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Characterization of the Interaction of Three Domains of NLRP2 (PYD, LRR, and NACHT) With EBP1 Protein

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CHARACTERIZATION OF THE INTERACTION OF THREE DOMAINS OF NLRP2 (PYD, LRR AND NACHT) WITH EBP1 PROTEIN

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Master of Science, Biology

By

Rakshya Bhatta

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ABSTRACT

NOD-like receptors (NLRs) are cytoplasmic proteins essential for various immune responses such as reactions to infectious diseases, metabolic and cellular damage, fetal development, and cancer. Among the 22 identified NLR proteins, research has highlighted the significant roles of NLRP2 in fetal development. NLRP2 is also classified as a maternal effect gene and the mutation to this gene can lead to DNA methylation imprinting defects, altered gene expression, and conditions such as recurrent miscarriages (RMs). The protein EBP1, which has been found to have roles in embryonic development has been implicated in the regulation of DNA methylation. Recent studies have also suggested that EBP1 could be a novel interacting partner of NLRP2. This has been observed through coimmunoprecipitation and Fluorescence Resonance Energy Transfer (FRET) techniques as well. In this research, I looked at the interaction between these two proteins in detail by examining which domains of NLRP2 are important for the interaction of this protein with EBP1. Thus, this study provides a detailed insight into possible mechanism by which EBP1 or other proteins could interact with NLRP2 and regulate DNA methylation.

KEYWORDS: NOD-like receptors, EBP1, DNA Methylation, PYD, LRR, NACHT, idiopathic recurrent miscarriages

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INTRODUCTION

The Immune System

The immune system is an intricate and dynamic network of cells, tissues and organs that work together to defend the body against pathogenic threats while maintaining immunological memory and homeostasis to maintain the organism's long-term health and survival. It comprises two main components: the innate immune system and the adaptive immune system. The adaptative immune system provides a highly specific response to pathogens with the help of T cells and B cells (Bonilla & Oettgen, 2010). The innate immune system is the body's first line of defense and provides rapid but non- specific responses to pathogens. The key components of the innate immune system include physical and chemical barriers like skin and mucus membrane, cellular defenses through neutrophils, NK cells and dendritic cells, pattern recognition receptors like TLRs and NLRs and the complement system (Kaur & Secord, 2019). My research is focusing on one of the important components of Innate immune system, Pattern Recognition Receptors (PRRs).

Pattern Recognition Receptors (PRRs)

Pattern recognition receptors are a group of protein sensors that are found in and on cells and can recognize antigen determinants of approximately all groups of pathogenic organisms. PRRs include Toll-like receptors (TLRs) on cell surfaces or within endosomes, Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs) in the cytoplasm, C-type lectin receptors (CLRs) on cell surfaces, and AIM2-like receptors (ALRs) in the cytoplasm (Kano et al., 2022).

Many of the PRR families are evolutionarily conserved. These molecules are expressed in macrophages, lung epithelial cells, dendritic cells, and recruited immune cells, and can also be detected in endothelial cells, stromal cells, and fibroblasts (Bonilla & Oettgen, 2010; Kawai & Akira, 2006). There are two signals these PRR receptors can sense, PAMPs and DAMPs (Muruve et al., 2008). Pathogen-associated molecular patterns (PAMPs) are highly conserved molecules that are only found in pathogens and are not associated with mammalian cells. These include genomic DNA, bacterial polysaccharides, single stranded RNA (ssRNA), double-stranded RNA (dsRNA), bacterial and viral proteins (Kumar et al., 2011). Damage associated molecular patterns (DAMPs) are host molecules that are released by damaged cells (Seong & Matzinger, 2004).

Upon binding to their specific ligands, PRRs undergo conformational changes that initiate signaling cascades. The signaling pathways lead to the activation of key transcription factors such as NF-κB, mitogen-activated protein (MAP) kinases and interferon regulatory factors (IRFs) that control the transcription of genes encoding pro-inflammatory factors including type I interferon and other inflammatory cytokines and chemokines (Kawai & Akira, 2010; Medzhitov & Janeway, 2002). This study mainly focuses on a Nod- Like Receptor (NLR), NLRP2 specifically.

NOD-Like Receptors (NLRs)

The nucleotide-binding oligomerization domain-like receptors (NLRs) are a specific subset of PRRs that are located in the cytoplasm and are particularly important for detecting intracellular threats. These receptors are expressed in various types of cells including dendritic

cells, endothelial cells, macrophages and others and can initiate inflammatory immune responses by forming inflammasomes to protect against infections and cellular stress (Martinon et al., 2002). NLRs have also been found to play important roles in reproduction and embryonic development (Van Gorp et al., 2014).

NLRs have a tripartite domain structure that includes N-terminal effector domain that facilitates protein-protein interactions, a central NACHT domain responsible for nucleotide-binding and oligomerization and C-terminal LRR domain involved in ligand recognition (Maruta et al., 2022). There are 22 known NLRs in humans. These NLRs are classified into several subfamilies like NLRA, NLRB, NLRC and NLRP, based on their N-terminal effector domains (Ting et al., 2008). NLRA has the acidic transactivation domain (AD), NLRB has the baculoviral inhibitory repeat-like domain (BIR), NLRC has the caspase activation and recruitment domain (CARD) and NLRP has the pyrin domain (PYD) (Kim et al., 2016). NLRP family includes several members, each playing distinct roles in immune regulation and inflammasome formation. NLRP1 responds to bacterial toxins and stress signals and is involved in the formation of the inflammasome, which activates caspase-1 and subsequently leads to the maturation of IL-1β and IL-18 (Tupik et al., 2020). NLRP3 is the most studied NLRP, and it recognizes microbial components, ATP, and crystalline substances resulting in inflammasome activation (Yang et al., 2019). NLRP12 is known to down-regulate the canonical and non-canonical NF-κB signaling pathways by inducing proteasome degradation of NF-κB related enzymes (L. Huang et al., 2023).

NLRP2, NLRP5 and NLRP7 are also known as maternal effect genes that are involved in early embryogenesis and fetal development and are known to regulate factors like RNA in the

oocyte that control embryonic development prior to activation of the embryonic genome (Begemann et al., 2018). Maternal variation caused by mutations in maternal effect genes (MEGs) is associated with a range of adverse outcomes in human such as hydatidiform moles, zygotic cleavage failure, offspring with multi-locus imprinting disorders as well as structural birth defects such as congenital heart defect (Mitchell, 2022). Nlrp5 (Mater) was the first-described maternal-effect gene, with maternal ablation causing developmental arrest at the two-cell stage in mice (Tong et al., 2000). NLRP2 and NLRP5 are MEGs also known to encode components of a multiprotein complex called the Subcortical Maternal Complex (SCMC) which has multiple functions in early developmental stages and is expressed in the oocyte and early embryo (Anvar et al., 2024; Van Gorp et al., 2014). NLRP7 is known to be involved in oocyte maturation, endometrial remodeling and placental development during early pregnancy (Carriere et al., 2021). In this research, we are focusing on NLRP2 specifically. Even though we know NLRP2 has roles in early embryogenesis, not much research has been done focusing on the mechanism and the effects of NLRP2 mutation in early fetal development.

NLRP2 and Its Functions

NLR Family Pyrin Domain Containing 2 (NLRP2) protein is a member of nucleotide-binding and leucine-rich repeat receptor (NLR) family that is involved in the innate immune system and plays a role in formation of inflammasomes to activate immune responses. NLRP2 is located on chromosome 19q13-42 and adjacent to NLRP7 (T. Zhang et al., 2024). NLRP2 is known to contain three main domains, an N-terminal pyrin effector domain (PYD), a centrally located nucleotide-binding and oligomerization domain (NACHT) and C-terminal leucine-rich

repeats (LRR) (Basingab et al., 2021). The structure of NLRP2 is also shown in Figure 1. A few studies have shown that NLRP2 can regulate caspase-1 and NF-kB activity by interacting with the components of the IKB kinase complex and it can modulate the NF-κB signaling pathway that is crucial for the expression of many inflammatory genes (Tilburgs et al., 2017). NLRP2 is expressed in many tissues including the brain and has a higher level of expression in the placenta, lungs and thymus (T. Zhang et al., 2024). Because it is highly expressed in the placenta, it is being studied in embryonic and fetal development. Very few studies have shown that NLRP2 might form the inflammasome like other NLRPs, but the current understanding of NLRP2 are limited. NLRP2 is also seen to regulate proinflammatory as well as anti-apoptotic responses in proximal tubular epithelial cells (Rossi et al., 2019). The dysregulation of NLRP2 has also been linked to several diseases like systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and type II diabetes (Chen et al., 2021; Jiang et al., 2023).

NLRP2 is an example of a Maternal Effect Gene (MEG). A study conducted on mouse model showed that out of 82 mammalian MEGs, 8 were associated with birth defects like craniofacial, heart, neural tube, and skeletal defects and NLRP2 was the only one that was a MEG associated with humans (Mitchell, 2022). All these eight genes were involved in Methylation and imprinting. NLRP2 is also found to be involved in maintaining the maternal-to-zygotic transition when control of embryonic development shifts from maternal RNA to zygotic genome activation (Anvar et al., 2024). In addition, research has also shown that deletion of NLRP2 gene from zygotes in mouse results in their early embryonic arrest (Peng et al., 2017). Genetic analysis of patients with recurrent miscarriages has identified mutations in NLRP2, suggesting a direct link between these mutations and reproductive failure (J.-Y. Huang et al.,

2013). Additionally, some in vitro fertilization (IVF) studies have observed poor outcomes in embryos carrying NLRP2 mutations (Arian et al., 2021).

EBP1 and Its Functions

EBP1 (ErbB3 binding protein 1), also known as PA2G4, interacts with the ErbB3 receptor, which is part of the ErbB family of receptor tyrosine kinases (Radomski & Jost, 1995). It plays a significant role in regulating cell proliferation, differentiation, and survival by modulating ErbB3 signaling pathways (Ko et al., 2019). EBP1 is also involved in the regulation of gene expression and RNA processing within the cell nucleus. It has been implicated in cancer biology, where its altered expression can influence tumor growth and progression (Bao et al., 2022) and is also associated with the cellular stress response, contributing to the development of resistance to certain cancer therapies (Y. Zhang et al., 2008).

The Ebp1 gene (PA2G4), consists of ten exons and encodes two splice variants: p48 EBP1 and p42 EBP1 (Hwang et al., 2020). Recent studies have shown that the long form, the p48 protein, acts as an oncoprotein by suppressing apoptosis and promoting cell proliferation through Akt activation and p53 degradation. In contrast, p42 EBP1, recognized as a potent tumor suppressor, inhibits PI3K activity by degrading the p85 subunit (Ko et al., 2014). Recently, it was found that EBP1 also has a role in embryonic development. Studies conducted on mice showed that the genetic ablation of Ebp1 resulted in embryonic lethality characterized by extensive cell death and the dysregulation of the transcriptional repression unit SUV39H1/DNMT1 (Ko et al., 2019). DNA methyltransferase 1 (DNMT1) is a major DNA methyl transferase that plays an important role in maintaining DNA methylation patterns in

mammalian cells (Jin & Robertson, 2013) and its activity is important in regulating gene expression, genomic imprinting and X-chromosome inactivation (Morris & Monteggia, 2014). EBP1 is known to bind to the promoter region of DNMT1 to inhibit its expression which represses DNA methylation (Ko et al., 2014). Thus, it may have some role in the diseases that are associated with genetic imprinting disorders during embryonic development, and it could function by interacting with maternal effect genes like NLRP2, NLRP5 and NLRP7, but its role and the actual mechanism is still unknown.

DNA Methylation and Recurrent Miscarriage

DNA methylation is an important epigenetic modification that involves the addition of a methyl group at the 5th carbon position of the cytosine ring within CpG dinucleotides leading to 5- methyl cytosine (Moore et al., 2013). It is a key regulatory mechanism in gene expression and embryonic development. DNA methylation patters are also hallmark of many cancers (Jones & Baylin, 2007). DNA methylation pattern are important in cell differentiation and proper functioning of developmental processes.

Recurrent miscarriage (RM) also known as recurrent pregnancy loss is the occurrence of three or more consecutive pregnancy losses before 20 weeks of gestation (El Hachem et al., 2017). There are many known causes of recurrent miscarriages including genetic factors, immunological factors, endocrine factors, infections or lifestyle. But, sometimes, despite medical evaluation, no identifiable cause is found and results in idiopathic recurrent miscarriage. Idiopathic recurrent miscarriages is a complicated reproductive problem worldwide due to the lack of information about their etiology and thus require further study of

novel factors that could provide scientific information for their prevention and targeted strategies (Arias-Sosa et al., 2018).

Other than the known factors for these miscarriages, epigenetic factors might be involved that are associated with DNA methylation. Research has shown altered DNA methylation patterns in the endometrial tissue as well as placental tissues of women with RMs (Zhou et al., 2021). Recent studies have shown NLRP2 may also be involved in reproductive processes, including embryonic development and implantation. The purpose of this research was to understand if there was any connection between NLRP2 and DNA methylation in the context of Idiopathic Recurrent Miscarriages.

Research Question and Hypothesis

NLRP2 has been known to be a maternal effect gene that has a role in embryonic development (Anvar et al., 2024). Mutations in NLRP2 are also associated with altered DNA methylation in the maternal oocyte, which could be associated with maternal DNA methylation imprinting disorders like recurrent biparental complete hydatidiform molar pregnancies (BiCHM)(Van Den Veyver & Al-Hussaini, 2006). Previous research in the Lupfer lab conducted via a Yeast-2 Hybrid screening method has identified proteins that interact with NLRP2 to regulate DNA methylation. In this research, I am looking at one of those proteins, EBP1 and how it interacts with NLRP2 to regulate DNA methylation. NLRP2 has 3 main domains, PYD, LRR and NACHT. Firstly, I wanted to confirm the interaction between NLRP2 and EBP1. The main question I wanted to address in this research was to validate if these two proteins really interact, and if they do, with which domains of NLRP2 does EBP1 interact?

MATERIALS AND METHODS

Cell Culture and Maintenance

Human Embryonic Kidney 293 cells expressing the SV40 large T antigen (HEK293T cells) were purchased from B.E.I. Resources. The cells were grown in a 75 ml flask in Dulbecco's modified Eagle's medium (DMEM) (CORNING, Cat. 10-013-CV) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin antibiotics. The flask was incubated at 37°C in a humidified atmosphere with 5% CO_2, changing the medium every 2-3 days and subculturing the cells when they reach approximately 80-90% confluence. For subculturing, the old media was aspirated, and the cells were gently washed with PBS to remove residual serum. 2 ml of trypsin-EDTA was added to the flask and incubated until the cells detached. Trypsin was neutralized by adding an equal volume of growth media and the cell suspension was pipetted up and down to break up any cell clumps. The cell suspension was transferred to a centrifuge tube and centrifuged at 300x g for 7 minutes. The supernatant was discarded, and the cells were resuspended in fresh growth media. Cells were counted using a hemocytometer and around 2 million cells were seeded into a new 75 ml flask with 10ml of growth media and incubated.

Primer Design

To achieve the PYD, LRR and NACHT domains truncation mutations, primers were designed using the NEBaseChanger tool. The complete coding sequence of the target genes (Table 1) were pasted into the NEBaseChanger interface, and "Deletion" was selected as the mutation type. The start and end positions of the nucleotide positions for a domain to be

truncated were specified, and the tool generated the appropriate primer sequences flanking the deletion region. The forward and reverse primers provided by NEBaseChanger were reviewed for accuracy and optimal PCR performance. The designed primers included a forward primer upstream of the deletion region and a reverse primer downstream of the deletion region.

Site-Directed Mutagenesis

For site-directed mutagenesis experiments targeting the PYD (Pyrin), LRR and NATCH domains of the NLRP2 gene, the pcDNA 3.1-NLRP2 plasmid served as the template DNA. Firstly, exponential amplification was conducted using the Q5 Hot Start High-Fidelity 2X master mix (New England Biolabs, Cat.M0494A), 10µM forward primer, 10µM reverse primer, template DNA and nuclease free water under conditions optimized for efficient amplification and fidelity. Appropriate cycling condition for each template for mutagenic PCR reaction was selected (Table 2. Institutional Biosafety Committee approval for the use of all recombinant plasmids can be found in Appendix: IBC Approval.

Transformation To E. *coli*

After the PCR reaction, a Kinase, Ligase & DpnI (KLD) reaction was performed to ligate and circularize the mutated PCR products. 2X KLD reaction Buffer (New England Biolabs, Cat. B0554) and 10X KLD Enzyme Mix (New England Biolabs, Cat. M0554A) were used to carry out the reaction. 5 µL of KLD mix was added to 50 µl of chemically competent *E. coli* cells and incubated on ice for 30 minutes. The cells were heat shocked at 42° C for 30 sec to allow the

plasmids to enter the bacterial cells, followed by incubation on ice for 5 minutes. 950 µL SOC media was added and the cells were shaken at 37° C for an hour to promote cell growth. The cells were plated on LB agar supplemented with Ampicillin and incubated overnight at 37°C. The next day, bacterial colonies containing the mutated plasmids were selected for further analysis.

Colony PCR

The selected bacterial colonies were lightly touched using a sterile pipette tip, and the cells were transferred into PCR tubes containing the master mix (Thermo Fisher Scientific, Cat. 12358010). To lyse the bacterial cells, the PCR tubes were heated at 95°C for 5 minutes. The PCR was then run with typical cycling conditions: an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, annealing temperature of 60°C for PYD, 58°C for LRR and 71°C for NACHT for 30 seconds, and initial extension at 72°C for 1 minute per kb of the expected product, concluding with a final extension at 72°C for 5 minutes. The PCR products were analyzed by agarose gel electrophoresis to verify the presence of the expected DNA fragment.

Plasmid Purification and Sequencing

The selected colonies were grown overnight in LB broth containing 100 µg/ml Ampicillin at 37°C. The plasmid DNA were purified with ZymoPURE II Plasmid Midiprep Kit (Aat. 4201, Zymo Research) according to manufacturer's protocol. Also to confirm the proper deletion of each of the domains and insertion of NLRP2 truncated genes into the plasmids, the

cloned plasmids were sequenced via Sanger sequencing at Eurofins. Once we confirmed the sequencing data, plasmid maps were constructed for NLRP2 with PYD knockout (Figure 2), LRR knockout (Figure 3) and NACHT knockout (Figure 4).

Transfection

HEK293T cells were seeded at 800,000 cells/ well on a 6 well plate ensuring they reached 70-90% confluency the next day. The following day, the growth medium was replaced with DMEM. In separate microcentrifuge tubes, the DNA-Lipofectamine complexes were prepared by diluting 4 µg DNA and 10 µL of Lipofectamine (Thermo Fisher Scientific, Cat. 15338030) in 250 µL of serum free DMEM medium and mixed gently. The solution was incubated for 15 minutes at room temperature to allow the complexes to form. The DNA-Lipofectamine complexes were added dropwise to the cells, distributing them evenly. The cells were incubated at 37°C in a CO_2 incubator for 4 hours, allowing for optimal gene expression. After 4 hours of transfection, the media was changed to normal growth medium to reduce cytotoxicity and the wells were incubated again for 24 hours.

Co-Immunoprecipitation

HEK293T cells were plated in a 6 well plate and co-transfected with different mutated NLRP2 (PYD k/o, LRR k/o and NATCH k/o) plasmids with the EBP1 plasmid. After 24 hours, the old media was removed, and 0.5 mL of cold lysis buffer (1% NP-40 in PBS with protease inhibitor (Thermo Scientific, Cat. A32953) and phosphatase inhibitor (MilliporeSigma, Cat. P2850)) was added to the cells in each well. The plate was then incubated on a shaker at 4˚C for

30 minutes. After 30 minutes, the cells were transferred to a Dounce homogenizer on ice and homogenized about 10 times to fully disrupt them. The homogenates were then centrifuged at 5000xg at 4°C for 10 minutes. Next, 300-500 µL of the supernatant was transferred into a 1.5 mL microcentrifuge tube, and 2 µg of the appropriate antibodies (Myc Tag (Myc.A7), Cat. 121316, Invitrogen; anti-HA, Cat. 50-173-5882, Proteintech) were added to each tube. Beta-Actin (Invitrogen, Cat. PA1-981) was used as control. The details of each antibody used are provided in Table 3**.** Then, 40 µL of Protein A/G beads (Santa Cruz Biotechnology Protein A/G+ Agarose, Cat. NC9371547) were added to each tube, and the tubes were incubated at 4°C overnight. The next day, the samples were centrifuged at 2000xg for 1 minute at 4˚C. The homogenates were removed, and the protein A/G beads were washed 3-5 times using cold PBS. Between each wash, each tube was centrifuged at 2000xg for 1 minute at 4˚C. Finally, 50 µL of 4X SDS-PAGE loading dye was added to the protein A/G beads containing (co)-immunoprecipitants, and the samples were boiled for 15 minutes.

Western Blot

The samples from co-immunoprecipitation were loaded into the wells of an SDS-PAGE gel along with a protein ladder (Thermo Fisher scientific, Cat. BP3603-500). 20-30 µg of the samples were loaded, and the gel was run for about 1.5 hours at 100V. The Polyvinylidene fluoride (PVDF) membrane was then activated using methanol and rinsed with transfer buffer before preparing the stack. The stack was prepared by sandwiching the gel and the PVDF membrane between two sponges and two filter papers. The transfer was conducted for 45 minutes at 45V. After the transfer, the membrane was blocked with blocking buffer (5% non-

fat milk in TBS + 0.5% Tween 20 (1xTBST) for one hour at room temperature. A 1:1000 dilution of primary antibody in blocking buffer was then added to the membrane, which was incubated overnight at 4°C. The next day, the membrane was washed three times with TBST and incubated with a 1:5000 dilution of the appropriate secondary antibodies for an hour. Then, the membrane was washed three times with TBST for five minutes each. Finally, the blots were visualized using Radiance Q Luminol Substrate HRP- based chemiluminescence (AC2101, Azure Biosystems) and an Azure c300 gel imager was used to detect the bands.

RESULTS

Truncated Genes Were Cloned Into a Plasmid Vector

Previous research performed in the Lupfer lab had suggested a potential interaction between the proteins NLRP2 and EBP1. NLRP2 is made of three main domains, PYD, LRR and NACHT. To find out which domains are important for this interaction, each domain was separately truncated from the gene, PCR was used to amplify these truncated gene sequences and the truncated genes in the pcDNA 3.1 plasmid vectors were ligated and transformed into E. *Coli*. Following positive colony PCR and sequencing to confirm the mutation, each plasmid was purified prior to transfection to HEK293 T cells. The expression of the mutated proteins was checked via SDS- PAGE gel, followed by western blot to compare the protein size. The sizes of truncated proteins were smaller than the original NLRP2 protein and thus the mutagenesis was successful (Figure 5).

NLRP2 and EBP1 Interact With Each Other

To find out if NLRP2 and EBP1 interacted with each other, Myc- tagged NLRP2 and HA-tagged EBP1 vectors were co-transfected into HEK293 T cells. After the transfection, co-immunoprecipitation was performed and using the tag-specific antibodies of anti- Myc and anti- HA, immunoblotting was performed. Anti- Myc was used for NLRP2, and anti- HA was used for EBP1 for western blot. If these two proteins interact with each other, the NLRP2 and EBP1 should pull down together during co-immunoprecipitation and thus would be visible together when performing the western blot using tag specific antibodies (anti- Myc or anti- HA). Looking

at the Myc-NLRP2-EBP1 blot and HA-EBP1- NLRP2 blot, we could see the interaction between NLRP2 and EBP1 in the Myc blot (Figure 6), but the bands look pretty faint. We could clearly see the interaction between the proteins in the HA blot with solid visible bands (Figure 7) after repeating the experiment three times. This experiment confirmed their interaction.

EBP1 Did Not Interact With Domain Truncated NLRP2

To find out if NLRP2 and EBP1 interacted with each other, each of the domains truncated Myc- tagged NLRP2 and HA- tagged EBP1 vectors were co-transfected into HEK293 T cells. After the transfection, co-immunoprecipitation was performed and using the tag-specific antibodies of anti- Myc and anti- HA, immunoblotting was done through western blot. For PYD truncated NLRP2 blotted with Myc antibody (Figure 8), we cannot see any specific bands for both NLRP2 or EBP1. Because the size of the NLRP2 protein decreased after truncation, the protein is now the same size as the proteins that shows us the non- specific bands in the blot. So, it is possible that the protein that we are looking for is hiding behind those non- specific bands. For PYD truncated NLRP2 with HA blot (Figure 9), we can see a band for EBP1, but we cannot see any band for NLRP2 which shows the proteins did not get coimmunoprecipitated and thus the PYD truncated NLRP2 and EBP1 did not interact with each other.

Similarly, For LRR truncated NLRP2 blotted with Myc antibody (Figure 10), we cannot see any specific bands for both NLRP2 or EBP1, neither can we for LRR truncated NLRP2 with HA blot (Figure 11). We can see the EBP1 band, but we cannot see any band for LRR k/o NLRP2 which shows the proteins did not interact with each other.

Finally, the same process was repeated for NACHT truncated NLRP2. And the exact same result was seen. We cannot see any specific bands for both NLRP2 or EBP1 with Myc blot (Figure 12) and with HA blot (Figure 13). We can see the EBP1 band, but we cannot see any band for NACHT k/o NLRP2 which shows the proteins did not interact with each other.

DISCUSSION

Many studies have been conducted on NLR proteins regarding inflammation and immunity, but not much research has been done on these proteins in the context of embryogenesis and development. Several studies have shown that NLR proteins are associated with initiating both pro and anti-inflammatory signaling cascades and the formation of inflammasomes (Sundaram et al., 2024). Like other NLRs, NLRP2 could have a role in innate immune responses, but the specific functions are still controversial. Although NLRP2 can initiate inflammasome and promote inflammation, it can also downregulate inflammatory signals (T. Zhang et al., 2023). So, NLRP2 is mostly studied in the context of reproductive health and fetal development. NLRP2 is a member of the maternal effect gene and is found to be involved in NF-kB signaling pathway at different developmental stages (Fontalba et al., 2007). Research conducted on mice showed that the deletion of NLRP2 gene in oocytes and zygotes resulted in early embryonic arrest in these mice (Peng et al., 2017). Mutation in these genes has been associated with altered DNA methylation in the maternal oocyte and upon fertilization, the altered DNA methylation results in altered gene expression (Anvar et al., 2024). Since NLRP2 is not a DNA methyl transferase, it most probably interacts with other proteins to regulate the methylation. A protein EBP1, which is involved with DNA methylation by binding to the promoter region of DNMT1 and repressing its transcriptional expression (Ko et al., 2019) was one of the proteins we studied. A study on this protein-protein interaction might help us further understand the epigenetic mechanism of DNA methylation that cause idiopathic recurrent miscarriages.

In previous experiments carried out by the Lupfer lab, the NLRP2 and EBP1 interaction was identified through a yeast two-hybrid assay of a human HeLa cell cDNA library followed by coimmunoprecipitation and FRET analysis. In my research, the NLRP2 interaction with EBP1 was confirmed through co-immunoprecipitation. I performed this technique by precipitating NLRP2 and checking for EBP1 in the blot and by precipitating EBP1 and checking for NLRP2 using anti Myc and anti HA antibodies respectively. I could clearly see the coimmunoprecipitation (Figure 6-7) which confirms the two proteins interact.

However, we could not see any specific interaction of EBP1 with domain truncated NLRP2 vectors. In the Myc blot, we observed a lot of non- specific bands which made it difficult for us to interpret the blot. Also, because the non- specific protein bands are so thick, it could be possible that there was an interaction, but they are hidden under these concentrated non-specific proteins. So, further experiments need to be carried out to find a way to get rid of these non- specific bands so that we can see the interaction if they are co-immunoprecipitated. Also, because we could not see results with single domain truncation mutations, we could try double truncation mutation and follow the same protocol.

In conclusion, the interaction between NLRP2 and EBP1 was confirmed, but from this experiment, we could not confirm which domain of NLRP2 was important for this interaction. It is also possible that all three domains of NLRP2 are needed for the interaction. Sometimes, domain truncation can cause the protein to undergo conformational changes that can make the protein unstable and lead to misfolding or destabilization of the remaining protein structure, preventing it from adopting the correct conformation necessary for interaction. So, in the future, we could test the stability of the protein after making the mutations. Further

experiments need to be carried out addressing the troubleshooting like thick non- specific bands, unsuccessful transfections and problems with HEK293 T cells to make this experiment more successful. In this project, we only did single domain truncation. In the future, double domain truncation could be carried out to see the interaction of EBP1 with only one domain and compare the result with the results we got in this experiment, EBP1 has 2 isoforms: p48 and p42. For the future experiments, we could examine if p48 or p42 isoform of EBP1 interacts with NLRP2. Also, like NLRP2, EBP1 also contains three domains (NLS, σ70, AHD and LX). We can do domain truncation mutation for EBP1 and see which domain of EBP1 interacts with NLRP2 protein. And finally, we can induce some of the mutations observed in human patients with imprinting disorders to determine the effect of these mutations on the interaction between NLRP2 and EBP1.

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Table 1. Forward and reverse primer sequence of PYD, LRR and NACHT domains of NLRP2

Primers	Sequences
PYD Leucine (11)- Glutamic Acid (94)	Forward Primer- CTG CAG GCT CTC CTG GAG CAG CTC Reverse Primer- TTC TCT GAC TTC ATC CTT TGC TCT
NACHT Tyrosine (207)- Glutamic Acid (375)	Forward Primer- TAC ACG GTG GTG CTG TAT GGT CCT Reverse Primer- CTC GTC TCC AAA GTG TCT CAG GAA
LRR Leucine (785)- Threonine (1015)	Forward Primer- CTG CGA TAT CTC GGG TTG GTG TCT Reverse Primer- TGT CCG GAG GGT GCC ACT GGA ACA

Table 2. PCR conditions for Site directed mutagenesis

Steps	Temperature	Time	Cycles
Initial Denaturing	98°C	30 sec	1
Denaturing	98°C	10 sec	25
Annealing	PYD- 60°C LRR- 58°C NACHT- 71°C	30 sec	25
Extension	72°C	3 minutes	25
Final Extension	72°C	2 minutes	1
Hold	4°C	Indefinitely	-

Table 3. Antibodies used for co-immunoprecipitation and western blots

Antibody	Epitope	Catalog	Company
c-Myc Monoclonal Antibody	Myc-Tag (Mouse) EQKLISEEDL	121316	Invitrogen
HA-Tag Polyclonal Antibody	HA-Tag (Rabbit)	50-173-5882	Proteintech
Beta-Actin Monoclonal Antibody	(Rabbit)	PA1-981	Invitrogen

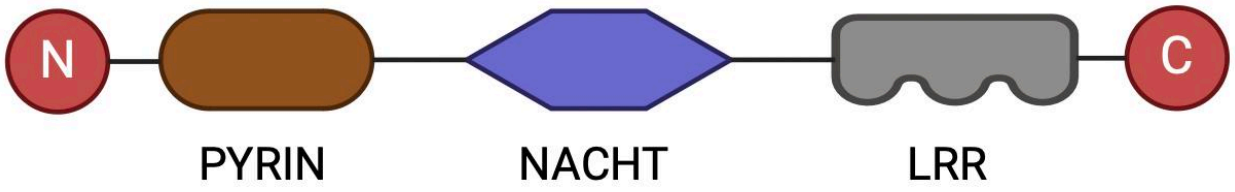

Figure 1. Domain structure of NLRP2. The figure shows its N- terminal Pyrin (PYD) domain, NACHT binding domain and C- terminal Leucine- Rich Repeat (LRR) domain

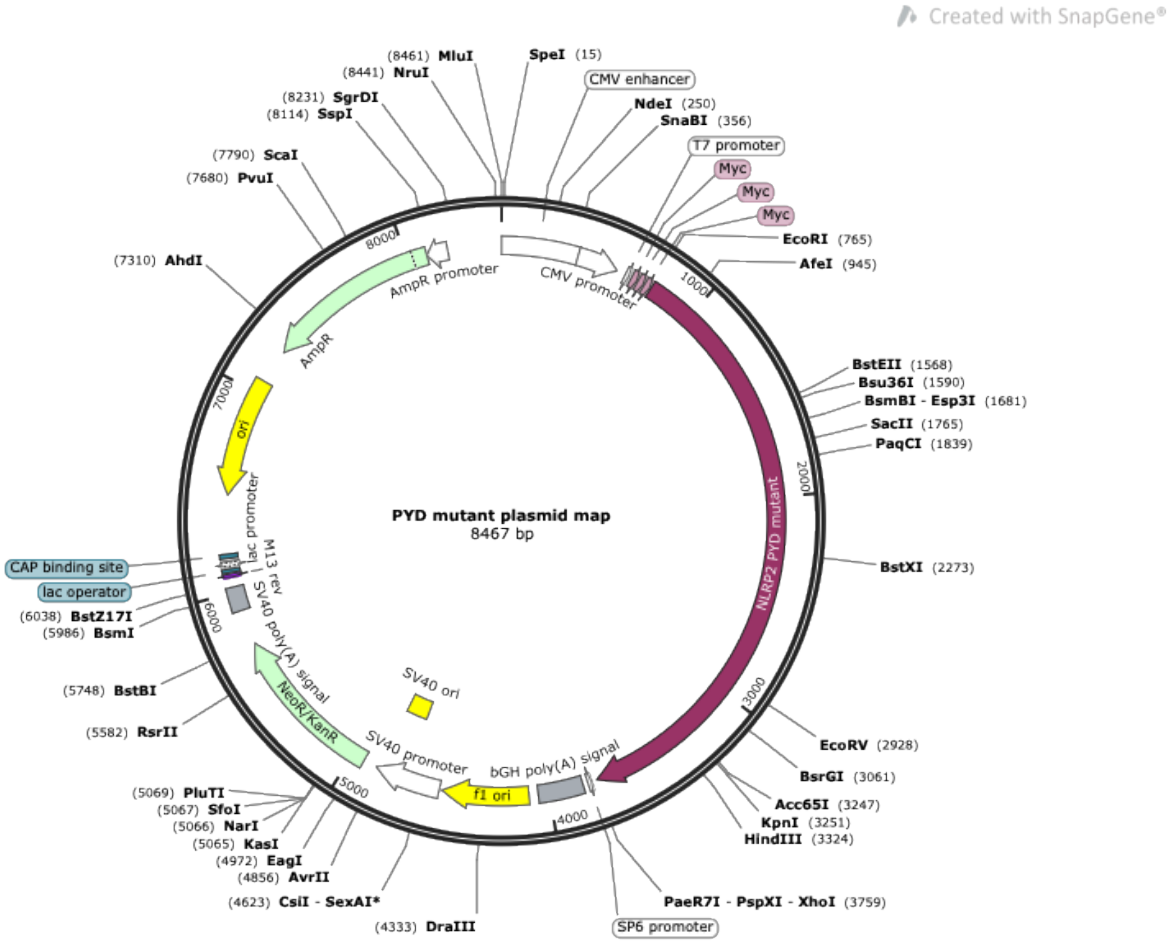

Figure 2. Plasmid map of PYD truncated NLRP2

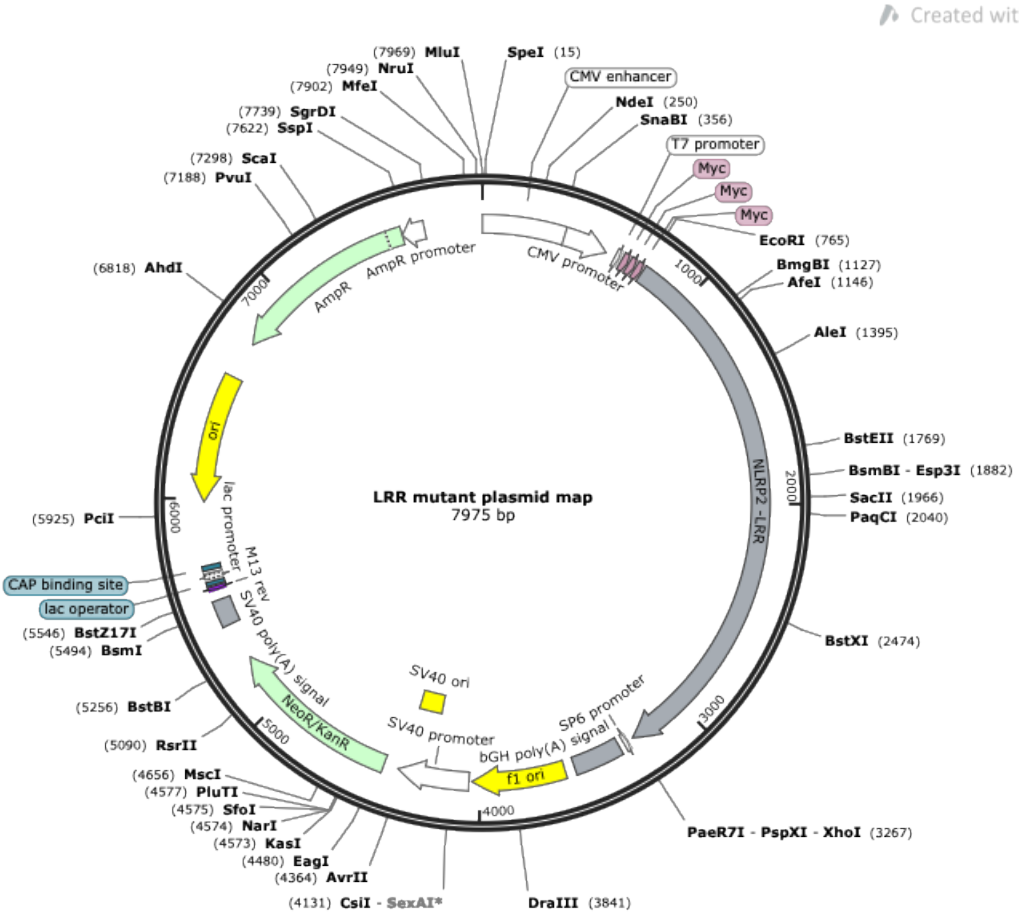

Figure 3: Plasmid map of LRR truncated NLRP2

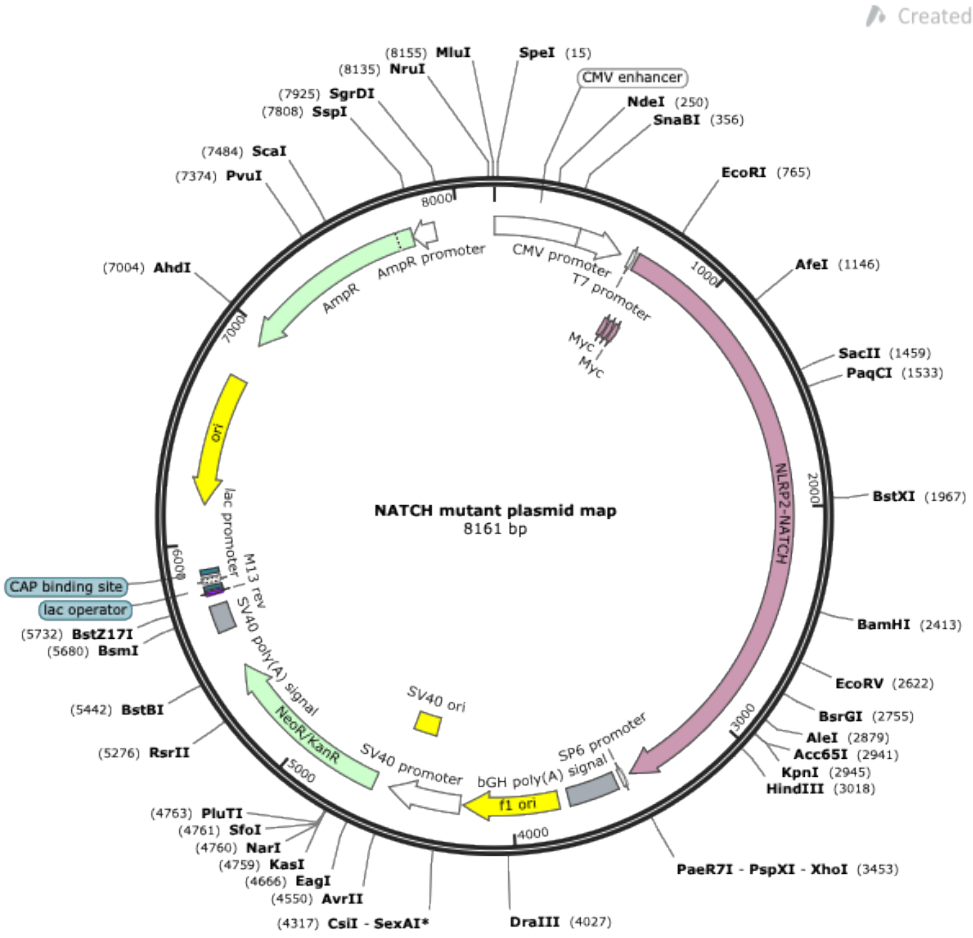

Figure 4. Plasmid map of NACHT truncated NLRP2

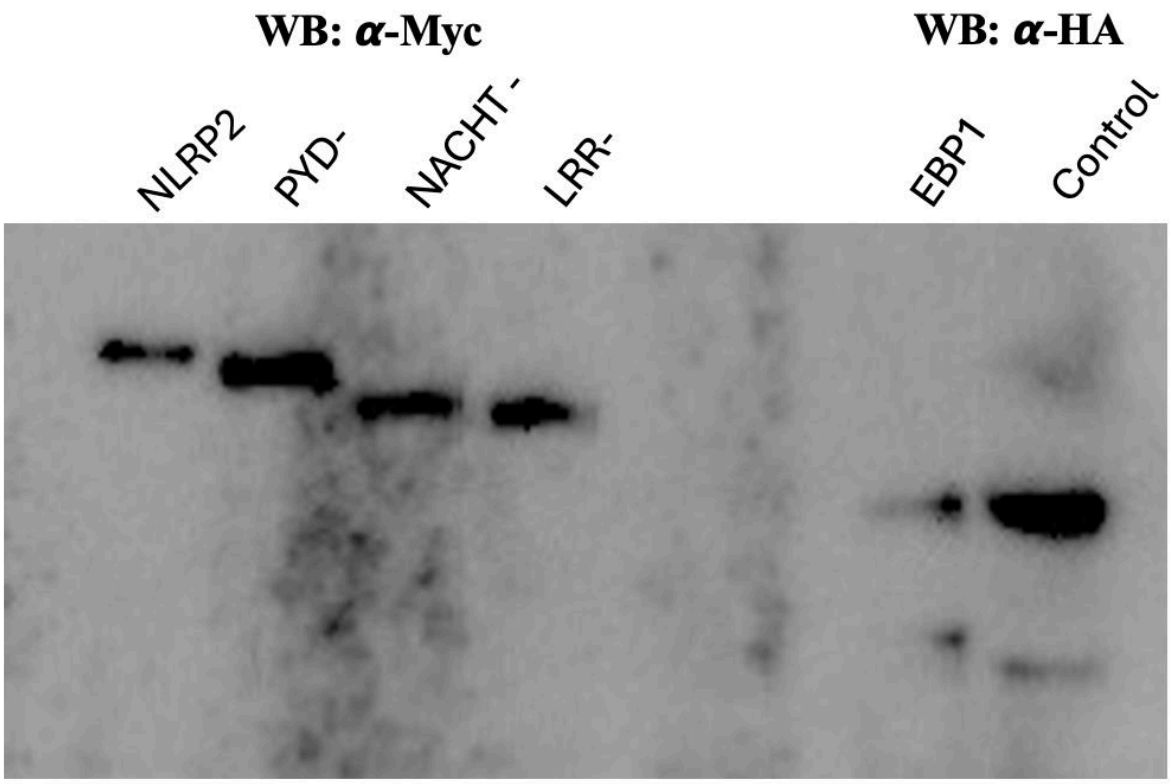

Figure 5. Western blot comparing size of proteins. Western blot shows the comparison of sizes of NLRP2 plasmid, EBP1 plasmid, PYD truncated plasmid, NACHT truncated plasmid and LRR truncated plasmid. The domain truncated NLRP2 plasmids are smaller than the regular NLRP2 plasmid which shows the cloning was successful.

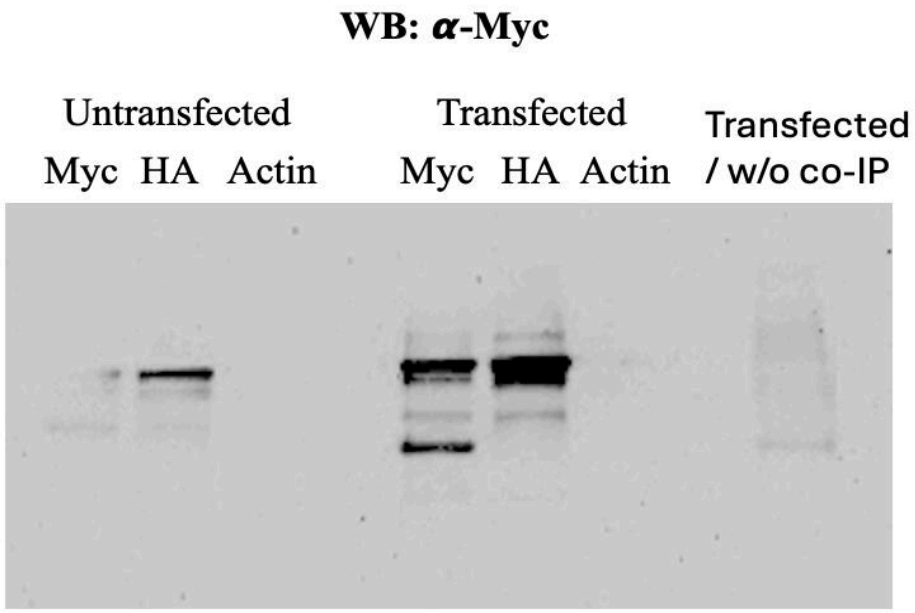

Figure 6. Co-immunoprecipitation western blot of EBP1 with NLRP2 (Myc antibody).
The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged NLRP2 and HA-tagged EBP1 in HEK293 T cells. Myc antibody was used for western blot analysis.

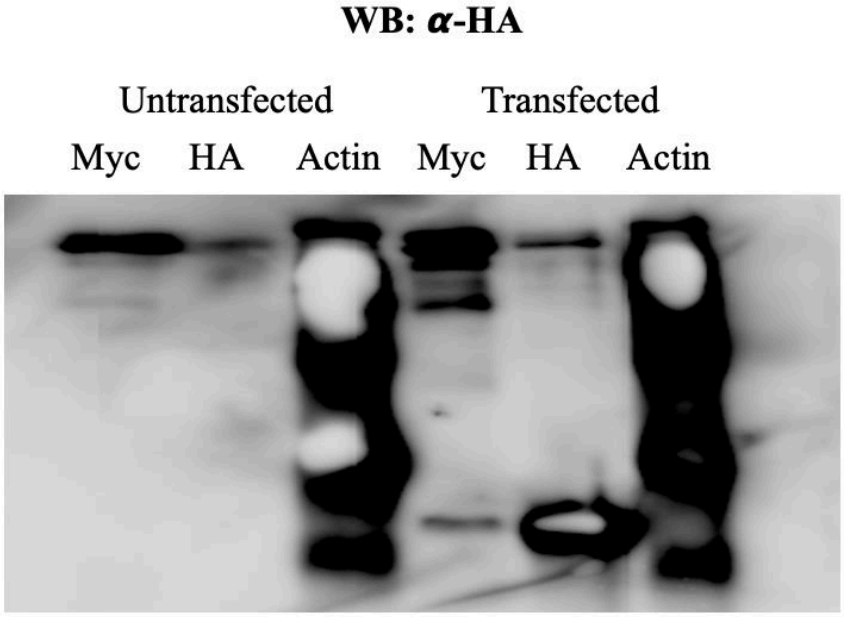

Figure 7. Co-immunoprecipitation western blot of EBP1 with NLRP2 (HA antibody).
The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged NLRP2 and HA-tagged EBP1 in HEK293 T cells. HA antibody was used for western blot analysis.

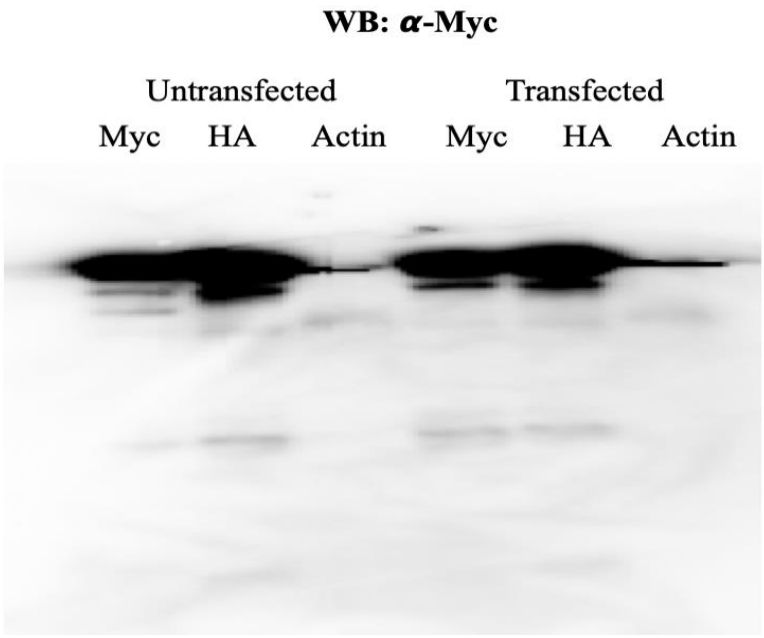

Figure 8. Co-immunoprecipitation western blot of EBP1 with PYD mutant (Myc antibody). The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged PYD truncated NLRP2 and HA-tagged EBP1 in HEK293 T cells. Myc antibody was used for western blot analysis.

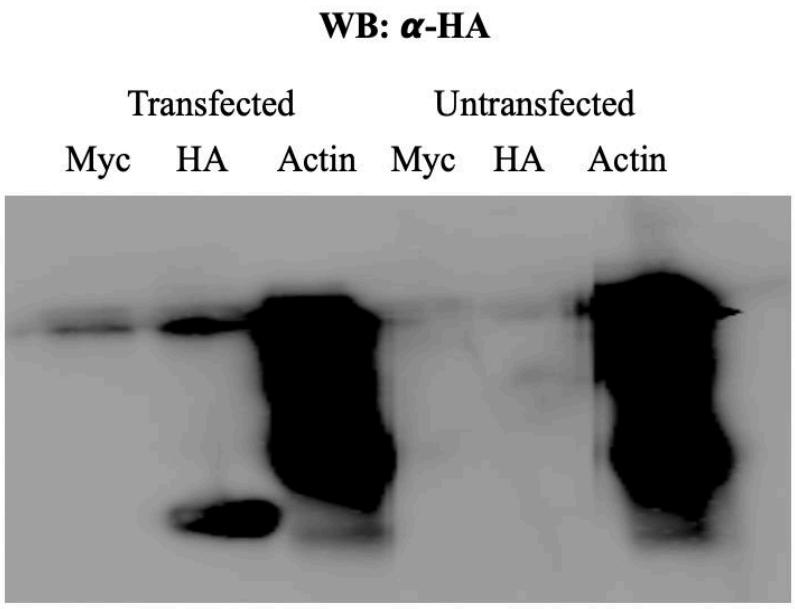

Figure 9. Co-immunoprecipitation western blot of EBP1 with PYD mutant (HA antibody). The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged PYD truncated NLRP2 and HA-tagged EBP1 in HEK293 T cells. HA antibody was used for western blot analysis.

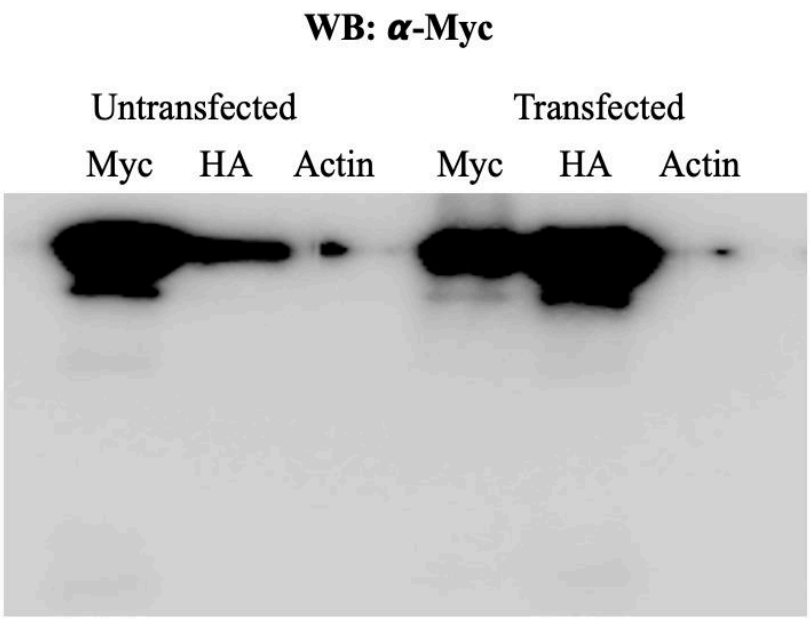

Figure 10. Co-immunoprecipitation western blot of EBP1 with LRR mutant (Myc antibody)
The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged LRR truncated NLRP2 and HA-tagged EBP1 in HEK293 T cells. Myc antibody was used for western blot analysis.

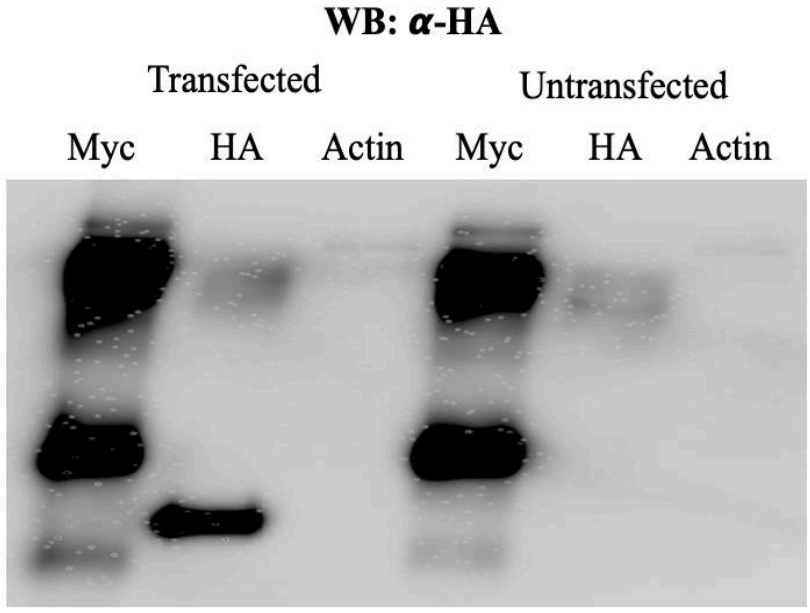

Figure 11. Co-immunoprecipitation western blot of EBP1 with LRR mutant (HA antibody). The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged LRR truncated NLRP2 and HA-tagged EBP1 in HEK293 T cells. HA antibody was used for western blot analysis.

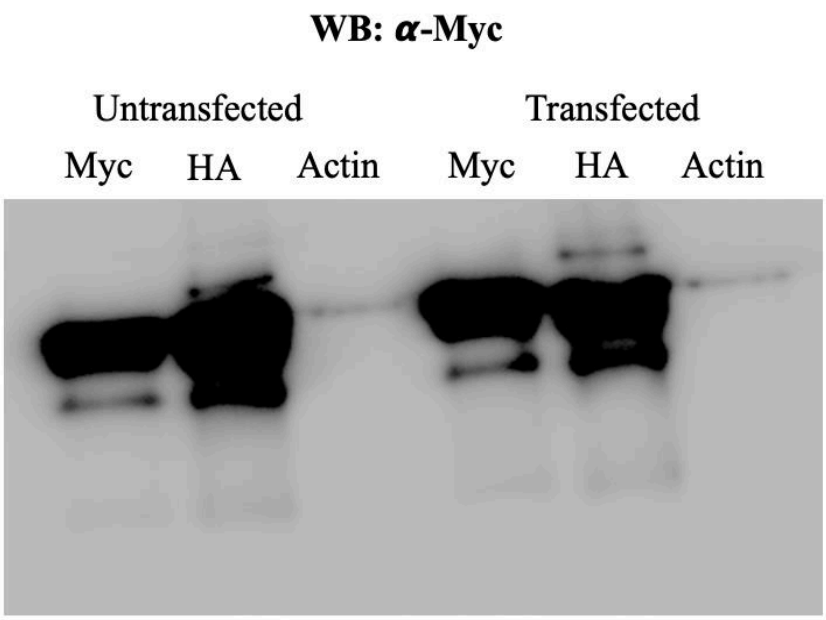

Figure 12. Co-immunoprecipitation western blot of EBP1 with NACHT mutant (Myc antibody). The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged NACHT truncated NLRP2 and HA-tagged EBP1 in HEK293 T cells. Myc antibody was used for western blot analysis.

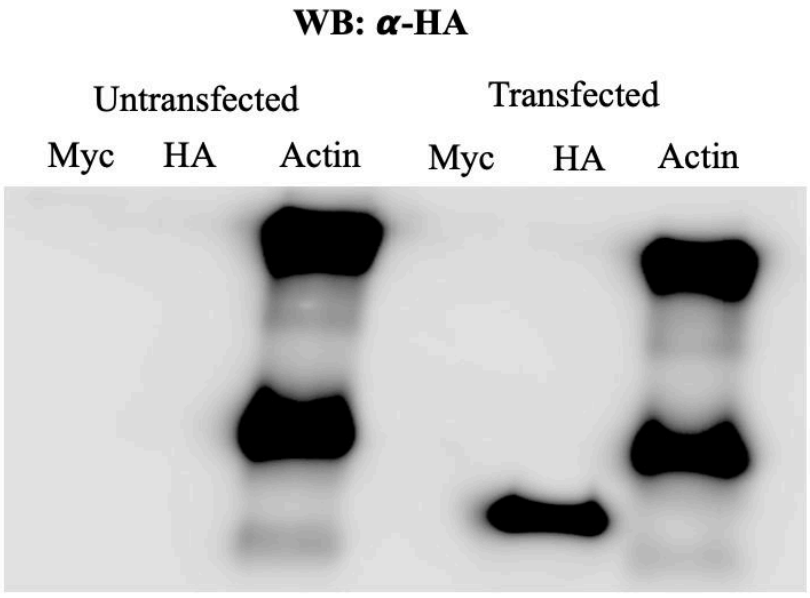

Figure 13. Co-immunoprecipitation western blot of EBP1 with NACHT mutant (HA antibody). The figure shows co-immunoprecipitation analysis of interaction between co-transfected Myc-tagged NACHT truncated NLRP2 and HA-tagged EBP1 in HEK293 T cells. HA antibody was used for western blot analysis.

APPENDIX: IBC APPROVAL

IBC approved 4/28/2022 to 4/27/2024

Missouri State University

MEMORANDUM OF UNDERSTANDING & AGREEMENT (MUA) FOR RECOMBINANT DNA EXPERIMENTS

All MUA'S can be submitted electronically to researchadministration@missouristate.edu or submitted as a hard copy to the ORA in Carrington 405. A signed copy must be provided. The *NIH Guidelines for Research Involving Recombinant DNA Molecules* should be used as a reference when completing this MUA (see http://oba.od.nih.gov/rdna/nih_guidelines_oba.html).

A. General Information

Date: April 22, 2022

Researcher Name: Christopher Lupfer

Researcher Title: Assistant Professor

Phone: 417-836-6887

Department: Biology

Office Bldg & Room #: Temple Hall, Room 254

Laboratory Bldg & Room #: Temple Hall, Room 232

Granting Agency: Planned grant applications to the National Institutes of Health

Grant Number (if applicable):

Title of Grant or Project: Mechanisms regulating NOD-like receptor function

B. Project Information

1. Describe the experiments involving recombinant DNA techniques. Your description is to be sufficiently complete so as to provide committee members an understanding of what you intend to do and how you will do it. A summary or abstract of your methods and materials section may also be provided if needed for clarity.

NOD-Like receptors are a class of innate immune receptors that have diverse functions in fighting infectious disease, cancer, and during fetal development. For example, NLRP3 and NLRC4 trigger the formation of the inflammasome, a macromolecular protease containing complex that cleaves the inactive forms of several inflammatory cytokines, including pro-IL-1β and pro-IL-18, into their active forms. Although the function of the inflammasome has been studied in numerous models of infection or inflammation, inflammasome activation in the absence of inflammation has never been examined. Furthermore, the mechanism of action of other NLR proteins like NLRP6 and NLRP12 have not been examined. Finally, NLRP2 and NLRP7 are structurally related to other NLRs, but appear to be important in fetal development and not during infection. To better understand the functions of NLRs, my lab will generate conventional overexpression plasmids (pCDNA3.1 or pCMV-Myc or similar vectors) and plasmids using the iDimerize™ Inducible Homodimer System (Clontech) or similar plasmids that have an inducible dimerization domain cloned in place of the protein interaction domains of caspase-1, its adaptor protein ASC or the pathogen sensing molecules NLRP3, NLRP6, NLRC4, NLRP12, NLRP2, NLRP7 and other NLRs as needed. Human and mouse cell lines such as HEK 293T cells, HeLa cells or RAW 264.7 macrophage cells (or similar cell lines) will be transfected with these plasmids and NF-kB signaling, inflammasome formation and caspase-1 activation examined by western blot. Cell death will also be determined as will the production of active IL-1β and IL-18 and other cytokines. Finally, novel protein interactions will be examined by immunoprecipitation and Western blot analysis of these tagged proteins that are activated in this system in the presence or absence of inflammation. Confocal microscopic imaging will elucidate the location and timing of the interactions. To examine protein interactions, the interacting protein will also need to be expressed in (pCDNA3.1 or pCMV-Myc or similar vectors). Current interactions of interest include Cul3, RPS8, COPS5, COPS6, IFIT1, SNAPIN, and many more. Ongoing studies will elucidate other novel interactions that cannot yet be listed, but will be human genes that interact with NLRs listed above.

In addition to these experiments, we will also generate vectors containing NLRP3, NLRC4, NLRP6, NLRP2, NLRP7 or NLRP12 for use in yeast-2-hybrid assays for the screening of novel interactions with these proteins and a library of cDNA from Clontech. The Clontech Match Maker 2-hybrid system or other similar plasmid system will be used.

2. Provide an assessment of the physical containment required for the experiments.

The DNA sequences to be cloned into the vectors are native to human or mice. The cells to be used in these experiments are of mouse (e.g. RAW 264.7) or human origin (e.g. HEK293T and HeLa cells). For bacterial propagation of the DNA, the cloned genes are not under control of bacterial promoters. For the yeast-2-hybrid system, all samples will be handled and disposed of as indicated below. Antibiotic or other selection markers are common to the field (e.g. Ampicillin for bacterial plasmids or histidine for yeast selection). Furthermore, the overexpression of these proteins is associated with increased cell death and therefore they are unlikely to provide any selective advantage. Therefore, standard precautions and decontamination procedures should be sufficient.

3. Describe the facilities and specific procedures which will be used to provide the required levels of containment.

Our laboratory in Temple 232 will be the site for all experiments. All cell culture equipment, bacterial culturing equipment, growth media and sterilization equipment are present in Temple 232. An autoclave is available in the microbiology prep room across the hall for decontamination.

All personnel working with recombinant DNA will wear disposable latex or nitrile gloves, a laboratory coat and eye protection. These must be worn at all times. Long pants and close toed shoes are also required. No eating (including chewing gum), drinking, applying cosmetics or contact lenses is allowed in the laboratory even when work with recombinant DNA is not taking place.

All purified recombinant DNA, transfected cells or transformed bacteria and yeast will be disposed of by incubation in a 10% bleach solution (final concentration) for a minimum of 5 minutes and/or by autoclaving the sample for 30 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags and autoclaved prior to being discarded. All work surfaces will be decontaminated prior to and following each procedure using 10% bleach with a contact time of at least 5 minutes. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

4. Describe the procedures and precautions to be followed if biohazardous organisms or agents are to be transported between laboratories.

If purified DNA plasmid or bacteria expressing the plasmids need to be transported, then samples must be sealed in a shatter resistant container (such as a threaded-cap polypropylene plastic test tube) and the outside of the container decontaminated with 10% bleach. The shatter resistant container must then be placed in a leak proof secondary container with absorbent material (small paint can filled with paper towels etc. and then properly sealed). Alternatively, a drop of purified DNA can be placed on a piece of filter paper and air dried. This filter paper can then be placed in a plastic bag and the bag placed in a shipping envelope. This has the advantage of avoiding the shipment of liquid cultures or samples.

5. Describe the waste disposal procedures expected to be used during this experiment.

All purified recombinant DNA, transfected cells or transformed bacteria and yeast will be disposed of by incubation in a 10% bleach solution (final concentration) for a minimum of 5 minutes and/or by autoclaving the sample for 30 minutes. All contaminated materials (pipet tips, gloves, tissue culture plates, old samples or cultures, etc.) will be disposed of in biohazard bags and autoclaved prior to being discarded. All work surfaces will be decontaminated prior to and following each procedure using 10% bleach with a contact time of at least 5 minutes. In addition to regular decontamination after every procedure, weekly decontamination of workbenches and other surfaces in the lab will also be performed using a 10% bleach solution.

6. Will this project involve environmental release? If yes, please provide a description of the release.

No.

7. Please list all students, staff and faculty involved with this project. CITI Biosafety training is mandatory for all personnel working with biohazards prior to final IBC approval.

Dr. Lupfer
Nayeon Son
Catherine Rippe
Rakshya Bhatta

8. The undersigned agree to certify the following conditions of the proposed research:

a. The information above is accurate and complete.
b. We agree to accept responsibility for training of all laboratory workers involved in the project.
c. We agree to comply with all appropriate requirements pertaining to shipment and of hazardous biological and recombinant DNA materials.
d. We are familiar with and agree to abide the provisions of the Missouri State University policies and procedures applicable to experiments involving recombinant DNA, the provisions of the current *NIH Guidelines for Research Involving Recombinant DNA Molecules,* and any other specific instructions pertaining to the proposed project.

Via email 4/28/2022

Principal Investigator	Date	Department Head	Date

9. The Institutional Biosafety Committee has determined, based on information provided the principal investigator, that:

a. No special medical surveillance (other than usual University health programs) is required for the project described in this MUA

b. The following specific medical surveillance procedures must be carried out, for individuals listed by name, before commencing the project described in this MUA:

10. We certify that the Missouri State University Institutional Biosafety Committee has reviewed the proposed project for recombinant DNA experiments for compliance with the current *NIH Guidelines for Research Involving Recombinant DNA Molecules* and Missouri State University's policies and procedures applicable to experiments involving recombinant DNA. The MSU IBC will monitor throughout the duration of the project the facilities, procedures, and the training and expertise of the personnel involved in the recombinant DNA activity.

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MSU IBC Chair or Representative Date