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The *Yersinia* Virulence Factor YopM Hijacks Host Kinases to Inhibit Type III Effector-Triggered Activation of the Pyrin Inflammasome

Lawton K. Chung[1,2], **Yong Hwan Park**[3], **Yueting Zheng**[1], **Igor E. Brodsky**[4], **Patrick Hearing**[1], **Daniel L. Kastner**[3], **Jae Jin Chae**[3], and **James B. Bliska**[1,2]

[1]Department of Molecular Genetics and Microbiology, School of Medicine, Stony Brook University, Stony Brook, NY 11794

[2]Center for Infectious Diseases, School of Medicine, Stony Brook University, Stony Brook, NY 11794

[3]Inflammatory Disease Section, Metabolic, Cardiovascular, and Inflammatory Disease Genomics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892

[4]Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104

Summary

Pathogenic *Yersinia*, including *Y. pestis,* the agent of plague in humans, and *Y. pseudotuberculosis,* the related enteric pathogen, deliver virulence effectors into host cells via a prototypical type III secretion system to promote pathogenesis. These effectors, termed *Yersinia* outer proteins (Yops), modulate multiple host signaling responses. Studies in *Y. pestis* and *Y. pseudotuberculosis* have shown that YopM suppresses infection-induced inflammasome activation, however the underlying molecular mechanism is largely unknown. Here we show that YopM specifically restricts the pyrin inflammasome, which is triggered by the RhoA-inactivating enzymatic activities of YopE and YopT, in *Y. pseudotuberculosis*-infected macrophages. The attenuation of a *yopM* mutant is fully reversed in pyrin knock-out mice, demonstrating that YopM inhibits pyrin to promote virulence. Mechanistically, YopM recruits and activates the host kinases PRK1 and PRK2 to negatively regulate pyrin by phosphorylation. These results show how a virulence factor can hijack host kinases to inhibit effector-triggered pyrin inflammasome activation.

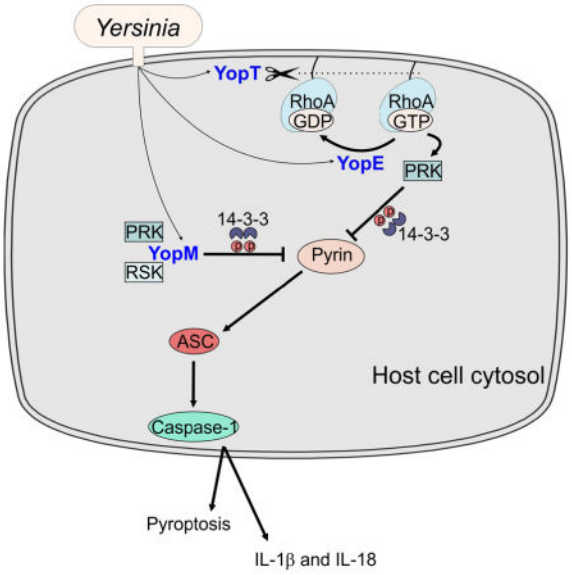
eTOC Blurb

Corresponding author: james.bliska@stonybrook.edu.

Author Contributions

L.C., YP, Y.Z., and J.C. performed all experiments; I.B., P.H., and D.K. provided essential reagents; L.C., J.C., and J.B. designed the experiments, analyzed and interpreted the data, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Pathogenic *Yersinia* secrete effectors called Yops, which can trigger or inhibit protective immune responses. Chung et al. demonstrate that both YopE and YopT inactivate RhoA, resulting in activation of the pyrin inflammasome. Consequently, *Yersinia* maintain virulence by delivering YopM, which hijacks host kinases to phosphorylate pyrin and inhibit inflammasome activation.



Introduction

Caspase-1 is activated by cytosolic multiprotein complexes known as inflammasomes and plays an important protective role in response to infection (Schroder and Tschopp, 2010). Following recognition of distinct pathogen-associated molecular patterns, inflammasomes are typically assembled through homotypic interactions among nucleotide-binding oligomerization domain leucine-rich repeat containing receptors (NLRs), apoptosis-associated speck-like protein containing a CARD (ASC), and caspase-1. The NLRP3 inflammasome senses disruptions in cellular homeostasis by exogenous ATP or bacterial pore-forming toxins, whereas NLRC4 and AIM2 inflammasomes respond to cytosolic contamination with bacterial components such as flagellin and double-stranded DNA, respectively. Recently, certain bacterial toxins that inhibit RhoA GTPases have been shown to activate the pyrin inflammasome through an indirect mechanism (Xu et al., 2014). RhoA activates the serine/threonine protein kinase C-related kinases (PRK or PKN) that bind to and phosphorylate pyrin (Park et al., 2016). Phosphorylated pyrin binds to 14-3-3 proteins, which negatively regulate the pyrin inflammasome (Park et al., 2016). Despite the specificity of different inflammasomes for their cognate signals or the requirement for ASC, assembly culminates in the activation of caspase-1 resulting in a form of cell death known as pyroptosis (Bergsbaken et al., 2009) as well as the maturation of IL-1β and IL-18 (Cerretti et al., 1992, Ghayur et al., 1997, Thornberry et al., 1992). The combined activity of these caspase-1 effector functions directs antimicrobial responses to promote clearance of pathogens. Therefore, counteracting caspase-1 activation is an important virulence strategy employed by bacterial pathogens.

Pathogenic *Yersinia* species (*Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*) utilize a contact-dependent type III secretion system (T3SS) to deliver *Yersinia* outer proteins (Yops) into the eukaryotic host cell cytosol (Cornelis, 2006). Yop effectors modulate a multitude of cellular processes upon cytosolic entry, including the disruption of phagocytosis and proinflammatory gene expression (Viboud and Bliska, 2005). For example, YopE and YopT contribute to inhibition of phagocytosis by inactivating Rho GTPases (Viboud and Bliska, 2005). Furthermore, inflammasome activation is also inhibited during *Yersinia* infection, specifically by the virulence effectors YopK and YopM. YopK has been shown to interact with components of the *Yersinia* translocation machinery, YopB and YopD, to restrict their aberrant translocation into infected host cells, thereby limiting activation of NLRP3 and NLRC4 inflammasomes (Brodsky et al., 2010, Zwack et al., 2015). The leucine-rich repeat (LRR)-containing protein YopM, another effector important for *Yersinia* virulence (Leung et al., 1990, Nemeth and Straley, 1997), inhibits caspase-1 activation (Chung et al., 2014, LaRock and Cookson, 2012). Recent studies showed that the virulence defect of a *Yersinia yopM* mutant was reversed in mice lacking caspase-1 (Chung et al., 2014, LaRock and Cookson, 2012) indicating that YopM promotes virulence by inhibiting caspase-1. There is evidence that YopM can act as a pseudosubstrate for caspase-1 (LaRock and Cookson, 2012), but this activity may not be essential for YopM to block inflammasome activation (Chung et al., 2014). Structurally, YopM consists of an N-terminal secretion signal followed by two α-helices that initiate folding into the LRR region and finally terminates in an unstructured C-terminal tail (Evdokimov et al., 2001). Because YopM lacks catalytic function and interacts with multiple host target proteins, it is thought to act as a scaffold. The LRR region binds to PRKs and the C-terminal tail binds to ribosomal S6 kinase (RSK) (McCoy et al., 2010, McPhee et al., 2010), resulting in kinase activation and increased phosphorylation of a heterologous substrate (Hentschke et al., 2010, McDonald et al., 2003). There have been no reported interactions between PRKs and RSKs under physiological conditions. Interestingly, the activities of several known targets of the individual kinases are not altered in the presence of YopM (Hentschke et al., 2010, McDonald et al., 2003), suggesting that complex formation redirects their substrate specificity. However, to date, endogenous targets of YopM-associated kinases remain enigmatic.

The pyrin inflammasome is negatively regulated by PRKs through the RhoA signal transduction pathway (Park et al., 2016). Here we demonstrate that the pyrin inflammasome is a key regulator of the innate immune response during *Yersinia* infection, and the specific target of YopM. Mice lacking functional pyrin are particularly susceptible to infection with a *Yersinia yopM* mutant, underscoring the importance of pyrin for host defense, and demonstrating that YopM inhibits pyrin to promote virulence. These results uncover a mechanism by which a pathogen hijacks host kinases and evades effector-triggered activation of caspase-1.

Results

YopM inhibits the pyrin inflammasome to counteract host defense against *Yersinia*

Bone marrow-derived macrophages (BMDMs) infected with wild-type (WT) *Yersinia* fail to mobilize caspase-1-dependent responses due to the activity of two effectors, YopK and

YopM (Brodsky et al., 2010, Chung et al., 2014, LaRock and Cookson, 2012, Zwack et al., 2015). Perplexingly, *Yersinia yopK* mutants translocate YopM into infected macrophages but fail to inhibit activation of caspase-1 (Brodsky et al., 2010, Chung et al., 2014, Zwack et al., 2015). Likewise, *yopM* mutants, that are competent for YopK translocation upon infection, are also unable to inhibit activation of caspase-1 in macrophages (Chung et al., 2014, LaRock and Cookson, 2012). These observations suggest that these effectors inhibit distinct pathways of caspase-1 activation during *Yersinia* infection. Previous studies have demonstrated that the NLRP3 and NLRC4 inflammasomes are important for activation of caspase-1 in macrophages infected with a *Yersinia yopK* mutant (Brodsky et al., 2010), but the specific inflammasome that is activated by a *yopM* mutant has not been identified. Hence, we investigated which inflammasomes respond to *Yersinia yopM* mutant infection by utilizing BMDMs from C57BL/6 mice deficient in various inflammasome components.

Consistent with previous studies, WT *Yersinia* (32777) did not induce caspase-1 activation, IL-1β or lactate dehydrogenase (LDH) release from LPS-primed WT BMDMs, while the *yopM* mutant (*ΔyopM*) failed to inhibit these processes (Figures S1A–C). The *yopM* mutant-induced caspase-1 cleavage, IL-1β and LDH release was substantially diminished in BMDMs from $Asc^{-/-}$ or $Mefv^{-/-}$ (the *Mefv* gene encodes pyrin) mice but not in BMDMs from $Nlrp3^{-/-}$, $Nlrc4^{-/-}$ or $Nlrp3^{-/-}Nlrc4^{-/-}$ mice (Figures 1A–F, and Figures S1A–C). There was slight but significant contribution of NLRP3 and NLRC4 for IL-1β secretion in response to the *yopM* mutant (Figure 1B). $Asc^{-/-}$ BMDMs infected with the *yopK* mutant (*ΔyopK*), which stimulates both NLRP3 and NLRC4 inflammasomes (Brodsky et al., 2010), exhibited a defect in cleavage of caspase-1 and secretion of IL-1β (Figures 1A and 1B), while caspase-1 processing and IL-1β release were largely absent in $Nlrp3^{-/-}Nlrc4^{-/-}$ BMDMs infected with the *yopK* mutant (Figures 1A and 1B). Levels of LDH release remained unchanged in $Nlrp3^{-/-}Nlrc4^{-/-}$ and $Asc^{-/-}$ BMDMs infected with the *yopK* mutant (Figure 1C), which may reflect caspase-11-dependent cell death (Casson et al., 2013). These findings demonstrate that *Yersinia yopM* and *yopK* mutants engage different inflammasomes. These findings also indicate that the pyrin inflammasome is activated during infection with a *Yersinia yopM* mutant, which in turn suggests that YopM specifically inhibits the pyrin inflammasome.

To date, several distinct YopM isoforms with different numbers of LRRs have been shown to inhibit caspase-1, including variants from *Y. pseudotuberculosis* YPIII ($YopM^{YPIII}$, 15 LRRs), *Y. pseudotuberculosis* 32777 ($YopM^{32777}$, 21 LRRs), and *Y. pestis* KIM ($YopM^{KIM}$, 15 LRRs) (Chung et al., 2014, LaRock and Cookson, 2012). Infection of BMDMs with *Yersinia* expressing different isoforms from *Y. pestis* Pestoides A ($YopM^{PestoidesA}$, 13 LRRs) or *Y. enterocolitica* ($YopM^{8081}$, 13 LRRs) (Figure S2A) revealed that inhibition of caspase-1 processing, IL-1β secretion, LDH release, and ASC foci formation is a conserved function of multiple YopM variants (Figures S2B–J). We conclude that most, if not all, YopM isoforms have the ability to restrict activation of caspase-1 by targeting the pyrin inflammasome.

A previous report presented evidence that the $YopM^{YPIII}$ isoform used a pseudosubstrate mechanism to directly inhibit caspase-1, which was mediated by a critical aspartic acid residue (amino acid 271) within a YLTD motif found in this effector (LaRock and Cookson,

2012). In the study of LaRock and Cookson, infection of BMDMs with a *Yersinia* strain lacking all known translocated effectors triggered activation of caspase-1, and this response was inhibited when wild-type YopM^{YPIII}, but not the corresponding YopM^{D271A} variant, was expressed in the "effectorless mutant" (LaRock and Cookson, 2012). Given our data indicating specificity of YopM and YopK for distinct inflammasomes (Figure 1), and our results showing that YopM isoforms with (e.g. YopM^{KIM}) or without (YopM^{32777}) a YLTD motif can inhibit the pyrin inflammasome (Figure S2), we sought to evaluate the ability of these effectors in restricting caspase-1 activation by the effectorless mutant strain. When YopM^{32777}, YopM^{KIM}, or a corresponding YopM^{KIMD271A} variant were expressed in a single *yopM* mutant, IL-1β secretion from infected BMDMs was significantly reduced (Figure S2K). Importantly, none of the YopM variants were able to significantly inhibit IL-1β secretion induced by infection with the effectorless mutant (Figure S2K). However, IL-1β secretion was significantly reduced when YopK was expressed in a single *yopK* mutant or the effectorless mutant (Figure S2K). Collectively, our data demonstrate that YopM specifically restricts the pyrin inflammasome independent of a YLTD motif, and, unlike YopK, is unable to inhibit NLRP3 and NLRC4 inflammasome activation by the effectorless mutant.

The conserved ability of distinct YopM isoforms to inhibit pyrin inflammasome activation led us to investigate the role of pyrin-dependent responses for *in vivo* protection against *Yersinia* infection. WT (32777) or *yopM* mutant *Yersinia* were used to intravenously infect C57BL/6 *Mefv*$^{+/+}$ or *Mefv*$^{-/-}$ mice and time-to-death was monitored. *Mefv*$^{+/+}$ mice were highly susceptible to infection with WT *Yersinia*, but not with the *yopM* mutant (Figure 2A). In contrast, *Mefv*$^{-/-}$ mice were susceptible to both WT *Yersinia* and the *yopM* mutant (Figure 2B). Furthermore, enumeration of bacterial burdens in the spleens and livers of *Mefv*$^{+/+}$ mice at 5 days post-infection showed an approximate one-log decrease of the *yopM* mutant relative to WT *Yersinia*, and the decreased colonization was reversed in *Mefv*$^{-/-}$ mice (Figures 2C and 2D). Taken together, these findings indicate an essential role for the pyrin inflammasome in controlling bacterial burdens of *yopM* mutant *Yersinia* and suggest that YopM promotes *in vivo* virulence by suppressing the pyrin inflammasome.

The catalytic activities of YopE and YopT trigger pyrin inflammasome activation

The primary targets of the effectors YopE and YopT are Rho GTPases, including RhoA, which regulates pyrin inflammasome activation (Park et al., 2016). Specifically, YopE mimics eukaryotic GTPase activating proteins (GAPs) by facilitating GTP hydrolysis (Black and Bliska, 2000), and YopT is a cysteine protease that cleaves the C-terminus of Rho GTPase, which results in its release from membranes (Shao et al., 2002, Shao et al., 2003). To examine whether pyrin inflammasome activation is triggered by YopE or YopT, we infected BMDMs with *yopM* mutant strains expressing catalytically dead YopE (YopE^{R144A}) and/or YopT (YopT^{C139A}). *Yersinia yopM* mutant-induced pyrin inflammasome activation was significantly reduced when both YopE and YopT were inactivated (Figures 3A–C), which corresponded to RhoA activation (Figure S3), while an intermediate phenotype was seen with each individual mutant (Figures 3A–C). In addition, consistent with previous observations (Chung et al., 2014), pyrin inflammasome activation required YopB, because a *Yersinia yopByopM* mutant defective in effector translocation

failed to activate caspase-1 (Figures 3A–C and 3G). Furthermore, the *yopM* mutant induced ASC foci formation, which was dependent on YopB, and YopE and YopT catalytic activities (Figures 3D–I). These findings indicate that BMDMs infected with *Yersinia* mobilize the pyrin inflammasome as an effector-triggered immune response following inactivation of RhoA by YopE or YopT.

The catalytic activities of YopE and YopT trigger immune responses that attenuate the *yopM* mutant *in vivo*

Our *ex vivo* studies suggest that YopM promotes virulence of *Yersinia in vivo* by inhibiting YopE- and YopT-induced activation of the pyrin inflammasome. To evaluate this possibility, we measured the degree of virulence attenuation of a *yopM* mutant *in vivo* in the presence or absence of the pyrin inflammasome-stimulating activities of YopE and YopT. Wild-type mice were infected as above except the number of bacteria was increased (~5000 CFU) to compensate for attenuation resulting from the $yopE^{R144A}yopT^{C139A}$ mutations. At this dose, a *yopM* mutant in the $yopE^{+}yopT^{+}$ background caused a delay in mortality by a median of one day compared to the wild-type strain (Figure 4A), which was statistically significant. Importantly, this delay was not observed upon deletion of *yopM* in the $yopE^{R144A}yopT^{C139A}$ background relative to the $yopE^{R144A}yopT^{C139A}$ parent (Figure 4B), demonstrating that YopM counteracts host responses triggered by YopE and YopT. Similar results were obtained when infections were done at a lower dose (~2000 CFU) (Figures S4A–B). Together, these data show that catalytic activities of YopE and YopT trigger activation of the pyrin inflammasome *in vitro* and drive immune responses important for killing of the *yopM* mutant *in vivo*.

YopM hijacks PRK to negatively regulate pyrin by phosphorylation

Recent studies show that RhoA-activated PRK negatively regulates pyrin by phosphorylation of two serine residues, creating a binding site for 14-3-3 proteins (Park et al., 2016). Since YopM binds to and sequentially activates PRK and RSK (McDonald et al., 2003), we hypothesized that YopM hijacks these kinases to bypass inactive RhoA to phosphorylate and inhibit pyrin. To assess this, $YopM^{32777}$ was purified as a fusion to glutathione S-transferase (GST) and incubated with lysates derived from HEK293T cells transfected to express murine pyrin containing an N-terminal Myc tag. The presence of Myc-pyrin, PRK2 or RSK1 in samples of starting material or fractions bound to GST-$YopM^{32777}$ (or GST alone as a control) was determined by Western blot. GST-$YopM^{32777}$, but not GST, interacted with Myc-pyrin, PRK2 and RSK1 (Figure 5A), suggesting that pyrin is a substrate of the YopM/PRK/RSK complex.

To directly examine whether pyrin is a substrate of YopM-associated kinases, we performed *in vitro* kinase assays. Proteins that associated with GST or GST-$YopM^{32777}$ in lysates of HEK293T cells, left untransfected or transfected to endogenously express Myc-pyrin, were incubated in the presence of [γ-^{32}P]ATP to analyze phosphorylation. Incorporation of γ-^{32}P into proteins by phosphorylation was assessed by SDS-PAGE and autoradiography (Figure 5B). Parallel kinase reactions were performed using cold ATP and analyzed by Western blot to verify the presence of PRK2, RSK1 and YopM, as well as pyrin (Figure 5B). A band corresponding to phosphorylated transfected pyrin was detected in the corresponding GST-

YopM^{32777} samples but not in the GST control (Figure 5B). Immunoprecipitation with a Myc antibody confirmed that the phosphorylated band was pyrin (Figure 5B). These results identify pyrin as a target of YopM-associated kinases and suggest that this complex might inhibit inflammasome activation during *Yersinia* infection.

To further evaluate the role of PRK and RSK activity in YopM-mediated suppression of the pyrin inflammasome, BMDMs were left uninfected or infected with WT *Yersinia* or the *yopM* mutant in the presence or absence of the PRK inhibitor PKC412 (Falk et al., 2014), RSK inhibitor BI-D1870 (Sapkota et al., 2007), or a combination of the two, and caspase-1 processing was measured. Uninfected BMDMs treated with inhibitors did not contain active caspase-1 (Figure 5C), indicating that kinase inhibition is not sufficient to activate the pyrin inflammasome. Inhibition of either PRK or RSK did not alter caspase-1 processing in BMDMs infected with the *yopM* mutant, but inhibition of PRK and not RSK, partially restored cleavage of caspase-1 in BMDMs infected with the wild-type strain (Figure 5C). We observed reduced phosphorylation of the known RSK target GSK-3β upon treatment of uninfected BMDMs with BI-D1870 (Figure S5), indicating that the inhibitor was effective at the concentration used. Importantly, the presence of kinase inhibitors did not negatively affect translocation of YopM (Figure 5C). To more precisely evaluate the contribution of PRK, secreted IL-1β was measured from *Yersinia*-infected BMDMs transiently transfected with scrambled control siRNAs or siRNAs targeting the most ubiquitously expressed and functionally redundant isoforms *Prk1* and *Prk2* (Palmer et al., 1995). Consistent with previous data, we observed a YopM-dependent inhibition of IL-1β release in control-transfected BMDMs (Figure 5D). In contrast, YopM failed to inhibit IL-1β in PRK1/PRK2 knock-down BMDMs (Figure 5D). These results indicate that during *Yersinia* infection, YopM hijacks PRK activity to restrict the pyrin inflammasome.

YopM enhances PRK-mediated pyrin phosphorylation

Our findings reveal that while YopM/PRK/RSK complexes are competent for pyrin phosphorylation (Figure 5A and 5B and Figure S5B), the activity of PRK appears to be specifically important for suppression of inflammasome activation (Figure 5C and 5D). To assess the contribution of YopM to PRK-dependent pyrin phosphorylation, we incubated recombinant PRK1 or PRK2 and GST or GST-YopM^{32777} with a purified N-terminal fragment of pyrin containing the critical serine residues important for inflammasome regulation (Masters et al., 2016, Park et al., 2016). Incubation of GST with PRK1 or PRK2 revealed an intrinsic ability of either kinase to phosphorylate pyrin, as measured by serine phosphorylation (Figure 6). Importantly, addition of GST-YopM^{32777} enhanced the abundance of phosphorylated serine (Figure 6), indicating that YopM interaction with PRKs enhances pyrin phosphorylation. Thus, our data show that YopM-associated PRK has increased ability to phosphorylate pyrin *in vitro*, suggesting that this post-translational modification negatively regulates activation of the pyrin inflammasome.

Discussion

Bacterial pathogens employ specialized secretion systems to deliver virulence effectors into the cytosol of host cells to disrupt a multitude of signaling pathways. While these features

are essential for pathogenesis, they may inadvertently trigger host responses. For example, cytosolic disruptions triggered by the *Yersinia* T3SS sets in motion the formation of inflammasomes which activate proinflammatory caspase-1. Therefore, limiting innate immune recognition of the T3SS in the host cell is vital towards promoting virulence of *Yersinia* and other bacterial pathogens with this type of specialized secretion system.

Here we determine that the pyrin inflammasome specifically recognizes inactivation of Rho-GTPases by YopE or YopT during *Yersinia* infection of macrophages. These results extend recent findings of how bacterial pathogens (Dumas et al., 2014, Gavrilin et al., 2012), and more specifically bacterial toxins that modify the switch I region of RhoA (Aubert et al., 2016, Xu et al., 2014), stimulate the pyrin inflammasome. For example, TcdB of *Clostridium difficile,* which glucosylates Thr 37 in switch I of RhoA, activates the pyrin inflammasome (Xu et al., 2014). In contrast, the Rho-inactivation domain (RID) of a *Vibrio* RTX toxin, which enhances GTP hydrolysis by RhoA, did not trigger activation of caspase-1, leading the authors to conclude that only inactivation of RhoA by covalent modification of switch I triggers the pyrin inflammasome (Xu et al., 2014). Both YopE GAP and YopT protease activities stimulate the pyrin inflammasome, but neither of these toxins covalently modify switch I of RhoA. However, YopE, by promoting GTP hydrolysis, and YopT, by catalyzing cleavage and detachment from the plasma membrane, prevent RhoA from activating downstream partners (Aktories, 2011), such as PRK (Zong et al., 1999), which negatively regulates pyrin by phosphorylation (Park et al., 2016). When RhoA is in the GTP bound conformation, it interacts, primarily via switch I, with the N-terminal homology region (HR1) of PRK2 leading to kinase activation (Zong et al., 1999). These data suggest that the pyrin inflammasome is activated in response to bacterial toxins that either directly (e.g. TcdB) or indirectly (e.g. YopE, YopT) prevent RhoA from interacting with and activating PRK at the plasma membrane. This conclusion is consistent with the known mechanism of *Clostridium botulinum* C3 toxin, which ADP-ribosylates RhoA on Asn 41 at the C-terminal border of switch I (Aktories, 2011). This modification does not directly prevent RhoA from interacting with downstream partners, but instead traps the GTPase in the cytosol in a complex with guanine-nucleotide dissociation inhibitor 1 (Aktories, 2011). Moreover, defects in geranylgeranylation lead to pyrin-dependent inflammatory diseases (Akula et al., 2016, Park et al., 2016), consistent with our results that general disruption of the RhoA signaling pathway stimulates inflammasome activation. Further studies are necessary to resolve why GTP hydrolysis in RhoA catalyzed by *Yersinia* YopE, but not *Vibrio* RID, triggers the pyrin inflammasome

YopE- and YopT-mediated activation of the pyrin inflammasome in *Yersinia*-infected macrophages is efficiently neutralized following delivery of YopM, thereby inhibiting ASC foci formation, caspase-1 processing, IL-1β secretion, and LDH release. A previous study presented evidence that YopM was unable to inhibit formation of ASC foci in *Yersinia*-infected macrophages (LaRock and Cookson, 2012). However, these studies were performed using *Yersinia yopK* mutants, which trigger activation of NLRP3 and NLRC4 inflammasomes (Brodsky et al., 2010) irrespective of the presence of YopM. Indeed, *Mefv*$^{-/-}$ macrophages are competent for caspase-1 activation by *yopK*, but not by *yopM* mutants. More importantly, the virulence defect of a *Yersinia yopM* mutant is rescued in *Mefv*$^{-/-}$ mice, indicating that pyrin is the major *in vivo* target of YopM. These data reveal

specificity of the distinct YopK and YopM effectors towards different inflammasome targets and establish the significance of pyrin-dependent responses for host defense against *Yersinia* infection.

While YopM shares homology to LRR-containing effectors such as IpaH of *Shigella* (Venkatesan et al., 1991) and SspH1 of *Salmonella* (Miao et al., 1999), it lacks the enzymatic domain found in these other bacterial virulence factors. Instead, YopM acts as a scaffold to bind various host proteins, including PRK2 and RSK1. *Yersinia* strains expressing deletion variants of YopM proteins unable to interact with PRK2 or RSK1 fail to inhibit activation of caspase-1 (Chung et al., 2014) and are attenuated *in vivo* (McCoy et al., 2010, McPhee et al., 2010). Our results provide an explanation for these previously published data by demonstrating that YopM usurps PRK activity to neutralize the pyrin inflammasome. We hypothesize that through binding and activation of PRK and RSK (McDonald et al., 2003), YopM bypasses YopE- and YopT-inactivated RhoA to maintain pyrin in a negatively regulated state by phosphorylation and binding of 14-3-3 proteins (Park et al., 2016). Reinforcing this hypothesis, we identify pyrin as an endogenous substrate to be phosphorylated by YopM-associated kinases, in particular PRK1 and PRK2 *in vitro*. Interestingly, we could not determine a role for RSK kinase activity in suppression of the pyrin inflammasome and propose instead that its interaction with YopM may be important for allosteric regulation *in vivo*. The relative contributions of particular PRK (PRK 1–3) and RSK (RSK 1–4) isoforms that can assemble with YopM (Hentschke et al., 2010), as well as the precise molecular interactions required for association of pyrin with the complex remain to be determined. The diversity of the LRR region of YopM also raises additional questions. To date, all YopM isoforms have been shown to inhibit the pyrin inflammasome, yet each isoform potentially interacts with a distinct range of host proteins. The purpose of this heterogeneity is still under investigation, but may enable specific isoforms to more efficiently restrict the pyrin inflammasome.

Although inhibition of the pyrin inflammasome appears to be a major virulence function, there exist additional mechanisms by which YopM promotes pathogenesis (Hofling et al., 2015). For example, YopM has been shown to autonomously translocate into both the cytosol and nucleus of eukaryotic cells and can also suppress transcription of the proinflammatory cytokine TNFα (Ruter et al., 2010, Scharnert et al., 2013, Hofling et al., 2014). Moreover, YopM is required for the induction of systemic levels of the anti-inflammatory cytokine IL-10 (McPhee et al., 2012, McPhee et al., 2010), possibly through interaction with nuclear helicases and RSKs (Berneking et al., 2016). Lastly, YopM plays a key role in promoting neutrophil survival via processes independent of RSK and caspase-1 catalytic activities (Stasulli et al., 2015). Taken together, these findings indicate that YopM could have multiple functions depending on the infected cell type, host protein interaction, or subcellular localization. For instance, the cytosolic fraction of YopM may inhibit inflammasome activation while the nuclear fraction may modify gene expression. How these features contribute to *Yersinia* virulence remain important questions, but nevertheless suggest that the overall role of YopM may be to restrict inflammation.

Interestingly, individuals with gain-of-function mutations of pyrin (Touitou et al., 2004) present with episodes of periodic inflammation, in a disease known as Familial

Mediterranean Fever (FMF). Another more-recently identified pyrin mutation leads to a disease that is clinically distinct from FMF, and therefore termed pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) (Masters et al., 2016). The prevalence of PAAND mutations remains to be determined, but the high carrier frequency of FMF in Mediterranean and Middle Eastern populations is suggested to be a result of a selective advantage in resistance to an unknown infection. It is intriguing to speculate that FMF or PAAND pyrin variants emerged to confer protection against *Yersinia* or other pathogens that actively evade the pyrin inflammasome. Use of knock-in mice encoding pyrin FMF variants (Chae et al., 2011) or monocytes from PAAND patients (Masters et al., 2016) in infection assays may provide insight into the origin for these gain-of-function mutations, and will surely provide insights into human disease as well as the evolution of host-pathogen interactions.

Experimental Procedures

Bacterial strains

Y. pseudotuberculosis strains used in this study are listed in Table S1. Details on strain construction are also listed in Supplemental Experimental Procedures.

Bone marrow isolation and culture conditions

Bone marrow was isolated from femur exudates of 8 week old wild-type C57BL/6 (Jackson Laboratories), *Nlrp3*$^{-/-}$ (Sutterwala et al., 2006), *Nlrc4*$^{-/-}$ (Lara-Tejero et al., 2006), *Nlrp3*$^{-/-}$*Nlrc4*$^{-/-}$ (Ip and Medzhitov, 2015), *Asc*$^{-/-}$ (Sutterwala et al., 2006) or *Mefv*$^{-/-}$ (Chae et al., 2003) mice, as previously described (Brodsky et al., 2010). At 18 hr prior to infection, BMDMs were seeded in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 10% L-cell-conditioned medium, 1 mM sodium pyruvate, 10 mM HEPES and 100 ng/ml *Escherichia coli* LPS (Sigma) in 6-well plates at a density of 0.8 x 10^6 cells/well except for fluorescence microscopy experiments, where they were seeded in 24-well plates at a density of 1.5 x 10^5 cells/well.

Macrophage infections

For BMDM infections, *Y. pseudotuberculosis* strains were grown overnight in Luria broth (LB) at 28°C. The following day, cultures were diluted 1:40 in LB containing 20 mM sodium oxalate and 20 mM $MgCl_2$ and grown at 28°C for 1 hr, then shifted to 37°C for 2 hr. For analysis of Yop secretion, cultures were grown at 28°C for 2 hr, then shifted to 37°C for 4 hr. LPS-primed BMDMs were left uninfected or infected with *Y. pseudotuberculosis* strains cultured under conditions as described above at a multiplicity of infection (MOI) of 30. Tissue culture plates were centrifuged at 95 x g for 5 min to promote contact of *Yersinia* with BMDMs and incubated at 37°C with 5% CO_2. At 90 min post-infection, supernatants were collected and processed for secreted IL-1β and LDH release, while cell lysates were harvested for Western blot analysis of host proteins. For infection experiments with kinase inhibitors BI-D1870 (10 μM) or PKC412 (5 μM) (Enzo Life Sciences), reagents were added simultaneously with bacteria to BMDMs at the start of infection. Transfection of BMDMs with scrambled control siRNAs or siRNAs targeting *Prk1* and *Prk2* were performed as previously described (Park et al., 2016).

Cytokine production

Levels of secreted IL-1β in supernatants collected from BMDMs were quantified using a commercially available ELISA kit (R&D Biosystems).

Cell death assays

Levels of LDH release in supernatants collected from BMDMs were analyzed using a commercially available cytotoxicity kit (Promega).

Western blotting of macrophage lysate

BMDMs were lysed in 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, with protease inhibitor cocktail (Roche) and resolved by SDS-PAGE using 4–12% or 12% NuPAGE Bis-Tris gels (Invitrogen). Proteins were transferred onto PVDF membranes and probed with rabbit polyclonal antibodies against caspase-1 (a gift from Gabriel Nuñez), YopM (a gift from Sue Straley), pyrin (Chae et al., 2003), or phospho-GSK-3β (Cell Signaling), a rabbit monoclonal antibody against GSK-3β (Cell Signaling), and mouse monoclonal anti-YopE 202.19 and 149.27 antibodies (unpublished data). Anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch) conjugated to HRP were used as secondary reagents. An HRP-conjugated β-actin antibody (Sigma) was used to control for loading. Proteins were visualized using chemiluminescent detection reagent (GE Healthcare).

Analysis of active RhoA

Levels of GTP-bound RhoA were assessed by incubating BMDM lysates and Rhotekin-RBD beads (Cytoskeleton), as previously described (Park et al., 2016).

Mouse infections

Y. pseudotuberculosis cultures were grown overnight in LB at 28°C and prepared as phosphate-buffered saline (PBS) suspensions containing approximately 2.0 x 10^4 or 5.0x10^4 colony forming units (CFU)/ml. A volume of 100 μl was delivered by tail vein injection into C57BL/6 or *Mefv*$^{-/-}$ mice. Time to death was monitored for 21 days, upon which the remaining mice were euthanized. At 5 days post infection, spleens and livers were collected, homogenized, serially diluted, and plated onto LB agar to enumerate bacterial colonization. All mice were handled in accordance with guidelines for the human care and use of experimental animals and the procedures used were approved by the Stony Brook University Institutional Animal Care and Use Committee.

Analysis of Yop secretion

Following growth of *Y. pseudotuberculosis* strains as described above, supernatants were clarified by centrifugation and precipitated with trichloroacetic acid. Samples were subsequently processed by Western blot using a mouse monoclonal anti-YopM antibody CE 6-1 (a gift from Sue Straley).

Fluorescence microscopy

BMDMs seeded onto glass coverslips were left uninfected or infected as described above, except that tissue culture medium was supplemented with 0.5 mM IPTG to induce GFP

expression in *Y. pseudotuberculosis* strains harboring p67GFP3.1. BMDMs were washed, fixed, permeablized and blocked, as previously described (Pujol and Bliska, 2003), and incubated with rabbit anti-*Yersinia* (Black and Bliska, 2000) or rat monoclonal anti-ASC (a gift from Gabriel Nuñez) antibodies. Binding of primary antibodies was visualized using FITC-conjugated anti-rabbit antibody (Sigma Immunochemicals) or Alexa Fluor 594-conjugated anti-rat antibody (Invitrogen) and DNA was stained with DAPI. Coverslips were mounted onto glass slides and imaged with an Axiovert S100 microscope (Zeiss). Three random fields of approximately 50 cells each were taken with a SPOT camera (Diagnostic Instruments) and processed with Adobe Photoshop.

Protein purification

E. coli strains harboring pGEX-2T vectors encoding YopM sequences from *Y. pseudotuberculosis* 32777, *Y. pestis* KIM and Pestoides A, or *Y. enterocolitica* 8081 were expressed and purified, as previously described (Chung et al., 2014).

GST pulldowns

To construct a vector for expression of murine pryin in mammalian cells, mRNA extracted from LPS-primed BMDMs was converted to cDNA using commercially available RNA isolation (Qiagen) and reverse transcriptase (Invitrogen) kits. A DNA sequence encoding murine pyrin isoform 1 (GenBank: NM_001161790.1) with an N-terminal c-Myc epitope was amplified using PCR and the cDNA template, and inserted into the pMIGR1 vector. HEK293T cells were transfected with the pMIGR1 vector encoding pyrin or empty vector using the Lipofectamine 3000 transfection reagent (Invitrogen). The following day, 10 μg of GST-YopM^{32777} or equimolar amounts of GST were added to 20 μl of GST-Bind Resin (Novagen) and incubated for 30 min at 4°C. Beads were washed three times with GST Bind/Wash Buffer (Novagen) and incubated with lysates derived from transfected HEK293T cells in lysis buffer containing 0.1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0 for 2 hr at 4°C on a rotating shaker. Bound proteins were washed three times in lysis buffer, boiled in sample buffer, separated by SDS-PAGE and Western blotted using mouse monoclonal c-Myc (Cell Signaling) or YopM (a gift from Sue Straley) antibodies or rabbit polyclonal anti-RSK1 or -PRK2 antibodies (Abcam).

Kinase assays

Pulldowns with GST or GST-YopM^{8081} were performed as described above except beads were incubated with lysates generated from nontransfected HEK293T cells. Bound proteins were washed three times in lysis buffer, followed by two washes in 20 mM HEPES pH 7.4 and 10 mM MgAc. Kinase assays were performed at room temperature, as previously described (McDonald et al., 2003). Parallel kinase reactions using cold ATP were performed, resolved by SDS-PAGE and analyzed by Western blot. *In vitro* kinase assays using N-terminal pyrin constructs were performed as previously described (Park et al., 2016).

Statistical analysis

Experimental data analyzed for significance were from at least three independent experiments using GraphPad Prism. Probability (*P*) values for IL-1β, LDH and fluorescence

microscopy experiments were calculated using one-way analysis of variance (ANOVA) or grouped two-way ANOVA with Tukey's post-test. *P* values from mouse survival experiments and organ burdens were calculated by log-rank or Mann-Whitney tests, respectively. *P* values of < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

AKTORIES K. Bacterial protein toxins that modify host regulatory GTPases. Nat Rev Microbiol. 2011; 9:487–98. [PubMed: 21677684]

AKULA MK, SHI M, JIANG Z, FOSTER CE, MIAO D, LI AS, ZHANG X, GAVIN RM, FORDE SD, GERMAIN G, CARPENTER S, ROSADINI CV, GRITSMAN K, CHAE JJ, HAMPTON R, SILVERMAN N, GRAVALLESE EM, KAGAN JC, FITZGERALD KA, KASTNER DL, GOLENBOCK DT, BERGO MO, WANG D. Control of the innate immune response by the mevalonate pathway. Nat Immunol. 2016

AUBERT DF, XU H, YANG J, SHI X, GAO W, LI L, BISARO F, CHEN S, VALVANO MA, SHAO F. A Burkholderia Type VI Effector Deamidates Rho GTPases to Activate the Pyrin Inflammasome and Trigger Inflammation. Cell Host Microbe. 2016

BERGSBAKEN T, FINK SL, COOKSON BT. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol. 2009; 7:99–109. [PubMed: 19148178]

BERNEKING L, SCHNAPP M, RUMM A, TRASAK C, RUCKDESCHEL K, ALAWI M, GRUNDHOFF A, KIKHNEY AG, KOCH-NOLTE F, BUCK F, PERBANDT M, BETZEL C, SVERGUN DI, HENTSCHKE M, AEPFELBACHER M. Immunosuppressive Yersinia Effector YopM Binds DEAD Box Helicase DDX3 to Control Ribosomal S6 Kinase in the Nucleus of Host Cells. PLoS Pathog. 2016; 12:e1005660. [PubMed: 27300509]

BLACK DS, BLISKA JB. The RhoGAP activity of the Yersinia pseudotuberculosis cytotoxin YopE is required for antiphagocytic function and virulence. Mol Microbiol. 2000; 37:515–27. [PubMed: 10931345]

BRODSKY IE, PALM NW, SADANAND S, RYNDAK MB, SUTTERWALA FS, FLAVELL RA, BLISKA JB, MEDZHITOV R. A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. Cell Host Microbe. 2010; 7:376–87. [PubMed: 20478539]

CASSON CN, COPENHAVER AM, ZWACK EE, NGUYEN HT, STROWIG T, JAVDAN B, BRADLEY WP, FUNG TC, FLAVELL RA, BRODSKY IE, SHIN S. Caspase-11 activation in response to bacterial secretion systems that access the host cytosol. PLoS Pathog. 2013; 9:e1003400. [PubMed: 23762026]

CERRETTI DP, KOZLOSKY CJ, MOSLEY B, NELSON N, VAN NESS K, GREENSTREET TA, MARCH CJ, KRONHEIM SR, DRUCK T, CANNIZZARO LA, et al. Molecular cloning of the interleukin-1 beta converting enzyme. Science. 1992; 256:97–100. [PubMed: 1373520]

CHAE JJ, CHO YH, LEE GS, CHENG J, LIU PP, FEIGENBAUM L, KATZ SI, KASTNER DL. Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice. Immunity. 2011; 34:755–68. [PubMed: 21600797]

CHAE JJ, KOMAROW HD, CHENG J, WOOD G, RABEN N, LIU PP, KASTNER DL. Targeted disruption of pyrin, the FMF protein, causes heightened sensitivity to endotoxin and a defect in macrophage apoptosis. Mol Cell. 2003; 11:591–604. [PubMed: 12667444]

CHUNG LK, PHILIP NH, SCHMIDT VA, KOLLER A, STROWIG T, FLAVELL RA, BRODSKY IE, BLISKA JB. IQGAP1 is important for activation of caspase-1 in macrophages and is targeted by Yersinia pestis type III effector YopM. MBio. 2014; 5:e01402–14. [PubMed: 24987096]

CORNELIS GR. The type III secretion injectisome. Nat Rev Microbiol. 2006; 4:811–25. [PubMed: 17041629]

DUMAS A, AMIABLE N, DE RIVERO VACCARI JP, CHAE JJ, KEANE RW, LACROIX S, VALLIERES L. The inflammasome pyrin contributes to pertussis toxin-induced IL-1beta synthesis, neutrophil intravascular crawling and autoimmune encephalomyelitis. PLoS Pathog. 2014; 10:e1004150. [PubMed: 24875775]

EVDOKIMOV AG, ANDERSON DE, ROUTZAHN KM, WAUGH DS. Unusual molecular architecture of the Yersinia pestis cytotoxin YopM: a leucine-rich repeat protein with the shortest repeating unit. J Mol Biol. 2001; 312:807–21. [PubMed: 11575934]

FALK MD, LIU W, BOLANOS B, UNSAL-KACMAZ K, KLIPPEL A, GRANT S, BROOUN A, TIMOFEEVSKI S. Enzyme Kinetics and Distinct Modulation of the Protein Kinase N Family of Kinases by Lipid Activators and Small Molecule Inhibitors. Biosci Rep. 2014

GAVRILIN MA, ABDELAZIZ DH, MOSTAFA M, ABDULRAHMAN BA, GRANDHI J, AKHTER A, ABU KHWEEK A, AUBERT DF, VALVANO MA, WEWERS MD, AMER AO. Activation of the pyrin inflammasome by intracellular Burkholderia cenocepacia. J Immunol. 2012; 188:3469–77. [PubMed: 22368275]

GHAYUR T, BANERJEE S, HUGUNIN M, BUTLER D, HERZOG L, CARTER A, QUINTAL L, SEKUT L, TALANIAN R, PASKIND M, WONG W, KAMEN R, TRACEY D, ALLEN H. Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. Nature. 1997; 386:619–23. [PubMed: 9121587]

HENTSCHKE M, BERNEKING L, BELMAR CAMPOS C, BUCK F, RUCKDESCHEL K, AEPFELBACHER M. Yersinia virulence factor YopM induces sustained RSK activation by interfering with dephosphorylation. PLoS One. 2010:5.

HOFLING S, GRABOWSKI B, NORKOWSKI S, SCHMIDT MA, RUTER C. Current activities of the Yersinia effector protein YopM. Int J Med Microbiol. 2015; 305:424–32. [PubMed: 25865799]

HOFLING S, SCHARNERT J, CROMME C, BERTRAND J, PAP T, SCHMIDT MA, RUTER C. Manipulation of pro-inflammatory cytokine production by the bacterial cell-penetrating effector protein YopM is independent of its interaction with host cell kinases RSK1 and PRK2. Virulence. 2014; 5:761–71. [PubMed: 25513777]

IP WK, MEDZHITOV R. Macrophages monitor tissue osmolarity and induce inflammatory response through NLRP3 and NLRC4 inflammasome activation. Nat Commun. 2015; 6:6931. [PubMed: 25959047]

LARA-TEJERO M, SUTTERWALA FS, OGURA Y, GRANT EP, BERTIN J, COYLE AJ, FLAVELL RA, GALAN JE. Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. J Exp Med. 2006; 203:1407–12. [PubMed: 16717117]

LAROCK CN, COOKSON BT. The Yersinia virulence effector YopM binds caspase-1 to arrest inflammasome assembly and processing. Cell Host Microbe. 2012; 12:799–805. [PubMed: 23245324]

LEUNG KY, REISNER BS, STRALEY SC. YopM inhibits platelet aggregation and is necessary for virulence of Yersinia pestis in mice. Infect Immun. 1990; 58:3262–71. [PubMed: 2401564]

MASTERS SL, LAGOU V, JERU I, BAKER PJ, VAN EYCK L, PARRY DA, LAWLESS D, DE NARDO D, GARCIA-PEREZ JE, DAGLEY LF, HOLLEY CL, DOOLEY J, MOGHADDAS F, PASCIUTO E, JEANDEL PY, SCIOT R, LYRAS D, WEBB AI, NICHOLSON SE, DE SOMER L, VAN NIEUWENHOVE E, RUUTH-PRAZ J, COPIN B, COCHET E, MEDLEJ-HASHIM M, MEGARBANE A, SCHRODER K, SAVIC S, GORIS A, AMSELEM S, WOUTERS C, LISTON A. Familial autoinflammation with neutrophilic dermatosis reveals a regulatory mechanism of pyrin activation. Sci Transl Med. 2016; 8:332ra45.

MCCOY MW, MARRE ML, LESSER CF, MECSAS J. The C-terminal tail of Yersinia pseudotuberculosis YopM is critical for interacting with RSK1 and for virulence. Infect Immun. 2010; 78:2584–98. [PubMed: 20368345]

MCDONALD C, VACRATSIS PO, BLISKA JB, DIXON JE. The yersinia virulence factor YopM forms a novel protein complex with two cellular kinases. J Biol Chem. 2003; 278:18514–23. [PubMed: 12626518]

MCPHEE JB, MENA P, BLISKA JB. Delineation of regions of the Yersinia YopM protein required for interaction with the RSK1 and PRK2 host kinases and their requirement for interleukin-10 production and virulence. Infect Immun. 2010; 78:3529–39. [PubMed: 20515922]

MCPHEE JB, MENA P, ZHANG Y, BLISKA JB. Interleukin-10 induction is an important virulence function of the Yersinia pseudotuberculosis type III effector YopM. Infect Immun. 2012; 80:2519–27. [PubMed: 22547545]

MIAO EA, SCHERER CA, TSOLIS RM, KINGSLEY RA, ADAMS LG, BAUMLER AJ, MILLER SI. Salmonella typhimurium leucine-rich repeat proteins are targeted to the SPI1 and SPI2 type III secretion systems. Mol Microbiol. 1999; 34:850–64. [PubMed: 10564523]

NEMETH J, STRALEY SC. Effect of Yersinia pestis YopM on experimental plague. Infect Immun. 1997; 65:924–30. [PubMed: 9038298]

PALMER RH, RIDDEN J, PARKER PJ. Cloning and expression patterns of two members of a novel protein-kinase-C-related kinase family. Eur J Biochem. 1995; 227:344–51. [PubMed: 7851406]

PARK YH, WOOD G, KASTNER DL, CHAE JJ. Pyrin inflammasome activation and RhoA signaling in the autoinflammatory diseases FMF and HIDS. Nat Immunol. 2016

PUJOL C, BLISKA JB. The ability to replicate in macrophages is conserved between Yersinia pestis and Yersinia pseudotuberculosis. Infect Immun. 2003; 71:5892–9. [PubMed: 14500510]

RUTER C, BUSS C, SCHARNERT J, HEUSIPP G, SCHMIDT MA. A newly identified bacterial cell-penetrating peptide that reduces the transcription of pro-inflammatory cytokines. J Cell Sci. 2010; 123:2190–8. [PubMed: 20554895]

SAPKOTA GP, CUMMINGS L, NEWELL FS, ARMSTRONG C, BAIN J, FRODIN M, GRAUERT M, HOFFMANN M, SCHNAPP G, STEEGMAIER M, COHEN P, ALESSI DR. BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms in vitro and in vivo. Biochem J. 2007; 401:29–38. [PubMed: 17040210]

SCHARNERT J, GREUNE L, ZEUSCHNER D, LUBOS ML, ALEXANDER SCHMIDT M, RUTER C. Autonomous translocation and intracellular trafficking of the cell-penetrating and immune-suppressive effector protein YopM. Cell Mol Life Sci. 2013; 70:4809–23. [PubMed: 23835836]

SCHRODER K, TSCHOPP J. The inflammasomes. Cell. 2010; 140:821–32. [PubMed: 20303873]

SHAO F, MERRITT PM, BAO Z, INNES RW, DIXON JE. A Yersinia effector and a Pseudomonas avirulence protein define a family of cysteine proteases functioning in bacterial pathogenesis. Cell. 2002; 109:575–88. [PubMed: 12062101]

SHAO F, VACRATSIS PO, BAO Z, BOWERS KE, FIERKE CA, DIXON JE. Biochemical characterization of the Yersinia YopT protease: cleavage site and recognition elements in Rho GTPases. Proc Natl Acad Sci U S A. 2003; 100:904–9. [PubMed: 12538863]

STASULLI NM, EICHELBERGER KR, PRICE PA, PECHOUS RD, MONTGOMERY SA, PARKER JS, GOLDMAN WE. Spatially distinct neutrophil responses within the inflammatory lesions of pneumonic plague. MBio. 2015; 6:e01530–15. [PubMed: 26463167]

SUTTERWALA FS, OGURA Y, SZCZEPANIK M, LARA-TEJERO M, LICHTENBERGER GS, GRANT EP, BERTIN J, COYLE AJ, GALAN JE, ASKENASE PW, FLAVELL RA. Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. Immunity. 2006; 24:317–27. [PubMed: 16546100]

THORNBERRY NA, BULL HG, CALAYCAY JR, CHAPMAN KT, HOWARD AD, KOSTURA MJ, MILLER DK, MOLINEAUX SM, WEIDNER JR, AUNINS J, et al. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature. 1992; 356:768–74. [PubMed: 1574116]

TOUITOU I, LESAGE S, MCDERMOTT M, CUISSET L, HOFFMAN H, DODE C, SHOHAM N, AGANNA E, HUGOT JP, WISE C, WATERHAM H, PUGNERE D, DEMAILLE J,

SARRAUSTE DE MENTHIERE C. Infevers: an evolving mutation database for auto-inflammatory syndromes. Hum Mutat. 2004; 24:194–8. [PubMed: 15300846]

VENKATESAN MM, BUYSSE JM, HARTMAN AB. Sequence variation in two ipaH genes of Shigella flexneri 5 and homology to the LRG-like family of proteins. Mol Microbiol. 1991; 5:2435–45. [PubMed: 1791758]

VIBOUD GI, BLISKA JB. Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu Rev Microbiol. 2005; 59:69–89. [PubMed: 15847602]

XU H, YANG J, GAO W, LI L, LI P, ZHANG L, GONG YN, PENG X, XI JJ, CHEN S, WANG F, SHAO F. Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome. Nature. 2014; 513:237–41. [PubMed: 24919149]

ZONG H, RAMAN N, MICKELSON-YOUNG LA, ATKINSON SJ, QUILLIAM LA. Loop 6 of RhoA confers specificity for effector binding, stress fiber formation, and cellular transformation. J Biol Chem. 1999; 274:4551–60. [PubMed: 9988689]

ZWACK EE, SNYDER AG, WYNOSKY-DOLFI MA, RUTHEL G, PHILIP NH, MARKETON MM, FRANCIS MS, BLISKA JB, BRODSKY IE. Inflammasome activation in response to the Yersinia type III secretion system requires hyperinjection of translocon proteins YopB and YopD. MBio. 2015; 6:e02095–14. [PubMed: 25691590]

Highlights

- Inactivation of RhoA by *Yersinia* effectors YopE and YopT triggers the pyrin inflammasome
- Pyrin activation is blocked by YopM, an effector that binds to RSK and PRK kinases
- YopM hijacks PRKs, which regulate pyrin by phosphorylation of 14-3-3 binding sites
- Inhibition of the pyrin inflammasome by YopM is essential for *Yersinia* virulence

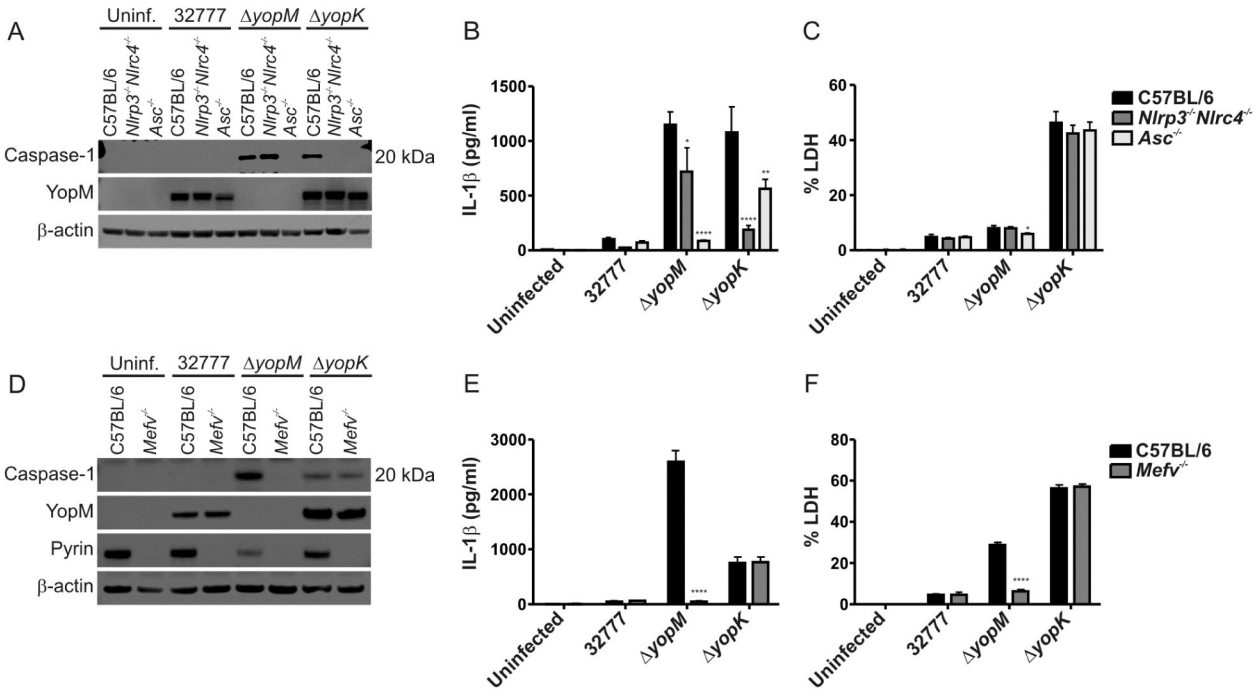

Figure 1. YopM inhibits activation of the pyrin inflammasome

LPS-primed wild-type or knock-out bone marrow-derived macrophages (BMDMs) were infected with the indicated *Y. pseudotuberculosis* strains for 90 min at a multiplicity of infection (MOI) of 30. **(A,D)** Caspase-1 processing in infected lysates was determined by Western blot (WB) analysis. **(B,E)** Secreted interleukin (IL)-1β was measured by ELISA and **(C,F)** cell-death was quantified by lactate dehydrogenase (LDH) release. Data in **(B–C)** and **(E–F)** represent average values ± SEM from three independent experiments compared to *yopM* mutant-infected BMDMs as analyzed by two- or one-way ANOVA, respectively. *, $P < 0.05$; ** $P < 0.01$; ****, $P < 0.0001$. See also Figure S1 and Figure S2.

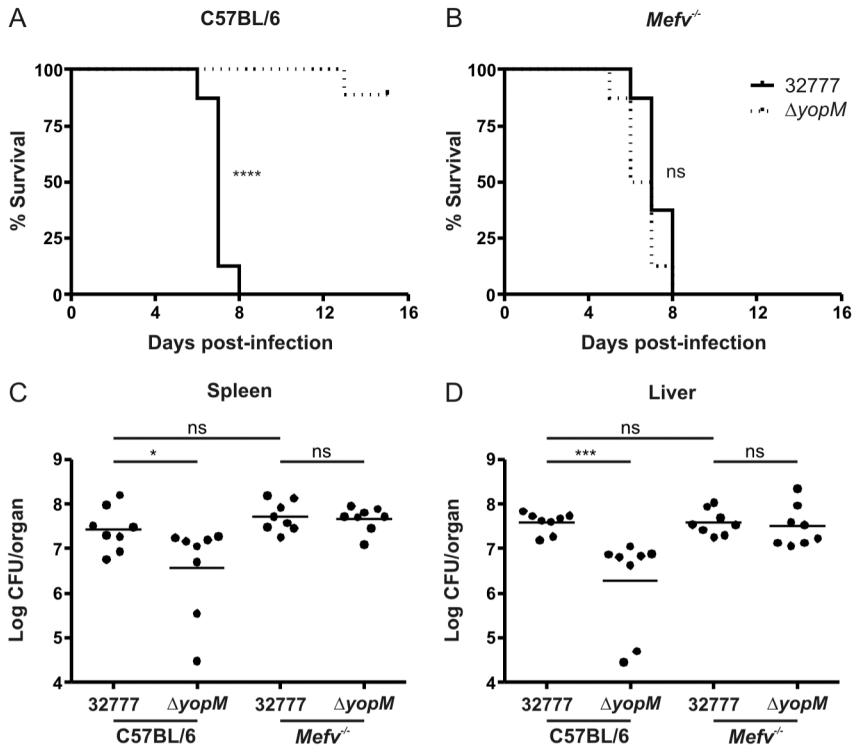

Figure 2. YopM inhibits pyrin to counteract host protection to *Yersinia*

12–16 week old C57BL/6 or *Mefv*$^{-/-}$ mice were infected intravenously by tail-vein injection with approximately 2000 CFU of wild-type *Y. pseudotuberculosis* or a *yopM* mutant. **(A–B)** Time-to-death of infected mice was monitored for 21 days. Results are plotted to day 15 and pooled from two independent experiments with groups of four to five mice (n = 8–9). **(C–D)** Enumeration of bacterial burdens in the spleens and livers of infected mice at 5 days post-infection by CFU assays. Results are pooled from two independent experiments with groups of four mice (n = 8). Results in **(A–B)** were analyzed using the log-rank test while data in **(C–D)** were analyzed by Mann-Whitney test. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

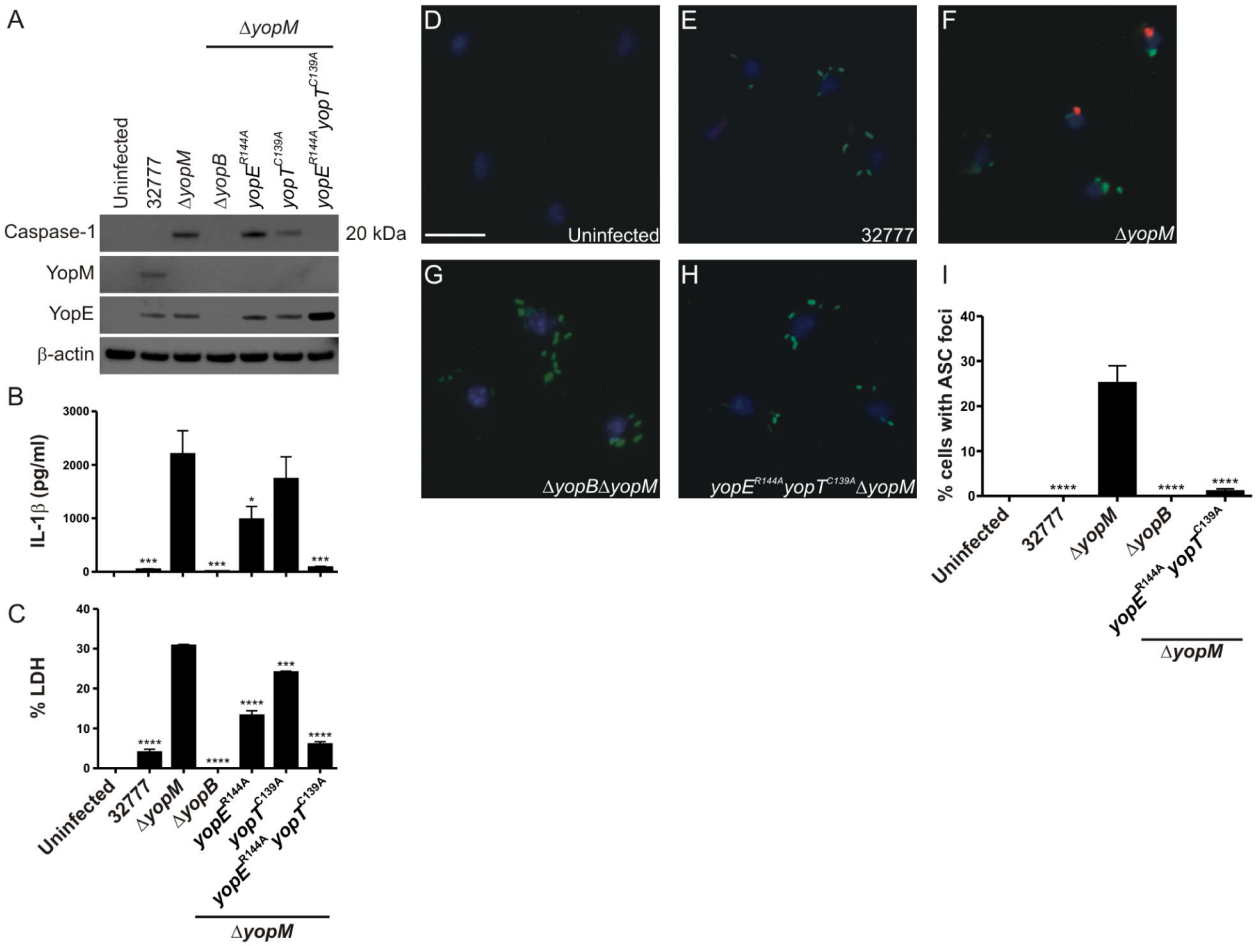

Figure 3. The catalytic activities of YopE and YopT stimulate the pyrin inflammasome

LPS-primed BMDMs were infected with the indicated *Y. pseudotuberculosis* strains for 90 min at an MOI of 30. **(A)** Caspase-1 processing in infected lysates was determined by WB analysis. **(B)** Secreted IL-1β and **(C)** cytotoxicity was quantified by ELISA or LDH release, respectively. **(D–H)** Fluorescence imaging of *Yersinia*-infected BMDMs. ASC, red; *Yersinia*, green; nuceli, blue. Scale bar represents 10 μm. **(I)** Quantification of BMDMs containing ASC foci from images in (**D–H**). Data in **(B–C)** and **(I)** represent average values ± SEM from three independent experiments compared to *yopM* mutant-infected BMDMs as analyzed by one-way ANOVA. *, $P < 0.05$; *** $P < 0.001$; ****, $P < 0.0001$. See also Figure S3.

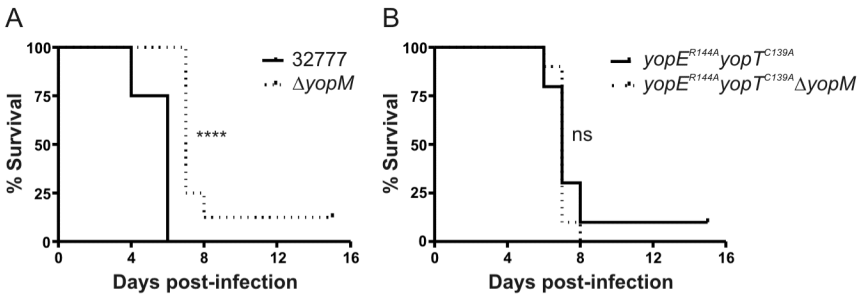

Figure 4. The catalytic activities of YopE and YopT drive responses that attenuate a *Yersinia yopM* mutant *in vivo*

C57BL/6 mice were infected intravenously via tail vein injection with ~5,000 CFU of the indicated *Y. pseudotuberculosis* strains and time-to-death was monitored for 21 days. Results are plotted to day 15 and pooled from two independent experiments with four to six mice per group (n = 8–10). Survival curves were analyzed using the log-rank test. ****, $P <$ 0.0001. See also Figure S4.

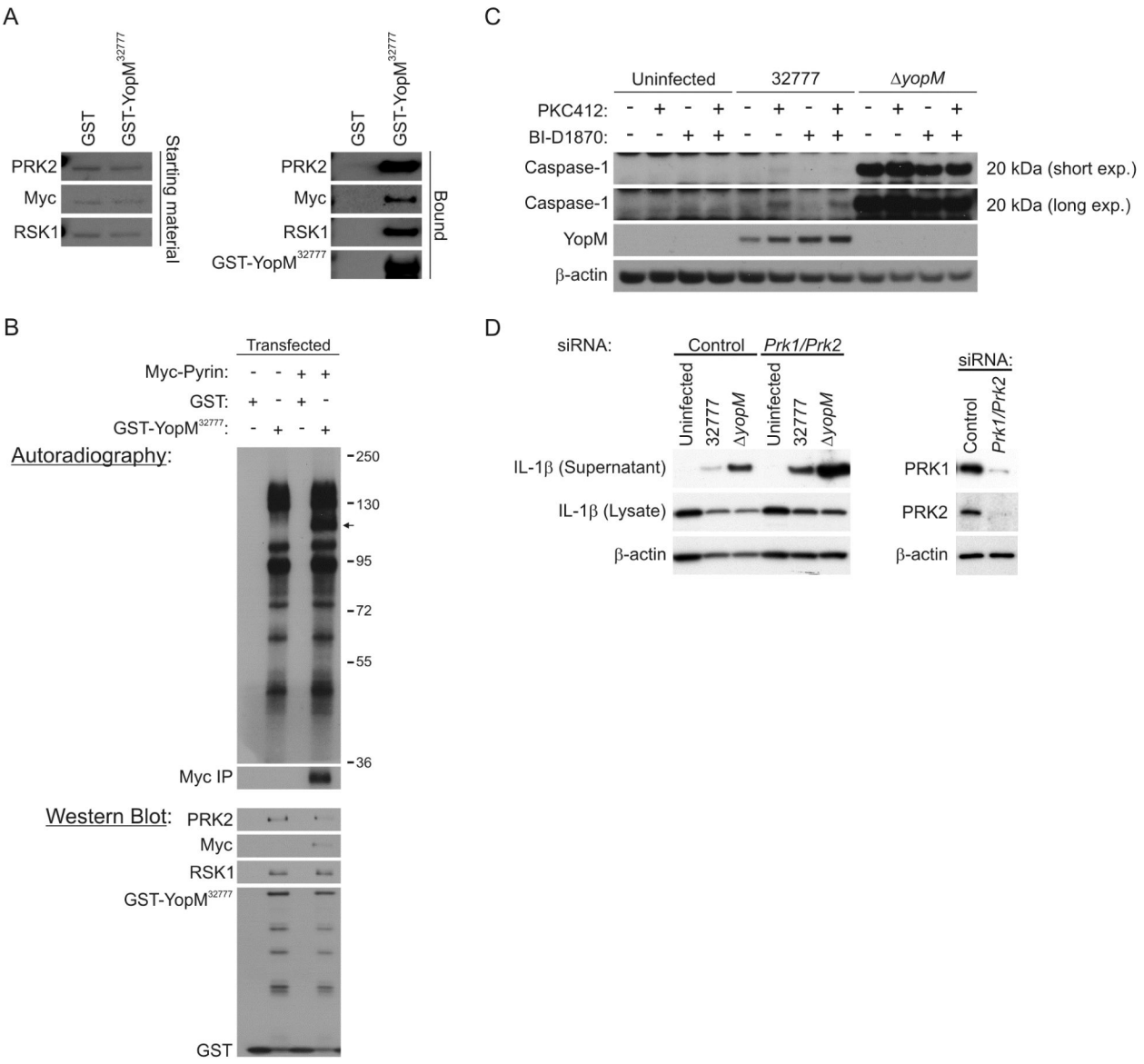

Figure 5. YopM associates with host protein kinases and hijacks PRK to negatively regulate pyrin by phosphorylation

(A) WB analysis was performed to detect host proteins that bound to GST or GST-YopM^{32777} in lysates (starting material) from HEK293T cells transfected to express Myc-tagged murine pyrin. **(B)** *In vitro* kinase assays were performed with proteins bound to GST or GST-YopM^{32777} in lysates of HEK293T cells that were transfected to express Myc-pyrin. Reactions performed in the presence of [γ-^{32}P]ATP were directly loaded or immunoprecipitated using an anti-Myc antibody prior to analysis by SDS-PAGE and autoradiography. Parallel kinase reactions were performed using cold ATP and analyzed by WB analysis to verify presence of PRK2, pyrin (Myc), RSK1 and YopM. Arrowhead indicates phosphorylated Myc-pyrin. **(C)** LPS-primed BMDMs were infected with the indicated *Y. pseudotuberculosis* strains for 90 min at an MOI of 30 in the presence or absence of PRK (PKC412) or RSK (BI-D1870) inhibitors and caspase-1 processing in

infected lysates was detected by WB analysis. **(D)** WB analysis of IL-1β in the supernatants and lysates of *Yersinia*-infected LPS-primed BMDMs that were transiently transfected with control siRNAs or siRNAs targeting PRK1 and PRK2 prior to infection. See also Figure S5.

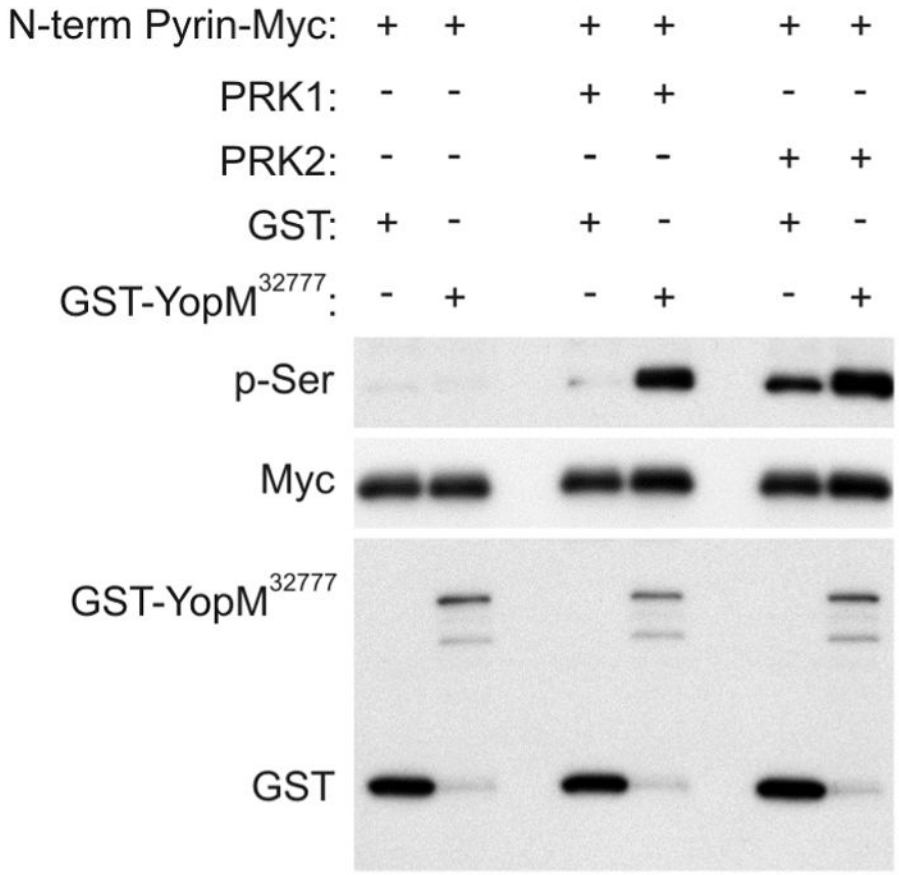

Figure 6. YopM increases PRK-mediated pyrin phosphorylation

Recombinant Myc-tagged N-terminal pyrin (amino acids 1–330) was incubated with either purified PRK1 or PRK2 and recombinant GST or GST-YopM^{32777}, after which phosphorylation of pyrin was assessed by WB analysis for phosphorylated serine.