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Dissecting The Functions Of Atr In Replication Fork Stability

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Dissecting The Functions Of Atr In Replication Fork Stability

Abstract

Genome maintenance is required for cellular viability, and failure to preserve genomic integrity is associated with an increased risk of diseases, such as cancer. To ensure genomic stability, cells have checkpoints that control cell cycle progression in the event of DNA damage or incomplete DNA replication. The DNA replication checkpoint is regulated by the ATR-CHK1 pathway that stabilizes stalled replication forks and prevents their collapse into DNA double-strand breaks (DSBs). Two distinct models have been proposed to explain how ATR stabilizes stalled forks: 1) through local modulation of fork remodelers, such as SMARCAL1 inhibition, and 2) through inhibition of CDK-dependent pathways, such as inhibition of the AURKA-PLK1 pathway, which prevent cell cycle progression. However, it remains unclear which stabilization function is essential for fork stability and whether specific sites in the genome depend on one function over the other.

In an effort to test if an essential part of fork stabilization is mediated through inhibiting CDK-dependent pathways, such as inhibiting premature activation of the AURKA-PLK1 pathway, we established a system to hyper-activate the AURKA-PLK1 pathway to determine if it is sufficient to cause fork collapse. We found that fork collapse was not achievable solely through Aurora A overexpression nor with overexpression of its co-activators, TPX2 and BORA, but rather that CDK1 activation was also required. To test if CDK1-activation is sufficient to promote fork collapse, we inhibited WEE1, which short-circuits the cell cycle checkpoint function of ATR without inhibiting its fork-proximal activity. Using flow cytometry based fork collapse assays and genome-wide detection of RPA accumulation using RPA ChIP-Seq, we show that WEE1 and ATR inhibition cause similar levels of fork collapse at overlapping genomic locations in a CDK1-depdendent manner under conditions of partial replication inhibition (low dose aphidicolin). Notably, treatment with WEE1 inhibitor (WEE1i) alone was also sufficient to cause replication fork collapse, and did so more rapidly and to a higher degree than treatment with ATR inhibitor (ATRi) alone. Interestingly, clear differences in site specificity were observed when WEE1i was combined with ATRi, suggesting that particular sites in the genome may be slightly more dependent on the local functions of ATR than others. Thus, cell cycle checkpoint abrogation by WEE1i is sufficient to cause replication fork collapse in a manner similar to ATRi; however, site-specific roles for ATR remain. Together our findings indicate that the cell cycle checkpoint of ATR is key in stabilizing replication forks at a majority of sites in the genome. These findings could be leveraged to develop cancer treatments that exploit combinations of oncogenic genomic breakage signatures with that of WEE1 or ATR inhibitors.

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DEDICATION PAGE

This doctoral thesis is dedicated to my family.

To my dad who has inspired me to work hard, think smart, be prepared and have fun while doing it. Your courage, strength and dedication is something I admire every day. Thank you for your guidance.

To my mom whose love, thoughtfulness and boundless care has always lifted me through hard times. Your independence, curiosity and love for life have served as a model after which I mold my life. Merci pour tous les mots encourageants et les mille caresses.

To my sister for all those times we laughed so hard that our legs gave out. And of course, for being the person I enjoy being totally weird with! You always know how to make me smile. Thank you for your support.

To my friends without whom I could not have gotten through the past 6 years. Your love, kindness and support gave me the strength to stand back up every time I was knocked down. ~ Friends are the family you choose… welcome to the family! ~

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ABSTRACT

DISSECTING THE FUNCTIONS OF ATR IN REPLICATION FORK STABILITY

Theonie Anastassiadis

Eric J. Brown

Genome maintenance is required for cellular viability, and failure to preserve genomic integrity is associated with an increased risk of diseases, such as cancer. To ensure genomic stability, cells have checkpoints that control cell cycle progression in the event of DNA damage or incomplete DNA replication. The DNA replication checkpoint is regulated by the ATR-CHK1 pathway that stabilizes stalled replication forks and prevents their collapse into DNA double-strand breaks (DSBs). Two distinct models have been proposed to explain how ATR stabilizes stalled forks: 1) through local modulation of fork remodelers, such as SMARCAL1 inhibition, and 2) through inhibition of CDK-dependent pathways, such as inhibition of the AURKA-PLK1 pathway, which prevent cell cycle progression. However, it remains unclear which stabilization function is essential for fork stability and whether specific sites in the genome depend on one function over the other.

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fork dynamics

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Introduction

The survival of an organism depends on its ability to faithfully and efficiently transmit genetic material from mother to daughter cell. During each cell cycle, human cells must accurately replicate over three billion base pairs of DNA in a short period of time and protect the genome from spontaneous or induced DNA damage that could result in alterations or loss of genetic information, ultimately contributing to cancer or age-related pathologies. Indeed, DNA is constantly under assault and can be damaged in a multitude of ways, including from replication-induced mismatches, chemically-induced crosslinks and adducts, base damage from ultraviolet light, and single- and double-strand breaks resulting from ionizing radiation or chemical reactions (Ciccia and Elledge, 2010). These DNA lesions affect the stability of the genome as well as impact its ability to be accurately duplicated. In response to these lesions, cells have evolved protective pathways that detect and repair damaged DNA, and pause cell cycle progression to give cells time to resolve the problematic lesion (Blackford and Jackson, 2017). Defects in these pathways lead to the accumulation of DNA damage over time, giving rise to an increased mutation burden that often results in age-related pathologies, such as cancer (Tubbs and Nussenzweig, 2017). Understanding the essential mechanisms at play during replication fork stabilization, collapse and repair in the context of the genome, of the proteome and of the molecular signaling cascades, will allow us to develop therapies for diseases induced by defects in replication and the checkpoint response, such as cancer, Seckel syndrome and Fanconi anemia (Zeman and Cimprich, 2014a).

DNA Replication

DNA replication is the process by which a doubled stranded DNA (dsDNA) molecule is duplicated to produce an identical copy. This process is divided into three phases: initiation, elongation and termination. Initiation occurs when the replicative DNA helicase unwinds the origin of replication; during elongation the replication fork copies the chromosome using semi-conservative DNA synthesis; and finally, termination results when converging replication forks converge (Siddiqui, On and Diffley, 2013). Specific regulation of the replication phases is crucial to assure accurate duplication of the genome to prevent loss or amplification of genetic information. Indeed, replication initiation is tightly controlled to ensure that the genome is only replicated once per cell cycle (Diffley, 1996; Blow and Dutta, 2005). In this section, we will elaborate on the sequence of events and factors involved in replication regulation.

Given that DNA replication must occur in approximately 6-12 hours in the majority of mammalian cells and that replicative polymerases have a processivity of approximately 1kb/min, origins spaced typically 50-150kb apart from one another must be used to complete replication over the entire genome. These origins of replication are mapped in bacteria and in yeast; however, mammalian origins of replication are not as easily identified (Prioleau and MacAlpine, 2016). Scientists have sought to identify the location of these origins for decades but it was not until recently that identification of origin locations grew from a few dozen to over 100,000 through the use of genome-wide technologies (Cadoret *et al.*, 2008; Sequeira-Mendes *et al.*, 2009; Mesner *et al.*, 2011; Besnard *et al.*, 2012). Identifying origins has allowed the field to increase its understanding of the genetic, epigenetic and proteomic features that characterize what defines an origin and what regulates their distribution and usage during replication.

Though these findings are still in their infancy, many advances have been made in our understanding of how the genetic sequence, chromatin milieu and nuclear architecture influence the location, timing and activation of origins.

Initiation of DNA replication is comprised of two major steps: origin licensing and origin firing. Origins are licensed in late mitosis and early G1 phases by loading the pre-replicative complex (pre-RC) onto chromatin (Bell and Stillman, 1992; Remus *et al.*, 2009; Riera, Tognetti and Speck, 2014). The pre-RC complex is comprised of the origin recognition complex (ORC) and the double hexameric minichromosome maintenance 2-7 (MCM2-7) helicase complex, that is recruited by CDC6 and the licensing factor CTD1 (Cvetic and Walter, 2006; Randell *et al.*, 2006; You and Masai, 2008). A greater number of origins are licensed than fired (Woodward *et al.*, 2006). The unfired origins are termed dormant origins and are only used to complete replication when the progression of a nearby replication fork is compromised as a result of fork slowing, stalling or collapse (Ge and Blow, 2010).

Origin firing occurs when the MCM2-7 helicases are activated in S phase to begin the elongation phase of replication. These events are controlled through a cell cycle mediated kinase signaling cascade, primarily regulated by DBF4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) (Heller *et al.*, 2011; Tanaka and Araki, 2013). CDK- and DDK-mediated recruitment of CDC45 and the GINS complex form the active CMG (CDC45-MCM-GINS) helicase that is required for DNA synthesis (Aparicio *et al.*, 2009; Ilves *et al.*, 2010; Fragkos *et al.*, 2015). Other recruited proteins that are key replisome components are Topoisomerase II binding protein 1 (TOPBP1), Treslin, and the DNA polymerases (Kumagai *et al.*, 2010, 2011; Boos *et al.*, 2011; Yeeles *et al.*, 2015). Following helicase unwinding and initiation of DNA synthesis, additional

components of the replisome, such as TIMELESS, TIPIN and Claspin, will be recruited (Gotter, Suppa and Emanuel, 2007; Yoshizawa-Sugata and Masai, 2007; Masai *et al.*, 2010). The ORC complex is removed from chromatin and degraded through ubiquitin-mediated proteolysis to prevent another MCM helicase from being loaded onto the ORC, which would lead to re-replication of that genomic loci (Méndez *et al.*, 2002).

Once origins have fired and DNA synthesis is initiated, replication forks move in opposite directions away from the origin. The helicase unwinds the duplex DNA, separating the parental strands, each of which will be used as a template for DNA synthesis (Masai *et al.*, 2010). Replicative polymerases are proteins that synthesize DNA in a 3' to 5' direction (Loeb and Monnat, 2008). Polymerase alpha initiates strand synthesis, polymerase epsilon synthesizes DNA on the leading strand, and polymerase delta synthesizes small stretches of DNA (Okazaki fragments) in between RNA primers laid by primases on the lagging strand (Lujan, Williams and Kunkel, 2016). These primers are eventually removed and Ligase 1 ligates Okazaki fragments to one another. There are many other factors involved in DNA replication that contribute to faithful genome replication and are adversely affected under conditions of replication stress. In addition, it is hypothesized that the replisome composition is altered or modified under different conditions of replication stress (Yoo *et al.*, 2004; Mailand *et al.*, 2006; Mamely *et al.*, 2006; Peschiaroli *et al.*, 2006; Karlene A. Cimprich and Cortez, 2008; De Piccoli *et al.*, 2012). By using various proteomic techniques such as isolation of proteins on nascent DNA (iPOND)-mass spectrometry (MS), we can better understand the replication dynamics involved in the response to replication stress, as will be discussed in this dissertation.

Finally, replication termination occurs when two converging replication forks meet or when a fork meets a chromatin end. The field has predominantly focused on elucidating the mechanisms involved in replication initiation and elongation, leaving much to still be understood about replication termination. During the process of termination, the following steps occur in most organisms: replication fork convergence, completion of DNA synthesis, replisome disassembly and decatenation (Dewar and Walter, 2017). When replication forks encounter each other, their replisomes are disassembled. Though this process has been shown to be mediated through ubiquitin signaling and to involve the ubiquitin selective protein segregase p97, it remains to be determined if the replisome is actively disassembled or not (Meerang *et al.*, 2011). The mechanism by which DNA synthesis is completed is also under debate. Some groups argue that the single-stranded gap remaining between the leading stand and the last Okazaki fragment is filled by the same mechanism utilized during elongation, whereas other groups argue this process requires replisome disassembly (Dewar and Walter, 2017). Finally, catenated DNA is created by replicating the last turn of the parental duplex DNA, which must be resolved before chromosome segregation. However, the topological stress needs to be managed until chromosome segregation takes place, leaving the field with important questions regarding the factors and pathways that are involved in decatenation and relief of topological stress (Postow *et al.*, 2001). If all phases are successfully executed, the cell will have achieved complete and accurate genome duplication, which it can pass on to the daughter cell.

However, as we will discuss, DNA replication can encounter numerous obstacles, many of which are a result of replication stress. Fortunately, cells have evolved intricate protective mechanisms, namely the DNA replication and DNA damage

checkpoints, that work to prevent these DNA replication perturbations from inducing disease-causing genomic instability.

Replication stress

Replication stress is a problem that cells often face during DNA replication that can have significant consequences on the stability of the genome, and ultimately, on cell survival. DNA replication renders cells particularly susceptible to DNA lesions that can block replication fork progression. Stalled forks can collapse into DSBs or be improperly repaired, leading to a loss of genetic information that gives rise to many diseases, such as cancer (Mcgowan, 2003; Paulsen and Cimprich, 2007). There is no uniform description of replication stress nor unique cellular markers that specifically define this phenomenon. As a result, the definition of replication stress is constantly evolving, though some broad characteristics are agreed upon by the field. Generally, replication stress is defined as the slowing or stalling of replication fork progression (Zeman and Cimprich, 2014a). Though physical structures change depending on the lesion, all replication stress-induced structures share a common feature: a tract of single-stranded DNA (ssDNA). This tract is generated either from a functional uncoupling of the DNA replicative helicase from the replicative polymerase, or from endonucleolytic processing of DNA that resects the DNA strand where a nick or gap was left unrepaired, forming a stretch of ssDNA. Prolonged exposure of ssDNA triggers the activation of the DNA replication checkpoint pathway, the details of which will be discussed in the following section (Byun *et al.*, 2005; MacDougall *et al.*, 2007).

Replication stress has both extracellular origins, such as exogenous DNA damaging agents, and intracellular origins, such as impaired polymerase progression through hard-to-replicate regions of the genome or DNA damaging agents generated

from metabolic and chemical reactions. Most exogenous sources of replication stress arise from chemical mutagens and ultraviolet (UV) light that generate DNA lesions that either block the helicase (e.g. DNA-topoisomerase adducts, intra-strand crosslinks, and bulky DNA adducts), or that block polymerase progression, (e.g. base pair adducts, polymerase inhibitors [such as aphidicolin], and chemicals [such as hydroxyurea] that deplete deoxyribonucleotides [dNTP] pools) (Ciccia and Elledge, 2010). Limiting exposure to these agents when it is possible should be a priority to prevent genomic instability that could drive malignant transformation of cells.

There also exists a plethora of endogenous sources of replication stress that cannot be mitigated by limiting exposure; instead, DNA damage repair pathways have naturally evolved to resolve these inevitable lesions. Ironically, nicks and gaps are naturally-occurring intermediates in many DNA repair pathways and if encountered by replication machinery, will stall the forks, eventually causing them to collapse. Though it remains controversial whether or not nicks and gaps behind the fork can cause fork stalling, nicks and gaps ahead of the replication fork can collapse into DSBs through duplex unwinding. Indeed, a nick or gap on the leading strand will cause the helicase to slide off the DNA, leading to fork collapse, and a nick or gap on the lagging strand could result in fork progression problems that cause fork stalling. Many unrepaired DNA lesions, such as inter-strand crosslinks and protein-DNA crosslinks that can be caused by metabolic pathway products (e.g. reactive aldehydes generated during alcohol metabolism) can also be barriers to replication fork progression (Mirkin and Mirkin, 2007; Dalgaard JZ, 2011).

Another cause of replication stress results from misincorporation of ribonucleotides in DNA (Williams, Lujan and Kunkel, 2016). DNA replicative

polymerases faithfully match base pairs but are less specific at discriminating between dNTPs and ribonucleotides, which they surprisingly often incorporate erroneously (Dalgaard, 2012). This replication defect is recognized and corrected through the ribonucleotide excision repair pathway (Reijns *et al.*, 2012; Sparks *et al.*, 2012). If not removed, the incorporated ribonucleotide will stall the polymerase and the DNA damage tolerance pathway will be required to bypass the lesion (Anglana *et al.*, 2003; Nick McElhinny *et al.*, 2010; Lazzaro *et al.*, 2012).

DNA replication requires many different components to be successfully completed, so when any are limiting, it can lead to polymerase slowing and eventual fork stalling (Poli *et al.*, 2012; Sørensen and Syljuåsen, 2012). Some well characterized insufficiencies are those of nucleotides and replisome factors. Depletion of these factors often result from aberrant regulation of replication initiation leading to an excess of origin firing that overwhelm the existing pools of factors (Beck *et al.*, 2012). It has been well documented that overexpression or constitutive activation of oncogenes, such as RAS, MYC or cyclin E, lead to increased origin firing, resulting in insufficient nucleotide levels or increased collision with transcriptional machinery (Halazonetis, Gorgoulis and Bartek, 2008; Bester *et al.*, 2011; Jones *et al.*, 2012; Burrell *et al.*, 2013).

Because replication and transcription machinery are both active during S phase and share the same substrate, they inevitably interfere with one another (Helmrich *et al.*, 2013). It has been reported that collision with the transcription complexes and RNA-DNA hybrid structures known as R loops causes replication stress (Bermejo, Lai and Foiani, 2012). This is supported by findings that identified "early replicating fragile sites" that are found in highly transcribed genomic regions, which have a high incidence of DSB formation (Barlow *et al.*, 2013). In addition, many studies using various visualization

techniques, including DNA combing and electron microscopy, have established that encounters between the transcription and replication complexes cause replication stress and eventual DNA damage (Paulsen *et al.*, 2009; Wahba *et al.*, 2011; Stirling *et al.*, 2012). DNA damage induced by replication-transcription interference is a consequence of either R loop formation or resulting topological stress (García-Muse and Aguilera, 2016). R loops occur when RNA polymerases pause due to secondary DNA structures or collide with the replication fork (Aguilera and García-Muse, 2012). Topological stress occurs when a replication fork approaches a transcribed region that cannot rotate freely, such as when it is tethered to the nuclear periphery (Bermejo *et al.*, 2011). If the region is not detached from the nuclear pore, accumulation of positive supercoiling in front of the fork will result in fork collapse and fork reversal (Bermejo *et al.,* 2011).

Finally, intrinsically difficult to replicate DNA sequences exist in the genome, such as secondary structure-forming DNA, DNA repeats, and common fragile sites (CFS), that pose a challenge to replication progression and that are associated with increased chromosomal rearrangements. CFS are defined genomic sites that display increased sensitivity to replication stress and, as visualized by metaphase spreads, are prone to breaking under conditions of replication stress (Casper *et al.*, 2002; Glover, Wilson and Arlt, 2017). The only unifying features between the different CFS are their megabase-spanning large size, their late replication timing in S phase, the presence of genes larger than 300kb in size, and their disposition to replication stress-induced breakage (Glover *et al.*, 2005; Debatisse *et al.*, 2012). All of these features have been proposed to contribute to CFS fragility though the reasons for their fragility is still under debate. One hypothesis is that because CFS carry very long genes, which take a long time to transcribe, the DSBs found at these sites result from collision of the replication

and transcription machinery (Helmrich *et al.*, 2013). However, because CFS fragility does not correlate with the expression of these genes in some cell lines and the rate of the replication fork is unperturbed, this hypothesis is unlikely (Le Tallec *et al.*, 2013). Another explanation of CFS fragility is their paucity of replication origins, preventing the ability of stalled forks to be rescued by replication initiation at other origins (Letessier *et al.*, 2011). The fact that CFS replicate in late S phase exacerbates this problem as dormant origins in areas surrounding CFS have a limited amount of time to initiate and complete replication. As these incompletely replicated genomic loci enter mitosis, the resulting aberrant replication intermediates are processed by nucleases, such as Mus81-Eme1 or ERCC1, resulting in DSBs and promoting disease-inducing genomic instability (Naim *et al.*, 2013; Ying *et al.*, 2013). Many deletions and chromosomal rearrangements found in cancers occur at these CFS, supporting their contribution to cancer-causing genome instability (Bignell *et al.*, 2010).

DNA repeats, such as trinucleotide repeats, short and long interspersed nuclear elements (SINEs and LINEs, respectively), and long terminal repeats (LTRs) also perturb DNA replication. A majority of the human genome is comprised of repetitive elements, some of which are remnants of viral sequence insertions during our evolution (Griffiths, 2001; Wildschutte *et al.*, 2016). Indeed, retroelements, such as LINEs and SINEs, can become increasingly transcribed during conditions of replication stress and insert themselves in genomic loci prone to instability, further promoting instability (Gualtieri *et al.*, 2013; Mourier *et al.*, 2014; Zaratiegui, 2017). Additionally, long stretches of repeats promote replication polymerase slippage, which can lead to the expansion or contraction of these repeats, causing gene dysfunction (McMurray, 2010; Kim and Mirkin, 2013). When expanded, several trinucleotide repeats (CGG, GAA, CTG) result in

genetic instability and are associated and contribute to the pathology of diseases such as Fragile X syndrome, Friedreich's ataxia and Myotonic dystrophy (Castel, Cleary and Pearson, 2010; Mirkin and Mirkin, 2014; Jones, Houlden and Tabrizi, 2017).

Finally, secondary structure-forming DNA are known to cause replication stress. Some of these sequences include the aforementioned tandem trinucleotide repeats, which can form hairpins or triplexes that can block fork progression. Recently, our laboratory has discovered additional hard-to-replicate tandem repeats that can cause secondary structures and that were shown to stall or collapse replication forks (Shastri, Tsai *et al.,* under revision). G-quadruplexes are secondary structures that form from GC-rich DNA sequences (Técher *et al.*, 2017). Structural stabilization of G-quadruplexes by chemical means or loss of the helicases required to unwind them increase DSB formation and cause a loss of genetic information at those sites (Bochman, Paeschke and Zakian, 2012; Paeschke *et al.*, 2013).

As discussed, there are many endogenous and exogenous sources of replication stress and more are still being discovered. In addition, levels of replication stress, as well as the source of stress, appear to elicit varying cellular responses as well as different patterns of genomic lesions and instability. Exposure to replication stress will activate the DNA replication checkpoint response to overcome these stresses and protect genomic integrity. Yet much remains to be elucidated when it comes to understanding how the cell copes with these different conditions and in particular which mechanisms are essential under different conditions, as will be discussed in Chapter 3.

DNA Replication Checkpoint

   To ensure genomic stability, cells have evolved detection and repair mechanisms that monitor DNA lesions and coordinate repair with cell-cycle progression (Blackford and Jackson, 2017). Indeed, cell cycle checkpoints control cell cycle progression and allow cells to pause the cell cycle to provide time for DNA damage to be repaired before cell division resumes (Zhou *et al.*, 2000; Kastan and Bartek, 2004a). If the damage cannot be resolved, checkpoint activation can trigger permanent cell cycle arrest (senescence) or cause cells to undergo programmed cell death (apoptosis) to prevent propagation of the mutated cell (Branzei and Foiani, 2009; Sperka, Wang and Rudolph, 2012). Once DNA damage is recognized by the cell, cellular signaling cascades are triggered to promote DNA repair. Different types of DNA damage activate cell cycle checkpoint pathways that regulate distinct repair mechanisms. More specifically, physical DNA lesions, such as DSBs, will trigger the ataxia telangiectasia mutated (ATM) and the DNA-dependent protein kinase (DNA-PK) kinase-regulated DNA damage checkpoint, whereas DNA replication stress will activate the ataxia telangiectasia and Rad3-related protein (ATR)-regulated DNA replication checkpoint (Maréchal and Zou, 2013; Blackford and Jackson, 2017). These signaling cascades will activate many downstream effector proteins as well as other cellular responses to mediate repair, ultimately resolving the DNA damage and resuming cell cycle progression.

   DNA replication checkpoint activation occurs in S phase and is essential to ensure faithful duplication of the genome. As discussed previously, this checkpoint is activated by replication stress, usually triggered by impediments that block DNA replication fork progression and is regulated by ATR, a phophoinositide 3-kinase-related kinase (PIKK). ATR was originally discovered as an essential budding yeast gene

through screens for S and G2 checkpoint defects and screens for hypersensitivity to hydroxyurea and methyl methanesulfonate (Allen *et al.*, 1994; Kato and Ogawa, 1994; Weinert, Kiser and Hartwell, 1994). The human gene was later cloned revealing its PIKK domain, adding it to the PIKK protein family along with ATM and DNA-PK (Bentley *et al.*, 1996; Cimprich *et al.*, 1996). However, unlike ATM and DNA-PK, ATR is essential for embryonic development and crucial for genomic integrity (E. J. Brown & Baltimore, 2003; E J Brown & Baltimore, 2000).

Many types of DNA replication fork obstructions can cause replication stress and activate ATR but they all share a similar resulting DNA structure at the fork, which consists of single-stranded DNA (ssDNA) (MacDougall *et al.*, 2007). The unwinding minichromosome maintenance (MCM) helicase, but not the DNA replicative polymerase, can overcome most DNA lesions leading to their uncoupling. This results in an accumulation of ssDNA between the helicase and the polymerase, which is rapidly coated by the ssDNA-binding protein complex, replication protein A (RPA) (Byun *et al.*, 2005). The tract of ssDNA can further be expanded by exonucleases. In addition to ssDNA tracts being formed from uncoupling of the helicase and polymerase, ssDNA tract can be generated by endonucleolytic processing of other forms of DNA lesions (Raderschall, Golub and Haaf, 1999). The RPA-ssDNA complex then serves as a platform to recruit the ATR-interacting protein (ATRIP), which is required for the interaction of ATR with the stalled replication fork but not sufficient for ATR activation at the fork (Cortez *et al.*, 2001; Zou and Elledge, 2003; Ball, Myers and Cortez, 2005; Kim *et al.*, 2005; Namiki and Zou, 2006).

Two proteins have been shown to be required to stimulate ATR activation, topoisomerase II binding protein 1 (TOPBP1) and Ewing tumour-associated antigen 1

(ETAA1). TOPBP1 is recruited to the to the ATR-ATRIP complex by a ssDNA-double-stranded DNA (dsDNA) junction (Michael *et al.*, 2000; Ellison and Stillman, 2003; Bomgarden *et al.*, 2004; Kumagai *et al.*, 2006; MacDougall *et al.*, 2007; Mordes *et al.*, 2008). This junction is also necessary for the loading of the RAD9-RAD1-HUS1 (9-1-1) checkpoint clamp complex by the RAD17/RFC2-5 clamp loader complex (Bermudez *et al.*, 2003; Delacroix *et al.*, 2007; Lee, Kumagai and Dunphy, 2007; Navadgi-Patil and Burgers, 2009). Once the 9-1-1 clamp is loaded on the ssDNA-dsDNA junction, it recruits TOPBP1 to the ATR-ATRIP-RPA coated ssDNA in conjunction with the MRE11-RAD50-NBS1 (MRN) complex and RAD9-RAD1-HUS1-interacting nuclear orphan (RHINO), although the mechanisms by which the MRN complex and RHINO contribute to TOPBP1 recruitment remain elusive (Cotta-Ramusino *et al.*, 2011; Duursma *et al.*, 2013; Lee and Dunphy, 2013; Lindsey-Boltz *et al.*, 2015). Once TOPBP1 is recruited, it interacts with the ATR-ATRIP complex through its ATR activation domain, which interacts with ATR to activate it. The second, and recently identified, ATR activator is ETAA1, which like TOPBP1 contains an ATR-activation domain but unlike TOPBP1 interacts with the RPA-ssDNA platform through direct binding of RPA (Bass *et al.*, 2016; Feng *et al.*, 2016; Haahr *et al.*, 2016). It remains to be determined if these activators recognize different ssDNA structures that require distinct repair signaling pathways to be resolved, possibly dictated through a specificity in downstream substrates of ATR. Once activated, ATR can phosphorylate its multitude of effector proteins to trigger signaling cascades that promote DNA repair by modulating many cellular processes and by coordinating cell cycle, DNA repair and DNA replication.

The functions of ATR in DNA replication and repair

Once activated, ATR stimulates multiple signaling cascades that protect genomic integrity by halting cell cycle progression, limiting firing of novel origins of replication, stabilizing the replication fork, and promoting replication fork repair and restart (Saldivar, Cortez and Cimprich, 2017).

ATR prevents cell cycle progression

By coordinating cell cycle arrest with replication, ATR prevents cells from entering prematurely into mitosis before replication defects are resolved and before replication is complete. ATR exerts its checkpoint function on the cell cycle primarily through its most prominent and well-characterized downstream effector, the checkpoint kinase 1 (CHK1) (Guo *et al.*, 2000; Hekmat-Nejad *et al.*, 2000; Liu *et al.*, 2000; Zhao and Piwnica-Worms, 2001). ATR activates CHK1 by phosphorylating its serine 317 and 345 sites. The interaction between ATR and CHK1 is facilitated by Claspin, an adaptor protein localized at the fork (Kumagai and Dunphy, 2000; Liu *et al.*, 2000; Zhao and Piwnica-Worms, 2001; Liu, Song and Zou, 2012). It has also been suggested that the timeless (TIM) and timeless-interacting protein (Tipin) complex might be involved in ATR activation of CHK1 through its recruitment to stalled forks. Indeed, TIM and Tipin loss lead to a decrease in the ability of ATR to activate CHK1 (Chou and Elledge, 2006; Errico, Costanzo and Hunt, 2007; Unsal-Kaçmaz *et al.*, 2007; Yoshizawa-Sugata and Masai, 2007; Smith, Fu and Brown, 2009). Once phosphorylated at the fork, CHK1 is released from chromatin to carry out its DNA damage response signaling to the rest of the nucleus.

CHK1 controls cell cycle progression by phosphorylating the three CDC25 phosphatases, ultimately leading to their degradation or sequestration (Löffler *et al.*, 2006). The CDC25 phosphatases remove the inhibitory phosphorylation of the cyclin-dependent kinases (CDKs). Therefore, CHK1-dependent inactivation of the CDC25 proteins prevents CDK activation and premature entry into mitosis (Piwnica-Worms *et al.*, 1998). CHK1 phosphorylation of CDC25A triggers its ubiquitin-mediated degradation, facilitated by the SCF$^{β-TRCP}$ E3 ubiquitin ligase complex (Busino *et al.*, 2003; Jin *et al.*, 2003). CDC25A degradation prevents CDK1 activation to inhibit entry into mitosis and prevents CDK2 activation to prevent origin firing (Chen, Ryan and Piwnica-Worms, 2003a). CHK1 also phosphorylates CDC25C, resulting in 14-3-3 binding and sequestration to the cytoplasm, which prevents CDK1 activation (Kasahara *et al.*, 2010). These events provide cells with sufficient time to respond to replication perturbations and finish replication before entry into mitosis.

ATR inhibits novel origin firing

The ATR-CHK1 axis also inhibits global origin firing under conditions of replication stress to prevent additional fork stalling until the stress is resolved (Diffley and Santocanale, 1998; Costanzo *et al.*, 2003; Karnani and Dutta, 2011; Yekezare, Gomez-Gonzalez and Diffley, 2013). ATR limits origin firing by preventing replication initiation by blocking activation of the MCM2-7 helicase. Helicase activation requires the loading of the pre-initiation complex, which is comprised of many factors, including CDC45, Treslin, the GINS complex and other replisome components. CDC45 binding for helicase activation depends on phosphorylation of Treslin by CDK2 and by phosphorylation of the MCM2-7 helicase by DBF4-dependent kinase (DDK) (Deegan, Yeeles and Diffley, 2016). Therefore, CDK2 and DDK inhibition prevents CDC45 loading, MCM2-7

activation and origin firing. The CHK1 yeast homologue was shown to phosphorylate Dbf4 to suppress DDK activity and ATR was shown to phosphorylate DDK in human cells, though this site was not shown to affect DDK activity. Further studies are required to determine if the ATR-CHK1 checkpoint can inhibit DDK-dependent CDC45 loading and origin firing (Heffernan *et al.*, 2007; Zegerman and Diffley, 2010). In addition, CHK1 prevents origin firing through CDC25A degradation resulting in CDK2 inhibition thus preventing CDC45 loading (Mailand *et al.*, 2000; Zhao, Watkins and Piwnica-Worms, 2002). Finally, CDC45 loading and origin firing was shown to be suppressed by CHK1-mediated phosphorylation of Treslin, which could be another mechanism by which the ATR-CHK1 axis controls origin firing (Guo *et al.*, 2015). Of note, the ATR-CHK1 checkpoint prevents origin firing globally but interestingly allows local origin firing (Diffley, 1996; Ge and Blow, 2010). These functions that initially appear at odds with one another actually promote genomic stability by allowing local dormant origins to fire to ensure complete replication in the region surrounding the stall site but prevent global and late-replicating origins from firing to avoid stalling of additional replication forks until the replication stress-inducing event is resolved. The mechanism by which local origins evade checkpoint suppression of origin firing remains to be elucidated; however, some evidence suggests that CDC45 might already be loaded onto local dormant origins in the replication problematic area thereby bypassing the step inhibited by the checkpoint (Thomson, Gillespie and Blow, 2010).

ATR controls dNTP levels

ATR also preserves genomic stability by preventing additional replication fork stalling events by inhibiting additional replication stress-inducing stressors, such as nucleotide insufficiency. Indeed, it was originally thought that safeguarding adequate

levels of nucleotides was ATR's primary genome-protecting function from studies in budding yeast, where lethality resulting from ATR homologue loss could be rescued by overexpressing ribonucleotide reductase (Rnr), the enzyme responsible for dNTP production (Huang, Zhou and Elledge, 1998; Zhao, Muller and Rothstein, 1998). In human, ATR is required for ribonucleoside-diphosphate reductase subunit M2 (RRM2) expression and to prevent its CDK-cyclin F mediated degradation (Zhang *et al.*, 2009; D'Angiolella *et al.*, 2012). Finally, mice carrying a hypomorphic mutation of ATR exhibited prolonged survival and reduced levels of genomic instability when crossed to mice expressing supra-physiological levels of RRM2 (Lopez-Contreras *et al.*, 2015). Though sometimes overlooked, ATR's function in maintaining replication-favorable levels of dNTPs is key in maintaining genomic stability.

ATR stabilizes stalled replication forks

In addition to its regulatory roles in cell cycle, origin firing and nucleotide levels, ATR is best known for its replication fork stabilizing functions. By stabilizing replication forks, ATR prevents stalled forks from collapsing into DSBs and allows them to reinitiate replication once the stalling agent has been eliminated (Lopes *et al.*, 2001; Tercero and Diffley, 2001). Indeed, ATR loss has been shown to increase levels of genomic instability as measured by elevated levels of DSBs and fork-associated recombination structures, such as reversed forks (E J Brown & Baltimore, 2000; Eric J Brown & Baltimore, 2003; Myung, Datta, & Kolodner, 2001). The mechanism by which this occurs has been under fierce debate in the field and many models have been proposed.

The first model suggests that fork collapse, as defined by the inability to restart replication, results from premature replisome disassociation (Cobb et al., 2005). ATR

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phosphorylates many replisome components under condition of replication stress, including DNA polymerases, the MCM2-7 helicase, the clamp loader RFC1-5 and the Claspin-timeless-tipin-AND1 complex (Block, Yu and Lees-Miller, 2004; Cortez, Glick and Elledge, 2004; Olson *et al.*, 2006; Göhler *et al.*, 2011). In addition, many replisome factors, such as FANCM and Claspin, are targeted for degradation following extended fork stalling (Yoo *et al.*, 2004; Mailand *et al.*, 2006; Mamely *et al.*, 2006; Peschiaroli *et al.*, 2006; Kee *et al.*, 2009). Finally, our laboratory has observed decreased levels of replisome components under conditions of replication stress in ATR-depleted cells and showed that suppression of RNF4, PLK1 or AURKA, which are involved in degradation pathways, rescues the replication restart defect seen in ATR-depleted cells (Ragland *et al.*, 2013). Together these data suggest that removal of components required for replication prevent the replication of restart once forks stall. Though there are studies supporting this model, recent genomic and proteomic data have contested this model (De Piccoli *et al.*, 2012; Dungrawala *et al.*, 2015). More studies are needed to better define in which context (cell cycle phase, organism, replication stress, etc) this model might be applicable.

A second model of fork stabilization proposes that fork collapse is driven by nucleolytic enzymes that recognize and cleave specific structures formed at stalled or processed forks (Alessandra Pepe and West, 2014a). Though direct regulation of structure-specific nucleases by ATR has yet to be shown, it is possible that ATR regulates fork remodeling processes, such as fork reversal. Indeed, the DNA annealing helicases and fork reversal enzyme SMARCAL1 is a substrate of ATR and once phosphorylated, SMARCAL1 displays reduced ability to reverse forks (Couch *et al.*, 2013a). The resulting reversed fork structures are susceptible to cleavage by structure-

specific endonuclease subunit SLX4 in complex with several structure-specific nucleases, such as SLX1, MUS81-EME1 and XPF-ERCC1 (Cotta-Ramusino *et al.*, 2005; Hanada *et al.*, 2007; Forment *et al.*, 2011; Wyatt *et al.*, 2013a, 2017; Duda *et al.*, 2016). In support of this model are studies that show that loss of SLX4 decreases DSB formation in ATR-inhibited cells (Couch *et al.*, 2013a; Ragland *et al.*, 2013). Of note, ATR's role in protecting forks from nuclease-dependent fork collapse is also reinforced by its indirect inhibition of CDK as some of the structure-specific endonuclease are activated by CDK (Domínguez-Kelly *et al.*, 2011). Indeed, in the absence of ATR, CDK levels are not regulated under conditions of replication stress, and nucleases are prematurely activated causing cleavage-induced fork collapse.

In line with this second model, it is also possible that DSB formation from fork collapse might actually be an intermediate generated during a process of homologous recombination (HR)-mediated replication restart. It was recently shown that the S phase specific endonuclease MUS81-EME2 is responsible for fork cleavage of stalled replication forks to allow for replication restart (Alessandra Pepe and West, 2014a). Therefore, it is possible that nuclease-dependent stalled fork cleavage is an attempt at replication restart in ATR-depleted cells.

A third model is that ATR stabilizes forks by regulating pathways that promote replication fork restart, such as template switching, DNA damage tolerance pathways, and HR-mediated fork restart. Indeed, ATR phosphorylates translesion polymerases, reversionless 1 (REV1) and Pol η, proposing that ATR allows DNA lesion bypass (Göhler *et al.*, 2011; Sale, 2013). Additionally, many players in restart pathway, such as HR and template switching, are substrates of ATR and are necessary for RAD51 recruitment to stalled and collapse forks, suggesting that ATR might regulate RAD51-

dependent restart-promoting pathways (Vassin *et al.*, 2009; Murphy *et al.*, 2014; Ahlskog *et al.*, 2016; Buisson *et al.*, 2017). Finally, ATR could protect forks by promoting replication restart by processing repair intermediates through the regulation of recombination repair factors, such as the BLM and WRN helicases, which are ATR substrates and involved in fork restart to prevent collapse (Davies *et al.*, 2004; Ammazzalorso *et al.*, 2010).

A final model has proposed that ATR stabilizes forks indirectly by preventing the exhaustion of RPA. Under conditions of replication stress and ATR inhibition, an excess of ssDNA is generated exceeding the amount of RPA available to coat it, thereby rendering the uncovered forks susceptible to nuclease cleavage (Toledo *et al.*, 2013; Toledo, Neelsen and Lukas, 2017). More studies are needed to build a stronger case for this model though it is not difficult to imagine that an insufficiency of protective factors could play an important role in triggering increased fork collapse after fork stalling.

Every model is backed by convincing experimental data and no model excludes the other so it is possible that the mechanism of fork stabilization by ATR might be context dependent or a combination of the different models. Additional studies will help further clarify the mechanism(s) by which ATR stabilizes replication forks and which of its functions are crucial for its fork stabilizing role (Chapter 3).

Repair of collapsed replication forks

Collapsed forks are defined as stalled forks unable to resume replication, and occur when ATR is unable to stabilize stalled forks or under conditions of prolonged replication stress. To repair a collapsed fork, it must often transition through a DSB structure intermediate. Many studies have shown that collapsed fork structures are

substrates of structure-specific nucleases that process them in DSBs (Fekairi *et al.*, 2009; Couch *et al.*, 2013a; Ragland *et al.*, 2013; Szakal and Branzei, 2013a; Wyatt *et al.*, 2013a; Sarbajna, Davies and West, 2014). Indeed, MUS81-EME2 is a nuclease complex that is only active in S phase and that is required for fork restart by cleaving the fork to induce DSB formation (Alessandra Pepe and West, 2014a). In the absence of the ATR-regulated checkpoint, aberrant CDK signaling promotes premature activation of G2/M nucleases, such as SLX4-SLX1 and MUS81-EME1, which process the unprotected fork into a DSB (Couch *et al.*, 2013a; Ragland *et al.*, 2013).

Once DSBs are formed, the ATM-CHK2-regulated DNA damage checkpoint pathway is activated. DSB repair occurs by homologous recombination, which uses the sister chromatid as a DNA template to prevent loss of genetic information (West, 2003). The DSB is resected by the MRN complex at the 5' end, recruiting ATM, and generating RPA-coated ssDNA overhangs (Lamarche, Orazio and Weitzman, 2010). Following its activation via autophosphorylation, ATM phosphorylates its substrates, among which are the H2AX histone variant and the CHK2 kinase (Rogakou *et al.*, 1998; Matsuoka *et al.*, 2000). Phosphorylated H2AX, γH2AX, regulates the repair process and CHK2 arrests the cell cycle (Paull *et al.*, 2000; Kuo and Yang, 2008; Chanoux *et al.*, 2009). Of note, under conditions of replication stress, ATR will also phosphorylate H2AX (Ward and Chen, 2001). γH2AX acts as a surrogate marker of DSB and recruits repair factors at the lesion. γH2AX spreads along the chromatin up to several hundreds of kilobases on either end of the break, either as a means to remodel the chromatin to enhance repair protein recruitment and accessibility to the site, or to prevent transcription-replication collision and formation of R loops because γH2AX-marked chromatin is transcriptionally silenced (Rogakou *et al.*, 1999; Chou *et al.*, 2010; Polo *et al.*, 2012; Britton *et al.*, 2014). Together

with the MDC1 adaptor protein, γH2AX recruits the RNF8 and RNF168 E3 ubiquitin ligases, which promote the ubiquitin-mediated recruitment of the BRCA1-RAP80 complex in a CtIP-dependent manner (Stucki *et al.*, 2005; Shao *et al.*, 2009; Ohta, Sato and Wu, 2011; Strauss and Goldberg, 2011). Following extended resection by MRN in coordination with BLM helicase and EXO1 or DNA2 nucleases, BRCA1 will then recruit BRCA2 via PALB2, which will mediate RAD51 loading onto the resected ssDNA overhangs, displacing RPA to form RAD51 filaments (Sy, Huen and Chen, 2009; Peterson *et al.*, 2011). RAD51 orchestrates strand exchange via D-loop formation and homologous recombination ensues to complete the repair (Liu *et al.*, 2010). Once the DSB is repaired, replication can continue and be completed by novel origin firing or by a nearby actively replicating fork.

G2-M cell cycle kinases

CDK-cyclin control of cell cycle

The cell cycle is a tightly regulated process that ensures genome duplication and cell division, which is controlled by mitogenic signaling and the pathways discussed previously that monitor DNA integrity. Cell cycle is comprised for four cell cycle phases (G0/G1, S, G2, M) that are coordinated by several cyclin-dependent kinases (CDKs) in complex with their cyclin partners whose expression levels cycle (hence their name) through the cell cycle (Murray, 2004). Different CDK-cyclin complexes and pathways control entry into each phase (Malumbres and Barbacid, 2009). Briefly, CDK4/6-cyclin D and CDK2-cyclin E are sequentially needed to have cells enter the cell cycle from a state of quiescence following mitogenic stimulation. These complexes in turn phosphorylate and inactivate the tumor suppressor protein retinoblastroma (Rb), which leads to the activation of the E2F-dependent transcriptional program that promotes S phase entry

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(Giacinti and Giordano, 2006). Progression through S phase is regulated by CDK2-cyclin E as well as CDK2-cyclin A in S phase and CDK1-cyclin A in G2. Finally, the CDK1-cyclin B complex drives cells into mitosis followed by cell division (Norbury and Nurse, 1992; Weinberg, 1995; Murray, 2004). We will focus on the players involved in entry into G2-M phase and their role in cancer.

<u>*Regulation of CDK1*</u>

CDK1 is the only CDK that is essential for cell cycle progression as it can compensate for the loss of all other cell cycle CDKs (CDK2, CDK4 and CDK6) (Santamaria and Ortega, 2006). As mentioned above, CDK1 is required for entry into mitosis and needs to be inactivated for cells to exit mitosis. In late mitosis, CDK1 is inactivated through proteasome-mediated degradation of cyclin B by the anaphase-promoting complex/cyclosome (APC/C) complex to promote mitotic exit (Gavet and Pines, 2010). Once CDK1 is inactive, cells can separate their chromosomes and undergo cytokinesis.

Cyclin levels are not the only regulators of CDKs. CDKs are also regulated by their phosphorylation levels. CDK1 is inactivated when phosphorylated at its Thr14 and Tyr15 sites by membrane associated tyrosine- and threonine-specific cdc2-inhibitory kinase (MYT) and WEE1, respectively, and it is activated by removal of these phosphates by the CDC25 phosphatases (Parker and Piwnica-Worms, 1992; Mueller *et al.*, 1995).

<u>*Regulation of the Aurora A- PLK1 pathway*</u>

The Aurora A (AURKA) and Polo-like kinase (PLK1) kinases carry out many functions in mitosis, such as centrosome maturation, bipolar spindle assembly and

chromosomal segregation, but our focus will be on their role and regulation in the context of cell cycle progression and entry into mitosis (Asteriti, De Mattia and Guarguaglini, 2015).

Aurora A is a serine-threonine kinase that is first expressed in late S phase (Walter *et al.*, 2000; Seki, Coppinger and Jang, 2008). Aurora A kinase promotes mitotic entry through phosphorylation and activation of the serine-threonine kinase PLK1 (Macůrek *et al.*, 2008). Indeed, PLK1 activates the CDK1-cyclin B complex by activating the CDC25C phosphatase which activates CDK1, as well as by inducing the degradation of WEE1, the negative regulator of CDK1 (Nigg, 2001; Seki, Coppinger and Jang, 2008; S. M. a Lens, Voest and Medema, 2010). The CDK1-AURKA-PLK1 mitotic entry pathway is regarded as a switch, which once activated is difficult to inhibit. Once the CDK1, Aurora A and PLK1 kinases are activated, they enter into a positive feedback loop that ultimately irreversibly drives mitotic entry (Seki, Coppinger and Jang, 2008; Seki, Coppinger, Du, *et al.*, 2008; Feine *et al.*, 2014).

Aurora A has an autophosphorylation site on its activation loop at conserved residue Thr288, which was thought to be a mark of activation until a recent study showed that it was possible for Aurora A to be autophosphorylated and not be active (Bischoff *et al.*, 1998; Walter *et al.*, 2000; Littlepage *et al.*, 2002; Bayliss *et al.*, 2003a; Eyers *et al.*, 2003; Haydon *et al.*, 2003; Hirota *et al.*, 2003a; Elena N. Pugacheva and Golemis, 2005; Seki, Coppinger and Jang, 2008; Moore *et al.*, 2010; Molli *et al.*, 2010; Huang *et al.*, 2011; Dodson and Bayliss, 2012a). Aurora A requires binding partners for enhanced activity, which are thought to dictate Aurora A substrate specificity and cellular localization (S. M. a Lens, Voest and Medema, 2010). In addition to these roles, binding of these partner proteins to Aurora A influences its activity and stability (1) by promoting

Aurora A autophosphorylation, (2) by shielding the autophosphorylation site from phosphatases through conformational change, and (3) by inhibiting Aurora A recognition by the ubiquitin E3 ligase APC/C with its co-activator Cdh1 (APC/C^{Cdh1}). Two well-characterized Aurora A binding proteins are Bora and TPX2 (Kufer *et al.*, 2002a; Eyers *et al.*, 2003; Eyers and Maller, 2004; Hutterer, Berdnik, Wirtz-Peitz, Zigman, *et al.*, 2006; Seki, Coppinger, Jang, *et al.*, 2008; Bruinsma *et al.*, 2014a). Bora localizes Aurora A in the nucleoplasm and plays an essential role in driving the G2/M transition by promoting Aurora A-mediated activation of PLK1 (Seki, Coppinger and Jang, 2008). TPX2 localizes Aurora A to mitotic spindles after nuclear envelope break down and protects Aurora A dephosphorylation at its Thr288 site by inducing a conformational change in its activation loop (Kufer *et al.*, 2002a; Bayliss *et al.*, 2003a; Eyers *et al.*, 2003; Li, Cao and Zheng, 2003; Tsai *et al.*, 2003a; Eyers and Maller, 2004; Dodson and Bayliss, 2012b). Aurora A is known to interact with several other binding proteins, including HEF1, PAK1, Arpc1b, and PUM2, which aid Aurora A with its other functions (Elena N. Pugacheva and Golemis, 2005; Zhao *et al.*, 2005; Molli *et al.*, 2010; Huang *et al.*, 2011).

Interestingly, the AURKA-PLK1 pathway regulates itself. Aurora A activates PLK1, which in turn is responsible for Aurora A degradation to drive mitotic exit and cytokinesis (Barr, 2004; Chan *et al.*, 2008a; van Leuken *et al.*, 2009; S. M. a Lens, Voest and Medema, 2010). Proteolytic degradation of Aurora A is tightly regulated by APC/C^{Cdh1} (van Leuken *et al.*, 2009). In late anaphase, the APC/C^{Cdh1} recognizes the degradation box (D-box) of Aurora A and a lack of phosphorylation at its Ser51 site in its A-box, leading to its proteasome-mediated degradation (Dutertre, Descamps and Prigent, 2002; Littlepage and Ruderman, 2002a; S. M. a Lens, Voest and Medema, 2010). PLK1 also controls Aurora A localization and function by regulating its binding

partners (Chan *et al.*, 2008b). Indeed, PLK1 regulates Aurora A binding partner preference, which in turn regulates its localization and function, through β-TRCP-dependent Bora degradation.

The AURKA-PLK1 pathway is important for recovery after DNA damage. Indeed, PLK1 is required for mitotic entry following G2 cell cycle arrest induced by DNA damage (van Vugt, Brás and Medema, 2004; Peng, 2013; Hyun *et al.*, 2014). PLK1 also regulates the activation of MUS81-EME1 and stimulates its association with the SLX4 endonuclease complex, promoting replication restart through DSB generation at stalled replication forks (Matos *et al.*, 2011; Gallo-Fernández *et al.*, 2012; Muñoz-Galván *et al.*, 2012; Szakal and Branzei, 2013a). Due to its prominent and impactful role in the cell cycle and in the checkpoint pathways, it is not surprising that the AURKA-PLK1 pathway is tightly regulated in various ways, such as by stabilization, degradation, localization, phosphorylation and partner protein interaction.

Cell cycle: a target of DDR

Under conditions of DNA damage, cell cycle checkpoints are activated to block cell cycle progression and allow time for DNA repair. As we previously discussed, the DNA damage checkpoints achieve cell cycle control through modulation of CDK activity. CHK1 phosphorylation inactivates the CDC25 phosphatases and activates WEE1 kinase to inhibit CDK1 and CDK2, forcing cells to arrest in G2 phase. In addition, the AURKA-PLK1 pathway is similarly inhibited during the checkpoint to prevent premature entry into mitosis (Parrilla *et al.*, 2016; Bruinsma *et al.*, 2017)

Cell cycle dysregulation in cancer

Cancer can be defined as uncontrolled cell proliferation that results from unregulated cell cycle activity, caused either by mutations in signaling pathways or in genes coding for cell cycle proteins. CDKs are frequently dysregulated in cancers, making them a therapeutic target (Otto and Sicinski, 2017). Interestingly, CDK1 activity is rarely deregulated in cancer, though studies have shown that it contributes to tumorigenesis (Otto and Sicinski, 2017). Indeed, CDK1 knockdown in liver was shown to prevent NRAS-driven liver tumor formation and CDK1 inhibition prevented KRAS-driven colorectal cancer xenograft growth in mice (Diril *et al.*, 2012; Costa-Cabral *et al.*, 2016).

WEE1 kinase is overexpressed in many cancers, such as melanoma and glioblastoma. Interestingly, heterozygous deletion of WEE1 in mammary tissue increased the incidence of mammary tumor formation, whereas homozygous deletion did not, suggesting that complete loss of WEE1 might oppose tumor formation (Mir *et al.*, 2010; Vassilopoulos *et al.*, 2015). Despite this finding, WEE1 is still considered an oncogene and is being actively pursued as a cancer therapeutic. A promising WEE1 inhibitor, AZD1775, in currently performing well alone and in combinatorial therapies in over twenty clinical trials, with WEE1 inhibition proving to be synthetic lethal with other compounds, such as PARP and HDAC inhibitors, in several cancers (Rajeshkumar *et al.*, 2011; Karnak *et al.*, 2014; Mueller *et al.*, 2014).

Aurora A is frequently amplified in prostate and breast cancers and is overexpressed in several other types of cancer (X. Wang *et al.*, 2006; Lin-Yu Lu *et al.*, 2008; Staff *et al.*, 2010b; Xu *et al.*, 2013; Wang *et al.*, 2014). Aurora A overexpression inactivates the DNA damage checkpoint during G2 phase and the spindle assembly checkpoint during mitosis, causing aneuploidy, centrosome amplification and premature onset of cytokinesis as well as form mammary tumors in mice, underscoring its role as

an oncogene (Dutertre, Descamps and Prigent, 2002; Marumoto *et al.*, 2002; Meraldi, Honda and Nigg, 2002a; S. M. a Lens, Voest and Medema, 2010). PLK1 is often overexpressed in tumors and correlates with poor prognosis (S. M. a Lens, Voest and Medema, 2010). The mechanism by which PLK1 contributes to tumorigenesis remains unclear though it is speculated that it compromises the cell cycle checkpoints, resulting in genomic instability through premature progression through the cell cycle (Osada and Simizu, 2000; Kanaji *et al.*, 2006). Interestingly, PLK1 loss in mice increases the incidence of tumors, suggesting PLK1 might play a tumor suppression role in certain contexts (L.-Y. Lu *et al.*, 2008). Both Aurora A and PLK1 inhibitors are being pursued in clinical trials and appear to be promising therapeutics (Otto and Sicinski, 2017).

Genomic instability in cancer and therapeutic implications

Two decades ago, Hanahan and Weinberg proposed that most cellular characteristics that enable cells to acquire malignant capabilities were the manifestation of six hallmarks (Hanahan and Weinberg, 2000). After a decade of new research and insight, they expanded their model to include additional hallmarks and to highlight that genome instability and mutation acquisition constitute enabling characteristics that promote the acquisition of these hallmarks (Hanahan and Weinberg, 2011). Genomic instability, which leads to increased mutability, bestows cancer cells with genetic changes that facilitate tumor progression. More specifically, certain acquired mutations will confer cells with a selective growth advantage, allowing their outgrowth in a tissue. As the cell undergoes aberrant and accelerated cell cycling, it becomes more vulnerable to DNA damage through DNA replication perturbations and failure of protective pathways to detect and repair DNA lesions. Eventually, mutations that allow checkpoint

bypass prevent cells from undergoing senescence and apoptosis and facilitate their clonal outgrowth and possible dissemination.

In support of genetic instability driving tumorigenesis, many studies have established that early tumorigenesis is associated with activation of a DNA damage response (DDR), which protects against development into malignancy (DiTullio *et al.*, 2002; Bartkova *et al.*, 2005; Karakaidos *et al.*, 2005). Furthermore, it was shown that oncogene expression results in DNA replication stress, which if unrepairable will trigger oncogene-induced senescence (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006; Fikaris *et al.*, 2006; Bartek, Bartkova and Lukas, 2007; Burhans and Weinberger, 2007). Finally, models in which repair pathway proteins, such as ATR and ATM, are lost display increased tumor incidence, suggesting their pivotal role in cancer prevention (Harper and Elledge, 2007; Jackson and Bartek, 2009; Ciccia and Elledge, 2010; Negrini, Gorgoulis and Halazonetis, 2010). Together, these data highlight the importance of the DNA replication and damage checkpoints in preventing genomic instability and disease.

The pattern of mutations found in specific cancers can be utilized to subtype them. Studies have shown that cancers that share similar genome alterations respond similarly to certain therapies and offer comparable prognostics. Therefore, increasing our understanding of how these patterns arise, whether it be from sites in the genome being more sensitive to certain stresses or to loss of specific protective repair components, can provide insight into how to treat these malignancies. Indeed, cancer cells are in a perpetual state of replication stress, forcing them to become more reliant on DNA replication checkpoints. Exploiting this reliance for therapeutic treatments through synthetic lethality allows for targeted cancer cell therapies that have minimal off target effects. Our work and that of others have shown that inhibition of the ATR-CHK1

pathway suppresses the growth of a broad spectrum of cancers and synergistically increases DSBs and cell death (Gilad *et al.*, 2010a; Murga *et al.*, 2011a; Toledo *et al.*, 2011; Ma *et al.*, 2012; Prevo *et al.*, 2012; Schoppy *et al.*, 2012a).

In addition, by further understanding how the genome is stabilized and by characterizing the features that render parts of the genome more sensitive to certain stabilization pathways will enhance our comprehension of tumor pathogenesis and our ability to develop novel and more targeted therapeutics. Using genome-wide and proteomic techniques to probe the genome landscape and the replication dynamics observed under conditions of replication stress in combination with compromised genome stabilization mechanisms will improve our understanding of the key pathways involved in driving tumor progression and inform our patient treatment courses, from discovering exploitable synthetic lethal interactions to identifying responsive patient cohorts.

Summary

Previous work from our laboratory has shown that fork collapse in ATR-deficient cells is mediated through the activation of the AURKA-PLK1 pathway, suggesting that a key part of ATR-mediated replication fork stabilization is a consequence of inhibiting premature activation of the AURKA-PLK1 pathway (Ragland *et al.*, 2013) (Figure 1.1). To test this hypothesis, we created a cell-based system in which we hyperactivated the AURKA-PLK1 pathway through kinase overexpression and co-expression of binding partners. In Chapter 2, we report that premature activation of this pathway was insufficient to induce fork collapse. We observed that CDK1 activation was limiting and without its activation, the AURKA-PLK1 pathway could not engage in a self-driven feedforward pathway to drive premature entry into mitosis, ultimately leading to fork

collapse. However, when WEE1 kinase, a negative regulator of CDK1, was inhibited, fork collapse occurred, suggesting that one major fork stabilizing function of ATR is to prevent premature activation of the mitotic CDK1-AURKA-PLK1 pathway to avoid premature entry into mitosis.

To further investigate the mechanisms essential in replication fork stabilization, we used genome-wide techniques and proteomics to determine whether ATR maintains fork integrity through its cell cycle checkpoint role or its local fork protection role, whether some sites of the genome are more dependent on one role over the other for genome stability and whether some replisome components or recruited factors play important roles in stabilization (Figure 1.2). In Chapter 3, we show that in the context of partial replication inhibition, WEE1 and ATR inhibition cause similar levels of fork collapse at overlapping genomic locations and that fork collapse at these sites is dependent on CDK1 and Aurora A kinase. Interestingly, while WEE1 inhibition is sufficient to cause replication fork collapse in a manner similar to ATR inhibition, WEE1 inhibition further promotes fork collapse through mechanisms distinct from ATR inhibition because WEE1 inhibition produces a distinct subset of sites compared to ATR inhibition. This could result from WEE1 inhibition leading to a depletion nucleotide levels, ultimately causing an additional source of replication stress. We show that the cell cycle checkpoint function of ATR is the essential mechanism by which ATR maintains fork integrity under conditions of replication stress.

Together, these studies provide a better understanding of the stabilization mechanisms of DNA replication forks under conditions of replication stress as well as the pathways driving fork collapse in checkpoint defective cells, giving us better insight on how to leverage these findings for more effective patient therapies.

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Figure Legends

Figure 1.1. Model figure for Chapter 2: ATR stabilizes forks by preventing the premature activation of the CDK1-AURKA-PLK1 pathway. When ATR is absent or inhibited, the AURKA-PLK1 pathway is prematurely activated and results in premature entry into mitosis as well as in the degradation or removal of replisome components, leading to fork stalling and inability to restart replication.

Figure 1.2. Model figure for Chapter 3: WEE1 inhibition inhibits ATR's cell cycle checkpoint function while leaving its local fork protection role intact. ATR stabilizes forks through its direct fork protection role and its cell cycle checkpoint role. WEE1 inhibition activates the CDK cell cycle controlled pathways that ATR inhibits as part of its cell cycle checkpoint role. However, WEE1 inhibition leaves ATR's direct fork protection role intact. Using WEE1 inhibition as a tool to dissect between ATR's two fork stabilizing functions, we showed that ATR's cell cycle checkpoint role is essential for fork stability.

Figure 1.1

Figure 1.2

Direct Fork Protection Role

Cell Cycle Checkpoint Role

P

ATR ATRIP · ATR ATRIP

MCMs

ε

RFC1-5

PCNA

Direct ATR fork substrates

SMARCAL1

ATR ATRIP · ATR ATRIP · ATR ATRIP

RPA

CHK1

CDC25A → CDK2 **Active**

WEE1

CDC25C → CDK1 **Active**

Origin firing

Cell cycle progression
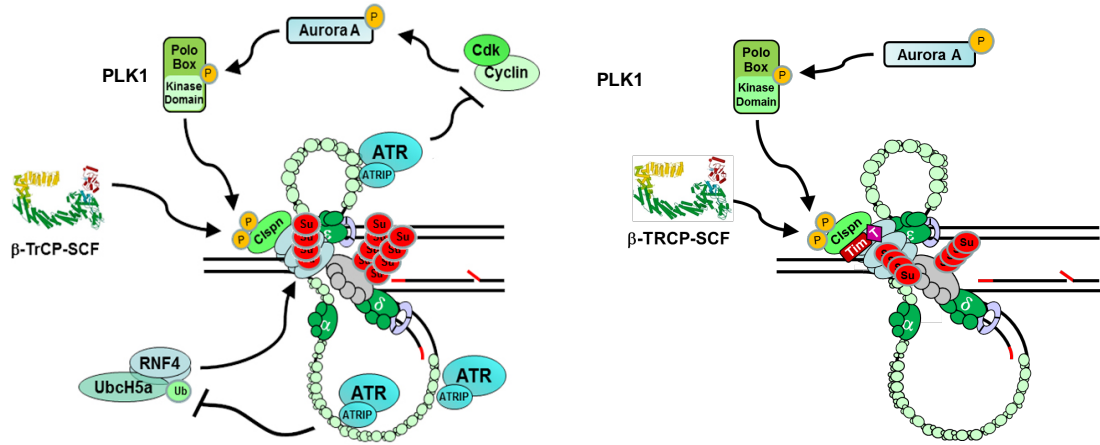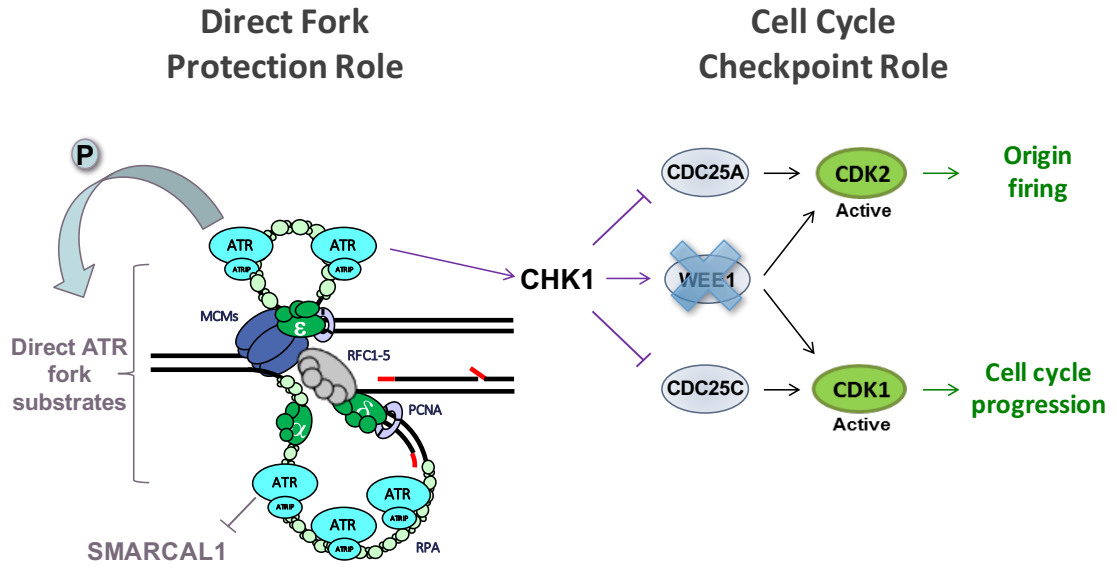
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CHAPTER 2: Premature activation of the AURKA-PLK1 pathway is insufficient to induce replication fork collapse

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Summary

Genome maintenance is required to maintain cellular viability, and thus failure to preserve genomic integrity is associated with an increased risk of cancer, accelerated aging and a higher incidence of other diseases. To ensure genomic stability, cells have checkpoints that control progression through the cell cycle. The S phase checkpoint is regulated by the ATR-CHK1 pathway that stabilizes stalled replication forks and prevents their collapse into DNA double-strand breaks (DSBs). Our laboratory has previously shown that fork collapse in ATR-deficient cells is mediated through the activation of the AURKA-PLK1 pathway, suggesting that a key part of ATR-mediated replication fork stabilization is a consequence of inhibiting premature activation of the AURKA-PLK1 pathway.

In an effort to test if premature activation of the AURKA-PLK1 pathway is sufficient to induce fork collapse, we established a system to hyper-activate the AURKA-PLK1 pathway. We are the first to show that Aurora A overexpression alone is insufficient to activate PLK1. Indeed, co-expression of Aurora A co-activators, TPX2 and BORA, is required for PLK1 phosphorylation. However, we found that achieving levels of pathway activation that lead to replication fork collapse as measured by γH2AX requires CDK1 activation. In addition, because the AURKA-PLK1 pathway is frequently dysregulated in a multitude of cancers, we hypothesized that this aberrant activation would increase reliance on the ATR-CHK1 pathway. Such an outcome would cause replication forks to collapse more readily following ATR-CHK1 inhibition. We found that Aurora A hyperactivation synergizes with ATR inhibition to increase DSB formation but does not with CHK1 inhibition. Together our findings indicate that hyperactivation of the

AURKA-PLK1 pathway is not sufficient to cause fork collapse but does lead to increased reliance on ATR-mediated fork stabilization functions.

Introduction

The faithful duplication of a cell's genome is ensured by the accuracy of the DNA replication process and by a series of cell cycle checkpoints that coordinate cell cycle progression with that of proper genomic replication and stability. One of these cell cycle checkpoints is the S phase DNA replication checkpoint, which is regulated by the Ataxia Telangiectasia and Rad3-related protein (ATR)- Checkpoint Kinase 1 (CHK1) pathway. This pathway is activated under conditions of replication stress, defined by increased uncoupling of the DNA helicase from the replicative polymerase, leading to the accumulation of RPA-coated single stranded DNA. The resulting stalled fork structure is stabilized through the recruitment of ATR, which prevents fork collapse into double strand breaks (DSB). It has been established that replication stress is intrinsically linked to genomic instability, but the underlying mechanisms remain unclear. Establishing these mechanisms would provide us with a better understanding of the driving forces behind tumorigenesis, as oncogene expression has been shown to result in replication stress, ultimately providing us with novel therapeutic avenues to explore.

Our work and that of others have aimed to better comprehend the signaling pathways involved in preventing fork collapse under conditions of replication stress and how those interplay with the ATR-CHK1 checkpoint pathway. Recently, our studies have shown that the Aurora A (AURKA)-Polo-like Kinase1 (PLK1) pathway plays an active role in replication fork collapse following ATR inhibition. Indeed, suppression of the AURKA-PLK1 pathway allows replication to restart after fork collapse in ATR-deficient

cells and can partially prevent fork collapse into DSBs. These data suggest that ATR stabilizes forks by preventing premature AURKA-PLK1 pathway activation.

The AURKA-PLK1 pathway plays several essential roles during cell division, including regulation of mitotic entry, centrosome duplication, spindle formation, chromosome segregation and cytokinesis (Glover *et al.*, 1995; Richter *et al.*, 2000; Hirota *et al.*, 2003a; Marumoto *et al.*, 2003; Marumoto, Zhang and Saya, 2005). The multitude of processes that Aurora A is involved in is tightly regulated by its association with co-activators, which include BORA, TPX2, astrin, ajuba and HEF1, that dictate Aurora A's localization, activation and substrate specificity (Kufer *et al.*, 2002b; Eyers *et al.*, 2003; Hirota *et al.*, 2003a; Elena N Pugacheva and Golemis, 2005; Hutterer, Berdnik, Wirtz-Peitz, Igman, *et al.*, 2006; Du *et al.*, 2008; Seki, Coppinger, Jang, *et al.*, 2008). Both AURKA and PLK1 are amplified in cancers, including breast, colorectal and ovarian cancers (Takahashi *et al.*, 2000, 2003; Weichert *et al.*, 2004; Kitajima *et al.*, 2007). Therefore, a better understanding of how dysregulation of the AURKA-PLK1 pathway impacts genome stability is key to discovering new therapeutic interventions.

Although our laboratory has shown that AURKA-PLK1 signaling is required for replication fork collapse in ATR-deficient cells, it has not been determined whether premature activation of the AURKA-PLK1 pathway is sufficient to promote fork collapse (Ragland *et al.*, 2013). It is known that ATR signaling inhibits the AURKA-PLK1 pathway in response to replication stress. Therefore, the ability of ATR inhibition to cause fork collapse might be the result of premature AURKA-PLK1 activation. Herein, we establish that overexpression of Aurora A or stabilized mutants of Aurora A are not sufficient to hyperactivate the AURKA-PLK1 pathway. Aurora A overexpression must be combined with expression of its co-activators, such as TPX2 or, preferably BORA, to achieve activation of the pathway as determined by increased phospho-PLK1 levels. Additionally,

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we show that lack of CDK1 activity limits the activation of the AURKA-PLK1 pathway and that when CDK1 is uninhibited through WEE1 inhibition, hyperactivation of the AURKA-PLK1 pathway is partially required to induce fork collapse into DSB, as measured by increased levels of γH2AX, and to accelerate inhibition of DNA replication restart following replication fork stalling with aphidicolin. Both the WEE1 inhibition-induced DSBs and inability to restart are rescued by Aurora A and PLK1 inhibition, suggesting that despite ATR activation, hyperactivation of the CDK1-AURKA-PLK1 pathway is a key component in promoting fork stalling and collapse into DSBs. Finally, we demonstrate that Aurora A overexpression results in an increased reliance on ATR for fork stabilization and is synthetic lethal with ATR inhibition, though surprisingly not with CHK1 inhibition. Altogether, our data suggests that ATR stabilizes replication forks and prevents their collapse into DSBs in part by inhibiting the premature activation of the CDK1-AURKA-PLK1 pathway, highlighting the importance of the ATR-CHK1 cell cycle progression checkpoint function for genomic integrity.

Results

Establishing a cell-based system to hyperactivate the Aurora A-PLK1 pathway

Aurora A overexpression is achieved in MCF10A and 293T cells through an inducible vector system.

To determine if hyperactivation of the Aurora A-PLK1 pathway is sufficient to promote fork collapse, a cell-based system was established in which the AURKA-PLK1 signaling pathway could be amplified. Two human cell lines were chosen to overexpress Aurora A: Human Embryonic Kidney 289T (HEK293T) cells for ease of plasmid transfection, and the non-tumorigenic breast epithelial cells MCF10A, which could be further used to study the contribution of AURKA-PLK1 pathway hyperactivation in breast

tumorigenesis. Using retroviral transduction, Aurora A was overexpressed, but over a period of 2-3 days, cells slowly downregulated Aurora A expression, and within a week, reestablished endogenous levels (data not shown). This result was unanticipated because Aurora A has been reported to be frequently amplified in a spectrum of cancers, including breast, colorectal, and ovarian cancers and was therefore expected to confer a survival advantage to the cells (Sen, Zhou and White, 1997; Bischoff *et al.*, 1998; Miyoshi *et al.*, 2001; Gritsko *et al.*, 2003; Vader and Lens, 2008; Staff *et al.*, 2010a). Because Aurora A overexpression was shown to induce aneuploidy and genomic instability, ultimately causing malignant transformation in the absence of checkpoints, we hypothesized that cells overexpressing Aurora A were either quickly eliminated in culture by being selected against due to their genomic instability or had cell intrinsic pathways that limited aberrant AURKA-PLK1 pathway amplification (Tatsuka *et al.*, 1998; Zhou *et al.*, 1998; Meraldi, Honda and Nigg, 2002b; Chung *et al.*, 2005; X. Wang *et al.*, 2006). To prevent cells from downregulating Aurora A expression over time as a result of constant Aurora A expression, cell lines that inducibly overexpressed Aurora A were created using retroviral transduction. Two MCF10A cell lines were generated and selected for by treating with puromycin to ensure that the majority of the cells expressed the puro-resistant Aurora A amplifying inducible construct. In both cell lines, Aurora A expression was induced within 6 hours of tamoxifen treatment and maximal expression was achieved at 24 hours of tamoxifen treatment (Figure 2.1).

Aurora A overexpression leads to Aurora A autophosphorylation at T288 but does not lead to PLK activation nor is sufficient to induce DNA damage.

Induction of Aurora A expression led to an increase in Aurora A autophosphorylation at the Thr^{288} site, a mark suggested to reflect Aurora A activation, indicating that the induced protein kinase is catalytically active and therefore able to

phosphorylate its downstream target PLK1 and activate the AURKA-PLK1 pathway (Figure 2.2A). However, despite Aurora A autophosphorylation, no increase in phospho-PLK1 levels was observed, indicating that Aurora A activation is not sufficient to hyperactivate the AURKA-PLK1 pathway (Figure 2.2A). In addition, Aurora A overexpression did not result in double strand break (DSB) formation, as determined by a lack of increased levels of the DSB surrogate marker γH2AX (Figure 2.2B). To ensure that the changes observed accurately reflected modulations in the nucleus and were not diluted out from cytoplasmic signals, markers of AURKA-PLK1 pathway activation were probed for in the insoluble nuclear fraction of the cell (Figure 2.2B). The chromatin fraction similarly displayed an increase in phospho-Aurora A levels without an increase in phopho-PLK1 levels.

Expression of a phosphomimetic mutant of Aurora A leads to Aurora A stability and autophosphorylation.

Aurora A expression peaks during mitosis and is rapidly degraded after G2/M phase through phosphorylation events that target the protein for degradation via the APC[Cdh1]-ubiquitin-proteasome pathway (Honda *et al.*, 2000; Katayama *et al.*, 2001; Littlepage and Ruderman, 2002b; Littlepage *et al.*, 2002; Taguchi *et al.*, 2002; Horn *et al.*, 2007). The ubiquitin ligase APC/C[Cdh1] recognizes two sequences that are required for Aurora A kinase proteolysis: the C terminus D box (destruction box), and the N terminus A box, which includes the serine 51 site (Castro *et al.*, 2002; Littlepage and Ruderman, 2002b). The serine 51 site of Aurora A is phosphorylated during M phase to stabilize Aurora A and is dephosphorylated upon mitotic exit by PP2A to control the timing of Aurora A destruction (Walter *et al.*, 2000; Littlepage *et al.*, 2002; Horn *et al.*, 2007). To ensure that the induced Aurora A protein was not being prematurely degraded

and prevented from binding to its co-activators, a phosphomimetic mutant at the serine 51 site was created that has previously been shown to be sufficient to stabilize the protein (Kitajima *et al.*, 2007). The inducible AURKA^{S51D}-ERT2 protein was highly expressed in MCF10A cells when treated with tamoxifen and led to elevated levels of T288 autophosphorylation (Figure 2.3). However, expression of the phosphomimetic mutant of Aurora A was still not sufficient to lead to increased levels of phospho-PLK1 levels or γH2AX (data not shown).

Co-expression of Aurora A with co-activators, TPX2 or BORA, results in an increase in PLK1 activation but not increased levels of DNA damage.

Aurora A is known to require binding partners for maximal activation or for activation at particular cellular locations as a means to tightly regulate the location and timing of its expression (Kufer *et al.*, 2002b; Hirota *et al.*, 2003a; Carmena, Ruchaud and Earnshaw, 2009; Li *et al.*, 2015). Its most well-characterized binding partners are TPX2 and BORA, which have been shown to significantly enhance Aurora A kinase activity, making them excellent candidates as co-activators of Aurora A in our system. Indeed, TPX2 has been shown to both promote Aurora A autophosphorylation and to protect it from dephosphorylation (Bayliss *et al.*, 2003a; Eyers *et al.*, 2003; Tsai *et al.*, 2003b; Eyers and Maller, 2004; Dodson and Bayliss, 2012a). We hypothesized that despite an increase in Aurora A autophosphorylation in our system, we were not achieving PLK1 phosphorylation due to insufficient levels of Aurora A coactivators. Because MCF10A cells are difficult to transfect, HEK293T cells that stably expressed the inducible AURKA^{S51D}-ERT2 construct were used. These cells were transiently transfected with mCherry-TPX2, resulting in robust TPX2 expression (Figure 2.4). Combining Aurora A overexpression with TPX2 expression led to a slight increase in phospho-PLK1 levels

(Figure 2.4). However, this slight increase in AURKA-PLK1 pathway activation did not result in an increase in γH2AX (Figure 2.5). It was unclear if the lack of DNA damage was due to insufficient hyperactivation of the AURKA-PLK1 pathway or if hyperactivation was achieved, but remained insufficient to cause fork collapse. As such, this question bears further investigation.

Unlike TPX2, which acts on Aurora A to keep it activated, BORA activates the AURKA-PLK1 pathway further downstream at the level of PLK1 activation (Hutterer, Berdnik, Wirtz-Peitz, Igman, *et al.*, 2006; Seki, Coppinger, Jang, *et al.*, 2008; Parrilla *et al.*, 2016). In this regard, BORA is a more promising pathway activator as the hurdle appears to be in achieving PLK1 phosphorylation. Indeed, BORA is known to interact with PLK1 and control the accessibility of the PLK1 activation loop for phosphorylation by Aurora A. Before BORA can bind to PLK1, it is primed through CDK1 phosphorylation at serine 252 (Feine *et al.*, 2014; Tavernier *et al.*, 2015; Thomas *et al.*, 2016). Moreover, in order to tightly regulate AURKA-PLK1 pathway activation at the G2/M phase of the cell cycle, BORA is degraded in early mitosis to prevent further pathway activation in following cell cycle phases. Proteolysis of BORA is mediated through its phosphorylation at a conserved site within the DSGxxT degron domain that promotes its interaction with the ubiquitin ligase SCF-β-TrCP, which catalyzes its ubiquitination and marks it for proteosomal degradation (Chan *et al.*, 2008b; Seki, Coppinger, Du, *et al.*, 2008). To maximize PLK1 binding to BORA and bypass BORA degradation, the double mutant BORA$^{S252D/T501A}$ was generated and transiently transfected in HEK293T cells that also stably expressed the inducible AURKA^{S51D}-ERT2 construct. Co-expression of the stabilization mutant with Aurora A^{S51D} overexpression led to an increase in phospho-

PLK1 levels but not to an increase in levels of DNA damage (Figure 2.6 and data not shown).

CDK1 activation allows for Aurora A-PLK1 activation and DNA damage.

Aberrant activation of the CDK1-AURKA-PLK1 pathway, which controls G2/M cell cycle entry could result in premature entry into mitosis and chromosome condensation followed by segregation. If DNA replication is not complete and cells prematurely go through cell division, this would result in severe genomic instability and cytogenetic defects. As a result, tight regulation of mitotic entry pathway activation exists. We hypothesized that despite overexpression of stabilized components of the AURKA-PLK1 pathway, hyperactivation of the pathway was restricted due to limitation by a key regulator. CDK1 is a key regulator of cell cycle progression, by modulating entry into M phase through activation of mitotic pathways (Nurse, 2000; Morgan, 2006). To test if CDK1 activation limits AURKA-PLK1 hyperactivation, WEE1, a negative regulator of CDK1, was inhibited to allow unrestricted CDK1 activation. Cells were treated with a WEE1 inhibitor, AZD1775, at concentrations of 300nM and 1uM for 3 hours, which resulted in an increase in phospho-PLK1 levels (Figure 2.7). Interestingly, WEE1 inhibition also led to an increase in γH2AX levels (Figure 2.7). These results suggest that CDK1 activation allows for AURKA-PLK1 pathway hyperactivation, which might be sufficient to cause fork collapse and DSB formation.

WEE1 inhibition-induced DSB formation depends on AURKA-PLK1 pathway activation.

To test if DSB formation induced by WEE1 inhibition is a result of AURKA-PLK1 pathway hyperactivation, cells were treated with WEE1 inhibitor for 30 min, 1 hr, 2 hr and 3 hr with or without Aurora A inhibitor. Levels of γH2AX were measured as a readout

for DSB formation (Figure 2.8). Interestingly, despite ATR-CHK1 pathway activation as early as 30 min, determined by increased phospho-CHK1 levels, forks still collapsed into DSBs as early as 1 hour, determined by increased γH2AX levels at 1 hour of WEE1 inhibitor treatment (Figure 2.8). Early DSB formation depends on AURKA-PLK1 pathway activation as γH2AX levels decreased upon Aurora A inhibitor treatment at 1 hr, 2 hr and 3 hr of WEE1 inhibitor treatment. By 3 hr of WEE1 inhibitor treatment, γH2AX levels decreased upon Aurora A inhibition, but not to basal levels, indicating that Aurora A-independent pathways might become activated and result in fork collapse. One possibility could be that, as it has previously been shown, WEE1 inhibition leads to CDK1-dependent degradation of RRM2, resulting in nucleotide shortage followed by fork stalling and collapse into DSBs (Beck *et al.*, 2010, 2012; Domínguez-Kelly *et al.*, 2011; D'Angiolella *et al.*, 2012; Pfister *et al.*, 2015). Indeed, RRM2 levels are stable at early time points of WEE1 inhibition, but by 3 hr of inhibition, RRM2 levels slightly decreased, suggesting that a shortage in nucleotide levels could contribute to WEE1 inhibition-induced DSBs after 3 hr of WEE1 inhibition (Figure 2.9). This would further explain why Aurora A inhibition can only partially rescue DSB formation. To further validate that WEE1 inhibition-induced DSB formation depends on AURKA-PLK1 pathway activation, cells were treated with WEE1 inhibitor for 30 min, 1 hr, 2 hr and 3 hr with or without PLK1 inhibitor (Figure 2.10). As seen with Aurora A inhibitor, PLK1 inhibition led to a decrease in γH2AX levels at early time points and to a partial decrease after 3 hr of WEE1 inhibition (Figure 2.10). These data suggest that DSB formation induced by WEE1 inhibition results in part from hyperactivation of the AURKA-PLK1 pathway.

WEE1 inhibition is sufficient to prevent DNA replication restart and is rescued by AURKA-PLK1 pathway inhibition

To study the impact of AURKA-PLK1 pathway hyperactivation on DNA replication, we utilized the replication restart assay developed by our laboratory. This assay allows us to study the ability of stalled forks to restart replication by monitoring the percent of cells that were originally replicating (BrdU positive) to continue replicating (EdU positive) after a period of inhibited replication achieved by using the replicative polymerase inhibitor, aphidicolin (Figure 2.11a) (Ragland *et al.*, 2013). In this assay, S phase cells were labeled with BrdU for 30 min before treating with aphidicolin and WEE1 inhibitor for 1 hr or 3 hrs. Cells were then washed and pulsed with EdU for 1 hr. BrdU-labeled S phase cells were assayed for their ability to restart replication by monitoring the percent of cells that were also EdU positive. At the high dose of WEE1 inhibition (1uM), WEE1 inhibition was sufficient to prevent replication restart in 11% of cells with 1 hr treatment and in 56% of cells with 3 hr treatment (Figure 2.11b). Impressively, even at the low 300nM dose of WEE1 inhibition, up to 25% of cells were unable to restart replication after 3 hrs of treatment. In comparison, WEE1 inhibition prevented replication restart 3 times faster than ATR inhibition, consistent with the fact that fork collapse caused by ATR loss could be due to premature activation of the AURKA-PLK1 pathway (data not shown). Indeed, ATR inhibition begins to prevent replication restart after 3 hrs of treatment, whereas WEE1 inhibition does it as early as 1 hr of treatment.

To determine if inhibition of replication restart by WEE1 inhibition was dependent on AURKA-PLK1 pathway activation, the restart assay described above was employed in the absence or presence of AURKA or PLK1 inhibitors. Aurora A and PLK1 inhibition rescued WEE1 inhibition-induced inability to restart replication, suggesting that despite ATR activation, premature activation of the AURKA-PLK1 pathway is involved in causing fork collapse and preventing replication restart (Figure 2.11c).

Aurora A overexpression is synthetic lethal with ATR inhibition but not CHK1 inhibition

Aurora A is a known oncogene amplified in many cancers. We and others have shown that oncogene-induced replication stress coupled with ATR suppression synergistically increases DSB formation (Gilad *et al.*, 2010b; Murga *et al.*, 2011b; Toledo *et al.*, 2011; Toledo, Murga and Fernandez-Capetillo, 2011; Schoppy *et al.*, 2012b; Zeman and Cimprich, 2014b). Additionally, inhibition of Aurora A-PLK1 signaling allows stalled forks to reinitiate replication in ATR-deleted cells. Conversely, as shown above, hyperactivation of the AURKA-PLK1 pathway through WEE1 inhibition promotes replication fork collapse and prevents replication fork restart, suggesting that hyperactivation of the pathway could lead to an increased reliance on ATR for fork stabilization and become synthetic lethal with ATR-CHK1 inhibition. To test this hypothesis, cells were transiently transfected with an AURKA^{S51D}-ERT2 construct and treated with tamoxifen to overexpress non-degradable Aurora A and with increasing doses of VE-822 to inhibit ATR. Immunoblot analysis of whole cell lysates demonstrated that ATR inhibition combined with Aurora A^{S51D} overexpression led to synergistic increases in γH2AX at all tested doses of ATR inhibition (Figure 2.12a).

Interestingly, when the same cells were treated with CHK1 inhibitor, only a slight increase in γH2AX levels was seen when combined with Aurora A overexpression (Figure 2.12b). This result could be explained by the fact that ATR has local fork protection functions that CHK1 does not, and thus CHK1 inhibition would be expected to retain some level of fork protection and consequently contribute to less fork collapse. Alternatively, synergy is not observed because CHK1 inhibition on its own causes maximal levels of γH2AX, preventing a visible synergistic increase when combined with

Aurora A overexpression. To test this possibility, this experiment would have to be repeated with lower doses of CHK1 inhibition. Together, these data suggest that patients with cancers that overexpress or amplify AURKA would benefit from therapies using ATR inhibitors.

Discussion

The results shown here are the first to establish that hyperactivation of the AURKA-PLK1 pathway through WEE1 inhibition is sufficient to cause replication fork collapse. This finding suggests that a crucial part of ATR-mediated replication fork stabilization is a function of its regulation of the AURKA-PLK1 pathway.

According to the literature that demonstrated that Aurora A overexpression or amplification alone was sufficient to transform cells, induce mitotic abnormalities and lead to tumorigenesis (Zhou *et al.*, 1998; Meraldi, Honda and Nigg, 2002b; Chung *et al.*, 2005; X. Wang *et al.*, 2006; X. X. Wang *et al.*, 2006; Zhang *et al.*, 2008), as well as the literature showing that Aurora A activated the PLK1 pathway (Macůrek *et al.*, 2008; Seki, Coppinger, Du, *et al.*, 2008; Bruinsma *et al.*, 2014b), we hypothesized that Aurora A overexpression alone would be sufficient to activate the AURKA-PLK1 pathway. We were surprised to find that Aurora A overexpression alone, including that of non-degradable Aurora A protein constructs, was not sufficient to lead to PLK1 activation. Autophosphorylation at the T288 site in Aurora A was observed, which was previously thought to be a mark of activation. However, it was later shown that non-phosphorylated Aurora A in complex with TPX2 at the mitotic spindle is catalytically active, suggesting that the autophosphorylation state of Aurora A might be an inaccurate surrogate for its activity (Dodson and Bayliss, 2012a). This finding could be context dependent, but based on our lack of PLK1 phosphorylation when Aurora A was overexpressed, it seems

that the increase in phosphorylation at T288 did not correlate with activation of the AURKA-PLK1 pathway. In addition, in the studies in which Aurora A overexpression alone was sufficient to transform cells, it is possible that either the cell lines were naturally altered to allow for AURKA-PLK1 pathway activation, such as through p53 mutation or activation of CDK1, or that the cell transformation was a consequence of the dysregulation of PLK1-independent functions of Aurora A. Our data are the first to show that Aurora A overexpression alone is not sufficient to activate the AURKA-PLK1 pathway. Instead, as was previously shown, activation of the AURKA-PLK1 pathway occurred through co-expression of Aurora A co-activators TPX2 and BORA. However, the level of activation required to result in fork collapse within hours could not be achieved without activating CDK1, which we believe to be the limiting switch for maximal pathway activation, as it occurs normally at the transition into M phase.

Model for fork collapse induced by AURKA-PLK1 pathway activation

Previously our laboratory has shown that when ATR is depleted, the CDK1-AURKA-PLK1 pathway is activated, resulting in fork collapse (Ragland *et al.*, 2013). We now show that activation of the CDK1-AURKA-PLK1 pathway using WEE1 inhibition is required for fork collapse, despite having an intact ATR-CHK1 checkpoint pathway. The mechanism by which activation of the CDK1-AURKA-PLK1 pathway can promote fork collapse is unknown, but we propose that it is mediated through the premature loss of replisome components. Replisome disengagement is a process that naturally occurs at sites of replication termination and during M phase transition and is regulated by the CDK1-AURKA-PLK1 pathway (Freire *et al.*, 2006; Mailand *et al.*, 2006; Mamely *et al.*, 2006; Peschiaroli *et al.*, 2006); therefore, aberrant activation of this mitotic fork termination pathway in S phase could lead to the premature removal of replication factors, resulting in fork collapse (Liu, Song and Liu, 2010; Dheekollu *et al.*, 2011).

Indeed, PLK1 has previously been implicated in DNA replication checkpoint termination and entry into mitosis through its βTrCP ubiquitin ligase-mediated degradation of Claspin, a known replisome component (Yoo *et al.*, 2004; Mailand *et al.*, 2006; Peschiaroli *et al.*, 2006). In addition, PLK1 was shown to control βTrCP-dependent degradation of FANCM, a replisome component, during mitosis (Kee *et al.*, 2009). Finally, our laboratory observed a general loss of replisome components under conditions of ATR inhibition and AURKA-PLK1 activation (Ragland *et al.*, 2013). Altogether, these data suggest that premature activation of the CDK1-AURKA-PLK1 pathway could lead to premature degradation of the replisome, causing fork collapse. Fork collapse from loss of replisome factors could be a consequence of exposing the bare DNA structure of a fork that occurs once DNA-bound protein complexes are removed, rendering it accessible to endonucleases that can cleave it into a DSB. In addition to stripping the fork, activation of AURKA-PLK1 pathway could be promoting the activation of fork-targeting nucleases. Indeed, studies have shown that PLK1 is required for the activation of MUS81-EME1 and its association with SLX4 and that these nucleases are responsible for fork cleavage to promote replication fork restart (Domínguez-Kelly *et al.*, 2011; Muñoz-Galván *et al.*, 2012; Kai J. Neelsen *et al.*, 2013; Szakal and Branzei, 2013a; Wyatt *et al.*, 2013b; Alessandra Pepe and West, 2014b). Future studies that determine which proteins are present at the fork under conditions of CDK1-AURKA-PLK1 pathway hyperactivation using iPOND2 could elucidate how CDK1-AURKA-PLK1 pathway hyperactivation results in fork collapse, which could occur either by loss of replisome proteins or the recruitment of endonucleases at the fork (Sirbu *et al.*, 2011; Sirbu, Couch and Cortez, 2012; Rivard *et al., unpublished*).

Clinical relevance

Aurora A is a known oncogene amplified in many cancers. Our findings indicate that Aurora A overexpression results in an increased reliance on ATR and is synthetic lethal with ATR inhibition. With ATR inhibitors already being assessed as cancer therapeutics in clinical trials, our data has promising potential for novel opportunities that can be rapidly implemented for the treatment of cancers with amplified AURKA, such as breast and ovarian cancers (Charrier *et al.*, 2011; Ma, Janetka and Piwnica-Worms, 2011; Reaper *et al.*, 2011; Toledo, Murga and Fernandez-Capetillo, 2011). Furthermore, oncogenes such as KRAS[G12D] and MYC, expression of which are synthetic lethal with ATR inhibition (Gilad *et al.*, 2010b; Murga *et al.*, 2011b; Toledo *et al.*, 2011; Schoppy *et al.*, 2012b; Zeman and Cimprich, 2014b), promote AURKA expression and activity (Macůrek *et al.*, 2008; Maris, 2009; Otto *et al.*, 2009; den Hollander *et al.*, 2010; Brockmann *et al.*, 2013; Yang *et al.*, 2013; Hilton and Shapiro, 2014; Dauch *et al.*, 2016; Richards *et al.*, 2016), therefore, broadening the number and types of cancers that can be targeted with ATR inhibitor therapies.

Future Directions

The model we propose is the first to suggest that ATR suppression leads to fork collapse as a result of aberrant activation of the CDK1-AURKA-PLK1 pathway. In light of our recent genome-wide work characterizing replication perturbed regions under conditions of ATR suppression combined with replication stress (Shastri, Tsai et al. 2017), it would be interesting to determine if these same sites are affected by hyperactivation of the CDK1-AURKA-PLK1 pathway. If so, these finding would suggest that the key role of ATR fork stabilization stems from its inhibition of premature cell cycle progression through activation of the CDK1-AURKA-PLK1 pathway. In addition, further studies designed to query the proteins and pathways involved in ATR-mediated replication fork stabilization through iPOND2 will clarify our fundamental understanding

of replication fork dynamics when the checkpoint response is activated. This will provide insight into how such findings can be utilized for cancer therapies.

Material and Methods

Cell Culture

MCF10A cells were cultured in Dulbecco's modified Eagle's medium-F12 (1:1) with 5% horse serum, insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), and epidermal growth factor (20 ng/ml), cholera toxin (100ng/ml), streptomycin (100 µg/mL) and penicillin (100 units/ml). HEK293T cells were cultured in a 3% O_2 and 6% CO_2 atmosphere at 37°C in Dulbecco modified Eagle's medium (Mediatech, MT10-013-CV) supplemented with 10% fetal bovine serum (Gemini BioProducts, 100-106), 2mM L-glutamine (Mediatech, 25-005-CI), and 100 µg/mL streptomycin, 100 units/ml penicillin (Invitrogen, 15140-122). MCF10A cells stably overexpressing Aurora A were generated by infecting them with retrovirus expressing the $AurA^{WT}$-ERT2 or $AurA^{S51D}$-ERT2 inducible fusion protein from the pBabe puro human Aurora A plasmid (Addgene plasmid #8510) followed by puromycin selection at 20ug/mL of puromycin for 3 days. For additional protein expression, cells were transiently transfected using Lipofectamine 3000 (Invitrogen) as per manufacturer's directions and using 24ug of indicated plasmid. Plasmid used were pmCherry-TPX2 (Addgene, plasmid 31227) and MGC Human BORA cDNA accession # BC025367 (Thermo Fisher MHS6278-202830791). All plasmid site directed mutagenesis was performed using the Agilent QuikChange Site Directed Mutagenesis Kit according the manufacturer's directions.

Human retrovirus creation and infection

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To generate retroviruses, HEK293T cells were transfected using the calcium phosphate method with the retroviral expression vector and a ψ2 vector. Control and pBabe puro human Aurora A retroviral vectors were purchased from Addgene (#1764 and #8510, respectively). For retroviral infection, MCF10A cells were infected with virus and polybrene (8ug/ml) using spin infection at 18C for 1 hr at 2500 rpm and left to incubate with the virus for 24hrs. The cell culture medium was then replaced and 24 hours after recovery in fresh medium, cells were selected with increasing concentration of puromycin.

Aurora A expression induction

Aurora A expression was induced in AurA-ERT2 infected cell lines by the addition of 0.5 uM 4-hydroxytamoxifen (4-OHT; EMD) to the culture medium for 6 or 24 hours. The 4-OHT was added at the time of plating and washed out 6 or 24 hours later. Cells were then cultured for an additional 24 hours after 4-OHT washout to afford effective expression of the Aurora A protein, at which time cells were collected for analysis. If cells were transfected with a plasmid for TPX2 or BORA co-expression, cells were transfected 24 hours after plating and 4-OHT was added 24 hours after that. Expression levels and DNA damage induction were assessed 24 hours after 4-OHT addition.

Chemical inhibitors

All chemical inhibitors were added to the cell medium 24 hours after 4-OHT addition for a treatment duration of 6 hours. The inhibitor used for ATR inhibition is VE-822 (SelleckChem) and for CHK1 inhibition is PF477736 (Tocris) at the doses indicated in the figure legends.

Chromatin extraction and fractionation

Extractions were performed as described by Mendez and Stillman (2000). Briefly, cells were collected by trypsin treatment and lysed in buffer A (10 mM HEPES at pH 7.9,

0.1% Triton-X, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol and protease inhibitors (Roche)) for 5 min at 4°C. Lysates were centrifuged at 1300g for 5 min. The resulting supernatant was mixed 1:1 with 2X Laemmli sample buffer and collected as the cytoplasmic fraction. Pellets were washed in buffer A once and incubated in buffer B (3 mM EDTA, 0.2mM EGTA, protease inhibitors (Roche)) for 30 min at 4°C. The samples were centrifuged at 1700g for 5 min, and the resulting supernatant was collected and mixed 1:1 with 2X Laemmli sample buffer to generate the nuclear-soluble fraction. Pellets from this extraction were washed in buffer B twice prior to a final centrifugation at 1700g for 5 min. The pellets were resuspended in buffer A and mixed 1:1 with 2X Laemmli sample buffer to generate the nuclear-insoluble fraction. All samples were boiled for 5 min prior to protein quantification as described below.

Western immunoblotting

Cells were resuspended in PBS and lysed with Laemmli sample buffer (final concentrations of 10% glycerol, 2% SDS, 60 mM Tris, pH 6.8) and boiled for 5 minutes. Protein concentration was determined by bicinchoninic acid protein assay (Thermo Fisher Scientific, 23225). The total protein quantities were normalized, loaded on a 4-20% gradient SDS-polyacrylamide gel (Bio-Rad, 456-1093) and separated by electrophoresis. Proteins were then transferred to 0.45 µM polyvinylidene fluoride (PVDF) membranes, blocked in 5% milk in TBST for 1 hour at RT, bound overnight to primary antibodies in 0.25% milk in TBST at 4°C, washed with TBST, bound to HRP-conjugated secondary antibody in 0.25% milk in TBST for 1 hour at RT, washed with TBST and then detected with chemiluminescence (Thermo Scientific, 34080). Blots were detected for phospho-PLK1 (Cell Signaling Technology, 5472S), phospho-S345 Chk1 (Cell Signaling Technology, 2348s), phospho-S139 H2AX (Millipore, 05-636), RFP antibody for TPX2 detection (Abcam, ab62341), Aurora A (Abcam, Ab13824), phospho-

T288 Aurora A (Cell signaling, 3079), MCM3 (Bethyl Laboratories, A300-124A), GAPDH (US Biological, G8140-01), BORA (Abcam, ab182149).

Figure Legends

Figure 2.1. MCF10A cells can inducibly overexpress AURKA. MCF10A cells expressing the parent vector (Empty) or AURKA^{S51D}-ERT2 as is (0) or selected with 1.5ug/mL of puromycin (1.5) were either not treated (-) or treated (+) with 4-OHT for 6 or 24hrs and detected for total AURKA levels by western blot. *endogenous AURKA, ** AURKA^{S51D}-ERT2. Two different AURKA^{S51D}-ERT2 overexpressing cell lines are used.

Figure 2.2. Aurora A overexpression is not sufficient to activate PLK1 or induce DSBs. **(A)** Whole cell lysates of MCF10A cells expressing the parent vector (Empty) or AURKA^{S51D}-ERT2 either not treated (-) or treated (+) with 4-OHT for 24hrs and in the presence or absence of 25uM anacardic acid for 4hrs (as an AURKA co-activator/binding partner surrogate) were analyzed by Western blot. Levels of phosho-PLK1, phospho-AURKA, total AURKA and γH2AX were assessed. **(B)** Western blot of chromatin fractions of samples described in A assessed for levels of phosho-PLK1, phospho-AURKA and total MCM3 levels. *endogenous AURKA, ** AURKA^{S51D}-ERT2. Two different AURKA^{S51D}-ERT2 overexpressing cell lines are used.

Figure 2.3. Induction and activation of chromatin-bound AURKA^{S51D}-ERT2 after 4-OHT treatment. MCF10A cells (10A), expressing the parent vector (Empty) or AURKA^{S51D}-ERT2 were either not treated (-) or treated (+) with 4-OHT for 24hrs and detected for chromatin-bound total and activated (phos-T288) AURKA by western blot. MCM3 is a loading control. *endogenous AURKA, ** AURKA^{S51D}-ERT2. Two different AURKA^{S51D}-ERT2 overexpressing cell lines are used.

Figure 2.4. Transient overexpression of TPX2 in stably expressing AURKA^{S51D}-ERT2 cells slightly increased phospho-PLK1 levels. HEK293T cells stably expressing the parent vector (Empty) or AURKA^{S51D}-ERT2 (AURKA), with and without co-expression of TPX2, were left untreated (-) or treated (+) with 4-OHT for 24hrs to induce the proper folding and localization of AURKA^{S51D}-ERT2. Samples were assessed for AURKA, phospho-PLK1 and TPX2 levels (with RFP antibody which recognizes the mCherry tag of TPX2) by western blot. *endogenous AURKA; ** AURKA^{S51D}-ERT2.

Figure 2.5. Transient co-overexpression of AURKA^{S51D}-ERT2 with TPX2 increases levels of AURKA autophosphorylation. HEK293T cells transfected with the parent vector (Empty) or AURKA^{S51D}-ERT2 (AURKA), with and without co-expression of TPX2, were left untreated (-) or treated (+) with 4-OHT for 24hrs to induce the proper folding and localization of AURKA^{S51D}-ERT2. Samples were assessed for total and autophosphorylated AURKA and phospho-PLK1 by western blot. GAPDH is a loading control. *endogenous AURKA; ** AURKA^{S51D}-ERT2. Two different AURKA^{S51D}-ERT2 overexpressing cell lines are used (#1 and #2).

Figure 2.6. Transient co-overexpression of AURKA^{S51D}-ERT2 with BORA activates PLK1. HEK293T cells transfected with the parent vector (Empty) or AURKA^{S51D}-ERT2 (AURKA), with and without co-expression of BORA mutants (BORA^{T501A} or BORA$^{T501A/S252D}$) were treated with 4-OHT for 24hrs to induce the proper folding and localization of AURKA^{S51D}-ERT2. Chromatin fractions of samples were assessed for induced AURKA expression and levels of phospho-PLK1 and MCM3 by western blot.

Figure 2.7. WEE1 inhibition is sufficient to activate the AURKA-PLK1 pathway and induce fork collapse. Western blot detection γH2AX, p-PLK1 and pCHK1 of whole cell lysates from cells treated with indicated dose of WEE1 inhibitor (WEE1i).

Figure 2.8. WEE1 inhibition-induced DSB formation depends on AURKA. Western blot detection γH2AX, p-PLK1, pCHK1 and GAPDH of whole cell lysates from cells treated with 3uM WEE1 inhibitor (WEE1i) for indicated times in the presence or absence of 10uM AURKA inhibitor (AURKAi).

Figure 2.9. WEE1 inhibition-induced DSB formation does not affect RRM2 levels. Western blot detection RRM2, MCM3 and GAPDH of whole cell lysates from cells treated with 3uM WEE1 inhibitor (WEE1^{INH}) for indicated times in the presence or absence of 10uM AURKA inhibitor (AURKA^{INH}).

Figure 2.10. WEE1 inhibition-induced DSB formation depends on PLK1. Western blot detection γH2AX, p-PLK1, pCHK1, total CHK1 and GAPDH of whole cell lysates from cells treated with 3uM WEE1 inhibitor (WEE1i) for indicated times in the presence or absence of 10uM PLK1 inhibitor (PLK1i).

Figure 2.11. AURKA and PLK1 inhibition prevent WEE1 inhibition-induced replication fork collapse. **(A)** Dual-labeling method to measure replication restart by flow cytometry. Pulse cells with BrdU to label S-phase cells before the fork collapse-inducing treatment of 5 uM aphidicolin (Aph) and subsequently monitor these cells for replication restart following treatment wash out by EdU pulse labeling. Flow cytometry plots display the level of EdU labeling (X-axis) of BrdU-positive cells across the cell cycle (PI stain on the Y-axis) **(B)** Representative examples of replication restart assessed by flow cytometry following WEE1 inhibitor (WEE1i) and 5uM Aphidicolin (Aph) treatment. Cells were pulsed with BrdU for 30 min, which was then washed off, followed by 1 or 3 hours of treatment with 5uM Aphidicolin and WEE1 inhibitor at indicated doses. Inhibitors were washed off and cells were pulsed with EdU for 1 hour. Flow cytometry plots of EdU labeling (X-axis) of BrdU-positive cells of indicated conditions are shown. **(C)**

Representative examples of replication restart assessed by flow cytometry following treatment of AURKA and PLK1 small molecule inhibitors (AURKAi and PLK1i, respectively). Cells were pulsed with BrdU for 30 min, which was then washed off, followed by 3 hours of treatment with WEE1 inhibitor and 5uM Aphidicolin with or without either AURKA or PLK1 inhibitors. Inhibitors were washed off and cells were pulsed with EdU for 1 hour. Flow cytometry plots of EdU labeling (X-axis) of BrdU-positive cells of indicated conditions are shown.

Figure 2.12. Overexpression of stabilized AURKA increases reliance on ATR but not on CHK1 for genome stability. **(A)** Cells transfected with the parent vector ("V") or AURKA^{S51D}-ERT2-expresssing vector ("A") were treated with 4-OHT for 3 hours to activate AURKA^{S51D}-ERT2 and then treated with indicated dose of VE-821 ATR inhibitor ("VE") for an additional 6 hours. Western blot detection of γH2AX, AURKA and GAPDH is shown. **(B)** Cells transfected with the parent vector ("V") or AURKA^{S51D}-ERT2-expresssing vector ("A") were treated with 4-OHT for 3 hours to activate AURKA^{S51D}-ERT2 and then treated with indicated dose of CHK1 inhibitor (PF477736) for an additional 6 hours. Western blot detection of phos-H2AX, AURKA and GAPDH is shown.

Figure 2.1

Figure 2.2

A.

Whole Cell Lysis

B.

Chromatin fraction
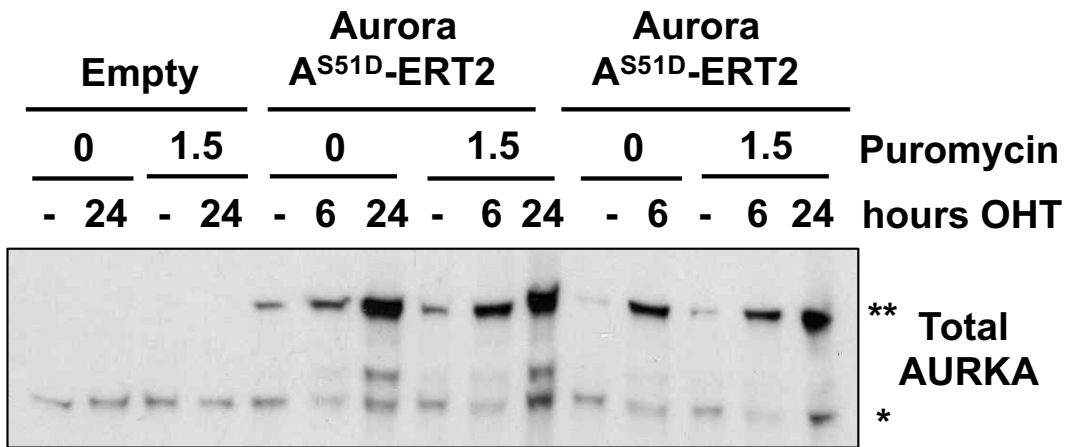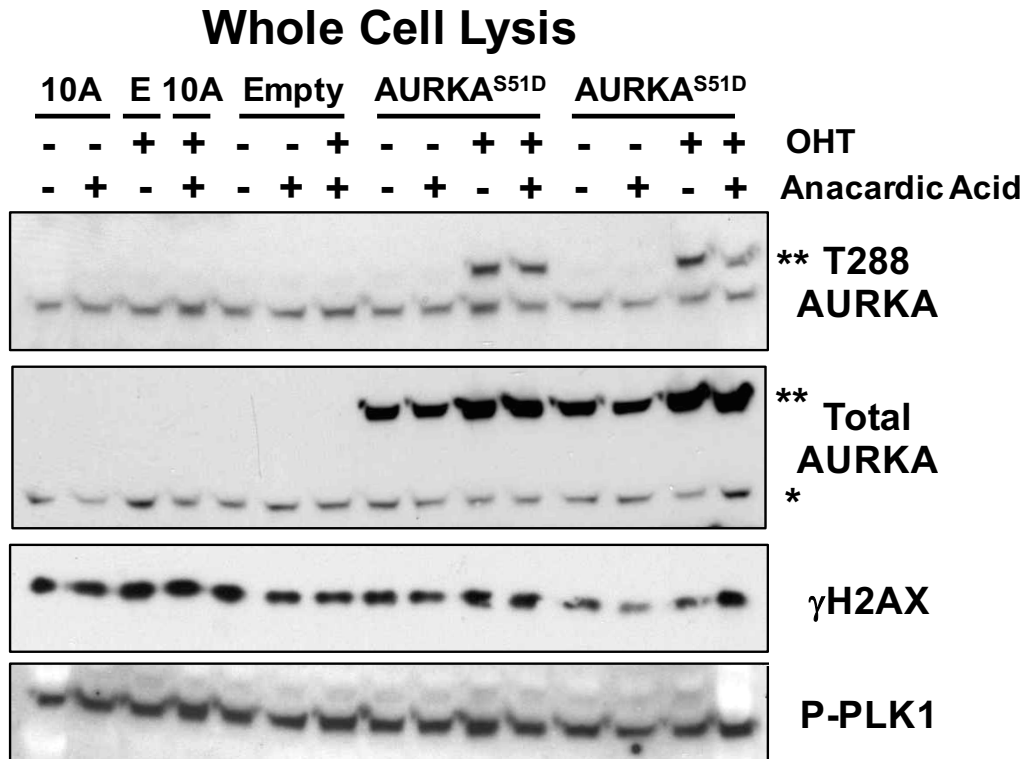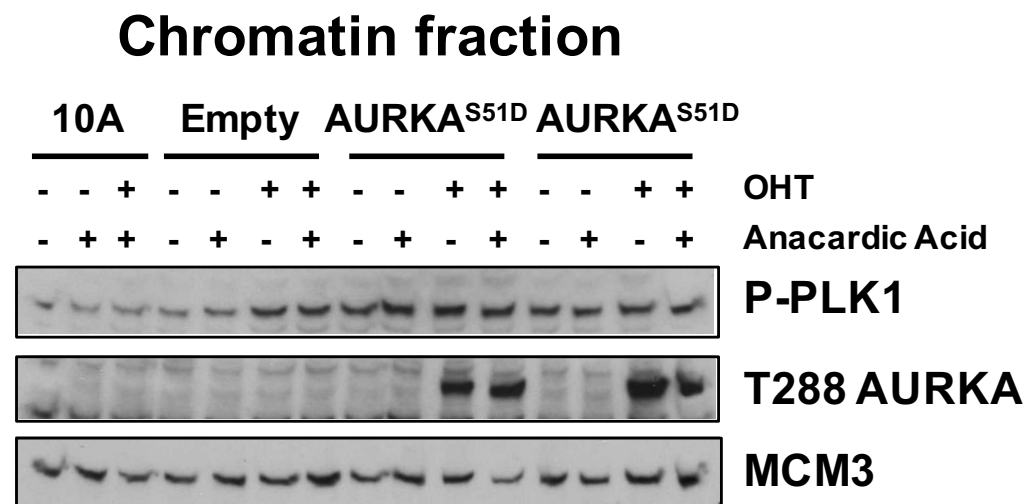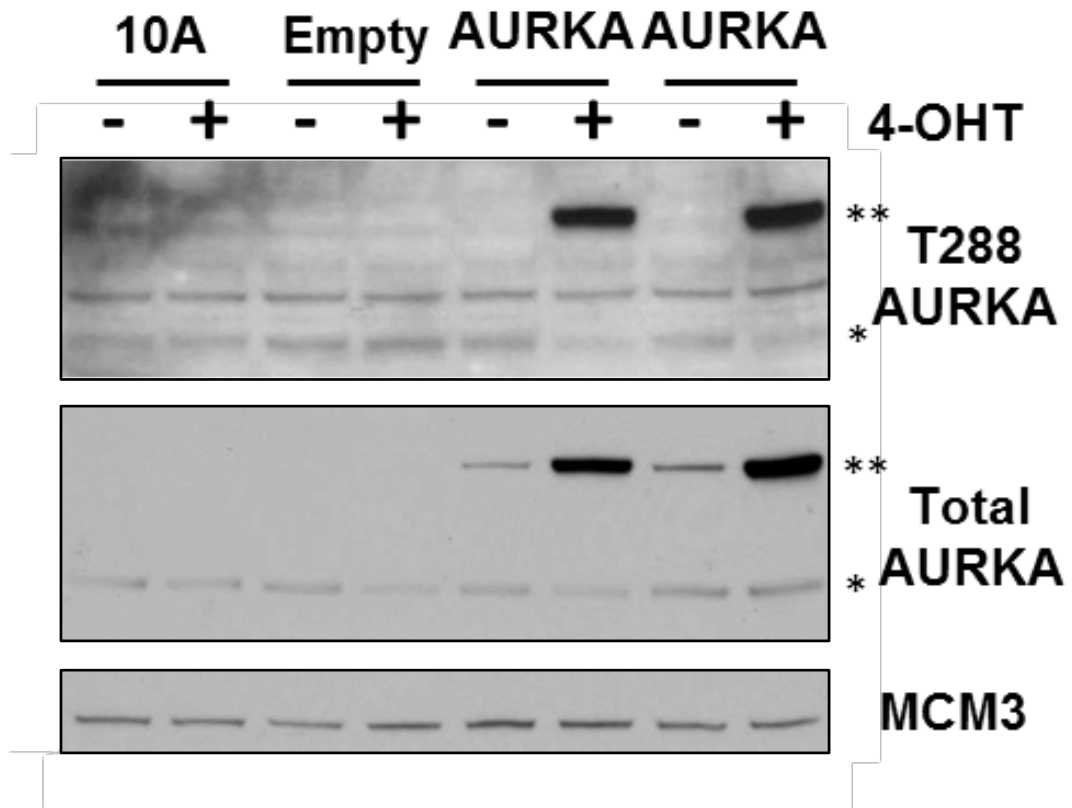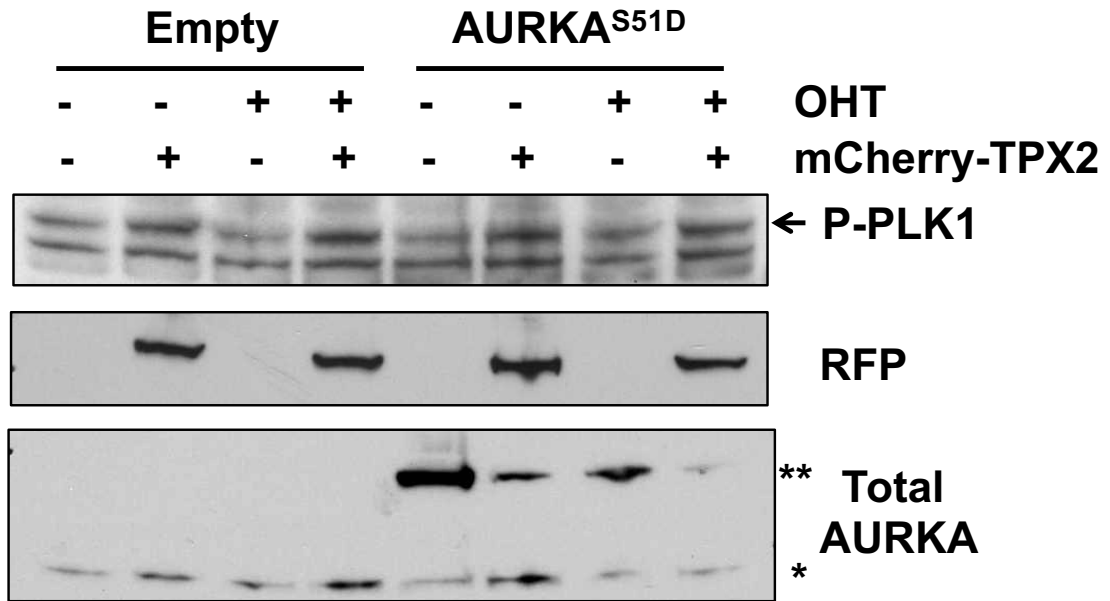
Figure 2.3

Figure 2.4

Figure 2.5
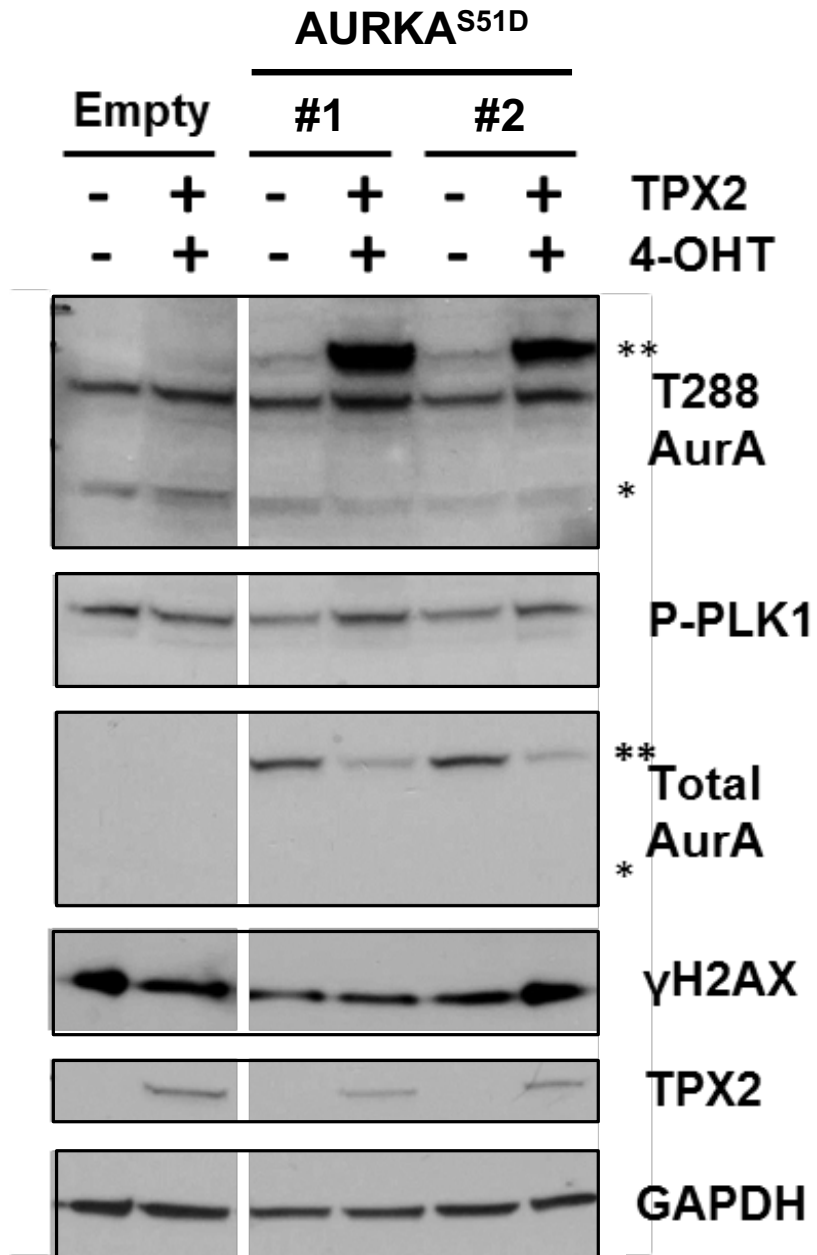
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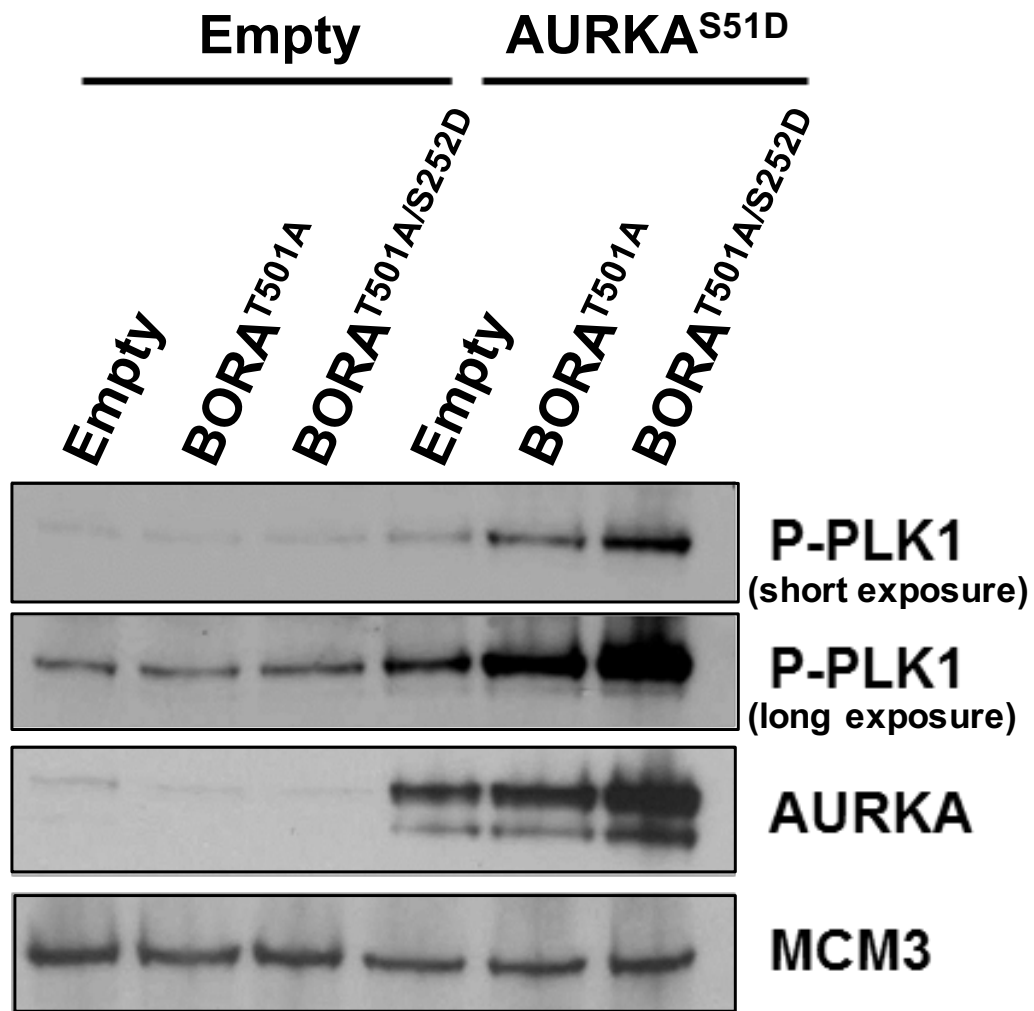
Figure 2.7

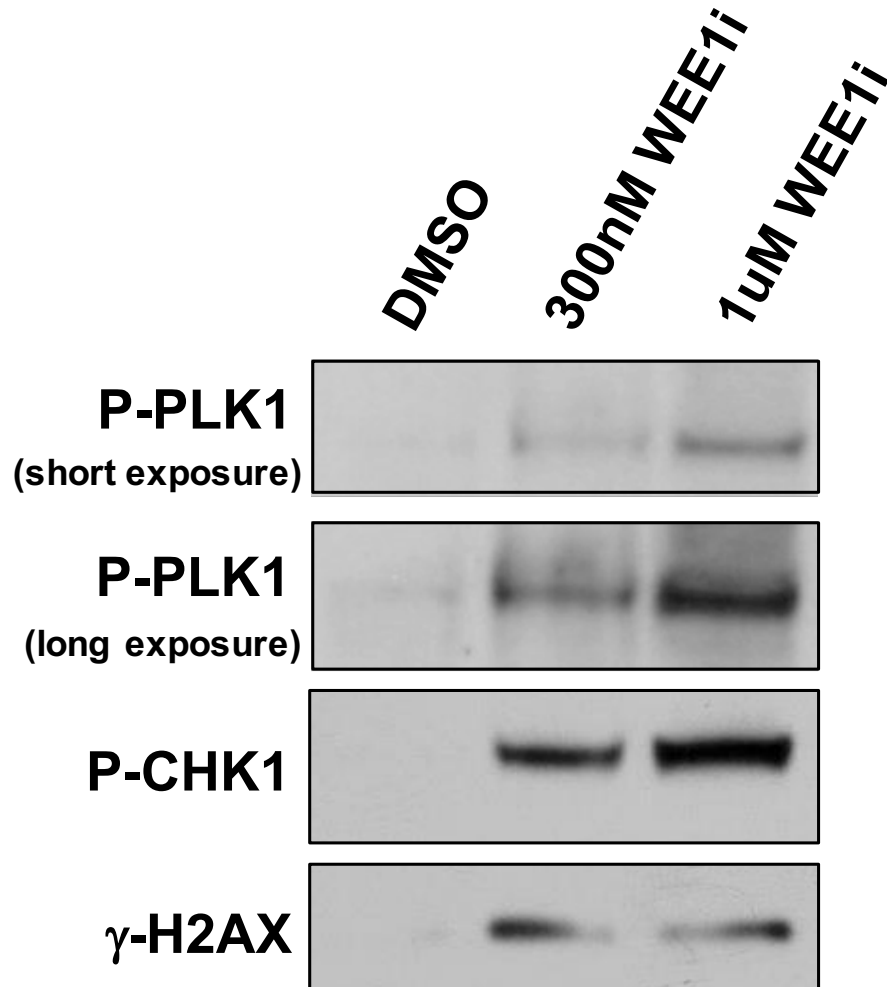# Figure 2.8
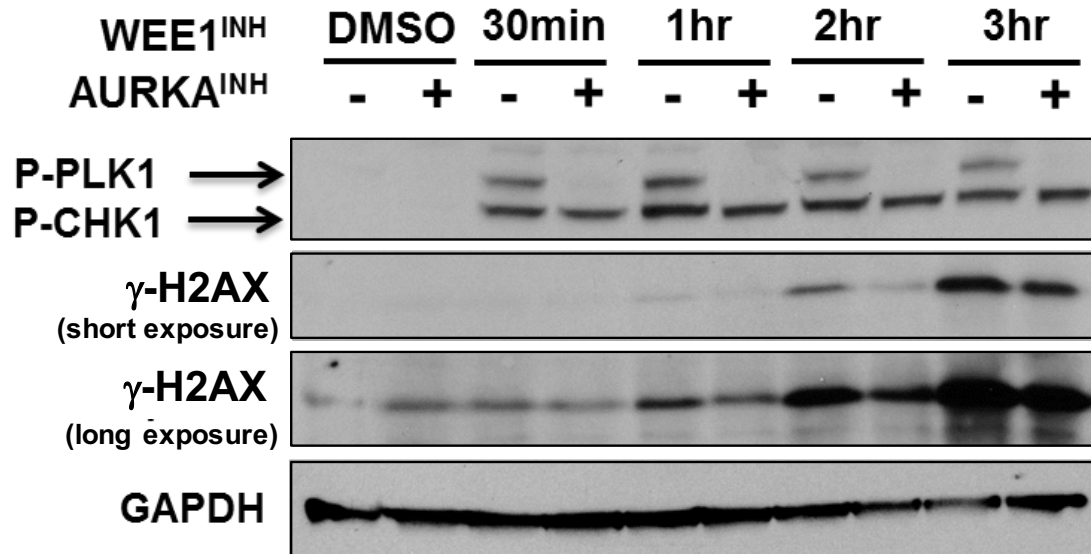
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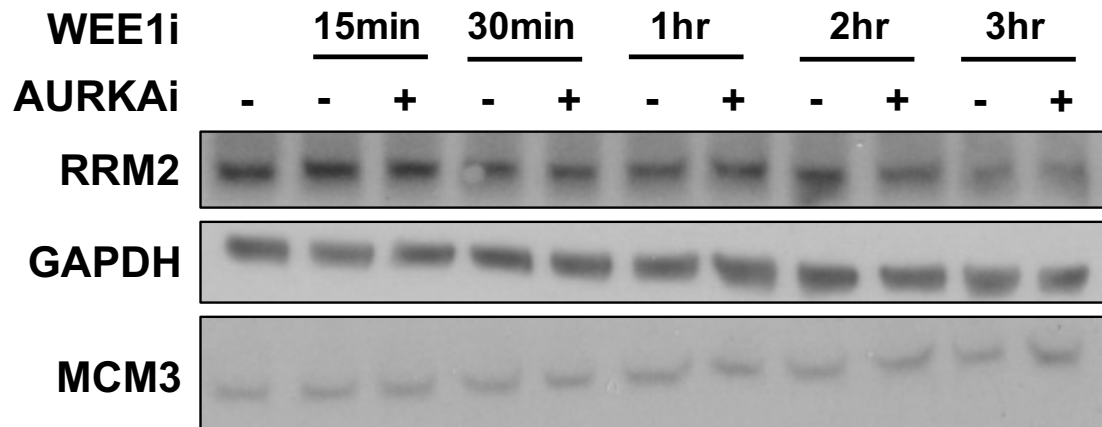
Figure 2.10

Figure 2.11

A.
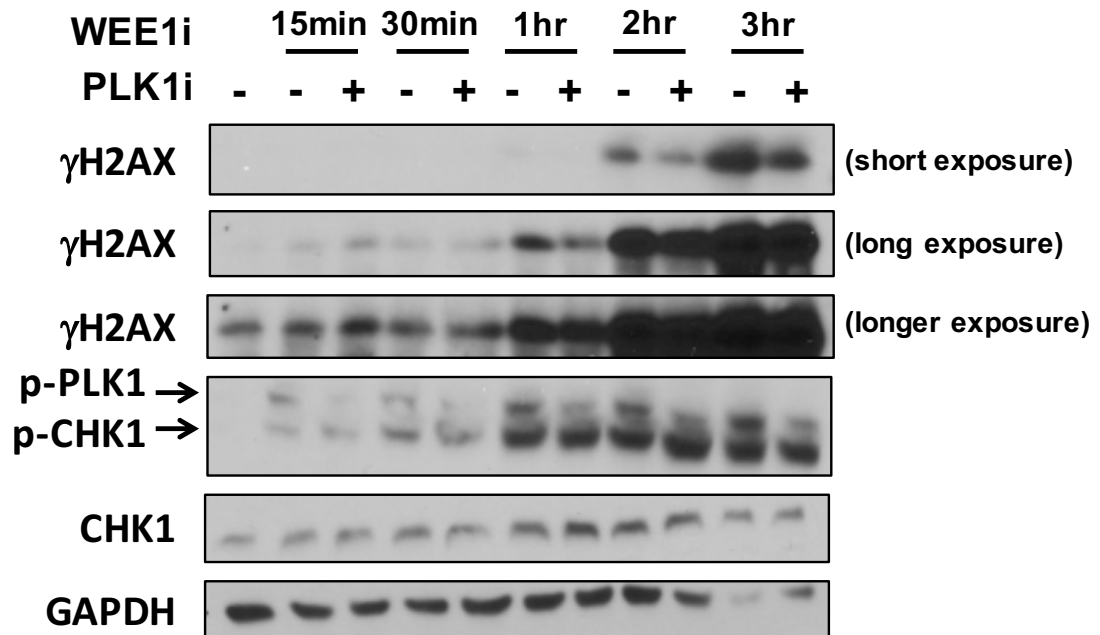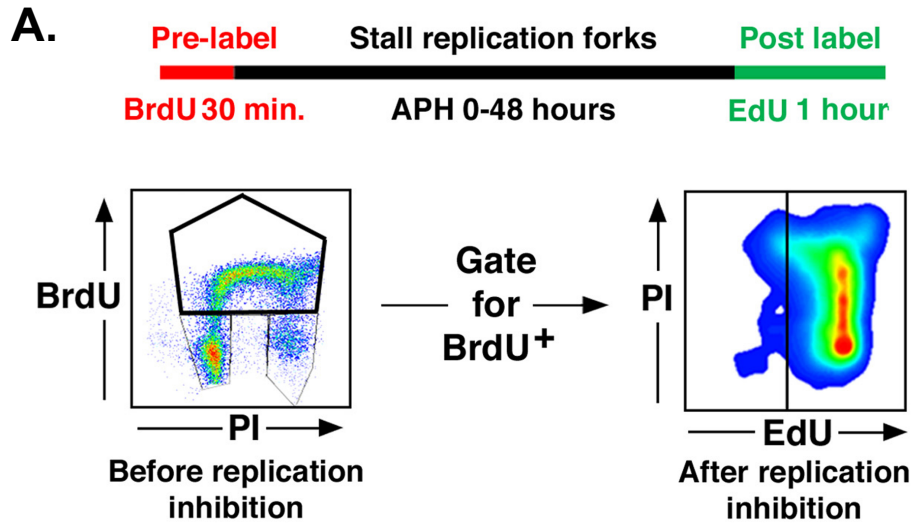
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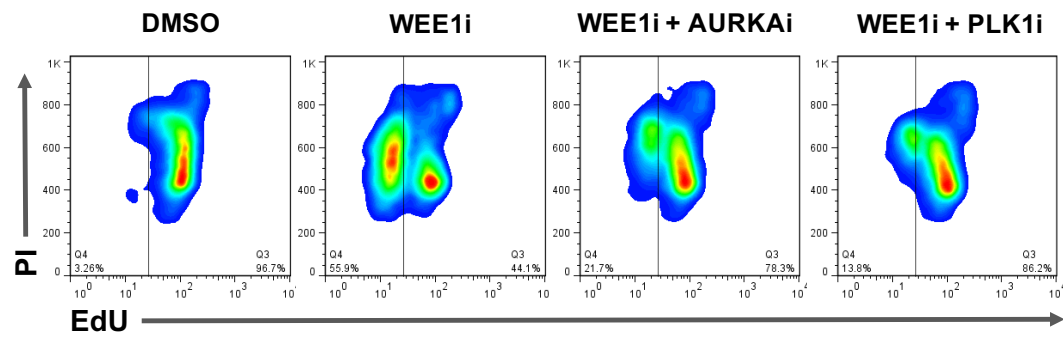
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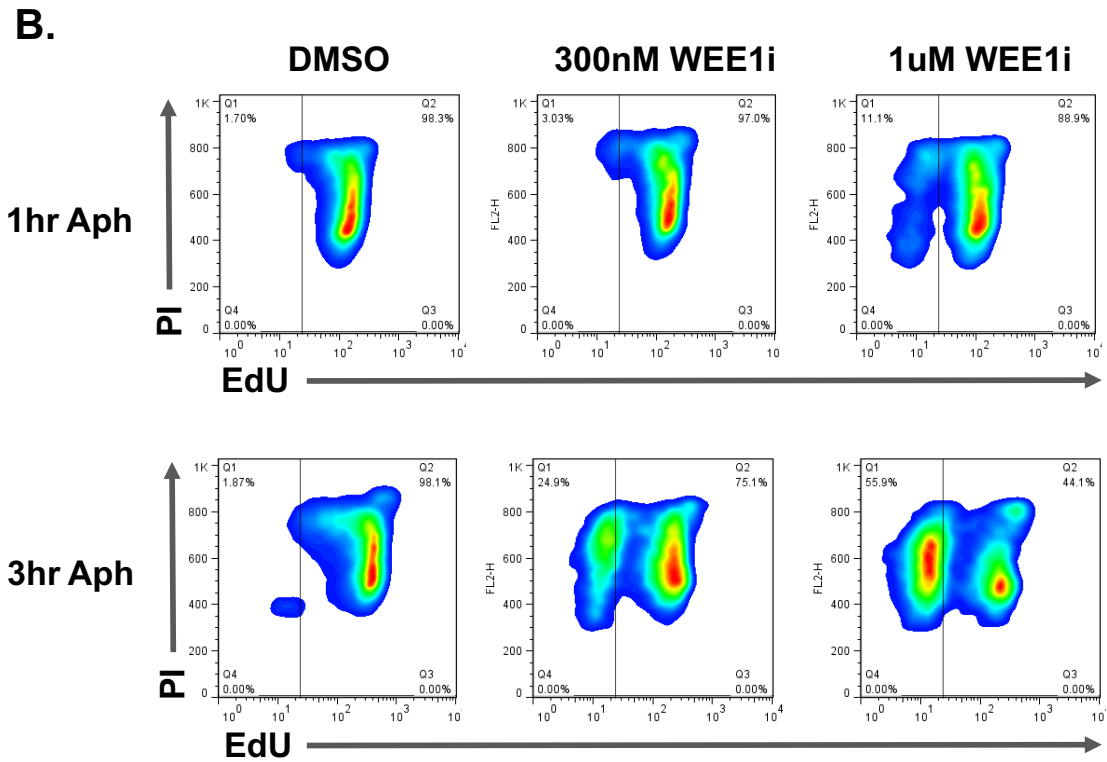
Figure 2.11

C.

Figure 2.12

A.
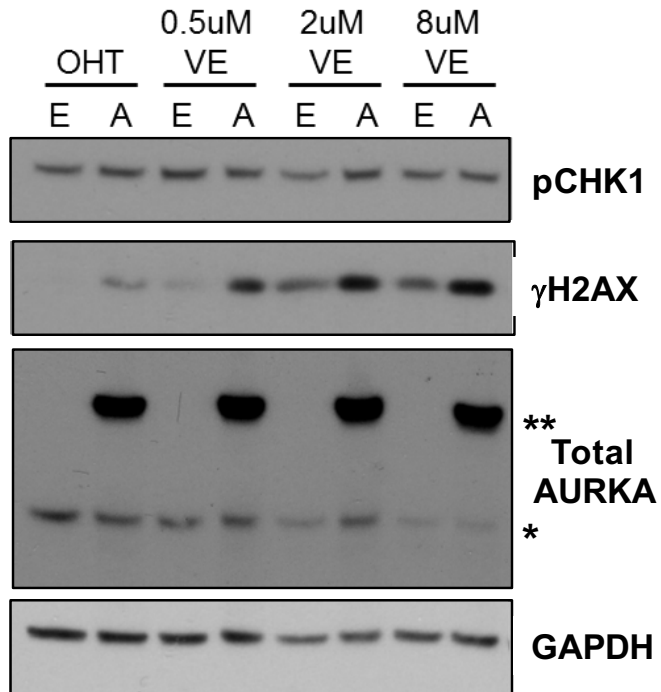
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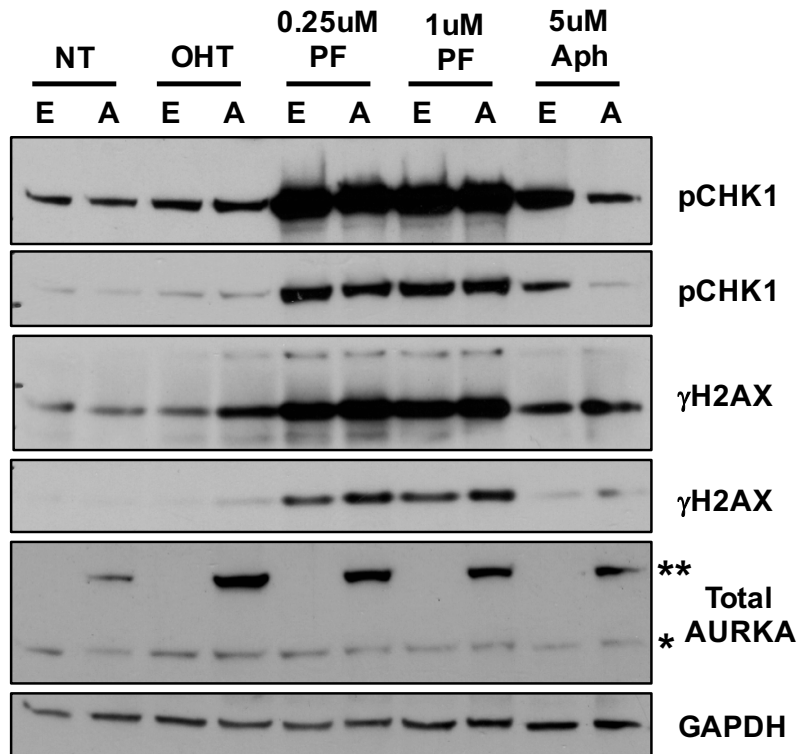
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CHAPTER 3: Cell cycle checkpoint function of ATR is required for replication fork stability.

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Summary

Checkpoints control cell cycle progression in the event of DNA damage or incomplete DNA replication. The DNA replication checkpoint is regulated by the ATR-CHK1 pathway, which also stabilizes stalled replication forks and prevents their collapse into DNA double-strand breaks. Two distinct models have been proposed to explain how ATR stabilizes stalled forks: 1) through local modulation of fork remodelers, and 2) through inhibition of CDK-dependent pathways, which prevents cell cycle progression. To test if CDK1-activation is sufficient to promote fork collapse, we inhibited WEE1, which short-circuits the cell cycle checkpoint function of ATR without inhibiting its fork-proximal activity. Using flow cytometry based fork collapse assays and genome-wide detection of RPA accumulation (RPA-ChIP Seq), we find that WEE1 and ATR inhibition cause similar levels of fork collapse at overlapping genomic locations in a CDK1-depdendent manner under conditions of partial replication inhibition (low dose aphidicolin). Notably, treatment with WEE1 inhibitor (WEE1i) alone was also sufficient to cause replication fork collapse, and did so more rapidly and to a higher degree than ATRi alone. Interestingly, clear differences in site specificity were observed when WEE1i was combined with ATRi, suggesting that particular sites in the genome may be slightly more dependent on the local functions of ATR than others. Thus, cell cycle checkpoint abrogation by WEE1i is sufficient to cause replication fork collapse in a manner similar to ATRi; however, site-specific roles for ATR remain.

Introduction:

Genome maintenance is essential for cell viability and disease prevention. To ensure genomic stability, cells have checkpoints that regulate cell cycle progression (Kastan and Bartek, 2004b). The intra-S phase DNA replication checkpoint is regulated by the ataxia telangiectasia and Rad3-related protein (ATR) pathway, which is activated by slowing or stalling of replication fork progression, generally referred to as replication stress (Zou and Elledge, 2003; Karlene A. Cimprich and Cortez, 2008). Replication stress can result in the accumulation of single-stranded DNA (ssDNA) through uncoupling of the DNA polymerase from the MCM2-7 helicase (Byun *et al.*, 2005; Zeman and Cimprich, 2014a). The resulting ssDNA is then coated by replication protein A (RPA), a ssDNA binding protein that recruits the ATR-ATRIP complex to stabilize uncoupled forks and prevent their collapse into double-stranded breaks (DSBs) (Cortez *et al.*, 2001; Zou and Elledge, 2003).

Previous studies have proposed that ATR stabilizes stalled forks either through regulation of cell cycle-dependent pathways that ultimately inhibit cell cycle progression and origin firing or through local modulation of fork remodelers (Costanzo *et al.*, 2003; E. J. Brown and Baltimore, 2003; Couch *et al.*, 2013b; Yazinski and Zou, 2016). ATR's cell cycle checkpoint function is primarily mediated through phosphorylation of its effector protein, CHK1 (Liu *et al.*, 2000; Zhao and Piwnica-Worms, 2001). Once activated, CHK1 phosphorylates CDC25A, marking it for proteasomal degradation, thereby preventing origin firing (Mailand *et al.*, 2000; Feijoo *et al.*, 2001; Zhao and Piwnica-Worms, 2001; Chen, Ryan and Piwnica-Worms, 2003b). CHK1 also phosphorylates CDC25C, causing it to bind to the 14-3-3 protein complex and sequestering it from CDK1, thereby inhibiting cell cycle progression (Peng *et al.*, 1997; Sanchez *et al.*, 1997; Liu *et al.*, 2000; Zhao and Piwnica-Worms, 2001). Additionally, CHK1 phosphorylates WEE1, a cell cycle

checkpoint kinase that blocks entry into mitosis and negatively regulates origin firing through inhibitory phosphorylation of CDK1 and CDK2, respectively (O'Connell *et al.*, 1997; Lee, Kumagai and Dunphy, 2001; Katsuno *et al.*, 2009; Kumar and Huberman, 2009; Sørensen and Syljuåsen, 2012). ATR has also been shown to protect forks from endonuclease complexes, such as SLX4-SLX1 or MUS81-EME1 that can cleave unprotected forks into DSBs, by inhibiting premature activation of CDK-dependent pathways (Castor *et al.*, 2013; Garner *et al.*, 2013; Ragland *et al.*, 2013; Szakal and Branzei, 2013b; Wyatt *et al.*, 2013a, 2017; Alessandra Pepe and West, 2014a; Sarbajna, Davies and West, 2014).

ATR locally stabilizes forks by directly regulating fork remodelers, such as SMARCAL1, which has been shown to play a role in fork protection (Couch *et al.*, 2013b), and Polη, which is involved in post-replicative repair (Göhler *et al.*, 2011). Additionally, ATR has many other fork-bound substrates that promote replication fork stability, though the mechanisms by which this occurs are still poorly understood (Karlene A Cimprich and Cortez, 2008; Saldivar, Cortez and Cimprich, 2017). While much progress has been made in delineating how ATR maintains fork stability, it remains unclear which of ATR's fork-stabilizing functions is essential for fork integrity. Whether ATR maintains fork integrity through its cell cycle checkpoint role or its local fork protection role, and whether some sites of the genome are more dependent upon one role over the other for genome stability remains to be shown.

To separate these two ATR functions, WEE1 inhibition (WEE1i) was used to bypass ATR's cell cycle checkpoint activity without affecting ATR's local replication fork functions. Wee1 inhibition permitted us to test if CDK activation was sufficient to promote replication fork collapse in a manner that emulates ATR inhibition. By comparing the

effects of WEE1i to those observed by ATR inhibition (ATRi), ATR's cell cycle checkpoint function could be distinguished from its local fork protection role.

Herein we show that in the context of partial replication inhibition (low dose aphidicolin), WEE1i and ATRi cause similar levels of fork collapse at overlapping genomic locations. Fork collapse at these sites was dependent on CDK1 and Aurora A kinase (AURKA), consistent with our previous study (Ragland *et al.*, 2013). In the absence of direct DNA polymerase inhibition, WEE1i was sufficient to cause replication fork collapse and did so more rapidly and to a higher degree than ATRi alone. Consistent with this observation, we found that WEE1i depleted ribonucleotide reductase (RRM2) levels to a greater extent than ATRi, leading to nucleotide insufficiency and, ultimately, to fork collapse. Surprisingly, while WEE1i was sufficient to cause fork collapse at many genomic loci that overlap with sites identified from ATRi and partial polymerase inhibition, WEE1i also produced a distinct subset of fork collapse sites not observed from ATRi, either in the presence or absence of partial DNA polymerase inhibition. In summary, while WEE1i is sufficient to cause replication fork collapse in a manner similar to ATRi, WEE1i further promotes fork collapse through mechanisms distinct from ATRi. Future studies will examine these genomic sites for features that render them vulnerable to fork collapse under these conditions. Our studies suggest that the cell cycle checkpoint function of ATR is essential to maintain fork integrity under conditions of low replication stress.

Results:

WEE1 and ATR inhibition results in similar levels of DNA damage under conditions of replication stress

Previous studies have proposed that ATR stabilizes stalled replication forks through both its cell cycle checkpoint function and its local role at the fork (Paulsen and

Cimprich, 2007; Couch *et al.*, 2013b; Zeman and Cimprich, 2014a). Our laboratory has previously shown that fork collapse under conditions of ATR loss is mediated through activation of the CDK1 signaling pathway (Ragland *et al.*, 2013). This suggests that ATR's role in regulating cell cycle progression to prevent premature activation of mitotic signaling pathways is important in fork stability. Thus, we hypothesized that ATR stabilizes stalled forks primarily through its cell cycle checkpoint role. By comparing WEE1 inhibition, which inhibits ATR's cell cycle checkpoint function while retaining its local fork protection function, to ATR inhibition, we can define which of ATR's functions are essential to stabilize stalled forks (Figure 3.1).

IC$_{50}$ concentrations of the WEE1 and ATR inhibitors were initially determined by looking at levels of phospho-PLK1 and phospho-CHK1 by immunoblot as readouts for WEE1 and ATR inhibitor efficiency, respectively (Supplemental Figure 3.1). In the passage-immortalized murine embryonic fibroblasts (MEFs) cell line, which is used in all experiment thereafter, the inhibitor IC$_{50}$ for WEE1 and ATR was found to be 1 μM and 0.26 μM, respectively. To ensure efficient substrate inhibition 3x the IC$_{50}$ was chosen resulting in a 3 μM and 1 μM dose used for WEE1 and ATR inhibitors, respectively.

To examine the fork stabilizing effects of the separate ATR functions, MEF cells were treated with either 3 μM of WEE1 inhibitor (WEE1i) or 1 μM of ATR inhibitor (ATRi) for 1, 2 and 4 hours (Figure 3.2). The treated cells were then assayed by flow cytometry for presence of γH2AX, a surrogate marker for DSB formation. Interestingly, WEE1 inhibition led to significantly higher levels of γH2AX compared to ATR inhibition. Because WEE1 inhibition displayed an increased capacity to cause replication fork collapse, additional inhibitor doses were examined that result in similar amounts of DNA damage as measured by levels of γH2AX assayed by flow cytometry. As expected, 500 nM of

WEE1i and 5 uM ATRi resulted in equivalent levels of γH2AX at 1, 2 and 4 hours of treatment (Figure 3.2).

WEE1 inhibition and to a lesser extent ATR inhibition induce RRM2 depletion

WEE1 inhibition has previously been shown to cause replication stress and subsequent fork collapse through RRM2 degradation-induced nucleotide depletion (D'Angiolella *et al.*, 2012; Pfister *et al.*, 2015). This finding led us to hypothesize that WEE1i-induced γH2AX levels were higher compared to ATR inhibition at 3x the IC$_{50}$ as a result of WEE1 inhibition being sufficient to cause replication stress on its own. To test if replication stress caused by WEE1 is mediated by nucleotide depletion resulting from RRM2 degradation, cells were treated with low or high doses of WEE1 and ATR inhibitors for 4 hours and examined for RRM2 protein levels (Figure 3.3). RRM2 levels were reduced in a dose-dependent manner with WEE1 inhibition and were almost undetectable at 3 μM WEE1i. Interestingly, ATR inhibition also led to a slight decrease in RRM2 levels in a dose-dependent manner, but to a lesser extent than WEE1 inhibition (Figure 3.3). The most prominent difference in RRM2 levels between WEE1 and ATR inhibition was seen at the 3x IC doses, which are also the doses at which we observe a large difference in γH2AX levels. However, RRM2 levels were comparable between 500 nM WEE1i and 5 μM ATRi, suggesting that the increase in γH2AX levels seen with WEE1i could result from replication stress induced by nucleotide depletion.

To test if replication stress contributed in part to the difference seen in γH2AX levels between 3 μM WEE1i and 1 μM ATRi, we treated MEF cells with the WEE1i or ATRi and with a low dose (0.2μM) of aphidicolin (APH), a DNA polymerase alpha and delta inhibitor, which is known to induce low levels of replication stress through fork slowing (Figure 3.4). When assayed by flow cytometry for levels of γH2AX, we found that

WEE1 and ATR inhibition following partial replication stress led to similar levels of γH2AX accumulation at all time points, again suggesting that the original difference observed between WEE1 and ATR inhibition was due to an inherent ability of WEE1i to cause replication stress. More importantly, these data together suggest that the cell cycle checkpoint role of ATR is important in preventing γH2AX accumulation in the absence or presence of replication stress. As expected, when the experiment was repeated with high dose of APH (5 μM), which induces complete fork stalling, WEE1i and ATRi under high replication stress conditions resulted in similar levels of γH2AX, indicating the requirement for the cell cycle checkpoint role of ATR in fork stabilization (Supplemental Figure 3.2).

WEE1 inhibition activates CDK1 and ATR signaling

To determine what other molecular signaling differences differentiate WEE1i and ATRi treatments, MEF cells were treated with the inhibitors for 4 hours and probed by immunoblot for substrates of the WEE1 and ATR signaling pathways (Figure 3.3). As expected, WEE1 inhibition significantly reduced phospho-CDK1 levels and increased phospho-PLK1 levels, both of which were unaffected by ATR inhibition at the 4 hour time point. Additionally, WEE1 inhibition alone significantly increased phospho-CHK1 levels, a readout of replication stress and ATR activation, again supporting that WEE1 inhibition alone can induce replication stress. Because WEE1 inhibition alone is sufficient to induce replication stress, it increases dependence on the ATR signaling pathway to prevent fork collapse. However, under conditions of WEE1 inhibition, the cell cycle checkpoint function of ATR is inhibited, leaving forks vulnerable to collapse, ultimately leading to DNA damage. Such an outcome is not observed with ATR inhibition alone since no exogenous replication stress is triggering a dependence on the ATR signaling

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pathway for fork stability, and thus, inhibition of the pathway does not lead to DNA damage. In contrast, aphidicolin-induced replication stress creates a dependence on the ATR signaling pathway such that both WEE1 and ATR inhibition results in similar levels of DNA damage.

WEE1 inhibition leads to greater DNA replication inhibition compared to ATR inhibition

Notably, phospho-CHK1 levels increase within 15 minutes of WEE1i treatment but RRM2 levels do not decrease until 3 hours of treatment, suggesting that there might be another mechanism by which WEE1 inhibition leads to fork collapse, such as premature mitotic entry. To test if WEE1 and ATR inhibition led to similar replication dynamic profiles, we looked at replication fork collapse and restart as a functional output of fork stability. MEF cells were treated with WEE1 or ATR inhibitors for 4 hours, after which the drugs were washed out (Figure 3.5a). The cells were then allowed to reinitiate DNA replication in the presence of EdU. Using flow cytometric analysis, we determined the percentage of S phase cells that did not incorporate EdU as a readout for cells that were unable to restart replication. As expected, 3 μM WEE1i prevented DNA replication restart more robustly than 1 μM ATRi, again suggesting that WEE1i alone is sufficient to cause replication stress that results in replication defects. Consistent with the DNA damage data, 5 μM ATRi and 0.5 μM WEE1i displayed similar levels of DNA replication restart capabilities.

The experiment was repeated in the context of low replication stress (0.2 μM APH) to determine if WEE1i-induced replication stress was responsible for the increased replication defects seen in WEE1i-treated cells (Figure 3.5b). Interestingly, unlike what was observed with DNA damage, additional replication stress did not equalize the

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amount of replication defects observed between 3 μM WEE1i and 1 μM ATRi, possibly indicating that different mechanisms might regulate the DNA damage and replication restart phenotypes observed with WEE1i treatment.

To test if high levels of replication stress could equalize the replication fork restart defects seen between WEE1i- and ATRi- treated cells, the replication restart assay was performed with 5 μM APH for 1, 2 and 4 hours of treatment (Figure 3.5c). At 2 hours of treatment, 3 μM WEE1i still had significantly increased inability to restart replication compared to 1 μM ATRi but by 4 hours of treatment, that difference disappeared. This data suggests that by 4 hours, the elevated levels of replication stress induced by 5 μM APH can level the WEE1i-induced replication stress in the ATRi-treated samples to cause similar replication defects. Because a replication defect difference is still seen by 2 hours of treatment between 1 μM ATRi and 3 μM WEE1i, at which point RRM2 levels are not decreased, it is possible that a nucleotide depletion independent mechanism is responsible for the replication defects observed. Together, these data establish that the cell cycle checkpoint role of ATR is important in preventing DNA replication inhibition in the absence or presence of replication stress.

CDK1 inhibition decreases γH2AX accumulation and rescues DNA replication inhibition induced by WEE1 and ATR inhibition

Based on the differences observed between 1 μM ATRi and 3 μM WEE1i in inducing DNA damage and causing replication restart defects in the context of replication stress, we hypothesized (1) that cell cycle checkpoint loss was inducing DNA damage and replication defects and (2) that different mechanisms might be regulating these phenotypes. To gain insight into the molecular mechanism by which WEE1 and ATR inhibition lead to DNA damage and DNA synthesis inhibition, we used a dual

CDK1/2 inhibitor, RO-3306, at a dose that inhibits CDK1 without affecting CDK2. We tested the effects of CDK1 inhibition on the ability of MEFs to restart replication under conditions of WEE1 or ATR inhibition with or without 0.2µM APH treatment. Cells were subsequently assayed for DNA damage and for the ability to restart DNA replication (Figures 3.6). CDK1 inhibition was able to rescue DNA damage across both concentrations of WEE1 and ATR inhibitors with no or low dose of APH (Figure 3.6a). In addition, CDK1 inhibition also allowed cells to restart replication under all conditions (Figure 3.6b). These data suggest that ATR stabilizes forks through inhibition of the CDK1 pathway, which acts as a major signaling axis in its cell cycle checkpoint function.

To further tease apart the signaling pathways through which WEE1 inhibition leads to DNA damage and replication restart defects, we next inhibited AURKA, which functions downstream of CDK1 (Hirota *et al.*, 2003b; Van Horn *et al.*, 2010). Using the same assays described above, MEF cells were examined for their ability to restart replication and to cause DNA damage after treatment with an AURKA inhibitor (Figure 3.6a). Under conditions of high-dose APH, AURKA inhibition rescued DNA replication that was unable to restart under WEE1 or ATR inhibition (Figure 3.6b). These data suggest that the inability of DNA replication to restart from WEE1 and ATR inhibition is mediated through the CDK1-AURKA signaling axis. Under conditions of no or low replication stress, we found that AURKA inhibition only rescues the block in DNA replication induced by WEE1 inhibition and, though not significant, displays some level of rescued DNA replication under conditions of ATR inhibition as well. Surprisingly, while AURKA inhibition rescued DNA replication restart, it did not rescue the DNA damage induced by WEE1 or ATR inhibition. This finding suggests that DSB formation occurs upstream of AURKA inhibition or through a different pathway downstream of CDK1, such as through regulation of endonuclease fork cleavage or through nucleotide metabolism.

In support of nucleotide levels having an effect on the DSB formation observed with WEE1i and ATRi, we observed that CDK1 inhibition restores RRM2 levels in WEE1i treated cells under conditions of no or low replication stress (Figure 3.6c,d). AURKAi was not sufficient to restore RRM2 levels (Figure 3.6c,d). These data suggest that in the context of WEE1i, CDK1i may be rescuing DSB formation through restoration of nucleotide levels. Because RRM2 levels do not appear to be altered with ATRi, we believe that other DSB forming mechanisms are at play downstream of CDK1, such as endonuclease fork cleavage.

RPA ChIP-Seq peaks are similar in WEE1 and ATR inhibited cells under conditions of replication stress

Our data indicates that ATR and WEE1 inhibition result in similar levels of DNA damage in a population of cells under conditions of replication stress. To better understand if those conditions led to similar fork stalling signatures at the genomic level, we performed RPA-chromatin immunoprecipitation (RPA-ChIP) on MEF cells following treatment with WEE1 or ATR inhibition in combination with low-dose APH to retrieve genomic sites that accumulate RPA. As mentioned previously, RPA is a protein that binds ssDNA that accumulates at stalled forks and at resected DSB ends resulting from collapsed forks; therefore, RPA accumulation defines regions sensitive to fork stalling or collapse.

RPA ChIP was performed on MEF cells treated for 18 hours with low-dose APH (0.2μM) and 1 μM ATRi or 0.5 μM WEE1i (ATRi+APH and WEE1i+APH, respectively). The lower doses of inhibitors were used for therapeutic relevance as WEE1 and ATR inhibitors are synthetic lethal when combined in mouse models (Simpkin lab data) and these doses displayed similar levels of DNA damage under conditions of replication

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stress. A higher dose of APH was not used in these experiments since complete replication inhibition would result in site mapping of the replication fork location at the time of treatment and not of sites that are specifically sensitive to WEE1 or ATR inhibition. RPA ChIP was similarly performed on DMSO-only treated (UT) cells as a treatment control, and pre-ChIP input DNA from each condition was isolated for normalization. We used next generation sequencing (NGS) to perform genome-wide mapping of the RPA ChIP retrievals (Figure 3.7a).

After normalizing the retrievals to the input to create ratio tracks, we identified sites that had statistically significant read enrichments (>4-fold over input, p-value $<10^{-3}$) in both of two biological replicates (Figure 3.7a). These sites were then compared to similarly normalized and evaluated DMSO-treated controls. In total, 216 and 913 sites of significant RPA enrichment were identified in ATRi+APH and WEE1i+APH conditions, respectively, that were not observed in the DMSO-treated controls (Figure 3.7b). As expected, the majority (135 of 144) of loci identified in the ATRi+APH-treated cells were found in the WEE1i+APH-treated cells. The 9 sites that appear to be unique to the ATRi+APH condition displayed some level of read accumulation in the WEE1i+APH coverage tracks and so would have to be confirmed as unique by qRT-PCR, which is more quantitative. If these sites are unique, this would indicate that a few sites in the genome depend on the local roles of ATR to maintain fork stability. Notably, WEE1i+APH treatment resulted in an additional 711 sites of significant RPA accumulation, consistent with previous observations that WEE1 inhibition alone may be sufficient to cause replication stress.

Intrigued by the differences in DNA damage and synthesis we observed between ATR and WEE1 inhibition alone (Figures 3.2 and 3.5a), we performed RPA ChIP-Seq to better understand the genomic profile of perturbed loci under these two conditions in the

absence of exogenous replication stress. The ChIP retrievals were derived as described previously in cells treated with low doses of WEE1 or ATR inhibitor without APH treatment. We identified 88 and 269 sites of significant RPA enrichment in ATRi and WEE1i conditions, respectively (Figure 3.7b). As expected, WEE1 inhibition alone produced 3 times more sites than ATR inhibition alone, with 196 of 269 of those sites unique to WEE1 inhibition and 17 of 88 being unique to ATRi inhibition. 67 of 88 of the ATRi sites overlapped with WEE1i sites. Future studies are required to confirm that unique sites are truly unique and if so to determine what features might distinguish them and make them more dependent on the local functions of ATR.

Since WEE1 inhibition alone causes replication stress, we found it appropriate to compare RPA ChIP retrievals from cells treated with WEE1i to those treated with ATRi+APH. Interestingly, only 112 out of 216 ATRi+APH sites were found to overlap in both conditions, with 151 sites unique to WEE1i and 95 unique to ATRi+APH (Figure 3.7b). This suggests that the replication stress induced by WEE1i affects sites differently than the stress induced by aphidicolin (see Discussion).

Finally, to assess if ATRi+APH treatment compared to WEE1i+APH treatment lead to similar genomic loci but with different signal intensity, we used Spearman correlation to measure how similar the signal intensity of each overlapping sites between both conditions was (Figure 3.7c; Sup. Fig. 3.3). We found that the majority of peaks shared relative similarity in peak intensity between all pairwise condition observed with a few exceptions highlighted in red (Figure 3.7c; Sup. Fig. 3.3).

Together these data indicate that under conditions of replication stress, the cell cycle checkpoint function of ATR is crucial in maintaining replication fork stability at specific loci. In the absence of replication stress, though the majority of sites depend on the cell cycle checkpoint function of ATR for fork stabilization, 25% of sites found in

ATRi-treated cells might depend more on the local functions of ATR for fork stabilization. Notably, the majority of shared sites between conditions showed similar peak signal intensities, suggesting that genome-wide effects observed at a cellular level, like the level of DNA damage, are likely a result of the number of sites affected by the treatment rather than by an increased frequency (amplified ChIP signal intensity) of specific sites breaking.

Discussion:

The results presented here are the first to demonstrate that in the absence and presence of partial polymerase inhibition the cell cycle checkpoint function of ATR is almost entirely responsible for maintaining fork stability. Additionally, we show that fork collapse is dependent on CDK1, which we hypothesize to be mediated through its control of endonucleases and/or of nucleotide levels, and that replication fork restart is dependent in part on the CDK1-AURKA pathway. Notably, WEE1i alone is sufficient to cause replication fork collapse, and did so more rapidly and to a higher degree than ATRi alone, which we propose results from WEE1i-induced RRM2 depletion. Furthermore, unique site specificity was observed when WEE1i was combined with ATRi, suggesting that particular sites in the genome are more dependent on the local functions of ATR than others. Our characterization of fork collapse induced by WEE1i and ATRi provides novel insight into how these inhibitors are affecting specific loci genome-wide and how these genomic fork collapse signatures could be used for the treatment of cancer.

Without replication stress, WEE1 inhibition significantly increased phospho-CHK1 and γH2AX levels compared to ATR inhibition alone and led to fork collapse at a set of unique loci compared to ATR inhibition with or without APH. Our findings are consistent

with previous reports in the literature that have shown that WEE1 depletion leads to a marked increase in γH2AX and phosphorylation of ATR targets such as CHK1 and RPA (Beck *et al.*, 2010, 2012; Domínguez-Kelly *et al.*, 2011; Heijink *et al.*, 2015). Together, these data suggest that WEE1 inhibition alone is sufficient to induce replication stress, causing a dependence on the ATR signaling pathway to prevent fork collapse. Under conditions of WEE1 inhibition, the cell cycle checkpoint function of ATR is inhibited leaving forks vulnerable to collapse, suggesting that ATR's local fork protection role is not sufficient to prevent fork collapse. DNA damage is not observed with ATR inhibition alone because no replication stress is triggering a dependence for the ATR signaling pathway for fork stability so inhibition of the pathway has no effect. In contrast, when cells are treated with aphidicolin, aphidicolin-induced replication stress creates a dependence on the ATR signaling pathway such that both WEE1 and ATR inhibition result in similar levels of DNA damage. The fact that WEE1 inhibition alone is sufficient to cause replication stress could explain the difference we observe in DNA damage levels when ATR and WEE1 are inhibited under conditions of no replication stress, which is not observed under conditions of replication stress.

Our genome-wide studies confirm the DNA damage data collected at a cell population level. Indeed, ATRi with or without replication stress affected the same genomic loci as those observed with WEE1i, highlighting the importance of the cell cycle checkpoint pathway in preventing replication fork collapse at all genomic loci observed. WEE1i alone gave rise to 25 unique loci. Interestingly, these sites were not affected when replication stress was added though it remains unclear why. As expected, with aphidicolin addition, 319 sites become uniquely sensitive to fork collapse and finally when both ATR and WEE1 are inhibited combined with aphidicolin a new set of 322

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sites appear to specifically be sensitive to that combination treatment, suggesting that the local functions of ATR might somehow help stabilize these sites under conditions of replication stress when WEE1 is inhibited. The mechanism by which this occurs is unclear and insight might be gained from studying features that characterize these sites. Additionally, future studies would include determining which of these sites collapse into DSBs by analyzing our genome-wide data generated using the Breaks Identified by TdT-Labeling (BrITL) technique, which detects DSBs as well as by understanding which of these sets of loci can be rescued by CDK1 or AURKA inhibition.

Studies have shown that WEE1 inhibition is lethal to cancer cells through dNTP starvation-induced replication stress because WEE1 inhibition promotes ubiquitin-mediated proteolysis of RRM2 (D'Angiolella *et al.*, 2012; Pfister *et al.*, 2015). Consistent with this, we observe a drastic decrease in RRM2 levels in our system under WEE1 inhibition, which we do not see with ATR inhibition. In addition, RRM2 loss is dose dependent, with increased RRM2 loss seen at the higher WEE1i dose, lending insight into the differences we saw in levels of DNA damage between ATR and WEE inhibition at the high doses. In support of RRM2 depletion contributing to WEE1i-induced fork collapse in our system is our finding that a CDK1 inhibitor, RO-3306, which rescues fork collapse, also restores RRM2 levels. However, some studies have reported that though nucleotide shortage induces WEE1i-induced DNA damage, it is not the only contributor (Beck *et al.*, 2012). Future studies, such as overexpression of non-degradable RRM2 mutants under WEE1i, are needed to investigate the extent to which RRM2 depletion contributes to WEE1i-induced replication stress in our system.

Notably, the most surprising finding in the genome-wide study was that only 54% of ATRi+Aph sites overlapped with WEE1i sites. This suggests that the replication stress induced by aphidicolin and WEE1 inhibition affect sites differently. Hydroxyurea (HU)

induces replication stress through nucleotide depletion, similarly to WEE1 inhibition. Interestingly, HU is known to deplete nucleotide pools unevenly, preferentially depleting dATP and dGTP (Collins, Oates and Collins, 1987). Based on these findings, it would be expected for HU, and in a similar manner WEE1 inhibition, to stall forks preferentially at sites that are enriched in adenine and guanine. Conversely, aphidicolin is a polymerase inhibitor and so would be expected to stall forks either more stochastically or in genomic loci where polymerases naturally have difficulty replicating DNA due to sequence-specific structure formations. It would be interesting to see if the sites unique to WEE1i are enriched in As and Gs and if the ones unique to ATRi+Aph are enriched in hard-to-replicate sequences.

Interestingly, only CDK1 inhibition, but not AURKA inhibition, rescues γH2AX induction by WEE1i and ATRi, yet both CDK1 and AURKA inhibition can rescue DNA replication inhibition that results from ATRi and WEE1i. Studies from our laboratory have previously shown that the CDK1-AURKA-PLK1 pathway is important in replication restart under conditions of ATR depletion and replication stress but that PLK1 inhibition was not sufficient to prevent DSB formation (Ragland *et al.*, 2013). Our data is in agreement with these findings but it still remains unclear why AURKAi is insufficient to prevent γH2AX accumulation. One possible model is that CDK1 activation recruits and activates the SLX4-endonuclease complex, which is known to cleave replication forks into DSBs (Froget *et al.*, 2007; Forment *et al.*, 2011; Gallo-Fernández *et al.*, 2012; Muñoz-Galván *et al.*, 2012; Szakal and Branzei, 2013b; Gritenaite *et al.*, 2014). SLX4 has been implicated in mediating replication fork collapse in partnership with the MUS81-EME1 complex (Fekairi *et al.*, 2009; Muñoz *et al.*, 2009; Wyatt *et al.*, 2013a, 2017). In support of this model, previous reports have shown that WEE1i leads to MUS81-EME1

recruitment at forks and that MUS81 and EME1 knockdown as well as CDK1 inhibition can reverse DSB formation triggered by WEE1 depletion (Domínguez-Kelly *et al.*, 2011; Beck *et al.*, 2012; Kai J Neelsen *et al.*, 2013; Pfister *et al.*, 2015). If endonuclease recruitment to the stalled fork is upstream of AURKA it would explain why CDK1 inhibition, but not AURKA inhibition, can prevent DSB formation.

Our work and that of others support a model of ATR-mediated fork stabilization that highlights the requirement for the cell cycle checkpoint function of ATR. Indeed, we propose that ATR's cell cycle checkpoint function is essential to maintain fork integrity under conditions of low replication stress through inhibition of premature CDK1 activation. Without ATR-mediated CDK1 regulation, forks will stall as a result of nucleotide deficiency through RRM2 degradation and collapse into DSBs through SLX4-MUS81-EME1 endonucleolytic fork cleavage. In addition, our data suggests that WEE1i is inducing replication stress through means other than cell cycle checkpoint bypass, making it an interesting cancer therapeutic candidate.

Of significance, ATR and WEE1 inhibitors are currently being used in clinical trials and are being proposed as a combinatorial therapy for a multitude of cancers. Our findings contribute to improving our understanding of how WEE1 inhibitors mediate toxicity, whether combination with ATR inhibitor is beneficial, and if novel synthetic lethal interactions can be utilized to increase treatment efficacy.

Material and Methods

Cell culture

MEF 4-3 Bcl-xL cells were generated by transducing retrovirus expressing the Bcl-xL protein from the pMIG Bcl-XL plasmid (Addgene plasmid #8790) into MEF 4-3 cells. Cells were cultured in a 3% O_2 and 6% CO_2 atmosphere at 37°C in Dulbecco modified

Eagle's medium (Mediatech, MT10-013-CV) supplemented with 10% fetal bovine serum (Gemini BioProducts, 100-106), 2mM L-glutamine (Mediatech, 25-005-CI), and 100 µg/mL streptomycin, 100 units/ml penicillin (Invitrogen, 15140-122).

Inhibitor treatment

All chemical inhibitors were added to the cell culture medium at the same time. The following chemical compounds were used: DMSO (Sigma, D2650), 1 or 5 µM ATR-45 (Charrier et al., 2011) for ATR inhibition, 0.5 or 3 µM MK-1775 (Selleckchem, Cat No. S1525) for WEE1 inhibition, 0.2 or 5 µM aphidicolin (Calbiochem, CAS 38966-21-1) for polymerase inhibition, 40 µM RO-3306 (Selleckchem, Cat No. S7747) for CDK1 inhibition, or 10 µM Aurora A Inhibitor I (Selleckchem, Cat No. S1451) for Aurora A inhibition. Treatment duration is indicated in figure legends.

DNA damage assay by flow cytometry

After inhibitor treatment, cells were collected by trypsin treatment and fixed in 70% EtOH overnight. Cells were stained with FITC-conjugated phospho-S139 H2AX antibody (Millipore, 16-202A) for 2 h, followed by staining with PI solution (50 µg/ml PI, 0.1% Triton X-100, 50 µg/ml RNase, and 5 mM EDTA in PBS) for 1 h. Cells were analyzed by flow cytometry using a FACScalibur (BD) and imaged and quantified using FlowJo (Tree Star) software.

DNA synthesis assay by flow cytometry

After cells were treated with indicated inhibitors for indicated times, chemical compounds were removed by washing 3x with PBS, 1x with medium at 37°C, and 3x with PBS. Cells were then incubated in 50 µM EdU for 1 h to label newly synthesizing DNA. Cells were collected for flow cytometric analysis by trypsin treatment and fixed in 70% EtOH overnight. EdU incorporation was detected using the Alexa Fluor-647 kit for flow cytometry per the manufacturer's instructions (Invitrogen, C10634). DNA content was

determined by staining cells with PI solution for 1 h. Cells were analyzed by flow cytometry using a FACS Calibur (BD) and imaged and quantified using FlowJo (Tree Star) software.

Immunoblotting

Cells were resuspended in PBS and lysed with Laemmli sample buffer (final concentrations of 10% glycerol, 2% SDS, 60 mM Tris, pH 6.8) and boiled for 5 minutes. Protein concentration was determined by bicinchoninic acid protein assay (Thermo Fisher Scientific, 23225). The total protein quantities were normalized, loaded on a 4-20% gradient SDS-polyacrylamide gel (Bio-Rad, 456-1093) and separated by electrophoresis. Proteins were then transferred to 0.45 µM polyvinylidene fluoride (PVDF) membranes, blocked in 5% milk in TBST for 1 hour at RT, bound overnight to primary antibodies in 0.25% milk in TBST at 4°C, washed with TBST, bound to HRP-conjugated secondary antibody in 0.25% milk in TBST for 1 hour at RT, washed with TBST and then detected with chemiluminescence (Thermo Scientific, 34080). Blots were detected for phospho-PLK1 (Cell Signaling Technology, 5472S), phospho-S345 Chk1 (Cell Signaling Technology, 2348s), phospho-S139 H2AX (Millipore, 05-636), total Chk1 (Santa Cruz Biotechnology, sc-8408), phospho-S33 RPA32 (Bethyl Laboratories, A300-246A), GAPDH (US Biological, G8140-01), tubulin (Cell Signaling, cs-3873T), phospho-Y14/15 CDK1 (Santa Cruz, sc-7989), RRM2 (Santa Cruz, sc-10846).

RPA ChIP-Seq

Chromatin ImmunoPrecipitation (ChIP) assays for RPA were performed from 15 million MEF Bcl-xL cells per experiment. Cells were crosslinked with 1% formaldehyde (Sigma, F1635-25ML) for 10 minutes at room temperature with gentle rocking and the reaction was quenched by glycine addition at a final concentration of 0.125 M for 10 minutes. The cell pellet was washed in 10 ml PBS and subsequently re-suspended in 1 ml cold

PBS. The cells were then lysed in lysis buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NPZ40, 0.25% Triton X-100) for 10 minutes on ice. Cell nuclei were recovered by centrifugation at 1,500 rpm for 5 minutes at 4°C and washed twice with washing buffer (10 mM Tris-Cl pH 8.1, 200 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0). The nuclei were rinsed without disturbing the pellet in 1 ml of shearing buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris pH 7.6) and after centrifugation were re-suspended in 1 ml of shearing buffer. Chromatin was fragmented by sonication at 4°C using a Covaris S220 (5% Duty Factor, 140 PIP, 200 cycles per burst) according to manufacturer's instructions resulting in an average fragment size of 200-2000bp. The samples were centrifuged at 14,000 rpm for 20 minutes at 4°C to remove insoluble cellular debris. The supernatant was transferred to a new tube and 10X RIPA buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris pH 7.6, 11% Triton X-100, 1.1% Na-DOC) was added to a final 1X concentration.

Protein A Dynabeads (Invitrogen, 10002D) were antibody bound at 4°C overnight with rotation to either 20 µg IgG (Sigma, I8765) for pre-clear beads or to 20 µg anti-RPA32 antibody (Millipore, NA19L) for IP beads in a solution comprised of 1 ml PBS, 10 µl 100 mg/ml BSA, 10 µg bridging antibody. Once antibody-bound, the beads were washed twice with PBS. The samples were then "pre-cleared" by incubating with IgG-bound Protein A-coated Dynabeads for 4 hours at 4°C with rotation. Once pre-cleared, 1/10th of the sheared chromatin was frozen for use as an input control. The remaining pre-cleared chromatin was added to RPA32-bound Dynabeads, and incubated overnight at 4°C with rotation. The next day, the beads were washed for 10 minutes with rotation at 4°C as follows: 2x with 1 ml of RIPA buffer, 2x with 1 ml of RIPA buffer + 0.3 M NaCl, 2x with 1 ml of LiCl buffer (0.25 M LiCl, 0.5% NP-40, 0.5% NaDOC, store at 4˚C), 1x with 1 ml of TE (pH 8.0) + 0.2 % Triton X-100, 1x with 1 ml of TE (pH 8.0). The DNA was then eluted

in 100 µl of TE buffer containing 0.6% SDS and 1 mg/ml Protease K, and reverse-crosslinked at 65°C overnight. The sonicated input control was reverse-crosslinked similarly. The DNA in all samples was extracted using phenol-chloroform and resuspended in TE buffer for ChIP-qPCR analysis or NGS library preparation.

ChIP-Seq Library preparation

500-1000ng of total ChIP DNA was used for library construction using the NEBNext DNA library Kit for Illumina (NEB, E6000S). Sequencing was performed using the Illumina HiSeq 2500 and 100bp single-end reads were obtained.

Sequence Alignment, Peak calling and Visualization

Sequence Alignment and Peak calling were performed as described in Tsai and Shastri et al, 2017. Sequenced reads were trimmed using trimmomatic (Bolger et al., 2014) to remove adapter sequences and then checked for quality control using fastqc (Leggett et al., 2013). Trimmed reads were aligned to Mus musculus mm10 genome using STAR Aligner with a maximum of 3 mismatches allowed (Dobin et al., 2013). Multi-mapping was allowed for reads that mapped to up to 100 different genomic locations and reads were de-duplicated to increase the complexity of the read population. Alignment-specific quality-control metrics were used, such as strand-cross-correlation (Landt et al., 2012), finger-plots (Ramírez et al., 2016) to gauge mutual back/level of enrichment across samples, Pearson and Spearman correlations of genomic and enriched regions across samples (≥0.6), principal component analysis (PCA) for clustering assessment, and a non-arbitrary estimate of signal over the input tracks (Liang and Keles, 2012). Black-listed regions in the mm10 genome were filtered out prior to peak-calling.

For enrichment analysis, an irreproducibility rate (IDR) analysis (Landt et al., 2012) from the ENCODE project with MACS2 peak-calling program (Zhang et al., 2008) was performed on biological replicates and inputs for each experimental condition to give the

final peak list per condition. The IDR thresholds were >0.05 for self-consistency and comparison of biological replicates, and >0.005 for pooled-consistency analysis. Peaks were then filtered for those with p-value <10^{-3} and with above 4-fold enrichment over input. Regions within 2 kb of one another were merged and peaks that intersected with the peaks called in the DMSO-control RPA ChIP-Seq condition were removed.

Replicates and statistical tests

Unless otherwise noted, all data represented in figures represent three to five independent experiments. Microsoft Excel was used for all statistical analysis. Error bars represent the standard error of the mean, and P-values were calculated using the Student's unpaired, 2-tailed t-test. $P \leq 0.05$ was considered significant.

Figure Legends

Figure 3.1. Model figure: WEE1 inhibition inhibits ATR's cell cycle checkpoint function while leaving its local fork protection role intact. ATR stabilizes forks through its direct fork protection role and its cell cycle checkpoint role. WEE1 inhibition activates the CDK cell cycle controlled pathways that ATR inhibits as part of its cell cycle checkpoint role. However, WEE1 inhibition leaves ATR's direct fork protection role intact. Using WEE1 inhibition as a tool to dissect between ATR's two fork stabilizing functions, we showed that ATR's cell cycle checkpoint role is essential for fork stability.

Figure 3.2. WEE1 inhibition leads to greater accumulation of DNA damage compared to ATR inhibition. First panel shows representative examples of cell cycle flow cytometric detection of γH2AX in cells treated with DMSO, 1 µM WEE1 inhibitor or 3 µM ATR inhibitor for 4 hours. γH2AX (y-axis) as a readout of DNA damage was immunodetected in asynchronous cells, co-stained for DNA content by PI (x-axis), and visualized by flow cytometry. Percentage of γH2AX-positive cells is indicated for each condition above the

gate. Second panel shows quantification of the % of γH2AX positive cells normalized to the number of cells to account for cell cycle population changes. Data were collected from minimum 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test.

Figure 3.3. WEE1 inhibition activates CDK1 and ATR signaling and leads to RRM2 depletion. WEE1 and ATR inhibitors induce signaling changes in ATR, CDK and RRM2 signaling pathways. Western blot detection of the indicated proteins in whole cell lysates of cells treated with or without Aphidicolin and ATR or WEE1 inhibitors for 4 h.

Figure 3.4. WEE1 and ATR inhibition results in similar levels of DNA damage under conditions of replication stress. Top panel consists of representative examples of cell cycle flow cytometric detection of γH2AX in cells treated with 0.2 μM Aphidicolin and DMSO, 3 μM WEE1 inhibitor or 1 μM ATR inhibitor for 4 hours. γH2AX (y-axis) as a readout of DNA damage was immunodetected in asynchronous cells, co-stained for DNA content by PI (x-axis), and visualized by flow cytometry. Percentage of γH2AX-positive cells is indicated for each condition above the gate. Bottom panel consists of the quantification of the % of γH2AX positive cells normalized to the number of cells to account for cell cycle population changes. Data were collected from minimum 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test.

Figure 3.5. WEE1 inhibition prevents DNA replication to a greater extent than ATR inhibition. **(A)** Top panel consists of representative examples of cell cycle flow cytometric detection of EdU in cells treated with DMSO, 0.5 μM or 3 μM WEE1 inhibitor or 1 μM or 5 μM ATR inhibitor for 4 hours, washed and then pulsed with EdU for 1 hr. EdU (y-axis)

as a readout of DNA synthesis was immunodetected in asynchronous cells, co-stained for DNA content by PI (x-axis), and visualized by flow cytometry. Percentage of EdU-positive and EdU-negative cells is indicated for each condition above and to the left of the gates, respectively. Second panel show the quantification of the % of S phase DNA content cells that are EdU negative. Data were collected from 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test. **(B)** First panel shows representative examples of cell cycle flow cytometric detection of EdU in cells treated with 0.2 μM Aphidicolin and DMSO, 0.5 μM WEE1 inhibitor or 1 μM ATR inhibitor for 4 hours, washed and then pulsed with EdU for 1 hr. EdU (y-axis) as a readout of DNA synthesis was immunodetected in asynchronous cells, co-stained for DNA content by PI (x-axis), and visualized by flow cytometry. Percentage of EdU-positive and EdU-negative cells is indicated for each condition above and to the left of the gates, respectively. Second panel shows quantification of the % of S phase DNA content cells that are EdU negative. Data were collected from 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test. **(C)** First panel shows representative examples of cell cycle flow cytometric detection of EdU in cells treated with 5 μM Aphidicolin and DMSO, 0.5 μM WEE1 inhibitor or 1 μM ATR inhibitor for 4 hours, washed and then pulsed with EdU for 1 hr. EdU (y-axis) as a readout of DNA synthesis was immunodetected in asynchronous cells, co-stained for DNA content by PI (x-axis), and visualized by flow cytometry. Percentage of EdU-positive and EdU-negative cells is indicated for each condition above and to the left of the gates, respectively. Second panel shows quantification of the % of S phase DNA content cells that are EdU negative. Data were collected from 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test.

Figure 3.6 CDK1 inhibition decreases γH2AX accumulation and rescues DNA replication inhibition induced by WEE1 and ATR inhibition. **(A)** CDK inhibition but not AURKA inhibition rescues WEE1 and ATR induced γH2AX. Quantification of the % of γH2AX positive cells normalized to the number of cells to account for cell cycle population changes. Data were collected from 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test. $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***; compared to vehicle control. **(B)** CDK and AURKA inhibition rescues WEE1 and ATR induced inhibition of DNA replication. Quantification of the % of S phase DNA content cells that are EdU negative. Data were collected from 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test. $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***; compared to Vehicle control. **(C)** CDK inhibition but not AURA inhibition can rescue signaling changes induced by WEE1 and ATR inhibition. Western blot detection of the indicated proteins in whole cell lysates of cells treated with ATR or WEE1 inhibitors and either CDK1 (RO-3306) or AURKA inhibitors for 4 h. **(D)** CDK inhibition but not AURA inhibition can rescue signaling changes induced by WEE1 and ATR inhibition under conditions of low replication stress. Western blot detection of the indicated proteins in whole cell lysates of cells treated with Aphidicolin and ATR or WEE1 inhibitors and either CDK1 (RO-3306) or AURKA inhibitors for 4 h.

Figure 3.7. RPA ChIP-Seq peaks overlap between WEE1- and ATR-inhibited cells with or without aphidicolin. **(A)** RPA ChIP-Seq ratio tracks of indicated condition over its input using 500 bp bin windows at indicated peak chromosomal location. The rows display the ratio tracks (RPA-ChIP/Input) for the conditions as follows: 1. WEE1i, 2. ATRi, 3. WEE1i+Aph, 4. ATRi+Aph, 5. DMSO-treated control (UT) **(B)** Venn diagram displaying

overlap of peaks identified from each indicated condition **(C)** Spearman correlation plots of peak signal intensity for overlapping sites between indicated conditions where W: WEE1i, WAp: WEE1i+Aph, A: ATRi, ApA: ATRi+Aph. Red dots indicate top and bottom 5% outliers.

Supplemental Figure 3.1. Determining the IC_{50} for WEE1 and ATR inhibitor in MEF cells. Western blot detection of the indicated proteins in whole cell lysates of cells treated with indicated doses of WEE1 inhibitor **(A)** or ATR inhibitor with 0.2uM Aphidicolin **(B)** for 4 h.

Supplemental Figure 3.2. WEE1 and ATR inhibition results in similar levels of DNA damage under conditions of replication stress. Top panel consists of representative examples of cell cycle flow cytometric detection of γH2AX in cells treated with 5 µM Aphidicolin and DMSO, 0.5 µM WEE1 inhibitor or 1 µM ATR inhibitor for 4 hours. γH2AX (y-axis) as a readout of DNA damage was immunodetected in asynchronous cells, co-stained for DNA content by PI (x-axis), and visualized by flow cytometry. Percentage of γH2AX-positive cells is indicated for each condition above the gate. Bottom panel consists of the quantification of the % of γH2AX positive cells normalized to the number of cells to account for cell cycle population changes. Data were collected from minimum 3 independent experiments. Error bars represent SEM and p values were calculated by paired 2-tailed Student's t test.

Supplemental Figure 3.3. RPA ChIP-Seq peaks have similar signal intensity between WEE1- and ATR-inhibited cells with or without aphidicolin. Spearman correlation plots on linear scale of peak signal intensity for overlapping sites between indicated conditions where W: WEE1i, WAp: WEE1i+Aph, A: ATRi, ApA: ATRi+Aph. Red dots indicate top and bottom 5% outliers.
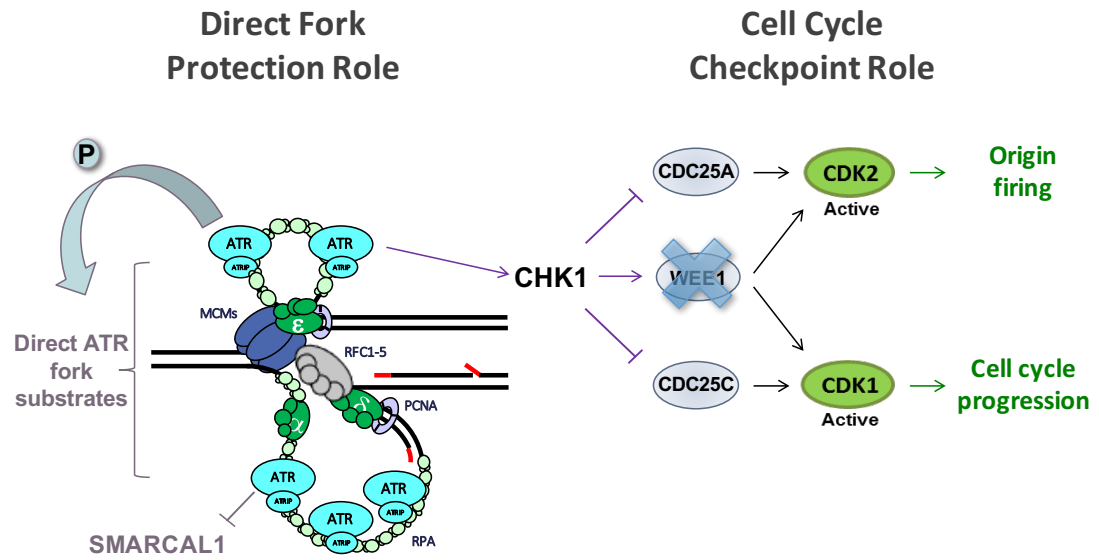
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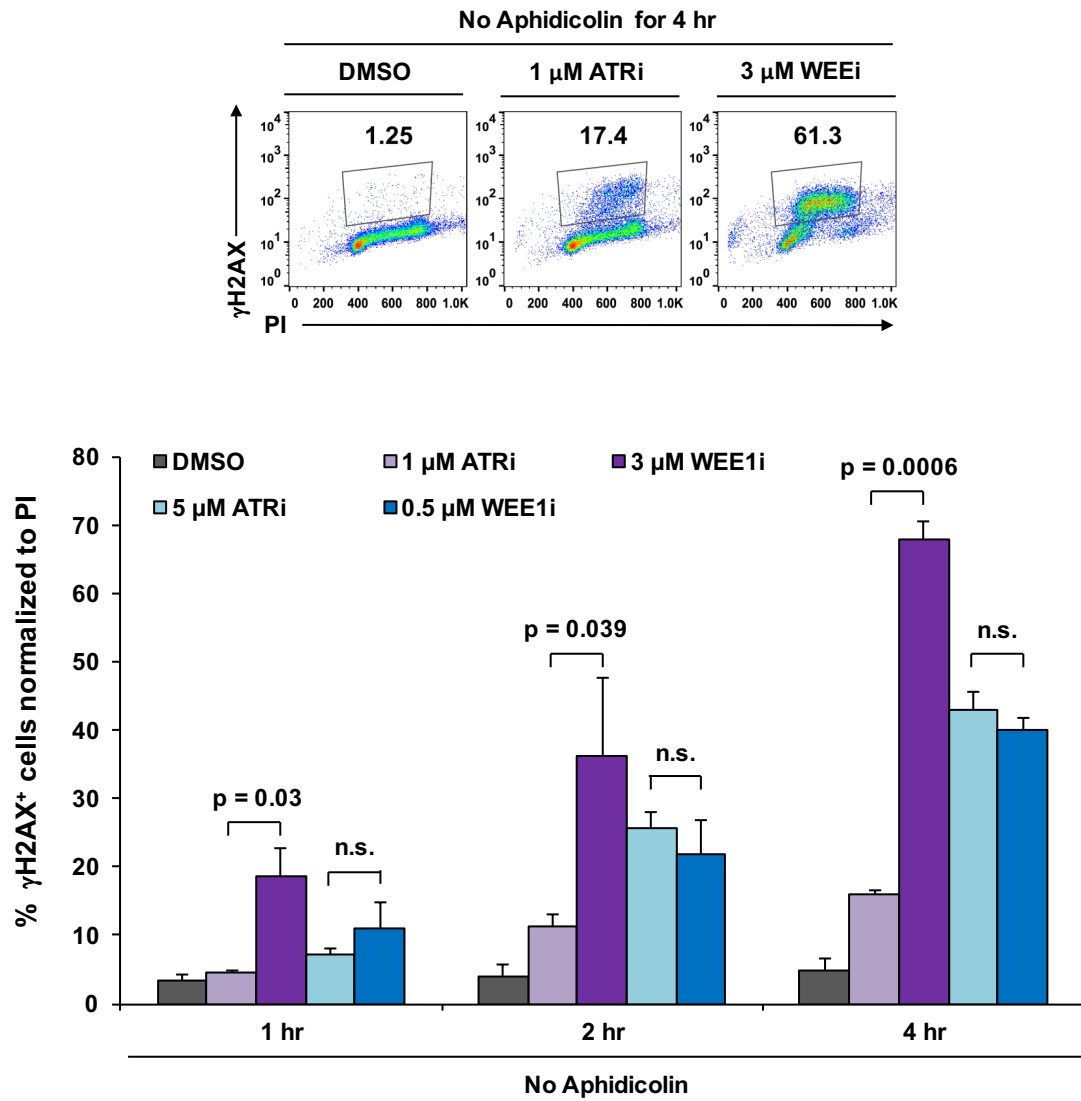# Figure 3.1

Figure 3.2

Figure 3.3
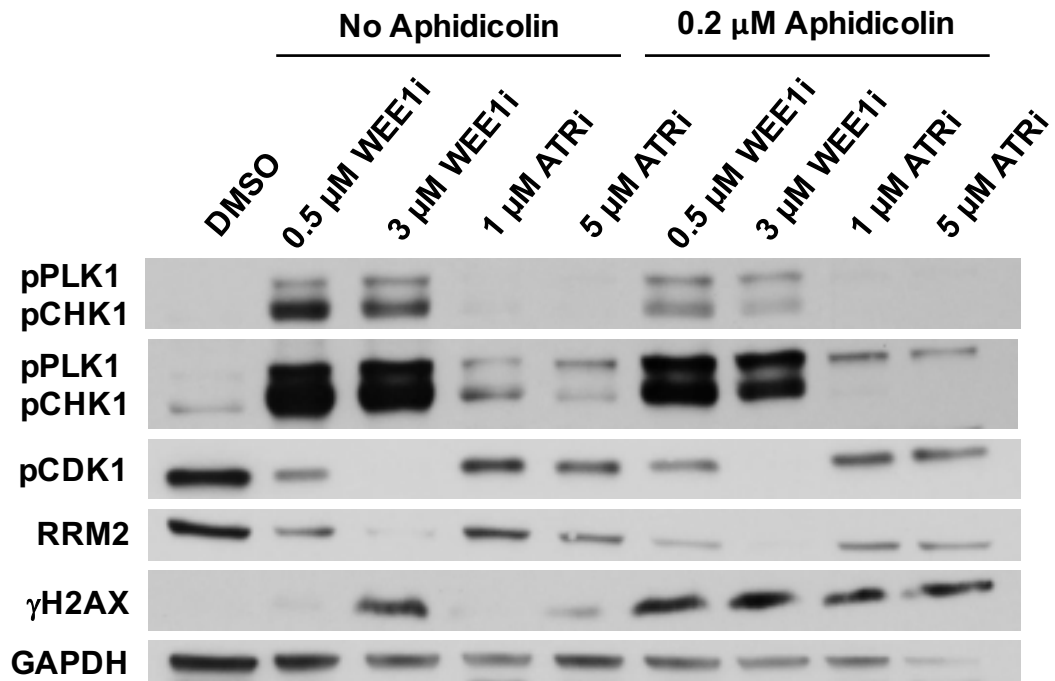
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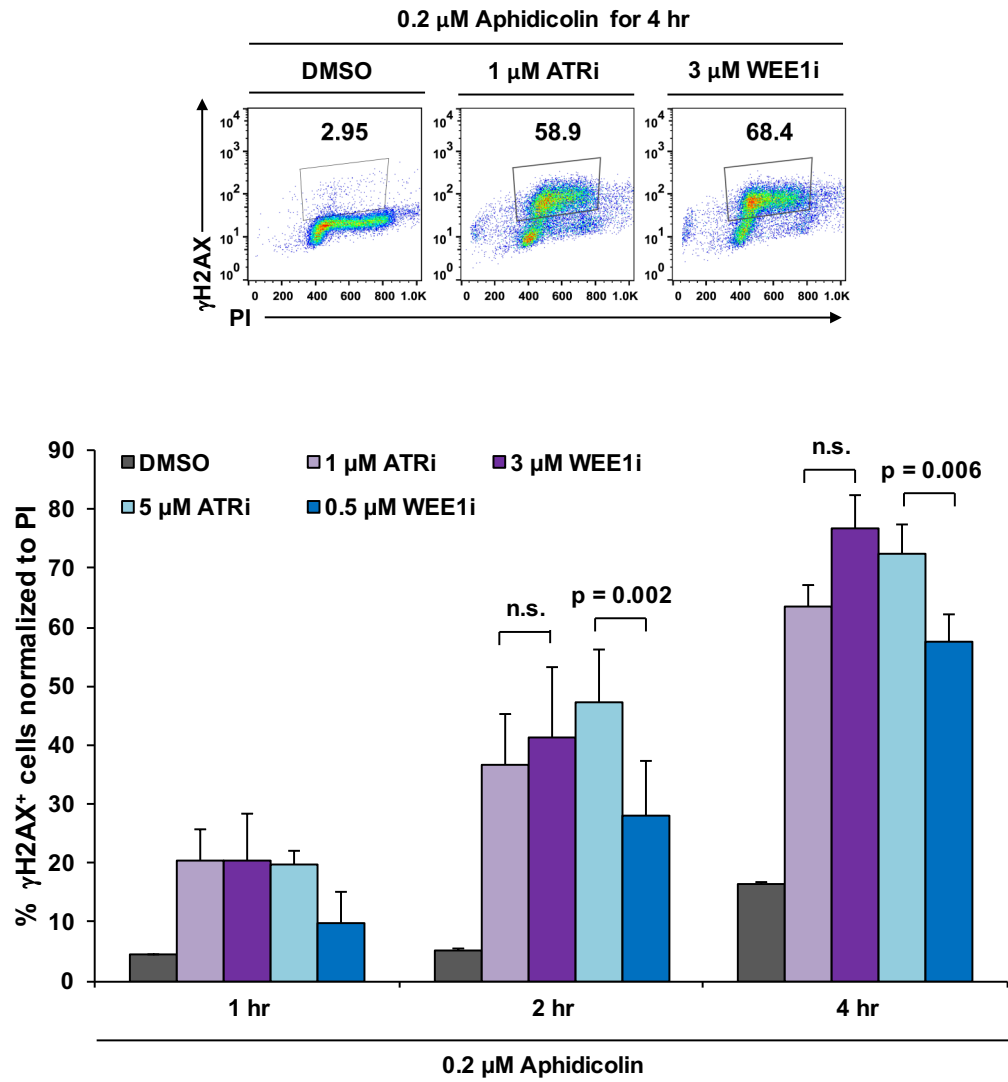
Figure 3.5A

A

Figure 3.5B

B

Figure 3.5C

C

5 µM Aphidicolin for 4 hr

DMSO   0.5 µM Wi   1 µM ATRi

32.5   11.2   5.59

1.95   19.5   24.5

143

Figure 3.6A

A

DNA Damage

Y-axis: % of γH2AX⁺ cells normalized to PI

Legend: □ Vehicle ■ AURKAi ■ RO-3306

X-axis categories: DMSO, 0.5 µM Wi, 3 µM Wi, 1 µM Ai, 5 µM Ai (No Aphidicolin 4 hr); DMSO, 0.5 µM Wi, 3 µM Wi, 1 µM Ai, 5 µM Ai (0.2 µM Aphidicolin 4 hr)
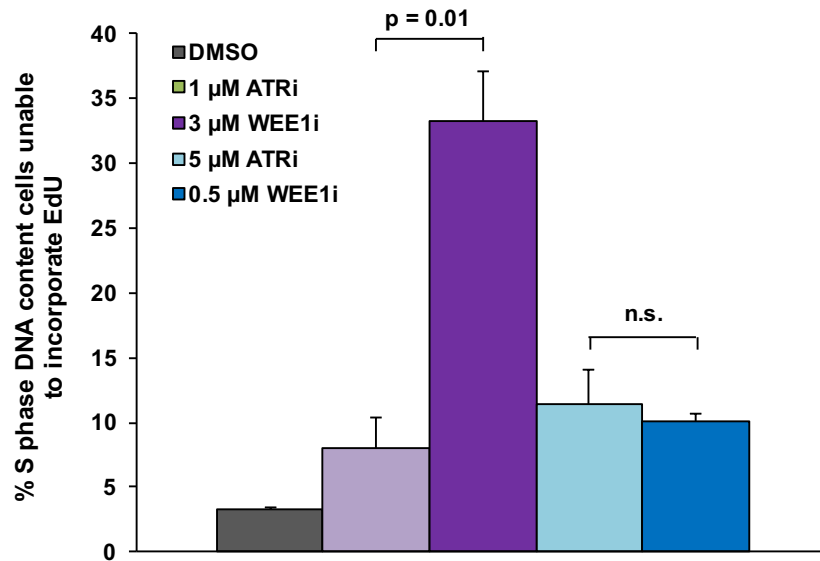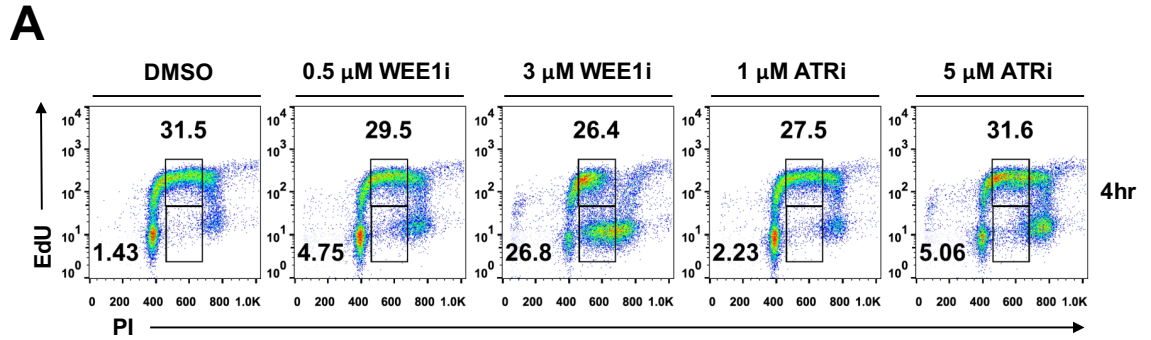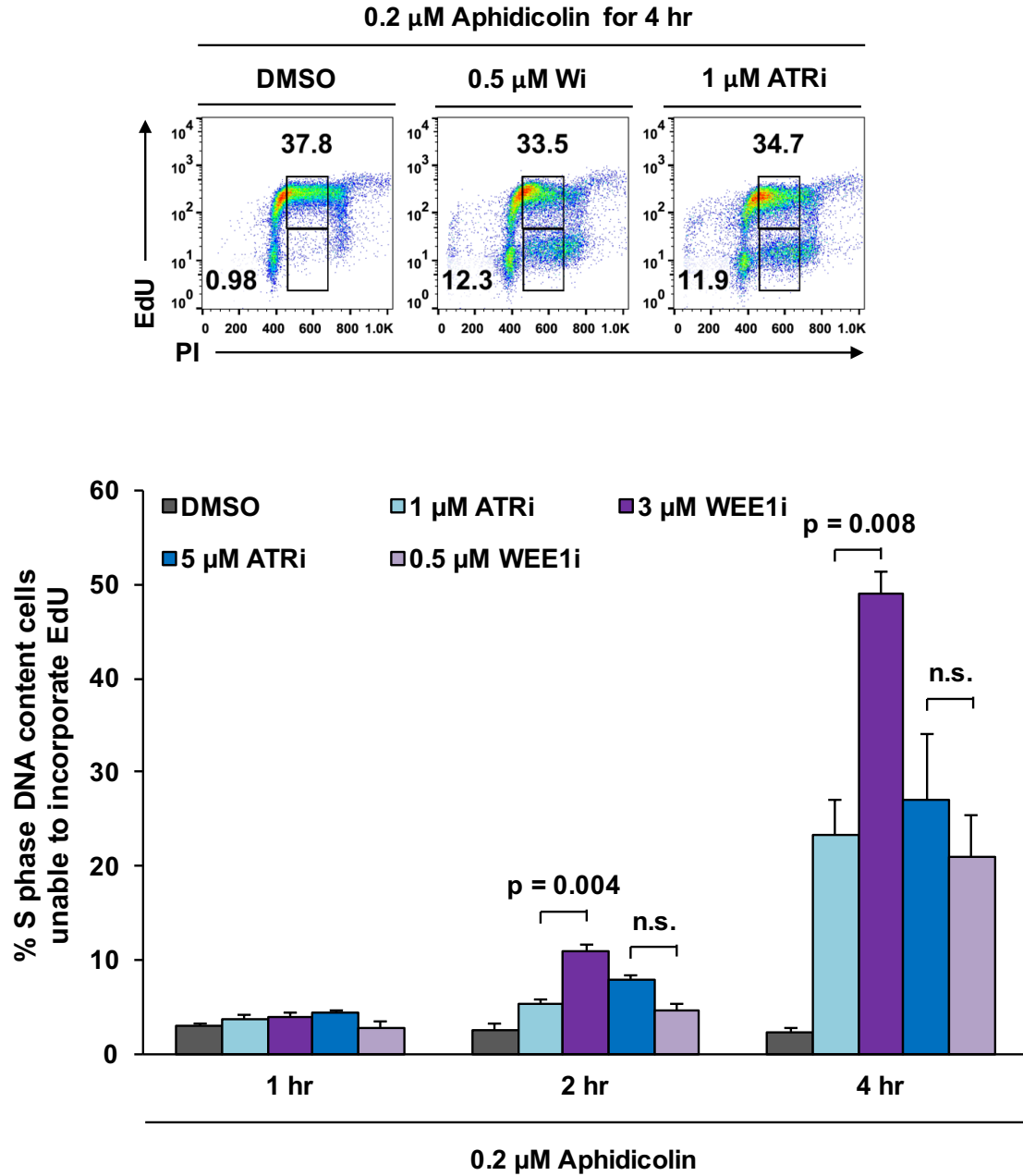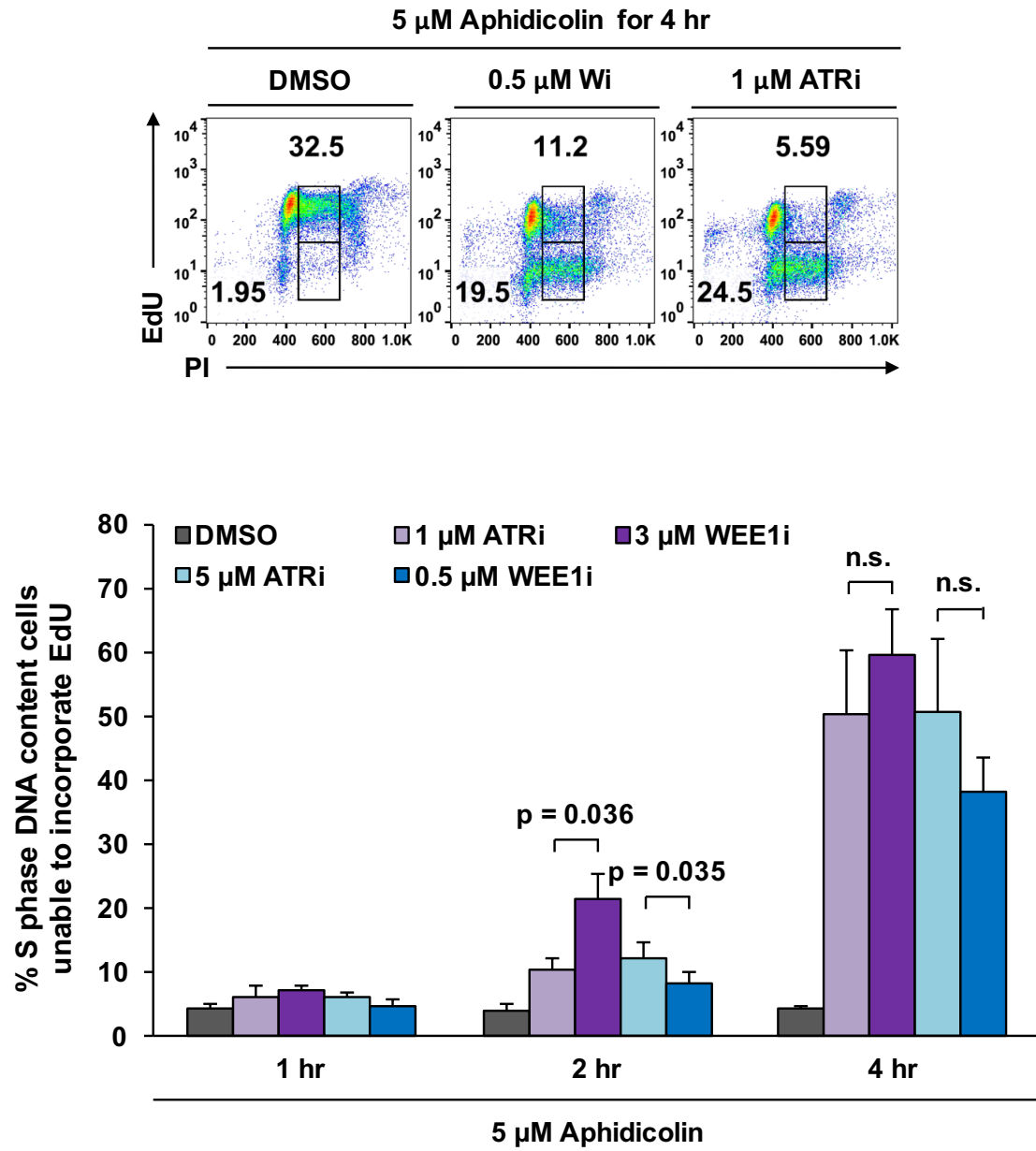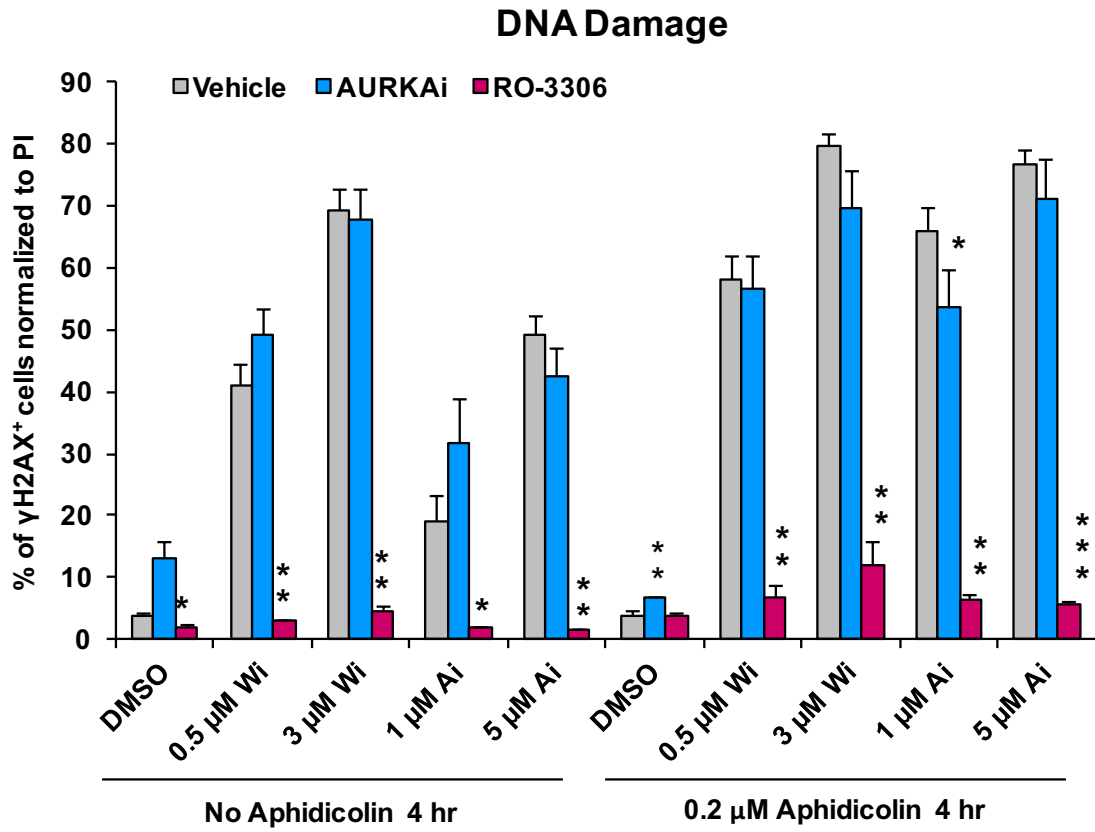
Figure 3.6B

B

Inhibition of DNA replication

145

Figure 3.6C

C

No Aphidicolin 4 hr

	Vehicle					40 μM RO-3306				
	-	+	-	-	-	-	+	-	-	-
3 μM WEE1i	-	-	+	-	-	-	-	+	-	-
1 μM ATRi	-	-	-	+	-	-	-	-	+	-
5 μM ATRi	-	-	-	-	+	-	-	-	-	+

pCHK1^{S345}

γH2AX^{S139}

pCDK1^{Y15}

pPLK1^{T210}

RRM2

GAPDH

	Vehicle					10 μM AURKAi				
0.5 μM WEE1i	-	+	-	-	-	-	+	-	-	-
3 μM WEE1i	-	-	+	-	-	-	-	+	-	-
1 μM ATRi	-	-	-	+	-	-	-	-	+	-
5 μM ATRi	-	-	-	-	+	-	-	-	-	+

pCHK1^{S345}

γH2AX^{S139}

pCDK1^{Y15}

pPLK1^{T210}

RRM2

GAPDH

Figure 3.6
D

0.2 µM Aphidicolin 4 hr

	Vehicle					40 µM RO-3306				
0.5 µM WEE1i	-	+	-	-	-	-	+	-	-	-
3 µM WEE1i	-	-	+	-	-	-	-	+	-	-
1 µM ATRi	-	-	-	+	-	-	-	-	+	-
5 µM ATRi	-	-	-	-	+	-	-	-	-	+

pPLK1^{T210}
pCHK1^{S345}

γH2AX^{S139}

pCDK1^{Y15}

RRM2

Tubulin

	Vehicle					10 µM AURKAi				
0.5 µM WEE1i	-	+	-	-	-	-	+	-	-	-
3 µM WEE1i	-	-	+	-	-	-	-	+	-	-
1 µM ATRi	-	-	-	+	-	-	-	-	+	-
5 µM ATRi	-	-	-	-	+	-	-	-	-	+

pPLK1^{T210}
pCHK1^{S345}

γH2AX^{S139}

pCDK1^{Y15}

RRM2

Tubulin

147

Figure 3.7

A

chr9:46,130,381-46,140,711

Figure 3.7

B

Figure 3.7
C

Supplemental Figure 3.1

A

µM WEE1i	10	3	1	0.3	0.1	0.03	0

pPLK1^{T210}

pPLK1^{T210}
(long exposure)

Tubulin

B

µM ATRi	0	0.03	0.1	0.3	1	3	10

pCHK1^{S345}
(short exposure)

pCHK1^{S345}
(long exposure)

pCHK1^{S345}
(long exposure)

Tubulin
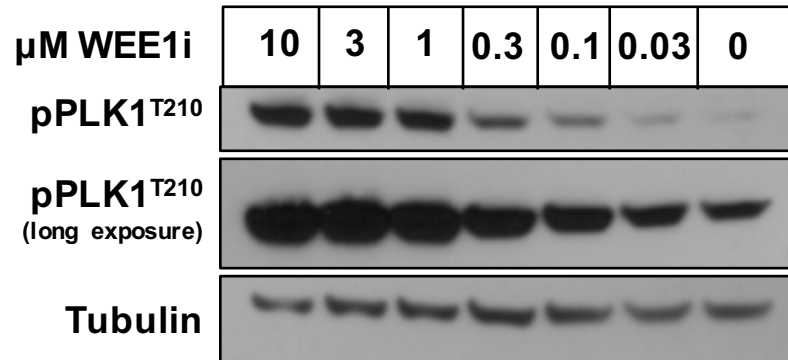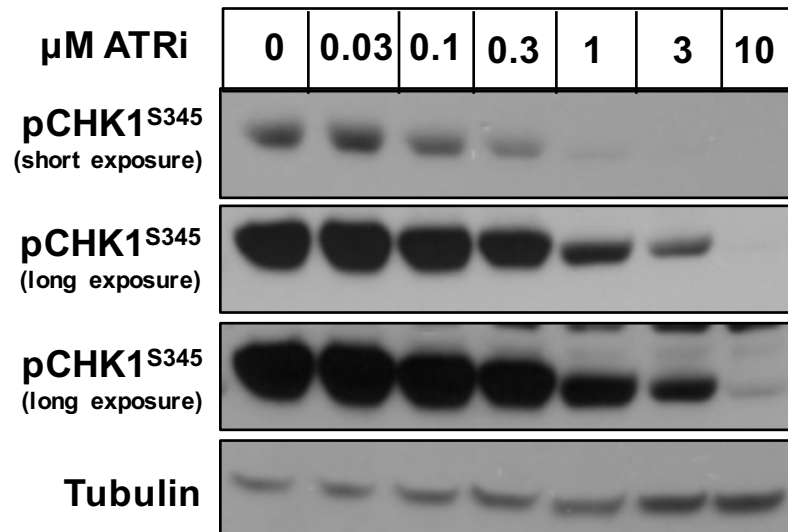
Supplemental Figure 3.2

Supplemental Figure 3.3

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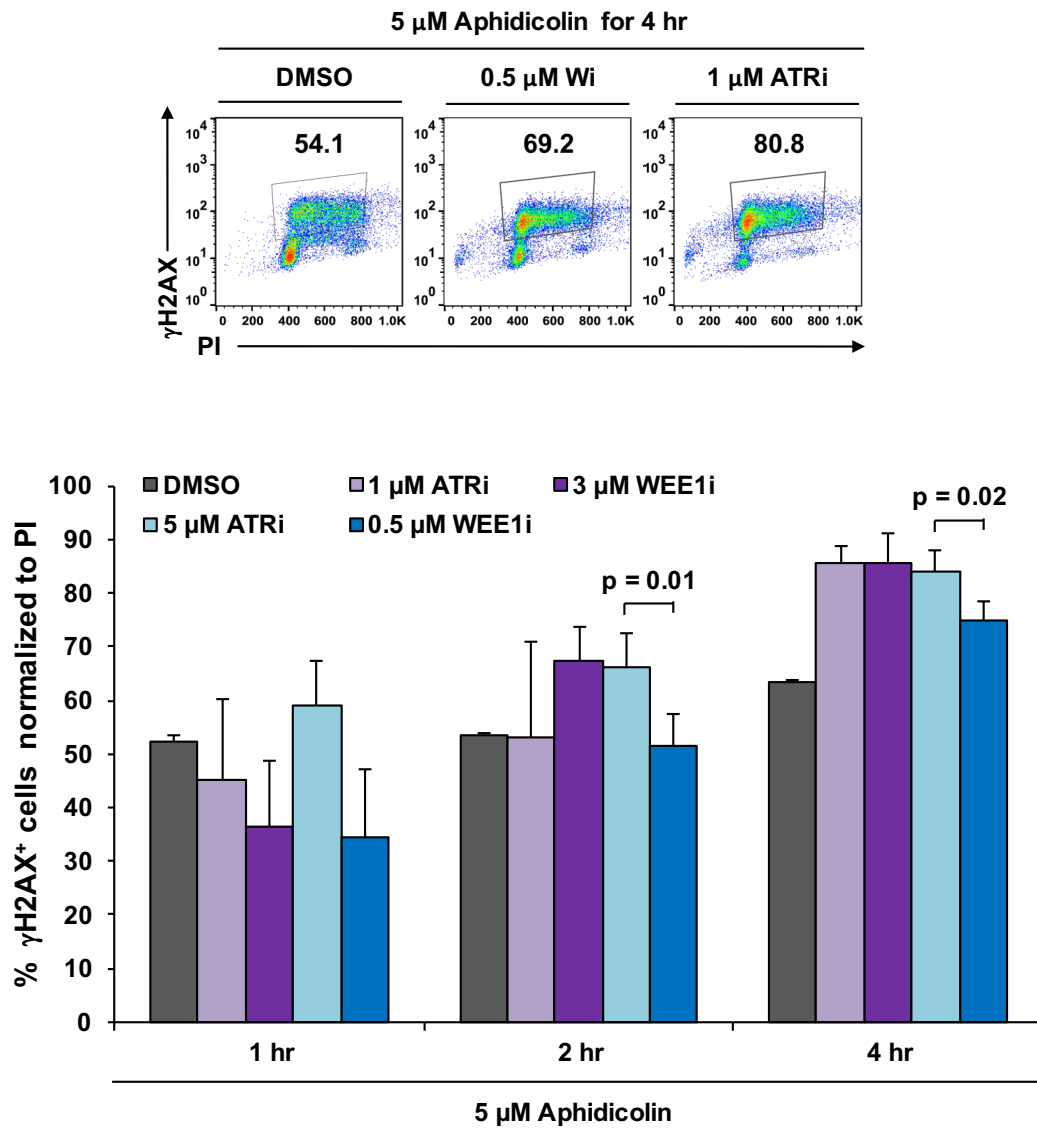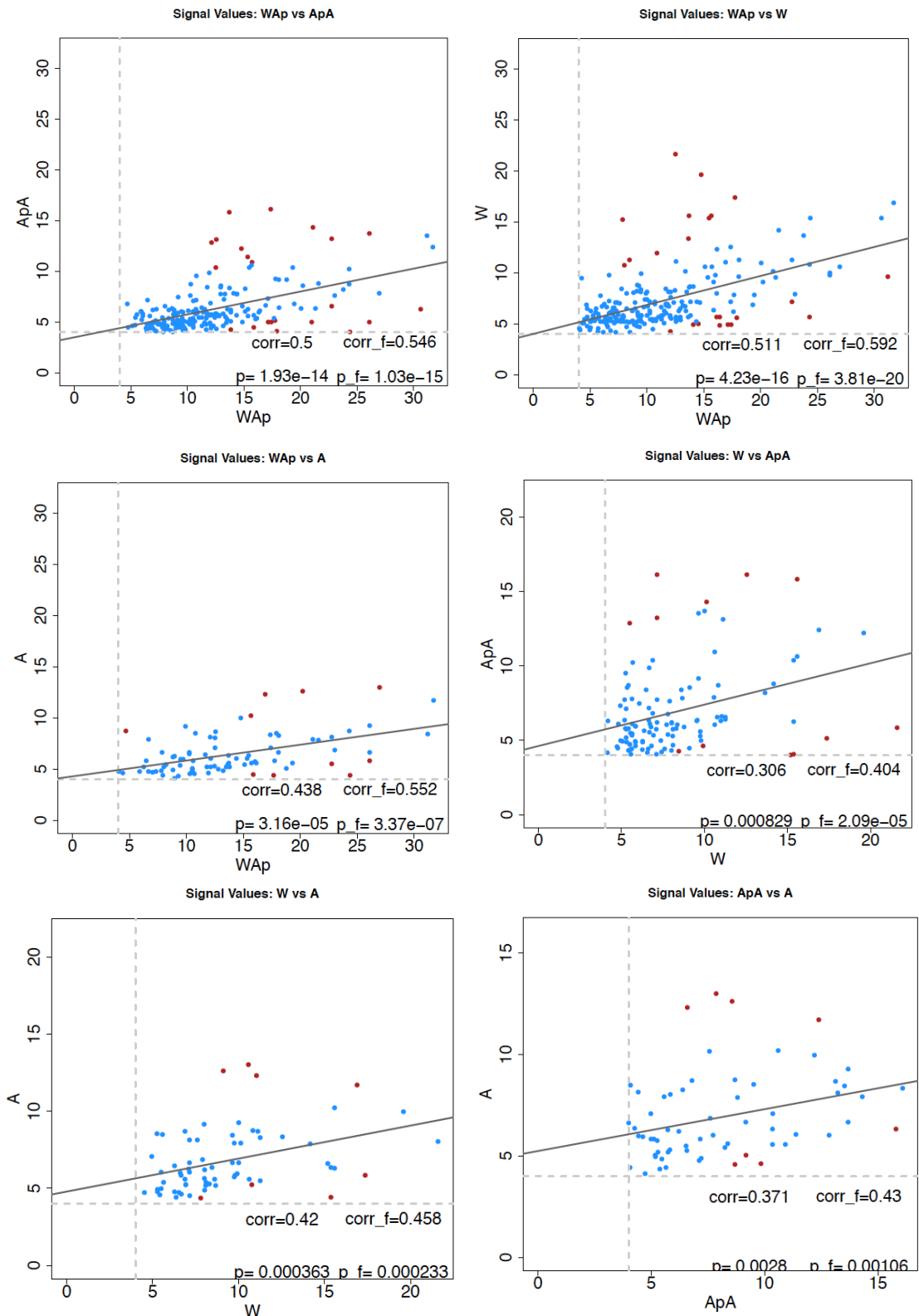
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CHAPTER 4: DISCUSSION

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The role of the Aurora A-PLK1 pathway in replication fork collapse

The impact played by the dysregulation of the Aurora A-PLK1 pathway in tumorigenesis has been clearly established because of the important functions it plays in the G2-M transition as well as in mitosis in preparation for cell division, such as in centrosome maturation, bipolar spindle assembly and chromosomal segregation (Asteriti, De Mattia and Guarguaglini, 2015). However, the role that the Aurora A-PLK1 pathway plays in genomic stability and particularly in regulating the DNA damage response still remains unclear. Amplification and overexpression of Aurora A promotes tumorigenesis (Bischoff *et al.*, 1998; Zhou *et al.*, 1998; Meraldi, Honda and Nigg, 2002a; X. Wang *et al.*, 2006). Therefore, increasing our understanding of the mechanisms by which Aurora A-induced tumorigenesis occurs will promote the development of novel and more efficient therapeutic cancer treatments (S. M. A. Lens, Voest and Medema, 2010; Otto and Sicinski, 2017). In addition, this knowledge will assist clinician in identifying patient populations that benefit from combinatorial treatments that take advantage of dependencies established in an Aurora A amplified tumor by capitalizing on synthetic lethal interactions.

Recent studies have shown that Aurora A overexpression or amplification abrogates the G2-M checkpoint in response to DNA damage (Marumoto *et al.*, 2002; Cazales *et al.*, 2005; Krystyniak *et al.*, 2006). Aurora A overexpression postpones cyclin B degradation and phosphorylates CDC25B, which prevents its sequestration by 14-3-3, thereby promoting CDK1 nuclear localization and activation, stimulating mitotic entry (Qin *et al.*, 2009). Conversely, Aurora A inhibition delays reentry of cells into mitosis (Marumoto *et al.*, 2002; Gorgun *et al.*, 2010). In addition, Aurora A activates PLK1, which drives mitotic entry and is required for mitotic entry following G2 cell cycle arrest induced

by DNA damage (Marumoto *et al.*, 2002; Macůrek *et al.*, 2008; Seki, Coppinger, Jang, *et al.*, 2008). Finally, inhibition of the Aurora A-PLK1 pathway under conditions of replication stress in ATR-deficient cells allows replication forks to resume replication (Ragland *et al.*, 2013). Together, these data indicate that the Aurora A-PLK1 pathway could prevent DNA damage repair through G2-M checkpoint bypass, suggesting that an important role of the DNA replication checkpoint is to inhibit premature activation of the Aurora A-PLK1 pathway (Figure 4.1). This is further supported by studies that demonstrate that the Aurora A-PLK1 pathway is inhibited in response to DNA damage as part of G2-M checkpoint activation (Medema *et al.*, 2000; Bassermann *et al.*, 2008; Qin *et al.*, 2013; Bruinsma *et al.*, 2017).

Herein we showed that despite fork collapse being mediated through the activation of the Aurora A-PLK1 pathway in ATR-deficient cells, it is not sufficient to induce fork collapse in the presence of ATR. We found that overexpression of Aurora A or stabilized mutants of Aurora A is insufficient to activate PLK1. Previous studies have shown that co-activators bind to Aurora A and dictate its cellular location as well as its substrate specificity. Some of these co-activators have been shown to regulate PLK1 activation and in line with these studies, we show that PLK1 activation, as determined by its phosphorylation at the T210 site, requires co-expression of Aurora A co-activators, TPX2 and BORA (Kufer *et al.*, 2002b; Bayliss *et al.*, 2003b; Eyers and Maller, 2004; Hutterer, Berdnik, Wirtz-Peitz, Zigman, *et al.*, 2006; Anderson *et al.*, 2007; Seki, Coppinger, Jang, *et al.*, 2008; Bruinsma *et al.*, 2013). Despite PLK1 activation, overexpression of Aurora A in combination with TPX2 or BORA remains insufficient to cause replication fork collapse. However, we did find that CDK1 activation is limiting in the activation of the Aurora A-PLK1 pathway by establishing that CDK1 activation

through WEE1 inhibition is able to hyperactivate the Aurora A-PLK1 pathway and is sufficient to induce fork collapse into DSBs. Additionally, WEE1 inhibition prevents DNA replication restart following replication fork stalling. These finding are expected in light of studies that showed that WEE1 inhibition increases origin firing, leading to nucleotide shortage followed by SLX4-MUS81 fork cleavage into DSBs (Domínguez-Kelly *et al.*, 2011; Beck *et al.*, 2012) as well as studies showing that WEE1 inhibition regulate ubiquitin-mediated RRM2 degradation through premature activation of CDK1 (D'Angiolella *et al.*, 2012; Pfister *et al.*, 2015). Notably, we found that both WEE1 inhibition-induced DSBs and inability to restart are rescued by Aurora A and PLK1 inhibition, which would suggest that nucleotide depletion is not the only mechanism by which WEE1 inhibition leads to fork collapse. In addition, our data would indicate that despite ATR activation, hyperactivation of the CDK1-AURKA-PLK1 pathway can promote fork stalling and collapse into DSBs, highlighting the importance of inhibiting the Aurora A-PLK1 pathway during DNA replication checkpoint activation to maintain genomic stability. Finally, we establish that Aurora A overexpression leads to an increased reliance on ATR for fork stabilization and is synergistic with ATR inhibition for DSB formation. Many studies have shown that oncogene-induced genomic instability is synthetic lethal with ATR inhibition (Gilad *et al.*, 2010b; Murga *et al.*, 2011a; Toledo, Murga and Fernandez-Capetillo, 2011; Schoppy *et al.*, 2012b). As we will discuss below, clinicians have begun adopting a novel therapeutic approach based on the concept of synthetic lethality, which ATR is proving to be an ideal candidate for. Our findings suggest that patients with Aurora A overexpressing or amplified cancers could benefit from ATR-targeted therapies. Interestingly, Aurora A has also been shown to be synthetic lethal with CHK1 inhibition in ovarian cancers though we did not observe this (Alcaraz-Sanabria *et al.*, 2017). This inconsistency could be due to a discrepancy in

different types of cancers or due to the fact that full AURKA-PLK1 pathway activation is required in order to see the synthetic lethality with CHK1 inhibition, which was likely not achieved in our system based on our data indicating that Aurora A overexpression alone is insufficient to lead to sufficient pathway activation that leads to fork collapse.

Together, data from the Aurora A-PLK1 pathway hyperactivation studies suggest that ATR stabilizes replication forks and prevents their collapse into DSBs in part by inhibiting the premature activation of the CDK1-AURKA-PLK1 pathway, highlighting the importance of the ATR-CHK1 cell cycle progression checkpoint function for genomic integrity (Figure 4.1). The importance of the ATR-mediated cell cycle regulation in the DNA replication checkpoint is further demonstrated in our mechanistic dissection of ATR-regulated fork stabilization.

ATR stabilizes stalled replication forks through its cell cycle function

The DNA replication checkpoint is integral to ensure genomic stability. Since its discovery, many studies have contributed to expand our understanding of the players, mechanisms and triggers involved during this response. One area of focus has been to elucidate the mechanisms by which ATR stabilizes stalled fork and prevents them from collapsing into DSBs (Saldivar, Cortez and Cimprich, 2017). It has been proposed that ATR stabilizes stalled forks either through regulation of cell cycle-dependent pathways that inhibit cell cycle progression and origin firing or through local modulation of fork remodelers (Costanzo *et al.*, 2003; E. J. Brown and Baltimore, 2003; Couch *et al.*, 2013b; Yazinski and Zou, 2016). The work presented herein provides a dissection of ATR fork integrity functions and shows that the cell cycle function of ATR is essential in stabilizing stalled fork in the presence or absence of replication stress (Figure 4.2). The cell cycle checkpoint function of ATR is required both to prevent fork collapse into DSBs,

as determined by γH2AX levels in a cell population DNA damage assay, as well as to prevent inability of cells to reinitiate replication.

Interestingly, our data suggests that there might be two separate mechanisms by which these functions are regulated. It appears that DSB formation is rescued by CDK1 inhibition but not by AURKA inhibition, suggesting a bifurcation of the regulatory pathway downstream of CDK1. CDK1 is known to phosphorylate and activate nucleases in order to resolve DNA junctions remaining after S phase to allow proper segregation of chromosomes and to prevent chromosomal aberrations, such as breaks and anaphase bridges (Boddy *et al.*, 2001; Matos *et al.*, 2011; Gallo-Fernández *et al.*, 2012; Dehé *et al.*, 2013; Szakal and Branzei, 2013b; Wyatt *et al.*, 2013a). Many structure-specific nucleases are involved in these processes but a central player is the MUS81 endonucleases. MUS81 is the catalytic subunit of a complex that can form either with EME1 or EME2 in mammalian cells (Ciccia, Constantinou and West, 2003; Öğrünç and Sancar, 2003). In addition to resolving recombination intermediates and under-replicated DNA in G2-M, MUS81-mediated nucleolytic cleavage is necessary for the restart of stalled replication forks (Hanada *et al.*, 2007; Alessandra Pepe and West, 2014a). The heterodimeric complex cleaves the stalled fork into a DSB that is then repaired by homologous recombination. Until recently, it was thought that all MUS81 functions were mediated through the MUS81-EME1 complex but recently it was reported that MUS81-EME2 is responsible for the processing of stalled replication forks to promote fork restart (Alessandra Pepe and West, 2014a). It remains unclear how this complex is regulated, how signaling pathway disruption could affect its function and why this function cannot be mediated by the MUS81-EME1 complex. In *S. cerevisiae*, MUS81 only has one binding partner, Mms4, which beckons the question why mammals evolved two separate

complexes that are activated at different cell cycle stages. Although the MUS81-EME1 complex is expressed through the cell cycle, it can only resolve Holliday junctions when bound to another endonuclease complex, SLX1-SLX4, which primarily occurs in G2-M in a CDK-dependent manner (Castor *et al.*, 2013; Garner *et al.*, 2013; Wyatt *et al.*, 2013). Additionally, premature CDK activation of the MUS81 complex leads to aberrant replication and increased levels of DNA crossovers (Domínguez-Kelly *et al.*, 2011; Choi *et al.*, 2013; Matos, Blanco and West, 2013; Szakal and Branzei, 2013b; Duda *et al.*, 2016). Interestingly, both complexes exhibit a preference for 3'-flap/fork DNA structures, yet only MUS81-EME2 can cleave a stalled fork to promote restart, whereas MUS81-EME1 activation in S phase results in DSB formation and inability to restart (A. Pepe and West, 2014). One possibility is that either these complexes process forks differently or they recruit different proteins to promote restart. Additional studies are required to better understand how these complexes are regulated and why they give rise to different outcomes when activated in S phase. In the context of our studies, one model that explains how DSB formation occurs when CDK1 is prematurely activated is that CDK1 activation aberrantly activates MUS81 resulting in stalled fork cleavage into DSBs and that the resulting break cannot be resolved appropriately to promote fork restart (Figure 4.3). Therefore, when WEE1i-induced CDK1 activation is inhibited with CDK1 inhibition, MUS81-EME1 is no longer active in S phase, letting MUS81-EME2 process the stalled forks and allow for restart to occur. It remains unclear why MUS81-EME2 cannot process and restart stalled forks when CDK1 is aberrantly activated in S phase. Alternatively, aberrant CDK activation can hyperactivate the MUS81-EME2 complex through a mechanism that is currently unknown and lead to inappropriate cleavage of stalled forks. Depletion studies of EME1 and EME2 would need to be performed to further elucidate the mechanism at play.

Furthermore, a recent paper found that CHK1, but not ATR, depletion in the absence of exogenous replication stress results in DNA breaks triggered by MUS81-EME2 and MRE11 aberrant activation (Técher *et al.*, 2016, 2017). In light of this study and others, it is possible that under conditions of ATR inhibition, CHK1 is still activated through DNA-PKcs, allowing the cell cycle checkpoint functions of ATR to be initiated. Consequently, we observe less DNA damage in ATRi cells compared to WEE1i cells in our system (Buisson *et al.*, 2015). With this in mind, it would be more accurate to compare ATRi+WEE1i to WEE1i to ensure that the cell cycle checkpoint roles of ATR are inhibited in the ATRi sample. This comparison allows us to assess the contribution of the local fork protection role of ATR, which appears to be minimal in our studies with 247 out of 269 WEE1i sites (92%) being affected in the ATRi+WEE1i sample compared to only 67 out of 88 ATRi sites (76%) found in WEE1i sample. Altogether, this only serves to further emphasize the importance of the cell cycle checkpoint functions of ATR in replication fork stability.

We also observe that AURKA inhibition can rescue the inability of cells to restart replication in WEE1i- and ATRi-treated cells under conditions of replication stress but it cannot rescue DSB formation. This implies that the AURKA-PLK1 pathway prevents replication fork restart downstream of fork cleavage (Figure 4.4). This finding is in agreement with a study previously published in our laboratory, in which the AURKA-PLK1 pathways suppresses replication reinitiation under conditions of ATR depletion and fork stalling (Ragland *et al.*, 2013). These findings suggest that aberrant activation of the AURKA-PLK1 pathway could mediate restart despite the inability to prevent endonuclease cleavage of stalled forks, as is observed under conditions of CDK1 inhibition. One mechanism independent of preventing DSB formation by which AURKA-

PLK1 inhibition rescues restart could be that of premature replisome disassembly, which then renders the bare forks accessible to endonucleases, which would naturally process them to promote replication restart (Figure 4.3). Additional studies are needed to determine if replisome factors are modified and/or removed under these conditions as well as to determine what factors might be recruited to these forks and whether or not these changes are shared in conditions of CDK1 inhibition.

In this study, we also sought to identify sites genome-wide that might be differentially sensitive to either of the fork stabilization functions of ATR. In agreement with the cell population data, the RPA ChIP-Seq data showed that the majority of sites identified depend on the cell cycle checkpoint function of ATR. Based on previous studies that established that ATR inhibition in the absence of exogenous replication stress did not result in DNA breaks, we expected and observed that ATRi-treated cells led to a minimal number of hard to replicate sites (Koundrioukoff *et al.*, 2013; Técher *et al.*, 2016). As expected, the number of sites increased with replication stress. Interestingly, the number of sites seen with WEE1 inhibition alone was significantly higher than for ATR inhibition alone. This could be a result of ATRi-treated cells still having some part of the cell cycle checkpoint pathway active as discussed above or a result of WEE1 inhibition inducing some inherent replication stress. Indeed, WEE1 inhibition has previously been shown to result in nucleotide depletion through RRM2 degradation, which is known to cause replication stress (Figure 4.3) (D'Angiolella *et al.*, 2012; Pfister *et al.*, 2015). This finding is in agreement with a study that found that dNTP addition suppressed fork slowing in CHK1-deficient cells but not in ATR-depleted cells, suggesting that nucleotide depletion is an additional mechanism involved in fork stalling under conditions of WEE1 inhibition (Técher *et al.*, 2016). Interestingly, though WEE1

inhibition increased the number of sites sensitive to fork collapse, it did not affect their intensity. One might expect that increased replication stress could result in an amplification in the breakage frequency of these sites as opposed to or in addition to an increase in the number of sites that are affected genome-wide. It would be interesting to see if lower doses of inhibitor could reveal a replication stress-induced increase in the frequency of sites undergoing fork collapse. If not, this could imply that there might be a cap in the frequency that certain sites in the genome can collapse before cell death-inducing mechanisms are activated or that specific sites in the genome have a predetermined replication stress threshold at which they collapse, resulting in increasing fork collapse sites as replication stress increases. Additional studies would need to be performed to determine if this occurs. As previously discussed, because ATR inhibition alone could still allow cell cycle functions of ATR to be activated through redundant upstream kinases, such as ATM and DNA-PK, it would be more appropriate to compare WEE1i to ATRi+WEE1i with or without aphidicolin. Indeed, when comparing these conditions, they lead to a similar number of total sites as well as a significant overlap between each other.

Another unexpected finding was that genomic loci sensitive to WEE1i and ATRi+Aph only overlapped by 54%. Based on the cell population DNA damage and restart assays, we anticipated them to almost completely overlap. This suggests that the replication stress induced by aphidicolin and WEE1 inhibition are distinct and result in different fork stalling patterns. By determining which features characterize these unique sites will provide us with insight into the mechanisms by which these two stressors result in replication perturbation. An interesting hypothesis to test would be to investigate whether the site unique to WEE1i treatment are enriched in adenines and guanines as

nucleotide depletion resulting from RRM2 degradation, based on HU data, would preferentially deplete dATPs and dGTPs (Collins, Oates, & Collins, 1987 and personal communications with Dr. D'Angiolella).

Together these data indicate an essential role for the cell cycle checkpoint function of ATR in stabilizing replication forks though the local role of ATR might also play stabilizing functions at a few sites in the genome (Figure 4.2). Future studies are required to determine what features render these sites vulnerable to fork stalling and collapse as well as what mechanisms lead to fork collapse.

Therapeutic implications and combinatorial treatments

As previously mentioned, ATR inhibition is actively being pursued in clinics to be used in combinatorial treatments with DNA damaging agents as well as with other proteins that synergize with ATR inhibition (Weber and Ryan, 2015; Blackford and Jackson, 2017; Sundar *et al.*, 2017). Originally, ATR inhibitors were not actively pursued as therapeutics because ATR knockout mice were embryonic lethal (Brown and Baltimore, 2000; de Klein *et al.*, 2000). It was then discovered that hypomorphic mutations were viable through the studies of Seckel syndrome (O'Driscoll *et al.*, 2003). Though ATR proved to be essential for cell viability and genomic stability, hypomorphic levels of ATR were not toxic for cells in the absence of replication stress. However, under conditions of replication stress, such as that induced by oncogenes, ATR loss proved to be lethal (Murga *et al.*, 2009, 2011a; Gilad *et al.*, 2010b; Toledo, Murga and Fernandez-Capetillo, 2011; Schoppy *et al.*, 2012b). These studies paved the way for applying the concept of synthetic lethality for the selective targeting of tumors (McLornan, List and Mufti, 2014; O'Neil, Bailey and Hieter, 2017). By targeting tumors with a mutational landscape that induces a reliance on the ATR-regulated cell cycle

checkpoint, ATR inhibitors specifically prevent tumor growth and viability without affecting surrounding healthy cells. ATR loss has been shown to be synthetic lethal with DNA damaging chemotherapeutic agents, p53, XRCC1, ERCC1, ATM, MYC, RAS and many other proteins (Reaper *et al.*, 2011; Sultana *et al.*, 2013; Mohni *et al.*, 2015; Hocke *et al.*, 2016; Kwok *et al.*, 2016; Sanjiv *et al.*, 2016; Williamson *et al.*, 2016). Studying the mechanisms by which ATR prevents genomic instability allows for the identification of new synthetic lethal interactors, such as proteins in the Aurora A-PLK1 pathway.

AURKA is frequently amplified in a spectrum of cancers, including ovarian, colorectal, and breast cancers and its overexpression correlates with poor prognosis and increased genomic instability. AURKA overexpression was recently shown to predict poor overall survival and early recurrence of triple negative breast cancer patients. By elucidating the mechanisms by which AURKA contributes to replication fork collapse and how its hyperactivation leads to an increased reliance on ATR, we can develop targeted therapies for AURKA-amplified cancers and use AURKA-PLK1 pathway hyperactivation as a prognostic indicator for ATR inhibitors, which are currently in clinical trials for several cancers. In addition, oncogene expression has been shown to increase dependency on ATR for genomic stability and cancer cell survival, thus promoting a synthetic lethal effect when treated with ATR inhibitors. Some of these oncogenes, such as KRAS^{G12D} and MYC, are known to promote AURKA expression and activity. Our finding showing that AURKA overexpression sensitizes cells to ATR inhibition suggests that AURKA-PLK1 pathway hyperactivation is one mechanism by which these oncogenes are synthetic lethal with ATR inhibition. Moreover, these findings indicate that AURKA amplification or overexpression could serve as a biomarker for ATR-based therapies.

Finally, it has been reported that chemically-induced and oncogene-induced replication stress give rise to different landscapes of fragile sites that only partially overlap with one another (Miron *et al.*, 2015). The underlying features that render some sites over others more prone to genomic instability under differing conditions remains unknown. Our studies contribute to our knowledge of which sites are more vulnerable to cell cycle checkpoint abrogation and could bring insight into how specific sites might result in lethal genomic instability. Determining genomic regions that are more sensitive to ATR or WEE1 inhibitors, both of which are currently in clinical trials for various cancers, will also allow us to determine if these loci contribute to the synthetic lethality observed with AURKA inhibition or oncogene expression. It is possible that a combination of specific sites achieved by combinatorial inhibition results in lethality. Alternatively, lethality could be due to an increase in the frequency of breakage at specific sites, which might give rise to fatal chromosomal translocations. Further characterization of these sites would allow us to determine if features specific to these sites, whether it be sequence, gene expression, chromatin modifications, etc., are key to tumor cell death while others have little effect. Together, these data could be used to develop more targeted cancer therapeutics that minimize off-target effects on healthy cells as well as be used as predictive biomarkers to maximize the efficacy of therapies in specific patient subpopulations.

Future Directions

The projects described herein leave many interesting avenues to pursue. For future directions regarding the AURKA-PLK1 pathway activation studies, it would be of interest to further test if cancer cells that have AURKA amplification or Aurora A overexpression are synthetic lethal with WEE1 inhibition. If this were the case, these

findings would support using WEE1i inhibitors to treat Aurora A overexpressing tumors in patients.

One interesting avenue to pursue based on the findings in Chapter 3 is to determine which features characterize RPA ChIP sites unique to each condition tested and to determine if sites unique to WEE1i compared to ATRi+Aph were enriched in adenines and guanines. If so, we would expect that supplementing dATPs and dGTPs to the WEE1i treatment media would prevent these sites from accumulating RPA. To gain an understanding of the mechanism by which these sites collapse, it would also be of interest to establish which sites can be rescued by AURKA or CDK1 inhibition as well as what might be the difference between these sites. Finally, determining which nucleases might be involved in fork cleavage into DSBs would be informative to understand whether cleaved forks are the results of premature activation of G2-M nucleases or whether fork cleavage is the result of aberrant activation of S phase-specific nucleases, such as MUS81-EME2 (Fekairi *et al.*, 2009; Muñoz-Galván *et al.*, 2012; Ragland *et al.*, 2013; Wyatt *et al.*, 2013b, 2017; Alessandra Pepe and West, 2014a).

Genome wide analysis of BrITL-Seq data

Though much of the RPA ChIP-Seq data still needs to be mined, this dataset lacks the ability to distinguish between stalled replication forks that have accumulated ssDNA and RPA-coated resected DSBs (Figure 4.5). Our laboratory has developed an assay, Breaks Identified by TdT-Labeling (BrITL), to isolate genomic loci at which DSBs occur genome-wide. The BrITL assay was performed on the same conditions as the RPA ChIP experiment with the addition to sample in the absence and presence of CDK1i to determine if fork cleavage into DSBs is CDK1-dependent. Genome wide

sequencing data analysis remains to be performed on these samples but characterizing the sites that overlap between RPA-Seq and BrITL-Seq would be interesting to determine if all of them are CDK1-dependent, and whether some conditions are more prone to having sites collapse into DSBs as opposed to being collapsed fork unable to restart replication.

Proteomic analysis of iPOND2 data

WEE1 inhibition leads to ATR-CHK1 activation within 15 min and to DSB formation by 1 hr. Studies have previously reported that WEE1 inhibition leads to an increase in MUS81-EME1 at the fork and results in fork cleavage and DSB formation (Beck *et al.*, 2010, 2012; Domínguez-Kelly *et al.*, 2011). In addition, WEE1 inhibition activates the AURKA-PLK1 pathway, which was shown to promote replication fork collapse following ATR inhibition (Ragland *et al.*, 2013). Finally, studies have shown that PLK1 can mediate degradation of Claspin and FANCM, two replisome components (Mamely *et al.*, 2006; Kee *et al.*, 2009). Based on these findings, we speculate that WEE1 inhibition induces many dynamic changes at the replication fork, possibly including premature removal of replisome components leading to bare fork structures that are cleaved by nucleases (Figure 4.3). Using isolation of Proteins On Nascent DNA (iPOND), we monitored protein changes that occurred after 30 min or 2 hrs of WEE1 inhibition by Western immunoblotting. We observed an increase in phosphorylation of ATR substrates at 30min of WEE1 inhibition and a decrease in protein ubiquitination at the fork at 2 hrs, suggesting that WEE1 inhibition causes replication stress that activates the ATR-CHK1 response rapidly and that following DSB formation, many previously ubiquitinated replisome components are removed or deubiquitinated (Figure 4.6).

To better understand how WEE1 inhibition affects replisome dynamics, we performed iPOND2-mass spectrometry (MS) on WEE1i, WEE1i+Aph, WEE1i+ATRi, WEE1i+CDK1i, WEE1i+Aph+CDK1i, WEE1i+ATRi+CDK1i, ATRi and ATRi+Aph for 2 hrs and WEE1i and WEE1i+Aph for 30 min. These samples will provide insight into how WEE1 inhibition alters protein recruitment or loss at the fork as well as their state of ubiquitination, how WEE1 inhibition synergizes with ATR inhibition and if it is CDK1-dependent. The analysis will entail monitoring changes in protein abundance and ubiquitination status at the replisome in the different conditions. Preliminary analysis suggests that WEE1 inhibition decreases ubiquitination-mediated degradation and upregulates metabolic pathways as well as repair pathway as expected from our preliminary findings. More specifically, WEE1i resulted in a downregulation of proteasome, aminoacyl-tRNA biosynthesis, spliceosome and FA pathway proteins and an upregulation of metabolic pathways, ribosome and carbon metabolism pathway proteins. WEE1i+Aph treatment lead to a downregulation of spliceosome, ubiquitin mediated proteolysis, cell cycle and RNA transport pathway proteins and an upregulation of metabolic pathways, aminoacyl-tRNA biosynthesis, spliceosome, FA and HR pathway proteins. WEE1i+ATRi+Aph resulted in a downregulation of carbon metabolism, metabolic pathways, and an upregulation in spliceosome, DNA replication, cell cycle, ribosome and nucleotide excision repair pathway proteins. ATRi leads to downregulation of carbon metabolism, mismatch repair, metabolic pathways, and DNA replication and to an upregulation in spliceosome, ribosome, RNA transport pathway proteins. ATRi+Aph results in downregulation of spliceosome, proteasome, carbon metabolism, and to an upregulation of ribosome, HR, RNA transport, FA and DNA replication pathway proteins. CDK1i in WEE1i treated cells compared to WEE1i treated cells leads to the downregulation of ribosome, carbon metabolism, metabolic pathways

and TCA cycle pathway protein and upregulates spliceosome, RNA transport, proteasome and ubiquitin mediated proteolysis as well as aminoacyl-tRNA biosynthesis. Detailed analysis of this wealth of data needs to be performed as does analysis of the data on the ubiquitination state of replisome components observed at the fork under these conditions. As expected, WEE1 inhibition activates and recruits repair pathway proteins at the forks as these are likely to stall and need repair under conditions of WEE1 inhibition. Additionally, WEE1i treatment appears to downregulate proteasome-mediated degradation pathway proteins, which could explain why we observed a reduction in levels of ubiquitination at the fork under WEE1 inhibition by Western immunoblotting.

Studies performed in human cells

All findings presented in Chapter 3 were performed in mouse cells; however, performing similar studies in human cell lines would provide therapeutically relevant information. Human breast cancer cells from the MDA-MB-231 cell line were treated with WEE1i and ATRi in the presence and absence of aphidicolin and BrITL-Seq was performed. Though the analysis is preliminary, new sites were identified and appeared enriched in A/T-rich repeats. It would be interesting to see if these sites could be rescued through supplementation of dATPs and dTTPs to determine if these sites occur from a lack of nucleotides or whether these sequences have intrinsic characteristics that render them inherently prone to breakage, such as being structure forming. Again, comparing sites unique to each condition and determining their features as well as establishing common features that promote DSB formation would be really interesting. These features might be able to explain why the human genome has cancer translocation hotspots or these features could be used to artificially create break spots in

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the genome by inserting them at a site of interest. Finally, if each treatment results in a unique breakage signature, these could be combined with breakage signatures unique to cancers, such as ones created from oncogenes, to specifically target cancer cells through synthetic lethality.

Figure Legends

Figure 4.1. Model figure: ATR stabilizes forks by preventing the premature activation of the CDK1-AURKA-PLK1 pathway. When ATR is absent or inhibited, the AURKA-PLK1 pathway is prematurely activated and results in premature entry into mitosis as well as in the degradation or removal of replisome components, leading to fork stalling and inability to restart replication.

Figure 4.2. Model figure the fork stabilizing functions of ATR. ATR stabilizes forks through its direct fork protection role (phosphorylation of substrates directly found at the replication fork) and through its cell cycle checkpoint role (signaling cascade triggered through activation of downstream effectors, not permanently localized at the fork, such as CHK1 and WEE1).

Figure 4.3. Signaling cascades activated by WEE1 inhibition that result in fork stalling. WEE1 inhibition has been shown to activate CDK1-AURKA-PLK1 pathway, which leads to fork stalling, to activate CDK2, which leads to aberrant origin firing and to lead to RRM2 degradation and endonuclease activation. All of these signaling pathways have been shown to lead to replication fork stalling.

Figure 4.4. Signaling cascades downstream of CDK1 that result in fork stalling and collapse. WEE1 inhibition activates CDK1 which in turn activates the AURKA-PLK1 pathway that is known to lead to fork stalling. CDK1 activation also leads to RRM2 degradation that is known to lead to fork stalling through nucleotide depletion. CDK1

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activation is also known to activate endonucleases such as MUS81-EME1 that have been shown to cleave forks. This figure summarizes the different pathways downstream of CDK1 that could contribute to fork stalling and eventual fork collapse.

Figure 4.5. Structures enriched in RPA ChIP assay compared to BrITL assay. The RPA ChIP assay isolates genomic loci that are enriched in RPA. As such, it enriches for and cannot distinguish between collapsed forks that are either unable to restart replication or that have collapsed into DSBs. The BrITL assay enriches for forks that have collapsed into DSBs. By comparing the peaks obtained with both assays, the sites at which DSBs occur as opposed to sites at which forks cannot restart replication can be determined.

Figure 4.6. WEE1 inhibition results in decreased protein ubiquitination and increased ATR substrates phosphorylation at the fork. **(A)** Representative Western blot of input or captured proteins following iPOND on 4-3 MEF cells treated with 3 μM WEE1i for 30 min or 2 hr and then labeled for 10 min with EdU. Levels of ATR substrate activation, such as phosho-MCM2, phospho-CHK1, phospho-RPA32, as well as levels of total MCM2 and total RPA70 were assessed. **(B)** Representative Western blot of input or captured proteins following iPOND on 4-3 MEF cells treated with 3 μM WEE1i for 30 min or 2 hr and then labeled for 10 min with EdU. Levels of protein ubiquitination was assessed.
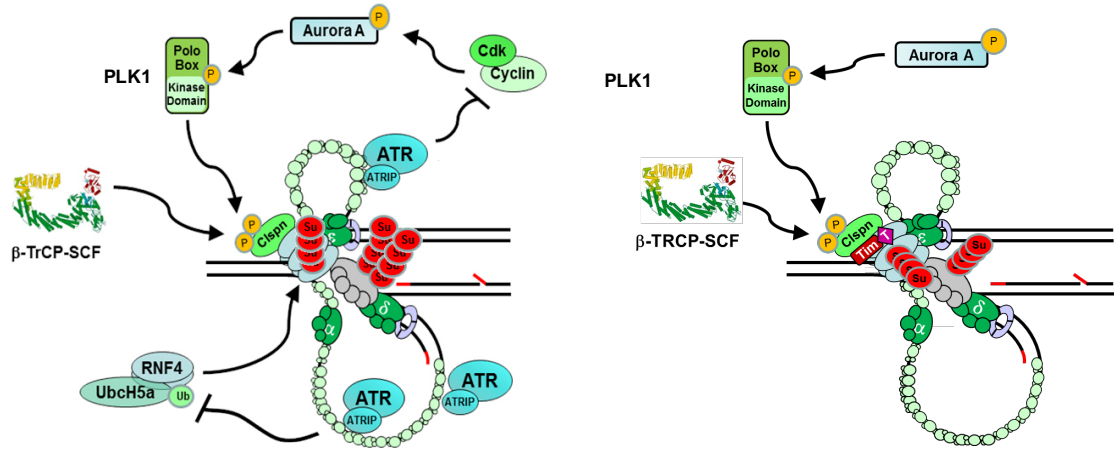
Figure 4.1

Figure 4.2
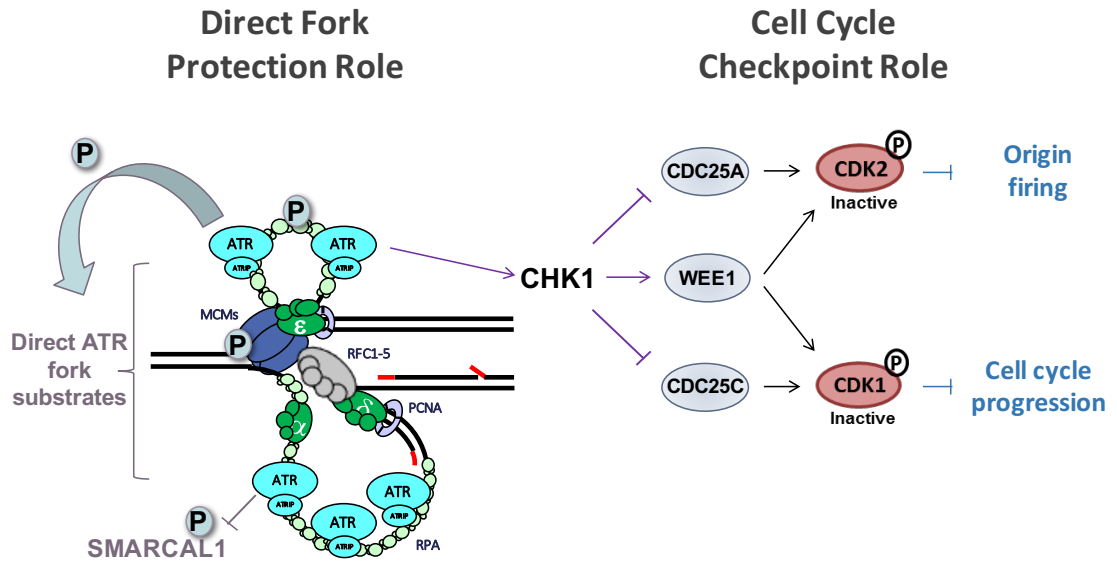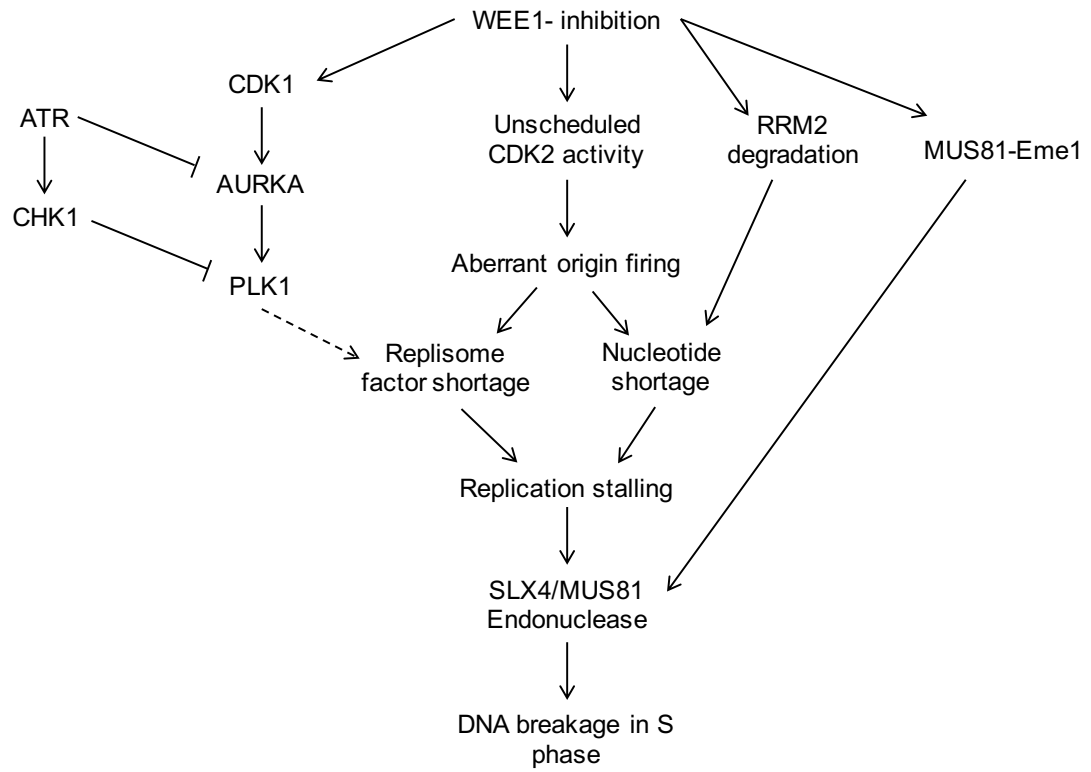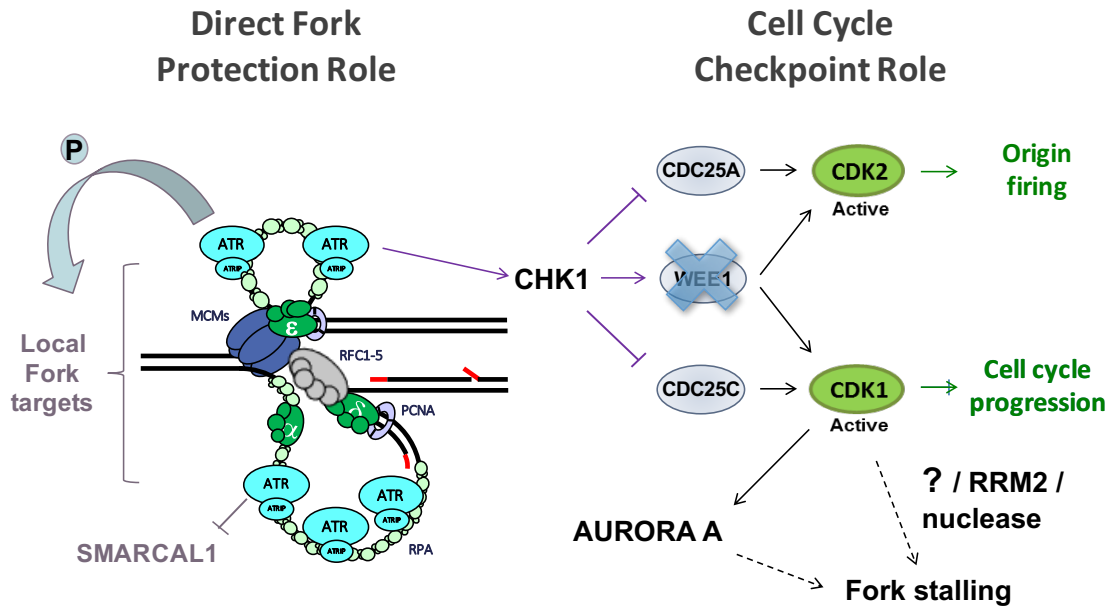
Figure 4.3

Figure 4.4

Figure 4.5

Figure 4.6

A.

B.
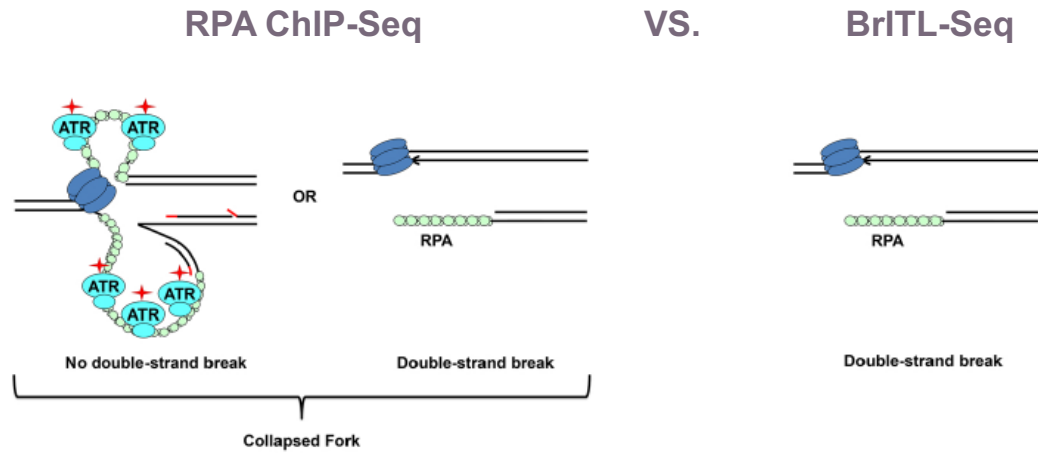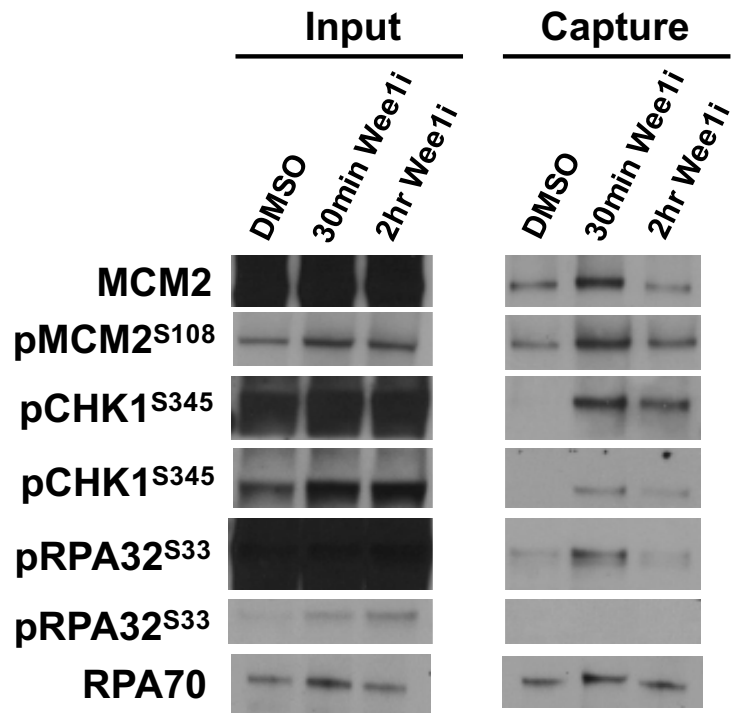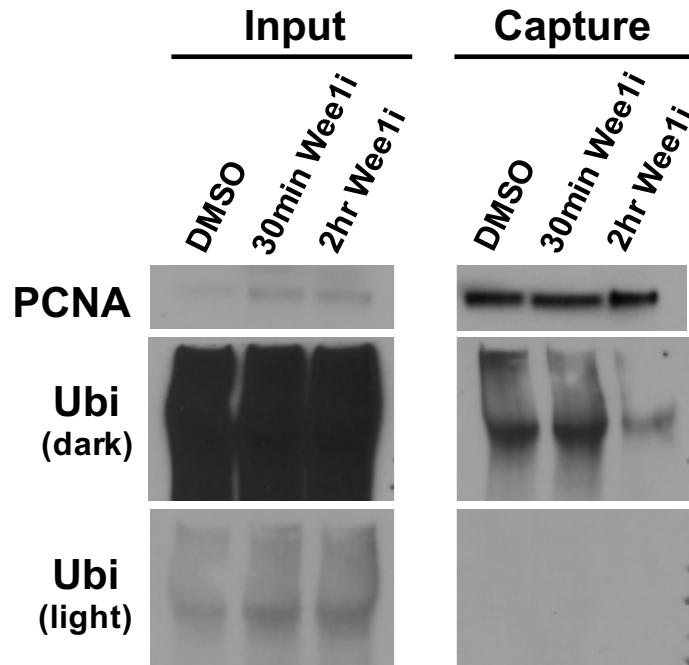

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