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Herpesviral regulation of the RAE-1 family of NKG2D ligands

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Herpesviral regulation of the RAE-1 family of NKG2D Ligands

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Abstract

Herpesviral regulation of the RAE-1 family of NKG2D Ligands

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Natural Killer (NK) cells are essential for control of viral infection and cancer. NK cells express NKG2D, an activating receptor that directly recognizes NKG2D ligands. These are expressed at low level on healthy cells, but are induced by stresses like infection and transformation. The physiological events that drive NKG2D ligand expression during infection are still poorly understood. Given the observation that the mouse cytomegalovirus (MCMV) encoded protein m18 is necessary and sufficient to drive expression of the RAE-1 family of NKG2D ligands I investigate m18, how it induces NKG2D ligand expression, and how the biology of NKG2D ligand regulation relates to the biology of herpesviruses. I demonstrate that RAE-1 is transcriptionally repressed by histone deacetylase inhibitor 3 (HDAC3) in healthy cells, and m18 relieves this repression by directly interacting with Casein Kinase II and preventing it from activating HDAC3. Accordingly, I found that HDAC inhibiting proteins from human herpesviruses induce human NKG2D ligand ULBP-1. Thus my findings indicate that virally mediated HDAC inhibition can act as a signal for the host to activate NK-cell recognition. Additionally, I characterize two unique proteins produced from the m18 ORF, and demonstrate tone of them is necessary and sufficient to induce the expression of RAE-1 ligands. Finally, I investigate a similarity between the reactivation control promoters of y-herpesviruses and NKG2D ligands, and investigate the potential of a drug that blocks NKG2D ligand induction to block y-herpesvirus reactivation.

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Chapter 1

Introduction to NKG2D ligand regulation

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Introduction to NKG2D ligand regulation

Natural Killer Cell Biology

The innate immune system provides a first line of defense against pathogens and malignancies. One of the greatest challenges in providing this initial defense is differentiating cells that are infected or malignant from healthy tissue. Natural killer (NK) cells are a crucial part of the innate immune system, and are particularly important for the control of intracellular pathogens, such as viruses, and cancers (Lodoen and Lanier 2006; Raulet and Guerra 2009). In order to distinguish healthy from unhealthy cells NK cells express on their surface a suite of germline encoded receptors. These receptors can inhibit or activate NK cell activity when presented with the appropriate ligand. The balance between inhibitory ligands (which are maintained at high levels on healthy cells) and activating ligands (which are absent or low on healthy cells) is integrated into the decision to activate an NK cell and drive it to kill the target cell and/or secrete pro inflammatory molecules to recruit a larger immune response (Smyth et al. 2005). NK cell activation driven by a lack of inhibitory signaling, is called “missing-self” recognition (Ljunggren and Kärre 1990). Conversely activation driven by an excess of activating receptors is called “induced-self” recognition. Both missing self and induced-self play important roles in NK cell surveillance of viral infections and cancers. These mechanisms work together to provide robust and redundant monitoring that prevents recognition of self and promotes the elimination of pathogens and cancers.

The best-studied inhibitory ligands are members of the major histocompatibility complex I (MHC-I) family. These molecules present virus and cancer peptides to CD8 expressing T cells. Pathogens and cancers often down regulate MHC-I in an effort to evade T cell control. In doing so they become exposed to control by NK cells through missing-self recognition. Missing-self recognition by NK cells in combination with CD8 expressing T cells provide the immune system with a hammer and anvil method of recognizing and eliminating these threats.

In contrast to NK cell inhibiting ligands, NK cell activating ligands are repressed on healthy cells. These ligands are induced by stresses like infection and transformation. Activating ligands may enhance NK cell recognition of cells that are already being recognized through missing-self recognition, or at high enough levels overcome inhibitory signaling to drive NK cell activation even in the presence of inhibitory ligands.

The best-studied family of activating ligands is recognized by the NK cell receptor NKG2D and is known as the NKG2D ligand family (Raulet 2003). This family is diverse containing two clades in humans (ULBP1-6, and MICA/B). Mice only have homologues of the ULBP family and this is subdivided into three sub-families (Raet1ɒ-ϵ, H60a-c, and MULT1) (Raulet et al. 2013). Each NKG2D ligand binds to NKG2D with a unique affinity, however expression of any single family member can be sufficient to drive NK cell killing (Champsaur and Lanier 2010).

NKG2D ligand regulation

The physiological that drive NKG2D ligand expression, as well as the molecular events that govern NKG2D ligand expression are still just beginning to be understood. From the work that has been done a few major themes have emerged in NKG2D ligand regulation. The most prominent of these is the multi-step regulation of these ligands. NKG2D ligands are actively regulated at nearly every step of expression from transcription to protein stability. Often multiple levels of repression must be released before ligands are efficiently displayed to NK cells. This may be an adaptation that allows for the specific presentation of NKG2D ligands in legitimate pathogenic states. Whereas a cell may transiently experience individual stressful states (such as amino acid starvation, genotoxic stress, or proliferative burst), the physiological stresses that drive expression of these ligands (viral infection and tumorogenesis) provide multiple types of stresses concurently. Thus the need for "multiple hits" may be a way to prevent inappropriate induction of these ligands and NK cell activation against otherwise healthy tissue. Additionally, this method of regulation may provide additional tunability to activating ligand regulation, or provide some qualitative character to ligand expression that dictates a particular type of NK response.

Another theme in NKG2D ligand regulation is redundancy. Often a stress that induces one ligand will induce multiple other (but not all) ligands. This redundancy may have developed as a way to prevent evasion by viruses and cancers. Many herpesviruses have developed multiple evasins, each of which has a greater ability to downregulate certain ligands or subfamilies of ligands (Griffin et al. 2010). Thus the expansion of NKG2D ligand diversity may be driven by host/virus competition.

Whatever the reason for its existence, the complicated multistage regulation of NKG2D ligands has provided a rich vein of study. Many groups have identified mechanisms by which NKG2D ligands are or can be regulated. In this text I organize these based on the way in which these mechanisms act on NKG2D ligand expression. The categories discussed are: transcriptional regulation, post-transcriptional RNA regulation, translation and protein level regulation, and soluble NKG2D ligand regulation.

Direct Transcriptional Regulators of NKG2D ligands

NKG2D ligand transcripts are very low in most healthy cells. Thus initiating transcription is an important first step in NKG2D ligand expression. Transcriptional regulators of NKG2D ligands and the physiological stresses are becoming better studied as new techniques for identifying and verifying these regulators are applied. Only four NKG2D ligand genes have defined promoter regions (*ULBP1*, *ULBP2, MICA*, *MICB*, and *Raet1e*). These are the only ligands for which direct transcriptional regulators have been identified.

E2F family transcription factors (Hyperproliferation)

The E2F family of transcription factors contains nine members (E2F1, 2, 3a, 3b, 4, 5, 5, 7 and 8). These can be further subdivided into activating (E2F1, 2, 3a) and repressive (E2F3b, 4, 5, 6, 7, 8) family members (Trimarchi and Lees 2002). The founding member of this family, E2F1, was originally discovered as a factor able to bind and activate the adenovirus E2 promoter (Kovesdi et al. 1987). E2F1 was later shown to drive non-viral cell cycle related genes such as c-Myc and DHFR.

In a healthy cell the E2F family contributes to cell cycle regulation. Activating E2F family members are bound by retinoblastoma (Rb) protein during the G1 phase of the cell cycle (Bagchi et al. 1991). This interaction prevents activating E2F transcription factors from driving transcription by retaining them in the cytosol. As the cell prepares to enter S phase Rb protein is phosphorylated by CDK4/6 complexes. This promotes the dissociation of Rb from activating E2F transcription factors and permits them to drive expression of S phase related genes (Reviewed in (Trimarchi and Lees 2002)). Activating E2Fs also drive expression of repressive E2Fs. Repressive E2Fs oppose transcription of activating E2Fs as well as their target genes creating a negative feedback loop. During G2-M all E2F family members (both activating and inhibitory) are targeted for degradation allowing the cycle to begin anew (Trimarchi and Lees 2002).

E2F transcription factors have been shown to bind the promoter of mouse NKG2D ligand *Raet1e* and promote the transcription of *Raet1e* in cells that are undergoing rapid proliferation . Overexpression of any of the activating E2F family members was sufficient to drive expression of *Raet1e*, and knockdown of any one of these was also sufficient to prevent expression of *Raet1e* (Jung et al. 2012).

Proliferation and E2F activation are functions of healthy tissue. However, RAE-1ε protein is not necessarily detectable in healthy proliferating tissues. One notable exception is fetal brain (Jung et al. 2012), which is not under surveillance by NK cells. This suggests that E2F may need to combine with some other transcription factor or epigenetic state to drive efficient *Raet1e* transcription in these contexts. Alternatively E2F levels may have to be elevated beyond their normal range to drive *Raet1e* transcription. The latter hypothesis is supported by the fact that transient overexpression of E2F was able to drive *Raet1e* expression.

It is also interesting that *Raet1e* expression persists past the end of the cell cycle. This may suggest that while *Raet1e* is upregulated by activating E2Fs it is not as efficiently downregulated as other cell cycle related genes. This would be true if the E2F binding sites in the *Raet1e* promoter efficiently bound activating, but not inhibitory E2F family members. This is possible because E2F family members do have unique binding preferences (Attwooll et al. 2004). The 2012 study did not evaluate the roll of repressive E2Fs in *Raet1e* expression, and it will be interesting in the future to understand any roll or lack of roll for repressive E2F family members in *Raet1e* expression in order to better understand how hyper proliferation drives NKG2D ligand expression.

c-Myc family transcription factors (Hyperproliferation)

c-Myc is a transcription factor activated downstream of growth factor receptors. It connects growth factor receptors to cell cycle activation by driving expression of cell cycle related genes including cyclins and E2F family members.

Predicted c-Myc binding elements can be found in many NKG2D ligand promoters including *Raet1e* in mice (Jung et al. 2012) as well as *ULBP1/3* in humans (Nanbakhsh et al. 2014). c-Myc binding sites in the human ULBP1/3 promoters can be enriched by chromatin immunoprecipitation (ChIP) against c-Myc indicating c-Myc does bind these elements. Additionally chemical inhibition of c-Myc decreased ULBP1/3 expression (Nanbakhsh et al. 2014). The mouse *Raet1e* promoter also contains a putative c-Myc binding site. c-Myc overexpression drove

expression from the *Raet1e* promoter, but mutation of the putative c-Myc binding site did not affect this activity. Ultimately the authors concluded that c-Myc drives *Raet1e* expression via induction of E2F transcription factors though they were unable to rule out a direct roll for c-Myc driving *Raet1e* expression in other contexts (Jung et al. 2012).

Like E2F c-Myc connects proliferative signals to NKG2D ligand regulation and may be most important in the context of cancers, which are known to secrete high levels of growth factors that would activate c-Myc (Normanno et al. 2006; Yamauchi et al. 2000). Additionally, c-Myc is a commonly mutated in cancers to become excessively expressed (Little et al. 1983; Dalla-Favera et al. 1983). Thus c-Myc could directly connect development of these cancers to NKG2D ligand regulation. Additionally, c-Myc may drive NKG2D ligand regulation in response to viral infection. Many viruses, including herpesviruses, use growth factor receptors as entry receptors. For example HCMV can use epithelial growth factor receptor (EGFR) as a receptor for entry, and stimulates signal through EGFR in the process (Chan et al. 2009)(Wang et al. 2003). EGFR can stimulate c-Myc activity (Cutry et al. 1989), and thus viral entry could induce expression of NKG2D ligands in a c-Myc dependent manner.

Sp family transcription factors

Specificity proteins (Sp) are members of the specificity protein/Krüppel-like factor (Klf) family of transcription factors. These are zinc-finger transcription factors. The founding member of this family Sp1, was originally identified for its ability to bind and drive transcription of the viral Sv40 promoter (Dynan and Tjian 1983). Many other Sp proteins have since been identified, but most work has focused on Sp1 and Sp3, which are ubiquitously expressed (Kaczynski et al. 2003). Sp proteins bind to GC boxes, and these elements can be found in a large number of genes. Sp1 and Sp3 can act as either repressors or activators of gene expression, though the former tends toward activation and the latter toward repression (Li et al. 2004).

Sp1 and Sp3 can both bind to both histone acetyl transferases (HATs) and histone deacetylases (HDACs). HAT activity promotes open chromatin and gene expression while HDACs do the opposite. The action of Sp factors as either repressor or activator at a given locus depends in part on the balance of recruitment of these enzymes at these sites (Li et al. 2004). Thus these factors often define loci that become activated in response to treatment with chemical histone deacetylase inhibitors (Wilson et al. 2006).

GC boxes have been found in the promoters of several human NKG2D ligands including MICA MICB and ULBP1. In the case of MICA and MICB Sp1 was bound to this site in HCT116 cells, and the Sp1 binding GC box was required to drive expression from these promoters in response to both heat shock and HCMV infection (Venkataraman et al. 2007). In the case of ULBP1 Sp3 was shown to bind this site in human foreskin fibroblasts (HFFs) and recruited HDAC3 in order to repress ULBP1 transcription (López-Soto et al. 2009). GC boxes can be found in the *Raet1e* promoter but these have not been previously analyzed for binding to Sp factors or regulation of *Raet1e*.

AP-2 (Development)

Despite similar naming AP-2 family transcription factors are not related to the AP-1 family. AP-2 proteins are expressed in the primitive ectoderm and neural crest of vertebrates, and deficiencies in AP-2α cause impairment in neural-crest-derived facial structures (Eckert et al. 2005). AP-2 family members are still expressed in adult tissues, although their function in this context is not well understood.

In NKG2D ligand regulation AP-2α has been shown to be capable of binding to the promoter of ULBP1 in lysates of HELA, but not HEK293 cells (López-Soto et al. 2006). This study suggested that an overlapping AP-2/CRE/GC box element within the ULBP1 promoter could bind AP-2, Sp1 or Sp3. As the AP-2 site negatively regulated promoter activity, and overexpression of AP-2α, β, or γ repressed activity from the ULBP1 promoter the authors concluded that AP-2 competes for binding to this overlapping element preventing Sp1 or Sp3 from accessing the site and driving ULBP1 transcription (López-Soto et al. 2006). It is unclear why AP-2 would naturally repress NKG2D ligand expression, but AP-2 has also been implicated in negatively regulating cell cycle, and growth signaling (Zeng et al. 1997). Thus AP-2 may provide a signal to stop growth, and evasion of this signal by cancers or viruses could release this repression and stimulate NKG2D ligand expression.

HSF-1 (Heat Shock)

Heat shock factor 1 (HSF1) is a transcription factor identified for its roll in driving expression of heat shock proteins (HSPs) in response to heat stress. Heat stress drives trimerization of HSF1 allowing it to translocate to the nucleus and bind heat shock response elements (HSE) in the promoters of HSPs. During their 2007 study of the MICA/MICB promoter architecture Gopalakrishnan et al discovered an HSE within the promoters of MICA and MICB. They showed that HSF1 binds this element in response to heat shock, and that mutation of this element disrupted the ability of the MICA or MICB promoter to drive expression of a luciferase reporter.

It is unclear why NKG2D ligand expression would be required in response to heat shock. However, heat shock also drives expression of MICA and MICB by inhibiting MARCH9 mediated ubiquitination of their cytoplasmic tails (Nice et al. 2009; Nice et al. 2010). Thus the concurent transcriptional, and post-translational regulation of MICA and MICB by heat shock demonstrates that there is a multilayered system driving MICA and MICB expression by heat shock.

CREB (Growth/Stress signaling)

Cyclic-AMP response element (CRE) binding protein (CREB) is a transcription factor of the CREB/ATF1/CREM family of transcription factors. This family has a leucine-zipper sequence for dimerization and can form homodimers and heterodimers within the family (Mayr and Montminy 2001). CREB can also form heterodimers with some AP-1 subunits to recognize either CREs or TPA Response Elements (TREs) (Hai and Curran 1991). The CREB family contains a kinase inducible domain (KID) and phosphorylation in the KID activates CREB. Thus CREB transcription is dependent on the activity of protein kinase pathways such as PKC (Yamamoto et al. 1988), CAMKII/IV (Matthews et al. 1994) and PI3K/Akt (Du and Montminy 1998).

CREB has specifically been implicated to be a transcription factor that helps to drive expression of human NKG2D ligands MICA/B and ULBP2. CRE sites exist in these promoters and CREB increases binding to these promoters when HEK-293T

cells are treated with the HDAC inhibitor LBH589 (Sauer et al. 2017). The physiological stimuli that drive CREB regulation have also been connected to NKG2D regulation. PI3K activation in particular has been shown to be required for *Raet1* family induction by mouse cytomegalovirus (MCMV) infection (Tokuyama et al. 2011), and RAS activation has been shown to drive expression of *Raet1* and ULBP family ligands (Liu et al. 2012).

ATF4 (ER Stress, Unfolded Protein Response, Amino Acid Starvation)

ATF4 is a member of the bZIP superfamily of transcription factors, in this superfamily it is most closely related to ATF1/CREB. ATF4 is connected to a variety of stress pathways through the translation initiation factor eIF2α. When eIF2α becomes phosphorylated in response to stress it globally suppresses translation of most transcripts, but preferentially increases translation of ATF4 (Harding et al. 2000).

In the context of NKG2D ligand regulation, ATF4 was identified as a positive regulator of ULBP1. ATF4 drives ULBP1 expression in response to amino acid starvation or ER stress induction with thapsigargin. ATF4 was shown by ChIP seq to bind to the ULBP1 promoter specifically under conditions of amino acid starvation (Gowen et al. 2015). Thus ATF4 connects NKG2D ligand regulation to both ER stress and amino acid starvation, both conditions commonly experience by infected and transformed cells.

Discussion

Transcriptional regulation of NKG2D ligands is complex and multivariate. The founding studies outlined above have identified a large number of transcription factor families that contribute to regulation of these ligands in a diverse set of cancers and tissues. Many of the families outlined above include a set of family members that bind to similar DNA sequences, but can either be repressive or activating. Thus, the picture is likely more complicated. As the field advances it will be important to remember the context in which each observation is made, especially with reference to the expression level of transcription factor family members, in order to synthesize an understanding of how NKG2D ligands are regulated in unique tissues, malignancies and stress conditions.

Transcription Factors that regulate NKG2D ligands

In addition to transcription factors that are known to directly bind NKG2D ligand promoters a number of transcription factors have been identified that interact in some way with NKG2D ligand transcript levels. The studies discussed below implicate transcription factors in NKG2D ligand regulation, but fall short of demonstrating a direct relationship.

AP-1 family transcription factors (Growth signaling)

AP-1 describes an activity of a family of proteins that drive expression of several genes containing AP-1 binding sites (5'-TGAG/CTCA-3') originally identified as TPA-responsive elements (TREs) (Angel and Karin 1991). The name AP-1 is used non-specifically to refer to a family of structurally and functionally related transcription factors from the Jun (Jun, JunB, JunD) and Fos (Fos, FosB, Fra1,Fra2) families as well as some members of the ATF (ATFa, ATF-2, ATF-3) and JDP (JDP-1 and JDP-2) families. Each AP-1 transcription factor is a dimer of proteins from this pool of subunits. The subunits dimerize through a leucine-zipper motif. Each subunit is

differentially expressed and regulated, and each dimer has a slightly different function (Hess et al. 2004). As the list of diverse functions and relationships within this pool is exhaustingly large this section will focus on TREs that have been identified in the promoters of NKG2D ligands as well as the specific AP-1 subunits that have been shown to alter NKG2D ligand expression.

TREs can be found in the promoters of the NKG2D ligand *Raet1e* from mice and MICA/B in humans (Venkataraman et al. 2007). This site has not directly been shown to be functional in human ligand regulation, but MICA expression increases in T lymphocytes when they are stimulated with TPA/anti-CD28 suggesting this TRE may be functionally relevant. The evidence for regulation of the mouse gene *Raet1e* by AP-1 is much better, though falls short of demonstrating a direct link.

Deletion of JunB in mouse embryos drives increased expression of *Raet1e* in mouse embryonic fibroblasts (MEF) and endotheliomas. Tissue specific knockout of JunB in mouse endothelium caused strong up-regulation of *Raet1e* expression in response to TPA. In JunB heterozygotes this induction was minimal and transient, however in full JunB epithelial knockouts TPA treatment drove a massive and sustained induction of *Raet1e* (Nausch et al. 2006).

JunB is an AP-1 subunit with very little transactivation activity, and is thought to in some cases act by sequestering the more active AP-1 subunits as a dominant negative regulator (Chiu et al. 1989). Thus it may be that activating AP-1 subunits are able to drive NKG2D ligand expression, and JunB expression is a natural check on this activity. This study supports this model, and may suggest that in tissues where JunB expression is naturally (or preternaturally) low, signaling through PKC can induce NKG2D ligand expression. As JunB is a tumor suppressor this system would create a rock/hard place style trap for cancer in which evasion of JunB's tumor suppressor activity permits the expression of NKG2D ligands.

NF-κB family (Inflamatory Signaling)

The nuclear factor κB (NF-κB) family of transcription factors are critical modulators of inflammation, immunity proliferation and survival. Prior to activation NF-κB family members are bound to inhibitory IκB proteins. This interaction retains these factors in the cytoplasm. Activation of an IκB kinase (IKK) drives phosphorylation and degradation of the IκB proteins, releasing NF-κB to translocate into the nucleus and drive transcription. The NF-κB family members include five proteins p105/p50 (NF-κB1), and p100/52 (NF-κB2), p65 (RelA), RelB, c-Rel. These come together as hetero or homodimers to form active transcription factor complexes. Up to 15 unique dimers can be formed from this pool (Oeckinghaus and Ghosh 2009), however not all have been shown to have a physiological function. Like Ap-1 transcription factors the combinatorial nature of NF-κB family, and the imprecise nature of referring to all dimers as "NF-κB" may contribute to seemingly contradictory literature.

Several studies have demonstrated that NF-κB related signaling is related to NKG2D ligand regulation. One group has demonstrated that after crosslinking of CD3 and CD28 MICA was upregulated in T lymphocytes. This group also demonstrated that this combination of treatment drove binding of p65/p50 to the first intron of MICA. They also showed that inhibition of IKK dampened, but did not eliminate, expression of MICA in response to CD3/CD28 crosslinking. Additionally

p65 overexpression was sufficient to drive increased expression of MICA in HeLa cells. Taken together this group concluded a direct role for NF-κB in MICA regulation (Molinero et al. 2004). However, their results do not rule out an indirect roll.

One other study of the roll of NF-κB in NKG2D ligand regulation showed that NF-κB signaling was related to a downregulation in expression of the mouse ligand H60a. Using a chemical inhibitor of NF-κB activity they were able to show a significant increase in H60a transcript levels, and demonstrated that this relied on an upstream regulatory element. However, the study stopped short of showing a direct link between NF-κB and H60a transcription (Peinado et al. 2013). These studies suggest that NF-κB may be involved in NKG2D ligand regulation, but its role may vary from ligand to ligand, or in different stress contexts.

STAT family transcription factors

The signal transducer and activator of transcription (STAT) family of transcription factors are regulators of cytokine and growth factor receptor signaling. These are transcription factors exist in the cytoplasm in an inactive state. Phosphorylation of inactive STAT proteins by receptor associated tyrosine kinases drives their dimerization and translocation into the nucleus.

STAT1 has been shown in multiple contexts to inhibit NKG2D ligand transcription, though each of these studies stops short of demonstrating a direct effect. Interferon-γ treatment of several tumor lines inhibited the expression of mouse NKG2D ligand H60 in a STAT1 dependent manner (Bui et al. 2006). Additionally the human ligands MICA and ULBP2 were similarly down regulated by IFN-γ treatment in several human melanoma lines (Schwinn et al. 2009) also in a STAT1 dependent fashion. In both cases IFN-γ treatment impaired the NKG2D dependent killing of tumors by NK cells (Schwinn et al. 2009; Bui et al. 2006). It is unclear whether these effects are direct results of STAT1 inhibiting NKG2D ligand expression or secondary effects.

The purpose of this inhibition is not clear. It should be noted that IFN-γ is secreted by activated NK cells, and also induces the expression of inhibitory NK cell ligands. Thus, persistent targets of NK cells become less stimulatory to NK cells; NKG2D ligand down regulation in response to IFN-γ appears to be part of this negative feedback loop.

IRF3

Interferon regulatory factor 3 (IRF3) is an activation induced transcription factor. It is a member of the Interferon regulatory factor family which mediate cellular host defense responses. IRF3 is activated by toll like receptor (TLR) or cytosolic DNA sensors (RIG-I, MDA-5, STING) by tank binding kinase 1 (TBK-1). After phosphorylation by TBK1 IRF3 can homo-dimerize, or hetero-dimerize with the related transcription factor IRF7 to bind to interferon stimulated regulatory elements (ISREs).

In the context of NKG2D ligand regulation IRF3 knockdown or heterozygosity was shown to inhibit the induction of RAE1 protein by genotoxic stress, as well as the reduce steady state levels of RAE1 on YAC1 cells. In addition inhibition of the upstream kinase TBK1, or knockdown of STING had the same effect (Lam et al. 2014). It is uncertain at what level this regulation happens as only RAE1 protein levels were measured.

Discussion of transcriptional regulators of NKG2D ligands

Transcription of NKG2D ligand genes is the first step toward expressing NKG2D ligands, and perhaps the most diverse. However it is one of the least well-understood parts of NKG2D ligand regulation. The proximal promoters of most NKG2D ligands still have not been accurately mapped, and no distal enhancers of NKG2D ligand expression have been identified. In the future many more transcription factors will likely be identified as new promoter regions are assigned, and perhaps enhancers uncovered. In addition our understanding of promoters, enhancers, chromatin, and transcriptional control in general is still being revised and improved. As that field and the tools used to analyze promoter enhancer biology improve so will our understanding of the elements that control NKG2D ligand transcription.

RNA stability and processing of NKG2D ligand transcripts

After transcription RNA is processed by splicing, polyadenylation, exported from the nucleus, and must then be engaged by ribosomes and translated. Alternative splicing, alterations in mature RNA stability and inhibition of translation by miRNA all regulate NKG2D ligands, and are discussed in detail below.

Alternate Splicing

Alternative splicing can provide a way in which protein-encoding genes can provide multiple functions. However, it can also add a level of on/off regulation to these genes if the alternatively spliced RNA lacks appreciable levels of function.

In the case of NKG2D ligands one example of splicing regulation has been identified in ULBP1. ULBP1 can be spliced in a canonical manner to produce functional protein, however, it can also be spliced into a unique form that is not translated into a functional protein (Gowen et al. 2015). The ratio between these forms is modulated in part by the RNA binding protein RBM4. RBM4 promotes the development of the functional ULBP1 transcript, and knockout of RBM4 resulted in the predominance of the non-functional transcript (Gowen et al. 2015).

RBM4 is expressed in most cell types under steady state conditions, but it has been shown to be regulated during reactive oxidative stress signaling *via* p38 kinase (Lin et al. 2007), thus ULBP1 splicing into a functional form may be more efficient under ROS conditions. RBM4 has also been shown to compete with another splicing factor polypryrimidine tract binding protein (PTB) to drive differential exon usage in α-Tropomyosin in muscle tissue (Lin and Tarn 2005). While this exact antagonism may not be relevant in ULBP1 splicing it is possible that other splice regulators preferentially promote non-functional ULBP1 splicing and compete with RBM4 to do so.

Two alternate splice isoforms exist for ULBP2. One of these is translated into a glycosylphosphatidylinositol (GPI) linked protein, while the other is an integral transmembrane protein (Fernández-Messina et al. 2011). The factors that mediate the switch between these isoforms have not been identified, and it is unclear what differences in regulation exist between the two forms. It has been proposed that each isoform is susceptible to a different type of enzyme for release as soluble ligand (discussed below).

Additionally, ULBP4 and 5 both have alternative splice isoforms that have been identified in tumor cells that eliminate their tansmembrane domains and allow them

to be secreted as soluble molecules (Eagle et al. 2009; Cao et al. 2007). Again the factors that mediate switching between these isoforms have not been identified, although the soluble forms seem to be either tumor or tissue specific (Cao et al. 2007; Eagle et al. 2009).

microRNA (Endogenous Repression and Viral Evasion)

Micro RNAs (miRNAs) are small non-coding RNA species that interfere with the expression of protein encoding genes. A miRNA is transcribed by RNA polymerase II into a pri-miRNAs. The Drosha-DGCR8 complex into pre-miRNA processes this species, before the pri-miRNA is exported from the nucleus into the cytosol. In the cytosol the protein Dicer converts pri-miRNA into a 22bp miRNA (Slezak-Prochazka et al. 2010). These miRNAs then associate with the RNA-induced silencing complex (RISC), which permits them to bind and silence mRNA transcripts either through cleavage and degradation of the RNA via the catalytic subunit argonaut, or by inhibiting translation (Filipowicz et al. 2008).

Several miRNAs have been identified to regulate the expression of the human ligands MICA/B (Yadav et al. 2009; Stern-Ginossar et al. 2008). These miRNAs inhibit translation of MICA/B transcripts, and some of them are inducible by IFN-γ. Additionally, the herpesvirus HCMV encodes a miRNA that binds to and degrades MICA/B RNA (Rodríguez-Rodero et al. 2007). In the case of the endogenous miRNAs the purpose of this system seems to be to act as a break on MICA/B expression. MICA/B can be upregulated at the transcript level, but without concurrent down regulation of these miRNA species protein cannot be efficiently presented to NKG2D.

DNA Damage stabilizes Raet1 transcripts

DNA damage induces the expression of the *Raet1e* family of NKG2D ligands in a manner dependent on the DNA damage induced kinases ATR and Chk1 (Gasser et al. 2005). Although expression of ligand RNA is increased in the presence of DNA damage it does not appear to be the result of a large increase in transcription. Rather *Raet1e* mRNA developes a longer half-life after cells are treated with DNA damaging agents. (Unpublished observations) The mechanism by which this happens is not well studied.

Discussion of post-transcriptional NKG2D ligand regulation

Post-transcriptional regulation is by its nature secondary to transcriptional regulation. Thus the above-mentioned mechanisms of control must be in a context in which NKG2D ligands are already being transcribed.

This is most clear in the context of the endogenous miRNA described above. These miRNA are induced by IFN-γ (Yadav et al. 2009). As was discussed above IFN- γ appears to signal to target cells to turn down NK cell activating receptors and turn up inhibitory receptors. Thus miRNAs appear to be a redundant form of control in this system. Alternatively, it is possible that these and related miRNA are responsible for the observation that IFN-γ reduces NKG2D expression.

In addition to this direct connection some of the pathways that have been shown to transcriptionally regulate NKG2D ligand expression also regulate miRNA processing and maturation. For example HDAC1, which inhibits transcription of some NKG2D ligands promotes the general processing of miRNAs via deacetylation of DGCR8 (Wada et al. 2012).

Translation and Protein level regulation of NKG2D ligands

After transcription and RNA processing NKG2D ligands must be translated by a ribosome transported into the ER, and shuttled to the cell surface, where they must remain stably in order to be recognized by NKG2D.

SPCS1 & SPCS2 (ER translocation)

NKG2D ligands are membrane-associated proteins, and undergo processing in the endoplasmic reticulum (ER) before shuttling to the surface of the cell. Transport of a nacent polypeptide into the ER can take place either co-translationally *via* the translocase Sec61, or post-translationally *via* TRC40. It is not known which strategy is predominantly used by NKG2D ligands, but the Sec61 pathway is used to translocate the majority of polypeptides.

Recently it was shown that two non-catalytic components of the signal peptide complex (the complex that cleaves signal peptides after translocation) regulate the expression of ULBP1. Knockout of SPCS1 and/or SPCS2 reduced the amount of ULBP1 that was able to reach the surface of the Hap-1 cell line (Gowen et al. 2015), counter intuitively knockout of either SPCS1 or SPCS2 did not effect the expression of any other membrane associated protein investigated in this study. Though it is likely it would affect some others. Little is known about the regulation of SPCS1/2, or their regulation of protein processing. However, it may be interesting in the future to determine if some cancer or virus associated stress signaling regulates these proteins.

Ubiquitination

Ubiquitination of membrane associated often takes place on their cytoplasmic tails and marks them for internalization or degradation. Only a few of the NKG2D ligands are transmembrane proteins, and thus susceptible to this form of regulation. These include MICA, MICB, ULBP4, ULBP5 in humans, and MULT1 in mice. The human ligand ULBP2 can be expressed as either a GPI linked protein or a transmembrane protein.

MULT1 stability at the cell surface has been shown to depend on ubiquitination of lysine within its intracellular tail. Interestingly, this ubiquitination happened efficiently at steady state, but was reduced in response to ultraviolet (UV) treatment or heat shock, resulting in increased levels of MULT-1 on the surface of cells exposed to these stresses (Nice et al. 2009). These residues were later shown to be modified by the Membrane-Associated RING-CH (MARCH) family of E3 ligases, specifically MARCH4 and MARCH9. MARCH9 was shown to release its repression of MULT-1 in response to heat shock (Nice et al. 2010).

There is also some evidence that ubiquitination is used to regulate the levels of human NKG2D ligand MICA. MICA has been shown to be downregulated in the human monocyte cell line THP-1 after treatment with histamine, and this downregulation is associated with ubiquitination of MICA and dependent on proteasome activity (Nagai et al. 2012). Additionally, MICA is targeted by a viral ubiquitin ligase during Kaposi Sarcoma Herpesvirus (KSHV) infection, and this event leads to intracellular retention, but not degradation of MICA (Thomas et al. 2008).

Soluble NKG2D ligands

Fully matured NKG2D ligands can be presented on the surface of a target cell or they can be released as soluble ligands. This release can take place in a variety of

ways including secretion, proteolytic release, and exosome mediated release (Reviewed in (G Chitadze et al. 2013)). The function of soluble NKG2D ligands is still not well understood. Many studies associated levels of soluble NKG2D ligands with decreased NKG2D expression on NK cells and less potent NK cell activity. In contrast one study in mice using an engineered form of the mouse ligand MULT-1 demonstrated that, at least in that system, soluble ligand increased NK cell potency and antitumor activity (Deng et al. 2015). Thus, the effect of soluble NKG2D ligands seems to depend on the ligand in question as well as possibly additional confounding factors.

Enzymatic release of NKG2D ligands

Enzymatic release is the most well studied mechanism that generates soluble NKG2D ligand generation. Ligands can be cut and released by a variety of extracellular enzymes, most commonly the A disintegrin and metalloproteases (ADAMs) (Reviewed in (G Chitadze et al. 2013)). ADAM family members are responsible for cleaving a large number of immunologically important proteins from the cell surface (Reviewed in (Reiss and Saftig 2009)). In the case of NKG2D ligands ADAM10 and 17 have been shown to drive shedding of MICA, MICB, and ULBP2. Differential activities of ADAM10 and 17 on these ligands have been reported, but recent study suggests that this heterogeneity may be a particularity of individual tumors rather than enzyme-substrate specificity (Guranda Chitadze et al. 2013).

ULBP2 has been shown to be generally a target of metalloprotease activity, but a specific enzyme has not been identified. Additionally, as discussed earlier, ULBP2 can be spliced to encode both integral membrane and GPI linked isoforms (Fernández-Messina et al. 2011). The GPI linked isoform can be released by Phosphoinositide phospholipase C (PI-PLC). In fact, in the same study PI-PLC was shown to release multiple GPI linked NKG2D ligands (ULBP1, 2 and 3) (Song et al. 2006).

Exosomal Release of NKG2D ligands

In addition to enzymatic cleavage, NKG2D ligands can be realeased *via* the exosomal secretion pathway. Exosomes, are small lipid vesicles secreted from cells as a form of cell-cell communication and transport. Exosomes derived from tumors and tumor lines (Hedlund et al. 2011; Clayton et al. 2008) as well as exosomes isolated from mature human DCs (Viaud et al. 2009) have been shown to contain NKG2D ligands.

Review of Soluble NKG2D ligand regulation

The study of soluble NKG2D ligands is relatively young, but the field is beginning to take shape. The majority of these studies have implicated that soluble NKG2D ligands down regulate NKG2D and/or decrease NK cell cytotoxicity. This effect is independent of whether they are released on exosomes, or via enzymatic release, or alternatively spliced into a secreted form. At the same time it has been demonstrated that soluble ligands have the potential to do the opposite (Deng et al. 2015). Understanding the differences between soluble ligands that inhibit NK function and soluble ligands that enhance it could allow for the development of new soluble NKG2D ligand based therapeutics, thus it will be important to study whether this

difference is the result of the ligands used, the mode of presentation or other confounding factors.

Summary of NKG2D ligand regulation

NKG2D ligand regulation is complicated by the sheer scope of the field. Many studies have spanned a wide variety of cell types and investigate the regulation of multiple different ligands. In addition, it is clear that individual ligands are regulated at multiple levels and thus a stress that drives NKG2D ligand expression in one context may not drive ligand expression in another. The past work on NKG2D ligand regulation has shed light on the types of stresses and stimuli that compel cells to promote or inhibit NK cell responses against themselves. Each form of NKG2D ligand regulation has a form of internal logic that connects into the lifecycle of viruses and cancers, and allows the cell to recognize these states and respond to them by recruiting an NK cell response. Future work will continue to improve our understanding of cell states considered “stressful” enough to warrant promoting NK cell activation.

As our understanding of NKG2D ligand regulation at every stage of expression becomes greater out we will be able to more clearly see the integrated circuits that come together to drive NKG2D ligand expression. Then we will be able to assess how sets of NKG2D ligands quantitatively or qualitatively convey the internal state of a target cell to an NKG2D expressing cell. This early work has already demonstrated certain stresses and signaling events act at multiple steps to induce or repress NKG2D ligand regulation. Heat shock drives MICA and MICB expression transcriptionally (Venkataraman et al. 2007), but also reduces the activity of the MARCH9 ubiquitine ligase that prevents MICA and MICB from being presented (Nice et al. 2009; Nice et al. 2010). IFN-γ drives a reduction in MICA and MICB transcript (Schwinn et al. 2009), but also upregulates miRNA expression that prevents existing transcripts from being translated (Yadav et al. 2009).

Early work has already showed that different ligands are able to drive functionally different effects on NK cells, including NKG2D signaling and recycling. In the future we may be able to combine NKG2D ligand regulation data with NK cell response data in a way that allows us to understand how ligands and combinations of ligands combine to qualitatively activate NK cell responses. Ultimately this will allow us to understand how NKG2D allows NK cells respond in qualitatively different ways to different physiological stresses.

NKG2D in viral infections

NKG2D surveillance has been best studied in response to cancer, however it is also an important part of recognizing viral infection. As indirect evidence of this fact many viruses encode proteins dedicated to evading NKG2D ligand recognition. However, it is also true that People with deficient NK cell responses including those with NKG2D polymorphisms predicted to reduce NKG2D signaling, are prone to pathogenic viral infections, particularly herpesvirus infections (Biron et al. 1989; Taniguchi et al. 2015). As a result, the majority of what is understood about viruses, their control by NKG2D, and evasion of NKG2D has been studied in the context of herpesviruses.

Herpesviruses

Herpesviruses are ancient viruses that have coevolved with their hosts for millennia. Herpesviruses are highly host tropic, and an individual family member may only be able to infect one or two different species. However as a family they include members that infect animals as diverse as humans and fish.

Herpesviruses are a family of large double stranded DNA viruses that establish life long infection in their hosts. Over 90% of the human population is infected with one or more of these viruses. Herpesviruses are consummate manipulators of their hosts and successfully transmit between hosts while also maintaining an ostensibly asymptomatic chronic infection. The maintenance of this detent with the host is in part made possible by the massive coding potential of these viruses which boast genomes between 100 and 250kb and can have upward of 250 open reading frames (ORFs) as well as many functional non-coding RNA elements. Our understanding of the gene coding potential of herpesviruses is in constant flux as new elements, alternative transcription start sites, and alternative splicing events are discovered. Furthermore, the majority of annotated genes lack function.

Herpesvirus control by NK cells and NKG2D

While herpesviruses normally are asymptomatic in their hosts, in immunocompromised individuals they can be pathogenic. This is true in patients specifically deficient in NK activity, or with polymorphisms predicted to reduce NKG2D signaling (Biron et al. 1989; Taniguchi et al. 2015). This suggests that in healthy hosts herpesvirus infections are held in detent in part through the activity of NK cells and NKG2D.

Induction of NKG2D ligand mRNA has been directly observed during infection for several herpesviruses including mouse cytomegalovirus (MCMV) (Lodoen et al. 2003), human cytomegalovirus (HCMV), and Kaposi sarcoma virus (KSHV). In addition herpes simplex virus one (HSV-1) and Rat cytomegalovirus (RCMV) have been shown to encode evasins that down-regulate NKG2D ligand expression suggesting that they are or were under selective pressure by NKG2D.

Molecular mechanisms of NKG2D ligand induction by viral infection

Many studies have observed NKG2D ligand induction during viral infection, and many others have identified viral factors that work to downmodulate the recognition of these ligands. However, little is known about the mechanisms by which NKG2D ligands are induced during viral infection. One key observation is that during MCMV infection it UV inactivated virus failed to induce the expression of NKG2D ligands indicating that viral gene expression is required for this induction (Tokuyama et al. 2011). It was also shown that the expression of NKG2D ligand in response to MCMV infection required active signaling through PI3K, though this seemed to be most important for translation of the ligands after transcriptional upregulation by infection. A group of authors from that study later performed a forward genetic screen showed a single viral gene (m18) was necessary and sufficient for this induction. That work would ultimately be published alongside some of the work in this thesis (Greene et al. 2016).

Issues addressed in this thesis

This thesis is primarily concerned with discerning the cause of NKG2D ligand induction by MCMV. In this work I identify the function of m18 as a viral histone

deacetylase (HDAC) inhibiting protein, which releases the RAE-1 family of NKG2D ligands from the repressive activity of HDAC3. Furthermore this activity is dependent on the presence of an Sp3 transcription factor binding-site within the *Raet1* promoter. I demonstrate that m18 directly interacts with the HDAC3 activating kinase CK2 and prevents it from phosphorylating and activating HDAC3. Thus, I demonstrate that MCMV infection, through m18, drives RAE-1 family NKG2D ligand expression by releasing repression by HDAC enzymes.

The m18 ORF was poorly characterized prior to this work, thus I also further characterize the m18 ORF. Most notably I demonstrate that it encodes at least two distinct stable protein products, and that it does so *via* an alternate splicing event. I demonstrate that the larger of these two forms which is decoded from the full length canonical ORF is both necessary and sufficient to induce expression of RAE-1 as described above.

Finally, I investigate the potential to take advantage of a *Raet1* promoter like system in the reactivation of y-herpesviruses in which the reactivation control locus can be induced using HDAC inhibitors in an Sp factor binding site dependent manner . I demonstrate that mithramycin, a chemical inhibitor of Sp transcription factor binding can effectively block y-herpesvirus reactivation and gene expression. Together, this thesis investigates the mechanism by which NKG2D ligands are induced during herpesvirus infection, and attempts to leverage that understanding to better understand, and potentially manipulate herpesvirus biology.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

Cell lines & Reagents

All cells cultured in DMEM with 10% FBS (Invitrogen) and 100u/mL Penicillin/Streptomycin (Invitrogen) unless otherwise noted. NIH 3T3 cells (ATC#CRL-1658) were obtained from the ATTC. HFF-1 cells (ATCC#SCRC-1041) were obtained from the ATTC. Mouse fibroblasts were generated as described (Gasser et al. 2005) and provided by Pr. D. Raulet. MCA-205 were received from Pr. L. Linear (UCSF). All cell lines tested negative for mycoplasma as described by Young et al 2010 (Young et al. 2010). Mithramycin A and Butyrate were purchased from Sigma. RGFP966 was purchased from Seleckchem. Antibodies recognizing Sp1 (product # 07-645), Acetylated Histone 3 (product # 06-599) were purchased from Millipore. Antibodies against histone 3 (clone D1H2), HDAC3 (Clone 7G685), and antibody recognizing CKII substrate (#8738) were purchased from Cell Signaling Technologies. Antibody recognizing CK2β (Product PA5-27416) was purchased from Thermo Fisher. Antibody against Sp3 (D-20) was purchased from Santa Cruz Biotechnology. Plasmid encoding HSV-1 ICP0 was provided by the Knipe Lab (Harvard). Plasmid encoding HCMV IE1 was provided by the Weinberger Lab (UCSF).

Transfection

Cells were transfected using FuGENE HD reagent (Promega) according to manufacturer's instructions.

RT-qPCR & qPCR

RNA from mouse fibroblasts or human foreskin fibroblasts was extracted in Trizol (Invitrogen). DNA was removed through treatment with RQ1 DNase (Promega), RNA abundance and quality was measured using a nanodrop ND-1000 to analyze 260/230 ratios. and 1µg of RNA was reverse transcribed for 45 minutes at 42°C using oligo(dT) primer (IDT) and SuperScript II (Invitrogen) in 20 µl total volume. cDNA was analyzed using an ABI7300 RT-qPCR System and cycled using a 95°C dissociation step for 15 seconds and a 60°C amplification step for 1 minute for 40 cycles. Samples were prepared as 1µl of prepared cDNA with 10µl of iTAQ universal Syber Green supermix (Invitrogen) with primers at a concentration of 300nM in a total reaction volume of 20µl. Cq values were determined using the Applied Biosystems 7300 SDS software. All samples were run as triplicates from the same pool of cDNA and the results averaged. Average Cq values were then normalized by $\Delta\Delta$CT against the indicated reference gene. Biological replicates were then used to calculate mean and standard deviation of values. Between 3 and 5 biological replicates were used in each experiment. Samples without RT were included to control for DNA contamination. RAE-1 primers were described previously (Tokuyama et al. 2011).

For DNA qPCR DNA was extracted from single cell suspensions of mouse tissue using Quiagen DNeasy Blood and Tissue Kit (Qiagen). DNA abundance and

quality was measured using a nanodrop ND-100 to analyze 260/290 ratios. Samples for use in qPCR were prepared as 2µL of isolated DNA with 10µL of iTAQ universal Syber Green supermix (Invitrogen) with primers at a concentration of 300nM, and cycled as described above with Cq values calculated as detailed above. Between 5-7 biological replicates were used in each experiment, tissues from uninfected mice were included to control for viral DNA contamination, and buffer processed without tissues were included to control for non-viral DNA contamination. MCMV gB primers were described previously by Khairallah and collegues. Standard curve for the calculation of absolute genome number was done using known quantity of purified MCMV BAC. Genome copy per gram tissue was calculated from weight of starting material and genome copies in each sample. Limit of detection was defined for each experiment and tissue as the copy number calculated from the average Cq from uninfected tissue.

Virus production, propagation, and infection

Transformation and induction of recombination was performed as described (Borst et al. 2007). E. coli strain GS1783 containing MCMV pSM3fr was provided by Dr. Caroline Kulesza (Fort Lewis College) and used to perform scarless BAC recombination as described by Tischer and colleagues (Tischer et al. 2006). The resulting BAC products were analyzed for anticipated mutation by PCR and EcoRI digestion. NIH 3T3 cells were transfected with BAC DNA, and supernatant was collected a week later. Supernatants were passaged twice in NIH 3T3 cells before use. All tissue culture infection experiments were performed at an MOI of 1.

Mice

BALB/cJ were purchased from The Jackson Laboratory. All mice were maintained under specific pathogen free conditions in the UC-Berkeley Animal Facilities. Mice used in experiments were between 3 and 8 weeks of age. All experimental procedures were conducted in accordance with the institution guidelines for care and use. Mice were infected with the indicated amount of virus. Liver, lung, and spleen homogenates were prepared at day five post infection, and viral titer was determined by qPCR specific for MCMV gB as described previously (Smith et al. 2008).

Luciferase Assay

Mouse fibroblasts were transfected with indicated constructs. Six separate transfections were averaged for each condition. At 24 hours post transfection passive lysis buffer (Promega #E1941) was used to lyse the cells. Lysates were transferred to an opaque assay plate (Corning) and D-Luciferin reagent was added to the plate. Luminescence was assessed over 10 seconds using an LMAX-II luminometer.

RAE-1 Staining

Mouse fibroblasts were harvested in 2mM EDTA in PBS and stained with monoclonal rat anti panRAE-1or Rat IgG_{2A} isotype control (R&D) followed by APC-conjugated goat anti-rat IgG (Jackson ImmunoResearch). All samples were co-stained with 7-AAD (BD) to exclude dead cells. Cells were analyzed by Flow cytometry using an LSR-Fortessa flow cytometer (BD).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear lysates were prepared as described by Jianping Ye (Pennington Biomedical Research Center, Louisiana State University). Oligonucleotides were labeled with ^{32}P-y-ATP using T4 kinase (NEB). Probes were purified on a G-50 column (G&E health care), and incorporated radioactivity was measured using a Beckman LS60001C scintillation counter. 4000cpm of labeled probe were added to nuclear lysates. Where indicated, competing unlabeled DNA probes were included in the reaction at a 1000:1 ratio. For super-shift assay 1ug of indicated Ab was added. Samples were run on a 5% native acrylamide gel. Gels were dried before being exposed in phosphofluor cassettes and analyzed using a Typhoon imager.

Affinity Purification and Peptide Sequencing by LC-MS/MS

Affinity-purification mass spectrometry (APMS) was used to identify candidate host-virus protein-protein interactions for the m18 protein. To this end, the annotated m18 orf was cloned into the pcDNA4TO expression vector encoding either an N-terminal or C-terminal 2X-StrepTag (m18-NS or m18-CS) for affinity purification and peptide sequencing by tandem liquid chromatography-mass spectrometry (LC-MS/MS) using methods identical to those previously reported ((Greninger et al. 2012). Briefly, 10 mg of vector were transfected into 15 cm cultures of HEK293T cells using a Transit-LT1 reagent (Mirus Bio, Madison, WI) at a 3:1 vol to mg plasmid, and the cells were grown for 48 h. Lysates were prepared in 0.1% NP40, 50 mM Tris HCl pH 8.0, 150mM NaCl, 1 mM EDTA. The M18 protein was captured on StrepTactin Sepharose, and then eluted with 1X desthiobiotin (IBA Technology, Gottingen Germany) as reported. To identify captured proteins by proteomic analysis, the protein samples were reduced with DTT, alkylated with iodoacetamide, and digested in solution with sequencing grade porcine trypsin (Promega) following an identical protocol to that reported (Greninger et al. 2012). The resulting peptides were subjected to LC-MS/MS on an LTQ-FT mass spectrometer (Thermo Scientific) equipped with a Nano-Acquity ultraperformance liquid chromatography system (Waters) for reversed-phase chromatography with a C18 column (BEH130; 1.7-µm bead size, 100 µm by 100µm), using identical acquisition parameters as reported (Greninger et al. 2012). MS data were searched using Protein Prospector software v. 5.10.17 (Chalkley et al. 2008) against the sequence of the m18 protein constructs and the NCBI Refseq human + virus database (downloaded Jan. 14, 2012) containing 131,459 sequences, concatenated with 131,459 additional randomized decoy sequences (Elias and Gygi 2007). A false discovery rate of <1% was obtained using protein score of 22, peptide score 15, protein expectation value 0.05 and a peptide expectation value of 0.001. Modifications allowed in the protein identification search were: fixed carbamidomethylation of Cys and the following variable modifications: oxidation of Met, start-Met cleavage, oxidation of the N-terminus, acetylation of the N-terminus, and pyroglutamate formation from Gln. HEK293T cells were chosen for these experiments to allow for identification of frequent background proteins and for specificity scoring, by comparison with a background dataset of unrelated picornavirus-host APMS experiments assayed in the same experimental system ((Greninger et al. 2012), PMC4332878).

Specificity scoring by Z-score was calculated using N=3 m18 APMS experiments, which included one m18-NS and two biological replicate m18-CS experiments, and a background dataset of 598 unrelated picornavirus-host APMS experiments. Additional peptides for the m18 protein constructs were identified by allowing additional missed cleavages and Ser/Thr phosphorylation. Phosphorylation sites are reported with a site localization (SLIP) score, where SLIP ≥ 6 corresponds to >95% confident site assignment (PMC3134073).

Western Blotting

Nuclear and cytoplasmic lysates were separated as above for EMSA analysis. Protein amounts were quantified using BCA assay (Thermo Scientific). Cell lysates were run on a 4-12% SDS-PAGE gradient gel and transferred to Immobilon-fl PVDF membranes (Millipore). Membranes were blocked with 5% Milk in PBS with 0.05%, or 1% BSA Tween before being probed with the indicated antibodies. Where phosphor-epitopes were being assayed, 1% BSA was used in place of 5% milk. Membranes were probed with Li-COR secondary antibodies and imaged on an Odyssey Li-COR imager.

CHiP

ChIP was performed essentially as previously described (Karijolich et al. 2014) with the exception being the use of a Fisher Scientific Sonic Dismembrator Model 100 to shear chromatin. *Raet1e* Promoter DNA was quantified by qPCR using previously described primers (Tokuyama et al. 2011). All samples were analyzed in triplicate.

Gene Cloning

Primers to genes of interest were designed using cDNA sequences available in the Uniprot database. Primers can be found.

Site Directed Mutagenesis

Site directed mutagenesis was carried out using the Quick-change site directed mutagenesis protocol (Stratagene, La Jolla CA). Primers used can be found below.

TCID50 assay

10,000 3T3 per well were infected using serial 1:10 dilutions of supernatant from infected cells. Dilutions ranged from no dilution to $1:10^9$ and eight replicate wells were used per dilution. The number of wells showing cytopathic effect were counted at day 7 post infection and used to calculate TCID50 using the Spearman-Karber method (Ramakrishnan 2016; Kärber 1931).

Immunofluorescence Assay

Mouse fibroblasts were plated onto glass slides before transfection with m18-RFP as described above. IFA was performed essentially as previously described using the following staining buffers (1%BSA in PBS for pHDAC1/3 & HDAC1/3, 1% Goat serum in PBS for AcH3 or H3) (21). Fluorescent signal intensity in the nucleus was quantified using FIJI software

Chapter 3

MCMV, m18, and NKG2D ligand regulation

SUMMARY

Herpesvirus infection drives expression of NKG2D ligands through an unknown mechanism. Using MCMV as a model system I have identified one mechanism by which herpesvirus infection drives NKG2D ligand expression. Previously it was demonstrated the induction of NKG2D ligand expression observed during MCMV infection required gene expression. Specifically the viral ORF m18 was shown to be both necessary and sufficient to drive NKG2D ligand expression. In this chapter I analyzed the activity of m18, and demonstrate that m18 drives NKG2D ligand expression by releasing Histone Deacetylase 3 (HDAC3) mediated repression of the *Raet1e* promoter. This repressive function is predicated activation by the cellular kinase CK2, which phosphorylates and activates HDAC3. The viral ORF m18 prevents this activating phosphorylation event, and increases acetylation at the *Raet1e* promoter. Furthermore, I demonstrate that this may be a conserved way to recognize herpesviruses as multiple HDAC inhibiting proteins from human herpesviruses induce the expression of ULBP1, a human ligand related to *Raet1e*.

Chapter 3
MCMV, m18, and NKG2D ligand regulation
RESULTS

Characterization of the m18 ORF

Previous studies have identified a CTL epitope produced from the m18 ORF (Holtappels et al., 2002), and peptides that correspond to this ORF have been identified in mass spectrometric analysis of MCMV virions (Kattenhorn et al., 2004). Otherwise little is known about m18 or its function. To characterize polypeptides produced from the m18 ORF we expressed a C-terminal hemagglutanin (HA)-tagged m18 protein in mouse fibroblasts & NIH 3T3s and analyzed cell lysates by western blot (Figure 3-1A). We observed a band at a size of ~180KDa, well above the predicted size of 110KDa, suggesting m18 is post-translationally modified. To evaluate m18 localization we expressed a C-terminal GFP m18 fusion protein in NIH 3T3s and analyzed localization of GFP by fluorescent microscopy. We found that the m18-GFP fusion protein localized mostly to the nucleus (Figure 3-1B). Importantly both m18-HA and m18-GFP constructs still induced expression of RAE-1 similarly to other m18 constructs (Figure 3-2).

The *Raet1e* promoter is activated by m18 through an Sp transcription factor-binding site.

To dissect the mechanism by which m18 induces RAE-1 expression, we measured m18's effect on *Raet1e* promoter activity. An expression vector encoding Firefly luciferase (FLuc) under the control of *Raet1e* promoter (Jung et al., 2012) was co-transfected into mouse fibroblasts with a vector encoding m18 or an empty control plasmid. Co-expression of m18 increased the activity of the *Raet1e* promoter relative to vector control (Figure 3-3B). Although E2F sites in the *Raet1e* promoter have been shown to drive RAE-1 expression during proliferation (Jung et al., 2012), these sites were dispensable for activation of the *Raet1e* promoter by m18 (Figure 3-4).

To identify the promoter elements required for m18 to drive expression from the *Raet1e* promoter, we generated a panel of serial 5' truncation mutants of the *Raet1e* promoter driving *FLuc* (Figure 3-3A) and co-transfected these with m18 expression vector or control vector. Promoters lacking 15 nucleotides at the 5' end of the promoter retained WT levels of induction in the presence of m18, but promoter activation by m18 was eliminated when 25 nucleotides were deleted (Figure 3-3B). These data indicate that the *Raet1e* promoter contains an m18 response element (m18RE) between -95 and -85 bp from the transcription start site.

Analysis of the m18RE for transcription factor (TF) binding sites using the JASPAR TF binding database (Mathelier et al., 2014) indicated a Specificity factor transcription factor family (Sp TF) binding site (Figure 3-3C) within the m18RE. To determine whether this site was required for m18 to drive expression from the *Raet1e* promoter we mutated this site (m18RE*) (Figure 3-3C) in the context of the rest of the *Raet1e* promoter. Promoter containing m18RE* showed significantly less promoter activity in the presence of m18 than the WT *Raet1e* promoter (Figure 3-3D), suggesting a role for Sp TFs in RAE-1 induction by m18.

We next assessed the ability of Sp TFs to bind the m18RE using an EMSA competition assay. Radiolabeled dsDNA oligonucleotides (oligos) of the m18RE or a

Sp consensus binding motif (Sp) were incubated with nuclear lysates in the presence or absence of excess non-radiolabeled oligos of m18RE, m18RE*, Sp, or mutant Sp (that lacks Sp factor binding ie. Sp*). These were then separated on a non-denaturing PAGE by electrophoresis, and the location of the radiolabeled oligo in the gel was measured. Incubation of m18RE or Sp without competing oligo showed high weight shifted bands indicating that these oligos bound factors in the nuclear lysate. The Sp oligo, but not Sp* eliminated the shift of ^{32}P-m18RE (Figure 3-3E, left panel) indicating that Sp but not Sp* can compete for binding with m18RE. Conversely, the m18RE oligo, but not m18RE*, can compete with ^{32}P-Sp (Figure 3-3E, right panel). Thus, the Sp consensus and m18RE oligos bind the same factors in nuclear lysate, suggesting that Sp TFs regulate *Raet1* transcription.

To assess Sp TF binding to the *Raet1e* promoter, we performed a chromatin immunoprecipitation (ChIP) analysis of Sp TF binding in MCA-205 cells. These cells encode only the *Raet1e* and *Raet1d* isoforms of *Raet1* genes allowing us to circumvent possible complications introduced by the presence of multiple highly homologous isoforms (Cerwenka et al., 2000). We first confirmed that MCA-205 cells have low basal RAE-1 expression and that RAE-1 expression is inducible by m18 (Figure 3-5). We used Sp1, Sp3, or control IgG antibodies to IP sheared chromatin/DNA complexes from these cells, and quantified the level of *Raet1e* promoter enrichment by qPCR. Samples immunoprecipitated using an antibody against Sp3 but not Sp1 showed significant enrichment of the *Raet1e* promoter over the IgG control, indicating that Sp3 occupies the *Raet1e* promoter. Interestingly, m18 expression did not alter the amount of Sp3 bound to the *Raet1e* promoter (Figure 3-3F). This indicates that Sp3 constitutively occupies the *Raet1e* promoter.

To determine whether Sp3 binding is required for m18 induction of RAE-1 expression, we co-transfected mouse fibroblasts with expression vectors encoding m18 and a dominant negative Sp TF (DN-Sp) and measured RAE-1 surface expression levels by flow cytometry. DN-Sp consists of an Sp1 protein DNA binding domain lacking the transactivation domain. DN-Sp binds to Sp1/3 binding sites and acts as a competitive inhibitor of promoter activation (Won et al., 2002). Co-expression of DN-Sp and m18 resulted in a decreased percentage of RAE-1 expressing cells (Figure 3-3G) suggesting that Sp factors are important for m18 to drive RAE-1 expression. To further test this hypothesis we transfected mouse fibroblasts with an expression vector encoding m18 and treated these cells with mithramycin, an inhibitor of Sp factor binding to DNA (Blume et al., 1991). Mithramycin treatment reduced RAE-1 induction by m18 (Figure 3-3H) further indicating that Sp TF activity is required for RAE-1 induction by m18.

The *Raet1e* promoter is repressed by HDAC3 in an Sp-dependent manner.

The human NKG2D ligand ULBP-1 is repressed by histone deacetylase 3 (HDAC3) in the absence of stress HDAC3 is recruited to the ULBP1 promoter by Sp3 (López-Soto et al., 2009). Additionally, HDAC inhibition induces RAE-1 expression (Gasser et al., 2005). Consistent with these findings, we observed that the chemical HDAC inhibitor butyrate activated the WT *Raet1e* promoter (Figure 3-6A). Interestingly, butyrate treatment failed to drive expression from the m18RE* mutant *Raet1e* promoter (Figure 3-6A). To assess whether chemical HDAC inhibition also requires Sp TFs to drive RAE-1 expression we treated mouse fibroblasts with butyrate in combination with

mithramycin. Mithramycin treatment reduced RAE-1 expression in response to butyrate treatment (Figure 3-6B), indicating that Sp TFs are also required to drive RAE-1 expression in response to HDAC inhibition.

Given that histone deacetylase inhibitors induce the expression of RAE-1 we wanted to identify which HDAC family member(s) must be inhibited to drive RAE-1 expression. To identify specific HDAC inhibitors that induce RAE-1 expression, cells were treated with a panel of HDAC inhibitors and analyzed for RAE-1 expression by flow cytometry. The pan-HDAC inhibitors TSA and NaB induced RAE-1 expression, as did the HDAC1/3 inhibitor MS-275 and the HDAC3 inhibitor RGFP966. In contrast, an HDAC1 inhibitor (4-(dimethylamino)-N-[6-(hydroxyamino)-6-oxohexyl]-benzamide), and an HDAC6,8 inhibitor (droxinostat) did not (Figure 3-6C). These results indicate that HDAC3 is involved in RAE-1 repression, likely through recruitment to the promoter by Sp3 (López-Soto et al., 2009) though additional HDACs may be involved.

m18 expression increases histone acetylation

As m18 and chemical HDAC inhibitors drive expression through the same promoter element in the *Raet1* promoter, we hypothesized that m18 acts as an HDAC inhibitor. HDACs modulate gene expression by deacetylating histones to maintain closed chromatin and repress gene expression. Thus one major prediction of this hypothesis is that histone acetylation should be increased in cells expressing m18.

To test the hypothesis that m18 expression increases histone acetylation we analyzed lysates of mouse fibroblasts transduced with m18 or vector control for acetylated histone 3 (AcH3) as well as bulk histone 3 (H3) by western blot. AcH3 levels were increased in m18 expressing mouse fibroblasts, as compared to those expressing vector control. H3 levels were unchanged (Figure 3-7A). To determine whether this effect was taking place as a direct result of m18 expression we transfected mouse fibroblasts with a construct encoding m18 C-terminally fused to red fluorescent protein (RFP), and assessed AcH3 levels by immunofluorescence assay (Figure 3-7B). We compared the fluorescent intensity of AcH3 staining in the nucleus of m18 expressing cells, to non-transfected cells in the same field of view, and found significantly higher levels of AcH3 in m18 expressing cells. AcH3 level was unchanged in cells transfected with RFP alone (Figure 3-7C). Expression of m18-RFP or RFP alone did not affect H3 levels (Figure 3-8). Finally, we assessed whether m18 expression increased acetylation at the *Raet1e* promoter by performing ChIP against AcH3 or H3 out of lysates from MCA-205 cells transduced with m18 or vector control. Analysis of *Raet1e* promoter enrichment showed an increase in AcH3 levels associated with the *Raet1e* promoter in m18 expressing cells while H3 levels were unchanged (Figure 3-7D). Together, these results indicate that m18 increases histone acetylation in cells, including at the *Raet1e* promoter.

CK2 inhibition connects m18 to HDAC3 regulation.

To investigate the mechanism by which m18 increases histone acetylation, we analyzed m18 binding to host proteins by immunoprecipitation (IP) followed by mass spectrometry. N- or C-terminally Strep-tagged m18 was transiently expressed in HEK293T cells, and native m18 complexes were captured by streptactin-affinity capture. Proteins were identified with peptide sequencing by LC-MS/MS. We used HEK293T for these assays as they allowed for direct comparison with similar virus-host AP-MS samples, and enabled us to identify high frequency background proteins found

in this system (previously reported in (Greninger et al., 2012)). To rank the specificity of the remaining candidate protein-protein interactions, a *Z*-score was calculated with three affinity experiments and 598 unrelated virus-host APMS experiments. This allowed identification of the most specific interaction as casein kinase II beta (CSNK2B) in APMS samples from human HEK293T cells. Peptide sequencing data from LC-MS/MS analysis covered 48-55% of the annotated m18 ORF protein sequence overall, and included phosphorylations at several S/T sites as well as peptides from the Strep-tag. We confirmed the interaction with CK2 in NIH-3T3 by IP-Western blot (Figure 3-9A).

CK2 is involved in many biological processes, including DNA damage signaling (Ghavidel and Schultz, 2001), apoptosis (Hellwig et al., 2010), and cell cycle progression (Homma and Homma, 2008). CK2 is a constitutively active kinase that phosphorylates HDAC3 at serine 424 (S424) activating it (Zhang et al., 2005). The activation of HDAC3 by CK2 has previously been demonstrated to help drive chromatin condensation during mitosis (Patil et al., 2016) and has been shown to repress expression of genes during beige fat thermogenesis (Shinoda et al., 2015) indicating that this interaction can functionally regulate both chromatin structure and gene expression. Thus, we hypothesized that CK2 activation of HDAC3 represses *Raet1* transcription in healthy cells, while inhibition of CK2 by m18 would result in less HDAC3 activity and thus induce *Raet1* transcription.

To test this hypothesis we investigated the ability of CK2 specific inhibitors to induce the expression of RAE-1. As CK2 acts as an anti-apoptotic factor (Hellwig et al., 2010) long-term inhibition of CK2 leads to apoptosis (Ruzzene et al., 2002). To circumvent this technical challenge, mouse fibroblasts were treated with zVAD caspase inhibitor in combination with the chemical inhibitor of CK2, TBBt, and cell surface RAE-1 expression was analyzed by flow cytometry. TBBt treatment induced RAE-1 expression in excess of zVAD alone (Figure 3-9B), indicating CK2 is a negative regulator of RAE-1 expression.

CK2 activates HDAC3 by phosphorylating HDAC3 at S424 (Zhang et al., 2005), thus CK2 inhibition by m18 should reduce levels of HDAC3 S424. To test this prediction we analyzed the HDAC3 S424 phosphorylation status in mouse fibroblasts expressing m18. We transfected mouse fibroblasts with a construct encoding m18 C-terminally fused to RFP or a control vector only expressing RFP, and assessed HDAC3 S424 levels by IFA (Figure 3-9E). We compared the fluorescent intensity of HDAC3 S424 staining in the nucleus of m18 expressing cells, to non-transfected cells in the same field of view, and found significantly higher levels of HDAC3 S424 in m18 expressing cells (Figure 3-9C,D). By the same measure overall levels of HDAC3 were unaffected by m18 transfection (Figure 3-10). This decrease was not observed in mouse fibroblasts transfected with control RFP vector. Together, these results indicate that m18 inhibits HDAC3 activity by binding to CK2 and reducing HDAC3 phosphorylation.

m18 is not required for MCMV growth *in vitro* or acutely *in vivo*

The fact that m18 ORF is highly conserved across strains of MCMV (Smith et al., 2008) suggests that m18 exert an essential function in MCMV life cycle. Such function is unlikely to be ligand upregulation as their expression would be detrimental for viral fitness (and accordingly the virus actively evades them). To assess whether m18 was

required for growth *in vitro* we infected NIH 3T3 cells with WT or Δ18 MCMV and assessed viral output by plaque assay 2,3,4,5 & 6 days post infection. Δ18 MCMV did not grow significantly different from WT MCMV in this assay (Figure 3-11A).

We then sought to determine whether m18 is critical for viral growth *in vivo*. As the Δ18 MCMV includes a large (3kB) genome deletion we generated an additional mutant MCMV to eliminate m18 expression with minimal alteration to the rest of the genome. This virus mutant ($MCMV^{m18stop}$) includes a set of two stop codons early in the m18 coding sequence preventing the production of the m18 protein. Like Δ18 MCMV, $MCMV^{m18stop}$ fails to induce RAE-1 expression in mouse fibroblasts (Figure 3-12A), but has no defect in IE-1 expression (Figure 3-12B) and growth was similar to wild type virus.

To assess the roll of m18 *in vivo* we infected BALB/c mice by intraperitoneal (i.p.) injection with WT MCMV or $MCMV^{m18stop}$, and assessed the levels MCMV five days later in the spleen, liver, lungs and salivary glands of these mice by qPCR (Khairallah et al., 2015). Mice infected with WT or $MCMV^{m18stop}$ had identical levels of MCMV in all organs assayed at this time (Figure 3-11B). This indicates that m18 elimination specifically effects growth in the salivary glands.

It is possible that a deleterious effect of losing m18 in the spleen, lung and liver was masked in WT mice by immune surveillance. To assess viral fitness in the absence of immune surveillance, we assessed the growth of WT MCMV and $MCMV^{m18stop}$ in $RAG^{-/-}$ $\gamma C^{-/-}$ mice. These mice lack T cells, B cells, and NK cells. Interestingly, these mice also showed identical levels of MCMV between the WT and mutant viruses in spleen, lung, and liver (Figure 3-11C). Indicating that m18 is dispensable for viral growth in the spleen, lung, and liver at this early time point, even in these mice. Salivary gland virus was not assessed in these mice at this time point, but it will be interesting in the future to see if the defect in salivary gland growth is dependent on the presence of T cells, B cells, or NK cells.

Model for m18 induced RAE-1 expression in MCMV infection

Together our results suggest a model for RAE-1 regulation in which HDAC3, constitutively activated by CK2, maintains *Raet1* in a repressed state. During MCMV infection m18 protein interacts with CK2 and prevents the activation of HDAC3, reducing its repressive activity. The *Raet1* promoter becomes unrepressed and constitutively bound Sp3 can recruit transcriptional machinery to transcribe *Raet1*. To circumvent this induction that would target infected cells for elimination by NK-Cells, MCMV encodes a number of highly efficient evasins that prevent NKG2D from recognizing RAE-1 during viral infection (Figure 3-13).

HDAC inhibitors of other herpesviruses induce expression of NKG2D ligands.

Some other herpesviruses also encode viral HDAC inhibitors. Two prominent examples of HDAC inhibiting viral proteins are IE1 from HCMV (Nevels et al., 2004) and ICP0 from HSV-1 (Gu et al., 2005). We sought to determine whether expression of these proteins would be sufficient to induce expression of human NKG2D ligands. We transfected human foreskin fibroblasts (HFFs) with plasmids expressing IE1 or ICP0 or empty vector and measured expression of human NKG2D-ligands by RT-qPCR compared to vector control. Both ICP0 and IE1 induced expression of human ULBP1 (Figure 3-14A, B). These results suggest that viral inhibition of HDACs is a common mechanism driving NKG2D ligand induction in humans and mice.

DISCUSSION

These results suggest a model in which RAE-1 expression is repressed in the absence of stress signals due to closed chromatin around the Raet1 locus. This repression is maintained by constitutive phosphorylation and activation of HDAC3 by CK2. However, during MCMV infection, m18 reduces phosphorylation of HDAC3 by directly interacting with CK2 an activator of HDAC3. As a result, HDAC3 becomes less activated and the chromatin around the Raet1 locus becomes acetylated and accessible. Sp3 is then able to recruit the general transcriptional machinery to transcribe Raet1 (Figure 3-13). These findings reveal how the chromatin environment contributes to silencing of Raet1 in unstressed cells, as well as how a single viral protein relieves this repression.

It is notable that HDAC inhibition is a feature common to a number of herpesviruses. Many of the known HDAC inhibiting proteins from herpesviruses are crucial to viral fitness (Gu et al., 2005; Nevels et al., 2004). Our work demonstrated that for viruses, encoding HDAC inhibiting proteins comes at the cost of inducing NKG2D ligand transcription. It is thus tempting to speculate that viruses being unable to replicate efficiently without inhibiting HDACs have had to evolve a plethora of proteins that reduce NKG2D ligand expression at the protein level (Jonjić et al., 2008).

While it is appreciated that viral HDAC inhibitors can improve viral fitness, the precise significance of virally encoded HDAC inhibitors in herpesvirus pathogenesis is still not well understood. Cellular HDAC enzymes have been shown to repress viral gene expression and, in some cases replication. In the context of lytic infection herpes viruses must overcome this repression using viral HDAC inhibitors (Nevels et al., 2004), in fact chemical HDAC inhibition can rescue a defect in viral replication caused by HCMV IE1 deficiency (Nevels et al., 2004). Additionally, viral gene repression by HDACs also promotes viral latency. HDACs have been shown to occupy the promoters of the immediate early genes that drive reactivation in HCMV and MCMV (Liu et al., 2010; Murphy et al., 2002), and have been shown to repress KSHV reactivation (Shin et al., 2014). In fact, inhibition of HDACs is sufficient to drive reactivation in latently infected KSHV cell lines (Miller et al., 1997). In these cases viral HDAC inhibitors may provide a way for the virus to promote its own reactivation from latency. Importantly, these proteins have been described in human viruses and their function in vivo has not been well established. We observe a tissue specific need for m18 in MCMV infection *in vivo.* Interestingly this defect is present in the salivary glands, which is one of the more immunoprivileged sites of MCMV replication. As NK cells are less active in the salivary glands (Tessmer et al., 2011) the drawback of inducing NKG2D ligand expression may be less acute in this location lessening any negative impact of expressing m18. It is unclear what m18 may be doing to promote viral growth in the salivary glands, and it will be interesting in the future to determine how m18 contributes to optimal replication or immunevasion within this niche.

Our data also provide the first evidence for the role of CK2 in NKG2D regulation. Given that CK2 activity can be modulated by many stress pathways, it will be of interest to assess whether CK2 contributes to NKG2D ligand regulation in situations such as DNA damage. Furthermore, CK2 is broadly involved in many cellular processes. Thus m18 inhibition of CK2 is likely to have additional effects on the cell. Chemical CK2 inhibitors enhance the anti-viral effect of Type I interferon signaling during HSV-1 infection (Smith et al., 2011), implicating CK2 as a key factor in host

response to viral infection. It will be interesting in the future to elucidate how manipulation of CK2 by m18 impacts the host during viral infection.

In addition to their role in viral infection, HDACs are also important for the control of cancer. Expression of Class I HDAC enzymes (including HDAC3) is increased in cancers (Glozak and Seto, 2007). Increased HDAC expression promotes carcinogenesis through down regulation of tumor suppressors such as p21 in an Sp1/Sp3 dependent manner (Wilson et al., 2010). As NKG2D ligand ULBP-1 is suppressed by HDAC3, it has been proposed that increased HDAC expression also contributes cancer's ability to evade NKG2D recognition (López-Soto et al., 2009). Several chemical HDAC inhibitors, including some used in this study, are being developed as anti-cancer drugs (González et al., 2008). While these drugs act in multiple ways, one effect may be to induce expression of NKG2D ligands leading to increased NK cell recognition and NK cell activation (González et al., 2008; López-Soto et al., 2009). Our study suggests that HDAC3-specific inhibitors may be particularly adept at leveraging this aspect of anti-cancer activity. CK2 is also highly upregulated in cancers (Litchfield, 2003), and one intriguing possibility is that this may contribute to NKG2D ligand repression and NK cell evasion.

The regulation of NKG2D ligands is an important pivot point in immune regulation. Active repression of NKG2D ligand transcription by HDACs provides a way for the host to repress NKG2D ligand expression in the absence of stress while allowing for the possibility of expression when experiencing stress. Our study suggests that this system may also provide a strategy for hosts to recognize and respond to viral infection. The rest of this dissertation will focus on additional characterization of the m18 ORF, as well as the investigation of a role for histone deacetylases and Sp transcription factors in the life cycle of γ-herpesviruses.

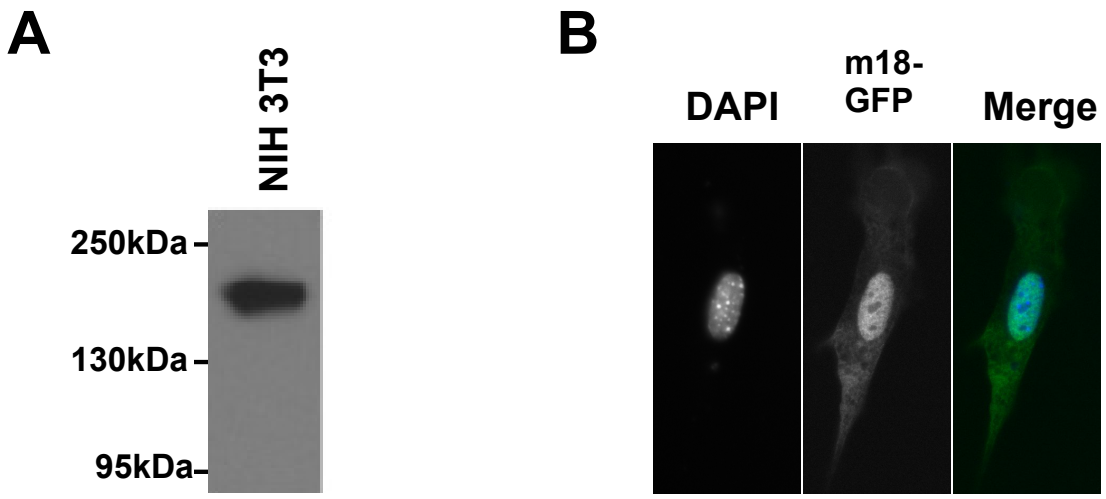


Figure 3-1: m18 produces a 180Kd nuclear localized protein
(A) NIH3T3 were transfected with an expression plasmid encoding an m18-HA fusion protein, and lysates were analyzed for m18-HA expression by western blot. (B) NIH 3T3 transfected with an expression plasmid encoding an m18-GFP fusion protein, and analyzed for localization of m18-GFP fusion protein by confocal microscopy.

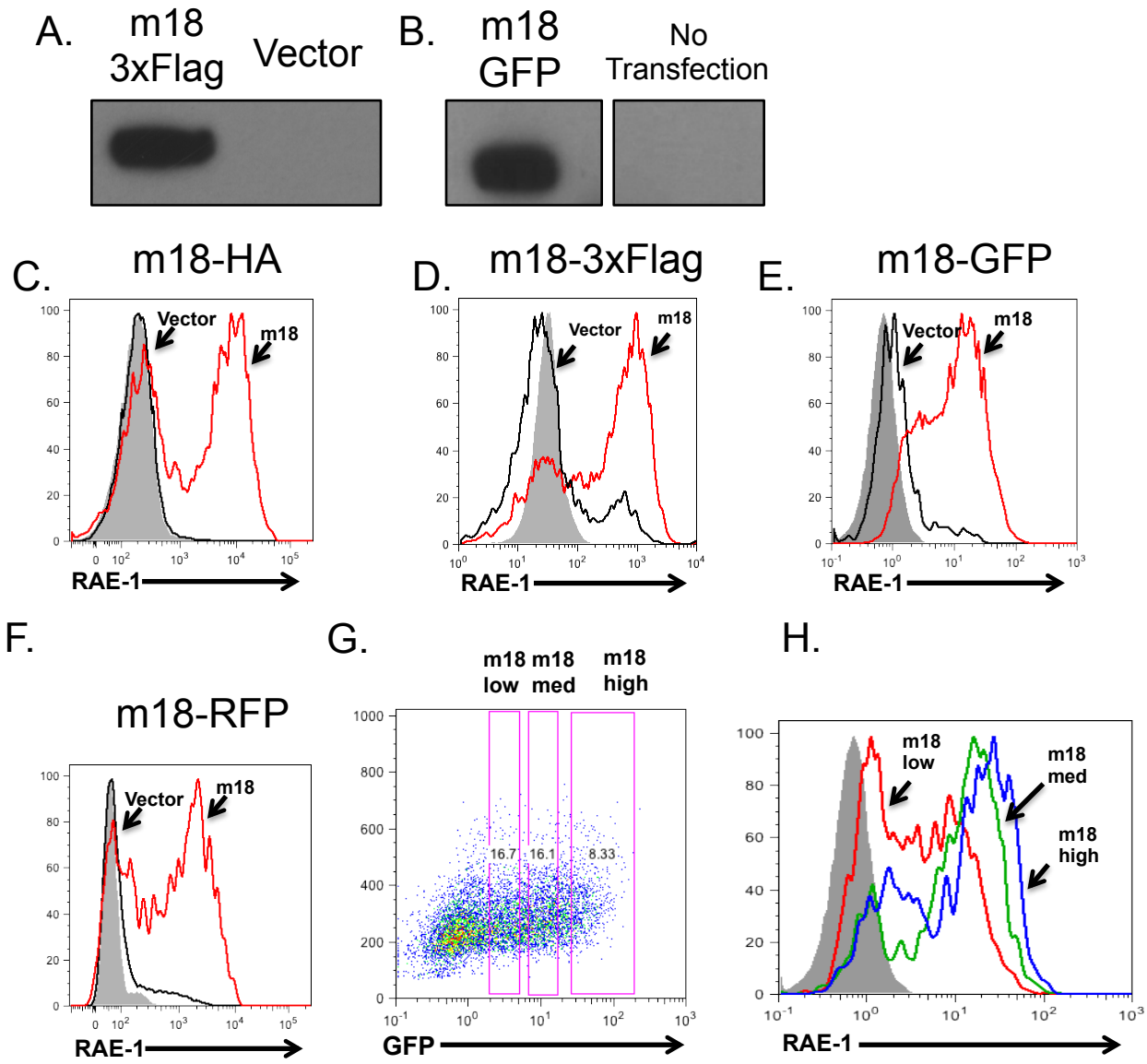


Figure 3-2: m18 fusion constructs express protein and induce RAE-1 expression.

(A,B) Lysates from mouse fibroblasts transfected with a mammalian expression construct encoding m18 C-terminally fused to 3xFlag epitope tag (A), mammalian expression construct encoding m18 C terminally fused to GFP (B) or vector control. (C-F) Mouse fibroblasts were transfected with the indicated constructs and analyzed for RAE-1 expression by flow cytomotry. (G) Mouse fibroblasts were transfected with a mammalian expression construct encoding m18 C-terminally fused to GFP, and analyzed for RAE-1 expression. From these data gates of GFP low, GFP medium, & GFP high (Left) were analyzed for RAE-1 expression (Right).

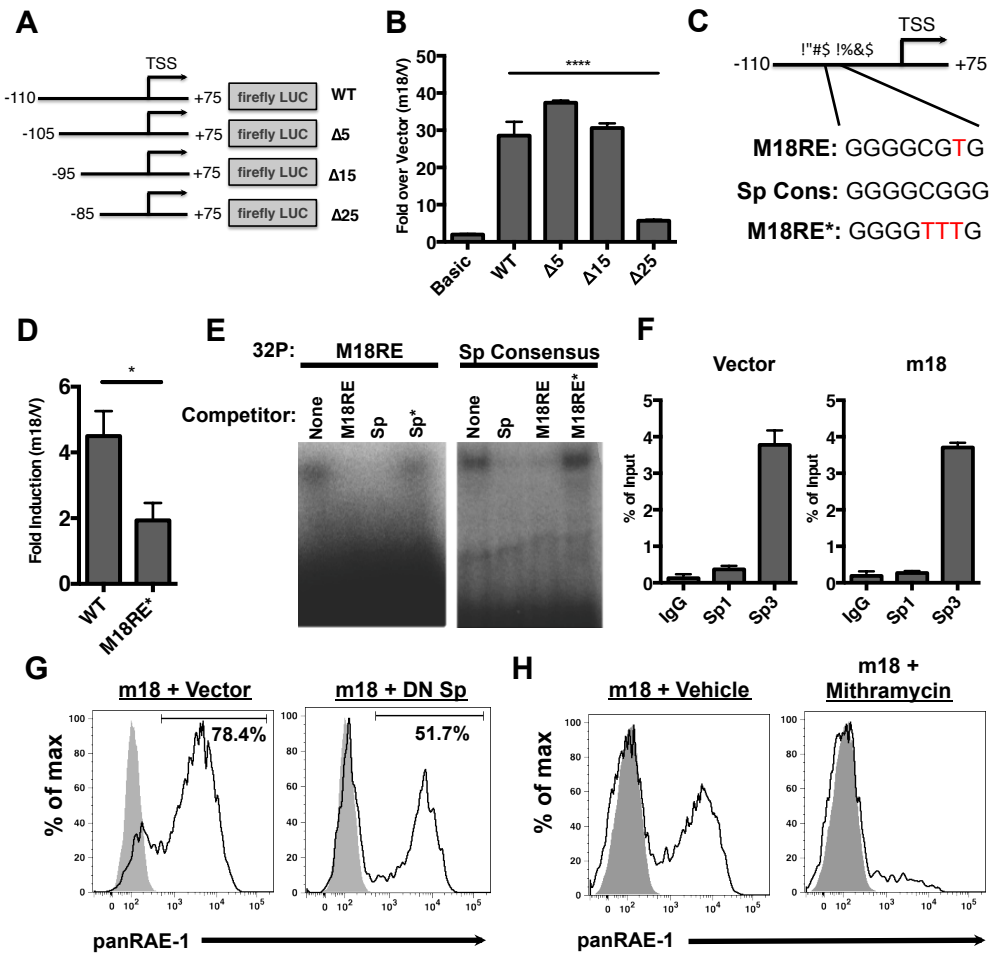


Figure 3-3: M18 drives Raet1e promoter activity via a Sp factor binding element.
(A) A graphic representation of Raet1e promoter luciferase constructs used in (B). (B) Raet1e promoter activity was measured by quantifying luminescence in lysates of cells transfected with the indicated luciferase promoter constructs in combination with m18 or vector control. Data are represented as fold increase in luminescence over vector control. Data are represented as mean±SEM. Data are representative of 3 independent experiments. ****, p<0.00005 (1-way ANOVA with Bonferroni's multiple comparison post-test) (C) Alignment of the WT m18RE (top), Sp consensus binding sequence (middle) and mutant m18RE (bottom). (D) Raet1e promoter activity was measured in cells co-transfected with a luciferase construct containing WT Raet1e promoter or Raet1e promoter containing a mutation in the Sp-binding site (m18RE*) and either m18 or vector control. Data shows fold increase in luminescence over vector control. Data are represented as mean (±SEM). Data are representative of 3 independent experiments. *,p<0.05 (Student's T-test). (E) EMSA was performed on mouse fibroblast nuclear extracts incubated with radio-labeled m18RE (left panel) or radio-labeled Sp consensus sequence (right panel). The indicated non-radiolabeled oligos were added in 1000-fold excess before separation by non-denaturing PAGE. Data is representative of 3 experiments. (F) ChIP was performed on MCA-205 cells using the indicated antibodies and enrichment of the Raet1e promoter was assayed by qPCR. Values were normalized to input chromatin. Data are representative of 3 independent experiments. (G) Mouse fibroblasts were co-transfected with m18-RFP and DN-Sp1 GFP or GFP vector control and cells expressing both GFP and RFP were analyzed for RAE-1 expression by flow cytometry. Data is representative of 3 experiments. (H) Mouse fibroblasts were transfected with m18-GFP and treated with Mithramycin or vehicle control were analyzed for expression of RAE-1 by flow cytometry. Data is representative of 3 experiments.

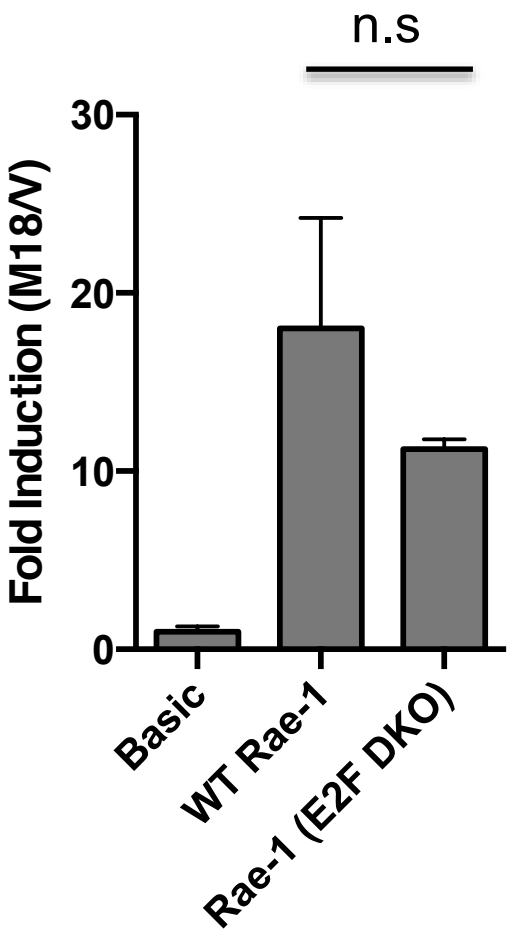


Figure 3-4: m18 induces transcription from the *Raet1e* promoter independent of E2F binding sites.
Mouse fibroblasts were transfected with m18 or vector control plasmids along with with luciferase reporter constructs containing the RAE-1 promoter (WT Rae-1) or a Rae-1 promoter with both E2F binding elements mutated (E2F DKO). Data are represented as fold difference between promoter construct transfected with m18 over promoter construct transfected with vector control. Values are shown as mean±SEM. Data are representative of 3 independent experiments. n.s.: Not significant (1-way ANOVA).

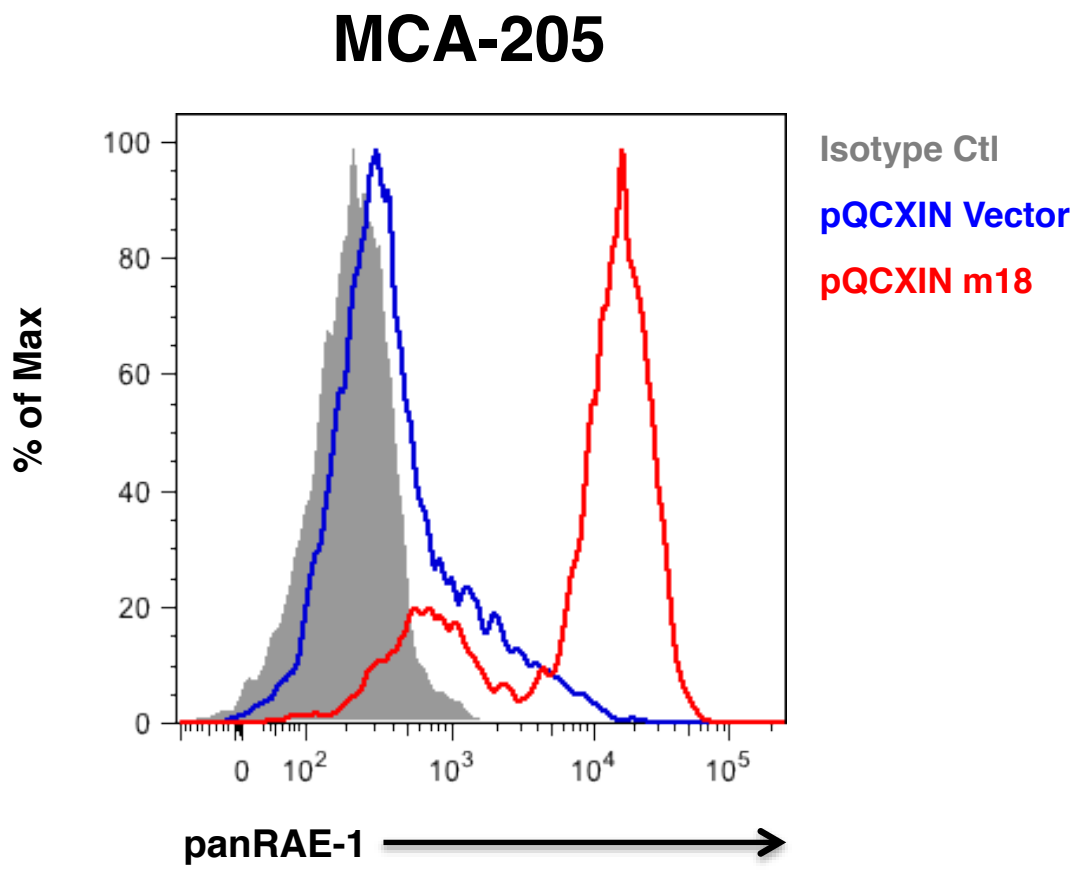


Figure 3-5: MCA-205 carcinoma cell line is inducible for RAE-1 expression by m18.
MCA-205 cells stably tranduced with either vector control or m18 & analyzed for RAE-1 expression by flow cytometry. Data are representative of 3 independent experiments.

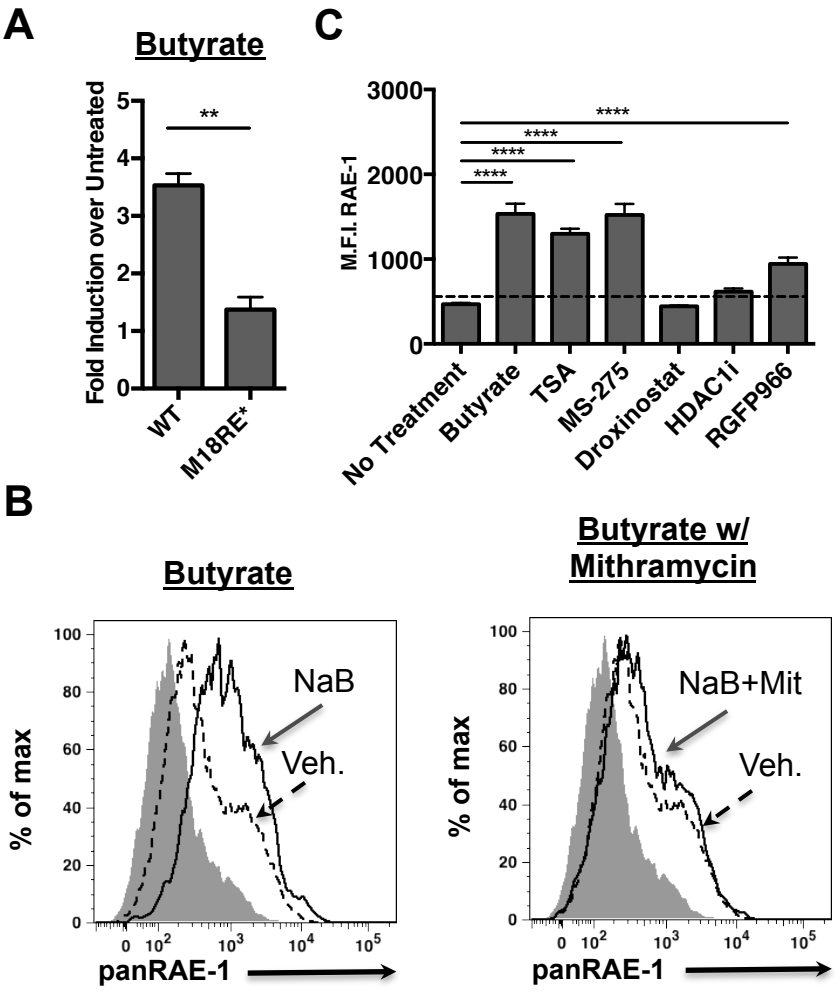


Figure 3-6: HDAC inhibition drives RAE-1 expression in an Sp factor dependent manner

(A) Raet1e promoter activity was measured in lysates from mouse fibroblasts transfected with either WT Raet1e promoter or the m18RE* promoter treated with sodium butyrate (NaB) (1mM). Data are expressed as fold change between butyrate treated and untreated promoter. Data are represented as mean±SEM. Data are representative of 3 independent experiments. **,p<0.005 (Student's T-test) (B) Cells treated with NaB (1mM) with or without Mithramycin (1.5uM) were analyzed for RAE-1 expression by flow cytometry. Data are representative of 5 independent experiments. (C) Cells were treated with HDAC inhibitors TSA (1nM) (pan-HDA-Ci), NaB (0.1mM) (Class I & IIa), MS-275 (800nM) (HDAC1,3), RGFP966 (640nM) (HDAC3) 4-(dimethylamino)-N-[6-(hydroxyamino)-6-oxohexyl]-benzamide (HDAC1i) (1μM) (HDAC1), or Droxinostat (3μM) (HDAC6,8) and analyzed for RAE-1 expression by flow cytometry. Data are represented as mean fluorescent intensity±SEM. Data are representative of 3 independent experiments. ****, p<0.00005 (1-way ANOVA with Bonferroni's multiple comparison post-test)

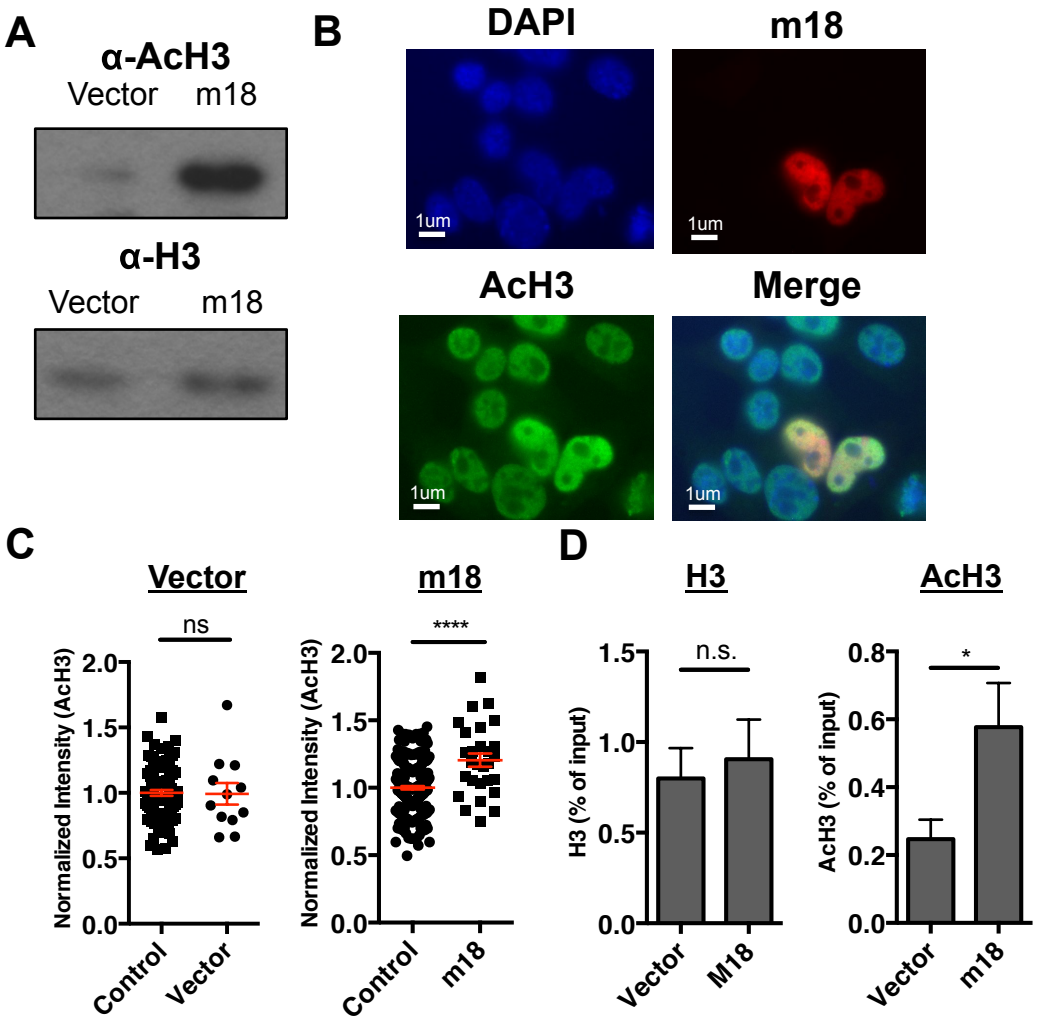


Figure 3-7: m18 expression increases levels of histone acetylation.
(A) Mouse fibroblasts stably expressing m18 or vector control were analyzed for H3 and AcH3 expression by western blot. Data are representative of 3 independent experiments. (B) Representative image of cells transiently transfected with vector encoding m18 with a C-terminal fusion to RFP (m18-RFP) and stained for AcH3. (C) Quantification of AcH3 levels in cells expressing m18-RFP or RFP control vector from compared to non-transfected controls in same field of view. Red bars are representative of mean±SEM. Data are representative of 3 independent experiments. ****P<0.00005. n.s., not significant. (Student's T-test) (D) ChIP was performed for AcH3 and H3 in MCA-205 mouse carcinoma cells stably expressing m18 or vector control. Data are normalized to input chromatin and represented as mean±SEM. Data are representative of 3 independent experiments. *, p<0.05, n.s., not significant (Student's t-test).

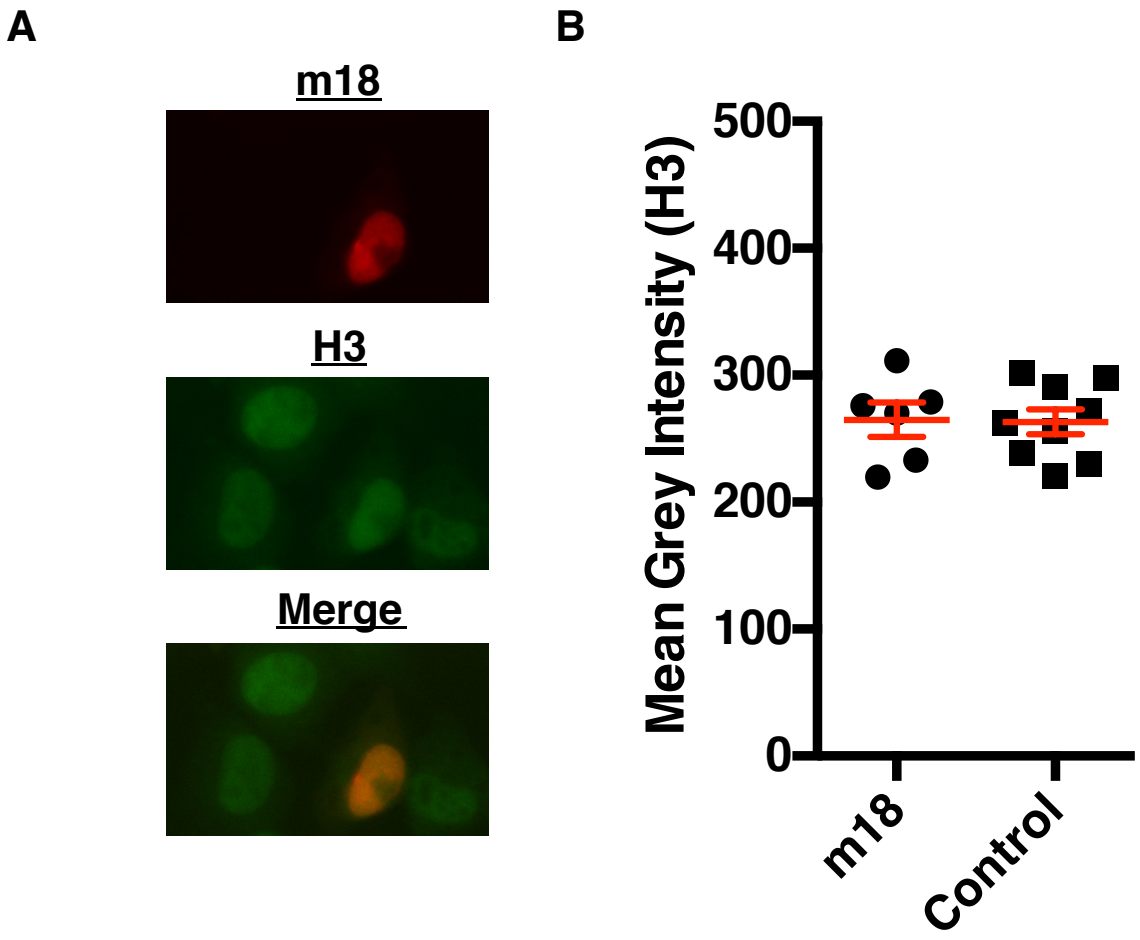


Figure 3-8: m18 expression does not change Histone 3 levels

(A) Representative picture of H3 staining in m18-RFP transfected cells.
(B) Nuclear staining of H3 was quantified in FIJI. Red bars are representative of mean±SEM. Data are representative of 3 independent experiments. n.s., Not significant (Students T-test).

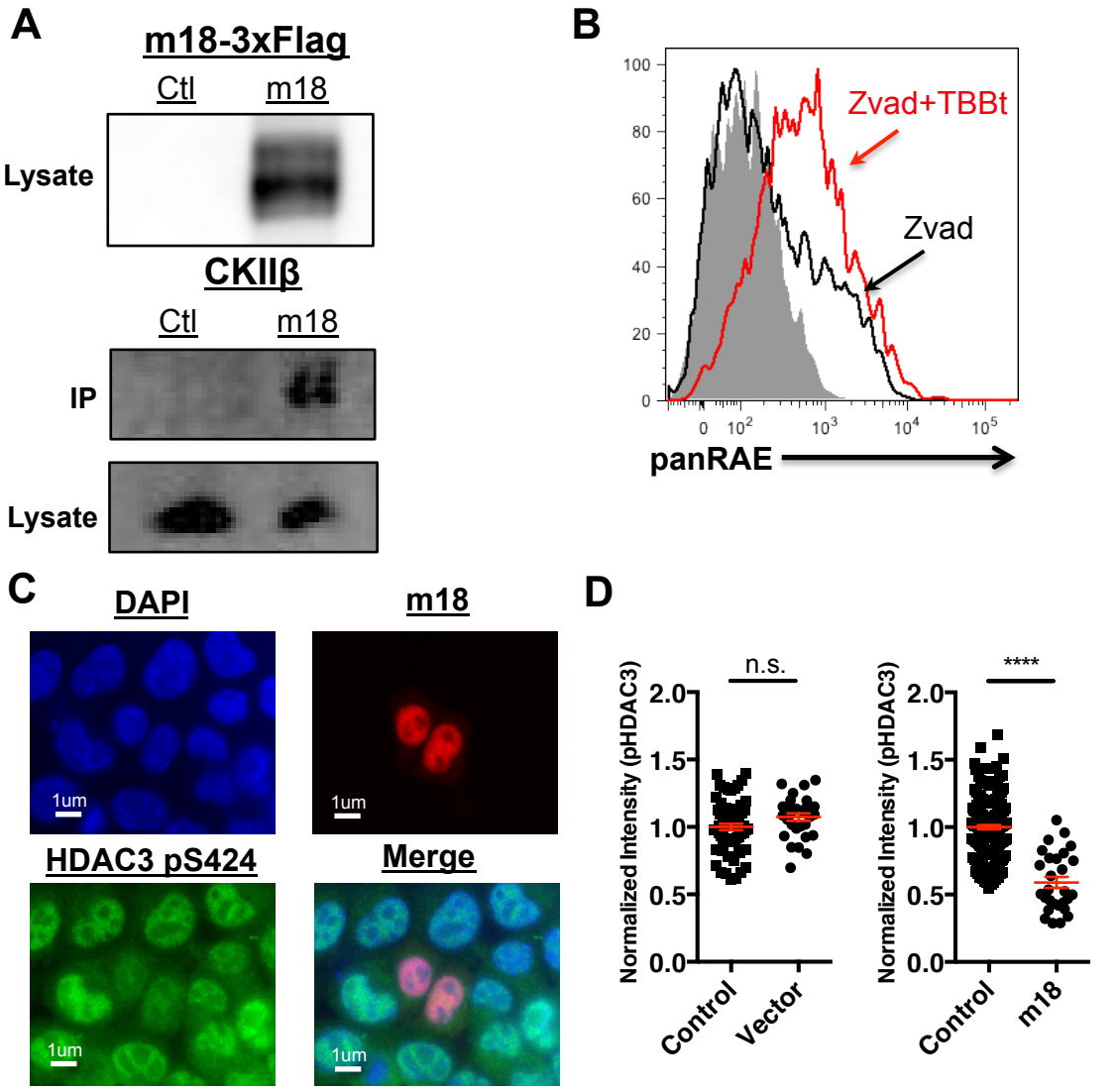


Figure 3-9: CK2 directly interacts with m18 and represses HDAC function

(A) Immunoprecipitation (IP) of m18 was performed in lysates of cells expressing m18-3xFlag or empty 3xFlag vector, and the products were analyzed for FLAG and CK2β by western blot. Data are representative of 3 independent experiments. (B) Mouse fibroblasts were treated with CK2 inhibitor TBBt in conjunction with zVAD or zVAD alone and analyzed for RAE-1 expression by flow cytometry. Data is representative of 3 independent experiments. (C) Representative image of fibroblasts expressing m18-RFP and stained for HDAC3 pS424. (D) Quantification of HDAC3 pS424 levels in cells expressing m18-RFP or RFP control vector from compared to non-transfected controls in same field of view. Red bars are representative of mean±SEM. Data are representative of 3 independent experiments. ****P<0.00005. n.s., not significant. (Student's T-test)

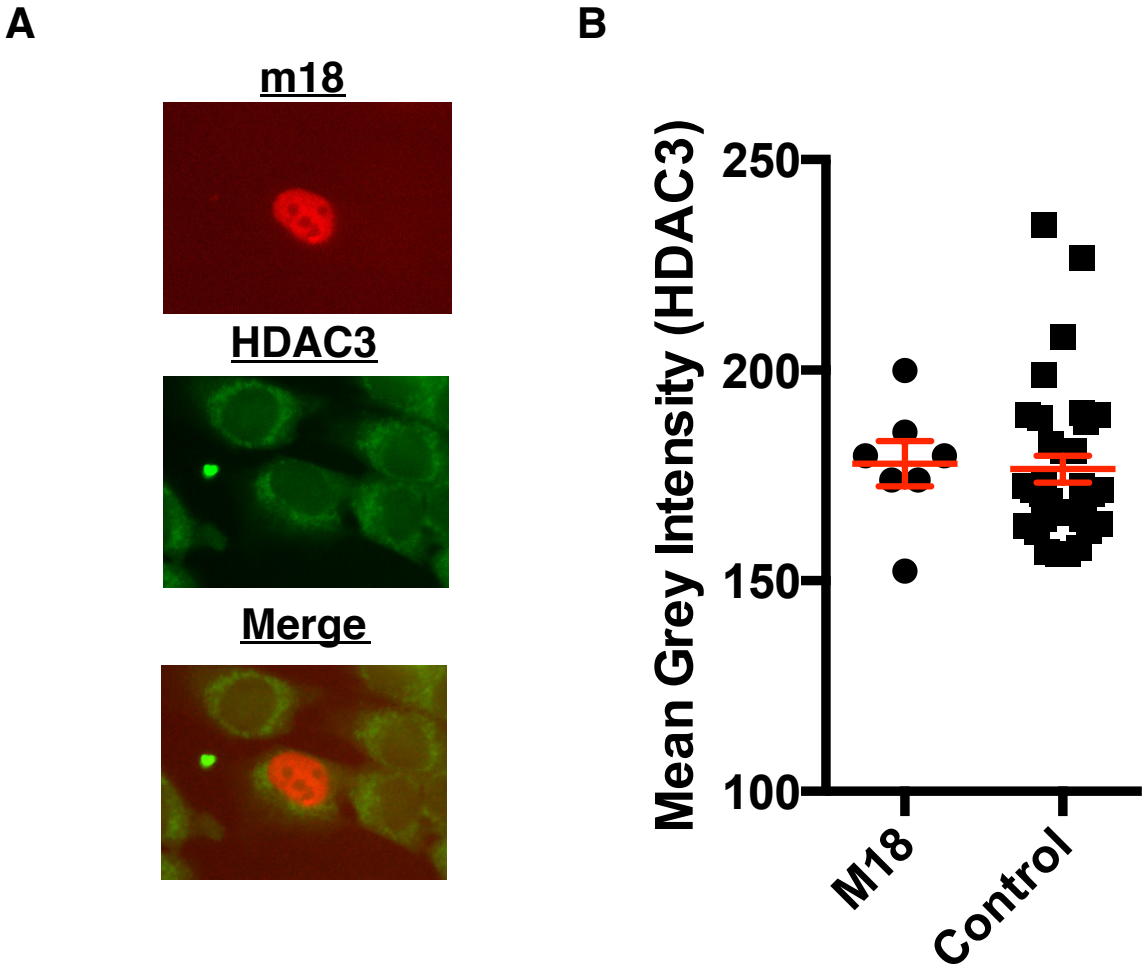


Figure 3-10: m18 expression does not change HDAC3 levels
(A) Representative picture of HDAC3 staining in m18-RFP transfected cells.
(B) Nuclear staining of HDAC3 was quantified in FIJI. Data are representative of mean±SEM. Data are representative of 3 independent experiments. n.s., Not significant (Students T-test).

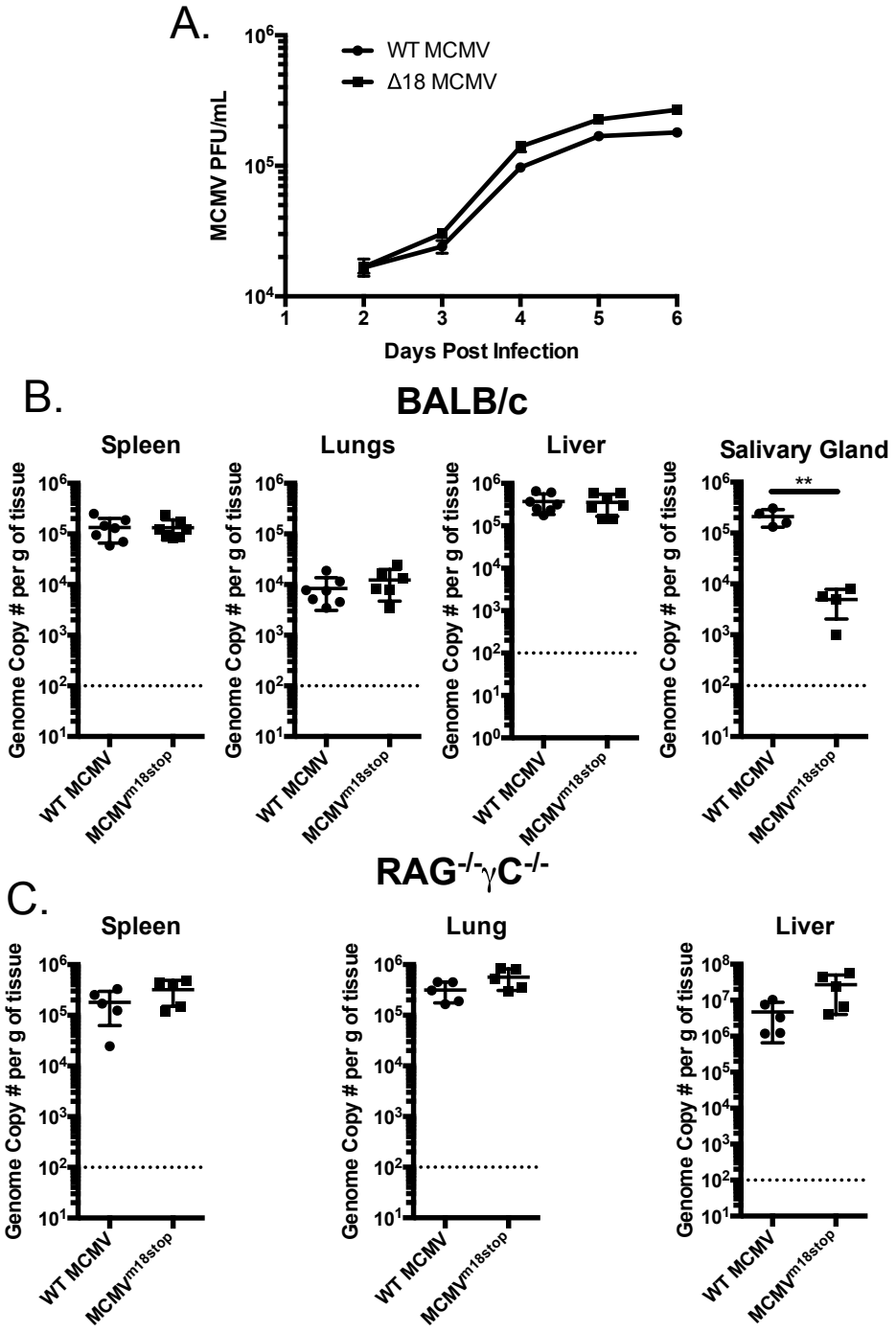


Figure 3-11: MCMV lacking m18 has no growth defect in vitro, but is deficient in the salivary gland in vivo.

(A) MCMV titers in supernatents of NIH-3T3 cells infected with either WT or Δ18 MCMV. (B) qPCR analysis of MCMV genome copy number from spleen, lung, liver, and salivary glands of BALB/c mice infected with 500,000 p.f.u. of WT or MCMVm18stop. (C) qPCR analysis of MCMV genome copy number from spleen, lung, and liver tissues of RAG-/-γC-/- Mice infected with 50,000 p.f.u. of WT or MCMVm18stop.

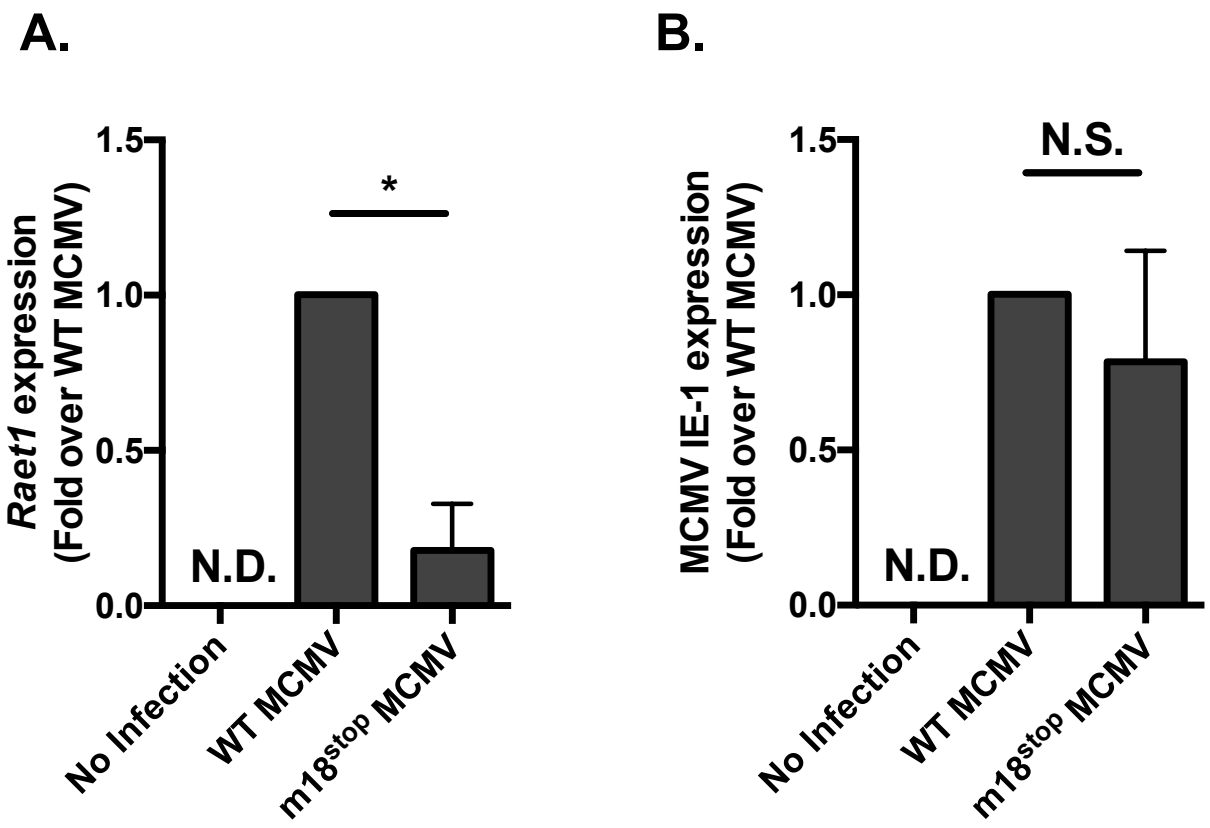


Figure 3-12: MCMVm18stop does not induce RAE-1 expression or cause a defect in IE-1 expression.
(A) RT-qPCR analysis of Raet1 expression in mouse fibroblasts infected with WTMCMV or MCMVm18stop. (B) RT-qPCR analysis of MCMV IE-1 expression in mouse fibroblasts infected with WTMCMV or MCMVm18stop. **P<0.05. n.s., Not significant (Students T-test).

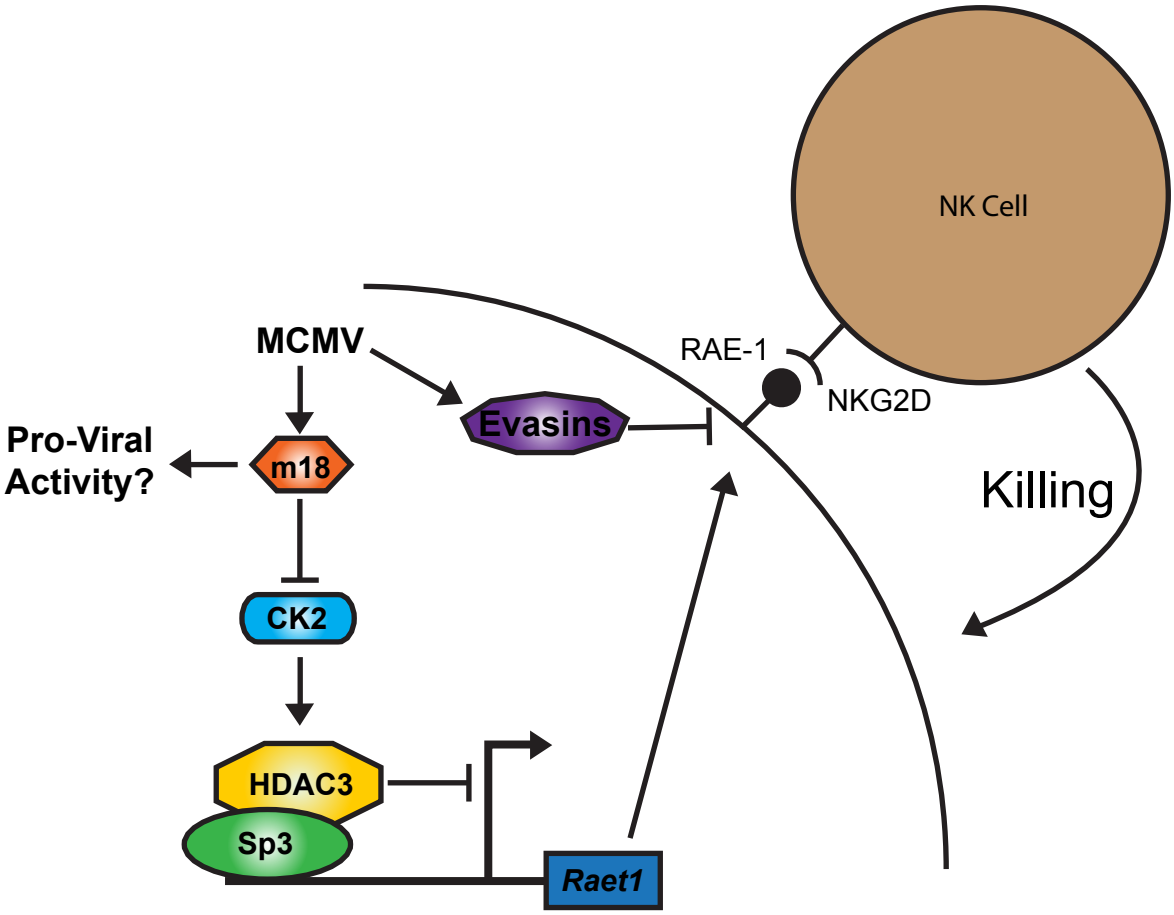


Figure 3-13: A model for RAE-1 induction by m18 during MCMV infection. In the absence of stimulus CK2 phosphoylates and activates HDAC3, which represses the Raet1 promoter. During MCMV infection m18 directly interacts with CK2 preventing activation of HDAC3 and allowing the Raet1 promoter to become de-repressed, and permiting the expression of Raet1. MCMV also encodes a variety of evasins that prevent the cell surface expression of RAE-1 in order to evade recognition and killing by NK cells. Together these systems allow the virus to inhibit HDAC3 activity, while evading the deleterious effects of inducing NKG2D ligands.

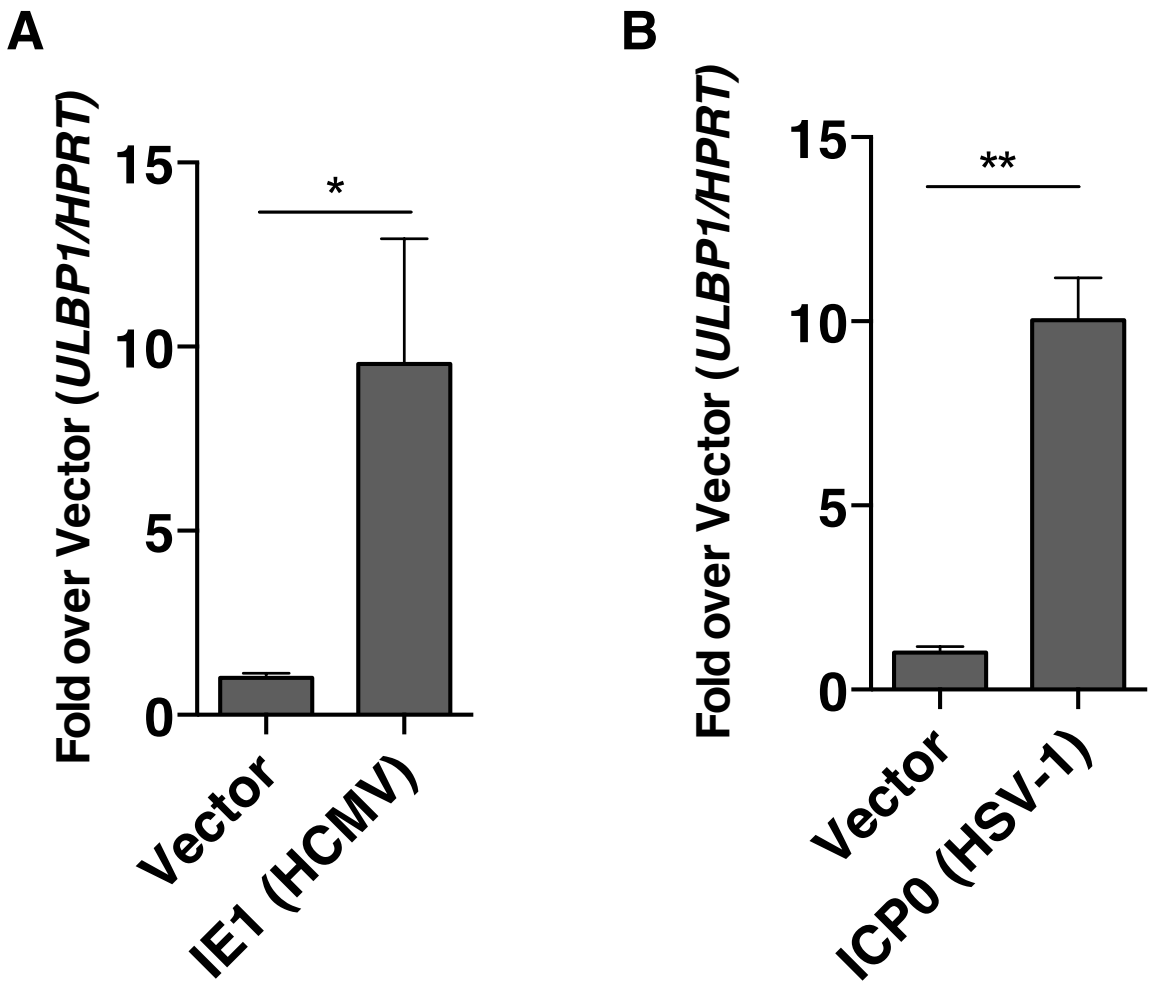


Figure 3-14: Virally encoded HDAC inhibitors from human herpesviruses induce human NKG2D ligand expression.

A, B) RT-qPCR analysis of ULBP1 expression in Human foreskin fibroblasts (HFFs) transfected with empty vector or IE-1 (HCMV), or ICP0 (HSV-1). Data are normalized to vector control and represented as mean±SEM. Data are representative of 3 independent experiments.

Chapter 4

An Alternate Splicing Event in the m18 ORF Produces Functionally Distinct Proteins

SUMMARY

In the previous chapter I analyzed the mechanism by which the m18 ORF induced the expression of the RAE-1 family of NKG2D ligands. However, I only briefly described the polypeptide(s) produced from this ORF. Further analysis of the protein products produced from this ORF demonstrated that there are actually two distinct polypeptides produced from this ORF. These products share an N-terminus, but have unique C-termini. In this chapter I demonstrate that the larger of these two peptides (m18-L) is decoded from the full length ORF, and the smaller protein (m18-S) is translated from an alternately spliced RNA. I demonstrate that the longer protein m18-L is both necessary and sufficient to drive RAE-1 expression; m18-S is dispensable for this activity.

Chapter 4
An Alternate Splicing Event in the m18 ORF Produces Functionally Distinct Proteins

RESULTS

The m18 ORF produces two products that share an N terminus and have distinct C-termini.

Previously we had characterized m18 expression using constructs tagged on the C-terminus. As many viruses encode alternate co-terminal transcripts we sought to determine if the m18 ORF encoded any transcripts that shared an N terminus we generated a construct encoding a 3xFlag epitope tag at the N-terminus, and an HA epitope tag on the protein C-terminus (Figure 4-1A). We then expressed these constructs in HEK293T cells and analyzed protein expression by western blot using antibodies against 3xFlag or HA. As previously shown blotting against the C-terminal HA tag showed a band around 180kD, much larger than the predicted size of 110kD (Figure 4-1B right). This 180kD band was also present in the cells expressing the N-terminally 3xFlag tagged construct indicating that this protein includes both the predicted N and C-termini of the m18 ORF. However, we also observed a smaller band of 68kD (Figure 4-1B left) specifically in the N terminally Flag tagged construct. This suggested that the m18 ORF encodes a second smaller protein that shared an N, but not a C-terminus with the predicted ORF. For ease of communication we designated the protein decoded from the full-length m18 ORF m18-L and the shorter product m18-S.

To determine which portions of the m18 ORF were required for the production of m18-S we generated serial deletions of the m18 ORF from the N or C terminus. 3xFlag epitope tags were included on the N-termini of these constructs to allow for visualization of both m18-L and m18-S. We transfected HEK293T cells with constructs encoding the m18 ORF lacking segments regions of length 300bp to 1500bp starting from either the 5' or the 3' end of the m18 ORF, and analyzed protein expression by western blot. As expected deletions of the 5' end of the m18 ORF decreased the size of both m18-L and m18-S. We were able to visualize a band for m18-S with deletions as large as 900bp (Figure 4-1C). Additionally deletions of up to 1500bp from the 3' end of m18 did not effect the production of m18-S indicating that this region is dispensable for the production of m18-S (Figure 4-1C).

Given the decrease in size of m18-S over the course of the first three deletion mutants the expected size of m18 5'Δ1200 would be ~5kD. Thus, even if this product exists we may not have been able to visualize it in our previous assay. To determine if the region of m18 ORF 5' end between base pairs 900 and 1200 was shared by m18-L and m18-S we generated constructs that included a the protein GFP fused to the N-terminus of m18, and made serial deletions between 300 and 1500 from the 5' end of this ORF. GFP adds ~30kD increasing the size of possibly vanishingly small protein products generated by the m18 ORF into the detectable range. We expressed these constructs in 293T

and analyzed the protein products produced by western blot. We were able to visualize m18-S for all deletions except m18 5'Δ1500 (Figure 4-1D). This indicates that an element important for the production of m18-S is located between m18 5'1200 and m18 5'1500.

The m18 ORF produces two distinct RNA products

One common method by which viruses produce diverse proteins with shared termini is by alternative splicing. Our above results are in line with a splicing model where a short splicing event between bp1200 (m18 5'Δ1200) and bp1624 (m18 3'Δ1500) generates a protein of 68kD. To determine if this RNA exists we isolated RNA from HEK293T cells transfected with a construct expressing the m18 ORF or an empty vector control. We then generated cDNA from this RNA by RT-PCR, and used this cDNA as a template for PCR amplification using primers that spanned between bp1116 and bp1518 of the m18 ORF. To ensure that no plasmid DNA was carried through the RNA isolation a no reverse transcriptase control was included. PCR products were separated on a 1.2% agarose gel using EtBr to visualize DNA. We observed a band at ~400bp from the full length ORF. We also observed a smaller band at about 240bp (Figure 4-2A). Neither of these bands was present in the vector transfected sample indicating that they were produced specifically in cells expressing the m18 ORF. Additionally they were not observed in samples that were not treated with reverse transcriptase indicating that these were produced off of cDNA templates, not contaminating DNA.

To determine the sequence of these species we excised the bands from the agarose gel and isolated the DNA fragments. We then used the Sanger method to sequence the DNA fragments. The forward primer used to amplify the fragments, was used as the primer for the Sanger reaction. The 400bp sequence aligned to the canonical m18 ORF as expected. The 240bp sequence aligned to the same region, but contained a 160bp gap (Figure 4-2B). An analysis of the local sequence revealed that the predicted excised region contained both canonical splice donor and splice acceptor sequences (Figure 4-2B, arrows). This suggests that the m18 ORF can produce two distinct RNA species differentiated by a splicing event.

To determine if RNA splicing between these elements is responsible for the generation of this RNA we generated m18 mutants that eliminated the predicted splice acceptor site from the m18 ORF. We altered nucleotide 1365 from A to G. We then transfected HEK293T with empty vector, vector containing the WT m18 ORF, or vector with the 1365T m18 ORF, isolated RNA, generated cDNA from this RNA by RT-PCR, and used this cDNA as a template for PCR amplification using the same primers decribed above. PCR products were separated on a 1.2% agarose gel using EtBr to visualize DNA. Mutation of nucleotide 1365 from A to G completely eliminated the production of the 250bp amplicon (Figure 4-2A). This suggests that RNA splicing using 1365AG as an acceptor sequence generates this RNA.

RNA spliced between bp1206 and bp1367 of the m18 ORF produces m18-S

We have demonstrated that m18 ORF produces two distinct proteins and that m18 ORF produces two distinct RNAs, but we have not established that

these necessarily correspond to one another. To establish that the RNA of this splicing event produces a protein of the same size as m18-S we generated a construct in which the m18-ORF lacked the same 160bp region absent in the spliced RNA. A 3xFlag epitope was also included on the N-terminus of this spliced m18 ORF (m18-S) construct. We then transfected constructs encoding WT 3xFlag m18 ORF, 3xFlag m18-S, or the 3xFlag m18-1365G ORF (in which the splice donor has been eliminated), and evaluated the expression of these constructs by western blot (Figure 4-3A). The m18-S ORF produces a peptide of identical size to that of m18-S (Figure 4-3A, middle lane). This is in line with the hypothesis that m18-S is produced from the spliced m18 mRNA identified above. Additionally, in m18-1365G the elimination of the splice donor eliminates production of the spliced RNA (Figure 4-2A) also eliminates production of m18-S (Figure 4-3A, right lane) suggesting that this may be important for the production of m18-S.

Notably, in this experiment a number of minor bands appear in cells expressing m18. This suggests there may be additional forms of m18 beyond m18-L and m18-S as described. However, these minor forms are significantly less stable than m18-L and m18-S and are not present in every analysis. In the future it may be interesting to identify these forms, and whether they correspond to other alternative splicing events, translational mistakes, or post-translational modifications of m18-L and m18-S. However, the remainder of this chapter focuses on differentiating m18-L and m18-S.

To further assess whether m18-S is produced as the result of the identified splicing event we investigated the use of a second frame stop codon by m18-S. The splice event identified above would shift the resulting translation product out of the canonical frame 1 and into frame 2, resulting in an alternate C-terminus, and a much shorter protein product. The predicted stop codon for this new RNA at 1447 would result in a protein of predicted size 68kD.

To determine if m18-S uses the stop codon at 1447 (TGA) we generated a mutant m18 ORF in which base pair 1446 was changed from G to T, (TGA to TTA) eliminating the stop codon in frame 2. We then transfected HEK293T with empty 3xFlag vector, 3xFlag vector with WT m18 ORF or, 3xFlag vector with m181446T isolated protein 24 hours later and analyzed protein expression by western blot, probing against flag. Expression and size of m18-L was not changed by the 1446T mutation, however, m18-S saw a 5-10kD increase in size (Figure 4-3C). This is in line with the expected size change of 6kD that would be expected if m18-S was permitted to read through the stop codon at 1447 and out to the next stop at 1491, a 68aa (6.2kD) increase in size.

As a second method to determine if m18-S uses this stop codon we generated a construct including both a 3xFlag tag on the N-terminus as well as a strep-tag in the second frame of the m18 ORF directly 5’ of the stop codon at 1447. This sequence did not introduce any stop codons in frame 1 of the m18-ORF. We then transfected this construct or 3xFlag with WT m18 ORF into HEK293T and analyzed protein expression by western blot, probing against either the flag or strep tag (Figure 4-3B). As expected visualizing the 3xFlag tagged N-terminus showed m18-L and m18-S expressed from both constructs

(Figure 3B Left). In contrast probing against the strep-tag visualized only one band. This band corresponded to the size of m18-S and was only present in cells expressing the construct that included the strep tag in the second frame of m18 (Figure 4-3B Right). Together these results demonstrate that m18-S uses the stop codon predicted by the alternatively spliced RNA identified above.

Mass Spectrometry confirms the presence of peptides terminating at 1447

Given that we predicted m18-S was being produced as a hybrid frame peptide between frames 1 and frame 2 of m18 we revisited our mass spectrometry data set described in chapter three to search for evidence that this form existed. We searched this data set for peptides that would be produced from frame 2 or frame 3 of m18 and we were able to identify one peptide with high confidence from frame two (YCHCCWTL*). Interestingly this peptide includes eight amino acids from the second frame of m18 and leads directly into the stop codon at 1447. This provides further evidence that the described splicing event leads to the production of a protein that terminates at the second frame stop codon at base pair 1447 of the m18 ORF.

The m18-L form is necessary and sufficient to induce RAE-1 expression

Previously we demonstrated that overexpression of the m18 ORF was sufficient to drive expression of the RAE-1 family of NKG2D ligands. This did not distinguish between the effects of m18-L or m18-S. To determine which of these forms are necessary and sufficient for the induction of RAE-1 we transfected mouse fibroblasts with a plasmid encoding GFP alone or along with one of the following constructs: 3xF m18-ORF, 3xF m18-1365G, or 3xF m18-S. We then analyzed expression of RAE-1 by flow cytometry in GFP positive cells 24 hours post transfection.

Cells transfected with constructs encoding WT m18 ORF or m18 1365G expressed equivalent levels of RAE-1 protein (Figure 4-4). In contrast, cells transfected with the m18-S ORF did not show increased levels of RAE-1 indicating that m18-S is dispensable for the induction of RAE-1. This suggests that m18-S is functionally distinct from m18-L and that m18-L is responsible for RAE-1 induction.

DISCUSSION

Our understanding of the coding potential of herpesviruses is continually evolving. Many herpesvirus ORFs have poorly characterized functions, but many more may produce transcripts or peptides that have not been previously annotated. Here I demonstrate that the viral ORF m18 has the potential to produce two distinct mRNAs, which in turn can be translated into distinct protein products. Furthermore I demonstrate that only one of these products is responsible for the induction of NKG2D ligands as described in chapter 3.

It will be important in the future to deconvolute the functions of these products and understand what each one does. I have already demonstrated that RAE-1 induction observed with this ORF is unique to the m18-L product, but the high efficiency of m18-S production suggests that it may play an important part in MCMV biology. One intriguing hypothesis is that m18-S acts as a negative regulator of m18-L, by sequestering some factor using their shared N-terminus. Even beyond a regulatory action for the protein the production of m18-S RNA reduces the available levels of m18-L transcript and may be a way to tune/abrogate NKG2D ligand induction by m18-L under certain conditions, or in specific tissues.

The mechanisms that regulate differential RNA splicing are still being actively investigated, but it is known that cell type is one of the major determinants of how RNA splicing variants are variegated. In order to better understand the roles of these two proteins it will also be important to characterize the contexts under which they are present *in vivo*. The potential for tissue specific regulation of m18-L, m18-S splicing may be particularly important given that m18 deficiency shows a specific defect in only one tissue during infection. This work can be done using tools already developed in this chapter. By analyzing m18-S RNA expression during MCMV infection, *in vitro* and *in vivo* and assessing the presence of the spliced RNA we will be able to better understand the contexts under which m18-L and m18-S are preferentially made. Additionally, mutant viruses that lack one or the other, using the same mutations described above to isolate one or the other, can be used to determine if either m18-L or m18-S specifically are crucial for MCMV growth in the salivary glands.

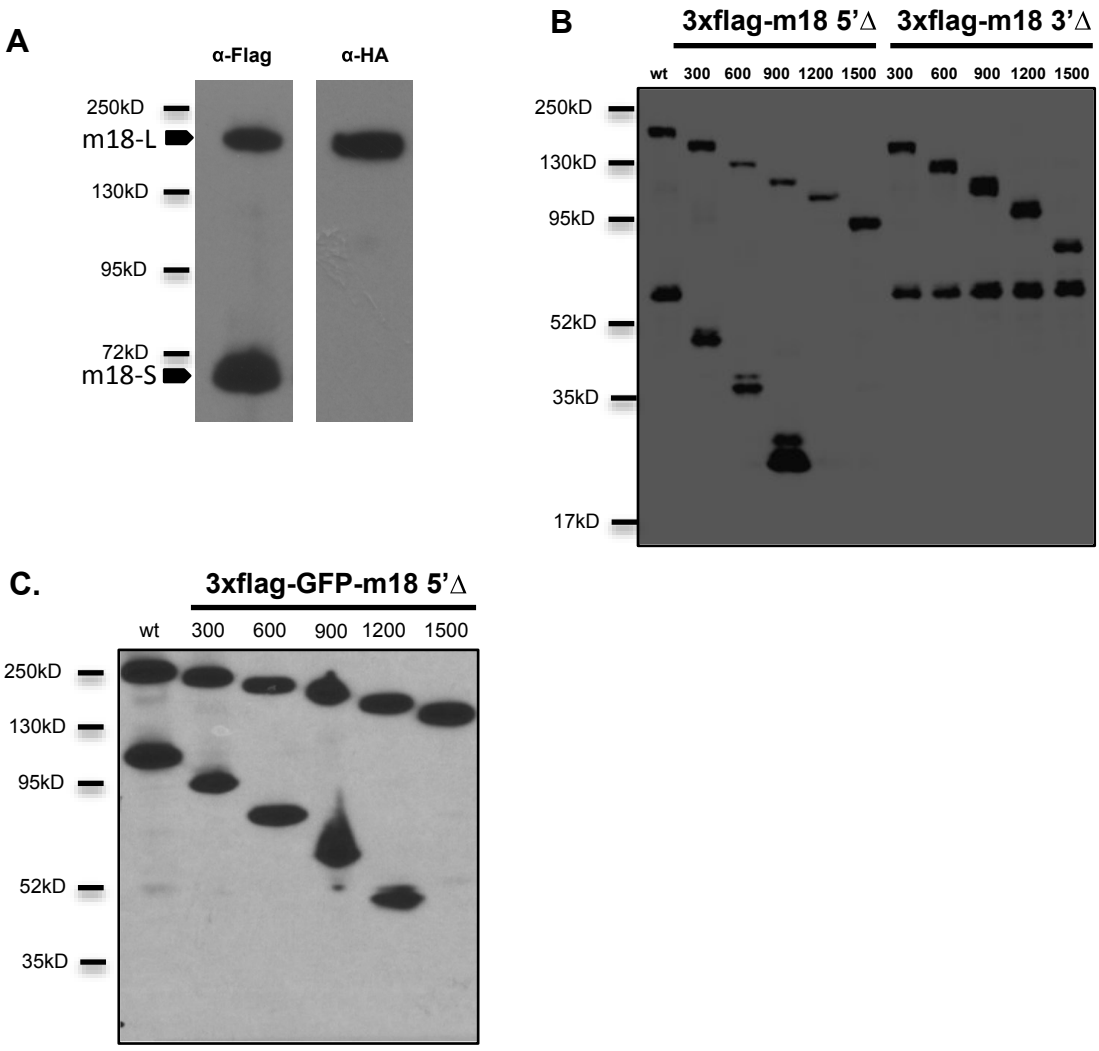
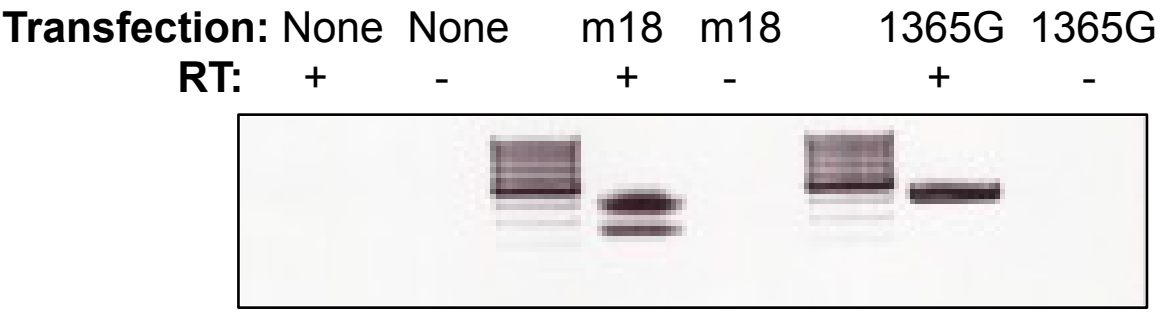


Figure 4-1: m18 produces two protein products that share an N-terminus
(A) HEK293T were transfected with plasmid encoding m18 N-termally tagged with a 3xFlag epitope tag (left) and C-terminally tagged with an HA epitope tag (right) and analyzed by western blot using antibody against the respective tags. (B) HEK293T were transfected with plasmids endocing variants of the m18 ORF N-terminally tagged with a 3xFlag epitope tag and analyzed by western blot using antibody against flag epitope. (C) HEK293T were transfected with plasmid encoding m18 N-terminally tagged with both a 3xFlag epitope tag and a GFP protein and analyzed by western blot using antibody against the flag epitope.

A.



B.

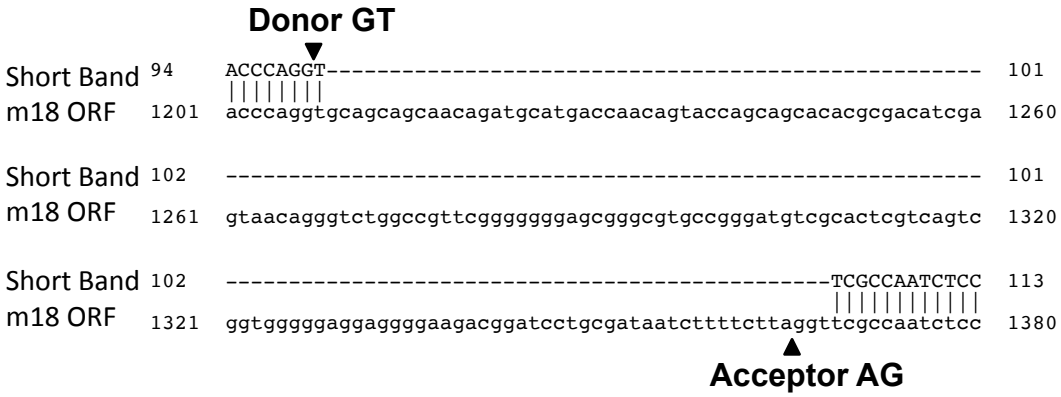


Figure 4-2: m18 ORF produces two RNAs that differ between bp1116 and 1518.

(A) RNA was isolated from HEK 293T transfected with the indicated m18 constructs and converted to cDNA using reverse transcriptase. This cDNA was used as a template to amplify the region between bp1116 and 1518 or the m18 ORF. (B) Allignment of the WT m18 ORF sequence (bottom) with the sanger sequencing from the 250bp band observed in (A). Hypothetical splice donor and acceptor sequences are marked.

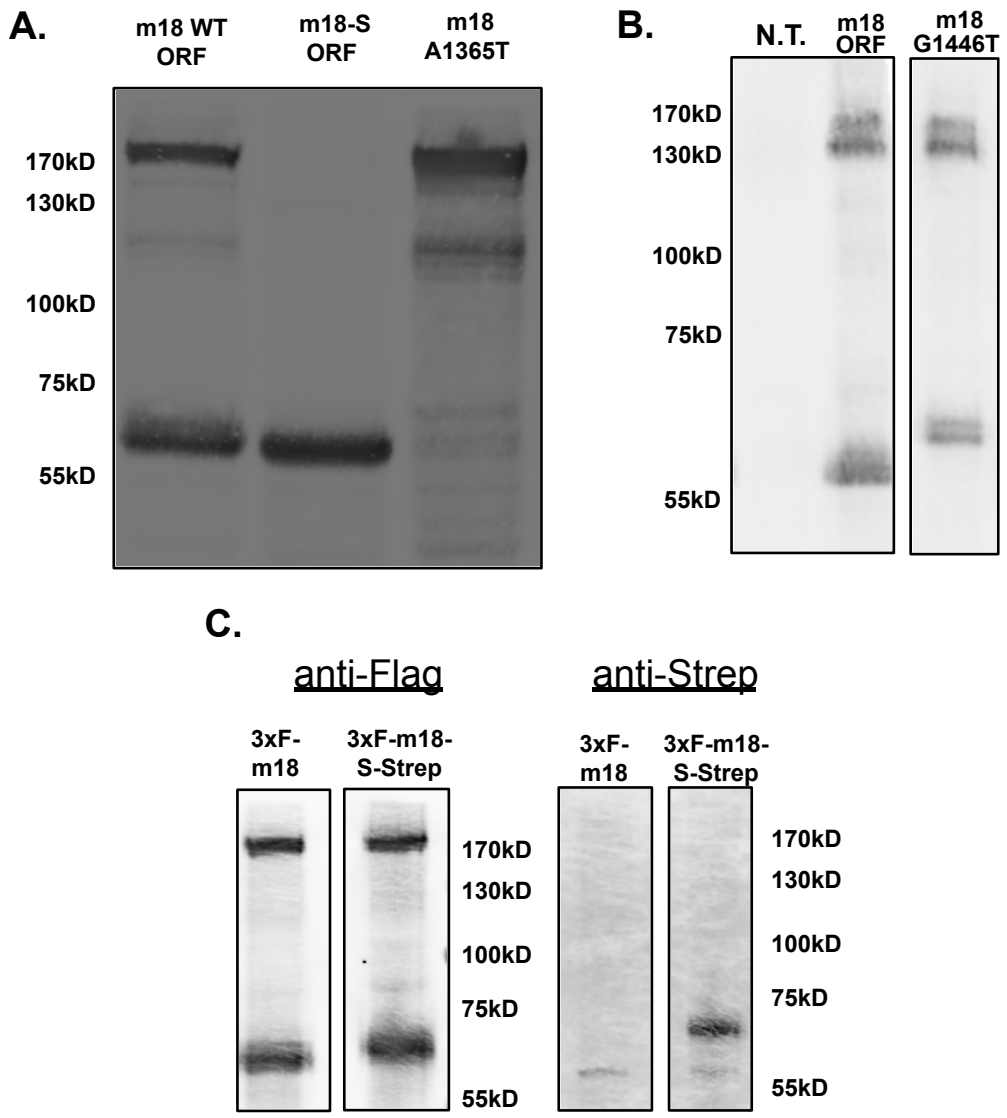


Figure 4-3: Alternatively spliced m18 is translated into m18-S
(A) HEK293T were transfected with N-terminally 3xFlag tagged constructs including the WT m18 ORF, a m18 ORF lacking the spliced out region identified above, or m18 mutated in the splice acceptor site. Lysates were analyzed by western blot for expression of corresponding proteins using antibody against 3xFlag tag. (B) HEK293T were transfected with N-terminally 3xFlag tagged constructs including the WT m18 ORF or m18 ORF with a point mutation at base pair 1446 that eliminates a stop codon in frame two. Lysates were analyzed by western blot for expression of corresponding proteins using antibody against 3xFlag tag. (C) HEK293T were transfected with N-terminally 3xFlag tagged constructs including the WT m18 ORF or a m18 ORF in which a strep tag had been inserted within frame two of the m18 ORF. Lysates were analyzed by western blot for expression of corresponding proteins using antibody against 3xFlag tag or Strep.

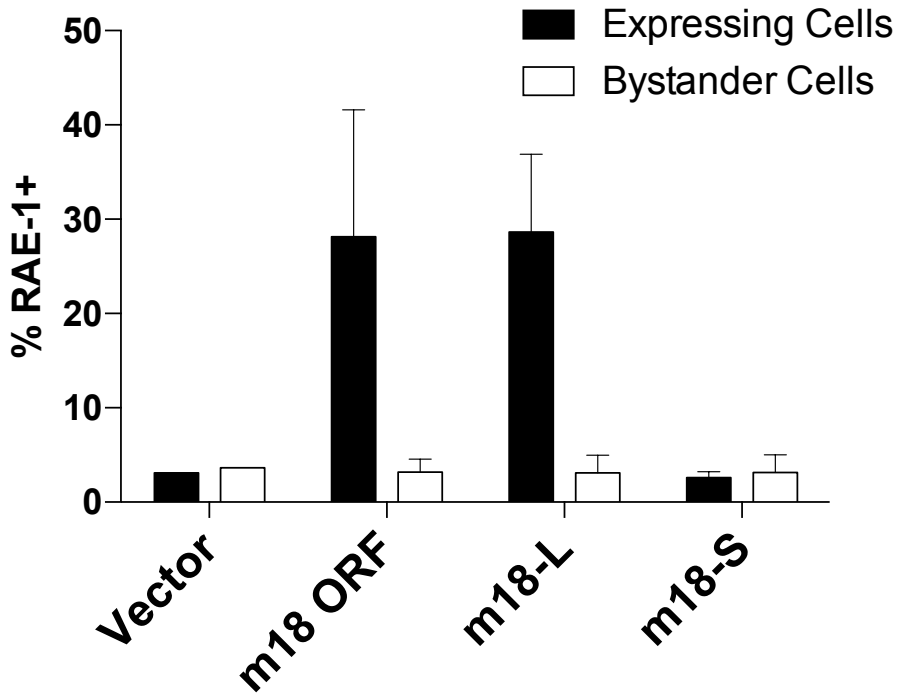


Figure 4-4: m18-L, but not m18-S induces RAE-1 expression.
Mouse fibroblasts were cotransfected with GFP expressing vectors and the indicated m18 expressing constructs. RAE-1 expression levels were measured 48 hours later by flow cytometry and analyzed seperately as cells that were transfected (GFP+) and bystanders (GFP-).

Chapter 5

Mithramycin treatment blocks reactivation and replication of the γ-herpesviruses KSHV and MHV-68

SUMMARY

In chapter 3 I describe a system by which NKG2D ligands are induced by release of HDAC mediated inhibition of the *Raet1e* promoter. Both the repression of this promoter, as well as the subsequent induction of the promoter is mediated by Sp transcription factors. This system of repression/derepression is used in a variety of cellular genes (Wilson et al. 2010), but notably it is also used to control herpesvirus IE gene expression, especially during reactivation from latency. Sp factors have been shown to bind the promoters of many viral IE genes including IE1/2 in HCMV (Lang et al. 1992), and Rta and Zta in EBV (Chang et al. 2005; Tsai et al. 2011), and Rta in KSHV (Shin et al. 2014). Additionally chemical inhibition of histone deacetylases has been shown to drive reactivation of KSHV and EBV (Shin et al. 2014; Luka et al. 1979), and in some cases the activity of these promoters. Some of these have even been shown to do so in an Sp transcription factor dependent manner (Shin et al. 2014). Given this we hypothesized that Sp factors are essential to the process of reactivation/replication and investigated the potential of a chemical Sp factor inhibitor (mithramycin) to block viral replication and reactivation. Interestingly, mithramycin effectively blocked reactivation of KSHV, possibly through the inhibition of the transcription of the reactivation master regulator ORF50 (Rta). Additionally, mithramycin inhibited the replication of MHV68, but did not alter the replication of MCMV. This suggests that some herpesviruses are more sensitive to blockade of Sp factor based transcription than others, and may suggest that γ-herpesviruses are particularly susceptible to this treatment.

RESULTS

Diverse stimuli drive KSHV reactivation in the iSLK rKSHV.219 system

KSHV reactivation can be driven by a variety of chemicals including histone deacetylase (HDAC) inhibitors (Shin et al. 2014), Phorbol Esters (Vieira and O'Hearn 2004), and reactive oxidative species (ROS) (Ye et al. 2011). To assess the effect of mithramycin treatment on reactivation with these stimuli we used a previously established model of KSHV reactivation (iSLK rKSHV.219) in which the KSHV genome contains GFP under a constitutive (EF-1α) promoter, and RFP under the KSHV PAN promoter, which is only active during reactivation (Vieira and O'Hearn 2004). The cell line in which rKSHV.219 is latently carried (iSLK) contains a doxycycline inducible expression cassette driving ORF50 expression. Reactivation can be driven using a combination of doxycycline and the HDAC inhibitor butyrate (Myoung and Ganem 2011). To determine whether other stimuli known to reactivate KSHV in other cell culture systems could be used to reactivate KSHV in the iSLK rKSHV.219 system we treated these cells with doxycycline in combination with various, known reactivating stimuli (Figure 5-1A-E, Figure 5-3, Figure 5-4)

Inhibitors of HDAC1 & 3 drive efficient KSHV reactivation in iSLK.219

It has been reported that histone deacetylase 1, 3, and 6 are most critical to keeping KSHV repressed in the KSHV infected PEL line BCBL-1 (Shin et al. 2014). To assess which HDAC are crucial to keeping KSHV repressed in iSLK rKSHV.219 we treated iSLK rKSHV.219 cells with doxycycline in combination with chemical inhibitors that targeted unique sets of HDACs. These included: panHDAC inhibitor (Butyrate), HDAC1/3 inhibitor MS-275, HDAC1 inhibitor (HDAC1i), HDAC3 inhibitor (RGFP966), and the HDAC6/8 inhibitor droxinostat. As previously reported the HDAC inhibitor butyrate drives efficient reactivation of rKSHV.219 in a dose dependent manner (Figure 5-1A). In line with what is known in BCBL-1 cells HDAC1i (Figure 5-1B) and HDAC1/3 inhibitor MS275 (Figure 5-1C) also drive reactivation exceptionally well. Interestingly, HDAC6/8 inhibitor droxinostat did not drive reactivation in our hands indicating that HDAC6 may be less important in repressing KSHV reactivation in this cell line then it is in the BCBL1 cell line (Figure 5-1D). Additionally, HDAC3 specific inhibitor RGFP966 drove reactivation poorly, and only at high concentrations (Figure 5-1E) suggesting that the role for HDAC3 may also less in this line then in BCBL1. Together, these results suggest that inhibitors of HDAC1 and HDAC3 most efficiently drive reactivation in this system, and that HDAC6 inhibition with droxinostat is not sufficient to reactivate KSHV in iSLK.219 cells.

PMA drives efficient KSHV reactivation in iSLK.219

Phorbol esters are small molecules that activate the cellular kinase PKC (Ryves et al. 1991). Phorbol ester stimulation has been used to drive KSHV reactivation in KSHV infected PEL lines (Vieira and O'Hearn 2004) . To determine if this stimulus also drives reactivation in the epithelial iSLK rKSHV.219 system we treated these cells with doxycycline in combination with the phorbol ester 12-*O*-Tetradecanoylphorbol-13-acetate (A.K.A. PMA, or TPA). PMA treatment effectively drove reactivation as measured by percentage of RFP positive cells. Reactivation was slightly less than that observed with sodium

butyrate in the same experiment (Figure 5-2) indicating that PMA treatment is able to drive reactivation in the iSLK rKSHV.219.

Reactive oxidative stress drives reactivation in the iSLK rKSHV.219 system

Reactive oxidative stress (ROS) has been shown to drive reactivation of KSHV in BCBL-1 cells (Li et al. 2011). To determine if ROS could drive reactivation in the iSLK rKSHV.219 system we treated iSLK rKSHV.219 cells with doxycycline in combination with hydrogen peroxide (H_2O_2) as a source of ROS. Peroxide treated iSLK.rKSHV.219 cells showed significantly increased levels of reactivation (Figure 5-3). These results indicate that ROS are capable of driving reactivation of KSHV in iSLK rKSHV.219 cells.

Mithramycin blocks diverse stimuli from driving KSHV reactivation

All KSHV reactivation stimuli must first drive expression of the master regulator of reactivation, ORF50 (Rta). The promoter of ORF50 is repressed in latent cells, in part by HDACs. HDAC inhibition can drive expression of the ORF50 promoter (Lu et al. 2003), but this activity is dependent on a GC box that binds to the transcription factors Sp1 and/or Sp3 (Ye et al. 2005). The iSLK system provides a small amount of ORF50 from the cell intrinsic doxycycline inducible promoter. Notably, butyrate treatment increases expression from this promoter even in iSLK lines with no KSHV (Figure 5-4). However, iSLK lines with KSHV produce 10 fold more ORF50 transcript in response to butyrate indicating that treatment likely enhances transcription from the endogenous locus as well (Figure 5-4).

We first investigated the ability of mithramycin treatment to block KSHV reactivation in response to our library of HDAC inhibitors. Mithramycin treatment abrogated KSHV reactivation in response to all HDAC inhibitors tested (Figure 5-3A-D). This suggests, as expected, that Sp factors are required for KSHV reactivation in response to HDAC inhibition. This fits with what is already known about the requirement for Sp1 to drive ORF50 expression in response to HDAC inhibitor treatment.

We then sought to determine if mithramycin blocked KSHV reactivation to other types of stimuli. Interestingly treatment with mithramycin also blocked the ability of PMA and ROS to enhance reactivation (Figure 5-6). Notably, PMA stimulation still drove low levels of reactivation at concentrations of mithramycin that abrogated reactivation driven by ROS and butyrate. This suggests that PMA induces KSHV reactivation in both Sp factor dependent and independent ways. This is the first observation that might implicate a roll for Sp1 in reactivation driven by these stimuli.

As this system relies on expression of RFP from the PAN promoter as a proxy for reactivation it is possible that mithramycin blocks expression from the PAN promoter, but does not effect the expression of other reactivation genes. To determine if reactivation was truly decreased in this system we analyzed the effect of mithramycin treatment on expression of KSHV ORF50 and ORF57 genes by RT-qPCR (Figure 5-7). ORF50 is considered a master regulator of KSHV reactivation. It is both necessary and sufficient to drive reactivation (Lukac et al. 1999). ORF57 is a KSHV early gene, necessary for the expression of late genes and directly controlled by ORF50 (Majerciak and Zheng 2015). The levels

of ORF50 and ORF57 were severely hampered by treatment with mithramycin. This trend held for all stimuli used, although the difference was not found to be significant for treatment with peroxide, which drove reactivation the least efficiently. These results suggest that mithramycin is blocking KSHV reactivation in the iSLK rKSHV.219 system at a very early stage in reactivation, possibly at the level of ORF50 expression.

Mithramycin blocks KSHV reactivation in BCBL1-PEL

Because iSLK.219 cells have an exogenous source of ORF50, it is possible that mithramycin treatment is exerting its effects in this system on the dox inducible element of iSLK.219 cells. To investigate whether mithramycin can block KSHV reactivation in a more physiological context we investigated the effect of mithramycin treatment on KSHV reactivation in BCBL1 primary effusion lymphoma cell line.

We reactivated iSLK.219 cells using our library of histone deacetylase inhibtitors, or with a combination of PMA along with the ionophore ionomyocin, in the presence or absence of mithramycin. We then isolated RNA from these cells and measured levels of KSHV late gene K8.1 by qPCR to assess reactivation (Figure 5-8A,B). Reactivation was much less efficient with butyrate then with PMA/ionomycin, but Mithramycin treatment effectively blocked K8.1 expression with both. We then repeated the experiment and assessed the levels of ORF50 transcript to determine if any block in reactivation was due to a loss of ORF50 expression. Reactivation in these experiments was more comparable between the two sets of stimuli, and we saw a reduction in ORF50 expression in all mithramycin treated conditions (Figure 5-8C,D).

One interesting observation from these experiments is that mithramycin treatment, for the most part did not reduce the levels of ORF50 or K8.1 observed in cells that were not treated with reactivation stimulating drugs. BCBL1 cells undergo a small but real amount of reactivation in culture (~1% of cells), and as a result ORF50, and K8.1 RNA can be detected with in the population even in the absence of stimulus. The mechanism of this is not well understood, but our data suggest that this reactivation is not effected by treatment with mithramycin.

Mithramycin blocks activity of the ORF50 promoter

Our observations in iSLK.219 and BCBL1 cells suggested that KSHV reactivation was being blocked by mithramycin mostly as a result of a reduction in ORF50 expression levels. Given that it is already known that Sp1 is bound to the ORF50 promoter and drives expression it is tempting to speculate that mithramycin blocks the activity of the promoter for this master regulator of reactivation.

To determine if this is the case we used a promoter-luciferase system as described in chapter 3, using the region from -295 to +100 of the transcriptional start site of ORF50 in front of the firefly luciferase gene instead of the *Raet1e* promoter. We expressed this construct in iSLK cells lacking kshv and treated these cells with doxycycline, butyrate, or PMA in combination with mithramycin and assessed the expression level of luciferase under these conditions (Figure 5-9). As expected butyrate treatment effectively stimulated expression from this construct, and mithramycin treatment abrogated this effect (Figure 5-9A).

Doxycycline treatment drove a small, but significant, increase expression of luciferase from this construct (Figure 5-9B). ORF50 has been shown to drive its own expression, and elements known to contribute to this are present within this construct, so this observation is not unprecedented. It is interesting that mithramycin blocks this activity as no role has been established for Sp1 in the ORF50 self-regulation loop. PMA did not induce expression above media alone. It is unknown which transcription factors PMA treatment stimulates within the ORF50 promoter, but this observation suggests that the transcription factor binding sites required for full response to PMA were not present in this construct (Figure 5-9C). Together these results suggest that mithramycin can block expression from the ORF50 promoter.

Mithramycin blocks MHV-68, but not MCMV replication *in vitro*

Having established that mithramycin can block KSHV reactivation in a variety of contexts we wanted to assess whether this drug was effective at blocking replication of other herpesviruses. To do this we grew murine herpesvirus 68 (MHV-68), and mouse cytomegalovirus (MCMV) in NIH-3T3 cells for five days in the presence or absence of mithramycin, and assessed the production of these viruses over that time period. 3T3 were pretreated with mithramycin for 2 hours prior to infection. Cells were incubated with virus for two hours, and excess virus removed. To assess whether mithramycin prevented MHV-68 or MCMV binding/entry into 3T3s we analyzed one set of samples for viral genome copy number immediately after washing off excess virus. Interestingly, mithramycin treated 3T3 cells shows a slight, but significant increase in both MCMV and MHV68 genome copy number associated with these cells (Figure 5-10A,B).

Supernatants from infected cells were isolated five days post infection and used to assess virus load by tissue culture infectious dose 50 assay, and viral genome copy number in these supernatants was assessed by isolating viral genomes and quantifying them using a tissue culture infectious dose 50% (TCID50) assay. MHV68 showed a striking mithramycin dependent defect in viral growth (Figure 5-10C). However, MCMV growth was essentially unaffected by the presence of mithramycin (Figure 5-10D). These results indicate that mithramycin can block the replication of herpesviruses other than KSHV, but this is not a general phenomenon and is virus specific.

DISCUSSION

Our results indicate that mithramycin is highly effective at blocking the reactivation of KSHV and the replication of MHV68 *in vitro*. Interestingly, this is not simply a general anti-viral activity, because MCMV growth was completely unaffected by the presence of mithramycin. We were unable to definitively establish a mechanism by which mithramycin acts on KSHV reactivation. However, we did note that mithramycin inhibition of reactivation is associated with a severe decrease in levels of the master regulator of reactivation, ORF50. Additionally, mithramycin is able to block transcription from the ORF50 promoter. These results suggest that mithramycin blocks KSHV reactivation *via* inhibition of ORF50 transcription. It is important to note that while mithramycin has been shown to bind and inhibit transcription from promoters similar to the ORF50 promoter, our data in this respect fall short of establishing a direct effect of mithramycin on the ORF50 promoter.

We have not done any work that would address the mechanism by which mithramycin inhibits MHV68 replication. MHV68 also encodes an ORF50 homologue that is critical for replication, and its promoter is similar to that of KSHV. If mithramycin is blocking reactivation/replication through interaction with a conserved element within this promoter that may explain why both KSHV and MHV68, but not MCMV are sensitive to treatment with this drug. In the future it will be important to assess MHV68 gene expression patterns, and MHV68 ORF50 promoter activity in the presence of mithramycin to evaluate these hypotheses.

It is interesting that although we originally hypothesized that mithramycin might be effective at hampering herpesvirus replication based on the action of the m18 protein of MCMV (described in chapter 3), MCMV replication was not effected by mithramycin treatment *in vitro*. However, it is important to note that even genetic m18 deficiency in MCMV does not alter MCMV growth *in vitro*. Indeed, MCMV is competent to replicate in most respects in the absence of this activity. Deficiency in m18 only causes defects in MCMV replication within the salivary gland. Thus the ability of MCMV to grow in the presence of mithramycin treatment *in vitro* does fall in line with our previous observations.

We have demonstrated that mithramycin is able to effectively block the replication of MHV68 and reactivation of KSHV. Mithramycin was at one time a FDA approved treatment for solid tumors, however it is no longer approved for use in the United States. As herpesviruses are not a large enough burden on human health to warrant life-threatening intervention for their eradication in most cases, and gancyclovir and other nucleotide analog anti-herpesvirals are still very effective and safe there is likely little direct application for the use of mithramycin in herpesvirus treatment. However, nucleotide analog resistant strains of herpesvirus are emerging (Drew et al. 1991), and new derivatives of mithramycin are being tested for increased efficacy and safety (Pérez et al. 2008). Thus this work could potentially be used as a basis for developing a safe and effective antiherpesviral drug with a unique mode of action.

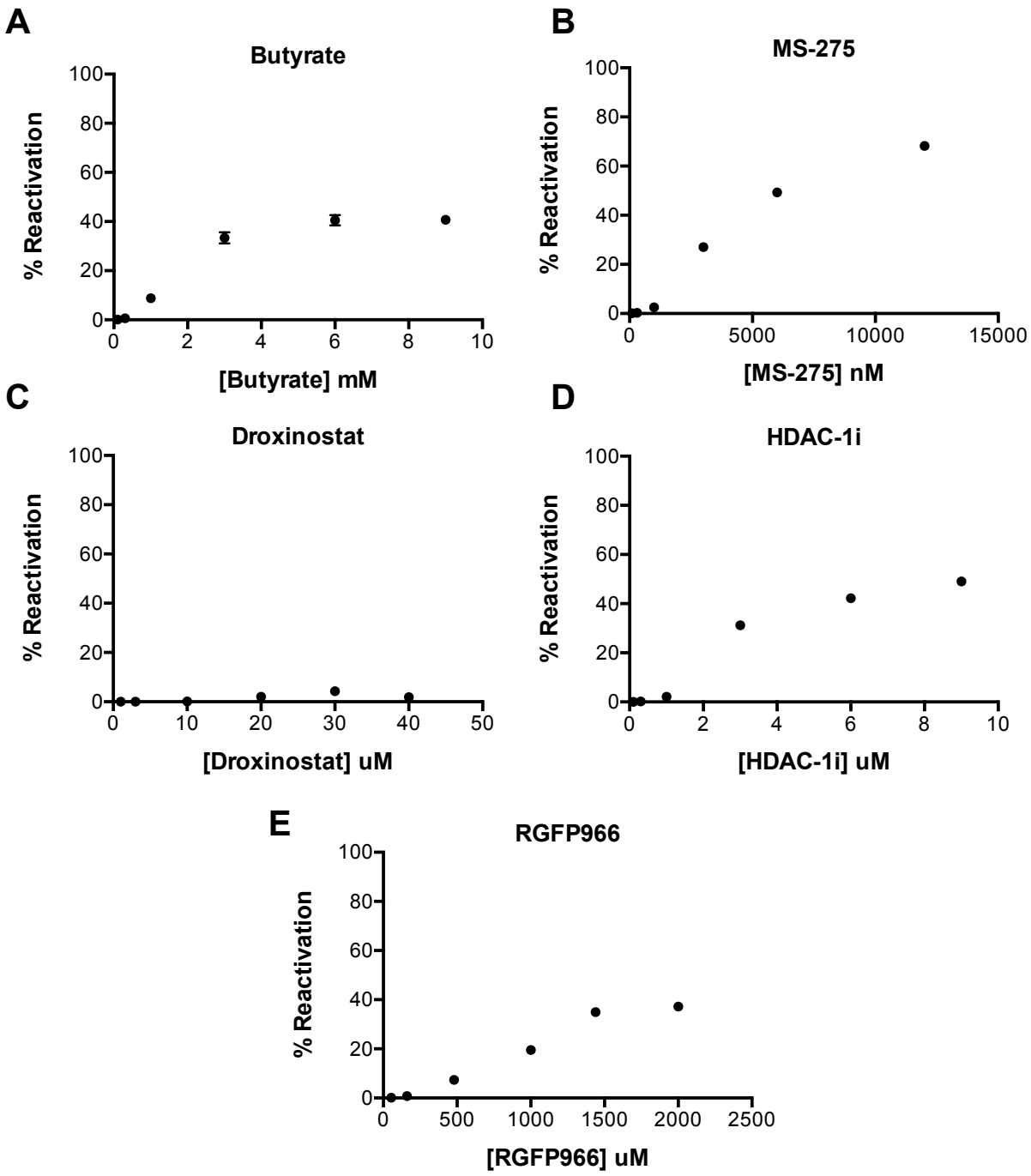


Figure 5-1: Specific HDAC inhibitors drive reactivation of KSHV with different efficiencies in iSLK.219

(A-E) iSLK.219 cells were treated with Doxycycline in combination with the indicated inhibitors at the indicated concentrations. Reactivation was measured as percentage of live cells expressing RFP by flow cytometry. Inhibitors used were specific for (A) all HDACs (Butyrate), (B) HDAC1/3 (MS-275), (C) HDAC6/8 (Droxinostat), (D) HDAC1 (HDAC1i), and (E) HDAC3 (RGFP966).

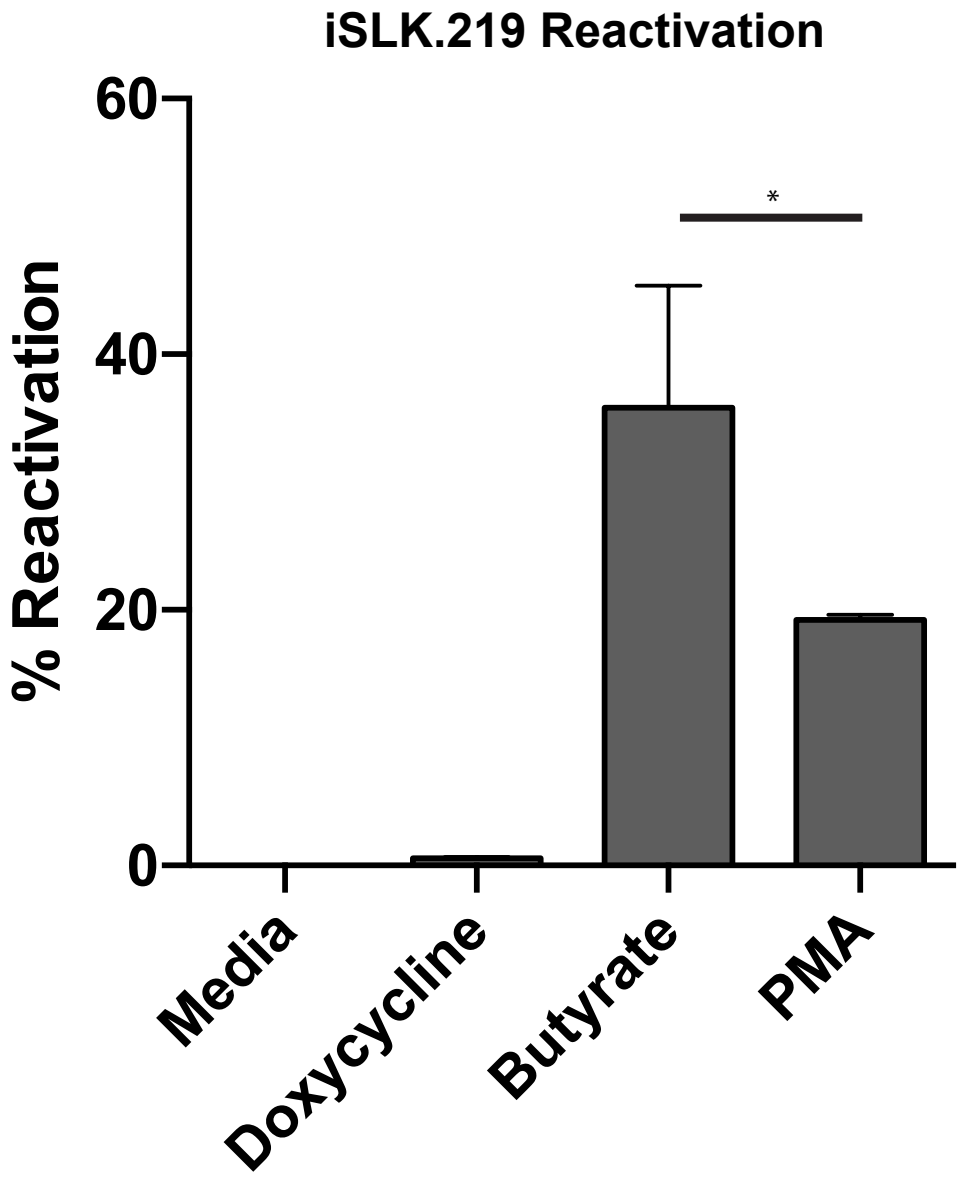


Figure 5-2: Treatment with PMA drives reactivation in iSLK.219
iSLK.219 cells were treated with doxycycline alone or in combination with the HDAC inhibitor butyrate or the phorbol ester PMA. Reactivation was measured as percentage of live cells expressing RFP at 24 hours post treatment. *, $p<0.05$

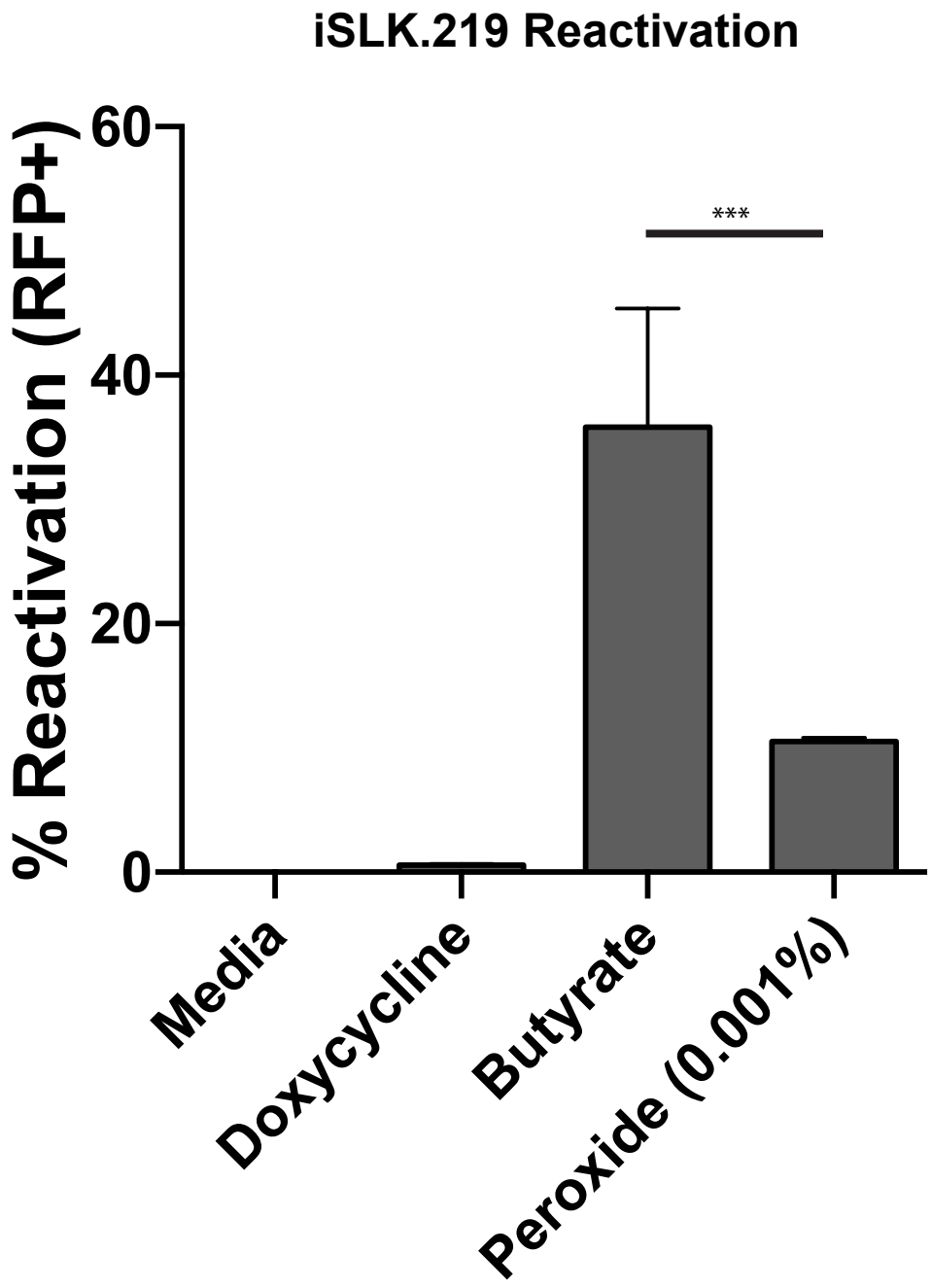


Figure 5-3: ROS drives reactivation in iSLK.219
iSLK.219 cells were treated with doxycycline alone or in combination with the HDAC inhibitor butyrate or the hydrogen peroxide (H2O2). Reactivation was measured as percentage of live cells expressing RFP at 24 hours post treatment. ***, $p<0.0005$.

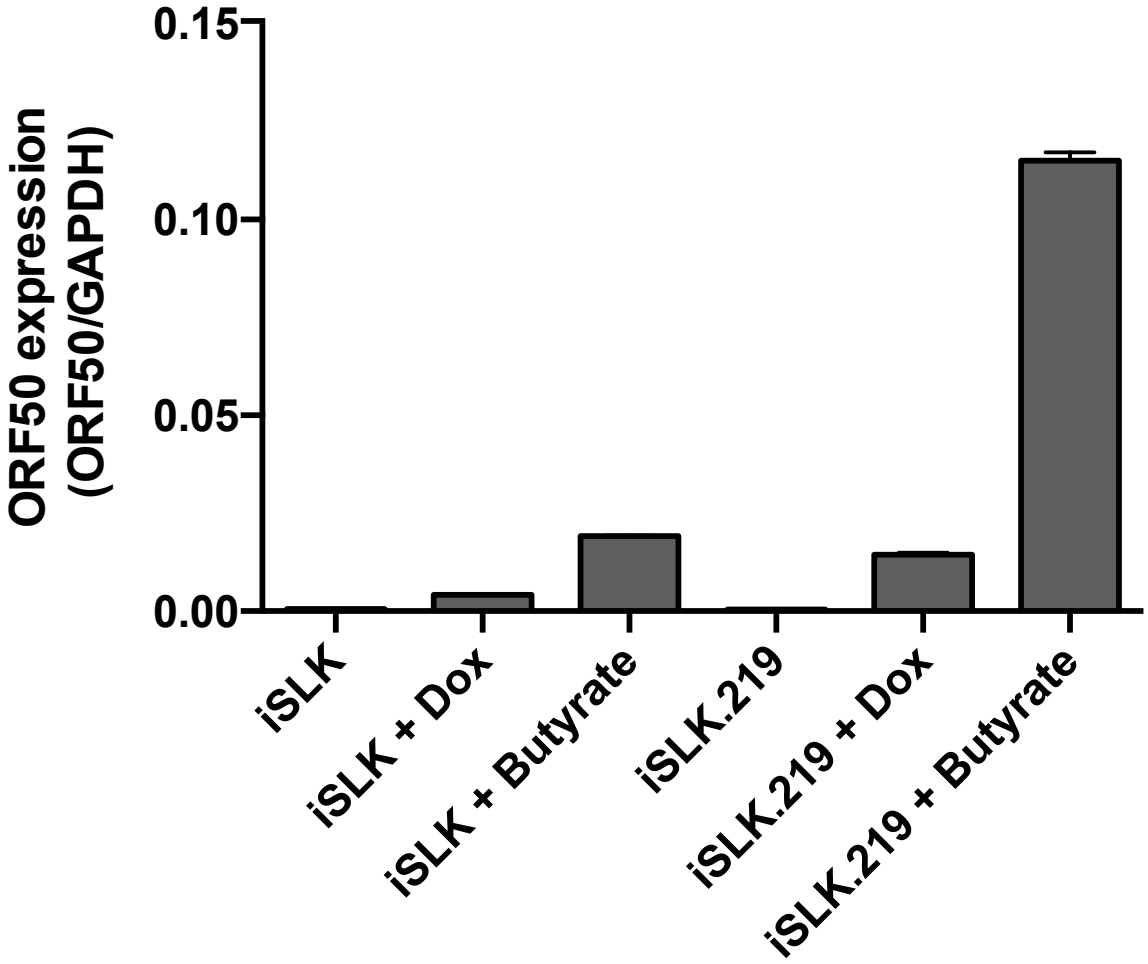


Figure 5-4: Butyrate drives increased expression of ORF50 in iSLK without KSHV

iSLK or iSLK.219 were treated with doxycycline alone or in combination with the HDAC inhibitor butyrate. Reactivation was measured as percentage of live cells expressing RFP at 24 hours post treatment.

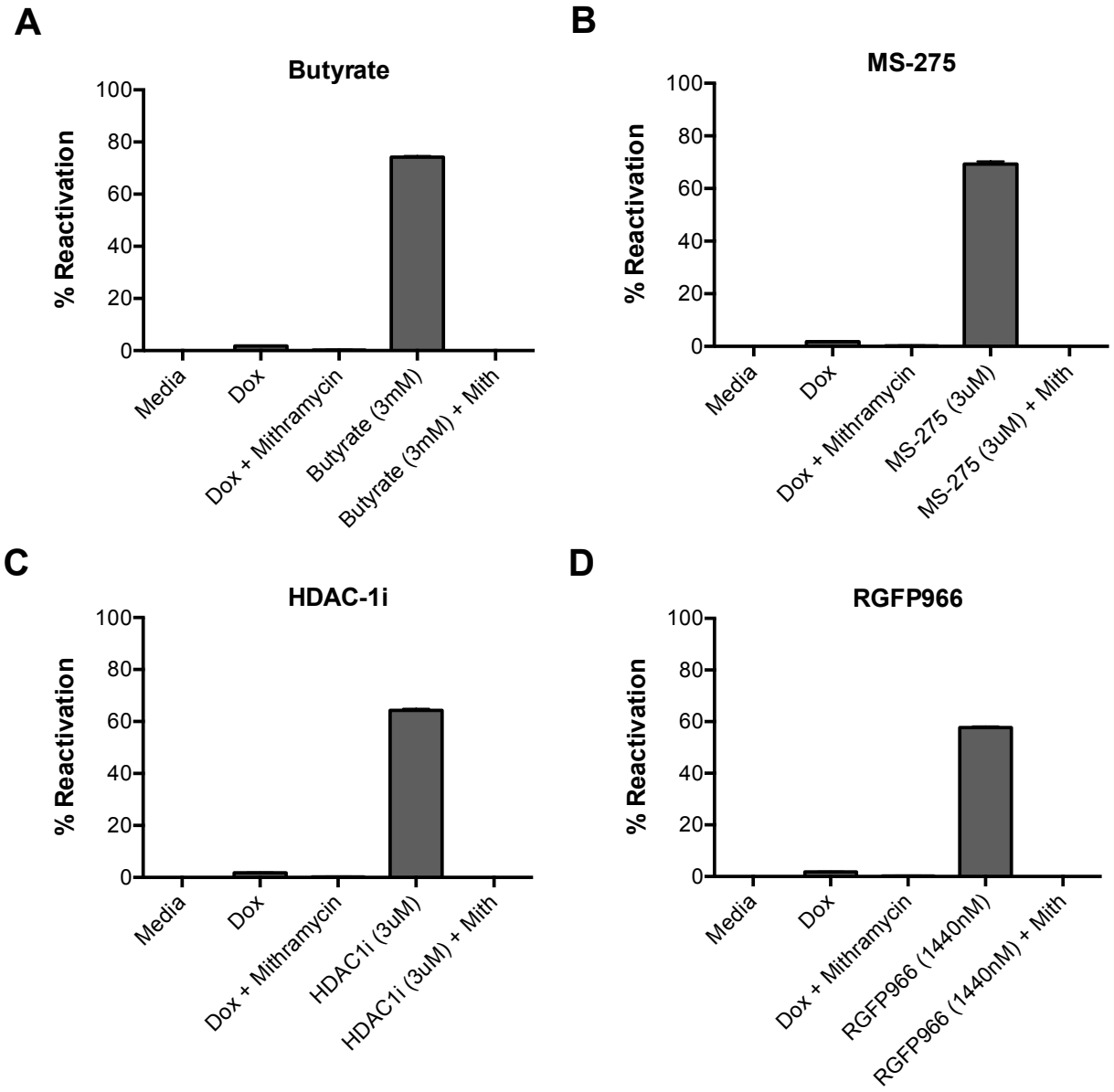


Figure 5-5: Mithramycin blocks HDAC mediated KSHV reactivation in iSLK.219

iSLK.219 cells were treated with doxycycline in combination with HDAC inhibitors against: (A) panHDAC (Butyrate), (B) HDAC1/3 (MS-275), (C) HDAC1 (HDAC1i), or (D) HDAC3 (RGFP966). Reactivation was measured in cells treated with mithramycin or vector control and reactivation was measured as percentage of live cells expressing RFP at 24 hours post treatment.

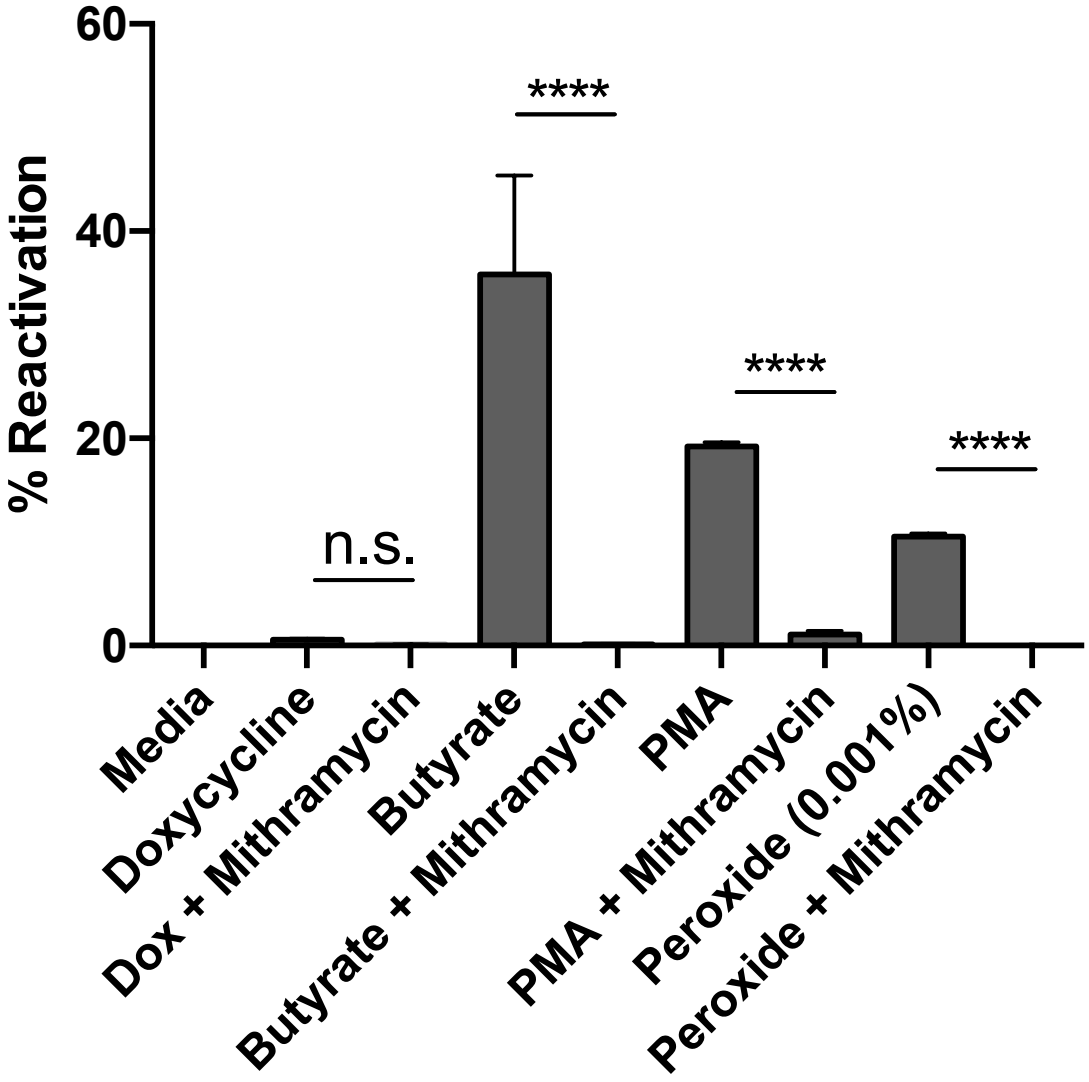


Figure 5-6: Mithramycin Treatment prevents diverse stimuli from driving KSHV reactivation in iSLK.219

iSLK.219 cells were treated with doxycycline alone or in combination with the HDAC inhibitor butyrate, the phorbol ester PMA, or the reactive oxidative stress peroxide (H2O2). Reactivation was then assessed in the presence or absence of mithramycin, and reactivation was measured as percentage of live cells expressing RFP at 24 hours post treatment. n.s., not significant, ****, $p<0.0001$. One way ANOVA with Tukey post test.

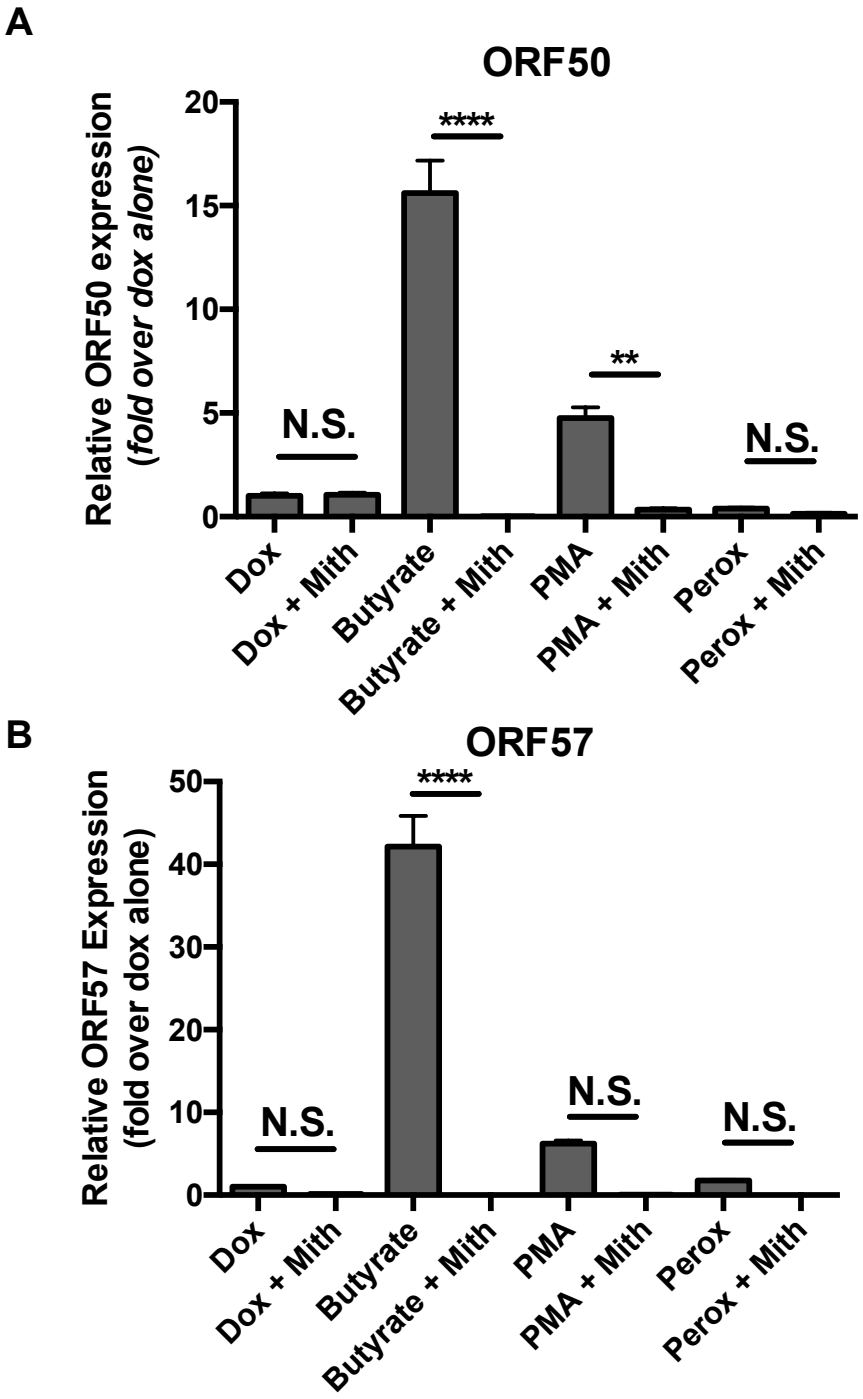


Figure 5-7: Mithramycin Treatment prevents diverse stimuli from driving KSHV gene expression in iSLK.219

iSLK.219 cells were treated with doxycycline alone or in combination with the HDAC inhibitor butyrate, the phorbol ester PMA, or the reactive oxidative stress peroxide (H2O2). Reactivation associated gene expression was then assessed in the presence or absence of mithramycin by RT-qPCRand values were normalized to 18s RNA levels in the same sample, then normalized to doxycycline treatment. N.S., not significant, ****, $p<0.0001$. One way ANOVA with Tukey post test.

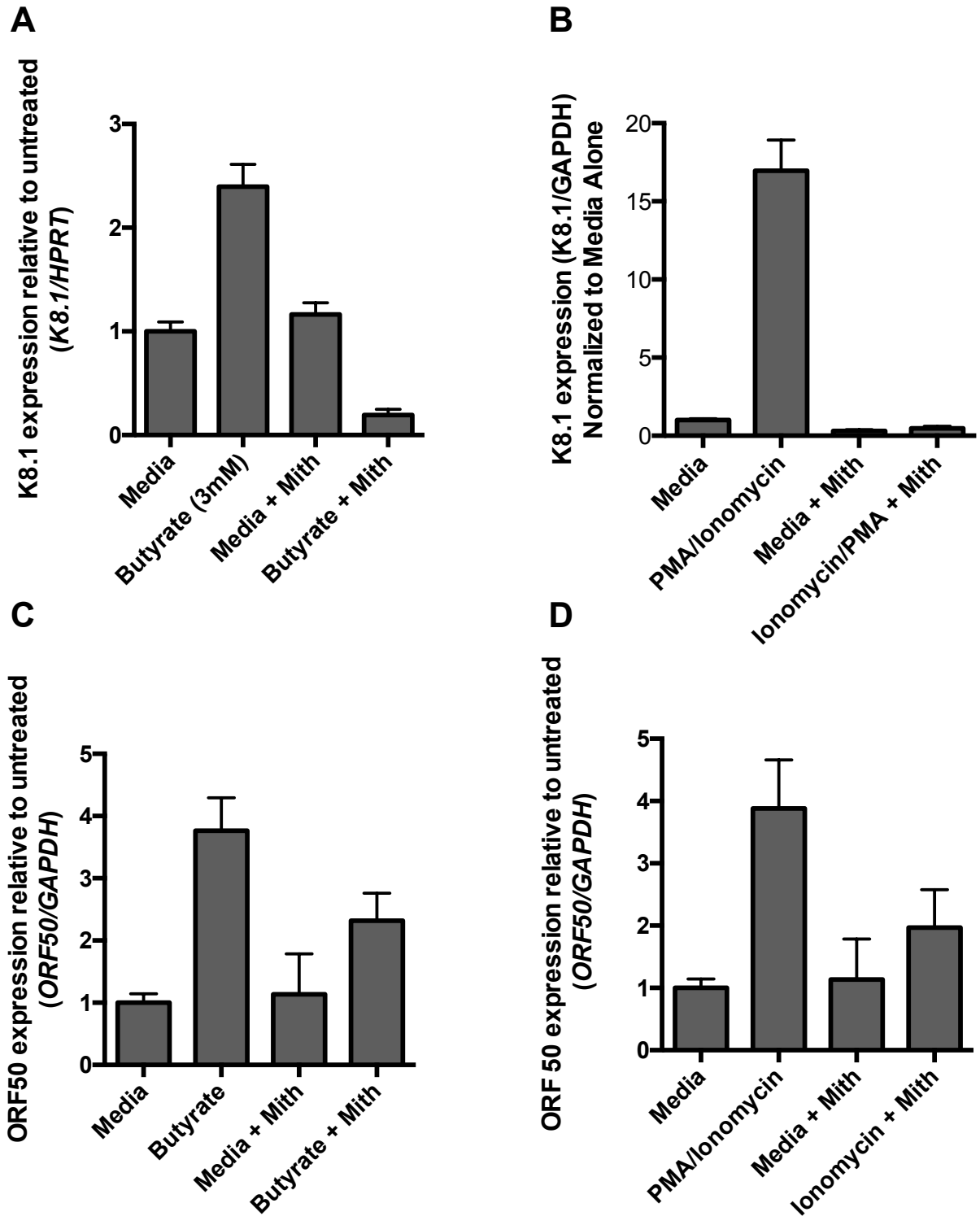


Figure 5-8: Mithramycin blocks reactivation and ORF50 expression in BCBL1 cells. BCBL1 cells were stimulated to reactivate by treatment with either PMA in combination with Ionomyocin (B,D) or Butyrate (A,C). 48 hours after treatment RNA was harvested and the levels of K8.1 (A,B) or ORF50 (C,D) RNA were assessed by RT-qPCR.

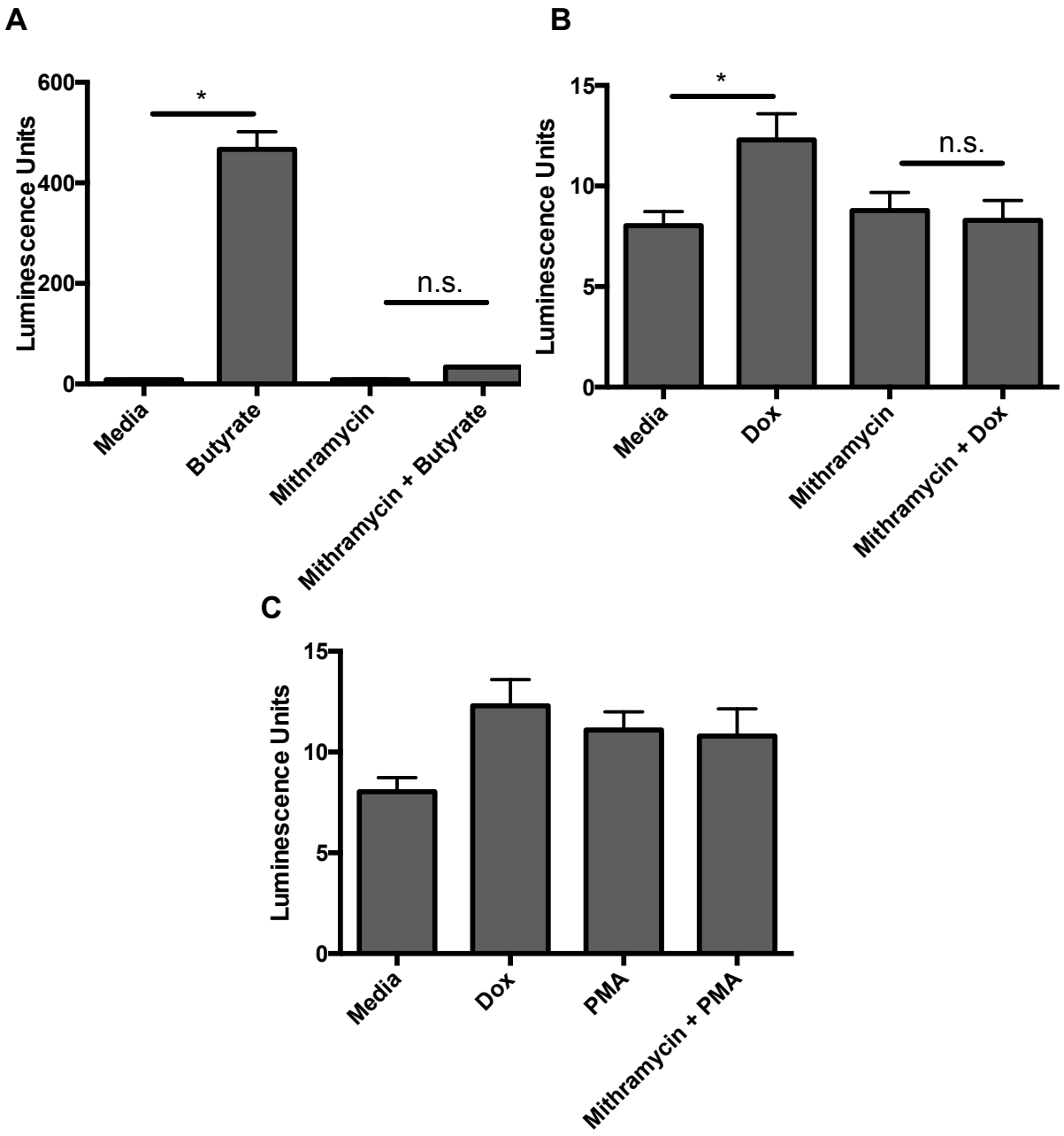


Figure 5-9: Mithramycin treatment prevents Butyrate from stimulating the ORF50 promoter.

iSLK cells were transfected with a construct encoding firefly luciferase under the control of the ORF50 promoter. 24 hours post transfection these cells were stimulated with doxycycline, butyrate, or PMA. Luciferase levels were assessed 24 hours after treatment by luminescence assay. n.s., Not significant, *, $p<0.05$ One way ANOVA with Tukey post test.

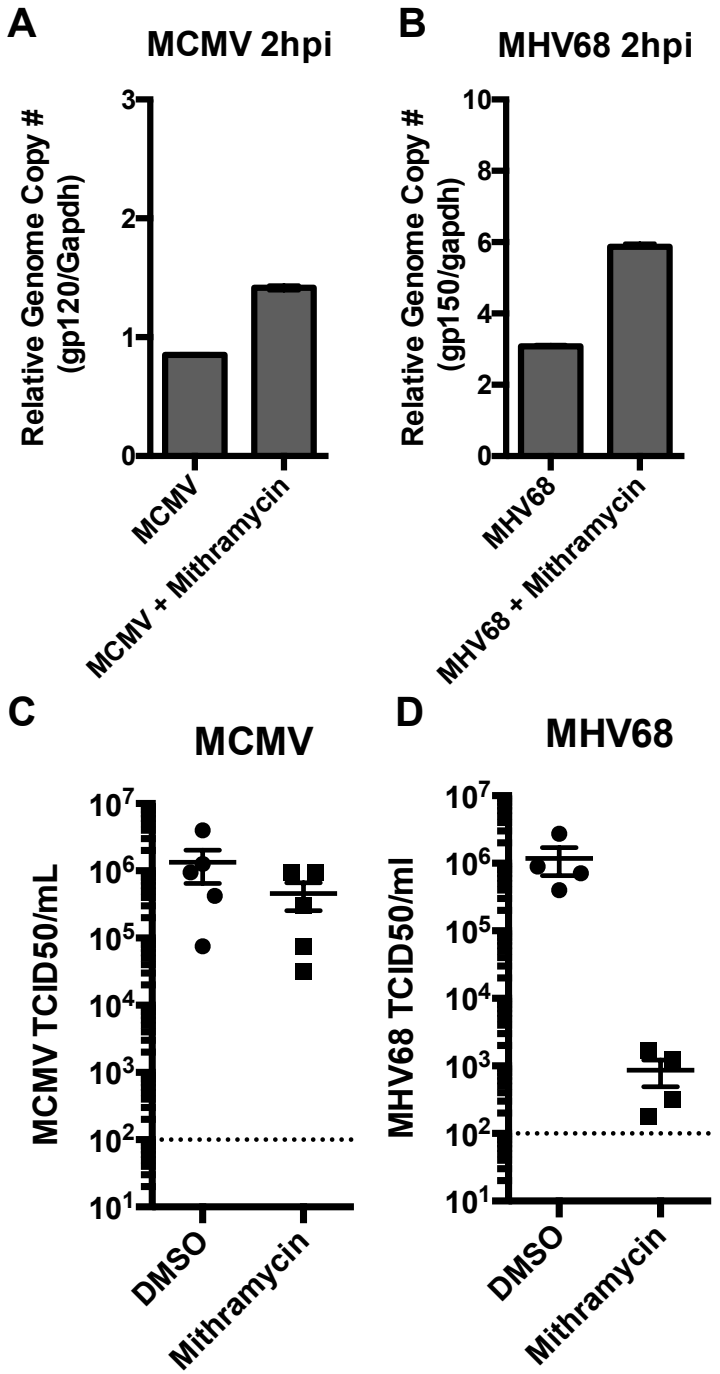


Figure 5-10: Mithramycin blocks MHV68, but not MCMV replication in NIH3T3
NIH3T3 cells were pretreated with mithramycin or DMSO for two hours before infection with either MHV68 (B,D) or MCMV (A,C) at an M.O.I. of 0.1. Mithramycin or DMSO was kept in culture durring infection. Genome copy number associated with cells was assessed by qPCR immediately after infection (A,B). Virus was then grown in the presence of mithramycin or DMSO for 5 days, and functional virus production was assessed by TCID50 assay (C,D).

Chapter 6

Conclusion

The work in this dissertation is an attempt to better understand how hosts recognize herpesvirus infection and connect that recognition to NKG2D ligand induction. This information, in turn, shines a light on herpesvirus biology, as the manipulation that drives NKG2D ligand induction is valuable enough to warrant the evolutionary price of carrying multiple genes that prevent the recognition of these ligands at the protein level. My work has highlighted the importance of a class of epigenetic modifying enzymes, histone deacetylases (HDACs) in the regulation of NKG2D ligands (Chapter 3), as well as related systems in the replication of herpesviruses (Chapter 5). In the course of this investigation I have also made minor contributions to our understanding of NKG2D ligand regulation, such as the implication of CK2 in the control of *Raet1e* transcription (Chapter 3). I have also made additional minor contributions to our understanding of MCMV biology by characterizing the multiple protein products of a previously uncharacterized MCMV ORF (Chapter 4).

Herpesviruses first encounter HDACs after entry into the cell and trafficking into the nucleus. The capsid associated herpesvirus DNA is devoid of any histones, and therefore unmodified. However, as soon as the linear DNA is released into the nucleus it becomes chromatinized (Lacasse and Schang 2012; Lieberman 2013). Histones are intercalated into the episome as it becomes circularized. The newly loaded histones are at first considered “open” chromatin, however they are quickly condensed in part through the action of HDACs (Toth et al. 2013). This first interaction may be considered antagonistic or synergistic with the virus depending on your perspective. If the viruses “goal” is to immediately replicate itself this interaction runs counter to that goal as it represses viral gene expression. However, herpesviruses include a latent life cycle stage stage in which the majority of the viruses’ genes are repressed. Thus, if the “goal” is to transition into latency this action is in line with that goal. When viewing this interaction as antagonistic you might consider HDACs a line of defense against viral infection. When viewing it as synergistic with the herpesvirus life-plan you might consider HDACs to have been co-opted by the virus. My data suggests that NKG2D ligand regulation has been tied into recognizing aberrantly low HDAC activity, which from context we know is associated with lytic replication. Thus, the recruitment of an NK cell response only becomes warranted during lytic replication. Whether this is because viruses have co-opted a host system to carry themselves silently, or because the host allows one anti-viral system to operate with a backup in case of failure is left to the interpreter.

Infected cell lineage also contributes to the balance between latency and lytic replication. It is well established there is strong cell type specificity to the entry into latency. HSV-1 for example will lyse human foreskin fibroblasts, but will enter latency within certain neuron culture preparations (Preston and Efstathiou 2007). Individual HDACs are expressed and regulated differently in unique tissues (de Ruijter et al. 2003). Thus, interactions between the histone deacetylase regulation of an infected cell and the infecting virus may contribute to the choice between lytic replication and latency. My own data reflect some

level of this in Chapter 5 where I note that HDAC3 specific inhibitor RGFP966, does not induce reactivation as well in the endothelial derived iSLK cell line as it does in the B cell derived BCBL1 line.

HDAC specific regulation also extends to NKG2D ligands. In my described system *Raet1e* is controlled mostly by HDAC3, and partially by HDAC1. One important aspect of NKG2D ligand that is currently lacking is a systematic understanding of how it differs in unique tissues. As mentioned in chapter 1 many of the transcription factors that are known to regulate NKG2D ligands are actually transcription factor families. Furthermore, as these families can form homo and hetero dimers, and individual subunits are expressed at different levels in unique tissues this complicates our understanding of how these transcription factors contribute to physiological NKG2D ligand regulation. Like these transcription factor families, HDACs have tissue specific expression patterns (de Ruijter et al. 2003), and regulation systems that are still not completely understood, and this will likely contribute to different cell types being more or less prone to express NKG2D ligands in response to infection.

In the case of NKG2D ligand regulation HDACs mediated repression not only provides a way for the host to recognize herpesviruses. It also provides a check on NKG2D ligand expression in healthy tissue. NKG2D can contribute to autoimmunity (Guerra et al. 2013), and constitutive engagement of NKG2D can desensitize NK cells by promoting downregulation of NKG2D (Song et al. 2006; Groh et al. 2002), thus it is important to keep these ligands controlled. It was previously known that HDAC3 controls the human ligand ULBP1(López-Soto et al. 2009), but my work demonstrates that HDAC mediated repression of NKG2D ligands is conserved in mouse *Raet1e* as well. A darker side to this repression is that it provides a way for cancers to turn off NKG2D ligand expression at the source. HDAC expression levels are very high in many different tumor types, and high HDAC expression is associated with poor prognosis (Barneda-Zahonero and Parra 2012). This increase may contribute to low levels of NKG2D ligands on the surface of these tumors, and thus contribute to NK cell evasion. Unlike the situation described for herpesviruses where HDAC antagonism is necessary to replicate, overexpression of HDACs by cancers is synergistic with their life plan, as a major tumor suppressor p21 is highly sensitive to HDAC activity (Gui et al. 2004). Thus this mechanism that provides repression of NKG2D ligand regulation at steady state, and recognition of herpesvirus infection can be coopted by cancers to evade NK cell control.

HDAC enzymes do not have intrinsic DNA or histone binding activity. Rather they must be recruited to loci by specific transcription factors, or chromatin binding molecules (Shahbazian and Grunstein 2007). In this thesis I describe an important roll for Sp3 in the repression of *Raet1e* by HDAC3, as well as in the induction of *Raet1e* in the presence of m18 or chemical HDAC inhibitors. It is also known that herpesviruses use Sp3 and its relative Sp1 in the control of their own gene expression, and in many studies the elements that bind these factors in herpesvirus promoters have been shown to be HDAC responsive elements (Ye et al. 2005; Tsai et al. 2011; Lang et al. 1992). In chapter 5 I sought to take advantage of this fact and use a drug that binds competitively to these

elements (mithramycin) to hamper herpesvirus replication (Blume et al. 1991). As expected mithramycin blocked replication of some herpesviruses (KSHV, MHV68), however surprisingly it had no effect on MCMV. It will be interesting in the future to understand why this specificity exists. It may also be interesting to investigate whether some NKG2D ligands are refractory to mithramycin treatment. Mithramycin, and its derivatives are in clinical trials to be used to treat cancer (Pérez et al. 2008). My work suggests that they may also down regulate NKG2D ligand expression. Thus, for example, HDAC inhibitors, which are being investigated as anti-cancer drugs in their own right (Wagner et al. 2010), may be anti-synergistic with this treatment. However, treatments that induce NKG2D ligands that are not sensitive to mithramycin treatment may be synergistic in their effects.

At the end of this thesis I hope I have convinced the reader of the importance of HDACs as an interface between the host immune response and herpesvirus biology. Active repression of NKG2D ligands by HDACs allows the host to prevent their expression in healthy cells, while allowing them to quickly become expressed if this system is disrupted. My work establishes that this system also provides a way to recognize herpesvirus infection, as it mirrors the way in which herpesviruses control their genes during latency and rapidly reactivate them to enter into lytic replication.

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