Regulation of Mitotic DNA Damage by the Retinoblastoma Tumor Suppressor Protein

A Major Qualifying Project

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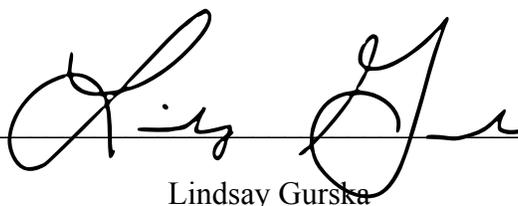
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Abstract

Function of the retinoblastoma protein, a master regulator of cell cycle progression, is compromised in the majority of cancers. Loss of pRB is known to promote DNA damage during interphase, but its impact during mitosis remains unclear. My work demonstrates a novel role for pRB such that its loss promotes high levels of DNA damage in mitotic cells. The data suggests that G2/M DNA damage checkpoints in pRB-deficient cells remain intact, and that damage acquired during interphase does not persist into mitosis. Instead, pRB-deficient cells acquire new damage during mitosis. More specifically, in pRB-deficient cells γH2AX DNA damage foci are present during normal mitotic timing, exacerbated in response to microtubule poisons that prolong mitotic arrest, and accumulate more quickly in pRB-deficient cells than in control cells. Together this data suggests that pRB functions to protect against DNA damage following delays in mitotic progression.

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I want to first thank my project advisor, Amity Manning, for giving me a new appreciation for research during these last two years. Through your guidance, I have learned so much about experimental design, data analysis, and scientific reading and writing. Thank you for continuously being enthusiastic about this project and giving me the confidence to excel in my future research endeavors.

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Chapter I: Retinoblastoma Protein as a Regulator of Genome Stability

Genomic Stability and Cancer

Genomic instability, characterized by alterations to a cell's genome, is a hallmark of cancer and has been shown to promote tumorigenesis (Meyerson & Pellman, 2011; Thompson & Compton, 2011). Several processes of the cell cycle can be disrupted in order to generate genomic instability, including DNA replication, chromosome segregation, DNA damage response pathways, and regulation of cell cycle progression (Shen, 2011). These processes are strictly regulated by cell cycle checkpoints, and it is the loss of function of the machinery required for these surveillance systems that onsets malignant transformation.

Overview of Retinoblastoma Protein

One of the best characterized regulators of the cell cycle is the retinoblastoma tumor suppressor protein (pRB). While the canonical role of pRB is as a negative regulator of E2F-dependent transcription and the G1/S phase transition, pRB additionally regulates the activity of various downstream targets important in later stages of the cell cycle. Several studies have shown the importance of pRB in regulating chromosome cohesion (Ren et al., 2002; Manning et al., 2010), chromosome condensation (Ren et al., 2002; Coschi et al., 2010), DNA replication (Ren et al., 2002; Srinivasan et al., 2007), DNA damage response (Ren et al., 2002; van Harn et al., 2010), and chromosome segregation (Ren et al., 2002; Hernando et al., 2004; Manning et al., 2010). It is no surprise then that pRB is either loss or functionally inactive most cancers (Horowitz et al., 1990). More specifically, the RB gene is often either deleted or the protein itself is functionally inactivated (i.e. constitutively hyperphosphorylated) due to either overexpression of RB-inactivating kinases or inactivation of negative regulators of these kinases (Damania and Pipas, 2008; Du and Searle, 2009). In some of the earliest studies of the RB pathway and cancer, Horowitz et al. showed that the RB gene is inactivated in all retinoblastomas, inactivated or alternatively spliced to result in a functional inactive protein in small cell lung cancer, and

functionally inactivated in most breast carcinomas and melanomas (Horowitz et al., 1990). Cancer genome sequencing has now confirmed that the RB1 gene is mutated in most retinoblastomas, osteosarcomas, and small cell lung cancer, and is mutated at lower frequencies in several other cancers (Dyson, 2016). Thus, it is important to understand the various roles of pRB with regard to maintaining genome stability to advance upon cancer knowledge and therapeutics.

Regulation of Chromatin Structure and Cell Cycle Progression

There are three major events during a cell cycle - cell growth, DNA replication, and nuclear division (Ferrari and Gentili, 2016). Cell growth and DNA replication occur during interphase, which consists of the stages G1, S, and G2, while nuclear division occurs during a process known as mitosis (Ferrari and Gentili, 2016). There are five sub-phases of mitosis - prophase, prometaphase, metaphase, anaphase, and telophase - followed by cytokinesis, or the physical separation of the daughter cells (Ferrari and Gentili, 2016). In order for a cell to divide, it must undergo an accurate duplication of its DNA during S phase. S phase produces identical DNA molecules, referred to as sister chromatids, that become and remain physically connected until anaphase by a process known as cohesion (Peters and Nishiyama, 2012). Cohesion is regulated by cohesin, a ring-shaped protein complex that loads onto DNA during replication to connect sister chromatids at the centromere and along their arms until chromosome segregation (Peters and Nishiyama, 2012). Cohesin is stabilized onto chromosomes by the protein soronin, which binds to a subunit of the cohesin complex (Schmitz et al., 2007). Cohesin is further regulated by Wapl, which when present in early mitosis competes with sororin for binding and releases cohesin from the chromosome arms of DNA (Kueng et al., 2006). Several other proteins are responsible for regulating cohesion, including separase, Polo-like kinase (Plk1), cyclin-dependent kinase 1 (Cdk1), and Aurora B (Losada et al., 2002; Sumara et al., 2002; Kueng et al., 2006; Hegemann et al. 2011). Defects in cohesion and its regulation machinery are underlying causes of chromosomal instability (CIN), as the inability for sister chromatids to accurately assemble on the mitotic spindle and symmetrically segregate into daughter cells results in aneuploidy (Peters and Nishiyama, 2012). Cohesion is also important in accurate DNA

repair through homologous recombination (Peters and Nishiyama, 2012). More specifically, cohesin is responsible for holding damaged ends of chromosomes together, as well as newly replicated strands behind the replication fork together to promote DNA repair surveillance and initiation of homologous recombination (Watrin and Peters, 2006). The cohesin complex has also been shown to be recruited to sites of DNA damage, and thus is responsible for initiating DNA repair (Strom et al., 2004). As a consequence, defects in cohesion can result in defects in the DNA damage repair pathway, and accumulation of DNA damage itself.

As the cell progresses into mitosis, the replicated chromatin undergoes a process known as condensation. During prophase, a protein complex called condensin II is recruited to the DNA in order to supercoil the chromatin for accurate segregation (Uhlmann, 2001; Coschi et al., 2010). Like cohesin, condensin II is also regulated by Plk1, Cdk1, and Aurora B (Ferrari and Gentili, 2016). Alongside this process, topoisomerase II is responsible for ensuring that the DNA does not get entangled (Uhlmann, 2001). Defects in chromosome condensation are another underlying cause of CIN, as they can produce segregation errors such as lagging chromosomes, which subsequently result in aneuploidy (Samoshkin et al., 2009).

Once the sister chromatids are appropriately attached and condensed, the cell continues with its division. During prometaphase and metaphase, Aurora B regulates the attachment of microtubules from the mitotic spindle to the kinetochores on the sister chromatids' centromere region (Cheeseman, 2014). Once attached, Aurora A coordinates the action of motor proteins like dynein and CENP-E so that the sister chromatids can assembly along the metaphase plate (Kim et al., 2010). Upon progression into anaphase, the anaphase promoting complex/cyclosome (APC/C) initiates cohesin removal from the centromere in order to promote chromosome segregation (Sivakumar and Gorbsky, 2015). Once the chromatids are positioned at the opposite poles of the cell, two new nuclear membranes form and the subsequent daughter cells physically separate (Pines and Rieder, 2001). All of the cell cycle is systematically regulated by strict checkpoints - the two most prominent being the G2/M checkpoint and the spindle assembly checkpoint (Ferrari and Gentili, 2016). The G2/M checkpoint, commonly recognized as the DNA damage checkpoint, is regulated by several key proteins including but not limited to ATR, ATM, CHK1, CHK2, Plk1, and Cdk1, while the spindle assembly checkpoint is regulated by a different set of proteins with the most prominent being Mad2 (Ferrari and Gentili, 2016). It is

often the inactivation or loss of function of these proteins that allow a cell to continue to divide in the presence of genomic changes, promoting tumorigenesis.

Function of pRB throughout the Cell Cycle

Before a cell can even initiate the cohesion process in S phase, however, it must promote the expression of all cell cycle proteins previously described – most of which are regulated by the pRB pathway. pRB is best known for its negative regulation of the E2F transcription factor family during the G1/S phase transition (Figure 1). Under normal conditions, pRB restricts a cell from entering S phase until hyperphosphorylation stimulated by Cdk4/Cdk6 activity (Weinberg, 1995). During this time, pRB is bound to E2F family members, repressing their ability to initiate the transcription of genes whose protein products are necessary for DNA replication (Weinberg, 1995). Once a cell has met the criteria to continue onto DNA replication, a growth factor stimulates cyclin-Cdk4/Cdk6 activity, subsequently hyperphosphorylating pRB so that it loses its attachment to the E2Fs (Weinberg, 1995). Following liberation, E2Fs can then promote the expression of the genes necessary for cell cycle progression and mitotic initiation (Weinberg, 1995).
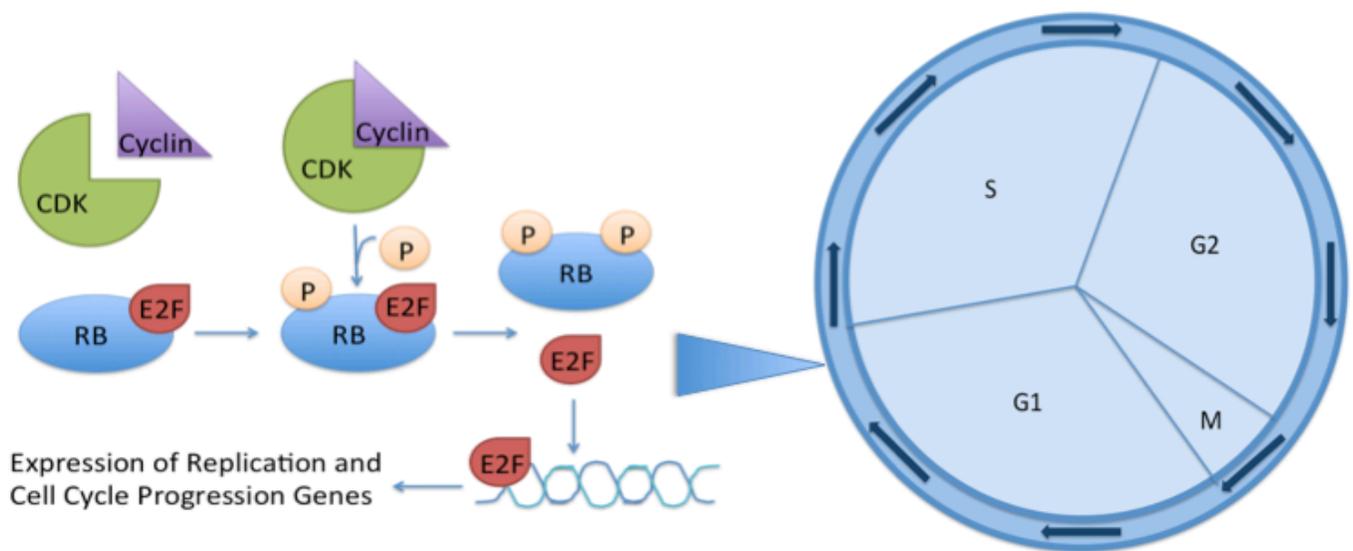

Figure 1: Schematic of the pRB-E2F Pathway during the G1/S Phase Checkpoint

Although pRB is best known for its function during interphase progression, there are several other roles for pRB that are less understood. Studies have shown that pRB-mutant cells have elevated levels of DNA damage (Pickering and Kowalik, 2006; van Harn et al., 2010; Bester et al., 2011), but the mechanism as to how

Table 1: Exploring Loss of pRB Cell Cycle Regulation in Promoting DNA Damage

Possible Promoter of DNA Damage	Result of Defect/Misregulation
Cohesion Defects	Cell loses ability to hold damaged ends of chromosomes together as well as newly replicated DNA strands together, thus disrupting DNA repair by limiting homologous recombination (Strom et al., 2004). Changes in the chromatin landscape can also alter the gene expression profile and/or expose normally methylated sites for DNA damage.
Condensation Defects	Condensin I is specifically recruited to sites of DNA damage, thus defects result in loss of repair initiation (Kong et al., 2011). Changes in the chromatin landscape can also alter the gene expression profile and/or expose normally methylated sites for DNA damage. In addition to the role of condensin I in DNA damage repair, RB has been shown to interact with condensin II (Longsworth et al., 2008), which has also been shown to have a role in DNA damage response (Floyd et al., 2013).
Nucleotide Deficiency	Failure to support normal DNA replication and repair (Bester et al., 2011).
Misregulated DNA Damage Repair Pathway	Failure to initiate and carry out normal DNA repair pathway. RB is phosphorylated in an ATM, Chk1/2 dependent manner in response to DNA damage, which promotes E2F repression and cell cycle arrest (Inoue et al., 2007). Thus, inactivation of RB limits DNA damage response and promotes accumulation of damage.
Misregulated Spindle Assembly Checkpoint	Misregulated progression of mitosis displays failure to initiate and carry out normal DNA repair pathway. Loss of correct spindle formation results in lagging chromosomes, micronuclei, and chromatin bridges (Manning et al., 2010). RB has also been shown to bind directly to the APC/C specificity factor Cdh1, thus inactivation of RB can limit the cell's ability to induce a cell cycle arrest when such defects arise (Binne et al., 2007).

pRB loss results in DNA damage is unclear. Furthermore, most of the damage studied due to pRB loss has been proposed to be from replication defects, further characterizing its importance in regulating S phase activity but not necessarily activity throughout the rest of the cell cycle. Other models suggest that cohesion defects (Manning et al., 2010), condensation defects (Coschi et al., 2010), nucleotide deficiency (Bester et al., 2011), misregulation of pRB-regulated DNA damage repair factors (Bosco et al., 2005; Genovese et al., 2006; Cook et al., 2015), and misregulation of pRB-regulated spindle assembly checkpoint proteins (i.e. Mad2) (Hernando et al., 2004) may play a role. Table 1 demonstrates how these changes may cause DNA damage.

Several other groups have begun to explore mitotic DNA damage. For example, the Mitchison group proposed that a prolonged mitotic arrest has the ability to deplete a cell's anti-apoptotic factors, which subsequently breaks the restraint of caspase activity and the function of caspase activated DNase (CAD) (Orth et al., 2012). CAD can then cleave DNA, thereby inducing DNA double stranded breaks (Orth et al., 2012). Other groups have explored the DNA damage response pathway itself with regard to mitotic damage, further accepting that under normal conditions, the DNA damage response pathway should be inactive during mitosis. For example, it has been shown that Plk1 actually functions to inhibit the DNA damage response pathway from turning on in response to mitotic damage (Benada et al., 2015), and that inducing the DNA damage response pathway during mitosis actually induces segregation errors (Bakhoum et al., 2014). However, no study to date considers the possibility of the effect pRB loss can have on mitotic damage.

Objectives & Hypotheses

This project aims to determine the correlation between pRB loss and DNA damage, specifically during mitosis. It is hypothesized that pRB-deficient cells acquire DNA damage during normal mitotic timing. Furthermore, similar to control cells undergoing a prolonged mitosis, it is hypothesized that pRB-deficient cells acquire caspase-dependent DNA damage that can be prevented by inhibiting caspase activity. Lastly, due to the canonical function of pRB in regulating cohesion and the accepted model that increasing cohesion can repair DNA damage of interphase pRB-deficient cells, it is further hypothesized that increasing cohesion will also

prevent the mitotic DNA damage phenotype. The broader goal of this project is to propose the molecular mechanism in which pRB regulates mitotic DNA damage in order to advance cancer therapy research for those malignancies in which pRB is lost or inactive.

Chapter II: Results

Prolonged Mitosis Induces DNA Damage in Control and pRB-Deficient Cells

Since previous literature has proposed that pRB-deficient cells experience an increase in mitotic chromosome segregation defects (Manning et. al., 2010), I set to determine whether pRB-deficient cells have an increase in mitotic DNA damage. In order to accomplish pRB knockdown, RNA interference via siRNA transfection was performed. Cells were arrested with the microtubule poison nocodazole for 8 hours to induce a mitotic arrest, and then were stained for γH2AX to monitor DNA double stranded break accumulation via immunofluorescence imaging. Only mitotic cells were scored, and they were considered to either have no damage, low damage, or high damage based on the number of γH2AX foci present (Figure 2). The data first suggests that pRB-deficient cells are more susceptible to DNA damage than control cells during normal mitotic events (Figure 3). Second, consistent with the literature (Orth et al., 2012), control cells undergoing a prolonged mitosis have an increase in DNA damage in comparison to control cells during normal mitotic timing (Figure 3). pRB-deficient cells undergoing a prolonged mitosis display this same pattern in comparison to control pRB-deficient cells, but they also have a larger percentage of cells exhibiting high damage in comparison to control cells undergoing abnormal mitoses (Figure 3). This suggests a novel role for pRB in the regulation of mitotic DNA damage and encourages further mechanistic analysis on how pRB loss promotes mitotic DNA damage.

pRB-Deficient Cells have an Intact G2/M DNA Damage Checkpoint

Since loss of pRB is accepted to be associated with DNA replication dependent damage, I next investigated whether this damage persists into mitosis and could thus account for the difference in levels of damage seen between control and pRB-deficient cells during abnormal mitoses. Control and pRB-deficient cells were treated with increasing concentrations of the DNA damaging agent Doxorubicin, and the mitotic index of
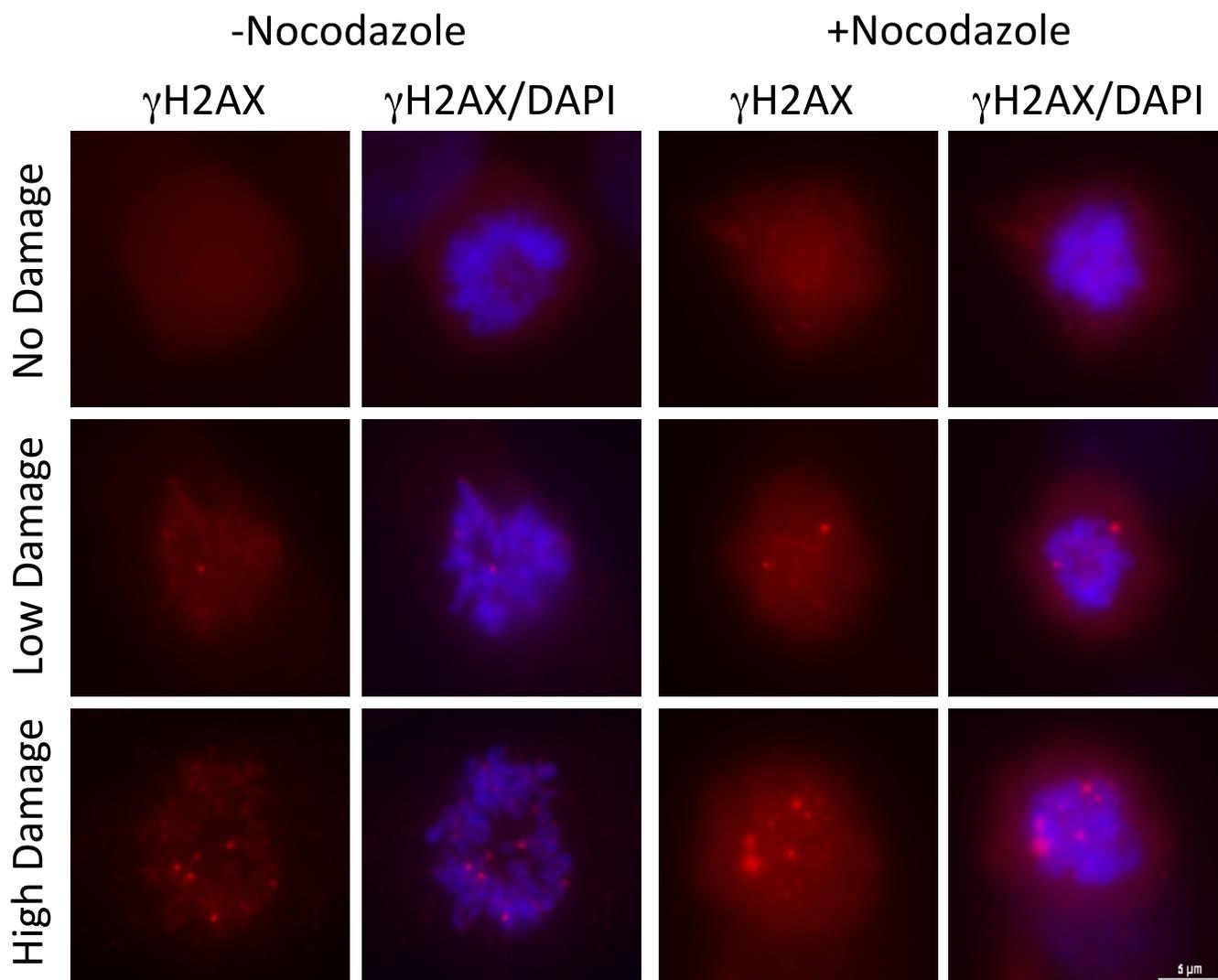
Figure 2: DNA Damage Scoring Parameters. Immunofluorescence imaging showing γH2AX foci categories for a cell in normal mitotic timing and a cell treated with the microtubule poison nocodazole. Cells were considered to have no damage if no γH2AX foci were present, low damage if 1-4 γH2AX foci were present, or high damage if 5 or more γH2AX foci were present. All experiments using siRNA transfection and these scoring parameters were performed a minimum of 3 replicates unless otherwise indicated, with a minimum number of 50 individual cells per replicate.
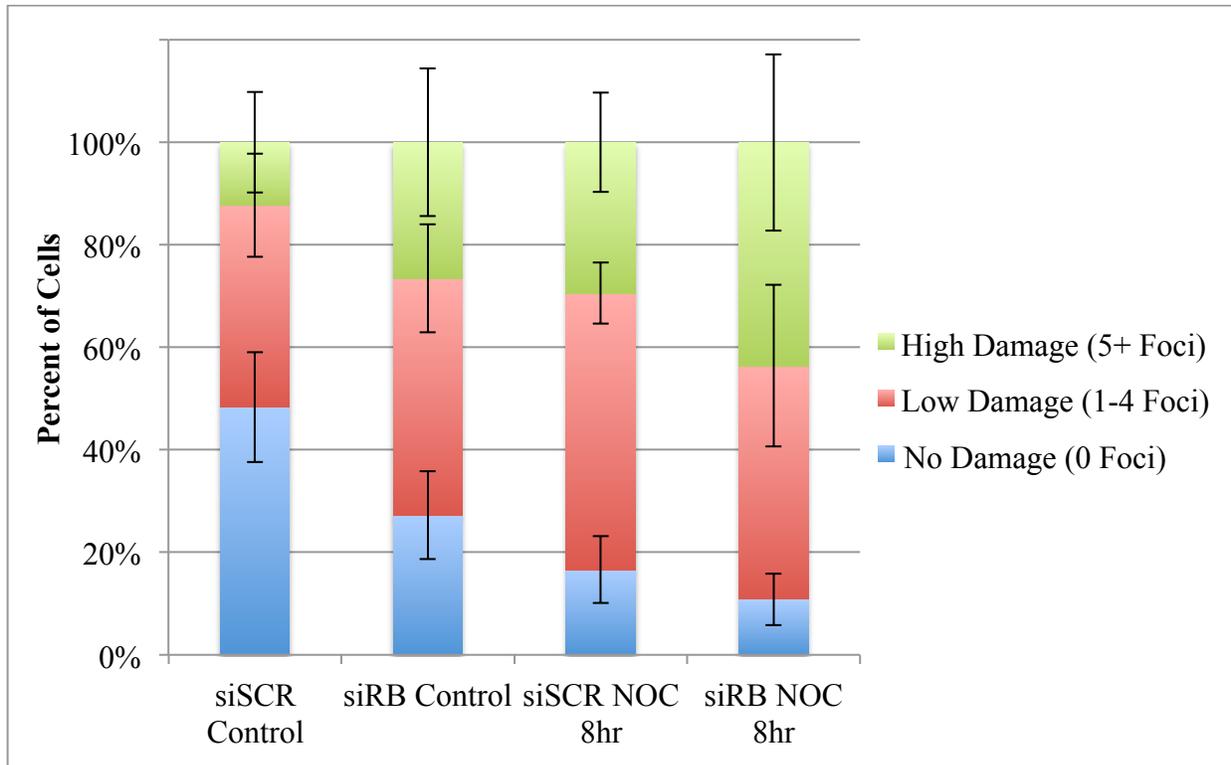
	siSCR Control vs. siRB Control	siSCR Control vs. siSCR NOC 8hr	siRB Control vs. siRB NOC 8hr	siSCR NOC 8hr vs. siRB NOC 8hr
No Damage (0 Foci)	**7.75E-05**	**9.91E-08**	**2.33E-05**	**0.0305**
Low Damage (1-4 Foci)	0.1453	**0.0006**	0.9084	0.1142
High Damage (5+ Foci)	**0.0168**	**0.0007**	**0.0203**	**0.0273**

Figure 3: Mitotic Arrest Induces γH2AX DNA Double Stranded Break Response in Control and pRB-Deficient Cells. Quantification of γH2AX foci scored after immunofluorescence imaging of RPE-1 cells. Cells were treated with either siSCR or siRB, and mitotic arrest was induced using the microtubule poison nocodazole for 16hr. P-values were calculated by a two-tailed t-test across conditions. Bolded values indicate significance. A total of 11 replicates were completed.

a minimum of 1000 cells imaged by immunofluorescence was calculated (Figure 4). Untreated conditions were the only conditions in which mitotic figures were observed, suggesting that the DNA damage resulting from Doxorubicin treatment activated the G2/M DNA damage checkpoint in both control and pRB-deficient cells (Figure 4). The data also suggests that pRB-deficient cells are still sensitive to lower concentrations of Doxorubicin and thus lower levels of DNA damage similar to control cells. These results indicate that the G2/M DNA damage checkpoint in pRB-deficient cells remains intact, suggesting that the damage accumulated in pRB-deficient cells during interphase is corrected before entry in mitosis.

pRB-Deficient Cells Accumulate DNA Damage at a Faster Rate than Control Cells during Abnormal Mitoses

Data thus far has suggested that pRB-deficient cells accumulate DNA damage during mitosis, and despite their known characterization for being susceptible to DNA damage acquired during interphase, pRB-deficient cells have intact DNA damage checkpoints and thus correct any damage before entering mitosis. Thus, it is important to determine how DNA damage is acquired in pRB-deficient cells once they enter mitosis. In order to characterize the time course at which pRB-deficient cells acquire mitotic DNA damage, control and pRB-deficient cells were treated with nocodazole for various time points and γH2AX foci accumulation was scored from immunofluorescence imaging to determine levels of DNA damage. First, the data again shows that control cells have a larger percentage of cells with no damage compared to pRB-deficient cells, a difference between the 8-hour nocodazole treated time point for both control and pRB-deficient cells, and a statistically significant difference in high levels of DNA damage between control and pRB-deficient cells during the 8-hour nocodazole treated time point (Figure 5). The data also shows the pRB-deficient cells arrested in mitosis begin to accumulate DNA damage as early as the 4-hour mitotic arrest time point, while control cells arrested in mitosis do not begin to accumulate statistically significant DNA damage until the 8-hour mitotic arrest time point (Figure 5). All together, the data suggests that pRB-deficient cells accumulate more DNA damage than control cells during normal mitotic timing, and accumulate more damage and at an exacerbated rate in comparison to control cells during a prolonged mitotic arrest.
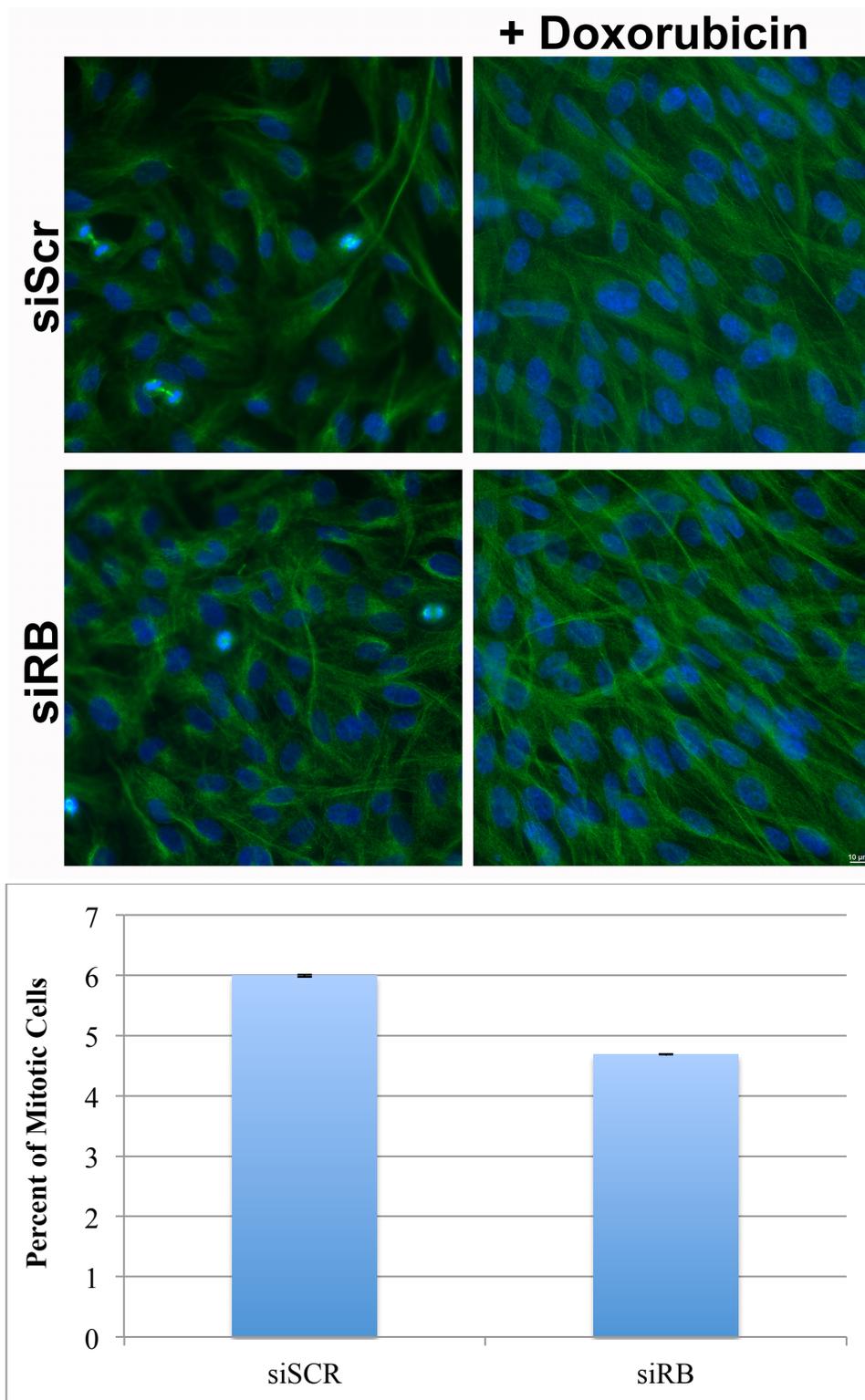
Figure 4: pRB-Deficient Cells Treated with the DNA Damaging Agent Doxorubicin were Competent to Arrest prior to Mitotic Entry. A) Representative immunofluorescence imaging of control and pRB-deficient cells treated with 500nM of Doxorubicin. B) Mitotic index of siSCR and siRB control conditions. A minimum of 1000 cells were scored per replicate for populations of control and pRB-deficient cells treated with four different concentrations of Doxorubicin (250nM, 500nM, 750nM, and 2μM). All Doxorubicin treated populations exhibited complete arrest with no apparent mitotic figures. A total of 3 replicates were completed.
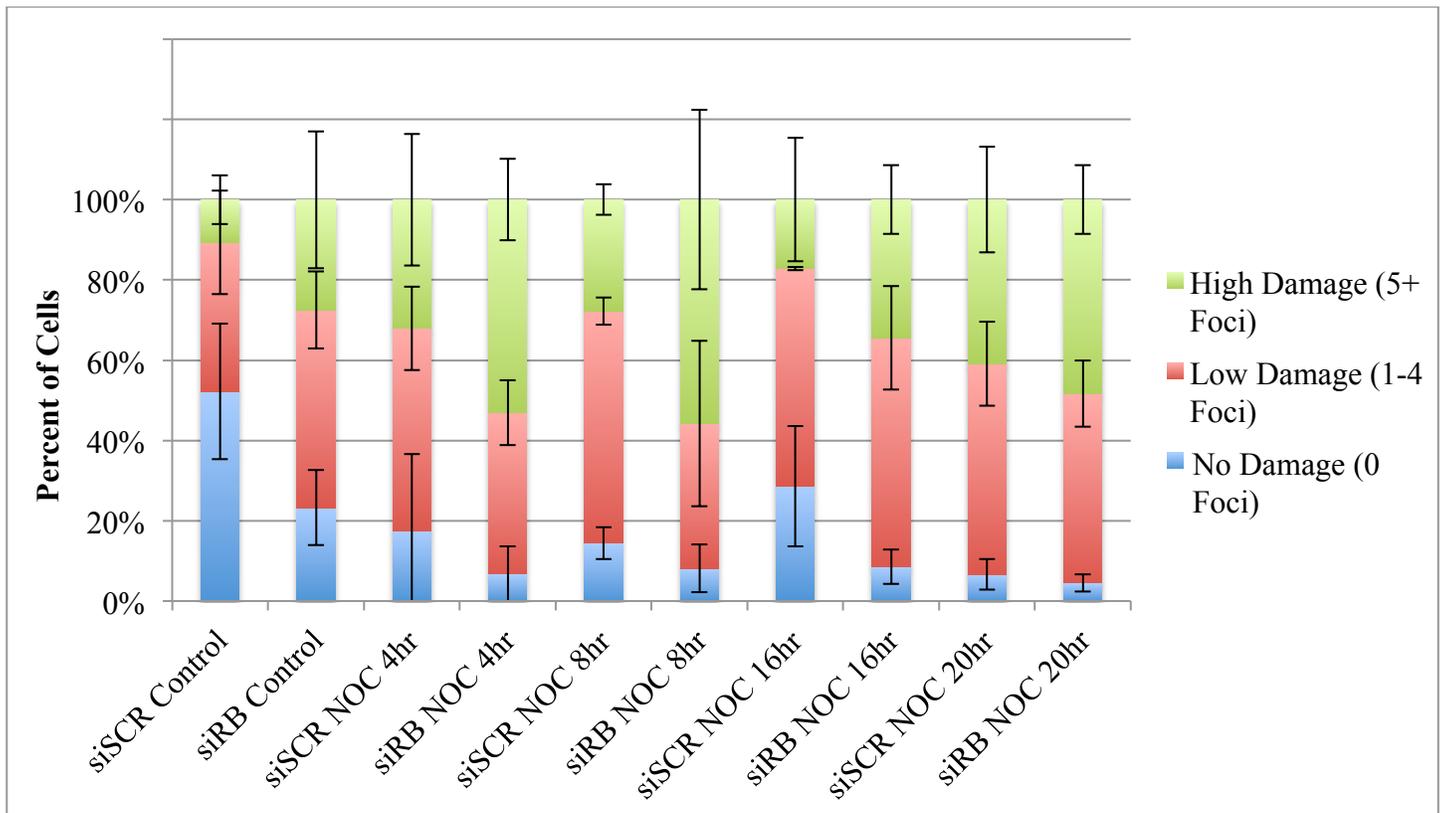
	siSCR Control vs. siRB Control	siSCR NOC 4hr vs. siRB NOC 4hr	siSCR NOC 8hr vs. siRB NOC 8hr	siSCR NOC 16hr vs. siRB NOC 16hr	siSCR NOC 20hr vs. siRB NOC 20hr
No Damage (0 Foci)	**0.0323**	0.4047	0.1331	0.0900	0.3614
Low Damage (1-4 Foci)	0.2105	0.2513	0.0826	0.7287	0.4593
High Damage (5+ Foci)	0.1699	0.131	**0.0482**	0.1648	0.3804
	siSCR Control vs. siSCR NOC 4hr	siSCR Control vs. siSCR NOC 8hr	siSCR Control vs. siSCR NOC 16hr	siSCR Control vs. siSCR NOC 20hr	
No Damage (0 Foci)	0.0775	**0.0056**	0.1433	**0.0029**	
Low Damage (1-4 Foci)	0.2388	**0.0249**	0.0828	0.1406	
High Damage (5+ Foci)	0.1011	**0.0056**	0.5317	**0.0150**	
	siRB Control vs. siRB NOC 4hr	siRB Control vs. siRB NOC 8hr	siRB Control vs. siRB NOC 16hr	siRB Control vs. siRB NOC 20hr	
No Damage (0 Foci)	**0.0502**	**0.0346**	0.0551	**0.0078**	
Low Damage (1-4 Foci)	0.2455	0.2910	0.3953	0.7626	
High Damage (5+ Foci)	0.0717	0.0910	0.5528	0.0722	

Figure 5: pRB-Deficient Cells Accumulate more γH2AX foci and do so at an Exacerbated Rate.
Quantification of γH2AX foci scored after immunofluorescence imaging of RPE-1 cells. Cells were treated with either siSCR or siRB, and mitotic arrest was induced using the microtubule poison nocodazole for 4hr, 8hr, 16hr, or 20hr. P-values were calculated by a two-tailed t-test across conditions. Bolded values indicate significance. Three replicates for all conditions, but additional fourth replicate for control, 8hr arrest, and 20hr arrest conditions.

Caspase Inhibition Partially Corrects Mitotic DNA Damage in pRB-Deficient Cells

At this point it has been proposed that pRB-deficient cells acquire DNA damage during prolonged mitoses and do so at an exacerbated rate in comparison to control cells. Previous literature has shown that in control cells, it is the partial activation of the caspase-dependent apoptotic pathway that causes DNA damage acquisition during prolonged mitoses (Orth et al., 2012). Inhibition of cleaved caspase activity was shown to restore DNA damage in cells undergoing a prolonged mitosis to control conditions (Orth et al., 2012), so I sought to determine if inhibition of caspase activity would also restore DNA damage in pRB-deficient cells undergoing a prolonged mitosis. Control and pRB-deficient cells were treated with nocodazole for 8 hours either in the presence or absence of the caspase inhibitor Z-VAD-FMK, and γH2AX foci accumulation was scored from immunofluorescence imaging to determine levels of DNA damage. The data shows that DNA damage in both control and pRB-deficient cells undergoing prolonged mitoses is partially corrected when caspase activity is inhibited (Figure 6). Thus, pRB-deficient cells are sensitive to caspase-dependent DNA damage during prolonged mitoses similar to control cells.

Enhancing Chromatid Cohesion does not correct Mitotic DNA Damage

Enhancing sister chromatid cohesion by inhibiting the negative regulator of cohesion Wapl has been shown to correct DNA damage in pRB-deficient interphase cells, and thus damage induced by replication stress (Manning et al., 2010). In order to determine if improper cohesion is also mechanistically responsible for regulating mitotic DNA damage in control and pRB-deficient cells, codepletion of pRB and Wapl using RNA interference was completed (Figure 7A), and γH2AX foci accumulation was scored from immunofluorescence imaging to determine levels of DNA damage. The data shows that mitotic DNA damage in both control and RB-deficient cells is not suppressed by depletion of Wapl (Figure 7B). This suggests that despite the accepted theory that pRB loss is associated with cohesion defects and chromosomal instability, mitotic DNA damage is not mechanistically regulated by chromatid cohesion.
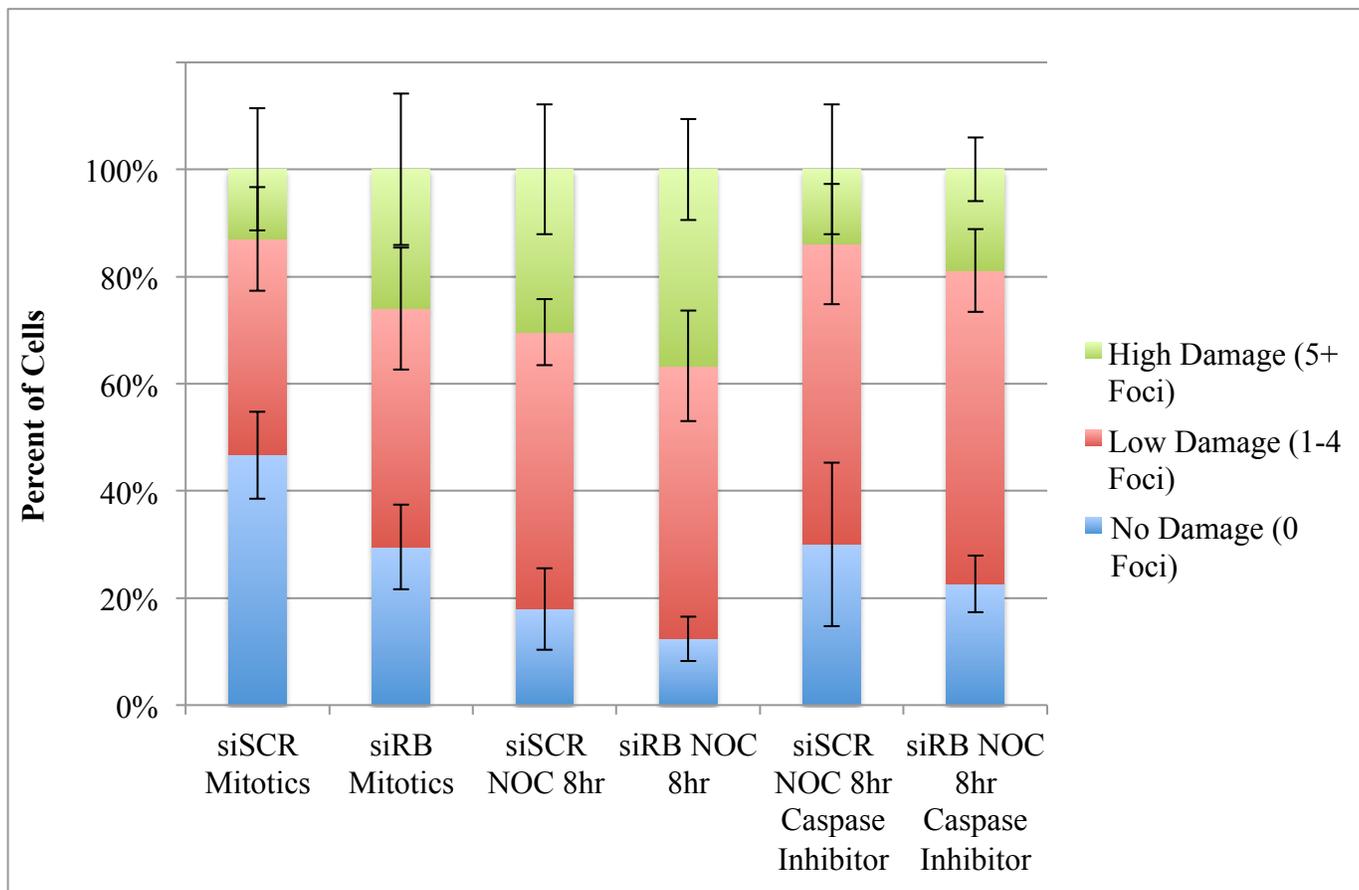
	siSCR Control vs. siRB Control	siSCR NOC 8hr vs. siRB NOC 8hr	siSCR Control vs. siSCR NOC 8hr	siSCR NOC 8hr vs. siSCR NOC 8hr Caspase Inhibitor	siRB Control vs. siRB NOC 8hr	siRB NOC 8hr vs. siRB NOC 8hr Caspase Inhibitor
No Damage (0 Foci)	**0.0017**	0.1173	**1.80E-05**	0.0903	**0.0002**	**0.0023**
Low Damage (1-4 Foci)	0.4740	0.8709	**0.0217**	0.3971	0.2857	0.1703
High Damage (5+ Foci)	0.0825	0.3008	**0.0173**	**0.0328**	0.1228	**0.0021**

Figure 6: Caspase Inhibition Partially Corrects DNA Damage acquired during Prolonged Mitosis. Quantification of γH2AX foci scored after immunofluorescence imaging of RPE-1 cells. Cells were treated with siSCR or siRB and mitotic arrest was induced using the microtubule poison nocodazole for 8hr or 20hr. Caspase inhibition was accomplished using the inhibitor Z-VAD-FMK. P-values were calculated by a two-tailed t-test across conditions. Bolded values indicate significance. A total of 7 replicates were completed.
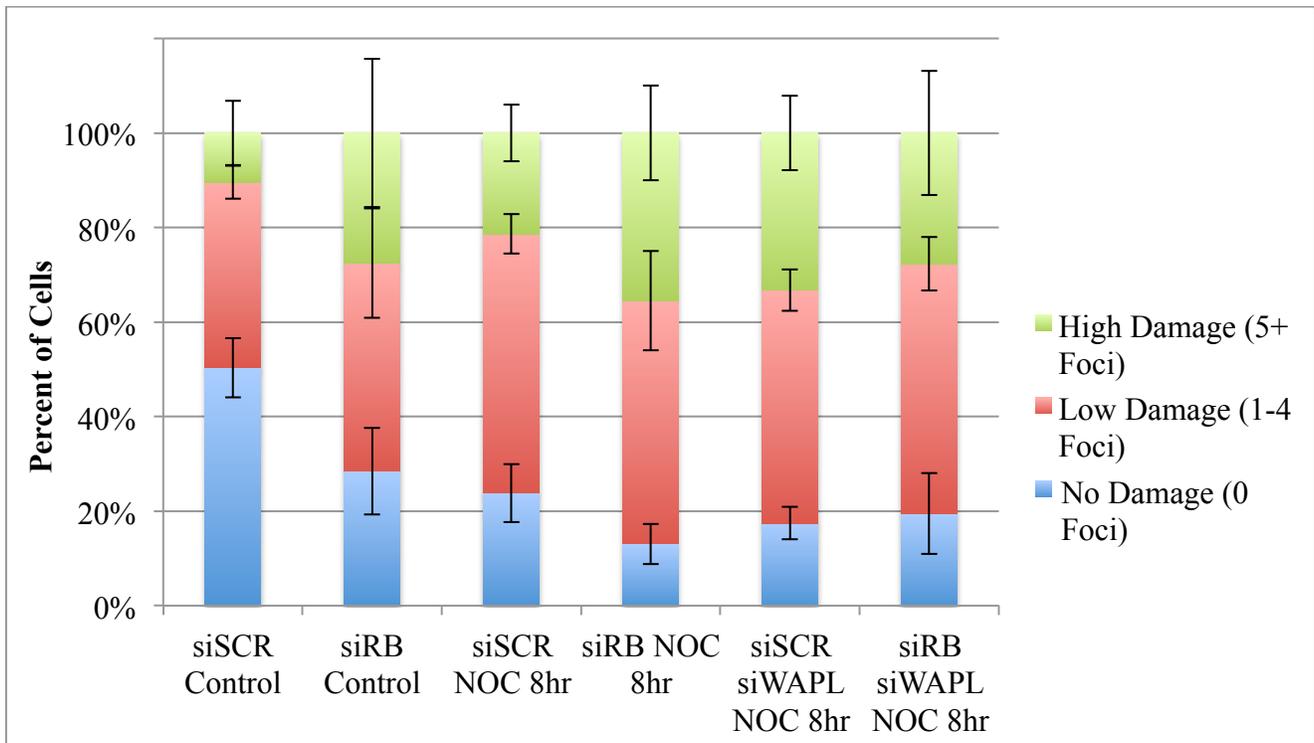
	siSCR Control vs. siRB Control	siSCR NOC 8hr vs. siRB NOC 8hr	siSCR Control vs. siSCR NOC 8hr	siSCR NOC 8hr vs. siSCR siWAPL NOC 8hr	siRB Control vs. siRB NOC 8hr	siRB NOC 8hr vs. siRB siWAPL NOC 8hr
No Damage (0 Foci)	**0.0059**	**0.0117**	**0.0063**	0.1926	**0.0017**	0.1001
Low Damage (1-4 Foci)	0.5174	0.6153	**0.0079**	0.1910	0.2330	0.7679
High Damage (5+ Foci)	0.1143	**0.0556**	0.1046	0.1049	0.2810	0.2359

Figure 7: Enhancing Cohesion via Depletion of Negative Regulator WAPL has no effect on Mitotic DNA Damage. Quantification of γH2AX foci scored after immunofluorescence imaging of RPE-1 cells. Cells were treated with siSCR, siRB, and/or siWAPL and mitotic arrest was induced using the microtubule poison nocodazole for 8hr or 20hr. P-values were calculated by a two-tailed t-test across conditions. Bolded values indicate significance. All conditions were performed a minimum of 3 replicates, with all siRB conditions completed with 3 additional replicates.

All data thus far has suggested that when RPE-1 cells are depleted of pRB and undergo a prolonged mitosis, they accumulate more DNA damage dependent γH2AX foci than control mitotics and do so at an exacerbated rate in comparison to control cells also undergoing a prolonged mitosis. The immunofluorescence data is complemented by western blot in HCT-116 cells (Figure 8A,B), confirming that an 8-hour mitotic arrest induced by the microtubule poison nocodazole accelerates DNA double stranded break acquisition in pRB-deficient cells. This data suggests that γH2AX accumulation in pRB-deficient cells undergoing prolonged mitoses is conserved in HCT116 cells, further promoting an opportunistic therapeutic phenotype for pRB-deficient cancers.

A

RB

P53

Tubulin

γH2AX

Tubulin

B

e in | 8 |————————————————————— ⊤ ——————————| ■ yH2AX |

Figure 8: γH2AX 6 **Cell Line.** A) Western b th nocodazole for the figure was generated by Ripu

Chapter III: Conclusions and Future Directions

pRB-Deficient Cells Acquire More DNA Damage than Control Cells during Normal Mitotic Timing and Abnormal Mitoses and do so at an Exacerbated Rate

Results have shown that pRB-deficient cells acquire more mitotic DNA damage than control cells, and that the level of DNA damage is exacerbated in response to the microtubule poison nocodazole. It has previously been recognized that control cells have increased levels of DNA damage during prolonged mitotic events (Orth et al., 2012), but the data considering the role of key cell cycle regulator pRB in regulating mitotic DNA damage is novel. Throughout a time course of induced mitotic arrest, it was demonstrated pRB-deficient arrested cells begin to acquire DNA damage as early as 4 hours, while control arrested cells do not begin to show significant changes in levels of DNA damage until 8 hours. In addition, despite DNA damage being present in both control and pRB-deficient cells during the 8 hours mitotic arrest time point, pRB-deficient cells acquire higher levels of DNA damage in comparison to their counterpart. Due to the evidence that pRB-deficient cells acquire mitotic DNA damage at higher rates than control cells, pRB-deficient cells have a novel mitotic regulatory role to be appreciated.

Mechanistically, the working model proposed for pRB-regulated acquisition of mitotic DNA damage originally theorized three different processes (Figure 9). First, it was hypothesized that loss of pRB accelerates the decrease in expression levels of anti-apoptotic factors to reach the threshold that activates caspase and DNase activity. The data in Figure 6 suggests that similar to the literature, caspase inhibition lowers the levels of DNA damage in control cells undergoing abnormal mitoses. This phenotype holds true in pRB-deficient cells, but there is also a smaller population of cells with damage in general when caspase activity is inhibited. This suggests a prominent role for pRB associated caspase activation, and requires further analysis of the levels of anti-apoptotic factors in pRB-deficient cells to more accurately describe the mechanism of DNA damage acquisition. Also required is a quantified protein analysis of cleaved caspase in control and pRB-deficient cells undergoing prolonged mitotic events.
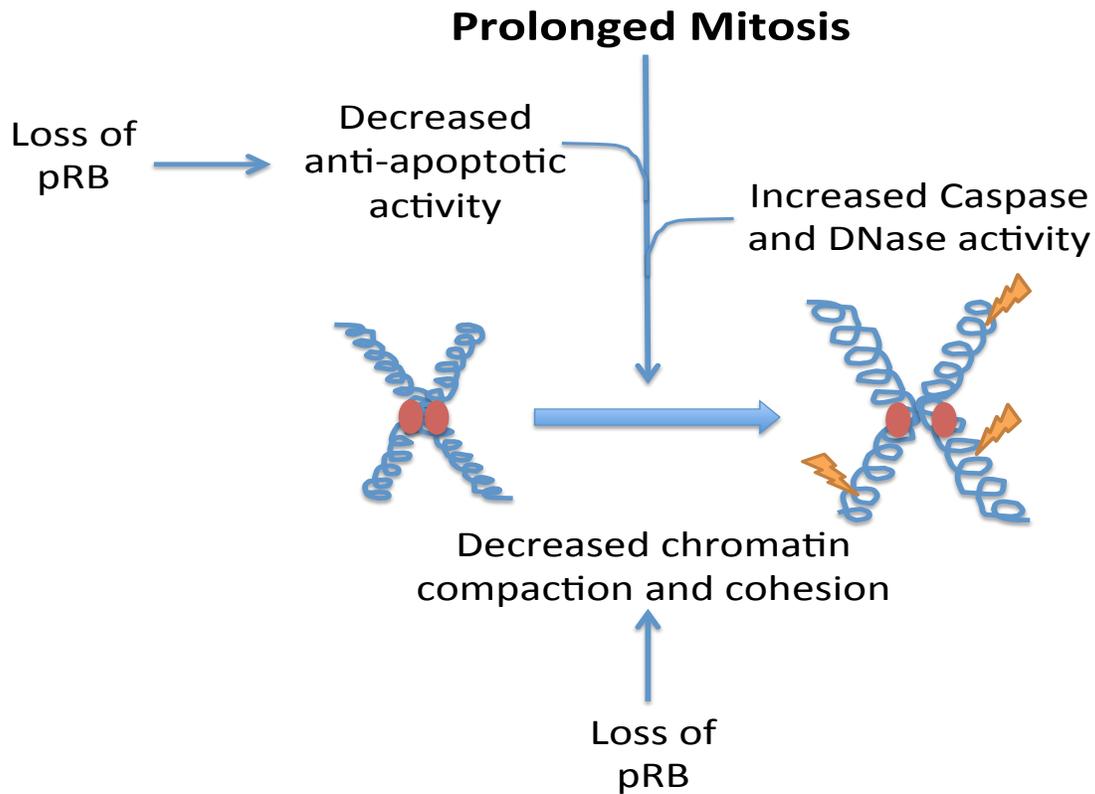
Figure 9: A Model of pRB-Regulated Acquisition of Mitotic DNA Damage

Alongside this and due to the regulatory function of pRB in maintaining accurate chromosome cohesion and condensation, it was also hypothesized that loss of pRB disrupts cohesion and/or condensation and thus leaves regions of the chromosome exposed for DNA damage (Figure 9). The data in Figure 7, which analyzed whether increasing chromatid cohesion would limit the acquisition of DNA damage in pRB-deficient cells undergoing prolonged mitoses, shows that the accepted role of pRB in regulating cohesion has no influence on mitotic DNA damage. In addition to these experiments, enhancing chromosome condensation should also be explored to determine if optimal function of pRB in condensation limits mitotic DNA damage.

Future Directions and Clinical Relevance

The data outlined above reveals a role for pRB in the regulation of mitotic DNA damage. Mechanistically, there are several options that still need to be experimentally optimized and investigated. First, the data above suggested that the G2/M DNA damage checkpoint in pRB-deficient cells remains intact due to

the inability to observe mitotic figures when such cells are treated with doxorubicin at doses ranges from 250nM to 2μM. This experiment could be verified with live cell microscopy, in which G2 pRB-deficient cells with DNA damage can be monitored on if they enter mitosis. Another important question to consider is was the range of doxorubicin appropriate, or more specifically what is the lowest concentration of doxorubicin that will still allow mitotic entry in both control and pRB-deficient cells. It is possible that this concentration would differ between control and pRB-deficient cells, suggesting that a higher amount of DNA damage in pRB-deficient cells is able to bypass the checkpoint in comparison to control cells. This would further support the literature, which shows how the phosphorylation of RB is regulated in an ATM Chk1/2 dependent manner in response to DNA damage (Inoue et al., 2007).

Alongside further analysis of DNA damage acquisition is exploring whether pRB-deficient cells that escape the mitotic arrest either correct DNA damage or activate the apoptotic pathway during the subsequent G1 phase of the cell cycle. It is hypothesized that since pRB regulates the G1/S phase checkpoint, correction of DNA damage will be compromised in pRB-deficient cells during the next cell cycle. This experiment could be accomplished using FACS analysis, in which a population of G1 cells washed out from cells previously arrested in mitosis will be analyzed for activation of the DNA damage response pathway. Specifically, the expression levels of proteins involved in nonhomologous end joining will be monitored to conclude whether the DNA damage acquired in mitosis has induced the DNA damage response for correction before subsequent DNA duplication and whether this holds in pRB-deficient cells.

Another possibility in relation to activation of the DNA damage response pathway that should be considered is if the repair pathway is being activated in response to prolonged mitotic arrest in control and pRB-deficient cells. It has been shown that activation of the DNA damage response pathway in mitosis induces chromosome segregation defects (Bakhoum et al., 2014). Similarly, pRB loss has been associated with chromosomal instability (Manning et al., 2010). Chromosomal instability may be an underlying cause or response to DNA damage, thus it is possible that pRB loss promotes DNA damage, which drives activation of the DNA damage response pathway, and subsequently promotes chromosomal instability.

The data above supports that the mitotic DNA damage phenotype observed in pRB-deficient mitotic cells is significant and conserved in a cancer cell line. Clinically, this is relevant as several cancers are characterized by pRB loss of function. To target this phenotype in cancer, I began to explore whether pRB-deficient cells are susceptible to mitotic arrest. To do this, control and pRB-deficient cells were treated with three different small molecules that induce mitotic arrest, and the viability of these cells was measured 3 days and 6 days after plating. There are several areas to improve on in this experiment, as the data collected showed variation due to the plate reader protocol set up as well as timing and concentration of small molecule inhibitor addition. If optimized for repeat experiments – in addition to chromosome condensation, caspase activation, and DNA damage response pathway analysis – the story behind pRB and mitotic DNA damage can be unraveled to provide therapeutic opportunities for pRB-deficient cancers.

Chapter IV: Methods

Cell Culture

Human RPE-1 cells and RPE-1 cells expressing a pIND shRB were maintained in Dulbecco's Modified Eagle Medium (DMEM), containing 10% Fetal Bovine Serum (FBS) and 50μg/mL streptomycin. Knockdown was accomplished in pIND RPE-1 cells by treating with 2μg/mL Doxycyclin hyclate (purchased from Sigma-Aldrich, Cat #D8191) for 48 hours.

shRB Sequence
TGCTGTTGACAGTGAGCGCGCACTTCGATATCTACTGAAATACTGAAGCCACAGA TGTATTTCAGTAGATATCGAACTGCTTGCCTACTGCCTCGGA
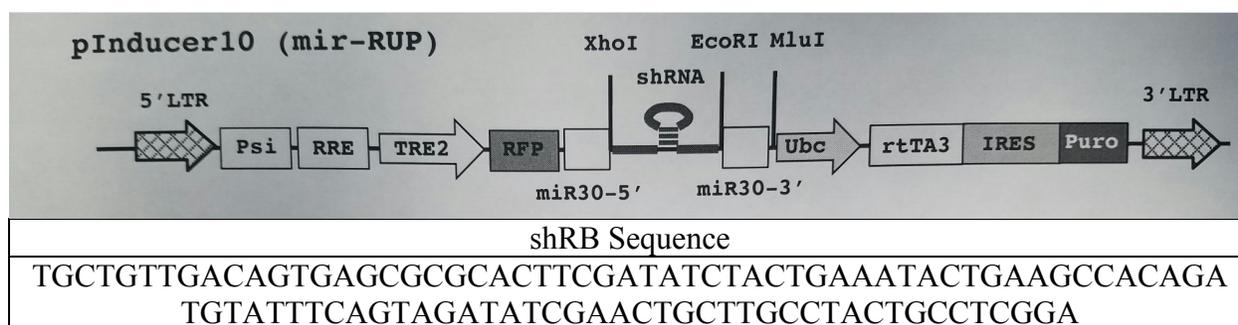
Figure 10: Vector map and short hairpin sequence for pIND RPE-1 cells. Received from Steve Elledge lab.

Antibodies

Antibodies used in this work included H2A.X (purchased from Cell Signaling, Cat. #2577L), Rb (4H1) (purchased from Cell Signaling, Cat. #9309L), and α-tubulin (DM1a) (purchased from Santa Cruz Biotechnology, Cat #sc-32293). All antibodies were used per the manufacturer's recommendations.

Small Molecules

Mitotic inhibitors used in this work included Nocodazole (purchased from Sigma-Aldrich, Cat #M1404, used at 100ng/mL), Monastrol (purchased from Sigma-Aldrich, Cat #M8515, used at 100μM), and Paclitaxel (purchased from Sigma-Aldrich, Cat #T7402, used at 10μM). DNA damaging agents used in this work included

Doxorubicin hydrochloride (purchased from Sigma-Aldrich, Cat #D1515, used at 250nM, 500nM, 750nM, and 2µM) and Etoposide (purchased from Selleckchem, Cat #S1225, used at 2µg/mL and 1µg/mL. The caspase inhibitor used was Z-VAD-FMK (purchased from Bio Connect, Cat #sc-3607, used at 100µM). All molecules were dissolved in Dimethyl Sulfoxide (DMSO).

siRNA Transfection

Approximately 60,000 RPE-1 cells were plated on coverslips in 12 well dishes the day of transfection and were grown without antibiotics. siRNA sequences in Table 2 were transfected into cells using Oligofectamine reagent through the following method: for one coverslip, the mixture of 3µL of desired siRNA incubated at room temperature for five minutes in 100µL of Opti-MEM reduced serum media per coverslip was combined with the mixture of 3µL of Oligofectamine reagent incubated at room temperature for five minutes in 100µL of Opti-MEM reduced serum media. This was incubated at room temperature for 20 minutes. Immediately after adding the desired amount of cells, the 200µL transfection mixture was added to the cells. Cells treated with nocodazole for 16 or 20 hours proliferated for approximately 36 hours, upon which media containing antibiotics and the desired small molecule inhibitor was added. Cells treated with nocodazole for 4 or 8 hours proliferated for approximately 48 hours, upon which media containing antibiotics and the desired small molecule inhibitor was added.

Immunofluorescence Staining and Microscopy

RPE-1 cells on glass coverslips were fixed for 20 minutes at room temperature in pre-warmed 4% paraformalydhyde/phosphate-buffered saline (PBS), extracted with 1X PBS/0.5% Triton X100 for 10 minutes, and blocked in TBS/BSA for 20 minutes. In between each step, cells were washed with 1X PBS. Cells were then incubated for 90 minutes in primary antibody, washed 5 minutes in TBS/BSA, and incubated in the dark

Table 2: siRNA Sequences. Purchased from Thermo Scientific

siRNA	Catalog Number	Target Sequence
ON-TARGETplus Human RB1	J-003296-23	GAACAGGAGUGCACGGAUA
	J-003296-24	GGUUCAACUACGCGUGUAA
	J-003296-25	CAUUAAUGGUUCACCUCGA
	J-003296-26	CAACCCAGCAGUUCGAUAU
ON-TARGETplus Human WAPAL	J-026287-09	GGAGUAUAGUGCUCGGAAU
	J-026287-10	GAGAGAUGUUUACGAGUUU
	J-026287-11	CAAACAGUGAAUCGAGUAA
	J-026287-12	CCAAAGAUACACGGGAUUA

for 45 minutes with Alexa Fluor 488 anti-mouse IgG and 546 anti-rabbit IgG (purchased from Life Technologies, Cat #A-11029 and #A-11010 respectively). Cells were washed for 5 minutes in TBS/BSA, and the slips were mounted onto slides using ProLong Gold antifade mountant (purchased from Life Technologies, Cat #P36934). At least 50 cells per condition were imaged using a Nikon Ti-E inverted fluorescence microscope, using the 60X oil-immersion lens in Z-series. The details on the microscope are as follows.

Dr. Manning has a dedicated microscopy room, adjacent to her wet lab research space, equipped with a Nikon Ti-E inverted fluorescence microscope on an antivibration table. The microscope is outfitted with Nikon objectives including: 10x CFI Plan Fluor for phase and immunofluorescence imaging; 20x and 40x CFI Plan Fluor extra long working distance objectives for immunofluorescence of multi well imaging plates; and a 60x Plan Apo oil immersion objective for high magnification imaging. Filter sets for DAPI, EGFP, DSRed, and Cy5 enable 4-channel imaging and capture with a Zyla sCMOS camera. An XY-encoded stage and NIS Elements software permit fully automated, semi-high throughput imaging of both slides and multi well plates. An InVivo Environmental incubator chamber allows for temperature and CO_2 control to enable multiday live cell imaging. Two image analysis stations running NIS Elements and Cell Profiler software compliment the imaging set up.

Quantification of DNA Damage

To quantify the amount of DNA damage in mitotic cells, cells were categorized through manual counting into three ranges of H2A.X foci. Cells were considered to have no damage if no yH2AX foci were present, low damage if 1-4 yH2AX foci were present, or high damage if 5 or more yH2AX foci were present.

Immunoblotting

For immunoblots, 10^7 cells/mL were collected per condition and were solubilized directly in 1X SDS-PAGE sample buffer. Total cell protein was then separated by size using SDS-PAGE and transferred to either PVDF membrane (RB immunoblots) or nitrocellulose (H2A.X). For RB immunoblots, primary antibodies were incubated overnight at 4 degrees Celsius in 5% milk, 1X TBS, 0.1% Tween 20. For H2A.X immunoblots, primary antibodies were incubated overnight at 4 degrees Celsius in 5% BSA, 1X TBS, 0.1% Tween 20. Primary antibody was then detected using HRP-conjugated secondary antibodies diluted in 1X TBS, 0.1% Tween 20 for one hour at room temperature. The signal was then detected using film.

Proliferation Assay

RPE-1 cells expressing pIND shRB were grown for 48 hours either in the absence or presence of Doxycyclin hyclate. 3000 cells/well were then plated on a 96 well dish in the presence of either no drug, nocodazole, monastrol, paclitaxel, doxorubicin, or etoposide. Doxycyclin hyclate was readded to desired cells upon plating. After three and six days, PrestoBlue Cell Viability Reagent (Invitrogen, Cat. #A13262) was added to cells and a viability reading was performed reading Alamar Blue at fluorescence 544 and emission 590.

References

Bakhoum, S.F., Kabeche, L., Murnane, J.P., Zaki, B.I., Compton, D.A. (2014). DNA-Damage Response during Mitosis Induces Whole-Chromosome Missegregation. *Cancer Discov.* 4:1281-1289

Benada, J., Burdova, K., Lidak, T., von Morgan, P., Macurek, L. (2015). Polo-like kinase 1 inhibits DNA damage response during mitosis. *Cell Cycle.* 14:219-231

Bester, A.C., Roniger, M., Oren, Y.S., Im, M.M., Sarni, D., Chaoat, M., Bensimon, A., Zamir, G., Shewach, D.S., Kerem, B. (2011). Nucleotide deficiency promotes genomic instability in early stages of cancer development. *Cell.* 145:435-446

Binné, U.K., Classon, M.K., Dick, F.A., Wei, W., Rape, M., Kaelin, W.G. Jr, Näär, A.M., Dyson, N.J. (2007). Retinoblastoma protein and anaphase-promoting complex physically interact and functionally cooperate during cell-cycle exit. *Nat Cell Biol* 9:225-32

Bosco, E.E. and Knudsen, E.S. (2005). Differential role of RB in response to UV and IR damage. *Nucl. Acids Res.* 33:1581-1592

Cheeseman, I.M. The Kinetochore. *Cold Spring Harb Perspect Biol.* 6:a015826

Cook, R., Zoumpoulidou, G., Luczynski, M.T., Rieger, S., Moquet, J., Spanswick, V.J., Hartley, J.A., Rothkamm, K., Huang, P.H., Mittnacht, S. (2015). *Cell Reports.* 10:2006-2018

Coschi, C.H., Martens, A.L., Ritchie, K., Francis, S.M., Chakrabarti, S., Berube, N.G., Dick, F.A. (2010). Mitotic chromosome condensation mediated by the retinoblastoma protein is tumor-suppressive. *Genes Dev* 24:1351-1363

Damania, B. and Pipas, J. (2008). DNA Tumor Viruses. New York: Springer Science

Du, Wei and Searle, J.S. (2009). The Rb Pathway and Cancer Therapeutics. *Curr Drug Targets.* 10:581-589

Dyson, N.J. (2016). Rb1: a prototype tumor suppressor and an enigma. *Genes Dev* 30:1492-1502

Ferrari, S. and Gentili, C. (2016). Maintaining Genome Stability in Defiance of Mitotic DNA Damage. *Front Genet* 7:10.3389/fgene.2016.00128

Floyd, S.R., Pacold, M.E., Huang, Q., Clarke, S.M., Lam, F.C., Cannell, I.G., Bryson, B.D., Rameseder, J., Lee, M.J., Blake, E.J., Fydrych, A., Ho, R., Greenberger, B.A., Chen, G.C., Maffa, A., Del Rosario, A.M., Root, D.E., Carpenter, A.E., Hahn, W.C., Sabatini, D.M., Chen, C.C., White, F.M., Bradner, J.E., Yaffe, M.B. (2013). The bromodomain protein Brd4 insulates chromatin from DNA damage signaling. *Nature.* doi:10.1038/nature12147

Genovese, C., Trani, D., Caputi, M., Claudio, P.P. (2006). Cell cycle control and beyond: emerging roles for the retinoblastoma gene family. *Oncogene.* 25:5201-5209

Hegemann, B., Hutchins, J.R., Hudecz, O., Novatchkova, M., Rameseder, J., Sykora, M.M., Lui, S., Mazanek, M., Lenart, P., Heriche, J.K. et al. (2011). Systematic phosphorylation analysis of human mitotic protein complexes. *Sci Signal* 4:rs12

Hernando, E., Nahle, Z., Juan, G., Diaz-Rodriguez, E., Alaminos, M., Hermann, M., Michel, L., Mittal, V., Gerald, W., Benezra, R., Lowe, S.W., Cordon-Cardo, C. (2004). Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. *Nature* 430:797-802

Horowitz, J.M., Park, S.H., Bogenmann, E., Cheng, J.C., Yandell, D.W., Kaye, F.J., Minna, J.D., Dryja, T.P., Weinberg, R.A. (1990). Frequent inactivation of the retinoblastoma anti-oncogene is restricted to a subset of human tumor cells. *Proc. Natl. Acad. Sci. USA* 87:2775-2779

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

Kim, Y., Holland, A.J., Lan, W., Cleveland, D.W. (2010). Aurora kinases and protein phosphatase I mediate chromosome congression through regulation of CENP-E. *Cell* 142:144-455

Kong, X., Stephens, J., Ball Jr., A.R., Heale, J.T., Newkirk, D.A., Berns, M.W., Yokomori, K. (2011). Condensin I Recruitment to Base Damage-Enriched DNA Lesions Is Modulated by PARP1. *PLoS ONE* 6:e23548

Kueng, S., Hegemann, B., Peters, B.H., Lipp, J.J., Schleiffer, A., Mechtler, K., Peters, J.M. (2006). Wapl controls the dynamic association of cohesin with chromatin. *Cell* 127:955-967

Longsworth, M.S., Herr, A., Ji, J.Y., Dyson, N.J. (2008). RBF1 promotes chromatin condensation through a conserved interaction with the Condensin II protein dCAP-D3. *Genes Dev* 22:1011-1024

Losada, A., Hirano, M., Hirano, T. (2002). Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev* 16:3004-3016

Meyerson, M. & Pellman, D. (2011). Cancer Genomes Evolve by Pulverizing Single Chromosomes. *Cell*. 144:9-10

Manning, A.L., Longsworth, M.S., Dyson, N.J. (2010). Loss of pRB causes centromere dysfunction and chromosomal instability. *Genes Dev* 24:1364-1376

Orth, J.D., Loewer, A., Lahav, G., Mitchison, T.J. (2012). Prolonged mitotic arrest triggers partial activation of apoptosis, resulting in DNA damage and p53 induction. *Mol. Biol. Cell.* 23:567-576

Peters, J.M., and Nishiyama, T. (2012). Sister Chromatid Cohesion. *Cold Spring Harb Perspect Biol* 4:a011130

Pickering, M.T. and Kowalik, T.F. (2006). Rb inactivation leads to E2F1-mediated DNA double-strand break accumulation. *Oncogene.* 25:746-755

Pines, J. and Rieder, C.L. (2001). Re-staging mitosis: a contemporary view of mitotic progression. *Nat. Cell Biol.* 3:E3-E6

Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R.A., Dynlacht, B.D. (2001). E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. *Genes Dev* 16:245-256

Samoshkin, A., Arnaoutov, A., Jansen, L.E., Ouspenski, I., Dye, L., Karpova, T., McNally, J., Dasso, M., Cleveland, D.W., Strunnikov, A. (2009). Human condensin function is essential for centromeric chromatin assembly and proper sister kinetochore orientation. *PLoS One* 4(8):e6381

Schmitz J., Wartin, E., Lenart, P., Mechtler, K., Peters, J.M. (2007). Sororin is required for stable binding of cohesin to chromatin and for sister chromatid cohesion in interphase. *Curr Biol* 17:630-636

Shen, Z (2011). Genomic instability and cancer: an introduction. *J Mol Cell Biol.* 3:1-3.

Sivakumar, S. and Gorbsky, G.J. (2015). Spatiotemporal regulation of the anaphase-promoting complex in mitosis. *Nat. Rev. Mol. Cell Biol.* 16:82-94

Srinivasan, S.V., Mayhew, C.N., Schwemberger, S., Zagorski, W., Knudsen, E.S. (2007). RB loss promotes aberrant ploidy by deregulating levels and activity of DNA replication factors. *J Biol Chem* 282:23867-23877

Strom, L., Lindroos, H.B., Shirahige, K., Sjorgren, C. (2004). Postreplicative Recruitment of Cohesin to Double-Strand Breaks is Required for DNA Repair. *Molecular Cell.* 16:1003-1015

Sumara, I., Vorlaufer, E., Stukenberg, P.T., Kelm, O., Redemann, N., Nigg, E.A., Peters, J.M. (2002). The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. *Mol Cell* 9:515-525

Thompson, S.L. & Compton, D.A. (2011). Chromosomes and Cancer Cells. *Chromosome Res.* 19:433-444

Uhlmann, F. (2001). Chromosome condensation: Packaging the genome. *Current Biology* 11:R384-R387

van Harn, T., Foijer, F., van Vugt, M., Banerjee, R., Yang, F., Oostra, A., Joenje, H., te Riele, H. (2010). Loss of Rb proteins causes genomic instability in the absence of mitogenic signaling. *Genes Dev* 24:1377-1388

Watrin, E. & Peters, J.M. (2006). Cohesin and DNA Damage Repair. *Experimental Cell Research.* 312:2687-2693

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. *Cell.* 81:323-330