Investigating the role of filamins in innate immune signaling and during VACV infection

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Abstract

Multiple signaling cascades are triggered after viral infection of mammalian cells, leading to an innate immune response to restrict viral replication and spread by inducing expression of proinflammatory and antiviral proteins. Vaccinia virus (VACV) is a poxvirus with a large double-stranded DNA genome, dedicating ~30% of this to encode non-essential proteins for replication *in vitro*, but aid immune evasion and viral virulence. C4 is such a protein, which is expressed early during infection and has been found to inhibit both NF-κB signaling and DNA-PK-mediated DNA sensing (Ember *et al.*, 2012; Scutts *et al.*, 2018). For both functions the C4 C-terminus is required. Unpublished data from this lab have shown that C4 with its N-terminus, which contributes to virulence *in vivo*, also binds filamins (FLNs). FLNs are large cytoskeletal proteins with actin-binding properties, also implicated in other cellular processes by acting as scaffold for signaling molecules (Zhou *et al.*, 2010). In this project, the role of FLNa and FLNb in innate immune signaling was investigated by luciferase reporter gene assays, whereas the role of FLNb in VACV restriction was also tested. Overexpression of only FLNa, but not FLNb, was found to inhibit multiple signaling pathways involved in type I interferon (IFN) response, including IRF-3 and JAK-STAT in a dose-dependent manner. Finally, although FLNb did not affect any of the signaling pathways under the conditions tested, it has been found on this project to restrict VACV replication and/or spread. Overall, we identified the effects of filamins on innate immune signaling pathways and the effect of absence of cellular FLNb on VACV infection.

1. Introduction

1.1 Vaccinia Virus

Vaccinia virus (VACV) is a member of the *Orthopoxvirus* genus, within the *Poxviridae* family. VACV is a large, enveloped virus with a linear, monopartite double-stranded DNA genome, which is common to this family. The genome of VACV is replicated in the cytoplasm of infected cells (Moss, 2007), which is a trait unusual among DNA viruses, specifically possessed by Poxviruses. VACV is widely known of being used as the live vaccine to eradicate a highly virulent and lethal disease, smallpox (Fenner *et al.*, 1988), which was caused by another virus in the same genus, Variola virus, until its eradication declared by the World Health Organization (WHO) in 1980. Research around VACV has continued after this, due to its potential as a vector for the expression of foreign genes and its capacity to accept foreign DNA with a size up to approximately 25 kilobase pairs (Smith *et al.*, 1983). This establishes VACV as a great tool for the generation of gene-recombinant polyvalent live vaccines able to induce both humoral and T-cell mediated immunity towards foreign antigens (Bennink *et al.*, 1984). Engineered VACV can be used in oncolytic virotherapies, and also acts as a very useful tool in the study of virus-host interactions (Cudmore *et al.*, 1995; Alcami & Smith, 1996). The genome of VACV is around 200 kilobase pairs encoding approximately 200 open reading frames (ORF) (Goebel *et al.*, 1990). Between one-third and one-half of these are dedicated to genes encoding proteins non-essential for the viral

replication in culture, but may enhance virulence and/or survival in mammalian hosts. These genes are mostly located in the terminal regions of the genome, while essential genes are found in a highly conserved central region. Many of these non-essential genes that enhance virulence encode immunomodulatory proteins, which act to manipulate and sabotage multiple immune signaling and response pathways, and thus the host innate immune response to viral infection (Seet *et al.*, 2003).

1.2 Innate Immune Signaling Pathways

The pattern recognition receptors (PRRs) within host cells detect VACV infection, and initiates an innate immune response to restrict the viral replication and spread. Such responses mainly consist of the production of pro-inflammatory and antiviral protein including cytokines, chemokines and IFNs, which have both direct antiviral effects and can indirectly render infected cells more resistant by inducing antiviral protein synthesis and other innate responses. In stimulating expression of these proteins, transcription factors of the interferon regulatory factors (IRFs) and nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) families play crucial roles (Lin & Zhong, 2015; Takeuchi & Akira, 2010). In the response of cells to interferons, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, stimulating the signaling through interferon stimulated gene factor 3 (ISGF3) and interferon stimulated response elements (ISRE) to induce interferon stimulated gene (ISG) expression also plays an important role (Ivashkiv & Donlin,

2014). Activator Protein 1 (AP-1) signaling also contributes to this innate immune response (Hess *et al.*, 2004). These pathways can be illustrated by Figure 1.

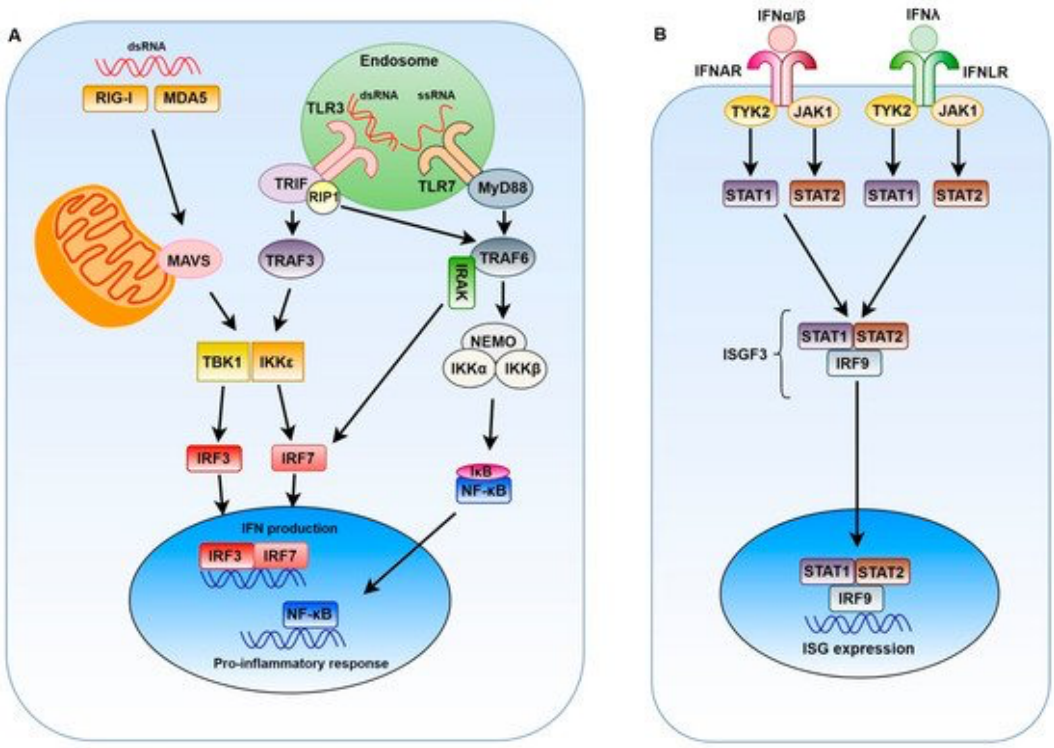

Figure 1. Innate Immune Signaling Pathways (Nelemans & Kikkert, 2019)

(A) IFN production is induced when nucleic acids are detected in the cell. Signaling through these pattern recognition receptors (PRRs) results in the translocation of NF-κB, IRF3 and IRF7 to the nucleus. These transcription factors then initiate production of IFNs. **(B)** Type I IFNs bind to their receptor composed of IFNAR1 and IFNAR2, resulting in the expression of ISGs.

A significant proportion of VACV genome is dedicated to encoding immunomodulators suppressing or evade this rapid innate response to enhance replication and spread by circumventing this mechanism. These proteins act in a wide variety of ways, with many functioning extracellularly to neutralize immune effectors including complement, IFNs, cytokines or chemokines, while others act intracellularly to inhibit apoptosis or innate immune signaling pathways mentioned above (Smith *et al.*, 2013).

1.3 VACV C4 Protein

One prototypical member of VACV non-essential gene that interferes with innate immune response is protein C4, encoded by gene C4L, with a size of 37.2 kDa. C4 is conserved within other *Orthopoxviruses,* suggesting an important function. C4 also shows 43% amino acid identity to VACV protein C16, an immunomodulatory protein affecting virulence (Fahy *et al.*, 2008). with similar structures on C-terminus and exhibiting similar functions. C4 has been found to be able to inhibit the DNA-PK-mediated DNA sensing pathway by binding to Ku proteins (Scutts *et al*, 2018), and the activation of NF-κB via an unknown mechanism at or downstream of the IKK complex (Ember *et al.*, 2012), and for both roles the C-terminus of C4 is required. Despite being the ninth VACV protein assigned this function, deletion of C4 from the VACV genome caused attenuation of virus virulence. However, C4 and C16 are functionally and structurally distinct at their N-terminus. Mass spectrometry data indicate that C4 binds to the cellular proteins filamin a (FLNa) and filamin b (FLNb). This interaction has been mapped on the N-terminus of C4 and it has been proven to be direct (unpublished data, Smith lab).

1.4 Filamin Proteins

Filamins (FLN) are large cytoskeletal proteins with actin-binding properties that can stabilize 3D actin filament networks and link them to cellular membranes, thus playing an important role in cell architecture and motility functions (Zhou *et al.*, 2010). The mammalian FLN family has three members: FLNa/b/c, which have

about 70% amino acid identity overall (van der Flier & Sonnenberg, 2001). All three FLNs are widely expressed during development; in adults, FLNc expression is predominantly limited to skeletal and cardiac muscle cells (Stossel *et al*., 2001). FLNs are elongated V-shaped dimer proteins with two polypeptide chains sized 240~280 kDa. Each monomer chain consists of an F-actin-binding domain at the N-terminus and a rod segment consisting of up to 24 homologous repeats of ~96 amino acid residues adopting an immunoglobulin-like fold (Ig repeats). Two hinge regions separate the 24 Ig repeats into two rod domains between repeats 15 and 16, and repeats 23 and 24. They dimerize via the repeat 24, which also anchors F-actin to the cell membrane. FLNs are also found to be implicated in other cellular processes by acting as a scaffold for signaling molecules. Additionally, experiments on FLNb knockout mouse embryonic fibroblast (MEF) cells showed a significant increase in plaque size compared to wild type cells when infected by VACV (unpublished data, Smith lab).

1.5 Aim

The aim of my project was to build on the existing work on C4 and FLNs by:

i. Characterizing the role of FLNa and FLNb in innate immune signaling pathways.

ii. Generating CRISPR/Cas9 knock-out HeLa cell lines for FLNa and FLNb.

iii. Identifying the effect of FLNs on viral replication and/or spread by employing the knock-out HeLa cell lines.

2. Materials and Methods

2.1 Maintaining and Passaging Cells

HEK293T (human embryonic kidney epithelial cell line) or HeLa (human cervical cancer cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, PAN Biotech) and 50µg/ml of penicillin/streptomycin (Pen/Strep, Gibco). Cells were incubated at 37°C, in 5% CO_2. For passaging them, cells were washed with Phosphate Buffered Saline (PBS, Sigma Aldrich) and then incubated with Trypsin-EDTA (0.125%, Gibco) at 37°C until they were detached. Detached cells were re-suspended in medium and split accordingly into a new tissue culture flask.

2.2 Plasmids

The different plasmids that were used in this project are the mentioned on the table below (Table 1).

Table 1: Plasmids used in this project.

Plasmid	Description
pcDNA3-FLAG (EV)	Mammalian Expression Vector, Amp Resistance
C6-TAP	TAP-tagged VACV C6
HA-FLNa	HA-tagged Filamin A
HA-FLNb	HA-tagged Filamin B
TRAF6-FLAG	FLAG-tagged TRAF6
B14-TAP	TAP-tagged VACV B14
Spir1-Myc	Myc-tagged Spir1
pRL-Luc	Renilla Luciferase Control Reporter
NFκB-Luc	NFκB Luciferase Reporter

Table 1: (cont.) Plasmids used in this project.

ISRE-Luc	ISRE Luciferase Reporter
ISG56.1-Luc	ISG56.1 Luciferase Reporter
AP1-Luc	AP1 Luciferase Reporter
RIGI-CARD	FLAG-tagged RIG-I
pSpCas9(BB)-2A-Puro (PX459)	Plasmid with Cas9 and cloning backbone for sgRNA

2.3 Transfection of Cells

Cells were transfected with either 3µl of PEI (Thermo Fischer) or LT1 (Mirus Bio) transfection reagent (HEK293T or HeLa cells, respectively) together with 50µl of OptiMEM (Gibco) per µg of DNA. OptiMEM and transfection reagent were mixed and incubated for 5mins at RT. Finally, the DNA was added into the mix and a further incubation for 20mins at RT was taking place. After the incubation the mixture was added dropwise onto monolayers.

2.4 Dual Luciferase Reporter Gene Assay

Luciferase reporter gene assays were performed with HEK293T cells, grown to 50-60% confluence and seeded into 96-well plates at $2.5x10^5$/well. The cells were transfected with the reporter plasmids containing the firefly luciferase gene (driven by the promoter of interest) and the constitutively active *Renilla* luciferase plasmid (Table 1)., For that, 70ng of the firefly luciferase reporter and 10ng of the *Renilla* luciferase plasmid were transfected per each well of a 96-well plate. Together with the above, up to 200ng/well of expression plasmid or pcDNA3.1 empty vector (EV) control were also used. The amount of expression

plasmid transfected varied to correct for protein expression levels, in order to achieve similar levels of expression for each. Transfections were carried out in 2% DMEM, and cells were then incubated for 24h. Cells were either unstimulated, or stimulated for 6h by the addition of IFNα (10^3U/ml), IL-1β (25ng/ml) or TNFα (50ng/ml), or for 24h by the addition of PMA (10ng/ml), then lysed using passive lysis buffer (Promega). Cells additionally transfected with the TRAF6-FLAG or RIGI-CARD plasmid (5ng/well) were not exogenously stimulated and were lysed 24h after transfection using passive lysis buffer (Promega). In both cases, 10ul of the lysates were used to measure both, Firefly and *Renilla* luciferase activities with a luminometer (LUMIstar Omega, BMG LABTECH).

2.5 Generation of Knock-out HeLa Cell Lines

Following existing protocol (Zhang Lab, MIT), Pre-designed guide RNA (by Dr. Georgana) for FLNa and FLNb, were phosphorylated and annealed, and finally cloned into the px459 CRISPR/Cas9 engineering vector. Plasmids were then expanded in Stbl3 Competent Cells (Thermo Fischer Scientific) at 30°C and sequenced. CRISPR/Cas9 Knockout were performed with monoclonal HeLa cells, grown to 50-60% confluence and seeded into 10cm dishes at $4x10^6$/plate. The cells were then transfected as indicated in section 2.3, with 3μg of expression plasmid or px459 empty vector (EV) control (Table 1). An untransfected control was also performed. After 2 days post-transfection, cells

underwent Puromycin (2µg/ml, Gibco) for 3 days. When the cells in untransfected control plates are completely killed, cells were trypsinized, resuspended and cultured in 10% DMEM with Pen/Strep. Cells were then seeded into 96-well plates at 0.5 cell/well and incubated for 3-4 weeks. Due to time limitations, validation of the single colonies was carried out by Dr Georgana.

2.6 Viral Infection and Plaque Measurement

Parental cell line, two EV controls and two confirmed FLNb knockout clones were seeded in a 6 well plate and reached 80% confluency. Cells were then infected with the GFP-A5 VACV in 200pfu (plaque forming units) /well. Infections were carried out in 500µl/well of 2% DMEM, and cells were then incubated for one hour at 37°C and 5% CO2, and plates were shacked every 10-15 mins. After one hour, monolayers were rinsed and overlaid with 2ml/well of Minimal Essential Medium (MEM, Gibco) solution. The cells were then incubated at 37°C and 5% CO2. The plaques formed were imaged using an Axiovision fluorescent microscope (Carl Zeiss AG) at 72 hours post-infection, with pictures of 30 plaques per sample taken. The plaque sizes (area) were then measured using ImageJ Software, with a sample size of 20 plaques per cell line.

2.7 SDS-PAGE and Immunoblotting

Samples were prepared by taking 40µl of cell lysate from each replicate, in total 120µl per condition and adding loading dye (0.5 M pH 6.8 Tris 250 mM, 6% SDS, 40% glycerol, 0.02% bromophenol blue, 8% v/v β-mercaptoethanol), before boiling at 95°C for 5 min. 15µl of cell lysate was run for each condition in reporter gene assays, while 30µl was run for the confirmation of knockout cell lines. Proteins were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 10% or 12% polyacrylamide resolving gels, under reducing conditions in Tris-Glycine SDS (TGS) running buffer (0.25 M Tris-HCl pH 8.6, 1.92 M glycine, 1% (w/v) SDS), and transferred at 25 V for 75 mins onto a Hybond ECL nitrocellulose membrane (GE Healthcare) pre-soaked in Tris-Glycine (TG) transfer buffer (20 mM Tris-HCl pH 8.3, 150 mM glycine, 20% methanol). After protein transfer the membranes were blocked in milk-PBST (Phosphate buffered saline supplemented with Tween-20) blocking solution (5% milk in Phosphate buffered Saline (PBS), with 0.1% TWEEN-20) for 30 min, and then incubated with primary antibodies at 4°C overnight. Primary antibodies used were rabbit anti-FLAG (1:2000 dilution, Sigma), mouse anti-FLNb (1:1000, Invitrogen), mouse anti-HA (1:1000, Sigma), mouse anti-Myc (1:2000, Sigma) and mouse anti-tubulin (1:5000, Upstate Biotech), diluted in milk-PBST blocking solution. Blots were washed 3 times in PBST for 5 mins, and then incubated with IR Dye-conjugated secondary antibodies (LI-COR Biosciences) at 1:10,000 dilutions

(again in milk-PBST blocking solution) for 60 min, then washed 3 times in PBST for 5 mins. Secondary antibodies used were goat anti-rabbit red and goat anti-mouse green, and the dried membranes were imaged using an Odyssey infrared scanner (LI-COR Biosciences).

2.8 Data and Statistic Analysis

2.8.1 Analysis of Reporter Gene Assays

The firefly luciferase activity was first normalized to the *Renilla* luciferase activity, and these data were then further normalized to either the corresponding non-stimulated control cells (transfected with the same plasmids), or to the non-stimulated EV control, producing two sets of data for each experiment. The graphs were then plotted as mean values ± standard deviation (SD) using GraphPad Prism 8.0.1, with which statistical analyses were also performed.

2.8.2 Analysis of Plaque Size Assays

The plaque sizes of each cell line were plotted as scatter dot plots with mean values ± standard deviation (SD) using GraphPad Prism 8.0.1, with which statistical analyses were also performed. Significance stars were calculated using unpaired, two-tailed Student's t-tests, with statistical significance demonstrated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3. Results

3.1 Reporter Gene Assays for Innate Immune Signaling Pathways

3.1.1 Dose-dependent effect of FLNa/b on NFκB Signaling, stimulated by TRAF6 Overexpression

In order to determine the effects and their dose-dependency of FLNa and FLNb proteins on the NF-κB signaling pathway, multiple luciferase reporter gene assays were performed. It was hypothesized that the effect of filamins on NF-κB signaling pathway would likely be due to their activity as scaffolds, and so filamins was expected to produce greater effect at higher concentrations. To investigate this, we conducted luciferase reporter gene assays to assess dose-dependent responses. Codon optimized plasmid constructs for full length (FL) Filamin a (FLNa) or Filamin b (FLNb) in different doses per well (200ng, 100ng, 50ng, 25ng, 10ng for FLNa and 100ng, 50ng, 25ng, 10ng, 5ng for FLNb), C6 (5ng/well) and B14 (50ng/well) were transfected into HEK-293T cells, along with a firefly luciferase reporter plasmid under the control of an NF-κB-dependent promoter sequence. As described in the introduction, VACV protein B14 is a potent inhibitor of the NF-κB pathway, and so was used as a positive control in these assays. The VACV C6 protein was used as a negative control. The B14 and C6 constructs were TAP-tagged, while FLNa/b were labelled with an N-terminal HA-tag. These allowed detection of protein levels by Western Blotting.

The cells were stimulated by transfection of TRAF6 (labelled with a FLAG-tag), and the effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to that of the corresponding non-stimulated cells transfected with the empty vector, referred to as non-stimulated EV (NS EV).

The transfection of TRAF6 produced an approximately 15-fold increase in reporter activity (demonstrated by the empty vector, EV) in the FLNa experiment, which was significantly reduced by ~12 fold with the addition of either B14 or 200ng/well FLNa transfection, and the level of NF-κB signaling inhibition by FLNa rose with increasing doses of the protein, indicating that FLNa had a dose-dependent inhibition effect on NF-κB signaling. Samples of cell lysate from both non-stimulated and stimulated cells were analyzed by immunoblotting and compared to a tubulin loading control, to confirm both the successful transfection of the plasmids used, and that similar levels of the B14, C6 and TRAF6 constructs were expressed. Western blotting confirmed the increasing expression of the FLNa protein in the cells as plasmid dose was increased (Fig. 2a).

The transfection of TRAF6 produced an approximately 30-fold increase in reporter activity (demonstrated by the empty vector, EV) in the FLNb experiment, which was significantly reduced by ~25-fold with the addition of B14, similar to the FLNa experiment. However, no significant effect of FLNb on the NF-κB signaling pathway was found. Corresponding Western Blots for this experiment were again run, demonstrating the expression levels of proteins transfected into cells (Fig. 2b).

In future assays determining the effects of filamins on innate immune signaling pathways, we decided to use 100ng, 50ng for FLNa and 50ng, 25ng for FLNb as doses of experiment.

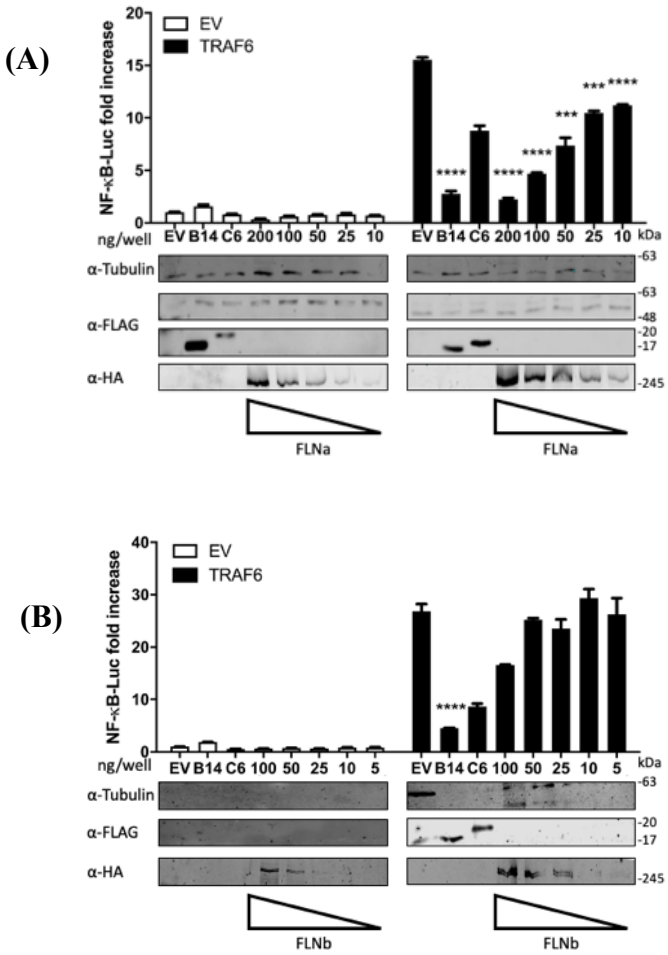

Figure 2. FLNa dose-dependently inhibits NF-κB signaling while FLNb does not.

(a,b) HEK-293T cells were transfected with plasmids encoding the indicated proteins, or with an empty vector (EV) control, along with an NF-κB promoter firefly luciferase reporter plasmid and a *Renilla* luciferase control plasmid. The cells were stimulated by transfection of TRAF6 (labelled with a FLAG-tag) and lysed 24h post-transfection, and the effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to that of the corresponding non-stimulated cells transfected with the empty vector, referred to as non-stimulated EV (NS EV). Data are represented as mean ± SD, and are representative of those from 3 experimental repeats. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.1.2 Determination of the effects of FLNa/b on NFκB Signaling, stimulated by real agonists IL-1β or TNFα

In order to confirm the previous data that FLNa inhibits the NF-κB signaling pathway whereas FLNb does not have this effect, multiple repeats of luciferase reporter gene assays were performed, with similar transfection protocols as in the dose-dependency assay experiments.

The cells were then stimulated by the addition of real agonists to the NF-κB signaling pathway, either interleukin-1β (IL-1β), or tumor necrosis factor alpha (TNFα), and the effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to either that of the non-stimulated cells transfected only with empty vector (NS EV), or to that of the corresponding non-stimulated cells transfected with the same plasmids, referred to as non-stimulated self (NS self).

The addition of IL-1β produced a poor stimulation with approximately 5-fold increase in reporter activity, which was unable to give any significant result. Therefore, this experiment is not included in this report.

The addition of TNFα produced an approximately 20-fold increase in reporter activity, which was significantly reduced by ~16-fold with the addition of B14 in both normalizations. No significant effect of FLNb was discovered in this experiment, which was similar to the results of the dose-dependency assays. However, for FLNa protein, a discrepancy was seen between the data graphed relative to NS EV, and that graphed relative to NS self. The data indicated dose-

dependency of inhibition by FLNa when compared to the NS EV data, but showed no significant inhibition by FLNa when compared to the NS self data (Fig. 3a, b). The corresponding Western Blots for this experiment were again run, demonstrating the expression levels of proteins transfected into the cells.

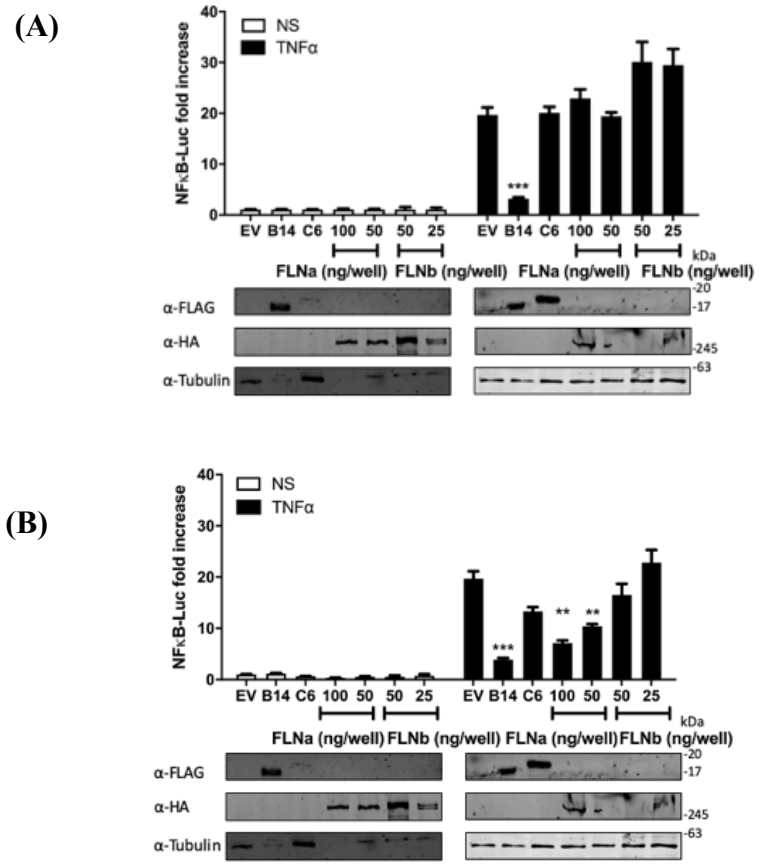

Figure 3. FLNa inhibits NF-κB signaling stimulated by TNFα when normalized to NS EV.
HEK-293T cells were transfected with plasmids encoding the indicated proteins, or with an empty vector (EV) control, along with an NF-κB promoter firefly luciferase reporter plasmid and a *Renilla* luciferase control plasmid. The cells were stimulated for 6h by the addition of real agonists to the NF-κB signaling pathway, either interleukin-1β (IL-1β), or tumor necrosis factor alpha (TNFα) 24h post-transfection and then lysed. The effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to either that of the non-stimulated cells transfected only with the same plasmids **(a, NS self)**, or to that of the corresponding non-stimulated cells transfected with the empty vector **(b, NS EV)**. Data are represented as mean ± SD, and are representative of those from 3 experimental repeats. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.1.3 Determination of the effects of FLNa/b on IRF-3 Signaling

To discover the effects of filamins on other signaling pathways, we also performed luciferase assays on IRF-3 signaling pathway. Codon optimized plasmid constructs for full length (FL) Filamin a (FLNa) or Filamin b (FLNb) in different doses per well (100ng, 50ng for FLNa and 50ng, 25ng, for FLNb), C6 (5ng/well) and Spir1 (50ng/well) were transfected into HEK293T cells, along with a firefly luciferase reporter plasmid under the control of an ISG56.1-dependent promoter sequence. As described in the introduction, VACV protein C6 is a potent inhibitor of the IRF-3 signaling pathway, and so was used as a positive control in these assays. The Spir1 protein, a potent activator of the pathway (unpublished data from this lab), was also used as a positive control. The C6 construct was labelled with a TAP-tag; the Spir1 construct possessed a Myc-tag, while FLNa/b were labelled with an N-terminal HA-tag. These allowed detection of protein levels by Western Blotting.

The cells were stimulated by transfection of RIG-I-CARD (labelled with a FLAG-tag), and the effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to that of the corresponding non-stimulated cells transfected with the empty vectors.

The transfection of RIG-I produced an approximately 40-fold increase in reporter activity (demonstrated by the empty vector, EV), which was significantly reduced by ~25-fold with the addition of C6, and significantly increased by ~40 fold with the addition of Spir1. The reporter activity was also significantly reduced by ~20

fold with the transfection of 100ng/well FLNa, indicating an inhibitory effect by FLNa on IRF-3 signaling pathway. No inhibition by FLNb was found. Samples of cell lysate from both non-stimulated and stimulated cells were analyzed by immunoblotting and compared to a tubulin loading control, to confirm both the successful transfection of the plasmids used, and that similar levels of the C6 and Spir1 constructs were expressed. Western blotting demonstrating the expression levels of the FLNa/b proteins in the cells were run (Fig. 4).

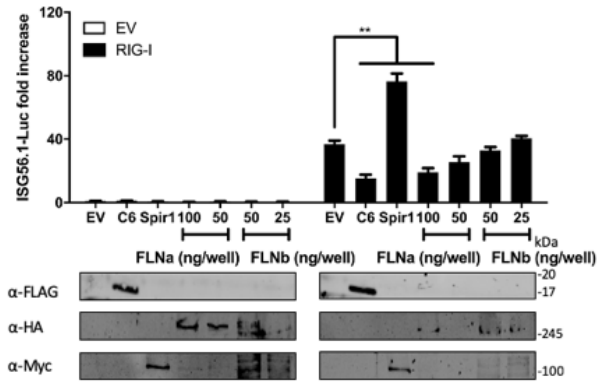

Figure 4. FLNa inhibits IRF-3 signaling stimulated by TNFα when normalized to NS EV.
HEK-293T cells were transfected with plasmids encoding the indicated proteins, or with an empty vector (EV) control, along with an ISG56.1 promoter firefly luciferase reporter plasmid and a *Renilla* luciferase control plasmid. The cells were stimulated by transfection of RIG-I-CARD (labelled with a FLAG-tag) and lysed 24h post-transfection. The effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to that of the corresponding non-stimulated cells transfected with the empty vector (NS EV). Data are represented as mean ± SD, and are representative of those from 3 experimental repeats. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.1.4 Determination of the effects of FLNa/b on JAK-STAT Signaling

Luciferase assay experiments on JAK-STAT signaling pathway are also done. Similar to previous experiments, we transfected codon optimized plasmid constructs for full length (FL) Filamin a (FLNa) or Filamin b (FLNb) in different doses per well (100ng, 50ng for FLNa and 50ng, 25ng, for FLNb) and C6 (5ng/well) into HEK-293T cells, along with a firefly luciferase reporter plasmid under the control of an ISRE-dependent promoter sequence. As described in the introduction, VACV protein C6 is a potent inhibitor of the ISRE signaling pathway, and so was used as a positive control in these assays. The C6 construct possessed a TAP-tag, while FLNa/b were labelled with an N-terminal HA-tag. These allowed detection of protein levels by Western Blotting. The cells were stimulated by the addition of a real agonist to the ISRE signaling pathway, interferon alpha (IFNα), and the effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to either that of the non-stimulated cells transfected only with empty vector (NS EV), or to that of the corresponding non-stimulated cells transfected with the same plasmids, referred to as non-stimulated self (NS self). The addition of IFNα produced an approximately 13-fold increase in reporter activity (demonstrated by the empty vector, EV), which was significantly reduced by ~7 fold with the addition of C6. No significant effect of FLNb was discovered in this experiment, which was similar to the results of the dose-dependency assays. However, for FLNa protein, a discrepancy was seen between the data graphed

relative to NS EV, and that graphed relative to NS self. The data indicated a significant inhibition of ~5-fold reporter activity decrease by FLNa when transfected 100ng/well when compared to the NS EV data, but showed no significant inhibition by FLNa when compared to the NS self data (Fig. 5a, b). The Western Blots for this experiment were again run, similar to the other experiments.

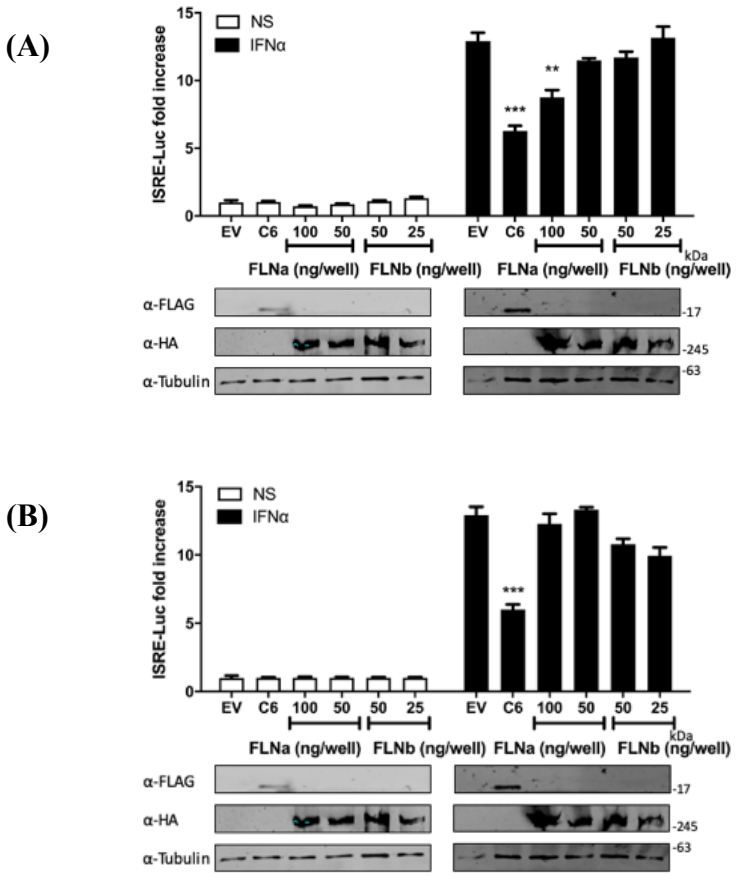

Figure 5. FLNa inhibits JAK-STAT signaling stimulated by IFNα when normalized to NS EV.
HEK-293T cells were transfected with plasmids encoding the indicated proteins, or with an empty vector (EV) control, along with an ISRE promoter firefly luciferase reporter plasmid and a *Renilla* luciferase control plasmid. The cells were stimulated for 6h by the addition of real agonist to the ISRE signaling pathway, interferon alpha (IFNα) 24h post-transfection and then lysed. The effects of the transfected proteins observed by indirectly measuring the levels of reporter gene transcription. The luminescence in the stimulated cells was then normalized to either that of the non-stimulated cells transfected only with the empty vector **(a, NS EV)**, or to that of the corresponding non-stimulated cells transfected with the same plasmids **(b, NS self)**. Data are represented as mean ± SD, and are representative of those from 3 experimental repeats. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3.2 Generation of FLNb Knockout HeLa Cell Lines and Plaque Assays

Human Embryonic Kidney cells (HEK293-T) were transfected with 4 different sgRNA plasmids (designed by Dr. Georgana) targeting various nucleotide sequences, 2 on FLNa and 2 on FLNb gene, both on exon 3. After 3 days, transfected cells were selected by incubation in the presence of puromycin and the clones were incubated for 3 further weeks. Due to time limit, the single cell selection and sequencing processes were performed by Dr. Georgana. The FLNa knockout cell lines were still at the process of sequencing validation, with no experiment conducted on these cell lines yet, and were therefore not included in this report. After Western Blot verification and sequencing of the FLNb knockout HeLa cell lines, 2 cell lines (KO13 and KO17) were selected by Dr. Georgana to be further confirmed. Two unedited HeLa cell lines transfected with px459 empty vector (EV) were also included as positive controls in the Western Blot experiments.

3.2.1 Western Blot Confirmation of Knockouts

Samples of cell lysate from both 2 FLNb knockout cell lines (KO13 and KO17) and 2 negative controls (EV1 and EV2), along with Parental HeLa cell lines as a negative control were analyzed by immunoblotting and compared to a tubulin loading control, to confirm that similar levels of cellular proteins were expressed. Western blotting confirmed that the expression of full length FLNb protein in cell lines transfected with EV were at similar levels to that of the Parental cell line,

whereas both FLNb KO13 and KO17 cell lines showed a complete absence of FLNb protein, indicating that the CRISPR/Cas9 knockout of FLNb in these 2 cell lines were successful.

3.2.2 Plaque Size Measurements

In order to discover the effects of the absence of FLNb on cellular defense against viral infection in terms of plaque size, multiple plaque assays were performed. GFP-tagged VACV in a dose of 200pfu (plaque forming units) per well was used to infect the Parental, the two EV and two FLNb knockout HeLa cell lines for one hour. The plaque sizes were measured at approximately 72 hours post-infection by fluorescent microscopy. In both experiments, the plaque size in two EV cell lines were similar to that in the parental cell line (~$2.5x10^5$ and ~$1.0x10^5 \mu m^2$ in the two experiments respectively), which was significantly increased in the KO17 cell line, giving an approximately doubled average plaque size in both experiments. However, for the KO13 cell line, a difference was seen between the data of the first and second repeat of the experiment. The data presented a significant increase of plaque size of ~$2.5x10^5 \mu m^2$ (2-fold) when compared to the Parental or EV cell lines in the first experiment, but showed no significant difference in plaque size from the Parental or EV cell lines in the second experiment (Fig. 6a&b). The data indicated that the absence of FLNb in HeLa cells weakens the defense effect against VACV infection and/or spread, and increases the plaque size after VACV infection, which agreed with the results of similar plaque assays performed on FLNb knockout mouse

embryonic fibroblast (MEF) cells (unpublished, Smith lab). Corresponding Western blots demonstrating the expression levels of FLNb in the cells were included.

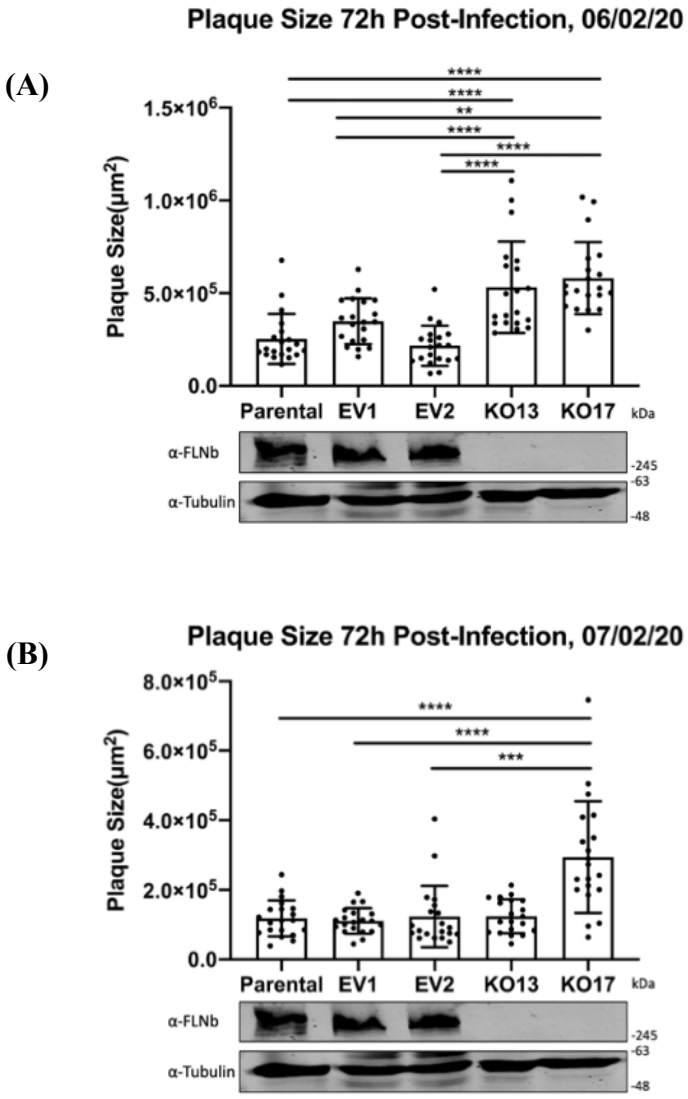

Figure 6. Absence of FLNb increases plaque size when cells are infected by VACV.

(a,b) The Parental, the two EV and two FLNb knockout HeLa cell lines were infected with 200pfu/well GFP-A5 VACV for one hour, and pictures of plaques are taken 72h post-infection by fluorescent microscopy. Data are represented as scatter dot plots with mean ± SD, and are representative of 20 plaques per cell line. Statistical significance was determined using an unpaired, two-tailed Student's *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4. Discussion

Multiple signaling pathways are activated after an infection by VACV is sensed by PRRs, rapidly leading to an innate immune response restricting the viral replication and spread. To evade this, VACV devotes between one third and half of its proteins encoded in the genome to act as immunomodulators, non-essential for viral replication in vitro, but aid immune evasion and virulence. VACV protein C4 is one among them, which inhibits NF-κB and DNA-PK signaling pathways, and also interacts with cytoskeletal protein, FLNs (unpublished result from this lab). In this study, we characterized the effects of FLNa protein on innate immune signaling, and the effect of FLNb on viral replication and/or spread, after infection of VACV on FLNb knockout HeLa cell lines generated.

In light of the observation that knockout of FLNb protein from MEF cells significantly increased plaque size after VACV infection, it was speculated that filamins have a role on immune response against viral infection (unpublished result, Smith lab). Given that filamins are also found to be implicated in other cellular processes by acting as a scaffold for signaling molecules, it was hypothesized that the mechanism of their effects on innate immune would be by their activity as scaffold, and as such the effects were expected to behave in a dose-dependent manner. We investigated this by transfecting varying amounts of FLNa/b plasmid into cells, and employing luciferase reporter gene assays to determine that the full-length (FL) FLNa protein has an inhibitory effect on the NF-κB pathway in cells

activated by either overexpression of TRAF6, a stimulatory component of NF-κB signaling with increased inhibitory activity with higher doses, or a real agonist to the pathway, TNFα. In contrast, no such effect is found with FLNb. However, when the cells were stimulated by another real agonist to NF-κB pathway, IL-1β, only a very poor stimulation of ~5-fold was achieved, which proved difficult to give any significant result, and was therefore not included in this report.

We also examined the effects of both FLNa and FLNb on other signaling pathways, in order to further elucidate the general effects of filamin proteins on innate immune. Similar to the result found with NF-κB pathway, FLNb has no significant effect on IRF-3 and JAK-STAT signaling, while a mild inhibitory effect by FLNa are seen on these two pathways. Nevertheless, a difference in inhibition level by FLNa between NF-κB, IRF-3 and JAK-STAT pathways was observed: A dose of 10ng/well of FLNa plasmid was sufficient to produce a significant inhibition on NF-κB pathway, while for IRF-3 and JAK-STAT pathways a minimal dose of 100ng/well was needed to achieve significance in inhibition. Similar experiments on AP-1 pathway were done, however, as the cells activated by the agonist to AP-1 pathway, PMA, only produced a 2-fold stimulation, it was not possible to produce any valid and significant result, and we decided not to include this experiment here as well. As such, the effects of filamins on both NF-κB pathway when stimulated by IL-1β, and the AP-1 signaling pathway require further investigation, which is possible by luciferase reporter gene assays with refined protocol.

To validate the effects of filamins on viral replication and/or spread after infection on human cell lines, we also performed plaque assays on FLNb knockout HeLa cell lines, as due to time limit the FLNa knockout cell lines were not available. A significant increase in plaque size 72 hours post-infection, compared to parental HeLa cell lines or cells transfected with the px459 empty vector (EV) was seen on FLNb knockout cell line 17 in both repeats of experiment, which accords well to unpublished previous results of similar experiment done on MEF cells found in this lab, while a discrepancy was seen between the experiments on cell line 13, on which an increase was observed in one repeat of experiment while not seen in the other. The plaque size were also significantly different between the two repeats, with one experiment achieving ~2.5x106 and the other achieving ~1.0x106 μm2 for average plaque size on the parental cell line. These discrepancies might be due to a change in titre and virulence of the virus strain, which experienced multiple cycles of freezing-defrosting, and experimental or sampling errors.

Considering these observations and previous work regarding filamin proteins, we proposed the activity as a scaffold of FLNb may be crucial in innate immune responses to viral infection, and was therefore targeted by VACV C4 protein which interact with it and inhibit multiple signaling pathways related with innate immune by protein-protein interaction. We also proposed that FLNa may possess a dose-dependent inhibitory effect on multiple innate immune signaling pathways by an unknown mechanism. However, this work is very preliminary thus far, and therefore more research is required before any reliable conclusions can be drawn.

Plaque assays as well as growth curve experiments can be done on both FLNb and FLNa knockout HeLa cell lines with wild type and ΔC4 vaccinia viruses, to further inspect the effect of the absence of filamins on the viral replication and/or spread after VACV infection. Other experiments by infecting the cells with a higher multiplicity of infection (MOI) and a shorter post-infection timescale (16-24h) of measurement can also be done to determine whether filamins affect viral replication or spread, or both processes during the infection. These viral infection assays can also be tested on other DNA viruses including Herpes Simplex Viruses (HSV), or RNA viruses e.g. influenza viruses, Zika viruses and measles viruses etc., to investigate the effects of filamins on the replication and/or spread of other viruses, which would be helpful in determination of the mechanisms of the effects of filamins on innate immune signaling pathways.

In order to confirm our observations and research further in the mechanisms of filamins acting on innate immune response, reporter gene assays on FLNa/b knockout cell lines could also be performed. Alternative methods to reporter gene assays should be used as well, such as quantitative PCR (qPCR) or enzyme-linked immunosorbent assay (ELISA) experiments.

In conclusion, overexpressing the cytoskeletal protein FLNa causes a dose-dependent inhibition of the NF-κB, IRF-3 and JAK-STAT signaling pathways involved in type I interferon response, while FLNb restricts viral replication and/or spread after VACV infection. These results imply FLNs to play important roles in innate immune, and further work on them are of particular interest, as development

of novel treatments and medicines targeting viral infection can be aided by finding out the exact effects and mechanisms of FLNs action in innate immune signaling.

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