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Regulation of Rab8 in Toll-Like Receptor Signalling Pathways

by
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Abstract

Small GTPases of the Rab family have wide ranging and essential roles in recruiting effectors to mediate membrane trafficking and receptor signalling events in mammalian cells. Nucleotide loading and activation/deactivation of the Rabs themselves are regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and other accessory proteins. While Rabs have important roles in innate immune cells, for host-pathogen interactions, receptor signalling and membrane trafficking, in many cases their GEFs and other accessory proteins have not been elucidated.

Pathogen-activated, Toll-like receptors (TLRs) initiate and modulate transcription of inflammatory cytokines and other innate immune responses which are important for immune defence but can also contribute to chronic disease. Macrophages are key cells of the innate immune system and previous work from our laboratory established a role for promiscuous Rab family member, Rab8a, in TLR signalling. Rab8a is activated in TLR pathways to recruit phosphoinositide 3-kinase gamma (PI3Kγ) as an effector which upregulates TLR-induced Akt/mTOR signalling to drive a biased program of anti-inflammatory cytokines. TLR-associated PI3Kγ has now emerged as a key mediator of macrophage programming (or polarisation) in inflammation and cancer and its cognate GTPase, Rab8a, has a prominent role in immunity and disease. Rab8a is itself recruited to macrophage ruffles and macropinosomes through its association with the TLR crosstalk-activated endocytic receptor LRP1. However, it is not yet known how Rab8a is activated in TLR pathways. This project set out to identify the essential Rab8a GEF(s) needed to activate Rab8a as part of the LRP1 complex.

Two well-known Rab8 GEFs, GRAB and Rabin8, which previously were uncharacterised in macrophages, were investigated in this project. GRAB was identified as part of the low density lipoprotein receptor-related protein 1 (LRP1) complex in pull-downs analysed by mass spectrometry. Co-immunoprecipitation and fluorescence microscopy showed that both GRAB and the structurally similar GEF, Rabin8, undergo LPS-inducible binding to Rab8a and are localised at sites of Rab8a enrichment. To carry out functional studies, stable knockouts (KOs) of Rabin8, GRAB, as well as a double KO were produced via CRISPR-Cas9 gene editing in macrophage cell lines. Nucleotide activation assays were developed for this project and live-cell imaging with KO cell lines showed that both GEFs contribute additively to TLR4-induced Rab8a GTP-loading, but they are not needed for Rab8a

membrane recruitment. Analysis of TLR signalling after double KO of both GEFs suggested redundant roles for Rabin8 and GRAB in activating Rab8a for PI3Kγ-dependent Akt/mTOR signalling. Live cell imaging utilising a fluorescent Akt1 reporter confirmed that LPS/TLR-induced Akt signalling is generated on macropinosomes and requires GRAB and Rabin8 GEF function.

Next, to investigate possible regulators of the Rab8 GEFs, pull-downs and mass spectrometry were performed and identified a known multi-Rab effector, oculocerebrorenal syndrome of Lowe (OCRL) as a Rabin8 binding protein in LPS activated macrophages. Follow-up experiments provided initial characterisation of a novel OCRL-Rabin8 interaction and indicate a possible recruitment mechanism for the Rab8 GEF during TLR signalling.

In conclusion, these results identified both GRAB and Rabin8 as essential activators of Rab8 downstream of TLR4 for inflammatory signalling in mouse macrophages. The results contribute these GEFs and other possible Rab recruiters to an expanding molecular complex that drives PI3Kγ/Akt/mTOR signalling for control of inflammation and innate immune responses.

Declaration by Author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications included in this thesis

Chapter 4

1. Wall AA, Luo L, Hung Y, **Tong SJ**, Condon ND, Blumenthal A, Sweet MJ and Stow JL (2017). Small GTPase Rab8a-recruited Phosphatidylinositol 3-Kinase γ Regulates Signaling and Cytokine Outputs from Endosomal Toll-like Receptors. Journal of Biological Chemistry. 2017;292(11): 4411-4422.

2. Luo L, Wall AA, **Tong SJ**, Hung Y, Xiao Z, Tarique AA, Sly PD, Fantino E, Marzolo MP and Stow JL. TLR Crosstalk Activates LRP1 to Recruit Rab8a and PI3Kγ for Suppression of Inflammatory Responses. Cell reports. 2018;24(11):3033-3044.

Chapters 4 – 6

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1. Luo L, Bokil NJ, Wall AA, Kapetanovic R, Lansdaal NM, Marceline F, Burgess BJ, **Tong SJ**, Guo Z and Alexandrov K. (2017) SCIMP is a transmembrane non-TIR TLR adaptor that promotes proinflammatory cytokine production from macrophages. Nature communications. 8: 14133; doi: 10.1038/ncomms14133.

2. Luo L, **Tong SJ**, Wall AA, Khromykh T, Sweet MJ and Stow JL. Development of SH2 probes and pull-down assays to detect pathogen-induced, site-specific tyrosine phosphorylation of the TLR adaptor SCIMP. (2017) Immunology and cell biology. 95(6): 564; doi:10.1038/icb.2017.10.

3. Wall AA, Luo L, Hung Y, **Tong SJ**, Condon ND, Blumenthal A, Sweet MJ and Stow JL (2017). Small GTPase Rab8a-recruited Phosphatidylinositol 3-Kinase γ Regulates Signaling and Cytokine Outputs from Endosomal Toll-like Receptors. Journal of Biological Chemistry. 292(11): 4411-4422; doi:10.1074/jbc.M116.766337.

4. Luo L, Wall AA, **Tong SJ**, Hung Y, Xiao Z, Tarique AA, Sly PD, Fantino E, Marzolo MP and Stow JL. (2018) TLR Crosstalk Activates LRP1 to Recruit Rab8a and PI3Kγ for Suppression of Inflammatory Responses. Cell reports. 24(11):3033-3044; doi:10.1016/j.celrep.2018.08.028

5. **Tong SJ**, Wall AA, Hung Y, Luo L, Stow JL. (2019) Guanine nucleotide exchange factors activate Rab8a for Toll-like receptor signalling. Small GTPases. 1-17; doi: 10.1080/21541248.2019.1587278

Contributions by others to the thesis

Professor Jennifer Stow

Contributed towards the conceptual design of the project, provided experimental advice and supported the editing of this thesis.

Dr Lin Luo

Contributed towards the conceptual design of the project, developed the GST probes used in this project, provided technical advice and assisted with the editing of this thesis.

Dr Adam Wall

Provided technical and experiment expertise and advice for all live- and fixed-cell microscopy imaging used in this project.

Tatiana Khromykh

Provided technical support with molecular cloning and tissue culture throughout the project.

Statement of parts of the thesis submitted to qualify for the award of another degree

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List of Abbreviations

Abbreviation	Meaning
ATCC	American type culture collection
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DAMP	Danger associated molecular patterns
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Egtazic acid
ERK1/2	Extracellular signal-regulated kinase 1/2
FBS	Foetal Bovine Serum
GAP	GTPase activating protein
GDI	Guanosine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
Gpp(NH)p	5'-Guanylyl imidodiphosphate
GST	Glutathione S transferase
GTP	Guanosine triphosphate
GTPα	Guanosine triphosphate analogue
GTTase	Geranylgeranyltransferase
HDR	Homology-directed repair
iBMMs	Immortalised bone-marrow derived macrophages
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL-4/6/12/13	Interleukin-4/6/12/13
Indels	Insertions and deletions
KO	Knockout
LPS	Lipopolysaccharide
LRP1	Low density lipoprotein receptor-related protein 1
MAL	MyD88 adaptor-like
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor κB
NHEJ	Nonhomologous end joining
OCRL	Oculocerebrorenal syndrome of Lowe protein 1
PAMPs	Pathogen associated molecular patterns
PCE	Protease cleavage elution
PI3Kγ	Phosphoinositide 3-kinase gamma
PPRs	Pattern recognition receptors
RBD	Rab binding domain
RasBD	Ras binding domain
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute
TIR	Toll-interleukin receptor
TLR	Toll-like Receptor
TNF	Tumour necrosis factor

TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β

Chapter 1

Introduction

1. Introduction

Macrophages

Macrophages are important innate immune cells that play key roles in coordinating host responses against pathogens and other sources of danger (Gordon and Taylor 2005, Xia and Triffitt 2006, Lacy and Stow 2011). These cells are differentiated from the bone marrow and are found either patrolling the circulatory system or residing in tissues throughout the body. As immune sentinels, macrophages express an array of cell surface receptors that allow for the detection of molecules associated with a wide range of pathogens (Gordon 2002). Along with pathogen clearance and initiating immune responses, macrophages are also important for maintaining tissue homeostasis, clearing cellular debris and promoting tissue repair (Mosser and Edwards 2008).

As long-lived cells of the innate immune system, macrophages not only stimulate and trigger pro-inflammatory responses upon pathogen detection, but also secrete signals to resolve inflammation. This dual role of promoting and constraining inflammation has been attributed to a gradient of polarising transcriptional programs between M1 or classically activated (pro-inflammatory) and M2 or alternately activated (anti-inflammatory) phenotypes (Mosser and Edwards 2008). These states determine both macrophage function and the cytokine signals they secrete, to communicate and coordinate with other immune cells for driving or dampening inflammation. Uncovering how these highly pleomorphic immune cells direct and regulate inflammation is essential for understanding innate immunity and inflammatory diseases.

Toll-Like Receptors (TLRs)

As primary immune surveillance cells, macrophages have distinct characteristics that make them ideally suited for detecting and destroying pathogens (Rosenberger and Finlay 2003). One such feature is the presentation of a large contingent of evolutionarily conserved pattern recognition receptors (PRRs), that recognise a wide range of specific pathogen-associated molecular patterns (PAMPs) (Akira and Uematsu *et al.* 2006). These PAMPs are common, highly conserved molecules or peptides that are essential for the survival of certain pathogen groups, making them ideal targets for immune recognition (Gordon 2002). PRRs are also able to recognise

another category of molecules called danger associated molecular patterns (DAMPs), normally consisting of altered endogenous, host proteins or lipids, activation by DAMPs allows macrophages to respond to tissue damage, facilitate the clearing of cellular debris and initiate wound healing (Koh and DiPietro 2011, Braza and Brouard *et al.* 2016).

Toll-like receptors (TLRs) are a prominent subset of PRRs, named after the *Toll* gene in *Drosophila* which was discovered by Nobelists Nusslein-Volhard and Wieschaus (Anderson and Jürgens *et al.* 1985). Initially described as a dominant factor for controlling *Drosophila* embryo development, comparisons between the *Toll* and IL-1 receptors introduced a link between the *Toll* genes and antifungal immunity (Lemaitre and Nicolas *et al.* 1996). Able to recognise different PAMPs and DAMPs associated with a wide range of pathogens, the TLR family, consisting of 10 known members in humans, constitutes an array of type I transmembrane proteins expressed in innate immune cells, epithelial cells and other cell types (O'Neill 2004, Trinchieri and Sher 2007) (Table 1.1). Individually, most TLRs are activated by one or a few PAMPs and DAMPs as agonists, and collectively they provide wide-ranging anti-pathogen and infection surveillance. The members of the TLR family are localised to different subcellular locations which in part helps to tailor immune responses to particular pathogens/immune stimulants (Table 1.1). Macrophages utilise these receptors to facilitate the screening of their extracellular environment, endosomal/phagosomal compartments and cytoplasm for pathogen signatures.

TLR4 is the prototypical TLR and is activated by lipopolysaccharide (LPS), a PAMP and coat component of Gram-negative bacteria (Hoshino and Takeuchi *et al.* 1999). LPS is presented to TLR4 on the macrophage cell surface in combination with the glycosylphosphatidylinositol (GPI)-anchored receptor CD14 and the co-receptor MD-2 (Park and Song *et al.* 2009, Gay and Symmons *et al.* 2014), triggering TLR4 dimerization and recruitment of the cytoplasmic signalling adaptors, Mal and MyD88 (Gay and Symmons *et al.* 2014). These adaptors then recruit sequential arrays of signalling kinases that drive the transcription of pro-inflammatory cytokines (TNF, IL-6, IL-12) and other anti-infective response outputs (Akira and Takeda 2004). TLR4 can also signal from a second set of adaptors, attracted by the different phospholipid environment of the endosomal, macropinosomal or phagosomal membranes after

receptor internalisation. The adaptors TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited to TLR4 in the endosomal/phagosomal system for signalling pathways that activate the transcription of anti-inflammatory or regulator mediators such as interferon β and IL-10 (Kagan and Su *et al.* 2008). Thus, TLR4 output is spatially and temporally regulated by the location specific recruitment of distinct signalling adaptors, as opposed to other TLRs which mainly signal from one site; for TLR5 this is the plasma membrane and TLR9 is only known to signal from endosomal compartments (O'Neill 2004, Gay and Symmons *et al.* 2014, Rosadini and Kagan 2017).

Upon activation, TLRs trigger an array of complex signalling and phenotypic changes in macrophages, through the activation of signal transducers such as mitogen activated protein kinases (MAPK) and the potent transcription factor nuclear factor κB (NF-κB) (Kawasaki and Kawai 2014). Depending on the resulting signalling cascade, macrophages can be activated to produce reactive oxygen species (ROS), pro- or anti-inflammatory cytokines, enhance phagocytosis/macropinocytosis or secrete chemokines for immune cell recruitment (Varin and Gordon 2009, West and Brodsky *et al.* 2011). The main roles of TLR-activated macrophages are to provide local innate immune defences and, via chemokines and cytokines, activate and recruit lymphocytes for adaptive immune responses. TLRs are one of the main groups of receptors contributing to inflammation as a protective, anti-infective response, but can also act in a pathognomonic context by triggering and maintaining inflammation in many chronic and inflammatory diseases.

Table 1.1. List of Toll-like receptors with their main ligands.

Receptor	Location	Ligand	References
TLR 1-2 (Heterodimer) TLR 1-6 (Heterodimer)	Plasma membrane	Bacterial Lipopeptides	(Takeuchi and Sato *et al.* 2002)
TLR 3	Endosomal	dsRNA	(Alexopoulou and Holt *et al.* 2001)
TLR 4	Plasma membrane	Bacterial LPS	(Hoshino and Takeuchi *et al.* 1999, Takeuchi and Hoshino *et al.* 1999)
TLR 5	Plasma membrane	Flagellin	(Gewirtz and Navas *et al.* 2001, Hayashi and Smith *et al.* 2001)
TLR 7/TLR 8	Endosomal	Viral ssRNA	(Diebold and Kaisho *et al.* 2004, Heil and Hemmi *et al.* 2004)
TLR 9	Endosomal	CpG DNA (Bacterial)	(Hemmi and Takeuchi *et al.* 2000)
TLR 10	Plasma membrane	No known ligand (Inflammatory modulator of TLR2)	(Oosting and Cheng *et al.* 2014)

Macrophage polarisation and inflammation

Upon activation, macrophages respond with diverse changes in gene and protein expression that are dictated by the microenvironment and immune stimuli they encounter. Broadly speaking, macrophages respond to immunological challenges by

transitioning into one of two polarised phenotypic states known colloquially as 'M1 and M2' (Mills and Kincaid *et al.* 2000). This classification is used for convenience to distinguish the different programs elicited by pro-inflammatory (M1) and anti-inflammatory or inflammation resolving M2 cells (Figure 1.1). However, it is also widely acknowledged that the M1 and M2 classification is an oversimplification that does not reflect the dynamic and continuously varied states macrophages take on during different immunological circumstances. A key reason for interest in macrophage polarisation is that inflammation (including TLR-induced inflammation) has emerged as an underlying or overt component of many chronic diseases, ranging from diabetes to neurodegenerative diseases (Serhan and Savill 2005, Crusz and Balkwill 2015, Zolezzi and Inestrosa 2017). This is in addition to the causative role played by TLRs and inflammation in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel diseases (Ospelt and Gay 2010). In many of these pathologies, uncontrolled inflammatory signalling contributes to disease progression and complicates therapeutic interventions, and it often does so through dysregulation of M1 and M2 macrophage programming (Wang and Liang *et al.* 2014).

As depicted in Figure 1.1, M1 macrophages are traditionally stimulated by pathogens through TLRs and/or pro-inflammatory cytokines from other immune cells such as interferon-γ (IFN-γ) and tumour necrosis factor (TNF) (Mosser and Edwards 2008). Characterised by an increased production of ROS and the inflammatory cytokines TNF, IL-12 and -6, M1 macrophages have enhanced antigen presentation and microbicidal capabilities for enhanced pathogen killing and clearance (Lawrence and Natoli 2011). In contrast, M2 macrophages are typically induced by immune regulatory cytokines such as IL-4 and IL-13 (Wang and Liang *et al.* 2014). These immune suppressive and wound-healing associated macrophages propagate an anti-inflammatory environment, and have an increased expression of arginase-1 that promotes the production of important tissue healing compounds such as polyamines and ornithine (Galván-Peña and O'Neill 2014). In mouse macrophages, work by Christoph Hölscher and colleagues have also shown IL-10 associated alternative macrophage activation that resulted in increased Arg1 gene expression using a tuberculosis infection model (Schreiber and Ehlers *et al.* 2009). Macrophages retain a high level of plasticity and are capable of switching between these M1 and M2 states

in response to signalling changes in the extracellular milieu (Wang and Liang *et al.* 2014). This flexibility allows for long-term participation during an inflammatory response, promoting or constraining inflammation when necessary to enhance pathogen clearing while minimising collateral tissue damage. Identifying the molecules and pathways associated with these phenotypic transitions is important for understanding the underlying machinery of how macrophages control and direct inflammation. As described below, a key focus of this project is to define the components of a Rab8/PI3Kγ complex that drives macrophages towards an M2-like state to constrain inflammation.

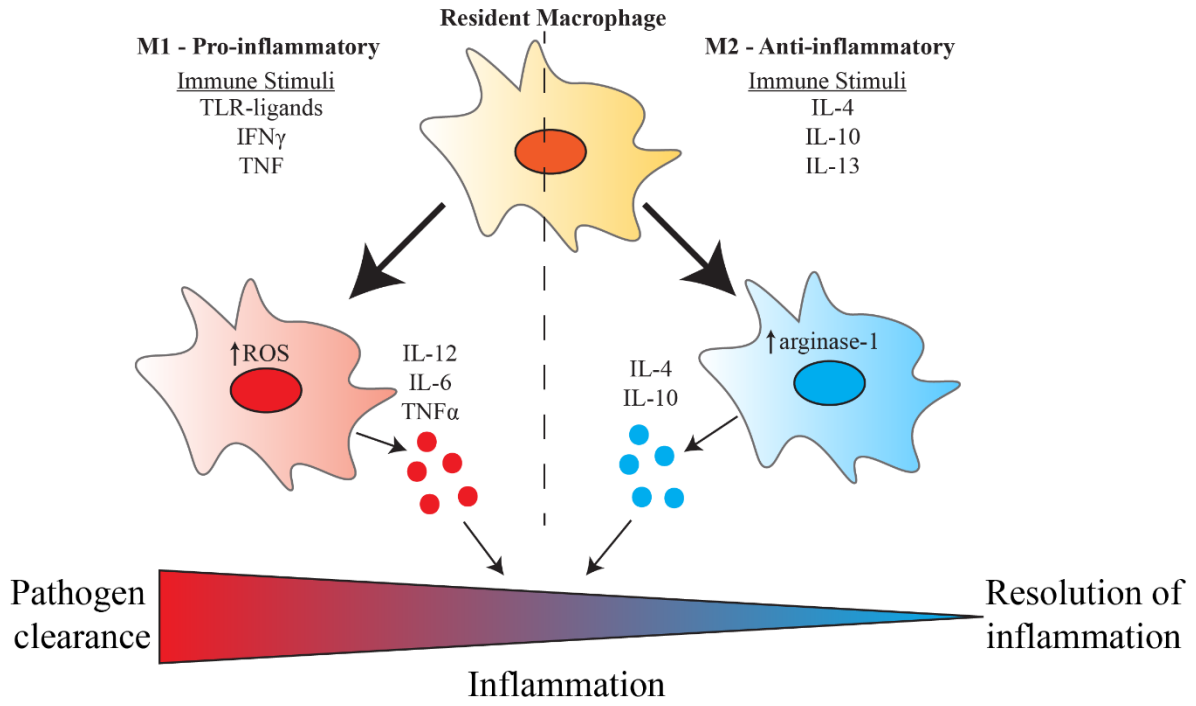


Figure 1.1. M1 vs M2 macrophage polarisation. *Macrophage activation by different immune stimuli elicits polarising immune phenotypes (M1 or M2) and cytokine outputs for promoting or resolving inflammation (adapted from Mosser & Edwards. Nat Reviews 2008.)*

Rab GTPases

Within the Ras superfamily of small (20-30 kDa) GTPases, Rabs are a family of molecular switches that function as master regulators for membrane/vesicle trafficking in eukaryotic cells (Zerial and McBride 2001, Stenmark 2009). Since they were first described in 1987 by Touchot and colleagues, and termed Ras genes from rat brain (Rab) (Touchot and Chardin *et al.* 1987), more than 60 different Rabs have been identified in mammalian cells and implicated in the facilitation of important cellular

functions such as motility, ciliogenesis, proliferation and cell signalling (Peränen 2011, Kelly and Horgan *et al.* 2012).

As molecular switches, Rab GTPases function by binding alternately to the nucleotides, guanosine triphosphate (GTP) and guanosine diphosphate (GDP) signifying the active and inactive states of the Rab protein respectively. In each state, Rabs interact with often discrete sets of proteins that dictate their recruitment and activation on specific cellular compartments. Figure 1.2 depicts the general pathway and highlights the key proteins that modulate Rab recruitment, activation and function. Briefly, newly synthesised Rabs are escorted to a protein prenyl transferase such as geranylgeranyltransferase (GGTase) for C-terminal prenylation (Pereira-Leal and Hume *et al.* 2001). The Rab is then recruited by various escort proteins and embeds into the target membrane through its prenylated tail (Pereira-Leal and Hume *et al.* 2001, Goody and Rak *et al.* 2005) and once at the membrane, it is activated by a guanine nucleotide-exchange factor (GEF) which catalyses the release of GDP from the nucleotide binding pocket (Kelly and Horgan *et al.* 2012). Due to the concentration of GTP being 10-fold higher than GDP in the cellular environment, this nucleotide release facilitates the binding of GTP onto the Rab resulting in activation (Müller and Goody 2018). Upon activation, the GTP-bound Rab recruits and interacts with specific effector proteins that bring about the desired function (Stenmark 2009). Common types of Rab effectors include membrane motors, membrane fusion proteins, signalling scaffolds and membrane tethers and while some Rabs are known to associate with a single effector, others are highly promiscuous with many possible effectors as binding partners at different cellular locations (Stenmark 2009).

The reverse and equally important part of the switch cycle for Rabs is turning off their function through GTP hydrolysis; a process that is facilitated by GTPase activating proteins (GAPs) (Zerial and McBride 2001). GAPs work in opposition to Rab GEFs, by promoting the hydrolysis of GTP into GDP and switching 'off' the Rab GTPase. This is often accompanied by the Rab being extracted from the membrane through a Rab GDP dissociation inhibitor (GDI), which specifically recognises GDP-bound 'off' Rabs, sequestering them in the cytoplasm for potential recycling (Müller and Goody 2018).

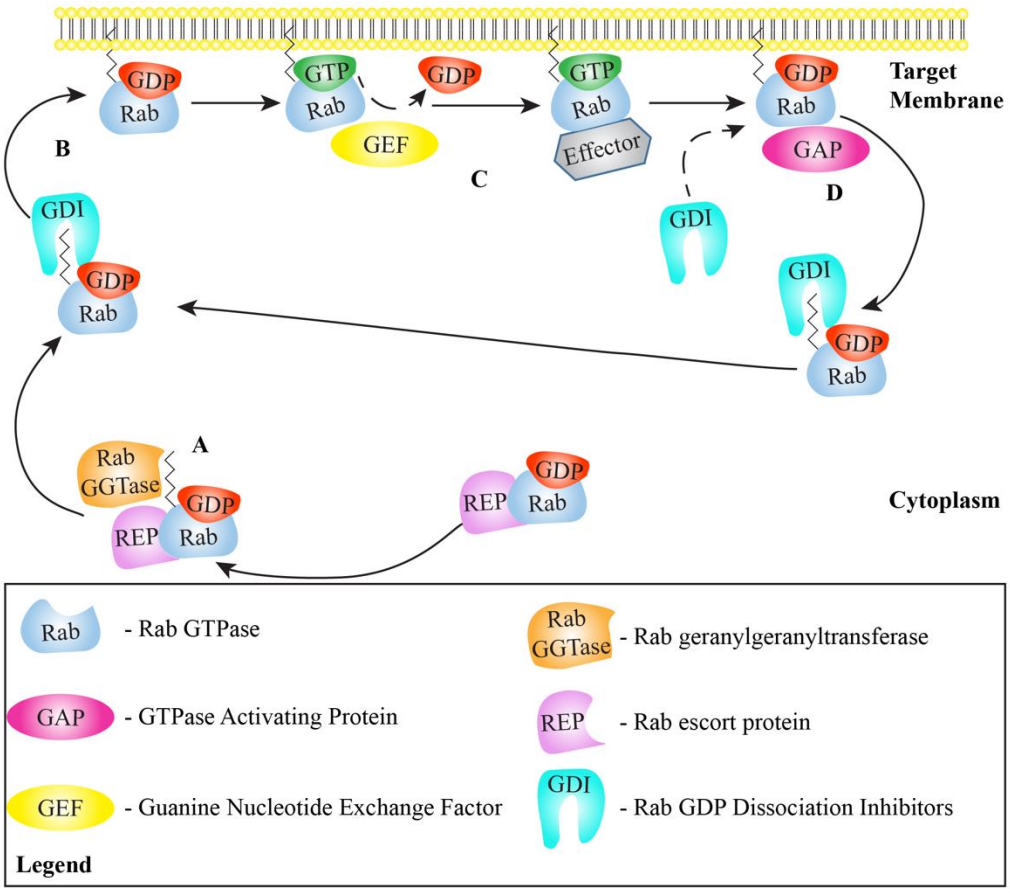


Figure 1.2. The Rab GTPase cycle. *Shown here are the general stages in a Rab cycle. (A) Through escort proteins, newly synthesised Rabs are sent for prenylation and sequestered in the cytoplasm by a Rab GDP dissociation inhibitor (GDI). (B) The Rab is recruited to its specific target membrane domains through various upstream adaptor proteins and is inserted via its prenylated tail. (C) Upon recruitment, the specific Rab GEF switches on the Rab by catalysing GDP-release, resulting in subsequent GTP-loading, allowing for Rab effector recruitment and function. (D) Once its function is completed the active Rab is switched off via GAP-mediated hydrolysis and the inactive GDP-bound Rab is recognised and extracted from the membrane by the GDI and sequestered back into the cytoplasm for recycling (Müller and Goody 2018).*

Earlier studies in yeast cells demonstrated that, in some pathways, a series of Rabs work in sequence to bring about a particular cell function. In such cases, some Rab effectors also act as GEFs to activate a subsequent Rab downstream, forming a 'Rab cascade' as first described by Novick and colleagues in 2002 (Ortiz and Medkova *et al.* 2002, Grosshans and Ortiz *et al.* 2006, Markgraf and Peplowska *et al.* 2007). This results in waves of different Rabs being sequentially recruited and activated on specific cellular organelles/compartments, each eliciting a distinct set of effectors to facilitate cellular events (Pfeffer 2013). This coordination is key for the Rab-dependent management and control of multi-step, multi-effector processes such as trafficking steps, which require directed vesicular transport (motor proteins), membrane lipid

modification (lipid kinases or phosphatases) and vesicle fusion (SNAREs). Collectively, the sequential recruitment and activation of Rabs and their downstream effectors provide directionality in these pathways.

In mammalian cells, there have been several well-defined pathways that are regulated by series of Rabs, although their overall coordination in these processes is less clear. An example is the complex networks of GTPases, including Rabs, that control the retrograde and anterograde trafficking and maturation of vesicles moving to and from the Golgi (Barr 2009). While an exact Rab cascade has not yet been determined for these processes, small glimpses of crosstalk events between Rabs linked to different stages of Golgi vesicle transport have been demonstrated. For instance, the medial Golgi-localised Rab33B has been shown to recruit the GEF complex Ric1-Rgp1 for Rab6 activation, which in turn mediates retrograde transport of mannose 6-phosphate receptors for processing, indicating a Rab handover event between different Golgi compartments (Pusapati and Luchetti *et al.* 2012). With roughly a third of mammalian Rabs associated with the Golgi network, it is reasonable to presume that more extensive Rab cascades contribute substantially to maintaining and coordinating the overall organisation and function of the Golgi complex (Goud and Liu *et al.* 2018). Endocytic and phagocytic processes are also governed by putative Rab cascades. For instance, work from our laboratory has demonstrated that during phagocytosis, a series of Rabs including Rabs 13 and 35, are recruited to facilitate the closure and maturation of the phagosome in macrophages (Yeo and Wall *et al.* 2016). Additionally, the well-known Rab endosomal markers, Rab5 on the early endosome, Rab11 on sorting/recycling endosome and Rab7 on late endosome/lysosome, are functionally linked by effector roles and by intervening by GEFs and GAPs to shepherd cargo through endocytic pathways (Markgraf and Peplowska *et al.* 2007).

The many roles Rabs play in essential cellular processes and pathways means that loss of Rab expression or function can have widespread biological consequences. There is a growing list of genetic and acquired diseases associated with altered Rab function or expression (Banworth and Li 2018). For instance, the autosomal recessive disorder ‘Carpenter syndrome’, which presents with severe birth defects including craniosynostosis and polysyndactyly, has been linked to nonsense mutations in the Hedgehog signalling regulator Rab23 (Jenkins and Seelow *et al.* 2007). Another

example is the neurodegenerative disorder, Parkinson's disease (PD). A hallmark of PD is the impaired intracellular transport and sorting of cargo-containing vesicles in dopamine neurons, which eventually causes the loss of axon projections and cell death (Abeliovich and Gitler 2016). Unsurprisingly, as master regulators of trafficking pathways, mounting evidence has linked a sub-set of Rabs in PD pathogenesis. Specifically, the leucine-rich repeat kinase 2 (LRRK2) phosphorylates several Rab family members, including Rabs 7, 8, 10 and 29, and PD-associated mutations of LRRK2 impair the function of these Rabs, contributing to the pathogenesis of this disease (MacLeod and Rhinn *et al.* 2013, Steger and Tonelli *et al.* 2016, Lis and Burel *et al.* 2018, Bonet-Ponce and Cookson 2019). Interestingly, Rab29 while a substrate of LRRK2 has also been shown to recruit and activate the kinase in turn, possibly exacerbating LRRK2 driven PD pathogenesis (Purlyte and Dhekne *et al.* 2018). Such findings indicate the potential for targeting Rab–mediated pathways as a therapeutic option for several diseases. However, the gross structural similarities between the members of this GTPase family and, with some Rabs being indispensable in other cell-types/functions, makes it challenging to target specific Rabs for drug treatment. As a result, alternative and indirect regulators that modulate Rab specific functions have been seen as promising new targets for clinical studies (Qin and Wang *et al.* 2017).

Rabs in innate immune cells

Immune cell function is dependent on highly organised and tightly controlled intracellular signalling and trafficking events (Gutierrez 2013, Luo and Wall *et al.* 2014, Yeo and Wall *et al.* 2016). From receptor expression, internalisation and degradation, to the secretion of inflammatory cytokines and chemokines, Rabs have been tightly associated with many vital immune cell processes (examples listed in Table 1.2) (Prashar and Schnettger *et al.* 2017). The complex vesicle trafficking pathways accompanying the formation and maturation of phagosomes during phagocytosis, for the destruction of pathogens and particles in innate cells, has been shown to involve many Rab GTPases (Yeo and Wall *et al.* 2016). These include Rabs 5, 8, 13, 31 and 35, which engage in the closure of phagocytic cups, the maturation of the phagosomes and the fusion with lysosomes for pathogen killing (Botelho and Grinstein 2011, Gutierrez 2013, Yeo and Wall *et al.* 2016). Furthermore, Rab11 in macrophages, also

mediates inflammatory cytokine secretion from Golgi to cell surface pathways that intersect with phagocytic cups (Murray and Kay *et al.* 2005) and with recycling endosomes (Stanley and Lacy 2010). Rabs are also engaged on phagosome membranes for receptor trafficking and signalling, with studies demonstrating the role of Rab11 in TLR4 signalling from the phagosome (Husebye and Aune *et al.* 2010). Other Rabs are also engaged for the trafficking of TLR4, with the recycling, cell surface expression and lysosomal degradation of the receptor being regulated by Rabs 11, 10 and 7 (Wang and Chen *et al.* 2007, Husebye and Aune *et al.* 2010, Wang and Lou *et al.* 2010). Aside from these trafficking roles, this project will extend the remit of Rab regulation in macrophages to a receptor signalling role focused on Rab8.

Table 1.2. Examples of known Rabs associated with immune functions

Rab	Function	References
Rab5	Early endosome fusion and phagosome maturation	(Vieira and Bucci *et al.* 2003)
Rab7	Enhanced pathogen clearance by promoting lysosomal targeting and fusion	(Bhattacharya and Ojha *et al.* 2006)
Rab8a	Mediates anti-inflammatory cytokine biasing downstream of TLR activation	(Luo and Wall *et al.* 2014)
Rab10	Regulates the delivery of TLR4 to the plasma membrane	(Wang and Lou *et al.* 2010)
Rab11	Regulates transport of TLR4 to phagosomes from early recycling compartments	(Husebye and Aune *et al.* 2010)
Rab12	Negatively regulates secretory granule trafficking in mast cells	(Efergan and Azouz *et al.* 2016)
Rab13	Regulates formation of LPS-induced macropinocytosis in macrophages	(Condon and Heddleston *et al.* 2018)
Rab27a	Neutrophil degranulation and exocytic secretion of TNFα	(Johnson and Hong *et al.* 2011)
Rab32	Delivery of antimicrobial cargo to bacteria containing vesicles	(Spanò and Galán 2012, Solano-Collado and Rofe *et al.* 2018)

Rab8

Identified as the mammalian homolog to the yeast GTP binding proteins Sec4p and Ytp2p, Rab8 heads a subset of Rab GTPases along with Rab10 and Rab13, which differ from other Rabs through having a CAAX box on their C-terminus in contrast to a CC or CXC motif (Peränen 2011). The cystine residues on these domains undergo prenylation, which is essential for allowing the recruitment and binding of these Rabs to membranes (Zerial and McBride 2001). Rab8 has 2 isoforms, Rab8a and Rab8b which share 83% identity in humans. These two isoforms of Rab8 have both separate and redundant functions, sharing interactions with some but not all accessory proteins and regulators (Peränen 2011). Rab8a, and to a lesser extent Rab8b, are highly promiscuous Rab family members with multiple known effectors and roles in many cellular pathways. Therefore, it is unsurprising that genetic ablation of Rab8a in mice is lethal, with newborns only surviving a matter of days (Sato and Mushiake *et al.* 2007). Nevertheless, these newborn mice have been used to demonstrate the need for Rab8a for apical trafficking in epithelial cells (Sato and Mushiake *et al.* 2007). A more extensive analysis of Rab8 knockouts subsequently showed that the deletion of Rab8b alone has no overt phenotype, but the double deletion of Rab8a and Rab8b produced a much more dramatic loss of apical integrity (loss of microvilli) in gastrointestinal tract epithelial compared with the Rab8a knockout alone (Sato and Iwano *et al.* 2014). The intestinal microvillus atrophy in the double knockouts indicate a synergistic and partially overlapping function of the two Rab8 isoforms. Ultimately, the functional differences between these two isoforms has still not been stringently defined, with some studies still linking them together under a single ‘Rab8’ banner. Rab8 is one of the most ubiquitous and multi-functional members of the Rab family with a relatively large list of effectors (examples listed in Table 1.3). The functions of Rab8 are mostly associated with vesicle trafficking and polarised trafficking for either endocytic, recycling or exocytic processes (Hattula and Furuhjelm *et al.* 2006, Peränen 2011). Rab8 is also prominently associated with cell surface protrusions on different cell types, including ruffles, filopodia, lamellipodia, phagocytic cups and primary cilia (Peränen 2011). Below describe several well-studied examples of Rab8 regulated pathways.

Table 1.3. Examples of known Rab8 effectors and their functions.

Effector	Function	Reference
JRAB/MICAL L2	Trafficking of tight-junction and adherent junction proteins (Polarisation)	(Yamamura and Nishimura *et al.* 2008)
OCRL (inositol polyphosphate 5-phosphatase)	Facilitate plasma membrane signalling	(Pirruccello and De Camilli 2012)
Sec15	Exocytosis and Ciliogenesis	(Wu and Mehta *et al.* 2005, Feng and Knödler *et al.* 2012)
Rab8IP (GC Kinase)	Trafficking of secretory vesicles in response to cellular stress	(Ren and Zeng *et al.* 1996)
Linker for activation of X cells (LAX)	Transport of CTLA-4 T-cell regulating receptor to the cell surface	(Banton and Inder *et al.* 2014)
Optineurin (FIP-2) Myosin VI	Vesicle Trafficking from TGN to Plasma Membrane	(Chibalina and Roberts *et al.* 2008)
PI3Kγ	LPS-induced mTOR and AKT signalling in macrophages	(Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017)
Rabaptin 5	Ciliogenesis	(Omori and Zhao *et al.* 2008)

Rab8 functions

Trafficking to and from the plasma membrane

The plasma membrane is a highly dynamic and compartmentalised cell structure that acts as a medium for many essential functions such as cell signalling, vesicular trafficking and cell adhesion (Laude and Prior 2004). Therefore, maintaining the plasma membrane is crucial for cell survival, requiring tightly controlled processes to

internalise, recycle and replenish membrane components (Cho and Stahelin 2005). Characterisation of Rab8 in multiple cell types has identified this Rab as a master regulator of trafficking to the plasma membrane, localising to dynamic ruffling cell surface structures, tubules and trafficking vesicles (Peränen 2011). Live-cell imaging of fluorescently tagged Rab8 in live cells demonstrated that this particular Rab is primarily on membranes that are rapidly tubulated and internalised from macropinosomes or endosomal vesicles from the plasma membrane and sorted back into recycling compartments (Hattula and Furuhjelm *et al.* 2006). This is followed by the recruitment of Rab11 to these recycling endosomes, which in turn recruits the Rab8 GEF Rabin8, facilitating the re-export of membrane vesicles back to the plasma membrane (Rowe and Suszko *et al.* 2008, Vetter and Stehle *et al.* 2015). Below are several specific examples of known cellular processes controlled by Rab8 membrane trafficking functions.

Apical Polarity

Approximately 60% of mammalian cells are epithelial-derived and form the building blocks of many organs and tissues, with their function intimately tied to cell polarity (Bryant and Mostov 2008). Multiple studies have identified Rab8 as a major contributor to the establishment and maintenance of epithelial cell polarisation by promoting directional transport of various apical and basolateral cargo proteins. One example was the use of a yeast two-hybrid screen with a GTP-Rab8 mutant construct against a mouse small intestine cDNA library and follow-up pull-down assays, Nakajo and colleagues demonstrated that the protein complex EH domain–binding protein 1–like 1 (EHBP1L1)-Bin1-dynamin is able to interact with active Rab8 (Nakajo and Yoshimura *et al.* 2016). The authors later used immunofluorescence and knockouts of EHBP1L1 and Bin1 to demonstrate the role this Rab8-binding complex plays in the sorting and delivery of apical protein cargo (the glycoprotein DPP4) in mouse and human epithelial cells. Another study by Henry and Sheff utilised the overexpression of mutant Rab8 constructs in MDCK cells and visually demonstrated disruptions to the polarised trafficking of basolateral proteins, such as VSV-G (Henry and Sheff 2008). Additionally, knockdown studies performed on MDCK cysts revealed that both Rab11a and Rab8a are critical components of the molecular machinery for regulating the polarised architecture of lumen generation via promoting polarised transport and

targeting of Cdc42 and apical membrane initiation site (AMIS) formation (Bryant and Datta *et al.* 2010, Datta and Bryant *et al.* 2011).

Ciliogenesis

One of the best characterised Rab8 functions is regulating ciliogenesis. Cilia are antenna-like protrusions from the cell surface and function as important signalling and sensory organelles (Satir and Christensen 2007). Experiments on mouse and human intestinal epithelial cells showed that Rab8 is the predominant Rab associated with cilia formation, and depletion of either isoform of Rab8 led to the inhibition or disruption of cilia formation (Nachury and Loktev *et al.* 2007, Sato and Mushiake *et al.* 2007, Feng and Knödler *et al.* 2012). Specifically, Sato and colleagues demonstrated that depletion of Rab8 in mouse intestinal epithelial cells showed an accumulation of apical proteins (Sato and Mushiake *et al.* 2007), while Nachury and colleagues showed that mutants of Rab8 alters the number and length of cilia in RPE cells (Nachury and Loktev *et al.* 2007). Other studies expanded this pathway to include Rab11, a known regulator and marker for recycling endosomes, and its interaction the Rab8 GEF Rabin8 to redirect Rab8 positive endosomes towards the primary cilia (Vetter and Stehle *et al.* 2015, Wang and Deretic 2015).

Neurite outgrowth

Neuronal cell development and morphology is essential for the function of the nervous system. One key aspect is the extension of neurites between neurons to connect and form neural networks. This process of neurite outgrowth requires a continuous supply of plasma membrane to generate these cell protrusions (Read and Gorman 2009, Villarroel-Campos and Gastaldi *et al.* 2014). Therefore, membrane trafficking is paramount to neuron maturation and function (Huber and Dupree *et al.* 1995, Villarroel-Campos and Gastaldi *et al.* 2014). Indeed, experiments conducted have shown that either knockdown or knockout of both Rab8 and its respective GEFs, GRAB and Rabin8, in mouse cortical neurons have resulted in significant reductions to neurite outgrowth, presumably through disruptions to the Rab8-Rab11 dependent transport of membrane vesicles for axon outgrowth (Furusawa and Asada *et al.* 2016, Homma and Fukuda 2016).

TLR signalling in macrophages

In macrophages, both Rab8a and 8b are distributed on membranes throughout the cell and exhibit prominent associations with cell surface ruffles and protrusions, as in other cell types. Earlier work in our laboratory showed that Rab8a is enriched on ruffles and on the macropinosomes that internalise from ruffles where it coincides with TLRs and their signalling adaptors (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). Live imaging shows Rab8a remaining on ruffle membranes as they form into macropinosomes before it is then rapidly shunted onto tubules emerging from the early macropinosomes as they mature and shrink (Wall and Luo *et al.* 2017). The Rab8a tubules are likely sorting membrane and cargo back to recycling endosomes for return to the cell surface. Rab8a is activated on the macropinosomes to recruit an atypical effector, namely PI3Kγ (Wall and Luo *et al.* 2017), through which Rab8a contributes to Akt/mTOR signalling and inflammatory outputs downstream of several TLRs. Neither Rab8a nor PI3Kγ are recruited directly to TLR complexes and in seeking other co-receptors, our group showed that the endocytic receptor, low density lipoprotein receptor-related protein 1 (LRP1) is activated by TLRs through a receptor crosstalk event, and that Rab8a is actually bound to LRP1 to enlist it for TLR signalling (Luo and Wall *et al.* 2018). LRP1 has also been shown in a number of studies, to help constrain inflammation in TLR pathways and in other contexts (Yancey and Blakemore *et al.* 2010). How Rab8a is recruited to LRP1 and how it is activated for its signalling roles are yet to be determined and will be the topic of this thesis (Figure 1.3).

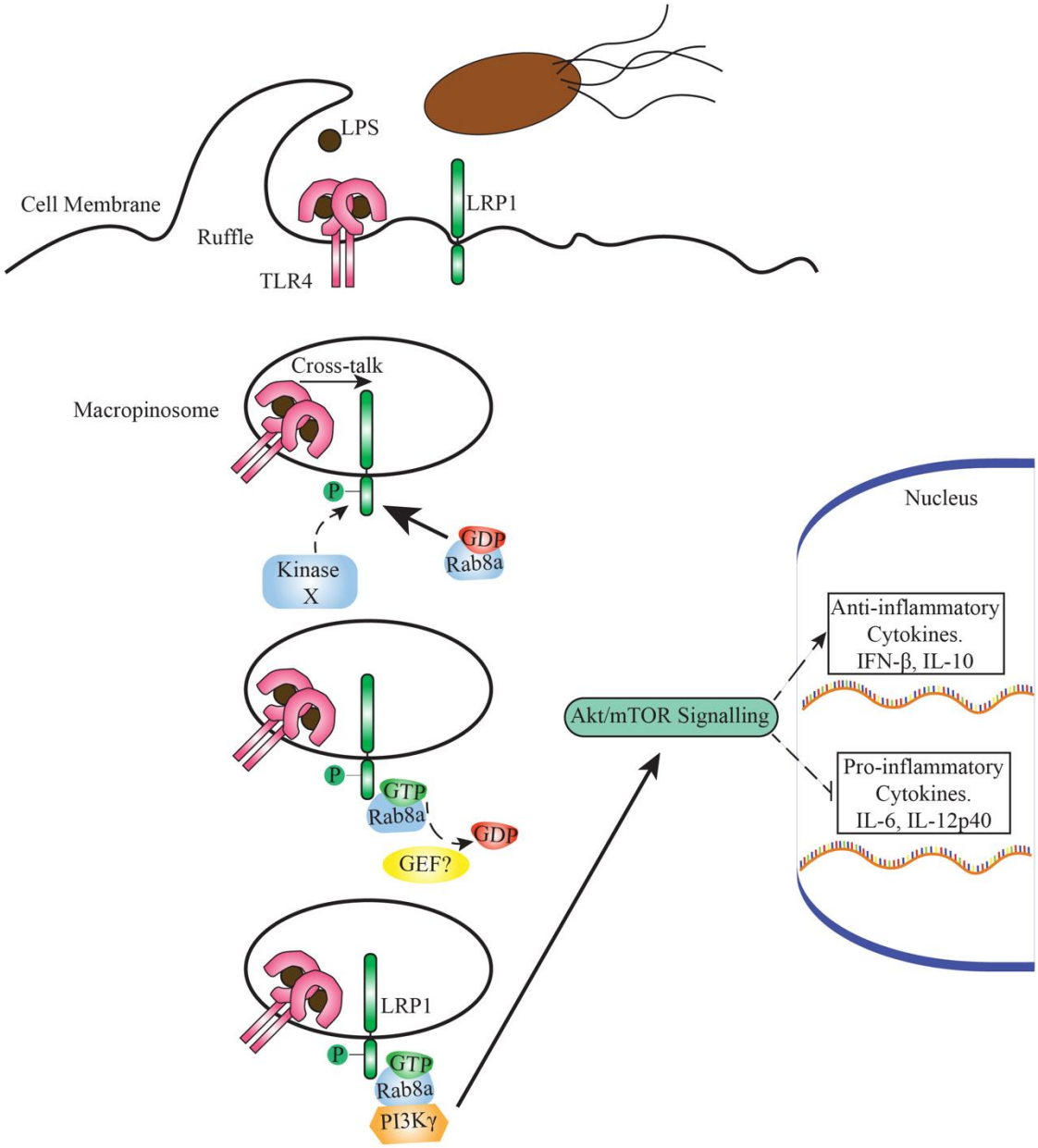


Figure 1.3. TLR4-Rab8a-PI3Kγ signalling pathway in macrophages. *Upon binding the ligand bacterial LPS, TLR4 is activated through an unknown kinase X it crosstalk phosphorylates and activates LRP1, which scaffolds and recruits Rab8a. Rab8a is then activated by an unknown GEF to recruit its effector PI3Kγ and elicits downstream Akt/mTOR signalling, biasing of cytokine outputs towards and anti-inflammatory state to constrain inflammation (Luo and Wall et al. 2014, Wall and Luo et al. 2017, Luo and Wall et al. 2018, Wall and Condon et al. 2018).*

Rab8 effectors

Rab8a has many known effectors in its disparate cellular roles (see Table 1.3). Here, I describe two of particular relevance to this project, that are Rab8 effectors in macrophages.

Phosphoinositide 3-kinase gamma (PI3Kγ)

PI3Kγ is a class 1B PI3K that is enriched in macrophages. It has the role of converting the phosphoinositide, phosphoiniositol (4, 5)-biphosphate [$PI(4,5)P_2$], to phosphoiniositol (3, 4, 5)-triphosphate [$PI(3,4,5)P_3$], which is an signalling phosphoinositide that attracts the signalling kinase Akt. PI3Kγ is traditionally recruited to G protein coupled receptors by the small GTPase Ras for signalling, however more recently it has been shown to act in receptor tyrosine kinase pathways and in TLR pathways (Luo and Wall *et al.* 2014, Kaneda and Messer *et al.* 2016). We showed that the Ras binding domain of the p110 catalytic subunit binds to GTP-Rab8a in macrophages, and that LRP1 and Rab8a enable the recruitment of this effector on early macropinosome membranes where it triggers Akt/mTOR signalling. Knockout of Rab8a in macrophage cell lines, or genetic ablation of PI3Kγ in mice, confirm the roles of this Rab-effector pair in inflammatory signalling (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). This signalling then drives transcriptional programs enhancing the synthesis and secretion of anti-inflammatory cytokines and curtailing pro-inflammatory cytokines. Overall, this helps to constrain inflammatory responses.

This immune suppressive function of PI3Kγ in macrophages has been further supported by findings from other studies in mice (Kaneda and Messer *et al.* 2016) and in humans (Stark and Sriskantharajah *et al.* 2015), where PI3Kγ deficiency is associated with immunodeficiency and hyper inflammation. Hence, PI3Kγ has emerged as a key determinant of an M2 polarised macrophage phenotype.

Oculocerebrorenal syndrome of Lowe protein 1 (OCRL)

Phosphoinositides are membrane bound molecules that shape important signalling domains for many important cellular functions. These phospholipids contain an inositol ring which, upon phosphorylation on its three different residues at positions 3, 4 or 5, forms signalling platforms on specific membrane locations for the recruitment of various effector proteins (Ooms and Horan *et al.* 2009). The phosphorylation states of these phosphoinositides are regulated by a range of phosphoinositide kinases, such as the PI3Ks described above and by inositol phosphatases. Oculocerebrorenal syndrome of Lowe protein 1 (OCRL) is one of 10 known mammalian inositol 5-phosphatases (Figure 1.4), named after it was revealed to be a key protein in an X-

linked genetic disease called oculocerebrorenal syndrome of Lowe, where mutations in the 5-phosphatase domain of OCRL result in neurological/renal/ocular defects due to disruptions in endolysosomal pathways (De Matteis and Staiano *et al.* 2017).

Found to preferentially hydrolyse phosphoiniositol (4, 5)-biphosphate [$PI(4,5)P_2$] into phosphoiniositol (4)-biphosphate [PI(4)P], OCRL regulates a wide range of important cellular processes. The main function of OCRL is to control the levels of available $PI(4,5)P_2$, which alters the lipid membrane environment and affects the recruitment and interactions of a number of metabolic, trafficking and signalling molecules to mediate a variety of important cellular functions (examples listed in Table 1.4). One key example is OCRL's role in regulating cytoskeletal reorganisation , by affecting the recruitment of actin-binding proteins such as talin and vinculin (Janmey and Lindberg 2004). In macrophages, OCRL-dependent $PI(4,5)P_2$ depletion on particular phagosome membranes have been shown as an important driver of phagosome maturation and attenuating downstream Akt signalling (Bohdanowicz and Balkin *et al.* 2012). With recent work from our laboratory demonstrating that OCRL is likewise on macrophage macropinosome membranes suggesting that this important regulator of phosphoinositide signalling might play a similar role during macropinocytosis (Wall and Condon *et al.* 2018).

OCRL is a promiscuous Rab interacting protein, known to bind to at least 17 Rab GTPases (Fukuda and Kanno *et al.* 2008) but notably including Rab8. Experiments in zebrafish (*Danio Rerio*) have identified Rab8 as an essential recruiter of OCRL to facilitate maintenance of primary cilia (Luo and West *et al.* 2012). Crystal structure analysis have revealed the nature of GTP-loaded Rab8 binding to OCRL via an interaction with its Rab binding domain (RBD) (depicted in Figure 1.4, residues 540–678) (Hagemann and Hou *et al.* 2012). Exploiting this active-Rab/effector interaction provides a means for utilising the RBD of OCRL, and other effectors, to capture GTP-Rab8 for activation assays as described in this thesis. Furthermore, OCRL is of interest in macrophages due to its potential for linking several members of the Rab8 subfamily through shared or sequential interactions.

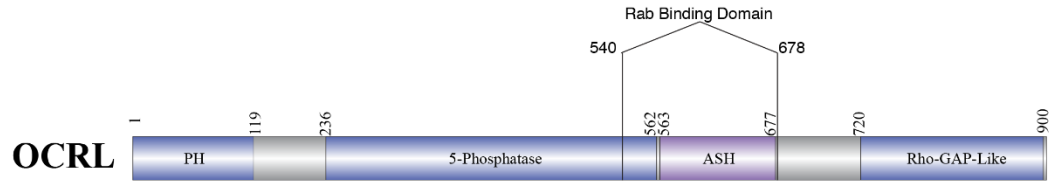


Figure 1.4. Functional domains of OCRL. *Depicted here are the known functional domains and Rab binding domain of OCRL. (Hagemann and Hou et al. 2012, De Matteis and Staiano et al. 2017)*

Table 1.4. List of known OCRL interacting proteins

Effector	Function	Reference
Clathrin	Facilitate clathrin vesicle uncoating after budding	(Nández and Balkin *et al.* 2014)
Rab5	Facilitates early endosome trafficking by inhibiting Rac-dependent actin polarisation	(Vicinanza and Di Campli *et al.* 2011)
Rab8	Exocytosis and ciliogenesis	(Hagemann and Hou *et al.* 2012, Luo and West *et al.* 2012)
Rab35	Promotes cytokinesis abscission	(Dambournet and Machicoane *et al.* 2011)
Arf6	Clathrin recruitment for pit assembly and budding	(Krauss and Kinuta *et al.* 2003)
APPL1	Receptor endocytosis and signalling through modulating Akt binding	(Pirruccello and De Camilli 2012)
Rac	Regulates Rac dependent polarisation and vesicular trafficking	(Faucherre and Desbois *et al.* 2003)

Guanine nucleotide exchange factors (GEFs)

As molecular switches, the functions of small GTPases are dependent on their ability to recruit specific effectors on targeted organelles at the appropriate time. This precise effector recruitment is dependent on structural changes undertaken by the GTPase, as it alternates between an 'on' GTP-bound state and an 'off' GDP-bound state, a process catalysed by specific GEFs and GAPs. As such, GEFs and GAPs have

become attractive alternative targets for altering GTPase activity through either enhancing/inhibiting their catalytic activity, or by modulating their recruitment to relevant membranes (Bos and Rehmann *et al.* 2007, Cherfils and Zeghouf 2013, Müller and Goody 2018). As effector binding and function only occurs when the GTPase is switched on, identifying the GEF(s) responsible for activating the GTPase is essential for understanding the regulatory machinery for any small GTPase-associated pathway. With over 150 members of the Ras-related superfamily of small GTPases, the GEFs responsible for regulating these proteins are categorised in equally diverse groups and subfamilies. Specifically, each subfamily of the Ras-like GTPases (Rho, Ras, Rab, Ran and Arf) have affiliated groups of GEFs that mostly share conserved catalytic domains for facilitating GEF-GTPase specific nucleotide exchange (Bos and Rehmann *et al.* 2007). Examples are the Rho GEFs which share a common Dbl homology (RhoGEF) domain (Cook and Rossman *et al.* 2014), and the Ras GEFs which possess a catalytic CDC25 homology GEF domain (Popovic and Rensen-de Leeuw *et al.* 2013). In this thesis, I will be focusing on the GEFs of the Rab family of Ras-related GTPases.

As with the other members of the Ras GTPase family, Rabs in general possess a slow intrinsic dissociation rate of nucleotides, as a consequence of having a high affinity for both GDP and GTP nucleotides (Bos and Rehmann *et al.* 2007). Therefore, to increase the response rate, GEFs act as catalysts to accelerate this process by several hundred-fold (Vetter and Wittinghofer 2001). Structural information for various Rab GEFs revealed that this occurs through an interaction between the switch I and II regions of a GDP-bound GTPase molecule and the catalytic GEF domains of the specific GEF, driving a conformational change of the Rab nucleotide binding site and releasing the bound GDP (Ishida and Oguchi *et al.* 2016). While this GEF-Rab interaction does not favour either GDP or GTP from reinserting into the empty nucleotide binding site, activation of the Rab occurs due to the 10-fold higher concentration of GTP to GDP in the cellular environment (Kelly and Horgan *et al.* 2012). Upon activation, the Rab is now available to recruit and bind its specific effector to facilitate downstream functions. There are currently several known families of Rab GEFs, classified by their catalytic GEF domain structures (Müller and Goody 2018). The two largest classes of Rab GEFs are the differentially expressed in normal and neoplastic cells (DENN) domain containing GEFs (18 members in humans) and the

vacuolar protein sorting 9 (VSP9) domain containing GEFs (9 members in humans), along with other smaller groups such as transport protein particle (TRAPP) complexes, which are multi-subunit GEFs, and the focus of this report, the Sec2-domain containing GEFs (Ishida and Oguchi *et al.* 2016). Despite the distinct separation and homology between these GEF families, there is interestingly no correlation between the GEF family members and Rab sub-families with the exception of the VPS9 proteins targeting the Rab5 family members (Carney and Davies *et al.* 2006). Several examples of each of these GEF family members and their target Rab is listed in Table 1.5.

In addition to targeting Rab GEFs directly, there is now a growing interest in identifying the molecular mechanisms that govern GEF activity as an alternative approach for modulating specific Rab functions. There are several known mechanisms for controlling GEF function, which revolves around either altering the recruitment and location of the GEF to a targeted cellular compartment, or modulating its catalytic activity through occluding/exposing its GEF domain to prevent/enhance Rab binding (Müller and Goody 2018). A well-known example is the recruitment of the GEF Sec2p (yeast homolog to the mammalian Rabin8) to secretory vesicles by the upstream GTPase Ypt32 in yeast cells (Mizuno-Yamasaki and Medkova *et al.* 2010). The authors demonstrated that preventing the recruitment, but not activity, of the GEF Sec2p to secretory vesicles prevented the activation of its GTPase Sec4p (yeast homolog to the mammalian Rab8) on the target membranes, inhibiting vesicle transport and maturation. Therefore, to elucidate additional components of the regulatory machinery that modulates Rab activation and function, the upstream proteins that dictate the recruitment and activation of its respective GEF must also be identified.

This project focuses on identifying the respective Rab8a GEF(s) that is/are responsible for activating Rab8a in the TLR4-Rab8a-PI3Kγ inflammatory signalling complex in macrophages.

Table 1.5. List of Rab GEF family members and examples of their known functions

GEF	Target Rab	Function	Reference
DENN-domain GEFs			
DENND1A	Rab35	Endosome membrane trafficking	(Allaire and Marat *et al.* 2010)
DENND1B	Rab35	Regulation of T-cell receptor signalling	(Yang and Hojer *et al.* 2016)
DENND2B	Rab13	Actin remodelling (cell trafficking and growth)	(Ioannou and Bell *et al.* 2015)
DENND3	Rab12	Autophagosome Trafficking	(Xu and Fotouhi *et al.* 2015)
DENND5A	Rab39	Exocytic trafficking and secretion of IL-1β in response to caspase-1	(Becker and Creagh *et al.* 2009, Yoshimura and Gerondopoulos *et al.* 2010)
DENND6 (FAM116)	Rab14	Regulation of cell-cell adhesion by trafficking proteases	(Linford and Yoshimura *et al.* 2012)
VPS9-Domain GEFs			
Rabex5	Rab5	Endosome maturation and membrane fusion	(Zhu and Liang *et al.* 2009)
Varp	Rab21	Dendrite formation in melanocytes	(Ohbayashi and Yatsu *et al.* 2012)
RIN1	Rab5	Epidermal growth factor receptor internalisation and degradation	(Balaji and Mooser *et al.* 2012)

RIN3	Rab31	Trafficking of receptor containing vesicles between the TGN and early endosomes	(Kajiho and Sakurai *et al.* 2011)
ALS2	Rab5	Endosome trafficking	(Otomo and Hadano *et al.* 2003)
TRAPP complex			
mammalian Trs130 (mTrs130)	Rab1	Early Golgi trafficking of COPI-coated vesicles	(Yamasaki and Menon *et al.* 2009)
Sec2p			
Rabin8	Rab8	Ciliogenesis	(Nachury and Loktev *et al.* 2007)
GRAB	Rab8	Neurite outgrowth	(Furusawa and Asada *et al.* 2016)

Rab8 GEFs

To date there are two main GEFs that specifically regulate Rab8 activity, the proteins Rabin8 and GRAB (Figure 1.5). Described below is a short literature review on these known Rab8 GEFs summarising what is currently known about these proteins.

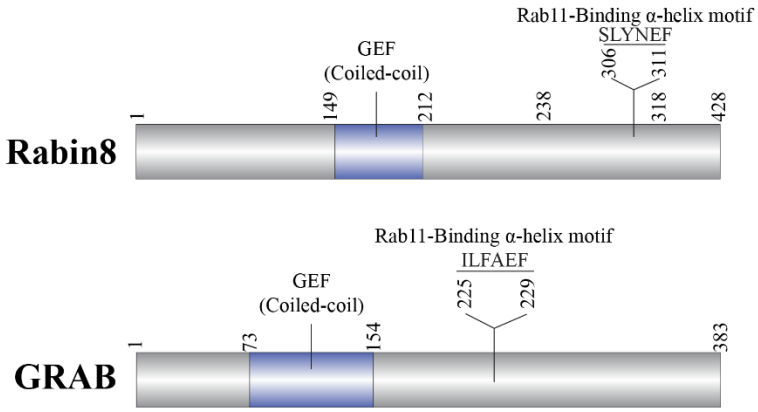


Figure 1.5. The Rab8 Sec2 GEFs Rabin8 and GRAB. *Depicted here are the known locations of the highly conserved functional GEF coiled-coil domains and Rab11-binding domains of Rabin8 and GRAB (Guo and Hou et al. 2013, Horgan and Hanscom et al. 2013, Vetter and Stehle et al. 2015).*

Rabin8 (Rab3IP)

The first identified and most well characterised GEF of Rab8, Rabin8, also known as Rab3-like interacting protein (Rab3IP), is best known for the pivotal role it plays in ciliogenesis, where it facilitates Rab8-mediated vesicle transport and fusion of Golgi-derived vesicles to the pericentrosomal area surrounding the centrosome (Chiba and Amagai *et al.* 2013, Patrussi and Baldari 2016). Belonging to the Sec2-GEF family of proteins, Rabin8 has a highly conserved Sec2p coiled-coil GEF domain where it predominately interacts with Rab8, however some studies hint at a possible GEF interaction with Rab10 and Rab3 (Sato and Fukai *et al.* 2007, Homma and Fukuda 2016, Patrussi and Baldari 2016). Crystal structures of these proteins revealed that Rabin8 preferentially forms a homodimer and interacts with Rab8 via the overlapping of two GEF coiled-coil domains (Figure 1.6). This interaction disrupts a magnesium binding site adjacent to the nucleotide binding pocket, resulting in a decreased affinity for the nucleotide (GDP) and the subsequent release of GDP, leaving the pocket open for GTP binding and Rab activation (Guo and Hou *et al.* 2013).

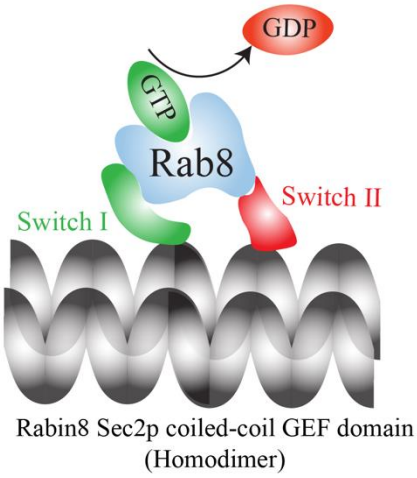


Figure 1.6. Interaction between Rab8a and Rabin8. *Structural studies have demonstrated that Rab8a and Rabin8 interaction occurs between the switch I and II regions of Rab8a and the homodimerised coiled-coil Sec2P GEF domains of two Rabin8 molecules. This binding interface causes a conformational change within the nucleotide binding pocket, resulting in the release of GDP and nucleotide exchange with GTP (Guo and Hou et al. 2013)*

As mentioned previously, the function and recruitment of Rabin8 has been well characterised during ciliogenesis. Identified as an effector of Rab11, Rabin8 forms a Rab11-FIP3-Rabin8 complex on recycling endosomes, which in turn recruits and activates Rab8 for docking and delivery of the vesicle to the ciliary base (Vetter and Stehle *et al.* 2015). This association between Rabin8 and Rab11 was further investigated in other studies, identifying a 69-residue sequence that is essential for Rab11a interaction (Knödler and Feng *et al.* 2010). This upstream recruitment of Rabin8 has been tightly associated with facilitating Rab8 localisation to the ciliary base, indicating that, at least in this context, Rabin8 contributes to both the activation and targeted recruitment of Rab8.

Rabin8 exists in a naturally auto inhibitory state and its GEF activity is tied to kinase driven phosphorylation to relieve this state (Wang and Ren *et al.* 2015). This study by Wang and colleagues demonstrated that Rabin8 GEF activation in response to epidermal growth factor (EGF) is regulated by the mitogen-activated protein kinase 1 (MAPK1) extracellular signal-regulated kinase 1 and 2 (ERK1/2). ERK1/2 is a central regulatory kinase for many cellular processes from cell proliferation to metabolism mostly through the MAPK cascade of Ras-Raf-MEK-ERK (Wortzel and Seger 2011). The authors identified four serine residues namely, 16, 19, 247 and 250 of Rabin8 that

are phosphorylated by ERK1/2 in HEK293 cells stimulated with EGF (Figure 1.7). This phosphorylation leads to an 'unfolding' of the GEF, releasing its inhibited state and exposing its GEF domain (Wang and Ren *et al.* 2015). This change in structural configuration allowed the binding and subsequent activation of Rab8, facilitating the recruitment of effectors for exocytic signalling and receptor trafficking functions. In another study, the serine/threonine kinase nuclear Dbf2-related protein 2 (NDR2) was also shown to phosphorylate Rabin8, but at serine-272 during ciliogenesis in RPE1 cells (Figure 1.8) (Chiba and Amagai *et al.* 2013). Interestingly, the authors demonstrated that the effects of this kinase did not alter Rabin8's GEF activity, but rather Rabin8 recruitment and localisation was altered by promoting its binding to Sec15, an essential component of the exocyst complex. This redirection of Rabin8 localisation eventually drove Rab8 activity towards the forming cilia (Chiba and Amagai *et al.* 2013). Taken together, these findings highlight the various effects of kinases that are used to differentially regulate Rabin8-Rab8 function.

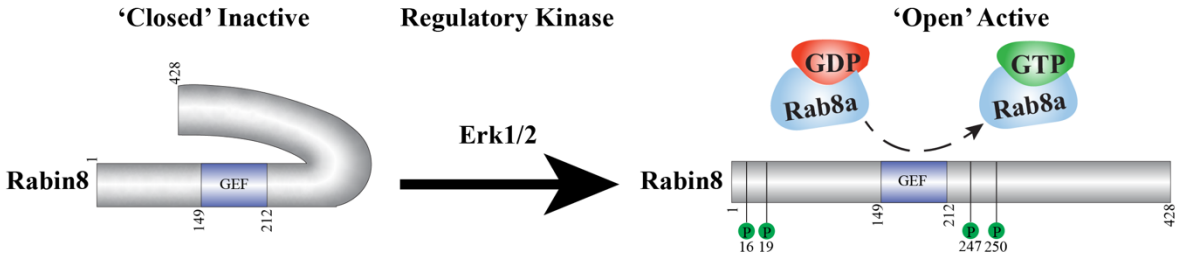


Figure 1.7. ERK1/2 kinase regulation of Rabin8 GEF function. *The ERK1/2 phosphorylation sites (ser16/19/247/250) identified on Rabin8 that when phosphorylated, drives a conformational change which 'unfolds' Rabin8 and relieves its autoinhibited state, exposing the Sec2p GEF domain for Rab8 binding and nucleotide exchange (Wang and Ren et al. 2015).*

GRAB (Rab3IL1)

Though not as well characterised as Rabin8, GRAB, also known as Rab3a interacting protein like 1 (Rab3IL1), is structurally very similar in its GEF interaction with Rab8 as Rabin8 (Guo and Hou *et al.* 2013). Sharing a 55% homology with Rabin8, structural analysis has shown that GRAB, likewise, has the Sec2p homologous coiled-coil GEF domain (See Figure 1.5), and interacts with Rab8 in a biochemically identical manner to Rabin8 (Guo and Hou *et al.* 2013).

Initially identified as a GEF for the neuron and endocrine specific Rab GTPase Rab3a, GRAB was first shown to regulate Rab3's role in the secretion of neurotransmitters and hormones (Luo and Saiardi *et al.* 2001, Matsumoto and Miki *et al.* 2004). Further insights into GRAB function have since demonstrated that this protein has a greater GEF specificity and activity for Rab8 compared to Rab3 (Yoshimura and Gerondopoulos *et al.* 2010). Additionally, like Rabin8, studies have identified GRAB as an effector for Rab11, suggesting a similar recruitment mechanism to Rabin8 (Horgan and Hanscom *et al.* 2013). This interaction is further elaborated with mediating axonal outgrowth, demonstrating a Rab11-GRAB-Rab8 pathway that was similar to the Rab11-Rabin8-Rab8a complex described earlier (Furusawa and Asada *et al.* 2016).

Interestingly, like Rabin8, GRAB's GEF function is shown to be regulated through phosphorylation by specific serine kinases. Multiple studies have implicated different kinases that regulate GRAB GEF function in various systems (Figure 1.8). For instance, the serine kinase c-Jun N-terminal kinase (JNK) has been identified to phosphorylate Ser^{169} of GRAB in myocytes, promoting the secretion of the important muscle repair cytokine IL-6, however, in this study GRAB GEF function has not yet been tested (Lee and Min *et al.* 2017). Interestingly in another publication, GRAB phosphorylation at $Ser^{169/180}$ by the neuron specific proline-directed serine/threonine membrane bound kinase Cdk5 is shown to actually decrease its GEF binding affinity to GDP-bound Rab8, modulating axon outgrowth (Furusawa and Asada *et al.* 2016). While it is unknown if GRAB also exists in an autoinhibitory state like Rabin8, it is clear that kinases are important regulators for both of these Rab8 GEFs.

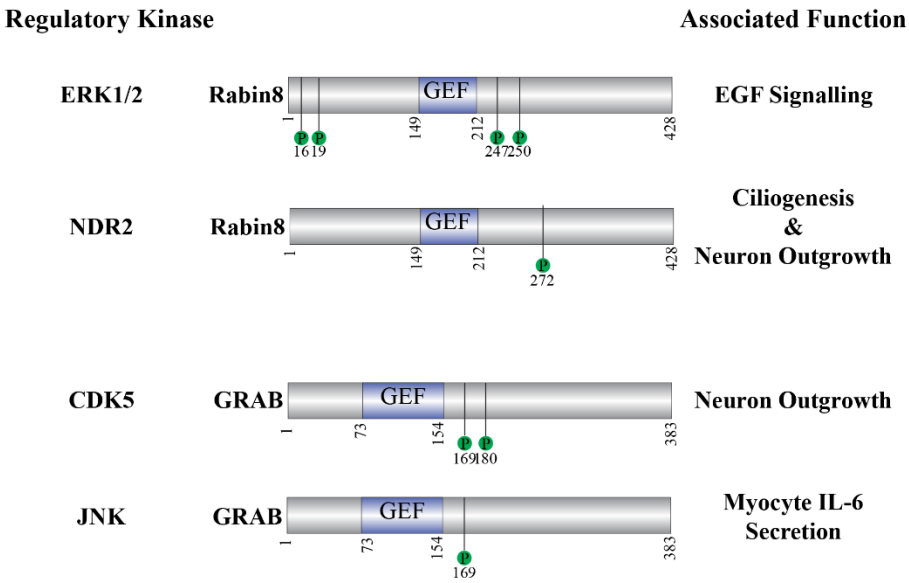


Figure 1.8. Kinase Regulation of Rabin8 and GRAB. *Several studies have identified kinase-mediated regulation of either Rabin8 or GRAB in various pathways. Shown here are examples of known kinases, along with their phosphorylation sites that regulate Rabin8 – or GRAB – associated pathways (Chiba and Amagai et al. 2013, Wang and Ren et al. 2015, Furusawa and Asada et al. 2016, Lee and Min et al. 2017).*

The similarities between Rabin8 and GRAB prevent the exclusion of either, in terms of Rab8 regulation in macrophage TLR signalling, making both of these proteins viable candidates for further investigation. This project will address and identify the regulatory GEFs, along with potential upstream modulators that activate the small GTPase Rab8a for its role in TLR-associated inflammatory signalling.

Chapter 2

Aims and Significance

2. Aims and Significance

Key roles of macrophages are to mount innate immune responses to kill pathogens and to recruit cells of the adaptive immune system for more extensive anti-microbial programs in order to ward off infection. Macrophages are engaged for these roles by pathogen-mediated activation of Toll-like receptors (TLRs), which generate signalling and transcriptional pathways leading to the secretion of inflammatory cytokines, chemokines and other effectors. TLR–mediated pathways are essential for effective immune defence but must also be tightly constrained to avoid excessive inflammation and tissue damage. In fact, poorly controlled inflammation, including from TLR responses, contributes to a heavy burden of chronic inflammatory and autoimmune diseases and, acutely to high morbidity and mortality in sepsis. Therefore, generating a greater understanding of TLR-mediated signalling pathways is a significant goal for biomedical research and the pharmaceutical industry in seeking new drug targets and approaches for managing inflammation and infection.

Rab GTPases and their attendant regulators are critical for enabling and controlling a wide range of cell functions. In innate immunity, Rab GTPases control key processes such as receptor trafficking, cytokine secretion, macropinocytosis, phagocytosis and receptor mediated signalling to promote and drive immune cell functions. In a detailed series of studies, our research group has identified and characterised an important role for Rab8 as a signalling regulator downstream of multiple TLRs (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017, Wall and Condon *et al.* 2018). TLRs activated by a range of pathogen-associated agonists, induce the crosstalk phosphorylation and activation of the endocytic receptor LRP1 (Luo and Wall *et al.* 2018). LRP1-bound Rab8a is activated to recruit the class 1B PI3K, PI3Kγ, as an effector which generates PI(3,4,5)P_3 enriched domains on surface ruffles and macropinosomes for the recruitment of the signalling kinase Akt. The LRP1/Rab8a/PI3Kγ complex enhances TLR-mediated Akt/mTOR signalling which biases the output of inflammatory cytokines towards anti-inflammatory responses. Accordingly, and in a series of subsequent papers in the literature, PI3Kγ has emerged as an important regulator for promoting M2 macrophage polarisation for the resolution of inflammation in animal models and humans (Stark and Sriskantharajah *et al.* 2015, De Henau and Rausch *et al.* 2016, Kaneda and Messer *et al.* 2016). In this context, there are several novel aspects of

Rab function, for instance, i) Rab8a is a novel GTPase for recruiting a PI3K effector, a role normally ascribed to Ras in other receptor pathways, ii) LRP1 is a novel scaffold for Rab8a and how Rab8a is activated as part of this receptor complex is not known, and finally, iii) in this novel signalling function for Rab8, the contributions of Rab8 GEFs and GAPs are unknown.

Inflammation in clinical settings is a widespread and rapidly growing problem. The recent decades have seen an increased burden of chronic diseases such as arthritis and diabetes where inflammation contributes almost universally to the underlying pathology and debilitating symptoms. The availability of specific PI3Kγ inhibitors for clinical intervention highlights the potential for drugging TLR pathways to control inflammation and macrophage polarisation in a number of diseases. Notably, PI3Kγ inhibitors are now being tested in clinical trials as a means to retain tumour associated macrophages in M1 states to enhance tumour cell killing (Kaneda and Messer *et al.* 2016). Identifying additional regulators of PI3Kγ inflammatory signalling function will therefore be beneficial to the development of new therapeutics for modulating inflammation in various diseases. As a direct activator of PI3Kγ immune signalling, targeting this particular function of Rab8a could be an alternative therapeutic for modulating this inflammatory pathway.

With a growing list of disease-causing mutations within the small GTPase family of proteins (for example the Ras oncogenes), clinical research into identifying therapeutic inhibitors for this important group of regulatory proteins is becoming increasingly important (Cook and Rossman *et al.* 2014, Simanshu and Nissley *et al.* 2017, Banworth and Li 2018). Unfortunately, biochemical and structural studies have revealed that small GTPases make challenging drug targets due to their small size, globular structure, and lack of well-defined binding pockets (Hong and Guo *et al.* 2015). In saying this, several small GTPase inhibitors have been proven to be able to compete and interfere with their activity, either through interfering with the nucleotide binding pocket (preventing GTP or GDP loading) or inhibiting protein-protein interactions with effectors or regulatory proteins such as GEFs and GAPs (Gao and Dickerson *et al.* 2004, Agola and Hong *et al.* 2012). In particular, in-vitro work by Waldmann and colleagues generated a stapled helical peptide, stRIP3, that could

compete and interfere with the interactions between Rab8 effector OCRL for binding to active-Rab8a (Spiegel and Cromm *et al.* 2014). While these molecular inhibitors have been demonstrated to be important molecular tools for interrogating the functions and characteristics of individual GTPase pathways, a majority of these studies have been limited to in-vitro experiments and the therapeutic efficacy has not yet been tested. Furthermore, being one of the more ubiquitous and multi-functional of the Rabs, direct inhibition of Rab8 itself might have wide ranging implications that could lead to unwanted side-effects.

As an alternative, the regulatory GEFs and GAPs of GTPases are often viewed as more attractive drug targets for manipulating the activity and function of a specific small GTPase for several reasons. From a structural standpoint, GEFs and GAPs are much larger and often have multiple functional domains with deeper, more defined binding pockets, making them easier targets for drug design (Cherfils and Chardin 1999). Indeed, preliminary data from our lab implicating AS160 as a GAP for Rab8 in the TLR pathway, is one example (A. Wall unpublished data). Targeting GEFs, kinases, expanding and identifying more components of this immune signalling pathway is warranted to allow greater insights into other potential targets for modulating inflammation.

Therefore, the key question I set out to address in this project is to find out how Rab8a is activated and regulated on the dorsal ruffles and macropinosome membranes, downstream of TLRs, where it functions in signalling pathways. Answering this question compels us to identify and characterise the upstream GEF(s) responsible for activating Rab8a and nucleotide exchange in macrophages. As the project evolved, it came to focus on two known Rab8a regulators, the GEFs Rabin8 and GRAB, exploring their roles in TLG signalling and examining upstream kinases and regulators for these GEFs.

Project Hypothesis

Preceding studies demonstrate that LPS and other TLR agonists activate Rab8a for the recruitment of PI3Kγ as an effector to modulate Akt/mTOR signalling and selective cytokine transcription (Luo and Wall *et al.* 2014). I propose that Rab8 guanine

nucleotide exchange factors (GEFs) will be required to facilitate GDP-GTP exchange on Rab8a to activate the GTPase for this role. Based on the typical roles of GEFs in other pathways, a Rab8a GEF in this instance could facilitate GDP exchange, direct membrane recruitment of Rab8a and control the selective recruitment of the effector PI3Kγ. Furthermore, a GEF would require additional interacting proteins to participate in either scaffolding or recruiting to the relevant membrane domains on ruffles and/or macropinosomes. It is therefore likely that one or more regulatory proteins are involved in recruiting the GEF(s) to TLR signalling membrane domains. Studies on Rab cascades also lead us to hypothesise that these accessory proteins could be effectors of an upstream Rab.

To elucidate answers to these questions, this project will be spilt into three distinct aims that are described below.

AIM 1

Identify the candidate GEF(s) for Rab8a activation in TLR-activated macrophages.

The first aim of this project will be to develop and utilise biochemical assays (protein pull-down, immunoprecipitation and Rab activation assays), novel knockout macrophage cell lines (CRISPR-Cas9) and live cell imaging to identify potential Rab8a GEFs in LPS-activated mouse macrophages.

AIM 2

Examine the functional role of GEF(s) in Rab8-mediated TLR-signalling

Utilising a combination of biochemical assays, fluorescence microscopy and gene manipulation, I will assess the phenotypic changes in Rab8 activation and Rab8a-associated TLR signalling in the absence of candidate GEFs, eg Rabin8 and/or GRAB, in LPS-treated macrophages, to determine which GEF is involved in regulating Rab8a-associated TLR inflammatory signalling pathways.

AIM 3

Investigate upstream regulators of Rab8a GEFs in TLR signalling

Using pull-downs and mass spectrometry, I seek to identify possible binding partners and regulators for the Rab8 GEF responsible for activating Rab8a (identified in Aim 2) in TLR-signalling pathways. Additionally, both candidate Rab8 GEFs, GRAB and Rabin8 have several regulatory kinases tied to their GEF activity. Using known inhibitors of these kinases and measuring the effects on Rab8 activation through the activation assay established in the previous aims, I can possibly identify novel kinases that modulate either Rabin8 or GRAB in TLR-activated macrophages.

Chapter 3

Materials and Methods

3. Materials and Methods

Plasmids

Microscopy

For generating the GFP-Rabin8 and GFP-GRAB plasmid, full-length mouse GRAB and Rabin8 were PCR amplified from mouse RAW 264.7 cell cDNA with the primers listed in Table 3.1. The respective genes were later inserted into a pEF6-GFP-C1 backbone using BamHI/NotI and SpeI/NotI respectively. pEGFP-C1 vector (Clonetech) with full-length mouse Rab5, Rab8a and Rab13 were generously provided by Prof M. Fukuda (Tohoku University). The Rab8a construct was later sub-cloned into ptd-Tomato-C1 as described in previous work (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). Full-length TRAM was cloned from RAW 264.7 cDNA into the Clontech vector pEGFP-N1 using BamHI/HindIII (Wall and Luo *et al.* 2017). The TagRFP-T-Akt1 construct was generously provided by James Burchfield (University of Sydney) (Norris and Yang *et al.* 2017).

Rab8 activation assay

For the Rab8 activation assays, the GST-OCRL-RBD construct was generated by sub-cloning a codon-optimised human OCRL-RBD (residues 539-901) into pGEX-6p-1 and expressed in E. coli BL21(DE3) bound to GSH-Sepharose beads and was used in previous studies (Wall and Luo *et al.* 2017). The original truncated codon-optimised OCRL-RBD construct (His6-TEV-OCRL, 539-901) was generously provided by Itzen and colleagues and used as the template for the activation probe (Hagemann and Hou *et al.* 2012). The GST-PI3Kγ-RBD (residues 1-356) was subcloned into pGEX-6P-1 from mouse cDNA obtained from the Facility for Life Science Automation at the Institute for Molecular Bioscience. Both these GST constructs (OCRL-RBD and PI3Kγ-RBD) used in this study was generated by my supervisor Lin Luo. For the GST-MICAL-L2-RBD probe, I PCR amplified the MICAL-L2-RBD from a FRET Rab13 biosensor developed by the McPherson laboratory (Montreal Neurological Institute) using the primers listed in table 3.1 (Ioannou and Bell *et al.* 2015). The amplified MICAL-L2-RBD was inserted into a pGEX-6P-1 vector via restriction enzyme digestion by BamHI/EcoRI.

Protein-protein interaction

For determining the direct protein-protein interactions between Rabin8 and OCRL, I generated a His-OCRL plasmid by subcloning the OCRL-RBD from the GST-OCRL-RBD construct (see Rab8 activation assay construct above) into a pOpine-His vector (addgene plasmid #26043). Using the primers listed in table 3.1, I PCR amplified the OCRL-RBD (residues 539-901) and inserted it into a pOpine-His vector with KpnI/HindIII. This construct was used in conjunction with GST-Rabin8 constructs (human Rabin8 WT, phospho-mimic [serine to aspartic acid] and phospho-deficient [serine to alanine] mutants in a pGEX-6P-1 vector), generously provided by Wei Guo and colleagues (Wang and Ren *et al.* 2015). The mutations were performed on serine residues 16/19/247/250.

CRISPR-Cas9

Several constructs were made for performing CRISPR knockout of GRAB and Rabin8. The first was cloning Rabin8 and GRAB guideRNA sequences into the Cas9 mammalian expression plasmid px458 [Addgene plasmid #48138, by (Ran and Hsu *et al.* 2013)] using primers ordered from IDT (Table 3.3). Sequences used were based on mouse gDNA information on the NCBI database and primer oligos targeting PAM CRISPR was designed as described by Ran et al 2013 with the help of an online CRISPR design tool by the Zhang laboratory (crispr.mit.edu, Broad Institute, MIT, USA).

Donor selection plasmids were made by PCR amplifying homologous regions (primers in Table 3.3) from RAW 264.7 genomic DNA that flank the target mutation site. The PCR products were then run on 1% agarose with 0.5 µg/ml ethidium bromide and gel purified using QIAquick Gel Extraction Kit (Qiagen, Germany). The purified 'arms' were digested with restriction enzymes per NEB digestion protocol (10X Cutsmart buffer for 1 hr at 37ºC). For GRAB, the enzymes HindIII/MluI was used for the left homologous 'arm' and SalI/NheI for the right homologous 'arm', as for Rabin8, MluI and BamHI was used for the left 'arm' and NheI/SalI was used for the right 'arm'. The purified 'arms' are then cloned into the selection vector pFloxNeoFlox (designed by Adam Wall) via the Gibson Assembly® Reaction (NEB, United States), the donor vectors were then digested via the same restriction enzymes to check for the correct

insertion of the homologous 'arms'. CRISPR selection resistance plasmid pFloxNeo-DTA was designed by Adam Wall (IMB, University of Queensland) (Wall and Luo *et al.* 2017).

Table 3.1. List of primers for the generation of various constructs used in this project

Primer Name	Sequence (5' – 3')	Construct
mRabin8 F	AATTACTAGTATGGCTAACGACCCCTTG	pEF6-EGFP-Rabin8
mRabin8 R	AATTGCGGCCGCCGAGTTCCTCTTTGAAATA	
mGRAB F	AATTGGATCCATGGAGATCCGAGAGAAG	pEF6-EGFP-GRAB
mGRAB R	AATTGCGGCCGCCGGCCTCCTGGGGGAAGAA	
hMICAL-L2-RBD F	AATTGGATCCGAGGAGCAGCAGCTGGAC	pGEX-6P-1-MICAL-L2-RBD
hMICAL-L2-RBD R	AATTGAATTCCTACTGGGAGGGGCTGCT	
His-OCRL-RBD F	AATTGGTACCATGGCTAATGATCCCTTG	pOpine-mod-His-OCRL
His-OCRL-RBD F	AATTAAGCTTTCAATCTTCTTCTGAACC	

Table 3.2. List of plasmids used in this project

Plasmid Name	Use	Source
td-Tomato-Rab8a	Live-cell/Fixed-cell Imaging	Adam Wall
EGFP-Rab8a	Live-cell/Fixed-cell Imaging	Mitsunori Fukuda
pEGFP-N1-TRAM	Live-cell Imaging	Adam Wall
pGEX-6P-1-Rab8a	Pull-down and mass spectrometry	Lin Luo
pEF6-GFP-C1-Rabin8	Live-cell/Fixed-cell Imaging	Generated in this project
pEF6-GFP-C1-GRAB	Live-cell/Fixed-cell Imaging	Generated in this project
pGEX-6P-1-OCRL-RBD	Pull-down/Rab8 activation	Lin Luo
pGEX-6P-1-PI3Kγ-RBD	Pull-down/Rab8 activation	Lin Luo
pGEX-6P-1-MICAL-L2-RBD	Pull-down/Rab8 activation	Generated in this project
pOpine-mod-His-OCRL-RBD	Pull-down	Generated in this project

Protein Expression

Recombinant GST-tagged proteins were produced by cloning genes of interest into a bacterial expression plasmid pGEX-6P-1 and expressed in *E. coli* BL21(DE3) via induction using 0.5 mM IPTG for 18 hr at 18ºC with constant agitation. The bacteria cells are then pelleted via centrifugation at 5, 000g and resuspended in ice-cold STE buffer (10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF). The cells were later lysed using a Constant Systems cell disruptor according to the manufacturer's instructions (Thermo Scientific, Australia). Bacterial lysates were centrifuged at 20, 000 rpm and the proteins were then applied to Glutathione Sepharose beads (GE Healthcare Life Science, Australia) for affinity purification. The beads were washed with buffer (1 M NaCl, 20 mM Tris pH 7.4) and stored in 20mM Tris pH 7.4 with 20% glycerol. His-tagged OCRL proteins were expressed similarly with different expression vectors (pET19-OCRL).

Cell culture and transfection

The mouse macrophage cell line RAW 264.7 (obtained from ATCC) was the primary cell-line used in this study. Cells were maintained in complete RPMI medium (Lonza, Australia) supplemented with 10% heat-inactivated Fetal calf serum (Thermo Trace, Australia) and 2 mM L-glutamine (Invitrogen) at 37ºC in humidified 5% CO_2. Primary mouse bone marrow-derived macrophages (BMMs) were differentiated from femur bone marrow cells for 7 days in RPMI medium supplemented with 10% heat-inactivated Fetal calf serum (Thermo Trace, Australia), 2 mM L-glutamine (Invitrogen), 20 unit/ml penicillin, 20 µg/ml streptomycin 100 ng/ml macrophage colony-stimulating factor-1 (CSF-1). For experimental procedures, all cells were treated with 10 ng/ml LPS unless stated otherwise. BMMs from TLR4$^{-/-}$ mice were kindly provided by Matt Sweet (IMB, The University of Queensland) (Hoshino and Takeuchi *et al.* 1999).

RAW 264.7 macrophages were transfected using Lipofectamine 2000™ (ThermoFisher Scientific, San Diego, CA) as per manufacturer's instruction. Cells were seeded either on 25mm coverslips or Mattek 35mm glass bottom dishes (Mattek Cooporation) at a density of $0.1x10^6$ cells/ml and incubated with transfection complexes for 2-4 hr before changing the medium, finally, cells were incubated overnight and then used for experiments.

Rab8 activation assay and immunoprecipitation

The Rab8 activation assay, based on capture and pull-down of GTP-Rab8a with GST-OCRL-RBD bound GSH-Sepharose resin, has been described previously (Wall and Luo *et al.* 2017). Briefly, cells +/- LPS (100 ng/ml, 15 min) were lysed in ice-cold lysis buffer A [25 mM TRIS pH 7.4, 150 mM NaCl, 5mM $MgCl_2$, 1% NP-40, PhosSTOP™ (Roche Applied Science, Switzerland), EDTA-free cOmplete Mini protease inhibitor (Sigma-Aldrich, Australia) and 5% glycerol], and centrifuged at 14,000 × g for 15 min. Lysates were applied to 30µl of 50% GSH-Sepharose bead slurry with bound with either GST-OCRL-RBD or GST-PI3Kγ-RasBD to capture active, GTP-loaded Rab8. Binding was performed in microspin columns (GE Healthcare) for 1 hr at 4ºC with constant agitation. For the loading calibration assay, cell lysates were applied to GSH-Sepharose resin and treated with lysis buffer containing either Gpp(NH)p (Sigma-Aldrich) at the described concentrations or 1 mM GDP (Sigma-Aldrich) for 15 min at

room temperature with constant agitation. The nucleotide exchange reaction was terminated by placing the sample on ice and adding 32 mL of 1 M MgCl2 (for a final concentration of 60 mM). The resin was later washed multiple times with ice-cold lysis buffer and bound proteins eluted by boiling at 95ºC for 5 min in 2X SDS-PAGE sample buffer (1M Tris pH7.4, 20% glycerol, 6mM EGTA, 2.5% SDS, 6% β-Mercaptoethanol). Eluted samples were analysed by immunoblotting. For quantification, experiments were performed independently in triplicates and due to the variations in basal GTP-Rab8 captured between separate control cell replicates (see for example control time 0 in Figure 5.5 for both representative blots), band intensities for individual repeat experiments were set relative to the amount of GTP-Rab8 captured in untreated control cell extracts (control time 0 or basal resting levels of GTP-Rab8 is set at 1) to correct for this variation. Therefore, the amount of GTP-Rab8 measured in each sample is set relative to the basal levels of GTP-Rab8 in control cells, and these relative changes were later used for statistical analysis.

For immunoprecipitations, LPS treated cells were lysed by passaging through a 27-gauge needles in ice-cold lysis buffer B [25mM TRIS pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 1% NP-40, 1% Triton X-100 (Sigma-Aldrich), PhosSTOP™ (Roche Applied Science, Switzerland), EDTA-free cOmplete Mini protease inhibitor (Sigma-Aldrich, Australia) and 5% glycerol], and centrifuged at 14,000 × g for 15 min. A small sample of cleared cell lysate was saved as input while the rest was mixed with antibody bound Pierce™ Protein G beads (ThermoFisher, Australia) for 1 hr at 4°C with constant agitation. The bound proteins were eluted from beads as described above, separated on 10% SDS-PAGE gels and analysed by immunoblotting. Pierce BCA Protein Assay Kits (#23225) were used to quantify total protein in cell lysates, according to the manufacturer's instructions.

Mass spectrometry

LPS treated immortalised bone marrow-derived mouse macrophages were lysed as above (Immunoprecipitation, lysis buffer B) and the lysates applied to GSH-Sepharose resin with bound GST-Rab8a or GST-Rabin8 (WT, phosphomimic and phosphodeficient mutants). The bound proteins were eluted using a single-step protease cleavage protocol using a commercially available PreScission protease

purification samples were analysed by mass spectrometry using a LC MS/MS with a Shimadzu Prominence Nano HPLC (Japan) coupled to a Triple TOF 5600 mass spectrometer (ABSCIEX, Canada) equipped with a nano electrospray ion source at IMB, The University of Queensland as described previously (Luo and Bokil *et al.* 2017). Protein identification was performed via database searching using ProteinPilot v4.5 (ABSCIEX, Canada) against the UniProt_Sprot_20130205 database (B106,000 entries of all species searched, FDR of 1%).

Immunoblotting

Cell lysates were fractionated using 10% SDS-PAGE gels and transferred onto to polyvinylidene difluoride (PVDF) membranes (Merck Immobilon-P®) using wet transfer apparatus (Bio-Rad). All protein gels were ran using a 1 X running buffer (25mM Tris, 192mM glycine and 1% Sodium Dodecyl Sulfate [SDS]) for ~1 hr at 120 volts. Western transfers were performed using a 1 X transfer buffer (25mM Tris, 192mM glycine and 20% methanol) at 30 volts overnight at 4°C (current maintained at 30mA).

The membranes were blocked in 5% skim milk in 1 X TBST buffer (20mM Tris, 150mM NaCl and 0.1% TWEEN® [Sigma Aldrich, #P1379], pH 7.4) for ~1 hr at room temperature and blotted with the appropriate antibodies overnight at 4°C with constant agitation. The membranes were wash three times with TBST and blocked with appropriate secondary antibody (in 5% skim milk in TBST) for ~1hr at room temperature. The membranes were washed three times in TBST, and results were visualized with an ECL kit (SuperSignal™ West Pico) and X-ray films (FujiFilm Super RX). Antibodies used are listed in the key resource table below. The specificity of commercial antibodies for Rabin8 and GRAB was verified for Western blotting and microscopy by testing on multiple samples with CRISPR KO controls.

CRISPR/Cas9-knockout (KO) of GRAB and Rabin8

CRISPR targeting guideRNAs were generated using published genome sequences (NCBI). The third exon of Rabin8 and the second exon of GRAB, which contained the start site and downstream coding regions were chosen to create a specific double-stranded break which would result in non-homologous end joining leading to a frameshift mutation, effectively creating a KO. The following primer pairs were ordered

from IDT: GRAB Forward TAATACGACTCACTATAGGCAGGCGTGACACATCCAG; GRAB Reverse TTCTAGCTCTAAAACCTGGATGTGTCACGCCTGC; Rabin8 Forward TAATACGACTCACTATAGAGAGAGAAGGGCTACGAA; Rabin8 Reverse TTCTAGCTCTAAAACTTCGTAGCCCTTCTCTCT. These primers were used to generate guideRNA sequences as per manufacturer's instructions (GeneArt™ Precision gRNA Synthesis Kit, ThermoFisher Scientific) and combined with purified *S.pyogenes* Cas9 nuclease protein (IDT) to form the genome targeting complex. The complex was co-transfected with a pFloxNeo resistance plasmid into RAW 264.7 cells using the Lipofectamine™ CRISPRMAX™ transfection reagent (ThermoFisher Scientific) and transfected cells were selected in RPMI medium with 1 mg/ml G418 (Geneticin, Invitrogen) and clonal lines were screened for loss of GRAB or Rabin8 protein expression by immunoblotting (see Figures 5.4 and 6.2).

Table 3.3. List of primers used for generating CRISPR constructs

Primer Name	**Sequence (5′ - 3′)**
px458 Rabin8 CRISPR I F	CACCGGGGCTTCGTAAACGCGAC
px458 Rabin8 CRISPR I R	AAACGTCGCGTTTACGAAGCCCC
px458 Rabin8 CRISPR II F	CACCGTACGAAGCCCATCTGTTC
px458 Rabin8 CRISPR II R	AAACGAACAGATGGGCTTCGTAC
px458 Rabin8 CRISPR III F	CACCGAGAGAGAAGGGCTACGAA
px458 Rabin8 CRISPR III R	AAACTTCGTAGCCCTTCTCTCTC
px458 GRAB CRISPR I F	CACCGCAGGCGTGACACATCCAG
px458 GRAB CRISPR I R	AAACCTGGATGTGTCACGCCTGC
px458 GRAB CRISPR II F	CACCGTCCATGGAGGAACTGCGC
px458 GRAB CRISPR II R	AAACGCGCAGTTCCTCCATGGAC
px458 GRAB CRISPR III F	CACCGCCATGGAGATCCGAGAGA
px458 GRAB CRISPR III R	AAACTCTCTCGGATCTCCATGGC
GRAB Left arm F	GAATAGGAACTTCGGTAAAGCTTATCCGATCTCCTCCCAGGAAGTGGAGG
GRAB Left arm R	CGGTATATATATATATATGTCGACATGGTTAGAAGGGCTCCGAGTTCCTA

GRAB Right arm F	CGGTATATATATATATATGTCGACATGGTTAGAAGGGCTCCGAGTTCCTA
GRAB Right arm R	AGATCTGCGATCGCAATCAATTGCTGGTTCCACAGGAGGGGTCAGGATA
Rabin8 Left arm F	TCGAGATTTAATTAAGATACGCGTGACGATCCCAGGCATTTCAACTGTGC
Rabin8 Left arm R	GAATAGGAACTTCGGTAAAGCTTATCCGATTCCGTGCTCAACCCAAAGAT
Rabin8 Right arm F	AGATCTGCGATCGCAATCAATTGCTGGTTGGTCAAAGTCTGCGGTGAGA
Rabin8 Right arm R	AGATCTGCGATCGCAATCAATTGCTGGTTGGTCAAAGTCTGCGGTGAGA

Fluorescence microscopy

For fixed cell imaging, RAW 264.7 cells were seeded on 2 mm glass coverslips at 0.1 X 10^6 cells/ml and left to adhere overnight. The cells were later transiently transfected with the appropriate constructs and left overnight to express. After which the cells were treated with/without LPS (30 min) and fixed in 4% PFA. After fixation the coverslips are then washed with PBS (3 times) and co-stained with Alexa-488 phalloidin (1:500 for 30 min). The coverslips were mounted on glass microscope slides and imaged using an upright Zeiss AxioImager equipped with Apotome2 and Axiocam 506 camera with a mercury light source. Images were captured using a 63X plan apochromat objective oil immersion lens.

For live cell imaging, RAW 264.7 macrophages were seeded onto 35 mm glass bottom MatTek dishes (MatTek Corporation, uncoated) at 0.1 X 10^6 cells/ml and left overnight to adhere. The cells were later transiently transfected with the appropriate constructs and left to express overnight. Prior to imaging, the media was changed from RPMI to Leibovitz L-15 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Thermo Trace, Australia) and 2 mM L-glutamine, which is a CO_2 independent media. The cells were treated with LPS and imaged using a Zeiss Axiovert 200 Inverted Microscope with CSU-X1 scanhead. For the two channels, GFP and RFP were sequentially imaged over 15-10 min at 5 sec intervals using a 63X LCI PlanN water immersion lens. Alternatively, single channel images were taken at 5 sec intervals for 20 min.

Membrane fractionation

RAW 264.7 cells treated with LPS (30 min) were lysed by passage through a 27-gauge needle in 500µl of fractionation buffer [20 mM HEPES pH 7.4, 10 mM KCl, 2mM $MgCl_2$, 1 mM EDTA, 1mM EGTA, 1mM DTT and EDTA-free cOmplete Mini protease inhibitor (Sigma-Aldrich, Australia)]. The extract was processed through centrifugation steps to clarify the suspension as follows: 720 x g for 5 min (nuclei pellet), 10, 000 x g for 5 min (mitochondria pellet), 100, 000 x g for 1 hr (membrane pellet) leaving a cytoplasmic supernatant. The supernatant (cytosolic fraction) was retained, while the membrane pellet was washed in 400µl of fractionation buffer by pipetting and recentrifuged at 100, 000 x g for 1 hr. Finally, the membrane pellet is resuspended in 0.1% SDS in TBS

and both cytosolic and membrane fractions were boiled in SDS-PAGE sample buffer and the samples were used for immunoblot analysis.

Image analysis software

Analysis of immunoblots and fluorescence imaging was performed using ImageJ software (version 2.0.0; NIH, USA). Adobe Photoshop CS6 was also used to crop regions of interest on images.

Statistics

Data are shown as arithmetic means ± s.e.m., unless otherwise stated. Data sets with normal distribution (assessed by Shapiro-Wilk test) were analysed with Student's t-test to directly compare one experimental variation. For multiple comparisons, Sidak's method was used for post-hoc analysis of Two-way ANOVA. All analysed experiments used biological and technical replicates (3 replicates each unless stated otherwise) to compute statistical significance. In all statistical analysis, a P value < 0.05 was considered statistically significant and calculated using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA).

Key resource table

Reagent	Source	Additional Information
Antibodies		
Mouse anti-Rab8	BD Biosciences, USA	WB 1:1000, #610845
Mouse anti-Flotillin-1	BD Biosciences, USA	WB 1:500, #610820
Rabbit anti-Rabin8 (Rab3IP)	ProteinTech, USA	WB 1:1000, #12321-1-AP
Rabbit anti-GRAB (Rab3IL1)	ProteinTech, USA	WB 1:1000, #17827-1-AP
Rabbit anti-OCRL1	ProteinTech, USA	WB 1:1000, #17695-1-AP
Rabbit anti-GAPDH	Cell Signalling Technology, USA	WB 1:4000, #14C10
Rabbit anti-pAkt (Ser473)	Cell Signalling Technology, USA	WB 1:1000, #9271
Rabbit anti-Akt	Cell Signalling Technology, USA	WB 1:1000, #9272
Rabbit anti-pERK1/2 (Thr202/Tyr204)	Cell Signalling Technology, USA	WB 1:3000, #9102
Rabbit anti-ERK1/2	Cell Signalling Technology, USA	WB 1:3000, #137F5
Rabbit anti-phospho p70S6K (Thr389)	Cell Signalling Technology, USA	WB 1:1000, #9205
Rabbit anti-phospho PRAS40 (Thr246)	Cell Signalling Technology, USA	WB 1:1000 #2997
Mouse anti-His	Cell Signalling Technology, USA	WB 1:1000, #2365
Mouse anti-Myc	Cell Signalling Technology, USA	WB 1:3000, IP 1:100, #9B11

Rabbit anti-GFP	ThermoFisher Scientific, Australia	WB 1:2000, #A6455
HRP-conjugated goat anti-rabbit IgG	ThermoFisher Scientific, Australia	WB 1:10 000, #G-21234
HRP-conjugated goat anti-mouse IgG	ThermoFisher Scientific, Australia	WB 1:5 000, #G-21040
Mouse anti-β-actin	Sigma Aldrich, Australia	WB 1:1000, #A1978
Tissue-culture		
RAW 264.7 mouse macrophage like cells derived from Abelson murine leukaemia virus-induced tumour	American Type Culture Collection	ATCC® TIB-71™
TLR4$^{-/-}$ and WT Bone Marrow-Derived Macrophages (BMMs)	Matthew Sweet, IMB, University of Queensland	Differentiated from TLR4$^{-/-}$ & paired WT mice femur bone marrow
Immortalised Bone Marrow-Derived Macrophages (BMMs)	Dr Ashley Mansell, Hudson Institute of Medical Research, Melbourne, Australia	Cells were immortalised with a J2 recombinant retrovirus (v-myc and v-raf oncogenes) (Halle and Hornung *et al.* 2008)
RPMI 1640 media	ThermoFisher Scientific, Australia	#21870
DMEM	ThermoFisher Scientific, Australia	#11995
Chemicals / Drugs		
U0126 (Erk1/2 Inhibitor)	Sigma Aldrich, Australia	#U120, used at 10μM

Gpp(NH)p/ GTP-analog	Sigma Aldrich, Australia	#G0635
GDP	Sigma Aldrich, Australia	#G7127

Chapter 4

Results 1: Identifying regulators of Rab8 activation in macrophages

4. Results 1

Introduction

In this project, I look to identify and characterise the regulatory proteins responsible for activating the small GTPase Rab8a to facilitate targeted TLR-associated immune signalling in macrophages. Previous work from the Stow laboratory has identified this GTPase to be a key modulator for controlling inflammatory responses (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). Functioning downstream of several TLRs, Rab8a was found to recruit the class IB PI3K, PI3Kγ as an effector, to facilitate inflammatory signalling and cytokine biasing to constrain inflammation (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). As a GTPase, Rab8a has to first be activated via GTP-loading to facilitate effector recruitment and downstream function. Therefore, while the inflammatory outputs of this Rab8a-PI3Kγ complex have been described, the regulatory proteins responsible for activating Rab8a in this pathway are yet to be identified. Hence, uncovering these modulators of Rab8a would expand the understanding of this important immune signalling cascade, and also reveal new targets for controlling specific PI3Kγ function in inflammation and macrophage polarisation.

Amongst the Rab family members, Rab8a is particularly multi-functional, with numerous, known effectors which act on different membranes in many cell types (Peränen 2011). Guanine nucleotide exchange factors (GEFs) (as described in the introduction chapter) typically activate Rabs by controlling the exchange of GDP for GTP in the nucleotide-binding site, which often promotes membrane association. Identifying the GEF(s) for Rab8a in macrophages is important for understanding how this GTPase is activated on specific membrane domains for TLR signalling. As mentioned earlier in the introduction, there are two well-known, structurally-similar and potent Rab8 specific GEFs: GRAB and Rabin8 (Yoshimura and Gerondopoulos *et al.* 2010, Guo and Hou *et al.* 2013, Wang and Ren *et al.* 2015). Both these GEFs have been understood to function in multiple Rab8 controlled trafficking pathways such as ciliogenesis for Rabin8 and neurite axon outgrowth for GRAB (Feng and Knödler *et al.* 2012, Furusawa and Asada *et al.* 2016). Interestingly, these Rab8 regulators might also have some functional overlap, with separate studies tying them to the same Rab8-associated neurite outgrowth pathway (Furusawa and Asada *et al.* 2016, Homma and

Fukuda 2016). However, despite their similarities, the exact roles and level of redundancy between GRAB and Rabin8 in Rab8 pathways are still not entirely known. GRAB and Rabin8 are, to date, unstudied in macrophages, or in the context of macropinosomes or TLR signalling pathways. In addition to these well-established Rab8 regulators, it is possible that there are unidentified GEFs for Rab8a. Therefore, an unbiased screening approach will be used to identify any potential GEF of Rab8a in these immune cells.

In this chapter, an investigation is launched to identify the regulatory GEF(s) for Rab8a in mouse macrophages. Using an unbiased protein affinity pull-down screen followed by mass spectrometry analysis, I identified several known Rab8a binding partners in TLR-activated macrophage lysates, that led to the detection of the Rab8 GEF GRAB. A combination of biochemical analysis with fluorescence microscopy was later used to confirm the interactions between Rab8a, GRAB and the closely related GEF Rabin8 in these immune cells. The results presented here formed part of a publication identifying the regulatory proteins that activate Rab8 for biasing TLR-signalling in macrophages (Tong and Wall *et al.* 2019).

Measuring Rab activation, namely the exchange of GDP for GTP, is fundamental to studies on GEFs. A principle readout for assessing GEF activity will be the quantity and rate of endogenous Rab8 GTP-loading. To date, there have been several methods used for measuring Rab GTPase activation, most were developed for measuring GTP loading and kinetics on purified proteins. Therefore, it is necessary for this project to develop a method for measuring the relative activation of Rab8a in cells, by comparing GTP-Rab8a levels under different conditions and in the presence or absence of putative GEFs. Therefore, part of this chapter describes the studies performed to establish a Rab8a activation assay. Thus, I set out to use the known Rab8 binding domains of three Rab8-binding effectors namely, MICAL-L2, PI3Kγ and OCRL, as probes to capture GTP-Rab8 in cell extracts and the results of these assays are described here.

Results

Characterisation of Rab8a in mouse macrophages

Recombinant Rab8a localisation in mouse macrophages

To establish the localisation and function of Rab8a as a subject of the current study, tdTomato-Rab8a was transiently transfected into RAW 264.7 macrophages, which were then activated by incubation with LPS prior to fixation. The cells were stained with fluorescent phalloidin to show actin-rich ruffles. In Figure 4.1A, tdTomato-Rab8a is found in a perinuclear location (consistent with its other roles in trafficking) and prominently on actin-rich ruffles at the cell surface. Some of these ruffles circularise consistent with ruffle closure to form macropinosomes (highlighted in insets). Rab8a is typically found on recycling membranes within cells in addition to its presence on the ruffles that are prominent on activated macrophages (Wall and Condon *et al.* 2018). Hence, despite the lack of antibodies available for staining endogenous Rab8a, but in many previous studies these recombinant Rab8a are believed to accurately reflect the subcellular distribution of this Rab in previous studies (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017, Eguchi and Kuwahara *et al.* 2018).

Next, GFP-Rab8a was transfected into RAW 264.7 cells for live cell imaging after LPS activation. This imaging confirmed the recruitment and enrichment of GFP-Rab8a to macropinosomes that derive from Rab8a containing ruffles (Figure 4.1B). Rab8a is tied to TLR signalling pathways through colocalisation with components of this pathway on ruffles and macropinosomes (Luo and Wall *et al.* 2014). Localising the TLR4 receptor itself is very difficult, but here I co-transfected fluorescently tagged Rab8a with the TLR4 adaptor TRAM. Live cell confocal imaging of LPS-treated RAW 264.7 cells co-expressing td-Tomato-Rab8a and the TLR adaptor GFP-TRAM showed that both proteins are enriched on the same macropinosome membranes, and therefore are available for joint immune signalling functions (Figure 4.1C).

This imaging serves to demonstrate the location of Rab8a on ruffles and newly formed macropinosomes in TLR-activated macrophages. These results were consistent with previous findings from our laboratory which have gone on to show that Rab8a on these membranes is engaged for regulation of signalling downstream of TLRs (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017).

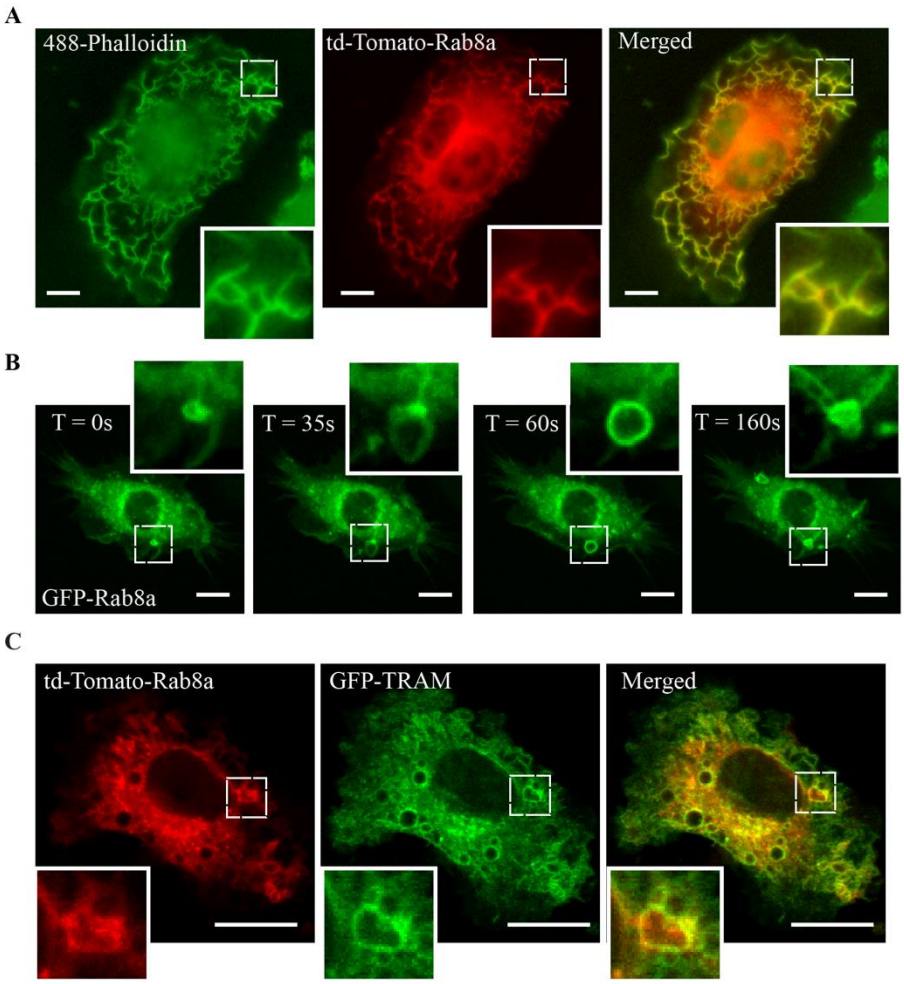


Figure 4.1 Rab8a localises to ruffles and is enriched on macropinosomes in LPS stimulated mouse macrophages. (A) Fluorescence microscopy images of fixed, LPS-treated (30 mins) RAW 264.7 cells transiently overexpressing td-Tomato-Rab8a and stained with Alexa488-phalloidin. (B) Live cell confocal spinning disc images of LPS-treated RAW 264.7 cells transiently transfected with GFP-Rab8a showing enrichment of Rab8a on macropinosomes and subsequent internalisation via tubules. (C) Live cell confocal image of RAW 264.7 macrophage cells transiently co-expressing GFP-TRAM and td-Tomato-Rab8a showing colocalization both constructs on a macropinosome. Movies were taken over 15 mins at 5 sec intervals. Scale bars, 10 µm.

Rab8a with its effector PI3Kγ functions to elicit Akt signalling downstream of TLRs

The class 1B PI3K, PI3Kγ, was earlier identified as a novel effector for Rab8a and as a modulator of Akt signalling downstream of TLR4 (Luo and Wall *et al.* 2014). This earlier study also demonstrated that knockdown of Rab8a or KO of PI3Kγ only affected Akt phosphorylation and not total Akt protein levels (Luo and Wall *et al.* 2014). In collaborative studies with other members of the laboratory, my experiments contributed to characterise the recruitment and function of Rab8a in response to other

members of TLRs. This immune signalling function is demonstrated here in LPS stimulated RAW 264.7 macrophage cells after CRISPR deletion of Rab8a [Figure 4.2A and Figure 4A in (Wall and Luo *et al.* 2017)]. This representative gel shows the selective loss of Rab8a protein in the Rab8a KO cells which is accompanied by reduction of LPS-induced phospho-Akt and no reduction of phospho-ERK1/2. This result implies that Rab8a is required for robust Akt signalling downstream of TLR4.

These experiments were then repeated in primary bone marrow derived macrophages (BMMs) from WT mice and PI3K$\gamma^{-/-}$ mice (Figure 4.2B). The loss of PI3Kγ resulted in a marked reduction of LPS-induced phospho-Akt, a modest reduction in phospho-mTOR and no reduction in phospho-ERK1/2. This result recapitulates the Akt signalling changes resulting from the loss of Rab8a, serving as one line of evidence that Rab8a and PI3Kγ jointly modulate Akt signalling downstream of TLR4 [Figures 5A and B from (Wall and Luo *et al.* 2017)]. These experiments also highlight a TLR-signalling role of Rab8a that can be used to assay for Rab8a activation and function in macrophages.

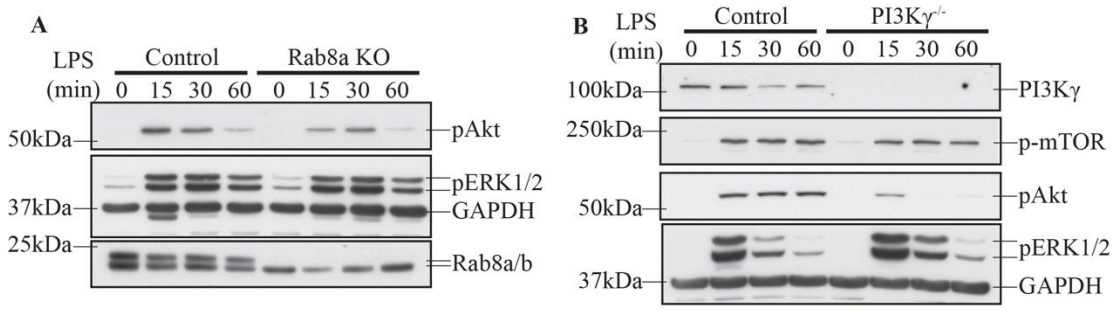


Figure 4.2. Rab8a with its effector PI3Kγ functions to elicit Akt signalling downstream of TLRs. *Immunoblots showing the levels of Akt and ERK1/2 phosphorylation using phospho Akt (Ser473) and phospho ERK1/2 (Thr202/Tyr204) specific antibodies on the lysates of (A) control and Rab8a KO RAW 264.7 cells or (B) control and PI3Kγ-/- BMMs, treated with LPS over a 60 min time course. These results were published in an article in JBC during this project (Wall and Luo et al. 2017). Experiments for Figure A was performed by Dr Adam Wall (IMB, University of Queensland), while I conducted the experiments for Figure B.*

The lipoprotein receptor LRP1 is involved in TLR-induced Akt activation.

After demonstrating that this Rab8a-PI3Kγ complex controls Akt phosphorylation as a subset of the TLR-signalling response, we turned our attention to understanding how the Rab8a complex is recruited for signalling from these immune receptors. Since

neither Rab8a nor PI3Kγ directly interact with TLRs, further studies were undertaken to identify how Rab8a is recruited for signalling on the ruffle-macropinosome membranes. The lipoprotein receptor LRP1 was subsequently identified as a crosstalk receptor that functions to scaffold and recruit Rab8a to TLRs upon activation, allowing the GTPase to be activated and in turn enlist its effector PI3Kγ to elicit Akt signalling (Luo and Wall *et al.* 2018). During the process of identifying this crosstalk receptor, RAW 264.7 cells CRISPR deleted for LRP1 (generated by Dr Adam Wall, IMB, University of Queensland) were made available for signalling studies performed by me as a collaboration. A loss of LRP1 resulted in decreased LPS-induced Akt phosphorylation compared to control cells without affecting the levels of phospho-ERK1/2 [Figure 4.3 and Figure 6A in (Luo and Wall *et al.* 2018)], recapitulating the effects of depleting Rab8a or PI3Kγ shown above.

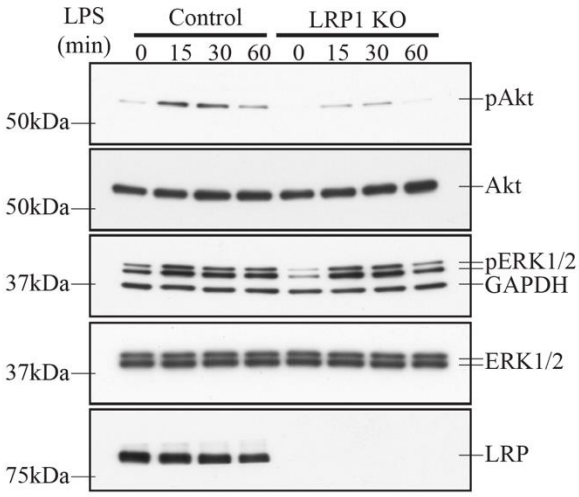


Figure 4.3. The lipoprotein receptor LRP1 is involved in TLR-induced Akt activation. *Immunoblot showing the levels of Akt (Ser473) and ERK1/2 (Thr202/Tyr204) phosphorylation using phospho-specific antibodies on the lysates of control and LRP1 CRISPR KO RAW 264.7 macrophage cells treated with LPS over a 60 min time course. Blots of total LRP1 (bottom-most blot) confirmed a loss of protein expression in the KO cell line, and total Akt, ERK1/2 and GAPDH were included as loading controls. This result was later used in a published article in Cell Reports (Luo and Wall et al. 2018).*

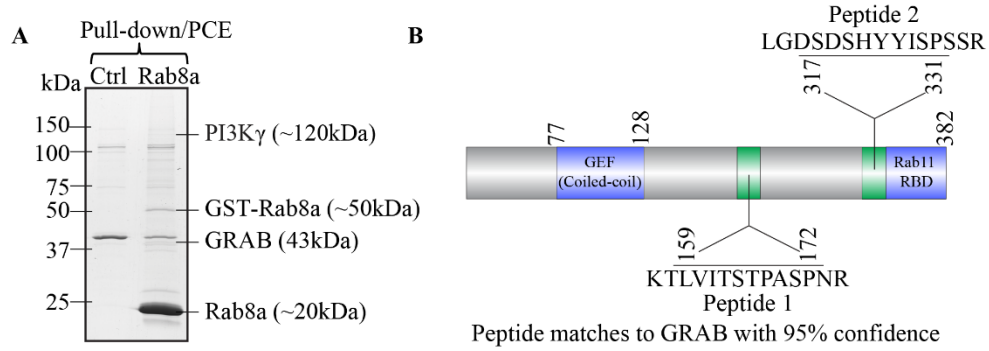
Taken together, these results demonstrate that Rab8a is on macrophage ruffles and macropinosomes where it is recruited by LRP1 and engages in Akt signalling downstream of TLR4. The unknown component of this complex is the GEF that activates Rab8a for this role; accordingly my project turned to the search for the activators and regulators of Rab8a.

An unbiased screen of Rab8 interacting partners in TLR4-activated macrophages

Rab GTPases are molecular switches that flip between two states, an 'on' GTP-bound state and an 'off' GDP-bound state, which are facilitated by their specific GEFs and GAPs respectively. In seeking to identify a Rab8a GEF responsible for activating Rab8a downstream of TLRs, I followed the strategy that successfully identified PI3Kγ and LRP1 as members of this Rab8a complex (Luo and Wall *et al.* 2014), namely to perform an unbiased screen for Rab8a binding partners in macrophage extracts using mass spectrometry. This approach has the potential to pull-down any known or novel Rab8a interacting GEF in these immune cells.

Immortalised bone marrow-derived macrophages (iBMMs) were used for this approach, offering the chance to use cells more closely resembling primary cells and still acquire large cell volumes required. Cells were grown to confluence in 15cm tissue culture dishes, incubated with LPS (30mins) and cell lysates were prepared. The lysates were applied to columns containing either glutathione Sepharose beads bound with GST alone (GST control) or beads bound with bacterially expressed GST-tagged full-length mouse codon optimised Rab8a (codon optimisation tool, IDT). The samples were later eluted using a single-step protease cleavage elution (PCE) technique with PreScission proteases (GE healthcare). This technique, previously published by the laboratory, uses proteases to perform targeted retrieval of the bait protein and any genuine binding partners, reducing the presence of unspecific contaminant proteins in the resulting eluent (Luo and King *et al.* 2014). The final eluates were separated on a gradient gel (Figure 4.4A) and a number of excised bands were analysed by mass spectrometry (IMB core facility). Results from the mass spectrometry identified many proteins, including several known Rab8a binding partners indicating that successful pull-downs had occurred. Among these pull-down proteins, several known influencers of Rab8 activity were identified including the effector PI3Kγ (Wall and Luo *et al.* 2017), the Rab GDP dissociation inhibitors (GDIs) alpha and beta (Ullrich and Stenmark *et al.* 1993, Shisheva and Chinni *et al.* 1999), the Rab stabilising chaperone MSS4 (Itzen and Pylypenko *et al.* 2006, Zhang and Zhang *et al.* 2015) and, in addition, the known Rab8 regulatory GEF, GRAB (Figures 4.4B and C) (Itzen and Pylypenko *et al.* 2006, Guo and Hou *et al.* 2013, Furusawa and Asada *et al.* 2016). Each of these proteins had at least one trypsin-digested peptide that were identified by the mass spectrometry

analysis with a confidence of 95% (Figure 4.4C), providing assurance that they are genuine binding partners. Importantly, the screen identified 2 peptides of the Rab8 GEF GRAB (depicted in Figure 4.4B), leading to a ProteinPilot score of 4 which equates to a protein identification confidence of 99.99% (Figure 4.4C). The identification of GRAB as a putative Rab8a binding partner is a novel finding as this GEF has not been empirically or functionally studied before in this cell type. With a candidate GEF now identified, the next step was to confirm this interaction with Rab8a and investigate the possible roles of GRAB in Rab8a macrophage function.



C

Protein name	Uniprot ID	Score	%Cov	%Cov(50)	%Cov(95)	Number of peptides (95%)
GRAB	Q8VDV3	4.00	7.31	7.31	7.31	2
MSS4	Q91X96	4.00	16.26	16.26	16.26	2
GDI alpha	P50396	4.00	6.71	4.47	4.47	2
GDI beta	Q61598	2.72	9.44	5.17	2.47	1
PI3K gamma	Q9JHG7	2.00	0.82	0.82	0.82	1

Figure 4.4. GRAB identified as a Rab8a binding partner in LPS activated macrophage lysate. *GSH-Sepharose beads with bound GST-Rab8a were used to pull-down proteins from LPS-treated (30 min) IBMM cell lysate and eluted with proteases. The elutes were separated on a 7-15% SDS-PAGE gradient gel. (A) A faint band between 150 kDa and 100 kDa indicating the presence of a previously identified effector PI3Kγ and another band above 37 kDa was identified as the Rab8a GEF GRAB (~43kDa), both of which were absent in the GST control sample. (B) Schematic of the two trypsin-digested peptides from GRAB identified from the mass spectrometry analysis mapped onto the peptide sequence of GRAB. (C) Sample list of known Rab8a interacting proteins identified in the mass-spectrometry analysis. The mass spectrometry 'score' is a measurement of peptide confidence from the ProteinPilot scoring algorithm: Score = -log(1-(PercentConfidence/100)). For example, a score of 2 = 99% confidence, whereas a score of 4 = 99.99% confidence. %Cov (coverage) refers to the percentage of all identified peptide(s) relative to total amino acid sequence, whereas %Cov (50%) and %Cov (95%) refer to peptide coverage with 50% and 95% confidence respectively.*

Rab8 GEFs Rabin8 and GRAB both interact with Rab8a in an LPS-inducible manner

Although not previously studied in macrophages, there is gene expression evidence showing that GRAB is present in both RAW 264.7 macrophages and mouse BMMs (BioGPS, http://biogps.org) (Lattin and Schroder *et al.* 2008, Hammer and Yum *et al.*

2010). Interestingly, data from these genome wide expression experiments also showed that the other Rab8 GEF, Rabin8, is likewise expressed in these same mouse macrophage cells. While Rabin8 was not picked up in the pull-downs or identified by the mass spectrometry analysis above, given the structural and functional similarities reported between GRAB and Rabin8, it was decided that both proteins will be studied and characterised in parallel with regards to possible roles in regulating Rab8a immune function in macrophages.

First, to affirm that both GRAB and Rabin8 are expressed in the RAW 264.7 macrophage-like cell line (the main cell line used in the project), immunoblots using the extracts of untreated RAW 264.7 cells were performed with commercially available antibodies. Results showed bands that correspond to the molecular weights of the respective proteins in each of three RAW 264.7 clonal lines (Rabin8 at 56kDa and GRAB at 43kDa), with no signal seen in the empty lane control confirming the specificity of the antibodies for these proteins (Figure 4.5A). This indicates that both proteins are indeed expressed in these macrophages, agreeing with previous mRNA expression studies (Hammer and Yum *et al.* 2010).

With both proteins present in these macrophages, co-immunoprecipitation studies were performed as an approach to both verify that GRAB binds to Rab8a and investigate if Rabin8 is likewise interacting with Rab8a in these macrophages. Attempts to use commercially available antibodies for either Rab8a or GRAB proved that neither was successful as a pull-down antibody, despite being able to detect these proteins. As an alternative approach, I stably expressed and reconstituted myc-Rab8a in a Rab8a KO cell line previously generated using CRISPR/Cas9 editing in RAW 264.7 cells (Wall and Luo *et al.* 2017). This allowed the use of the myc antibody for Rab8a immunoprecipitations. These cells (RAW 264.7 macrophages expressing myc-Rab8a) were treated with and without LPS (30 min), lysed and the resulting lysates applied to columns containing myc IgG coated Pierce™ Protein G beads. The final eluents were separated on SDS-PAGE gels for immunoblotting (Figures 4.5B and C). The results on this representative gel, show that myc-Rab8a was enriched on the beads and that it successfully co-immunoprecipitated endogenous GRAB but, notably, only in LPS-activated cells. The same samples were used to screen for Rabin8 in myc-Rab8a precipitations (Figure 4.5C) and Rabin8 was likewise co-immunoprecipitated

only in LPS-activated cells. Based on the immunoblotting of GRAB and Rabin8 in the 'input' lanes in the gels in Figures 4.5B and C, it is evident that their protein expression levels in these mouse macrophages remain unchanged after acute LPS treatment; moreover, co-immunoprecipitation of either protein with myc-Rab8a indicated that both proteins have LPS-inducible interactions with Rab8a upon macrophage activation.

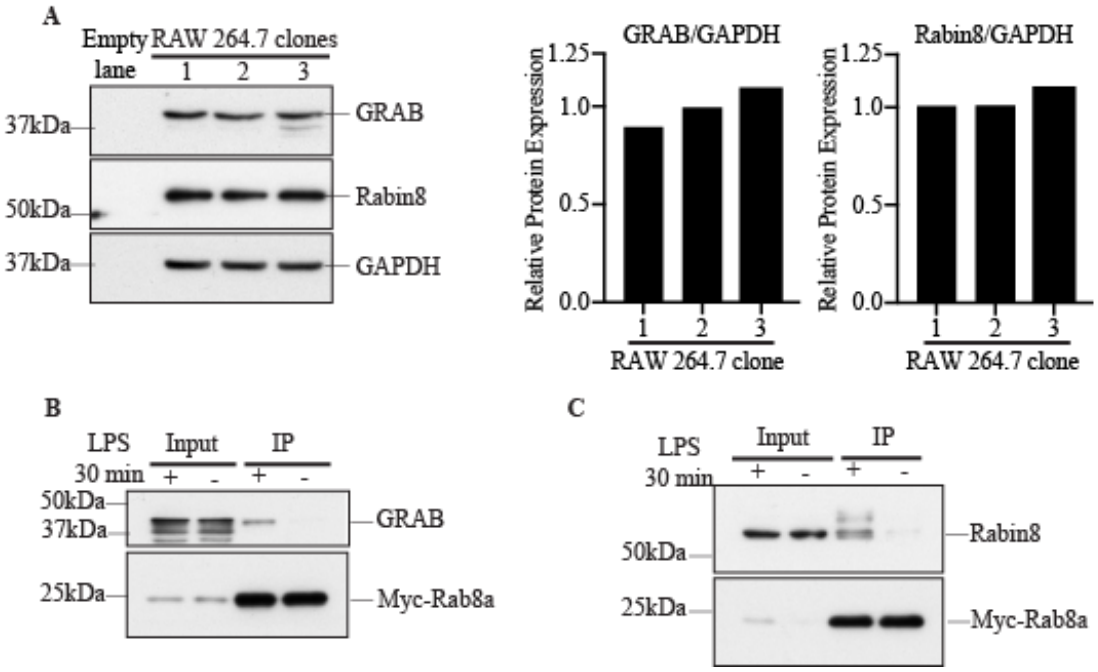


Figure 4.5. Both Rab GEFs GRAB and Rabin8 are expressed in RAW 264.7 macrophage cells and have an LPS inducible interaction with Rab8a. *(A) Immunoblots showing the endogenous expression of Rab8 GEFs Rabin8 and GRAB in RAW 264.7 macrophage cells. Blots were done using the lysates of three different untreated RAW 264.7 cell lines for assessing the relative expression of each protein. Levels of relative Rabin8 and GRAB protein expression were quantified by using the densitometric ratio between the band intensities of Rabin8 or GRAB and GAPDH. (B/C) Representative blots showing the immunoprecipitation of (B) GRAB or (C) Rabin8 using anti-myc antibodies from cell lysates of Rab8a KO cells +/- LPS (30 min) reconstituted with myc-Rab8a.*

Taken together, these results highlight two key findings: i) GRAB as well as Rabin8 are Rab8a binding partners in LPS-activated macrophages. ii) The LPS-inducible interaction of both proteins with Rab8a imply a possible TLR related function with Rab8a in these immune cells. This supports the data from the previous pull-down and mass spectrometry analysis which identified GRAB as a Rab8a binding partner in LPS treated cell extracts and additionally, shows that Rabin8 can also interact with Rab8a under similar conditions. Henceforth I investigated both GRAB and Rabin8 in experiments to determine if either of these proteins is responsible for activating Rab8a during TLR-activation in macrophages.

Localisation of Rabin8 and GRAB in mouse macrophages

It has been previously shown that Rab8a is localised and enriched on macrophage dorsal ruffles and early macropinosomes in response to immune stimuli such as LPS (Wall and Condon *et al.* 2018) (see Figure 4.1A and B). With both Rabin8 and GRAB now identified as candidate Rab8a partners, I next wanted to examine the localisation and distribution of these proteins in macrophages. Again, I found the relevant antibodies were not suitable for staining endogenous GRAB or Rabin8. Therefore, I generated GFP-tagged constructs of both GRAB and Rabin8 for expression and localisation in cells. Full-length GRAB and Rabin8 were amplified from RAW 264.7 macrophage cDNA and ligated into a pEF6-GFP-C1 plasmid (Figure 4.6A). Proper insertion of either GRAB or Rabin8 was screened and confirmed by PCR of purified plasmids (Figure 4.6B, primer sequences in Table 2.1 in Materials and Methods), and later also confirmed with sequencing. LPS treated or untreated (30 min) RAW 264.7 macrophages transiently expressing either GFP-Rabin8 or GFP-GRAB were fixed and imaged to examine their cellular distribution. Both proteins demonstrated diffuse labelling in the cytoplasm with some localisation in the cell periphery on ruffle membranes, which became more pronounced upon LPS-treatment (Figures 4.6C and D). This reveals that GRAB and Rabin are likely widespread in their intracellular distributions and that they can be recruited to membranes, including ruffle membranes of interest in this project.

To see if Rab8a is present on these same ruffle membranes, the GFP-GRAB and Rabin8 were transfected in tandem with td-Tomato-Rab8a (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). RAW 264.7 macrophages co-transfected with either GFP-GRAB or GFP-Rabin8 with td-Tomato Rab8a, were treated with LPS (30 min), fixed and co-stained with phalloidin to visualise F-actin as a cell marker. The imaging demonstrated that both GFP-GRAB and GFP-Rabin8 have similar but non-identical staining patterns when compared individually to recombinant Rab8a (Figures 4.6E and F). td-Tomato Rab8a was on perinuclear and peripheral membranes overlaid with widespread distributions of the GFP-labelled proteins. Importantly, both GRAB and Rabin8 were observed together with Rab8a, colocalised on dorsal and peripheral ruffle membranes (as highlighted in the insets). Thus, these findings show that GRAB and

Rabin8 are widespread in macrophages, reflecting a similar distribution to that of Rab8a and in keeping with the fairly ubiquitous expression of all three proteins in multiple cell types and on multiple membrane domains. The colocalisation of GRAB or Rabin8 with Rab8a supports the findings from pull-downs and coprecipitation earlier in the chapter which show binding of these proteins. Moreover, the convergence of both GRAB or Rabin8 with Rab8a suggests the potential for binding and functional interactions throughout the cell, including on the ruffles where Rab8a functions in TLR signalling.

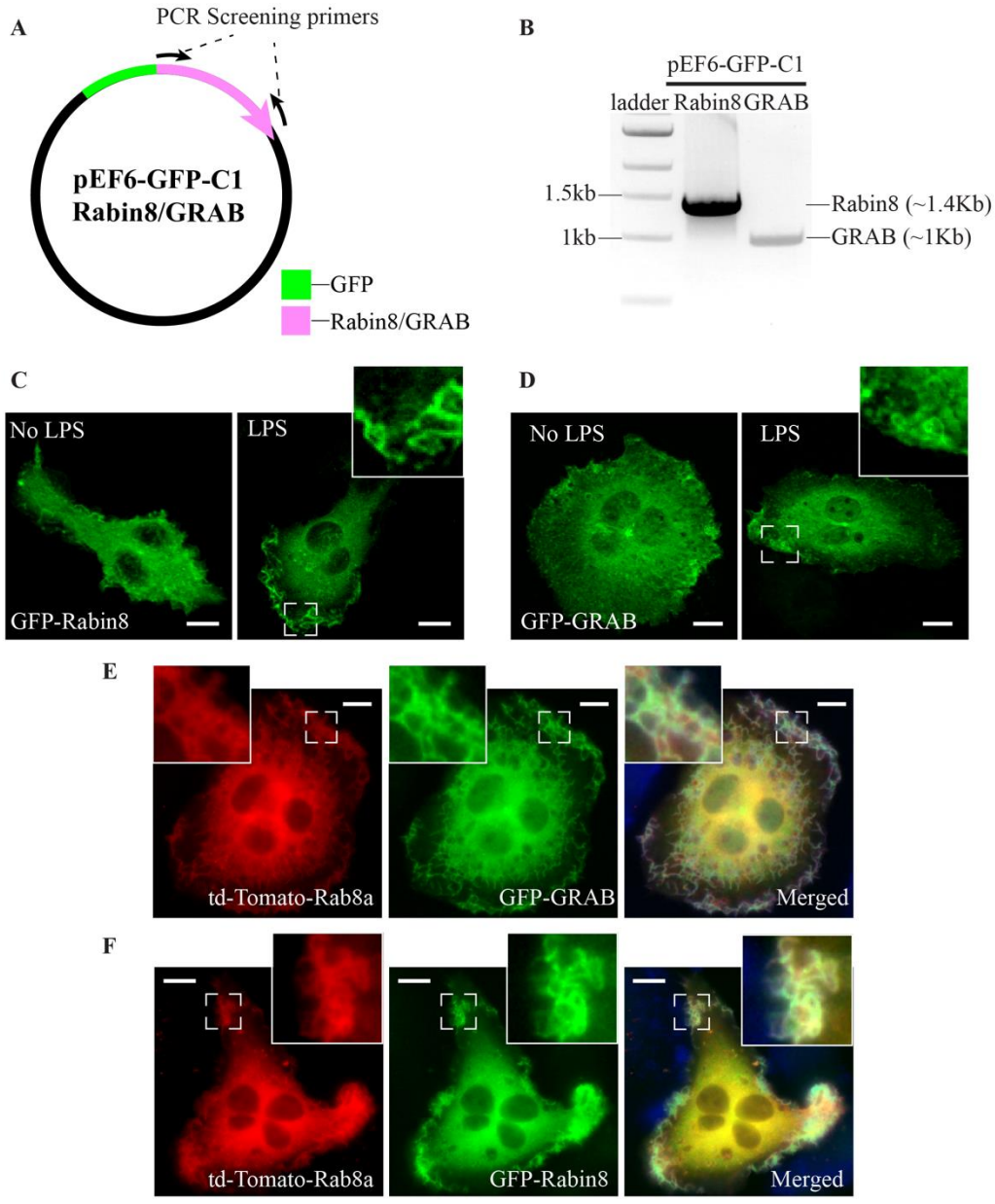


Figure 4.6. The Rab8 regulatory GEFs Rabin8 and GRAB co-localises with Rab8a on membrane ruffles in LPS-activated macrophages. *(A) Illustration of pEF6-GFP-C1 mammalian expression plasmid with either PCR amplified mouse Rabin8 or GRAB genes from RAW 264.7 macrophage cDNA. (B) Agarose gel showing the results*

of a PCR screen for either the Rabin8 or GRAB inserts in the respective constructs (PCR was performed using the screening primers depicted in [A] and listed in Table 3.1 in the Materials and Methods chapter). (C/D) Fluorescence microscopy images of fixed +/- LPS (30 min) RAW 264.7 cells transiently overexpressing either (C) GFP-Rabin8 or (D) GFP-GRAB. Magnified inserts highlight membranes and possible macropinosomes enriched with either GFP-Rabin8 or GFP-GRAB. (E/F) Fluorescence microscopy images of fixed LPS-treated RAW 264.7 cells transiently co-expressing either (E) GFP-GRAB or (F) GFP-Rabin8 with td-Tomato-Rab8a and co-stained with Alexa350-phalloidin. Magnified inserts highlight actin rich peripheral membranes displaying strong colocalisation of both td-Tomato-Rab8a and either GFP-GRAB or GFP-Rabin8 with the actin marker phalloidin. Scale bars, 10µm.

With GRAB and Rabin8 established as Rab8a binding proteins in macrophages, the next steps involve investigating Rab activation in order to assay and establish GEF function. Thus, I turned my attention to establish a robust assay for quantifying endogenous Rab8 activation and GTP-loading.

Measuring Rab8 activation

Generation of active Rab8 capture constructs

Establishing the activation of Rab GTPases on cell membranes for my experiments, requires tools and protocols that can reliably assess changes to the GTP-loading of endogenous Rabs. One approach for identifying Rabs in their GTP-bound states is to use probes or binding proteins that have unique interactions with Rabs only in their GTP-bound states. These probes (often effector binding protein domains) can be used for labelling or 'capture' of Rabs in pull-downs.

For assessing Rab8 activation in macrophages, I set out to develop Rab-GTP probes based on known Rab8 effectors with the help of my co-supervisor Dr Lin Luo (Figure 4.7A). The first probe involved the production of a GST-tagged construct using the Rab binding domain (RBD) of a known, multi-Rab effector, the inositol 5-phosphatase, oculocerebrorenal syndrome of Lowe protein 1 (OCRL) (Hagemann and Hou *et al.* 2012). The truncated $OCRL_{539-901}$ construct containing the RBD was generously provided by Hou and colleagues (Max-Plank-Institute of Molecular Physiology, Germany) and it has been previously used in structural studies characterising the interaction between Rab8 and OCRL (Hou and Hagemann *et al.* 2011). The second probe uses the RBD of another multi-Rab effector, the molecule interacting with CasL-like 2 (MICAL-L2) (Fukuda and Kanno *et al.* 2008). The known RBD of hMICAL-L2 (residues 806 – 903) was sub-cloned from a WT Rab13 biosensor generously

provided by Prof Peter McPherson (MNI, McGill University) (Ioannou and Bell *et al.* 2015). The third probe is made up of the Ras binding domain (RasBD) of PI3Kγ which we have previously identified as a Rab8a effector (Luo and Wall *et al.* 2014). The RasBD of mPI3Kγ (residues 1 – 356) was PCR amplified from mouse cDNA obtained from the facility of life science automation (IMB, University of Queensland). As validated Rab8 effectors, all three of these proteins interact specifically with only GTP-Rab8 (among other Rabs). The principle of this assay is to exploit these probes as GST-fusion proteins for pulling-down Rab8 (and other interacting, activated Rabs) in the 'active' GTP-bound conformation, allowing us to quantify the relative levels of activated endogenous Rab8 (by immunoblotting) in macrophage extracts (Figure 4.7C).

All three domains were GST-tagged through inserting them into a pGEX-6P1 vector, the fusion proteins were bacterially expressed and bound to glutathione Sepharose beads. Samples were taken from each construct, boiled, separated on SDS-PAGE gels and the relative expressions were analysed via Coomassie staining. While both the PI3Kγ and OCRL constructs were able to express relatively efficiently and cleanly, the production of GST-MICAL-L2-RBD was always accompanied by a large contamination of GST (Figure 4.7B). This GST contamination was initially thought to be a result of an impure bacteria colony with a portion of the cells expressing the empty vector. However, this GST contamination persisted even after re-streaking and testing single pure colonies from the original bacteria culture, indicating perhaps inefficient translation or premature cleavage of the GST tag during expression (Figure 4.7B, left gel col 1 and 2). With GST having a similar molecular weight to Rab8 (~22kDa), the shear amount of GST present impaired Western blotting, I believe as a result of artificially trapping the antibodies or masking the epitopes of the target Rab. Therefore, I took additional approaches in an attempt to remove the excess free GST before proceeding.

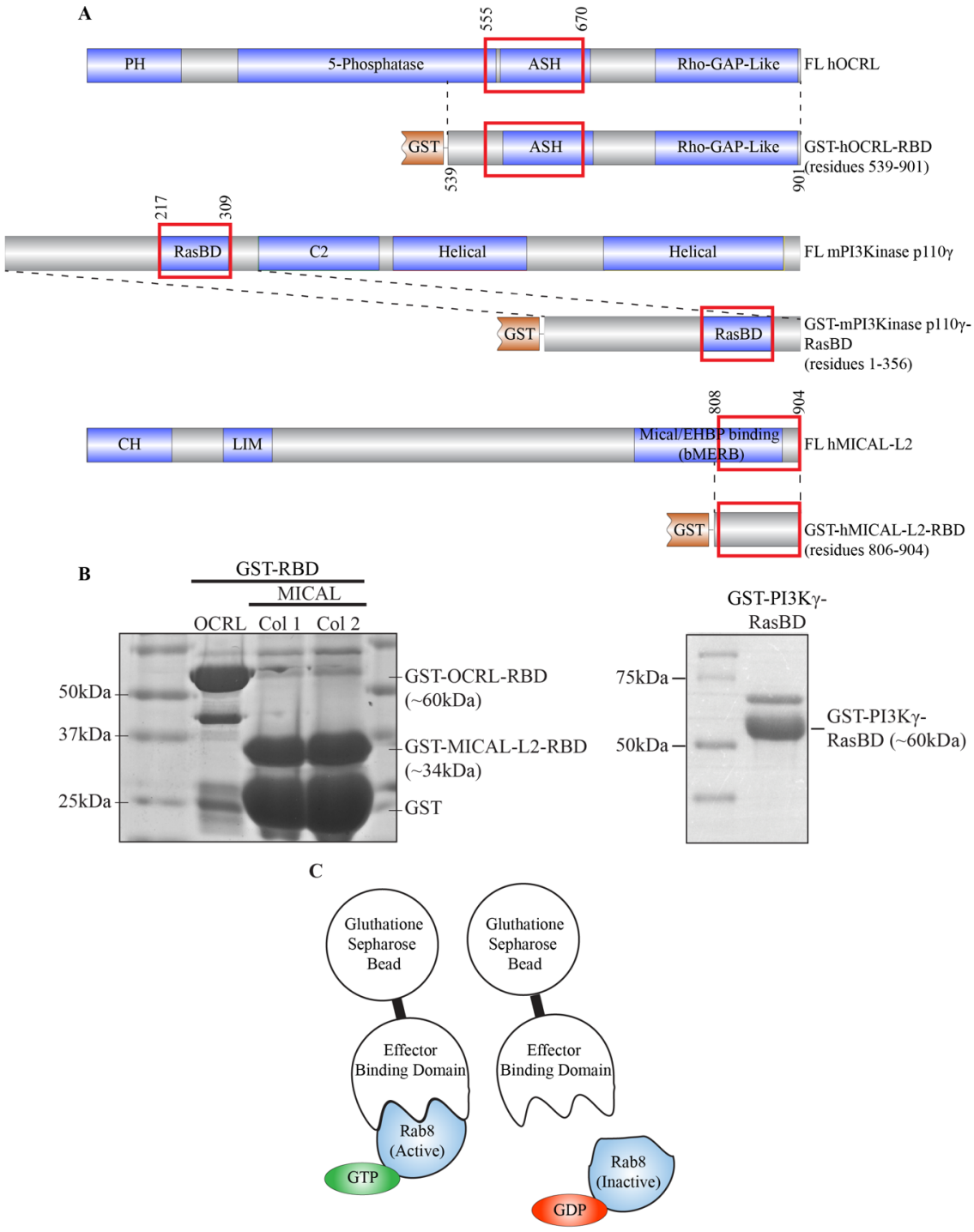


Figure 4.7. Generation of Rab activation probes using the Rab binding domains of known multi-GTPase effectors. *(A) Schematics depicting three different bacterially expressed GST-tagged constructs and domains used for each protein compared to the full-length (FL) protein. Namely, the Rab binding domains (RBD) of human OCRL (residues 539-901) and human MICAL-L2 (residues 806-903) and the Ras binding domain of mouse PI3Kγ (residues 1-356). Red boxes denote the published Rab binding domains for each of these effectors. (B) Coomassie stained gels showing the expression of the above-mentioned GST-constructs. While there were good expression efficiencies of all three fusion proteins, there was a substantial GST contamination observed in the GST-MICAL-L2-RBD samples despite screening multiple pure bacterial colonies (col 1 and 2 samples in the left gel), 10µl of bound bead slurry was used per lane. (C) Illustration of the GST-Rab GTPase activation capture constructs using GSH Sepharose beads coated with GST-tagged RBD.*

Removal of unbound GST with a single-step protease elution method

I employed a single-step protease cleavage protocol previously utilised in this chapter for improving the specificity of protein pull-down elutes for mass spectrometry analysis (Figure 4.4) (Luo and King *et al.* 2014). This method employs a PreScission protease protocol marketed by GE Healthcare together with the commercially available pGEX-6P1 bacterial expression vector that contains a cleavage site between the GST affinity tag and the target fusion protein. This site is recognised by a modified human rhinovirus 3C protease, which binds and cleaves off the affinity tag for recovery and elution of the bait with any specifically bound protein (Walker and Leong *et al.* 1994). Compared to boiling (the conventional elution protocol), utilising this cleavage method to elute the MICAL-L2-RBD would leave all free GST bound to the glutathione Sepharose beads during sample recovery, effectively removing the contamination (Figure 4.8A). The gels in figure 4.8B compare the recovery of MICAL-L2-RBD bait protein off the GST-MICAL-L2-RBD resin using either the traditional boiling technique in sample buffer or protease cleavage. As anticipated, the samples that underwent protease cleavage have markedly lower levels of GST contamination compared to the boiled samples (Figure 4.8B). However, the resulting eluant is substantially diluted due to the addition of buffers required to perform this elution method (Figure 4.8B, right blot). After successful removal of the contaminant GST, all three constructs were ready for testing as probes for the Rab8 activation assay.

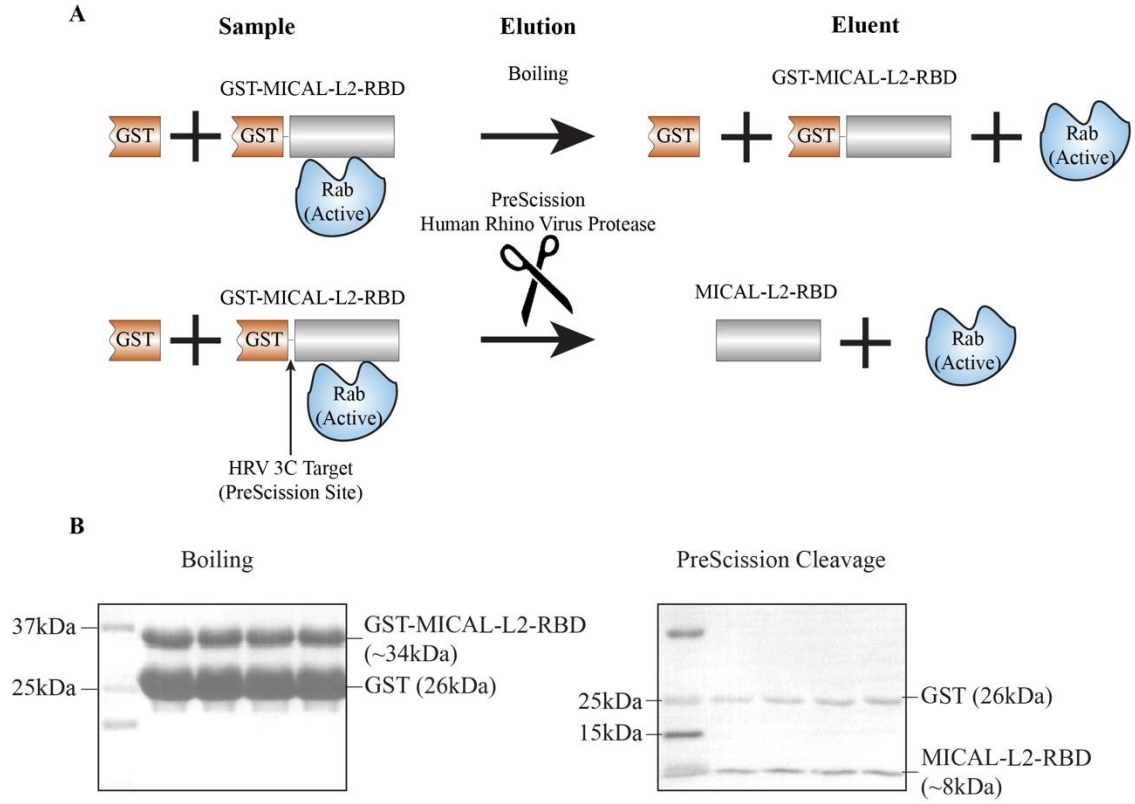


Figure 4.8. Removal of GST contamination through protease cleavage elution. *(A) Schematic diagram illustrating the predicted outcomes when using different elution methods. Initial methods involving elution through boiling would cause all proteins bound to the resin (specifically or unspecifically bound) to be eluted out, resulting in a large amount of contaminant free-GST in the sample, whilst using a PreScission cleavage method (which targets the sites between the GST-tag and RBD) would only elute out the MICAL-RBD and bound proteins, leaving both the GST-Tag and contaminant GST in the sample columns resulting in a purer eluent for analysis. (B) Ponceau staining comparing the amount of free unwanted GST between the elution methods of boiling the beads (left blot) and using protease mediated elution (right blot). 30µl of GST-MICAL-L2-RBD bound bead slurry was eluted per sample and 4 replicates of each elution method were tested.*

Validation of Rab8 activation probes

The first task was to establish that these probes exclusively bind to Rab8 in its GTP-loaded form. RAW 264.7 cell macrophages lysates were incubated with either 1mM GDP or 500µM of the non-hydrolysable GTP-analog, Gpp(NH)p, and applied to columns containing each of the Rab8 activation probes. Promisingly, all three probes demonstrated the ability to pull-down and enrich GTP-loaded Rab8a/b with little or no pull-down of the GDP-incubated samples, as indicated on representative gels (Figure 4.9). Both Rabs 8a and 8b (from here on, Rabs 8a and 8b will be referred together as Rab8 if not otherwise mentioned) are anticipated to bind to these probes as the Rab8 antibody used binds to both homologs. The OCRL-RBD and mPI3Kγ-RasBD produced robust capture of Rab8. The MICAL-L2-RBD probe still suffered from being less

abundant (more dilute) as a probe and capturing less Rab, also still in the presence of a low level of contaminant GST, probably visible due to unspecific antibody binding. Though diluted, the MICAL-L2-RBD still demonstrated a clear ability to discriminate between GDP-loaded and GTP-Rab8 from the macrophage lysates, preferentially pulling-down GTP-bound Rab8, making it a valid Rab activation probe. I did not attempt to blot with other Rab antibodies at this stage to determine whether other GTP-Rabs in the macrophages extracts also bind, but this could be done in the future. Thus, I successfully produced three probes that can bind to and capture GTP-Rab8.

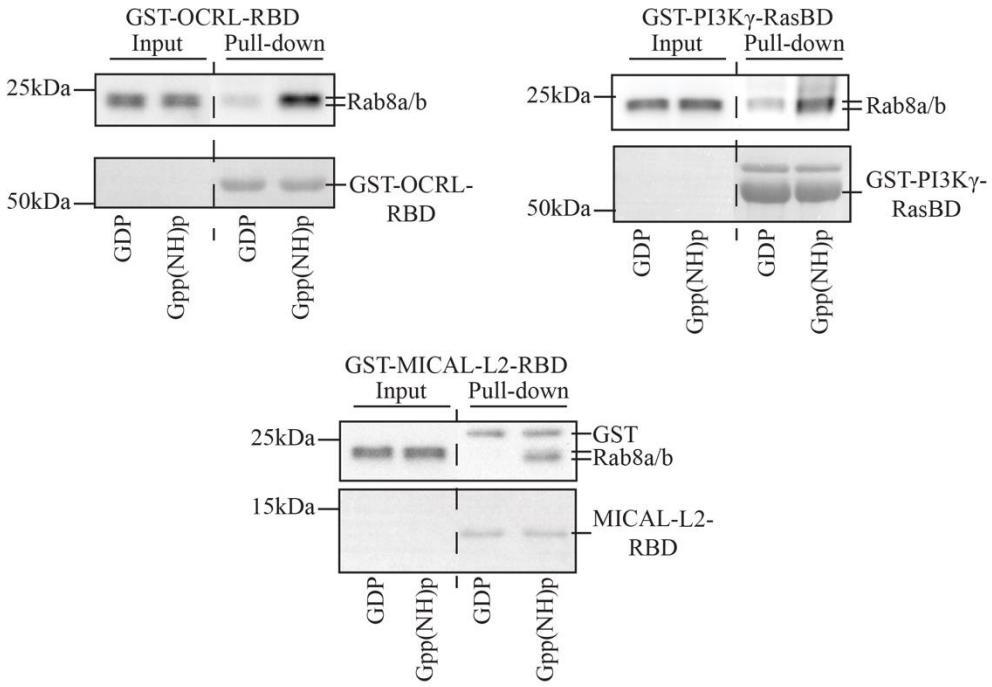


Figure 4.9. Testing of active Rab8 capture constructs with GDP/Gpp(NH)p loaded RAW 264.7 cell lysates. *Immunoblots showing the levels of captured active GTP-Rab8 from the lysates of RAW 264.7 were loaded with either GDP (1mM) or the non-hydrolysable GTP analog, Gpp(NH)p (500µM) and applied to GSH-Sepharose beads using either GST-OCRL-RBD, GST-PI3Kγ-RasBD or GST-MICAL-L2-RBD bound beads for pull-downs.*

I next sought to test these probes for the capture of endogenous GTP-Rab8 after macrophage activation. We had established that LPS activates Rab8a-mediated signalling (Luo and Wall *et al.* 2014). Other macrophage processes likely to engage Rab8 are macropinocytosis and phagocytosis, which can be initiated through stimulation with macrophage colony stimulating factor (M-CSF) (Yoshida and Gaeta *et al.* 2015) and human Ig-G coated latex beads respectively (Yeo and Wall *et al.* 2016). Therefore RAW 264.7 cells were treated with different activators under the

following conditions; LPS (100 ng/ml, 30 min), human-IgG conjugated latex beads (2µl of a 1/10 dilution, 30 min) and macrophage colony stimulating factor (M-CSF) (20ng/ml, 30 min). After treatment, the cells were lysed and the resulting lysates applied to the resin-bound probes for GTP-Rab capture, which was analysed using Western blotting. Phosopho-ERK1/2 and phospho-Akt was blotted in the respective samples to signify activation of the cells, GAPDH was blotted as a loading control and the base levels of the probes and Rab8 were examined on the gels. There was typically some level (varied) of Rab8 pulled-down from the lysates of non-activated cells, which could indicate constitutive or other means of Rab8 activation occurring in the cells but in general, the probes were able to recover increased amounts of GTP-Rab8 from cells activated by each different condition.

The OCRL-RBD construct detected increased levels of GTP-Rab8 in response to each of these stimulants, most markedly in LPS treated cells (Figure 4.10A, left column). Interestingly, with the exception of the IgG-bead treated cells (Figure 4.10C, middle column), the PI3Kγ-RasBD probe was also able to detect an induced activation of Rab8 in both M-CSF and LPS treated macrophages (Figures 4.10A and B). Unfortunately, with the MICAL-L2-RBD construct, I could not discern any differences in Rab8 GTP-loading from any of the conditions (Figure 4.10, entire right column). However, it is clear that the concentration of the probe itself was much lower than for the other 2 probes. I tried to overcome the dilution of this probe by loading more of the reaction mixture on the gels, however this still showed lower amounts of both probe and captured Rab.

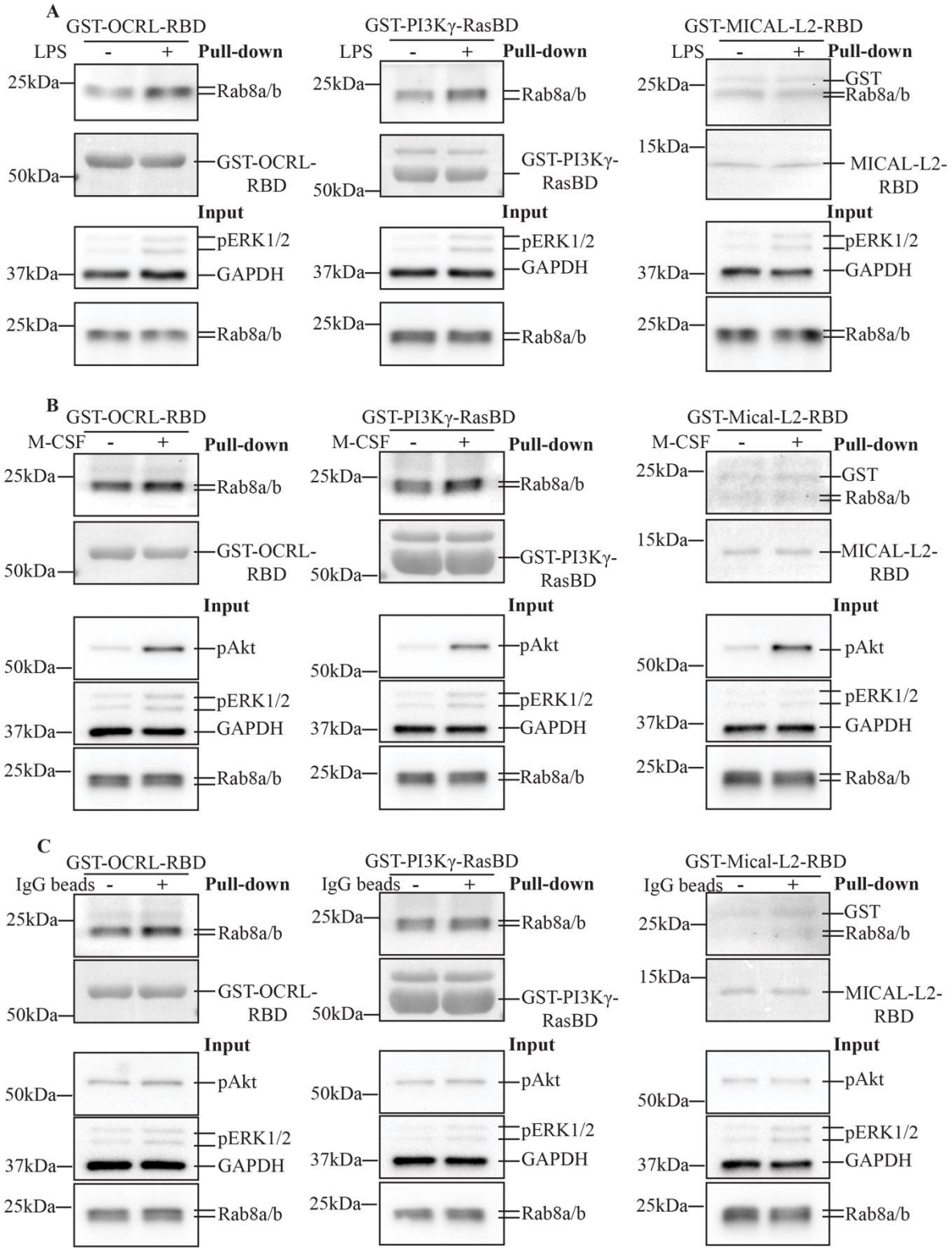


Figure 4.10. OCRL-RBD and PI3Kγ-RasBD constructs are able to detect Rab8 activation in macrophages responding to various stimuli. *Immunoblots showing the levels of active GTP-Rab8 that was pulled down from the extracts of stimulated RAW 264.7 macrophages using three different Rab8 activation probes. RAW 264.7 cells were treated with either (A) LPS (100 ng/ml, 15 min), (B) M-CSF (20 ng/ml, 30min) or (C) IgG-coated beads (30min) and the resulting lysates applied to GSH-Sepharose beads bound with either GST-OCRL-RBD, GST-PI3Kγ-RasBD or GST-MICAL-L2-RBD probes. GAPDH was used as an input and protein loading control while phospho-ERK1/2*

(Thr202/Tyr204) and -Akt (Ser473) were measured to observe for macrophage activation in response to the various stimuli. These experiments were performed multiple times and the blots shown here are representative.

Further optimisation and characterisation will be needed to render all three probes as useful tools for detecting Rab8 activation in cell lysates. However, these preliminary results highlight three observations; i) all three of these Rab activation probes are able to selectively bind to GTP-loaded Rab8, ii) of the three constructs, both the OCRL-RBD and PI3Kγ-RasBD probes demonstrate the most promise as potential tools for assessing Rab or Ras activation in cells and iii) Rab8 is indeed activated in macrophages stimulated with either LPS, M-CSF or IgG opsonised beads, consistent with previous studies highlighting its multiple roles in immune signalling and endocytic pathways (Luo and Wall *et al.* 2014, Yeo and Wall *et al.* 2016, Condon and Heddleston *et al.* 2018). Of the three probes tested, the OCRL-RBD construct displayed the most sensitivity for detecting changes to Rab8 GTP-loading, and thus this probe was pursued for further studies in this project.

Optimisation and validation of the OCRL-RBD Rab8 activation probe

To further optimise this activation assay, its ability to detect quantitative activation was tested. RAW 264.7 mouse macrophage lysates were loaded with either GDP (1mM) or increasing amounts of a non-hydrolysable GTP-analogue (1, 10 or 100µM) and was then applied to the OCRL-RBD probes. As expected, Western blotting revealed the amount of active Rab8a captured correlates with higher concentrations of GTP, proving the validity of this assay as a quantitative readout for Rab8 GTP-loading (Figure 4.11A). Next, varying concentrations of lysate from RAW 264.7 cells treated with LPS (100ng/ml, 30mins) were applied to the probe. On a representative gel, the highest concentrations of lysate showed increased capture of LPS-activated Rab8a but at lower concentrations, the activated Rab8 was inconsistently captured or not detected on the gels (Figure 4.11B). Incubation times for lysates-probe binding were also tested. Results showed that incubating the lysates with the GST-RBD-OCRL beads at 1 hr captured more activated Rab8a compared to a short incubation time of 10 min (Figure 4.11C). Taken together these conditions served to optimise the assay for detecting GTP-Rab8 in RAW 264.7 cell lysates and prepared the assay for investigating biological questions.

Rab8 is activated downstream of TLR4 in LPS-activated macrophages

To measure physiological activation of Rab8 using the assay, an LPS time course was performed on RAW cells and Rab8 GTP-loading was assessed using the same optimised parameters from earlier. Interestingly, results shown in Figure 4.11D showed a 'wave' of increased GTP-loaded Rab8 in response to LPS-stimulation over a 15-30min period. This corresponds to the pattern of Akt phosphorylation (see Figure 4.2A, control), and is consistent with the timing for the binding of the GTP-Rab8a effector PI3Kγ and its activity.

The Rab8 activation experiment was repeated using primary bone marrow derived macrophages (BMMs) from TLR4$^{-/-}$ and wild-type mice. This was done to demonstrate Rab8a activation in primary macrophages and to determine whether this is downstream of LPS/TLR4. In wild-type, control BMMs the assay detected LPS-induced Rab8, despite a much higher baseline of Rab8 activation in non-LPS treated cells. In the absence of TLR4, there was less basal activated Rab8 and LPS did not induce an increase, in fact there was much less active Rab8a captured from these lysates (Figure 4.11E). This confirms that the RAW 264.7 macrophage cell line and primary macrophages have the same response in showing LPS-induced activation of Rab8, and this immune associated GTP-loading of Rab8 is indeed downstream of TLR4. These results prove the efficacy of this Rab8 activation assay as a reliable system for measuring changes in endogenous Rab8 activation within these cells, and the optimised version of this probe was utilised in a published article during my PhD of which I contributed as a co-author (Wall and Luo *et al.* 2017). With a Rab8 activation assay now established, I was ready to investigate the GEF activity for GRAB and Rabin8 in macrophage TLR-signalling.

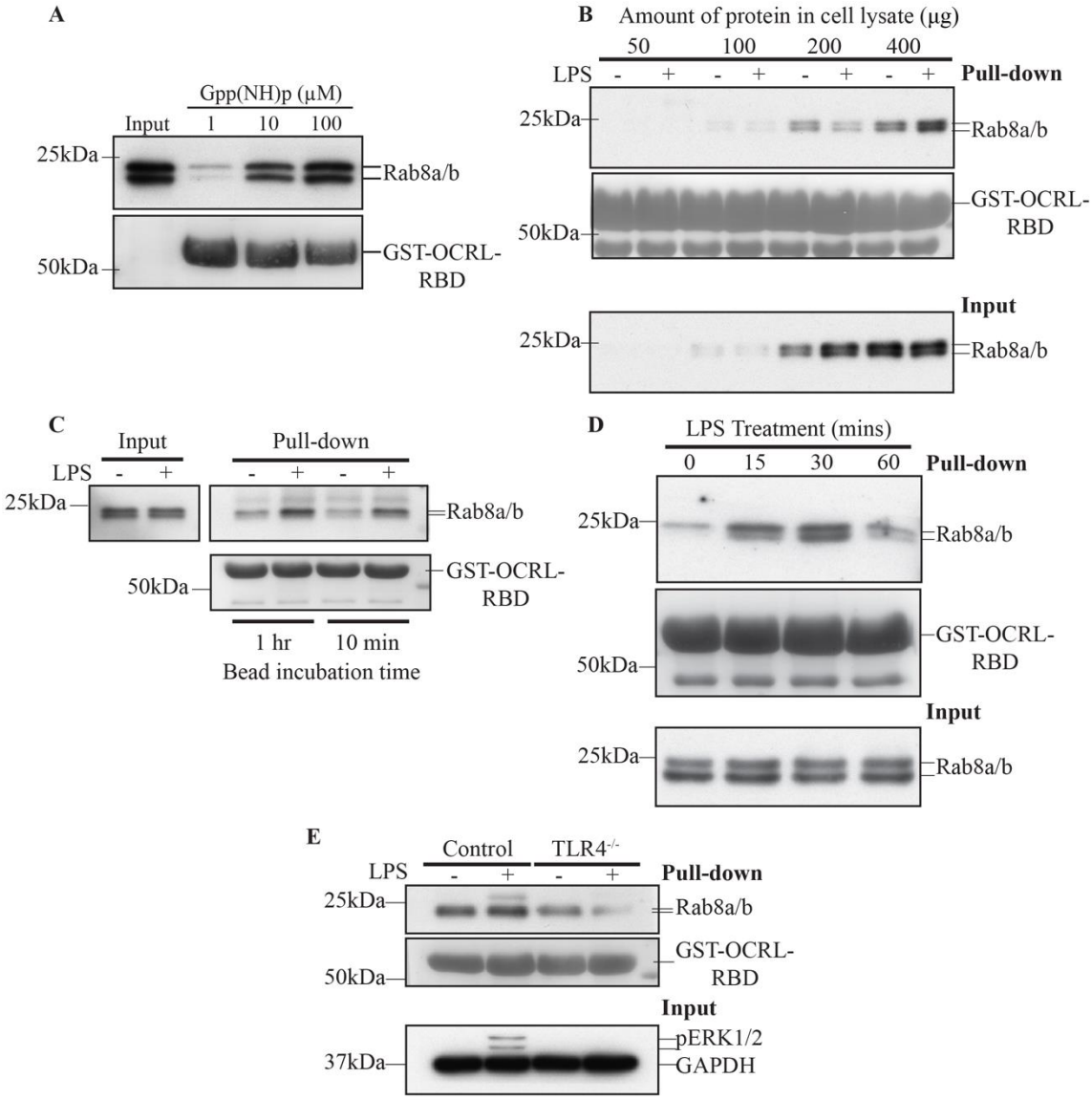


Figure 4.11. Calibration and validation of the OCRL-RBD Rab8 activation probe. *Immunoblots showing the levels of active GTP-Rab8 that was pulled down from RAW 264.7 macrophage lysates using GST-OCRL-RBD bound glutathione Sepharose beads. Results showing pull-downs of active Rab8 using the OCRL-RBD probes on; (A) GDP/GTP loaded cell lysates, (B) a calibration using different concentrations of lysate from +/- LPS (15 min) treated cells, (C) lysate from +/- LPS (15 min) treated cells incubated with probes for either 1 hr or 10 min, and (D) lysate from cells following a 60 min LPS time course. (E) +/- LPS (30 min) control and TLR4-/- mouse BMM lysate. The total volume used per pull-down was kept at a 400μl with 30μl of bead slurry, and pull-downs in C and D used 400μg of protein as per the calibration in (B).100 ng/ml of LPS was used for all activation assay treatments shown here.*

Discussion

Having discovered an interesting and novel role for Rab8a in TLR signalling, a key pursuit for our research was to identify how Rab8a is activated for this role. In the context of our earlier work, Rab8a activation is relevant for its scaffolding by the crosstalk activated endocytic receptor LRP1, and for the recruitment of its effector, PI3Kγ. In its other cellular roles, Rab8a is activated by one or both of the two known Rab GEFs, GRAB and Rabin8 and these proteins became the focus for this chapter after emerging from studies on Rab8a binding partners. A second focus for this chapter then became the establishment of an activation assay to measure Rab8a nucleotide activity and subsequently, GEF activity.

Initial results demonstrate relevant aspects of Rab8a in macrophages. Upon cell stimulation with bacterial LPS, Rab8a becomes enriched on cell surface ruffles and macropinosomes, where it is poised to participate in TLR4 signalling, as demonstrated by the colocalisation of Rab8a and the TLR4 adaptor, TRAM (Figure 4.1C). It is on these membrane domains where Rab8a plays an important role during TLR-signalling, driving anti-inflammatory cytokine secretion to constrain inflammation through its effector PI3Kγ (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). Indeed, in signalling assays, deletion of either Rab8a, its effector PI3Kγ or its associated recruiter LRP1, in RAW 264.7 macrophages reduced LPS triggered Akt phosphorylation, a key signalling kinase within this inflammatory pathway (Figure 4.2 and 4.3).

Pull-down of GST-Rab8a was used to 'fish' for Rab binding partners in activated macrophage lysates. Interestingly, this revealed other potential proteins of interest, such as the GDIs (Ullrich and Stenmark *et al.* 1993, Shisheva and Chinni *et al.* 1999) and the Rab escort protein, MSS4 (Itzen and Pylypenko *et al.* 2006) as binding partners in macrophages. These proteins could be further pursued, and they may in the future flesh out the regulatory repertoire for Rab8a in TLR signalling. PI3Kγ emerged from this screen and it is a known LPS-induced Rab8 effector (Luo and Wall *et al.* 2014), confirming that this pull-down captured proteins based around LPS-activated Rab8a. GRAB was also captured as a binding partner for Rab8a and it is a known GEF for this Rab (Guo and Hou *et al.* 2013) and its expression in macrophages was confirmed, along with the expression of the other known Rab8 GEF, Rabin8. The

pull-down and mass spectrometry identification of only GRAB, in the first instance, suggest it might be the sole GEF for Rab8a in the context of interest. However, for comparison, both GRAB and Rabin8 were studied in parallel. Co-immunoprecipitation experiments using RAW cells expressing myc-Rab8a verified that GRAB bound to Rab8a in activated cell extracts, validating the results observed from the earlier pull-down experiments and mass spectrometry analysis. Rabin8 was also found to bind to myc-Rab8a. Both GRAB and Rabin8 showed similar, LPS-induced binding to myc-Rab8a. The reason for not picking up Rabin8 in the initial pull-downs is not clear.

In LPS-activated macrophages, the production and expression of GFP-labelled GRAB and Rabin8 showed they are widespread in cells, as expected for these normally soluble proteins, but both are recruited to cell surface ruffles where they colocalised with tagged Rab8a. This is in accord with their binding to Rab8a in extracts and in intact cells, both GRAB and Rabin8 are on the membrane domains where they are seemingly both available to interact with and activate this GTPase to facilitate TLR signalling. Indeed, high-resolution crystal structures have shown that both GRAB and Rabin8 bind via the same domains to Rab8 (Sato and Shirakawa *et al.* 2007, Guo and Hou *et al.* 2013). Functional studies performed individually on GRAB and Rabin8 showed that both operate in some of the same complexes. For instance, both GEFs are known to form a complex with GTP-loaded Rab11 to facilitate Rab8 recruitment for the subsequent targeting of exocytic membrane vesicles (Horgan and Hanscom *et al.* 2013, Wang and Deretic 2015). Both GEFs have also been found to function in the same pathways, such as regulating the transport of membrane vesicles for neurite outgrowth (Furusawa and Asada *et al.* 2016, Homma and Fukuda 2016). These known functional similarities between GRAB and Rabin8 provided a background for continuing to study both as potential Rab8a GEFs in TLR-activated macrophages.

One key measurement for assessing GEF function is by quantifying endogenous Rab activation (GTP-binding). As mentioned previously, protocols for gauging endogenous Rab activity are limited. The first of these methods used radioactive phosphate isotopes ($^{32}P_i$) to label the pools of GTP/GDP within cells. After which, the respective GTPase protein is immunoprecipitated and thin-layer chromatography was used to measure the ratios of bound radiolabelled GTP/GDP bound protein was measured (Gibbs and Schaber *et al.* 1987). However, extensive safety precautions was required

for this technique due to the use of millicurie levels of $^{32}P_i$, and the added need to culture the cells in phosphate-depleted media could alter cellular responses, made this technique obsolete as other assays were developed (Taylor and Resnick *et al.* 2001). Another technique uses monoclonal antibodies, which only recognises the GTP-bound configurations of specific GTPases, however few of these antibodies have ever been proven to be effective with rare examples such antibodies reported for GTP-Rab6 and GTP-Rho (Nizak and Monier *et al.* 2003, Goffinet and Chinestra *et al.* 2008). While it is not entirely known why there is a lack of these active-configuration specific antibodies for small GTPases, the small size, globular structure and relatively high similarities between the GTPase family members may prove to be challenging targets for developing these specific antibodies. The final and most common method for measuring GTPase activation in cells is through the use of effector binding domains to selectively bind and detect GTP-bound GTPases in lysates or protein solutions. Indeed, commercial GTPase activation kits, such as the G-LISA® assays developed by Cytoskeleton Inc, utilised various effectors to generate activation probes for members of the Rho, Ras and Arf sub-family of GTPases. Interestingly, despite being the largest subfamily of GTPases to date there is no available commercial assay for measuring the activation of endogenous Rabs in cellular systems.

In saying this, there have been instances of endogenous Rab activation being measured in specific studies, for example the use of a GST-tagged Rab8 effector called synaptotagmin-like protein 1 (Slp1) to pull-down and assess the role of Rab7 in apoptosis, indicating that it is possible to generate such assays for Rab family members (Romero Rosales and Peralta *et al.* 2009). For Rab8 in particular, work by Peränen and colleagues identified a Rab8/Rab27 effector called synaptotagmin-like protein 1 (Slp1) (Hattula and Furuhjelm *et al.* 2006), which was used in a later study by the Guo laboratory to demonstrate changes to endogenous Rab8 activity and Rabin8 GEF function in HeLa cells (Wang and Ren *et al.* 2015). While effective for assessing Rab8 activation, being a specific effector for Rabs 8 and 27 limited the use of this Slp1 construct for just these two Rabs.

For this project, due to the absence of a commercially available GTP-Rab8 specific antibody, we set about generating three Rab8 activation probes using the RBDs of the

multi-Rab effectors, OCRL and MICAL-L2, and the RasBD of PI3Kγ. In some of its other roles, Rab8a shares effectors with other Rabs. For instance, OCRL and MICAL-L2, are effectors also for Rabs 1, 3, 5, 13 and 35 (Fukuda and Kanno *et al.* 2008) and probes based on their RBDs would potentially have the ability to pull-down activated versions of Rab8a but also these other Rabs. In contrast, Rab8a binds to the RasBD of PI3Kγ as an effector specifically identified in TLR signalling, which is directly relevant to the context of interest. However, it should be noted that the RasBD of PI3Kγ has not been directly tested, in our hands, for binding to other Rabs. The assay we developed relied on these probes for strong and selective binding to GTP-loaded Rab8a, rather than for their specificity to Rab8a *per se*, since Rab8a will be detected in the eluate by immunoblotting.

Initial experiments with GDP/GTP-analogue loaded GTPases in RAW 264.7 macrophage lysates demonstrated that all three constructs are able to pull-down Rab8 in the presence of GTP, indicating a binding preference for GTP-loaded Rab8 (Figure 4.9). By this measure, the probes are successful in detecting activated Rab8 in cell extracts. OCRL-RBD and PI3Kγ-RasBD constructs were then able to detect a robust increase in GTP-Rab8 after LPS activation of cells. This verified previous findings showing that Rab8 activity is indeed induced upon TLR4-stimulation (Wall and Condon *et al.* 2018) as a key precept for the current project. However, this LPS-associated increase in GTP-Rab8 levels was not observed using the MICAL-L2-RBD probe. This is likely due to the extra dilution of this probe during purification (evident from the difference in 'bait' intensity between the MICAL-L2-RBD and the OCRL-RBD or PI3Kγ-RasBD, Figure 4.10A ponceau stains). While preliminary testing using GDP/GTP-analogue loaded macrophage lysates demonstrated that the MICAL-L2-RBD was still sensitive enough to detect a difference in GTP-Rab8 levels (Figure 4.9), this indicates that the current protocol for the MICAL-L2-RBD probe is not sensitive or concentrated enough to detect the subtler change in Rab8 GTP-loading during LPS-TLR4 activation.

I also tested the capabilities of these Rab8 activation probes to detect changes in GTP-Rab8 in lysates from macrophages activated with other stimuli, namely M-CSF (stimulates macropinocytosis) (Yoshida and Gaeta *et al.* 2015)] and IgG opsonised

beads (stimulates phagocytosis). While LPS induced the clearest (biggest) induction of Rab8 activation, M-CSF and IgG produced smaller increments in GTP-Rab8a capture after cell treatments. This is consistent with the prominent TLR-induced recruitment of Rab8a to macropinosomes, in keeping with this as one of the main roles of Rab8a in macrophages. Since we know that Rab8a is active downstream of multiple TLRs (Wall and Luo *et al.* 2017), it would be fruitful in the future to test other TLR ligands in the activation assay. The OCRL-RBD construct appeared to be more effective in capturing GTP-Rab8a than the PI3Kγ-RasBD in treated cells. This might reflect the differential sensitivity of the binding domains. With these results, it was clear that the OCRL-RBD was the most sensitive and robust of the activation probes and was selected for further use in the project.

In cells, the GTP-/GDP-loaded states of Rabs are often transient in nature, catalytically driven by the regulatory partners they interact with, such as GEFs and GAPS. However, by themselves GTP-bound Rabs also have an intrinsic, albeit slow, GTP hydrolytic activity that naturally switches off the Rab (Nottingham and Pfeffer 2015, Pylypenko and Hammich *et al.* 2018). This calls attention to the incubation times I used for the probes during capture of GTP-bound Rab8 from macrophage lysates. Given the slow nucleotide exchange on Rabs, unsurprisingly, the results showed that incubating the lysates with the GST-RBD-OCRL beads for 1 hr showed better capture of GTP-Rab8 than 10 min (Figure 4.11C) and the longer incubation time was employed throughout my studies.

During the optimisation of the GST-tagged OCRL-RBD, I confirmed that Rab8 was indeed activated in macrophages treated with LPS and this occurred in a peak at 15-30 mins, which coincides with the LPS induced AKT phosphorylation pattern in Figure 4.3, which ties the Rab8a activation with its recruitment of the PI3Kγ effector for signalling. Furthermore, this LPS-induced Rab8 activation was abolished in TLR4$^{-/-}$ BMMs, verifying that this Rab8 GTP-loading is directly tied to immune receptor activation. These results confirm that Rab8 is activated upon macrophage stimulation with LPS, resulting in downstream PI3Kγ function to elicit TLR-associated signalling responses.

In conclusion, this chapter describes the identification of the candidate Rab8 specific binding partners GRAB and Rabin8 as potential activators of Rab8 in TLR-associated pathways. In developing and establishing a reliable Rab8 activation assay, I can now further investigate Rab8 activity in macrophages and test the roles of GRAB and Rabin8 in TLR-associated Rab8 activation. By creating a useful activation assay using two RBD probes, the assay can now be used much more widely to examine activation of Rab8 (and other Rabs) in vitro and in cell extracts.

Chapter 5

Results 2: Deletion of GRAB and Rabin8 and its effects on Rab8 activation and localisation

5. Results 2

Introduction

In macrophages, Rab8a localises dynamically on cell surface ruffles and becomes enriched on early macropinosome membranes. This recruitment of Rab8a becomes enhanced when macrophages are activated by various TLR ligands (Wall and Luo *et al.* 2017). In the previous chapter, with help from my supervisor Dr Lin Luo, I developed a Rab8 activation probe for pull-downs of GTP-Rab8 in the extracts of LPS activated RAW 264.7 macrophages and we used the assay to show that LPS increases GTP-Rab8 in macrophage extracts (see Figure 4.11D). However, the GEF responsible for nucleotide exchange to GTP-load Rab8a for this signalling function is yet to be characterised.

In the previous chapter, using protein pull-down and immunoprecipitation experiments, I initially identified GRAB and subsequently demonstrated the presence of GRAB and Rabin8 as candidate Rab8 GEFs in LPS-activated macrophages. Cellular imaging in LPS-activated RAW 264.7 cells, using fluorescently tagged constructs, demonstrated that both GRAB and Rabin8 colocalise with Rab8a on macrophage surface ruffles from which macropinosomes arise. This indicates that both proteins are available to interact with Rab8a on relevant membranes tied to its TLR-signalling function. Now, further functional studies are needed to examine if either GRAB or Rabin8 are indeed the GEFs responsible, specifically, for TLR-induced Rab8 activation. In order to assign GRAB or Rabin8 to the role of GEF activation for this specific function of Rab8a, I needed to deplete these proteins within our cells of interest.

Gene knockouts (KO) in mice are traditionally problematic for Rabs and their accessory proteins. The Rab8a KO is embryonically lethal in mice and mouse KO models for GRAB and Rabin8 are not available (Sato and Mushiake *et al.* 2007). Most phenotypic studies on GRAB or Rabin8 have been performed using siRNAs (Fukuda 2008, Wang and Ren *et al.* 2015, Eguchi and Kuwahara *et al.* 2018). Given the difficulty of transfection in RAW 264.7 cells and other macrophage lines, siRNAs are not an ideal approach for assaying cell functions. More recently our laboratory has had success using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) gene editing system to generate the

complete deletion of Rabs8a and 13 and LRP1 in RAW 264.7 cell lines (Wall and Luo *et al.* 2017, Condon and Heddleston *et al.* 2018, Luo and Wall *et al.* 2018). Therefore, using these methods and improved CRISPR protocols developed by Ran and colleagues (Ran and Hsu *et al.* 2013), I set out to generate KO RAW 264.7 cell lines of both, GRAB and Rabin8, to investigate the functional roles of these proteins during TLR-Rab8a-signalling.

This chapter describes the generation of GRAB and Rabin8 CRISPR-Cas9 KO RAW 264.7 macrophage cell-lines for investigating the roles these proteins play in TLR-associated Rab8 activation and function. Using the optimised Rab8 activation probe from the previous chapter and live-cell confocal imaging of Rab8a, I will assess changes to both LPS-induced Rab8 activation and localisation in macrophages in these CRISPR-KO cell-lines.

Results

Generation of GRAB and Rabin8 CRISPR KO cell-lines

To begin characterising the functional roles of GRAB and Rabin8 in TLR-activated macrophages, I proceeded to generate KO RAW 264.7 macrophage cell lines for each of these proteins. Utilising previously published protocols by Ran and colleagues (Ran and Hsu *et al.* 2013), Dr Adam Wall from our laboratory established regimes for using the CRISPR-Cas9 system to KO (completely and selectively) Rab8a (Wall and Luo *et al.* 2017) and other proteins such as LRP1 in the RAW 264.7 mouse macrophage cell line (Luo and Wall *et al.* 2018). I therefore set out to make CRISPR KO RAW 264.7 cell lines for GRAB and Rabin8.

However, despite the previous successes of this CRISPR-process, it was still a challenge to perform gene editing in our macrophage cell lines. Some of the complicating factors include the difficulty of transfecting macrophages, since these cells react very strongly to foreign proteins and genetic material, resulting in immune activation followed by programmed cell death (Chow and Deo *et al.* 2016, Nagata and Tanaka 2017) and the variation in CRISPR targeting efficiencies due to gene/sequence specific 'off' target rates. In all, both GRAB and Rabin8 turned out to be much harder to target than the previous gene editing done in the laboratory. Several methods were trialled, and alternative approaches were performed over a period of 8 months, the methodology and the workflow used to ultimately, successfully generate the GRAB and Rabin8 KO cell lines is described below.

Construction of CRISPR-Cas9 protein complex and selection plasmids

Using an online CRISPR guideRNA design tool (originally portrayed on crispr.mit.edu) as developed by the Zhang laboratory (Broad Institute, MIT), three different CRISPR target sites for each gene were selected (Figure 5.1A). These targeting sequences called guideRNAs were designed to recognise regions within the second exon of mouse GRAB and third exon of mouse Rabin8 (based on the 'off' and 'on' target scores by the online design tool, Appendix 2). Each of these guideRNAs contain a 20-nucleotide sequence that is homologous to the target gene, termed 'spacer', and a protospacer adjacent motif (PAM), which the CRISPR-Cas9 complex uses for binding, sequence recognition and cleaving (Figure 5.1A). To maximise the chance of generating KO cell lines, three different methods were tried for generating the

CRISPR-Cas9 ribonucleoprotein (RNP) functional unit as set out in Figure 5.1B. Method 1 uses the cDNA mammalian expression vector px458 (Addgene plasmid #48138), a Cas9 expressing plasmid from the Feng Zhang laboratory (McGovern Institute, MIT) that endogenously expresses the complex after transfection within the cell, while methods 2 and 3 involve generating an exogenous Cas9 RNP complex, through mixing the purified Cas9 nuclease from *S.pyogenes* (IDT) with guide-RNA oligos using either a guide-RNA synthesis kit (method 2) or directly made by IDT (method 3). By varying the way these RNP complexes were made and introduced to the RAW 264.7 cells, we predicted that at least one of these approaches would minimise immune cell reactivity, increasing the possibility of attaining successful incorporation of a gene KO.

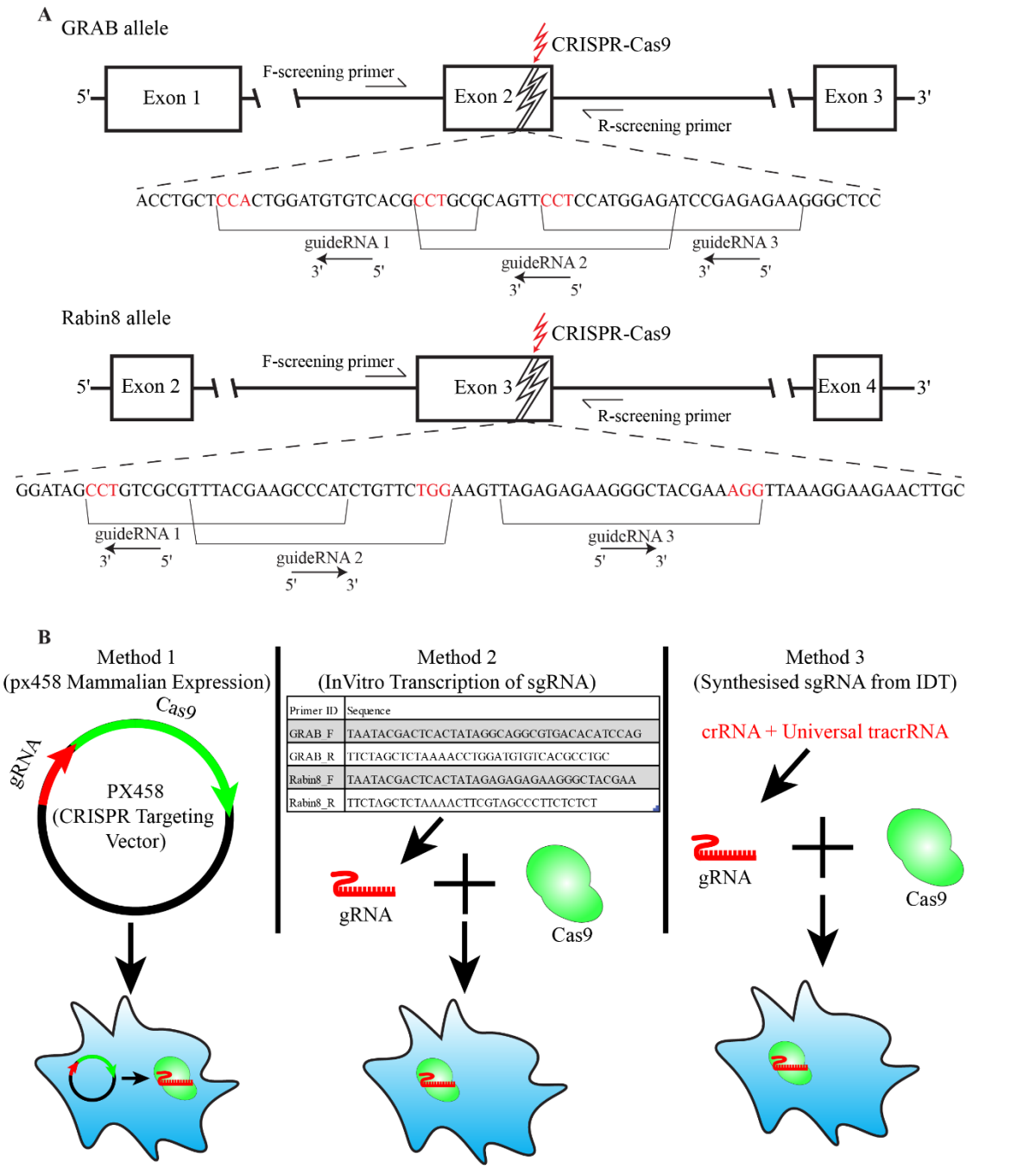


Figure 5.1. Design and production of the Cas9-gRNA complex for generating GRAB and Rabin8 KO RAW 264.7 macrophage cell lines. *(A) Schematics denoting the guideRNA target locations on the alleles of GRAB and Rabin8 (exons 2 and 3 respectively). These guideRNA sequences are composed of a CRISPR -Cas9 protospacer adjacent motif (PAM, in red), and the upstream homologous 20bp 'spacer' sequence used for CRISPR-Cas9 targeting. Each of these guideRNAs were designed and selected using an online CRISPR guideRNA design tool (crispr.mit.edu, by the Zhang Lab, Broad institute). (B) Illustration depicting the 3 methods that were used in an attempt to minimise macrophage sensitivity to the foreign protein/genetic material. In method 1, Rabin8 and GRAB gRNA homologous sequences were cloned into the Cas9-gRNA complex mammalian expression vector px458, which was directly transfected and expressed in RAW 264.7 cells. In methods 2 and 3, synthetic gRNA oligos were produced either via a synthesis kit (method 2) or ordered from IDT (method 3). The gRNA oligos were later complexed with pre-ordered, purified S. pyogenes Cas9 proteins (from IDT), which is later directly transfected into RAW 264.7 cells.*

In conjunction with these RNP complexes, two further methods were utilised to select for the KO mutant cell lines after transfection in order to encourage the generation of different mutation outcomes: i) nonhomologous end joining (NHEJ) resulting in insertions/deletions (indels); ii) homology-directed repair (HDR), whereby a donor cDNA sequence (selection cassette) is inserted at the CRISPR cut-site, both of which closely follow protocols established by others (Ran and Hsu *et al.* 2013). In line with these methods, 2 resistance plasmids were generated, with guidance from Dr Adam Wall in our laboratory, using a neomycin resistance plasmid pFloxNeoFlox to be co-transfected with the Cas9-guideRNA complex (Figure 5.2).

NHEJ is an endogenous DNA repair mechanism that is prompted after the CRISPR-Cas9 RNP complex cleaves a double-stranded break in the target gene. This process has a high rate of error as it ignores sequence homology and simply ligates the 2 strands of DNA back together, often resulting in indels (Burma and Chen *et al.* 2006). This results in a random frameshift mutation, effectively silencing the gene. Co-transfection of the resistance plasmid (containing GFP) serves as a visual aid for comparison of transfection efficiency and for the identification of cells that have been successfully transfected with the Cas9-guide RNA complex, that can then be picked and screened for mutations. Single colonies of resistant cells were picked and used for further screening.

For HDR, after the CRISPR-Cas9 directed cleavage, the co-transfected donor resistance plasmid provides a template that contains ~2kb homologous ‘arms’ that flank a neomycin resistance cassette (Figure 5.2, right plasmid). During the gene repair process, the presence of the template facilitates the insertion of the selection cassette into the site of mutation, allowing for the stable expression of resistance in addition to disrupting the target gene. A downstream diphtheria toxin A-chain is included to negatively select any mutants where a random insertion of the resistance cassette has occurred (‘suicide’ sequence). Due to the inherent genomic variation of the RAW 264.7 cell line, the relatively long (~2kb) homologous ‘arms’ were cloned from RAW 264.7 genomic DNA to maximise the chances of accurate HDR occurring. With the integration of the resistance plasmid, the resulting cells will remain in selection and be grown as a mixed population of CRISPR-mutated cells.

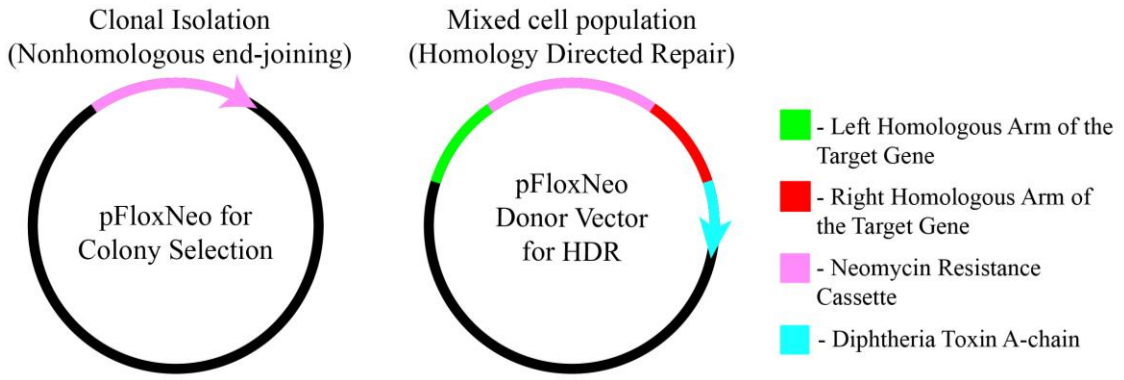


Figure 5.2. Illustration of resistance selection vectors for CRISPR-Cas9 mutant selection. *Figure depicts the 2 different selection plasmids used for generating knockout mutants. The first vector (left) is a simple pFloxNeo plasmid meant for transient expression of neomycin resistance to identify positively transfected cells. This was used for the positive selection and isolation of single colonies for expansion and generation of clonal gene knockout cell-lines. The second vector (right), is the pFloxNeo donor vector containing the homologous sequence 'arms' (red and green) which flank the target sequence site, along with a diphtheria toxin sequence for negative selection of random plasmid insertion. The integration of the resistance cassette into the cleaved gene enables the stable expression of neomycin resistance, allowing for the resulting cells to be kept in selection and thus a mixed-cell population of mutant cells.*

Transfection and selection of GRAB and Rabin8 knockout mutants

With the components ready, the KO mutants were generated by mixing the different Cas9 RNP complexes with the respective resistance plasmid and co-transfecting them into RAW 264.7 cells using the Lipofectamine™ CRISPRMAX™ (Invitrogen) transfection reagent (Figure 5.3A). For the NHEJ transfections, selection (G418, 1 mg/ml) was applied 24 hr post transfection and removed after 96 hr. The surviving single cells were left to recover and expand into colonies for picking. For the HDR transfections, neomycin was applied after 72 hr post transfection, to allow time for resistance gene integration and expression. This mixed population of transfected cells were continuously kept in neomycin selection to maintain the expression of the integrated resistance cassette. After the selection process of either method, resulting clonal cell lines (NHEJ) and mixed populations (HDR) were first screened for successful mutations via a T7 endonuclease cleavage assay (New England Biolabs).

Using screening primers flanking the target CRISPR cut sites (see Figure 5.1A, and Table 2.2 in Materials and Methods), I amplified these mutant regions using gDNA extracted from each of the mutant cell lines and a wild-type control. The amplified sequences were denatured and annealed back together and treated with a T7

endonuclease enzyme. Essentially, if a successful mutation has occurred, it would result in a mismatch that produces a 'bulb' in the annealed DNA sequence which is recognised by the nuclease and cleaved, leading to a shortened DNA fragment that can be visualised on an agarose gel. In Figure 5.3B, the results of a T7 endonuclease assay on gDNA extracted from mixed population cell lines (obtained using the px458 CRISPR vector with HDR selection, see Figures 5.1B and 5.2) is shown. By comparing the band intensities of cleaved product (arrows 2 and 4) between each of the guideRNAs, I show that the targeting CRISPR RNAs, GRAB guideRNA 1 and Rabin8 guideRNA 3, had the highest mutation efficiency. This finding demonstrates the variability in CRISPR mutation efficiencies (even within the same target exon) and importantly, allowed me to focus on one guideRNA for either gene, streamlining downstream protocols.

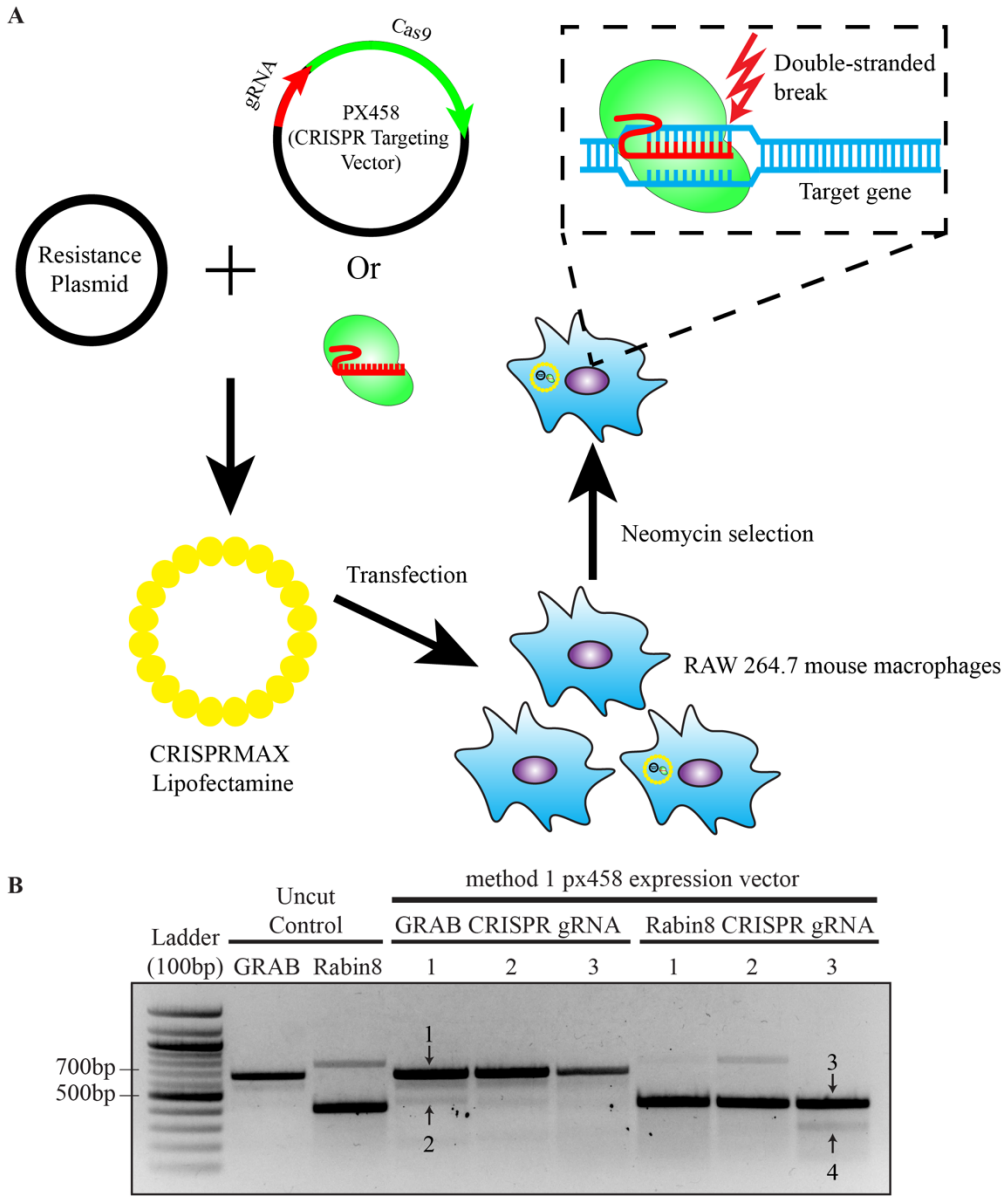


Figure 5.3. Transfection and screening for generating CRISPR-Cas9 KO RAW 264.7 macrophage cell lines. *(A) Schematic describing the general workflow used to transfect, select and screen for transfected macrophages for creating the knockout cells. First the Cas9 nuclease complex or the px458 CRISPR mammalian expression vector is mixed with either the NHEJ or HDR selection plasmids. The selection vectors with the CRISPR-Cas9 are mixed with the cationic liposome reagent CRISPR-MAX. Next, the resulting transfection complex is then added to the macrophage cell line. Finally, positively transfected cells are then placed in antibiotic selection (G418, 1 mg/ml). These selected cells are later screened for CRISPR-mediated mutations via Western blotting. (B) RAW 264.7 cells were co-transfected with the CRISPR mammalian expression vector px458 with the various guideRNAs and the HDR selection vector. After selection, gDNA was extracted from each mixed population cell line and the T7 endonuclease assay performed. A representative agarose gel showing the results from the T7 endonuclease cleavage screen. Uncut controls of wild-type GRAB and Rabin8 show the original size of the screening primer amplicons to be ~700bp for GRAB and 400bp for Rabin8 (arrows 1 and 3 respectively). Positive mutants are identified via cleaved PCR fragments (arrows 2 and 4) from T7 endonuclease digestion.*

Multiple rounds of transfection and selection of both clonal and mixed population cell lines were performed. Six 'mixed population' cell lines were generated by HDR, these represented transfected lines kept under selection but containing a mixture of transfected, knockdown and unaffected cells. Some of these populations do show reduction or loss of either GRAB or Rabin8 upon Western blotting (see in Figure 5.4, GRAB CRISPR cell line Mix 1 and Rabin8 CRISPR cell line Mix 1). In addition, I generated various KO and knockdown clonal cell lines (through the NHEJ method) which were cloned from the original mutant cell population by single cell selection to produce clonal cell lines (see examples in Figure 5.4, with arrows denoting GRAB CRISPR clonal cell line 4 and Rabin8 CRISPR clonal cell lines 1 and 4). This one GRAB KO and two Rabin8 KO clonal cell lines demonstrated a complete deletion of GRAB or Rabin8 protein expression respectively, indicating that these are homozygous KO colonies. Sequencing was later used to confirm the mutations. These clonal KO cell lines became the primary subjects to investigate the role of GRAB and Rabin8, in the regulation of Rab8a activation and function, within an LPS/TLR-associated pathway. The initial test was to assess if LPS-induced Rab8 activation is affected in the absence of either of these GEFs.

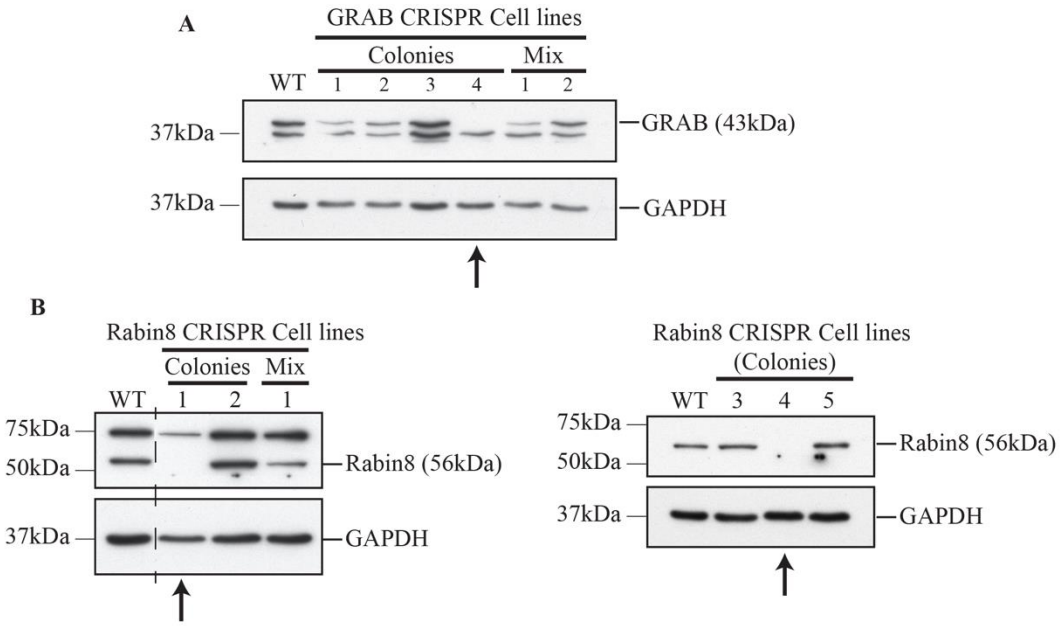


Figure 5.4. Screening CRISPR-Cas9 mutant cell lines for protein expression of target gene knockouts. *Western blot screening of CRISPR transfected cell lines for assessing protein expression levels of either (A) GRAB or (B) Rabin8. Blots show the presence of homozygous complete knockout colonies (arrows denoting GRAB colony 4 and Rabin8 colonies 1 and 4) and knockdown mixed cell populations (GRAB CRISPR mix 1 and Rabin8 CRISPR mix 1, left blot third lane). px458 plasmid transfected mixed CRISPR mutant population cell lines are referred to as 'Mix', while 'Colonies' refer to lines generated from picked isolated colonies.*

Both GRAB and Rabin8 contribute to LPS-induced Rab8 activation in mouse macrophages

To assess Rab8a activation, I employed the previously optimised GTPase activation assay for GST OCRL-bead capture of GTP-Rab8 (see Figure 4.11). Lysates from LPS treated/untreated (100 ng/ml, 15 min) control cells and from GRAB KO and Rabin8 KO RAW 264.7 cells were applied to the GST-OCRL-RBD probes and the amount of GTP-Rab8 captured was compared. GRAB and Rabin8 were both detected in the lysates before capture. Results show that the control and KO macrophages have variable basal levels of GTP-Rab8 in the capture eluates, but detectable increases were found after LPS treatment in all cases signifying LPS-induced Rab8a activation. The GRAB KO and Rabin8 KO cells both showed a modest but significant ($P<0.05$) decrease in the amount of GTP-Rab8a captured from LPS activated cells (Figure 5.5). While activation of Rab8 was affected, the phosphorylation of the MAP kinase ERK1/2 remained unchanged between the control and KO cells, suggesting that other pathways were unaffected by the deletion of these proteins. These results demonstrate that both GRAB and Rabin8 contribute to Rab8 activation in an LPS-dependent manner in mouse macrophages. Furthermore, they reveal that both GRAB and Rabin8 have activities consistent with the role of Rab8 GEFs in activated macrophages. At this stage, both proteins are possible GEFs that contribute to one or more Rab8-mediated functions triggered by LPS/TLR4.

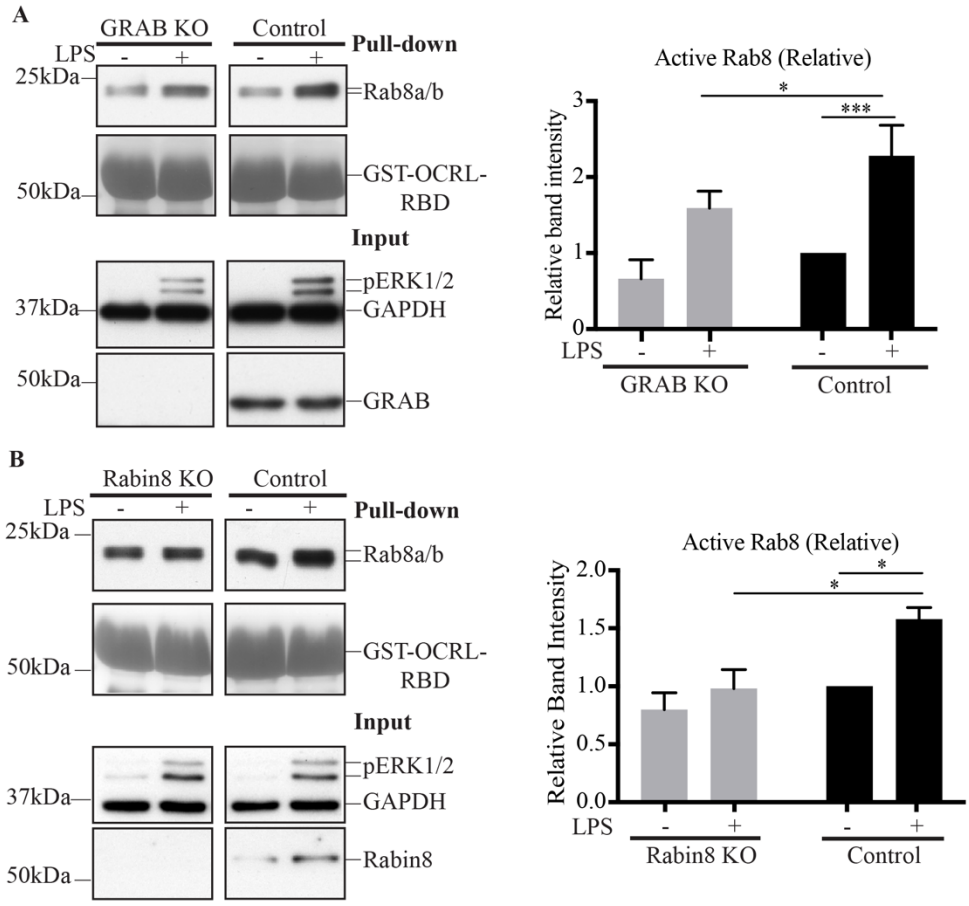


Figure 5.5. Loss of either GRAB or Rabin8 significantly reduces LPS induced Rab8 GTP-loading in activated macrophages. *Immunoblots showing the levels of active Rab8 pulled-down from the lysate of control and (A) GRAB or (B) Rabin8 KO cells +/- LPS (100 ng/ml, 15 min) using GST-OCRL-RBD bound GSH-Sepharose beads. Levels of active Rab8 were quantified by using the densitometric ratio between the band intensities of captured Rab8 and GAPDH. Significance was measured via two-way analysis of variance (ANOVA) (*P<0.05, ***P<0.001, n= 3).*

Rab8a localisation is unaffected in the absence of either GRAB or Rabin8

With both GRAB and Rabin8 now shown to be functioning as Rab8 GEFs in LPS activated macrophages, I wanted to examine whether the depletion of either GEF affected Rab8a membrane localisation in LPS-treated RAW 264.7 cells. To investigate this, I performed confocal live cell imaging on LPS-treated GRAB KO, Rabin8 KO and control RAW 264.7 cells transiently expressing GFP-Rab8a to assess the localisation of Rab8a in these CRISPR cell lines. GFP-Rab8a was found enriched on ruffles and newly formed macropinosomes when it was expressed in control cells or in GRAB KO or Rabin8 KO cells (Figure 5.6). Live cell imaging demonstrated that there were no striking morphological differences between the control and GRAB KO cells (Figure 5.6A) and the cells exhibited similar membrane ruffling dynamics and rates of

macropinocytosis in response to LPS. In Figure 5.6A, GFP-Rab8a was seen on ruffles, internalising macropinosomes and tubules emerging from the macropinosomes and the loss of GRAB did not affect this localisation. In Figure 5.6B, GFP-Rab8a was also seen on dynamic ruffles, macropinosomes and tubules and its overall distribution throughout the cell was similar in the Rabin8 KO and control cells. The loss of Rabin8 also did not affect LPS-induced ruffling or macropinocytosis, as assessed on movies. Thus, despite reducing Rab8a activation, the loss of either GRAB or Rabin8 did not affect the ability of Rab8a to be recruited to and enriched on ruffle or macropinosome membranes.

Conventionally, GEFs play a key role in stabilising their GTPases on target membranes by facilitating activation or GTP-loading, which prevents GDI recognition and extraction from the membrane, retaining the Rab on relevant cellular compartments for effector interaction (Blümer and Rey *et al.* 2013, Pfeffer 2013). As a further measure of GEF activity I therefore set up an assay to measure GFP-Rab8a retention on the macropinosome membranes. Using live cell imaging, I tracked GFP-Rab8a positive macropinosomes in GRAB KO, Rabin8 KO and respective control cells, and measured the time GFP-Rab8a was spent enriched on these membrane domains before being lost by tubulation or dissociation during macropinosome maturation (Figure 5.6C). The results revealed that the deletion of GRAB did not significantly impact the time GFP-Rab8a is spent enriched on macropinosome membranes (Figure 5.6D). Similar results were obtained with Rabin8 KO cells, with no measurable reduction in macropinosome membrane retention of GFP-Rab8a (Figure 5.6D). Thus, these observations indicate that GRAB and Rabin8 do not conform with the role of GEFs as stabilisers of Rab membrane association. While this seems to indicate that the deletion of either GRAB or Rabin8 does not affect Rab8a localisation and membrane retention, it must be noted that these findings were obtained with cells overexpressing GFP-Rab8a. While this recombinant fluorescent Rab construct (GFP-Rab8a) is believed to accurately portray the localisation of its endogenous counterpart, more experiments will be needed to thoroughly interrogate the impact of deleting either GRAB or Rabin8 on the membrane recruitment of endogenous Rab8 in these macrophage cells.

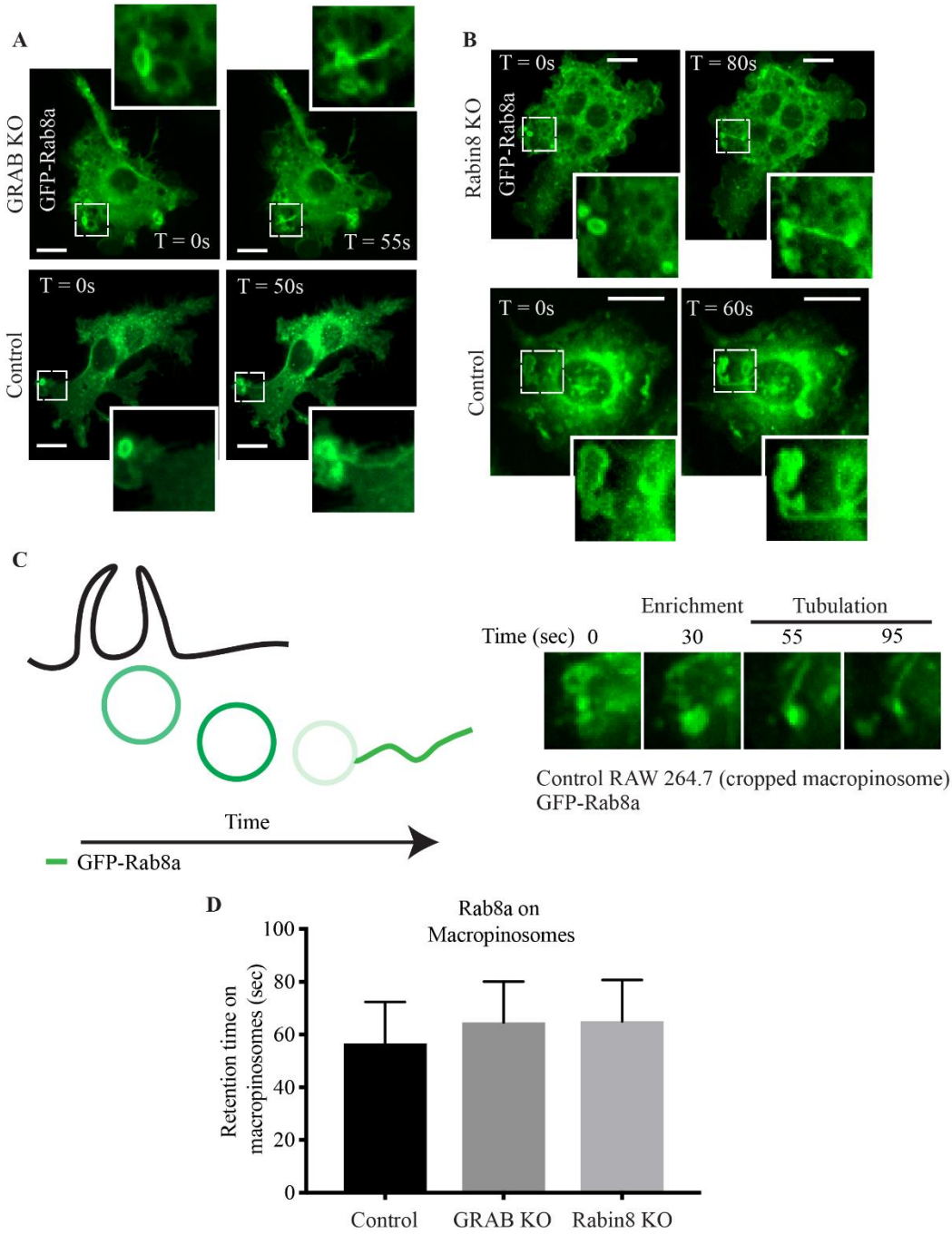


Figure 5.6. Absence of either GEF, GRAB or Rabin8 does not affect Rab8a localisation and enrichment on macrophage macropinosomes. *Live cell confocal spinning disc imaging comparing LPS-treated control with either (A) GRAB KO or (B) Rabin8 KO cells transiently overexpressing GFP-Rab8a showing recruitment and localisation to macropinosomes and tubules. (C) Illustration accompanied with live cell confocal images of GFP-Rab8a in LPS-treated RAW 264.7 control cells depicting the dynamics of GFP-Rab8a enrichment observed on macropinosomes where GFP-Rab8a becomes enriched on macropinosome membranes (30 sec frame) and is later sorted off on tubules that emanate from these endosomes (55-95 sec frames). (D) Graph showing the measurement of Rab8a membrane retention times on macropinosomes in LPS-treated control, GRAB KO and Rabin8 KO RAW 264.7 cells. Quantifications were performed by observing the total number of timeframes Rab8a is spent enriched on each macropinosome, and the macropinosomes from 5 cells of each cell line were used for quantification (n=5). Scale bars, 10µm.*

Discussion

In the first result chapter, I demonstrated that both known Rab8 GEFs, GRAB and Rabin8, have LPS-inducible interactions with Rab8a in LPS-activated mouse macrophages. With the identification of both GRAB and Rabin8 as candidate proteins of interest, I turned my attention to generating macrophage cell lines depleted of each protein and assess this affected TLR-associated Rab8 activation and function. As members of the innate immune system, macrophages are well designed to seek, identify and eliminate any substance foreign in the microenvironment. As a result, genetic and experimental manipulation of these cells becomes challenging due to the adverse reactions they have when exposed to various reagents.

A recent study demonstrated that simply adding the transfection reagents Lipofectamine 2000 or 3000 (Invitrogen) to either RAW 264.7 cells or BMMs triggered type I interferon signalling responses not present in HEK 293T (human embryonic kidney cells) or L929 (murine fibroblast) cell lines (Guo and Wang *et al.* 2019). This reactivity often results in a loss of cell viability and transfection efficiency, leading to the development of commercial reagents/protocols designed to maximise transfection efficiencies specifically for this cell type (Maeß and Wittig *et al.* 2014, Lai and Xu *et al.* 2018). In anticipation of these difficulties, a multi-pronged approach was organised for the creation of stable KO cell lines in macrophages using the CRISPR-Cas9 system. This involved generating different versions of the CRISPR-Cas9 RNP complex, along with the use of different selection vectors for the isolation of mutant colonies and mixed population cell lines. The resultant cell lines obtained from these protocols showed that of the CRISPR complexes trialled, method 3 – using ordered synthetic gRNA oligos and Cas9 protein from IDT (Figure 5.1B) - proved to have the highest efficiency in generating mutant KO lines in RAW 264.7 macrophage cells (positive KO lines in Figure 5.4, black arrows highlighting GRAB colony 4 and Rabin8 colonies 1 and 4). The other two methods were only successful in producing partial knockdowns of either GRAB or Rabin8. While it has not been empirically tested in this study, I predict this difference in efficacy is related to a combination of the transfection efficiencies and the RNPs being relatively immunologically silent.

Having successfully obtained GRAB KO and Rabin8 KO stable cell lines, these became available for functional studies throughout the project. In all cases the loss of

either of these proteins had no observable (or measurable) effect on cell growth or morphology. The functional studies carried out in this chapter first sought to measure LPS-induced Rab8 activation, utilising the activation assay previously established. In GRAB KO cells there was still both basal and LPS-induced GTP-Rab8 captured from lysates, but less of the LPS-induced GTP-Rab8a was present compared to control cells. This provided evidence that GRAB was contributing to, but not wholly responsible for, Rab8 activation by LPS or other stimuli (basal activation) in these cells. When Rab8 activation was measured in Rabin8 KO cells there was a modest reduction in LPS-induced Rab8 suggesting that this GEF also contributes to some of the Rab8 activation in stipulated cells. A notable shortcoming of this assay that became evident from multiple experiments, was the variable amounts of basal and activated GTP-Rab8 obtained in different experiments. This variation remained despite many repetitions and attempts to further standardise the assay and significant results were obtained by internally controlled quantification within each experiment (more information detailed in the Materials and Methods chapter). Nonetheless, it was not surprising to find both GRAB and Rabin8 contributing to Rab8 activation. Both GEFs are present in the cells and could be contributing to multiple pools of Rab8 functioning with different effectors in LPS activated cells, or they can act redundantly in the same Rab8 function.

In light of this, although Rab8 has multiple roles in trafficking (Peränen 2011), cellular processes such as macropinocytosis and ruffling - which require dynamic and rapid membrane internalisation and replenishment - did not seem to be affected by loss of either GEF (Figure 5.6). With clear membrane trafficking defects observed in other cells when either of these GEFs are deleted, seems to imply that in macrophages, there is another Rab or GTPase might have a similar membrane trafficking function to Rab8. Another possibility could be a single KO of either GRAB or Rabin8 was not sufficient to influence Rab8a's trafficking functions, as while decreased, a detectable level of GTP-Rab8 was still present in either KO cell line (Figure 5.5). Both GRAB and Rabin8 therefore remain as candidate GEFs for Rab8a in the project moving forward.

A second discriminating factor could be the role of GRAB or Rabin8 in Rab8a membrane association and thus I observed and measured GFP-Rab8a localisation in live cells after KO of GRAB or Rabin8. In many instances, GEFs are important for both

activating and targeting Rabs to relevant membranes for their function. An example being the targeted mislocalisation of mutated GEFs Rabex-5 or Rabin8 to mitochondrial membranes in Cos-7 cells, which in turn leads to the mistargeting of the respective Rabs - Rab5a from endosomes to mitochondria and Rab8a from endosomes and plasma membrane to mitochondria (Blümer and Rey *et al.* 2013). Surprisingly, in my experiments, deletion of either GRAB or Rabin8 as Rab8 GEFs, did not affect GFP-Rab8a localisation on ruffles, macropinosomes or tubules. Thus, the loss of these GEFs in my experiments did not demonstrate a noticeable loss of membrane targeting for Rab8a. This suggests that Rab8a remains bound to these membranes independently of these GEFs or that GRAB and Rabin8 are not solely responsible for recruiting and retaining Rab8a to macropinosome and surface ruffle membranes in macrophages. Research has shown that other factors including escort proteins, GDIs, post-translational modifications (prenylation and methylation) of Rab8a and Rab8b all contribute to targeted membrane binding (Leung and Baron *et al.* 2007). Additionally, we know that Rab8a is in a complex with the membrane protein LRP1 (Luo and Wall *et al.* 2018) which we believe is important for scaffolding the Rab8a-PI3Kγ complex on macropinosomes. Therefore, it is perhaps not surprising that the GEFs, in this instance, are not wholly responsible for Rab8 membrane targeting. I also measured the retention of GFP-Rab8a on the macropinosome membrane in live cells. By this measure also, neither GRAB nor Rabin8 was required to maintain a normal level of GFP-Rab8a on the macropinsomes. Thus, membrane retention time might also be dictated more by the LRP1 binding than by the GEFs. In future experiments, we could use the LRP1 KO cells to examine Rab8a membrane targeting and retention.

Given the similarities between GRAB and Rabin8, and the fact that both GEFs appear to activate Rab8 in response to LPS, this may indicate potential redundancy between these proteins within macrophages. Therefore, to address this, the obvious next step is to produce a double GRAB and Rabin8 KO CRISPR cell line to fully interrogate the roles these GEFs play in macrophages responding to LPS.

Chapter 6

Results 3: Investigating the roles of GRAB and Rabin8 in Rab8a associated TLR signalling

6. Results 3

Introduction

Macrophages employ pattern recognition receptors of the TLR family to detect molecular signals from different classes of pathogens, including bacteria and viruses (Akira and Takeda *et al.* 2001). Upon activation, ligand-bound TLRs, in combination with their signalling adaptors, initiate complex signalling pathways that result in transcriptional activation for the synthesis and secretion of inflammatory cytokines and chemokines. This immune response is a culmination of signal transduction events mediated by various kinases such as MAP kinases (MAPKs), Interleukin-1 receptor-associated kinases (IRAKs) and Akt downstream of TLRs (Gay and Symmons *et al.* 2014, Kawasaki and Kawai 2014). As mentioned in the previous chapters, work from our laboratory introduced the small GTPase Rab8a as a key player that contributes to signalling induced by TLRs (Luo and Wall *et al.* 2014), and we now know that this occurs directly through LRP1 as a crosstalk receptor to TLRs and also to scaffold Rab8a. Activated Rab8a then recruits PI3Kγ, which in turn recruits and activates the signalling kinase Akt to produce PI(3,4,5)P_3 from PI(4,5)P_2 (Bellacosa and Chan *et al.* 1998). Further to this, we showed that other kinases such as mTOR (Delgoffe and Pollizzi *et al.* 2011), are also activated downstream of Rab8a/PI3Kγ. Under the influence of LRP1/Rab8a/PI3K, phospho-Akt and phospho-mTOR are increased which go on to drive and modulate transcription of inflammatory cytokines, resulting in an anti-inflammatory or M2-like status (Wall and Luo *et al.* 2017). While several major components of this immune signalling complex have been described, the regulatory GEF that activates Rab8a for this particular pathway remains unknown.

Two Rab8 specific GEFs, GRAB and Rabin8, were found to have LPS-inducible interactions with Rab8a in RAW 264.7 macrophage cells (Figures 4.5B and C). I went on to generate CRISPR-Cas9 deletion of these proteins and was able to demonstrate that both GRAB and Rabin8 do function as Rab8 GEFs in immune activated macrophages (Figure 5.5). Imaging indicated that the absence of either GRAB or Rabin8 did not affect Rab8a membrane recruitment or retention at sites for TLR/Rab8a signalling on ruffles and macropinosomes (Figure 5.6). To determine whether GRAB and Rabin8 contribute to signalling downstream of Rab8a, I set out to measure LPS-induced signalling in control cells compared to Rabin8 and GRAB KO cells.

To measure and pinpoint the precise location of TLR-driven Akt signalling on macropinosomes, I employed an imaging approach to visualise in real-time the spatial-temporal localisation and distribution of the major signalling kinase Akt in live LPS-activated macrophage cells. David James and James Birchfield at the University of Sydney produced an enhanced fluorescent probe (TagRFP-Akt) to detect Akt in live cells, particularly after its activation and recruitment to membranes (Norris and Yang *et al.* 2017). This improved reporter replaces the eGFP with TagRFP, a brighter and more stable fluorophore with different structural properties that allows for improved recruitment dynamics. This study utilised this probe in murine fibroblast-derived adipocytes, demonstrating that despite encoding a human Akt gene, this construct can be used equally well in mouse cells as a signalling reporter. This probe was generously provided to us to examine Akt in LPS-stimulated macrophages. Live cell imaging was performed on RAW 264.7 cells expressing the TagRFP-hAkt1 probe, to visualise the real-time localisation of Akt after LPS stimulation and to explore the roles of GRAB and Rabin8 on this signalling pathway. The findings reported here reveal that in macrophages, GRAB and Rabin8 both contribute to Rab8a activation in response to LPS to facilitate downstream TLR signalling and that this signalling is occurring on macropinosome membrane domains.

Results

Deletion or overexpression of either GRAB or Rabin8 does not affect Rab8-associated TLR-signalling

In the first result chapter, I described previous work which demonstrated that the deletion of either Rab8a and its effector PI3Kγ decreases Akt signalling downstream of TLR-activation (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017) (see Figure 4.2). Having identified Rab8 GEFs, GRAB and Rabin8 as activators of Rab8a in LPS-stimulated macrophages (see Figure 5.5), I next aimed to test if genetic deletion of either one of these Rab8 GEFs impacted on PI3Kγ-dependent TLR-signalling function. GRAB KO, Rabin8 KO and control RAW 264.7 cells were treated with LPS over a 60 min time course and their lysates were analysed by Western blotting to compare levels of Akt phosphorylation. In control cells, Akt was phosphorylated in response to LPS resulting in increased levels of phospho-Akt from 15 to 60 min in control cells (Figure 6.1A). A side-by-side quantification and comparison with control cells demonstrated that GRAB KO and Rabin8 KO cells had similar patterns of Akt activation/phosphorylation in response to LPS, with peaks in phospho-Akt at 15 and 30 min (Figure 6.1A). There was no statistical significance the LPS-mediated Akt response across either GRAB KO or Rabin8 KO cells when compared with control cells (Figure 6.1A graph). Additionally, deletion of either GRAB or Rabin8 had no effect on phospho-ERK1/2 levels induced by LPS which was consistent with previous observations in Rab8a KO cells (see Figure 4.1). Therefore, despite a decrease in Rab8a activation upon LPS stimulation (see Figure 5.5), knockout of either GRAB or Rabin8 did not affect Akt signalling downstream of TLR4.

As an additional test, recombinant GFP-GRAB or GFP-Rabin8 were transiently overexpressed in RAW 264.7 macrophages and cells were activated with LPS over the same time course. Cells overexpressing GFP-GRAB had similar phospho-Akt and phospho-ERK1/2 responses to LPS when compared to the control cells, despite a significant overexpression of GFP-GRAB protein (Figure 6.1B). A similar pattern of Akt phosphorylation was observed in GFP-Rabin8 overexpressing cells when compared to control cells (Figure 6.1C). These results indicate that neither the deletion nor the overexpression of either GRAB or Rabin8 is sufficient to affect Rab8a-driven TLR

signalling. This result was surprising given that both GEFs contributed to Rab8 activation in response to LPS.

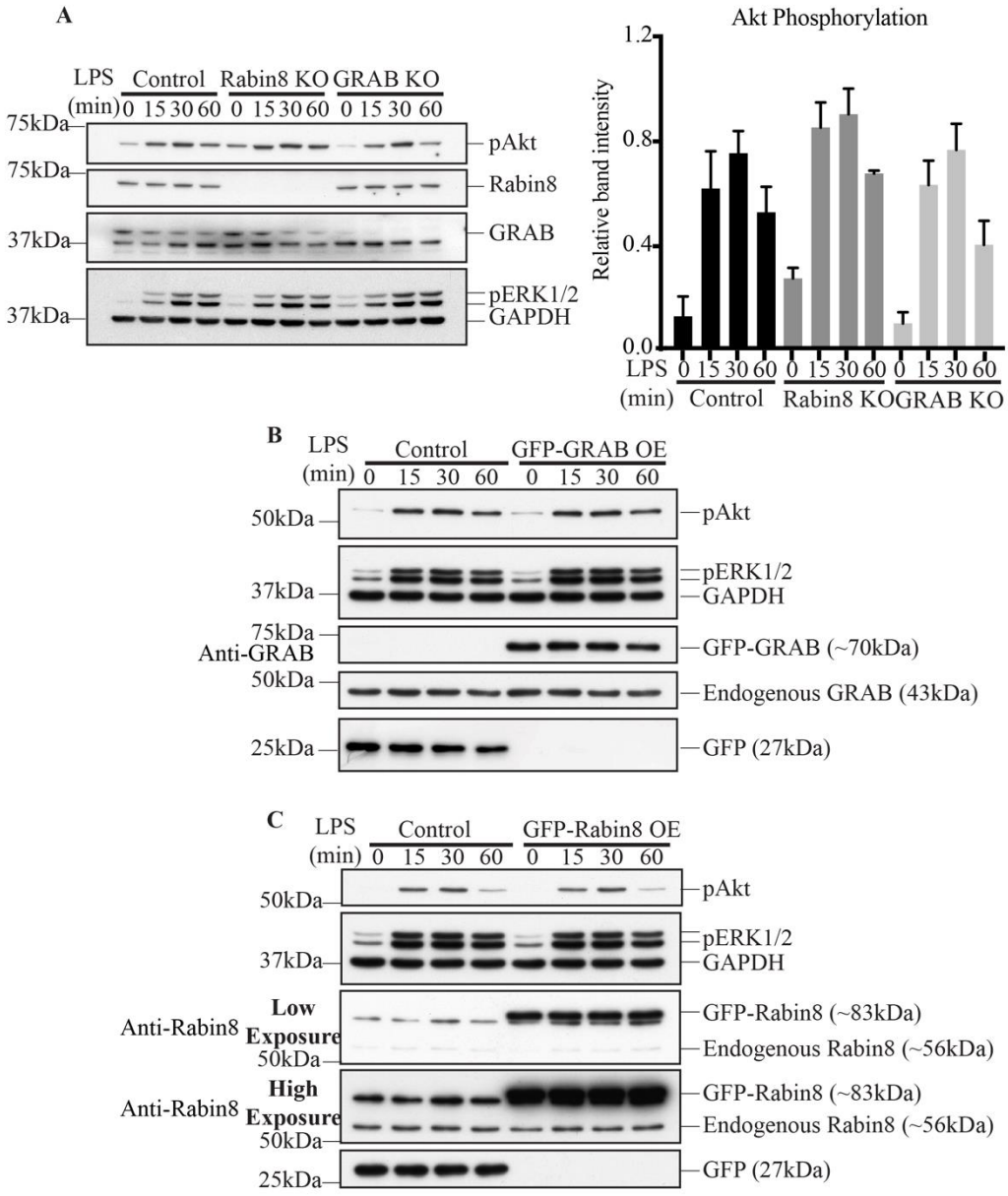


Figure 6.1. Absence or overexpression of either Rabin8 or GRAB does not affect Rab8a-associated TLR signalling. *(A) Immunoblots showing the comparative levels of Akt (Ser473) phosphorylation, using phospho-specific antibodies, between the lysates of control, Rabin8 KO and GRAB KO cells treated with LPS over a 60 min time course. The immunoblots shown here are representative and levels of Akt phosphorylation were quantified using the densitometry ratio between the band intensities of GAPDH and phospho-Akt levels. Significance was measured via two-way analysis of variance (ANOVA) (n= 3). (B/C) Levels of Akt phosphorylation was also assessed in RAW 264.7 cells transiently overexpressing either (B) GFP-GRAB or (C) GFP-Rabin8 and treated with LPS over a 60 min time course.*

The striking similarities between GRAB and Rabin8 in both localisation and LPS-dependent Rab8 GEF activity in macrophages suggests that there might be a compensatory or redundant relationship between these proteins. This is also consistent with reports in literature showing that GRAB and Rabin8 can function in the same cellular pathways (Furusawa and Asada *et al.* 2016, Homma and Fukuda 2016). Therefore, as an approach to examine possible complementary roles for GRAB and Rabin8, I generated a double KO cell line in which both GRAB and Rabin8 are deleted.

Double knockouts reveal additive functions for GRAB and Rabin8 in LPS-associated activation

To investigate redundancy or overlap in the roles of GRAB and Rabin8, I repeated the CRISPR-Cas9 KO protocols described above using constructs to delete Rabin8 in the GRAB KO cell line. Clonal cell lines were produced that displayed varying levels of Rabin8 protein expression (Figure 6.2A). After rounds of testing, several clonal cell lines were observed to have no or greatly decreased Rabin8 protein expression compared to the controls (an example shown in Figure 6.2A, colony 1), effectively making them a double Rabin8 and GRAB KO cell line (double KO). Initial characterisation tests showed that these double KO cells had no growth defects and were morphologically similar to control wild-type RAW 264.7 cells (data not shown).

The Rab activation assay was performed on double KO cells alongside the original GRAB KO cells and control cells, all of which were subjected to a 60 min LPS time course. The assay captured increasing levels of GTP-Rab8 over the LPS time course in control cells with a peak at 15-30 min (Figure 6.2B), consistent with earlier results (see Figure 4.11D). The GRAB KO cells had a noticeable decrease in LPS-induced GTP-Rab8 at the 15- and 30-min marks, whereas in the double KO cells, a greater reduction of GTP-Rab8 was observed across the whole time course, surprisingly including the untreated cells at time 0 (Figure 6.2B). Phospho-ERK is patently induced by LPS across all cell lines with no significant difference in the double KO cells compared to control cells. Given the enhanced reduction in the levels of GTP-Rab8 in the double KO, this agrees with previous work suggesting that TLR4-induced ERK1/2 signalling is predominantly independent of Rab8 activity.

For ease of quantification, the activation assay was repeated on these cell lines using a single time point for LPS treatment (15 min). Notably, the results highlight that not only was LPS-induced Rab8 activation significantly impaired in both the GRAB KO and double KO cells, but the basal level of GTP-Rab8 in the double KO was also significantly decreased when compared to the control. Taken together, these results suggest that both GRAB and Rabin8 not only contribute additively to LPS-dependent Rab8 activation, but also act to maintain a basal level of constitutively active Rab8 in these cells. Thus, I show in terms of Rab8 activation in response to LPS/TLR4, genetic deletion of both GRAB and Rabin8 has a measurable effect, over and above that seen with KO of GRAB alone. This stands as important new evidence for cooperative GEF activity shared by GRAB and Rabin8 for the activation of Rab8.

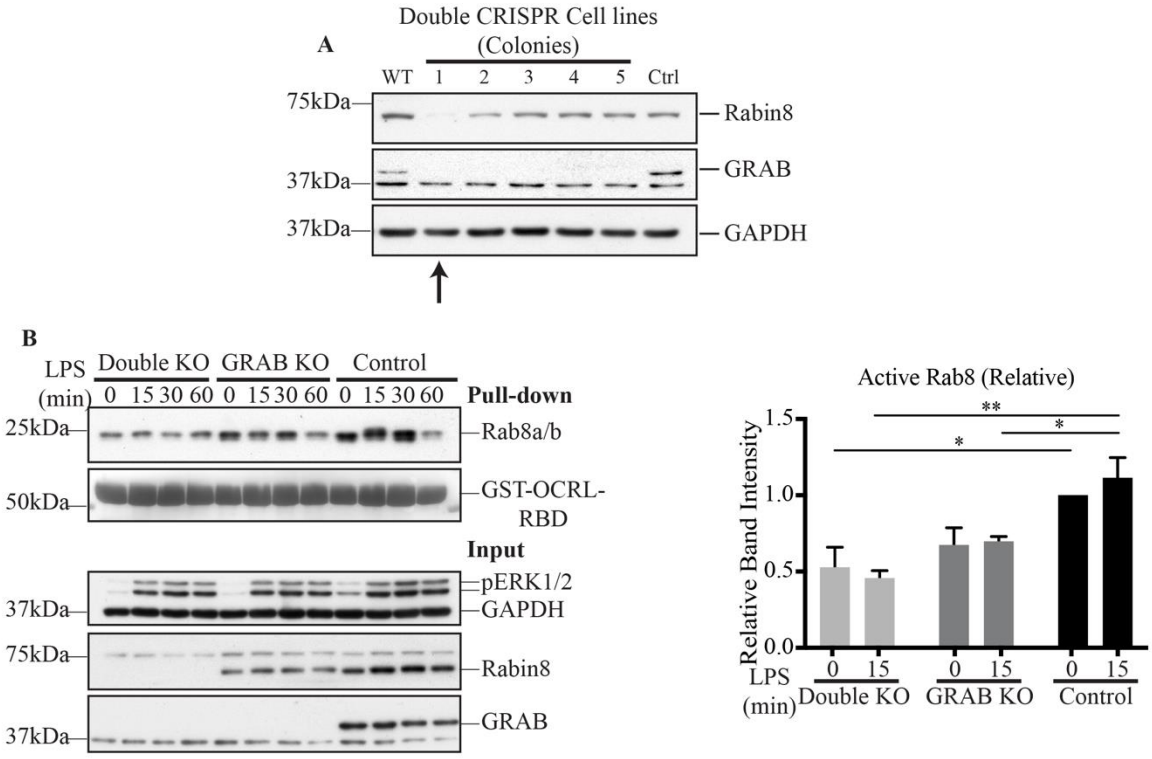


Figure 6.2. Both GRAB and Rabin8 contribute additively to LPS induced Rab8 activation. *(A) Western blot screen for protein expression levels of both Rabin8 and GRAB in Double KO CRISPR-Cas9 KO colonies demonstrating a positive homozygous knockout (denoted by arrow, colony 1). Wild-type control RAW 264.7 lysate is referred to as 'WT', while a CRISPR empty vector control colony (transfected with the CRISPR RNP but no guide RNA) is referred to as 'Ctrl'. (B) Immunoblots showing the relative amount of Rab8 that was pulled-down using GSH-Sepharoase beads bound with GST-OCRL-RBD from the lysate of double KO, GRAB KO and control cells treated with LPS over a 60 min time course. Repeat activation assays of untreated and 15 min LPS treatments was used for quantification. Levels of active Rab8 were quantified by using the densitometric ratio between the band intensities of captured Rab8 and GAPDH. Significance was measured using via two-way analysis of variance (ANOVA) (*P<0.05, **P<0.01, n= 3).*

Analysis of Rab8a membrane localisation and retention in the GRAB and Rabin8 double knockout cells

Now, with a cell line depleted of both Rab8 GEFs, I next examine whether Rab8 localisation and membrane association is impacted in these cells. Confocal live cell imaging was performed on LPS-treated double KO and control cells transiently transfected with GFP-Rab8a. Initial observations showed no striking morphological difference between the double KO and control cells, akin to imaging of the single KO cells (see Figure 5.6) where GFP-Rab8a still localises to surface ruffles and is enriched on early macropinosomes and resulting tubules. Thus, the localisation of GFP-Rab8a in the double KO cells was similar to control cells (Figure 6.3A). As an additional assessment to measure Rab8a membrane retention, I tracked GFP-Rab8a positive macropinosomes in both the control and double KO cells and measured the time GFP-Rab8a spent enriched on these membranes. Quantification demonstrated no significant change in GFP-Rab8a membrane retention and enrichment on macropinosomes between the double KO and control cells (Figure 6.3A graph). Even after the deletion of both GRAB and Rabin8, GFP-Rab8a localisation, recruitment and enrichment on membranes remained unaffected.

As these results were obtained by visualising overexpressed recombinant GFP-Rab8a, I wanted to assess the membrane association of endogenous Rab8 in these double KO cells as an additional confirmation of this result. To do this, I performed a membrane fractionation assay (see Materials and Methods) in which LPS-activated cells were mechanically lysed for preparation of post-nuclear supernatants which were centrifuged to obtain total membrane and cytosol fractions. Gels and Western blots show actin and flotillin as cytosol and membrane markers respectively and reveal that Rab8 is found mostly in membrane fractions compared to cytosol, and the relative membrane amount is similar across control and double KO cell fractions (Figure 6.3B). Therefore, Rab8 membrane recruitment and retention appear to be independent of both GRAB and Rabin8.

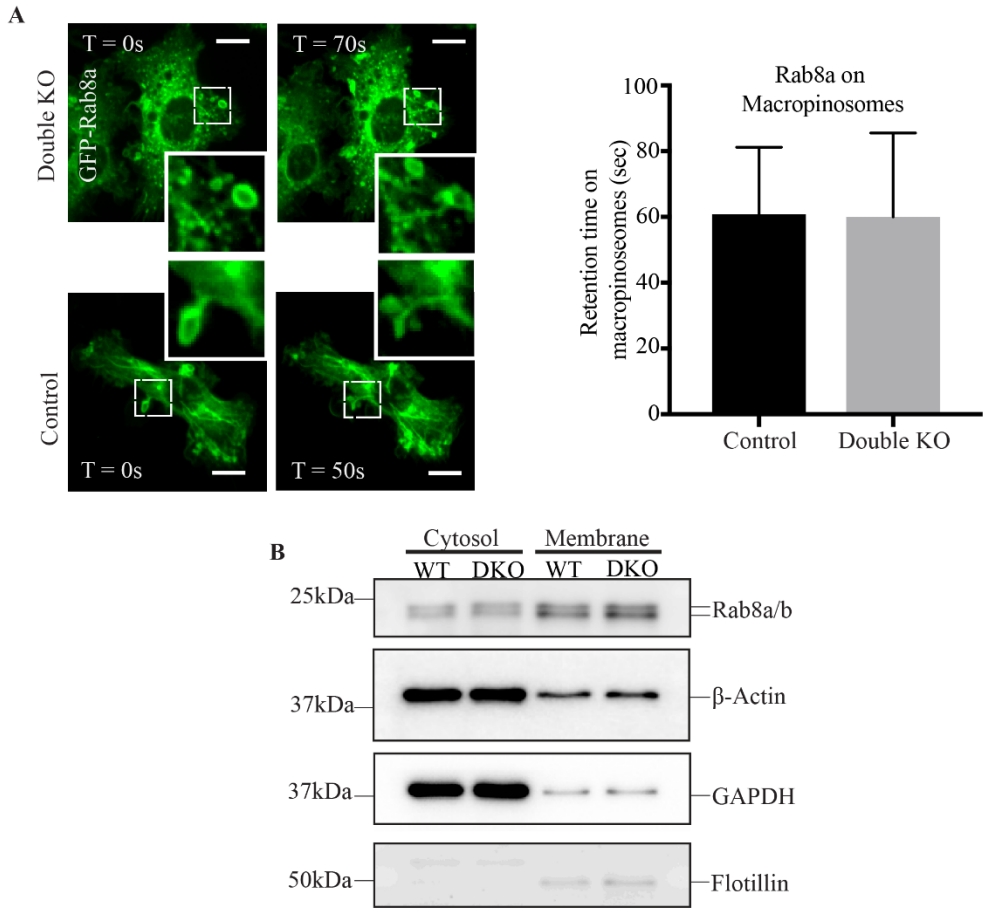


Figure 6.3. Neither Rabin8 nor GRAB controls Rab8 membrane recruitment, localisation or enrichment in macrophages. *(A) Live cell confocal spinning disc imaging of LPS-treated double KO and control cells overexpressing GFP-Rab8a showing recruitment to macropinosomes and tubules. Quantification of Rab8a retention on macropinosomes was measured by the number of timeframes Rab8a is spent enriched on each macropinosome prior to tubulation or dissociation. 5 cells of each cell line were used for the quantifications (n=5). Scale bars, 10µm. (B) Control RAW 264.7 and Double KO cells were treated with LPS (30 min) and a membrane fractionation assay was performed. The resulting membrane and cytosolic fractions from both cell-lines were analysed by immunoblotting. Immunoblots showing the relative levels of Rab8 in both fractions along with the soluble and membrane markers GAPDH and flotillin respectively with β-actin as a loading control.*

GRAB and Rabin8 both contribute to GTP-Rab8a-mediated Akt signalling.

I next assayed Rab8a mediated LPS/TLR signalling in the double KO cells. Over an LPS time course, the double KO cells display reduced levels of phosphorylated Akt compared to control cells, and quantification revealed that this is a significant decrease at the peak times (15 and 30 min) of Akt activation (Figure 6.4A). I also measured the phosphorylation of signalling kinases downstream of Akt, including the proline-rich Akt substrate of 40kDa (PRAS40) and the mTOR substrate p70S6 kinase (Figure 6.4B) (Luo and Wall *et al.* 2014). Both kinases showed reduced levels of phosphorylation in

double KO cells and these reductions were also significant according to the quantification of both proteins over three experiments (Figure 6.4B graph). There was no significant change in phospho-ERK by comparison, which supports previous results indicating that Rab8 is not a major contributor to TLR-associated ERK1/2 phosphorylation (Wall and Luo *et al.* 2017). Thus, the double KO cells indeed show clear changes in LPS-induced TLR4 signalling.

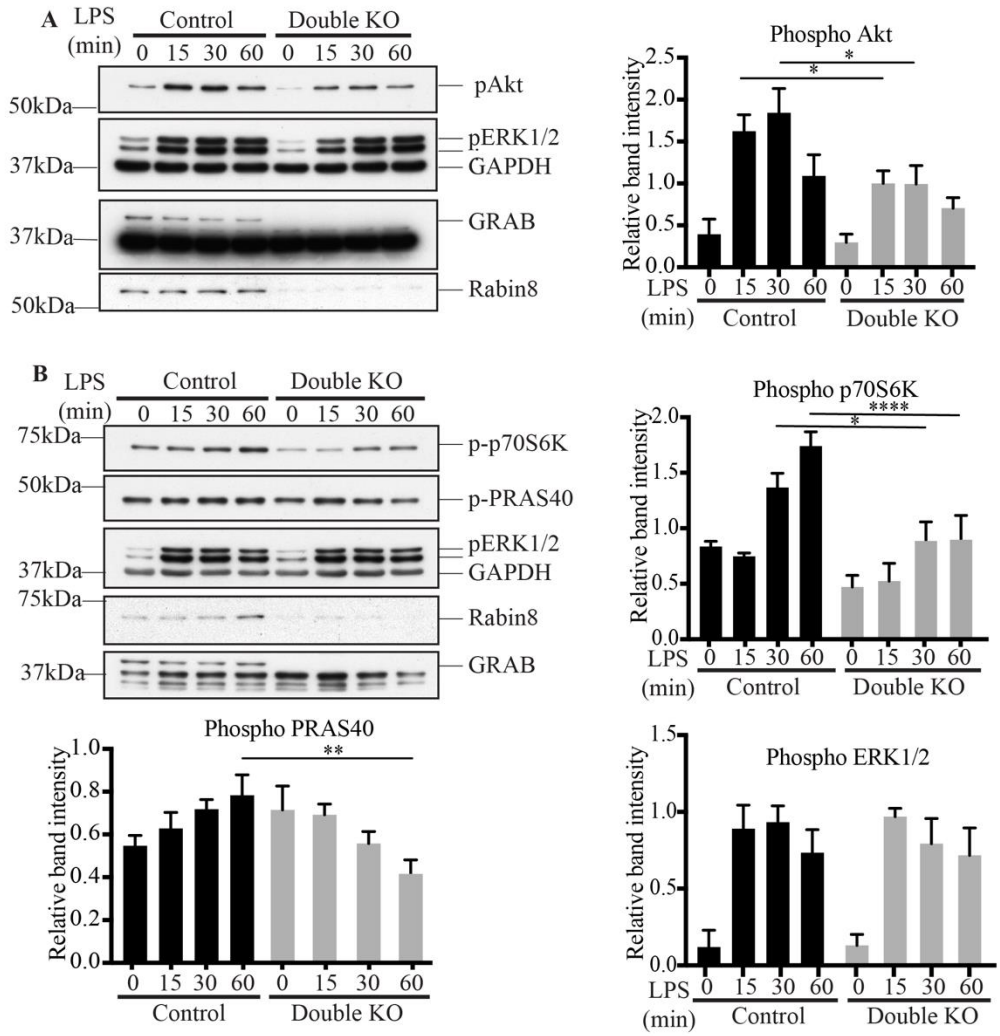


Figure 6.4. Loss of both GRAB and Rabin8 perturbs Rab8a-associated TLR signalling. *Control and Double KO cells were treated with LPS over a 60 min time course and the samples were analysed for levels of phosphorylation of key signalling substrates. Representative immunoblots of the phosphorylation levels of (A) Akt (Ser473) and (B) p70S6K (Thr389) and PRAS40 (Thr246). Quantification of immunoblots was performed using the densitometric ratio between the band intensities of phosphorylated proteins and GAPDH. The gels are representative, and significance was measured via two-way analysis of variance (ANOVA), (*P<0.05, **P<0.01, ****P<0.0001, n= 3 each).*

To ensure these signalling changes are specifically due to the loss of GRAB and Rabin8 function, I reintroduced GFP-tagged versions of both proteins into the double KO cells by transient co-transfection and the expression of GFP-GRAB and GFP-Rabin8 in these rescue cells was confirmed by Western blot (Figure 6.5). The double KO cells were transfected with GFP alone as a negative control (Figure 6.5). Akt signalling was measured after LPS activation (15 min) and it demonstrates increased phosphorylation of Akt in the rescue cells compared to the controls. This confirms that reduced TLR4 signalling in the double KO cells is specifically due to the loss of GRAB/Rabin8. We can now conclude that Rab8a function in this TLR signalling pathway requires *both* GRAB and Rabin8 as GEFs, for optimal Rab8a activation and signalling.

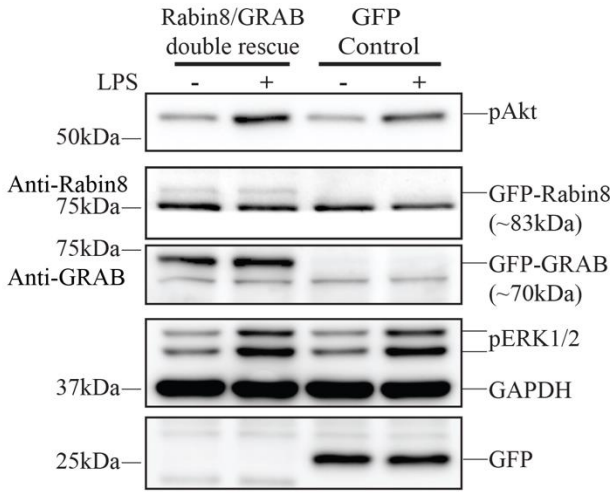


Figure 6.5. Reintroduction of both Rabin8 and GRAB recovers LPS-induced Akt phosphorylation in double KO macrophages. *(A) Double KO cells were transiently transfected with either an empty GFP construct (Control) or co-transfected with GFP-Rabin8 and GFP-GRAB and treated with LPS (15 min). Immunoblots comparing the levels of phosphorylated Akt and ERK1/2, along with Rabin8, GRAB, GFP and GAPDH between control and rescue double KO cells.*

Visualising signalling through Akt enrichment on macropinosome membranes

In order to pinpoint the location of Rab8a-PI3Kγ-mediated signalling and to further test the involvement of GRAB and Rabin8, I introduced an image-based method to visualise Akt membrane recruitment in response to receptor activation. The recombinant TagRFP-hAkt1 reporter (Norris and Yang *et al.* 2017) introduced previously can be used to pinpoint sites of Akt enrichment in response to receptor signalling (Figure 6.6A). I transfected cells with TagRFP-hAkt1 to perform live-cell spinning-disc imaging. Based on initial experiments, I co-transfected cells with soluble

GFP as a marker of the cytosol, to discriminate between soluble and membrane-bound TagRFP-hAkt1. The representative images of live-cell microscopy on LPS-treated macrophages depicts overlapping pools of cytoplasmic TagRFP-hAkt and soluble GFP (Figure 6.6B). In addition, TagRFP-hAkt is enriched around macropinosomes at the leading edge of the cell (inset). By simultaneous capture of both RFP and GFP channels, a ratiometric heat map of the TagRFP-hAkt labelling relative to the GFP background was produced, highlighting the most intense staining due to specific enrichment of the Akt probe on the macropinosome membranes, compared to other sites in the cell. This provides new evidence to support earlier findings from our group indicating that macropinosomes are signalling centres in activated macrophages (Wall and Condon *et al.* 2018).

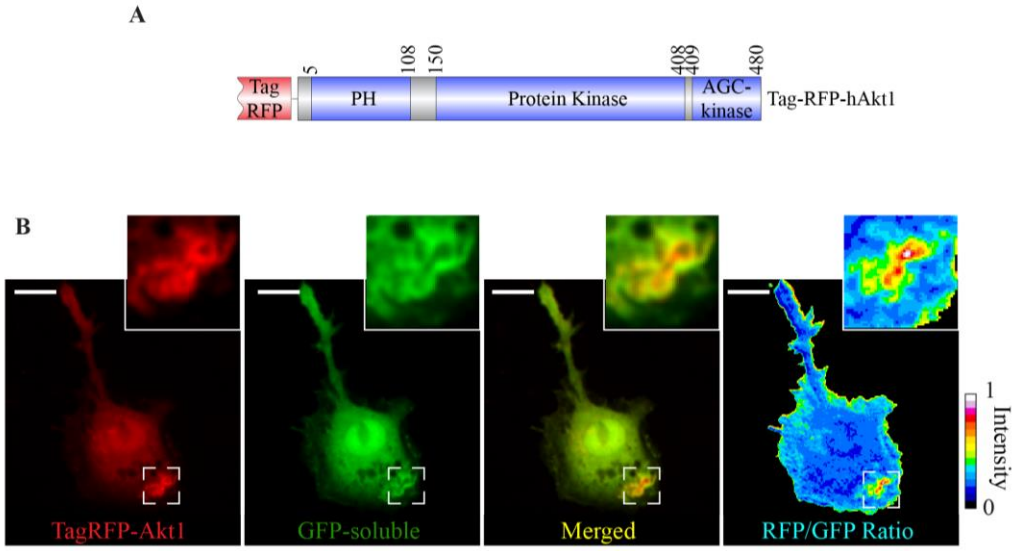


Figure 6.6. Optimisation of imaging protocols for demonstrating live cell Akt enrichment on macropinosome membrane domains. *(A) Schematic of TagRFP-hAkt1 probe used to visualise Akt enrichment on signalling membranes. (B) Image from confocal live cell imaging of an LPS-treated RAW 264.7 cell co-expressing TagRFP-Akt1 and soluble GFP. Using GFP as a soluble protein marker, a ratio metric 'heat-map' was generated by dividing the RFP signal over GFP, which allows for a clearer visualisation of Akt membrane enrichment on macropinosomes (inserts) as an analysis tool.*

Rab8 GEFs are involved in Akt signalling on macropinosomes

The TagRFP-hAkt1 reporter was then used to bridge Akt signalling to GTP-Rab8a-mediated complexes downstream of TLRs. Live cell imaging was performed on the double KO and control cells after transient co-expression of GFP and the TagRFP-hAkt1 reporter. Cells were imaged untreated and then activated with LPS to continue

live cell imaging of the same cells with simultaneous capture of both RFP and GFP channels. In the absence of LPS, small flashes of TagRFP-hAkt1 appeared randomly and continuously over the plasma membrane indicating basal cell signalling activity. Both control and double KO cells performed constitutive macropinocytosis which was captured in the imaging with some enrichment of TagRFP-hAkt1 to the macropinosomes (not shown).

Upon LPS activation, macropinocytosis was upregulated in both control and KO cells. The LPS treated control cell demonstrated enrichment of TagRFP-hAkt1 (at 105s) upon closure of the macropinosomes (Figure 6.7, left panels). Following macropinosome closure, the Akt signal is lost indicating the inherent dynamism and duration of the signalling period. In double KO cells, the macropinosomes still form (over a 120s interval, Figure 6.7, right panels); however, a significantly reduced proportion of macropinosomes show enrichment of TagRFP-hAkt1 (Figure 6.7, graph). Notably, the flashes of Akt are still present in the cells as confirmation of the reporter activity. This result shows that the control and double KO cells have similar levels of basal Akt reporter activity. Counting macropinosomes under these conditions in both cell types confirmed that there is a significant loss of TLR-mediated Akt enrichment on macropinosomes in the double KO cells. This finding agrees with previous biochemical analysis that demonstrated a reduction in Akt phosphorylation in the double KO cells in response to LPS (Figure 6.4A). Here I confirm through live-cell microscopy that Rab8a-controlled Akt signalling is occurring on macrophage macropinosomes, denoting these cellular compartments as important sites for TLR and Rab8a-mediated signalling.

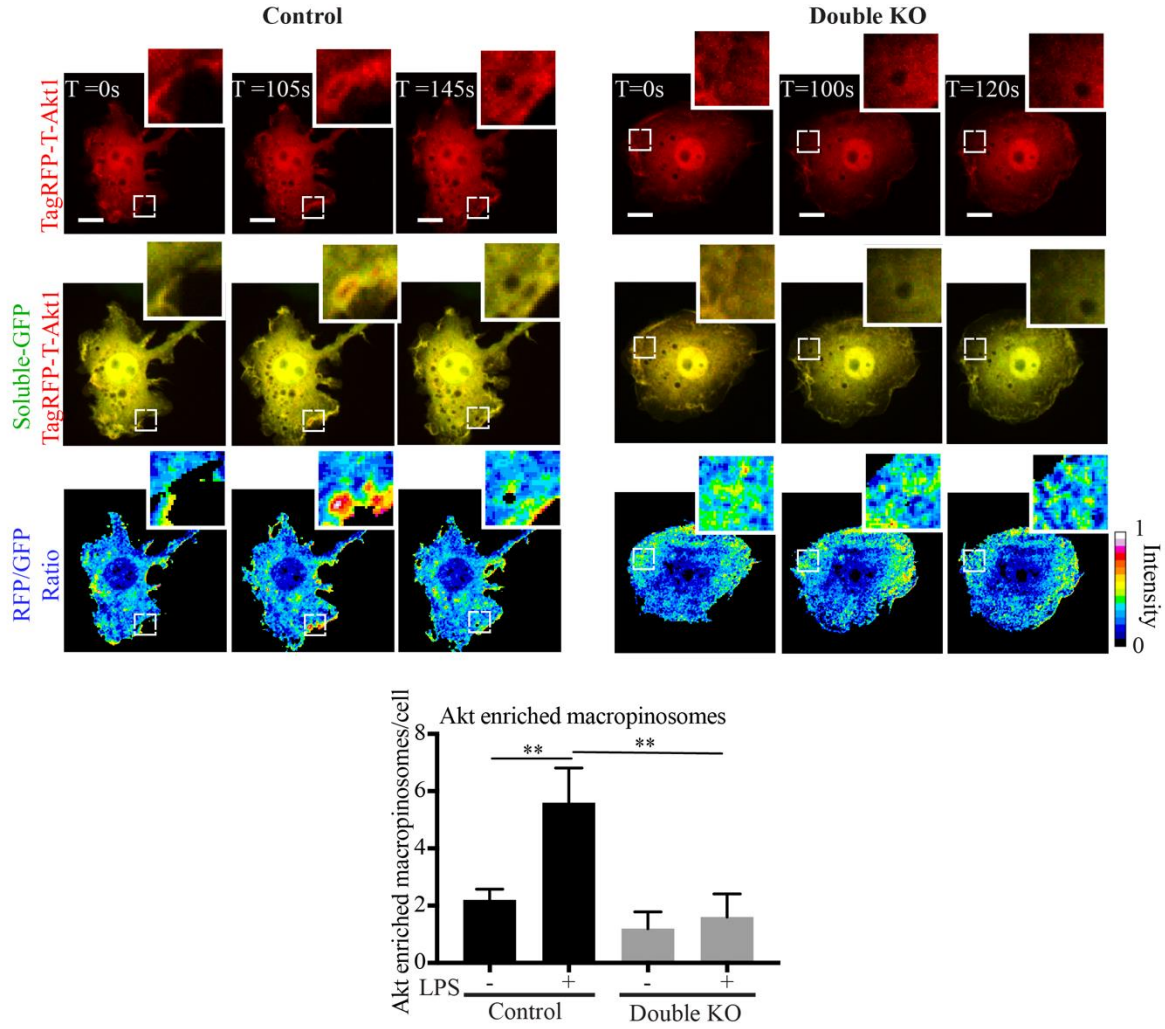


Figure 6.7. The Rab8 GEFs GRAB and Rabin8 jointly controls TLR-associated Akt immune signalling on macrophage macropinosome membranes. *Control and Double KO cells were transiently co-transfected with C1-GFP and TagRFP-T-Akt1 and live cell spinning disc confocal microscopy was performed. A ratiometric 'heat map' of Akt intensity was generated by using the ratio of TagRFP-Akt1 signal intensity to the soluble GFP to identify regions of Akt membrane enrichment. The cells were imaged with and without LPS. Macropinosomes with Akt enrichment were counted as number of events per cell, expanded panels show examples of macropinosomes observed with and without Akt enrichment in the control and Double KO cells respectively. Movies were taken over 15 min with 5 sec intervals and quantification was performed by counting the number of Akt enriched macropinosomes observed per cell over a sample of 5 cells for each cell line. Significance was measured via Student's t-test. (*P<0.05, **P<0.01 and n= 5 cells). Scale bars, 10μm.*

Discussion

In this chapter, the roles of two Rab8 GEFs, GRAB and Rabin8, were examined for their possible involvement in Rab8a-mediated signalling downstream of TLR4/LRP1. Previous work in our group revealed that this pathway activates Rab8a to recruit the effector PI3Kγ, which in turn modulates membrane lipids for the recruitment and enhanced activity of signalling kinases, Akt and mTOR (Luo and Wall *et al.* 2014). Ultimately, this signalling is important for driving a biased cytokine response to downregulate inflammation.

The experiments here first tested the loss of individual GEFs, GRAB or Rabin8 in CRISPR KO cell lines. However, LPS-induced signalling assayed in these KO cell lines showed no differences compared to control macrophages. This was surprising, given that previous results revealed a decrease in LPS-induced Rab8a activation in both KO cell lines (Figure 5.3). Thus, by this measure, both GRAB and Rabin8 have GEF activity towards Rab8 in macrophages, but this might be for Rab8 functions unrelated to TLR signalling. The next approach then involved making a CRISPR double KO line, using the GRAB KO as a background for additional deletion of Rabin8. This was successful and double KO lines were produced which show no detectable expression of either GEF (Figure 6.2).

When the double KO line was assayed, there was, pleasingly, a step wise reduction in Rab8 activation from the single to the double loss of GEF expression. The GRAB and Rabin8 double KO had significantly less activation of Rab8 and also reduced basal activation in unstimulated cells. The signalling results also showed, now, a clear change in the phosphorylation of Akt, PRAS40, p70S6K in response to LPS (Figure 6.4). These changes implicate GRAB and Rabin8 as drivers of Rab8 activity upstream of TLR-associated Akt and mTOR signalling. In the past we have shown that Rab8 and PI3Kγ influence the LPS induced phosphorylation of Akt and of mTOR (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). Previous studies have shown that Rab8a-recruited PI3Kγ goes on to bias the synthesis and secretion of pro- and anti-inflammatory cytokines through Akt/mTOR. Although cytokine responses remain to be measured directly, we predict that the double KO of GRAB and Rabin8 would similarly

alter the cytokine output from LPS-induced macrophages by driving pro-inflammatory cytokine production resulting in a hyper inflammatory status.

The reintroduction of both GFP-GRAB and GFP-Rabin8 in the double KO cells rescued the phosphorylation of Akt in response to LPS, confirming that this phenotype is specifically due to the loss of these proteins (Figure 6.5). These results demonstrate that GRAB and Rabin8 are indeed GEFs for Rab8a in the TLR signalling pathway. GRAB and Rabin8 may additionally have roles in other Rab8-mediated cellular functions which may contribute to the basal levels of Rab8a activation seen in these cells.

A notable factor in these experiments is that only the double KO of both GEFs produced a measurable change in Rab8 mediated TLR signalling, while the loss of either GEF alone did not impair Akt signalling. In other cell types, studies have mostly assayed trafficking roles of Rab8 where knockdown or knockout of a single GEF had a measurable effect. More specifically, knockdown or mutagenesis of Rabin8 had a considerable impact in ciliogenesis or neurite outgrowth (Chiba and Amagai *et al.* 2013, Homma and Fukuda 2016). Furthermore, another study demonstrated that knockdown of GRAB reduced axon outgrowth in mouse neuron cells (Furusawa and Asada *et al.* 2016). A possible explanation for this difference could be in the relative amount of Rab8 activation required for trafficking versus signalling functions. Vesicle trafficking may be readily perturbed by loss of a single GEF because it requires a near maximal level of active Rab to recruit effectors for large scale vesicle deployment or movement. Our live imaging highlights that TLR-mediated Akt signalling occurs in very short local bursts upon PI3K recruitment, and this may require only small local levels of active Rab8a. Consequently, knockout of both GEFs was necessary to perturb active Rab8 below activation threshold. This is also consistent with the stepwise levels of GTP-Rab8 recovered after single and double GEF KO (Figure 6.2B). The effects of GRAB and Rabin8 are most likely to be additive, rather than compensatory since there was no change in the protein levels of either GRAB or Rabin8 in response to the opposite knockdown (Figure 6.1). This observation hints to a functional overlap between GRAB and Rabin8, jointly regulating Rab8a TLR-signalling in macrophages.

Following Rab8a activation is signalling through the Akt/mTOR pathway for cytokine modulation through the effector PI3Kγ. The serine/threonine kinase Akt promotes cell survival and proliferation, and it has long been studied for its association with various diseases such as cancer and diabetes (Manning and Cantley 2007). In our studies, Akt emerges as an important signalling kinase during TLR-activation in macrophages (Luo and Wall *et al.* 2014); where it is recruited and signals from upon TLR activation is poorly understood. In macrophages, Akt signalling is difficult to detect since it occurs at low levels and is very dynamic. Whereas in Cos-7 cells for example, endogenous Akt can be readily immunostained all over the plasma membrane in response to EGF receptor activation (Wang and Wu *et al.* 2006). Similarly in adipocytes, insulin stimulation induces marked recruitment of Akt to the plasma membrane where it can be detected by membrane fractionation (Carvalho and Eliasson *et al.* 2000). David James and James Birchfield at the University of Sydney designed an Akt reporter that allows detection of Akt in live cells, which they used to demonstrate dramatic and dynamic oscillations of insulin-induced Akt recruitment to the plasma membrane in 3T3-L1 adipocytes (Norris and Yang *et al.* 2017). By utilising this fluorescent Akt1 reporter, I was able to detect and localise TLR-driven Akt recruitment in live macrophages for the first time (Figure 6.6B). Unlike the sustained Akt responses to EGF and insulin (Norris and Yang *et al.* 2017), our live imaging showed very small focal flashes of Akt on macrophage plasma membranes and forming macropinosomes. The live imaging showed that macropinosomes internalising from the cell surface are a key site for TLR-induced Akt and this was further confirmed by loss of Akt labelling in double GEF KO cells. Moreover, this data corroborates earlier findings from our group using a Rab8a FRET biosensor to show that Rab8a is indeed activated on macropinosome membranes (Wall and Condon *et al.* 2018). Together these findings place a firm focus on the early macropinosome as a key site for GRAB/Rabin8/Rab8a-mediated Akt signalling in response to TLR4 activation in macrophages.

Here, I define a newly found joint function of GRAB and Rabin8 in regulating macrophage TLR-associated Akt signalling on macropinosome membranes through controlling Rab8 activation. I describe an expansion of the Rab8a complex that participates in TLR signalling in macrophages by identifying the GEFs, GRAB and

Rabin8, as modulators for activating Rab8a in this context (Figure 6.8). These findings highlight the mechanistic importance of Rab regulation in modulating macrophage responses in infection and inflammation.

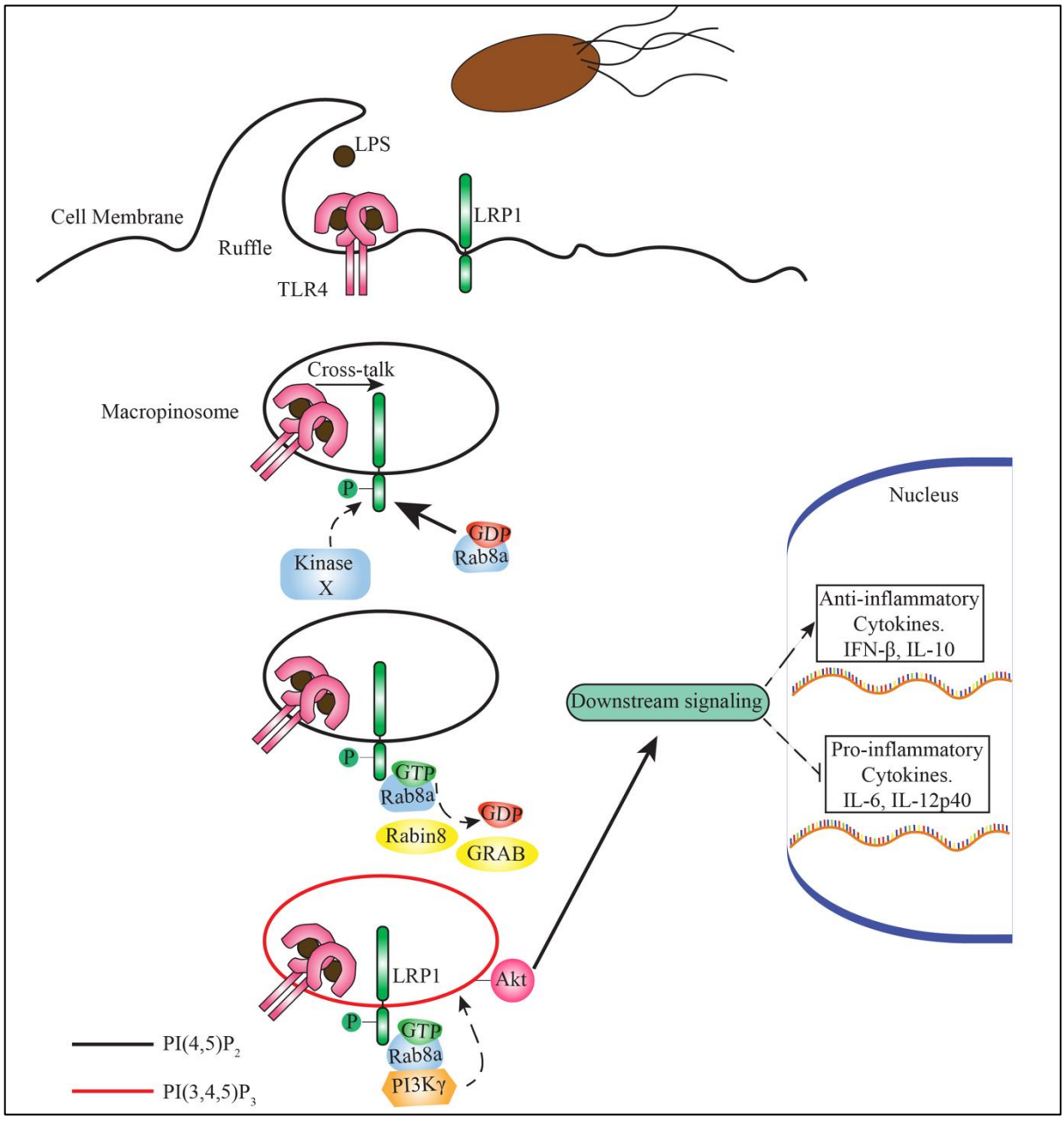


Figure 6.8. TLR4-associated Rab8a activation and signalling is jointly regulated by the GEFs GRAB and Rabin8. *The new proposed TLR4-LRP1-Rab8a signalling pathway. This introduces the Rab8 regulatory GEFs, GRAB and Rabin8, as activators of Rab8a for the recruitment of PI3Kγ, and can now be regarded as new components of the LRP1-Rab8a complex. Based on findings in this project, I propose that upon GEF-driven activation of Rab8a, PI3Kγ phosphorylates and converts PI(4,5)P2 to PI(3,4,5)P3 on macropinosome membranes for the recruitment and phosphorylation of Akt, facilitating a biasing of downstream immune signalling and cytokine secretion towards an anti-inflammatory state.*

Chapter 7

Results 4: Investigating regulators of Rabin8 in macrophage TLR pathways

7. Results 4

Introduction

Rab8 is a ubiquitous and multifunctional GTPase that is able to interact with numerous effectors (Peränen 2011). Therefore, regulatory mechanisms are needed to discriminate between these effectors to ensure the appropriate Rab-effector interaction takes place to elicit a desired function. A key part of this regulatory machinery controls the recruitment and activation of the Rab GEF on relevant membrane domains, which in turn, dictate the spatio-temporal activation of the Rab for location and context specific effector recruitment (Müller and Goody 2018). In the previous chapters, I identified two Rab8 GEFs, GRAB and Rabin8, which jointly contribute to modulating Rab8 activation and its downstream effector, PI3Kγ, for TLR4-associated signalling on macropinosome membranes. Identifying the proteins involved in the recruitment and activation of these Rab8 GEFs, would further expand the regulatory network controlling TLR4-Rab8a-PI3Kγ signalling in macrophages. Through recent collaborations with the Guo Laboratory (University of Pennsylvania), we have access to Rabin8 constructs and studies from the Guo group provide insights into Rabin8 activation that might be relevant in our context (Wang and Ren *et al.* 2015). Therefore, the experiments in this chapter have a refined focus on Rabin8 and on identifying potential regulatory partners for this particular Rab8 GEF in LPS activated mouse macrophages.

One known mechanism for regulating GEF activity and specificity is through kinase driven phosphorylation of serine and/or tyrosine residues near the GEF domains of these proteins, which can either promote or inhibit their interactions with their cognate GTPase (Cherfils and Zeghouf 2013, Patel and Karginov 2014, Kulasekaran and Nossova *et al.* 2015). Wei Guo and colleagues demonstrated that Rabin8 exists in an auto-inhibited state, which can be relieved via the phosphorylation of four serine residues (16, 19, 247 and 250) adjacent to its functional GEF domain. Phosphorylating these residues causes Rabin8 to enter an ‘open’ state allowing Rab8 to interact with its GEF domain (Wang and Ren *et al.* 2015). Furthermore, ERK1/2 was identified as the kinase responsible for phosphorylating these residues to activate Rabin8 during EGF signalling and functionally ERK1/2 phosphorylation was found to influence Rab8-associated transferrin recycling in retinal pigment epithelial cells (Wang and Ren *et al.*

2015). Given that ERK1/2 is also markedly activated in LPS-treated macrophages (see Figure 4.2), I set out to examine if ERK1/2 could be likewise responsible for activating Rabin8 GEF activity to facilitate TLR4-Rab8a signalling. Therefore, preliminary studies described here used an ERK1/2 inhibitor and our GEF KO cells in signalling experiments.

Another important regulatory mechanism for modulating GEF function is through the specific and targeted recruitment of these proteins to the appropriate membrane domains for Rab activation. While GEFs can be targeted individually to membranes, an emerging concept is that of Rab cascades, whereby the GEF of one Rab is recruited as an effector of another upstream Rab (Mizuno-Yamasaki and Rivera-Molina *et al.* 2012). Having such cascades involved in trafficking and signalling on macrophage membranes is an attractive but yet unproven concept, since sequences of multiple Rabs are known to be recruited for the formation of macropinosomes and phagosomes (Egami and Taguchi *et al.* 2014, Yeo and Wall *et al.* 2016). The intervening GEFs could quite nicely coordinate these GTPase arrays. For instance, Rab13, is largely recruited and localised to macrophage surface ruffles and is responsible for the formation of large LPS-induced macropinosomes (Condon and Heddleston *et al.* 2018). Rabs 8 and 13 are related as members of the Rab8 subfamily and they share some regulatory proteins. The studies shown in this chapter explore the relationship between Rabs 8 and 13 on macropinosome membranes. Furthermore, a new unbiased screen for novel Rabin8 binding partners was conducted and one of the binding partner candidates emerging from this work raises the spectre of having an unconventional linker that coordinates the activities of Rabin8, Rab8 and Rab13.

Results

Investigating kinase mediated activation of Rabin8 in macrophages

Using a mitogen-activated protein kinase 1 (MEK1) inhibitor, U0126, which prevents the phosphorylation and activity of the downstream serine/threonine kinase ERK1/2, Wang and colleagues demonstrated reduced Rabin8 GEF activity after EGF stimulation of HeLa cells (Wang and Ren *et al.* 2015). In order to examine and optimise the inhibition of ERK1/2 phosphorylation in macrophages, a dose response test of U0126 was performed on LPS-treated RAW 264.7 cells. RAW 264.7 cells were pre-treated for 1 hr with the appropriate concentration of U0126 or DMSO and stimulated with LPS (30 min). The cells were lysed, and extracts were examined by Western blot analysis using a phospho-specific ERK1/2 antibody. The results show potent and complete inhibition by U0126 and at 10µM, U0126 completely abolishes ERK1/2 activation even in the presence of LPS (Figure 7.1A), which matches the working concentration utilised in other studies (Hotokezaka and Sakai *et al.* 2002, Wang and Ren *et al.* 2015). Given the importance of ERK1/2 in multiple essential cell survival pathways, the cytotoxicity of U0126 on RAW 264.7 was tested to see if acute treatment would affect macrophage survivability and metabolism using an MTT cell viability assay. Dose dependent treatments were performed on RAW 264.7 cells for over 4hrs and the results showed that treatment with any of the U0126 doses tested did not appear to affect cell viability (Figure 7.1B). With the working concentration of U0126 now established at 10µM, I next wanted to assess the effects of ERK1/2 inhibition on LPS-associated Rabin8 GEF function.

A Rab8 activation assay was performed over a 60 min LPS time course with RAW 264.7 macrophages treated with either DMSO (control) or U0126 (10µM). As expected, U0126 abolished ERK1/2 phosphorylation in both the untreated (time 0 min) and LPS-induced samples (Figure 7.1C). Despite this, U0126 had little or no effect on Rab8 activation across the time course (Figure 7.1C), particularly at the peak time point, where earlier results showed that Rabin8 KO did reduce Rab8 activation (see Figure 5.5A). This preliminary result suggested that ERK1/2 may not be involved in activating Rabin8 for its GEF activity in response to LPS. Independently, we decided to assess whether ERK1/2 is required for Rab8-mediated signalling in the LPS treated macrophages.

I next tested U0126 treatment on LPS/TLR4-induced signalling in RAW cells. In addition, I made use of the GEF KO cell lines produced and used for signalling assays in Chapters 4 and 6. Based on the earlier finding that Akt signalling was reduced in the double KO cells (see Figure 6.5), here, I coupled the GRAB KO cells with the U0126 inhibitor to see if this produces an effective 'double KO' result. Control and GRAB KO cells +/- LPS (15 min) were treated with either DMSO or U0126 (10μM) and Western blots were used on the cells lysates to observe changes in Akt phosphorylation as a read out of Rab8-PI3Kγ signalling. Repeated experiments produced quantitative data showing that LPS-induced Akt phosphorylation was unaffected by loss of GRAB alone (as expected) or by ERK1/2 inhibition, alone or in combination with GRAB KO (Figure 7.1D). From these results, it appears that the MAPK, ERK1/2, while important for regulating Rabin8 GEF activity in other cell types, is not involved in activating Rabin8 for Rab activation nor for regulating Akt signalling in response to LPS in macrophages.

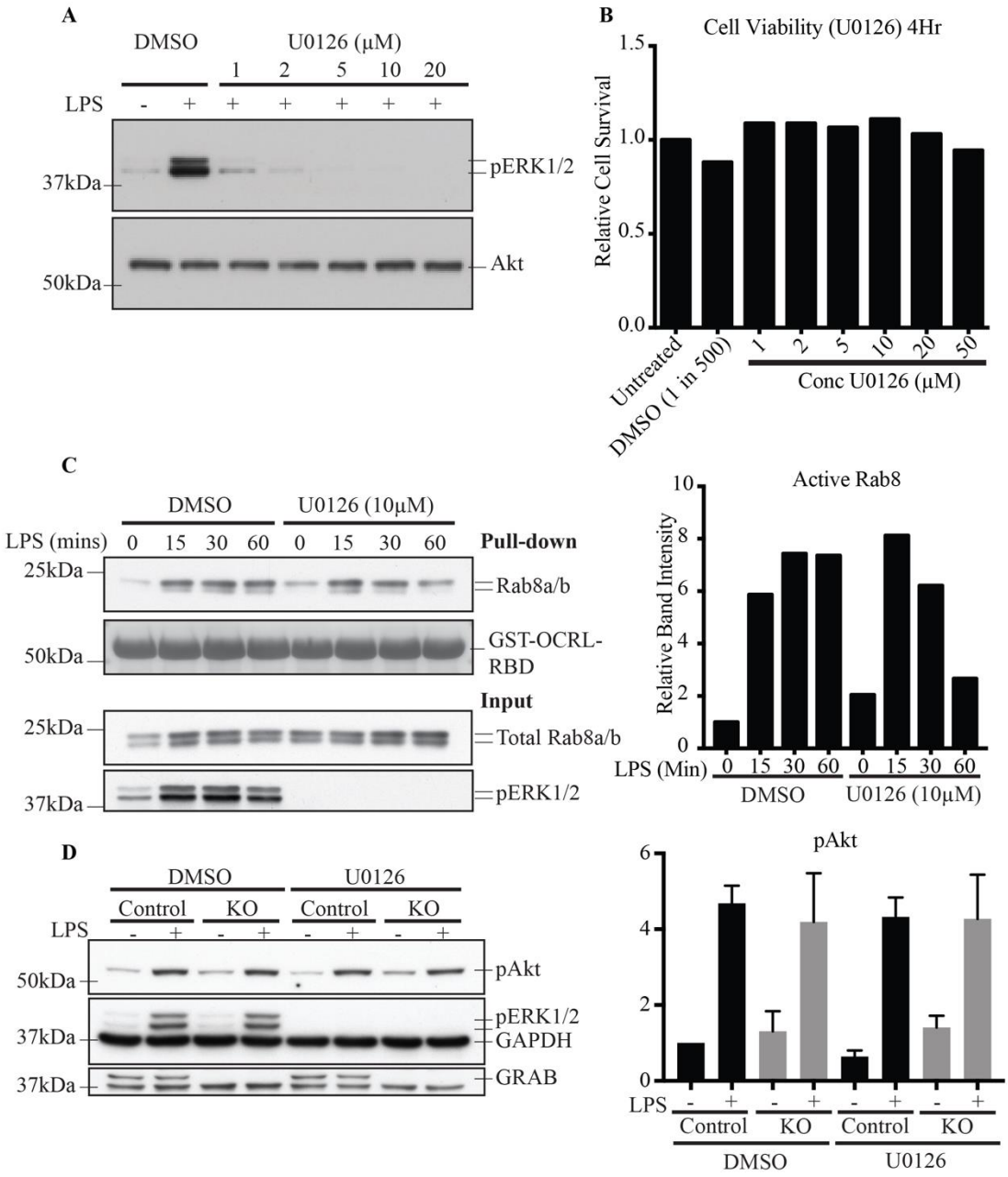


Figure 7.1. The MAP kinase ERK1/2 does not regulate Rabin8-Rab8a associated TLR-signalling. *(A) RAW 264.7 cells were treated with either vehicle (DMSO) or varying concentrations of U0126, ranging from 1μM to 20μM, before being incubated with LPS (100ng, 30min). Immunoblotting was performed on the resulting cell extracts and analysed for ERK1/2 phosphorylation and total AKT levels. (B) Graph showing the results of an MTT cell viability assay performed to test the effects of varying concentrations of the ERK1/2 inhibitor U0126 on the RAW264.7 macrophage cells. Relative cell survival was quantified using the untreated cell samples as the baseline. (C) RAW264.7 cells treated for 1hr with either vehicle (DMSO) or 10μM U0126 before being subjected to a 60-minute time course of LPS and the cell extracts were analysed for Rab8 activation, as well as, ERK1/2 (Thr202/Tyr204) phosphorylation and GAPDH. Graph quantifying the relative band intensity of GTP-bound active Rab8 recovered from the Rab8 activation pull-down via ratiometric analysis between the levels of Rab8 in the pulldown sample against total Rab8 from the input. Result is representative of one experiment. (D) Control and GRAB KO cells were treated with LPS and the samples were analysed for levels of phosphorylation Akt. Quantification of immunoblots was performed using the densitometric ratio between the band intensities of phosphorylated Akt and GAPDH (n=3). The gels are representative, and significance was measured via two-way analysis of variance (ANOVA).*

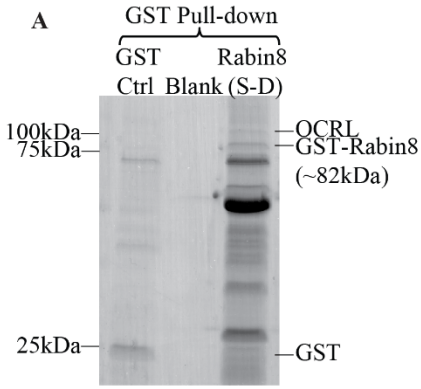
Due to both these early negative results for ERK1/2 and time constraints for the project, the ERK1/2 studies were not pursued further. However, we propose that an alternative kinase is responsible for phosphorylating Rabin8 in macrophages, since relieving the auto-inhibition of Rabin8 is likely to be a ubiquitous requirement for its GEF activity. I attempted to examine the serine phosphorylation of Rabin8 in LPS treated cells using immunoprecipitation and a pan phospho-serine antibody. Unfortunately, this antibody was of no use in this context and future studies will need to measure Rabin8 serine phosphorylation using alternative approaches.

Identification of a new Rabin8 binding partner in mouse macrophages

The Guo group provided us with their GST-tagged Rabin8 constructs in the pGEX-6P1 bacterial expression plasmid. These included a phospho-mimic mutant (S to D, serine to aspartic acid) and a phospho-deficient mutant (S to A, serine to alanine) to represent and mimic the active 'open' and inactive 'closed' states respectively, along with a wild-type construct (Wang and Ren *et al.* 2015). The availability of these reagents suggested another approach for examining Rabin8 activation, namely, use of pull-downs with the phospho-mimetic Rabin8 to identify activating or recruiting binding partners.

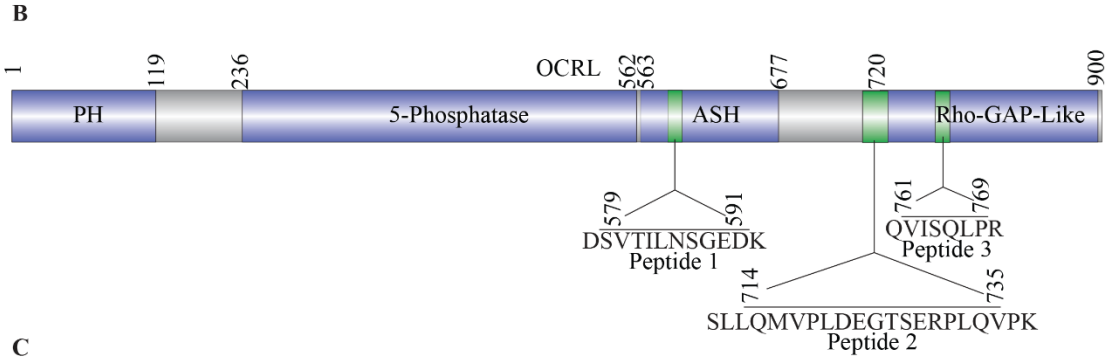
Using the phospho-mimic Rabin8 construct as bait, I performed a pull-down experiment using the lysate of LPS-treated (30 min) iBMMs. As with the previous experiments identifying GRAB as a Rab8a binding partner (see Figure 4.4), the use of iBMMs for this pull-down as opposed to primary BMMs was to satisfy the need for the large number of cells required. Briefly, LPS-treated (30min) iBMM whole cell extracts were applied to Sepharose beads bound with either GST alone or the GST-Rabin8 mutant construct (phospho-mimic). The bound samples were eluted via GST cleavage using a PreScission Protease protocol as described in previous chapters (GE Healthcare Life Sciences). Mass spectrometry analysis was then performed on the resulting eluant to identify both known and novel binding partners and the resulting peptides aligned with a number of known Rabin8 interacting partners including Rab8a and Rab8b, along with Rab11 (Vetter et al. 2015), indicating that the pull-down was indeed successful (Figure 7.2C). Interestingly, in addition to these known binding partners of Rabin8, the results revealed that the inositol polyphosphate 5-phosphatase, OCRL is also a potential binding partner for the phospho-mimic Rabin8

in these macrophages (Figure 7.2). While OCRL is well known to interact with Rab8 as an effector, (Hagemann and Hou *et al.* 2012, Pirruccello and De Camilli 2012), an interaction between Rabin8 and OCRL has not previously been reported.



Protein	Uniprot ID	Species	Score	%Cov (95%)	No. of peptides (95%)
Oculocerebrorenal syndrome of Lowe (OCRL)	Q6NVF0	Mus musculus	5.65	4.55	3

Confidence (%)	Sequence	Predicted MW (Da)	ΔMass
99.0	QVISQLPR	922.53	0.01
99.0	SLLQMVPLDEGTSERPLQVPK	2306.23	-0.0002
97.9	DSVTILNSGEDK	1276.61	-3.64E-06



C

Protein name	Uniprot ID	Score	%Cov	%Cov(50)	%Cov(95)	Number of peptides (95%)
Rabin8	Q68EF0	14.29	42.29	35.98	30.61	11
OCRL	Q6NVF0	5.65	7.99	4.56	4.56	3
Rab8a	P55258	3.55	10.63	10.63	10.63	2
Rab8b	P61028	3.55	10.63	10.63	10.63	2
Rab11	Q61598	4	24.54	11.11	11.11	2

Figure 7.2. The Rab effector OCRL is a novel Rabin8 binding protein. *RAW 264.7 macrophage lysate was used for protein pull-down experiments with GST-tagged Rabin8 (phospho-mimic mutant) and analysed with mass spectrometry. (A) The elutes were separated on a 7-15% SDS-PAGE gradient gel, a faint band at 100 kDa indicating the presence of a novel Rabin8 binding partner, the multi-Rab effector OCRL which is absent in the GST control sample. Table shows the identified peptide sequences of OCRL identified during the analysis with a 5.65 score, with 3 different peptide chains identified with >95% confidence by the analysis. (B) Schematic of identified peptides (in green) over entire OCRL sequence. (C) Example list of identified known Rabin8 interacting proteins from the mass-spectrometry analysis. The mass spectrometry score is a measurement of peptide confidence from the ProteinPilot scoring algorithm: Score = -log(1-(PercentConfidence/100)). For example, a score of 2 = 99% confidence, whereas a score of 4 = 99.99% confidence. %Cov (coverage) refers to the percentage of all identified peptide(s) relative to total amino acid sequence, whereas %Cov (50%) and %Cov (95%) refer to peptide coverage with 50% and 95% confidence respectively.*

OCRL localisation in LPS-activated macrophages

As part of characterising OCRL as a potential binding partner, its localisation in macrophages was determined. Live-cell confocal imaging of LPS-treated RAW 264.7 cells transiently expressing mCherry-OCRL demonstrated that OCRL localising on membrane ruffles and macropinosomes (Figure 7.3A). This was further supported by a publication from our laboratory, demonstrating that OCRL is indeed located on macrophage macropinosomes (Wall and Condon *et al.* 2018).

RAW 264.7 cells were then co-transfected with mCherry-OCRL and GFP-Rab8a, and the imaging showed strong co-localisation of OCRL with Rab8a on the same macropinosomes, indicating that OCRL is spatially and temporally in the right place for taking part in a Rab8a-mediated TLR signalling pathway (Figure 7.3B). Co-transfection of Rabin8 and OCRL was not successful in initial attempts due to the difficulty of expressing either or both of these proteins.

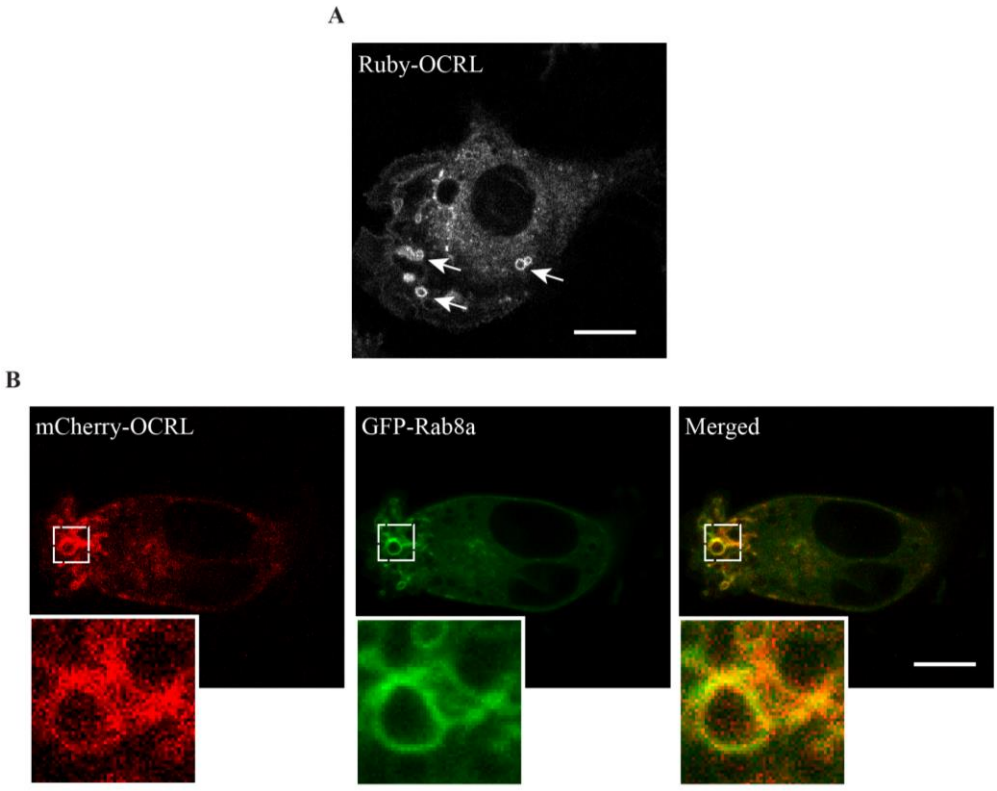


Figure 7.3. OCRL is recruited to Rab8a positive macropinosomes. *Images taken from live cell confocal imaging of an LPS-treated RAW 264.7 cell expressing either (A) mCherry-OCRL or co-expressing (B) mCherry-OCRL and GFP-Rab8a. (A) Upon LPS activation, RAW 264.7 macrophage cells have enhanced macropinocytic activity and OCRL is found to be enriched on a subset of these macropinosomes (arrows). (B) Simultaneous imaging of both OCRL and Rab8a demonstrate that both proteins are present on the same macropinosome membranes related to TLR-signalling. Scale bars, 10µm*

OCRL interacts directly with Rabin8 independently of Rab8

I next wanted to verify the interaction of the OCRL with Rabin8 in these RAW 264.7 macrophages. Here GST pull-downs were performed from LPS-treated iBMM extracts with phospho-mimic Rabin8 and phospho-deficient Rabin8 mutants and the wild type Rabin8 construct. The pull-downs were then blotted for OCRL, which was detected in all three pull-downs, but it was dramatically increased in the phospho-mimic Rabin8 pull-down (Figure 7.4A). The phospho-mimic Rabin8 is essentially an active form that binds to the Rab8 GTPase. Therefore, there is a likelihood of having both Rabin8 and Rab8 in tertiary complexes, and either could recruit OCRL.

To investigate this further, a pull-down experiment was performed using purified, bacterially expressed versions of Rabin8 and OCRL. While using a full-length OCRL construct would be optimal for use in this experiment, the size and complexity of OCRL made it challenging for bacteria expression. Indeed, many studies expressing full-length OCRL have utilised insect cells (Sf9 cells) instead of bacteria expression systems (Erdmann and Mao *et al.* 2007, Mao and Balkin *et al.* 2009). Therefore, a bacterially expressible and codon-optimised OCRL-RBD ($OCRL_{539-901}$) used in Chapter 5 for my activation assays was made with a His-Tag using a different bacteria expression vector pOpine (Figure 7.4B and C). This His-OCRL RBD was then applied to resin bound GST-Rabin8. Western blot analysis was performed with anti-His antibodies, and this served to confirm the presence and binding of His-OCRL to GST-Rabin8 (Figure 7.4E). This result demonstrates a novel genuine, direct interaction between purified proteins, the Rab8 GEF Rabin8 and the multi-Rab effector OCRL, that is independent of the GTPase, Rab8.

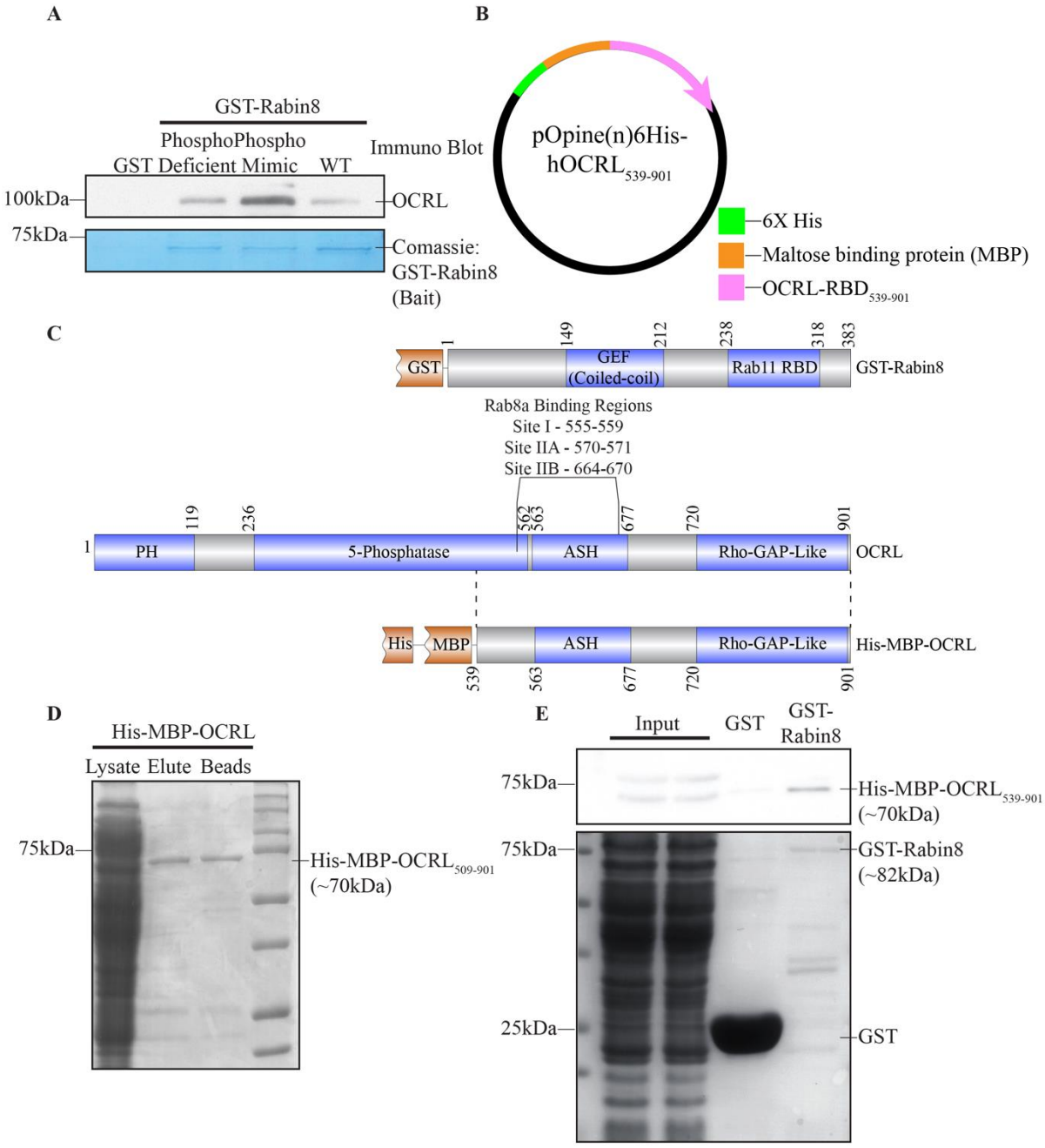


Figure 7.4. OCRL has an enhanced interaction with constitutively active Rabin8. *(A) Immunoblot analysis was performed on mass spectrometry samples using OCRL-specific antibodies to confirm presence of the protein. (B) Illustration of the pOpine (6X)His-MBP-OCRL bacterial expression plasmid. (C) Schematics of the bacterially expressed GST-Rabin8 and His-OCRL-RBD constructs. (D) and a commassie stained SDS-PAGE gels showing the results of protein expression and purification using TALON® metal affinity resin. (E) Bacteria expressed (6X)His-Maltose binding protein-tagged OCRL was loaded onto GST-tagged Rabin8 beads and analysed via immunoblotting using OCRL-specific antibodies, demonstrating direct interaction between Rabin8 and OCRL.*

This finding is intriguing, potentially setting up a scenario where the GEF is in a complex with the GTPase and its effector, with all three proteins interacting at some point (discussed below). While uncommon, Rab effector-GEF interactions have been previously reported, exemplified by an interaction between the Rab5 effector, Rabatin-5 and the Rab5 GEF, Rabex-5, which form a complex that stabilises active Rab5 on endosomes to facilitate fusion (Horiuchi and Lippé *et al.* 1997). A second consideration is that OCRL is a promiscuous effector, known to be recruited by Rab8 and also by Rab13. Therefore, a second model might be that an upstream Rab13-OCRL complex recruits Rabin8 as a GEF for Rab8 as part of a Rab cascade.

We thus set out to examine the relationship between Rabs 13 and 8 on ruffle and macropinosome membranes using live cell imaging on co-transfected cells. LPS-treated RAW 264.7 cells transiently co-expressing td-Tomato-Rab8a with either GFP-Rab13 or GFP-Rab5a were imaged (Figure 7.5A). In the movie segment shown in Figure 7.5B, GFP-Rab13 is consistently and stably associated with ruffle membranes and with macropinosomes forming from them, while tdTomato-Rab8a is gradually enriched on the newly forming macropinosome. Therefore, Rab13 is present and enriched on the membranes prior to the enrichment of Rab8a and they both overlap and co-localise transiently on the new macropinosome. This visually confirms that Rab13 would be positioned to recruit proteins prior to Rab8a activation.

Imaging with GFP-Rab5a and tdTomato-Rab8a demonstrated that upon Rab8a enrichment on macropinosomes, Rab5a is subsequently recruited to these membranes where it replaces Rab8a on maturing macropinosomes (Figure 7.5C). Notably, Rab5 is recruited from the cytoplasm, since it is not found on the ruffles. Taken together, these results place two GTPases in quick succession before and after Rab8a on early stage macropinosomes where my results (see Figures 6.6B and 6.7), along with our previous studies indicate that PI3K/Akt signalling occurs in TLR pathways (Wall and Condon *et al.* 2018). Thus, these Rabs are poised to act in a cascade and interestingly all three Rabs have the ability to bind OCRL (Fukuda and Kanno *et al.* 2008).

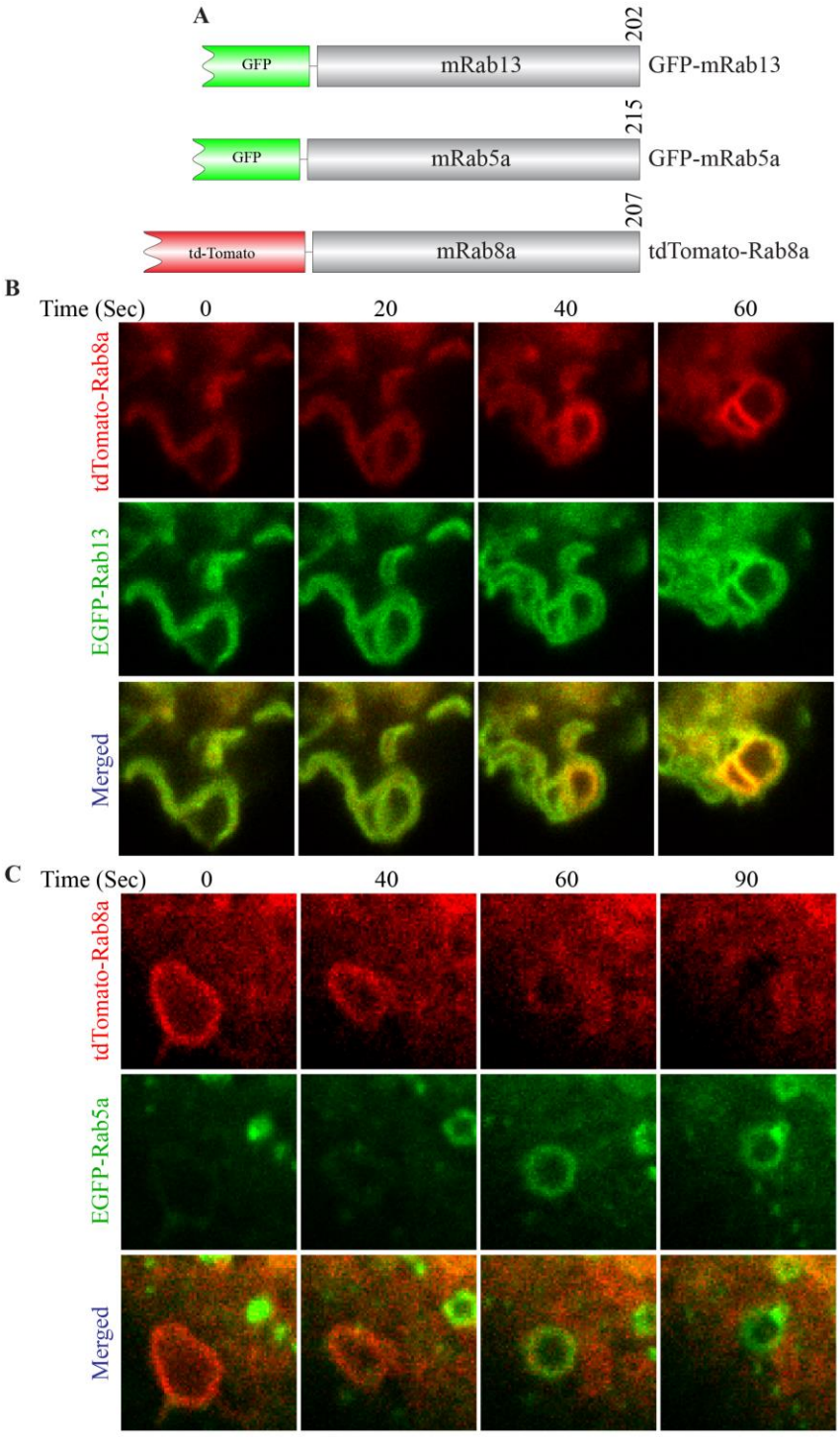


Figure 7.5. Rabs 13, 8a and 5 are sequentially recruited during macropinocytosis in mouse macrophages. *(A) Schematic of the fluorescent constructs used to visualise the recruitment and localisation of Rab13 and Rab5a with Rab8a. (B/C) Representative time lapse images shown here were from live cell confocal images of LPS-treated RAW 264.7 macrophages co-expressing either (B) GFP Rab13 or (C) GFP-Rab5 with td-Tomato Rab8a.*

Further studies are now required to further examine the interactions and conjoint functions of Rabin8 as part of dynamic and likely multi-functional Rab complexes. Other students will now take up projects stemming from the base established by my findings.

Discussion

This chapter describes preliminary investigations into the regulators of the Rab8 GEF, Rabin8 in mouse macrophage TLR signalling pathways. Two main aspects of Rabin8 regulation were examined by experiments here, firstly looking at ERK1/2 as potential kinases for activating Rabin8 in LPS-activated macrophages. The results discount ERK1/2 as Rabin8 activators in this context but set up future studies that can explore Rabin8 phosphorylation and seek other kinases responsible for GEF activation. The second level of Rabin8 regulation I examined, used an unbiased screen to identify the effector and inositol polyphosphate 5-phosphatase, OCRL as a part of a possible recruiting mechanism for Rabin8. Excitingly, OCRL could act through a novel effector-GEF interaction and/or as part of a multi-Rab cascade for recruiting Rabin8. Rab13 and Rab5 were shown to flank Rab8 on macropinosomes and they could be part of such a cascade. While time constraints did not allow me to pursue these studies further, my results have established fruitful and interesting findings that will be the basis for future studies to elucidate how GEFs contribute to Rab-mediated TLR signalling and macropinocytosis.

While the function and regulation of the Rab GTPase family of proteins is important and has been well established, the proteins involved in regulating Rabs such as GEFs and GAPs are still poorly understood. One fundamental question is how GEF activity is regulated to ensure that inappropriate Rab activation does not occur. It has been suggested that one common method for hindering or promoting GEF activity is through altering the efficiencies for binding to the target Rab, via occluding/exposing its GEF binding domain. Indeed, several GEFs including TIM (RhoA GEF) (Yohe and Rossman *et al.* 2007), Rabin8 (Wang and Ren *et al.* 2015) and intersectin (Cdc42 GEF) (Kintscher and Wuertenberger *et al.* 2010) have been reported to exist in 'folded', auto-inhibited states that block GTPase binding. Therefore, interaction with various regulators is required for relieving this inhibited state and promoting GEF function. For Rabin8, kinase driven activation by ERK1/2 was found to be responsible for activating this Rab8 GEF in response to EGF signalling in HeLa cells, producing dramatic effects on Rab8 activation (Wang and Ren *et al.* 2015). Interestingly, while it is not known if GRAB naturally exists in an auto-inhibited state like Rabin8, other kinases have also been reported to alter its GEF activity (Furusawa and Asada *et al.* 2016). Therefore, establishing the kinase(s) responsible for controlling the TLR-

associated interactions between Rab8 and the GEFs GRAB and Rabin8 would further expand our understanding of the regulatory pathways that govern Rab8 macrophage immune functions. As a preliminary kinase of interest, I found that inhibition of ERK1/2 phosphorylation did not affect LPS-induced Rab8 activation or downstream Rab8a associated signalling functions (see Figure 7.1). While not empirically tested here, other studies have showed that U0126 specifically inhibits ERK1/2 phosphorylation and not other kinases such as JNK and p38 phosphorylation (Cohen and Lawrence *et al.* 2010). Future work could systematically test the inhibitors of other kinases known to regulate GRAB and Rabin8 GEF functions (see Figure 1.6) and identify those involved in TLR associated GEF and Rab8a activation in macrophages.

In addition to modulating Rab nucleotide binding and effector recruitment, GEFs often, but not always, help to anchor the GTPase on target membrane domains, thereby promoting location-specific function (Grosshans and Ortiz *et al.* 2006). The precise enlistment of the GEFs themselves to the proper cellular compartments plays an important role in driving selective Rab-effector functions. With GRAB and Rabin8 now confirmed as Rab8 GEFs that function downstream of TLR4, investigating how these GEFs are being recruited and activated on macropinosome membranes will be essential to understanding the regulator machinery that controls Rab8 activity in macrophages. As mentioned in the introduction chapter, Rab11 is the most well characterised recruitment partner for Rabin8 in Rab8 pathways (Knödler and Feng *et al.* 2010, Horgan and Hanscom *et al.* 2013). However, given its primary association with recycling and exocytic complexes (Welz and Wellbourne-Wood *et al.* 2014), it is unlikely that Rab11 is playing a similar role during this particular Rab8a immune signalling pathway. Protein pull-down assays with mass spectrometry analysis using GST-Rabin8 constructs has identified a novel Rabin8 interacting protein, the multi-Rab effector OCRL. Further experiments with bacterially expressed proteins have proven that this interaction is direct and independent of Rab8. This surprising pairing raises two interesting scenarios (Figure 7.6). The first (Rabin8 recruiting OCRL as a Rab8a effector), implies that there will be two competing Rab8a effectors present on these LPS-induced macropinosomes, PI3Kγ and potentially OCRL. Both effectors preferentially act on the same substrate, the phospholipid PI(4,5)P_2. PI3Kγ phosphorylates it to become PI(3,4,5)P_3, while OCRL dephosphorylates it to produce

PI(4)P_1 (Zhang and Jefferson *et al.* 1995, Vanhaesebroeck and Waterfield 1999). These phosphoinositides are essential for controlling the signalling and maturation of macropinosomes and other endocytic pathways (Bohdanowicz and Balkin *et al.* 2012, Bohdanowicz and Grinstein 2013) and for recruiting signalling kinases such as Akt. If validated, this might suggest a dual role for Rab8a in controlling the phosphoinositide environment on these signalling membranes, mediating Akt signalling and potentially influencing the maturation of the macropinosome, and Rabin8 recruitment of OCRL might be an effector recruitment factor that modulates this process.

For the second scenario, our previous studies have identified multiple Rabs associated with macrophage ruffles and macropinosome membranes, including Rabs 13, 31 and 5, along with Rab8 (Yeo and Wall *et al.* 2016, Wall and Luo *et al.* 2017, Condon and Heddleston *et al.* 2018). Most notably, Rab13 is implicated in the formation of LPS-induced ruffles and the formation of large macropinosomes, indicating that this Rab is likely upstream of Rab8 TLR-signalling functions. Live cell imaging confirmed this arrangement with Rab13 shown to be localised and enriched on macrophage membrane ruffles prior to macropinosome formation and Rab8a enrichment (see Figure 7.5B). Being a multi-Rab effector, OCRL is also known to interact with Rab13 (Fukuda and Kanno *et al.* 2008). Therefore, a possible model is that OCRL is recruited as an upstream effector of Rab13 where it is poised to subsequently recruit Rabin8 to newly formed macropinosomes to facilitate Rab8 activation (as depicted in Figure 7.6). This scenario establishes a potential Rab cascade that links the regulators of macropinocytosis and TLR-signalling pathways.

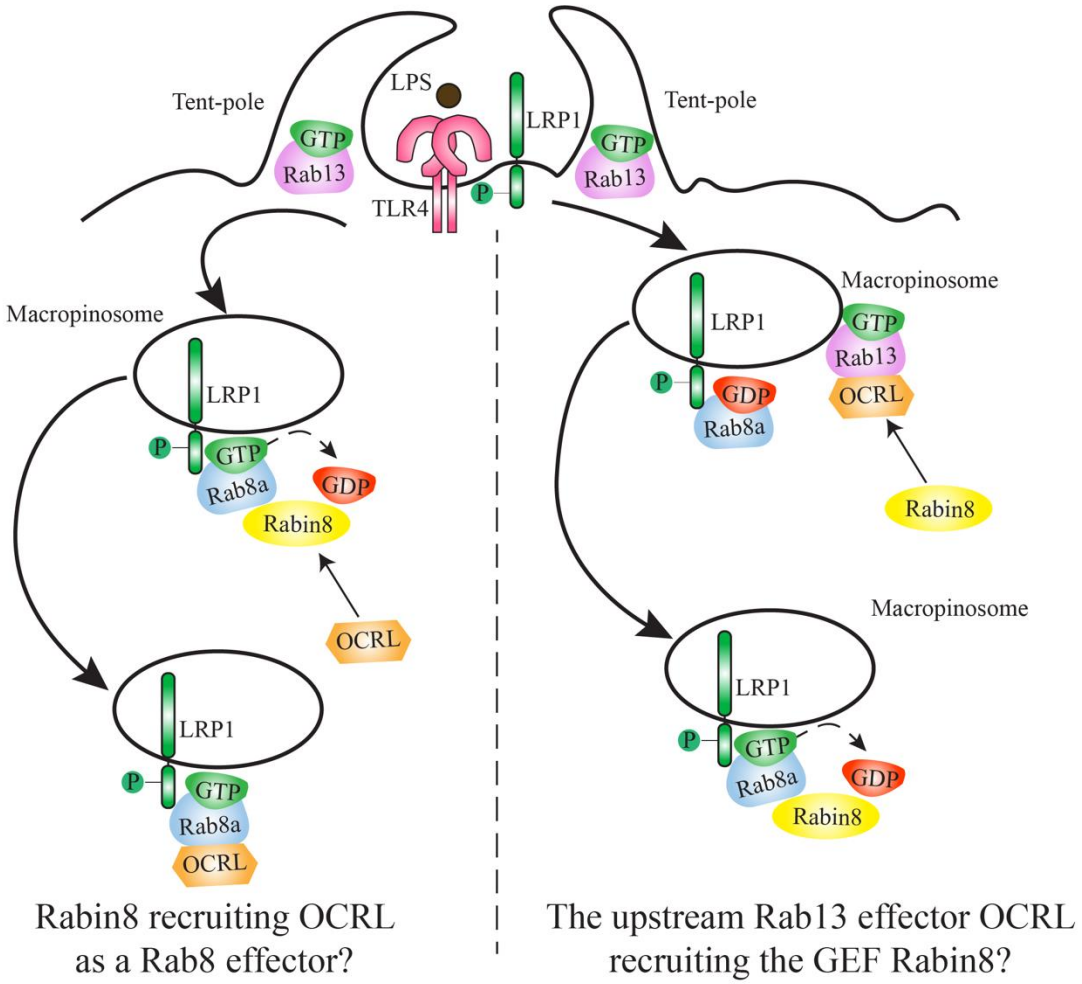


Figure 7.6. Proposed models for a Rabin8-OCRL interaction in macrophages. *Proposed models for an OCRL-Rabin8 interaction in Rab8a-TLR signalling pathways. In the first scenario (left pathway), Rabin8 functions as a novel recruiter of the Rab8 effector OCRL to macrophage macropinosome membranes. Upon activation of Rab8a by Rabin8, this Rabin8-OCRL interaction is proposed to place OCRL in close proximity to the newly active Rab8a for Rab-effector function. In the second scenario (right pathway), with Rab13 known to function upstream of Rab8a on macrophage ruffles, OCRL is initially recruited as a Rab13-effector and recruits Rabin8 to macropinosome membrane to facilitate Rab8 activation.*

A final piece of the recruitment picture is the relationship of the GEFs and Rab8a with the TLR crosstalk receptor LRP1. We know from our earlier studies that LRP1 binds to both GDP and GTP-bound Rab8a and LRP1 is needed for LPS-induced Rab8a activation (Luo and Wall *et al.* 2018). This implies that the GEFs may also be recruited to a complex scaffolded by LRP1 and unpublished evidence from Lin Luo in the group shows that Rabin8 appears in LRP1 pull-downs in macrophages (not shown). Future studies will address the full context for the recruitment and activation of both GEFs, Rabin8 and GRAB to dynamic and likely complex arrays of proteins that regulate Rab8 and its effectors for TLR signalling.

Chapter 8

Final Discussion and Future Directions

8. Final Discussion and Future Directions

Significance and overview

With inflammation now recognised as a component of an increasing number of chronic and acute diseases, it has never been more important to understand the mechanisms that control inflammatory cytokines and other mediators. TLR-activated macrophages are major contributors to inflammation in many settings including infection and chronic disease; TLR pathways are therefore targets for developing new therapeutic strategies to modulate inflammation in different diseases and tissues (Zhu and Yang *et al.* 2014, Ullah and Sweet *et al.* 2016). Alzheimer's disease and metabolic diseases, such as diabetes (melitis, Type I , II) are now known to have major inflammatory components that will require new drugs and therapies (Lien and Zipris 2009, Bieghs and Trautwein 2013, Zolezzi and Inestrosa 2017). TLR pathways are also critical for infection control, immune surveillance and pathogen destruction (Kawai and Akira 2005). In this area too, new therapies are necessary to counteract antibiotic resistance and new viral and bacterial outbreaks. Finally, there are also roles for TLRs in ageing (Lavelle and Murphy *et al.* 2010, Panda and Qian *et al.* 2010). TLR signalling pathways contain potential drug targets in the form of signalling kinases, GTPases, lipid kinases and phosphatases and signalling adaptors.

A major underlying goal for my project was to develop a deeper understanding of the TLR signalling machinery. Specifically, this project was focused on the Rab GTPase, Rab8a, which had earlier been implicated in signalling and cytokine regulation downstream of TLRs by work from our laboratory. The TLR-induced, LRP1-Rab8a-PI3Kγ-Akt signalling axis emerged from this work, as a key driver of anti-inflammatory, M2-like phenotypes in macrophages (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). While my experiments have focussed on mouse macrophages, PI3Kγ has a similar effect downstream of TLRs in both mice and humans (Kaneda and Messer et al. 2016). Indeed, druggable proteins in this pathway already include PI3Kγ which is inactivated by the γ specific inhibitor, IPI-549, currently in clinical trials for several cancer types (Tolcher and Hong *et al.* 2016, Sullivan and Hong *et al.* 2018). Its use therein is predicated on *in vitro* and animal studies which show that loss or inhibition of PI3Kγ enhances proinflammatory responses and maintains macrophages in a cancer killing, M1 state (Kaneda and Cappello *et al.* 2016). Early results from these

clinical trials confirm enhanced proinflammatory responses resulting from use of the inhibitor in human subjects (De Henau and Rausch *et al.* 2016). This is reflected in other studies using mouse models (Kaneda and Messer *et al.* 2016) and in humans (Takeda and Maher *et al.* 2019) where PI3Kγ is inactivated through genetic ablation or mutation. The application of PI3Kγ inhibitors in cancer for manipulating TLR-mediated signalling affirms the importance of these pathways and as potential targets in inflammatory disease. This also highlights the significance of my project in studying Rab8a as a key recruiter of PI3Kγ in macrophages. The ability to use drugs or biologicals to manipulate Rab8a activation will be an interesting prospect for the future and one that depends on a deeper understanding of Rab8a activation and its GEF activators.

At the outset of my project, the molecules that activate Rab8a in TLR pathways had not been identified. Nevertheless, based on other known evolutionarily conserved Rab regulatory pathways, there was a likely possibility that one or more GEFs would be required for this purpose. The specific goal of my project was thus primarily to finding the GEF regulator(s) for Rab8a in TLR pathways. Several approaches were employed for this research, including protein pull-downs and mass spectrometry screens, CRISPR knockout of proteins, novel Rab activation assays, biochemical analysis of cell signalling and the use of fluorescent reporters to visualise real-time signalling events in cells. The main outcome of this project was the identification of GRAB and Rabin8 as GEFs for Rab8a in TLR signalling. These are two new molecules that I have added to the TLR pathway that influence inflammation, thus extending the repertoire of potential molecular targets available for future biomedical and pharmacological studies. We would expect that specifically targeting GRAB and Rabin8 with inhibitors or activators would modulate Akt/mTOR signalling and inflammatory programs. Moreover, other studies performed in conjunction with my project explored other molecules in this pathway, such as OCRL, that if validated would also be novel additions to this signalling axis and provide further opportunities for manipulating disease-associated processes. The following sections further discuss the overall findings and insights that emerged from my studies along with possible future directions.

Rab8 activation

As master regulators of many cellular pathways, mounting evidence has linked the aberrant activation of small GTPase family members to the pathogenesis of various diseases (Cook and Rossman *et al.* 2014, Simanshu and Nissley *et al.* 2017, Banworth and Li 2018). As molecular switches, all GTPases cycle between a GTP-bound 'active' and GDP-bound 'inactive' state and alternating between these configurations is critical for regulating their function. The ability to reliably detect and quantify the activation of specific GTPases in cellular systems is critical for elucidating the identity and role of GTPase regulators. Experimental activation assays have been traditionally used to assess the GTP-bound status of many subfamilies of endogenous GTPases. One approach for such assays is the use of effector binding domains as probes that define GTP-bound states of GTPases. For instance, using an assay based on the Ral-binding domain of the effector Ral binding protein 1, Chen and colleagues were able to identify a Ral GAP complex that regulates RalA activity for insulin-associated Glut4 translocation to the plasma membrane (Chen and Leto *et al.* 2011). In another study, Koo and colleagues used the CRIB domain of Pak1, a Rac1 effector, to demonstrate that Arf6 is recruiting the Rac GEF, kalirin, to facilitate Rac activation on the plasma membrane for actin remodelling to generate membrane ruffles and protrusions for cell migration (Koo and Eipper *et al.* 2007). In TLR-Src-associated pathways, a role for RhoA was established using the RBD of the RhoA effector rhothekin to show induction of RhoA GTP loading (Manukyan and Nalbant *et al.* 2009). A final example is the use of a bead-based system bound with the GTPase effector binding domains of Pak1 (Rac1 effector), rhotekin (Rho effector), RalGDS (Rap1 effector) and RILP (Rab7 effector) was to demonstrate GTPases involved in a new multi-GTPase cascade for Hantavirus infection in HeLa cells (Buranda and BasuRay *et al.* 2013). Taken together, these examples demonstrate the broad applications of these assays as biological tools for investigating the molecular mechanisms that modulate the activation and inactivation of GTPases.

As mentioned in chapter 4, there is currently no commercially available Rab8 activation probe or assay available. Thus, to identify and validate the regulatory Rab8 GEFs in the macrophage TLR4 signalling complex, I generated three different Rab activation probes using the RBDs of known Rab8 effectors: OCRL, MICAL-L2 and PI3Kγ. Using an optimised protocol of the OCRL-RBD probe in combination with my

CRISPR GEF KO cell lines, I was able to demonstrate a step-wise decrease in Rab8 activation with the subsequent deletions of GRAB and Rabin8 in macrophages (see Figure 6.2B). This assay proved invaluable in validating both Rab8 GEFs GRAB and Rabin8 as joint activators of Rab8a during TLR4-signalling in macrophages (Tong and Wall *et al.* 2019). Other uses for this assay have been implemented by our group. For instance, we measured Rab8a activation downstream of multiple TLRs in macrophages, implicating Rab8a in innate immune responses to a wide range of pathogens (Wall and Luo *et al.* 2017). Future studies could expand the use of the assay to examine Rab8a activation in macrophages, or indeed in other cells types, under varying infectious or metabolic conditions. We have not tested the efficacy of the assay for measuring Rab8a activation in other cell processes where it functions, such as vesicle traffic to and from recycling endosomes or apical cell surfaces (Peränen 2011) but potentially such applications would also be possible.

In the context of this project, all three probes were only screened and tested for their ability to detect Rab8 activation; however, work by Fukuda and colleagues have shown that the Rab effectors OCRL and MICAL-L2 can interact with a broader array of different Rabs (Fukuda and Kanno *et al.* 2008). Thus, the assays I set up using probes from these proteins could be optimised for examining the activation of additional Rabs by blotting the protein pull-down samples with other Rab antibodies. Ultimately, if successful these three probes are predicted to have the combined ability to detect the activation of Rabs 1, 3, 5, 6, 8, 10, 13, 15, 22, 35 and 36 (Fukuda and Kanno *et al.* 2008), along with Ras – effectively establishing a multi-Rab/GTPase activation assay for systems analysis. This would allow users to perform screens in parallel for the activation of these GTPases in cells responding to specific stimuli.

GEFs in TLR signalling

TLR signal transduction is comprised of highly complex networks of trafficking and signalling pathways which require the precise, sequential recruitment of signalling complexes and adaptors to direct and propagate downstream events to elicit tailored immune responses (Kawasaki and Kawai 2014). Therefore, it is unsurprising that as master regulators of cellular trafficking and signalling pathways, small GTPases and their associated GEFs have been implicated in various immune signalling processes.

For instance, the RhoA GEF, GEF-H1 has been shown to be activated downstream of TLR4 in LPS-treated endothelial cells, and through RhoA, promotes the activation of the transcription factor NFκB for driving IL-8 expression and secretion (Guo and Tang *et al.* 2012). Another RhoA GEF, protein kinase A anchoring protein 13 (AKAP13), is also demonstrated to facilitate NFκB function downstream of TLR2 in HEK293 cells, resulting in increased IL-8 and monocyte chemoattractant protein-1 (MCP-1) expression (Shibolet and Giallourakis *et al.* 2007). Similarly, in LPS-activated RAW 264.7 macrophages, TLR4-associated actin-cytoskeleton reorganisation results in the activation of Rac1 by its GEF, Cdc42, which enhances phagocytosis (Kong and Ge 2008). The TLR-induced activation of RhoA by different GEFs suggests this as a mechanism for selectively managing a variety of receptor-driven, RhoA-dependent, actin-mediated responses such as phagocytosis, macropinocytosis or cell migration.

Previous work by our laboratory identified the small Rab GTPase Rab8a as an essential component of a TLR-signalling complex, which ultimately biases cytokine output through the signalling kinase PI3Kγ (Luo and Wall *et al.* 2014, Wall and Luo *et al.* 2017). My findings reported here, identified the Rab8 GEFs GRAB and Rabin8 as joint activators of Rab8a that facilitate PI3Kγ effector-mediated TLR-signalling. From our previous findings, is it also likely that Rabin8 and GRAB regulate Rab8a/PI3Kγ signalling from not only TLR4, but other additional TLRs as well (Wall and Luo et al. 2017). While neither GRAB nor Rabin8 were observed to influence Rab8a recruitment and enrichment on cell membranes in macrophages, these results highlight both GRAB and Rabin8 as new members of this regulatory TLR-signalling machinery and uncovers a redundant relationship between these GEFs as activators of Rab8a to drive specific PI3Kγ-associated TLR-signalling outputs.

For both GRAB and Rabin8, directly influencing Rab8a in a *signalling* role is unique, as previous studies on these GEFs have been more focused on the regulation of various Rab8-associated *trafficking* functions in processes such as ciliogenesis (Feng and Knödler *et al.* 2012), growth of axons in neuronal cells (Furusawa and Asada *et al.* 2016, Homma and Fukuda 2016) and the polarised transport of cellular components/vesicles (Hattula and Furuhjelm *et al.* 2002). Furthermore, at other times when Rab8 has been shown to influence receptor signalling, it was through controlling

the trafficking and localisation of the receptor itself rather than downstream signalling kinases (Nagabhushana and Chalasani *et al.* 2010, Esseltine and Ribeiro *et al.* 2012, Finetti and Patrussi *et al.* 2015). In saying this, during the course of my PhD, another surprising Rab8 GEF was identified associated with TLR trafficking (Sellier and Campanari *et al.* 2016), namely, a trimeric protein complex containing chromosome 9 open-reading frame 72 - Smith–Magenis syndrome chromosome region candidate 8 - WD repeat domain 41 (C9ORF72-SMCR8-WDR41).

First identified from genetic abnormalities observed in patients with amyotrophic lateral sclerosis (ALS) and fronto-temporal dementia (FTD), this C9ORF72-SMCR8-WDR41 complex has been mostly tied to endosomal trafficking pathways, particularly during the stages involving lysosome fusion and function (Sullivan and Zhou *et al.* 2016). Structural studies of the components in this complex have discovered that two of the proteins, C9ORF72 and SMCR8, contain Rab GEF DENN domains (Zhang and Iyer *et al.* 2012, Levine and Daniels *et al.* 2013). A preliminary *in vitro* nucleotide exchange assay using purified recombinant proteins demonstrated that Rab8a GTP-release was enhanced by the addition of C9ORF72 alone or in complexes with SMCR8 and WDR4 (Sellier and Campanari *et al.* 2016). Taken together, these studies highlight a rather uncommon phenomenon where a structurally unrelated DENN domain GEF is able to facilitate nucleotide exchange in Rab8a, instead of a typical Sec2 GEF such as GRAB or Rabin8 (Ishida and Oguchi *et al.* 2016). This opens up potential possibilities for novel GEF-Rab interactions, suggesting that different subfamilies of GEFs are able to cross-activate previously unrelated Rab GTPase subfamilies. To validate this, protein crystallisation and structural studies will be required to further investigate how Rab8a interacts with this new GEF complex for facilitating nucleotide exchange.

Of particular relevance to this project, the C9ORF72-SMCR8-WDR4 complex was also linked to TLR-signalling, by evidence demonstrating that inactivation of one or more of these proteins resulted in increased endosomal TLR-signalling and excessive inflammatory cytokine output (McAlpine and Sun *et al.* 2018). The authors attribute this finding to activation of Rab8 for TLR receptor trafficking (reduced degradation) suggesting that multiple GEFs could activate Rab8a for different roles in TLR trafficking and signalling. It is worth noting that none of the components of this C9ORF72-SMCR8-WDR41 complex were identified in GST-Rab8a pull-downs and

mass spectrometry in LPS-treated BMM cell lysates (performed in Chapter 4). Furthermore, TLR-PI3Kγ-associated Akt signalling observed in our double KO (GRAB and Rabin8) cells mimicked the levels of signalling previously seen in the Rab8a KO cells (see Figures 6.4A and 4.2A). Thus, it is unlikely another Rab8a GEF, other than GRAB and Rabin8, is influencing Rab8a-PI3Kγ signalling in this context. The TLR-induced deployment of multiple GEFs for Rab8, Rabin8, GRAB versus C9ORF72-SMCR8-WDR41, is analogous to the recruitment of multiple RhoA GEFs discussed above and it illustrates how small GTPases are used in various roles for carefully controlled responses elicited in these innate immune pathways.

Rab8 and its GEFs and membrane recruitment in macrophages

Controlling the precise recruitment of Rabs to the appropriate membrane domains is fundamental for driving specific Rab effector mediated functions (Grosshans and Ortiz *et al.* 2006). As mentioned previously, this targeted membrane recruitment of the Rab can be orchestrated by a host of different binding partners such as GEFs, GDIs, GAPs, as well as escort proteins and prenylation enzymes (Müller and Goody 2018). Of these interacting partners, the Rab GEFs have been shown to influence both the recruitment and retention of Rabs onto their respective cellular compartments for effector binding and function (Cherfils and Zeghouf 2013). This was exemplified by an innovative strategy employed by Blumer and colleagues which used recombinant GEFs with mitochondrial membrane tags, Rabex-5, DrrA and Rabin8, to mistarget their respective Rabs (Rab5, Rab1A and Rab8 respectively) onto mitochondrial membranes in order to uncouple the Rabs functionally from their endogenous settings (Blümer and Rey *et al.* 2013). This strategy relied on GEFs with joint responsibilities for membrane targeting and activation of the specific Rabs.

In my studies, despite a significant reduction in Rab8 activation observed in the double GRAB and Rabin8 KO macrophages (see Figure 6.2B), imaging and biochemical approaches showed that Rab8a macropinosome membrane recruitment, enrichment and retention remained unaffected by its activation state or by the loss of the GEFs (see Figure 6.3). This indicated that in macrophages, Rab8a membrane recruitment and retention is independent of both GRAB and Rabin8. Another example in the literature demonstrates a similar scenario for the closely related Rab8 family member,

Rab13 whose membrane recruitment to vesicles is also independent of its GEF and prenylation. This study by Ioannou and colleagues demonstrated that constitutively inactive Rab13 or nonprenylated Rab13 mutants were still recruited to endocytic vesicle membranes, in keeping with the location of active or wild type Rab13 (Ioannou and Girard *et al.* 2016). This was presumed to occur through overriding protein-protein interactions that anchored Rab13 irrespective of its activation state or prenylation. Curiously, the relevant Rab13 GEF, DENND2B, was shown in previous work to be required for Rab13 recruitment to the plasma membrane (Ioannou and Bell *et al.* 2015). This highlights two different contexts with only one requiring the GEF for Rab13 membrane recruitment. Thus, my work concurs with such findings in illustrating that Rab8 membrane attachment is uncoupled from GEF function and location, at least in the context of macrophage ruffles/macropinosomes. Indeed it would be interesting to explore Rab13 and DENND2B in macrophage ruffles where its recruitment precedes Rab8a (Condon and Heddleston *et al.* 2018).

It is thus likely that protein-protein interactions control Rab8 membrane recruitment in our macrophage system, with the most obvious candidate being the TLR crosstalk receptor LRP1. As mentioned previously, our group has reported that Rab8a is able to directly interact with this receptor in a LPS-dependent manner to perform its PI3Kγ-signalling function (Luo and Wall *et al.* 2018). Additionally, protein pull-down experiments from this study also showed that this Rab8a-LRP1 interaction is independent of the activation state of Rab8a, agreeing with observations in the Rabin8/GRAB double KO cells (activation independent Rab8 membrane enrichment, see Figure 6.3A). Hence, this LRP1-mediated recruitment of Rab8a could could be the GRAB and Rabin8 independent recruitment mechanism for Rab8a enrichment on macropinosomes observed in LPS-treated double KO macrophages. Future experiments can assess possible changes to Rab8a membrane enrichment and recruitment in the absence of LRP1 using LRP1 KO RAW 264.7 cells previously generated in our laboratory by Dr Adam Wall. Aside from LRP1, other studies have demonstrated that Rab8a can also be recruited to other receptor complexes to perform functions through trafficking effectors. For instance, Rab8a is recruited to T-cell receptors and to cilia sensory receptors (Wang and Morita *et al.* 2012, Finetti and Patrussi *et al.* 2015) helping to determine the trafficking for those receptor complexes.

Live cell imaging on the double KO cells also showed the presence of Rab8a on constitutive macropinosomes irrespective of LPS stimulation, indicating that there is also at least one other protein-protein interaction that positions Rab8a on macropinosome membranes besides through LRP1. Finding additional novel binding partners for Rab8a on macropinosomes could be approached in the future by performing pull-downs with GST-Rab8a and mass spectrometry analysis on BMM lysates (not treated with LPS in this case), as done previously by the group (Luo and Wall *et al.* 2014). Based on the hypothesis that Rab8a is part of a Rab cascade, other binding partners could also include upstream Rab effectors or accessory proteins.

The macropinosome is a hub for immune receptor signalling

As vital components of the innate immune system, macrophages are part of the first line of defence against pathogens, distinguished by their ability to rapidly detect foreign molecules and elicit robust inflammatory responses. Mediated by immune receptors such as TLRs, this surveillance system is dependent on receptor-ligand recognition and rapid downstream signalling pathways. While the individual components of TLR-signalling pathways have been characterised (Kagan and Su *et al.* 2008, Kawasaki and Kawai 2014), the exact signalling compartments and membrane domains that are involved in TLR signalling have yet been fully elucidated (Barton and Kagan 2009). Activated TLRs are recruited to and cluster in specific lipid microdomains that go on to form signalling platforms to facilitate receptor signal transduction (Varshney and Yadav *et al.* 2016). Membrane cholesterol levels, and cholesterol-depleting statins, can thus influence pro-inflammatory signalling downstream of TLRs (Chansrichavala and Chantharaksri *et al.* 2010). The endosomal compartments housing TLR signalling are still not fully defined.

A distinct characteristic of macrophages as immune sentinels is their prodigious macropinocytic capacity, which facilitates the indiscriminate uptake and rapid sampling of the extracellular environment (Kerr and Teasdale 2009). As large portals of entry into the cell, macropinosomes are in a prime position for TLR-ligand recognition, activation and signalling. Accordingly, I demonstrated that the TLR adapter TRAM as part of TLR complexes, is localised on macropinosome membranes

upon macrophage activation (see Figure 4.1C). Additionally, I demonstrated using a fluorescent reporter that upon stimulation with LPS, the signalling kinase Akt is enriched on macropinosomes and this enrichment is affected by the absence of the Rab8 GEFs GRAB and Rabin8 (see Figure 6.7), pinpointing the macropinosome as a site of Rab8a controlled TLR4-PI3Kγ signalling. Through these experiments, I established the early macropinosome as the cellular compartment where this TLR4-Rab8a-PI3Kγ signalling complex is functioning. However, studies from our group have implicated this same Rab8a-PI3Kγ signalling complex with other TLRs, including the endosomal TLRs, TLR9 and TLR3 (Wall and Luo *et al.* 2017). This raises an important question: do these endosomal TLRs signal from the same macropinosome compartment as the extracellular receptor TLR4? Future work is necessary to repeat the live cell imaging using the fluorescent Akt reporter with control and double GRAB and Rabin8 KO cells treated with the TLR9 ligand CpG or the TLR3 ligand Poly (I:C) to address this question. If validated, these results highlight macrophage macropinosomes as a central signalling site for multiple TLRs, and perhaps other immune receptors.

Identification of a novel Rab effector-GEF interaction between OCRL and Rabin8

The focus of this project was to identify the upstream regulatory GEFs that activate Rab8a and drives its TLR signalling function in macrophages. In understanding that both GEFs GRAB and Rabin8 jointly contribute to Rab8 activity in this TLR signalling complex, we ventured further upstream to investigate the regulatory partners that recruit and activate these Rab8 GEFs. Mass-spectrometry analysis and subsequent bacterially expressed recombinant protein pull-down experiments validated the multi-Rab effector OCRL as a genuine Rabin8 binding partner (see Figures 7.2 and 7.4). Live cell imaging with fluorescent Rab8a and OCRL constructs have shown that both these proteins are actively recruited to and enriched on macrophage macropinosomes at similar times in response to LPS (see Figure 7.3B). As mentioned in chapter 7, this led to the hypothesis that two different scenarios exist for the potential involvement of OCRL. Either 1) Rabin8 helps to recruit OCRL as a Rab8 effector, or 2) OCRL recruits Rabin8 to macropinosomes to act as the Rab8a GEF (see Figure 7.6).

If Rabin8 is recruiting OCRL as an alternative effector for Rab8a on macropinosomes, this places Rab8a in an interesting situation where, through both its effectors PI3Kγ and OCRL, it would be acting to alter the phosphoinositide environment on the macropinosome membrane. This would allow Rab8a to potentially direct the maturation, trafficking and signalling outcomes of the macropinosome (Di Paolo and De Camilli 2006) as well as controlling signalling. To test this, future work will include performing live cell imaging in Rabin8 KO cells where I would predict seeing loss of OCRL on macropinosome membranes. However, as both OCRL (phosphatase) and PI3Kγ (kinase) compete for the same substrate [$PI(4,5)P_2$] with opposite outcomes, this puts into question whether OCRL is also able to interact with GRAB in macrophages. A study by Bohdanowicz and colleagues demonstrated that inhibition of Rab5 associated recruitment of OCRL to late phagosomes enhanced Akt signalling due to the increase in conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ by phospholipid kinases, skewing cell signalling outputs and endosome maturation processes (Bohdanowicz and Balkin *et al.* 2012). My findings showed that in both single GRAB or Rabin8 KO cells, PI3Kγ associated Akt signalling remained unchanged, suggesting there was no competition by OCRL in the absence of either GEF, possibly indicating the balance between PI3Kγ and OCRL function remained relatively intact. If this OCRL-GEF interaction is exclusive only to Rabin8, an increase in PI3Kγ-associated Akt signalling due to the lack of OCRL competition would have been expected. I was not able to assess if GRAB is likewise able to bind to OCRL in macrophages; however, now with a bacterially expressed GST-OCRL construct, another potential future experiment will be to use this OCRL construct in a pull-down experiment to assess if GRAB is also likewise able to interact with OCRL. Additionally, live cell imaging of fluorescent OCRL can be performed in the double KO cells to see if OCRL recruitment to macropinosomes is affected in the absence of both GEFs.

For the second scenario, live cell imaging with recombinant Rab13 and Rab8a constructs have shown that Rab13 is indeed spatially and temporally upstream of Rab8a macropinosome enrichment in macrophages (see Figure 7.5B). As a multi-Rab effector, OCRL has the ability to bind to multiple Rabs including Rab13 and Rab8a. Therefore, in this context, OCRL could be first recruited to the macropinosome as a Rab13 effector which then acts to recruit Rabin8 to these macropinosomes for

downstream Rab8a activation. If this proves accurate, it would describe a novel Rab cascade that ties in Rab13-associated macropinocytosis (Condon and Heddleston *et al.* 2018), and Rab8a TLR-signalling function (Luo and Wall *et al.* 2014). To test this, future work will involve Rab8 activation assays performed in CRISPR Rab13 KO macrophages will determine if Rab13 is functionally upstream of Rab8a in this TLR-signalling pathway. Additionally, live cell imaging with fluorescent Rabin8 in Rab13 KO cells can be used to investigate if the absence of Rab13 affects Rabin8 recruitment to macropinosomes. Thus, the early studies carried out in this project presage interesting scenarios for OCRL involvement in TLR signalling and macrophage innate immune responses.

Conclusions

In this study, I describe an expansion of the TLR4-LRP1-Rab8a-PI3Kγ complex that contributes to inflammatory signalling in macrophages by identifying two GEFs responsible for activating Rab8a in this context. I demonstrated that the Rab8 GEFs, GRAB and Rabin8, interact with Rab8a in macrophages in an LPS/TLR4 inducible fashion but gene deletion *in vitro* showed that neither GEF is required for Rab8a membrane localisation on macropinosome membranes. The absence of either GRAB or Rabin8 affects LPS induced Rab8 GTP-loading, and deletion of both GEFs together impairs the phosphorylation of key kinases and signalling molecules in the LPS-induced Akt/mTOR pathway. Using a fluorescent Akt probe, I visually confirmed that Akt enrichment occurs on macropinosomes in response to LPS and this recruitment of Akt is diminished in the absence of both GEFs, defining the macropinosome as the exact site of activation for this TLR-signalling complex. Furthermore, using biochemical techniques, I identified a novel Rab8 GEF-effector interaction between Rabin8 and OCRL, introducing an exciting new regulatory protein for Rab8 function in macrophages.

Through the TLR4-LRP1-Rab8a-PI3Kγ complex, GRAB and Rabin8 are predicted to control inflammatory cytokines, since the other members of the complex both individually and as a cohort, constrain pro-inflammatory cytokine output and enhance anti-inflammatory cytokines (Fukao and Koyasu 2003, Stark and Sriskantharajah *et al.* 2015, Luo and Wall *et al.* 2018). In this context, the complex controls macrophage

polarization towards the M2, inflammation-resolving phenotype. Thus, GRAB and Rabin8 are anticipated to also be inflammation constraining regulators and further cytokine assays on the KO cell lines or future KO mice will be able to confirm this. These Rab GEFs add to a complex mixture of other Rab GEFs and small GTPases that influence TLR trafficking and signalling. Their respective distributions and possible association with macropinosomes will be interesting and important to resolve through future imaging studies.

In conclusion, this thesis defines a previously unknown function for GRAB and Rabin8 in regulating macrophage TLR signalling for the control of inflammatory responses, further developing our understanding of the regulatory machinery that controls inflammatory signalling in macrophages.

Chapter 9

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9. REFERENCES

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Chapter 10

Appendices

Appendix 1. University of Queensland Animal Ethics Letter

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA

Office of Research Ethics
Director
Nicole Shively

Animal Ethics Approval Certificate

13-Feb-2018

Please check all details below and inform the Animal Ethics Unit within 10 working days if anything is incorrect.

Activity Details

Chief Investigator:	Professor Jennifer Stow, Institute for Molecular Bioscience
Title:	Cytokine Trafficking and Phagocytosis in macrophages
AEC Approval Number:	IMB/535/17
Previous AEC Number:	IMB/026/15/NHMRC/ARC (NF)
Approval Duration:	12-Mar-2018 to 12-Mar-2021
Funding Body:	ARC, NHMRC
Group:	Molecular Biosciences
Other Staff/Students:	Lin Luo, James Xiao, Darren Brown, Samuel Tong, Yu Hung, Adam Wall, Rebecca Mills
Location(s):	St Lucia Bldg 80 - QBP, IMB

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Mice - genetically modified	CD14-/-	Adults	Mix	Institutional Breeding Colony	25	25
Mice - genetically modified	Lyn-/-	Adults	Mix	Institutional Breeding Colony	50	50
Mice - genetically modified	TLR4 -/-	Adults	Mix	Institutional Breeding Colony	40	40
Mice - genetically modified	Lynup/up	Adults	Mix	Institutional Breeding Colony	50	50
Mice - genetically modified	P110y-/-	Adults	Mix	Institutional Breeding Colony	105	105
Mice - genetically modified	Lyn kinase dead	Adults	Mix	Institutional Breeding Colony	50	50
Mice - genetically modified	tbc1d1-/-	Adults	Mix	Institutional Breeding Colony	15	15
Mice - genetically modified	CD11b	Adults	Mix	Institutional Breeding Colony	25	25
Mice - genetically modified	PI3Kgamma	Adults	Mix	Institutional Breeding Colony	20	20
Mice - non genetically modified	C57BL/6	Adults	Mix	Institutional Breeding Colony	170	170

Permits

Provisos

Approval Details

Description	Amount	Balance

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Mice - genetically modified (CD11b, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	25	25
Mice - genetically modified (CD14-/-, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	25	25
Mice - genetically modified (Lyn kinase dead, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	50	50
Mice - genetically modified (Lyn-/-, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	50	50
Mice - genetically modified (Lynup/up, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	50	50
Mice - genetically modified (P110y-/-, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	105	105
Mice - genetically modified (PI3Kgamma, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	20	20
Mice - genetically modified (tbc1d1-/-, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	15	15
Mice - genetically modified (TLR4 -/-, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	40	40
Mice - non genetically modified (C57BL/6, Mix, Adults, Institutional Breeding Colony)		
9 Feb 2018 Initial approval	170	170

Please note the animal numbers supplied on this certificate are the total allocated for the approval duration

Please use this Approval Number:

1. When ordering animals from Animal Breeding Houses
2. For labelling of all animal cages or holding areas. In addition please include on the label, Chief Investigator's name and contact phone number.
3. When you need to communicate with this office about the project.

It is a condition of this approval that all project animal details be made available to Animal House OIC.
(UAEC Ruling 14/12/2001)

The Chief Investigator takes responsibility for ensuring all legislative, regulatory and compliance objectives are satisfied for this project.

This certificate supercedes all preceeding certificates for this project (i.e. those certificates dated before 13-Feb-2018)

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Appendix 2. CRISPR guide RNA on and off target scores

Table 10.1. Off and on target scores for CRISPR guideRNAs (IDT gRNA analysis)

CRISPR gRNA	Sequence (5′ - 3′)	On-target	Off-target
Rabin8 CRISPR I	ATGGGCTTCGTAAACGCGACAGG	33	98
Rabin8 CRISPR II	TTTACGAAGCCCATCTGTTCTGG	56	75
Rabin8 CRISPR III	TAGAGAGAAGGGCTACGAAAGG	34	56
GRAB CRISPR I	CGCAGGCGTGACACATCCAGTGG	25	71
GRAB CRISPR II	TCTCCATGGAGGAACTGCGCAGG	58	71
GRAB CRISPR III	CTTCTCTCGGATCTCCATGGAGG	61	24

Appendix 3. Uncropped blots for Figures 4.5B and C

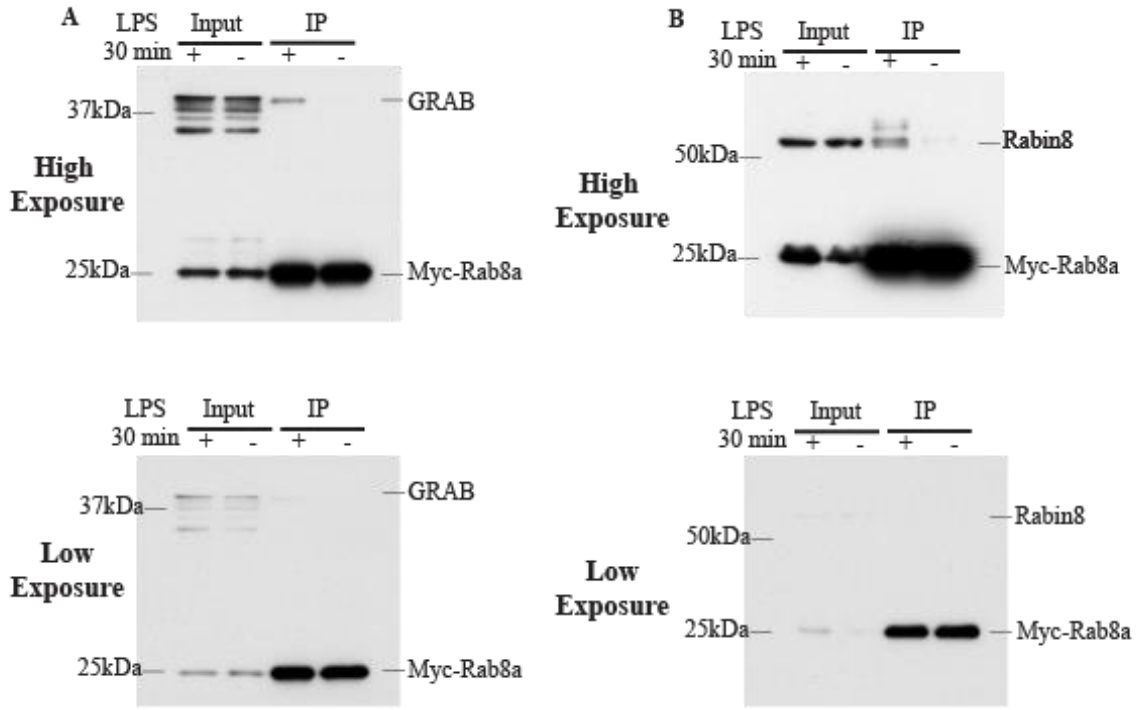

Figure 10.1. Uncropped immunoprecipitation blots of Figures 4.5B and C.