

UC Berkeley

UC Berkeley Electronic Theses and Dissertations

Title

Genetic and Proteomic Dissection of the Macrophage Response to Mycobacterium tuberculosis Infection

Permalink

<https://escholarship.org/uc/item/57p6f35t>

Author

McCall, Rita Mae

Publication Date

2024

Peer reviewed|Thesis/dissertation

Genetic and Proteomic Dissection of the Macrophage Response to *Mycobacterium tuberculosis* Infection

By

Rita McCall

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Microbiology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Jeffery Cox, Chair

Professor Britt Glaunsinger

Professor Daniel Portnoy

Professor Sarah Stanley

Spring 2024

Abstract

Genetic and Proteomic Dissection of the Macrophage Response to *Mycobacterium tuberculosis* Infection

By

Rita McCall

Doctor of Philosophy in Microbiology

University of California, Berkeley

Professor Jeffery Cox, Chair

During *Mycobacterium tuberculosis* infection, macrophages serve key functions as both a niche for bacterial growth and as potent inducers of inflammation to influence the outcome of *M. tuberculosis* infection. After macrophages phagocytose *M. tuberculosis*, the permeabilization of the phagosomal membrane by ESX-1 signals the start of a battle between antimicrobial host responses and subversion of these defenses by the bacteria. To further characterize the macrophage responses to the *M. tuberculosis* infection, we previously conducted a large post-translational modification (PTM) proteomics experiment that identified proteins modified in response to infection. Here I investigate a host protein, MYCBP2, that is ubiquitinated and phosphorylated during *M. tuberculosis* infection in primary macrophages. I found that MYCBP2 deficient macrophages are more susceptible to *M. tuberculosis* and express less inflammatory cytokines during infection and in response to stimulus via a p38 MAPK-dependent mechanism. I also provide the first evidence that MYCBP2 can regulate viral growth in macrophages. As a complementary approach to our PTM proteomics data, I used proximity-based labeling to identify host proteins recruited to the phagosomal membrane after membrane permeabilization. In this dissertation, I present multiple approaches to conducting proximity labeling experiments during *M. tuberculosis* infection and explore some proteins of interest arising from these datasets. Lastly, I explore the possible role of RNF213 in *M. tuberculosis* infection. This dissertation adds to our knowledge of how the macrophage responds to infection by *M. tuberculosis*.

Table of Contents

Dedication.....	ii
Acknowledgements.....	iii
Chapter 1: An introduction to the molecular interactions of <i>M. tuberculosis</i> and the macrophage.....	1
Chapter 2: The role of MYCBP2 in inflammation and infection.....	6
Chapter 2 Introduction.....	6
Chapter 2 Results.....	8
Chapter 2 Discussion.....	11
Chapter 2 Methods.....	13
Chapter 2 Figures.....	17
Chapter 3: Proximity labeling of the <i>M. tuberculosis</i> phagosome.....	26
Chapter 3 Introduction.....	26
Chapter 3 Results.....	28
Chapter 3 Discussion.....	30
Chapter 3 Methods.....	33
Chapter 3 Figures and tables.....	39
Chapter 4: A reflection on the unique ubiquitin ligase RNF213.....	57
Chapter 4 Introduction and results.....	57
Chapter 4 Discussion.....	57
Chapter 4 Methods.....	58
Chapter 4 Figures.....	60
Chapter 5: Conclusions and speculations.....	61
References.....	63

*To my parents,
who taught me how to do difficult things*

Acknowledgments

A motto oft repeated at my alma mater was, “Science is a collaborative effort.” Though in college my friends and I recited it to poke fun at our department handbook, it rings very true as I sit down to write my acknowledgments. Throughout my six years at Berkeley, I experienced several challenges including a global pandemic with lab shutdowns and shift work, a six-week UAW strike, many distressing global events, and personal ups and downs. Overcoming these challenges was only possible with the support of my community at Berkeley and beyond.

First I would like to thank my advisor, Jeff Cox, whose endless optimism and love for science was a source of inspiration throughout my PhD. I’m appreciative of Jeff for giving me the freedom to drive my project while also providing support anytime I needed it. I have thoroughly enjoyed being a member of the Cox Lab thanks to many wonderful people who have passed through over the years. I’ll reflect fondly on the countless Jupiter excursions, entertaining lunchtime chats, and fun conferences and lab retreats. I am grateful for all the amazing SRAs and undergraduates we had over the years who kept the lab running smoothly, especially superstars Laura Britt, Linda Del Cid, Jessica Robles, and Daniel Licht. I am lucky to have overlapped with Christine Qabar, Ellie Wouters, and Chris Rae who have been excellent labmates. I’m thankful to my undergraduate mentee of four semesters, Sena Gul, for being an A+ scientist and making mentoring such a fun experience. I am especially grateful to Huntly Morrison, Guillaume Golovkine, and Allison Roberts for all of the help and mentorship they provided throughout my PhD. Huntly taught me so much about how to navigate grad school, design a project, and enjoy life while being a grad student. Guillaume taught me most of what I know about working with mice and tuberculosis and was always so generous with his time when I had questions. Lastly, Allison Roberts was a superb mentor to me throughout my entire PhD. She has spent so much time listening to my ideas, providing expert advice, and modeling how to be a careful, thorough scientist.

Outside of my lab, I am grateful to members of my qualifying and thesis committees for providing their time and support throughout my graduate school career: Sarah Stanley, Dan Portnoy, Britt Glaunsinger, Matt Welch, and Michi Taga. I am also grateful to have spent weekly lab meetings with the Stanley and Vance labs who provided valuable feedback throughout the years. I am also thankful for my scientific mentors from college and the NIH who I continued to lean on for support during graduate school: Leslie Gregg-Jolly, Shannon Hinsaleasure, Robin Shields-Cutler, Stephen Leppla, and Andrei Pomerantsev. Leslie and Shannon have been excellent role models for me as women in STEM. As the first person in my family to earn a traditional four-year degree and also the first to attend graduate school, I relied heavily on Leslie and Shannon for advice throughout college and afterward. I would not have been here without them.

I'm thankful for my cohort, especially Nicholas Karavolias, Clare Lou, Erin Baggs, and Lorenzo Washington, for being a great support network, especially as we adjusted to Berkeley. Special thanks to Nicholas for being my rock during the first year of grad school, making Berkeley instantly feel like home, and for trusting me to pierce his ears. I'm also very grateful for the "Immuno Girls" in MCB (Shaina Carroll, Victoria Chevee, Kayla Dinshaw, Victoria Rael, Lisa Strong, and Elizabeth Turcotte) for adopting me, sharing snacks on the picket line, always coming through with reagents, Bachelor nights, and being great sounding boards throughout this whole process.

I'm so thankful to have had a community outside of the university to help me escape the grad school bubble during my time here. Thanks to Kaitlyn Zhou, Dexter Crowley, Laurel Moffat, Ty Dewey, Noah Schlager, Felipe Torres, Jacqueline Torres, and Prakriti Shrestha for all the adventures throughout the years. I am also so lucky to have had my college best friend, Malachi Wickman, here in the Bay for most of my PhD. Malachi has always been one of my biggest supporters and grad school was made so much better by our dog walks, baking days, and many rant sessions. I'm also thankful for my friends who supported me from afar during my degree: Nicole Potchen, Ivy Kuhn, Queenster Nartey, Angela Wang, Sam Murphy, Sarah Corsa, Toni Ray, Emma Lange, Emily Howe, and Joey Kathan.

I would not be here at Berkeley today without the support and encouragement of my family Annie, Jim, and Bretta. My parents worked so very hard to give my sister and me the opportunities they never dreamed of for themselves. Their hard work and perseverance are why I was able to explore academia in the first place. They have been my biggest cheerleaders throughout my academic journey, always celebrating my wins and comforting me during the losses. Though unfamiliar with science and academia, they helped me so much with their frequent check-ins and encouragement.

Lastly, I have so much gratitude for my little Berkeley family: my sweet pup, Olive, and my incredible boyfriend, Nathan. I adopted Olive before my fourth year and she has been a source of love, peace, and joy every day since. Thanks to Olive for giving me a reason to leave the lab and loving me even when I had to work past dinner time. And Nathan! Nathan and I met at the beginning of my second year of grad school and he has been there for me every day since. Thanks to Nathan for the pep talks, rides to the lab, serving as my audience for practice presentations, picking up my slack when needed, taking me on amazing trips, and cheering me up on the bad days. I am so lucky to have had such a supportive partner these last five years. I'm not sure how other people get through a PhD without a Nathan by their sides.

Chapter 1: An introduction to the molecular interactions of *M. tuberculosis* and the macrophage

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB), is the world's deadliest infectious disease. In 2022 alone, approximately 1.3 million people died from TB¹. Both in the U.S. and globally, TB is a disease of inequality. In the U.S. the disease disproportionately affects people of color and the incarcerated population and abroad lower and middle-income countries have a much higher disease burden². Current antibiotic regimens for TB are lengthy and have low compliance and the vaccine only protects against some forms of the disease. Therefore, there exists a considerable need for therapeutics to treat TB. The bacterium is unique and therapeutics face a unique challenge due to its distinctive cell wall and slow replication time. Fundamental research into the bacterium and its interaction with its host is critical for gaining new insights into its biology and future therapeutic development.

A hallmark of TB infection is the granuloma, an organized collection of cells in the lung where *M. tuberculosis* resides. This is composed of macrophages, epithelioid cells, neutrophils, dendritic cells, B-cells, and T-cells³. *In vivo*, macrophages are the first cells that encounter *M. tuberculosis*⁴ and exist both as a niche and antagonist of the bacterium through the eventual recruitment of CD4+ T-cells which activate macrophages to kill the bacteria⁵. Once phagocytosed, *M. tuberculosis* uses a type VII secretion system, ESX-1, to permeabilize the phagosome which sets off several host cell responses⁶. Below we highlight some key interfaces between pathogen and host in the macrophage.

Phagosome Maturation Arrest

First observed in 1971⁷, phagosome maturation arrest is a pivotal step in the pathogenesis of *M. tuberculosis*. Normally, upon phagocytosis, foreign objects are phagocytosed and phagosomes are fused with increasingly acidic endosomes, and eventually the lysosome where the contents are recycled by the cell. *M. tuberculosis* prevents the fusion of a small GTPase, Rab7, from binding and excludes vacuolar ATPase (vATPase), which normally acidifies the phagosomal compartment^{8,9}. This inhibits fusion with late endosomes and acidification of the phagosomal compartment, ultimately allowing the survival of *M. tuberculosis*. *M. tuberculosis* utilizes many effectors to stop this essential process. For example, the mycobacterial protein PtpA has been demonstrated to bind directly to host vATPase and thereby prevent its association with the phagosomal compartment¹⁰. Another mycobacterial protein, SapM, can remove PI3P from the phagosomal membrane which is important for proper endosomal processing^{11,12}. There are many other known effectors in addition to the aforementioned proteins but the exact mechanism of phagosome maturation arrest continues to be defined.

Pattern Recognition Receptors of *M. tuberculosis*

Permeabilization of the phagosome leads to the detection of the bacterium by cytosolic sensors and pattern recognition receptors^{13–17}. *Ex vivo* in murine macrophages, the NLRP3 and AIM2 inflammasomes lead to activation of IL-1 β which is critical for control of *M. tuberculosis* infection *in vivo*¹⁸. *M. tuberculosis* activates NLRP3 during infection via plasma membrane damage but NLRP3-deficient mice can control pulmonary TB^{19–21}. Another inflammasome, AIM2, was initially detected as a sensor for *M. tuberculosis* DNA in murine macrophages and an activator of IL-1 β ¹⁶. The authors also demonstrated that AIM2-deficient mice succumbed quickly to infection. However, in human macrophages, AIM2 induced by *M. tuberculosis* does not lead to IL-1 β induction²². It's still unclear to what extent these inflammasomes matter in the context of human TB infection.

Another important cytosolic sensor of *M. tuberculosis* is cGAS which senses mycobacterial dsDNA in the macrophage. Mice lacking cGAS are still able to control *M. tuberculosis* infection, indicating other sensors are also required to control infection. Production of c-di-GMP by cGAS activates STING which in turn activates autophagy and induction of type I interferon¹³. We expand upon the role of autophagy below. Type I interferon is induced in *M. tuberculosis*-infected macrophages via ESX-1²³. Though type I interferons cannot restrict *M. tuberculosis* growth *ex vivo*, this signaling pathway promotes mycobacterial growth *in vivo*^{24,25}. Importantly, it's still not understood how mycobacterial dsDNA is released from the bacterium and sensed by cGAS. *M. tuberculosis* also activates the cytosolic sensor NOD2 which senses MDP after membrane permeabilization and is important for long-term control of *M. tuberculosis* infection *in vivo* and inflammatory cytokine release^{26–28}.

RIG-I and MDA5 are well-studied RNA cytosolic sensors that have also been implicated in the sensing of *M. tuberculosis*. RIG-I and its activation of MAVS are required for complete type I interferon induction *ex vivo* and control of infection *in vivo*²⁹. The sensor MDA-5 has also been demonstrated to sense *M. tuberculosis* RNA and appears to provide an advantage to the bacteria as activated macrophages and mice lacking the sensor have decreased *M. tuberculosis* growth³⁰. Both of these RNA sensors can induce type I interferon similar to cGAS³⁰.

Finally, toll-like receptors (TLR) are also important in sensing *M. tuberculosis*. TLR2, TLR4, and TLR9 all regulate host response to infection. TLR2 can sense multiple *M. tuberculosis* antigens and is important for differentiation into a Th1 response³¹. Similarly, TLR4 also detects multiple components of *M. tuberculosis* though its role is less clear and it seems to regulate innate immune signaling in a manner that benefits both bacteria and host³². TLR9 senses mycobacterial DNA and functions with TLR2 to regulate Th1 responses³³.

Autophagy

Autophagy refers to the process by which the cell encapsulates cargo in a double membrane-bound vesicle which is then delivered to the lysosome for degradation. Selective autophagy of pathogens, termed xenophagy, is an important method of host control of infection that some bacteria have evolved to target³⁴. Autophagy was first proposed to play a role in the control of mycobacteria in 2004 when studies with *Mycobacterium tuberculosis* variant *bovis* BCG found an increase in autophagy targeting of bacteria under autophagy-inducing starvation conditions³⁵. Later work demonstrated that cytosolic sensing of extracellular *M. tuberculosis* DNA targets the bacteria to autophagy after phagosome membrane permeabilization by ESX-1^{13,36}. A subpopulation of *M. tuberculosis* containing permeabilized phagosomes is targeted by ubiquitin ligases Parkin, Smurf1, and TRIM16^{37–39} as well as cytosolic β -galactoside-binding receptors^{17,40–42}. These proteins recruit autophagy adaptors which bind LC3 leading to phagophore formation and eventual degradation^{36,37,43,39}. Canonical autophagy genes restrict *M. tuberculosis* growth *ex vivo*^{36,44,45} in human and murine macrophages.

Although it is clear autophagy restricts *M. tuberculosis* growth at the macrophage level, the role of autophagy *in vivo* has been hotly contested. One of the first canonical autophagy genes tested *in vivo* was Atg5. Mice lacking this gene succumbed quickly to *M. tuberculosis* infection, indicating a role of autophagy *in vivo* during *M. tuberculosis* infection^{36,46}. A few years later, a study of additional core autophagy genes suggested they were not required to control *M. tuberculosis* infection⁴⁷, and thus autophagy per se did not play an important role *in vivo*⁴⁸. However, with more thorough gene editing and longer survival experiments, our group demonstrated that canonical autophagy is important for long-term control of *M. tuberculosis* infection⁴⁵. At the macrophage level, many questions remain as to how exactly autophagy plays a role in infection. Why is only a population of bacteria targeted to autophagy? What are the targets of the ubiquitin ligases? Are additional ubiquitin ligases and autophagy adaptors needed for targeting *M. tuberculosis* to autophagy?

ESCRT and Membrane Repair

ESCRT machinery comprises four complexes ESCRT-0, -I, -II, -III, and additional accessory proteins. These complexes work sequentially to deliver ubiquitylated substrates to lysosomes via multivesicular bodies^{49,50}. It is also involved in endolysosomal membrane repair⁵¹. In 2004, researchers found that Hrs, a member of the ESCRT-0 complex, was excluded from the phagosomal membrane by pathogenic mycobacteria⁵². Another study found that ESCRT proteins were required to restrict mycobacteria growth in *Drosophila* cells⁵³. However, both of these studies were performed with bacteria other than *M. tuberculosis* and it is unclear what role ESCRT played during actual *M. tuberculosis* infection. More recent work found that cells lacking

ESCRT proteins Hrs and Tgs101 are more susceptible to *M. tuberculosis* infection⁵⁴ confirming ESCRT's importance in pathogen control. Building on this finding, researchers found that 80% of bacteria were colocalized with ESCRT-III proteins 3 hours post-infection, but cells infected with Δ esxA *M. tuberculosis* lacking a functional ESX-1 system only had around 20% colocalization⁵⁵, indicating ESCRT recruitment was dependent on phagosome permeabilization. The authors also found that *M. tuberculosis* antagonizes ESCRT-III recruitment to the phagosome via the ESX-3 effector. Besides recruitment to the phagosome, ESCRT-0 and -I proteins have previously been described to play a role in antigen presentation during *M. tuberculosis* infection⁵⁶ demonstrating the importance of these proteins in the outcome of infection. Recently, groups have shown that ESCRT can repair small ruptures in the phagosomal membrane early in *Mycobacteria* infection^{21,57}.

Reactive Nitrogen and Oxygen Species

A key macrophage function during *M. tuberculosis* is the production of nitric oxide (NO) and ROS. These small molecules directly antagonize the bacterium. Macrophages produce NO when activated by IFN- γ . NO is synthesized by the gene *Nos2* and is mycobactericidal in macrophages⁵⁸. *Nos2* deficient mice are extremely susceptible to *M. tuberculosis*, succumbing to infection within 50 days post-infection⁵⁹. *M. tuberculosis* has evolved mechanisms to limit NO damage via its proteasome⁶⁰.

The host produces ROS via the NADPH oxidase, mitochondria, and peroxisome to kill intracellular pathogens. Recent work found human macrophages lacking the peroxisome, which produces ROS in the cytosol, are more susceptible to *M. tuberculosis* infection in an ESX-1-dependent manner⁶¹. Components of the phagosomal associated NADPH complex are required for *M. tuberculosis* resistance in mice early in infection but loss of these genes does not impact the outcome of infection⁶². On the bacterial side, *M. tuberculosis* has evolved defenses to these ROS, one being the enzyme KatG that directly detoxifies ROS⁶³. Additional studies need to be done to clarify the exact role of ROS in *M. tuberculosis* infection.

Macrophage Inflammatory Signals

Though cytokines and chemokines cannot restrict *M. tuberculosis* growth in the macrophage, they are essential for recruiting other innate and adaptive immune cells during infection. Cytokines and chemokines secreted by infected macrophages include but are not limited to; TNF- α , IL-6, IL-12 family, IL-23, IL-10, IL-1 β , CCL2, CCL3, CCL4, CCL5, MIP-1 proteins, and RANTES⁶⁴. Though all play important roles during infection, some are of particular importance. TNF- α is a key cytokine and a potent activator of inflammation. Its secretion by macrophages regulates antimicrobial peptide production and chemokine secretion in macrophages and can also regulate programmed cell death^{65,66}. As additional evidence of the role of TNF signaling in TB pathogenesis, mice

lacking the TNF receptor, TNFR1, on their monocytes and macrophages cannot control *M. tuberculosis* infection⁶⁷. Another key cytokine is IL-1 β which is released by inflammasomes and caspase-independent mechanisms during infection. Macrophages express IL-1 β upon *M. tuberculosis* infection and this expression can be suppressed by induction of type I interferon⁶⁸. *In vivo*, IL-1 β deficient mice succumb quickly to infection⁶⁹. A disruption in inflammatory signaling can have severe consequences for the host.

Conclusion

The macrophage response to *M. tuberculosis* is complex as the host employs many mechanisms to thwart infection. Here we present some key players in the macrophage response to *M. tuberculosis*, however, more exist that were beyond the scope of this review including changes in metabolism and utilization of lipid droplets. In this dissertation, I present three projects to further define macrophage-mediated control of infection. In the first project, I identify a protein that is post-translationally modified in response to infection and limits *M. tuberculosis* growth. This protein is also a mediator of inflammatory signaling states in the macrophage, though how this connects to *M. tuberculosis* infection is unclear. In the second project, I use a proximity-based labeling strategy to identify host proteins recruited to the phagosome during *M. tuberculosis* infection. And lastly, I present a brief exploration of the possible role of unique ubiquitin ligase in *M. tuberculosis* infection.

Chapter 2: The role of MYCBP2 in inflammation and infection

Additional contributors: Sena N. Gul¹, Allison W. Roberts¹, Michael Cronce², Huntly M. Morrison¹, Guillaume Golovkine¹, Jeffery S. Cox¹

¹Department of Molecular and Cell Biology, University of California, Berkeley ²Department of Bioengineering, University of California, Berkeley

The following work is currently in preparation as a manuscript for submission to a journal.

INTRODUCTION

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB), is a leading cause of death worldwide. In 2022, approximately 10 million people acquired a TB infection and 1.3 million people died of the disease¹. Upon infection of the host, *M. tuberculosis* initially resides in phagosomes inside alveolar macrophages⁴. Once phagocytosed by the alveolar macrophage, *M. tuberculosis* replicates inside the cell and disseminates to other macrophages, neutrophils, and dendritic cells^{70–74}. Macrophages are a key niche for *M. tuberculosis* replication and therefore the bacterium has evolved many ways to thwart the phagocyte's anti-microbial defenses, such as phagosome maturation arrest⁷. Macrophages are also highly important for host survival during infection and as mediators of the immune response. Decisions between hyper-inflammatory necrosis and anti-inflammatory apoptosis are controlled at the macrophage level which has outcomes on host survival^{75,76,73}. Additionally, macrophages are not only the initial replicative niche of *M. tuberculosis* but also their executioners as they induce T-cells to make IFN- γ and consequently kill the bacteria^{77–79}. Given its importance, much work has been focused on deciphering the macrophage transcriptional response to *M. tuberculosis* infection. Recent work in our lab used post-translational proteomics to identify host proteins modified in response to *M. tuberculosis* infection⁸⁰. We reasoned that monitoring changes at the post-translational level could uncover novel host responses as some known mechanisms of control, such as autophagy, are primarily regulated at the post-translational level. In a preliminary analysis of proteins that were phosphorylated and/or ubiquitylated in response to *M. tuberculosis* infection, we identified a ubiquitin ligase, MYCBP2, that was both phosphorylated and ubiquitylated over the course of a 24-hour macrophage infection, suggesting its activity is altered during innate immune responses.

Although MYCBP2 has not been implicated previously in host control of *M. tuberculosis* infection it has been well studied for its ubiquitin ligase-dependent and independent activities in different species. Also known as Phr1 and Pam in mice and humans, MYCBP2 is conserved across many organisms. Its studied orthologs are highwire in *Drosophila*, RPM-1 in *Caenorhabditis elegans*, and Esrom in Zebrafish. It is a large ubiquitin ligase, unique in its noncanonical ubiquitylation of threonines instead of lysines⁸¹. MYCBP2 regulates axon degeneration and synapse development in mouse

neurons^{82–84} and orthologs of MYCBP2 have been found to have similar functions^{85–88}. MYCBP2 has also been shown to regulate mTOR^{89,90}, p38 MAPK⁹¹, cAMP^{92,93}, and ephrin signaling⁹⁴ in various cell types. Though studied most extensively in neurons, MYCBP2 is highly expressed in both the brain and thymus and to a lesser extent, peripheral blood leukocytes⁹⁵. MYCBP2's role in cells other than neurons remains mysterious. A key finding supporting its role in immunity was that MYCBP2 deficient macrophages are more anti-inflammatory in response to innate immune stimulus⁹⁶. Other studies also point to an inflammatory role of MYCBP2 in cells beyond the macrophage. For example, a similar pro-inflammatory role for MYCBP2 was found in a breast cancer cell line⁹⁷ and another group found a correlation between high MYCBP2 expression and immune cell infiltration in thyroid cancer⁹⁸. Macrophage-mediated control of inflammation plays a role in immunity and other pathologies, including cancer. Though some work has been done on investigating MYCBP2's role in inflammation, no studies have investigated its role in infection. Interestingly, MYCBP2 has been found in multiple host-virus studies as directly interacting with viral proteins^{99–102} indicating a possible antiviral role, though this idea has not yet been tested.

The inflammatory signaling potential of macrophages was initially proposed as a binary distinction between pro-inflammatory (M1) versus anti-inflammatory (M2) conditions. In reality, inflammatory states exist across a spectrum of cellular states that represent differences in cytokines, chemokines, and metabolism^{103–105}. Though research has uncovered the true heterogeneity of macrophage signaling, M1/M2 categorization is still useful for classifying macrophage phenotypes. Historically, M1 macrophages are associated with a damaging, or killing, response and M2 macrophages are associated with a healing response^{103,106}. Pro-inflammatory signals recruit adaptive immune cells leading to sustained inflammation and elimination of pathogens. Common pro-inflammatory macrophage markers include CD80, CD86, CD16, and iNOS. Anti-inflammatory signals dampen the immune response, promote tissue repair and angiogenesis, and express markers such as Arg1, CD206, and IL-10¹⁰⁷. Given the pressures from pro-inflammatory signaling, some pathogens have evolved to exploit the macrophage's signaling state. For example, *Salmonella typhimurium* inhibits NADPH oxidase¹⁰⁸, *Brucella* activates STAT6¹⁰⁹, and *Coxiella burnetii* stimulates macrophages to produce an M2 response¹¹⁰. In *M. tuberculosis* infection, both pro-and anti-inflammatory cells are present in the granuloma¹¹¹ and M1 macrophage signaling is associated with a protective response against infection^{112–115}. Early in infection, pulmonary macrophages isolated from mice express M1 markers, while later in infection they express more M2 markers¹¹⁶. Similarly, monocytes isolated from TB patients were found to be more M2-like¹¹⁷. Some *M. tuberculosis* proteins have been shown to polarize macrophages towards an M2 phenotype^{118,119}. Balance of inflammation in TB disease is crucial to control and the mechanism by which the host mediates this balance is still not entirely clear.

Given its modification in response to *M. tuberculosis* infection and its implication in macrophage signaling, we asked whether MYCBP2 was important for the control of *M. tuberculosis* infection. Here we report that MYCBP2 deficient cells exhibited decreased control of *M. tuberculosis* *ex vivo* and increased susceptibility to infection *in vivo*. We also demonstrated that MYCBP2-deficient cells are less susceptible to murine norovirus. Our further studies of the role of MYCBP2 uncovered additional evidence of its control of inflammatory states. Our work highlights additional functions of MYCBP2 in the macrophage and demonstrates the first evidence of its role in pathogen control.

RESULTS

MYCBP2 is required for control of *M. tuberculosis* but not *L. monocytogenes* *ex vivo*

Our previous proteomics work identified many macrophage proteins post-translationally modified in response to infection with *Mycobacterium tuberculosis*⁸⁰. To begin to identify new factors that affect *M. tuberculosis* resistance, we carried out a directed CRISPR/Cas9 screen on genes encoding a subset of the modified host factors including *Mycbp2*, *Ifi205a*, *Tmem230*, *Tdpoz3*, and *Tnfaip3*. We found that cells lacking a ubiquitin ligase, MYCBP2, were more susceptible to *Mycobacterium marinum* infection (Supplemental Figure 1). MYCBP2 had changes in both phosphorylation and ubiquitylation during *M. tuberculosis* infection. To investigate MYCBP2's function during infection, we edited conditionally immortalized macrophages (CIMs)¹²⁰ and infected them using *M. tuberculosis* expressing the *luxBCADE* operon. We found that MYCBP2 knockout (KO) CIMs had an increase in bacterial growth over the course of infection (Figure 1A). To independently test the role of MYCBP2, we infected primary macrophages from MYCBP2-deficient mice. Because MYCBP2 is embryonically lethal¹²¹ we used floxed MYCBP2 mice⁸² that have cre expression under the control of the *LysM* promoter. We also measured an increase in bacterial growth in *Mycbp2^{fl/fl}*, *LysMcre^{+/+}* CFU from macrophages compared to *Mycbp2^{fl/fl}*, *LysMcre^{-/-}* controls (Figure 1B).

To test if MYCBP2 controls the replication of other intracellular bacterial pathogens, we infected BMDMs with *Listeria monocytogenes*. Compared to *M. tuberculosis*, *L. monocytogenes* *ex-vivo* infections occur over a much shorter period and the bacteria escape the phagosome and spread to neighboring cells using actin-based motility¹²². We compared bacterial growth inside WT and MYCBP2 deficient cells over the course of an 8-hour infection but did not detect differences in *L. monocytogenes* growth between the two (Figure 1C). Therefore, we conclude that MYCBP2-mediated control of infection *ex vivo* is likely not broadly restrictive of other intracellular pathogens and likely only specific to some pathogens, like *Mycobacteria*.

MYCBP2 deficient macrophages are resistant to MNV infection

Antiviral responses to *M. tuberculosis* are detrimental to host survival^{24,36,68,123} and we have previously identified cell intrinsic host factors that mediate the balance between anti-bacterial and anti-viral immunity in the context of *M. tuberculosis* infection^{124,125}. MYCBP2 has been previously identified as interacting with several viral proteins^{99–102}. Therefore, we hypothesized that loss of MYCBP2 could impact the host response to viral infection. We chose to test murine norovirus due to its ability to infect macrophages. Using MNV-1 we performed TCID50s with bone marrow macrophage cell lines and observed that MYCBP2 deficient cells had a decrease in viral burden 24 hours after infection (Figure 2A). Infected MYCBP2-deficient macrophages had a decrease in pro-inflammatory cytokines IL-12 and TNF- α , consistent with our *M. tuberculosis* data (Figure 2B). To test the possible role of MYCBP2 in virus entry, we evaluated virus levels early after infection and found no difference between the genotypes (Figure 2C). We next asked whether differences in replication could account for the growth differences between our genotypes. We used high-throughput microscopy to enumerate MNV replication complexes seven hours post-infection and observed no significant differences in replication (Figure 2D). Therefore, we concluded that the MYCBP2-dependent difference in MNV growth is independent of defects in replication or binding.

MYCBP2 deficient mice are more susceptible to *M. tuberculosis* infection

We next asked whether MYCBP2 played a role in pathogen resistance or susceptibility *in vivo*. As we had evidence the protein itself was post-translationally modified in response to *M. tuberculosis*, we chose to focus on this pathogen for our *in vivo* studies. To determine the *in vivo* role of MYCBP2 in *M. tuberculosis* resistance, we infected (1) WT, *LysMcre*^{+/+}, (2) *Mycbp2*^{fl/fl}, *LysMcre*^{-/-}, (3) *Mycbp2*^{fl/fl}, *LysMcre*^{+/-}, and (4) *Mycbp2*^{fl/f}, *LysMcre*^{+/+} mice with ~100 CFU of wildtype Erdman *M. tuberculosis*. We monitored both CFU burden in the lung and survival during infection. We included multiple copies of *LysMcre* as we and others had previously demonstrated that more than one copy of *LysMcre* can, at times, be required for efficient knockout of the target⁴⁵. Additionally, we included a control of WT mice with *LysMcre*^{+/+}, as *LysMcre* has been demonstrated to induce some type I interferon¹²⁶. We did not observe a difference in bacterial burden in the lungs between genotypes (Figure 3A). This differs from our *ex vivo* findings of an increase in *M. tuberculosis* growth in cultured macrophages. Differences between *ex vivo* and *in vivo* phenotypes are not surprising due to the extreme differences in environment between the two conditions. Despite no difference in CFU between the genotypes of mice, *Mycbp2*^{fl/fl}, *LysMcre*^{+/+} mice succumbed earlier to *M. tuberculosis* infection as compared to controls (Figure 3B), indicating MYCBP2 is required for control of *M. tuberculosis* infection. These results suggest that MYCBP2's role *in vivo* is predominantly immunomodulatory rather than participating directly in anti-bacterial killing in macrophages.

Inflammation during *M. tuberculosis* infection

Previous work identified MYCBP2 as a mediator of inflammatory responses in the macrophage⁹⁶. To test whether MYCBP2 deficient macrophages exhibited differences in inflammatory signaling during *M. tuberculosis* infection, we assessed cytokine levels secreted by infected macrophages using ELISAs and cytokine bead arrays. We observed that MYCBP2 deficient macrophages showed a decrease in secreted pro-inflammatory cytokines including IL-6 and TNF- α at 24 hours post-infection (Figure 4A). To ask whether these differences were maintained at the transcriptional level, we used a panel of markers for both pro- and anti-inflammatory genes. We demonstrated that MYCBP2 deficient macrophages had a decrease in pro-inflammatory mRNA levels at 24hpi (Figure 4B). MYCBP2 deficient cells exhibited a decrease in transcription of *Il6* and *Il1b* and an increase in transcription of *Il10* and *CD206*. Importantly, qPCR analysis indicated that these differences in cytokine levels were due to changes in mRNA levels of these genes, indicating that MYCBP2 plays a role in macrophage signal transduction during *M. tuberculosis* infection.

MYCBP2 inhibits cytokine response to TLR signaling

Having established that MYCBP2 deficient macrophages during *M. tuberculosis* infection had a decrease in pro-inflammatory cytokines, we asked whether this was pathogen-specific or more broadly indicative of macrophage activation states. To test this, we used cytokine bead arrays with TLR2, 3, and 4 agonists to profile a number of cytokines concurrently. We observed that in response to TLR2 and TLR4 stimulation, MYCBP2 deficient macrophages secreted lower levels of cytokines including IL-6, IL-27, TNF- α , and IL-1 α as well as an increase in the secretion of the anti-inflammatory cytokine IL-10 (Figure 5A-E). We also observed that MYCBP2-deficient macrophages had a decrease in IFN- β secretion in response to TLR3 stimulation (Figure 5F). We next used TLR4 agonist LPS to investigate transcriptional differences between MYCBP2 WT and deficient macrophages during stimulation at both 6 and 24 hours post-stimulation. We observed an enrichment in pro-inflammatory cytokines in the WT and an enrichment in anti-inflammatory cytokines in the deficient macrophages 24 hours post-stimulus (Figure 5G). This data demonstrates that MYCBP2 is involved in innate signaling broadly and functions to promote an anti-bacterial inflammatory response.

Autophagy does not play a role in MYCBP2's regulation of the inflammatory response

Previous work has suggested a role for MYCBP2 in regulating autophagy via ubiquitination of TSC2^{127,89}. Autophagy proteins have previously been demonstrated to regulate and be regulated by various cytokines¹²⁸. Therefore, we asked whether differences in autophagy induction were responsible for differences in cytokine

signaling. To identify possible differences in autophagy in MYCBP2 deficient macrophages, we induced starvation and investigated differences in phosphorylated p70S6K and LC3 lipidation, two readouts of mTOR function¹²⁹. We did not observe differences in LC3 lipidation or phosphorylation of p70S6K (Figure 6), suggesting that MYCBP2 deficient macrophages do not have differences in mTOR activation in macrophages.

MYCBP2 inflammatory signaling is mediated by p38 MAPK

MYCBP2 has previously been implicated in regulating p38 MAPK signaling^{130,131,91}. Given p38 MAPK's role in inflammatory signaling¹³², we asked whether MYCBP2 also regulates p38 MAPK in macrophages. We first investigated whether MYCBP2 deficient macrophages had a change in phosphorylation of p38 MAPK when treated with a TLR agonist. Using phospho-p38 antibodies in Western blots we did not measure a difference in phosphorylation of p38 MAPK upon LPS stimulation (Figure 7A). We next asked whether inhibiting p38 MAPK in WT and MYCBP2 deficient macrophages could rescue the difference in inflammatory signaling. We treated MYCBP2 deficient and WT BMMs with the p38 MAPK inhibitor SB203580 and stimulated with TLR2,3,4 agonists. We observed that differences in pro-inflammatory cytokines between WT and MYCBP2 deficient cells were greatly reduced in the presence of SB203580 (Figure 7B-F). These results suggest that inflammatory signaling deficiencies in MYCBP2 deficient cells are mediated by p38 MAPK signaling. However, we have not yet ruled out possible adverse effects of SB203580 accounting for some of these differences. Our findings differ from previous reports that MYCBP2 is a negative regulator of the p38 MAPK pathway in neurons.

DISCUSSION

Studies of the transcriptional changes in *M. tuberculosis*-infected macrophages have yielded key discoveries¹³³ but missed host processes controlled at the post-transcriptional level. Using our PTM proteomics data we previously uncovered an additional autophagy adaptor involved in the control of *M. tuberculosis*⁸⁰. Here we identify another post-translationally modified protein, MYCBP2, that plays a role in the control of infection.

Robust, pro-inflammatory immune responses are crucial for the control of *M. tuberculosis* infection¹³⁴. The pro-inflammatory signaling control via MYCBP2 may be necessary for long-term control of *M. tuberculosis* infection. For example, recent work demonstrated that mice lacking all three IRGM genes have less pro-inflammatory signaling both early and late in infection, but no difference is observed in bacterial growth until late in infection¹³⁵. Similar to our MYCBP2 findings, differences in IRGM KO mice survival emerge only very late (200+ days) in infection. At the macrophage level, MYCBP2 deficient cells are moderately more permissive for *M. tuberculosis* growth over

the course of a five-day infection. These cells also have less pro-inflammatory cytokine secretion one day post-infection. MYCBP2's more subtle and long-term impact on *M. tuberculosis* control in the chronic stage of infection may explain why we saw no differences in growth with the fast-growing pathogen *L. monocytogenes*. The discrepancies between our *ex vivo* and *in vivo* *M. tuberculosis* growth are likely due to the complexities of the environmental milieu *in vivo*. Future work is needed to understand MYCBP2's role at the host level of control of *M. tuberculosis* infection.

MYCBP2 has previously been found to interact with several viral proteins^{99–102} but no studies have investigated a possible role in viral replication. We found that MYCBP2 promotes the replication of MNV in macrophages. MNV, like other viruses, is restricted by the antiviral cytokine, IFN- β ^{136,137}. Given the decrease of IFN- β in poly(I:C) stimulated MYCBP2 deficient macrophages, we hypothesized that these macrophages should demonstrate an increased susceptibility to MNV. However, we observed the opposite effect with MYCBP2 deficient cells leading to a 10-fold decrease in MNV replication. We did not observe differences in viral replication or binding between MYCBP2 deficient and WT macrophages, indicating that MYCBP2-mediated susceptibility to MNV is independent of these early stages of the viral life cycle. It is well documented that viruses can hijack host proteins for their survival and replication^{138–140}. It's possible that MNV, or other viruses, utilize MYCBP2 at some point in their life cycle which is perhaps why loss of MYCBP2 reduces the viral burden of MNV.

We also investigated MYCBP2's role in immunity independent of infection. Previous work has demonstrated a role for MYCBP2 in regulating inflammatory signaling of the macrophage⁹⁶. Our work builds upon these past findings by demonstrating additional MYCBP2-mediated differences in cytokine secretion during stimulation with TLR2, 3, and 4 agonists at both the secretory and translational levels. In addition, our RNA-seq data demonstrate a large transcriptional change in cells lacking MYCBP2 during TLR4 stimulation. Previous studies suggested that these inflammatory differences could be due to differences in p38 MAPK signaling. Here we found no differences in phosphorylation of p38 MAPK between WT and MYCBP2 deficient cells in response to stimulus. However, when cells were stimulated in the presence of p38 MAPK inhibitor SB203580, the differences between WT and MYCBP2 deficient macrophages were eliminated, similar to previous findings with IL-10 stimulated macrophages⁹⁶. Because all cytokine levels were reduced in the presence of SB203580, we cannot rule out that this reduction of cytokine signaling is not simply due to the pleiotropic effects of the inhibitor on cytokine response.

To understand how MYCBP2 impacts inflammatory signaling, we investigated other pathways previously shown to be regulated by MYCBP2 including the regulation of autophagy. Canonical and non-canonical autophagy processes influence inflammation outcomes of the macrophage^{141,142} as well as control growth of *M. tuberculosis*⁴⁵. Therefore we hypothesized that differences in immune signaling were

mediated by MYCBP2-dependent autophagy differences. However, we found no differences in LC3 induction or phosphorylation of p70S6K between wildtype and MYCBP2 deficient cells. Therefore, we conclude that in macrophages, MYCBP2-mediated inflammatory signaling is not due to differences in autophagy.

MYCBP2 is a large protein involved in many cell processes with both ubiquitin ligase-dependent and independent functions. As a ubiquitin ligase, MYCBP2 has been found to function in a complex with FBXO45 and SKP1^{143–145} but it's unclear if this complex is active in macrophages. Future work could unravel the possible role of these proteins in inflammatory signaling. Although it's been well studied for its function as a ubiquitin ligase, MYCBP2 also has ubiquitin ligase-independent activities. MYCBP2 functions as a GEF for Ran in rat dorsal root ganglia¹⁴⁶ and recent work has identified ubiquitin ligase-independent activities of MYCBP2 in ephrin signaling⁹⁴. Our work further solidifies MYCBP2's reputation as a signaling hub with a diverse biological role¹⁴⁷. Future work is needed to identify the exact mechanism of how this protein regulates inflammatory signaling and whether its regulation is independent of its function as a ubiquitin ligase.

METHODS

Mice

All mice were housed in specific pathogen-free conditions and treated using procedures described in an animal care protocol (AUP-2015-11-8096) approved by the Institutional Animal Care and Use Committee of the University of California Berkeley, in adherence with guidelines from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. MYCBP2 (Phr1) floxed mice were a generous gift from Elisabetta Babetto. These floxed mice were crossed with B6.129P2-Lyz2tm1(cre)Ifo/J mice (Strain #004781) from Jackson Laboratories. Genotypes were determined using genotyping services from Transnetyx.

Cell lines

Conditionally immortalized macrophages were generated using guides from the Brie library as previously described¹²⁰. Editing efficiency was determined using TIDE analysis¹⁴⁸. Bone marrow-derived macrophages were harvested from 5-8-week-old male and female mice. Briefly, femurs were crushed using mortar and pestle, and cells were differentiated in DMEM (Gibco), 10% FBS, 5mM L-glutamine, 5mM sodium pyruvate, and 10% MCSF for 7 days prior to being frozen and aliquoted for later use.

Ex vivo M. tuberculosis infections

200,000 macrophages/well were seeded in a 24-well plate and incubated at 37°C and 5% CO₂ overnight. A culture of WT Erdman *M. tuberculosis* EF2 was inoculated in 7H9 (BD Biosciences) supplemented with 10% Middlebrook OADC (BD), 0.5% glycerol, and

0.05% Tween-80 so that it would be between 0.2 - 0.8 OD on the day of infection. Luciferase experiments were performed with WT Erdman expressing the *luxCDABE* operon. After 3 PBS washes and sonication at 90% amplitude for 2 x 30 seconds, macrophages were infected via centrifugation at 400 x g for 10 minutes. After spinning, cells were washed and fresh media was added. The monolayer was monitored daily for cell death and media exchanges were performed simultaneously. For luciferase experiments, luminescence was measured daily at 32°C on the SpectraMax L (Molecular Devices). For CFU infections, cells were lysed in water + 0.5% Triton-X 100 at the indicated time point. Serial dilutions were plated on 7H10 plates supplemented with OADC and glycerol as mentioned above. Plates were incubated at 37°C for 21 days before counting.

Mycobacterium marinum infections

Mycobacterium marinum expressing mCherry was grown in 7H9 supplemented with 10% Middlebrook OADC, 0.5% glycerol, and 0.2% Tween80 to an OD of 0.2- 0.8 shaking at 30°C. Cultures were prepared like *M. tuberculosis* above. Bacteria were added at an MOI of 2. Infected macrophages were incubated at 33°C and 5% CO₂ for the duration of the experiment. At indicated time points, media was removed and cells were fixed in 4% paraformaldehyde (PFA). Before high throughput microscopy analysis, cells were stained with DAPI 1:1000 and Phalloidin Alexa 488 (Invitrogen) 1:250.

In vivo M. tuberculosis infections

8-12-week-old male and female mice were infected with ~100 CFU of WT *M. tuberculosis* Erdman using the Glas-Col system as previously described⁴⁵. Mice were monitored weekly for weight changes. For survival experiments, 10-12 mice per genotype were sacrificed upon losing 15% of their maximum weight. For lung CFU, 4 to 6 mice per genotype were sacrificed at indicated time points. To determine inoculum, 24 hours after infection 3 to 5 mice were sacrificed. Lungs were homogenized in PBS + 0.05% Tween-80 and lysates were plated in various dilutions on 7H10 plates supplemented with 10% OADC and 0.5% glycerol and containing polymyxin B (200,000 U l⁻¹), carbenicillin (50 mg l⁻¹), trimethoprim lactate (20 mg l⁻¹) and amphotericin B (5 mg l⁻¹). Plates were incubated at 37°C for 26 days prior to counting.

Cell culture

For cytokine analysis, BMDMs were stimulated with LPS, PAM, or HMW Poly(I:C) (Invivogen) for 4-24 hours at varying concentrations as indicated. At indicated time points, supernatants were collected in fresh plates or tubes and frozen at -80°C for later analysis. When indicated, RNA was collected using the RNeasy Mini Kit (Qiagen). Supernatants were analyzed via ELISA and cytokine bead arrays. ELISAs were performed as previously described¹²⁰ with the addition of TNFα reagents antibodies

#14-7325-81 and #13-7326-81 from Thermofisher and recombinant mouse TNF from Biotechne (#410-MT). Cytokine bead arrays were performed with the BioLegend Legendplex Mouse Inflammation Panel according to the manufacturer's instructions with a few slight modifications. Supernatants were incubated with beads overnight at 4°C instead of 2h at room temperature and plates were not shaken during incubations. ELISAs and cytokine bead arrays performed with *M. tuberculosis* supernatants were adapted to be BSL3 compliant.

For starvation assays, bone marrow macrophages were seeded at 800,000 cells/well in a 6-well dish and incubated at 37°C and 5% CO₂ overnight. To induce starvation, media was removed and Gibco EBSS (Thermo Fisher Scientific) was added to cells for 4h. Bafilomycin A1 (Invivogen) was added 2h before the end of the assay to a final concentration of 300 nM. Cells were collected as described in the Western Blots section.

RNA-sequencing

Bone marrow macrophages were seeded at 400,000 cells/well in a 12-well dish and incubated overnight at 37°C and 5% CO₂. The following day, cells were stimulated with 100 ng/mL of LPS (Invivogen, San Diego, CA). At 6 and 24 hours post infection RNA was collected by lysing cells in Buffer RLT from the RNeasy Kit (Qiagen). RNA was first processed using the RNeasy Kit, followed by DNase digestion (New England Biolabs), and RNA clean-up using the ZymoChem RNA Clean and Concentrator kit. 200 ng of RNA was sent to Novogene for processing and data analysis.

Western blots

Lysates were harvested in RIPA buffer (Thermofisher) supplemented with protease (Sigma) and phosphatase inhibitor (Roche), flash frozen, and stored at -80°C. Lysates for LC3 analysis were kept at 4°C instead. Protein concentrations were calculated using the Micro BCA Protein Assay Kit or the Pierce 660 assay (Thermofisher). Fifteen to 20 ug of protein was added to 4-20% Miniprotean TGX gels (Biorad). After running, gels were transferred to PVDF and probed with indicated antibodies diluted in Intercept Blocking Buffer (Licor). Blots probed with phosphorylation antibodies were washed with TBS + 0.05% Tween-20 while all other blots were washed with PBS + 0.05% Tween-20. PVDF membranes were imaged using the Licor Odyssey system and quantified using ImageJ (National Institutes of Health). The following primary antibodies were used in this study: α-LC3 1:1000 (Sigma cat#L7543), α-phos p38 1:1000 (Cell Signaling cat#4511), α-actin 1:1000 (Cell Signaling cat#3700), α-phos 70S6K 1:750 (Cell Signaling cat#9234). All secondary antibodies were IRDye purchased from Licor and added at a 1:5000 dilution in blocking buffer.

Listeria macrophage infection

Bone marrow macrophages were seeded on 14 coverslips in 60 mm dishes and incubated at 37°C and 5% CO₂ overnight. *Listeria monocytogenes* strain 10403S was grown at a slant overnight at 30°C in BHI media supplemented with 200 ug/mL streptomycin. On the day of infection, *L. monocytogenes* was washed in PBS and added to cells at a MOI of 0.25. Gentamicin (50 ug/mL) was added 1 hour post-infection. At indicated time points coverslips were collected, vortexed, and dilutions were plated on LB agar plates containing streptomycin.

MNV infection

For TCID₅₀, ELISA, and replication experiments, bone marrow macrophages were seeded at 40,000 cells/mL in 96 well plates overnight. The following morning, MNV was added to cells at an MOI of 0.2 and cells were incubated at 37°C and 5% CO₂ for 7 hours for replication and 24 hours for TCID₅₀ experiments. The supernatant was added to WT BMMs for TCID₅₀ calculation as previously described¹⁴⁹. For binding experiments, BMMs were seeded at 200,000 cells/mL for two days before infection. On the day of infection, MNV was added at an MOI of 1.2. Cells were incubated at 4°C for 45 minutes and then collected for RT-qPCR.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 9.

ACKNOWLEDGMENTS

Mycbp2^{fl/fl} mice were a generous gift of Elisabetta Babetto. We thank members of the Cox, Stanley, and Vance labs for helpful discussions. Erika Oki and Jessica Robles assisted with mouse colony maintenance.

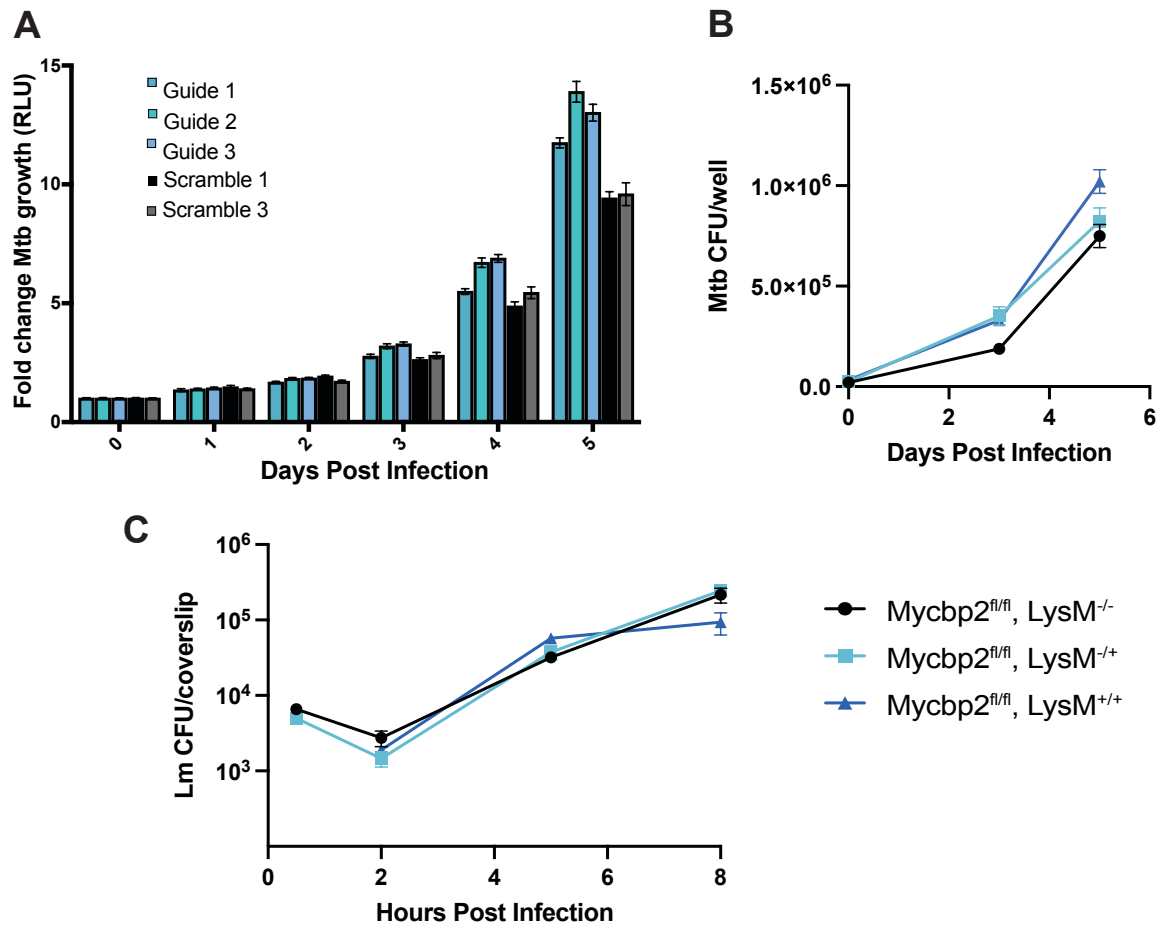


Figure 1 Mycbp2 deficient cells are susceptible to *M. tuberculosis* but not *L. monocytogenes*. (A) CIMs edited with guides targeting Mycbp2 were infected with *M. tuberculosis* expressing the *luxBCADE* operon at an MOI of 2 and growth was measured daily via luminescence. Data are compared to luminescence at day 0. (B) Mycbp2 floxed BMMs with zero, one, or two copies of LysM Cre were infected with *M. tuberculosis* (B) or *L. monocytogenes* (C). At indicated time points cells were lysed and plated for CFU. All data is representative of at least two experiments. Data was found to be statistically insignificant using Mann-Whitney tests. Error bars represent SEM.

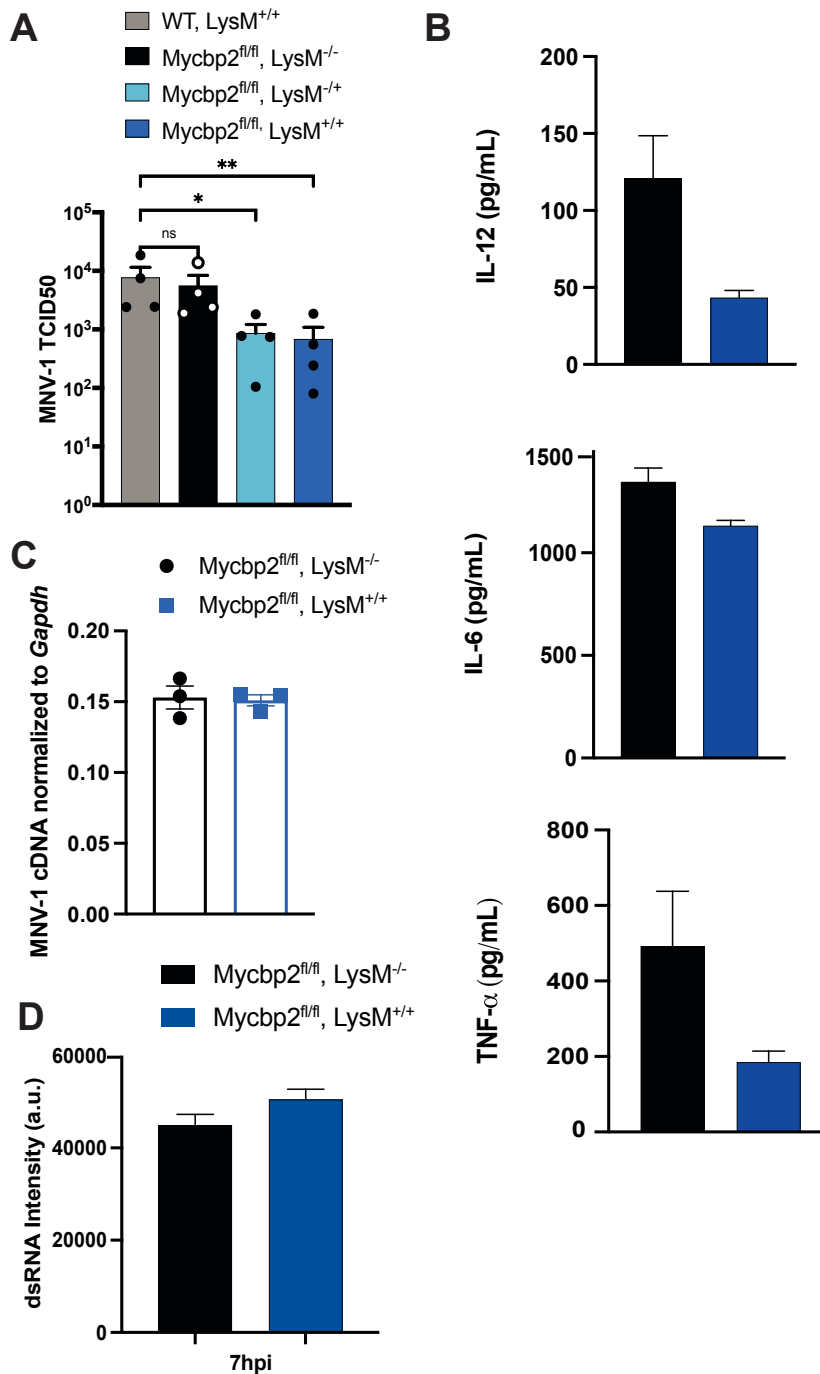


Figure 2 Mycbp2 deficient cells are resistant to MNV-1. Mycbp2 deficient BMMs were infected with 2.5×10^5 PFU/mL of MNV-1 for 24 hours. Supernatants of infected cells were (A) added to WT BMMs and incubated for 48 hours for TCID50 or (B) analyzed for cytokine secretion with ELISAS specific for IL-6, IL-12, or TNF-α. (C) BMMs were incubated with MNV-1 for 30 minutes at 4°C followed by RT-qPCR to assay MNV genome copy levels (D) BMMs were infected with MNV-1 for 7h. After fixation,

cells were incubated with J2 dsDNA antibodies to detect MNV-1 replication complexes. Data was analyzed using a Kruskal-Wallis test. * $P < 0.05$, ** $P < 0.005$. Error bars represent SEM.

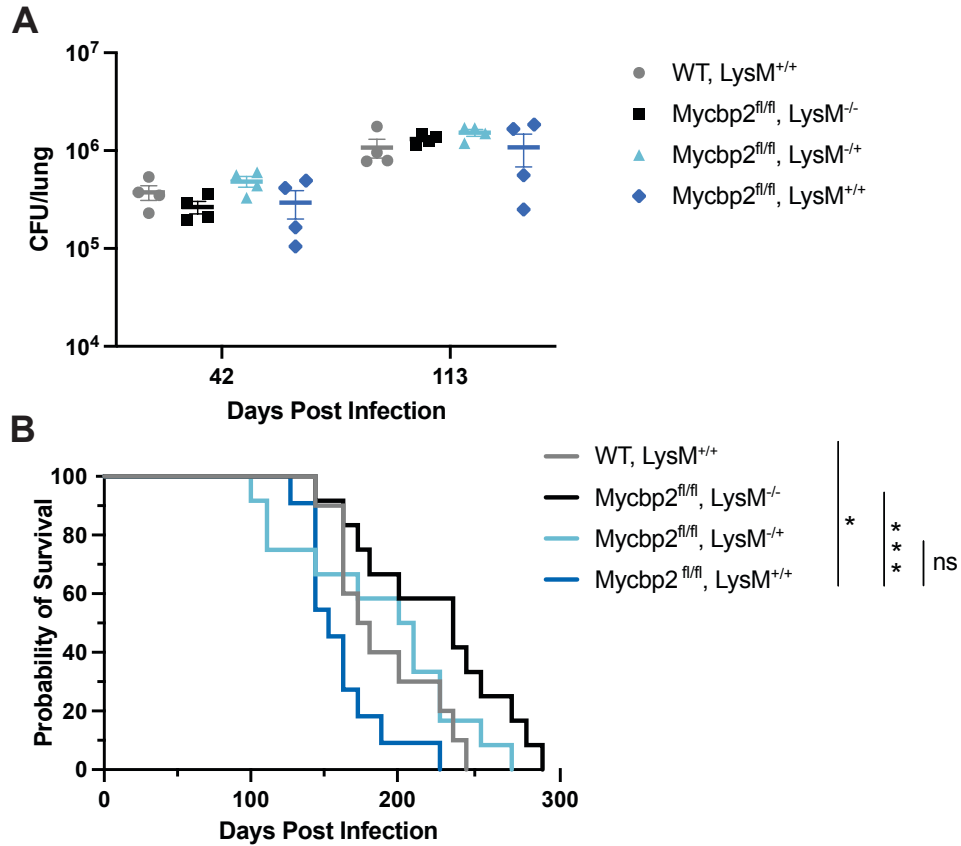


Figure 3 Mycbp2 deficient mice have similar bacterial loads but succumb earlier to *M. tuberculosis* infection compared to WT mice. Mice were infected with approximately 100 CFU of *M. tuberculosis*. (A) At indicated time points four mice per genotype were harvested and lungs were lysed and plated for CFU. (B) Infected mice were monitored for survival during infection and were euthanized after losing 15% of their maximum body weight. Data is representative of one to two experiments. For statistical analysis, samples were compared to Mycbp2^{fl/fl}, LysM^{+/+} by Mantel-Cox test. *P<0.05, ***P<0.001.

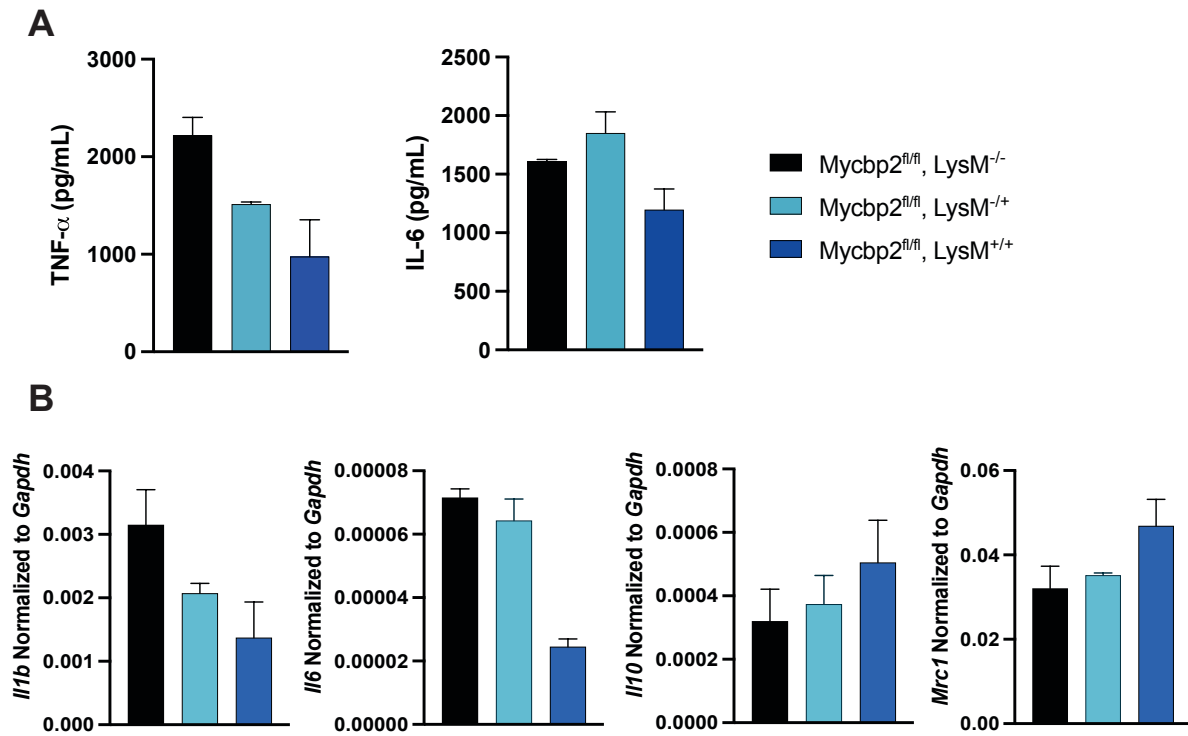


Figure 4 Mycbp2 deficient macrophages are less pro-inflammatory during *M. tuberculosis* infection. Mycbp2 floxed BMMs with none, one, or two copies of LysM Cre were infected with WT Erdman *M. tuberculosis* for 24 hours at an MOI of 1. At 24 hours post infection supernatants were analyzed using a cytokine bead array (A) and RNA was harvested, reverse transcribed, and analyzed for inflammatory markers using qPCR (B). Data is representative of one to two experiments. Data was found to be statistically insignificant using Mann-Whitney tests. Error bars represent SEM.

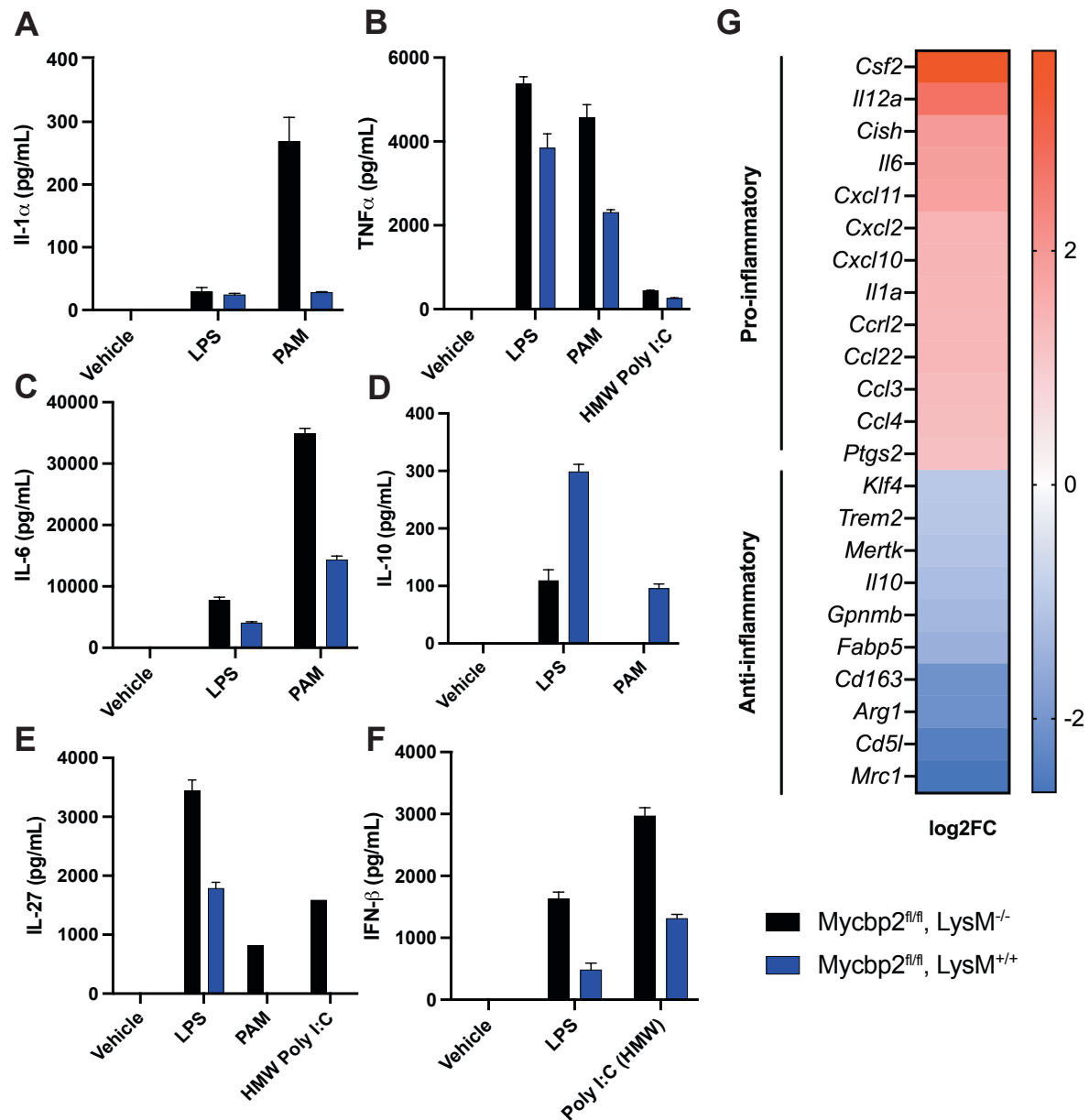


Figure 5 Mycbp2 deficient cells are less pro-inflammatory in response to TLR2, TLR3, and TLR4 agonists. BMMs were stimulated with LPS (100 ng/mL), Pam3CSK4. (1 ug/mL), or Polyinosinic:polycytidylic acid (poly (I:C)) (HMW) (20ug/mL) for 18-24 hours. (A) Supernatants were analyzed with cytokine bead arrays for pro- and anti-inflammatory cytokines. (B) LPS stimulated macrophages were harvested and RNA was analyzed using RNA sequencing. Heatmap shows some select pro-and anti-inflammatory genes detected. Positive log2FC reads were enriched in WT compared to Mycbp2^{fl/fl}, LysM^{+/+} cells. Data is representative of two to three biological replicates. Data was found to be statistically insignificant using Mann-Whitney tests. Error bars represent SEM.

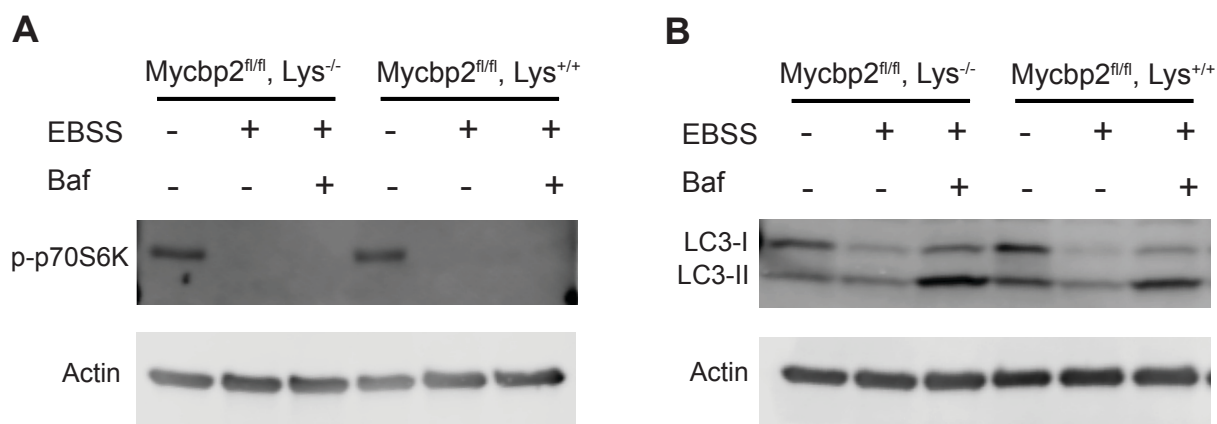


Figure 6 Mycbp2 does not regulate autophagy in macrophages. BMMs were cultured in normal media conditions or starved by replacing media with EBSS for four hours. Two hours prior to end of the experiment, bafilomycin (300nM) was added to indicated samples. Lysates were harvested and analyzed via Western for (A) phosphorylation of P70S6K or (B) LC3 lipidation.

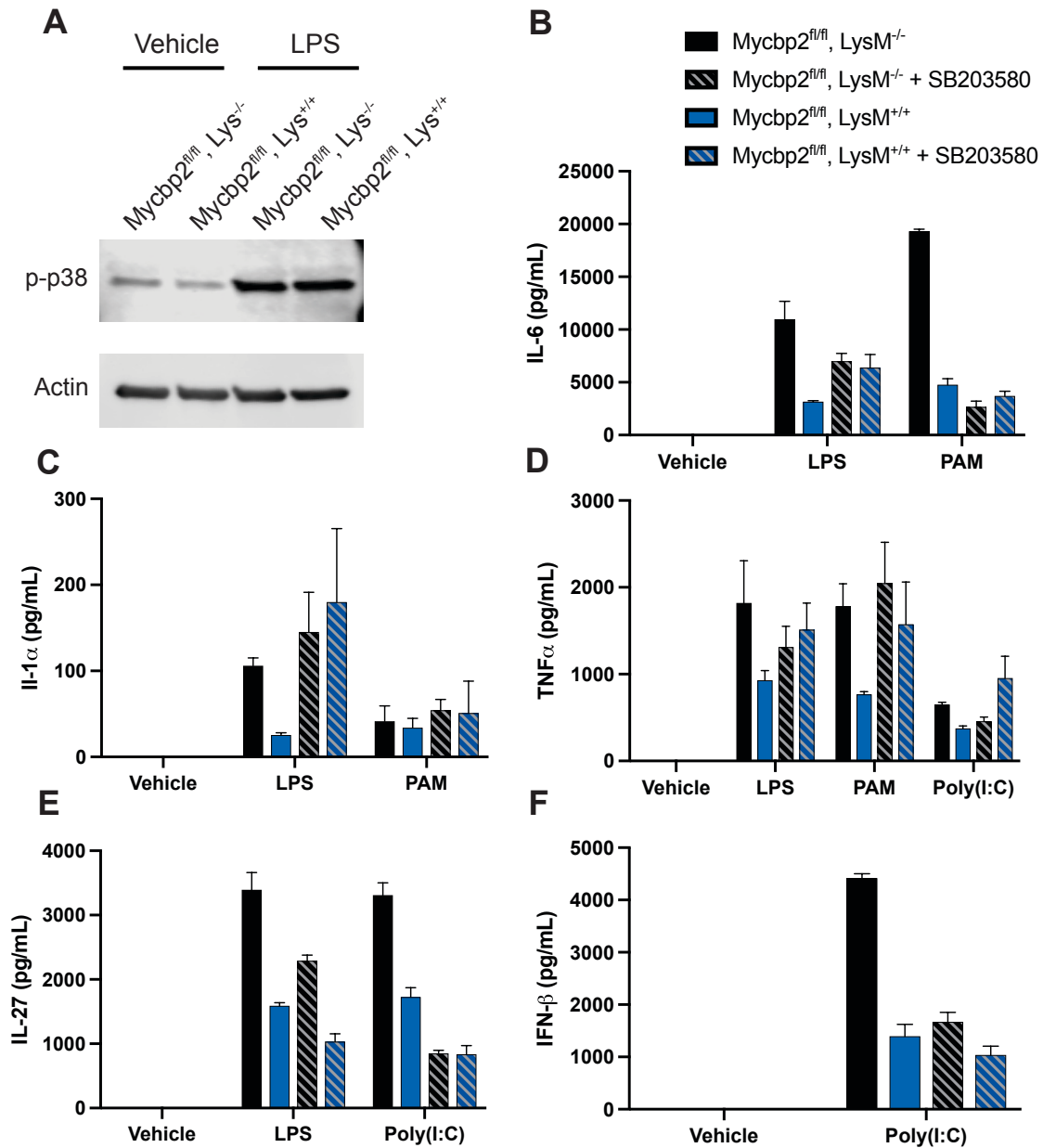
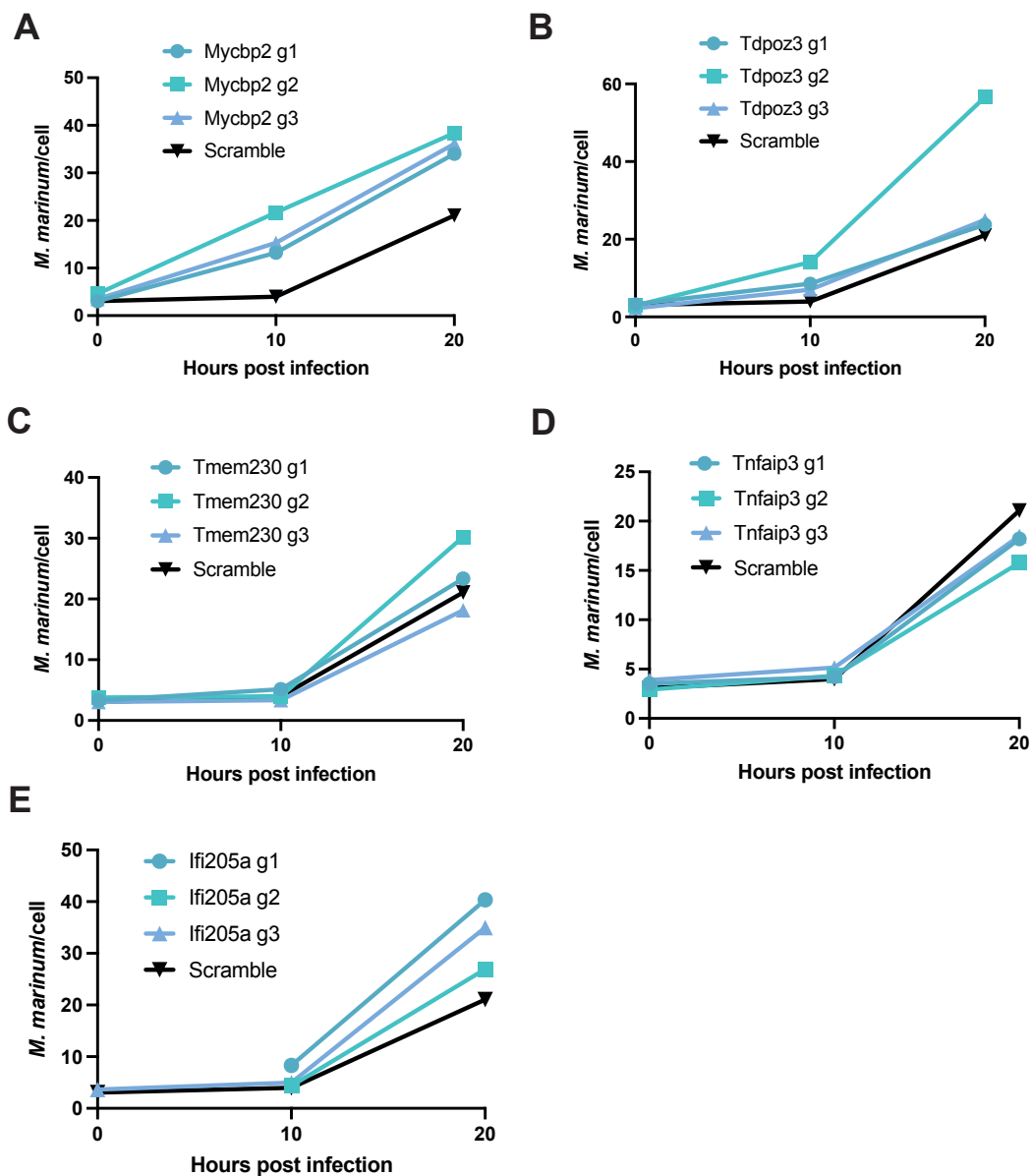


Figure 7 Mycbp2 mediated inflammatory control is dependent on p38 MAPK function. (A) BMMs were stimulated with 1 ug/mL of LPS for 4 hours. Cells were analyzed via SDS-PAGE and western blot with antibodies raised against phosphorylated p38 MAPK. (B-F) BMMs were stimulated with vehicle, 100 ng/mL LPS, 1 ug/mL Pam3CKS4, or 20 ug of poly(I:C) HMW in the presence or absence of 10 uM SB203580 for 18 h. Supernatants were analyzed via a cytokine bead array assay. Data was found to be statistically insignificant using Mann-Whitney tests. Error bars represent SEM.



Supplemental Figure 1 Small screen of proteins post-translationally modified in response to *M. tuberculosis*. CRISPR/Cas9 was used to knock out genes in CIMs (Mycbp2, Tdpoz3, Tmem230, Tnfaip3, and Ifi205a) corresponding to five host proteins found to have significant changes in either phosphorylation or ubiquitylation during *M. tuberculosis* infection in primary macrophages. CIMs were infected with *M. marinum* expressing mCherry at an MOI of 2 for the indicated time points. Growth of bacteria was measured using high throughput microscopy and compared to a scramble control.

Chapter 3: Proximity labeling of the *M. tuberculosis* phagosome

Additional contributors: Sena N. Gul¹, Elizabeth Reinhart Wouters¹, Martin Gordon², Ben J. Polacco², Donovan Trinidad³, Oren Rosenberg³, Nevan J. Krogan², Matthew D. Champion⁴, Jeffery S. Cox¹

¹Department of Molecular and Cell Biology, University of California, Berkeley ²Quantitative Biosciences Institute, University of California, San Francisco ³Department of Medicine, Division of Infectious Diseases, University of California, San Francisco ⁴Department of Chemistry and Biochemistry, University of Notre Dame, Indiana

INTRODUCTION

Permeabilization of the phagosome by the Type VII secretion system ESX-1 is crucial for the virulence of *M. tuberculosis* as detailed in previous chapters. As the intracellular niche for *M. tuberculosis*, the phagosome has garnered much interest throughout decades of study as the site of many interactions between host and bacteria. Permeabilization of the phagosome leads to the recruitment of many host proteins. Host glycans exposed by phagosome perforation are bound by cytosolic lectins including galectins-3, 8, and 9^{17,40–43}. Ubiquitin ligases PARKIN³⁷, SMURF1³⁹, and TRIM16³⁸ are also recruited to the phagosomal membrane in an ESX-1-dependent manner. Additionally, autophagy adaptors that bind both ubiquitin and LC3 have been found to specifically colocalize with *M. tuberculosis* with a functional ESX-1 system during infection^{36,37,39,80}. The aforementioned proteins are associated with phagosomal damage and ultimate degradation of the bacteria, but some proteins attempt to repair the damage rather than degrade. As mentioned in an earlier chapter, recent work has found that ESCRT proteins are recruited to perforated phagosomes and can repair small perturbations in the phagosomal membrane^{21,57}. Other host proteins recruited to mycobacterial phagosomes include the immunomodulatory protein CISH¹⁵⁰ and cytoskeleton-associated protein coronin 1¹⁵¹.

To identify additional proteins recruited to the phagosome, studies have relied on purification techniques such as density-gradient centrifugation that often has contamination from other organelles, confounding studies of the phagosomal proteome. Initial phagosome proteome studies have yielded important discoveries such as the exclusion of vacuolar ATPase from the *Mycobacterium* phagosome⁹. In recent years, phagosome isolation methods have advanced with separation techniques such as magnetically labeled bacteria¹⁵² but these techniques are time-intensive and may not accurately reflect the true biology of the phagosome.

Proximity labeling is a technique that utilizes engineered enzymes in conjunction with biochemical reactions to identify proteins in the vicinity of a protein of interest¹⁵³. These techniques can identify more transient protein interactions compared to popular techniques like affinity purification and yeast two-hybrid assays which detect interactions based on affinity, not proximity. We hypothesized that proximity labeling could provide an alternative way to capture the phagosomal proteome. Two main enzymes, biotin ligases and peroxidases are the center of current proximity labeling

technologies. Peroxidases, such as APEX2, can quickly label their substrates in approximately one minute, but labeling requires H₂O₂ which can be toxic in some systems. Biotin ligases, like BioID, are not toxic but use long labeling times of over 16 hours, though recent advances have partially mitigated this requirement¹⁵⁴. Biotin ligases label lysines while peroxidases label tyrosines, as lysines are typically more abundant, this may limit available substrates in peroxidase-based studies¹⁵⁵. Both classes of enzymes have been used in recent host-pathogen studies. APEX2 has been used with the intracellular pathogen, *Chlamydia trachomatis* to identify novel interactions with the host^{156,157} and TurboID has recently been used with the human cytomegalovirus to identify a role for RhoGEF in viral reactivation¹⁵⁸. For our studies of the *M. tuberculosis*-containing phagosome, we chose to use APEX2 due to its quick labeling time and previous uses in lysosomal damage studies¹⁵⁹.

Equally important to enzyme choice is choosing the protein of interest to fuse with the enzyme. In our work, we took two initial approaches: 1) adding protein directly to bacteria and 2) fusing the enzyme to a host protein. We initially chose to fuse the APEX2 enzyme with the amphipathic helix of MirA, a *Mycobacterium marinum* protein responsible for the actin-based motility of the bacteria¹⁶⁰. The Welch Lab had shown that the amphipathic helix was responsible for the localization of MirA to the surface of *M. marinum*, and we reasoned that the expression of MirA would also colocalize to the surface of *M. tuberculosis*. This approach was unsuccessful as we were unable to get a large amount of colocalization between construct and bacterium so we turned to the more typical method of expressing APEX2 with a protein of interest from the host.

Because we lack knowledge of how proteins are partitioned to the outer surface, we pivoted to utilizing host proteins that localize to the *M. tuberculosis*-containing phagosome. We chose to use Galectin-8 (Gal-8), a cytosolic lectin that detects damaged vesicles because research has shown it is recruited to the phagosome during perforation, a large proportion of *M. tuberculosis* is labeled with Gal-8 during infection^{41,161}, and the APEX2-GAL8 construct previously published was freely available¹⁵⁹. As a control, we utilized a strain of *M. tuberculosis* lacking a functional ESX-1 system. We hypothesized that the APEX2-GAL8 would be dispersed throughout the cell as a cytoplasmic control in mutant-infected cells. We reasoned that proteins only detected in WT and not mutant infected samples would be proteins specifically recruited near Gal-8 and, therefore, the permeabilized phagosome during *M. tuberculosis* infection.

We identified proteins recruited to the perforated phagosome and Gal-8 during *M. tuberculosis* infection using APEX2-GAL8 and multiple mass spectrometry methods. We explore some possible proteins of interest and discuss limitations and future considerations of our data.

RESULTS

Exogenous APEX2 labeling of bacteria

In proximity labeling experiments, the majority of the population of interest should be labeled by the peroxidase in order to characterize accurate proteomes. We initially hypothesized that labeling the bacteria themselves could allow us to efficiently identify host proteins interacting with *M. tuberculosis* during infection. We created a construct containing the amphipathic domain of MirA, the protein responsible for actin-based motility of *M. marinum*¹⁶⁰, and added the APEX2 peroxidase as well as a V5 tag (Figure 1A and Supplemental File 1). We then transformed *Escherichia coli* BL21(DE3)-Gold with the construct and purified the protein. We added the lysate in various concentrations to *M. tuberculosis* and measured the colocalization of the construct and bacteria via confocal microscopy. We observed very little colocalization with varying concentrations of the construct (Figure 1B) suggesting that this method required further optimization to be successful.

APEX2-GAL8 constructs colocalize with *M. tuberculosis* in an ESX-1-dependent manner and are functionally active

As an alternative approach, we used lentivirus to transduce THP-1 with a construct containing APEX2-GAL8. We wanted to use a construct that would colocalize with *M. tuberculosis* during infection with wild-type bacteria but not with bacteria lacking a functional ESX-1 system. We infected THP1s with WT and Δ eccC Erdman *M. tuberculosis*, which lacks a functional ESX-1 system¹⁶², and then used confocal microscopy to determine colocalization with APEX2-GAL8. We found clear puncta of APEX2-GAL8 that colocalized with *M. tuberculosis* during infection with WT. Infection with Δ eccC bacteria lacked APEX2-GAL8 colocalization, with the construct likely being more diffuse through the cell (Figure 2A-B). To test if the APEX2 peroxidase was active, we labeled cells with biotin phenol and hydrogen peroxide as described in the methods. We then performed a western blot analysis using whole-cell lysates and used a α -streptavidin antibody to identify biotinylated proteins. With this antibody, we observed that our APEX2-GAL8 protein biotinylated a large number of proteins. Cells not exposed to hydrogen peroxide only detected endogenously biotinylated proteins indicating our peroxidase could properly biotinylate proteins (Figure 2C).

APEX2-GAL8 colocalization signal peaks at 6 hours post-infection

We next wanted to determine the time point at which we would see the highest signal of our construct colocalized with *M. tuberculosis*. We infected APEX2-GAL8 THP1s with an MOI of 2, 5, and 10 for 6, 12, and 24 hours. We then used high-throughput microscopy to determine when the peak signal was colocalized with *M. tuberculosis*. We determined that the signal peak was 6 hours post-infection and MOI 5 and 10 had similar amounts of colocalization (Figure 2D). Therefore, we decided to use an MOI of 5

at 6 hours post-infection for labeling. We next infected three separate biological replicates of APEX2-GAL8 THP1s with WT Erdman or $\Delta eccC$ *M. tuberculosis* and labeled the infected cells with biotinyl tyramide and H₂O₂ for analysis at 6 hours post-infection.

Different mass spec approaches and analysis pipelines yield differing results

For an initial analysis, we utilized 2D MuDPit mass spectrometry to identify proteins enriched in WT-infected THP1s. To rank proteins we used a confidence score for APEX2 data previously reported¹⁶³. We detected positive controls like ubiquitin in our dataset, indicating that we likely detected proteins recruited near the *M. tuberculosis* phagosome (Table 1). However, more robust analysis using MaxQuant software proved difficult due to the low abundance of detected proteins.

To expand the number of proteins detected, we next tried an approach using LC-MS/MS which captured a more robust sample of proteins compared to 2DMudPIT. We then used artMS to analyze data from the three biological replicates. We observed a strong enrichment of proteins involved in neutrophil degranulation including antimicrobial proteins (Figure 3). However, using this approach we no longer detected expected positive controls in our top hits, such as ubiquitin as detected previously (Tables 2 and 3).

We also analyzed the LC-MS/MS data using the Fragpipe program. Using this analysis, we identified that one $\Delta eccC$ infected biological replicate had considerable variance from the other samples we therefore decided to drop the experiment from our dataset (Supplemental Figure 1). An advantage of FragPipe is that it allows us to visualize *M. tuberculosis* proteins in addition to human proteins. We hypothesized that our APEX2-GAL8 dataset would detect possible *M. tuberculosis* effector proteins at the phagosomal membrane. However, we did not identify any *M. tuberculosis* proteins enriched in all three biological replicates that were also detected in this mass spectrometry (Figure 4).

Network propagation analysis finds similarities with protein-protein interaction

After identifying a list of proteins identified near Gal-8 during *M. tuberculosis* infection, we sought to utilize machine learning to prioritize proteins for follow-up studies. We used network propagation analysis¹⁶⁴ in combination with the *M. tuberculosis* and host protein-protein dataset generated in our lab previously¹²⁴. We used a normalized betweenness method to identify proteins of interest. Significant proteins with high normalized betweenness values include STK24, PSG9, NUFIP2, and RGS20 (Figure 5 and Table 4).

Initial follow up: Investigating a role of PGRMC1, ANKRD13a, and OSTF1 in mycobacterial control

From our artMS analysis, we picked three initial genes to perform a preliminary analysis investigating a possible role in *M. tuberculosis* infection. These proteins PGRMC1, ANKRD13a, and OSTF1 were also previously identified as having significant changes in post-translational modifications in response to *M. tuberculosis* infection⁸⁰. PGRMC1 and OSTF1 had significant increases in phosphorylation early during infection. ANKRD13a also had a significant decrease in ubiquitylation in *M. tuberculosis*-infected macrophages at 4 and 8 hours post-infection with no significant changes in abundance. Additional PGRMC1 and OSTF1 are known to interact with proteins important in *M. tuberculosis* infection, the *M. tuberculosis* effector ESAT-6, and host ubiquitin ligase, Cbl^{124,165}. We were interested in ANKRD13a because it is a ubiquitin-binding protein and we hypothesized it may bind to ubiquitin at the phagosomal membrane¹⁶⁶.

For initial analysis, we chose to use CIMs¹²⁰ for ease of genetic editing to investigate these genes. Due to its less restrictive biosafety level designation, we infected edited CIMs with *Mycobacterium marinum*, a closely related intracellular pathogen. We infected these macrophages with *M. marinum* and monitored bacterial growth and actin tail formation throughout infection using high throughput microscopy (Figure 6). We compared each guide to a density and plate location-matched scramble control to minimize confounding effects on a possible phenotype. We did not observe differences in bacterial growth or actin tail colocalization of OSTF1 and PGRMC1 knockouts. However, ANKRD13a knockouts increased in bacterial growth in one guide and both guides showed a decrease in actin tail colocalization, suggesting less bacteria had escaped from the phagosome in these cells.

DISCUSSION

In this study, we explored multiple techniques for labeling the *M. tuberculosis* phagosome and encountered challenges in each method. Initial attempts to exogenously label the bacteria were unsuccessful as we saw low colocalization between the bacteria and the APEX2 protein. Though this method has been successfully used before¹⁶⁷ we were also unsure if the purification process could impact peroxidase function activity. We explored the idea of expressing a protein from *M. tuberculosis* itself but it is very difficult to express proteins from *M. tuberculosis* to the outside of the cell. Recent work has successfully identified cytoplasmic and cell wall proteins of *M. tuberculosis* using APEX2¹⁶⁸. However, the researchers could not specifically localize the construct to a surface-associated protein with this technique. Extensive optimization is needed to attempt this exogenous APEX2 approach in the future.

We initially chose to utilize MudPIT because of its increased sensitivity¹⁶⁹. However, a lower-than-expected number of proteins suggested this technique and the methods used to prepare our samples were not ideal for APEX2 experiments. Conversely, using LC-MS/MS we detected many more proteins than expected. Future optimization should involve more stringent wash conditions to reduce background. Many studies include a cytoplasmic control APEX2 to detect background proteins. We reasoned that our condition infected with Δ eccC should perform similarly to a cytoplasmic control as Gal-8 is most likely dispersed in the cytosol rather than recruited to sites of lysosomal or phagosomal damage. However, it could be useful to validate this hypothesis using a cytoplasmic APEX2 control in the future.

We detected proteins expected to be at the phagosome such as ubiquitin and Gal-8 in our initial studies using MudPIT. However, we did not see other known proteins recruited to the phagosomal membrane such as autophagy adaptors and other galectins. We did not detect any of these proteins as significant hits in our artMS or Fraggpipe datasets. The intracellular milieu of the perforated phagosome may limit what we can detect. The APEX2 labeling radius is known to be altered by glutathione concentration and protein density and also the short half-life of the biotin phenol radical can limit possible hits¹⁷⁰. Another possibility is that the unusually high number of proteins detected in our LC-MS/MS studies obfuscated potentially relevant hits.

We identified three proteins of particular interest in the artMS analysis, to begin an initial study as they were also post-translationally modified during *M. tuberculosis* infection⁸⁰; PGRMC1, OSTF1, and ANKRD13A. We knocked out the genes in CIMs and infected them with *M. marinum*. We saw no differences in actin tail colocalization or bacterial growth between PGRMC1, OSTF1, and scramble controls. We observed a decrease in actin tail colocalization in ANKRD13a KO and an increase in bacterial growth in one guide.

We were interested in ANKRD13a due to its ubiquitin-binding domain demonstrated to bind to K63 ubiquitin linkages¹⁶⁶. It is well known that ubiquitin is present at the perforated phagosome and many ubiquitin-binding proteins such as autophagy adaptors are recruited to *M. tuberculosis* during infection. Therefore, we hypothesized that ANKRD13a may be involved in the host response to *M. tuberculosis* infection by binding ubiquitin and possibly recruiting other host proteins. Little is known about ANKRD13A, however recent work has demonstrated its role in cell death via binding to RIP1¹⁷¹. Additionally, ANKRD13a has previously been shown to interact with the ubiquitin ligase RNF11 and EGFR during EGF receptor endocytosis¹⁷². ANKRD13a's known interaction with RNF11 is interesting as RNF11 has been implicated in regulating several proteins that in turn regulate the host response to *M. tuberculosis* infection including components of ESCRT-0, SMURF1, NEDD4, and ITCH¹⁷³. If ANKRD13a is indeed regulating anti-microbial ESCRT-0 and SMURF1 proteins upstream of RNF11, we would expect less containment of bacteria in

ANKRD13a KO cells. However, the decrease in actin tail colocalization in ANKRD13a KO cells suggests that fewer bacteria are escaping the phagosome in the absence of ANKRD13a. In most cases, a decrease in phagosome escape leads to a decrease in bacterial growth, though we observed an increase in bacterial growth in at least one guide. Future repeats of these experiments are necessary to determine the true phenotype of ANKRD13a KO cells.

Although we did not observe phenotypes in our initial studies with PGRMC1 and OSTF1, it is still possible they may be playing a role in infection that was obfuscated by the techniques we chose to use. Previous work on both proteins indicates they may play a role in *M. tuberculosis* infection. PGRMC1 is a heme-binding protein implicated in many different roles with little mechanistic insight¹⁷⁴. We became interested in this protein as it was identified in our *M. tuberculosis*-host protein-protein interaction studies as interacting with the important *M. tuberculosis* antigen ESAT-6¹²⁴. Little is known about the role of PGRMC1 in macrophages. A previous study found that RAW cells depleted of PGRMC1 had less pro-inflammatory signaling in response to necrotic debris¹⁷⁵. In neural cells, PGRMC1 has been demonstrated to suppress TNF- α expression¹⁷⁶.

OSTF1, also known as SH3P2, was one of the most highly differentially detected proteins between WT and Δ eccC infected THP1s in our artMS analysis. It has also been previously described as near the *M. tuberculosis* phagosome but no follow-up studies have been done. It is most well characterized for its role in osteoclast formation¹⁷⁷. OSTF1 was previously demonstrated to interact with Cbl¹⁶⁵ but little work has been done to characterize this interaction. Cbl is a ubiquitin ligase known for playing a role in the host response to *M. tuberculosis* infection and directly interacting with the LpqN protein¹²⁴. OSTF1 may be bound to Cbl which is recruited to *M. tuberculosis*, however, we did not detect Cbl as an enriched protein in our dataset. We also determined that OSTF1 was significantly phosphorylated at 2 and 4 hours following *M. tuberculosis* infection in mouse bone marrow-derived macrophages at Serine 203⁸⁰. Phosphorylation of human OSTF1 at serine 202 inhibits interaction between the protein and Myo1E¹⁷⁸ and it's likely the mouse OSTF1 phosphorylation has a similar function. Human OSTF1 regulates localization of Myo1E to endocytic sites¹⁷⁹ and Myo1E is known to regulate exocytosis of cytosolic vesicles¹⁸⁰. OSTF1 may be required for proper endocytic or vesicular trafficking via regulation of Myo1E during infection but future work is needed to support this hypothesis.

Beyond these proteins identified in our artMS analysis, our Fragpipe analysis identified additional proteins of interest that we have not yet studied. One such protein is HMGB1, which is significantly enriched in the WT-infected THP1s. HMGB1 is a DNA binding protein¹⁸¹, known to be a danger-associated molecular pattern (DAMP) elicited during infections¹⁸². HMGB1 has been shown to regulate autophagy via binding of Beclin1¹⁸³ in the cytosol. Adults with active pulmonary TB have increased HMGB1

signaling¹⁸⁴ but it's not clear the role that the signal is playing in infection. As HMGB1 is well known for being a secreted protein in times of inflammatory stress, it's interesting to ask why we found it in our screen as a protein near Gal-8 during WT *M. tuberculosis* infection. We also identified LAMTOR1 as a possible, though not top, hit in multiple iterations of our analysis. LAMTOR1 is a component of the Ragulator complex and activates mTOR at the lysosomal surface. LAMTOR1 is recruited near Gal-8 during lysosomal damage¹⁵⁹. It is also important for localization of the autophagy adaptor TAX1BP1 to the *Salmonella* Typhimurium and Group A *Streptococcus* containing phagosomes¹⁸⁵. However, its role in *M. tuberculosis* pathogenesis has not yet been studied.

Another approach we took to analyze our artMS dataset was network propagation¹⁶⁴. We initially used our artMS data analysis in comparison to our previous host-*M. tuberculosis* protein-protein interactions dataset¹²⁴. Significant hits include STK24, PSG9, NUFIP2, and RGS20. None of these high betweenness proteins detected had previously been studied in *M. tuberculosis* infection. However, NUFIP2 is a stress granule protein known to be recruited to sites of lysosomal damage¹⁸⁶. Recent work demonstrated that stress granules can plug sites of endolysosomal damage. Though this work did not examine the role of NUFIP2, they found that stress granules are required for the restriction of *M. tuberculosis*¹⁸⁷. The identification of NUFIP2 in our network propagation may indicate that stress granules are detected near Gal-8 during infection. Lastly, Because the protein-protein interaction dataset was performed with cell lysates and a collection of secreted *M. tuberculosis* proteins, it's possible some of these hits are not an accurate representation of *in vivo* realities. Future work should use additional datasets from our lab, such as the PTM proteomics, to strengthen the findings in our network.

Here we have demonstrated multiple approaches to label the *M. tuberculosis* phagosome. Though our initial work has some promising hits, further refinement of the infection methods and data analysis is required to create a high-confidence dataset.

METHODS

Cell lines THP1s were purchased from ATCC and cultured in RPMI (Gibco) supplemented with 10% FBS, 5 mM L-glutamine, 5 mM sodium pyruvate, and 10 mM HEPES. Conditionally immortalized macrophages were edited and cultured as previously described¹²⁰ using gRNA sequences from the Brie library. All cells were incubated at 37°C and 5% CO₂.

Construction of exogenous APEX2

A gene block containing the amphipathic helix, linker, and APEX2 was ordered from IDT. Geneblock was ligated into the pH3c-LIC vector using SLiCE cloning¹⁸⁸ (Supplemental File 1). BL21(DE3)-Gold (Agilent) competent cells were transformed with

the vector. For protein production, 5 mL of stationary overnight culture was added to 1 L of 2x YT broth and incubated for 4 hours at 220 rpm at 37°C. After 4 hours, 20 uM of IPTG was added and culture was returned to the incubator for 7 hours. Cultures were spun at 6000 rev/min in a Sorvall centrifuge for 15 minutes and sent to the Rosenberg Lab at UCSF for purification.

Exogenous APEX2-GAL8 labeling of M. marinum

Two days before the experiment, *M. marinum* expressing mCherry was inoculated into 7H9 (BD) supplemented with 0.5% glycerol. Prior to incubations, glass coverslips were coated with 50 ug/mL poly-D-lysine and incubated at 37°C for 2 hours. On the day of the experiment, cultures were washed 3 times in DPBS. The pellet was then resuspended with varying concentrations of purified construct ranging from 0.3-30 ug. Bacteria and protein were incubated on ice for 1 hour and then resuspended in 1 mL of cold PBS. Samples were added to poly-D-lysine coated coverslips and spun for 10 minutes at 350 x g, followed by a 30-minute incubation at room temperature. Bacteria were then fixed with 4% PFA and rinsed with PBS. Slides were stained with an anti-V5 antibody (Invitrogen #46-0705) 1:300 for 3 hours at room temperature, followed by incubation with an Alexa Fluor 488 (Invitrogen) secondary antibody. Slides were imaged at 60-100x on a Nikon Eclipse TE2000-E spinning disc confocal microscope.

Construction of APEX2-GAL8 Construct

The APEX2-GAL8 plasmid deposited into Addgene (#149519) by the Deretic lab¹⁵⁹ was used as a PCR template with primers 5'-GTGGAATTCTGCAGATATCAAgccaccatggactacaaggat- 3' and 5'-GCCACTGTGCTGGATATCAActaccagctccttacttcca - 3' for cloning into the pLenti CMV Puromycin DEST (Addgene #17452) plasmid¹⁸⁹ using SLiCE cloning¹⁸⁸. Lenti-X 293T cells were reverse transfected with the pLenti-APEX2-GAL8 plasmid, pMD2.G, and psPAX2 and THP1s were transduced two days later. THP1s were incubated for 2 days prior to selection with puromycin at a concentration of 4 ug/mL.

Proximity labeling during M. tuberculosis infection

THP1s expressing the APEX2-GAL8 were seeded at 4 million cells/well in a 4-well OmniWell plate 3 days prior to infection and differentiated with 20 ng/mL phorbol 12-myristate 12-acetate (PMA) (Invivogen). WT Erdman and Δ eccC Erdman¹⁶² *M. tuberculosis* were grown rolling at 37°C to an OD of 0.6-0.8 in 7H9 (BD) supplemented with 10% Middlebrook OADC (BD), 0.5% glycerol, and 0.05% Tween-80. Bacteria were washed three times in DPBS prior to sonication at 90% amplitude for 2 x 30 seconds. Bacteria were added at an MOI of 5 unless otherwise indicated in DMEM + 10% horse serum (Gibco) and infected via centrifugation for 10 minutes at 400 x g. After spinning, the media was replaced and cells were incubated at 37°C and 5% CO₂ for 5.5 hours. At

5.5 hours, fresh media containing 500 μ M of biotinyl tyramide (Adipogen) was added and cells were returned to the incubator. 6 hours post-infection, cells were removed from the incubator and labeled following the previously described protocol¹⁷⁰. Briefly, 1 mM of H₂O₂ was added for 1 minute before washing with a quenching buffer (10 mM sodium ascorbate, 5 mM Trolox, 10 mM sodium azide in DPBS) five times. Cells were scraped, spun down, decanted, and stored at -80°C overnight to lyse. The following day, cells were thawed on ice and resuspended in RIPA buffer (Thermofisher) containing 1X protease inhibitor cocktail (Roche), 1 mM PMSF, 5 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 10 mM sodium azide, and 10mM sodium ascorbate. Lysed cells were spun at 15,000 x g for 10 minutes at 4°C. The supernatant was saved and analyzed via the Pierce 660 nm assay to determine concentration.

Elution of biotinylated proteins

Pierce Streptavidin Magnetic Beads (Thermofisher) were washed with the aforementioned supplemented RIPA buffer twice. 900 μ g of protein was added to 125 μ L beads and lysates were rotated at room temperature for 1 hour. Following the hour, beads were pelleted on a magnetic rack and washed in 1 mL volumes twice with the supplemented RIPA buffer, once with 1M KCl, once with 0.1M Na₂CO₃, once with 2M urea in 10 mM Tris-HCl 8.0, and twice again with supplemented RIPA buffer. Sixty μ L of 3X protein loading buffer supplemented with 2 mM biotin and 20 mM dithiothreitol (DTT) was added to each tube and samples were boiled at 95°C for 30 minutes as previously described¹⁷⁰. Samples were then transferred from the BSL3 following approved protocols. After transferring, beads were collected on a magnetic rack and eluate was collected for downstream processing.

Validation of colocalization of APEX2-GAL8 construct

THP1s were seeded at 200,000 cells/mL and differentiated with 20 ng/mL of PMA for 3 days prior to infection. WT and Δ *eccC* *M. tuberculosis* cultures expressing GFP were prepared as described earlier and added to cells at an MOI of 2. Six hours post-infection, cells were fixed with 4% PFA. Following fixation, cells were permeabilized and blocked in PBS with 0.05% saponin and 5% fetal bovine serum. Cells were then stained with anti-FLAG M2 (Sigma #F3165) 1:500 for 3 hours at room temperature followed by a secondary antibody conjugated to a fluorophore at 1:1000 and DAPI 1:1000. Slides were imaged at 60-100x on a Nikon Eclipse TE2000-E spinning disc confocal microscope and analyzed in ImageJ (National Institutes of Health).

Validation of peroxidase activity of APEX2-GAL8 construct

To validate peroxidase function, APEX2-GAL8 THP1s were first differentiated in 20 ng/mL PMA for 3 days. Cells were incubated in media either with or without 500 mM

biotinyl tyramide for 30 minutes followed by the addition of 1 mM H₂O₂ for 1 minute. Lysates were collected and concentration was calculated using the Pierce 660 nm assay. 20 ug of lysate was run at 200V for 45 minutes on a 4-20% Miniprotean TGX gel (Biorad) and the gel was transferred to PVDF. After blocking, blots were incubated with 1:5000 IRDye 800 Streptavidin (Licor) for 45 minutes, washed with PBS + 0.05% Tween20, and imaged on the Licor Odyssey system.

High-throughput microscopy colocalization of APEX2-GAL8 over time

APEX2-GAL8 THP1s were seeded at 350,000 cells/mL in a 24-well plate and differentiated with 20 ng/mL PMA for three days. On the day of infection, *M. tuberculosis* expressing GFP was infected at an MOI of 2, 5, or 10 as previously described. Samples were fixed with PFA at indicated time points and stained with the anti-FLAG M2 as described above. Perkin Elmer plates were imaged on the Opera Phenix High-Content Screening System as previously described⁴². Colocalization of APEX2-GAL8 with *M. tuberculosis* was performed using a spot finder assay. The total number of APEX2-GAL8 puncta detected was divided by the number of spots colocalized with Mtb to identify the total percent colocalized.

2D MudPIT Mass spectrometry (section contributed by Rob Maxwell)

Mass spectrometry was performed at the Proteomics/ Mass Spectrometry Laboratory at the University of California, Berkeley. Multidimensional protein identification technique (MudPIT) was performed as described^{169,190}. Briefly, a 2D nano-LC column was packed in a 100-μm inner diameter glass capillary with an integrated pulled emitter tip. The column consisted of 10 cm of Polaris c18 5-μm packing material (Varian) and 4 cm strong cation exchange resin (Partisphere, Hi Chrom). The column was loaded and conditioned using a pressure bomb. The column was then coupled to an electrospray ionization source mounted on a Thermo-Fisher LTQ XL linear ion trap mass spectrometer. An Agilent 1200 HPLC equipped with a split line to deliver a flow rate of 1 ul/min was used for chromatography. Peptides were eluted using a 4-step gradient with 4 buffers. Buffer (A) 5% acetonitrile, 0.02% heptafluorobutyric acid (HFBA), buffer (B) 80% acetonitrile, 0.02% HFBA, buffer (C) 250mM NH₄AcOH, 0.02% HFBA, (D) 500mM NH₄AcOH, 0.02% HFBA. Step 1: 0-80% (B) in 70 min, step 2: 0-50% (C) in 5 min and 0- 45% (B) in 100 min, step 3: 0-100% (C) in 5 min and 0- 45% (B) in 100 min, step 4 0-100% (D) in 5 min and 0- 45% (B) in 160 min. Collision-induced dissociation (CID) spectra were collected for each m/z. Protein identification, quantification, and analysis were done with Integrated Proteomics Pipeline-IP2 (Bruker Scientific LLC, Billerica, MA, <http://www.bruker.com>)) using ProLuCID/Sequest¹⁹¹, DTASelect2^{192,193}, and Census^{194,195}. Spectrum raw files were extracted into ms1 and ms2 files from raw files using RawExtract 1.9.9 (<http://fields.scripps.edu/downloads.php>) 10, and the tandem mass spectra were searched against human database^{193,196}.

LC-MS/MS

Eluted proteins were reduced, alkylated, and digested with trypsin as earlier described¹⁹⁷. Samples were analyzed in technical triplicate on a QE-HF mass spectrometer (Thermo Fisher) coupled to a nano-LC (Waters, Billerica, MA) running a TOP15 data-dependent method. Label-free quantification (LFQ) of RAW files and database searching were compared to the human and Erdman *M. tuberculosis* proteomes obtained from UniProt.

Mass spectrometry analysis

Data was analyzed using MaxQuant¹⁹⁸, artMS¹⁹⁹, and/or Fragpipe (<https://fragpipe.nesvilab.org/>). Protein groups were visualized using Metascape²⁰⁰.

Network propagation (section contributed by Ellie Reinhart Wouters)

Unstimulated monocytes from a Ugandan cohort of resistor vs latently infected individuals were previously assessed for chromatin accessibility (ATAC-seq), methylation, genetics (SNP), and proteomic enrichment. The last data set was an *M. tuberculosis*-infected RNA-seq data set. Each of the individual networks was separately mapped within the human interactome data from STRING v11.5 database²⁰¹. The network was restricted to nodes with a combined experimental confidence of ≥ 400 . Then the Laplacian heat diffusion algorithm operated heat diffusion with a permutation test to filter out false-positive high diffusion score nodes ($p\text{-value} < 0.05$). Genes that were significantly scored with each separate dataset were combined and the resultant subset narrowed down the network from 19,076 human proteins to 5205 genes with hypothesized importance to the resistor vs latent infection phenotype.

Building on the previous network-propagated subnetwork, we combined the APEX dataset with the *M. tuberculosis*-host interaction network previously generated by the Cox lab, identifying interactions between TB-secreted proteins and macrophage proteins¹²⁴. We performed an exhaustive shortest paths analysis on all pairs—each gene differentially enriched ($\text{MIST} > 0.7$) or unique to WT-infected macrophages paired with its closest interactor gene. We then produced a network containing all the shortest paths. On this subnetwork, key linker proteins are identified based on various network measures by the number of shortest paths passing through as well as their betweenness measure on the entire subnetwork. The underlying network topology is controlled for by normalizing actual measures compared to permuted gene sets on the same network ($p\text{-value} < 0.05$). Because each shortest path links an *M. tuberculosis* protein to a human gene involved in phagosome-specific responses to *M. tuberculosis* infection, the specific *M. tuberculosis* proteins linked to our key linker proteins are possible drivers of phagosome remodeling.

All network analysis was carried out in R v4.2.2 and subnetworks were visualized using Cytoscape²⁰².

Mycobacterium marinum infections

Mycobacterium marinum expressing mCherry was grown in 7H9 supplemented with 10% Middlebrook OADC, 0.5% glycerol, and 0.2% Tween80 to an OD of 0.2- 0.8 shaking at 30°C. Cultures were prepared like *M. tuberculosis* above with the exception of sonication. Bacteria were added at an MOI of 2. Infected macrophages were incubated at 33°C and 5% CO₂ for the duration of the experiment. At indicated time points, media was removed and cells were fixed in 4% paraformaldehyde (PFA). Prior to high throughput microscopy analysis, cells were stained with DAPI and Phalloidin Alexa 488 (Invitrogen) 1:250.

ACKNOWLEDGMENTS

Initial mass spectrometry experiments were performed at the Vincent J. Coates Proteomics/Mass Spec Laboratory at UC Berkeley. We would like to thank Bisco Hill, Huntly Morrison, James Olzmann, Tess Branon, and Jonathan Budzik for their technical advice. Additionally, we would like to thank members of the Cox, Stanley, and Vance labs for helpful discussions. This project was supported by the Host Pathogen Mapping Initiative.

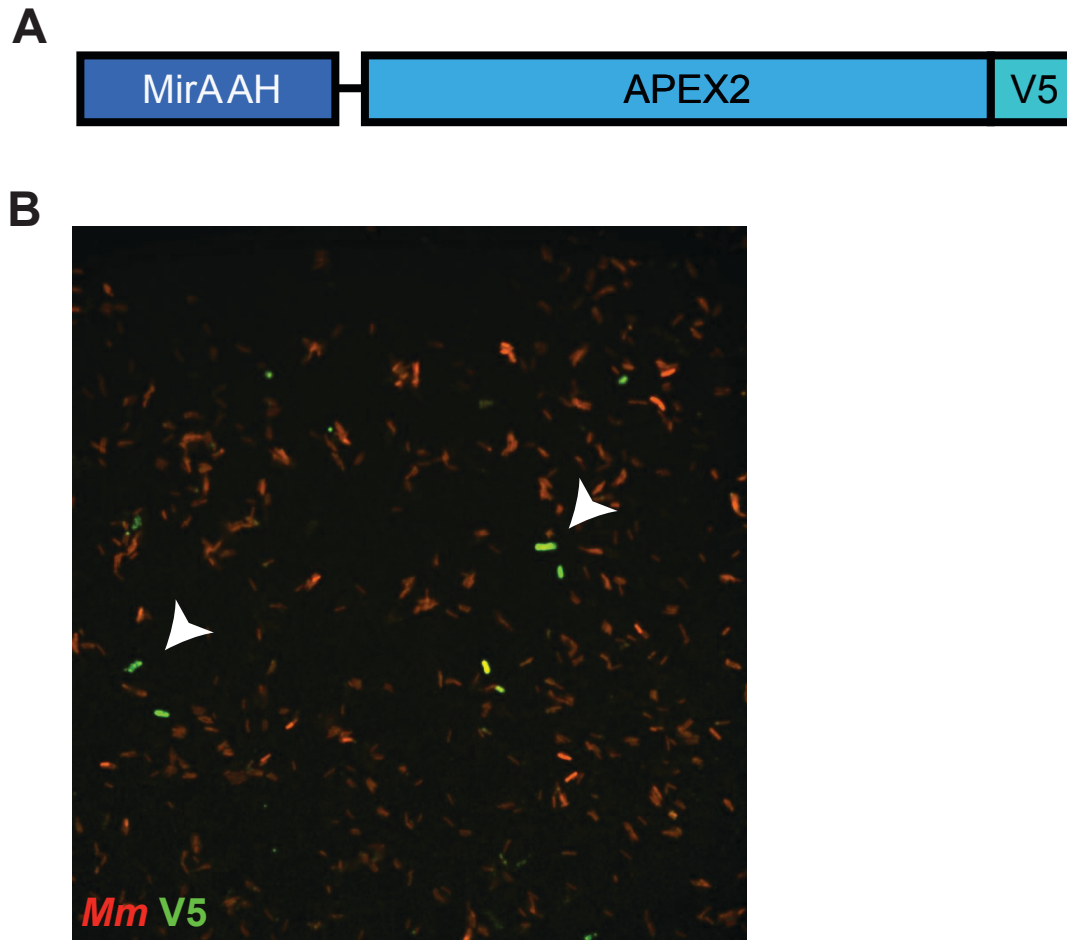


Figure 1 Exogenous labeling of mycobacteria with APEX2. (A) Representative map of exogenous APEX2 construct. (B) *M. marinum* expressing mCherry were incubated with the above construct and imaged at 60x using confocal microscopy. Antibodies raised against the V5 tag were used to detect the APEX2 fusion protein. Arrows indicate examples of colocalization of construct with bacterium.

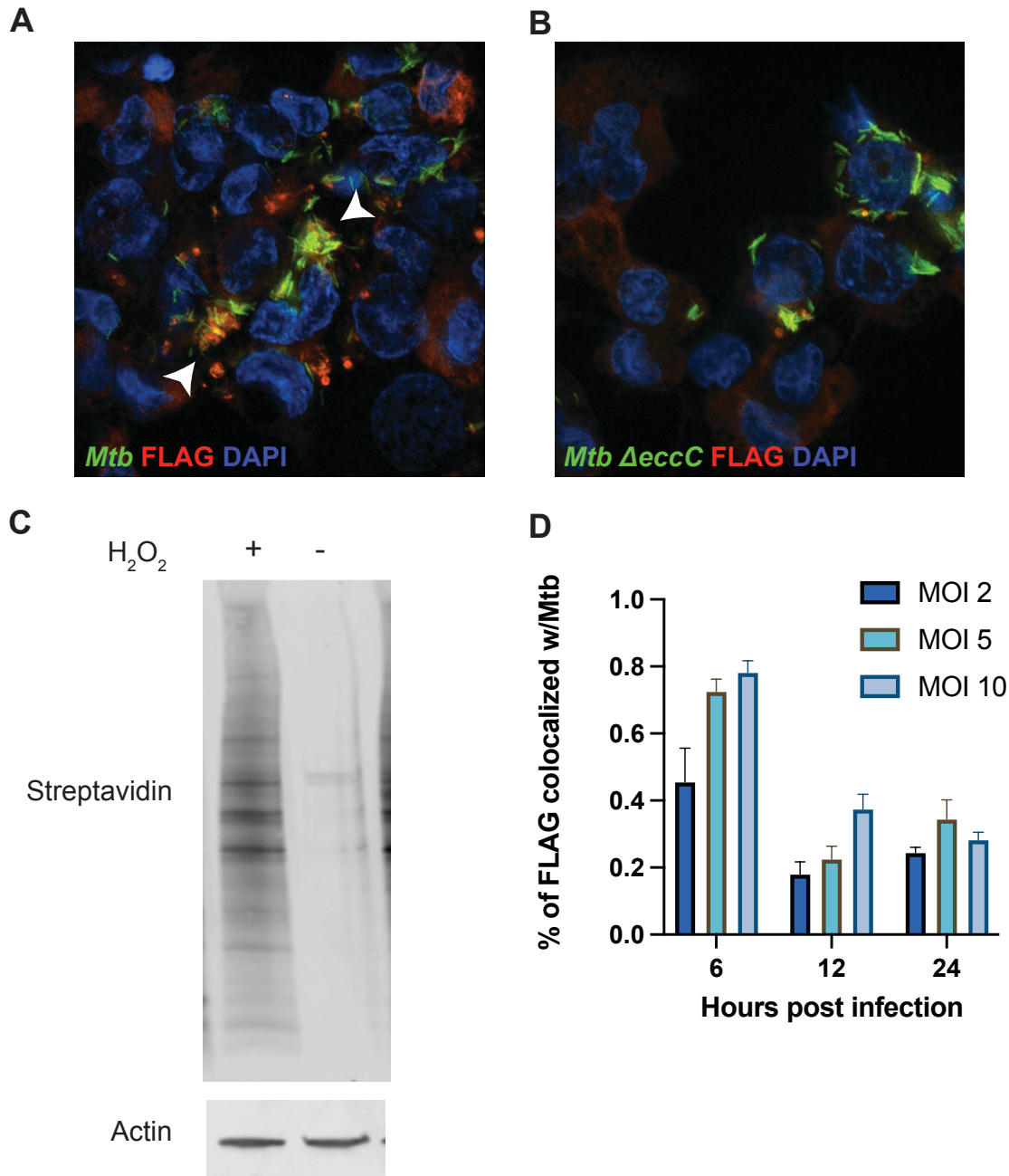


Figure 2 Validation of APEX2-GAL8. (A-B) THP1s expressing the GAL8-APEX2 construct were infected with both WT (A) and $\Delta eccC$ (B) Erdman *M. tuberculosis* at an MOI of 2. At 6 hours post infection, cells were fixed, incubated with an anti-FLAG antibody to detect the GAL8-APEX construct, and imaged at 60x on a confocal microscope. Arrows indicate examples of colocalization. (C) GAL8-APEX2 THP1s incubated for 30 minutes with biotin phenol were treated with or without H_2O_2 for 1 minute. Cells were quenched, lysed, and run on SDS-PAGE. Following transfer, blot was incubated with anti-streptavidin to detect biotinylated proteins. (D) APEX2-GAL8

THP1s were infected with WT Erdman *M. tuberculosis* for the indicated time points and MOIs. Cells were stained as above and analyzed for colocalization differences using high-throughput microscopy. Error bars represent SEM.

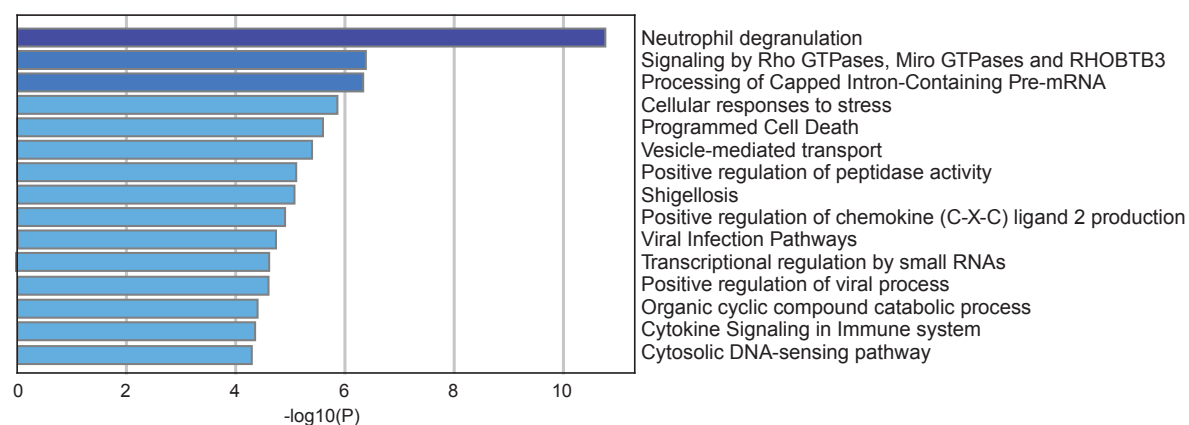


Figure 3 Metascape analysis of imputed and enriched proteins detected in artMS dataset. Proteins detected only in WT infected THP1s or found to be enriched with a $\log_2FC > 1$ in WT infected THP1s as compared to $\Delta eccC$ infected THP1s were analyzed for gene and pathway enrichment using Metascape.

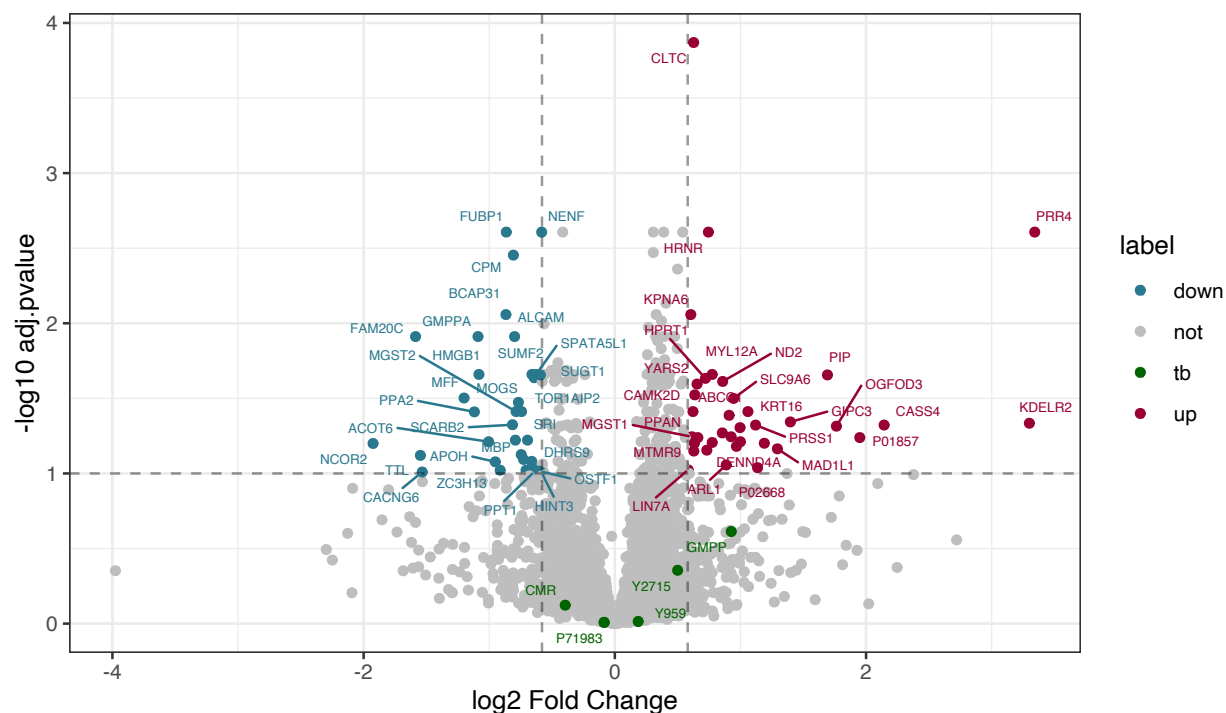


Figure 4 Volcano plot of proteins enriched APEX2-GAL8 proteins analyzed via **Fragpipe**. Human and TB proteins (indicated in green) detected in APEX2-GAL8 THP1s infected with WT and Δ eccC Erdman *M. tuberculosis*. Proteins labeled in blue were enriched in WT infected THP1s while proteins in red were enriched in mutant infected THP1s. Labeled samples had a $\log_2\text{FC} > 0.5$ and $p\text{-value} < 0.1$.

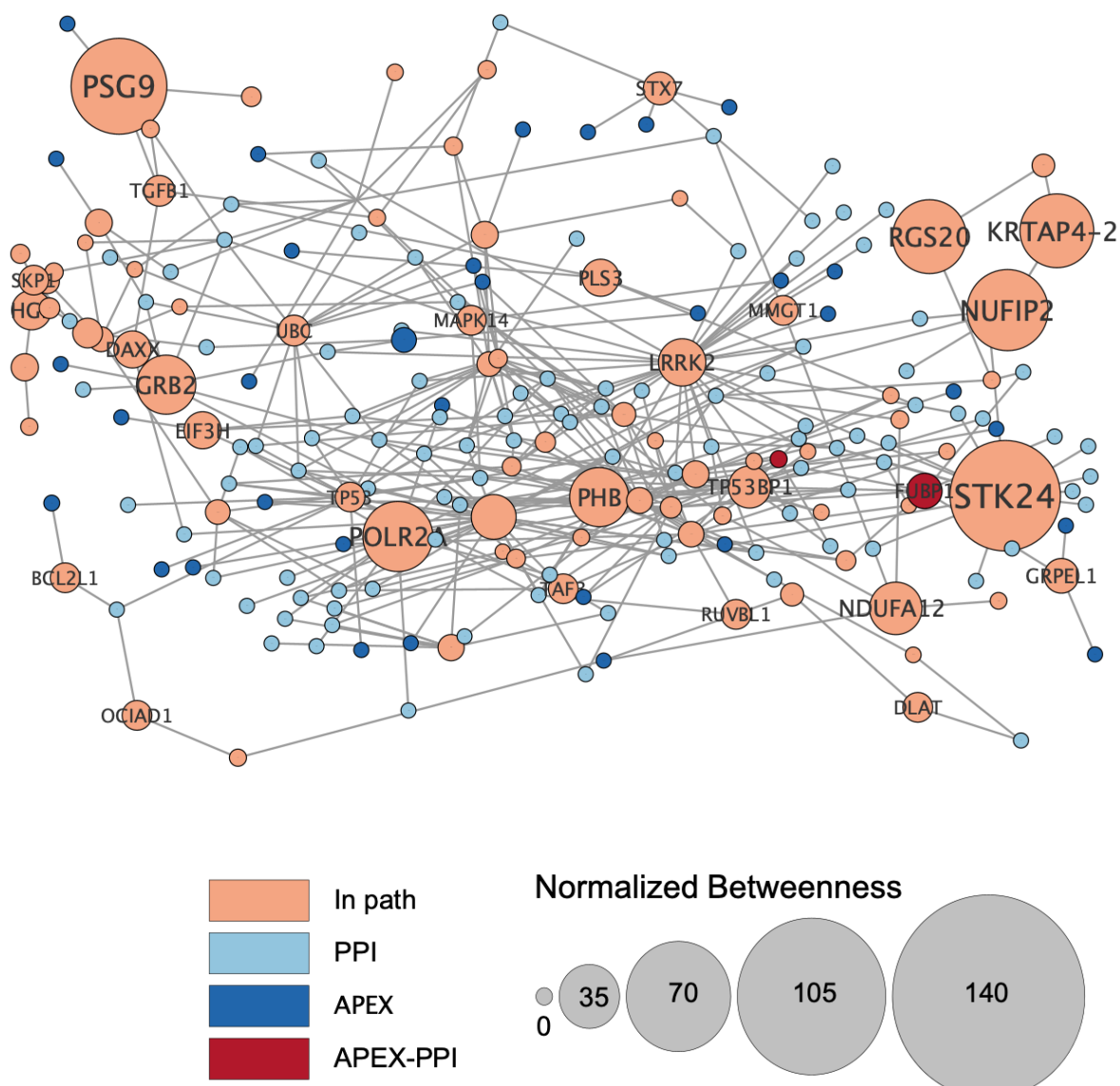


Figure 5 Network propagation of artMS dataset. Representative subnetwork of network propagation analysis. Circles represent proteins with a normalized betweenness of >12 plus first neighbors. Betweenness is represented by circle size. PPI represent human proteins detected in Penn et al. Human-Mtb protein protein interaction studies. APEX proteins represent proteins detected in this study. APEX-PPI are proteins detected in both datasets. In path represents proteins that are in pathways protein-protein interactions of hits from both datasets. Graph created using Cytoscape.

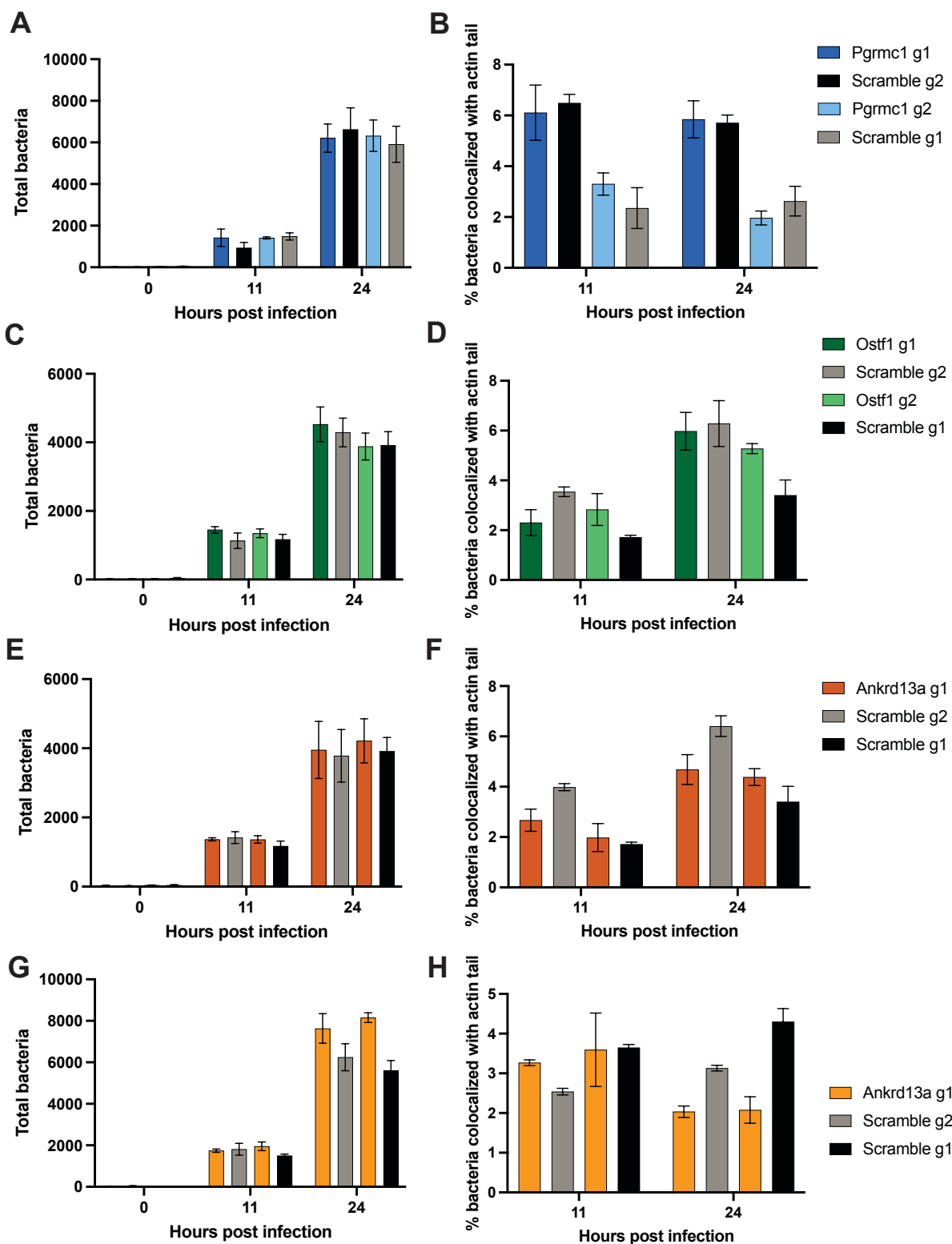


Figure 6 OSTF1, ANKRD13A, and PGRMC1 have limited role in *M. marinum* infection. Three proteins detected as imputed in our initial artMS analysis were

knocked out using CRISPR/Cas9 editing of CIMs. CIMs with scramble control were infected with *M. marinum* expressing mCherry for 0, 11, or 24 hours at an MOI of 2. At indicated time points, plates were fixed and analyzed for growth (A,C,E,G) and actin tail colocalization (B, D, F, H) using high-throughput microscopy. ANKRD13a experiments were performed on separate days and are therefore represented in distinct graphs. Error bars represent SEM.

Table 1 Proteins enriched in WT infected APEX2-GAL8 THP1s analyzed using 2DMudPIT.

Accession Number	Gene Name	Confidence Score (AU)
P62987	UBA52	0.025
P0CG47	UBB	0.02270742
O00370	LINE-1 retrotransposable element ORF2 protein	0.01505882
Q01518	CAP1	0.01221053
O00214	LGALS8	0.01072555
Q9BQE3	TUBA1C	0.01024499
Q14847	LASP1	0.00996169
P0CG48	UBC	0.00817518
Q3ZCM7	TUBB8	0.00675676
P05141	SLC25A5	0.00671141
Q05639	EEF1A2	0.00647948
P41218	MNDA	0.00638821
Q02878	RPL6	0.00625
P67809	YBX1	0.00617284
P12236	SLC25A6	0.00604027
Q86UX7	FERMT3	0.005997
P12235	SLC25A4	0.00536913
P61586	RHOA	0.00518135
P08134	RHOC	0.00518135
P13796	LCP1	0.00510367
P47914	RPL29	0.00503145
Q06830	PRDX1	0.00502513
P23528	CFL1	0.00481928
Q8TCT9	HM13	0.00477454
P46940	IQGAP1	0.0047073
Q02543	RPL18A	0.00454545
P39023	RPL3	0.0044665
P68104	EEF1A1	0.004329
Q5VTE0	EEF1A1P5	0.004329
O43707	ACTN4	0.0039517
P08238	HSP90AB1	0.0038674
Q6IAA8	LAMTOR1	0.00372671
Q562R1	ACTBL2	0.0037234
P07477	PRSS1	0.00364372

Table 2 Proteins detected as imputed in WT infected APEX2-GAL8 THP1s using artMS analysis.

Accession Number	Gene	iLog2C	iP-value
Q92882	OSTF1	2.30329121	0.0111393
P28799	GRN	2.26969226	0.0113486
P49593	PPM1F	2.20924368	0.0121283
Q9BVL2	NUP58	1.24379048	0.0128758
O00264	PGRMC1	3.49362825	0.0148823
Q92538	GBF1	2.61504309	0.0159745
Q9H1I8	ASCC2	0.57162315	0.016279
Q6ZQR2	CFAP77	1.20346001	0.0200877
Q07955	SRSF1	2.38785926	0.0207301
Q9NYQ7	CELSR3	2.88712803	0.0208144
Q8TEU7	RAPGEF6	2.42189628	0.0217039
P32942	ICAM3	2.17514157	0.0227799
P19404	NDUFV2	0.91203475	0.0228614
Q13435	SF3B2	3.29964832	0.023574
Q92547	TOPBP1	4.2068733	0.0248476
Q8NEY4	ATP6V1C2	4.242508	0.0263608
Q8NF91	SYNE1	1.68797816	0.0271567
Q9Y5Y6	ST14	3.20026442	0.027582
Q9P0L1	ZKSCAN7	0.91399184	0.0280299
Q13546	RIPK1	1.85070681	0.0280853
Q8IXI2	RHOT1	1.3974863	0.0295035
Q96QV6	H2AC1	1.72500084	0.0310302
P16104	H2AX	1.72500084	0.0310386
Q99747	NAPG	3.96503023	0.0338472
P30043	BLVRB	1.87031047	0.0341171
P63261	ACTG1	1.23673342	0.0342699
Q8IZ07	ANKRD13A	3.31327049	0.0358707
Q9Y5Q0	FADS3	1.75397424	0.0364289
O00330	PDHX	2.98870483	0.0369683
Q9UBR2	CTSZ	3.35136213	0.0372956
Q9P1Y6	PHRF1	3.30055353	0.0373348
Q9ULD2	MTUS1	2.95943899	0.0377727
O14737	PDCD5	1.91762466	0.0379379
Q9Y6H1	CHCHD2	1.95690523	0.0387622
Q96DB5	RMDN1	1.36626258	0.0397425

B7ZBB8	PPP1R3G	2.19287392	0.0424742
Q9Y3B4	SF3B6	1.70945856	0.0429077
Q96J84	KIRREL1	1.77804332	0.0452311
P18887	XRCC1	3.36217473	0.0452371
Q7Z3D6	DGLUCY	3.8654457	0.046813
Q8WXE0	CASKIN2	2.30642034	0.0492954

Table 3 Proteins enriched in WT infected THP1s using artMS analysis.

Accession Number	Gene	Log2FC	p-value	Adjusted p-value
O77932	DXO	4.500295	0.074712	0.861387
Q8WWB5	PIH1D2	3.554441	0.221861	0.897657
Q9H0K1	SIK2	2.735326	0.11463	0.897657
Q9UMX0	UBQLN1	2.435489	0.049111	0.831392
Q6UUV9	CRTC1	2.330557	0.002722	0.592396
P42694	HELZ	2.128159	0.277813	0.92373
O75449	KATNA1	2.126933	0.081038	0.881864
O75381	PEX14	2.125339	0.025944	0.791032
Q03252	LMNB2	2.044756	0.233288	0.902514
P01584	IL1B	1.996183	0.006682	0.64537
Q15392	DHCR24	1.981225	0.043011	0.810589
Q9UNA1	ARHGAP26	1.949935	0.033808	0.791032
P9WMJ7	P9WMJ7	1.926567	0.138347	0.897657
P62312	LSM6	1.917956	0.100611	0.897657
P30566	ADSL	1.860616	0.048366	0.831392
Q01469	FABP5	1.776404	0.039112	0.791305
P62699	YPEL5	1.774138	0.068846	0.859677
P52789	HK2	1.773786	0.001063	0.351377
Q96LI5	CNOT6L	1.737538	0.182829	0.897657
O95153	TSPOAP1	1.713472	0.008472	0.64537
O75368	SH3BGRL	1.710376	0.055819	0.833729
Q96CU9	FOXRED1	1.710215	0.0154	0.733176
P09429	HMGB1	1.658922	0.00016	0.23243
P52565	ARHGDIA	1.624631	0.144487	0.897657
B1AJZ9	FHAD1	1.573025	0.030561	0.791032
Q9UID3	VPS51	1.561246	0.160057	0.897657
P54687	BCAT1	1.557777	0.063991	0.849049
Q7L2J0	MEPCE	1.556695	0.052224	0.831392
Q9Y6B6	SAR1B	1.54354	0.063829	0.849049
Q04721	NOTCH2	1.537444	0.35792	0.926616
P02686	MBP	1.513667	0.048114	0.831392
Q8NBK3	SUMF1	1.512848	0.082383	0.883854
Q12756	KIF1A	1.510715	0.001787	0.468971
Q9HCS7	XAB2	1.443064	0.299997	0.926095
Q8NCE2	MTMR14	1.434556	0.058815	0.849049
P15924	DSP	1.424921	0.265536	0.92373

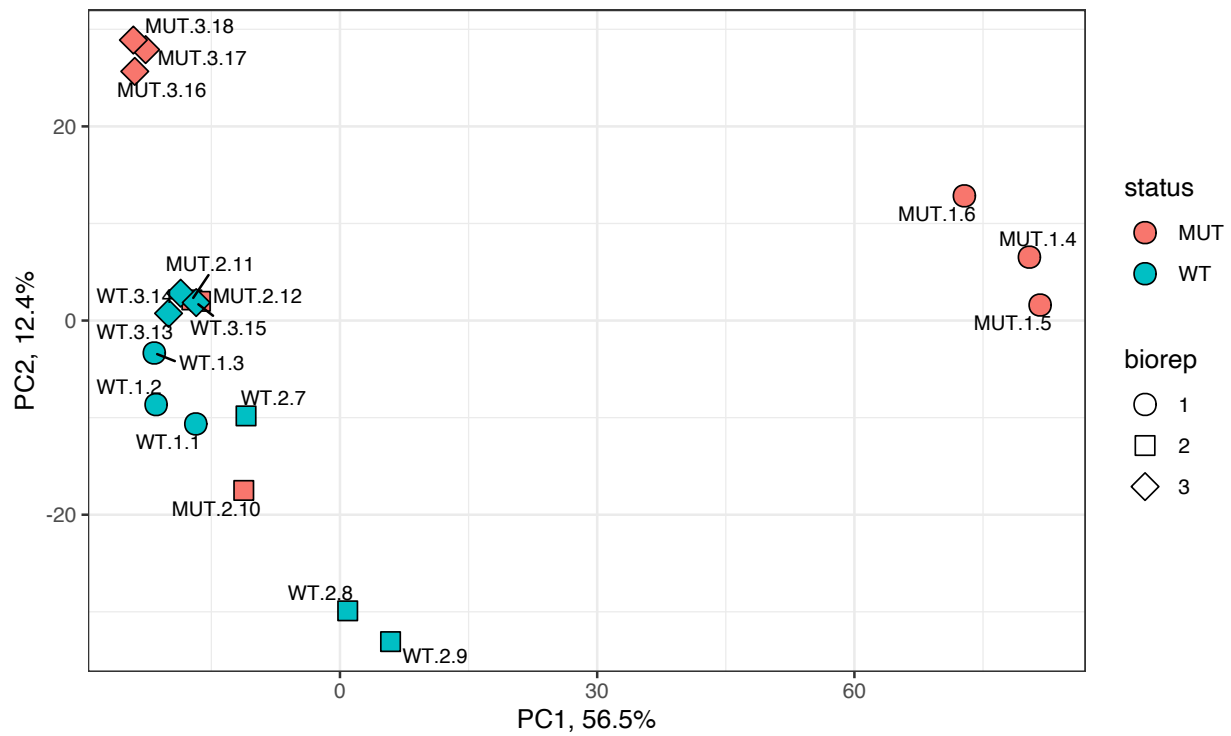
Q12846	STX4	1.410461	0.116276	0.897657
Q9Y5T5	USP16	1.409548	0.031196	0.791032
P10412	H1-4	1.407814	0.364795	0.926616
Q86VY4	TSPYL5	1.402007	0.029916	0.791032
Q9BPX6	MICU1	1.395466	0.39792	0.926949
Q9BT25	HAUS8	1.377965	0.052161	0.831392
O60333	KIF1B	1.375447	0.055446	0.832234
Q15283	RASA2	1.370051	0.496465	0.946046
P14174	MIF	1.358479	0.010667	0.670043
O96000	NDUFB10	1.355813	0.090431	0.897532
Q16630	CPSF6	1.346716	0.080346	0.881864
Q8TEH3	DENND1A	1.33907	0.080564	0.881864
Q9UK61	TASOR	1.337566	0.497141	0.946744
P61289	PSME3	1.324445	7.01E-05	0.213592
Q9NXG6	P4HTM	1.321306	0.111733	0.897657
Q8N257	H2BC26	1.310072	0.20126	0.897657
Q96M96	FGD4	1.307354	0.274027	0.92373
Q8N684	CPSF7	1.30505	0.00488	0.64537
Q9H2L5	RASSF4	1.303812	0.106307	0.897657
P19474	TRIM21	1.282436	0.118421	0.897657
A2RRP1	NBAS	1.281627	0.022139	0.791032
Q96KP4	CNDP2	1.274678	0.561351	0.95943
P09110	ACAA1	1.272457	0.002001	0.468971
Q7L1W4	LRRC8D	1.266178	0.026263	0.791032
O75746	SLC25A12	1.260979	0.037599	0.791305
P08571	CD14	1.259539	0.15345	0.897657
P78362	SRPK2	1.259104	0.119873	0.897657
Q8NCY6	MSANTD4	1.255762	0.372442	0.926616
Q9H3K6	BOLA2	1.252529	0.033224	0.791032
P31689	DNAJA1	1.241433	0.010659	0.670043
Q8NHL6	LILRB1	1.235373	0.173372	0.897657
P29466	CASP1	1.234082	0.374351	0.926616
Q8IUN9	CLEC10A	1.21996	0.089123	0.890354
Q96I99	SUCLG2	1.21723	0.028566	0.791032
O75323	NIPSNAP2	1.2117	0.023688	0.791032
Q9GZT8	NIF3L1	1.210569	0.079845	0.881864
Q8WX94	NLRP7	1.199894	0.057293	0.844122
Q6WKZ4	RAB11FIP1	1.198138	0.503717	0.94679
Q99720	SIGMAR1	1.197814	0.02679	0.791032

P18754	RCC1	1.197472	0.042705	0.810589
Q9H1Y0	ATG5	1.190507	0.185913	0.897657
Q8NFF5	FLAD1	1.189459	0.65471	0.966169
Q8NBM4	UBAC2	1.185916	0.073638	0.859677
Q71UM5	RPS27L	1.185543	0.070962	0.859677
P61626	LYZ	1.18511	0.187338	0.897657
P51798	CLCN7	1.177478	0.192685	0.897657
P52434	POLR2H	1.17365	0.048073	0.831392
Q9NYL2	MAP3K20	1.167751	0.007651	0.64537
P08708	RPS17	1.161153	0.170052	0.897657
Q9Y5H0	PCDHGA3	1.15706	0.206017	0.897657
Q12912	IRAG2	1.150443	0.170874	0.897657
P84090	ERH	1.148007	0.008225	0.64537
Q02413	DSG1	1.147832	0.003611	0.64537
Q7Z3K3	POGZ	1.147613	0.141273	0.897657
O15523	DDX3Y	1.145239	0.22473	0.897657
Q8WTV0	SCARB1	1.135342	0.025947	0.791032
Q96AE4	FUBP1	1.133207	0.019433	0.779105
A8MVU1	A8MVU1	1.127856	0.001153	0.351377
P40123	CAP2	1.122294	0.070184	0.859677
P98194	ATP2C1	1.110645	0.303085	0.926095
Q9Y2Q5	LAMTOR2	1.099308	0.024552	0.791032
O15511	ARPC5	1.096025	0.035138	0.791032
Q96EE3	SEH1L	1.090974	0.021144	0.791032
P26447	S100A4	1.082227	0.084313	0.885005
P78417	GSTO1	1.074385	0.188442	0.897657
Q99798	ACO2	1.071861	0.009825	0.670043
A8MXV4	NUDT19	1.071287	0.369765	0.926616
P16930	FAH	1.070683	0.1188	0.897657
Q7L2H7	EIF3M	1.069722	0.037539	0.791305
Q5T1V6	DDX59	1.065351	0.010838	0.670043
O60934	NBN	1.057897	0.030848	0.791032
Q92526	CCT6B	1.05602	0.822992	0.970798
Q16531	DDB1	1.052497	0.030232	0.791032
Q6PJG6	BRAT1	1.051005	0.1471	0.897657
O00161	SNAP23	1.050923	0.035307	0.791032
Q96G23	CERS2	1.046636	0.017783	0.749577
Q71DI3	H3C14	1.038352	0.371499	0.926616
Q96MH6	TMEM68	1.036276	0.208481	0.897657

P22392	NME2	1.028384	0.300545	0.926095
Q969Q5	RAB24	1.027076	0.213054	0.897657
Q8IUG5	MYO18B	1.027061	0.039095	0.791305
P09104	ENO2	1.025546	0.778246	0.966169
P49585	PCYT1A	1.024113	0.305316	0.926095
Q9NQE9	HINT3	1.02054	0.026947	0.791032
P06730	EIF4E	1.016309	0.293766	0.926095
P37837	TALDO1	1.014216	0.23024	0.902053
Q9NYB9	ABI2	1.010038	0.189359	0.897657
P80723	BASP1	1.009928	0.005204	0.64537
P67936	TPM4	1.00179	0.149959	0.897657
O15118	NPC1	1.001277	0.070742	0.859677

Table 4 Proteins identified in network propagation analysis between APEX2-PPI datasets.

Symbol	Normalized Betweenness	Betweenness p-value
EGFR	201	0.472528
STK24	78	0.004995
PSG9	66	0.003996
NUFIP2	54	0.026973
RGS20	48	0.03996
KRTAP4-2	48	0.00999
POLR2A	45	0.467533
PHB	36	0.075924
GRB2	36	0.744256
NDUFA12	30.56604	0.007992
LRRK2	26.36025	0.057942
H4C6	24.24868	0.066933
TP53BP1	22.11702	0.407592
HGF	20	0.02997
DAXX	18	0.074925
EIF3H	18	0.040959
PLS3	18	0.010989
FUBP1	16.5	9.99E-04
GRPEL1	16	0.021978
STX7	14.55319	0.075924
TGFB1	12.9148	0.061938
UBC	12.90631	0.437562
NME2	12	0.085914
SKP1	12	0.45055
RUVBL1	12	0.315684
TAF3	12	0.037962
MMGT1	12	0.081918
DLAT	12	0.073926
OCIAD1	12	0.014985
MAPK14	12	0.184815
BCL2L1	12	0.090909



Supplemental Figure 1 PCA plot demonstrating variation of $\Delta eccC$ Erdman biological replicate 1. PCA analysis of features between biological and technical replicates of mass spectrometry results from infected APEX2-GAL8 THP1s infected with WT or $\Delta eccC$ Erdman (MUT) *M. tuberculosis*. Numbers following bioreplicate number indicate fraction run.

Ggggatgccgaatataccaatgtttcgcaatggatcagtgggcgagttttccaaattagtcagttcattcttaacctggtgaa
cgcgccgagcggaattgctgtttccgggggcggtgggggctctggcggcggaggcagtggaagtcttaccctaactgtg
agtgctgattaccaggacgccgttgagaaggcgaagaagaagctcagaggcttcacgctgagaagagatgcgctcct
ctaagtctccgtttggcattccactctgctggaacctttgacaagggcacgaagaccggtggacccttcggaaccatcaag
caccctgccgaactggctcacagcgctaacaacggtcttgacatcgctgttaggcttttgagccactcaaggcggagttc
cctattttgagctacgccgatttctaccagttggctggcgttgttgccgttgaggtcacgggtggacctaaggttcattccacc
ctggaagagaggacaagcctgagccaccaccagagggctcgcttggccgatcccactaagggttctgaccatttgagag
atgtgtttggcaaagctatggggcttactgaccaagatatcggtgctctatctgggggtcacactatggagctgcacacaag
gagcgttctggatttgagggctccctggacctctaatacctcttattttcgacaactcatacttcacggagttgttgagtgtgaga
aggaaggtctccttcagctaccttctgacaaggctctttgtctgacctgtattccgccctctcggtgacaaatatgcagcgga
cgaagatgccttcttctgattacgctgagggtcaccaaaagctttccgagcttgggttctgatgccggcaagcccatcc
ccaacccccctgctgggcctggacagcacc

Supplemental File 1 Gene block sequence of AH-Linker-A

Chapter 4: A reflection on the unique ubiquitin ligase RNF213

Additional contributors: Elizabeth Turcotte (Vance Lab - UC Berkeley)

INTRODUCTION AND RESULTS

Work from our lab and others has demonstrated that *M. tuberculosis*-containing phagosomes are ubiquitylated during infection^{37,39}. Previous work from our lab, highlighted in Trevor Parry's thesis, demonstrated that *M. tuberculosis* itself is ubiquitylated. Trevor found that 10% *M. marinum* isolated from RAW 264.7 macrophages were ubiquitylated after isolation from the macrophage and this phenotype was dependent upon ESX-1. This ubiquitin was mainly K48 branched rather than K63. It is unknown which ubiquitin ligase is responsible for directly ubiquitylating the bacteria and this result has not been repeated.

In previous work in our lab, we identified a ubiquitin ligase, RNF213, that has an ESX-1-dependent increase in ubiquitylation during *M. tuberculosis* infection^{80,125}. RNF213 is a massive 500kDa ubiquitin ligase that has recently been found to directly ubiquitylate bacteria. Otten *et. al.* found that RNF213 could directly ubiquitylate LPS of *Salmonella* Typhimurium²⁰³. This stunning result demonstrated that non-proteinaceous substrates can be ubiquitylated by human E3 ubiquitin ligases. RNF213 was also found to colocalize with and control *Listeria monocytogenes*²⁰⁴. This apparent antimicrobial activity of RNF213 is known to be targeted by at least one pathogen, *Chlamydia trachomatis* which uses its GarD effector to exclude the ligase from inclusion membranes²⁰⁵. RNF213-mediated control of infection is not limited to bacteria, as it has also been demonstrated to be antiviral and antiparasitic^{204,206,207}. Because RNF213 is post-translationally modified in response to *M. tuberculosis* infection and has recently been demonstrated to act as an antimicrobial ubiquitin ligase that can directly ubiquitylate bacteria, we asked whether it was responsible for directly ubiquitylating *M. tuberculosis* during infection.

To investigate this question, we infected RNF213 deficient THP1s with WT Erdman *M. tuberculosis* expressing GFP for 6 hours. Cells were fixed with 4% paraformaldehyde and stained for total and linkage-specific ubiquitylation. We then imaged the cells on the OperaPhenix high-throughput microscope and used an analysis pipeline to determine colocalization. We did not see a significant difference between total ubiquitylation (FK2) nor linkage-specific ubiquitylation chains (K48 and K63). We did see a slight, though not statistically significant, increase in K63 ubiquitin linkages in RNF213 KOs compared to WT though this result should be repeated.

DISCUSSION

Previous members in our lab performed proteomics on the bacterium, specifically investigating lysine linkages, and found one periplasmic and five membrane proteins ubiquitylated during infection. We concluded that it would be difficult for ubiquitin ligases

to access these proteins through the mycolic acid layer and therefore the K48 labeled bacteria are likely damaged. However, in light of RNF213 developments, this should be revisited. It is possible that RNF213 or another yet-to-be-discovered ubiquitin ligase can directly ubiquitylate mycolic acids which would not have been detected in our original proteomics experiment.

Given that RNF213 is post-translationally modified in response to phagosome perforation and new findings of its role in host-pathogen interactions have been coming to light, I suspect RNF213 is somehow involved in *M. tuberculosis* infection. It's possible that our methods obscure differences in the actual ubiquitylation of *M. tuberculosis*. Future work should determine if RNF213 is colocalized with *M. tuberculosis* during infection. If it is colocalized, isolating bacteria from infected macrophages lacking RNF213, lysing with urea, and then staining bacteria with ubiquitin antibodies could give insight into whether or not RNF213 is directly ubiquitylating the bacteria. Additionally, although we did not see a change in ubiquitylation in THP1s, we did not assess the differences in growth over the course of infection. Given its antiviral role, it's possible that this ubiquitin ligase could be playing a role other than direct ubiquitylation of *M. tuberculosis* during infection.

METHODS

Cell lines THP1s were purchased from ATCC and cultured in RPMI (Gibco) supplemented with 10% FBS, 5 mM L-glutamine, 5 mM sodium pyruvate, and 10 mM HEPES. CRISPR/Cas9+ edited RNF213 KO THP-1s were a gift from the Vance Lab. Cells were incubated at 37°C and 5% CO₂.

Mycobacterium tuberculosis infection THP-1s were seeded in 24-well plates at 350,000 cells/well and differentiated with 20 ng/mL phorbol 12-myristate 12-acetate (PMA) (Invivogen) for three days. WT Erdman *M. tuberculosis* was grown rotating in roller bottles at 37°C to an OD of 0.6-0.8 in 7H9 (BD) supplemented with 10% Middlebrook OADC (BD), 0.5% glycerol, and 0.05% Tween-80. Bacteria were washed three times in DPBS prior to sonication at 90% amplitude for 2 x 30 seconds. Bacteria were added at an MOI of 2 unless otherwise indicated in DMEM + 10% horse serum (Gibco) and infected via centrifugation for 10 minutes at 400 x g. After spinning, the media was replaced and cells were incubated at 37°C and 5% CO₂ for 6 hours. At 6 hours, media was removed and cells were fixed in 4% paraformaldehyde.

Immunofluorescence Following fixation, cells were permeabilized and blocked in PBS with 0.05% saponin and 5% fetal bovine serum. Cells were stained with the following antibodies for two hours at room temperature: anti-FK2 1:400 (Enzo), anti-K63 1:200 (EMD Millipore Rabbit APU), and anti-K48 1:200 (Millipore 1:1 glycine Rabbit APU). Following primary stains, secondary Alexa Fluor antibodies were added at 1:4000 with

DAPI 1:1000. Plates were imaged on the Opera Phenix High-Content Screening System. Colocalization of ubiquitin linkages with *M. tuberculosis* was performed using spot finder assay. The total number of ubiquitin puncta detected was divided by the number of spots colocalized with *M. tuberculosis* to identify the total percent colocalized.

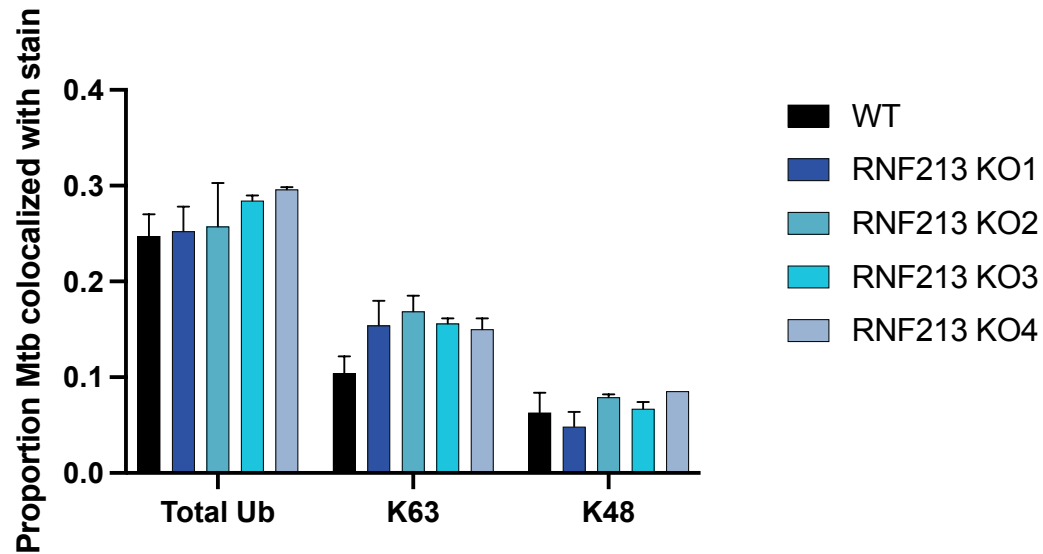


Figure 1 RNF213 knockout THP1s do not have differences in ubiquitylation.

THP1s lacking RNF213 and a WT control were infected with *M. tuberculosis* expressing GFP at an MOI of 2 for 6 hours. Following fixation, cells were stained for total and linkage specific ubiquitin and analyzed via high-throughput microscopy.

Chapter 5: Conclusions and speculations

As an intracellular pathogen, *M. tuberculosis* has evolved under pressure to subvert sophisticated host defense systems. Over the last century, researchers have made incredible progress in deciphering how the host responds to infection but much about these processes remains to be discovered. In my dissertation, I initially used a model system of *ex vivo* macrophages to study the molecular interactions between bacteria and host. Using previously published proteomics data, I identified MYCBP2 as a novel regulator of the host response to *M. tuberculosis* infection. I demonstrated that MYCBP2 regulates inflammatory signaling states of the macrophage during *M. tuberculosis* infection and TLR stimulation. I also showed the first evidence of MYCBP2's role in viral infection.

My dream hypothesis when I started this project was that MYCBP2 could be responsible for directly ubiquitylating *M. tuberculosis* during infection. We have observed in our lab that outer surface proteins of *M. tuberculosis* have fewer lysines, the normal residue target of ubiquitin ligases, and MYCBP2 ubiquitylates threonines instead of canonical lysines. Therefore, I postulated that perhaps MYCBP2 could be responsible for directly ubiquitylating the bacteria, this theory was further buoyed by recent evidence that RNF213, as mentioned in the previous chapter, can directly ubiquitylate non-proteinaceous substances on the outside of bacteria²⁰³. I changed my hypothesis over time and no longer think this is the role of MYCBP2 during infection. One of my initial reasons for becoming interested in the protein was that it was ubiquitylated and phosphorylated during *M. tuberculosis* infection. However, ubiquitin ligases known to ubiquitylate *M. tuberculosis* or its phagosome during infection, such as PARKIN or SMURF1, are not post-translationally modified over the course of infection⁸⁰.

Instead, I hypothesize and have shown in Chapter 2, that MYCBP2 is important for regulating the signaling states of the macrophage and that this has implications for *M. tuberculosis* growth. I don't think this is specific to *M. tuberculosis* and think a broader study of bacterial pathogens will uncover other susceptible bacteria. I did not investigate metabolism changes between WT and MYCBP2 deficient cells but postulate this is a plausible candidate to explain why MYCBP2 deficient macrophages have less bacterial growth. Glycolysis limits *M. tuberculosis* growth and M1 macrophages rely on glycolysis, therefore if MYCBP2 deficient cells have less glycolysis, this may benefit the bacterium^{208,209}. Substrate identification studies are a logical next step if MYCBP2 modulates signaling via its ubiquitin ligase activities. However, studies into MYCBP2's substrates are complicated by the protein's large size. If it is ligase-dependent, performing these experiments in macrophages would help identify exactly how it regulates signaling states. I believe its unique ubiquitylation and massive size indicate its special role in biology and I look forward to future insights into its role in immunity and beyond.

Besides MYCBP2, I also spent considerable time developing proximity labeling approaches for the *M. tuberculosis* phagosome. This project involved much trial and error and self-directed learning. Though I cannot yet conclude that I have developed a high-confidence data set, I have narrowed down the best approaches to these experiments. Future repeats of these experiments should use APEX2-GAL8 and APEX2-Cytosol with WT *M. tuberculosis* compared to APEX2-GAL8 with $\Delta eccC$ *M. tuberculosis* processed with stringent wash conditions and analyzed via LC-MS/MS followed by Fragpipe analysis. If these results still do not return a strong dataset with positive controls, then perhaps the Gal-8 protein is not specific enough for this assay. Although I found that the majority of the signal should have been located near *M. tuberculosis* when I stopped the infection, this finding could be complicated by experimental methods. This relies upon appropriate staining, quality imaging, and a sound analysis pipeline. There are always opportunities for improvement in each of these areas. If future researchers want to attempt a similar experiment, I would be interested to see how results compare with other proteins recruited to the *M. tuberculosis* phagosome, such as PARKIN or TAX1BP1. Although my APEX data set requires some refining, I still think there are interesting proteins to explore in my datasets. I hope future researchers can enlighten us on why these proteins are near Gal-8 during infection.

Overall, my dissertation adds to our knowledge of how the macrophage responds to *M. tuberculosis* infection and highlights additional host proteins involved in broader host-pathogen interactions.

References

1. Global Tuberculosis Report 2023. <https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2023>.
2. Asabor, E. N. & Vermund, S. H. Confronting Structural Racism in the Prevention and Control of Tuberculosis in the United States. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **73**, e3531–e3535 (2020).
3. Ramakrishnan, L. Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol.* **12**, 352–366 (2012).
4. Cohen, S. B. *et al.* Alveolar macrophages provide an early Mycobacterium tuberculosis niche and initiate dissemination. *Cell Host Microbe* **24**, 439–446.e4 (2018).
5. O'Garra, A. *et al.* The Immune Response in Tuberculosis. *Annu. Rev. Immunol.* **31**, 475–527 (2013).
6. Stanley, S. A., Raghavan, S., Hwang, W. W. & Cox, J. S. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13001–13006 (2003).
7. Armstrong, J. A. & Hart, P. D. RESPONSE OF CULTURED MACROPHAGES TO MYCOBACTERIUM TUBERCULOSIS, WITH OBSERVATIONS ON FUSION OF LYSOSOMES WITH PHAGOSOMES. *J. Exp. Med.* **134**, 713–740 (1971).
8. Via, L. E. *et al.* Arrest of Mycobacterial Phagosome Maturation Is Caused by a Block in Vesicle Fusion between Stages Controlled by rab5 and rab7*. *J. Biol. Chem.* **272**, 13326–13331 (1997).
9. Sturgill-Koszycki, S. *et al.* Lack of Acidification in Mycobacterium Phagosomes Produced by Exclusion of the Vesicular Proton-ATPase. *Science* **263**, 678–681 (1994).
10. Wong, D., Bach, H., Sun, J., Hmama, Z. & Av-Gay, Y. Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H⁺-ATPase to inhibit phagosome acidification. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 19371–19376 (2011).
11. Vergne, I. *et al.* Mechanism of phagolysosome biogenesis block by viable Mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 4033–4038 (2005).
12. Upadhyay, S., Mittal, E. & Philips, J. A. Tuberculosis and the art of macrophage manipulation. *Pathog. Dis.* **76**, fty037 (2018).
13. Watson, R. O. *et al.* The cytosolic sensor cGAS detects Mycobacterium tuberculosis DNA to induce type I interferons and activate autophagy. *Cell Host Microbe* **17**, 811–819 (2015).
14. Wassermann, R. *et al.* Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1. *Cell Host Microbe* **17**, 799–810 (2015).
15. Collins, A. C. *et al.* Cyclic GMP-AMP Synthase Is an Innate Immune DNA Sensor for Mycobacterium tuberculosis. *Cell Host Microbe* **17**, 820–828 (2015).
16. Saiga, H. *et al.* Critical role of AIM2 in Mycobacterium tuberculosis infection. *Int. Immunol.* **24**, 637–644 (2012).
17. Wong, K.-W. & Jacobs Jr, W. R. Critical role for NLRP3 in necrotic death triggered by Mycobacterium tuberculosis. *Cell. Microbiol.* **13**, 1371–1384 (2011).

18. Mayer-Barber, K. D. *et al.* Cutting Edge: Caspase-1 Independent IL-1 β Production Is Critical for Host Resistance to Mycobacterium tuberculosis and Does Not Require TLR Signaling In Vivo. *J. Immunol.* **184**, 3326–3330 (2010).
19. Mishra, B. B. *et al.* Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. *Cell. Microbiol.* **12**, 1046–1063 (2010).
20. Dorhoi, A. *et al.* Activation of the NLRP3 inflammasome by Mycobacterium tuberculosis is uncoupled from susceptibility to active tuberculosis. *Eur. J. Immunol.* **42**, 374–384 (2012).
21. Beckwith, K. S. *et al.* Plasma membrane damage causes NLRP3 activation and pyroptosis during Mycobacterium tuberculosis infection. *Nat. Commun.* **11**, 2270 (2020).
22. Arnett, E. *et al.* Cutting Edge: Cytosolic Receptor AIM2 Is Induced by Peroxisome Proliferator-activated Receptor γ following Mycobacterium tuberculosis Infection of Human Macrophages but Does Not Contribute to IL-1 β Release. *J. Immunol. Baltim. Md 1950* **212**, 765–770 (2024).
23. Stanley, S. A., Johndrow, J. E., Manzanillo, P. & Cox, J. S. The Type I IFN Response to Infection with Mycobacterium tuberculosis Requires ESX-1-Mediated Secretion and Contributes to Pathogenesis¹. *J. Immunol.* **178**, 3143–3152 (2007).
24. Manca, C. *et al.* Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN- α/β . *Proc. Natl. Acad. Sci.* **98**, 5752–5757 (2001).
25. Antonelli, L. R. V. *et al.* Intranasal Poly-IC treatment exacerbates tuberculosis in mice through the pulmonary recruitment of a pathogen-permissive monocyte/macrophage population. *J. Clin. Invest.* **120**, 1674–1682 (2010).
26. Ferwerda, G. *et al.* NOD2 and Toll-Like Receptors Are Nonredundant Recognition Systems of Mycobacterium tuberculosis. *PLOS Pathog.* **1**, e34 (2005).
27. Divangahi, M. *et al.* NOD2-Deficient Mice Have Impaired Resistance to Mycobacterium tuberculosis Infection through Defective Innate and Adaptive Immunity¹. *J. Immunol.* **181**, 7157–7165 (2008).
28. Pandey, A. K. *et al.* NOD2, RIP2 and IRF5 Play a Critical Role in the Type I Interferon Response to Mycobacterium tuberculosis. *PLOS Pathog.* **5**, e1000500 (2009).
29. Cheng, Y. & Schorey, J. S. Mycobacterium tuberculosis–induced IFN- β production requires cytosolic DNA and RNA sensing pathways. *J. Exp. Med.* **215**, 2919–2935 (2018).
30. Bullen, C. K. *et al.* MDA5 RNA-sensing pathway activation by Mycobacterium tuberculosis promotes innate immune subversion and pathogen survival. *JCI Insight* **8**, e166242.
31. Faridgohar, M. & Nikoueinejad, H. New findings of Toll-like receptors involved in Mycobacterium tuberculosis infection. *Pathog. Glob. Health* **111**, 256–264 (2017).
32. Pattanaik, K. P., Sengupta, S., Jit, B. P., Kotak, R. & Sonawane, A. Host-mycobacteria conflict: Immune responses of the host vs. the mycobacteria TLR2 and TLR4 ligands and concomitant host-directed therapy. *Microbiol. Res.* **264**, 127153 (2022).
33. Bafica, A. *et al.* TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *J. Exp. Med.* **202**, 1715–

- 1724 (2005).
34. Gatica, D., Lahiri, V. & Klionsky, D. J. Cargo recognition and degradation by selective autophagy. *Nat. Cell Biol.* **20**, 233–242 (2018).
 35. Gutierrez, M. G. *et al.* Autophagy Is a Defense Mechanism Inhibiting BCG and *Mycobacterium tuberculosis* Survival in Infected Macrophages. *Cell* **119**, 753–766 (2004).
 36. Watson, R. O., Manzanillo, P. S. & Cox, J. S. Extracellular M. tuberculosis DNA Targets Bacteria for Autophagy by Activating the Host DNA-Sensing Pathway. *Cell* **150**, 803–815 (2012).
 37. Manzanillo, P. S. *et al.* PARKIN ubiquitin ligase mediates resistance to intracellular pathogens. *Nature* **501**, 512–516 (2013).
 38. Chauhan, S. *et al.* TRIMs and Galectins Globally Cooperate and TRIM16 and Galectin-3 Co-direct Autophagy in Endomembrane Damage Homeostasis. *Dev. Cell* **39**, 13–27 (2016).
 39. Franco, L. H. *et al.* The ubiquitin-ligase Smurf1 functions in selective autophagy of M. tuberculosis and anti-tuberculous host defense. *Cell Host Microbe* **21**, 59–72 (2017).
 40. Beatty, W. L., Rhoades, E. R., Hsu, D. K., Liu, F.-T. & Russell, D. G. Association of a macrophage galactoside-binding protein with Mycobacterium-containing phagosomes. *Cell. Microbiol.* **4**, 167–176 (2002).
 41. Bell, S. L., Lopez, K. L., Cox, J. S., Patrick, K. L. & Watson, R. O. Galectin-8 Senses Phagosomal Damage and Recruits Selective Autophagy Adapter TAX1BP1 To Control Mycobacterium tuberculosis Infection in Macrophages. *mBio* **12**, e01871-20.
 42. Morrison, H. M. *et al.* Deficiency in Galectin-3, -8, and -9 impairs immunity to chronic Mycobacterium tuberculosis infection but not acute infection with multiple intracellular pathogens. *PLOS Pathog.* **19**, e1011088 (2023).
 43. Thurston, T. L. M., Wandel, M. P., von Muhlinen, N., Foeglein, Á. & Randow, F. Galectin-8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* **482**, 414–418 (2012).
 44. Aylan, B. *et al.* ATG7 and ATG14 restrict cytosolic and phagosomal Mycobacterium tuberculosis replication in human macrophages. *Nat. Microbiol.* **8**, 803–818 (2023).
 45. Golovkine, G. R. *et al.* Autophagy restricts Mycobacterium tuberculosis during acute infection in mice. *Nat. Microbiol.* **8**, 819–832 (2023).
 46. Castillo, E. F. *et al.* Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E3168–E3176 (2012).
 47. Kimmey, J. M. *et al.* Unique role for ATG5 in PMN-mediated immunopathology during M. tuberculosis infection. *Nature* **528**, 565–569 (2015).
 48. Behar, S. M. & Baehrecke, E. H. Autophagy is not the answer. *Nature* **528**, 482–483 (2015).
 49. Raiborg, C. & Stenmark, H. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458**, 445–452 (2009).
 50. Schmidt, O. & Teis, D. The ESCRT machinery. *Curr. Biol.* **22**, R116–R120 (2012).

51. Skowrya, M. L., Schlesinger, P. H., Naismith, T. V. & Hanson, P. I. Triggered recruitment of ESCRT machinery promotes endolysosomal repair. *Science* **360**, eaar5078 (2018).
52. Vieira, O. V. *et al.* Acquisition of Hrs, an Essential Component of Phagosomal Maturation, Is Impaired by Mycobacteria. *Mol. Cell. Biol.* **24**, 4593–4604 (2004).
53. Philips, J. A., Porto, M. C., Wang, H., Rubin, E. J. & Perrimon, N. ESCRT factors restrict mycobacterial growth. *Proc. Natl. Acad. Sci.* **105**, 3070–3075 (2008).
54. Mehra, A. *et al.* Mycobacterium tuberculosis Type VII Secreted Effector EsxH Targets Host ESCRT to Impair Trafficking. *PLOS Pathog.* **9**, e1003734 (2013).
55. Mittal, E. *et al.* Mycobacterium tuberculosis Type VII Secretion System Effectors Differentially Impact the ESCRT Endomembrane Damage Response. *mBio* **9**, e01765-18 (2018).
56. Portal-Celhay, C. *et al.* Mycobacterium tuberculosis EsxH inhibits ESCRT-dependent CD4⁺ T-cell activation. *Nat. Microbiol.* **2**, 1–9 (2016).
57. López-Jiménez, A. T. *et al.* The ESCRT and autophagy machineries cooperate to repair ESX-1-dependent damage at the Mycobacterium-containing vacuole but have opposite impact on containing the infection. *PLOS Pathog.* **14**, e1007501 (2018).
58. Chan, J., Xing, Y., Magliozzo, R. S. & Bloom, B. R. Killing of virulent Mycobacterium tuberculosis by reactive nitrogen intermediates produced by activated murine macrophages. *J. Exp. Med.* **175**, 1111–1122 (1992).
59. MacMicking, J. D. *et al.* Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5243–5248 (1997).
60. Darwin, K. H., Ehrt, S., Gutierrez-Ramos, J.-C., Weich, N. & Nathan, C. F. The Proteasome of Mycobacterium tuberculosis Is Required for Resistance to Nitric Oxide. *Science* **302**, 1963–1966 (2003).
61. Pellegrino, E. *et al.* Peroxisomal ROS control cytosolic Mycobacterium tuberculosis replication in human macrophages. *J. Cell Biol.* **222**, e202303066 (2023).
62. Cooper, A. M., Segal, B. H., Frank, A. A., Holland, S. M. & Orme, I. M. Transient Loss of Resistance to Pulmonary Tuberculosis in p47phox^{-/-} Mice. *Infect. Immun.* **68**, 1231–1234 (2000).
63. Ng, V. H., Cox, J. S., Sousa, A. O., MacMicking, J. D. & McKinney, J. D. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. *Mol. Microbiol.* **52**, 1291–1302 (2004).
64. Guirado, E., Schlesinger, L. S. & Kaplan, G. Macrophages in Tuberculosis: Friend or Foe. *Semin. Immunopathol.* **35**, 563–583 (2013).
65. Roach, D. R. *et al.* TNF Regulates Chemokine Induction Essential for Cell Recruitment, Granuloma Formation, and Clearance of Mycobacterial Infection1. *J. Immunol.* **168**, 4620–4627 (2002).
66. Roca, F. J., Whitworth, L. J., Redmond, S., Jones, A. A. & Ramakrishnan, L. TNF Induces Pathogenic Programmed Macrophage Necrosis in Tuberculosis through a Mitochondrial-Lysosomal-Endoplasmic Reticulum Circuit. *Cell* **178**, 1344–1361.e11 (2019).
67. Segueni, N. *et al.* Innate myeloid cell TNFR1 mediates first line defence against primary Mycobacterium tuberculosis infection. *Sci. Rep.* **6**, 22454 (2016).
68. Mayer-Barber, K. D. *et al.* Innate and Adaptive Interferons Suppress IL-1 α and

- IL-1 β Production by Distinct Pulmonary Myeloid Subsets during *Mycobacterium tuberculosis* Infection. *Immunity* **35**, 1023–1034 (2011).
69. Mayer-Barber, K. D. *et al.* Caspase-1 independent IL-1 β production is critical for host resistance to mycobacterium tuberculosis and does not require TLR signaling in vivo. *J. Immunol. Baltim. Md 1950* **184**, 3326–3330 (2010).
 70. Divangahi, M. *et al.* Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. *Nat. Immunol.* **10**, 899–906 (2009).
 71. Wolf, A. J. *et al.* Mycobacterium tuberculosis Infects Dendritic Cells with High Frequency and Impairs Their Function In Vivo¹. *J. Immunol.* **179**, 2509–2519 (2007).
 72. Lerner, T. R. *et al.* Mycobacterium tuberculosis replicates within necrotic human macrophages. *J. Cell Biol.* **216**, 583–594 (2017).
 73. Repasy, T. *et al.* Bacillary replication and macrophage necrosis are determinants of neutrophil recruitment in tuberculosis. *Microbes Infect. Inst. Pasteur* **17**, 564–574 (2015).
 74. Dallenga, T. *et al.* *M. tuberculosis*-Induced Necrosis of Infected Neutrophils Promotes Bacterial Growth Following Phagocytosis by Macrophages. *Cell Host Microbe* **22**, 519-530.e3 (2017).
 75. Park, J. S., Tamayo, M. H., Gonzalez-Juarrero, M., Orme, I. M. & Ordway, D. J. Virulent clinical isolates of Mycobacterium tuberculosis grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. *J. Leukoc. Biol.* **79**, 80–86 (2006).
 76. Behar, S. *et al.* Apoptosis is an innate defense function of macrophages against Mycobacterium tuberculosis. *Mucosal Immunol.* **4**, 279–287 (2011).
 77. Orme, I. M., Roberts, A. D., Griffin, J. P. & Abrams, J. S. Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection. *J. Immunol. Baltim. Md 1950* **151**, 518–525 (1993).
 78. Flynn, J. L. *et al.* An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J. Exp. Med.* **178**, 2249–2254 (1993).
 79. Leemans, J. C. *et al.* Macrophages Play a Dual Role during Pulmonary Tuberculosis in Mice. *J. Infect. Dis.* **191**, 65–74 (2005).
 80. Budzik, J. M. *et al.* Dynamic post-translational modification profiling of Mycobacterium tuberculosis-infected primary macrophages. *eLife* **9**, e51461 (2020).
 81. Pao, K.-C. *et al.* Activity-based E3 ligase profiling uncovers an E3 ligase with esterification activity. *Nature* **556**, 381–385 (2018).
 82. Bloom, A. J., Miller, B. R., Sanes, J. R. & DiAntonio, A. The requirement for Phr1 in CNS axon tract formation reveals the corticostriatal boundary as a choice point for cortical axons. *Genes Dev.* **21**, 2593–2606 (2007).
 83. Babetto, E., Beirowski, B., Russler, E. V., Milbrandt, J. & DiAntonio, A. The Phr1 ubiquitin ligase promotes injury-induced axon self-destruction. *Cell Rep.* **3**, 1422–1429 (2013).
 84. Virdee, S. An atypical ubiquitin ligase at the heart of neural development and programmed axon degeneration. *Neural Regen. Res.* **17**, 2347–2350 (2022).
 85. Zhen, M., Huang, X., Bamber, B. & Jin, Y. Regulation of Presynaptic Terminal Organization by *C. elegans* RPM-1, a Putative Guanine Nucleotide Exchanger with a RING-H2 Finger Domain. *Neuron* **26**, 331–343 (2000).
 86. Schaefer, A. M., Hadwiger, G. D. & Nonet, M. L. *rpm-1*, A Conserved Neuronal

- Gene that Regulates Targeting and Synaptogenesis in *C. elegans*. *Neuron* **26**, 345–356 (2000).
87. Wan, H. I. *et al.* Highwire Regulates Synaptic Growth in *Drosophila*. *Neuron* **26**, 313–329 (2000).
 88. D'Souza, J. *et al.* Formation of the retinotectal projection requires Esrom, an ortholog of PAM (protein associated with Myc). *Development* **132**, 247–256 (2005).
 89. Han, S. *et al.* Pam (Protein associated with Myc) functions as an E3 Ubiquitin ligase and regulates TSC/mTOR signaling. *Cell. Signal.* **20**, 1084–1091 (2008).
 90. Han, S. *et al.* The E3 Ubiquitin Ligase Protein Associated with Myc (Pam) Regulates Mammalian/Mechanistic Target of Rapamycin Complex 1 (mTORC1) Signaling in Vivo through N- and C-terminal Domains. *J. Biol. Chem.* **287**, 30063–30072 (2012).
 91. Holland, S. *et al.* The Ubiquitin Ligase MYCBP2 Regulates Transient Receptor Potential Vanilloid Receptor 1 (TRPV1) Internalization through Inhibition of p38 MAPK Signaling. *J. Biol. Chem.* **286**, 3671–3680 (2011).
 92. Scholich, K., Pierre, S. & Patel, T. B. Protein Associated with Myc (PAM) Is a Potent Inhibitor of Adenylyl Cyclases*. *J. Biol. Chem.* **276**, 47583–47589 (2001).
 93. Pierre, S. C., Häusler, J., Birod, K., Geisslinger, G. & Scholich, K. PAM mediates sustained inhibition of cAMP signaling by sphingosine-1-phosphate. *EMBO J.* **23**, 3031–3040 (2004).
 94. Chang, C. *et al.* Ubiquitin ligase and signalling hub MYCBP2 is required for efficient EPHB2 tyrosine kinase receptor function. *eLife* **12**, RP89176 (2024).
 95. Guo, Q., Xie, J., Dang, C. V., Liu, E. T. & Bishop, J. M. Identification of a large Myc-binding protein that contains RCC1-like repeats. *Proc. Natl. Acad. Sci.* **95**, 9172–9177 (1998).
 96. Pierre, S. *et al.* Myc binding protein 2 suppresses M2-like phenotypes in macrophages during zymosan-induced inflammation in mice. *Eur. J. Immunol.* **48**, 239–249 (2018).
 97. Neff, R. A. *et al.* Dysfunction of ubiquitin protein ligase MYCBP2 leads to cell resilience in human breast cancers. *NAR Cancer* **5**, zcad036 (2023).
 98. Wang, G. *et al.* MYCBP2 expression correlated with inflammatory cell infiltration and prognosis immunotherapy in thyroid cancer patients. *Front. Immunol.* **13**, (2022).
 99. Davis, Z. H. *et al.* Global Mapping of Herpesvirus-Host Protein Complexes Reveals a Transcription Strategy for Late Genes. *Mol. Cell* **57**, 349–360 (2015).
 100. Gordon, D. E. *et al.* A SARS-CoV-2 protein interaction map reveals targets for drug repurposing. *Nature* **583**, 459–468 (2020).
 101. Boudreaault, S. *et al.* Reovirus μ 2 protein modulates host cell alternative splicing by reducing protein levels of U5 snRNP core components. *Nucleic Acids Res.* **50**, 5263–5281 (2022).
 102. Khadka, S. *et al.* A Physical Interaction Network of Dengue Virus and Human Proteins. *Mol. Cell. Proteomics MCP* **10**, M111.012187 (2011).
 103. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J. & Hill, A. M. M-1/M-2 Macrophages and the Th1/Th2 Paradigm1. *J. Immunol.* **164**, 6166–6173 (2000).
 104. Mantovani, A. *et al.* The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* **25**, 677–686 (2004).
 105. Martinez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage

- activation: time for reassessment. *F1000Prime Rep.* **6**, 13 (2014).
106. Ley, K. M1 Means Kill; M2 Means Heal. *J. Immunol.* **199**, 2191–2193 (2017).
 107. Yunna, C., Mengru, H., Lei, W. & Weidong, C. Macrophage M1/M2 polarization. *Eur. J. Pharmacol.* **877**, 173090 (2020).
 108. Vazquez-Torres, A. *et al.* Salmonella Pathogenicity Island 2-Dependent Evasion of the Phagocyte NADPH Oxidase. *Science* **287**, 1655–1658 (2000).
 109. Wang, Y. *et al.* *Brucella* induces M1 to M2 polarization of macrophages through STAT6 signaling pathway to promote bacterial intracellular survival. *Res. Vet. Sci.* **145**, 91–101 (2022).
 110. Benoit, M., Barbarat, B., Bernard, A., Olive, D. & Mege, J.-L. *Coxiella burnetii*, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur. J. Immunol.* **38**, 1065–1070 (2008).
 111. Marakalala, M. J. *et al.* Inflammatory signaling in human Tuberculosis granulomas is spatially organized. *Nat. Med.* **22**, 531–538 (2016).
 112. Khan, A. *et al.* Human M1 macrophages express unique innate immune response genes after mycobacterial infection to defend against tuberculosis. *Commun. Biol.* **5**, 1–19 (2022).
 113. Huang, L., Nazarova, E. V., Tan, S., Liu, Y. & Russell, D. G. Growth of *Mycobacterium tuberculosis* in vivo segregates with host macrophage metabolism and ontogeny. *J. Exp. Med.* **215**, 1135–1152 (2018).
 114. Cambier, C. J. *et al.* Mycobacteria manipulate macrophage recruitment through coordinated use of membrane lipids. *Nature* **505**, 218–222 (2014).
 115. Queval, C. J. *et al.* STAT3 Represses Nitric Oxide Synthesis in Human Macrophages upon *Mycobacterium tuberculosis* Infection. *Sci. Rep.* **6**, 29297 (2016).
 116. Redente, E. F. *et al.* Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation. *J. Leukoc. Biol.* **88**, 159–168 (2010).
 117. Lastrucci, C. *et al.* Tuberculosis is associated with expansion of a motile, permissive and immunomodulatory CD16⁺ monocyte population via the IL-10/STAT3 axis. *Cell Res.* **25**, 1333–1351 (2015).
 118. Zhang, Y. *et al.* *Mycobacterium tuberculosis* Heat-Shock Protein 16.3 Induces Macrophage M2 Polarization Through CCRL2/CX3CR1. *Inflammation* **43**, 487–506 (2020).
 119. Parveen, N. *et al.* Endocytosis of *Mycobacterium tuberculosis* Heat Shock Protein 60 Is Required to Induce Interleukin-10 Production in Macrophages. *J. Biol. Chem.* **288**, 24956–24971 (2013).
 120. Roberts, A. W. *et al.* Cas9⁺ conditionally-immortalized macrophages as a tool for bacterial pathogenesis and beyond. *eLife* **8**, e45957 (2019).
 121. Burgess, R. W. *et al.* Evidence for a Conserved Function in Synapse Formation Reveals *Phr1* as a Candidate Gene for Respiratory Failure in Newborn Mice. *Mol. Cell. Biol.* **24**, 1096–1105 (2004).
 122. Radoshevich, L. & Cossart, P. *Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis. *Nat. Rev. Microbiol.* **16**, 32–46 (2018).
 123. Ji, D. X. *et al.* Type I interferon-driven susceptibility to *Mycobacterium tuberculosis* is mediated by IL-1Ra. *Nat. Microbiol.* **4**, 2128–2135 (2019).
 124. Penn, B. H. *et al.* An Mtb-Human Protein-Protein Interaction Map Identifies a

- Switch Between Host Anti-Viral and Anti-Bacterial Responses. *Mol. Cell* **71**, 637-648.e5 (2018).
125. Johnson, J. R. *et al.* Comparative analysis of macrophage post-translational modifications during intracellular bacterial pathogen infection. 2020.05.27.116772 Preprint at <https://doi.org/10.1101/2020.05.27.116772> (2020).
 126. Pépin, G. *et al.* Cre-dependent DNA recombination activates a STING-dependent innate immune response. *Nucleic Acids Res.* **44**, 5356–5364 (2016).
 127. Bento, C. F., Ashkenazi, A., Jimenez-Sanchez, M. & Rubinsztein, D. C. The Parkinson's disease-associated genes ATP13A2 and SYT11 regulate autophagy via a common pathway. *Nat. Commun.* **7**, 11803 (2016).
 128. Cadwell, K. Crosstalk between autophagy and inflammatory signalling pathways: balancing defence and homeostasis. *Nat. Rev. Immunol.* **16**, 661–675 (2016).
 129. Klionsky, D. J. *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition)1. *Autophagy* **17**, 1–382 (2021).
 130. Nakata, K. *et al.* Regulation of a DLK-1 and p38 MAP Kinase Pathway by the Ubiquitin Ligase RPM-1 Is Required for Presynaptic Development. *Cell* **120**, 407–420 (2005).
 131. Park, E. C., Glodowski, D. R. & Rongo, C. The Ubiquitin Ligase RPM-1 and the p38 MAPK PMK-3 Regulate AMPA Receptor Trafficking. *PLOS ONE* **4**, e4284 (2009).
 132. Yang, Y. *et al.* Functional Roles of p38 Mitogen-Activated Protein Kinase in Macrophage-Mediated Inflammatory Responses. *Mediators Inflamm.* **2014**, 352371 (2014).
 133. Ehrt, S. *et al.* Reprogramming of the Macrophage Transcriptome in Response to Interferon- γ and Mycobacterium tuberculosis. *J. Exp. Med.* **194**, 1123–1140 (2001).
 134. Kaufmann, S. H. & Dorhoi, A. Inflammation in tuberculosis: interactions, imbalances and interventions. *Curr. Opin. Immunol.* **25**, 441–449 (2013).
 135. Wilburn, K. M. *et al.* Differential Requirement for IRGM Proteins during Tuberculosis Infection in Mice. *Infect. Immun.* **91**, e00510-22.
 136. Mumphrey, S. M. *et al.* Murine Norovirus 1 Infection Is Associated with Histopathological Changes in Immunocompetent Hosts, but Clinical Disease Is Prevented by STAT1-Dependent Interferon Responses. *J. Virol.* **81**, 3251–3263 (2007).
 137. Karst, S. M., Wobus, C. E., Lay, M., Davidson, J. & Virgin, H. W. STAT1-Dependent Innate Immunity to a Norwalk-Like Virus. *Science* **299**, 1575–1578 (2003).
 138. Davey, N. E., Travé, G. & Gibson, T. J. How viruses hijack cell regulation. *Trends Biochem. Sci.* **36**, 159–169 (2011).
 139. Speckhart, K., Williams, J. M. & Tsai, B. How DNA and RNA Viruses Exploit Host Chaperones to Promote Infection. *Viruses* **13**, 958 (2021).
 140. Jassey, A. & Jackson, W. T. Viruses and autophagy: bend, but don't break. *Nat. Rev. Microbiol.* 1–13 (2023) doi:10.1038/s41579-023-00995-y.
 141. Levine, B., Mizushima, N. & Virgin, H. W. Autophagy in immunity and inflammation. *Nature* **469**, 323–335 (2011).
 142. Deretic, V. Autophagy in inflammation, infection, and immunometabolism. *Immunity* **54**, 437–453 (2021).

143. Liao, E. H., Hung, W., Abrams, B. & Zhen, M. An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. *Nature* **430**, 345–350 (2004).
144. Saiga, T. *et al.* Fbxo45 Forms a Novel Ubiquitin Ligase Complex and Is Required for Neuronal Development. *Mol. Cell. Biol.* **29**, 3529–3543 (2009).
145. Desbois, M. *et al.* PAM forms an atypical SCF ubiquitin ligase complex that ubiquitinates and degrades NMNAT2. *J. Biol. Chem.* **293**, 13897–13909 (2018).
146. Dörr, A. *et al.* MYCBP2 Is a Guanosine Exchange Factor for Ran Protein and Determines Its Localization in Neurons of Dorsal Root Ganglia. *J. Biol. Chem.* **290**, 25620–25635 (2015).
147. Grill, B., Murphey, R. K. & Borgen, M. A. The PHR proteins: intracellular signaling hubs in neuronal development and axon degeneration. *Neural Develop.* **11**, 8 (2016).
148. Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* **42**, e168 (2014).
149. Hwang, S. *et al.* Murine Norovirus: Propagation, Quantification and Genetic Manipulation. *Curr. Protoc. Microbiol.* **33**, 15K.2.1-15K.2.61 (2014).
150. Queval, C. J. *et al.* Mycobacterium tuberculosis Controls Phagosomal Acidification by Targeting CISH-Mediated Signaling. *Cell Rep.* **20**, 3188–3198 (2017).
151. Ferrari, G., Langen, H., Naito, M. & Pieters, J. A Coat Protein on Phagosomes Involved in the Intracellular Survival of Mycobacteria. *Cell* **97**, 435–447 (1999).
152. Hoffmann, E., Machelart, A., Song, O.-R. & Brodin, P. Proteomics of Mycobacterium Infection: Moving towards a Better Understanding of Pathogen-Driven Immunomodulation. *Front. Immunol.* **9**, 86 (2018).
153. Qin, W., Cho, K. F., Cavanagh, P. E. & Ting, A. Y. Deciphering molecular interactions by proximity labeling. *Nat. Methods* **18**, 133–143 (2021).
154. Branon, T. C. *et al.* Efficient proximity labeling in living cells and organisms with TurboID. *Nat. Biotechnol.* **36**, 880–887 (2018).
155. Bosch, J. A., Chen, C.-L. & Perrimon, N. Proximity-dependent labeling methods for proteomic profiling in living cells: an update. *Wiley Interdiscip. Rev. Dev. Biol.* **10**, e392 (2021).
156. Dickinson, M. S. *et al.* Proximity-dependent proteomics of the Chlamydia trachomatis inclusion membrane reveals functional interactions with endoplasmic reticulum exit sites. *PLOS Pathog.* **15**, e1007698 (2019).
157. Olson, M. G. *et al.* Proximity Labeling To Map Host-Pathogen Interactions at the Membrane of a Bacterium-Containing Vacuole in Chlamydia trachomatis-Infected Human Cells. *Infect. Immun.* **87**, e00537-19 (2019).
158. Medica, S. *et al.* Proximity-dependent mapping of the HCMV US28 interactome identifies RhoGEF signaling as a requirement for efficient viral reactivation. *PLOS Pathog.* **19**, e1011682 (2023).
159. Jia, J. *et al.* Galectins control mTOR in response to endomembrane damage. *Mol. Cell* **70**, 120-135.e8 (2018).
160. Hill, N. S. & Welch, M. D. A glycine-rich PE_PGRS protein governs mycobacterial actin-based motility. *Nat. Commun.* **13**, 3608 (2022).
161. Lerner, T. R. *et al.* Phthiocerol dimycocerosates promote access to the cytosol and intracellular burden of Mycobacterium tuberculosis in lymphatic endothelial cells. *BMC Biol.* **16**, 1 (2018).

162. Rosenberg, O. S. *et al.* Substrates Control Multimerization and Activation of the Multi-Domain ATPase Motor of Type VII Secretion. *Cell* **161**, 501–512 (2015).
163. Bersuker, K. *et al.* A Proximity Labeling Strategy Provides Insights into the Composition and Dynamics of Lipid Droplet Proteomes. *Dev. Cell* **44**, 97–112.e7 (2018).
164. Cowen, L., Ideker, T., Raphael, B. J. & Sharan, R. Network propagation: a universal amplifier of genetic associations. *Nat. Rev. Genet.* **18**, 551–562 (2017).
165. Szymkiewicz, I., Destaing, O., Jurdic, P. & Dikic, I. SH3P2 in complex with Cbl and Src. *FEBS Lett.* **565**, 33–38 (2004).
166. Tanno, H., Yamaguchi, T., Goto, E., Ishido, S. & Komada, M. The Ankrd 13 family of UIM-bearing proteins regulates EGF receptor endocytosis from the plasma membrane. *Mol. Biol. Cell* **23**, 1343–1353 (2012).
167. Zhen, Y., Haugsten, E. M., Singh, S. K. & Wesche, J. Proximity Labeling by a Recombinant APEX2–FGF1 Fusion Protein Reveals Interaction of FGF1 with the Proteoglycans CD44 and CSPG4. *Biochemistry* **57**, 3807–3816 (2018).
168. Jaisinghani, N. *et al.* Proteomics from compartment-specific APEX2 labeling in *Mycobacterium tuberculosis* reveals Type VII secretion substrates in the cell wall. *Cell Chem. Biol.* (2023) doi:10.1016/j.chembiol.2023.10.013.
169. Washburn, M. P., Wolters, D. & Yates, J. R. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **19**, 242–247 (2001).
170. Hung, V. *et al.* Spatially resolved proteomic mapping in living cells with the engineered peroxidase APEX2. *Nat. Protoc.* **11**, 456–475 (2016).
171. Won, M. *et al.* ANKRD13a controls early cell-death checkpoint by interacting with RIP1 independent of NF- κ B. *Cell Death Differ.* **29**, 1152–1163 (2022).
172. Mattioni, A. *et al.* Ring Finger Protein 11 acts on ligand-activated EGFR via the direct interaction with the UIM region of the ANKRD13 protein family. *FEBS J.* **287**, 3526–3550 (2020).
173. Mattioni, A., Castagnoli, L. & Santonico, E. RNF11 at the Crossroads of Protein Ubiquitination. *Biomolecules* **10**, 1538 (2020).
174. McGuire, M. R. & Espenshade, P. J. PGRMC1: An enigmatic heme-binding protein. *Pharmacol. Ther.* **241**, 108326 (2023).
175. Lee, S. R. *et al.* Loss of PGRMC1 Delays the Progression of Hepatocellular Carcinoma via Suppression of Pro-Inflammatory Immune Responses. *Cancers* **13**, 2438 (2021).
176. Intlekofer, K. A. *et al.* Progesterone receptor membrane component 1 inhibits tumor necrosis factor alpha induction of gene expression in neural cells. *PLOS ONE* **14**, e0215389 (2019).
177. Reddy, S. *et al.* Isolation and characterization of a cDNA clone encoding a novel peptide (OSF) that enhances osteoclast formation and bone resorption. *J. Cell. Physiol.* **177**, 636–645 (1998).
178. Tanimura, S. *et al.* ERK signaling promotes cell motility by inducing the localization of myosin 1E to lamellipodial tips. *J. Cell Biol.* **214**, 475–489 (2016).
179. Willet, A. H., Chen, J.-S., Ren, L. & Gould, K. L. Membrane binding of endocytic myosin-1s is inhibited by a class of ankyrin repeat proteins. *Mol. Biol. Cell* **34**, br17 (2023).

180. Paul, P. *et al.* A Genome-wide Multidimensional RNAi Screen Reveals Pathways Controlling MHC Class II Antigen Presentation. *Cell* **145**, 268–283 (2011).
181. Agresti, A. & Bianchi, M. E. HMGB proteins and gene expression. *Curr. Opin. Genet. Dev.* **13**, 170–178 (2003).
182. Lotze, M. T. & Tracey, K. J. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* **5**, 331–342 (2005).
183. Tang, D. *et al.* Endogenous HMGB1 regulates autophagy. *J. Cell Biol.* **190**, 881–892 (2010).
184. Lui, G. *et al.* HMGB1/RAGE Signaling and Pro-Inflammatory Cytokine Responses in Non-HIV Adults with Active Pulmonary Tuberculosis. *PLoS ONE* **11**, e0159132 (2016).
185. Lin, C.-Y. *et al.* LAMTOR2/LAMTOR1 complex is required for TAX1BP1-mediated xenophagy. *Cell. Microbiol.* **21**, e12981 (2019).
186. Jia, J. *et al.* Membrane Atg8ylation, stress granule formation, and MTOR regulation during lysosomal damage. *Autophagy* **19**, 1893–1895.
187. Bussi, C. *et al.* Stress granules plug and stabilize damaged endolysosomal membranes. *Nature* **623**, 1062–1069 (2023).
188. Zhang, Y., Werling, U. & Edelman, W. Seamless Ligation Cloning Extract (SLiCE) Cloning Method. *Methods Mol. Biol. Clifton NJ* **1116**, 235–244 (2014).
189. Campeau, E. *et al.* A Versatile Viral System for Expression and Depletion of Proteins in Mammalian Cells. *PLoS ONE* **4**, e6529 (2009).
190. Liu, H., Lin, D. & Yates, J. R. Multidimensional Separations for Protein/Peptide Analysis in the Post-Genomic Era. *BioTechniques* **32**, 898–911 (2002).
191. Xu, T. *et al.* ProLuCID: an improved SEQUEST-like algorithm with enhanced sensitivity and specificity. *J. Proteomics* **129**, 16–24 (2015).
192. Cociorva, D., L Tabb, D. & Yates, J. R. Validation of tandem mass spectrometry database search results using DTASelect. *Curr. Protoc. Bioinforma.* **Chapter 13**, Unit 13.4 (2007).
193. Tabb, D. L., McDonald, W. H. & Yates, J. R. DTASelect and Contrast: Tools for Assembling and Comparing Protein Identifications from Shotgun Proteomics. *J. Proteome Res.* **1**, 21–26 (2002).
194. Park, S. K., Venable, J. D., Xu, T. & Yates, J. R. A quantitative analysis software tool for mass spectrometry-based proteomics. *Nat. Methods* **5**, 319–322 (2008).
195. Park, S. K. R. *et al.* Census 2: isobaric labeling data analysis. *Bioinformatics* **30**, 2208–2209 (2014).
196. Eng, J. K., McCormack, A. L. & Yates, J. R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**, 976–989 (1994).
197. Avila-Cobian, L. F. *et al.* Amber-codon suppression for spatial localization and in vivo photoaffinity capture of the interactome of the *Pseudomonas aeruginosa* rare lipoprotein A lytic transglycosylase. *Protein Sci.* **32**, e4781 (2023).
198. Tyanova, S., Temu, T. & Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat. Protoc.* **11**, 2301–2319 (2016).
199. Bioconductor - artMS.
<https://bioconductor.org/packages/release/bioc/html/artMS.html>.
200. Zhou, Y. *et al.* Metascape provides a biologist-oriented resource for the analysis

- of systems-level datasets. *Nat. Commun.* **10**, 1523 (2019).
201. Szklarczyk, D. *et al.* The STRING database in 2021: customizable protein–protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res.* **49**, D605–D612 (2021).
 202. Shannon, P. *et al.* Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. *Genome Res.* **13**, 2498–2504 (2003).
 203. Otten, E. G. *et al.* Ubiquitylation of lipopolysaccharide by RNF213 during bacterial infection. *Nature* **594**, 111–116 (2021).
 204. Thery, F. *et al.* Ring finger protein 213 assembles into a sensor for ISGylated proteins with antimicrobial activity. *Nat. Commun.* **12**, 5772 (2021).
 205. Walsh, S. C. *et al.* The bacterial effector GarD shields *Chlamydia trachomatis* inclusions from RNF213-mediated ubiquitylation and destruction. *Cell Host Microbe* **30**, 1671–1684.e9 (2022).
 206. Hernandez, D., Walsh, S., Saavedra Sanchez, L., Dickinson, M. S. & Coers, J. Interferon-Inducible E3 Ligase RNF213 Facilitates Host-Protective Linear and K63-Linked Ubiquitylation of *Toxoplasma gondii* Parasitophorous Vacuoles. *mBio* **13**, e01888–22.
 207. Tian, H. *et al.* RNF213 modulates γ -herpesvirus infection and reactivation via targeting the viral Replication and Transcription Activator. *Proc. Natl. Acad. Sci. U. S. A.* **120**, e2218825120.
 208. Liu, Y. *et al.* Metabolic reprogramming in macrophage responses. *Biomark. Res.* **9**, 1 (2021).
 209. Braverman, J., Sogi, K. M., Benjamin, D., Nomura, D. K. & Stanley, S. A. HIF-1 α Is an Essential Mediator of IFN- γ –Dependent Immunity to *Mycobacterium tuberculosis*. *J. Immunol.* **197**, 1287–1297 (2016).