Recruitment of TBK1 to cytosol-invading Salmonella induces WIPI2-dependent antibacterial autophagy

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Abstract

Mammalian cells deploy autophagy to defend their cytosol against bacterial invaders. Anti-bacterial autophagy relies on the core autophagy machinery, cargo receptors, and “eat-me” signals such as galectin-8 and ubiquitin that label bacteria as autophagy cargo. Anti-bacterial autophagy also requires the kinase TBK1, whose role in autophagy has remained enigmatic. Here we show that recruitment of WIPI2, itself essential for anti-bacterial autophagy, is dependent on the localization of catalytically active TBK1 to the vicinity of cytosolic bacteria. Experimental manipulation of TBK1 recruitment revealed that engagement of TBK1 with any of a variety of Salmonella-associated “eat-me” signals, including host-derived glycans and K48- and K63-linked ubiquitin chains, suffices to restrict bacterial proliferation. Promiscuity in recruiting TBK1 via independent signals may buffer TBK1 functionality from potential bacterial antagonism and thus be of evolutionary advantage to the host.

Keywords anti-bacterial autophagy; PI(3)P; Salmonella; TBK1; WIPI

Introduction

Anti-bacterial autophagy provides potent cell-autonomous immunity against bacterial attempts to colonize the cytosol of mammalian cells (Kuballa et al., 2012; Deretic et al., 2013; Randow et al., 2013). The defense of the gut epithelium against bacteria in particular is crucially dependent on anti-bacterial autophagy, since mice lacking the essential autophagy gene Atg5 in enterocytes suffer from tissue invasion by commensal bacteria and from increased pathology upon infection with Salmonella enterica serovar Typhimurium (S. Typhimurium), a specialized enteropathogen (Benjamin et al., 2013).

Macro-autophagy, hereafter autophagy, is an evolutionarily conserved quality control and degradation pathway that engulfs cytosolic content into double-membrane vesicles called autophagosomes. Autophagosome biogenesis requires the concerted activity of about 15 core AuTophaGy genes (ATGs), among them the VPS34 lipid kinase complex (Mizushima et al., 2011). VPS34 produces membrane patches rich in phosphatidylinositol 3-phosphate (PI(3)P) that recruit PI(3)P-binding proteins such as WIPI and DFCP1 to the site of phagophore formation (Axe et al., 2008). In contrast to the non-selective engulfment of cytosol into starvation-induced autophagosomes, anti-bacterial autophagy is mediated by cargo receptors including NDP52, optineurin, and p62 (Thurston et al., 2009; Zheng et al., 2009; Wild et al., 2011). Cargo receptors bind members of the LC3/GABARAP family of ubiquitin-like proteins on the autophagosomal membrane and specific “eat-me” signals associated with cytosol-invading bacteria, thereby selectively tethering bacteria to phagophore membranes (Weidberg et al., 2011; Rogov et al., 2014).

Salmonella enterica serovar Typhimurium reaches the cytosol from a vesicular compartment, the Salmonella-containing vacuole (SCV). Damage to the limiting membrane of the SCV during bacterial escape exposes host glycans otherwise hidden inside the vacuole as ligands for a family of cytosolic lectins, the galectins (Dupont et al., 2009; Paz et al., 2010). By binding the cargo receptor NDP52, galectin-8 provides an “eat-me” signal for anti-bacterial autophagy (Thurston et al., 2012). The dense layer of poly-ubiquitylated proteins that accumulates on cytosol-exposed S. Typhimurium serves as an alternative “eat-me” signal, which is sensed by multiple cargo receptors, namely NDP52, optineurin, and p62 (Perrin et al., 2004; Thurston et al., 2009; Zheng et al., 2009; Wild et al., 2011). Failure of “eat-me” signals to associate with cytosolic bacteria or interference with cargo receptor function prevents efficient anti-bacterial autophagy and allows hyper-proliferation of cytosolic S. Typhimurium (Boyle & Randow, 2013).
Restricting the proliferation of S. Typhimurium also requires the kinase TBK1, a member of the IKK (inhibitor of nuclear factor κB kinase) family (Radtke et al., 2007; Thurston et al., 2009). The antibacterial function of TBK1 is distinct from its well-characterized role of inducing type I interferons by phosphorylating IRF3 in virally infected cells (Randow et al., 2013; Wu & Chen, 2014). TBK1 accumulates in the vicinity of cytosol-exposed bacteria together with its adaptors proteins Nap1, Sintbad, and their binding partner NDP52 (Fujita et al., 2003; Ryzhakov & Randow, 2007; Thurston et al., 2009; Verlhac et al., 2015). TBK1 also associates with optineurin and it has been reported to phosphorylate both optineurin and p62, thereby enhancing their affinity for LC3B and ubiquitin, respectively (Morton et al., 2008; Wild et al., 2011; Pilli et al., 2012; Heo et al., 2015; Richter et al., 2016). While these findings imply that TBK1 strengthens the tethering function of cargo receptors, TBK1 has also been suggested to promote autophagosome maturation (Pilli et al., 2012).

Here we show that in order to restrict Salmonella proliferation TBK1 activity is required in the proximity of cytosolic bacteria for the recruitment of WIPI2, a PI(3)P-binding upstream autophagy component itself essential for anti-bacterial autophagy. To investigate the recruitment requirements for TBK1 in restricting bacterial proliferation, we deployed a TBK1 variant unable to bind any of its known adaptors. Recruitment of TBK1 to S. Typhimurium via any of several eat-me signals, including galectin-8 and K48- or K63-linked ubiquitin, is sufficient to provide TBK1 functionality for antibacterial autophagy, suggesting that robust and promiscuous recruitment of TBK1 to cytosol-invading bacteria may be beneficial in thwarting potential bacterial evasion attempts.

Results

The autophagic capture of Salmonella requires enzymatically active TBK1 in the bacterial vicinity

TBK1 is essential for anti-bacterial autophagy but its precise function in the pathway, as well as its mode of activation, remain poorly understood. TBK1 comprises an N-terminal kinase domain, a ubiquitin-like domain, and two C-terminal coiled-coils. To explore the role of TBK1 in antagonizing S. Typhimurium replication inside host cells we utilized TBK1 knockout mouse embryonic fibroblasts (MEFs). We confirmed previous findings of unrestricted proliferation of S. Typhimurium in Tbkl−/− MEFs, a phenotype complemented with wild-type but not catalytically inactive TBK1K38M (Figs 1A and EV1A) (Pomerantz & Baltimore, 1999; Radtke et al., 2007). We have previously shown that TBK1 physically associates with those intracellular Salmonella that are positive for the TBK1 adaptor proteins Nap1 and Sintbad and the autophagy cargo receptor NDP52 (Thurston et al., 2009). To test whether the function of TBK1 in anti-bacterial autophagy requires interactions with its adaptors proteins we truncated TBK1 at its C-terminus (TBK1ΔC, hereafter referred to as TBK1ΔC), thereby generating a molecule deficient in binding to all its known adaptors, that is Nap1, Sintbad, Tank, and optineurin (Fig EV1B) (Goncalves et al., 2011), while maintaining kinase activity as indicated by the activation of an ISRE reporter (Fig EV1C). Complementation of Tbk1−/− MEFs with TBK1ΔC failed to restrict proliferation of S. Typhimurium (Figs 1A and EV1A). The double mutant lacking catalytic activity and adaptor binding had a phenotype no more severe than either single mutant in the Salmonella assay (Figs 1A and EV1A). We therefore conclude that the catalytic activity of TBK1 and its ability to bind adaptor proteins are equally important to protect cells against S. Typhimurium, most
likely because adaptor binding controls TBK1 spatially and/or temporally.

Precisely how TBK1 restricts bacterial proliferation is controversial; the enhanced bacterial load in TBK1-deficient cells has been suggested to be caused by either the cells’ inability to maintain SCV integrity (Radtke et al, 2007) or the cells’ inability to execute antibacterial autophagy (Thurston et al, 2009). The TBK1 inhibitor MRT68843, which inhibits poly(I:C)-induced ISRE reporter activity similar to the related TBK1 inhibitor MRT68601 (Newman et al, 2012) (Fig EV1D), was used to analyze the relationship between TBK1 activity and autophagy. As expected, Atg5<sup>−/−</sup> cells failed to suppress proliferation of S. Typhimurium (Fig 1B). Addition of MRT68843 increased bacterial replication only 1.5-fold in Atg5<sup>−/−</sup> MEFs but more than eightfold in cells complemented with ATG5, consistent with TBK1 controlling antibacterial autophagy due to its kinase activity. To substantiate this finding, we next investigated where in the anti-bacterial autophagy pathway TBK1 acts. By complementing Tbk1<sup>−/−</sup> MEFs, we confirmed that lack of TBK1 increased the percentage of ubiquitin-coated cytosolic S. Typhimurium at 4 h post-infection (Fig 1C) (Radtke et al, 2007; Thurston et al, 2009). TBK1<sub>K38M</sub> and TBK1<sub>AC</sub>, which are catalytically inactive and deficient in binding adaptor proteins, respectively, did not complement the ubiquitin phenotype, in line with the lack of these alleles to control proliferation of S. Typhimurium in the cytosol of host cells (Fig 1A and C).

The recruitment of WIPI2, itself essential for anti-bacterial autophagy, is controlled by TBK1

The anti-bacterial autophagy attack can be visualized by assessing the association of S. Typhimurium with LC3B, a mammalian Atg8 ortholog. However, complementation of Tbk1<sup>−/−</sup> MEFs with wild-type TBK1 did not significantly alter the percentage of GFP:LC3B-posS. Typhimurium at 1 h post-infection, nor did complementation with TBK1<sub>AC</sub> or TBK1<sub>K38M</sub> (Fig 2A). Such apparently normal recruitment of LC3B to S. Typhimurium in cells failing to restrict bacterial proliferation (Fig 1A and C) may be due to conjugation of LC3 to the remnants of SCV membranes rather than anti-bacterial phagophores, a phenotype well-documented for MEFs with defects in upstream autophagy components such as FIP200 or ATG9 (Kageyama et al, 2011). The phenotype in Tbk1<sup>−/−</sup> MEFs therefore points to an upstream defect in the autophagy pathway.

Phagophore formation requires the PI3 kinase VPS34 to generate PI(3)P as a recruitment signal for WIPI proteins, the mammalian ortholog. However, complementation of Tbk1<sub>K38M</sub> or Tbk1<sub>AC</sub> with defects in upstream autophagy components such as FIP200 or ATG9 did not complement the ubiquitin phenotype, in line with the lack of these alleles to control proliferation of S. Typhimurium (Kageyama et al, 2011), we tested whether TBK1 was similarly required for the recruitment of WIPI proteins to bacteria. We found that GFP-tagged WIPI1 and WIPI2B but not WIPI3 and WIPI4 accumulated on S. Typhimurium, as did endogenous WIPI2 (Figs 2B and C, and EV2A and B). The recruitment of WIPI1 and WIPI2B was sensitive to wortmannin treatment and abrogated by mutations in their PI(3)P-binding sites (GFP:WIPI1<sub>F70C</sub> and GFP:WIPI2B<sub>E545C</sub>). Importantly, accumulation of WIPI1 and WIPI2B also required expression of wild-type TBK1 and was not supported by either catalytically inactive TBK1<sub>K38M</sub> or TBK1<sub>AC</sub> deficient in binding adaptor proteins (Fig 2B). In contrast, recruitment of DFCP1 did not require TBK1, although it was also sensitive to wortmannin treatment and mutational inactivation of its PI(3)P-binding site (DFCP1<sub>F797E</sub>) (Fig 2D–F). WIPI proteins also bind PI(3,5)P<sub>2</sub> (Baskaran et al, 2012). However, the accumulation of PI(3,5)P<sub>2</sub> on S. Typhimurium was independent of TBK1, as revealed by the normal recruitment of GFP:MLIN<sub>2</sub>*<sub>2</sub>, a PI(3,5)P<sub>2</sub>-specific probe (Li et al, 2013b) (Fig 2G and H). We therefore conclude that TBK1 and VPS34 independently control the recruitment of WIPI1 and WIPI2 to S. Typhimurium and that TBK1 functionality requires catalytic activity as well as its C-terminal adaptor-binding coiled-coil domain.

To further investigate the mechanism of how TBK1 recruits WIPI1 and WIPI2 to cytosol-invading bacteria, we depleted cells of optineurin, the only known TBK1 substrate in anti-bacterial autophagy (Wild et al, 2011). Cells lacking optineurin recruited WIPI1 and WIPI2B normally to S. Typhimurium, suggesting that phosphorylation of a substrate other than optineurin is essential for WIPI1/2 recruitment in anti-bacterial autophagy (Fig EV2C). We also tested the interdependence of WIPI1 and WIPI2B recruitment and found that neither protein was required for the recruitment of the other (Fig EV2D).

We next investigated whether WIPIs are essential to protect cells against bacterial proliferation. Cells lacking WIPI2 failed to restrict proliferation of S. Typhimurium, confirming a recent finding (Dooley et al, 2014), while the presence of WIPI1 was not required (Fig 3). We therefore conclude that the recruitment of WIPI2 to cytosol-invading bacteria is likely an essential function of TBK1 in cell-autonomous defense.

NDP52-mediated recruitment of TBK1 to S. Typhimurium suffices to restrict bacterial proliferation

Since the C-terminal domain of TBK1 is required to restrict bacterial proliferation and mediates adaptor binding (Fig EV1A) we thought to repair TBK1<sub>AC</sub> by fusing it directly to individual adaptor proteins. This strategy enables the evaluation of individual adaptors in the TBK1-mediated restriction of S. Typhimurium and structure–function analyses without interference from potentially redundant adaptor function. As cytosol-exposed S. Typhimurium recruit Nap1 but not Tank (Thurston et al, 2009), we compared these two adaptors by fusing them to TBK1<sub>AC</sub>. Consistent with their differential recruitment to cytosolic Salmonella, TBK1<sub>AC</sub>–Tank did not restrict Salmonella proliferation in Tbk1<sup>−/−</sup> MEFs; in contrast, TBK1<sub>AC</sub>–
Figure 2.

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Nap1 reduced bacterial replication as efficiently as full-length TBK1 (Figs 4A and EV3A). Complementation of Tbk1<sup>−/−</sup> MEFs with TBK1<sub>AC</sub>-Nap1 but not with TBK1<sub>AC</sub>-Tank also restored localization of GFP-WIPI1 to Salmonella (Fig 4B).

How does Nap1 contribute to TBK1 function? Considering the overall structural similarity between Nap1 and Tank (Fig 4C) (Ryzhakov & Randow, 2007), efficient complementation with TBK1<sub>AC</sub>-Nap1 but not TBK1<sub>AC</sub>-Tank suggested a role for the N-terminal coiled-coil region of Nap1. Indeed, TBK1<sub>AC</sub>-Nap1<sub>N85</sub>, in contrast to TBK1<sub>AC</sub>-Nap1, did not prevent hyper-proliferation of S. Typhimurium in Tbk1<sup>−/−</sup> MEFs (Figs 4A and EV3A). Complementation with TBK1<sub>AC</sub>-Nap1<sub>N85</sub> also restricted bacterial proliferation; the N-terminal 85 residues of Nap1 are therefore required and sufficient to provide functionality to TBK1<sub>AC</sub>. Nap1<sub>N85</sub> forms a coiled-coil that contributes to the dimerization of TBK1, which is abrogated in NDP2<sub>L374A</sub> (Kim et al., 2013; Li et al., 2013a). NDP2 binds ubiquitin via its C-terminal zinc finger and structural information on the interaction has been recently published (Xie et al., 2015). We also determined the solution structure of the C-terminal ubiquitin-binding zinc finger of NDP2 by NMR spectroscopy, which confirmed the existence of an α-helix and a two-stranded β-sheet (Fig 5A) (Xie et al., 2015). The zinc ion is coordinated by residues His<sub>446</sub> and His<sub>447</sub> as well as residues Cys<sub>422</sub> and Cys<sub>425</sub>, which are located in the helix and in the loop connecting the two β-strands, respectively. This fold is found in several ubiquitin-binding proteins with the NDP2 structure most similar to the C-terminal ubiquitin-binding Zn fingers of Nemo and optineurin (Fig 5B). Addition of mono-ubiquitin to the NDP2 zinc finger produced changes in the chemical shift primarily of residues in the helix (Fig 5C and Appendix Fig S1). Analysis of the chemical shift changes as a function of ubiquitin concentration revealed a dissociation constant of 60 μM (Appendix Fig S2). Perturbed ubiquitin residues upon binding to NDP2 are centered on the so-called Ile44 patch in ubiquitin, a common binding site for UBDs (Fig 5B). These data suggest that the NDP2 zinc finger interacts with ubiquitin similar to other UBD domains, that is via binding of the exposed face of the helix to the hydrophobic Ile44 patch on ubiquitin. Consistent with such a binding mode and a crystallographic analysis of the NDP2 zinc finger bound to ubiquitin (Xie et al., 2015), residues on the exposed face of the helix are highly conserved among NDP2 orthologs (Fig 5D). To test our model, we mutated Asp<sub>439</sub>, a conserved residue with a large ubiquitin-induced chemical shift change located on the exposed helix surface (Appendix Fig S3). NDP2<sub>D439K</sub> failed to bind ubiquitin (GST-4xUB) similar to NDP2<sub>D439K</sub> (Xie et al., 2015), while the interaction with GST-galectin-8 was maintained (Fig 5E). In contrast, NDP2<sub>L374A</sub> bound GST-4xUb but not GST-galectin-8. As expected, the double-mutant NDP2<sub>L374A</sub>/D<sub>439K</sub> did not bind to either ligand. We used the NDP2 mutants specifically deficient in binding to galectin-8 or ubiquitin to test (i) whether the recruitment of TBK1 to cytosol-invading Salmonella via bacteria-associated danger signals is essential to restrict bacterial growth and (ii) whether recruitment via both galectin-8 and ubiquitin is required. Bacterial proliferation in Tbk1<sup>−/−</sup> MEFs was potently restricted by both TBK1<sub>AC</sub>-NDP2<sub>D439K</sub> and TBK1<sub>AC</sub>-NDP2<sub>L374A</sub>, which selectively bind galectin-8 or ubiquitin, respectively (Figs 5F and EV3B). In contrast, TBK1<sub>AC</sub>-NDP2<sub>L374A</sub> + D<sub>439K</sub>, which binds neither galectin-8 nor ubiquitin,
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was inactive in this assay. We therefore conclude that the recruitment of TBK1 to cytosol-invading Salmonella via galectin-8 or the bacterial ubiquitin coat is essential to restrict bacterial proliferation and that either signal, that is galectin-8 or the ubiquitin coat, suffices in recruiting TBK1. To directly test this prediction for Salmonella, we fused ubiquitin-binding domains to TBK1AC. The ubiquitin coat of S. Typhimurium contains different linkage types; at minimum, M1- and K63-linked chains are present but their functional contribution to antibacterial autophagy remains unknown (van Wijk et al., 2012). NDP52 can bind M1-, K48-, and K63-linked ubiquitin chains (Wild et al., 2011) with similar affinities for di-ubiquitin molecules and tetra-ubiquitin chains that are M1-linked (Xie et al., 2013). To investigate whether NDP52 binds longer chains in a linkage-specific manner, we exposed NDP52 to an equimolar mixture of M1-, K48-, and K63-linked ubiquitin tetramers (Fig 7A). Control proteins of known linkage specificity (Trempe et al., 2005; Kulathu et al., 2009; Rahighi et al., 2009b) precipitated their ligands selectively; the UBAN domain of Nemo bound M1-linked chains, the NZF domain of TAB2 K63-linked chains, and the UBA domain of Mudi1 K48-linked chains. In contrast, NDP52 bound equally well to all three ubiquitin chains, suggesting lack of linkage specificity for NDP52, at least among the tested linkages types.

To directly test the importance of individual ubiquitin-linkage types in recruiting TBK1 to the ubiquitin coat of cytosolic

Figure 4. Recruitment of TBK1 to Salmonella enterica serovar Typhimurium via NAP1 or NDP52, but not TANK, restricts bacterial proliferation and recruits WIPI1.

A–D Analysis of Tbk1 ΔC MEfs complemented with the indicated Flag-tagged Tbk1ΔC–adaptor fusion proteins. S. Typhimurium replication kinetics (A, D). Infected cells were lysed at the indicated time points post-inoculation (p.i.), and bacteria were enumerated by their ability to form colonies on agar plates. Mean and SD of triplicate MEF cultures and duplicate colony counts. Data are representative of at least two repeats. Statistical significance to Con was determined using one-way ANOVA with Dunnett’s multiple comparisons test. (B) Percentage of GFP:WIPI1-positive S. Typhimurium at 1 h p.i. in the indicated complemented MEFs. Mean and SEM of four independent experiments. >200 bacteria counted per coverslip. *P < 0.05, ***P < 0.001, one-way ANOVA with Dunnett’s multiple comparisons test. (C) Tbk1ΔC-NAP1–NDP52 complex. N- and C-termini, domains and binding partners are indicated.

was inactive in this assay. We therefore conclude that the recruitment of TBK1 to cytosol-invading Salmonella via galectin-8 or the bacterial ubiquitin coat is essential to restrict bacterial proliferation and that either signal, that is galectin-8 or the ubiquitin coat, suffices in recruiting TBK1. To directly test this prediction for Salmonella, we fused ubiquitin-binding domains to TBK1AC. The ubiquitin coat of S. Typhimurium and enabled the recruitment of GFP:WIPI1 to bacteria (Figs 6 and EV4A).

Recruitment of TBK1 to S. Typhimurium via K48- or K63-linked ubiquitin chains suffices to restrict bacterial proliferation

To test whether the bacterial ubiquitin coat also provides recruitment signals for TBK1 sufficient to protect cells against hyper-proliferation of Salmonella, we fused ubiquitin-binding domains to TBK1AC. The ubiquitin coat of S. Typhimurium contains different linkage types; at minimum, M1- and K63-linked chains are present but their functional contribution to antibacterial autophagy remains unknown (van Wijk et al., 2012). NDP52 can bind M1-, K48-, and K63-linked ubiquitin chains (Wild et al., 2011) with similar affinities for di-ubiquitin molecules and tetra-ubiquitin chains that are M1-linked (Xie et al., 2013). To investigate whether NDP52 binds longer chains in a linkage-specific manner, we exposed NDP52 to an equimolar mixture of M1-, K48-, and K63-linked ubiquitin tetramers (Fig 7A). Control proteins of known linkage specificity (Trempe et al., 2005; Kulathu et al., 2009; Rahighi et al., 2009b) precipitated their ligands selectively; the UBAN domain of Nemo bound M1-linked chains, the NZF domain of TAB2 K63-linked chains, and the UBA domain of Mudi1 K48-linked chains. In contrast, NDP52 bound equally well to all three ubiquitin chains, suggesting lack of linkage specificity for NDP52, at least among the tested linkages types.

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S. Typhimurium, we fused TBK1ΔC to ubiquitin-binding proteins of defined specificity. Complementing Tbk1Δ/C0/C0 MEFs with TBK1ΔC:Nemo restricted the proliferation of S. Typhimurium (Figs 7B and EV5A). The UBAN domain of Nemo selectively binds M1-linked ubiquitin (Rahighi et al, 2009a), while full-length Nemo engages additional chain types due to combined contributions from its UBAN
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Figure 6. Recruitment of TBK1 to Salmonella enterica serovar Typhimurium via galectin-8 restricts bacterial proliferation and recruits WIPI2.

A, B Analysis of Tbk1<sup>−/−</sup> MEFs complemented with the indicated Flag-tagged TBK1<sub>AC</sub>–galectin fusion proteins. S. Typhimurium replication kinetics (A). Infected cells were lysed at the indicated times post-inoculation (p.i.), and bacteria were enumerated by their ability to form colonies on agar plates. Mean and SD of triplicate MEF cultures and duplicate colony counts. Data are representative of at least two repeats. Statistical differences to Tbk1<sup>−/−</sup> MEFs expressing TBK1<sub>AC</sub> are shown.

Discussion

Our data establish that the recruitment of TBK1 to S. Typhimurium is essential to restrict bacterial proliferation by autophagy and that a variety of bacteria-associated "eat-me" signals, including galectin-8 and K48- and K63-linked ubiquitin chains, can mediate TBK1 recruitment. Promiscuity in recruiting TBK1 may be of evolutionary advantage to the host by providing a backup mechanism against potential bacterial interference. We find that the localization of TBK1 activity to S. Typhimurium is required to control an upstream step in anti-bacterial autophagy, namely the recruitment of the ATG18 orthologue WIPI2 to cytosol-invading bacteria.

TBK1 is essential for cell-autonomous immunity against both viral and bacterial infections. In anti-viral immunity, TBK1 acts downstream of several important receptors—Toll-like receptors, RIG-like receptors, and cGAS—to phosphorylate IRF3 (Wu & Chen, 2014). IRF3 controls expression of type I interferons and other anti-viral genes that establish the so-called anti-viral state of increased resistance against a broad spectrum of viruses. In cell-autonomous anti-bacterial immunity, TBK1 specifically protects the host cytosol independently of IRF3 (Radtke et al., 2007; Thurston et al., 2009). While originally thought to control vesicular integrity and thus access of bacteria to the cytosol (Radtke et al., 2007), the unchanged recruitment of galectins to S. Typhimurium in cells lacking TBK1 revealed in this study demonstrates that TBK1 protects cells against cytosol-invading S. Typhimurium by controlling autophagy. Consistent with TBK1 controlling anti-bacterial autophagy is the enzyme’s ability to phosphorylate functionally important sites in cargo receptors, namely the LC3-interacting region (LIR) of optineurin (Wild et al., 2011) and the ubiquitin-binding site of p62 (Pilli et al., 2012), thereby enhancing their affinity for LC3 and ubiquitin, respectively. While the spatial and temporal control of the tethering function of cargo receptors appears as an important contribution of TBK1 to anti-bacterial autophagy, we found that in Tbk1<sup>−/−</sup> MEFs, LC3 is
still recruited to S. Typhimurium. Apparently normal LC3 recruitment while failing to control the growth of cytosolic S. Typhimurium also occurs in MEFs deficient in FIP200 and ATG9, where it is caused by conjugation of LC3 to the damaged membrane of SCVs rather than to de novo phagophores attacking bacteria (Kageyama et al., 2011). Lack of TBK1, and failure to phosphorylate the LIR motif in optineurin, could result in a similarly subtle but nevertheless functionally important mislocalization of LC3.

However, irrespective of the precise membrane localization of LC3 in Tbk1−/− MEFs, the lack of WIPI2 recruitment, whose essential role in anti-bacterial autophagy we confirm (Dooley et al., 2014), provides a sufficient and more parsimonious explanation for the failure of autophagy to control bacterial proliferation. WIPI2 is recruited to bacteria via PI(3)P, which initially suggested insufficient PI(3)P in the bacterial vicinity may hinder anti-bacterial autophagy in Tbk1−/− MEFs. During autophagy PI(3)P is produced by the VPS34 lipid kinase complex and inhibition of VPS34 with wortmannin or knockout of ATG14L, a subunit of the VPS34 complex, abrogates anti-bacterial autophagy (Kageyama et al., 2011). However, a defect in PI(3)P production in Tbk1−/− MEFs can be ruled out since DFCP1, a bona fide probe for the autophagic pool of PI(3)P, is recruited normally to S. Typhimurium. A defect in PI(3,5)P2 levels (Baskaran et al., 2012), an alternative recruitment signal for WIPI2, can also be excluded since recruitment of GFP:MLIN*2, a PI(3,5)P2-specific probe, did not depend on TBK1 either. We therefore conclude that an essential function of TBK1 in anti-bacterial autophagy is to control WIPI2 recruitment to cytosol-invading Salmonella independently of PIP-related signals. We propose that WIPI2 detects the coincidence of TBK1 and VPS34-generated signals on cytosol-invading bacteria in order to direct autophagy toward cognate cargo and away from cellular structures. The requirement for both TBK1 kinase activity and TBK1 localization to S. Typhimurium for the recruitment of WIPI2 furthermore suggests that the TBK1 substrates crucial for anti-bacterial autophagy are also located in the vicinity of the invading bacterium. Further experiments will be required to reveal their identity.

Anti-bacterial autophagy targets cytosol-invading bacteria in response to several distinct “eat-me” signals (Boyle & Randow, 2013). The earliest known “eat-me” signal comprises galectin-8, which detects damage to SCV membranes by binding...
cytosol-exposed host glycans liberated by S. Typhimurium during its entry into the cytosol (Thurston et al., 2012). Galectin-8 is a selective ligand for the cargo receptor NDP52, which is physically linked to TBK1 via Nap1 and Sintbad, two homologous adaptor proteins (Ryzhakov & Randow, 2007; Thurston et al., 2009, 2012). The bacterial ubiquitin coat that develops subsequent to SCV damage comprises another “eat-me” signal (Perrin et al., 2004). Two E3 ligases, Parkin and LRSAM1, have been suggested to generate the bacterial ubiquitin coat, possibly in response to different bacterial species (Huett et al., 2012; Manzanillo et al., 2013). The ubiquitin “eat-me” signal is detected by several cargo receptors, of which optineurin and NDP52 provide independent physical links to TBK1. To overcome the redundancy in TBK1 recruitment regarding both “eat-me” signals and cargo receptors, we generated TBK1ΔC, a TBK1 allele unable to bind any of its known adaptors and thus unable to sense invading bacteria. TBK1ΔC failed to complement Tbk1−/− MEFs, suggesting that adaptor binding is essential for TBK1 function in anti-bacterial autophagy. Importantly, TBK1 functionality was reconstituted by fusing TBK1ΔC directly to NDP52 or optineurin but not to mutants deficient in binding “eat-me” signals, thus providing strong evidence that TBK1 recruitment to “eat-me” signals is essential for autophagy against cytosol-invading S. Typhimurium. Fusing TBK1ΔC to NDP52 alleles selectively deficient in ubiquitin binding or directly to galectin-8 demonstrated that glycan-driven recruitment suffices to provide TBK1 functionality for anti-bacterial autophagy. In contrast, NDP52ΔC/ΔA, a mutation that destabilizes the C-terminal zinc finger, has been reported not to support anti-bacterial autophagy because of a defect in phagosome maturation, an apparently TBK1-independent function of NDP52 (Verlhac et al., 2015).

Similarly, fusing TBK1ΔC to optineurin or NDP52 deficient in galectin-8 binding revealed that ubiquitin-driven recruitment also suffices. Why would such apparent redundancy in recruiting TBK1 to invading bacteria be needed? Glycan- and ubiquitin-encoded signals can provide functionality to TBK1 for cytosolic defense against invading bacteria at different stages of infection. Recruitment of TBK1 via galectin-8 enables autophagy to catch invading bacteria as soon as they appear in the cytosol but would fail against bacteria that thwart the attack long enough for cytosol-exposed glycans to become degraded by cytosolic glycosidases or against those bacteria that proliferate and thereby outgrow the vacuolar membrane remnants. The ubiquitin coat, on the other hand, requires time to develop but could be maintained even on moderately proliferating bacteria for long enough that ultimately anti-bacterial autophagy may succeed. We therefore suggest that redundancy in TBK1 recruitment via the galectin-8 and ubiquitin route equips anti-bacterial autophagy with sufficient robustness to efficiently combat cytosol-invading bacteria throughout the invasion process.

Additional sturdiness to TBK1 recruitment is provided by the ability of NDP52 to bind M1-, K48-, and K63-linked ubiquitin chains similarly (Xie et al., 2015; this study), which enables anti-bacterial autophagy to sense a variety of ubiquitin-derived “eat-me” signals, for example those generated by different E3 ligases on bacterial and host-derived substrates. The ubiquitin coat on S. Typhimurium comprises, at minimum, M1- and K63-linked chains (van Wijk et al., 2012). By fusing linkage-specific ubiquitin-binding domains to TBK1ΔC, we demonstrate that the ubiquitin coat on S. Typhimurium also contains K48-linked chains and that recruitment of TBK1 via K48- and K63-linked chains suffices for anti-bacterial autophagy. The inability of the UBAN domain in NemoΔZnF to recruit TBK1 implies that M1-linked ubiquitin chains may not be a sufficient signal to provide functionality to TBK1. However, we refrain from explicitly ruling out that M1-linked chains could provide such a signal since the combined effect of zinc finger and adjacent UBAN domain on Nemo’s affinity and specificity for M1 and other linkage types is insufficiently understood. Nevertheless, not all signals found on cytosol-invading S. Typhimurium are sufficient to provide functionality to TBK1ΔC, as demonstrated by the failure of NDP52 and optineurin to recruit TBK1 functionality to bacteria-associated LC3 via their LIR motifs.

Taken together, in this study, we demonstrated that recruitment of TBK1 to cytosol-invading bacteria is essential for anti-bacterial autophagy and that multiple recruitment signals provide TBK1 functionality for anti-bacterial autophagy throughout distinct stages of bacterial invasion. We suggest that such robustness in providing TBK1 functionality to anti-bacterial autophagy protects the pathway from potential bacterial antagonism and is of evolutionary advantage to the host.

Materials and Methods

Antibodies

Antibodies were from Enzo Life Science (ubiquitin FK2), Sigma (Flag M2), Abcam (WIP12, ab101985; TBK1, ab40676), Dabco (HRP-conjugated reagents), and Invitrogen (Alexa-conjugated anti-mouse and anti-goat antisera).

Bacteria

Salmonella enterica serovar Typhimurium (strain 12023) was grown overnight in LB and subcultured (1:33) in fresh LB for 3.5 h prior to infection. MEF cells in 24-well plates were infected with 5×10⁶ bacteria overnight in LB and subcultured (1:33) in fresh LB for 3.5 h prior to infection. MEF cells in 24-well plates were infected with 5×10⁶ bacteria

Cell culture

Tbk1−/− MEFs (gift from Kate Fitzgerald, University of Massachusetts), Atg5−/− MEFs (gift from Noboru Mizushima, University of Tokyo), and 293ET cells (gift from Brian Seed, Harvard University), confirmed as mycoplasma free, were grown in IMDM supplemented with 10% FCS at 37°C in 5% CO₂. MEFs expressing GFP-tagged LC3B were obtained by limiting dilution of retrovirally transduced cells. MEFs expressing GFP-tagged WIPI proteins, DFCP1, or MLN82 were transduced with retrovirus and selected in blasticidin at 5 µg/ml. To complement Tbk1−/− MEFs, cells were transduced with retrovirus and selected in puromycin at 1.5 µg/ml.
LUMIER assays

Binding assays with pairs of putative interactors, one fused to luciferase and the other fused to GST or Flag, were performed in Lumier lysis buffer (150 mM NaCl, 0.1% Triton X-100, 20 mM Tris–Cl (pH 7.4), 5% glycerol, 5 mM EDTA and protease inhibitors). GST fusion proteins were immobilized on beads before incubation with the luciferase-tagged binding partner for 2 h. For Flag-based assays, both proteins were expressed in 293ET cells and immobilized using Flag-agarose. After washing in lysis buffer, proteins were eluted with glutathione or Flag peptide in Renilla lysis buffer (Promega). Relative luciferase activity represents the ratio of activity eluted from beads and present in lysates.

Ubiquitin-binding assay

Ubiquitin chains and assay conditions have been described previously (Komander et al, 2009).

Western blot

Post-nuclear supernatants from 1 × 10^8 MEF cells or 293ET cells expressing the indicated fusion proteins were separated on 12% denaturing Bis-Tris gels. Proteins were transferred to PVDF membrane and incubated with relevant primary and secondary antibodies. Visualization following immunoblotting was performed using ECL detection reagents (Amersham Bioscience).

Microscopy

MEFs were grown on poly-L-lysine pre-treated glass cover slips prior to infection. Following infections, cells were washed twice with warm PBS and fixed in 4% paraformaldehyde in PBS for 20 min. Cells were washed twice in PBS and then quenched with PBS pH 7.4 containing 1 M glycine and 0.1% Triton X-100 for 30 min prior to blocking for 30 min in PBTB (PBS, 0.1% Triton X-100, 2% BSA). Where required, cover slips were incubated with primary antibody for 2 h, followed by secondary antibodies and DAPI (4’,6-diamidino-2-phenylindole) for 1 h in PBTB before being mounted (Vector Laboratories). Confocal images were taken with a 100×1.4 objective on a Zeiss 710 microscope.

Plasmids

MSP or closely related plasmids were used to express proteins in mammalian cells. For TBK1AC fusions (N685), genes encoding full-length or deletion constructs of murine Nemo, Nap1, Tab2, or human NDP52, galectin-1, galectin-8, optineurin or Tank were amplified by PCR and ligated BspHI to NotI, in frame with TBK1AC. Mutations were generated by PCR and verified by sequencing. Open reading frames for LC3B, DFCP1, WIP1, WIP2B, WIP3, and WIP4 were amplified from human cDNA. PI(3)P-binding mutants in WIPI (FRRG-FTTG) were introduced by mutational PCR, that is WIP1RR226/227TTT and WIP2RR224/225TTT. Plasmids encoding GFP-DFCP1 double FYVE mutant (FYVE*) and GFP-ML1N*2 have been described (Ridley et al, 2001; Li et al, 2013b).

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Further construct information</th>
<th>References</th>
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<tbody>
<tr>
<td>TBK1</td>
<td>Full-length TBK1</td>
<td></td>
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<tr>
<td>TBK1AC</td>
<td>TBK1 N685, lacks the C-terminal adaptor-binding region</td>
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<tr>
<td>TBK1K38M</td>
<td>TBK1 catalytic mutant</td>
<td>Pomerantz and Baltimore (1999)</td>
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<tr>
<td>TBK1AC K38M</td>
<td>TBK1 N685 N685 K38M</td>
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<td>TBK1AC Nap1</td>
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<tr>
<td>TBK1AC Nap1 AN85</td>
<td>TBK1 N685 fused to Nap1 AN85, Nap1 lacks the CC for binding NDP52</td>
<td>Ryzhakov and Randow (2007)</td>
</tr>
<tr>
<td>TBK1AC Nap1 N85</td>
<td>TBK1 N685 fused to N85 of Nap1</td>
<td>Ryzhakov and Randow (2007)</td>
</tr>
<tr>
<td>TBK1AC Tank</td>
<td>TBK1 N685 fused to full-length Tank</td>
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<td>Gurung (2003)</td>
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<td>TBK1AC NDP52 D439K</td>
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<td>TBK1 N685 fused to Mud1 UBA AA293–332</td>
<td>Trempe et al (2005)</td>
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</table>

RNA interference

About 5 × 10^4 MEFs were reverse-transfected with 6 pmol of siRNA (Optn siRNA, Invitrogen) or 40 pmol siRNA against WIP11 (Oligo
TBK1 induces WIPI2-dependent xenophagy

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ID$s$ MSS225230, MSS225231, and MSS225232, Invitrogen) or WIPI2 (Oligo ID$s$ MSS232888, MSS232890, and MSS232891, Invitrogen), using Lipofectamine RNAiMAX (Invitrogen). Control siRNAs were purchased from Invitrogen. siRNA transfection was repeated after 2 days, and experiments were performed 4 days after the first siRNA treatment.

siOPTN #29 5'-GAAGCUAAAUAAUCUAGCU
siOPTN #30 5'-GCCCUCGAGUAUCCGAUU

Quantitative PCR

RNA was extracted (Qiagen) from siRNA-treated MEF cells and converted into cDNA (SuperScript III reverse transcriptase kit, Invitrogen). Control siRNAs were (Oligo IDs MSS232888, MSS232890, and MSS232891, Invitrogen), or WIPI2 (IDs MSS225230, MSS225231, and MSS225232, Invitrogen) or WIPI2 (Oligo IDs MSS232888, MSS232890, and MSS232891, Invitrogen), using Lipofectamine RNAiMAX (Invitrogen). Control siRNAs were purchased from Invitrogen. siRNA transfection was repeated after 2 days, and experiments were performed 4 days after the first siRNA treatment.

siOPTN #29 5'-GAAGCUAAAUAAUCUAGCU
siOPTN #30 5'-GCCCUCGAGUAUCCGAUU

Quantitative PCR

RNA was extracted (Qiagen) from siRNA-treated MEF cells and converted into cDNA (SuperScript III reverse transcriptase kit, Invitrogen) according to the manufacturer’s protocol. SYBR Green qPCR kit (Applied Biosystems) was used to quantify gene expression using the following primer pairs:

muWirp2 5'-ATGAACTGCGAGCCGACGC-3'
and 5'-CTTGAGAGAAATCTCTCTAC-3'
muRsp9 5'-CTGACAGGGCCAAGTGACGC-3'
and 5'-TGCGCTTTGGCGATGACACA-3'

Data were normalized to Rsp9 levels in each sample after relative cDNA levels were calculated from a standard curve.

NMR

NDP52 zinc finger domain samples for NMR spectroscopy experiments were typically at 1.0 mM in 90% H2O and 10% D2O in PBS containing 10 mM 2-mercaptoethanol. All spectra were acquired with either a Bruker Advance 700 or a DRX600 spectrometer at 20°C and referenced relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for proton and carbon signals, or liquid ammonium for nitrogen. Assignments were obtained using standard NMR methods using 15C/15N-labeled and 15N-labeled samples. Distance constraints were derived from 2D NOESY spectra recorded on 1.5 mM samples with a mixing time of 100 ms. The three-dimensional structure of the domain was calculated using the standard torsion angle dynamics-simulated annealing protocol in the program CNS 1.2. Structures were accepted where no distance violation was > 0.25 Å and no dihedral angle violations > 5°. To map the binding interface between the NDP52 zinc finger and ubiquitin, a series of 1H,15N HSQC spectra of 15N-labeled zinc finger domain in the presence of increasing molar ratios of unlabeled ubiquitin were recorded; a reciprocal titration was carried out with the unlabeled zinc finger domain added to 15N-labeled ubiquitin. Dissociation constants were obtained by fitting the concentration dependence of the normalized chemical shift changes to a single site-binding model.

Accession numbers

Coordinates have been deposited under PDB code 5AAQ and 5AAZ.

Statistical analysis

Student’s t-test or one-way ANOVA with Dunnett’s multiple comparisons test were used as indicated. *P < 0.05, **P < 0.01 and ***P < 0.001.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

TLMT, KBB, BJR, MK, SB, AK, JN, AF, and SAM planned, performed, and analyzed the experiments; MA and MB performed and analyzed NMR experiments; DK provided reagents and advice; and TLMT and FR designed the overall research and wrote the manuscript.

Expanded View for this article is available online.


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