Defining the mechanisms contributing to genomic instability following RB loss

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Abstract

The vast majority of human cancers exhibit loss or functional inactivation of the retinoblastoma protein. For over 30 years studies have descried the canonical role of RB in controlling the G_1-to-S transition through the binding and regulation of E2F transcription factors. While previous work has shown the loss of RB results in of genomic instability, mechanistic insights into the underlying defects arising from this loss of RB have yet to be understood. Here I asses the role RB loss in the acquisition of DNA damage in RPE-1 cells during mitosis. I show that loss of RB leads to increased DNA damage in mitosis and that damage is occurring at centromeres. Furthermore, the damage seen during mitosis can be decreased by increasing key components of chromatin, Cohesin and Condensin. Finally, the changes to chromatin structure resulting from loss of RB lead to increased rates of transcription promoting increased levels of centromeric Aurora B and DNA damage. In cancer cells we also see increased centromeric Aurora B levels resulting in the loss of microtubule stability and generation of segregation errors. This persistent loss or gain of whole chromosomes is known as nCIN and has been shown to promote intra-tumor heterogeneity and drug resistance. In non small cell lung cancer cells, we show that chromosome segregation errors are sensitive to changes in microtubule dynamics and through increasing chromosome cohesion we are able to suppress nCIN and reduce intra-tumor heterogeneity. Suppression of nCIN was subsequently found to reduce the incidence of acquired drug resistance. These findings suggest mechanisms to suppress nCIN in RB deficient cancers may allow us to develop more effective therapeutic treatments.

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Chapter 1: Introduction

Chromosome alignment and segregation

Mitosis is a complex and highly regulated process by which a eukaryotic cell accurately segregates all its genetic material to form two genetically identical daughter cells. The onset of mitosis is defined by nuclear envelope breakdown, where the cell tightly compacts its chromatin to form condensed structures known as the mitotic chromosomes. The chromosomes then interact with dynamic microtubules that are nucleated by two organelles known as centrioles. Positioned at either side of the cell, the centrosomes anchor the two poles of the mitotic spindle. In order for chromosomes to make directed movements needed for accurate chromosome segregation, spindle microtubules must first attach to a specialized proteinaceous structure on

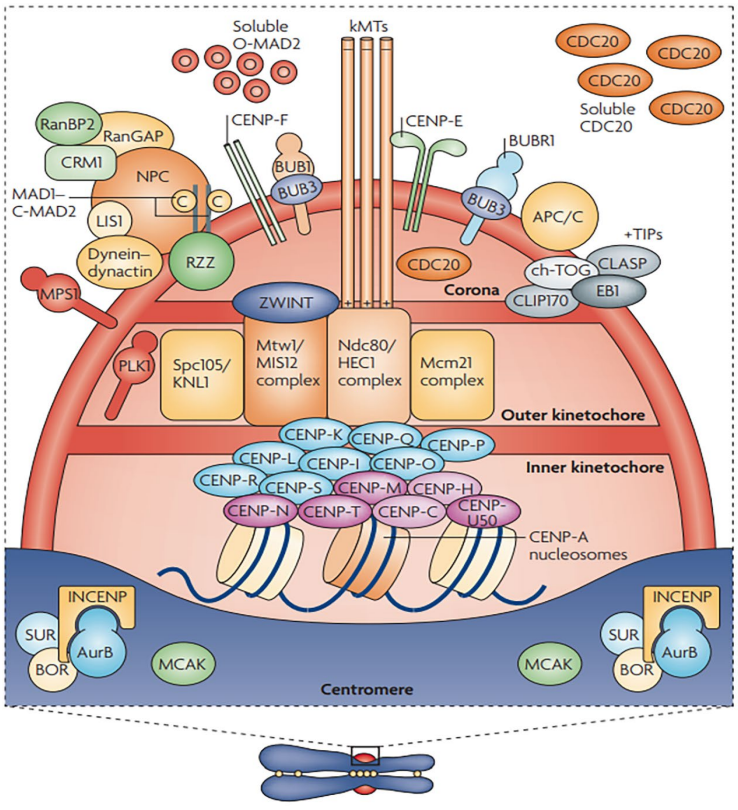

Figure 1.1: Structure of the Centromere-Kinetochore region. Proteins of the inner kinetochore and centromeric region to include those involved in establishment of stable microtubule attachments, centromere size, and spindle assembly checkpoint signaling (Adapted by permission from Springer Nature: *Nat Rev Mol Cell Biol* (The spindle-assembly checkpoint in space and time, Musacchio A and Salmon E) 2007).

the chromatin. This structure, known as a kinetochore, encompasses over 100 proteins that assemble at a region of each chromosome known as the centromere (Figure 1.1) (Brinkley and Stubblefield 1966, Musacchio and Salmon 2007).

In the early stages of chromosome congression motor proteins called chromokinesins that are located on chromosome arms, and the motor protein CENP-E, that is located at the kinetochore, work in concert to move chromosomes away from spindle poles (Schaar, Chan et al. 1997, Wandke, Barisic et al. 2012, Vicente and Wordeman 2015). Both types of molecular motors have plus-end directed motor activity to walk the associated chromosomes towards the plus end of nearby or laterally associated microtubules (i.e., away from the spindle pole) (Walczak, Cai et al. 2010). This movement positions the chromosome closer to the center of the cell to facilitate the formation of stable end-on attachments with microtubules (Figure 1.2). When the plus end of a

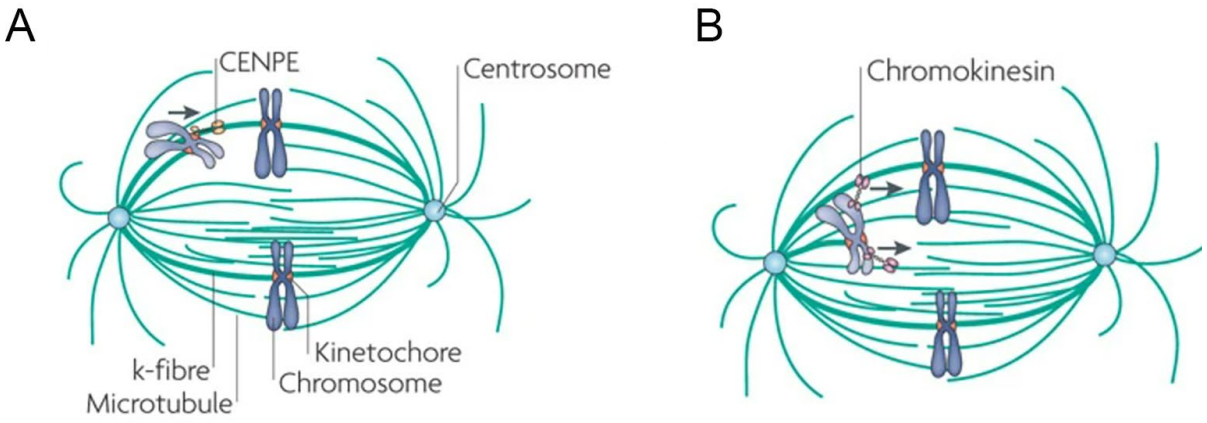

Figure 1.2: Chromosome congression with microtubule and motor-based forces. A) Plus end-directed CENP-E moves chromosomes towards the spindle equator. B) Chromokinesin motor proteins, found along chromosome arms drag a chromosome towards the spindle equator (Adapted by permission from Springer Nature: *Nat Rev Mol Cell Biol* (Mechanisms of chromosome behavior during mitosis, Walczark E., et al) 2010).

microtubule engages with a kinetochore, it is able to form a stable attachment through associations with a key complex of proteins, knowns as the KMN network, that make up the kinetochore-microtubule binding interface (Cheeseman, Chappie et al. 2006, Santaguida and Musacchio 2009). The KMN network, comprised of KNL1, Mis12, and the NDC80 complex, bind

to the plus end of a microtubule in a manner whereby microtubule dynamics are suppressed, and the now-stabilized microtubule can continue to grow and shrink (albeit at a decreased rate) while maintaining its attachment to the kinetochore (McKinley and Cheeseman 2016, Nagpal and Fukagawa 2016, Pesenti, Weir et al. 2016). Each human kinetochore is able to bind over 20 microtubules, which are bundled laterally and known as kinetochore fibers, or k-fibers (McDonald, O'Toole et al. 1992). The coordinated depolymerization of a kinetochore fiber is the dominant driving force for directed chromosome movement during mitosis. Initially, only a single kinetochore achieves end-on attachment, a state known as monotely (Rieder and Salmon 1998). Occasionally, both kinetochores of a replicated sister chromatid pair may form end on k-fiber attachments with microtubules that are nucleated from a single spindle pole, a state known as

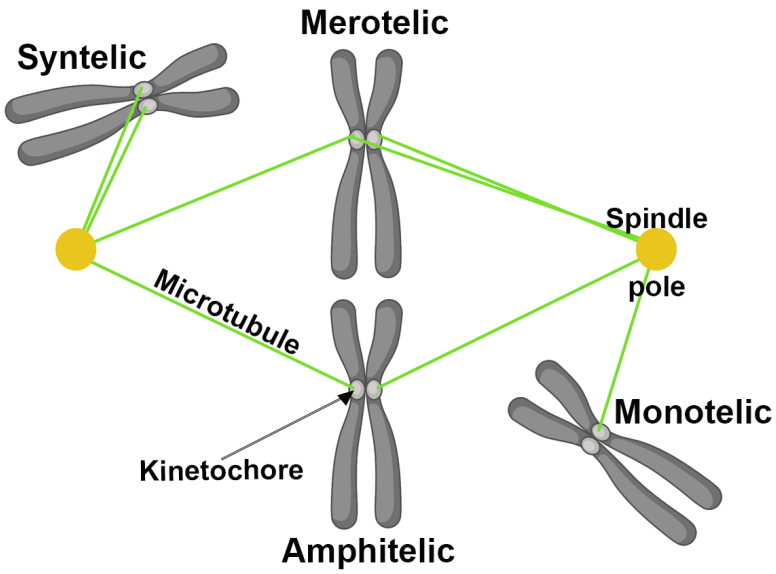

Figure 1.3: Types of microtubule attachments. Various microtubule attachments made on the way to forming a stable bioriented, amphitelic attachment. Syntelic and monotelic attachments will activate the spindle assembly checkpoint (created with biorender.com).

syntely (Figure 1.3). In the cases of both monotely and syntely, k-fiber depolymerization that drag the chromosome towards the spindle pole are antagonized by the anti-poleward forces described above (Cimini, Moree et al. 2003, Lampson, Renduchitala et al. 2004). The combination of forces positions the pair of chromosomes near the center of the spindle where they are more likely to

encounter and form stable attachments with microtubules nucleated at the opposite spindle pole. Chromosomes that remain in this state of attachment to a single spindle pole sustain a molecular “wait” signal known as the spindle assembly checkpoint (Musacchio and Hardwick 2002, Taylor, Scott et al. 2004). When the sister chromosome of a monotonically or syntelicly attached pair achieves stable end-on attachments with microtubules from the opposite spindle pole, that chromosome pair is said to be biallelic. The balance of forces generated by the k-fibers attached to each sister pair drive congression and alignment of the chromosome pair at the center of the cell.

The presence of a single unattached, monotelic or syntelic attached chromosome will activate the spindle assembly checkpoint (SAC) (Rieder, Cole et al. 1995, Musacchio and Salmon 2007, London and Biggins 2014, Kuhn and Dumont 2017). Sensing lack of microtubule attachments or lack of the inter-kinetochore tension that would be generated by biallelic attachment, the mitotic checkpoint complex (Mad1, Mad2, Bub1, Bub3, BubR1, and Msp1) assembles at the kinetochore, inhibiting the anaphase-promoting complex (APC), and preventing exit from mitosis (Fang, Yu et al. 1998, Sudakin, Chan et al. 2001). Once the SAC has been satisfied the APC, a ubiquitin ligase, targets proteins for proteosome-mediated degradation (Fang, Yu et al. 1998, Wassmann and Benezra 1998, Tang, Bharadwaj et al. 2001, D'Angiolella, Mari et al. 2003, Morrow, Tighe et al. 2005). Securin is one such critical degradation target. Destruction of Securin allows for activation of the protease Separase which cleaves the cohesin complex that holds replicated sister chromosomes together (Uhlmann 2001, Ishiguro and Watanabe 2007). In the absence of cohesin, microtubule depolymerization forces felt at each kinetochore are unrestrained and the chromosomes are rapidly dragged towards the spindle pole from which their k-fiber originates (Shannon, Canman et al. 2002).

During the transition from syntelic to biallelic attachment, microtubules from the syntelicly attached kinetochore must first be released. However, if some of the syntelicly-attached microtubules remain associated with the kinetochore as new biallelic microtubule attachments are made, that single kinetochore will have stable end-on attachments with microtubules associated with both spindle poles, a situation referred to as merotely (Cimini, Moree et al. 2003, Gregan, Polakova et al. 2011). By virtue of the subset of biallelic attachments made, a merotelicly attached chromosome generates inter-kinetochore tension and can congress and align at the center. As a result, merotely does not activate the SAC. As a result, cells with merotelic attachments can progress into anaphase. However, because of its improper attachment, merotelic chromosomes do not get dragged towards one spindle pole and instead lag within the cleavage furrow. Lagging chromosomes are at risk of being segregated into the wrong daughter cells, leading to the two daughter cells having an unequal number of chromosomes, a feature known as aneuploidy (Figure1.4) (Teixeira and Heim 2005, Ricke, van Ree et al. 2008). Unresolved merotelic attachments have been identified as being one of the primary sources of segregation errors in cancer cells (Cimini, Moree et al. 2003, Cimini 2008, Compton 2011, Gregan, Polakova et al. 2011).

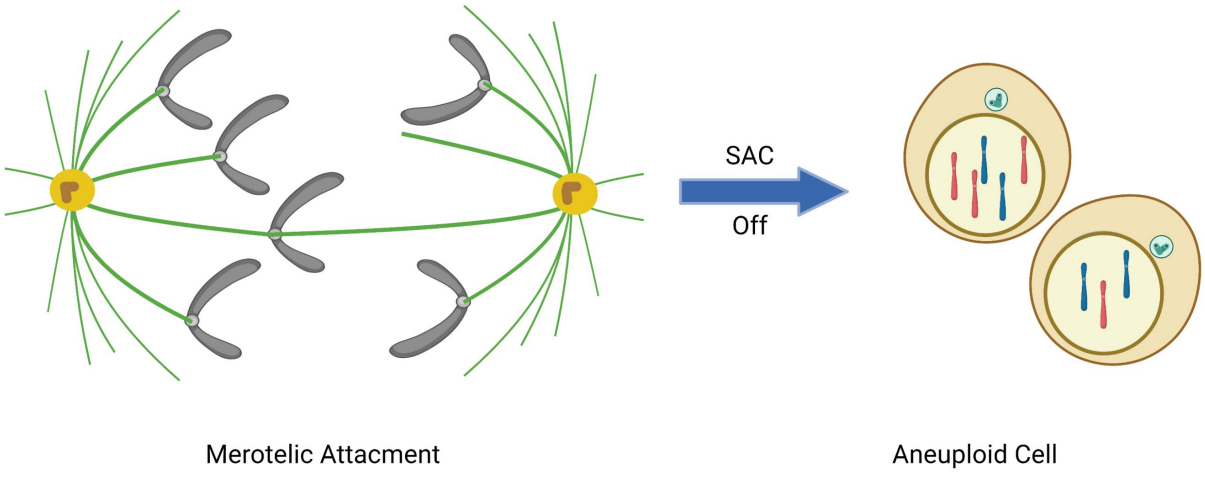

Figure 1.4: Consequence of merotelic attachments. A single kinetochore attaches to microtubules that arise from both poles of the spindle. This mal-attachment is still able to generate enough inner-kinetochore tension thus satisfying the SAC. As a consequence of this, both sister chromatids will be segregated into the same daughter cell resulting in aneuploidy (created with biorender.com).

Error Correction and SAC activation through Aurora B kinase

Intrinsic mechanisms exist within the cell before anaphase onset to correct merotelic attachments and ensure proper chromosome segregation. The correction of mal-attached microtubules is in part regulated by a centromere-localized serine/threonine kinase called Aurora B (AurB) (Glover, Leibowitz et al. 1995, Carmena, Ruchaud et al. 2009). Aurora B is part of a larger complex known as the chromosomal passenger complex (CPC). The other components of this complex are INCENP, Survivin, and Borealin (Cooke, Heck et al. 1987, Earnshaw and Bernat 1991, Adams, Wheatley et al. 2000, Kaitna, Mendoza et al. 2000, Kelly and Funabiki 2009, van der Waal, Hengeveld et al. 2012). Within the CPC Borealin and Survivin bind to the N-terminus of INCENP and are involved in bringing the CPC to the inner centromere (Mackay and Earnshaw 1993, Jeyaprakash, Klein et al. 2007, Yue, Carvalho et al. 2008), while the catalytic activity of the CPC is mediated by Aurora B, which binds to the c-terminal IN-box of INCENP (Figure 1.5) (Adams, Wheatley et al. 2000).

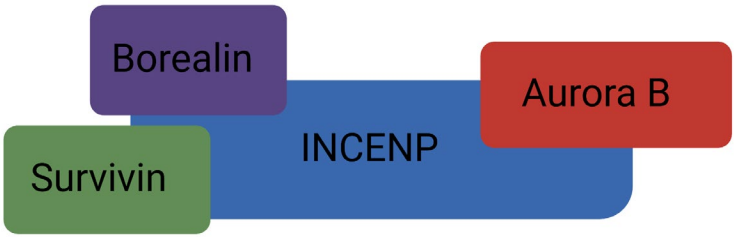

Figure 1.5: Components of the CPC. Borealin and Survivin bind to the N-terminus of INCENP, while Aurora B binds at the C-terminus of INCENP (created with biorender.com).

The Aurora B kinase is the master regulator of kinetochore-microtubule dynamics during mitosis. In mitosis Aurora B, along with the other members of the CPC are concentrated at the inner centromere, a highly specialized region on the chromosome where spindle microtubules and the kinetochore interact to form stable attachments. Through the phosphorylation of Hec1/NDC80, Aurora B destabilizes improper kinetochore-microtubule attachments thereby lowering the kinetochores binding affinity for microtubules (DeLuca, Gall et al. 2006, DeLuca, Lens et al. 2011,

Zaytsev, Sundin et al. 2014, Zaytsev and Grishchuk 2015, Yoo, Choi et al. 2018). In this way, phosphorylation of NDC80 by Aurora B is essential in releasing merotelic attachments to enable formation of biallelic microtubule attachments. Phosphomimetic mutants of Ndc80 fail to support microtubule attachments, while non-phosphorylatable mutants hyperstabalize attachments resulting in an increase of syntelic and merotelic attachments (Umbreit, Gestaut et al. 2012, Wimbish and DeLuca 2020). Studies using small molecule inhibitors of Aurora B have also shown that inhibition of Aurora B leads to stabilization of microtubules, thus decreasing the frequency at which improper attachments are removed (Hauf, Cole et al. 2003). Conversely over expression of Aurora B causes the continuous disruption of kinetochore-microtubule attachments, which can result in the premature release of the k-fiber (Munoz-Barrera and Monje-Casas 2014).

In addition to Ndc80, other Aurora B substrates have also been implicated in assisting in error correction. The SKA complex is one of those substrates that has been shown to be key in maintaining stable kinetochore-microtubule attachments. It is localized to the outer kinetochore in an KMN-dependent manner where it is regulated by Aurora B. Aurora B has been shown to negatively regulate the association of the SKA complex with Ndc80 (Welburn, Grishchuk et al. 2009, Chan, Jeyaprakash et al. 2012, Schmidt, Arthanari et al. 2012, Abad, Medina et al. 2014, Redli, Gasic et al. 2016). The mitotic centromere-associated kinesin (MCAK) is another target of Aurora B. MCAK is needed to regulate the depolymerization of microtubules. In order to act upon microtubules, it must first concentrate at the centromere (Kline-Smith, Khodjakov et al. 2004). Phosphorylation of MCAK by Aurora B was found to be essential in localizing MCAK to the centromere (Andrews, Ovechkina et al. 2004, Lan, Zhang et al. 2004, Knowlton, Lan et al. 2006, Tanno, Kitajima et al. 2010, Ems-McClung, Hainline et al. 2013, Shrestha and Draviam 2013). Finally, Aurora B has been shown to have an antagonistic relationship with the protein phosphatase 1 (PP1) and PP2A-B556 (a family of protein phosphatases) (Liu, Vleugel et al. 2010,

Foley and Kapoor 2013). Phosphorylation of Aurora B is countered by these two protein phosphatases.

Regulation of kinetochore-microtubule attachments requires the phosphorylation of substrates located at the outer kinetochore, but Aurora B along with the other components of the CPC are located at the inner kinetochore during mitosis. To explain how Aurora B is able to regulate proteins located at the outer kinetochore, researchers have proposed a model known as “spatial positioning”, in which it is posited that active Aurora B emerges from the inner kinetochore as a diffusible gradient to phosphorylate its substrates (Figure 1.6) (Tanaka, Rachidi et al. 2002,

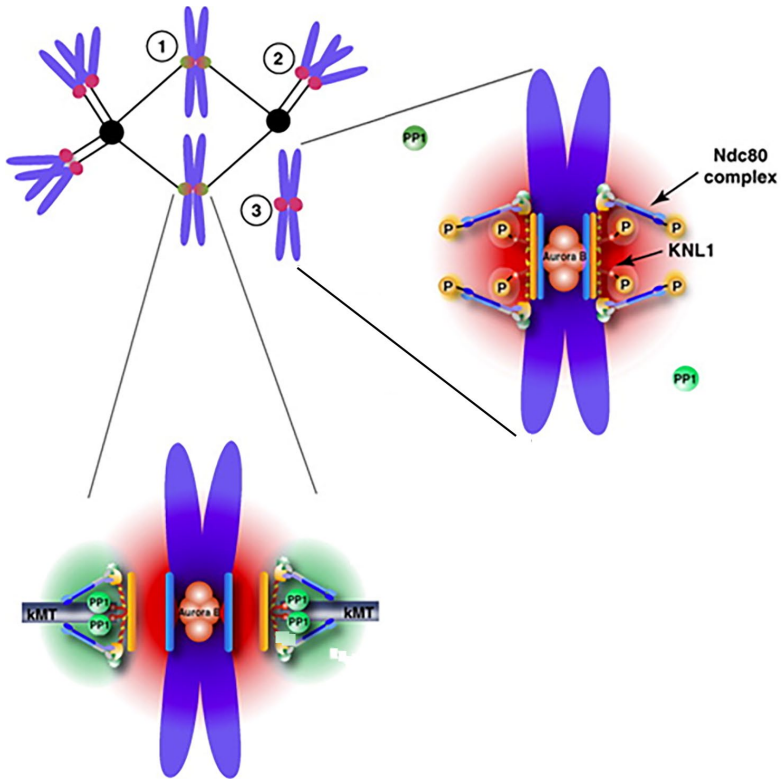

Figure 1.6: Spatial positioning model for Aurora B kinase. Cartoon depiction of a spindle with (1) bioriented chromosomes (2) syntelically attached chromosome (3) or an unattached chromosome. At the unattached chromosome a phosphorylation gradient is generated with Aurora B concentrated at the inner centromere. Phosphorylation targets within the Aurora B gradient are phosphorylated owing to their position within this gradient (red). Here tension is low, which destabilizes kinetochore microtubules. These substrates are then dephosphorylated at correct attachments, where tension is high, because they are positioned farther from the kinase gradient. Recruitment of PP1 to the outer kinetochore provides a counteracting gradient of dephosphorylation (green) (Adapted by permission from Elsevier: *Trends Cell Biol* (Sensing centromere tension: Aurora B and the regulation of kinetochore function, Lampson M and Cheeseman I) 2011).

Lampson and Cheeseman 2011). “Spatial positioning” reasons that in the early stages of mitosis Aurora B is recruited to and activated at the inner centromere before kinetochore-microtubule attachments have been established. In this case kinetochores lack pulling forces that are generated from attached microtubules and Aurora B is therefore located physically close enough to the kinetochore to phosphorylate kinetochore-localized substates. As attachments are made between the spindle microtubules and kinetochores, pulling forces generated from the microtubule attachments stretch the kinetochore away from the inner centromere and the Aurora B gradient. The physical separation of centromere-localized Aurora B from the kinetochore results in decreased phosphorylation of kinetochore substates, and as described above, results in stabilization of kinetochore-microtubule attachments (Tanaka, Rachidi et al. 2002, Kelly, Sampath et al. 2007, Liu, Vader et al. 2009, Uchida, Takagaki et al. 2009, Welburn, Vleugel et al. 2010, Wang, Ballister et al. 2011).

The contribution of Aurora B to the SAC is less well understood than its role in correcting erroneous microtubule attachments. It is postulated that Aurora B occupies a position upstream in recruitment pathway of SAC effectors. This is due to the fact that inhibition of Aurora B inhibits the recruitment of all proteins belonging to the SAC (Kallio, McCleland et al. 2002, Hauf, Cole et al. 2003). Interestingly studies have also shown that the relocation of the CPC to the spindle midbody during anaphase is needed to silence the SAC (Vazquez-Novelle and Petronczki 2010, Rattani, Vinod et al. 2014).

Recruitment of centromere localized Aurora B

The CPC can first be visualized on pericentromeric heterochromatin during interphase. Targeting

of the CPC to heterochromatin involves HP1α binding a PXVXL/I motif found between residues 167-171 in INCENP (Nozawa, Nagao et al. 2010, Kang, Chaudhary et al. 2011, Ruppert, Samejima et al. 2018). Activation of Aurora B is needed in order to target the CPC to the inner centromere, in such that active Aurora B phosphorylates Histone 3 at Serine 10 (H3S10), which removes HP1α so the CPC can concentrate at the centromere (Hsu, Sun et al. 2000, Murnion, Adams et al. 2001, Fischle, Tseng et al. 2005, Hirota, Lipp et al. 2005, Ruppert, Samejima et al. 2018). Centromeric enrichment of the CPC then occurs through the mitotic specific phosphorylation of two histone tails, Histones 3 and 2A (H3 and H2A) by the mitotic kinases Haspin and Bub1, respectively (Yamagishi, Honda et al. 2010). Haspin is recruited to centromeric chromatin by the cohesin regulator Pds5, then subsequently phosphorylates H3 at Threonine 3 (pH3T3) (Zhou, Liang et al. 2017). Bub1 is recruited by a member of the KMN network, KNL1. Here centromere localized Bub1 can then phosphorylate H2A at Threonine 120 (pH2A120). The region where these two phosphorylation events overlap defines the inner centromere (Polioudaki, Markaki et al. 2004, Dai and Higgins 2005, Kawashima, Tsukahara et al. 2007, Bassett, Wood et al. 2010, Kawashima, Yamagishi et al. 2010, Kelly, Ghenoiu et al. 2010, Wang, Dai et al. 2010, Yamagishi, Honda et al. 2010). The BIR domain of the Survivin subunit of the CPC binds to pH3T3 and the Borealin subunit of the CPC binds to Shugoshin 1 (Sgo1), which interacts with pH2A120 (Kawashima, Tsukahara et al. 2007, Jeyaprakash, Basquin et al. 2011, Du, Kelly et al. 2012). Together these interactions serve to localize the CPC, and Aurora B activity, to the centromere. At the metaphase to anaphase transition Aurora B is removed from chromosomes and translocated the central spindle and midbody.

In addition to the histone marks that have been shown to recruit Aurora B to the inner centromere, other modes of recruitment have also been identified. For example, post-translational modifications of the CPC and direct binding of Borealin to double stranded DNA, have been shown

to aid in the recruitment of Aurora B to the inner centromere (Pereira and Schiebel 2003, Klein, Nigg et al. 2006, Jelluma, Brenkman et al. 2008, Colnaghi and Wheatley 2010, Tsukahara, Tanno et al. 2010, Chu, Yao et al. 2011, Nakajima, Cormier et al. 2011). More recently, centromeric transcription has been identified as a potential mechanism that can recruit Aurora B to the inner centromere. Long non-coding RNA transcripts are expressed from centromere sequences and have been shown to directly interact with Aurora B and also to indirectly recruit Aurora through binding of Sgo1. Experiments in *Xenopus laveis* egg extracts show a reduction in Aurora B localization at the inner centromere and activity after treatment with RNase or transcription inhibitors. Ectopic transcription has also been found to be associated with mis-localization of Aurora B whereby excess transcripts are believed to draw Aurora B away from the centromere (Jambhekar, Emerman et al. 2014, Blower 2016, Perea-Resa, Bury et al. 2020).

Chromatin structure and function

The dynamic nature of chromatin structure is regulated through the post-translational modifications of the histones around which DNA is wrapped. Acetylation of H3 and H4, or di- or trimethylation of H3K4 are considered to be activating marks that promote chromatin accessibility. As such, these marks are enriched in euchromatic regions that are transcriptionally active. In contrast, mono methylation of H3K9 or H3K27 are considered to be repressive marks that are enriched in heterochromatin inactive genes (Rea, Eisenhaber et al. 2000, Jenuwein and Allis 2001, Litt, Simpson et al. 2001, Nakayama, Rice et al. 2001, Noma, Allis et al. 2001, Schotta, Ebert et al. 2002, Huisinga, Brower-Toland et al. 2006). In addition to epigenetic regulation, two critical protein complexes, Cohesin and Condensin, contribute to defining the chromatin structure within a cell (Hagstrom and Meyer 2003, Heidinger-Pauli, Mert et al. 2010, Tada, Susumu et al. 2011, Aragon, Martinez-Perez et al. 2013).

Cohesin is a four-subunit complex that forms a ring complex and includes SMC1, SMC3, Rad21, and STAG/SA subunits. SMC1 and SMC3 belong to a family of ATPases, whose sequence contains a large coiled-coil domain that folds back onto itself around a central hinge domain. At the other end of the coiled-coil domain is an ATPase "head." The hinge domains of the SMC proteins bind to each other, and the ATPase "heads" of both proteins bind to Rad21 to form the cohesin ring. STAG/SA interacts with Rad21 to maintain the integrity of the complex (Ciosk, Shirayama et al. 2000, Rollins, Korom et al. 2004, Peters, Tedeschi et al. 2008, Nasmyth and Haering 2009, Seitan and Merkenschlager 2012, Brooker and Berkowitz 2014). Cohesin is loaded onto chromosomes before replication in S-phase, by the accessory proteins Scc2 and Scc4 (Ciosk, Shirayama et al. 2000, Gillespie and Hirano 2004, Watrin, Schleiffer et al. 2006). One model suggests cohesin associates with chromatin by trapping chromosomes inside of its ring-like structure (Figure 1.7A). However, this model is problematic as the space within the cohesion ring is barely large enough to entrap the replicated chromosomes and would not permit passage of any chromatin-associated proteins. An alternative model positions a single chromosome within

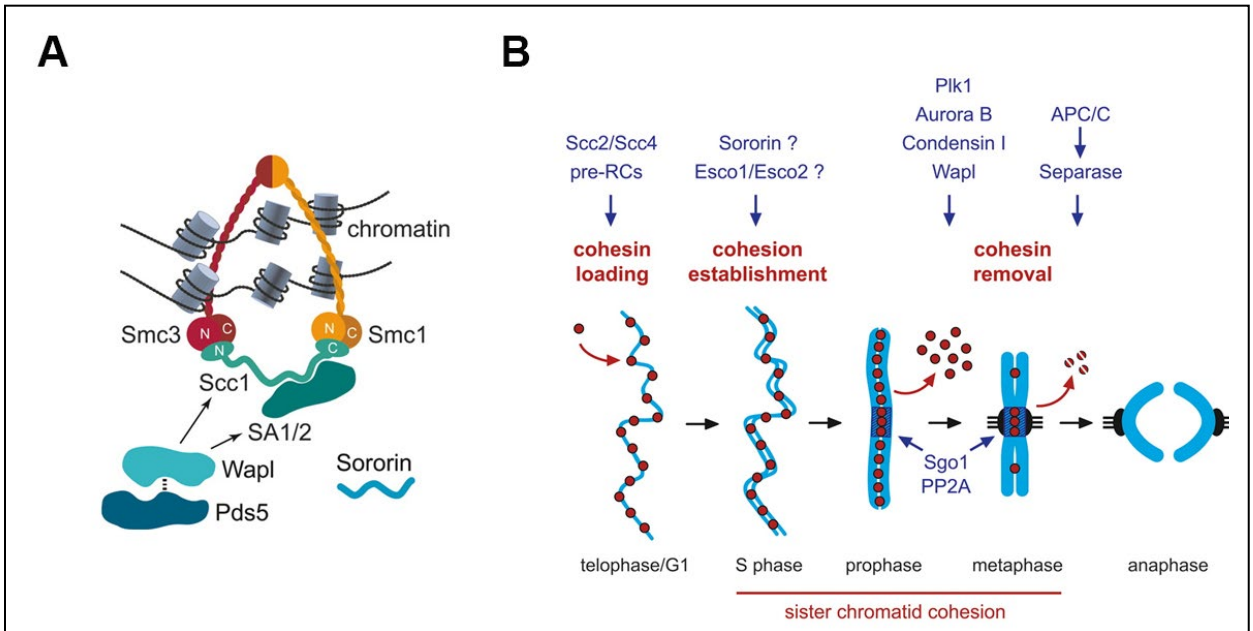

Figure 1.7: Cohesin structure and regulation A) Model for how the cohesin complex associates with chromatin. B) Regulation of sister chromatid cohesin throughout the cell cycle (Adapted by permission from Elsevier: *Cell* (Wapl controls the dynamic association of cohesin with chromatin, Kueng S et al., 2006)) and (Adapted by permission from Cold Spring Harbor Press: *Gene and Dev* (The cohesin complex and its role in chromosome biology, Peters J., et al 2008))

the cohesin ring such that individual cohesion rings interact to sustain chromosome cohesion (Srinivasan, Scheinost et al. 2018, Srinivasan, Petela et al. 2019).

The affinity of cohesin for chromatin is dynamic and changes throughout the different phases of the cell cycle. During S phase cohesin binding is mediated through the acetylation of Smc3 by Eco1 and 2. Acetylation of Smc3 recruits Sororin to bind the cohesin regulator Pds5. When Sororin-bound Pds5 associates with Cohesin it remains stably associated with chromatin (Zhang and Pati 2012). Cohesin is removed from DNA in two cycles (Figure 1.7B). The initial removal of the cohesin complex is termed the “prophase pathway” and relies on the negative regulator of cohesin, Wapl. Wapl competes with Sororin for Pds5 binding and once Sororin is displaced, cohesion association with chromatin becomes more dynamic (Gerlich, Koch et al. 2006, Rolef Ben-Shahar, Heeger et al. 2008, Unal, Heidinger-Pauli et al. 2008, Zhang, Shi et al. 2008, Rowland, Roig et al. 2009, Haarhuis, Elbatsh et al. 2014). At the onset of mitosis Sororin is phosphorylated by Plk1, Cdk1, and Aurora B which disrupts Sororin’s interaction with Pds5 and allows Wapl to bind. This results in the removal of cohesin from chromosome arms. At this point centromeric cohesin is protected from phosphorylation by PP2A and also by Sgo1, which protects centromeric cohesin directly by preventing Wapl from binding. Centromeric cohesin must be maintained through metaphase to resist the pulling forces of microtubules (Waizenegger, Hauf et al. 2000, Nishiyama, Ladurner et al. 2010, Buheitel and Stemmann 2013, Nishiyama, Sykora et al. 2013). Upon satisfaction of the SAC at the metaphase-to-anaphase transition, the remaining cohesin is removed by APC-dependent proteasomal cleavage of Rad21, releasing sister chromatids (Cohen-Fix, Peters et al. 1996, Uhlmann, Lottspeich et al. 1999, Zou, McGarry et al. 1999, Lin, Luo et al. 2016). Defects in cohesin regulation can lead to premature chromosome segregation if cohesion is released to early, or to lagging chromosomes and chromatin bridging if cohesion is not resolved at anaphase onset. Both defects can contribute to chromosome

segregation errors. In addition to, cells with compromised or reduced cohesion exhibit a decrease in rigidity at the centromeres which can in turn promote merotelic attachments and mis-segregation of chromosomes (Cimini, Moree et al. 2003, Tang, Sun et al. 2004, Bakhoum, Genovese et al. 2009, Carvalhal, Tavares et al. 2018).

Condensin complexes, which are also composed of proteins belonging to the SMC family of chromosomal ATPases, additionally contribute to chromatin structure. Condensin complexes are essential for chromosome compaction and segregation in mitosis (Nasmyth 2001, Cuylen, Metz et al. 2011, Goloborodko, Imakaev et al. 2016). In eukaryotic cells there are two isoforms of condensin (Condensin I and II) present and have distinct roles throughout the cell cycle (Figure 1.8A) (Hirota, Gerlich et al. 2004, Green, Kalitsis et al. 2012, Hirano 2012, Piskadlo and Oliveira 2016). Structurally both condensin complexes contain Smc2 and Smc4 subunits, but each

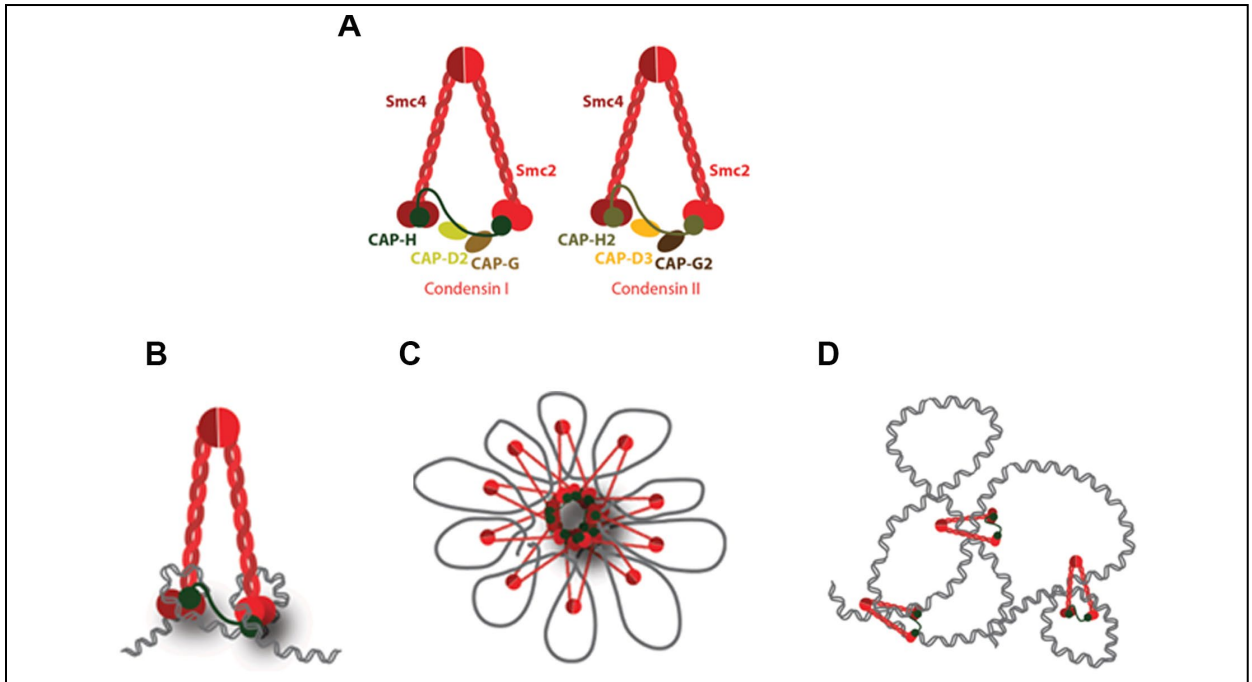

Figure 1.8: Structure and function of condensin A) Structure of eukaryotic condensin isoforms. Purposed roles of condensin in DNA compaction to include B) DNA supercoiling C) loop holder and D) topological linker (Adapted by permission from Piskadlo, E. and R. A. Oliveira (2016). "Novel insights into mitotic chromosome condensation." F1000Res 5)

contains three different auxiliary CAP proteins. Condensin I contains CAP-D2, CAP-G, and CAP-H, while Condensin II contains CAP-D3, CAP-G2, and CAP-H2 (Ono, Losada et al. 2003, Hirota, Gerlich et al. 2004, Ono, Fang et al. 2004). Prior to the beginning of mitosis, the kinase Mps1 phosphorylates CAP-H2. This phosphorylation helps to recruit Condensin II to chromatin in order to promote chromosome condensation. Unlike Condensin II, which is nuclear, Condensin I is cytoplasmic and cannot interact with chromatin until nuclear envelope breakdown. Following nuclear envelope breakdown, the phosphorylation of CAP-H by Aurora B induces mitotic chromosomal localization of Condensin I (Takemoto, Murayama et al. 2007, Abe, Nagasaka et al. 2011, Kagami, Nihira et al. 2014). The chromatin association of Condensin I antagonizes and helps remove the cohesin complex from chromosome arms (Hirota, Gerlich et al. 2004).

Like cohesin the condensin complexes have enzymatic activity, which is used to condense chromatin (Figure 1.7B-D). Condensin introduces positive supercoils into double stranded DNA (Kimura, Rybenkov et al. 1999, Hirano 2005, Hirano 2012). Continued supercoiling of the DNA by Condensin increases torsional stress that needs to be relieved. Torsional relief is mediated by topoisomerase II (Samejima, Samejima et al. 2012, Gilbert and Allan 2014). Similar to cohesin, the precise mechanism that allows for condensin to facilitate chromosome compaction remains unclear. More recently condensinopathies have been identified where biallelic mutations of the condensin subunits cause microcephaly. It was purposed that as a consequence of mutations in the condensin subunits, impaired decatenation followed by chromosomal segregation errors resulted in decreased cell proliferation and increased cell death leading to microcephaly (Martin, Murray et al. 2016, Sakai, Mochizuki et al. 2018, Dyson, Segura et al. 2021).

Chromosomal Instability

Maintaining genomic stability throughout the cell cycle is essential for cellular integrity. Most cancers cells are considered to be genomically unstable. Genomic instability encompasses changes to the genomic sequence as well as the instability in chromosome structure and number. The later, termed chromosomal instability (CIN), can be further separated into structural CIN (sCIN) and numerical CIN (nCIN). sCIN is characterized by an increased rate of acquiring chromosomal amplifications, deletions, inversions, duplications, and translocations, while nCIN is defined by the increased frequency of the loss of gain of whole chromosomes. Early studies in colon cancer cell lines gave us our first insights into potential mechanisms underlying CIN (Lengauer, Kinzler et al. 1997). It was shown that colon cancer cells displaying a phenotype known as microsatellite instability (a result of sCIN) still had a chromosome count that did not vary, while the aneuploid cancer cells had final chromosome counts that deviated from the model chromosome number up to 66%. Since then, other cancer types have been found to be CIN, such as breast and lung cancer cells (Haruki, Harano et al. 2001, Yoon, Wersto et al. 2002). Despite these initial findings over more than 20 years ago and the widespread prevalence of CIN in human cancers, the molecular changes that lead to chromosome mis-segregation remain unclear (Weaver, Silk et al. 2007, Baker, Jin et al. 2009, Choi, Seo et al. 2009, McClelland, Burrell et al. 2009, Lee, Endesfelder et al. 2011).

Most solid tumors are found to be aneuploid, a consequence of mitotic segregation errors that results in an unequal number of chromosomes being distributed between the two daughter cells (Weaver and Cleveland 2006). When the error in chromosome segregation is the result of a persistent underlying defect in mitotic regulation, resulting in a high rate of whole chromosome mis-segregation over subsequent cell divisions, these cells now become CIN (as mentioned above). The distinction between stable aneuploidy (the result of a rare error in chromosome

segregation) and CIN (the result on ongoing segregation errors) is important as both the cellular consequences and the tools used to identify each is different. Aneuploidy represents a karyotype in which there is an abnormal number of chromosomes, while CIN represents a condition in which there is an increased rate of chromosomes that mis-segregate (e.g. every 1 in 5 mitoses) (Nicholson and Cimini 2013). Aneuploidy can be measured through various methods that enable average chromosome number within a population of cells, such as fluorescence *in situ* hybridization, spectral karyotyping, and genotyping. To measure CIN, that rate of chromosome mis-segregation events must be able to be determined through clonal assays that allow for the numerical heterogeneity of a given chromosome to be quantified between individual cells within a population (Geigl, Obenauf et al. 2008, van Jaarsveld and Kops 2016, Godek and Compton 2018). While gain or loss of a chromosome in an aneuploid cell can result in the respective gain of an oncogene or loss of a tumor suppressor, CIN and the resulting intra-tumor heterogeneity produced from CIN has been additionally linked to tumor adaptation, progression, resistance to therapeutic treatments, relapse, and poor patient outcomes (Gerlinger and Swanton 2010, Lee, Endesfelder et al. 2011).

As described above, the fidelity of chromosome segregation is sensitive to the regulation of centromere structure (cohesin and condensin) and kinetochore formation. While the experimental reduction or mis-regulation of the cohesin complex has been shown to promote chromosome mis-segregation (Barber, McManus et al. 2008, Remeseiro, Cuadrado et al. 2012, Covo, Puccia et al. 2014) and acute depletion of cohesin regulators such as Sgo1 or overexpression of Securin and Separase induce aneuploidy (Jallepalli, Waizenegger et al. 2001, Wirth, Wutz et al. 2006, Zhang, Ge et al. 2008, Iwaizumi, Shinmura et al. 2009), the incidence of mutations of these proteins in cancer is quite low (Kon, Shih et al. 2013) compared to that seen in other genes involved in regulating mitosis, suggesting that perturbations to these pathways do not underlie nCIN in

cancer. Alternatively, the dominant defect seen in cancer cells with nCIN is the presence of merotelic attachments. Experimentally it was determined that by changing microtubule attachment dynamics by increasing the average time a microtubule stays attached to a kinetochore or by depleting those proteins that stabilize interactions at the kinetochore allows merotelic attachments to persist (Cimini, Fioravanti et al. 2002, Thompson and Compton 2008). As described above, merotelic attachments satisfy both the microtubule occupancy and tension requirements needed to satisfy the SAC, meaning cells can progress into anaphase even without correcting these mal attachments (Cimini 2008). Left uncorrected, these mal-attachments can result in lagging chromosomes during anaphase and chromosome mis-segregation.

The occurrence of a merotelic attachment is determined by a difference in the rate of formation vs the rate of correction. The rate at which merotelic attachments form can be significantly increased by disrupting spindle geometry. In cancer spindle geometry is sometimes compromised by the presence of extra centrosomes (Pihan, Purohit et al. 1998, Ganem, Godinho et al. 2009, Chan 2011). Cells with more than 2 centrosomes will cluster the extra centrosomes together to form a bipolar spindle. The change from a multipolar to a bipolar spindle increases the rates of merotely, possibly overwhelming the error correction capacity. Alternatively, merotely can occur when the chromatin that underlies kinetochores is mis-regulated in a way that disrupts the back-to-back structure of the sister chromatids (Coschi, Martens et al. 2010, Manning, Longworth et al. 2010, Coschi, Ishak et al. 2014, Kleyman, Kabeche et al. 2014, Manning, Yazinski et al. 2014, Dick, Goodrich et al. 2018). This is seen following experimental manipulation of Cohesin and Condensin and also thought to result from the loss of the RB tumor suppressor. This provides an explanation for the elevated rate of chromosome mis-segregation in cancers with extra centrosomes or RB loss, respectively. In other cancers that do not exhibit extra centrosomes or RB loss, merotelic attachments may result from a defect in the error correction pathway itself.

Consistent with this, multiple studies have found that MT stability is enhanced in cancer cells with high nCIN (Bakhoum, Genovese et al. 2009, Bakhoum and Compton 2012, Ertych, Stolz et al. 2014). In this way, defects in Aurora B regulation and activity are likely to underlie nCIN in many cancer contexts.

Summery

The majority of cancer cells exhibit loss or functional inactivation of RB. For over 30 years studies have described RB's canonical role in regulating the G_1-S phase transition through the binding and regulation of E2F transcription factors. Recent studies done by our lab and others have shown that loss of RB promotes DNA damage and chromosomal instability (Hernando, Nahle et al. 2004, Coschi, Martens et al. 2010, Manning, Longworth et al. 2010, van Harn, Foijer et al. 2010). While it is clear that loss of RB promotes mitotic infidelity, the mechanisms underlying how RB loss compromises mitotic fidelity are less well understood. In chapter 2 I define how loss of RB promotes mitotic DNA damage in RPE-1 cells. I demonstrate that changes to chromatin structure led to increased accessibility to nucleases and transcription factors which in turn corrupt centromere and kinetochore integrity and regulation. In chapter 3 I describe how the molecular changes that result from RB loss and that correspond with nCIN impact mechanisms of drug resistance in a panel of non small cell lung cancer cells. nCIN has been clinically and experimentally linked to acquired drug resistance though the impact of modulating nCIN on drug response is not well understood. In chapter 3, I use validated methods to modulate nCIN and demonstrate that suppression of CIN, either in vitro or in vivo, limits the emergence of drug tolerant cells. Together, these findings provide enhanced understanding of the mechanistic basis for mitotic errors in cancer lacking RB and suggest that modulation of these defects may be of therapeutic benefit.

Chapter 2: Loss of RB increases mitotic DNA damage

Introduction

Cell cycle checkpoints constitute a complex network of signal transduction pathways that monitor replication and DNA damage throughout the cell cycle. DNA damage can arise from both endogenous (cellular metabolic processes) and or exogenous sources (environmental factors). Sources of endogenous DNA damage include oxidation, hydrolysis, alkylation, and mismatched base pairs; sources of exogenous DNA damage include ionizing radiation, ultraviolet radiation, and chemical agents. At the G_1/S phase transition the cell will check for appropriate levels of growth factors and DNA damage before it irreversibly commits to replicating the DNA in S-phase. Damage to the DNA that occurs after S-phase as a result of errors that occurred during replication or from other exogenous factors will be sensed by the G_2/M checkpoint. Here the cell will arrest temporarily to ensure that all the chromosomes have been accurately replicated and any damage present is repaired prior to entering mitosis.

If cells bypass the DNA damage checkpoint in G2, genome stability can be compromised in several distinct ways. First, mitotic cells are unable to effectively repair DNA damage so existing damage will persist through cell division and be propagated from one cell into both daughter cells (Zhou and Elledge 2000, van Gent, Hoeijmakers et al. 2001, Bartek and Lukas 2003, Krempler, Deckbar et al. 2007). Second, when cells enter mitosis with DNA damage, the key DNA damage response (DDR) kinase ATR becomes activated. However, in addition to initiating a signal cascade for DNA damage repair, ATR kinase activity also impacts key mitotic regulators of microtubule dynamics such that activation of the DDR during mitosis increases microtubule stability, limits correction of merotelic attachments, and promotes whole chromosome segregation

errors (Bakhoum, Kabeche et al. 2014, Bakhoum, Kabeche et al. 2017). Finally, the presence of merotelic attachments and resulting lagging chromosomes have been implicated in the generation of de novo DNA damage as cells progress through anaphase (Janssen, van der Burg et al. 2011, Crasta, Ganem et al. 2012).

Loss of the RB tumor suppressor promotes replication stress and increased levels of DNA damage during G2 and mitosis (Hernando, Nahle et al. 2004, van Harn, Foijer et al. 2010, Manning, Benes et al. 2014). Our prior work, as well as that of other groups, has additionally implicated RB in the regulation of mitotic fidelity such that loss of RB compromises chromatin compaction and cohesion, leading to high rates of chromosome segregation errors (Gonzalo, Garcia-Cao et al. 2005, Isaac, Francis et al. 2006, Coschi, Martens et al. 2010, Manning, Longworth et al. 2010, Manning, Yazinski et al. 2014). However, whether these assaults to genomic stability share a common underlying defect, or RB loss independently impacts both DNA damage and chromosome segregation pathways remain unclear.

My work demonstrate that RB deficient cells exhibit an intact DNA damage checkpoint and arrest robustly in G2 following exposure to DNA damaging agents. However, once in mitosis, RB deficient cells begin to accumulate de novo DNA damage. This DNA damage is enriched at heterochromatic regions of centromeres and telomeres, is accumulated in a caspase-dependent manner, and is exacerbated by delayed mitotic progression. Consistent with previously described defects in chromatin compaction, I find that RB deficient cells exhibit an increase in centromere transcription during mitosis and that enhanced chromosome cohesion or condensin is sufficient to suppress acquisition of mitotic DNA damage and promote mitotic fidelity. Together, these data implicate increased chromatin accessibility as the underlying defect that permits acquisition of

mitotic DNA damage and functionally links centromere deregulation with chromosome segregation errors in RB deficient cells.

Results

RB deficient cells contain an intact G_2/M checkpoint

Previous studies have shown that loss of RB leads to defects in mitotic progression and an increase in chromosome segregation errors. Inactivation of RB has also been shown to cause replication-dependent damage in S-phase, raising the possibility that mitotic defects associated with loss of RB arise as a consequence of DNA damage that is not repaired prior to mitotic entry. If the DNA damage that is not prior to mitotic entry is allowed to enter into mitosis, chromosomes containing multiple breaks will not distribute evenly between the two daughter cells causing them to no longer be diploid (van Gent, Hoeijmakers et al. 2001, Krempler, Deckbar et al. 2007). To test this possibility and determine the origin of the mitotic defects, I first assessed the integrity of the G_2/M DNA damage checkpoint in RB deficient cells.

To evaluate if RB deficient cells have an intact G_2/M DNA damage checkpoint both control (siControl) and RB depleted (siRB) retinal pigment epithelial (hTERT RPE-1) cells were treated with increasing concentrations of the DNA damaging agent doxorubicin. Immunofluorescence microscopy was used to quantify both the mitotic index and the amount of DNA damage per cell as indicated by the presence of the canonical DNA damage marker γH2AX (Figure 2.1A). Consistent with previous studies, I find that loss of pRB, even in the absence of DNA damaging agents, results in an increase in DNA damage. However, both control and RB-depleted cells exhibit similar sensitivity to an increasing range of doxorubicin such that both conditions exhibit approximately a 2-fold increase in DNA damage following 24h exposure to 250nM doxorubicin

(Figure 2.1B). Furthermore, consistent with the increase in DNA damage seen with doxorubicin treatment, both control and RB-depleted conditions exhibit a robust cell cycle arrest at G_2 following

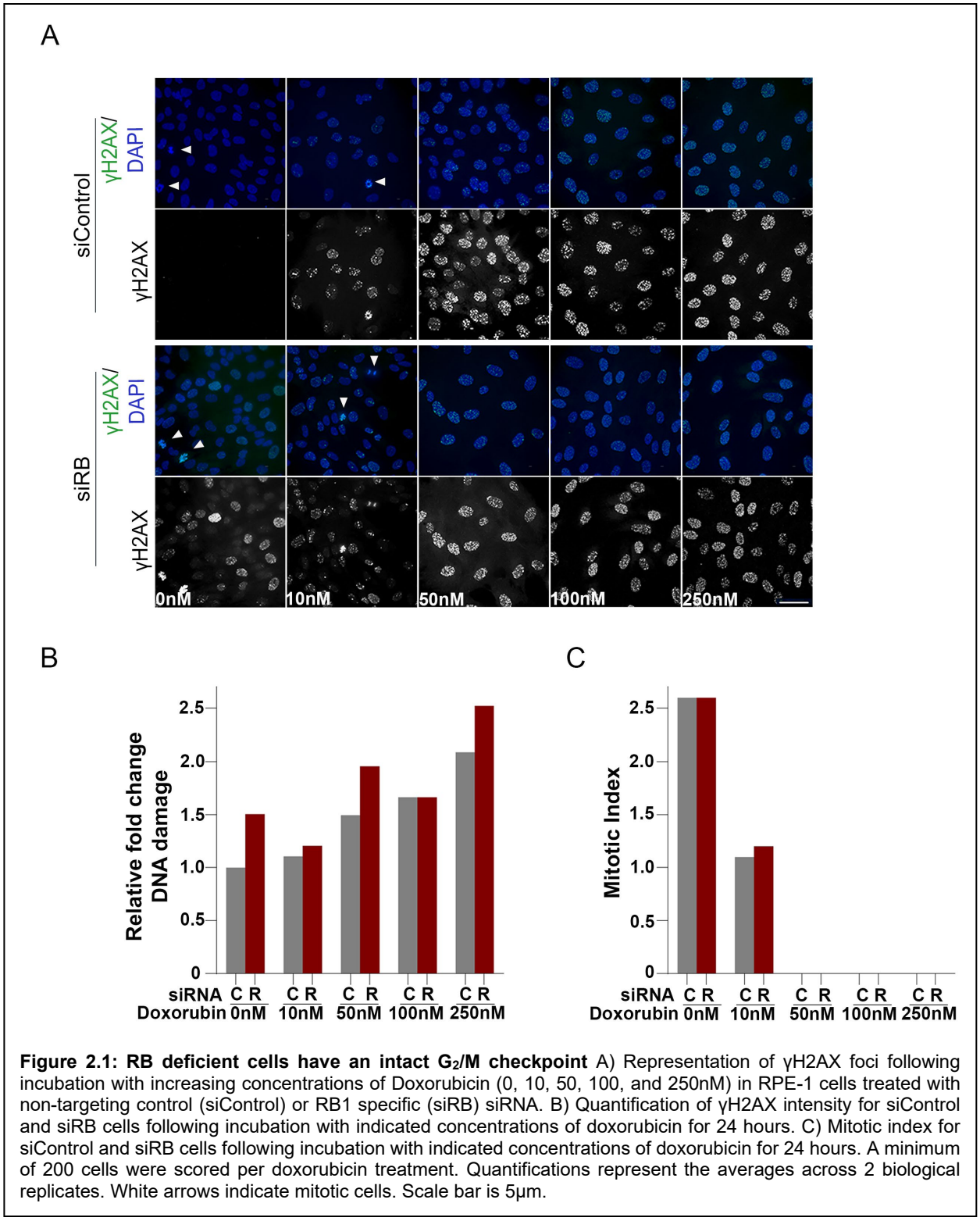

Figure 2.1: RB deficient cells have an intact G_2/M checkpoint A) Representation of γH2AX foci following incubation with increasing concentrations of Doxorubicin (0, 10, 50, 100, and 250nM) in RPE-1 cells treated with non-targeting control (siControl) or RB1 specific (siRB) siRNA. B) Quantification of γH2AX intensity for siControl and siRB cells following incubation with indicated concentrations of doxorubicin for 24 hours. C) Mitotic index for siControl and siRB cells following incubation with indicated concentrations of doxorubicin for 24 hours. A minimum of 200 cells were scored per doxorubicin treatment. Quantifications represent the averages across 2 biological replicates. White arrows indicate mitotic cells. Scale bar is 5μm.

doxorubicin treatment as low as 50nM, with no difference in the mitotic index between control and pRB deficient cells (Figure 2.1C). These data indicate that, at the population level, similar levels of DNA damage induce comparably checkpoint-dependent arrest in both control and RB-depleted cells.

Next, I asked whether individual RB-deficient cells in G_2 are competent to repair DNA damage prior to mitotic entry. To monitor the presence and resolution of DNA damage I performed live cell imaging of RPE-1 cells engineered to express RFP tagged Histone H2B (RFP-H2B) and GFP tagged 53BP1 (53BP1-GFP). Images were captured every 5 minutes for 24h. Individual cells were tracked using RFP-H2B to identify mitotic entry based on chromatin condensation (Figure 2.2A).

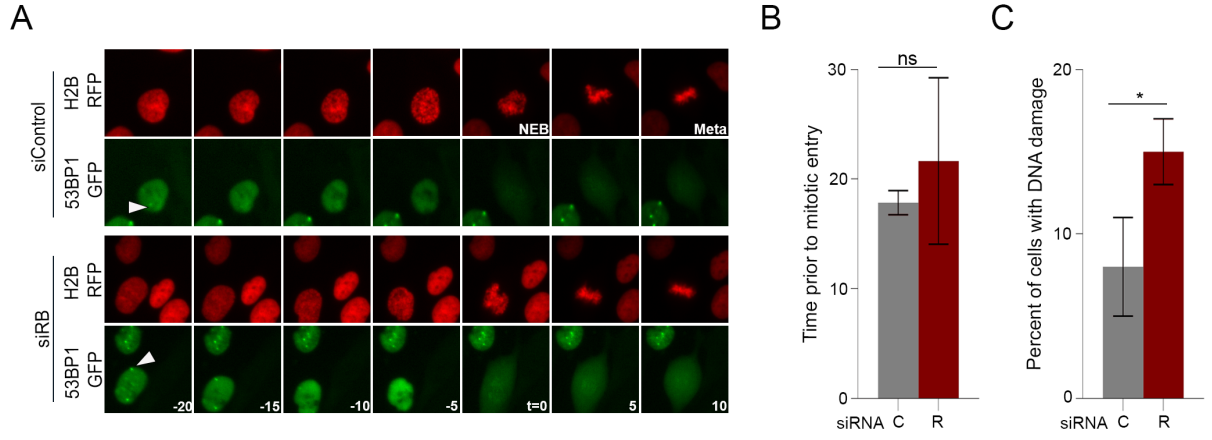

Figure 2.2: Resolution of G2 DNA damage A) Representative stills from live cell imaging of RPE-1 cells expressing RFP tagged H2B (chromatin) and GFP tagged 53BP1 (double stand breaks) treated with a non-targeting control (siControl) or RB1 specific (siRB) siRNA. White arrows indicate foci followed. B) Quantification of the time it took a cell to resolve all 53BP1 foci prior to mitotic entry C) Quantification of cells with G2 DNA damage. A minimum of 50 cells were scored for 3 individual replicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Prior to mitotic entry, cells spend 2-4h in G2 stage of the cell cycle. Therefore, to identify cells that exhibit DNA damage during G2, GFP-53BP1 was assessed for the 4h prior to mitotic entry for each of 100 mitotic cells. 53BP1 is recruited to sites of DNA double stand breaks such that the formation of 53BP1-positive foci is an indication of DNA damage and the dissipation of 53BP1 foci indicates repair of DNA damage. 30 or more G2 cells that were positive for 53BP1 foci were

tracked to determine the timing of foci resolution with respect to mitotic entry (i.e., chromatin condensation).

Consistent with the described increase in replication-dependent DNA damage in cells lacking RB (Bester, Roniger et al. 2011, Manning, Yazinski et al. 2014) , I similarly find that RB-depleted cells have a higher frequency of 53BP1 foci during G2 than control cells (Figure 2.2C). However, tracking of G2 cells that are positive for 53BP1 foci revealed that the duration of time between the resolution of 53BP1-positive DNA damage foci and mitotic entry (as indicated by chromosome compaction) is comparable in both control and RB depleted cells. (Figure 2.2B) Importantly, consistent with the G2/M checkpoint challenge with doxorubicin, these analyses indicate that, similar to control cells, cells lacking RB (siRB) resolve their damage prior to mitotic entry.

Loss of RB renders mitotic cells more sensitive to the acquisition of DNA damage during mitosis

Given that my data indicate RB deficient cells exhibit a robust DNA damage checkpoint in G2 that precludes cells from entering mitosis with unresolved DNA damage, I next hypothesized that loss of RB leads to the acquisition of *de novo* DNA damage. To test if loss of pRB leads to *de novo* DNA damage, control (siControl) and pRB-depleted (siRB) mitotic cells were analyzed by immunofluorescence microscopy and the number of γH2AX-positive DNA damage foci per mitotic cell quantified. Cells containing four or fewer yH2AX foci were considered undamaged while those with 5 or more yH2AX foci were scored as positive for DNA damage (Figure 2.3A). Consistent with previous work showing that RB deficient mitotic cells have a higher incidence of DNA damage (Manning, Yazinski et al. 2014), I find that cells lacking RB are more likely to exhibit DNA damage during normal mitotic progression than are control mitotic cells (0h nocodazole treatment).

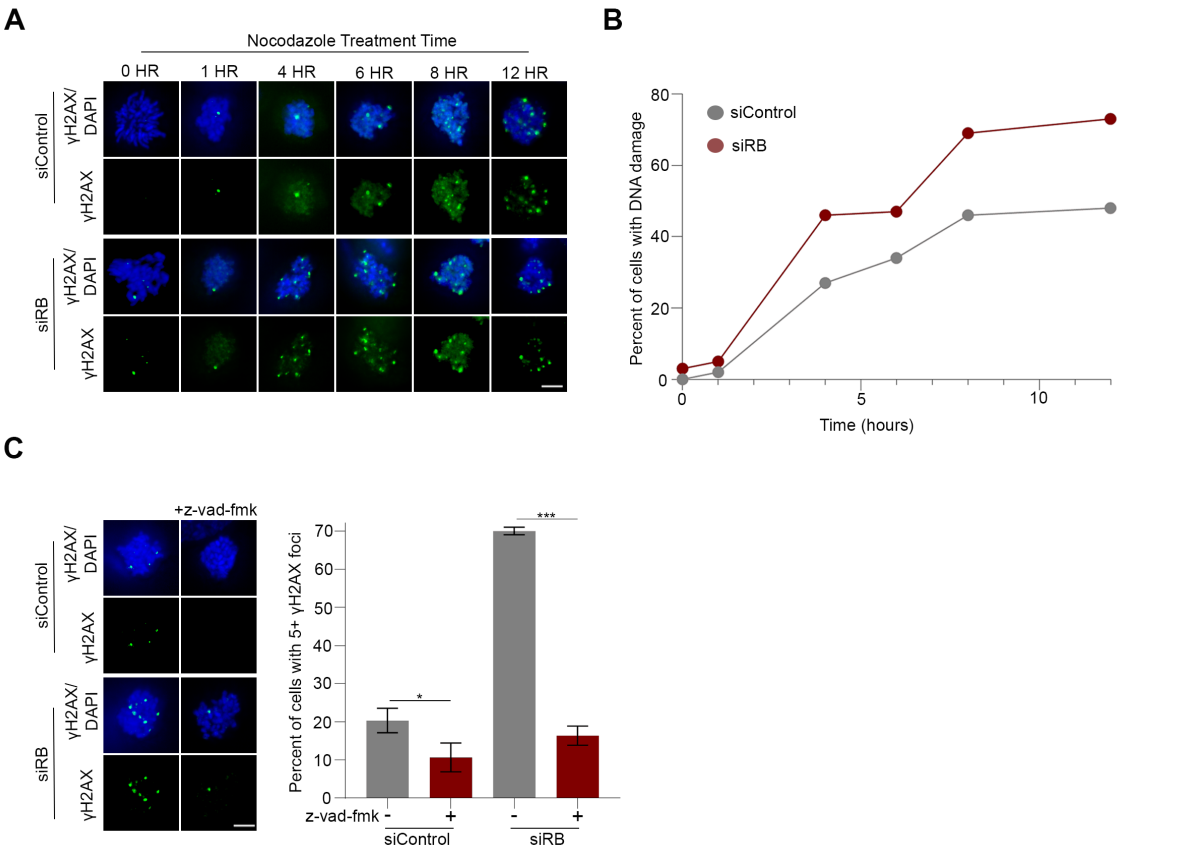

Figure 2.3 Loss of RB promotes the acquisition of mitotic caspase dependent DNA damage. A) Representation of γH2AX foci following incubation with 100 ng/ml nocodazole for 0, 1, 4, 6, 8, and 12 hours in RPE-1 cells treated with non-targeting control (siControl) or RB1 specific (siRB) siRNA. B) Quantification of the percent of cells without DNA damage over the course of the noc treatment. Data represents the average of 2 individual replicates. C) Representation of γH2AX foci following concurrent incubations with 100 ng/ml nocodazole for 4 hours in RPE-1 cells treated with non-targeting control (siControl) or RB1 specific (siRB) siRNA with or without z-vad-fmk. D) Quantification of the percent of cells with and without DNA damage in mitosis following treatment with or without z-vad-fmk. A minimum of 50 cells were scored for 3 individual replicates. Scale bar is 5μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Work from the Mitchison Lab had previously shown that de novo DNA damage can accumulate during prolonged mitoses when caspase-activated DNase (CAD) induces double strand breaks. Therefore, to test whether RB loss sensitizes cells to acquisition of de novo mitotic DNA damage, I assessed the levels of yH2AX foci in control and RB depleted cells following 1, 4, 6, 8, or 12 hours of nocodazole-induced mitotic arrest. As described by the Mitchison group, I find that control cells accumulate mitotic DNA damage as the duration of mitosis increases. However, the rate at

which RB deficient cells accumulate DNA damage during mitotic arrest was strikingly higher than that of the control population: Within 4 h of nocodazole-induced mitotic arrest 30% of control and 50% of RB depleted cells exhibit DNA damage and by 12h of mitotic arrest, nearly 80% of RB deficient cells, compared to only 50% of control cells, were positive for DNA damage (Figure 2.3A & B).

To next test whether the DNA damage that accumulates in RB-deficient mitotic cells is caspase dependent, cells were arrested in mitosis with nocodazole and treated concurrently with the caspase inhibitor z-vad-fmk. Cells where then analyzed for γH2AX foci as described above. Following z-vad-fmk treatment, the percent of mitotic cells scoring positive for DNA damage decreased from nearly 70% to less than 20% (Figure 2.3C & D). This dramatic decrease indicates that the majority of DNA damage that accumulates in RB-deficient mitotic cells is caspase dependent.

Compromised chromatin structure following RB loss leaves centromeres susceptible to DNA damage

Mitotic chromatin compaction is protective and limits the accessibility of DNA to nucleases (Groth, Rocha et al. 2007, Luijsterburg and van Attikum 2011, Takata, Hanafusa et al. 2013). During a prolonged mitotic delay, mechanisms to retain condensed chromatin structure can become compromised, leaving chromatin susceptible to caspase-activated nucleases (Orth, Loewer et al. 2012). RB has been implicated in the regulation of chromatin compaction and cohesion, particularly at centromere and telomere heterochromatin (Garcia-Cao, Gonzalo et al. 2002, Gonzalo, Garcia-Cao et al. 2005, Isaac, Francis et al. 2006, Longworth, Herr et al. 2008, Coschi,

Martens et al. 2010, Manning, Longworth et al. 2010), suggesting that RB's loss might render these regions of chromatin particularly sensitive to nuclease activity.

To test this possibility, high resolution imaging of individual chromosomes was performed and the localization of yH2AX-labelled DNA damage foci, with respect to ACA-labelled centromeres and chromosome ends (telomeres), was assessed. (Figure 2.4A). The percentage of cells with centromere localized γH2AX foci was then quantified. Consistent with a model whereby RB functions to protect or otherwise limit CAD access to heterochromatin, I find that RPE-1 cells expressing an shRB construct to induce RB depletion display an enrichment of DNA damage at centromeres, with an average of 8 chromosomes per RB-deficient cell exhibiting centromere-proximal DNA damage, compared to only one chromosome per control cell (Figure 2.4B).

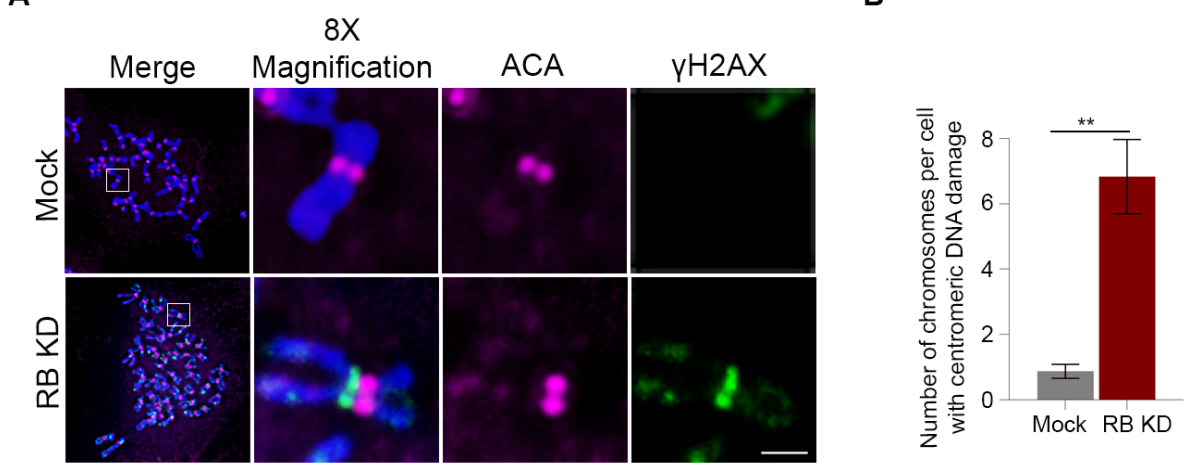

Figure 2.4: DNA damage occurs at centromeres with loss of RB A) Representation of γH2AX foci in unfixed metaphase spreads treated without doxycycline (Mock) or with doxycycline (RB KD). Single chromosome is magnified 4X from merged image. B) Quantification of the number of centromeres per cell with DNA damage in Mock and RB KD conditions. Minimum of 30 spreads were scored for each of 3 individual replicates. Scale bar 5μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

To directly test if susceptibility to DNA damage is linked to the compromised chromatin cohesion and compaction seen in RB deficient cells (Manning, Longworth et al. 2010, Coschi, Ishak et al. 2014, Manning, Yazinski et al. 2014) I enhanced chromosome cohesion or condensation via depletion of their respective negative regulators. Following siRNA-mediated depletion of Wapl (negative regulator of cohesin complex) MCHPH1 (negative regulator of condensing complex) individually or in combination with RB depletion, I assessed the impact of each on DNA damage

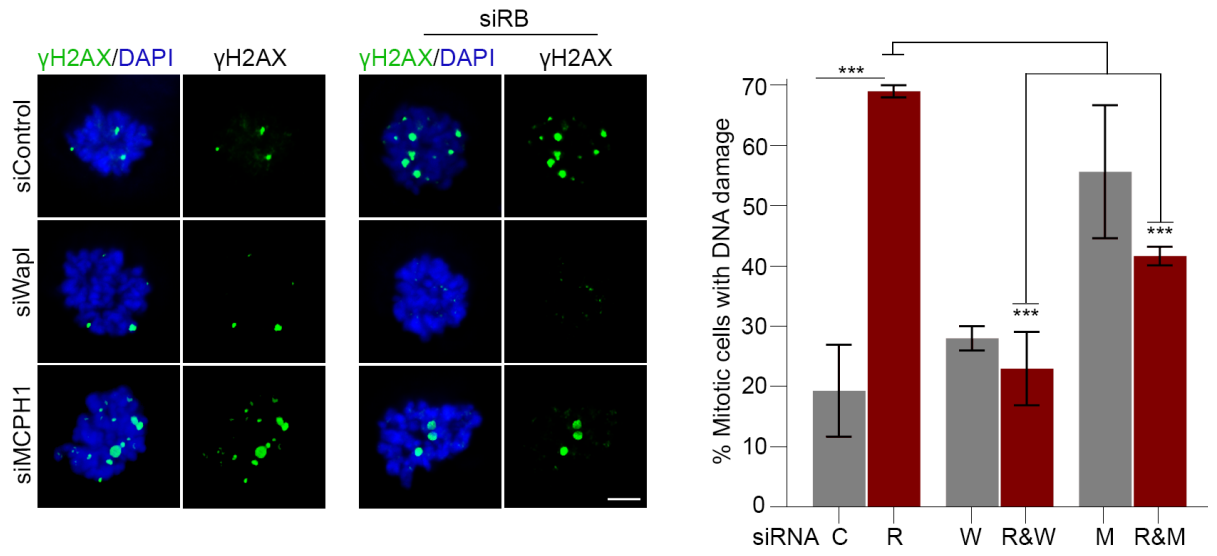

Figure 2.5: Mitotic DNA damage in RB deficient cells is rescued upon increasing Cohesin and Condensin A) Representation of γH2AX foci in RPE-1 mitotic arrested cells after treatment with non-targeting control (siControl), RB1 specific (siRB), Wapl specific (siWapl), MCPH1 specific (siMCPH1), or concurrent depletions of siRB/siWapl, and siRB/siMCPH1. B) Quantification of the percent of cells from panel A with γH2AX foci upon treatment with siControl (C), siRB (R), siWapl (W), siRB/siWapl (R&W), siMCPH1 (M), and siRB/siMCPH1(R&M). Minimum of 30 cells per condition were scored in each of 3 individual replicates. Scale bar, 5μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

levels in RB depleted cells. Following enhancement of cohesion, via depletion of Wapl, levels of DNA damage in RB deficient cells were reduced to that seen in RB- proficient control cells. Similarly, enhancement of condensation, via depletion of MCPH1, reduced the percent of RB deficient cells that were positive for DNA damage, albeit to a lesser degree (Figure 2.5A & B). Together, these data indicate the compromised chromatin cohesion and compaction, long-accepted consequences of RB loss, underlie the susceptibility of these cells to enhanced mitotic DNA damage.

CPC localization is sensitive to transcriptional changes resulting from RB loss

Chromosome compaction is typically thought to silence transcription during mitosis. However, recent work in by Palozola et al show that there is a basal level of transcription that occurs throughout mitosis (Palozola, Donahue et al. 2017, Palozola, Liu et al. 2017). Subsequent studies describe centromeric transcription as being required for the maintenance of both the cohesin complex and the CPC-containing AurB kinase at centromeres (Liu, Qu et al. 2015, Chen, Zhang et al. 2022). Given the described role for RB in regulating both the condensin and cohesion complexes (Longworth, Herr et al. 2008, Manning, Longworth et al. 2010, Bester, Roniger et al. 2011, Manning, Yazinski et al. 2014), I next speculated that RB depletion may not only render centromeres more accessible to CAD activity, but also to transcription machinery.

To understand the impact of RB loss on mitotic transcription I assessed bulk nascent RNA levels in addition to centromere-derived transcripts. First, control and RB-depleted RPE-1 cells were arrested in mitosis for 4 hours with nocodazole while concurrently being incubated with 5-Ethynyl Uridine (EU). This nucleotide analog is incorporated into newly synthesized RNA transcripts and can be visualized by using click chemistry to append a fluorescence probe. Immunofluorescence analysis indicates that loss of pRB leads to increased levels of EU-labelled RNA, indicating an increase in mitotic transcription (Figure 2.6A & B).

To specifically assess centromere transcription, I next used qPCR to quantify levels of centromere-derived transcripts from mitotic cells. Consistent with changes in overall nascent RNA levels, I find that loss of RB increases transcription of the centromeric and pericentromeric transcripts K111, K222, and P82H by 5-20-fold compared to that seen in control cells (Figure 2.6C).

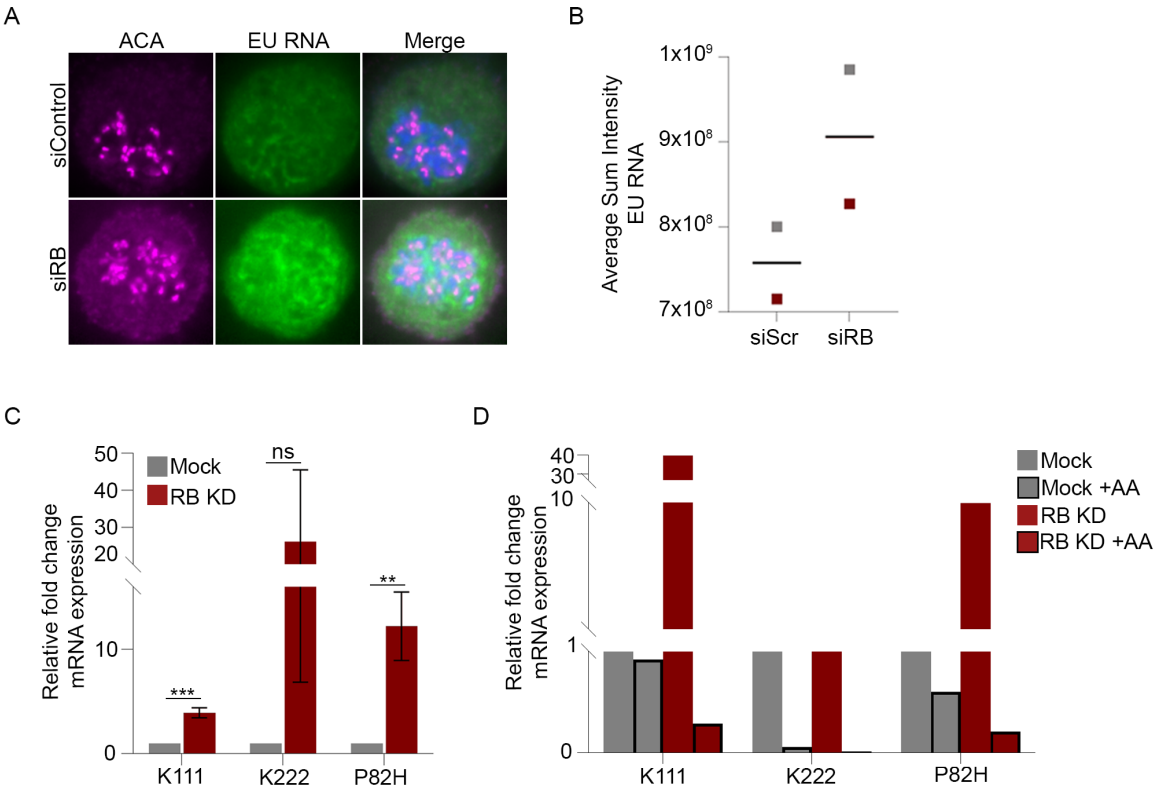

Figure 2.6: Loss of RB increases bulk and centromere derived RNA levels A) Representation of global transcription levels (EU RNA) during mitosis in RPE-1 cells treated with non-targeting control (siControl) or RB1 specific (siRB) siRNA. B) Average sum intensity of EU RNA for 2 individual replicates. C) qPCR analysis of centromere and pericentromere transcripts from mitotic shake offs in Mock and RB knockdown cells for 3 individual replicates, run in biological triplicate. D) qPCR analysis of centromere and pericentromere transcripts from mitotic shake offs in Mock and RB knockdown cells with and without treatment with 50 mg/ml α-amanitin. Data represents 1 replicate run in biological triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Multiple studies have shown that centromeric transcription is needed for proper targeting of the CPC to centromere and for full activation of Aurora B. Pulldowns using Aurora B, INCENP, and Survivin antibodies were able to bind to centromeric RNAs in mouse (Ferri, Bouzinba-Segard et al. 2009) and HeLa cells (Ideue, Cho et al. 2014). Knockdown of α-satellite RNA in HeLa cells or inhibition of transcription using triptolide in *Xenopus laevis* egg extracts was shown to cause a reduction in the CPC to the inner centromere and misregulation of the CPC component Aurora B (Ideue, Cho et al. 2014, Blower 2016). Given that centromeric and pericentromeric transcription

is increased with loss of RB and Aurora B localization and activation is sensitive to centromeric transcription, I hypothesized that centromeric Aurora B levels will increase with loss of RB.

To next determine if the increased centromere transcription in RB deficient cells is relevant to CPC localization, I used quantitative immunofluorescence approaches to assess the levels of centromere-localized Aurora B, a key subunit of the CPC. Following shRB-induced depletion of RB cells were arrested in mitosis with nocodazole and additionally exposed to the RNA polymerase II inhibitor alpha amanitin, or not, for four hours. To assess centromeric Aurora B levels, NIS-elements Advanced Research software was used to perform line scans in a single focal plane through individual ACA-stained kinetochore pairs where the area under the curve in the Aurora B-stained channel indicates centromere/kinetochore-localized Aurora B. These data indicate that mitotic cells lacking RB have, on average, a 2.5-fold increase in centromere Aurora B levels (Figure 2.7A). However, exposure to 50ug/ml of α-amanitin, a concentration sufficient to inhibit mitotic transcription (Figure 2.6D) reduced centromere Aurora B levels to those seen in control cells. (Figure 2.7A & B).

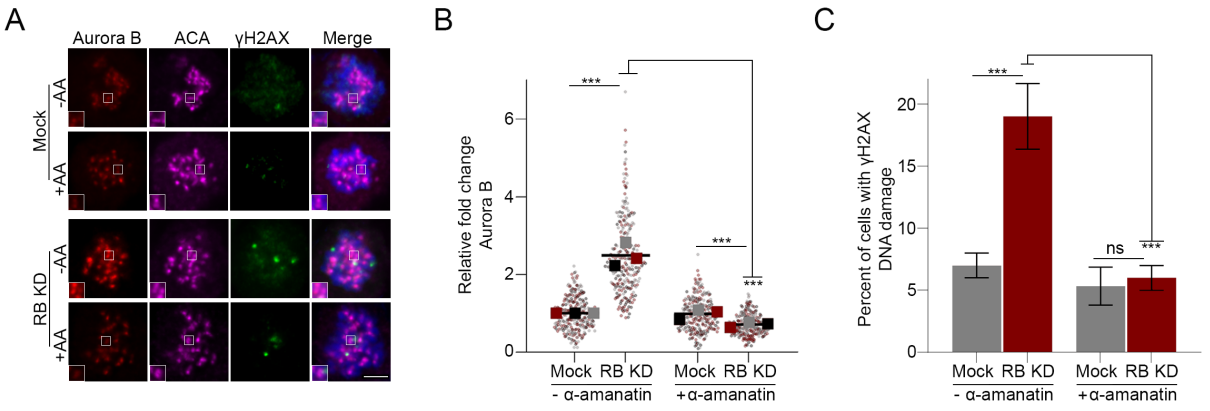

Figure 2.7: Decreasing mitotic transcription in RB deficient cells decreases centromere Aurora B and DNA damage E) Representation of centromere Aurora B and mitotic DNA damage (γH2AX) in Mock or RB knockdown RPE-1 cells with or without treatment with 50 mg/ml α-amanitin. F) Quantification of centromere Aurora B from images in panel E. G) Quantification of mitotic DNA damage from images in panel E. Insets of individual kinetochore pairs at 2X magnification. Scale bars, 5μm. A minimum of 90 kinetochore pairs were measured (3/cell for 30 cells), for each of 3 biological replicates. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

In addition to regulation of Aurora B localization, centromere transcription has also been implicated in genome stability such that high levels of transcription can promote DNA damage (Zou, McGarry et al. 1999, Zhu, Pao et al. 2011, Kishikawa, Otsuka et al. 2016, Kishikawa, Otsuka et al. 2018, Zhu, Hoong et al. 2018, Zhu, Chen et al. 2019). To assess whether high levels of centromere transcription that follow RB loss may be functionally linked to the centromere DNA damage described above, I next assessed yH2AX-labelled DNA damage foci in mitotic RB depleted cells in the presence and absence of the RNA polymerase II inhibitor, α-amanitin, and find that inhibition of transcription reduced the percent of RB deficient cells exhibiting DNA damage by nearly 4-fold (Figure 2.7C). Taken together these data indicate that RB loss both promotes centromere localization of Aurora B and renders centromeres susceptible to DNA damage in a transcription dependent manner.

Discussion

Prior to entry into mitosis the cell uses cell cycle checkpoints to monitor and subsequently repair DNA damage. Repairing DNA damage prior to mitotic entry is essential for maintaining mitotic fidelity as the cell has limited ability to repair damage once in mitosis (Bakhoum, Kabeche et al. 2017). Failure to recognize and repair DNA damage prior to the onset of mitosis promotes further genomic instability by propagating the damage from one cell into both daughter cells. Given the previously described role for the DNA damage response pathway in perturbing chromosomes segregation fidelity, these data suggest that the chromosome segregation errors seen in RB deficient cells is functionally linked to the de novo DNA damage during mitosis.

Loss of RB promotes accumulation of mitotic DNA damage

De novo DNA damage has been previously described by the Mitchison lab who reported that a

prolonged mitosis results in partial activation of the apoptotic cascade. They detect that low levels of cytochrome c are released during prolonged mitoses, inducing Caspase Activated DNases that lead to the formation of double strand breaks. Here I report similar findings in RPE-1 cells that experience a prolonged mitosis. I show that these cells acquire DNA damage in a time dependent manner, and that loss of RB accelerates this phenotype (Figure 2.3A and B). As with data presented in Orth et al., (Orth, Loewer et al. 2012) I find that damage resulting from prolonged mitosis is sensitive to caspase activity, as inhibition with a pan-caspase inhibitor prevents the accumulation of de novo DNA damage (Figure 2.3C).

Compromised mitotic chromatin structure during mitosis increases DNA damage

My data further suggests that the acceleration of the acquisition of DNA damage in RB deficient mitotic cells is tied to compromised chromatin structure. Mitotic chromatin is highly compact and less susceptible to the actions of exogenous and endogenous sources of DNA damage (Takata, Hanafusa et al. 2013, Nair, Shoaib et al. 2017). Loss of cohesin and condensin are reported phenotypes that results from depletion of RB (Garcia-Cao, Gonzalo et al. 2002, Gonzalo, Garcia-Cao et al. 2005, Isaac, Francis et al. 2006, Longworth, Herr et al. 2008, Coschi, Martens et al. 2010, Manning, Longworth et al. 2010). These two structural protein complexes are important for the maintenance of the “closed” chromatin structure. My data suggests that a “relaxed” chromatin confirmation that results from RB loss renders DNA more accessible to the activity of CAD, as increasing cohesin (Wapl knockdown) or condensin (MCPH1 knockdown) recuses the damage seen with loss of RB (Figure 2.5). Consistent with RB’s known role in regulating heterochromatin, I find that centromeres and telomeres are particularly susceptible to DNA damage following loss of RB (Figure 2.4).

RB loss increases centromeric transcription through defects in chromatin structure

Centromere compaction limits access to transcription machinery and corruption of centromere compaction leads to an increase in the expression of non-coding centromere transcripts. For example, loss of the methyltransferase Suv420, leads to loss of heterochromatin-associated Cohesin, which in turn promotes genomic instability resulting from chromosome mis-segregation errors (Hahn, Dambacher et al. 2013, Saksouk, Simboeck et al. 2015). Consistent with my data suggesting DNA damage arises in RB deficient mitotic cells due to compromised chromatin structure, I find that RB deficient cells have a high level of centromere transcription. These transcripts have functional roles in tethering mitotic regulators, such as Aurora B, to the centromere. (Jambhekar, Emerman et al. 2014, Blower 2016). The level of Aurora B at centromeres is critical to regulating the dynamics of microtubule attachments. Too much or too little Aurora B disrupts the highly dynamic nature of microtubules, resulting in chromosome mis-segregation. Consistent with changes in centromere transcription, my data indicate that loss of RB leads to ~ 2.5-fold increase in Aurora B at centromeres (Figure 2.7). Although not yet tested, I propose that this increase in transcription, and corresponding increase in Aurora B centromere localization, is functionally tied to the high rate of segregation errors seen in RB deficient cells.

Increased centromeric transcription compromises mitotic fidelity in RB deficient cells

Consistent with Aurora B localization being sensitive to centromere transcription, I find that inhibition of transcription reduces centromere Aurora B levels to that seen in control cells (Figure 2.7). Unexpectedly, I also find that levels of DNA damage also decrease with inhibition of mitotic transcription, suggesting that high transcription that results from RB underlies both Aurora B dependent regulation of mitotic fidelity and compromised DNA integrity. Work from other groups demonstrate that high rates of transcription can lead to the formation of G-quadruplex DNA structure (Kim and Jinks-Robertson 2012, Armas, David et al. 2017) and that GC rich sequences,

like those at the centromeric repeats, are particularly sensitive to their formation (Mukherjee, Sharma et al. 2019, Masai and Tanaka 2020, Wu, Niu et al. 2021). G quadruplex DNA is thought to be especially sensitive to DNA damage (McLuckie, Di Antonio et al. 2013, De Magis, Manzo et al. 2019, Masai and Tanaka 2020, Robinson, Raguseo et al. 2021), suggesting the formation of such aberrant DNA structures may underlie the mitotic defects in RB deficient cells.

Chapter 3: Suppression of chromosome instability limits acquired drug resistance

This chapter includes material from the following published manuscript:

Crowley EA, Hermance NM, Herlihy CP, Manning AL. Suppression of Chromosome Instability Limits Acquired Drug Resistance. Mol Cancer Ther. 2022 Oct 7;21(10):1583-1593. doi: 10.1158/1535-7163.MCT-22-0263.

Abstract

Numerical chromosome instability, or nCIN, defined as the high frequency of whole chromosome gains and losses, is prevalent in many solid tumors. nCIN has been shown to promote intra-tumor heterogeneity and corresponds with tumor aggressiveness, drug resistance and tumor relapse. While increased nCIN has been shown to promote the acquisition of genomic changes responsible for drug resistance, the potential to modulate nCIN in a therapeutic manner has not been well explored. Here we assess the role of nCIN in the acquisition of drug resistance in non small cell lung cancer. We show that generation of whole chromosome segregation errors in non small cell lung cancer cells is sensitive to manipulation of microtubule dynamics and that enhancement of chromosome cohesion strongly suppresses nCIN and reduces intra-tumor heterogeneity. We demonstrate that suppression of nCIN has no impact on non small cell lung cancer cell proliferation *in vitro*. However, suppression of nCIN alters the timing and molecular mechanisms that drive acquired drug resistance. These findings suggest mechanisms to suppress nCIN may serve as effective co-therapies to limit tumor evolution and sustain drug response.

Introduction

Whole chromosome instability, or numerical CIN (nCIN), is generated by underlying defects in mitosis (Weaver, Silk et al. 2007, Baker, Jin et al. 2009). The aneuploidy that results from mitotic segregation errors promotes intra-tumor heterogeneity and is a driving force in cancer that contributes to tumor evolution and drug resistance (Weaver, Silk et al. 2007, Baker, Jin et al. 2009, Choi, Seo et al. 2009, McClelland, Burrell et al. 2009, Sotillo, Schvartzman et al. 2010, Ippolito, Martis et al. 2021, Lukow, Sausville et al. 2021). Chromosome segregation is exquisitely sensitive to the regulation of dynamic microtubule attachments and defects that either increase or decrease the stability of microtubule attachments can corrupt mitotic fidelity and contribute to nCIN (Bakhoum, Genovese et al. 2009, Godek, Kabeche et al. 2015). Conversely, perturbations that reduce nCIN are proposed to limit acquired drug resistance and may hold therapeutic potential.

Aurora B kinase is a master regulator of kinetochore-microtubule dynamics during mitosis. Its overexpression is common in many cancer contexts and recent analyses of over 10,000 cancer genomes from the Cancer Genome Atlas show that, across cancer subtypes, Aurora B expression corresponds with degree of aneuploidy (Taylor, Shih et al. 2018). Increased expression of Aurora B also correlates with poor patient prognosis in a variety of cancer contexts (Nagy, Lánczky et al. 2018). Aurora B is a component of the Chromosome Passenger Complex (CPC). The CPC localizes to the centromere where Aurora B kinase activity regulates localization and activity of numerous kinetochore components responsible for binding and stabilizing kinetochore microtubule attachments, thereby promoting satisfaction of the spindle assembly checkpoint and regulating proper chromosome segregation (Hindriksen, Lens et al. 2017). Consistent with this function, both decreased and increased Aurora B activity at the centromere results in chromosome segregation errors (Hauf, Cole et al. 2003, Munoz-Barrera and Monje-Casas 2014,

González-Loyola, Fernández-Miranda et al. 2015, Abe, Sako et al. 2016, Huang, Lampson et al. 2018, Broad, DeLuca et al. 2020).

Here we demonstrate that in a panel of non small cell lung cancer (NSCLC) cell lines, where Aurora B is highly expressed and rates of chromosome segregation are high, the experimental reduction of centromere-localized Aurora B levels, or the independent stabilization of kinetochore microtubule attachments, is sufficient to minimize genomic heterogeneity and limit whole chromosome amplifications. We additionally find that suppression of nCIN limits acquired drug resistance both *in vitro and in vivo* and that sustained nCIN is rate limiting for tumor relapse.

Results

Chromosome segregation errors in NSCLC cells correspond with high expression and enhanced mitotic centromere localization of Aurora B

Non small lung cancer (NSCLC) cells frequently exhibit high levels of whole chromosome instability (nCIN), a feature that has been experimentally linked to acquired drug resistance (Baker, Jin et al. 2009, Choi, Seo et al. 2009, McClelland, Burrell et al. 2009, Swanton, Nicke et al. 2009, Sotillo, Schvartzman et al. 2010, Lukow, Sausville et al. 2021). To understand the molecular changes that correspond with segregation errors and nCIN in NSCLC cells we first identified a panel of NSCLC cell lines that exhibit mitotic defects and chromosome copy number heterogeneity consistent with nCIN (A549, H1299, and PC9; (Manning, Benes et al. 2014)). Using immunofluorescence microscopy to assess chromosome segregation we confirm that NSCLC lines A549, H1299, and PC9 all exhibit high rates of lagging chromosomes that are positive for the centromere marker ACA. The presence of such lagging chromosomes during anaphase are indicative of mitotic chromosome segregation errors (Figure 1A). Frequent high rates of whole

chromosome segregation errors during mitosis contribute to genomic heterogeneity within a cell population that can be assessed using FISH-based approaches to measure population-level numerical heterogeneity (NH) for individual chromosomes. Using centromeric probes for chromosomes 6, 7, 8, and 10 we find that, consistent with frequent mitotic errors, populations of A549, H1299 and PC9 cells exhibit NH scores ranging from ~7-25% (Figure 1B). In contrast, the non-transformed, human bronchial epithelial cell line HBEC3-KT exhibits neither frequent mitotic errors, nor high numeric heterogeneity (Figure 3.1 A & B).

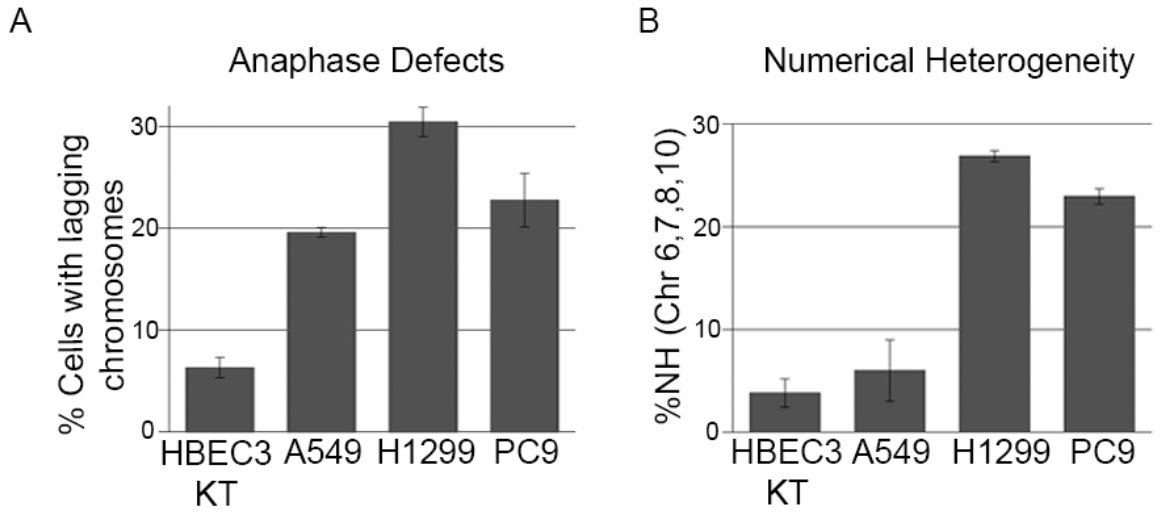

Figure 3.1: NSCLC cells exhibit frequent mitotic defects A) Quantification of anaphase lagging chromosomes in HBEC3-KT and NSCLC cell lines B) HBEC3-KT and NSCLC cell populations labelled with centromere enumeration FISH probes for chromosomes 6, 7, 8, and 10 and quantified for numerical heterogeneity (NH). A minimum of 300 cells for each chromosome probe were scored and averaged per cell line (Image from Crowley EA et al., 2022)

Aurora B kinase, an important regulator of mitotic chromosome segregation and the mitotic spindle assembly checkpoint, is commonly overexpressed in non small cell lung cancer, where nCIN is also prevalent (Vischioni, Oudejans et al. 2006, Takeshita, Koga et al. 2013, Yu, Zhou et al. 2018). Consistent with these earlier studies, we find that Aurora B kinase is highly expressed in our panel of NSCLC cells, when compared to the normal lung epithelial cell line HBEC3-KT

(Figure 3.2A). Aurora B regulation of mitotic chromosome segregation is largely dependent on its

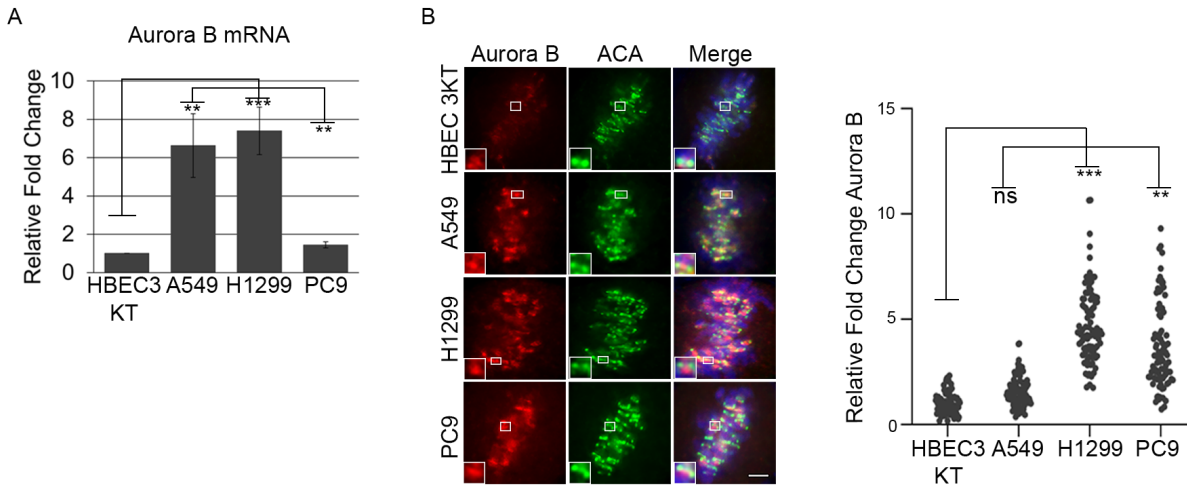

Figure 3.2: NSCLC cells display increased expression of Aurora B kinase A) qPCR analysis of Aurora B mRNA levels in NSCLC cells (A549, H1299, PC9) compared to normal human bronchial epithelial cells (HBEC3-KT). Error bars indicate standard deviation between three biological replicates. Statistical analyses were performed between averages of biological replicates. B) Representation and quantification of centromere-localized Aurora B in NSCLC and HBEC3-KT cell lines. A minimum of 30 kinetochore pairs (3 per cell for 10 or more cells). Insets are of individual kinetochore pairs at 4x magnification. Scale bar is 5μm. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Image from Crowley EA et al., 2022)

localization at centromeres where it phosphorylates key substrates that regulate the stability of kinetochore-microtubule attachments (Lampson, Renduchitala et al. 2004, Cimini, Wan et al. 2006, Welburn, Vleugel et al. 2010, Gregan, Polakova et al. 2011, Liang, Zhang et al. 2020) Importantly, we find that centromere localization of Aurora B in our panel of NSCLC cell lines mirrors overall expression levels of this kinase such that Aurora B localization at centromeres is increased, on average, two to five-fold over that seen in HBEC3-KT cells (Figure 3.2B).

Centromere localization and activity of Aurora B in NSCLC is sensitive to cohesion

Aurora B localization is sensitive to changes in cohesin regulation such that redistribution of the cohesin complex from pericentromere enrichment to distribution along chromosome arms leads to a concurrent redistribution of Aurora B along chromosome arms and a reduction in its centromere localization (Haarhuis, Elbatsh et al. 2013, Kleyman, Kabeche et al. 2014, Meppelink,

Kabeche et al. 2015, Zhou, Liang et al. 2017) Therefore, to test the impact of high centromere Aurora B levels in the mitotic defects observed in these NSCLC lines without altering overall Aurora B protein levels, we first experimentally manipulated the distribution of the cohesin complex. Enhancement of chromosome cohesion along chromosome arms was achieved using si- and sh-RNA approaches to deplete Wapl, a well-characterized negative regulator of the cohesin complex (Sherwood, Takahashi et al. 2010) (Figure 3.3A & B). Wapl associates with the

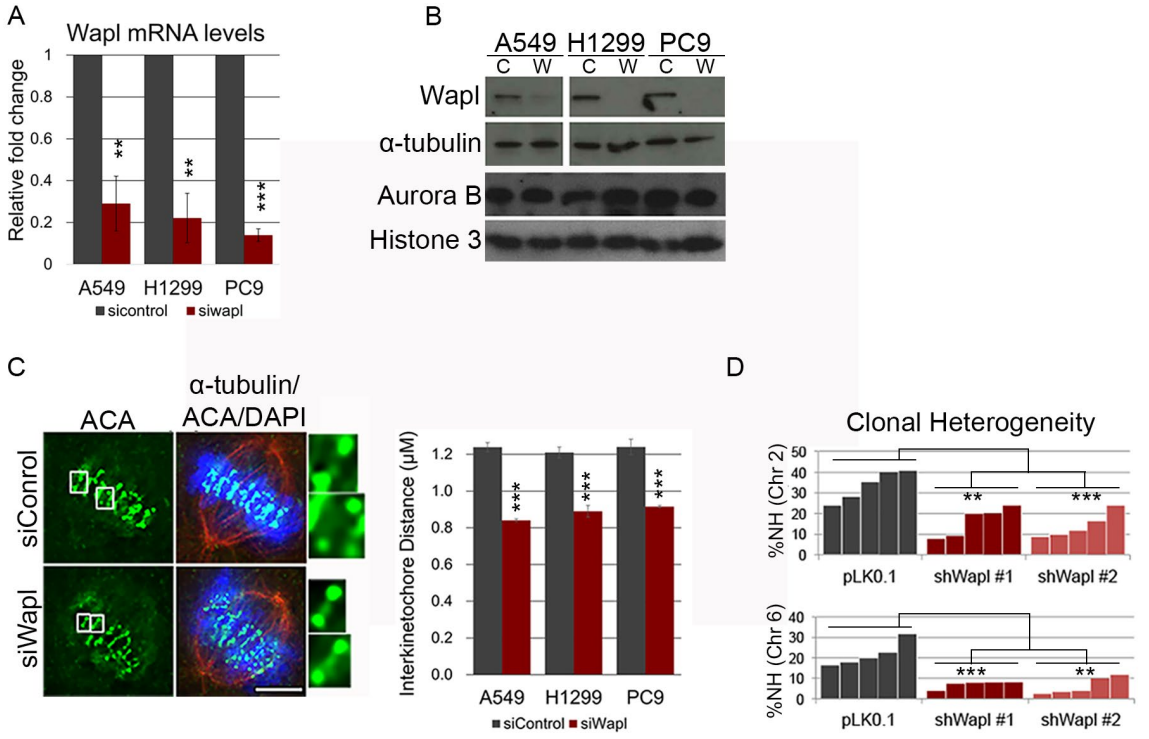

Figure 3.3: Increased cohesion reduces clonal heterogeneity in NSCLC cells A) qPCR analysis of Wapl mRNA levels in control (Mock) and Wapl-depleted (Wapl KD) NSCLC cells. B) Western blot analysis of Aurora B and Wapl protein levels in control (C) and Wapl depleted (W) NSCLC cell lines C) Representation of PC9 cells and quantification of inter-centromere distances in (A539, H1299, and PC9 cells) with and without Wapl depletion (siWapl) and siControl, respectively). Insets are of individual kinetochore pairs at 4x magnification. Scale bar is 5μm. A minimum of 90 kinetochore pairs were measured (3/cell for 30cells), for each of 3 biological replicates. Error bars indicate standard deviation between biological replicates and statistical analyses were performed between averages of biological replicates. D) Quantification of chromosome copy number heterogeneity (NH) in clonal populations of PC9 cells carrying an empty expression vector (pLKO.1) or one of two different shRNA constructs targeting Wapl for depletion (Wapl KD #1 and #2). Chromosome copy number was scored in a minimum of 300 cells for 5 clonal populations of each condition. Statistical analyses were performed between conditions where each clone was considered as a biological replicate, *p<0.5,**: p<0.01, ***: p<0.001. (Image from Crowley EA et al., 2022)

cohesin complex to regulate its dynamic association with chromatin throughout the cell cycle (Kueng, Hegemann et al. 2006) such that Wapl depletion blocks cohesin complex dissociation from chromosomes during early mitosis. We used immunofluorescence imaging of metaphase

cells to identify and measure inter-kinetochore distances as a readout of functional centromere cohesion. This demonstrated that Wapl knockdown enhanced cohesion, as evidenced by a reduction in interkinetochore distance (Figure 3.3C).

Using quantitative immunofluorescence, we find that depletion of Wapl perturbs Aurora B localization such that centromere-localized Aurora B levels in all three NSCLC lines is reduced

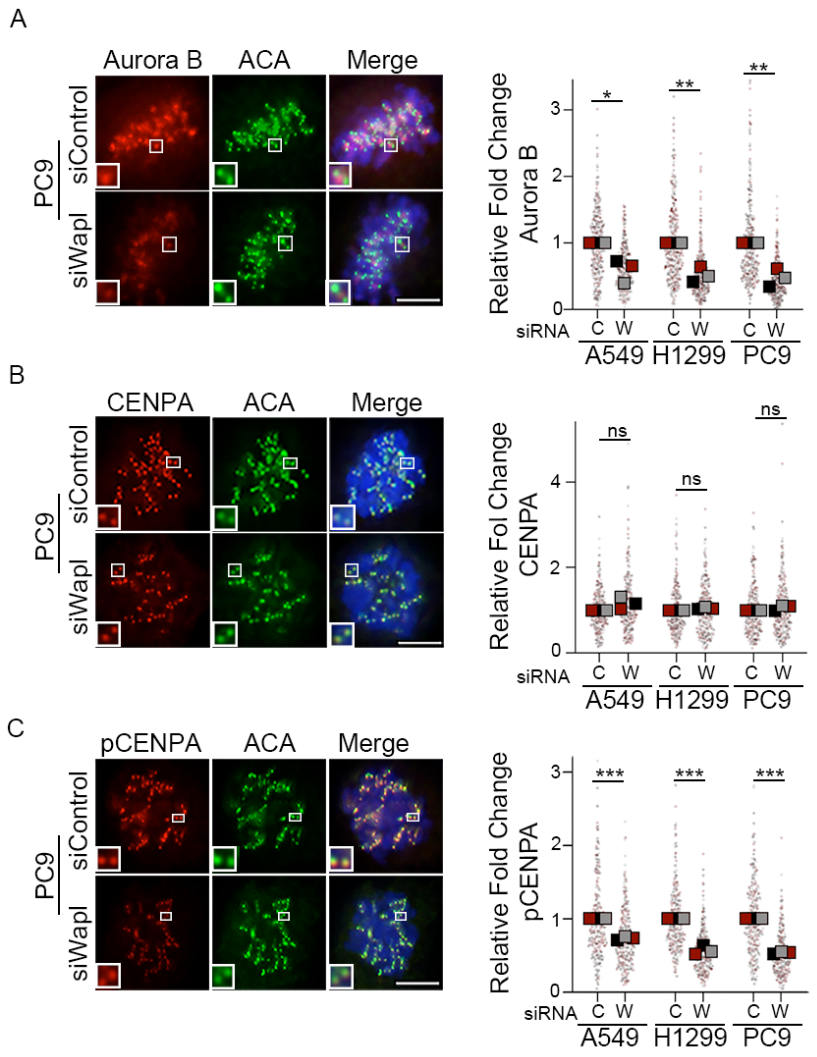

Figure 3.4: Enrichment of cohesion in NSCLC cells decreases Aurora B localization and activity at the centromere A-C) Representation and quantification of centromere localized Aurora B, CENPA (total) and pCENPA (pSer7) in PC9 cells treated with non-targeting (siControl) or Wapl specific (siWapl) siRNA. Insets are of individual kinetochore pairs at 4x magnification. Scale bars are 5μm. A minimum of 90 kinetochore pairs were measured (3/cell for 30 cells), for each of 3 biological replicates. Error bars represent standard deviation between biological replicates and statistical analyses were performed between averages of biological replicates *: p< 0.05, **: p<0.01; ***: p<0.001. (Image from Crowley EA et al., 2022)

by ~50% when Wapl is depleted (Figure 3.4A and Figure 3.5A). Western blot analysis of mitotic cells indicates that this change in Aurora B localization does not arise due to Wapl-dependent changes in Aurora B expression (Figure 3.3B). Consistent with reduced centromere localization and activity of Aurora B, we see comparable reduction in the phosphorylation, but not localization, of key Aurora B substrate CENPA (Total and CENPA pS7: 3.4B & C, Figure 3.5B & C).

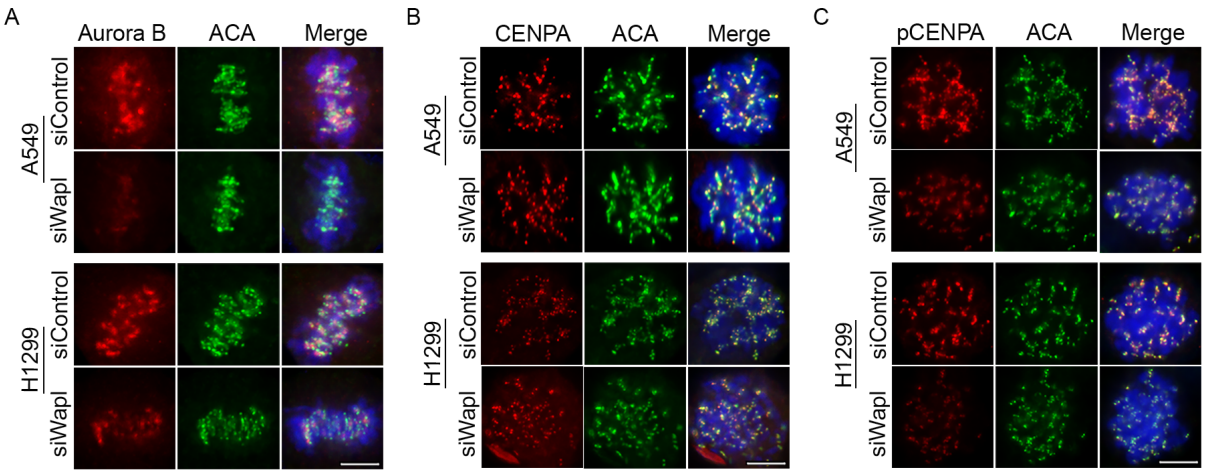

Figure 3.5: Enhanced cohesion limits Aurora B localization and activity at mitotic centromeres. Representation and quantification of centromere-localized A) Aurora B, B) CENPA (total), and C) pCENPA (pSer7) following Wapl depletion (siWapl) or not (siControl) in A549 and H1299 cells. A minimum of 90 kinetochore pairs were measured (3/cell for 30 cells) for each of 3 biological replicates. Scale bar is 5μm. (Image from Crowley EA et al., 2022)

Modulation of Aurora B activity suppresses kinetochore-microtubule dynamics and chromosome segregation errors

Aurora B kinase is the master regulator of microtubule dynamics such that Aurora B localization and kinase activity promotes the dynamic turnover of kinetochore microtubule attachments. The fidelity of chromosome segregation has been shown to be sensitive to both increased and decreased Aurora B activity, and hence corresponding decreased and increased stability of kinetochore-microtubule attachments (Hauf, Cole et al. 2003, Cimini 2007, Bakhoum, Genovese et al. 2009, Liu, Vader et al. 2009, Munoz-Barrera and Monje-Casas 2014). Consistent with this

activity, we find that kinetochore fibers in Wapl-depleted cells, where Aurora B localization at centromeres had been reduced, exhibit decreased sensitivity to cold-induced microtubule depolymerization compared to control cells (Figure 3.6A). This suggests kinetochore microtubule

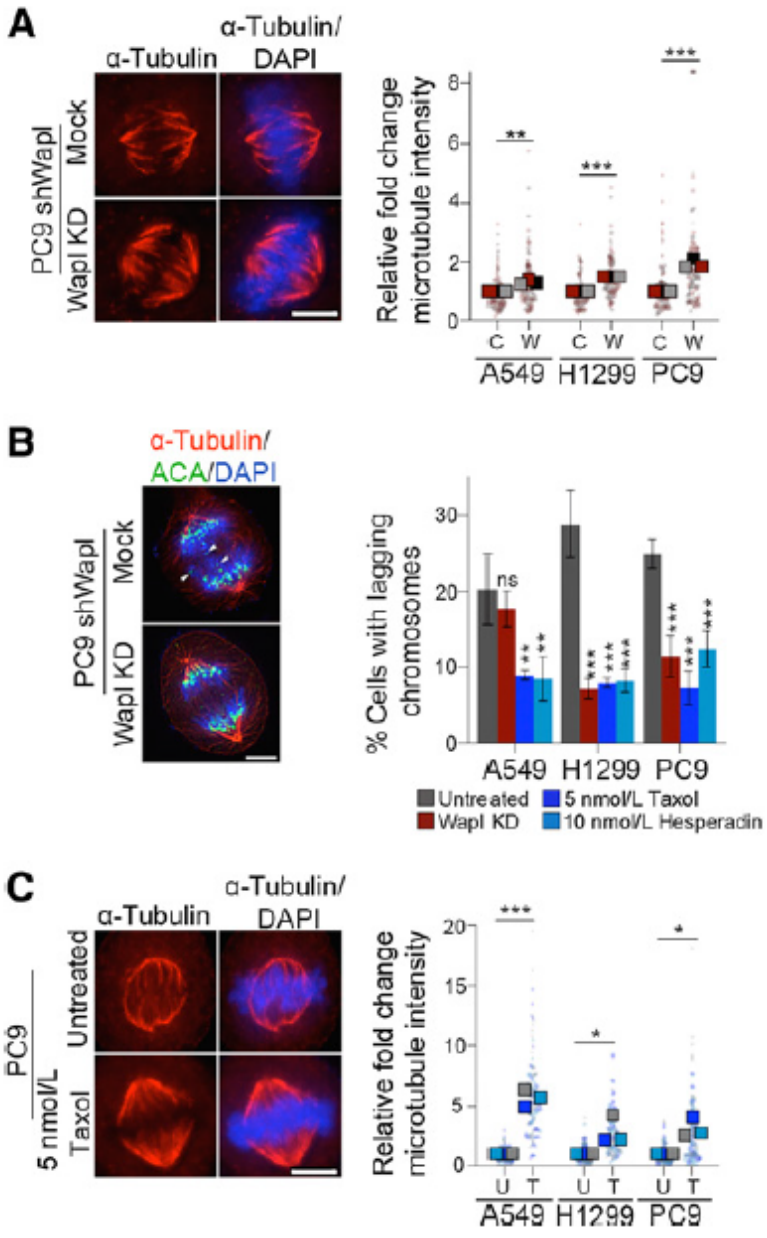

Figure 3.6: Anaphase segregation errors in NSCLC cells are reduced by microtubule stabilizing perturbations A) Representation and quantification of cold stable microtubules in PC9 cells depleted of Wapl B) Representation and quantification of anaphase lagging chromosomes in NSCLC cells following Wapl depletion, or treatment with 5nM Taxol, or 10nM Hesperadin. White arrow heads indicate individual lagging chromosomes. C) Representation and quantification of cold stable microtubules in PC9 cells treated with 5nM Taxol. Scale bars are 5μm. A minimum of 30 metaphase or anaphase cells were scored per population for each of 3 biological replicates. Error bars represent standard deviation between biological replicates and statistical analyses were performed between averages of biological replicates *: p< 0.05, **: p<0.01; ***: p<0.001. (Image from Crowley EA et al., 2022)

attachments are stabilized by the redistribution of Aurora B away from the centromere that occurs following Wapl depletion. We next assessed mitotic fidelity in these cells and find that enhancement of chromosome arm cohesion, via Wapl depletion, is sufficient to reduce the incidence of lagging chromosomes during anaphase in both H1299 and PC9 cells (Figure 3.6B). Similarly, partial inhibition of Aurora B activity with the small molecule inhibitor Hesperidin, is sufficient to suppress mitotic defects in all three NSCLC lines (Figure 3.6B).

To directly test the role of microtubule dynamics in the high frequency of mitotic errors observed in this panel of NSCLC cells, we treated cells with a microtubule-stabilizing concentration of Taxol prior to assessing mitotic fidelity. We find that following acute treatment (1h) with 5nM Taxol, or long-term treatment (6 weeks) with 1nM Taxol, cells exhibit both resistance to cold- induced

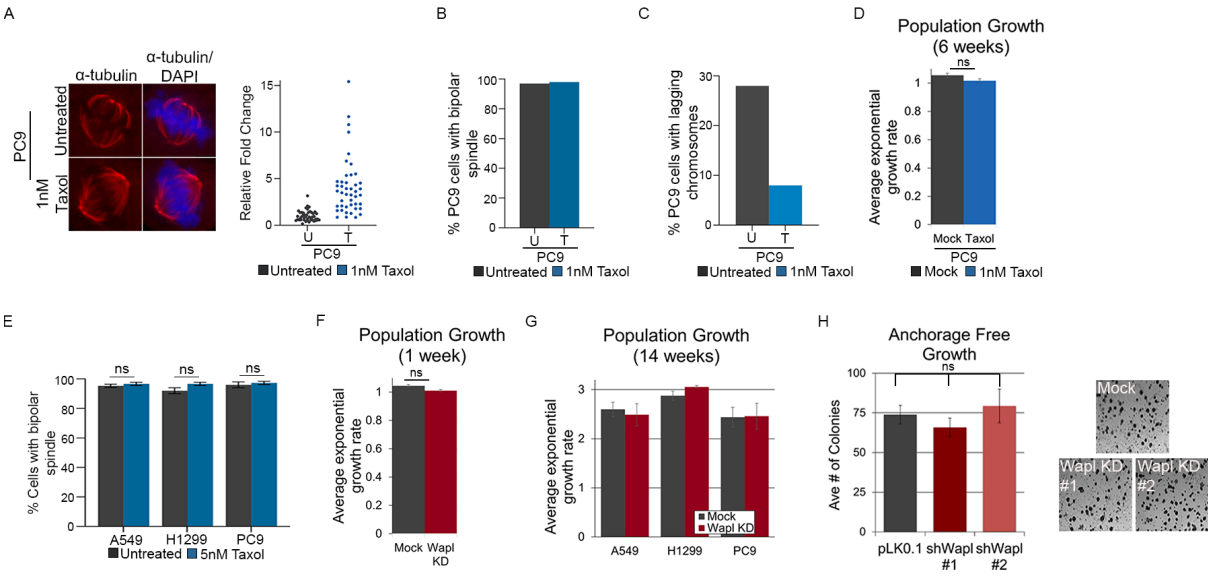

Figure 3.7: Suppression of nCIN does not compromise cell proliferation A-C) Representation and quantification of A) cold stable microtubules, B) bipolar spindles and C) anaphase lagging chromosomes in PC9 cells before or following acclimation to grow in 1nM Taxol. D) Average exponential growth rate of PC9 cells following 6 weeks of exposure to 1nM Taxol E) Quantification of bipolar spindles in NSCLC lines with and without treatment with 5nM Taxol for 1h. F-G) Average exponential growth rate of PC9 cells following F) 1-week acute depletion of Wapl, or G) 14 weeks of constitutive Wapl depletion. H) Representation and quantification of average colony number formed in control (PLK0.1) and Wapl depleted (Wapl KD#1 & #2) cell lines cultured under anchorage-free conditions. Error bars represent standard deviation between biological replicates and statistical analyses were performed between averages of biological replicates *: p< 0.05, **: p<0.01; ***: p<0.001. (Image from Crowley EA et al., 2022)

microtubule depolymerization and reduction in anaphase lagging chromosomes, without perturbations to overall spindle structure, comparable to that seen following Wapl depletion (Figure 3.6B & C, Figure 3.7)

To next assess if enhanced cohesion and reduction of anaphase defects is sufficient to suppress nCIN, PC9 cells were engineered to constitutively express one of two different shRNA hairpin constructs designed to target Wapl mRNA for depletion. Single cell clones were derived from parental and Wapl-deficient PC9 cells and analyzed for numerical heterogeneity (NH). Individual clones derived from PC9 cells expressing an empty PLK0.1 vector exhibit NH values of 18-31% for chromosome 6, and up to 20-40% for chromosome 2. NH for both chromosomes was reduced ~2-3-fold in Wapl-deficient PC9 cell clones (chromosome 6: 3-12%, chromosome 2: 5-24%) (Figure 3.3D), indicating that enhanced cohesion is sufficient to suppress nCIN. Together, these data support a model whereby increased Aurora B activity and/or highly dynamic kinetochore microtubule attachments underlie nCIN in NSCLC cells.

Suppression of nCIN limits drug tolerance in NSCLC cells

Activating mutations within the EGFR gene that drive tumor cell proliferation are present in nearly a quarter of all NSCLCs (Gazdar 2009). Patients with such mutations are commonly treated with EGFR tyrosine kinase inhibitors (TKIs) (Gerber 2008). However, the majority of patients treated with EGFR TKIs ultimately develop resistance with nearly 60% of resistant or relapsed tumors exhibiting resistance-conferring mutations in the EGFR gene, making this the most frequent mechanism of EGFR TKI resistance (Stewart, Tan et al. 2015). PC9 cells exhibit an activating deletion in exon 19 of EGFR that drives cell proliferation and renders them sensitive to EGFR TKIs (Arao, Fukumoto et al. 2004). Like tumors in patients, these cells commonly acquire

resistance to EGFR TKIs via acquisition of a secondary Threonine to Methionine mutation in EGFR (T790M) (Stewart, Tan et al. 2015). While nCIN in NSCLC, and other cancer contexts, has been correlated with acquired drug resistance (Sotillo, Schvartzman et al. 2010, Vargas-Rondón, Villegas et al. 2017, Yu, Zhou et al. 2018), the impact of nCIN on mutation-based mechanisms of acquired drug resistance remain unclear.

Drug response is sensitive to cell proliferation rates and the impact of aneuploidies that result from nCIN have alternatively been demonstrated to promote tumor cell growth or to reduce proliferative capabilities (Bakhoum and Compton 2012, Chunduri and Storchová 2019). Therefore, we first assessed proliferative capacity of PC9 cells with and without constitutive nCIN/Wapl depletion (Mock and Wapl KD, respectively) or following acclimation to microtubule-stabilizing concentrations of Taxol. Importantly, in all clones tested, proliferation rates with or without Wapl depletion or Taxol treatment, when grown in the absence of TKI treatment, were comparable (Figure 3.7D & F). Similarly, anchorage independent colony formation assays of growth in soft agar revealed similar colony number and size, irrespective of Wapl/nCIN status (Figure 3.7H), indicating that suppression of nCIN alone does not alter drug naïve PC9 cell growth.

Next, we assessed the response of cells, with and without Wapl depletion or those acclimated to grow in microtubule-stabilizing doses of Taxol, to the EGFR TKI Gefitinib. EGFR activity results in phosphorylation of EGFR and downstream targets (such as ERK) and promotes cell proliferation. Western blot analyses indicate that PC9 cells with either mock or induced Wapl depletion or treated with 1nM Taxol are initially similarly responsive to Gefitinib: all populations show dramatic reduction of EGFR-dependent phosphorylation (phospho EGFR: Tyr1068 & phospho ERK: Tyr 202/Tyr 204) (Figure 3.8A). Nevertheless, following 4 weeks of continuous

treatment with a sub-lethal dose of Gefitinib drug-tolerant cells slowly form colonies that can be detected with crystal violet stain. Following long-term exposure to Gefitinib, Wapl-depleted and Taxol-treated PC9 cells exhibit a dramatic reduction in the number and size of drug tolerant colonies that arise while under Gefitinib treatment (Figure 3.8B & C).

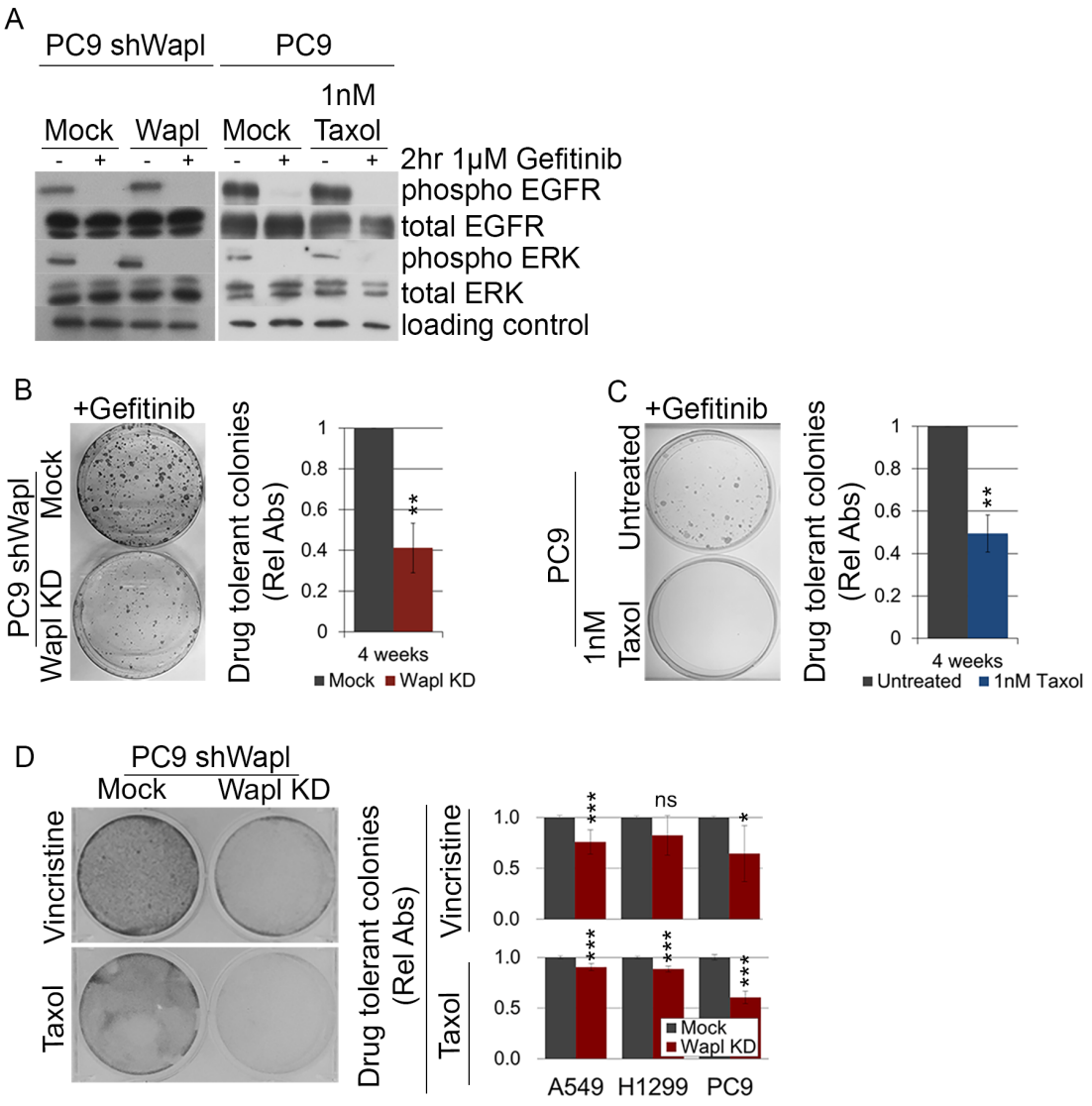

Figure 3.8: Suppression of nCIN limits drug tolerance A) Total and phosphorylated levels of EGFR and ERK in PC9 cells with induced depletion of Wapl or 1nM Taxol treatment following Gefitinib treatment. B & C) Crystal Violet staining and quantification of drug tolerant colonies of control and Wapl depleted PC9 cells following extended culture of Wapl depleted (B) or Taxol treated (C) cells in presence of 1μM Gefitinib D) Crystal Violet staining and quantification of drug tolerant colonies of control and Wapl depleted NSCLC cells following extended culture in the presence of Vincristine or Taxol. Representative images and quantification reflect data from three biological replicates. Error bards represent standard deviation between replicates and statistical analyses were performed between averages of biological replicates *: p< 0.05, **: p<0.01; ***: p<0.001. (Image from Crowley EA et al., 2022)

While low dose Taxol can stabilize microtubule dynamics and reduce nCIN, therapeutic approaches exploit higher dosing and/or prolonged exposure to compromise cell proliferation. Nevertheless, resistance to other clinically relevant therapeutics such as Vincristine and Paclitaxel have been reported in NSCLC (Kavallaris, Kuo et al. 1997, Engelman, Zejnullahu et al. 2007, McDermott, Pusapati et al. 2010, Nurwidya, Takahashi et al. 2014, Lee, Chen et al. 2017, Zhou, Zhu et al. 2018). Therefore, to next test whether the impact of suppression of nCIN on drug tolerance is specific to Gefitinib or instead may similarly limit the outgrowth of cells tolerant to other therapeutic agents, we performed *in vitro* drug tolerance assays in A549, H1299, and PC9 cells with and without constitutive depletion of Wapl in the presence of either Vincristine or Paclitaxel. Over the course of 14 weeks the concentration of these drugs was doubled every 4 days until drug tolerant cells emerged. Comparative measures of cell numbers in each condition were approximated using crystal violet staining. Following long-term treatment with either Vincristine or Paclitaxel, all three NSCLC lines constitutively depleted of Wapl show reduction in the number of drug-tolerant cells (Figure 3.8D). Importantly, cells constitutively depleted of Wapl for the duration of the drug sensitivity assay show no change in proliferation rates, suggesting decreased tolerance to Vincristine or Paclitaxel cannot be accounted for by changes in proliferation rate following Wapl depletion (Figure 3.7G). These data suggest that nCIN permits acquisition of drug tolerance to promote continued proliferation.

Chromosome Instability informs mechanism of TKI drug resistance

To better understand the relationship between nCIN-dependent genomic changes that may promote or permit resistance to drug therapy we characterized four drug-tolerant clones from each of the parental and Wapl-depleted populations that were exposed to long-term Gefitinib treatment. Clones were selected and expanded under culture conditions that maintained 1μM Gefitinib and mock or induced Wapl depletion, as appropriate. Importantly, in both Wapl-depleted

and Wapl-proficient contexts, drug tolerant PC9 cells that persist following long term exposure to Gefitinib continue to exhibit features consistent with Wapl status: single colonies derived from Mock-depleted cells have frequent anaphase defects and a high measure of NH and whole chromosome amplifications while Wapl-depleted cells have reduced anaphase defects and less numerical heterogeneity (Figure 3.9A-C).

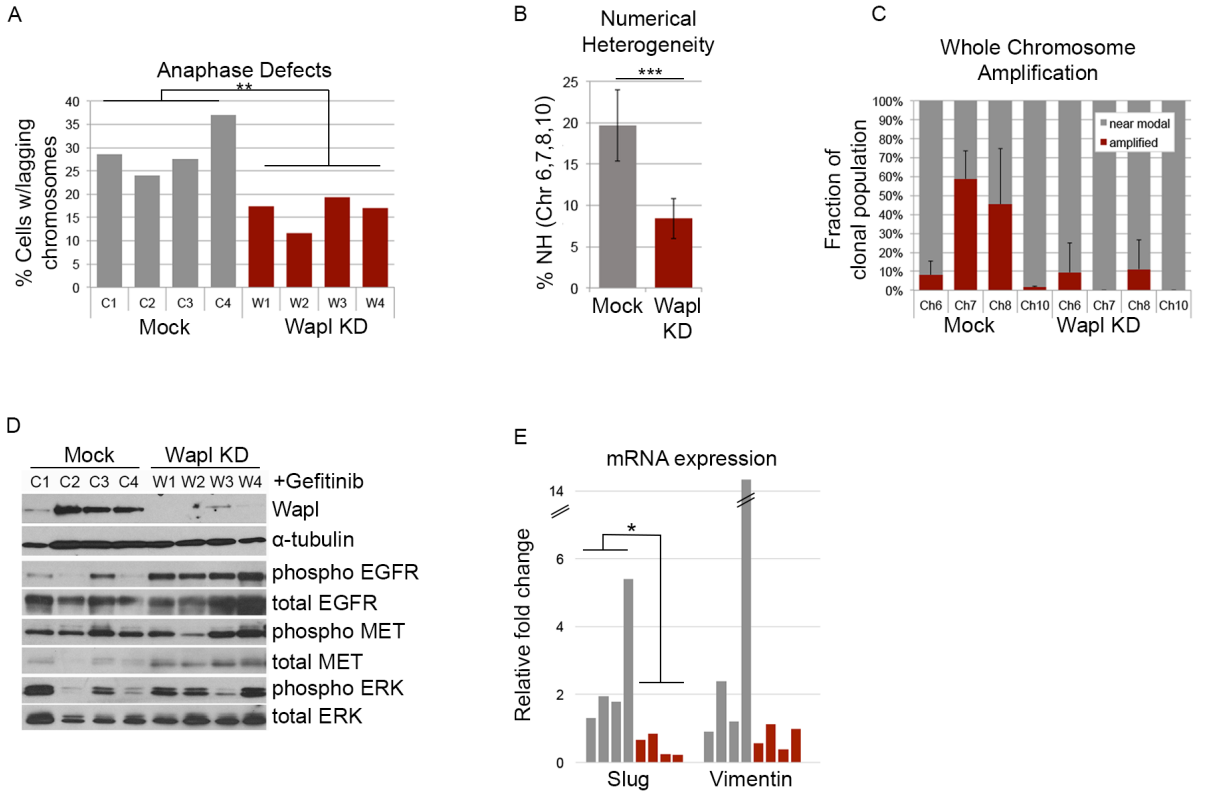

Figure 3.9: Suppression of nCIN influences mechanisms of drug tolerance in PC9 cells. A) Quantification of anaphase cells exhibiting lagging chromosomes in clonal populations of Gefitinib-tolerant cells with (Wapl KD) or without (Mock) induced Wapl depletion. B & C) Gefitinib-tolerant PC9 clonal populations derived with or without Wapl depletion labelled with centromere enumeration FISH probes for chromosomes 6, 7, 8, and 10 and quantified for chromosome copy number. Represented as average numerical copy number across all chromosomes assessed (B) and fraction of cells with modal or amplified copies of the indicated chromosomes (C). A minimum of 300 cells per condition per centromere probe were scored. D) Western blot showing Wapl levels and EGFR pathway status in four each of mock and Wapl-depleted Gefitinib-tolerant clones. E) qPCR analysis of mRNA levels indicative of EMT pathway activation (Slug, Vimentin) in Mock and Wapl-depleted Gefitinib-tolerant clones. Error bars represent standard deviation between clones. Each clonal population (n=4 per condition) was considered as a biological replicate for statistical analysis, *: p< 0.05, **: p<0.01; ***: p<0.001. (Image from Crowley EA et al., 2022)

Cells that acquire tolerance or and ultimately resistance to TKI activity do so by restoring or

bypassing EGFR kinase function to activate downstream pathway components and promote proliferation (Stewart, Tan et al. 2015). Resistant clones that emerge early following TKI inhibition likely do so through selection of pre-existing sub clonal resistance-conferring mutations while those that emerge later reflect the acquisition of de novo mutations (Hata, Niederst et al. 2016). Following 4 weeks of exposure to Gefitinib we find that one of the eight clones (Mock clone C3) exhibits a T790M mutation, indicative of a pre -existing or early mutation that was selected for over the short course of Gefitinib exposure. The remaining seven gefitinib-tolerant clones did not yet exhibit known resistance-conferring mutations in EGFR. This is consistent with late emerging, de-novo drug resistance and provides an opportunity to examine nCIN-dependent molecular changes that may precede or promote the transition from a drug tolerant to drug resistant state.

Although the emergence of drug tolerant clones is decreased significantly by Wapl depletion, molecular characterization of EGFR pathway function indicates that drug tolerant clones that do arise from both Mock- and Wapl-depleted populations similarly exhibit increased EGFR expression and phosphorylation of ERK, a downstream target in the EGFR pathway (Figure 3.9D). In contrast, Wapl-depleted PC9 clones, but not Mock-depleted PC9 clones, exhibit EGFR auto phosphorylation (Figure 3.9D). This phosphorylation is dependent on EGFR kinase activity and suggests drug-tolerant clones that lack nCIN (Wapl KD) primarily arise through the acquisition of resistance-conferring mutations that impair TKI binding or otherwise restore EGFR activity, while clones that sustain nCIN (Mock) likely exploit other mechanisms of drug tolerance (Engelman, Zejnullahu et al. 2007, Gerber 2008, Gazdar 2009, Stewart, Tan et al. 2015).

To explore possible mechanisms by which EGFR activity may be restored in clones that lack nCIN, we examined EGFR transcript sequences from each clone. We find that one clone (W2)

expresses a new V689M activating mutation in EGFR (Massarelli, Johnson et al. 2013), while the remaining three clones exhibit a shift in allelic expression such that 10-30% of the detected EGFR transcripts lack the activating exon 19 deletion (vs 0-10% of EGFR transcripts in clones that have nCIN). The deletion in exon 19 of EGFR both promotes cell proliferation and confers sensitivity to Gefitinib as the mutant protein has a higher affinity for Gefitinib than does the wild type of protein (Harrison, Vyse et al. 2020). As such, increased expression of EGFR transcripts that lack the deletion and/or gain of an independent activating mutation are consistent with changes that would increase tolerance to Gefitinib.

Alternative pathways reported to promote TKI resistance in NSCLC include amplification of EGFR, amplification of the receptor tyrosine kinase MET (Engelman, Zejnullahu et al. 2007), and/or activation of epithelial to mesenchymal transition (EMT) (Tulchinsky, Demidov et al. 2019, Yochum, Cades et al. 2019, Zhu, Chen et al. 2019). To understand if these alternative pathways could explain tolerance of Mock- depleted PC9 clones to Gefitinib treatment, we first assessed copy number of chromosome 7, on which both EGFR and MET genes are located. Drug naïve PC9 cells have a modal copy number of four for chromosome 7, as do drug tolerant Wapl-depleted clones. In contrast, nearly 60% of drug tolerant Mock-depleted cells have >8 copies of chromosome 7 (Figure 3.9C, Figure 3.10). We next looked at expression of key EMT transcription any of the Wapl-depleted clones (Figure 3.9E). The gene that encodes for SLUG, SNAI2, is factor, SLUG and found it to be upregulated 2-fold in 3 out of 4 Mock-depleted clones but not in located on chromosome 8p, adjacent to the centromere. In support of a model whereby nCIN influences the mechanism of TKI resistance, single cell analysis of chromosome 8 copy number indicates that the same 3 Mock-depleted clones with increased SLUG expression also exhibit amplification of chromosome 8 (Figure 3.9C, Figure 3.10). Consistent with SNAI2 amplification and increased

Slug expression, Vimentin, a widely used marker of EMT and a Slug target gene is also increased 2 to 12-fold in Mock-depleted clones (Figure 3.9E).

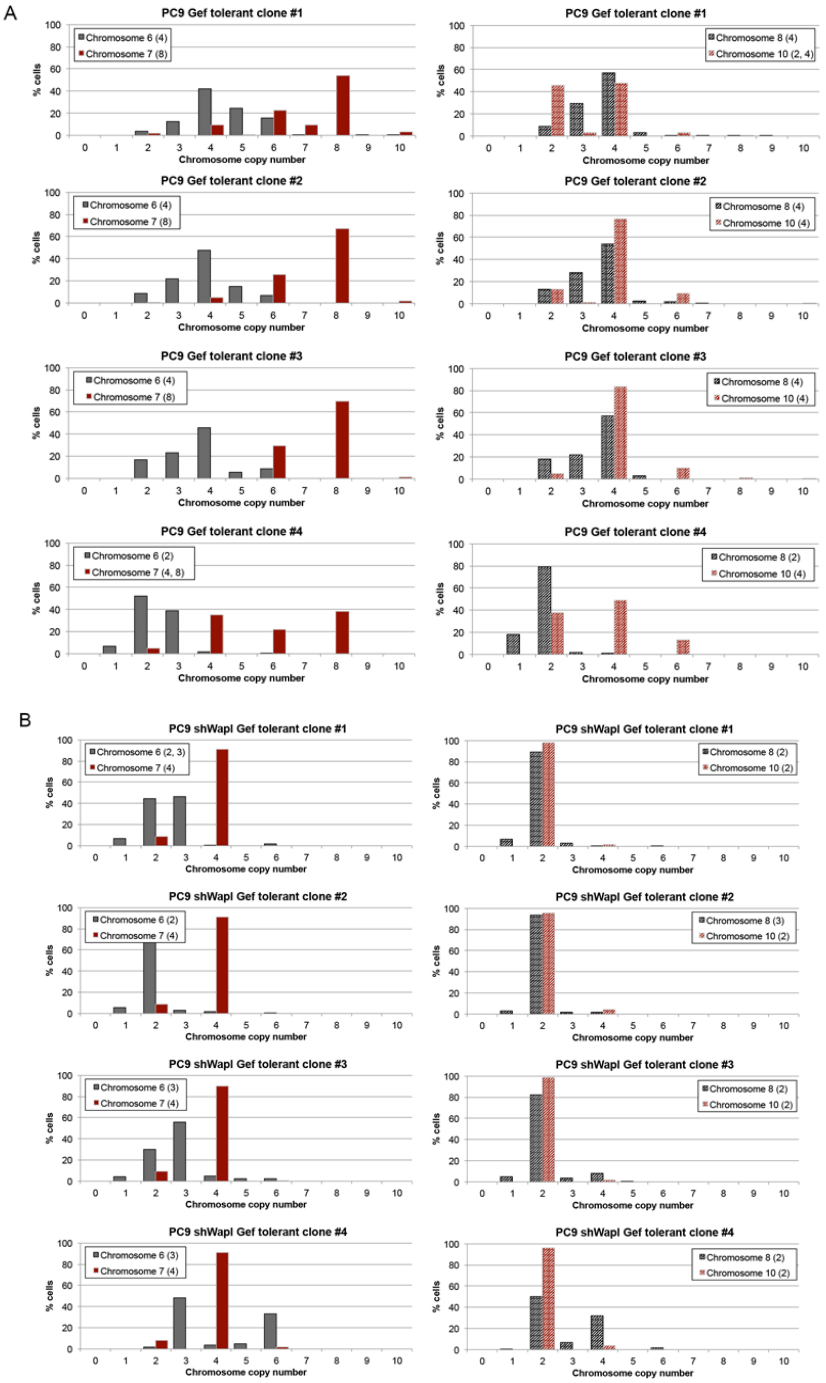

Figure 3.10: nCIN promotes acquisition of drug tolerance-promoting chromosome amplifications. A & B) FISH analysis with centromere enumeration probes to measure chromosome copy number in individual cells for chromosomes 6, 7, 8 and 10 in Mock (A) or Wapl-depleted (B) Gefitinib-tolerant clones. Modal copy number for each chromosome is indicated in parentheses. Copy number of each chromosome probe was scored in a minimum of 300 cells for each cell line. (Image from Crowley EA et al., 2022)

Together these data suggest that nCIN influences the mechanism of acquired drug tolerance. Without nCIN, mutation-driven mechanisms of drug resistance dominate, but the acquisition of such mutations are slow, particularly in drug treated populations with limited proliferation, and as result drug tolerant colonies are slow to emerge. Through whole chromosome gains and losses hundreds to thousands of genes may become mis-regulated in a single cell division. In this way nCIN has the capacity to promote cellular changes that contribute to drug tolerance. These data support a model whereby re-establishment of Wapl expression/nCIN, or expansion of clones that fail to silence Wapl/sustain nCIN, is limiting for acquired drug resistance and tumor relapse (Figure 6).

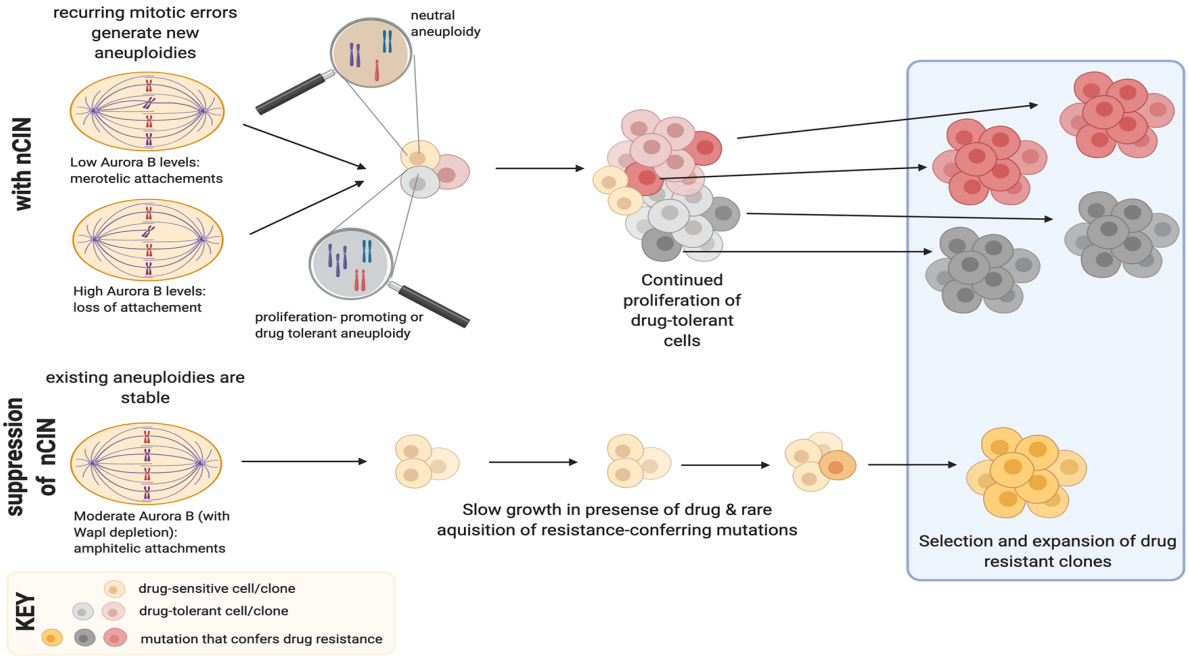

Figure 3.11: Mitotic defects that promote CIN enable the generation of drug tolerant aneuploidies that permit continued growth and increased incidence of acquired drug resistance. (Image from Crowley EA et al., 2022)

Discussion

Clonal mutations that enable resistance to targeted or chemotherapeutic approaches pose a clinical challenge and remain a major cause of death in many cancer types (Redmond, Papafili et al. 2015). Clinically and experimentally, the degree of intra-tumor genomic heterogeneity, and underlying defects in mitotic cell division have been functionally linked to tumor evolution, drug resistance, and metastasis (Rajagopalan and Lengauer 2004, Gao, Furge et al. 2007, Choi, Seo et al. 2009, McClelland, Burrell et al. 2009, Ippolito, Martis et al. 2021, Lukow, Sausville et al. 2021). In the context of NSCLC, identification of driver mutations in EGFR and the initial clinical success of TKI treatment has been hampered by rapid and prevalent acquisition of drug resistance (Gazdar 2009). Here, we present mechanistic evidence that nCIN may arise from mis-regulation of cohesin-sensitive Aurora B kinase activity at centromeres and deregulation of spindle microtubule dynamics. We propose that subsequent chromosome amplifications contribute to a high incidence of acquired resistance to therapy. The reduction in emergence of drug tolerant clones following experimental suppression of nCIN demonstrate that whole chromosome copy number changes create a favorable environment for continued proliferation while resistance-conferring mutations are attained.

Whole chromosome segregation errors enable tumor evolution and drug resistance

Our analysis examined isogenic PC9 cells that primarily differ, at least initially, in their nCIN status. Our results indicate that nCIN contributes to drug resistance by allowing for the generation of whole chromosome amplifications that harbor key drug tolerance genes (like EGFR, MET, and SNAI2/SLUG) and promote continued proliferation in the presence of TKI. In the absence of an increased mutation rate, this continued proliferation is key to enable the acquisition of replication-dependent mutations that confer robust drug resistance and tumor relapse. Such adaptive mutability may be particularly relevant to the mis-segregation and subsequent selection for

amplification of chromosome 7, which contains both MET and EGFR genes. Increased EGFR gene copy number both promotes proliferation (Stewart, Tan et al. 2015) and, by virtue of having more EGFR gene templates for replication-acquired mutation, increases the apparent mutability of individual cells (Andersson, Slechta et al. 1998). In addition to a role for nCIN in promoting continued proliferation prior to acquired drug resistance, nCIN has also been linked to metastasis, chronic inflammation and tumor immunity (Tijhuis, Johnson et al. 2019), suggesting that, independent of limiting drug resistance, suppression of nCIN may be of therapeutic benefit to limit overall tumor growth and progression. These findings are consistent with previous studies showing selective pressure for cancer cells to sustain nCIN and demonstrating that high levels of nCIN can drive the generation and selection of drug-resistant clones (Orr, Talje et al. 2016, Salgueiro, Buccitelli et al. 2020, Ippolito, Martis et al. 2021, Lukow, Sausville et al. 2021).

Although our studies indicate that key chromosome amplifications are selected for early during Gefitinib treatment of nCIN cells, chromosome amplifications have not been shown to be a common mechanism of resistance to TKI in patients (Stewart, Tan et al. 2015) and resistance-conferring amplifications are often found to pre-exist exposure to TKI (Turke, Zejnullahu et al. 2010). These data suggest that nCIN-driven chromosome amplifications may not be a direct driver of resistance *per se*, but instead that nCIN may contribute to drug tolerance and, by permitting or promoting early proliferation, enable the eventual acquisition of drug resistance-conferring mutations. Consistent with a model whereby enhanced proliferation is a necessary initial step in acquired drug resistance, drug tolerant nCIN clones isolated in this study exhibit amplifications that promote cell survival and proliferation.

In the absence of nCIN, whole chromosome amplifications cannot drive drug tolerance. Instead, our data implicate the acquisition of additional activating mutations and/or changes in allelic expression of EGFR as factors that promote continued proliferation. PC9 cells are heterozygous for an activating deletion in exon 19 of EGFR that both promotes cell proliferation and confers sensitivity to Gefitinib (Harrison, Vyse et al. 2020). Consistent with published work showing shifts in allelic expression that favor the wildtype EGFR allele contribute to TKI resistance in cell lines and tumors (Tabara, Kanda et al. 2012), gefitinib-tolerant clones that lack nCIN exhibit an increase in EGFR transcripts that contain an intact exon 19 over EGFR with an exon 19 deletion.

High mutation rates may preclude the need for nCIN in acquired drug resistance

In the absence of nCIN, resistance is limited to the clonal amplification of cells with pre-existing amplifications or mutations, and those that acquire chromosome amplifications through rare segregation errors. An increase in mutation rate may negate the need for nCIN by increasing the frequency at which resistance-conferring mutations are generated in each cell cycle. Consistent with this view, high mutation rates and nCIN have been found to be mutually exclusive in various cancer contexts (Lengauer, Kinzler et al. 1998).

Wapl depletion is sufficient to suppress nCIN *in vivo*

The data presented here demonstrates that *in vitro,* maintaining nCIN acts as a rate limiting step such that nCIN permits the continued proliferation of drug tolerance cells, while suppression of nCIN limits tumor evolution and allows the sustained response of therapeutic treatment. *In vivo* work completed by Nicole Hermance supports the conclusions of work performed *in vitro*. Cells derived from Wapl-deficient tumors exhibit fewer anaphase defects and display less intra-tumor

chromosome numerical heterogeneity. Together these data indicate that suppression of nCIN in NSCLC cells is not in itself sufficient to limit tumor initiation and growth *in vivo*.

nCIN is a driver of drug resistance *in vivo*

To test the role of nCIN in tumor relapse *in vivo*, it was found that in tumors expressing the inducible PC9 shWapl hairpin, both the induced cohort and the uninduced cohort exhibited a similar initial response in tumor recession to the TKI inhibitor Gefitinib. Continued long term exposure to TKI treatment showed that mock-depleted tumors had a greater propensity to relapse as compared to Wapl depleted tumors. Relapsed tumors in the induced cohort also we found to be re-expressing Wapl. Onset of relapse was also delayed in Wapl depleted tumors compared to those without Wapl depletion. When looking at the frequency of the resistance-conferring EGFR T790M mutation in relapsed tumors from both cohorts, the appearance of this mutation was similar between both cohorts. Interestingly, anaphase defects were similarly prevalent in all relapsed tumors, regardless of Wapl status within the cohorts, indicating a selective pressure to maintain nCIN during acquisition of drug resistance.

nCIN as a therapeutic target

Work from several groups has shown that presence of nCIN can promote acquisition of drug resistance and is a mechanism to evade oncogene addiction (Sotillo, Schvartzman et al. 2010, Salgueiro, Buccitelli et al. 2020). Our data additionally show that suppression of segregation errors in a cancer context is achievable and that reduction of nCIN can limit cell proliferation and impact mechanisms of acquired drug resistance. Indeed, due to its role in regulation of chromosome segregation, Aurora B is a provocative drug target, and its inhibition has recently been shown to be efficacious in limiting proliferation of TKI-resistant NSCLC cells (Bertran-

Alamillo, Cattan et al. 2019). Highly aneuploid cells, such as those that have experienced a whole genome doubling event, often have correspondingly high degree of nCIN. Consistent with data presented here showing that high Aurora B activity can drive nCIN in a small panel of NSCLC cells, recent work has shown that Aurora B expression is among the most highly expressed genes in highly aneuploid cancer cells (Quinton, DiDomizio et al. 2021). Together these data propose that pathways that promote nCIN may serve as valuable drug targets, alone, or as co-therapies to enhance or prolong response to targeted therapeutic approaches.

Chapter 4 Conclusions and future directions

Loss of RB leads to genomic instability

The RB1 gene is the first characterized human tumor suppressor, identified over 30 years ago. The first cellular function of pRB to be described and to date the most thoroughly studied, is its role as a negative regulator of the cell cycle. Almost all human cancers have lost or functionally inactivated RB (Burkhart and Sage 2008, Mandigo, Tomlins et al. 2022). Since the initial discovery of RB's tumor suppressive function, it has been shown that outside of cell cycle regulation, loss of RB has profound effects on many more cellular processes. Involvement in these pathways, which include differentiation, senescence, cell death, and genome stability, may represent additional tumor suppressive functions of RB. The complexity of understanding RB's role in tumor initiation and progression is further highlighted by the fact that there are over 300 proteins in the RB interactome and as a field we have just begun to understand the consequences when these interactions are disrupted. In chapter 2 I sought to determine how loss of RB compromises genome stability during mitosis.

RB deficient cells are subject to increased mitotic DNA damage following caspase activation

While loss of RB is known to compromise the G_1/S checkpoint, leading to high levels of replication-dependent DNA damage and compromised homologous recombination based DNA damage repair, (Marshall, Roes et al. 2019), I now show that the G2/M checkpoint is sufficient to restrain mitotic entry while damage is repaired, regardless of RB status. Work shown here and in previous studies indicate that loss of RB leads to a higher incidence of mitotic cells with DNA damage (Figure 2.2). My data demonstrate that this damage is further exacerbated when the cells are

arrested in mitosis. While control cells also accumulate damage if mitosis is unnaturally extended, RB depleted cells do so at a faster rate, accumulating more damage at earlier time points (Figure 2.2B). Accumulation of mitotic DNA damage in normal human cells had been previously described by the Mitchison Lab who proposed that the DNA damage results from partial activation of caspase activated DNase (CAD). My data is consistent with this model (Figure 2.2C and D). Nevertheless, a mechanistic understanding of how a partial apoptosis is initiated remains unknown. One theory is that cessation of transcription during mitosis combined with continued protein turn over as a result of the prolonged mitotic arrest allow for anti-apoptotic factors to decrease to an extent that they are no longer able to block apoptotic factors (Colin, Hain et al. 2015, Di Cesare, Verrico et al. 2017, Wang and Poon 2022). Another possible mechanism is that cleavage of Cap-H, a condensin I subunit disrupts chromatin compaction, leaving the decondensed chromatin now accessible to the activity of CAD (Lai, Wong et al. 2011). Consistent with this second model, mitotic RB deficient cells exhibit defects in chromatin cohesion and condensation, suggesting that this deregulation of chromatin structure may leave cells sensitive to CAD activity.

Deregulation of heterochromatin upon loss of RB promotes DNA damage

The regulation of chromatin compaction and cohesin during mitosis is important to ensure mitotic fidelity. Compaction of mitotic chromatin protects against nucleases that can cause DNA damage (as seen in Figure 2.2) and establishment of cohesin is needed to provide the physical rigidity that chromosomes need in order to withstand the pulling forces of spindle microtubules. Previous studies have implicated RB in the regulation of chromatin compaction and cohesin. Loss of RB compromises the chromatin association of both the Condensin II and the cohesin protein complexes (Manning, Longworth et al. 2010, Manning, Yazinski et al. 2014). An increase in DNA damage at the telomeres was also seen. Our data indicate that the DNA damage at centromeres

results from defects in chromatin cohesin and compaction. This model is supported by rescue experiments showing DNA damage levels can be moderated by increasing cohesin or compaction (through the depletion of Wapl or MCPH1 respectively) (Figure 2.5).

Increased centromere transcription following loss of RB leads to increased centromere localization of Aurora B

RB is lost or functionally inactivated in nearly all human cancers, and homozygous deletion, or inactivation of the RB1 gene is prevalent in osteosarcomas, breast cancers, small cell, and non small cell lung cancers (Viatour and Sage 2011). Overexpression of Aurora B kinase is also a common occurrence in numerous cancer types, including non small cell lung cancer (Tatsuka, Katayama et al. 1998, Chieffi 2018). A consensus behind the mechanism driving the overexpression of Aurora B in cancer cells has yet to be reached (Smith, Bowers et al. 2005).

When cells enter mitosis transcription factors and RNA polymerases, including RNA polymerase II, are evicted from chromatin. Thus mitosis has historically been considered transcriptionally silent (Gottesfeld and Forbes 1997). Recently it was discovered that there is active transcription occurring at the centromere during mitosis. This is important because transcription is needed to recruit Sgo1 at the centromere, maintain centromeric cohesin, recruit the CPC to the centromere, and regulate the activity of Aurora B (Liu, Qu et al. 2015, Blower 2016, Perea-Resa, Bury et al. 2020, Chen, Zhang et al. 2021, Chen, Zhang et al. 2022). A simple explanation could just be the rate at which Aurora B is transcribed in these cells, as it is already known that cancer cells have increased rates of transcription and translation (Laham-Karam, Pinto et al. 2020). Supporting this conclusion studies have shown that centromeric transcription regulates Aurora B localization to the inner centromere and its activation (Blower 2016). I also show that experimental depletion of RB leads to increased transcription and significantly higher levels of centromeric Aurora B (Figure

2.6). As in the Blower study I show that transcription is important for the localization of Aurora B as inhibition of transcription using α-amanitin decreased the amount of centromere localized Aurora B (Figure 2.7).

Increased Aurora B at the centromere perturbs proper chromosome segregation

We show that non small cell lung cancers (NSCLC) are highly nCIN as indicated by the prevalence of segregation errors and a high degree of numerical heterogeneity (Figure 3.1A and B). nCIN is driven by disruptions in microtubule dynamics with key regulators normally being mis-regulated. In agreement with published data, we find that in our panel of NSCLC line show that Aurora B is highly overexpressed when compared to normal lung cells (Figure 3.2A) and that these cells have increased Aurora B at the centromere (Figure 3.2B).

We show that increased Aurora B at the centromere creates unstable microtubule attachments. These unstable microtubule attachments can be reduced by redistributing Aurora B to chromosome arms as was shown with increasing cohesion (Wapl KD) or through the addition of Taxol (Figure 3.6A and C). Stabilization of microtubule dynamics subsequently reduces the high rate of chromosome mis-segregation events (Figure 3.6B). In conclusion this work leverages our understanding of the molecular changes in RB deficient cells to design therapeutic strategies to suppress nCIN. We believe that increased Aurora B at centromeres is driving the destabilization of microtubule dynamics leading to increased segregation defects and drug tolerance. Through increasing cohesin or inhibiting transcription we were able to decrease Aurora B at centromeres. The increase in cohesin and resulting reduction of Aurora B at centromeres, or alternatively chemical inhibition of Aurora B activity, stabilizes microtubule dynamics and leads to a reduction in segregation defects. Independently stabilization of microtubules using the microtubule-binding drug Taxol similarly suppressed segregation defects in these cancer cell lines, suggesting the

suppression of nCIN is tied to regulation of microtubule dynamics and not an independent function of Aurora B.

Future Directions

Through experiments presented here we now have a greater understanding of the mechanisms contributing to genomic instability following RB loss. We show that deregulation of centromere structure following RB loss results in transcriptional changes, DNA damage, perturbation of kinetochore composition, and compromised mitotic fidelity. More data is needed to better understand how these independent defects are functionally linked.

Data published in chapter 2 shows that deregulation of the centromere following loss of RB leads to increased DNA damage during mitosis, increased centromeric transcription, and elevated levels of centromeric Aurora B. To better understand the consequences of increased transcription and Aurora B levels at centromeres, assays to assess microtubule dynamics and monitor mitotic chromosome segregation errors following inhibition of transcription are needed. The molecular mechanisms of RB loss were investigated in non small cell lung cancers in chapter 3. In Non small cell lung cancer cells, the RB gene remains intact, however the RB protein is hyperphosphorylated. Therefore, to confirm findings from chapter 2 are consistent with mechanism that contribute to nCIN in cancer cells that are RB null, the experiments presented in chapter 2 should also be done in Saos-2 cells (RB null), or other similar cancer cell contexts.

I show that loss of RB leads to DNA damage at the centromere, but the mechanism behind the DNA damage has yet to be shown. R-loops, nucleic acid structures composed of an RNA-DNA

hybrid and a displaced single stranded DNA, occur during transcription and can promote genomic instability (Aguilera 2002). During mitosis the DNA damage response element ATR is recruited to centromeres through Aurora A-regulated association with CENP-F. As ATR is activated at centromeres it then phosphorylates and activates Aurora B (Kabeche, Nguyen et al. 2018). I show that RB loss leads to increased levels of Aurora B at the centromere. I propose that this increase in Aurora B is linked to an accumulation of R loops following increased transcription rates with loss of RB. To test this possibility, the amount of R loops formed in cells with and without RB must be determined. To next link ATR activity to the increased levels of Aurora B seen at centromeres of RB depleted cells, ATR inhibitors could be used and changes in Aurora B localization, microtubule dynamics, and mitotic fidelity monitored. Although ATR has been established as an essential protein, several studies have demonstrated that the inhibition of the kinase activity of ATR in tumor cells may increase the sensitivity of these cells to genotoxic agents (Karnitz and Zou 2015, Weber and Ryan 2015). Decreased sensitivity to therapeutic agents in cancer cells is an ongoing problem and therefore targeting R-loops may present some therapeutic potential.

Chapter 5 Materials and Methods

Cell culture

PC9, H1299, A549, and HBEC3-KT (ATCC) cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium. hTERT immortalized RPE-1 (RPE-1) cells were grown in Dulbecco's Modified Essential Medium (DMEM). RPE-1 cells stably expressing H2B-RFP and 53BP1-GFP were provided by Neil Ganem of Boston University Medical School and were cultured as the parental RPE-1 cells were. All cell culture medium was supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were maintained at 37°C and 5% CO2. High resolution immunofluorescence imaging with DNA stain (DAPI) was used to monitor and confirm cell lines are free of *Mycoplasma* contamination.

Metaphase arrest was induced using nocodazole at a final concentration of 100ng/ml for 4 hours unless otherwise indicated. DNA damage was induced using the specified concentrations of Doxorubicin for 12 hours. In EU-labeling experiments cells were co-cultured with nocodazole and 5-EU substate at a final concentration of 0.25μM for 4 hours. Inhibition of RNA polymerase transcription was completed using a final concentration of 50ng/ml α-amanitin for the duration of the metaphase arrest. Aurora B inhibition was performed by adding Hesperadin for 1 hour at a final concentration of 10nM. Microtubule stabilization was achieved through the addition of Paclitaxel for 1 hour at a final concentration of 5nM.

Protein expression and depletion

Short term knock down of RB1, Wapl, and MCPH1 was achieved through transient transfection of 50nM pool siRNAs (a pool of 4 siRNAs to Wapl and RB1; Dharmacon-ON-TARGETplus Human

Wapl siRNA-SMARTpool) using RNAiMax transfection reagent (ThermoFisher) according to the manufacturer's instructions. Transfection with a SMARTpool of four non-targeting siRNA sequences was used as a negative control for all depletions. Alternatively, cells were infected with a lentiviral construct containing a shRNA hairpin for constitutive (pLK0.1-Puro) depletion or a doxycycline inducible shRNA hairpin (tet-pLK0-Puro) for the depletion of Wapl and RB1. Stable hairpin-expressing clones were selected with Puromycin for 7-10 days. Induced depletion was achieved by the addition of 2μg/ml doxycycline for a minimum of 48 hours. Mock controls are completed by the absence of doxycycline in the given cell line being used. Sequences for all siRNA and shRNA constructs represented can be found in table 5.1.

Table 5.1: Genes and target sequences used for si-and shRNA

Gene Name	Target Sequence
siRB #1	GAACAGGAGUGCACGGAUA
siRB #2	GGUUCAACUACGCGUGUAA
siRB #3	CAUUAAUGGUUCACCUCGA
siRB #4	CACCCAGCAGUUCGAUAU
siWapl #1	GGAGUAUAGUGCUCGGAAU
siWapl #2	GAGAGAUGUUUACGAGUUU
siWapl #3	CAAACAGUGAAUCGAGUAA
siWapl #4	CCAAAGAUACACGGGAUUA
siMCPH1	CUCUCUGUGUGAAGCACCAUU
shWapl #1	GAATGATTCCAATCGTAAATA
shWapl #2	AGCTACTTGATAGCATATAAA
Inducible-shWapl	TTCGTCATGCATTCGGCATTT
Inducible-shRB	AGCAGTTCGATATCTACTGAAA

Confirmation of protein expression and depletion

For quantification of mRNA expression levels of Wapl, Aurora B, Slug, Vimentin, K111, K222, and P82H and/or expression in cell lines and drug tolerant clones, RNA was extracted from cell lines using Trizol reagent according to the manufacturer's instructions. cDNA synthesized from 2μg of total RNA and gene expression determined using the ΔΔ cycle threshold method normalized to either *GAPDH* or *b-actin.* Sequences for qPCR primers can be found in table 5.2.

Table 5.2: target genes and primer sequenced used for qPCR	
Gene Name	**Sequence**
Aurora B Forward	CAG TGG GAC ACC CGA CAT C
Aurora B Reverse	GTA CAC GTT TCC AAA CTT GCC
Beta Actin Forward	ACA CCT TCT ACA ATG AGC
Beta Actin Reverse	ACG TCA CAC TTC ATG ARG
GAPDH Forward	CTA GCT GGC CCG ATT TCT CC
GAPDH Reverse	CGC CCA ATA CGA CCA AAT CAG A
K111 Forward	AAG AGC ACC AGG ATG CTT AAT GCC
K111 Reverse	AGT GAC ATC CCG CTT ACC ATG TGA
K222 Forward	TGT ATT GTG GTA ACT GGG TAT ATG T
K222 Reverse	GTG ACC ACC CGT CTG TCG
P82H Forward	ATG TTT GCA TTC AAC TCA CAG AG
P82H Reverse	CAA CAC AGT CCA AAT ATC CAG TTG
Slug Forward	CGA ACT GGA CAC ACA TAC AGT G
Slug Reverse	CTG AGG ATC TCT GGT TGT GGT
Vimentin Forward	AGT CCA CTG AGT ACC GGA GAC
Vimentin Reverse	CAT TTC ACG CAT CTG GCG TTC
WAPL Forward	TGG TTT GAA ATT GGG CCT GTT C
WAPL Reverse	AAC GGA CTA CCC TTA GCA CAA

To assess protein levels, whole cell extracts were prepared using 2x Laemmli buffer with b-Mercaptoethanol, run on an SDS-PAGE gel, and transferred to PVDF membrane. Membranes were blocked in 1xTBST/ 5% milk and incubated in antibodies diluted in 1xTBST/5% milk at 4°C overnight. Membranes were incubated for 1 hour in corresponding secondary antibody, and signal detected using ProSignal Pico.

Immunofluorescence and Fluorescence *in situ* hybridization (FISH)

Cultured cells were grown on coverslips, fixed, and stained for Aurora B and ACA as previously described (Kleyman, Kabeche et al. 2014, Herlihy, Hahn et al. 2021). Cells were fixed with 3.5% paraformaldehyde for 15 minutes, washed with PBS, and quenched twice with 500mM ammonium chloride in PBS for 10 minutes. Subsequently cells were then washed with TBS-BSA + 0.5%

TritionX-100 for 5 minutes, washed with PBS, then permeabilized with Methanol at -20°C for 5 minutes prior to blocking with TBS +5% BSA. Primary and secondary antibodies were diluted in TBS-BSA + 0.1% TritionX-100 or TBS-BSA + 0.1% TritionX-100 + 0.2 mg/ml DAPI. CENP-A stained coverslips were fixed in Methanol -20°C for 10 minutes and blocked with TBS + 5% BSA. Primary and secondary antibodies were diluted in TBS +5% BSA or TBS + 5% BSA + 0.2mg/ml DAPI. pCENPA stained coverslips were fixed with 2% paraformaldehyde for 10 minutes, extracted with TBS + 5% BSA + 0.5% TX-100 for 5 minutes, and blocked with TBS + 5% BSA. Primary and Secondary antibodies were diluted in TBS +5% BSA or TBS + 5% BSA + 0.2mg/ml DAPI. Interphase and nocodazole arrested prometaphase cells stained to monitor DNA damage was done using γH2AX Ser139. Cells were fixed with 4% paraformaldehyde for 20 minutes, extracted with 0.2% TritionX-100 in PBS for 10 minutes, and blocked with TBS + 5% BSA. Primary and secondary antibodies were diluted in TBS +5% BSA or TBS + 5% BSA + 0.2mg/ml DAPI. Coverslips were then mounted onto slides using Prolong Antifade Gold. A list of antibodies used throughout the experiments performed can be found in table 5.3.

Table 5.3: Antibodies used for experiments conducted in chapter 2 and 3

Name	Company	Catalog Number
ACA	Antibodies Inc	15-234
Alpha-tubulin	Santa Cruz	sc-32293
Aurora B	BD Biosciences	611083
CENPA	Enzo Life Sciences	ADI-KAM-CC006-E
CenpA S7p	Upstate	07-232
EGFR (phospho)	Cell Signaling	3777S
EGFR (total)	Cell Signaling	3777S
ERK (phospho)	Cell Signaling	9101S
ERK (total)	Cell Signaling	9102L
γH2AX (ser139)	Cell Signaling	2577
Histone H3	Abcam	176842
MET (phospho)	Epitomics	2319-1
MET (total)	Cell Signaling	3127
Wapl	Bethyl	A300-268A

For visualization of EU-labeled RNA the Click-iT RNA imaging kit was used following the manufacturer's specifications. Alexa Flour 488 was utilized in the Click-iT reaction performed on fixed cells that had previously been incubated with or without 0.25mM EU.

To assess kinetochore microtubule stability cells were incubated in ice cold RPMI for 5 minutes (PC9 and A549) or 10 minutes (H1299) prior to fixation in ice cold methanol. Primary and antibodies were diluted in TBS + 5% BSA and TBS + 5% BSA + 0.2mg/ml DAPI, respectively.

Metaphase spreads were prepared as in (). Mitotic cells were collected via shake-off after treatment with 100ng/ml nocodazole for 3 hours and then incubated in 75mM KCl for 15 minutes at 37°C. Cells were then cytospun onto slides at 1800 RPM for 10 minutes, incubated in KCM buffer 1(0mM Tris pH 8.0; 120mM KCl; 20mM NaCl; 0.5mM EDTA; 0.1% Triton X-100) for 10 minutes before adding antibody. Primary and secondary antibodies were diluted in KCM + 1% BSA. Cells were then post fixed with 4% paraformaldehyde for 10 minutes prior to counter staining with TBS + 5% BSA + 0.2mg/ml DAPI.

FISH with centromeric probes and quantification of numerical heterogeneity was performed as previously described in (Manning, Benes et al. 2014) using α satellite-specific probes for chromosomes 2,6,7,8 and 10. Clonal Numerical Heterogeneity (NH) of a population was determined by scoring >300 cells per clone/tumor for chromosome probe copy number to determine the modal copy number and the fraction of cells deviating from that number (i.e., the numerical heterogeneity). A given copy number with greater than 20% prevalence in a population

was considered to be a stable subclone and not included in the numerical heterogeneity score. Data is represented for individual chromosomes, or alternatively, as average NH across all chromosomes measured for a given sample.

Fixed cell images were captured using a Zyla sCMOS camera mounted on a Nikon Ti-E microscope, with a 60X Plan Apo oil immersion objective and 0.3 μm z-stacks. To assess centromeric protein levels, NIS-elements Advanced Research software was used to perform line scans in a single focal plane through individual ACA-stained kinetochore pairs where the area under the curve indicates the region of centromere/kinetochore-localized staining. Kinetochore microtubule intensity was assessed by measuring the intensity across a line positioned parallel to metaphase-aligned chromatin. γH2AX levels were assessed by counting the number of foci per cell or gating on DAPI to determine the sum intensity of the γH2AX labeled channel. Global transcription levels were determined by determining the sum intensity for the EU-labeled channel. For intensity measurements a minimum of 3 kinetochore pairs per 30 cells, per condition (90 kinetochore pairs/condition) were measured in each of 3 biological replicates. DNA damage, global transcription, anaphase defects and kinetochore microtubule intensity were assessed in a minimum of 30 cells per condition, in each of 3 biological replicates. For figure generation, images were prepared using NIS Elements deconvolution software and represented as projections of 5 central plains. Insets represent a single focal plane.

Live Cell Imaging

RPE-1 cells stably expressing H2B-RFP and 53BP1 GFP were seeded into a 12-well plastic bottom plate and imaged according to Mercadante et al (Mercadante, Crowley et al. 2019). In summery the plate containing the RPE-1 fluorescently label cells were imaged as a single z-stack

(pixel size of 0.67 μm at 20x), in an environmental chamber maintained at 37 °C and 5% humidified CO_2. NIS elements acquisition software with HCA jobs was used to capture multi-well, multi-coordinate images. Images were captured every 5 minutes for up to 48 hours. Analysis was performed on at least 50 mitotic cells. Resolution of 53BP1-GFP foci was determined by identifying nuclear envelope breakdown and determining the time prior to this point that the first 53BP1-GFP foci appears. Percentage of cells with DNA damage in G2 was quantified by determining out of the total number of cells scored, how many had 53BP1-GFP foci present in G2.

Growth and survival analyses

Proliferation rates of parental cell lines and/or cells stably expressing a doxycycline inducible construct to target Wapl for depletion were plated following presence or absence of 2 □g/ml doxycycline for 2 days (acute depletion) or for 14 weeks (sustained depletion) in the presence or absence of 1nM Taxol, as indicated. Following depletion of Wapl, cells were collected and counted for 5 consecutive days and growth rates calculated. Anchorage-free proliferation assays were performed with or without constitutive shRNA-depletion of Wapl. 4 x 10^4 cells were mixed with 0.4% agarose in growth medium, plated in a 6 well dish containing a solidified layer of 0.5% agarose in growth medium and placed at 4° for 15 minutes to allow solidification. Cells were fed once a week with 2% agarose in growth medium. Colonies were imaged and counted at 4 weeks. For *in vitro* drug tolerance assays 1x10^4 PC9 shWapl cells were plated in a 10 cm dish with or without 2μg/ml doxycycline. Cells were treated with 1μM Gefitinib (Selleckchem) and medium replaced twice per week. At 4 weeks cells were washed in 1xPBS and fixed and stained with 0.5% (w/v) crystal violet (acros organics, #548-62-9) in 25% methanol (v/v) (Sigma, #67-56-1). To quantify the relative number of drug tolerant cells, crystal violet was resuspended in 10% (v/v) glacial acetic acid and absorbance measured. To determine tolerance to Vincristine or Taxol cells

were first subjected to 48h of doxycycline-induced Wapl depletion then exposed to repetitive cycles of Vincristine or Taxol treatment for 48h, followed by recovery in drug-free medium for 48h, where drug concentration was doubled every cycle. Concentrations ranged from 1nM to 3nM (A549) or 12nM (PC9 and H1299) of Taxol, and 4nM to 8nM (H1299) or 32nM (PC9 and A549) of Vincristine. Cells were maintained sub-confluent and split, as needed, during recovery days. Following final drug concentration cells were stained and analyzed as described above.

Statistical analyses

Experimental data were analyzed with a Student t-test unless indicated otherwise. Individual measurements from experiments where multiple measurements were made per replicate are represented as superplots, with per-replicate averages and standard deviation between three biological replicates superimposed. All error bars represent standard deviation between biological replicates and statistically significant differences are labeled with *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

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