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It's A Hard NACHT Life: Understanding How NLRP12 Ticks

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IT'S A HARD NACHT LIFE: UNDERSTANDING HOW NLRP12 TICKS

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillments

Of the Requirements for the Degree

Master of Science, Biology

By

Abbigale Julia Brown

December 2019

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IT'S A HARD NACHT LIFE: UNDERSTANDING HOW NRLP12 TICKS

Biology

Missouri State University, December 2019

Master of Science

Abbigale Julia Brown

ABSTRACT

The protein NOD- like receptor pyrin domain containing 12 (NLRP12) comes from a family of protein receptors with a wide range of functions including fertility as well as anti-inflammatory properties. The biological role of NLRP12 is poorly understood: research on the mechanisms behind its function and/or activation remains contradictory between different cell models. Current research suggests its involvement in a multi-protein complex named the inflammasome. The alternative hypothesis that has also been proposed is that NLRP12 is not a part of the inflammasome, rather it negatively regulates a transcription factor known as NF- κ B down stream of Toll-like receptors. NLRP12 is important because if it is mutated, research has found it can cause severe inflammation in the colon (colitis) and even lead to certain types of colorectal cancer. Other experiments have shown that NLRP12 is also responsible for causing a wide range of autoimmune diseases in humans. Thus, if we can control NLRP12, we might be able to provide better treatments for these debilitating diseases. I propose that by performing a yeast-2-hybrid screen, that I can find new proteins that can unveil NLRP12's function(s). My research has identified three ubiquitin associated proteins that interact with NLRP12; including: CUL3, RNF2, and COPS5 proteins. The functions of all three of these proteins lead me to believe NLRP12 may participate in a ubiquitin pathway to regulate inflammation. In addition, I found NLRP12 also interacted with IFIT1, which is known to interfere with virus infection and is also associated with ubiquitination. This new information could help provide details to unveiling how NLRP12 functions in relation to different proteins it interacts with in vivo.

KEYWORDS: NOD-like receptors, yeast-2-hybrid, inflammation, colitis, anti-inflammatory, ubiquitin, NF- κ B, autoimmune disease.

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

ACKNOWLEDGEMENTS

A wonderful project such as this one that I have just completed did not happen on its own. First, I would like to thank my wonderful PI and mentor Dr. Christopher Lupfer for his insight, knowledge, and support that was the driving force behind this project. I appreciate all you have done to get this project where it is, in addition to helping me grow as a scientist along the way.

Second, I would like to thank Missouri State Graduate College and the Biology Department and its dedicated faculty and staff that helped to fund this project and make it possible to get where it is now. I will miss all the smiles found within the halls of Temple Hall.

Third, I would like to thank my graduate thesis committee members Dr. Kyoungtae Kim and Dr. Laszlo Kovacs who have gone above and beyond in their constant encouragement and support over my four years spent here.

Lastly, I would like to thank my loving husband Mason Brown, and my wonderful dog Nova for their patience, endless love, and support on both the good days and the bad ones. My favorite part of my day is coming home to them. I love you both very much.

I would like to dedicate this thesis to my husband who has allowed and encouraged me to grow as not only a wife, but also as a scientist. His constant encouragement has helped me to strive to find humility, kindness, and integrity in myself and encourage those around me to do the same. Without him I would not be here.

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INTRODUCTION

According to a study done by the National Institutes of Health, nearly twenty percent of the entire U.S. population have some type of inflammatory disease (Pahwa, R. and Jialal, 2018). Crohn's disease and inflammatory bowel syndrome (IBS) are included in this list and are responsible for causing chronic pain and discomfort. Recent research has found that chronic colon inflammation is now one of the leading causes of colorectal cancer (Wang, ZH. and Fang, JY. 2014). In other words, inflammatory diseases such as Crohn's and IBS might be the least of our worries, as they contribute to the development of life-threatening cancer. Research has tried to find new ways for the immune system to alleviate chronic inflammation naturally, instead of the use of steroids or other drugs; giving new hope to patients in its wake.

It is no secret that cancer is on the rise. According to the Center for Disease Control, cancer is the second leading cause of death behind heart disease in the United States. Advancing technology and interminable research has helped us to come a long way battling the numerous types of cancer and its victims in which it ensnares.

The Immune System

Overview. The immune system is comprised of both the innate and adaptive immune response systems that are independent parts that work together in a synergistic manner for a common goal: protect the host from succumbing to infection. It does this through recognition via cellular receptors known as pattern recognition receptors (PRRs). This super-family of receptors are located both intracellularly and extracellularly, which aids in a wide range of pathogen recognition. These receptors also help to detect non-self-antigens that can be categorized as

damage associated molecular patterns (DAMPs), which could include damage signals such as sudden ion influx that indicate cellular structural damage. Antigens could also register as pathogen associated molecular patterns (PAMPs), which are pathogen components such as lipids, proteins, and nucleic acids. Both DAMPs and PAMPs reiterate that detection of non-self-antigens is crucial to stimulate the correct response and prevent unnecessary damage to the host (Jacobs, SR. and Damania, B. 2012).

Pattern Recognition Receptors. The innate immune system is comprised of several germline-encoded recognition proteins. These proteins are combined into one super-family called pattern recognition receptors (PRRs). PRRs are expressed in several cell types and there are several families encompassed within the super-family. They are expressed in several innate immune cells including monocytes, macrophages, dendritic cells, endothelial cells, and neutrophils. Some PRRs have also been found in several adaptive immune cells as well. (Tuncer, S. et al. 2014). Family members include transmembrane proteins called Toll-like receptors (TLRs), retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and cytoplasmic receptors known as NOD-like receptors (NLRs) (Jacobs, SR. and Damania, B. 2012). These receptors are responsible for detecting both PAMPs and DAMPS. Altogether, these proteins are the scaffold to how the innate immune system functions (Lupfer, CR. and Kanneganti, KD. 2011). If one of these mechanisms malfunctions (i.e. activated excessively) the whole system breaks down, potentially leading to inflammatory diseases. For example, dysregulation of nucleotide-binding oligomerization domain proteins 1 and 2 (NOD1 and NOD2) leads to the development of Crohn's disease (CD) (Caruso, R. et al. 2014). It has been studied that Inflammatory Bowel Syndrome (IBS) and CD have been linked to overzealous innate signaling of the Nuclear Factor

kappa-light-chain of activated B cells (NF- κ B) and Mitogen associated protein kinase/extracellular signal related kinase (MAPK/ERK) pathways (Caruso, R. et al. 2014).

NOD-like Receptors (NLRs). Mammalian NLRs share structural homology with plant innate receptors known as R proteins. These proteins help plants detect and respond to infection (Tuncer, et al. 2014). These R proteins and NLRs have a homologous three leaflet structure that includes an N-terminal effector domain that mediates protein-protein interactions necessary for function, a central nucleotide binding domain (NBD) that contains a Walker A and B motif which are highly conserved regions necessary for ATP hydrolysis, and a C-terminus leucine rich repeating (LRR) domain that is thought to be necessary for ligand binding and autoregulation (Ye, Z. et al. 2007). Research has found that there are 22 known NLR proteins and, since their discovery in 2005, nearly half remain to be characterized. These 22 proteins are further divided into four subfamilies based on their effector domains: NLRA, NLRB, NLRP which have Pyrin domains, and NLRC which has a CARD effector domain. NLRP proteins, specifically NLRP12, is what I am most interested in. The NLR proteins can also be categorized based on their function including: inflammasome assembly, signal transduction, transcription activation, and autophagy (Tuncer, S. et al. 2014; Kim, Y. et al. 2016). Inflammasomes are multi-protein complexes that form upon stimulation with certain DAMPs. The protein complex consists of the NLR protein associating with apoptosis associated speck protein (ASC) via either CARD-CARD or PYRIN-PYRIN domain oligomerization, Caspase-1, which upon association recruits inactive forms of cytokine proteins including pro-IL-1 β or pro-IL-18 which are activated via inflammasome formation. The release of these pro-inflammatory cytokines stimulates a robust immune response based upon the source of the stimuli (Chen, Y. 2014). Some NLRs, NOD1, NOD2, NLRP6, and NLRP12, have been found to activate or suppress the NF- κ B inflammatory

signaling pathway via signal transduction. Autophagy, which is the recycling of the cellular components via autodigestion, has not been shown to include very many NLR proteins in its pathway. However, NOD1 and NOD2 are among the only two NLR proteins to be associated with the autophagy pathway to date. It has been shown that NOD1 and NOD2 can induce the autophagy pathway via association with autophagy-related gene (ATG) protein ATG16LI (Tuncer, S. et al. 2014; Kim et al. 2016). ATG16LI associates with ATG12 and ATG5 to form the ATG12 conjugation system that is required for autophagosome formation (Naser, SA. et al. 2012). Lastly, a fourth category, not previously mentioned, associates NLR proteins having inhibitory effects on inflammatory signaling pathways. One specific NLR associated with this function is NLRP12 (Lupfer, CR. and Kanneganti, KD. 2013). How NLRP12 performs this function (amongst others) is what I am most interested in.

NLRP12 and Its Many Functions

Overview of Structure and Function(s). NLR Family Pyrin Domain Containing 12 (NLRP12) is a protein that has been studied extensively for nearly two decades. However, the way that NLRP12 functions is still under scrutiny. It is believed that NLRP12 promotes inflammatory signaling in neutrophils upon differentiation via the MAPK/ERK signaling pathway (Wang Q. et al. 2018). On the other hand, it has also been seen that NLRP12 deficient mice develop large cancerous growths within their colorectal epithelium. It was concluded that NLRP12 could be responsible for modulating certain cytokines which have been found to promote cancerous growths; allowing them to spiral out of control (Zaki, MH. et al. 2011).

In addition, recent research depicts that NLRP12 does not suppress inflammation, but it exacerbates it by forming a common multi-protein complex called an inflammasome (Vladimer,

GI. et al. 2012). Regardless of the study, it appears that NLRP12 has been found to serve several different functions throughout different types of immune cells. However, it has not been studied why these discrepancies in data exist. Therefore, I believe that future research should address the possibility that NLRP12's function varies based upon the splice form it has originated from upon its formation. NLRP12's full-length cDNA open-reading frame encodes 10 exons that can be spliced into at least four different variations. The first isoform is NLRP12 with all 10 exons (full-length), the second isoform lacks exon 9, the third lacks exons 7 and 8, and the last isoform lacks exons 7 through 9. Interestingly, the only differences between each of the isoforms lies within differential splicing of the LRR domain (Williams, KL. et al. 2003; Lich, JD. et al. 2007). Thus, I conclude that each of these isoforms must bind to a different ligand that determines how NLRP12 will function. I believe more research should compare these isoforms with the same in vitro and in vivo experiments that have been completed so far. In addition, more research should look to see if there are more than four isoforms and if these isoforms can be modified epigenetically based upon which cell NLRP12 is being expressed.

As previously mentioned, NLRP12 has been found to have several different functions. The question remains if NLRP12 performs all these functions at once, or how it is spliced prior to translation into a functional protein determines its fate. NLRP12 has three distinct domains it shares with its family members: Pyrin, NACHT, and Leucine Rich Repeat (LRR). Its NACHT domain allows the activation of the binding complex through ATP-dependent oligomerization. The Pyrin domain, which is found at the N-terminus, mediates homotypic protein-protein interactions for downstream signaling. Lastly, the LRR domain is found on the C-terminus and functions in ligand sensing and autoregulation (Tuncer, S. et al. 2014). These domains work together to allow NLRP12 to be functional. Although research has identified the different

domains of NLRP12, not much has been done to determine the mechanisms that allow proteins to interact with NLRP12 upon its activation. More research still needs to be done to see which domain(s) may be involved within these different interactions.

Inflammasome Formation or Anti-Inflammatory Regulator. Inflammasome formation concurs via associations between several NLRs (NLRP1, NLRP3, NLRP6, NLRC4, and NLRP12) (Zhong, Y. et al. 2013) via their CARD or PYRIN domain via the CARD or PYRIN domain of apoptosis associated speck protein (ASC) that acts as an adaptor protein to connect the NLR protein with pro-caspase-1 protein. Upon these associations, the pro form of IL-1 β is then cleaved into its active form and released into circulation (Chen, GY. 2014). Recent research has focused around the conundrum of whether NLRP12 takes part in the formation of an inflammasome complex. NLRP12 was one of the first NLR proteins to be believed to form an inflammasome (Wang, L. et al. 2002). In 2016, Cai, S. et al conducted similar experiments with human macrophages, neutrophils, and dendritic cells. They observed a decrease in pro-inflammatory cytokines (IL-18 and IL-1 β). These results suggest NLRP12 inflammasome activation (Cai, S. et al. 2016). Whether or not NLRP12 was acting alone in the cytokine production or other inflammasome were contributing to the cytokine storm was not evaluated. In vivo results have shown NLRP12 deficient mice succumb to *Yersinia pestis* infection and exhibited lower amounts of inflammasome associated pro-inflammatory cytokines IL-18 and IL-1 β . These results indicated that upon infection of *Y. pestis* NLRP12 serves a pro-inflammatory role to reduce the bacterial load and provide bacterial resistance (Vladimer, GI. et al. 2012). A year later another article was published claiming the opposite was true. Allen, I. et al, claimed that upon stimulation with *Klebsiella pneumoniae* or *Mycobacterium tuberculosis*, mice showed no significant difference in the release of IL-1 β in NLRP12 deficient mice compared to wild

type mice for both their in vitro and in vivo experiments (Allen, IC. et al. 2013). They claimed that NLRP12 does not play a role in inflammasome formation in response to *K. pneumoniae* and *M. tuberculosis*. These results are supported by recent findings in NLRP12 deficient mice with *Brucella abortus* and *Salmonella typhimurium* infection which showed NLRP12 deficient mice were found to have increased bacterial resistance. In this model, it appears NLRP12 served as a negative regulator of pro-inflammatory signaling via NF- κ B/MAPK/ERK signaling pathways (Silveira, TN et al. 2017; Zaki, MH. et al. 2014). Nevertheless, research is still grasping for straws trying to pin down the exact mechanism NLRP12 mediates during pathogenic infections. One problem in comparing these studies is that different background strains of mice and different bacteria were used, which may play a role in the differences observed. However, it may take some time to be able to examine all possibilities of functionality in certain infections.

Immune Cell Migration. A newer function of NLRP12 that has been introduced is that NLRP12 is serving a role in the recruitment of innate cells during the early stages of infection. Several studies have been conducted and, in response to *K. pneumoniae* infection, NLRP12 deficient mice showed a decrease in both dendritic and neutrophil recruitment chemokines (CXCL1, CCR7, and CXCR4 ligands) (Cai, S. et al. 2016). Interestingly, similar findings were found in NLRP12 deficient mice in response to influenza A virus infection. However, the outcome was beneficial to the deficient mice. The NLRP12 deficient mice survived lethal flu infection because of the lack of neutrophil recruitment via CXCL1 secretions (Hornick, E. et al. 2018). I believe the difference in outcomes must stress the importance of NLRP12 as a modulator (whether it be pro or anti-inflammatory) when it comes to pathogenic infections. NLRP12 must play a role in controlling how potent or mild an immune response must be based on the type of infection and location of that infection.

NLRP12 Diseases and Altered Pathologies

Familial Cold Autoinflammatory Syndrome Type 2 (FCAS2). NLRP12 (PYPAF-7/NALP12/Monarch-1) was one of the first NLRs to be discovered. However, currently it is one of the most dynamic NLRs when it comes to how it functions in vivo. Specifically, it has been found that certain missense mutations in the NLRP12 gene also causes a disease called Familial Cold Autoinflammatory Syndrome Type 2 (FCAS2) and certain skin diseases; such as atopic dermatitis (Zamoshnikova, A. et al. 2016; Jeru et al. 2008; Lukens et al. 2015). The characteristics of FCAS2 include recurrent fever, rash, and/or joint pain when exposed to cold temperatures (Xia et al. 2016). The cause of such diseases is a result in a malfunction in modulation of inflammation, specifically NF- κ B signaling pathway within myeloid cells as well as T cells (Thaiss, CA. and Elinav, E. 2015). The result is an overzealous and recurrent immune response. Treatment with anti-IL-1, which has shown to work for NLRP3 related diseases, have had limited success in FCAS2. Inquiry shows that patients treated with IL-1 inhibitor therapies return within less than 18 months resistant to the drug administered. (Zhong, Y. et al. 2013). Jeru et al. proposes that NLRP12 inflammatory disease must be caused by overreaction of the NLRP12 inflammasome (increased CASP-1 production) as well as increased migration of innate immune cells (Jeru, I et al. 2011). However, atopic dermatitis and FCAS2 are not the only autoimmune diseases that are found to be associated with dysfunctional NLRP12 protein expression.

Dysfunctional Regulatory T Cell Diseases. Recent research has veered the focus of NLRP12 inflammatory disease from experiments in innate immune cells to adaptive immune cells in vivo. Gurung, P. and Kanneganti, KD. mentioned that the cause of these diseases comes from dysregulation in T cells, but most research for these diseases have been done in innate

immune cells. Preliminary data showed an overexpression of NLRP12 in T cells compared to that of other innate immune cells (ie. DCs and macrophages). In addition, adoptive transfer experiments showed NLRP12 deficient T cells given to mice resulted in disease pathologies which resembled that of atopic dermatitis and colitis; never shown in mice experiments to date. More importantly, Gurung, P. and Kanneganti, KD. believe IL-4 blocking treatments would be more beneficial to these patients because mice experiments showed increased Th2 cytokine production which resulted in similar disease pathology as mentioned above. Furthermore, experiments with NLRP12 deficient mice showed increased EAE and, upon anti- IL-4 treatment, showed remarkable improvement proving the overproduction of such cytokines can cause other autoimmune diseases as well (Gurung, P. and Kanneganti, KD. 2015; Lukens, JR. et al. 2016; Thaïss, CA. and Elinav, E. 2015).

Cancer Tumorigenesis. One of the more commonly known diseases associated with NLRP12 deficiencies is it has been shown to cause colorectal inflammation. Both Zaki, MH. et al. and Allen, I. et al. have conducted NLRP12 deficient mice models that display its role in modulating colon inflammation. However, their mechanisms show that the key factor in modulation of cancer tumorigenesis lies within NF- κ B signaling regulation. First, Zaki, MH. et al. described NLRP12 deficient mice had increased canonical NF- κ B signaling (Figure 1) (IL-6, I κ B α , TNF α , STAT3, and ERK), which they believed to contribute to the progression of colon tumorigenesis. They believe this mouse model demonstrates a mechanism of irregular response between the host immune system and microflora in the gut. In other words, the immune system is initiating a hypersensitive response that is unnecessary and causing hyperinflammation in the gut. Research has shown that constant inflammation is a major contributor to cancer tumorigenesis (Zaki, MH. et al. 2011). Similarly, Allen, I. et al showed that NLRP12 deficient

mice were highly susceptible to colitis and tumor production, but the mechanism was different. Their data showed an increase in non-canonical NF- κ B signaling (Figure 2) via cancer inducing genes of CXCL12 and CXCL12. In 2014, Allen, I et al. characterized that the non-canonical NF- κ B pathway is regulated by the degradation of TRAF3 that leads to the activation of NIK and NLRP12 mediation of this pathway is crucial in prevention of disease progression (Allen, I. et al. 2014).

NLRP12 Known Protein-Protein Interactions

Interleukin-1 Receptor Associated Kinase 1 (IRAK1). IRAK1 is a well-known serine/threonine kinase protein that initiates downstream NF- κ B signaling via TLR and IL-1R pathways. In preliminary data, Dr. Christopher Lupfer displayed NLRP12 associates with Fas-associated protein 1 (FAF-1) and with IRAK1. Additionally, recent research has shown that NLRP12 expression is reduced upon both TLR signaling and Tumor-necrosis factor (TNF) signaling pathways. Interestingly, NLRP12 interferes with both TLR 2 and 4 activation via its association with IRAK1 (Figure 1). It was found that this association prevented IRAK1 from hyperphosphorylation that is required for associating with TRAF6 and thereby inhibiting pathway progression (Williams, KL. et al. 2015) and leading to IRAK1 degradation through the 26S proteasome (Lich, JD. et al. 2007). This mechanism is a great example that displays how NLRP12 modulates the canonical NF- κ B signaling pathway. However, the exact mechanisms behind how NLRP12 associates with IRAK-1 is still to be determined. Recent research has also investigated the possibility of NLRP12 inhibiting canonical signaling downstream of IRAK-1 with a similar mechanism. It was found that NLRP12 regulates the non-canonical signaling pathway through NIK degradation (Lich, JD. et al. 2007).

NIK and TRAF3 in Non-Canonical NF- κ B Signaling. NF- κ B inducing kinase (NIK) is a well-known protein kinase that is unique in its function within the N-F κ B pathway. Unlike canonical signaling, which has many upstream effectors that can stimulate downstream signaling and activation, non-canonical signaling only has one known regulatory kinase (NIK) (Lich, JD et al. 2007; Chen, Y. 2014). How NLRP12 mediates non-canonical signaling via NIK is through its proteasomal degradation (Arthur, JC. et al. 2010) upon interaction with NLRP12's NOD and LRR domains (Tuncer, S. et al. 2014). After NIK becomes activated, it recruits IKK α and p100, which leads to proteasomal modification of the NF- κ B subunit into p52, which translocates to the nucleus to induce transcription of certain chemokines including CXCL12/13 and CCL5 (Lich, JD. et al. 2007). Importantly, these chemokines are known to be a key factor in tumorigenesis (Allen, I. et al. 2012). NLRP12 has concurrently been shown to interact with TNF receptor associated factor 3 (TRAF3), which is also linked with the non-canonical NF- κ B signaling pathway. It has been shown that NLRP12 associates with TRAF3 via the TRAF2-TRAF3-CIAP1-CIAP2 complex, which is known to be responsible for constant NIK ubiquitination and degradation that is required for its modulation to keep its NF- κ B processing of p100 at basal levels and serve as a negative feedback loop of NIK activation levels (Razani, B. et al. 2011; Sun, SC. 2011).

Other Known Protein Interactions with NLRP12. Since NLRP12's discovery nearly twenty years ago, research has implicated numerous proteins that mediate its function. In addition to the three already discussed, there are several more that are worth mentioning. One of the first known proteins to associate with NLRP12 was B lymphocyte-induced maturation protein-1 (Blimp-1). This zinc-finger protein has come to be known as the master regulator of the regulator via histones 3 acetylation (Lord, C. et al. 2009; Shi, F. et al. 2016). NLRP12 not only

has its own modulator, it also has its own chaperone complex of Hsp70/90 that prevents its degradation while it tags other proteins for proteasomal degradation (Arthur, JC et al. 2007). All of which suggest several different functional applications of NLRP12 and its many isoforms that could be differentially expressed in several immune cells. One of the most compelling interactions that could lead to several breakthroughs in NLRP12 activation is its association with NOD1 and NOD2. These NLR family proteins are mostly known for immunity to different bacterial antigens. However, it has been seen that this complex formulation can be stimulated via viral antigens as well. NOD1 and NOD2 can interact with RIPK2 via their CARD domains and recruit several E3 ubiquitin ligases (TRAFs, CIAP1/2, ITCH, and XIAP) which leads to K63 ubiquitination of RIPK2 which leads to downstream AP-1 and NF- κ B transcription factor activation (Zhong, Y. et al. 2013). However, NLRP12 has been known to associate with NOD 1 and 2 via a linker protein (FAF-1). The function of this relationship remains undetermined (Wagner, RN. 2009). Lastly, NLRP12 has been known to associate with TRIM25 (another E3 ubiquitin ligase) after viral infection through the RIG-I pathway (Chen, ST. 2019). Other NLRs are known to associate to NOD1 and 2 via the autophagy protein ATG16L via which has been shown to be critical in autophagosome formation via the NODosome formation so the question remains if NLRP12 could as well (Coutermarsh-Ott, S. et al. 2016).

NF- κ B Inflammatory Signaling Pathway

The nuclear factor kappa B (NF- κ B) was first discovered by Ranjan Sen in 1986. NF- κ B is a family of proteins that include: RelA, RelB, c-Rel, p50/p105, and p52/p100. All of which, in their inactive form, bind to the protein I κ B via their Rel homology domain (RBD) (Ghosh, S.

and Hayden, M. 2018). The NF- κ B signaling pathway can be divided into two categories: classical (canonical) and alternative (non-canonical) pathways.

Canonical Signaling Pathway. Extensive research has been done on both the classical and alternative NF- κ B signaling pathways. Lich, J. et al. describes that classical pathway activation occurs very rapidly following ligand binding of several cell membrane receptors, including TLRs, which subsequently promotes NF- κ B activation. Brown, K. et al. stated that upon the activation of IKKs ($\alpha/\beta/\lambda$) the IKKs form a complex with NF- κ B essential modulator (NEMO) and become capable of phosphorylating I κ B (Inhibitor of κ B) which induces the release of several NF- κ B subunits to translocate to the nucleus to initiate transcription of necessary cytokines and other pro-inflammatory genes (Brown, K. et al. 2008). NF κ B also activates gene expression of NF- κ B genes such as p100 and I κ B α (Lich, J. 2007). If this were not complicated enough, there is an entirely different pathway that includes hundreds more proteins that research still is unveiling (Figure 1).

Non-Canonical Signaling Pathway. More research has focused on the non-canonical pathway in recent years. However, it is still considered the least understood NF- κ B signaling pathway. Non-canonical signaling has been found to be important for B and T cell survival, development, and lymphoid organogenesis (Brown, K. et al. 2008). The non-canonical pathway focuses mainly on the further processing of p100 instead of I κ B α degradation (Figure 2). In addition, the NF- κ B-inducing kinase (NIK), with the help of I κ B α , work to induce proteolytic processing of p100 into p52. It has been shown that NIK is under constitutive degradation itself under normal conditions by TNF receptor associated factor-3 (TRAF3) (Sun, SC. 2013). Once NIK becomes stabilized, the non-canonical NF- κ B pathway can continue.

Modulating the Pathway.

As complicated as the NF- κ B pathways are, it is critical that they are kept in check. There are several post-translational methods that have been found to modulate these signaling pathways. In layman terms: these modulators act as sequenced traffic lights to regulate signals and prevent excessive inflammation. Post-translational modifications involved in the NF- κ B signaling pathway include ubiquitination, de-ubiquitination, SUMOylation and many more. I will only discuss ubiquitination because it is directly relevant to my research.

Ubiquitination. Briefly, ubiquitination is the covalent attachment of a protein (ubiquitin) to a substrate protein. Upon attachment of this small protein (8.5 kDA), several outcomes may occur. These may include control of cell cycle division, differentiation, DNA repair or its most pivotal role: protein stability. Ubiquitination is ubiquitous throughout the cell and evolutionary history. Ubiquitin is attached to a protein of interest via a series of activation (E1 enzyme), conjugation (E2 enzyme), and ligation (E3 enzyme) proteins. It acts as a cell's bucket brigade as the small ubiquitin protein is passed along the three enzymes ending with ubiquitin being attached to the protein of interest (Swatek, K. and Komander, D. 2016). One common purpose for ubiquitination/poly-ubiquitination of a protein is because the protein may become destined for degradation via the proteasome (ie. The woodchipper of the cell). This process is called the Ubiquitin-proteasome system (UPS) and its main function is to regulate protein abundance and turnover. The mechanism behind such a task lies within ubiquitin's seven lysine residues (Lys6, 11, 27, 29, 48, and 63). For the sake of simplicity I will only be discussing Lys48 (K48) and Lys63 (K63) ubiquitination mechanisms and their downstream relevance (Collins, P. et al. 2016). Upon poly-ubiquitination, the ubiquitin tags are stacked one on top of the other in a sequential fashion. The purpose for this mechanism is modulation of how active a protein will

become which is beneficial in signaling pathways that may contribute to forceful immune responses (ie. NF- κ B/MAPK/ERK). Interestingly, K48 poly-ubiquitination serves as a degradation signal in the canonical NF- κ B pathway to the 26S proteasome. One example in this pathway (Figure 1) is upon recognition of the pro-inflammatory cytokine IL-1, via the IL-1 receptor (IL-1R), the protein MyD88 associates with IL-1R and IRAK1/4 which allows TRAF6 (E3 Ligase) to associate and through K48 ubiquitination, TRAF6 tags IRAK1/4 for degradation via the 26S proteasome in order for the NEMO complex to be recruited for the pathway to continue (Courtois. G. and Fauvarque, MO. 2018). K63-poly-ubiquitination was the first lysine residue to be discovered and is known to be associated with modulating protein activity as well as many other functions that range from protein-protein interactions to endocytosis. In other words, K63 acts as a flag or signal to other proteins downstream that give them a “go” signal to either associate or mediate other cellular functions. K63 ubiquitin chains are also said to be favored with de-ubiquitin proteins such as USP48. Ubiquitin-Specific Proteins (USP) are especially important in the NF- κ B signaling pathway. USP48 is known to bind with COP9 signalosome to stabilize NF- κ B subunit p65 by removing K48 ubiquitin chains within the nucleus. Actions such as this help to enhance or dampen pro-inflammatory genes during an infection (Collins, P. et al. 2016).

NLRP12 Involvement. Recent research has shown that NLRP12 is able to alter the signaling within both the canonical and non-canonical NF- κ B signaling pathways (Ting, JP et al. 2010). Lich, J. et al. also reported that there is a possibility that NLRP12 modulates both the classical and alternative pathways. Research has found that NLRP12 induces the degradation of NIK by binding to it, which in turn modulates any further downstream NF- κ B signaling. Additionally, NLRP12's NACHT domain directly destabilizes NIK, but more research needs to

be done to deliberate what affects it has on downstream signaling (Lich, J. et al. 2007). Finally, NLRP12 can affect the ubiquitination and degradation of proteins in the NF- κ B pathway. Chen, SG. et al. believe that NLRP12 regulates RIG-I activation via the interaction with TRIM25 to disrupt K63 ubiquitination and activation of RIG-I (Chen, SG. et al. 2019). However, these findings may be only one of many protein interactions involved to modulate different types of ubiquitination within this pathway.

Type 1 Interferon Signaling

One of the major anti-viral signaling pathways is the Type I IFN signaling pathway (IFN-I). This pathway depends on several transcription factors, mainly NF- κ B and IRF3/7. Signaling of this pathway begins with PRR signaling cascades like TLR7, which is induced by ssRNA or RIG-I that is activated via dsRNA/5'tripohosphate RNA. These stimulate TRAF3 activation and thus formation of the IKK ϵ and TBK1 complex which stimulates transcription for type 1 interferons (IFN- α/β) that are highly potent signaling proteins that propel the immune system into high throttle. Upon viral infection, other stress pathways are also induced (JNK and p38) which promotes IFN- β production. IFN- α/β then bind to their receptor in an autocrine and paracrine manner that results in STAT1/2 pathway activation. Interferon stimulated genes (ISGs), which are important for creating an unfavorable environment for any viral invader, are then produced. (Seth, RB. et al. 2006; Li, K. and Zhong, B. 2018).

One ISG, known as interferon-induced proteins with tetratricopeptide repeats 1 (IFIT1), has been found to be responsible for directly binding to and regulating RNA viral proteins and functional RNAs, preventing viral replication from occurring. IFIT1 is strongly induced by type 1 interferons and type 3 interferons, which activate the transcription factor STAT1/2 whose job

is inducing transcription of IFIT genes (Fensterl, V. and Sen, GC. 2015). Recent research has shown that NLRP12 can directly interact with TRIM25, which in turn disrupts RIG-I activation (Chen, SG. et al. 2019). I believe there is a possibility NLRP12 could also interact with other proteins downstream of RIG-I and other PRRs such as IFIT1 to cease anti-viral signaling when it is no longer necessary.

Scientific Question

Based on previous research, NLRP12 has been known to associate with IRAK1 and other proteins. My questions include: Does NLRP12 associate with other proteins upstream of IRAK1 upon its activation? What proteins associate with NLRP12 to induce its activation? Do a wide variety of proteins associate with NLRP12 or is it just ubiquitin or ubiquitin-like proteins? If NLRP12 is known to destabilize IRAK1, causing it to be degraded by proteasome degradation to prevent further downstream signaling, what is stabilizing NLRP12 to prevent it from being degraded? Under what conditions does NLRP12 need to be downregulated or destabilized? How does NLRP12 control both TCR and TLR signaling pathways of NF κ B? All these questions point to our lack of knowledge regarding the protein-protein interactions of NLRP12.

Scientific Hypothesis

NLRP12 is not a ubiquitin ligase or protease itself, or a kinase or phosphatase. Thus, there must be additional proteins that interact with NLRP12 to induce its activation. Furthermore, there are 4 known isoforms of NLRP12, and I hypothesize that these isoforms are activated by different stimuli and may interact with different proteins following activation. Such differences could account for the different, and sometime contradictory, functions proposed for NLRP12.

MATERIALS AND METHODS

Cloning of NLRP12

Using the cDNA template purchased from Addgene, I conducted a polymerase chain reaction to isolate the NLRP12 gene using gene specific NLRP12 primers (Table 1) and a high-fidelity fusion polymerase (Phusion High-Fidelity, ThermoScientific). I was able to isolate the NLRP12 insert necessary to clone into the pGBKT7 vector (Figure 3 and 4). The PCR conditions are shown in Table 2. The pGBKT7 vector must be prepped for cloning of NLRP12 using specific restriction enzymes that must match both the insert and the plasmid vector (Figure 3 and 4). I used FastDigest enzymes (ThermoScientific) for *NdeI* and *EcoRI* according to the manufactures protocol. After gel purification of the digested PCR product and plasmid (Figure 5), the pieces were ligated via T4 ligase (Promega) according to the manufactures protocol. Then, the ligated product was transformed into the β 10 *E. coli* strain. Transformed *E. coli* was plated on LB plates containing 50 μ g/ml Kanamycin.

Screening *E. coli* for Positive Clones

After the vector was introduced into bacteria, I picked as many colonies as I could to test to see if NLRP12 had been successfully cloned into the vector. I did this by diluting the bacteria into RNAase free water (1:10) and conducting a PCR with primers for the T7 and M13 sequences in the vector plasmid (Table 1). Dream Taq was used as the polymerase (ThermoScientific) according to the manufacture's recommendations. The PCR settings are shown in Table 2. Plasmid from colonies that were positive by PCR (Figure 6) for successful NLRP12 cloning were then purified and a restriction digest was conducted using *NdeI* and *EcoRI*

to confirm the NLRP12 insert was cloned successfully by examining digested plasmids on a 1% agarose gel and looking for the correct ~3200bp size bands (Figure 7). Finally, vectors purified from *E. coli* that showed the correct band visualized by agarose gel were sequenced via Sanger sequencing using T7 and M13 primers for sequencing.

Western Blot

Western blot was used to detect protein expression in yeast cells prior to conducting the 2-hybrid screen. Y2H Gold yeast transformed with the pGBKT7-NLRP12 vector were grown over night in SD-Trp medium and the cells then isolated by centrifugation at 2000 x g for 10 minutes. Yeast cells were combined with a 1:1 ratio of NaOH to sample which mediated lysis of the yeast cells. These samples were then boiled in SDS loading buffer. A standard western blot was conducted using an anti-myc epitope antibody that detected the myc tag in frame with the N-terminal region of NLRP12. Blots were imaged using Clarity Western ECL Substrate (Bio-Rad, A170-5060) and an Azure c300 digital gel imager. All antibodies that were used can be found in Table 3.

Yeast-2-Hybrid Screen

A yeast-2-hybrid screen was performed using the NLRP12-pGBKT7 plasmid introduced into Y2H Gold yeast (Frozen-EZ Yeast Transformation II Kit, Zymogen) and mating these yeast with a human cDNA library in Y187 yeast (Clontech, Normalized HeLa cDNA library) according to the Matchmaker Gold Yeast-2-Hybrid protocol. Positive yeast colonies were grown in SD-Leu-Trp broth and plasmids purified via the EZ yeast plasmid kit (Zymogen). Purified plasmids contained the cDNA of the potential interacting proteins for NLRP12, and the cDNA

was amplified using the insert screening amplimer primers (Table 1) via PCR using DreamTaq polymerase and the PCR conditions in Table 2. The results of the PCR are shown in Figure 8. Amplified PCR products were sequenced by Sanger sequencing and BLASTN used to determine the gene for each positive cDNA clone.

IFIT1 and Cloning of NLRP12 into a Mammalian Expression Vector

The pCDNA3.1 3xFlag IFIT1 plasmid was purchased from Addgene as a bacterial stab. The bacteria were grown overnight in LB broth containing 100µg/ml Ampicillin, and the plasmid was purified. NLRP12 was cloned into the pCDNA3.1 3xMyc plasmid using Gibson cloning (NEB Gibson cloning kit) according to the manufacturer's instructions. Primers used for the PCR amplification of the plasmid and NLRP12 insert are shown in Table 1. The PCR conditions are shown in table 2. After the Gibson cloning reaction, the plasmid was transformed into β 10 *E. coli* and plated on LB plates with 100µg/ml ampicillin. Several colonies were screened by PCR by diluting the bacteria into RNAase free water (1:10) and conducting a PCR with primers for the T7 and BGH reverse sequences in the vector plasmid (Table 1). Dream Taq was used as the polymerase (ThermoScientific) according to the manufacture's recommendations. The PCR settings are shown in Table 2. Finally, vectors purified from *E. coli* that showed the correct band visualized by agarose gel electrophoresis were sent off to be sequenced via Sanger sequencing.

Transfection of Plasmid into HEK 293 T Cells

Human embryonic kidney (HEK) 293 T cells were plated in 12-well tissue culture plates at 300,000 cells per well in 1mL DMEM plus 10% fetal bovine serum (FBS), 100U penicillin,

and 100U streptomycin. The next day, 100 μ L of transfection buffer was added to a 1.5 mL tube for each sample. Next, 2 μ g of plasmid was added to the same 1.5 mL tube for each sample. Lastly, 0.6 μ L of the XFECT polymer (Takara/Clontech) was added to each 1.5 mL tube for each sample and mixed softly by flicking the tubes. The tubes were then left at room temperature to incubate for 10 minutes. 100 μ L of each DNA/XFECT sample was added to the matching culture well dropwise. The plates were then left in a 37°C 5% CO₂ incubator for 24 hours.

Western Blot Verification of Protein Expression in HEK 293 T Cells

Twenty-four hours after transfection, I examined protein expression of NLRP12 and IFIT1 in HEK 293 T cells. The IFIT1 protein has a 3xFlag epitope and NLRP12 protein has a 3xMyc epitope within mammalian expression vectors that were transfected into HEK 293 T cells. All samples were collected by adding RIPA lysis buffer to plated cells in order to lyse them. Next, 4x SDS loading dye was added to the cells. Samples were boiled using a heat block at 95°C. After about 30 minutes, the samples were removed from the heat and electrophoresed on an 8% polyacrylamide gel. The gel was then transferred onto a PVDF (Polyvinylidene difluoride). After the transfer was complete, the membranes were blocked in 5% powdered milk in TBS+0.5% Tween 20 (wash buffer) for approximately 60 minutes. The blocking milk was then removed, and the primary antibody diluted in fresh blocking buffer (1:2000) was added and allowed to incubate while slowly rocked at 4°C overnight. The next day, the primary antibody was removed, and the membranes were washed with 1x TBST (Wash Buffer) three times before the secondary antibody was added (1:5000 dilution in blocking buffer) and incubated for at least 2 hours at room temperature. The membranes were then washed six more times with wash

buffer. Blots were imaged using Clarity Western ECL Substrate (Bio-Rad, A170-5060) and an Azure c300 digital gel imager. All antibodies that were used can be found in Table 3.

Co-Immunoprecipitation after Transfection into HEK 293 T Cells

The purpose of a co-immunoprecipitation (CO-IP) experiment is to observe or capture and purify the protein of interest interacting with other proteins or complexes. One can do this by using target-specific antibodies to detect each protein or complex of interest and observing the interactions via SDS-Page gel electrophoresis and Western Blotting. In my case I first overexpressed NLRP12 (pcDNA3-myc-CUL3 mammalian expression vector with c-Myc epitope tag Addgene plasmid #:19893 was a gift from Yue Xiong)) and IFIT1 (pCDNA3.1-3xFlag-IFIT1 mammalian expression, Addgene plasmid #: 53554) within HEK 293 T Cells. I seeded the cells in in 60x15 mm tissue culture dishes in DMEM plus 10% fetal bovine serum (FBS), 100U penicillin, and 100U streptomycin. After 24 hours, I transfected the cells and let them incubate for 24 hours (See Transfection of Plasmid into HEK 293 T cells). I then removed the media from the dishes and added 0.5 mL of cold 1% NP40 in PBS with phosphatase and protease inhibitors (Pierce, ThermoScientific). From this point on I kept the samples on ice. I shook the plates for 30 minutes at 4°C. I then removed the lysate from the plates and put them in 1.5 mL centrifuge tubes. I spun the cells at 5,000 x g for 10 minutes at 4°C. Next, I put the supernatant into new 1.5 mL centrifuge tubes and added 40 μ L of protein A/G beads and shook the cells again at 4°C for 1 hour. Following the 1-hour incubation period I spun the samples at 2,000 x g for 1 minute at 4°C and put the supernatant into a third 1.5 mL centrifuge tube and added 2 μ g of antibody (See Table 3). I then incubated the samples at 4°C while shaking for 1 more hour. Afterwards, I added 40 μ L of A/G beads and put the samples back on the shaker at 4°C and let them shake overnight. The

next day, I proceeded to wash the samples 5 times by spinning them at 2,000 x g at 4°C and remove the supernatant and adding 1mL of ice cold DPBS. I repeated the wash steps as necessary and after the last wash was removed, I added 40 μ L of 4x SDS loading dye and proceeded with a Western Blot as above.

RESULTS

Yeast-2-Hybrid Screen Reveals PPI's for NLRP12

Based on the plethora of functions ascribed to NLRP12, I hypothesized that there must be novel proteins that interact with NLRP12 to facilitate these functions. To examine protein-protein interactions, I used a yeast-2-hybrid screening system. In the yeast-2-hybrid, the GAL4 transcription factor is split into its activation (AD) and binding domains (BD). The BD is cloned in frame with the bait protein (NLRP12) and the AD is cloned in frame to the potential interacting proteins or prey proteins (Human cDNA library) (Figure 9). In this instance we did not know which proteins interacted with NLRP12, so we screened through the cDNA library. If the two proteins interact (NLRP12 and a prey protein), the BD and AD of GAL4 are indirectly brought together to activate transcription of reporter genes (AUR1-C, α -galactosidase, HIS3, and ADE2 genes) which change the phenotype of the yeast cell. The goal is to grow yeast on media lacking amino acids, nucleotides, or containing Aureobasidin A or α -gal and colonies that grow and turn blue should indicate positive interactions. Yeast that grows on media lacking histidine and adenine indicates a very strong interaction between the bait and prey proteins. In order to begin this quest, I first needed to clone the full NLRP12 sequence into a cloning vector (pGBKT7). Unfortunately, I ran into problems doing so and had to resort to separating the full NLRP12 insert into two halves. (Figure 3). I successfully cloned the first 1570 bp of NLRP12 (which contained the protein interaction domains) (Figures 4-7) and a Y2H screen was performed. The screen originally revealed eleven proteins, but only six were confirmed (Figure 8 and Table 4). Three of the confirmed proteins: Ring-Finger Protein 2 (RFN2), Cullin 3 (Cul3), and COP9 signalosome subunit 5 (COPS5) all have ubiquitin-related functions and became main

proteins of interest. All these interactions have been confirmed in yeast through an individualized yeast-two-hybrid screen. (Figure 10). Furthermore, the screen revealed a strong interaction with Interferon Induced Protein with Tetratricopeptide Repeats 1 (IFIT1). IFIT1 (ISG56) functions as an inhibitor of viral replication downstream of the RIG-I signaling pathway. IFIT1 has a helix-turn-helix domain that is known to allow a wide variety of protein-protein interactions (Liu, XY. et al. 2011). Importantly, its overexpression has been shown to inhibit expression of IRF3, NF κ B, and IFN- β promoters via disruption of the MITA/TBK1 complex (Li, Y. et al. 2009). Thus, I believe that NLRP12 interacts with IFIT1 and proteins of the ubiquitin conjugations system to enhance IFIT1 activity, which in turn enhance the interference with the inflammatory signaling pathway, preventing transcription of interferon-stimulating genes (ISGs).

Co-Immunoprecipitation Confirm Protein Interaction

In order to confirm the interaction between NLRP12 and IFIT1 that was determined via previous Yeast-2-Hybrid results (Table 4). I conducted a co-immunoprecipitation experiment using human HEK 293 T cells transfected with both IFIT1 and NLRP12 (full-length) mammalian expression vectors (Figure 11 and 12). Preliminary data showed successful overexpression of both mammalian expression vectors (Figure 13) via western blot. In addition, co-immunoprecipitation experiments were performed twice to ensure the results were consistent. These experiments revealed via western blot that IFIT1 and NLRP12 (full-length) do not interact via overexpression in HEK 293 T cells (Figure 14). However, further experiments could be conducted to ensure this is the case.

DISCUSSION

NLRP12 is a protein that is still misunderstood. After nearly 20 years of research, several studies have been done to try and identify which proteins interact with NLRP12 upon its activation. There have been a lot of speculations amongst scientists that NLRP12 is responsible for modulating intestinal homeostasis. It has been found that NLRP12 is highly expressed in the gut and acts as a modulator of intestinal inflammation and/or tumorigenesis (Chen, G. 2019). Allen, I. et al. took it a step farther to say that NLRP12 modulates intestinal inflammation via regulation of the non-canonical NF- κ B and MAPK signaling pathways through the degradation of NIK via the proteasome-dependent pathway. He went on to show that NLRP12 deficient mice were found to have increased activity of NIK-regulated genes such as Cxcl2 and Cxcl3. He believed that dysregulation of this signaling pathway was causing the NLRP12 deficient mice to develop cancer within their colonic mucosal tissues. (Allen, I et al. 2013). In summation, he believes NLRP12 is responsible for modulating a critical checkpoint for inflammation and tumorigenesis via the modulation of NIK in the non-canonical signaling pathway of NF- κ B.

Furthermore, recent research from Chen, L. et al. conducted cohort studies with monozygotic twins to find that any deficiency in NLRP12 can lead to a change in (or loss of) the commensal growth in the gut that leads to basal inflammation. Their studies showed that these symptoms could be reversed with antibody treatment for pro-inflammatory cytokines or with treatment of *Lachnospiraceae* commensals (Chen, L. et al. 2017). Findings such as this may be the key to helping those that suffer from different inflammatory bowel diseases. However, the mechanism behind which pro-inflammatory cytokines and other signaling pathways that may be exacerbating the problem were not mentioned and more research must be done to determine if it

is NLRP12 alone causing the basal inflammation, or if it may be other proteins that work with NLRP12 that may be contributing to the problem. Zaki, MH. et al. believe NLRP12's main job in the gut is to maintain homeostasis that may get out of hand if NF- κ B/ERK/STAT3 signaling is not modulated correctly (Zaki, MH. et al. 2011). All in all it seems that when it comes to colonic inflammation in the gut, NLRP12 plays a key role in its prevention. However, most research in NLRP12 has focused on the gut, it would be interesting to see a shift to other types of inflammatory diseases outside of the gut that may lead to similar inflammatory pathologies.

Likewise, other research has suggested that NLRP12 has a pro-inflammatory role as an inflammasome, which is responsible for the secretion of pro-inflammatory cytokines upon stimulation of several viral DAMPs and PAMPs (Vladimer, GI et al. 2012). Research has connected NLRP12 to modulating anti-viral signaling pathways before, but specific protein-protein interactions with NLRP12 remains unidentified. In addition, research seems to be lacking in understanding of the functionality NLRP12 may serve amongst different types of infections. I believe that research should first compare intracellular pathogens with extracellular pathogen infections and observe whether NLRP12 exhibits a consistent function in either infection. For example, Allen, IC et al. and Vladimer, GI et al. saw different results because the types of cells and mice used were not consistent. I am not sure of their reasoning behind this, but maybe someone can redo these same experiments and compare both extracellular pathogens to each other and see if their results are consistent with one another.

I have conducted a yeast-two-hybrid screen in hopes of identifying any proteins that interact with NLRP12 and may be responsible for its functions. I identified several potential interactions with NLRP12 (SNAPIN, CUL3, RNF2, COPS5, and IFIT1). However, only IFIT1 among them has been examined in human cells. Normally, IFIT1 proteins are known for binding

to viral RNA and another IFIT protein: IFIT3. These interactions have been found to be critical for inhibition of viral replication of viruses that lack a 2'-O methylation cap on their RNA (Johnson, B. et al. 2018). It has also been shown that IFIT1 interacts with both IFIT2 and IFIT3 to enhance its own reactivity to prevent viral translation initiation factors from binding to the methylated RNA, preventing replication (Habjan, M. et al. 2013). I hypothesized that once the body senses a virus is no longer a threat, NLRP12 becomes activated and associates with IFIT1 directly and with E3 ubiquitin ligases; then through K48 ubiquitination, IFIT1 is tagged for destruction. Once IFIT1 is degraded, the antiviral (IFN) signaling pathway is inhibited. However, coimmunoprecipitation in human cells did not demonstrate an interaction between NLRP12 and IFIT1. To make a final determination on this interaction, additional research is needed. I believe these results occurred because the full-length NLRP12 insert was cloned into the mammalian expression vector. However, only the front portion of NLRP12 (effector domain) revealed the novel interaction between NLRP12 and IFIT1 in yeast. Therefore, I believe if the effector domain truncated version of NLRP12 were cloned into the mammalian expression vector and the co-immunoprecipitation experiment was done again that the results might turn out differently. In addition, recent research has indicated that NLRP12 activation can be induced via LPS or Nitric oxide (NO) (Lich, JD. et al. 2007). I think other experiments should be conducted in which 293 T cells are first stimulated with LPS or NO to induce NLRP12 activation first and then co-immunoprecipitation experiments could be conducted to see if the same results could be obtained in human cells and yeast cells.

I identified three other proteins. It has also been suggested that TRIM25 directly interacts with NLRP12 during viral infection and K63 ubiquitination of RIG-I was disrupted which prevented further pro-inflammatory signaling from commencing (Chen, ST. et al. 2019). I

believe the interaction between NLRP12 and IFIT occurs downstream of RIG-I and TRIM25. Maybe NLRP12 and other proteins (E3 ligases) interact to disrupt the interaction of IFIT1 with IFIT3 or other IFIT proteins to terminate the anti-viral signaling pathway. In addition, it may also signal to other adaptive immunity cells to discontinue pro-inflammatory signaling once the virus is cleared and promote cell repair from any damage that took place.

For the other interacting proteins, CUL3, RNF2, and COPS5 have the potential to explain how NLRP12 works with ubiquitin ligases to disrupt the continuation of NF- κ B signaling after an infection is cleared. First, Cullin 3 (CUL3) is a specific E3 ubiquitin ligase that is a part of the Cullin Ring ubiquitin E3 ligase family (CRL). CRL is a multi-protein complex that consists of the entire E1, E2, E3 bucket brigade that is required for the addition of ubiquitin onto a target protein in a process known as ubiquitination (Wang-Shick, R. 2017). Regulation and the magnitude of proteins involved in this pathway is vastly complex and is beyond the scope of this thesis. The goal of an E3 ubiquitin ligase is to associate with E1 and E2 proteins in combination with the target protein complex. Upon association, ubiquitination occurs. Interestingly, the process of ubiquitination is not a simple process. Ubiquitin can be added one time (mono-ubiquitination) or up to several times (poly-ubiquitination) (Collins, P. et al. 2016). Regulation of this system is also complex and will not be discussed in detail here. Yet, CUL3 has been found to associate with a protein called Cullin Associated and Neddylation Dissociated 1 (CAND1)/(TRIP120A) that is responsible for inhibiting the neddylation (a process analogous to ubiquitination) of CUL3 by the Skp1, Cullin 1, F box protein complex (SCF). Effectively, this prevents the ubiquitination of phosphorylated I κ B α by SCF (beta-TrCP) and leads to inhibition of the NF- κ B signaling pathway (Min, KW. et al. 2003). In my opinion, it is not surprising that NLRP12 was found to associate with CUL3 in this way because NF- κ B is sequestered by I κ B α

in its inactive state. Nonetheless, once IKK phosphorylates I κ B α at serine residues 32 and 36, a destruction motif is then recognized by SCF (beta-TrCP); targeting I κ B for degradation which then frees NF- κ B and the signaling pathway continues (Figure1) (Collins, P. et al. 2016). I believe NLRP12 associates with CUL3 and other E3 ligases to target NF- κ B subunits within this pathway for degradation which would inhibit the pathway from continuing.

On the other hand, Ring finger protein 2 (RNF2) has an entirely different role than CUL3. Although it is still an E3 ligase, RNF2's main function is modulating the K-119 residue ubiquitination on histone H2A. Not much is known specifically about RNF2's function, but it has been found to form many complexes with BMI1 and UB2D3 when modulating histone H2A (Bentley, ML. et al. 2011). In other words, recent research has found RNF2's main responsibility is in chromatin modification or gene expression. RNF2 has also been found to work with MDM2 (E3 ligase) in tumor suppression at the DNA level via stabilization of replication fork progression. Klusmann, I. et al. noted that if either ubiquitin ligase (RNF2 or MDM2) was suppressed, the other could pick up the slack for replication fork progression (Klusmann, I. et al. 2018)

Third, COP9 Signalosome Subunit 5 (COPS5) protein is only one of eight E3 ligases to form the COP9 signalosome which is like the 19S subunit of the 26S proteasome. Fundamentally, the COP9 signalosome is found to positively regulate E3 ubiquitin ligases; specifically, those within the CRL family (CUL3) (Wei, N. et al. 2008). It has recently been discussed that the COP9 signalosome may be a future target for cancer drug research. Schlierf, A. et al. believe the key is that most cancers associated with malfunctions within the COP9 signalosome are due to a disruption in the management of the several E3 ligases involved. A disruption in these proteins leads to improper management of several oncogenes and tumor

suppressors that require close modulation (Schlierf, A. et al. 2016). Their goal is to control the CRL proteins involved, then the signalosome may be more likely to be controlled which would lead to better modulation of the oncogenes and tumor suppressors. I believe in order to understand the mechanism behind how NLRP12 and COPS5 interact a few more missing proteins need to be identified before it can be fully elucidated. I believe the key to understanding the mechanism behind how NLRP12 interacts and facilitates modulation of either the canonical or non-canonical signaling pathway lies within the realm of ubiquitination and other forms of post-translational modifications via several E3 ligases and other associated proteins.

In summation, I believe my hypothesis regarding NLRP12 interacting with certain proteins to inhibit pro-inflammatory signaling was preliminarily confirmed for RNF2, CUL3 and COPS5. Although IFIT1 interacted with NLRP12 in yeast, this interaction was not confirmed in human cells and additional research is needed to verify or refute these interactions in human cells to elucidate their functions.

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Table 1. The following primer sequences were used for all cloning experiments.

Primers	Sequences
NLRP12 NdeI for	GAA TGA CAT ATG ATG CTA CGA ACC GCA GGC AG
NLRP12 Sall rev	GAT CTT GTC GAC TCA GCA GCC AAT GTC CAA ATA AG
T7 for	TAA TAC GAC TCA CTA TAG GG
M13 rev	CAG GAA ACA GCT ATG ACC
Yeast Amplimer for	CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC C
Yeast Amplimer rev	GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG AT
NLRP12 (Gibson) for	TCC TGA TAT CCG CGG TAC CGA TGC TAC GAA CCC CAG
NLRP12 (Gibson) rev	CCT CTA GAT GCA TGC TCG AGT CAG CAG CCA ATG TCC AAA T
3x Myc (Gibson) for	CTC GAG CAT GCA TCT AGA G
3x Myc (Gibson) rev	CGG TAC CGC GGA TAT CAG

Table 2. The following information is the protocols used for all PCR, Colony PCR, and Yeast Colony PCR experiments.

Protocol	Denaturation	Annealing	Extension	Number of Cycles	Final Extension
PCR (Cloning)	98°C for 10 sec	67°C for 20 sec	72°C for 2 min	34	72°C for 10 min
Colony PCR (Cloning)	95°C for 3.5 min	55°C for 30 sec	72°C for 2 min	34	72°C for 10 min
Yeast Colony PCR	95°C for 3.5 min	55°C for 30 sec	72°C for 2 min	34	72°C for 10 min

Table 3. The following antibodies were used for all western blot and CO-IP experiments.

Name of Antibody	Detects	Catalog #	Company
Myc-Tag Polyclonal Antibody	Myc-Tag EQKLISEEDL (Mouse)	16286-1-AP	Proteintech
Flag DYKDDDDK Tag	DYKDDDDK Flag Epitope (Mouse)	MA1-91878	Invitrogen

Table 4. The following proteins were shown to interact with NLRP12 via the Y2H screen.

Protein Abbreviation	Protein Name	Brief Description
SNAPIN	SNAP associated protein	SNARE protein responsible for vesicle docking and fusion and regulates neurotransmitter release
SPATA22	Spermatogenesis associates 22	Protein required for homologous recombination in meiosis I
IFIT1	Interferon-induced Proteins with Tetratricopeptide Repeats	Inhibition of Viral Replication
RNF2	Ring Finger Protein-2	E-3 ubiquitin-protein ligase
CUL3	Cullin 3	Ubiquitin -protein ligase
COPS5	COP9 signalosome subunit 5	Ubiquitin regulator

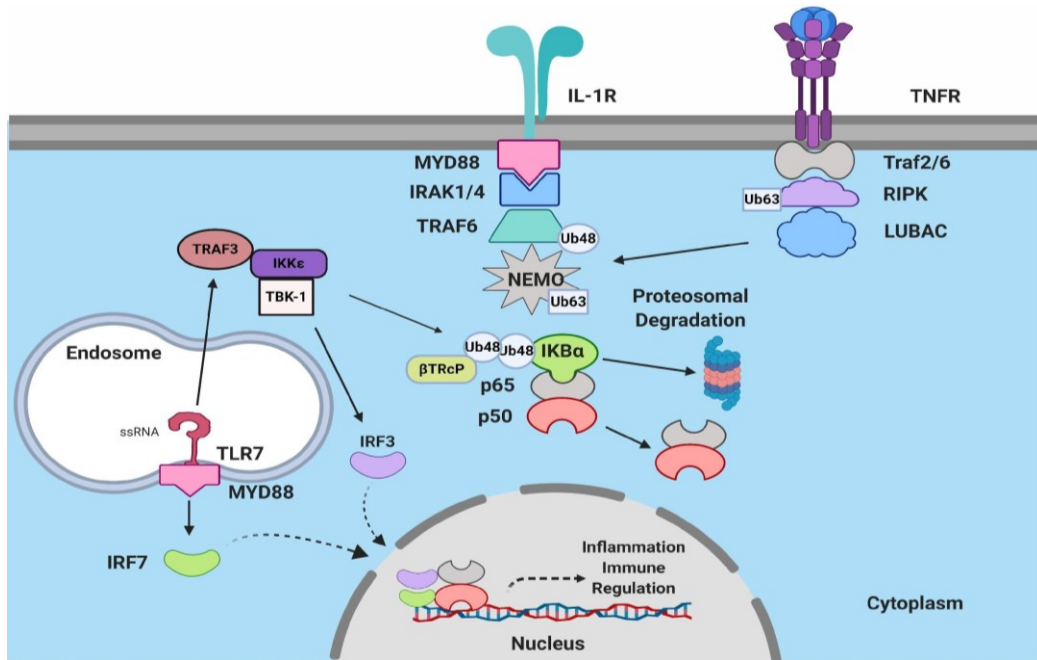


Figure 1. A diagram of the classical NF- κ B signaling pathway that depicts IRAK1 and TRAF3's roles within this pathway. Which also shows where NLRP12 could intervene within this pathway.

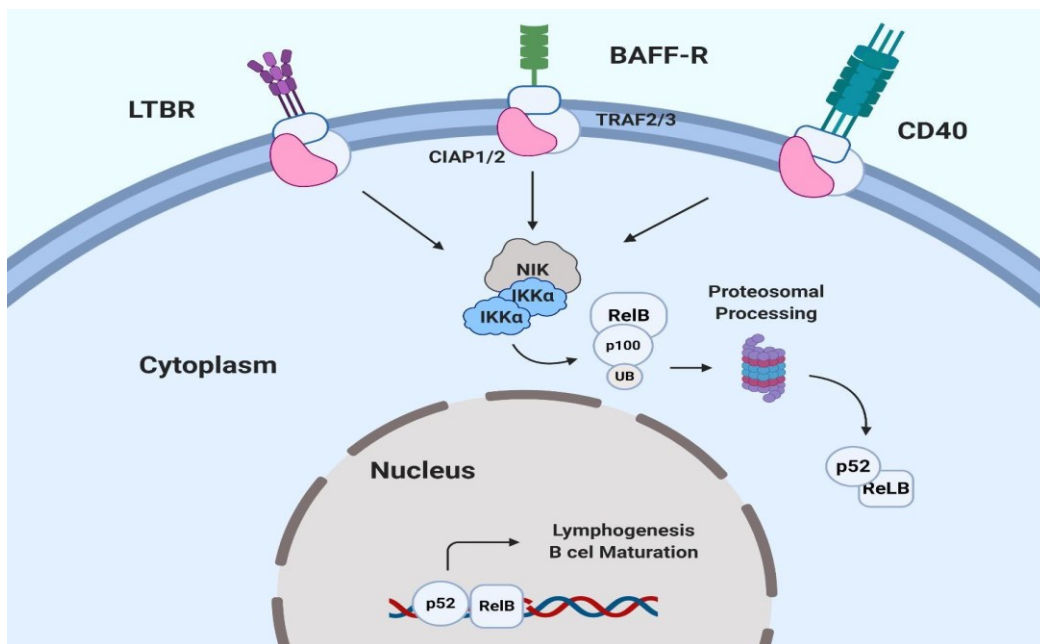


Figure 2. A diagram of the alternative NF- κ B signaling pathway that depicts TRAF3's and NIK's roles within the pathway. Which in turn shows where NLRP12 could intervene within the pathway.

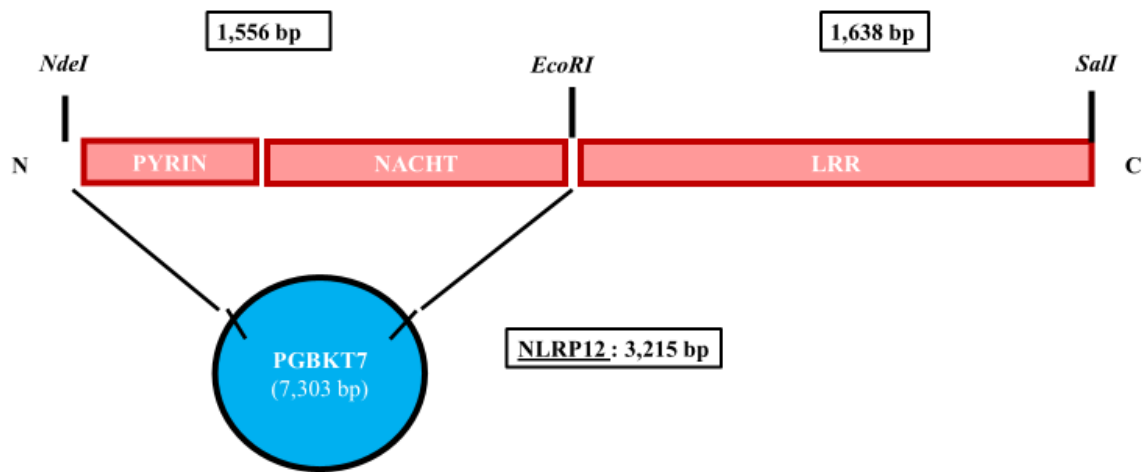


Figure 3. In order to conduct a Y2H screen, NLRP12 had to be cloned into the PGBKT7 vector. Only the front section of NLRP12 was successfully cloned.

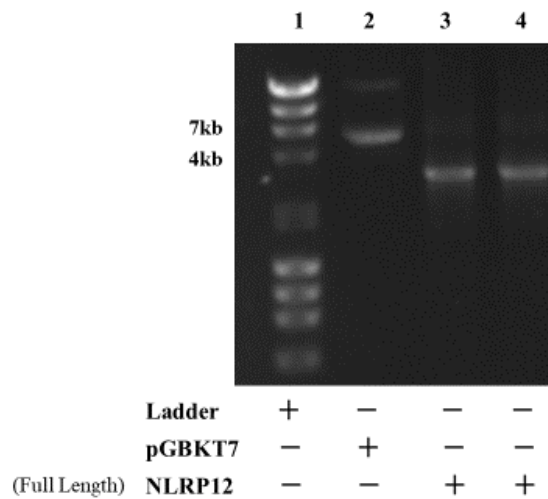


Figure 4. PCR gel electrophoresis for NLRP12 using human NLRP12 forward and reverse primers indicated about a 3300 bp NLRP12 insert.

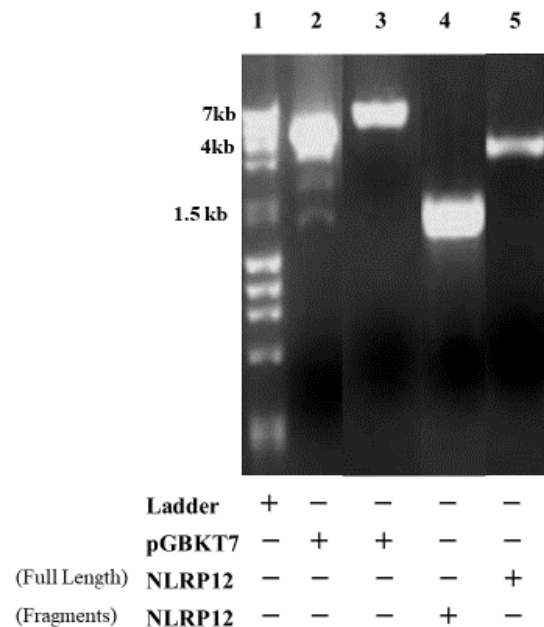


Figure 5. A gel electrophoresis image of a restriction enzyme digest showing the isolation of the PGBKT7 plasmid and NLRP12 insert which were then cut out from the gel and purified for ligation.

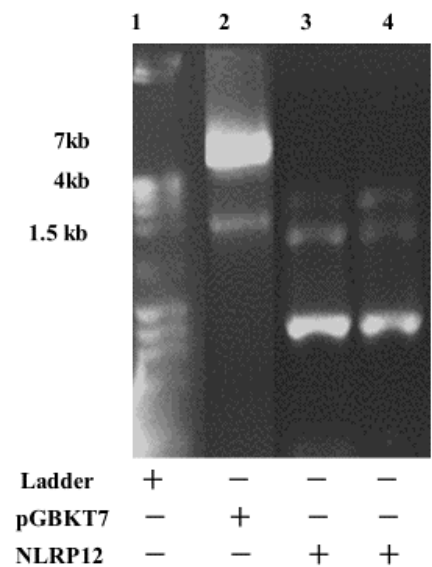


Figure 6. A gel electrophoresis image of picked colonies that were thought to contain the successful NLRP12 insert within the PGBKT7 plasmid. The image reveals successful NLRP12/PGBKT7 clones.

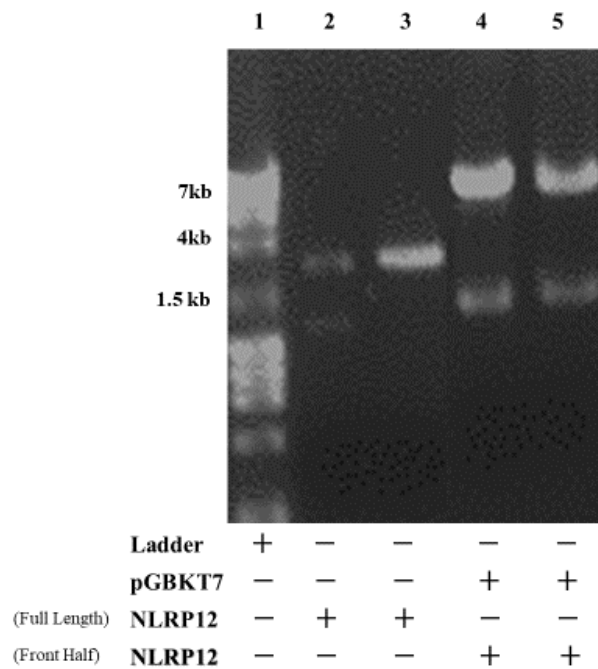


Figure 7. A gel electrophoresis image of a restriction enzyme digest. A restriction enzyme digest was completed a second time to ensure the NLRP12 insert was successfully cloned into the PGBKT7 plasmid.

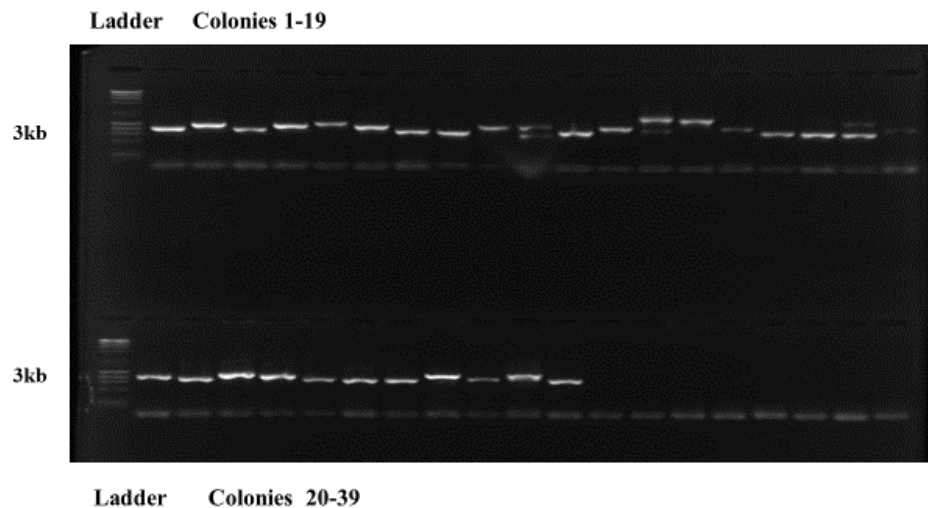


Figure 8. A colony PCR was conducted on 40 yeast colonies from the Y2H screen. These samples were then sent for sequencing to obtain their identities.

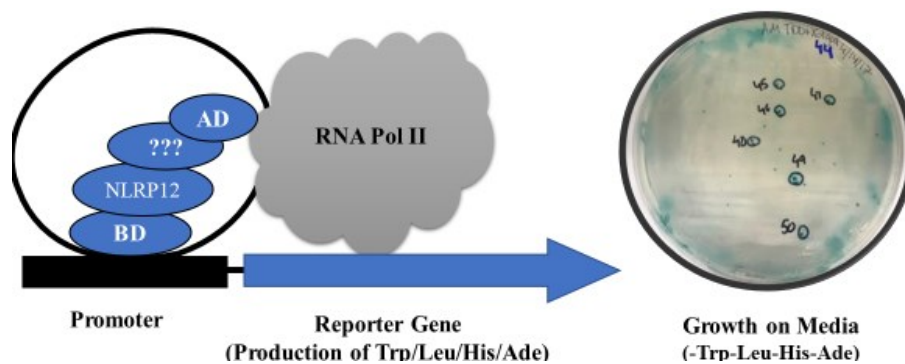


Figure 9. A diagram depicting an unknown protein interacting with NLRP12 via Y2H screen promotes activation of reporter genes that encode the production of Tryptophan, Leucine, Histidine, and Adenine allowing the yeast containing both plasmids to grow on media lacking all four amino acids.

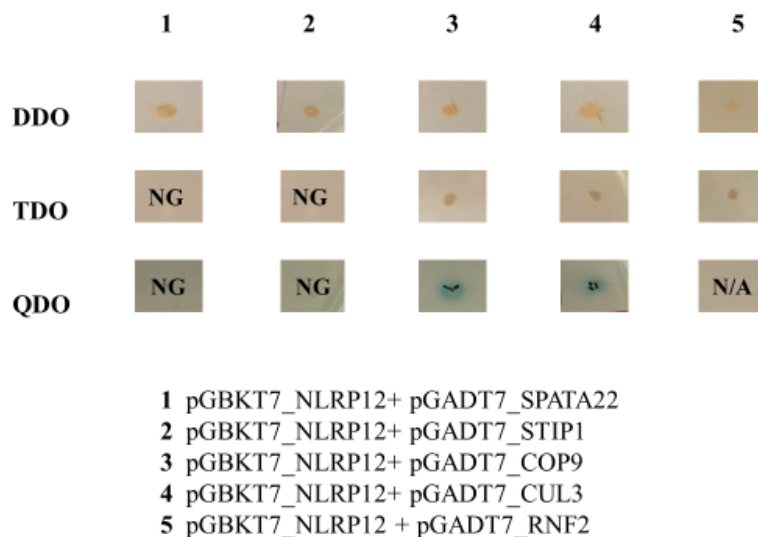


Figure 10. An image of Y2H plates against NLRP12 and other proteins (STIP1, SPATA22, RNF2, CUL3, and COP9) that were shown to interact with NLRP12 in the first Y2H screen. These plates confirmed a strong interaction between NLRP12 and COP9, RNF2, and CUL3.

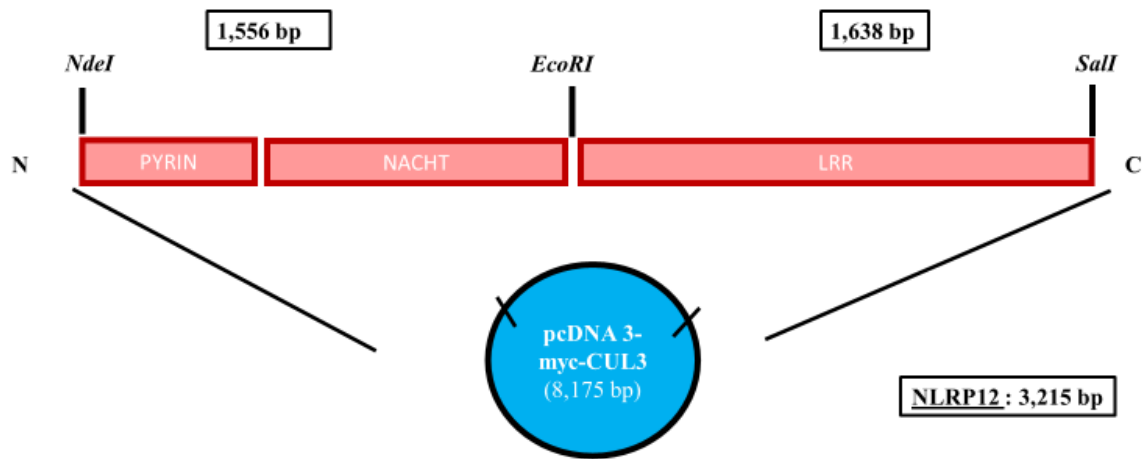


Figure 11. A diagram depicting the specific mammalian expression vector that NLRP12 (full-length) was cloned into.

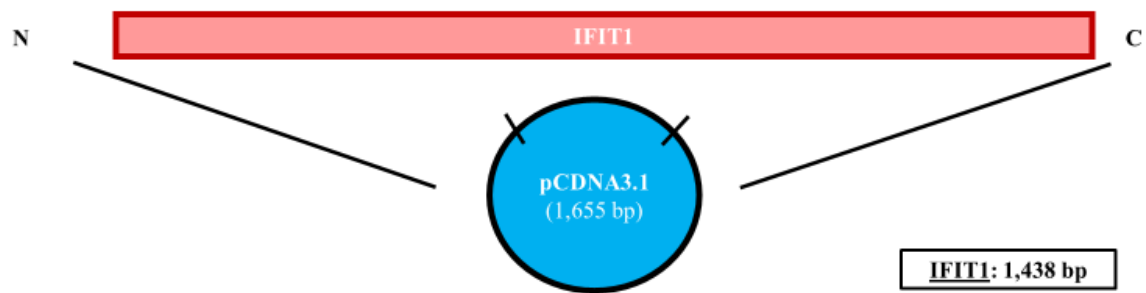


Figure 12. A diagram depicting the specific mammalian expression vector containing the IFIT1 gene.

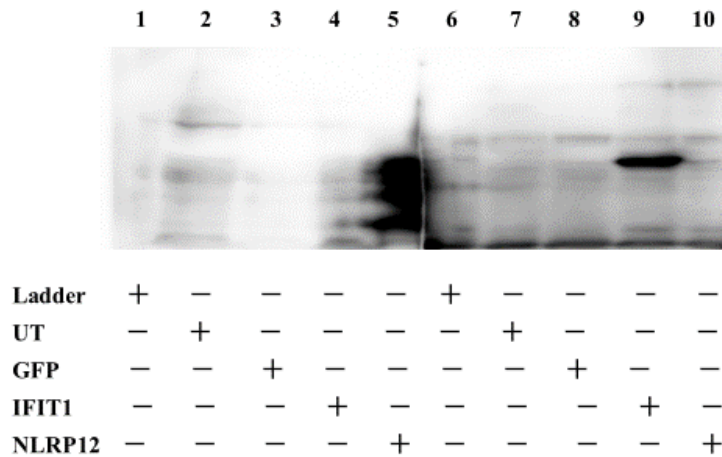


Figure 13. A western blot using both anti-Flag (IFIT1, right half) and anti-Myc (NLRP12, left half) antibodies revealed overexpression of both proteins was observed.

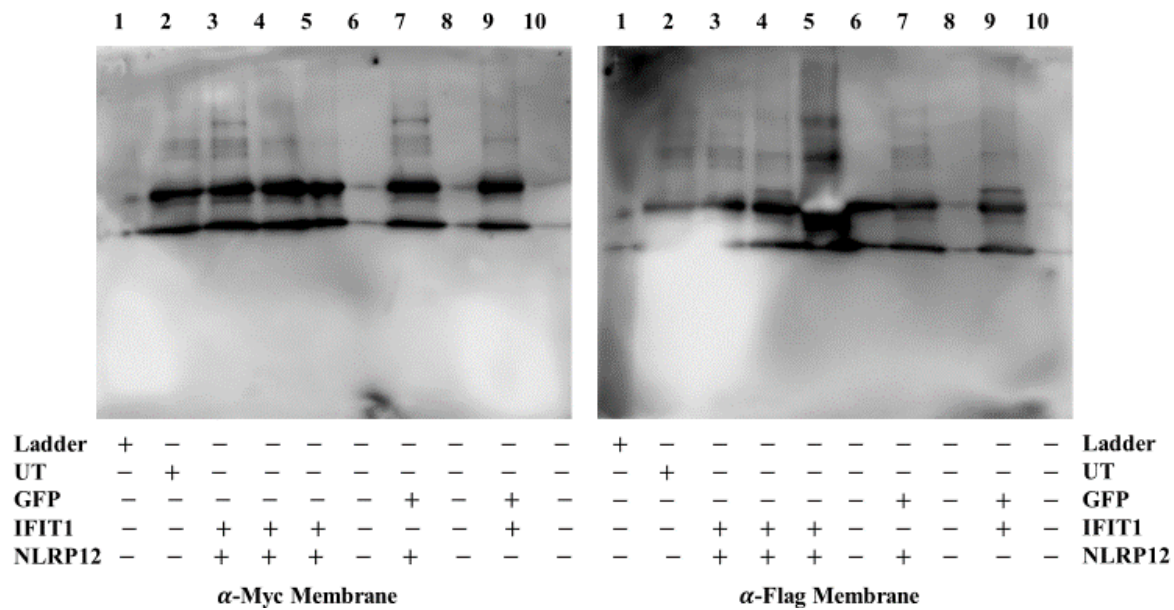


Figure 14. The CO-IP experiment revealed IFIT1 and NLRP12 do not interact via overexpression of each protein within HEK 293 T Cells which was observed via Western Blot. Lanes 2, 3 and 7 were immunoprecipitated with Myc antibody, lanes 4 and 9 were immunoprecipitated with Flag antibody, and lane 5 was immunoprecipitated with Tubulin (control) antibody.