:10.1016/j.ccell.2018.03.007 (2018).

13 Reider, C. L. & Cole, R. N. Entry into Mitosis in Vertebrate Somatic Cells Is Guarded by a Chromosome Damage Checkpoint That Reverses the Cell Cycle When Triggered during Early but Not Late Prophase. *Journal of Cell Biology* **142**, 1013-1022 (1998).

14 Petsalaki, E. & Zachos, G. DNA damage response proteins regulating mitotic cell division: double agents preserving genome stability. *FEBS J* **287**, 1700-1721, doi:10.1111/febs.15240 (2020).

15 Matos, D. A., Zhang, J. M., Ouyang, J., Nguyen, H. D., Genois, M. M. & Zou, L. ATR Protects the Genome against R Loops through a MUS81-Triggered Feedback Loop. *Mol Cell* **77**, 514-527 e514, doi:10.1016/j.molcel.2019.10.010 (2020).

16 Shiotani, B. & Zou, L. ATR signaling at a glance. *J Cell Sci* **122**, 301-304, doi:10.1242/jcs.035105 (2009).

17 Stiff, T., Walker, S. A., Cerosaletti, K., Goodarzi, A. A., Petermann, E., Concannon, P., O'Driscoll, M. & Jeggo, P. A. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. *EMBO J* **25**, 5775-5782, doi:10.1038/sj.emboj.7601446 (2006).

18 Buis, J., Wu, Y., Deng, Y., Leddon, J., Westfield, G., Eckersdorff, M., Sekiguchi, J. M., Chang, S. & Ferguson, D. O. Mre11 nuclease activity has essential roles in DNA repair and genomic stability distinct from ATM activation. *Cell* **135**, 85-96, doi:10.1016/j.cell.2008.08.015 (2008).

19 Duursma, A. M., Driscoll, R., Elias, J. E. & Cimprich, K. A. A role for the MRN complex in ATR activation via TOPBP1 recruitment. *Mol Cell* **50**, 116-122, doi:10.1016/j.molcel.2013.03.006 (2013).
20 Lee, J. & Dunphy, W. G. The Mre11-Rad50-Nbs1 (MRN) complex has a specific role in the activation of Chk1 in response to stalled replication forks. *Mol Biol Cell* **24**, 1343-1353, doi:10.1091/mbc.E13-01-0025 (2013).
21 Gatei, M., Kijas, A. W., Biard, D., Dork, T. & Lavin, M. F. RAD50 phosphorylation promotes ATR downstream signaling and DNA restart following replication stress. *Hum Mol Genet* **23**, 4232-4248, doi:10.1093/hmg/ddu141 (2014).
22 Mordes, D. A., Glick, G. G., Zhao, R. & Cortez, D. TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. *Genes Dev* **22**, 1478-1489, doi:10.1101/gad.1666208 (2008).
23 Choi, J. H., Lindsey-Boltz, L. A., Kemp, M., Mason, A. C., Wold, M. S. & Sancar, A. Reconstitution of RPA-covered single-stranded DNA-activated ATR-Chk1 signaling. *Proc Natl Acad Sci U S A* **107**, 13660-13665, doi:10.1073/pnas.1007856107 (2010).
24 Haahr, P., Hoffmann, S., Tollenaere, M. A., Ho, T., Toledo, L. I., Mann, M., Bekker-Jensen, S., Raschle, M. & Mailand, N. Activation of the ATR kinase by the RPA-binding protein ETAA1. *Nat Cell Biol* **18**, 1196-1207, doi:10.1038/ncb3422 (2016).
25 Lee, Y. C., Zhou, Q., Chen, J. & Yuan, J. RPA-Binding Protein ETAA1 Is an ATR Activator Involved in DNA Replication Stress Response. *Curr Biol* **26**, 3257-3268, doi:10.1016/j.cub.2016.10.030 (2016).
26 Bass, T. E., Luzwick, J. W., Kavanaugh, G., Carroll, C., Dungrawala, H., Glick, G. G., Feldkamp, M. D., Putney, R., Chazin, W. J. & Cortez, D. ETAA1 acts at stalled replication forks to maintain genome integrity. *Nat Cell Biol* **18**, 1185-1195, doi:10.1038/ncb3415 (2016).
27 Feng, S., Zhao, Y., Xu, Y., Ning, S., Huo, W., Hou, M., Gao, G., Ji, J., Guo, R. & Xu, D. Ewing Tumor-associated Antigen 1 Interacts with Replication Protein A to Promote Restart of Stalled Replication Forks. *J Biol Chem* **291**, 21956-21962, doi:10.1074/jbc.C116.747758 (2016).
28 Achuthankutty, D., Thakur, R. S., Haahr, P., Hoffmann, S., Drainas, A. P., Bizard, A. H., Weischenfeldt, J., Hickson, I. D. & Mailand, N. Regulation of ETAA1-mediated ATR activation couples DNA replication fidelity and genome stability. *J Cell Biol* **218**, 3943-3953, doi:10.1083/jcb.201905064 (2019).
29 Bass, T. E. & Cortez, D. Quantitative phosphoproteomics reveals mitotic function of the ATR activator ETAA1. *J Cell Biol* **218**, 1235-1249, doi:10.1083/jcb.201810058 (2019).
30 Lyu, K., Kumagai, A. & Dunphy, W. G. RPA-coated single-stranded DNA promotes the ETAA1-dependent activation of ATR. *Cell Cycle* **18**, 898-913, doi:10.1080/15384101.2019.1598728 (2019).
31 Difilippantonio, S., Celeste, A., Fernandez-Capetillo, O., Chen, H. T., Reina San Martin, B., Van Laethem, F., Yang, Y. P., Petukhova, G. V., Eckhaus, M., Feigenbaum, L., Manova, K., Kruhlak, M., Camerini-Otero, R. D., Sharan, S., Nussenzweig, M. & Nussenzweig, A. Role of Nbs1 in the activation of the Atm kinase revealed in humanized mouse models. *Nat Cell Biol* **7**, 675-685, doi:10.1038/ncb1270 (2005).
32 Gatei, M., Sloper, K., Sorensen, C., Syljuasen, R., Falck, J., Hobson, K., Savage, K., Lukas, J., Zhou, B. B., Bartek, J. & Khanna, K. K. Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *J Biol Chem* **278**, 14806-14811, doi:10.1074/jbc.M210862200 (2003).
33 Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G. C., Lukas, J. & Jackson, S. P. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* **8**, 37-45, doi:10.1038/ncb1337 (2006).
34 Myers, J. S. & Cortez, D. Rapid activation of ATR by ionizing radiation requires ATM and Mre11. *J Biol Chem* **281**, 9346-9350, doi:10.1074/jbc.M513265200 (2006).
35 Zhu, J., Peterson, S., Tessarollo, L. & Nussenzweig, A. Targeted disruption of the Nijmegen breakage syndrome gene NBS1 leads to early embryonic lethality in mice. *Current Biology* **11**, 105-109 (2001).
36 Luo, G., Yao, M. S., Bender, C. F., Mills, M., Bladl, A. R., Bradley, A. & Petrini, J. H. Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc Natl Acad Sci U S A* **96**, 7376-7381, doi:10.1073/pnas.96.13.7376 (1999).

37 Stewart, G. S., Maser, R. S., Stankovic, T., Bressan, D. A., Kaplan, M. I., Jaspers, N. G., Raams, A., Byrd, P. J., Petrini, J. H. & Taylor, A. M. The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* **99**, 577-587, doi:10.1016/s0092-8674(00)81547-0 (1999).
38 Waltes, R., Kalb, R., Gatei, M., Kijas, A. W., Stumm, M., Sobeck, A., Wieland, B., Varon, R., Lerenthal, Y., Lavin, M. F., Schindler, D. & Dork, T. Human RAD50 deficiency in a Nijmegen breakage syndrome-like disorder. *Am J Hum Genet* **84**, 605-616, doi:10.1016/j.ajhg.2009.04.010 (2009).
39 Carney, J. P., Maser, R. S., Olivares, H., Davis, E. M., Le Beau, M., Yates, J. R., 3rd, Hays, L., Morgan, W. F. & Petrini, J. H. The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell* **93**, 477-486, doi:10.1016/s0092-8674(00)81175-7 (1998).
40 Uchisaka, N., Takahashi, N., Sato, M., Kikuchi, A., Mochizuki, S., Imai, K., Nonoyama, S., Ohara, O., Watanabe, F., Mizutani, S., Hanada, R. & Morio, T. Two brothers with ataxia-telangiectasia-like disorder with lung adenocarcinoma. *J Pediatr* **155**, 435-438, doi:10.1016/j.jpeds.2009.02.037 (2009).
41 Minocherhomji, S., Ying, S., Bjerregaard, V. A., Bursomanno, S., Aleliunaite, A., Wu, W., Mankouri, H. W., Shen, H., Liu, Y. & Hickson, I. D. Replication stress activates DNA repair synthesis in mitosis. *Nature* **528**, 286-290, doi:10.1038/nature16139 (2015).
42 Zou, L. & Elledge, S. J. Sensing DNA Damage Through ATRIP Recognition of RPA-ssDNA Complexes. *Science* **300**, 1542-1548 (2003).
43 Nguyen, H. D., Yadav, T., Giri, S., Saez, B., Graubert, T. A. & Zou, L. Functions of Replication Protein A as a Sensor of R Loops and a Regulator of RNaseH1. *Mol Cell* **65**, 832-847 e834, doi:10.1016/j.molcel.2017.01.029 (2017).
44 Zhong, H., Bryson, A., Eckersdorff, M. & Ferguson, D. O. Rad50 depletion impacts upon ATR-dependent DNA damage responses. *Hum Mol Genet* **14**, 2685-2693, doi:10.1093/hmg/ddi302 (2005).
45 Holland, A. J., Fachinetti, D., Han, J. S. & Cleveland, D. W. Inducible, reversible system for the rapid and complete degradation of proteins in mammalian cells. *Proc Natl Acad Sci U S A* **109**, E3350-3357, doi:10.1073/pnas.1216880109 (2012).
46 Taylor, A. M. R., Rothblum-Oviatt, C., Ellis, N. A., Hickson, I. D., Meyer, S., Crawford, T. O., Smogorzewska, A., Pietrucha, B., Weemaes, C. & Stewart, G. S. Chromosome instability syndromes. *Nat Rev Dis Primers* **5**, 64, doi:10.1038/s41572-019-0113-0 (2019).
47 Pines, J. Mitosis: a matter of getting rid of the right protein at the right time. *Trends Cell Biol* **16**, 55-63, doi:10.1016/j.tcb.2005.11.006 (2006).
48 Rai, R., Hu, C., Broton, C., Chen, Y., Lei, M. & Chang, S. NBS1 Phosphorylation Status Dictates Repair Choice of Dysfunctional Telomeres. *Mol Cell* **65**, 801-817 e804, doi:10.1016/j.molcel.2017.01.016 (2017).
49 Di Fiore, B. & Pines, J. How cyclin A destruction escapes the spindle assembly checkpoint. *J Cell Biol* **190**, 501-509, doi:10.1083/jcb.201001083 (2010).
50 Sana, S., Keshri, R., Rajeevan, A., Kapoor, S. & Kotak, S. Plk1 regulates spindle orientation by phosphorylating NuMA in human cells. *Life Sci Alliance* **1**, e201800223, doi:10.26508/lsa.201800223 (2018).
51 Kettenbach, A. N., Schweppe, D. K., Faherty, B. K., Pechenick, D., Pletnev, A. A. & Gerber, S. A. Quantitative phosphoproteomics identifies substrates and functional modules of Aurora and Polo-like kinase activities in mitotic cells. *Sci Signal* **4**, rs5, doi:10.1126/scisignal.2001497 (2011).
52 Volkening, L., Vatselia, A., Asgedom, G., Bastians, H., Lavin, M., Schindler, D., Schambach, A., Bousset, K. & Dork, T. RAD50 regulates mitotic progression independent of DNA repair functions. *FASEB J* **34**, 2812-2820, doi:10.1096/fj.201902318R (2020).
53 Rhee, H. W., Zou, P., Udeshi, N. D., Martell, J. D., Mootha, V. K., Carr, S. A. & Ting, A. Y. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. *Science* **339**, 1328-1331, doi:10.1126/science.1230593 (2013).

54 Nakajima, H., Toyoshima-Morimoto, F., Taniguchi, E. & Nishida, E. Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J Biol Chem* **278**, 25277-25280, doi:10.1074/jbc.C300126200 (2003).
55 Rao, Q., Liu, M., Tian, Y., Wu, Z., Hao, Y., Song, L., Qin, Z., Ding, C., Wang, H. W., Wang, J. & Xu, Y. Cryo-EM structure of human ATR-ATRIP complex. *Cell Res* **28**, 143-156, doi:10.1038/cr.2017.158 (2018).
56 Banani, S. F., Lee, H. O., Hyman, A. A. & Rosen, M. K. Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol* **18**, 285-298, doi:10.1038/nrm.2017.7 (2017).
57 Alberti, S., Gladfelter, A. & Mittag, T. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. *Cell* **176**, 419-434, doi:10.1016/j.cell.2018.12.035 (2019).
58 Flory, P. J. Thermodynamics of High Polymer Solutions. *The Journal of Chemical Physics* **10**, 51-61, doi:10.1063/1.1723621 (1942).
59 Huggins, M. L. Some Properties of Solutions of Long-chain Compounds. *J. Phys. Chem* **46**, 151-158 (1942).
60 Ruff, K. M., Roberts, S., Chilkoti, A. & Pappu, R. V. Advances in Understanding Stimulus-Responsive Phase Behavior of Intrinsically Disordered Protein Polymers. *J Mol Biol* **430**, 4619-4635, doi:10.1016/j.jmb.2018.06.031 (2018).
61 Jiang, H., Wang, S., Huang, Y., He, X., Cui, H., Zhu, X. & Zheng, Y. Phase transition of spindle-associated protein regulate spindle apparatus assembly. *Cell* **163**, 108-122, doi:10.1016/j.cell.2015.08.010 (2015).
62 Huang, Y., Li, T., Ems-McClung, S. C., Walczak, C. E., Prigent, C., Zhu, X., Zhang, X. & Zheng, Y. Aurora A activation in mitosis promoted by BuGZ. *J Cell Biol* **217**, 107-116, doi:10.1083/jcb.201706103 (2018).
63 Tiwary, A. K. & Zheng, Y. Protein phase separation in mitosis. *Curr Opin Cell Biol* **60**, 92-98, doi:10.1016/j.ceb.2019.04.011 (2019).
64 Trivedi, P., Palomba, F., Niedzialkowska, E., Digman, M. A., Gratton, E. & Stukenberg, P. T. The inner centromere is a biomolecular condensate scaffolded by the chromosomal passenger complex. *Nat Cell Biol* **21**, 1127-1137, doi:10.1038/s41556-019-0376-4 (2019).
65 Rai, A. K., Chen, J. X., Selbach, M. & Pelkmans, L. Kinase-controlled phase transition of membraneless organelles in mitosis. *Nature* **559**, 211-216, doi:10.1038/s41586-018-0279-8 (2018).
66 Nott, T. J., Petsalaki, E., Farber, P., Jervis, D., Fussner, E., Plochowietz, A., Craggs, T. D., Bazett-Jones, D. P., Pawson, T., Forman-Kay, J. D. & Baldwin, A. J. Phase transition of a disordered nuage protein generates environmentally responsive membraneless organelles. *Mol Cell* **57**, 936-947, doi:10.1016/j.molcel.2015.01.013 (2015).
67 Patel, A., Lee, H. O., Jawerth, L., Maharana, S., Jahnel, M., Hein, M. Y., Stoynov, S., Mahamid, J., Saha, S., Franzmann, T. M., Pozniakovski, A., Poser, I., Maghelli, N., Royer, L. A., Weigert, M., Myers, E. W., Grill, S., Drechsel, D., Hyman, A. A. & Alberti, S. A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell* **162**, 1066-1077, doi:10.1016/j.cell.2015.07.047 (2015).
68 Altmeyer, M., Neelsen, K. J., Teloni, F., Pozdnyakova, I., Pellegrino, S., Grofte, M., Rask, M. D., Streicher, W., Jungmichel, S., Nielsen, M. L. & Lukas, J. Liquid demixing of intrinsically disordered proteins is seeded by poly(ADP-ribose). *Nat Commun* **6**, 8088, doi:10.1038/ncomms9088 (2015).
69 Pak, C. W., Kosno, M., Holehouse, A. S., Padrick, S. B., Mittal, A., Ali, R., Yunus, A. A., Liu, D. R., Pappu, R. V. & Rosen, M. K. Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein. *Mol Cell* **63**, 72-85, doi:10.1016/j.molcel.2016.05.042 (2016).
70 Harmon, T. S., Holehouse, A. S., Rosen, M. K. & Pappu, R. V. Intrinsically disordered linkers determine the interplay between phase separation and gelation in multivalent proteins. *Elife* **6**, doi:10.7554/eLife.30294 (2017).
71 Frey, S. & Gorlich, D. FG-Rich Repeats of Nuclear Pore ProteinsForm a Three-Dimensional Meshworkwith Hydrogel-Like Properties. *Science* **314**, 815-817 (2006).

72 Frey, S. & Gorlich, D. A saturated FG-repeat hydrogel can reproduce the permeability properties of nuclear pore complexes. *Cell* **130**, 512-523, doi:10.1016/j.cell.2007.06.024 (2007).
73 Meszaros, B., Erdos, G. & Dosztanyi, Z. IUPred2A: context-dependent prediction of protein disorder as a function of redox state and protein binding. *Nucleic Acids Res* **46**, W329-W337, doi:10.1093/nar/gky384 (2018).
74 Erdos, G. & Dosztanyi, Z. Analyzing Protein Disorder with IUPred2A. *Curr Protoc Bioinformatics* **70**, e99, doi:10.1002/cpbi.99 (2020).
75 van der Linden, E., Sanchez, H., Kinoshita, E., Kanaar, R. & Wyman, C. RAD50 and NBS1 form a stable complex functional in DNA binding and tethering. *Nucleic Acids Res* **37**, 1580-1588, doi:10.1093/nar/gkn1072 (2009).
76 Kilic, S., Lezaja, A., Gatti, M., Bianco, E., Michelena, J., Imhof, R. & Altmeyer, M. Phase separation of 53BP1 determines liquid-like behavior of DNA repair compartments. *EMBO J* **38**, e101379, doi:10.15252/embj.2018101379 (2019).
77 Lee, J. H. & Paull, T. T. in *DNA Repair, Part A Methods in Enzymology* 529-539 (2006).
78 Molliex, A., Temirov, J., Lee, J., Coughlin, M., Kanagaraj, A. P., Kim, H. J., Mittag, T. & Taylor, J. P. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* **163**, 123-133, doi:10.1016/j.cell.2015.09.015 (2015).
79 Ribbeck, K. & Gorlich, D. Phase Separation by Low Complexity DomainsPromotes Stress Granule Assembly and DrivesPathological Fibrillization. *EMBO J* **21**, 2664-2671 (2002).
80 Strom, A. R., Emelyanov, A. V., Mir, M., Fyodorov, D. V., Darzacq, X. & Karpen, G. H. Phase separation drives heterochromatin domain formation. *Nature* **547**, 241-245, doi:10.1038/nature22989 (2017).
81 Overbeek, J. T. G. & Voorn, M. J. Phase separation in polyelectrolyte solutions; theory of complex coacervation. *J. Cell. Physiol* **49**, 7-22 (1957).
82 Shin, Y., Berry, J., Pannucci, N., Haataja, M. P., Toettcher, J. E. & Brangwynne, C. P. Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. *Cell* **168**, 159-171 e114, doi:10.1016/j.cell.2016.11.054 (2017).
83 Saito, M., Hess, D., Eglinger, J., Fritsch, A. W., Kreysing, M., Weinert, B. T., Choudhary, C. & Matthias, P. Acetylation of intrinsically disordered regions regulates phase separation. *Nat Chem Biol* **15**, 51-61, doi:10.1038/s41589-018-0180-7 (2019).
84 Minton, A. P. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J Biol Chem* **276**, 10577-10580, doi:10.1074/jbc.R100005200 (2001).
85 Mitchison, T. J. Colloid osmotic parameterization and measurement of subcellular crowding. *Mol Biol Cell* **30**, 173-180, doi:10.1091/mbc.E18-09-0549 (2019).