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INTERACTIONS BETWEEN IFI16 AND CHROMATIN SILENCING PROTEINS

A Thesis in Biochemistry and Molecular Biology

by

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Abstract

Interferon-inducible protein 16 (IFI16) has classically been described as a cytosolic viral DNA pattern recognition receptor (PRR) that begins a signaling pathway to lead to the production of interferon β (IFN-β) in response to viral infection. However, it has also been shown to have other, noncanonical antiviral roles, such as promoting the silencing of integrated HIV. DNA editing protein APOBEC3A (A3A) and the chromatin remodeling complex human silencing hub (HUSH) have both been shown to also be involved in the silencing of HIV, specifically via chromatin methylation. However, the DNA sensor that is used for this response has not been shown. We propose that IFI16 is that sensor. In this study, we conduct immunoprecipitation (IP) experiments with a nuclease to show evidence of direct protein-protein interactions between IFI16 and APOBEC3A. We also show evidence of a similar, although more complex and interesting, relationship between IFI16 and TASOR, a member of the HUSH complex, which only occurs in the absence of nucleic acid. These findings suggest that IFI16 exists in pathway(s) with APOBEC3A, TASOR, or both that could result in the silencing of viral DNA. These suggest implications in the treatment of retroviruses such as HIV with new targets for antiretroviral therapies (ARTs).

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Abbreviations

A3A: APOBEC3A

AIDS: Acquired Immunodeficiency Syndrome

APOBEC3A: Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3A

ART: Anti-Retroviral Therapy

HIV: Human Immunodeficiency Virus

HUSH: Human Silencing Hub

IFI16: Interferon Gamma-Inducible Protein 16

IFN: Interferon

IP: Immunoprecipitation

LTR: Long Terminal Repeat

MAMP: Microbe-Associated Molecular Pattern

MGE: Mobile Genetic Element

PMA: Phorbol Myristate Acetate

PRR: Pattern Recognition Receptor

SAINT: Significance Analysis of Interactions

Introduction

Innate Immune System/PRRs

The innate immune system is the body's first response against infection by pathogens. In this phase of fighting infection, the body has many strategies for slowing the replication of pathogens. These include sustaining barriers to infection, such as skin and mucosal layers, and secreting proteins such as antimicrobial peptides that are able to attack pathogen structures that are discernable from host structures (reviewed by Romo et al. 2016). These attacks can even include certain cell-mediated actions, such as those carried out by macrophages, specialized cells that can destroy pathogens through a process known as phagocytosis. These systems are often much quicker than those of the adaptive immune system, which can make more specialized but slower attacks on pathogens. The adaptive immune system differs from the innate immune system in that adaptive responses to pathogens get stronger with subsequent insults or infections by the same pathogen. However, these responses require going through steps such as identifying the pathogen, replicating the proper cells and producing effectors such as antibodies to circulate through the body. While these steps are being carried out, pathogens are continuing to replicate at rates that would cause the infection to overtake the host. Therefore, the innate immune responses are necessary to slow the replication and spread of pathogens until the adaptive system can clear the pathogens (Turvey et al. 2010).

Viruses are an important threat the innate immune system works to control. Viruses are a wide group of microbes that vary greatly but are often much smaller than the cells that they infect and generally exist as nucleic acid genomes surrounded by a

protein coat. The genome that is contained in the viral particles can be made of a wide variety of nucleic acid, such as single- or double-stranded RNA or DNA. These microbes are obligate intracellular parasites, meaning that they must enter a cell in order to reproduce (Doherty et al. 2007). Through various means, viruses place their genome into host cells, where the viral information is transcribed and translated, producing new viral particles. Although they vary wildly, every virus must enter a cell, create more copies of their genome, produce mRNA transcripts for viral proteins and use the host's ribosomes to translate these transcripts into protein. These pathogens can cause severe clinical symptoms and as such are important targets of the immune system.

All immune responses must be tightly controlled, lest they destroy the host they are meant to protect. Adaptive immune effectors identify their targets through small portions, called an epitope, of a pathogen molecule, called an antigen. Therefore, when these cells encounter their specific epitope, they start the processes necessary to prime an adaptive response (Sykulev et al. 1996). However, overzealous adaptive immune attacks can mistakenly identify host molecules, such as the myelin that covers certain vital nerve cells, as an antigen and mount an immune response to it. As a result, the powerful attacks that are meant to clear the body of pathogens instead lead to autoimmune disorders, such as multiple sclerosis. In a similar way, the innate immune system must be regulated and its targets controlled carefully. While the adaptive system recognizes its targets through highly specific receptors for specific portions of molecules, the innate system recognizes broad ranges of molecules, called microbe-associated molecular patterns (MAMPs), that are associated with pathogens (Turvey et al. 2010). When one of these receptors, called

pattern recognition receptors (PRRs), encounters a suitable MAMP, the cell carries out an innate immunity response. These effects employed by the innate immune system can cause pronounced physiological symptoms resulting from the arrest of cellular processes and inflammation of infected tissue. Therefore, a PRR that lacks proper specificity can lead to an unnecessary and overactive response and can unduly damage the host and impede its recovery from the infection. This collateral damage makes it necessary to be able to clearly distinguish between that which the immune system needs to harm and that which it needs to protect.

Important effectors of the innate immune system produced as a result of PRR signaling are small proteins known as cytokines. Cytokines are secreted by immune cells to signal to other cells to coordinate an immune response (Romo et al. 2016). One such cytokine, interferon β (IFN-β), is produced rapidly in response to viral infection and can be produced by any nucleated cell in the body (Deonarain et al. 2000, Lin et al. 2014). IFN-β signals nearby cells and induces expression of interferon stimulated genes (ISGs) to induce an antiviral state in those cells in order to curb the infection. The induction of an antiviral state causes a cell to repress viral transcription and translation and degrade viral DNA (Ivashkiv et al. 2014). However, the process of inhibiting viral transcription and translation also impede host processes. Therefore, antiviral responses can cause very prominent clinical symptoms due to the arrest of normal cellular function. A successful recognition of a viral infection can also lead to the destruction of the infected cells (Lin et al. 2014). These negative effects of antiviral cytokines make it obvious that the proper amount of cytokine production is vital. Too little response, and the pathogenic viruses or

cells can easily out-replicate human cells. Too large a response and the immune system can damage the host more than the pathogen would. Therefore, it is important for the immune system to carefully regulate its responses to potential pathogens.

Because the structure of viral particles is relatively simple compared to other pathogens such as bacteria and fungi, the problem of identifying viruses as "nonself" is not a trivial one. However, a MAMP that is available in all viruses is their nucleic acid. Some viruses have RNA genomes while others have DNA, which need to be transcribed into RNA in order to produce viral proteins. The problem is that all nucleated host cells have their own DNA genomes and use RNA for a variety of essential cell functions. This problem is even more complicated when it comes to identifying integrated harmful DNA. Therefore, it is very important to be able to differentiate between an HIV RNA genome and an mRNA transcript that is on its way to a ribosome to create a normal host protein like actin.

One way of detecting nucleic acid, specifically DNA, as viral and not endogenous is through the use of cytosolic DNA PRRs. If a PRR is localized in the cytosol and recognizes DNA, either the cell's nuclear membrane is compromised, or the cell has been infected (Paludan et al. 2013). Either way, the cell must respond and this event has been shown to lead to production of IFN-β (Vance et al. 2016). The act of a cytosolic PRR binding such DNA can trigger innate immune responses, such as the secretion of cytokines (Turvey et al. 2010). This means that the cell must be able to identify the DNA MAMP and that the DNA is foreign. If the cell was to misidentify its own DNA and

mount an immune response to it, the cell would harm the organism even in the absence of pathogen.

One important PRR for viral DNA is IFI16, a member of the PYHIN family. The PYHIN family is composed of structurally similar intracellular DNA sensors (Unterholzner et al. 2010). These proteins, also known as AIM2-like receptors (ALRs), have both a pyrin domain, which helps them bind to other proteins, and at least one HIN 200 domain, which allow them to bind to DNA. IFI16 has been shown to be integral to the production of the cytokine IFN-β in response to viral infection (Unterholzner et al. 2010). When IFI16 senses exogenous DNA, it starts a signaling pathway that induces the production of IFN-β.

Many DNA sensors have been identified, such as AIM2, cGAS, and TLR9 in addition to IFI16 (Vance et al. 2016). However, among these, IFI16 tends to stand out as more interesting than the others in terms of viral infections. While AIM2 has been shown to induce the creation of inflammasomes which can lead to pyroptosis, a form of programmed cell death (Sagulenko et al. 2013), and cGAS and TLR9 have been shown to lead to the production of IFN-β (Jønsson et al. 2017, Puig et al. 2011, Hoshino et al. 2006), IFI16 is unique in that it can induce both responses (Monroe et al. 2014, Kerur et al. 2011, Unterholzner et al. 2010, Jakobsen et al. 2013). This wide range of antiviral responses makes IFI16 a very attractive target as the sensor initiating a specific antiviral pathway.

In fact, IFI16 has been shown to have other functions in addition to those listed above. Johnstone and colleagues (2000) have shown that IFI16 can bind to and modify

the tumor suppressor protein p53. p53 is a transcription factor that has been shown to help regulate the cell cycle and is often mutated in many types of human cancers (reviewed in Soussi et al. 1994). While other HIN-200 proteins have been shown to bind p53 indirectly via adapter proteins, IFI16 has been shown to specifically bind p53 directly and modify the way in which it influences gene expression (Johnstone et al. 2000). This shows that IFI16 has the capability to alter signaling pathways, suggesting that this could happen in other pathways as well. This further indicates that IFI16 is interesting as an antiviral DNA sensor, as it has a variety of functions against viral infection.

IFI16 has also been identified as a restriction factor for many viruses, including HIV (Hotter et al. 2019). While IFI16 has classically been identified as having antiviral activity by inducing production of IFN-β, it has been shown as being able to restrict HIV through a pathway separate from that which leads to interferon production. Hotter and colleagues (2019) showed that IFI16 could repress the production of viral products and suggested that this was due to binding the transcription factor Sp1. However, these findings do not exclude the possibility of this repression occurring via chromatin silencing, which was not investigated by the researchers.

In the studies discussed above, IFI16 has been shown to be involved in many pathways to combat viral infections. We believe that this is because a viral sensor such as IFI16 could have multiple roles as an inducer of antiviral responses. As the innate immune system makes broad responses to broad classes of threats, it is entirely possible and quite likely that a PRR that senses the presence of viral pathogens may be able to initiate multiple responses to those pathogens. Therefore, while IFI16 has been shown to

initiate pathways leading to various changes in a cell, we believe that it has more, undescribed antiviral properties.

AS described above, Hotter and colleagues (2019) that IFI16 can inhibit the transcription of HIV proteins in a manner independent of its IFN-β production pathway. This would suggest that IFI16 is capable of repressing retroviruses in one of its novel and undescribed functions. This action has been shown to require the pyrin domain, which is needed for protein-protein interactions and not the HIN domain, which allows for protein-DNA interactions. Thus, it is very possible that this novel action of IFI16 is carried out by its activation of other proteins through its pyrin interactions which can directly or indirectly inhibit the transcription of integrated viruses.

Chromatin Silencing and Viral Infection

The *Retroviridae* family, the most famous member of which is HIV, is a very important group of viruses that can integrate their genetic information into the host genome using an enzyme known as integrase (Scherdin et al. 1990). After entering the cell, these viruses copy their RNA genome into complementary DNA (cDNA) using a very important enzyme known as reverse transcriptase. This cDNA is inserted into the host cell's genome where it is transcribed and translated by the cell's own molecular machinery. This allows the virus to be passed to the cell's progeny because replication of the cell's genome also replicates the viral DNA that is integrated.

Not only do cells need to respond to protect other cells from infection, but they also need to protect themselves from harmful DNA, which can disrupt the host genome.

An important form of harmful DNA is that which is integrated into the host's genome. Recent integration events, such as HIV infection, require the activation and transcription of the integrated viral genome, at this stage called provirus, in order to cause widespread infection and horrible disease states. Integration events like these that occurred long ago in germline cells and have been stably passed from generation to generation pose a threat of their own in that they can still be mobile and reintegrate into the genome at a different location (Miki et al. 1998). Elements like these, called retrotransposons or generally mobile genetic elements (MGEs), can integrate somewhat randomly, allowing for the possibility that they will disrupt an important gene, causing disease in the host. Therefore, it is important to repress any viral DNA that has been integrated into the genome, lest it cause disease. This repression can be done many ways, although the most important is through chromatin silencing.

Just as cells can control the level of transcripts of their genes in order to respond to external or internal stimuli, viral genes vary in level of expression (reviewed in Gale et al. 2000). Most viruses exhibit different expression profiles at different periods of infection, as some genes may be more important for helping stabilize the infection and neutralizing host defenses while other genes are needed only when the virus is ready to start producing new viral particles. These genes are usually divided into early and late genes, depending on what stage in the replication cycle they are transcribed. While the virus can regulate these expression levels through various means, the cell can also modify viral transcript or protein production. This means that infected cells can vary greatly in their amount of viral product production. The problem of fighting viral infections with

the immune system is complicated when viral activity inside of an infected cell reaches the point where it no longer produces viral particles, making infection difficult to detect.

One of the biggest problems with treating HIV is the latent reservoir, which is created when infected cells stop producing viral particles (Chun et al. 1995). This obviously prevents the infected cell from having to deal with the stress of producing virus and keeps the virus from being able to spread to neighboring cells. However, this produces a collection of cells known as the latent reservoir, a reservoir containing provirus that is unidentifiable by immune responses and that can begin producing virus again at a later time (Finzi et al. 1997). When this latency reversal happens, it allows the virus to spread again, but it also allows the immune system to identify this cell as being infected by the virus. Therefore, latency reversal and the chromatin modification associated with it have been important targets for anti-retroviral therapies (ARTs), drug therapies that target HIV in patients. In order to more accurately target this process with ARTs, we must first learn much more about the processes that induce and reverse latency in HIV-infected cells.

Cellular genomic DNA does not exist as a loose collection of double-stranded DNA molecules, but in a highly organized and regulated system. This organization includes DNA wrapping around proteins known as histones (reviewed in Isenberg 1979). An octamer of these histones forms the center that DNA wraps around, forming a structure known as a nucleosome. In this structure, the DNA closely binds to the histones while the N-termini of the histones project away from the nucleosomes as a “tail”.

Nucleosomes play an important role in not only organizing genomic information, but also controlling the expression of that genomic information. Control over protein production is very important for cells within a larger, multicellular organism. Not only do a wide variety of cell types need to be created using the same genomic information, but also cells change over the course of their lifespan and in response to extracellular signals. There are many ways a cell can control this: post-translational modification or degradation of proteins, degradation of mRNA transcripts before they are translated into proteins, and regulation of transcript levels. One important way to control the level of mRNA transcripts of a gene is to tightly pack nucleosomes around the gene in question (reviewed in Cedar and Bergman 2009). The histones in a nucleosome sterically hinder the action of RNA polymerases that would normally transcribe the information into pre-mRNA. Therefore, tight packing of nucleosomes causes more of the DNA in that region to be more associated with histones and leaves less accessible DNA between nucleosomes.

The packing of nucleosomes is a highly controlled process that relies on modifications of the histone cores, specifically in the N-terminal tail regions (reviewed in Cedar and Bergman 2009). Specific amino acid residues on these tails can be post-translationally modified with methyl or acetyl moieties. Acetyl groups can be added to lysine residues through the action of enzymes known as histone acetyltransferases. These acetylated lysines are recognized by proteins, including chromatin remodeling complexes, which contain a specific domain, called a bromodomain (Dhalluin et al. 1999). These chromatin remodeling complexes act to separate the nucleosomes and thus

allow for increased expression of DNA in that area. Methylation of histones acts in a similar fashion with a different set of specialized proteins. Methyl transferases, such as SETDB1, add methyl groups to lysines or arginines in histones (Schultz et al. 2001). These modifications are then read by proteins containing domains known as chromodomains (Bannister et al. 2001). Chromatin remodeling proteins that contain chromodomains can function to pack nucleosomes more closely and thus decrease gene expression in that area, silencing any genes that are present.

This process is also very important as a defensive response against infecting retroviruses or MGEs. Retroviruses pose an interesting threat due to the fact that they insert their genetic information into the host cell's genome and thus a healthy, normally functioning cell can translate and transcribe viral genes, producing new viral particles that can spread the infection. The danger of MGEs come from the possibility of their moving within the genome, possibly disrupting genes that are important to normal functioning of the organism (reviewed in Payer and Burns 2019). In both of these cases, the accessibility and normal function of information in the host genome can lead to disease states. However, because these viral elements have been integrated into the genome and are thus associated with histones and chromatin, the same control that the cell has over its own gene products can be applied to these sequences. Thus, activation of the machinery involved in chromatin silencing can be a powerful tool in the immune response against certain viruses.

One group of proteins that have been shown to be important to the silencing of chromatin and maintaining HIV latency is known as the Human Silencing Hub (HUSH)

complex (Tchasovnikarova et al. 2015, Liu et al. 2018). HUSH is a complex made up of the proteins periphilin, MPP8 and TASOR, also known as FAM208A. These members were shown to bind to chromatin methylated with the H3K9me3, meaning that three methyl groups are attached to the third histone at the 9th lysine residue (biochemically referred to as "K"), histone mark around the retroelement L1 (Liu et al. 2018). This suggests that these proteins are involved in the mechanism of chromatin remodeling that leads to methylation and repression of MGEs. In myeloid KBM7 cells transduced with a lentiviral GFP vector, a vector that is meant to act like HIV, HUSH members were found to be the cause of epigenetic silencing of the integrated vector (Tchasovnikarova et al. 2015). This complex was found to recruit the methyl transferase SETDB1 in order to condense chromatin around integrated virus.

HUSH has also been shown to directly interfere with the production of HIV particles (Chogui et al. 2018, Yurkovetsky et al. 2018, Tchasovnikarova et al. 2015). Methylation ChIP-qPCR experiments, experiments that assess the association between histone methylation and specific segments of DNA, performed by Tchasovnikarova and colleagues showed that removing HUSH components greatly reduced the amount of methylation and therefore silencing of HIV viral genes. HIV encodes a protein called Vpx that has been shown by Chogui, Yutkovestsky and their respective colleagues to degrade TASOR, presumably as a way to evade the HUSH complex's antiviral action. This TASOR degradation then leads to a rise in viral activation, further suggesting that TASOR and the HUSH complex are necessary for the full repression of integrated HIV. Furthermore, removing each HUSH member individually from a CD4+ T cell line

infected with an HIV-1 reporter vector caused reactivation of transcription of the vector. This shows that these proteins work together as the HUSH complex to silence integrated HIV provirus as well as the previously shown silencing of retroelements.

Another protein, APOBEC3A (A3A), has classically been described as a cytosine deaminase and has been shown to be important in restricting foreign DNA, both transfected DNA and integrated DNA as a model for MGEs (Steinglein et al. 2010). Chen and colleagues (2006) were able to show that A3A inhibits adeno-associated virus and retroelements much more than any other members of the APOBEC3 family. However, these researchers assumed that this antiviral activity was due to the cytosine deaminase role of A3A that they had discovered. However, they were not able to see any evidence of cytosine deamination in the repressed retroelements, suggesting that A3A is active against retroelements in a different way. This was explored more by Bogerd and colleagues (2006), who showed that a mutant form of A3A that cannot carry out cytosine deamination, was still able to repress retroelements. A later paper by Taura and colleagues (2018) found that removing A3A from cells with stably infected HIV-1 reporter lead to reactivation of HIV-1 transcription. This was shown to be cytosine deaminase independent and instead relied on the recruitment of methyltransferases, such as SETDB1. This paper also showed that A3A binds to the region of the HIV LTR (Long Terminal Repeat) that allows for the binding of the transcription factor Sp1, the transcription factor shown in a study discussed above to be acted upon by IFI16 in its similar repressing mechanism. Taura and colleagues were also able to show that the LTR is marked by H3K9me3 marks, the same marks that TASOR was shown to interact with

in a study discussed previously, much more in cells with A3A than those that lacked A3A.

Seeing as how HUSH members and A3A have been shown to have similar functions in response to HIV and MGEs and associate with the same histone marks, it is possible that these work together to accomplish their function. Both HUSH and A3A recruit SETDB1 to the location of the viral or MGE DNA in order to induce the repression of that DNA. Both have been shown to be necessary for the maintenance of HIV latency. We believe that HUSH and A3A either interact together as partners or are activated by the same upstream factor in the event of retroviral infection. We focused on IFI16 as that upstream protein and explored its ability to work with HUSH and A3A. Literature has shown that IFI16 is multifunctional as an antiviral protein and can repress transcription and activation of retroelements in a way that is reminiscent of chromatin silencing. Through many studies, IFI16 has emerged as a very likely candidate for initiating a pathway that leads to repression of HIV, likely through chromatin modification and silencing. Likewise, A3A and HUSH have been identified as strong repressors of HIV and identified as doing these through the same specific histone marks. However, the matter in which these proteins sense viral DNA has not been shown. We hypothesize that the apparent connection between the sensor and the effectors is not a coincidence and that this occurs via an undescribed pathway.

There is some evidence in the literature that IFI16 and our proteins of interest interact. A proteomics screen done by a group from Princeton showed evidence of interactions between IFI16, multiple HUSH members and members of the APOBEC

family (Diner et al. 2015). In this screen, primary human foreskin fibroblasts (HFFs) were either uninfected or infected with herpes simplex virus 1 (HSV-1) and lysed for immunoprecipitation to isolate proteins that were bound to IFI16. The IFI16-associated proteins were identified via mass spectrometry and assigned a specificity score based on the significance analysis of interactions (SAINT) algorithm. This SAINT score offers a quantified probability of true protein-protein interactions. Thus, the higher the SAINT score, the more likely the two proteins interact (Choi et al. 2011). The screen found high SAINT probability scores for interactions between IFI16 and APOBEC3B and APOBEC3C in cells infected with HSV-1 (Figure 1). The screen also found high scores in infected cells for TASOR (also known as FAM208A) and MPP8 (MPHOSPH8) in their interaction with IFI16. These data suggest that IFI16 does bind to these proteins *in vivo*, and in fact, they interact more readily when the cell has been infected. This indicates that when the cell is mounting an antiviral response, the cellular environment changes in such a way that these proteins bind more and supports our hypothesis that IFI16 may interact with APOBEC family members and members of the HUSH complex.

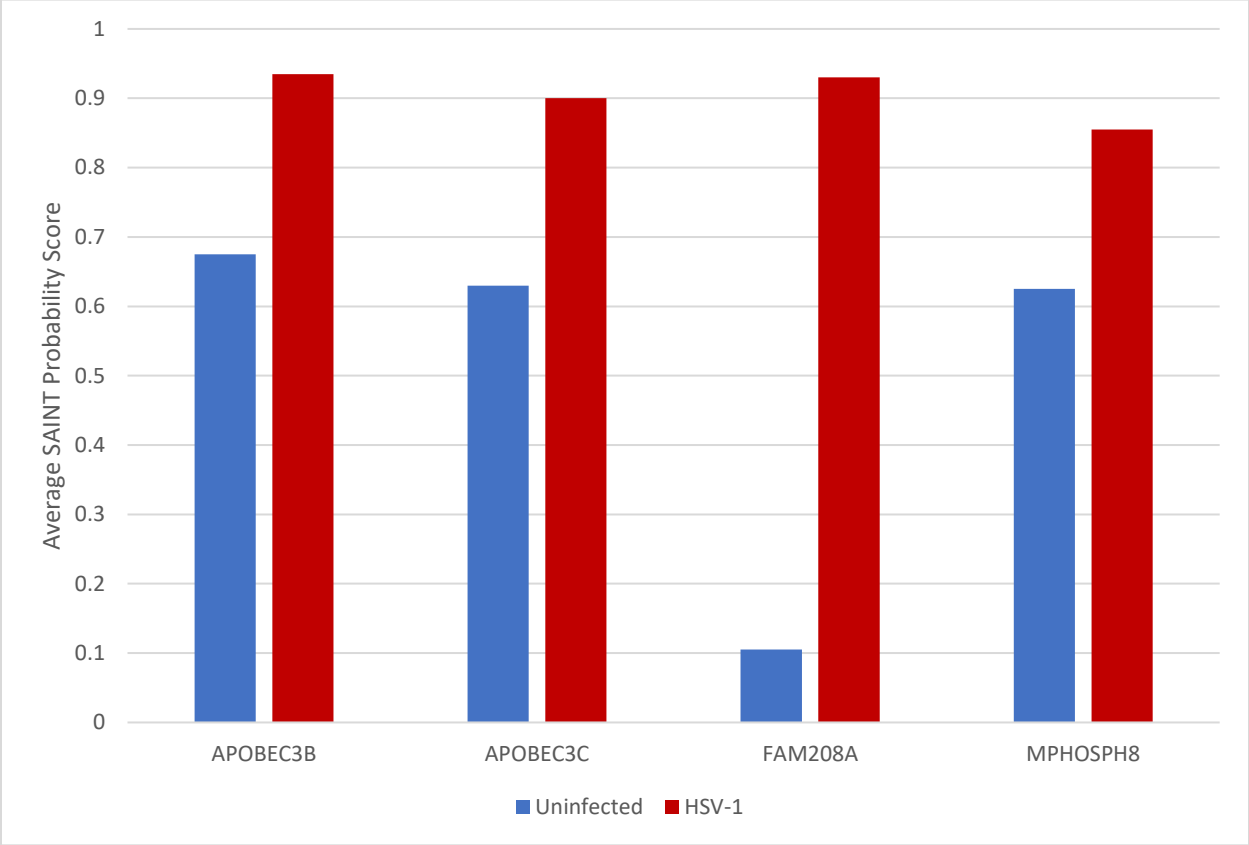

Figure 1: SAINT probability scores for interactions between IFI16 and proteins of interest

Probability scores calculated for interactions between IFI16 and several APOBEC family and HUSH complex proteins. Cells were either uninfected or infected with HSV-1. FAM208A = TASOR; MPHOSPH8 = MPP8. Data adapted from Diner et al. 2015.

This proteomics screen used HFFs to explore IFI16 and did not validate any of the specific interactions between IFI16 and our proteins of interest. It is important to note that HFFs have a very specific protein expression profile, one that is very different from our model system, monocyte-like THP-1 cells. The most notable difference between these two is that while HFFs do not produce A3A, THP-1 cells do (Goujon et al. 2013). Therefore, although the screen did not reveal A3A as a binding partner of IFI16, it did reveal APOBEC3B and APOBEC3C, which are very structurally similar to A3A (Salter et al. 2016). THP-1 cells also can produce much more IFI16 when stimulated into macrophage-like cells (Unterholzner et al. 2010), allowing for the possibility of more interactions with binding partners. Also, previous work on IFI16 has been largely done in THPs, such as the initial identification of IFI16 as a PRR (Unterholzner et al. 2010), and much of the work further describing IFI16's biological functions (Unterholzner et al. 2010, Kerur et al. 2011, Jønsson et al. 2017, Jakobsen et al. 2013). Therefore, in order to best understand IFI16's biological functions, it is best to study it in the cell type in which it is best characterized, THP-1 cells, instead of HFF cells.

Experimental Goals

The main objective of this study was to see if IFI16 acts as an initiator of chromatin silencing pathways through interactions with A3A and HUSH complex members. Since IFI16 has been shown to act as a viral PRR and induce antiviral responses, influence transcription and act in signaling pathways (Unterholzner et al. 2010, Kerur et al. 2011, Johnstone et al. 2000), A3A and HUSH have been shown to

silence chromatin in response to viral infection, but with an unknown DNA sensor (Tchasovnikarova et al. 2015, Steinglein et al. 2010), and it has been suggested that IFI16 interacts with both APOBEC proteins and HUSH members (Diner et al. 2015), we hypothesized that IFI16 could be a viral DNA sensor that leads to this chromatin silencing. This would be a novel function of IFI16 and could help us understand the body's response to retroviruses such as HIV and the pathways that lead to the formation of latent reservoirs, allowing for the development of new targeted therapies for treating such infections.

In order to test our hypothesis, we first validated our model system to find the presence and normal levels of all of our proteins of interest through a BCA assay and western blotting. We then investigated the interactions between IFI16 and A3A and HUSH member TASOR through immunoprecipitation (IP) and western blotting. We then investigated the nature of interactions between these proteins through the use of nucleases.

Methods

Cell Culture

Because our experiment is investigating the action of the human innate immune system in response to viruses, we used immortalized human cell lines for our experiments. These cell lines originally came from human cells that have been modified such that they can grow outside of an organism and can replicate limitlessly. HEK 293T cells (HEK standing for Human Embryonic Kidney) originated from human embryonic kidney cells and thus do not usually act like immune cells. THP-1 cells, however originated from a leukemia patient and are monocyte-like. Therefore, these cells act like immune cells and can even be differentiated into macrophage-like cells, which changes the activity and gene expression profile of the cell. This stimulation occurs by adding phorbol 12-myristate 13-acetate (PMA) and has been shown to increase cytokine production and IFI16 levels (Daigneault et al. 2010, Unterholzner et al. 2010). Although these cells can be grown outside of an organism, they must still be supplied with proper nutrients and environmental conditions. This comes in the form of R10 (containing RPMI media (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 0.1% β-mercaptoethanol (Invitrogen), 1% penicillin/streptomycin/glutamine (PSG) (Invitrogen), 1% non-essential amino acids (NEAA) (Invitrogen), 5 mL/500mL sodium pyruvate (Invitrogen), 0.1% normocin (Invivogen)) for THP-1 cells or D10 (containing DMEM media (Invitrogen), 10% fetal bovine serum (FBS) (Invitrogen), 0.1% β-mercaptoethanol (Invitrogen), 1% penicillin/streptomycin/glutamine (PSG) (Invitrogen), 1% non-essential amino acids

(NEAA) (Invitrogen), 5 mL/500mL sodium pyruvate (Invitrogen), 0.1% normocin (Invivogen)) for HEK293T cells and an incubator that is kept at 37°C and 5% CO_2.

THP-1 or HEK 293T cells were counted with a hemocytometer and $2.5x10^5$ cells were added to wells of a 12-well plate in 1 mL of R10 (THP-1) or D10 (HEK293T) media. Some THP-1 cells were stimulated with PMA at 5 ng/mL for 3 days. All cells were incubated at 37°C at 5% CO_2 before being collected.

Stimulation and lysis

Some THP-1 cells were stimulated with PMA at 5 ng/mL for 3 days in order to differentiate them into macrophage like cells (Shannon et al. 2018). All cells were transfected with a double stranded DNA stimulus (HSV60 or Vac70) in order to induce an antiviral response such as increased IFN-β production (Unterholzner et al. 2010). We accomplished this with Lipofectamine 2000 (Invitrogen) in order to deliver the stimulant to the cytoplasm of the cells (Figure 2). In this process, 12 μg of DNA was diluted in 250 μL total of Opti-MEM (Invitrogen). This was added to a mixture of 10 μL of Lipofectamine 2000 in 250 μL total of Opti-MEM. Mock transfection mixtures were prepared by combining a mixture of 10 μL of Lipofectamine 2000 and 240 μL of Opti-MEM with an additional 250 μL of Opti-MEM. All transfection mixtures were incubated between 20 minutes and 6 hours before being added to cells according to the manufacturer's instructions.

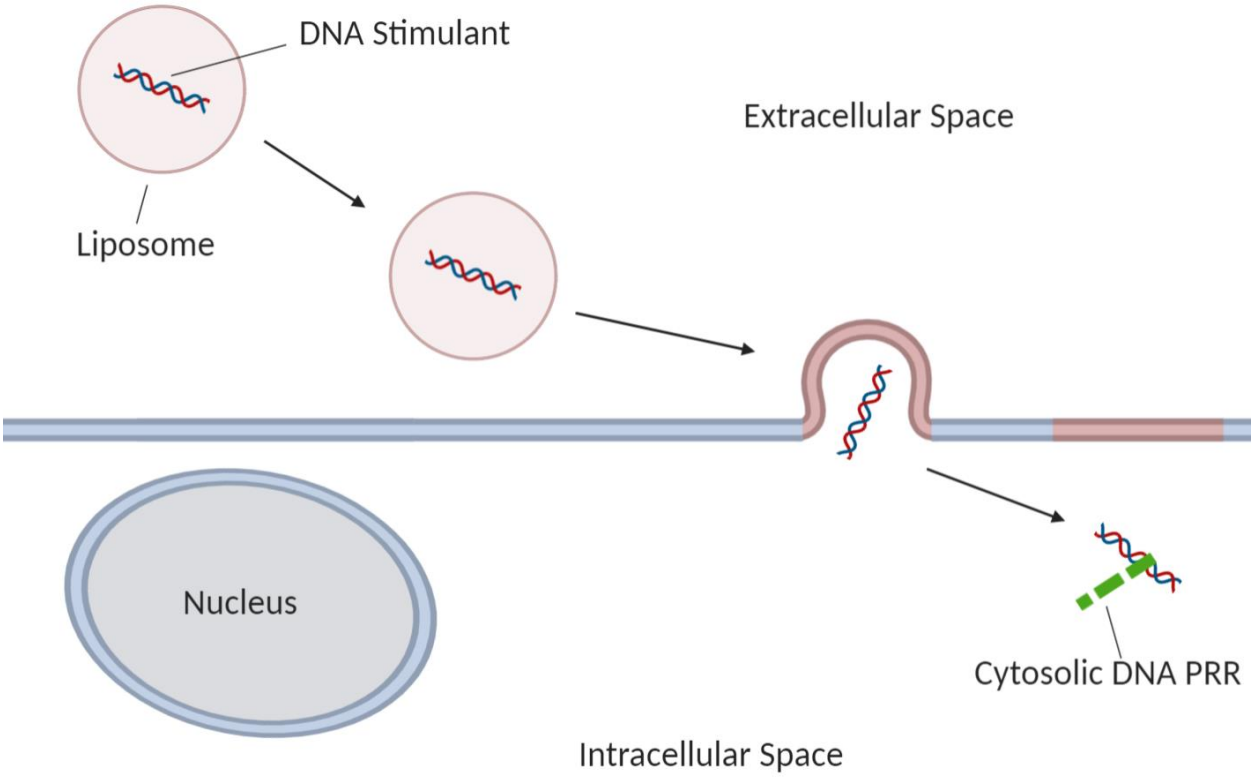

Figure 2: Transfection of DNA into cytoplasm as a model for viral infection

Lipofectamine 2000 incubated with nucleic acid forms liposomes containing the nucleic acid stimulant. These liposomes fuse with the cell membrane and deliver the nucleic acid to the cytosol of the cells, where they can interact with cytosolic DNA PRRs.

To collect cells, all cell suspensions were centrifuged at 1800 rpm for 5 mins in order to form a pellet of cells at the bottom of the tubes so that all media could be removed. All of these cells were then resuspended and collected with 250 μL lysis buffer made from 10 mL of NP-40 Lysis Buffer (Boston Bio Products), which is able to disrupt both cellular and nuclear membranes, and 1 Pierce™ Protease Inhibitor Mini Tablet (Thermo Scientific) for 1 hour at 4°C with constant agitation in order to release all proteins from the cell. The lysates were centrifuged at 14,000 rpm and 4°C for 10 minutes in order to pellet all cellular debris at the bottom of the tube and whole cell lysate samples (WCL) were taken from the supernatant and frozen at -20°C in order to preserve the proteins. When working with lysates, it was important to keep everything as cold as possible because the cells have naturally occurring proteases that will start to degrade the proteins of interest once the cell membranes are disrupted. Therefore, the samples were kept cold to slow the action of these proteases and preserve the proteins of interest. At the end of this process, we hoped to have all intracellular components in our samples.

Nucleic Acid-Free Lysis

Some of the IPs that we carried out were done with lysates with a nuclease called benzonase added to it. This nuclease destroys any RNA or DNA present in the sample without affecting proteins (Eaves and Jeffries 1962). This was done in order to investigate the nature of interactions between proteins in the experiments. All of the proteins of interest in this experiment have been shown to interact with DNA; thus it is possible that two proteins show signs of interaction purely because they interact with the

same piece of DNA and do not truly bind together. By adding a nuclease, we would expect to only see protein-protein interactions.

When performing our IP experiment in the presence of a nuclease, a cold addition as described by Chogui et al. (2018) was carried out. To the lysis buffer, benzonase was added to a final concentration of 750 units/mL and $MgCl_2$ was added to a final concentration of 1 mM. Then the cells were lysed in this nuclease buffer for 1 hour at 4°C with constant agitation. At the end of this procedure, we expect to have all molecules present in the cell sample without any stable membranes or nucleic acid.

Quantification of Proteins in Cell Lysates

In order to validate using the cell lines as a model for studying the interactions of the proteins of interest, we carried out a BCA (bicinchoninic acid) assay to determine the total protein concentrations of lysates for the purpose of normalizing these values. This assay uses the property of BCA that causes it to change color in the presence of protein. This assay involves mixing BCA with various known concentrations of a control protein (albumin) in order to develop a relationship between protein concentration and amount of color change (measured with a plate reader). This relationship is used along with the amount of color change in mixtures with our lysates in order to determine the concentrations of the WCLs.

Whole cell lysates from PMA-stimulated and unstimulated THP-1 and HEK 293T cells were quantified for overall protein concentration according to the instructions in the Pierce® BCA Protein Assay Kit (Thermo Scientific).

The samples were read for absorbance at 562 nm with a plate reader. The BSA concentrations of the standards were plotted against absorbance in an Excel scatter plot. A regression line relating concentration and absorbance was fitted against these data and used to estimate concentrations of the experimental samples. All samples run on the BCA were diluted to the same total protein concentration before being run on an SDS-PAGE and immunoblotted in order to compare the intensities of the resulting bands and thus the relative concentrations of the proteins of interest.

Co-Immunoprecipitation

This experiment relied heavily on a technique known as co-immunoprecipitation (IP). This technique takes advantage of the highly specific nature of antibody binding and the difference in density between the bulk lysate and agarose beads. In this experiment, antibodies that bind to a protein of interest (in this case IFI16) and to agarose beads are added to the lysate. Then the samples are centrifuged, which pulls the agarose beads to the bottom of the sample. These beads are separated from the supernatant and washed, which should leave only the protein of interest and any proteins that pulled down with that protein, presumably because they physically interacted (any proteins that would bind non-specifically to the agarose beads are removed in a preliminary step known as a "preclear", where beads are spun down in the absence of antibodies).

Co-Immunoprecipitations were performed in order to isolate IFI16 along with any of its binding partners (Figure 3). Lysates generated as described above were treated with 20 μL of Control Agarose Resin beads (Thermo Scientific) and incubated rocking at 4°C

for 30 mins in order to separate all proteins that would bind non-specifically to the beads used. These samples were centrifuged at 4°C for 60 seconds and the supernatants were separated from the beads and saved. The supernatants were treated with 10 μL of anti-IFI16 antibodies (Santa Cruz Biotechnology, sc-8023) and incubated rocking at 4°C overnight in order to allow the antibodies to bind to the IFI16. To the samples, 20 μL of Protein G Agarose beads (Thermo Scientific) were added and incubated rocking at 4°C for 60 mins to allow the beads to bind to the antibodies. The beads were washed by centrifuging at 4°C for 60 seconds and adding 500 μL of lysis buffer before incubating rocking at 4°C for 5 mins. Two more washing steps were performed in order to ensure that all proteins that would not bind to the antibodies or other proteins that are bound. The final samples were centrifuged at 4°C for 60 seconds and the supernatant was removed. The samples (referred to below as immunoprecipitates) were frozen at -20°C until needed.

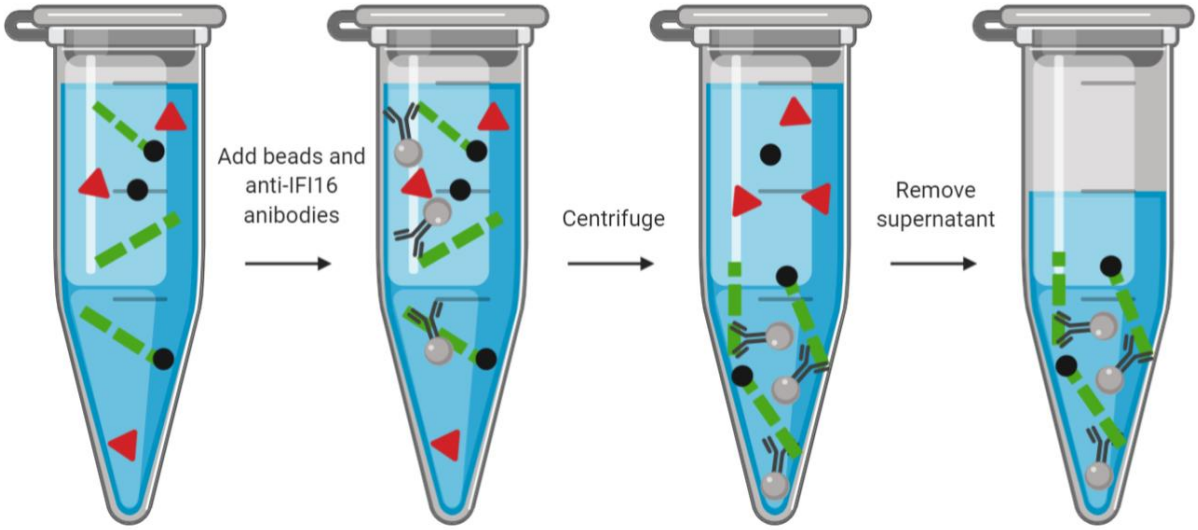

Figure 3: Schematic for Co-Immunoprecipitation

The basic steps for a co-immunoprecipitation procedure. The tube in the left most step contains the whole cell lysate, including IFI16 (green rectangles), IFI16-associated proteins (black circles) and non IFI16-associated proteins (red triangles). In the next step, agarose beads (grey circles) and IFI16-specific antibodies (black "Y" shapes) are added and the antibodies bind to the IFI16. In the third step, the tube is centrifuged, separating the beads, antibodies, IFI16 and any IFI16-associated proteins from unassociated proteins. In the right most step, the unassociated proteins are removed and the sample contains only IFI16 and its associated proteins.

SDS-PAGE and Immunoblotting

One of the most important techniques in molecular biology, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and immunoblotting, also known as Western blotting, allows us to investigate the presence, and in some cases the concentration, of proteins in a sample. The SDS-PAGE itself works by linearizing the proteins in sample while adding negative charges so that it can be forced through an acrylamide gel mesh with an electric current. This gel offers more resistance to larger proteins and thus resolves the proteins in the solution according to size, which can be determined by comparing to a protein ladder that contains proteins of known sizes. However, protein sizes are not always reliable from cell type to cell type and many proteins can be the same size, so this technique is important but not sufficient for identifying specific proteins. In order to do this, we rely heavily on the highly specific nature of antibodies. We first transfer the proteins from the gel to a nitrocellulose membrane (purely because it is much more durable than the gel) and add generic proteins to any portion of the membrane not already bound by proteins with blocking buffer. The membrane used binds protein easily and because antibodies are proteins, an unblocked membrane would nonspecifically bind antibodies, which would make protein detection impossible. The primary antibody (i.e., the antibody specific to the protein of interest) is added to the membrane and allowed to bind to its protein. Because these antibodies are made in animals, they have a portion that is specific to the animal used to produce the antibody. Thus, the secondary antibody, which contains an enzyme (in this case horseradish peroxidase) that can be identified through a specific reaction, must be

specific for the animal that produced the primary antibody (e.g., anti-mouse or anti-rabbit). This secondary antibody, when added to the membrane, binds to the primary antibody and thus indirectly to the protein of interest. Then the membrane is developed using a kit that provides the substrate for the enzyme linked to the secondary antibody and allows for luminescence at the location of the secondary antibody and by extension the protein of interest. This luminescence is detected by an imager and combined with an image containing the protein ladder in order to verify the size of the protein of interest and confirm its presence.

SDS-PAGE mixtures were prepared by combining 30 μL of whole cell lysate, immunoprecipitate, or BCA protein normalized sample with 10 μL of loading buffer (100 mM Diothiothreotol (DTT), a substance that allows the proteins to be fully denatured, in 4X Laemmli, which helps denature the proteins and add an overall negative charge so that it can be separated via electrophoresis). Mixtures were boiled at 95°C for 5 mins and centrifuged at 14,000 rpm for 30 sec to collect droplets on the side of the tube. Thirty microliters of sample and ten microliters of Broad Spectra Protein Ladder marker were added to individual wells of a 4-20% polyacrylamide gel (Bio Rad). The gel was run in the presence of running buffer (Tris-Glycine-SDS Buffer) at 120 V until the dye front reached the bottom of the gel to allow for the separation of the proteins in the sample by size. The gels were removed from their casing and transferred onto a nitrocellulose membrane using an iBlot system in the presence of transfer buffer (Tris-Glycine buffer with 2% Methanol) to be better manipulated.

The membranes were blocked shaking for 30 mins at room temperature with blocking buffer (5% milk powder in TBST w/v). This step was important for blocking any exposed parts of the membrane, which could non-specifically bind the antibodies that are used for their specificity. The membranes were treated with 10 mL of primary antibody solution (1:1,000 primary antibody (anti-IFI16, Santa Cruz Biotechnology, sc-8023) in blocking buffer) in a sealed bag and incubated rocking at 4°C overnight to allow the antibodies to bind to their specific proteins in the membrane. The membranes were washed three times with TBST for 5 mins each rocking at room temperature to remove any unbound antibodies. The washed membranes were then treated with 10 mL of secondary antibody solution (1:10,000 secondary antibody (goat-anti-mouse, Santa Cruz Biotechnology, sc-516102) to blocking buffer) in a sealed bag and incubated rocking at 4°C for 1 hour to allow the secondary antibodies to bind to the primary antibodies. Three more washing steps were performed as previously described to remove any unbound secondary antibodies and the membranes were developed according to the SuperSignal™ West Dura Kit (Thermo Scientific) and imaged on an Amersham Imager 600 in order to visualize the presence of the secondary antibodies, which indirectly indicate the presence of the protein of interest.

Results

Protein Expression Levels

In order to validate our experimental model of examining our proteins of interest in THP-1 cells and determine the baseline relative expressions of our target proteins in the three cell types used, we performed an immunoblot on whole cell lysates after normalizing the amounts of proteins loaded on the gel. The samples used were THP-1 cells, which act as a human monocyte model, THP-1 cells stimulated with PMA, which are macrophage-like (Unterholzner et al. 2010, Tschukiya et al. 1980), and HEK293T cells, which are non-immune cells and thus are expected to produce fewer proteins important for immune responses. We expect THP-1 cells to have all of our proteins of interest and HEK293T cells to be missing one or more (Unterholzner et al. 2010, Goujon et al. 2013, Chen et al. 2016, Chougui et al. 2018). The BCA assay is important for finding out the total concentration of proteins in the lysates so that they can be diluted to the same concentration. This assay uses a protein dependent colorimetric reaction with bicinchoninic acid (BCA) to relate protein concentration to color intensity (Smith, 1985). Once the protein levels were normalized across the three samples, an equal mass of each protein sample was run on an SDS-PAGE and immunoblotted (IB) for 6 different proteins: HUSH members TASOR, MPP8, and PPH as well as APOBEC3A (A3A), IFI16 and the ubiquitous metabolic enzyme GAPDH as a loading control. Because the overall protein levels were normalized, differences between amounts of specific proteins will be due to actual differences in amount of that protein, not just due to differences in sample sizes.

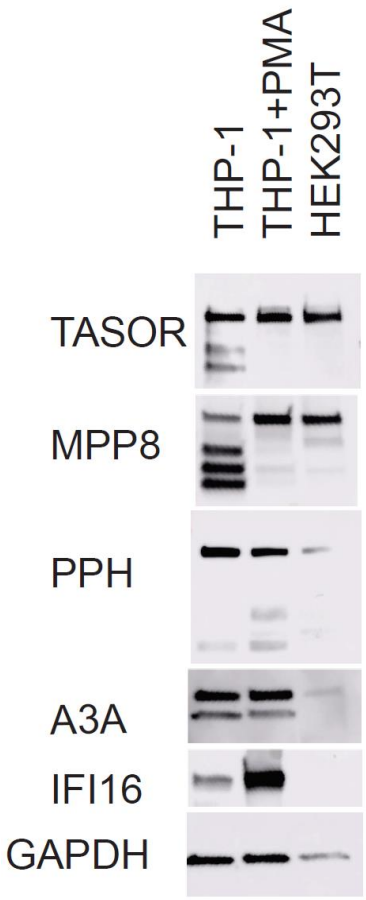

Figure 4: Expression levels of proteins of interest in the model cell types

Indicated cell types were normalized for total protein concentration using a BCA assay and run on an immunoblot for different proteins of interest. TASOR (189 kDa), MPP8 (105 kDa) and PPH (53 kDa) are HUSH complex members, A3A is APOBEC3A (28 kDa). GAPDH (37 kDa) is a metabolic enzyme and is unrelated to immune responses, therefore it is used as a loading control.

This immunoblot shows that all five proteins appear in different levels or forms in all three cell types. Because the total protein levels were normalized, a darker band indicates that that cell type produced that protein in larger quantities compared to all other proteins. Bands at different sizes or more bands could indicate that the cell type in question undergoes some sort of splice form variation or post-translational modification to the protein, creating proteins of different molecular weights. The unstimulated THP-1 cells produced all three HUSH complex members as well as A3A and IFI16 (Figure 4). THP-1 cells stimulated with PMA behaved similarly to the unstimulated condition with the notable exception of producing much more IFI16, which could indicate that these cells are able to have a stronger immune response after differentiation by producing more IFI16, which has been shown in previous literature (Daigneault et al. 2010, Unterholzner et al. 2010). These cells also seemed to have TASOR and MPP8 in slightly different splice forms than the unstimulated lane, suggesting that these proteins may also behave differently in an immunologically primed cell type. The non-immune HEK293T cells expressed TASOR and MPP8 but minimal PPH and no A3A or IFI16. However, they also weakly expressed the loading control GAPDH, indicating that the cells likely were experiencing metabolic stress at the time they were sampled and as such would be producing proteins somewhat unusually. These data suggest that THP-1 cells, both unstimulated and stimulated, which are often used for studies involving IFI16, are suitable for our study of the interactions between IFI16 and HUSH components or A3A.

IFI16 Interactions with APOBEC3A

In order to determine protein-protein interactions we carried out co-immunoprecipitation (IP) experiments. These experiments utilized agarose beads bound to antibodies for a protein of interest (IFI16), which can be centrifuged and pulled out of the solution. When these beads are pulled out of solution, they pull out the antibodies that pull down not only IFI16, but also any proteins that IFI16 was physically interacting with. These samples were run on an SDS-PAGE and then immunoblotted for proteins of interest. Thus, the presence of a protein in an IFI16 IP sample indicates that the protein is capable of interacting with IFI16.

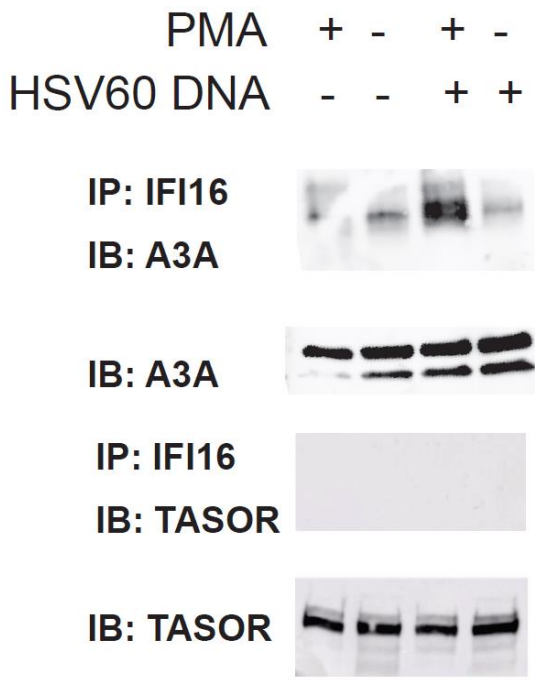

Figure 5: Co-immunoprecipitation and immunoblot without nuclease

THP-1 cells were either stimulated with PMA into macrophage-like cells (PMA +) or not (PMA -) and were either transfected with herpes simplex virus DNA (HSV60 DNA +) or not (HSV60 DNA -). These cells were lysed and the whole cell lysates and IFI16 co-immunoprecipitation (IP) were immunoblotted (IB) for APOBEC3A (A3A) or TASOR.

This immunoblot indicates an interaction between IFI16 and A3A in THP-1 cells under all our stimulation conditions. PMA was used to stimulate cells in order to stimulate them to macrophage-like cells and HSV60 DNA was transfected into cells in order to initiate responses to exogenous DNA. It is important to note that these samples were not normalized for protein concentration and thus some bands may be darker or lighter because they had more cells or more concentrated overall proteins, not necessarily because the specific protein was more abundant in the sample. Also, differing levels of interactions seen could be due to differing levels of IFI16 or the protein of interest, for both need to be present in high concentration to see a high level of interaction. If a protein is visible both in the WCL IB and the IP, then we can conclude that the protein interacts with IFI16 and comes out of solution with it in the IP. Therefore, this immunoblot suggests that IFI16 and A3A physically interact (Figure 5). This supports our hypothesis in that when cells are challenged with exogenous DNA, the DNA PRR IFI16 increasingly interacts with known chromatin silencer A3A, suggesting that IFI16 bridges the gap between the recognition of viral infection and the effector function of repressing chromatin associated with viral DNA.

The previous experiment suggested that IFI16 interacts with A3A, however both proteins have been known to associate with DNA, thus both proteins could have pulled down with the same piece of DNA instead of pulling down because they interacted with each other. In order to explore this idea and further probe IFI16's interactions, we performed another experiment with an IFI16 IP and subsequent IB for proteins of interest. However, this time the cells were lysed in the presence of benzonase. Benzonase

is a nuclease, degrading both DNA and RNA while not affecting proteins (Moreno, 1991). This experiment will tell us if IFI16 and A3A do actually interact with each other or if they both just bind DNA.

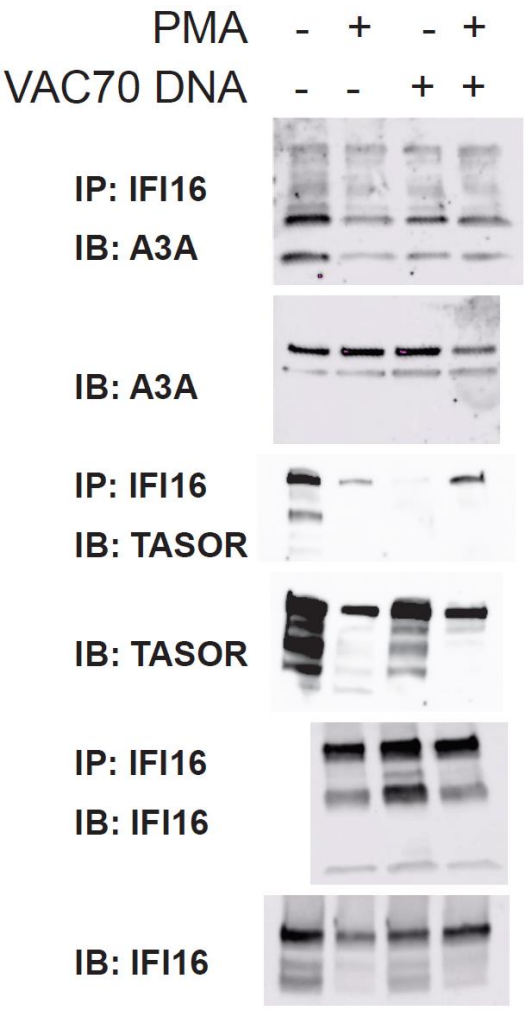

Figure 6: Co-immunoprecipitation and immunoblot with nuclease

THP-1 cells were either stimulated with PMA into macrophage-like cells (PMA +) or not (PMA -) and were either transfected with vaccinia virus DNA (VAC70 DNA +) or not (VAC70 DNA -). These cells were lysed in the presence of the nuclease benzonase and the whole cell lysates and IFI16 co-immunoprecipitation (IP) were immunoblotted (IB) for APOBEC3A (A3A), TASOR or IFI16.

This immunoblot shows evidence of IFI16-A3A interaction in the absence of nucleic acid. These samples were not normalized for protein concentrations and thus should not be analyzed for quantity/band darkness but simply for the presence of the protein in the sample. In this experiment, VAC70 DNA was used as the exogenous DNA stimulant. Due to an experimental error, the unstimulated IFI16 IP and IB sample was lost; however, this sample was just verifying that the IFI16 IP did pull down IFI16, so its absence does not nullify the experiment.

This experiment verifies our previous finding that IFI16 pulls down with A3A in all of our treatment conditions (Figure 6). This suggests that IFI16 directly interacts with A3A and not through a DNA intermediate. This supports our hypothesis that IFI16 could activate A3A in response to viral infection and strengthens the theory that this interaction occurs as part of the same signaling pathway.

IFI16 Interactions with TASOR

Our first IP experiment did not show any evidence of IFI16-TASOR interaction (Figure 5). However, when we repeated this experiment with a nuclease that degrades all nucleic acid, we saw evidence of IFI16-TASOR interaction (Figure 6). This suggest that IFI16 is not able to bind to TASOR in the presence of nucleic acid but is free to interact when nucleic acid is absent. This might suggest that either binding to nucleic acid causes IFI16 to change in confirmation in some way that excludes TASOR binding, or that TASOR binds to IFI16 at a site that would preferentially bind nucleic acid. Neither of these possibilities disproves our hypothesis and the fact that IFI16 and TASOR are able

to interact in some condition helps to support our hypothesis that IFI16 and TASOR belong in the same pathway.

Discussion

The aim of this study was to explore the novel roles of IFI16 in chromatin silencing pathways. IFI16 is a known viral sensor in the innate immune system. This PRR has been shown to lead to antiviral responses, most famously the production of the antiviral cytokine IFN-β. However, previous studies have shown that IFI16 is also necessary for the silencing of integrated retroviral and MGE DNA. Therefore, we hypothesized that this pathway involves IFI16 acting as a sensor for other antiviral responses, such as the chromatin-silencing dependent silencing of HIV by proteins A3A and TASOR, of the HUSH complex. In order to explore this hypothesis, we performed IP and western blotting experiments in order to identify physical interactions between IFI16 and these proteins of interest. However, because IFI16 and the proteins of interest have been shown to independently bind DNA, we had to repeat these experiments with a nuclease. This allowed us to specifically investigate direct protein-protein interactions and not indirect protein-DNA-protein interactions.

Understanding the repression of retroviral elements is underscored by one of the most important emerging infectious diseases crises, the AIDS crisis. This crisis is exacerbated by the inherent difficulty in treating HIV infections. The retrovirus integrates into human cells, making them very hard to identify and attack. This hidden nature of the virus coupled with the fact that it tends to be tropic to CD4+ T cells, which are major players in the immune system, to make normal immune responses against the infection wholly ineffective at clearing the infection. Therefore, we must apply creative strategies towards treatment of these patients.

One very important idea of treatment revolves around attacking the latent reservoir. This reservoir is made of cells in which the integrated provirus has stopped actively producing virus. These cells are the true hidden infections, as a cell with viral DNA inside its nucleus looks exactly like a normal cell from the outside. However, these cells can reactivate and begin production of viral particles. This means that we will not be able to stop an HIV infection unless we can either clear this latent reservoir (by reactivating them all, making them possible targets of the immune system to be killed) or by stopping them from ever reactivating. In order to accomplish either of these goals, we need to better understand how these cells induce and maintain the latent infection of HIV provirus.

Understanding the pathway leading to repression of retroelements is not only important to understanding the disease states of HIV infection, but that of MGEs. These elements do not encode for viral particles that are in danger of spreading infection but have the unique property of being able to excise from the genome and reintegrate at a different site upon reactivation. This movement can cause the genetic sequence to become inserted into an important gene, causing a mutated and likely nonfunctional protein product. Therefore, this is another case in which improper repression of genetic sequences can lead to disease. Perhaps, if we better understood the signaling which results in the repression of pathogenic DNA, we can help maintain that and prevent both HIV latency reversal and MGE activation.

IFI16 Interacts with APOBEC3A in a Nuclease Independent Manner

Our data suggest that APOBEC3A interacts with the viral DNA PRR IFI16 both in the presence and absence of nuclease. We showed this through two separate IP and western blotting experiments (Figure 5, Figure 6). This indicates that IFI16 and A3A interact with each other and not just with the same DNA. This suggests that IFI16 and A3A might interact as two pieces of the same signaling pathway. IFI16 has previously been shown to sense viral DNA and lead to the repression of HIV and A3A has been shown to be important to the repression of HIV as well. However, no one has shown that the two are directly related and that they do not act in two separate pathways. Therefore, our data help to suggest that the action of repressing integrated HIV is accomplished via a pathway involving IFI16 as a viral sensor and A3A as an initiator of chromatin repression.

Our data also show interaction between IFI16 and A3A in both infected and uninfected cells. This suggests that the physical interaction is not specific to the sensing of viral DNA. However, it is possible and likely that this interaction can activate or change A3A in some way only when IFI16 has sensed viral DNA, causing it to localize to the nucleus and induce chromatin remodeling in response to viral infection and not in response to normal cell functioning.

IFI16 Interacts with TASOR in the Absence of Nucleic Acid

Our data suggest a more complex relationship between IFI16 and TASOR. In an experiment without nuclease, no interaction between the two was seen (Figure 5).

However, when the same experiment was done in the presence of a nuclease, an interaction revealed itself. These data suggest something very peculiar: IFI16 and TASOR cannot interact with each other when DNA is present. The full significance of this is not yet apparent, but it warrants more work be done on the topic.

Our data also show that this interaction is possible in varying strengths across the stimulation conditions, including when stimulated and unstimulated by viral DNA. This suggests something similar to what was suggested with A3A. Although it is possible that IFI16 only interacts with HUSH in the nucleus, a location that IFI16 might localize to only after the sensing of viral DNA, our experiments involved the lysing of all cellular membranes, mixing all cytosolic and nuclear proteins synthetically. The variance in the strength of interaction might be explained by the varying amounts of IFI16 and TASOR produced in the cells under each condition, for both high levels of IFI16 and TASOR are needed in order to have high levels of IFI16-TASOR interactions.

Although there is certainly more to discover on this topic, our data suggest that, under complicated conditions, IFI16 is able to interact with a member of the HUSH complex. This furthers our hypothesis that IFI16 may be an initiator of chromatin silencing pathways, whether it be using A3A, HUSH, or a combination of both.

Future Directions and Relevance

Our experiments suggested that the interaction between IFI16 and TASOR is inhibited by the presence of nucleic acid. Because our experiment sent out to explore the interaction of these proteins in response to DNA, this relationship should be explored and

understood more deeply. A good experiment to further this direction would be to repeat the IPs that we performed with the mutant IFI16 proteins used by Hotter et al. (2019), which are missing different portions of the protein. This would help to elucidate the actual site of binding to TASOR and might help to understand whether the nucleic acid dependent inhibition of IFI16-TASOR binding is due to nucleic acid outcompeting TASOR for IFI16 or for some other reason. We also showed that A3A interacts with IFI16 with and without nuclease, suggesting that they bind outside of their shared binding of DNA, suggesting that they might exist within the same signaling pathway. The next step in this research would be to show that this binding, and the binding with TASOR, leads to a change in function for A3A and HUSH, similar to that done by Johnstone et al. (2000). This would likely be done by looking for increased methylation among integrated MGEs or HIV LTR when cells overexpress IFI16 with and without A3A and TASOR, similar to the experiments performed by Tchasovnikarova et al. (2015). Data from these follow up experiments would help to prove that IFI16 activates A3A and HUSH.

Many pieces of literature suggest that IFI16 can act as a multipurpose antiviral receptor. IFI16 sensing viral DNA and then, due to some set of factors that we are currently unaware of, initiating one of many signaling pathways that leads to some antiviral response is not an unreasonable model. If we could better understand the way that this model works, then we could investigate the modulation of IFI16 signaling as a targeted antiviral therapy.

Specifically, if a direct connection can be made between IFI16 and modulated methylation of integrated viral DNA, then IFI16 and its interactions will be very

important targets for future ART development. If the remodeling activity can be blocked in such a way that A3A or HUSH cannot carry out their function, possibly by interfering with IFI16's interactions and activation, it would be as if the cells did not have A3A or HUSH, which has been shown to reverse HIV latency (Taura et al. 2018, Tchasovnikarova et al. 2015). If all infected cells can be reversed out of latency, then it is possible to destroy all of the infected cells and help treat patients who are suffering from HIV infection.

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