Endogenous Retroviruses as a Key Modulator of Immune Response to Cancer Therapies

by

Andrew Kang-Kang Lee

Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________
Approved:

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Chuan-Yuan Li, Supervisor

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Xiao-Fan Wang

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David Kirsch

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Kris Wood

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Scott Floyd

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Endogenous retroviruses (ERVs) are ancient deactivated viral elements that have integrated into the human genome over the course of millennia of evolution. ERVs are normally kept transcriptionally repressed, but recent evidence has demonstrated that ERV transcription can be upregulated in response to a variety of stimuli and pharmacological treatments. Furthermore, ERV transcription in the form of double-stranded RNA (dsRNA) was shown to upregulate a host of immune signaling pathways, with implications for the future of cancer therapy. In this study, we utilize the transcriptional corepressor KRAB-Associated Protein 1 (KAP1), a chromatin modulator responsible for suppressing ERV transcription sites, as a tool to investigate ERV expression in cancer therapy.

We demonstrate that radiotherapy, historically believed to mediate tumor cell repression through direct cell killing, can also upregulate ERV transcription, which stimulates downstream interferon production and interferon-stimulated genes through the MDA5/MAVS innate antiviral immunity pathway, a separate pathway from cGAS/STING signaling. KAP1 depletion enhances the effect of radiation, as ERV and interferon transcription is significantly upregulated. Additionally, KAP1 depletion enhances the effect of radiation-induced anti-tumor response *in vivo* in two separate

tumor models. Our findings indicate a novel and understudied pathway of radiotherapy-induced tumor control.

We also demonstrate that KAP1 depletion is sufficient to provoke interferon signaling and immune effects. KAP1 depletion upregulates ERV transcription, driving a similar effect to that seen with irradiation. Furthermore, KAP1 depletion is sufficient to inhibit tumor growth of B16F10 tumors in *in vivo* studies, but this effect is dependent on an intact host immune system. Tumor growth inhibition *in vivo* is driven by increased recruitment of immune cells to the tumor microenvironment, along with upregulated of interferon-stimulated genes. Patient data support our findings, as mRNA analysis of TCGA patient cohorts reveals that *Trim28* expression is negatively associated with survival and immune recruitment. Taken as a whole, our work indicates KAP1 as a crucial modulator of ERVs, with significant consequences for cancer therapy.

Dedication

Dedicated with love to all who have supported me through this long journey, and to all who I will support into the future through their own journeys.

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List of abbreviations

APC – antigen-presenting cell

ATM – ataxia telangiectasia mutated

CARD – caspase recruitment domain

CAR-T – chimeric antigen receptor T-cell therapy

CD28 – cluster of differentiation 28

cGAMP – cyclic GMP-AMP

cGAS – cyclic GMP-AMP synthase

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

DAMP – damage-associated molecular pattern

DBD – DNA-binding domain

DNMT – DNA methytransferase

DNMTi – DNA methyltransferase inhibitor

DsDNA – double-stranded DNA

DsRNA – double-stranded RNA

EBV – Epstein-Barr virus

ERV – endogenous retrovirus

HDAC – histone deacetylase

HDACi – histone deacetylase inhibitor

HERV – human endogenous retrovirus

HP1 – heterochromatin protein 1

HPV – human papilloma virus

HTLV-1 – human T-cell leukemia virus

IFN – interferon

IR – ionizing radiation

IRF3 – interferon regulatory factor 3

ISG – interferon-stimulated gene

KAP1 – KRAB-associated protein 1

KRAB – Kruppel-associated box

KRAP-ZFP – Kruppel-associated box domain zinc finger proteins

LSD1 – lysine-specific histone demethylase 1A

LTR – long terminal repeat

MAVS – mitochondrial antiviral signaling protein

MDA5 – melanoma differentiation-associated protein 5

MeCP – methyl-CpG-binding protein

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NuRD – nucleosome deacetylase remodeling complex

PAMP – pathogen-associated molecular pattern

PD-1 – programmed death 1 receptor

PHD – plant homeodomain

PP1β – protein phosphatase 1β

PRR – pattern recognition receptor

RBCC – Ring B-box coiled coil domain

RIG-1 – retinoic acid-inducible gene 1

RT – radiotherapy

RT-qPCR – quantitative reverse transcription PCR

SETDB1 – SET domain bifurcated histone lysine methyltransferase 1

SgRNA – single guide RNA

STING – stimulator of interferon genes

TBK1 – TANK-binding kinase 1

TE – transposable element

TIF1β – transcriptional intermediary factor 1 beta

TIL – tumor-infiltrating lymphocyte

TIP – tracking tumor immunophenotype

TLR3 – toll-like receptor 3

TRIM28 – tripartite motif-containing 28

TSS – TIF1 signature sequence

ZNF – zinc finger

1. Introduction

1.1 History of endogenous retroviruses

The human genome consists of over 3 billion base pairs of DNA, but only about 1.5% of the genome is composed of protein-coding genes (Lander et al., 2001). The remaining proportion is comprised of introns, regulatory sequences, regions of noncoding RNA, repetitive DNA sequences, and genomic transposable and repetitive elements (Kellis et al., 2014). Transposable and repetitive elements make up about 45% of the genome by themselves (Lander et al., 2001). One major family of transposable elements (TEs) are endogenous retroviruses (ERVs), which make up approximately 8% of the total human genome, representing ancient deactivated viral elements integrated into the genome over the course of evolution (Figure 1) (Grandi & Tramontano, 2018).

ERVs were initially discovered in the late 1960s through studies of Mendelian inheritance of a viral-transformed phenotype in the domestic fowl (Gallus gallus) and laboratory mouse (Mus musculus) (Weiss, 2006), and later confirmed in the 1970s through the discovery of reverse transcriptase (Baltimore, 1970; Temin & Mizutani, 1970). The vast majority of human ERVs (HERVs) are evolutionarily ancient, having integrated into our genome an estimated 35-60 million years ago, with the exception of the relatively young HERV-K group (Tonjes et al., 1996).

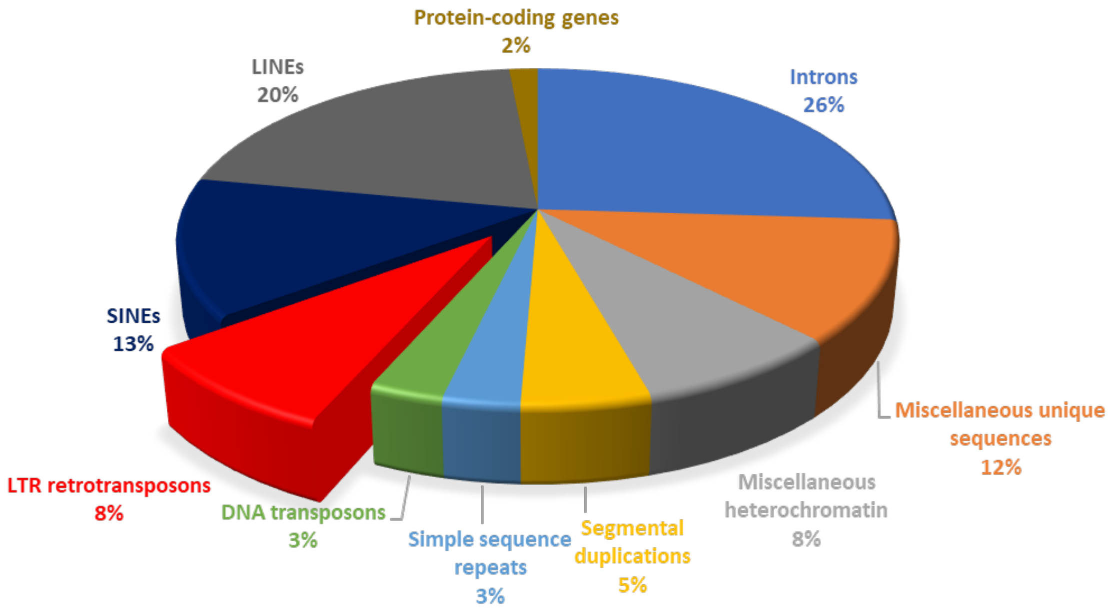


Figure 1: Components of the human genome.
Representation of various genetic elements comprising the human genome. Adapted from (Gregory, 2005). LTR retrotransposons make up approximately 8% of the total human genome.

1.1.1 Genetic structure of an endogenous retrovirus

The general structure of an intact ERV is similar to that of an exogenous retrovirus, with gag, pol, and env gene regions flanked by regions of long terminal repeats (LTRs) (Figure 2) (Vargiu et al., 2016). The flanking LTRs are comprised of a U3 region which contains the TATA promotor and transcriptional enhancers, an R region responsible for post-transcriptional processing, and a U5 region necessary for proper polyadenylation of the RNA transcript (Thompson, Macfarlan, & Lorincz, 2016). In the case of an exogenous retrovirus, initiation of transcription would allow for gag, pol, and env transcription and subsequent creation of infectious viral particles (Bolinger & Boris-

Lawrie, 2009), but thus far there have been no HERVs discovered which are replication competent, due to mutations accumulated over time (G. R. Young, Stoye, & Kassiotis, 2013).

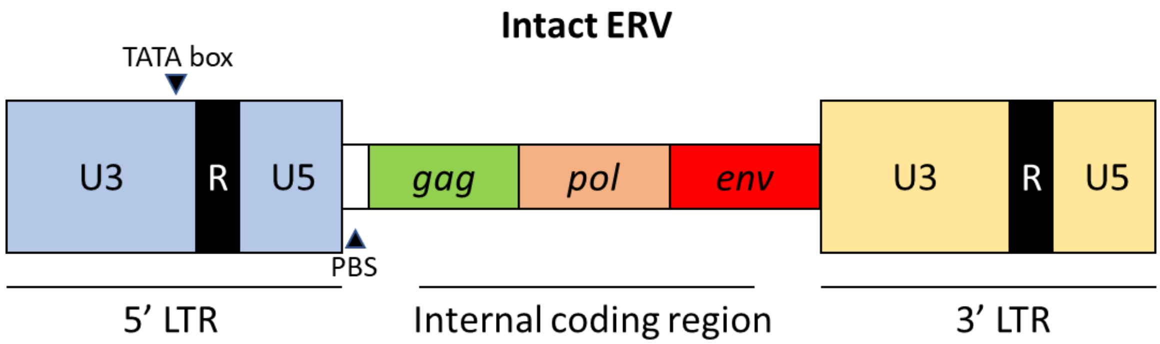


Figure 2: Structure of an intact ERV.
Overview of the structure of an intact ERV. The internal coding region contaings the primer binding site, flanked by LTRs with U3, R, and U5 regions. Adapted from (Thompson et al., 2016).

Most ERVs have lost their principal coding domains through mutational decay and recombinational deletion, leaving behind only a "solo LTR" (Thompson et al., 2016). A few more evolutionarily-recent ERVs still retain minor protein-coding potential (Villesen, Aagaard, Wiuf, & Pedersen, 2004), but the vast majority of ERVs have been deactivated through the combination of genetic mutations and evolutionary pressure and are no longer able to produce competent infectious particles. However, ERVs are still capable of affecting biological phenotype through DNA transposition, double-stranded RNA (dsRNA) sensing pathways, and possibly other biological mechanisms (Benit, Lallemand, Casella, Philippe, & Heidmann, 1999; Chiappinelli et al., 2015).

Therefore, cells must still maintain control over the ERV regions in its genome, as unchecked ERV regions can exert biological actions through these alternative methods.

1.2 Biological consequences of ERVs

1.2.1 DNA retrotransposition

One of the primary mechanisms by which ERVs can affect biological phenotype, even without the ability to generate viral particles, is through DNA retrotransposition (Cordaux & Batzer, 2009). By nature of retroviral integration into the genome, ERV regions are flanked by LTRs which serve as control centers, containing functional promoters, enhancers, and other regulatory elements (Cohen, Lock, & Mager, 2009). These LTRs are capable of replication and transposition to other regions of the genome, possibly with detrimental effects (Havecker, Gao, & Voytas, 2004).

Typically, the U3 promoter region of a 5′ LTR drives the production of downstream mRNA by RNA polymerase II. The mRNA transcript is then converted to proviral double-stranded DNA (dsDNA) by reverse transcriptase, followed by re-integration into the DNA by integrase (Thompson et al., 2016). This can be considered a "copy-and-paste" mechanism, which can be contrasted to class II and III DNA transposons found in non-human genomes – other genomic elements which utilize transposases to migrate around the genome, more akin to a "cut-and-paste" mechanism (Munoz-Lopez & Garcia-Perez, 2010). The reintegration of ERV sequences into the genome not only increases their copy number over time, but also has other potential effects such as creating alternative promotors for genes already present, affecting genomic plasticity, or potentially affecting carcinogenesis (Platt, Vandewege, & Ray, 2018).

One of the simplest scenarios is where an ERV LTR reintegrates directly upstream of a gene, and the alternative promoter present within the LTR drives increased mRNA transcription. One of the best-known examples of this phenomenon is the human gene *CYP19A1,* which encodes P540 aromatase but has a MER21C LTR promotor driving its expression within the placenta in a tissue-dependent manner (van de Lagemaat, Landry, Mager, & Medstrand, 2003). As shown by *CYP19A1*, in the long term, the cell can adapt to and even utilize alternative promotors created in this fashion, but in the short term, unexpected upregulation of proteins can upset the microenvironment within the cell and lead to a disease state.

Alternatively, genomic plasticity and mutagenesis can be argued to be a more common and biologically important effect of ERV retrotransposition. As reintegration by integrase is a random process, it is possible for the ERV to be inserted within a gene exon and lead to elimination or mutation of the downstream protein product (Gagnier, Belancio, & Mager, 2019). Additionally, ERVs may be inserted within regions of "junk DNA" and drive the expression of a previously dormant gene, or even contribute to *de novo* gene creation (Franke et al., 2017). ERV retrotransposition has been a source of genomic plasticity for millions of years and is an important component of human genetic evolution over the course of time (Benit et al., 1999).

ERVs may also play a role in carcinogenesis. Multiple exogenic oncogenic viruses have been discovered and linked to cancer including Epstein-Barr virus (EBV) for Burkitt's lymphoma, human papilloma virus (HPV) for cervical cancer, and human T-cell leukemia virus 1 (HTLV-1) for leukemia (Bosch, Lorincz, Munoz, Meijer, & Shah, 2002; Matsuoka & Jeang, 2011; L. S. Young & Murray, 2003). However, as these oncogenic viruses drive transformation through their downstream protein products, and most ERVs are unable to produce functional proteins, it is unlikely that ERVs could drive oncogenic transformation through a similar pathway. It has been demonstrated in mice that insertional activation of oncogenes such as *Wnt1* or *Notch1* with murine ERVs can promote carcinogenesis (Fan & Johnson, 2011), but this has yet to be shown to be the case in humans. Upregulated levels of HERVs have been associated with cancer (Downey et al., 2015; Gonzalez-Cao et al., 2016), but whether it is a causative or a resultant factor of carcinogenesis is still a matter of debate.

1.2.2 Double stranded DNA and RNA particles

Aside from retrotransposition, ERVs can affect biological function even without mRNA being translated to proteins. Proviral dsDNA generated from reverse transcriptase is not necessarily reintegrated into the genome but may instead be exported to the cytosol where it can be recognized as a damage-associated molecular pattern (DAMP) or pathogen-associated molecular pattern (PAMP) (D. Tang, Kang, Coyne, Zeh, & Lotze, 2012). This can have positive or negative effects, as recognition of DAMPs and PAMPs by pattern recognition receptors (PRRs) is an important aspect of cellular defense against DNA damage and pathogens (Mogensen, 2009), but PRR-associated sensing pathways have been linked with autoimmunity, implying that aberrant signaling may be a factor in autoimmunity diseases such as type 1 diabetes and systemic lupus erythematosus (Nelson, Rylance, Roden, Trela, & Tugnet, 2014; van der Werf, Kroese, Rozing, & Hillebrands, 2007).

DsDNA is not the only molecular pattern from ERVs that can drive a signaling response. Recent studies have indicated that bidirectional transcription of ERVs regions can generate complementary mRNA strands, which can associate to form double-stranded RNA (dsRNA). While dsRNA is unable to reintegrate into the genome, it can instead be exported to the cytosol where it can also be sensed by PRRs, simulating a viral infection state and inducing a downstream type I interferon (IFN) signaling response (Chiappinelli et al., 2015).

1.3 Genomic regulation of ERVs

As uncontrolled ERVs can have significant unexpected and detrimental effects on the cellular environment, it is important for the cell to maintain control over ERV transcription. The primary regulatory mechanism for ERVs is epigenetic repression – by preventing transcription, both retrotransposition-mediated mutagenesis and dsDNA/dsRNA expression are blocked (Hurst & Magiorkinis, 2017). This is mediated through two currently known mechanisms: DNA methylation at CpG sites mediated by DNA methyltransferases (DNMTs) (Moore, Le, & Fan, 2013) and heterochromatin formation through histone methylation mediated by KRAB-associated protein 1 (KAP1) (Groner et al., 2010).

The vast majority of methylated CpG sites within the human genome are associated with inhibition of repetitive elements, as approximately 90% of methylated CpG sites are located within the 45% of the human genome containing transposable and repetitive elements (Beisel & Paro, 2011). This methylation is catalyzed by DNMTs, which are also responsible for propagating methylation marks through DNA replication (Hermann, Gowher, & Jeltsch, 2004). The precise mechanism as to how DNA methylation inhibits transcription is still a subject of some debate, but it is believed that methylated CpG sites attract methyl-CpG-binding proteins (MeCPs) such as MeCP2 and form a protein complex that inhibits access to transcriptional machinery (Deaton & Bird, 2011; Wade, 2001). DNA methylation within LTR regions thus blocks transcription of the LTR-promoted mRNA transcript.

Heterochromatin formation is another mechanism by which ERVs are inhibited. Krüppel-associated box domain zinc finger proteins (KRAB-ZFPs), which make up the largest family of transcriptional regulators in humans, bind to ERV regions and recruit KAP1 to initiate the process of heterochromatin formation (Groner et al., 2010). Once recruited, KAP1 serves as a scaffolding protein, assembling a complex of heterochromatin-associated proteins including Heterochromatin Protein 1 (HP1), SET Domain Bifurcated Histone Lysine Methyltransferase 1 (SETDB1), and the Nucleosome Deacetylase Remodeling (NuRD) complex (Ryan et al., 1999; Schultz, Ayyanathan, Negorev, Maul, & Rauscher, 2002; Schultz, Friedman, & Rauscher, 2001). These proteins catalyze the conversion of euchromatin to heterochromatin, inhibiting transcription of DNA in that genomic region (Figure 3) (Iyengar & Farnham, 2011).

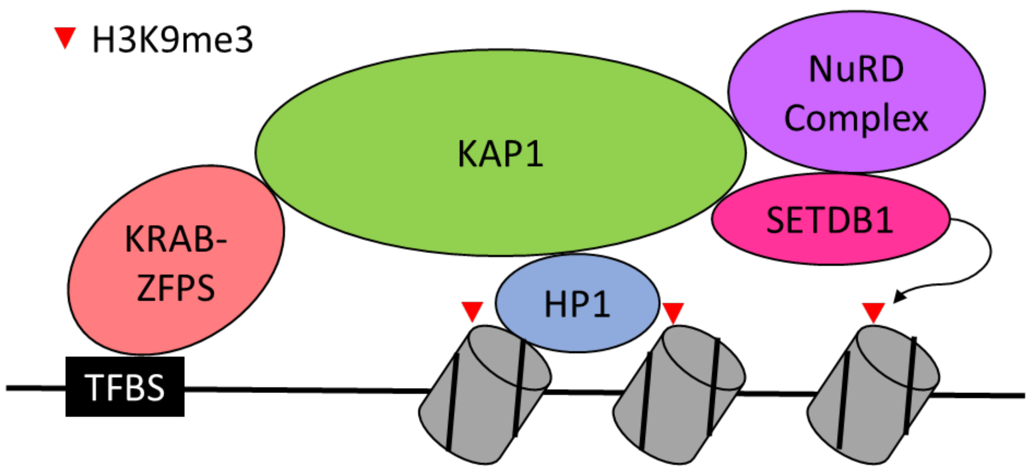


Figure 3: Heterochromatic repression by KAP1 complex.
KRAB-ZFPs recruit KAP1 to transcription factor binding sites, where KAP1 serves as a scaffold for heterochromatin remodeling factors HP1, SETDB1, and the NuRD complex.

DNA methylation and heterochromatin formation through histone methylation are closely linked, with significant interplay between the two mechanisms. Similar to DNA methylation, heterochromatic marks are preserved through DNA replication (Budhavarapu, Chavez, & Tyler, 2013), and DNMT1 actively recruits the histone methyltransferase G9a during the process of DNA replication (Esteve et al., 2006).

Additionally, KAP1-mediated heterochromatin formation has been demonstrated to initiate *de novo* DNA methylation at its targeted sites (Rowe et al., 2013), so it is apparent that both mechanisms work in parallel to reliably inhibit transcription of ERVs.

1.4 Mechanisms and consequences of ERV deregulation and expression

DNA methylation and heterochromatin are highly effective at inhibiting ERV transcription, but there are also mechanisms to disable the inhibition and allow for expression of ERV transcripts. DNA demethylation via DNA methyltransferase inhibitors (DNMTis) has been demonstrated to cause an increase in ERV transcripts (Chiappinelli et al., 2015; Sheng et al., 2018), and a similar increase is seen upon loss of heterochromatic repression through various histone-related mechanisms (Izquierdo-Bouldstridge et al., 2017). Recent studies of the consequences of ERV induction, especially in regard to cancer, have primarily focused on the response of DAMP and PAMP PRRs to dsDNA and dsRNA particles, due to the difficulty and randomness associated with studying insertional mutagenesis related to retrotransposon activity (Kassiotis, 2014).

1.4.1 DNA demethylation via DNMTis

DNMTis such as azacytidine and decitabine have been FDA approved for cancer therapy since 2004 (X. Yang, Lay, Han, & Jones, 2010), but their mechanism of action was a matter of debate until recently. It was known that DNMTis integrated into DNA as a cytosine analog and catalyzed DNA hypomethylation via DNMT degradation and loss of methylation marks (Stresemann & Lyko, 2008), but it wasn't discovered until 2015 that the global hypomethylation also stimulates ERV transcription (Chiappinelli et al., 2015; Roulois et al., 2015). The resulting dsRNA particles are sensed by cytosolic PRRs including Toll-like Receptor 3 (TLR3), Retinoic Acid-Inducible Gene 1 (RIG-1), and Melanoma Differentiation-Associated Protein 5 (MDA5) (Chattopadhyay & Sen, 2014; Saito & Gale, 2008; Wu et al., 2013). Recognition of dsRNA by PRRs activates a "viral mimicry" state and triggers a signaling cascade that promotes type I IFN expression, activation of downstream IFN-stimulated genes (ISGs), and subsequent immune response (Chiappinelli et al., 2015; Roulois et al., 2015). More on this pathway will be discussed in chapter 2.

1.4.2 Loss of heterochromatic repression

Loss of heterochromatic repression has also been correlated with ERV transcription and subsequent immune response in a multitude of studies. The presence of activating histone acetylation marks at H3K27 stimulate ERV transcription and downstream ISGs in gliomas, mediated through the same dsRNA and PRR signaling pathway as DNMTis (Krug et al., 2019). The addition of activating histone methylation marks at H3K9 via depletion of DNA methyltransferase Lysine-Specific Histone Demethylase 1A (LSD1) demonstrate a similar effect in melanoma (Sheng et al., 2018). Complete depletion of histone H1 in breast cancer also corroborates these results, with increases in ERV transcription and downstream IFN activation (Izquierdo-Bouldstridge et al., 2017). It is likely that upregulation of ERV transcription is of relevance to cancer treatment, as IFN and ISG activation is associated with better patient prognosis and improved responses to immunotherapy (Cheon, Borden, & Stark, 2014).

1.5 The multifaceted role of KAP1 in heterochromatic repression

Loss of KAP1 and subsequent heterochromatic relaxation has also been shown as a mechanism to upregulate ERV transcription and immune response (Tie et al., 2018). However, KAP1 is unique in that it is capable of derepressing heterochromatin in response to DNA damage, without the need for knockdown or pharmacological manipulation. Depending on its structural conformation and post-translational modifications, KAP1 can adjust the chromatin compaction from heterochromatin to euchromatin and back again at a moment's notice (X. Li et al., 2007), which holds implications for ERV transcript expression.

1.5.1 Discovery and structure of KAP1

KAP1, also known by Tripartite Motif-Containing 28 (TRIM28) and Transcriptional Intermediary Factor 1 Beta (TIF1β), was discovered in 1996 as an interacting partner for KRAB-ZFPs (Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1996; Moosmann, Georgiev, Le Douarin, Bourquin, & Schaffner, 1996). KAP1 is part of a family of roughly 60 human TRIM genes and is structurally similar to three other TRIM proteins: TIF1α, TIF1γ, and TIF1δ (Ozato, Shin, Chang, & Morse, 2008). However, despite the structural similarity, KAP1 shares limited functional overlap with its other family members, as only the TIF1 proteins are capable of transcriptional repression, and KAP1 is the only known TRIM protein to interact with KRAB-ZFPs to facilitate heterochromatic repression (Peng, Feldman, & Rauscher, 2002). KAP1's structure is composed of three distinct regions: an N-terminal tripartite motif (TRIM), a central region containing the TIF1 signature sequence (TSS) domain as well as an HP1-binding domain, and a C-terminal combination plant homeodomain (PHD) and bromodomain (Ryan et al., 1999).

The N terminus TRIM region contains the RBCC domain, which itself consists of a RING (Really Interesting New Gene) finger, two B-box zinc fingers (ZNFs), and a coiled coil. The RBCC domain is necessary and sufficient for KAP1 to bind to the KRAB repression domain located on KRAB-ZFPs and localize to the correct regions of the genome (Peng et al., 2002). Oligomerization of KAP1 is necessary, as RBCC-KRAB binding only occurs if KAP1 is in a homotrimerized state (Peng et al., 2000).

The central region contains the TSS domain as well as the HP1-binding domain. The role of the TSS domain is unknown in KAP1, but deletion of the domain in related family protein TIF1γ abrogates transcriptional repression (Venturini et al., 1999), implying a role in KAP1-mediated transcriptional repression as well. The central region of KAP1 also contains an HP1-binding domain, a hydrophobic P*x*V*x*L peptide necessary for binding to the chromoshadow domain of HP1 (Lechner, Begg, Speicher, & Rauscher, 2000). The remainder of the central region is rich in prolines, glycines, and serines, and no known structures have been discovered. It has been suggested that the central region may exist in a highly flexible state, to allow KAP1 to adapt to a large variety of protein complex structures (Iyengar & Farnham, 2011).

The C-terminal region contains the PHD and bromodomain, collectively termed the PB domain. The PB domain serves as the primary enabler for transcriptional repression, and both domains are necessary for full repression (Schultz et al., 2001). The bromodomain is responsible for interaction with two chromatin-modifying enzymes: Mi2α, a component of the NuRD complex, and SETDB1, a methyltransferase specific to H3K9 trimethylation (Schultz et al., 2002; Schultz et al., 2001). The PHD domain functions as an intramolecular E3 ligase that SUMOylates the bromodomain at lysines 554, 575, 676, 750, 779, and 804 (Zeng et al., 2008). SUMOylation of the bromodomain is essential for effective binding to SETDB1, and also stimulates the methyltransferase function of SETDB1 once bound, mediating the transcriptional repression effect through generation of trimethylated histones (Ivanov et al., 2007).

1.5.2 KRAB-ZFP-directed binding to DNA

As HP1, SETDB1, and the NuRD complex do not have DNA-binding domains (DBDs), KAP1 functions as a necessary scaffold to localize the heterochromatin complex to targeted regions of the genome (Iyengar & Farnham, 2011). However, KAP1 itself also has no DBD – instead, KAP1 is recruited to the genome by a large family of KRAB-ZFP transcription factors, which allows for high specificity without the necessity of multiple KAP1 proteins with different DBDs (Lorenz et al., 2010).

KRAB-ZFPs are the largest class of DNA-binding transcription factors in the human genome, with over 400 KRAB-ZFP genes discovered (Huntley et al., 2006). The majority of KRAB-ZFPs specifically target TEs, and retrotransposons in particular (P. Yang, Wang, & Macfarlan, 2017), unpinning the importance of keeping ERVs under strict control. It is suspected that the rise of KRAB-ZFPs represents an evolutionary arms race, with retroviral infection and genomic integration necessitating the development of transcription factors to repress viral transcription. It has also been proposed that KRAB-ZFPs themselves may play a role in harnessing their TE targets for evolutionary advantage (Ecco, Imbeault, & Trono, 2017).

KRAB-ZFPs are characterized by a KRAB domain and the presence of an array of C_2H_2 ZNFs (Lupo et al., 2013). The KRAB domain is responsible for effective binding to KAP1, thus recruiting the heterochromatin machinery, while the ZNFs are responsible for targeting of specific sequences of DNA (Groner et al., 2010). Thus, through the 400+ family members of KRAB-ZFPs, KAP1 is efficiently recruited to a wide variety of locations within the genome, primarily centered around TEs and retrotransposons.

1.5.3 Heterochromatin complex

Once assembled at the KRAB-ZFP-targeted region, the heterochromatin complex centered around KAP1 is responsible for initiating chromatin condensation and generating a repressive state (Sripathy, Stevens, & Schultz, 2006). The components of the complex consist of the NuRD complex and SETDB1, which bind to the bromodomain, and HP1, which binds to the HP-binding domain of KAP1 (Iyengar & Farnham, 2011).

The NuRD complex consists primarily of proteins involved in histone deacetylation, including Histone Deacetylase (HDAC) 1 and 2, as well as histone-binding proteins RBBP7 and RBBP4 (Basta & Rauchman, 2015). Histone acetylation is traditionally associated with relaxed chromatin and decondensation (Gorisch, Wachsmuth, Toth, Lichter, & Rippe, 2005; Toth et al., 2004), so the role of the NuRD complex is deacetylation of histone lysines to induce chromatin compaction. However, it has been shown that pharmacologic inhibition of HDACs only partially negates KAP1-mediated repression (Schultz et al., 2001), so it is suspected that the NuRD complex may only play a minor role in effecting chromatin compression.

SETDB1 is a methyltransferase that specifically targets lysine 9 on histone 3 for trimethylation (Schultz et al., 2002). In contrast to histone acetylation, histone methylation is traditionally associated with compressed chromatin and transcriptional repression (Zhang & Reinberg, 2001), so SETDB1-mediated H3K9 trimethylation also induces chromatin compaction, primarily through an HP1-dependant mechanism.

HP1 has high affinity for H3K9 trimethylation and is responsible for folding H3K9me3-containing chromatin into a higher order structure (G. Li & Reinberg, 2011). Briefly, HP1 forms a symmetric dimer and bridges two H3K9me3 histones together, compacting nucleosomes together and generating the traditional compressed chromatin state associated with heterochromatin (Machida et al., 2018). Thus, we have an overall model where KRAB-ZFPs recruit KAP1 to specific regions of the genome, bringing in heterochromatin remodeling factors and generating a heterochromatin state to repress transcription.

1.5.4 Role in DNA-damage response

As mentioned earlier, KAP1 has a secondary function in derepressing heterochromatin in response to DNA damage. Double-strand DNA breaks must be repaired quickly and efficiently or the cell risks generating errors and mutations during DNA replication, but DNA repair factors are unable to access damaged DNA within heterochromatin (Fortuny & Polo, 2018). KAP1 mediates the process of derepressing heterochromatin to allow access to DNA repair proteins, prompted by upstream signaling by Ataxia Telangiectasia Mutated (ATM) (White et al., 2012).

ATM is a serine/threonine kinase responsible for detecting DNA double-strand breaks and phosphorylating key downstream proteins to initiate the DNA damage response (Marechal & Zou, 2013). Upon detection of double-stranded DNA damage, ATM phosphorylates KAP1 at serines 473 and 824 (White et al., 2012). The addition of the phosphorylation generates a motif that interferes with SUMO interactions as well as with KAP1 oligomerization (Stoll et al., 2019). Upon deoligomerization, KAP1 is no longer able to bind to KRAB-ZFP KRAB domains via its RBCC domain, nor SETDB1 via its SUMOylated bromodomain (X. Li et al., 2007). Once the KAP1-associated heterochromatin complex is no longer functional, chromatin decondensation and DNA repair factors are able to access the damaged DNA (Figure 4) (White et al., 2012).

When DNA repair is complete, Protein Phosphatase 1β (PP1β) dephosphorylates KAP1, allowing KAP1 to restore its oligomerized structure and generate heterochromatin (X. Li et al., 2010). However, during the process of DNA repair while KAP1 is phosphorylated, transcription and gene expression of KAP1-regulated genes is increased, a finding supported by studies involving S824D phosphomimetic mutant versions of KAP1 (X. Li et al., 2007).

We hypothesized that induction of DNA damage and subsequent ATM and KAP1-mediated heterochromatin release could drive a temporary upregulation not only of gene products, but also ERV transcription and downstream IFN and ISG activation, as a novel mechanism for the role of DNA damage in cancer treatment. We also hypothesized that depletion of KAP1 may induce an immune response, mediated through increased dsRNA in the form of unregulated ERV transcription. We first investigated whether radiation-induced DNA damage could drive ERV transcription and provoke a downstream immune response in cancer treatment.

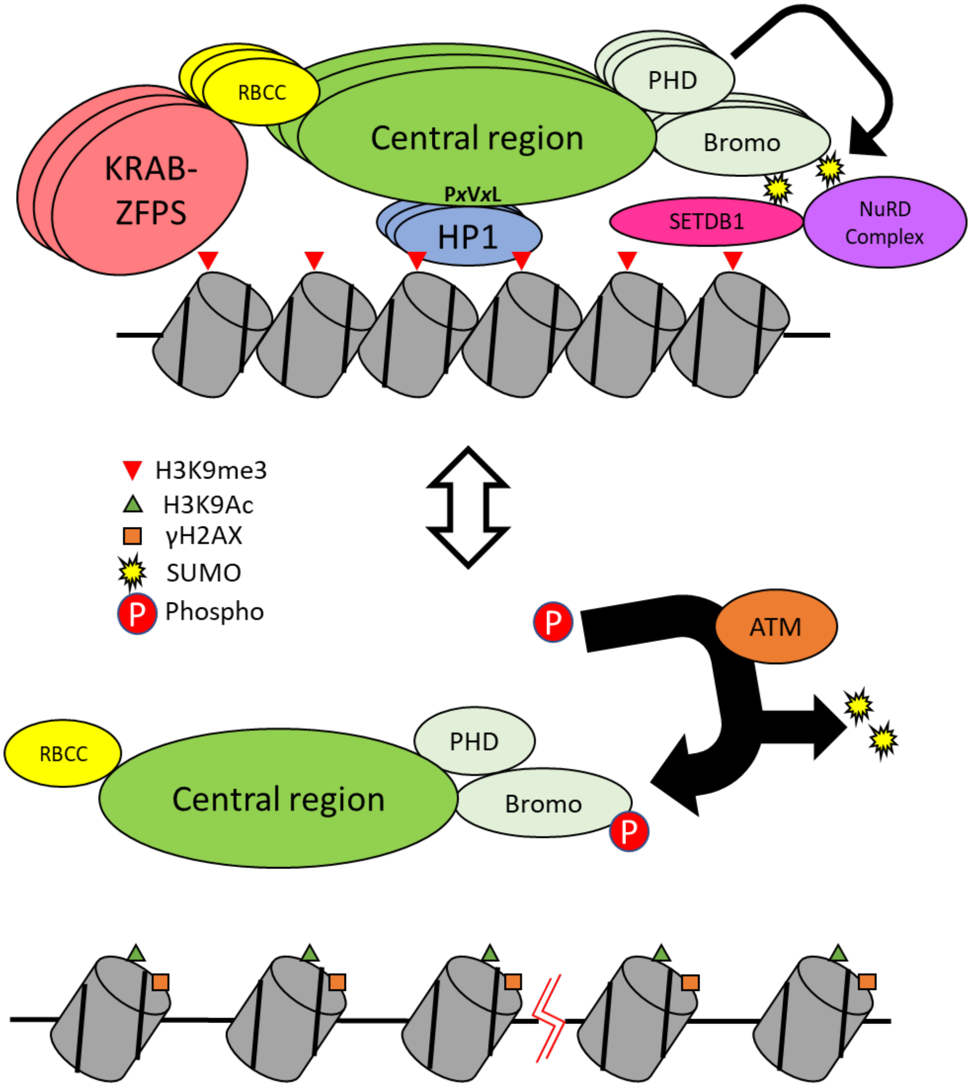


Figure 4: KAP1 conformational shift in response to DNA damage
SUMOylation of the bromodomain of homotrimerized KAP1 enhances histone deacetylation and methylation action of NuRD complex and SETDB1. Phosphorylation by ATM in response to DNA damage deoligomerizes KAP1 and allows access to the DNA for DNA repair factors.

2. Radiation induces ERV expression and provokes an immune response in cancer therapy

Note: Chapter 2 was modified from a manuscript entitled "Endogenous retrovirus activation as a key mediator of anti-tumor immune response in radiotherapy" published in Radiation Research. The authors were Andrew Lee, Xuhui Bao, Dong Pan, Mengjie Hu, Fang Li, and Chuan-Yuan Li. Xuhui Bao assisted with performing *in vivo* mouse tumor inoculations. Dong Pan assisted with performing immunofluorescence and analysis of J2-stained cells. I performed all other experiments and analysis.

2.1 Introduction

2.1.1 Radiation as a treatment modality

Radiotherapy (RT) continues to play a large role in cancer therapy, with over 50% of cancer patients receiving RT over the course of treatment (Barton et al., 2014). Traditionally, RT is utilized for local tumor control, with large doses of ionizing radiation (IR) used to kill cancer cells and shrink the tumor, often in conjunction with surgical resection. The established radiobiology paradigm states that exposure to IR induces DNA single and double-strand breaks within the targeted cells, which are responsible for IR-induced tumor cell death and subsequent tumor control (Eriksson & Stigbrand, 2010; Moding, Kastan, & Kirsch, 2013; Vignard, Mirey, & Salles, 2013). Accordingly, there is much evidence for IR-induced DNA strand breaks causing deleterious effects such as cell cycle arrest, or tumor cell death via apoptosis, necrosis, or autophagy (Brugarolas et al., 1995; Garcia-Barros et al., 2003; Nehs et al., 2011; Paglin et al., 2001).

However, not all IR-induced tumor control can be attributed to direct cell killing. It was discovered back in the late 1970s that the efficacy of radiotherapy was significantly attenuated in immunocompromised mice, indicating a role of immunoeffector cells in mediating tumor response (Jurin & Suit, 1972; Suit & Kastelan, 1970). Additionally, the abscopal effect, where a distant unirradiated tumor shrinks despite not receiving IR, suggested there was more to IR-induced tumor control than direct cell killing (Demaria et al., 2004). Indeed, it was recently demonstrated that a significant proportion of IR-induced tumor control could be attributed to immunological control of the tumor, triggered by DAMP-associated PRR signaling in response to cytosolic DNA fragments as a function of the cellular innate immune system (X. Li et al., 2013).

2.1.2 DNA damage response dsDNA-mediated immune response

IR generates a great deal of DNA strand breaks within a cell, which can lead to chromosomal aberrations and micronuclei formation due to improper DNA replication (Durante & Formenti, 2018). These micronuclei are then relocated to the cytoplasm and undergo nuclear envelope collapse, whereby the dsDNA fragments are detected by the PRR cyclic GMP-AMP synthase (cGAS) (Mackenzie et al., 2017). Detection of dsDNA fragments by cGAS catalyzes the production of cyclic GMP-AMP (cGAMP) from ATP and GTP. cGAMP then binds to Stimulator of Interferon Genes (STING), forming a signaling complex (T. Li & Chen, 2018). The STING signaling complex provides a scaffold for TANK Binding Kinase 1 (TBK1) to phosphorylate Interferon Regulatory Factor 3 (IRF3) and Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (Nf-κB), key transcription factors necessary to stimulate downstream type I IFN transcription (Tanaka & Chen, 2012).

Type I IFN activation is normally associated with viral infection, thus it is believed that activation of the cGAS/STING pathway induces a "viral mimicry" state, stimulating anti-tumor T cell-mediated immunity and contributing to tumor inhibition through the canonical antiviral immune response (Z. Ma & Damania, 2016). Furthermore, the downstream activated ISGs also stimulate tumor immunity, with genes such as *CXCL9* and *CXCL10* recruiting antigen-presenting cells (APCs) and T cells to the tumor (Cheon et al., 2014). As multiple studies have demonstrated that IR can induce a potent immune-mediated anti-tumor response, there are a quickly growing number of clinical trials being initiated focused on testing the combination of RT and immunotherapy (Cushman, Caetano, Welsh, & Verma, 2018; Y. Wang et al., 2018).

2.1.3 ERV-linked dsRNA-mediated immune response

While most immune signaling studies have focused on the DNA damage, dsDNA, and the cGAS/STING signaling pathway, there is an alternative sensing pathway involving dsRNA and the PRRs RIG-1 and MDA5 that leads to the same induction of type I IFNs and downstream ISGs (Chiappinelli et al., 2015; Roulois et al., 2015). Instead of DAMP-associated PRRs however, RIG-1 and MDA5 are more accurately labeled PAMP-associated PRRs, as they detect cytoplasmic dsRNA, which is generally produced as a result of pathogenic viral infection (Jensen & Thomsen, 2012). RIG-1 recognizes short blunt ends of viral dsRNA, while MDA5 recognizes longer strands of dsRNA (Reikine, Nguyen, & Modis, 2014).

Upon detection of cytoplasmic dsRNA, RIG-1 and MDA5 will form a protein complex with the dsRNA termed a "translocon" and migrate to the mitochondria (Barral et al., 2009). There, the complex activates the Mitochondrial Antiviral Signaling Protein (MAVS) by binding to its caspase recruitment domain (CARD) and initiating downstream signaling (Wu & Hur, 2015). The activated MAVS signaling complex initiates a signaling cascade through phosphorylation of TBK1, IRF3, and NF-κB (Liu, Chen, Wei, Shan, & Wang, 2011), the same effectors as the cGAS/STING pathway, thus activating transcription of the same downstream IFNs and ISGs (Figure 5).

We hypothesized that aside from the activation of the cGAS/STING pathway through cytoplasmic dsDNA, IR also would cause increased ERV transcription due to DNA damage-induced KAP1-mediated heterochromatic relaxation, thus generating cytoplasmic dsRNA and activating the MDA5/MAVS/TBK1 pathway. Though it has been previously shown that radiation can induce upregulation of ERV transcripts (J. R. Lee, Ahn, Kim, Jung, & Kim, 2012), to our knowledge, it has not yet been demonstrated if IR-induced ERV transcription is sufficient to stimulate IFN activation, or if there is potential to induce anti-tumor immunity during radiotherapy. In this study, we evaluated if ionizing radiation exposure could cause increased activation of ERVs, as well as if KAP1 could regulate this process. Furthermore, we determined if ERV activation contributes to a radiation-induced type I interferon response, as well as whether the dsRNA-induced type I interferon response was relevant for radiotherapy in immunocompetent murine mouse models. Our work demonstrates that KAP1 is a potential therapeutic target to enhance the radiation-induced immune response against tumor cells.

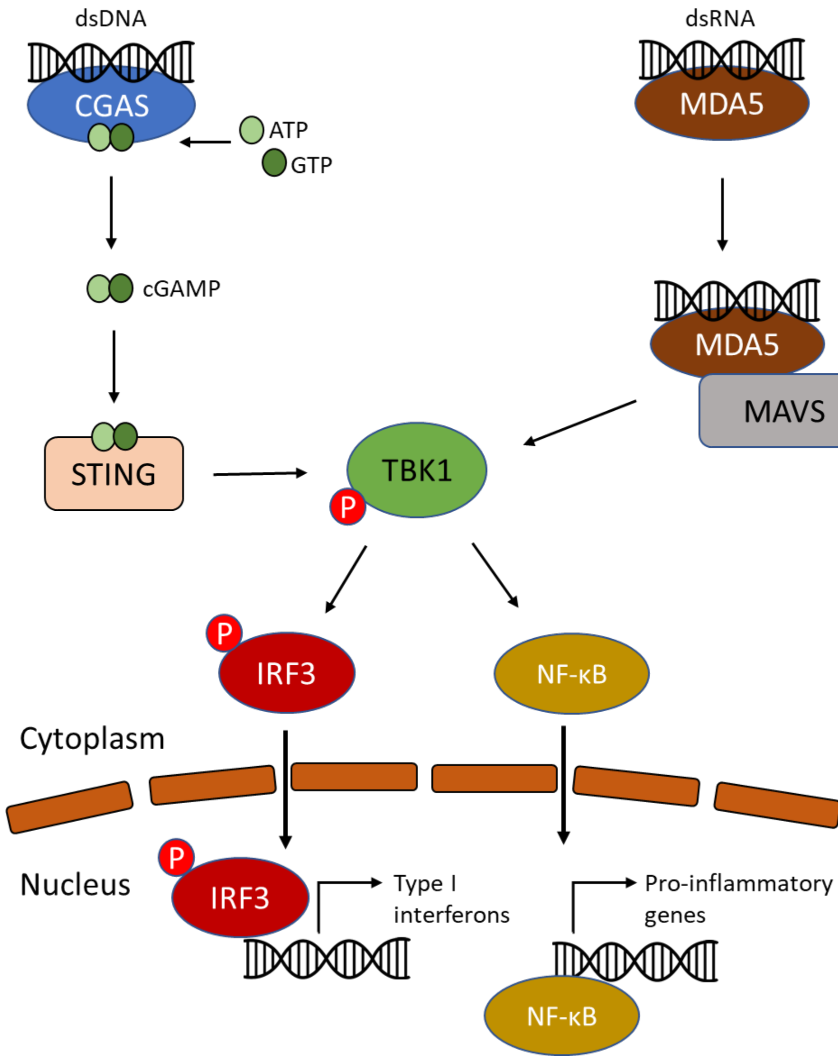


Figure 5: cGAS/STING and MDA5/MAVS dsDNA/dsRNA sensing pathways. cGAS/STING and MDA5/MAVS phosphorylate TBK1 in response to dsDNA/dsRNA, activating downstream transcription factors IRF3 and NF-κB and promoting transcription of type I IFNs and pro-inflammatory genes.

2.2 Materials and methods

2.2.1 Cell culture

HEK 293T, A549, HeLa, B16F10, and 4T1 cell lines were obtained from the Cell Culture Facility at Duke University (Durham, NC) and routinely cultured at 37°C and

5% CO_2 in supplemented DMEM - high-glucose Dulbecco's modified Eagle medium (Sigma-Aldricht® LLC, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Corning® Inc, Corning, NY) and 1x penicillin/streptomycin (Gibco®, Thermo Fisher Scientific™ Inc, Waltham, MA). Cell stocks were stored at -80°C in a solution of 50% heat-inactivated fetal bovine serum, 40% supplemented DMEM, and 10% dimethylsulfoxide (DMSO) (Sigma-Aldricht® LLC, St. Louis, MO).

Cells were routinely passaged via trypsinization by 0.25% Trypsin-EDTA (Gibco®, Thermo Fisher Scientific™ Inc, Waltham, MA) every 2 days. Monthly tests for mycoplasma contamination were conducted using PCR. Contaminated cells were treated for 2 weeks with Plasmocin™ (InvivoGen, San Diego, CA) according to manufacturer's protocol.

2.2.2 *in vitro* ionizing radiation

in vitro irradiation was performed in an X-RAD 320 irradiator (Precision X-ray Inc., North Branford, CT) according to dosage calculations conducted by the Duke Shared Irradiator Resource. Cells were irradiated as an adherent monolayer culture in a single X-ray dose at a dose rate of 2.31Gy/min. Sham irradiated cells were transported and kept in the same environment for the same period of time as irradiated cells.

2.2.3 Immunofluorescence staining and imaging

Cells were cultured in 35mm glass bottom plates (MatTek, Ashland, MA) prior to irradiation and staining. Cells were fixed in 4% PFA, followed by membrane permeabilization with 0.1% Triton X-100 (Sigma-Aldrich® LLC, St. Louis, MO). After blocking in 1% BSA and 22.52mg/mL glycine in PBST (1x Phosphate Buffered-saline, 0.1% Tween® 20), cells were incubated overnight at 4°C in primary antibody in 1% BSA in PBST. After 3 washes in PBS, cells were incubated in the dark for 1 hour with Alexa Fluor secondary antibodies (Invitrogen, Carlsbad, CA), followed by 3 more washes in PBS. Cellular fluorescence was imaged on a TCS SP5 inverted confocal microscope (Leica, Wetzlar, Germany) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD) (Schneider, Rasband, & Eliceiri, 2012). J2 antibody was purchased from Scicons (Szirák, Hungary).

2.2.4 CRISPR/Cas9 plasmid generation

pLentiCRISPR plasmids were provided by Fang Zhang (Addgene plasmid #52961; http://n2t.net/addgene:52961; RRID:Addgene_52961), and sgRNA sequences were designed using CHOPCHOP (Table 1) (Labun et al., 2019) and synthesized by IDT (Coralville, IA). All-in-one CRISPR/Cas9 plasmid generation was carried out using a protocol described previously (Sanjana, Shalem, & Zhang, 2014). Briefly, pLentiCRISPR plasmid was digested using Esp3I (BsmBI) restriction enzyme (Thermo Fisher Scientific™ Inc, Waltham, MA) according to manufacturer's protocol. The backbone fragment was then gel purified from a 1% agarose gel using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific™ Inc, Waltham, MA) according to manufacturer's protocol. Oligos were phosphorylated and annealed using T4 PNK and Ligation Buffer (New

England Biolabs, Ipswich, MA) before ligation overnight to the purified plasmid backbone with T4 DNA Ligase (New England Biolabs, Ipswich, MA).

Ligated product was transformed into $CaCl_2$-transformed competent bacteria for plasmid expansion. Bacteria were grown for 16 hours at 37°C shaking at 250 RPM in Luria broth (Invitrogen, Carlsbad, CA). Plasmid extraction was performed using GenElute™ Plasmid Midiprep Kit (Sigma-Aldrich® LLC, St. Louis, MO) according to manufacturer's protocol. Plasmids are eluted into 0.1x Tris-EDTA (TE) buffer (Sigma-Aldrich® LLC, St. Louis, MO). Extracted plasmid purity and concentration was checked using a Synergy™ H1 Hybrid Reader (BioTek Instruments, Winooski, VT) before storage at -20°C for future use.

2.2.5 Viral vectors and transduction

Virus generation was performed in HEK 293T cells using psPAX2 and pMD2.G plasmids provided by Didier Trono (Addgene plasmid #12259; http://n2t.net/addgene:12259; RRID:Addgene_12259 and Addgene plasmid12260; http://n2t.net/addgene:12260; RRID:Addgene_12260).

Transfection mixture consists of 20µg CRISPR/Cas9 plasmid, 15µg psPAX2 packaging plasmid, and 6µg pMD2.G envelope plasmid in 1mL of final concentration 1x Hepes-buffered saline. 50µL of 2.5M $CaCl_2$ is then added dropwise while vortexing, and the mixture is incubated at room temperature for 20 minutes before being added dropwise to monolayer cultured HEK 293T cells at 70-80% confluency. 12-14 hours later, transfection media is replaced with 10.5mL of fresh supplemented DMEM. 8 hours later, transfected plates are moved to 32°C. Supernatant is harvested 16 hours later and filtered through a 0.45μm filter prior to aliquot and storage at -80°C for future use.

Target cells are infected with a 1:1 mixture of filtered supernatant and fresh supplemented DMEM, with the addition of 10μg/mL polybrene. After 16 to 24 hours, the supernatant is replaced with fresh supplemented DMEM, and treated with either puromycin or geneticin (Gibco®, Thermo Fisher Scientific™ Inc, Waltham, MA) for selection depending on resistance gene. Working concentrations for puromycin and geneticin were determined empirically for each cell line used. Cells were passaged for 2 weeks in selective media prior to harvest and western blotting for detection of knockouts.

2.2.6 Western blots

Harvested cells are lysed in RIPA buffer with protease inhibitor cocktail (Sigma-Aldrich® LLC, St. Louis, MO) for 1 hour, constantly rocking at 4°C. The supernatant is extracted and protein concentration is checked using a Synergy™ H1 Hybrid Reader (BioTek Instruments, Winooski, VT) against a known BSA curve. Samples are then diluted into 2x Laemmli Sample Buffer (Bio-Rad, Hercules, CA) before denaturation by boiling for 5 minutes. Protein samples are stored at -20°C for future use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were made inhouse, at a range of 8-12% acrylamide, according to standard protocols.

Equal amounts of protein are loaded into individual wells of SDS-PAGE gels and run at a constant voltage of 120V using a PowerPac™ Basic (Bio-Rad, Hercules, CA) in 1x running buffer. 10x running buffer is composed of 144g glycine (Sigma-Aldrich® LLC, St. Louis, MO), 30.6g Tris base (Sigma-Aldrich® LLC, St. Louis, MO), and 10g SDS in 1L dH_2O. Protein is then transferred onto methanol-treated 2µm PVDF membranes (Bio-Rad, Hercules, CA) at a constant voltage of 70V for 2 hours at 4°C in 1x transfer buffer. 10x transfer buffer is composed of 29g glycine, 58g Tris base, and 3.7g SDS in 1L dH_2O. After transfer, membranes are blocked in 5% milk in Tris-buffered saline with 1% Tween-20 (TBST) (Sigma-Aldrich® LLC, St. Louis, MO) for 1 hour. Membranes are incubated with primary antibody constantly rocking at 4°C for 16 hours in antibody dilution buffer (4% BSA, 1% sodium azide). Appropriate antibody dilutions are determined empirically per antibody.

After primary antibody incubation, membranes are washed 3 times for 5 minutes each in 5% milk in TBST, followed by incubation in HRP-conjugated secondary antibody in antibody dilution buffer for 1 hour at room temperature. Membranes are then washed 3 more times for 5 minutes each in TBST, with 1 final rinse in 1x PBS before immersion in SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific™ Inc, Waltham, MA) to visualize protein bands. Antibodies used are as follows: KAP1 (Bethyl Laboratories Inc., Montgomery, TX), IFIH1/MDA5 and GAPDH

(ProteinTech, Rosemont, IL), and TBK1, Phospho-TBK1 (Ser172), and MAVS (Cell Signaling Technologies, Danvers, MA).

2.2.7 Single clonal selection

Single clonal selection was conducted to find individual clonal CRISPR knockout cells from a mixed population. Virally infected cells were counted, and 100 cells were diluted into 20mL supplemented DMEM and split into a 96 well plate at 180μL per well. Cells were grown for 2 weeks before single colonies were transferred into 6-well plates for analysis by western blot. Knockout cells used for this study consisted of 3 individual confirmed single clone knockouts combined into one mixture.

2.2.8 *in vitro* growth assay

Cells were harvested and counted, and 10^4 live cells were seeded into individual wells of 6-well plates. Cells were harvested and live cells counted in 24-hour intervals and represented by fold-change over initial seeding density.

2.2.9 Clonogenic assay

Cells were harvested and counted, with varying numbers of cells seeded depending on irradiation dose. 200 cells were seeded for sham and 2Gy X-ray irradiation, 400 cells were seeded for 4Gy X-ray irradiation, 1000 cells were seeded for 6Gy X-ray irradiation, and 2×10^4 cells were seeded for 8Gy X-ray irradiation. Cells were irradiated in a single dose as previously described, followed by incubation under standard cell growth conditions for 14 days.

Plates were stained for 5 minutes in 0.5% crystal violet (Sigma-Aldrich® LLC, St. Louis, MO) in 80% methanol solution. Following staining, plates were rinsed 6x in distilled water prior to imaging and counting of colonies. Colonies were counted if there were a total of 50 cells or more. Plating efficiency was calculated by the average colonies observed in vector control cells treated with sham irradiation divided by 200. Surviving fraction of cells treated with irradiation was then calculated by (colonies counted) / [Cells seeded x (plating efficiency / 100)].

2.2.10 Quantitative reverse transcription PCR (RT-qPCR)

Extraction of total RNA from cells was performed using the E.Z.N.A.® Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA) following manufacturer's protocol, including DNAse treatment. RNA was eluted into dH_2O and stored at -80°C for future use. Synthesis of complementary DNA library was performed on 1μg of RNA using the SuperScript® II Reverse Transcriptase Kit with random hexamers (Invitrogen, Carlsbad, CA). Quantitative PCR amplification was performed with 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK) on a ViiA 7 Real-Time PCR System (Applied Biosystems®, Foster City, CA).

Target genes were normalized to *GAPDH* in human cells and *ACTB* in mouse cells. Endogenous retroviral primers were selected as described elsewhere (Chatterjee et al., 2018). All other primers were generated using the NCBI Primer Design Tool (Ye et al., 2012). Primers were synthesized by IDT and are listed in Table 1.

2.2.11 Murine tumor xenograft model

All animal experiments were approved by the Duke University Institutional Animal Care and Use Committee. Specific-pathogen-free C57BL/6 and Balb/C female mice were purchased from Jackson Laboratory (Bar Harbor, ME) while NOD *scid* gamma (NSG) and nude female mice were obtained from the Division of Lab Animal Resources at Duke University (Durham, NC). Upon receipt, up to five mice per cage were house in a specific-pathogen-free facility under constant temperature at 25°C and a 12:12 hour light-dark schedule and fed a standard *ad libitum* mouse diet with water.

After at least 4 days of acclimatization, tumor cells were injected into the right hindlimb of each mouse, and tumor sizes were tracked every other day. Mice treated with irradiation had their right hind limb irradiated with the remainder of the body under lead shielding in an XRAD 320 (Precision X-ray Inc.) with a single 8Gy X-ray dose at a dose rate of 3.28 Gy/min. Mice were euthanized when tumor size reached endpoint, set at 2000 mm^3 as defined by volume = 0.5 x length x $width^2$. Lungs were extracted at endpoint and weighed prior to preservation in 10% formalin.

2.2.12 Statistical analysis

Quantitative data are expressed as mean ± the standard error of the mean (SEM). Comparisons between groups were performed using two-tailed unpaired Student's *t* test or two-way analysis of variance (ANOVA) as appropriate. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using JMP Pro version 14.0 software.

Table 1: Primers used for CRISPR/CAS9 and RT-qPCR

Gene	Species	Usage	Forward primer	Reverse primer (if applicable)
TRIM28	Human	sgRNA	GTTCGCATCCTGGGCGTCGG	
TRIM28	Human	sgRNA	AATTATTTCATGCGTGATAG	
TRIM28	Mouse	sgRNA	CGCCGCAGCGAATAATTCGG	
TRIM28	Mouse	sgRNA	TATGCGTGATAGTGGCAGTA	
MAVS	Mouse	sgRNA	GCCACCAGACATCCTCGCGA	
MAVS	Mouse	sgRNA	GAGGACAAACCTCTTGTCTG	
GAPDH	Human	qPCR	AGGGCTGCTTTTAACTCTGGT	CCCCACTTGATTTTGGAGGGA
MER21C	Human	qPCR	GGAGCTTCCTGATTGGCAGA	ATGTAGGGTGGCAAGCACTG
MER57B1	Human	qPCR	CCTCCTGAGCCAGAGTAGGT	ACCAGTCTGGCTGTTTCTGT
MLT1C49	Human	qPCR	TATTGCCGTACTGTGGGCTG	TGGAACAGAGCCCTTCCTTG
ACTB	Mouse	qPCR	GAAATCGTGCGTGACATCAAA	TGTAGTTTCATGGATGCCACA
IFIT1	Mouse	qPCR	CAAGGCAGGTTTCTGAGGAG	GACCTGGTCACCATCAGCAT
CXCL10	Mouse	qPCR	GCCGTCATTTTCTGCCTCA	CGTCCTTGCGAGAGGGATC
ISG15	Mouse	qPCR	CTAGAGCTAGAGCCTGCAG	AGTTAGTCACGGACACCAG
CCL5	Mouse	qPCR	CAAGTGCTCCAATCTTGCAGTC	TTCTCTGGGTTGGCACACAC

IFNB1	Human	qPCR	GCGACACTGTTCGTGTTGTC	GCCTCCCATTCAATTGCCAC
IFNL2	Human	qPCR	TCCAGTCACGGTCAGCA	CAGCCtCAGAGTGTTTCTTCT

2.3 Results

2.3.1 Ionizing radiation stimulates ERV transcription in the form of dsRNA, initiating an immune response

Previous studies have demonstrated upregulation of ERV transcripts in response to radiation (J. R. Lee et al., 2012) but did not investigate whether those transcripts could effect a downstream innate immunity response. Thus, we first sought to confirm if increased ERV transcription due to radiation could be detected in the form of cytosolic dsRNA, which is our hypothesized effector molecule responsible for initiating the innate antiviral immune response.

Using the J2 antibody which is specific for dsRNA (Weber, Wagner, Rasmussen, Hartmann, & Paludan, 2006), we immunostained A549 human lung cancer cells that had been treated 5 days prior with either sham or 8Gy of X-ray irradiation. As predicted, we observed significant upregulation of cytosolic J2 staining in the irradiated A549 cell population (Figure 6A-B). Similar findings were also observed in B16F10 mouse melanoma cancer cells treated with 8Gy of X-ray irradiation, demonstrating general applicability in mammalian cells (Figure 6C-D). In both cell lines, relative integrated density was significantly upregulated by 10 to 20-fold, indicating a dramatic increase in dsRNA levels.

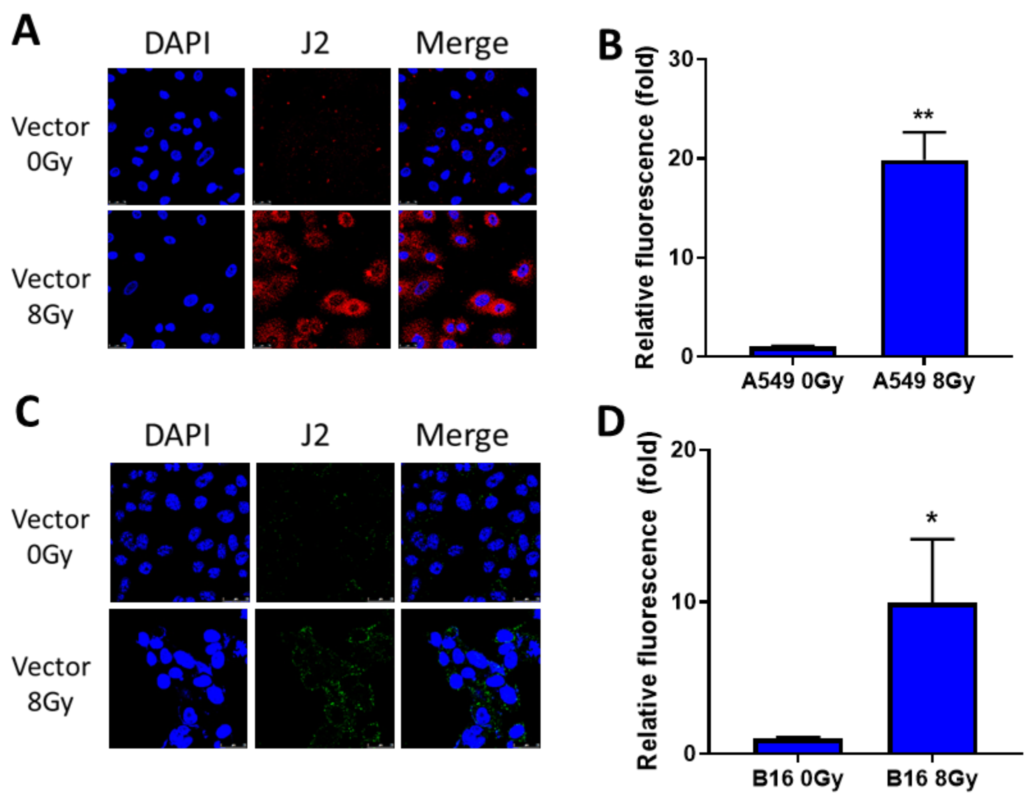


Figure 6: Radiation induces J2 expression *in vitro* in A549 and B16 cells.
(A-B) Representative immunofluorescence images and quantification, respectively, of J2 antibody staining of vector control A549 cells 5 days after treatment with sham or 8Gy X-ray irradiation. (C-D) Representative immunofluorescence images and quantification, respectively, of J2 antibody staining of vector control B16F10 cells 5 days after treatment with sham or 8Gy X-ray irradiation. Error bars represent SEM. *p<0.05, **p<0.01, as determined by unpaired Student's T test.The upregulation of cytosolic dsRNA was consistent with increased ERV transcripts, as RT-qPCR of RNA transcripts indicated that expression of transcripts specific to ERVs was significantly upregulated in irradiated A549 cells (Figure 7A). MER21C, MER57B1, and MLT1C49 represent a selection of HERV LTRs located within the human genome (Chatterjee et al., 2018). DsRNA upregulation also correlated with induction of downstream ISGs, as mRNA expression

of Interferon Induced Protein With Tetratricopeptide Repeats (IFIT1), C-X-C Motif Chemokine Ligand 10 (CXCL10), Interferon-Stimulated Gene 15 (ISG15), and C-C Motif Chemokine Ligand (CCL5) were all substantially upregulated in irradiated B16F10 cells (Figure 7B). IFIT1, CXCL10, ISG15, and CCL5 all represent ISGs previously shown to stimulate host immune activation (Araujo et al., 2018; Jeon, Yoo, & Chung, 2010; McDermott et al., 2012; Tokunaga et al., 2018).

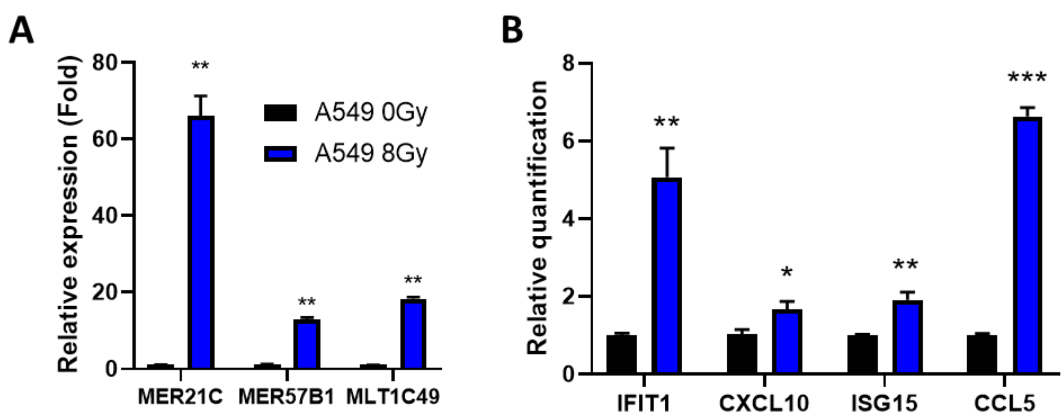


Figure 7: Radiation upregulates ERV and ISG transcription.
(A) Quantification of selected ERV mRNA by RT-qPCR in vector control A549 cells 5 days after treatment with sham or 8Gy X-ray irradiation. (B) Quantification of selected ISG mRNA by RT-qPCR in vector control B16F10 cells 5 days after treatment with sham or 8Gy X-ray irradiation. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001 as determined by unpaired Student's T test.

2.3.2 Knockout of the transcriptional corepressor KAP1 sensitizes cells to IR

As described previously, KAP1 is responsible for restoring heterochromatin marks at ERV regions once DNA repair is complete (White et al., 2012). Depletion of KAP1 has been demonstrated to result in the inability of the cell to restore certain heterochromatin marks, specifically H3K9 trimethylation (Jang et al., 2018). Therefore, we hypothesized that knockout of KAP1 would lead to enhanced effects of IR-induced immune signaling, due to heterochromatin relaxation generating increased ERV transcription in the form of dsRNA.

Using CRISPR/Cas9 gene editing, we successfully knocked out KAP1 in a variety of cancer cell lines (Figure 8A). Single clone knockouts were selected for each cell line, and experiments were performed using a mixture of three individual single clone knockouts. Interestingly, in our hands, KAP1 knockout does not appear to have any effect on *in vitro* proliferation rates (Figure 8B), in contrast to other studies which have shown substantial decreases in proliferation rates (Addison et al., 2015). The effect on cellular proliferation may be cell-type specific, and multiple conflicting studies means the true effect of KAP1 depletion on cellular proliferation remains an open question within the field (Burck et al., 2016).

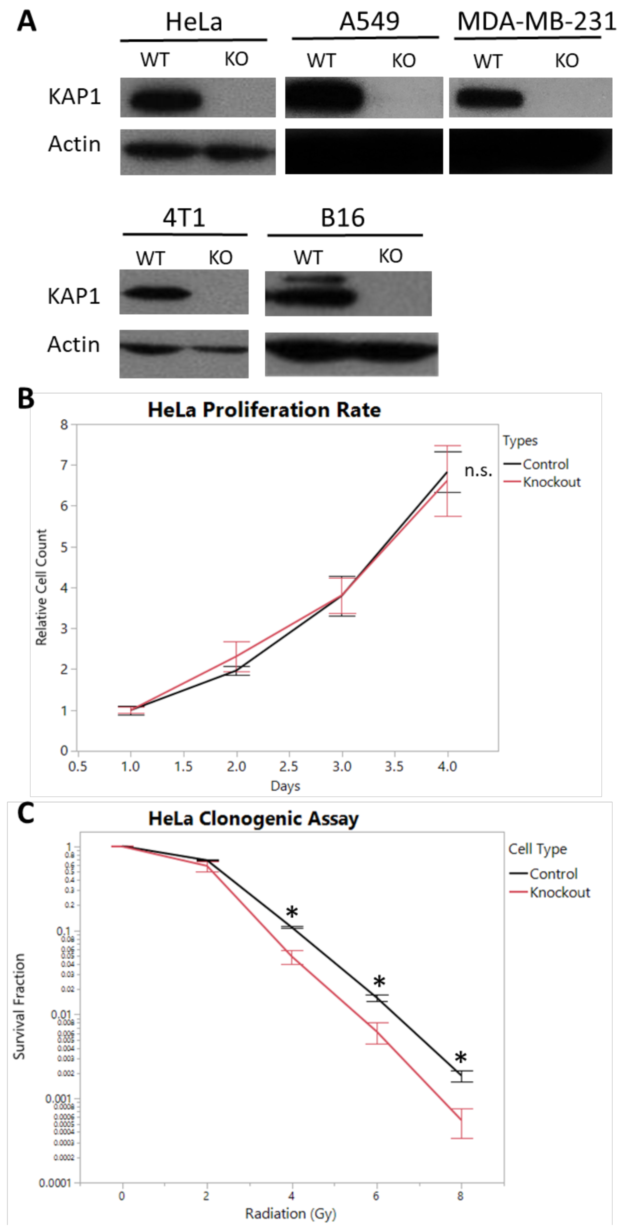


Figure 8: CRISPR-Cas9 knockout of KAP1.

(A) Representative western blots of CRISPR-Cas9-mediated KAP1 knockouts in HeLa, A549, MDA-MB-231, B16F10, and 4T1 cell lines. (B) *in vitro* proliferation rates of HeLa vector control cells compared to KAP1 knockouts. (C) Radiation clonogenic cell survival assay of HeLa vector control compared to KAP1 knockouts subjected to 0, 2, 4 6, or 8Gy X-ray irradiation. Error bars represent SEM. *p<0.05, ns = not significant, as determined by unpaired Student's T test.

However, KAP1 depletion does have a significant effect on radiation sensitivity, as determined by clonogenic assay (Figure 8C). A significant difference in survival appears in the linear region of the survival curve at 4, 6, and 8Gy X-ray irradiation, but there is no difference in the "shoulder" region of the curve. Based on the linear-quadratic model of radiation biology, this implies that disparity in survival and colony formation is not due to deficiency in DNA damage repair processes, but rather through other cellular effects as a response to radiation. This is an odd result, as KAP1 is known to play a significant role in the DNA damage repair process through mediation of heterochromatin relaxation (Cann & Dellaire, 2011). However, immunofluorescence imaging of γ-H2AX foci after 8Gy X-ray irradiation over 24 hours post-IR demonstrates that while there is an initial delay in DNA damage repair, KAP1 knockout cells are still DNA repair-competent, and counts of γ-H2AX foci are on par with vector control by the 24-hour mark (Figure 9). Therefore, we hypothesized that the difference in radiation sensitivity could instead be due to increased ERV transcription and innate antiviral immune signaling.

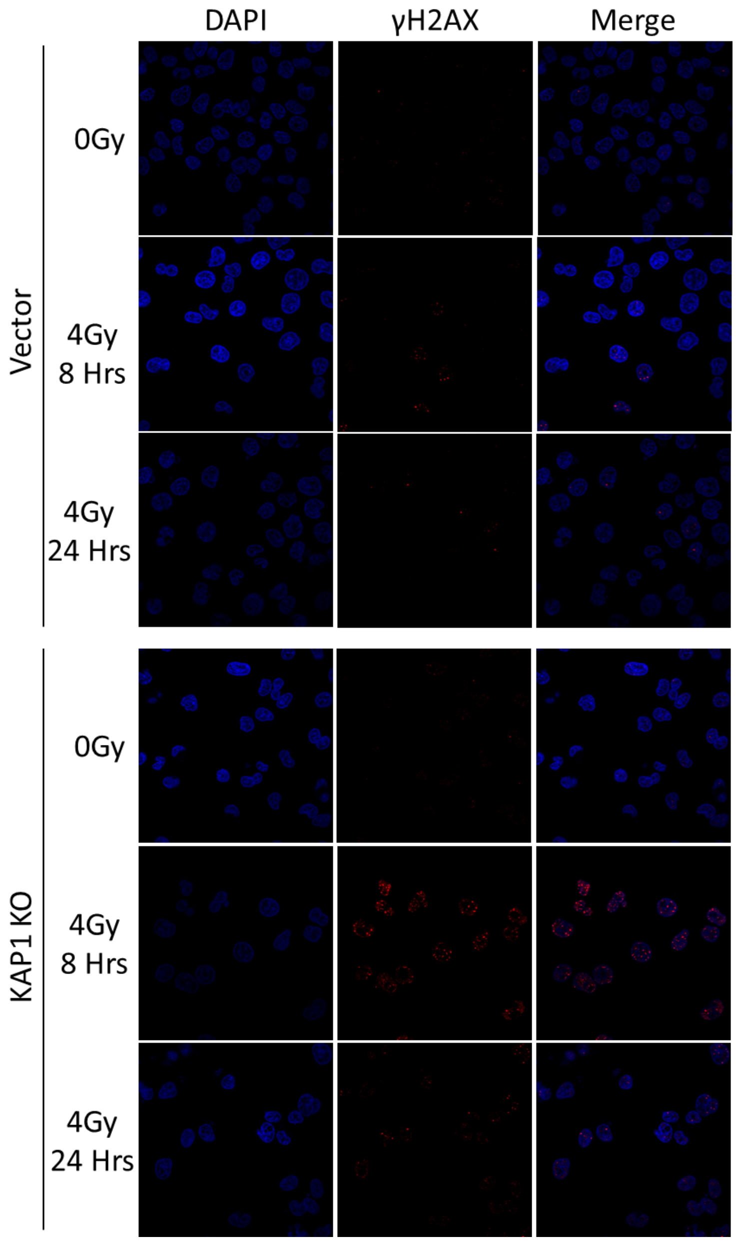


Figure 9: γH2AX staining in irradiated cells.

Representative immunofluorescence images of γH2AX staining as a marker for DNA damage over time in HeLa vector control or KAP1 knockout cells treated with 0Gy or 8Gy X-ray irradiation. KAP1 knockout cells are initially deficient in DNA damage repair, but return to control levels of γH2AX staining by 24 hours.

2.3.3 KAP1 knockout enhances IR-induced ERV transcription, type I IFN production, and ISG activation

To confirm our hypothesis, we treated KAP1 knockout A459 and B16F10 cells with sham or 8Gy of X-ray irradiation and immunostained with J2 antibody 5 days later. While KAP1 knockout alone generated a small but significant increase in J2 stain fluorescence, the combination of KAP1 knockout and 8Gy X-ray irradiation had a large multiplicative effect on the order of 20 to 40-fold relative integrated density increase (Figure 10). Additionally, RT-qPCR analysis of ERV transcripts recapitulate these results, with expression levels of MER21C, MER57B1, and MLT1C49 RNA transcripts significantly upregulated after combination KAP1 knockout and 8Gy X-ray irradiation (Figure 11). These results suggest a synergistic effect of KAP1 knockout with irradiation driving dsRNA expression, possibly due to cellular inability to restore heterochromatin marks after DNA repair has completed.

As demonstrated earlier (Figure 7B), radiation alone was sufficient to drive expression of downstream ISGs including CXCL10 and ISG15. Mechanistically, cytosolic dsRNA is sensed by MDA5, which then activates MAVS and initiates a signaling cascade involving phosphorylation of TBK1 (Fang et al., 2017; Reikine et al., 2014). To confirm this pathway was activated, we performed western blots on irradiated and KAP1 knockout B16F10 cells for key members of the signaling pathway (Figure 12A). MDA5 protein level is upregulated in all samples aside from non-irradiated vector control, implying that a positive feedback loop may be present where dsRNA induction drives more MDA5 expression. Notably, levels of phosphorylated TBK1 are higher in both irradiated samples, indicating IR-induced activation of the TBK1 signaling cascade.

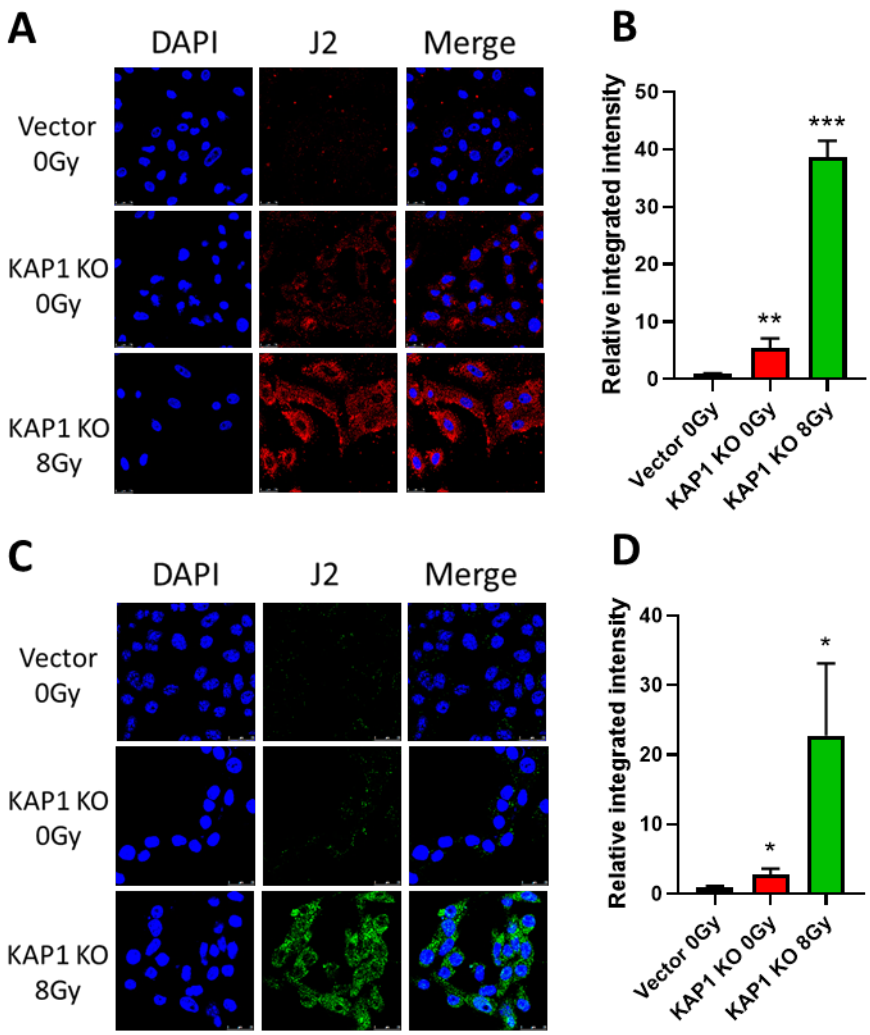


Figure 10: KAP1 knockout enhances J2 staining in irradiated A549 and B16 cells.

(A-B) Representative immunofluorescence images and quantification, respectively, of J2 antibody staining of A549 vector control or KAP1 knockout cells treated with sham or 8Gy X-ray irradiation. (C-D) Representative immunofluorescence images and quantification, respectively, of J2 antibody staining of B16F10 vector control or KAP1 knockout cells treated with sham or 8Gy X-ray irradiation. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001 as determined by unpaired Student's T test.

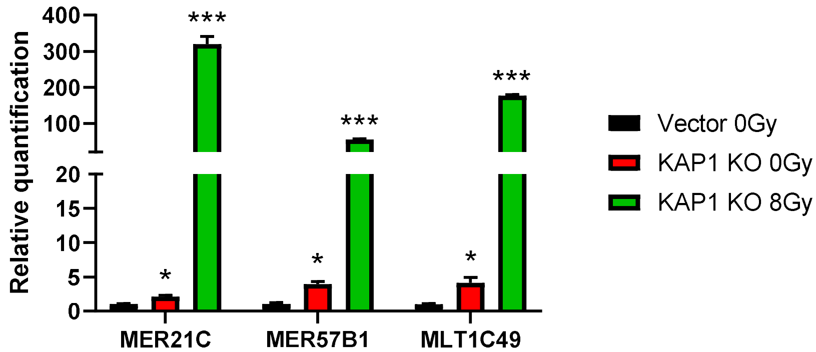


Figure 11: KAP1 knockout enhances ERV transcription in irradiated A549 cells. Quantification of selected ERV mRNA by RT-qPCR in A549 vector control or KAP1 knockout cells 5 days after treatment with sham or 8Gy X-ray irradiation. KAP1 knockout has a small effect on ERV transcription alone, and strongly enhances ERV transcription in combination with radiation. Error bars represent SEM, *p<0.05, ***p<0.001 as determined by unpaired Student's T test.

Phosphorylated TBK1 activates transcription factors such as IRF3, NF-κB, p300, and CREB-binding protein (CBP) in order to upregulate transcription of type I IFNs and downstream ISGs (Liu et al., 2011; X. Ma et al., 2012; Yoneyama et al., 1998). Therefore, we performed RT-qPCR to quantify mRNA expression levels of downstream IFNs and ISGs. As shown in (Figure 12B), KAP1 knockout alone is sufficient to induce a small but significant increase in Interferon Beta 1-Alpha (IFNβ), and the effect is significantly enhanced with the addition of radiation. Interestingly, we also saw a similar significant increase in expression of Interleukin 28-A (IL28A, also known by Interferon Lambda-3) in KAP1 knockout cells, which was also enhanced by irradiation. IL28A is part of the type III interferon family, which is known to be upregulated in response to viral infection (Donnelly & Kotenko, 2010; Jewell et al., 2010), supporting our hypothesis that radiation and KAP1 knockout effects are mechanistically functioning through stimulation of the innate antiviral immunity pathway.

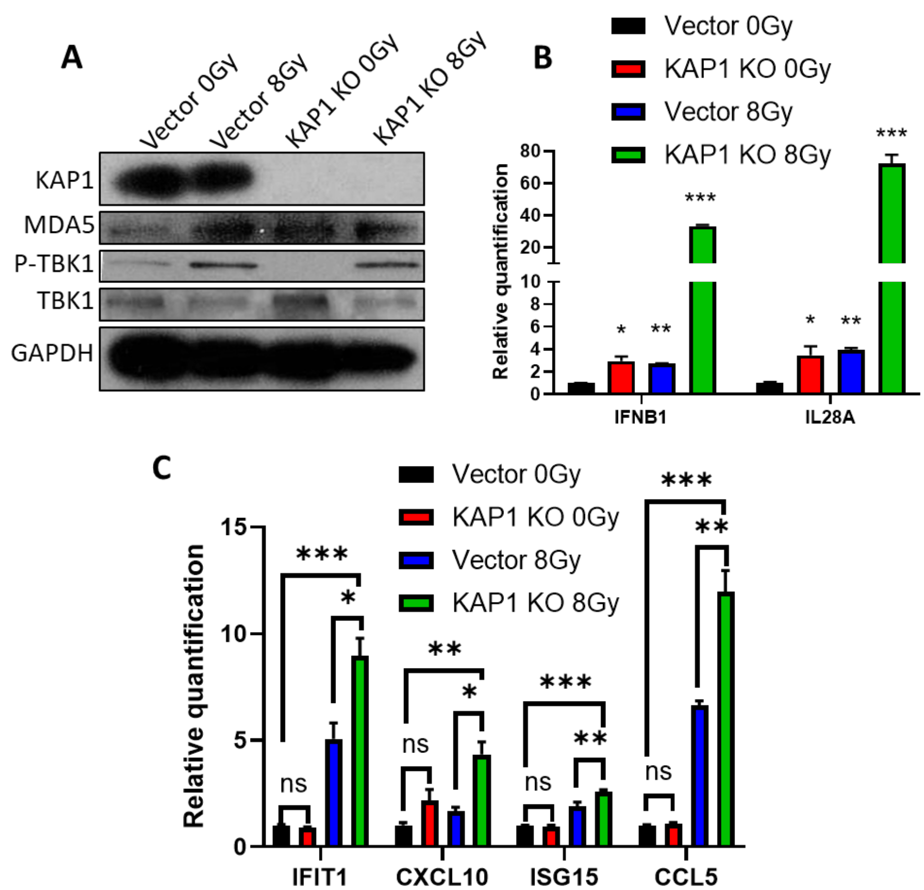


Figure 12: The combination of radiation and KAP1 knockout increases TBK1 phosphorylation, IFN expression, and ISG expression.

(A) Western blots staining for MDA5/MAVS pathway members in B16F10 vector control or KAP1 knockout cells 5 days after treatment with sham or 8Gy X-ray irradiation. (B) Quantification of selected IFN mRNA by RT-qPCR in A549 vector control or KAP1 knockout cells 5 days after treatment with sham or 8Gy X-ray irradiation. (C) Quantification of selected ISG mRNA by RT-qPCR in B16F10 vector control or KAP1 knockout cells 5 days after treatment with sham or 8Gy X-ray irradiation. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001, ns = not significant, as determined by unpaired Student's T test.

Despite the significant increase in type I and type III IFN production, KAP1 knockout alone was insufficient to effect a significant change in downstream ISG transcription (Figure 12C), which is somewhat unsurprising considering the limited magnitude change in IFN transcription. However, KAP1 knockout in combination with irradiation significantly upregulates downstream ISG transcription over that of irradiated cells alone, indicating a synergistic effect between KAP1 knockout and radiation-induced ISG induction.

Upregulation of ISG expression may provide an explanation for why KAP1 knockouts are radiation sensitive despite not being deficient in DNA damage repair. Many ISGs are associated with cell death pathways, where upregulation is associated with apoptosis and autophagy (de Veer et al., 2001). For example, ISG15 is known to promote apoptosis in cervical cancer via p53 (M. J. Zhou et al., 2017), while IFIT1 is known to associate with IFIT2 to mediate apoptosis through the mitochondrial pathway (Stawowczyk, Van Scoy, Kumar, & Reich, 2011). Because cytoplasmic dsRNA co-opts the innate antiviral immunity pathway, the artificial state of viral infection caused by excessive ERV transcription and dsRNA expression may be upregulating IFNs and ISGs and driving the cell towards apoptosis.

2.3.4 Loss of MAVS inhibits TBK1 phosphorylation and downstream ISG induction

Aside from the MDA5/MAVS pathway, the cGAS/STING pathway is also capable of phosphorylating TBK1 as part of its signaling cascade (Z. Ma & Damania, 2016). However, it has been previously demonstrated that MAVS depletion reduces the ISG response to radiation (Ranoa et al., 2016), indicating that in the context of radiation alone, the cGAS/STING pathway is not completely responsible for the effects of ISG induction. In order to confirm that the KAP1-knockout effect of increased ISG expression is also due to the MDA5/MAVS pathway and not the cGAS/STING pathway, we used CRISPR/Cas9 to generate a KAP1/MAVS double knockout in B16F10 cells. Western blot analysis shows that depletion of MAVS abrogates TBK1 phosphorylation in irradiated KAP1 knockout cells, without any change in overall TBK1 levels (Figure 13A). Additionally, RT-qPCR analysis demonstrates that mRNA expression levels of downstream ISGs are downregulated upon MAVS knockdown (Figure 13B). These results indicate that a substantial portion of ISG activation is due to MDA5 and MAVS signaling through phospho-TBK1, as opposed to the cGAS/STING signaling pathway.

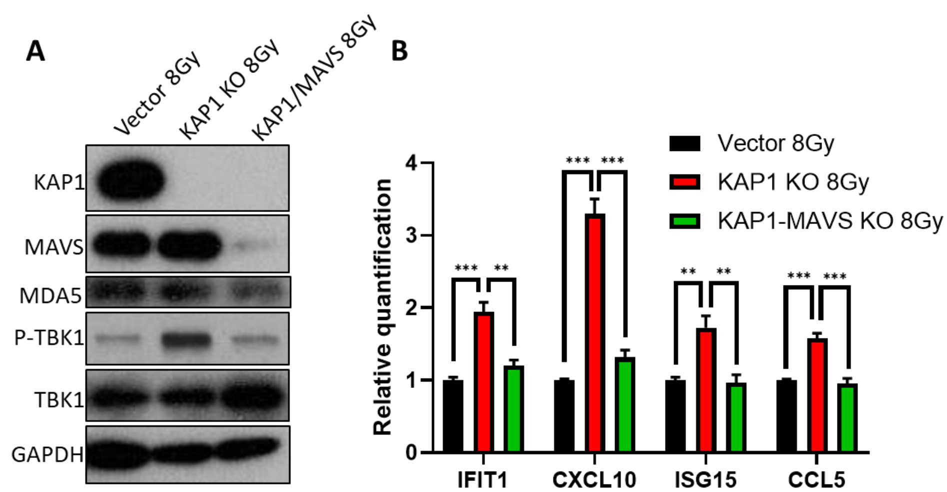


Figure 13: MAVS knockout abrogates the effect of KAP1 knockout.
(A) Western blots staining for MDA5/MAVS pathway members in B16F10 vector

control, KAP1 knockout, or KAP1/MAVS double knockout cells 5 days after treatment with 8Gy X-ray irradiation. (B) Quantification of selected ISG mRNA by RT-qPCR in B16F10 vector control, KAP1 knockout, or KAP1/MAVS double knockout cells 5 days after treatment with 8Gy X-ray irradiation. **p<0.01, ***p<0.001, ns = not significant, as determined by unpaired Student's T test.

2.3.5 KAP1 knockout in B16F10 cells enhances tumor response to radiotherapy in mice

While it is clear that KAP1 knockout in combination with irradiation can stimulate the innate antiviral immunity pathway and downstream IFN and ISG expression, it was unknown whether this would have a phenotypically visible effect in animals. Therefore, we next moved to an *in vivo* mouse model to determine the effects of ISG activation on tumor growth in mice with a competent host immune system. We inoculated 10^5 B16F10 vector control or KAP1 knockout cells subcutaneously into the right hindlimb of syngeneic C57 black mice. Tumors received either sham or 8Gy X-ray irradiation 8 days post-inoculation.

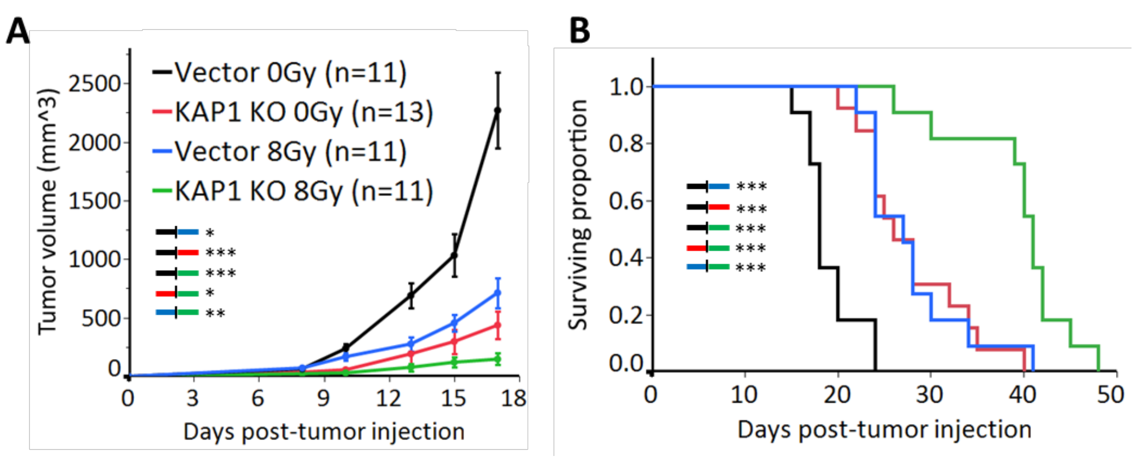


Figure 14: KAP1 knockout inhibits B16F10 melanoma growth and enhances survival in syngeneic C57BL/6 mice.

(A-B) Tumor growth curve and overall survival curve, respectively, in immunocompetent C57BL/6 mice inoculated with 10^5 B16F10 vector control or KAP1 knockout cells and treated with sham or 8Gy X-ray irradiation 8 days post-inoculation. Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001, as determined by two-way ANOVA (A) or log-rank test (B).

Surprisingly, for B16F10 tumors, KAP1 knockout alone is sufficient to cause a significant delay in tumor growth and commensurate prolonged survival (Figure 14), despite *in vitro* data only showing minor effects of KAP1 knockout alone. KAP1 knockout tumor growth and survival is roughly on par with that of irradiation-treated vector control tumors, with the combination group showing the largest effect of tumor growth delay and survival benefit. KAP1 knockout being sufficient to inhibit *in vivo* tumor growth is a striking result, especially since B16F10 is known to be a poorly immunogenic tumor line (J. Wang, Saffold, Cao, Krauss, & Chen, 1998), and indicates that targeting ERVs and dsRNA expression could be relevant in treating human cancers.

2.3.6 KAP1 knockout in 4T1 cells synergizes with radiotherapy to inhibit tumor growth and metastasis

To confirm if the effect of KAP1 knockout could be extended to a different tumor model, we switched to the 4T1 mouse breast cancer model, which is known to be highly aggressive and metastatic (Heppner, Miller, & Shekhar, 2000). We inoculated 5×10^5 4T1 vector control of KAP1 knockout cells subcutaneously into the right hindlimb of syngeneic BALB/c white mice. Tumors received either sham or 8Gy X-ray irradiation 8 days post-inoculation. In contrast to the B16F10 tumor model, neither KAP1 knockout nor irradiation alone is sufficient to inhibit tumor growth or enhance survival (Figure

15A-B). The combination of KAP1 knockout and irradiation is the only group to show a significant difference in tumor growth and survival, implying a synergistic effect between KAP1 knockout and radiotherapy.

As 4T1 is highly metastatic and is known to metastasize to the lungs (Pulaski & Ostrand-Rosenberg, 2001), we extracted the lungs at tumor endpoint to determine if KAP1 knockout or irradiation had an effect on lung metastases. Interestingly, KAP1 knockout alone was sufficient to inhibit the formation of lung metastases with or without radiation, as determined by average lung mass comparison and counted metastatic surface nodules (Figure 15C-E). This suggests a scenario where KAP1 knockout-mediated 4T1 immune stimulation is insufficient to recruit the host immune system to reduce tumor mass within the primary tumor, but is sufficient to attract the immune system to find and destroy migrating metastatic cells before they can develop into secondary tumors.

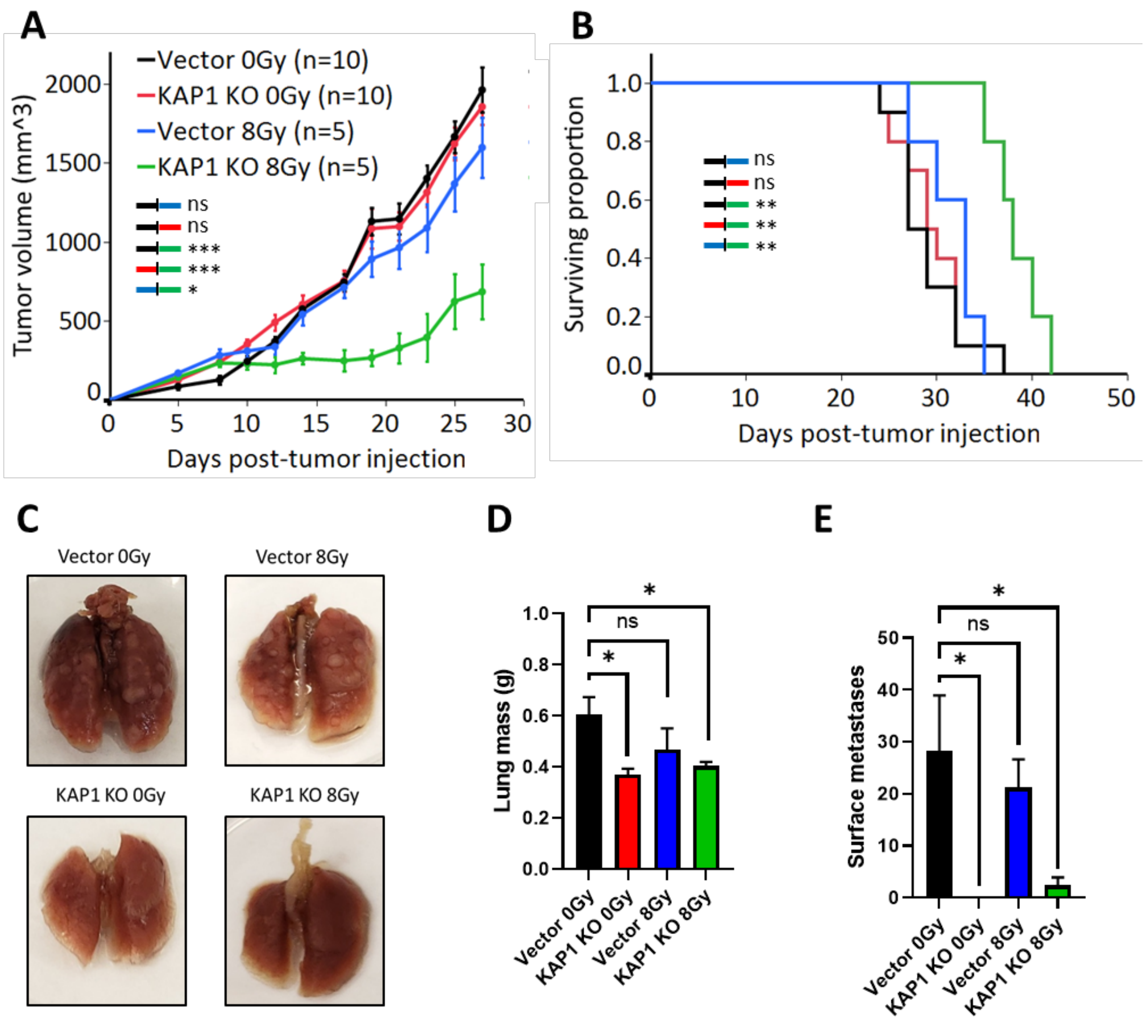


Figure 15: KAP1 knockout in combination with radiation inhibits tumor growth and metastasis in an *in vivo* 4T1 mouse model.

(A) Tumor growth curves and overall survival, respectively, in immunocompetent Balb/C mice inoculated with $5x10^5$ 4T1 vector control or KAP1 knockout cells and treated with sham or 8Gy X-ray irradiation 8 days post-inoculation. (C) Representative images of lungs extracted from mice whose tumors had reached endpoint. (D-E) Quantification of lung mass and counted visible metastases, respectively (n = 5 per group). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001, ns = not significant, as determined by two-way ANOVA (A), log-rank test (B), or unpaired Student's T test (D-E)

2.4 Discussion

The discovery of the cGAS/STING dsDNA sensing pathway and its role in regulating tumor immunity in response to irradiation was a paradigm shift for the radiation biology field, introducing a novel regulatory pathway and providing a rational mechanism for prior decades of studies into the abscopal effect and other non-targeted effects of radiation therapy (Vanpouille-Box, Formenti, & Demaria, 2018). However, the excitement over the cGAS/STING pathway means that other pathways functioning competitively or in parallel to activate IFN and ISG signaling have remained understudied. Here, we demonstrate that radiation-induced ERV induction in the form of dsRNA plays a substantial role in modulating the immune response to radiation, and has major implications for how we understand the interplay between radiation therapy and immunotherapy.

Previous studies have shown that ionizing radiation induces greater transcription of repetitive and transposable elements (Koturbash et al., 2016), as well as dsRNA-induced activation of downstream type I IFN and ISG signaling (Ranoa et al., 2016). KAP1 is an important regulator of this process, as loss of KAP1 in combination with radiation generates substantially more cytoplasmic dsRNA, as well as greater downstream induction of type I IFN and ISG signaling. This effect is mediated through the MDA5/MAVS/TBK1 pathway, as demonstrated by the abrogation of effect upon knockout of MAVS. MAVS appears to be the central effector protein for all dsRNA-related sensing pathways (Wu & Hur, 2015), but there may be other redundant signaling pathways activated by dsRNA-related PRR activation, which merits future study.

Knockout of KAP1 was also relevant *in vivo*, as KAP1-deficient B16F10 and 4T1 tumors in combination with radiation therapy grew significantly slower in syngeneic mouse models. Additionally, KAP1 knockout had a very significant effect on metastasis in the 4T1 model, with KAP1 knockout alone able to block metastasis to the lungs by tumor endpoint, suggesting that KAP1 knockout in an *in vivo* model even without is sufficient to drive immune-mediated tumor killing. Our B16F10 model also suggests immune anti-tumor activation driven by KAP1 knockout, which will be expanded upon further in Chapter 3.

Taken as a whole, our results indicate an appealing potential mechanism of KAP1-mediated dsRNA induction in response to radiation, where KAP1 phosphorylation by ATM in response to DNA damage leads to heterochromatin relaxation and subsequent dsRNA transcription (**Figure 16**). Recent studies in DNA repair support this hypothesis, as it has been shown that DNA-damage response RNAs (small non-coding RNAs induced by double-strand DNA breaks) are generated from bidirectionally-transcribed long non-coding RNAs, which are able to anneal and form dsRNA (Michelini et al., 2017). KAP1 normally blocks this process, through inhibition of formation of the MRE11-RAD50-NBS1 DNA repair complex which recruits RNA polymerase II (Michelini et al., 2017; Zhou, Kawamura, Yanagihara, Kobayashi, &

Zhang-Akiyama, 2017), as well as regulation of RNA polymerase II pause and pause release itself (Bunch et al., 2014). Knockdown of KAP1, or phosphorylation by ATM in response to DNA damage, was sufficient to restore transcription of gene transcripts as well as long non-coding RNAs.

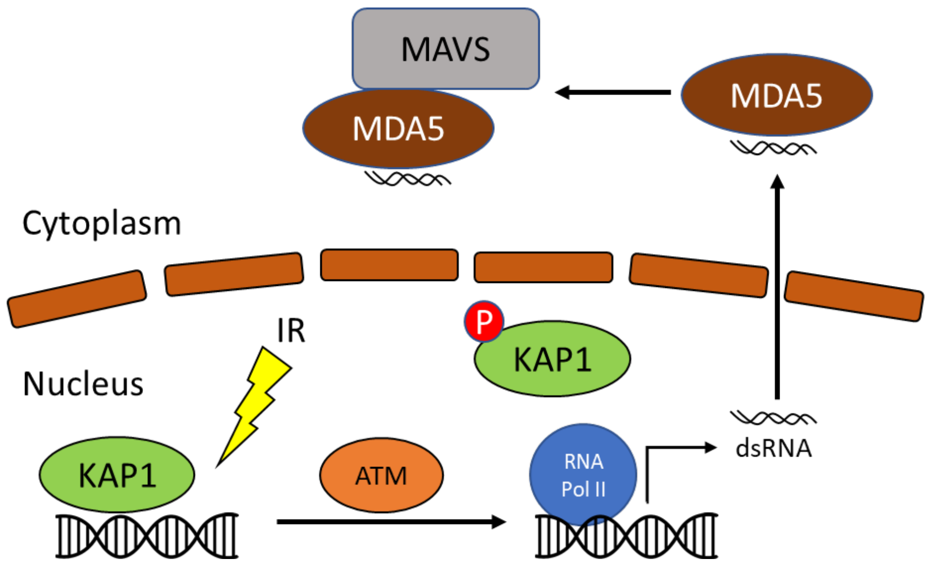


Figure 16: Proposed model for radiation induction of dsRNA.
Radiation induces DNA double-strand breaks, triggering ATM to phosphorylate KAP1 to allow for DNA repair of heterochromatic regions. RNA polymerase II bidirectionally transcribes mRNA at regions of relaxed heterochromatin, generating dsRNA which is then exported to the cytoplasm to be sensed by MDA5 and triggering the MDA5/MAVS innate antiviral immunity pathway.

Previously, the mechanism of radiation-induced dsRNA and subsequent type I IFN and ISG activation was unclear, and most research focused instead on the cGAS/STING pathway, with the straightforward mechanism of DNA damage -> micronuclei -> cytoplasmic dsDNA. We have demonstrated here that the cGAS/STING pathway is not a complete explanation for immune response after radiation, and the

MDA5/MAVS/TBK1 pathway serves as an alternative and complementary signaling pathway to induce an immune response post-radiation. More research is needed to determine the relative effects of each pathway, as well as whether there are other undiscovered signaling pathways activated post-radiation, in order to fully understand the complex landscape of immune signaling in response to radiation.

3. KAP1 knockout is sufficient to drive an immune response in immunocompetent mouse models, and synergizes with checkpoint blockade immunotherapy

Note: portions of chapter 3 are adapted from a manuscript entitled "Endogenous retrovirus activation as a key mediator of anti-tumor immune response in radiotherapy" published in Radiation Research. The authors were Andrew Lee, Xuhui Bao, Dong Pan, Mengjie Hu, Fang Li, and Chuan-Yuan Li. Xuhui Bao assisted with performing flow cytometry and *in vivo* mouse tumor inoculations. Dong Pan assisted with analysis regarding the TIP database and immune cell recruitment. I performed all other experiments and analyses.

3.1 Introduction

Cancer treatment in the clinic focuses heavily on combination therapies, combining multiple treatment modalities such as radiation, surgery, and immunotherapy to eliminate cancer and improve patient survival (Bayat Mokhtari et al., 2017). Combination therapies can produce synergistic effects far greater than individual treatment modalities alone, and much research is focused on discovering or elucidating combination therapies that will lead to better patient outcomes (Johnson & Win, 2018; Palmer & Sorger, 2017). Our previous work demonstrates that KAP1 modulates the innate antiviral immunity pathway response to radiation, suggesting that KAP1 may be closely related to immunotherapy, possibly by sharing similar mechanistic pathways.

This raises the possibility of enhancing immunotherapy cancer treatment with combination therapy targeting KAP1 or related dsRNA factors.

The success of immunotherapy has provoked a dramatic paradigm shift in cancer therapy in both the clinic and in the lab, with research focus shifting to methods of harnessing the body's own immune system to recognize, target, and destroy tumor cells (Couzin-Frankel, 2013; Mellman, Coukos, & Dranoff, 2011). Strategies are highly varied, and include chimeric antigen receptor T-cell therapy (CAR-T) which focuses on modifying T-cells to recognize cancer cells more efficiently (June, O'Connor, Kawalekar, Ghassemi, & Milone, 2018), immune checkpoint blockade antibody therapy which focuses on inhibition of immune checkpoint signaling (Postow, Callahan, & Wolchok, 2015; Topalian, Drake, & Pardoll, 2015), and neoantigen induction which focuses on increasing the mutational load of tumor cells (Schumacher & Schreiber, 2015). The full breadth of immunotherapy research is impossible to cover within the span of this dissertation, so this chapter introduction will focus on immunotherapy fields where KAP1 is likely to have a substantive effect: immune checkpoint blockade and cytokine induction.

3.1.1 Immune checkpoint blockade

Immune checkpoints are molecules responsible for regulation of the immune system. They are necessary for self-tolerance, in order to prevent the immune system from indiscriminately attacking host cells and leading to autoimmunity. However, tumor cells have evolved to co-opt immune checkpoint expression to escape immune surveillance and enable greater proliferation. Inhibition of immune checkpoint molecules via monoclonal antibody therapy has shown very promising results in the clinic (Pardoll, 2012; Sharma & Allison, 2015), and the FDA has currently approved monoclonal antibody therapies targeting two such checkpoint molecules: Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) and Programmed Death 1 receptor (PD-1).

3.1.1.1 CTLA-4

CTLA-4, also known as cluster of differentiation 152 (CD152), is a protein receptor molecule responsible for downregulating immune responses (Chambers, Kuhns, Egen, & Allison, 2001). First discovered in 1987, CTLA-4 is a homolog of the protein cluster of differentiation 28 (CD28), which is responsible for co-stimulatory signaling necessary for effector T cell activation and proliferation (Balzano, Buonavista, Rouvier, & Golstein, 1992). Found exclusively on the surface of T cells, CTLA-4 and CD28 have the same affinity for B7-1 and B7-2 proteins ligands, expressed on the surface of antigen-presenting cells (APCs), but while CD28 acts as a stimulatory signal, CTLA-4 acts as an inhibitory signal, suppressing T cell activation and proliferation (Chambers et al., 2001).

Currently there is one monoclonal antibody that is FDA approved to target CTLA-4 in cancer – Ipilimumab (*Yervoy*®) was approved by the FDA in 2011 for the treatment of melanoma and is undergoing multiple clinical trials for use in other cancers

(Seidel, Otsuka, & Kabashima, 2018). Ipilimumab functions by specifically blocking the CTLA-4 receptor, thus upregulating effector T cell activation and promoting an enhanced overall immune response (Tarhini, Lo, & Minor, 2010).

3.1.1.2 PD1/PD-L1

PD-1, and its associated ligands Programmed-death Ligand 1 and 2 (PD-L1, PD-L2) also serve as negative regulators of immune response (Keir, Butte, Freeman, & Sharpe, 2008). PD-1 is a receptor located on the surface of a large variety of immune cells, most importantly CD8+ tumor-infiltrating lymphocytes (TILs), or cytotoxic T cells (Riley, 2009). The interaction of PD-1 with its associated ligand leads to cytotoxic T cell exhaustion and loss of function (Jiang, Li, & Zhu, 2015). Normally this serves to protect the body's cells from indiscriminate attack from cytotoxic T cells, but many tumors have evolved to express PD-L1, inhibiting cytotoxic T cell killing and allowing the tumor to grow unchecked by the body's immune system (Thommen & Schumacher, 2018).

Currently there are 6 FDA-approved antibodies targeting the PD-1 receptor, the most well-known being pembrolizumab (*Keytruda*®), approved by the FDA in 2014 (H. T. Lee, Lee, & Heo, 2019). Pembrolizumab and other related anti-PD1 antibodies function by blocking the PD-1 receptor, thus removing the inhibitory signal of tumor-expressed PD-L1 and enabling cytotoxic T cells to destroy tumor cells (Alsaab et al., 2017).

CTLA-4 and PD-1 receptor inhibition have a synergistic effect, as CTLA-4 inhibition focuses on enhancing overall T cell activation, while PD-1 inhibition focuses on preventing cytotoxic T cell exhaustion. Thus, there have been a large number of clinical trials testing CTLA-4 and PD-1 inhibition in combination, many of which have demonstrated exceptional results in tumor control and patient survival (Das et al., 2015; Rotte, 2019). However, checkpoint inhibition can also lead to autoimmunity as immune cells attack non-tumor cells within the body, so checkpoint inhibition remains a delicate balancing act between beneficial anti-tumor immunotherapy and harmful autoimmunity (A. Young, Quandt, & Bluestone, 2018).

3.2.2 Cytokine therapy

Cytokines are a broad group of small peptides such as interferons and interleukins that are important for signaling as immune-modulating agents (S. Lee & Margolin, 2011). Multiple cytokines have been discovered that have an immunostimulatory effect, as well as direct anti-tumor effects, and were investigated decades ago as a precursor to current immunotherapy techniques (Dinarello, 2007; Vilcek & Feldmann, 2004). However, cytokine therapy suffered from a multitude of problems, including short half-life times and limited effectiveness as monotherapies, and have been mostly eclipsed by the advent of immune checkpoint therapy. The only two cytokines that are currently FDA approved for clinical use are Interferon Alpha (IFNα) and Interleukin 2 (IL-2) (S. Lee & Margolin, 2011).

IFNα was initially approved for treatment of hairy cell leukemia in 1986, and has subsequently been approved for use in follicular non-Hodgkin lymphoma, melanoma, and Kaposi's sarcoma (Golomb et al., 1986; Quesada & Gutterman, 1986). IL-2 was approved for the treatment of renal cell carcinoma in 1992, followed by metastatic melanoma in 1998 (Atkins, 2002). Both therapies have significant immunostimulatory effects, as IFNα is known to directly activate natural killer (NK) cells, dendritic cells (DC), and T-cell lymphocytes (Jewett & Bonavida, 1995), while IL-2 is necessary for NK and T-cell expansion (Becknell & Caligiuri, 2005). However, both therapies at effective doses have significant clinical toxicity, and thus are disfavored compared to less toxic targeted therapies and newer immunotherapies (S. Lee & Margolin, 2011). Much of the current research on cytokine therapy revolves around combination therapy with immune checkpoint inhibitors, aiming to supplement immune checkpoint inhibition with immunostimulatory cytokines (Waldmann, 2018).

3.2.3 Potential KAP1-mediated immune effects

Our previous work demonstrated that KAP1-mediated ERV transcription and dsRNA could drive production of downstream IFNs and ISGs, which are known to stimulate tumor immunity. Other studies have also demonstrated an interferon response in response to KAP1 depletion (Tie et al., 2018). Therefore, we hypothesized that KAP1 depletion may drive an IFN-mediated immune response effective at inhibiting tumor growth. In this study, we investigated the effects of KAP1 depletion alone on IFN and ISG stimulation, as well as effects on tumor growth and immune cell recruitment.

3.2 Materials and methods

Any methods not found here can be found in Chapter 2.2 Materials and methods.

3.2.1 TCGA analyses of human patient data

Patient survival analyses were performed using the online GEPIA survival analysis platform (http://gepia.cancer-pku.cn/) (Z. Tang et al., 2017). mRNA co-enrichments relative to *Trim28* mRNA expression levels in skin cutaneous melanoma (SKCM) patients were downloaded from cBioPortal (q value < 0.01) (Cerami et al., 2012; Gao et al., 2013) and subjected to pathway enrichment analysis by PANTHER through the Gene Ontology Resource website, utilizing Reactome pathway classifications (http://geneontology.org/) (Ashburner et al., 2000; Fabregat et al., 2018; Fabregat et al., 2017; Mi, Muruganujan, Ebert, Huang, & Thomas, 2019; The Gene Ontology, 2019). All P values were corrected for False Discovery Rate (FDR). Immune infiltration scores were obtained from the Tracking Tumor Immunophenotype (TIP) online platform pancancer analysis (Xu et al., 2018) and linked to *Trim28* mRNA expression by patient ID (http://biocc.hrbmu.edu.cn/TIP/index.jsp).

3.2.2 Soft agar colony formation assay

The soft agar colony formation assay was performed as previously described (Borowicz et al., 2014). Briefly, 6-well plates were layered with a base layer of 1mL of

0.6% agar (Invitrogen, Carlsbad, CA) and allowed to solidify. 2500 cells were then seeded into a 1mL solution of 0.3% agar and layered above the 0.6% layer. Upon solidification, 500µL of supplemented DMEM was added to each well, and plates were incubated under standard cell growth conditions for 2-3 weeks, with media changes every 3 days until visible colonies could be observed.

Plates were stained overnight with 500µL of 0.005% crystal violet in 80% methanol per well prior to imaging and counting. Colonies were counted if there were a total of 50 cells or more.

3.2.3 Tumor extraction and digestion

Mouse tumors were mechanically excised using scalpels and dissociated to a single cell suspension in cell staining buffer (2% heat-inactivated FBS in PBS) using a modified version of a triple enzyme mouse tumor digestion protocol described elsewhere. Briefly, 100mg of minced tumor fragments were incubated shaking at 37°C for 20 minutes in 250µL enzyme digestion buffer (10µg/µL collagenase and 20 µg/µL DNAse in 1x Hank's Balanced Salt Solution (HBSS)). Following digestion, cells were passed through a 70µm nylon mesh filter, followed by treatment with 200µL of Red Blood Cell Lysis Buffer (Roche, Basel, Switzerland) shaking at room temperature for 10 minutes. Cells were passed again through a 70µm nylon mesh filter and washed 3 times in cell staining buffer prior to preparation for flow cytometry.

3.2.4 Flow cytometry

10^6 cells were blocked in CD16/32 antibody for 10 minutes on ice in the dark, following manufacturer's recommended dilution. Cells were then incubated in primary antibody for 30 minutes on ice in the dark, following manufacturer's recommended dilution. Following primary antibody incubation, cells were washed in cell staining buffer twice and then sorted on a BD FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA). Data was analyzed using FlowJo version 10.6 software (Ashland, OR). Protein antibodies used are as follows: LIVE-DEAD stain (Thermo Fisher Scientific®, Waltham, MA); and TruStain FcX™ CD16/32, CD45, CD8A, CD4, and NK1.1 (Biolegend, Carlsbad, CA).

3.2.5 PD1 treatment

Anti-PD1 antibody and matched isotype control were obtained from Bio X Cell (West Lebanon, NH). 100ng of antibody or isotype control were diluted into sterile PBS and injected into mice via intraperitoneal injection on days 5, 8, and 11 post-tumor inoculation.

3.3 Results

3.3.1 *Trim28* mRNA levels correlate with patient survival and immune cell recruitment in TCGA cancer cohorts

In our prior *in vivo* studies regarding KAP1 knockout, we demonstrated that KAP1 knockout alone was sufficient to delay tumor growth in B16F10 tumor models, as well as inhibit metastasis in 4T1 tumor models. We hypothesized that if we saw such large effects in mouse models, then it was likely that KAP1 also plays a role in patient survival in human cancer therapy as well. To test this hypothesis, we turned to The Cancer Genome Atlas (TCGA).

The TCGA project is a large-scale cancer genomics study characterizing over 20,000 primary cancers and matched samples across 33 different cancer types. The project utilized high throughput genomic assays to collect a substantial amount of data spanning a large breadth of possible prognostic genomic and epigenomic factors, including whole genome sequences, mutations, mRNA sequences, miRNA sequences, and DNA methylation (Cancer Genome Atlas Research et al., 2013).

To determine if variation in *Trim28* mRNA expression was relevant to patient survival, we analyzed patient survival rates across the 33 TCGA cancer types, comparing patients with high *Trim28* expression versus low *Trim28* expression (high defined as 75th percentile, low defined as 25th percentile). We discovered that *Trim28* expression levels had a significant effect on survival when compared across all 33 cancers in the TCGA dataset, with high *Trim28* mRNA levels strongly correlating with worsened patient survival (Figure 17). There was a greater effect in specific cancers such as lung adenocarcinoma (LUAD) and skin cutaneous melanoma (SKCM), and while not every cancer type showed a significant effect of survival, the trend was consistent for the majority of tumor types analyzed.

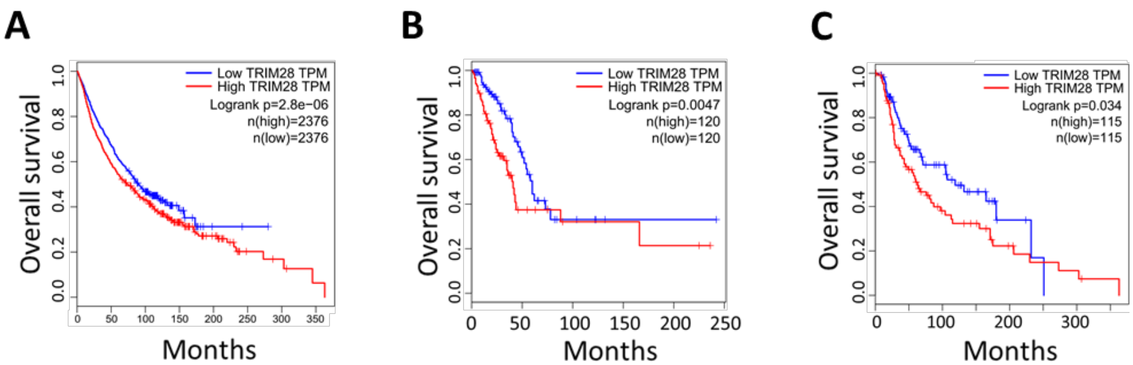


Figure 17: *Trim28* mRNA expression levels correlate with patient survival in TCGA cancer cohorts.

(A-C) Kaplan-Meier survival curves for total 33 cancer cohorts (A), lung adenocarcinoma (LUAD) (B), and skin cutaneous melanoma (SKCM) (C), separated into *Trim28*-high vs *Trim28*-low by 75th versus 25th quartile mRNA expression levels. High KAP1 is significantly associated with poorer patient survival by log-rank test.

To elucidate the biological pathways affected by KAP1, we decided to perform gene ontology analysis to detect correlations in gene expression compared to *Trim28* mRNA levels. We identified the most significantly under-expressed genes in *Trim28*-high tumor samples from SKCM patients (q<0.01) and subjected them to Reactome pathway enrichment analysis. We discovered that *Trim28* was negatively associated with a wide variety of immune regulatory, cytokine signaling, and antigen presentation pathways (Figure 18A), implying that KAP1 mechanistically affects patient survival by downregulating immune-regulatory signals that would otherwise be activating the patient's immune system.

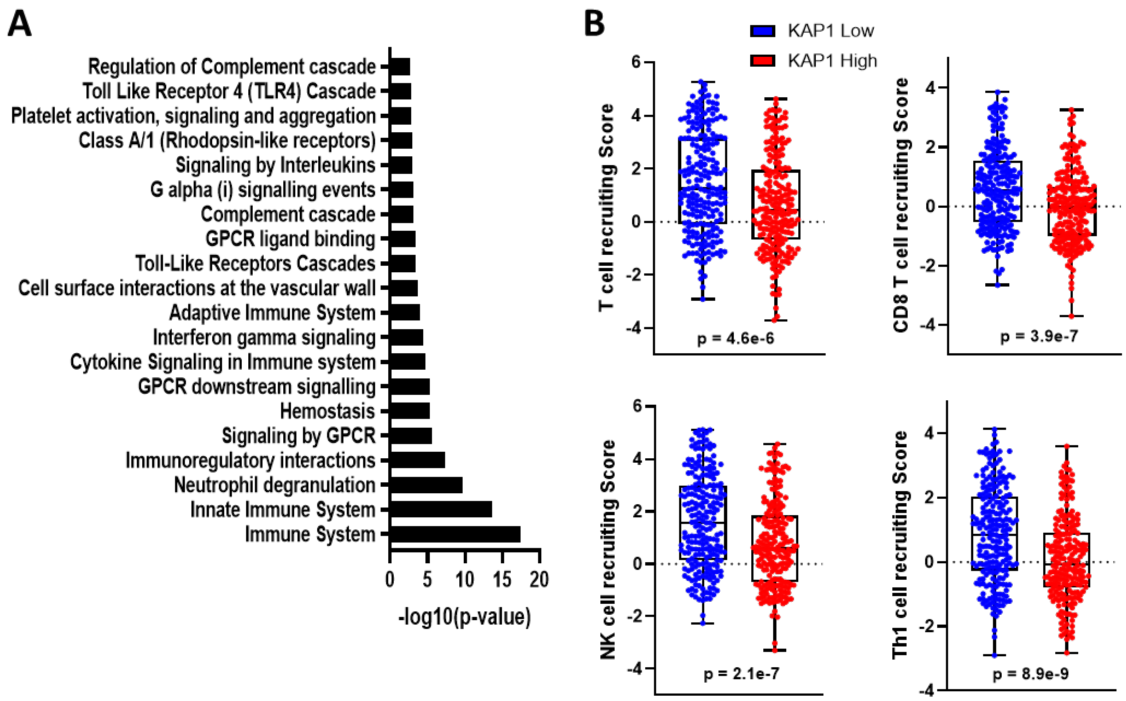


Figure 18: Pathway analysis and immune infiltration scores of *Trim28*-low patients.
(A) REACTOME pathway analysis of the most significantly downregulated genes in TCGA SKCM *Trim28*-high patients. (B) Immune effector cell recruitment score comparison between *Trim28*-high and *Trim28*-low TCGA SKCM patients. P values are determined by unpaired Student's T test and corrected for false discovery rate.

We hypothesized that depressed immune activation correlated with high levels of KAP1 would lead to decreased tumor infiltration of immune cells, thus allowing the tumor to grow unchecked and negatively affecting patient survival. We analyzed genes specific to immune and T-cell recruitment in the SKCM patient cohort using the Tracking Tumor Immunophenotype (TIP) online database (Xu et al., 2018) (http://biocc.hrbmu.edu.cn/TIP/index.jsp). Gene expression data were analyzed and different immune effector subsets were identified and categorized using the CIBER-

SORT method (Newman et al., 2015). Each patient was assigned immune filtration scores, which were then correlated to that patient's *Trim28* mRNA expression levels. Our analysis demonstrated that average scores for immune infiltration and recruitment were significantly reduced in *Trim28*-high SKCM patients (Figure 18B), supporting our hypothesis that high levels of KAP1 negatively affects the ability for the immune system to infiltrate and eliminate tumors. We decided to use our KAP1-knockout cell lines to determine if we could see effects of KAP1 knockout without radiation, both *in vitro* and *in vivo*.

3.3.2 KAP1 knockout inhibits the ability for tumor cells to grow in the soft agar colony formation assay

As shown previously (Figure 8B), KAP1 knockouts did not have any effect on cell proliferation rates *in vitro*. To more accurately simulate conditions that would be found *in vivo*, we used the soft agar colony formation assay to measure the ability of KAP1 knockouts to grow in an anchorage-independent environment, a hallmark of tumorigenesis and cancer aggression (Borowicz et al., 2014). We found that KAP1 knockout significantly inhibited the ability of HeLa cervical cancer tumor cells to form colonies in soft agar (Figure 19), indicating that the loss of KAP1 induces a phenotype where cancer cells have reduced ability to proliferate in semi-solid conditions.

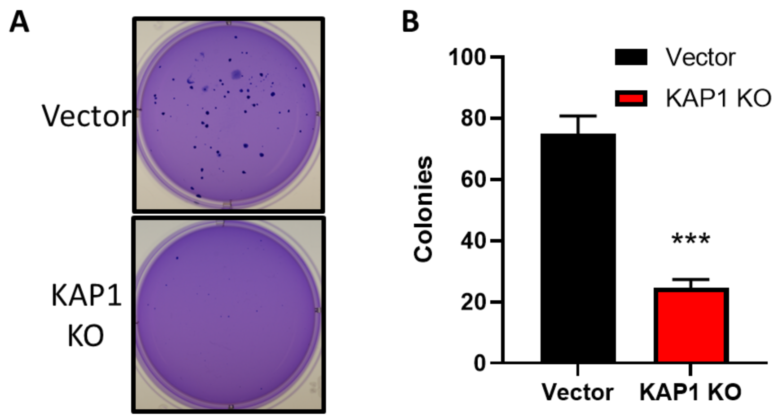


Figure 19: KAP1 knockout inhibits soft agar colony formation of HeLa cells. (A-B) Representative images and quantification, respectively, of soft agar colonies in 0.3% agar stained with crystal violet 14 days after seeding with 200 HeLa vector control or KAP1 knockout cells. Error bars represent SEM. ***$p<0.001$ as determined by unpaired Student's T test.

3.3.3 KAP1 knockouts may have altered chromatin patterns due to HP1 mislocalization, promoting enhanced overall ERV transcription

In chapter 2, I proposed a mechanism where KAP1 relaxes heterochromatin in response to DNA damage, thus driving ERV transcription and downstream type I IFN and ISG expression. However, it is possible that KAP1 knockout alone in the absence of radiation is sufficient to drive phenotypic changes, as evidenced by soft agar colony formation inhibition and increased ERV transcription in KAP1 knockout cells. We performed immunofluorescence staining against HP1 in KAP1 knockout HeLa cells and discovered that HP1 was severely mislocalized in KAP1 knockout cells compared to control cells (Figure 20). Rather than a clean alignment with DAPI as in control cells, HP1 was randomly distributed throughout the nucleus in various clusters, implying significant heterochromatin dysregulation. However, HP1 is merely one marker for heterochromatin, and more in-depth research remains to be done to confirm that heterochromatin mark locations are indeed significantly affected by KAP1 knockout.

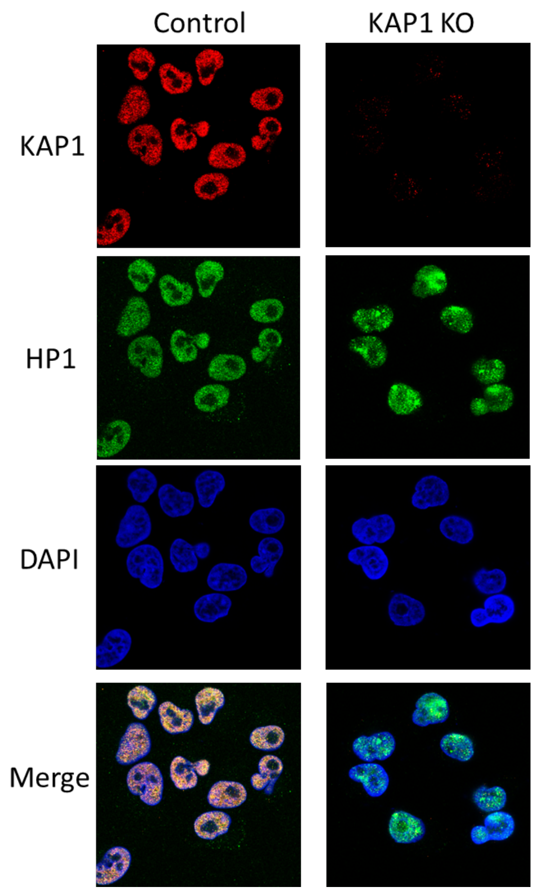


Figure 20: Mislocalization of HP1 in HeLa KAP1 knockout cells. Representative immunofluorescence images of HP1 staining as a marker for heterochromatin in HeLa vector control and KAP1 knockout cells. Depletion of KAP1 alters the structural distribution of HP1 throughout the nucleus.

Heterochromatin dysregulation implies an abnormal transcription and gene expression profile and has been implicated in multiple diseases. While we had demonstrated previously that A549 KAP1 knockouts had elevated levels of ERV transcripts, we wanted to know if ERV transcription due to KAP1 knockout was affected across multiple cell lines and could be generalized. Indeed, our results showed that in HeLa cervical cancer cells and MDA-MB-231 triple negative breast cancer cells, knockout of KAP1 trended towards elevation of multiple known ERV transcripts (Figure 21), although the specific upregulated ERV transcripts varied between cancer types. This suggests that KAP1 knockout upregulating ERV transcription may be a highly generalizable effect, and applies across multiple cell lines and species.

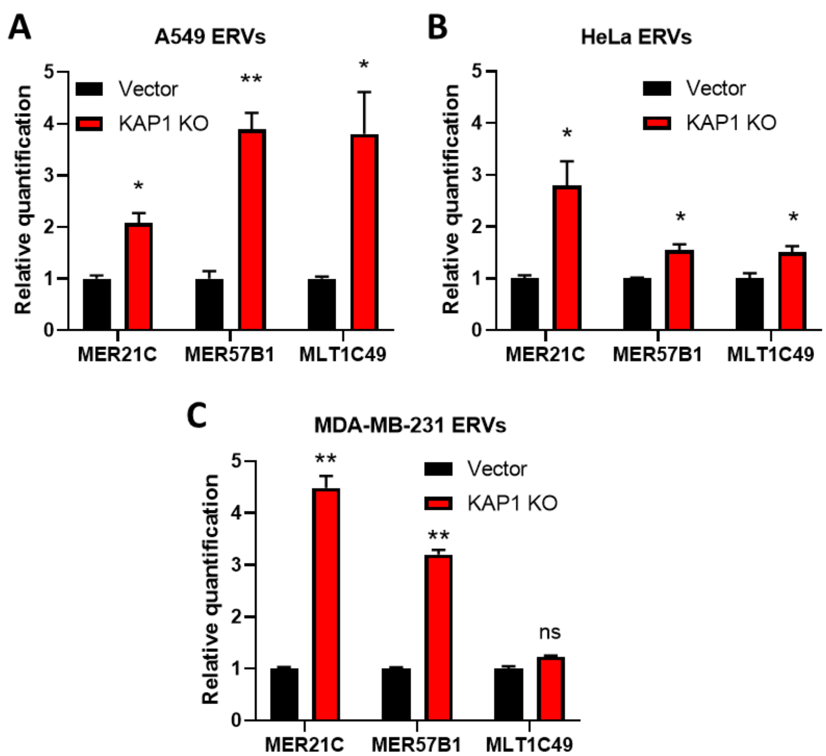


Figure 21: ERV expression is enhanced by KAP1 knockout in multiple cell lines.

(A-C) Quantification of selected ERV mRNA by RT-qPCR in A549 (A), HeLa (B), and MDA-MB-231 (C) vector control or KAP1 knockout cells. KAP1 depletion induces a significant increase in transcription of selected ERVs. Error bars represent SEM. *p<0.05, **p<0.01, ns = not significant, as determined by unpaired Student's T test.

3.3.4 KAP1 knockout B16F10 *in vivo* tumors show growth delay in immunocompetent mice, but not in immunocompromised mice

Our previous *in vivo* studies with B16F10 cells showed that KAP1 knockout B16F10 tumors grew slower than control B16F10 tumors in syngeneic C57 black mice, even in the absence of radiation (Figure 14). To confirm those results, we repeated the experiment with a different cohort of mice and saw a similar effect as before of tumor growth inhibition and overall survival enhancement (Figure 22). Because of our TCGA cancer patient data analysis, we hypothesized that the effect of KAP1 knockout was likely an immune-mediated effect, and so we decided to perform confirmation *in vivo* studies in immunodeficient mice. If the effect is immune-mediated, then we should see no difference in tumor growth rates in immunodeficient mice.

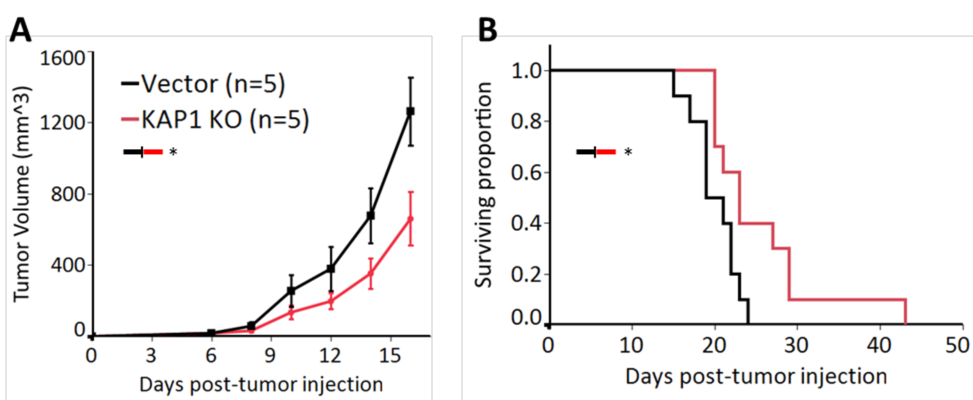


Figure 22: KAP1 knockout inhibits tumor growth and enhanced survival in a syngeneic C57BL/6 mouse model.
(A-B) Tumor growth and overall survival curves, respectively, in C57BL/6 mice inoculated with 10^5 B16F10 vector control or KAP1 knockout cells. *p<0.05, as determined by two-way ANOVA (A) or log-rank test (B).

First, we performed tumor xenograft studies, inoculating 5x10^5 HeLa vector control or KAP1 knockout cells subcutaneously into the right hindlimb of NU/J nude mice. Nude mice are athymic, and thus they are immunosuppressed due to lack of T cell maturation (Szadvari, Krizanova, & Babula, 2016). As we hypothesized, we did not see a significant difference in tumor growth rates or survival (Figure 23A-B), although we did observe that HeLa KAP1 knockout tumors trended slightly smaller than vector control tumors; this may be related to the soft agar colony formation assay, where KAP1 knockouts weren't as effective at initiating colony growth in 3-dimensional anchorage-independent environments.

Next, we inoculated 10^5 B16F10 vector control or KAP1 knockout cells subcutaneously into the right hindlimb of NOD scid gamma (NSG™) mice, to confirm whether our *in vivo* B16F10 KAP1 knockout tumor growth inhibition was mediated by immune effects. NSG™ mice are highly immunodeficient, lacking T cells, B cells, and NK cells, and are the most immunodeficient mice available for research (Shultz et al., 2014). As expected, there was also no significant difference in NSG™ tumor growth rates or survival between vector control and KAP1 knockout B16F10 tumors (Figure 23C-D), indicating that KAP1 knockout-mediated tumor suppression requires an intact immune system.

Finally, to support our original radiation studies regarding KAP1, we repeated our radiation *in vivo* studies by inoculating 10^5 B16F10 vector control or KAP1 knockout cells subcutaneously into the right hindlimb of nude mice, followed by treating the tumors with sham or 8Gy of X-ray radiation 8 days post-injection. As shown in (Figure 23E-F), both irradiated vector control tumors and non-irradiated KAP1 knockout tumors are unable to induce a response, as tumor growth rates and survival are not significantly different from control. The exception is the combination of KAP1 knockout and radiation, which did show significant improvement in tumor growth and survival; however, this effect is likely due to the innate radiation sensitivity of KAP1 knockout cells, as opposed to an immune-mediated effect. Taken as a whole, our results demonstrate that KAP1-knockout tumor inhibition is intrinsically linked to an intact host immune system.

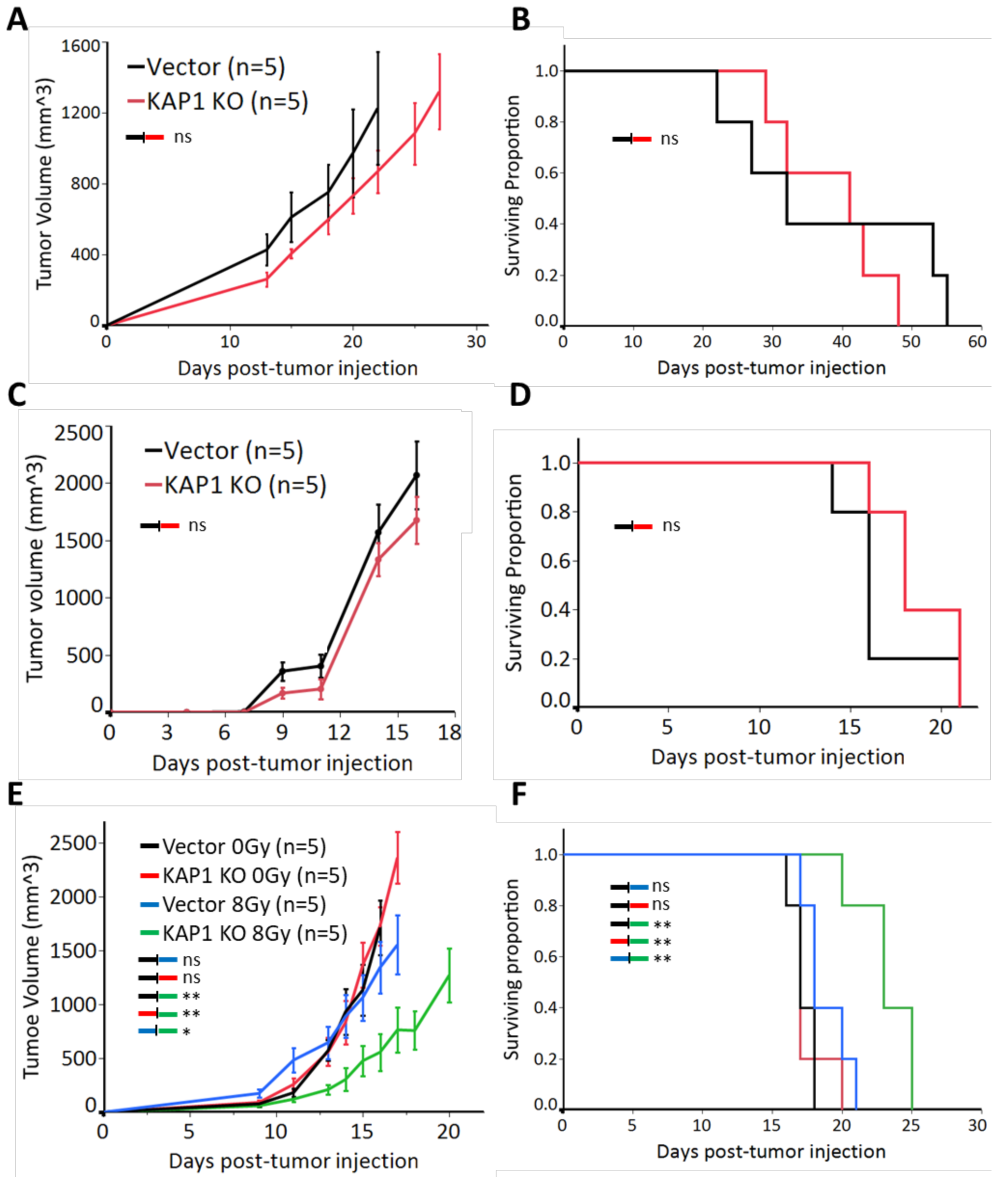


Figure 23: KAP1 knockout-mediated tumor suppression requires an intact host immune system.

(A-B) Tumor growth and overall survival curves, respectively, in nude mice inoculated with $5x10^5$ HeLa vector control or KAP1 knockout cells. (C-D) Tumor growth and overall survival curves, respectively, in NSG mice inoculated with 10^5 B16F10 vector control or KAP1 knockout cells. (E-F) Tumor growth and overall survival curves, respectively, in nude mice inoculated with 10^5 B16F10 vector control or KAP1 knockout cells and treated with sham or 8Gy X-ray irradiation 8 days post-inoculation. Error bars represent SEM. *p<0.05,**p<0.01, ns = not significant, as determined by two-way ANOVA (A, C, E) and log-rank test (B, D, F).

3.3.5 KAP1 knockout B16F10 *in vivo* tumors show greater CD45+/CD8+ leukocyte population

To delve deeper into the mechanism behind KAP1 knockout-mediated tumor growth inhibition, we decided to investigate immune infiltration of *in vivo* tumors, hypothesizing that KAP1 knockout-mediated IFN or ISG production may be attracting tumor-infiltrating lymphocytes to the tumor microenvironment. We inoculated 10^5 vector control or KAP1 knockout B16F10 cells subcutaneously into the right hindlimb of syngeneic C57 mice, followed by harvesting of the tumors at day 11 prior to tumors reaching endpoint size. Tumors were disaggregated and analyzed via flow cytometry to determine immune populations within the tumor.

Flow cytometry analysis of dissociated tumor cells revealed that there was no significant difference in the proportion of live CD45+ cells between vector control and KAP1 knockout tumors (Figure 24), despite the minor increase in the KAP1 knockout population. However, within that CD45+ population, there was a significant increase of CD8+ cells in the KAP1 knockout tumors, suggesting an increased cytotoxic immune cell population compared to vector control tumors. Interestingly, even though NK cells are traditionally associated with the immune response to viral infection (Jost & Altfeld, 2013), we did not see a significant difference in NK1.1+ expressing cells between our tumor populations, implying that KAP1 knockout primarily mediates its effects through recruitment of cytotoxic T cells, and not NK cells. It may be that NK cells serve as an initial frontline defense, giving way to cytotoxic T cell infiltration over time, but more research is needed to determine if NK cells play any role in mediating KAP1 knockout-induced tumor suppression.

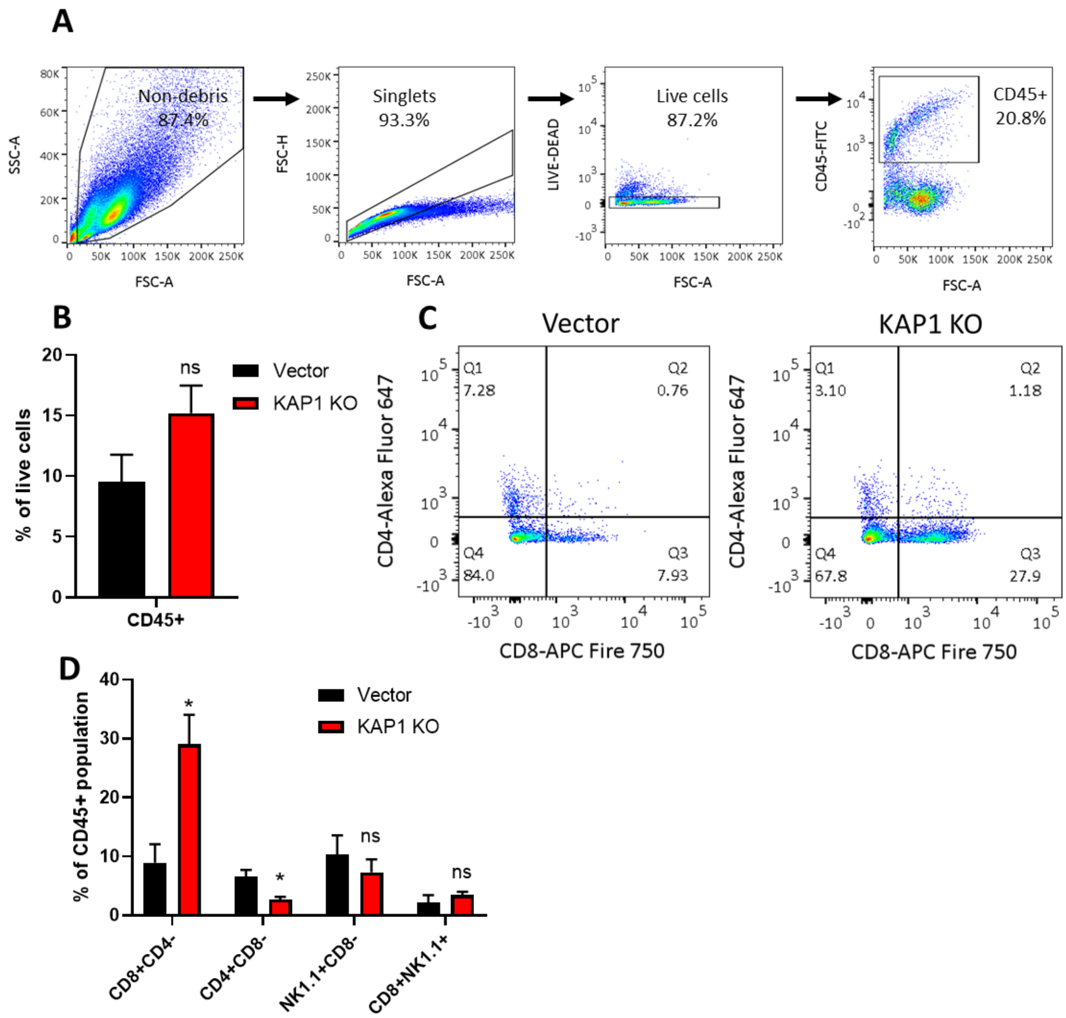


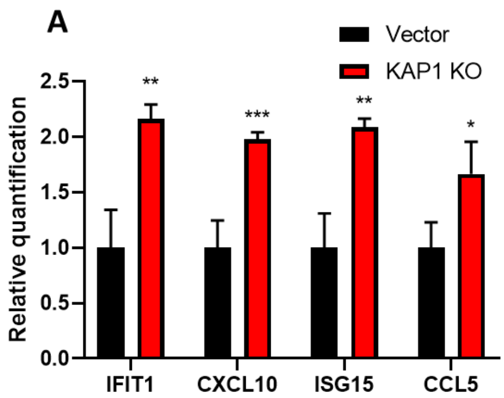
Figure 24: Flow cytometry analysis of immune infiltrate in a syngeneic C57BL/6 mouse model.

Mice were inoculated with 10^5 B16F10 vector control or KAP1 knockout cells; tumors were harvested 11 days post-inoculation and subjected to flow cytometry analysis. (A) Gating strategy for selection of live single cell CD45+ leukocyte population. (B) Quantification of CD45+ cell proportions between B16F10 vector control and KAP1 knockout tumors. (C) Representative flow cytometry comparison of CD8+ cells versus

CD4+ cells among the total CD45+ cell population between B16F10 vector control and KAP1 knockout tumors. (D) Quantification of various immune cell marker proportions between B16F10 vector control and KAP1 knockout tumors. Error bars represent SEM. *p<0.05, ns = not significant, as determined by unpaired T Student's T test.

3.3.6 ISG expression is upregulated in KAP1 knockouts *in vivo* and correlates to MDA5 and phospho-TBK1

We next performed RT-qPCR on our harvested tumor samples for ISGs. ISG expression is upregulated in response to IFN signaling, so we suspected that cytotoxic T cells may have been recruited to the tumor through IFN and ISG signaling. Our analysis showed significant upregulation of ISGs in KAP1 knockout tumors compared to vector control tumors (Figure 25), indicating significant IFN activity within the tumor. Additionally, proteins levels of MDA5 and phosphorylated TBK1 were upregulated in KAP1 knockout tumors, and their protein levels correlated with CXCL10 induction on an individual tumor basis, demonstrating a correlative link between dsRNA-mediated innate antiviral immunity pathway induction and induction of ISGs.



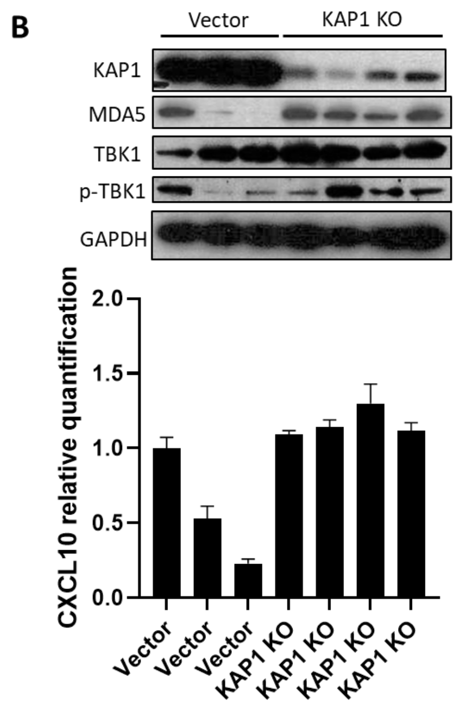


Figure 25: ISG expression from B16F10 *in vivo* tumor samples.
(A) Quantification of selected ISG mRNA by RT-qPCR in B16F10 tumors harvested from syngeneic C57BL/6 mice 11 days post-inoculation. (B) Western blot staining for MDA5/MAVS pathway members in B16F10 tumors (upper), linked to individual tumor-level quantification of CXCL10 mRNA expression by RT-qPCR (lower). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001, as determined by unpaired Student's T test.

However, it is unknown whether these ISGs are produced by the tumor cells, or by infiltrating immune cells responding to IFN signaling. We demonstrated previously that radiation can induce significant ISG upregulation in KAP1 knockout B16F10 cells *in vitro*, but KAP1 knockout alone was insufficient to drive a similar effect (Figure 12C). It is plausible that a stressor of some sort is needed to induce sufficient ERV transcription to see downstream ISG induction, such as radiation or transplantation into a mouse, but it may also be the case that KAP1 knockout tumors themselves aren't producing ISGs, but rather immune cells being recruited to the tumor microenvironment. More research is needed to distinguish specific cellular signaling pathways.

3.3.7 KAP1 knockout synergizes with anti-PD1 checkpoint inhibition to inhibit tumor growth *in vivo*

Seeing as KAP1 knockout tumors have greater recruitment of cytotoxic T cells to the tumor, we hypothesized that checkpoint inhibition therapy with anti-PD1 antibody would synergize with KAP1 knockout, as anti-PD1 therapy inhibits immune regulation and enhances the effect of cytotoxic T cells. We inoculated 10^5 vector control or KAP1 knockout B16F10 cells subcutaneously into the right hindlimb of syngeneic C57 black mice. At each of days 5, 8, and 11 post-tumor inoculation, we administered either isotype control or anti-PD1 antibody via intraperitoneal injection.

We found that as before, there was a significant difference in tumor growth rates and survival between vector control and KAP1 knockout tumors (Figure 26). Anti-PD1 therapy induced a modest but significant reduction in tumor growth in both vector control and KAP1 knockout tumors, but oddly only promoted a significant survival benefit in mice with KAP1 knockout tumors. The effect on tumor growth in KAP1 knockouts was relatively minor but still statistically significant, suggesting that anti-PD1 checkpoint inhibition therapy interacts synergistically with KAP1 knockout.

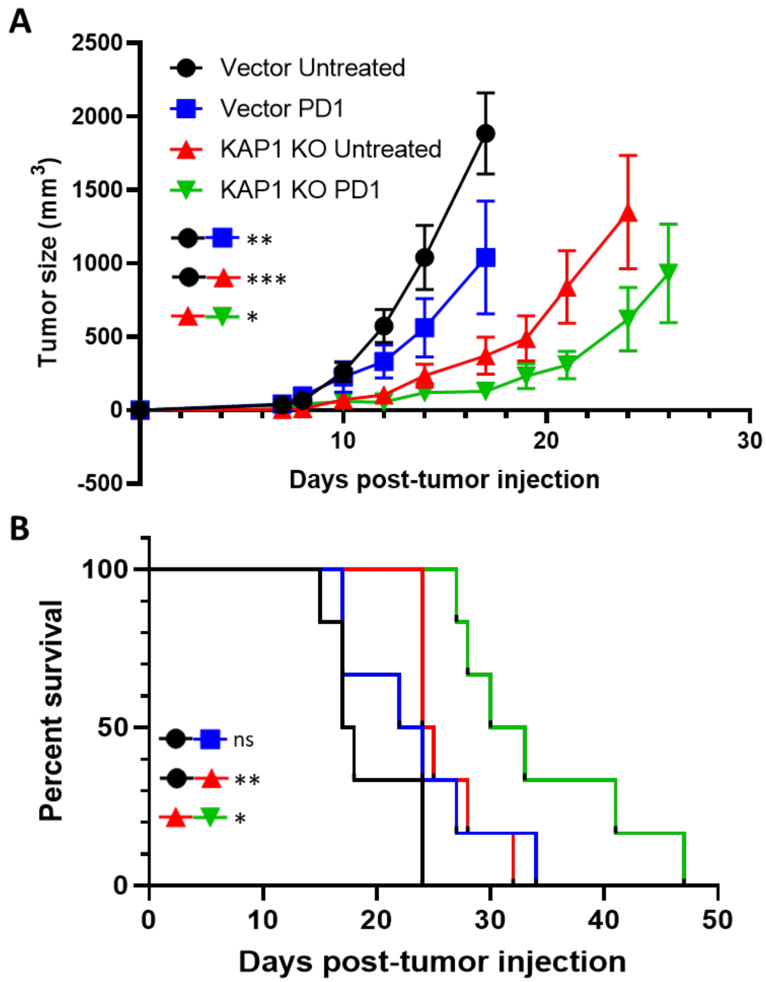


Figure 26: KAP1 knockout synergizes with anti-PD1 checkpoint blockade. (A-B) Tumor growth and overall survival curves, respectively, of C57BL/6 mice inoculated with 10^5 B16F10 vector control or KAP1 knockout cells and treated with 100ng anti-PD1 antibody via intraperitoneal injection on days 5, 8, and 11 post tumor inoculation. N = 5 per group. Error bars represent SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns = not significant, as determined by two-way ANOVA (A) or log-rank test (B).

3.4 Discussion

Immunotherapy in cancer treatment has been hailed as a miracle worker in the clinic, inducing durable responses and improving outcomes in a significant fraction of patients. However, as with all monotherapies, immunotherapy has its limits, and so a great deal of research has gone into seeking synergistic combination therapies to improve the efficacy of immunotherapy, as well as seeking methods to induce a response in a greater proportion of patients. Here, we demonstrate that depletion of KAP1 modulates immune cell populations within a tumor, suggesting that targeting KAP1 and its related pathways may be a strong strategy to enhance the effects of immunotherapy.

TCGA data analysis indicates that higher levels of *Trim28* transcription is associated with poorer survival, likely due to impaired immune cell recruitment to tumor sites. Certain interferons are known to promote immune activation and recruitment, suggesting a link between dsRNA-mediated IFN production and immune-mediated tumor control. Our *in vivo* studies support this link, as KAP1 depletion inhibits tumor growth in immunocompetent mice, but not in immunodeficient mice, indicating that tumor control requires an intact host immune system. Additionally, KAP1 depletion is correlated with an increased CD8+ T cell population and upregulated ISG production within the tumor, suggesting upregulation of IFN production as the causative factor.

We propose a mechanism where depletion of KAP1 leads to enhanced ERV transcription in the form of dsRNAs, thus constitutively activating the MDA5/MAVS innate antiviral pathway and driving type I IFN production. Type I IFNs then act as signaling cytokines, initiating autocrine or paracrine signaling to stimulate an immune response and upregulate expression of downstream ISGs. Thus, KAP1 depletion may be similar in a way to cytokine therapy, where induction and secretion of IFNs from KAP1-depleted tumor cells stimulates an immune response that leads to tumor killing.

There are currently no drugs or small molecules that specifically target KAP1, likely due to expected toxicity, as KAP1 is a ubiquitous protein throughout the body, and is essential for heterochromatin formation during embryonic development (Cammas et al., 2000). However, the chemotherapeutic doxorubicin has been previously reported to upregulate ATM-mediated KAP1 phosphorylation, as well as subsequent histone eviction from the chromatin, potentially upregulating ERV transcription (X. Li et al., 2007; Pang et al., 2013). Thus, in addition to targeting KAP1 directly, it is likely also worthwhile to investigate therapies that target KAP1-mediated heterochromatic repression, in order to induce greater ERV transcription and subsequent dsRNA-induced downstream immune responses. Inducing heterochromatic relaxation of ERV regions normally repressed by KAP1 is a novel direction for therapeutic drug design and has high potential to synergize with immunotherapy in the clinic to improve patient outcomes in cancer therapy.

4. Conclusions

4.1 Summary

The overarching goal of this dissertation was to study the relevance of endogenous retroviruses in cancer therapy, more specifically immune therapy. To that end, I elected to study ERVs through manipulation of KAP1, the protein responsible for heterochromatic repression at ERV regions of the genome. Prior studies had indicated that pharmacological treatments such as DNMTis and HDAC inhibitors could upregulate ERV transcription and dsRNA, inducing immune effects through upregulation of the IFN response (Chiappinelli et al., 2015; Krug et al., 2019). However, the role of KAP1 in repressing dsRNA was understudied, especially in the context of radiation therapy.

My first project revealed that aside from the cGAS/STING dsDNA sensing pathway, ionizing radiation could also stimulate IFN production through the MDA5/MAVS dsRNA sensing pathway, modulated by KAP1. The impetus for this direction of study was the discovery that J2 immunofluorescence staining, which is specific for dsRNA, was significantly upregulated in the cytoplasm of irradiated cells. Investigation of possible dsRNA pathways affected by radiation suggested KAP1 as a key modulator, and indeed, we found that depletion of KAP1 enhanced the dsRNA and IFN response to radiation. The enhanced IFN response was confirmed to be due to the MDA5/MAVS pathway, as depletion of MAVS abrogated KAP1 knockout-mediated

TBK1 phosphorylation and subsequent ISG upregulation. *In vivo* studies further demonstrated possible clinical relevance of KAP1 depletion in conjunction with radiotherapy, as KAP1 depletion significantly inhibited tumor growth and enhanced survival in both C57BL/6 and Balb/C mice treated with radiation. This led us to propose a mechanism where KAP1 normally represses ERV transcription via heterochromatin, but radiation and DNA damage-induced heterochromatin relaxation increases ERV transcription and IFN production, driving immune-mediated tumor inhibition. While the exact mechanism remains to be confirmed, our work demonstrates a secondary innate immunity pathway alongside the cGAS/STING pathway that must be taken into consideration during radiotherapy.

The follow up to this project focused on investigating the immune consequences of KAP1 depletion, as KAP1 depletion in B16F10 melanoma tumors inhibited tumor growth even without radiotherapy, implying that KAP1 depletion alone could stimulate an immune response. Analysis of TCGA patient data supported this notion, as *Trim28* mRNA levels were negatively associated with overall survival and immune recruitment in multiple cancer types. We demonstrated that an intact immune system was necessary for KAP1 depletion-mediated tumor growth inhibition, as KAP1 depletion in tumors xenografted into immunodeficient mice failed to inhibit tumor growth and survival compared to immunocompetent mice. Furthermore, KAP1 depletion appears to stimulate immune cell recruitment in immunocompetent mice, as proportions of CD8+

lymphocytes were increased in KAP1 knockout tumors compared to vector control, along with an increase in ISG transcription. Taken as a whole, our results suggest a mechanism where KAP1 depletion promotes ERV transcription, thus activating the MDA5/MAVS pathway and driving type I IFN expression. IFN autocrine and paracrine signaling then promotes immune cell recruitment to the tumor, inhibiting tumor growth and enhancing survival.

4.2 Future directions

The study of ERVs, KAP1, and their relevance to immunotherapy and cancer treatment is by no means complete. There remain multiple avenues of research still unexplored, including mechanistic analysis, relative contribution, and combination studies with other cancer therapies.

4.2.1 KAP1 re-expression and heterochromatin analysis

Though we have presented a highly plausible mechanism for KAP1 modulation of ERV transcription, more work remains to be done to confirm whether heterochromatin relaxation is sufficient to upregulate ERV transcription, or if there are potentially other mechanisms in play not yet investigated. To this end, we plan to re-express mutated forms of KAP1 in our KAP1 knockouts – namely the S824A mutant, which cannot be phosphorylated, and the S824D mutant, which constitutively mimics a phosphorylated state (X. Li et al., 2007). We expect that expression of a non-phosphorylatable mutant will block ATM-mediated KAP1 phosphorylation and ERV transcription in response to radiation, while the phospho-mimic mutant may upregulate ERV transcription even without radiation present. We also plan to investigate chromatin accessibility in our KAP1 knockout lines with ATAC-seq (Buenrostro, Wu, Chang, & Greenleaf, 2015). Using DNA Tn5 transposase and high throughput sequencing, we would investigate open regions of chromatin in our KAP1 knockout and reconstituted lines, expecting to see greater accessibility at ERV regions normally kept repressed by KAP1.

4.2.2 Relative contributions of MDA5/MAVS and cGAS/STING

While we have demonstrated that the MDA5/MAVS pathway contributes significantly to IFN production and immune response post-irradiation, it is undeniable that the cGAS/STING pathway also contributes to the overall response, as evidenced by the many studies implicating cGAS/STING in the response to radiation. The question therefore remains, what proportion of the effect is due to cGAS/STING activation, and what proportion is due to MDA5/MAVS activation? We plan to address this question through more knockouts – depletion of STING will leave just the MDA5/MAVS response to radiation, while depletion of MAVS will leave just the cGAS/STING response to radiation. Both pathways activate type I IFN production via phosphorylation of TBK1, so it is likely that previous studies may have misattributed the cause of IFN production and subsequent immune response due to not taking the existence of multiple pathways into account. It is also possible that there are other PRR pathways contributing to IFN activation that have remained understudied; for instance, TLR3 is also known to sense dsRNA, activating a separate signaling cascade that may be relevant for cancer therapy (Roers, Hiller, & Hornung, 2016).

4.2.3 Importance of MDA5/MAVS pathway to *in vivo* tumor control

We also plan to investigate whether MAVS depletion can abrogate the *in vivo* phenotype of tumor and metastasis suppression in KAP1 knockout xenograft models. We hypothesized that activation of the MDA5/MAVS pathway through dsRNA stimulated IFN production and thus led to an immune response *in vivo*. Therefore, we expect that MAVS depletion in KAP1 knockouts will inhibit IFN production and immune response, downregulating the immune response and allowing the tumor to grow more effectively. Additionally, *in vivo* studies of MAVS knockout tumor xenografts will allow us to determine whether the MDA5/MAVS pathway is inherently activated to affect tumor growth and immune response, or if a stimulus such as radiation or KAP1 depletion is necessary to bring out its effects.

4.2.4 Origin of ISGs – tumor cells or immune cells?

In our research, we have used ISG induction as a downstream indicator for IFN production, and we have seen ISG upregulation in response to radiation, suggesting autocrine IFN signaling. Interestingly, we also saw ISG upregulation in our KAP1 knockout B16F10 *in vivo* tumor samples, despite there not being a significant difference in ISG transcription in our *in vitro* KAP1 knockout studies. We plan to address this by investigating ISG transcription in specific tumor subpopulations as opposed to the tumor as a whole. Using fluorescence-activated cell sorting (FACS), we can separate out the immune cell population from the tumor cell population and assay each population for ISG induction via RT-qPCR. ISG upregulation in immune cells would indicate that IFN paracrine signaling is responsible for immune recruitment to the tumor microenvironment, while ISG upregulation in tumor cells would indicate that IFN autocrine signaling of tumor cells is responsible for immune recruitment, possibly through enhanced antigen presentation. It is possible that the truth is a combination of both, which would open multiple avenues of research for the role of IFN and ISG signaling in cancer immunotherapy.

4.2.5 Combination studies with oncolytic viruses

The MDA5/MAVS pathway also holds implications for a cancer therapy that has recently seen a resurgence in interest with the advent of immune checkpoint blockade – oncolytic viruses (OVs). OVs are believed to mediate their therapeutic effect by selectively killing tumor cells, as well as stimulating antitumor immunity through the secretion of viral antigens, and over 100 clinical trials are currently underway investigating the therapeutic benefits of oncolytic virus therapy (Lawler, Speranza, Cho, & Chiocca, 2017). However, the contribution of the MDA5/MAVS pathway to immune activation suggests that viral killing of the tumor may not be necessary to stimulate an immune response; instead, tumor cell viral infection and upregulation of the

MDA5/MAVS innate antiviral signaling pathway for immune cell recruitment may be sufficient to confer significant effects, allowing for smaller doses and less toxicity.

4.3 Conclusion

Cancer treatment and immunotherapy are quickly evolving fields with many interconnecting factors affecting patient response to treatment and therapeutics. With increasing focus on combination therapies to drive more durable responses in the clinic, a comprehensive understanding of pathways targeted by cancer therapies is more important than ever. ERVs, dsRNA, and KAP1 represent only a small fraction of the entire field of research but provide an important mechanistic link between radiotherapy and immunotherapy that should not be ignored. Moving forward, more research is needed to develop therapies that take advantage of the MDA5/MAVS signaling pathway as a part of combination therapies that improve the health and survival of cancer patients in the clinic.

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Biography

Andrew K Lee completed his undergraduate degree in Chemistry and Economics at Emory University College of Arts and Sciences in May of 2009. In August of 2013, he began his Ph.D studies at Duke University Graduate School in the department of Pharmacology and Cancer Biology. He joined lab of Dr. Chuan-Yuan Li in the fall of 2014.

First author publications:

Lee AK, Pan D, Bao X, Hu M, Li F, Li CY. Endogenous retrovirus activation as a key mechanism of anti-tumor immune response in radiotherapy. Radiat. Res. Feb 2020, doi: 10.1667/RADE-20-00013. [Epub ahead of print]

Oral presentations:

2019 Radiation Research Society annual meeting, San Diego, CA

Title: Epigenetic regulation of endogenous retrovirus expression modulates the induced anti-tumor immune response to radiotherapy.

Awards and honors:

Radiation Research Society Scholar-in-Training travel award (2017, 2019)

Duke Scholar in Molecular Medicine – Oncology and Regenerative Medicine (2016-2017)

NASA Space Radiation Summer School (2016)