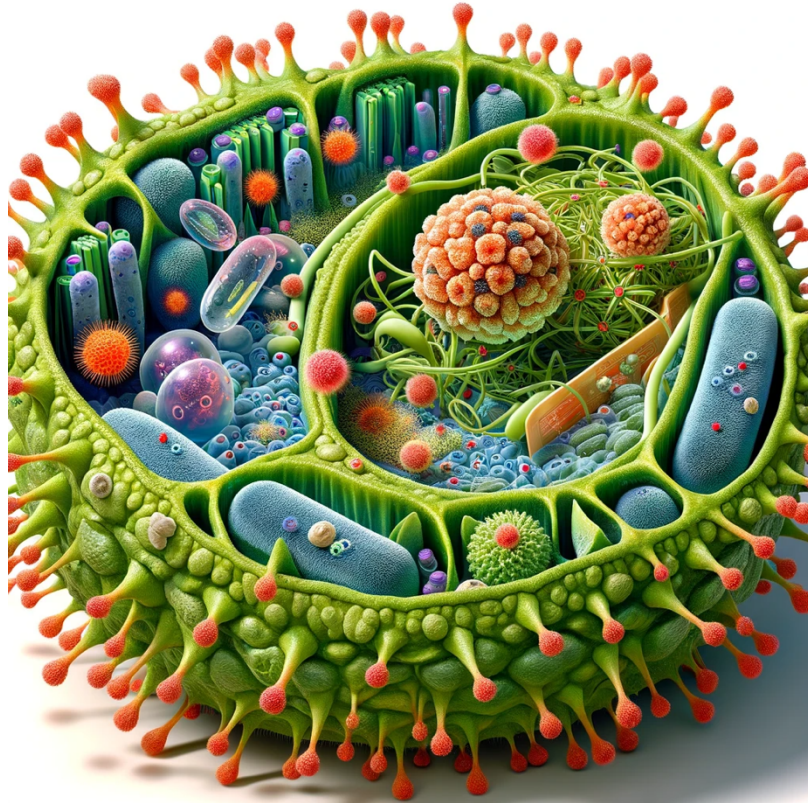


Characterization of molecular mechanisms governing selective autophagy



title prompt - DALL·E

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the degree of

Doctor of Philosophy

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Abstract

This thesis delves into the molecular underpinnings of autophagy, a critical cellular mechanism for maintaining cellular homeostasis and defence against pathogens, through the lens of novel protein-protein interactions and structural insights. We pioneer the use of the AF2-multimer protein modelling approach to accurately identify both canonical and non-canonical AIM/LIR motifs, crucial for autophagy receptor and adaptor function. This innovative methodology not only sheds light on the interaction between ATG8/LC3 proteins and various autophagy components but also highlights its potential in uncovering ATG8-LIR/AIM associations across kingdoms, thus offering new perspectives on the cross-talk between autophagy pathways and pathogen virulence factors. Exploration into the ribosomal protein RPLP2 unveils its unexpected role within autophagic dynamics, characterized by a novel non-canonical binding motif facilitating its interaction with ATG8. This interaction points to RPLP2 as a potential selective receptor in ribophagy, expanding our understanding of autophagy's selectivity and complexity. Moreover, the application of the AF2-multimer prediction pipeline to viral proteomes reveals diverse strategies by which viral effectors can manipulate host autophagy, providing insights into the intricate dance between host defence mechanisms and viral evasion strategies. Lastly, the thesis presents a comprehensive study on Joka2, a selective cargo receptor in plants, elucidating its structure-function relationships through AlphaFold2 predictions, circular dichroism spectroscopy, and negative stain electron microscopy. The challenges encountered in structural analysis underscore the dynamic nature of autophagy-related proteins and the complexity of achieving high-resolution structural data. Together, our results contribute significantly to the understanding of autophagy's molecular basis, offering novel insights into the structural and functional nuances of autophagy components and their interactions with pathogens. This work not only broadens our comprehension of autophagy but also opens avenues for future research in cellular biology, biochemistry and structural biology.

Declaration of originality

I declare that this thesis is a result of my own work. Unless explicitly stated otherwise, all the data have been collected and analysed by me. Any work completed by others has been cited or acknowledged as appropriate. This work has not been previously submitted to this or any other institute for any degree, diploma, or other qualification.

Tarhan Ibrahim

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Abbreviations

ACR	autophagy cargo receptor
AF2	AlphaFold2
AF2-multimer	AlphaFold2-multimer
AI	artificial intelligence
AIM	ATG8-interacting motif
ATG	autophagy-related gene
ATI	ATG8-interacting
BNP	blue native PAGE
EHM	extra-haustorial membrane
ER	endoplasmic reticulum
ETI	effector triggered immunity
GG	Golden gate
HR	hypersensitive response
LDS	LC3-docking site
LIR	LC3-interacting motif
MSA	multiple sequence alignment
NSEM	negative stain electron microscopy
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen associated molecular patterns
PAS	phagophore assembly site
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PIKC3	phosphatidylinositol 3 kinase complex
PM	plasma membrane
PRR	pattern recognition receptors
PTI	PAMP triggered immunity
PVX	potato virus X
ROS	reactive oxygen species
RPM	rounds per minute
SA	salicylic acid
SDS	sodium dodecyl sulphate
sLIM	short linear interaction motif
SYMMV	soy yellow mosaic virus
SVBV	strawberry vein binding virus
TICV	tomato infectious chlorosis virus
TORC	target of rapamycin complex
TuMV	turnip mosaic virus
UPS	ubiquitin-proteasome system
VTMoV	velvet tobacco mottle virus
NLR	nucleotide-binding leucine-rich repeat receptors

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1. Introduction

Autophagy, an essential mechanism for all eukaryotes

As a highly regulated and conserved mechanism, autophagy is an indispensable tool for the maintenance of cellular life. Compared to the ubiquitin-proteasome system (UPS), autophagy is a cellular process of large-scale destruction. Autophagy targets larger substrates, such as damaged organelles and protein aggregates for recycling (Figure1). With implications in cellular homeostasis, growth and development, stress response and innate immunity, autophagy is crucial [1-3]. In mammals, impaired autophagic function can lead to diabetes, heart failure and a range of neurodegenerative diseases [4]. In cancer, the enhancement of autophagic activity can play a major role in disease development [5]. In plants, autophagy is a known target for plant viruses to encourage infection. However, this relationship can also be described as a “tug of war”, where the plant can defend itself against infectious pathogens by promoting autophagic degradation of effector proteins (Figure1) [6].

Due to its involvement in cellular health, autophagy is a major target for pharmacological interference. Modulators of upstream signalling events to induce or suppress autophagy initiation have been developed [7]. However, these modulators lack specificity to a particular autophagy core protein and have broader effects in the cell, which has hampered their clinical development [8]. The development of more specific and potent modulators of autophagy will enhance our ability to treat a wide variety of diseases. Specialised designs will allow tailoring of autophagy earlier, towards disease prevention.

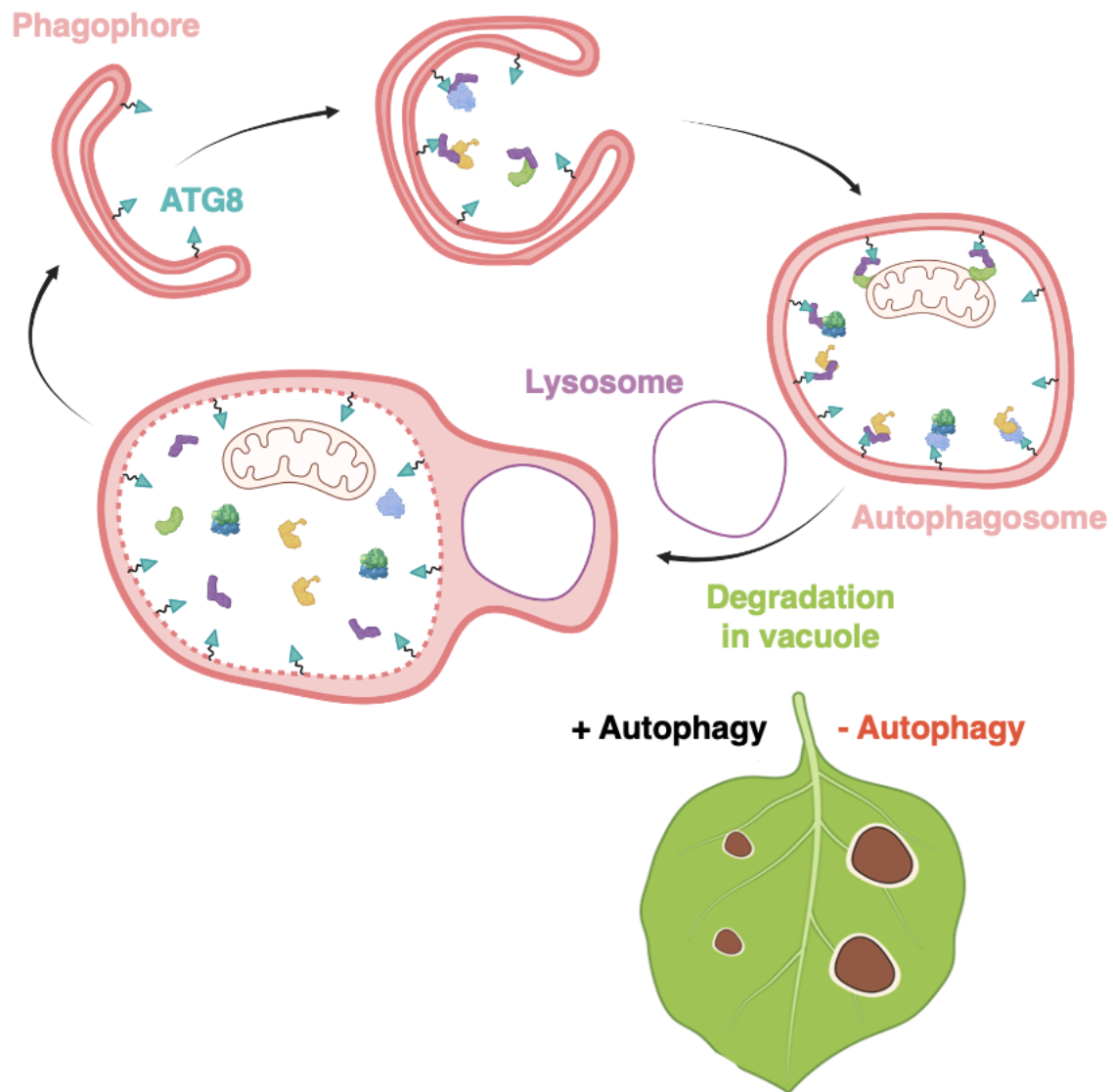


Figure1. Autophagy is a multi-step intracellular degradation pathway involved in cellular homeostasis. Upon autophagy activation, an isolation membrane, phagophore, is formed, which is decorated with ATG8. This structure sequesters cargo and simultaneously is supplied with membranes to mature into an autophagosome. In mammals, the autophagosome fuses with the lysosome to degrade internalised material. In plants and yeast, the acidic nature of the vacuole is utilised. In plants, autophagy is essential for immunity and in the absence of autophagy, enhanced pathogen growth is observed. Made using BioRender.

Autophagy relies on the successive recruitment of conserved machinery

Broadly, autophagy has been classified under two forms, micro- and macro-autophagy. Microautophagy is mediated by lysosomal action in mammals, and vacuolar action in plants, where cytoplasmic cargo is directly engulfed by membrane invaginations into these compartments. On the other hand, for macroautophagy, the site of sequestration of cargo is away from these organelles. It involves the formation of cytosolic vesicles that are trafficked to the degradation structures (Figure1) [9]. Macroautophagy is the main pathway and the most studied, hence from here on will be referred to as autophagy.

Autophagy is regulated by the autophagy-related (ATG) proteins. More than 30 are involved in the canonical pathway in yeast, where they were first discovered [10]. Most of the ATG proteins have orthologs in plants [11]. Autophagy is carried out in de novo formed, double-membrane vesicles called autophagosomes. Cytosolic cargo to be degraded are captured in these structures, which then fuse with the vacuole (in plants and yeast) or lysosome (in mammals) when fully matured. The enzymatic nature of these compartments destroys internalised cargo, involving autophagic machinery (Figure1). This process can be split into five steps, (1) development of the isolation membrane (phagophore), (2) capture of cellular components for cellular degradation, (3) phagophore elongation, (4) autophagosome maturation and (5) closure and fusion of autophagosomes [10, 12].

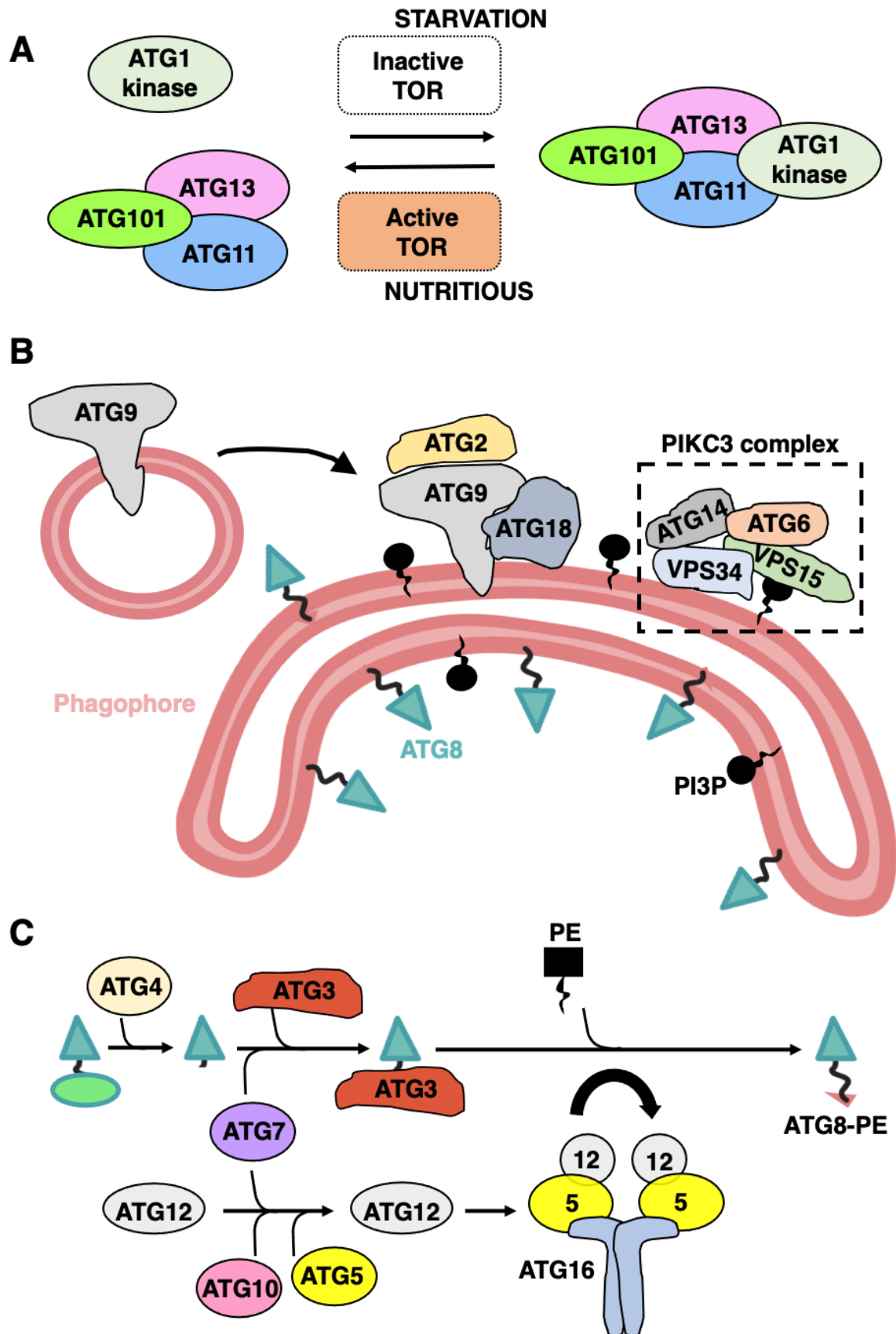


Figure2. Core autophagy proteins and their complexes in plants and animals.

(A) the ATG1-kinase complex. Under basal, nutritious conditions, TORC signalling is active, hence autophagy is suppressed through the disassociation of the ATG1-kinase complex. Upon starvation, TORC signalling is reduced, which allows phosphorylation of ATG1, which in return phosphorylates the ATG13-11-101 trimer and they form the active ATG1-kinase complex, which initiates phagophore development and further recruitment of ATG proteins. **(B)** The ATG9 transmembrane and PIKC3 complex at the phagophore. ATG9-trafficked membranes are delivered to the elongating phagophore through the formation of ATG9-18-2 transmembrane complex. This complex localises to the isolation membrane by interacting with PI3P, which is produced by the PIKC3 complex. This complex is formed by ATG14-6 and VPS34-15. **(C)** ATG8-PE conjugation systems. ATG4 removes a C-terminal “cap” of ATG8, which allows activation of ATG8 through ATG7. Activated ATG8 is then transferred to ATG3. ATG7, together with ATG10, is also responsible for the activation of ATG12-5-16 dimer complex. ATG3-ATG8 and this heterodimer yield a PE covalently linked to ATG8, which localises ATG8 to the isolation membrane. *Adapted from [13].*

Table1. Core autophagy proteins in their complexes and cross-kingdom nomenclature. *Adapted from [13].*

Plants	Mammals
<i>ATG1-kinase (ULK) complex</i>	
ATG1	ULK1/2
ATG13	ATG13
ATG11	FIP200
ATG101	ATG101
<i>ATG9-ATG2-ATG18 transmembrane complex</i>	
ATG2	ATG2A
ATG9	ATG9A
ATG18	WIP1/2
<i>PIKC3 complex</i>	
ATG6	BECN1
ATG14	ATG14L
VPS15	VPS15
VPS34	VPS34
<i>ATG8-PE conjugation systems</i>	
ATG3	ATG3
ATG4	ATG4A-D
ATG5	ATG5
ATG7	ATG7
ATG8	LC3A-C and GABARAPs
ATG10	ATG10
ATG12	ATG12
ATG16	ATG16L1

Initiation of autophagy (step 1)

Target of rapamycin (TOR) is a conserved serine/threonine kinase and forms the TOR signalling complex (TORC) [14]. TOR can form two structurally and physiologically distinct complexes, TORC1 and TORC2, and only TORC1 has been identified in plants. In *A. thaliana*, this complex is comprised of TOR, RAPTOR (regulatory-associated protein of TOR) and LST8 (lethal with SEC13 protein 8) [15]. Under nutrient-rich (basal) conditions, TORC signalling acts to prevent the induction of autophagy. TORC gets phosphorylated by RAPTOR-binding proteins, such as ribosomal protein S6 kinase β -1 (S6K1) [16], and in turn hyper-phosphorylates core autophagy proteins, namely ATG13, to prohibit nucleation of autophagy. Stress conditions, such as cellular starvation, trigger responses to reduce TORC activity, hence induce autophagy. This results in the formation of the ATG1-kinase complex (ULK complex in mammals) between dephosphorylated ATG1, ATG13, ATG11 and ATG101 [17], which recruits other ATG proteins to the phagophore assembly site (PAS) and forms the class III phosphatidylinositol 3-kinase (PIKC3) [18] and ATG2-ATG9-ATG18 complex [19, 20]. Phosphatidylinositol-3-phosphate (PtdIns-3-P) and PtdIns-4-P lipids are products of the PIKC3 complex and are present in the membrane that will eventually yield the phagophore. Through PtdIns-3-P (PI3P), more ATG proteins (ATG8-ATG9-ATG12 complex, ATG18) can localise to this membrane and drive nucleation (Figure2 and Table1– for ATG proteins and complexes) [21, 22].

Development and elongation of the phagophore (steps 2 and 3)

Nucleation is completed via the elongation of source membrane into the phagophore and the sequestration of cargo. The membranes of the phagophore and those required for the elongation and maturation have been a critical area of research. Current known

donors include the lipids from ER, COPII vesicles, endosomes, and the ATG9-system vesicles from the Golgi apparatus [23]. However, new sources of membrane, depending on the cargo being degraded, are still being uncovered. For example, using cryo-electron tomography, phagophore contacts with the nuclear and mitochondrial membranes were observed, suggesting a possible supply of lipids for elongation [24]. ATG8 and ATG12 are ubiquitin-like proteins and essential for the elongation process. ATG12 forms the dimeric ATG12-ATG5-ATG16 complex, which is an E3 enzyme recruited by PI3P [25]. Together with ATG3 and ATG4, the enzymatic complex is responsible for phosphatidylethanolamine (PE) conjugation to ATG8. ATG8-PE is inserted into the developing membrane, which allows for cargo recruitment and further ATG complexes being assembled (Figure2 and Table1) [26, 27].

Closure and fusion of the autophagosome (steps 4 and 5)

Although both utilise their vacuoles for degradation, yeasts and plants have different modes of autophagosome maturation. In yeast, the maturation happens near the autophagosome, and the fusion is facilitated through SNARE proteins and ATG8 working together [28, 29]. Using knock-out strains of fission yeast, Rop1 was identified as crucial for phagophore expansion into autophagosomes. In *rop1D* strains, induction of autophagy did not accumulate ATG8-decorated structures. Negative-stain electron microscopy demonstrated Rop1 formed lipoprotein particles of extreme curvature, hence suggesting its function relies on its ability to generate membrane curvature in the developing phagophore [30]. In plants, autophagosomes are formed all around the cell, therefore; require trafficking to the vacuole [31]. The mechanisms of trafficking and subsequent fusion remain unclear. A newly identified autophagy adaptor, CFS1, interacts with ATG8, on either side of the autophagosome membrane, and is essential

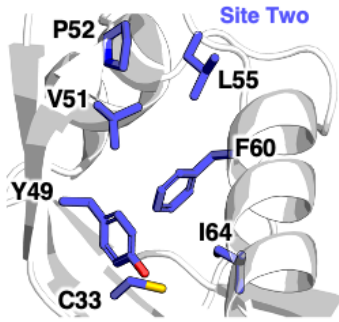
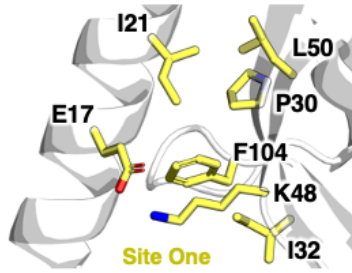
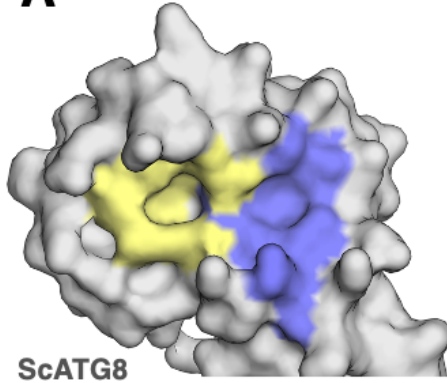
for the delivery to the vacuole. CFS1 also regulated autophagosome connections to components of the ESCRT complex. A disruption in these interactions in *A. thaliana* was seen to affect autophagic flux and render plants sensitive to stress [32]. Blocking lipidation of ATG8 in mammals leads to accumulation of open autophagic structures [33].

ATG8-family of proteins

Although yeast has only a single copy of ATG8, in plants (ATG8A–I) and humans (LC3/GABARAP family) ATG8 has diversified into multiple isoforms, forming family-specific clades [34]. For example, the Solanaceae family ATG8s cluster into four distinct well-supported monophyletic clades that do not contain any other plant families (Figure3). This family specific diversification of ATG8 is hypothesized to allow for further specificity in autophagic recycling and clearance, however, there are minor structural differences between isoforms and species (Figure3) [35]. Ubiquitin-like ATG8-family proteins play central roles in virtually all steps of autophagy, such as autophagosome development and fusion. Using overexpression assays, ATG8-PE was shown to drive autophagosome maturation [33]. Lipidated ATG8 is associated with the membrane and recruits non-ATG proteins, such as SH3P2 in *A. thaliana*, to promote phagophore elongation [11]. Further maturation into autophagosomes is facilitated by ATG4-mediated deconjugation of PE from ATG8. In mammals, ATG8 recruits PLEKHM1 to the autophagosome membrane and facilitates fusion with the lysosome [36]. Recently, ATG8 was discovered to have a non-canonical function in the recovery of Golgi apparatus under heat stress in plants [37].

Another crucial function of ATG8 is the recruitment of cargo into the developing autophagosomes for subsequent clearance. ATG8 is bound to autophagy cargo receptors (ACRs) via their ATG8/LC3-interacting motifs/regions (AIMs/LIRs), which are bound to ubiquitinated cargo at their ubiquitin associating (UBA) domains [38]. AIMs/LIRs, structurally described as short linear interaction motifs (sLIMs), occupy two pockets on the surface of ATG8 [39]. These pockets are described as hydrophobic pockets 1 and 2 (HP1, HP2) or W- and L- pockets, respectively. These pockets are formed by a set of residues that are conserved in all ATG8-family proteins (Figure3) [40]. The HP1 is generally more conserved than HP2 among the ATG8 members. HP1 is formed by residues found in $\alpha 2$ α -helix and β -strands $\beta 1$ and $\beta 2$. The same β -strands together with $\alpha 3$ and $\alpha 4$ helices form the second pocket [41]. Residues in both pockets include a mixture of all amino acid characteristics, which provides the platform for hydrophobic, electrostatic and hydrogen bond interactions with AIM/LIR-containing proteins [40, 41] (Figure3).

A



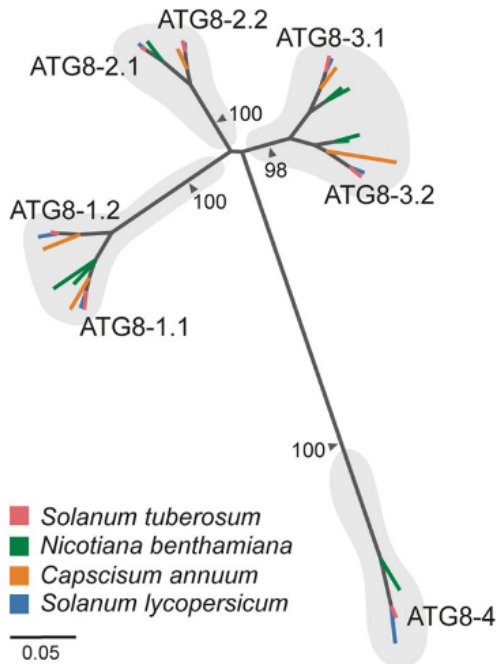
HsLC3A	M P P P Q K I P S V R P F K Q R K S L A I R Q E	E V A G I R	30
HsLC3B	- - - - - M P S D R P F K Q R R S F A D R C K E	E V Q Q I R	24
HsLC3C	- - - - - M P S E K T F K Q R R T F E Q R V E	E V R L I R	24
HsGABARAP	- - - - - M K F V Y K E E H P F E K R R S	E G E K I R	21
HsGABARAPL1	- - - - - M K F Q Y K E D H P F E Y R K E	E G E K I R	21
ScATG8	- - - - - M K S T F K S E Y P F E K R K A	E S E R I A	21
AtATG8E	- - - - - M N K G S I F K M D N D F E K R K A	E A G R I R	23
AtATG8F	- - - - - M A K S S F K Q E H D L E K R R A	E A A R I R	22
NbATG8F	- - - - - M A K S S F K Q E H D F E K R R A	E A A R I R	22
AtATG8A	- - - - - M A K S S F K I S N P L E A R M S E	E S S R I R	22
NbATG8A	- - - - - M A K S S F K L E H P L E R R Q A	E A A R I R	22
StATG8CL	- - - - - M A K S S F K L E H P L E R R Q A	E A A R I R	22

HsLC3A	A K F P N K I	P V V V E R Y P R E T F L P P L D K T	K F L V	60
HsLC3B	D Q H P S K I	P V I I E R Y K G E K Q L P V L D K T	K F L V	54
HsLC3C	E Q H P T K I	P V I I E R Y K G E K Q L P V L D K T	K F L V	54
HsGABARAP	K K Y P D R V	P V I V E K A P K - A R I G D L D K K	K Y L V	51
HsGABARAPL1	K K Y P D R V	P V I V E K A P K - A R V P D L D K R	K Y L V	51
ScATG8	D R F K N R I	P V I C E K A E K - S D I P E I D K R	K Y L V	51
AtATG8E	E K Y P D R I	P V I V E K A E K - S E V P N I D K K	K Y L V	53
AtATG8F	E K Y P D R I	P V I V E K A E K - S D I P T I D K K	K Y L V	52
NbATG8F	E K Y S D R I	P V I V E K A E K - S D I P N I D K K	K Y L V	52
AtATG8A	E K Y P D R I	P V I V E K A G Q - S D V P D I D K K	K Y L V	52
NbATG8A	E K Y P D R I	P V I V E K A E R - S D I P D I D K K	K Y L V	52
StATG8CL	E K Y P D R I	P V I V E K A E R - S D I P D I D K K	K Y L V	52

HsLC3A	P Q E L T M T Q F L S I I R S R M V L R A T E A F Y L L V N	90
HsLC3B	P D H V N M S E L V K I I R R R L Q L N P T Q A F F L L V N	84
HsLC3C	P D H V N M S E L I K I I R R R L Q L N A N Q A F F L L V N	84
HsGABARAP	P S D L T V G Q F Y F L I R K R I H L R A E D A L F F F V N	80
HsGABARAPL1	P S D L T V G Q F Y F L I R K R I H L R P E D A L F F F V N	80
ScATG8	P A D L T V G Q F V Y V I R K R I M L P P E K A I F I F V N	80
AtATG8E	P S D L T V G Q F V Y V I R K R I K L S A E K A I F I F V D	82
AtATG8F	P A D L T V G Q F V Y V I R K R I K L S A E K A I F I F V D	81
NbATG8F	P A D L T I G Q F V Y V I R K R I K L S A E K A I F I F I D	81
AtATG8A	P A D L T V G Q F V Y V V R K R I K L G A E K A I F V F V K	81
NbATG8A	P A D L T V G Q F V Y V V R K R I K L S A E K A I F I F V K	81
StATG8CL	P A D L T V G Q F V Y V V R K R I K L S A E K A I F I F V K	81

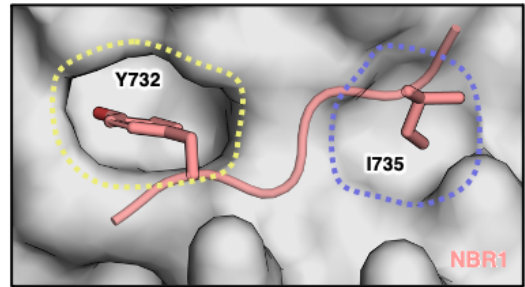
HsLC3A	N K S L V S M S A T M A E I Y R D Y K D E D G	F V Y M T Y A	120
HsLC3B	Q H S M V S V S T P I A D I Y E Q E K D E D G	F L Y M V Y A	114
HsLC3C	G H S M V S V S T P I S E V Y E S E K D E D G	F L Y M V Y A	114
HsGABARAP	N V - I P P T S A T M G Q L Y Q E H H E E D	F L Y I A Y S	110
HsGABARAPL1	N T - I P P T S A T M G Q L Y E D N H E E D Y	F L Y V A Y S	110
ScATG8	D T - L P P T A A L M S A I Y Q E H K D K D G	F L Y V T Y S	110
AtATG8E	N V - L P P T G E L M S S V Y E D K K D E D G	F L Y I T Y S	112
AtATG8F	N V - L P P T G A L M S S V Y E E K K D D D G	F L Y V T Y S	111
NbATG8F	N V - L P P T G A I M S A I Y D E K K D E D G	F L Y V T Y S	111
AtATG8A	N T - L P P T A A L M S A I Y E E H K D E D G	F L Y M T Y S	111
NbATG8A	N I - L P P T A A M M S A I Y E E H K D E D G	F L Y M T Y S	111
StATG8CL	N I - L P P T A A M M S A I Y E E H K D E D G	F L Y M T Y S	111

B



C

Canonical



Non-canonical

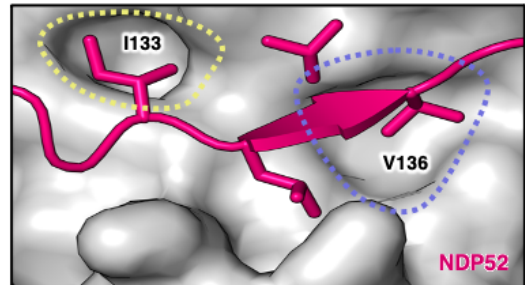


Figure3. Structural conservation of the ATG8-family of proteins. **(A)** The surface of ATG8 has two pockets which can be occupied by ATG8-interacting proteins. The hydrophobic pockets, sites 1 and 2 (yellow and slate, respectively), are formed by residues that are conserved among the ATG8-family of proteins. **(B)** Phylogenetic tree of solanaceous ATG8 isoforms, *adapted from* [35]. **(C)** The pockets are occupied by short linear motifs found in the intrinsically disordered regions of ATG8-binding proteins. The motifs can be described as canonical, where aromatic and hydrophobic residues occupy pockets 1 and 2, respectively. For example, autophagy cargo receptor NBR1 utilises a tyrosine and isoleucine to bind to ATG8. Non-canonical interaction has also been described. NDP52 occupies ATG8 pockets using two hydrophobic residues, isoleucine, and valine, and does not require an aromatic amino acid at this interface. *Adapted from* [42].

ATG8-interacting short linear motifs

All ATG8-binding proteins carry sLIMs in their intrinsically disordered regions (IDRs) that can be described as typical (canonical) or atypical (non-canonical) AIMs/LIRs [39]. For typical interactions, the core AIM/LIR sequence (W,Y,F)(X)(X)(L,I,V) consists of an aromatic amino acid followed by any two amino acids and a hydrophobic residue. The first aromatic and last hydrophobic amino acids bind to the hydrophobic pockets, W (site one) and L (site two), as described previously (Figure3) [43]. Sequences flanking the core AIM/LIR typically consist of acidic, negatively charged residues, which enhance AIM/LIR docking by forming polar interactions with the positively charged, basic residues surrounding the pockets [44]. In some cases, a neighbouring serine/threonine residue is phosphorylated to enhance docking into the pockets of ATG8 [45]. Non-canonical AIMs/LIRs that mediate ATG8 binding have also been discovered, expanding the spectrum of residues that facilitate binding to ATG8. For example, NBR1 utilises a canonical Y(aromatic)xxI(hydrophobic) motif to interact with

GABARAP [46], whereas NDP52 has an atypical $\text{I}(\text{hydrophobic})\text{xxV}(\text{hydrophobic})$ LIR to bind LC3 [47] (Figure3).

Selective autophagy is facilitated by cargo receptors

Although initially described only as a bulk process, autophagy can be selective through autophagy cargo receptors [48]. Bulk autophagy is induced in response to widespread matters concerning the whole organism, such as nutrient deprivation, and depends on reduced TORC signalling. Selective autophagy functions more in cellular maintenance and homeostasis. As opposed to bulk autophagy, it can occur in the presence of TORC signalling, where autophagic machinery is directed to specific cargo by the action of cargo receptors [49]. Autophagosome biogenesis is mediated in the vicinity of these cargo [50]. Specific autophagic cargoes can include, damaged mitochondria and chloroplasts, stalled ribosomes, and invading viruses. These specialised processes are referred to as mitophagy [51], chlorophagy [52], ribophagy [53] and virophagy [54], respectively.

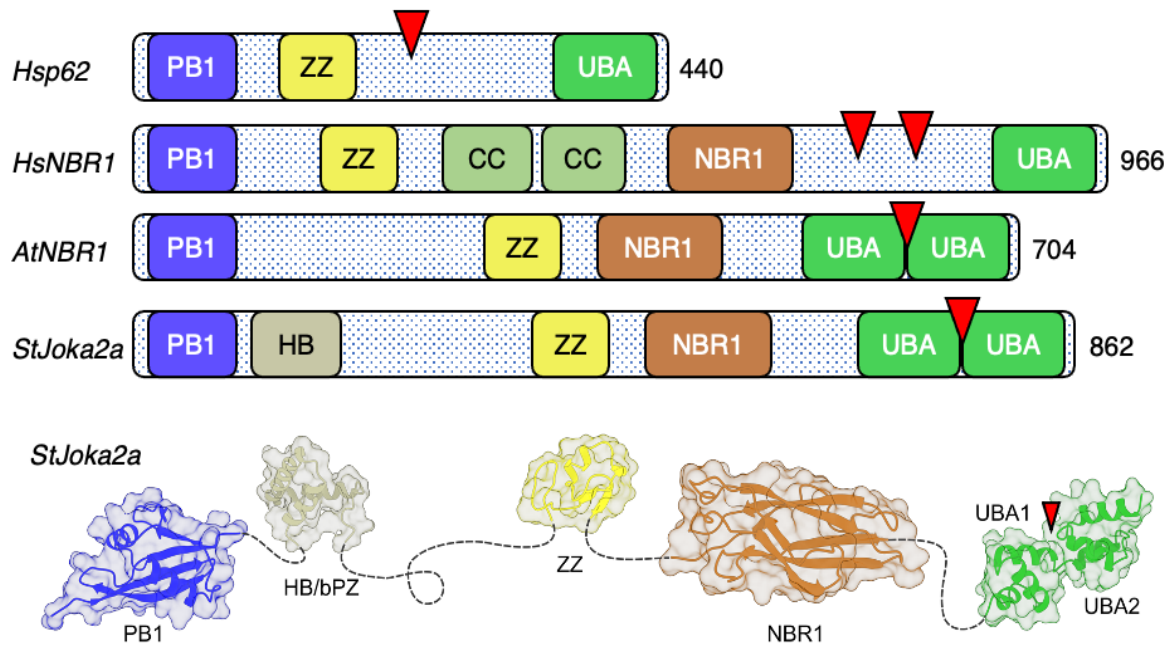


Figure4. Molecular architecture of the NBR1- and p62- like autophagy cargo receptors. Plant ACRs Joka2 and NBR1 have the same molecular architecture, however, differ in size. All ACRs share an N-terminal PB1 domain (blue) albeit belonging to distinct species. This is the site of oligomerization. Followed by the PB1 is a zinc-finger domain (yellow). Between the two, various nuclear localisation and exclusion signals have been described. Plant ACRs have two ubiquitin-associating domains (UBAs – green), which sandwich the ATG8-interacting motif (AIM/LIR – red triangle). Mammalian ACRs have one UBA domain and can have various AIMs/LIRs that are distant from UBA domains. Mammalian NBR1 houses two coiled-coil (CC – light green) domains that are sights of major protein-protein interactions (PPIs). The NBR1 domain (brown) is a beta-sandwich also described in PPIs. *Adapted from [55].*

Cargo selectivity is established through specialised autophagy cargo receptors (ACRs). They link cargo to the ATG8-family proteins localised at the developing phagophore through their AIMs/LIRs (Figure4 – red triangles) and consequently get degraded in autophagosomes. NBR1 and p62 are well studied examples of ACRs and conserved among eukaryotes. Relatives of these proteins, namely Joka2, are prominent regulators of plant cellular health and protection against infectious

pathogens (Figure4) [56, 57]. Other examples in mammals include NDP52 and TAX1BP1. The latter can form biomolecular condensates with ubiquitinated proteins and recruit ATG9 vesicles through adaptors for autophagosome formation [58]. All ACRs share a ubiquitin recognition domain (UBA), which have a tri-helical structure and bind poly-ubiquitin signals found on cargo destined for degradation [50]. Hence, ACRs must interact with ATG8/LC3 and cargo simultaneously, which is allowed by their flexible and oligomeric structures.

NBR1- and p62-like ACRs sequester and enrich cargo in larger condensates [59]. Utilising their PB1 domain, they can form high-order molecular structures. The presence of opposing surface charges, acidic and basic, allows the PB1 domain of one ACR to interact with the opposite side of another. Jakobi et al. 2020 showed in vitro that PB1 domains of *A. thaliana* and mammalian NBR1 assemble in a helical manner to form filamentous structures [60]. However, this was with the PB1 domain only and not the full-length protein, hence, the reality of these structures in vivo is not confirmed. NBR1 and p62 is known to form large scaffolds and facilitate downstream signalling to regulate responses beyond autophagy [61, 62]. These oligomeric scaffolds can bind proteins involved in autophagy and other cellular functions, such as MAPKs (Tumtas et al. unpublished), through their zinc-finger (ZZ) and NBR1 domains and contribute to plant innate immunity as a signalling scaffold.

Plant innate immunity

Plants lack mobile immune cells and rely on innate immunity of individual cells, therefore, each cell must detect the pathogen and not rely on immunological memory. To compensate for the absence of adaptive immunity, plants have evolved a wide

variety of immune receptors (Figure5). These immune receptors are arranged in two layers and recent advances in the field suggest the interplay between the various layers of immune response yields greater immunity (Figure5) [63].

The first layer of immune response is known as pattern-triggered immunity (PTI), which detects the pathogen at early stages of the infections. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are detected at the cell surface by pattern-recognition receptors (PRR), triggering a cascade of events which yield a strengthened cell wall via callose and pectin deposition. The MAPK signalling pathway regulates gene expression to benefit the plant and is an essential part of the PTI response (Figure5) [64-66].

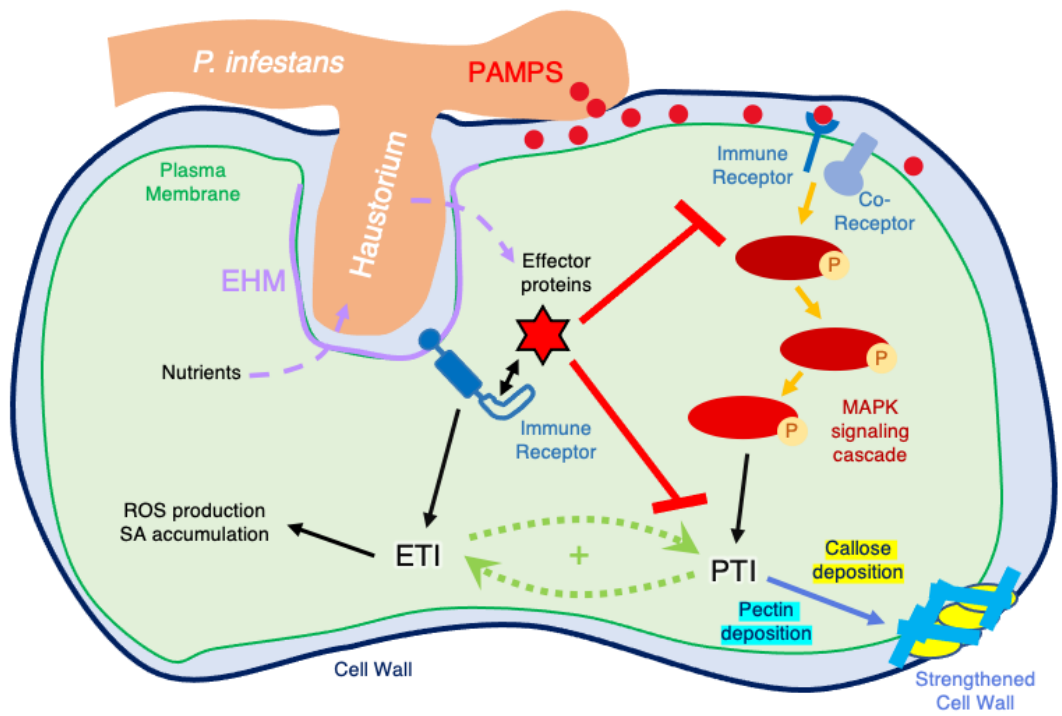
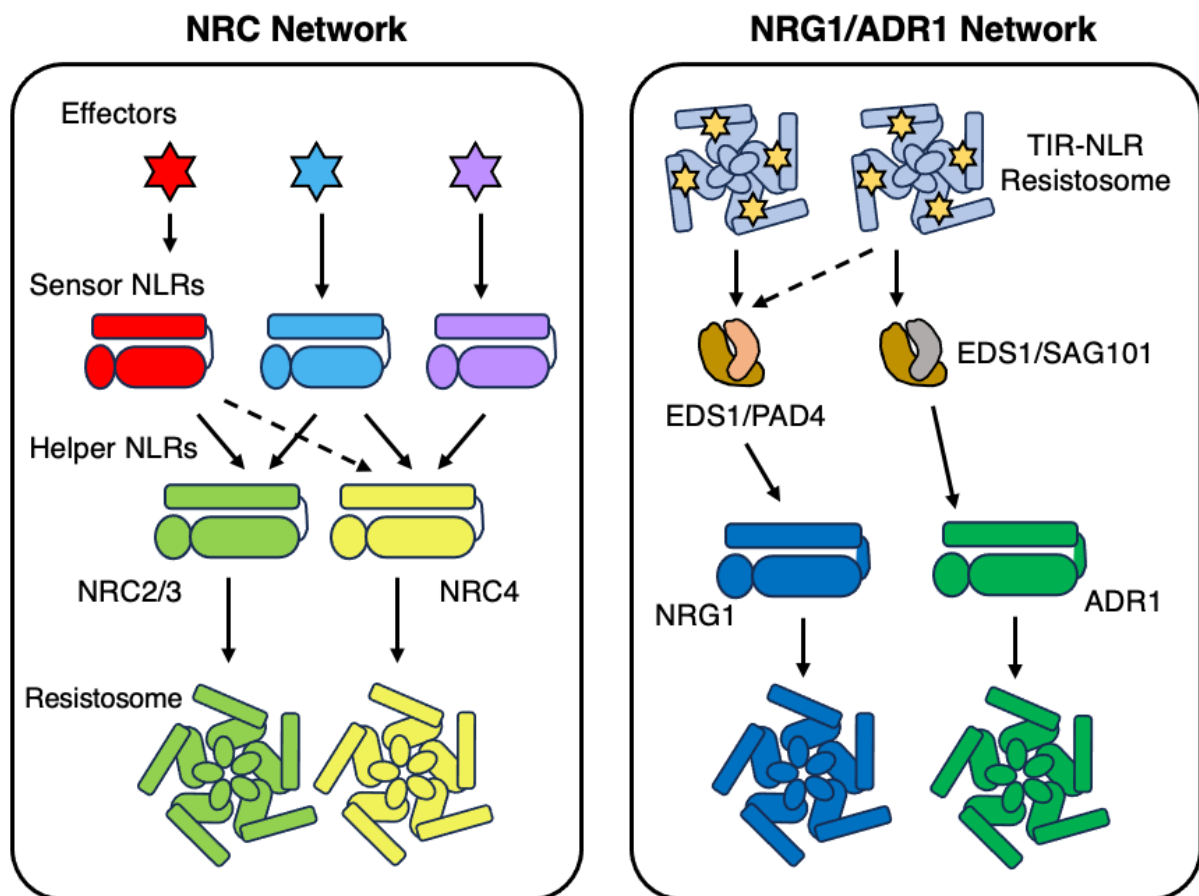
A**B**

Figure5. Overview of the plant innate immune system upon *P. infestans* infection. (A) A mutually potentiated multi-layered innate immune response is key to providing successful immune response towards invading pathogens. Here I am using *P. infestans* as a model organism to describe plant immune responses. Upon invasion, the oomycete attacker releases pathogen-associated molecular patterns (PAMPs - red dots) into the extracellular space between plant cells. These are recognised at the cell surface by PRRs (pattern recognition receptors), which are immune receptors that work as pairs (blue/light blue at PM). Their activation triggers a MAPK (mitogen associated protein kinases) signalling cascade, which leads to transcription events causing PTI. Consequently, callose and pectin (yellow/cyan, respectively) are deposited to the cell wall to strengthen the structure and prevent further pathogen entry. The haustorium invaginate the PM and begin nutrient uptake and effector release. The extra-haustorial membrane (EHM - lilac membrane) is unique in protein and lipid composition, which allows efficient diffusion. Effectors impair with pathogen triggered immunity (PTI) by targeting immune receptors and downstream signalling events. In response, plants have evolved effector triggered immunity (ETI), which is activated by intracellular receptors, known as NLRs. Salicylic acid (SA) and reactive oxygen species (ROS) accumulation via ETI, leads to cell death, stopping the spread of the pathogen in healthy cells. PTI and ETI are positive regulators of each other, which generates a stronger immune response. *Adapted from* [55]. (B) A large repertoire of intracellular receptors has evolved to detect effectors targeting host machinery. NLRs can be roughly split into the NRC and NRG1/ADR1 network. The NRC branch work in pairs, a sensor recognises the effector and triggers helper NLRs to form a resistosome. The second branch rely on downstream lipase-like protein dimers EDS1/PAD4 and EDS1/SAG101. These interact with helpers NRG1 and ADR1 to initiate resistosome formation. *Adapted from* [67].

Pathogens have evolved an arsenal of effector proteins that are deployed to inhibit PTI responses. For example, filamentous pathogen *P. infestans* release effector proteins, such as cell wall-degrading enzymes, toxins, and various cysteine-rich proteins to increase their chances of survival in the plant cell [68].

In return, plants evolved effector-triggered immunity (ETI), facilitated by intracellular immune receptors, broadly known as the NLR (nucleotide-binding leucine-rich repeat receptors) network (Figure5). Briefly, inactive NLRs can sense intracellular effectors and trigger a signalling cascade, which yields active oligomers of NLRs. The oligomers, also known as resistosome, can accumulate at the plasma and chloroplastic membrane and the plant-pathogen interface to initiate disease resistance through hypersensitive cell death response (HR) [69].

HR prevents dispersal of infection between pathogen-affected and uninfected plant tissue. HR is achieved by ROS production and the accumulation of senescence signalling molecule salicylic acid (SA). For example, Potato Rx sensor mediates a well characterised HR response to Potato Virus X (PVX), inducing cell death in response to PVX coat protein. This is achieved through the activation of the NRC branch of the NLR network, leading to resistosome formation [70]. An alternative NLR network is known as the NRG1/ADR1 branch. The bacterial effector avrRps4 is recognised by receptors, which triggers EDS1-dependent formation of the resistosome (Figure5) [71]. EDS1 is a lipase that interacts with either SAG101 (senescence regulator) or PAD4 (SA-generating signal) leading to host cell death and pathogen restriction by formation of NRG1 or ADR1 resistosome's [72, 73]. By studying ETI in the absence of PTI, it was shown the two pathways work together to produce a stronger defence response [74] (Figure5).

Beyond direct pathogen suppression, autophagy has been implicated as a regulator of NLR-dependent immunity. In mammals, NLRP3, a mammalian NLR, senses PAMPs and forms an inflammasome (mammalian resistosome) to recruit caspases to trigger

cell death. Upon activation, NLRP3 gets ubiquitinated and can recruit the ACR p62, leading to autophagic degradation of the inflammasome, which can act as a regulatory mechanism of immune responses [75, 76]. In plants, *atg* knock-out variants (e.g., VPS34, ATG3, ATG6, and ATG7) exhibited an enhanced spread of HR when challenged with Tobacco Mosaic virus (TMV) [77].

Selective autophagy in plant innate immunity

Although the internal clearance of pathogens is well-known in mammals, the role of autophagy in plant resistance is not fully grasped. However, the involvement of autophagy to safeguard plants against infectious diseases has been shown. *Atg* mutant lines of *A. thaliana* were more susceptible to oomycete fungi and viral pathogens. Over-expression of ATG proteins, such as ATG8, could rescue this immune deficient phenotype and in wild-type plants lead to increased tolerance [77-79]. In return, these pathogens are known to release effector proteins that specifically target autophagic machinery. Although the importance of autophagy in this “tug of war” is evident, the molecular mechanisms governing these responses are not very well understood.

The Irish potato famine pathogen *P. infestans* (oomycete fungi) form finger-like structures in the plant cell (haustorium) and via a unique membrane EHM (extra haustorial membrane), facilitate uptake of nutrients and release of effectors to drive pathogenic survival and adjust plant responses (Figure5). Autophagic machinery, aided by ACRs Joka2 and NBR1, localise to the host-pathogen interface. Overexpression of ATG9 and Joka2 enhanced plant immunity towards *P. infestans*, demonstrating the involvement of selective autophagy in infection control. In return,

the pathogen counteracts autophagic upregulation by releasing the effector PexRD54. It localises to defence-related autophagosomes and displaces cargo from developing vesicles by binding to ATG8, through a higher affinity AIM [56, 57]. *Serendipita indica* is a root endophytic fungus, which interact with plants in a beneficial manner. Autophagy mutant plants exhibited higher colonisation levels compared to wild-type plants, where the latter showed increased numbers of ATG8-labelled autophagosomes, suggesting enhanced autophagy upon infection [80].

Plant viruses are targeted by autophagy and have evolved effector proteins to mediate immune evasion. For example, Turnip mosaic virus (TuMV) HcPro is removed by NBR1-mediated autophagy. In turn, TuMV evolved viral proteins VPg and 6K2 to counteract NBR1-driven host defences by blocking the degradation of HcPro [81]. Autophagic clearance of viruses is termed virophagy and is discussed in more depth in Chapter 4, where we studied how viruses modulate autophagy and vice-versa through ATG8 interactions.

Importance of studying crop diseases

Plants are the base of the global food chain and thus shape our planet's nature. With the current severity of global warming, preventing crop diseases is essential to increase yields. Our survival and evolution into more complex civilizations relies on our ability to efficiently harness plant resources. Consequently, plants form a major part of the global economy and any pathogenic threat to them is a major concern for our livelihood. Plant pathogens claim 20% to 40% of our annual crop yield. This, together with global warming, has led to 10% of the population suffering from undernourishment. In the early 2010's, predictions suggested an increase of ~100%

in food production will be required to meet our demands by 2050. The current trends indicate we will not be able to achieve this [82-86].

Hence, studying crop diseases and improving our current knowledge is essential. We employ a wide variety of techniques to determine molecular mechanisms of disease and develop intervening strategies. Drug development for human diseases has been accelerated by studying the structure and interactions of molecules with their protein targets [87]. Structural biology and developments in the field of computational biology must be applied to plant disease understanding and manipulation. For example, using engineered nanobody insertions NLR proteins, known as pikobodies, can be designed to recognise specific effectors and elicit HR [88]. Such innovative strategies must be applied to developing probes for autophagy and our goal has been to study core autophagy protein ATG8 as a target protein, see Chapter 3 and 4 for studies of ATG8 interactions.

Structural studies of autophagy using AF2 and AF2-multimer

The molecular mechanisms governing defence-related autophagy are studied extensively using cellular biology and biochemical techniques. However, detailed information is lacking and contradicting studies have been published. This is due to the targeting of core autophagy proteins in the studies, where multiple molecular machineries might be altered. Structural biology specifically allows the study of the proteins involved in these processes and the complexes they form. The most prominent technique used has been X-ray crystallography, generally using peptides of proteins to visualise complexes. This lacks dynamic and full-length information. The incorporation of cryo-electron microscopy (CryoEM), where transient and dynamic

complexes can be studied, into this field of research will and has enhanced our knowledge. For example, the NLR resistosome could not be studied with X-ray crystallography and only small domain complexes were visualised. The incorporation of cryoEM and purification of these proteins from their native sources has led to visualization of the resistosome, which were hypothesised for many years, and provided insight into how NLRs might trigger HR [89, 90].

The study of ACRs has been hampered by their extremely flexibility and tendency to get degraded because of their function. The challenges and our solutions to the issue of studying ACRs using cryoEM are discussed in further detail at Chapter 5. Recent developments in the field of artificial intelligence (AI) and structural predictions by DeepMind, yielded state-of-the-art technologies such as AlphaFold2 (AF2) [91] and AF2-multimer [92]. They allow structural insights into proteins that have struggled in wet-lab studies and make structural biology more accessible to all interested, which has accelerated new research development and advancements in molecular understanding of biological processes.

Limitations in structure prediction pre-AF2

The protein folding problem addresses how a protein's amino acid sequence dictates its three-dimensional structure. This has been described as a “problem” as proteins must correctly fold into specific shapes that satisfy the energy limitations and function correctly. Calculating the electrostatic potential and energies of bonds has been a rate limiting factor in structure prediction [93]. In addition to this, the sampling problem, which describes the number of possible configurations available for one amino acid sequence, has been a significant obstacle [94]. A common structure prediction

software is RosettaFold (RosettaCommons) [95], which relies on ab-initio algorithms. Ab initio approaches are based on the thermodynamics hypothesis that the native protein structure presents the lowest free energy possible. The limitation of ROSETTA is suggested to be in the sampling algorithms rather than the energy function [96]. AF2 (DeepMind, Google) uses a new hybrid deep learning approach, which led to AF2 being top ranked at the 14th Community Wide Experiment on the Critical Assessment of Techniques for Protein Structure Prediction (CASP14) ahead of BAKER (David Baker's Rosetta development). It contains a convolutional neural network that is trained by PDB structures to predict the distances between residues. The authors of AF2 describe the improvements on other prediction software as "incorporating novel neural networks architectures and training procedures based on the evolutionary, physical and geometric constraints of protein structures" [91].

AF2 pipeline

AF2 uses an amino acid sequence as an input, which it uses to generate a multiple sequence alignment (MSA). The MSA is used to detect mutation prone sequences and their correlation as well as producing a template structure via identifying proteins with similar structures. This is known as pair representation. This is not a novel approach and shared with other software. The advantage of AF2 comes at how these are processed using the evoformer and structure modules (Figure6).

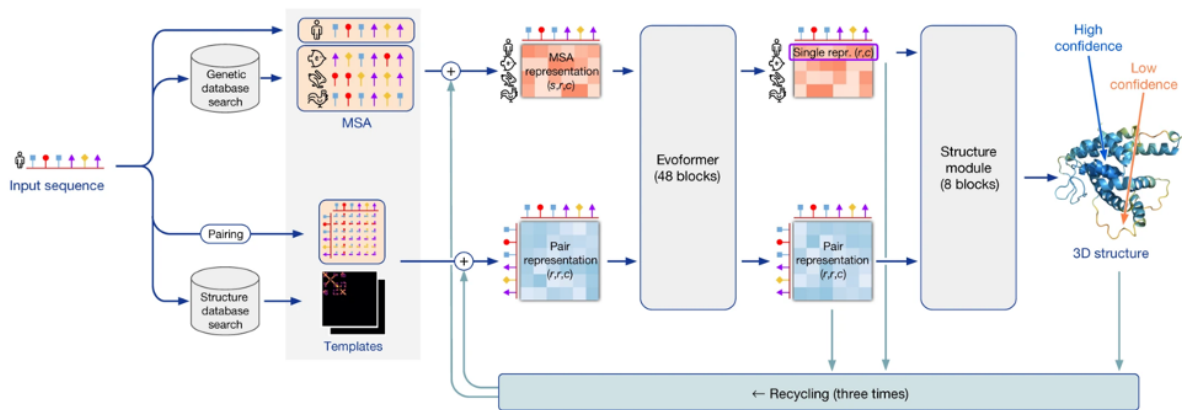


Figure6. AlphaFold2 architecture. Arrows show the information flow among the various components. An input protein sequence is matched against genetic and structure databases to gather templates (similar sequences and structures). These are combined to form a multiple sequence alignment (MSA) and pair representation, which are then processed through a neural network (Evoformer, 48 blocks) to refine the predictions. A final structure module (8 blocks) constructs the 3D structure with indicated confidence levels. The prediction may iterate three times for accuracy. *Adapted from Jumper et al., 2021.*

The evoformer extracts information from MSA and templates. It consists of two transformers, the MSA and pair representation transformer. These exchange information between the MSA and templates back and forth throughout the network and improves the MSA assessment and template structure. The outputs from the evoformer are processed in the structure block. Here the orientation of the protein backbone is optimised with considerations to residue rotations and translations, localising each amino acid side chain within a frame. This is followed by local refinement and energy minimisation to produce the final model [91].

Limitations of AF2

AF2 has difficulty predicting IDRs and loop regions of proteins. These are hypervariable and flexible, therefore making it difficult to extract evolutionary

constraints from the MSA. The output structure is a single conformer and different forms of a structure cannot be predicted. Post-translational modifications, complexes with DNA/RNA and metal ions cannot be predicted using AF2 now. The RosettaFold team developed an improvement to their algorithm, RoseTTAFold All-Atom, which can predict full biological assemblies containing proteins, nucleic acids, small molecules, metals, and covalent modifications [97]. The algorithm allows input information about the molecular composition of the biomolecular assembly to be modelled, including amino acid and nucleic acid sequences, metal ions, small molecule bonded structure, and atomic geometry of covalent modifications between small molecules and proteins. Predicting novel structures with no homology to known and target sequences is also a challenge as it relies on MSAs. Finally, it struggles with point mutations. It fails to compute defects in protein folding upon a mutation [98]. The team behind AF2 recently developed AlphaMissense, which can predict the effects of single point mutations on protein structure and function [99]. It is trained on human and primate variant population frequency data. AlphaMissense predicts the pathogenicity of all possible single amino acid changes at a given position from the input amino acid sequence. It is trained to do structural predictions, as per AF2, and is improved upon by protein language modelling to predict the identity of the amino acids masked at random positions in the MSA.

Current algorithms beyond AF2

AF2 received a Z-score, a metric used to assess prediction models, of over 240 at CASP14, which has not been achieved by any other prediction technology yet. However, in the absence of AF2, CASP15 demonstrated the prowess of alternative algorithms in the field. A large language model-based approach has been developed

by Meta AI, ESMFold [100]. Unlike AF2, ESMFold relies on a single input sequence; it does not perform multiple sequence alignments and requires no template structure. Although not as accurate as AF2, ESMFold is faster and can predict accurate structures for proteins with limited sequence homology. This is possible due to the vast training of their language model, with 15 billion parameters, to understand the underlying structure that creates the patterns in the sequences across evolution. D-I-TASSER was developed for CASP15 and in ~50 modelling targets showed a 20% improvement in accuracy compared to AF2 [101]. D-I-TASSER introduced a new protocol for generating MSAs, DeepMSA2, and a deep learning approach to their folding simulation system [102]. DeepMSA2 improves on previous MSA methods as it generates 10 potential MSAs using various sub-methods and ranks them based on the structure model-associated confidence score.

AF2-multimer

AF2-multimer is an expansion of AF2 and is optimised to predict protein-protein complexes. For multimer, AF2 developers modified the pair MSA alignments to surface cross-chain genetic information. AF2 is trained on cropped segments of full-length proteins due to limited memory and compute considerations. In multimer, the cropped regions were improved in chain coverage and crop diversity to capture multiple chains in a given complex and binding interfaces between chains [92]. An exciting alternative to AF2-multimer, DMFold-Multimer, was showcased at CASP15 and was the highest-ranking model for complex prediction [103]. DMFold-Multimer is based on the end-to-end modelling module from AF2-multimer and improves upon it by integrating the DeepMSA2 algorithm from D-I-TASSER. Beyond accuracy, the main advantage of AF2 and the AF2-multimer pipeline is user compatibility and ease of access. AF2 (and

-multimer) can be run using any computer anywhere using ColabFold with Google servers, whereas other methods must be accessed through their GitHub packages and run locally.

Aims and objectives

The main goal of my PhD was to characterise molecular mechanisms governing autophagy at the level of core ATG protein ATG8. I investigated ATG8-interacting motifs, discovering new interaction sequences in proteins and subsequently, study how they are utilised in newly established ribophagy and virophagy systems to modulate ATG8. I investigated the ATG8-interacting cargo receptor Joka2 from *S. tuberosum*. My aim was to structurally examine Joka2 and gain insights into how a full-length cargo receptor organizes itself in the plant cell. I also investigated individual domains of Joka2 to define mechanisms governing protein-protein interactions. To this extent, I pursued the following objectives:

(1) outline a method to accurately identify typical and atypical ATG8-interacting motifs – Chapter 3.

Current prediction tools for AIMs/LIRs do not produce a visual complex output. They generate many AIM/LIR options within a protein with no information on secondary structure and can only identify typical and not atypical binding motifs. Our aim was to produce a pipeline that would eliminate false AIMs/LIRs and allow atypical binding partners to be identified. To achieve this, we incorporated AF2-multimer as a preliminary step to our wet-lab approaches. I wanted to see if AF2-multimer can correctly predict the interface at the hydrophobic pockets of ATG8 (LIR docking site) and AIMs/LIRs of binding partners. Following AF2-multimer visualisation of the

interface, as a further confirmation, we analysed the phylogeny of the interface, thinking the more conserved the AIM/LIR sequence, the more likely it evolved to interact with ATG8 in a functional manner. These were followed by molecular and biochemical characterisation, mainly using co-immunoprecipitation assays, and cellular approaches, such as fluorescence microscopy, to determine the impact on autophagic activity upon mutational tampering of the interface. We established a pipeline that is successful in predicting typical and atypical candidates screened from literature, as well as, predicting previously unknown motifs. Interestingly, cross-kingdom interactions could also be predicted using our pipeline.

(2) with respect to ATG8, characterise adaptors and modulators of ribophagy and virophagy – Chapter 4.

We established the ability of our pipeline to identify novel AIM/LIRs and its accuracy in cross kingdom interactions. Pathogens, such as viruses, are known to manipulate host autophagy to their own benefit. To identify viral ATG8 modulators, we employed an AI proteomics approach, where we applied our pipeline to over 100 viral proteomes to identify novel interactors of ATG8. This yielded various candidates with great structural and functional variety, which we validated in the wet lab using co-immunoprecipitation and in vivo autophagy assays. We then applied this method to screening ribosomes to determine an adaptor for selective recycling of ribosomes. We determine the RPLP stalk protein, RPLP2, as a direct interactor of ATG8 and that it acts to regulate autophagic activity. These were later validated using IP-MS. In this chapter, I applied our AF2-multimer approach to uncover the various ways viruses act to manipulate host mechanisms and whether selective ribosomal degradation is possible through RPLPs.

(3) characterise the domain biochemistry and full-length structure of cargo receptor Joka2 – Chapter 5.

Full-length structures of ACRs are not available. This avenue of research has suffered from their intrinsic flexibility, ability to form transient, large complexes with a wide variety of proteins and their tendency to get degraded due to their function and instability. To tackle these issues, we set out to optimise the extraction of Joka2 using *N. benthamiana* as an expression and purification system and biochemical modifications to Joka2 oligomerisation. I employed AF to predict a Joka2 model to clarify domain boundaries and important interface. The novel helical bundle domain, unique to plant cargo receptors, was discovered as a result. I then studied the in vitro ability of the PB1 domain to oligomerise, which I then mutate in the full-length protein to achieve more soluble protein. Our aim is to produce in vitro scaffolds for high resolution structure determination of Joka2 and partners. We use the cutting-edge affinity grid design to directly purify Joka2 on cryoEM grids and limit the time Joka2 is exposed to non-native conditions. Overall, I studied Joka2 domain organisation and biochemistry whilst attempting to achieve ground-breaking advances in structural exploration of cargo receptors.

2. Materials and methods

Cloning of plasmid constructs

Cloning into *Escherichia coli* compatible vectors

Joka2 domains, PB1, HB and UBAs, were amplified using polymerase chain reaction (PCR) with overhangs suitable for Gibson assembly [104] or Golden gate [105] cloning vectors. Joka2 domains were initially cloned into pMAT10 vector expressing an N-terminal six his-tag followed by maltose binding protein (MBP) and a thrombin protease site. Further cloning of Joka2 domains was performed using the modified, Golden-gate compatible, pOPIN expression vector, provided by the Banfield lab (JIC, Norwich) [106]. The Golden gate digestion/ligation reactions and cycling conditions were carried out as described by Engler et al. (2008) and Iverson et al. (2016), using enzymes BsaI (level one), BbsI (level zero) and T4 DNA ligase (NEB) [105, 107].

Cloning into *Nicotiana benthamiana* compatible vectors

Fluorescently tagged Joka2 wild-type and ATG8 constructs were previously cloned into third-generation pK(GFP/BFP/RFP/HA/Myc)(C'/N')3S and Gateway vectors [35, 56, 57, 108]. The HB domain truncated Joka2 constructs were generated with PCR followed by Gibson assembly in to pK(G/B)C3S vectors. Joka2 UBA domain fragments, including wild-type and individual mutants (Table2) were ordered from GeneWiz and cloned in to pK(G/HA)C3S vectors. The SpyTag003-4xMyc-Joka2-GFP constructed was generated using separate vectors containing the SpyTag003-4xMyc fragment (provided by Banfield lab), Joka2 and fluorescent-optimized GFP, which were all PCR amplified separately and joined in the pKGC3S vector using Gibson assembly.

Fragments of Joka2 wild-type and RR mutant with a C-terminal strep and Spytag002 were ordered from TwistBio and cloned into Gibson assembly vector pJK268c_mRFP1 (TSL SynBio, Norwich) with a p19 module. C-terminal GFP-tagged ATI2 construct was generated by Gibson assembly of PCR fragment amplified from *S. tuberosum* cDNA into pKGC3S GFP expression vector. The DNA fragment for ATI2 AIM1 peptide was custom synthesised and inserted into the pKGN3S vector to generate an N-terminal GFP expression construct. ATI2-AIM1, ATI2-AIM2, and ATIAIM1+2 mutant fragments were generated using site-directed mutagenesis (SDM) PCR amplification from wild-type construct. ATG3 AIM peptide DNA fragments were custom synthesised and inserted into the pKRN3S vector to generate an N-terminal RFP expression construct using Gibson assembly [42]. For ribophagy, RPLP constructs were custom synthesised and inserted into pK(G/R)N3S vectors, with an N-terminal GFP fluorophore. For virophagy, including plant virus and Covid19 proteins, fragments were custom synthesised and inserted into Golden gate vectors pJK001c_mRFP1 and pJK268c_mRFP1 with an N-terminal GFP and Myc tag. The pJK001c_mRFP1 vector lacks a p19 module. For all mentioned above, fragments were custom synthesised and ordered from GeneWiz. All primers were purchased from Integrated DNA technologies. All constructs listed in Table2 were confirmed using Sanger sequencing services provided by Eurofins. All cloning was performed in DH5 α *E. coli* strain.

Table2. Plasmid constructs used in this study. Construct in bold were previously cloned by members of the Bozkurt lab.

Construct Name	Comments
StJoka2^{WT}-GFP/BFP	<i>N. benthamiana</i> , pK7 vector
StJoka2 ^{DHB} -GFP/BFP	<i>N. benthamiana</i> , pK7 vector
SpyTag003-4xMyc-StJoka2-GFP	<i>N. benthamiana</i> , modified pK7 vector
StJoka2 ^{WT} -Strep-Spytag002	<i>N. benthamiana</i> , pJK268c_mRFP1
StJoka2 ^{RR} -Strep-Spytag002	<i>N. benthamiana</i> , pJK268c_mRFP1
SpyCatcher002-Cys	<i>E. coli</i> , Addgene #102829
SpyTag-sfGFP	<i>E. coli</i> , Addgene #184226
StJ2a-UBAs^{WT}-GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2a-UBAs ^{V818A} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2a-UBAs ^{W821A} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2a-UBAs ^{DoubleA} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2a-UBAs ^{I824A} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2a-UBAs ^{DoubleAcidic} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2a-UBAs ^{I824Mswitch} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{WT} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{V690A} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{W693A} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{DoubleA} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{M696A} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{DoubleAcidic} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
StJ2b-UBAs ^{M696Iswitch} -GFP/HA	<i>N. benthamiana</i> , pK7 vector
NtRPLP2 ^{WT/aim} -GFP/RFP	<i>N. benthamiana</i> , pK7 vector
NtRPLP0 ^{WT/aim} -GFP/RFP	<i>N. benthamiana</i> , pK7 vector
Covid19_4Myc-Orf9b ^{WT/aim}	<i>N. benthamiana</i> , pJK268c_mRFP1
BFP/RFP/GFP-StATG8CL	<i>N. benthamiana</i> , pK7 vector
EV-RFP/GFP	<i>N. benthamiana</i> , pK7 vector
GFP-StATG8variants (1.1-4.0)	<i>N. benthamiana</i> , Gateway (TSL)
RFP-StATG3peptide ^{WT/aim}	<i>N. benthamiana</i> , pK7 vector
GFP-StATI2^{WT/aim}	<i>N. benthamiana</i> , pK7 vector

GFP-StATI2peptide ^{WT/aim(s)}	<i>N. benthamiana</i> , pK7 vector
VTMoV_P1 ^{WT/aim} -GFP	<i>N. benthamiana</i> , pJK001c_mRFP1
TICV_rPPR5 ^{WT/aim} -GFP	<i>N. benthamiana</i> , pJK268c_mRFP1
SVBV_Orf5 ^{WT/aim} -GFP	<i>N. benthamiana</i> , pJK001c_mRFP1
TuMV_P3 ^{WT/aim} -GFP	<i>N. benthamiana</i> , pJK001c_mRFP1
TICV_P9 ^{WT/aim} -GFP	<i>N. benthamiana</i> , pJK001c_mRFP1
SYMMV_P7 ^{WT/aim} -GFP	<i>N. benthamiana</i> , pJK001c_mRFP1
6His-SUMO-PB1	<i>E. coli</i> , pOPIN GG vector suite
6His-MBP-PB1	<i>E. coli</i> , pMAT10 (not codon optimised)
6His-HB	<i>E. coli</i> , pOPIN GG (not codon optimised)

Plant and pathogen maintenance

Wild-type and genetically edited *Nicotiana benthamiana* were grown at 25°C on a mixture of soil and vermiculite (3:1) in a growth chamber, with a photoperiod of 16-hours of light and 8-hours dark. Humidity of chambers were kept at 50%.

Phytophthora infestans wild-type field strain #88069 was grown on rye sugar agar (RSA) plates in an 18°C dark room for at least two-weeks prior to use for infection of leaves.

Transient gene expression

Expression with *E. coli* compatible vectors

Proteins were produced in *E. coli* BL21 (DE3) cells, made in the Bubeck lab, or using C41 (DE3) cells (a gift from the Meier lab). Cultures were grown in Terrific Broth media (Sigma) at 37°C, 220 RPM until the OD₆₀₀ reached 0.5-0.6. Protein expression was induced at 18°C for 16 hours or 30°C for 5 hours. PB1 and HB domain constructs were

expressed using 0.5mM IPTG. For SpyCatcher002-Cys and SpyTag-sfGFP constructs the expression was as previously described [109, 110].

Expression with *N. benthamiana* compatible vectors

Agrobacterium tumefaciens-mediated transient expression (agroinfiltration) experiments were performed on 3- to 5-week-old *N. benthamiana* plants, depending on the ensuing experimental technique. Electrocompetent *A. tumefaciens* strain GV3101 were transformed with constructs of interest and grown on spectinomycin or kanamycin and gentamycin (SG/KG) plates for 48 hours. *Agrobacterium* were prepared for agroinfiltration by re-suspension and washes in water and agroinfiltration buffer (10mM MgCl₂, 5mM 2-(N-morpholino)-ethane sulfonic acid, pH 5.6).

Protein purification

Following transient expression in *E. coli*, cells were harvested at 3800xg for 10 minutes and stored at -80°C. Pellets were re-suspended in 50mM HEPES, pH 7.3-8.0, 250-500mM NaCl, 0.1% Triton-X detergent, +/- 5% glycerol and one complete EDTA-free protease inhibitor tablet per 50mL of buffer. A buffer:pellet (vol/vol) ratio of 5:1 was maintained. Following sonication, soluble protein content was harvested at 40000xg for 45 minutes and HisTrap HP His tag protein purification columns (Cytiva) were used to capture his-tagged proteins (all *E. coli* construct expressed a poly-histidine tag). Eluants were collected using an imidazole gradient applied by an AKTA Pure (GE Healthcare). Fractions with significant amounts of desired protein, were concentrated and further purified using Superdex (GE) gel filtration columns depending on the size of protein. Tag cleavage was achieved using thrombin protease (Sigma) and purified

HRV 3C protease (modified by Otting lab, Addgene plasmid #162795). Imidazole and gel filtration elution profiles were analysed using SDS-PAGE.

Co-immunoprecipitation (coIP) assays

Following transient expression in *N. benthamiana*, samples were harvested 2-3 days post agroinfiltration. Detached leaves, with their veins removed, were weighed out to 0.25g and placed into 2 mL Eppendorf tubes with three 2.8mm ceramic beads (Qiagen) and immediately flash-frozen with liquid nitrogen (LN2). Frozen leaf tissue was ground into fine powder using a Genogrinder with an LN2 cooled holder. 700mL of extraction buffer (Table3) was applied to the sample followed by three vortex and sonication (water bath) cycles. Resuspended sample was centrifuged at 10000xg for 10 minutes and the resultant supernatant for a further 15 minutes at 13000xg, 4°C. For the immunoprecipitation, 200mL of bead mix (12mL colour trap bead suspension (ProteinTech/Chromotek) and 188mL of IP buffer) was incubated with the soluble protein content harvested for 1 hour. Subsequently, beads were washed with IP buffer three times, in each case adding 1mL of buffer and 30 gentle rotations followed by a 1000xg centrifugation to harvest beads. Following the final wash, all liquid was aspirated using a 0.3mm syringe. The beads were eluted in 20ml elution buffer (25% of 4X Laemmli buffer (BioRad), 10% of 1M DTT (Sigma), 65% of dH₂O). All samples were boiled at 70°C for 10 min and spun down at max speed for 5 minutes prior to SDS-PAGE analysis.

Table3. Buffers used for coIP assays.

GTEN	10% (v/v) glycerol, 25 mM Tris pH 8.3, 1 mM EDTA, 150 mM NaCl
Extraction	GTEN, 0.15% IGEPAL (v/v), 2% Polyvinylpolypyrrolidone (PVPP – w/v), 10 mM DTT, 0.5% 1X protease inhibitor cocktail (Sigma Aldrich – v/v)
Immunoprecipitation (IP)	GTEN, 0.075% IGEPAL (v/v)

Western blotting

Samples were loaded onto SDS-PAGE gels ranging from 8 to 16% and separated at 120V for ~90 minutes. For Joka2, 8% gels were used. For ATG8 co-expression assays, 12-16% gels were used. Separated protein content was transferred to polyvinylidene difluoride (PVDF) membranes, which were then blocked with 5% milk powder in tris buffered saline (TBS). Membranes were incubated with primary antibody solution (3% TBST) for 1 hour and subsequently washed with TBST (TBS + 5% Tween) for 30 minutes. This was repeated with secondary antibody solution for detection. Chemiluminescence detection was performed with SuperSignal West Pico Plus kit (Thermo-Fisher) and imaged with the LiCOR Odyssey Fx. Colour detection was done using the colorimetric AP conjugate substrate kit (BioRad). Primary antibodies used for this study were α Myc/HA (Abcam), α GFP/RFP (Chromotek) and α NBR1 (Abcam).

Blue Native PAGE

Six leaf disks expressing protein(s) of interest were harvested at 2-3 days post agroinfiltration using a cork borer hole punch size of 4 or 5 and flash-frozen in LN2. Samples were ground using a Genogrinder with an LN2 cooled holder. Powder was

resuspended in 200mL of 50mM Tris pH 7.5, 50mM NaCl, 5mM MgCl₂, 10% glycerol, 10mM DTT, 1X protease inhibitor and detergent of choice (Table4).

Table4. Detergents used for protein extraction from *N. benthamiana*.

Digitonin	Mild, non-ionic
CHAPS	Zwitterionic
DDM	Non-ionic
NP40	Non-ionic
IGEPAL	Non-ionic

Following vortexing, samples were centrifuged at 15000xg for 15min, 4°C. Subsequently, BNP loading buffer was added to 50mL of supernatant. BNP loading buffer was composed of 50mL protein extract, 2.5ml BNP additive (5% original stock - 5mL if 1% digitonin is used)), 22.5ml MilliQ H₂O and 25ml of 4x Sample Buffer. Ideal sample composition varies from sample to sample. These were loaded onto Native PAGE (4-16% or 3-12%), Bis-Tris (Invitrogen) gels and ran using XCell SureLock Mini-Cell tank. Buffers used to run Native PAGE are detailed at Table5.

Table5. Native PAGE running buffers.

Native PAGE anode buffer	50mL 25X stock, 950mL H ₂ O
Dark cathode buffer	(0.02% G-250) 10mL running buffer (20X); 10mL cathode additive (20X); 180mL dH ₂ O
Light cathode buffer	(0.002% G-250) 10mL running buffer (20X); 1 mL Cathode Additive (20X); 189mL dH ₂ O

Outer and inner chambers were filled with anode and cathode buffers, respectively. Samples were run at 150V for 40 minutes in the cold room. Buffers were refreshed

and gel moved to room temperature and ran for a further 1.5 hours at 200V. Separated protein was transferred using a semi-dry system (BioRad Transblot Turbo). PVDF membrane was washed with 8% acetic acid for 15 minutes to fix protein, which was removed with further H₂O washes. The following steps were same for western blotting, previously described.

Mass spectrometry sample preparation

Proteins to be analysed were expressed in *N. benthamiana* using agroinfiltration and 1 gram of leaf sample was harvested 2 days after. Following LN2 freezing and grinding as previously described for colP assays, extraction buffer detailed in Table6 was used to harvest protein content.

Table6. Mass spectrometry sample extraction buffer.

	Concentration	Stock Conc.
<i>IGEPAL</i>	0.3%	100% (cut the tip)
<i>DTT</i>	10 mM	1 M
<i>PMSF</i>	1 mM	1 M
<i>PVPP</i>	2 % (w/v)	powder
<i>Protease inhibitor cocktail</i>	0.5%	100%
<i>GTEN (pH 8.3)</i>	Top up to final volume with GTEN buffer.	

Proteins of interest were captured on GFP beads, like described in colP and washed with wash buffer five times (with (3) and without (2) 0.15% IGEPAL in GTEN). Cleaned beads with adhered protein were incubated with Trypsin working solution (20mL trypsin stock, 100ng/uL add 780mL 50mM ABC (ammonium bicarbonate) and 10% Acetonitrile) for 10 minutes at room temperature and 37°C overnight. Produced peptides were recovered by incubation with folic acid:acetonitrile (5%:50%) solution

and sonication. Resultant supernatant was transferred to a Lo-bind Eppendorf. Peptide content was dried into powder form using a vacuum drier at room temperature. Mass spectrometry experiments and analysis experiments were performed in collaboration with Ed Tate lab member Jack W. Houghton.

Confocal fluorescence microscopy

Abaxial epidermal cells of live leaves were imaged using Leica resonance inverted confocal microscopes (FILM facility, ICL) at the 63X water immersion objective. 2 days post infiltration/infection, leaf disks were collected from *N. benthamiana* leaves and mounted between slides and cover slip using a droplet of water. GFP and RFP probes were excited using 488 and 561 nm laser diodes and their fluorescent emissions detected at 495 to 550 and 570 to 620 nm, respectively. BFP fluorophore was excited at 405nm laser diode and emissions were sequentially detected at 450-480. Maximum intensity projections of Z-stack images were presented in each figure. Image analysis was performed using ImageJ (Fiji) and Inkscape.

Quantitative analysis of fluorescent micrographs

For quantifying ATG8 puncta, maximum-intensity projection of each confocal microscopy image was generated using Fiji. The total cell surface area was measured after removing, if any, the stomata, or pavement cells from the images using the “Freehand selections” tool of Fiji, if the cells were not expressing ATG8. Max projection micrographs were split into smaller areas and puncta were counted by eye. The relative number of puncta per 10,000-15,000 μm^2 was then calculated to plot a graph. The statistical significance of any differences in means was found using the Wilcoxon test in R [42]. For co-localisation studies, the ATG8-puncta were quantified in the same

manner. ATG8-puncta and puncta formed in co-expressed protein fluorescent channel were overlayed to determine ATG8-puncta occupied by other protein of interest. This was normalised as a percentage of the total ATG8-puncta.

Infection assays using *P. infestans*

Cultures grown on RSA plates were coated with cold water and incubated at 4°C for 2 hours, to allow the release of zoospores from sporangia. Zoospore numbers were counted on a hemocytometer. The final concentration of zoospores was adjusted to 50000 spores/mL. 10uL droplets of this final solution were added onto detached leaves incubated in humid conditions in a 22°C room. 6 to 8 days following infection, leaves were imaged by a camera and the necrotic lesion was quantified using ImageJ. For infection confocal microscopy, on-plant leaf infections were performed.

Hypersensitive response (HR) cell-death assays

Constructs were co-infiltrated on to *N. benthamiana* leaves in small circles. Test and control constructs were infiltrated into the same leaf to control for inter-leaf differences in expression. HR lesions were imaged using an EOS 7D DSLR camera at 7 days post agroinfiltration. In addition, fluorescent images were taken with LiCOR Odyssey Fx at excitation wavelength 800nm. Images were analysed in ImageStudio software and ImageJ. Cell death signal caused by the syringe mark was subtracted from measurement of the whole infiltrated region to obtain the true signal at 800nm. This subtraction was also performed on the area of agroinfiltration. The true signal was divided by the true area to obtain the signal:area ratio, then normalised to the highest value. Plots and statistical analysis performed in Excel and R.

Structural and bioinformatic analysis

Protein sequences used for structural predictions were obtained from UniProt (Appendix A). Homologs of proteins used were found using BLAST [111] and their sequences were analysed using Clustal Omega [112]. AlphaFold2 (AF2) [91] and AF2-multimer [92] were used through a subscription to ColabFold, a Google developed user interface [113]. Superposition of AlphaFold2 predictions on known structures was performed using the align command in PyMOL (The PyMOL Molecular Graphics System, Version 2.3.5 Schrödinger, LLC). Predicted complexes were investigated using PyMOL and ChimeraX (UCSF). Where indicated, predictions of AIM/LIR interactions are coloured according to the AlphaFold2 produced per-residue confidence metric called the local distance difference test (pLDDT), which corresponds to the model's predicted score on the IDDT-C α metric [91]. The scale ranges from 0 to 100, where 100 corresponds to values of highest confidence. Models were coloured by this score in PyMOL using a script generated by J. Murray and D. Pretorius (Imperial College London). Viral proteomes were screened against ATG8 using the ColabFold batch mode. Structure-based homology was performed using SWISS-MODEL on the Biozentrum ExPASy database [114], DALI server [115] and FoldSeek [116].

Circular Dichroism (CD) Spectroscopy

CD spectroscopy was performed in the X-ray crystallography facility at ICL, with the help of Dr. Marc Morgan, using the Chirascan CD Spectrometer. In brief, 300uL of sample at 0.2 mg/mL was used to perform measurements under typical conditions (20°C, 190-260nm wavelength range, 0.5nm intervals, 1s per point) using a UV quartz cuvette, as previously described [55].

Negative stain Transmission Electron Microscopy (NSEM)

Samples were negative stained using 2% uranyl acetate on 300 mesh Cu or Carbon grids (Agar Scientific). Grids were plasma cleaned and incubated with sample for 1 minute. Following washes with buffer sample was in, grids were incubated with 2% UrAc. Stained sample and grids were imaged using a FEI Technai T12 Spirit transmission electron microscope, at the centre for structural biology (CSB, ICL). For the imaging of oligomers, images were obtained at 52k magnification at -2.5 defocus. For images at 100k magnification, defocus was increased to -1, as previously described [55].

Cryo-electron microscopy grid preparation and screening

Grids used in this thesis were provided by Feng Wang and David Agard (USCF). R1.2/1.3 Au Quantifoil grids were functionalised with graphene oxide (GO), PEG and maleimide [117]. SpyCatcher002-Cys was purified as previously described [109, 110]. pDEST14-Cys-SpyCatcher002 was a gift from Mark Howarth (Addgene plasmid #102829). 10mL of SpyCatcher002-Cys (10mg/mL) was incubated on grids for 10 minutes in a humid chamber (Tupperware with wet towel) to form the affinity layer of the functionalised grids. The grid was then washed with 25mM Tris (pH7.3) and 100mM NaCl (3 drop and blot). A total of 20mL 3% BSA in the same buffer was applied to grids (10mL/10min) in humid chamber to block non-specific binding to GO and PEG background. The grid was then washed with 25mM Tris (pH7.3) and 100mM NaCl (3 drop and blot). Following Spytagged sample (Joka2^{RR}-Strep-Spytag002 from leaf lysate) application, incubation, and washes (using FlowCell [118], grids were frozen using a Vitrobot Mark IV (FEI). More details on the extraction and application of protein sample can be found in Chapter 3. All grids had a blot force of -2 and Vitrobot chamber

was set to 25°C, with 95% humidity. The blot time was alternated between 4 and 5 seconds to optimise ice thickness. Data was collected on the inhouse Glacios (Thermo Fischer) (CSB, ICL) and Krios I at the electron bioimaging centre (eBIC), Diamond Light Source (DLS), Didcot. Details on collection parameters can be found at Chapter 5. Data processing was performed using CryoSPARC version 4.1 [119]. From a small subset of the dataset, Joka2 particles were picked using blob picker. Following multiple rounds of 2D classification, selected particles were used to train a TOPAZ model [120]. The whole dataset was re-picked with TOPAZ. After cleaning of particles using 2D classification, ab-initio reconstruction was used to produce a 3D model. Visualisation of models was done using ChimeraX 1.6.1 (UCSF). Models were refined using non-uniform refinement.

3. AlphaFold2-multimer guided high accuracy prediction of canonical and non-canonical ATG8-binding motifs

Summary

Autophagy is a vital cellular process for maintaining balance and defending against pathogens in eukaryotic cells. It relies on the binding of cargo receptors and adaptors to ATG8/LC3 proteins, facilitated by a conserved AIM/LIR sequence. This sequence is found in both autophagy components and pathogen virulence factors. However, due to the prevalence of the canonical AIM/LIR sequence in proteins, the identification of functional AIM/LIR motifs has posed a considerable challenge. In this context, in Chapter Three we demonstrate the efficacy of protein modelling using AF2-multimer in identifying AIM/LIR motifs, both canonical and non-canonical, with remarkable accuracy. We further reveal that AF2-multimer can be adapted to detect additional functional AIM/LIR motifs by analysing protein sequences containing mutations in the primary AIM/LIR residues. Through a comprehensive approach that combines protein modelling, phylogenetic analysis of protein sequences, and protein-protein interaction experiments, we provide evidence that AF2-multimer accurately predicts the physiologically relevant canonical AIM motif in the ATG8-interacting protein 2 (ATI-2) and unveils a previously uncharacterized non-canonical AIM motif in ATG3. Notably, our pipeline identifies AIM/LIR motifs within virulence factors. This finding suggests that AF2-multimer can predict cross-kingdom ATG8-LIR/AIM associations. In conclusion, our study posits that the AF2-guided exploration of autophagy adaptors and receptors will significantly expedite our comprehension of the molecular underpinnings of autophagy across diverse biological domains.

Introduction

AIM/LIR-ATG8/LC3 interactions

ATG8-related protein interactions are mediated through a crucial structural motif known as the AIM/LIR (ATG8-interacting motif/LC3-interacting region). AIM/LIRs play a universal role in binding to the evolutionary conserved ATG8 LIR docking site (LDS) [121]. This LDS is two pockets formed by the N-terminal arm and the UBL (ubiquitin-like) domain of ATG8 proteins, highlighting its central role in mediating these interactions. Structural studies involving isolated AIM/LIR peptides bound to ATG8 proteins have consistently revealed that AIM/LIRs typically adopt an extended β -sheet conformation when binding to the LDS. The core AIM/LIR motif, (W/Y/F)₀-(X₁X₂)-(L/I/V)₃, is essential for this interaction. Hydrophobic residues within the core AIM/LIR, with TRP in the X₀ position, exhibit particularly strong binding. In fact, replacing Y with W in the NBR1 LIR motif resulted in a remarkable 7.5-fold increase in binding affinity [46]. Additionally, the X₁ and X₂ positions within the core LIR are typically occupied by acidic or hydrophobic residues, with occasional allowance for basic residues at the X₂ position. Notably, P and G residues, which could disrupt the β -sheet structure of the AIM/LIR when complexed with the LDS, are usually absent from the X₁ and X₂ positions, ensuring structural stability.

The determinants of selectivity in ATG8 proteins are partially understood. The amino acids surrounding the LDS can vary between ATG8 proteins, which may lead to AIM/LIR recognition specificity. The core AIM/LIR is flanked by N- and C-terminal sequences that contribute significantly to binding affinity. Interestingly, residues immediately N-terminal to the core AIM/LIR often include acidic D or E, as well as S

or T residues that can undergo phosphorylation [45]. Approximately 25% of LIR motifs feature an S or T residue in the pivotal X_{-1} position, further emphasizing the diversity within these motifs. N-terminal acidic residues near the core AIM/LIR are functionally significant. These acidic residues engage in electrostatic interactions with basic residues within the LDS, further enhancing the binding specificity. Moreover, several ATG8-interacting proteins feature C-terminal extended AIM/LIRs, which play a crucial role in enhancing binding strength and specificity. For instance, ULK1 and ATG13 LIRs contain C-terminally extended regions that specify their preference for binding to the GABARAP superfamily, showcasing the versatility of AIM/LIR interactions [122]. In species expressing functionally distinct ATG8s, selectivity is finely regulated by subfamily or subset-specific salt bridges, hydrogen bonds, or hydrophobic interactions. Many LIR motifs include a negatively charged residue in the X_{+7} position, facilitating electrostatic interactions with conserved R residues [121]. This not only ensures selectivity but also increases binding affinity. Lastly, non-canonical AIM/LIR sequences introduce intriguing variations, lacking either the aromatic X_0 residue or the hydrophobic X_{+3} residue. A notable example is found in NDP52 (ILVV), which lacks the aromatic X_0 residue, preventing engagement with HP1 but exhibits selective and robust binding to LC3C [47]. Indeed, the presence or absence of specific residues within AIM/LIRs can profoundly impact their binding affinities and selectivity, underscoring the intricate nature of AIM/LIR-mediated autophagy-related protein interactions.

Challenges in studying ATG8-interacting motifs and AIM-containing proteins

For the progression of autophagy, adaptors, and modulators of ATG8 must interact with ATG8 in a hierarchical, transient manner to allow numerous ATG8 interactions.

Proteins that interact with ATG8 for their autophagic function must contain an AIM/LIR sequence. Various prediction algorithms have been developed to identify these short sequences in proteins, such as LIRcentral [123], iLIR [124] and hfAIM [125]. These perform well in predicting canonical AIM/LIRs. However, they usually produce many candidates, with the number of candidates correlated to the length of the amino acid sequence of the protein of interest. In the core, AIM/LIRs are four-residue motifs and match the consensus (W/Y/F)-(XX)-(L/I/V), therefore, there are many candidates in a protein sequence that can match this. The predicted typical motifs are ranked based on a confidence score, but ATG8-binding is usually mediated through multiple AIM/LIRs. Hence, this means that many candidates are screened in the wet lab to determine the functional AIM/LIR(s), which significantly slows down research progress. The functionality of motifs relies on their structural characteristics, such as being in disordered regions and not constrained to secondary structure [39]. The websites mentioned do not provide structural information in their predictions and lack a visual output with ATG8-variants, where the motif is seen to occupy the hydrophobic pockets. Non-canonical AIM/LIRs cannot be predicted using these platforms, which has hampered their identification and limited most of our knowledge to proteins with canonical motifs. Identification of atypical sequences has relied on nuclear magnetic resonance (NMR), crystal structure analysis, various truncations and mutants and activity assays with the cloned variants of the protein of interest. A technology where binding assays can be repeated many times with various truncations and mutants will limit time spent in finding interactors and allow discovery of new information in ATG8-binders and its consequences of autophagic function.

Results

AF2-multimer predicts experimentally validated canonical AIM/LIR motifs with high accuracy

Amino acids forming the hydrophobic pockets of ATG8 that are occupied by the AIM/LIRs are conserved among the ATG8 family of proteins [40]. This observation indicates that there are consistent binding patterns that regulate ATG8-LIR/AIM interactions, and AF2-multimer may have the capability to predict these patterns. To determine the capacity of AF2-multimer in predicting AIM/LIR motifs, we decided to run AF2-multimer using a subset of proteins that carry experimentally validated, canonical (W/Y/F)-(XX)-(L/I/V) AIM/LIR motifs along with their corresponding ATG8 from plants, humans, and yeast.

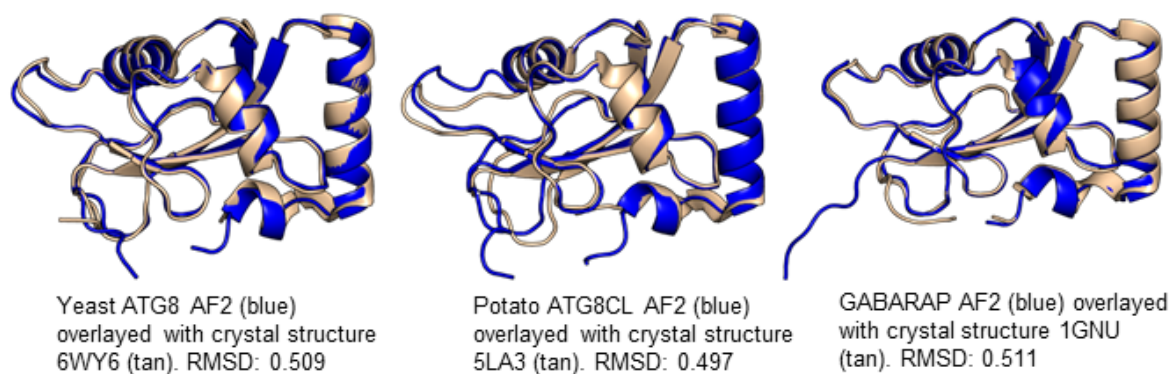


Figure7. AF2-multimer predicted ATG8 models. Predictions for yeast ATG8, potato ATG8CL and human GABARAP have an RMSD of close to 0.5Å compared to their crystal structures 6WY6 [126], 5L83 [127] and 1GNU [128], respectively.

We initially compared the ATG8 models generated in AF2-multimer to previously determined crystal structures of respective ATG8s. AF2-predicted models of plant ATG8CL, human GABARAP, and yeast ATG8 proteins display highly similar backbone

and side chain conformations to wet-lab determined structures. All tested ATG8 AF2-models have root mean square deviation (RMSD) scores close to 0.5Å (ScATG8: 0.51Å; HsGABARAP: 0.51Å; StATG8CL: 0.50Å), suggesting AF2-multimer can be used to identify ATG8-binding proteins (Figure7). We tested 36 proteins with experimentally validated canonical AIM/LIRs and AF2-multimer predicted ~90% of them accurately (33 out of 36 – Appendix A). AIM/LIRs are found in structurally disordered regions, which allows flexible and dynamic protein interaction interfaces. AF2-multimer predicted ~70% of our total dataset to be disordered at their AIM/LIR (Figure8). Of the partially disordered 15 candidates, 13 were experimentally validated, however, AF2-multimer could only predict 10 (Appendix A). All AF2-multimer predictions done for this work can be found at Appendix F.

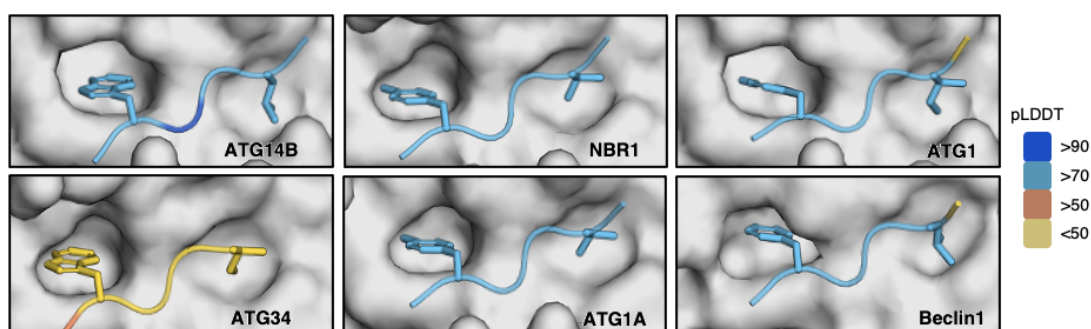


Figure8. AF2-multimer predicted AIM/LIR sequences are predominantly found in intrinsically disordered regions. In complex with ATG8, AIM/LIRs can be predicted with high confidence, as opposed to low confidence models in the absence of ATG8 binding. ATG8-family are coloured in grey. The AIM/LIR of ATG14B, NBR1, Beclin1 (human), ATG1, ATG34 and ATG1A (yeast) are coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low).

Although AF2-multimer showed high accuracy with most of our dataset, it failed to predict experimentally identified AIM/LIR motifs of plant cargo receptor Joka2 and

human Calreticulin proteins. To determine why, we analysed the confidence scores determined by the predicted local distance difference test (pLDDT) at the interface. Joka2's AIM sequence (WDPI) had a low score threshold (~50), which could account for the failure (Figure9C). However, the Calreticulin LIR had a good score (>70) and AF2-multimer still could not place the LIR into the hydrophobic pockets of ATG8 (Figure9B). We then assessed whether this is a consequence of the AF2-model for Calreticulin being incorrect. We compared the prediction to the crystal structure 3O0W [129] by superimposition and saw a high correlation accounting for an RMSD of 0.33Å (Figure9). Together, these indicate that AF2-multimer's inability to identify the functional LIR is not a consequence of structural mis-prediction.

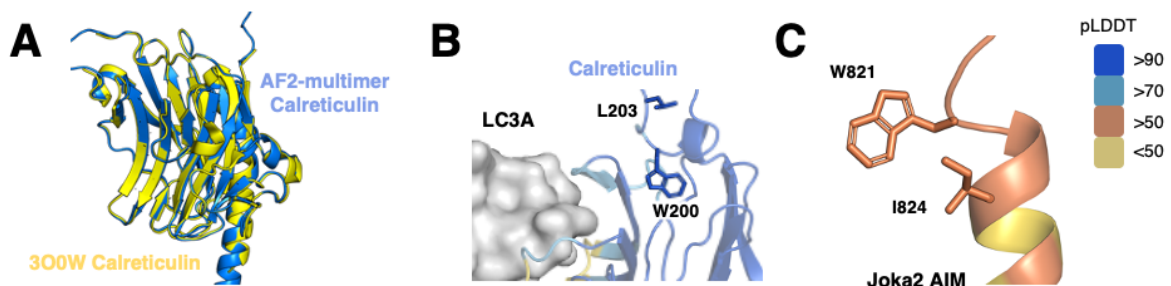


Figure9. The AIM/LIR sequences of Calreticulin and Joka2 are confined between structured regions. (A) The 3O0W crystal structure (yellow) of Calreticulin aligned with the AF2-multimer model (blue) to demonstrate high accuracy. (B) The typical validated AIM of Calreticulin (W₂₀₀XXL₂₀₃) is not found near the LDS of LC3A. (C) The Joka2 AIM, W₈₂₁XXI₈₂₄, is found in a structured region. LC3A is coloured grey. For panels B and C, Calreticulin and Joka2 are coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low).

Upon closer inspection of Joka2 and Calreticulin models, we observed that the AIM/LIRs were embedded in short, disordered regions (5 and 6 amino acids long, respectively) that are flanked by α -helices and β -strands, respectively (Figure9B/C).

Hence, ATG8 docking might be hindered by the limited flexibility in these regions. In cases like these, AF2-multimer predictions most likely fail due to additional requirements for AIM/LIR-ATG8 association, such as posttranslational modifications, intramolecular interactions, or interactions with other proteins, which may relax this interface. In this context, DVL2 is an interesting example in our dataset. AF2-multimer revealed a novel canonical LIR, FVDV, in the human autophagy substrate, as opposed to the experimentally discovered WLKI [130]. Gao et al. 2010, demonstrated that mutating the WLKI motif to ALKA, hindered, but did not prevent, LC3 binding. This suggests additional LC3-interacting regions in DVL2, as indicated by AF2-multimer. The AF2-determined FVDV LIR is in the C-terminal disordered region, whereas the WLKI motif is constricted between two β -strands of DVL2 (Figure10).

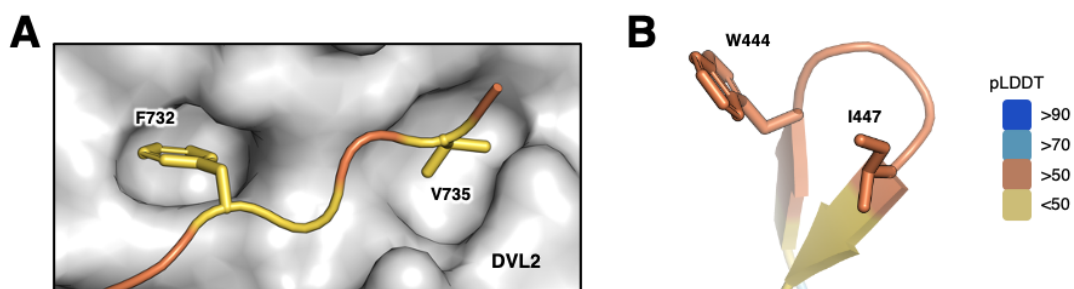


Figure10. AF2-multimer predicts an alternative LIR for DVL2. (A) AF2-multimer prioritises the LIR FVDV in the ATG8 complex. The LIR is predicted with low confidence. . ATG8 is coloured in grey. (B) The typical validated LIR of DVL2 is in the loop of a β -sheet. DVL2 is coloured based on the pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low).

This is a similar situation to that we observed for Joka2 and Calreticulin and may explain why AF2-multimer did not identify this sequence as the primary interface between LC3 and DVL2 and showed a preference to FVDV. This LIR has not been

validated in the wet-lab and requires further research. Overall, using a pool of proteins with validated canonical AIM/LIRs, we established AF2-multimer as a powerful tool for predicting canonical AIM/LIR sequences with a high level of accuracy [42]. We also demonstrate the limitations to this approach, such as conditional ATG8 binding due to post-translational modifications and emphasise the need for further experimental validation of all predictions.

AF2-multimer can predict multiple AIM/LIR motifs

AF2-multimer demonstrated great accuracy in predicting interfaces in proteins with a dominant AIM/LIR. Next, we were interested in the performance of AF2-multimer in cases where multiple AIM/LIRs, that are not restricted by surrounding secondary structure, act to regulate ATG8 binding. To this extent, we investigated the human E3 ubiquitin ligase NEDD4 [131] with two validated LIRs and plant ATG8-interacting protein 2 (ATI2) [132], which had two predicted AIMs that we set-out to validate.

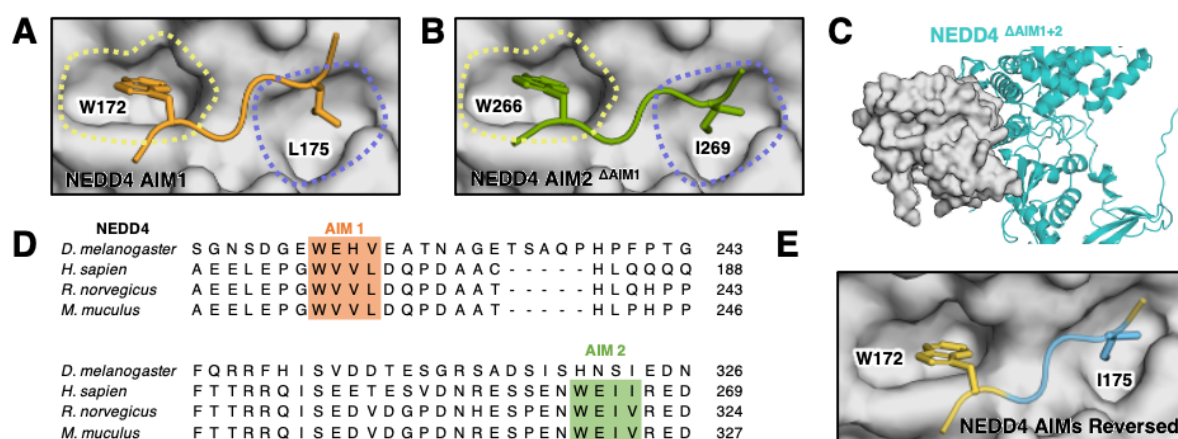


Figure11. NEDD4 has two functionally validated LIRs and AF2-multimer prioritises the upstream LIR. However, will not predict false motifs once both LIRs are truncated or mutated. AF2-multimer prioritises the upstream LIR (AIM1) found in NEDD4. **(A)** AIM1 of NEDD4 (orange). **(B)** AIM2 (green), the previously validated AIM,

is identified upon the truncation of AIM1. Dotted lines indicate hydrophobic pockets of ATG8. **(C)** Once both AIMs are removed, AF2-multimer does not predict an LIR-LDS interactions. **(D)** Both AIM1 (orange) and AIM2 (green) are highly conserved. This alignment was generated using Clustal Omega. **(E)** When AIM1 and AIM2 are swapped around, AF2-multimer prioritises the upstream, now AIM2, AIM. This prediction is coloured based on the pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low).

NEDD4 was previously reported to carry two LIR motifs that bind LC3B. Using deletion analysis, Sun et al. 2017 showed that LIR1 (WVVL) was not necessary for LC3 binding, whereas the deletion of both LIR1 and LIR2 (WEII) impaired LC3 binding. This suggests LIR2 is more critical for LC3 binding, however, it is possible LIR1 is also functional. AF2-multimer predicted LIR1 over LIR2 in the pockets of LC3B (Figure11A). To determine if AF2-multimer could also place LIR2 into the pockets, we truncated LIR1 and re-ran the prediction. Like previously shown, LIR2 was in the LDS of LC3B (Figure11B). During experimental validation, both LIRs were truncated to demonstrate complete loss of LC3B interaction with NEDD4 [131]. To validate this in AF2-multimer, we re-ran the prediction with a NEDD4 sequence lacking both LIRs. As expected, no interaction interface was predicted (Figure11C). Hence, AF2-multimer would not predict alternative or non-functional, false AIM/LIRs. To determine why AF2-multimer prioritised LIR1 over LIR2 we performed phylogenetic analysis of both LIR sequences. This revealed that both are conserved (Figure11D). Consistent with this and our predictions, a different study using surface plasmon resonance (SPR) showed that both LIR1 and LIR2 peptides bind LC3B with similar dissociation constants [133]. In the previous study, various truncations were made to NEDD4 to determine where the functional LIR is located, hence the LIR1 truncate lacked another functional domain within proximity. LIR activity can be dependent on binding and actions of other domains

[134], which can explain why LIR1 was deemed not essential. Whereas with LIR peptides, there is no restrictive confirmation and usually they show greater affinity compared to full-length proteins. We next assessed whether this prioritisation of LIR1 is a consequence of it being upstream of LIR2 in the NEDD4 sequence. For this, we swapped the two LIRs. LIR2 (X₄WEIIX₄) replaced LIR1 (X₄WVVLX₄) and vice versa in the sequence. This swap resulted in AF2-multimer predicting LIR2 (now LIR1 in sequence) as the primary LIR (Figure11E). Hence, AF2-multimer prioritises the first functional AIM/LIR in protein sequences. Therefore, whilst studying AIM/LIR candidates with AF2-multimer, it is necessary to repeat predictions with truncated or mutated AIM/LIRs, to determine all functional candidates.

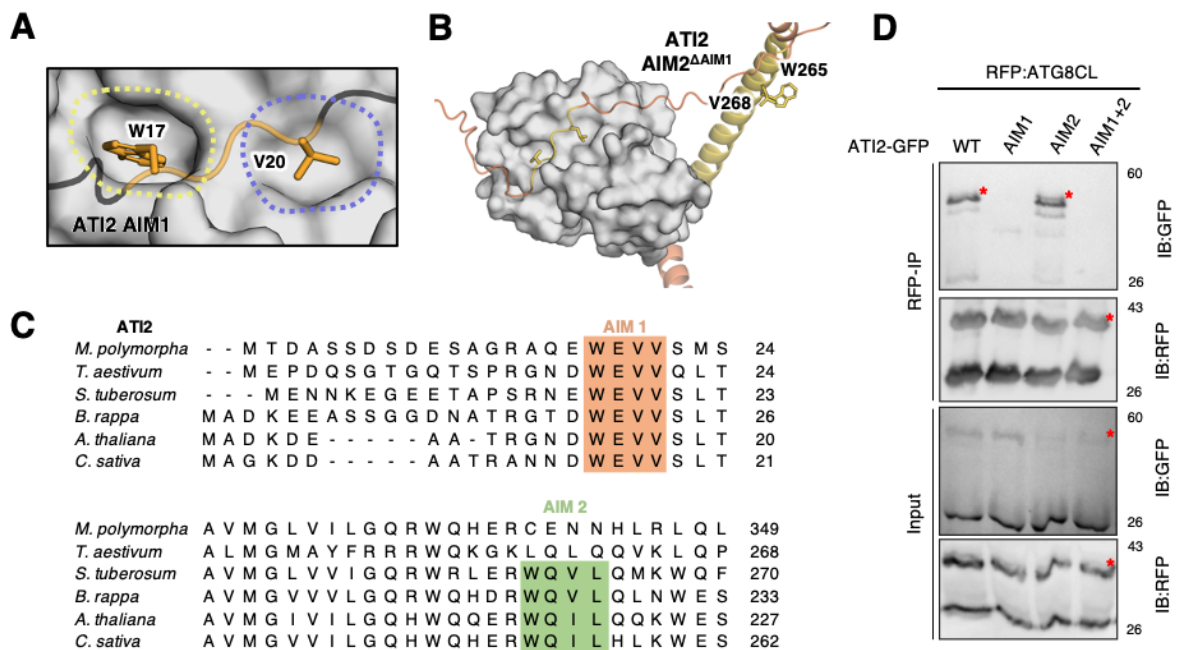


Figure12. AF2-multimer accurately predicts the functional AIM found in AT12 from multiple candidates. (A) AT12 AIM1 in LDS of ATG8CL (grey). Dotted lines indicate hydrophobic pockets of ATG8. **(B)** Once AIM1 is mutated, AF2-multimer predicts an alternative AIM and not the previously predicted AIM2, which is localised to an α -helix. **(C)** AIM1 is conserved in all plant species screened, whereas AIM2 is less conserved. **(D)** Binding assay with RFP-ATG8CL and AT12 variants (WT, AIM1

mutant, AIM2 mutant, AIM1+2 double mutant). AIM2 mutation does not alter ATI2-ATG8 interactions. Red asterisks indicate expected band size. Uncropped blots – Appendix B.

This approach was then applied to the plant protein ATI2, which has two predicted AIMS. A previous yeast-two-hybrid analysis of ATI-2 and ATG8F proteins from *A. thaliana* indicated that the N-terminal AIM1 in ATI2 is necessary and sufficient for ATG8 interaction [135]. We examined whether AF2-multimer could predict the functional AIM of potato ATI2 and supported this with the biochemical characterisation of the interface. Consistent with the published yeast-two-hybrid model, AF2-multimer identified AIM1 of potato ATI2 as associating with the pockets of potato ATG8CL variant. Following truncation of AIM1, we repeated the prediction and observed no interface between downstream ATI2 AIM2 and ATG8CL (Figure12A/B). The second AIM of ATI2 is placed in an α -helix and not the typical random coil for functional AIM/LIRs, which could explain why it is not necessary for ATG8 interactions. MSA analysis revealed that AIM1 is highly conserved but AIM2 was only conserved in closely related dicot plants but not in distantly related plant species (Figure12C). All together, these suggest that AIM2 may not be a functional AIM. To validate the AF2-multimer predictions, we generated GFP-tagged ATI2 constructs harbouring AIM1 (WEVV to AEVA), AIM2 (WQVL to AQVA) and AIM1+2 double mutations (AEVA and AQVA) in plant cells. We performed a co-immunoprecipitation assay with RFP-ATG8CL and validated that the AIM1 and AIM1+2 double mutants ATI2 cannot interact with ATG8CL, confirming AIM1 (WEVV) as the primary functional motif. However, as expected, the AIM2 mutant could interact with ATG8CL like wild type ATI2 (Figure12D). Overall, AF2-multimer prioritises the first functional AIM/LIR in a protein containing

several functional AIM/LIRs but will not predict false AIM/LIRs, as shown in the case of NEDD4 and AT12, respectively.

Previously we demonstrated the power of AF2-multimer with proteins harbouring two functional AIM/LIRs. Next, we wanted to determine the boundaries of AF2-multimer and investigated the human RTN3 protein, which was reported to have six functional LIRs that could interact with the GABARAP-family [136]. AF2-multimer identified LIR3 (FEVI) as the main LIR in our first run with RTN3 and GABARAP-L1, which was in line with previous structural data. However, when we re-ran AF2-multimer with an *in silico* LIR3 truncated RTN3, we did not observe any GABARAP interaction. Our phylogenetic analysis showed that only LIR3 was conserved and the remaining LIRs were not. This is a potential limitation to our AF2-multimer approach as we screen the outputs using conservation analysis and AF2-multimer predicts the structures based on an initial MSA it generates in the first place.

AF2-multimer can predict noncanonical AIM/LIRs

Current prediction software lacks the ability to predict non-canonical AIM/LIRs, which has hampered their discovery. To determine if AF2-multimer could overcome this challenge, we used NDP52 and TAX1BP1 proteins with previously characterised non-canonical LIRs as a model for multimer predictions. AF2-multimer accurately identified the validated noncanonical LIR motifs of NDP52 (ILVV) and TAX1BP1 (MLVV) with high confidence. For a typical interaction the first hydrophobic pocket is occupied by an aromatic residue. Here, I and M residues occupy these pockets, and the final V occupies the latter pocket of LC3. Our predictions also showed the central LEU and VAL residues forming additional contacts with surrounding hydrophobic surfaces. In

addition to NDP52 and TAX1BP1, we ran a prediction with ATG40, which is a cargo receptor for specialised autophagy of the endoplasmic reticulum (ER-phagy). ATG40 carries an atypical YDFM, where the second pocket is occupied by an unusual MET residue [137]. AF2-multimer was also successful in this case and we show that it can extend its ability to predict functional AIM/LIRs to identify non-canonical interactors (Figure13).

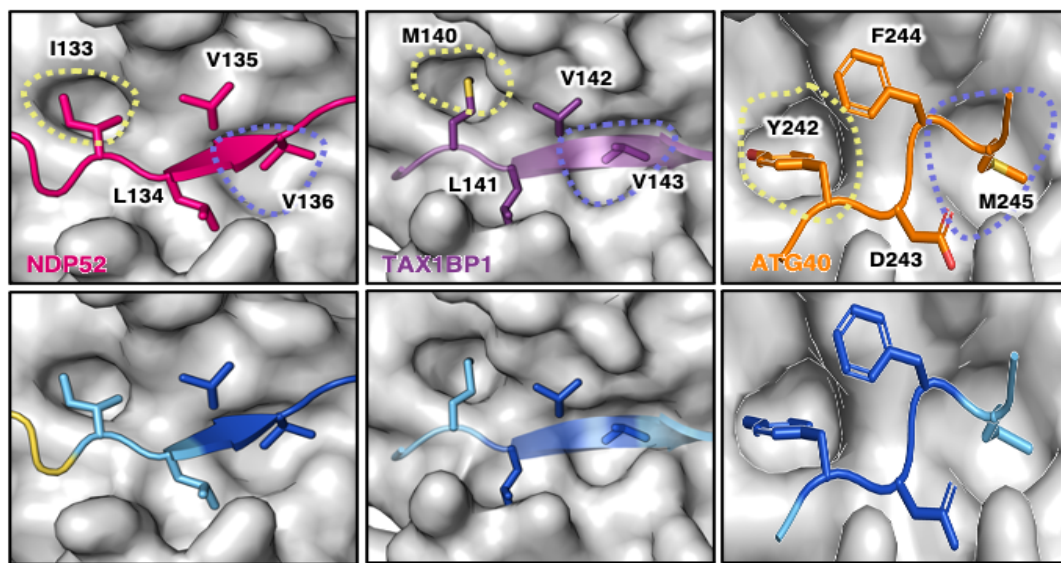


Figure13. AF2-multimer can accurately predict experimentally validated non-canonical AIM/LIRs with great accuracy. The top three panels show NDP52 (pink), TAX1BP1 (purple) and ATG40 (orange) with ATG8 (grey). The lower panels show the same interface coloured based on the pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). Dotted lines indicate hydrophobic pockets of ATG8.

In plants, the *A. thaliana* C53 was shown to bind ATG8 through several shuffled (non-canonical) AIMs with the consensus sequence IDWG/D as well as the canonical YEIV [138]. AF2-multimer predictions yielded an interface between the canonical YEIV and ATG8 pockets. Upon the in-silico mutation of YEIV to AEIA, AF2-multimer predicted one of the shuffled AIMs in C53 as the ATG8 binder. The aromatic TRP residue of the

shuffled AIM occupied the W-site as expected. However, the second pocket was occupied by a neighbouring V (WDVSV), suggesting an additional non-canonical AIM neighbouring the shuffled AIM (Figure14).

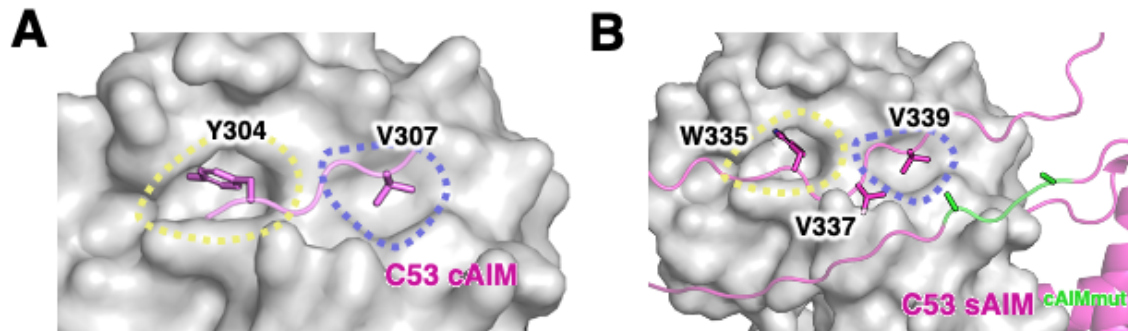


Figure14. AF2-multimer predicted AtC53-ATG8 interface. (A) Although AtC53 was validated to interact with ATG8 via shuffled AIMS, AF2-multimer predicts a canonical YXXV AIM. (B) Once the canonical AIM is mutated, AF2-multimer can predict an extended version of the shuffled AIM found in C53. ATG8 is coloured in grey. C53 is shown in pink, and the canonical AIM (cAIM) mutant is shown in green. Dotted lines indicate hydrophobic pockets of ATG8.

Considering the success of AF2-multimer in predicting both canonical and atypical motifs, we next applied this pipeline to previously uncharacterised AIM/LIR residues. We chose ATG3 as a case study, as it is known to interact with ATG8 to mediate its lipidation, but how remains controversial in different organisms. In yeast, a canonical AIM was reported to mediate this interaction [139]. However, this motif is conserved in neither plants nor humans. Interestingly, AF2-multimer predicted a non-canonical AIM/LIR in plants and humans with the consensus W-XX-I sequence (Figure15). This prediction is consistent with the atypical motif found in the malaria parasite ATG3 [140].

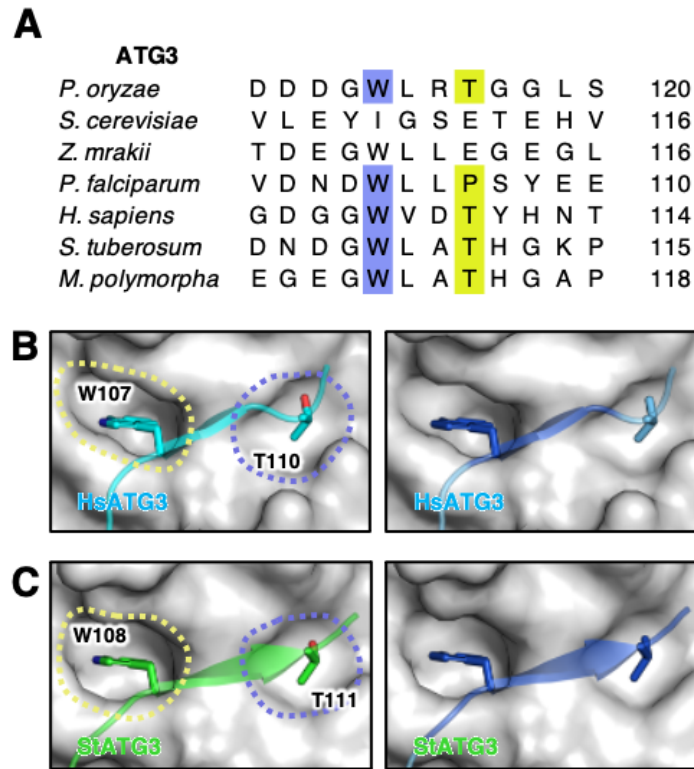


Figure15. ATG3 is predicted to carry a conserved atypical AIM by AF2-multimer.

(A) The non-canonical AIM is conserved in yeast, plants, and mammals. **(B/C)** W occupies hydrophobic pocket one and a charged THR the second in both humans and potato. Left hand panel of B and C show human and yeast ATG8 coloured in cyan and green, respectively. Right hand side panels are coloured based on the pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). Dotted lines indicate hydrophobic pockets of ATG8.

To validate our observation, we cloned the wild type and mutant AIM sequence region of potato ATG3 as a peptide ($X_{17}\underline{W}LATX_4$ or $X_{17}\underline{A}LAAX_4$) attached to an RFP tag and performed pull-down assays with the potato ATG8CL in plant cells. Wild-type ATG3 AIM peptide binds ATG8, and this interaction was severely disrupted in the mutant peptide, validating the AF2-multimer model (Figure16). Recently, this non-canonical ATG3-ATG8 interaction was validated in humans using X-ray crystallography, also corroborating our results (Farnung et al. 2023).

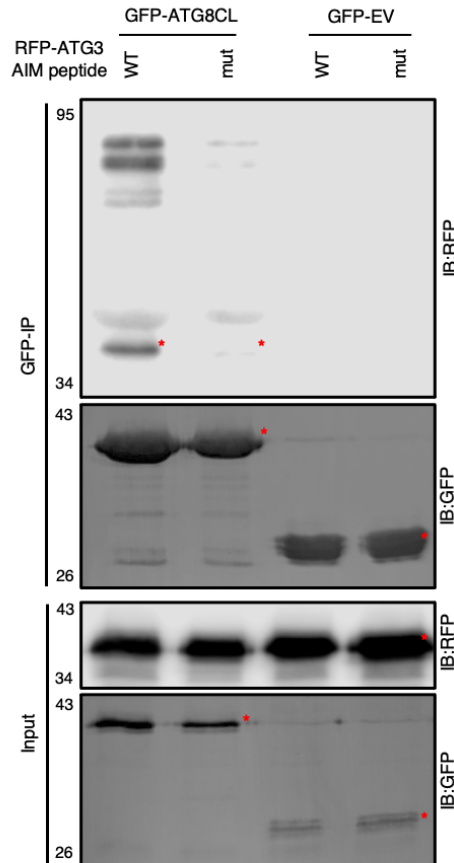


Figure16. ATG3 carries a functional non-canonical AIM as predicted by AF2-multimer. Either GFP-ATG8CL or GFP-control were co-expressed with RFP-ATG3 peptides of the AIM region (WXXT) and its mutant (AXXA). No interaction was observed in the control lanes. The AIM mutant shows significantly less ATG8 interaction compared to wild type peptide. Red asterisks indicate expected band size. Uncropped blots – Appendix C.

Previously, our lab demonstrated that overproduction of functional AIM peptides blocks autophagosome formation in plants [108], which we use as proxy to estimate AIM peptide-ATG8 interaction in planta. Overexpression of the ATG3 wild type peptide, reduced ATG8CL puncta numbers, blocking autophagy via saturating ATG8 pockets (Figure17).

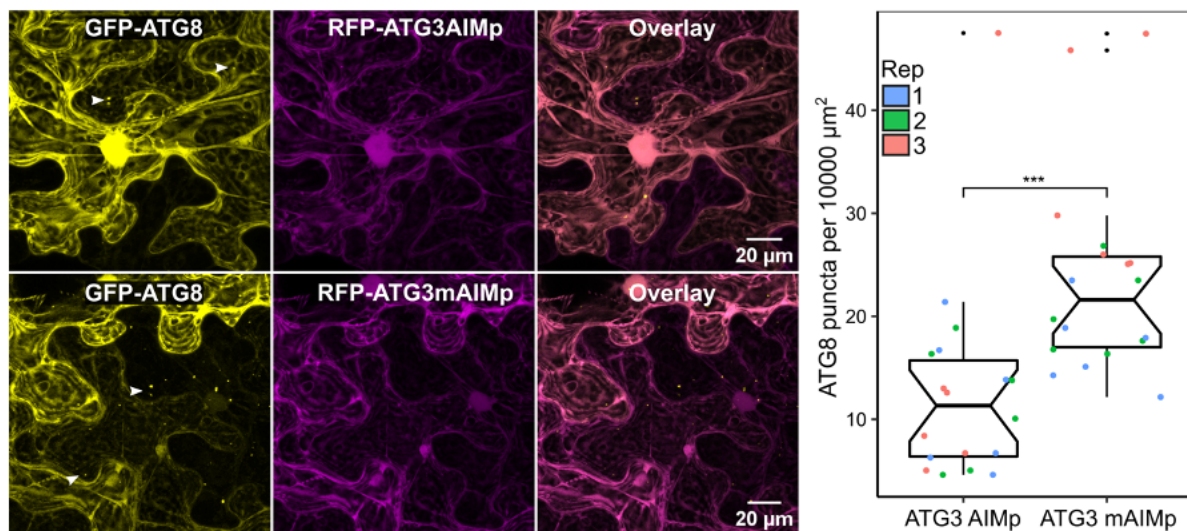


Figure17. ATG3 AIM peptide block autophagosome biogenesis, suggesting strong ATG8 interactions. Fluorescent micrographs from left to right: GFP-ATG8 (yellow), RFP-ATG3 peptide (pink) and Overlayed. White arrows indicate example puncta that was counted with different sizes. Images shown are maximal projections of 18 frames with 1 μ m steps. Quantification of ATG8CL autophagosome puncta per 10,000 μ m² with WT and mutant (m) ATG3 AIM revealed a significantly higher number of puncta in sample co-expressed with mutated peptide compared to WT. A Wilcox test using RStudio determined the puncta number were significantly different ($p < 0.01$). Raw files with puncta numbers and analysis RStudio code – Appendix D and E, respectively.

These results demonstrate the non-canonical binding pattern of ATG3 and re-emphasises the power of AF2-multimer in predicting both canonical and non-canonical AIM/LIRs. We also show, when combined with phylogenetic and molecular analysis, this AI-guided approach can provide new autophagy understanding.

AF2-multimer predicts cross-kingdom AIM/LIR-ATG8 interactions in host–pathogen interactions

Plant and human pathogens deploy intracellular effectors to target host autophagy machinery. We investigated whether AF2-multimer could identify cross kingdom ATG8-

AIM/LIR contacts during host-pathogen interactions. To this extent, we ran predictions with three pathogenic effectors that were discovered to manipulate host ATG8/LC3 proteins utilising experimentally validated AIM/LIRs. This included the RavZ protein secreted into human cells by the bacterial pathogen *Legionella pneumophila* [134], Matrix protein 2 (M2) deployed by the Influenza A virus [141], and the PexRD54 protein secreted into plant cells by the Irish potato famine pathogen *P. infestans* [56, 127]. These were all established to carry typical AIM/LIRs and AF2-multimer successfully predicted the correct residues occupy the hydrophobic pockets of the ATG8 family (Figure18).

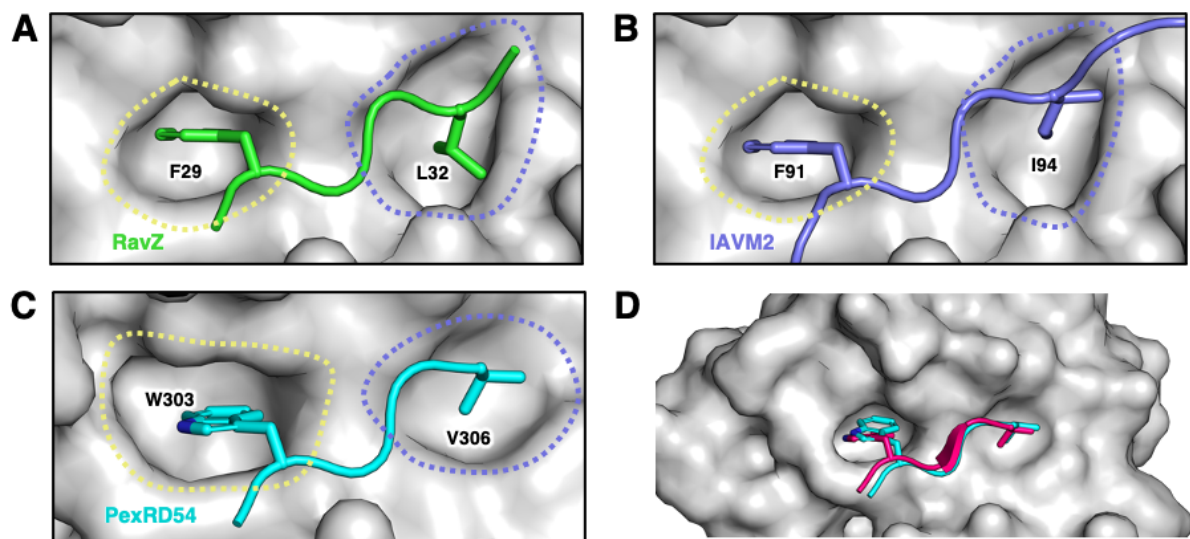


Figure18. AF2-multimer can predict cross-kingdom AIM/LIR-ATG8 interactions. (A-C) AF2-multimer models for RavZ (green) from malaria, M2 protein (slate blue) from influenza and PexRD54 (cyan) from the late blight pathogen, respectively. (D) Overlay of PexRD54 AIM peptide crystal structure (5l83 (pink) – Maqbool et al. 2016) with the AF2-model (cyan) showing high accuracy of AF2-multimer. ATG8 variants are coloured in grey. Dotted lines indicate hydrophobic pockets of ATG8.

From two and eight AIM/LIR candidates in PexRD54 and RavZ, respectively, AF2-multimer was able to identify the functional AIM/LIR validated experimentally, which

re-emphasises its ability to distinguish between functional and non-functional AIM/LIRs among multiple candidates. For PexRD54, the predicted model perfectly aligned with a crystal structure solved for the effector AIM peptide and ATG8 (Figure18D). Most commonly, the highest ranked AF2-model for the prediction would have an accurate AIM/LIR-LDS interface between the validated candidates. However, in the case of M2, the highest ranked AF2-model does not show a proper LIR (FVSI) fit in the pockets, although other models have the residues PHE and ILE perfectly placed at the ATG8 interface (Figure19).

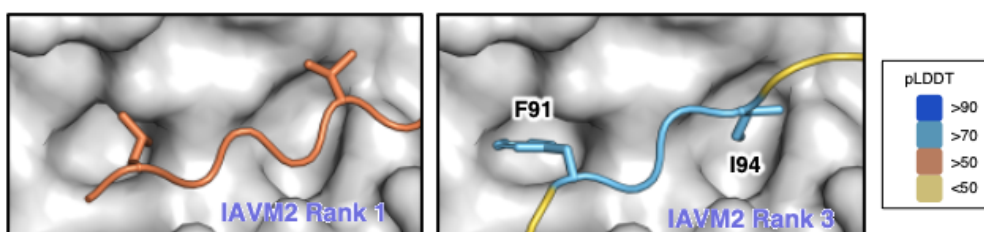


Figure19. Highest ranked AF2-multimer model does not predict a correct interface between M2 protein from influenza and LC3. The third highest ranked model has a high accuracy prediction of the experimentally validated canonical FXXI AIM. M2 protein is coloured based on the pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). LC3 is coloured in grey.

The confidence score for the LIR-LC3 interface in the highest ranked model was much lower compared to the other models (Figure19). This suggests that the confidence scores around the AIM/LIR region are more reliable compared to the overall protein model when we are using this tool to determine AIM/LIR motifs. Furthermore, we conducted an analysis on the virulence factor PbUIS3 in *Plasmodium berghei*. PbUIS3 does not possess a typical LIR motif. Instead, it forms a connection with LC3 through an undisclosed and concealed interface [142].

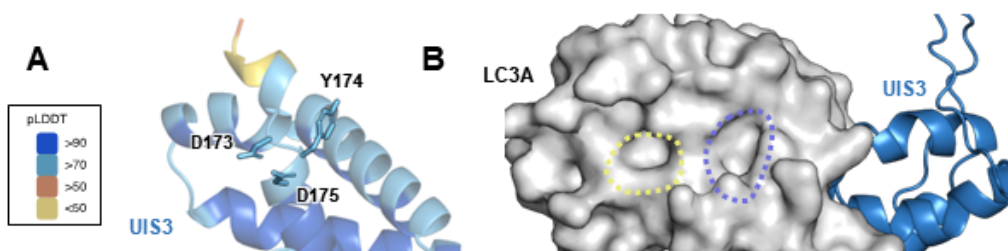


Figure20. UIS3 validated LDS contacts cannot be predicted by AF2-multimer. (A)

The experimentally studied residues D173, Y174 and D175 are localised to α -helices and as per limitations of AF2, **(B)** are not predicted at the AIM/LIR-LDS interface. LC3A is coloured in grey. UIS3 is coloured based on the pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). Dotted lines indicate hydrophobic pockets of ATG8.

Nevertheless, when we examined the AF2-multimer models, none of them displayed a connection between PbUIS3 residues and the surface pockets of LC3. Instead, they indicated the presence of an alternative binding interface. Based on these models, the previously identified PbUIS3 residues (D173, Y174, D175, E205, and K209), known to be crucial for LC3 binding, are situated within two distinct α -helices (Figure20A). Importantly, these α -helices do not interact with LC3A (Figure20B), suggesting that mutations in these locations might indirectly hinder LC3 binding. Despite limitations, such as conditional ATG8 binding that can prevent AIM/LIR predictions, our work demonstrates that AF2-multimer can accurately predict cross-kingdom AIM/LIR–ATG8 interactions.

Discussion

We present evidence that AF2-multimer excels at predicting both typical and atypical AIM/LIR motifs with remarkable accuracy. In a dataset comprising 36 proteins, each carrying at least one experimentally confirmed canonical AIM/LIR motif, AF2-multimer accurately identified 33 functional AIM/LIR motifs from a pool of 310 candidate

sequences that matched the canonical AIM/LIR consensus (W/Y/F)-(XX)-(L/I/V). AF2-multimer also demonstrates the capability to predict multiple functional AIM/LIR motifs within a single protein. However, it tends to prioritize the N-terminal AIM/LIR. Nevertheless, this limitation can be overcome by re-running AF2-multimer using truncated or mutated sequences of the initial AIM/LIR. AF2-multimer accurately predicted four out of five experimentally validated noncanonical AIM/LIR motifs. Current AIM/LIR prediction algorithms could not predict non-canonical AIM/LIR motifs. AF2-multimer accurately identified a previously unknown functional noncanonical AIM motif in potato ATG3, which we experimentally validated through ATG8 binding and autophagy assays. Moreover, AF2-multimer performed well in identifying AIM/LIR motifs in three out of four tested pathogen virulence factors targeting ATG8 in both plant and human hosts, revealing its ability to predict cross-kingdom ATG8–LIR/AIM associations. AF2-multimer proves highly effective in discerning true AIM/LIR motifs from numerous other non-functional motifs within a protein of interest, minimizing the need for experimental validation. These predictions can be bolstered by incorporating evolutionary insights from phylogenetic analyses of protein sequences to assess AIM/LIR conservation, particularly when planning time-consuming validation experiments. The AF2-guided AIM/LIR prediction workflow encompasses structural prediction of ATG8 and candidate proteins, phylogenetic analysis of predicted AIM/LIR motifs, and experimental validation through in vitro/in vivo binding assays (Figure 21).

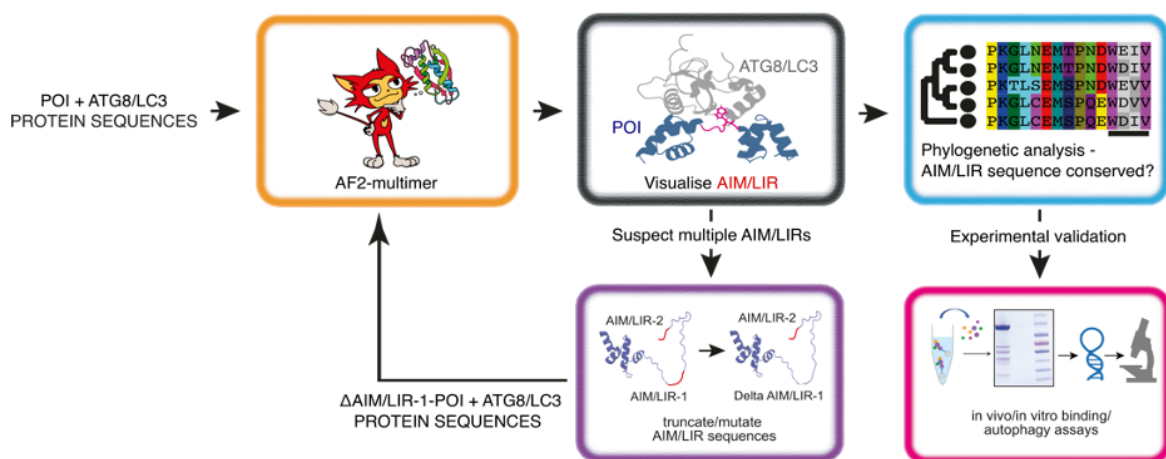


Figure21. AF2-multimer pipeline for AIM/LIR discovery. We run AF2-multimer predictions in the ColabFold document with ATG8 and a sequence of interest. The initial prediction is validated via various rounds of predictions where AIM/LIRs are truncated. Predicted AIM/LIRs are then assessed based on their conservation. AF2-multimer predicted conserved AIM/LIRs are then validated using in vivo and in vitro binding and autophagy assays. *Adapted from [42].*

One significant advantage of AF2-multimer is its ability to offer spatial resolution of the AIM/LIR-ATG8 interaction. It not only provides structural alignment of LIR/AIM residues on the AIM docking site but also highlights additional associations through flanking LIR/AIM residues. This aspect is crucial for understanding the role of flanking residues in AIM/LIR specificity towards ATG8 isoforms, contributing valuable insights into evolutionary studies on autophagy regulation. This is particularly essential for deciphering the mechanisms of action of noncanonical AIM/LIR motifs, which are often challenging to predict due to the absence of distinctive features. Notably, by combining AF2-multimer with experimental approaches and phylogenetic analysis, we successfully identified and validated a previously undisclosed functional noncanonical AIM motif within an intrinsically disordered region of plant ATG3. As previously reported, a recurring pattern among canonical and noncanonical AIMS is their frequent

location within intrinsically disordered regions. Our AF2-multimer predictions align with this pattern, as most AIM/LIR sequences we analysed are found within these regions.

In our assessments, AF2-multimer accurately predicts the ATG8–AIM interface with varying confidence levels depending on the models. However, a high-confidence structural prediction of the entire protein is not always necessary to identify an ATG8 interface, as these regions may adopt ordered or disordered states depending on their interacting partners. The top-ranked AF2-multimer models correctly identified functional AIM/LIRs in all tested proteins, except for the M2 protein from Influenza A virus. In the case of M2, while the rank one model had a lower confidence score than other models, it still correctly depicted the LIR–LC3 binding interface. Therefore, models with higher confidence scores in regions encompassing AIM/LIR residues provide more informative insights when determining AIM/LIR–ATG8-binding interfaces.

A current limitation of AF2-multimer is its inability to provide information regarding the ATG8-binding affinity of the AIM/LIR motif. For instance, phosphorylation of flanking residues around the LIR/AIM has been shown to enhance ATG8-binding affinity. Still, AF2-multimer does not consider these post-translational modifications. However, since AF2-multimer effectively identifies AIM/LIRs even without factoring in post-translational modifications, comparing the side chain interactions of flanking AIM/LIR residues revealed by AF2-multimer could offer further insights into AIM/LIR specificity towards various ATG8 members.

Despite its advantages, there is room for improvement in AF2-multimer, as it cannot predict all AIM/LIR residues. In three cases, involving the plant autophagy cargo receptor Joka2/NBR1 and the human proteins Calreticulin and DVL2, AF2-multimer could not predict the experimentally proposed AIM/LIRs. It is possible that ATG8/LC3 binding in such proteins is facilitated by post-translational modifications and/or interactions with other proteins, which AF2-multimer currently does not account for. Intriguingly, AF2-models indicate the existence of alternative binding interfaces for these proteins, raising the question of whether AF2-multimer can be employed to predict additional, novel ATG8-binding interfaces. For example, AF2-multimer predicted a previously unrecognized LC3-binding interface in the malaria virulence factor PbUIS3, despite its lack of a typical LIR motif. According to AF2-multimer models, the known residues (D173, Y174, D175, E205, and K209) required for LC3 interaction in PbUIS3 do not reside in the LC3-binding interface. This suggests that they might indirectly influence LC3 binding, a hypothesis further supported by the absence of conservation of these residues in other *Plasmodium* species. This finding challenges the conventional notion that AIM/LIR motifs are located within conserved, structurally disordered regions. Thus, aside from identifying validated AIM/LIRs, AF2-multimer could become a valuable tool for uncovering new ATG8-binding interfaces, shedding light on cases where ATG8/LC3 interaction remains elusive and inspiring new experimental hypotheses.

An imperfection to our pipeline is the need to individually assess the models generated by AF2-multimer using visualisation software, such as ChimeraX. To speed up the screening of AF2-models we are developing a script that can run through our batch results and identify residues occupying the hydrophobic pockets of ATG8 and close by

regions with their confidence scores and distance as further outputs. Using these criteria, we will further our accuracy and limit the experimental validation process, leading to accelerated discovery of new biology.

Since we developed and published our AF2-guided pipeline [42], many have described their own interpretations of AI-aided discovery of new protein-protein interactions. Cristiana et al. 2023 purified autophagosomes to identify new candidates for selective cargo receptors. Their autophagosome proteomics data set was validated by modelling the ATG8-cargo receptor complexes using AF2-multimer and identifying their LIR-LDS interface [143]. Homma et al. 2023 used the power of AF2-multimer in predicting cross-kingdom interactions to characterise interactions between six defence-related hydrolases of tomato and seven tomato pathogens at the plant-pathogen interface [144]. Using two pairs of known effector-hydrolase complexes (P69B-Epi1a and Pip1-EpiC2B) they validated AF2-multimers accuracy in predicting existing cross-kingdom complexes with high pLDDT scores for the complex. To determine whether AF2-multimer would make false predictions or not, they swapped the effectors between the pairs and re-ran predictions, which showed a low pLDDT confidence prediction for the interface. To find novel interactions during pathogen invasion, they screened 11,274 protein pairs and identified 15 non-annotated effectors that are predicted to obstruct the active site of hydrolases. To validate AF2-multimer predictions of novel effector interactions, they performed inhibition assays of hydrolases with a fluorescent readout. The research from both groups corroborates the usefulness of AF2-multimer as a tool for guiding the discovery of new biology.

In conclusion, we introduce an AF2-multimer-based framework for AIM/LIR motif identification, which can aid in the discovery of novel autophagy receptors, adaptors, modulators, and pathogen virulence factors targeting ATG8 family of proteins. This framework promises to address critical questions in both plant and human autophagy, including the mechanisms underlying selective autophagy, its role in cellular and environmental stress responses, its connection to genetic diseases, the factors determining degradative versus secretory autophagy, and the strategies pathogens employ to manipulate autophagy for their benefit. The AI-guided pursuit of autophagy modulator discovery will significantly accelerate our understanding of autophagy's molecular basis across all life kingdoms.

4. AI-guided discovery of ATG8 adaptors and modulators in specialised autophagy of ribosomes and viruses

Summary

This study explores the interaction between ribosomal stalk protein RPLP2 and ATG8, shedding light on RPLP2's novel non-canonical binding motif. Employing an AF2-multimer prediction pipeline, a distinctive FSLF motif in the C-terminal region was validated and confirmed RPLP2's direct interaction with ATG8. Further investigations unveiled RPLP2's pivotal role in autophagy dynamics, demonstrating its localization to ATG8-marked autophagosomes. The study also raises intriguing questions about RPLP2's selectivity in the ribophagy processes. Expanding the scope, the AF2-multimer-guided pipeline was applied to decipher how viral effectors manipulate host autophagy via ATG8. Screening viral proteomes identified 93 potential effectors, showcasing diverse structural and functional strategies. Experimental validation of select viral proteins, highlighted their successful modulation of autophagic activity. An in-depth examination of Velvet tobacco mottle virus (VTMoV) revealed the movement protein P1 as an ATG8 interactor with a canonical AIM. Surprisingly, P1-ATG8 interaction induced cell death in *N. benthamiana*, however, we ruled out toxicity as the cause using sensitive tobacco plants, emphasizing the role of autophagic modulation in immune recognition. In summary, this research unveils RPLP2's role as a potential selective ribophagy receptor and uncovers diverse viral strategies in manipulating host autophagic pathways. The findings provide novel insights into cellular processes and host-pathogen interactions, opening avenues for further exploration in autophagy modulation and immune responses.

Introduction

Viruses are intracellular pathogens that interact with host physiology and cause disease

Viruses are obligate parasites that depend on host machinery throughout their replication cycle. Viruses are formed by a nucleic acid core encapsulated by coat protein and/or capsid [145]. The transmission of plant viruses, unlike animal viruses, is rarely through direct contact and relies on insect vectors, pollen, or seeds [146]. Upon initial contact, the viral particle disassembles to release the viral genome, followed by expression, replication, cell-to-cell, and long-distance movement. Although of great variety and divergence, plant viruses can share common features, such as genomes arrangement [147, 148]. The largest plant virus family, Potyviridae, have a monopartite genome, expressing their proteome from a single molecule of nucleic acid. As opposed to a monopartite genome, multipartism allows elasticity in gene regulation, which can be beneficial in responding to environmental changes. A multipartite genome allows for large genome sizes without necessarily increasing particle sizes, which is beneficial for movement between cells using the plasmodesmata. Plant viruses' express movement proteins that increase the size exclusion limits of the plasmodesmata to enable cell-to-cell movement of viral particles [149]. For a successful infection, extensive host-virus interactions take place, which commonly lead to disorders and disease. The annual economic impact of plant viruses is over \$30 billion, where some viruses can wipe out fields resulting in 100% yield loss [150]. Consequently, farmer revenues are reduced, and food prices soar with reduced availability. Majorly affected crops include staple food crops such as cassava, maize, rice, and banana, which can lead to the starvation of a significant portion of the world's population who depend on these plants for their subsistence. Hence, a complete

understanding of plant-viral interactions is essential for discovering new ways of sustainable agriculture and ensuring food security.

Autophagy is employed to defend towards invading viruses

The PTI/ETI-dependent plant innate immune system plays a critical role in responding to viruses. For example, the tobacco mosaic virus (TMV) replicase protein is recognised by intracellular receptors from the NRG1/ADR1 branch of the tobacco NLR network and this interaction initiates cell death (HR) to prevent cell-to-cell spread [151]. Autophagy is an additional, yet critical mechanism employed during antiviral PTI/ETI immunity. In BECLIN1 (a regulator of PI3K phosphorylation, yeast ATG6 homolog) knockout transgenic plants, HR could not be controlled upon TMV infection and unrestricted cell death was observed [77]. The silencing of core autophagy components ATG3 and ATG7 (regulators of ATG8 modifications), reduced plant resistance to tomato yellow leaf curl virus (TYLCV) [152]. Plant autophagy cargo receptor NBR1 was shown to directly capture capsid proteins of cauliflower mosaic virus and mediate their autophagic destruction [153]. The importance of autophagy during plant-viral interactions, necessitates more research in to elucidating the molecular mechanisms governing these responses.

Viruses modulate autophagy to disrupt immune responses

Due its involvement in cellular maintenance and antiviral immunity, autophagy is a major target for invading viruses [154]. Viruses can target autophagy to (1) block or suppress viral degradation, (2) counteract antiviral autophagy receptors and (3) facilitate destruction of host proteins. (1) Barley stripe mosaic virus (BSMV) γ B protein outcompetes ATG8 for ATG7 binding to prevent ATG8-lipidation and subsequent

autophagosome formation, which promotes infection (Yang et al., 2018). (2) NBR1 cargo receptor targets turnip mosaic virus (TuMV) HCPro for degradation. To counteract this response, TuMV blocks NBR1 flux in an unknown manner (Hafren et al., 2018). (3) TuMV effector VPg can facilitate the removal of plant suppressor of gene silencing 3 to allow viral expression (Cheng et al., 2017). RNA interference (RNAi) or RNA silencing are the main antiviral defence mechanism triggered by virus-derived double-stranded DNA (dsDNA) [155]. dsDNA's are processed by the DICER-LIKE (DCL) RNase III-like enzymes, DCL1-4, to produce micro- and small-interfering- RNA (miRNA and siRNA). These RNA duplexes can act to improve plant growth and development, initiate genome-wide DNA methylation, and guide a family of Argonaute (AGO) nucleases to viral RNA/DNA, suppressing spread. To counteract Argonaute activity, the poliovirus family encode a viral silencing suppressor F-box protein P0, which recruits ATG8-interacting proteins 1 and 2 to degrade AGO through autophagy [156].

Ribosomal recycling via ribophagy

The eukaryotic ribosome is a conserved complex of four ribosomal RNAs (rRNAs) and ~80 ribosomal proteins. The 40S small subunit contains the 18S rRNA and 33 ribosomal proteins, while the 60S large subunit comprises three rRNAs and 46 ribosomal proteins. Together, they mediate the proper decoding of the genome and ensure correct cellular functionality [157]. Within the cytoplasm there are many millions of ribosomes, accounting for 10% of the protein mass of a typical mammalian cell [158]. Very little is known about the turnover of ribosomes and its consequences on cellular homeostasis. The ubiquitin-proteasome system can degrade excess ribosomal proteins. However, once they are assembled, large ribosomes require

alternative modes of destruction. Ribosomes are almost always found in autophagosomes when studied using electron microscopy or mass spectrometry [159]. Autophagosomes are formed in the cytoplasm and engulf ribosomes as part of bulk autophagy. However, this process was also shown to be selective (ribophagy). The large and small subunit proteins are degraded during nutrient starvation in an ATG8-dependent manner, indicating its reliance on the ATG8 conjugation pathway during selective autophagy. How ribosomes are linked to ATG8-dependent selective autophagy is currently unknown.

In human ATG5 knockout cells, starvation induced degradation of the ribosomal protein RPL28 (large subunit) was reduced but RPS3 (small subunit) remained unaffected [158]. This highlights potential differences between large and small subunit degradation pathways, which was also observed in yeast. Depending on the inducing stimulus, these subunits can be degraded to similar degrees. For example, using sodium arsenite, which induces stress granule formation, An and Harper, 2017 demonstrated that ribosome degradation was induced more specifically, and the subunits were similarly affected in ATG5 knockout cells. How ribophagy is triggered by sodium arsenite remains unknown.

There is limited information on ribophagy in yeast and mammals, and almost none in plants. A selective ribophagy receptor is yet to be identified. Their validation process has been a controversial topic in the field of ribophagy. In humans, NUFIP1, nuclear FMR1 interacting protein 1, localised from the nucleus to cytosolic autophagosomes when autophagy was triggered upon Torin1 (TOR inhibitor 1) treatment of mammalian cells [160]. NUFIP1 depletion prevented autophagic degradation of ribosomal proteins

even in the presence of Torin1. NUFIP1 is a direct interactor of LC3B and all together these observations lead Wyant et al. 2018 to suggest NUFIP1 as selective ribophagy receptor. However, An et al. 2020 produced data showing NUFIP1 deletion had no effect on ribosomal protein abundance and knock-out cells showed no change in autophagic flux of key ribosomal proteins [161]. They did not observe re-localisation of NUFIP1 to the nucleus upon recovery of cellular stress and established that the reduction of ribosomal proteins was due to NUFIP1's role in 60S biogenesis. Another study showed that NUFIP1 translocated from the nucleus to cytoplasm upon mechanical stress without triggering ribophagy [162].

We screened ribosomal components for their ability to interact with ATG8 and their relevance to ribosomal activity and identified the ribosomal stalk proteins as a potential adaptor candidate for ATG8-dependent autophagy. The ribosomal stalk has been described in autophagy, however, to date a direct interaction with ATG8 or whether RPLPs can selectively control ribosomal degradation has not been described.

The ribosomal stalk

The ribosomal stalk is made up of RPLP0 and two hetero dimers of RPLP1 and RPLP2, which are bound to RPLP0 (Figure22). RPLP1 and RPLP2 have a ~70 amino acid residue structured N-terminal followed by a disordered region. The disordered C-term is highly conserved in all RPLP1/2 proteins across kingdoms. The N-terminal structures are variable in sequence and length, however, maintain an overall acidic charge [163, 164]. Silencing of all ribosomal stalk proteins lead to unfunctional ribosomes. In the absence of RPLP1 and RPLP2, the ribosome proceeded with protein synthesis relying on RPLP0 alone [163]. However, a different subset of mRNAs was

translated. C-terminal truncations of RPLP1/2 severely disrupted protein translation. In all species the ribosome stalk is long and flexible. In most structures of the ribosome, the stalk is usually not resolved. A crystal structure of the human 80S ribosome (4V6X) had density for N-terminus of RPLP1/2, but not for the C-terminus disordered region [165]. AF2-multimer predicted structure of the RPLP1/2 dimer reveals a highly disordered C-terminus, as previously described. When RPLP proteins were inhibited using short hairpin RNAs in human cancer cells, an increase in autophagic activity was confirmed by increases in LC3 puncta and p62 protein levels, which could be reversed with the autophagy inhibitor 3-methyladenine [166].

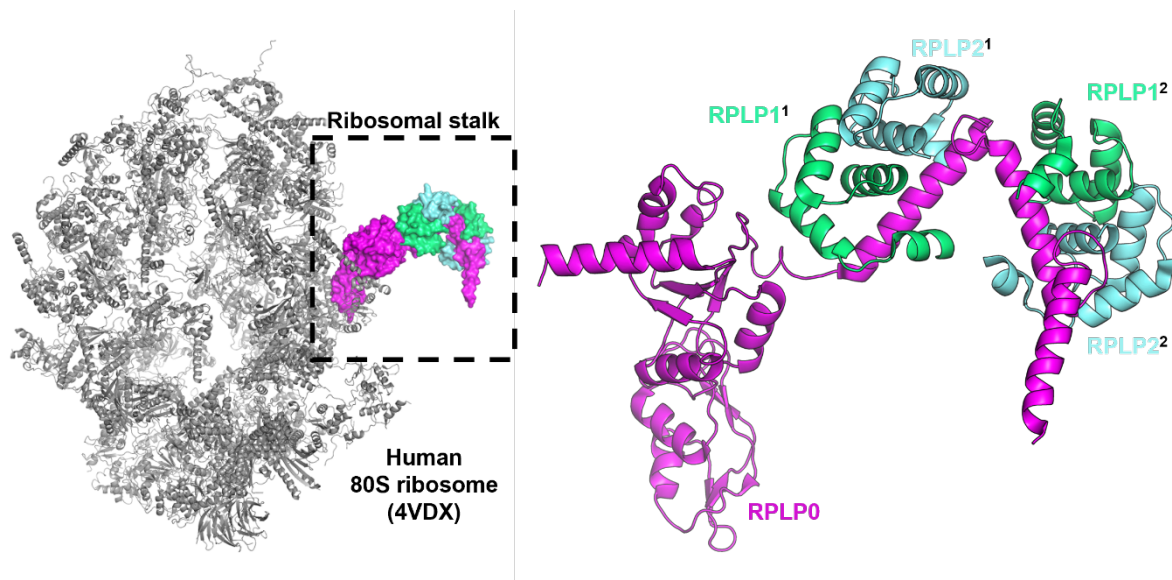


Figure22. The ribosomal P-stalk is composed of RPLP0 and two copies of each RPLP1 and RPLP2 arranged as two hetero-dimers bound to RPLP0. A crystal structure of the human 80S ribosome (grey – 4V6X [165]) could resolve the N-terminal soluble domains of the RPLP stalk: RPLP0 (pink), RPLP1 (green) and RPLP2 (cyan). C-terminal extensions for neither were available in the structure due to extreme flexibility.

Identifying ATG8 adaptors and modulators for ribophagy and virophagy

A receptor for the selective degradation of ribosomes is currently not discovered in plants. We identified RPLP proteins using our AF2-multimer pipeline and set out to determine whether it was a direct interactor of ATG8 or not and if so, what is the functional relevance for autophagic activity? To take a proteomics guided approach to cross-kingdom interactions, we chose viruses as a model pathogen, as they rely on host machinery and have been shown to interact with autophagy. Here we used AF2-multimer to generate an AI-guided proteomics data set for ATG8 interactors, which we then proceeded to validate in the wet lab to establish molecular mechanisms of viral autophagic modulation.

Results

AF2-multimer accurately predicts previously uncharacterised ATG8-interacting motifs in viral modulators

Inspired by our successful validation of ATG8 interactors in various organisms, we directed our AF2-multimer guided pipeline to find answers for the question: how do viral effectors modulate host autophagy through ATG8 during host-pathogen interactions? Our starting point was to screen human and plant viral proteomes, obtained from Uniprot [167], against ATG8 to fish for potential effectors. We have screened ~100 viral proteomes, with more than 400 viral proteins, and identified 93 that occupy the hydrophobic pockets on ATG8, then selected 11 for further investigation (Figure23).

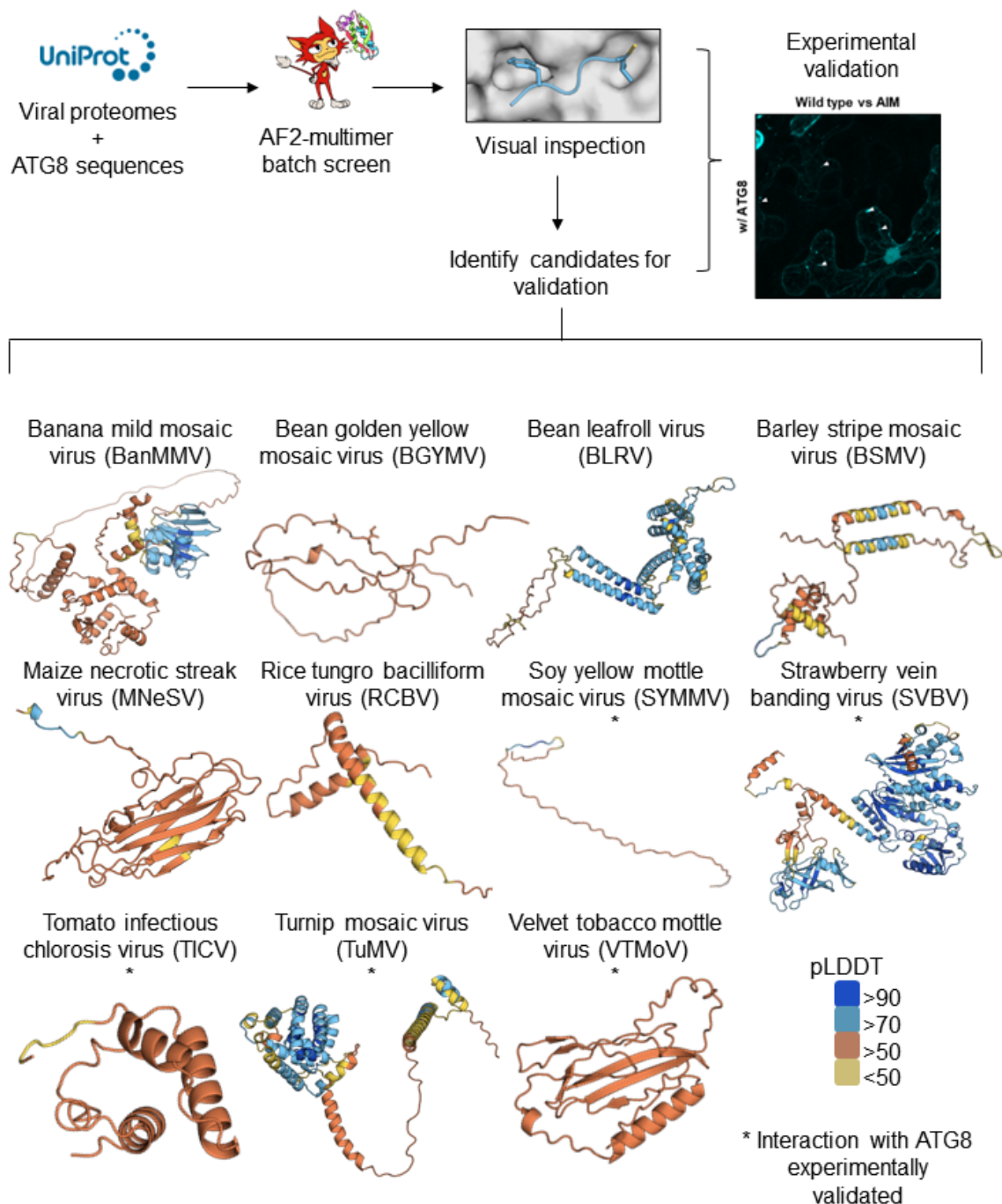


Figure23. AF2-multimer guided pipeline for discovery of viral ATG8 modulators.

Viral proteomes obtained from UniProt were batch screened against Atg8 using AF2-multimer. Following visual inspection of the AIM/LIR-LDS docking, we identified 11 candidates for further validation. Experimental validation was performed using in vivo and in vitro binding and autophagy activity assays. Viruses with asterisks have been experimentally validated. All viral proteins are coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low).

Interestingly, viral modulators identified show great structural and functional variety (Figure23). This is important to emphasise, as it illustrates AF2-multimers ability to not prioritise any specific structures or functionality when fishing out these proteins from the viral proteome. This also indicates that during host-pathogen interactions, viruses employ a variety of strategies to modulate autophagy. The predicted models for viral effectors show a range confidence scores (Figure23). However, all five predictions taken to the next step have been successfully validated in the lab, with effects on autophagic activity. For example, to validate AF2-predicted SVBV and TuMV viral protein interactions with ATG8, we performed in planta binding assays. We cloned the wild type and AIM mutant versions of ORF5 polyprotein from SVBV and P3 from TuMV into our GFP Golden gate expression vectors. Wild type ORF5 and P3 interact with ATG8 using canonical AIMS WEML and YTFL, respectively, as predicted by AF2-multimer (Figure24C and Figure24D, respectively). These interactions were significantly less in the AIM mutants, where the AIM residues entering the pockets were mutated to alanines.

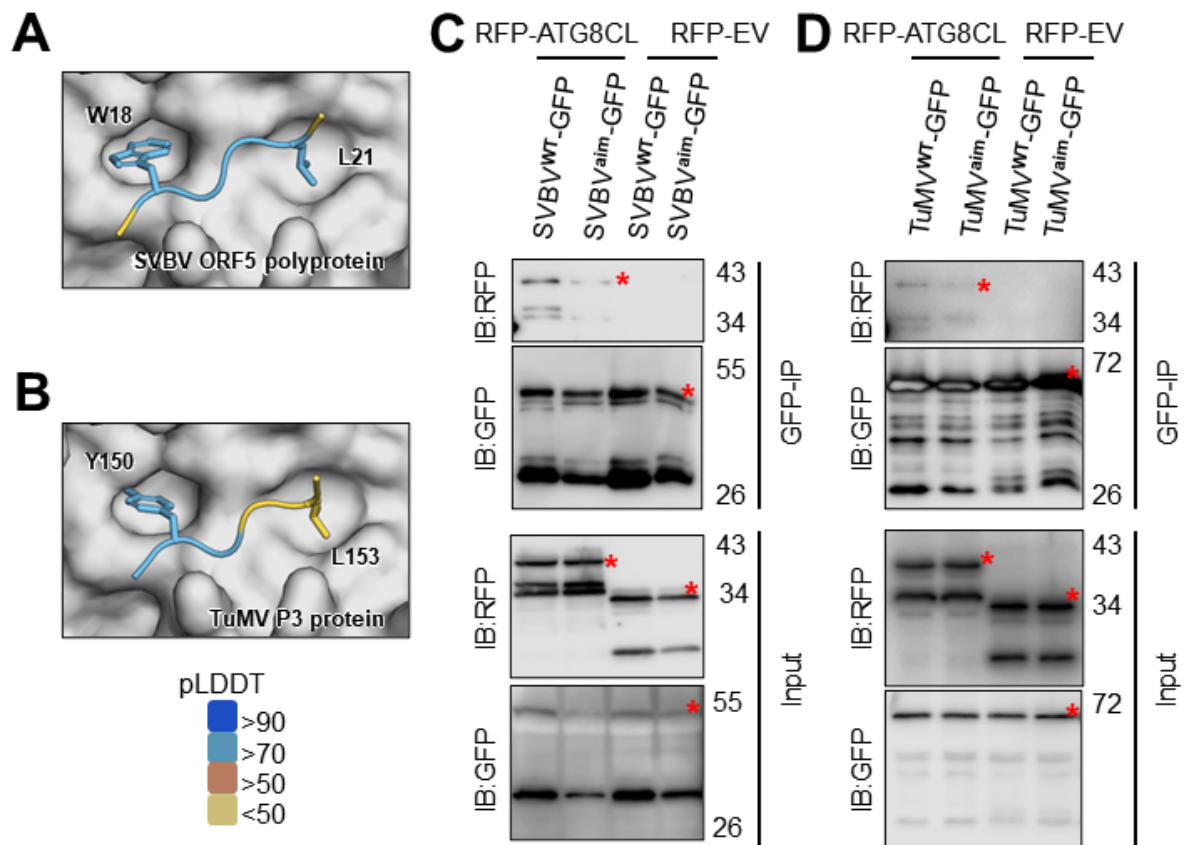


Figure24. SVBV and TuMV utilise viral proteins with canonical AIMs to modulate ATG8. (A) SVBV ORF5 protein AIM region in the ATG8 LDS as predicted by AF2-multimer. (B) AF2-multimer predicted TuMV P3 AIM-ATG8 LDS interface. (C, D) Co-immunoprecipitation assays with SVBV ORF5 and TuMV P3, respectively. RFP-ATG8CL and an RFP-EV control were co-expressed with GFP-tagged viral modulators in the plant cells. No signal was observed in the control lanes. Wild type modulators interacted with ATG8 as predicted by AF2-multimer. Red asterisks indicate expected band size. ORF5 and P3 ATG8 interfaces are coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). ATG8 is shown in grey. *Adapted from [168].* Uncropped blots – Appendix G.

Another discovery from the AF2-multimer viral screen is the SYMMV P7 protein. AF2 predicts this protein to be completely random coil with low confidence. However, AF2-multimer can predict a high confidence canonical (FVIV) interface for ATG8-P7 interaction (Figure25A). To validate this interface, we co-expressed wild type or AIM

mutant P7 with RFP-ATG8CL in the plant cells and studied ATG8-autophagosome puncta for co-localisation. Wild type SYMMV P7 localises to ATG8 puncta, however, the AIM mutant (FVIV to AVIA) shows less signal intensity from puncta (Figure25B/C). We then determined the effect of P7 on autophagic activity by quantifying the number of autophagosomes in the cell. SYMMV P7 significantly increases number of ATG8-labelled autophagosomes compared to the AIM mutant version and a GFP control. The P7 AIM mutant and GFP control show no difference in autophagosome numbers (Figure25D) [168]. These indicate that SYMMV utilises P7 as a modulator of autophagy to potentially enhance autophagic flux. All together these are great examples of how we can discover effectors and characterise their interactions with ATG8 to gain insight into viral evasion strategies.

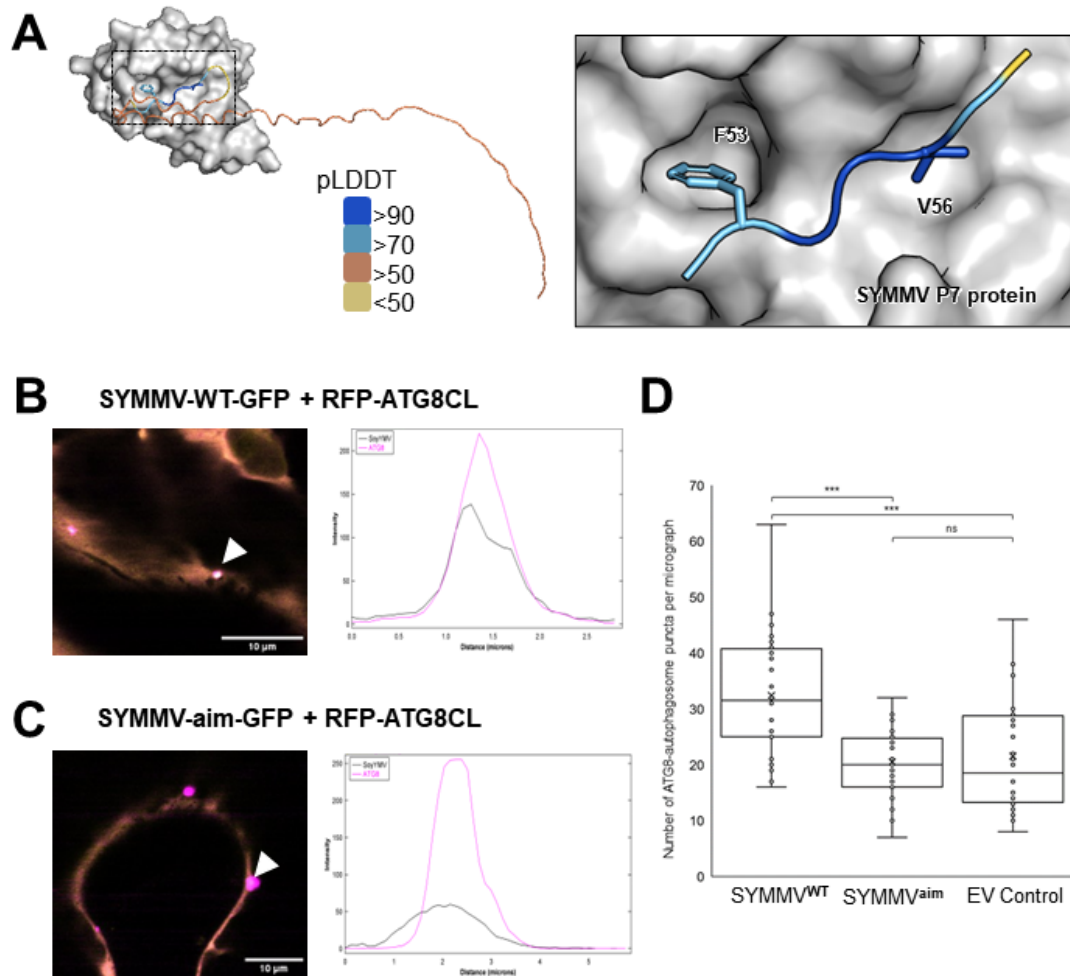


Figure25. SYMMV P7 protein interacts with ATG8 using a canonical AIM to enhance number of ATG8-decorated autophagosomes. (A) SYMMV P7 protein is predicted to be completely unfolded, with a low confidence score. At the ATG8 LDS interface, P7 has a high confidence score. **(B)** Wild type P7 colocalises to ATG8 puncta as signal intensity overlaps at puncta indicated with white arrow. **(C)** AIM mutant P7 cannot localise to ATG8 puncta. Signal intensity from P7^{AIM} is severely reduced at ATG8 puncta. **(D)** Number of ATG8 puncta is significantly higher when ATG8 is co-expressed with SYMMV wild type protein compared to the AIM mutant (Wilcox test, W=59, p<0.05) and an EV-GFP control (Wilcox test, W=52, p<0.05). White arrows indicate example puncta investigated. Images shown are maximal projections of 18 frames with 1µm steps and overlaid channels of GFP (yellow) and RFP (pink). Viral proteins are coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). *Adapted from [168].* Raw puncta numbers and analysis – Appendix H and G, respectively.

Velvet tobacco mottle virus deploys movement protein P1 to modulate ATG8

An interesting member of our viral dataset is velvet tobacco mottle virus (VTMoV) discovered in Australia to infect various plants in the *Nicotiana* family [169]. From the VTMoV proteome, AF2-multimer predicted a putative movement protein, P1, as an interactor of ATG8. P1 utilises a canonical (WSTV) AIM and the confidence score for the model at this region is low (Figure26A/B). To validate the functionality of the canonical motif, we cloned wild type and AIM mutant (WSTV and ASTA, respectively) P1 into a GFP expression vector and co-expressed them with RFP-ATG8CL in the plant cell to perform binding assays. The wild type P1 protein interacts with ATG8, however, the AIM mutant had a stronger interaction, which has been validated through various repeats (Figure26C). This intriguing observation was investigated with AF2-multimer. In the P1^{AIM}-ATG8 model, we observed a closer association of the two proteins where the secondary structure elements of P1 seem to “wrap around” ATG8, whereas in the P1^{WT}-ATG8 model, significant interactions were observed only at the hydrophobic pockets of ATG8 (Figure26D). Hence, we believe mutating the AIM region exposes alternative binding sites of P1. It must be stated that, AF2 cannot predict direct effects of single-point mutations on protein structure [170].

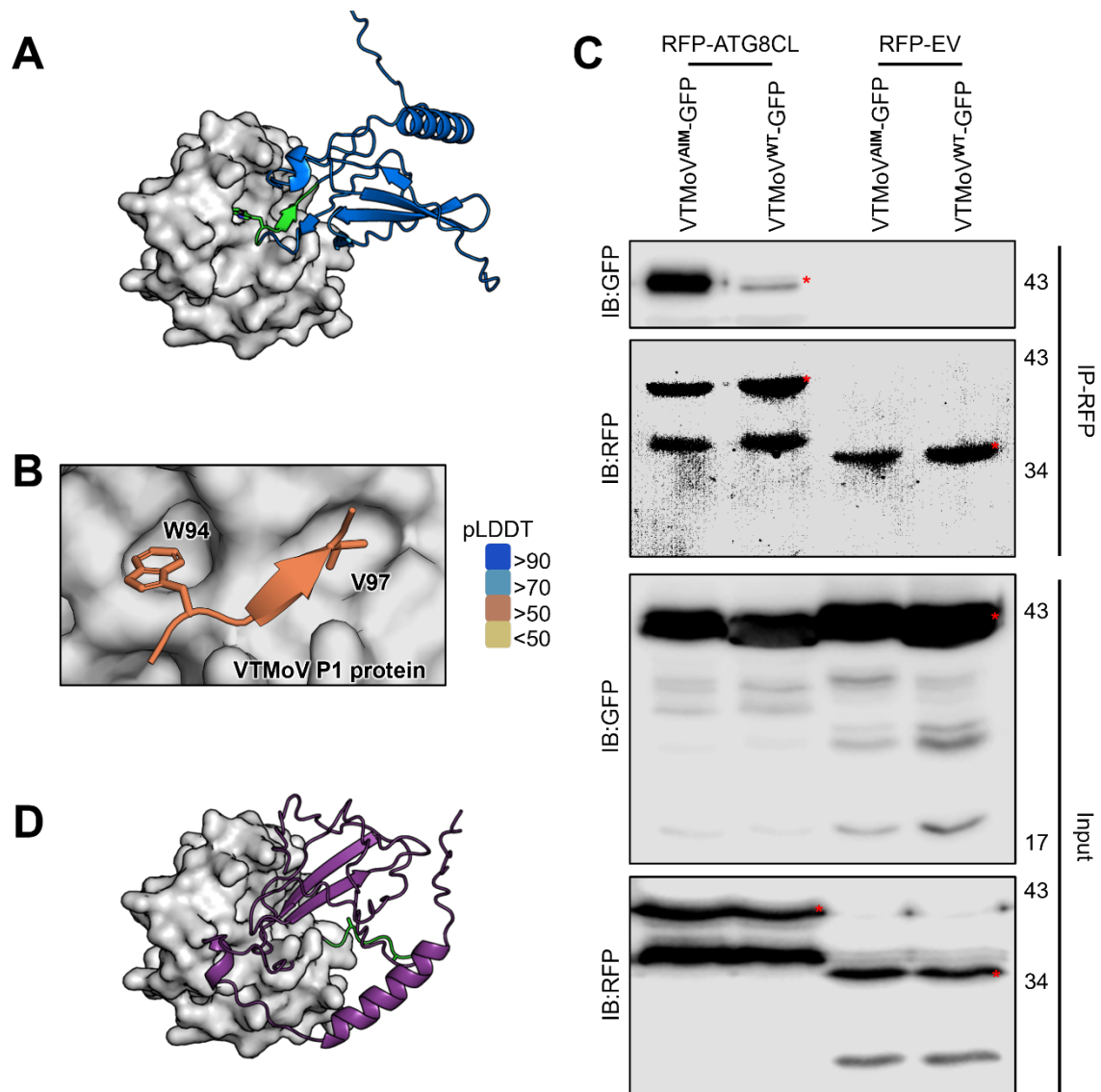


Figure 26. VTMoV P1 protein is a modulator of ATG8 via a canonical AIM. (A) The P1-ATG8 complex. P1 (blue) canonical AIM (green) is localised to the LDS of ATG8 (grey). The remainder of P1 doesn't form significant interactions with ATG8. **(B)** P1-ATG8 AIM-LDS interface. P1 uses a canonical WSTV AIM to dock onto ATG8. P1 is coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). **(C)** P1 AIM mutant interacts with ATG8 more compared to wild type P1. RFP-ATG8CL or an RFP-EV control were co-expressed with either wild type or AIM mutant P1. AIM mutant had a stronger band in the RFP IP for ATG8 compared to wild type P1. Red asterisks indicate expected protein band size. **(D)** ATG8-P1 AIM mutant complex. As opposed to wild type P1, AIM mutant P1 (purple) forms alternative interactions with ATG8 and seems "wrap" around ATG8 LDS. Uncropped blots – Appendix I.

VTMoV P1-ATG8 complex triggers HR-like immune responses in wild type *Nicotiana benthamiana*

Whilst investigating P1-ATG8 interactions in planta, we came across an interesting phenotype, where P1^{WT} and ATG8 co-expression in *N. benthamiana* was causing discolouration of the leaves. The discolouration was like that observed for HR, which indicates an activation of immune responses. Considering the ability of VTMoV to infect the *Nicotiana* family, we suspect this is HR-like cell death and believe the P1 protein directly, or its actions indirectly, are being recognised by the NLR-network.

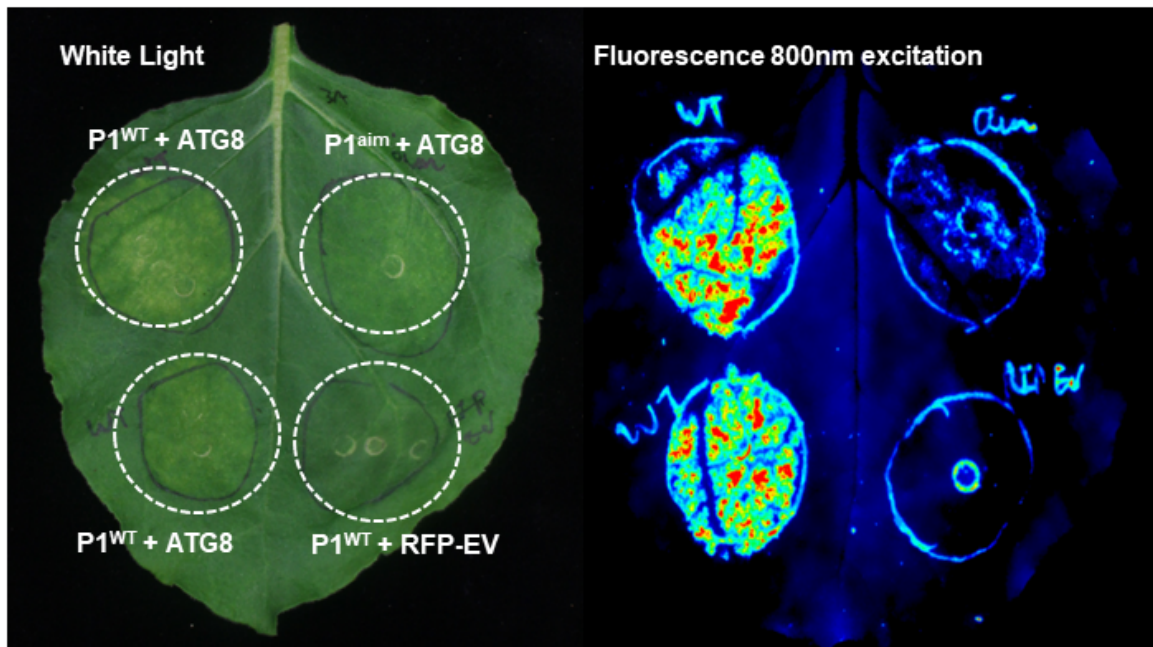


Figure 27. Experimental set-up for the analysis of discolouration caused by P1-ATG8 co-expression. Leaves were co-infiltrated with P1 wild type or AIM mutant and ATG8 or RFP control and left to develop yellowing for seven days. The results were investigated under white light and using a fluorescence detector. Fluorescence detections were performed with an excitation wavelength of 800nm to detect microscopic cell death using a LiCOR (see chapter two).

To validate if the yellowing on leaves were HR, we set-up a HR assay, as shown in Figure27. We co-expressed in small patches GFP-P1^{WT} or GFP-P1^{AIM} with RFP-ATG8 and after 7 days measured the level of cell death using the fluorescent signal emitted at 800nm by dying tissue with LICOR Odyssey Fc (Figure27). After 7 days post co-infiltration, cell death was observed for P1^{WT} (Figure28A), however, HR response was significantly reduced in the AIM mutant (Figure28B and D). To determine if cell death was dependent on co-expression of P1 with ATG8, we repeated the HR assay with and without ATG8 for P1^{WT} (Figure28C). When P1 was co-expressed with just the RFP tag, no cell death was observed. P1 expression with ATG8 generated fluorescent signal associated with cell death, as expected (Figure28E). Together these suggest that AIM-LDS docking between P1 and ATG8 is essential for immune recognition. To further validate the dependence on the AIM-LDS interaction, we co-expressed the P1-ATG8 complex with a high affinity AIM peptide from the *P. infestans* effector PexRD54, which was previously shown to saturate the hydrophobic pockets of ATG8 and inhibit autophagy (Figure29). When autophagy was blocked by the AIM peptide, no cell death was observed with P1^{WT}+ATG8 co-expression. When the complex was expressed in the presence of a mutated AIM peptide that could not bind ATG8, significant cell death was observed (Figure29). Hence, AIM-LDS docking and autophagic activity are essential for the recognition of P1 protein by plant defences. Overall, these results suggest that P1 protein on its own can evade immune recognition. Once it forms a complex with ATG8, specifically via hydrophobic pocket docking, P1-ATG8 is recognised by intracellular immune receptors that subsequently trigger HR. Hence, autophagic modulation of P1 leads to its recognition and counteracting immune responses.

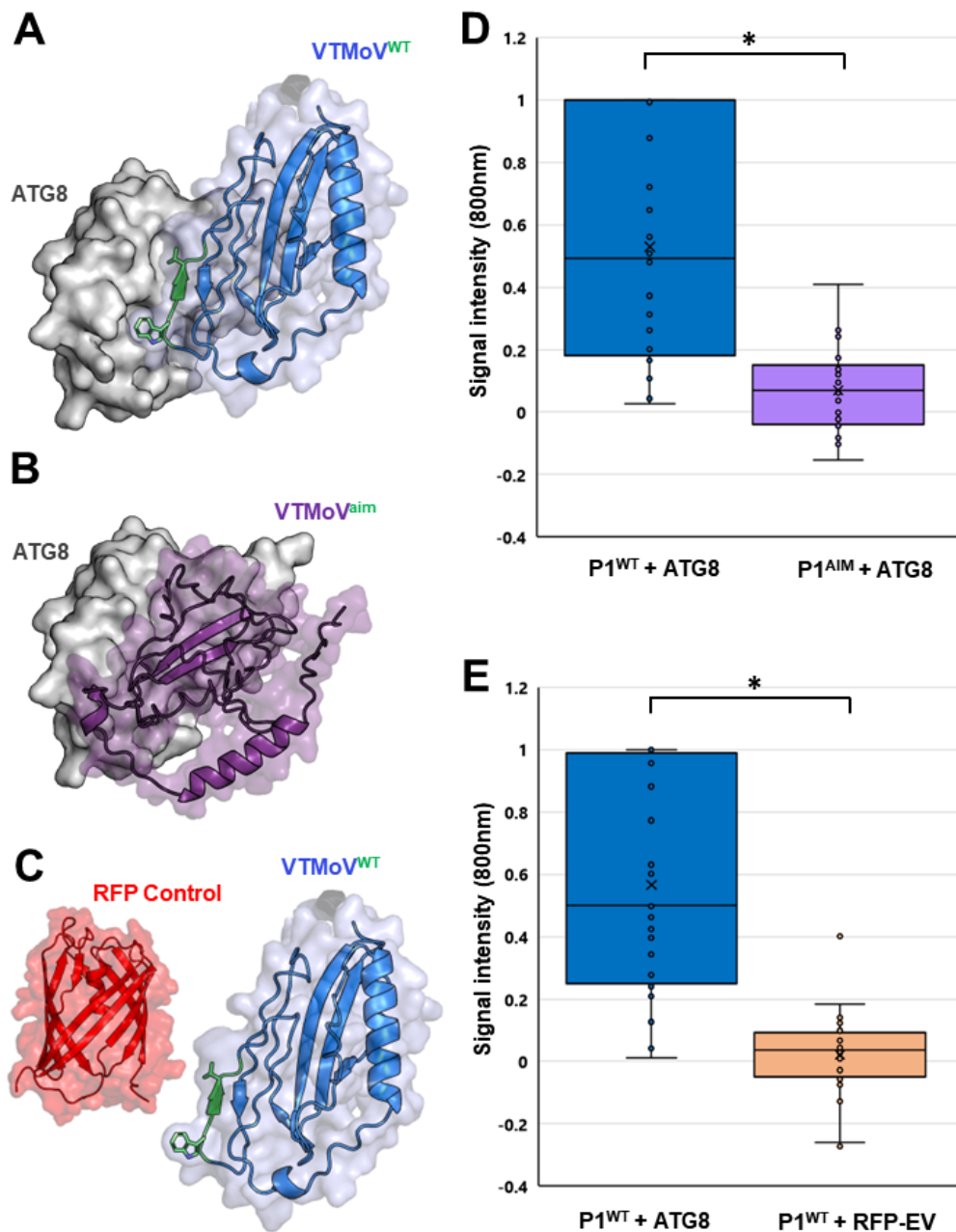
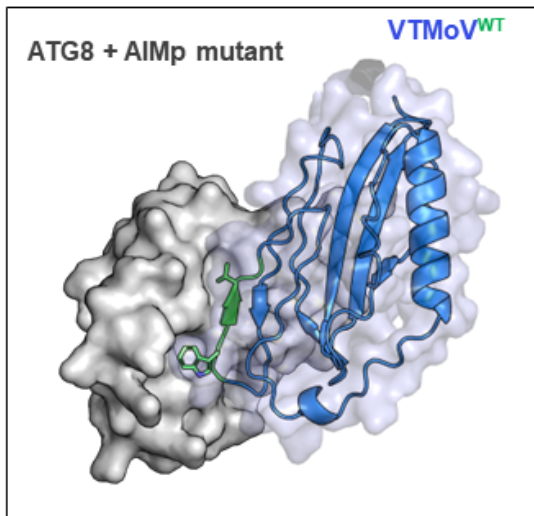


Figure 28. VTMoV P1 AIM-ATG8 LDS interaction leads to HR-like cell death. (A) P1 wild type (blue) AIM (green)-ATG8 (grey) LDS complex. **(B)** P1 AIM mutant (purple)-ATG8 (grey) complex. **(C)** P1 wild type with RFP control. **(D)** Cell death is significantly reduced when the P1 AIM cannot dock into the ATG8 LDS. A Wilcox test found that the mean signal from P1^{WT} (0.493) was significantly different to that from P1^{AIM} (0.069) (W=506, $p < 0.01$) when co-expressed with ATG8. **(E)** ATG8 complex formation with P1 wild type is necessary for cell death. A Wilcox test found that the mean signal from P1^{WT} with ATG8 (0.501) was significantly different to that from P1^{WT} with EV:RFP (0.036) (w=542, $p < 0.01$). Raw data of normalised HR intensities – Appendix J.

No inhibition of autophagy



Inhibition of autophagy

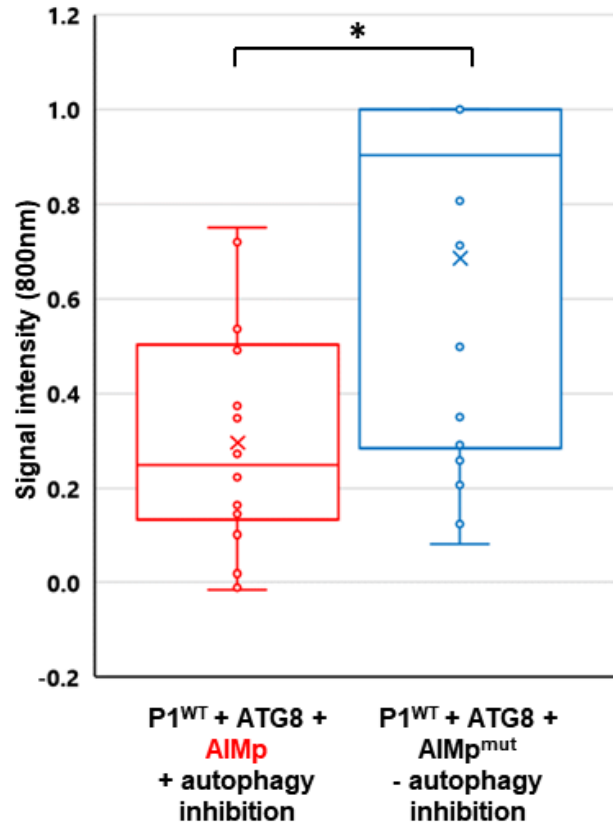
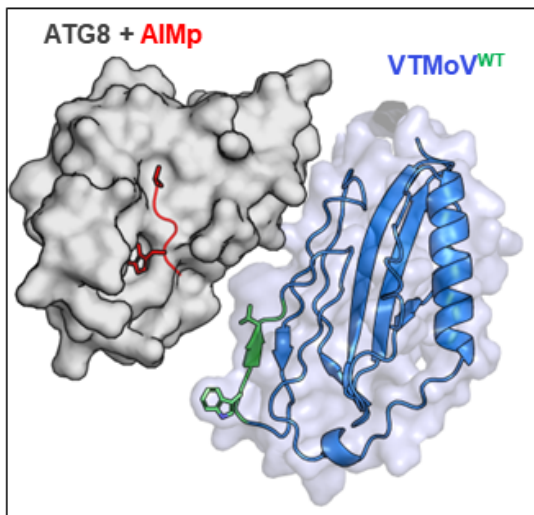


Figure29. Autophagy inhibition abolishes VTMoV P1-ATG8 dependent cell death. The P1^{WT}-ATG8 complex was either expressed with PexRD54 mutant or wild type AIM peptide (AIMp). Mutant AIMp does not block autophagy and the P1^{WT}-ATG8 AIM-LDS complex can be formed. Wild type AIMp saturates ATG8 LDS and blocks autophagy. A Wilcox test found that the mean fluorescence signal when autophagy was blocked (0.296) was significantly different to when it was not blocked (0.684) ($W=69$, $p < 0.01$). ATG8 is coloured grey. P1 protein is coloured blue with a green AIM. AIMp is shown in red. Raw data for normalised HR intensities – Appendix K.

We established that autophagy was necessary for the HR using an autophagy inhibitor. We then questioned whether the degradation of P1 generated toxic peptides that caused cell death and our observation was not an immune response. To eliminate this possibility, we repeated the HR assay in *Nicotiana tabacum*, as from previous experience we believe tobacco is more sensitive to cell death responses. No visible discolouration or cell death associated fluorescent signals were observed in three repeats (Figure30). Although, protein expression levels may not be equal in tobacco and *N. benthamiana*, these results indicate that toxicity is not the cause of the cell death we observe, and it is a specific immune response found in *N. benthamiana* and lost in *N. tabacum*. Protein expression in the different *Nicotiana* can be compared using immunoblotting following transient expression for the same number of days.

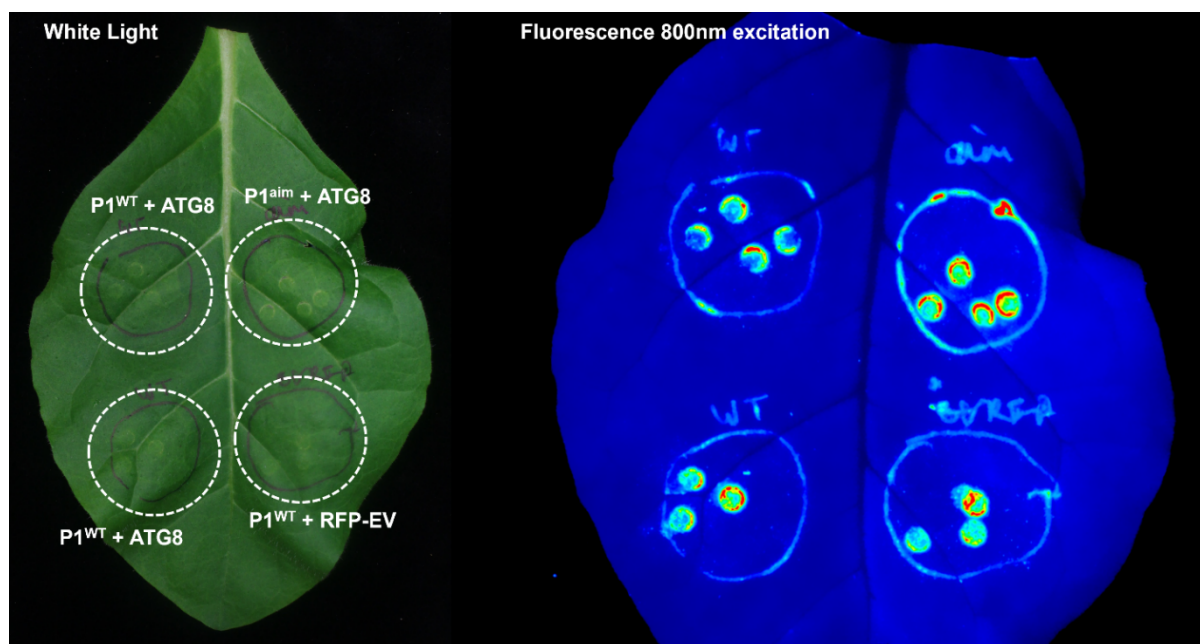


Figure30. VTMoV P1-ATG8 complex does not trigger immune responses in tobacco plants. No cell death related fluorescence at 800nm was observed.

VTMoV P1 associated cell death is TIR-NLR-dependent

HR-related cell death is a consequence of NLR activity. As we established P1-ATG8 complex was leading to immune specific HR, our next goal was to establish mode of

recognition. To determine if the cell death was dependent on the NRC network of the NLR family, we repeated the HR assays in NRC2/3/4 knock-out plants, which lack the NRC-mediated immune signalling for cell death. P1^{WT}-ATG8 complex formation led to cell death in NRC2/3/4 knock-out plants, as in wild type *N. benthamiana* (Figure31). Hence, P1-ATG8 associated HR is not mediated by the NRC network.

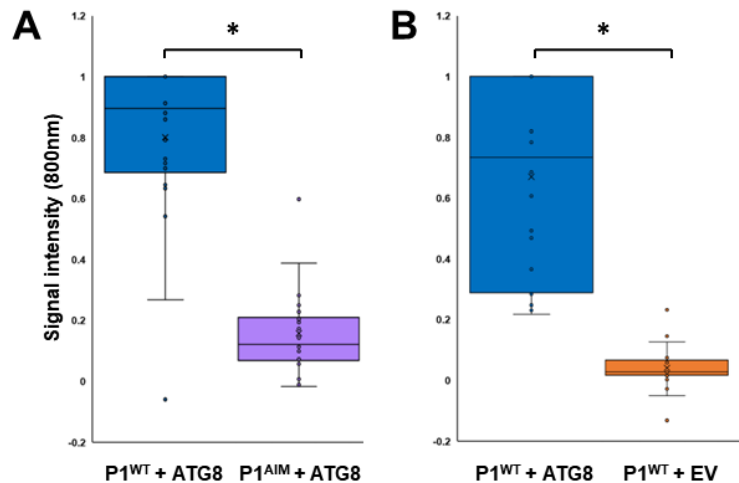


Figure31. VTMov P1-ATG8 associated cell death is not NRC2/3/4 dependent. (A) P1^{WT} and P1^{aim} were co-expressed with RFP-ATG8 and **(B)** EV-RFP in NRC2/3/4 transgenic triple knockout mutants of *N. benthamiana*. A Wilcox test found that the mean fluorescence signal when P1^{WT} was expressed with RFP-ATG8 (0.670) was significantly different to when P1^{aim} was expressed with RFP-ATG8 (0.040) (W=69, p<0.01). Raw data for normalised HR intensities – Appendix L. As previously shown (Figure28), the cell death detected was dependent on P1-ATG8 complex formation as in the absence of ATG8 no cell death was observed (B).

The other main branch of the NLR family is the NRG1/ADR1 branch, which is associated with the signalling protein EDS1. To establish if P1-ATG8 complex is recognised by the NRG1/ADR1 branch, we repeated the HR assay in NRG1/ADR1 transgenic double knock-out *N. benthamiana*. No significant cell death was observed in NRG1/ADR1 KO transgenic plants (Figure32). Hence, we suggest the P1-associated cell death is dependent on the recognition of the P1-ATG8 AIM-LDS

complex via the NRG1/ADR1-dependent NLR networks. We additionally assessed whether another viral protein from SYMMV, P7, would also cause cell death to further eliminate the possibility of viral protein degradation causing toxic cell death. No cell death was observed for SYMMV P7-ATG8 in wild-type nor transgenic plants (Figure32).

Overall, using AF2-multimer, we generated an AI-proteomics dataset for viral ATG8 interactors from plant viruses. We expanded on this innovation with experimental validation of direct ATG8 interactions using coIP and co-localisation assays, as well as later mass spectrometry corroboration. We take two viral proteins from SYMMV and VTMoV, P7 and P1, respectively, into further experimentation and demonstrate their ability manipulate ATG8 activity. P7 acts to increase autophagic activity and its cargo are yet to be determined. We are using IP-MS approaches with all viral proteins studied to build a network of their plant protein interactome. P1 was seemed to give HR only in complex with ATG8 and we suspect their autophagic activities of certain plant proteins lead to induction of immune responses, i.e., P1 may act on guarded complexes. We then applied this AI-proteomics approach to ribosomes aiming to identify an adaptor to ATG8 that could facilitate selective ribosome degradation through autophagy.

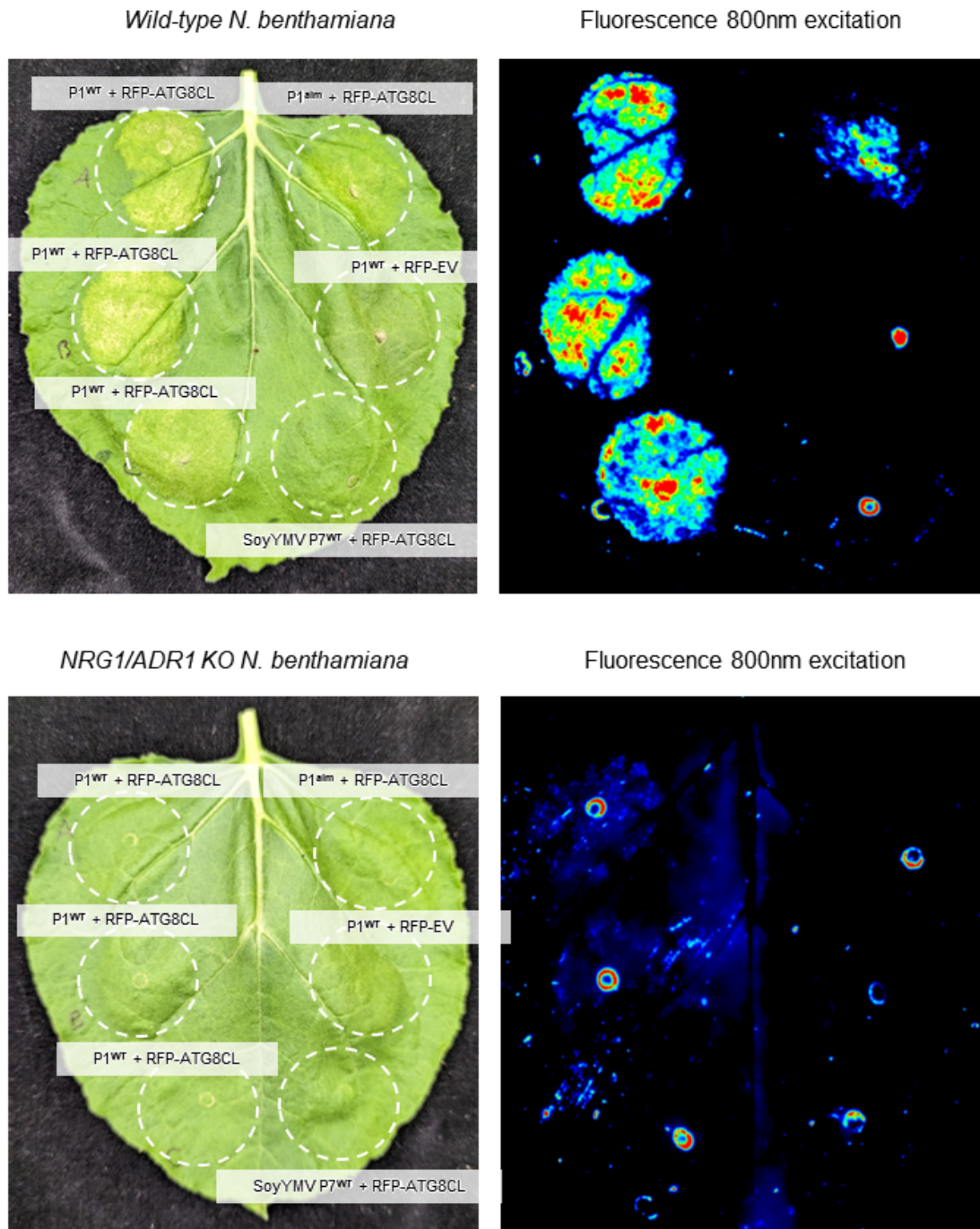


Figure32. No VTMoV P1-ATG8 associated cell death is observed in NRG1/ADR1 KO transgenic plants. Wild type plants were used as a control, which displayed previously described cell death patterns with VTMoV P1. SYMMV P7 was used as an additional control for viral protein toxicity. No cell death was observed for P7. Left-hand side of all leaves were infiltrated with three patches of VTMoV P1^{WT}-ATG8. Right-hand side of leaves were infiltrated with VTMoV P1^{aim}-ATG8, P1^{WT}-EVRFP and SYMMV P7^{WT}-ATG8, respectively.

Ribosomal stalk protein RPLP2 utilises a non-canonical motif to interact with ATG8

An AF2-screen of ribosomal proteins yield RPLP proteins as a potential direct ATG8 interactor with good confidence. To determine whether RPLP2 can directly interact with ATG8, I used the AF2-multimer guided pipeline we developed. I ran an AF2-multimer prediction with RPLP2 and ATG8 and phylogenetically analysed the interface. The AF2-model predicted an atypical interaction motif (FSLF) in the C-terminal disordered region of RPLP2. I analysed the homologs of RPLP2 from plants and mammals, which reveal the non-canonical AIM as a highly conserved AIM (Figure33). To experimentally validate these observations, I cloned the RPLP2 protein and the AIM mutant (FSLF to ASLA) into our expression vectors and performed a binding assay with ATG8 in the plant cell. Wild type RPLP2 interacts with ATG8, as predicted by AF2-multimer. This interaction was hampered with the AIM mutant of RPLP2 (Figure34). As these were studied in planta it is not possible to eliminate a potential secondary intermediate receptor, however, the severe loss of interaction in the AIM mutant is a good indicator of direct interaction. To further validate the functionality of the AIM region, I cloned a peptide of the AIM containing the C-terminal disordered region, X₆FSLFX₁, together with its mutant (X₆ASLAX₁). I co-expressed the peptides with ATG8 in the plant cell and observed complete loss of ATG8 interaction with the mutant peptide in the colP, whereas the wild type RPLP2 peptide had high affinity for ATG8 (Figure34). Using our AF2-multimer pipeline, I corroborated the ribosomal stalk protein as a direct interactor of ATG8 and validated its atypical binding interface. This was also later validated by Nicoll Mathews using IP mass spectrometry, where ATG8 pull downs had a high abundance of RPLP2. This is a clear example of how AF2-multimer, together with proteomics data, can lead to the accurate discovery

of new biology. The next question was to understand how RPLP2 affects autophagy and whether it can direct ribosomes to autophagy compartments selectively as part of ribophagy.

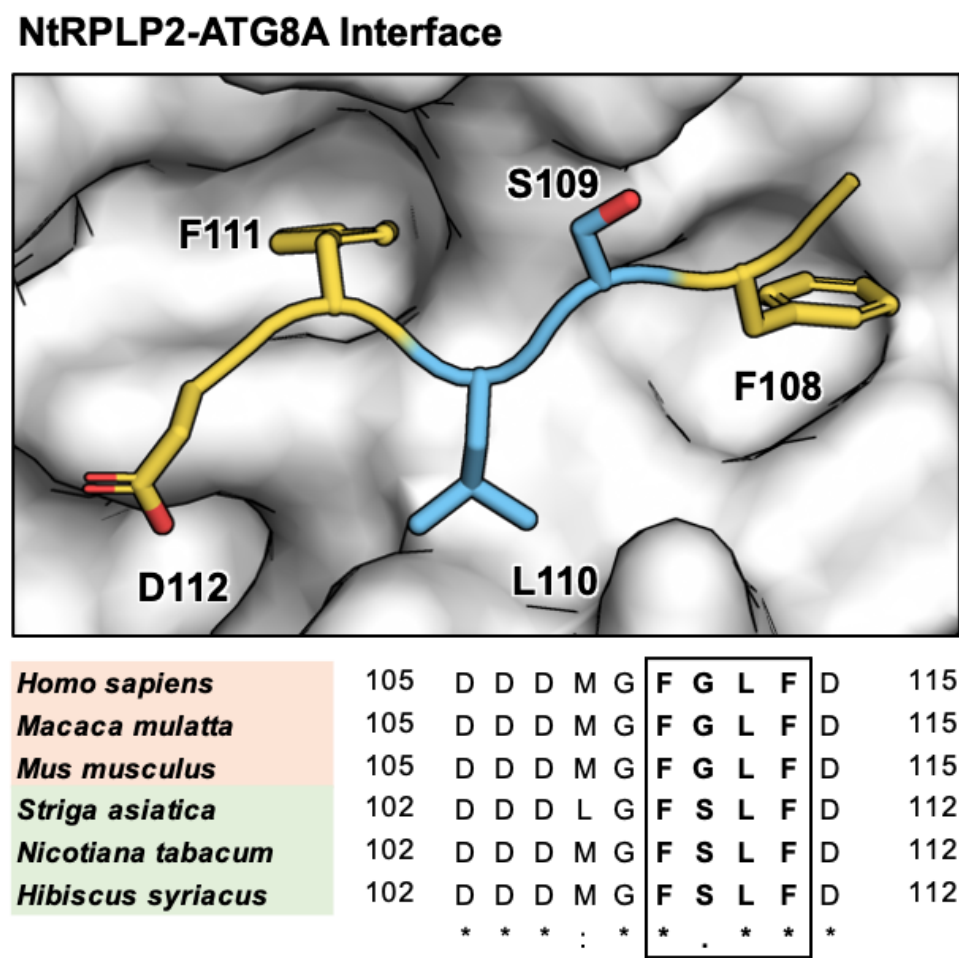


Figure33. AF2-multimer predicted non-canonical RPLP2-ATG8 interface is highly conserved in mammals and plants. The atypical F₁₀₈SLF₁₁₁ motif makes interactions with the LDS of ATG8A. RPLP2 AIM is coloured based on their pLDDT confidence score generated by AF2-multimer: >90 dark blue (high), >70 light blue (good), >50 brown (average) and <50 yellow (low). ATG8A is shown in grey. The alignment was generated using Clustal Omega. Mammalian species are coloured in pink and plants in green.

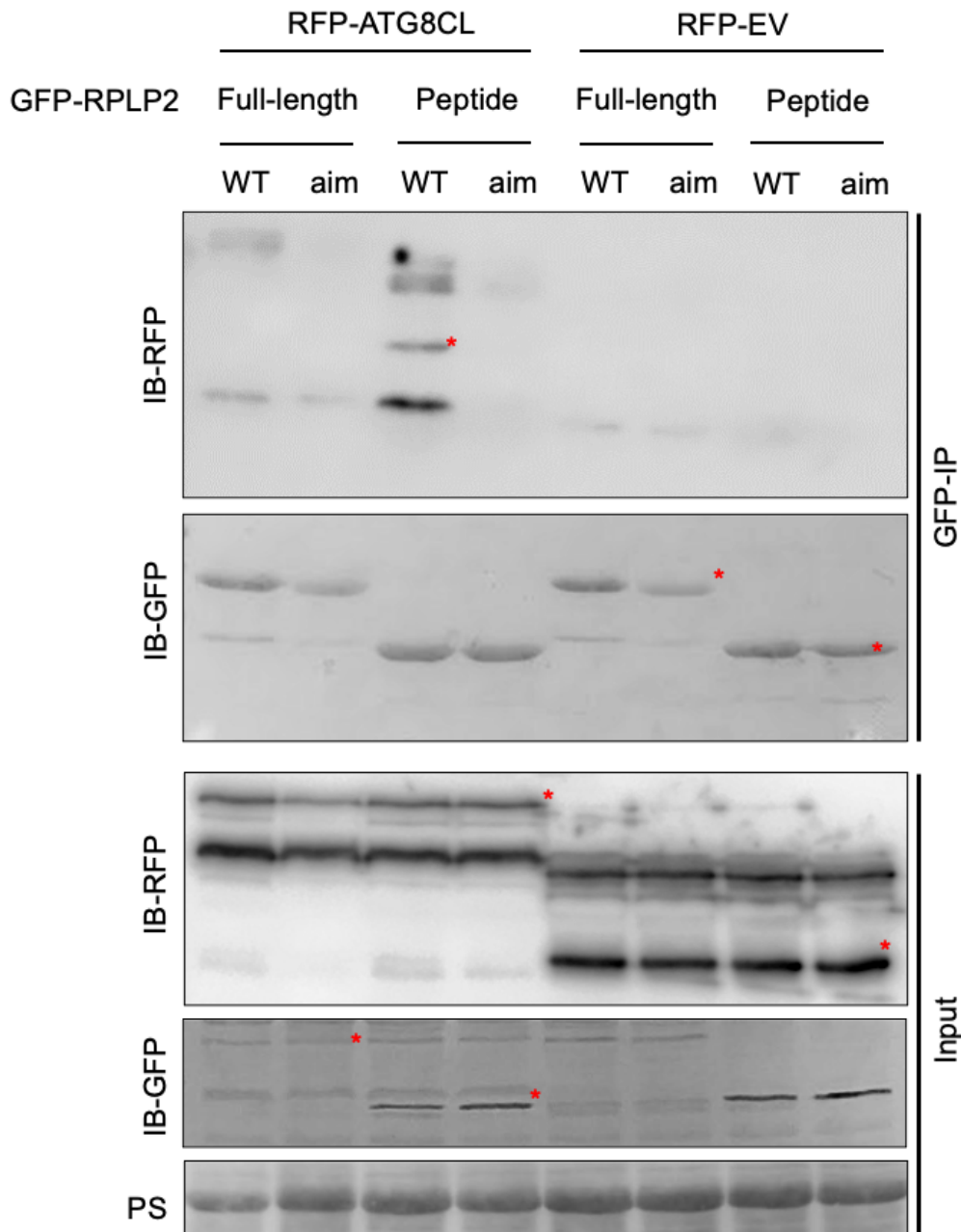


Figure34. RPLP2 interacts with ATG8 using a non-canonical AIM. RFP-ATG8CL or an RFP-EV control were co-expressed with GFP-RPLP2 wild type or AIM mutant with both the full-length protein and AIM peptides. Wild type RPLP2 and its AIM peptide interact with ATG8CL. This interaction is hampered in the ASLA AIM mutant. No RPLP2 bands were observed in the RFP-EV control. Red asterisks indicate expected band sizes. Uncropped blots – Appendix M.

RPLP2 localises to ATG8 puncta and drives autophagosome biogenesis

Having confirmed the non-canonical association, I then investigated the effects of RPLP2-ATG8 interaction on the subcellular dynamics of autophagy. To determine if RPLP2 can localise to autophagosomes, I transiently co-expressed BFP-ATG8CL and RFP-RPLP2 (WT (FSLF) or AIM mutant (ASLA)) and observed sharp RPLP2 signal from ATG8CL puncta. This localisation was hampered with the AIM mutant. This indicates that RPLP2 can localise to ATG8CL-marked autophagosomes, and its non-canonical C-terminal AIM is necessary for autophagosome localisation (Figure35).

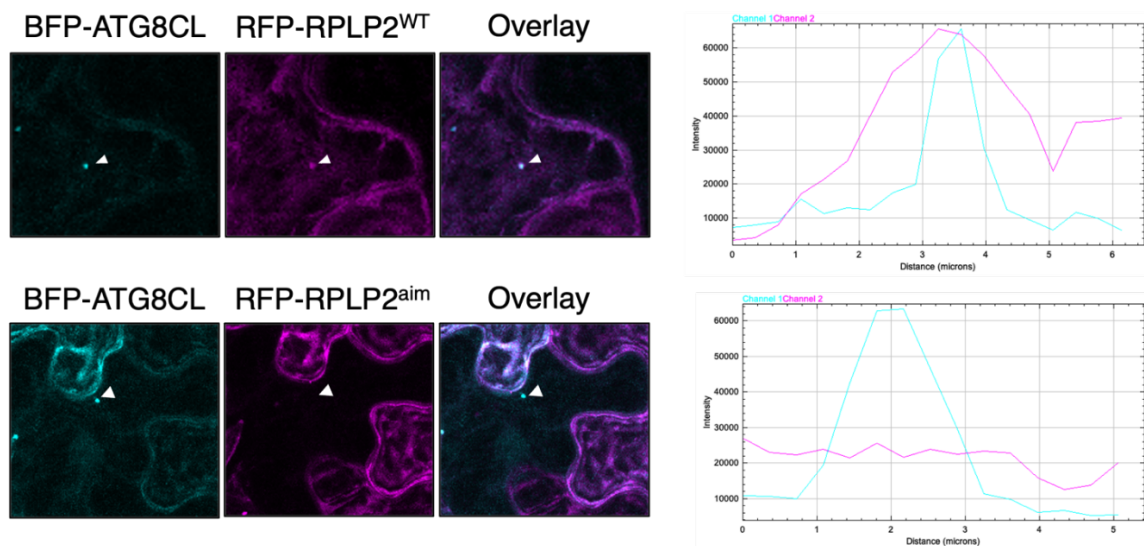


Figure35. RPLP2 localises to ATG8-decorated autophagosomes via its non-canonical AIM. Fluorescent micrographs from left to right: BFP-ATG8 (cyan), RFP-RPLP2 (pink) and Overlayed. White arrows indicate example puncta investigated. Images shown are maximal projections of 20 frames with 1 μ m steps. The right-hand side panel illustrates the overlap of signal intensity. For wild type RPLP2 the signal overlaps with the ATG8 signal, indicating co-localization. This overlap is lost in the AIM mutant RPLP2.

Increase in ATG8CL-labelled puncta is widely used as a functional readout of autophagic activity. When I co-expressed wild type RPLP2 with ATG8CL, the number of autophagosomes in the cell were significantly higher compared to when ATG8 was

co-expressed with the AIM mutant (Figure36). This indicated that RPLP2 induces the formation of more ATG8-decorated autophagosomes.

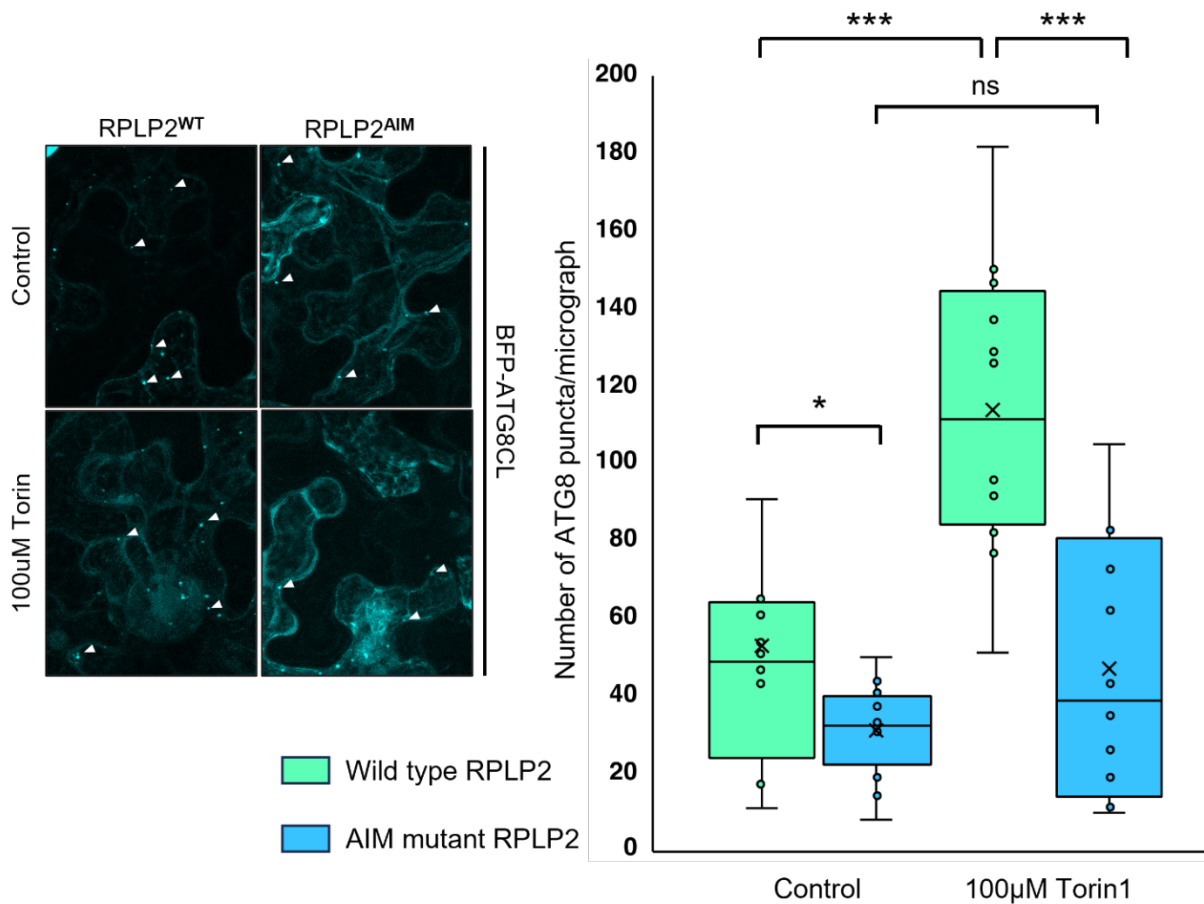


Figure36. RPLP2-ATG8 interactions increase ATG8-decorated autophagosomes in the cell. The left-hand side panel represents BFP-ATG8 micrographs when co-expressed with either wild type or AIM mutant RPLP2 and exposed to Torin1 treatment. White arrows indicate example puncta counted. Images shown are maximal projections of 48 frames with 1µm steps. Under control conditions, number of ATG8-puncta was significantly higher when ATG8 was co-expressed with wild type, compared to AIM mutant of RPLP2 (Wilcox test, $W=108.5$, $p<0.05$). This was also observed when Torin1 was used to increase autophagic flux (Wilcox test, $W=130$, $p<0.001$). Number of puncta significantly increased when RPLP2^{WT}-ATG8 were co-expressed and exposed to Torin1 compared to basal cellular state (Wilcox test, $W=129.5$, $p<0.001$). This difference between basal and Torin1-induced autophagy was not observed in AIM mutant RPLP2-ATG8 co-expressing cells (Wilcox test, $W=86.5$, $p>0.05$). Raw puncta number and analysis – Appendix N.

To further confirm that RPLP2- and ATG8- labelled endomembrane compartments are indeed autophagosomes, I investigated the effects of autophagy enhancer Torin1 (TORC inhibitor), widely used in mammalian cells, on number of ATG8 puncta. Under basal conditions, RPLP2^{WT}-ATG8 co-expression had significantly higher puncta compared to RPLP2^{AIM}-ATG8 co-expression in the cell. This was also true in the presence of Torin1-dependent increased autophagic flux (Figure36). Number of puncta counted for RPLP2^{WT}-ATG8, was significantly higher in the presence of Torin1 compared to basal conditions. There was no significant increase in ATG8 puncta when co-expressed with RPLP2^{AIM} under increased autophagic flux (Figure36). Together these indicate that RPLP2-ATG8 labelled puncta are indeed autophagosomes and via its non-canonical AIM, RPLP2 can interact with ATG8 to increase number of ATG8-autophagosomes. Overall, I confirmed the ribosomal protein RPLP2 as an adaptor between ribosomes and ATG8. However, a question remains whether RPLP2 is a selective autophagy receptor or an adaptor for bulk autophagy.

Discussion

Discovery of viral ATG8 modulators

Using the AF2-multimer guided pipeline we established, we set out to identify adaptors and modulators of ribosome recycling and viral manipulation of autophagy. We had previously shown that AF2-multimer can predict previously characterised pathogen ATG8 modulators. Recently, Homma et al. 2023 demonstrated its power in distinguishing between correct effector-receptor pairs through pLDDT confidence scores and used their pipeline to discover new effectors from tomato pathogens. To determine ATG8-based autophagy host-pathogen modulators, we chose plant viruses as a model organism. Viral interactions with host autophagy have been studied to

show both host and pathogen beneficial outcomes. We produced an ATG8-based interaction database with viral proteins. As we could not screen over 100 viruses using experimental techniques, we fished out viral proteins from their proteome using AF2-multimer by running predictions with ATG8-family members. This yielded 11 candidates with high scores at the AIM-LDS interface. The viral proteins had great structural and functional variety, which emphasises AF2-multimers ability to avoid functional or specific motif-based bias in identifying these proteins. This is also a great insight into the range of methods viruses employ to manipulate autophagy through ATG8 modulation, which can make them go undetected.

Viral proteins containing functional AIMS were validated from SYMMV, SVBV, TuMV and VTMoV. The viral ATG8 interactors from SVBV and TuMV, a reverse transcriptase and serine protease, respectively, had not been linked to autophagy, which demonstrates the ability of our approach to explore new avenues of plant-virus interactions. SYMMV P7 protein has a functional AIM that it uses to bind ATG8 and increase number of autophagosomes. The function of P7 here is unknown and a BLAST search of the virus taxa yielded no similarity to proteins of known function. Our goal now is to establish how P7 utilises the increased number of autophagosomes. For example, it could be directing host proteins to degradation or preventing degradation of SYMMV viral proteins. To this extent we have done mass spectrometry with a pull-down sample of P7 in the plant cell. Interestingly this has shown various ABC transporter complexes to associate with P7 and these have been described in plant immunity previously. P7 proteins from other viruses are known to form oligomers and facilitate ion transport. Our goal going ahead is to establish the link between ABC transporters and P7, and how the modulation of ATG8 affects viral infectivity. For this,

I am building a robust plant-pathosystem with a SYMMV agroinfection construct and the P7 protein expressed in the well-established PVX-*N. benthamiana* infection model, to determine how it affects PVX spread in the plant.

Interestingly, unlike other viral modulators investigated, the ATG8-interactor from VTMoV, P1, showed significant discolouration of *N. benthamiana* leaves when overexpressed, suggesting the activation of immune responses. We established this was not a toxic response by performing an overexpression assay in tobacco plants, which are known to be highly responsive to cellular stress. The cell death was specific to *N. benthamiana* and was only present when P1 was co-expressed with ATG8 and autophagic activity was not suppressed. These suggest that the autophagic activation through the formation of P1-ATG8 complexes triggered cell death. If the cell death observed is HR, it should be reliant on the NLR network. Using knock-out plants, we discovered that the TIR-NLR network is involved in the immune response. In follow up microscopy experiments, P1 overexpression led to chloroplast accumulation at the nucleus, which is a typical indicator of immune response activation (data not shown). Although the AIM mutant was more tightly bound, it did not show cell death. Upon further investigation, I discovered an alternative AIM by doing in silico truncations in AF2-multimer with ATG8. Most likely, the mutation of AIM1 (WSTV) exposes the secondary AIM to bind, which may carry out an alternative function through ATG8-binding. AF2-multimer was ineffective in identifying AIMS that require additional modifications to be functional, as demonstrated previously with Joka2. I am currently working to establish if this secondary AIM-ATG8 interaction is indeed occurring in the AIM1 mutant (WSTV to ASTA) using double mutants.

VTMoV belongs to the Sobemovirus family and their P1 proteins have great sequence variety but maintain structural similarities. P1 proteins from the Sobemovirus genus are implicated in viral movement and silencing suppression. P1 knock outs of Sobemoviruses are unable to spread systemically. The function of P1 in VTMoV has not been established. Our new mass spectrometry data set for a P1 pull down enriched Dicer-like proteins (DCLs) that form RNA silencing complexes. Hence, suggesting P1 acting to regulate RNA silencing. Going ahead, my goal is to establish the mechanism behind the potential silencing suppression activity of P1 and if this is achieved through ATG8 modulation and autophagy. The TIR-NLR dependent cell death will be studied to establish what the NLR network is guarding. They could be guarding the DCLs and once they are driven to autophagosomes through P1-ATG8, cell death is triggered to prevent viral growth and spread.

RPLP2 as a potential selective adaptor between ribosomes and autophagy

Screening of key ribosomal proteins against ATG8, as we did with viral proteomes, yielded the ribosomal stalk, specifically RPLP2, as a direct indicator of ATG8. The AIM found in RPLP2 was surrounded by serine and acidic residues, which is typical for a functional ATG8 interaction [45]. Using plants as a model organism, my goal was to validate RPLP2 as a direct ATG8 interactor. This was achieved using coIP and co-localisation assays with the full-length protein and the AIM region as a peptide to validate its functionality. Interestingly, RPLP2 overexpression increased number of ATG8-decorated autophagosomes. This is most likely due to the RPLP2-ATG8 complex initiating autophagosome formation, as these structures are known to form in the vicinity of cargo [50]. Although this could be due to blockage of vacuolar fusion of autophagosomes, we observe RPLP2 in the vacuole suggesting successful fusion and

activity of autophagy. These observations suggest that RPLP2 is a direct interactor of ATG8 and may act as a cargo to initiate autophagy. We further confirmed this by using an autophagy activator Torin1 [171], which further increased number of ATG8 puncta in the presence of wild type RPLP2 but not its mutant. This is typical as Torin1 is known to increase ATG8 (LC3) puncta in the cell. The difference between puncta numbers under control conditions was less compared to initiation of autophagy using Torin1, which may be a bulk response. Hence, none of these observations are sufficient to conclude that RPLP2 can act in a selective manner to clear ribosomes. Hence, I aim to silence RPLP2 in the plant cell and study ribosomal protein abundance in the presence and absence of autophagic activity.

The work in this chapter provides a step towards understanding mechanisms of ribo- and viro-phagy. We elucidated the importance of integrating AI-guided approaches into our research and its potential to accelerate discovery of new biology. This work pinpoints the ribosomal stalk and its RPLP protein family as a potential regulator of ribosome activity through ATG8-dependent autophagic clearance. We are yet to determine the selectivity of this process and what conditions lead to RPLP-ATG8 reliant ribosomal degradation. We also provide a step towards uncovering the diverse strategies by which plant viruses antagonise host autophagy and immune responses. Our goal is to develop clear mechanisms for biotechnology interventions that aim to introduce robust antiviral immunity to new crop cultivars.

5. Towards the structural characterisation of Joka2

Summary

Joka2, a selective cargo receptor implicated in plant autophagy, has been investigated for its structural characteristics. AlphaFold2 predictions to visualise Joka2 domain boundaries identified a novel helical bundle (HB) domain proximal to the PB1 domain in Joka2. Experimental validation of this domain revealed stable alpha-helices through expression, purification, and circular dichroism spectroscopy. Functionally, the helical bundle did not influence Joka2-dependent immune responses. The PB1 domain of Joka2, responsible for oligomerization, was studied using negative stain electron microscopy (NSEM). The presence of high salt hindered oligomerization, suggesting a role for acid-base ionic interactions. We established a protocol to assemble PB1 filaments in vitro and aimed to use these as scaffolds to solve structures of Joka2. Full-length structural studies faced challenges due to Joka2 degradation, flexibility, and heterogeneity. To enhance stability, autophagy-blocking tools were employed. The Joka2^{RR} mutant, with PB1 domain mutations, showed improved stability and solubility. Despite optimization efforts, cryoEM trials exhibited challenges with aggregation and contamination. Implementing a flow-cell device improved lysate application and wash steps, reducing membrane and rubisco contamination. However, ice thickness variability and sample heterogeneity persisted, leading to limited particle availability, and resulting in low-resolution 3D models. In conclusion, our study provides insights into the oligomerization and structural aspects of Joka2, shedding light on its dynamic nature and presenting challenges in obtaining high-resolution structures of this selective cargo receptor.

Introduction

Joka2 is an essential part of plant immune responses

Joka2, the main autophagy cargo receptor found in *S. tuberosum*, was identified as a major contributor to immunity against *P. infestans* (Figure 37A) [56, 57]. In turn, the late blight pathogen has evolved an effector, PexRD54, that subverts host immune responses by depleting Joka2 from autophagosomes [57, 172]. Over-expression of Joka2 produced increased immunity towards *P. infestans* and Joka2-decorated autophagosomes are known to localise to the haustoria (plant-pathogen interface). These findings and the presence of effectors targeting Joka2-dependent defence-related autophagy suggests an important role for the protein in plant innate immunity. How Joka2 contributes to immunity and mediation of defence-related autophagy via Joka2 remains a mystery. Joka2 interacts with the ATG8CL isoform of the diverse ATG8 family in plants. They co-localise in autophagosomes at the plant-pathogen interface, which is known to be a hot-spot for autophagosome biogenesis. Beyond *P. infestans*, Joka2 contributes to immunity towards viruses, by targeting capsid and effector proteins to autophagosomes.

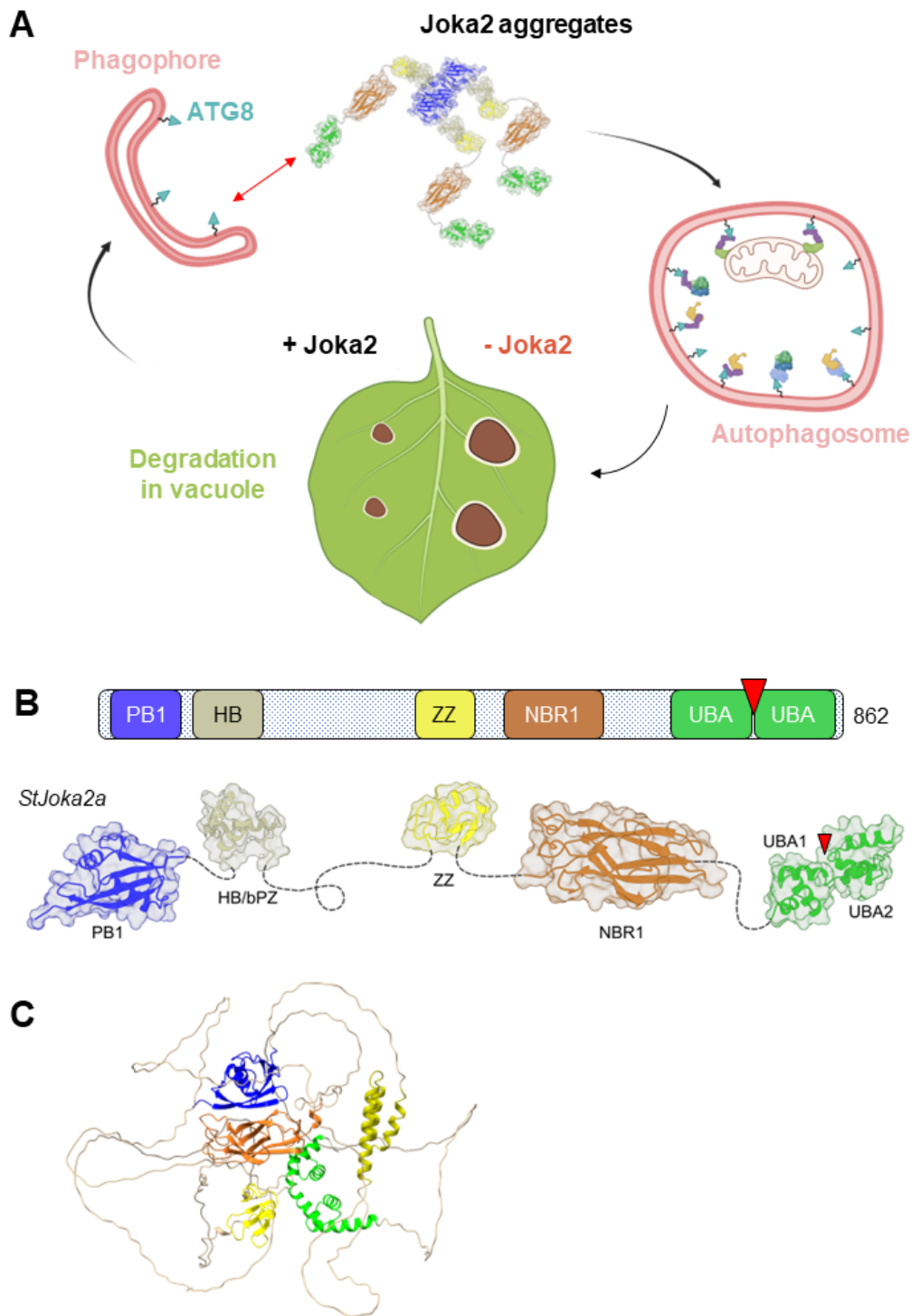


Figure37. Joka2 is a multi-domain autophagy cargo receptor implicated in plant immunity against pathogens. (A) Joka2 forms aggregates and localises to phagophores via UBA domain interactions with ATG8 (cyan), indicated by red arrow. The resultant autophagosome localises to vacuoles and leads to immunity because of autophagic degradation. **(B)** The domain organisation of Joka2. **(C)** AF2 prediction of

full-length Joka2. Almost half of Joka2 is intrinsically disordered and the folded domains seem to congregate. PB1: blue, HB: tan, ZZ: yellow, NBR1: orange and UBAs: green.

Joka2, a hybrid homolog of *H. sapiens* p62 and *A. thaliana* NBR1

The molecular architecture of Joka2 was defined by its relation to cargo receptors p62 (mammalian) and NBR1 (mammalian/*A. thaliana*) (Figure 37B). Joka2 and homologs use their N-terminal phagocytosis oxidase/Bem1P (PB1) domain for complex formation. The basis for the oligomerisation via the PB1 domain is the presence of opposing surface charges; acidic and basic. Hence, these PB1 domains are described as AB-type. The mammalian NBR1 homolog cannot utilise its non-AB-type PB1 domain but oligomerises via the presence of coiled-coil domains [59]. PB1 oligomerisation was demonstrated by Jakobi et al. 2020 in vitro. They show the PB1 domains to assemble in a helical manner to form filamentous structures. However, this was shown for the PB1 domain only and not full-length protein [60]. It is hypothesised that Joka2 can form mesh-like structures to capture cargo and form aggregasomes that initiate autophagosome biogenesis. For the PB1-based oligomerisation, a double arginine motif (RR), forming the basic patch, and an acidic OPCA motif (consisting of acidic Glu and Asp) were shown to be essential via site-directed mutagenesis. Cargo is recruited to mesh-like Joka2 aggregates via its ubiquitin associating domains (UBAs), which are also conserved in homologs. These contain the typical ubiquitin-binding motif MGF, which binds to poly-ubiquitinated proteins destined for degradation [173]. Specific to the plant selective receptors, two UBA domains are present (UBA1 and UBA2), however, only the more C-terminal one (UBA2) has been shown to bind poly-ubiquitin [174]. The function of UBA1 remains unknown. In mammalian orthologs of Joka2, the AIM/LIR is localised distant from the UBAs and modulates ATG8

recognition and subsequent localisation to autophagosomes. However, in plants, the two UBA domains “sandwich” the AIM (Figure 37B), potentially suggesting a function for their structural arrangement in this manner. UBA1 and AIM are close in space indicating a possible steric hinderance of that site. I demonstrated this using UBA constructs lacking UBA1, which pulled down more ATG8CL compared to the complete UBAs [175]. The AIM has the consensus hydrophobic sequence Y/W-XX-I/L (where X is any amino acid). For Joka2, the AIM sequence is WDPI, and this aligns to the first alpha-helix of UBA2, to which the poly-ubiquitin MGF sequence also aligns on the opposing end. These observations were made in the AF2 prediction. The AIM sequence has been described to exist in a flexible disordered confirmation and arrange into a beta-strand upon ATG8 docking. The *P. infestans* effector PexRD54 also contains an AIM (WEIV) and has a higher affinity to ATG8, hence its ability to displace Joka2 from autophagosomes.

The zinc-finger (ZZ) domain has been implicated in protein-protein and DNA interactions for all orthologs [176]. The NBR1 domain functionality remains unknown, however, is known to form protein-protein interactions [177]. Tumtas et al. (Bozkurt lab – unpublished) has shown this domain to interact with MAPKs implicated in plant innate immunity [178]. Joka2 may be acting as a signalling scaffold upon immune activation to regulate MAPK activity and downstream responses.

Structural studies of Joka2 and orthologs

Majority of the amino acid sequence for Joka2 is intrinsically disordered. To date, four functional domains have been identified in Joka2 using sequence homology and biochemistry. The PB1 domain of relatives has been shown to form filaments. ZZ,

NBR1 and UBA domains of relatives were structurally characterised using X-ray crystallography. Binding dynamics of NBR1 and p62 UBA domains with ATG8 and ubiquitin were established using colPs and NMR spectroscopy. Although individual domains are well characterised for Joka2 and its relatives, full-length information is not present, which may elucidate how these domains are arranged to allow oligomerisation and promote transient complexes with other proteins.

AlphaFold predictions of Joka2

Development of state-of-the-art AI technologies in structural biology has aided in the studies of proteins with limited structural knowledge as well as proteins that were troublesome in structural analysis. DeepMind has created a database together with the PDB where they predicted the structures of all known proteins. Autophagy cargo receptors, such as Joka2, have been challenging for experimental structural biology. The AF2 predicted structure provides great insights into why this has been the case. As the protein is highly dynamic and disordered, AF2 cannot accurately predict the Joka2 structure (Figure37C). The domains with structural information in the PDB, the dataset used to train AF2, had high accuracy. However, the intrinsically disordered region (IDR) had very low confidence and appear as a halo, where the domains are forced into the centre. This most likely does not represent the real organisation of Joka2 in planta.

Aims and objectives of Joka2-related work

There are no full-length structures for autophagy cargo receptors. This has limited our understanding of how these proteins arrange in the cell to facilitate their functions across all species. We hypothesise that Joka2 can act as a platform to stabilise

protein-protein complexes during immunity, for example, by binding MAPK signalling proteins. To establish how Joka2 interacts with these proteins and facilitates their function, we set out to optimise the extraction of Joka2 from its plant source and solve structures of native complexes, which can then be applied to relatives NBR1 and P62 from other organisms. We wish to study binding partners and interfaces to then probe these for functional validation and future bioengineering approaches in improving plant immune responses. Joka2 suffers from severe degradation and aggregation in the cell and during its extraction, due to its autophagic function and instability. To understand the overall protein characteristics and domain arrangement an AF2 prediction was performed. The predicted model led to the discovery of a new domain, potentially involved in solubility of Joka2, using protein expression, purification, and subsequent functional and biophysical assays. I then studied the PB1 domain to establish a protocol for its oligomerisation in vitro to form stable scaffolds for full-length Joka2 to aid with its instability. To prevent degradation of Joka2 we attempted to block autophagic activity and use a double Arg mutant preventing oligomerisation to improve solubility whilst extractions. We trialled capturing Joka2 using affinity grids from plant lysate to reduce its degradation in buffer and analyse our approaches using cryoEM. Consequent analysis revealed sample heterogeneity and flexibility in the Joka2 complexes immobilised on the grids. We addressed these issues with certain image processing approaches and produced a future outline to biochemically stabilise a Joka2 complex and reduce heterogeneity.

Results

AlphaFold2 predicted novel helical bundle domain

The lack of full-length structures for selective cargo receptors and the major breakthroughs in structural predictions made by AlphaFold2 prompted me to use this tool and visualise a full-length model for Joka2. My aim was to use this model as a guide in our biochemical and structural studies and reconsider the domain boundaries we have used to define the domain architecture. Most of the protein is predicted as a random coil. This presumably allows the vast protein-protein interactions of Joka2 and is likely stabilised in higher order structures. The PB1, ZZ, NBR1 and UBAs of Joka2 have been studied structurally, with various PDB depositions, and are conserved in other cargo receptors, such as NBR1 and p62. Hence, AF2 predicted these with high accuracy.

A

Affinity Chromatography

Size Exclusion Chromatography

W1 W2 W3 E1 E2

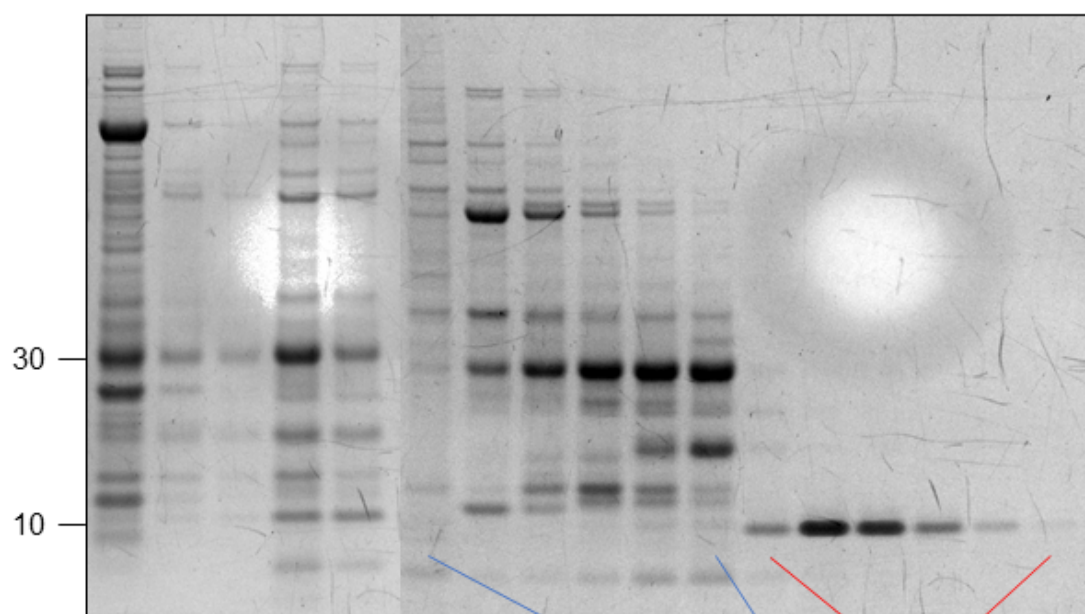
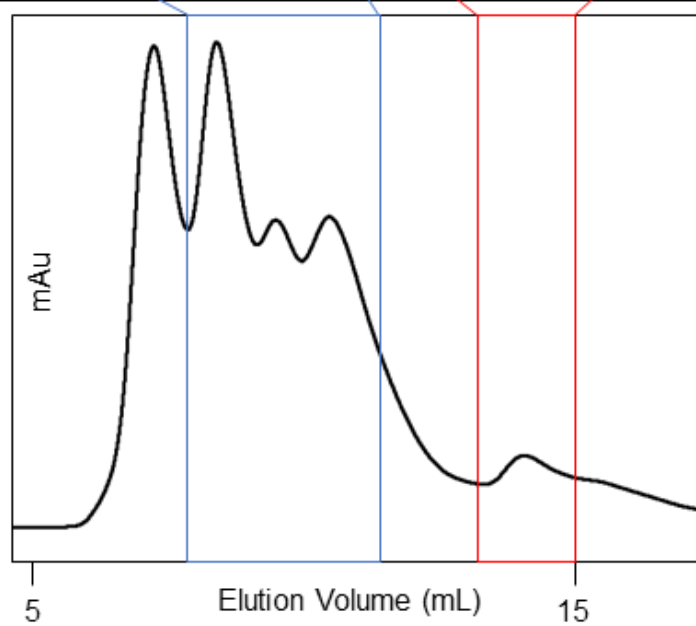
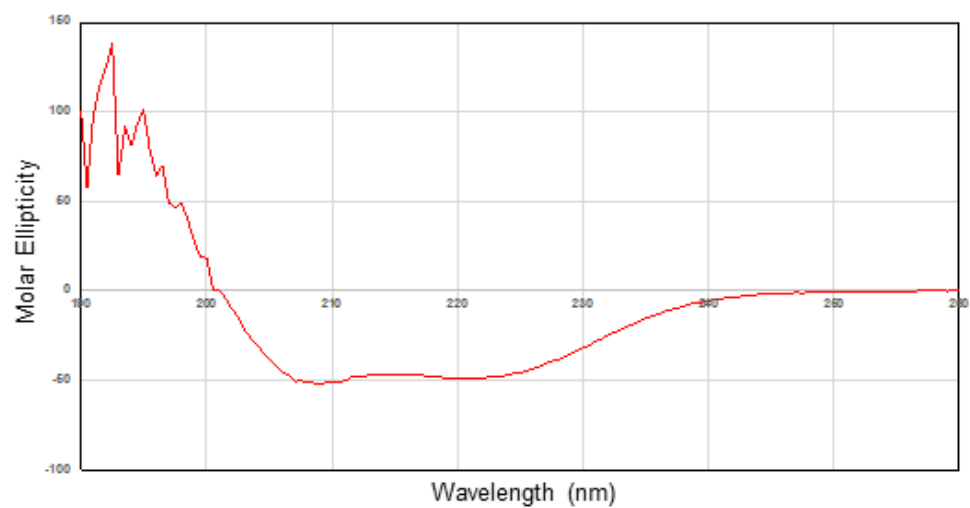
**B****C**

Figure38. Novel helical bundle domain identified in Joka2. (A) the sequence of the helical bundle can be expressed and purified from *E. coli*. Washes one-to-three (W1-3) and elutions one and two (E1-2) are shown. Size exclusion chromatography was done using a Superdex S75 Increase column. The low mAu band around 15mL accounts for the ~10kDa band observed on the SDS-PAGE. The other fractions analysed seem to be contaminations separated from the elution. The helical bundle is composed of three alpha-helices and accounts for a molecular weight of around 10kDa according to ExPASy. (C) CD spectroscopy of the purified helical bundle. The signal outline indicates an alpha-helical structure with no other secondary structure. Peaks at 193nm, -222nm and -208nm. The signal is an average of three replicates.

Interestingly, the AF2 model showed an unknown alpha-helical structure composed of three alpha-helices, located near the PB1 domain, with low confidence. To validate if the sequence corresponding to this predicted structure can form alpha-helices, I cloned this sequence into an N-terminal poly-histidine tagged vector for expression and purification from BL21 (DE3). Our construct was expressed and purified from *E. coli* following a protocol for a typical soluble protein and elutes at the expected range during size exclusion chromatography (Figure38A/B). Hence, this sequence most likely corresponds to a stable structure. To determine the secondary structure in the purified protein, we performed CD spectroscopy, which illustrated the expected peaks for an alpha-helical protein with no other secondary structure elements, validating the AF2 prediction (Figure38B/C). After validating a novel domain in Joka2, I was interested in the functional relevance with respect to Joka2's role in plant immunity.

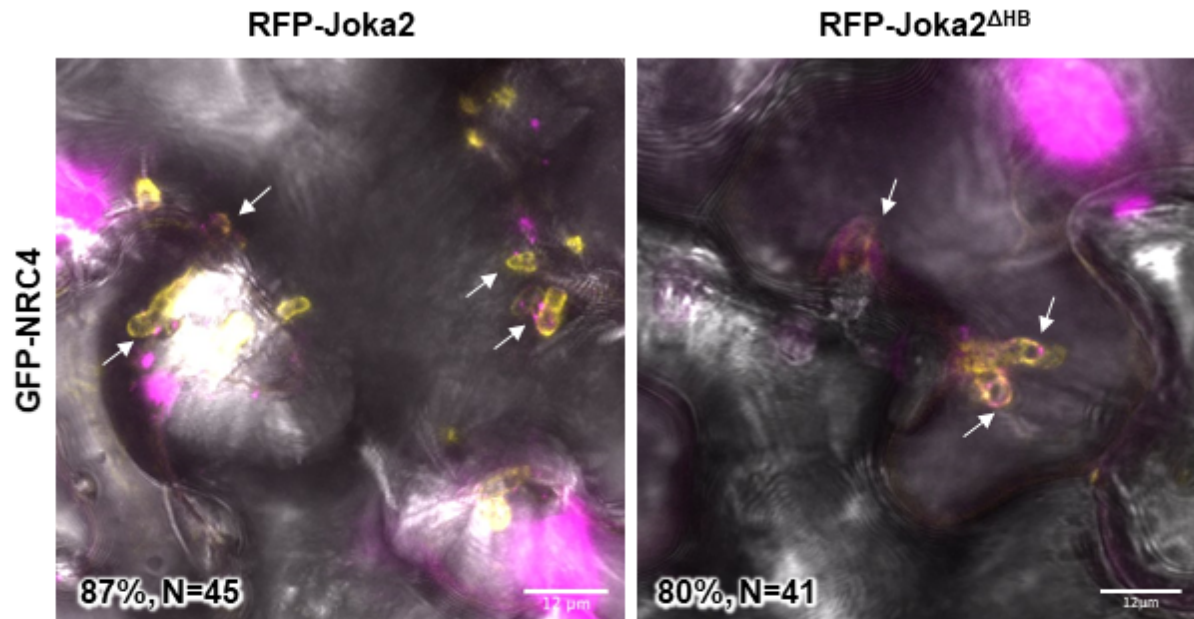


Figure39. Joka2 lacking the helical bundle can localise to the haustoria and produce an immune response. Micrographs are representations of 20 slices and are overlay images of the GFP, RFP and light channel. White arrows indicate haustoria. Pink puncta are RFP-Joka2 localised to haustoria. GFP-NRC4 localises to the haustoria and was used as a label. For RFP-Joka2 wild type 45 haustoria were counted. 41 were counted for the delta-helical bundle construct. N represent number of haustoria counted and the percentage value is the number haustoria with Joka2 puncta. Scalebar: 12μm.

Joka2 localises to the plant-pathogen interface (haustoria) during *P. infestans* infection [57]. To determine the role of the helical bundle in localisation to haustoria, we performed confocal microscopy to analyse Joka2 puncta comparing the wild type protein to a helical bundle truncate. I co-expressed Joka2 with a marker of the cytoplasm, an NLR protein (GFP-NRC4), which typically localises to the haustoria [179]. Plants co-expressing Joka2-RFP and GFP-NRC4 were infected with *P. infestans* and haustoria were visualised using a confocal microscope (Figure39). Joka2 lacking the helical bundle retains its ability to translocate to the haustorial membrane (N=41, 80%). Wild type Joka2 formed puncta around the haustoria as

expected (N=45, 87%). Hence, the helical bundle is not involved in the localisation of Joka2 at the plant-pathogen interface. Although the helical bundle truncate could localise to haustoria, I wanted to determine if it maintained a comparable immune response to the wild type protein. To this extent, I performed an infection assay on leaves expressing Joka2 wild type on one side and helical bundle truncate on the other half. The growth of necrotic lesions caused by *P. infestans* were similar on either side of the leaves, hence indicating that the helical bundle has no functional relevance during immune responses (results not shown). However, these experiments were done in plants expressing endogenous Joka2 and relatives, which can oligomerise and bring along overexpressed Joka2 to these puncta, via its PB1 domain. To eliminate the effect of endogenous protein, the experiments will be repeated in Joka2 knock-out plants.

Joka2 PB1 domain is responsible for oligomerisation and in vitro filament formation

Joka2 homolog NBR1 from *Arabidopsis thaliana* and humans was shown to form oligomers through its PB1 domain [60]. When purified, the PB1 domain could form filaments. Our goal was to see if Joka2 could form such filaments and optimise the process to form stable scaffolds for later structural biology approaches with Joka2, Joka2 domains and binding partners. To establish PB1 filaments for Joka2, I purified a poly-histidine SUMO-tagged PB1 domain construct from *E. coli* and imaged filaments using negative stain EM (NSEM). No filamentation was observed with the SUMO-PB1 tagged protein, as the SUMO-tag prevented PB1-PB1 interactions. Following 3C protease incubation, the SUMO-tag was isolated using size exclusion chromatography, and the PB1 domain alone was dialysed into a low salt buffer

(500mM to 50mM) to simulate PB1-PB1 ionic interactions (Figure40). Using NSEM, I observed filament formation for the Joka2 PB1 domain like that of NBR1 (Figure40C). With 500mM NaCl in the buffer, no oligomerisation is observed (Figure40B), supporting the theory that acid-base ionic interactions are the basis of observed oligomerisation.

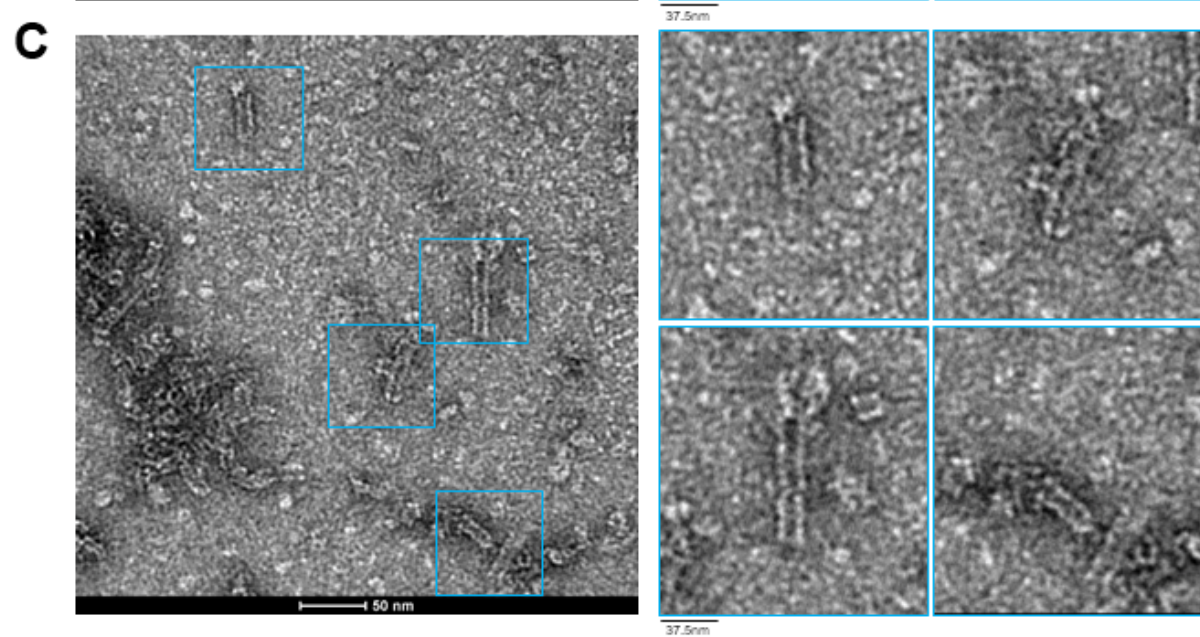
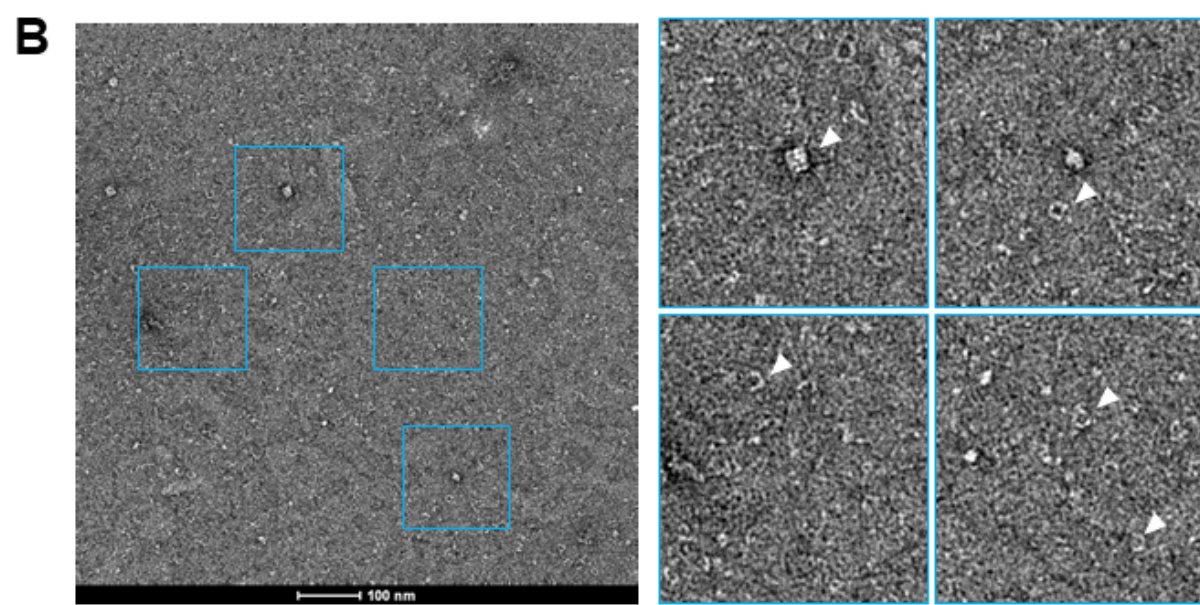
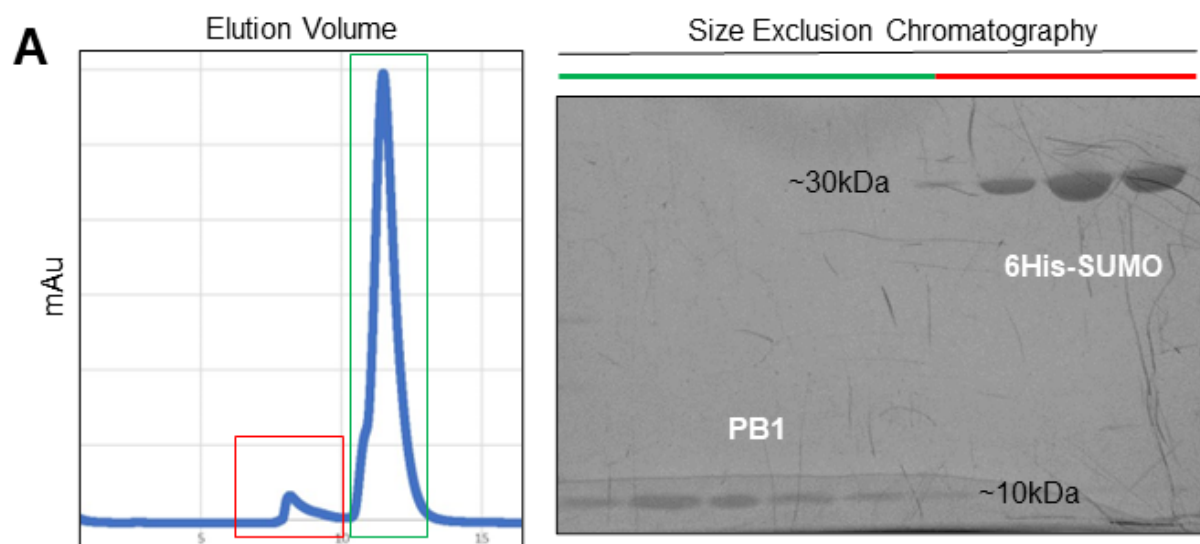


Figure40. Joka2 PB1 domain forms filaments in vitro. (A) Elution profile and its SDS-PAGE analysis following gel filtration of PB1 and SUMO-tag after tag cleavage. The PB1 domain appears less and is lost in the void. **(B)** NSEM analysis of high salt conditions. In the presence of 500mM NaCl almost no oligomerisation is observed. Scalebar 100nm. **(C)** NSEM analysis of post-dialysis low salt conditions. The PB1 domain can form acid-base interactions and initiate filamentation. Scalebar 50nm. Scalebars for zoomed in images (B) and (C): 37.5nm.

Advancements in full-length Joka2 structural studies

Autophagy cargo receptors are flexible proteins capable of binding various partners simultaneously to carry out their function, consequently leading to their degradation. They must form oligomers and functional aggregates with their cargo to promote autophagosome biogenesis in their vicinity. Joka2 is heavily degraded in the cell, which is further exacerbated during its extraction and purification. This is due to the autophagy process and protein instability. Oligomerisation of Joka2 drives it to aggregate and most of the protein is lost to the insoluble fraction during centrifugations. Together, these have hampered structural studies of cargo receptors. There are no full-length structures for NBR1 and p62 or their relative Joka2.

To reduce the degradation of Joka2 caused by autophagy, we utilised the PexRD54 AIM peptide tool, known to block autophagy [108]. I co-expressed RFP-AIM peptide with Joka2 and attempted a small-scale extraction followed by immunoblotting to determine effects on Joka2 levels. I also tested additional tools such as p19, an inhibitor of vector silencing, and hpVPE, silencer of vacuolar processing enzymes to increase protein abundance in the plant cell. Joka2 co-expressed with RFP-AIM peptide and p19 was more abundant and less degradation products were present compared to Joka2 co-expressed with RFP-AIMpeptide only and the peptide tool with

hpVPE (Figure41). Hence, for later purification attempts I co-expressed Joka2 with the AIM peptide and p19 tools to achieve higher protein levels.

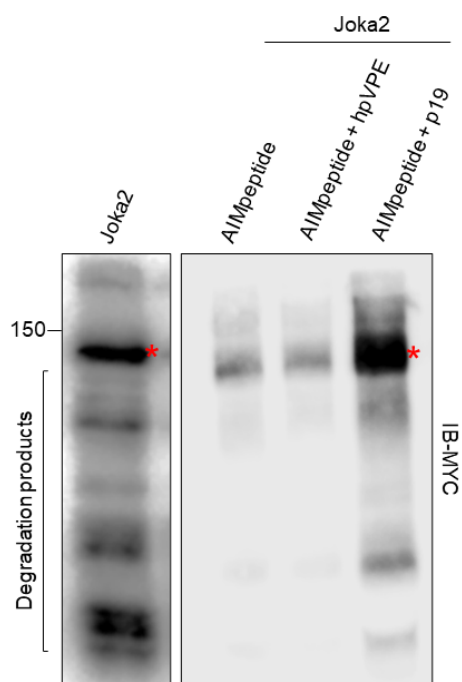


Figure41. Autophagy inhibition and vectoral silencing inhibition improves Joka2 levels in the plant cell. Blot on left-hand-side represent Joka2 (red asterisk) without any treatment and the degradation products. The right-hand-side blot represent Joka2 with various treatments, from left-to-right: RFP-AIM peptide, RFP-AIM peptide+hpVPE (vacuolar silencing) and RFP-AIM peptide+p19 (silencing inhibitor). Red asterisks indicate expected band size for Joka2. Blots were detected with MYC tag (Chromotek 9E1) antibody. Uncropped blots – Appendix O.

To tackle the oligomerisation and solubility challenges, I mutated two R (18 and 19) residues in the PB1 domain known to facilitate the formation of NBR1 aggregates. I performed an expression and extraction assay with Joka2^{WT} and Joka2^{RR}, the oligomerisation mutant. I compared the solubility and oligomerisation of Joka2 using reducing and native gels, with various detergents. Joka2^{RR} was more abundant in the plant cell compared to Joka2^{WT} (Figure42). This is most likely due to Joka2^{WT} shifting to the insoluble phase during extraction and centrifugations (demonstrated in a

separate trial in Figure43). For Joka2^{RR}, digitonin extraction seemed to promote the formation of a stable complex, whereas other detergents screened promoted monomeric forms of Joka2, most likely too harsh on native complexes and/or oligomers. Hence going ahead, we used the Joka2^{RR} construct and a digitonin-like detergent, DDM (due to availability), for future extractions.

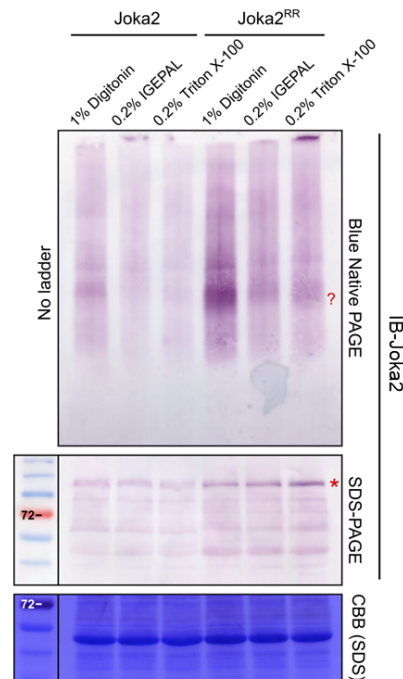


Figure42. Joka2 PB1 R₁₈R₁₉ to A₁₈A₁₉ mutant increases stability in planta and enhances extraction yield. Although equal loading, determined by Coomassie brilliant blue (CBB) staining of rubisco in the lower panel coloured blue, Joka2^{WT} is less abundant in the plant cell compared to Joka2^{RR} as demonstrated by the middle blot where a regular SDS-PAGE analysis was performed. The blue native page of the same sample for SDS-PAGE, for both Joka2^{WT} and Joka2^{RR}, shows a stable complex with digitonin only. IGEPAL and Triton promote monomeric forms of Joka2. Red asterisks indicate expected protein size. SDS-PAGE and Blue native gels were immunoblotted with a native Jok2 antibody. Although a ladder was loaded on to the native page it could not be observed on the gel and show accurate sizes. Red question mark indicates possible stable complex. Uncropped blots – Appendix P.

I made a streptavidin tagged construct of Joka2^{RR} and attempted an affinity extraction.

Joka2^{WT/RR}-Strep was bound to StrepTactin resin from lysate following a series of centrifugations to remove contaminants. After various washes, Joka2^{RR} was stable and available in supernatant in equal amounts, whereas wild type was lost into the insoluble phase more in each centrifugation (Figure43). Using immunoblotting, I was able to identify Joka2^{RR} in the elution fraction, however, the size of the protein eluted seemed to represent a stable degradation product of Joka2 around 70kDa (Figure43). Although this stable protein could be a candidate for further structural studies, my goal was to stabilize and extract the full-length protein to establish domain organisation of complete Joka2 and its ability to bind various targets transiently. To this extent, I added a SpyTag to the Joka2^{RR} construct to allow its direct purification with potential binders on a cryoEM grid to limit exposure to non-plant conditions with immediate freezing in liquid ethane. To determine if the SpyTag and SpyCatcher (immobilised on the grid) I purified was functional or not, I immobilised SpyCatcher on maleimide beads (Cube biotech) and determined if this affinity resin could bind SpyTag-GFP (Figure44). The maleimide beads functionalised with SpyCatcher were able to specifically concentrate SpyTag-GFP. This was supported with a size exclusion chromatography elution shift analysis of proteins on their own following incubation on ice. SpyTag-SpyCatcher incubated together, eluted from the column earlier, indicating successful complex formation. Overall, validation using maleimide beads suggests this approach of capturing spy-tagged proteins with SpyCatcher grids is successful.

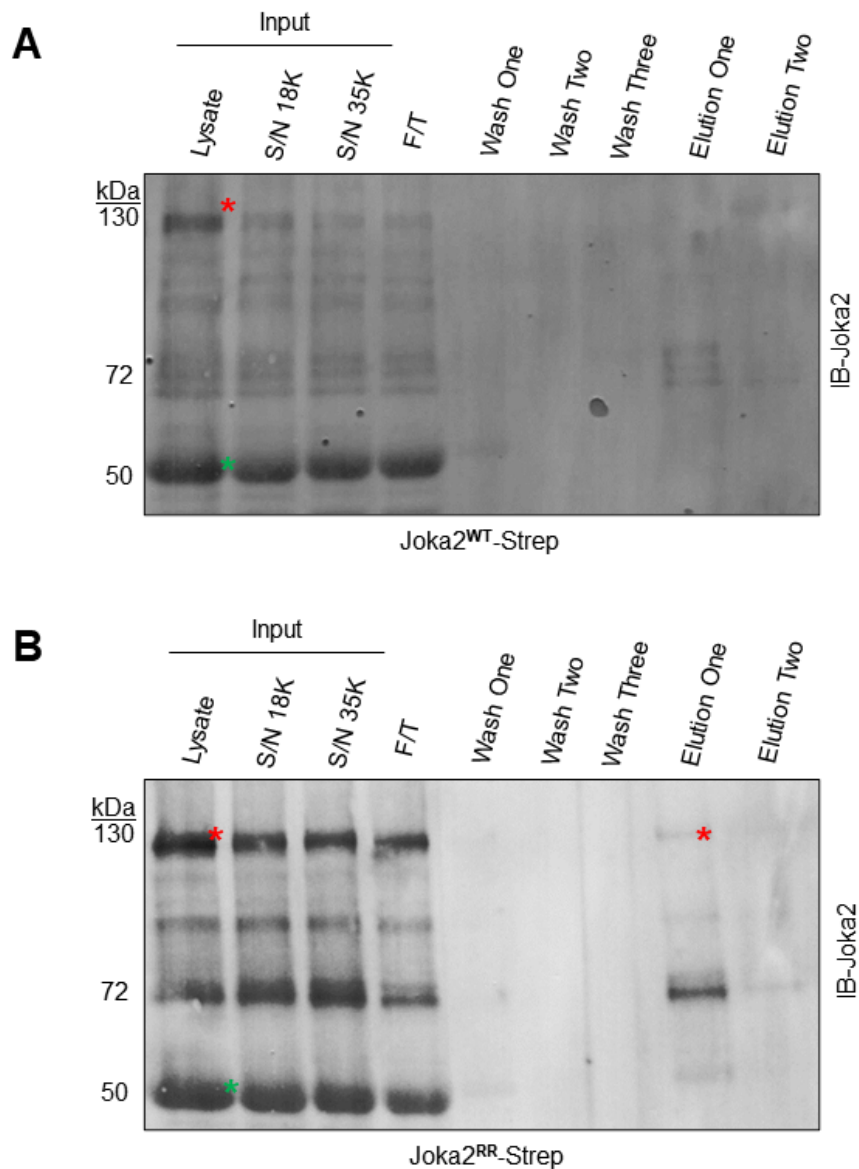


Figure43. Joka2^{RR} is more stable during purification attempts compared to Joka2^{WT}. Samples were extracted (lysate) then centrifuged twice at 18 and 35 thousand RPM (S/N 18K and 35K). The last supernatant was applied to beads and the flowthrough collected (F/T). No Joka2 was observed in the wash fractions. Elution one and two were done using 50mM biotin each. Joka2^{WT} significantly decreases during centrifugations, but Joka2^{RR} does not. Most of the full-length Joka2 (red asterisks) remains in the flow-through and the beads are bound by a stable 72kDa Joka2 fragment. Green asterisks represent rubisco, specified in this case to demonstrate equal loading and stability of soluble protein during extraction. Rubisco is detected because of polyclonal primary antibody non-specificity. Uncropped blots – Appendix Q.

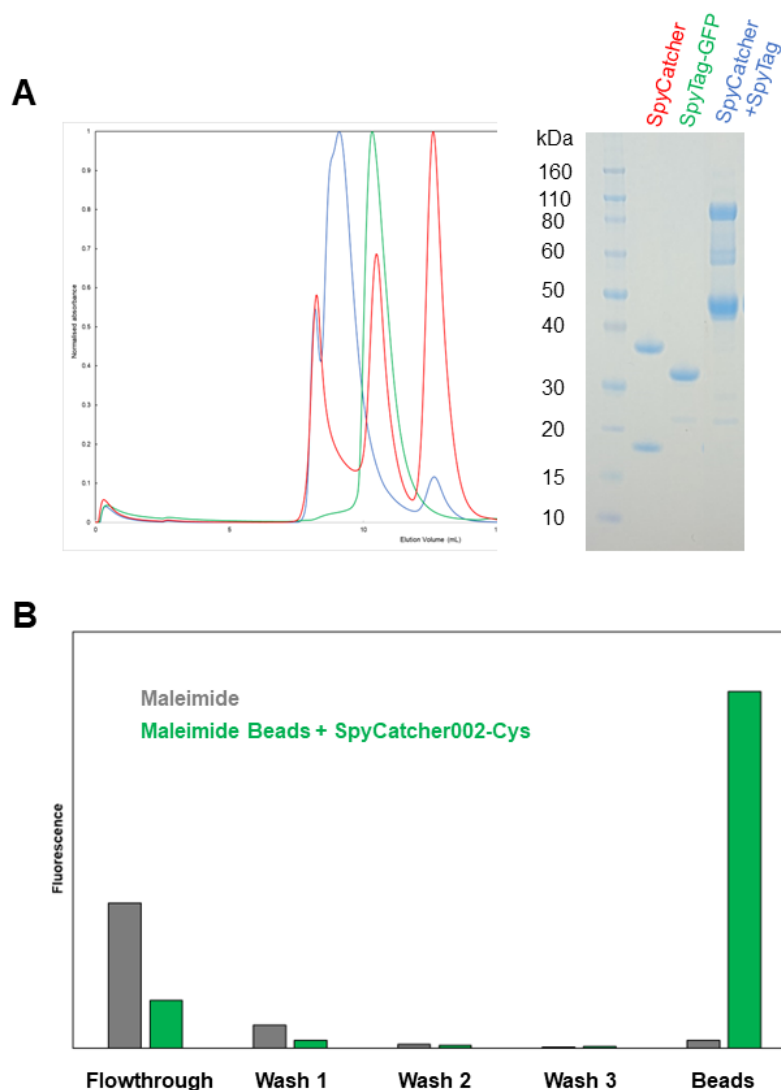


Figure44. Analysis of the SpyTag-SpyCatcher system. (A) Proteins were analysed using a Superdex Increase S75. SpyTag (green) and SpyCatcher (red) elute at 10 and 14mL, respectively. When incubated together, the elution profile is shifted to ~8mL, indicating a higher molecular weight complex. SpyCatcher elution profile had various peaks. The SDS-PAGE of the protein shows monomer and potential dimer. A covalent bond is formed between SpyTag and SpyCatcher, which shows a significant shift in the SDS-PAGE. **(B)** SpyCatcher has a free cysteine residue that is used to immobilise it onto maleimide beads. These beads (green) and beads with no SpyCatcher (grey) were incubated with SpyTag-GFP. The flowthrough fraction of beads with no SpyCatcher had more fluorescence compared to SpyCatcher beads. SpyCatcher beads had significantly more GFP fluorescence compared to empty beads, which had almost no fluorescence.

Following described optimisations, cryoEM trials were performed with Joka2^{RR}-SpyTag co-expressed with AIM peptide and p19 tools. Digitonin-like detergent DDM was used in the extraction from plant tissue. Instead of affinity resins, 4 μ L of lysate was directly applied to functionalised SpyCatcher grids. Following the first trial, micrographs contained aggregation, membranes, and non-specific rubisco binding on the grids (Figure45).

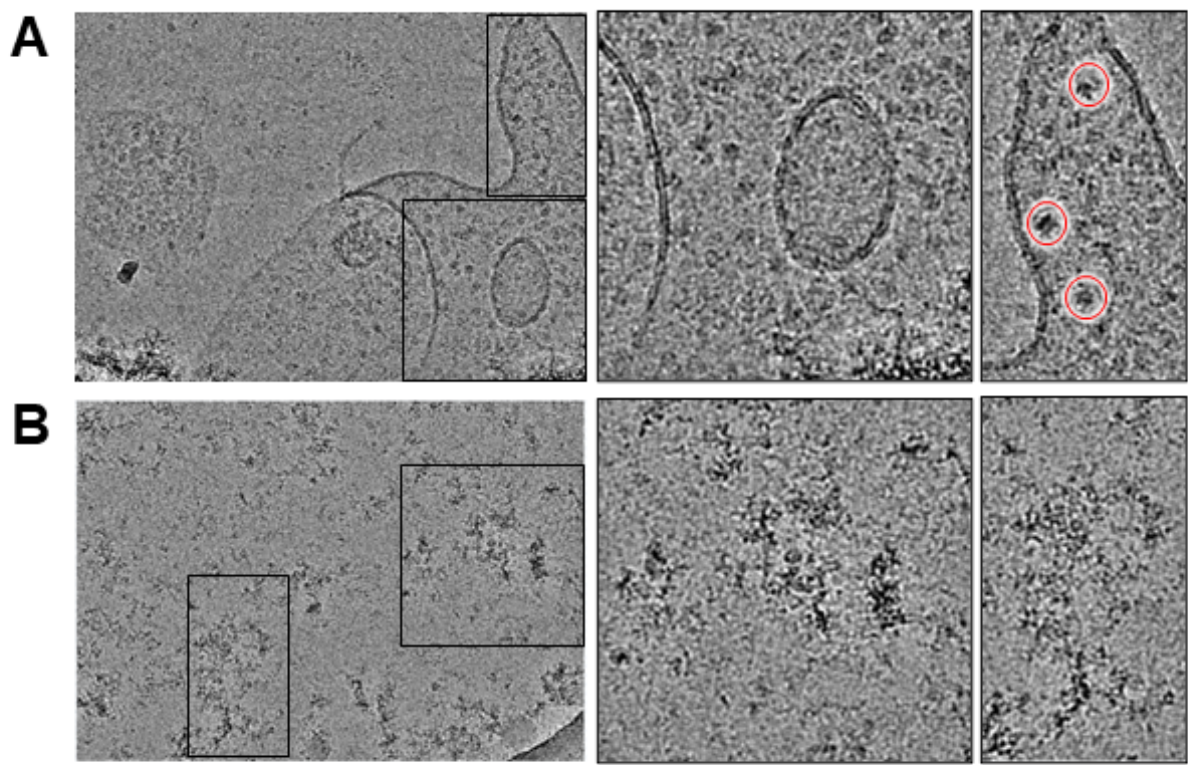


Figure45. Lysate application to grids with limited washing leads to membrane and rubisco contamination. (A) Large pieces of membranes were covering the holes with small vesicles present. Individual particles of rubisco contamination highlighted with red circles. **(B)** Heavy protein aggregation was observed on the grids. Whether this was Joka2, or other cellular proteins is not known. Scale bar for original micrographs: 210nm. Inset scalebars: 105nm.

This is most likely associated with the washing technique. There are no purification steps prior to application of the lysate to the grid. This brings along membranes and a

high protein content, which can bind to background modifications on the functionalised grid, such as graphene oxide and maleimide. Washes were performed using a drop-blot technique, where the grid was sequentially touched onto three droplets of buffer and blotted in between each. To improve washing conditions and lysate application to grids, I used a flow-cell device developed by Ramlaul et al. (2023) (Figure46).

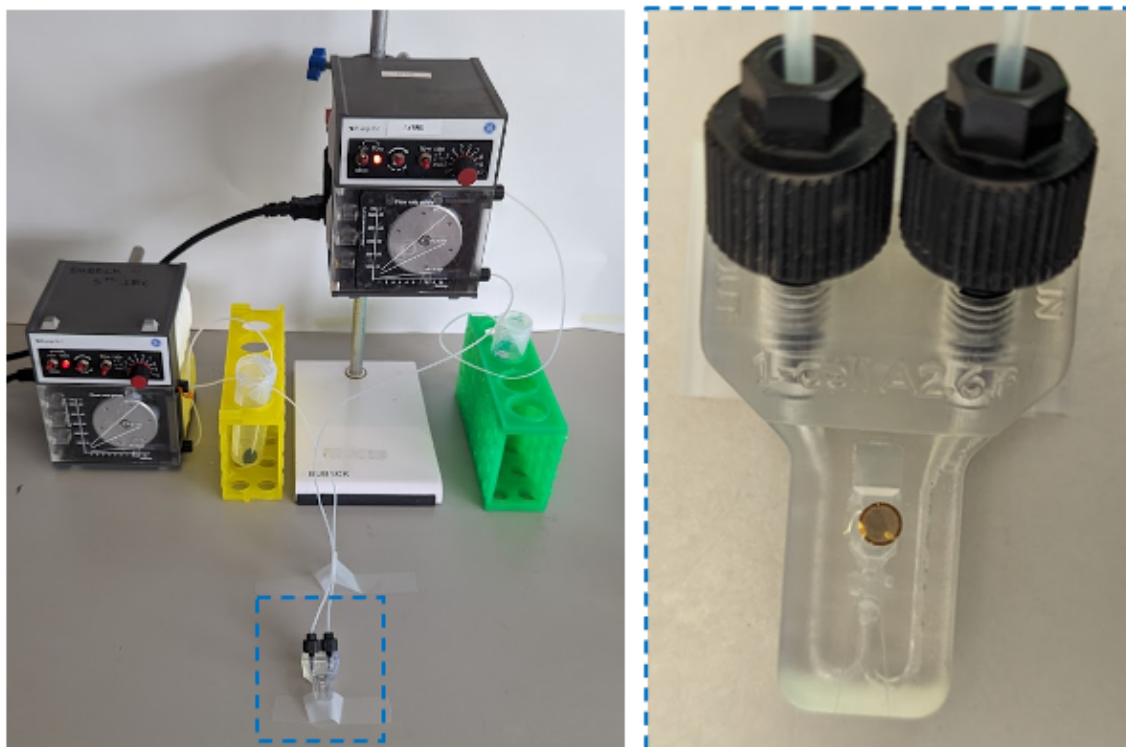


Figure46. Flow-cell set-up to improve and speed-up on grid lysate application and wash steps. Two peristaltic pumps were attached to the flow-cell to maintain a 1mL/minute sample flow rate.

This device allows rate controlled, fast application of more lysate on to grids, which might reduce the aggregation observed and improve particle numbers. It also allows fast washes of large volumes of buffer rather than drop-blot, which could remove non-specific binding to SpyCatcher grids. The flow-cell was attached to two peristaltic pumps and the rate was adjusted to 1mL/minute, which allowed quick incubation and wash times and did not damage the grids. 10mL of plant lysate was directly applied to

grids and immediately washed with 20mL of buffer using the flow-cell set-up. Micrographs from this sample preparation were clean with no membrane and rubisco observed (Figure47).

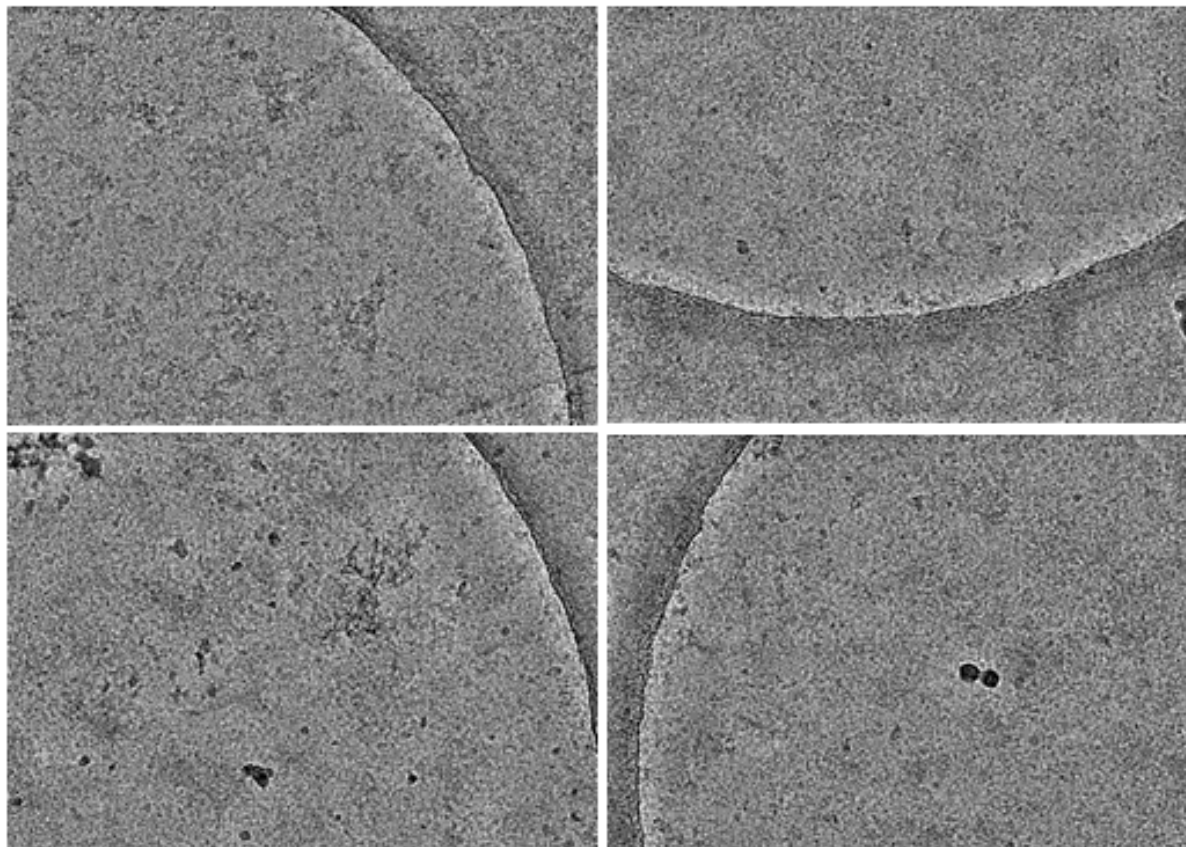


Figure47. Flow-cell lysate and wash methods improved grid contamination. No membranes or rubisco were spotted on grids. However, the images show dry holes with grainy contrast and aggregated protein. Scalebar: 210nm.

Although less, aggregates were still present, which can be a consequence of ice thickness. Grid atlases showed good ice distribution (Figure48), however, upon closer inspection of the micrographs, ice thickness was not accurate, and dryness was observed. This is a consequence of the functionalised grids with graphene oxide (GO), PEG and maleimide layers. The GO and PEG layers can scatter electrons, which alters the grey scale for ice thickness in the micrographs, hence, although the hole appears to contain ice, it does not. The squares in the atlases are rounded off, which

usually is an indicator of ice presence and the more rounded the edges, the thicker the ice. For the functionalised grids, this is also not accurate as there is an issue with wetting, which is a consequence of liquid distribution over the grid. We cannot glow discharge these grids and they have an inherent charge due to the functionalisation, which alters with the spread of liquid.

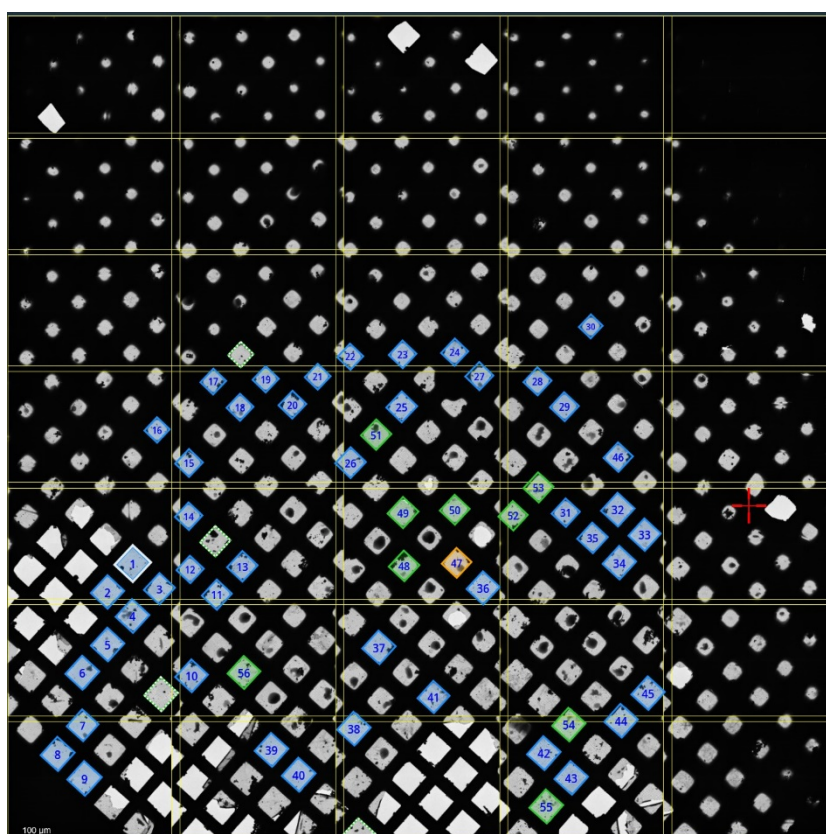


Figure48. Functionalised grid atlases showing an ice gradient. Rounded holes indicate thick ice. Squares with no ice have sharp edges. This atlas is from a data collection session and numbered squares indicate imaged regions.

To overcome sample- and ice- dependent limited particle availability, ~40,000 micrographs were collected for processing in CryoSPARC 4.1 (Figure49). Following motion correction and CTF refinement, blob picking was used to generate a set of particles. Potential Joka2-like particles, within the box size of 320Å (estimated Joka2

particle size is $\sim 120\text{\AA}$ in diameter), were selected and used to train a TOPAZ model, which was applied to the whole data set for particle picking.

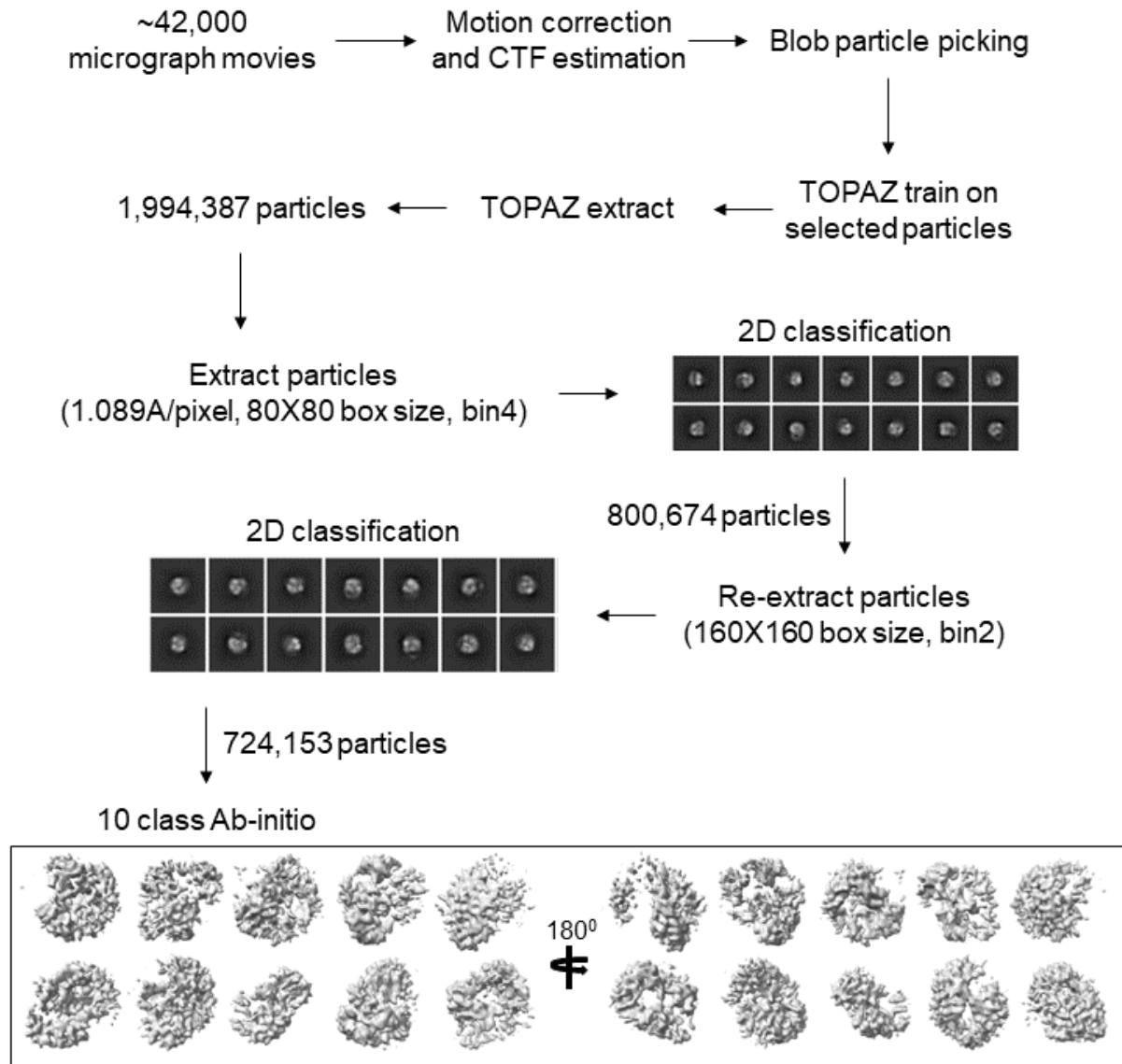


Figure49. Joka2 dataset processing pipeline. Over 40,000 micrographs were collected at eBIC, Krios I equipped with a BioQuantum K3 camera at with a total dose of $\sim 50\text{ e}^-/\text{\AA}^2$. Following motion correction and CTF estimation in CryoSPARC Live, blob particle bicker was used to generate a set of particles used for TOPAZ training and subsequent extraction. ~ 2 million particles were picked and extracted at a box size of 80 pixels (bin4). 2D classification at 80 and 160 pixels were used to clean the particle set. Just over 700,000 particles were used in ab-initio 3D model generation. A 10-class ab-initio yielded 10 different classes that could not be greatly distinguished.

Subsequently, particles were analysed using 2D classification and ab-initio reconstruction. 2D classes and 3D models generated for the sample were noisy and had great variety. Joka2 flexibility and sample heterogeneity yielded low resolution models, which could not be improved by increasing the number particles assigned per class. Direct application of sample from lysate is most likely responsible for the heterogeneity of Joka2 particles and degradation due to deceptive ice thickness also contributes to low models generated. Within the plant lysate, Joka2 is most likely forming a range of interactions with various proteins. Although the PB1 domain had a non-oligomerisation mutation, other domains could still form complexes with proteins, such as NBR1 domain with MAPK's. Oligomerisation between the PB1 and UBA domains is also known to contribute to Joka2 aggregate formation [180].

Discussion

For Joka2, my main goal was to solve its structure to address the questions: (1) how are domains organised to allow Joka2 function and (2) how complexes form with binding partners? Joka2 can interact with MAPKs, and this is dependent on its solubility (separation from aggregates) [178]. We set out to purify Joka2 from its native source to visualise functional complexes. To this extent, I had to optimise the degradation and solubility issues faced in earlier extraction attempts. Beyond biochemical probing, I used AF2 to produce models of Joka2 as a guide in our optimisation attempts. Consequently, I observed a new domain, unique to plant cargo receptors, which I validated using expression, purification, and CD spectroscopy. In planta experiments demonstrated no functional relevance for the helical bundle domain, however, the endogenous wild type protein cannot be ignored, and we must

repeat these experiments in a silencing background to avoid endogenous Joka2 from recruiting the mutants via PB1-PB1 oligomerisation.

I used AF2-multimer to study how the helical bundle might be associated with other Joka2 domains and to gain an insight into its function. HB could interact with itself, forming dimers, and the PB1 domain, which indicates it might be involved in regulating the oligomerisation events. Upon closer inspection of our SDS-PAGE analysis of the purification of the helical domain, we observe higher molecular weight bands with great intensity, ~30kDa, which may be oligomers of the helical bundle domain. These observations are supported by the discoveries of Gonzalo A. Dominguez in the Bozkurt lab, where he demonstrated the importance of this domain in regulation of Joka2 solubility from its aggregasomes. Truncation of the HB domain led to increased solubility of monomeric Joka2 species. However, there is no direct evidence to this in terms of Joka2 function, and further biochemical and biophysical studies are required to establish helical bundle dependent Joka2 in planta behaviour. A similar helical bundle was identified in human NBR1 near the UBA domain (opposite end compared to Joka2) and was established as a membrane contact site, affecting NBR1 localisation to membranes upon its removal. This helical bundle was described as conditional and was folded only in the presence of membranes. PMIPred [181] and DREAMM [182] membrane binding prediction servers suggest the helical bundle contains a membrane sensing and binding region, however, this has not been experimentally validated. AF2 predictions of human p62 and NBR1 do not contain a helical bundle, whereas the Arabidopsis NBR1 did. An integrated, folded HB domain near the PB1 may be only functionally relevant to plant specific cargo receptors.

Subsequently, I focused on the PB1 domain to build scaffolds to solve structures of Joka2. The PB1 domain of NBR1 forms filaments in vitro, which I demonstrated to be the case for Joka2 as well. Our goal was to use the PB1 filaments in vitro to stabilise Joka2 complexes, preventing extreme flexibility. In the end, this was not attempted but other immobilisation strategies were explored to prevent troublesome extraction. Affinity purification of Joka2 using beads was successful, but the most stable species purified was a ~70kDa stable degradation product. The streptavidin tag used was on the C-terminus, hence this fragment must be consisting of at least the UBA and NBR1 domains. For future efforts, the exact identity of this fragment can be determined using mass spectrometry. This region of the protein, together with its binding partners, such as MAPK, which binds to NBR1 domain, can be used answering our questions to a certain extent.

Developments in the field of sample extraction and application to EM grids improved our attempts to solve the structure of Joka2 from in planta. To avoid degradation during extraction and increase protein yield, I decided to use the SpyTag-SpyCatcher system established in the lab of David Agard [117]. This system was coupled with the developments from Chris Aylett's group with the FlowCell system [118]. FlowCell improved direct sample application and washing of EM grids to prevent contamination from crude plant lysate we observed in previous data collections with the SpyTag-SpyCatcher system. We collected a dataset for Joka2^{RR}-SpyTag immobilised onto SpyCatcher modified grids using a Titan Krios at eBIC. Over 40,000 movies were collected to account for limited number of particles per hole and screen the differential ice thickness across the grid. Although grid atlases represented an ice gradient, the micrographs and later image processing revealed lack of ice and degradation due to

likely exposure to the air-water interface. This is a consequence of using functionalised grids, where they have background scattering and lack of wetting. To tackle this, I will optimise detergent conditions. For example, increasing the DDM concentration or a different detergent, may adjust the charges on the surface to improve solvent distribution. This can also be improved during freezing, with adjusting the blot time and force. Hence, using atlases as a guide for ice thickness is not suitable for these affinity grids. I performed a range of 3D model generation techniques in CryoSPARC. Increasing model numbers did not improve resolution of models and kept on yielding different models with a similar core structure. Hence, Joka2 was most likely immobilised on the grids yet could not be reconstructed to high resolution due to heterogeneity and degradation.

Going ahead, my goal is to optimise the Joka2 sample in the plant cell. I aim to induce immune responses using *P. infestans* extracted PAMPs. Joka2 was shown to become more soluble under these conditions in a helical bundle domain dependent manner by Gonzalo Dominguez. Hence, we aim to promote the soluble form of Joka2 and co-express it with MAPK proteins to produce a stable complex that we could capture with an additional purification step of the MAPKs. This should yield a mostly homogeneous sample and improve sample stability.

6. Conclusions

In this work, I developed a novel AI-integrated pipeline for the discovery of short linear interaction motifs (sLIMs), specifically found in ATG8-interacting proteins. Our AF2-multimer guided method can identify typical motifs with great accuracy, and compared to other methods, identify atypical and cross-kingdom ATG8 interactions, leading to discovery of new biology. Expanding on this, we investigated specialised autophagy processes virophagy and ribophagy. We employed an AI-proteomics approach with our pipeline to screen viral and ribosomal proteome against ATG8. We experimentally validated the effects of candidates on ATG8 and autophagic activity to establish a system of ATG8 modulation during viral infections and ribosomal recycling. I explored the ATG8-interacting cargo receptor Joka2 to establish a novel domain and oligomeric scaffolds of its PB1 domain.

Our AF2 pipeline accurately predicts and validates functional sLIMs from various organisms. For ATG8 adaptors and modulators, the discovery of atypical modules has allowed fast-forward discovery of novel biology. We were able to validate a unique non-canonical AIM in ATG3, shedding light into its ATG8 priming activity during initiation and elongation of selective autophagy compartments. Another non-canonical AIM was discovered in the RPLP stalk of the ribosome, and we validated RPLP2 as a direct interactor of ATG8, presenting a new candidate for the controversial field of selective ribophagy. These findings provide further insights into the molecular mechanisms of autophagy in the cell. We expand on this novelty in cross-kingdom manipulation of autophagy, aiming elucidate various molecular techniques pathogens employ to redirect host autophagy to their own benefit. With our findings I present here, we work towards understanding the manipulation of selective autophagy to

hypothesise bioengineering approaches. We have now produced mass spectrometry data from pull-downs of viral effectors to understand their interactome in the host. Interestingly, we have identified proteins previously described in immune responses, like ABC transporters, to be direct interactors of viral effectors, which simultaneously interact with ATG8 to induce autophagy. We hypothesise that viral effectors can redirect host immune proteins to degradation to improve viral infection. We aim to prove this using viral-plant pathosystems, like the well-established PVX-*N. benthamiana* model system.

An interesting observation during the investigation of VTMoV P1 effector, immune-dependent cell death was observed, and we linked this to the TIR-NLR network facilitated by the lipases EDS1/PAD4, leading to activation of NRG1 and/or ADR1 NLRs. This brings a new perspective to how viral effectors can affect ETI responses. Through expression trials and mass spectrometry, we discovered P1 to interact with silencing complexes (DCLs). We observe increased expression when proteins are co-expressed with P1, suggesting it is a silencing inhibitor. How it achieves this is not yet clear, but we hypothesise it interacts with DCLs and ATG8 simultaneously to facilitate its degradation or targeting away from the nucleus. The cell death we observe might be consequence of inhibited silencing control over a long period of time leading to increased NLR accumulation.

An important challenge to overcome in my PhD was optimisation of sample preparation for cryoEM analysis of Joka2. We set to determine the structure of Joka2 to understand its ability to encompass a range of complexes and their functions. Lack of full-length structures for cargo receptors, is a good indicator of the toughness of the

task. Hampered by degradation, flexibility, and heterogeneity, it was clear that creative approaches are required to solve this problem. Initially aiming to use scaffolds of the PB1 in vitro filaments, we later focused on new developments in the field of sample preparation for cryoEM.

For Joka2 extraction, I tried the streptavidin-biotin affinity system, and purified Joka2, however, a degradation product, ~70kDa, was more abundant. To avoid this, we used affinity grids to directly capture Joka2 on the grids from fresh lysate and immediately flash freeze preserving its native state. This was supplemented with non-oligomerisation mutations, co-expression with autophagy blocking peptides and an optimised detergent in extraction buffer. In our cryoEM analysis, we observed degradation due to issues with ice and heterogeneity of particles. To limit these, in next trial, I will co-express Joka2 with MAPK3 to prioritise the capture of signalling Joka2 complexes. I will further stabilise complexes by triggering immune responses by using *P. infestans* treatment. Joka2-MAPK3 interactions are known to increase and Joka2 solubility promoted in the plant cell upon immune activation. As a back-up, I will prioritise the ~70kDa stable product but also make truncations of Joka2 in the disordered regions to produce a more stable protein.

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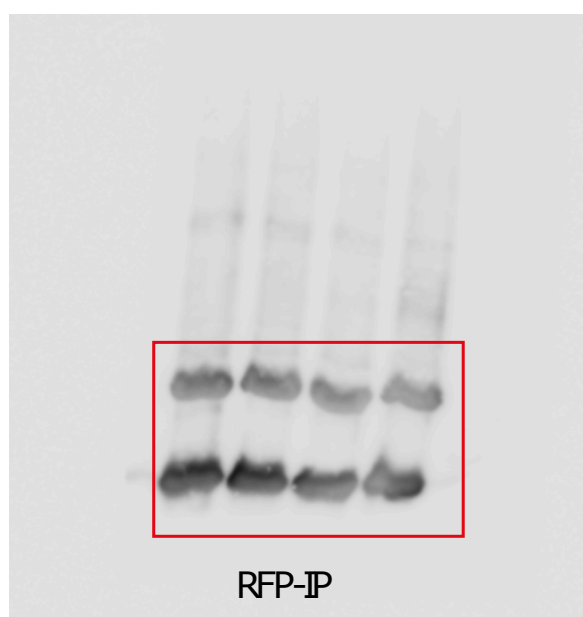
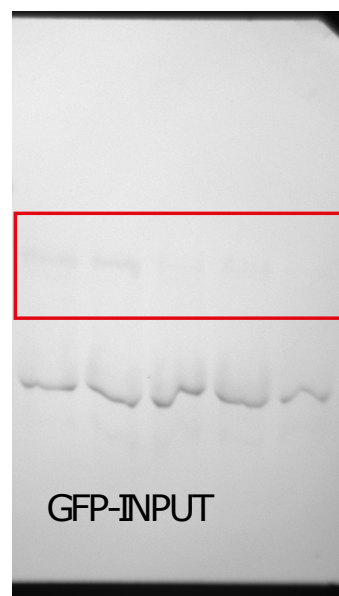
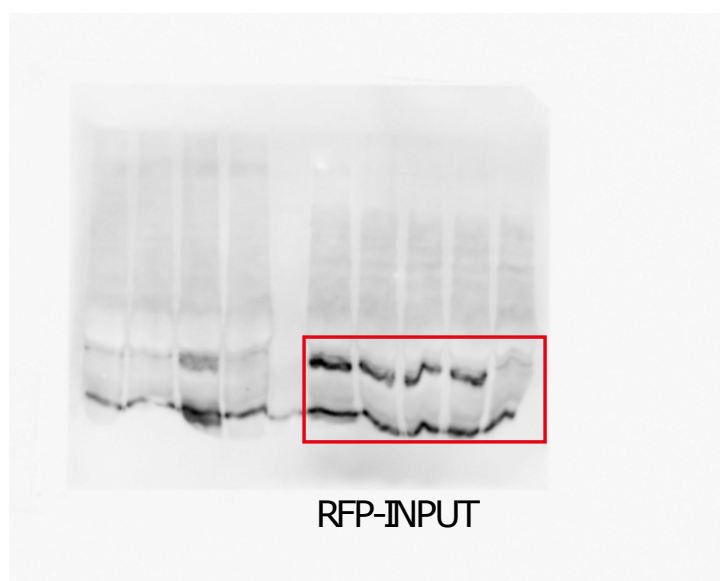
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Appendices

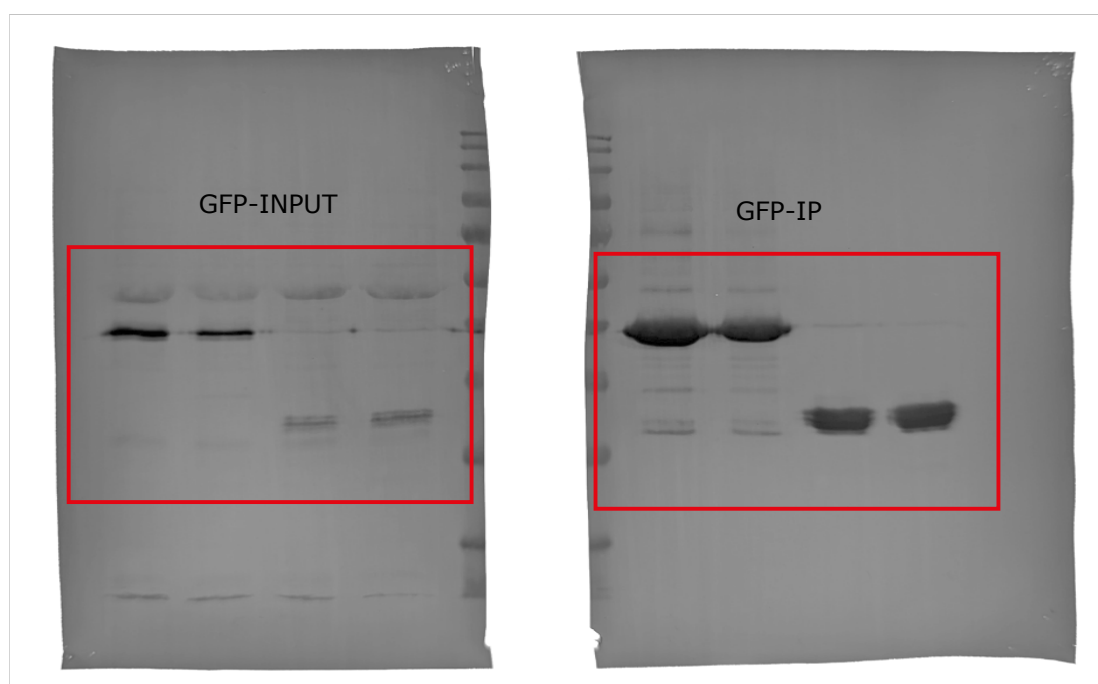
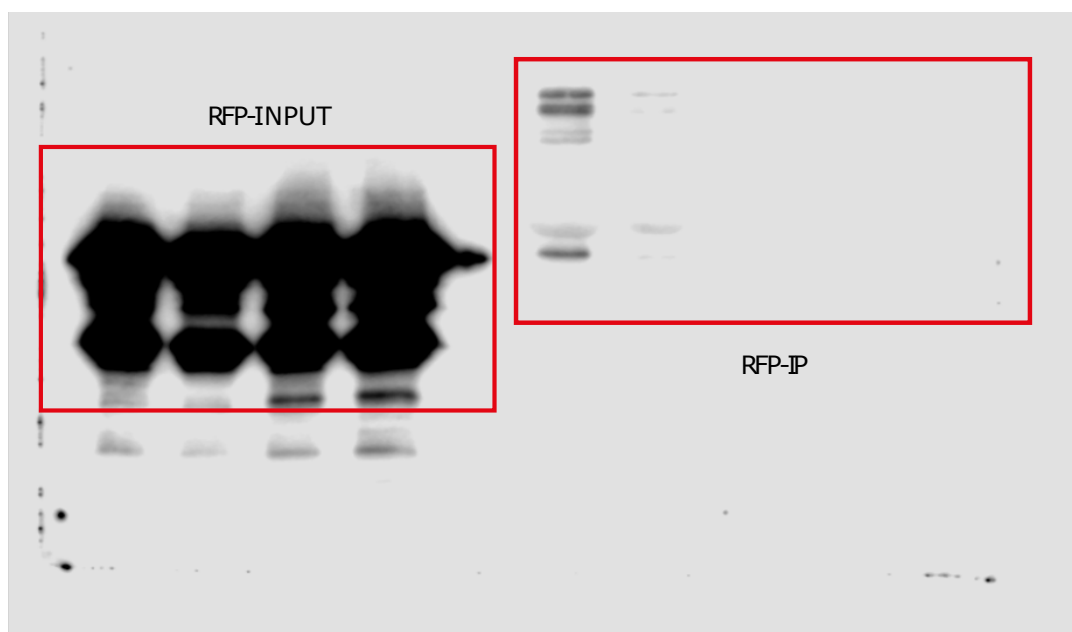
Appendix A: ATG8-interacting proteins used in chapter three. Protein names and their Uniprot IDs are presented in first two rows. ATG8 protein used in AF2-multimer predictions, and its source is listed. The AIM/LIR identified, its type and whether it was experimentally validated or not is described.

Protein	UniProt	Organism	ATG8-family Client	AIM/LIR Sequence	AIM/LIR-type	AF2 Structure of AIM/LIR	Experimentally Verified	AF2-multimer Verified
ATG1A	Q84C95	A. thaliana	ATG8A	YVLV	Canonical	Disorder	Yes	Yes
ATG4B	Q8M1Y0	A. thaliana	ATG8A	WQLL (other AIMS)	Canonical	Disorder	No - site not identified	Yes
C53	Q9FG23	A. thaliana	ATG8A	YEIV (other AIMS)	Canonical (and Non-canonc	Disorder	Yes	Yes - both canonical and non-canonical
DSK2	Q9SJI9	A. thaliana	ATG8E	3 AIMS	Canonical	Helical (all 3)	Partial - all AIMS mutated simultaneously	No
LON2	Q64948	A. thaliana	ATG8E	YVVV* (other AIMS)	Canonical	Partial Disorder	No - site not identified	Yes - other AIM found; non-canonical
ATG7	Q5ZKY2	Chicken	ATG8	MDM	Non-canonical	Disorder	No	Yes
ATG13	Q75143	Human	LC3A	FVMI	Canonical	Disorder	Yes	Yes
ATG14	Q6ZNE5	Human	GABARAP	WEVL	Canonical	Disorder	Yes	Yes
ATG4B	Q9Y4P1	Human	GABARAP	FELL (other AIMS)	Canonical	Disorder	Yes	Yes
ATG7	Q9S352	Human	LC3A/GABARAP	MDM	Non-canonical	Disorder	No - site not identified	Yes
BNIP3L/H	Q60238	Human	GABARAP	WVEL	Canonical	Disorder	Yes	Yes
Calciculin	P27797	Human	GABARAP	WDFL	Canonical	Partial Disorder	Yes	No - other interactions identified
CCPG1	Q9ULG6	Human	GABARAP	WTVI	Canonical	Partial Disorder	Yes	Yes
DVL2	Q14641	Human	LC3A	WLKI	Canonical	Beta-sheet	Partial - mutant partly loses binding	Yes - other AIM found; canonical
FAM134B	Q9H6L5	Human	GABARAP	FELL	Canonical	Disorder	Yes	Yes
FKBP8	Q14318	Human	LC3B	FEVL	Canonical	Disorder	Yes	Yes
FUNDC1	Q8IVP5	Human	LC3A	YEVL	Canonical	Disorder	Yes	Yes
FYCO1	Q9BQS8	Human	LC3A	FDII	Canonical	Disorder	Yes	Yes
KBTB6	Q86V97	Human	GABARAP	WVRV	Canonical	Disorder	Yes	Yes
NBR1	Q14596	Human	GABARAP	VIII	Canonical	Disorder	Yes	Yes
NDP52	Q13137	Human	LC3C	LLVV	Non-canonical	Partial Disorder	Yes	Yes
NEDD4	P46934	Human	LC3B	WEII (other AIMS)	Canonical	Disorder	Yes	Yes - other AIM found
Opiateuim	Q96CV9	Human	LC3A	FVEI	Canonical	Disorder	Yes	Yes
PLEKHM1	Q9Y4G2	Human	LC3A	WVNV	Canonical	Disorder	Yes	Yes
SEC62	Q99442	Human	GABARAP	FEMI	Canonical	Disorder	Yes	Yes
Sequeosome-1 (p62)	Q13501	Human	LC3A	WTHL	Canonical	Partial Disorder	Yes	Yes
STBD1	Q9S210	Human	GABARAP	WEMV	Canonical	Disorder	Yes	Yes
TAX1BP1	Q86VP1	Human	LC3A	MLVV	Non-canonical	Partial Disorder	Yes	Yes
TBC1D25	Q3MI6	Human	GABARAP	WDII	Canonical	Disorder	Yes	Yes
TBC1D5	Q92609	Human	GABARAP	FTV	Canonical	Disorder	Yes	Yes
TP53NP2	Q8IXH6	Human	LC3A	WLLI	Canonical	Partial Disorder	Yes	Yes
ULK1	Q75385	Human	GABARAP	FVMV	Canonical	Disorder	Yes	Yes
JAV-M2	A4ZNC0	Influenza	LC3A	FVSI	Canonical	Disorder	Yes	Yes
Rauz	Q5ZUV9	Legionella	LC3A/LC3B/GABARAP	2 LIRs	Canonical	Disorder	No - site not identified	Yes - FDL AIM sequence
ATG7	A0A1U9L192	N. benthamiana	ATG8A	LDW	Non-canonical	Disorder	No	Yes
Becn1-1	Q4QXM1	N. benthamiana	ATG8A	FVVL	Canonical	Disorder	Yes	Yes
PexRD54	DONBE6	P. infestans	ATG8CL	WEV	Canonical	Disorder	Yes	Yes
UIS3	Q38SD7	Plasmodium	LC3A/GABARAP	DYD[30aa]E[3aa]K	Non-canonical	Partial Disorder	Yes	No - other interactions identified
AT2	Q8VY98	Potato	ATG8CL	WEVV (other AIM)	Canonical	Disorder	Yes - other organism	Yes
Joka2	IM1BJF6	Potato	ATG8CL	WDPI	Canonical	Partial Disorder	Yes	No - other interactions identified
YukJ	Q8Z286	Salmonella	LC3A/LC3B	WDEI	Canonical	Helical	No - site not identified	Partial - other interactions observed
ATG1	P53104	Yeast	ATG8	YVVV	Canonical	Partial Disorder	Yes	Yes
ATG19	P56193	Yeast	ATG8	WEEL	Canonical	Disorder	Yes	Yes
ATG32	B3L1Z3	Yeast	ATG8	WQAI	Canonical	Partial Disorder	Yes	Yes
ATG34	Q12292	Yeast	ATG8	WEEL	Canonical	Disorder	Yes	Yes
ATG39	Q06159	Yeast	ATG8	WNLV	Canonical	Partial Disorder	Yes	Yes
ATG40	Q99325	Yeast	ATG8	YDFM	Non-canonical	Disorder	Yes	Yes
ATG7	P38862	Yeast	ATG8	FEWEDSEDEA	Non-canonical	Partial Disorder	Partial	Partial - other interactions verified

Appendix B: Uncropped blots from colP in Figure12 panel D.



Appendix C: Uncropped blots from colP in Figure16.



Appendix D: Raw puncta number counted for ATG3 peptide analysis in Figure17.

condition	puncta pe	rep
Maimp	26.00775	1
Maimp	29.78306	1
Maimp	45.82012	1
Maimp	25.08119	1
Maimp	47.43988	1
Maimp	25.16879	1
WTAIMP	47.50026	1
WTAIMP	12.58439	1
WTAIMP	13.00387	1
WTAIMP	5.033757	1
WTAIMP	8.389596	1
WTAIMP	6.711677	1
Maimp	19.71555	2
Maimp	16.77919	2
Maimp	26.84671	2
Maimp	23.49087	2
Maimp	17.61815	2
Maimp	16.35971	2
WTAIMP	13.78709	2
WTAIMP	16.35328	2
WTAIMP	10.06751	2
WTAIMP	18.87659	2
WTAIMP	5.033757	2
WTAIMP	4.614278	2
Maimp	18.87659	3
Maimp	14.26231	3
Maimp	23.49087	3
Maimp	17.90774	3
Maimp	12.16491	3
Maimp	15.10127	3
WTAIMP	21.39347	3
WTAIMP	6.711677	3
WTAIMP	13.84283	3
WTAIMP	16.71679	3
WTAIMP	4.614278	3
WTAIMP	6.292197	3

Appendix E: RStudio code used to analyse puncta numbers in Figure17.

```
.y. group1 group2    p  p.adj p.format p.signif method  
<chr> <chr> <chr>   <dbl>  <dbl> <chr>   <chr>   <chr>  
1 puncta WTAImp Maimp 0.000241 0.00024 0.00024 ***    Wilcoxon
```

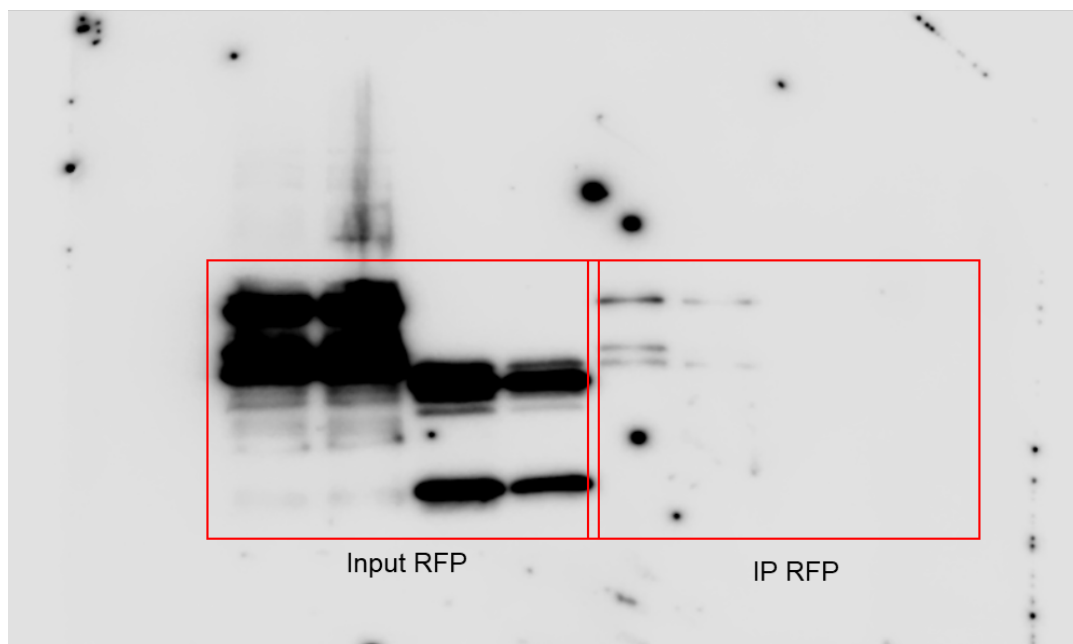
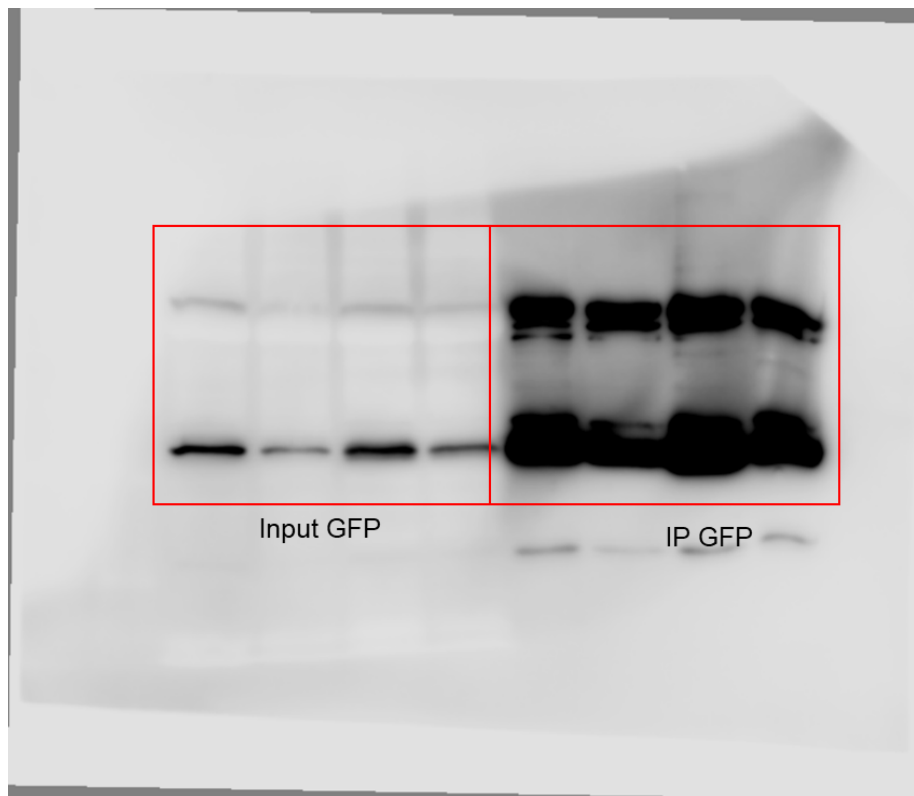
Appendix F: All AF2-multimer predictions for the analysis of Appendix A and figures shown in chapter three.

<https://figshare.com/articles/dataset/AF2->

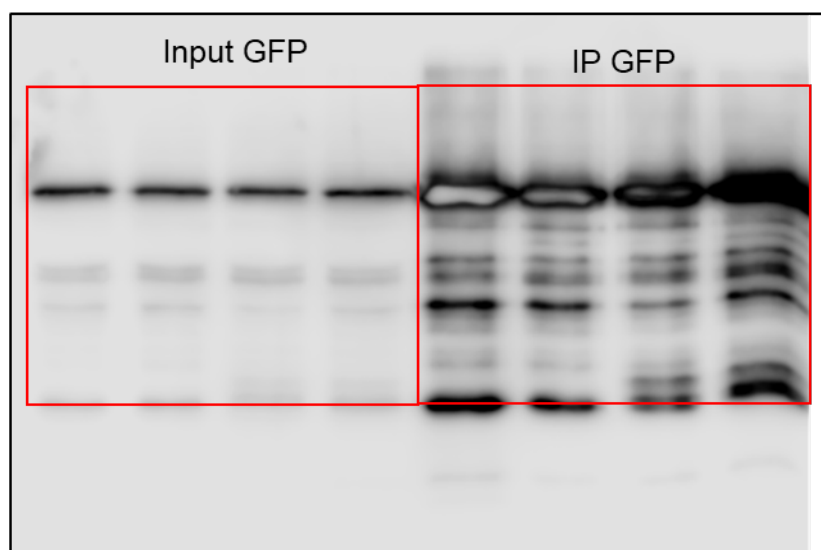
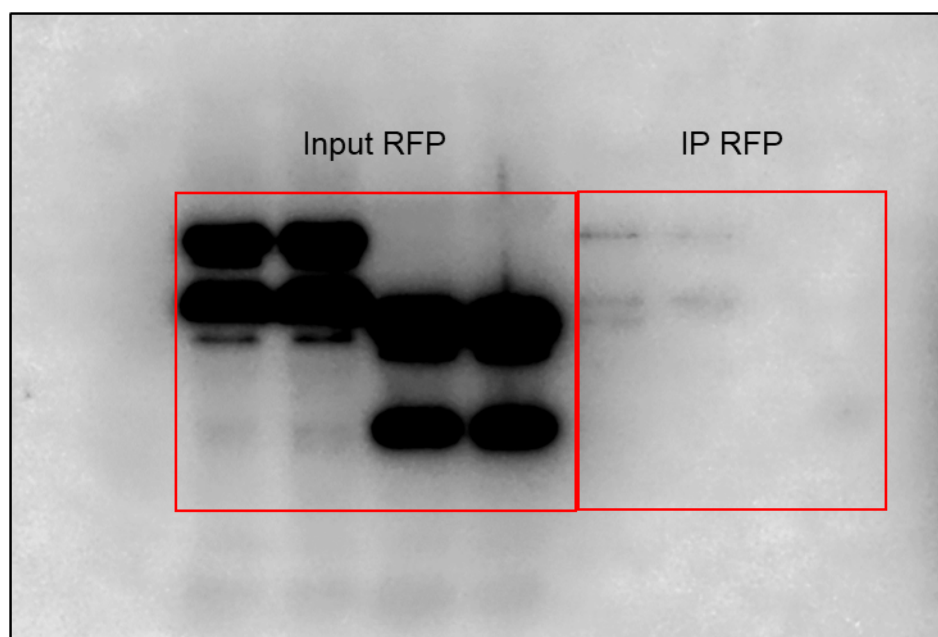
[multimer guided high accuracy prediction of typical and atypical ATG8-
binding motifs - AF2-multimer DATA/21533769/1](#)

Appendix G: Uncropped blots from Figure24 panels C and D.

Panel C:



Panel D:



Appendix H: Puncta number counted for SYMMV P7 in Figure25 panel D.

condition	rep	puncta_count	condition	rep	puncta_count	condition	rep	puncta_count
SoyYMV P7 WT	1	63	SoyYMV P7 mutant	1	18	Control	1	36
SoyYMV P7 WT	1	47	SoyYMV P7 mutant	1	32	Control	1	38
SoyYMV P7 WT	1	45	SoyYMV P7 mutant	1	19	Control	1	27
SoyYMV P7 WT	1	40	SoyYMV P7 mutant	1	28	Control	1	36
SoyYMV P7 WT	1	32	SoyYMV P7 mutant	1	39	Control	1	29
SoyYMV P7 WT	1	42	SoyYMV P7 mutant	1	24	Control	1	25
SoyYMV P7 WT	1	31	SoyYMV P7 mutant	1	16	Control	1	46
SoyYMV P7 WT	1	41	SoyYMV P7 mutant	1	20	Control	1	30
SoyYMV P7 WT	2	16	SoyYMV P7 mutant	2	21	Control	2	15
SoyYMV P7 WT	2	25	SoyYMV P7 mutant	2	23	Control	2	12
SoyYMV P7 WT	2	28	SoyYMV P7 mutant	2	12	Control	2	17
SoyYMV P7 WT	2	25	SoyYMV P7 mutant	2	14	Control	2	21
SoyYMV P7 WT	2	20	SoyYMV P7 mutant	2	20	Control	2	8
SoyYMV P7 WT	2	25	SoyYMV P7 mutant	2	18	Control	2	17
SoyYMV P7 WT	2	17	SoyYMV P7 mutant	2	10	Control	2	28
SoyYMV P7 WT	2	19	SoyYMV P7 mutant	2	17	Control	2	22
SoyYMV P7 WT	3	39	SoyYMV P7 mutant	3	23	Control	3	14
SoyYMV P7 WT	3	21	SoyYMV P7 mutant	3	29	Control	3	10
SoyYMV P7 WT	3	37	SoyYMV P7 mutant	3	21	Control	3	20
SoyYMV P7 WT	3	34	SoyYMV P7 mutant	3	16	Control	3	13
SoyYMV P7 WT	3	25	SoyYMV P7 mutant	3	14	Control	3	15
SoyYMV P7 WT	3	43	SoyYMV P7 mutant	3	25	Control	3	14
SoyYMV P7 WT	3	37	SoyYMV P7 mutant	3	26	Control	3	11
SoyYMV P7 WT	3	26	SoyYMV P7 mutant	3	7	Control	3	13

Appendix G: Data analysis of Appendix H.

\$Control					
Shapiro-Wilk normality test					
data: X[[i]]					
W = 0.92276, p-value = 0.06725					
\$SoyYMV P7 mutant`					
Shapiro-Wilk normality test					
data: X[[i]]					
W = 0.98209, p-value = 0.931					
\$SoyYMV P7 WT`					
Shapiro-Wilk normality test					
data: X[[i]]					
W = 0.94748, p-value = 0.2387					
P-value for all is over 0.05, therefore, data is normally distributed.					

Welch Two Sample t-test

data: WT and aim

t = 4.3306, df = 38.886, p-value = 0.0001011

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

6.350192 17.483142

sample estimates:

mean of x mean of y

32.41667 20.50000

data: WT and control

t = 3.4803, df = 45.477, p-value = 0.001117

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

4.583236 17.166764

sample estimates:

mean of x mean of y

32.41667 21.54167

data: aim and control

t = -0.40778, df = 41.336, p-value = 0.6855

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

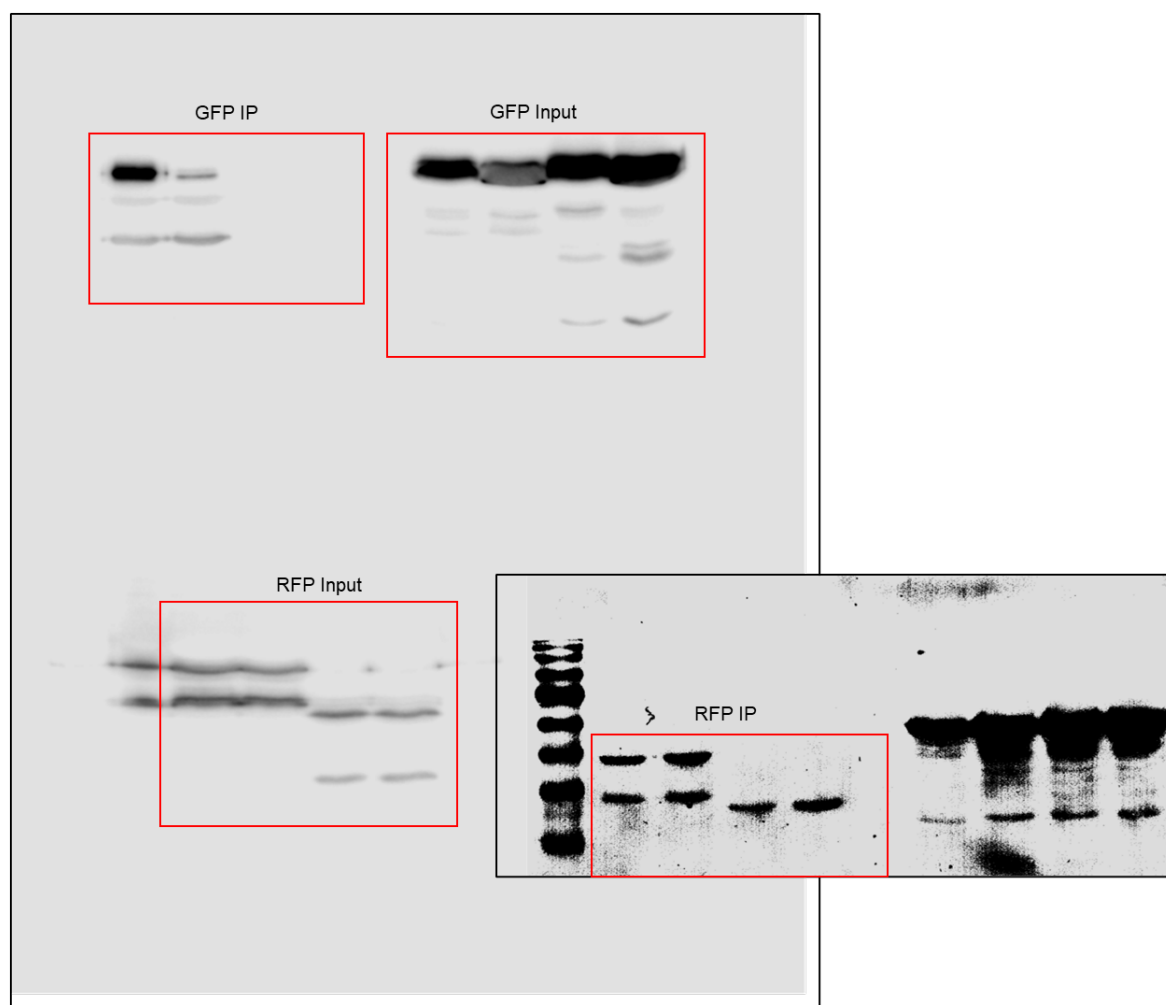
-6.199240 4.115906

sample estimates:

mean of x mean of y

20.50000 21.54167

Appendix I: Uncropped blots from VTMoV P1 colIP in Figure26 panel C.



Appendix J: Normalised HR intensity data from Figure28 panels D and E.

Panel D:

RFP-ATG8CL		
TobMotV:P1wt-GFP	TobMotV:P1aim-GFP	
1	0.074456146	Rep One
0.719903808	0.136793333	
0.042883846	-0.0447729	
0.261829986	0.095161195	
0.560326533	-0.043687025	
0.027695658	0.062599299	
0.646642323	-0.002011299	
0.216800064	0.008260335	
0.20148577	0.409052951	
1	0.150430247	Rep Two
0.877025339	0.120014855	
0.507393316	-0.102620059	
1	0.174005695	
1	-0.103362121	
0.045954746	0.133919079	Rep Three
0.479251745	-0.022721011	
0.165069631	-0.082559349	
1	0.18227875	
0.993218247	0.242284117	
0.37234325	0.262271693	
1	0.147717616	
0.312106963	-0.154011791	
0.106877621	0.036503454	
0.172851896	-0.017688935	

Panel E:

TobMotV:P1wt-GFP		
RFP-ATG8CL	EV-RFP	
1	-0.01534335	Rep One
0.772802353	0.069042711	
0.042142636	-0.012936261	
0.27663868	0.008504102	
0.222979011	-0.015070909	
0.126561969	0.102782074	
0.602122542	0.044420488	
0.343164452	0.140273198	
0.504497448	0.123025311	
1	-0.028178153	Rep Two
1	-0.065795721	
0.461450382	-0.273129626	
0.881440489	-0.259006549	
1	0.09857205	
0.012587936	0.048064024	Rep Three
0.209734752	-0.056280822	
0.424643973	-0.075003982	
0.395657358	0.18318866	
0.630827402	0.401052542	
1	-0.128043182	
1	0.067343574	
0.955647669	0.07016067	
0.240730784	0.027869878	
0.497304694	0.071988191	

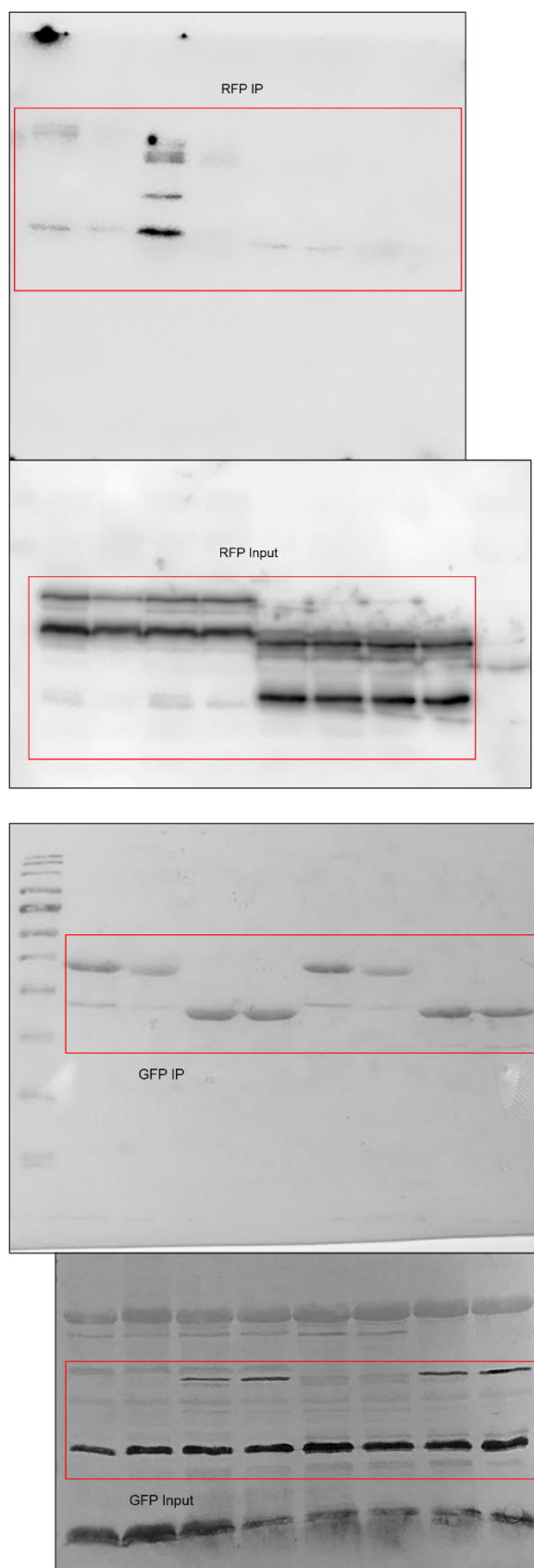
Appendix K: Normalised HR intensity data from Figure29.

VTMoV:P1wt-GFP			
	AlMp wt	AlMp mutant	
REP ONE		0.1011	0.2584
		-0.0099	1.0000
		-0.0145	1.0000
		0.1635	0.3498
		0.0186	0.1237
		0.5353	1.0000
REP TWO		0.4904	0.2052
		0.7196	1.0000
		0.1671	0.0816
		0.7498	1.0000
		0.5384	1.0000
		0.2715	0.7124
REP THREE		0.3475	1.0000
		0.2231	0.2905
		0.3725	1.0000
		0.1444	0.4972
		0.1486	0.8068
		0.3603	1.0000

Appendix L: Normalised HR intensity data from Figure31.

RFP-ATG8CL		
TobMotV:P1wt-GFP	TobMotV:P1aim-GFP	
0.6448	0.05859	
0.912711585	0.12046	
1	0.388455898	
1	0.24952018	
0.267551604	-0.011486654	
1	0.098530892	
0.730865101	0.28171566	
-0.059641235	0.193197234	
0.5417	-0.0166	
1.0000	0.1130	
1.0000	0.1732	
0.8597	0.0751	
1.0000	0.5978	
1.0000	0.0576	
0.6991	0.0721	
1.0000	0.2039	
0.7158	0.1939	
0.6330	0.2281	
0.7915	0.0072	
1.0000	0.1217	
0.8807	0.1076	
1.0000	0.1430	

Appendix M: Uncropped blots from Figure34.



Appendix N: Puncta numbers counted for RPLP2 and their analysis in Figure36.

Condition	RPLP2_WT	RPLP2_aim		Condition	RPLP2_WT	RPLP2_aim
Control	51	32		100µM Torin	129	19
Control	17	8		100µM Torin	82	35
Control	44	41		100µM Torin	126	26
Control	11	31		100µM Torin	182	10
Control	91	31		100µM Torin	147	11
Control	18	14		100µM Torin	96	12
Control	43	44		100µM Torin	51	73
Control	47	37		100µM Torin	150	62
Control	65	33		100µM Torin	137	83
Control	134	33		100µM Torin	92	43
Control	61	50		100µM Torin	97	105
Control	54	19		100µM Torin	77	84
mean	53	31.08333333		mean	113.8333333	46.9166667

Analysis:

\$aim	F test to compare two variances				
Shapiro-wilk normality test	data: data1\$puncta_count by Condition				
data: x[[i]]	F = 0.12869, num df = 11, denom df = 11, p-value = 0.001989				
w = 0.94343, p-value = 0.5438	alternative hypothesis: true ratio of variances is not equal to 1				
	95 percent confidence interval:				
	0.03704651 0.44702483				
	sample estimates:				
\$wt	ratio of variances				
Shapiro-wilk normality test	0.1286884				
data: x[[i]]	independent				
w = 0.89795, p-value = 0.1493					
Hence data is normal distribution					

wilcoxon rank sum test with continuity correction

data: CTRL_WT and CTRL_aim

w = 108.5, p-value = 0.03754

alternative hypothesis: true location shift is not equal to 0

wilcoxon rank sum exact test

data: Torin_WT and Torin_aim

w = 130, p-value = 0.0003713

alternative hypothesis: true location shift is not equal to 0

wilcoxon rank sum test with continuity correction

data: Torin_WT and CTRL_WT

w = 129.5, p-value = 0.0009961

alternative hypothesis: true location shift is not equal to 0

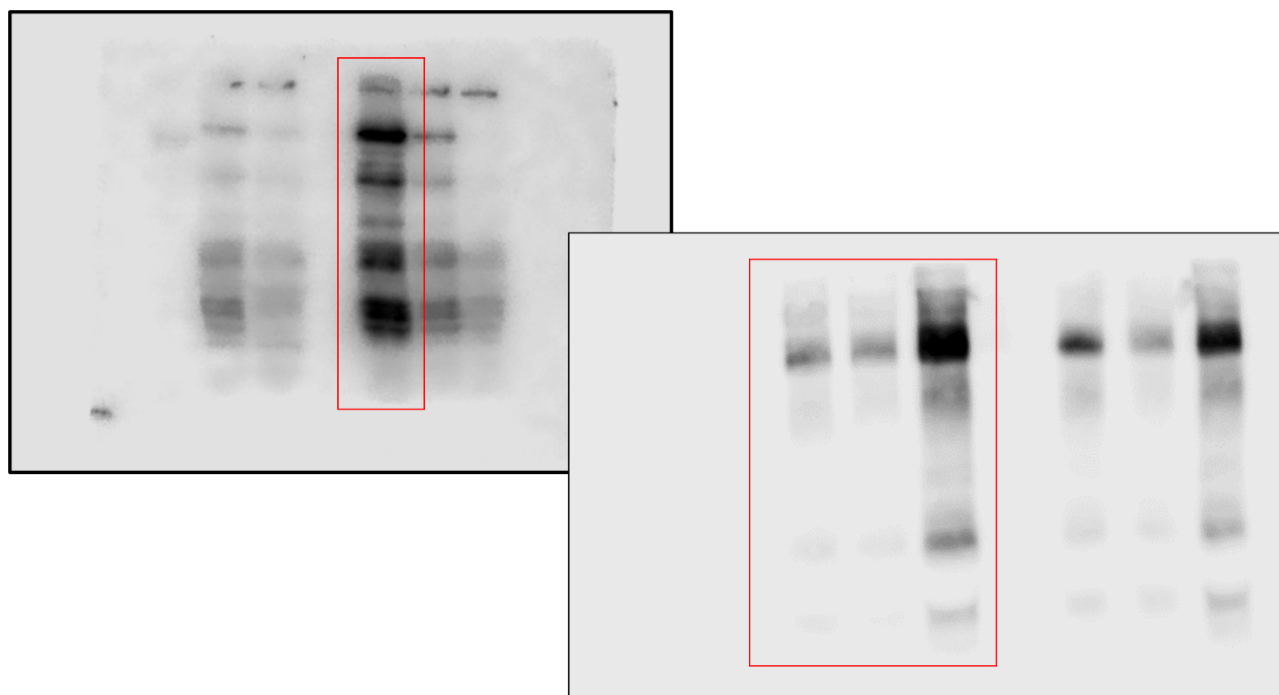
wilcoxon rank sum test with continuity correction

data: Torin_aim and CTRL_aim

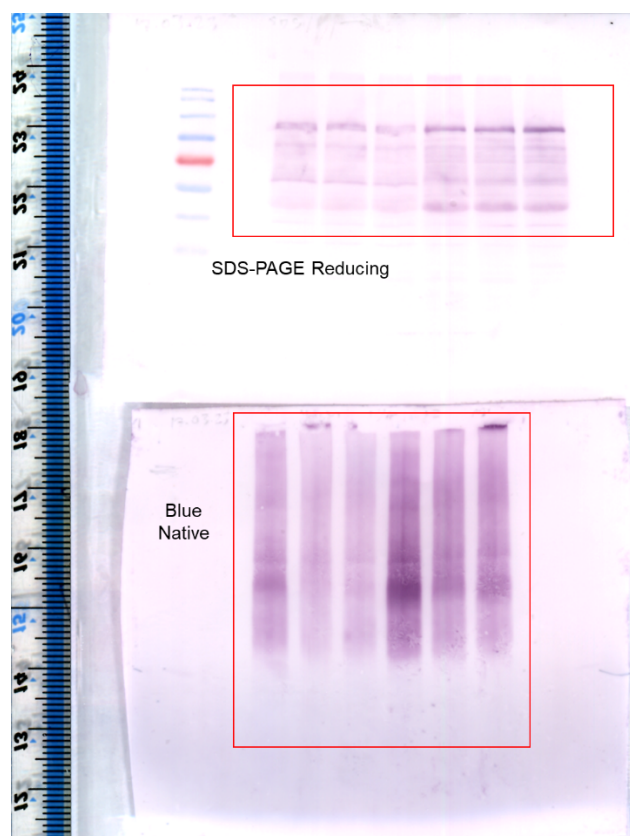
w = 86.5, p-value = 0.4186

alternative hypothesis: true location shift is not equal to 0

Appendix O: Uncropped blots from Figure41.



Appendix P: Uncropped blots from Figure42.



Appendix Q: Uncropped blots from Figure43.

