VICTORIA UNIVERSITY OF WELLINGTON

DOCTORAL THESIS

**Characterisation of the *Bartonella*
quintana YopJ Homologue**

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Abstract

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Bartonella is a genus of re-emerging bacterial pathogens that typically cause asymptomatic, intra-erythrocytic bacteraemia in their reservoir hosts and are highly specialised to evade host immunity. One of the many mechanisms by which *Bartonella* spp. modulate the host immune system is the type-IV-secretion system, a protein complex that delivers effector proteins directly into host cells to modulate their function.

Some *Bartonella* species, including *B. quintana*, the causative agent of trench fever, possess an effector protein that is homologous to the effector YopJ of *Yersinia* species. *Yersinia* YopJ inhibits the MAPK and NF- κ B pathways, and YopJ homologues in other species have similar effects, though through different targets. Very little is known about the function of the *B. quintana* YopJ homologue, but it may play a role in immune modulation by the bacteria.

My aim was to characterise the function of the *B. quintana* YopJ homologue. I had evidence that it inhibits the NF- κ B pathway, so I investigated which step of signalling activation is the target of this inhibition. I also sought to determine whether it impacts signalling pathways other than NF- κ B, and to identify the specific host protein that it targets.

I performed these investigations using ELISA, high-throughput fluorescence microscopy, Western blotting, LC/MS proteomic screening, and a yeast two-hybrid screen.

I found that the *B. quintana* YopJ homologue inhibits the NF- κ B pathway at or upstream of IKK activity, may also impact JNK signalling, the cell cycle, and the mTOR complex, and interacts with the host protein DCNL1, a component of neddylation machinery. Additionally, I determined that this interaction does not impact the neddylation of the protein Cullin-1.

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List of Abbreviations

AMP ^r	<u>A</u> mpicillin <u>r</u> esistance
ANOVA	<u>A</u> nalysis of <u>v</u> ariance
Bep	<u>B</u> artonella <u>e</u> ffector <u>p</u> rotein
BID domain	<u>B</u> artonella <u>i</u> ntracellular <u>d</u> elivery <u>d</u> omain
BLAST	<u>B</u> asic <u>L</u> ocal <u>A</u> lignment <u>S</u> earch <u>T</u> ool
bp	<u>b</u> ase <u>p</u> air
Bq-YopJ	<u>B</u> artonella <u>q</u> uintana <u>Y</u> opJ homologue (protein)
Bq-yopJ	<u>B</u> artonella <u>q</u> uintana <u>Y</u> opJ homologue (gene)
cDNA	<u>c</u> omplementary <u>D</u> N <u>A</u>
CRL	<u>C</u> ullin- <u>R</u> ING- <u>l</u> igase
DAPI	<u>4</u> ', <u>6</u> - <u>d</u> iamidino-2-phenyl <u>i</u> ndole
DCNL1	<u>d</u> efective in <u>c</u> ullin <u>n</u> eddylation protein 1- <u>l</u> ike protein <u>1</u>
DCUN1D1	<u>d</u> efective in <u>c</u> ullin <u>n</u> eddylation <u>1</u> - <u>d</u> omain containing <u>1</u> (gene)
ddH ₂ O	<u>D</u> ouble <u>d</u> istilled <u>w</u> ater
DMSO	<u>D</u> imethyl <u>s</u> ulfoxide
DNA	<u>D</u> eoxyribonucleic <u>a</u> cid
EDTA	<u>E</u> thylenediaminetetraacetic <u>a</u> cid
ELISA	<u>E</u> nzyme-linked immunosorbent <u>a</u> ssay
FCS	<u>F</u> oetal <u>c</u> alf <u>s</u> erum
G418 ^r	<u>G</u> 418 <u>r</u> esistance
gDNA	genomic <u>D</u> N <u>A</u>
GFP	<u>G</u> reen <u>f</u> luorescent protein
HEK293	<u>H</u> uman <u>E</u> mbryonic <u>K</u> idney <u>2</u> 93 cells
IκBα	<u>I</u> nhibitor of NF-κB <u>a</u> lpha
IKK	<u>I</u> κB <u>k</u> inase
IL-8	<u>I</u> nterleukin <u>8</u>
KAN ^r	<u>K</u> anamycin <u>r</u> esistance
kb	<u>k</u> ilobase
LB	<u>L</u> ysogeny <u>b</u> roth
LC/MS	<u>L</u> iquid <u>c</u> hromatography <u>m</u> ass <u>s</u> pectrometry
LC/MS/MS	<u>L</u> iquid <u>c</u> hromatography tandem <u>m</u> ass <u>s</u> pectrometry
LPS	<u>L</u> ipopolysaccharide

MAPK	<u>M</u> itogen- <u>a</u> ctivated p <u>r</u> otein <u>k</u> inase
MS	<u>m</u> ass <u>s</u> pectrometry
MS/MS	tandem <u>m</u> ass <u>s</u> pectrometry
NF-κB	<u>N</u> uclear <u>f</u> actor <u>k</u> appa-light-chain-enhancer of activated <u>B</u> cells
PBS	<u>P</u> hosphate <u>b</u> uffered <u>s</u> aline
PCR	<u>P</u> olymerase <u>c</u> hain <u>r</u> eaction
PRR	<u>P</u> attern <u>r</u> ecognition <u>r</u> eceptor
RING	<u>R</u> eally <u>i</u> nteresting <u>n</u> ew gene
RNA	<u>R</u> ibon <u>u</u> cleic <u>a</u> cid
rRNA	<u>r</u> ibosomal <u>R</u> NA
rpm	<u>r</u> evolutions per <u>m</u> inute
RPMI	<u>R</u> oswell <u>P</u> ark <u>M</u> emorial <u>I</u> nstitute medium
SDS	<u>S</u> odium <u>d</u> odecyl <u>s</u> ulfate
SDS-PAGE	<u>S</u> DS poly <u>a</u> crylamide gel <u>e</u> lectrophoresis
TAE	<u>T</u> ris/ <u>a</u> cetic acid/ <u>E</u> DTA
TE	<u>T</u> ris/ <u>E</u> DTA
TLR	<u>T</u> oll- <u>l</u> ike <u>r</u> eceptor
TNF-α	<u>T</u> umour <u>n</u> ecrosis factor <u>a</u> lpha
Tris	<u>T</u> ris(hydroxymethyl)aminomethane
YopJ	<u>Y</u> ersinia <u>o</u> uter protein <u>J</u>

Chapter 1

Introduction

1.1 *Bartonella*

Bartonella is a genus of Gram-negative alphaproteobacteria that infect mammals, are transmitted by biting arthropods, and cause intracellular infection of red blood cells (intra-erythrocytic bacteraemia). Each *Bartonella* species infects one, or a few closely related, host species, called the reservoir host¹. *Bartonella* infections are widespread and have been found in most mammalian species tested¹. Incidental infection, which involves an infection in a non-reservoir host, has even been reported in non-mammals^{2–4}. Tables 1.1 and 1.2 show lists of host species where *Bartonella* spp. DNA has been detected in the bloodstream. This list continues to grow; in 1990 there were only two named *Bartonella* species, today there are more than thirty⁵.

1.1.1 Infection in Reservoir Hosts

Bartonella infections in reservoir hosts are highly prevalent, occurring in up to 50 % of feral and domestic cats, over 40 % of wild rodents, and over 90 % of some wild ruminants⁶. These infections are often apparently asymptomatic, long-lasting, and cause intra-erythrocytic bacteraemia, the hallmark of *Bartonella* infection of a reservoir host⁷. One exception is *B. apis*, a recently described honeybee gut symbiont that is not known to cause mammalian bloodstream infection⁸.

Bartonellae are transmitted between reservoir hosts by biting arthropod vectors, such as fleas, lice, sandflies, keds, and biting flies⁹. When a vector takes a blood meal, it inoculates the bacteria into the dermis, either depositing *Bartonella*-laden faeces onto the skin, which are rubbed or scratched into the bite wound, or inoculating the bacteria directly, depending on the vector. Once

past the skin, the bacteria enter lymphatic capillaries and are drained into lymph nodes, where they resist phagocytosis, suppress an immune response, and finally drain into the blood¹⁰.

There is still uncertainty surrounding the mechanisms by which *Bartonella* species establish chronic infection; one theory is that the bacteria first infect another cell type, such as endothelial cells, from which they can reseed into the blood¹. A blood seeding niche explains the relapsing nature of some *Bartonella* infections, but as of yet there is no strong evidence to suggest what type of cell it is, or even that it is universal across *Bartonella* species.

1.1.2 Phylogenetics

Recent genome sequencing projects have suggested that there are three major branches of the *Bartonella* family tree, called groups A, B, and C⁴⁰. Within each group, the species share a similar infection strategy and array of virulence factors (expanded upon in Section 1.3 below). Group A includes the species *B. bovis* and *B. schoenbuchensis*, and primarily infects ruminants⁴⁰. Group B includes the species *B. rochalimae* and *B. clarridgeiae*, and infects wild canids, cats, rats, and squirrels⁴⁰. Group C includes the species *B. henselae* and *B. quintana*, and infects humans, cats, dogs, and rodents⁴⁰. Not all *Bartonella* species neatly fit into these groups. For instance, both *B. australis* and *B. bacilliformis*, whose reservoir hosts are kangaroos and humans, respectively, are highly divergent and not included in these three groups⁴⁰. Figure 1.1 shows a phylogenetic tree of selected *Bartonella* groups (Section 1.3).

TABLE 1.1: **Reported mammalian carriers of *Bartonella*.** Most of these were detected by PCR only so are likely to be incidental, rather than reservoir, infections.

Host	<i>Bartonella</i> species (if known)	Reference
MAMMALS		
Badger		[11]
Bat		[12]
Beluga Whale	<i>B. henselae</i>	[13]
Bobcat	<i>B. henselae</i> , <i>B. koehlerae</i>	[14]
Camel	<i>B. bovis</i>	[15]
Cat	<i>B. henselae</i>	[16]
Cattle	<i>B. bovis</i> , <i>B. chomelii</i> , <i>B. schoenbuchensis</i>	[17]
Cheetah		[18]
Coyote	<i>B. vinsonii</i> subsp. <i>berkhoffi</i> , <i>B. rochalimae</i> , <i>B. henselae</i>	[19]
Deer		[20]
Dog	<i>B. henselae</i> , <i>B. vinsonii</i> subsp. <i>berkhoffi</i> , <i>B. washoensis</i> , <i>B. elizabethae</i>	[21]
Donkey	<i>B. henselae</i>	[22]
Elk		[20]
Fox	<i>B. rochalimae</i>	[11]
Horse		[22]
Human	<i>B. bacilliformis</i> , <i>B. quintana</i> , <i>B. henselae</i>	[23]
Kangaroo	<i>B. australis</i>	[24]
Lion	<i>B. henselae</i>	[18]
Marten		[25]
Mouse	<i>B. grahamii</i> , <i>B. taylorii</i>	[26, 27]
Mole		[27]
Mongoose	<i>B. henselae</i>	[14]
Monkey	<i>B. quintana</i>	[28]
Moose		[29]
Opossum		[30]
Pig	<i>B. henselae</i> , <i>B. koehlerae</i> , <i>B. vinsonii</i> subsp. <i>berkhoffi</i>	[31]
Porpoise	<i>B. henselae</i>	[32]
Puma		[33]
Rabbit	<i>B. alsatica</i>	[34]
Raccoon	<i>B. henselae</i> , <i>B. koehlerae</i>	[35]
Rat	<i>B. tribocorum</i>	[36]
River Otter		[14]
Sea Otter	<i>B. washoensis</i> , <i>B. clarridgeiae</i> , <i>B. henselae</i>	[14]
Seal	<i>B. grahamii</i>	[37]
Sheep	<i>B. melophagi</i>	[38]
Shrew	<i>B. elizabethae</i> , <i>B. rattimassiliensis</i>	[39]
Skunk	<i>B. rochalimae</i>	[14]
Squirrel	<i>B. durdenii</i> , <i>B. volans</i>	[39]
Vole		[27]
Wolf	<i>B. rochalimae</i>	[11]

TABLE 1.2: **Non-mammalian animals reported to have detectable *Bartonella* DNA in the bloodstream.** These are likely to be incidental, rather than reservoir, infections.

Host	<i>Bartonella</i> species (if known)	Reference
BIRDS		
Blackbird	<i>B. henselae</i>	[2]
Bluebird		[3]
Loon	<i>B. koehlerae</i>	[2]
Martin		[3]
Mockingbird	<i>B. henselae</i>	[2]
Swallow		[3]
Woodpecker	<i>B. koehlerae</i>	[2]
REPTILES		
Sea Turtle	<i>B. henselae</i> , <i>B. vinsonii</i> subsp. <i>berkhoffii</i>	[4]

1.2 *Bartonella* Infection in Humans

Most human infections are caused by three species, *B. henselae*, *B. bacilliformis*, and *B. quintana*¹, though at least 17 *Bartonella* species have been reported to cause human disease⁴¹. Humans are the reservoir host for *B. bacilliformis* and *B. quintana*; infections by all other *Bartonella* species are incidental, and are generally zoonotic, resulting from contact with animals or biting arthropods. Both *B. henselae* and *B. quintana* are part of group C, while *B. bacilliformis* does not belong to any of the groups, though is most closely related to the ruminant-associated group A⁴⁰.

1.2.1 *B. henselae*

Cats are the reservoir host for *B. henselae*, although its DNA has been detected in the bloodstream of a wide range of other incidental hosts, as shown in Tables 1.1 and 1.2.

In humans, *B. henselae* causes cat scratch disease (CSD), so named because it is transferred to humans by bites or scratches from infected cats. CSD presents as swelling of the lymph nodes and is usually accompanied by inflammation at the wound site, though other symptoms such as fever, headache, and fatigue are possible²³. In most cases CSD is self-limiting and will resolve without treatment over the course of 2 to 6 months²³. *B. henselae* DNA has been detected in cat fleas in New Zealand, although the annual incidence of CSD in New Zealand is unknown. In the United States, which likely has a similar

incidence, the case rate is 4.7 per 100,000 persons < 65 years of age^{42,43}. Apart from CSD, *B. henselae* can cause relapsing fever, bacteraemia, and endocarditis in some people²³.

In an immunocompromised host, such as a person suffering from AIDS, chronic alcoholism, or undergoing immunosuppressive therapy, *B. henselae* infection can be life-threatening. In these cases it can cause bacillary angiomatosis, or vasoproliferative lesions on the skin. Blood-filled lesions in the liver, bacillary peliosis hepatis, may also occur, along with chronic bacteraemia, a long-term bacterial infection of the blood²³.

1.2.2 *B. bacilliformis*

B. bacilliformis is one of two *Bartonella* species for which humans are the reservoir host. The infection occurs only at high altitudes in the Andes, which is the range of its arthropod vector, *Lutzomyia verrucarum*, a species of sand fly. *B. bacilliformis* causes Carrion's disease, which occurs in two distinct phases: an acute phase, also known as Oroya fever, and a chronic phase, known as verruga peruana (Peruvian wart)⁴⁴.

Carrion's disease has been present in Peru since before the arrival of Europeans, with verruga peruana lesions identified on a mummy from the Tiahuanaco (Tiwanaku) culture⁴⁵. The first known account of what was likely Carrion's disease was published in 1571 by Spanish conquistador Pedro Pizarro, where he refers to both native Peruvians and Spaniards suffering from a disease called "berrugas"⁴⁶.

Carrion's Disease is named after Daniel Alcides Carrión, a Peruvian medical student who demonstrated the link between the acute and chronic phases of the disease. He inoculated himself with tissue from a verruga peruana lesion, and subsequently developed, and died from, Oroya fever⁴⁷.

The symptoms of Oroya fever begin with malaise, headache, chills, fever, muscle pain, and joint pain, progressing into lymphadenopathy and severe haemolytic anaemia. *B. bacilliformis* is unique among *Bartonella* species, in that the intraerythrocytic bacteraemia results in lysis of red blood cells and potentially fatal anaemia. Oroya fever has mortality rates of 44 – 88 % if untreated, though this is reduced to 0.7 % with treatment⁴⁴. In verruga peruana, the bacteria chronically infect endothelial cells, leading to the characteristic nodular lesions on the arms, legs, face, and torso⁴⁴.

1.2.3 *B. quintana*

B. quintana is the second *Bartonella* species for which humans are the reservoir, and is the focus of this project. It is transmitted by the body louse (*Pediculus humanus humanus*) and causes the disease trench fever.

B. quintana has a long history with humanity in both war and peace, stretching back thousands of years. Its DNA has been detected in the dental pulp of a person who died ~2000 BCE in south-eastern France, and in erythrocytes on the bones of a young adult who died in Roman Gaul in the second century CE^{48,49}. It has also been found in dental pulp from burials across Europe, including France, Italy, Russia, and Ukraine, dating from the first century CE through the 1850s⁵⁰. The pathogen was also identified in a mass grave of bubonic plague victims from the Middle Ages⁵¹.

B. quintana has also been detected in the graves of soldiers from the war of the Spanish succession and from Napoleon's Grand Army during their retreat from Russia^{52,53}. There have also been descriptions of diseases that may be trench fever including a reference to a "five day fever" in the medieval poem *Ysengrimus*, and reports of a similar "relapsing fever" in Belfast in the nineteenth century^{54,55}.

Trench fever was first identified by British doctors during World War I, where it affected between 380,000 and 1,000,000 troops and was responsible for 15 % of medical evacuations due to disease⁵⁶. It returned during World War II and once again caused large scale epidemics, particularly among German troops⁵⁷. Since its discovery, trench fever has also been reported in civilian populations across the world, including North America, Europe, Africa, The Middle East, Australia, and Asia⁵⁷⁻⁶². In modern times, *B. quintana* infection is also known as urban trench fever, and has emerged as a significant public health problem among populations experiencing poverty, homelessness, and alcoholism; it is also common among displaced people⁶³⁻⁶⁵. There were three outbreaks of urban trench fever in 2020, in Denver, Colorado; Winnipeg, Canada; and Marseille, France; there was also an outbreak in Seattle, Washington in 2018⁶⁶⁻⁶⁹. Another group who are at high risk of *B. quintana* infection are immunocompromised patients, such as those with AIDS; this was particularly prevalent during the epidemic in the 1980s and 1990s⁷⁰.

There have been no confirmed cases of urban trench fever in New Zealand, but *B. quintana* endocarditis has been reported in dogs in New Zealand⁷¹. Culture-negative infective endocarditis is a well-established clinical manifestation of *B. quintana* infection, and is also a significant health problem in New Zealand^{63,72}. In New Zealand, the causative organism of culture-negative endocarditis is only identified in around 10 % of cases, so the lack of confirmed *B. quintana* infections may be due to a failure of diagnosis rather than the absence of the bacteria⁷³.

The "classical" symptoms of trench fever include headache, pain in the shins, and relapsing fever⁵⁷. These bouts of fever typically last 1 – 3 days and recur every 4 – 6 days⁷⁴. These symptoms are the origin of two of the other names for trench fever: shin bone fever, due to the pain in the shins, and quintan or five-day fever, due to the characteristic interval of fever relapses. The second of these is also the origin of the name of the species, *B. quintana*⁶³. Trench fever is typically not fatal, though it can result in chronic health problems, and there is anecdotal evidence of decades-long asymptomatic bacteraemia^{74–76}. Other possible symptoms include dizziness, back pain, conjunctivitis, sweating, leucocytosis, and splenomegaly⁷⁵. Like *B. henselae*, *B. quintana* can also cause bacillary angiomatosis in immunocompromised patients⁷⁰.

Diagnosis of *B. quintana* infection is difficult. Culturing the bacteria from blood is slow, often requiring 20 – 30 days of growth⁶³. For this reason, the most widely-used diagnostic techniques are serological assays, though these can be cross reactive with *B. henselae*. PCR amplification and sequencing, of either citrate synthase genes or 16S rRNA, are also used⁶³. The treatment for trench fever is a combination of gentamicin for two weeks and doxycycline for four weeks. *B. quintana* endocarditis requires a longer course of gentamicin for two weeks in combination with ceftriaxone and possibly doxycycline for six weeks⁷⁷.

1.3 *Bartonella* Virulence Factors

Bartonellae possess a wide variety of virulence factors, though each species and group have a unique array as shown in Figure 1.1 below.

FIGURE 1.1: **Distribution of virulence factors among *Bartonella* species.**

The virulence factors found in each species is shown as boxes, with a coloured box indicating that a virulence factor is found in that species. The gains and losses of virulence factors are depicted above and below the nodes in the tree. Dashed boxes represent assumed pseudogenes. Dark brown boxes in the virB column indicate multiple gene copies. ** Partial gain, or gain followed by partial loss. LCA refers to the last common ancestor. This figure was modified from figures 1a and 5b in reference [40], in accordance with the Creative Commons Attribution (CC BY 4.0) license.

1.3.1 Flagella

Flagellation is almost ubiquitous in groups A and B but is not present in group C⁴⁰. The flagella of *B. bacilliformis* are the best characterised. They are involved in adherence to red blood cells and are required for their invasion^{78,79}.

1.3.2 Lipopolysaccharide

Bacterial lipopolysaccharide (LPS) is a pathogen-associated molecular pattern of Gram-negative bacteria, which is recognised by Toll-like receptor 4 (TLR4). TLR4 activation triggers the NF- κ B pathway, leading to activation and recruitment of the innate immune system⁸⁰; *Bartonella* spp. have adapted to avoid this. The LPS from *B. quintana* is the best characterised. Not only does it not activate TLR4, it is an antagonist to TLR4 activation⁸¹. LPS molecules from *B. henselae* and *B. bacilliformis* are not immunogenic, which suggests that this modified LPS is a shared feature among the *Bartonella* genus^{82,83}. This likely enables the bacteria to persist in the host bloodstream, even at high titres, without triggering the symptoms of septic shock.

1.3.3 Type-V-Secretion Systems

Type-V-secretion systems include autotransporters (Type V_a), two-partner secretion systems (Type V_b), and Trimeric autotransporter adhesins (Type V_c)⁸⁴. They are found in all *Bartonella* groups, and have probably been vertically inherited from the last common ancestor of all *Bartonella* species⁴⁰.

Type-V_a-Secretion Systems

There are three type-V_a-secretion systems present in all *Bartonella* species: *autoA*, *autoB*, and *iba*, though only *iba* has been characterised^{40,85}. In *B. tribocorum*, *iba* is highly expressed when the bacteria start to invade endothelial cells in vitro, and is downregulated after erythrocyte invasion, which suggests that it is involved in establishing erythrocyte infection⁸⁵.

Type-V_b-Secretion Systems

There is one type-V_b-secretion system present in some *Bartonella* species. Filamentous hemagglutinin (Fha) genes are found in some species within group C, including *B. henselae* and *B. tribocorum*, but are not present in the reduced genome of *B. quintana*^{1,40}. It is a two-partner secretion system, with *fhaC* (also called *hecB*) encoding the protein that mediates secretion of the filamentous haemagglutinin, encoded by *fhaB*⁸⁶. There is currently not enough evidence to determine the function of Fha in *Bartonella* but the importance of homologues to virulence in other species suggests that it may play a role in the virulence of *Bartonella* as well⁸⁶.

Type-V_c-Secretion Systems

The type-V_c-secretion proteins, also known as trimeric autotransporter adhesins, are found in many Gram-negative pathogens, where they often assist with bacterial binding to the extracellular matrix of host cells¹. All *Bartonella* species have trimeric autotransporter adhesins, though the best studied is *Bartonella* adhesin A (BadA) of *B. henselae*⁴⁰. BadA is a large protein that coats the surface of *B. henselae*, and is involved in host cell adhesion, inhibition of phagocytosis, and upregulation of vascular growth factors^{87,88}.

1.3.4 The Type-IV-Secretion System

Type-IV-secretion systems are related to conjugation machinery and are found in many Gram-negative bacteria⁸⁹. They usually mediate the transfer of macromolecules (DNA or proteins) into eukaryotic host cells⁹⁰. There are three type-IV-secretion systems found in the genus *Bartonella*: Trw, which is only found in group C and in *B. australis*; Vbh, which is present in groups A and C, and is sometimes encoded on a plasmid rather than chromosomally; and VirB/D4, which is present in all members of groups B and C⁴⁰.

Trw

The Trw type-IV-secretion system does not apparently secrete any effectors, but rather is involved in adhesion to erythrocytes, host specificity, and mediates erythrocyte invasion⁹¹. The presence of Trw is mutually exclusive with the flagella; each *Bartonella* species (and group) has one or the other but not both, suggesting that they may play similar roles in virulence⁴⁰.

Vbh

The VirB-homologue (Vbh) type-IV-secretion system seems to be closely related to VirB/D4⁹². Its role in virulence is unclear, though its presence as the only T4SS in some species suggests that it is functional, although redundant with VirB/D4^{1,92}.

VirB/D4

For the *Bartonella* species that possess the VirB/D4 type-IV-secretion system, it is an important virulence factor. It is involved in immune regulation, suppression of apoptosis, and is essential for the establishment of intraerythrocytic bacteraemia that is the hallmark of *Bartonella* reservoir host infection^{93,94}. The

Bartonella VirB/D4 type-IV-secretion system primarily secretes effector proteins into mammalian host cells¹, though in some species it may also secrete plasmid DNA into host cells⁹⁵. It is encoded by 11 structural genes, including VirB2 – VirB11 and VirD4. These are located in a single genetic locus, adjacent to the *Bartonella* Effector Proteins (Beps). The genetic arrangements of the VirB/D4 Type 4 secretion locus for *B. henselae*, *B. tribocorum*, and *B. quintana* are shown for comparison in Figure 1.2.

1.3.5 Other Virulence Factors

Bartonella species in group A possess multiple copies of a potential variant of a type-V-secretion system called parallel beta-helix repeat protein (PbH1). This protein is possibly derived from a type-V-secretion system but has lost its autotransporter domain⁴⁰.

Another protein of interest is a homologue of cholera toxin A (CtxA) found in *Bartonellae* belonging to group A and some members of group B⁴⁰. In *Vibrio cholerae*, CtxA is known as the cholera toxin, and is a major mediator of the disease. CtxA works in concert with CtxB, forming an AB toxin, with CtxB mediating binding and uptake by the host cell, and CtxA carrying out ADP ribosylation of the G α subunit of heterotrimeric G protein⁹⁶. There is no known *Bartonella* homologue of CtxB⁴⁰. *V. cholerae* CtxA modifies adenylate cyclase and constitutively activates it, causing a build-up of cAMP in the cell and subsequent Cl⁻ anion secretion⁹⁷.

Interestingly, the type-IV effector BepA of *B. henselae* also modifies adenylate cyclase, and CtxA is most prevalent in group A, which lacks the VirB type-IV-secretion system and BepA^{40,98}. This raises the question of whether CtxA and BepA perform similar roles in different *Bartonella* species, though some species in group B possess both CtxA and the VirB type-IV-secretion system⁴⁰.

1.3.6 *Bartonella* Effector Proteins

The Beps are the primary *Bartonella* effector proteins and while they vary between species, all but BepA1 of *B. quintana* possess at least one Bep Intracellular Delivery (BID) domain, which mediates secretion via the type-IV-secretion system⁹⁹. Some Beps contain a Filamentation Induced by cAMP (FIC) domain⁹⁹. FIC domains usually catalyse an AMPylation reaction, a post-translational modification in which an adenosine monophosphate (AMP) moiety is attached to a target protein¹⁰⁰, though there is only experimental evidence of this in one Bep, Bep2 of *B. rochalimaea*¹⁰¹. The FIC domain of BepC in both

B. henselae and *B. quintana* is not enzymatically active, but is required for the effector to function, possibly playing a role in substrate binding^{102,103}. Another domain found in the Beps is a functional tyrosine phosphorylation motif similar to the EPIYA motif of the *Helicobacter pylori* effector CagA⁹⁹. Tyrosine phosphorylation is generally restricted to multicellular organisms, where it plays an important role in a wide array of cellular signalling processes¹⁰⁴. Pathogenic bacteria make use of tyrosine phosphorylated effector proteins to subvert and modulate these host processes¹⁰⁴.

The best characterised Beps are those of *B. henselae*, of which there are seven, and have the following effects: BepA, inhibition of apoptosis; BepB, unknown; BepC, BepF, and BepG, actin modulation and invasome formation; BepD, inhibition of TNF- α secretion and stimulation of IL-10 secretion; BepE, protection of host cells from damage caused by other Beps^{103,105–109}.

When compared to *B. henselae*, *B. quintana* has a reduced arsenal of only six Beps. Two of these (BepA1 and BepA2) are homologues of *B. henselae* BepA, but with the FIC and BID domains occurring on separate proteins. *B. quintana* also lacks homologues of *B. henselae* effectors BepB and BepD, while the BepF homologue has undergone gene duplication. Given the close phylogenetic relationship between *B. henselae* and *B. quintana*, this variation in Bep repertoire suggests that the Beps are highly host-adapted, with Bep function and host targets not necessarily conserved⁴⁰.

Some *Bartonella* species, including *B. quintana*, possess a protein that is homologous to the *Yersinia* type-III-secreted intracellular effector YopJ.

FIGURE 1.2: **VirB/D4 operon in three representative group C *Bartonella* species.**
Assembled by aligning the *B. henselae* (NZ_LN879429.1) and *B. tribocorum* (NC_010161.1) annotated NCBI genomes with consensus sequences for the conserved domains, and the *B. quintana* JK-31 genome (NZ_KL411732.1) in the Geneious software package¹¹⁰.

1.4 YopJ

The YopJ family of effectors are a group of homologous acetyltransferases that are generally secreted by the type-III-secretion systems of a wide array of bacterial plant and animal pathogens^{111,112}. YopJ homologues tend to inhibit immune activation, though each have distinct cellular targets¹¹¹. In animal pathogens, YopJ homologues tend to interfere with the NF- κ B pathway, the MAPK pathway, or both¹¹¹. In plant pathogens, the function of YopJ homologues is far more diverse and less well-characterised; they can either play a role in inhibition of innate immunity or may exacerbate disease symptoms, depending on host species^{112,113}. YopJ homologues have a conserved catalytic triad of histidine, aspartate/glutamate, and cysteine residues, which catalyse the transfer of an acetyl group from acetyl coenzyme A (acetyl-CoA) to the target protein[114, 115]. This reaction involves a eukaryotic cofactor, inositol hexakisphosphate, which is also called IP6 or phytic acid¹¹⁶. Figure 1.3 shows an alignment of several diverse YopJ homologues and indicates the catalytic triad, acetyl-CoA binding domains, and phytic acid binding domains. The YopJ/YopP homologues of the *Yersinia* genus are the namesake and best characterised member of the effector family.

FIGURE 1.3: Alignment of YopJ homologues. Aligned using the the Geneious software package¹¹⁰. Sequences from Uniprot or NCBI gene. AopP from *Aeromonas salmonicida* (Uniprot accession: Q8GMP6), AvrA from *Salmonella enterica* serotype typhimurium (Uniprot accession: E8XKZ3), AvrRxv from *Xanthomonas euvesicatoria* (Uniprot accession: Q08678), Bq-YopJ from *B. quintana* JK-31 (GenBank: KEC61478.1), YopJ from *Yersinia pseudotuberculosis* (Uniprot accession: A0A0N9NCU6). Red boxes show the catalytic triad, blue boxes show acetyl-CoA binding domains, black boxes show phytic acid binding domains. The catalytic triad was identified base on reference [115]. The acetyl-CoA binding domains and phytic acid binding domains were identified based on the Uniprot database¹¹⁷.

1.4.1 *Yersinia*

The best known member of the *Yersinia* genus, and arguably the most famous bacterial pathogen in history, is *Y. pestis*, which is responsible for the bubonic plague. Few diseases have had a greater effect on history than epidemic bubonic plague, as it played a role in the collapse of the Byzantine Empire during the Justinian plague, and later killed up to a third of the European population during the 14th century CE. The latter epidemic even produced a measurable drop in atmospheric CO₂ levels, as a result of forest growth on fallow farmland^{118–120}.

Plague is usually spread to humans from an animal reservoir, usually rodents, via fleas, but can also be transmitted by handling infected tissues and by respiratory droplets¹²¹.

Bubonic plague presents with the rapid onset of fever, chills, weakness and headache, followed by the development of a painful bubo on a lymph node, usually on the groin, armpit, or neck¹²¹. Untreated bubonic plague has a fatality rate of 50 – 90 % and can kill in as little as 2 – 4 days after the onset of symptoms^{119,121}. In some cases, plague can spread to the lungs, causing secondary pneumonic plague, or can colonise the lungs directly by inhalation of respiratory droplets, causing primary pneumonic plague. Regardless of how it accesses the lungs, pneumonic plague is incredibly deadly, with a fatality rate of almost 100 % if not treated within a day of the onset of symptoms¹²². There are two other *Yersinia* species that cause disease in humans, *Y. enterocolitica* and *Y. pseudotuberculosis*, which cause a common but minor gastrointestinal disease called yersiniosis¹²¹.

1.4.2 *Yersinia* Effector Proteins

All three pathogenic *Yersinia* species possess a type-III-secretion system, encoded on a 70-kb virulence plasmid¹²³. The type-III-secretion system has a similar function to the type-IV-secretion system of *Bartonella*, secreting proteins directly into host cells, but is evolutionarily unrelated, having most likely originated from the bacterial flagella¹²³. The *Yersinia* effector proteins are called Yersinia Outer Proteins (Yops). There are eight known Yops: two translocator Yops (YopB, and YopD), which embed in the host plasma membrane and transport the six effector Yops (YopE, YopH, YopJ, YopO, YopT, and YopM) into the cytoplasm^{123,124}. The effector Yops interfere with host signalling pathways involved in immune activation, phagocytosis, and apoptosis¹²⁴.

1.4.3 *Yersinia* YopJ

The YopJ of *Yersinia* inhibits the activation of both the NF- κ B pathway and the MAPK pathway by acetylating IKK, MEK2, and TAK1, thereby preventing their phosphorylation^{115,125}. Inhibition of phosphorylation interrupts binding and interactions with signalling partners. The downstream effects of blocking these pathways include inhibition of pro-inflammatory cytokine secretion and killing of innate immune cells by apoptosis, pyroptosis, and necroptosis^{126–129}.

1.4.4 Other YopJ Homologues

AvrA

Salmonella enterica can cause multiple diseases depending on the serotype, but is best known for causing typhoid and severe diarrhoea¹³⁰. It has a type-III-secretion system and an effector that is homologous to YopJ, AvrA¹³¹.

AvrA inhibits the activation of the JNK pathway by acetylating the activation loop of MAPKK4¹³². The downstream effects of this are inhibition of apoptosis and the NF- κ B pathway^{131,132}.

AvrRxv

Xanthomonas is a genus of plant pathogens that infect over 400 different hosts, including many food crops¹³³. It has a type-III-secretion system and an effector that is homologous to YopJ, AvrRxv¹³¹. While plants lack an NF- κ B pathway they do have the MAPK pathway¹³⁴.

AvrRxv interacts with the ARI1 of tomato, an adaptor protein involved in many signalling pathways including MAPK^{135,136}. The downstream effect of this interaction is the elicitation of the hypersensitive response, a localised programmed cell death^{112,135}.

1.4.5 YopJ Biochemistry

The mechanism by which YopJ family effectors acetylate substrate proteins has been fully resolved based on both YopJ of *Yersinia* as well as HopZ1a the YopJ homologue from the plant pathogen *Pseudomonas syringae*^{137,138}. The first requirement for acetylation by a YopJ family effector is the binding of the eukaryotic cofactor IP6. Upon binding, IP6 causes conformational changes in the protein which opens a binding site for acetyl-CoA¹³⁷. IP6 is not present in prokaryotic cells, so this requirement ensures that YopJ homologues are only active within host cells. Acetyl-CoA may then bind, and therefore bring its acetyl group into the active site of the enzyme¹³⁷. This acetyl group is then attacked by the cysteine of the catalytic triad, forming an acetyl-YopJ covalent intermediate¹³⁸. The CoA molecule is then released and the acetyl group is transferred to the substrate protein¹³⁸.

1.4.6 *Bartonella* YopJ

There has been very little research into the YopJ homologues in *Bartonella*. Unlike other bacterial species that possess a YopJ homologue, *Bartonella* spp. lack a type-III-secretion system. Table 1.3 shows the identification of homologues of YopJ from *B. quintana* (Bq-YopJ), identified using BLAST. Also listed are the percent identities between homologues and, for *Bartonella* species with a homologue, their phylogenetic group. Intriguingly, *Bartonella* YopJ homologues are found only in groups B and C, which are the groups that possess the VirB/D4 type-IV-secretion system. This co-occurrence provides indirect evidence that *Bartonella* YopJ homologues could be secreted via type-IV-secretion.

TABLE 1.3: **YopJ homologues most closely related to *B. quintana* YopJ.** Results presented for a tBLASTn search using the *B. quintana* JK-31 *yopJ* gene as a query. Only the highest identity match from each species is included.

Species	Percent Identity	<i>Bartonella</i> group (if applicable)
<i>Bartonella quintana</i>	100 %	C
<i>Bartonella alsatica</i>	61.15 %	C
<i>Bartonella grahamii</i>	58.33 %	C
<i>Serratia symbiotica</i>	57.78 %	–
<i>Bartonella</i> sp. JB63	56.44 %	B
<i>Bartonella</i> sp. JB15	56.44 %	B
<i>Bartonella clarridgeiae</i>	55.17 %	B
<i>Bartonella kosoyi</i>	54.72 %	C
<i>Bartonella tribocorum</i>	54.40 %	C
<i>Yersinia enterocolitica</i>	53.93 %	–
<i>Bartonella krasnovii</i>	53.70 %	C
<i>Yersinia pseudotuberculosis</i>	53.56 %	–
<i>Yersinia pestis</i>	53.56 %	–
<i>Edwardsiella anguillarum</i>	53.48 %	–
<i>Salmonella enterica</i>	52.40 %	–
<i>Bartonella</i> sp. AR 15-3	52.09 %	B
<i>Bartonella</i> sp. 1-1C	51.88 %	B
<i>Bartonella elizabethae</i>	50.63 %	C

The YopJ protein amino acid sequences were aligned for several of the homologues identified by BLAST, and these are shown in Figure 1.4. All of the *Bartonella* YopJ homologues in the database maintain the conserved catalytic triad, but in some cases the acetyl-CoA and phytic acid binding domains are less conserved, though these may still be functional. One noteworthy domain that was not identified in any of the homologues is the BID domain, which is almost ubiquitous among the other *Bartonella* effector proteins and marks them for type-IV-secretion.

The function of *Bartonella* YopJ homologues are unknown and understudied. There is substantial sequence variation between them, as Figure 1.4 shows. Intriguingly, the *yopJ* genes in some *Bartonella* species have undergone gene duplication, including *B. clarridgeiae*, which has four copies; *B. tribocorum*, which has three copies; and *B. grahamii* which has two copies. The duplication of the *yopJ* gene in these species is further indirect evidence that YopJ is functional in *Bartonella*. Additionally, while the *yopJ* duplicates in *B. tribocorum* and *B. grahamii* are in close proximity on the genome, the *B. clarridgeiae* *yopJ* duplicates are scattered across the genome. The cause of this

distribution is unknown but it could be due to gene transfer agents that are active in group B (which includes *B. clarridgeiae*), but not in group C (which contains *B. tribocorum* and *B. grahamii*). Given this variation, it is possible that like the Beps, *Bartonella* YopJ homologues are host adapted and may have varying cellular targets.

The only *Bartonella* YopJ homologues that have been characterised at all are those of *B. tribocorum* and *B. quintana*. *B. tribocorum* has three YopJ homologues, which have been shown to be important in maintaining bacteraemia in rats¹³⁹. This study showed that a *B. tribocorum* strain that lacks the three *yopJ* genes has an impaired ability to persist in the blood, and interestingly a *B. tribocorum* strain with neither YopJ homologues nor Beps is incapable of establishing a bacteraemic infection at all¹³⁹. This could suggest some redundancy in the functions of the Beps and YopJ in *Bartonella* species.

There is evidence that *B. quintana* YopJ inhibits the NF- κ B pathway based on an IL-8 ELISA that I performed on transfected tissue culture cells during my Honours project; but beyond that, its function and cellular target are unknown¹⁴⁰.

FIGURE 1.4: Alignment of *Bartonella* YopJ homologues.

Including *Y. pseudotuberculosis* YopJ and the canonical YopJ catalytic domain for reference. Aligned using the the Geneious software package¹¹⁰. Sequences from NCBI gene. Red boxes show catalytic triad, blue boxes show acetyl-CoA binding domains, black boxes show phytic acid binding domains. The catalytic triad was identified base on reference [115]. The acetyl-CoA binding domains and phytic acid binding domains were identified based on the Uniprot database¹¹⁷.

1.5 Research Questions

The goal of this project was to further characterise the *B. quintana* YopJ homologue and to develop a model for how it causes the inhibition of NF- κ B signalling. Based on this goal, and informed by my previous work, I formed the following research questions:

- Which step of the NF- κ B pathway does the *B. quintana* YopJ homologue inhibit?
- Does the *B. quintana* YopJ homologue interfere with any cellular processes or signalling, apart from the activation of NF- κ B?
- What is the target host protein of the *B. quintana* YopJ homologue?
- How does acetylation of that host protein lead to NF- κ B inhibition?

To answer these questions, I assessed the effect of Bq-YopJ on various steps of NF- κ B activation; carried out a yeast two-hybrid screen to identify host proteins that interact with Bq-YopJ; and performed a proteomic screen to identify host cell proteins that differed in abundance in the presence of Bq-YopJ.

Chapter 2

Materials and Methods

2.1 *Escherichia coli* Culture

E. coli liquid cultures were grown in LB broth with appropriate selection antibiotics at 37 °C, 200 rpm.

E. coli solid cultures were plated on LB agar plates with appropriate supplements, and grown at 37 °C.

2.1.1 Strains

The *E. coli* strains used in this project are listed in Table 2.1 below.

TABLE 2.1: *E. coli* strains used in this project

Strain	Function	Source
OneShot™ TOP10	Cloning	Invitrogen
DH5α	Cloning	invitrogen

2.1.2 Growth Media

All bacteriological media were autoclaved at 121 °C for at least 15 minutes, and were cooled below 50 °C before supplements were added.

Solid Media

LB Agar was purchased as a pre-mixed powder from Life Technologies, and was prepared according to the manufacturer's instructions.

Liquid Media

LB broth was purchased as a pre-mixed powder from Accumedia, and was combined with water to reconstitute, according to the manufacturer's instructions.

2XYT broth was purchased as a pre-mixed powder from BD, and was reconstituted with water according to the manufacturer's instructions.

Selection Antibiotics

Ampicillin was purchased from Roche and was added as a 1000× solution directly to growth media, for a final concentration of 100 µg/mL.

Kanamycin was purchased from Fluka and was added as a 1000× solution directly to growth media, resulting in a final concentration of 50 µg/mL.

2.1.3 Transformation

Transformations were performed according to the instructions of the suppliers of the *E. coli* strains listed in Table 2.1.

2.1.4 Frozen Bacterial Stocks

Preparing frozen stocks

Frozen stocks of *E. coli* strains were prepared by combining overnight liquid cultures of *E. coli* with sterile 80 % glycerol for a final concentration of 40 % glycerol. The stocks were mixed and frozen at -80 °C in 1 mL cryovials (Tarsons).

Thawing frozen stocks

A small scrape of frozen stock was collected with a sterile loop and was either inoculated into LB broth or streaked onto an LB agar plate.

2.2 DNA Manipulation

2.2.1 Plasmids used in this Project

The plasmids used in this project are listed in Table 2.2 below.

TABLE 2.2: **Plasmids used in this project**

Plasmid	Properties	Source
pcDNA3.1-Bq-YopJ-GFP	Mammalian expression vector encoding <i>B. quintana yopJ</i> gene, C-terminal GFP fusion, AMP ^r , G418 ^r	[140]
pcDNA3.1-Bq-YopJ-C197A-GFP	Mammalian expression vector <i>B. quintana yopJ</i> Mutant gene, C-terminal GFP fusion, AMP ^r , G418 ^r	[140]
pGBKT7	Yeast two-hybrid bait vector, KAN ^r , <i>TRP1</i>	Clontech
pGADT7	Yeast two-hybrid prey vector, AMP ^r , <i>LEU2</i>	Clontech
pGBKT7-53	Yeast two-hybrid control bait vector with murine p53 insert, KAN ^r , <i>TRP1</i>	Clontech
pGBKT7-Bq-YopJ-GAL4BD	Yeast two-hybrid bait vector with Bq- <i>yopJ</i> insert, KAN ^r , <i>TRP1</i>	This study

2.2.2 Mammalian Expression Plasmids Used

The two mammalian expression plasmids, pcDNA3.1-Bq-YopJ-GFP and pcDNA3.1-Bq-YopJ-C197A-GFP were produced during my Honours project¹⁴⁰. Since they were used extensively in this project, their construction is reviewed here. The plasmid pcDNA3.1-Bq-YopJ-GFP encodes the wild type Bq-*yopJ* gene with a green fluorescent protein (GFP) tag, to confirm expression. It also includes a consensus mammalian ribosome binding (Kozak) sequence. The pcDNA3.1-Bq-YopJ-C197A-GFP plasmid also includes the GFP tag and Kozak sequence, but rather than the wild type Bq-*yopJ* gene it encodes a catalytically inactive mutant form of Bq-YopJ in which the invariant cysteine has been replaced with an alanine, and is therefore not catalytically active. These plasmids were produced using the CT-GFP Fusion TOPO TA Expression Kit (Life Technologies), as is summarised below.

The wild type Bq-*yopJ* insert for the pcDNA3.1-Bq-YopJ-GFP plasmid was amplified by PCR from a stock of *B. quintana* strain JK-31 genomic DNA (kindly provided by Dr. Jane Koehler). JK-31 is a clinical *B. quintana* isolate from a homeless patient in San Francisco¹⁴¹. For this amplification, the primers yopJ_pcDNA_F and yopJ_pcDNA_R were used. Their nucleotide sequences appear in Table 2.5. The Bq-*yopJ* catalytically inactive mutant insert for the pcDNA3.1-Bq-YopJ-C197A-GFP plasmid was synthesised as a double-stranded DNA fragment by Integrated DNA Technologies (IDT); its sequence was based on that of Bq-*yopJ* of the reference *B. quintana* strain Toulouse (NCBI accession GCA_000046685.1), which has slight genetic differences to JK-31. Additionally, silent mutations were introduced into the sequence to adjust the G/C content to optimise the synthesis of this gene. The consensus mammalian ribosome binding (Kozak) sequence was added to each insert, introduced as part of the PCR primer for wild type Bq-*yopJ* or included in the synthetic gene for the mutant. The sequence of both inserts appear in Appendix Figure A.1, and the resultant amino acid sequences appear in Appendix Figure A.2.

The TOPO cloning kit uses adenosine 3' overhangs on inserts to insert them into the plasmid backbone without restriction digestion. Adenosine overhangs are usually introduced to the inserts by *Taq* polymerase during PCR. The Bq-*yopJ* catalytically inactive mutant synthetic gene lacked these overhangs, so they were introduced by incubating the synthetic gene in PCR reaction buffer with active polymerase at 68 °C for 10 minutes.

The TOPO cloning reaction was performed according to the manufacturer's instructions, and the resultant plasmids were transformed into chemically competent TOP10 *E. coli* by heat-shock at 42 °C for 30 seconds. The colonies produced by the TOPO cloning were screened by colony PCR (see Table 2.3) and sequenced using the T7 promoter and GFP reverse primers. The sequence of these primers is listed in Table 2.5.

2.2.3 Construction of the Yeast Two-Hybrid Bait Plasmid

The yeast two-hybrid bait plasmid, pGBKT7-Bq-YopJ-GAL4BD, was produced for use with the Matchmaker[®] Gold Yeast Two-Hybrid System, as is described below.

The Bq-*yopJ* gene was amplified by PCR from the same stock of *B. quintana* strain JK-31 genomic DNA as for the mammalian expression plasmids, using the primers YopJ_His_F and YopJ_Rev (see Table 2.5), which introduced NdeI and BamHI restriction sites respectively. This PCR was carried out using high fidelity PCR (Table 2.4). The pGBKT7 plasmid backbone was provided, purified, with the matchmaker kit. Throughout this cloning, a plasmid-only (no insert) control was included to ensure proper digestion by the restriction enzymes and a lack of re-ligation of the empty plasmid.

A sample of the Bq-*yopJ* PCR product from a high fidelity PCR reaction was checked by agarose gel electrophoresis to confirm that the band was of the correct size. The remaining PCR product was purified using the DNA Clean & Concentrator kit (Zymo research) and quantified using a NanoDrop ND-1000 Spectrophotometer (ThermoFisher).

Both the purified PCR product and the pGBKT7 plasmid were then digested with NdeI and BamHI (New England Biolabs), then the two concentrated using the DNA Clean & Concentrator kit (Zymo research) and combined at a 5:1 plasmid:insert ratio. The DNA mixture was then ligated using T4 ligase (ThermoFisher) overnight in a thermic cycler, alternating between 10 °C and 30 °C for 30 seconds each cycle. An additional, unligated control was included to confirm that the plasmid was fully digested.

The ligated plasmid was transformed via electroporation into DH5 α *E. coli*. Colonies from the resultant plates were picked, inoculated into LB broth with kanamycin, and incubated overnight at 37 °C with shaking at 200 rpm. The plasmids in these cultures were extracted using the Monarch[®] Plasmid Miniprep Kit (New England Biolabs), and digested with NdeI and BamHI. The digested plasmid was screened for inserts by agarose gel electrophoresis. Frozen stocks were produced of any DH5 α strain that contained a plasmid with an insert of the correct size as described in Section 2.1.4, and a sample of the plasmid was sequenced (Macrogen, Seoul, South Korea) to confirm that the Bq-*yopJ* gene had been successfully inserted.

2.2.4 Polymerase Chain Reaction

Colony PCR

Colony PCR was used for screening colonies after transformation. It was performed using Biomix Red (Bioline) following the manufacturer's instructions and the standard cycling conditions shown in Table 2.3 below.

TABLE 2.3: **Colony PCR cycling conditions**

Number of cycles	Temperature	Time
1	94 °C	10 minutes
30	95 °C	30 seconds
	45 °C	30 seconds
	72 °C	1 minute
1	72 °C	5 minutes
1	4 °C	Hold

High Fidelity PCR

High fidelity PCR was performed using Q5 polymerase (New England Biolabs) following the manufacturer's instructions and the standard cycling conditions shown in Table 2.4 below.

TABLE 2.4: **High fidelity PCR cycling conditions**

Number of cycles	Temperature	Time
1	98 °C	30 seconds
30	98 °C	10 seconds
	60 °C	30 seconds
	72 °C	30 seconds
1	72 °C	2 minutes
1	4 °C	Hold

2.2.5 DNA Primers

The primers used in this project are listed in Table 2.5 below.

TABLE 2.5: **Oligonucleotide primers used in this project.** Restriction sites in sequence underlined.

Primer name	Restriction site	Sequence
yopJ_pcDNA_F	–	5'- ACC ATG GGG ATG AAA CCA CAA AAT TCA AAC AGC ACC CC -3'
yopJ_pcDNA_R	–	5'- A AAT CTC TCA AAA AGA GTT TCC TCC TTT TTG TTA ATG -3'
T7 Promoter	–	5' - TAA TAC GAC TCA CTA TAG GG - 3'
GFP_Reverse	–	5' - GGG TAA GCT TTC CGT ATG TAG C - 3'
YopJ_His_F	NdeI site	5'- GCG <u>CAT</u> ATG AAA CCA CCA AAT TCA AAC AGC A -3'
YopJ_Rev	BamHI site	5'- GCG <u>GGA TCC</u> TCA TAA TTT AAG CAA AGA TTT ATA TTC AGA AAT CCT -3'
T7 Sequencing Primer	–	5'- TAA TAC GAC TCA CTA TAG GGC -3'
3' AD Sequencing Primer	–	5'- AGA TGG TGC ACG ATG CAC AG -3'

2.2.6 DNA Gel Electrophoresis

Gels for DNA electrophoresis were 1 % agarose (HyderaGene) in 1× TAE and were stained with RedSafe (iNtRON Biotechnology).

They were run in 1× TAE at 100 V for 60 minutes using 6× loading dye (Thermo Fisher), and Hyperladder 1kb (Bioline).

2.2.7 DNA Sequencing

Prepared plasmids were quantified using a NanoDrop spectrophotometer (ThermoFisher Scientific) and 20 µL was sent to Macrogen (South Korea) for

Sanger sequencing. The sequence data were viewed and analysed using the Geneious 9 software package (Biomatters Ltd).

2.3 Mammalian Cell Culture

2.3.1 HEK293 Cell Maintenance

HEK293 Media

Human embryonic kidney (HEK293) cells¹⁴² were grown in RPMI 1640 with L-glutamine and Phenol Red (Thermo Fisher), supplemented with 10 % foetal calf serum.

HEK293 Passage

Cells were cultured in T75 flasks (CellStar), and grown until 75 – 80 % confluent before passaging. The cells were washed with warm PBS and were treated with trypsin (TrypLE; Thermo Fisher) for three minutes at 37 °C to detach from the flasks. The cell suspension was then centrifuged at 400 × g for 5 minutes, and the pellet was resuspended in fresh media. New flasks were seeded with the cell suspension diluted at 1:5 or 1:10 dilution with fresh cell medium.

2.3.2 HEK293 Frozen Stocks

Freezing HEK293 Cells

HEK293 cells were suspended in a freezing media of 8 % DMSO, 42 % FCS, and 50 % RPMI, and transferred to 1 mL cryovials (Tarsons) with one third of a 75 – 80 % confluent T75 flask per vial. The vials were slowly frozen in a CoolCell[®] LX freezing container (Biocision) at -80 °C overnight, before being transferred to liquid nitrogen for long term storage.

Defrosting HEK293 Cells

A vial was removed from liquid nitrogen storage and defrosted in a 37 °C water bath, then cells were gently centrifuged at 300 × g for 5 minutes to remove the DMSO, resuspended in warm growth medium and seeded into a T75 flask.

2.3.3 Transfection

Transfections were performed using the Lipofectamine 3000 reagent (Life Technologies). Mammalian expression plasmids were produced in TOP10 *E. coli* by inoculating a scrape from a frozen stock into 5 mL of LB broth with ampicillin, and incubating it overnight at 37 °C with shaking at 200 rpm. The following day the plasmid was extracted from the bacteria using the Monarch[®] Plasmid Miniprep Kit. The purified plasmid was quantified using a NanoDrop ND-1000 Spectrophotometer (ThermoFisher), and 5000 ng was concentrated into 10 µL using the DNA Clean & Concentrator kit (Zymo research), for a final plasmid concentration of 500 ng/µL.

The day before transfection, a cell count was performed and 1×10^6 HEK293 cells were seeded into 6-well plates. The following day, cells were typically ~70 % confluent. The transfection reagent was prepared with 2500 ng of plasmid for each well, according to the manufacturer's instructions, then was slowly pipetted, dropwise, onto the cells. The cells were incubated with the transfection reagent for 16 hours then split, and reseeded at a density depending on their intended use.

2.3.4 TNF- α Stimulation

A final concentration of 100 ng/mL recombinant human TNF- α protein (Abcam) was added to wells containing RPMI 1640 with 0.5 % foetal calf serum. The TNF- α was diluted in serum-free RPMI 1640 to increase the pipetting volume, and therefore accuracy.

2.3.5 Cell Counting and Live:Dead Staining

Both routine cell counting and live:dead counts of transfected cells were performed by staining the cells with 0.4 % trypan blue (Sigma) and counting them using a haemocytometer (Hawksley). For routine counting, only cells that fully excluded the stain were counted. For live:dead counting, cells that excluded the stain and those that internalised it were counted separately.

2.3.6 Preparation of HEK293 Lysates

Three different lysis methods were used throughout the course of this project, using a detergent, urea, or direct suspension of the cells into Tris-glycine SDS sample buffer.

Urea Lysis

Urea lysis was used for liquid chromatography and mass spectrometry analysis of the cells.

The cells were harvested using TrypLE as described under HEK293 Passage. Once pelleted, the cells were washed once with PBS, then centrifuged at $300 \times g$. The supernatant was removed from the cells and the pellet was stored at -80°C until use.

The frozen cell pellets were resuspended in 100 μL of urea lysis buffer (8 M urea in 40 mM Tris-HCl (pH 7.6)) per ~ 1 million cells, then sonicated using a Bandelin Sonopuls probe sonicator set to 3 W with cycles of 10 seconds on and 10 seconds off, for 2 minutes.

The lysates were then centrifuged at $12,000 \times g$ for 20 minutes at 4°C . The supernatants were transferred to fresh tubes and were either used immediately or stored at -20°C .

Detergent Lysis

Detergent lysis was used for preparation of lysates for Western blotting early in the project, but subsequently was replaced with sample buffer lysis, below. The cells were washed with cold PBS, then RIPA buffer (Sigma, R0278) was added to the wells. The cells were scraped from each well using the plunger of a 5 mL syringe and transferred to a microfuge tube.

The tubes were vortexed for 30 minutes then centrifuged at $15,000 \times g$ for 20 minutes, both at 4°C . The supernatants were transferred to new tubes and stored at -80°C .

Sample Buffer Lysis

Sample buffer lysis was used for Western blotting later in the project. This method was used because it minimised processing of the lysates, and did not remove insoluble cell fractions, both of which increased the total protein recovered. Additionally, as it was a very rapid, this procedure was well suited to lysing cells after a time course of TNF- α stimulation.

The cells were washed once with PBS then 100 μL of sample buffer (2 $\times$ NovexTM Tris-Glycine SDS Sample Buffer; ThermoFisher) per ~ 1 million cells was added to the wells. The cells were scraped up using a pipette tip and transferred to a microcentrifuge tube. The lysates were very sticky and viscous, so could be easily collected by scraping with a pipette tip while applying gentle suction. The samples were then heated to 95°C for 5 minutes.

The lysates were sonicated as described for urea lysis to fragment the DNA and reduce viscosity, and were either used immediately or stored at -20 °C .

2.4 Enzyme Linked Immunosorbent Assay (ELISA)

2.4.1 Cell Supernatant Production

HEK293 cells were transfected as described in Section 2.3.3. One well of a six-well plate was prepared for each of the three conditions, which included cells that were transfected with Bq-*yopJ* and Bq-*yopJ* Mutant, and non-transfected cells. After transfection, each of these wells was split into eight wells of a 24-well plate and incubated overnight to allow the cells to attach to the plate and to secrete baseline levels of IL-8.

After incubation, 100 µL of cell culture supernatant was collected from each well and stored at -80 °C . Each well was then stimulated with recombinant human TNF-α protein (Abcam) at a final concentration of 100 ng/mL. The TNF-α was diluted in RPMI 1640 to replace the 100 µL just removed from the wells. The cells were then incubated for 24 hours, after which another 100 µL of cell culture supernatant was harvested and stored at -80 °C .

2.4.2 IL-8 ELISA

The IL-8 ELISA assay was performed using the BD OptEIA Human IL-8 ELISA set, according to the manufacturer's instructions. It was performed using 96-well ELISA plates purchased from Jet Biofil. A standard curve was produced with purified, recombinant IL-8.

The standard curve was generated and statistical analyses were performed using the GraphPad Prism 6 software package.

2.5 Immunofluorescence Microscopy

2.5.1 Coverslip Preparation

Glass coverslips (22 mm × 22 mm, labserv) were incubated in a sterile 0.01 % (w/v) poly-(l)-Lysine solution in water (Sigma, P4832) for five minutes and then allowed to air-dry. They were then placed into the wells of 6-well plates. Transfected HEK293 cells were seeded and grown directly on the coverslips.

2.5.2 Paraformaldehyde Fixation

To fix the cells, a 2× fixative solution (4 % (w/v) sucrose, 8 % (w/v) paraformaldehyde in PBS) was added directly to an equal volume of cell culture media in the wells of 6-well plates and incubated at room temperature for 10 minutes with gentle rotation.

The wells were washed with ice-cold PBS three times, then treated with 1.5 mg/mL glycine in PBS at room temperature for 10 minutes to quench aldehyde groups. The glycine was then removed and permeabilisation buffer (10 % (v/v) FCS, 0.1 % (v/v) Triton X-100 in PBS) was added. The permeabilisation buffer was incubated in the wells on ice for 20 minutes with gentle rotation. The coverslips were carefully removed from the 6-well plates and the edge of each was blotted against a Kimwipe (Kimtech) to remove excess liquid. Each coverslip was placed, cell side up, on a square of parafilm in the bottom of a well of a fresh 6-well plate. The coverslips were blocked immediately

2.5.3 Fluorescence Staining

NF-κB p65 Stain

Microscopy blocking buffer (1 % (w/v) bovine serum albumin, 10 % (v/v) normal goat serum, 0.3 M glycine, in PBS-Tween) was gently pipetted onto the surface of the coverslip, and incubated for 30 minutes at room temperature with gentle rocking. The blocking buffer was removed, and replaced with anti-NF-κB p65 antibody diluted 1:200 in blocking buffer. The coverslips were then incubated overnight at 4 °C with gentle rocking.

DAPI Stain

DAPI (4', 6-diamidino-2-phenylindole), a DNA-binding fluorescent probe, was used to counterstain the cell nuclei. A 1 mg/ml DAPI solution in water (Thermo Fisher) was diluted to a working concentration in PBS, at a 1:1000 dilution. After the coverslips had been washed following the NF-κB p65 staining, the diluted solution was added to cover the samples, which were incubated in the dark, at room temperature, for 5 minutes. The coverslips were then washed with PBS three times to remove excess DAPI.

2.5.4 Coverslip Mounting

Coverslips were mounted onto glass slides (Interlab). First, a small drop (10 – 20 μ L) of SlowFade Gold antifade reagent (Thermo Fisher) was placed onto a clean microscope slide. The coverslips were then lowered onto the mountant, avoiding trapping air bubbles. The edges of the coverslips were then sealed with clear nail polish.

2.5.5 High-Throughput Microscopy

Slides were imaged using an IN Cell Analyzer 6500 high throughput microscope (Cytiva), and digital images were recorded. A total of 256 fields were imaged from each slide, and each sample was performed with three biological replicates, with three slides per sample.

2.5.6 Image Processing

Images were segmented using the IN Carta image analysis software (Molecular Devices), and the intensity of the NF- κ B p65 stain from both the nucleus and cytoplasm of each cell were exported to Excel (Microsoft). The intensities of NF- κ B p65 stain in each segment within each cell were compared. Cells with higher stain intensity in the nucleus, relative to the cytoplasm were recorded as having p65 localised to the nucleus. Statistical analysis and graphing was performed using the GraphPad Prism 6 software package. The differences between conditions were analysed using one-way ANOVA with Tukey's multiple comparisons test.

2.6 Protein Manipulation

2.6.1 SDS Polyacrylamide Gel Electrophoresis

Pre-cast gradient Novex™ WedgeWell™ 10-20 % Tris-Glycine mini gels, with 10 or 12 wells, were purchased from Thermo Fisher, or Mini-PROTEAN TGX 4 – 20 % gels (15 well) were purchased from BioRad. Cell lysates were combined with 2× SDS Sample buffer (Thermo Scientific), and dithiothreitol (DTT) was added to a final concentration of 50 mM. The Lysates were then heated at 90 °C for 5 minutes. Samples were then loaded onto gels at the equivalent of 4×10^4 cells per well. The gels were run in Tris-glycine SDS electrophoresis buffer (120 mM glycine, 40 mM Tris, and 0.1 % SDS in ddH₂O), according to the manufacturer's instructions.

The SeeBlue™ Plus2 Pre-stained Protein Standards (ThermoFisher) were included in at least one well of all gels.

2.6.2 Western Blotting

After a protein gel had been run, the cassette was removed from the gel tank, and the gel carefully separated from the plates. The transfer block was assembled as follows: sponge, filter paper (Thermo Scientific), gel, PVDF membrane (Merck), filter paper, sponge. Each component was saturated with transfer buffer before it was added, except for the PVDF membrane, which was activated in 100 % methanol then soaked in transfer buffer for 2 – 5 minutes or until it easily sank in transfer buffer.

The transfer assembly was placed in the transfer tank and a 300 mA current was applied for 1 hour at room temperature with a -80 °C icepack submerged in the transfer buffer. The block was disassembled and the membrane trimmed and rinsed once with PBS-Tween (0.1 % Tween 20 in PBS). After the rinse, the membrane was transferred to a small container and blocked for at least one hour in Western blot blocking buffer (5 % skim milk in PBS-Tween).

Antibody specific for the protein of interest was diluted 1:1000 in Western blot blocking buffer and incubated on the membrane at 4 °C overnight with gentle rocking. For the loading controls, the primary antibody was incubated at 4 °C for one hour with gentle rocking.

After primary antibody binding, the membrane was washed three times in PBS-Tween, with at least five minutes per wash. The loading control antibodies were directly conjugated to horseradish peroxidase (HRP) so could be imaged immediately, while blots with all other antibodies were incubated

with a secondary HRP-conjugated antibody. The secondary antibodies were diluted 1:7000 in PBS-Tween and incubated on the membrane at room temperature for one hour. Before imaging, the membranes were again washed three times with PBS-Tween, with five minutes per wash.

The blots were developed with chemiluminescence, using either Pierce™ ECL Western Blotting Substrate (ThermoFisher) or Western Lightning® Ultra extreme sensitivity chemiluminescence substrate (PerkinElmer), as per the manufacturer's instructions. The blots were then imaged with an Amersham Imager 600 (Cytiva).

2.6.3 Quantifying Western Blots

The Western blot bands were quantified using the Fiji Is Just ImageJ (FIJI) software package¹⁴³. Each lane was selected and plotted using the analyse:gels tool. The trough of each band was identified and a line was drawn over the top of each, allowing for the Wand tool to calculate the area of the trough as a number of pixels.

The area of every test band was divided by the area of a loading control band to give the ratio of test protein:loading control. This ratio was used for statistical analysis and graphing using the GraphPad Prism 6 software package. Quantified western blots were compared using repeat measures two-way ANOVA for blots of protein abundance over time, and t-test for comparing conditions without a time dimension.

2.6.4 Antibodies

The antibodies used in this project are listed in Table 2.6 below. Antibodies were shipped from the manufacturers at 4 °C , then transferred to fresh tubes in 10 µL aliquots, and frozen for long-term storage at -20°C. The aliquots of the immunofluorescence antibody with an Alexa Fluor® 647 tag were additionally stored in an opaque container to avoid photobleaching of the fluorophore.

TABLE 2.6: Antibodies used in this project

Target Protein	Species	Use (Tag)	Source	Clone Number
Human I κ B α	Mouse	Western blotting	Cell Signalling Technology	
Human I κ B α	Rabbit	Western blotting	Abcam	
Human Phospho-I κ B α	Mouse	Western blotting	Cell Signalling Technology	
Rabbit IgG	Goat	Western blotting secondary (HRP)	Abcam	# E130
Mouse IgG	Goat	Western blotting secondary (HRP)	Abcam	
Human DCNL1	Rabbit	Western blotting	Abcam	# EPR13492
Human Lam-inB1	Rabbit	Western blotting control (HRP)	Abcam	# EPR8985(B)
Human GAPDH	Mouse	Western blotting control (HRP)	Abcam	# 6C5
Human CAND1	Rabbit	Western blotting	Abcam	# EPR14241
Human Rbx1	Rabbit	Western blotting	Abcam	
Human NF- κ B p65	Rabbit	Immunofluorescence microscopy (Alexa Fluor [®] 647)	Abcam	
Human NF- κ B p65	Rabbit	Western blotting	Abcam	
Human Cullin-1	Mouse	Western blotting	Invitrogen	
Human NF- κ B p105/p50	Mouse	Western blotting	Abcam	# ABM20H5
Human Nedd8	Rabbit	Western blotting	Abcam	# Y297

2.7 Mass Spectrometry

2.7.1 Sample Preparation

Four lysate samples from transfected HEK293 cells were prepared per condition, as described in Section 2.3.6, and were quantified using a Qubit® Protein Assay (Life Technologies) as per the manufacturer's instructions.

After quantification, 30 µg of total protein for each sample was loaded into a microcentrifuge tube, and total volume was brought to 30 µL total volume in urea lysis buffer (8 M urea dissolved in 40 mM Tris-HCl (pH 7.6)). Dithiothreitol (DTT) was added to each tube to a final concentration of 5 mM. The samples were then incubated in the dark for one hour at room temperature. Iodoacetamide (IAA) was then added to a final concentration of 10 mM and was incubated at room temperature for 30 minutes. Each sample was then diluted 1:3 in 100 mM Tris-HCl (pH 8.5) and calcium chloride was added to a final concentration of 1 mM.

Mass spec grade Trypsin (Promega) was added to each protein sample for a final ratio of 0.02 µg Trypsin per µg of sample protein, and was incubated overnight at 37 °C .

Trypsin digestion was halted by the addition of formic acid to a final concentration of 4 % (v/v) and the peptide samples were desalted immediately.

2.7.2 C18 Desalting Tips

Samples were desalted using one 100 µL C18 desalting tip (Varian, OMIX) per peptide sample. The tip was pre-wet in 100 % acetonitrile then equilibrated with C18 wash buffer (0.2 % (v/v) trifluoroacetic acid in LC/MS grade water) by aspirating up and down three times. The peptides were bound to each tip by slowly aspirating the samples up and down 20 times, ensuring no air was forced through the C18 resin in the process. Once the peptides were bound, the tip was rinsed three times in C18 wash buffer and the peptides were eluted in 20 µL of C18 elution buffer 1 (0.1 % (v/v) formic acid, 50 % (v/v) acetonitrile in LC/MS grade water) by aspirating 15 times.

Each peptide sample was rebound to the desalting tips and washed as before, then eluted again, this time in C18 elution buffer 2 (0.1 % (v/v) formic acid, 70 % (v/v) acetonitrile in LC/MS grade water). The two eluates were pooled in a low binding microcentrifuge tube (ThermoFisher Scientific), dried down in a Speedvac (Labconco), then resuspended in 10 µL of LC/MS buffer (0.1 % (v/v) formic acid, 2 % (v/v) acetonitrile in LC/MS grade water).

The samples were centrifuged for 10 minutes at $16,000 \times g$, then 9 μL of each was transferred into a labelled 2 mL glass LC/MS vial with a 205 μL tapered insert (Thermo Scientific).

The peptide samples were stored at 4 °C until they were loaded into the High Performance Liquid Chromatography (HPLC) unit.

2.7.3 LC/MS

The peptide samples were analysed using an UltiMate 3000 RSLCnano chromatography system and an Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer (ThermoFisher Scientific). The Xcalibur™ software package (Version 2.1.0, ThermoFisher Scientific) was used to control the LC/MS machinery and acquire the LC/MS data. The vials were held at 10 °C in the autosampler until they were injected into the machine.

The samples were injected into an Acclaim™ PepMap™ 100 C18, 5 μm 0.3 $\times$ 5 mm trap column (catalogue number: 160454, ThermoFisher Scientific) at a flow rate of 8.0 $\mu\text{L}/\text{min}$. The peptides were then separated using an Acclaim™ PepMap™ 100 C18, 2 μm , 100 Å, 75 μm $\times$ 15 cm analytical column (catalogue number: 164941, ThermoFisher Scientific) with a 0.3 $\mu\text{L}/\text{min}$ flow rate. The solvent gradient was produced by mixing 0.1 % (v/v) formic acid in ddH₂O with 0.1 % (v/v) formic acid, 80 % (v/v) acetonitrile, in ddH₂O. The solvent gradient was 3 % from 0 to 5 min, 30 % from 5 – 70 min, 50 % from 70 – 82 min, 95 % from 82 – 88 min, then 3 % from 88 – 99 min.

The peptides were ionised by nanoelectrospray (Nanospray Flex, ThermoFisher Scientific) with the 25 μM ion transfer capillary tube set to 275 °C and 1.8 kV. The Orbitrap settings were as follows: AGC target 5.0×10^3 , Maximum Injection Time 50 ms, charge state 2 – 7, data type Profile, detector type OT, resolution 120,000, scan range 375 – 1500 m/z.

Data-dependent MS/MS (ddMS/MS) scans were acquired using ion trap (IT) with the following settings: AGC target 5.0×10^3 , scan rate: Rapid, Maximum Injection Time 300 ms, data type Centroid, detector type IT, scan range Auto: m/z Normal.

The 20 highest intensity ions from each scan were selected for high-energy collision-induced dissociation (HCD) fragmentation using the linear quadrupole ion trap with an isolation window of 1.6 m/z and the HCD collision energy at 30 %. Dynamic exclusion was included with mass tolerance of 10 ppm and an exclusion duration of 60 s.

2.7.4 Protein Identification

The spectra were searched against a modified SwissProt human protein sequence database that had the sequence of Bq-YopJ added to it to confirm its expression. The search was performed using the SequestHT search engine in Proteome Discoverer 2.4 (ThermoFisher Scientific) with the following settings: peptide length range of 6 – 144, max 2 missed trypsin cleavages allowed, precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.5 Da, static modifications: carboxyamidomethylation of cysteine (+57.021); dynamic modifications: oxidation (+15.995 Da) at M, carbamylation (+43.066 Da) at K, acetylation (+42.011 Da) at K, S, and T; deamidation (+0.984 Da) at N and Q, and peptide terminus modification of carbamylation (+43.066 Da) at the N-terminus.

The files were searched against the protein sequence database and a decoy database (Percolator node) with the false discovery rate filter set to 0.01, plus a relaxed false discovery rate of 0.05. Proteins were identified with high peptide confidence and a minimum peptide number of 1. Proteins identified that shared common peptides were grouped for export.

Venn diagrams of identified proteins were produced using the web tool Venny¹⁴⁴.

2.7.5 Quantitation of Specific Proteins

Label-free quantitation was performed using Proteome Discoverer 2.4 (ThermoFisher Scientific). Study factors were defined as “condition”, “biological replicate” and “technical replicates”, with condition being transfection with either Bq-YopJ or Bq-YopJ Mutant. The files from the protein identification were imported and each was assigned to the appropriate study factors. The comparison was defined as Bq-YopJ Mutant vs Bq-YopJ. While this was an error and they should have been reversed, it did not impact the result, and was corrected during analysis.

Proteins with a log₂ fold change of 1 or more (representing $\geq$ 2-fold difference) and a P-value > 0.05 were counted as significantly differentially expressed.

2.7.6 Gene Ontology

Gene ontology searches were performed using g:Profiler¹⁴⁵ and DAVID¹⁴⁶, two online gene ontology tools.

For g:Profiler, the differentially represented proteins were converted from

Uniprot accession codes to gene names, then searched using the g:GOST functional profiling tool with default settings (P-value cutoff: 0.05).

For DAVID, the differentially represented proteins were loaded in as Uniprot accession codes, with isoform information removed due to incompatibility with DAVID. The search was performed using the default settings, ontologies with a P-value > 0.05 on the functional annotation chart were considered to be significant.

2.7.7 Functional Analysis

Functional analysis was performed using the online Search Tool for Retrieval of Interacting Genes/Proteins (STRING)¹⁴⁷ with the following parameters: full network, edges set to confidence, all interactions used, minimum required interaction score 0.4 (medium confidence), disconnected nodes hidden.

2.8 Yeast Two-Hybrid System

The Matchmaker Gold Yeast Two-Hybrid System (Takara Bio) was used to screen for human proteins that interacted with Bq-YopJ.

2.8.1 Yeast Culture

Liquid cultures of yeast were grown in YPDA broth (Takara Bio) or an appropriate selective broth such as SD/-Trp (See Table 2.8) at 30 °C, 200 rpm.

Solid cultures of yeast were plated on YPDA agar (Takara Bio) plates or an appropriate selective media such as SD/-Trp, and grown at 30 °C. It took 2 – 5 days for yeast colonies to grow on plates.

Yeast Strains

The yeast strains used in this project are listed in Table 2.7 below.

TABLE 2.7: Yeast strains used in this project

Strain	Function	Source
Y2HGold	Yeast two-hybrid bait strain, mating type a	Takara Bio
Y187	Yeast two-hybrid prey strain, mating type α	Takara Bio

Yeast Media

All yeast media were autoclaved at 121 °C for at least 15 minutes, and were cooled below 50 °C before supplements were added. All yeast media were provided as part of the Matchmaker[®] Gold Yeast Two-Hybrid System (Takara Bio), and could be prepared as either solid or liquid as needed.

A full list of yeast media is provided in Table 2.8.

TABLE 2.8: Yeast media used in this project

Name	Function
YPDA	General growth media
SD Base	Minimal media, needs to be supplemented with amino acids and nucleosides
SD/-Trp	Minimal media, lacking tryptophan
SD/-Leu	Minimal media, lacking leucine
SD/-Trp/X	Minimal media, lacking tryptophan, supplemented with X- α -Gal
SD/-Trp/-Leu	Minimal media, lacking tryptophan and leucine, also called double drop out (DDO)
SD/-Trp/-Leu/X	Minimal media, lacking tryptophan and leucine, supplemented with X- α -Gal, also called DDO/X
SD/-Trp/-Leu/X/A	Minimal media, lacking tryptophan and leucine, supplemented with X- α -Gal and aureobasidin A, also called DDO/X/A
SD/-Trp/-Leu/-Ade/-His/X/A	Minimal media, lacking tryptophan, leucine, adenine, and histidine, and supplemented with X- α -Gal and aureobasidin A, also called quadruple drop out (QDO)/X/A

2.8.2 Yeast Transformation

Yeast was transformed using the Yeastmaker™ Yeast Transformation System 2 (Takara Bio).

Chemically competent Y2HGold yeast cells were prepared as per the manufacturer's instructions, and 50 µL of competent cell mixture was combined in a cold microcentrifuge tube with 100 ng of pGBKT7-Bq-YopJ-GAL4BD, 5 µL of Yeastmaker Carrier DNA (provided with the kit), and 500 µL polyethylene glycol/lithium acetate solution (also provided with the kit). This mixture was incubated in a 30 °C water bath for 30 minutes, then 20 µL of DMSO was added, and the tube mixed. The cells were then heat-shocked in a 42 °C water bath for 15 minutes, and rescued with 1 mL of YPD Plus media (provided with the kit) and plated on SD/-Trp agar plates in a serial dilution. These plates were incubated for 3 – 5 days at 30 °C, then colonies were picked and resuspended in freezing medium (YPDA broth with 25 % (v/v) glycerol) and stored at -80 °C.

2.8.3 Yeast Two-Hybrid Screen

The yeast two-hybrid screen was carried out using the Matchmaker® Gold Yeast Two-Hybrid System (Takara Bio), as per the manufacturers instructions using a Mate & Plate™ universal human cDNA prey library (Takara Bio). Plasmids were extracted from yeast using the Easy Yeast Plasmid Isolation Kit (Takara Bio), according to the manufacturer's instructions. The yeast two-hybrid bait plasmid was constructed as described in Section 2.2.3. In a yeast two-hybrid screen, two yeast strains, one of mating type a and the other of mating type α are mated. One of the strains codes for a protein of interest called the bait, the other codes for a potential interaction partner (or library of potential interaction partners) of the bait, called the prey. The bait protein is conjugated to the binding domain of the GAL4 transcription factor, and the prey protein is conjugated to the activator domain of the GAL4 transcription factor. When the two strains mate, the bait and prey come together in the diploid cell. If they interact, the two halves of the GAL4 transcription factor come together and activate the transcription of reporter genes. These reporter genes allow the diploid yeast to grow on selective media and turns the colonies blue in the presence of X- α -Gal. However, before mating the strains it is important to ensure that the bait alone does not activate the reporter genes and is not significantly toxic to yeast.

Once the bait yeast strain, Y2HGold with pGBKT7-Bq-YopJ-GAL4BD, had been produced, the two control experiments were performed. The first was a test for autoactivation of the reporter genes by the bait, in which Y2HGold yeast with the pGBKT7-Bq-YopJ-GAL4BD plasmid was grown on SD/-Trp/X media, which selects for the plasmid pGBKT7-Bq-YopJ-GAL4BD and causes colonies to turn blue if one of the reporter genes is activated. It was also grown on SD/-Trp/X/A media, which additionally contains aureobasidin A, which kills yeast cells that do not express one of the reporter genes. Y2HGold yeast with the pGBKT7-Bq-YopJ-GAL4BD plasmid was compared to Y2HGold yeast with the pGBKT7-53 plasmid, which encodes the murine p53 protein and is known not to activate the reporter genes.

The second control experiment was to determine if the bait protein was toxic to yeast, Y2HGold yeast with the pGBKT7-Bq-YopJ-GAL4BD plasmid was plated on SD/-Trp and the size of the colonies was compared to Y2H gold yeast with the pGBKT7 plasmid (no insert). Smaller colonies indicate a toxic bait protein.

Once the control experiments were complete, the bait yeast strain, Y2HGold with pGBKT7-Bq-YopJ-GAL4BD, was mated with a Mate & Plate Library - Universal Human (Normalized; Takara Bio). Both strains were grown together in 2×YPDA media for 20 hours at 30 °C with gentle shaking (~30 rpm). After 20 hours the culture was microscopically examined for the presence of yeast zygotes, then centrifuged at 1000 × g for 10 minutes, and resuspended in 10 mL of 0.5×YPDA with kanamycin, and the total volume was recorded. SD/-Trp/-Leu plates, that select for both the bait and prey plasmids, were streaked with 100 µL of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions from the mated culture, allowing for the calculation of the number of screened clones. The rest of the mated culture was plated onto fifty-nine 150 mm SD/-Trp/-Leu/X/A plates, which select for both the bait and prey plasmid and the activation of one of the reporter genes, and enable a visual screen for a second reporter gene (and therefore interaction of bait and prey). These plates were incubated at 30 °C for 3 – 5 days, then the colonies were patched onto a SD/-Trp/-Leu/-Ade/-His/X/A high stringency agar plate. This high stringency agar requires the activation of two additional reporter genes for the yeast to survive.

To extract the prey plasmid from diploid yeast, strains that activated the reporters were subcultured on SD/-Trp/-Leu/X media three times, each time

selecting a blue colony, to ensure that no non-interacting prey strains were mistakenly extracted. The plasmids were then extracted using the Easy Yeast Plasmid Isolation Kit (Cat. No. 630467, Takara Bio). These extracts contained a mix of bait and prey plasmid, so they were transformed into DH5 α *E. coli* by electroporation, and plated on LB plates with ampicillin, which selects for the pGADT7 prey plasmid. The plasmids were then extracted from *E. coli* using the Monarch[®] Plasmid Miniprep Kit (New England Biolabs), and sequenced as described in Section 2.2.7.

Finally, the prey protein was tested to ensure that it could not activate reporter genes by itself. This was achieved by co-transforming Y2HGold yeast with one of the extracted prey plasmids and either the pGBKT7-Bq-YopJ-GAL4BD plasmid or the pGBKT7 plasmid (no insert). These transformations were performed as described in Section 2.8.2. Each strain was plated on both SD/-Trp/-Leu/X agar and SD/-Trp/-Leu/-Ade/-His/X/A agar, both of which select for both the bait and prey plasmid. Yeast grown on SD/-Trp/-Leu/X media will form blue colonies if the reporter genes are activated, and yeast can only survive on SD/-Trp/-Leu/-Ade/-His/X/A agar if the reporters are active. White colonies on the SD/-Trp/-Leu/X agar and no colonies on the SD/-Trp/-Leu/-Ade/-His/X/A agar indicate lack of reporter activation by the prey protein alone.

2.9 Buffers and Solutions

Phosphate Buffered Saline (PBS)

PBS tablets were purchased from Thermo Fisher or Gibco, and were dissolved in ddH₂O according to the manufacturer's instructions, and sterilised by autoclaving.

Tris/EDTA (TE) Buffer

TE buffer was made with 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8) in ddH₂O, then autoclaved to sterilise.

Tris/Acetic Acid/EDTA (TAE) Buffer

The 1× TAE buffer, used for pouring and running agarose gels, was purchased as a 50× solution from ThermoFisher and was diluted to 1× in ddH₂O.

2× Fixative Solution

The 2× fixative solution was prepared fresh before each use, and contained 4 % (w/v) sucrose, and 8 % (w/v) paraformaldehyde in PBS.

Permeabilisation Buffer

Permeabilisation buffer was 10 % (v/v) foetal calf serum and 0.1 % (v/v) Triton X-100 (Sigma), in PBS. It was filter sterilised and stored at 4 °C .

PBS-Tween

PBS-Tween was 0.1 % (v/v) Tween 20 (Abcam) in PBS.

Microscopy Blocking Buffer

The blocking buffer for microscopy was made fresh before each use and contained 1 % (w/v) bovine serum albumin, 10 % (v/v) normal goat serum, 0.3 M glycine, in PBS-Tween.

RIPA Buffer

RIPA buffer was purchased from Sigma (R0278).

Tris-Glycine SDS Electrophoresis Buffer

Tris-glycine SDS electrophoresis buffer was 120 mM glycine, 40 mM Tris, and 0.1 % (w/v) SDS in ddH₂O. It was prepared as a 5× stock and diluted before use.

Tris-Glycine SDS Sample Buffer

2× Novex™ Tris-Glycine SDS Sample Buffer was purchased from ThermoFisher. DTT was added to a final concentration 0.5 mM before use.

Western Blot Transfer Buffer

Transfer buffer was 120 mM glycine, 40 mM Tris, dissolved in ddH₂O with 20 % (v/v) methanol. It was chilled to 4 °C before use.

Western Blot Blocking Buffer

The blocking buffer for Western blotting was 5 % (w/v) skim milk powder (Becton Dickinson) in PBS-Tween.

Urea Lysis Buffer

Detergent free urea lysis buffer was 8 M urea dissolved in 40 mM Tris-HCl (pH 7.6).

C18 Wash Buffer

The wash buffer for C18 desalting tips was 0.2 % (v/v) trifluoroacetic acid in LC/MS grade water.

C18 Elution Buffer 1

The first elution buffer for C18 desalting tips was 0.1 % (v/v) formic acid, 50 % (v/v) acetonitrile in LC/MS grade water.

C18 Elution Buffer 2

The second elution buffer for C18 desalting tips was 0.1 % (v/v) formic acid, 70 % (v/v) acetonitrile in LC/MS grade water.

LC/MS Buffer

The buffer for LC/MS analysis of peptide samples was 0.1 % (v/v) formic acid, 2 % (v/v) acetonitrile in LC/MS grade water.

Chapter 3

Investigating the Effects of *Bartonella quintana* YopJ on the NF- κ B Pathway

3.1 Introduction

3.1.1 The NF- κ B Signalling Pathway

The nuclear factor- κ B (NF- κ B) family of transcription factors regulates the expression of hundreds of genes involved in innate and adaptive immune responses. When activated, NF- κ B transcription factors induce the expression of many cytokines and chemokines, including IL-8 and TNF- α ¹⁴⁸. NF- κ B signalling also plays a key role in regulating apoptosis, activation, and differentiation of diverse immune cells¹⁴⁹. The mammalian NF- κ B proteins belong to the Rel protein family, and include RelA (also known as p65), RelB, c-Rel, the p105 subunit (also known as NF- κ B1, which is cleaved into the p50 subunit), and the p100 subunit (also known as NF- κ B2, which is cleaved into the p52 subunit). These proteins can form homo- or heterodimers to mediate gene transcription¹⁴⁹.

There are two NF- κ B pathways: the canonical, which broadly activates an immune response, and the alternative, which is more specific to activating the adaptive immune response^{150,151}. Functionally, the canonical pathway is involved in all responses, while the alternative response is supplementary and involved in the regulation of specific adaptive immune functions^{150,151}.

The canonical pathway is activated by diverse ligands, including those that interact with pattern recognition receptors (PRRs), the TNF- α receptor, and various cytokine receptors¹⁴⁹. In the canonical pathway, the main NF- κ B

transcription factor is a heterodimer made of two components, p65 and p50, although the p50 and c-Rel heterodimer may also play a role¹⁴⁹. In the resting state, p65 and p50 are bound to the inhibitor I κ B α in the cytoplasm of the cell. Activation occurs when cytokine receptors bind to their ligands, including TNF- α and IL-1, or when PRRs, such as the Toll-like receptors (TLRs), bind to molecular signatures of pathogens¹⁴⁸. This signalling leads to activation of the I κ B α kinase complex (IKK), which phosphorylates I κ B α at two N-terminal serine residues. Once phosphorylated, I κ B α is ubiquitinated by the ubiquitin ligase complex SCF ^{β -TrCP}, then proteasomally degraded¹⁵². This releases the p50/p65 dimer, exposing a nuclear localisation signal, which leads to rapid nuclear translocation¹⁵². Once in the nucleus, p50/p65 binds to a specific DNA element, known as the κ B enhancer, where it is a potent activator of gene expression (Figure 3.1)¹⁵². This mechanism, with the proteolytic release of the inhibitor-bound transcription factor, allows the NF- κ B pathway to respond incredibly quickly to stimuli, with NF- κ B dimers localising to the nucleus within minutes of pathway activation.

In contrast with p65, p50 is derived from the proteolytic processing of a precursor protein called p105¹⁵⁰. In its unprocessed form, p105 has an inhibitory effect on NF- κ B activation through I κ B-like C-terminal ankyrin repeats, and is fully degraded by the same machinery as I κ B α upon activation of IKK^{150,153}.

Though NF- κ B signalling is found in all cells, the genes under its control vary between different cell types¹⁵⁴. Activation of NF- κ B in innate immune cells causes production of cytokines and chemokines, including TNF- α and IL-8¹⁵⁴. It also causes the differentiation of macrophages into the pro-inflammatory M1 phenotype and maturation of dendritic cells¹⁴⁹. In neutrophils, which usually undergo apoptosis within 48 hours of release from the bone marrow, NF- κ B suppresses apoptosis, facilitates neutrophil extracellular trap (NET) formation, and promotes adhesion to endothelial cells¹⁵⁴. NF- κ B also promotes endothelial cells to produce chemokines and adhesion molecules including ICAM-1, VCAM-1, and E-selectin, to recruit circulating immune cells to the site of inflammation¹⁵⁴.

Beyond innate immunity, NF- κ B is important in T-cell receptor signalling and the activation of naïve T-cells¹⁴⁹. It also is involved in T-cell differentiation, particularly for the T-helper cells Th1, Th2, and Th17¹⁴⁹. In B-cells, NF- κ B is activated by the B-cell receptor in addition to TNF- α receptor and TLRs, and is involved in B-cell activation, proliferation, and survival¹⁵⁵.

FIGURE 3.1: **Overview of the NF-κB pathway.**

Activation of the NF-κB pathway by stimulation by TNF- α , IL-1, or the toll-like receptors results in the activation IκB kinase (IKK), leading to the phosphorylation of IκB α . The phosphorylated IκB α is ubiquitinated and degraded, resulting in release of NF-κB (p65 + p50). Free NF-κB localises to the nucleus and modulates gene expression, resulting in expression of IL-8, TNF- α , and other cytokines, depending on cell type.

3.1.2 Pathogen Inhibition of NF-κB

Activation of NF-κB is essential for initiation of the innate immune response when pathogens are detected. In non-immune cells, NF-κB is needed for the secretion of chemokines that recruit innate immune cells; these not only control the infection directly, but also activate the adaptive immune response^{149,150}. In addition, NF-κB is an extremely rapid response that is activated by a wide variety of stimuli, including signatures of diverse microbial pathogens¹⁴⁹. Because NF-κB plays such a central role in pathogen defense, it is an important target of inhibition by pathogens that need to suppress the immune response to enable colonisation and transmission¹⁵⁶. As a result, convergent evolution has led to many different mechanisms of pathogen subversion of NF-κB, arguably the host cell pathway most frequently targeted by pathogens¹⁵⁷.

Viral and bacterial pathogens have both evolved multiple mechanisms to alter NF- κ B signalling, including inhibition of signalling, alteration of post-translational modification, and degradation of inhibitors¹⁵⁶. Some pathogens produce proteases that degrade NF- κ B components directly¹⁵⁶. Viral and bacterial proteins are not structurally related, but frequently have the same targets; because of their essential role in the activation and regulation of NF- κ B signalling, the IKK complex and I κ B α and their post-translational modifications, are frequently targeted directly by microbial pathogens. At the same time, structurally related microbial factors often have diverse cellular targets, as is seen with the secreted YopJ family of bacterial effectors¹¹¹.

Because of the strong selection pressure for pathogens to suppress NF- κ B, different pathogens have evolved diverse mechanisms of NF- κ B suppression, with effectors that inhibit different aspects of the pathway to reach the same goal¹⁵⁶. Between the NF- κ B inhibitory bacterial and viral effector proteins that have been characterised, essentially every component of the NF- κ B pathway is a target¹⁵⁶.

Examples of different effectors that inhibit NF- κ B activation include A52R, of the Vaccinia virus, and OspI a secreted effector of *Shigella flexneri*, which both inhibit signal transduction from surface receptors^{158,159}. YopJ of *Yersinia* spp. acetylates the activation loops of IKK α and IKK β , preventing the phosphorylation and degradation of I κ B α ; the inhibitor becomes stabilised and NF- κ B activation is prevented¹⁶⁰. VopS (also called VP1686) of *Vibrio parahaemolyticus* AMPylates p65, preventing it from binding to DNA and initiating transcription¹⁶¹. ORF006 of the cowpox virus prevents the degradation of p105 by a direct interaction¹⁶².

Like other pathogens, *B. quintana* is highly adapted to survival in the host and evasion of the immune response; because of the central role that NF- κ B plays in immune activation, I hypothesised that it is a likely target of inhibition. Additionally, previously characterised YopJ homologues have been reported to target the NF- κ B pathway so I wanted to explore the role of the *B. quintana* YopJ homologue in evasion of host immunity¹¹¹.

This was supported by work done during my Honours project, which showed that when the *B. quintana* YopJ homologue (Bq-YopJ) was expressed in tissue culture cells, it inhibited the TNF- α -induced secretion of the chemokine IL-8 into tissue culture medium, as would be expected if NF- κ B activation were

blocked¹⁴⁰. However, given the variation in mechanisms for pathogen mediated NF- κ B inhibition, even among YopJ homologues, it remains unknown how Bq-YopJ targets the NF- κ B pathway.

3.1.3 Rationale

My preliminary results, from my Honours project, suggested that the *B. quintana* YopJ homologue (Bq-YopJ), when overexpressed in tissue culture cells, inhibited NF- κ B activity. My goal for this part of my project was to dissect the impact of Bq-YopJ on different steps of NF- κ B activation. To do this I opted to transfect mammalian expression plasmids, cloned with either the *B. quintana* wild type *yopJ* gene (pcDNA3.1-Bq-YopJ-GFP), or a catalytically inert point mutation version (pcDNA3.1-Bq-YopJ-C197A-GFP), into HEK293 cells. The DNA and amino acid sequences of these inserts appear in Appendix Figures A.1 and A.2. This allowed me to overexpress Bq-YopJ, or a mutant version, and to assess the effects of Bq-YopJ without potential interference from other *B. quintana* effectors, as pathogens often have redundant mechanisms for NF- κ B suppression¹⁵⁶. The catalytically inactive mutant form of Bq-YopJ, henceforth called Bq-YopJ Mutant, served as a negative control. In Bq-YopJ Mutant the cysteine in the acetyltransferase catalytic triad was replaced with an alanine, a mutation that has been shown to inactivate other YopJ homologues¹¹⁴. I used the Bq-YopJ Mutant as a negative control, instead of non-transfected cells or an empty vector, because it controlled for any innate immune response to exogenous double stranded DNA (the plasmid) or bacterial proteins in general. Both plasmids also coded for a C-terminal GFP tag, which served as a marker for expression.

The overall aim of this chapter was to identify the steps of NF- κ B activation that were inhibited by Bq-YopJ, but not the catalytic mutant. To achieve this I decided to start at the end of the pathway and work backwards, testing each step. This approach provided the most direct path to identify the earliest point where Bq-YopJ interfered with NF- κ B inhibition.

3.2 Results

3.2.1 Bq-YopJ Inhibits TNF- α -Mediated Secretion of IL-8

During my honours project I had constructed mammalian expression constructs, including the catalytic mutant, transfected HEK293 cells, and performed an IL-8 ELISA following TNF- α stimulation. These data had shown that heterologous expression of Bq-YopJ inhibited TNF- α -mediated IL-8 secretion. I first repeated this experiment, confirming that NF- κ B was impacted by Bq-YopJ.

IL-8 is a chemotactic cytokine that is under transcriptional control of the NF- κ B transcription factor, so the levels of IL-8 in cell culture supernatant indicated whether heterologous expression of *B. quintana* YopJ inhibited activation of the NF- κ B pathway¹⁶³.

Cells were transfected with Bq-*yopJ* or the Bq-*yopJ* Mutant. The Bq-*yopJ* Mutant was included to determine the effect on the cells of the expression plasmid and a bacterial protein, but one without catalytic activity. Non-transfected cells were also included to determine whether the Bq-YopJ Mutant had any other unknown enzymatic activity, despite its mutated acetyltransferase catalytic domain.

After transfection, the cells were split into eight wells of a 24-well plate the following day. After giving the cells another day to recover and attach to the plate, media samples from unstimulated cells were collected and frozen. The NF- κ B pathway was activated by the addition of TNF- α , at a final concentration of 100 ng/mL¹⁶⁴. After stimulation I collected and froze samples of cell culture media from each well. Figure 3.2 shows this experiment in schematic form.

The results from the TNF- α stimulated samples are summarised in Figure 3.3.

FIGURE 3.2: **Diagram showing the workflow for the IL-8 ELISA.**

I transfected the cells in one well of a 6-well plate, then split that well into eight wells of a 24-well plate the following day. After 24 hr, pre-stimulation samples were recovered from the wells and frozen. I then activated the NF- κ B pathway in the cells by stimulating them with 100 ng/ml TNF- α for 24 hours. After stimulation I collected and froze another sample of cell culture media from each well.

After stimulation, two of the eight non-transfected samples, and one of the Bq-YopJ Mutant samples, had IL-8 concentrations that exceeded the highest concentrations of the standard curve, so their concentrations could not be determined. The remaining six non-transfected samples showed an average of 558.2 pg of IL-8 per mL of media. This was not significantly different from the remaining seven Bq-YopJ Mutant samples, which had an average of 457.2 pg of IL-8 per mL of media (P-value = 0.2012). This could be in part due to excluding the replicates that were above the standard curve, reducing both means. By contrast, the samples from cells transfected with Bq-*yopJ* had an average of 194.5 pg of IL-8 per mL of media, with a P-value of 0.0003 when compared to Bq-*yopJ* Mutant and of < 0.0001 when compared to the non-transfected samples. This showed that Bq-YopJ inhibits the secretion of IL-8 in response to TNF- α secretion, likely via the NF- κ B pathway.

The unstimulated samples showed very low concentrations of IL-8. Prior to addition of TNF- α , the non-transfected samples had an average of 10.65 pg of IL-8 per mL of media, the Bq-YopJ Mutant samples had an average of 11.34 pg of IL-8 per mL of media, and the Bq-YopJ samples had an average of 10.97 pg of IL-8 per mL of media. The results of the IL-8 ELISA of unstimulated samples is summarised in Appendix Figure A.3.

FIGURE 3.3: **Bq-YopJ inhibits the secretion of IL-8 in response to TNF- α stimulation.**

HEK293 cells were either non-transfected, or transfected with plasmids encoding Bq-YopJ, wild type, or the Bq-YopJ Mutant. Following 24 hours of TNF- α stimulation, cell media was tested for IL-8 secretion by ELISA. Error bars show standard deviation. For the non-transfected condition $n = 6$, For the Bq-YopJ condition $n = 8$, For the Bq-YopJ Mutant condition $n = 7$. I calculated statistical differences with one-way ANOVA, including Tukey's multiple comparisons test. ***: P-value = 0.0003. ****: P-value < 0.0001.

3.2.2 Bq-YopJ Inhibits the Nuclear Localisation of p65

After having confirmed that Bq-YopJ causes general inhibition of TNF- α -induced IL-8 secretion, I decided to examine the nuclear localisation of p65, which occurs prior to the production of IL-8. The NF- κ B transcription factor is a heterodimer, usually comprised of two proteins, p65 and p50. When the canonical NF- κ B pathway is activated, the heterodimer transcription factor is released from its inhibitor, I κ B α , in the cytoplasm. This unveils the nuclear localisation signal on p65, which then rapidly localises to the nucleus, where it activates transcription of a variety of immunity-related genes, including IL-8.

I compared the nuclear localisation of p65 in cells transfected with plasmids expressing Bq-YopJ and Bq-YopJ Mutant, both with and without TNF- α stimulation. I did not include non-transfected cells, as IL-8 secretion levels did not differ significantly between non-transfected cells and those transfected with the Bq-YopJ Mutant. The unstimulated samples were important for establishing the baseline for p65 localisation.

I transfected, then split the cells from each condition (Bq-*yopJ* or Bq-*yopJ* Mutant) onto six coverslips, at a low density to reduce clumping and increase the number of cells with clear edges. I then stimulated the cells with a final TNF- α concentration of 100 ng/mL for 30 minutes, a protocol adapted from Fehr et al¹⁶⁴. I then fixed, permeabilised, and stained the cells with an anti-p65 fluorescent antibody and DAPI (a fluorescent DNA stain). I then mounted the coverslips onto slides and imaged them. By comparing the p65 stain intensity between the cytoplasm and nucleus of each cell, I determined whether the p65 was nuclear or cytoplasmic. Figure 3.4 shows an overview of the experimental design.

FIGURE 3.4: **Diagram showing the workflow for p65 nuclear localisation assay.**

I transfected HEK293 cells with Bq-*yopJ* or Bq-*yopJ* Mutant then split each condition onto six coverslips. I then stimulated the cells with a final TNF- α concentration of 100 ng/mL, fixed, permeabilised, and stained the cells with an anti-p65 fluorescent antibody and DAPI (a fluorescent DNA stain). After staining, I mounted the coverslips onto slides and imaged using an IN Cell Analyzer 6500 high throughput microscope. By comparing the p65 stain intensity between the cytoplasm and nucleus of each cell I determined whether the p65 was nuclear or cytoplasmic.

To increase the sample size, I used a high-throughput confocal microscope and image analysis software to determine whether p65 was localised to the nucleus or the cytoplasm. This allowed cells to be counted from 256 fields from each slide, for an average of 3728.5 cells counted per slide. I used the DAPI stain to segment the nuclei of the cells, then compared the intensity of fluorescence from the p65 antibody stain between the nucleus and cytoplasm of each cell. For this automated workflow, every cell with a higher average intensity in the nucleus rather than the cytoplasm was designated as having nuclear p65. I then compared the percent of cells with nuclear p65 between the conditions. Figure 3.5 summarises these results.

According to the automated count, the mean percentages of nuclear p65 in the unstimulated samples were 37.77 % for Bq-YopJ and 47.46 % for the Bq-YopJ Mutant, which was not a statistically significant difference (P-value = 0.2217). After TNF- α stimulation, the mean percentage of nuclear p65 was 71.78 % for the Bq-YopJ samples, and 94.51 % for the Bq-YopJ Mutant samples, a statistically significant difference (P-value = 0.0046).

FIGURE 3.5: **Bq-YopJ inhibits nuclear localization of NF- κ B.** Each point represents one slide, with an average of 3728.5 cells assessed per slide. Three slides were produced for each condition. Error bars represent standard deviation. I calculated statistical significance with one-way ANOVA, including Tukey's multiple comparisons test. ** = P-value < 0.0046.

While automated counting is a powerful tool and the results shown in Figure 3.5 were robust, manual examination of the data revealed that many cells occupied a phenotype where p65 was dispersed throughout the cellular compartments and detected in both the nucleus and cytoplasm. The automated count does not capture this phenotype. Figure 3.6 below shows examples of these three phenotypes.

FIGURE 3.6: **p65 localisation phenotypes.**

A) Representative image from a slide of cells transfected with a plasmid encoding Bq-YopJ and stimulated with TNF- α , NF- κ B p65 in red. B) Same image overlaid with DAPI in blue. The white arrow indicates a cell with mostly cytoplasmic p65, the orange arrow indicates a cell with mostly nuclear p65, and the blue arrow indicates a cell with a dispersed phenotype.

To verify the results of the automated count, I manually counted >75 cells from each replicate and divided them into the three phenotypes. Figure 3.7 shows the results of this manual count.

The phenotype of dispersed (i.e., both nuclear and cytoplasmic localisation) p65 was prevalent in both of the TNF- α stimulated samples, representing 39.56 % of cells in the Bq-YopJ sample and 39.27 % in the Bq-YopJ Mutant sample. This difference was not statistically significant (P-value > 0.9999). The percentage of cells with only nuclear p65 in the stimulated samples was 21.70 % in cells transfected with Bq-*yopJ* wild type and 51.68 % in cells transfected with Bq-*yopJ* Mutant. This difference was statistically significant (P-value = 0.0039). The percentage of cells with only cytoplasmic p65 in the stimulated conditions was 38.73 % in cells transfected with Bq-*yopJ* wild type and 9.05 % in cells transfected with Bq-*yopJ* Mutant, a statistically significant difference (P-value = 0.0043). There were no significant differences observed between the unstimulated cells.

FIGURE 3.7: **p65 localisation phenotypes by manual count.**

Each bar shows the average of three replicates with > 75 cells counted from each replicate. I calculated statistical differences with two-way ANOVA, including Tukey's multiple comparisons test. There were no significant differences between the unstimulated conditions. TNF- α -stimulated cells with nuclear p65 (indicated in blue) differed significantly for those transfected with Bq-*yopJ* wild type vs the Bq-YopJ Mutant (P-value = 0.0039). TNF- α -stimulated cells with cytoplasmic p65 (yellow) also differed significantly for those transfected with Bq-*yopJ* wild type vs Bq-YopJ Mutant (P-value = 0.0043). No significant difference was seen for TNF- α -stimulated cells with dispersed p65 (green) when comparing those transfected with Bq-*yopJ* wild type vs Bq-YopJ Mutant (P-value = 0.9999).

Both the automated cell count and the manual cell count demonstrated that cells transfected with wild type Bq-*yopJ* have reduced nuclear localisation of p65 after TNF- α stimulation, compared to cells transfected with Bq-*yopJ* Mutant. This suggests that Bq-YopJ inhibited NF- κ B activation at or upstream of this point in the pathway.

3.2.3 Bq-YopJ Inhibits the Degradation of I κ B α

The degradation of the inhibitor of NF- κ B, I κ B α , reveals the nuclear localisation signal on p65, enabling it to migrate to the nucleus. When the NF- κ B pathway is activated, I κ B α is phosphorylated by IKK, marking it for ubiquitination by SCF ^{β -TrCP} and subsequent degradation in the proteasome. Once I κ B α has been degraded, the now unbound NF- κ B dimer localises to the nucleus, where it mediates gene expression. Because Bq-YopJ inhibited nuclear localisation of p65, I next examined whether Bq-YopJ inhibits I κ B α degradation.

The degradation of I κ B α occurs very rapidly after NF- κ B activation, and is dynamic. To detect alterations in the degradation of I κ B α , I compared the total I κ B α in lysates from HEK293 cells that had been transfected with Bq-*yopJ* and Bq-*yopJ* Mutant. Cells were stimulated with TNF- α , and lysates were recovered at five minute intervals, starting from just prior to addition of TNF- α (timepoint 0), until twenty minutes after TNF- α stimulation. Cells were harvested and lysed, then separated via SDS-PAGE, transferred to a membrane, and immunoblots were performed, using antibodies to I κ B α and LaminB1. Figure 3.8 shows this sample preparation in schematic form. LaminB1 was included in the Western blots as a loading and transfer control, selected because it has a different mass to I κ B α , enabling the inclusion of loading controls for all lanes of the gel. This method allowed me to compare amounts of I κ B α in unstimulated cells and at subsequent time points after TNF- α stimulation when either Bq-YopJ or Bq-YopJ Mutant were present. Figure 3.9 shows a representative I κ B α Western blot.

FIGURE 3.8: **Diagram showing the sample preparation for all Western blotting experiments.**

HEK293 cells were transfected with plasmids expressing Bq-YopJ or Bq-YopJ Mutant, then split into five wells and incubated 24-48 hours to enable attachment. Cells were stimulated with TNF- α , with lysates collected at five minute intervals, from just prior to TNF- α addition to twenty minutes after. Lysates were stored at -20 °C or -80 °C or processed immediately. I used this general workflow with minor changes to the number of wells and the stimulation times for all Western blotting experiments.

The bands for both $\text{I}\kappa\text{B}\alpha$ and the LaminB1 control were quantified using the image analysis software FIJI¹⁴³, resulting in a total pixel intensity of the band directly proportional to the total amount of $\text{I}\kappa\text{B}\alpha$ or LaminB1 in the sample. I then divided the total pixel intensity of $\text{I}\kappa\text{B}\alpha$ in each lane by the total pixel intensity of LaminB1 in the same lane, accounting for any variation in the amount of total protein loaded into each lane.

FIGURE 3.9: Western blot of $\text{I}\kappa\text{B}\alpha$ with TNF- α stimulation over time.

A representative blot is shown. I blotted for $\text{I}\kappa\text{B}\alpha$ first, followed by detection and imaging of the membrane, then blotted for LaminB1 so the controls are from the same lane. Note that there are slight differences in the intensity of the loading controls, demonstrating the importance of standardising $\text{I}\kappa\text{B}\alpha$ to the loading control.

When comparing the degradation of $\text{I}\kappa\text{B}\alpha$ following TNF- α stimulation, I normalised the $\text{I}\kappa\text{B}\alpha$:LaminB1 total pixel intensity ratio to time-point 0, enabling the observation of only the change in $\text{I}\kappa\text{B}\alpha$ levels following TNF- α stimulation. I repeated this Western blot a total of six times; Figure 3.10 summarises the results of the experiment.

At ten minutes following TNF- α stimulation, the trend of $\text{I}\kappa\text{B}\alpha$ degradation had significantly deviated between the lysates from cells transfected with Bq-*yopJ* vs those transfected with Bq-*yopJ* Mutant, with the Bq-YopJ cell lysate showing reduced degradation of $\text{I}\kappa\text{B}\alpha$ (P-value = 0.0065). This shows that Bq-YopJ inhibits $\text{I}\kappa\text{B}\alpha$ degradation.

I also compared the baseline amount of I κ B α in the lysates using the time-point 0 samples. I did this because if Bq-YopJ increases the total amount of I κ B α , it will reduce NF- κ B nuclear localisation by increasing the amount of I κ B α that needs to be degraded before p65 is released to localise to the nucleus. Figure 3.11 shows the results of this comparison.

No significant difference was detected in the amount of I κ B α at time-point 0 between the two conditions (P-value 0.4496), suggesting that Bq-YopJ does not affect the total I κ B α in unstimulated host cells.

This experiment demonstrated that Bq-YopJ inhibition of NF- κ B activation occurs at or upstream of the degradation of I κ B α . It also showed that inhibition is likely not due to an overall change in the expression of I κ B α .

FIGURE 3.10: **Graph of quantified and normalised I κ B α in Western blots with TNF- α stimulation over time.**

For each blot, the I κ B α was quantified as a ratio of pixel intensity over the loading control. Time points were then normalised to the value for the time point 0 to observe the dynamics of I κ B α degradation. Each point represents the average of six replicates. Error bars represent standard deviation. I calculated statistical difference between the two conditions using a repeated measures two-way ANOVA. ** = P-value < 0.01

FIGURE 3.11: **Graph of $I\kappa B\alpha$ prior to $TNF-\alpha$ stimulation in Western blots, compared to control.**

The amount of $I\kappa B\alpha$, calculated as the relative pixel intensity compared by the LaminB1 loading control, was calculated from time-point 0, just prior to addition of $TNF-\alpha$ to the cells. Each point represents one replicate. Error bars represent standard deviation. The mean of the Bq-YopJ condition is 1.582. The mean of the Bq-YopJ Mutant condition is 2.344. The means are not significantly different, P-value = 0.4496.

3.2.4 Bq-YopJ Likely Inhibits the Phosphorylation of $I\kappa B\alpha$

During $NF-\kappa B$ activation, $I\kappa B\alpha$ is phosphorylated by IKK; the dual phosphoryl groups tag it for ubiquitination and degradation. I decided to investigate whether Bq-YopJ inhibited the degradation of $I\kappa B\alpha$ by preventing its phosphorylation by IKK. This was detected using an antibody specific for a phosphorylated $I\kappa B\alpha$ epitope.

I compared the amount of phosphorylated $I\kappa B\alpha$ in lysates from HEK293 cells that had been transfected with Bq-*yopJ* and Bq-*yopJ* Mutant. The experimental procedure was essentially the same as appears in Figure 3.8, with the exception that the longest $TNF-\alpha$ stimulation time-point was taken 30 minutes after addition of $TNF-\alpha$, rather than 20 minutes. I made this change to capture any longer term changes, while maintaining high resolution in the first 15 minutes. Unfortunately, I only produced one replicate of this blot, which appears in Figure 3.12.

FIGURE 3.12: Western blot of phosphorylated I κ B α following TNF- α stimulation over time.

The lysates of transfected HEK293 cells were prepared as described. An antibody specific for phosphorylated I κ B α was used first. After detection and imaging of the membrane, it was then blotted with antibody for LaminB1, so the controls are from the same lane. Note that there are slight differences in the intensity of the loading controls, demonstrating the importance of standardising I κ B α to the loading control.

As expected, phosphorylated I κ B α was not detected in either sample at time-point 0, indicating that NF- κ B is not activated in these cells prior to addition of TNF- α . There appears to be far less phosphorylated I κ B α in the cells transfected with Bq-*yopJ*, particularly at five and ten minutes following TNF- α stimulation. To ensure that this difference was not due to variable amounts of total protein, I quantified the bands and the ratio of phosphorylated I κ B α :LaminB1 pixel intensity over time. This graph appears in Figure 3.13.

This graph shows that the amount of phosphorylated I κ B α at the five and ten minute time-points is reduced in the Bq-YopJ transfected cell lysates, compared to the Bq-YopJ Mutant transfected cells. By 15 and 30 minutes after TNF- α stimulation, the difference had disappeared. Because additional replicates were not done, the differences in levels of phosphorylated I κ B α at 5 and 10 minute time points, while apparent, could not be evaluated for statistical significance.

This experiment shows that Bq-YopJ inhibition of NF- κ B activation occurs at or upstream of the phosphorylation of I κ B α by IKK.

FIGURE 3.13: Graph of phosphorylated IκBα Western blot at time points following TNF-α stimulation.

Each point represents the ratio of phosphorylated IκBα:LaminB1 loading control, measured as total pixel intensity of the bands.

One replicate was done, so there are no error bars.

3.2.5 Bq-YopJ May Reduce the Baseline Abundance of p65

Bacterial effectors use many varied mechanisms to inhibit the NF-κB pathway, with some effectors, such as YopJ from *Yersinia*, inhibiting more than one component of the pathway. To thoroughly investigate the inhibition of NF-κB by Bq-YopJ, I tested whether it alters the amount of p65 in the cells, in addition to its localisation. This would not be a novel mechanism of inhibition of NF-κB activation, as there are known bacterial effectors that directly target p65, including CT441 of *Chlamydia trachomatis*, which cleaves p65¹⁵⁶.

I used the experimental outline shown in Figure 3.8, with TNF-α stimulation followed by harvesting cell lysates over a time-course, to detect whether Bq-YopJ affects total p65 protein levels. I used Bq-YopJ Mutant as a negative control for NF-κB inhibition and GAPDH as a loading and transfer control for this Western blot, as it differed in mass to enable blotting for both p65 and the GAPDH in the same gel lanes. Unfortunately, I only produced one replicate of this blot, it appears in Figure 3.14.

FIGURE 3.14: **Western blot of p65 with TNF- α stimulation over time.**

Lysates were collected at 5 minute intervals following TNF- α stimulation. I blotted for p65 first, imaged the membrane, then blotted for GAPDH so the controls are from the same lanes. Note that there are slight differences in the intensity of the loading controls, demonstrating the importance of standardising p65 to the loading control.

There is not any clear change in the amount of p65 in response to TNF- α stimulation in either of the two conditions. There does seem to be a difference in the baseline amount of p65, however, with less p65 in the Bq-YopJ samples. To confirm the lack of a difference in the amount of p65 over time following TNF- α stimulation, I quantified the bands and graphed the ratio of p65:GAPDH pixel intensity over time, normalised to time-point 0. This removed the total amount of p65 as a factor so I could more clearly observe the trend. This graph appears in Figure 3.15 and shows no clear difference in the amount of p65 over time with TNF- α stimulation between cells transfected with Bq-*yopJ* or Bq-*yopJ* Mutant.

To better assess whether the baseline amount of p65 was impacted by Bq-YopJ, I graphed the ratio of p65:GAPDH pixel intensity over time, which is a measure of how much p65 is in the sample relative to the total amount of protein. In this graph, the pixel intensity ratios were not normalised to time-point 0. I then performed linear regression to determine if the elevation of the line of best fit was significantly different. I used this analysis method because with only one replicate of this blot it was the best way to assess statistical significance, though it does assume a linear trend which is not a certainty. This graph appears in Figure 3.16, and shows that the gradient of the trend-lines are not significantly different (P-value = 0.6353) but that the elevation of the lines is significantly different (P-value = 0.0171). However, the experiment should be repeated to establish whether this result can be replicated. Though the elevations of the lines were different, this represents only a single replicate.

These results suggest that Bq-YopJ may reduce the amount of p65 in host cells, though it does not explain the mechanism by which this occurs. Bq-YopJ could cause degradation of p65 or it could inhibit its transcription at a basal level; in either case, reduced levels of p65 in a cell could result in reduced nuclear localisation and expression of NF- κ B-activated genes.

FIGURE 3.15: **Graph of quantified and standardised p65 Western blot following TNF- α stimulation over time.**

Each point represents the ratio of p65:GAPDH on the p65 Western blot as a percent of time-point 0, measured as total pixel intensity of the bands. No error bars are included, as it represents one replicate.

FIGURE 3.16: Graph of quantified p65 western blot with TNF- α stimulation over time.

Each point represents the ratio of p65:GAPDH on the p65 Western blot, measured as total pixel intensity of the bands. No error bars are included, as it represents one replicate. I compared the gradients and elevations of the two trend-lines by linear regression. The gradients are not significantly different, P-value = 0.6353. The elevations are significantly different *: P-Value = 0.0171

3.2.6 Bq-YopJ Does Not Inhibit the Degradation of p105 or its Processing to p50

The result for p65 made me question whether p50, the other component of the NF- κ B heterodimer, was similarly inhibited by Bq-YopJ. Additionally, the finding that I κ B α degradation is inhibited suggested that the degradation of p105 could be inhibited too, particularly because p105 has a similar inhibitory function to I κ B α ¹⁵².

Under normal, non-activated conditions, p105 is basally processed into p50 independent of NF- κ B activation¹⁵². Upon TNF- α stimulation however, p105 is phosphorylated by IKK, ubiquitinated by SCF $^{\beta}$ -TrCP, and completely degraded by the proteasome, just like I κ B α ¹⁵². This SCF $^{\beta}$ -TrCP-dependant proteasomal degradation of p105 does not produce p50¹⁵². Also, in addition to dimerising with p65 to form NF- κ B, p50 can also form functional homodimers. Both p105 and p50 are core to NF- κ B signalling, so it is not surprising that they are targets for NF- κ B suppression by pathogens. The Cowpox virus effector

ORF006 inhibits NF- κ B through interaction with p105, as does M013 of Myxoma virus and G1R of Variola virus^{156,162}.

I used the experimental outline in Figure 3.8 with a TNF- α stimulation time course, followed by blotting the lysates with an antibody that recognises both p105 and p50, which are differentiated based on their size. I added a 30 minute time-point in addition to the 20 minute time-point to increase the likelihood of capturing longer-term changes in degradation of p105. I used cells transfected with Bq-*yopJ* Mutant as a negative control to compare to cells transfected with the wild type Bq-YopJ. The cellular protein GAPDH was included as the loading and transfer control for this Western blot, as its mass was different to those of p105 and p50. I repeated this experiment three times, with a representative blot shown in Figure 3.17.

FIGURE 3.17: **Western blot of p105 and p50 with TNF- α stimulation over time.**

I blotted for p105/p50 first, as the same antibody detected both, then imaged the membrane, and blotted for GAPDH so the controls are from the same lanes. Note that there are slight differences in the intensity of the loading controls, demonstrating the importance of standardising p105/p50 to the loading control.

p105

The Western blot in Figure 3.17 shows that Bq-YopJ may inhibit the degradation of p105, with the difference most visible at the 30 minute time-point. There may also be less p105 at time-point 0 in cells transfected with wild type Bq-YopJ compared to those transfected with Bq-*yopJ* Mutant.

To confirm a difference in the amount of p105 over time with TNF- α stimulation, I quantified the bands and determined the ratio of p105:GAPDH pixel intensity over time, as a percent of time-point 0. This removed the total amount of p105 as a factor to more clearly observe the trend. Figure 3.18 shows this graph.

There is no significant difference in the amount of p105 between the Bq-YopJ and Bq-YopJ Mutant samples during the time course (P-value = 0.0872). The graph shows that the amount of p105/GAPDH between the two conditions may be starting to diverge at the later time-points so it is possible that Bq-YopJ has a weak effect that was not captured in this experiment.

FIGURE 3.18: Graph of quantified p105 western blot with TNF- α stimulation over time.

Each point represents the average ratio of p105:GAPDH on three p105 Western blots, expressed as a percent of time-point 0. Error bars represent standard deviation. I calculated statistical difference between the two conditions using a repeated measures two-way ANOVA, there was no significant difference between the two conditions, P-value = 0.0872. Note that the repeats for Bq-YopJ Mutant at 20 and 30 minutes are so close that the error bars are too small to show on the graph.

To confirm a difference in the basal amount of p105, I quantified the bands and graphed the ratio of p105:GAPDH pixel intensity for time-point 0 of all three replicates. Figure 3.19 shows this graph.

The difference in p105 at time-point 0 between the Bq-YopJ condition and the Bq-YopJ Mutant condition did not reach statistical significance (P-value = 0.2060). Perhaps with a larger sample size this difference would be significant, but there is not currently enough evidence to conclude that Bq-YopJ reduces the basal amount of p105 in host cells. Additionally, because p105 is under the transcriptional control of NF- κ B, reduced basal p105 could result from NF- κ B inhibition, rather than being the cause of it¹⁴⁸.

FIGURE 3.19: Graph of p105 in westerns blot compared to control.

Each point represents one replicate. Error bars represent standard deviation. Comparing time-point 0 from the TNF- α time-course. The mean from cells transfected with Bq-*yopJ* is 0.4172, and the mean from cells transfected with Bq-*yopJ* Mutant is 0.8285. An unpaired t-test to compare the means showed that they are not significantly different, P-value = 0.2060.

p50

The Western blot in Figure 3.17 does not seem to show any difference in p50, either basally or in response to TNF- α between the Bq-YopJ condition and the Bq-YopJ Mutant condition. To confirm this lack of an effect by Bq-YopJ I once again quantified the Westerns and graphed their results.

To test for a difference in the amount of p50 over time with TNF- α stimulation I quantified the bands and graphed the ratio of p50:GAPDH pixel intensity over time, as a percent of time-point 0. This controlled for the total amount of p50 in the sample so I could more clearly observe the trend. Figure 3.20 shows this graph.

There is no significant difference in the amount of p50 over time with TNF- α stimulation between the Bq-YopJ condition and the Bq-YopJ Mutant condition. This is unsurprising given that there was no difference in p65 over time either. This confirms that Bq-YopJ does not cause degradation of either component of the NF- κ B transcription factor in a TNF- α -dependent manner.

FIGURE 3.20: **Graph of quantified p50 western blot with TNF- α stimulation over time.**

Each point represents the average ratio of p105:GAPDH on three p105 Western blots, expressed as a percent of time-point 0. Error bars represent standard deviation. I calculated statistical difference between the two conditions using a repeated measures two-way ANOVA, there was no significant difference between the two conditions, P-value = 0.5487.

Because the p65 Western showed reduced baseline p65 I decided to test baseline p50 as well. To test this I quantified the bands and graphed the ratio of p50:GAPDH pixel intensity for time-point 0 of all three replicates. Figure 3.21 shows this graph.

There was no significant difference in the amount of p50 at time-point 0 between HEK293 cells transfected with Bq-*yopJ* to those transfected with Bq-*yopJ* Mutant (P-value = 0.9171). This indicates that though Bq-YopJ may inhibit TNF- α -induced degradation of p105, it does not affect its basal processing to p50.

FIGURE 3.21: Graph of average p50 in Westerns blot compared to control.

Each point represents one replicate. Error bars represent standard deviation. Comparing time-point 0 from the TNF- α time-course. The mean from cells transfected with Bq-*yopJ* is 0.9682, and the mean from cells transfected with Bq-*yopJ* Mutant is 0.9239. An unpaired t-test to compare the means showed that they are not significantly different, P-value = 0.9171.

3.3 Discussion

Modulation of host immunity is vital for the survival of all pathogens; they must evade or suppress the immune system to establish an infection for long enough to spread to new hosts. The NF- κ B pathway is core to immune activation in animals, so it is an important target for inhibition by animal pathogens. *B. quintana* is highly adapted to evading host immunity, and inhibiting NF- κ B is likely to be an important part of its strategy.

After activation of the NF- κ B pathway, Bq-YopJ inhibited the secretion of IL-8, the nuclear localisation of p65, the degradation of I κ B α , and the phosphorylation of I κ B α . This supports the conclusion that Bq-YopJ targets IKK, a component of the TNF- α receptor, or the signal transduction components and adaptors that link them. These mechanisms would not be novel, as YopJ of *Yersinia* blocks stimulatory phosphorylation of IKK, OspI of *S. flexneri* inhibits TRAF6 (an adaptor of the TNF- α receptor), and an unknown factor of *Neisseria meningitidis* causes host cells to shed the TNF- α receptor^{159,165,166}. A summary of these mechanisms appears in Figure 3.22.

FIGURE 3.22: **Overview of the NF-κB pathway and Bq-YopJ Inhibition.**

Asterisks in the figure above show the steps that were shown in this chapter to be inhibited by Bq-YopJ. Question marks indicate the possible targets of Bq-YopJ inhibition. See Figure 3.1 for more information on NF-κB activation. YopJ is YopJ from *Yersinia*, OspI is from *S. flexneri*, UNMF is an unknown factor from *Neisseria meningitidis*.

The kinase that phosphorylates I κ B α , IKK, is itself activated by phosphorylation by the TAK1 adaptor protein. *Yersinia* Yop acetylates the phosphorylation sites on IKK β , one of the three subunits of IKK¹⁶⁵. Because acetylation blocks the phosphorylation of these sites, IKK is blocked from activation. The next step in characterising Bq-YopJ inhibition of NF- κ B activation will be to test whether TAK1-mediated phosphorylation of IKK is inhibited. This test could be a Western blot with an antibody specific for phosphorylated IKK α / β . If phosphorylation of IKK is also inhibited, the target of Bq-YopJ could be the kinase that phosphorylates IKK, TAK1, or one of the components of the TNF- α receptor¹⁵². It is important to consider that TAK1 is the convergence point for multiple cell surface receptors, so if Bq-YopJ inhibits the NF- κ B pathway upstream of TAK1, its inhibitory effects may be receptor-specific. There are, however, some additional adaptor proteins that activate NF- κ B in response to signals from multiple receptors, such as TRAF6, the target of the *Shigella flexneri* effector, OspI. TRAF6 is an adaptor essential for NF- κ B activation in response to signals from the TNF- α , IL-1, and Toll-like receptors¹⁶⁷. Therefore, future experiments could also evaluate the effect of Bq-YopJ on NF- κ B when it is activated by alternative receptors, such as TLR4.

TRAF6 is part of the tumor necrosis factor receptor-associated factor (TRAF) family, which is associated with almost all of the receptors that activate NF- κ B^{168,169}. There are seven TRAF proteins. All of these, apart from TRAF7, share a C-terminal TRAF domain, and all besides TRAF1 share a RING ubiquitin ligase domain¹⁶⁸. The TRAFs play many roles in NF- κ B signalling, including as adaptor proteins and ubiquitin ligases; they are responsible for the activation of TAK1 and recruitment of IKK¹⁶⁸.

Another important family of adaptor proteins are the receptor-interacting proteins (RIPs), of which there are seven¹⁷⁰. The RIPs are kinases and are involved in the signalling of many immune receptors, including the TNF- α receptor and the IL-1 receptor¹⁷⁰. RIPs 1 – 4 are known to be involved in NF- κ B signalling, working with the TRAFs to recruit and activate IKK^{169,170}. Other important adaptor proteins include TRADD, MyD88, and IRAK, though they are less widespread across different receptor signalling cascades. TNF receptor type 1-associated death domain protein (TRADD) recruits both RIP1 and TRAF2 to the TNF- α receptor upon TNF- α binding¹⁵². Myeloid differentiation primary response protein 88 (MyD88) and IL-1R-associated kinase (IRAK) are involved in signalling from the TLRs and IL-1R, while MyD88 directly binds to the cytoplasmic tails of the receptor proteins and

recruits members of the IRAK family of proteins¹⁷¹. In the IL-1 receptor and TLRs, MyD88 recruits IRAK4, which in turn recruits IRAK1, which activates TRAF6¹⁷².

These adaptor proteins are common targets for inhibition of NF- κ B activation by pathogens, i.e., OspI inhibition of TRAF6¹⁵⁹. The effector NS5a of the Hepatitis C virus targets both TRAF2 and MyD88, M45 of the Human cytomegalovirus binds to RIP1, and A52R of the Vaccinia virus targets IRAK2 and TRAF6¹⁵⁶.

Previous studies of effector proteins have often overlooked the role of p105 in NF- κ B signalling, despite its importance to the pathway as both an inhibitor of NF- κ B nuclear localisation and as a precursor to the NF- κ B transcription factor p50¹⁵³. Direct interaction with p105 is sufficient for NF- κ B inhibition by Cowpox virus effector ORF006¹⁶². Bq-YopJ likely inhibits I κ B α phosphorylation by IKK, and it would be worthwhile to confirm that this is also the case for p105, particularly because a difference in p105 degradation between the Bq-YopJ and the Bq-YopJ Mutant lysates over a longer period was possible. This could be determined using an antibody that recognises phosphorylated-p105, and could be accompanied by additional repeats of the p105 blots over a longer period. Additional repeats of the phosphorylated I κ B α blot would also be valuable.

It would be useful to determine if Bq-YopJ inhibition of NF- κ B is receptor specific. Given the number of proteins involved and the complexity in the signalling of each receptor, identifying receptor specificity would narrow down the potential targets of Bq-YopJ immensely. To determine receptor specificity, additional IL-8 ELISA experiments could be done, but rather than stimulating the cells with TNF- α , NF- κ B activation could be triggered with other cytokines, such as IL-1 or IL-17, or with a pathogen-associated molecular pattern, such as LPS via TLR4.

There are other mechanisms by which Bq-YopJ could inhibit NF- κ B in addition to preventing the canonical signalling cascade that should also be considered. Upon release from I κ B, p65 is phosphorylated on Serine 276 by protein kinase A; this phosphorylation is required for its transcriptional activity¹⁶⁹. Inhibiting this phosphorylation would prevent the expression of NF- κ B activated genes. Phosphorylation of another p65 residue, Serine 468, by glycogen synthase kinase-3 β has the opposite effect, reducing p65 activity¹⁷³. For these reasons it would be beneficial to examine the phosphorylation of p65 at these different

residues when functional Bq-YopJ is or is not present.

Beyond phosphorylation of p65, NF- κ B is also regulated by cross-talk with other signalling pathways, including ERK, MAPK, and CREB, as well as chromatin modification¹⁶⁹. NF- κ B regulation is complex, so it is important to consider that Bq-YopJ could inhibit the pathway by multiple mechanisms.

Another area of future investigation could be the interaction between Bq-YopJ-induced suppression of the NF- κ B pathway and the TLR-4 antagonistic LPS of *B. quintana*. TLR-4 activation stimulates both the NF- κ B pathway and the MAPK pathway^{174,175}. Normal activation of TLR4 paired with inhibition of the NF- κ B pathway would likely cause apoptosis in host cells due to the suppression of the anti-apoptotic effects of NF- κ B but continued activation of pro-apoptotic effects of MAPK^{149,176}. This could mean that the TLR-4 antagonistic LPS of *B. quintana* prevents Bq-YopJ from inducing apoptosis in host cells.

Interestingly, the LPS of *Yersinia pestis* may, like that of *B. quintana*, be non-immunogenic¹⁷⁷. YopJ of *Y. pestis* is reported to cause apoptosis in macrophages¹²⁷, so it is also possible that the combination of Bq-YopJ and TLR-4 antagonistic LPS in *B. quintana* is redundancy in mechanisms of immune evasion.

3.4 Summary

The overall aim of this chapter was to identify the point of the NF- κ B pathway that Bq-YopJ inhibits. Each of these experiments tested one step of the NF- κ B pathway in the hopes of pinpointing the exact target of Bq-YopJ inhibition. The results indicate that Bq-YopJ inhibits the NF- κ B pathway at or upstream of the IKK-mediated phosphorylation of I κ B α . While these experiments confirmed that Bq-YopJ inhibits NF- κ B activation, the exact early step at which inhibition took place was not identified. There are many adaptors and signalling proteins that relay the signal from the TNF receptor to IKK, and we were not able to identify the target of Bq-YopJ using this approach. Therefore, I next decided to perform a proteomics screen, to further assess any baseline changes in protein abundance due to Bq-YopJ, and protein interaction studies, to identify the host cell Bq-YopJ target.

Chapter 4

Exploring the Effects of *Bartonella quintana* YopJ by Proteomic Screen

4.1 Introduction

4.1.1 Host - Pathogen Proteomics

Proteomics is the study of the proteome, the complete set of proteins expressed by a cell or tissue, with the goal of understanding the roles of specific proteins within the whole system¹⁷⁸. Usually, proteomics involves comparing the relative abundances of proteins between conditions.

In the context of host-pathogen interactions, proteomics can be approached from the perspective of the pathogen, specifically, how the proteome of the pathogen changes during infection; or of the host, examining how the host cell proteome changes during infection¹⁷⁹. The host perspective can give insights into changes directly induced by the pathogen, and how the host responds to, and combats, the pathogen.

A more targeted approach to the host perspective is to examine the effect of a specific bacterial component on the host proteome. This can be done either by introducing that bacterial component to the cells in the absence of bacteria, or by comparing the effects of wild-type bacteria to a mutant strain that has the component knocked out¹⁸⁰.

Examining the effects of a bacterial effector protein on the host proteome can give clues about the function of the effector without any pre-existing knowledge about it. Even with prior knowledge, it is valuable to take a broad approach in case the effector has multiple functions or downstream effects. In

the case of the *B. quintana* YopJ homologue, it is useful to test its effects more broadly, as it could affect more than just the NF- κ B pathway.

4.1.2 Liquid Chromatography Mass Spectrometry

One way to measure changes in the proteome is liquid chromatography mass spectrometry (LC/MS), in which enzymatically digested samples of mixed proteins are separated by hydrophobicity on a resin column, then detected by mass spectrometry¹⁷⁸. This produces data for the mass, hydrophobicity, and abundance of every peptide in the sample. Additionally, LC/MS can include tandem mass spectrometry (MS/MS), in which peptides are fragmented and the fragments re-analysed to determine their exact amino acid sequence.

This allows each peptide to be matched to a protein in a protein database, giving information about all of the proteins in the sample and their relative abundances. Finally, changes in the proteome due to a treatment, such as the presence of a bacterial component, are determined by comparing the abundances of each protein between the treatments. This yields a list of proteins that were enriched (over-represented) or depleted (under-represented) by the treatment. These lists can give clues about the effects of the treatment based on the functions of the altered proteins.

4.1.3 Rationale

To study the effect of Bq-YopJ on the proteome of mammalian cells, I transfected a mammalian expression plasmid encoding the Bq-*yopJ* gene (pcDNA3.1-Bq-YopJ-GFP) into HEK293 cells, so that the cells produced Bq-YopJ directly. This allowed me to examine the effects of Bq-YopJ without potential interference from other *B. quintana* components. I used a mammalian expression plasmid with the catalytically inactive mutant form of Bq-YopJ (Bq-YopJ Mutant; pcDNA3.1-Bq-YopJ-C197A-GFP) as a negative control. I used the Bq-YopJ Mutant instead of non-transfected cells or an empty vector, because it controlled for any effect on the proteome due to exogenous double-stranded DNA (the plasmid) or expression of a bacterial protein in general. Both the Bq-*yopJ* and Bq-*yopJ* Mutant plasmids also encoded proteins with a C-terminal GFP translational fusion, which served as a marker for expression.

The overall aim of this part of the project was to cast a wide net and assess the effects of Bq-YopJ on host cellular proteins in general, and use these

insights to identify pathways and proteins of interest that may be important to the function of Bq-YopJ.

4.2 Results

4.2.1 Ensuring Comparability

Transfection Efficiency

Forty eight hours after each transfection, immediately before I harvested the cells, I examined the cells with a fluorescence microscope. I checked for the presence of the C-terminal green fluorescent protein (GFP) tag to confirm that the transfection was successful and that the cells were transcribing Bq-YopJ (or Bq-YopJ Mutant). Figure 4.1 shows a representative transfection.

Not all transfected cells showed high levels of GFP, but after each transfection at least 40 % of cells produced visible amounts of GFP. It is likely that more produced smaller amounts that could not be visualised. This was considered sufficient to confirm a successful transfection.

FIGURE 4.1: **A representative transfection.**

Images of cells transfected with the Bq-*yopJ*-containing mammalian expression plasmid. A: GFP fluorescence channel. B: bright field image of the cells. C: A + B overlaid. The presence of GFP in a cell is a marker for successful transfection and protein production. In this example there is clearly variation in the amount of GFP in each cell but the GFP (and therefore the Bq-YopJ protein) is widespread.

Live/Dead Staining

When I harvested the cells, I collected a small volume of cell suspension and performed a live/dead stain using trypan blue stain. Trypan blue is unable to cross the intact membranes of live cells but can enter dead cells which have damaged membranes; this results in live cells appearing colourless under a microscope while dead cells appear blue. Counting colourless and blue cells and calculating the ratio of live to dead cells gives an estimate of the general health and viability of the population. Figure 4.2 shows the ratios of live to dead cells compared between conditions.

FIGURE 4.2: HEK293 cell viability when transfected with Bq-*yopJ* or the Bq-*yopJ* mutant.

Each point represents the count from one biological replicate, Error bars represent standard deviation. The mean for cells transfected with Bq-*yopJ* is 13.35 live cells per dead cell. The mean for cells transfected with Bq-*yopJ* Mutant is 15.18 live cells per dead cell. There was no significant difference between the mean viability between the two conditions according to an unpaired t test, P-value = 0.4638. It is unlikely that cells transfected with Bq-*yopJ* are significantly less healthy than cells transfected with Bq-*yopJ* Mutant.

The average ratio of live cells to dead, for cells expressing Bq-YopJ, was 13.35, while the ratio for cells expressing the Bq-YopJ Mutant was 15.18 live cells per dead cell. This difference was not statistically significant (P-value = 0.4638). Though this does not necessarily mean that there is no difference, it suggests that cells transfected with Bq-*yopJ* are significantly less healthy than

cells transfected with Bq-*yopJ* Mutant, and they are therefore comparable for proteomic analysis.

4.2.2 Protein Identification

I prepared four biological replicates of lysates from HEK293 cells transfected Bq-*yopJ* or Bq-*yopJ* Mutant. I harvested the cells 48 hours after transfection, which gave them sufficient time to express the protein and for the protein to affect the cells.

I included at least two technical replicates of each of these samples by LC/MS/MS and analysed the resultant spectral data using the Proteome Discoverer 2.4 software package (ThermoFisher Scientific). Henceforth the replicates will be referred to with the following structure: Bqx_y_z. Where *x* is either 'wt', short for wild type Bq-YopJ or 'Cat' short for catalytic Mutant Bq-YopJ, *y* is a number representing the biological replicate, and *z* is a number representing the technical replicate.

I searched against the SwissProt Human database¹¹⁷, with the Bq-YopJ protein added to confirm it was expressed. This analysis identified the proteins present in each sample, and produced a table of all of the identified proteins from each replicate.

I then assessed the reproducibility of the LC/MS/MS runs by comparing the number of proteins identified in each run using Venn diagrams produced by the web tool Venny¹⁴⁴. Figures 4.3 and 4.4 show these comparative Venn diagrams for cells expressing the wild type Bq-YopJ (Fig. 4.3) or the BQ-YopJ Mutant (Fig. 4.4).

I also visually examined the chromatograms of each replicate, which show the relative abundance of peptides over time as they are eluted from the C18 column by the solvent gradient. Practically, this shows the peptides in the sample separated by hydrophobicity. Gaps in the chromatogram can indicate problems with the run, such as air bubbles, and could produce artificial differences in protein abundance if replicates with gaps are used for quantitation. Figure 4.5 shows two chromatograms, one with a gap and one without. If the chromatogram for a replicate showed a gap, I ran another replicate until I had at least two acceptable technical replicates for each biological replicate.

FIGURE 4.3: Venn diagrams comparing proteins identified in replicate LC/MS/MS runs of Bq-YopJ samples.

I used the technical replicates marked with a red asterisk for quantitation.

Comparisons of identified proteins in the technical replicates of the Bq-YopJ condition for each biological replicate. (A) Bq-YopJ biological replicate 1 (two technical replicates). (B) Bq-YopJ biological replicate 2 (three technical replicates). (C) Bq-YopJ biological replicate 3 (three technical replicates). (D) Bq-YopJ biological replicate 4 (two technical replicates).

FIGURE 4.4: Venn diagrams comparing proteins identified in replicate LC/MS/MS runs of Bq-YopJ Mutant samples.

I used the technical replicates marked with a red asterisk for quantitation.

Comparisons of identified proteins in the technical replicates of the Bq-YopJ Mutant condition for each biological replicate. (A) Bq-YopJ Mutant biological replicate 1 (three technical replicates). (B) Bq-YopJ Mutant biological replicate 2 (two technical replicates). (C) Bq-YopJ Mutant biological replicate 3 (two technical replicates). (D) Bq-YopJ Mutant biological replicate 4 (three technical replicates).

(A) Unacceptable chromatogram with gap (indicated by the red arrow)

(B) Acceptable chromatogram

FIGURE 4.5: Representative LC/MS chromatograms.

(A) shows the chromatogram from BqCat_1_1, where the red arrow indicates a gap in the chromatogram that meant that I had to run another technical replicate. (B) shows the chromatogram from BqCat_4_1, as an example of what the chromatograms should look like.

I selected two technical replicates of each biological replicate by first ensuring the chromatograms were acceptable, then selecting the replicates with the highest number of identified proteins and the greatest overlap in identified proteins. For two of my biological replicates in each condition (Bqwt_1, Bqwt_4, BqCat_2, and BqCat_3), the first two runs produced acceptable results so I did not run additional replicates.

My chosen replicates contained 5058 unique proteins across all samples. All of the samples from cells transfected with Bq-yopJ had peptides from Bq-YopJ detected by MS/MS, as did four of the eight samples from cells transfected with Bq-yopJ Mutant. The remaining four Bq-yopJ Mutant samples had peaks of the same size and hydrophobicity, but were not fragmented and detected by MS/MS. This difference is most likely due to the change in the active site of Bq-YopJ Mutant, resulting in some peptides having a different mass and sequence that would not be recognised as originating from Bq-YopJ.

4.2.3 Quantitation

Once I had selected two technical replicates of each biological replicate, I performed the protein quantitation to test for differences in the amounts of specific proteins in each condition. I used the Proteome Discoverer 2.4 software package to compare the number of peptides from each identified protein between the Bq-YopJ samples and Bq-YopJ Mutant samples. This gave the relative abundance of each identified protein in the Bq-YopJ samples when compared to the Bq-YopJ Mutant samples. Henceforth I will refer to proteins that were more abundant in the Bq-YopJ condition as over-represented and proteins that were more abundant in the Bq-YopJ Mutant condition (and therefore less abundant in the Bq-YopJ condition) as under-represented. For the quantitation, I assigned proteins that were at least two fold over- or under-represented with a P-value of > 0.05 as being significantly differentially represented. Figure 4.6 shows a volcano plot of all of the identified proteins, their relative abundances, and the P-value of the differences.

There were a total of 57 differentially represented proteins that met these criteria, with 31 over-represented and 26 under-represented in cells transfected with Bq-YopJ. Appendix Tables A.1 and A.2 show complete lists of these proteins, respectively.

FIGURE 4.6: Volcano plot showing differential protein representation between cells transfected with Bq-yopJ vs Bq-yopJ Mutant.

Each point represents one identified protein. P-value cut-off = 0.05. Fold change (FC) cut-off was set at Log2 Ratio = 1, which represents a two-fold difference in protein abundance. 31 proteins were significantly over-represented in cells transfected with Bq-YopJ (red), and 26 were significantly under-represented in cells transfected with Bq-YopJ (green). Some points on the graph are overlapping.

To get a better idea of what cellular compartments were impacted in cells transfected with Bq-YopJ, I manually checked the UniProt entries for each over- and under-represented protein and graphed their primary cellular location. In cases where a protein had more than one cellular location, I selected the location where the protein exhibits its activity. If there was no clear single primary location, I classified the protein as unknown. Figure 4.7 depicts this information about the cellular localisation of the identified proteins.

I also manually checked the UniProt entries for each over- and under-represented protein and assigned a functional category to each, based on reports in the literature. Figure 4.8 shows the overall functional categories for the differentially represented proteins.

FIGURE 4.7: Pie charts showing the cellular distribution of differentially represented proteins.

Primary cellular locations of each differentially represented protein. In cases when a protein had more than one cellular location I selected the location where the protein exhibits its activity. If there was no clear single primary location I classified the location as unknown. (A) shows the cellular locations of proteins over-represented in cells transfected with Bq-YopJ, showing that 6/31 localise to the cytoplasm and cytosol, 1/31 localises to the cytoskeleton, 5/31 localise to the endoplasmic reticulum, 2/31 localise to the Golgi apparatus, 5/31 localise to the mitochondrion, 9/31 localise to the nucleus, 2/31 localise to the plasma membrane, and 1/31 localises to an unknown compartment. (B) shows the cellular locations of proteins under-represented in cells transfected with Bq-YopJ, showing 7/26 localise to the cytoplasm and cytosol, 2/26 localise to the cytoskeleton, 1/26 localises to the endoplasmic reticulum, 1/26 localises to the endosome, 2/26 localise to the extracellular region or are secreted, 1/26 localises to the mitochondrion, 6/26 localise to the nucleus, 4/26 localise to the plasma membrane, and 2/26 localise to an unknown compartment.

FIGURE 4.8: Pie charts showing the functional categories of differentially represented proteins.

Functional categories of each differentially represented protein. (A) shows the functional categories of proteins over-represented in cells transfected with Bq-YopJ, 1/31 is involved in apoptosis, 5/31 are involved in biosynthesis, 1/31 are involved in the cell cycle, 1/31 are associated with the cytoskeleton, 8/31 are involved in DNA repair or binding, 1/31 is involved in cellular respiration, 4/31 are involved in RNA binding or transcription, 4/31 are involved in signalling pathways, 4/31 are involved in intracellular transport, 2/31 are of unknown function.

(B) shows the functional categories of proteins under-represented in cells transfected with Bq-YopJ, 2/26 are involved in biosynthesis, 2/26 are involved in the cell cycle, 2/26 are associated with the cytoskeleton, 4/26 are involved in DNA repair or binding, 1/26 is involved in cellular respiration, 4/26 are involved in RNA binding or transcription, 6/26 are involved in signalling pathways, 3/26 are involved in intracellular transport, and 2/26 are of unknown function.

4.2.4 Gene Ontology Search

I performed gene ontology (GO) searches in both g:Profiler¹⁴⁵ and DAVID¹⁴⁶, searching against the lists of proteins over- and under-represented in cells transfected with Bq-*yopJ* separately. These searches identify the molecular functions and biological processes that each protein in a list is involved in, as well as their subcellular localisation. This produces information about common functions shared between some or all of the proteins in each input list. The results of these searches appear in Tables 4.1 and 4.2 below.

TABLE 4.1: **g:Profiler gene ontology results**

(A) shows the statistically significant ontologies for the 31 proteins that were over-represented in cells transfected with Bq-*yopJ*. (B) shows the statistically significant ontologies for the 26 proteins that were under-represented in cells transfected with Bq-*yopJ*. The number of genes for each ontology is non-exclusive, so the same gene can appear in multiple categories.

(A) Over-represented in cells transfected with Bq-*yopJ*

Ontology	P-value	Number of genes
CELLULAR COMPARTMENT		
intracellular membrane-bounded organelle	0.0081	28
intracellular organelle	0.012	29

(B) Under-represented in cells transfected with Bq-*yopJ*

Ontology	P-value	Number of genes
MOLECULAR FUNCTION		
enzyme binding	0.025	10

The g:Profiler gene ontology results showed very few ontologies significantly over- or under-represented in cells transfected with Bq-*yopJ*. Two were over-represented, including intracellular membrane-bounded organelle and intracellular organelle, which are similar and fairly broad categories of cellular compartment. The only ontology identified for the under-represented proteins was a molecular process, enzyme binding.

This result from the g:Profiler GO search shows the lack of any clear response to Bq-YopJ in unstimulated cells. In the context of an effector protein, this would likely benefit *B. quintana* during infection, as more subtle changes to the host cell are less likely to result in the host cell detecting and responding to the effector.

The DAVID gene ontology search identified more significant ontologies than the g:Profiler search. Proteins localised in both the nucleus and mitochondrion cellular compartments were over-represented in cells transfected with Bq-YopJ. This is in agreement with Figure 4.7, which showed that nearly half (14/31) of proteins over-represented in Bq-YopJ-expressing cells were localised to the nucleus or mitochondrion. Both are also intracellular membrane-bounded organelles, so this was in agreement with the g:Profiler search. Also over-represented were two biological processes involved in the organisation and development of the inner ear, although this has no known involvement in *B. quintana* infection.

Among the ontologies identified with the under-represented proteins was both the phosphoprotein keyword and the kinase activity molecular function. This was particularly noteworthy because not only are these required for NF- κ B function, but *Yersinia* YopJ interferes with both by blocking phosphorylation sites on kinases¹⁶⁵. Also of particular note is the cellular response to lipopolysaccharide, which includes activation of the NF- κ B pathway¹⁵⁰. There were three proteins identified with the "cellular response to lipopolysaccharide" ontology, MAPK8, HAVcr2, and ADAM9.

Mitogen-activated protein kinase 8 (MAPK8), also called c-Jun N-terminal kinase 1 (JNK1) is a key component of the JNK signalling pathway, one of three MAPK pathways in mammals, and is associated with the response to stress¹⁸¹. It can be activated by many of the same mechanisms as the NF- κ B pathway, including cytokine receptors via TAK1^{181,182}. JNK signalling is required for the activation of the caspase apoptosis cascade by the mitochondrial pathway¹⁸¹.

Hepatitis A virus cellular receptor 2 (HAVcr2), also called T-cell immunoglobulin mucin receptor 3 (TIM-3), is reported to normally only be expressed by immune cells but is also found in some cancers¹⁸³. This may explain its presence in HEK293 cells, which are immortalised but not directly cancer-derived. The primary function of HAVcr2 is negative regulation of T-cell function, though it may have some pro-inflammatory function as well, in particular stimulating innate immune cells^{183–185}. As the name suggests, HAVcr2 is implicated in hepatitis A infection, though it is also involved in hepatitis C and HIV^{186,187}. These all cause chronic viral infections and suppress the immune system; however, these infections are associated with over-expressed HAVcr2 while it was under-represented in cells transfected with Bq-YopJ^{186,187}.

A disintegrin and metalloproteinase domain-containing protein 9 (ADAM9) is an outer membrane protein that is involved in a wide array of processes, including inflammation and immunity, by catalysing the shedding of cell surface receptors^{188,189}. Its direct link to the response to LPS is its involvement in the NF- κ B-dependent shedding of Angiotensin-I converting enzyme in response to LPS stimulation of endothelial cells¹⁹⁰. This is not the only role that ADAM9 plays in immunity, as it is responsible for the shedding of members of the TNF- α receptor family and the IL-11 receptor¹⁸⁸. However, NF- κ B activation in pancreatic cancer cells leads to increased expression of ADAM9, so the under-representation of ADAM9 could possibly be a downstream effect of basal NF- κ B suppression rather than a targeted function of Bq-YopJ¹⁹¹.

TABLE 4.2: **DAVID gene ontology results** (A) shows the statistically significant ontologies for the 31 proteins that were over-represented in cells transfected with Bq-*yopJ*. (B) shows the statistically significant ontologies for the 26 proteins that were under-represented in cells transfected with Bq-*yopJ*. The number of genes for each ontology is non-exclusive, so the same gene can appear in multiple categories.

(A) Over-represented in cells transfected with Bq-*yopJ*

Ontology	P-value	Number of genes
UNIPROT KEYWORDS		
Nucleus	0.0033	16
CELLULAR COMPARTMENT		
nucleus	0.019	15
mitochondrion	0.05	6
BIOLOGICAL PROCESS		
inner ear receptor stereocilium organization	0.031	2
cochlea development	0.039	2

(B) Under-represented in cells transfected with Bq-*yopJ*

Ontology	P-value	Number of genes
UNIPROT KEYWORDS		
Alternative splicing	0.03	19
Phosphoprotein	0.035	16
UNIPROT SEQUENCE FEATURES		
splice variant	0.04	15
MOLECULAR FUNCTION		
kinase activity	0.039	3
protein binding	0.04	17
BIOLOGICAL PROCESS		
cellular response to lipopolysaccharide	0.01	3
positive regulation of gene expression	0.049	3

4.2.5 Functional Association Analysis of Differentially Represented Proteins

I performed functional association analysis using the online tool STRING¹⁴⁷ on both my over- and under-represented protein lists. This analysis identified proteins that are known, or predicted, to "contribute jointly to a specific biological function"¹⁴⁷. Figure 4.9 shows the results of this analysis. Unlike gene ontology searches, which identify the processes in which each protein plays a role, functional annotation analysis identifies interactions between the input proteins. This produces a network of proteins with joint function, and can identify specific proteins of interest from a list. These proteins of interest can then be further investigated.

FIGURE 4.9: Function networks of differentially represented proteins.

Proteins with no interaction are not shown. The thickness of the line between nodes represents the confidence of the interaction. (A) Clusters from the list of over-represented proteins. Highlighted pink is a cluster of mitochondrial and ribosomal proteins. Highlighted orange is a cluster based around a ubiquitin/ribosomal protein which includes important regulatory proteins, involved in the same pathways as the orange cluster in B.

(B) Clusters from the list of under-represented proteins. Highlighted orange is a cluster of important regulatory proteins, involved in the same pathways as the orange cluster in A. Highlighted grey are two connections of low confidence, unlikely to be biologically relevant.

There were two clear functional networks in the over-represented proteins, one of mitochondrial and ribosomal proteins, the other based around a ubiquitin/ribosomal protein which includes the important regulatory proteins GSK3 β (GSK3B) and Cyclin-2A. There were three clusters among the under-represented proteins, one of which included the regulatory proteins MAPK8 and RPTOR. The remaining two were of low confidence and contained only two proteins.

Glycogen synthase kinase-3 β (GSK-3 β) was over-represented in cells transfected with Bq-*yopJ*. It is a serine/threonine kinase that is involved in many signalling pathways, notably Wnt and Hedgehog, though the β isoform in particular is associated with NF- κ B¹⁹². The relationship between GSK-3 β and NF- κ B is generally positive, as GSK-3 β knockout mice are phenotypically very similar to p65 knockout mice, and inhibition of GSK-3 β has been shown to reduce nuclear localisation of p65^{192,193}. However, GSK-3 β also inactivates p65 via phosphorylation of serine 468¹⁷³.

Cyclin-A2 was over-represented in cells transfected with Bq-*yopJ*. It is an important regulator of the cell cycle though it may also play roles in immune regulation and apoptosis^{194,195}.

MAPK8 (JNK1), was also identified by the DAVID gene ontology search as having involvement in the "cellular response to lipopolysaccharide", and was described in Section 4.2.4.

Regulatory-associated protein of mTOR (RAPTOR, or RPTOR) is a component of the mammalian target of rapamycin complex 1 (mTORC1) and was under-represented in cells transfected with Bq-*yopJ*. It is a major regulator of protein synthesis and cellular metabolism.

4.3 Discussion

To learn more about the effect of the *B. quintana* YopJ homologue on host cells, I carried out a proteomic analysis of transfected host cells using LC/MS. I compared the abundance of host proteins in lysates of cells transfected with Bq-*yopJ* to lysates of cells transfected with Bq-*yopJ* Mutant. I assigned all proteins that were at least two fold ($\log_2$ ratio ≥ 1) over- or under-represented and had a P-value of > 0.05 as being significantly differentially represented. This assigned 31 proteins as over-represented in cells transfected with Bq-*yopJ* and 26 proteins under-represented in cells transfected with Bq-*yopJ*.

There were no clear patterns among these proteins so I performed deeper analysis of the subcellular localisation and functional categorisation of each protein. I then performed gene ontology searches and functional association analyses to identify patterns within these protein lists and to identify specific proteins of interest.

This analysis identified a number of immunity-associated mechanisms as potentially being impacted by Bq-YopJ, including apoptosis, the cell cycle, and the mTOR pathway.

4.3.1 Apoptosis

Apoptosis is a common target of modulation by bacterial pathogens, including many YopJ homologues¹⁹⁶. Some bacterial effector proteins inhibit apoptosis in host cells that they have invaded, a process that would kill the pathogens¹⁹⁶. Other extracellular pathogens, such as *Yersinia*, secrete an effector, YopJ, that induces apoptosis in host immune cells, dampening the immune response^{127,196}.

MAPK8, also called JNK1, was under-represented in cells transfected with Bq-*yopJ*, and is a key component of the JNK signalling pathway. JNK1 was identified by the STRING analysis as a protein of interest and was one of three proteins reported by the DAVID search to be involved in the "cellular response to lipopolysaccharide".

The JNK signalling pathway is one of three MAPK pathways in mammals, and is associated with the response to stress¹⁸¹. It can be activated by many of the same mechanisms as the NF- κ B pathway, including cytokine receptors via TAK1^{181,182}. JNK signalling is required for the activation of the caspase apoptosis cascade by the mitochondrial pathway, while NF- κ B is generally anti-apoptotic and inhibits this activity^{148,181,182}. Inhibition of apoptosis is a

known function of the *B. henselae* effector protein BepA, and the BID domain of *B. quintana* BepA2¹⁹⁷. Bacterial pathogens often have redundancy in the functions of their effector proteins, so if *B. quintana* BepA2 inhibits apoptosis, Bq-YopJ may have a similar effect; however, *Yersinia* YopJ induces apoptosis while also reducing the abundance of JNK1^{127,198}. The role of JNK signalling encompasses more than just apoptosis, however, it also has significant cross-talk with the NF- κ B pathway, and is required for T-cell function^{182,199}.

In addition to its role in NF- κ B signalling, GSK-3 β is also associated with JNK signalling. Both can signal to the same apoptosis pathways, and inhibition of GSK-3 triggers JNK-mediated apoptosis in pancreatic cancer cells^{200,201}. Taken together, there seem to be both pro- and anti- apoptotic changes in the proteome of cells expressing Bq-YopJ. Whether these effects are part of the function of Bq-YopJ or are the result of the host cells responding to it, is unknown.

Cyclin-A2 was over-represented in cells transfected with Bq-*yopJ*, and activates cyclin-dependant kinase 2 (CDK2). Inhibition of CDK2 has been shown to induce apoptosis in T-cells, so Cyclin-A2 may have anti-apoptotic effects^{194,202}. If Bq-YopJ activity stabilises Cyclin-A2, it may inhibit apoptosis. Additionally, the gene ontology searches identified that mitochondrial proteins were over-represented in cells transfected with Bq-*yopJ*, and the STRING analysis identified a cluster of mitochondrial proteins. Mitochondria, in addition to their primary function of cellular respiration, are vital in many stress response and apoptosis pathways. Given the complexity of these pathways and the contradictory effects on the proteome of Bq-YopJ, JNK signalling and apoptosis are interesting avenues for future research.

There are commercially available kits that can test many of these pathways. JNK signalling can be measured by immunoblotting for c-Jun phosphorylation of serine 63. Mitochondrial function can be examined using MitoSOX Red Mitochondrial Superoxide Indicator (ThermoFisher Scientific), a dye that produces a fluorescent signal in response to superoxide, a by-product of oxidative phosphorylation. Apoptosis itself can be detected using luciferase based tests for caspase cleavage.

4.3.2 The Cell Cycle

The cell cycle is a known target of bacterial effectors; the type-III-secreted effector cycle inhibiting factor (Cif) of enteropathogenic *E. coli* blocks the

progression of the cell cycle in host cells²⁰³. Because Cyclin-A2 was over-represented in cells transfected with Bq-*yopJ*, it is possible that Bq-YopJ prevents its degradation, which would cause the cell cycle to stall²⁰⁴. Interestingly, Cyclin-A2 is also a regulator of GSK3 β with Cyclin-A2 inhibiting an inhibitor of GSK3 β ²⁰⁴. The over-representation of GSK3 β could be at least in part due to the over-representation of Cyclin-A2.

An important immunity-related function of the cell cycle is the expansion of adaptive immune cells following antigen presentation. Interestingly, dogs experimentally infected with *B. vinsonii*, another *Bartonella* species with a YopJ homologue, had decreased amounts of CD8+ lymphocytes when compared to uninfected dogs²⁰⁵. The YopJ homologues of *B. quintana* and *B. vinsonii* share 51.38 % identity (according to a BLAST search), and while their precise functions remain unknown, both would similarly benefit from the inhibition of the cell cycle.

4.3.3 The mTOR Pathway

mTORC1 is a major regulator of protein synthesis and cellular metabolism and is another known target of bacterial effector proteins. *Legionella pneumophila* possesses a pair of effector families, Lgt and SidE, which positively and negatively regulate mTORC1 respectively²⁰⁶. Modulating mTORC1 allows bacterial pathogens to manipulate the metabolism of host cells to their advantage, granting the bacteria access to free amino acids for consumption²⁰⁶. Interestingly, GSK-3 inhibitors have been found to counteract the effects of mTORC1/2 inhibitors on the growth of cancers, so GSK-3 function may support mTORC1/2 inhibition²⁰⁷.

4.3.4 Validation Experiments

The results of this proteomic screen need to be validated by other methods. The first step in validating these results would be to perform Western blots on lysates from cells transfected with Bq-*yopJ* (or the Bq-*yopJ* Mutant control) for each protein, and comparing the band intensity between conditions. This should be performed for the proteins of interest identified by the STRING analysis, GSK-3 β , JNK1 (MAPK8), Cyclin-A2, and RAPTOR, and also possibly HAVcr2 and ADAM9.

Depending on the results of the validation experiments further experiment into the effects of Bq-YopJ on the associated signalling pathways should be

performed. These could include assays for the function of each of the proteins of interest, for example, JNK1 activity can be measured by immunoblotting for that target of its phosphorylation, c-Jun. Phosphorylation of p65 by GSK-3 β can be assayed by immunoblotting for phosphorylated p65 (serine 468). Additionally, GSK-3 β is activated by phosphorylation of tyrosine 216, so Western blotting for this phosphorylated residue can give an indication of its activation. The function of metalloproteases, including ADAM9, can be measured using gelatin zymography, in which non-denatured lysates are separated by SDS-PAGE in a gelatin-containing gel. After electrophoresis, the gel is incubated in a digestion buffer then stained with Coomassie blue. Active proteases will digest the gelatin and produce light bands on the otherwise dark gel²⁰⁸.

The proteomic screen presented in this chapter only examined the abundances of proteins in baseline, unstimulated cells. This limited the ability to observe effects on proteins that are degraded or expressed in response to a stimulus, such as the components of the NF- κ B pathway. A good future experiment would examine the effects of Bq-YopJ on the proteome after the activation of the NF- κ B pathway. This could be achieved by stimulating the cells with TNF- α before lysis. Additionally, the proteome of cells transfected with Bq-*yopJ* (or the Mutant control) could be examined at various time-points after TNF- α stimulation to capture short- and long-term differences, for example, 5 minutes, 30 minutes, and one hour.

4.4 Summary

The results of this proteomics screen have revealed additional cellular processes that may be affected by Bq-YopJ in addition to NF- κ B, namely JNK signalling, apoptosis, the cell cycle, and mTOR. The proteins GSK-3 β , JNK1 (MAPK8), Cyclin-A2, and RAPTOR stand out as particularly promising candidates for future work. It is worth noting however, that the overall differences between the two conditions were quite small, which indicates that the effects of Bq-YopJ are subtle in unstimulated cells, and do not cause significant disruption to unrelated processes.

Chapter 5

Identifying a Potential Host Target of *Bartonella quintana* YopJ by Yeast Two-Hybrid Screen

5.1 Introduction

5.1.1 Protein-Protein Interactions

Effector proteins that alter host cell signalling or other processes must physically interact with host proteins, regardless of their specific enzymatic activity. Identifying the host protein or proteins that an effector interacts with can provide valuable information about its mechanism of action, and is vital to fully characterise its function. When effectors have an enzymatic function, interacting proteins may include the targets of their enzymatic activity. All other YopJ homologues are acetyltransferases, so it is very likely that this enzymatic activity is conserved in Bq-YopJ, but YopJ homologues acetylate diverse cellular host protein targets¹¹¹. One method that can identify novel protein-protein interactions is the yeast two-hybrid screen, which has been used to characterise bacterial effectors. Specifically, yeast two-hybrid screens have been used to identify interaction partners, including acetylated host cell proteins, of other YopJ homologues²⁰⁹.

5.1.2 The Two-Hybrid Principle

The yeast two-hybrid system is a well-established technique that can identify protein-protein interactions between a known protein (also known as the bait) and a usually unknown target protein (prey). It can be used to test for an interaction between two proteins that are hypothesised to interact, or it can be used as a screen to identify novel interactions by using a genetic library as

the prey. For this project, Bq-YopJ was used as the bait to identify interactions with a universal human cDNA library.

The bait and prey are expressed on separate expression plasmids. Each plasmid is maintained in the yeast host strain with selection cassettes, such as *TRP1* and *LEU2*, which enable tryptophan and leucine biosynthesis, respectively. The plasmids are engineered so that the bait protein is translationally fused with the yeast GAL-4 DNA binding domain (GAL4BD). The library of prey genes are cloned such that they form translational fusions with the GAL-4 activation domain (GAL4AD). Each plasmid is transformed into different yeast strains, one of mating type *a* and the other of mating type α .

When the two strains are combined and mate, the plasmids, and thus the expressed bait and prey fusion proteins, are brought together in the resulting diploid cells. If the bait and prey proteins interact, the GAL-4 DNA binding domain and the GAL-4 activation domain are brought together, resulting in transcriptional activation of reporter genes. The Takara Matchmaker Gold Yeast Two Hybrid system was used in this project, which utilises four reporter genes for the screen. These included *AUR1-C*, which encodes a protein that confers resistance to the antifungal agent aureobasidin A; *ADE2*, which enables adenine biosynthesis; *HIS3*, which allows for histidine biosynthesis; and *MEL1*, which processes X- α -Gal into a blue product. The four genes are under the control of three independent Gal-4-responsive promoter elements. The protein-binding sites in the promoters are distinct but all related to the consensus sequence recognised by Gal-4. Importantly, the interaction between the bait and prey does not need to be long lasting, as even a transient enzyme-substrate interaction is sufficient to activate transcription of the reporter genes.

Expression of these reporter genes allows the diploid yeast cells to grow on selective media, and will turn the colonies blue. If there is no interaction between the bait and prey, the GAL-4 activation and DNA-binding domains remain apart, transcription of the reporter genes will not be activated, and the yeast will die on selective media.

Figure 5.1 shows the yeast two-hybrid principle in schematic form.

FIGURE 5.1: **Schematic of the Yeast Two-Hybrid Principle.**

(A) If a prey protein (violet) interacts with the bait (green), the GAL-4 activation and DNA binding domains are brought together and expression of the reporter genes will be activated, resulting in a diploid cell that is blue and able to survive on selective media.

(B) If the prey protein does not interact with the bait (red), the reporter genes will not be expressed, resulting in a diploid cell that is not viable on the selective media.

5.1.3 Selective Media and Reporter Genes

The yeast two-hybrid screen and related control experiments involve many selection cassettes, reporter genes, and media types. These are summarised in Table 5.1 below.

The bait plasmid, pGBKT7, encodes the *TRP1* gene as a selection cassette. Enabling the yeast to produce tryptophan, and therefore grow on tryptophan deficient ($-$ Trp) media. The prey plasmid, pGADT7, encodes the *LEU2* gene as a selection cassette, enabling the yeast to produce leucine, and therefore grow on leucine deficient ($-$ Leu) media.

The four reporter genes are encoded on the chromosome of the mating type **a** Y2HGold yeast strain. The first two reporter genes, *AUR1-C* and *MEL1*, are under the control of the same Gal4-responsive promoter sequence. *AUR1-C* encodes the enzyme inositol phosphoryl ceramide synthase, which grants resistance to the antifungal agent aureobasidin A, enabling the yeast to grow on media that contains aureobasidin A ($/A$). *MEL1* encodes a-galactosidase, an enzyme that processes the substrate X- α -Gal into a blue product, turning the colonies blue when they are grown on media that contains X- α -Gal ($/X$).

The other two reporter genes, *HIS3* and *ADE2*, are each under the control of unique Gal4-responsive promoter sequences. *HIS3* and *ADE2* enable the yeast to produce histidine and adenine respectively, and therefore grow on histidine deficient (-His) or adenine deficient (-Ade) media. The screen is initially performed on media with selection by a single reporter gene (and screening by another), then additionally screened on media which selects for three reporter genes. This reduces the rates of false negative results in the initial screen, while maintaining the higher stringency of the second selection.

TABLE 5.1: Selective media and reporter/selective genes that are used in the yeast two-hybrid screen

Media	Composition	Supplements	Selection <i>Gene</i> (selects or screens for)
SD/-Trp	Lacks tryptophan	–	<i>TRP1</i> (pGBKT7)
SD/-Leu	Lacks leucine	–	<i>LEU2</i> (pGADT7)
SD/-Trp/X	Lacks tryptophan	X- α -Gal	<i>TRP1</i> (pGBKT7)
SD/-Trp/-Leu	Lacks tryptophan and leucine	–	<i>TRP1</i> (pGBKT7), <i>LEU2</i> (pGADT7). Found in diploid cells.
SD/-Trp/- Leu/X	Lacks tryptophan and leucine	X- α -Gal	<i>TRP1</i> (pGBKT7), <i>LEU2</i> (pGADT7), <i>MEL1</i> (active GAL4)
SD/-Trp/- Leu/X/A	Lacks tryptophan and leucine	Aureobasidin A, X- α -Gal	<i>TRP1</i> (pGBKT7), <i>LEU2</i> (pGADT7), <i>AUR1-C</i> (active GAL4), <i>MEL1</i> (active GAL4)
SD/-Trp/-Leu/- Ade/-His/X/A	Lacks tryptophan, leucine, adenine, histidine	Aureobasidin A, X- α -Gal	<i>TRP1</i> (pGBKT7), <i>LEU2</i> (pGADT7), <i>AUR1-C</i> (active GAL4), <i>HIS3</i> (active GAL4), <i>ADE2</i> (active GAL4), <i>MEL1</i> (active GAL4)

5.1.4 Rationale

Identifying the cellular target of the *B. quintana* YopJ homologue would reveal a lot about its mechanism of action, and potentially give clues to novel effects on pathways other than NF- κ B. I decided to perform a yeast two-hybrid screen to identify candidate human proteins that interact with Bq-YopJ. A commercially-available normalised universal human cDNA-GAL4AD fusion prey library (Takara Bio) was used to screen a wide array of human proteins. The library was encoded on the pGADT7 plasmid and expressed in the mating type α yeast strain Y187. The Bq-*yopJ*-GAL4BD plasmid was constructed in pGBKT7 and expressed in the mating type a yeast strain Y2HGold. The screen was performed using the Matchmaker® Gold Yeast Two-Hybrid System kit from Takara Bio. The specific goal of this part of the project was to identify candidate host proteins that interact with Bq-YopJ, and are potential targets of its enzymatic activity.

5.2 Results

5.2.1 Bait Plasmid Cloning

I produced a Bq-*yopJ*-encoding bait plasmid in pGBKT7, which was provided with the Matchmaker® Gold Yeast Two-Hybrid System kit and already encodes the GAL4 binding domain. I used high fidelity PCR to amplify the Bq-*yopJ* gene from *B. quintana* strain JK-31 genomic DNA, which was kindly provided by Dr. Jane Koehler. The PCR primers introduced an NdeI restriction site on one end of the insert and a BamHI site on the other. Both the amplified insert and plasmid backbone were digested with both NdeI and BamHI, then combined, and ligated. Both the insert and plasmid were quantified prior to ligation to ensure a 5:1 ratio of plasmid:insert.

After ligation, the plasmids were transformed into DH5α *E. coli* and plated on selective plates. Throughout this process, I included a plasmid-only control and a ligase-free control. These controls confirmed that the initial restriction digestion was complete and that empty plasmid were not re-ligating.

The resultant colonies were inoculated into liquid cultures, and incubated overnight at 37 °C with shaking. A frozen stock of each culture was prepared, then the plasmids were extracted from the remaining culture, and digested with NdeI and BamHI. These digested plasmid were screened for the presence of an insert by agarose gel electrophoresis. I did not use colony PCR for screening because I found it to have a high rate of false positives. Figure 5.2 shows the agarose gel with successfully inserted Bq-*yopJ* genes.

FIGURE 5.2: Screening of prey plasmid clones.

L = Hyperladder 1kb (Bioline). 1 – 12 are clones selected from a transformation plate. Yellow boxes indicate the clones that were selected for sequencing. The red arrow indicates that clone 9 had a point mutation that was revealed by sequencing. The blue arrow indicates that clone 12, which lacked point mutations was selected for use in the yeast two-hybrid screen.

The plasmid clones that were found to contain an insert of the correct size were sequenced to confirm the successful amplification and insertion of the gene. One clone, number 9, was found to contain a point mutation so was discarded. The remaining sequenced clones all encoded identical Bq-yopJ sequences so one, number 12, was arbitrarily selected for transformation into yeast. The sequence of this insert aligned with the *B. quintana* JK-31 Bq-yopJ gene appears in Appendix Figure A.5.

5.2.2 Control Experiments

Before performing the yeast two-hybrid screen it was important to confirm that Bq-YopJ was compatible with the system. This meant ensuring that it did not, by itself, activate expression of the reporter genes, and that it was not toxic when expressed in yeast cells. Either of these findings would indicate that the protein is not a suitable bait.

Autoactivation Test

For the autoactivation test, haploid Y2HGold yeast strains with prey plasmids (pGBKT7) expressing either Bq-YopJ-GAL4BD or murine p53-GAL4BD fusions were used. The plasmid with murine p53 is provided with the Matchmaker Gold yeast two-hybrid kit as a negative control, as it does not activate expression of the reporter genes. I plated both strains on three types of agar. These included SD/-Trp agar, which selected only for the presence of the pGBKT7 plasmid; SD/-Trp/X agar, which selected for pGBKT7 but also contains X- α -Gal, which turns blue with reporter gene activation; and SD/-Trp/X/A agar, which selected for reporter gene activation with aureobasidin A and contained X- α -Gal. A bait protein that does not autoactivate the reporter genes should produce white colonies on both the SD/-Trp agar and SD/-Trp/X agar but should not grow on the SD/-Trp/X/A agar, because it lacks resistance to aureobasidin A. A bait protein that autoactivates the reporter genes would produce white colonies on the SD/-Trp agar/ and blue colonies on the SD/-Trp/X and SD/-Trp/X/A agar plates, because it will have resistance to aureobasidin A and will produce MEL1, an enzyme that processes X- α -Gal into a blue product. The yeast expressing Bq-YopJ-GAL4BD showed the same phenotype as the murine p53 negative control, indicating that Bq-YopJ-GAL4 alone does not activate the reporter genes.

Figure 5.3 summarises the results of the autoactivation test.

FIGURE 5.3: Autoactivation Test of Bait Plasmid.

Each row shows two serial dilutions (1/10 and 1/100) of Y2HGold yeast containing pGBKT7 with (Left) Bq-YopJ or (Right) murine p53 (control), grown in SD/-Trp broth and plated on varying media. The top row (A & B) shows yeast grown on SD/-Trp, showing that the yeast are viable with both plasmids. The middle row (C & D) shows yeast grown on SD/-Trp supplemented with X- α -Gal, which turns blue when MEL1, encoded by a reporter gene, is expressed. The white colour of the colonies indicates no detectable reporter gene activity. The bottom row (E & F) shows yeast grown on SD/-Trp supplemented with X- α -Gal and aureobasidin A. The lack of growth indicates minimal reporter gene activity.

Toxicity Test

For the toxicity test, I grew haploid Y2HGold yeast carrying the Bq-*yopJ*-GAL4BD pGBKT7 bait plasmid on media that selected only for the plasmid. I compared the size of the colonies with those of the haploid Y2HGold yeast strain carrying pGBKT7 without any insert, so not expressing any bait protein. I plated both strains onto SD/-Trp plates to select for plasmid maintenance, then compared colony size by visual inspection. Both strains grew similarly sized colonies, suggesting that Bq-YopJ was not significantly toxic to yeast. Figure 5.4 shows photographs of these plates.

FIGURE 5.4: Toxicity Test of Bait Plasmid.

SD/-Trp plates with Y2HGold haploid yeast strain containing the bait plasmid pGBKT7 with (A) Bq-YopJ-GAL4BD or (B) no insert. Both plates are a 1/50 dilution of an overnight SD/-Trp broth culture. Both images show the same total surface area on their respective plates. Red circles indicate isolated colonies of similar size.

Colonies on both plates are of a similar size, indicating that Bq-YopJ-GAL4BD is not toxic to yeast.

5.2.3 Screening

Once the control experiments had been completed, I proceeded to carrying out the screen. For this yeast two-hybrid screen, two types of selection plates were used. The SD/-Trp/-Leu/X/A plates enable the identification of diploid cells that have both plasmids and express *AUR1-C* and *MEL1*, which results in blue colonies. This lower-stringency selection media was used in the first round of the screen. Higher-stringency selection plates additionally lack adenine and histidine, and enable for a selection based on the expression of three reporter genes (*AUR1-C*, *HIS3*, and *ADE2*) and a colour-based screen of reporter gene *MEL1*. For this assay, the first round of screening was done on the lower-stringency plates, and any colonies obtained were subsequently plated onto the higher-stringency plates.

The Bq-YopJ bait yeast strain was mated with a haploid strain carrying the universal human cDNA prey library in 45 mL of 2× YPDA broth. The mated culture was then centrifuges at $1000 \times g$ and the cells resuspended in 10 mL of 0.5× YPDA broth. The mated culture of diploid cells (11.8 mL) was then plated onto fifty-nine 150 mm SD/-Trp/-Leu/X/A selective plates (200 µL per plate). These plates select for both plasmids, pGBKT7 and pGADT7, through tryptophan and leucine deficiency respectively, and for resistance to aureobasidin A; they also screen for processing of X-α-Gel processing to a blue product. This means that only diploid yeast (containing both plasmids), that also activate the *AUR1-C* reporter gene will grow; further, they will turn blue if the *MEL1* reporter gene is activated. After incubating the plates, clones that contain a prey protein that interacts with Bq-YopJ-GAL4BD may be identified as blue colonies that grow on the selective media. It is possible for weakly interacting proteins to form small white colonies as well. Figure 5.5 summarises the workflow for the yeast two-hybrid screen, and Figure 5.6 shows the colonies that grew on my screening plates.

FIGURE 5.5: **Schematic of the Yeast Two-Hybrid Screen.**

The bait strain, which expresses the prey protein (Bq-YopJ-GAL4BD, shown in white) is mated with the prey library. After mating, a small aliquot of mated culture is plated in a serial dilution on SD/-Trp/-Leu plates. This selects for cells that contain both the bait and prey plasmids (i.e., diploid yeast cells), and allows for calculation of the library titre. The remaining mated culture is plated on SD/-Trp/-Leu/X/A selective plates, which select for diploid cells and the activation of the *AUR1-C* reporter gene, and screen for the activation of the *MEL1* reporter gene. After the plates have been incubated and colonies have formed, blue colonies on the SD/-Trp/-Leu/X/A selective plates represent clones of cells that contain a bait protein that facilitated activation of the reporter genes by interacting with the bait.

There were three blue colonies and one white colony on my screening plates. Two of the blue colonies, henceforth called Positive Clone 1 and Positive Clone 2, came from the same plate. The third blue colony (Positive Clone 3) and the white colony (Borderline Clone) were the only colonies on their respective plates. The remaining 56 screening plates had no colonies or any detectable growth. There was also a colony on one of the titre plates (see Section 5.2.4 for more information) that showed resistance to adenine deficiency, indicating possible expression of one of the reporter genes, so I labelled this colony as the DDO Extra Clone (Appendix Figure A.4). Adenine deficiency causes colonies of yeast strains that are unable to synthesise adenine, to turn pink, this does not significantly impact the viability of the cells. The media provided with the matchmaker kit may have been old, so the adenine in the media may have degraded.

FIGURE 5.6: Yeast Two-Hybrid selective plates with colonies. The red arrows on plates 1 and 2 (A & B) show Positive Clones 1, 2, and 3. The yellow arrow on plate 3 (C) shows a Borderline Clone, that grew on the selective media but did not turn blue.

5.2.4 Number of Clones Screened

It is important when screening a protein prey library for interactions that all of the proteins in the library have the opportunity to interact with the bait. The kit recommends that screening at least one million diploid clones will give adequate coverage to be confident that every protein in the library was tested at least once²¹⁰. To confirm that I had screened enough diploid clones, I performed a library titre to calculate the number of clones that I had screened. After I mated the Bq-YopJ-GAL4BD containing Y2HGold bait strain and cDNA Library-GAL4AD containing Y187 prey strain, immediately before plating the mated culture onto the screening plates, I serially diluted the mated culture, plating 100 µL of each dilution onto plate. These were plated onto SD/-Trp/-Leu plates, which select for both pGBKT7 and pGADT7 plasmids but none of the reporter genes. As a result, any of the diploid cells that contain both plasmids, i.e diploid yeast, can grow on it.

I counted the colony forming units (CFU) and calculated the total number of mated clones screened. The formula below explains the calculation.

$$\frac{\text{CFU on plate}}{100 \mu\text{L plated}} \times \text{dilution factor} \times 10 \left(\text{to change units to } \frac{\text{CFU}}{\text{mL plated}} \right) \times \text{mated culture volume} = \text{number of clones screened}$$

There were 85 colonies on the 1/100 dilution plate, and the total volume of mated culture plated onto screening plates was 11.8 mL. The calculation below shows the number of clones screened. Appendix Figure A.4 shows a photograph of this plate.

$$\frac{85 \text{ CFU on plate}}{100 \mu\text{L plated}} \times 100 \text{ (dilution factor)} \times 10 \left(\text{to change units to } \frac{\text{CFU}}{\text{mL plated}} \right) \times 11.8 \text{ mL mated culture volume} = 1,003,000 \text{ clones screened}$$

I screened just over one million clones and therefore there is a high probability that every protein in the library was screened.

5.2.5 High Stringency Testing

To be sure that the identified Clones represented true interactions, I next tested them on high stringency SD/-Trp/-Leu/-Ade/-His/X/A agar, which selects for both plasmids through tryptophan and leucine deficiency, and for the activation of three reporter genes: *ADE2* and *HIS3* through adenine and histidine deficiency, and *AUR1-C* through aureobasidin A resistance. It also screens for activation of the *MEL1* reporter gene through X- α -Gal processing to a blue product. I patched the five Clones onto a single SD/-Trp/-Leu/-Ade/-His/X/A plate and observed their growth. A photograph of this plate appears in Figure 5.7.

FIGURE 5.7: **Positive Clone Patches on High Stringency Agar.** SD/-Trp/-Leu/-Ade/-His/X/A with patches of the clones from the screening plates, Positive Clones 1 – 3 are labelled Hits 1 – 3, the Borderline Clone is labelled "Borderline", and the DDO Extra Clone from the titre plates that was resistant to adenine deficiency is labelled "DDO X". Only Positive Clones 1, 2, and 3 grew and produced a blue product, so these were analysed further.

The Borderline Clone and the DDO Extra Clone did not grow on the high stringency SD/-Trp/-Leu/-Ade/-His/X/A agar, but Positive Clones 1, 2, and 3 did, and were blue. I abandoned the Borderline Clone and the DDO Extra Clone, and continued forward with only Positive Clones 1 – 3 which had robustly activated all four reporter genes.

5.2.6 Sequencing the Positive Clones

I extracted the plasmids from Positive Clones 1 – 3 and transformed them into *E. coli* to amplify the plasmid. I grew the transformed *E. coli* with only ampicillin selection, because the prey plasmid pGADT7 contains an ampicillin resistance cassette. The bait plasmid, pGBKT7, contained only a kanamycin cassette so was readily lost in the absence of selective pressure. This allowed me to separate the prey plasmid from the bait plasmid for sequencing with the T7 Sequencing Primer and 3' AD Sequencing Primer (see Table 2.5). After sequencing I performed BLAST searches to identify the proteins that had interacted with Bq-YopJ. The results of these BLAST searches appear in Appendix Figures A.6 – A.11.

All three Positive Clones aligned with a single gene, known as defective in cullin neddylation 1-domain containing 1 (*DCUN1D1*), which encodes the protein defective in cullin neddylation protein 1-like protein 1 (DCNL1). Strangely, the reverse sequencing primer for Positive Clones 1 and 2 aligned with a region of the gene annotated as an intron. Since this was from a cDNA library, there may be alternative splicing or the annotations are incorrect. Given the close proximity of Positive Clones 1 and 2 on the screening plate, and the similarity in their sequence, it is likely that they are descended from two daughter cells of a single diploid clone. Despite this relationship, they are still considered to be independent positive hits, as the reporter activation occurred independently in each. Appendix Figure A.12 shows an alignment of each sequencing read for all three Positive Clones with the *DCUN1D1* gene. Three independent clones encoding DCNL1 is very strong evidence that there is a biologically relevant interaction with Bq-YopJ. However, it was important to next ensure that DCNL1 alone is not sufficient to activate the reporter genes.

5.2.7 Confirmation of Positive Interaction

To confirm that the interaction between Bq-YopJ and DCNL1 was robust, and that DCNL1 alone is not sufficient to activate the reporter genes, I co-transformed a *DCUN1D1*-containing prey plasmid with either a Bq-*yopJ*-containing bait plasmid, or a bait plasmid with no insert, into haploid Y2HGold yeast. I then made serial dilutions of the transformants and plated them on SD/-Trp/-Leu/X and SD/-Trp/-Leu/-Ade/-His/X/A plates. SD/-Trp/-Leu/X media selects for cells with both plasmids through tryptophan and leucine deficiency, and screens for the activation of the *MEL1* reporter gene. High stringency SD/-Trp/-Leu/-Ade/-His/X/A media selects for cells with both plasmids through tryptophan and leucine deficiency, the activation of the *HIS3*, *ADE2*, and *AUR1-C* reporter genes, and screens for the activation of the *MEL1* reporter gene. The bait plasmid without an insert produces no protein for DCNL1 to interact with, so activation of the reporter genes would indicate that DCNL1 alone activates the GAL4 promoter, which would suggest that it does not interact with Bq-YopJ. Figure 5.8 shows photographs of these plates.

(A) *DCUN1D1* and Empty Plasmid on SD/-Trp/-Leu/X Agar (B) *DCUN1D1* and Empty Plasmid on SD/-Trp/-Leu/-Ade/-His/X/A Agar (C) *DCUN1D1* and Bq-*yopJ* Plasmids on SD/-Trp/-Leu/-Ade/-His/X/A Agar

FIGURE 5.8: Confirmation of Positive Interaction with DCNL1

The white colonies on the SD/-Trp/-Leu/X plate (A) and the lack of growth on the SD/-Trp/-Leu/-Ade/-His/X/A plate (B) indicates that DCNL1 alone is not sufficient to activate expression of the reporter genes, while the blue colony on the SD/-Trp/-Leu/-Ade/-His/X/A plate in (C) confirms that the Bq-*yopJ* and *DCUN1D1* plasmids together do activate the reporter genes. The SD/-Trp/-Leu/X plate with the Bq-*yopJ* + *DCUN1D1* strain was identical to the SD/-Trp/-Leu/-Ade/-His/X/A plate, so I have not shown it.

The strain with DCNL1 and Bq-YopJ formed a blue colony on the SD/-Trp/-Leu/-Ade/-His/X/A plate, again confirming that the combination of Bq-YopJ and DCNL1 was sufficient to activate the reporter genes. The strain with DCNL1 and an empty bait vector produced white colonies on the SD/-Trp/-Leu/X media, indicating that it contained both plasmids (required to grow on the media) but did not activate the reporter gene (*MEL1*) required to turn the colonies blue. The empty bait vector strain did not grow on the SD/-Trp/-Leu/-Ade/-His/X/A plates at all, suggesting that it did not activate the three reporter genes required for survival, which confer adenine biosynthesis, histidine biosynthesis, and aureobasidin A resistance.

This result supports the idea that the interaction between Bq-YopJ and DCNL1 is real, at least in this model system, because DCNL1 alone does not activate the reporter genes.

5.3 Discussion

The yeast two-hybrid screen produced three independent clones of DCNL1 with two unique cDNA sequences, all of which passed high stringency screening. Additionally, the results of the control experiments showed no autoactivation of the reporter genes by either Bq-YopJ or DCNL1. Together this is very strong evidence that the interaction between Bq-YopJ and DCNL1 is real, and is the strongest result that is possible from a yeast two-hybrid screen. The interaction between Bq-YopJ and DCNL1 still needs to be independently validated, however.

Protein interaction in yeast cells is good evidence of interaction in mammalian cells, as both are eukaryotic, but additional verification is always needed to confirm the interaction. The yeast two-hybrid screen is not as robust as more specific assays, such as co-immunoprecipitation assays or bimolecular fluorescence complementation (BiFC) in mammalian cells. It is also important to consider that a physical interaction between Bq-YopJ and DCNL1 does not necessarily mean that DCNL1 is acetylated by Bq-YopJ. Therefore, further experiments to detect Bq-YopJ-mediated acetylation of DCNL1 are needed, such as an in vitro acetylation reaction followed by mass spectrometry.

DCNL1 is a component of a regulatory mechanism called neddylation²¹¹. Neddylation is a post-translational modification in which the ubiquitin-like

protein neural precursor cell expressed developmentally downregulated protein 8 (Nedd8) is attached to target proteins²¹¹. Nedd8 and neddylation are structurally and functionally very similar to ubiquitin and ubiquitination; the two proteins are 59 % identical, and both involve three similar protein complexes called E1, E2, and E3 enzymes; DCNL1 is part of the Nedd8 E3 enzyme complex²¹¹. The key difference between them is that, while ubiquitination is primarily a marker for proteasomal degradation, neddylation is not. Neddylation is, however, involved in regulation of other systems, it can activate or inhibit the target, or mark it for degradation²¹². The best characterised targets of neddylation are the Cullin family. The Cullins are scaffold proteins for Cullin-RING-ligase (CRL) ubiquitin ligase complexes, and are activated by neddylation^{211,213}.

There is known involvement of neddylation in immunity and NF- κ B signalling. A small molecule inhibitor of the Nedd8 E1 enzyme (MLN4924) has been shown to attenuate the downstream effects of NF- κ B activation²¹⁴. One process that is required for NF- κ B activation that relies on neddylation is the activity of the SCF ^{β -TrCP} ubiquitin ligase complex, which is responsible for the degradation of both I κ B α and p105^{215,216}. This is by no means the only way that neddylation regulates NF- κ B, but it is the best characterised, so provides the best starting point for investigation of the effects of Bq-YopJ on neddylation²¹².

Some YopJ homologues target multiple host proteins, for example YopJ of *Yersinia* spp. interacts with MAPKK1, MAPKK3, MAPKK4, MAPKK5, MAPKK6, and IKK β ¹⁶⁵. The yeast two-hybrid screen reported only a single robust interaction for Bq-YopJ, to a completely unrelated protein. Unlike these kinases, DCNL1 is not directly involved in MAPK or NF- κ B signalling, which suggests that it may indirectly inhibit NF- κ B, given the results presented in Chapter 3.

5.4 Summary

This yeast two-hybrid screen revealed the human protein DCNL1 as a potential cellular target for Bq-YopJ, a result as robust as this method can provide, although more work would be beneficial to confirm the interaction.

Additionally, this result made me question how Bq-YopJ interacting with DCNL1 could cause the inhibition of NF- κ B activation that I reported in Chapter 3, so I decided to investigate whether Bq-YopJ interferes with the neddylation of Cullin-1 and the activity of the SCF ^{β -TrCP} ubiquitin ligase complex. This is the best characterised regulation of NF- κ B via neddylation, so was a good starting point for exploring the effects of Bq-YopJ on DCNL1 activity.

Chapter 6

Investigating the Effects of *Bartonella quintana* YopJ on DCNL1 and Neddylation

6.1 Introduction

6.1.1 Neddylation and Immunity

The yeast two-hybrid screen identified three independent hits for the protein DCNL1 as a potential interaction partner of Bq-YopJ. DCNL1 is part of a ligase complex for the ubiquitin-like protein Nedd8, a process called neddylation²¹¹. Post-translational modifications, such as ubiquitin and Nedd8, play vital roles in many cellular processes, including the regulation of inflammation²¹⁷. Because of their central role in immune regulation, the alteration of these post-translational modifications is emerging as a theme among secreted bacterial effectors²¹⁷.

The full extent of immune regulation by neddylation is an area of active research. It has been reported that general inhibition of neddylation reduces inflammation. This includes reduced secretion of pro-inflammatory cytokines and the inhibition of LPS-induced NF- κ B activation in immune cells.^{214,218}. Many studies of neddylation have been carried out using the pharmacological agent MLN4924, which potently inhibits E1 Nedd8-activating enzymes, thereby inhibiting the transfer of Nedd8 onto target proteins. Studies with this agent have shown that neddylation is a critical regulator of function in a wide range of immune cells, including B and T cells, macrophages, and endothelial cells during inflammation^{219–222}.

The best characterised role of neddylation in NF- κ B signalling is through the activation of Cullin-1 by neddylation²¹¹. Cullin-1 is part of the SCF ^{β -TrCP} ubiquitin ligase complex, which is responsible for the ubiquitination and subsequent proteasomal degradation of I κ B α and p105²¹¹. Neddylation of Cullin-1 is not the only way that neddylation regulates NF- κ B signalling. IKK γ , also called NEMO, is a component of the IKK complex, and can be neddylated, resulting in inhibition of signalling²²³. A-kinase-interacting protein 1 (AKIP1, also called BCA3) is involved in NF- κ B regulation but its exact role is unclear, as are the effects of its neddylation²¹¹. AKIP1 has been reported to enhance transcription of NF- κ B regulated genes by promoting nuclear localisation and phosphorylation of p65 at serine 276, but, when neddylated, may instead inhibit the transcription of these genes and recruit SIRT1, a histone deacetylase, to induce chromatin restructuring^{224,225}. The first study did not investigate the effects of neddylation, but its precise role, while unclear, is likely to be important²²⁴. TRAF6, a ubiquitin ligase and component of many immune receptors, including the IL-1 receptor, the IL-17 receptor, and the TLRs, must be neddylated to function properly^{167,226,227}.

The role of neddylation in immunity is complex, and the full role of DCNL1 remains uncertain; while it has only been demonstrated to neddylate the Cullins, overexpression of DCNL1 in human cells results in an increase in total neddylation^{211,228}. Regardless, the neddylation of Cullin-1 and the activity of the SCF ^{β -TrCP} ubiquitin ligase complex is the best characterised link between DCNL1 and NF- κ B activation.

6.1.2 The Mechanics of Neddylolation

The neddylation of different target proteins involves slightly different components, but Figure 6.1 shows the general process. Free Nedd8, a small protein of about 9 kDa, is first chemically activated and bound by the Nedd8 E1 enzyme, which then recruits the Nedd8 E2 enzyme²¹¹. The Nedd8 E2 enzyme transfers the Nedd8 moiety from the E1 to its own active site, then transports the Nedd8 to the target protein²¹¹. The E3 ligase then transfers the Nedd8 from the E2 onto the target protein²¹¹.

FIGURE 6.1: **Overview of Neddylation.**

The Nedd8 E1 enzyme chemically activates and binds to free Nedd8. The E2 enzyme collects the Nedd8 from the E1 and transports it to a target. Finally, the E3 enzyme transfers the Nedd8 from the E2 to the target protein, such as Cullin-1²¹¹.

DCNL1 is part of the Nedd8 E3 enzyme complex, and its function is to bring together the E3 ligase, the Nedd8-loaded E2 enzyme, and the target into the optimal orientation for Nedd8 transfer^{211,229}. This role makes DCNL1 a major regulator of neddylation²¹¹. The best-characterised substrates for neddylation are the Cullins, a family of structural proteins that are the scaffolds for Cullin-RING E3 ubiquitin ligase (CRL) complexes²¹³. The activity of CRLs is positively regulated by conjugation of a Nedd8 polypeptide onto the Cullin proteins. The CRL family of ubiquitin ligases is involved in ubiquitination of a wide array of cellular proteins, including regulatory proteins in the NF- κ B pathway. The primary CRL that is involved in regulation of NF- κ B signalling is the ubiquitin ligase enzyme complex, SCF $^{\beta}$ -TrCP, which is responsible for ubiquitination of phosphorylated I κ B α and phosphorylated p105 during NF- κ B activation^{215,216}. The Cullin-1 component must be neddylated for SCF $^{\beta}$ -TrCP function²³⁰.

The Cullin scaffold for SCF ^{β -TrCP} is Cullin-1, and its neddylation and the assembly of the SCF ^{β -TrCP} complex are intrinsically linked. Figure 6.2 shows this process in schematic form.

FIGURE 6.2: Cullin-1 Neddylation and SCF ^{β -TrCP} Assembly. Nedd8 activating enzyme (NAE), the E1 enzyme, chemically activates and binds to free Nedd8. UBC12, the E2 enzyme, collects the Nedd8 from NAE and transports it to a Cullin-1 molecule²¹¹. DCNL1, Cullin-1, and RING-box protein 1 (Rbx1) are bound by Cullin-associated NEDD8-dissociated protein 1 (CAND1) which prevents neddylation until the substrate-recognition module of S-phase kinase-associated protein 1 (Skp1) and β -TrCP bind²³⁰. After CAND1 has dissociated, DCNL1 brings the Nedd8-loaded UBC12, Cullin-1, and another Rbx1 molecule together, and the Rbx1 transfers Nedd8 from UBC12 to Cullin-1²¹¹.

The E1 enzyme is Nedd8 activating enzyme (NAE) which activates and binds free Nedd8 for collection by the E2 enzyme UBC12²¹¹. DCNL1, Cullin-1, and the ligase RING-box protein 1 (Rbx1) are both bound by Cullin-associated NEDD8-dissociated protein 1 (CAND1)²³⁰.

CAND1 regulates CRL assembly and Cullin-1 neddylation; it can only bind to non-neddylated Cullin-1 but blocks its neddylation when bound²³⁰. In general CAND1 has an inhibitory effect on CRL assembly, but its role is more complicated and more important than simply an inhibitor, as it seems to promote cycling of different substrate-recognition modules²³⁰. CAND1 is of specific interest because it also interacts with DCNL1, forming stable CAND1-Cullin-DCNL1 complexes, which likely serve as a reservoir for these proteins ready for neddylation as soon as CAND1 dissociates²³¹.

The substrate-recognition module of SCF ^{β -TrCP} is β -TrCP, which attaches to Cullin-1 via S-phase kinase-associated protein 1 (Skp1)²³⁰. Once the substrate-recognition module has replaced CAND1 in binding to Cullin-1, DCNL1 brings together the Nedd8-loaded UBC12, Cullin-1, and another Rbx1 molecule (which functions as both a ubiquitin ligase and a Nedd8 ligase), inducing Rbx1 to transfer Nedd8 from UBC12 to Cullin-1²¹¹. Once Cullin-1 has been neddylated, the SCF ^{β -TrCP} ubiquitin ligase is active and ready to ubiquitinate phosphorylated I κ B α and p105, which results in their proteasomal degradation^{215,216}. Neddylation is therefore likely important for NF- κ B activation, as the degradation of ubiquitinated I κ B α frees the transcription factors p65 and p50 to migrate to the nucleus and induce gene expression.

6.1.3 Microbial Interference with Neddylation

The connection between neddylation and immune regulation are well established, so it is unsurprising that it is a target for suppression by viral and bacterial pathogens^{219–222}. The bacterial type-III-secreted effector Cif of Enteropathogenic *E. coli* deamidates Nedd8, abolishing the activity of neddylated CRLs and suppressing both the cell cycle and NF- κ B signalling²³². *ChlaDub1* and *ChlaDub2* are two *Chlamydia trachomatis* enzymes which have been shown to have de-neddylase activity, removing Nedd8 from target proteins²³³. Additionally, *ChlaDub1* has been demonstrated to inhibit NF- κ B by preventing I κ B α degradation²³⁴. These proteins target Nedd8 directly, and do not share the acetyltransferase activity of Bq-YopJ, so the hypothesised inhibition of NF- κ B through acetylation of DCNL1 and inhibition of the Nedd8 ligase complex is novel.

6.1.4 Rationale

My working hypothesis was that Bq-YopJ interacted with DCNL1, interfering with the neddylation of Cullin-1, and therefore inhibiting the activity and stability of the SCF ^{β -TrCP} ubiquitin ligase.

To test this, I first evaluated the abundance of components of the SCF ^{β -TrCP} complex and neddylation machinery in the presence of Bq-YopJ or the catalytically inactive variant. I performed Western blots on lysates of transfected cells, using antibodies to DCNL1, Rbx1, CAND1, and Cullin-1. I assessed abundance in unstimulated cells and in response to TNF- α stimulation, because post-translational modifications (e.g. acetylation and neddylation) are often associated with protein degradation in signalling pathways such as NF- κ B. I considered that Bq-YopJ could selectively interfere with TNF- α -stimulated signalling as well as signalling during homeostasis.

I also performed Western blots of lysates using an antibody to Cullin-1 that enabled differentiation between neddylated and non-neddylated Cullin-1; and a Western blot using an antibody to the Nedd8 peptide that allowed me to examine the ratio of bound to free Nedd8.

The overall aim of this part of the project was to determine whether Bq-YopJ affects the neddylation of Cullin-1 and the abundance of associated proteins.

6.2 Results

6.2.1 Western Blots of DCNL1 and Interacting Proteins

To explore the impact of Bq-YopJ on neddylation components, I compared the abundance of DCNL1, Rbx1, CAND1, and Cullin-1 in lysates from HEK293 cells that had been transfected with Bq-*yopJ* or the Bq-*yopJ* Mutant.

Cells were stimulated with TNF- α and lysates collected over a time-course, at five minute intervals from just prior to addition of TNF- α (time-point 0) to twenty minutes, with a final time-point at thirty minutes. After cells were lysed, proteins were separated by SDS-PAGE and Western blots were done using antibodies to the test proteins and loading and transfer controls. The experimental design was identical to that in Figure 3.8 which was used for the NF- κ B Westerns. The host cell enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in most of the Western blots as a loading/transfer control, as its mass placed it roughly in the centre of the membranes, facilitating optimal transfer. The exception to this is that LaminB1 was included as a control for CAND1, as both proteins are similar in size, resulting in a similar efficiency of transfer.

This method allowed the comparison of the amount of each protein present in unstimulated cells (time-point 0) and to visualise changes in response to TNF- α stimulation, which activates NF- κ B signalling. Figure 6.3 shows a representative Western blot for each of DCNL1, Rbx1, CAND1, and Cullin-1, along with their loading and transfer controls.

Based on the representative Western blots, there was no clear difference in DCNL1, CAND1, or Cullin-1 protein levels over time during the TNF- α time-course, comparing transfected cells expressing Bq-YopJ and the Bq-YopJ Mutant. Rbx1 may be slowly degraded over time after TNF- α stimulation in cells transfected with Bq-*yopJ*, but not Bq-*yopJ* Mutant. There may also be more Rbx1 and less Cullin-1 at time-point 0 in the cells transfected with Bq-*yopJ* compared to those transfected with Bq-*yopJ* Mutant.

FIGURE 6.3: **Western blots of DCNL1 and associated neddylolation proteins with TNF- α stimulation over time.**

Lysates from transfected cells were collected following TNF- α stimulation, as described in the text. Protein abundance was assessed by blotting for the proteins of interest first, detecting and imaging the membrane, then blotting for the loading control. Therefore the controls are from the same lanes. (A) shows DCNL1 and the loading control GAPDH; (B) shows the DCNL1-interacting protein Rbx1 with the loading control GAPDH; (C) CAND1 and loading control LaminB1 are shown; LaminB1 was used as a control with CAND1 because they are similar in mass and likely to transfer with similar efficiency. (D) shows Cullin-1 and the loading control GAPDH from the same lanes; Cullin-1 appears as a doublet, with the smaller band representing non-neddylolated Cullin-1 and the slightly larger band as Cullin-1 conjugated to Nedd8 (neddylolated).

6.2.2 Quantifying the Western Blots

To more accurately compare the amount of the test proteins over time after TNF- α stimulation between the Bq-YopJ and Bq-YopJ Mutant conditions, I quantified the bands of each test protein control using the image analysis software FIJI¹⁴³. This gave a total pixel intensity of each band directly proportional to the amount of transferred protein that was detected. I then divided the total pixel intensity of test protein by the total pixel intensity of the control in the same lane. This accounted for variability between lanes in the amount of total protein loaded. For the Cullin-1 bands, I combined the neddylated and non-neddylated bands and quantified the total Cullin-1.

To compare the amount of the test proteins over time after TNF- α stimulation, I normalised the test protein:control total pixel intensity ratio to time-point 0, removing variability in the baseline amount of the test protein, leaving only the change in the amount of the protein due to stimulation. I repeated each Western blot a total of three times for DCNL1, Rbx1, and CAND1. I repeated the Cullin-1 western six times. Figure 6.4 summarises the results of these experiments.

There were no significant differences in the amounts of all four neddylation proteins over time following TNF- α stimulation.

In all, these results indicate that Bq-YopJ has no effect on the amounts of the proteins in response to TNF- α stimulation. This may be because Bq-YopJ activity does not result in protein degradation. Further, the interactions between DCNL1 and Rbx1, CAND1, or Cullin-1 were not assessed using this approach.

To identify the basal abundances of the test proteins, I calculated the ratios of test protein:loading control pixel intensity of time-point 0 for each replicate of each Western blot. Figure 6.5 shows these graphs.

FIGURE 6.4: Graphs of quantified Western blots of DCNL1 and associated proteins with TNF- α stimulation over time.

Each point represents the average ratio of the test protein to the loading control from three Western blots for DCNL1, Rbx1, and CAND1, and six Western blots for Cullin-1. All are expressed as a percent of time-point 0. Error bars represent standard deviation. I calculated statistical differences using repeated measures two-way ANOVAs. None of the time-points were significantly different between the two conditions for any of the test proteins. (A) shows the graph for the DCNL1 blots. (B) shows the graph for the Rbx1 blots; there may be a slight downward trend in both conditions. (C) shows the graph for the CAND1 blots. (D) shows the graph for the Cullin-1 blots, showing the total Cullin-1 (neddylated + non-neddylated).

FIGURE 6.5: Graph of average DCNL1 in Western blots compared to control.

Each point represents one replicate, expressed as the ratio of test protein to loading control, from the time-point 0 from the TNF- α time-course (i.e., prior to addition of TNF- α). Error bars represent standard deviation. I used an unpaired t-test to compare the means. There were no significant differences between the Bq-YopJ condition and the Bq-YopJ Mutant condition. (A) Graph of baseline DCNL1, Bq-YopJ mean is 1.667, Bq-YopJ Mutant mean is 1.590, P-value = 0.9155. (B) Graph of baseline Rbx1, Bq-YopJ mean is 1.975, Bq-YopJ Mutant mean is 1.375, P-value = 0.1052. While there was less Rbx1 in the cells expressing the Bq-YopJ mutant, this did not reach statistical significance. (C) Graph of baseline CAND1 versus LaminB1, Bq-YopJ mean is 12.56, Bq-YopJ Mutant mean is 10.54, P-value = 0.8414. (D) Graph of baseline Cullin-1 showing the total Cullin-1 (neddylated + non-neddylated), Bq-YopJ mean is 1.499, Bq-YopJ Mutant mean is 1.725, P-value = 0.4911.

There were no statistically significant differences in the mean ratios of the test protein to the loading controls when comparing cells expressing Bq-YopJ or the Bq-YopJ Mutant. The only protein that approached a statistically significant difference was Rbx1, with a mean of 1.975 for the Bq-YopJ condition, and 1.375 for the Bq-YopJ Mutant condition and a P-value of 0.1052. Rbx1 forms part of the SCF ^{β -TrCP} complex, in addition to interacting with DCNL1 directly, so a Bq-YopJ-mediated effect on its abundance by Bq-YopJ is possible, but more evidence is needed. It is also possible that an increase in Rbx1 in cells transfected with Bq-*yopJ* is due to the cells compensating for the effects of Bq-YopJ, rather than a targeted function of the effector.

6.2.3 Cullin-1 Neddylation

To test the differences in levels of neddylated Cullin-1, for each lane I calculated the ratio of the total pixel intensity of neddylated Cullin-1 to the total pixel intensity of non-neddylated Cullin-1. This ratio of neddylated Cullin-1 to non-neddylated Cullin-1 could detect any Bq-YopJ-dependent changes in the neddylation of Cullin-1. I used the same six Westerns as the previous experiment. Figure 6.6 summarises the results of this experiment.

FIGURE 6.6: **Graph of the ratio of neddylated to non-neddylated Cullin-1 with TNF- α stimulation over time.**

Each point represents the average ratio of neddylated:non-neddylated Cullin-1 from six Cullin-1 Western blots. Error bars represent standard deviation. I calculated statistical difference between the two conditions using a repeated measures two-way ANOVA, there was no significant difference between the two conditions, P-value = 0.6295.

There was no significant difference in the neddylation of Cullin-1 in response to TNF- α stimulation between cells transfected with Bq-*yopJ* or Bq-*yopJ* Mutant. Time-point 0 of this graph shows the baseline neddylation of Cullin-1. This time-point alone is presented in Figure 6.7 to more closely examine the neddylation of Cullin-1 in unstimulated cells.

The mean ratio of baseline neddylated:non-neddylated Cullin-1 was 0.6714 in the Bq-YopJ condition and was 0.7408 in the Bq-YopJ Mutant condition. This difference was not statistically significant (P-value = 0.5271).

The results of this experiment indicate that Bq-YopJ does not affect the neddylation of Cullin-1 within the limits of what I can detect. This data suggests that it is unlikely that Bq-YopJ-induced suppression of the NF- κ B pathway occurs via the SCF $^{\beta$ -TrCP ubiquitin ligase complex. This is in agreement with results presented in Chapter 3 that suggest that Bq-YopJ inhibits phosphorylation of I κ B α , which occurs upstream of SCF $^{\beta$ -TrCP function.

FIGURE 6.7: Graph of average baseline neddylolated to non-neddylolated Cullin-1 in Western blots.

Each point represents one replicate. Error bars represent standard deviation. Comparing time-point 0 from the TNF- α time-course. I used an unpaired t-test to compare the means, they are not significantly different, P-value = 0.5271.

6.2.4 Total Nedd8

There are many proteins other than Cullin-1 that are targets for neddylolation, including the other Cullins. One way to test whether neddylolation of another protein is impacted by Bq-YopJ is to do a Western blot of lysates to detect Nedd8, and examine the intensity of the free Nedd8 band. I compared lysates from HEK293 cells stimulated with TNF- α for 30 minutes transfected with Bq-*yopJ* to non-transfected HEK293 cells and those transfected with Bq-*yopJ* Mutant. Figure 6.8 shows this Nedd8 Western blot.

FIGURE 6.8: **Western blot of Nedd8 after 30 minutes of TNF- α stimulation.**

L = SeeBlue plus two protein Ladder, 1 = Untransfected HEK293 cells without TNF- α stimulation, 2 = Untransfected HEK293 cells with TNF- α stimulation, 3 = HEK293 cells transfected with Bq-*yopJ* and stimulated with TNF- α , 4 = HEK293 cells transfected with Bq-*yopJ* Mutant and stimulated with TNF- α

The most intense band is neddylated Cullins, which are all a similar mass. There are a few other bands of various neddylated proteins and a low mass band of free Nedd8. The free Nedd8 bands are not very distinct because the protein is so small that it spread horizontally. Despite this, the band representing free Nedd8 from lysates of cells expressing Bq-YopJ appears to be slightly darker than the bands from lysates of cells expressing Bq-YopJ Mutant or non-transfected cells.

By comparing the free Nedd8 band to the neddylated Cullin band I can determine if there is a change in the amount of free Nedd8 in the sample, and therefore a change in total neddylation. I quantified the bands and graphed the pixel intensity ratio of free Nedd8:neddylated Cullins bands. The larger this ratio the more free Nedd8 there is relative to the amount of neddylated Cullins

Figure 6.9 shows this graph. This is only a single replicate, but there may be more free Nedd8 in the Bq-YopJ sample than the Bq-YopJ Mutant sample and the non-transfected samples, suggesting that some neddylation is inhibited.

FIGURE 6.9: Graph of the ratio of free Nedd8 to neddylation Cullins.

Each bar represents the ratio of free Nedd8:neddylation Cullins for a single replicate from a Nedd8 Western blot, so a larger bar indicates more free Nedd8 relative to neddylation Cullins. This is a single replicate so there are no error bars.

6.3 Discussion

The goal of this part of the project was to test the working hypothesis that Bq-YopJ interacts with DCNL1 and interferes with the neddylation of Cullin-1, and therefore the activity and stability of the SCF $^{\beta}$ -TrCP ubiquitin ligase complex. This hypothesis was formed as a result of the yeast two-hybrid screen in Chapter 5 identifying DCNL1 as a potential interaction partner of Bq-YopJ, and the results of Chapter 3 that Bq-YopJ causes inhibition of the NF- κ B pathway. There are also reports of the importance of neddylation in NF- κ B signalling, though not through DCNL1 specifically²¹⁹⁻²²².

Neddylation is involved in a wide array of cellular processes, and though I did not detect any Bq-YopJ-mediated inhibition of neddylation of Cullin-1, many other neddylation proteins play a role in immunity, including Cullin-2, -3, and -5²³⁵⁻²³⁷. This result does suggest that if Bq-YopJ acetylates DCNL1, which remains to be shown, the effect is very specific and does not inhibit neddylation in general. However, it is important to confirm that Bq-YopJ interacts with DCNL1, as we were unable to validate the yeast two-hybrid results, or to show that neddylation is impacted by Bq-YopJ.

While not presented here, I attempted many more experiments to further

validate and characterise the interaction between Bq-YopJ and DCNL1. These did not yield results within the timeframe of this project.

These experiments included an *in vitro* acetylation assay, using a reaction mix that contained purified acetyl-CoA as the substrate, phytic acid, and recombinant Bq-YopJ and DCNL1 proteins, produced in *E. coli*¹¹⁶. However, despite multiple attempts to optimise expression of Bq-YopJ, a functional or soluble recombinant protein was never produced.

I tried to perform a co-immunoprecipitation assay from transfected HEK293 cell lysates, but could not detect an interaction. This may be because the interaction is highly transient or the native abundance of DCNL1 was too low to produce a detectable result. Indeed, many of the original publications that explored the function and interactome of DCNL1 reported experiments that were done with either cells transfected with *DCUN1D1* or with purified DCNL1^{231,238,239}.

To this end, I stably transfected a histidine-tagged version of *DCUN1D1* into HEK293 cells to aid in purification. The tagged protein was purified from lysates of the transfected cells and analysed by both LC/MS and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry to detect any acetylation or other post-translational modifications. However, detectable amounts of His-DCNL1 protein could not be recovered. Many of the problems with these experiments derived from a faulty *Yersinia yopJ* positive control, which was constructed in the same mammalian expression plasmid as the Bq-*yopJ* vector. Based on experiments during my Honours project I believed that this control was functional but had a frame shift in the GFP tag; it was not until I tested lysates of cells transfected with this control plasmid by LC/MS did I discover that it didn't produce protein at all and that those early experiments had produced false positive results.

The next steps to take beyond confirming the interaction between Bq-YopJ and DCNL1 is analysing the neddylation of other proteins to determine if any are affected by Bq-YopJ. The best starting point for this is the other Cullins, given that they are the only reported neddylated substrates of DCNL1²¹¹. These experiments would be very similar to the Cullin-1 Western blot in Figure 6.3D, and should be performed for Cullins -2, -3, and -5, which are also important in immune regulation^{235–237}. Comparing the relative intensity of the neddylated and non-neddylated bands for each Cullin in lysates from cells transfected with Bq-YopJ or Bq-YopJ Mutant would determine if the neddylation of each Cullin was impacted.

It is also possible that Bq-YopJ disrupts the protein interactions between DCNL1 and Rbx1 or CAND1, without causing degradation or inhibiting Cullin-1 neddylation. DCNL1 has been shown to be mono-ubiquitinated, which has no effect on Cullin neddylation, but does impact the assembly of CRLs, including the SCF ^{β -TrCP} complex; acetylation by Bq-YopJ could interfere with other post-translational modifications as well²⁴⁰. This could be investigated through co-immunoprecipitation experiments, comparing the amount of Rbx1 and CAND1 that precipitate along with DCNL1 between lysates from cells transfected with Bq-*yopJ* or Bq-*yopJ* Mutant by immunoblotting.

6.4 Summary

Based on these experiments, it is unlikely that Bq-YopJ inhibits the SCF ^{β -TrCP} ubiquitin E3 ligase complex by preventing neddylation of Cullin-1, though it may inhibit the neddylation of another protein. Bq-YopJ does not seem to cause degradation of DCNL1, Cullin-1, the ubiquitin/Nedd8 ligase Rbx1, or the regulatory protein CAND1. It also does not appear to impact the baseline abundance of these proteins, though additional repeats are required for Rbx1. It is also possible that Bq-YopJ alters a different aspect of DCNL1 function, such as the mono-ubiquitination of DCNL1, which is not reported to effect neddylation but does alter the assembly of CRLs²⁴⁰.

Chapter 7

Discussion

7.1 Project Purpose

Bartonella quintana is an understudied pathogen. Current research has not yet shown its full impact on human health or explained how it evades the immune system to cause long-term infection. The *B. quintana* YopJ homologue may play an important role in this immune evasion. By understanding how it works there is a lot that we can learn not only about *B. quintana* infection, but also about immune regulation itself.

The specific goal of this project was to characterise the *B. quintana* YopJ homologue and to develop a model for how it causes the inhibition of NF- κ B signalling.

7.2 Research Summary

In the experiments described in Chapter 3, I assayed the effects of Bq-YopJ on the major stages of NF- κ B activation, starting at the end result of the pathway and working backwards. Based on these results, it is most likely that Bq-YopJ inhibits the NF- κ B pathway at or upstream of the phosphorylation of I κ B α by IKK, though the exact early step at which inhibition takes place remains unclear. It could block phosphorylation of IKK by TAK1, preventing IKK activation, or it could inhibit the ability of one or more immune receptors from activating TAK1. A likely component of these receptors that could be targeted is the TRAF protein family, which are found in many receptors and are vital for the activation of TAK1¹⁶⁹.

In the experiments described in Chapter 4, I performed a proteomic screen to identify what cellular mechanisms are impacted by Bq-YopJ. The additional mechanisms that it identified were JNK signalling, apoptosis, the cell

cycle, and mTOR signalling. I also identified four specific proteins of interest: GSK-3 β , JNK1 (also called MAPK8), Cyclin-A2 and RAPTOR. GSK-3 β is most associated with NF- κ B, while JNK1, Cyclin-A2 and, RAPTOR represent interesting links to other cellular mechanisms. These results need validation, but they are a good starting point for future work into the effects of Bq-YopJ on apoptosis, the cell cycle, and metabolism.

In the experiments described in Chapter 5, I performed a yeast two-hybrid screen to identify human proteins that interact with Bq-YopJ. I identified the protein DCNL1 as an interacting partner of Bq-YopJ. DCNL1 is involved in neddylation, which is an important cellular regulatory system known to be crucial for NF- κ B activation specifically, and immune regulation generally²¹⁴. These results require further validation but are as robust as the technique can provide.

In Chapter 6 I explored one of the possible mechanisms of Bq-YopJ activity, interfering with the SCF $^{\beta}$ -TrCP ubiquitin E3 ligase by preventing Cullin-1 neddylation. These experiments were designed to test my hypothesis that this was the likely mechanism of DCNL1-dependent inhibition of NF- κ B activation. The results did not, however, provide support for this hypothesis, as no difference in neddylation of Cullin-1 was detected in cells transfected with Bq-YopJ. As a result, I have refined and developed a new hypothesis, as described in Section 7.3, which may be tested in the future.

7.3 Potential Mechanisms of Inhibition of NF- κ B Activation

One of the goals of this project was to develop a model for how Bq-YopJ inhibits the NF- κ B pathway. I have developed four possible models, described below. Each is based on the assumption that Bq-YopJ is modulating the neddylation of different host proteins. It's important to note that DCNL1 works with many different Nedd8 ligases which recognise different host proteins, but that it has only been shown to be involved in the neddylation of Cullins²¹¹. Bq-YopJ-mediated acetylation of DCNL1 could impact its ability to interact with some ligases and not others.

7.3.1 Inhibition via Cullin-1

When the yeast two-hybrid screen identified DCNL1 as a top candidate for interaction with Bq-YopJ, my hypothesis was that Bq-YopJ acetylation of DCNL1 would prevent interaction with binding partner Rbx1 and subsequent neddylation of Cullin-1. This would prevent the assembly of the SCF ^{β -TrCP} ubiquitin E3 ligase, meaning that phosphorylated I κ B α and p105 would not be ubiquitinated and degraded²¹⁵. If I κ B α is not degraded, it remains sequestered with p65 and p50, preventing their nuclear translocation and transcription factor activity. This mechanism can also explain the over-representation of GSK3 β in cells expressing Bq-YopJ as reported in Chapter 4; neddylation of GSK3 β is degraded by Nedd8 Ultimate Buster 1 (NUB1), so inhibition of neddylation could cause it to accumulate²⁴¹. Current research has not shown the E3 ligase responsible for GSK3 β neddylation however, and DCNL1 is only known to facilitate neddylation of Cullins. Additionally, whether or not increasing basal GSK3 β would result in inhibition or activation of NF- κ B is unclear.

Figure 7.1 shows this mechanism in graphical form.

This mechanism is unlikely given the result presented in Chapter 3, where abundance levels of phosphorylated I κ B α were shown to be reduced by Bq-YopJ. If Bq-YopJ led to reduced ubiquitination and degradation of I κ B α , we would expect to see an increased abundance. Furthermore, results reported in Chapter 6 suggested that Cullin-1 neddylation was not inhibited by Bq-YopJ.

FIGURE 7.1: **Mechanism of inhibition of NF-κB activation by suppression of Cullin-1 neddylation.**

Bq-YopJ acetylation of DCNL1 would inhibit the neddylation of Cullin-1 and therefore the formation of the SCF^{β-TrCP} ubiquitin E3 ligase. Without SCF^{β-TrCP}, phosphorylated IκBα and p105 would not be ubiquitinated and therefore not degraded or processed. This would cause phosphorylated IκBα to accumulate and prevent the release and subsequent nuclear localisation of p65 and p50. This could also inhibit the neddylation and therefore degradation of GSK3β which itself can inhibit p65 from initiating gene transcription.

7.3.2 Inhibition via Cullin-3 & IKK β

One way that Bq-YopJ mediated acetylation of DCNL1 could inhibit the NF- κ B pathway is by inducing the neddylation of Cullin-3. Increasing neddylation of Cullin-3 would stabilise the Cullin-3, Rbx1, Kelch-like ECH-associated protein 1 (KEAP1) ubiquitin E3 ligase complex²¹¹. This E3 complex ubiquitinates IKK β , one of the three subunits of IKK, and marks it for degradation. This would reduce the total amount of functional IKK in the cell, and therefore reduce the phosphorylation of I κ B α ²³⁶.

Figure 7.2 shows this mechanism in graphical form.

This mechanism relies on the same DCNL1:Rbx1 interaction as the previous one, so assumes that Bq-YopJ acetylation of DCNL1 affects its interactions only with specific Cullins. In this case, the phenotypes observed would result from an enhancement of Cullin neddylation. This would also reduce the amount of IKK β in the cell, though if this were significant enough to inhibit NF- κ B, I would have expected to detect the change in the proteomic screen. However, no differences in protein levels were seen for any of the IKK subunits. Additionally, it is unlikely that Bq-YopJ would act to increase the activity of DCNL1 by acetylating it, and the Nedd8 blot shown in Figure 6.9 in Chapter 6 was suggestive of Bq-YopJ increasing relative free Nedd8, therefore inhibiting neddylation, though this blot would need to be repeated.

FIGURE 7.2: **Mechanism of inhibition of NF- κ B activation by induction of Cullin-3 neddylation.**

Bq-YopJ acetylation of DCNL1 would induce the neddylation of Cullin-3, stabilising the Cullin-3, Rbx1, KEAP1 ubiquitin E3 ligase. This would increase the ubiquitination and degradation of IKK β inhibiting the activity of IKK. This would prevent the phosphorylation of I κ B α and p105, preventing the release, and subsequent nuclear localisation of p65 and p50.

7.3.3 Inhibition via TRIM40 & IKK γ

Another potential way for acetylation of DCNL1 to cause suppression of NF- κ B is by stabilising an interaction between the Nedd8 ligase TRIM40 and DCNL1. TRIM40 mediates the neddylation of IKK γ (also called NEMO), which inhibits the activity of the IKK complex²²³. This would prevent the phosphorylation of I κ B α and p105, and therefore their ubiquitination and degradation, and the nuclear translocation of p65 and p50.

Figure 7.3 shows this mechanism in graphical form.

This mechanism is more direct than the previous two, and though it focuses on the inhibition of IKK, it does not propose its degradation. This means that it does not conflict with the inhibition of I κ B α suggested in Chapter 3 or the proteomic screen described in Chapter 4. It also does not involve Rbx1 or any of the Cullins, which is in line with the results presented in Chapter 6. However, DCNL1 is only known to facilitate neddylation of Cullins and has not been demonstrated to interact with TRIM40, though this does not necessarily mean that these interactions do not take place²¹¹. Also, It is uncertain whether Bq-YopJ induced acetylation of DCNL1 would increase neddylation, rather than inhibit it, as was suggested by the Nedd8 Western blot in Chapter 6.

FIGURE 7.3: Mechanism of inhibition of NF-κB activation by induction of TRIM40 mediated IKK neddylation.

Bq-YopJ acetylation of DCNL1 would induce the neddylation of IKKγ via interaction with TRIM40. This would deactivate IKK and inhibit the phosphorylation of IκBα and p105, preventing their ubiquitination and degradation. Without degradation of IκBα and p105 the release and nuclear localisation of p65/p50 will not occur.

7.3.4 Inhibition via Cullin-5 and the TRAFs

One likely mechanism involves inhibition of the neddylation of Cullin-5 and its involvement in the ubiquitination of the TNF receptor-associated factor (TRAF) family of proteins. The TRAFs are components of the TNF- α receptor, IL-1 receptor, and IL-17 receptor, and are involved in both NF- κ B and JNK signalling; additionally, six of the seven TRAFs (2 – 7) have ubiquitin E3 ligase activity^{167,242–244}. Upon activation of the NF- κ B pathway, TRAF6 is known to be auto-polyubiquitinated in a Cullin-5 dependent manner as part of signal transduction²³⁷. The reported interaction between TRAF6 and Cullin-5 occurs via the TRAF-C domain, which is present in TRAF 1 – 6; therefore, while other TRAF family members have not been reported to interact with Cullin-5, this interaction could be conserved²³⁷. Additionally, TRAF6 is most associated with the TLRs, the IL-1, and IL-17 receptors. In this project, NF- κ B was activated via the TNF- α receptor, which reportedly uses TRAF2 instead of TRAF6¹⁵². This model assumes that TRAF2 interacts with Cullin-5, but this has not been shown experimentally. Bq-YopJ-mediated acetylation of DCNL1 could disrupt the neddylation of Cullin-5, and therefore suppress the ubiquitination of the TRAFs and inhibit NF- κ B activation²³⁷. This mechanism assumes that neddylation is inhibited, so like the mechanism via Cullin-1, it can also explain the over-representation of GSK3 β in cells expressing Bq-YopJ that I observed in Chapter 4.

Figure 7.4 shows this mechanism in graphical form.

This mechanism is consistent with my experimental results, as it would result in inhibition of I κ B α phosphorylation, enrichment of GSK3 β abundance, as identified in the proteomic screen, and it assumes the inhibition of neddylation, as suggested by the Western blot of Nedd8. Targeting the TRAFs to cause inhibition of NF- κ B signalling is not a novel mechanism; as there is an effector in *Shigella flexneri*, OspI, which inhibits NF- κ B activation by preventing TRAF6 ubiquitination¹⁵⁹. Additionally, TRAF6 has been proposed as a target of *Yersinia* YopJ²⁴⁵.

Though there is still significant work required before this mechanism is fully characterised, future experiments should be designed to test this new hypothesis for Bq-YopJ induced inhibition of NF- κ B activation.

FIGURE 7.4: **Mechanism of inhibition of NF-κB activation by suppression of Cullin-5 neddylation.**

Bq-YopJ induced acetylation of DCNL1 would inhibit the neddylation of Cullin-5, and therefore prevent its activation. This would inhibit the polyubiquitination, and therefore activation of TRAF proteins. The TRAF proteins are required for activation of IKK so inhibiting their function will prevent phosphorylation of IκBα and p105, preventing their ubiquitination and degradation. Without the degradation of IκBα and p105 the release and nuclear localisation of p65/p50 will not occur. This could also inhibit the neddylation and therefore degradation of GSK3β which itself can inhibit p65 from initiating gene transcription.

There is another potential mechanism by which Bq-YopJ could inhibit TRAF6. TRAF6 must itself be neddylation to function, so Bq-YopJ mediated acetylation of DCNL1 could disrupt neddylation of TRAF6, and therefore suppress the activation of the NF- κ B pathway²²⁷. This is less likely than the mechanism involving Cullin-5, because DCNL1 is only known to interact with Cullins. However, it is possible that DCNL1 does interact with other neddylation substrates. Additionally, current research has yet to determine the Nedd8 ligase responsible for TRAF6 neddylation. This is one area where future experiments, to determine the mechanism by which Bq-YopJ inhibits NF- κ B activation, could shed light on human cell biology and innate immune activation.

7.4 Future Directions

7.4.1 Validating the Yeast Two-Hybrid

The yeast two-hybrid screen is a useful tool, but as it is a screen, its findings need to be validated using independent approaches. As described in Chapter six, I attempted multiple experiments to validate the yeast two-hybrid results. Some of the issues encountered are also described, including difficulty in detecting DCNL1 using mass spectrometry techniques, and being unable to express and purify soluble, recombinant Bq-YopJ.

I believe that optimising the experiments that utilise the stably transfected histidine-tagged *DCUN1D1* is the best way forward. The focus of this optimisation should be on purification of DCNL1, as this would improve the chances of detecting acetylation by bringing the amount of protein up to a detectable level. I attempted to purify tagged DCNL1 from lysates from HEK293 cells stably transfected with histidine tagged-*DCUN1D1*, using both magnetic beads and binding columns. It is likely that the issues stemmed from low expression of the protein, though the entire population of stably transfected cells likely produced the protein, each only produced a very small amount. This could be remedied by switching to an established protein expression cell line, such as Expi293, which is non-adherent and therefore can be grown at a higher density. It may also be necessary to use transient transfections, which tend to produce higher expression levels than stable transfections, due to higher copy numbers.

Once tagged DCNL1 can easily be produced, the tagged-DCNL1-producing cells should additionally be transiently transfected with Bq-*yopJ* or the Bq-*yopJ* Mutant. After allowing the cells sufficient time to produce the protein and for Bq-YopJ to have its effects, the cells should be lysed and the tagged-DCNL1 purified and analysed by mass spectrometry. A validating result would identify a shift in the mass of DCNL1 by 43.045 Da, the mass of an acetyl moiety. The protein could additionally be fragmented to identify the specific modified amino acid.

It is possible that Bq-YopJ is not an acetyltransferase, so optimising co-immunoprecipitation would provide an avenue for confirming interaction without relying on acetylation. One major hindrance in co-immunoprecipitation was the lack of an anti-Bq-YopJ antibody. The previously attempted immunoprecipitations used the C-terminal GFP tag to extract Bq-YopJ, but did not have particularly high binding. This could be solved by producing a mammalian expression plasmid encoding Bq-*yopJ* with a different tag, such as HA or streptavidin, as this would allow for pull down assays without relying on potentially low efficiency antibodies for purification. Antibodies for these tags could be used to detect the presence of these proteins by immunoblotting, however. This experiment should include additional control plasmids, two that express just the protein tags, histidine for DCNL1 and HA or streptavidin for Bq-YopJ, and possibly a positive control of a protein shown to interact with DCNL1, such as one of the Cullins. The interaction between Bq-YopJ and DCNL1 is likely far more transient than that of DCNL1 and the Cullins, but it would be very useful to confirm that the proteins in the samples have not been denatured.

Additionally, such a tagged-Bq-*yopJ* encoding mammalian expression plasmid could overcome the difficulties in producing functional, purified Bq-YopJ. As of yet, functional Bq-YopJ has only been produced in eukaryotic cells (mammalian and yeast). *E. coli* does not seem to be able to produce it in a soluble form, despite having tried of a GST-tag, which increases solubility, and the co-expression of six different chaperone proteins, which assist in protein folding. Optimising production in mammalian cells could avoid these problems.

Another option would be a Bimolecular Fluorescence Complementation (BiFC) experiment, in which half of a fluorescent protein is attached to Bq-YopJ and DCNL1, so that their interaction brings the two halves together and produces the complete fluorescent protein. BiFC can be performed in human cells but the proteins would be overexpressed, which means that it could still detect non-biologically relevant interactions. This could also be performed as an in vitro experiment if production of functional Bq-YopJ is optimised. AlphaScreen (Perkin Elmer) is a bead-based assay for detecting protein-protein interactions; two bead types, donor and acceptor, are coated with the proteins of interest (Bq-YopJ and DCNL1). The donor bead is excited with a 680 nm laser, which causes it to release excited singlet oxygen; if the acceptor bead is in very close proximity to the donor bead, such as when the proteins coating each bead interact, the energy in the singlet oxygen is transferred

to the acceptor bead, which then produces a light emission of 520 – 620 nm. This emission can be detected with a luminometer, and the intensity of the emission is directly proportional to the interaction between the two proteins of interest²⁴⁶. The benefit of this approach is that it is capable of detecting very transient interactions.

7.4.2 Exploring the Effects of Bq-YopJ on Cullin-5 and TRAF2/6

The hypothesis for Bq-YopJ-mediated inhibition of NF- κ B activation that I have proposed focuses on the TRAF protein family, especially TRAF2 and TRAF6, and their signal transduction by Cullin-5-dependent autoubiquitination. This hypothesis could be tested through analyses of both the NF- κ B pathway and Cullin-5, with two good starting experiments being comparing TRAF6 ubiquitination and Cullin-5 neddylation in cells transfected with Bq-*yopJ* or the Bq-*yopJ* Mutant control. The first step would involve an immunoprecipitation of TRAF2 and TRAF6, followed by Western blotting the eluted samples with an anti-ubiquitin antibody and comparing the band intensity between the conditions. This would determine if Bq-YopJ inhibits TRAF auto-ubiquitination. It would also be useful to simultaneously blot for Nedd8 in case Bq-YopJ inhibits TRAF neddylation directly; however, the endogenous neddylation of TRAF6 is reported to be too low to detect by immunoblotting²²⁷, so TRAF6 and Nedd8 would need to be overexpressed by transient transfection. The next experiment would be to analyse the neddylation of Cullin-5 in cells transfected with Bq-YopJ, to determine if it inhibits neddylation; as before, this would be done by Western blotting of lysates with an antibody to Cullin-5, which can distinguish neddylated vs non-neddylated Cullin-5, based on size. Other experiments could investigate the downstream effects of TRAF6 activation, such as phosphorylation of IKK by TAK1 and the phosphorylation of p65 at serine 276 by protein kinase A. This phosphorylation of p65 occurs after its release from I κ B α and is of particular interest because it has been demonstrated to be reliant on TRAF6 neddylation²²⁷. Testing for effects on serine 276 phosphorylation of p65 can be done by Western blotting using an antibody specific to phosphorylated p65 (serine 276) and comparing the intensity of the bands from lysates of cells transfected with either Bq-*yopJ* or Bq-*yopJ* Mutant.

7.4.3 Following up on GSK3 β , JNK1, Cyclin-A2, and RAPTOR

The proteomic screen showed that the abundance of GSK3 β , JNK1, Cyclin-A2, and RAPTOR may be impacted by Bq-YopJ; these need further confirmation and investigation. The first step for all of these is validating the change in abundance by Western blotting lysates from cells transfected with Bq-*yopJ* (or the Mutant control) for each protein, and comparing the band intensities between conditions.

An experiment for testing the effect of Bq-YopJ on GSK3 β would also be to investigate phosphorylation of p65. GSK3 β has been reported to constitutively phosphorylate serine 468 of p65 to decrease its activity, so it would be valuable to test whether this was impacted by Bq-YopJ¹⁷³. This could be achieved by Western blotting lysates from cells transfected with Bq-*yopJ* (and a control) with an anti-phosphorylated p65 (serine 468) antibody and comparing the band intensity. This test would be in addition to the investigation of inducible phosphorylation of p65 (Serine 276) detailed in Section 7.4.2 above.

JNK signalling is important in apoptosis so it is possible that Bq-YopJ has an effect on apoptosis²⁴⁷. YopJ of *Yersinia* spp. induces apoptosis in immune cells, so apoptosis is a known result of the activity of YopJ family effectors¹²⁷. Additionally, the BID domain of *B. quintana* BepA2 has been shown to be anti-apoptotic, so Bq-YopJ may have a redundant effect¹⁹⁷. Investigating JNK signalling can be achieved by testing whether TNF- α induced phosphorylation of c-Jun, the target of JNK1, is impacted by Bq-YopJ by Western blotting for phosphorylated c-Jun and comparing lysates from cells transfected with Bq-*yopJ* or the Bq-*yopJ* Mutant.

Additionally, there are many apoptosis assay kits available, including luciferase-based tests for caspase cleavage and simple Western blots for pro- and active caspase.

Beyond confirming an increase in Cyclin-A2 in cells transfected with Bq-*yopJ* by Western blotting, it would be useful to show whether Bq-YopJ causes the cell cycle to stall.

One such assay that could detect this measures the stage of the cell cycle that cells are in by measuring the change in the DNA content of the cells; first by staining the DNA of a population of cells with a fluorescent dye, then analysing them by flow cytometry. The amount of DNA, and therefore fluorescence, indicates what stage of the cell cycle each cell is in, and if there is a stall

in the cell cycle, the appropriate fluorescence phenotype will be enriched²⁴⁸.

RAPTOR is a part of mTORC1 which is a kinase that phosphorylates a protein called p70 as part of a signalling cascade in response to insulin. One way to test whether Bq-YopJ impacts mTORC1 signalling would be to see whether it prevents insulin-induced phosphorylation of p70. The design of this experiment would be very similar to the I κ B α phosphorylation assay that I performed in Chapter 3 but with insulin stimulation rather than TNF- α stimulation, and Western blotting for phosphorylated p70 rather than I κ B α .

7.4.4 Investigating Bq-YopJ Secretion

This project focused solely on the function of Bq-YopJ once it is inside a host cell, but much remains unknown about how it gets there. All other YopJ homologues are secreted by type-III-secretion systems, but *B. quintana* has a type-IV-secretion system, which is functionally similar but evolutionarily unrelated. There is some indirect evidence that Bq-YopJ is secreted via the type-IV-secretion system; YopJ homologues are only found in those *Bartonella* species that have the VirB type-IV-secretion system, and is absent in those that lack it. However, the other *Bartonella* effector proteins, the Beps, have a conserved delivery domain (the BID domain) which is not present in Bq-YopJ⁹⁹.

The first step to confirming that Bq-YopJ is secreted via the VirB type-IV-secretion system will be to produce a mutant strain of *B. quintana* that lacks a functional VirB type-IV-secretion system. This can be done using a suicide plasmid, such as pEX18Gm, carrying the flanking regions of a truncated version of one of the core genes of the VirB type-IV-secretion system, such as VirB4. This plasmid will also carry a marker for positive selection; in the case of pEX18Gm this would be gentamicin resistance, which would select for integration of the plasmid into the bacterial genome. A negative selection marker, such as SacB, which processes sucrose into a toxic product, would enable selection of cells that had subsequently lost the integrated plasmid. This plasmid loss will result in the truncated gene replacing the wild type gene 50 % of the time (and reverting to wild type the other 50 %). This results in a markerless knockout strain.

This knockout strain can be used along with either an anti-Bq-YopJ antibody, or with tagged Bq-YopJ introduced to the cells in a broad host range plasmid, to examine the localisation of Bq-YopJ in mammalian cells when they interact with VirB knockout bacteria compared to wild type bacteria. This could include immunofluorescence microscopy and Western blotting as readouts for the experiment.

I began work on producing a VirB knockout strain of *B. quintana* during this project, but due to the slow growth of the bacteria and the difficulty involved in transforming them, I did not manage to produce a mutant strain before the end of the project. I did, however, produce a broad-host-range complementation plasmid which encoded histidine-tagged Bq-YopJ, and optimised the transformation of broad-host-range plasmids into *B. quintana*.

7.5 Concluding Remarks

The primary goals of this project were to characterise Bq-YopJ and to develop a model for how it causes the inhibition of NF- κ B signalling.

I have made significant advancements in characterising Bq-YopJ; I identified that Bq-YopJ inhibits the NF- κ B pathway at or upstream of IKK phosphorylation of I κ B α , identified a candidate cellular target of Bq-YopJ, and identified multiple previously unknown avenues of future research.

I have also developed a new hypothesis for how Bq-YopJ causes inhibition of NF- κ B activation based on my observations, which is that Bq-YopJ inhibits the function of DCNL1, preventing neddylation of Cullin-5 which is responsible for activating TRAF proteins, vital components in NF- κ B signalling.

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Appendix A

Appendix Figures and Tables

FIGURE A.1: DNA alignment of *B. quintana yopJ* homologue and Mutant mammalian expression plasmid inserts. Aligned using the the Geneious software package¹¹⁰. Sequence of the *yopJ* genes for the two *B. quintana* strains JK-31 (NCBI accession GCA_000708815.1) and Toulouse (NCBI accession GCA_000046685.1) included for reference.

Site of point mutation in active site highlighted by a red box. The wild type insert was generated by PCR amplification of genomic DNA while the Mutant was synthesised as a double-stranded DNA fragment. Because of this the precise wild type sequence was determined by sequencing, which could not reliably detect the start and end of the insert, so it appears truncated. This is also the source of the many silent mutations at the end of the YopJ Mutant gene, which were required to improve synthesis of the gene.

FIGURE A.2: Predicted amino acid sequence alignment of *B. quintana yopJ* homologue and Mutant protein sequences, encoded by mammalian expression plasmids.

Aligned using the the Geneious software package¹¹⁰. Sequence of the YopJ proteins for the two *B. quintana* strains JK-31 and Toulouse included for reference.

Site of mutation in active site highlighted by a red box. The wild type insert was generated by PCR amplification of genomic DNA while the Mutant was a synthetic gene. Because of this the precise wild type sequence was determined by sequencing, which could not reliably detect the start and end of the insert, so it appears truncated.

FIGURE A.3: **IL-8 ELISA of unstimulated samples.** Error bars show standard deviation. $n = 8$. I calculated statistical differences with one-way ANOVA, including Tukey's multiple comparisons test. ****: P -value < 0.0001 .

FIGURE A.4: **Potential Extra Yeast 2-Hybrid Hit from Titre Plate.**

Pink colonies are a symptom of adenine deficiency, and the ability to synthesise adenine was mediated by one of the reporter genes. The red arrow shows a colony that shows a white phenotype, so may have had activated reporters. The colony was further investigated to see if other reporter genes were activated.

TABLE A.1: **Proteins over-represented in cells transfected with Bq-yopJ**, according to the LC/MS proteomic screen in chapter 4. The abundance ratio was artificially capped at 100 by the proteome discoverer software.

Protein	Uniprot accession number	Abundance ratio	Adjusted P-Value
Probable ATP-dependent RNA helicase DDX28	Q9NUL7	100	1.29E-15
3-beta-hydroxysteroid-delta(8),delta(7)-isomerase	Q15125	6.67	1.29E-15
Cat eye syndrome critical region protein 2	Q9BXF3-1	100	1.29E-15
Protein FAM162A	Q96A26	8.47	1.29E-15
Fanconi anemia group A protein	O15360	100	1.29E-15
Non-histone chromosomal protein HMG-17	P05204	2.04	1.29E-15
pentatricopeptide repeat-containing protein 1, mitochondrial	O75127	11.0	1.29E-15
Homeobox protein Hox-A13	P31271	100	1.29E-15
PML-RARA-regulated adapter molecule 1	Q96QH2-1	100	1.29E-15
Isoform A1-A of Heterogeneous nuclear ribonucleoprotein A1	P09651-2	2.36	1.29E-15
Ubiquitin-60S ribosomal protein L40	P62987	3.91	1.29E-15
Pre-mRNA-splicing factor 38B	Q5VTL8	100	1.29E-15
propionyl-CoA carboxylase beta chain, mitochondrial	P05166-1	100	1.29E-15
Protein FAM47A	Q5JRC9	5.35	1.29E-15
Glycogen synthase kinase-3 beta	P49841-1	100	1.29E-15
sigma intracellular receptor 2	Q5BJF2	2.23	8.48E-14
Cytochrome c oxidase subunit 3	P00414	2.82	2.36E-08
Zinc finger protein 43	P17038	2.04	3.60E-07
Zinc transporter SLC39A7	Q92504	3.04	6.10E-07
Putative zinc finger protein 66	Q6ZN08	2.22	8.75E-06
Isoform 2 of Neuroepithelial cell-transforming gene 1 protein	Q7Z628-2	2.02	1.71E-05
Protein bassoon	Q9UPA5	2.07	4.61E-05
transcription elongation factor A protein-like 3	Q969E4	2.38	0.00018
Isoform 2 of Meckel syndrome type 1 protein	Q9NXB0-2	2.16	0.00029
Glycosylphosphatidylinositol anchor attachment 1 protein	O43292-1	2.17	0.00055
Zinc finger protein 384	Q8TF68-1	2.19	0.00057
acyl-CoA desaturase	O00767	2.43	0.00081
Cyclin-A2	P20248	2.01	0.010

TABLE A.2: **Proteins under-represented in cells transfected with Bq-yopJ**, according to the LC/Ms proteomic screen in chapter 4. The abundance ratio was artificially capped at 0.01 by the proteome discoverer software.

Protein	Uniprot accession number	Abundance ratio	Adjusted P-Value
Protein os-9	Q13438-1	0.01	1.29E-15
MICOS complex subunit MIC13	Q5XKP0	0.37	1.29E-15
PRKR-interacting protein 1	Q9H875	0.01	1.29E-15
C-Maf-inducing protein	Q8IY22-1	0.01	1.29E-15
ATPase family AAA domain-containing protein 2B	Q9ULI0	0.01	1.29E-15
Meiosis-specific coiled-coil domain-containing protein MEIOC	A2RUB1	0.01	1.29E-15
Aurora kinase A	O14965	0.01	1.29E-15
Prolactin-inducible protein	P12273	0.01	1.29E-15
Hepatitis A virus cellular receptor 2	Q8TDQ0	0.01	1.29E-15
Apolipoprotein L4	Q9BPW4-1	0.01	1.29E-15
Histone H1.5	P16401	0.01	1.29E-15
disintegrin and metalloproteinase domain-containing protein 9	Q13443	0.01	1.29E-15
Maturin	Q8N3F0-1	0.01	1.29E-15
PHD finger protein 20	Q9BVI0	0.01	1.29E-15
RNA polymerase-associated protein LEO1	Q8WVC0-1	0.01	1.29E-15
Diacylglycerol kinase epsilon	P52429	0.01	1.29E-15
Isoform 8 of Intersectin-1	Q15811-8	0.01	1.29E-15
Keratin, type II cytoskeletal 1	P04264	0.40	3.91E-13
Mitogen-activated protein kinase 8	P45983-1	0.31	1.33E-12
TBC1 domain family member 19	Q8N5T2	0.42	1.72E-11
polyadenylate-binding protein 4-like	P0CB38	0.40	4.78E-10
Tyrosyl-DNA phosphodiesterase 2	O95551-1	0.47	1.49E-05
Ankyrin-2	Q01484	0.40	0.00028
Regulatory-associated protein of mTOR	Q8N122	0.47	0.0030
Selenide, water dikinase 2	Q99611	0.49	0.0051
Nesprin-1	Q8NF91	0.50	0.016

Homo sapiens cDNA FLJ31773 fis, clone NT2RI2008045, highly similar to DCN1-like protein 1Sequence ID: [AK056335.1](#) Length: 1731 Number of Matches: 1Range 1: 9 to 863 [GenBank](#) [Graphics](#)[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
1561 bits(845)	0.0	852/855(99%)	1/855(0%)	Plus/Plus
Query 70	GCCGGGGGAGGAGGGGAGGCCCTGGAGGACCAACATGAACAAGTTGAAATCATCGCA	129		
Sbjct 9	GCCGGAGGAGGAGGGGAGAGGCCCTGGAGGACCAACATGAACAAGTTGAAATCATCGCA	68		
Query 130	GAAGGATAAAGTTCGTCACTTTATGATCTTCACACAATCTAGTGAAAAACAGCAGTAAG	189		
Sbjct 69	GAAGGATAAAGTTCGTCACTTTATGATCTTCACACAATCTAGTGAAAAACAGCAGTAAG	128		
Query 190	TTGCTTTTCTCAAAATGACTGGAAGTTAGATGTTGCAACAGATAATTTTTCCAAAATCC	249		
Sbjct 129	TTGCTTTTCTCAAAATGACTGGAAGTTAGATGTTGCAACAGATAATTTTTCCAAAATCC	188		
Query 250	TGAACTTTACATACGAGAGAGTGTAAAAGGATCATTGGACAGGAAGAAGTTAGAACAGCT	309		
Sbjct 189	TGAACTTTATATACGAGAGAGTGTAAAAGGATCATTGGACAGGAAGAAGTTAGAACAGCT	248		
Query 310	GTACAATAGATACAAAGACCTCAAGATGAGAATAAAATTGGAATAGATGGCATACAGCA	369		
Sbjct 249	GTACAATAGATACAAAGACCTCAAGATGAGAATAAAATTGGAATAGATGGCATACAGCA	308		
Query 370	GTTCTGTGATGACCTGGCACTCGATCCAGCCAGCATTAGTGTGTTGATTATTGCATGGAA	429		
Sbjct 309	GTTCTGTGATGACCTGGCACTCGATCCAGCCAGCATTAGTGTGTTGATTATTGCATGGAA	368		
Query 430	GTTCAAGAGCAGCAACACAGTGCAGTTCTCCAAACAGGAGTTTCATGGATGGCATGACAGA	489		
Sbjct 369	GTTCAAGAGCAGCAACACAGTGCAGTTCTCCAAACAGGAGTTTCATGGATGGCATGACAGA	428		
Query 490	ATTAGGATGTGACAGCATAGAAAACTAAAGGCCAGATACCCAAGATGGAACAAGAATT	549		
Sbjct 429	ATTAGGATGTGACAGCATAGAAAACTAAAGGCCAGATACCCAAGATGGAACAAGAATT	488		
Query 550	GAAAGAACCAGGACGATTTAAGGATTTTACCAGTTTACTTTTAAATTTGCAAGAATCC	609		
Sbjct 489	GAAAGAACCAGGACGATTTAAGGATTTTACCAGTTTACTTTTAAATTTGCAAGAATCC	548		
Query 610	AGGACAAAAAGGATTAGATCTAGAAATGGCCATTGCCTACTGGAACCTAGTGCTTAATGG	669		
Sbjct 549	AGGACAAAAAGGATTAGATCTAGAAATGGCCATTGCCTACTGGAACCTAGTGCTTAATGG	608		
Query 670	AAGATTTAAATCTTAGACTTATGGAATAAATTTTGTGGAACATCATAACGATCAAT	729		
Sbjct 609	AAGATTTAAATCTTAGACTTATGGAATAAATTTTGTGGAACATCATAACGATCAAT	668		
Query 730	ACCAAAAGACACTTGGAATCTTCTTTTAGACTTCAGTACGATGATTGCAGATGACATGTC	789		
Sbjct 669	ACCAAAAGACACTTGGAATCTTCTTTTAGACTTCAGTACGATGATTGCAGATGACATGTC	728		
Query 790	TAATTATGATGAAGAAGGAGCATGGCCTGTTCTTATTGATGACTTTGTGGAATTTGCACG	849		
Sbjct 729	TAATTATGATGAAGAAGGAGCATGGCCTGTTCTTATTGATGACTTTGTGGAATTTGCACG	788		
Query 850	CCCTCAAATTGCT-GGACAAAAAGTACAACAGTGTAGCACTAAAGGAACCTTCTAGAATG	908		
Sbjct 789	CCCTCAAATTGCTGGGACAAAAAGTACAACAGTGTAGCACTAAAGGAACCTTCTAGAATG	848		
Query 909	TACATAGTCTGTACA	923		
Sbjct 849	TACATAGTCTGTACA	863		

FIGURE A.6: Top BLAST result for the forward sequencing primer of Positive Clone 1.

A BLAST match for Positive Clone 1 (query) to a cDNA encoding DCN1 (subject). The alignment reached 99 % identity.

Sequence ID: [NM_001308101.2](#) Length: 7895 Number of Matches: 1

Range 1: 1652 to 2568 [GenBank](#) [Graphics](#)

▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1661 bits(899)	0.0	912/918(99%)	1/918(0%)	Plus/Plus
Query 1	GTCTACATTATGCACACTACAATTGGACTTCATTATGCTTTTGAAATGCTTATCTGCCTG	60		
Sbjct 1652	GTCTACATTATGCACACTACAATTGGACTTCATTATGCTTTTGAAATGCTTATCTGCCTG	1711		
Query 61	TCACATAAGTTAAACTATTTAATTTGTTTGTGATGTTTGGATTGCTACACAATAACAATA	120		
Sbjct 1712	TCACATAAGTTAAACTATTTAATTTGTTTGTGATGTTTGGATTGCTACACAATAACAATA	1771		
Query 121	TTCTAAATTTAGGCATGAGGGTttttttttgttttttttttacttttttttGTCATCGCA	180		
Sbjct 1772	TTCTAAATTTAGGCATGAGGGT-tttttttgtttttgttttttTACTTTTTTTTGTCTATCGCA	1830		
Query 181	CTATGGAAACACAATGGAGTTCCTCTTAATTTATAAGAAAGATAGTTGCAGTTAAATTTTGA	240		
Sbjct 1831	CTATGGAAACACAATGGAAATTCCTCTTAATTTATAAGAAAGATAGTTGCAGTTAAATTTTGA	1890		
Query 241	AAATGGTTGTAATGAGCCATGAAGTTCATCTTTATAATATAGGTAAGTCTCTTTCAGAC	300		
Sbjct 1891	AAATGGTTGTAATGAGCCATGAAGTTCATCTTTATAATATAGGTAAGTCTCTTTCAGAC	1950		
Query 301	AAATAGTCCATTTTCGATGACTTATTATTTTGTGAAATGCTTTAAGTCTAATCACTG	360		
Sbjct 1951	AAATAGTCCATTTTCGATGACTTATTATTTTGTGAAATGCTTTAAGTCTAATCACTG	2010		
Query 361	TGGTTGCTAAATATTACTTCAGGAGCAAGATTTTCAAAACAGCATACAGATGCAAAA	420		
Sbjct 2011	TGGTTGCCAAATATTACTTCAGGAGCAAGATTTTCAAAACAGCATACAGATGCAAAA	2070		
Query 421	TACCAATCTGGCTTCAGTCTCTTTACTGTTTCGTTTCAGTCTGAGTTAGTCTAGTTTC	480		
Sbjct 2071	TACCAATCTGGCTTCAGTCTCTTTACTGTTTCGTTTCAGTCTGAGTTAGTCTAGTTTC	2130		
Query 481	TCATCAAAAGCAGAACGCTATCTGTATGTATtttttttCATCAAGCCCCATGAGCTGCT	540		
Sbjct 2131	TCATCAAAAGCAGAAATGCTATCTGTATGTATTTTTTTCATCAAGCCCCATGAGCTGCT	2190		
Query 541	TTTATGCTGAAAAATGGTCATTTCCCTGTTCACTTACTGACATGTGAAGAGGGTTTCTTG	600		
Sbjct 2191	TTTATGCTGAAAAATGGTCATTTCCCTGTTCACTTACTGACATGTGAAGAGGGTTTCTTG	2250		
Query 601	CTTTCTTAAACATTTCCGTAAAGCAGGCTAGAATGTAATACCTTCAAAATGTTTGATGATT	660		
Sbjct 2251	CTTTCTTAAACATTTCCGTAAAGCAGGCTAGAATGTAATACCTTCAAAATGTTTGATGATT	2310		
Query 661	ATGGTCTTTTGATAGGAATAGATTCTGCTTGGGATATATATCAAGGCACTCTCTAAGGTC	720		
Sbjct 2311	ATGGTCTTTTGATAGGAATAGATTCTGCTTGGGATATATATCAAGGCACTCTCTAAGGTC	2370		
Query 721	TAGGGTTGATATTAAACAAAGGAATGACTTAGAATAGCAGTACATTTATGCAAAATATGG	780		
Sbjct 2371	TAGGGTTGATATTAAACAAAGGAATGACTTAGAATAGCAGTACATTTATGCAAAATATGG	2430		
Query 781	AAATTATTTTAAAGAAACAATGACATATCAAACTGCTTTTTACATGATTTTGAATAGAC	840		
Sbjct 2431	AAATTATTTTAAAGAAACAATGACATATCAAACTGCTTTTTACATGATTTTGAATAGAC	2490		
Query 841	TAGAAAGCTTTCCCTATAGACATATTAATTTCCAATCATAACTTTAATTCAAGAAATGCA	900		
Sbjct 2491	TAGAAAGCTTTCCCTATAGACATATTAATTTCCAATCATAACTTTAATTCAAGAAATGCA	2550		
Query 901	GTTTTACCaaaaa	918		
Sbjct 2551	GTTTTACCaaaaa	2568		

A BLAST match for Positive Clone 1 (query) to an mRNA of the *DCUN1D1* gene (subject). The alignment reached 99 % identity.

Homo sapiens defective in cullin neddylation 1 domain containing 1 (DCUN1D1), transcript variant 1, mRNA

Sequence ID: [NM_020640.4](#) Length: 7849 Number of Matches: 1

Range 1: 13 to 171 [GenBank](#) [Graphics](#)
[▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
289 bits(156)	2e-75	158/159(99%)	0/159(0%)	Plus/Plus
Query 69	GCCGGGGGAGGAGGGGAGAGGCCCTGGAGGACCAACATGAACAAGTTGAAATCATCGCA			128
Sbjct 13	GCCGGAGGAGGAGGGGAGAGGCCCTGGAGGACCAACATGAACAAGTTGAAATCATCGCA			72
Query 129	GAAGGATAAAGTTCGTCAAGTTTATGATCTTCACACAATCTAGTGAAAAACAGCAGTAAG			188
Sbjct 73	GAAGGATAAAGTTCGTCAAGTTTATGATCTTCACACAATCTAGTGAAAAACAGCAGTAAG			132
Query 189	TTGTCTTTCTCAAAATGACTGGGAAGTTAGATGTTGCAAC			227
Sbjct 133	TTGTCTTTCTCAAAATGACTGGGAAGTTAGATGTTGCAAC			171

FIGURE A.8: Top BLAST result for the forward sequencing primer of Positive Clone 2.

A BLAST match for Positive Clone 2 (query) to an mRNA of the *DCUN1D1* gene (subject). The alignment reached 99 % identity. The sequencing read of this primer was of low quality, so only a short section was robust.

Homo sapiens defective in cullin neddylation 1 domain containing 1 (DCUN1D1), transcript variant 2, mRNA
Sequence ID: [NM_001308101.2](#) Length: **7895** Number of Matches: **1**

Range 1: 1652 to 2568 [GenBank](#) [Graphics](#) [▼ Next Match](#) [▲ Previous Match](#)

Score	Expect	Identities	Gaps	Strand
1661 bits(899)	0.0	912/918(99%)	1/918(0%)	Plus/Plus
Query 1	GTCTACATTATGCACTACATAAATTGGACTTCATTATGCTTTTGAAATGCTTATCTGCCTG	60		
Sbjct 1652	GTCTACATTATGCACTACATAAATTGGACTTCATTATGCTTTTGAAATGCTTATCTGCCTG	1711		
Query 61	TCACATAAGTTAAACTATTTAATTGTTTTGAATGTTTTGGATTGCTACACAATACAATA	120		
Sbjct 1712	TCACATAAGTTAAACTATTTAATTGTTTTGAATGTTTTGGATTGCTACACAATACAATA	1771		
Query 121	TTCTAAATTTAGGCATGAGGGttttttttgtttttttttacttttttttGTCATCGCA	180		
Sbjct 1772	TTCTAAATTTAGGCATGAGGG-tttttttgtttttttttacttttttttGTCATCGCA	1830		
Query 181	CTATGGAAACACAAATGGAGTTCTCTTAATTTATAAGAAAGATAGTTGCAGTTAAATTTTGA	240		
Sbjct 1831	CTATGGAAACACAAATGGAGTTCTCTTAATTTATAAGAAAGATAGTTGCAGTTAAATTTTGA	1890		
Query 241	AAATGGTTGTAATGAGCCATGAAGTTCAATCTTTATAATATAGGTAAGTCTTTTCAGAC	300		
Sbjct 1891	AAATGGTTGTAATGAGCCATGAAGTTCAATCTTTATAATATAGGTAAGTCTTTTCAGAC	1950		
Query 301	AAATAGTCCATTTTCGATGACTTATTATTTTGTGAAATTGCTTTAACTGCTAATCACTG	360		
Sbjct 1951	AAATAGTCCATTTTCGATGACTTATTATTTTGTGAAATTGCTTTAACTGCTAATCACTG	2010		
Query 361	TGGTTGCTAAATTTTACTTCAGGAGCAAAGATTTTCAACAAGCATACACGATGCAAAA	420		
Sbjct 2011	TGGTTGCTAAATTTTACTTCAGGAGCAAAGATTTTCAACAAGCATACACGATGCAAAA	2070		
Query 421	TACCAATCTGGCTCTTAGTCTCTTACTGTTTTCGTTTTCACTCAGATTAGCTCAGTTTTC	480		
Sbjct 2071	TACCAATCTGGCTCTTAGTCTCTTACTGTTTTCGTTTTCACTCAGATTAGCTCAGTTTTC	2130		
Query 481	TCATCAAAGCAGAACTATCTTGATGTATtttttttCATTACAAGCCCCATGAGCTGCT	540		
Sbjct 2131	TCATCAAAGCAGAACTATCTTGATGTATtttttttCATTACAAGCCCCATGAGCTGCT	2190		
Query 541	TTTATGCTGAAATGGTCATTCCCTGTTCACTTACTGACATGTGAAGAAAGGTTTCTTG	600		
Sbjct 2191	TTTATGCTGAAATGGTCATTCCCTGTTCACTTACTGACATGTGAAGAAAGGTTTCTTG	2250		
Query 601	CTTTCTTAAACATTTCCGTAAAGGCAGGCTAGAAATGTAATACTTCAATGTTTGATGATT	660		
Sbjct 2251	CTTTCTTAAACATTTCCGTAAAGGCAGGCTAGAAATGTAATACTTCAATGTTTGATGATT	2310		
Query 661	ATGGTCTTTTGATAGGAATAGATTCTGCTTGGGATATATATCCAGGCACTCTCTAAGGTC	720		
Sbjct 2311	ATGGTCTTTTGATAGGAATAGATTCTGCTTGGGATATATATCCAGGCACTCTCTAAGGTC	2370		
Query 721	TAGGGTTGATATTAAACAAAGGAATGTACTTAGAATAGCAGTACATTTTATGCAAAATATGG	780		
Sbjct 2371	TAGGGTTGATATTAAACAAAGGAATGTACTTAGAATAGCAGTACATTTTATGCAAAATATGG	2430		
Query 781	AAATTATTTTAAAGAAACAATGACATATCAAAACTGCTTTTACATGATTTTGAAATAGAC	840		
Sbjct 2431	AAATTATTTTAAAGAAACAATGACATATCAAAACTGCTTTTACATGATTTTGAAATAGAC	2490		
Query 841	TAGAAAGCTTTCCCTATAGACATATTAATATTCCAATCATAACTTTAATTCAAGAAATGCA	900		
Sbjct 2491	TAGAAAGCTTTCCCTATAGACATATTAATATTCCAATCATAACTTTAATTCAAGAAATGCA	2550		
Query 901	GTTTTACCAaaaaaaaaa	918		
Sbjct 2551	GTTTTACCAAAAGAAAAA	2568		

FIGURE A.9: Top BLAST result for the reverse sequencing primer of Positive Clone 2.
A BLAST match for Positive Clone 2 (query) to an mRNA of the *DCUN1D1* gene (subject). The identity of this alignment reached 99 %.

Homo sapiens cDNA, FLJ95287, highly similar to Homo sapiens RP42 homolog (RP42), mRNA
 Sequence ID: [AK314480.1](#) Length: **809** Number of Matches: **1**

Range 1: 45 to 356 [GenBank](#) [Graphics](#)

▼ [Next Match](#) ▲ [Previous Match](#)

Score	Expect	Identities	Gaps	Strand
577 bits(312)	5e-162	312/312(100%)	0/312(0%)	Plus/Plus
Query 79	TCATCGCAGAAGGATAAAGTTCGTCAGTTTATGATCTTCACACAATCTAGTGAAAAACA	138		
Sbjct 45	TCATCGCAGAAGGATAAAGTTCGTCAGTTTATGATCTTCACACAATCTAGTGAAAAACA	104		
Query 139	GCAGTAAGTTGTCTTTCTCAAAATGACTGGGAAGTTAGATGTTGCAACAGATAATTTTTTC	198		
Sbjct 105	GCAGTAAGTTGTCTTTCTCAAAATGACTGGGAAGTTAGATGTTGCAACAGATAATTTTTTC	164		
Query 199	CAAAATCCTGAACCTTATATACGAGAGAGTGTAAGGATCATTGGACAGGAAGAAGTTA	258		
Sbjct 165	CAAAATCCTGAACCTTATATACGAGAGAGTGTAAGGATCATTGGACAGGAAGAAGTTA	224		
Query 259	GAACAGCTGTACAATAGATACAAAGACCCCTCAAGATGAGAATAAAATTGGAATAGATGGC	318		
Sbjct 225	GAACAGCTGTACAATAGATACAAAGACCCCTCAAGATGAGAATAAAATTGGAATAGATGGC	284		
Query 319	ATACAGCAGTTCTGTGATGACCTGGCACTCGATCCAGCCAGCATTAGTGTGTTGATTATT	378		
Sbjct 285	ATACAGCAGTTCTGTGATGACCTGGCACTCGATCCAGCCAGCATTAGTGTGTTGATTATT	344		
Query 379	GCATGGAAGTTC 390			
Sbjct 345	GCATGGAAGTTC 356			

FIGURE A.10: Top BLAST result for the forward sequencing primer of Positive Clone 3.

A BLAST match for Positive Clone 3 (query) to a cDNA annotated as RP42 (subject). RP42 is another name for DCNL1. This sequencing read was of lower quality so not as much sequence was recovered; however, the identity match of this alignment is 100 %.

FIGURE A.11: **Top BLAST result for the reverse sequencing primer of Positive Clone 3.**
A BLAST match for Positive Clone 3 (query) to an cDNA encoding DCN1 (subject).(subject). The identity of this alignment reached 99 %.

FIGURE A.12: Alignment of Positive Clone sequencing reads with the *DCUN1D1* gene. The dashed black box on the *DCUN1D1* gene represents the region annotated as the exon