

Subcellular Barriers to Viral Infection and Gene Delivery

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Abstract

Viruses are obligate intracellular parasites that survive by gaining entry into cells and suppressing subsequent antiviral responses. Pestiviruses encode a single polyprotein which is processed by proteases to produce 12 major viral proteins. The N-terminal protease, N^{pro} cleaves itself from the N-terminus of the polyprotein and targets IRF3 for degradation to suppress interferon signalling. This study shows that N^{pro} also inhibits apoptosis by inhibiting the IRF3-dependent Bax pathway and uses mitochondria and peroxisomes as sites for IRF3 regulation. Mass spectrometry was used to identify proteins that interact with N^{pro}. Of the 55 proteins identified most were ribosomal proteins and ribonucleoproteins including RHA, YBX1, DDX3, DDX5, eIF3, IGF2BP1, IEBP3, and PABP-1. These are components of the translation machinery, ribonucleoprotein particles (RNPs), and stress granules (SGs) which can halt translation and prevent translation of viral genomes.

Sindbis virus (SINV) infection induces eIF2 α phosphorylation, which leads to SG assembly. SINV infection also stimulates autophagy. We showed that the autophagy protein ATG16L1 not only regulates eIF2 α phosphorylation and the translation of viral and antiviral proteins, but also controls SG assembly. SINV capsid was recruited by ribonuclear proteins TIA1, YBX1, and VCP, to a single perinuclear body, which co-localised with protein-kinase R (PKR) and RIG-I. Loss of ATG16L1 inhibited SG assembly and the capsid remained in small foci containing YBX1, TIA1 with RIG-I. It is possible that ATG16L1 is required for SG assembly, and for attenuating the innate immune response.

A non-canonical autophagy pathway called LC3-associated phagocytosis (LAP) targets LC3 to phagosomes and endosome membranes during uptake of pathogens. LAP is dependent on the WD domain of ATG16L1 and this study has used a mouse lacking the WD domain (δ WD) to investigate the roles played by LAP during influenza virus infection in vivo. The growth, fertility and tissue homeostasis of δ WD mice were comparable with wild type mice, but δ WD mice were extremely sensitive to IAV resulting in fulminant pneumonia, lung inflammation and high mortality rates. A conditional mouse model (δ WD^{phag}) where δ WD was expressed solely in myeloid cells showed that protection against IAV infection of lung was independent of phagocytes. This establishes non-canonical autophagy pathways in non-phagocytes such as epithelial cells as a novel innate defence mechanism that can restrict IAV infection.

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Abbreviations

ATF4: Activating transcription factor 4

Bax: BCL2 associated X

BDV: Border disease virus

BMDM: Bone marrow derived macrophage

BVDV: Bovine viral diarrhea virus

CARD: Caspase activating and recruiting domain

CHIKV: Chikungunya virus

CLR: C-type lectin receptors

CSFV: Classical swine fever virus

Cyt c: Cytochrome c

DC: Dendritic cells

DENV: Dengue virus

DMEM: Dulbecco's Modified Eagle's Medium

dsRNA: Double stranded RNA

DTT: Dithiothreitol

ER: Endoplasmic reticulum

eIF2 α : Eukaryotic initiation factor 2

eIF3i: Eukaryotic initiation factor 3

FCR: Fc receptor

GCN2: General control nonderepressible 2 GCN2

GNL3: Guanine nucleotide binding protein 3

GOI: gene of interest

GST: Glutathione S-transferase

HA: Hemagglutinin

HEK-293: Human embryonic kidney

HeLa: Henrietta Lacks

HBSS: Hanks balanced salt solution

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HPD: High-salt pulldown buffer

hnRNP: Heterogeneous nuclear RNPs

HRI: Heme-regulated eIF2 α kinase

HRP: Horse radish peroxidase

HSV: Herpes simplex virus

IAV: Influenza A virus

IBV: influenza B virus

IEBP3: Interleukin enhancer binding factor 3

IFN: Interferon

IH: Immunohistochemistry

IIP: Interferon inhibiting protein

IKK ϵ : I kappa-B kinase

IPTG: Isopropyl-B-D-thiogalactopyranoside

IRES: Internal ribosome entry site

IRF3: Interferon regulatory factor 3

ISG: Interferon stimulated gene

ISRE: Interferon sensitive response element

KLH: Keyhole limpet hemocyanin

LamR: Laminin receptor

LAP: LC3 associated phagocytosis

LC3: Microtubule-associated protein light-chain 3

LPD: Low-salt pulldown buffer

LysM: Lysozyme M

Lyz2: Lysozyme 2 gene

M1: Matrix 1

M2: Matrix 2

MAVS/IPS-1: Mitochondrial antiviral signalling protein

MEF: Mouse embryonic fibroblast

MEM: Minimum Essential Media

MERS: Middle East Respiratory Syndrome

MDA-5: Melanoma differentiation-associated protein 5

MDBK: Madin-Darby Bovine Kidney

MHV: Mouse hepatitis coronavirus

MMTAG2: Multiple myeloma tumour protein 2

moi: multiplicity of infection

NA: Neuraminidase

NaA: Sodium arsenate

NP: Nucleoprotein

N^{pro}: N-terminal protease

ORF: Open reading frame

OVA: Ovalbumin

PABP-1: Polyadenylate-binding protein 1

PAMP: Pathogen-associated molecular pattern

PKR: Protein kinase R

PMSF: Phenylmethylsulfonyl fluoride

PRR: Pattern recognition receptor

PtdSer: Phosphatidylserine

RdRp: RNA-dependent RNA polymerase

RHA: RNA helicase A

RIG-I: Retinoic acid-inducible gene-I-like

RLR: RIG-I like receptor

RNP: Ribonucleoprotein

ROS: Reactive oxygen species

saRNA: Self amplifying RNA

SARS-CoV-2: Severe acute respiratory syndrome, coronavirus 2

SFV: Semliki Forest Virus

SGs: Stress granules

SinV: Sindbis virus

ssRNA: Single stranded RNA

SQSTM1: Sequestosome 1/p62

TBK1: Tank binding kinase-1

TFA: Trifluoroacetic acid TFA

TLRs: Toll like receptors

TMEV: Theiler's murine encephalomyelitis virus

TPEN: N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine

TRASH: trafficking, resistance, and sensing heavy metal

UPLC: Ultra-performance liquid chromatography

UTR: Untranslated regions

VEEV: Venezuelan equine encephalitis virus

vRNA: Viral RNA

vRNP: Viral ribonucleoprotein

WNV: West Nile virus

YBX-1: Y-box binding protein

ZIKV: Zika virus

YBX-1: Y-box binding protein

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Chapter 1

Virus-Host interaction Papers

Chapter 1 Virus-Host interaction Papers

1.1. The Pestivirus N Terminal Protease, Npro Redistributes to Mitochondria and Peroxisomes Suggesting New Sites for Regulation of IRF3 by Npro

This chapter is an adaption of work that has been published (Jefferson et al) [1] and appears with permission of the author and named contributors.

1.1.i. Introduction

For millennia viruses and their hosts have coevolved, producing an immunological arms race between the two. Viruses are molecular genetic “parasites” [2] that have shaped and afflicted all living organisms. Viruses have evolved to hijack the cell’s machinery in order to self-replicate [5]. This hijacking has led to the infected host having to develop an array of immune responses to dampen viral infection. Viruses have evolved strategies to evade host antiviral cellular immune [3], [4], [5]. This thesis by publication describes several viruses and surrogate viruses plus their interactions with innate immune pathways in eukaryotic cells. The innate immune system is complex. Unravelling complex virus-immune interactions is critical in order to unpick the molecular mechanics of the innate system so that this may lead to the development of new therapeutics, new technologies and reduction of viral disease burden in the world.

The innate immune system is the front line of defence against viruses and other pathogens. Pathogens are detected through cell surface and cytoplasmic recognition molecules such as pathogen-associated molecular patterns (PAMPs). PAMPs encompass several non-self molecules including nucleic acids, carbohydrates and polypeptides seen on the virus [6]. PAMPs are recognised by complimentary pattern recognition receptors (PRRs), which can be either membrane bound like Toll like receptors (TLRs) or are present in the cytoplasm. TLR3 recognises double stranded RNA (dsRNA), a viral replication intermediate that is produced by positive-strand RNA and DNA viruses. TLR3 is also activated by ssRNA containing stem looped structures [7]. TLR7 and TLR8 recognises ssRNA when internalisation of the virus and its replication releases the viral RNA into endosomes, where TLR7 and TLR8 reside. TLR3, TLR7 and TLR8 recognise RNA motifs and are preferentially located in intracellular compartments, such as the endoplasmic reticulum (ER), endosomes, lysosomes, and

endolysosomes. Viral nucleic acids are also recognised by retinoic acid-inducible gene-I-like receptors (RLRs). RLRs are part of the RNA helicase family. This RNA helicase family includes retinoic acid-inducible gene-I-like (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and protein kinase R (PKR), that can bind dsRNA [8]. RIG-I recognizes 5'-di-/triphosphate blunt-ended dsRNA and 5'-triphosphate ssRNA, which are unique patterns to pathogens. MDA5 recognizes long dsRNA. PAMPs binding to PRRs produces a signal cascade resulting in the infected cell to release Type 1 and Type 3 interferons (IFNs). IFN β induces the expression of other IFNs and hundreds of different genes, collectively termed interferon stimulated genes (ISGs), both in the infected cell, and in neighbouring cells. Some ISGs degrade RNA and others decrease protein synthesis to halt viral replication [9]. Secreted IFNs alerts and activates immune cells like macrophages and dendritic cells (DCs) that, phagocytose the infected cell, arresting the potential spread of the virus [10]. A third role of IFN is to signal the cell and neighbouring cells to undergo apoptosis for macrophage engulfment [11] (Figure 1.1.1).

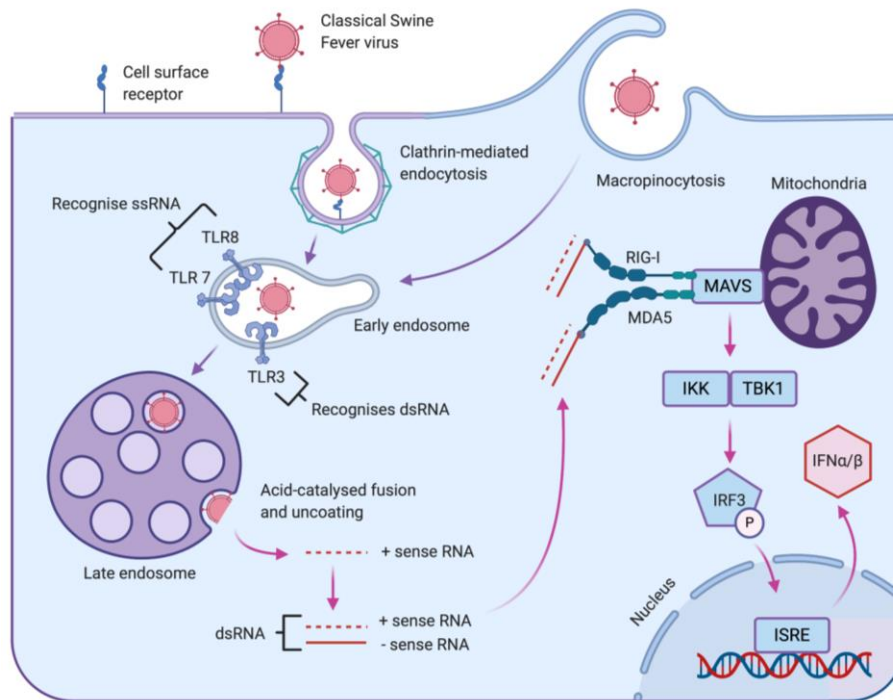


Figure 1.1.1 dsRNA sensing activating an innate immune response. IRF3 plays essential roles in interferon production and apoptosis [12, 13]. Upon Flavivirus entry and release, vRNA is recognised by PRRs such as TLR3 plus members of the RNA helicase family Rig-I and MDA-5. Binding of viral dsRNA to helicases, activates the caspase activating and recruiting domains (CARD) on Rig-I and MDA-5. Rig-I and MDA-5 translocate to mitochondria, to interact with the mitochondrial antiviral signalling protein (MAVS/IPS-1) [14, 15] via its own CARD domain. MAVS forms a multi-layered protein complex containing several cytosolic protein kinases. This platform recruits I kappa-B kinase (IKK ϵ) and tank binding kinase-1 (TBK1), which phosphorylates IRF3. Phosphorylated IRF3 translocates to the nucleus where it binds to interferon sensitive response elements (ISRE), which switch on interferon stimulated genes (ISGs) [16]

1.1.i.a Pestivirus Classification and Genome

Pestiviruses such as, Classical Swine Fever Virus (CSFV), Bovine Viral Diarrhea Virus (BVDV) and Border Disease Virus (BDV) are animal viruses belonging to the *Flaviviridae* family. They cause major livestock losses worldwide and have a significant economic impact. Pestiviruses are small single-stranded, positive-sense RNA viruses with a genome size of approximately 12.3 kb for CSFV [17], BDV [18] and 12.5 kb for BVDV [19] (Figure 1.1.2, A). The genomes encode for 12 mature proteins that are co- and post-translationally processed from a single RNA into a large poly protein consisting of N-terminal protease (N^{pro}), capsid, E^{RNS} , E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Figure 1.1.2, B) [20]. These viruses inhibit both apoptosis and the cellular innate immune system [21]. In this study we wanted to investigate how N^{pro} acts on these cellular processes.

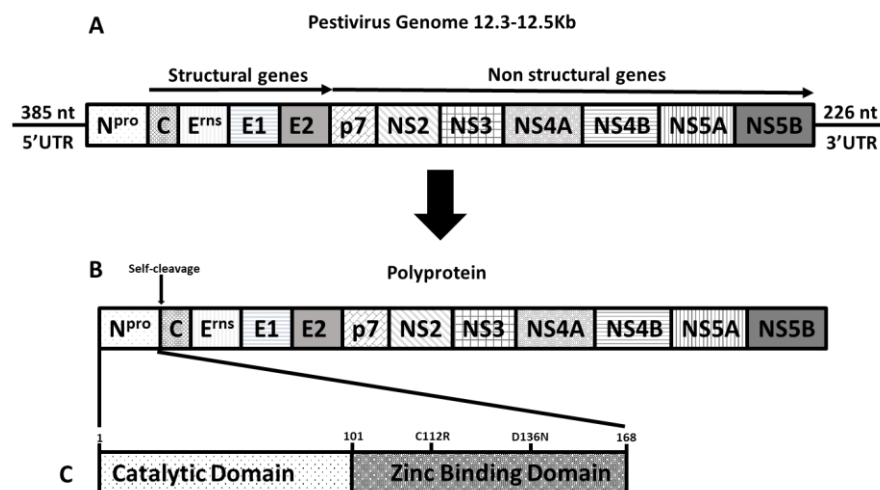


Figure 1.1.2. Pestivirus genome and N^{pro} domains. A. Organization of the pestivirus genome. B. Pestivirus polyprotein. C. Protein coding domains of N^{pro} .

1.1.i.b Pestiviruses interferon inhibitory viral protein N^{pro}

N^{pro} is exclusively expressed by CSFV, BVDV and BDV strains in contrast to other viruses in the *Flaviviridae* family. N^{pro} is a 168-amino-acid autoprotease located at the N-terminus, which cleaves itself from the polyprotein during translation of the viral mRNA between itself and the capsid protein. N^{pro} is a cysteine autoprotease that cleaves between amino acids 168 and 169 [22] (Figure 1.1.2, C). The remaining core and structural proteins associates with its viral RNA (vRNA) genome to package and form new virions [23]. N^{pro} itself is not associated with virions and is not required for virus replication [24]. CSFV and BVDV infection was known to inhibit the activation and synthesis of IFN as well as preventing apoptosis [25]. Previous work, showed that these pestiviruses are able to evade cellular innate immune responses during infection, by inhibiting the induction of α/β interferon [26, 27]. The investigation showed inhibition of IFN synthesis by CSFV and BVDV by degradation of interferon regulatory factor 3 (IRF3) by N^{pro}. This did not require the autocatalytic activity of N^{pro}. When N^{pro} is expressed on its own in cells it can block type 1 interferon production by promoting loss of IRF3 [21]. N^{pro} consists of two domains. These domains are, a catalytic N-terminal domain and a zinc-coordinating C-terminal TRASH domain (Figure 1.1.2, C). It was found that N^{pro} directly binds to IRF3 through the zinc-binding trafficking, resistance, and sensing heavy metals (TRASH) motif that's encoded within it [28]. The zinc binding TRASH motif in N^{pro} involves several residues including C112 and D136 (Figure 1.1.3). When mutations were introduced within these residues to produce C112R and D136N, N^{pro} no longer bound zinc resulting in no interaction with IRF3 [29]. Preventing IRF3 interacting with IFN- α/β genes inhibits an innate immune response.

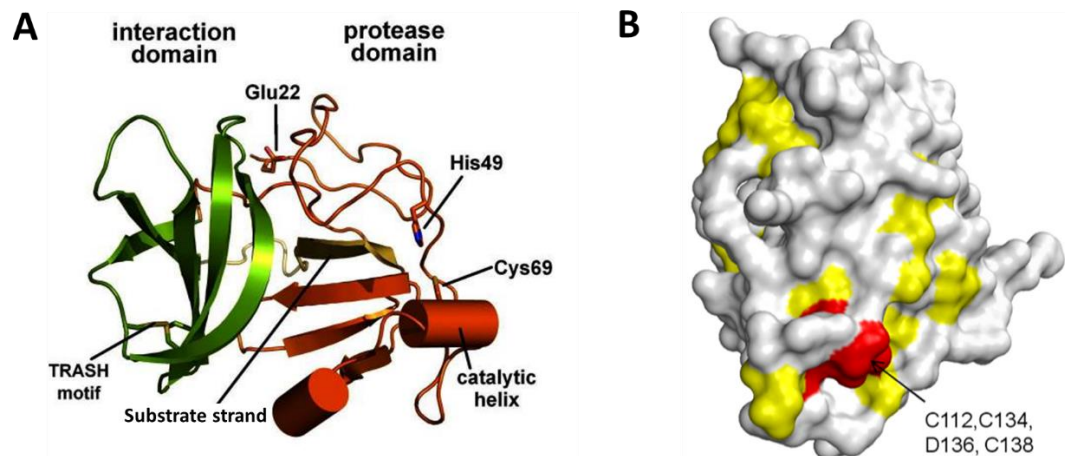


Figure 1.1.3. Structure of N^{pro} and distribution of residues involved in N^{pro} mediated degradation of IRF3. **A.** N^{pro} protein structure when folded revealing its two domains. The protease domain indicated in red and the interaction domain in green [30]. **B.** 3D modelling showing residues C112, C134, D136 and C138 that are essential for N^{pro} mediated IRF3 degradation indicated in red [29].

1.1.i.c Role of IRF3 in an innate immune response

IRF3 is a transcription factor that is required to turn on interferon synthesis. It is found in the cytoplasm, but on activation by phosphorylation and dimerization, it is translocated into the nucleus, where it binds upstream of the interferon gene. Activated IRF3, additionally interacts with pro-apoptotic protein BCL2 associated X apoptosis regulator (Bax). Upon activation Bax translocates to the mitochondria and initiates the intrinsic apoptotic pathway [31] (Figure 1.1.4). Through activation of IRF3 and the induction of interferons, to its role played in initiating apoptosis in response to viral infection, mitochondria play a central role the host's defence system [32]. RIG-I and MDA5 relocate to mitochondria upon sensing vRNA, and these receptors also travel to peroxisomes [33] (Figure 1.1.5). Peroxisomes are cytosolic single lipid membraned organelles encapsulating a granular matrix [34] that also have MAVS localised on the membrane. Peroxisomes have been well characterised as having functions in lipid and reactive oxygen species (ROS) metabolism [34, 35] but also have antiviral response roles (Figure 1.1.5). MAVS is also located on the outer mitochondrial membrane that highlights the pivotal role that mitochondria play within the hosts' innate immune signalling.

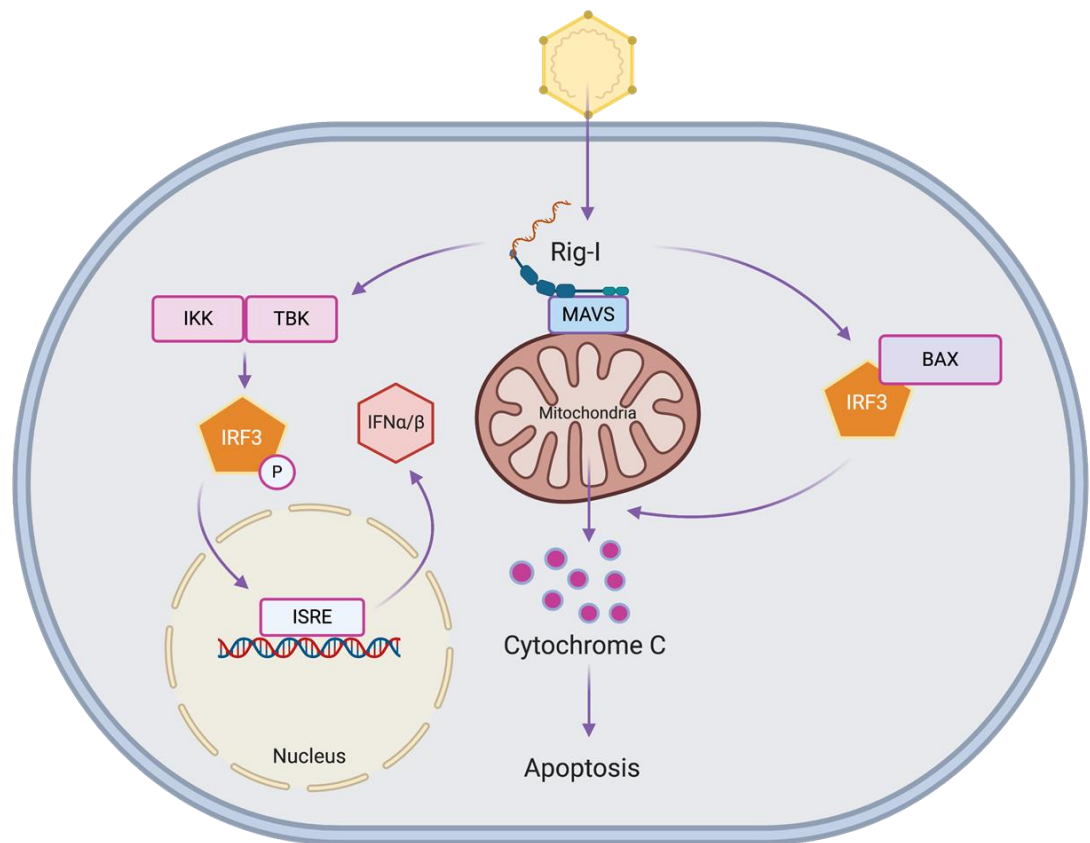


Figure 1.1.4. Bax is essential for dsRNA-induced apoptosis. Intracellular dsRNA produced by replicating viruses in the cytoplasm is detected by RNA helicases RIG-I and MDA5. Protein kinases TBK1/IKKε are activated and transcription factor IRF3 is phosphorylated and binds ISRE to drive transcription of many ISGs promoting an antiviral response [36]. At the same time IRF3 promotes a pro-apoptotic action independent of its transcriptional role through RLR signalling. IRF3 interacts with pro-apoptotic Bax where they translocate to the mitochondria activating the intrinsic apoptotic pathway releasing cytochrome c (Cyt c), triggering caspase activation [31, 37].

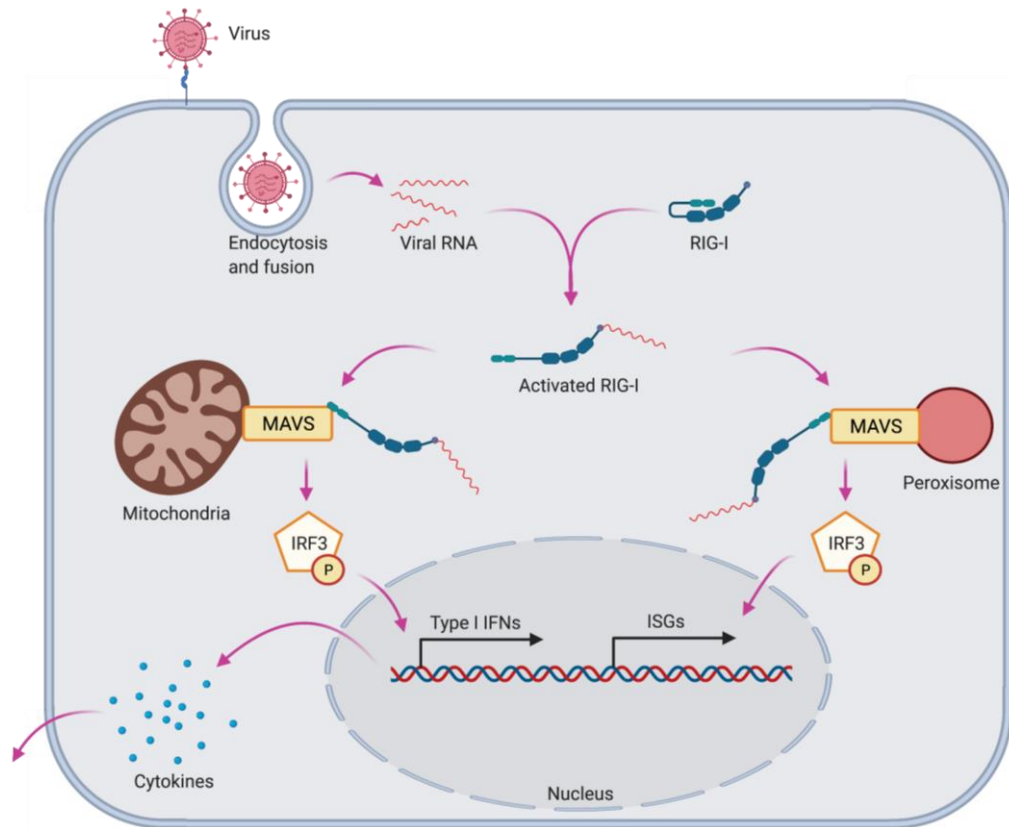


Figure 1.1.5. Viral activation of MAVS on mitochondria and peroxisomes. Upon infection, vRNA is detected by RIG-I and MDA5 that activates MAVS localised on the mitochondria and peroxisome membrane where it activates IRF3 to initiate an interferon response [33].

1.1.i.d Pestiviruses are an important model for pathogenic human viruses

Pestiviruses are also of interest because they are homologous to the human flavivirus Hepatitis C Virus (HCV). Growth of HCV was hampered due to lack of an efficient cell culture-based infection systems. As HCV is the biggest causative agent of liver cirrhosis and liver cancer in man, pestiviruses are a model for the life cycle and innate immune evasion mechanisms of HCV. For example, a BVDV model was created where N^{pro} had been replaced by HCV NS3. N^{pro} is a cysteine protease that cleaves itself from the N-terminal of the core protein. N^{pro} is not required for viral replication so it can be replaced in order to study other viral proteins. NS3 is a protease for HCV and this BVDV-HCV chimeric virus was made to develop an assay to discover anti-HCV protease inhibitors to arrest HCV replication [38].

1.1.i.e Industrial applications of N^{pro}

N^{pro} is interesting because its autoprotease activity has also been used in industry. In viral infection, pestivirus proteins are translated as a single polyprotein. The N^{pro} protein cleaves itself off from the following capsid protein. Proteolytic activity of N^{pro} that leads to this cleavage is hydrolysis between Cys₁₆₈ and Ser₁₆₉ in the polyprotein where Ser₁₆₉ has been determined to be the N-terminus of the capsid protein [23]. This specific cleavage site has been exploited to make true recombinant proteins, leaving an authentic N-terminus. Removing the need for any additional chemical or enzymatic processing [39]. N^{pro} fusion technology is used in industry for large scale protein expression for molecules like proinsulin, hepcidin, interferon- α 1, keratin-associated protein 10-4, monocyte chemoattractant protein-1, toxic gyrase inhibitor and many more [40]. The autoprotease activity has been used to enhance refolding and expression of recombinant fused peptides and proteins during bacterial protein expression [40].

Having been shown to be an antagonist of IRF3, cells lines expressing N^{pro} have been developed to grow novel viruses in mammalian cell lines where the IFN system is inhibited [41]. More recently N^{pro} from BVDV has been used in the development of self-amplifying RNA (saRNA) vaccines for severe acute respiratory syndrome, coronavirus 2 (SARS-CoV-2). The RNA vaccine is exposed to the innate immune response sensing by TLR's on endosomes and RNA sensing by RIG-I, MDA5 and PKR, which induce a type 1 interferon response inhibiting the vaccine. These nucleic acid vaccines incorporate interferon inhibiting proteins (IIPs) such as N^{pro} and encode them within an RNA construct, co-expressed with the viral sequence for SARS-CoV-2 spike protein. The spike protein is used as it plays a role in both virus entry and viral RNA release into the cytoplasm. A vaccine expressing the spike protein should induce antibodies against it that will block viral entry [42]. Incorporating IIPs into these saRNA vaccines can lead to a 14 fold increase in antibody titres when compared without the incorporation of IIPs [43].

These diverse and unique roles of this viral protein make it essential to understand how it acts when expressed alone in host cells. In this study we investigated the subcellular site of action of N^{pro} in order to understand its function in inhibiting interferon and apoptosis.

1.1.ii Materials and Methods

1.1.ii.a Tissue culture and reagents

Mouse embryonic fibroblast (MEFs), HeLa and Madin-Darby Bovine Kidney (MDBK) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (1X) + GlutaMAX™-I [+]
1g/L D-Glucose [+]
Pyruvate (Life Technologies) with 10% fetal calf serum (Heat Inactivated) (Life Technologies) and 100U/ml Penicillin Streptomycin (Life Technologies), in 5% CO₂ at 37°C. MDBK cells were grown in Minimum Essential Media (MEM) with glutamate (Life Technologies) and 10% BVDV free serum. MEF cell lines stably expressing N^{pro}Cherry or mutant N^{pro}Cherry proteins were selected using Geneticin™ selective antibiotic (G418 Sulphate) (Life Technologies) at 500uG/ml [1].

1.1.ii.b Cell lysis preparation

Cells were cultured on 6-well plates containing 150,000 cells per well 12 h treatment or transfection. Cells were washed twice with PBS before being scraped in 50µl M-PER™ mammalian protein extraction reagent (Life Technologies) containing complete protease inhibitor (Sigma) and incubated on ice for 30 mins. Cell debris was separated by centrifuge, spun for 10 mins at 13,000 rpm at 4°C [1].

1.1.ii.c BCA Assay

Quantitation of total protein was determined using the Pierce BCA Protein Assay Kit (Life Technologies). 10 µl of standards and samples (1:10 dilution in water) in a 96-well plate along with BCA working reagent (made up according to manufacturer's instructions). After 30 mins at 37°C incubation, the plate was measured using a Wallac EnVision 2013 Multilabel plate reader (PerkinElmer) [1].

1.1.ii.d Western Blotting

All lysates were made up to 30 µg protein in 6 x sample preparation buffer and boiled for 5 mins at 95°C before loading. Samples were separated on 4-12% SDS-PAGE gradient gels (Life Technologies) in MOPS SDS running buffer (Life Technologies). Proteins were transferred to

Immobilon-FL PVDF membrane (Millipore) and then IRF3 was detected on the membrane using anti-IRF3 (Abcam, ab25950). N^{pro} Rabbit polyclonal antibody to detect DS14 was produced in rabbits immunised with synthetic peptide KTNKQKPMGVEEPVYDATGKPLFGDPS, corresponding to amino acids 11 to 37 of the Alfort strain of CSFV linked to ovalbumin (OVA) and keyhole limpet hemocyanin (KLH) as described previously [21]. Equal protein loading was confirmed by anti-beta-actin monoclonal antibody AC-15 (Sigma, A5441) staining. Membranes were left overnight to incubate at 4°C with the primary antibodies and then washed and probed using IRDye[®]-labelled secondary antibodies (Li-Cor Biosciences) at 1:10,000 dilution or HRP conjugated antibodies (Jacksons Laboratories) at 1:2,000. Proteins detected by the labelled secondary antibodies were visualised on the Odyssey infrared system, or enhanced chemiluminescence detection of horse radish peroxidase (HRP) activity. Membranes were stripped and re-blotted using Re-blot plus strong solution (10x) (Millipore, 2504). Bands were quantified using Odyssey software or Scion image software. <http://scion-image.software.informer.com/> [1].

1.1.ii.e BVDV Infection

MDBK cells seeded on coverslips in 24 well plates at density of 15,000 cells per plate and infected with BVDV Kyle non-cytopathic strain of BVDV, from J. Brownlie, Royal Vet College, South Mimms UK. After absorption for 1 h, cells were fed with fresh media and incubated overnight at 37°C. MDBK cells were then transfected with plasmids encoding Bax-GFP using JetPRIME (Polyplus) according to the manufacturer's instructions. Cells were incubated for a further 24 h to allow sufficient plasmid expression before being treated with staurosporine or etoposide (Sigma) for 4 h or left untreated and then fixed in ice cold 80% acetone. BVDV glycoproteins were stained with hyperimmune anti-BVDV serum V182 and a secondary anti-cow Cy3 (Jackson ImmunoResearch Europe, 111-165-003) [1].

1.1.ii.f Cell stress and Apoptosis reagents

MEF cells were treated with sodium arsenate (NaA) at 100uM or staurosporine at 20nM or hydrogen peroxide at 200uM (All Sigma), or transfected with sheared poly IC (Roche) at 100ug/ml using Lipofectamine 2000 (Life Technologies) for 4 h. In other experiments, cells were treated with MG132 (Sigma) at 10um or cycloheximide (Sigma) at 20ug/ml. Cells were

then stained with anti-ubiquitin monoclonal antibody FK2 (Enzo, PW8810), anti-rabbit peroxisome antibody PMP70 (Sigma, P0497) and DS14 Rabbit polyclonal antibody [1].

1.1.ii.g Caspase 3/7 Apoptosis Assay

Cells were grown in black-walled 96-well plates with a transparent bottom. All treatments were performed in biological triplicates and the results shown represent the average and s.d. of the three independent wells. Following treatment of cells with inducers of apoptosis, caspase activity was measured using the Apo-ONE Caspase-3/7 assay (Promega) in 96-well plates and the addition of the fluorescent substrate Z-DEVD. Fluorescence was measured using Envision plate-reader (PerkinElmer). Fold induction of enzyme activity was obtained by dividing the activity in the treated samples by the activity obtained in the untreated cells [1].

1.1.ii.h Plasmids and Transfections

N^{pro}mCherry was generated from pcDNA3 N^{pro} [21] and cloned in-frame into pcDNA3mCherry (Invitrogen). N^{pro}mCherry point mutants C112R and D136N were created using the Quick Change kit (Stratagene). Plasmids were transfected into cells with jetPRIME (Polyplus) and cell lines stably expressing N^{pro} mCherry or mutant proteins were selected with G418 (1 mg/ml, Invitrogen). Plasmids encoding tBid-GFP [44] and BAX-GFP (from Stephen Tait, Beatson Institute of Cancer Sciences, University of Glasgow) and IRF3-GFP (from John Hiscott, MacGill University, Canada) were transfected into cells using Eugene HD (Roche). MDBK cells were transfected with plasmids encoding Bax-GFP using jetPRIME (Polyplus) according to the manufacturer's instructions [1]

University, Canada and STING-GFP pMRX from J.Zou Akira lab, Dept of Host Defence, Osaka, Japan, Ubiquitin-GFP (Addgene). The peroxisome marker PTS1-dsRed (pMF578) was from Marc Fransen, KU Leuven, Belgium [35]. The mitochondrial associated membrane marker hu PSS-1 p1420 was from Annie Colberg-Poley, Children's National Medical Centre, Washington DC [45] [1].

1.1.ii.i Immunofluorescence and image analysis

Cells were fixed in 4% Paraformaldehyde and permeabilised with Triton-X100 (Sigma). Cells were stained with anti-ubiquitin monoclonal antibody FK2 (Enzo, PW8810) or anti-rabbit peroxisome antibody PMP70 (Sigma, P0497) at 1:000 dilution in 30% goat-gelatin buffer overnight at 4°C. Primary antibodies were visualized using Alexa fluorescent conjugated anti-rabbit (Life Technologies, A-21206), anti-mouse (Life Technologies, A-21202) secondary antibodies and donkey anti-mouse Cy5 (Jackson Immunoresearch, 715-175-151). For BVDV staining, MDBK cells fixed in ice cold 80% acetone and BVDV glycoproteins were stained with bovine hyperimmune serum V182 against BVDV (a gift from J. Brownlie, Royal Vet College, South Mimms) and visualised with a secondary anti-cow Cy3 (Jackson Labs, 101-005-003). MitoTracker® Red CMXRos and Mitotracker Far Red (Molecular Probes, Invitrogen,) was used at a final concentration of 400nM and applied to live cells 30 minutes before fixation. Cells were stained with DAPI (Life Technologies, 10116287) at 1:5000 PBS dilution for 5 minutes, to visualise the nucleus and then mounted in Fluoromount G (Southern Biotech). The fixed cell images were obtained at x63 magnification on a Zeiss Axioplan 2 or a Zeiss laser-scanning confocal microscope LSM510. Images were analysed using the Axioplan software version 4.8. For pixel density analysis, images were analysed using Imaris x64 7.2.3 software (Bitplane Software Incorporated). Punctate structures within the images were identified using the “spots” function, and larger structures, such as nuclei, were identified using the “surfaces” function within the software. These object layers were used to create the rendered images and to assimilate data concerning vesicle diameter and number [1].

1.1.iii Results

1.1.iii.a Inhibition of Caspase-dependent Apoptosis by N^{pro} Correlates with Degradation of IRF3

Infection with CSFV or N^{pro} expressed alone results in the degradation of IRF3 [21]. A plasmid expressing wild type N^{pro} fused with mCherry was constructed. In addition, a plasmid expressing N^{pro} with mutations in the TRASH domain at cysteine 112 (C112R) or aspartic acid 136 (D136N) residue within the zinc binding TRASH domain. These residues have been previously shown to be important in the binding of IRF3 during viral infection, leading to a loss of IRF3 binding to N^{pro} and preventing IRF3 degradation and interferon inhibition [46]. MEF cells were transfected with N^{pro}mCherry and its mutants with DS14 anti N^{pro} and anti IRF3 antibodies being used to detect expression levels of the two (Figure 1.1.6, A western). Stable MEF cell lines could not be generated expressing N^{pro} due to the toxicity of expressing N^{pro} and the plasmid would be expelled after a few days. In MEF cells expressing wild type N^{pro}, virtually no detectable levels of IRF3 present when compared to un-transfected control lysates expressing endogenous levels of IRF3. Since transfection rates are not 100% there is residual presence of IRF3. Rates of transfection could be observed and calculated by the expression of mCherry in cells and counting frames of x20 field of views. There was detectable levels of IRF3, but reduced in cells expressing N^{pro} mutations C112R and D136N. Quantification of IRF3 bands against actin showed expression of N^{pro} C112R and N^{pro}D136N led to approximately 50% of the IRF3 levels seen in control cells (Figure 1.1.6 graph). This indicates that these mutations lead to less efficient degradation of IRF3 when compared to cells expressing wild type N^{pro}. The two residues at cys112 and asp136 have been shown to be part of a site for co-ordination of zinc [1, 28].

The ability of N^{pro} protein on its own to inhibit apoptosis from several different stimuli was investigated [47]. We wanted to show whether N^{pro} or mutant N^{pro} can protect cells from apoptosis. Cells were challenged for 4 h with inducers of caspase-dependent apoptosis (Figure 1.1.6, B). Caspase 3/7 activity was measured after 4 h using the fluorescent substrate Z-DEVD and compared to untreated cells normalised to 1. Cells expressing wild type N^{pro} had reduced caspase activation by staurosporine, interferon, poly IC, NaA and H₂O₂ compared to control cells not expressing N^{pro}. Cells expressing mutant N^{pro} at position C112R, significantly reversed inhibition of caspase activation by 50% when compared to cells expressing wild type

N^{pro}. The significance p values for caspase activation by each drug treatment for cells expressing viral protein compared to the drug treatment of control cells was tabulated below (Figure 1.1.6, B). The most significant values showing inhibition of apoptosis by N^{pro} was with NaA and hydrogen peroxide. Both chemicals trigger apoptosis via a reduction in mitochondrial membrane potential, cytochrome c release, and caspase activation [48]. Sodium arsenate is also an inducer of the cell stress and we chose this chemical to look at the role of N^{pro} in inhibition of apoptosis, both because mitochondria play a central role in IRF3 signalling and because virus infection activates the cell stress pathway [1, 49].

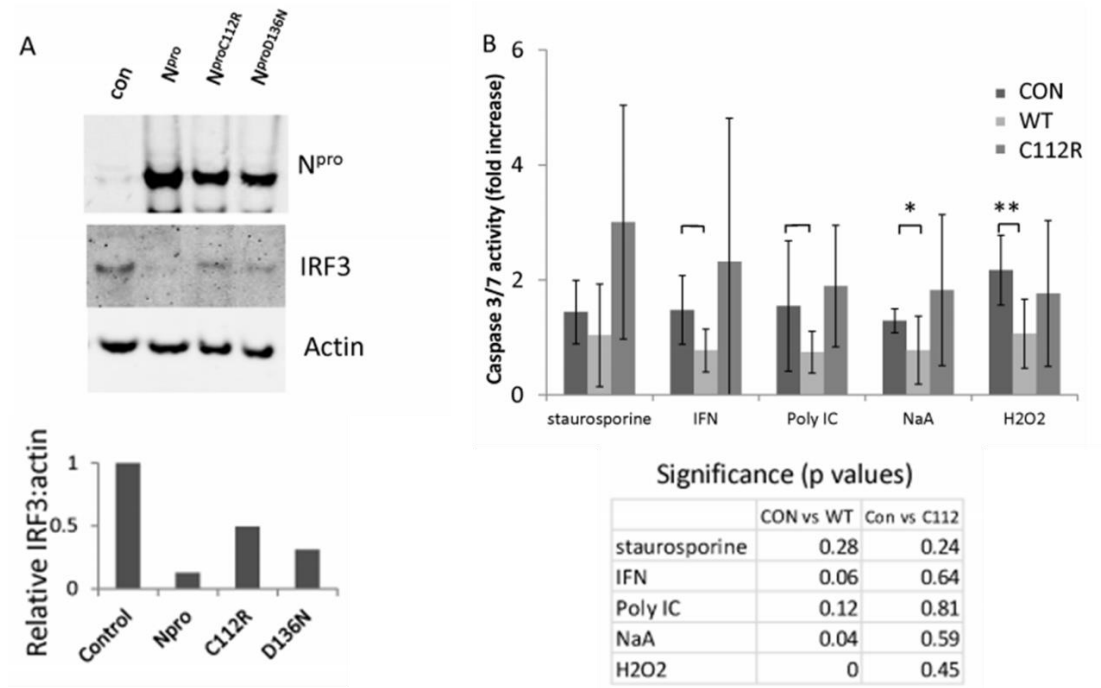


Figure 1.1.6. Inhibition of apoptosis by N^{pro} correlates with degradation of IRF3. **A.** Western blotting of lysates from MEF cell expressing N^{pro}cherry with antibodies against N^{pro}, IRF3 and actin shows loss of IRF3 from cells expressing N^{pro}. Lane 1: Control MEF cells not expressing plasmid. Lane 2: MEF cells stably expressing N^{pro}mCherry. Lane 3: MEF cells expressing N^{pro} C112R mCherry mutant. Lane 4: MEF cells expressing N^{pro} D136N mCherry mutant. Graph shows relative intensities of IRF3 compared to actin for the image scanned with Scion image software <http://scion-image.software.informer.com> (representative of Western blot repeated three times for each lane). **B.** N^{pro} but not N^{pro} C112R inhibits caspase 3/7 activity. MEF cells stably expressing wild type N^{pro} or mutant N^{pro} C112R mCherry were treated with staurosporine, interferon, dsRNA (poly I:C), sodium arsenate (NaA) or hydrogen peroxide (H2O2) for 4 hrs. Fold increase in caspase activity was normalized to negative (untreated cells) for each cell line and analysed with a two-sided t-test with unequal variance relative to the un-transfected cells for each cell line, and significance values are: bars marginally significant (0.05–0.1), * significant (0.01–0.05), ** highly significant (0–0.01) (n = 5 to 8). The table shows significance of drug treatment relative to control treated cells for cells expressing WT N^{pro} or N^{pro} C112R and analysis was conducted using similar tests as described above. Western blot and fold change were completed by Matthew Jefferson. Caspase assay was carried out by Penny Powell and statistics were done by Irina Mohorianu as named on the paper Jefferson et al [1].

1.1.iii.b N^{pro} Inhibits Fragmentation of Mitochondria following Cell Stress

When the hosts innate immune system detects a viral infection, apoptosis can be induced by IRF3-mediated activation of Bax [31]. When apoptosis is activated, Bax and Bid re-localise to mitochondria. Bid is an activator of Bax inducing a conformational change where Bax is inserted into the outer mitochondrial membrane at mitochondrial fission sites [50]. We asked if the mechanism of apoptosis inhibition by N^{pro} involved the intrinsic mitochondrial pathway which is dependent on Bax. In control cells, Bax is a highly abundant cytosolic protein and is loosely bound to the outer mitochondrial membrane [51, 52] (Figure 1.1.7, a–c). tBid localised in the cytoplasm and nucleus (Figure 1.1.7, g and k). After induction of cell stress with NaA, Bax and tBid redistributed to small, punctate perinuclear structures that also co-localised with mitochondria, observed using Mitotracker (MT) (Figure 1.1.7, d–f and h–j respectively). This mitochondrial fragmentation is well documented after cell stress, and Bax and Bid remain associated with mitochondria following fragmentation, and promote apoptotic cell death [52]. Loss of integrity by Bax in the outer mitochondrial membrane generates Bax pores where release of Cyt c into the cytosol promotes caspase activation [1, 52, 53].

In cells expressing N^{pro} alone following treatment with NaA, N^{pro} also co-localised with mitochondria (Figure 1.1.7, l–n). In comparison to the spherical, perinuclear puncta seen in cells expressing Bax or tBid alone, the mitochondria remained tubular and spread throughout the cytoplasm. N^{pro} expression stabilized the tubular mitochondrial network and the cells were protected from apoptosis. In addition, this shows that since levels of IRF3 are depleted (Figure 1.1.6, A), N^{pro} association with mitochondria is independent of its binding to IRF3 [1].

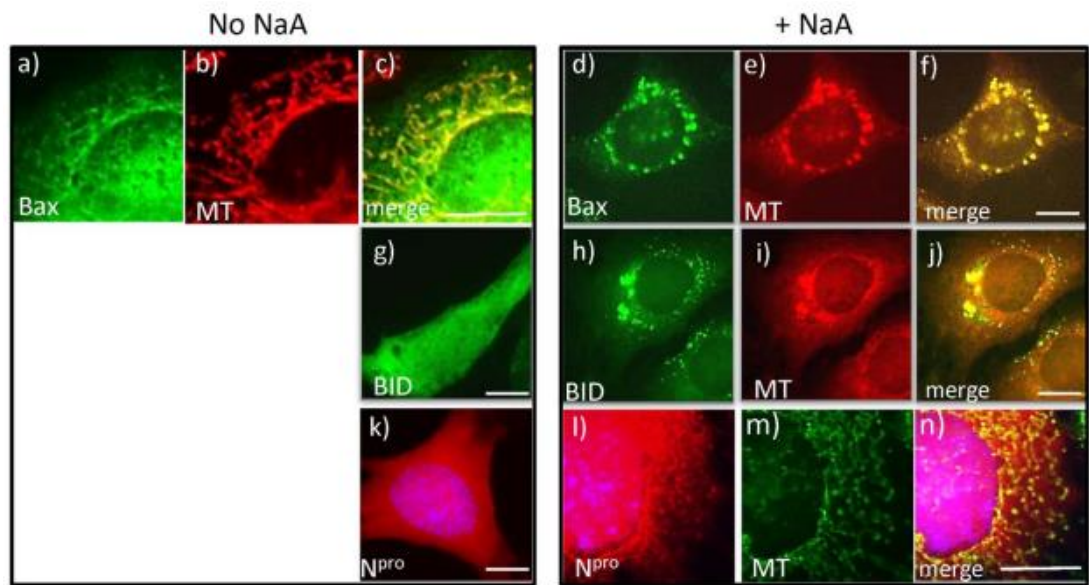


Figure 1.1.7. N^{pro} is recruited to mitochondria after cell stress and protects from apoptosis. MEF cells were transfected with Bax-GFP alone (**a–f**) or tBid-GFP alone (**g–j**) or N^{pro}mCherry alone (**k–n**) and either untreated (No NaA, left panel) or treated with NaA for 4 h (+ NaA, right panel). Cells were incubated with Mitotracker (MT) CM Ros (**b,e,i**) or Green MT (**m**) for 30 mins before fixation. Imaging completed by Jefferson et al [1].

1.1.iii.c Is IRF3 degradation required to prevent redistribution of Bax?

To see if Bax localisation occurred during virus infection when compared to N^{pro} on its own, the primary MDBK cells were infected with BVDV Kyle strain (a non-cytopathic strain). In control MDBK cells treated with NaA for 4 h, Bax-GFP relocated to spherical perinuclear punctae and cells died by apoptosis, seen by chromatin condensation (Figure 1.1.8, a-b). In contrast, in MDBK cells infected with BVDV for 24 h, Bax distribution remained cytoplasmic and partly mitochondrial (Figure 1.1.8, c-d) and cells were protected from apoptosis [1].

When both Bax-GFP and N^{pro}mCherry were co-expressed in MEFs, Bax distribution remained cytoplasmic and a fraction on the mitochondria, even after addition of NaA (Figure 1.1.8, e-f). This demonstrates, that when cell stress is induced in the presence of N^{pro} and the absence of IRF3, cytoplasmic Bax does not redistribute to spherical fragmented mitochondria. Therefore, cells are protected from apoptosis in the presence of wild type N^{pro}. However, in cells expressing the N^{pro} C112R mutant which is unable to bind and target IRF3 for degradation or protect cells completely against apoptosis (Figure 1.1.6), Bax located to spherical perinuclear punctae (Figure 1.1.8, g), and the cells died. N^{pro} redistributed to mitochondria and cytoplasmic puncta (Figure 1.1.8, b and d) [1].

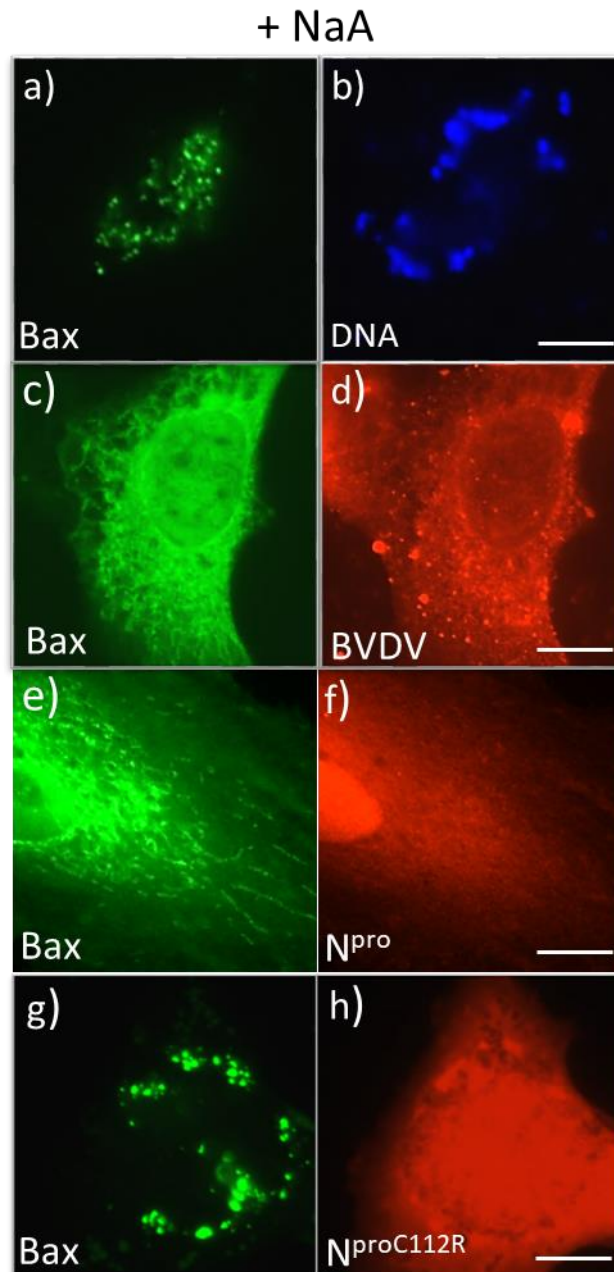


Figure 1.1.8. Protection from apoptosis induced by NaA requires IRF3. MDBK primary cells, transfected with Bax-GFP (**a–b**) alone or infected with BVDV overnight and then transfected with Bax-GFP (**c–d**), were treated with NaA for 4 h. BVDV was detected with bovine hyperimmune serum V182 and detected with Cy3 rabbit anti bovine antibody (red) and DNA was stained with DAPI. MEF cells co-expressing Bax-GFP and either wild type N^{pro}mCherry (**e–f**) or mutant N^{pro} C112R mCherry (**g–h**) were treated with NaA. Imaging by Jefferson et al [1].

1.1.iii.d N^{pro} indirectly inhibits Bax signalling through degradation of IRF3.

These experiments show through these co-expression experiments, the induction of cell stress with NaA, to mimic stress during viral infection, N^{pro} redistributes and associates with mitochondria (Figure 1.1.9, A). This not only blocks the innate immune pathways but also promotes cell survival through the degradation of IRF3 (Figure 1.1.9, B). IRF3 degradation prevents the pro-apoptotic effects on Bax (Figure 1.1.9, C). This was confirmed by the expression of mutant N^{pro} C112R, which did not result in the degradation of IRF3 and Bax relocates to spherical fragmented mitochondria. Recently work has shown that IRF3 binding to Bax is required for a conformational change in Bax to take place to allow binding to mitochondria (Figure 1.1.9, D) and regulate the permeability of the outer mitochondrial membrane [31]. Bax signalling results in cytochrome c release to initiate caspase 3 activating apoptosis. This demonstrates that both virus and the single viral protein N^{pro}, inhibit apoptosis through preventing Bax redistribution to the mitochondrial membrane [1].

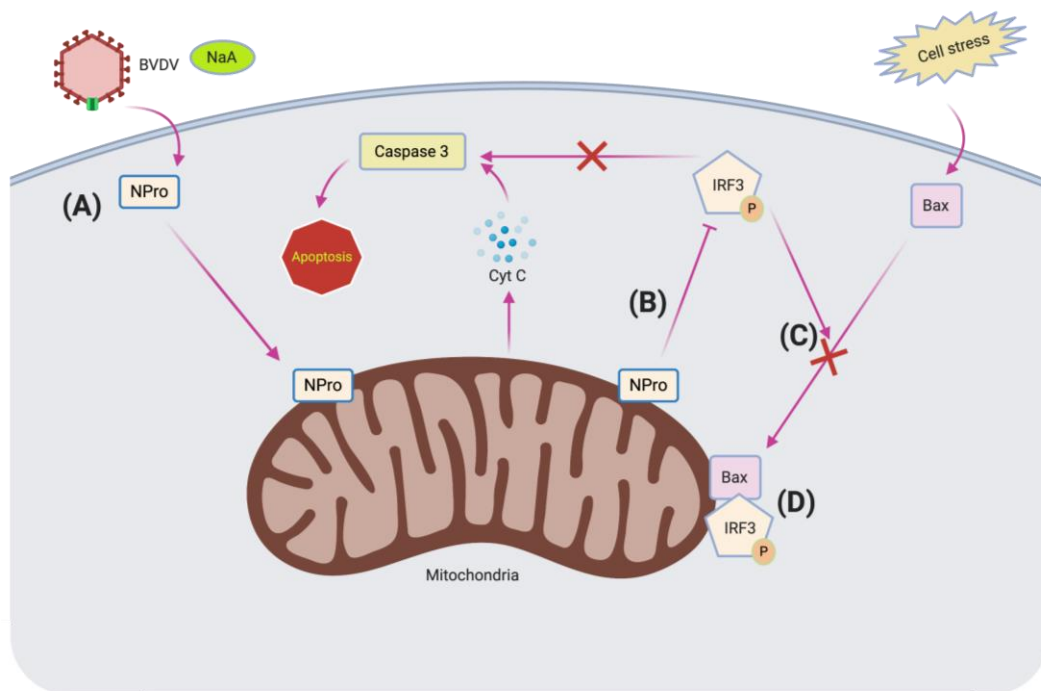


Figure 1.1.9. Schematic representation of the inhibitory actions of N^{pro} during cell stress. A. N^{pro} redistributes as a result of cell stress. **B.** N^{pro} blocks and degrades IRF3. **C.** IRF3 is unable to bind to Bax and initiate conformational changes. **D.** Bax-IRF3 binding to the mitochondrial membrane doesn't occur in the presence of N^{pro}.

1.1.iii.e N^{pro} Rapidly Redistributes with IRF3 to mitochondria and cytoplasmic puncta

Previous work in other labs have said that IRF3 is targeted by N^{pro} to proteasomes for degradation. We used a time course after stress with NaA to follow IRF3 degradation and co-localisation with N^{pro}. Cellular re-localisation of N^{pro} co-expressed with IRF3-GFP was examined by inducing a stress response with NaA to mimic a viral infection. A live cell time course with cells stably expressing N^{pro}mCherry were transfected with IRF3-GFP, then visualised by confocal microscopy after the addition of NaA (Figure 1.1.10). At 30 mins post-stimulation, N^{pro} and IRF3 were present in the cytosol but also accompanying tubular mitochondrial structures, visualised with Mitotracker and as small punctate co-localised on and around the mitochondria. (Figure 1.1.10, A). To visualise that IRF3 was seen in these distinctly small punctate structures with N^{pro} at 30 mins (Figure 1.1.10, B), to then after 60 mins of treatment, these co-localised structures not only increased with number but also in size (Figure 1.1.10, C). After 60 mins, N^{pro} also remained associated with tubular mitochondria but the size of N^{pro} cytoplasmic puncta increased. IRF3 at this stage was almost exclusively co-localised with N^{pro} in larger puncta in the cytoplasm, or in the nucleus (Figure 1.1.10, C). Further work will determine the identity of these puncta by co-staining with antibodies to lysosomes or autophagosomes and other subcellular compartments [1].

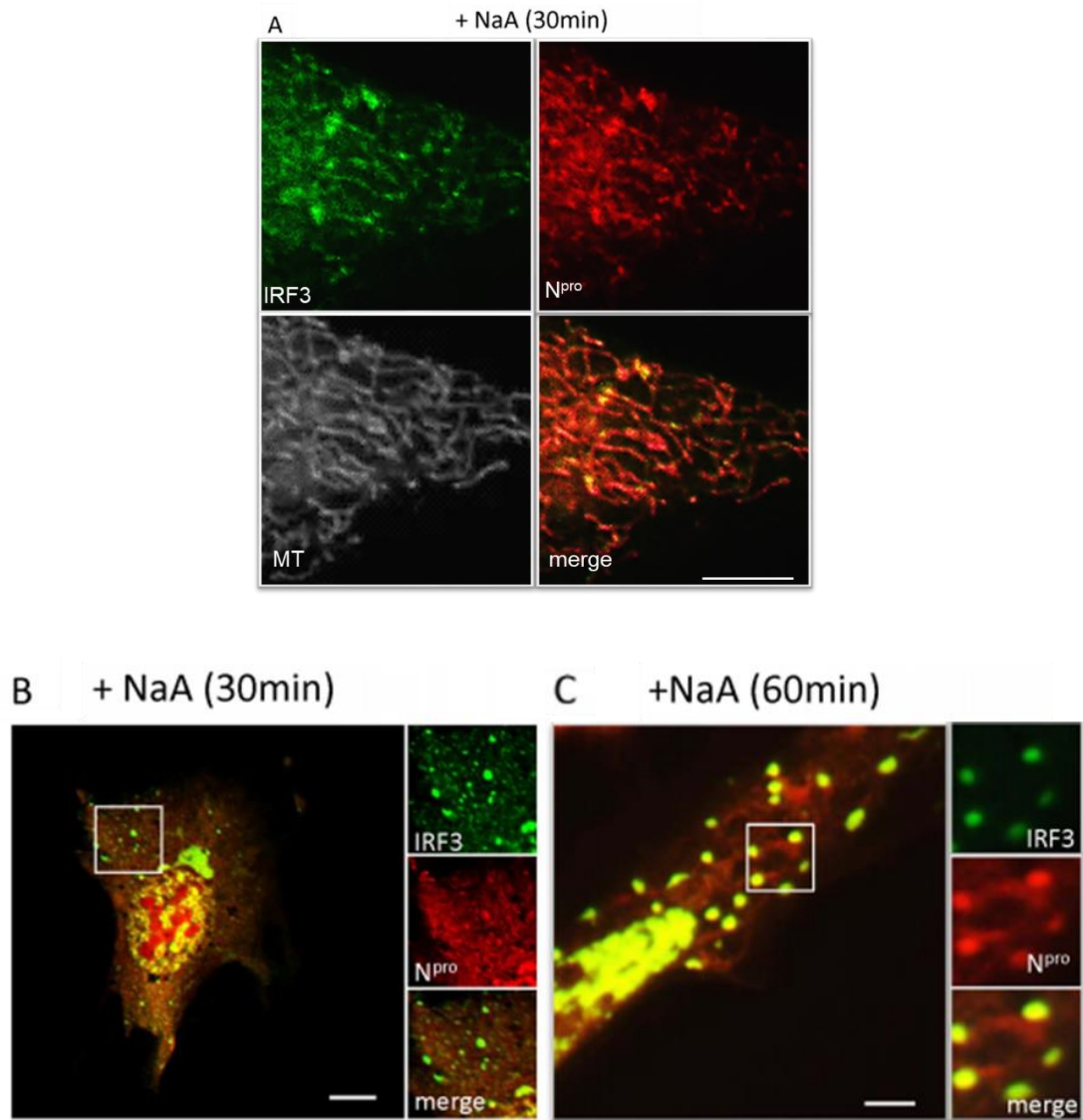


Figure 1.1.10. N^{pro} rapidly redistributes with IRF3 to mitochondria and cytoplasmic puncta following induction of stress. A-B. MEF cells co-expressing N^{pro} mCherry (red) and IRF3-GFP (green) with and without Mitotracker, were treated with NaA for 30 minutes and examined by confocal microscopy. **C.** Treatment of MEF cells co-expressing N^{pro} mCherry and IRF3-GFP for 60 minutes. Imaging by Jefferson et al [1].

1.1.iii.f N^{pro} Redistributes with IRF3 to Mitochondria and Peroxisomes

IRF3 and MAVS are key regulators of interferon signalling and have been shown located on both mitochondria and peroxisomes in response to cell stress [33]. In un-stimulated control cells, peroxisomes were visualised by immunostaining with PMP70. The peroxisome presented themselves as abundant small cytoplasmic punctate and when N^{pro}mCherry was co-expressed there was no change in regard to punctate size or numbers of peroxisomes (Figure 1.1.11, Ai). Once a stress response was initiated with NaA, N^{pro} accumulated in cytoplasmic puncta as previously seen in Figure 1.1.10 and many of these puncta co-distributed with peroxisomes (Figure 1.1.11, Aii). Using Imaris software, a statistical analysis was performed by creating digital rendering of pixel densities on fluorescent puncta observed, using the same cells as shown in Figure 1.1.11, Aii (Figure 1.1.11, Aiii). The average number of N^{pro} granules per cell treated with NaA for 4 h, versus the average number of N^{pro} granules associated with PMP70 per cell was compared (Figure 11, B). Statistical analysis in the form of a box plot (Figure 1.1.11, C) of the number of puncta containing PMP70 and containing N^{pro} was done. A distribution of 75% in the upper end of the box gave a ratio of 0.85 puncta with N^{pro} and PMP70 when compared to the total number of N^{pro} positive puncta. This reveals that most of the N^{pro} puncta observed are associated with peroxisomes after 4 h of treatment with NaA. The larger cytoplasmic puncta remained unidentified by sub-cellular markers [1].

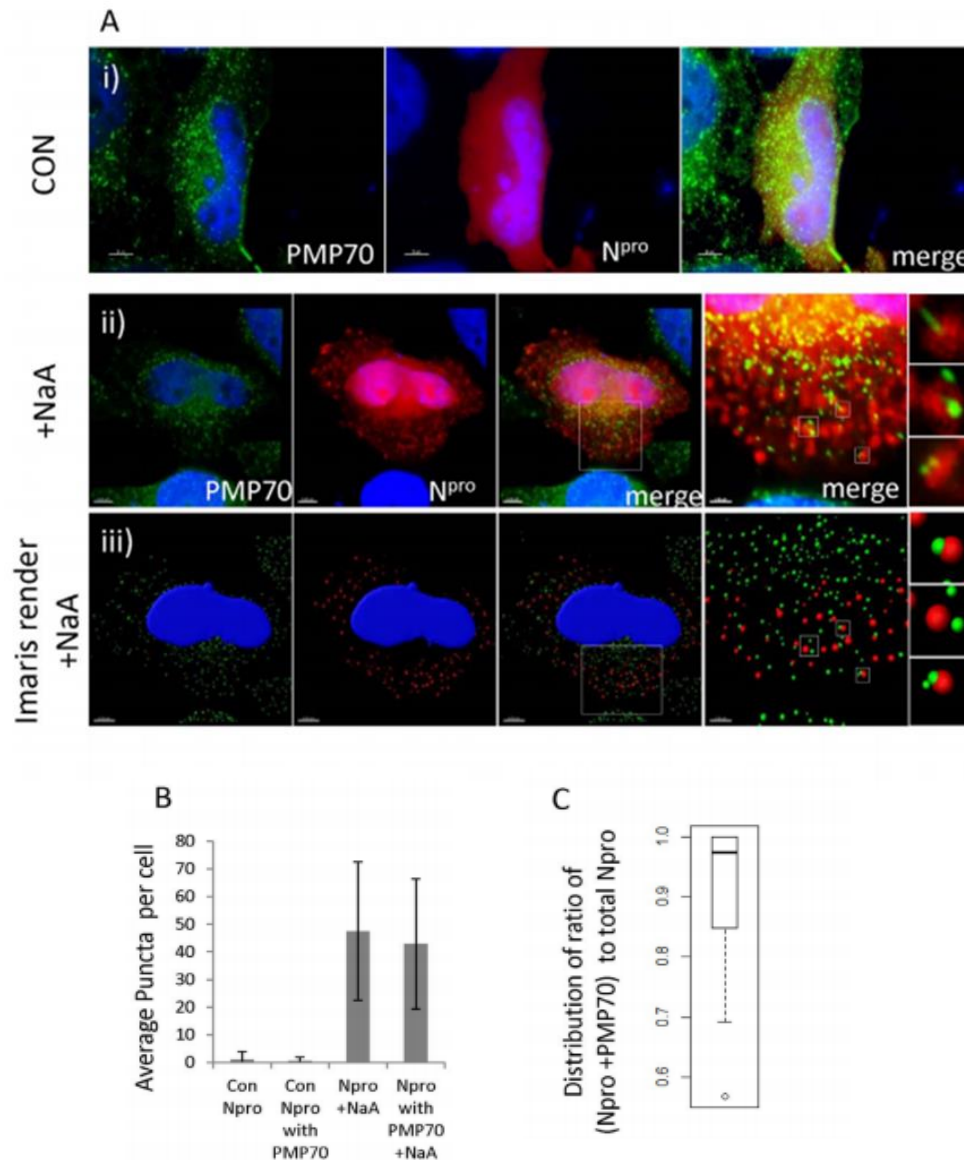


Figure 1.1.11. N^{pro} co-distributes with peroxisomes marker PMP70. **A.** Peroxisomes were localized by immunostaining with anti-peroxisome marker PMP70 in MEF cells expressing N^{pro}mCherry **(i)** control cells (CON) **(ii)** treatment with +NaA for 4 h **(iii)** Digital rendering using Imaris software is shown for the images in ii) (scale bars are 5 μ m). **B.** Statistical analysis of average number of N^{pro} puncta per cell and average number co-distributing with PMP70 after NaA treatment for 4 h. **C.** Boxplot: distribution of the fraction of N^{pro} distributing with PMP70 compare to total number of N^{pro} granules. A Welch two sample t-test was applied, two-sided, not paired, (assuming that the variances are unequal) and showing $t = 0.5867$, $df = 25.712$, $p\text{-value} = 0.5625$ with the lower end of the box = 25% of the data, bold line in the middle = median, Upper end of the box = 75%, lower whisker = 5%. Imaris rendering was performed by Matthew Whelband. Statistical analysis was performed by Irina Mohorianu, as both named on the paper. Imaging by Jefferson et al [1].

1.1.iii.g N^{pro} and IRF3 Co-distributes to Cytoplasmic Peroxisomes Associated with Ubiquitin

Knowing that in the presence of N^{pro} the degradation of IRF3 is increased by the proteasome [26], the site of ubiquitin accumulation in cells expressing N^{pro} following stress was determined. In control cells co-expressing N^{pro}mCherry and IRF3-GFP together and then immunostained for polyubiquitin (Figure 1.1.12, Ai), IRF3 and N^{pro} were seen throughout the cytoplasm and at a perinuclear site, which also contained a small amount of ubiquitin. After the addition of NaA, it rapidly induced the formation of small (block arrow) and large (arrow) N^{pro} puncta, that was also IRF3 positive in both forms localized with ubiquitin (Figure 1.1.12, Aii). The small punctate structures observed were confirmed as peroxisomes using an anti-peroxisome antibody PMP70 (Figure 1.1.12, Aiii). The large puncta that were also present remain unidentified as these failed to show co-staining peroxisome marker PMP70. To compare, mutant N^{pro} C112R also redistributed to these puncta (not shown), suggesting that redistribution was independent to IRF3 binding. However, it cannot be excluded that the redistribution of IRF3 to peroxisomes may depend on its binding to N^{pro}. These results indicate that IRF3 and N^{pro} redistribute together to peroxisomes and larger cytoplasmic stress granules where ubiquitin accumulates. These puncta did not co-stain with LAMP1, so these remain unidentified but they are probably lysosomes [1].

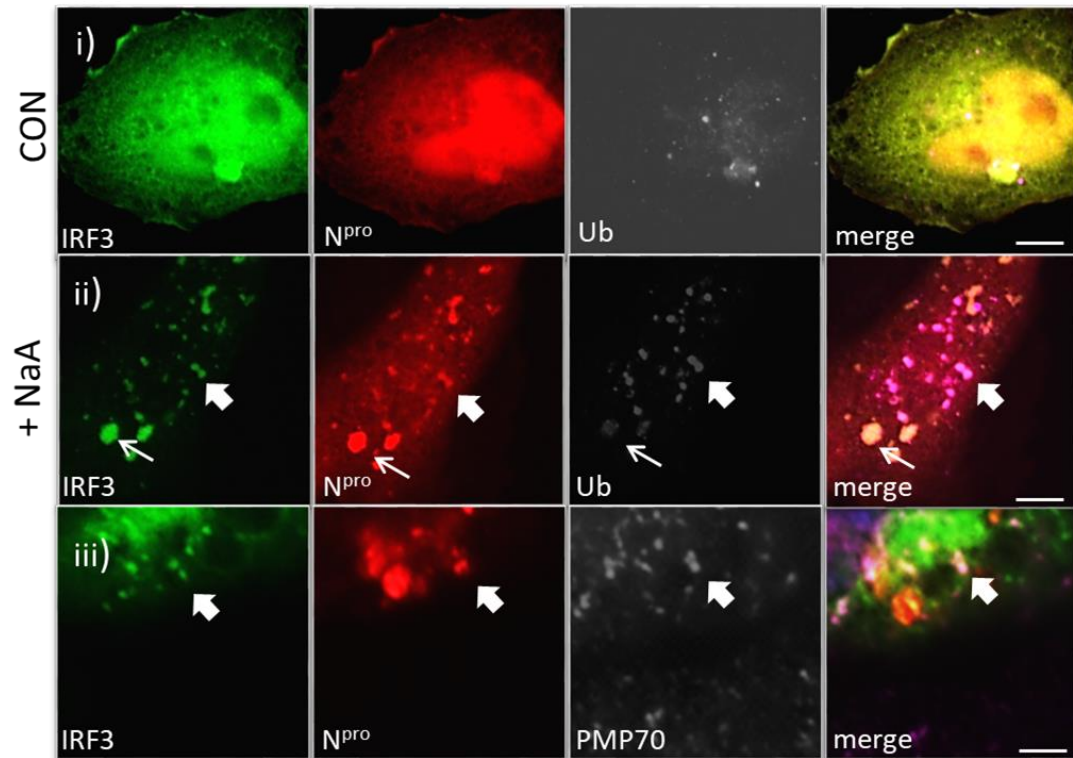


Figure 1.1.12. N^{pro} and IRF3 co-distribute to cytoplasmic peroxisomes containing ubiquitin. Cells expressing IRF3-GFP and N^{pro}mCherry **(i)** Control cells (CON) were stained for ubiquitin with monoclonal antibody FK2 and visualized with anti-mouse Cy5. **(ii)** Cells treated with NaA for 60 mins and ubiquitin was stained with monoclonal antibody FK2 and visualized with anti-mouse Cy5. **(iii)** Cells treated with NaA for 60 mins and peroxisomes were stained with anti-rabbit PMP70 and visualized with anti-rabbit Cy5. Imaging by Jefferson et al [1].

1.1.iv. Discussion

IRF3 is essential in interferon signalling and also for activation of apoptosis. Pestivirus protein N^{pro}, inhibits interferon signalling by degrading IRF3 during virus infection with CSFV and when expressed alone in cell lines [21]. These properties of N^{pro} are cross universal, as we demonstrate here in mouse and human cell lines. IRF3 was partially degraded in the presence of mutant N^{pro} C112R and D136N, suggesting that two or more residues are necessary to completely inactivate the motif [28]. The apoptotic pathway was also inhibited by the expression of wild type N^{pro}, but not by the mutant N^{pro}. Recent work shows that direct binding of IRF3 to Bax activates apoptosis, so N^{pro} can also inhibit this pathway. Thus the dual effects of IRF3 on interferon signalling and apoptosis can be inhibited by a single viral protein N^{pro} [1].

Cellular stress initiated by both NaA and BVDV showed subcellular re-distribution of N^{pro} to tubular mitochondria. In the presence of N^{pro}, mitochondrial fragmentation was prevented. This prevented the Bax-dependant intrinsic apoptotic pathway and release of Cyt c. In comparison, mutant N^{pro} are unable to bind IRF3 efficiently and did not protect against apoptosis and mitochondrial fragmentation. But it does not rule out that this mutation interferes with a direct action of N^{pro} on mitochondrial fission. More recent studies have shown that longer incubation times of 48 hrs with flaviviruses, leads to mitochondrial fragmentation, which are then cleared by selective mitochondrial autophagy (mitophagy). Flavivirus-induced mitophagy inhibits mitochondrion-dependent apoptosis allowing a persistent infection [1, 54].

These data adds to published work at the time of this study, that IRF3-dependent activation of Bax is an important part of the intrinsic mitochondrial apoptotic pathway [31]. The BH3 domain of IRF3 binds to cytosolic Bax and induces translocation of Bax to mitochondrial membranes to activate apoptosis in response in the presence of Sendai virus and double stranded RNA [55]. In our model we show that N^{pro} degrades IRF3, inhibiting IRF3 interaction with Bax and compromising the activation of apoptosis and stress when induced with NaA. This was also seen when cells were infected with BVDV where Bax redistribution to mitochondrial membranes was also inhibited. Mitochondria are important sites to enable persistent infection of viruses. Many large DNA viruses like human adenoviruses, target the mitochondrial pathway by encoding bcl2 homologues to manipulate apoptosis [56]. This

work shows that smaller RNA viruses with limited coding, can manipulate both the innate immune and apoptosis pathway, by targeting the mitochondria for its role in IRF3 signalling [1].

Immunofluorescence data showed N^{pro} localised to the mitochondrial tubular network and punctate structures identified as peroxisomes using marker PMP70. These were in close proximity to mitochondria but did not stain with the mitochondrial marker, Mitotracker. Peroxisomes are also important platforms for regulating innate immune signalling in response to viral infection. Peroxisomes harbour several other essential antiviral proteins like adaptor transmembrane protein RIG-I [34], antiviral signalling protein MAVS [57] and LSm14A, a sensor for both RNA and DNA [58]. When vRNA is released into the cytosol and detected by RIG-I, RIG-I translocates to peroxisomes to activate MAVS. Here it activates the early expression of ISGs and IFNs that function as antiviral effectors [59]. Additionally, MAVS recruitment to peroxisomes modulates the NF- κ B signalling pathway [60], previously shown that N^{pro} interacts with I- κ B α , the inhibitor of NF- κ B [1, 61].

N^{pro}'s ability to interact with peroxisomes demonstrates another unique ability at inhibiting the host's antiviral pathway. The important role played by peroxisomes is also demonstrated by the fact that other viruses have co-evolved to target this site. HCV encodes a multifunctional serine protease NS3-4A, which localises to the peroxisome membrane where it targets and cleaves peroxisomal MAVS, resulting in its degradation [62]. Dengue virus (DENV), West Nile virus (WNV), Zika virus (ZIKV) all target the peroxisome platform through different mechanisms to alter peroxisome mediated immune signalling [63]. HCV, DENV, WNV and ZIKV are also positive single stranded RNA virus, belonging to same *Flaviviridae* family as CSFV and BVDV. Other examples outside the *Flaviviridae* family include influenza virus which uses its non-structural protein NS1, to bind to a multifunctional peroxisomal enzyme [64]. NS-1 showed the same punctate immunofluorescence structure as transfected N^{pro}, which was also shown to inhibit interferon production [1, 64].

Rapid relocation of N^{pro} to peroxisomes containing ubiquitin was observed during microscopy analysis. The co-localisation of IRF3 and N^{pro} with ubiquitin in peroxisomes suggests a link between IRF3 binding to N^{pro} and rapid IRF3 degradation. The mechanism still remains unclear, but a recent report showed that N^{pro} is able to bind both inactive and activated IRF3, for ubiquitination and proteasomal degradation [1, 65, 66].

In conclusion, our study shows that the pestivirus protein N^{pro} disrupts the host innate immune response by associating with IRF3 on mitochondria and peroxisomes. This is probably through the binding of Bax to IRF3, and the inhibition of the IRF3-induced conformational change in Bax by N^{pro}. It would be interesting to determine if all three proteins bind simultaneously to mitochondria, or whether N^{pro} binds to mitochondria independently of IRF3. Identifying co-localisation of ubiquitin with peroxisomes containing IRF3 in the presence of N^{pro}, possibly leads to enhanced degradation of IRF3 by ubiquitin targeting to lysosomes or proteasomes. N^{pro} co-distributed with peroxisomes but the larger puncta seen were unidentified by sub-cellular markers used. These may be lysosomes or autophagosomes and will require additional surveillance to identify the protein N^{pro} is co-localised with. By targeting IRF3 for enhanced degradation, N^{pro} is also able to inactivate innate immune pathways for both apoptosis and interferon production. This creates conditions perfect for a persistent infection and allowing the spread to neighbouring cells.

N^{pro} is a multifunctioning protein and in the next chapter I investigated new host proteins that bind to N^{pro} that might be targets for antiviral therapies to prevent viral persistence [1].

1.2. Host Factors That Interact with the Pestivirus N terminal Protease, N^{pro}, Are Components of the Ribonucleoprotein Complex

This chapter is an adaption of work that has been published (Jefferson et al) [67] and appears with permission of the author and named contributors.

1.2.i. Introduction

1.2.i.a Pestivirus translation and replication

The pestiviruses genome is a positive-sense, single stranded RNA, containing one open reading frame (ORF) consisting of structural and non-structural proteins within a single polyprotein. The fast synthesis of its own proteins is necessary for rapid inhibition of the host's innate immune response and for initiating virus replication [68]. Replication occurs in the cytoplasm with no nuclear component. The virus matures in intracellular vesicles derived from the endoplasmic reticulum (ER) [69]. The ORF is flanked by untranslated regions (UTRs) and at the 5' UTR there is an internal ribosome entry site (IRES) [70]. Translation is initiated at the IRES, which binds the 40s ribosomal subunit and eIF3, to direct cap-independent translation. This does not require the translational initiation factors eIF4B and eIF4F [71]. The motifs and domains within the IRES is conserved amongst the pestiviruses [72] and other groups of viruses belonging to the *Flaviviridae* family, like HCV [73]. Deletion of N^{pro} has no effect on viral replication or virulence [24] (Figure 1.2.1) [67].

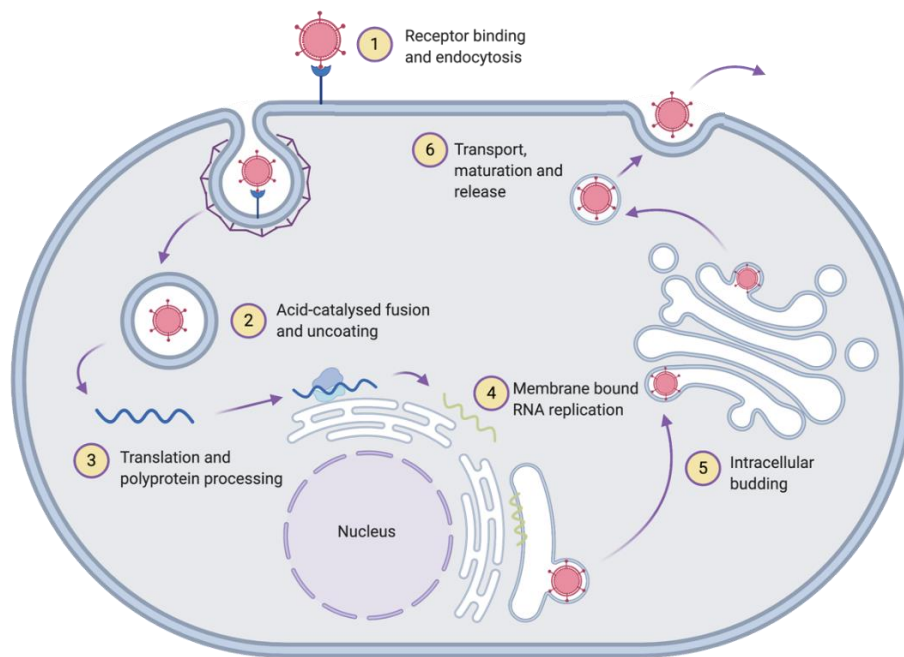


Figure 1.2.1. Pestivirus life cycle. **1.** Attachment of pestivirus envelope protein to the host's cell surface receptor, is one method of internalisation by clathrin-mediated endocytosis. **2.** In endosomes, the viral envelope fuses with the host's endosomal membrane, and the viral capsid disassembles. RNA genome is released into the cytoplasm. **3.** At the ER, positive-sense genomic ssRNA is translated by host ribosomes into a polyprotein. The polyprotein is cleaved into all structural and non-structural proteins. **4.** The viral RNA-dependent RNA polymerase, replicates the viral genome in specialised ER derived membrane compartments where a dsRNA genome is generated. **5.** Virus assembly occurs at the ER where virions bud to be transported to the Golgi. **6.** New virions are released by exocytosis.

1.2.i.b Stress Granules impose a barrier to viral protein translation

Viruses are reliant on the host's translational machinery to translate viral genes during infection in order to replicate and maintain a persistent infection. Viral infections interfere with normal cell function, resulting in cell stress responses at multiple levels. These include ER-associated stress, mitochondrial stress as a result of oxidative stress and cytoplasmic stress producing stress granules (SGs) [74]. The stress response results in inhibition of translation and stalled mRNAs, which are stored as non-membraneous ribonucleoprotein (RNP) aggregates called SGs [75]. SGs play an essential role in the regulation of gene expression by halting mRNA translation. Signalling may lead to an antiviral response, which makes them a target of viruses [76]. The innate immune response to infection takes place through viral sensing of dsRNA by PRRs like RNA helicases and PKR. Not only is PKR an RNA sensor that can bind dsRNA, but it is also a cell stress sensor [77]. When activated it phosphorylates initiation factor eIF2 α , to arrest translation and promote SG formation [78]. RNA helicases RIG-I, MDA5, MAVS and PKR can co-localise with SGs induced by viruses, forming antiviral stress granules (avSG). These may function as a platform for RNA sensing and IFN response, or a method of inhibiting protein synthesis of IFN induced genes [79, 80] (Figure 1.2.2) [67].

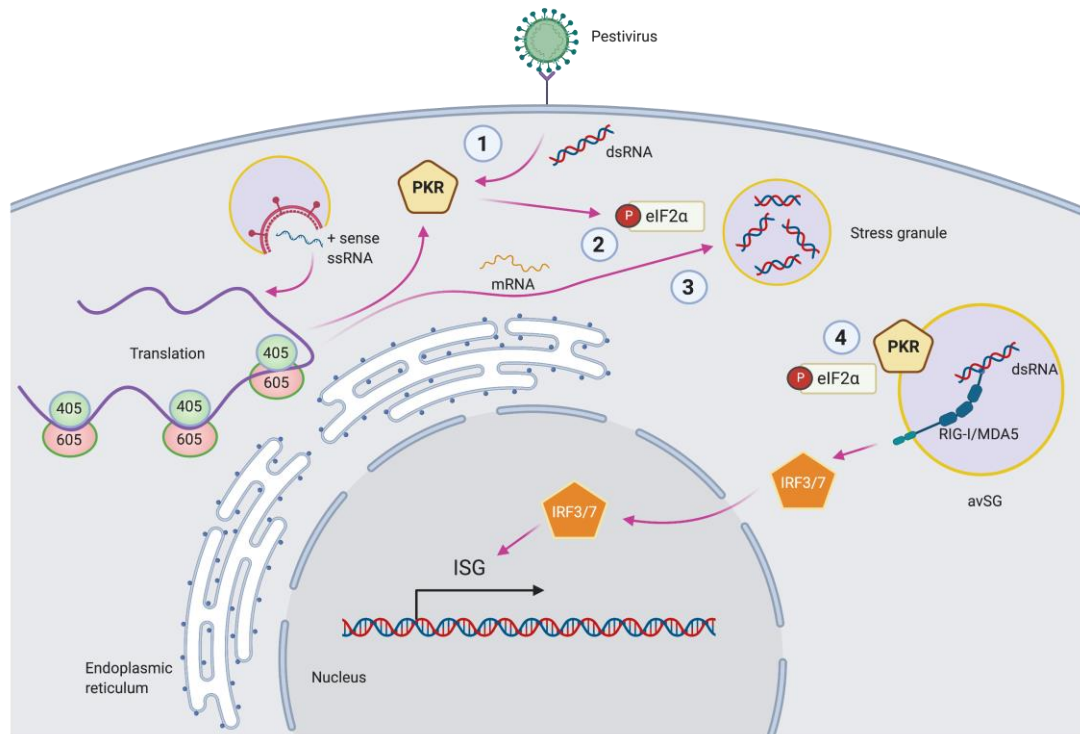


Figure 1.2.2. Viruses can induce SG formation by activating PKR. 1. PKR is activated by cell stress as a result of viral infection or as a viral sensor, binding dsRNA. 2. Activated PKR phosphorylates eIF2α and promotes RNP aggregation and SG formation. 3. RNA-protein aggregates halting translation of viral mRNA. 4. RNA helicases RIG-I, MDA5, MAVS and PKR relocate to SGs forming an avSG providing a platform for RNA sensing and IFN stimulation.

DExD/H-box helicases such as DHX9 from now on referred to as RNA helicase A (RHA), can bind vRNA acting through MAVS/IPS-1 [81]. RHA antiviral signalling activates NF-κB signal transduction pathway to induce IFNα/β genes [82, 83]. Most viruses encode proteins that block these innate responses to dsRNA [84]. Our previous work with pestivirus N^{pro} implicated its role in the degradation and loss of IRF3, which is a central regulator of interferon transcription and initiator of apoptosis [1]. Loss of IRF3 as a result of N^{pro}, inhibits dsRNA-induced interferon alpha and beta expression. N^{pro} was shown to directly interact with IRF3 by co-immunoprecipitation and by mutating the TRASH domain of N^{pro} [1, 26, 67].

Does N^{pro} interact with additional binding partners to inhibit an innate immune response?

Many viral structural proteins are multi-functional. CSFV has only 12 proteins but it can persist in pigs, causing long term disease. N^{pro} has many interesting properties. It is an RNA binding protein; it has autocatalytic activity and it's a Zn binding protein. N^{pro} targets IRF3 for degradation when expressed in cells alone, and as shown in the previous chapter, lack of IRF3 can also inhibit apoptosis.

We were interested in identifying cellular proteins that interact with N^{pro} that may explain viral persistence and life cycle. Using mass spectrometry from various pulldown assays we investigated new targets of this viral protein. Interestingly host proteins binding to N^{pro} were found to be RNA-binding proteins involved in translation. This is interesting because virus infection stops host protein translation in order to translate its own proteins. When host protein translation is halted through activation of PKR, non-membraneous stress granules are formed containing translation machinery and stalled transcripts. Following the data that N^{pro} bound to RNA proteins involved in translation, we investigated formation of stress granules in pestivirus infection.

1.2.ii. Materials and Methods

1.2.ii.a Plasmids and Transfections

TIA1-mCherry, pEGFP-RNA helicase A (RHA), V5-tagged YBX-1 V5, DDX1-V5, and DCP1a, and Myc-tagged DDX3 were transfected into cells with Fugene HD (Roche). Transfected cells were given 24 h to express labelled proteins prior to stimulation with 100 μ M NaA. Plasmids used were obtained from the following sources: pDDX3-myc was from Andrew Bowie and Martina Schoder [85]. pEGFP-RHA full length (amino acids 1 to 1270) and pEGFP-RHA R1163A with a mutation at amino acid 1163 was gifted by T. Nakajima, Department of Genome Science, Institute of Medical Science, St. Marianna University School of Medicine, Kanagawa, Japan [86]. pDDX1-V5, pDCP1a-V5, and YBX-V5 were from S. Ishura, University of Tokyo [87]. The mCherry-tagged construct TIA1 was from T. Eisinger-Mathason, University of Virginia, as described in reference [88]. La-V5 was generated by T. Csorba (University of East Anglia, Norwich, United Kingdom) [67].

1.2.ii.b GST Pulldown analysis

Bacteria (*Escherichia coli*, BL21) were transformed with plasmids encoding glutathione S-transferase (GST) or GST-N^{pro}, cultured for 4 h, and then induced with 100 mM isopropyl-B-dithiogalactopyranoside (IPTG) for 4 h. Bacteria were lysed with the Bug Buster Protein extraction reagent (Merck). Lysates were clarified by centrifugation, re-suspended in low-salt pulldown buffer (LPD) (20 mM Tris-HCl [pH 7.6], 200 mM NaCl, 0.5% NP-40, 0.5 mM dithiothreitol [DTT], and 0.4 mM phenylmethylsulfonyl fluoride [PMSF]) and 1 mg/ml protease inhibitors, and added to 3 ml of 50% glutathione–Sepharose bead slurry (Amersham Biosciences) overnight at 4°C. Beads were washed 7 times in a high-salt pulldown buffer (HPD) (500 mM NaCl and 1% Triton) to remove non-specific proteins and re-equilibrated with LPD with or without 1 mM zinc chloride to obtain beads binding either GST alone or GST-N^{pro} [67].

HEK 293 cells were lysed in MPER buffer (Pierce), and supernatants were clarified by centrifugation. Lysates were pre-cleared by adding glutathione beads overnight at 4°C, and beads were separated by centrifugation. Pre-cleared supernatants were added to GST-Sepharose beads or GST-N^{pro}-Sepharose beads as appropriate. Beads were washed seven

times in LPD buffer, and proteins were analysed by SDS-PAGE. For mass spectrometry analysis, proteins were eluted from the beads with 10 mM or 30 mM reduced glutathione [67].

For V5 antibody pulldowns, HEK 293 cells were transfected with plasmids expressing protein with a V5 tag for 24 to 48 h, and cells were lysed in MPER with 1 mM ZnCl and incubated on ice for 30 min. In some experiments, a zinc chelator, N,N,N',N'-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), was used. Lysates were pre-cleared with 50% slurry protein G-Sepharose for 1 h, before addition of anti-V5 antibody followed by protein G Sepharose beads. Beads were washed seven times in LPD, re-suspended in sample preparation buffer, and then analysed by SDS-PAGE followed by Western blotting [67].

1.2.ii.c Identification of virus-host protein partners by mass spectrometry

All mass spectrometry and protein library assembly was done at the Johns Innes Centre proteomics lab by Gerhard Saalbach.

Proteins were eluted from washed beads by adding 2-fold-concentrated SDS-gel sample loading buffer and heating to 80°C for 10 min. The supernatant was loaded onto a Novex gel (10% Bis-Tris SDS gel, Life Technologies) and run about 1/3 of the length of the lane. The lanes were cut out and stained with InstantBlue (Expedeon) in separate trays until bands were just visible. The area covering the bands was cut into 5 to 6 slices, which were washed, reduced, and alkylated and treated with trypsin according to standard procedures. Peptides were extracted with 5% formic acid–50% acetonitrile, dried down, and re-dissolved in 0.1% trifluoroacetic acid (TFA). For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, a sample aliquot was applied via a nanoAcquity (Waters) ultra-performance liquid chromatography (UPLC) system running at a flow rate of 250 nl min⁻¹ to an LTQ-Orbitrap mass spectrometer (ThermoFisher). Peptides were trapped using a pre-column (Symmetry C₁₈, 5 µm, 180 m by 20 mm; Waters), which was then switched in-line to an analytical column (BEH C₁₈, 1.7 m, 75 µm by 250 mm; Waters) for separation. Peptides were eluted with a gradient of 3 to 40% acetonitrile in water–0.1% formic acid at a rate of 0.67% min⁻¹. The column was connected to a 10 µm SilicaTip nanospray emitter (New Objective) attached to a nanospray interface (Proxeon) for infusion into the mass spectrometer. The mass spectrometer was operated in positive ion mode at a capillary temperature of 200°C. The source voltage and focusing voltages were tuned for the

transmission of Met-Arg-Phe-Ala (MRFA) peptide (m/z 524) (Sigma-Aldrich). Data-dependent analysis was carried out in Orbitrap-ion trap parallel mode using collision-induced fragmentation (CID) on the 6 most abundant ions in each cycle. The Orbitrap was run with a resolution of 30,000 over the MS range from m/z 350 to m/z 1,800 and an MS target of 10^6 and 1-s maximum scan time. Collision energy was 35, and an isolation width of 2 was used. Only monoisotopic 2+ and 3+ charged precursors were selected for MS2 fragmentation stage. The MS2 was triggered by a minimal signal of 1,000 with an automatic gain control target of 3×10^4 ions and 150-ms scan time using the chromatography function for peak apex detection. Dynamic exclusion was set to 1 count and 60-s exclusion with an exclusion mass window of ± 20 ppm. MS scans were saved in profile mode, while MS/MS scans were saved in centroid mode [67].

Raw files were processed using the software program MaxQuant, version 1.3.0.5 [89] (<http://maxquant.org>), to generate recalibrated peak list files, which were used for a database search using an in-house Mascot 2.4 server (Matrix Science Limited). Mascot mgf files were generated from MaxQuant apl files using a suitable Perl script. Mascot searches were performed on the Sprot_sptrembl20121031.fasta database with taxonomy set to human using trypsin/P with 2 missed cleavages, 6-ppm precursor tolerance, 0.6-Da fragment tolerance, carbamidomethylation (C) as fixed, and oxidation (M) and acetylation (protein N terminus) as variable modifications. Mascot search results were imported and evaluated in the program Scaffold 3.6.1 (Proteome Software), resulting in a false-discovery rate of 0% for both peptides and proteins. For the detection of GST and CSFV N^{pro}, the protein sequences of those proteins were added to a custom database with 1,000 random *E. coli* sequences (downloaded from uniprot.org) as a background, and the searches were performed in the same way as described above [67].

The mass spectrometry proteomics data has been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [90] with the data set identifier PXD000115 and doi number 10.6019/PXD000115 (PRIDE accession numbers 28015 to 28026) [67].

1.2.ii.d Western Blotting

Membranes were probed with antibodies rabbit anti-YBX1 (Abcam, ab12148), anti-RHA antibody (Abcam, ab54593), anti-V5 MAb (Life Technologies, R960-25), and DS14 anti-N^{pro} [67].

1.2.ii.e Immunofluorescence

HEK293 cells were fixed in 4% Paraformaldehyde and permeabilised with 0.2% Triton X-100. Cells were then blocked in goat serum, gelatin quench. Primary antibodies were diluted into 0.2% Triton X-100 goat gelatin quench and incubated for 24 h at 4°C. Antibodies used were anti-RHA monoclonal antibody (Abcam, ab54593), anti-V5 (Life Technologies, R960-25), anti-Myc 9E10 MAb, anti-YB1 (Abcam, ab12148), and anti-ubiquitin FK2 (Enzo, BML-PW8810) [67].

1.2.iii. Results

1.2.iii.a Host proteins interacting with N^{pro} are components of ribosomes and ribonucleoprotein particles

Orbitrap mass spectrometry was run and compiled by Gerhard Saalbach at the John Innes Centre [67]. It was used to identify novel cellular proteins interacting with N^{pro} from the CSFV strain Alfort. Purified recombinant GST-N^{pro} protein was incubated with HEK 293 cell lysates that had either been untreated or transfected with synthetic dsRNA, poly I:C for 4 h, both to induce expression of early response genes that may be important following viral infection. Table 1 shows 45 proteins that were specifically eluted from GST- N^{pro} compared to GST alone, with either low (10 mM) or high (30 mM) glutathione concentration and with or without poly I:C. The table is in order of the number of peptides found for each protein. Full list is given in the PRIDE partner repository, dataset identifier PXD000095 and DOI 10.6019/PXD000095 PRIDE accessions 28008. Many of the hits were ribosomal proteins and dsRNA binding proteins which form RNPs in the cytoplasm and are involved in RNA metabolism. Transfection of dsRNA poly I:C into cells for 4 h before lysis made very little difference to the proteins detected, indicating that binding to N^{pro} was not dependent on dsRNA, normally only present in cells during viral infection and replication. As well as ribosomal proteins, interacting proteins included Y-box binding protein (YBX-1), La autoantigen, RNA helicase A (RHA), multiple myeloma tumour protein 2 (MMTAG2), interleukin enhancer binding factor 3 (IEBP3), guanine nucleotide binding protein 3 (GNL3) and polyadenylate-binding protein 1 (PABP-1). These proteins are involved in RNA metabolism, including translation and mRNA degradation and they are normally found distributed throughout the cytoplasm of cells [91]. It may be that N^{pro} interacts with one or two of these RNA binding proteins, and the other proteins are pulled down in the same complex. No direct binding of N^{pro} to IRF3 was seen, though targets had been previously identified using yeast 2-hybrid interaction as well as IRF7, Hax-1 or I- κ B α [26, 28, 61, 92, 93] were not identified. This may reflect differences between yeast 2-hybrid and protein pulldown-mass spectrometry. This may be due to endogenous levels of these proteins are low in HEK 293 cells when compared to immune cells, or additional proteins or conditions are required for binding. Our previous work has shown that N^{pro} is able to bind and degrade IRF3 in HEK 293 cells [1, 67].

Peptide abundance rank	Accession no.	Protein identified	Mol mass ^b (kDa)	Association with N ^{pro} at GSH concn (mM) of:							
				10				30			
				No pI-C		pI-C		No pI-C		pI-C	
				% ID ^c	No. of peptides	% ID	No. of peptides	% ID	No. of peptides	% ID	No. of peptides
1	P09211	Glutathione S-transferase	23	100	7	100	10	100	9	100	9
2	P61247	40S ribosomal protein S3a	30	100	6	100	4	100	3	100	4
3	P15880	40S ribosomal protein S2	31	100	3	100	1	100	3	100	3
4	Q00839	Heterogeneous nuclear ribonucleoprotein U	91	100	6	100	2	100	4	100	4
5	P67809	Y-box-binding protein 1 (YBX-1)	36	100	4	100	4	100	5	100	4
6	P62424	60S ribosomal protein L7a	30	100	3	85	1	100	3	100	5
7	P19338	Nucleolin	77	100	4	100	2	100	3	100	4
8	P62750	60S ribosomal protein L23a	18	100	4	100	4	100	2	100	3
9	P39023	60S ribosomal protein L3	46	100	3	99	1	100	2	83	1
10	P62263	40S ribosomal protein S14	16	100	2	100	1	100	2	100	2
11	P05455	La autoantigen, Sjogren syndrome type B antigen (SS-B)	47	100	3	100	2	100	3	100	3
12	Q5JR94	40S ribosomal protein S8	24	100	2	99	1	100	1	100	3
13	Q59F19	Ribosomal protein L12	21	100	2	100	2	100	2	100	2
14	P26373	60S ribosomal protein L13	24	100	2	99	1	100	1	100	3
15	P62244	40S ribosomal protein S15a	15	100	2	100	2	100	2	99	1
16	P46782	40S ribosomal protein S5	23	100	2	100	2	100	2	100	3
17	P62277	40S ribosomal protein S13	17	100	3	100	2	100	2	99	1
18	Q59ES8	Heterogeneous nuclear ribonucleoprotein M	65	100	3	93	0	100	3	100	2
19	P62081	40S ribosomal protein S7	22	100	1	46	0	100	1	99	1
20	P83731	60S ribosomal protein L24	18	100	2			99	0	100	3
21	Q08211	ATP-dependent RNA helicase A (RHA, DEAH box protein 9)	141	100	2			100	4	83	1
22	P18621	60S ribosomal protein L17	21	100	2	100	2	100	2	100	2
23	P61254	60S ribosomal protein L26	17	100	1	98	1	99	0	100	1
24	Q53HK9	Ribosomal protein P0	34	100	1	85	1	100	1	100	2
25	P18124	60S ribosomal protein L7	?	85	1	99	1	99	1	100	2
26	P62899	60S ribosomal protein L31	14	100	2	100	2	100	2	100	2
27	P39019	40S ribosomal protein S19	16			100	2	87	1	100	2
28	P38159	Heterogeneous nuclear ribonucleoprotein G (hnRNP G)	42	100	2			100	2	83	1
29	Q9BU76	Multiple myeloma tumor-associated protein 2	29	100	2	85	1	99	1	100	2
30	Q12906	Interleukin enhancer-binding factor 3 (TCP80)	95	100	2			100	2	100	2
31	P84098	60S ribosomal protein L19	23	100	2			100	1	60	0
32	P83881	60S ribosomal protein L36a	12	98	1	84	0	100	1	83	1
33	P61313	60S ribosomal protein L15	24	98	1	33	0	87	1	99	1
34	P36578	60S ribosomal protein L4	48	85	1			99	1	100	1
35	P31943	Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	49	100	3	85	1	36	0	48	0
36	Q9BVP2	Guanine nucleotide-binding protein-like 3	62	100	1	85	1	100	2		
37	P61978	Heterogeneous nuclear ribonucleoprotein K (hnRNP K)	51	85	1			100	2	100	2
38	Q8NC51	Plasminogen activator inhibitor 1 RNA-binding protein	45	85	1	85	1			100	2
39	P08579	U2 small nuclear ribonucleoprotein B ^o (SU2 snRNP B)	25	99	1			100	2	70	0
40	Q9Y3D9	28S ribosomal protein S23 (mitochondrial)	22	100	1	85	1	48	0		
41	P11940	Polyadenylate-binding protein 1 (PABP-1)	71	99	1					100	2
42	P27635	60S ribosomal protein L10	25					100	2	83	1
43	O60832	H/ACA ribonucleoprotein complex subunit 4	58	60	0			99	1		
44	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	37	56	0			99	1		
45	Q59GL1	Synaptotagmin binding, cytoplasmic RNA interacting protein variant	60					100	1		

Table 1.2.1. N^{pro} interacts with ribosomal and ribonucleoproteins, components of ribonuclear particles (RNPs).

Interaction of N^{pro} with cellular proteins was identified by mass spectrometry analysis. Recombinant GST-tagged N^{pro} was used to pull down cellular binding partners from HEK293 cells. Cells were either control with no. pI-C or transfected with poly (I·C) (dsRNA) for 4 h before lysis. Beads were eluted with low (10 mM) or high (30 mM) glutathione (GSH). Proteins binding to GST alone were used for a control. The accession number and molecular mass for each protein are shown in order of abundance of peptides found. ^b Molecular mass. ^c ID, identity. Mass spectrometry and sequence analysis was performed by Gerhard Saalbach, named on the paper [67]

1.2.iii.b Zinc is required for N^{pro} binding partners

N^{pro} is a metalloprotein coordinating a single zinc ion through a putative metal binding domain called a TRASH motif at Cys112-X21-Cys134-X3-Cys138 [94]. This domain is thought to be required for IRF3 interaction and degradation [28]. To determine which proteins were interacting with the zinc binding TRASH domain in the C-terminus end of N^{pro}, a second experiment, where recombinant protein was bound to lysates either in the presence of zinc or in the presence of a chelator of zinc, TPEN (deposited in PRIDE accessions 28009). A total of 55 cellular proteins was found to bind N^{pro} (Table 2), of which 9 were also detected in Table 1 (number in brackets in column 1 of Table 2). There were 28 additional proteins in the presence of zinc and only two extra proteins bound when zinc was absent (desmoglein and desmoplakin). 25 proteins bound irrespective of the presence of zinc. The proteins that required zinc were involved in RNA translation, and included PRRs, RNA helicases DDX5 and DHX15, eukaryotic initiation factor eIF3i, and tRNA ligases. These are RNPs, which are present in polysomes, SGs and P bodies [95]. Whether in the presence or absence of zinc, identical proteins were bound to N^{pro}. But the number of peptides increased in the presence of zinc, showing increased affinity, and suggesting some interaction with the TRASH motif in N^{pro}. Heterogeneous RNP (hnRNP) M, U, A/B/C, H and G were found as cellular binding partners. These proteins are involved in mRNA nuclear export, localisation, translation, and stability, important in both the nucleus and the cytoplasm. Many of these factors are also involved in post transcriptional gene regulation [67].

Peptide abundance rank ^b	Accession no.	Protein identified	Mol mass ^c (kDa)	Binding to N ^{PRO} with:			
				No Zn		Zn present	
				% binding	No. of peptides	% ID ^d	No. of peptides
1 (21)	Q08211	ATP-dependent RNA helicase A; DEAH box protein 9	141	100	30	100	50
2	P28161	Glutathione S-transferase	26	100	11	100	9
3	C7DJS2	Glutathione S-transferase	17	100	2	100	2
4	P52272	Heterogeneous nuclear ribonucleoprotein M	78	100	6	100	6
5 (2)	P61247	40S ribosomal protein S3a	30	100	1	100	7
6	P23396	40S ribosomal protein S3	27	100	2	100	7
7	P54577	Tyrosine-tRNA ligase	59			100	9
8	B5BTY4	ATP-dependent RNA helicase DDX3X	73	100	2	100	6
9	B1AHM1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	73	99	1	100	5
10	Q9NZI8	Insulin-like growth factor 2 mRNA-binding protein 1	63	100	2	100	5
11	P12956	Lupus Ku autoantigen protein p70	70	39	0	100	5
12 (38)	Q8NC51	Plasminogen activator inhibitor 1 RNA-binding protein	45	62	0	100	2
13	P46782	40S ribosomal protein S5	23	78	1	100	5
14	P62701	40S ribosomal protein S4	30	78	1	100	3
15	Q9Y262	Eukaryotic translation initiation factor 3 subunit L (eIF3L)	67	35	0	100	3
16	Q92900	ATP-dependent helicase RENT1	124	99	1	100	3
17 (41)	P11940	Polyadenylate-binding protein 1 (PABP-1)	71	25	0	100	3
18	P61011	Signal recognition particle 54-kDa protein; (SRP54)	56	18	0	100	2
19	O76094	Signal recognition particle 72-kDa protein (SRP72)	75			100	3
20	Q9Y285	Phenylalanine-tRNA ligase alpha subunit	58			100	3
21 (45)	Q59GL1	Synaptotagmin binding, cytoplasmic RNA interacting protein	60			100	3
22	Q0VAC0	Heterogeneous nuclear ribonucleoprotein A1	34	100	2	99	1
23	B3KNR3	Glutathione S-transferase	19	99	1	99	1
24 (19)	P62081	40S ribosomal protein S7	22	98	1	100	2
25 (30)	Q12906	Interleukin enhancer-binding factor 3; TCP80	95	78	1	100	3
26	P13435	Splicing factor 3B subunit 2	100	78	1	100	2
27	Q9UHB9	Signal recognition particle 68-kDa protein	71			100	3
28	P04844	Ribophorin II (RPN-II)	69			100	2
29	P49458	Signal recognition particle 9-kDa protein; SRP9	10			100	2
30	Q9H6T3	RNA polymerase II-associated protein 3	76			100	3
31 (11)	P05455	La autoantigen; Sjogren syndrome type B antigen (SS-B)	47			100	3
32	Q02543	60S ribosomal protein L18a	21			100	3
33	P37108	Signal recognition particle 14-kDa protein	15			100	2
34	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2	34	78	1	100	2
35	P62913	60S ribosomal protein L11	20	78	1	100	2
36	P62266	40S ribosomal protein S23	16	41	0	100	2
37	P62277	40S ribosomal protein S13	17			100	3
38	Q02413	Desmoglein 1	114	100	3		
39	Q6NVW7	Importin subunit alpha	58			100	2
40	Q9BUJ2	Heterogeneous nuclear ribonucleoprotein U-like protein 1	96			100	2
41	P14923	Desmoplakin 3	82	100	1		
42	P46778	60S ribosomal protein L21	19			100	1
43	Q96EY7	Pentatricopeptide repeat-containing protein 3 (mitochondrial)	79			100	1
44	P26640	Valine-tRNA ligase	140			99	1
45	F8W7C6	60S ribosomal protein L10	19			100	2
46	O43143	ATP-dependent RNA helicase DHX15 (DEAH box protein 15)	91			100	2
47	P17844	ATP-dependent RNA helicase DDX5; DEAD box protein 5	69			100	2
48 (44)	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	37			100	2
49	Q13347	Eukaryotic translation initiation factor 3 (eIF-3-beta)	37			100	2
50	Q53GX7	Threonyl-tRNA synthetase variant	83			100	2
51	Q99729	Heterogeneous nuclear ribonucleoprotein A/B	36			100	1
52	Q53F35	Acidic (leucine-rich) nuclear phosphoprotein 32	29			100	1
53	P62851	40S ribosomal protein S25	14			99	1
54	Q9Y5A9	YTH domain family protein 2; high-glucose-regulated protein 8	62			99	1
55	P18077	60S ribosomal protein L35a	13			99	1

Table 1.2.2. N^{PRO} binding to cellular proteins through TRASH domain by zinc chelation.

Cellular proteins associated with N^{PRO} in the presence or absence or zinc, detected by Orbitrap mass spectrometry analysis. b The accession number and molecular mass for each protein are shown in order of abundance of peptides found. Proteins also identified in Table 1 are indicated by numbers in parentheses. c Molecular mass. d ID, identity. Mass spectrometry and sequence analysis was performed by Gerhard Saalbach, named on the paper [67]

1.2.iii.c BVDV infection inhibits stress granule formation

From the mass spectrometry performed above there was 55 proteins identified with the highest hits and most of these were identified as ribosomal proteins and ribonucleoproteins including RHA, YBX1, DDX3, DDX5, eIF3, IGF2BP1, IEBP3, and PABP-1. These are components of the translation machinery, ribonucleoprotein particles (RNPs), and stress granules (SGs) which can halt translation and prevent translation of viral genomes. A ribonucleoprotein is a complex of RNA and RNA-binding proteins. These complexes have many roles within the cell including control of transcription, translation and regulating gene expression. YBX1 is an RNA binding protein and regulates RNA translation and SG formation [96]. RHA is also involved in gene regulation and is also associated with SGs. The RNPs shown to interact with N^{pro}, described above are known to aggregate during cell stress, into RNA granules known as SGs. SGs regulate initiation, termination and decay of mRNAs [97]. Viruses like ZIKV, IAV and Chikungunya virus (CHIKV), have been shown to actively inhibit SG assembly at certain times post-infection [98-102]. We investigated whether pestivirus infection also modulates SG formation. Arsenate treatment is a widely used for oxidative stress to induce SG formation and in the next experiments cells were treated with NaA in the presence and absence of BVDV (Figure 1.2.3, A). TIA1, an RNA binding protein which nucleates stress granule formation, was used as a SG marker [98]. TIA1 is not only a translational silencer but is also a key protein in the prion-like aggregation of further mRNPs to SGs [97]. YBX1, shown above to interact with N^{pro}, is a major component of cellular RNPs, which accumulates in granules following cellular stress. MDBK cells expressing YBX1 or TIA1 alone were treated with NaA for 4 h showed YBX1 and TIA1 accumulation in SGs and cells died by apoptosis, as seen by DNA fragmentation (Figure 1.2.3, A i and iii). BVDV infected cells were incubated for 48 h. YBX1 and TIA1 remained cytoplasmic following NaA treatment for 4 h (Figure 1.2.3, A ii and iv) and did not accumulate in SGs or in virus replication centres. BVDV infected cells were protected from apoptosis following NaA treatment. This was determined by a caspase 3/7 assay and quantified (Figure 1.2.3, B). There was a decrease in caspase activity after NaA treatment in BVDV infected cells when compared to uninfected cells. BVDV is able to inhibit apoptosis enabling it to persist longer in viable cells [67].

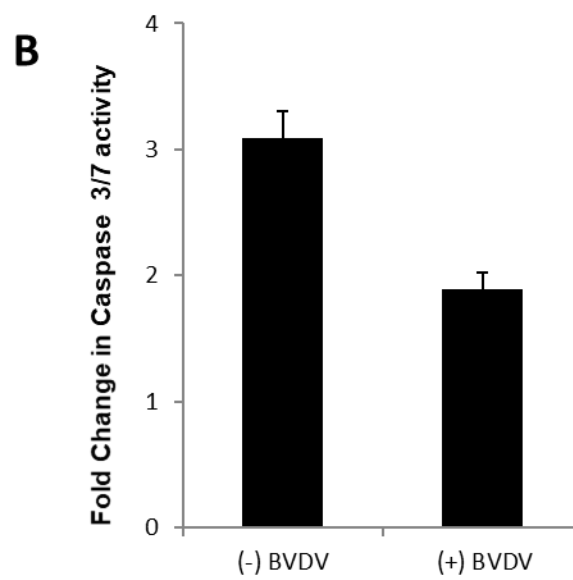
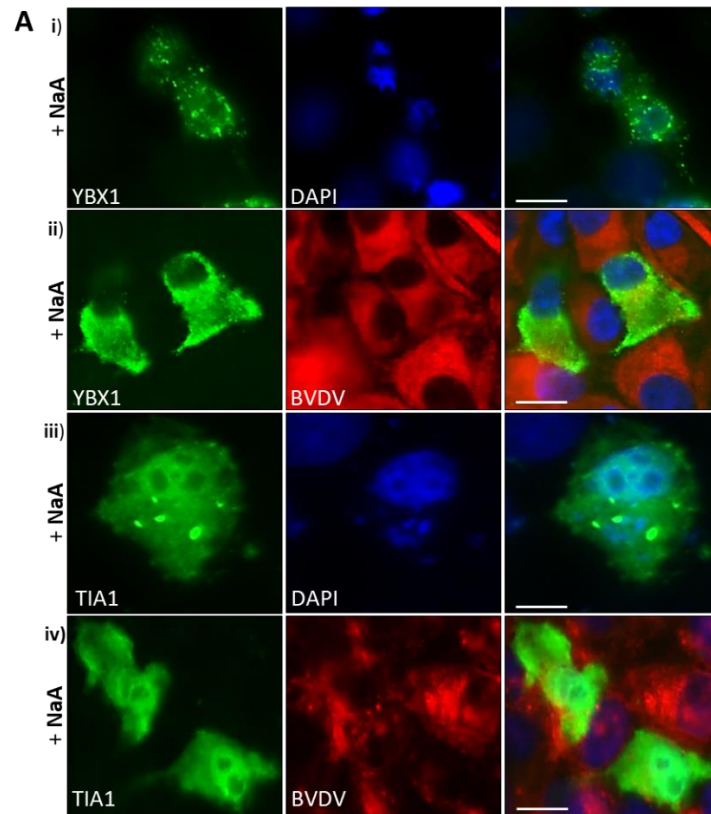


Figure 1.2.3. Does pestivirus infection affect stress granule formation? **A.** MDBK cells infected with BVDV are protected from stress-induced apoptosis. **(i)** Control MDBK cells transfected with YBX1-V5 and treated with NaA for 4 h. **(ii)** Cells infected with BVDV for 48 h post infection (hpi), transfected with YBX1-V5 overnight, and treated with NaA for 4 h. YBX-V5 was detected with anti-V5 MAb and anti-mouse Alexa 488 secondary antibody. Virus was detected with V182 hyperimmune serum and anti-bovine cy5 secondary antibody. **(iii)** MDBK cells transfected with TIA1-mCherry plasmid and treated with NaA for 4 h. (TIA was pseudo coloured green for consistency). **(iv)** MDBK cells infected with BVDV for 48 hpi, transfected with TIA1-mCherry, and treated with NaA for 4 h. Virus was detected with V182 hyperimmune serum and anti-bovine cy5 secondary antibody, and TIA1 was pseudo coloured green. **B.** BVDV infection decreases stress-induced caspase 3/7 activation. MDBK cells, either uninfected (-) or infected with (+) BVDV non-cytopathic (ncp) Kyle for 48 h, were stressed with NaA for 4 h. The graph shows the fold increase in caspase activity normalized to that of control cells and significance with one -sided t test value, P 0.00004. Imaging by Jefferson et al [67].

1.2.iii.d N^{pro} interaction with YBX1 does not prevent formation of stress granules

N^{pro} binds to RNPs. RNPs like YBX1, are a major component of SGs. Virus infection inhibits SG formation and with N^{pro}'s inhibitory actions previously stated, might be a candidate for the individual protein responsible for SG inhibition. Fluorescently tagged GFP-N^{pro} or N^{pro}mCherry were expressed in cells. N^{pro} distributed to the nucleus and cytoplasm (Figure 1.2.4, A, i) and subsequently redistributed to cytoplasmic granules following stress with NaA (Figure 1.2.4, A, ii). These cells were protected from apoptosis, as we have described in detail in our previous work [1], indicating SG assembly in the absence of apoptosis activation. When N^{pro}mCherry was expressed in BVDV-infected cells, N^{pro} relocated to an area associated with the viral replication complex (Figure 1.2.4, A, iii). In the next experiments, we focused on possible functional roles of N^{pro}, by investigating its binding to, localisation with, and effect on host proteins we have identified in Tables 1 and 2, outside the context of viral infection and in the absence of the other viral proteins. We investigated whether N^{pro} redistributed with YBX-1 in stressed cells. When co-expressed together, N^{pro} and YBX1 were both located to cytoplasm in unstressed cells (Figure 1.2.4, B, i). Cells expressing N^{pro}mCherry and YBX1 treated with NaA for 4 h showed N^{pro} redistribution to cytoplasmic granules containing YBX1 (Figure 1.2.4, B, ii), indicating that expression of N^{pro} itself does not inhibit the formation of SG complexes in cells. Cells expressing the mutant N^{pro}C112R protein, perinuclear SG were

formed containing YBX1 but these granules no longer co-localised with the N^{pro} C112R. These were observed in smaller cytoplasmic foci (Figure 1.2.4, B, iii, insert). These results indicate that wild type N^{pro} binds to YBX1 through its TRASH domain, since N^{pro} with the C112R mutation disrupts the zinc binding TRASH domain and no longer co-distributes with YBX1 to SGs. N^{pro} relocation to the SG complex is attributable to its binding to host proteins, such as YBX1. During infection, however, SG formation is inhibited and YBX1 remains cytoplasmic (Figure 1.2.3, A), whereas N^{pro} redistributes to the replication complex after infected cells are stressed (Figure 1.2.4, A). This suggests that, since YBX1 is not recruited to the replication complex during infection, it is not required for viral replication, but it may have an important function bound to N^{pro} at sites of viral genome translation in the cytoplasm. Though N^{pro} doesn't directly have an involvement in pestivirus replication, it may be interacting with host factors to prevent translational inhibition through binding with YBX1 and preventing YBX1 initiating SG formation [67].

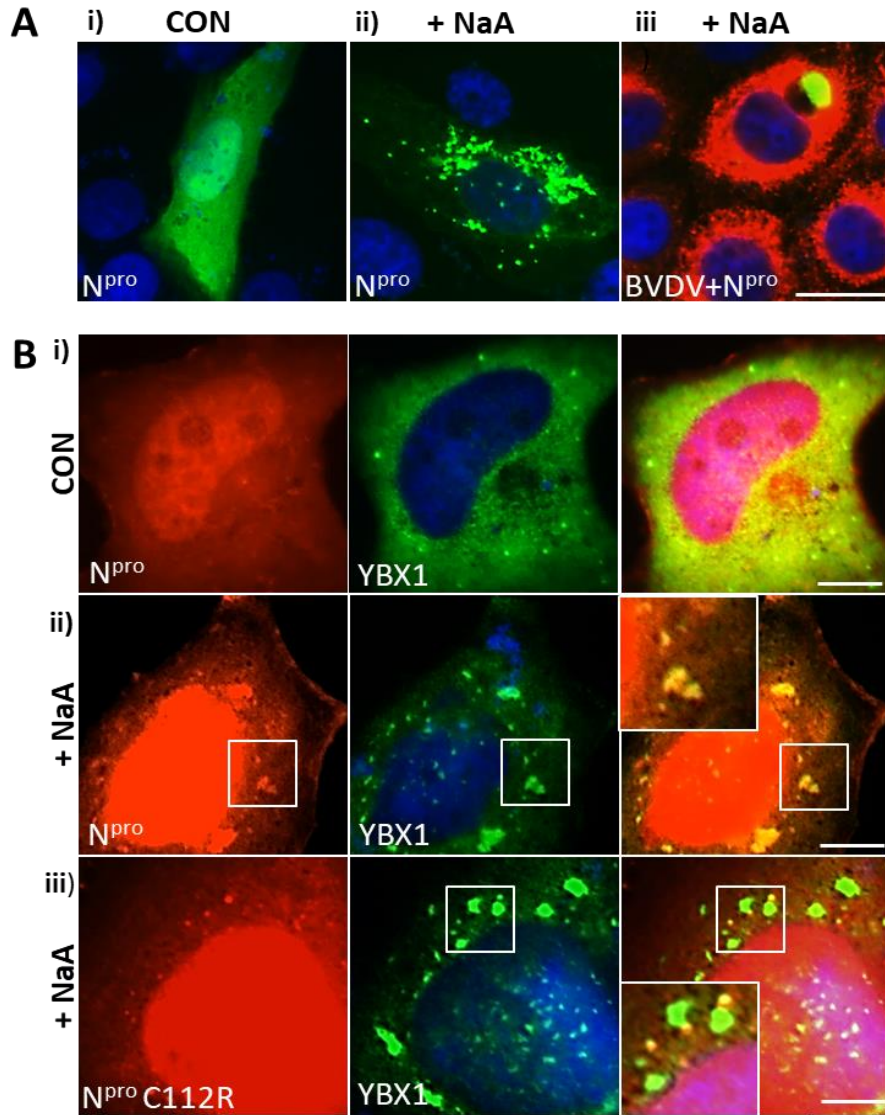


Figure 1.2.4. Interaction and co-localisation of N^{pro} with Y-box protein 1 (YBX-1) requires the zinc-binding TRASH motif. A. N^{pro} localisation in BVDV-infected MDBK cells. **(i)** Control MDBK cells transfected with N^{pro}-GFP. **(ii)** MDBK cells transfected with N^{pro}-GFP and treated with NaA for 4 h. **(iii)** Cells infected with BVDV for 48 hpi, transfected with N^{pro}-GFP for 24 h, and treated with NaA for 4 h. Virus was detected with V182 bovine hyperimmune serum and anti-bovine cy5 secondary antibody. **B.** N^{pro} co-distributes with YBX1 to large cytoplasmic stress granules. Cells stably expressing N^{pro}-mCherry and transfected with YBX-1-V5 were either untreated or treated with NaA for 4 h. YBX1 was detected using anti-V5 MAb and anti-mouse Alexa 488 secondary antibody, and DNA was stained with 4', 6 diamidino-2-phenylindole (DAPI) (blue). **(i)** Control untreated HeLa cells, **(ii)** Cells stably expressing N^{pro}-mCherry, transfected with YBX1-V5 and treated with NaA for 4 h. **(iii)** Cells stably expressing mutant N^{pro} C112R-mCherry and transfected with YBX1-V5 and treated with NaA for 4 h. Imaging by Jefferson et al [67].

1.2.iii.e N^{pro} interaction with YBX1 requires zinc and oxidative cell stress

YBX1 was a top hit from pulldowns using N^{pro} and were shown to co-localise with one another in SGs by imaging, but do they directly bind one another. Demonstrating that N^{pro} directly binds YBX1 would be a novel interaction. The binding of N^{pro} to YBX1 was confirmed biochemically by co-immunoprecipitation assay using either recombinant GST-N^{pro} (Figure 1.2.5, A) or lysates from HEK 293 cells expressing N^{pro}mCherry (Figure 1.2.5, B). Cell lysates were passed over glutathione beads bound to GST or GST-N^{pro} that had been washed in zinc buffer or washed with a zinc chelator, TPEN (Figure 1.2.5, A). Proteins pulled down were separated by gel electrophoresis and blotted with an anti-YBX 1 antibody. YBX1 was pulled down with N^{pro} recombinant protein specifically in the presence of zinc and not when the zinc had been chelated. This confirmed that N^{pro} interacts with YBX1 through its zinc-binding TRASH motif. Cells stably expressing N^{pro}mCherry were co-transfected with a plasmid encoding YBX1 tagged with V5 to investigate binding in cells (Figure 1.2.5, B). Cells were treated with NaA for 4 h to promote the formation of SG. Proteins pulled down with YBX1 using anti-V5 antibody were separated on a gel and probed with anti-N^{pro} antibodies. N^{pro} was detected bound to YBX1 in cells that had been stressed, but not in unstressed cells (Figure 1.2.5, B). This may reflect the increased affinity or abundance of the complex bound together in SG in cells [67].

In cells treated with NaA for 4 h there was accumulation of N^{pro} protein (Figure 1.2.5, C, i). Oxidative stress also increased the levels of YBX1 (Figure 1.2.5, C, ii, lanes 1 and 2). In the presence of N^{pro}, increased levels of YBX1 were precipitated both with and without arsenate treatment (Figure 1.2.5, E, ii, lanes 3 and 4). These results show that both induction of stress and expression of N^{pro} led to the accumulation of YBX1. The requirement for YBX1 during BVDV infection was attempted by silencing of YBX1 in infected MDBK cells, but because of the extremely low rates of transfection of these cells and the high level of expression of YBX1 it was not possible. Pestiviruses have a narrow tropism to their host species and therefore gene knockdown experiments are not possible using knocked-out MEFs [67].

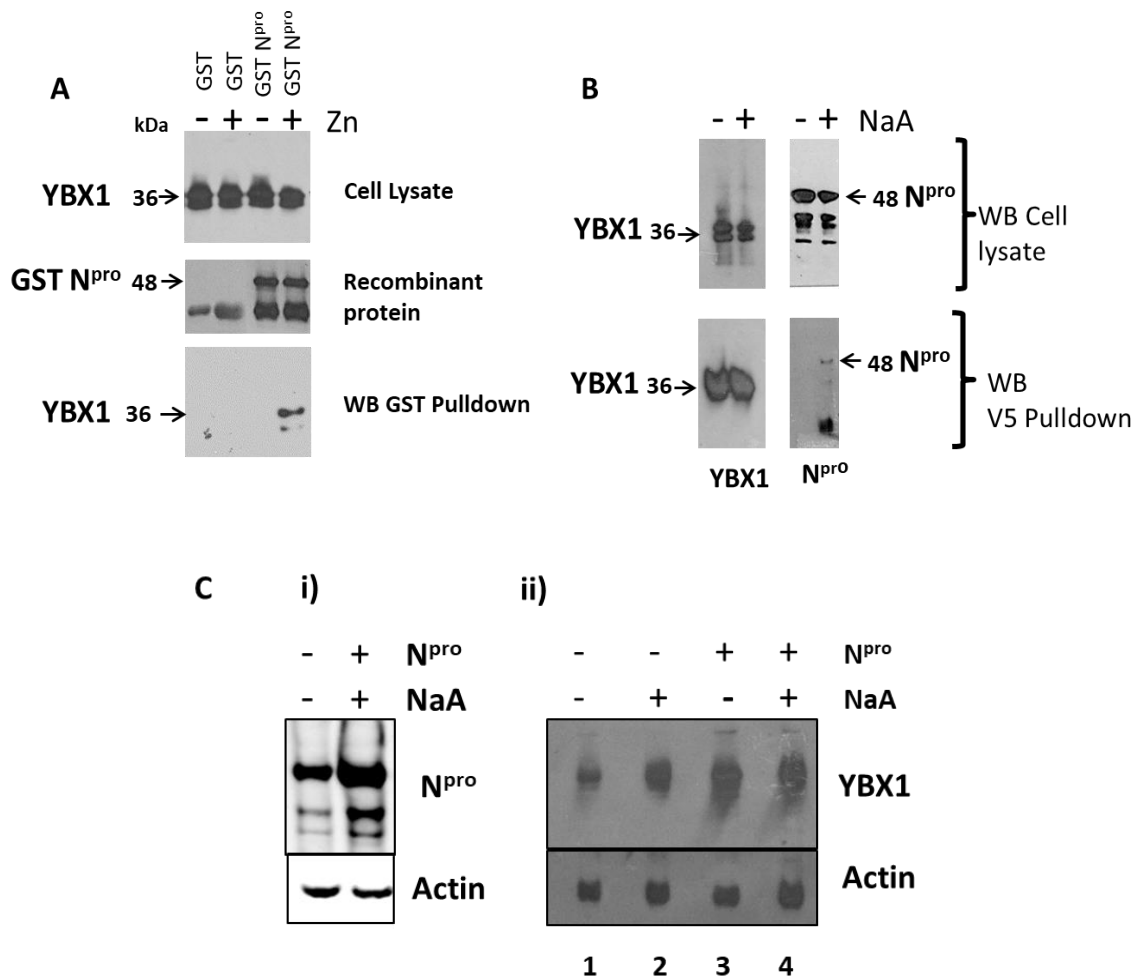


Figure 1.2.5. Co-immunoprecipitation of N^{pro} with Y-box protein 1 (YBX-1) requires zinc and cell stress. **A.** Protein pull-down of HeLa cell lysates with recombinant N^{pro} shows interaction with endogenous cellular YBX1 requiring zinc. The GST and GST-N^{pro} recombinant proteins were washed in zinc buffer or zinc chelator, TPEN. Glutathione-Sepharose beads were used to pull down GST-N^{pro} and its co-binding proteins. Top panel, endogenous YBX1 detected in cell lysates at 36kDa with an anti-YBX1 antibody; middle panel, GST and GST-N^{pro} recombinant proteins detected using anti-GST antibody and anti-N^{pro}, respectively; bottom panel, HeLa cell lysates incubated with GST and GST-N^{pro} proteins were pulled down with glutathione-Sepharose beads, washed extensively, and separated by SDS-PAGE. Proteins were transferred to membranes and probed with rabbit YBX1 antibody, which was detected with anti-rabbit-HRP by enhanced chemiluminescence (ECL). **B.** Interaction of N^{pro} with YBX1 increases after stress. Cells stably expressing N^{pro}-mCherry were transfected with YBX1-V5, and cells were untreated (-) or treated (+) with sodium arsenate (NaA) for 4 h. Lysates were pulled down with anti-V5 antibody bound to Sepharose beads. Top panels, Western blots of cell lysates either untreated (-) or treated (+) with NaA to detect YBX-V5 with anti-V5

monoclonal antibody (left) or N^{pro} with rabbit anti-N^{pro} antibody (right) using anti-mouse or anti-rabbit HRP antibodies, respectively. Bottom panels, immunoprecipitation of proteins using an anti-V5 antibody bound to Sepharose beads, followed by transfer of proteins to membranes and Western blotting with anti-V5 (left panel) or anti-N^{pro} (right panel) antibodies. **C.** N^{pro} and YBX accumulate following stress. **(i)** Lysates from control cells (-) or cells expressing N^{pro}-mCherry (N^{pro}), either untreated (-NaA) or stressed for 4h (NaA), were blotted with antibodies against N^{pro} and actin. **(ii)** Lysates from control cells (-) or cells expressing N^{pro} (+N^{pro}) either untreated (-) or treated with NaA (+) for 4 h were blotted with anti-YBX1 and anti-actin antibodies. Westerns by Jefferson et al [67].

1.2.iii.f Co-localisation of interacting host factors with N^{pro} after stress

As an assay for N^{pro} interaction with factors identified by mass spectrometry, oxidative stress was used to determine co-redistribution to granules. We co-expressed N^{pro} mCherry with TIA1, DDX1, DDX3 and DCP1a. Co-distribution of these proteins in the cytoplasm and their redistribution with N^{pro} to SG, indicating co-binding (Figure 1.2.6). DDX1 was also found in smaller cytoplasmic foci. DDX1 and DDX3 are RNA helicases with roles in transcriptional and post-transcriptional RNA metabolism including: RNA splicing, translation, DNA repair, and transcription [103]. The de-capping enzyme DCP1a, a component of P bodies which are related to SGs but contain other proteins involved in mRNA decay [97], co-distributed with N^{pro} to SGs (Figure 1.2.6, iv). The imaging experiments, together with the proteomic data, show that N^{pro} interacts with important anti-viral RNA binding proteins in the cytoplasm, required for translation. Pestivirus infection prevents granule aggregation by an unidentified mechanism, but expression of N^{pro} alone does not. During infection, the interaction may have an effect on viral or host translation [67].

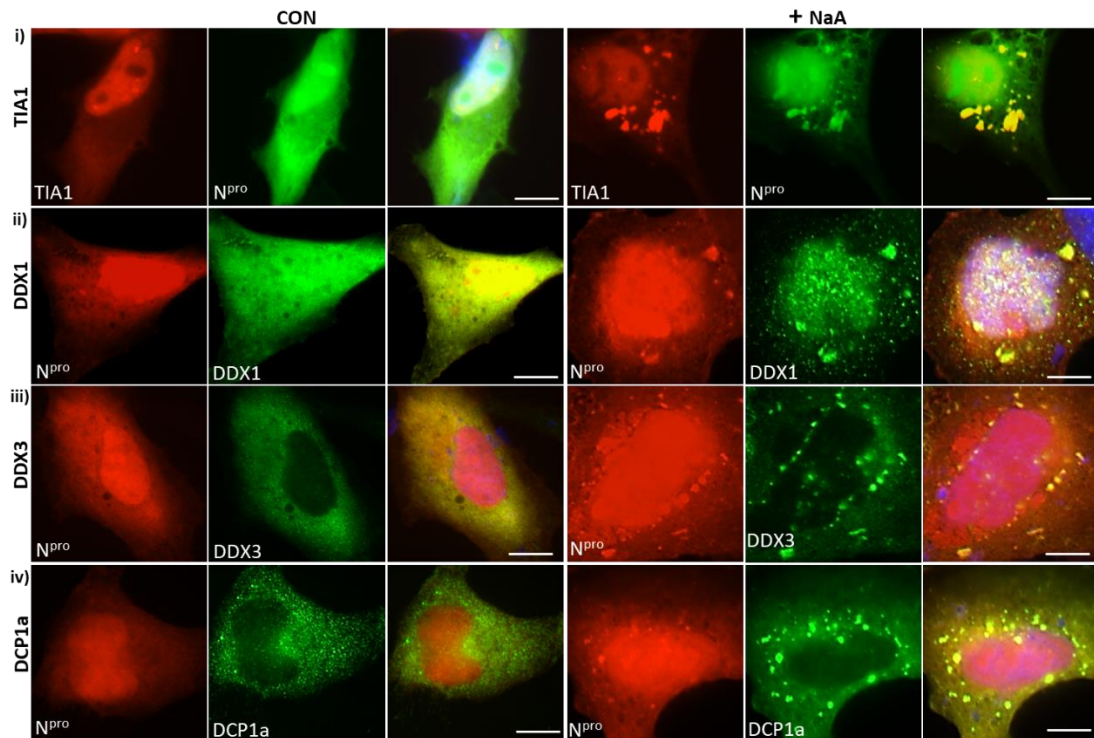


Figure 1.2.6. Pestivirus N^{pro} forms a complex with RNPs and redistributes to stress granules.

N^{pro} redistribution with TIA1, DDX1, DDX3, and DCP1a following stress. Cells were either untreated (CON) or subjected to oxidative stress for 4h (+NaA). Cells expressing N^{pro}-GFP were co-transfected with TIA1-mCherry (i). Cells expressing N^{pro}-mCherry were co-transfected with DDX1-V5 (ii), DDX3-Myc (iii), or DCP1a-V5 (iv), which were detected with anti-V5 or anti-Myc MAbs and secondary anti-mouse Alexa 488 antibodies. Imaging by Jefferson et al [67].

1.2.iii.g Characterising RNPs in stress granules

To determine if these aggregates being seen are in fact SGs they need to be characterised by screening known associated RNPs with SGs. SGs are complex structures made up of RNA-binding proteins, 40S ribosomal subunits, but the composition can vary depending on the stress response [97]. Using immunofluorescence, we can distinguish what type of SG is forming under our experimental conditions. We show that DDX1 and DDX3 aggregate into granules which also comprise YBX1 and TIA1 (Figure 1.2.7, i -iii). Whereas DCP1a formed a ring-like structure around the TIA1 containing granules (Figure 1.2.7, iv) demonstrating a separate domain for this protein [67].

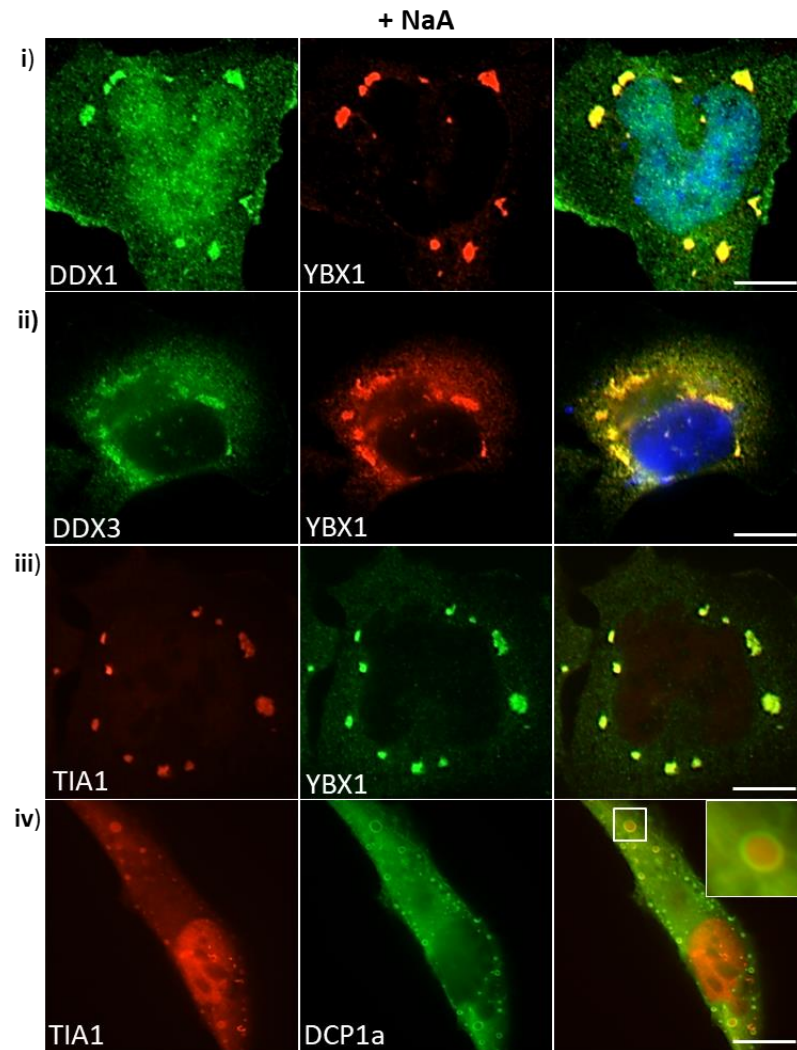


Figure 1.2.7. RNPs composition within stress granules. Co-distribution of YBX1 and TIA1-containing particles with RNA helicases DDX1 and DDX3. HeLa cells were transfected with plasmids encoding DDX1-V5 (**i**) or DDX3-Myc (**ii**) and treated with NaA for 4 h. Endogenous YBX1 was detected with rabbit anti-YBX1 and an anti-rabbit Alexa 594 secondary antibody, and DDX1 and DDX3 were detected with V5 and Myc MAbs, respectively, and an anti-mouse Alexa 488 secondary antibody. HeLa cells were co-transfected with a plasmid encoding TIA1-mCherry and YBX1-V5 (**iii**) or DCP1a-V5 (**iv**), detected using anti-V5 with anti-mouse Alexa 488 secondary antibody. Imaging by Jefferson et al [67].

1.2.iii.h RNA helicase A and La autoantigen redistribute with N^{pro} from the nucleus to cytoplasmic stress granules

Another important protein shown to interact with N^{pro} is RNA helicase A (RHA). RHA (also known as DEAH box protein 9, DHX9) binds dsRNA; it is a DExD/H box helicase with both DNA and RNA helicase activity [104]. Its anti-viral properties include binding to the RNA-induced silencing complex (RISC) to help load dsRNA onto Argonaute for miRNA production [105]. To confirm interaction between RHA and N^{pro}, cells were transfected with a plasmid encoding RHA tagged to HA. Cell lysates were incubated with beads bound to GST or GST-N^{pro} recombinant protein and washed as described. RHA was pulled down with beads containing GST-N^{pro} but not GST beads alone (Figure 1.2.8, A), showing specific binding of RHA by recombinant N^{pro}. When HEK 293 cells stably expressing cDNA for GST-N^{pro} were co-transfected with plasmid encoding RHA-HA, GST-N^{pro} was pulled down by GST in cells overexpressing RHA but not in cells without RHA (Figure 1.2.8, B). This indicates an increased efficiency of GST binding and N^{pro} pull down in the presence of RHA, possibly due to conformational change [67].

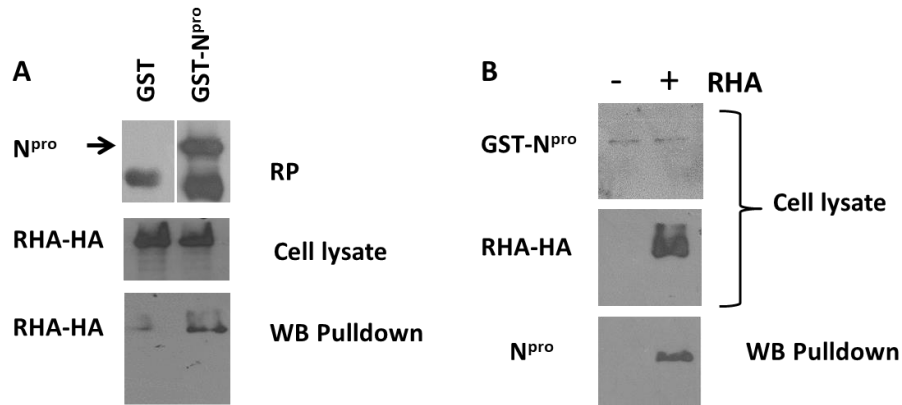


Figure 1.2.8. Co-immunoprecipitation assay to show RNA helicase A (DHX9) binds to N^{pro}.

A. RHA tagged with HA was transfected into HEK293 cells, and lysates were incubated with GST or GST-N^{pro} recombinant proteins. Co-binding proteins were pulled down with glutathione Sepharose beads. Top panel, Western blot of recombinant protein with anti-GST antibody (RP); middle panel, Western blot of cell lysates expressing RHA with anti-HA antibody detected with anti-mouse HRP secondary antibody; bottom panel, Western blot of proteins pulled down with glutathione-Sepharose beads and detected with anti-HA antibody.

B. GST-N^{pro} expressed alone (-) or with RHA-HA (+) in HEK293 cells was pulled down using glutathione-Sepharose beads from cells lysates. Top panel, Western blot of cell lysates with anti-N^{pro} antibody; middle panel, Western blot of cell lysates with anti-HA antibody; bottom panel, Western blot of proteins pulled down with glutathione beads using anti-N^{pro} antibody. Western blotting by Jefferson et al [67].

Next, we investigated the localisation of RHA in cells expressing N^{pro}. HeLa cells were co-transfected with RHA-GFP and N^{pro}mCherry. In untreated cells RHA localised to the nucleus (Figure 1.2.9, A, top left panels), and there was little staining in the cytoplasm, whereas N^{pro} expression was both nuclear and cytoplasmic. When these cells were subjected to oxidative stress, RHA relocated from the nucleus to cytoplasmic SGs (Figure 1.2.9, A, right top panels + NaA) and these granules co-localised with N^{pro}. Since pestiviruses replicate in the cytoplasm with no nuclear component, we used a mutant RHA-GFP (R1163A) which blocks its nuclear translocation [86] to investigate the effect on RHA which is expressed exclusively in the cytoplasm. In unstressed cells mutant RHA-GFP R1163A was only seen in the cytoplasm (Figure 1.2.9, A, bottom right panels). Treatment with NaA led to large cytoplasmic SGs that co-localised with N^{pro}mCherry, clearly indicating RHA binding in a complex to N^{pro} in cytoplasmic SGs (Figure 1.2.9, A, bottom left panels). In order to investigate RHA re-localisation in BVDV infected cells, MDBK cells were infected with virus and transfected with RHA-GFP (Figure 1.2.9, B). In control BVDV-infected cells, RHA-GFP distributed to the nucleus. When cells were stressed, there was no change in the distribution of RHA-GFP, which remained in the nucleus (Figure 1.2.9, B, top right panel.) This confirms the results in Figure 1.2.3 that SGs are not formed when BVDV-infected cells are stressed. RHA, TIA1 or YBX1 do not redistribute into SGs. Similarly, mutant RHA R1163A -GFP remained cytoplasmic both before and after treatment with NaA and did not relocate to SGs (Figure 1.2.9, B, lower panels). Mass spectrometry analysis showed La autoantigen interacted with N^{pro} (Table 1). La autoantigen is an RNA-binding protein that binds several vRNAs, including those of poliovirus and human immunodeficiency virus (HIV) to enhance their translation in vitro [106]. La autoantigen also re-distributes from nucleus to cytoplasmic granules with N^{pro} following induction of stress (Figure 1.2.9, C, right panel). La-GFP was predominantly nuclear before stress (Figure 1.2.9, C, left), but after treatment it can be seen in small cytoplasmic dots co-localising with N^{pro} (Figure 1.2.9, C, right panel). Components of ribonucleoparticles made up from both cytoplasmic and nuclear proteins that bind to N^{pro} in cells and which are redistributed to a cytoplasmic complex together after stress [67].

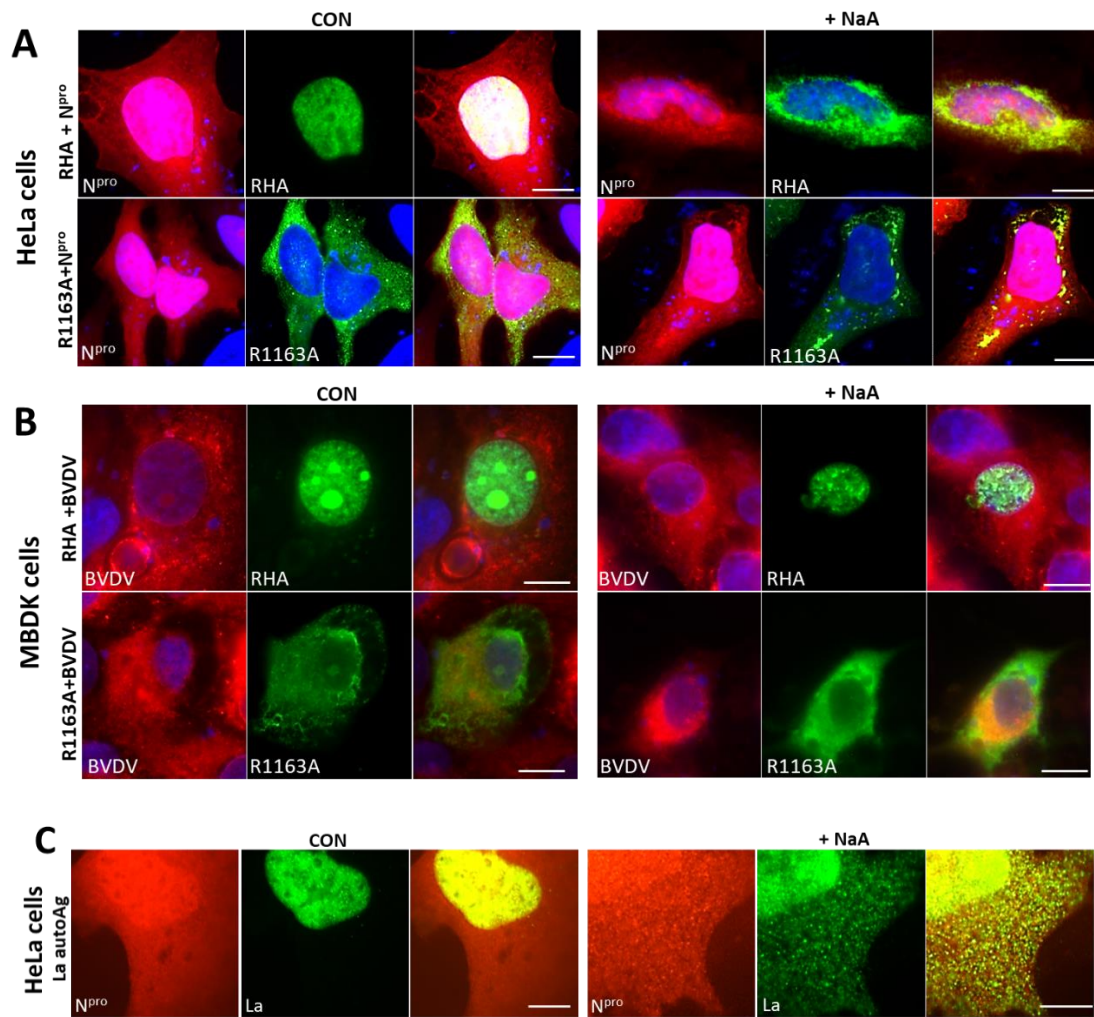


Figure 1.2.9. RNA helicase A (DHX9) binds to N^{pro} and redistributes from the nucleus to cytoplasmic stress granules following stress. RNA helicase A (DHX9) redistributes from the nucleus to cytoplasmic stress granules with N^{pro} following stress. **A.** HeLa cells were co-transfected with N^{pro}-mCherry and either RHA-GFP (RHA + N^{pro}) or mutant RHA R1163A-GFP (R1163A + N^{pro}). Cells were either untreated (CON) or subjected to oxidative stress (+NaA). **B.** MBDK cells were infected with BVDV for 48 h and transfected with either RHA-GFP or mutant RHA R1163A GFP for 24 h. Cells were either untreated (CON) or subjected to oxidative stress (+NaA). **C.** HeLa cells were co-transfected with N^{pro}mCherry and La autoantigen-GFP. Cells were either untreated (CON) or subjected to oxidative stress (+NaA). Imaging by Jefferson et al [67].

1.2.iv. Discussion

Pestiviruses must hijack host cellular translation machinery in order to express viral genes. As pestiviruses are cytoplasmic RNA viruses they must also evade the hosts' innate immune response that include stress, apoptosis, and interferon induction. In this study, BVDV was shown to inhibit SG formation, adding to its repertoire of evasion strategies for this virus [21], [1], [107]. Viral infection and replication is detected by innate immune sensors inducing a cellular stress response leading to the formation of cytoplasmic granules known as SGs, where viral mRNA can be stored and halt translation. The activation of kinases such as PKR, phosphorylates eIF2 to shut off host translation and limit viral replication [108]. As a result many viruses have evolved a range of mechanisms to control the assembly of different RNA granules. Pestiviruses prevent mRNA decay and translational silencing in their host cell in order to survive and ensure viral replication [109]. West Nile virus (WNV) was the first member of the *Flaviviridae* family shown to block SG assembly [110]. WNV replication takes place within pockets generated in the ER membrane by the virus, masking the dsRNA from PKR sensing [111]. Many RNA viruses from other virus families also inhibit SG formation during infection like Influenza A virus (IAV) from the *Orthomyxoviridae* family. IAV inhibits SG assembly through its non-structural protein NS1, suppressing PKR activity [112]. This is not always the case for other virus families where SGs are induced at either early or late phases of infection for them to be inhibited at later times in infection [113]. Mouse hepatitis coronavirus (MHV) induces SG formation to inhibit host protein synthesis allowing enhanced viral replication of MHV [67, 114].

For this investigation, cells were either unstressed or stressed using a synthetic dsRNA poly(I·C) or were treated with and without the presence of zinc (Table 1 & 2). In the absence of stress and other viral proteins, proteomic analysis found that N^{pro} bound to many RNA binding proteins, such as the 40S and 60S ribosomal proteins. RNPs and components of the translational machinery that include YBX-1, RHA (DHX9), IGF2BP1, DEAD box helicases, eIF3, PABP-1, and heterogeneous nuclear RNPs (hnRNPs) [91]. Binding was not dependent on the presence of vRNA or dsRNA as the same peptides were detected in either conditions [67].

In BVDV-infected cells that were stressed with NaA, N^{pro} redistributed from the cytoplasm to the replication complex. RNP proteins remained cytoplasmic and SGs were not seen. In uninfected cells expressing N^{pro} alone, N^{pro} bound to RNPs and ribosomal proteins in the cytoplasm, and co-localised with SG complexes. N^{pro}C112R expressing a mutation in the C-

terminal TRASH motif was no longer assembled into SGs. This shows that N^{pro} alone, is not the viral protein responsible for preventing the redistribution of RNPs to SGs in BVDV-infected cells. In comparison to the leader (L) proteins expressed by Picornaviruses, like Theiler's murine encephalomyelitis virus (TMEV) where expression of the leader (L) protein alone is sufficient to inhibit stress granule assembly [115, 116]. The L protein shares similar structural and mechanistic characteristics as N^{pro} containing a zinc binding motif and inhibits alpha/beta IFN production [117]. N^{pro} may recruit these RNPs at some point during infection for vRNA translation. Or possibly to stall the translation of host proteins, but only at early stages of infection, since cells persistently infected with pestiviruses do not show translational arrest, and cells are protected from apoptosis [105]. Even in conditions where there is extensive eIF2 α phosphorylation from PKR sensing, CSFV has been shown to promote translation and become independent of eIF2 α [118]. Alternative protein complexes can be formed with other eukaryotic initiation factors to promote translation, so instead of eIF3/eIF3 complex an eIF5B/eIF3 complex is used [118, 119]. eIF3 was identified by N^{pro} pulldown in the presence of zinc (Table 2) but no other eukaryotic initiation factors were detected with high affinity. The proteomic content of RNPs to which N^{pro} binds are described here, but how the proteomic composition is linked to function or changes in different cellular compartments is still largely unknown [67].

In this investigation, N^{pro} was shown to interact with YBX1, but this is not just unique amongst pestiviruses as other members of the *Flaviviridae* family have also been shown to bind YBX1. In HCV infection, YBX1 is a novel binding partner of NS3 which redistributes to lipid droplets for virus assembly to control the balance between infectious particle production and vRNA replication [120]. From another genera, Flavivirus DENV requires YBX1 as part of its replication cycle for assembly and secretion. When YBX1 was silenced, intracellular vRNA levels increased but the release of viral particles from the host cell was impeded [121]. Other viral families also utilise YBX1 for their vRNA synthesis. In alphavirus infections, SinV protein nsP3 is also found in YBX1 protein-RNA complexes playing a role in vRNA synthesis [122]. N^{pro} was identified as interacting with many of the same proteins within YBX1 interactome, which included IGFBP2, DDX3, ILF2, and RHA. In a similar study, NS1 of influenza B virus (IBV) validated several of the same proteins identified using N^{pro}, including YBX1 and RHA [123]. Cellular RNPs are multifunctional proteins, and there are now many examples of how they play a role in the loading of vRNA and particle assembly, with YBX1 forming a macromolecular complex to modulate virus production [121, 124].

From our proteomic data we showed interacting partners RHA and La autoantigen that contribute towards RNP particles. When stress was applied to cells using NaA, both RHA and La autoantigen was transported from the nucleus to the cytoplasm (Figure 1.2.9). The cellular reorganisation of RHA with N^{pro} seen in this study is similar to that seen during picornavirus foot and mouth disease virus (FMDV) infection, where RHA plays an important part in its replication [125]. At the time of our analysis, little was known about pestiviruses relationship with RHA and whether RHA is recruited to vRNA. It had been shown that during BVDV infection, RHA bound to both the 5' and 3' UTRs of pestivirus RNA that are the translational sites, indicating a pro-viral effect [126]. In another study, RHA was silenced in cells and then infected with CSFV. A 65-70% reduction in viral replication was observed confirming RHA of having a pro-viral role in pestiviruses [127]. In other members of the *Flaviviridae* family, DENV binding to the 3' UTR mediates an antiviral effect [128]. RHA, now known as DExH-box helicase 9 has multifunctional roles. Linked to dsRNA sensing by binding to PKR to establish an IFN response, making it a perfect candidate for viruses to sequester and prevent an antiviral response [81, 129]. RHA is phosphorylated by PKR when activated upon viral infection [130], and also binds to nuclear factor kappaB p65, a central regulator of the inflammatory response [67, 83].

Further analysis of our proteomic data revealed other N^{pro}-interacting proteins that may have pro-viral roles, for example DEAD-box helicase 3 (DDX3). DEAD-box helicases have been shown to play a part in vRNA recognition and form part of the immune signalling pathways [131]. DDX3 interacts with IKK ϵ to mediate IRF3 phosphorylation to initiate an IFN response [132]. DDX3 can reside in SGs and it is from here that interferon signalling may be occurring. The DEAD-box helicase family make up a large family of proteins playing a pivotal role in innate immune sensing. In a virus-host arms race, viruses have evolved an array of strategies to evade detection or utilise their multifunctional capacity to aid in replication. DDX3 binding to vaccinia virus K7 inhibiting TBK1/IKK ϵ activation [85]. TBK1/IKK ϵ activation is also inhibited during Hepatitis B virus (HBV) infection [133]. Binding of DDX3 to the 3' UTR of HCV helps promotes virus replication and assembly [134, 135]. A broad study using 70 viral immune modulators from 30 viral species, corroborated many of the interactors we found in Tables 1 and 2, demonstrating that many other viruses also target these cellular pathways [67, 136].

To conclude, N^{pro} forms a complex with ribosomal and RNPs in the cytoplasm which serves to recruit the machinery for translation and production of viral particles. Pestiviruses themselves inhibit oxidative stress-induced SG formation, preventing inhibition of cellular protein synthesis and promote viral protein synthesis. The modulation of viral replication by these interacting RNPs isn't fully understood in pestivirus infections. Using data generated by closely related family members of pestiviruses like HCV, may narrow the mechanistic actions of this relationship with RNPs. There is still a lot to learn about the innate immune response in relation to viral defence. This complex system has many layers and multiple systems all working in conjunction with one another with the one goal of removing the viral threat [67].

In the next series of experiments, we looked at the role of stress granules in controlling innate immune responses. We chose Sindbis virus, which is an alphavirus very similar to flaviviruses. This virus has been shown to induce stress granules on infection. We investigated the role of SG formation in the innate immune response to SINV. SINV infection also induces autophagy, so we also investigated if autophagy has a role in SG formation.

1.3. The Autophagy Protein ATG16L1 Is Required for Sindbis Virus-Induced eIF2 α Phosphorylation and Stress Granule Formation

This chapter is an adaption of work that has been published (Jefferson et al) [137] and appears with permission of the author and named contributors.

1.3.i. Introduction

1.3.i.a Sindbis virus (SINV) Classification

Sindbis virus (SINV) is a member of the *Togaviridae* family, within the alphavirus genus. Other alphaviruses within this family include Semliki Forest Virus (SFV) and Chikungunya virus (CHIKV). SINV is transmitted by arthropods, specifically mosquitoes, these types of viruses are referred to as arboviruses [138]. Alphaviruses are capable of infecting a wide range of hosts that include vertebrates and insects. One entry is the conserved cell-surface laminin receptor (LamR) [139]. The LamR is also a route of entry for pestivirus CSFV [140]. In humans SINV causes a mild fever or in some cases arthralgia syndrome. The *Togaviridae* family originally consisted of Group A (alphaviruses) and Group B (flaviviruses) as both groups are transmitted by arthropods. Pestiviruses were later added to this family based on their similar structural properties. SINV has been studied extensively due to its ease of growth in cell culture and low risk to human health. With these two factors in mind and the fact that these viruses were all originally classified within the same family, alphaviruses became a model for pestiviruses and later HCV research. With developments in genome sequencing and replication cycles, alphavirus genomes are expressed by two ORFs generating a subgenomic message. Compared to flaviviruses [141] that have large ORFs expressing the whole genome as one polyprotein [142]. Pestiviruses lack of arthropod transmission led to the joint classification being dissolved. SINV remained a member of the *Togaviridae* family and all pestiviruses, flaviviruses and a new genus hepaciviruses for HCV were made to make up the *Flaviviridae* family [137, 143, 144].

1.3.i.b SINV Genome

Alphaviruses are single-stranded positive-sense RNA viruses with a genome of 11.7 kb [145]. The full-length virus genome contains a 5' cap and 3' polyadenylated tail serving directly as mRNA in the host cell. The genome contains two ORFs, with four non-structural proteins (nsP 1–4), that are translated from the 49S full length genomic RNA. These then form the virus replication complex [146]. The nsP4 contains the core RNA-dependent RNA polymerase (RdRp) domain at the C-terminal end. The RdRp participates in replicating the genomic RNA via a negative strand RNA and transcribing the 26S subgenomic RNA [147]. The subgenomic 26S RNA is translated into structural proteins, the capsid and envelope glycoproteins E1 and E2. Two strains of SINV was used to conduct this study, an original AR339 Sindbis cell culture adapted strain [148] and AR339 with a mCherry fused capsid [149]. (Figure 1.3.1) [137].

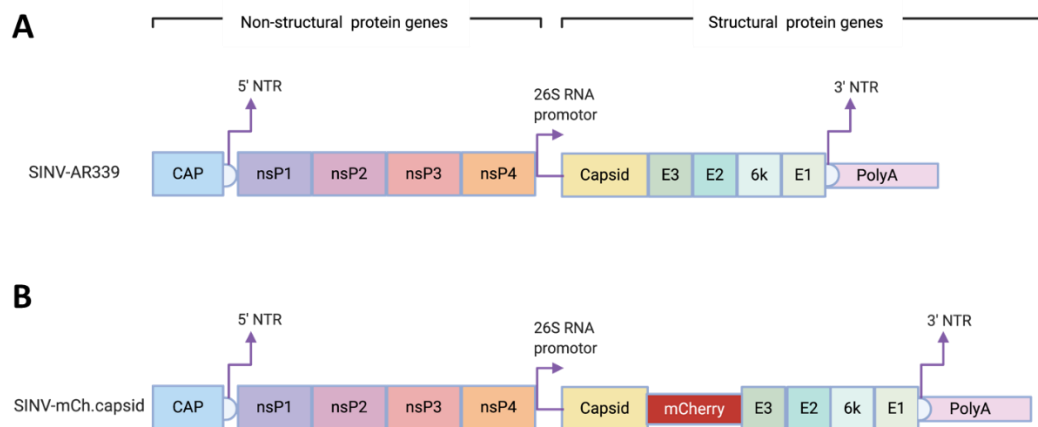


Figure 1.3.1. Schematic of SINV genome organisation. The virus genome is a single stranded RNA molecule. **A.** SINV-AR339 Sindbis virus, cell culture adapted strain. **B.** SINV TR339-mCherry infectious clone carries a mCherry tag fused to the Capsid protein.

1.3.i.c Alphavirus Replication

Entry of alphaviruses into cells is carried out by the E2 component of the virion with the host receptor. Receptor-mediated endocytosis is the predominant mode of entry into the cell, where once internalised is delivered to endosomes [150]. Low pH in the endosome allows viral E1 protein to dissociate from E2. As the pH drops in the endosome, E1 drives fusion between the viral envelope and the endosomal membrane [151]. The nucleocapsid is then released into the cytoplasm [152]. Viral replication can begin once the nucleocapsid has disassembled and releases its RNA, where the full-length virus genome serves as an mRNA molecule. Non-structural proteins are transcribed as two polyproteins p123 and p1234. These non-structural proteins along with host factors form the initial replication complex, where nsP2 performs several proteolytic cleavages. During early stages of infection, a full-length negative strand genome is produced by the replication complex. This template serves for the replication of the viral genome and synthesis of the 26S subgenomic RNA, where structural proteins are encoded. nsP4 forms the RNA dependent RNA polymerase and participates in replicating the genomic RNA via the negative strand RNA and transcribing the 26S subgenomic RNA [147]. A polyprotein is formed of the structural proteins where the capsid protein is first to be cleaved by autoproteolysis. The cleaved capsid protein binds to full length 49S SINRV RNA genome and assembles into core particles. The remaining polyprotein containing structural proteins is chaperoned to the ER and Golgi for further processing and maturation. Glycoproteins E1-E2 form heterodimers and are then transported from the ER by the Golgi complex to the plasma membrane [153]. E2 precursor is cleaved before virus budding by host furin protease generating mature E2 and E3 proteins. This cleavage results in a conformational change that weakens the E1-E2 complex allowing it to interact with the nucleocapsid proteins. E1-E2 glycoproteins form an envelope around the nucleocapsid triggering the budding process, resulting in the exocytosis of viral particles (Figure 1.3.2) [137].

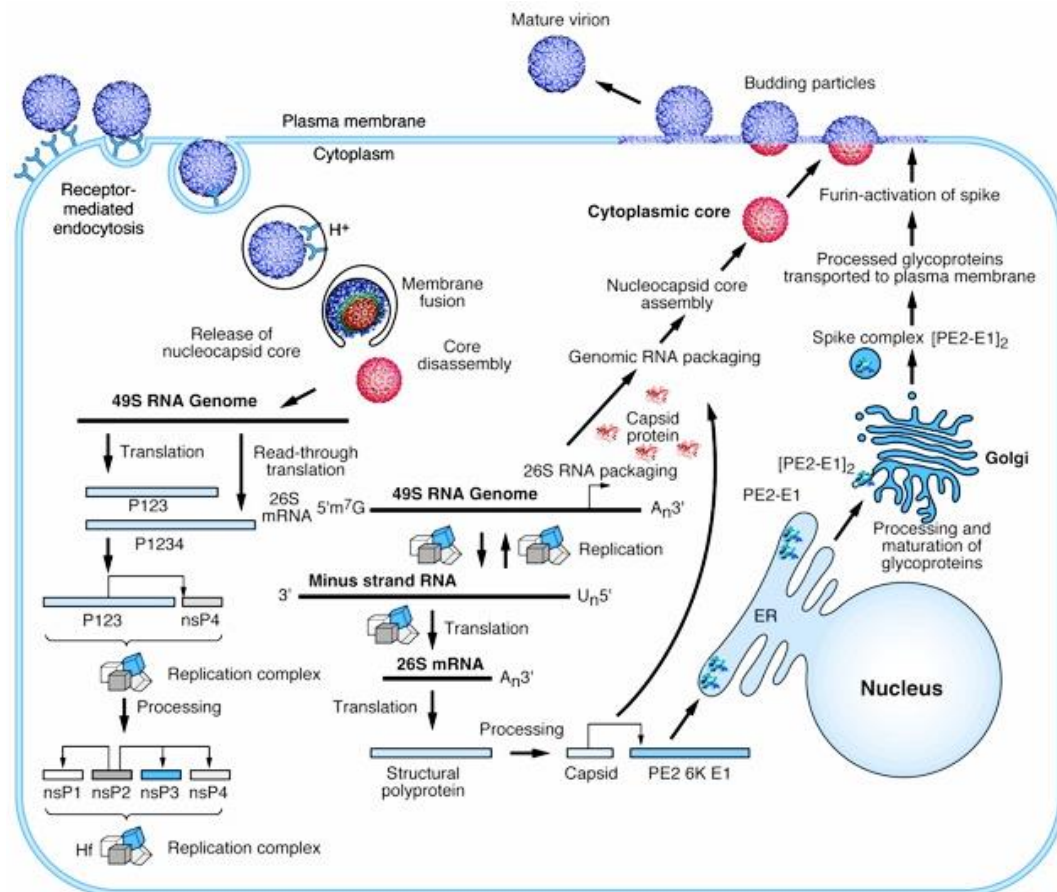


Figure 1.3.2. Alphavirus entry, replication and assembly. One route of entry by SINV at the plasma membrane is by receptor-mediated endocytosis. Transport by early endosomes occurs where a low pH environment triggers fusion with the intracellular membrane of the endocytic vesicle [150]. The SINV viral particle undergoes nucleocapsid disassembly and releases its genomic RNA to the cytoplasm. SINV contains two ORFs generating non-structural and structural polyproteins. Polyprotein P1234 encoding non-structural proteins is cleaved releasing nsP4 where both form an unstable replication complex is made to make negative-strand RNA. Complete cleavage generating nsP1, nsP2, nsP3 and nsP4 inactivates negative-strand synthesis and a stable replication complex forms switching to positive-strand genomic and subgenomic RNA [154]. A leaky termination codon is located after nsP3 generating P123 polyprotein in order to reduce excess nsP4. Excess nsP4 can destabilise the replication complex and result in inefficient RNA replication [155]. The structural poly protein made from the subgenomic RNA undergoes cleavage. Autoproteolytic cleavage of the capsid protein from the structural polyprotein occurs. The capsid protein is free to associate with newly synthesised full length genomic RNA and is packaged into nucleocapsid-like particles [156, 157]. The E3 protein directs the remaining polyprotein to the ER where it is processed

by host signal peptidase. E2 glycoprotein precursor pE2 interacts with E1 glycoprotein after the 6K protein has directed the downstream processing of E1 [158]. Here they form heterodimer spike complexes and then transported from the ER to the cell surface via the Golgi complex. PE2 precursor is cleaved by host furin protease generating mature E2 and E3 proteins weakening the interaction between the E1-E2 spike heterodimer [153]. The capsid protein interacts with the E2 protein to initiate budding where the E1-E2 heterodimer forms an envelope around the nucleocapsid particles [159]. (Image source: Richard J Kuhn [157]).

1.3.i.d Alphavirus vaccine technology

Alphavirus vaccine vectors contain elements from both SINV and Venezuelan equine encephalitis virus (VEEV) backbones, generating a construct with robust antigen-specific immune responses [160]. Alphavirus vectors are referred to as replicons due to their self amplifying RNA (saRNA), as the replicon contains the non-structural genes that encodes the viral replicase complex. The saRNA contains a CAP and poly A tail to provide stability and to prevent degradation within the host as well as a 5' and 3' UTR to help with RNA replication. The native subgenomic promoter is still present to drive the expression of the heterologous gene of interest (GOI). Each alphavirus has its own desirable properties in terms of function, safety and potency. VEEV demonstrates a natural lymphotropism [161] that makes it ideal for vaccines but there are safety concerns due to the pathogenesis of wild-type VEEV in humans [162]. SINV is not associated with serious human diseases and can be used in containment level 2 labs compared to VEEV that requires containment level 3 biosafety. By combining the positive attributes of the alphaviruses, a versatile replicon is created. saRNA vaccines are a powerful nucleic acid technology due their versatility and potency. The self-amplification properties yield higher protein expression from a single dose. No need to grow up live viruses or attenuate viruses for vaccine development making it safer and cheaper. There's less inflammation generated in the host as the replicon only encodes your antigen of choice. The alphavirus replicon allows for fast cloning of genetic material from pathogens making it possible to make a rapidly producible and scalable vaccine. A prime example is the interferon inhibiting protein (IIP) VEEV replicon expressing the surface spike glycoprotein encoded by SARS-COV-2 [163] (Figure 1.3.3). A library of replicons was generated using viral proteins from a range of viruses known to inhibit an IFN response. These IIPs enhanced the protein expression of SARS-COV-2 spike glycoprotein in vitro and in vivo. IIPs stabilisers increased doses of RNA and allowing enhanced expression of RNA [164]. This means saRNA vaccines containing IIPs within the replicon generate more copies of RNA once in the

cytoplasm and this leads to higher yields of spike glycoprotein. The spike glycoprotein is then presented on the cell surface where it is recognised as foreign material and initiates an immune response leading to antibody production [164].

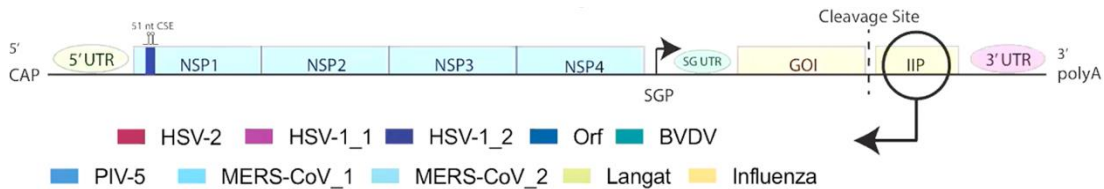


Figure 1.3.3. Interferon inhibiting protein (IIP) VEEV replicon. An alphavirus expressing saRNA with a virally derived IIP allowing high expression levels of your viral antigen of choice. A library of viral proteins known to inhibit IFN response, Herpes simplex virus (HSV), N^{pro} from BVDV and Middle East Respiratory Syndrome (MERS) was tested for efficiencies [163].

1.3.i.e Studying Hepatitis C Virus (HCV) with Alphaviruses

HCV research was hindered by the lack of an infectious cell culture system so the full life cycle of HCV in an experimental setup was difficult. One of the main problems in culturing viable viral progeny was linked to the nature of the host cell, human hepatocytes are highly differentiated and very difficult to maintain in cell culture [165]. Hepatocytes change their transcriptional profiles and lose their differentiation when plated in cell culture [166]. Without a robust system in place the mechanisms of viral attachment, cellular entry, and intracellular steps in HCV RNA replication, virus assembly, and virion release could not be studied. Following development of SINV subgenomic delivery systems meant that HCV RNA replicons could be made, leading to successful replicating chimeras expressing HCV viral proteins. With the added benefit of being able to study HCV proteins safely without the risk to human health working with live HCV models. Chimeric alphaviruses have been crucial in the development of direct acting antiviral drugs to target specific HCV proteins required for replication. HCV NS3 protease cleaves its viral polyprotein at specific sites to release the components required for the HCV replication machinery making it an ideal candidate to study. Enzyme inhibition of NS3 screening can occur in tissue culture for the development of an anti-HCV therapeutic [167]. Chimeric viruses do not show you the full aspects of HCV life cycles, but they have generated crucial information from their creation in the pursuit of treatment regimes.

1.3.i.f SINV and the Innate Immune Response

The innate immune response is crucial for controlling the early stages of alphavirus disease and activation of a type I IFN response [168]. SGs are targeted by many RNA viruses that will either induce SG formation to halt host protein synthesis in the case of alphaviruses and MHV [114, 169]. Or RNA viruses like BVDV and IAV will inhibit SG formation to enhance the synthesis of its own proteins [67, 112]. Meaning SGs themselves can be either pro- or anti-viral, as they may sequester either factors needed for viral replication or innate factors required for anti-viral defences [170]. Virus-induced SG can moderate the induction of Type I interferon by recruiting RIG-I, MAVS, and PKR to co-localise with the SG. Forming antiviral stress granules (avSG) that act as a platform for RNA sensing and IFN stimulation (Figure 1.3.4). PKR is activated through cell stress and dsRNA sensing of alphavirus infection, resulting in the phosphorylation of eIF2 α [171]. Phosphorylated eIF2 α regulates activating transcription factor 4 (ATF4) which is a transcription factor for a number of autophagy genes like ATG16L1, resulting in the activation of autophagy [137, 172]

1.3.i.g Autophagy and the Innate Immune system

Autophagy also known as canonical autophagy is an intracellular degradation process activated by cellular stresses, like starvation and pathogen infection. Autophagy is crucial in maintaining cellular homeostasis but it also plays an important role in the innate immune response of host defense and elimination of viruses [173]. Autophagy is a stress response and in classical autophagy it's a self-degradation process of removing cytosolic components via lysosomes to release core materials required for cell homeostasis [174]. When autophagy is activated through the stress of infection, virions and viral components are sequestered in the cytosol for lysosomal degradation in a process termed xenophagy [175, 176]. Autophagy and autophagy proteins play both anti and pro-viral roles in the pathogenesis of viruses [176]. Autophagy and autophagy proteins can control IFN signalling. Excessive IFN signalling can lead to immune-mediated tissue damage and autoimmune disorders and therefore require tight regulation to maintain immune homeostasis [177]. Autophagy protein ATG5 can upregulate IFN signalling through TLR7 mediated pathways [178]. Whereas the ATG5-ATG12 complex inhibits RNA virus-induced IFN production by binding to the CARD domain of RIG-I and MDA5. This prevents the downstream activation of IRF3/7 leading to suppression of IFN gene activation [179]. Viruses, as with other parts of the innate immune pathways

have evolved to target and inhibit the autophagy machinery and circumvent an autophagic response [180]. Autophagy is activated during alphavirus [181] and HCV infection [182] demonstrating another model role for SINV to study HCV. When autophagy is activated a double-membraned vesicle called an autophagosome is formed encapsulating virions and viral components. Autophagosomes deliver the viral contents to lysosomes for degradation [183]. Autophagosomes have been shown to deliver viral PAMPs to PRRs to initiate an antiviral response by IFN induction [184]. An important step in autophagy is the recruitment of autophagy protein microtubule-associated protein light-chain 3 (LC3-I) to the membranes of the autophagosome. ATG16L1 interacts with ATG5-ATG12 to direct the conjugated LC3-1 with phosphatidylethanolamine (PE) onto autophagosomes. Lipidation of LC3-1 occurs to form LC3-II that is a central protein in the autophagy pathway and as such is used as a marker for autophagy [185].

In this study, we investigated the innate immune response to SINV, focussing on the formation of stress granules following infection, and the effect on host and virus translation. We investigated the role of autophagy protein ATG16L1 in the control of the innate response, including SG formation and interferon signalling.

1.3.ii. Materials and Methods

1.3.ii.a Cell lines

MEF and BHK cells were grown in DMEM with GlutaMAX. MEFs were isolated from E13.5 embryos and immortalized by serial passaging. ATG16L1 ^{-/-} MEF cells were generated from ATG16L1 FL/FL mice crossed with Rosa26-LacZ mice [186]. The *Atg16L1* gene was deleted with Adenovirus expressing Cre recombinase and isolated by FACS through activation of LacZ expression after deletion of upstream stop sequences by the Cre recombinase. Matched parental control MEF cells were made from *Atg16 FL/FL* embryos without treatment with Cre recombinase. Immunostaining of ATG16L1 was used to assess absence of ATG16L1 protein, and staining of LC3 and WIPI used to show absence of autophagosomes [137, 186].

1.3.ii.b Sindbis Infection

SINV laboratory strain AR339 was from John Fazakerley, The Pirbright Institute. SINV mCherry.capsid virus was made from an infectious clone by in vitro transcription of the cDNA for dsTE12Q with mCherry fused to the n-terminus the capsid protein and a kind gift from B. Levine, University of Texas Southwestern Medical Centre [146]. cDNA was linearised with Xho 1, the RNA was transcribed using SP6 RNA polymerase and transfected into BHK cells. The supernatant containing virus was collected after 3 days, and stored at -80 °C. Cells were incubated with virus diluted in media to moi 2–5 for 1 h, before washing with fresh media and incubating at 37 °C. Cells were incubated for the times stated in individual experiments [137].

1.3.ii.c Antibodies for Western Blotting and Immunostaining

Rabbit anti-SINV antibody (1 in 200) was a gift from Sondra Schlesinger, Washington University St Louis. Other antibodies were as listed below. They were used at the dilutions indicated and they were incubated either for 2 h at room temperature, or at 4 °C overnight. Rabbit anti- YBX1 (Abcam, ab12148, 1 in 300); rabbit anti-VCP (Cell Signalling Technologies, 2648); mouse anti-G3BP1 (BD, 611126, 1 in 200); goat anti-TIA1 (Santa Cruz, sc-1751, 1 in 200); rabbit anti-PKR (Abcam, ab32052); rabbit anti-RIG-I (Abcam, ab45428, 1 in 200); rabbit anti-dsRed (Clontech, 632496, 1 in 1000); mouse anti-ATG16L1 (MBL M150–3, 1 in 1000); mouse anti-actin (Sigma-Aldrich, Gillingham, A5441, 1 in 7000); rabbit anti LC3 A/B (Cell

Signalling, 4108 1 in 1000). The antibody against phosphorylated eIF2 α (Cell Signalling 3398, 1 in 1000). Poly I:C (Roche) was transfected into cells with lipofectamine (Invitrogen) for 4 h [137].

1.3.ii.d Western Blotting

For phosphorylated proteins, cells were lysed in 1% hot SDS in Tris pH 8.0 with PhosSTOP phosphatase inhibitors (Roche). Proteins were separated by SDS-PAGE (4–12% acrylamide) and transferred to PVDF membranes (0.45 μ m transfer membrane, ThermoFisher Scientific). Membranes were blocked with 5% (w/v) BSA in TBS containing 0.5% Tween 20 for 1 h. Membranes were probed with anti-SINV, LC3 and actin antibodies as described. Proteins were detected with IRDye[®]-labelled secondary antibodies at 1:10,000 dilution. Proteins detected by the labelled secondary antibodies were visualized on the Odyssey CLX infrared system [137].

1.3.ii.e Immunofluorescence

MEF cells were grown on coverslips and infected with SINV at a multiplicity of infection (moi) 5 for the stated times and fixed in 4% paraformaldehyde for 10 min. Cells were blocked in goat serum in gelatin quench and permeabilised with 0.2% Triton X-100. Primary antibodies were diluted to the concentrations listed above into 0.2% Triton X-100, in goat gelatin quench and incubated 4 °C overnight. Cells were washed three times 0.1% Tween before incubation with fluorescent secondary antibodies, for 2 h room temperature [137].

1.3.ii.f Statistical Analysis

The data was analysed by an unpaired student t test using Graphpad Prism 8 (www.graphpad.com/scientific-software/prism) [137].

1.3.iii. Results

1.3.iii.a ATG16L1 is required for SINV-Induced Phosphorylation of eIF2 α and the Switch to Viral Protein Synthesis

To investigate the effect of ATG16L1 on virus protein expression and eIF2 α phosphorylation, wild type and ATG16L1-deficient MEF cells were infected with SINV at a moi of 2. A time course of viral protein expression from 0–8 hpi using rabbit anti-SINV antibody showed that viral protein translation commenced by 6 hpi in both cell lines (Figure 1.3.4, A). The enveloped glycoprotein p62 (E2/3) together with the capsid protein fused to mCherry fluorescent protein (mCh.capsid), is seen as the major band at 62 kDa. The envelope protein E1 is detected at 45kDa and cleaved capsid protein at 30kDa. This anti-SINV antibody detected a non-specific, cross reacting band at this molecular weight at 0 hpi. The p62/capsid proteins increased 12-fold by 8 hpi in wild type cells. In contrast, there was only a 2-fold increase in p62/capsid proteins in ATG16L1 knockout cells (Figure 1.3.4, A, bar chart). In wild type cells, SINV infection triggered phosphorylation of elongation factor eIF2 α by 6 hpi. There was little phosphorylation of eIF2 α detected by 6hpi in ATG16L1 knockout cells (Figure 1.3.4, B). On this blot, SINV expressing mCherry capsid is shown at 62kDa in wild type cells using an anti-dsRed antibody. There is a low intensity capsid band at 62kDa in ATG16L1 knockout cells, confirming the lower viral protein translation in these cells. The graph below shows the levels of eIF2 α phosphorylation from three independent blots (+/-SE) at each time point, with 3-fold more eIF2 α phosphorylated protein in wild type compared to ATG16L1 knockout cells. These results suggest that ATG16L1 is required both for eIF2 α phosphorylation and for viral protein synthesis [137].

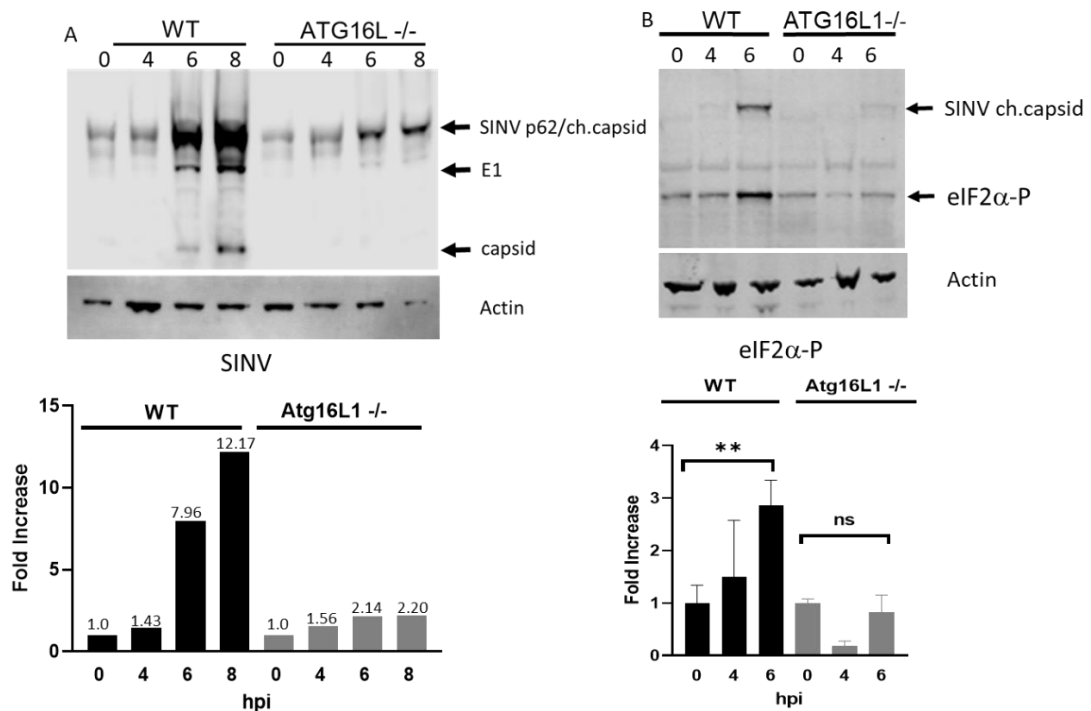


Figure 1.3.4. eIF2α phosphorylation and virus protein production is diminished in ATG16L1^{-/-} cells. **A.** Western blot of SINV-infected lysates at 0–8 hpi detected with anti-SINV antibody. Bar chart below shows fold increase in p62/capsid proteins relative to actin. **B.** SINV expressing cherry capsid detected with anti-dsRed antibody and phosphorylated eIF2α detected with rabbit monoclonal phospho-eIF2α (Ser51). Bar chart below shows the fold increase in eIF2α -P normalised to actin for 3 independent blots with +/-SE ** $p < 0.01$, ns is non-significant. Westerns by Jefferson et al [137].

1.3.iii.b Host Protein Rearrangements Following Entry of SINV Capsids

Canonical SG are induced by NaA and they are comprised of host proteins TIA1, G3BP1 and YBX1. In MEF cells, these RNA-binding proteins redistributed from the cytoplasm and nucleus to several perinuclear bodies after treatment with NaA (Figure 1.3.5, A). In contrast, SG formed after infection with SINV wild type strain AR339 condensed into a single, large perinuclear granule, which also localised with host RNA binding proteins YBX1 and TIA1 (Figure 1.3.5, B). As such, these granules are defined as canonical SG. Host proteins redistributed from the nucleus and cytoplasm and formed SGs by 4–8 hpi. The SG formed after virus infection was seen as a single perinuclear body. Similarly, during infection of the recombinant SINV mCherry.capsid, the virus capsid redistributed with YBX1 and TIA1 and also with VCP and G3BP1 into a single perinuclear granule (Figure 1.3.5, C) [137].

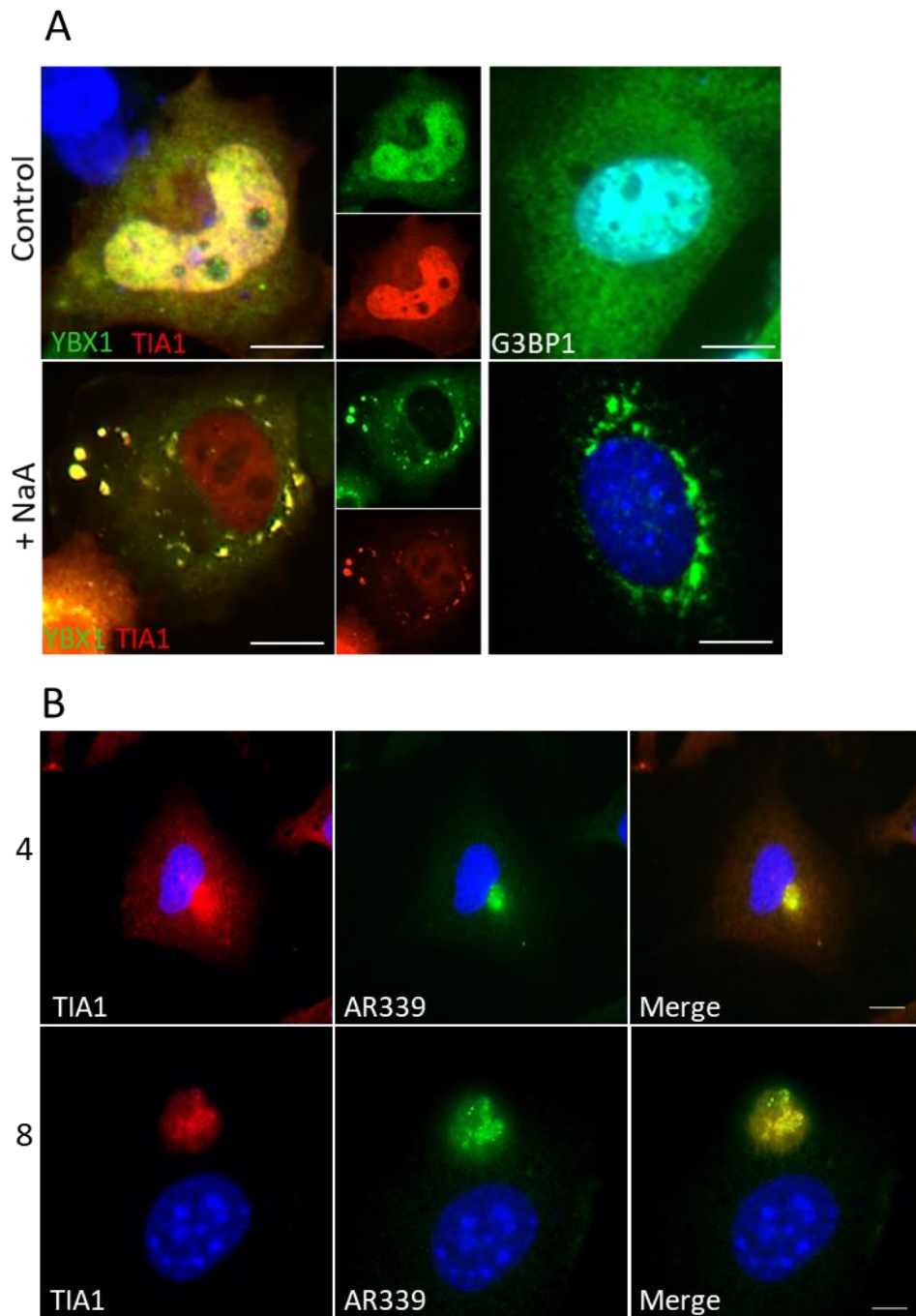


Figure 1.3.5. RNA binding proteins redistributed with SINV capsid early in infection. Host RNA-binding proteins YBX1, TIA 1, G3BP1 and VCP were detected by immunostaining. **A.** Control MEF cells and cells treated with 100 nM NaA for 2 h. **B.** MEFs infected with SINV strain AR339 for 4 and 8 hpi. Scale bar= 10mm. Imaging by Jefferson et al [137].

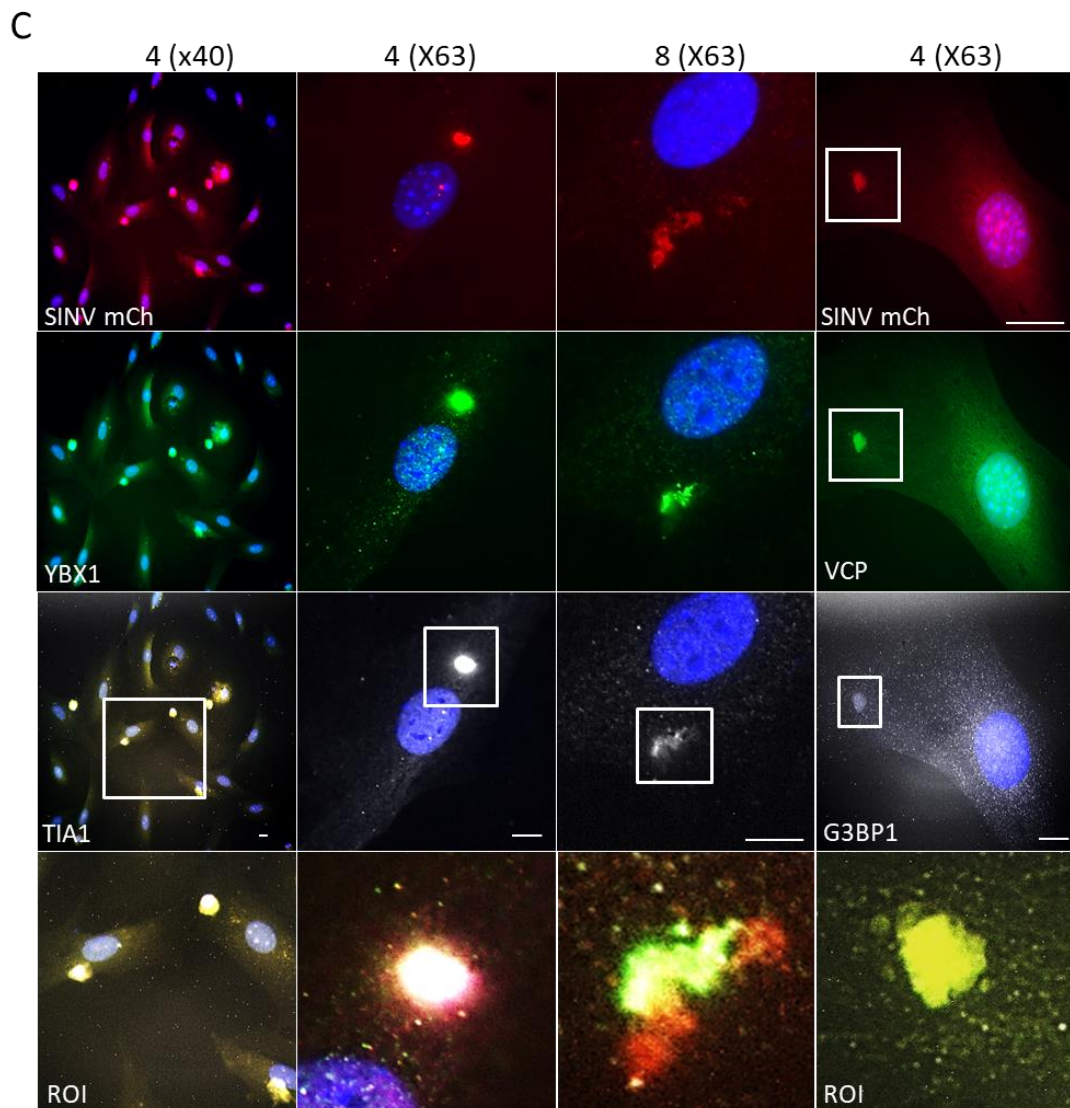


Figure 1.3.5, C. RNA binding proteins redistributed with SINV capsid early in infection. Host RNA-binding proteins YBX1, TIA 1, G3BP1 and VCP were detected by immunostaining. Control MEF cells infected with SINV Cherry capsid for 4hpi (low power x40), 4 hpi (x63) and 8 hpi (x63). Region of interest (ROI) are from merged images of white squares. Scale bar= 10mm. Imaging by Jefferson et al [137].

1.3.iii.c Autophagy protein ATG16L1 is required for stress granule formation

RNA-binding proteins VCP, YBX1 and TIA1 were initially found in the nucleus and cytoplasm in both WT and ATG16L1 ^{-/-} cells (Figure 1.3.6, A). The capsid redistributed with the RNA-binding proteins as early as 2 hpi into small cytoplasmic puncta which coalesced into a single large perinuclear body in WT cells by 8 hpi (Figure 1.3.6, B, WT). In ATG16L1 ^{-/-} cells, characterised in detail in Rai et al. [187], capsid redistributed with YBX1, TIA1 and VCP in the cytoplasm. By 2 hpi, a large perinuclear granule was not formed by 8 hpi (Figure 1.3.6, B, ATG16L1^{-/-}). In ATG16L1^{-/-} cells, host RNA-binding factors and capsid were seen as numerous small puncta throughout the cytoplasm. When the number of cells having either a large capsid-containing perinuclear SG in wild type cells, or small cytoplasmic puncta containing capsid in ATG16L1^{-/-} were quantitated, there was a significant difference in large and small granules between both cell types. The same percentage of cells were infected in both cell types (Figure 1.3.6, C). Although the initial infection was the same in both cell types, viral protein synthesis shown in Figure 1.3.6, A was higher in wild type cells. In wild type cells, SG formation does not halt translation of viral proteins. In contrast, in ATG16L1 knockout cells, viral protein synthesis was restricted, despite low levels of eIF2 α phosphorylation and absence of perinuclear SG formation. These experiments followed labelled capsid at early time points before the start of virus replication. We detected dsRNA with antibody J2 in separate replication complexes [137].

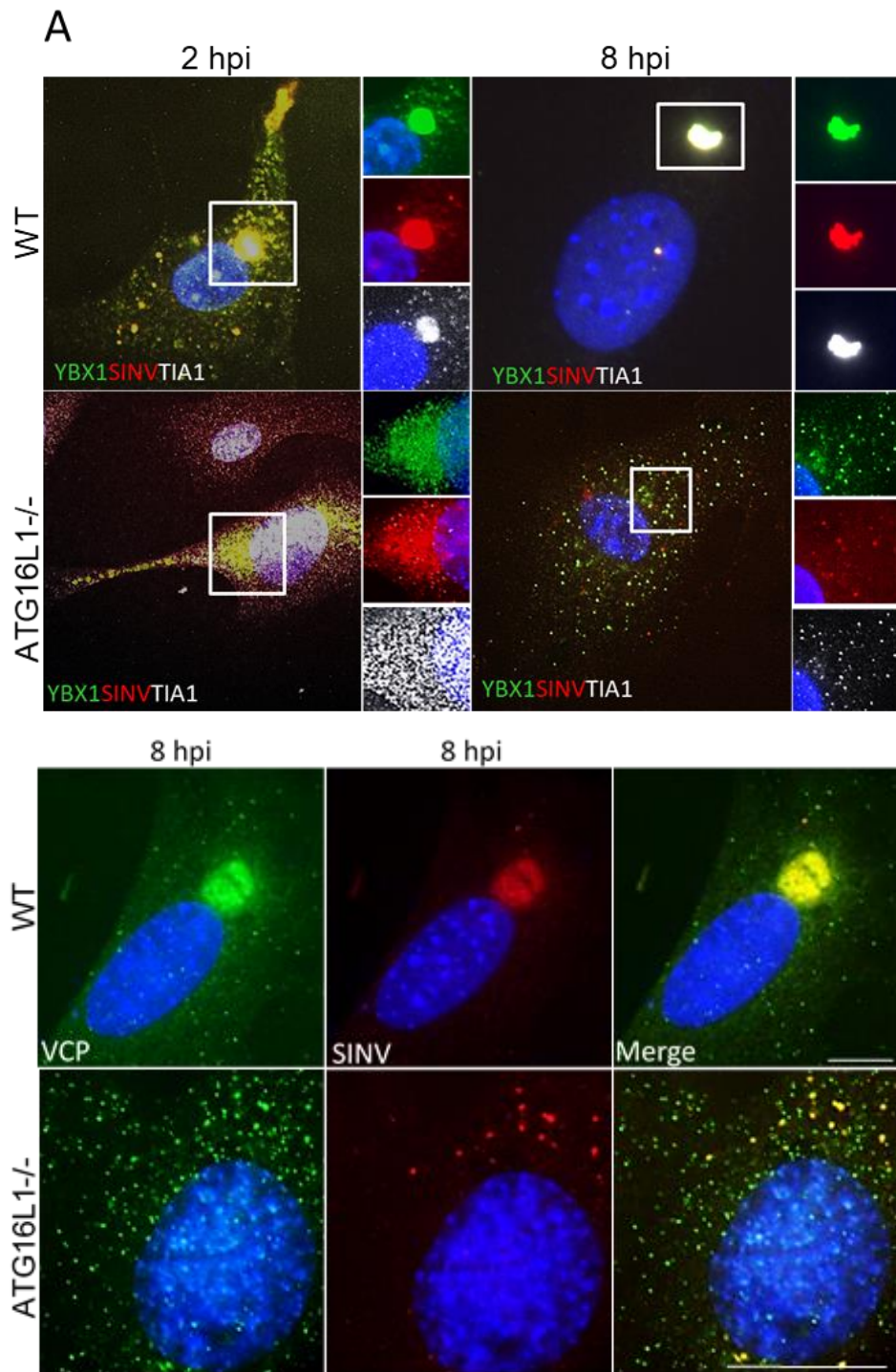


Figure 1.3.6. A. Perinuclear stress granules do not form in ATG16L1^{-/-} cells. Host proteins YBX1, TIA1 and VCP were stained in WT and ATG16L1^{-/-} MEF cells. **A.** Distribution of host proteins in WT and ATG16L1 MEFs cells infected with SINV mCh.capsid for 2 and 8 hpi.

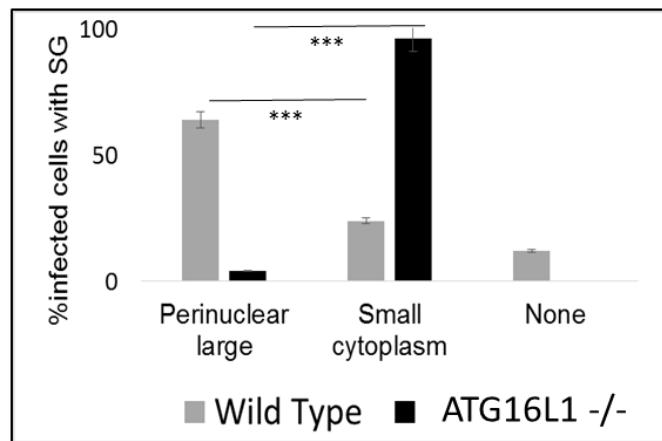
B

Figure 1.3.6. B. Perinuclear stress granules do not form in ATG16L1-/-cells. The bar chart shows percentage of infected cells with either large perinuclear SG or small cytoplasmic puncta from 5 independent fields of view of WT and ATG16L1-/- cells at 8 hpi at 40× magnification. +/- SE and *** $p < 0.001$. None indicates a small number of infected cells with no SG. Imaging by Jefferson et al [137].

1.3.iii.d Turnover of Host Translation Factors

Inhibition of degradation by autophagy may explain the accumulation of the many small cytoplasmic puncta containing capsid and RNA-binding proteins seen in ATG16L1-/- cells. To investigate whether autophagy affects turnover of these host translation factors, we investigated protein levels during activation of autophagy by SINV in wild type cells (Figure 1.3.7). During autophagy, cytoplasmic LC3 (LC3I) is conjugated to phosphatidylethanolamine to form membrane-bound LC3II, by proteins, including ATG16L1 that is part of an ubiquitination-like conjugation system. By 4 hpi, LC3I had been converted to the faster migrating LC3II form, demonstrating that SINV indeed activates autophagy early in infection. In ATG16L1 -/- cells, LC3I was not converted to LC3II during infection, confirming that these cells lack autophagy and demonstrating that the puncta in Figure 1.3.6, A are not autophagosomes. There was no change in levels of YBX1, VCP and TIA1 over the time course of 0 to 8 hpi in wild type cells, indicating that they are not degraded by autophagy during this time period. In ATG16L1-/- cells, protein levels for TIA1, YBX1 and VCP also remained constant. Therefore, inhibition of autophagy does not lead to the accumulation of these host translation proteins, and the small cytoplasmic puncta seen in ATG16L1 knockout cells are not due to a block in degradation of these factors [137].

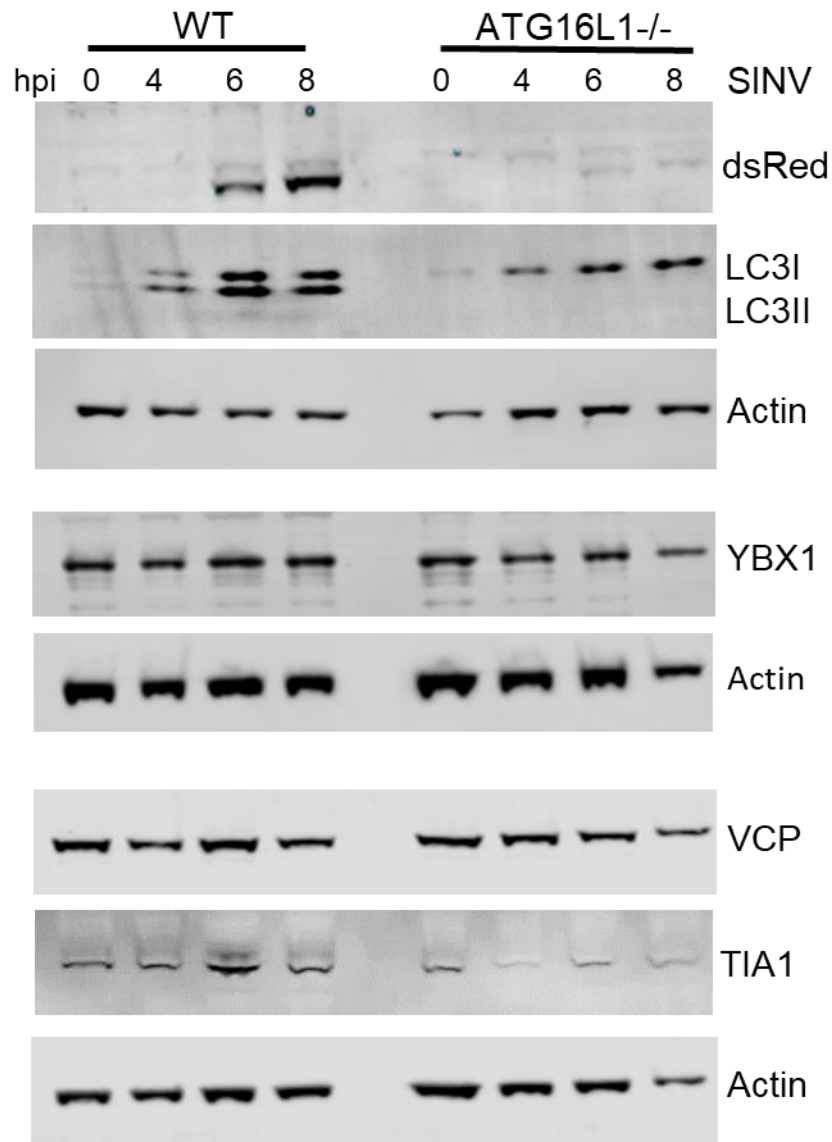


Figure 1.3.7. Host translational RNA-binding proteins are not degraded by autophagy induced by SINV infection. A time course of SINV infection from 0–8 hpi in wild type (WT) and *ATG16L1*^{-/-} MEFs (*ATG16L1*^{-/-}). Western blot of protein lysates with antibodies against LC3, YBX1, TIA1 and VCP, with actin showing equal lane loading for each gel. Westerns by Jefferson et al [137].

1.3.iii.e Innate Responses to SINV in ATG16-Deficient Cells

We examined the localisation of dsRNA viral sensors PKR and RIG-I. In wild type cells, by 2 hpi, both PKR and RIG-I had relocated from the cytoplasm to a single capsid-containing perinuclear SG (Figure 1.3.8, A, WT), which remained for up to 8 hpi. In ATG16L^{-/-} cells, PKR and RIG-I also redistributed with capsid but they were smaller granules, less tightly packed and which became dispersed throughout the cytoplasm by 8 hpi (Figure 1.3.8, A, ATG16L1^{-/-}). These small cytoplasmic puncta at 8hpi contained SINV capsid, RIG-I and TIA1 (Figure 1.3.8, B), marking them as canonical SG [137].

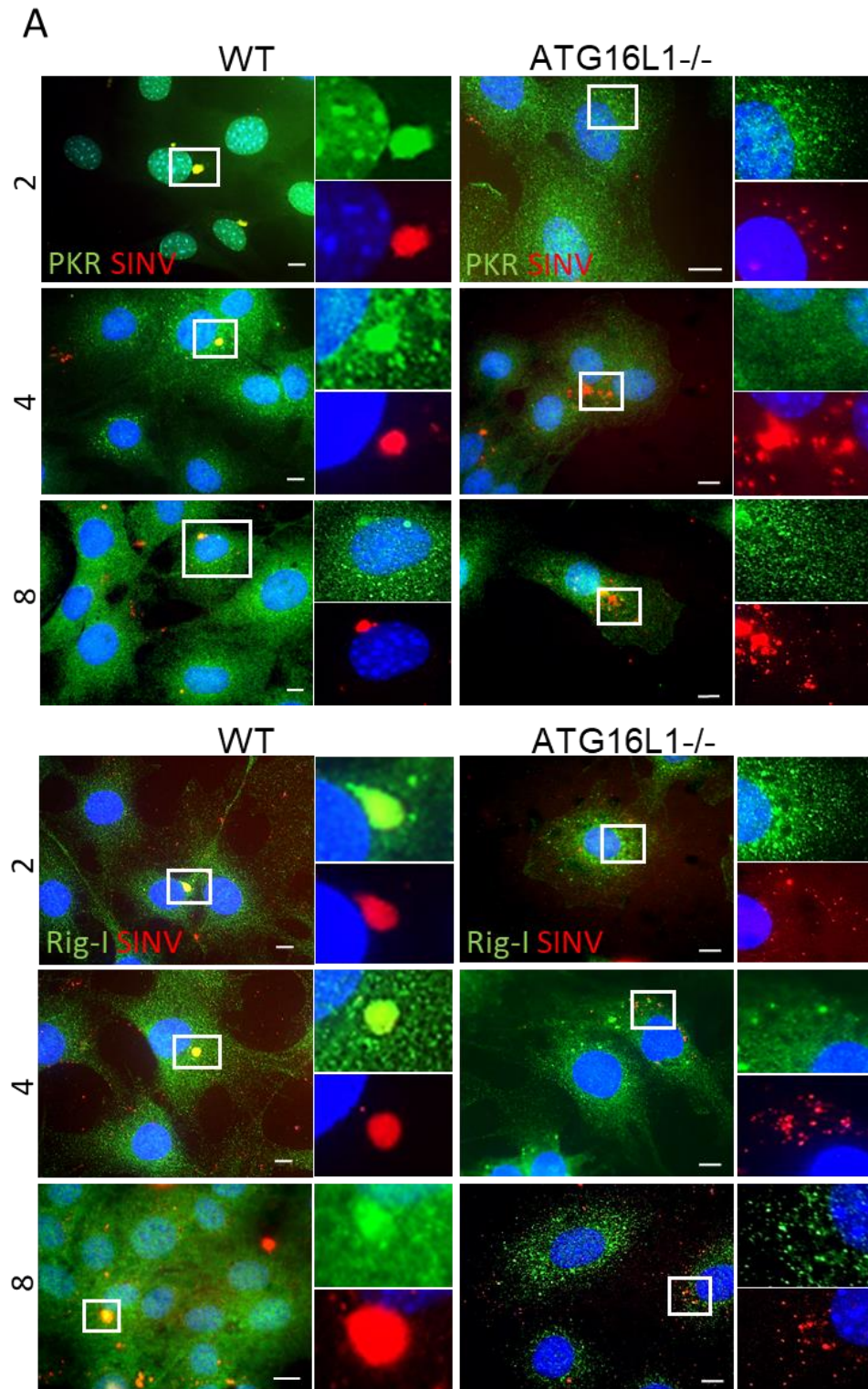


Figure 1.3.8. Viral dsRNA sensors PKR and RIG-I redistribute with viral capsid. Cells were immunostained for PKR, RIG-I and TIA-1. **A.** Wild type (WT) and ATG16L1^{-/-} cells infected with SINV mCh.capsid for a time course of 2, 4 and 8 hpi.

B

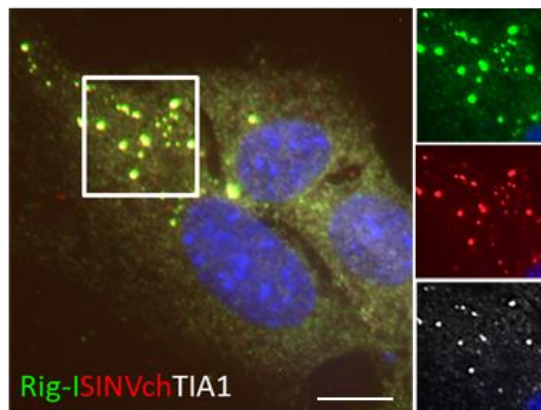


Figure 1.3.8. Viral dsRNA sensors PKR and RIG-I redistribute with viral capsid. Cells were immunostained for PKR, RIG-I and TIA-1. **B.** ATG16L1^{-/-} infected with SINV mCh.capsid for 8 hpi. Imaging by Jefferson et al [137].

Interferon synthesis is required to induce several interferon stimulated genes or ISGs [186]. Type 1 interferon secretion was measured by Jasmine Buck in our lab after SINV infection or with dsRNA (poly I:C) treatment of wild type and ATG16L1 ^{-/-} cells, using the Affymetrix Luminex IFN β assay kit. Both dsRNA (poly I:C) treatment and SINV infection increased interferon secretion in ATG16L1 knockout cells by 1- to 4-fold over wild type cells [137]. To examine if this was due to early induction of interferon synthesis, ISG mRNA expression was analysed, using total RNA isolated from SINV infected cells. Transcription of ISGs, MDA-5, RIG-I, PKR, ISG-15, ISG-56 and IP-10 were analysed by semi-quantitative RT-PCR. SINV infection led to a rapid increase in mRNA for ISGs RIG-I, PKR, MDA-5, with the greatest increase being ISG15 and ISG56 mRNA, which were undetectable before infection [137]. Real-time quantitative RT-PCR was used to measure relative differences between wild type and ATG16L1 knockout cells for IFN beta, ISG15 and IFIT1 (ISG56). When cells were infected with SINV, there was 100-fold greater IFN beta, ISG15 and ISG56 mRNA detected in cells lacking ATG16L1 compared to wild type cells [137].

In summary, interferon translation occurs in wild type cells in the presence of SG, at a time when eIF2 α is phosphorylated and translation factors had been sequestered into SG during virus infection. However, there is a potentiation in interferon production and ISG transcription in ATG16L1 knockout cells, when RNA-binding host translation factors are scattered throughout the cytoplasm, and when there was little eIF2 α phosphorylation and low viral protein synthesis.

1.3.iv. Discussion

In this investigation we showed SINV infection requires ATG16L1 for the efficient phosphorylation of eIF2 α and for high levels of viral protein synthesis. ATG16L1 is also required for the formation of a singular perinuclear SG that contains capsid at early infection. During viral infection, dsRNA or viral proteins produced activate eIF2 α kinases, leading to eIF2 α phosphorylation resulting in host and viral translational arrest. Stalled RNPs aggregate together from viral infection stress to form SGs [188]. eIF2 α can be phosphorylated by over four different kinases, PERK, PKR, heme-regulated eIF2 α kinase (HRI), and general control nonderepressible 2 (GCN2) [189]. PKR is activated by dsRNA during viral infection, although GCN2 can also be activated by SINV RNA genomes [190]. Host translational arrest would block viral replication for most viruses, but this does not affect the translation of alphavirus proteins. SINV is resistant to high levels of phosphorylated eIF2 α due to a stable hairpin loop in the 26S subgenomic promoter that acts as a translational enhancer. This enables SINV to promote its own translation by stalling the ribosome on the initiation codon to enhance translation of structural proteins [171, 191]. In this study, when there was a diminished eIF2 α phosphorylation in ATG16L1 deficient cell, there was lower viral protein translation. This demonstrates that the translation enhancer requires eIF2 α to be phosphorylated for virus translation to begin [137].

SINV infection promotes both stress and autophagy [76, 149]. Previous work has shown that phosphorylation of eIF2 α is essential for both starvation and virus-induced autophagy [192]. Using cells expressing a mutant eIF2 α (S51A) that is non-phosphorylatable, autophagy was not induced in response to starvation [193]. In our work we showed autophagy protein ATG16L1 is essential for eIF2 α phosphorylation and to initiate SG formation. Phosphorylated eIF2 drives the translation of ATF4 that is master regulator of autophagy genes, enabling autophagy to be maintained [172, 188]. ATG16L1 is a well characterised protein with a main role in canonical and non-canonical autophagy. Non-canonical autophagy is the phagocytosis of extracellular pathogens into single membraned vesicles in a process known as LC3 associated phagocytosis (LAP). ATG16L1 is also involved in endocytosis, exocytosis and endosomes/lysosome repair [194, 195]. This study highlights another role for ATG16L1 in eIF2 α kinase signalling. Additional work conducted by our group showed ATG16L1 was important for initiating virus protein translation, and for attenuating an anti-viral response [137]. Other autophagy proteins have been shown to negatively regulate interferon signalling through ATG5–ATG12 interacting with the CARD domains of RIG-I and MAVS [196].

SGs can act as antiviral platforms for sensing viral dsRNA and during SINV infection RLR sensors, PKR and RIG-I localized to virus-induced SG very early in infection (Figure 1.3.8) [197]. In wild type cells, RNA binding proteins TIA1, YBX1 and VCP redistributed with PKR and RIG-I co-localising into a single perinuclear body containing SINV capsid. In ATG16L1 deficient cells, RIG-I and PKR were observed as scattered puncta with TIA-1 and capsid throughout the cytoplasm and did not combine into a single SG. Western blot analysis of RNA binding proteins showed that these were not degraded by autophagy in wild type cells and that accumulation of cytoplasmic granules was not due to inhibition of autophagy. Formation of large perinuclear SGs in wild type cells had no effect on viral protein synthesis as higher yields are detected. This would suggest that SG assembly is not an important anti-viral defense against SINV infection. Alphaviruses have developed an array of mechanisms to inhibit different parts of the host's antiviral responses. Alphaviruses have evolved to use RNA structural motifs as a mechanism to prevent host recognition of its viral dsRNA by RLRs [198]. Alphavirus VEEV capsid proteins are not only important in virion assembly but can also inhibit IFN induction, activation of ISGs and transcription. At a later stage the VEEVs nsp2 protein can inhibit translation [137, 199]

Visual representation of the results we found in this study and how this may look as a new pathway for the role of ATG16L1 during SINV infection.

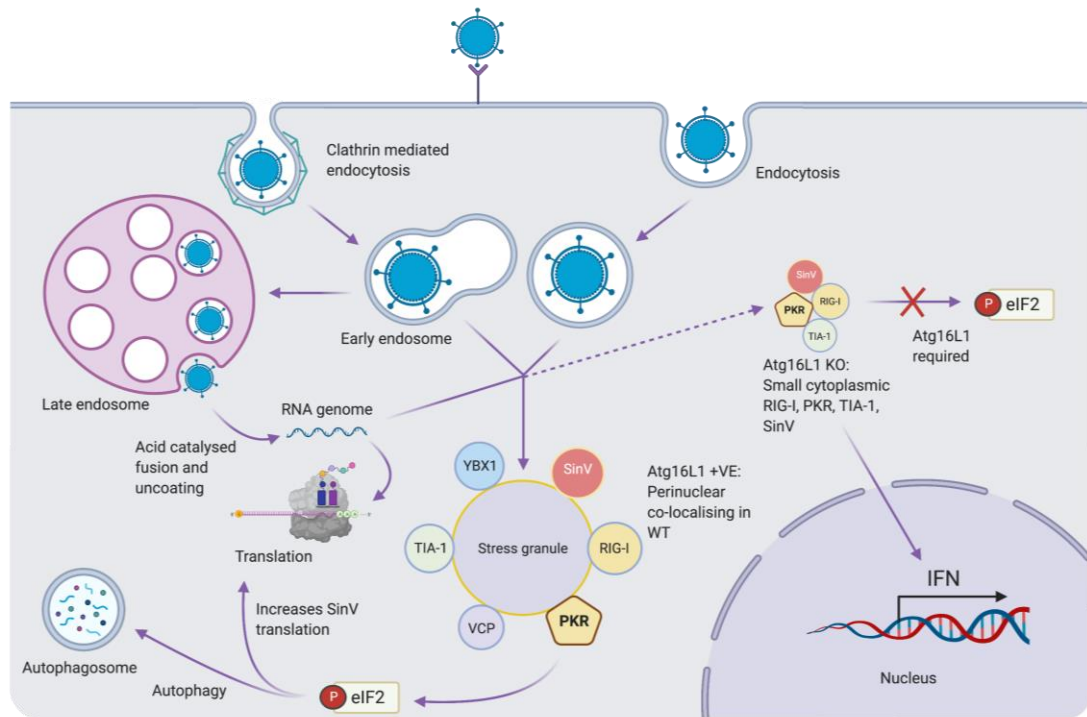


Figure 1.3.9. SINV activation of innate immune response. Viral entry of SINV into wild type MEFs expressing ATG16L1. PKR detects SINV dsRNA and induces eIF2α phosphorylation. High levels of viral protein translation and activation of autophagy occurs through ATF4 signalling. The viral capsid protein redistributes to a single perinuclear SG comprising host RNA-binding proteins YBX1, TIA1, VCP, in addition to PKR and RIG-I in wild type cells. In ATG16L1^{-/-} cells, there is no eIF2α phosphorylation, resulting in low viral protein synthesis. Host RNA-binding proteins also fail to coalesce into a singular perinuclear SG, but instead co-localises with viral capsid in numerous small cytoplasmic puncta.

To conclude, we show that SG assembly occurred early during infection from 2 hpi and that ATG16L1 was required. It has been well established that SINV disassembles SGs late in infection from 8–16 hpi [200]. Our study shows a relationship between the inhibition of SG assembly in the absence of ATG16L1 and that it is not the assembly of SG that enhances anti-viral responses, but the lack of eIF2α phosphorylation that decreases viral protein synthesis.

1.4. Summary of Virus-Host interaction Papers

Virus infections and the subsequent diseases they cause are a major problem for humanity and livestock, both in terms of economics and health. Understanding the relationship of the interaction between viruses and the innate immune system is critical to understanding how viruses overcome these barriers to cause a persistent infection without killing the host. When RNA viruses infect mammalian cells, innate immune pathways including autophagy, apoptosis and the IFN signalling are induced to eliminate the virus. Viruses interact with the hosts innate immune systems to subvert these pathways. In this chapter we investigated the methods in which flaviviruses, and alphaviruses can manipulate IFN pathways, stress and apoptosis signalling. We showed the pestivirus, N-terminal protease (N^{pro}) controlled apoptosis by interaction with IRF3 on mitochondria and peroxisomes. It has been shown that IRF3 binds to cytosolic Bax and induces translocation of Bax to mitochondrial membranes to activate apoptosis. Since N^{pro} targets IRF3 for degradation, our study shows that inhibition of apoptosis by N^{pro} is through inhibition of Bax translocation to mitochondria. N^{pro} acts in multi-functional ways within the host to prevent cell death and activation. We determined if there were more host target proteins for N^{pro} that were involved with the innate immune response. Using protein pull-down and mass spectrometry we determined new protein targets of N^{pro} and that these were identified as RNPs and RNA proteins. These are involved in host protein translations. Following cellular stress, they become components of stress granules. BVDV inhibited the formation of SGs but not through N^{pro} , as cells transfected with this single viral protein were still able to form SGs in response to sodium arsenate. As an RNA binding protein, we show N^{pro} binds host RNA binding proteins and aggregates together into SGs with YBX1, DDX3, RHA and TIA1. This indicated that it may have an effect on host translation. N^{pro} binds to these cellular proteins through its TRASH domain by zinc chelation, indicating that the interaction is specific. Pestiviruses must hijack host cellular translation machinery in order to express viral genes.

In the next series of experiments, we used the alphavirus Sindbis virus to induce stress granules to investigate their role in innate immune signalling, namely interferon production. We showed that eIF2 α phosphorylation was essential for SG formation. SG contain viral pattern recognition sensors, double stranded RNA-activated protein-19 kinase R (PKR) and RIG-I and are essential for interferon production. In cells where SG assembly is inhibited, then interferon expression is potentiated. We show that the protein ATG16L1 is essential for virus induced phosphorylation of eIF2, viral protein synthesis and the formation of SG.

Identifying new host proteins in mechanisms of virus infection is essential in the further understanding and development of vaccines and therapeutics. By targeting either host or viral proteins with drugs you can prevent the virus from establishing a persistent infection within the host. This body of work also demonstrates

Chapter 2

Generation of a mouse model to study the role played by non- canonical autophagy during viral infection

Chapter 2 Generation of a mouse model to study the role played by non-canonical autophagy during viral infection.

2.1 The ATG5-binding and coiled coil domains of ATG16L1 maintain autophagy and tissue homeostasis in mice independently of the WD domain required for LC3-associated phagocytosis

This chapter is an adaption of work that has been published (Rai, Jefferson et al) [187] and appears with permission of the author and named contributors.

2.1.i Introduction

2.1.i.a Non-canonical autophagy and LC3 associated phagocytosis (LAP)

Non-canonical autophagy uses ubiquitin-like ATG16L1:ATG12–ATG5 complex to drive LC3 conjugation to phosphatidylethanolamine (PE) on single membraned endo-lysosome compartments [201]. There are two forms of non-canonical autophagy LC3-associated endocytosis (LANDO) and LC3 associated phagocytosis (LAP) [202]. LC3 attachment to single membraned vesicles has been shown to occur in cultured cells during viral infections, micropinocytosis and phagocytosis [203]. LAP occurs in phagocytic cells like macrophages where LC3 is conjugated to phagosomes. This generates a single-membraned structure called a LAPosome, where it is thought the pathogen is rapidly removed to lysosomes providing a barrier to infection [202, 204]. LAPosomes are formed during phagocytosis of extracellular pathogens that activate cell surface receptors like TLRs [205]. Upon activation, LC3 recruitment is signalled on the cytosolic side of the phagosome membrane where it facilitates fusion with lysosomes [206].

2.1.i.b The LC3 associated phagocytosis (LAP) pathway

A main distinction between canonical autophagy and LAP is that LAP isn't reliant on mTOR signalling through nutrient availability. Neither is it regulated by the autophagy preinitiation complex mTOR-ULK1-FIP200-ATG13-ATG101 [202, 207]. Activation of LAP is initiated through cell surface receptor recognition of pathogens or dead cells at the point of phagocytosis. These plasma membrane receptors recognise a wide spectrum of ligands. As previously stated PRRs including TLRs and, C-type lectin receptors (CLRs) recognise PAMPs. TIM4 receptor binds to phosphatidylserine (PtdSer) displayed by dead cells [208]. Other

receptors known to initiate LAP are immunoglobulins (Ig) bound to antigens [206] and Fc receptors (FCR) [209]. Though a series of receptors and ligands have been identified that activate LAP, it is still not entirely clear how activation recruits LAP components like Rubicon to the phagosome [204] (Figure 2.1.1). TLR signalling recruits Rubicon to the forming phagosome where it is required for the activity of the class III Phosphatidylinositol-3 kinases (PI3K) complex. The PI3K complex consists of Beclin-1, VPS34, VPS15 and UVRAG [210]. Rubicon is a Beclin-1 binding protein where it stabilises class III PI3K complex. VPS34 subunit phosphorylates PtdIns on the endo-lysosome membrane to produce phosphatidylinositol 3-phosphate (PI3P) [206, 207]. Generation of PI3P is required for downstream events of LAP, including production of reactive oxygen species (ROS) by the recruitment of the NADPH oxidase-2 (NOX2) complex. The NOX2 complex consisting of RAC1, p47, p40, p67 and p22 associates with the phagosome membrane where PI3P has created binding sites. Rubicon also binds the p22 subunit to activate the NADPH oxidase and stabilises the NOX2 complex. Here the NOX2 complex is responsible for the production of ROS in the lumen of the LAPosome [207]. ROS is required for the recruitment of the LC3 conjugation system, where ROS induces binding of the WD domain of ATG16L1 to the phagosome membrane. Membrane associated ATG16L1 recruits the LC3 conjugation machinery ATG16L1:ATG5-ATG12, ATG7 and ATG3 allowing LC3 conjugation to PE [211] (Figure 2.1.1).

2.1.i.c LC3-associated endocytosis (LANDO)

As previously stated, there are two forms of non-canonical autophagy, LC3-associated endocytosis (LANDO) and LC3 associated phagocytosis (LAP). LAP only occurs in phagocytic cells where LC3 is conjugated to phagosomes, whereas LANDO occurs in non-phagocytic cells and LC3 is conjugated to endosomes and lysosomes. Having first been shown by Oliver Florey (but not called LANDO) [173], LC3 lipidation was seen to occur on endo-lysosomal membranes when cells were treated with lysosomotropic drugs chloroquine or monensin. Endo-lysosomal LC3 lipidation is activated by chloroquine and monensin in a V-ATPase-dependent manner by raising the endo-lysosomal pH resulting in osmotic stress [173]. At a later point work by Douglas Green's lab [212], identified LC3 conjugation to endosomal membranes during receptor mediated endocytosis and coined the response LANDO. They demonstrated that LANDO was required for receptor recycling in microglia cells for the removal of toxic aggregated proteins such as β -amyloid [213]. This is still a new and emerging pathway with limited molecular mechanistic insight. Both LAP and LANDO conjugate LC3

onto single membraned vesicles so it is possible that mechanism in which they conjugate LC3 may be similar.

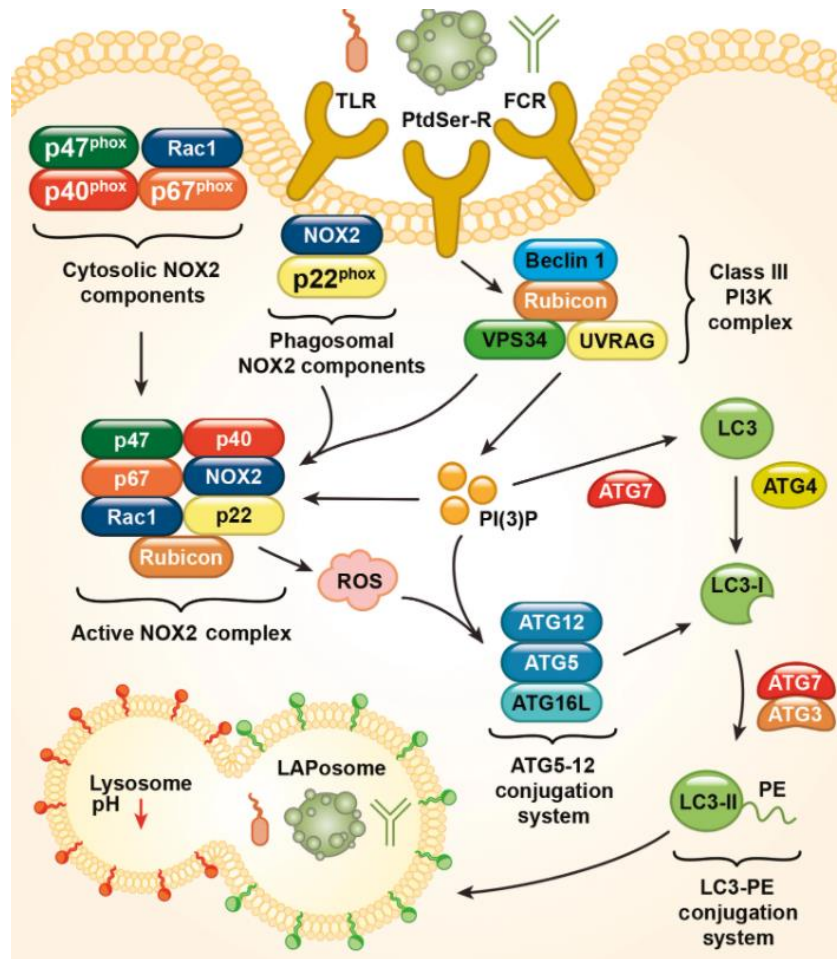


Figure 2.1.1. Molecular mechanism of LC3-associated phagocytosis (LAP). LAP is activated by phagocytosed cargo interacting with immune receptors TLRs, PtdSer-R or Fc receptors (FCR) [209]. A single membraned phagosome containing the extracellular phagocytic cargo is formed inside the cell. Engagement of receptors recruits Rubicon, where it activates UVRAG containing the Class III PI3K complex (Beclin-1-VPS34-UVRAG-Rubicon). Once the Class III PI3K complex assembles and associates with the phagosome, PI3P is then generated at the LAPosome. Engagement of receptors also incorporates the NOX2 complex (NOX2 and p22^{phox}) onto to the phagosome. Rubicon secondly binds the p22^{phox} subunit to activate the NADPH oxidase complex and to stabilise the NOX2 complex (p47^{phox}, p40^{phox}, p67^{phox}, and Rac1). PI3P has three important roles within LAP. PI3P recruits the downstream conjugation system ATG16L1:ATG5-ATG12 with E3-ligase activity and the LC3-PE conjugation

System. PI3P also stabilises the NOX2 complex along with Rubicon leading to the production of ROS. Both ROS and PI3P drives the lipidation of LC3I with PE to make LC3II where it is translocated to the single membraned LAPosome. LC3-II is essential for the fusion to the lysosome and maturation of LAPosome [209, 214].

2.1.i.d The WD domain of ATG16L1 is required for LAP/LANDO

Using a yeast two-hybrid screen for yeast ATG12 binding partners, identification of a new autophagy protein ATG16 was found [215]. A mammalian ortholog was later discovered through co-immunoprecipitation with mouse ATG5 [216]. In yeast the ATG16 protein structure is very simple and only consists of an N-terminal ATG5 binding domain and a C-terminal coiled coil domain (CCD) (Figure 2.1.2). Whereas in the mammalian ATG16L1 there is a linker region after the CCD domain followed by an additional 40 tryptophan-aspartic acid repeat called the WD-40 domain (Figure 2.1.2). ATG16L1 interacts with ATG5-ATG12 through the ATG5 binding domain to mediate the conjugation of PE to LC3. The CCD domain binds FIP200 and is also responsible for binding to WIPI on the phagophore membrane through glutamate residues E226 and E230. The ATG5 binding domain and the CCD domain in yeast and higher eukaryotes is sufficient to facilitate autophagy [217]. The WD-40 repeats represent a large proportion of the protein and since they are found in higher eukaryotes it is possible that the WD domain has been acquired during evolution of complex organisms [216]. The full function of the WD-40 repeats is still not fully understood but progress was made during a collaboration with Oliver Florey. Here ATG16L1 constructs were made lacking the WD domain and LC3 was no longer recruited to the endo-lysosomal membrane during non-canonical autophagy [211]. Then later our study demonstrated that macrophages isolated from a mouse model lacking the WD domain was required for the LAP [187].

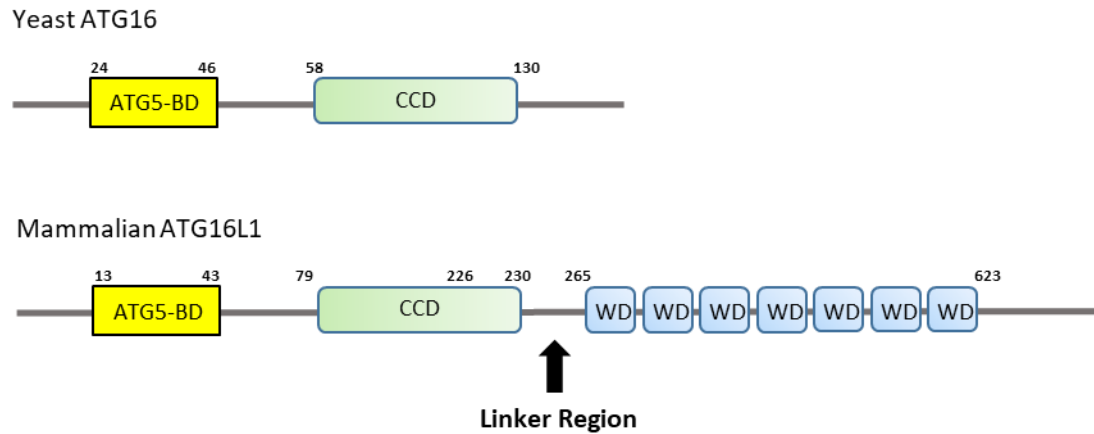


Figure 2.1.2. ATG16 protein structure. Schematic representation of mammal ATG16L1 and yeast ATG16 protein structure. The yeast and the mammalian ortholog both share a ATG5 binding domain and CCD domain. The mammalian ATG16L1 distinguishing feature is the addition of a WD-40 repeat which is separated from the CCD domain by a linker region.

2.1.i.e Generation of a δ WD mouse model to study the role played by LAP/LANDO *in vivo*

LAP and LANDO both share similar downstream pathways with conventional autophagy. Therefore analysis of the specific roles played by non-canonical autophagy proteins *in vivo* has been challenging because most deletions of autophagy proteins remove both pathways and lead to neonatal lethality [218]. Both autophagy and non-canonical autophagy use the E3-ligase like activity of the ATG16L1:ATG5-ATG12 complex to conjugate LC3 to PE on endo-lysosome membranes. In autophagy this is achieved through the binding of WIPI to sites located on the CCD domain. Cell culture experiments show that non-canonical autophagy has an additional requirement for the WD repeat domain of ATG16L1. To determine the role of the WD repeat domain of ATG16L1 on LAP *in vivo*, a mouse model lacking the WD domain was designed by Maryam Arasteh, a PhD student in our group who introduced two stop codons directly after the E230 glutamate residue of the CCD needed for WIPI binding. Thereby preventing the translation of the linker and the WD repeats, creating the δ WD mouse model [187] (Figure 2.1.3). This would create a system where systemic loss of LAP and LANDO was experienced in all tissue. But importantly the mice would still harbour a truncated form of the ATG16L1 containing the ATG5 binding domain and the CCD required for WIPI binding and canonical autophagy. These mice should be able to activate autophagy, survive neonatal lethality and maintain tissue homeostasis.

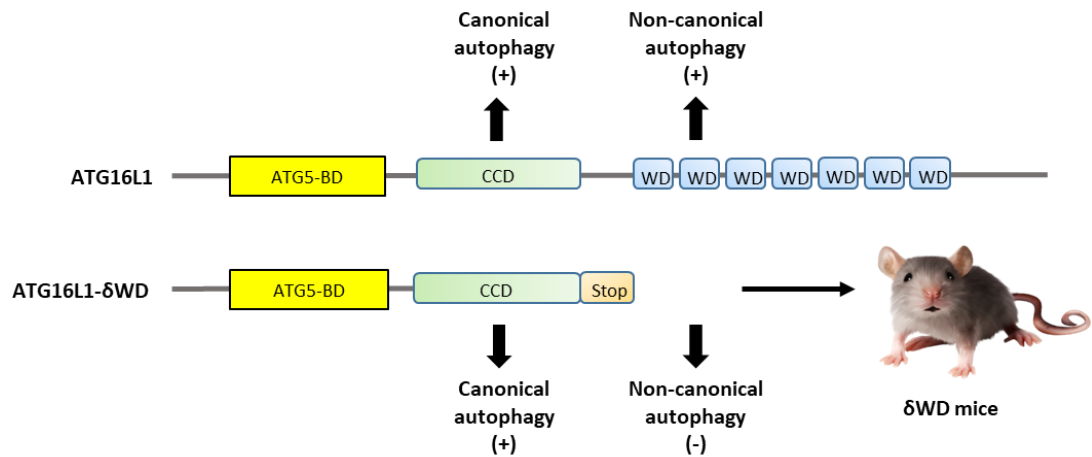


Figure 2.1.3. Generation of the δ WD mouse model lacking the WD domain of ATG16L1. The ATG5 binding domain and the CCD is only required for canonical autophagy. Whereas the WD domain of ATG16L1 is required for non-canonical autophagy. The stop codons prevent translation of the WD domain generating a δ WD mouse model with systemic loss of non-canonical autophagy in all tissues [187].

At the beginning, the effects of deleting the WD domain of ATG16L1 ‘in vivo’ were not known and even though the mice could maintain autophagy it was possible that δ WD mice might fail to thrive or show major defects in tissue development. LAP is a new and emerging arm of the innate immune system. If the δ WD mouse model did not show major defects in growth or development, it could be developed as a novel *in vivo* infection model to determine the role played by LAP during infection.

In the results presented below tissue development and homeostasis in the δ WD model were compared to wild type littermate controls.

2.1.ii. Materials and Methods

2.1.ii.a Mice

All experiments were performed in accordance with UK Home Office guidelines and under the UK Animals (Scientific procedures) Act 1986. These studies used adult male and female mice of approximately 2–3 months of age from the first cross of 129 and C57BL/6. The generation of δ WD mice (*Atg16L1* ^{δ WD/ δ WD}) has been described previously [187]. Generation of δ WD and *Atg16L1*^{fl/fl} [219]. Generation of δ WD^{phag} and *Atg16L1*^{fl/fl}-LysM-Cre mice is described in detail in Figure 2.2.4-6. Comparisons were made using age and sex-matched littermate control mice for each individual genotype. The growth rate of mice was estimated by recording weights each week. Mice were killed by schedule-1 procedures and dissected to harvest brain, lung, liver, kidney, gastrocnemius muscle, skin, femur and tibia. The weights of the organs were recorded before fixing or freezing [187]. Generation and breeding of mice was approved by the University of East Anglia Animal Welfare and Ethical Review Body and performed under UK Home Office Project License 70/8232 [220].

All influenza infection studies were performed at the University of Liverpool by James Stewart and Parul Sharma. Influenza infection studies were approved by the University of Liverpool Animal Welfare and Ethical Review Body and performed under UK Home Office Project License 70/8599. Studies used 2-3 m old male and female mice that had been back-crossed to C57BL/6J. Mice were maintained under specific pathogen-free barrier conditions in individually ventilated cages (Greenline GM500, Techniplast) at a temperature of 22°C ($\pm$ 2°C), humidity 55% ($\pm$ 10%), light/dark cycle 20 12/12 hours (7 am to 7 pm), food CRM(P) and RO or filtered water ad lib. Colonies were screened using the Charles River surveillance plus PRIA health screening profile every 3 months to ensure SPF status [220]. For IAV infection, animals were randomly assigned into multiple cohorts, anaesthetised lightly by the i.m. route with 150 mg/kg ketamine (Ketavet, Zoetis UK Ltd) and separate cohorts inoculated intranasally with 10³ PFU IAV strain X31 in 50 μ l sterile PBS. Mice were infected between 9 and 11 AM. Animals were sacrificed at variable time points after infection by cervical dislocation. Tissues were removed immediately for downstream processing. Sample sizes of n = 6 were used as determined using power calculations and previous experience of experimental infection with these viruses. For survival analysis, a humane endpoint was determined using a scoring matrix that included excessive (>20%) weight loss [220].

2.1.ii.b Cells and cell culture

Cells were taken from mice at embryonic day 13.5 of littermate control and δ WD mice. The embryos were harvested from time mated females at 13.5 d.p.c. (day post-coitum), positive pregnancies were confirmed by weight checks every 2 days. Individual embryos were separated from placenta and embryonic sac where the head, heart and liver were removed. The tail was removed and kept for genotyping each embryo. The remaining tissue was minced with a razor blade and incubated with 0.05% trypsin at 37°C for 30 min. After trypsin digestion, cell suspension was passed through 40 μ m filter and centrifuged at 1000 g for 10 mins at room temperature. Cell pellets were collected and placed in a 10 cm tissue culture dish containing DMEM supplemented with 10% FBS and 1% pen strep. Cells were incubated 37 °C and 5 % CO₂ until confluent. MEFs were generated by serial passage until spontaneous immortalisation was achieved [187, 220].

Skin fibroblasts were collected from δ WD, δ WD^{phag} and littermate control mice. Bodies were sterilised with 70% ethanol and the fur was shaved off the abdomens with a sharp razor blade. The skin was cut with scissors into pieces of approximately 1 cm² and collected in a petri dish. The dermal side of the skin was scraped with a sterile scalpel to remove adipose tissue and fat tissue. Skin pieces were flattened inside empty wells of a sterile 6 well plate, with the dermis facing down. The plates were dried in the tissue culture hood for 15-20 minutes, to ensure the skin adheres to the well surface. Adding carefully as to not dislodge the skin pieces, 3 ml of DMEM media (with 10% FBS 1% pen strep, 50 ng/ml Kanamycin) was added to each well. Plates were placed in the incubator at 37 °C, 5 % CO₂ untouched for 2 days to avoid dislodging the skin pieces. Skin fibroblasts started to grow out from the tissue fragments from 2-5 days. After 7 days the skin is removed and cultured until confluent [187].

BMDMs were generated from bone marrow isolated from femur and tibia flushed with RPMI 1640 (Sigma) [187]. Macrophages were generated by culturing adherent cells in RPMI 1640 containing 10% FCS and CSF1/M-CSF (Peprotech, 30 ng/ml) for 6 d. Bone marrow derived macrophages (BMDMs) were isolated from δ WD and littermate control mice [187, 220].

2.1.ii.c Autophagy and LC3-associated phagocytosis

Autophagy was activated by incubating cells in Hanks balanced salt solution (HBSS) (ThermoFisher) to create starvation for 2 h at 37°C. LAP was stimulated in bone marrow-derived macrophages by Pam3CSK4-coupled beads. Carboxyl-modified beads (polybead carboxylate 3.0 µm) were conjugated with Pam3csk4 (Invivogen) by following the manufacturer's protocol (Bangs Laboratories, Inc. Tech Note 205, III.). Pam3CSK4-coupled beads were added to macrophage cultures at a ratio of 10:1 (bead/cell) for 1.5 h before being fixed and the location of LC3 analysed by immunofluorescence microscopy [187]. Non-canonical autophagy was stimulated in BMDMs by incubation with Zymosan A (Alexa Fluor 594-labelled; ThermoFisher, Z23374) for 30 min. Non-canonical autophagy was stimulated in MEFs and skin fibroblasts with monensin (Sigma) 100 µM for 2 h at 37°C [187, 220].

2.1.ii.d Tissue western blotting

Dissected tissue was snap-frozen in liquid nitrogen, ground to a fine powder under liquid nitrogen and lysed in RIPA buffer (150 mM sodium chloride, 1% TritonX-100 [Sigma], 0.5% sodium deoxycholate [Sigma], 0.1% sodium dodecyl sulfate [Fisher Bioreagents], 50 mM Tris, pH 8.0) containing protease and phosphatase (Sigma) inhibitors followed by homogenisation and freeze thaw. Samples were clarified by centrifugation (10,600 g, 10 min at 4°C). Supernatants containing 10 µg protein were boiled in Laemmli buffer followed by SDS-PAGE using 4–12% gradient gels (Expedeon). The resolved proteins were electro-blotted onto nitrocellulose membrane (Bio-Rad), blocked in 5% skimmed milk in 1X TBS (50 mM Tris pH 7.5, 150 mM NaCl), for 1 h, room temperature. Membranes were then probed with primary antibodies ATG16L1 (MBL, M150-3), SQSTM1/p62 (Abcam, ab91526), GAPDH (Abcam, ab9482) and LC3A/B (Cell Signalling Technology, 4108). Secondary antibodies anti-mouse and rabbit HRP labelled (Cell Signalling Technology, 7074S and 7076S). Blots were visualised by exposure to Supersignal West Pico chemiluminescent substrate (ThermoFisher Scientific) [187].

2.1.ii.e Cell western blotting

Extracted protein (30 µg) was separated on a precast 4–12% gradient SDS-PAGE gel (ThermoFisher) and transferred to Immobilon PVDF (Millipore) for blotting. Membranes were probed using antibodies for ATG16L1 (MBL, M150-3), SQSTM1/p62 (Abcam, ab91526),

LC3A/B (Cell signalling, 41085) and Actin (Sigma, A5441). Primary antibodies were detected using IRDye labelled secondary antibodies, anti-mouse, rabbit and guinea pig (LI-COR biosciences, 926–32,211, 926–68,020) at 1:10,000 dilution [187, 220].

2.1.ii.f Fluorescence imaging

Cells were fixed at -20°C in ice cold methanol for 7 min, then blocked in 5% goat serum, 0.3% Triton X-100 in PBS (Sigma). Cells were incubated overnight at 4°C with anti LC3 A/B (Cell Signalling Technology, 4108), in 1% BSA, 0.3% Triton X-100, PBS. Washed cells were incubated with secondary antibody anti-rabbit-Alexa Fluor 488 (Life Technologies, A11008) and counter-stained with 4', 6 diamidino-2-phenylindole (DAPI; ThermoFisher Scientific, 10,116,287) and mounted on slides with Fluoromount-G from Southern biotech (ThermoFisher Scientific). Cells were imaged on a Zeiss Imager M2 Apotome microscope with a 63X, 1.4 NA oil-immersion objective using 365 ± 40 nm excitation and 445 ± 25 nm emission for DAPI, 470 ± 20 nm excitation and 525 ± 25 nm emission for LC3. Images were obtained using a Zeiss Axioplan microscope with bright field [187, 220].

2.1.ii.g Genotyping

Extraction genomic DNA: Ear biopsies were incubated overnight at 55°C in 50 μl of DNA lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, 100 $\mu\text{g}/\text{ml}$ Proteinase K and nuclease free water). Tail biopsies were incubated in 100 μl of DNA lysis buffer. Lysates were vortexed vigorously and diluted 1:20 in nuclease free water.

Table 2.1.1. Primer information for genotyping

Primer name	Primer sequence (5'-3')	Genotyping PCR details: Using the DreamTaq PCR kit as per manufacturer's instructions (ThermoFisher), 3 µl of the 1:20 DNA dilution was used per 50 µl total reaction mix.
290	CAAATATGCCTTCAGAACTG	
291	GCTGTAGTTCCAATCCCTAA	
223	CTGAACAGTTAAGTTCCTAG	
226	CCAAGAGACACTGACATAGG	Touchdown PCR Program (Annealing temperature decreases from 65°C to 56°C at the rate of 1°C/cycle)
15B	GACGGAAATCCATCGCTCGACCAG	
19B	GACATGTTCAGGGATCGCCAGGCG	

95°C – 3 minutes	}	10 Cycles
95°C – 45 seconds		
65°C – 45 seconds (decreases 1°C/cycle)		
72°C – 1 minute	}	25 Cycles
95°C – 45 seconds		
55°C – 45 seconds		
72°C – 1 minute		
72°C – 10 minutes	}	
04°C – forever (hold)		

PCR products were mixed with 10 µl of 5X Orange G DNA loading dye (50% Glycerol+ 1X TAE buffer, 0.25g Orange G dye and distilled water) was added to each 50 µl genotype PCR product. Samples were separated by electrophoresis on 2 % agarose in TAE buffer gels and stained with ethidium bromide, photographed in ultraviolet light by ChemiDoc-It® 2 810 Imager.

2.1.iii. Results

2.1.iii.a Generating a mouse model lacking the WD domain and linker domains of Atg16L1

Mice lacking the WD and linker domain of *Atg16L1* were designed and generated by Julia Maryam Arasteh (Arasteh, 2012). A targeting vector was designed for exon 6 of *Atg16L1* to insert 2 stop codons after glutamate residue E230 of the CCD domain (Figure 2.1.4, A). Homologous recombination within embryonic stem cells was verified by Southern blotting and RNA analysis of the F1 generation showed that most mice carried the correct sequence and expressed the E230 residue [187]. This mouse strain was termed *atg16l1^{E230}*, hereafter referred to as δ WD mice. Confirmation of the correct genetics for the δ WD mice from the breeding was carried out using the primer design system by Julia Maryam Arasteh (Arasteh, 2012) (Figure 2.1.4, B). Timed matings were setup to harvest embryos at E13.5 to generate a MEF cell line for the δ WD mice. Each embryo that was processed was genotyped by removing the tail tissue and collecting established cells from culture. An example of genotyping mice to identify littermate control and δ WD mice is shown in Figure 2.1.4, B. DNA samples were generated from the tail tissue and MEFs originating from the same experimental embryo. Primer set 290 X 291 was used to detect the presence of wild type *Atg16L1* sequence or the δ WD insertion. Lane 1 and 3 shows one PCR product for the wild type *Atg16L1* allele at 291 bp in both tail and MEF DNA samples, identifying it as a homozygous littermate control. In lanes 2 and 4 a single PCR product for δ WD is observed 639 bp, identifying it as a homozygous δ WD. The increase in size is a result of the cassette insertion into exon 6 to introduce the two stop codons. If two PCR products are of both seen at 291 and 639 bp then that would be classified as a heterozygous mouse carrying one wild type allele and one δ WD allele.

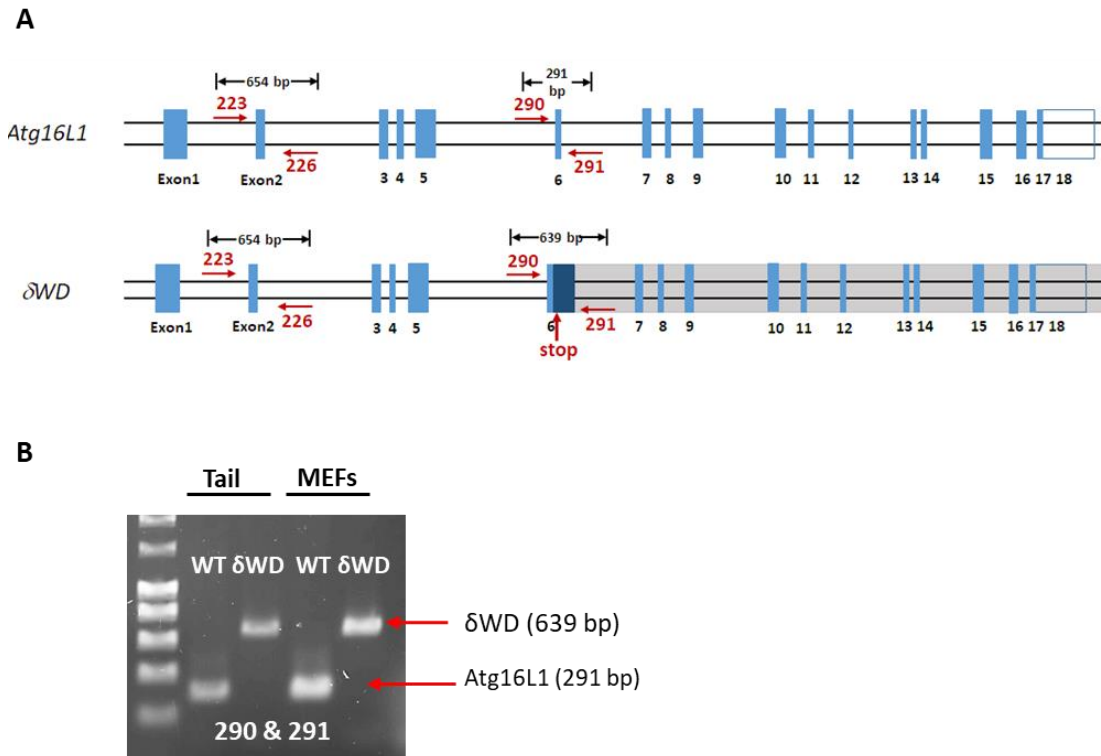


Figure 2.1.4. Genotyping δ WD mice. **A.** Wild type allele *Atg16L1* is identified using primers flanking exon 2 (223 X 226) and exon 6 (290 X 291). The δ WD allele was generated by inserting a cassette containing a stop codon into exon 6. **B.** The insertion increases the size of the frame, increasing the PCR product of exon 6 from 291 bp to 639 bp. DNA was extracted from both tail and MEFs of the same embryo. Genotyping by Jefferson et al [187].

2.1.iii.b Protein characterisation of litter mate controls and δ WD

To compare how the genetics of the mouse translated in terms of protein, expression of ATG16L1 (Figure 2.1.5, A) and δ WD (Figure 2.1.5, B) was determined. MEFs from littermate control (wild type) and δ WD (E230) mice were analysed by western blot. Full-length ATG16L1 splicing isoforms ATG16L1- β 68 kDa and ATG16L1- α 66 kDa bands were seen in the wild type MEFs. Expression of the truncated ATG16L1^{E230} could be observed migrating at 27 kDa. The truncated ATG16L1^{E230} which consists of the ATG5 binding and CCD domain of ATG16L1 gave the expected size after the deletion of the WD domain (Figure 2.1.5, C) [187].

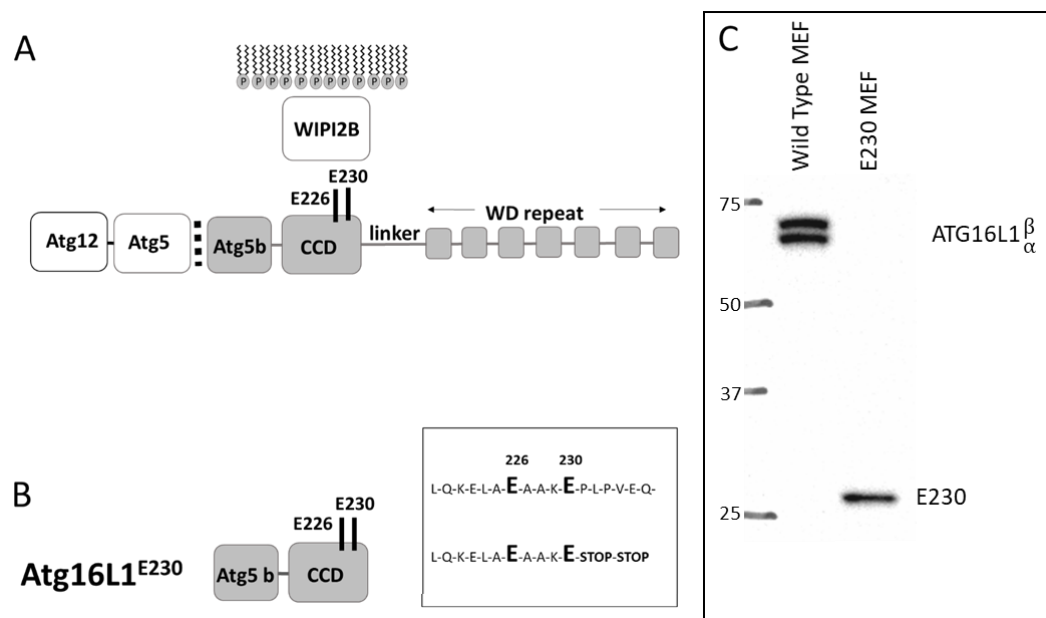


Figure 2.1.5. ATG16L1 protein and domain interactions. **A.** Domain structure of ATG16L1. The N-terminal ATG5 binding domain (ATG5b) binds the ATG12—ATG5 conjugate. The coiled coil domain (CCD) binds WIPI2B through glutamates E226 and E230. A linker domain separates the CCD from the seven WD repeats of the WD domain. **B.** The *Atg16L1*^{E230} (δ WD) had two stop codons inserted into exon 6 immediately after glutamate E230 to preserve binding sites for WIPI2B but prevent translation of the linker and WD domain. **C.** MEFs were isolated from E13.5 embryos. 30 μ g of total cell was analysed by western blot using an antibody specific for ATG16L1. Western by Jefferson et al [187].

2.1.iii.c Characterisation of autophagy and LAP in cells from δ WD

Skin Fibroblasts and MEFs isolated from littermate control, δ WD and δ WD^{E226} (Figure 2.1.6, A- B) were incubated with HBSS to activate autophagy and determine if the N-terminal ATG5 binding domain and CCD domain were sufficient for autophagy to occur. The δ WD^{E226} model was used as a double-negative control for both autophagy and LAP. The δ WD^{E226} mice have an extra 14 bp deletion into the CCD removing glutamate E230 required for WIPI binding. WIPI binding is essential to bring the ATG12–ATG5–ATG16L1 complex to the autophagosome and conjugate LC3 to the phagophore membrane during starvation [201]. Cells from the littermate controls were able to activate autophagy during starvation with HBSS. The same was true for cells generated from the δ WD mouse which expresses an intact CCD domain but lacks the WD domain. In contrast MEFs and skin fibroblasts from δ WD^{E226} mice were unable to generate LC3 puncta following starvation (Figure 2.1.6, A-B, lower panels). These results demonstrate that autophagy requires the E226 and E230 glutamate residues in the CCD domain for WIPI2 binding but autophagy does not require the WD domain [201]. To determine the role of the WD domain during LAP in phagocytic cells, BMDMs were isolated from littermate control, δ WD and δ WD^{E226}. BMDMs were incubated with Pam3CSK4 (a mimic of bacterial lipopeptides)-coupled polystyrene beads to follow the translocation of LC3 to phagosomes (Figure 2.1.6, C). LC3 was recruited to phagosomes around the coupled beads in macrophages from littermate control mice, but in macrophages from δ WD and the autophagy-defective δ WD^{E226} mice, LC3 was not recruited to phagosomes. Both strains lack the WD domain of ATG16L1 and these findings further supported previous observations that the WD domain is required for LAP in myeloid cells [211, 221].

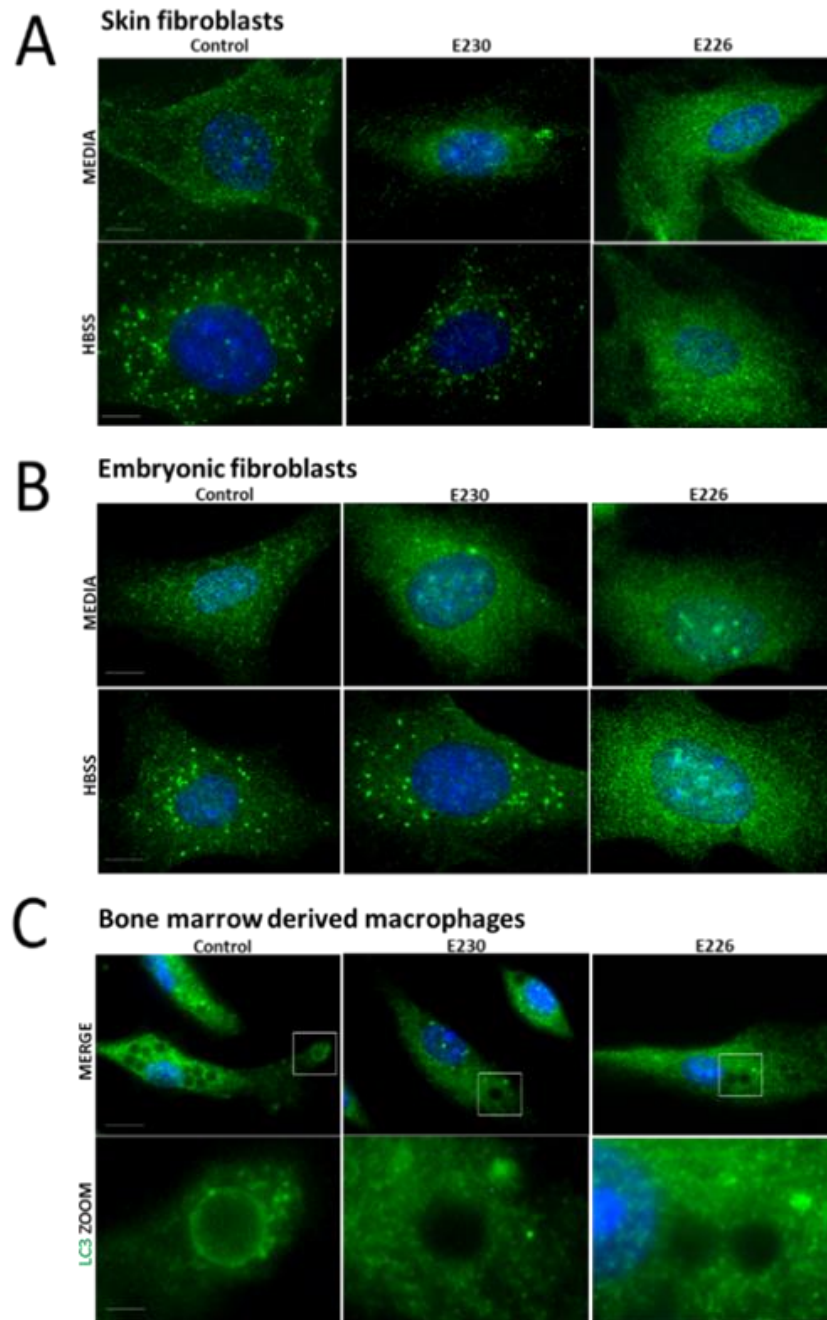


Figure 2.1.6. Role played by WD and linker domains of ATG16L1 during autophagy and LC3 associated phagocytosis (LAP). **A.** Skin fibroblasts, **B.** MEFs, from littermate controls, δ WD (E230) and δ WD^{E226} (E226) were incubated in complete media or HBSS for two hours to induce autophagy. Cells were immunostained for endogenous LC3 (green). **C.** BMDMs from littermate controls, δ WD (E230) and δ WD^{E226} (E226) were incubated with Pam3csk4-coupled polystyrene beads for 1.5 hours in complete media to induce LAP and immunostained for endogenous LC3 (green). Boxed areas highlight regions of interest. Internalised beads are enlarged and shown in the lower panel. Magnification 63X, scale bars 10 μ m. Imaging by Jefferson et al [187].

2.1.iii.d Characterisation of autophagy in cells from δ WD mice

The above studies have shown that the truncated form of ATG16L1 in δ WD mice was able to recruit LC3 onto autophagosomes in response to starvation. In the next experiment western blots were used to determine if cells from δ WD mice were still capable of clearing autophagy substrates such as SQSTM1/p62 (sequestosome 1) and LC3 (Figure 2.1.7). Littermate control MEFs and MEFs from mice lacking ATG16L1 (FL KO) were used for a positive and an autophagy-negative control. In the control and δ WD MEFs it was difficult to detect p62 indicating that degradation of p62 was occurring in these 'autophagy positive' cell lines. In comparison, MEFs lacking ATG16L1 (FL KO), there was a strong signal for p62 even before starvation. This is also true for the double-negative δ WD^{E226} MEFs where both autophagy and LAP function have been removed. MEFs from control mice expressed both α and β isoforms of ATG16L1 at 68 and 66 kDa, and these were absent from FL KO, δ WD^{E226} and δ WD MEFs. The blots also show processing of LC3I to LC3II as second assay for autophagy. The FL KO and δ WD^{E226} MEFs were unable to generate lipidated LC3II after starvation while production of LC3II was seen in littermate control and δ WD MEFs [187]. Importantly, conversion of LC3I to LC3II correlated with reduced levels of p62.

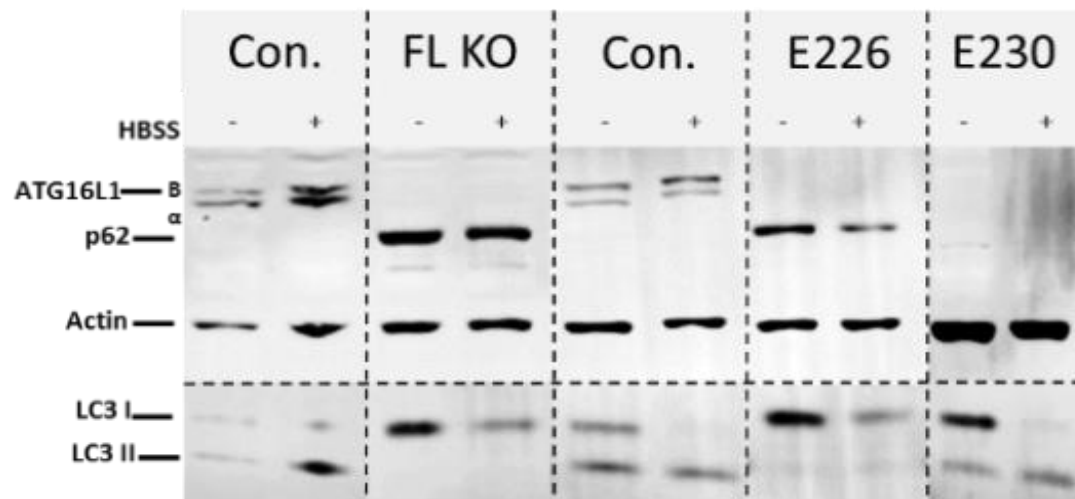


Figure 2.1.7. Role of WD domain in autophagy. MEFs from mice lacking ATG16L (FL KO), δ WD^{E226} (E226) and δ WD (E230) and appropriate littermate controls were incubated in complete media or HBSS for two hours to induce autophagy. Cell lysates were analysed by western blot using antibodies specific for the indicated proteins. Western by Jefferson et al [187].

2.1.iii.e Mouse growth and survival of LAP deficient δ WD mice

The mice used in this study were analysed from 2-3 months of age. LAP-deficient δ WD mice survived the postnatal lethality unlike that reported for complete *Atg16^{-/-}* mice [222]. The δ WD were visually very similar to their littermate control apart from the δ WD mice would splay their legs more when being held by the tail that could be related to balance. The δ WD mice had similar growth rates and sizes to littermate controls whereas the δ WD^{E226} mice which have defects in autophagy, were visually smaller and grew at a slower rate (Figure 2.1.8, A, B). The δ WD mice were fertile and followed Mendelian frequency from heterozygous mating's. Reproductive capacity was comparable to littermate control litters from homozygous matings (data not shown). The survival rate of δ WD mice and littermate controls was similar to C57/BL6 wild-type mice with life spans of at least 24 months being reached (data not shown). The δ WD^{E226} mice did not follow Mendelian frequency but would survive postnatal lethality. It is not known why the δ WD^{E226} do not show postnatal lethality but unlike the δ WD mice and its own littermate control, the δ WD^{E226} mice did not thrive and died within 5–7 months of age [187].

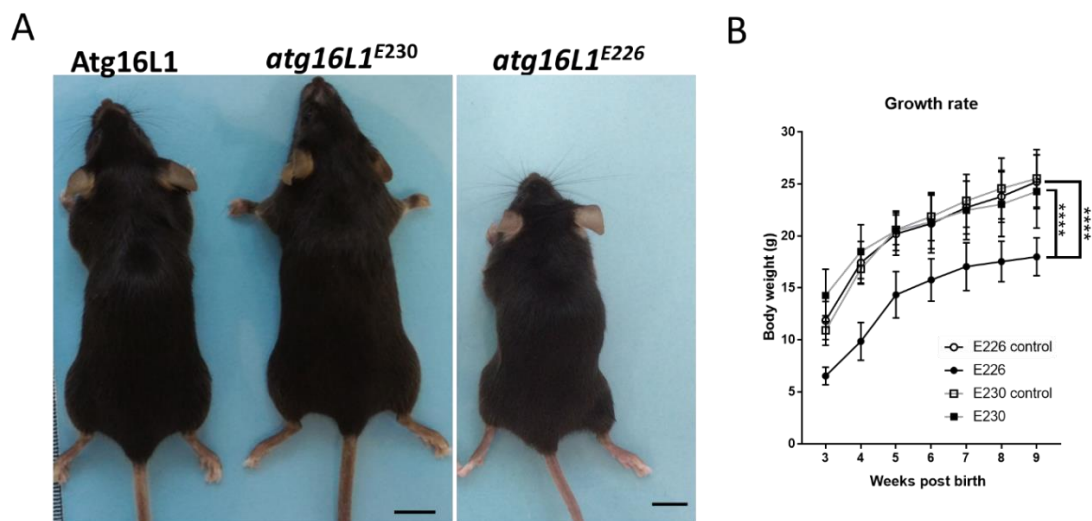


Figure 2.1.8. Phenotype of δ WD^{E226} and δ WD mice. **A.** Representative pictures of mice at ~3 months of age (scale bar 1cm). **B.** Body weight of mice and littermate controls fed on chow diet. Mice were weighed at indicated times and each point is generated from at least 6 individuals. (n =11 and 6 for δ WD) and control respectively. n=11 and 8 for δ WD^{E226} and control respectively). Statistical analysis was by unpaired t test. Error bars represents $\pm$ SEM. ****-P<0.0001. Imaging by Jefferson et al [187].

2.1.iii.f The role of the WD domain in maintaining tissue homeostasis

All internal organs from the δ WD LAP-deficient mice were surveyed to check tissue homeostasis and this has been published [187]. Shown here is a representative example of the tissue analysis carried out using liver tissue from δ WD LAP-deficient mice as an example with controls. Livers from the LAP-deficient δ WD mice were similar in size to littermate controls. The livers from autophagy-defective δ WD^{E226} mice showed the most obvious defect. The δ WD^{E226} displayed an abnormally enlarged liver (hepatomegaly) (Figure 2.1.9, A). The average δ WD^{E226} liver weighed 2.6 g, compared to 1.6 g of control mice. Liver weight was expressed as percentage of body weight (Figure 2.1.9, B) to compensate for the smaller size of the δ WD^{E226} mouse. The δ WD^{E226} livers were more than twice the size (11%) of littermate controls (5%) [187]. This data is consistent with a liver specific *Atg7*^{-/-}-knock-out autophagy model that also showed hepatomegaly. The *Atg7*^{-/-} strain was defective in the clearance of damaged organelles from the liver implicating autophagy's role in maintaining tissue homeostasis [223]. Another study where *Atg5* was restored in only the neuronal tissue of *Atg5*^{-/-} mice also demonstrated multiple abnormalities in tissues including hepatomegaly and that mouse was also small in size [218]. Importantly, here was no hepatomegaly in the δ WD LAP-deficient mice having a mean liver weight of 1.8 g that was comparable to littermate controls. Three biological replicas for each littermate control, δ WD and δ WD^{E226} were blotted to determine autophagy function in liver tissue by measuring autophagy substrates p62 and LC3. Full-length ATG16L1 was present in control mice livers displaying both α and β isoforms of ATG16L1 but absent from δ WD and δ WD^{E226} livers (Figure 2.1.9, C). Liver lysates from the δ WD^{E226} mouse showed raised levels of LC3I and p62 compared to control and δ WD mice, suggesting impaired clearance of autophagy substrates '*in vivo*' (Figure 2.1.9, C). There were no raised levels of autophagy substrates in the control and δ WD mice suggesting that autophagy is active in the δ WD liver allowing normal tissue homeostasis to be maintained. This was further supported by immuno-histological analysis of liver sections where p62 inclusions were absent in hepatocytes in either the δ WD and littermate control. p62 inclusions were present in hepatocytes of δ WD^{E226} mouse liver sections indicative of accumulative damaged proteins and organelles (Figure 2.1.9, D) [187].

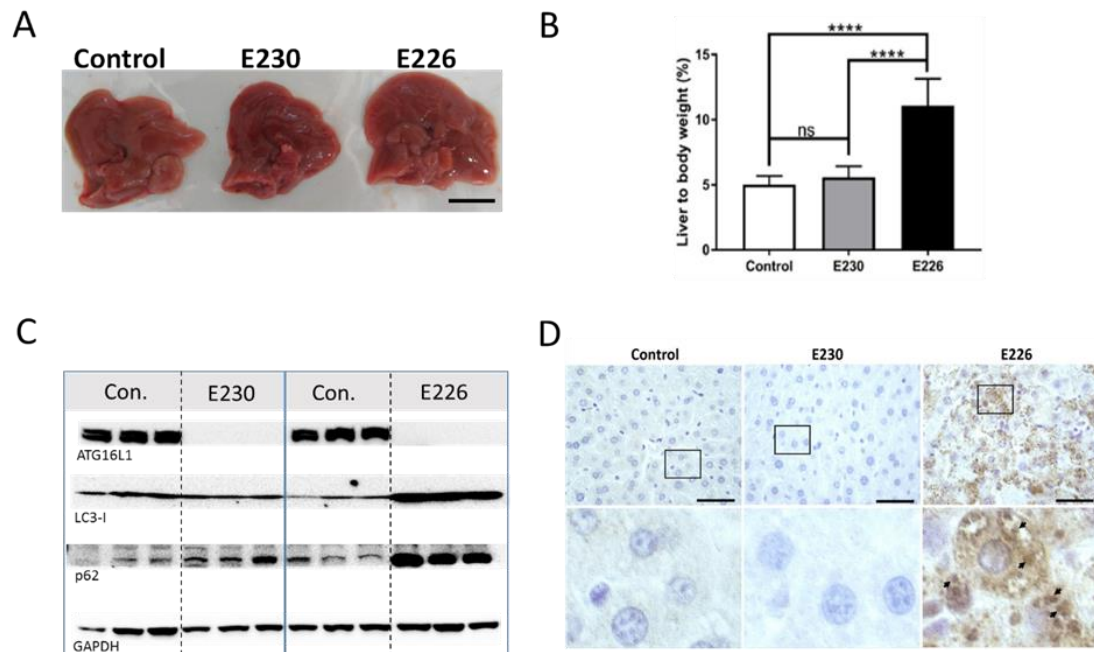


Figure 2.1.9. Analysis of autophagy substrates in liver. **Panel A.** Representative livers at ~2 months (scale bar 1cm). **B.** Liver weight expressed as percentage body weight at 2-3 months of age. [E230 (δ WD) $n=9$, control $n=8$; E226 (δ WD1^{E226}) $n=9$, control $n=7$]. **C.** Western blot of liver lysates from three representative mice. Membranes strips taken from appropriate molecular weight range were analysed separately using indicated antibodies. **D.** Representative histochemical sections of livers immunostained for p62. Enlarged regions of interest are shown in lower panel. Arrows: p62 inclusions. In all figures data from littermate controls for E230 and E226 were pooled. Statistical analysis was by unpaired t test. Error bars represents $\pm$ SEM. ****- $P<0.0001$, ***- $P<0.001$, ns non-significant. Image magnification 40X, scale bars 50 μ m. Western blot performed by Timothy Pearson and histology imaging Shashank Rai [187].

The lungs, muscle, kidneys, spleen and brain of LAP-deficient δ WD mice were of comparable size to littermate controls. Western blots of representative tissue lysates did not show raised levels of LC3I or p62. This was also consistent with immuno-histological analysis showing the absence of p62 inclusions in tissue sections. As seen in the liver, tissue homeostasis in remaining tissues required autophagy, but did not require the WD domain of ATG16L1 and was, maintained independently of LAP. Brain lysates from the LAP-deficient δ WD mice had comparable levels of LC3I and p62 to littermate controls and brains lacked p62 inclusions. Motor coordination appeared normal in the majority of the δ WD mice, but some developed a head tilt suggesting defects in the inner ear or possible ear infection [187].

2.1.iv. Discussion

Mice lacking the WD and linker domains of ATG16L1 termed δ WD were generated to study the roles played by autophagy and LAP in maintaining tissue homeostasis *in vivo*. But to also generate a novel *in vivo* infection model to observe how the loss of LAP would affect the host during infection. The characterisation of δ WD mice showed that BMDMs isolated from δ WD were unable recruit LC3 to the phagosomes and could not activate LAP. These mice however retained both glutamates E226 and E230 in the WIPI binding site in the CCD and could therefore activate autophagy. This was confirmed when cells from the δ WD mouse could generate LC3 positive autophagosomes and convert LC3I to LC3II during starvation. The results also suggested that the ATG5 binding and CCD of ATG16L1 could maintain tissue homeostasis in the absence of the WD domain. Body weights were collated over time and the δ WD mice grew at the same rate as littermate controls. The δ WD mice were also fertile and demonstrated Mendelian frequency in the reproduction of δ WD from heterozygous crosses. Litter sizes and survival rates were also comparable with wild type mice (data not shown) [187]. Tissue analysis did not show obvious defects in liver, kidney, brain or muscle homeostasis. As these δ WD mice are autophagy positive but LAP deficient, this would suggest that autophagy instead of LAP plays a major role in reducing tissue damage *in vivo* [187]. Structurally the WD domains of ATG16L1 make up a third of the total protein and are unique to higher eukaryotes [216]. There are seven WD domains in total on the C-terminal of ATG16L1 where they form a complex of 7 bladed β -propellers. Surprisingly, the WD domains were not required for maintaining tissue homeostasis *in vivo* as demonstrated from δ WD tissue examination. The role of tissue homeostasis has therefore been retained during evolution in just the ATG5 binding and the coiled-coiled domains which are conserved in yeast [216].

The WD domain is not required for tissue homeostasis but it may be required to protect complex eukaryotes from infection. LAP has been described as an important component of phagocytosis. Macrophages isolated from δ WD mice were unable to activate LAP when challenged with Pam3csk4-coupled polystyrene beads and Oliver Florey's group have shown that the WD domain is essential for the recruitment of LC3II to endocytic compartments swollen by osmotic stress [187, 211]. These included newly formed macropinosomes, phagosomes containing apoptotic corpses or latex beads [211]. Similar signals may be generated in endosomes containing viruses and bacteria. Bone marrow derived dendritic

cells from δ WD mice were stimulated with yeasts *S. cerevisiae* and *Kazachstania unispora* through a Dectin-2 and Syk-dependent manner that resulted in reduced secretion of TNF and IL1B [221]. Implicating the role of LAP in the regulation of dendritic inflammatory cytokine responses to pathogens, in our δ WD model. In another study with the δ WD mice, dendritic cells showed a reduction in MHC class II antigen presentation [211].

It is possible that the WD provide a protein-protein/ligand interaction platform on ATG16L1 for the regulation of different cellular functions unrelated to autophagy and possibly interact with a number of receptors that are involved in pathogen recognition. For example the WD domain has previously been shown to have a role in xenophagy clearing *Staphylococcus aureus* infection. The human transmembrane protein 59 (TMEM59) is an endosomal protein that encodes a WD binding motif that promotes LC3 recruitment to endosomes [224]. The same motif is found in the diverse TLR2 that recognises a variety of PAMPs from viruses, fungus, bacteria, and parasites that promotes LC3 labelling of phagosomes [224, 225]. When macrophages were challenged with zymosan from *Tlr2*^{-/-} mice there was reduced translocation of LC3 to phagosomes [226].

The evidence that the WD domain of ATG16L1 has evolved in higher eukaryotes to provide a specialised role independent of autophagy is growing. However, the bulk of studies on LAP have been carried out using cells in tissue culture. These include analysis of phagosomes containing pathogens or apoptotic cells, or endocytic compartments showing signs of damage from osmotic imbalances [173, 187, 211]. During the course of this work a different LAP^{-/-} mouse model was developed by Doug Green's lab in the USA. LAP in phagocytic cells requires Rubicon and NOX2 [207]. Rubicon has been shown to be recruited to the LAPosome where it's required for the activity of a Class III PI3K complex (Rubicon-Beclin-1, UVRAG, and VPS34). Using myeloid cells derived from *Rubicon*^{-/-} mice, they were able to show that *Rubicon*^{-/-} macrophages were LAP-deficient and that *Rubicon*^{-/-} mice had defects in the clearance of bacterial and fungal pathogens as well as the removal of dying and apoptotic cells [207]. The *Rubicon*^{-/-} model has flaws as a LAP model because Rubicon is involved in other processes outside of LAP. For example Rubicon affects NF- κ B signalling and acts as a negative regulator of IFN signalling and cellular antiviral functions [227]. NOX2 has also been shown to be involved in the translocation of LC3 to TLR or FCR engaged phagosomes [228]. A *NOX2*^{-/-} mouse model has been generated and *NOX2*^{-/-}-macrophages were also unable to undergo LAP because of defects in ROS production [207]. NOX2 like Rubicon is a

multifunctional protein that not only generates ROS for LAP but is also an immune modulator, plays a critical role in the initiation of antigen presentation and is an activator of many signalling pathways [229]. This is illustrated by the observation that targeted loss of Rubicon and NOX2 from mice resulted in slow growth rates and an increased susceptibility to an autoimmune disease like systemic lupus erythematosus because of defects in the clearing of apoptotic cells [230].

In summary the *Rubicon*^{-/-} mice do not provide a good model for studying infection in vivo because they have defects in tissue homeostasis and immune function including. For example, increased level of antibodies against double-stranded DNA, anti-nuclear antibodies and raised circulating cytokine levels. In contrast our δ WD mice have not shown defects in tissue development, elevated cytokines or anti-nuclear antibodies thus far.

2.1.iv.a Proposed molecular mechanism of LAP

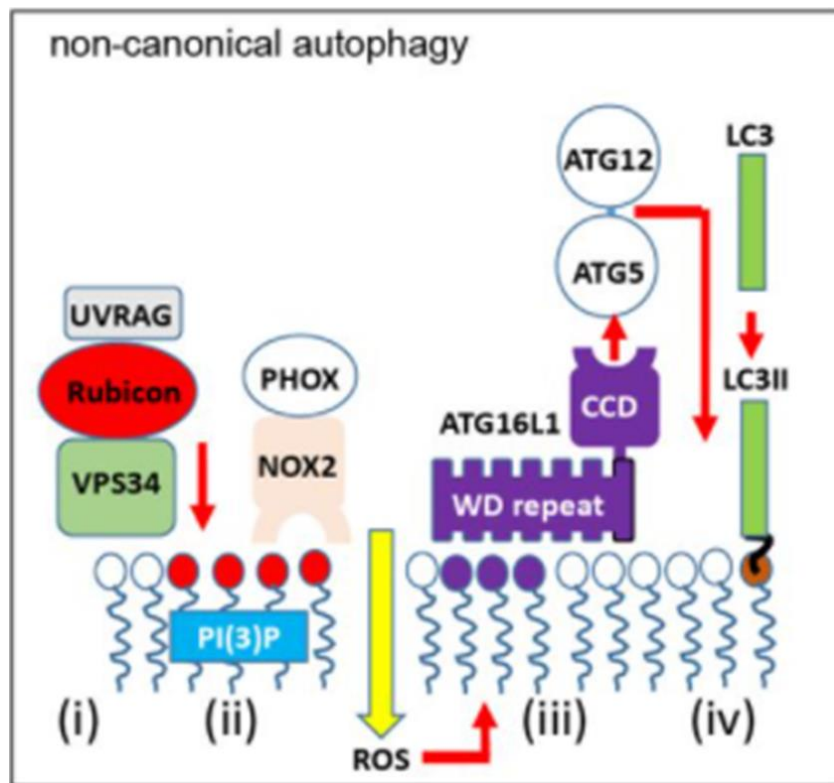


Figure 2.1.10. Model for the WD domain of ATG16L1 to induce LAP. During non-canonical autophagy processes LAP and LANDO, signalling is initiated by the ligation of cell surface receptors and from the lumen of the endosome/phagosome. Recruitment of the class III PI3K complex containing UVRAG, Rubicon and VPS34 (i). The VPS34 subunit phosphorylates lipids in endo-lysosome membranes to generate PI3P sites for binding the multicomponent PHOX (p47phox, p40phox, p67phox, and Rac1) to the NOX2 complex (ii) Rubicon stabilises the PHOX:NOX complex to generate ROS. Production of ROS induces binding of the WD domain of ATG16L1 to endo-lysosome membranes (iii) Bound ATG16L1 leads to the recruitment of the LC3 conjugation machinery (ATG16L1:ATG5-ATG12, ATG7, ATG3) (iii) Conjugation of LC3 to PE (iv)

2.2. Non canonical functions of ATG16L1 in epithelial cells limit lethal infection by influenza A virus

This chapter is an adaption of work that has been published (Wang, Jefferson et al) [220] and appears with permission of the author and named contributors.

2.2.i Introduction

2.2.i.a Influenza A virus (IAV) as a model to study the role played by LAP/LANDO during viral infection

IAV was used in this study to determine the role played by LAP/LANDO during viral infection. IAV is known to enter cells by endocytosis and activate non-canonical processes where LC3 is recruited to vesicle membranes. The recruitment of LC3 to vesicles following IAV infection was shown to be dependent on the WD domain of ATG16L1 *in vitro* [211] but the role played by the WD domain during IAV infection *in vivo* was unknown. In this chapter we use the δ WD to study the importance of the WD domain for LAP/LANDO in controlling IAV infection by analysing viral replication within respiratory tissue, innate immune responses and the effects on morbidity. Understanding the molecular mechanism between host and virus is extremely relevant as this virus inflicts a heavy toll on society resulting in hundreds of thousands of global deaths.

2.2.i.b Influenza A Virus Classification

Influenza A virus (IAV) belongs to the influenzavirus genera within the *Orthomyxoviridae* family [157]. IAV consists of many strains and are named according to their hemagglutinin (HA) and neuraminidase (NA) genes. There are 3 different genera of influenzaviruses A, B and C that can all infect humans with influenzaviruses A and B being the most common. The main focus is on IAV as out of all the influenzaviruses, IAV undergoes the most amount of genetic re-assortment and is responsible for the most seasonal outbreaks. The main strains of IAV that routinely circulate seasonally amongst people are H1N1 and H3N3. There are 18 different subtypes of HA and 11 different NA subtypes for IAV alone. These subtype strains can undergo antigenic drift when mutations arise in the glycoprotein receptors of influenza [231]. But a foremost concern with influenza is antigenic shift, involving a major change in HA or NA glycoprotein combinations giving rise to a new IAV subtype in humans [232]. This can occur when animals act as reservoirs for strains of influenza that can then gain the ability

to infect humans. There have been multiple pandemics this century including the Spanish influenza H1N1 pandemic in 1918 believed to have originated from waterfowl, Asian influenza H2N2 in 1957, the Hong Kong H3N2 swine influenza in 1968 and the 2009 outbreak of H5N1 avian influenza [233, 234]. New combinations of HA/NA can create more virulent and significant threats to the human immune system as there has been no pre-exposure to gain immunity.

These new emerging influenza pandemics with high incidences of viral pneumonia, morbidity and mortality can result in millions of global deaths and that's why surveillance is extremely important. The increasing risk of an influenza driven pandemics highlights the need to understand IAV and host interactions. The majority of influenza infection occurs in the lower respiratory tract that results in flooding of alveolar spaces causing acute respiratory distress syndrome and respiratory failure that can lead to death. Death comes as result of a two-pronged attack from not only from the influenza virus but also from the host's response to the influenza. IAV infects airway and alveolar epithelium causing damage from the intrinsic pathogenicity of individual virus strains as well as the level of response from hosts innate/inflammatory responses [220]. The host responds to the influenza with the production of cytokines, but if there is an overreaction a cytokine storm can develop resulting in excessive inflammation and damage to the lungs [235]. For this study the low-pathogenicity murine-adapted X31 (H3N2) subtype of IAV was used to study the effects of IAV on δ WD mice. These mice are deficient in non-canonical autophagy and we can determine the importance that LAP/LANDO have as a barrier to infection.

2.2.i.c Influenza A Virus genome structure and organisation

IAV is a negative-sense, RNA virus belonging to the *Orthomyxoviridae* family [157]. The IAV genome is segmented and consists of eight single-stranded negative-sense viral RNAs (vRNA) (Figure 2.2.1, A). The eight vRNAs consists of polymerase basic protein 2 (PB2), polymerase basic 1 protein (PB1), polymerase acidic protein (PA), haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 protein (M) and non-structural protein (NS) that contain 11 open reading frames [236]. Each vRNA is flanked by NCRs with a centre containing the coding region. The NCRs promotes replication and transcription by the viral heterotrimeric polymerase complex that consists of PB1, PB2 and PA [237, 238]. HA and NA encode the glycoproteins with M vRNA encoding for M1 and M2 structural proteins. The vRNA NS1 encodes the non-structural genes that make NS1 and a spliced variant NS2 also known as the nuclear export protein (NEP). NEP facilitates the export of newly synthesised vRNPs from the host's nucleus [239]. The vRNAs are packaged as viral ribonucleoprotein (vRNP) complexes within the virion. These vRNP's are bound to a viral polymerase complex (PB1, PB2 and PA), proceeded by multiple copies of nucleoprotein (NP) that bind non-specifically to the vRNA phosphate sugar backbone [157]. By binding to the backbone, the bases are exposed to aid in replication [88]. Contained within the virion a layer of M1 protein surrounds the genome and forms the protein capsid. This ensures structural integrity to the core binding the vRNAs within the core [240]. On top of the M1 protein is a host derived lipid envelope that contains HA and NA. and the M2 channel protein [241] (Figure 2.2.1, B).

2.2.i.d IAV entry

HA binds to sialic acid on the surface of the epithelial cell. IAV is internalised by multiple endocytic pathways that include receptor-mediated and clathrin-mediated endocytosis, micropinocytosis and the clathrin-independent caveolae pathway [242, 243] which converge on mature endosomes that have an acid pH [244]. The low pH leads to HA cleavage and a conformational change exposing the fusion peptide that initiates fusion of the viral envelope fusion with the endosomal membrane [245]. Using the ion channel of the M2 matrix protein, protons are transferred from the endosome into the core of virus [246]. Acidification weakens the M1 protein layer allowing the release of vRNPs into the cytoplasm [247, 248].

Understanding how the airway defends against viral infection is more important and relevant than ever before. Within a small window of time we have seen new and emerging viruses from animal reservoirs to infect humans and causing global pandemics. Sometimes these manifest as highly infectious strains but result in relatively low morbidity. In 2009 an IAV strain, H1N1 (Swine Flu) emerged and infected as many as 700 million-1.4 billion people, resulting in anywhere between 150,000–575,000 deaths [249]. Unlike the avian H1N1 (Spanish Flu) of 1918 that killed anywhere from 17-50 million+ deaths, equivalent to 3-5% of the world's population then [250]. At this point in time we are living through the SARS COV-2 pandemic that has currently claimed an estimated 1.7 million+ deaths with over 70 million cases with no end in sight. These cases underline the urgency in our need to understand how the airways defend against viral infections in relation to which cells types and sub cellular barriers are in play.

In this study we will look at the role of non-canonical autophagy as a barrier to IAV infection and what cell types are involved *in vivo*.

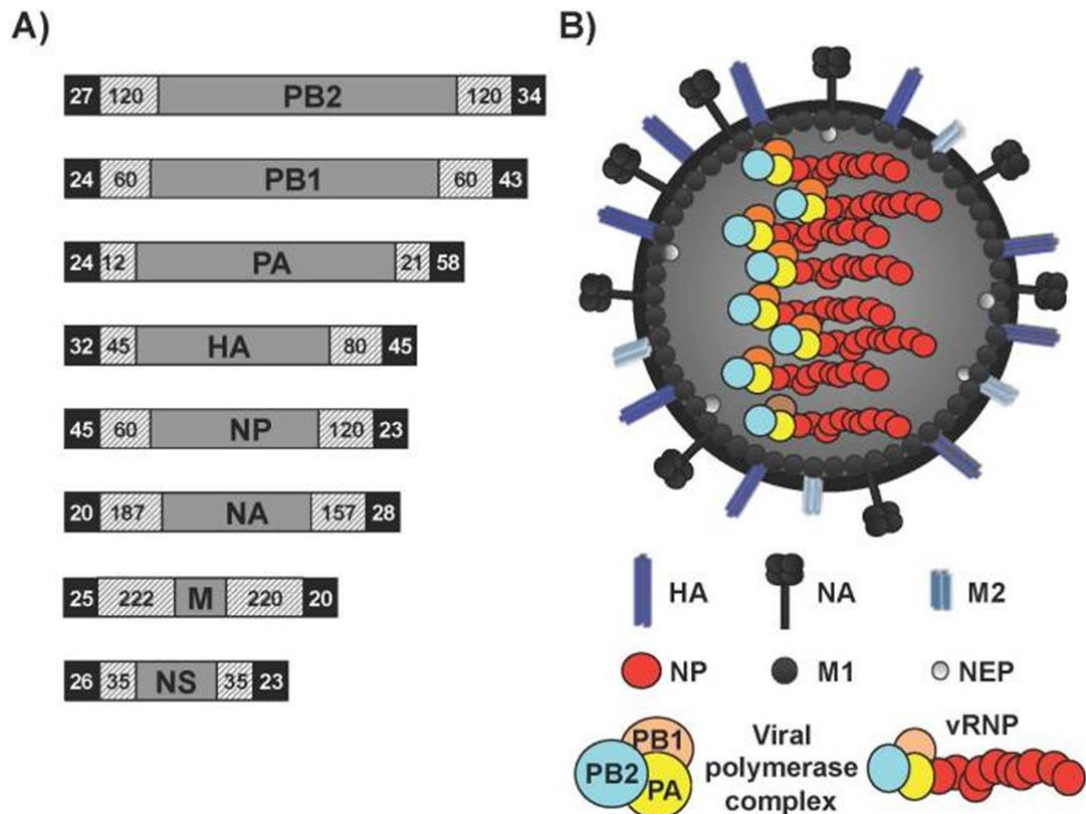


Figure 2.2.1. Influenza A virus (IAV) genome organisation and virion structure: A. Genome organisation. IAV consists of eight single-stranded, negative-sense, viral RNA (vRNA) segments PB2, PB1, PA, HA, NP, NA, M and NS. Black boxes indicate the 3' and 5' NCRs. Each vRNA contains its own 3' and 5' packaging signal represented by the hatched boxes. The packaging signals present on each vRNAs drives efficient encapsidation into emerging virions.

B. Schematic of Virion structure: IAV viral glycoproteins hemagglutinin (HA) and neuraminidase (NA) are encased within lipid bilayer around the IAV genome. HA is required to bind to sialic acid-containing receptors. NA is required for exocytosis from infected cells. Lining the lipid membrane surrounding the IAV genome is the inner surface envelop matrix 1 (M1) protein. M1 has roles in virion assembly and budding. Surrounding the virion is the transmembrane ion channel protein matrix 2 (M2). Studded within the lipid bilayer is the nuclear export protein (NEP). NEP exports the viral ribonucleoprotein (vRNP) complexes. Within the core of the virus is eight vRNA segments that are all individually encapsidated by the viral nucleoprotein (NP). Each vRNP complex is capped with the viral RNA-dependent RNA polymerase (RdRp) complex. The RdRp consists of three polymerase subunits PB2, PB1 and PA. RdRp with NP are the minimal components required for viral replication and transcription [237]

2.2.ii. Results

2.2.ii.a The WD and linker domains of ATG16L1 are required for non-canonical autophagy in both phagocytic and non-phagocytic cells.

The studies described previously in chapter 2.1 have shown that the WD domain of ATG16L1 was required for LAP in phagocytic cells. During the course of these studies a non-canonical pathway called LANDO was described in non-phagocytic cells. This may be related to the pathway described by Oliver Florey and colleagues where LC3 is recruited to endosomes and lysosomes in tissue culture cell lines incubated with lysosomotropic drugs. The first part of this study was designed to determine if LANDO-like pathways could be detected in MEFs isolated from mice and that these pathways were defective in cells from δ WD mice. As described in chapter 2.1, MEFs and BMDMs from littermate control mice expressed full-length α and β isoforms of ATG16L1 at 66, 68 kDa (Figure 2.2.2, A, western), and generated PE-conjugated LC3II during recruitment of LC3 to autophagosomes following starvation in HBSS (Figure 2.2.2, C, left panel). The MEFs also recruited LC3 to endo-lysosome compartments swollen by monensin (Figure 2.2.2, A, left panel) indicative of a LAP-like pathway in non-phagocytic cells. Bone marrow-derived macrophages (BMDM) from the control mice also activated LAP to recruit LC3 to phagosomes containing zymosan monensin (Figure 2.2.2, A, right panel). MEFs from δ WD mice expressed a truncated ATG16L1 at 30 kDa (Figure 2.2.2, B, western). BMDM from δ WD mice generated LC3II and autophagosomes in response to starvation (Figure 2.2.2, C, right panel) but failed to recruit LC3 to swollen endo-lysosome compartments from monensin treatment or phagosomes containing zymosan. This data confirms a defect in non-canonical autophagy and LAP in both phagocytic and non-phagocytic cells in the δ WD mice [220].

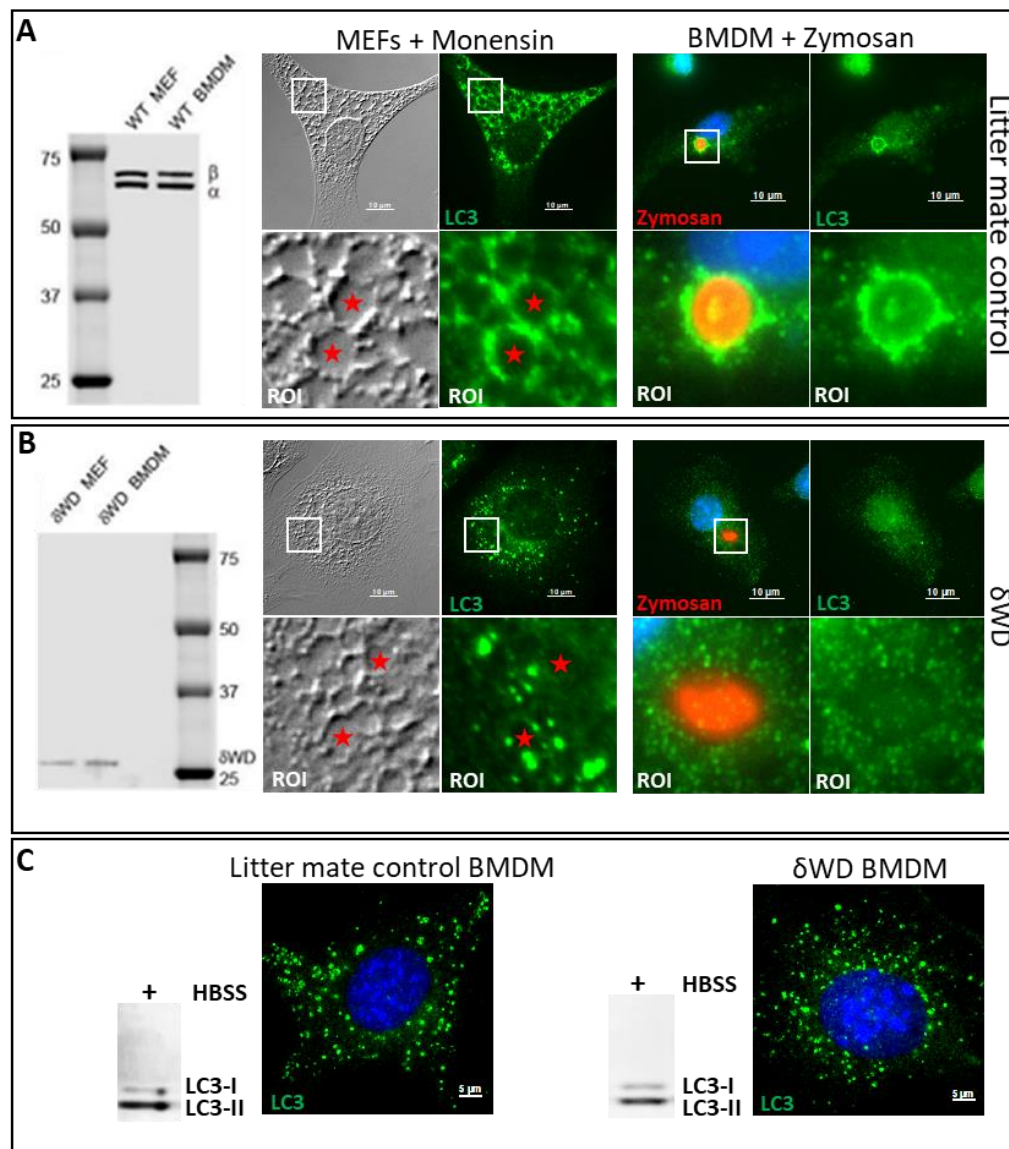


Figure 2.2.2. Deletion of the WD domain of ATG16L1 causes loss of non-canonical autophagy and LAP but retains autophagy. **A.** Western: WT MEFs and BMDMs from littermate control express α and β isoforms of ATG16L1 at 68, 66 kDa. **A.** MEFs left panel: LC3 (green) is recruited to endo-lysosomes following induction of non-canonical autophagy by monensin. BMDM right panel: LC3 (green) is recruited to phagosomes following engulfment of zymosan by BMDMs. **B.** Western: MEFs and BMDMs from Δ WD mice express a 27 kDa truncated ATG16L1. **B.** Left panel: MEFs do not recruit LC3 (green) to endo-lysosomes following incubation with monensin. Right panel: BMDMs do not recruit LC3 (green) to phagosomes containing zymosan. **C.** Left western: WT BMDMs convert LC3I to LC3II and generate LC3 puncta during autophagy induced by HBSS. **C.** Right western: BMDMs from Δ WD mice still convert LC3I to LC3II and generate LC3 puncta following autophagy induced by HBSS [220] Imaging and westerns by Jefferson et al [220].

2.2.ii.b Mice with systemic loss of the WD and linker domains of ATG16L1 are highly sensitive to IAV infection.

IAV enters airway and lung epithelial cells by endocytosis, and in tissue culture IAV induces non-canonical autophagy leading to ATG16L1-WD domain-dependent conjugation of LC3 to the plasma membrane and perinuclear structures. To test whether non-canonical autophagy has a host defence function in the host *in vivo*, δ WD mice were infected with IAV. We used a low-pathogenicity murine-adapted IAV H3N1, X31 that does not normally lead to extensive viral replication throughout the lungs, or cause the cytokine storm and death typically seen after infection with highly pathogenic viral strains H1N1, PR8 [251] [241]. The results (Figure 2.2.3) showed that δ WD mice declined drastically and showed severe signs of clinical illness (rapid breathing, piloerection). They also displayed rapid weight loss compared to littermate controls. Littermate controls nearly lost 20% body weight, while δ WD mice showed 27% loss weight loss by day 7 (Figure 2.2.3, A). The δ WD mice had increased mortality with survivors recovering more slowly from infection, when compared to littermate controls that suffered no losses and recovered faster. The sharp decline in δ WD mortality is a direct result of weight loss, and mice reaching 30% weight loss were culled in accordance with home office regulations (Figure 2.2.3, B). The increased weight loss was associated with an approx. log increase in lung virus titre at 5 days post-infection (d.p.i.) (Figure 2.2.3, C). Histopathology and immunohistochemistry (IH) analysis of lungs from δ WD mice showed severe viral pneumonia with large numbers of IAV-positive cells (Figure 2.2.3, D) [220].

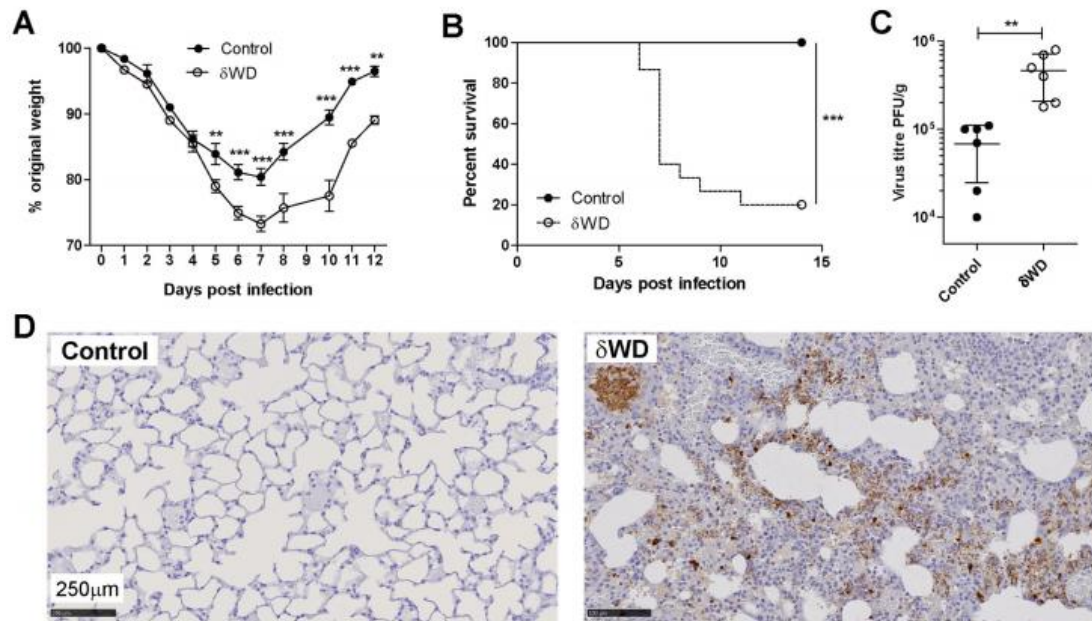


Figure 2.2.3. Systemic loss of non-canonical autophagy increases susceptibility to IAV infection. Littermate control and δ WD mice were challenged intra-nasally with IAV strain X31 (103 pfu). **A.** Mice were monitored for weight loss at indicated time-points. (n = 8). Data represent the mean value $\pm$ SEM. Comparisons were made using a repeated-measures two-way ANOVA (Bonferroni post-test). **B.** Survival was assessed at indicated time points (n = 15). Comparisons were made using log-rank (Mantel-Cox) test. **C.** IAV titre in lungs was determined by plaque assay at 5 d.p.i. (n = 6). Data for individual animals are shown, bars represent the mean $\pm$ SD. Mann Whitney U test was used to determine significance. **D.** The presence of IAV antigen was assessed by IH at 7 d.p.i. (representative images from n = 6). This representation of IAV challenge in δ WD mice was performed by Parul Sharma and James Stewart at the University of Liverpool. Weight loss data was from Parul Sharma. Virus titre was performed by Yingxue Wang. Histology was performed by Anja Kipar [220].

2.2.ii.c Generating a mouse model lacking the WD domain of ATG16L1 in phagocytic cells to determine which cell type is important for protection against IAV infection.

The role of the WD domain in ATG16L1 during IAV infection was demonstrated in Figure 2.2.3 where an increase in clinical illness and IAV lung titre was shown. The δ WD mouse model expresses the truncated form of ATG16L1 in all cells and tissues of the mouse. During IAV infection, many cells types will come into contact with the virus, including lung epithelial cells, macrophages, neutrophils and dendritic cells. To determine which cell type may play an important role in controlling IAV infection by non-canonical autophagy/LAP, a myeloid cell specific δ WD mouse (δ WD^{phag}) was generated using a mouse with myeloid cell-specific expression of Cre recombinase purchased from Jackson labs [252]. The Cre expression is under the control of the murine lysozyme 2 gene (*Lyz2*) promoter that is specific to cells of myeloid lineage, including macrophages, monocytes, and granulocytes [252]. When crossed with a strain containing a sequence of interest that has been flanked with *loxP* sites, Cre-mediated recombination results in a deletion of that sequence in a targeted gene. This system would enable a specific knock out of the WD domain in myeloid cells only allowing remaining cells and tissues to express full length ATG16L1. This would enable us to determine the role of the WD domain in phagocytic cells during IAV infection [220].

2.2.ii.d Genome mapping and PCR primers for Atg16L1 Strains

The Cre-LoxP system is an extremely important tool when generating tissue specific or knockout mouse models. When generating a Cre-LoxP tissue specific or knockout models you need mice that are either heterogeneous *loxP*-flanked on one allele with the other allele carrying your gene of interest with the transgene Cre. Or for a full knockout both alleles must be flanked with *loxP* and Cre recombinase positive [253]. The parental mouse models used in this study to generate the δ WD^{phag} were generated based on a Cre-LoxP system designed by Maryam Arasteh a previous member of our group [219]. An *Atg16L1*^{fl/fl} mouse was made where exon 2 of *Atg16L1* was flanked by *loxP* sites. Removal of exon 2 leads to a frame shift resulting in a premature stop codon [219] (Figure 2.2.4, ii). Primer set 223 and 226 are either side of the two *loxP* sites, resulting in a 801 bp band for *Atg16L1*^{fl/fl} mice when compared to the wild type *Atg16L1* strain of 654 bp (Figure 2.2.4, i). The δ WD mouse was designed with an insertion of a stop codon within exon 6 of *Atg16L1* that is covered by primers 290 and 291. Without the insertion this would produce a 291 bp product but with the insertion a 639 bp product is produced, indicating that the mouse was a δ WD (Figure 2.2.4, iii) [220].

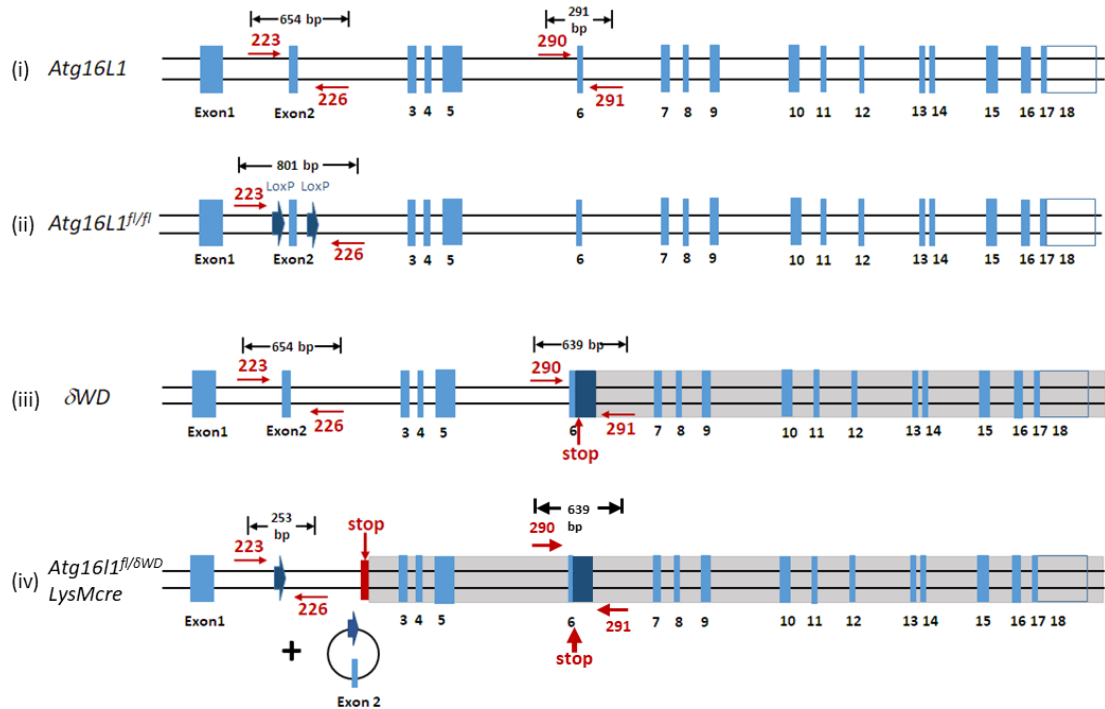


Figure 2.2.4. Genome map of wild type *Atg16L1* and derivatives to show PCR primer binding sites to characterise δ WD^{phag} for genotyping. (i) Wild type allele *Atg16L1* is identified using primers flanking exon 2 (223, 226) and exon 6 (290 and 291). (ii) The *Atg16L1^{fl/fl}* *loxP* sites flanking exon 2 in *Atg16L1* increase the PCR product of exon 2 from 654 bp to 801 bp. Cre-recombinase expressed by the *Atg16L1* leads to removal of exon 2 by Cre recombinase reducing the PCR product of exon 2 from 801 bp to 253 bp. (iii) The δ WD allele was generated by inserting a cassette containing a stop codon into exon 6. The insertion increases the size of the frame. Increasing the PCR product of exon 6 from 291 bp to 639 bp. (iv) Crossing of mouse strains *Atg16L1^{fl/fl}* and δ WD mouse that contains a Cre transgene (not depicted). PCR products generated from primer sets 223 and 226 are a 801, 654, 253 bp. Primer design and mouse models *Atg16L1^{fl/fl}* and δ WD were designed generated by Maryam Arasteh and Ulrike Mayer [220].

2.2.ii.e Genetic background and breeding of the δ WD LysM-Cre mouse model (δ WD^{phag}).

To investigate the role of LAP in myeloid cells only, LysM-Cre/*loxP* system was used to generate mice with specific loss of the *Atg16L1* allele in myeloid cells macrophages, monocytes, and granulocytes [252]. A homozygous δ WD ^{δ WD/ δ WD} mouse was first crossed with a LysM-Cre model mouse [252]. The LysM-Cre mouse carries both full length *Atg16L1* alleles and all offspring generated were heterozygous δ WD ^{δ WD/*Atg16L1*-LysM-Cre}. The δ WD ^{δ WD/*Atg16L1*-LysM-Cre} mouse was then backcrossed with a homozygous δ WD ^{δ WD/ δ WD} mouse to make a homozygous δ WD ^{δ WD/ δ WD-LysM-Cre}, removing the full length *Atg16L1*. To generate the myeloid specific mouse model termed δ WD^{phag} mouse, a crossing of mouse strains *Atg16L1*^{fl/fl} [219] with the homozygous δ WD ^{δ WD/ δ WD-LysM-Cre} was performed (Figure 2.2.5). When the δ WD ^{δ WD/ δ WD-LysM-Cre} was crossed with the homozygous *Atg16L1*^{fl/fl}, two genetic outcomes are generated. All pups will be heterozygous δ WD ^{δ WD/*Atg16L1*-fl/fl} but only a proportion will carry the LysM-Cre. Mice that are δ WD ^{δ WD/*Atg16L1*-fl/fl} LysM-Cre positive will have the Cre recombinase in myeloid cells, leading to the removal of the *loxP* flanked *Atg16L1* leaving one δ WD on the other allele. One δ WD allele is sufficient for autophagy but not LAP, generating a specific LAP knockout in myeloid cells only, termed the δ WD^{phag} mouse. In LysM-Cre negative mice, the *loxP* flanked *Atg16L1* is not excised from the genome and will express full length *Atg16L1* and was used as a littermate control [220].

δ WD^{phag} mice: Cre recombinase is only activated in myeloid cells of δ WD^{phag} mice

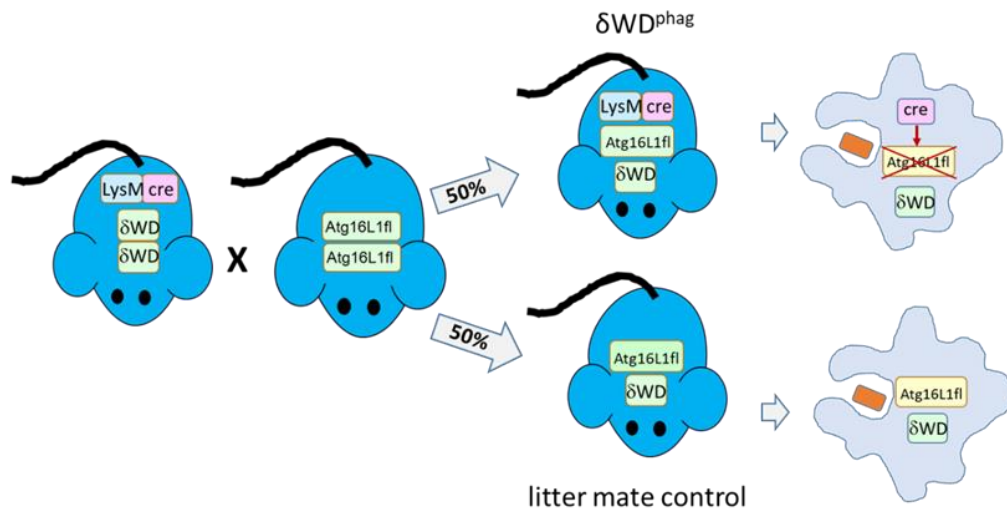


Figure 2.2.5. Generation of δ WD^{phag} mice breeding strategy. Homozygous δ WD mice carrying LysM-Cre were crossed with *Atg16L1*^{fl/fl} mice. 50% of the progeny are *Atg16L1*^{fl/ δ WD} and carry LysM-Cre (δ WD^{phag}). The other 50% are *Atg16L1*^{fl/ δ WD} and don't carry LysM-Cre, that are referred to as the littermate controls. Cre recombinase expressed in myeloid cells of these mice inactivates *Atg16L1* by removing exon 2 from *Atg16L1*^{fl/ δ WD} making the mouse the final δ WD^{phag}. The myeloid cells only express δ WD. Cre recombinase is not expressed in non-myeloid tissues and *Atg16L1* is preserved to power autophagy. 50% of progeny provide littermate controls because they lack LysM-Cre and preserve *Atg16L1* in all tissues. Mouse models *Atg16L1*^{LysM-Cre/ δ WD} *Atg16L1*^{fl/ δ WD} were generated jointly by myself and Weijiao Zhang. Figure design Yingxue Wang [220].

Confirmation of the correct genetics for the $\delta\text{WD}^{\text{phag}}$ from the breeding was carried out using the primer design system discussed in Figure 2.2.4 [219]. An example of genotyping mice to identify $\delta\text{WD}^{\text{phag}}$ and littermate control mice that lack Cre (Figure 2.2.6, A). DNA samples were processed from tail tissue and BMDMs (M ϕ) from the same experimental mouse. Using primer set 223 X 226 to detect the presence of full length *Atg16L1* or the δWD insertion. Lane 1 and 3 shows a PCR product for the *Atg16L1*^{fl/fl} allele at 800 bp in both tail and M ϕ DNA samples as well as a PCR product for δWD at 654 bp. Primer set 15B X 19B was used to identify the presence of the Cre gene. This mouse didn't produce a PCR product for Cre meaning it is a littermate control. In lane 2 and 4 a PCR product for the *Atg16L1*^{fl/fl} allele at 800 bp in both tail and M ϕ DNA samples was seen as well as a PCR product for δWD at 654 bp. More importantly a 253 bp PCR product is seen in the M ϕ of lane 4 that also corresponds to the presence Cre, identified by a 600 bp PCR product. This indicates that this is a $\delta\text{WD}^{\text{phag}}$ mouse where exon 2 is being removed only in the M ϕ sample only to produce the 253 bp PCR product (Figure 2.2.6, A).

To compare how the genetics of the mouse translated in terms of protein and tissue specificity, expression of ATG16L1 and δWD was determined. Skin fibroblasts and BMDM were isolated from the same littermate control and $\delta\text{WD}^{\text{phag}}$ mice observed from the DNA profile, then analysed by western blot. Skin fibroblasts and BMDM from littermate control mice lacking Cre express full length ATG16L1 at 68, 66 kDa and the truncated δWD at 27 kDa. The $\delta\text{WD}^{\text{phag}}$ mice that express Cre was indicated by the removal of full length ATG16L1 from BMDM but not skin fibroblasts also confirming that δWD was only expressed in myeloid cells (Figure 2.2.6, B) [220].

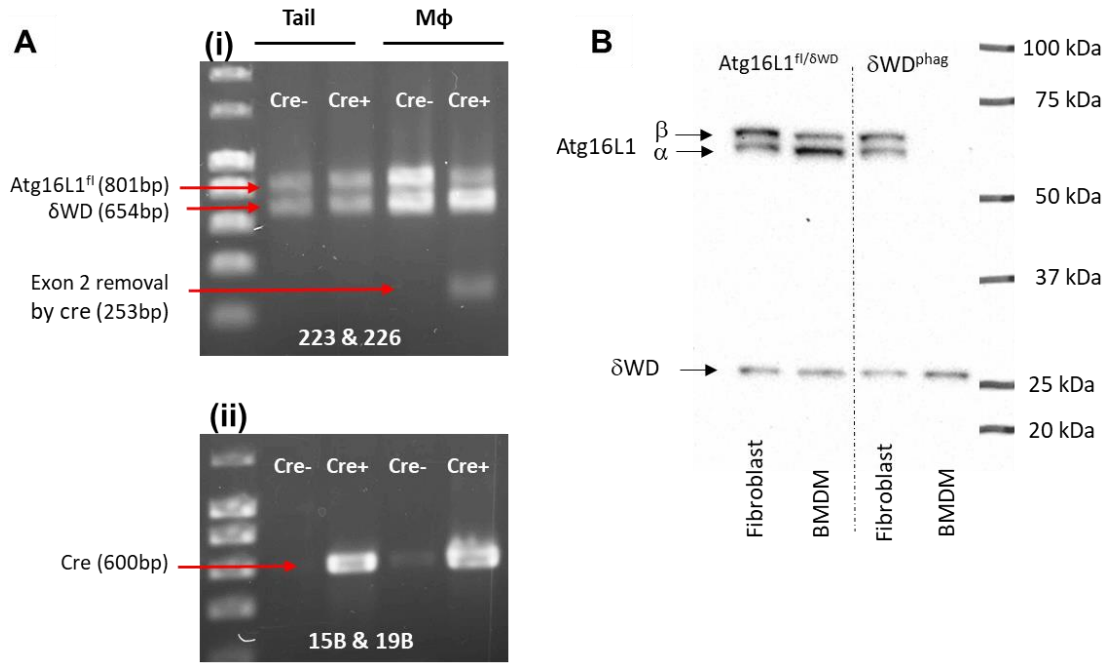


Figure 2.2.6. Genotyping $\delta\text{WD}^{\text{phag}}$ mice. **A.** DNA extracted from mouse tail tissue and bone marrow derived macrophages (MΦ) from the same mice was analysed by PCR. **(i).** Samples from littermate controls (Cre-) and $\delta\text{WD}^{\text{phag}}$ mice (Cre+). The 801 bp and 654 bp products from PCR primers 223 X 226 corresponding to the *Atg16L1^{fl}* allele and *Atg16L1^{δWD}* allele. The 253 bp PCR product seen in macrophage DNA of cre+ $\delta\text{WD}^{\text{phag}}$ strains indicates specific removal of exon 2 from *Atg16L1* in myeloid cells. **(ii).** 15B X 19B PCR primers verify presence of Cre recombinase (Cre+). **B.** Tissue specific expression of *ATG16L1* and δWD . Skin fibroblasts and BMDM were isolated from the same littermate controls and $\delta\text{WD}^{\text{phag}}$ mice, then analysed by western blot. Skin fibroblasts and BMDM from littermate control mice lacking Cre- (*Atg16L1^{fl/δWD}*) express full length *ATG16L1* at 68, 66 kDa and the truncated δWD at 27 kDa. The $\delta\text{WD}^{\text{phag}}$ mice express Cre+ indicated by the removal of full length *ATG16L1* from BMDM but not skin fibroblasts. Genotyping and western by Jefferson et al [220].

2.2.ii.f In vitro characterisation of non-canonical autophagy/LAP in cells from $\delta\text{WD}^{\text{phag}}$ mice

Non-canonical autophagy/LAP was characterised in $\delta\text{WD}^{\text{phag}}$ mice using skin fibroblasts and BMDMs isolated from littermate controls and $\delta\text{WD}^{\text{phag}}$ mice. Skin fibroblast cells from both control and $\delta\text{WD}^{\text{phag}}$ mice were treated with monensin for 2 hrs to activate non-canonical autophagy. In littermate control and $\delta\text{WD}^{\text{phag}}$ fibroblasts, LC3 can be visually seen as being recruited to swollen endo-lysosomal membranes (Figure 2.2.7, A, B). Indicating that non-canonical autophagy was active in fibroblasts isolated from littermate control and $\delta\text{WD}^{\text{phag}}$ mice. To demonstrate the presence of LAP, BMDMs isolated from littermate control and $\delta\text{WD}^{\text{phag}}$ mice were incubated with labelled Zymosan. LAP was confirmed in BMDMs from littermate control mice. Zymosan particles had been taken up by the control macrophages and encapsulated within phagosomes that were LC3 positive (Figure 2.2.7, C). BMDMs from $\delta\text{WD}^{\text{phag}}$ mice showed that the macrophages had taken up the Zymosan particles but failed to recruit LC3 to phagosomes. LC3 remained as small cytoplasmic puncta and not as an LC3 positive ring around the Zymosan (Figure 2.2.7, D) [220].

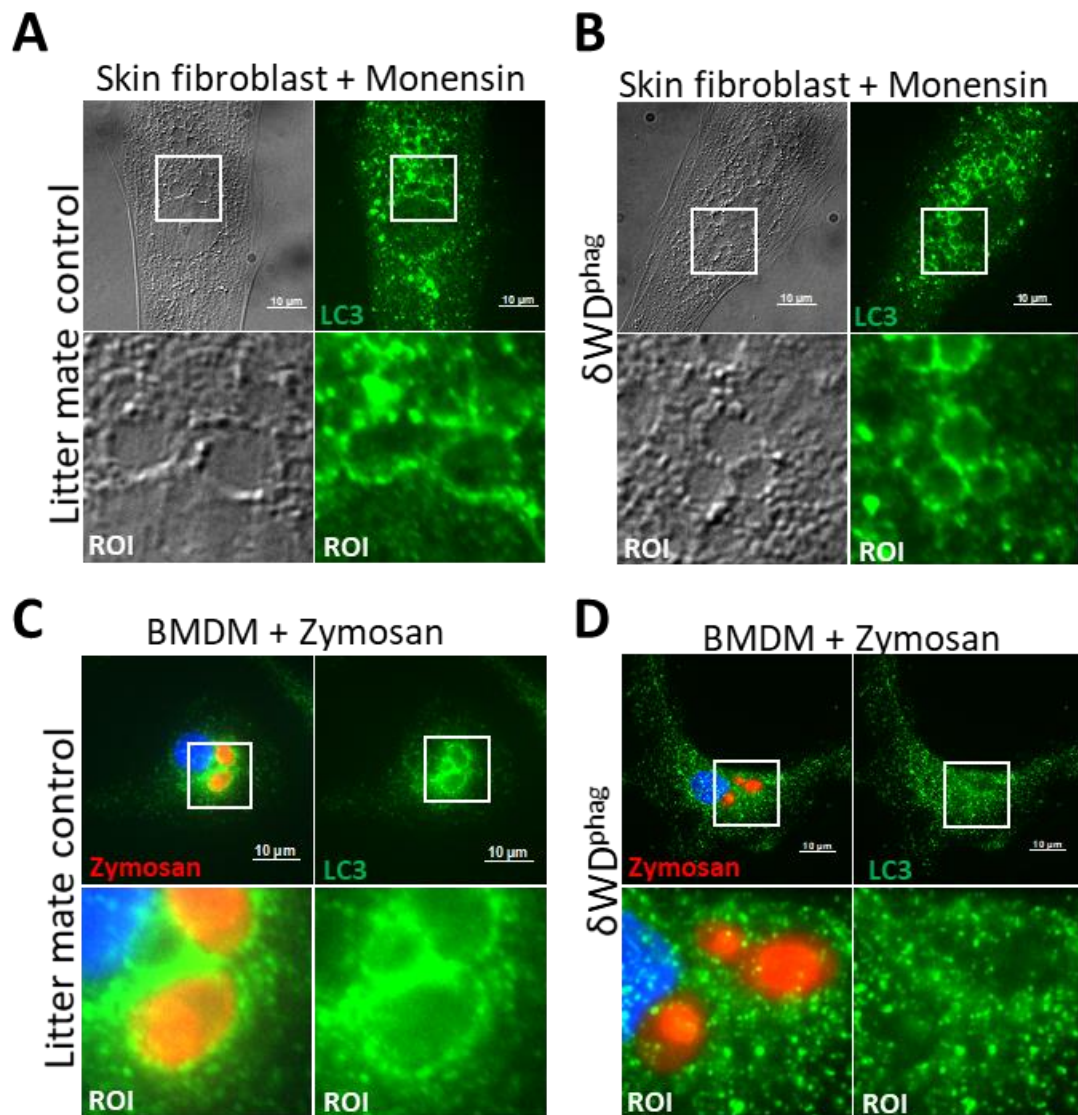


Figure 2.2.7. Functional analysis of non-canonical autophagy and LAP in cells from $\delta\text{WD}^{\text{phag}}$ mouse line. A-B: non-canonical autophagy. Skin fibroblasts isolated from $\delta\text{WD}^{\text{phag}}$ and litter mate controls were incubated with monensin for 2 hrs to induce non-canonical autophagy. Immunostaining of LC3 using Alexa Fluor 488 green was used to show swollen vesicles an indicator of non-canonical autophagy. **C-D: Analysis of LAP.** BMDMs isolated from $\delta\text{WD}^{\text{phag}}$ mice and litter mate controls were incubated with Zymosan for 30 min, fixed and immunostained for LC3 with Alexa Fluor 488 green. LAP was indicated by the recruitment of LC3 to phagosomes containing Zymosan Alexa Fluor 594-labelled. Imaging by Jefferson et al [220].

2.2.ii.g The WD domain and linker domains of ATG16L1 limit IAV infection independently of phagocytic cells

The $\delta\text{WD}^{\text{phag}}$ mice were challenged with IAV to determine if non-canonical autophagy/LAP in myeloid cells provided a barrier to IAV infection. After infection with IAV, $\delta\text{WD}^{\text{phag}}$ mice showed comparable weight loss and virus titres to those seen in littermate control mice (Figure 2.2.8, A and B). This is the opposite as to what was seen in the δWD where severe weight loss and morbidity was caused by IAV infection. The sensitivity of δWD mice to IAV seen in earlier experiments was not therefore due to the loss of non-canonical autophagy from myeloid cells, making it likely that the activity of the WD and linker domains of ATG16L1 protect against lethal IAV infection in non-myeloid tissue [220]. Taken together the results demonstrate for the first time that the WD and linker domains of ATG16L1 allow non-canonical autophagy to provide a novel innate defence mechanism against lethal IAV infection within the epithelial barrier in vivo [220].

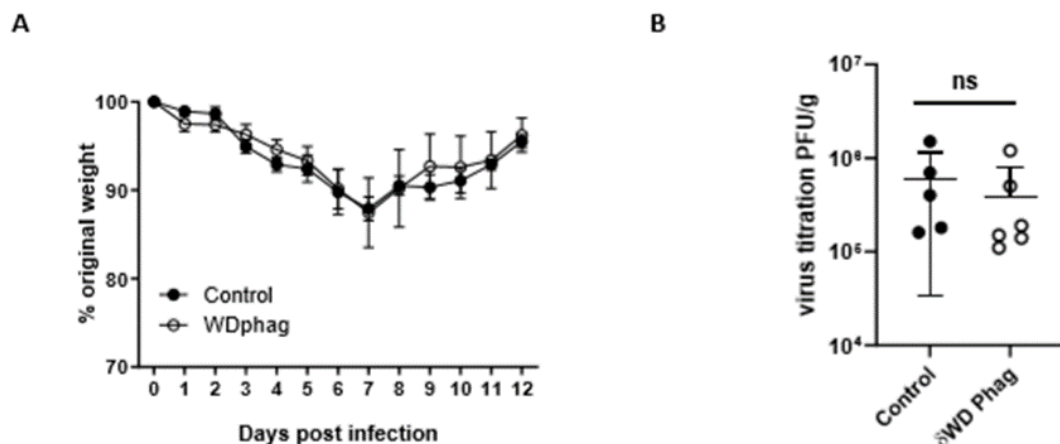


Figure 2.2.8. Non canonical autophagy suppresses IAV replication independently of LAP in phagocytes in vivo. $\delta\text{WD}^{\text{phag}}$ mice lack non-canonical autophagy in myeloid cells. Offspring negative for LysM-Cre were used as littermate controls. Mice (n = 6 per group) were challenged intranasally with IAV X31 (103 pfu). **A.** Mice were monitored for weight loss at indicated time-points. Data represent the mean value $\pm$ SEM. Comparisons were made using a repeated-measures two-way ANOVA (Bonferroni posttest). **B.** IAV titre in lungs was determined by plaque assay at 5 d.p.i. (n = 6). Data for individual animals are shown, bars represent the mean $\pm$ SD. Mann-Whitney U test was used to determine significance. This representation of IAV challenge in $\delta\text{WD}^{\text{phag}}$ mice was performed by Parul Sharma and James Stewart at the University of Liverpool. Virus titration was performed by Yingxue Wang [220].

2.2.iii. Discussion

We have generated two mouse models to study the role played by non-canonical autophagy during infection *in vivo*. The δ WD mice lacking the linker and WDs domains of ATG16L1 were characterised as shown in the first part of this chapter. These mice maintained tissue homeostasis, grew normally and had normal levels of autophagy [187] and were therefore suitable for infection studies. Further analysis of the δ WD mice by members of our group revealed that these mice do not have a pro-inflammatory phenotype, as seen in other non-canonical deficient models like the Rubicon knockout model [207]. In contrast to littermate control mice, δ WD showed an intense sensitivity to IAV with almost a tenfold increase in viral lung titre and as a result of the severe infection, dramatic weight loss was experienced giving rise to high morbidity rates. Pathology of the mice indicated extensive viral replication throughout the lungs, fulminant pneumonia and lung inflammation. These severe contributing factors is what lead to the high mortality and death rates that are generally seen after infection with more virulent strains [254]. These symptoms are indicative of what is seen with highly pathogenic strains of IAV such as the 1918 'Spanish' Influenza. Data not shown here but a dysregulation of cytokines was also seen in the δ WD mice to levels that are usually seen during cytokine storms, another contributory factor causing death [220].

The role played by LAP in myeloid cells in protection against IAV infection was determined using the δ WD^{phag} mouse that only expresses the δ WD allele in cells of myeloid lineage (macrophages, dendritic cells, neutrophils, granulocytes and lymphocytes), while the remaining cells and tissues express full length ATG16L1 [220]. The specific loss of LAP in phagocytic cells was confirmed using *in vitro* LAP and autophagy assays in skin fibroblasts and BMDM. The δ WD^{phag} mice failed to show an increase in sensitivity to IAV infection and were comparable with control mice and suggested that LAP in myeloid cells does not protect against infection. Importantly the results suggested for the first time that non-canonical autophagy in cells other than phagocytes can protect the airway against IAV infection. This was an unexpected observation. Activation of non-canonical autophagy in phagocytic cells leads to LAP where TLR signalling and reactive oxygen species (ROS) recruit LC3 to phagosomes would provide a powerful way to remove viruses [220]. Many *In vitro* studies have shown the importance of LAP in phagocytes in response to pathogens for example, activation of TLR2 and NOX2 by *Legionella dumoffii* translocation of LC3 to single-membraned vacuole [255]. Or *Listeria monocytogenes* activates acid sphingomyelinase

leading to recruitment of LC3 to phagosomes [256]. LAP is such a pivotal tool in the defence against pathogens that the GP63 metalloprotease of *Leishmania major* and melanin of *Aspergillus fumigatus* prevent recruitment of NOX2 to phagosomes to inhibit LAP [220, 257-259]. Maybe the difference we see in response to a pathogen is due to the fact that these studies have focused on microbes *in vitro* with macrophages whereas our *in vivo* viral model of IAV encounters epithelial barriers.

When IAV is administered to the mice intranasally it results in the rapid infection of predominantly airway and pulmonary epithelial cells [260]. Further work has been conducted with our δ WD mouse to determine if the epithelium is responsible for providing the barrier for IAV infection with non-canonical autophagy. Fibroblasts and epithelial cells establish themselves as the primary physical barrier of the host against a variety of pathogens. Due to the front line nature of these cells they are equipped with an array of innate immune signalling pathways and cytoplasmic sensors to engage with host-pathogen interactions [261]. IAV infections in *ex vivo* experiments on precision-cut lung slices and MEFs from δ WD mice gave a 5-10 fold greater interferon responses and virus titres [220]. This can be further clarified in the future when airway epithelial cells can be derived from the δ WD mice and grown in culture. It might be feasible that non-canonical autophagy in epithelial cells is able to reduce IAV replication by increasing transfer of endocytosed virus to lysosomes for degradation. Transmembrane protein TMEM59 was identified as containing a WD domain binding motif that binds to ATG16L1 and facilitates increased fusion with lysosomes by additional recruitment of LC3 onto the same single-membrane endosomes where TMEM59 is present. This promotes a more efficient lysosomal targeting of endo-lysosome compartments [224]. It might be possible the non-canonical autophagy system has evolved at epithelial barriers for a fast and effective innate mechanism for new pathogenic strains of viruses, as in the case for IAV emerging strains. Particularly useful where there is no acquired immunity from the previous strains. It would be interesting to see how our ATG16L1 variants would react to different strains of influenza and if the same or different responses were seen. Also if the outcomes could be changed with targeted use of drug treatment on non-canonical autophagy to increase resistance at the epithelial barrier [220].

2.3. Summary of Generation of a mouse model to study the role played by non-canonical autophagy during viral infection papers

Pandemic respiratory virus infections are a huge concern at a time when the majority of the global population live in densely populated towns and cities. Respiratory viruses like Influenza A Virus (IAV) and Severe Acute Respiratory Syndrome (SARS) virus have accounted for all the major pandemics in the last century and there is an urgent need for a better understanding of how the airways defend against these viral infections. Both IAV and SARS cause respiratory infections that can progress to aggressive inflammatory responses where a cytokine storm and viral pneumonia can result in high mortality rates. Recent work using cells in culture has shown that pathogens entering cells by endocytosis can activate a non-canonical autophagy pathway called LC3-associated endocytosis (LAP) where endosomes are labelled with autophagy protein LC3 and transported to the lysosome for degradation. The C-terminal WD domain of autophagy protein ATG16L1 is required for non-canonical autophagy/LAP. In this study two stop codons were introduced into the ATG16L1 gene of mice to prevent translation of the WD domain. This mouse model was termed δ WD and used to determine the role played by LAP/non-canonical autophagy during IAV infection of the airways. The δ WD mice grew at comparable rates to their littermate controls and were fertile. The liver, brain, kidney and muscle of δ WD mice developed normally showing that mice can maintain tissue homeostasis independently of the WD domain. However, the δ WD mice were extremely sensitive to low pathogenicity IAV resulting in high viral lung titre, fulminant pneumonia and severe lung inflammation leading to high mortality. During IAV infection, many cells types come into contact with the virus including lung epithelial cells, macrophages, neutrophils and dendritic cells. A myeloid cell specific δ WD mouse (δ WD^{phag}) was made to determine if LAP in phagocytes provided a barrier to infection. The δ WD^{phag} mice showed no difference in IAV susceptibility from their littermate controls indicating that IAV infection was controlled independently of phagocytes. This suggests that LAP within non-phagocytic cells, such as the airway epithelium, restricts IAV infection to prevent lethal inflammation 'in vivo'.

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