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Exploration of Ataxia Telangiectasia and Rad3-Related's (ATR's) Role in Cell Death Regulation: Implications in Development, Cancer, and Stroke

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by

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ABSTRACT

Exploration of Ataxia Telangiectasia and Rad3-Related's (ATR's) Role in Cell Death Regulation: Implications in Development, Cancer, and Stroke

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From gametogenesis until death an organism's genome is under constant bombardment from endogenous and exogenous sources of DNA damage. To maintain genomic integrity amid this damage, cells have evolved responses which allow them to either preserve viability for recovery or initiate self-destructive pathways depending on the severity of DNA damage. One protein involved in initiating and carrying out these responses is the protein kinase ataxia telangiectasia and Rad3-related (ATR). ATR is known primarily for its regulatory role in initiating the checkpoint-signaling cascade following DNA damage and replicative stress. These signaling events lead to cell cycle arrest, DNA repair, or apoptosis when damage is too extreme. In addition to these kinase-dependent roles, ATR also is capable of directly blocking the intrinsic apoptotic pathway through structural sequestration of the proapoptotic protein tBid. The sum of these regulatory events is a delicate balancing act resulting in either cell death or cell survival depending on the severity of the damage and the differentiation state of the cell in question. In the following studies, we sought to investigate the complex interplay of ATR's kinase and structural roles in determining cellular fate. First, we investigated the structural role of prolyl isomerization of ATR across development by using mouse models of two isomerically locked forms of ATR which were previously shown to lock cytoplasmic ATR into a single isomer. Studies showed that ATR which is locked in ATR-L (*trans*-ATR, hATR-P429A/mATR-P432A) is embryonically lethal and that heterozygotes tend to have neurological and other developmental

abnormalities. This contrasts with ATR-H (*cis*-ATR, hATR-S428A/mATR-S431A), which is viable, but naturally prone to cancer development. Next, we used various *in vitro* stroke-like conditions to test if ATR inhibition could serve as a therapeutic target for stroke. We found that ATR inhibition is protective in non-dividing neuron-like cells; whereas, it potentiates death in cycling glial and immune-like cycling cells. Thus, ATR inhibition could likely be a target for both neuron sparing and immunosuppressive anti-stroke therapeutic strategies. Taken together, these studies provide insightful information into the structural and pathological roles of ATR in development and disease.

DEDICATION

I would like to dedicate the work herein to my family and friends. Without them enduring my incessant complaining, late night rantings, and random distraught messages I would never have been able to complete this journey.

A special dedication goes to my grandmother, Lula Cartwright, and my late grandmother, Hazel Triplett. Throughout this process, their words kept me going even in the roughest of times; for that I am forever grateful.

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CHAPTER 1

INTRODUCTION

Cellular responses to DNA damage

An organism's genome is under constant assault from both endogenous and exogenous sources of DNA damage. In order to maintain genomic integrity despite this damage, cells must respond in order to either repair the damage incurred or to eliminate critically damaged cells. This response, fittingly deemed the DNA damage response (DDR), is initiated by three members of the PIKK (phosphatidylinositol-3-kinase-like family) family of proteins: ATM (ataxia-telangiectasia mutated), ATR (ataxia-telangiectasia and rad3-related), and DNA-PK (DNA-dependent protein kinase). These three protein kinases work together to facilitate changes in the cellular environment to either promote cell cycle arrest and DNA repair for continued cell survival or to promote cell death to eliminate severely damaged cells.

While the studies contained within this dissertation are focused on the role of ATR in the modulation of cell death, in the following sections we will present an overview of DNA damage signaling (DDS), various mechanisms of cell death, and some general information pertinent to the contained studies. Lastly, we will generalize some of the findings contained within each of our studies.

DNA damage signaling and cell-cycle arrest

Following DNA damage, cells will initiate signaling in order to arrest cell cycle progression and, ultimately, to determine whether a cell should repair its DNA or be eliminated by undergoing cell death. While all three members of the PIKK family mentioned previously are

involved in regulation of cell cycle arrest, ATR and ATM are the primary regulators as shown in Figure 1.1.

The primary response of either ATR or ATM in cell cycle regulation and DDS is determined by two main factors: 1) the stage of the cell cycle and 2) the type of DNA damage present (Ciccia and Elledge 2010; Marechal and Zou 2013; Sancar et al. 2004; Sirbu and Cortez 2013). ATM functions primarily through its effector kinase Chk2 (checkpoint kinase 2) to signal DNA damage caused by DNA double strand breaks (DSBs). While ATM is active throughout the entirety of the cell cycle, it is predominantly responsible for signaling during the G1 phase and into the G1/S transition (Sancar et al. 2004; Sirbu and Cortez 2013). Upon DNA damaging during these phases, ATM phosphorylates its main substrate Chk2 on Thr68 leading to its activation and priming of the cell for cell cycle arrest (Falck et al. 2001; Marechal and Zou 2013). Following activation, Chk2 phosphorylates two main cell cycle substrates: cdc25A and p53. Phosphorylation of cdc25A on Ser123 by Chk2 limits the activity of cdc25A by targeting it for ubiquitination and proteasomal degradation. This prevents cdc25A, a protein phosphatase, from removing inhibitory phosphorylations from CDK2 (cyclin dependent kinase 2), which is required for progression from the G1 phase to the S phase of the cell cycle (Falck et al. 2001; Mailand et al. 2000). In addition, ATM and Chk2 phosphorylate p53 at Ser15 and Ser20. These phosphorylations serve to dissociate p53 from its negative regulator MDM2 as well as to activate its transcriptional activity. These events result in p53-mediated transcriptional upregulation of p21 ($p21^{WAF1/Cip1}$), a direct inhibitor of not only CDK2, but also an indirect inhibitor of CDK1 (cyclin dependent kinase 1) (Falck et al. 2001; Maya et al. 2001). These events lead to cell cycle arrest as CDK1 and CDK2 are necessary to promote cell cycle progression (Bartek and Lukas 2003).

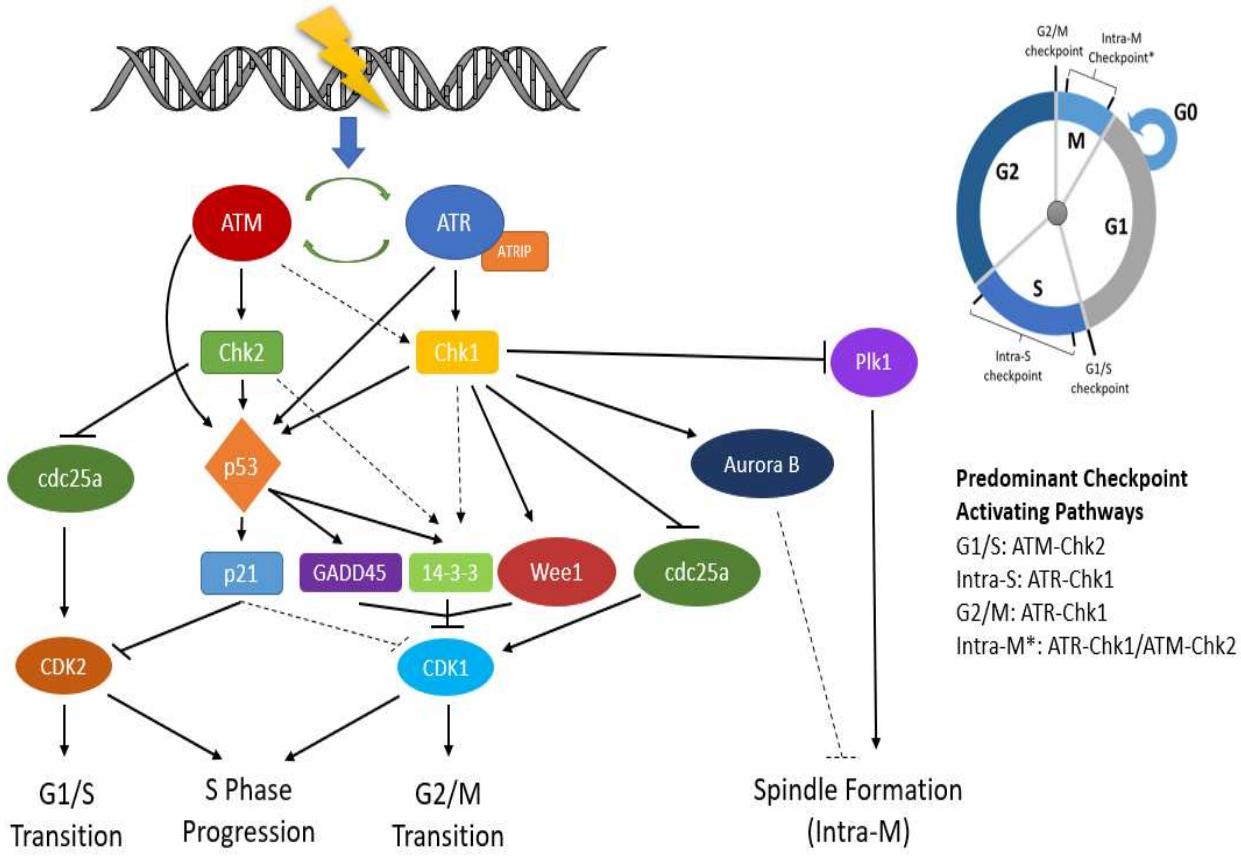


Figure 1.1 Summary of DNA damage response checkpoint activation
(Modified from Cartwright *et al*. 2018)

Whereas ATM is responsible for the G1/S checkpoint, ATR is responsible for activating the intra-S and G2/M checkpoints. Upon activation by single stranded DNA (ssDNA) generated under replication stress or DSB end resection, ATR activates its effector kinase Chk1 through phosphorylation at Ser317 and Ser345 (Zhou et al. 2002; Zou and Elledge 2003). Similar to Chk2, Chk1 also works through phosphorylating cdc25A at Ser123 and p53, but only at Ser20, not Ser15. Each of these results in effects similar to that seen by Chk2 during cell cycle arrest at the G1/S transition; however, the effects of Chk1 carry through into an intra-S checkpoint as well as into the G2/M transition (Bartek and Lukas 2003; Zhou et al. 2002). ATR activity through its substrate Chk1 also serves to facilitate this process through modulation of Wee1. Once activated

by Chk1, Wee1 phosphorylates CDK1, inhibiting this protein's kinase activity (Harvey et al. 2005). In this way, ATR induces the suppression of CDK1 activity through indirectly facilitating its suppression and prevention of its activation.

In addition to p53's induction of $p21^{WAF1/Cip1}$, p53 also upregulates two other important regulators of cell cycle progression: proteins GADD45a and 14-3-3. Each of these proteins regulate the G2/M checkpoint; however, do so through differing mechanisms. GADD45a binds directly to and suppresses the activity of CDK1, preventing the G2/M transition from occurring (Yang et al. 2000). 14-3-3 works through inhibition of cdc25C, another regulatory phosphatase, through facilitating the nuclear export of cdc25C and, subsequently, its removal from its target substrate, CDK1. This leads to accumulated inactive CDK1 and stalling of the cell cycle at the G2/M transition (Peng et al. 1997).

While individual stalling of the cell cycle is important to note, one must consider that any activation of ATR or ATM is likely to invoke subsequent DDR and cell cycle arrest regardless of cell cycle phase. This is due to known cross activation within the pathways, as shown in Figure 1.1. Noting this, information regarding ATR and ATM activation should be taken as a tentative guideline as to what is predominantly seen (Myers and Cortez 2006; Serrano et al. 2013; Stiff et al. 2006).

Apoptosis and other mechanisms of cell death

When damage is too extreme for survival, cells must have programs in place to ensure they can be removed before they can undergo cancerous transformation. The most common of these processes is apoptosis, a highly-regulated and energy-dependent form of cell death. Apoptosis is common to both cycling and non-cycling cells; however, the process differs slightly

between the two due to the absence of Chk1 in non-cycling cells (Kemp and Sancar 2016). These processes are summarized in Figure 1.2 A & B.

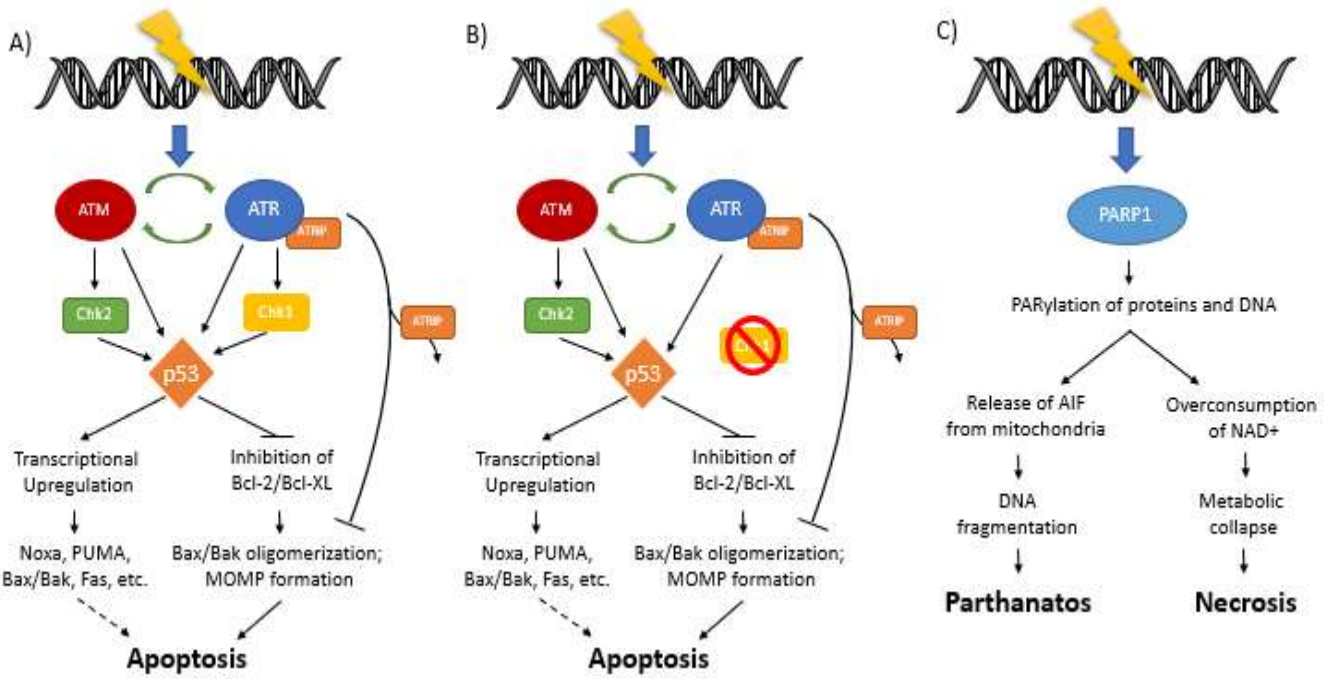


Figure 1.2 Mechanisms of cell death in cycling and non-cycling cells
A) Mechanisms leading to apoptosis after DNA damage in cycling cells. B) Mechanisms leading to apoptosis in non-cycling cells. C) Mechanisms of PARP1 driven parthanatos and necrosis. (Modified from Cartwright *et al*. 2018)

Upon DNA damage, ATR and ATM phosphorylate p53 at Ser15. This phosphorylation leads to the activation of p53's transcriptional activity which results in upregulation of numerous pro-apoptotic genes including Bax/Bak, Puma, Noxa, Fas-ligand, and others (Riley et al. 2008; Zilfou and Lowe 2009). Puma and Noxa, members of the Bcl-2 family of proteins, inhibit the anti-apoptotic proteins Bcl-2 and Bcl-XL. Under normal conditions, these proteins are bound to the pro-apoptotic proteins Bax and Bak. By releasing Bax and Bak from their negative regulators, they are allowed to oligomerize and induce mitochondrial outer membrane permeabilization (MOMP) and subsequently apoptosis (Brady and Attardi 2010; Vaseva and Moll 2009). In addition to this, apoptosis is further promoted through Fas-ligand mediated

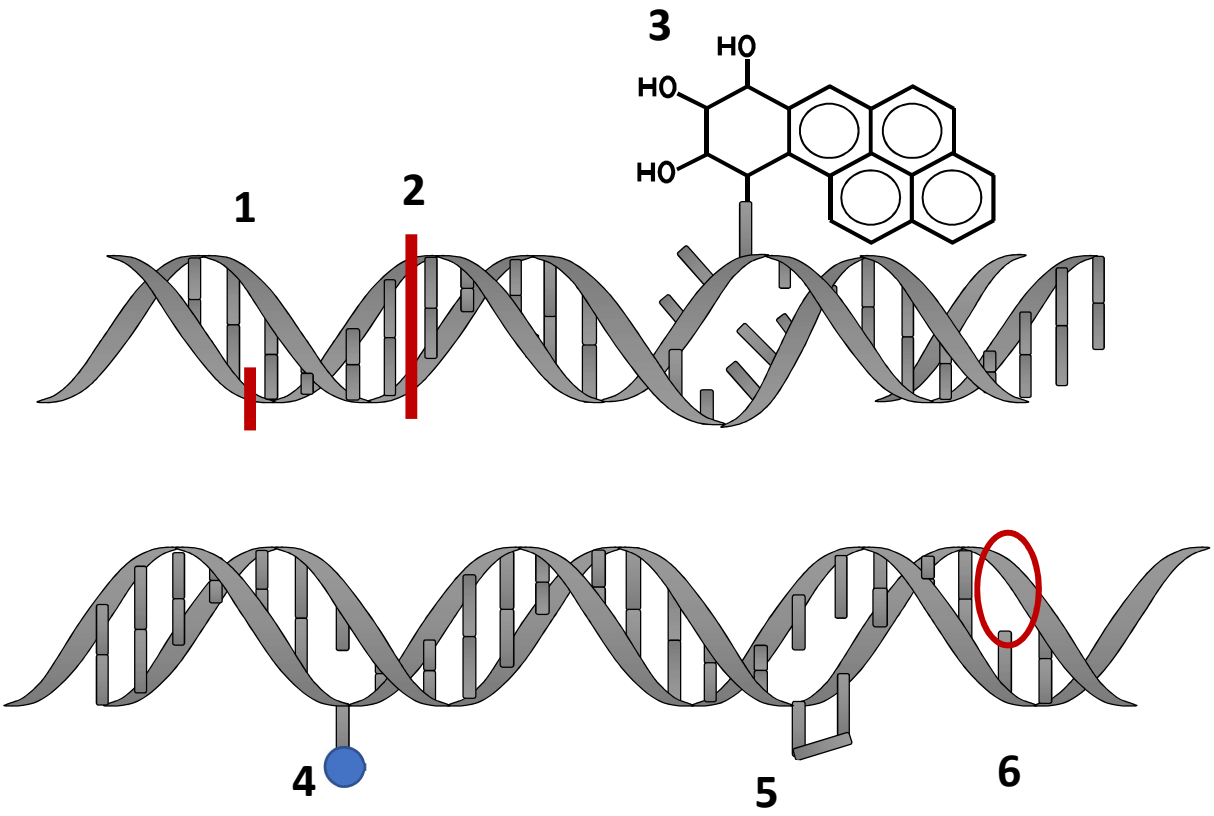
upregulation. This receptor functions to facilitate p53-dependent extrinsic apoptosis following DNA damage (Brady and Attardi 2010). In addition to its transcriptional roles in apoptosis, p53 also is capable of directly promoting MOMP through direct displacement of Bax and Bak from their suppressing proteins (Chipuk et al. 2005; Leu et al. 2004). While these pathways support the involvement of ATR and ATM's activation of p53 in promotion of apoptosis, it is important to also note that their roles have been noted in both direct and indirect suppression of apoptosis (Hilton et al. 2016; Imanishi et al. 2014; Kemp and Sancar 2016; Korwek et al. 2012). This is notable, and expected, as the decision to undergo apoptosis should be highly regulated under a variety of checks and balances.

In addition to apoptosis, another DNA damage-induced form of cell death is due to overactivation of the protein PARP1 (poly-ADP ribose polymerase 1). Under cases of extreme oxidative damage or calcium overload, PARP1 can become hyperactivated leading to either parthanatos or necrosis (Wei and Yu 2016). Overviewed in Figure 1.2 C, PARP1 hyperactivation can lead to AIF (apoptosis inducing factor) release from mitochondria. Following release, AIF translocates to the nucleus where it induces DNA fragmentation in a non-specific fashion. Alternatively, necrosis from PARP1 hyperactivation occurs under extreme stress leading to overconsumption of NAD^+ and subsequent metabolic collapse (Weaver and Yang 2013).

DNA Damage Repair

In cases where DNA damage is not extreme enough to induce cell death, the cell will attempt to restore genomic integrity through repair of the damaged DNA. The pathway of repair engaged is dependent on the lesion or lesions present. A summary of potential lesions and their major repair pathways is shown in Figure 1.3. For the sake of brevity, we will only discuss the

pathways relevant to the works addressed in this dissertation: base excision repair and nucleotide excision repair.



DNA Repair Pathway	Types of Damage
BER	Alkylation, spontaneous depurination or depyrimidination, deamination, oxidation, single-strand breaks
NER	Bulky adducts (benzo(a)pyrene, photoproducts, etc.), intrastrand crosslinks
MMR	A>G mismatches, T>C mismatches, trinucleotide expansions, base deletions
NHEJ/Alt-NHEJ	Double-strand breaks
HR	Double-strand breaks
ICLR	Inter- and intrastrand crosslinks

Figure 1.3 Types of DNA lesions and their repair pathways
1-Single-strand break (SSB), 2-Double-strand break (DSB), 3- Bulky DNA adduct (BA) (Reactive aromatic hydrocarbons, reactive lipids, reactive proteins), 4- Direct base oxidation (8-oxoguanine, thymine glycol, etc.), 5- Pyrimidine dimer (UV or oxidatively induced), 6-Abasic site (Modified from Cartwright *et al*. 2018)

Base Excision Repair. Bases excision repair (BER) most commonly repairs and resolves DNA lesions resulting from oxidation, alkylation, or spontaneous depurination/depyrimidination (abasic site formation). The initial step of classic BER is cleavage of the N-glycosylic bond of the modified base by various DNA glycosylases. Then the base is removed by APE1 (apurinic exonuclease 1) resulting in the formation of an apurinic/apyrimidinic (AP) site where the damaged base was once positioned. Following AP site formation, PARP1 processes the AP site through an addition of a polymer of ADP-ribose which serves as a scaffold for recruitment of WRN (RecQ/Werner protein), a helicase and exonuclease, which will further process the site before DNA poly β (DNA polymerase β) fills in the missing base. The break in the repaired strand is then ligated by DNA ligase I or DNA ligase III (Fig.1.4 A) (Fromme and Verdine 2004; Sancar et al. 2004; Wei and Yu 2016).

In addition to classic BER, an alternative form of BER exists where, instead of forming an AP site, the region is instead resected. In this instance, APE2 (apurinic exonuclease 2) will remove the damaged base and bases adjacent to the damaged base. This forms a short patch of ssDNA which is repaired by similar mechanisms to that seen in nucleotide excision repair (NER). In brief, PCNA (proliferating cell nuclear antigen) and RFC (replication factor C) are recruited to the ssDNA where they then recruit one of three DNA polymerases: DNA pol δ (DNA polymerase delta), DNA pol ε (DNA polymerase epsilon), or DNA pol κ (DNA polymerase kappa). One of these DNA polymerases then will fill in the gap generated by APE2 on the initially damaged strand. Following gap resynthesis, either DNA ligase I or DNA ligase III will finish the repair by religation of the damaged strand (Sancar et al. 2004; Schärer 2013).

While classic BER does not tend to activate DDR unless the damage is extreme, the alternative form of BER is found to activate DDR through the ATR pathway. This is due to the

ssDNA gap that is generated through APE2 end resection and occurs before the DNA can be repaired.

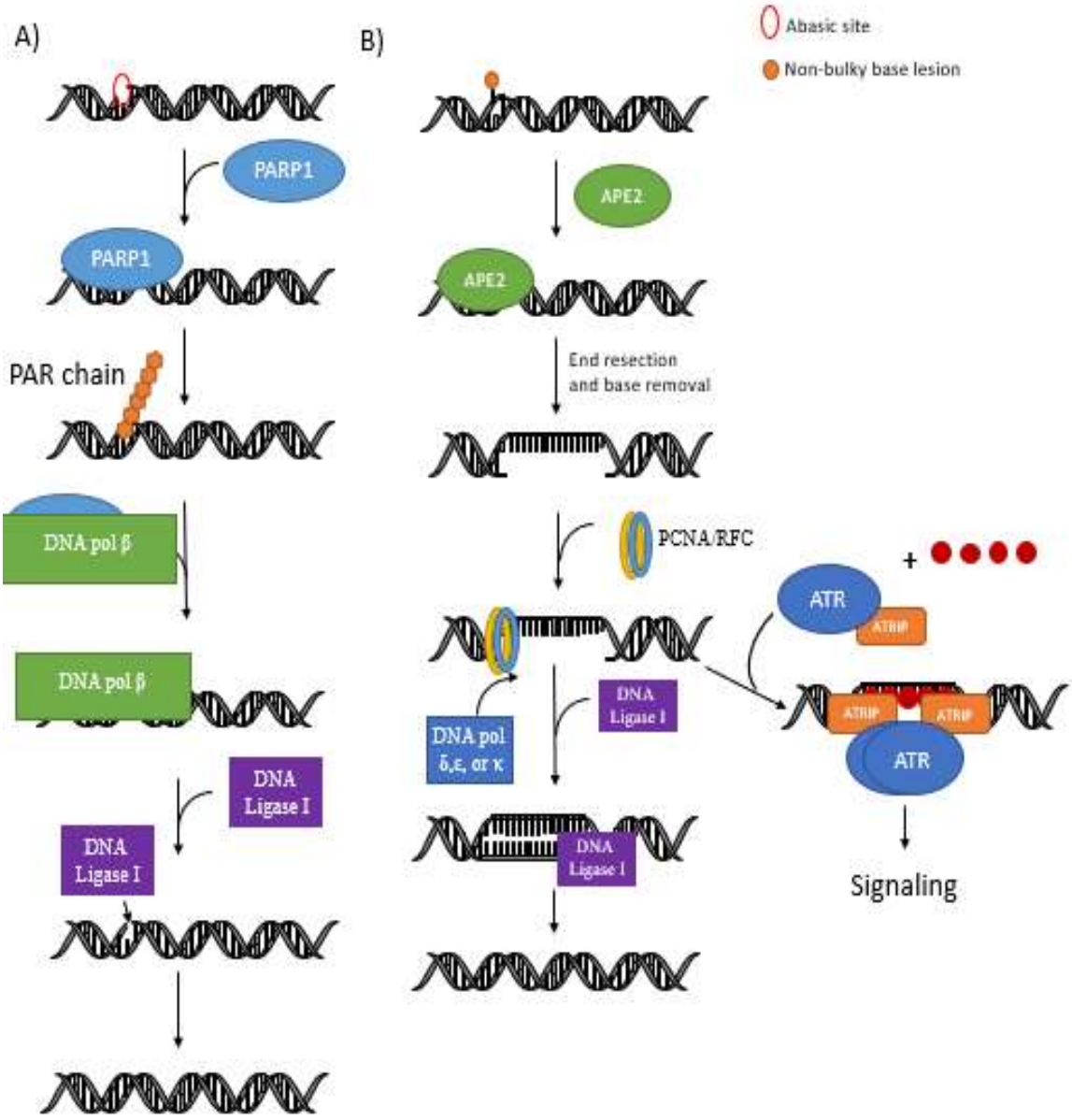


Figure 1.4 Base Excision Repair (BER)
A) PARP1-mediated BER of an abasic site. Initial AP site generation is omitted. B) APE2-mediated BER/short-patch NER and resultant ATR activation (Modified from Cartwright *et al*. 2018)

Nucleotide Excision Repair. Nucleotide excision repair (NER) most commonly repairs and resolves DNA lesions resulting from UV-induced photoproducts, crosslinking agents, and other bulky adducts that can distort the helical structure of DNA. Two main forms of NER exist: global genome repair (GG-NER) and transcription-coupled repair (TC-NER) (Figure 1.5) (Mu and Sancar 1997; Schärer 2013; Sugasawa et al. 1998).

While the downstream mechanisms of bulky lesions repaired by both GG-NER and TC-NER are similar, the initiation and initial recognition of damage vary between the two. In GG-NER, the lesion is recognized either by XPC (Xeroderma pigmentosum complementation group C)-HR23B or XPC-HR23B in combination with DDB1-DDB2/XPE (Damage specific DNA binding protein 1 or 2-Xeroderma pigmentosum complementation group E) to enhance affinity for the lesion. Once damage is recognized by the XPC-HR23B complex, it recruits transcription factor IIH (TFIIH) which mediates strand unwinding and ssDNA generation. This single strand is rapidly bound by replication protein A (RPA) to stabilize the intermediate and allow for verification of the DNA lesion by XPA (Xeroderma pigmentosum complementation group A). Once the lesion is verified, XPG (Xeroderma pigmentosum complementation group G) and XPF-ERCC1 (Xeroderma pigmentosum complementation group F-excision repair cross-complementation group 1) will excise the DNA segment containing the bulky lesion by performing 3’ and 5’ incisions, respectively (Gaillard and Wood 2001; O'Donovan et al. 1994; Schärer 2013). TC-NER differs only in the initial recognition step which is performed by ERCC6/CSB (excision repair cross-complementing group 6/Cockayne syndrome B) and results from recognition of stalled RNA polymerase II. ERCC6/CSB facilitates ubiquitin-mediated removal of RNA polymerase II and recruitment of TFIIH (Gaillard and Aguilera 2013; Schärer

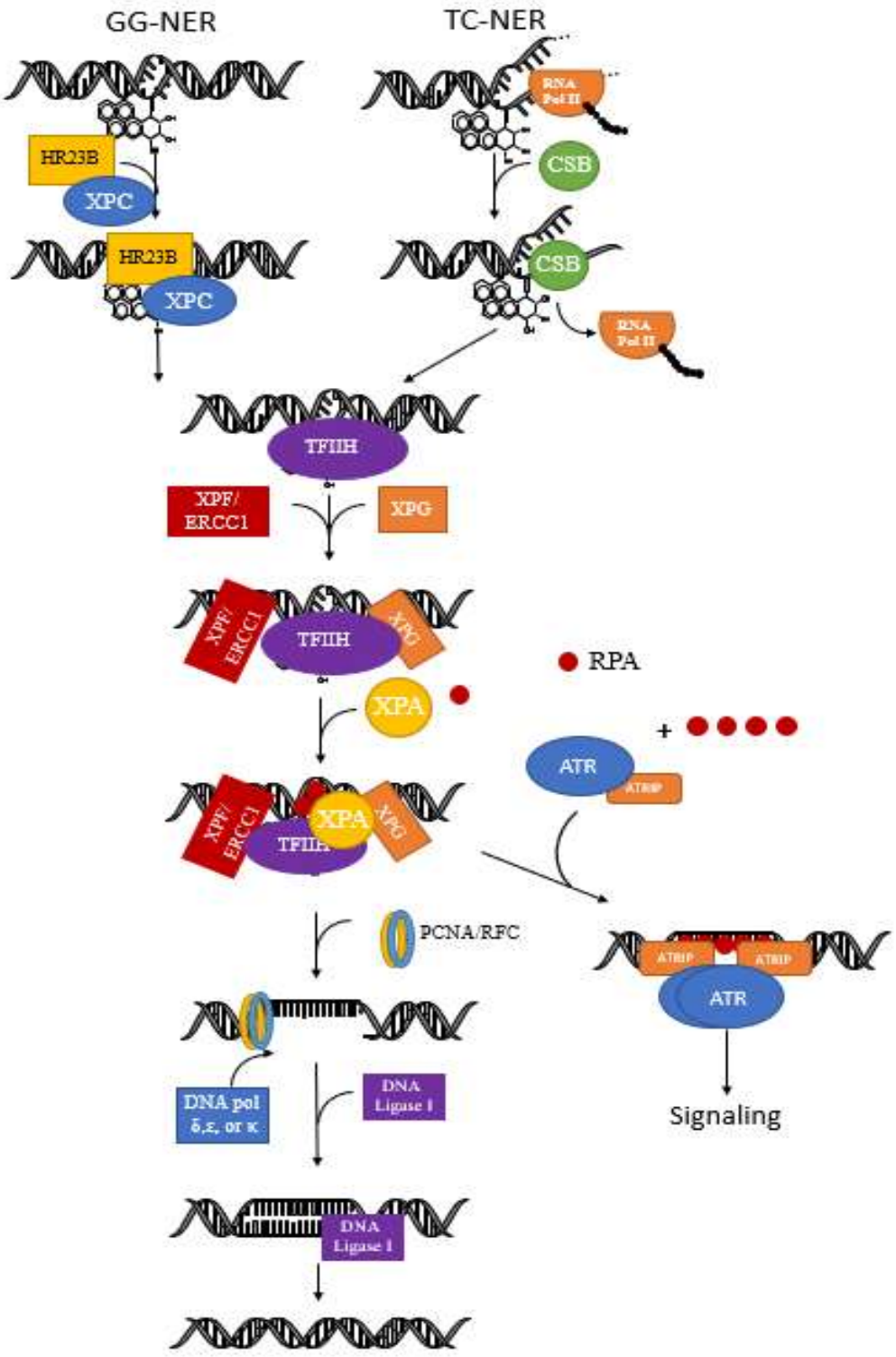


Figure 1.5 Nucleotide Excision Repair (NER)
A) NER and resultant ATR activation (Modified from Cartwright *et al*. 2018)

2013). Once the damaged DNA is removed, the gap will be filled as mentioned previously in reference to alternative BER.

Unlike with alternative BER, the DDR through ATR is always activated during this process. In fact, ATR is necessary to facilitate NER through facilitation of XPA nuclear import and stability (Lee et al. 2014; Li et al. 2013).

Ataxia telangiectasia and rad-3 related (ATR)

Ataxia telangiectasia and Rad-3 related (ATR), is one of the three main protein kinases responsible for eliciting the DDR. Primarily nuclear in distribution, ATR is responsible for regulating a variety of cellular processes ranging from cell cycle arrest, to DNA repair and cell death (Cimprich and Cortez 2008; Hilton et al. 2016; Kemp and Sancar 2016; Sancar et al. 2004; Wang-Heaton 2016; Zeman and Cimprich 2014). Nuclear ATR typically exists in complex with its binding partner ATRIP (ATR interacting protein). Together, they bind to RPA coated ssDNA during periods of replication stress or DNA damage. This binding leads to a conformational shift during which ATR undergoes autophosphorylation and activation (Zou and Elledge 2003). Subsequent to this activation, the ATR-ATRIP complex goes on to phosphorylate hundreds of proteins influencing everything from direct cell cycle progression to transcription (Matsuoka et al. 2007; Wagner et al. 2016). In contrast to its nuclear functions, cytoplasmic ATR is not bound to ATRIP and does not possess kinase activity. Instead, cytoplasmic ATR is found to regulate apoptosis through structural sequestration of the pro-apoptotic protein t-Bid (truncated-Bid) (Hilton et al. 2016).

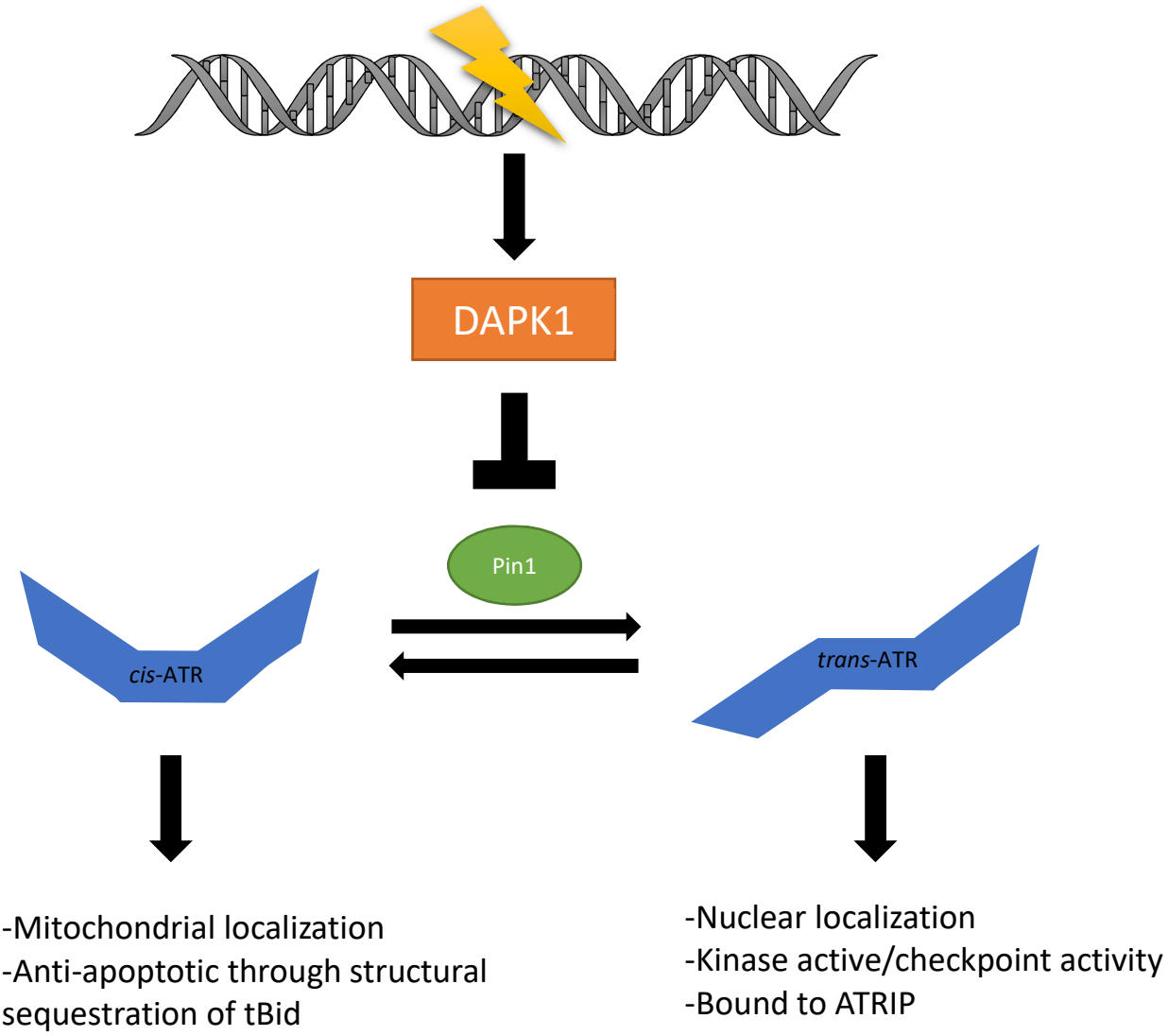


Figure 1.6 Prolyl isomerization of ATR

Prolyl-isomerization of ATR and direct anti-apoptotic activity

The differences between nuclear and cytoplasmic ATR can be explained further by looking at the structural changes that occur in the protein following DNA damage (Figure 1.6). Previous findings by our lab has shown that under normal circumstances the cell only possesses a single prolyl isoform of ATR, ATR-L/*trans*-ATR. This isoform is predominantly nuclear and is dependent on Pin1 (peptidylprolyl *cis/trans* isomerase NIMA-interacting 1) to be maintained.

Pin1 function through isomerizing proline groups following a phosphorylated serine residue which in the case of ATR is Ser428. However, following DNA damage, Pin1 is inactivated by DAPK1 (Death associated protein kinase 1) through phosphorylation of Pin1 on Ser71. With loss of Pin1 activity, the total amount of ATR as well as the ATR-H/*cis*-ATR form increases in the cytoplasm. While not binding to ATRIP or having kinase activity, ATR-H possesses an intrinsic anti-apoptotic activity. During the prolyl isomeric shift from ATR-L to ATR-H a novel BH3 domain is revealed. This exposed domain allows ATR-H to bind to and sequester t-Bid, thus preventing apoptosis (Hilton et al. 2016).

This S428P429 site and its isomeric shift is of interest due to the fact that previous investigation into the phosphorylation status of cytoplasmic ATR pSer428 revealed a lower phosphorylation status of cytoplasmic ATR in ovarian carcinoma was associated with poor prognosis and an increased resistance to chemotherapeutics (Lee et al. 2015). In this way, these findings could support a role for the ATR prolyl isomeric shift in chemotherapeutic/DNA damage tolerance.

ATR has multiple roles during development

In addition to its roles in DDR, it is important to note that ATR is essential to organismal development. Previous studies have shown that embryonic mice lacking ATR die prior to E7.5/8.5 and that conditional knockout in adult animals leads to rapid stem cell depletion and accelerated ageing (Brown and Baltimore 2000; Ruzankina et al. 2007). Tangential to this, ATR has been shown to be necessary for cilia signaling, organ development, post-natal nervous system development up to adolescent stages, and to overall survival (Lang et al. 2016; Lee et al. 2012; Ruzankina et al. 2007; Stiff et al. 2016).

Known roles of ATR in responding to calcium overload and oxidative damage: an implication in ischemic stroke

During ischemic stroke, the cells of the nervous system are assaulted by a barrage of excitatory neurotoxic compounds, calcium overload, and oxidative stress (Chen et al. 2011; Kritis et al. 2015; Li et al. 2018; Rodrigo et al. 2013). These events subsequently lead to DNA damage and cellular death. ATR is known to be activated by oxidative DNA damage (Martin et al. 2012; Moreno et al. 2019; Yan et al. 2014) and our previous studies also have implicated its involvement in preventing calcium overload-induced cell death (Wang-Heaton 2016). Given that the effects of ATR inhibition have been found to differ between cycling and noncycling cells we sought to understand whether those results may extend past UV irradiation and into other disease states such as ischemic stroke. The studies presented in Chapter 3 support the link presented here and show potential implications for ATR inhibition in the therapeutic treatment of ischemic stroke.

Findings of these studies

These studies further elaborate upon the roles of ATR in development and its influence on cell death under various conditions. Through the studies presented in Chapter 2, we show that cytoplasmic-ATR prolyl isomerization is required for organismal survival and development; however, in contrast, one structural isoform, ATR-H, also can potentially promote spontaneous tumorigenesis. Then, in Chapter 3, we show that inhibition of ATR's kinase activity differentially affects cycling and non-cycling cell populations. Together, these studies show that ATR is a complex regulator of both development and disease and that this regulation is dependent on both its structural and kinase functions. While further studies need to be

performed, the preliminary findings presented in Chapters 2 and 3 present new avenues into the exploration of ATR as a target in the treatment of human disease.

CHAPTER 2

ISOMERICALLY LOCKED FORMS OF ATAXIA TELANGIECTASIA AND RAD3-RELATED (ATR) HAVE DIFFERING ROLES IN DEVELOPMENT AND DE NOVO CARCINOGENESIS

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Keywords: ATR, cancer, embryonic lethality, oncogenesis, prolyl isomerism

ABSTRACT

Ataxia telangiectasia and Rad3-related (ATR) is a multifunctional protein kinase that functions in a variety of cellular processes ranging from cell cycle arrest, to DNA repair and apoptosis. Previous studies have shown that cytoplasmic ATR assumes two prolyl isomeric forms depending on the state of cellular stress. Under basal conditions, cytoplasmic ATR assumes the *trans* isomer (*trans*-ATR/ATR-L) and under stress conditions it assumes the anti-apoptotic *cis* isomer (*cis*-ATR/ATR-H). In this study, we sought to investigate the role of cytoplasmic ATR isomerically locked into either the *cis*- or *trans*- isoform. Using mice with isomerically locked ATR-S431A (*cis*-ATR/ATR-H) or ATR-P432A (*trans*-ATR/ATR-L), we investigated the role of each isoform on phenotypic development. We found that the *trans* isomer ATR-P432A is embryonically lethal and that ATR-P432A/WT heterozygotes tend to have neurological and other developmental abnormalities. In contrast, the *cis* isoform ATR-S431A produced viable homozygotes; however, mice possessing one or both alleles locked in the *cis* isoform were prone to spontaneous cancer development. These findings indicate that ATR prolyl isomerization plays a critical role in development and maintenance of the interconversion between the two isoforms is necessary for maintaining organismal homeostasis.

INTRODUCTION

Ataxia telangiectasia and Rad3-related (ATR), a member of the PIKK (phosphatidylinositol-3-kinase-like) family, is a protein kinase known for its roles in regulating cell cycle progression, DNA repair, and maintenance of genomic stability following both endogenous and exogenous DNA damage (1-5). Nuclear ATR exists in a complex with the ATR-interacting protein (ATRIP) allowing it to sense DNA damage and replication stress through binding to RPA-coated ssDNA leading to its activation (4). When activated, ATR phosphorylates hundreds of proteins allowing it to induce cell cycle arrest, modify transcription and DNA repair, or even induce cell death in cases where damage is too extreme (2, 6-8). Recent studies have shown that, in addition to these nuclear roles, using a previously unknown BH3 binding domain cytoplasmic ATR functions directly at the mitochondria where it suppresses the intrinsic apoptotic pathway through structural sequestration of tBid. This shift in function comes via an isoform switch due to the actions of Pin1 (peptidylprolyl *cis*/*trans* isomerase NIMA-interacting 1). Pin1, a prolyl isomerase, isomerizes human ATR at proline 429 (mouse proline 432) causing ATR to shift from *cis*-ATR (ATR-H) to *trans*-ATR (ATR-L). Under basal conditions ATR is present in the *trans*- isomer and is primarily nuclear. However, upon DNA damage-induced phosphorylation of DAPK1, Pin1 is inactivated which allows cytoplasmic ATR to return to the *cis*- isomer which exposes its BH3 domain allowing it to bind tBID (9).

ATR is essential in organismal development. Deletion of ATR or removal of its kinase activity leads to early embryonic lethality in mice before embryonic day E7.5 and E8.5, respectively (10, 11). In addition to its roles in embryonic development, it also has been shown to be indispensable in post-natal cilia signaling, organogenesis, neuronal growth, and overall survival (12-16). In humans, ATR mutations can lead to different phenotypes depending on the

degree of mutation. Seckel syndrome 1, also known as bird-headed dwarfism (Seckel type) or microcephalic primordial dwarfism, is an autosomal recessive disorder resulting from splice variant mutations leading to hypomorphic ATR. This disorder is characterized by congenital malformation, mental retardation, and shortened life span which have all been attributed to increased replication stress resulting from low ATR levels (17, 18). ATR mutations also have been implicated in cancer development with certain ATR germline mutations predisposing individuals to higher cancer risk (19-21). Other studies have found that ATR signaling is essential for continued cell replication and that ATR signaling functions to promote or dissuade cancer in a dose-dependent manner (5, 22, 23). As a result, ATR inhibition has become a target of great interest for therapeutic treatment and is currently being used in multiple clinical trials both alone and as an adjuvant to other therapies (24).

In this study, we investigate the effect of locking cytoplasmic ATR into either the *cis*- or *trans*- prolyl isomer. We provide evidence that mice with cytoplasmic ATR locked in the *trans*-isomer are embryonically lethal and that heterozygotes of this line show congenital and neuronal abnormalities. In addition, they show similar signs of advanced aging to those seen in Seckel mice and mice which have had tamoxifen-induced Cre conditional knockout of the entire ATR gene (16, 18). In contrast, we show that mice expressing ATR isomerically locked in the *cis*-isomer show no outward deleterious developmental defect, but are prone to spontaneous cancer development. These studies reveal novel insight into the role of ATR prolyl isomerization during development and normal aging.

METHODS

Animals

Isomerically locked ATR mice (ATR S431A/cis-ATR/ATR-H or ATR-P432A/trans-ATR/ATR-L) were generated by the Cincinnati Children's Hospital Medical Center CCHMC Transgenic Animal & Genome Editing Core. Cas9 editing was used through direct injection into fifteen C57BL/6 embryonic stem cells (ESCs). The guides used (with mutation sites highlighted) are:

5'-CTCAACAGGAGAATCTCAGTGGTAATAATGATGAGGTGGCCCTAAGAGGCGTAAACTCA-3' (S431A)

-and-

5'-CTCAACAGGAGAATCTCAGTGGTAATAATGATGAGGTGAGCGCTAAGAGGCGTAAACTCA-3' (P432A)

Five founders possessing appropriate mutation profiles were identified and mice were bred until suitable progeny for continuing the lines were identified.

Animals are housed in climate-controlled rooms with a standard 12hr light/dark cycle. Mice are either individually housed or cohoused, when appropriate, in large plastic cages and allowed access to food and water *ad libitum*. Humane care was provided to comply with the federal legislation (Animal Welfare Acts) and the "Principles of Laboratory Animal Care" and the "Guide for Care and Use of Laboratory Animals". All procedures and protocols were approved by the East Tennessee State University Committee on Animal Care.

Mouse breeding

Mouse colonies were maintained by crossing heterozygous mice with wild types to prevent background mutational accumulation. Mice for breeding studies were identified by differential genotyping as described below. Individuals used for breeding studies were no younger than 12 weeks and no older than 32 weeks. For timed studies, females were exposed to soiled male bedding (Whitten effect) to induce estrus. Estrus was determined by visual staging at

which point they were placed with males. The following morning, they were checked for vaginal plugs and removed from the male's cage if they were positive. This was considered day E0.5.

Cell culture, transfections, and treatments

HCT-116 $ATR^{+/+}$ and HCT-116 $ATR^{flox/-}$ cells were obtained from either the American Type Culture Collection (ATCC) or Coriell Cell Repositories, respectively. Cultures were maintained in McCoy's 5A media (VWR) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific). All lines were supplemented with antibiotics (100 units/mL penicillin, 100 units/mL streptomycin, VWR) and maintained at 37ºC under a constant atmosphere of 5% CO_2.

An original construct, pcDNA3-FLAG-ATR, was a generous gift from Dr. Stephen Elledge. Mutagenesis on this parent construct was previously described (9). FLAG-ATR (wt), FLAG-ATR (S428A), and FLAG-ATR (P432A) were all transfected into HCT116 ATR flox/- cells using jetPEI transfection reagent (Polyplus) by the company's protocol. Cells were allowed to express constructs for 48hr prior to UV treatment. UV treatment dose was delivered by a 254nm lamp at a flounce of 0.83J/m^2/sec. 40J/m^2 was administered and cells were allowed to recover for 2-hour post-treatment. Cells then were harvested by scraping and suspended in lysis buffer (50mM Tris-HCl, pH7.8, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1x protease/phosphatase inhibitor cocktail [Thermo]).

Differential genotyping and sequencing

DNA for genotyping was obtained through a digest of a 1cm segment of tail or partial embryo per the given protocol, EZNA DNA kit (Omega Biotek). Isolated DNA then was amplified by using DreamTaq polymerase (ThermoFisher) and the following primers: Forward 5'- GACTCATGTAACACCTCATGCA-3', Reverse 5'-ACCCAAATTAAACAGGCATGC-3'.

The protocol was as follows: 94ºC for 2 min (initial denaturing), [94ºC for 15 seconds (denaturing), 54ºC for 15 sec (annealing), 72ºC for 2 min (elongation)] x38, 72ºC for 1 min (final elongation). Products then were digested with one of three restriction enzymes (AfeI, HaeII, or SfoI (NEB)) for 8hr at 37ºC before the restriction enzyme was denatured at 60ºC for 10min. Digests were electrophoresed for 30min at 100 V in 2% agarose gels in in Tris-acetate-EDTA buffer(40 mM Tris, 20 mM acetate, 1 mM EDTA, pH 8.3). No loading dye was used to allow for easier resolution of DNA bands with ethidium bromide (0.1 μg/mL). Gels were imaged with UV excitation in either a Fuji Film imager LAS-400 or an Amerscham Imager 680 (GE). Positive cleavage with AfeI (AGC˅GCT) revealed the presence of the S431A mutation whereas SfoI cleavage (GGG˅CCC) showed the presence of the P432A mutation. Digestion with HaeII (RGCGC˅Y) revealed the presence of a mutation but does not delineate which specific mutant is present. PCR cleanup and extraction of select gel bands were performed according to kit protocols (NucleoSpin Gel and PCR Cleanup, Macherey-Nagel). Confirmatory sequencing of certain mice was performed by dye-terminator sequencing on a Beckman CEQ 8000 automated DNA sequencer.

Echocardiography

M-mode echocardiography was performed using a ToshibaAplio 80 Imaging System (Tochigi) with a 12MHz transducer. Mice were anesthetized using induction of 2% isoflurane (Isothesia) with titrated maintenance under forced oxygen (0.5 L/min). Body temperature was maintained at 37ºC using a heating pad. Echocardiographic assessments were performed to assess heart placement, ventricle function, and heart rate.

Immunoblotting and antibodies

2xSDS loading buffer (0.5 M Tris-HCL pH 6.8, 4.4% w/v SDS, 20% glycerol, 0.2% bromophenol blue, 200 mM DTT) was added to cell lysates and then the mixture was heated at 95ºC for 5min. Samples were then loaded onto 8% SDS-PAGE gels for resolution of the proteins in question. Proteins were transferred from gels onto PVDF membranes (Millipore). Antibodies raised against actin (Cell Signaling), ATR (Bethyl, Clone 137A), Chk1 (Cell Signaling), and pChk1 Ser345 (Cell Signaling) were used. The chemiluminescent signal from HRP-conjugated secondary antibodies (ThermoFisher) was detected and captured using either the Fuji Film imager LAS-400 or an Amerscham Imager 680 (GE).

Tissue fixation, sectioning, and staining for phenotyping study

Mice were allowed to develop with no outside influence for a period of one year at which point they were euthanized by CO_2. Following euthanasia, mice underwent full body necropsy and were assessed for anything of gross pathological significance. Control and abnormal tissues were collected and immediately washed twice with ice-cold PBS. Following these rinses, the tissue was placed in 10% formaldehyde buffered with PBS for 24-48hr depending on tissue thickness. For cancer specimens, the tissue was rinsed and placed in 70% ethanol before being paraffin embedded. These sections were then cut at 20 μm with a knife microtome IEC horizontal before further processing. For embryos, the tissue was rinsed and allowed to sit in 30% sucrose (cryoprotectant) overnight. These specimens were then embedded in OCT (Fisher) and coronally sectioned using a cryostat CM3050SS (Leica). Both embryonic and cancer sections were hematoxylin and eosin stained to reveal morphological features. Gross anatomical images were acquired with a 16-megapixel, f/1.6 (OIS) 77-degree LG G7 mounted camera (LG). H&E histological images were acquired with an AMG FD1000 Evos microscope (Life Technologies).

RESULTS

Design and confirmation of cis-ATR (ATR-S431A) and trans-ATR (ATR-P432A) knock-in

To explore the role of isomerically locking cytoplasmic ATR, mice were generated which possess mutations in the Pin1 isomerization domain of ATR. Sterically locking ATR into the *cis*-isomer (*cis*-ATR/ATR-H) was accomplished through Cas9 gene editing resulting in the substitution of the serine in position 431 with alanine (S431A). Sterically locking ATR into the *trans*- isomer (*trans*-ATR/ATR-L) was accomplished by substitution of proline in position 432 with alanine (P432A). These substitutions allow for the steric locking of cytoplasmic ATR into one prolyl isomer over another due to the biochemical nature of the original Pin1 motif (Ser/Thr-Pro) as previously shown (9). Briefly, Pin1 requires phosphorylation on either a serine or threonine residue prior to a proline to have catalytic activity at the motif. By generating the ATR-S431A mutant, we can eliminate the catalytic activity of Pin1 on cytoplasmic ATR due to the phosphorylation of the serine residue being required for Pin 1 activity. This results in an inability of Pin1 to convert the *cis*-proline to *trans*-proline effectively locking ATR into the *cis*-isomer (ATR-H). In contrast, the ATR-P431A mutant takes advantage of the fact that all amino acids other than proline assume the *trans*-isomer in proteins (25). By substituting proline for alanine, we ensure that ATR is locked into the *trans*-isomer. The design and confirmatory sequencing of the various mutants are shown in Figure 1A & 1B, respectively.

Normal genotyping for the remaining experiments was carried out by PCR amplification of a common region followed by differential digest. For normal genotyping, HaeII was used. Both modifications to the ATR sequence have the HaeII restriction site allowing for a single enzyme to be used to distinguish between wildtype and mutated alleles (Fig.1C). The wildtype allele (459bp) is cleaved into two fragments (255 bp, 204 bp) by a single cut. For differential

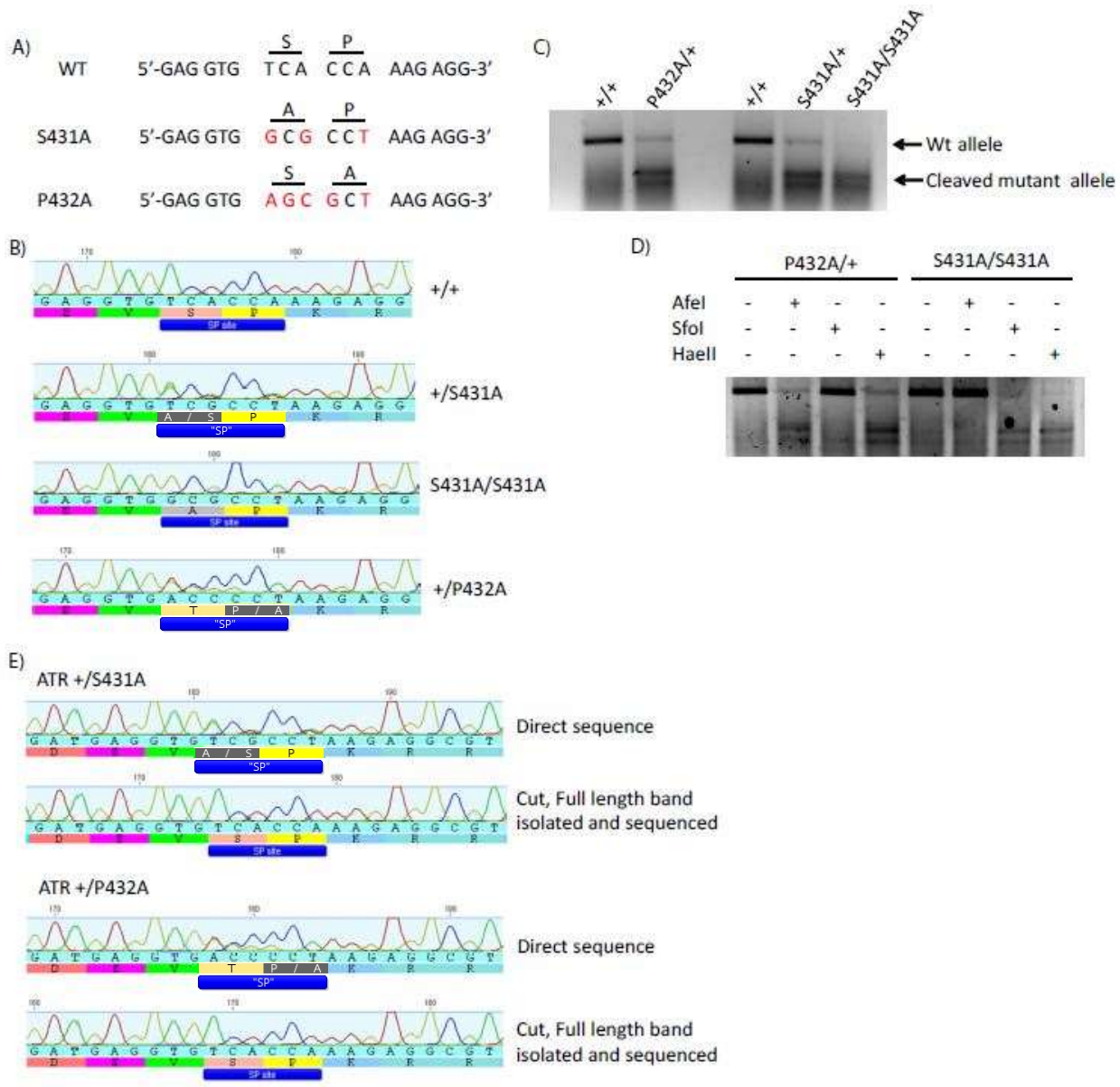

Figure 2.1: Targeted knock-in of ATR-S431A (*cis*-ATR/ATR-H) and ATR-P432A (*trans*-ATR/ATR-L)

A) Schematic showing the mutational substitutions to wild type mouse ATR. By introducing three base changes (in red), the serine 431 to alanine mutation was achieved with no change to the adjacent proline. A total of 5 bases (in red) were changed in the proline 432 to alanine. This resulted in no change to the adjacent serine. Changes to bases outside of the amino acid to be targeted were performed to insert restriction sites for differential genotyping. B) Confirmatory dye-terminator sequencing confirmed the presence of four different genotypes for the mice from F1 crossing of founder offspring. C) Example digest of PCR products from mice cleaved with the HaeII restriction enzyme. It is seen that the larger fragment (459bp) is cleaved into a 204 and 255 bp fragment denoting the presence of a mutated allele. D) Example differential PCR digest showing that AfeI can be selectively used to identify the ATR-P432A mutation while SfoI can be used to identify the ATR-S431A mutation. E) Confirmational sequencing of digested DNA showing that presence of the isolated full-length (wild type) band was not just due to inadequate digestion.

genotyping, AfeI and SfoI were used. AfeI recognizes a restriction site in only the P432A mutation whereas SfoI recognizes only the S431A mutation (Fig.1D). These enzymes were used to confirm the genotypes of lines.

One potential issue that arose during the sequencing that is common to dye terminator-based reactions is a potential misread of heterozygous genotypes due to double peaks. To ensure that these were indeed heterozygotes and not additional mutations in the gene, direct PCR sequences, as well as those of the full-length PCR product after digestion, were obtained. It was found that after digestion and analysis of the purified full-length fragment the ambiguity disappeared, and the sequence presented fully as wild type. This indicates that the other contributing allele was heterozygous and able to be cleaved by HaeII. This implies the automated readout of either SP or TP indicates the ATR-S431A/+ (ATR-*cis*/+) and ATR-P432A/+ (ATR-*trans*/+) genotypes, respectively (Fig. 1E).

Isomerically locked ATR mutants differ in developmental effects and embryonic lethality

Upon maintenance of mouse colonies, it was discovered that no mice bearing the ATR-P432A/P432A genotype were present. To determine if this was due to breeding error or if the ATR-P432A/P432A genotype was embryonically lethal a formal breeding study was established. Breeding showed that there was no statistical difference in litter size between the different lines (Table 2.1). A subset of these litters was then used to assess for embryonic lethality. Criteria for

Average litter size				
Genotype Crossing			Number of litters	Average Litter Size
+/+	x	+/+	7	6.50 ± 0.94
S431A/+	x	S431A/+	10	6.57 ± 1.13
P432A/+	x	P432A/+	15	7.53 ± 1.63

Table 2.1: Average litter size
S431A/+ = ATR-*cis*/+
P432A/+ = ATR-*trans*/+

inclusion into this study included litters sizes having five or more pups and litters from the first two crossings as pup number is known to decrease with breeder age and pregnancy number. Litters in which there were ambiguous or missing reads were excluded from these studies. It was found that cis-ATR (ATR-S431A/+) crossings exhibited normal Mendelian distribution (1:2:1, +/+:S/+:S/S) in the ratio of offspring genotypes. In contrast, *trans*-ATR (ATR-P432A/+) crossings exhibited a non-Mendelian distribution (1:2:0, +/+:P/+:P/P) where no mice homozygous for the ATR-P432A allele were present (Table 2.2).

Offspring number and sex ratio					
+/+ x +/+					
	Total Number	% Offspring	Number (Male)	Number (Female)	Ratio (M:F)
+/+	46	100%	22	24	1:1.09
S431A/+ x S431A/+					
	Total Number	% Offspring	Number (Male)	Number (Female)	Ratio (M:F)
+/+	15	23%	8	7	1.14:1
S431A/+	36	55%	20	16	1.25:1
S431A/S431A	14	22%	9	5	1.8:1
P432A/+ x P432A/+					
	Total Number	% Offspring	Number (Male)	Number (Female)	Ratio (M:F)
+/+	37	33%	21	16	1.31:1
P432A/+	76	67%	41	35	1.17:1
P432A/P432A	0	0%	-	-	-

Table 2.2: *trans*-ATR (ATR-P432A) homozygosity is embryonically lethal whereas *cis*-ATR (ATR-S431A) is not
S431A/+ = ATR-*cis*/+, S431A/S431A = ATR-*cis*/*cis*
P432A/+ = ATR-*trans*/+, P432A/+ = ATR-*trans*/*trans*

Early embryonic lethality, prior to E7.5, is well established for ATR knockout mice (10). To investigate whether this was true for ATR-P432A/P432A mice, timed pregnancies studies were performed to assess the state of embryos and their genotypes at various stages. Hematoxylin and eosin (H&E) staining of day E7.5/8.5 implanted embryos were used to assess

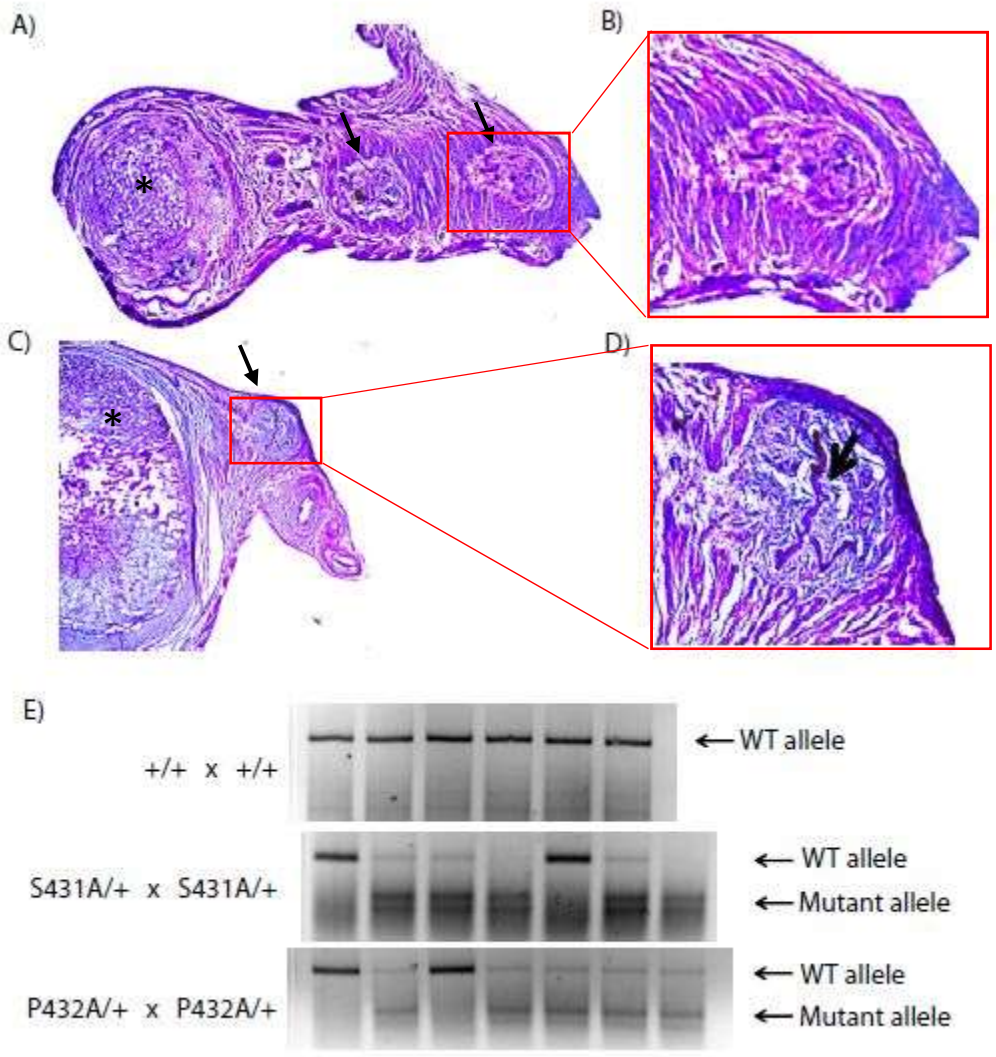

Figure 2.2: *trans*-ATR (ATR-P432A) is embryonically lethal before E7.5/8.5
A) & C) H&E staining of day E7.5/8.5 showing normal (stars) and partially reabsorbed embryos (arrows). B & D) Show enlarged views of these embryos appearing in A and C, respectively. B) is earlier in the reabsorption process and still possesses a near embryonic shape. D) has been completely reabsorbed and the only remnant of the embryo is the compacted trophoblastic layer indicated by the black arrow. E) Characteristic genotyping of embryos from crossing indicated which were cleaved with HaeII. In ATR-P432A heterozygote crossing no homozygotes are seen at day E13.5 indicating that ATR-P432A homozygosity is likely lethal at the peri-implantation or implantation stage. S431A/+ = ATR-*cis*/+, P432A/+ = ATR-*trans*/+

reabsorption following implantation. Results imply that by E7.5/8.5 several embryos have undergone full reabsorption by the uterine horn (example images in Fig. 2.2A-D). To confirm that no animals bearing the ATR-P432A/P432A genotype were present past this point, embryos were harvested at E13.5 and genotyped (Table 2.3). This resulted in a ratio and percentage of offspring that was like that seen previously. This leads us to believe that the ATR-P432A/P432A genotype is peri-implantationally lethal (E4.5-7.5) and aligns with the previous findings seen in ATR knockout mice (10, 26). This also explains our findings where it is seen that litter sizes do not differ substantially across genotypes as these embryos likely die and are reabsorbed prior to or shortly after implantation allowing for normally developing embryos to implant in their place.

E13.5 P432A/+ Crossing		
	Total Number	% of Offspring
+/+	18	64%
P432A/+	10	36%
P432A/P432A	0	0%

Table 2.3: ATR-*trans*/+ (ATR- P432A/+) crossings show no homozygote embryos at E13.5

Prior studies have shown that loss of ATR or it's kinase activity leads to cell death through increased replication stress and a failure to initiate the checkpoint signaling cascade (1, 2, 5, 10). To investigate whether ATR-P432A/P432A lethality was due to a loss of checkpoint activity, HCT116 ATR flox/- cells were transfected with human analogs of the different sterically locked ATR mutants (mouse ATR S431A/human ATR S428A, mouse ATR P432A/human ATR P428A) and treated with UV to induce replication stress. Results implicate little to no difference in the activation of checkpoint kinase 1 (Chk1), the main target of ATR, as indicated by the phosphorylation of Chk1 at Ser345 (Fig. 2.3A). This observation implies that only the cytoplasmic function of ATR is changed by sterically locking these mutants and the nuclear function, where ATR is always in the *trans*-isomer, is unaffected. Similar results show

unaffected checkpoint activation in combinations of the different ATR isomers mimicking heterozygotes (Fig. 2.3B). These results imply that sterically locking ATR into either prolyl

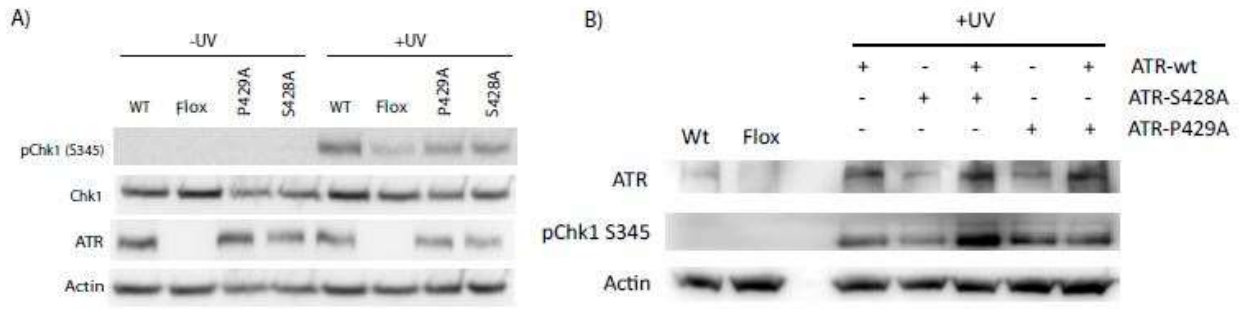

Figure 2.3: DNA damage signaling is unaffected by isomerically locking cytoplasmic ATR Cells transfected with ATR appear to still maintain normal checkpoint kinase activity. HCT116 ATR flox/- cells were transfected with indicated constructs for 48hr prior to UV exposure with a 2hr recovery. A) Transfection of a single construct does not cause activation of the DNA damage signaling response as indicated by a lack of pChk1 (S345) in control (-UV) lanes. Upon UV damage and recovery, wild type and both mutant ATRs showed appropriate phosphorylation of Chk1. B) ATR mutants co-transfected with wild type yielded results similar to the previous study with the exception of ATR-wt/ATR-P429A which showed a reduction in phosphorylation of Chk1 in comparison to ATR-wt/ATR-S428A co-transfection relative to total ATR and phosphor-Chk1 levels. ATR-S428A = *cis*-ATR, ATR-P429A = *trans*-ATR

isomer has no consequence on ATR nuclear function and that loss of embryonic viability must be through some other unknown means.

cis-ATR (ATR-S431A) mice are naturally prone to cancer development

To investigate the differences in phenotypic development, we allowed mice to develop with no outside influence for a period of one year. At the end of that year, mice were euthanized to assess their phenotypes. Additional phenotypes leading to other abnormal outcomes or death were noted.

Results from necropsies revealed that mice of the ATR-S431A/+ (ATR-*cis*/+) genotype had increased rates of *de novo* hyperplasia (Table 2.4). These hyperplasias were either benign (lipoma) or malignant (lymphoma, prostate). The mice with likely prostate cancer and lipomas

Mouse Genotype	Total # mice assessed	Total # with cancer	Percentage with cancer (inflammation)	Types of Cancer/Hyperplasia Seen
+/+	10 (0)	0	0% (0%)	-
S431A/+ǂ	11 (8)	5*	40% (80%)	Lymphoma [2], Lipoma [2], Prostate (likely) [1]
S431A/S431Aǂ	10 (8)	2**	20% (80%)	Ovarian
P432A/+	26 (15)	0	0% (58%)	-

Table 2.4: Cancer/hyperplasia incidence
Mice of the ATR-S431A line exhibit spontaneous cancer development in comparison to both wild type and ATR-P432A/+ mice.
ǂ $p<0.05$ by Fisher-exact test for comparison to +/+
*3 males, 2 females
**Cancerous or Precancerous lesions
(Number with) Enlarged Peyer's patches indicative of increased systemic inflammation, (percent)
S431A/+ = ATR-*cis*/+, S431A/S431A = ATR-*cis*/*cis,* P432A/+ = ATR-*trans*/+
[Number with indicated hyperplasia]

were asymptomatic at the time of euthanasia and collection. The mice with lymphomas were moribund having extreme infiltrates to various organs depending on the mouse in question.

Figure 2.4A shows one of the mice which presented with lymphoma. The liver shows gross hepatomegaly with multiple white nodules indicative of metastatic, infiltrating lymphoma. In addition to the liver, the lungs and spleen (highly enlarged) also possessed nodules but were not processed for further assessment. Hematoxylin and eosin staining of liver sections revealed the presence of immune infiltrate into the liver parenchyma resulting in compaction and, in some instances, complete replacement of normal liver architecture (Fig. 2.4B). Another mouse with the same genotype is shown in Figure 2.5. This mouse presented with abnormal liver morphology, enlarged spleen, an enlarged Peyer's patch (.7cm diameter, normal = .1-.2cm), and a primary lymph node tumor (1cm in diameter, normal = .1-.2cm) (Fig.2.5A). The liver possessed large areas of focal necrosis, without immune infiltrate (Fig2.5B&C) indicating dysfunction potentially unrelated to the presence of lymphoma. Other areas of the liver indicate the beginnings of immune cell infiltration (Fig. 2.5D, F & G).

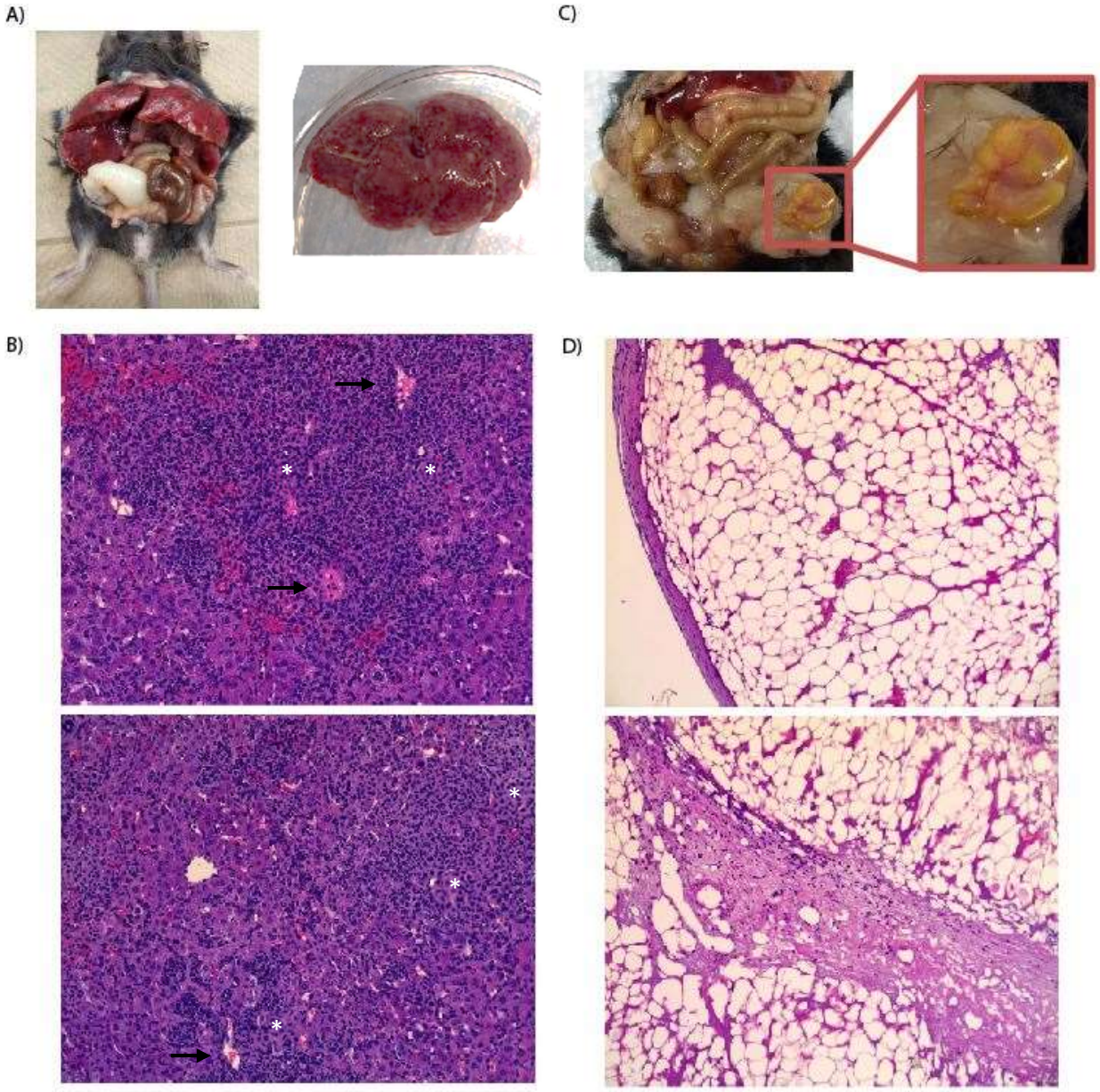

Figure 2.4: Varying cancer phenotypes of *cis*-ATR (ATR-S431A) mice (1)
A) A male mouse (ATR S431A/+, 12 months) exhibiting a highly inflamed abdominal cavity. To the right is the excised liver showing gross hepatomegaly with various white nodules indicative of lymphomic infiltrates. B) 20x images of H&E-stained liver show huge populations of immune cells around central veins (arrows) with infiltration and compaction of sinusoids (stars). Two representative images are shown. The primary source of the lymphoma could not be determined. C) Another male mouse (ATR S431A/+, 12 months) with a primary liposarcoma in its gonadal fat pad. D) 10x (top) & 20x (bottom) images of the H&E stained lipoma. The top panel shows a high degree fat necrosis with an atypical fibrotic cap formed from atypical adipocytes. The bottom panel shows atypical adipocytes forming a fibrous structure within the lipoma itself. This fibrosus is surrounded by fat necrosis. S431A/+ = ATR-*cis*/+, S431A/S431A = ATR-*cis*/*cis*

However, of note, abnormal liver cells also are present (Fig2.5E) which could indicate another malignancy, such as the beginning stages of hepatocellular carcinoma may be present. In addition to the liver pathology, the lymph node (Fig 2.5H&I) shows complete loss of the normal node architecture. Furthermore, the densely stained areas could potentially indicate extramedullary hematopoiesis. While the bone marrow is the typical site of hematopoiesis, lymph nodes, as well as other organs, have been known to exhibit extramedullary hematopoiesis under cases of extreme inflammation or neoplasm (27). This also was seen in sections of the Peyer's patch, shown in Figure 2.5 J & K. Here the normal morphology of the Peyer's patch was absent, and the patch was so enlarged that it was causing near obstruction of the small intestine.

Interestingly, all mice presenting with overt hyperplasic phenotypes were of the heterozygous *cis*-ATR (ATR-S431A/ATR-H) line. This line is expected to have a higher intrinsic level of anti-apoptotic function due to the properties of ATR-H described previously (9). Thus, cancer development in this line likely could be attributed to prevention of intrinsic apoptosis, through tBid sequestration, during transformation. This would allow for precancerous cells to escape apoptosis and progress into malignancy. Given that this ATR is locked into the *cis*-isomer, it is also possible that less ATR-L is present. This would result in less proapoptotic signaling resulting from DNA damage and replicative stress due to the cytosolic *cis*-ATR (ATR-H) form being found to not possess kinase activity (9).

While hyperplasic phenotypes were only apparent in the *cis*-ATR (ATR-S431A/ATR-H) line, both ATR mutant lines exhibited enlarged Peyer's patches in their gastrointestinal tract (Table 2.4). This is commonly associated with infection or chronic systemic inflammation (28, 29). Since sentinel mice for this colony were always negative for pathogens, it is highly likely

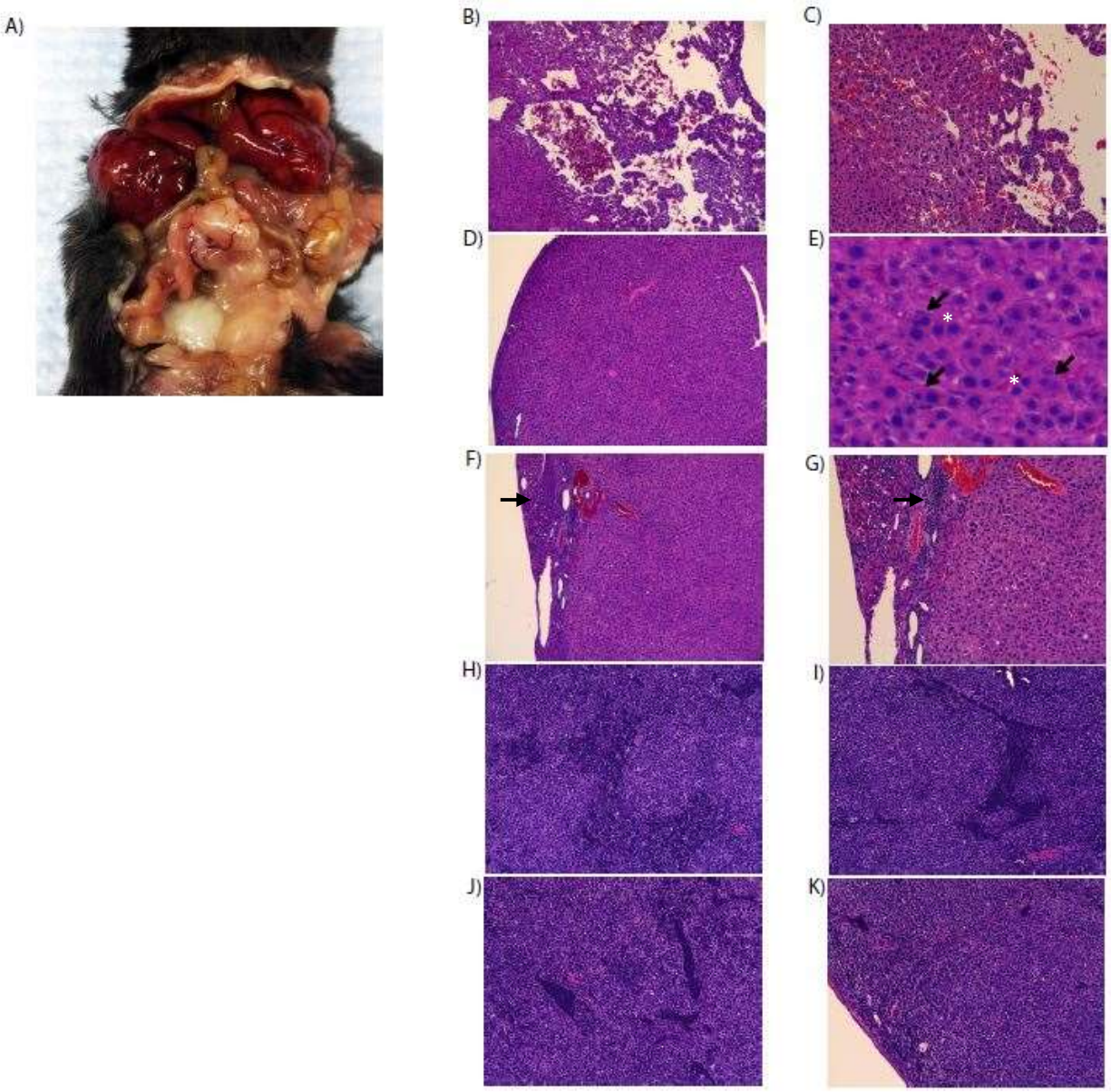

Figure 2.5: Varying cancer phenotypes of *cis*-ATR (ATR-S431A) mice (2)
A) Male mouse (S431A/+, 11 months) exhibiting a variety of abdominal pathologies. B) 10x and C) 20x images of focal liver necrosis. Entire portions of the liver have been evacuated leaving behind cyst-like structures. Surprisingly, these were devoid of immune infiltration. D) 10x liver image showing almost normal liver with some immune cell infiltration. E) A closer look at this section at higher magnifications (60x) shows a variety of atypical cells. Multinucleated cells are marked with arrows. Cells recently undergoing division are marked with stars. F) 10x H&E of liver showing immune cell infiltrate (arrows). G) 20x image of the same section revealing in better detail the immune cell infiltrate. H & I) 20x images of the primary lymph node tumor. The normal architecture has been completely taken over by lymphoma. Normal germinal centers, medullary cords, and paracortex have been completely replaced. J & K) 20x images of the enlarged Peyer's patch seen in A. The entire normal architecture has been replaced by lymphoid tissue. S431A/+ = ATR-*cis*/+

that both lines possess basal systemic inflammatory characteristics. This, however, would need to be confirmed by serum cytokine analysis.

trans-ATR (ATR-P432A) mice exhibit premature aging phenotypes alongside other developmental abnormalities

After one year of development, heterozygous *trans*-ATR (ATR-P432A/ATR-L) mice exhibit a wide array of developmental and age-dependent phenotypes. The first of these is evident in that some mice (~2-3%), have spontaneous pronounced hair loss, alopecia (Fig.2.6A). Alopecia in mice can result from numerous factors including over grooming, auto-immune type effects (alopecia areata), and/or accelerated aging-induced loss of follicular stem cells (30-32). Given that this condition was noted in mice from both individual and group housing, it is likely this phenomenon is not due to an overgrooming effect. Theories of alopecia areata stemming from an auto-immune disorder could support these findings due to the increased inflammation seen in these lines; however, accelerated aging-associated loss of follicular stem cells cannot be ruled out as mice with conditional ATR knockouts have previously been found to have progressive alopecia after induction of ATR knockout (16). Accelerated aging is also likely due to the presence of progressive kyphosis, arching of the spine due to weakness in the spinal bones or supporting muscles, in several mice (~43%) from this line (Fig. 2.6B). While kyphosis can be either a developmental or age-associated phenotype, young mice from this line do not show obvious signs of kyphosis which leads us to believe that this is an accelerated aging phenotype instead of a developmental abnormality (33). This is supported further by that fact that kyphosis is seen in mice from other DNA repair protein mutant backgrounds as well as ATR conditional knockout mice (16, 34, 35).

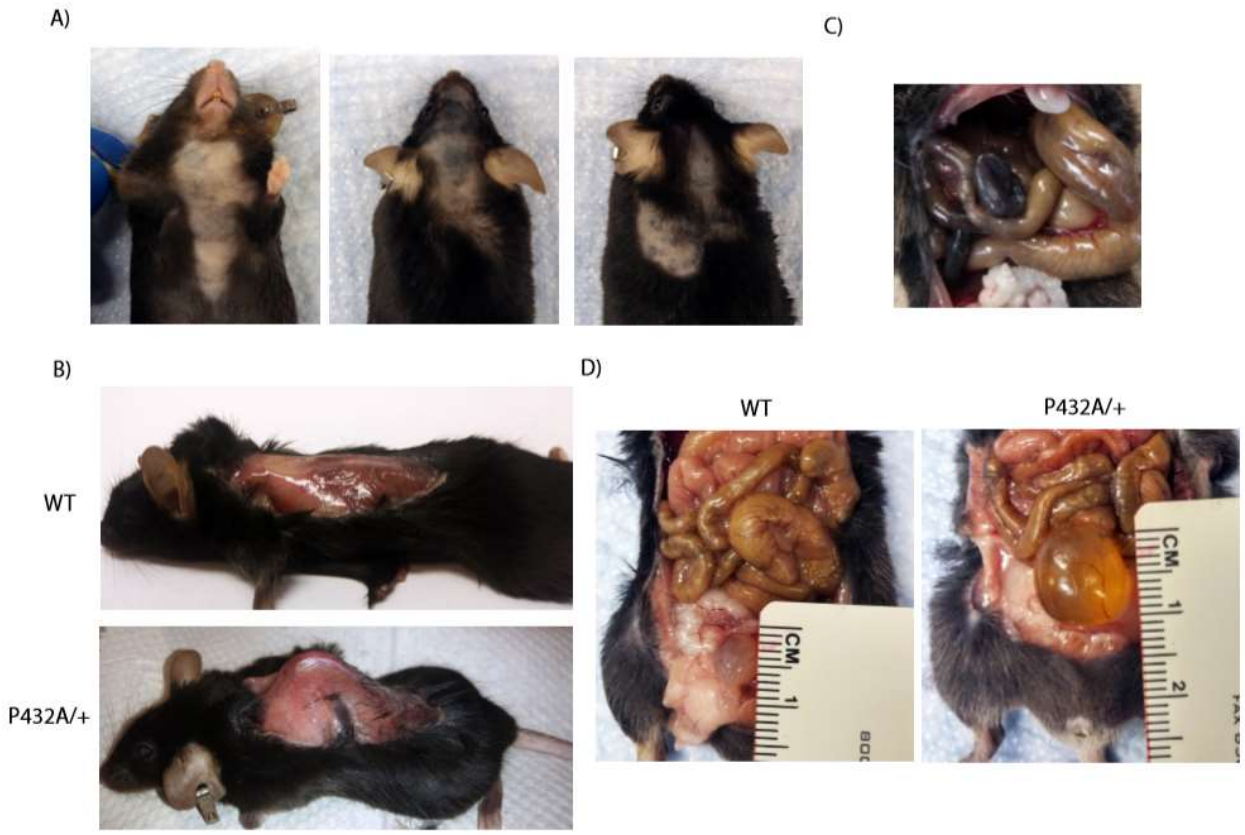

Figure 2.6: Heterozygous *trans*-ATR (ATR-P432A/+) mice express various deleterious phenotypes
A) Alopecia areata is seen in some mice of the ATR-P432A/+ genotype. B) Several mice of the ATR-P432A/+ genotype exhibit marked kyphosis. Here two female litter mates (12 months) are shown for comparison. C) Spontaneous death occurred in several mice. In all mice necropsied, the presence of a necrotic bowel was found. D) Male mice of the ATR-P432A/+ genotype frequently have distended/engorged bladders. Here two male litter mates (12 months) are shown for comparison. P432A/+ = ATR-*trans*/+

In addition to age-dependent phenotypes, heterozygous *trans*-ATR mice display various developmental abnormalities. The most extreme of these is the repeat occurrence of necrotic bowel-like symptoms leading to intestinal obstruction and death (Fig. 2.6 C). This was noted in a small fraction of heterozygous *trans*-ATR mice (~4-5%) but was completely absent in controls. While intestinal banding, a congenital malformation, was present in some mice, it was absent in others. Blockage from congenital banding is known to be able to cause intestinal obstruction and can lead to necrotizing enterocolitis (36-38). However, this only provides support for instances

where banding is present. Another potential cause could stem from issues in the enteric nervous system (ENS). Studies have shown that defects in the retinoblastoma protein prevent proper ENS formation and can lead to intestinal obstructions through failure to move fecal matter (39). Given that ATR is known to have roles in central nervous system (CNS) development, it is highly possible that it also has roles in ENS development as well (14, 15). In addition, ~50% of *trans*-ATR heterozygous males have highly engorged bladders in comparison to wild type controls (Fig.2.6 D). Control of urination, also called micturition, is regulated by ENS (40-42). Therefore, this sex-linked phenomenon provides further evidence of changes in ENS development in heterozygous *trans*-ATR mice. A lack of appropriate signaling due to either malformation or miss signaling could lead to urinary retention and the bladder distention seen in these mice. Along with these findings, *trans*-ATR heterozygous males were found to be more aggressive. Aggressive behavior was documented as an increase in fighting between littermates leading to mice having to be individually housed. While no formal behavioral assays were performed, it was found that male *trans*-ATR heterozygotes were five times more likely to have to be removed from cohousing than either wildtype or *cis*-ATR mice of either genotype. Improper CNS development can result in aggressive behaviors, especially in transgenic animals, and could provide a causal link between CNS developmental abnormalities and the behavior seen in male *trans*-ATR mice (43-45).

Additionally, male *trans*-ATR heterozygotes were found to have cardiac abnormalities when evaluated by echocardiography. Tachycardia (~500-550bpm) was present in all male *trans*-ATR mice evaluated (n=8) and this increased heart rate was completely refractory to isoflurane-based anesthesia. This is in stark comparison to a known decrease in heart rate (~430-475bpm) of wild type mice exposed to isoflurane-based anesthetics (46, 47). Cardiac placement is also

shifted in some *trans*-ATR heterozygote mice. These findings agree with the previous finding in zebrafish which show that ATR is necessary for appropriate loop formation during cardiac development and ATR alteration, such as Seckel mutation, resulted in abnormal cardiac development and reduced function (13). Interestingly, clinical cases of Seckel syndrome were reported which show complex cardiac complications stemming from cardiac malformation (48, 49). These findings further support the critically important role of interconversion between *cis*- and *trans*-isoforms being necessary for normal development and organismal maintenance. A summary of all findings can be found in Table 2.5.

Findings present in heterozygous *trans*-ATR mice	
Finding	Percentage or comparison
Alopecia	~2-3%
Aggression	5x more likely to have to be separated than wt or *cis*-ATR mice
Bladder enlargement/Micturition issues	~50% of males
Kyphosis	~43%
Tachycardia	100% of males tested

Table 2.5 Findings present in heterozygous *trans*-ATR mice

Both cis-ATR (ATR-S431A) and trans-ATR (ATR-P432A) female mice exhibit features indicating elevated reproductive stress

During necropsy examinations, it was found that mutant female mice from both lines possessed increased rates of ovarian and uterine horn abnormalities (Table 2.6). For uterine horns, this abnormality was mainly enlargement or swelling as indicated in Figure 2.7 B in comparison to the wild type control (Fig. 2.7A). No cysts or other gross abnormalities were seen. Gross evaluation of ovaries showed multiple phenotypes ranging from apparently normal to potentially precancerous. Many ovaries possessed follicular cysts and enlarged corpus luteum, others, like that shown in Figure 2.7B, exhibit endometroid or hemorrhagic cysts as indicated by the engorgement of blood seen both under gross examination as well as histological examination

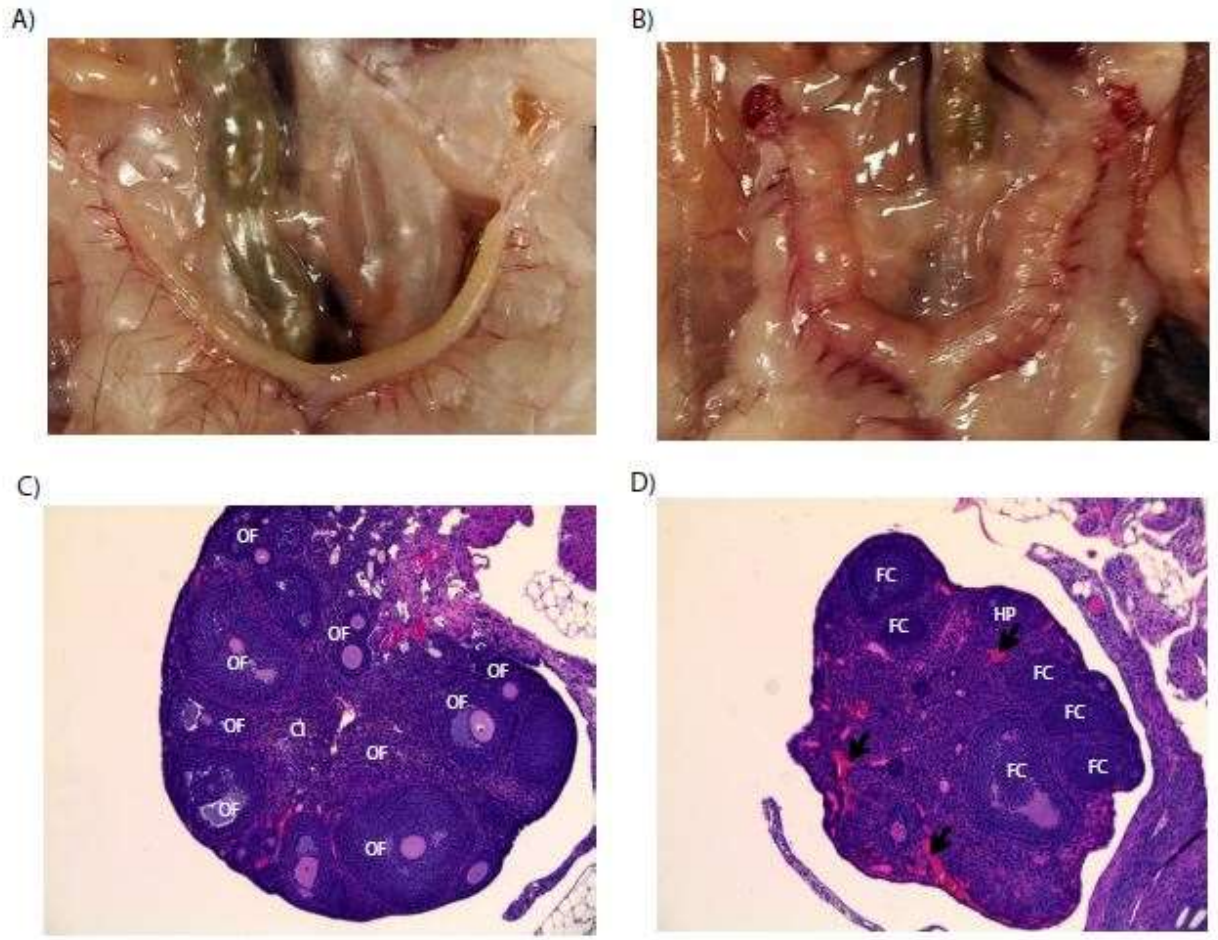

Figure 2.7: Ovary/uterine inflammation and follicular cysts are present in both *cis*-ATR (ATR-S431A) and trans-ATR (ATR-P432A) mice

A) and B) show gross anatomy of the reproductive tract of two female litter mates (12 months). Panel B This ATR-S431A/S431A mouse is representative of most females of both the S431A line (ATR-S431A/+ & ATR-S431A/S431A) and the P432A line (ATR-P432A/+). Notice how the ovaries are engorged and the uterine horns are thickened (a consequence of chronic inflammation). C) 20x H&E of a wild type ovary from panel A. Normal follicular development is noted by the presence of oocyte formations (OF) and normal corpus luteum (Cl). D) 20x H&E of the ATR-S431A/S431A ovary shown in B. The ovary is misshapen and has lost size due to rupture during the embedding process. An abnormal number of follicular cysts (FC) are seen. The ovary appears to be hyperplasic (HP) in some regions and hemorrhagic (arrows) in others. S431A/+ = ATR-*cis*/+, S431A/S431A = ATR-*cis*/*cis,* P432A/+ = ATR-*trans*/+

Mouse Genotype	Total # mice assessed	Total # with abnormal ovaries/uterine horns	Percentage
+/+	10	0	0%
S431A/+ ɪ	8	5	62.5%
S431A/S431A ɪ	4	3	75%
67S431A/+ or S431A/S431A ɪ	12	8	67%
P432A/+ ɪ	10	8	80%

Table 2.6: Incidence of abnormal/cystic ovaries and uterine horns by gross examination
S431A/+ = ATR-*cis*/+, S431A/S431A = ATR-*cis*/*cis,* P432A/+ = ATR-*trans*/+
ɪ $p<0.05$ by Fisher-exact test for comparison to +/+

(Fig. 2.7D, arrows). Ovarian cyst formation can be due to a variety of factors, but most are attributed to either trauma, developmental abnormalities, or chronic inflammation (50-53). Developmental abnormalities in cilia function in the absence of DNA damage repair protein MRE11 have been found to contribute to kidney cyst formation (54). While not shown, renal cysts were present in some mice from these lines, though uncommon, and make show a potential link between known ATR cilial dysfunction and pathogenesis of both ovarian and renal cysts (13). Other studies have shown that increases in genomic stress leading to increased inflammation can lead to the formation of cysts and cancerous lesions. For example, BRACA1 (breast cancer type 1 susceptibility protein 1) mutant mice develop ovarian and uterine cysts typically resulting in cancer formation (55) whereas NEK8 (NIMA regulated kinase 8), a regulator of cytoskeletal structure, knockout mice show increased ATR activation and replicative stress leading to chronic high levels of inflammation resulting in cyst formation (56). Given that chronic inflammation involved in polycystic ovarian syndrome (PCOS) and other similar disorders predisposes individuals to cancer development it is possible that these ATR mutations likely have the same effect (53, 54). Additionally, cytoplasmic ATR phosphorylation at the Pin1 motif previously has been linked to prognosis of ovarian cancer. These studies showed that

higher levels of phosphorylated ATR Ser428 were associated with poorer disease outcomes (57). Since pATR-S428 is a substrate for Pin1 and leads to its conversion into the *trans*- isomer it suggests that the *trans*- isomer is likely involved in tumor tolerance to chemotherapeutic treatment; however, its role here in precancerous cyst formation is unknown. In contrast, *cis*-ATR (non-phosphorylated ATR-S428A) likely is cancerous through the direct anti-apoptotic activity previously described (9). This leads us to believe that while the phenotypes between ATR *cis* and *trans* mice are similar for ovarian cyst development, the underlying mechanisms driving these phenotypes are likely different.

DISCUSSION

To investigate the effects of sterically locking the prolyl isomerization site of ATR on organismal development we first began by constructing a mouse model wherein cytoplasmic ATR is locked into either the *cis* or the *trans* prolyl isomer through a single amino acid substitution. Studies revealed that mutations locking ATR into the *cis*-isomer were embryonically viable whereas no mice bearing only the *trans*-isomer of ATR were found. ATR knockout has previously been shown to cause early embryonic lethality and loss of kinase activity is well associated with cell death in cycling cells (1, 5, 10). We questioned whether embryonic lethality could be due to a loss of kinase activity in *trans*-ATR mice; however, upon investigation, it was found that *trans*-ATR possesses the same kinase activity as the wild type when constructs bearing the human form of the mutant were expressed in an ATR-deficient cell line and exposed to UV irradiation. Further investigation revealed that no mice bearing the homozygous *trans*-ATR genotype were present at E13.5 and that sections from E7.5 uteri post implantation from breeding of heterozygote *trans*-ATR mice revealed notable embryonic reabsorption sites. This could imply that the ability to switch between the two isoforms is

necessary for development at the implantation stage, the same stage at which ATR knockout embryos die (10). Another potential, though unexplored explanation, could involve the fact that ATR is involved in male meiotic recombination. Conditional knockout and Seckel syndrome models have shown that loss of ATR or its kinase activity can lead to reduced sperm counts with various functional defects (58, 59). Additionally, male mice expressing a single ATR kinase dead allele were found to be sterile due to ATR exchange dynamics limiting ATR activation kinetics and subsequent checkpoint activity (60). This could provide a potential explanation as human ATR deficient cells doubly-transfected with both wild type and *trans*-ATR constructs showed reduced checkpoint activation when compared to either wild type or *trans*-ATR transfected cells alone; however, further studies to explore this theory need to be performed.

In addition to potential aberrations in embryonic development, heterozygote *trans*-ATR mice exhibit a wide variety of developmental and age-related phenotypes. This is in contrast to mice carrying the *cis*-ATR which appear to develop normally, but are prone to neoplasia occurrence. The *trans*-ATR form presents with various phenotypes typical to either adult ATR conditional knockout or ATR Seckel syndrome mice; however, it is interesting because these mice only express a single, or at most a subset, of the phenotypes seen in either condition. While these phenotypes may vary, they most likely have roots in underpinning neurological development defects. Because ATR is essential in CNS development, it is likely that it also has roles in ENS development (12, 14, 15). Defects in ENS signaling could explain the phenotypes seen whereby micturition is inhibited as well as formation of the spontaneous necrotic bowel (36, 37, 39, 40). While we can speculate that these phenotypes likely result from a change in ATR signaling, further studies investigating the development of these phenotypes need to be performed.

The role of *cis*-ATR in carcinogenesis is likely defined by *cis*-ATR's direct anti-apoptotic activity through structural sequestration of the pro-apoptotic protein tBid (9). Cancers/neoplasms develop when cells fail to initiate appropriate cell cycle arrest or apoptosis. Through blocking apoptosis, *cis*-ATR likely contributes to cancer by promoting tolerance to internal signals for apoptosis. In support of this, low phosphorylation of cytoplasmic ATR at the Pin1 site has been linked to advanced stage ovarian carcinoma and poor prognosis in regards to chemotherapeutic outcome (57). Phosphorylation is required for *cis*-ATR to be isomerized to the *trans*-ATR isoform by Pin1. Therefore, a lack of phosphorylation on the Pin1 motif likely results in a higher propensity for forming the *cis*-ATR conformation. This could give pre-cancerous cells the tolerance to apoptotic signaling necessary to develop into full blown neoplasms. In addition to this, both *cis*-ATR and *trans*-ATR mice seem to exhibit chronic inflammatory symptoms. This could be due to genomic instability which is known to induce inflammation in a DNA damage-dependent manner (61-64). Furthermore, ATR mutations have been linked to modulation in the tumor immune microenvironment leading to promotion of tumor development and growth (65). Further investigation into the roles of each ATR prolyl isoform in these areas are needed, but current findings allude to the presence of inflammatory subtypes common to both lines. Why this contributes to neoplastic development in *cis*-ATR mice, but not in *trans*-ATR mice, still needs to be clarified, but is likely is due to the potential kinase deficiency seen in heterozygote *trans*-ATR mentioned previously.

The results of this study, summarized in Figure 2.8, reveal the importance of the cell's ability to shift between *cis* and *trans* ATR prolyl isomers and give insight into specific isomer functions. Future investigation will hopefully reveal the exact mechanisms by which the two

isoforms differ in their phenotypic development and help to explain some of the differences seen both between the different mouse lines and within the *trans*-ATR line.

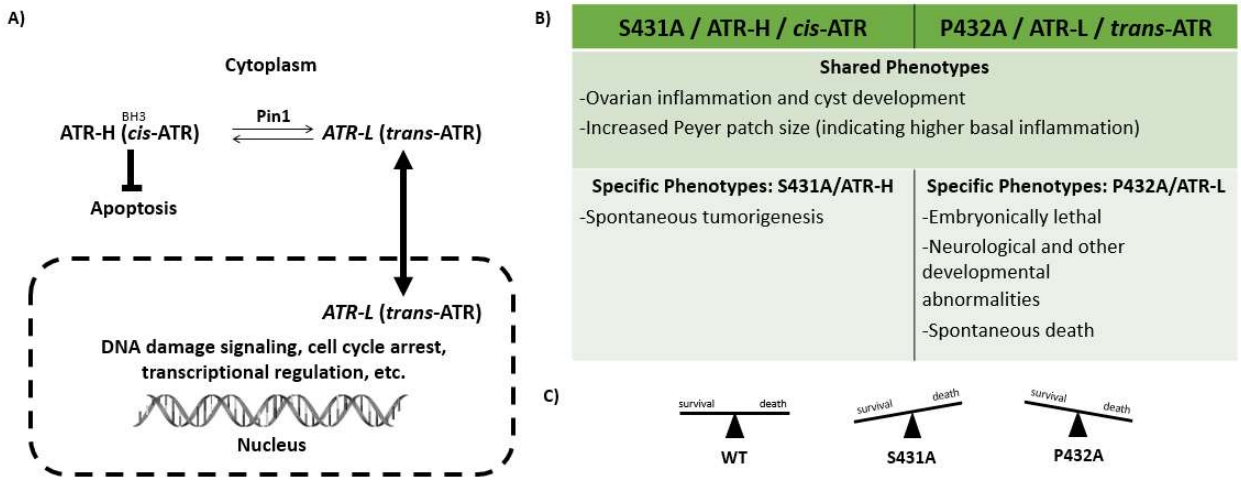

Figure 2.8: Structurally locking cytoplasmic ATR has varying effects on organismal and phenotypic development.
A) Summary of the effect of locking ATR into either the *cis*- (ATR-S431A/ATR-H) or *trans*- (ATR-P432A/ATR-L) form. B) Summary of the findings of this study. C) Potential implication of each isoform on cellular fate.

REFERENCES

1. Cimprich, K. A., and Cortez, D. (2008) ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* **9**, 616-627
2. Zeman, M. K., and Cimprich, K. A. (2014) Causes and consequences of replication stress. *Nature cell biology* **16**, 2-9
3. Wu, X., Shell, S. M., Yang, Z., and Zou, Y. (2006) Phosphorylation of nucleotide excision repair factor xeroderma pigmentosum group A by ataxia telangiectasia mutated and Rad3-related-dependent checkpoint pathway promotes cell survival in response to UV irradiation. *Cancer research* **66**, 2997-3005
4. Zou, L., and Elledge, S. J. (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science (New York, N.Y.)* **300**, 1542-1548
5. Brown, E. J., and Baltimore, D. (2003) Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes & development* **17**, 615-628
6. Matsuoka, S., Ballif, B. A., Smogorzewska, A., McDonald, E. R., 3rd, Hurov, K. E., Luo, J., Bakalarski, C. E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S. P., and Elledge, S. J. (2007) ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science (New York, N.Y.)* **316**, 1160-1166
7. Surova, O., and Zhivotovsky, B. (2013) Various modes of cell death induced by DNA damage. *Oncogene* **32**, 3789-3797
8. Wagner, S. A., Oehler, H., Voigt, A., Dalic, D., Freiwald, A., Serve, H., and Beli, P. (2016) ATR inhibition rewires cellular signaling networks induced by replication stress. *Proteomics* **16**, 402-416
9. Hilton, B. A., Li, Z., Musich, P. R., Wang, H., Cartwright, B. M., Serrano, M., Zhou, X. Z., Lu, K. P., and Zou, Y. (2016) ATR Plays a Direct Antiapoptotic Role at Mitochondria, which Is Regulated by Prolyl Isomerase Pin1. *Molecular cell* **61**, 487
10. Brown, E. J., and Baltimore, D. (2000) ATR disruption leads to chromosomal fragmentation and early embryonic lethality. *Genes Dev* **14**, 397-402
11. de Klein, A., Muijtjens, M., van Os, R., Verhoeven, Y., Smit, B., Carr, A. M., Lehmann, A. R., and Hoeijmakers, J. H. (2000) Targeted disruption of the cell-cycle checkpoint gene ATR leads to early embryonic lethality in mice. *Current biology : CB* **10**, 479-482
12. Lang, P. Y., Nanjangud, G. J., Sokolsky-Papkov, M., Shaw, C., Hwang, D., Parker, J. S., Kabanov, A. V., and Gershon, T. R. (2016) ATR maintains chromosomal integrity during postnatal cerebellar neurogenesis and is required for medulloblastoma formation. *Development (Cambridge, England)* **143**, 4038-4052
13. Stiff, T., Casar Tena, T., O'Driscoll, M., Jeggo, P. A., and Philipp, M. (2016) ATR promotes cilia signalling: links to developmental impacts. *Human molecular genetics* **25**, 1574-1587
14. Lee, Y., Shull, E. R., Frappart, P. O., Katyal, S., Enriquez-Rios, V., Zhao, J., Russell, H. R., Brown, E. J., and McKinnon, P. J. (2012) ATR maintains select progenitors during nervous system development. *The EMBO journal* **31**, 1177-1189
15. Enriquez-Rios, V., Dumitrache, L. C., Downing, S. M., Li, Y., Brown, E. J., Russell, H. R., and McKinnon, P. J. (2017) DNA-PKcs, ATM, and ATR Interplay Maintains Genome Integrity during Neurogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **37**, 893-905

16. Ruzankina, Y., Pinzon-Guzman, C., Asare, A., Ong, T., Pontano, L., Cotsarelis, G., Zediak, V. P., Velez, M., Bhandoola, A., and Brown, E. J. (2007) Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell stem cell* **1**, 113-126
17. Alderton, G. K., Joenje, H., Varon, R., Borglum, A. D., Jeggo, P. A., and O'Driscoll, M. (2004) Seckel syndrome exhibits cellular features demonstrating defects in the ATR-signalling pathway. *Human molecular genetics* **13**, 3127-3138
18. Murga, M., Bunting, S., Montaña, M. F., Soria, R., Mulero, F., Cañamero, M., Lee, Y., McKinnon, P. J., Nussenzweig, A., and Fernandez-Capetillo, O. (2009) A mouse model of ATR-Seckel shows embryonic replicative stress and accelerated aging. *Nat Genet* **41**, 891-898
19. Tanaka, A., Weinel, S., Nagy, N., O'Driscoll, M., Lai-Cheong, J. E., Kulp-Shorten, C. L., Knable, A., Carpenter, G., Fisher, S. A., Hiragun, M., Yanase, Y., Hide, M., Callen, J., and McGrath, J. A. (2012) Germline mutation in ATR in autosomal- dominant oropharyngeal cancer syndrome. *American journal of human genetics* **90**, 511-517
20. Janku, F., Kaseb, A. O., Tsimberidou, A. M., Wolff, R. A., and Kurzrock, R. (2014) Identification of novel therapeutic targets in the PI3K/AKT/mTOR pathway in hepatocellular carcinoma using targeted next generation sequencing. *Oncotarget* **5**, 3012-3022
21. Zighelboim, I., Schmidt, A. P., Gao, F., Thaker, P. H., Powell, M. A., Rader, J. S., Gibb, R. K., Mutch, D. G., and Goodfellow, P. J. (2009) ATR Mutation in Endometrioid Endometrial Cancer Is Associated With Poor Clinical Outcomes. *Journal of Clinical Oncology* **27**, 3091-3096
22. Durocher, F., Labrie, Y., Soucy, P., Sinilnikova, O., Labuda, D., Bessette, P., Chiquette, J., Laframboise, R., Lepine, J., Lesperance, B., Ouellette, G., Pichette, R., Plante, M., Tavtigian, S. V., and Simard, J. (2006) Mutation analysis and characterization of ATR sequence variants in breast cancer cases from high-risk French Canadian breast/ovarian cancer families. *BMC cancer* **6**, 230
23. Gilad, O., Nabet, B. Y., Ragland, R. L., Schoppy, D. W., Smith, K. D., Durham, A. C., and Brown, E. J. (2010) Combining ATR suppression with oncogenic Ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner. *Cancer Res* **70**, 9693-9702
24. Curtin, N., and Pollard, J. (2018) Targeting ATR for Cancer Therapy: Profile and Expectations for ATR Inhibitors. In *Targeting the DNA Damage Response for Anti-Cancer Therapy* (Pollard, J., and Curtin, N., eds) pp. 63-97, Springer International Publishing, Cham
25. Wedemeyer, W. J., Welker, E., and Scheraga, H. A. (2002) Proline Cis−Trans Isomerization and Protein Folding. *Biochemistry* **41**, 14637-14644
26. Papaioannou, V. E., and Behringer, R. R. (2012) Early embryonic lethality in genetically engineered mice: diagnosis and phenotypic analysis. *Veterinary pathology* **49**, 64-70
27. Kim, C. H. (2010) Homeostatic and pathogenic extramedullary hematopoiesis. *J Blood Med* **1**, 13-19
28. Schulz, O., Ugur, M., Friedrichsen, M., Radulovic, K., Niess, J. H., Jalkanen, S., Krueger, A., and Pabst, O. (2013) Hypertrophy of infected Peyer's patches arises from global, interferon-receptor, and CD69-independent shutdown of lymphocyte egress. *Mucosal Immunology* **7**, 892

29. Jung, C., Hugot, J.-P., Barreau, F., #233, #233, and rick. (2010) Peyer's Patches: The Immune Sensors of the Intestine. *International Journal of Inflammation* **2010**
30. Zoller, M., Zhao, K., Kutlu, N., Bauer, N., Provaznik, J., Hackert, T., and Schnolzer, M. (2018) Immunoregulatory Effects of Myeloid-Derived Suppressor Cell Exosomes in Mouse Model of Autoimmune Alopecia Areata. *Frontiers in immunology* **9**, 1279
31. Gilhar, A., Schrum, A. G., Etzioni, A., Waldmann, H., and Paus, R. (2016) Alopecia areata: Animal models illuminate autoimmune pathogenesis and novel immunotherapeutic strategies. *Autoimmunity Reviews* **15**, 726-735
32. Militzer, K., and Wecker, E. (1986) Behaviour-associated alopecia areata in mice. *Laboratory animals* **20**, 9-13
33. Roghani, T., Zavieh, M. K., Manshadi, F. D., King, N., and Katzman, W. (2017) Age-related hyperkyphosis: update of its potential causes and clinical impacts-narrative review. *Aging Clin Exp Res* **29**, 567-577
34. Moskalev, A. A., Shaposhnikov, M. V., Plyusnina, E. N., Zhavoronkov, A., Budovsky, A., Yanai, H., and Fraifeld, V. E. (2013) The role of DNA damage and repair in aging through the prism of Koch-like criteria. *Ageing Research Reviews* **12**, 661-684
35. Karikkineth, A. C., Scheibye-Knudsen, M., Fivenson, E., Croteau, D. L., and Bohr, V. A. (2017) Cockayne syndrome: Clinical features, model systems and pathways. *Ageing research reviews* **33**, 3-17
36. Zachariah, S. K. (2011) Adult necrotizing enterocolitis and non occlusive mesenteric ischemia. *J Emerg Trauma Shock* **4**, 430-432
37. Yang, K.-H., Lee, T.-B., Lee, S.-H., Kim, S.-H., Cho, Y.-H., and Kim, H.-Y. (2016) Congenital adhesion band causing small bowel obstruction: What's the difference in various age groups, pediatric and adult patients? *BMC Surg* **16**, 79-79
38. Dimitrios, C., George, A. A., Dimosthenis, Z., and Nikolaos, X. (2008) Intestinal obstruction due to an anomalous congenital band. *Saudi J Gastroenterol* **14**, 36-37
39. Fu, M., Landreville, S., Agapova, O. A., Wiley, L. A., Shoykhet, M., Harbour, J. W., and Heuckeroth, R. O. (2013) Retinoblastoma protein prevents enteric nervous system defects and intestinal pseudo-obstruction. *J Clin Invest* **123**, 5152-5164
40. de Groat, W. C., Griffiths, D., and Yoshimura, N. (2015) Neural control of the lower urinary tract. *Compr Physiol* **5**, 327-396
41. Hou, X. H., Hyun, M., Taranda, J., Huang, K. W., Todd, E., Feng, D., Atwater, E., Croney, D., Zeidel, M. L., Osten, P., and Sabatini, B. L. (2016) Central Control Circuit for Context-Dependent Micturition. *Cell* **167**, 73-86.e12
42. Hill, W. G. (2015) Control of urinary drainage and voiding. *Clin J Am Soc Nephrol* **10**, 480-492
43. Vaz, R., Hofmeister, W., and Lindstrand, A. (2019) Zebrafish Models of Neurodevelopmental Disorders: Limitations and Benefits of Current Tools and Techniques. *Int J Mol Sci* **20**, 1296
44. Sala, M., Braida, D., Lentini, D., Busnelli, M., Bulgheroni, E., Capurro, V., Finardi, A., Donzelli, A., Pattini, L., Rubino, T., Parolaro, D., Nishimori, K., Parenti, M., and Chini, B. (2011) Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism. *Biological psychiatry* **69**, 875-882
45. Takahashi, A., and Miczek, K. A. (2014) Neurogenetics of aggressive behavior: studies in rodents. *Curr Top Behav Neurosci* **17**, 3-44

46. Ho, D., Zhao, X., Gao, S., Hong, C., Vatner, D. E., and Vatner, S. F. (2011) Heart Rate and Electrocardiography Monitoring in Mice. *Curr Protoc Mouse Biol* **1**, 123-139
47. Stypmann, J. (2007) Doppler ultrasound in mice. *Echocardiography (Mount Kisco, N.Y.)* **24**, 97-112
48. Sorof, J. M., Dow-Smith, C., and Moore, P. J. (1999) Severe hypertensive sequelae in a child with Seckel syndrome (bird-like dwarfism). *Pediatric Nephrology* **13**, 343-346
49. Rappen, U., and von Brenndorff, A. I. (1993) [Cardiac symptoms in 2 patients with Seckel syndrome]. *Monatsschr Kinderheilkd* **141**, 584-586
50. Knauf, Y., Köhler, K., Knauf, S., and Wehrend, A. (2018) Histological classification of canine ovarian cyst types with reference to medical history. *J Vet Sci* **19**, 725-734
51. Lee, H. J., Bahr, J. M., Bitterman, P., Basu, S., Sharma, S., Abramowicz, J. S., and Barua, A. (2018) Polycystic Ovarian Condition May Be a Risk Factor for Ovarian Tumor Development in the Laying Hen Model of Spontaneous Ovarian Cancer. *J Immunol Res* **2018**, 2590910-2590910
52. Kelly, C. C., Lyall, H., Petrie, J. R., Gould, G. W., Connell, J. M., and Sattar, N. (2001) Low grade chronic inflammation in women with polycystic ovarian syndrome. *The Journal of clinical endocrinology and metabolism* **86**, 2453-2455
53. Savant, S. S., Sriramkumar, S., and O'Hagan, H. M. (2018) The Role of Inflammation and Inflammatory Mediators in the Development, Progression, Metastasis, and Chemoresistance of Epithelial Ovarian Cancer. *Cancers (Basel)* **10**, 251
54. Chaki, M., Airik, R., Ghosh, A. K., Giles, R. H., Chen, R., Slaats, G. G., Wang, H., Hurd, T. W., Zhou, W., Cluckey, A., Gee, H. Y., Ramaswami, G., Hong, C.-J., Hamilton, B. A., Cervenka, I., Ganji, R. S., Bryja, V., Arts, H. H., van Reeuwijk, J., Oud, M. M., Letteboer, S. J. F., Roepman, R., Husson, H., Ibraghimov-Beskrovnaya, O., Yasunaga, T., Walz, G., Eley, L., Sayer, J. A., Schermer, B., Liebau, M. C., Benzing, T., Le Corre, S., Drummond, I., Janssen, S., Allen, S. J., Natarajan, S., O'Toole, J. F., Attanasio, M., Saunier, S., Antignac, C., Koenekoop, R. K., Ren, H., Lopez, I., Nayir, A., Stoetzel, C., Dollfus, H., Massoudi, R., Gleeson, J. G., Andreoli, S. P., Doherty, D. G., Lindstrad, A., Golzio, C., Katsanis, N., Pape, L., Abboud, E. B., Al-Rajhi, A. A., Lewis, R. A., Omran, H., Lee, E. Y. H. P., Wang, S., Sekiguchi, J. M., Saunders, R., Johnson, C. A., Garner, E., Vanselow, K., Andersen, J. S., Shlomai, J., Nurnberg, G., Nurnberg, P., Levy, S., Smogorzewska, A., Otto, E. A., and Hildebrandt, F. (2012) Exome capture reveals ZNF423 and CEP164 mutations, linking renal ciliopathies to DNA damage response signaling. *Cell* **150**, 533-548
55. Chodankar, R., Kwang, S., Sangiorgi, F., Hong, H., Yen, H.-Y., Deng, C., Pike, M. C., Shuler, C. F., Maxson, R., and Dubeau, L. (2005) Cell-Nonautonomous Induction of Ovarian and Uterine Serous Cystadenomas in Mice Lacking a Functional Brca1 in Ovarian Granulosa Cells. *Current Biology* **15**, 561-565
56. Choi, H. J. C., Lin, J.-R., Vannier, J.-B., Slaats, G. G., Kile, A. C., Paulsen, R. D., Manning, D. K., Beier, D. R., Giles, R. H., Boulton, S. J., and Cimprich, K. A. (2013) NEK8 links the ATR-regulated replication stress response and S phase CDK activity to renal ciliopathies. *Mol Cell* **51**, 423-439
57. Lee, B., Lee, H. J., Cho, H. Y., Suh, D. H., Kim, K., No, J. H., Kim, H., and Kim, Y. B. (2015) Ataxia-Telangiectasia and RAD3-Related and Ataxia-Telangiectasia-Mutated Proteins in Epithelial Ovarian Carcinoma: Their Expression and Clinical Significance. *Anticancer research* **35**, 3909-3916

58. Pacheco, S., Maldonado-Linares, A., Marcet-Ortega, M., Rojas, C., Martínez-Marchal, A., Fuentes-Lazaro, J., Lange, J., Jasin, M., Keeney, S., Fernández-Capetillo, O., Garcia-Caldés, M., and Roig, I. (2018) ATR is required to complete meiotic recombination in mice. *Nature Communications* **9**, 2622
59. Widger, A., Mahadevaiah, S. K., Lange, J., ElInati, E., Zohren, J., Hirota, T., Pacheco, S., Maldonado-Linares, A., Stanzione, M., Ojarikre, O., Maciulyte, V., de Rooij, D. G., Tóth, A., Roig, I., Keeney, S., and Turner, J. M. A. (2018) ATR is a multifunctional regulator of male mouse meiosis. *Nature Communications* **9**, 2621
60. Menolfi, D., Jiang, W., Lee, B. J., Moiseeva, T., Shao, Z., Estes, V., Frattini, M. G., Bakkenist, C. J., and Zha, S. (2018) Kinase-dead ATR differs from ATR loss by limiting the dynamic exchange of ATR and RPA. *Nature Communications* **9**, 5351
61. Härtlova, A., Erttmann, S. F., Raffi, F. A. M., Schmalz, A. M., Resch, U., Anugula, S., Lienenklaus, S., Nilsson, L. M., Kröger, A., Nilsson, J. A., Ek, T., Weiss, S., and Gekara, N. O. (2015) DNA Damage Primes the Type I Interferon System via the Cytosolic DNA Sensor STING to Promote Anti-Microbial Innate Immunity. *Immunity* **42**, 332-343
62. Ohnishi, S., Ma, N., Thanan, R., Pinlaor, S., Hammam, O., Murata, M., and Kawanishi, S. (2013) DNA damage in inflammation-related carcinogenesis and cancer stem cells. *Oxid Med Cell Longev* **2013**, 387014-387014
63. Dunphy, G., Flannery, S. M., Almine, J. F., Connolly, D. J., Paulus, C., Jonsson, K. L., Jakobsen, M. R., Nevels, M. M., Bowie, A. G., and Unterholzner, L. (2018) Non-canonical Activation of the DNA Sensing Adaptor STING by ATM and IFI16 Mediates NF-kappaB Signaling after Nuclear DNA Damage. *Molecular cell* **71**, 745-760.e745
64. Carroll, B. L., Pulkoski-Gross, M. J., Hannun, Y. A., and Obeid, L. M. (2016) CHK1 regulates NF-kappaB signaling upon DNA damage in p53- deficient cells and associated tumor-derived microvesicles. *Oncotarget* **7**, 18159-18170
65. Chen, C. F., Ruiz-Vega, R., Vasudeva, P., Espitia, F., Krasieva, T. B., de Feraudy, S., Tromberg, B. J., Huang, S., Garner, C. P., Wu, J., Hoon, D. S., and Ganesan, A. K. (2017) ATR Mutations Promote the Growth of Melanoma Tumors by Modulating the Immune Microenvironment. *Cell reports* **18**, 2331-2342

CHAPTER 3

INHIBITION OF ATAXIA TELANGIECTASIA AND RAD3-RELATED (ATR) HAS DIFFERENTIAL EFFECTS ON CYCLING AND NON-CYCLING CELL POPULATIONS EXPOSED TO CALCIUM OVERLOAD AND OXIDATIVE DAMAGE

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ABSTRACT

Calcium overload and oxidative damage are two marked outcomes of ischemic stroke. Ischemic stroke is one of the major leading causes of death and disability worldwide. During ischemic stroke, blood flow to a region of the brain is restricted by thromboembolism leaving the tissue supplied by the occluded vessel oxygen and nutrient deprived. Upon natural or assisted removal of the embolus, the brain is then reperfused. The initial hypoxia, as well as the reperfusion, leads to the release of excitatory neurotoxic components, oxidative stress, and destructive inflammation. Because current therapeutic options are limited, we sought to investigate the potential use of Ataxia telangiectasia and Rad3-related (ATR) inhibition as a potential treatment. Investigations of ATR inhibition, using various calcium overload and oxidative damage conditions, revealed differential effects between populations of cycling and non-cycling cells. In general, ATR inhibition was protective against these insults in non-cycling cells and detrimental to cycling cells. We also determined the upstream activation of ATR signaling in non-cycling cells to oxidative damage be both base excision repair and nucleotide excision repair dependent. These results indicate that ATR inhibition could serve as a potential therapeutic option by limiting neuronal death while simultaneously suppressing immune cell proliferation, and subsequent inflammation, following transient ischemic stroke.

INTRODUCTION

Stroke is one of the top five causes of death and major disability worldwide (1-3). Despite this, there are few therapeutic interventions and all available interventions have narrow clinical windows (4). Of all strokes, ischemic strokes account for the majority, 87% (1). During an ischemic stroke, blood flow to a region of the brain is restricted by a thromboembolism leaving the tissue supplied by the occluded vessel oxygen and nutrient deprived. Upon natural or assisted removal of the embolus, the brain is reperfused. The initial hypoxia, as well as the reperfusion, lead to the release of excitatory neurotoxic compounds, calcium overload, oxidative stress, and destructive inflammation (5-10). These events lead to widespread DNA damage and neuronal cell death in the infarct region.

Neuronal inflammation, calcium overload, and oxidative stress drive reactive nitrogen and oxygen signaling. This leads to calcium overload and the generation of reactive species which are capable of damaging proteins, lipids, and DNA. Calcium overload and oxidative DNA damage has been shown to induce cell death, cause base modifications that are mutagenic, induce replication stress, and inhibit/modify transcription dependent on the type of oxidative lesion (7, 11-13). One protein activated following hypoxia and oxidative damage is the protein kinase ataxia telangiectasia and rad3-related (ATR) (14-16). ATR is a member of the PIKK (phosphatidylinositol-3 kinase-like) family which has roles in regulating cell cycle progression, DNA repair, and apoptosis (17-21). Recent studies have shown that inhibition of ATR's kinase activity in non-cycling cells is protective against the lethal effects of transcription stress and advanced DNA damage (22, 23). This contrasts with the fact that ATR's kinase activity in cycling cells is required not only for replication but also for suppression of both caspase-2- and caspase-3-dependent apoptotic pathways following DNA damage (24-26).

Previous attempts at targeting calcium overload and oxidative DNA damage have focused on the individual components leading to the damage; however, they tend to therapeutically 'miss the mark' due to only being useful against one or two of individual factors (4). Here we show evidence that inhibition of ATR signaling is neuroprotective, as well as immunosuppressive, in various calcium overload and oxidative damage conditions. We further provide evidence that this protection is likely due to the differential actions of ATR inhibition on cycling and non-cycling cell populations. Lastly, we define ATR activation following oxidative damage in non-cycling cells to be both APE2-, a base excision repair (BER) protein, and nucleotide excision repair (NER)-dependent. These results give hope that ATR inhibitors could serve as a therapeutic option following ischemic stroke or other similar conditions.

METHODS

Cell culture

C6 (ATCC CCL-107, rat glioma), HMC3 (ATCC CRL-3304, human microglia), and SH-SY5Y (ATCC CRL-226, human neuroblastoma) cell lines were all obtained from the American Type Culture Collection (ATCC). C6 cells were maintained in F-12 media (Corning) supplemented with 10% fetal bovine serum (Thermo Scientific). HMC3 and SH-SY5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Scientific). All lines were supplemented with antibiotics (100 units/mL penicillin, 100 units/mL streptomycin, VWR) and maintained at 37ºC under a constant atmosphere of 5% CO_2.

Cell differentiation

C6 cells were grown to 60-70% confluency. Then, complete media was replaced with serum-free F-12 that was supplemented with 100 nM paclitaxel (SigmaAldritch). This media was refreshed on the second day with more media of the same composition. On the fourth day and every two days after that, the media was replaced with serum-free F-12. A washout period of six days post-paclitaxel treatment was allowed before experiments were performed.

SH-SY5Y were grown to 60-70% confluency. Then, complete media was replaced with 1% serum DMEM supplemented with freshly made all-*trans* retinoic acid dissolved in DMSO to a final in dish concentration of 10μM. This media was changed every two days with the media of the same composition until day ten. At day ten the media was replaced with 1% serum in DMEM with no further supplementation. A washout period of two days was allowed before any experiments were performed.

Cell treatments and transfections

All treatments were done for the times indicated in the sections where they are presented. The ATR inhibitor, VE-822 (MedKoo), was dissolved in DMSO to make a stock concentration of 80 μM which was then diluted in culture media to 80 nM before treatment. All listed VE-822 treatments were initiated 1 hr prior to other treatments. Glutamate (SigmaAldritch) was dissolved in equimolar NaOH to a final stock concentration of 1M. Before treatment, glutamate was diluted in media appropriate for the cells to be treated. Media was removed from cells and replaced with media containing glutamate. Similar treatments were performed for both hydrogen peroxide (VWR) and ionomycin (Cayman). For glucose deprivation and oxygen-glucose deprivation with reperfusion (OGDR), glucose and serum-free DMEM (Corning) was used. Cells were washed with 1x PBS after the removal of normal media and addition of the glucose and

serum-free media. For oxygen depletion studies, 4 hr hypoxia ($O_2/N_2/CO_2$, 1:94:5) treatments were achieved through the use of an oxygen depletion regulation system (ProOx C31, BioSpherix). Following hypoxia, media was replaced with complete media for the times indicated.

For DNA damage signaling assays, Triptolide (300nM, ApexBio) was administered 1hr prior to further treatment. For shAPE2 (SantaCruz) mediated knockdown of APE2, transfection of shAPE2 plasmid was done by using jetPEI transfection reagent (Polyplus) by the company's protocol. Selection with puromycin (1µg/mL, Gibco) was done for three days before further treatment.

Immunoblotting and antibodies

Cells were collected by first scraping in media and spinning down all cells at 10,000rpm for 5min. Then, the cells were resuspended in lysis buffer (50mM Tris-HCl, pH7.8, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1x protease/phosphatase inhibitor cocktail [Thermo]) and the mixture incubated on ice for approximately 20min before centrifugation (10,000rpm at 4ºC) to pellet cellular debris. 2xSDS loading buffer was added to cell lysates and the mixture was heated at 95ºC for 5min. Samples were loaded onto appropriate percentage SDS-PAGE gels for resolution of the proteins in question. Proteins were transferred from gels onto PVDF membranes (Millipore). Primary antibodies raised against actin (Cell Signaling), APE2 (ThermoFisher), Chk1 (Cell Signaling), cleaved Caspase 3 (c-caspase 3, Cell Signaling), cleaved PARP1 (c-PARP1, Millipore), Ki-67 (Cell Signaling), NeuN (Millipore), p-Chk2 T68 (Cell Signaling), and p-p53 S15 (Cell Signaling) were used. The chemiluminescent signal from HRP-conjugated secondary antibody (ThermoFisher) was detected and captured using either the Fuji Film imager LAS-400 or an Amerscham Imager 680 (GE).

Viability assays

MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assays (Alfa Aesar) were used to assess viability following hydrogen peroxide, glutamate, and OGDR treatments per manufacturer's instruction. Absorbance at 490 nM was determined using a PowerWave XS2 spectrophotometer (BioTek). Differences in cell viability were statistically determined using GraphPad Prism by one-way ANOVA with significance set at $p<0.05$ and the standard deviation is shown.

Trypan blue exclusion assays were used to determine the cell death following ionomycin treatment. Cells were harvested by trypsinization followed by low-speed centrifugation (500 rpm, 5 min). The supernatant was removed, and the cell pellet was resuspended in 0.1mL of 0.4% trypan blue solution. Death was assessed by counting the number of blue cells versus the total number of cells. Differences in cell death were statistically determined using GraphPad Prism by one-way ANOVA with significance set at $p<0.05$ and the standard deviation is shown.

Colony formation and viability staining with crystal violet were used to determine the effects of ATR inhibition on the growth of HMC3 cells and survival of differentiated C6 and SH-SY5Y cells. HMC3 cells were plated and allowed to reattach overnight. A control plate was then fixed and saved until the end of treatment. The remaining plates were either treated with 80 nM VE-822 or with the same amount of DMSO for six days. Then the cells were fixed with 4% PFA (paraformaldehyde, EM Biosciences) for 10min at room temp before being stained with 4% crystal violet for 10 min at room temp. Plates were then destained with ddH_2O until no crystal violet could be liberated from the plate. The same procedure was performed to ascertain viability in differentiated C6 and SH-SY5Y cells.

RESULTS

ATR inhibition has opposite effects on the survival of cycling and non-cycling cells exposed to calcium overload

Strokes involve a complex interplay of excitatory neurotoxicity and direct oxidative damage. To tease apart the effect of ATR inhibition on these various components, we decided to address them individually *in vitro*. Using cell lines representative of different types of cells within the brain we looked first at the effects of two different excitotoxic calcium overload models: glutamate and ionomycin.

Glutamate is a multifunctional excitatory neurotransmitter capable of acting on multiple ionotropic and metabotropic receptors. During ischemia, glutamate is continuously released due to a feedforward depolarization of neurons resulting in the concentration of glutamate in the extracellular portion of the ischemic lesion. This glutamate activates ionotropic receptors which either directly (NMDA receptors) or indirectly (AMPA/KAR receptors) lead to an increase of intracellular calcium. These mechanisms are further augmented by activation of metabotropic glutamate receptors which lead to the activation of PLC (phospholipase C), generation of IP_3 (inositol 1,4,5-trisphosphate), and the subsequent release of calcium from the endoplasmic reticulum. The sum result of these events is an increase in cytoplasmic calcium concentration which leads to mitochondrial dysfunction, the generation of reactive oxygen species (ROS), and cell death by either apoptosis or necrosis (5, 7, 8, 27, 28).

To assess the effects of ATR inhibition, we first looked at the consequence of ATR inhibition on cellular viability post glutamate exposure. In human microglial cell 3 (HMC3), a cycling macrophage/microglial like cell line, ATR inhibition reduced viability both alone and in conjunction with glutamate treatment at both early (Fig. 3.1A) and late (Fig. 3.1B) timepoints. This was confirmed by an increase in apoptotic markers, cleaved caspase-3 (c-Caspase 3) and

cleaved PARP1 (c-PARP1), seen by western blot analysis at twenty-four hours (Fig. 3.1C). The absence of these markers at the earlier timepoint is likely due to mitochondrial dysfunction, without apoptosis initiation, and necrotic cell death. While early timepoints are known to induce necrosis at the dosage used, later timepoints are known to exhibit a mostly apoptotic phenotype due to accumulated oxidative damage from mitochondrial dysfunction and antioxidant depletion (5, 8, 28).

In contrast to the findings with cycling HMC3 cells, ATR inhibition was found to be protective when similar experiments were performed with non-cycling neuron-like cells. For these experiments, SH-SY5Y and C6 cells were differentiated using all-*trans* retinoic acid and paclitaxel, respectively. Both methods are known to induce a neuron-like phenotype upon differentiation (29, 30). Confirmation of a non-cycling state was established by western blot analysis for Ki-67 and Chk1 (checkpoint kinase 1) (Fig. 3.S1). Both Ki-67 and Chk1, defined markers for cycling cells, were highly depleted following retinoic acid treatment of SH-SY5Y and paclitaxel treatment of C6 indicating exit from the cell cycle and entrance into a non-cycling state (22, 31). Seen in Figure 3.1D and 3.1E, ATR inhibition was protective against the toxic effects of glutamate-induced calcium overload in differentiated SH-SY5Y both at four hours and twenty-four hours. Again, this was confirmed in the western blot analysis for c-PARP1 and c-Caspase 3 at twenty-four hours (Fig. 3F). Interestingly, whereas both cycling HMC3 and differentiated SH-SY5Y lines had marked decreased viability at the four-hour timepoint, differentiated C6 cells were tolerant to the initial viability loss induced by glutamate exposure (Fig. 3.1G); however, there was still a significant protection conferred by ATR inhibition here as well as in the twenty-four-hour timepoint (Fig. 3.1H). These results were confirmed by western blot assessment of c-PARP1 and c-Caspase3 in which both four-hour and twenty-four-hour

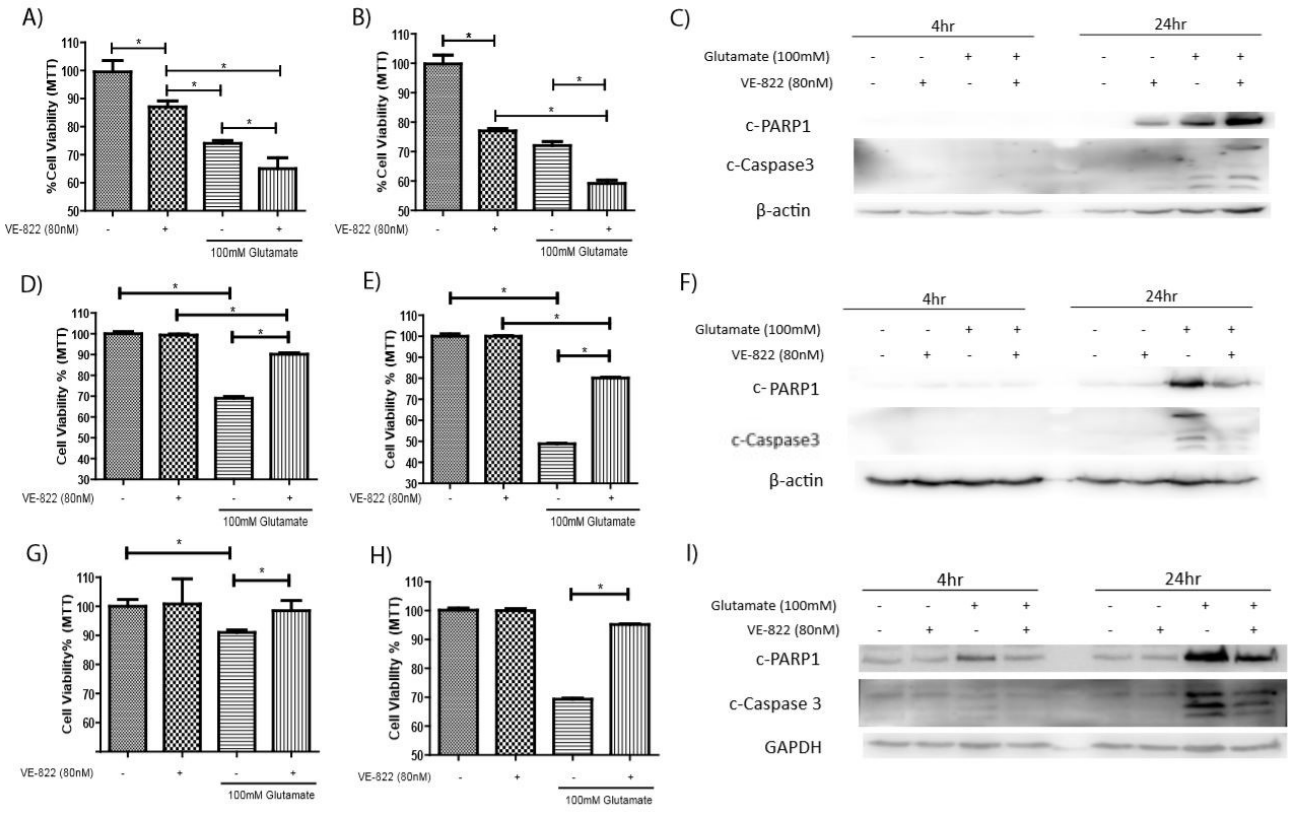

Figure 3.1: ATR inhibition differentially affects cycling and non-cycling cells exposed to glutamate-induced calcium overload

A & B) HMC3 cells were treated with glutamate, ATR inhibitor (VE-822), or a combination. Cellular viability was assessed by MTT at 4hr (A) and 24hr (B). Under both conditions, MTT results shows an increase in cell death in combination treatment compared to glutamate alone. C) Results of MTT were confirmed by analysis of whole cell extracts of similarly-treated HMC3. Increases in c-PARP1 and c-Caspase 3 are indicative of increased apoptotic cell death in ATR inhibitor-treated groups. D & E) Differentiated SH-SY5Y cells were treated with glutamate, ATR inhibitor (VE-822), or a combination. Cellular viability was assessed by MTT at 4hr (D) and 24hr (E). A protective effect against glutamate-induced calcium overload was seen at both timepoints. F) Results of MTT were confirmed by analysis of whole cell extracts of similarly-treated SH-SY5Y cells. Decreases in c-PARP1 and c-Caspase 3 are indicative of decreased apoptotic cell death in ATR inhibitor-treated groups. G & H) Differentiated C6 cells were treated with glutamate, ATR inhibitor (VE-822), or a combination. Cellular viability was assessed by MTT at 4hr (G) and 24hr (H). A protective effect against glutamate-induced calcium overload was seen at both timepoints. I) Results of MTT were confirmed by analysis of whole cell extracts of similarly-treated C6 cells. Decreases in c-PARP1 and c-Caspase 3 are indicative of decreased apoptotic cell death in ATR inhibitor-treated groups. One-way ANOVAs were performed for all MTT tests listed. $p<0.05$

timepoints revealed a reduction in apoptotic markers in ATR inhibitor-treated samples (Fig. 3.1I).

These observations indicate that ATR inhibition has differing effects between cycling immune-like cells and non-cycling neuronal-like cells. ATR inhibition alone appears to be enough to reduce viability, prevent clonal expansion, and induce apoptosis over time in cycling HMC3 (Fig. 3.S2A) while it has no effect on the viability of non-cycling cells (Fig. 3.S2B&C). These results resemble those previously reported by other labs which showed that ATR kinase activity is essential in cycling cell populations, but dispensable in non-cycling cells (22-24).

Because glutamate is known to induce oxidative damage in addition to calcium overload, ionomycin, a calcium ionophore, also was employed to investigate calcium-overload induced cell death in these cell populations. Whereas glutamate is known to induce both apoptosis and necrosis, the dosage of ionomycin used is well known to induce a pure necrotic phenotype at early time points (32). Treatment of HMC3 cells with ionomycin in the presence of ATR inhibition led to a near three-fold increase in necrotic cell death as determined by trypan blue staining (Fig. 3.2A). Similar to the findings with glutamate, both differentiated SH-SY5Y (Fig. 3.2B) and C6 cells (Fig.3.3C) were protected from the calcium overload-induced necrosis by ATR inhibition. These results suggest that ATR inhibition could serve to suppress the immune response to calcium-overload stimuli post-stroke while also directly sparing neurons from this mode of cell death.

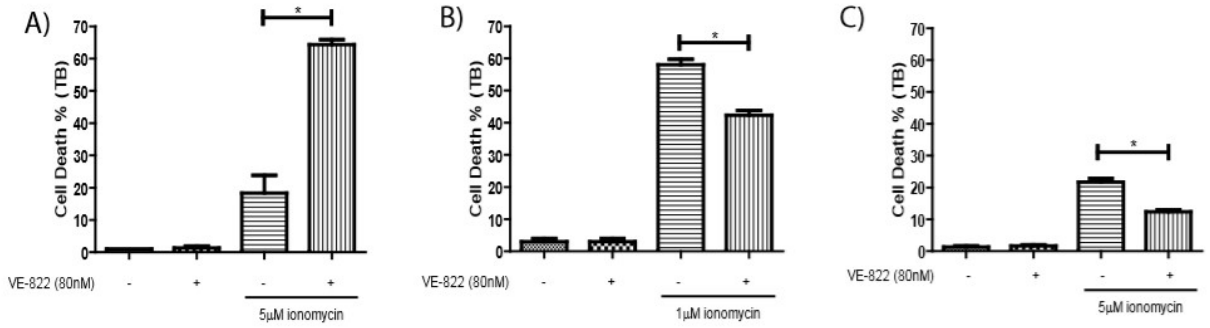

Figure 3.2: ATR inhibition differentially affects cycling and non-cycling cells exposed to ionomycin-induced calcium overload
HMC3 (A), differentiated SH-SY5Y (B), or differentiated C6 (C) cells were pretreated with ATR inhibitor (80nM VE-822) and then exposed to ionomycin for 4hrs. ATR inhibition was detrimental to necrosis inducing calcium overload in cycling HMC3 cells. This contrasts with a protective effect of ATR inhibition seen in differentiated SH-SY5Y and differentiated C6 cells. Cell viability was assessed by the trypan blue exclusion assay. One-way ANOVAs were used to access statistical significance. $p<0.05$

ATR inhibition has opposite effects on the survival of cycling and non-cycling cells exposed to oxidative stress

Another component common to all strokes is oxidative damage resulting from ischemia and reperfusion, in cases where clot evacuation is possible. To study these events, we employed both direct oxidative damaging agents as well as an *in vitro* model mimicking ischemia/reperfusion.

Cycling HMC3 cells showed decreased viability when exposed to a moderate, apoptosis-inducing, dose of hydrogen peroxide. This loss of viability was only slightly increased by ATR inhibition at four hours but was significantly increased at twenty-four hours (Fig. 3.3A&B). Increases in apoptotic cell death were seen at both four hours and twenty-four hours by western blot analysis for apoptotic markers (Fig. 3.3C). As seen previously with the calcium-overload models, ATR inhibition alone was sufficient to induce loss of viability and induce apoptosis in

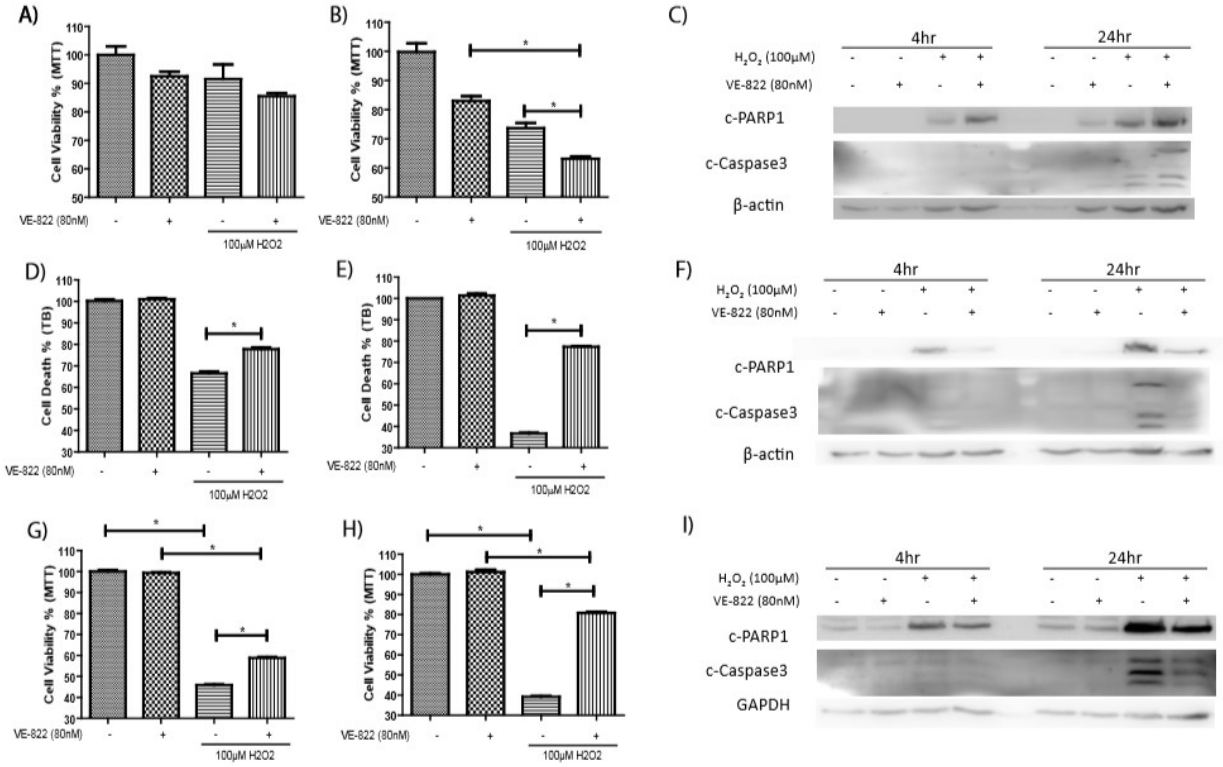

Figure 3.3: ATR inhibition differentially affects cycling and non-cycling cells exposed to direct oxidative damage

A & B) HMC3 cells were treated with hydrogen peroxide, ATR inhibitor (VE-822), or a combination. Cellular viability was assessed by MTT at 4hr (A) and 24hr (B). While no change in viability was seen at 4 hours there was an increase in cell death at 24hr. C) Results of MTT differed from western blot analysis of similarly treated HMC3 in which increases in the apoptotic markers c-PARP1 and c-Caspase 3 were seen at both 4hr and 24hr. D & E) Differentiated C6 cells were treated with hydrogen peroxide, ATR inhibitor (VE-822), or a combination. Cellular viability was assessed by MTT at 4hr (D) and 24hr (E). There was statistical protection at both 4hr and 24hr. F) Results of the MTT were confirmed by analysis of whole cell extracts of similarly treated SH-SY5Y. Decreases in c-PARP1 and c-Caspase 3 are indicative of decreased apoptotic cell death in ATR inhibitor treated groups. G & H) Differentiated C6 cells were treated with hydrogen peroxide, ATR inhibitor (VE-822), or a combination. Cellular viability was assessed by MTT at 4hr (G) and 24hr (H). There was statistical protection at both 4hr and 24hr. The increase in cellular viability seen in G over H is likely due to transient loss of viability (mitochondrial respiration dependent) followed by recovery. I) Results of MTT were confirmed by analysis of whole cell extracts of similarly treated C6. Decreases in c-PARP1 and c-Caspase 3 are indicative of decreased apoptotic cell death in ATR inhibitor treated groups. One-way ANOVAs were performed for all MTT tests listed. $p<0.05$

these cycling cells adding to the evidence that ATR inhibition could be an immunosuppressive following stroke.

Similar to calcium-overload models, ATR inhibition was protective against direct oxidative damage in differentiated C6 (Fig. 3.3D-F) and SH-SY5Y cells (Fig. 3.3G-I) exposed to hydrogen peroxide. Of note, both differentiated lines appear to have an initial reduction in viability in the ATR inhibitor treated group (Fig. 3.3D & G) that partially recovers at twenty-four hours (Fig. 3.3E & G). While not reflected in the western blot analyses for apoptotic markers, this shows that the initial reduction in viability was likely transient. As apoptosis is a time-consuming process, it is likely the initial burst of oxidative damage induced mitochondrial dysfunction, but this dysfunction did not lead to apoptosis in ATR inhibitor-treated samples. In this way, ATR inhibition likely prevents apoptosis initiation allowing for adequate repair of DNA damage and removal of damaged mitochondria. This is supported by the fact that many proteins involved in oxidative DNA repair are markedly upregulated in neurons following oxidative insults, such as stroke (7, 33, 34). Thus, ATR inhibition of apoptosis initiation would allow time for upregulation of repair mechanisms and clearance of damaged macromolecules from the system.

To confirm these results, we employed an oxygen-glucose deprivation with reperfusion (OGD-R) model to study the effects of stroke-like conditions on these cell lines. This model employs a period of oxygen and glucose deprivation in which the cells are starved, imitating the ischemia induced by artery blockage. Then, nutrients and oxygen are restored to simulate reperfusion following blockage removal. Results show that ATR inhibition in HMC3 cells leads to reduced viability at both four hours and twenty-four hours of reperfusion following a four-hour ischemic period (Fig. 3.4A & B). In contrast, ATR inhibition in differentiated SH-SY5Y

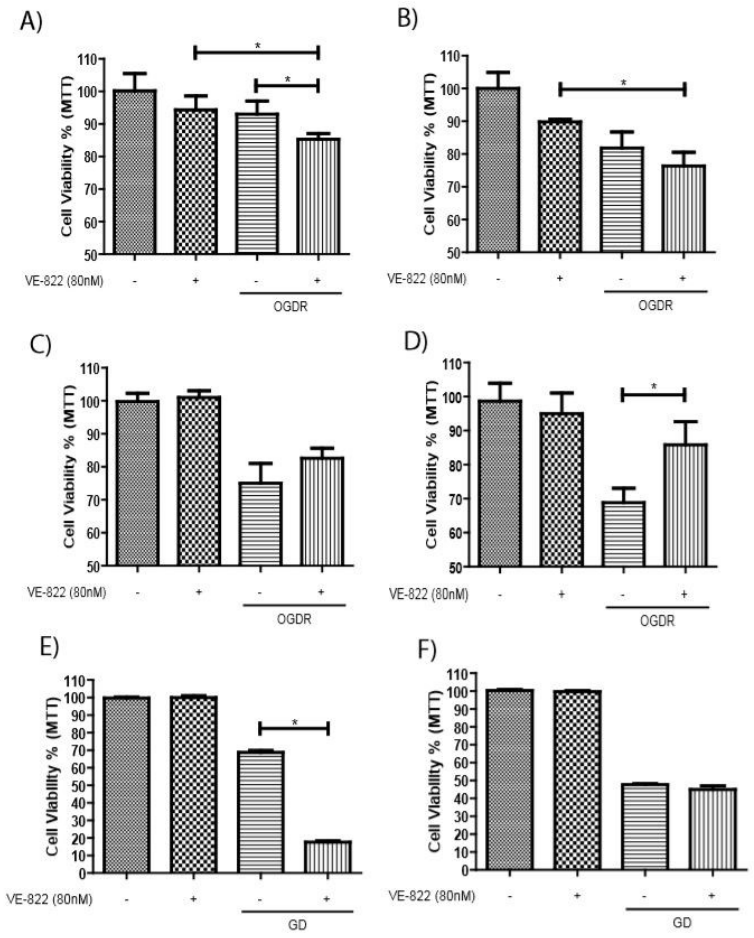

Figure 3.4: ATR inhibition differentially affects cycling and non-cycling cells exposed to an *in vitro* ischemia/reperfusion model

A& B) HMC3 cells were exposed to oxygen-glucose deprivation for 4hr followed by either a 4hr (A) or 24hr (B) reperfusion period. A trend in the MTT assay data showing increased loss of viability with combination treatment was seen. C & D) Differentiated SH-SY5Y cells were exposed to oxygen-glucose deprivation for 4hr followed by either a 4hr (C) or 24hr (D) reperfusion period. MTT readings showed ATR inhibition trended to be protective at 4hr, but this was not significant. Results were significantly protective at 24hr. E) Cycling SH-SY5Y were exposed to glucose deprivation alone for 24hr with or without ATR inhibition. F) Differentiated SH-SY5Y cells were exposed to glucose deprivation alone for 24hr with or without ATR inhibition. ATR inhibition is deleterious for the survival of cycling cells under glucose deprivation, but appears to have no effect on non-cycling cells. One-way ANOVAs were used to access statistical significance. $p<0.05$

showed modest, but not significant, increases in viability at four hours post-reperfusion (Fig.3.4C); however, a significant level of protection was seen at twenty-four hours post-reperfusion (Fig 3.4D). Glucose deprivation (GD) alone also was assessed in cycling (Fig. 3.4E) and differentiated (Fig. 3.4F) SH-SY5Y cells. It was found that ATR inhibition greatly potentiates viability loss associated with GD in cycling cells, but it has no real effect on the viability of differentiated cells. Taken together, these results support the previous findings that ATR inhibition is neuroprotective and immunosuppressive following oxidative injury.

ATR activation following oxidative damage is different between cycling and non-cycling cells

Given that the previous results indicated differences between cycling and non-cycling cell populations, we sought to investigate whether differences exist in DNA damage signaling between the two states upon ATR inhibition. For this purpose, we employed hydrogen peroxide as a direct oxidative agent to avoid off-target effects commonly known to be involved with the other treatments presented previously. It was found that cycling HMC3 cells and differentiated SH-SY5Y cells exhibited decreased p53 phosphorylation at Ser15 when pretreated with ATR inhibitor before hydrogen peroxide treatment (Fig. 3.5A). p53 phosphorylation was chosen as a marker for ATR signaling because Chk1, the main substrate for ATR, is nearly absent in non-dividing cells (22). Additionally, p53-Ser15 phosphorylation activates the transcriptional properties of p53 leading to transcription of both proapoptotic and cell-cycle regulatory proteins (35, 36). Interestingly, while p53-Ser15 phosphorylation was decreased in both cells upon ATR inhibition, phosphorylation of Chk2 (checkpoint kinase 2) at Thr68 by ATM (Ataxia telangiectasia mutated) was increased in cycling cells and decreased in non-cycling cells. This is indicative of increased replicative stress, resultant replication fork collapse, and generation of DNA double-strand breaks induced from ATR inhibition with DNA damage (18, 24, 37).

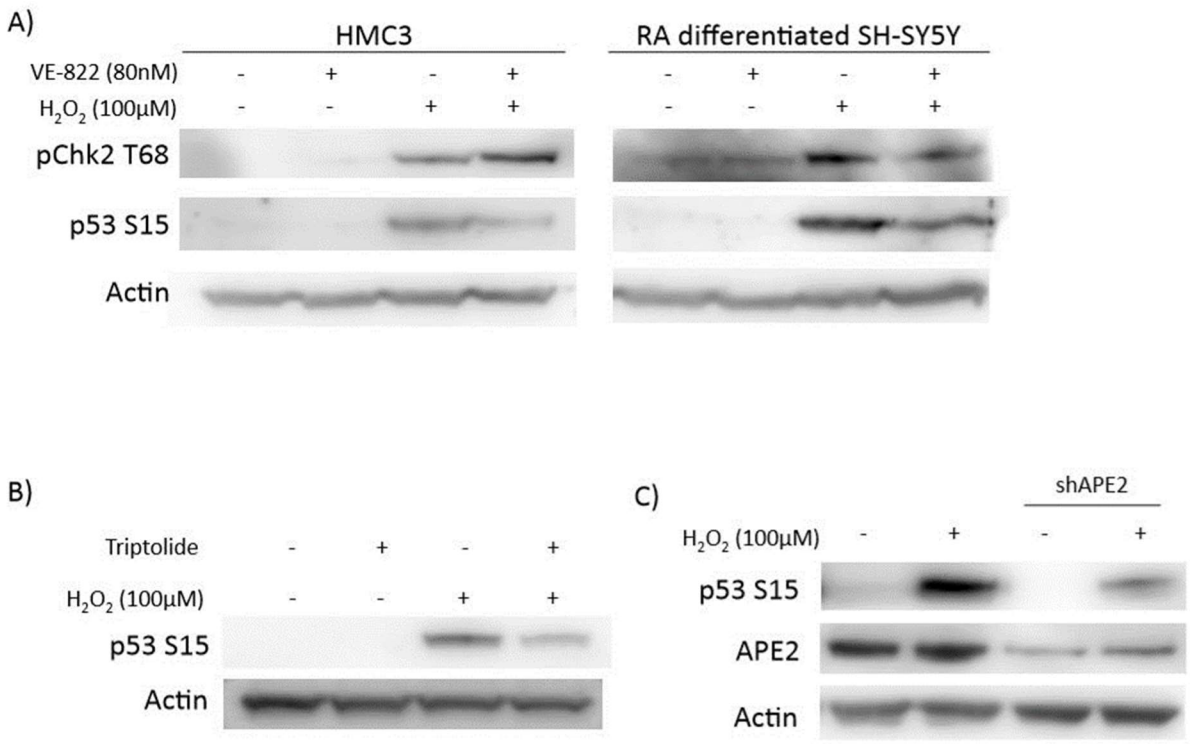

Figure 3.5: DNA damage signaling is different in HMC3 and RA differentiated SH-SY5Y cells following oxidative damage
A) Cycling and non-cycling cells express different DNA damage signaling profiles under ATR inhibition and oxidative damage. Note that while p-p53 Ser15 phosphorylation is decreased in both cell lines, there is an opposite effect on phosphorylation of Chk2, indicating a compensatory role of ATM in cycling cells that isn't present in non-cycling cells. B) Inhibition of the TC-NER protein XPB with triptolide (300 nM) reduces the phosphorylation status of p53 following hydrogen peroxide treatment indicating transcription stress as an activator of ATR. C) shRNA knockdown of the BER protein APE2 has similar effects to triptolide treatment on p53 phosphorylation status. This implicates that both BER and NER pathways are involved in oxidative signaling in non-cycling cells.

Replication does not occur in differentiated cells, so activation of ATM is dependent on transcription-induced strand breaks after oxidatively-induced abasic site generation and direct activation by ROS, which are both additional mechanisms of ATM activation in cycling cells (37, 38). While a reduction in p53 S15 phosphorylation could be seen as anti-apoptotic in cycling cells, it is known that ATR inhibition upon replication stress in cycling cells leads to apoptosis through a caspase-3 dependent mechanism that has been attributed to ATM signaling (25, 39). In

contrast, recent studies have shown that ATR inhibition is protective against transcription stress and generalized DNA damage in non-cycling/resting cells (22, 23).

Because replication stress, the primary activator of ATR, isn't present in non-cycling cells we sought to investigate which pathways were involved in ATR activation post oxidative DNA damage in these cells. Previous reports have implicated that both nucleotide excision repair (NER) as well as single-strand break end resection by APE2 (apurinic exonuclease 2), a component of base excision repair (BER), could be involved (40-46). To investigate the effects of oxidative damage-induced activation of ATR in non-cycling cells, we first used triptolide to inhibit the NER pathway. TC-NER (transcription coupled-NER) is the pathway which responds to RNA polymerase stalling in response to bulky DNA lesions, whereas GG-NER (global genome-NER) response to helix-distorting DNA adducts in currently untranscribed regions. By using triptolide, an inhibitor of both GG-NER and TC-NER through inhibition of XPB (xeroderma pigmentosum type B), it was found that p53 Ser15 phosphorylation is reduced after hydrogen peroxide exposure in differentiated SH-SY5Y (Fig. 3.5B). This indicates that blocking NER generation of single-stranded DNA (ssDNA) by inhibiting the XPB DNA helicase can reduce, but not abolish, ATR activation. Investigation of shRNA-mediated knockdown of APE2 also revealed a reduction in p53 Ser15 phosphorylation (Fig. 3.5C). This indicates that not only is NER involved, but BER as well through APE2 generation of ssDNA. Dual inhibition of BER and NER pathways was attempted but lead to extreme death following hydrogen peroxide treatment that was likely due to excessive damage accumulation from inhibition of repair. As a result, signaling could not be tested.

These observations demonstrate differences in activation of ATR following oxidative DNA damage. They also show that pathways which are known to be minor activators of ATR in

cycling cells are highly involved in its activation in non-cycling cells. A summary of these proposed activations and the resultant signaling is shown in Fig. 3.6A.

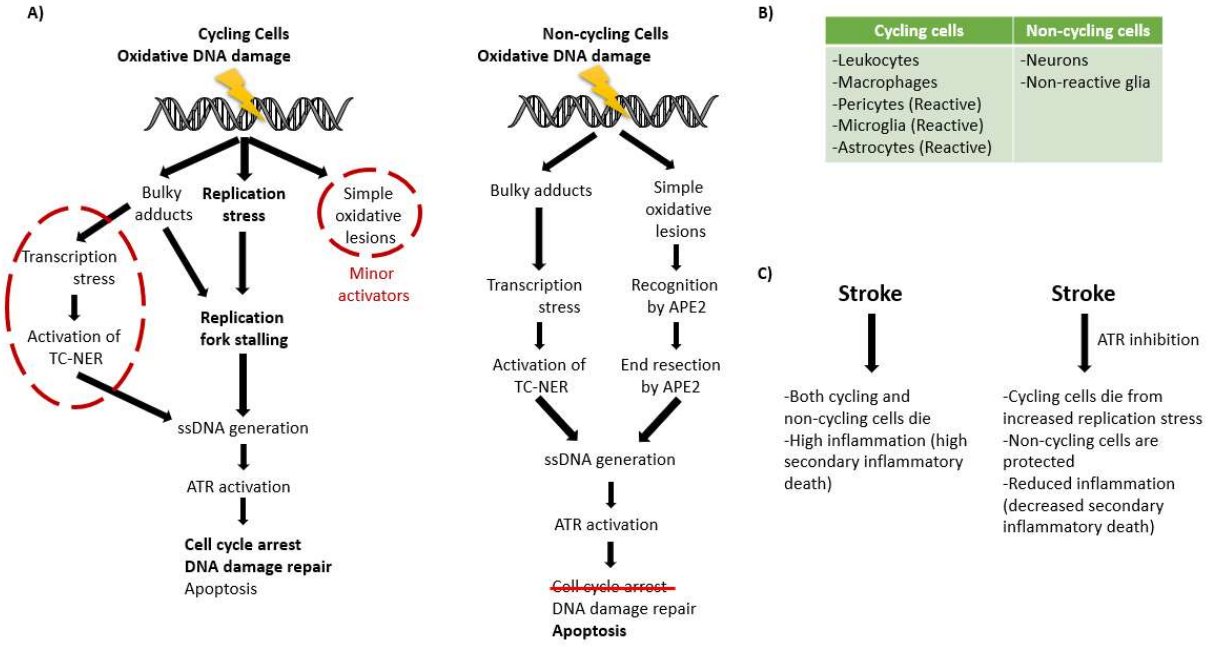

Figure 3.6: Summary of differential ATR signaling and outcomes of ATR inhibition in cycling and non-cycling cells after DNA damage.
A) ATR is differentially activated in cycling and non-cycling cells. The outcomes of this signaling are also noted. B) Examples of cycling and non-cycling cell populations in the brain. C) Stroke outcomes with and without ATR inhibition.

DISCUSSION

During a stroke, the brain is assaulted by excitatory neurotoxic compounds, calcium overload, oxidative stress, and destructive inflammation (5-10). Here we provide evidence that ATR inhibition following various stroke-like insults has radically different effects among cycling and non-cycling cell populations representative of cells within the brain post-stroke (Fig. 3.6B). Using various *in vitro* models, we demonstrate that ATR inhibition is protective in differentiated cells which mimic neurons and non-reactive glia. In contrast, ATR inhibition directly suppresses cell division and potentiates pro-death signaling in cycling immune- and glia-like cells.

Direct protection of neurons is the ideal therapeutic strategy following stroke. This maintains tissue integrity and provides the highest likelihood of functional protection and recovery (47). Here we show that ATR inhibition is capable of sparing neuron-like cells exposed to a variety of common *in vitro* stroke-like insults. ATR is well known to induce apoptosis in non-cycling cells exposed to DNA damage and transcription stress (22, 23). While previous reports have shown this effect to be independent of p53 activation (22), here we show that suppression of ATR kinase activity leads to decreased p53 phosphorylation at Ser15, implying reduced p53 proapoptotic activation. This corresponds to findings showing p53 Ser15 phosphorylation to be required for p53-induced apoptosis (48). While not investigated, ATR also is known to induce a caspase 2-dependent, p53-independent, form of apoptotic cell death (26). This form of death is typically suppressed in cycling cells by Chk1; however, Chk1 is not present in non-cycling cells and, therefore, could contribute to the apoptosis seen. Inhibition of ATR, in this instance, would serve to directly prevent activation of the apoptotic pathway induced by caspase 2 following DNA damage.

In the absence of neuronal sparing, suppression of the inflammatory response serves as a second therapeutic option following stroke (9, 10). Neuron stress and death within the infarct region leads to the release of multiple proinflammatory factors leading to time-dependent recruitment of various immune and glial cells (49). These cells then continue to propagate cell death in the infarct region and surrounding tissue by either directly damaging neurons through the release of oxygen and nitrous oxide radicals (neutrophils, macrophages, and microglia) or through the release of proinflammatory/proapoptotic cytokines (natural killer cells, macrophages, microglia, astrocytes, and cytotoxic t-cells), such as tumor necrosis factor alpha (TNFα) (10, 49, 50). Additionally, the release of cytokines such as TNFα and interferon-γ (INFγ) can lead to

further release of glutamate from neurons and further amplification of the excitotoxic environment following stroke. Our studies suggest that ATR inhibition can hinder the proliferation of these cycling cells and this inhibition combined DNA damage leads to increased cell death in these immune/microglia-like cells in comparison to DNA damage alone. While further studies need to be done to look at reduced inflammatory effects, such as cytokine release, these results are promising in the use of ATR inhibitors to suppress at least the immune cell proliferation at the ischemic lesion.

Separate from these effects, ATR inhibition also could be implicated in other potentially protective mechanisms. For one, ATR and ATM have been found to assist in the balancing of excitatory and inhibitory neurotransmitters. While ATM binds to excitatory glutamate vesicles, ATR is found to bind to inhibitory GABA (γ-aminobutyric acid) vesicles and inhibition of either leads to decreased reuptake of neurotransmitters at the synaptic cleft (51). This could indicate a role for ATR inhibition in the prevention of GABA reuptake and, as previous studies have shown that GABAnergic signaling is protective in cases of ischemic injury (47, 52-54), these increases in GABA could lend to a protective effect. Another potential mechanism comes from the ATR-induced bystander effect. In this phenomenon, cells which experience DNA damage trigger the induction of DNA damage signaling in neighboring cells without damage (55, 56). Following a stroke, this could potentially lead to the expansion of the lesion and increased recruitment of inflammatory cells. Therefore, prevention of this effect could be beneficial to stroke treatment.

Alongside the cellular outcomes of ATR inhibition, we also demonstrate that DNA damage signaling is different between cycling and non-cycling cells. Activation of ATR is well known to be primarily replication stress driven in cycling cells (21, 24, 37); however, replication is not present in non-cycling cell populations. To address this, we looked at pathways which

were implicated in ATR activation while also involved in the repair of oxidative DNA damage. Oxidative DNA damage can come in multiple forms: direct base modifications (eg. 7,8-dihydro-8-oxoguanine [8-oxoG], 5,6-dihydroxy-5,6-dihydrothymine [thymine glycol], 8,5'cyclo-2'deoxyadeonsine [cdA], and 8,5'-cyclo-2'deoxyguanosine [cdG]) and protein/lipid adducts. While most direct modifications are either directly reversed or repaired by BER, a select subset (cdA, cdG) is known to block transcription and require the NER pathway to be resolved (43-45, 57). In contrast, protein and lipid adducts must be removed by NER exclusively (12, 46). Here we demonstrate that both the BER protein APE2 as well as the NER pathway are involved in ATR activation following oxidative damage. This could implicate the presence of multiple kinds or complex lesions generated through oxidative DNA damage under stroke-like conditions. These findings align with those of others who have shown either BER or NER to be involved in the activation of ATR and, while controversial within the field, our data indicate the involvement of both repair pathways.

These findings expand the existing view of ATR regulation of cellular fate following stroke and imply that ATR inhibition could serve as a target for both neuronal sparing and immunosuppressive therapeutic strategies. While these results are promising, future studies need to be conducted to further explore the role of ATR inhibition in immunosuppression and to test the potential outcomes of ATR inhibition in *in vivo* stroke models.

REFERENCES

1. Benjamin, E. J., Virani, S. S., Callaway, C. W., Chamberlain, A. M., Chang, A. R., Cheng, S., Chiuve, S. E., Cushman, M., Delling, F. N., Deo, R., de Ferranti, S. D., Ferguson, J. F., Fornage, M., Gillespie, C., Isasi, C. R., Jimenez, M. C., Jordan, L. C., Judd, S. E., Lackland, D., Lichtman, J. H., Lisabeth, L., Liu, S., Longenecker, C. T., Lutsey, P. L., Mackey, J. S., Matchar, D. B., Matsushita, K., Mussolino, M. E., Nasir, K., O'Flaherty, M., Palaniappan, L. P., Pandey, A., Pandey, D. K., Reeves, M. J., Ritchey, M. D., Rodriguez, C. J., Roth, G. A., Rosamond, W. D., Sampson, U. K. A., Satou, G. M., Shah, S. H., Spartano, N. L., Tirschwell, D. L., Tsao, C. W., Voeks, J. H., Willey, J. Z., Wilkins, J. T., Wu, J. H., Alger, H. M., Wong, S. S., and Muntner, P. (2018) Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. *Circulation* **137**, e67-e492
2. Feigin, V. L., Norrving, B., and Mensah, G. A. (2017) Global Burden of Stroke. *Circulation research* **120**, 439-448
3. (2018) Global, Regional, and Country-Specific Lifetime Risks of Stroke, 1990 and 2016. *New England Journal of Medicine* **379**, 2429-2437
4. Feigin, V. L., Norrving, B., George, M. G., Foltz, J. L., Roth, G. A., and Mensah, G. A. (2016) Prevention of stroke: a strategic global imperative. *Nature reviews. Neurology* **12**, 501-512
5. Chen, H., Yoshioka, H., Kim, G. S., Jung, J. E., Okami, N., Sakata, H., Maier, C. M., Narasimhan, P., Goeders, C. E., and Chan, P. H. (2011) Oxidative stress in ischemic brain damage: mechanisms of cell death and potential molecular targets for neuroprotection. *Antioxidants & redox signaling* **14**, 1505-1517
6. Rodrigo, R., Fernandez-Gajardo, R., Gutierrez, R., Matamala, J. M., Carrasco, R., Miranda-Merchak, A., and Feuerhake, W. (2013) Oxidative stress and pathophysiology of ischemic stroke: novel therapeutic opportunities. *CNS & neurological disorders drug targets* **12**, 698-714
7. Li, P., Stetler, R. A., Leak, R. K., Shi, Y., Li, Y., Yu, W., Bennett, M. V. L., and Chen, J. (2018) Oxidative stress and DNA damage after cerebral ischemia: Potential therapeutic targets to repair the genome and improve stroke recovery. *Neuropharmacology* **134**, 208-217
8. Kritis, A. A., Stamoula, E. G., Paniskaki, K. A., and Vavilis, T. D. (2015) Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/study. *Frontiers in cellular neuroscience* **9**, 91
9. Drieu, A., Levard, D., Vivien, D., and Rubio, M. (2018) Anti-inflammatory treatments for stroke: from bench to bedside. *Therapeutic advances in neurological disorders* **11**, 1756286418789854
10. Fu, Y., Liu, Q., Anrather, J., and Shi, F. D. (2015) Immune interventions in stroke. *Nature reviews. Neurology* **11**, 524-535
11. Yang, J. L., Mukda, S., and Chen, S. D. (2018) Diverse roles of mitochondria in ischemic stroke. *Redox biology* **16**, 263-275
12. Tudek, B., Zdzalik-Bielecka, D., Tudek, A., Kosicki, K., Fabisiewicz, A., and Speina, E. (2017) Lipid peroxidation in face of DNA damage, DNA repair and other cellular processes. *Free radical biology & medicine* **107**, 77-89

13. Yang, J. L., Tadokoro, T., Keijzers, G., Mattson, M. P., and Bohr, V. A. (2010) Neurons efficiently repair glutamate-induced oxidative DNA damage by a process involving CREB-mediated up-regulation of apurinic endonuclease 1. *The Journal of biological chemistry* **285**, 28191-28199
14. Martin, L., Rainey, M., Santocanale, C., and Gardner, L. B. (2012) Hypoxic activation of ATR and the suppression of the initiation of DNA replication through cdc6 degradation. *Oncogene* **31**, 4076-4084
15. Moreno, N. C., Garcia, C. C. M., Rocha, C. R. R., Munford, V., and Menck, C. F. M. (2019) ATR/Chk1 Pathway is Activated by Oxidative Stress in Response to UVA Light in Human Xeroderma Pigmentosum Variant Cells. *Photochemistry and photobiology* **95**, 345-354
16. Yan, S., Sorrell, M., and Berman, Z. (2014) Functional interplay between ATM/ATR-mediated DNA damage response and DNA repair pathways in oxidative stress. *Cellular and molecular life sciences : CMLS* **71**, 3951-3967
17. Cimprich, K. A., and Cortez, D. (2008) ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* **9**, 616-627
18. Zeman, M. K., and Cimprich, K. A. (2014) Causes and consequences of replication stress. *Nature cell biology* **16**, 2-9
19. Hilton, B. A., Li, Z., Musich, P. R., Wang, H., Cartwright, B. M., Serrano, M., Zhou, X. Z., Lu, K. P., and Zou, Y. (2016) ATR Plays a Direct Antiapoptotic Role at Mitochondria, which Is Regulated by Prolyl Isomerase Pin1. *Molecular cell* **61**, 487
20. Wu, X., Shell, S. M., Yang, Z., and Zou, Y. (2006) Phosphorylation of nucleotide excision repair factor xeroderma pigmentosum group A by ataxia telangiectasia mutated and Rad3-related-dependent checkpoint pathway promotes cell survival in response to UV irradiation. *Cancer research* **66**, 2997-3005
21. Zou, L., and Elledge, S. J. (2003) Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science (New York, N.Y.)* **300**, 1542-1548
22. Kemp, M. G., and Sancar, A. (2016) ATR Kinase Inhibition Protects Non-cycling Cells from the Lethal Effects of DNA Damage and Transcription Stress. *The Journal of biological chemistry* **291**, 9330-9342
23. Beyaert, M., Starczewska, E., Perez, A. C. G., Vanlangendonck, N., Saussoy, P., Tilman, G., De Leener, A., Vekemans, M. C., Van Den Neste, E., and Bontemps, F. (2017) Reevaluation of ATR signaling in primary resting chronic lymphocytic leukemia cells: evidence for pro-survival or pro-apoptotic function. *Oncotarget* **8**, 56906-56920
24. Brown, E. J., and Baltimore, D. (2003) Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes & development* **17**, 615-628
25. Myers, K., Gagou, M. E., Zuazua-Villar, P., Rodriguez, R., and Meuth, M. (2009) ATR and Chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. *PLoS genetics* **5**, e1000324
26. Sidi, S., Sanda, T., Kennedy, R. D., Hagen, A. T., Jette, C. A., Hoffmans, R., Pascual, J., Imamura, S., Kishi, S., Amatruda, J. F., Kanki, J. P., Green, D. R., D'Andrea, A. A., and Look, A. T. (2008) Chk1 suppresses a caspase-2 apoptotic response to DNA damage that bypasses p53, Bcl-2, and caspase-3. *Cell* **133**, 864-877
27. Murugan, M., Ling, E. A., and Kaur, C. (2013) Glutamate receptors in microglia. *CNS & neurological disorders drug targets* **12**, 773-784

28. Rueda, C. B., Llorente-Folch, I., Traba, J., Amigo, I., Gonzalez-Sanchez, P., Contreras, L., Juaristi, I., Martinez-Valero, P., Pardo, B., Del Arco, A., and Satrustegui, J. (2016) Glutamate excitotoxicity and Ca2+-regulation of respiration: Role of the Ca2+ activated mitochondrial transporters (CaMCs). *Biochimica et biophysica acta* **1857**, 1158-1166
29. Cheung, Y. T., Lau, W. K., Yu, M. S., Lai, C. S., Yeung, S. C., So, K. F., and Chang, R. C. (2009) Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology* **30**, 127-135
30. Chao, C. C., Kan, D., Lo, T. H., Lu, K. S., and Chien, C. L. (2015) Induction of neural differentiation in rat C6 glioma cells with taxol. *Brain and behavior* **5**, e00414
31. Bologna-Molina, R., Mosqueda-Taylor, A., Molina-Frechero, N., Mori-Estevez, A.-D., and Sánchez-Acuña, G. (2012) Comparison of the value of PCNA and Ki-67 as markers of cell proliferation in ameloblastic tumors. *Med Oral Patol Oral Cir Bucal* **18**, e174-e179
32. Gwag, B. J., Canzoniero, L. M., Sensi, S. L., Demaro, J. A., Koh, J. Y., Goldberg, M. P., Jacquin, M., and Choi, D. W. (1999) Calcium ionophores can induce either apoptosis or necrosis in cultured cortical neurons. *Neuroscience* **90**, 1339-1348
33. Van Houten, B., Santa-Gonzalez, G. A., and Camargo, M. (2018) DNA repair after oxidative stress: current challenges. *Current opinion in toxicology* **7**, 9-16
34. Narciso, L., Parlanti, E., Racaniello, M., Simonelli, V., Cardinale, A., Merlo, D., and Dogliotti, E. (2016) The Response to Oxidative DNA Damage in Neurons: Mechanisms and Disease. *Neural plasticity* **2016**, 3619274
35. Beckerman, R., and Prives, C. (2010) Transcriptional regulation by p53. *Cold Spring Harb Perspect Biol* **2**, a000935-a000935
36. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes & development* **13**, 152-157
37. Maréchal, A., and Zou, L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol* **5**, a012716
38. Guo, Z., Deshpande, R., and Paull, T. T. (2010) ATM activation in the presence of oxidative stress. *Cell Cycle* **9**, 4805-4811
39. Kodama, M., Otsubo, C., Hirota, T., Yokota, J., Enari, M., and Taya, Y. (2010) Requirement of ATM for rapid p53 phosphorylation at Ser46 without Ser/Thr-Gln sequences. *Mol Cell Biol* **30**, 1620-1633
40. Wallace, B. D., Berman, Z., Mueller, G. A., Lin, Y., Chang, T., Andres, S. N., Wojtaszek, J. L., DeRose, E. F., Appel, C. D., London, R. E., Yan, S., and Williams, R. S. (2017) APE2 Zf-GRF facilitates 3'-5' resection of DNA damage following oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 304-309
41. Willis, J., Patel, Y., Lentz, B. L., and Yan, S. (2013) APE2 is required for ATR-Chk1 checkpoint activation in response to oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 10592-10597
42. Lin, Y., Bai, L., Cupello, S., Hossain, M. A., Deem, B., McLeod, M., Raj, J., and Yan, S. (2018) APE2 promotes DNA damage response pathway from a single-strand break. *Nucleic acids research* **46**, 2479-2494
43. Brooks, P. J., Wise, D. S., Berry, D. A., Kosmoski, J. V., Smerdon, M. J., Somers, R. L., Mackie, H., Spoonde, A. Y., Ackerman, E. J., Coleman, K., Tarone, R. E., and Robbins,

J. H. (2000) The oxidative DNA lesion 8,5'-(S)-cyclo-2'-deoxyadenosine is repaired by the nucleotide excision repair pathway and blocks gene expression in mammalian cells. *The Journal of biological chemistry* **275**, 22355-22362
44. Menoni, H., Hoeijmakers, J. H., and Vermeulen, W. (2012) Nucleotide excision repair-initiating proteins bind to oxidative DNA lesions in vivo. *The Journal of cell biology* **199**, 1037-1046
45. Melis, J. P., van Steeg, H., and Luijten, M. (2013) Oxidative DNA damage and nucleotide excision repair. *Antioxidants & redox signaling* **18**, 2409-2419
46. Maddukuri, L., Speina, E., Christiansen, M., Dudzinska, D., Zaim, J., Obtulowicz, T., Kabaczyk, S., Komisarski, M., Bukowy, Z., Szczegielniak, J., Wojcik, A., Kusmierek, J. T., Stevnsner, T., Bohr, V. A., and Tudek, B. (2009) Cockayne syndrome group B protein is engaged in processing of DNA adducts of lipid peroxidation product trans-4-hydroxy-2-nonenal. *Mutation research* **666**, 23-31
47. Tuttolomondo, A., Di Sciacca, R., Di Raimondo, D., Arnao, V., Renda, C., Pinto, A., and Licata, G. (2009) Neuron protection as a therapeutic target in acute ischemic stroke. *Current topics in medicinal chemistry* **9**, 1317-1334
48. Chao, C., Herr, D., Chun, J., and Xu, Y. (2006) Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. *The EMBO journal* **25**, 2615-2622
49. Grønberg, N. V., Johansen, F. F., Kristiansen, U., and Hasseldam, H. (2013) Leukocyte infiltration in experimental stroke. *J Neuroinflammation* **10**, 115-115
50. Brown, G. C., and Vilalta, A. (2015) How microglia kill neurons. *Brain research* **1628**, 288-297
51. Cheng, A., Zhao, T., Tse, K. H., Chow, H. M., Cui, Y., Jiang, L., Du, S., Loy, M. M. T., and Herrup, K. (2018) ATM and ATR play complementary roles in the behavior of excitatory and inhibitory vesicle populations. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E292-e301
52. Gilby, K. L., Sydserff, S. G., and Robertson, H. A. (2005) Differential neuroprotective effects for three GABA-potentiating compounds in a model of hypoxia-ischemia. *Brain research* **1035**, 196-205
53. Ravindran, J., Shuaib, A., Ijaz, S., Galazka, P., Waqar, T., Ishaqzay, R., Miyashita, H., and Liu, L. (1994) High extracellular GABA levels in hippocampus--as a mechanism of neuronal protection in cerebral ischemia in adrenalectomized gerbils. *Neuroscience letters* **176**, 209-211
54. Shuaib, A., Mazagri, R., and Ijaz, S. (1993) GABA agonist "muscimol" is neuroprotective in repetitive transient forebrain ischemia in gerbils. *Experimental neurology* **123**, 284-288
55. Burdak-Rothkamm, S., Rothkamm, K., and Prise, K. M. (2008) ATM acts downstream of ATR in the DNA damage response signaling of bystander cells. *Cancer research* **68**, 7059-7065
56. Burdak-Rothkamm, S., Short, S. C., Folkard, M., Rothkamm, K., and Prise, K. M. (2007) ATR-dependent radiation-induced gamma H2AX foci in bystander primary human astrocytes and glioma cells. *Oncogene* **26**, 993-1002
57. Wang, Y. (2008) Bulky DNA lesions induced by reactive oxygen species. *Chemical research in toxicology* **21**, 276-281

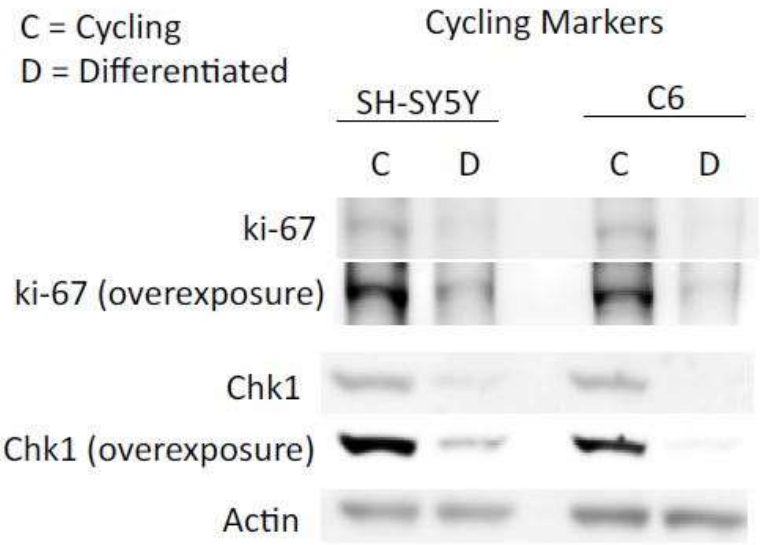

Figure 3.S1: C6 and SH-SY5Y are appropriately differentiated into a non-cycling state Western confirming entrance into a non-cycling state for both all-*trans* retinoic acid treated SH-SY5Y and paclitaxel treated C6. Depletion of Ki-67 and Chk1 are both noted on cell cycle exit and entrance into a non-cycling state.

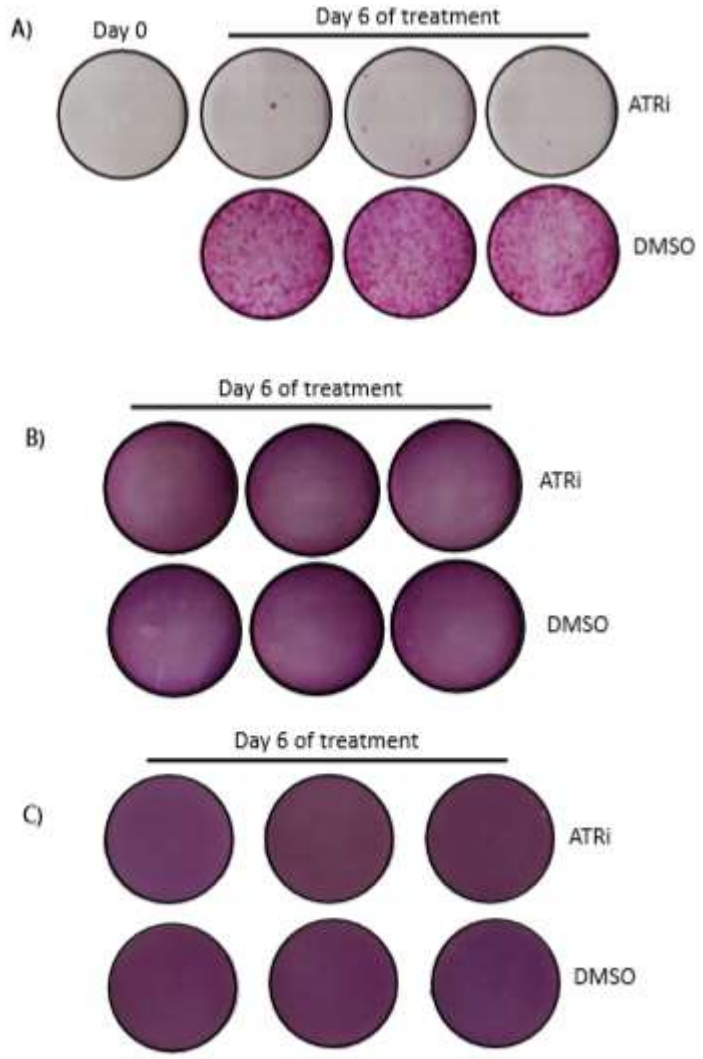

Figure 3.S2: ATR inhibition prevents colony formation in cycling HMC3 cells, but has no effect on the viability of differentiated C6 or SH-SY5Y
A) HMC3 cells were plated, allowed to attach, and then treated for six days with ATR inhibitor (80nM VE-822). Colonies developed in the control (DMSO) group, while few were present in the inhibitor group. B & C) Taxol-differentiated C6 and RA-differentiated SH-SY5Y show no difference in viability after six days of ATR inhibitor treatment. This viability assay is based on crystal violet staining.

CHAPTER 4

OVERALL DISCUSSION

Each day cells are assaulted by an assortment of intrinsic and extrinsic genomic insults. As a result, cells must respond by either repairing the damage done or by initiating cell death in order to maintain overall organismal integrity. One of the proteins involved in this response, ATR, has been shown, both in the enclosed studies and others, to have regulatory roles at multiple points within these response pathways (Cimprich and Cortez 2008; Hilton et al. 2016; Wu et al. 2006; Zeman and Cimprich 2014; Zou and Elledge 2003). These studies help to further define the role of ATR in the regulation of cell death and provide potential avenues for further investigation into how ATR structural changes and kinase modulation could impact clinical outcomes.

Implications of sterically locking ATR into a single prolyl isomer

Previous findings from our lab implicate prolyl isomerization in ATR's anti-apoptotic activity (Hilton et al. 2016). In the studies presented in Chapter 2, we expand on those findings to show that the ability to shift between prolyl isoforms is essential to organismal development and that possessing one prolyl isoform over another may even predispose an organism to cancer development.

By using mouse models expressing locked prolyl isoforms in the cytoplasm, we show that when ATR is restricted to its lower form, ATR-L (*trans*-ATR, ATR-P432A/P432A), that the organism is unable to develop. This is shown through controlled breeding between mice heterozygous for the prolyl isomer-locked ATR-L form. During this study, no offspring were found, either postnatally or at E13.5, to be of the homozygous ATR-L genotype leading us to

conclude that this mutation was likely embryonically lethal.

In contrast, ATR locked into its higher form, ATR-H (*cis*-ATR, ATR-S431A/S431A), are embryonically viable. Additionally, heterozygous ATR-H mice appear to be prone to spontaneous lymphoma development when compared to both ATR-L heterozygotes and to ATR-wt controls. This incidence appears to be sex-dependent as only males were found to possess potential lymphomas.

Interestingly, females of both lines exhibited evidence of reproductive stress as indicated by cystic ovaries and/or inflamed uterine horns. These findings typically coincided with increased Peyer's patch size which could indicate a global inflammatory phenotype. This could lend an explanation to both the findings of increased reproductive stress as well as the lymphoma see in some of the ATR-H males.

These results suggest that ATR prolyl isomerization can affect a wide variety of cellular and organismal processes. Further mechanistic studies into how each prolyl isoform can impact these processes need to be performed in order to truly tease apart the function of each independent isoform. Additionally, studies involving exogenous stressors, such as applied UV-irradiation should be used to determine whether these prolyl isoforms have separate functions only under basal conditions or if they can function to independently influence DNA repair, apoptosis, DNA damage signaling, or DNA damage driven tumorigenesis.

Potential for ATR-inhibition based therapeutic intervention in ischemic disease

It is well established that ATR inhibition in cycling cells leads to cell death through increased replication stress and subsequent DNA damage (Brown and Baltimore 2003; Cimprich and Cortez 2008; Kemp and Sancar 2016; Ruzankina et al. 2007; Zeman and Cimprich 2014); however, recent reports have implicated that ATR inhibition is protective in non-cycling cells

exposed to UV-irradiation (Kemp and Sancar 2016). To determine if these findings were limited to UV-irradiation or if they could be extrapolated to other types of clinically relevant DNA damage, we used cell lines mimicking cycling neuronal immune cells (HMC3) and non-cycling neuronal cells (differentiated C6 and SH-SY5Y).

When exposed to treatments simulating ischemic stroke and oxidative DNA damaging conditions, cycling cells were found to have increased cell death in the presence of ATR inhibition whereas non-cycling cells were partially protected from these insults. These results indicate that inhibition of ATR following ischemia-induced damage could serve as a therapeutic target post-stroke. The reason behind this is two-fold. First, ATR inhibition causes neuronal sparing following ischemic insult or oxidative damage. This would lead to primary protection of the neurons themselves. Secondly, ATR inhibition induces cell death in cycling immune cells which could potentially suppress the hyperinflammatory, pro-death, state post-ischemic/oxidative insult. This would prevent further insult to already damaged neurons allowing more neurons to be spared.

Additionally, this study addresses the mechanistic underpinnings of ATR activation in these non-cycling neuronal cells. While replication stress is the major known initiator of ATR signaling in cycling cells exposed to oxidative DNA damage, less is known about ATR activation in non-cycling cells. Previous reports have implicated both BER and NER pathways in this activation (Brooks et al. 2000; Lin et al. 2018; Melis et al. 2013; Menoni et al. 2012; Wallace et al. 2017; Willis et al. 2013; Yan et al. 2014). To determine which pathway was involved, we inhibited both at their initial steps of resection either through chemical or siRNA-mediated means. It was found that both pathways were involved, and that no singular DNA damage response pathway was responsible for ATR activation post oxidative insult.

These findings implicate that modulation of ATR signaling following ischemic stroke could be potentially beneficial; however, further investigation in animal models of ischemic stroke needs to be performed to confirm the true therapeutic potential of ATR inhibition. Additionally, these studies only address ATR activation and apoptosis following oxidative insult. Further exploratory studies need to be done in order to determine how DNA repair is affected and if the neurons spared in this way are functionally viable.

REFERENCES

Alderton GK, Joenje H, Varon R, Borglum AD, Jeggo PA, O'Driscoll M. 2004. Seckel syndrome exhibits cellular features demonstrating defects in the atr-signalling pathway. Human molecular genetics. 13(24):3127-3138.

Bartek J, Lukas J. 2003. Chk1 and chk2 kinases in checkpoint control and cancer. Cancer cell. 3(5):421-429.

Beckerman R, Prives C. 2010. Transcriptional regulation by p53. Cold Spring Harb Perspect Biol. 2(8):a000935-a000935.

Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, Chiuve SE, Cushman M, Delling FN, Deo R et al. 2018. Heart disease and stroke statistics-2018 update: A report from the american heart association. Circulation. 137(12):e67-e492.

Beyaert M, Starczewska E, Perez ACG, Vanlangendonck N, Saussoy P, Tilman G, De Leener A, Vekemans MC, Van Den Neste E, Bontemps F. 2017. Reevaluation of atr signaling in primary resting chronic lymphocytic leukemia cells: Evidence for pro-survival or pro-apoptotic function. Oncotarget. 8(34):56906-56920.

Bologna-Molina R, Mosqueda-Taylor A, Molina-Frechero N, Mori-Estevez A-D, Sánchez-Acuña G. 2012. Comparison of the value of pcna and ki-67 as markers of cell proliferation in ameloblastic tumors. Med Oral Patol Oral Cir Bucal. 18(2):e174-e179.

Brady CA, Attardi LD. 2010. P53 at a glance. Journal of cell science. 123(Pt 15):2527-2532.

Brooks PJ, Wise DS, Berry DA, Kosmoski JV, Smerdon MJ, Somers RL, Mackie H, Spoonde AY, Ackerman EJ, Coleman K et al. 2000. The oxidative DNA lesion 8,5'-(s)-cyclo-2'-deoxyadenosine is repaired by the nucleotide excision repair pathway and blocks gene expression in mammalian cells. The Journal of biological chemistry. 275(29):22355-22362.

Brown EJ, Baltimore D. 2000. Atr disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev. 14(4):397-402.

Brown EJ, Baltimore D. 2003. Essential and dispensable roles of atr in cell cycle arrest and genome maintenance. Genes & development. 17(5):615-628.

Brown GC, Vilalta A. 2015. How microglia kill neurons. Brain research. 1628(Pt B):288-297.

Burdak-Rothkamm S, Rothkamm K, Prise KM. 2008. Atm acts downstream of atr in the DNA damage response signaling of bystander cells. Cancer research. 68(17):7059-7065.

Burdak-Rothkamm S, Short SC, Folkard M, Rothkamm K, Prise KM. 2007. Atr-dependent radiation-induced gamma h2ax foci in bystander primary human astrocytes and glioma cells. Oncogene. 26(7):993-1002.

Carroll BL, Pulkoski-Gross MJ, Hannun YA, Obeid LM. 2016. Chk1 regulates nf-kappab signaling upon DNA damage in p53- deficient cells and associated tumor-derived microvesicles. Oncotarget. 7(14):18159-18170.

Chaki M, Airik R, Ghosh AK, Giles RH, Chen R, Slaats GG, Wang H, Hurd TW, Zhou W, Cluckey A et al. 2012. Exome capture reveals znf423 and cep164 mutations, linking renal ciliopathies to DNA damage response signaling. Cell. 150(3):533-548.

Chao C, Herr D, Chun J, Xu Y. 2006. Ser18 and 23 phosphorylation is required for p53-dependent apoptosis and tumor suppression. The EMBO journal. 25(11):2615-2622.

Chao CC, Kan D, Lo TH, Lu KS, Chien CL. 2015. Induction of neural differentiation in rat c6 glioma cells with taxol. Brain and behavior. 5(12):e00414.

Chen CF, Ruiz-Vega R, Vasudeva P, Espitia F, Krasieva TB, de Feraudy S, Tromberg BJ, Huang S, Garner CP, Wu J et al. 2017. Atr mutations promote the growth of melanoma tumors by modulating the immune microenvironment. Cell reports. 18(10):2331-2342.
Chen H, Yoshioka H, Kim GS, Jung JE, Okami N, Sakata H, Maier CM, Narasimhan P, Goeders CE, Chan PH. 2011. Oxidative stress in ischemic brain damage: Mechanisms of cell death and potential molecular targets for neuroprotection. Antioxidants & redox signaling. 14(8):1505-1517.
Cheng A, Zhao T, Tse KH, Chow HM, Cui Y, Jiang L, Du S, Loy MMT, Herrup K. 2018. Atm and atr play complementary roles in the behavior of excitatory and inhibitory vesicle populations. Proceedings of the National Academy of Sciences of the United States of America. 115(2):E292-e301.
Cheung YT, Lau WK, Yu MS, Lai CS, Yeung SC, So KF, Chang RC. 2009. Effects of all-trans-retinoic acid on human sh-sy5y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology. 30(1):127-135.
Chipuk JE, Bouchier-Hayes L, Kuwana T, Newmeyer DD, Green DR. 2005. Puma couples the nuclear and cytoplasmic proapoptotic function of p53. Science (New York, NY). 309(5741):1732-1735.
Chodankar R, Kwang S, Sangiorgi F, Hong H, Yen H-Y, Deng C, Pike MC, Shuler CF, Maxson R, Dubeau L. 2005. Cell-nonautonomous induction of ovarian and uterine serous cystadenomas in mice lacking a functional brca1 in ovarian granulosa cells. Current Biology. 15(6):561-565.
Choi HJC, Lin J-R, Vannier J-B, Slaats GG, Kile AC, Paulsen RD, Manning DK, Beier DR, Giles RH, Boulton SJ et al. 2013. Nek8 links the atr-regulated replication stress response and s phase cdk activity to renal ciliopathies. Mol Cell. 51(4):423-439.
Ciccia A, Elledge SJ. 2010. The DNA damage response: Making it safe to play with knives. Molecular cell. 40(2):179-204.
Cimprich KA, Cortez D. 2008. Atr: An essential regulator of genome integrity. Nat Rev Mol Cell Biol. 9(8):616-627.
Curtin N, Pollard J. 2018. Targeting atr for cancer therapy: Profile and expectations for atr inhibitors. In: Pollard J, Curtin N, editors. Targeting the DNA damage response for anti-cancer therapy. Cham: Springer International Publishing. p. 63-97.
de Groat WC, Griffiths D, Yoshimura N. 2015. Neural control of the lower urinary tract. Compr Physiol. 5(1):327-396.
de Klein A, Muijtjens M, van Os R, Verhoeven Y, Smit B, Carr AM, Lehmann AR, Hoeijmakers JH. 2000. Targeted disruption of the cell-cycle checkpoint gene atr leads to early embryonic lethality in mice. Current biology : CB. 10(8):479-482.
Dimitrios C, George AA, Dimosthenis Z, Nikolaos X. 2008. Intestinal obstruction due to an anomalous congenital band. Saudi J Gastroenterol. 14(1):36-37.
Drieu A, Levard D, Vivien D, Rubio M. 2018. Anti-inflammatory treatments for stroke: From bench to bedside. Therapeutic advances in neurological disorders. 11:1756286418789854.
Dunphy G, Flannery SM, Almine JF, Connolly DJ, Paulus C, Jonsson KL, Jakobsen MR, Nevels MM, Bowie AG, Unterholzner L. 2018. Non-canonical activation of the DNA sensing adaptor sting by atm and ifi16 mediates nf-kappab signaling after nuclear DNA damage. Molecular cell. 71(5):745-760.e745.

Durocher F, Labrie Y, Soucy P, Sinilnikova O, Labuda D, Bessette P, Chiquette J, Laframboise R, Lepine J, Lesperance B et al. 2006. Mutation analysis and characterization of atr sequence variants in breast cancer cases from high-risk french canadian breast/ovarian cancer families. BMC cancer. 6:230.
Enriquez-Rios V, Dumitrache LC, Downing SM, Li Y, Brown EJ, Russell HR, McKinnon PJ. 2017. DNA-pkcs, atm, and atr interplay maintains genome integrity during neurogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience. 37(4):893-905.
Falck J, Mailand N, Syljuasen RG, Bartek J, Lukas J. 2001. The atm-chk2-cdc25a checkpoint pathway guards against radioresistant DNA synthesis. Nature. 410(6830):842-847.
Feigin VL, Norrving B, George MG, Foltz JL, Roth GA, Mensah GA. 2016. Prevention of stroke: A strategic global imperative. Nature reviews Neurology. 12(9):501-512.
Feigin VL, Norrving B, Mensah GA. 2017. Global burden of stroke. Circulation research. 120(3):439-448.
Fromme JC, Verdine GL. 2004. Base excision repair. Advances in protein chemistry. Academic Press. p. 1-41.
Fu M, Landreville S, Agapova OA, Wiley LA, Shoykhet M, Harbour JW, Heuckeroth RO. 2013. Retinoblastoma protein prevents enteric nervous system defects and intestinal pseudo-obstruction. J Clin Invest. 123(12):5152-5164.
Fu Y, Liu Q, Anrather J, Shi FD. 2015. Immune interventions in stroke. Nature reviews Neurology. 11(9):524-535.
Gaillard H, Aguilera A. 2013. Transcription coupled repair at the interface between transcription elongation and mrnp biogenesis. Biochimica et biophysica acta. 1829(1):141-150.
Gaillard PHL, Wood RD. 2001. Activity of individual ercc1 and xpf subunits in DNA nucleotide excision repair. Nucleic acids research. 29(4):872-879.
Gilad O, Nabet BY, Ragland RL, Schoppy DW, Smith KD, Durham AC, Brown EJ. 2010. Combining atr suppression with oncogenic ras synergistically increases genomic instability, causing synthetic lethality or tumorigenesis in a dosage-dependent manner. Cancer Res. 70(23):9693-9702.
Gilby KL, Sydserff SG, Robertson HA. 2005. Differential neuroprotective effects for three gaba-potentiating compounds in a model of hypoxia-ischemia. Brain research. 1035(2):196-205.
Gilhar A, Schrum AG, Etzioni A, Waldmann H, Paus R. 2016. Alopecia areata: Animal models illuminate autoimmune pathogenesis and novel immunotherapeutic strategies. Autoimmunity Reviews. 15(7):726-735.
Global, regional, and country-specific lifetime risks of stroke, 1990 and 2016. 2018. New England Journal of Medicine. 379(25):2429-2437.
Grønberg NV, Johansen FF, Kristiansen U, Hasseldam H. 2013. Leukocyte infiltration in experimental stroke. J Neuroinflammation. 10:115-115.
Guo Z, Deshpande R, Paull TT. 2010. Atm activation in the presence of oxidative stress. Cell Cycle. 9(24):4805-4811.
Gwag BJ, Canzoniero LM, Sensi SL, Demaro JA, Koh JY, Goldberg MP, Jacquin M, Choi DW. 1999. Calcium ionophores can induce either apoptosis or necrosis in cultured cortical neurons. Neuroscience. 90(4):1339-1348.
Härtlova A, Erttmann SF, Raffi FAM, Schmalz AM, Resch U, Anugula S, Lienenklaus S, Nilsson LM, Kröger A, Nilsson JA et al. 2015. DNA damage primes the type i interferon

system via the cytosolic DNA sensor sting to promote anti-microbial innate immunity. Immunity. 42(2):332-343.
Harvey SL, Charlet A, Haas W, Gygi SP, Kellogg DR. 2005. Cdk1-dependent regulation of the mitotic inhibitor wee1. Cell. 122(3):407-420.
Hill WG. 2015. Control of urinary drainage and voiding. Clin J Am Soc Nephrol. 10(3):480-492.
Hilton BA, Li Z, Musich PR, Wang H, Cartwright BM, Serrano M, Zhou XZ, Lu KP, Zou Y. 2016. Atr plays a direct antiapoptotic role at mitochondria, which is regulated by prolyl isomerase pin1. Molecular cell. 61(3):487.
Ho D, Zhao X, Gao S, Hong C, Vatner DE, Vatner SF. 2011. Heart rate and electrocardiography monitoring in mice. Curr Protoc Mouse Biol. 1:123-139.
Hou XH, Hyun M, Taranda J, Huang KW, Todd E, Feng D, Atwater E, Croney D, Zeidel ML, Osten P et al. 2016. Central control circuit for context-dependent micturition. Cell. 167(1):73-86.e12.
Imanishi S, Umezu T, Ohtsuki K, Kobayashi C, Ohyashiki K, Ohyashiki JH. 2014. Constitutive activation of the atm/brca1 pathway prevents DNA damage-induced apoptosis in 5-azacytidine-resistant cell lines. Biochemical pharmacology. 89(3):361-369.
Janku F, Kaseb AO, Tsimberidou AM, Wolff RA, Kurzrock R. 2014. Identification of novel therapeutic targets in the pi3k/akt/mtor pathway in hepatocellular carcinoma using targeted next generation sequencing. Oncotarget. 5(10):3012-3022.
Jung C, Hugot J-P, Barreau F, #233, #233, rick. 2010. Peyer's patches: The immune sensors of the intestine. International Journal of Inflammation. 2010.
Karikkineth AC, Scheibye-Knudsen M, Fivenson E, Croteau DL, Bohr VA. 2017. Cockayne syndrome: Clinical features, model systems and pathways. Ageing research reviews. 33:3-17.
Kelly CC, Lyall H, Petrie JR, Gould GW, Connell JM, Sattar N. 2001. Low grade chronic inflammation in women with polycystic ovarian syndrome. The Journal of clinical endocrinology and metabolism. 86(6):2453-2455.
Kemp MG, Sancar A. 2016. Atr kinase inhibition protects non-cycling cells from the lethal effects of DNA damage and transcription stress. The Journal of biological chemistry. 291(17):9330-9342.
Kim CH. 2010. Homeostatic and pathogenic extramedullary hematopoiesis. J Blood Med. 1:13-19.
Knauf Y, Köhler K, Knauf S, Wehrend A. 2018. Histological classification of canine ovarian cyst types with reference to medical history. J Vet Sci. 19(6):725-734.
Kodama M, Otsubo C, Hirota T, Yokota J, Enari M, Taya Y. 2010. Requirement of atm for rapid p53 phosphorylation at ser46 without ser/thr-gln sequences. Mol Cell Biol. 30(7):1620-1633.
Korwek Z, Sewastianik T, Bielak-Zmijewska A, Mosieniak G, Alster O, Moreno-Villanueva M, Burkle A, Sikora E. 2012. Inhibition of atm blocks the etoposide-induced DNA damage response and apoptosis of resting human t cells. DNA repair. 11(11):864-873.
Kritis AA, Stamoula EG, Paniskaki KA, Vavilis TD. 2015. Researching glutamate - induced cytotoxicity in different cell lines: A comparative/collective analysis/study. Frontiers in cellular neuroscience. 9:91.
Lang PY, Nanjangud GJ, Sokolsky-Papkov M, Shaw C, Hwang D, Parker JS, Kabanov AV, Gershon TR. 2016. Atr maintains chromosomal integrity during postnatal cerebellar

neurogenesis and is required for medulloblastoma formation. Development (Cambridge, England). 143(21):4038-4052.
Lee B, Lee HJ, Cho HY, Suh DH, Kim K, No JH, Kim H, Kim YB. 2015. Ataxia-telangiectasia and rad3-related and ataxia-telangiectasia-mutated proteins in epithelial ovarian carcinoma: Their expression and clinical significance. Anticancer research. 35(7):3909-3916.
Lee HJ, Bahr JM, Bitterman P, Basu S, Sharma S, Abramowicz JS, Barua A. 2018. Polycystic ovarian condition may be a risk factor for ovarian tumor development in the laying hen model of spontaneous ovarian cancer. J Immunol Res. 2018:2590910-2590910.
Lee TH, Park JM, Leem SH, Kang TH. 2014. Coordinated regulation of xpa stability by atr and herc2 during nucleotide excision repair. Oncogene. 33(1):19-25.
Lee Y, Shull ER, Frappart PO, Katyal S, Enriquez-Rios V, Zhao J, Russell HR, Brown EJ, McKinnon PJ. 2012. Atr maintains select progenitors during nervous system development. The EMBO journal. 31(5):1177-1189.
Leu JI, Dumont P, Hafey M, Murphy ME, George DL. 2004. Mitochondrial p53 activates bak and causes disruption of a bak-mcl1 complex. Nature cell biology. 6(5):443-450.
Li P, Stetler RA, Leak RK, Shi Y, Li Y, Yu W, Bennett MVL, Chen J. 2018. Oxidative stress and DNA damage after cerebral ischemia: Potential therapeutic targets to repair the genome and improve stroke recovery. Neuropharmacology. 134(Pt B):208-217.
Li Z, Musich PR, Cartwright BM, Wang H, Zou Y. 2013. Uv-induced nuclear import of xpa is mediated by importin-alpha4 in an atr-dependent manner. PloS one. 8(7):e68297.
Lin Y, Bai L, Cupello S, Hossain MA, Deem B, McLeod M, Raj J, Yan S. 2018. Ape2 promotes DNA damage response pathway from a single-strand break. Nucleic acids research. 46(5):2479-2494.
Maddukuri L, Speina E, Christiansen M, Dudzinska D, Zaim J, Obtulowicz T, Kabaczyk S, Komisarski M, Bukowy Z, Szczegielniak J et al. 2009. Cockayne syndrome group b protein is engaged in processing of DNA adducts of lipid peroxidation product trans-4-hydroxy-2-nonenal. Mutation research. 666(1-2):23-31.
Mailand N, Falck J, Lukas C, Syljuasen RG, Welcker M, Bartek J, Lukas J. 2000. Rapid destruction of human cdc25a in response to DNA damage. Science (New York, NY). 288(5470):1425-1429.
Maréchal A, Zou L. DNA damage sensing by the atm and atr kinases. Cold Spring Harb Perspect Biol. 5(9):a012716.
Martin L, Rainey M, Santocanale C, Gardner LB. 2012. Hypoxic activation of atr and the suppression of the initiation of DNA replication through cdc6 degradation. Oncogene. 31(36):4076-4084.
Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y et al. 2007. Atm and atr substrate analysis reveals extensive protein networks responsive to DNA damage. Science (New York, NY). 316(5828):1160-1166.
Maya R, Balass M, Kim ST, Shkedy D, Leal JF, Shifman O, Moas M, Buschmann T, Ronai Z, Shiloh Y et al. 2001. Atm-dependent phosphorylation of mdm2 on serine 395: Role in p53 activation by DNA damage. Genes & development. 15(9):1067-1077.
Melis JP, van Steeg H, Luijten M. 2013. Oxidative DNA damage and nucleotide excision repair. Antioxidants & redox signaling. 18(18):2409-2419.

Menolfi D, Jiang W, Lee BJ, Moiseeva T, Shao Z, Estes V, Frattini MG, Bakkenist CJ, Zha S. 2018. Kinase-dead atr differs from atr loss by limiting the dynamic exchange of atr and rpa. Nature Communications. 9(1):5351.
Menoni H, Hoeijmakers JH, Vermeulen W. 2012. Nucleotide excision repair-initiating proteins bind to oxidative DNA lesions in vivo. The Journal of cell biology. 199(7):1037-1046.
Militzer K, Wecker E. 1986. Behaviour-associated alopecia areata in mice. Laboratory animals. 20(1):9-13.
Moskalev AA, Shaposhnikov MV, Plyusnina EN, Zhavoronkov A, Budovsky A, Yanai H, Fraifeld VE. 2013. The role of DNA damage and repair in aging through the prism of koch-like criteria. Ageing Research Reviews. 12(2):661-684.
Moreno NC, Garcia CCM, Rocha CRR, Munford V, Menck CFM. 2019. Atr/chk1 pathway is activated by oxidative stress in response to uva light in human xeroderma pigmentosum variant cells. Photochemistry and photobiology. 95(1):345-354.
Mu D, Sancar A. 1997. Model for xpc-independent transcription-coupled repair of pyrimidine dimers in humans. The Journal of biological chemistry. 272(12):7570-7573.
Murga M, Bunting S, Montaña MF, Soria R, Mulero F, Cañamero M, Lee Y, McKinnon PJ, Nussenzweig A, Fernandez-Capetillo O. 2009. A mouse model of atr-seckel shows embryonic replicative stress and accelerated aging. Nat Genet. 41(8):891-898.
Murugan M, Ling EA, Kaur C. 2013. Glutamate receptors in microglia. CNS & neurological disorders drug targets. 12(6):773-784.
Myers JS, Cortez D. 2006. Rapid activation of atr by ionizing radiation requires atm and mre11. The Journal of biological chemistry. 281(14):9346-9350.
Myers K, Gagou ME, Zuazua-Villar P, Rodriguez R, Meuth M. 2009. Atr and chk1 suppress a caspase-3-dependent apoptotic response following DNA replication stress. PLoS genetics. 5(1):e1000324.
Narciso L, Parlanti E, Racaniello M, Simonelli V, Cardinale A, Merlo D, Dogliotti E. 2016. The response to oxidative DNA damage in neurons: Mechanisms and disease. Neural plasticity. 2016:3619274.
Ohnishi S, Ma N, Thanan R, Pinlaor S, Hammam O, Murata M, Kawanishi S. 2013. DNA damage in inflammation-related carcinogenesis and cancer stem cells. Oxid Med Cell Longev. 2013:387014-387014.
O'Donovan A, Davies AA, Moggs JG, West SC, Wood RD. 1994. Xpg endonuclease makes the 3' incision in human DNA nucleotide excision repair. Nature. 371(6496):432-435.
Papaioannou VE, Behringer RR. 2012. Early embryonic lethality in genetically engineered mice: Diagnosis and phenotypic analysis. Veterinary pathology. 49(1):64-70.
Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. 1997. Mitotic and g2 checkpoint control: Regulation of 14-3-3 protein binding by phosphorylation of cdc25c on serine-216. Science (New York, NY). 277(5331):1501-1505.
Rappen U, von Brenndorff AI. 1993. [cardiac symptoms in 2 patients with seckel syndrome]. Monatsschr Kinderheilkd. 141(7):584-586.
Ravindran J, Shuaib A, Ijaz S, Galazka P, Waqar T, Ishaqzay R, Miyashita H, Liu L. 1994. High extracellular gaba levels in hippocampus--as a mechanism of neuronal protection in cerebral ischemia in adrenalectomized gerbils. Neuroscience letters. 176(2):209-211.
Riley T, Sontag E, Chen P, Levine A. 2008. Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol. 9(5):402-412.

Rodrigo R, Fernandez-Gajardo R, Gutierrez R, Matamala JM, Carrasco R, Miranda-Merchak A, Feuerhake W. 2013. Oxidative stress and pathophysiology of ischemic stroke: Novel therapeutic opportunities. CNS & neurological disorders drug targets. 12(5):698-714.
Roghani T, Zavieh MK, Manshadi FD, King N, Katzman W. 2017. Age-related hyperkyphosis: Update of its potential causes and clinical impacts-narrative review. Aging Clin Exp Res. 29(4):567-577.
Rueda CB, Llorente-Folch I, Traba J, Amigo I, Gonzalez-Sanchez P, Contreras L, Juaristi I, Martinez-Valero P, Pardo B, Del Arco A et al. 2016. Glutamate excitotoxicity and ca2+-regulation of respiration: Role of the ca2+ activated mitochondrial transporters (camcs). Biochimica et biophysica acta. 1857(8):1158-1166.
Ruzankina Y, Pinzon-Guzman C, Asare A, Ong T, Pontano L, Cotsarelis G, Zediak VP, Velez M, Bhandoola A, Brown EJ. 2007. Deletion of the developmentally essential gene atr in adult mice leads to age-related phenotypes and stem cell loss. Cell stem cell. 1(1):113-126.
Sala M, Braida D, Lentini D, Busnelli M, Bulgheroni E, Capurro V, Finardi A, Donzelli A, Pattini L, Rubino T et al. 2011. Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: A neurobehavioral model of autism. Biological psychiatry. 69(9):875-882.
Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annual review of biochemistry. 73:39-85.
Savant SS, Sriramkumar S, O'Hagan HM. 2018. The role of inflammation and inflammatory mediators in the development, progression, metastasis, and chemoresistance of epithelial ovarian cancer. Cancers (Basel). 10(8):251.
Schärer OD. 2013. Nucleotide excision repair in eukaryotes. Cold Spring Harb Perspect Biol. 5(10):a012609-a012609.
Schulz O, Ugur M, Friedrichsen M, Radulovic K, Niess JH, Jalkanen S, Krueger A, Pabst O. 2013. Hypertrophy of infected peyer's patches arises from global, interferon-receptor, and cd69-independent shutdown of lymphocyte egress. Mucosal Immunology. 7:892.
Shuaib A, Mazagri R, Ijaz S. 1993. Gaba agonist "muscimol" is neuroprotective in repetitive transient forebrain ischemia in gerbils. Experimental neurology. 123(2):284-288.
Serrano MA, Li Z, Dangeti M, Musich PR, Patrick S, Roginskaya M, Cartwright B, Zou Y. 2013. DNA-pk, atm and atr collaboratively regulate p53-rpa interaction to facilitate homologous recombination DNA repair. Oncogene. 32(19):2452-2462.
Sidi S, Sanda T, Kennedy RD, Hagen AT, Jette CA, Hoffmans R, Pascual J, Imamura S, Kishi S, Amatruda JF et al. 2008. Chk1 suppresses a caspase-2 apoptotic response to DNA damage that bypasses p53, bcl-2, and caspase-3. Cell. 133(5):864-877.
Sirbu BM, Cortez D. 2013. DNA damage response: Three levels of DNA repair regulation. Cold Spring Harb Perspect Biol. 5(8):a012724.
Sorof JM, Dow-Smith C, Moore PJ. 1999. Severe hypertensive sequelae in a child with seckel syndrome (bird-like dwarfism). Pediatric Nephrology. 13(4):343-346.
Stiff T, Casar Tena T, O'Driscoll M, Jeggo PA, Philipp M. 2016. Atr promotes cilia signalling: Links to developmental impacts. Human molecular genetics. 25(8):1574-1587.
Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, O'Driscoll M, Jeggo PA. 2006. Atr-dependent phosphorylation and activation of atm in response to uv treatment or replication fork stalling. The EMBO journal. 25(24):5775-5782.

Stypmann J. 2007. Doppler ultrasound in mice. Echocardiography (Mount Kisco, NY). 24(1):97-112.
Sugasawa K, Ng JM, Masutani C, Iwai S, van der Spek PJ, Eker AP, Hanaoka F, Bootsma D, Hoeijmakers JH. 1998. Xeroderma pigmentosum group c protein complex is the initiator of global genome nucleotide excision repair. Molecular cell. 2(2):223-232.
Surova O, Zhivotovsky B. 2013. Various modes of cell death induced by DNA damage. Oncogene. 32(33):3789-3797.
Takahashi A, Miczek KA. 2014. Neurogenetics of aggressive behavior: Studies in rodents. Curr Top Behav Neurosci. 17:3-44.
Tanaka A, Weinel S, Nagy N, O'Driscoll M, Lai-Cheong JE, Kulp-Shorten CL, Knable A, Carpenter G, Fisher SA, Hiragun M et al. 2012. Germline mutation in atr in autosomal-dominant oropharyngeal cancer syndrome. American journal of human genetics. 90(3):511-517.
Tibbetts RS, Brumbaugh KM, Williams JM, Sarkaria JN, Cliby WA, Shieh SY, Taya Y, Prives C, Abraham RT. 1999. A role for atr in the DNA damage-induced phosphorylation of p53. Genes Dev. 13(2):152-157.
Tudek B, Zdzalik-Bielecka D, Tudek A, Kosicki K, Fabisiewicz A, Speina E. 2017. Lipid peroxidation in face of DNA damage, DNA repair and other cellular processes. Free radical biology & medicine. 107:77-89.
Tuttolomondo A, Di Sciacca R, Di Raimondo D, Arnao V, Renda C, Pinto A, Licata G. 2009. Neuron protection as a therapeutic target in acute ischemic stroke. Current topics in medicinal chemistry. 9(14):1317-1334.
Van Houten B, Santa-Gonzalez GA, Camargo M. 2018. DNA repair after oxidative stress: Current challenges. Current opinion in toxicology. 7:9-16.
Vaseva AV, Moll UM. 2009. The mitochondrial p53 pathway. Biochimica et biophysica acta. 1787(5):414-420.
Vaz R, Hofmeister W, Lindstrand A. 2019. Zebrafish models of neurodevelopmental disorders: Limitations and benefits of current tools and techniques. Int J Mol Sci. 20(6):1296.
Wagner SA, Oehler H, Voigt A, Dalic D, Freiwald A, Serve H, Beli P. 2016. Atr inhibition rewires cellular signaling networks induced by replication stress. Proteomics. 16(3):402-416.
Wallace BD, Berman Z, Mueller GA, Lin Y, Chang T, Andres SN, Wojtaszek JL, DeRose EF, Appel CD, London RE et al. 2017. Ape2 zf-grf facilitates 3'-5' resection of DNA damage following oxidative stress. Proceedings of the National Academy of Sciences of the United States of America. 114(2):304-309.
Wang-Heaton H. 2016. A novel role of human DNA damage checkpoint protein atr in suppressing ca2+ overload-induced parp1-mediated necrosis. East Tennessee State Univeristy.
Wang Y. 2008. Bulky DNA lesions induced by reactive oxygen species. Chemical research in toxicology. 21(2):276-281.
Weaver AN, Yang ES. 2013. Beyond DNA repair: Additional functions of parp-1 in cancer. Frontiers in oncology. 3:290.
Wedemeyer WJ, Welker E, Scheraga HA. 2002. Proline cis−trans isomerization and protein folding. Biochemistry. 41(50):14637-14644.
Wei H, Yu X. 2016. Functions of parylation in DNA damage repair pathways. Genomics, proteomics & bioinformatics. 14(3):131-139.

Widger A, Mahadevaiah SK, Lange J, ElInati E, Zohren J, Hirota T, Pacheco S, Maldonado-Linares A, Stanzione M, Ojarikre O et al. 2018. Atr is a multifunctional regulator of male mouse meiosis. Nature Communications. 9(1):2621.
Willis J, Patel Y, Lentz BL, Yan S. 2013. Ape2 is required for atr-chk1 checkpoint activation in response to oxidative stress. Proceedings of the National Academy of Sciences of the United States of America. 110(26):10592-10597.
Wu X, Shell SM, Yang Z, Zou Y. 2006. Phosphorylation of nucleotide excision repair factor xeroderma pigmentosum group a by ataxia telangiectasia mutated and rad3-related-dependent checkpoint pathway promotes cell survival in response to uv irradiation. Cancer research. 66(6):2997-3005.
Yan S, Sorrell M, Berman Z. 2014. Functional interplay between atm/atr-mediated DNA damage response and DNA repair pathways in oxidative stress. Cellular and molecular life sciences : CMLS. 71(20):3951-3967.
Yang JL, Mukda S, Chen SD. 2018. Diverse roles of mitochondria in ischemic stroke. Redox biology. 16:263-275.
Yang JL, Tadokoro T, Keijzers G, Mattson MP, Bohr VA. 2010. Neurons efficiently repair glutamate-induced oxidative DNA damage by a process involving creb-mediated up-regulation of apurinic endonuclease 1. The Journal of biological chemistry. 285(36):28191-28199.
Yang K-H, Lee T-B, Lee S-H, Kim S-H, Cho Y-H, Kim H-Y. 2016. Congenital adhesion band causing small bowel obstruction: What's the difference in various age groups, pediatric and adult patients? BMC Surg. 16(1):79-79.
Yang Q, Manicone A, Coursen JD, Linke SP, Nagashima M, Forgues M, Wang XW. 2000. Identification of a functional domain in a gadd45-mediated g2/m checkpoint. The Journal of biological chemistry. 275(47):36892-36898.
Zachariah SK. 2011. Adult necrotizing enterocolitis and non occlusive mesenteric ischemia. J Emerg Trauma Shock. 4(3):430-432.
Zeman MK, Cimprich KA. 2014. Causes and consequences of replication stress. Nature cell biology. 16(1):2-9.
Zhou XY, Wang X, Hu B, Guan J, Iliakis G, Wang Y. 2002. An atm-independent s-phase checkpoint response involves chk1 pathway. Cancer research. 62(6):1598-1603.
Zighelboim I, Schmidt AP, Gao F, Thaker PH, Powell MA, Rader JS, Gibb RK, Mutch DG, Goodfellow PJ. 2009. Atr mutation in endometrioid endometrial cancer is associated with poor clinical outcomes. Journal of Clinical Oncology. 27(19):3091-3096.
Zilfou JT, Lowe SW. 2009. Tumor suppressive functions of p53. Cold Spring Harb Perspect Biol. 1(5):a001883.
Zoller M, Zhao K, Kutlu N, Bauer N, Provaznik J, Hackert T, Schnolzer M. 2018. Immunoregulatory effects of myeloid-derived suppressor cell exosomes in mouse model of autoimmune alopecia areata. Frontiers in immunology. 9:1279.
Zou L, Elledge SJ. 2003. Sensing DNA damage through atrip recognition of rpa-ssdna complexes. Science (New York, NY). 300(5625):1542-1548.

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Serrano MA, Li Z, Dangeti M, Musich PR, Patrick S, Roginskaya M, **Cartwright BM**, Zou Y. DNA-PK, ATM and ATR collaboratively regulate p53-RPA interaction to facilitate homologous recombination DNA repair. Oncogene. 2013 May 9;32(19):2452-62. PubMed PMID: 22797063

Stuart CA, Howell ME, **Cartwright BM**, McCurry MP, Lee ML, Ramsey MW, Stone MH. Insulin resistance and muscle insulin receptor substrate-1 serine hyperphosphorylation. Physiol Rep. 2014 Dec 1;2(12). PubMed PMID: 25472611

Hilton BA, Li Z, Musich PR, Wang H, **Cartwright BM**, Serrano M, Zhou XZ, Lu KP, Zou Y. ATR Plays a Direct Antiapoptotic Role at Mitochondria, which Is Regulated by Prolyl Isomerase Pin1. Mol Cell. 2015 Oct 1;60(1):35-46 PubMed PMID: 26387736

Stuart CA, Lee ML, South MA, Howell ME, **Cartwright BM**, Ramsey MW, Stone MH. Pre-Training Muscle Characteristics of Subjects Who Are Obese Determine How Well Exercise Training Will Improve Their Insulin Responsiveness: Exercise training and muscle of obese subjects. J Strength Cond Res. 2016 Jun 28. PubMed PMID: 27379957

Hilton BA*, Liu J*, **Cartwright BM**, Liu Y, Breitman M, Wang Y, Jones R, Tang H, Rusinol A, Musich PR, Zou Y. Progerin sequestration of PCNA promotes replication fork collapse and mislocalization of XPA in laminopathy-related progeroid syndromes. FASEB J. 2017 May 17. PMID: 28515154

Cartwright BM, Musich P, Zou Y. *DNA Damage: Cellular Responses, Repair, and Cancer Treatment.* Regulation of Signal Transduction in Human Cell Research. Springer Nature. 2018 DOI: 10.1007/978-981-10-7296-3_6

Li Z*, Wang H*, **Cartwright BM**, Hilton BA, Makinwa Y, Musich PR, Chen Q, Yu X, Zou Y. ATR inhibits Ca^{2+} overload-induced necrotic cell death through phosphorylation-mediated inhibition of PARP1. (in preparation)

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