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Innate immunity kinase TAK1 phosphorylates Rab1 on a hotspot for posttranslational modifications by host and pathogen

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TGF-β activated kinase 1 (TAK1) is a critical signaling hub responsible for translating antigen binding signals to immune receptors for the activation of the AP-1 and NF-κB master transcriptional programs. Despite its importance, known substrates of TAK1 are limited to kinases of the MAPK and IKK families and include no direct effectors of biochemical processes. Here, we identify over 200 substrates of TAK1 using a chemical genetic kinase strategy. We validate phosphorylation of the dynamic switch II region of GTPase Rab1, a mediator of endoplasmic reticulum to Golgi vesicular transport, at T75 to be regulated by TAK1 in vivo. TAK1 preferentially phosphorylates the inactive (GDP-bound) state of Rab1. Phosphorylation of Rab1 disrupts interaction with GDP dissociation inhibitor 1 (GDI1), but not guanine exchange factor (GEF) or GTPase-activating protein (GAP) enzymes, and is exclusive to membrane-localized Rab1, suggesting phosphorylation may stimulate Rab1 membrane association. Furthermore, we found phosphorylation of Rab1 at T75 to be essential for Rab1 function. Previous studies established that the pathogen *Legionella pneumophila* is capable of hijacking Rab1 function through posttranslational modifications of the switch II region. Here, we present evidence that Rab1 is regulated by the host in a similar fashion, and that the innate immunity kinase TAK1 and *Legionella* effectors compete to regulate Rab1 by switch II modifications during infection.

chemical genetics | posttranslational modification | kinase substrates | vesicle trafficking | Rab GTPases

Cellular response to microbial infection is a complex, coordinated process that is initiated by innate immune receptors at the cell surface in response to cytokines or pathogen-associated molecular patterns. Pattern recognition receptors trigger cellular-response cascades culminating in activation of two master transcriptional programs, AP-1 and NF-κB, which drive cytokine production and recruitment of immune cells. In particular, Toll-like receptor (TLR) signaling cascades require the intricate orchestration of activation of downstream pathway components through the formation of diverse complexes and intensive reliance on posttranslational modifications for regulation. Ultimately, these pathways converge on the activation of an essential kinase, TGF-β activated kinase 1 (TAK1), which is responsible for translating receptor activation for the activation of these master transcriptional programs (1).

In the early phase following activation of receptors such as TLR2 or -4, an unusual nondegradative ubiquitin scaffold is assembled, leading to the activation of TAK1, also known as MAP3K7. Once activated, TAK1 serves two roles. First, TAK1 acts as a canonical MAPKKK by phosphorylating the MAPKKs MKK4/7 and MKK3/6. These MKKs phosphorylate and activate p38 and JNK, respectively, initiating AP-1–mediated transcription. Second, TAK1 provides a priming phosphorylation to IKKβ, which colocalizes with TAK1 to M1-poly-Ub chains generated by TRAF6 upon TLR activation (2, 3). Activation of IKKβ leads to the activation IKKα, degradation of IκBα, and finally, activation of NF-κB–driven transcription. The known direct substrates of TAK1 are limited to these protein kinases, TAK1 binding protein 1 (TAB1), and an additional protein kinase, AMPK (4, 5). TAK1 is primarily viewed as an initiator of kinase signaling cascades that lead to transcription factor activation.

Kinases often serve as signaling relays, transferring phosphorylation down a cascade of kinases, but also commonly function as direct effectors of biochemical processes via phosphorylation of enzymes from many classes. Given the importance of TAK1 as the terminal output of pattern-recognition receptor activation, we wondered if TAK1 might possess direct substrates beyond the three characterized classes of downstream kinases. Only one study has characterized a small number of downstream targets of TAK1 using quantitative phosphoproteomics (6). Although no direct TAK1–substrate relationships were established, Gene Ontology term enrichment of TAK1-regulated phosphoproteins suggested involvement in GTPase regulation and membrane organization. Other studies have suggested a role for TAK1 in directly regulating protein degradation to prevent accumulation of reactive oxygen species (7). Thus, we turned to the analog-specific kinase covalent capture methodology (8, 9) to identify direct TAK1 substrates in vitro. Through this method, we identified hundreds of candidate substrates.

Significance

Rab GTPases regulate vesicle traffic within the cell by switching between active (GTP-bound) and inactive (GDP-bound) states. The switch II region of Rab proteins undergoes a significant conformational change to switch between states. Rab1 is hijacked during intracellular *Legionella pneumophila* infection by bacterial effector-mediated posttranslational modifications of the switch II region, a unique mechanism for regulation of Rab function. We present new evidence that Rab1 is endogenously modified within switch II by TGF-β activated kinase 1 (TAK1), a kinase crucial for responding to infection. We show phosphorylation of Rab1 is necessary for normal Rab1 function. Interestingly, phosphorylation of Rab1 is competed during *Legionella* infection, adding to evidence that *Legionella* target substrates of the innate immunity kinase TAK1.

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The authors declare no conflict of interest.

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Data deposition: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (www.proteomexchange.org) via the PRIDE partner repository (dataset identifier PXD004213).

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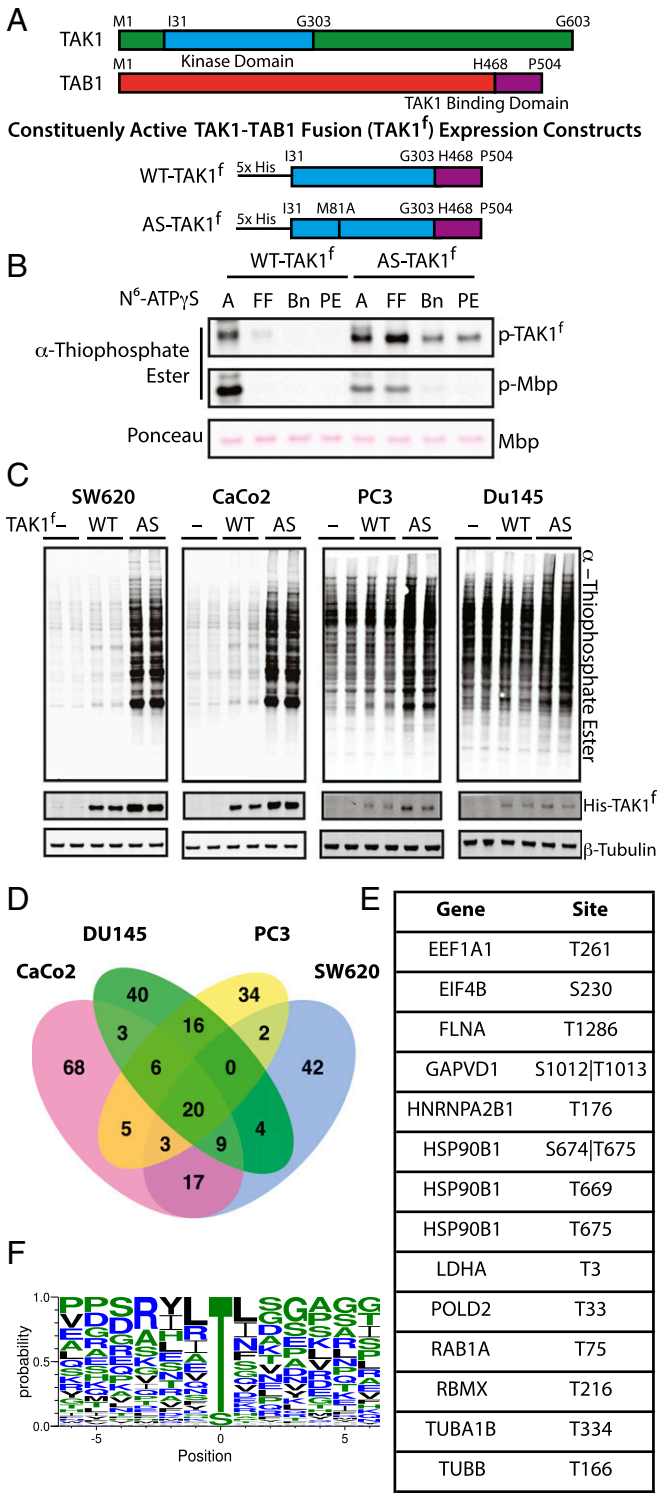
We decided to focus, in particular, on a novel phosphorylation site within a dynamic region of small Ras-like GTPase Rab1. Rab proteins are the largest family of small Ras-like GTPases and serve to regulate many steps of membrane trafficking. These proteins act as molecular switches, cycling through active GTP-bound and inactive GDP-bound states. Two regions of the GTPases, termed switch I and switch II, undergo significant conformational shifts between these states, altering the ability of the protein to bind interactors. Rab nucleotide state, and therefore signaling, is tightly regulated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). By binding Rab proteins in the cytoplasm, GDP dissociation inhibitors (GDIs) sequester inactive Rab proteins to further control Rab activation. Although the C-terminal tails of Rab proteins are geranylgeranylated to mediate insertion into membranes, there are few examples of regulation of Rab proteins, or small GTPases in general, by posttranslational modification of the core GTPase domain. Although phosphorylation of Rab proteins, and other Ras-like GTPases, has been previously observed on the C-terminal tail and other outlying regions, there is little consensus on the regulatory effect of these phosphorylations (10–13). The strongest examples of small GTPase regulation by post-translational modification are when the core GTPase domain is modified, rather than tails. Such modifications are almost exclusively the result of infection by pathogens, such as phosphorylation of switch I of immunity-related GTPases by a secreted *Toxoplasma gondii* kinase in mice (14) or AMPylation and phosphocholination of the switch region of Rab1 by *Legionella pneumophila* (15, 16).

Here, we demonstrate that TAK1 phosphorylation of Rab1 within the dynamic switch II region is key to Rab1 signaling. Phosphorylation of Rab1 is necessary for normal Rab1 function in maintaining Golgi structure, and disrupts interaction with GDI1, allowing for activation of Rab1. More interestingly, TAK1-mediated phosphorylation of Rab1 competes with *L. pneumophila* during intracellular infection. We believe Rab1 is a newly recognized hotspot for regulation via posttranslational modifications, both by a bacterial pathogen and now by TAK1, a host kinase responsible for responding to infection.

Results

Identification and Validation of TAK1 Substrates. We first sought to identify direct substrates of TAK1 using a chemical genetic, analog-specific (AS) kinase approach (Fig. S1*A*) (8, 9). Mutation of a single bulky residue within the active site of a kinase, termed the active-site gatekeeper residue, to alanine or glycine expands the native ATP binding pocket. This mutation allows the kinase to accept N^6-substituted ATPγS analogs, bulky variants of ATP that fit in the newly expanded active site but not the active sites of WT kinases, creating an AS kinase. The AS-kinase transfers the γ-thiophosphate of the ATP analog to its substrates. This thiophosphorylation acts as a uniquely reactive chemical handle that can be alkylated for detection of substrates by Western blotting or used to affinity-purify and identify substrate proteins by liquid chromatography (LC)-MS/MS. We generated a constitutively active form of TAK1 by expressing and purifying from insect cells a fused TAK1 construct containing the kinase domain of TAK1 fused to the TAK1-activating domain of binding partner TAB1 (Fig. 1*A*) (17). We will refer to this fusion construct as $TAK1^f$, with WT indicating no mutations to the gatekeeper residue. AS-$TAK1^f$ was generated by mutation of gatekeeper methionine 81 to alanine. We tested the specificity and preference of AS-$TAK1^f$ for N^6-substituted ATPγS analogs through an in vitro kinase assay using myelin basic protein (MBP) as a generic substrate (Fig. 1*B*). AS-$TAK1^f$ used both ATPγS and N^6-furfuryl-ATPγS efficiently for autophosphorylation and transphosphorylation of MBP. In contrast, WT-$TAK1^f$ was largely incapable of using any bulky ATP analogs. N^6-furfuryl-ATPγS was used for lysate-labeling



Gene	Site
EEF1A1	T261
EIF4B	S230
FLNA	T1286
GAPVD1	S1012|T1013
HNRNPA2B1	T176
HSP90B1	S674|T675
HSP90B1	T669
HSP90B1	T675
LDHA	T3
POLD2	T33
RAB1A	T75
RBMX	T216
TUBA1B	T334
TUBB	T166

Fig. 1. Characterization of AS-$TAK1^f$ and identification of TAK1 substrates. (*A*) Schematic of full-length TAK1, TAB1, and constituently active fusion constructs of WT-$TAK1^f$ and AS-$TAK1^f$ used for protein purification. (*B*) In vitro kinase assay with $TAK1^f$, MBP, and bulky N^6-substituted ATPγS analogs (A, ATPγS; Bn, N^6 benzyl; FF, N^6 furfuryl; PE, N^6 phenethyl). Thiophosporylation was evaluated by Western blot. (*C*) Lysate from SW620, CaCo2, PC3, and Du145 cells were labeled with no kinase (–), His-tagged WT-$TAK1^f$ (WT), or His-tagged AS-$TAK1^f$ (AS) in biological duplicate. (*D*) Venn diagrams of phosphoproteins identified in four cell cancer lines, colorectal (SW620, CaCo2) and pancreatic (DU145, PC3). (*E*) Phosphosites exclusive to and identified in all eight individual AS-$TAK1^f$–labeled samples. (*F*) TAK1 consensus motif derived from all phosphopeptides identified in ≥2 samples.

experiments to ensure any detected thiophosphorylation was the result of AS-TAK1^f activity.

We next sought to identify proteins selectively thiophosphorylated by AS-TAK1^f in lysates from four cell lines from two cancer types, colorectal (CaCo2, SW620) and pancreatic (PC3, DU145). These cell lines were selected because TAK1 has been shown to be particularly important in colorectal and prostate cancers (18, 19). Lysates were individually labeled by spiking in N^6-furfuryl-ATPγS and purified AS-TAK1^f, WT-TAK1^f, or with no added kinase. A portion of each sample was analyzed by Western blot, where an obvious increase in thiophosphorylation is observed exclusively in the AS-labeled colorectal cell line samples; the contrast between AS and WT or no-kinase conditions is less obvious in the pancreatic cell lines, as these cell lines displayed much higher background thiophosphorylation (Fig. 1*C*). We attribute this difference, in part, to variability between cell lines in the background proteome activity toward the ATPγS analog. The remainder of the thiophosphorylated lysates were digested, thiophosphorylated peptides covalently captured, converted to phosphorylated peptides upon elution from resin, and analyzed by MS. Despite differences in levels of thiophosphorylation by Western blot, many more phosphopeptides were identified in all AS-TAK1^f–labeled samples versus controls. (Datasets S1–S4). Thus, although Western detection of thiophosphorylation is a useful tool, it is limited in comparison with MS results as observed in this study and others (20).

The data were filtered by cell type to exclude background phosphopeptides from the WT-TAK1^f and no-kinase conditions (21), leaving phosphopeptides exclusive to AS-TAK1^f. In total, 269 phosphoproteins yielding 424 phosphopeptides were identified as candidate TAK1 substrates (Fig. S1*B*). A list of all candidate substrate phosphopeptides identified is available in Dataset S5. The difference in peptide versus protein number is a result of the identification of multiple phosphopeptides per protein and, in some cases, a single peptide identified multiple times with differing sites of phosphorylation. Whereas all cell lines shared a set of 20 substrate proteins, generally substrates were shared more frequently between cell lines of the same origin, with many substrates uniquely identified in a single cell line (Fig. 1*D*). Although the stochastic nature of shotgun LC-MS/MS identification may explain some of the lack of overlap, we believe the method of capture used is able to identify cell-type–specific substrates. Conservation of a substrate across cell types may be indicative of a central, conserved function, and therefore a useful means to triage substrates for further study. Fourteen phosphopeptides were identified in all AS-TAK1^f samples analyzed (Fig. 1*E*). To further analyze the substrate preferences of TAK1, we generated a TAK1 consensus sequence from phosphopeptides identified in at least two samples (Fig. 1*F*) (22). We observed a strong preference for phosphorylation of threonine, with some preference for aliphatic −1 and +1 residues.

To corroborate our MS results, a subset of substrates identified by MS were selected for further validation (Fig. S2*A*). We assessed the ability of TAK1 to phosphorylate substrates overexpressed with N-terminal Flag or GST tags and immunoprecipitated from HEK-293Ts by in vitro radioactive kinase assay with WT-TAK1^f. As a positive control, we sought to confirm that our WT-TAK1^f would strongly phosphorylate a known substrate, MKK6. MKK6 was not identified by MS because of the presence of cysteine in tryptic MKK6 peptides containing TAK1 phosphorylation sites, as Cys-containing peptides are permanently retained on the capture resin (9). TAK1 strongly phosphorylated kinase-dead MKK6 as shown by the incorporation of ^{32}P in the WT-TAK1^f–labeled sample (Fig. 2*A*). Of the eight candidate substrates tested, only PSMC4 was not strongly phosphorylated by WT-TAK1^f. The remaining seven substrates were strongly phosphorylated by TAK1 (Fig. 2 *A*–*C*). Interestingly, WT-TAK1^f was clearly able to phosphorylate more than the single identified site on three substrates (Vinexin, EIF4B, EIF3I), as shown by incorporation of ^{32}P into the nonphosphorylatable T to A mutants (Fig. 2*B*). It is possible additional phosphosites in these

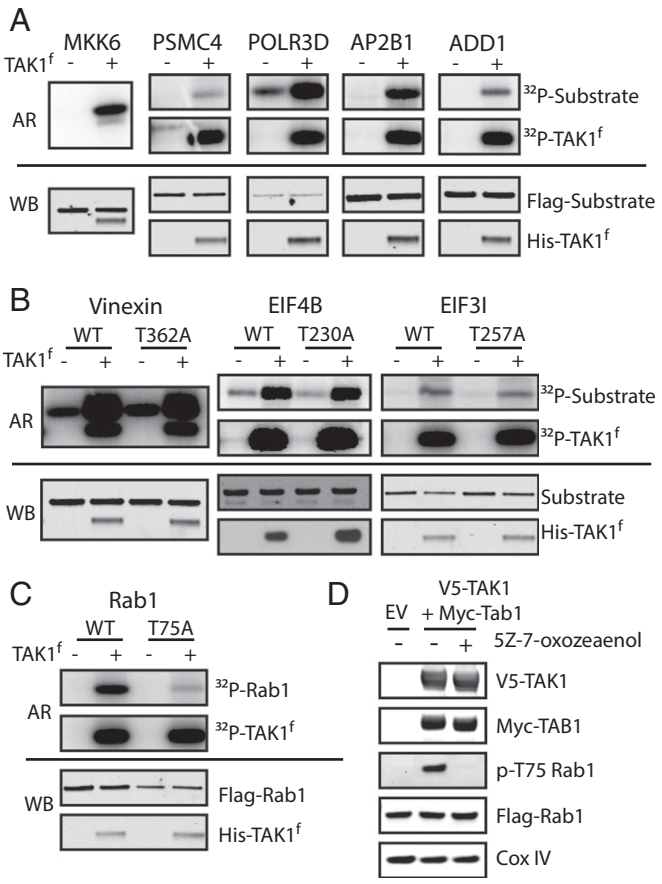


Fig. 2. Validation of TAK1 substrates in vitro and in vivo. (*A*–*C*) In vitro radioactive kinase assays with GST- or Flag-tagged TAK1 substrates. Substrates immunoprecipitated from HEK-293Ts were incubated with purified WT-TAK1^f (300 nM) and γ[^{32}P]ATP, run on a gel, and imaged by autoradiography (AR). Aliquots removed before γ[^{32}P]ATP addition were used to assess loading by Western blot (WB). MKK6 (kinase dead) is a known substrate and positive control. (*D*) HEK-293s stably expressing Flag-Rab1 were transfected with either empty vector (EV) or full-length V5-TAK1 and Myc-TAB1 were analyzed by Western blot for pT75 Rab1. One condition was dosed with 2.5-μM TAK1 inhibitor 5z-7-oxozeaenol for 1 h.

proteins are within regions not amenable for detection by trypsin-based LC-MS/MS and were therefore not detected. Given the high rate of substrate validation, we have high confidence in the validity of the candidate substrates identified by MS.

TAK1 Selectively Phosphorylated GDP-Bound Rab1. Rab1 was the sole protein tested that was phosphorylated by WT-TAK1^f at a single site, T75, within its switch II region, in all four cell lines (Fig. 2*C*). Switch II is a conserved region within the catalytic domain of GTPases that undergoes a conformational shift between the active (GTP-bound) and inactive (GDP-bound) states (23) and is not a common site of posttranslational modification. This selective phosphorylation motivated us to carry out in vivo validation with a polyclonal antibody raised against pT75 Rab1. In HEK-293 cells stably expressing Flag-Rab1, overexpression of full-length V5-TAK1 and activating partner Myc-TAB1 (24) led to increased phosphorylation of Rab1 T75. Addition of a TAK1-selective inhibitor, 5z-7-oxozeaenol (25), eliminated phosphorylation of Rab1 (Fig. 2*D*). The increased abundance of pT75 Rab1 following TAK1 overexpression and loss of phosphorylation upon inhibition of TAK1 was also observed by LC-MS/MS analysis of immunoprecipitated Rab1 (Fig. S2*B*). A similar increase in phosphorylation of endogenous Rab1 is observed upon TAK1 overexpression in normal HEK-293T, as is a decrease in phosphorylation after TAK1 inhibition

(Fig. S2*C*). The modulation of pT75 Rab1 levels upon manipulation of TAK1 catalytic activity by overexpression and inhibition suggests Rab1 is a direct substrate of TAK1 in vivo.

Because switch II occupies two distinct conformations, we hypothesized that the nucleotide state of Rab1 may influence the ability of TAK1 to phosphorylate T75. Radiometric in vitro kinase assays with GST-Rab1 purified from a bacterial expression system and WT-TAK1^{f} showed preferential phosphorylation of the inactive, GDP-locked Rab1S25N (26) and reduced phosphorylation of active-state mimetic Rab1Q70L (27) (Fig. 3*A*). Correspondingly, TAK1 preferentially phosphorylated Rab1 loaded with GDP by nucleotide exchange versus nonhydrolyzable GTPγS (Fig. 3*B*). The preference of TAK1 for GDP-Rab1 may be explained by available structural data of the yeast homolog of Rab1, Ypt1. Alignment of the structure of GDP (PDB ID code 2BCG) and GTP mimetic GppNHp- (PDB ID code 1YZN) bound Ypt1 shows the switch II region to be flipped outward from the body of the protein, exposing T75-equivalent residue T72 (Fig. 3*C*). Our findings suggest that T75 is only accessible for phosphorylation by TAK1 when GDP binding to Rab1 causes the switch II region to become disordered (23). Interestingly, the intracellular pathogen *L. pneumophila* is well documented to hijack the function of Rab1 in infected cells by posttranslational modification of nearby switch II residues, including adenylylation, also known as AMPylation, of Y80 and phosphocholination of S79 (Fig. 3*D*) (15, 16, 28).

Phosphorylation of Rab1 Disrupts Interaction with GDI but Not GAP or GEF. During activation, switch II becomes more ordered and T75 is flipped inward toward the core of Rab1 (Fig. 3*C*). It is possible that phosphorylation may block Rab1 from binding GTP and localizing to the membrane by sterically hindering the conformational shift of switch II. To determine whether phosphorylation may disrupt GTP binding, we assayed the nucleotide affinities of Rab1 mutants using 2′-deoxy-3′-O-(*N*-methylanthraniloyl) (mant)-dGDP (mant-GDP) –loaded Rab1, which forms a fluorescent complex. EDTA was used to catalyze nucleotide exchange while titrating unlabeled GDP or GTP (29), with a reduction in fluorescence caused by mant-GDP displacement corresponding to affinity for the titrated nucleotide. All constructs tested, including WT-Rab1 phosphorylated by preincubation with TAK1 and ATP, maintained a similar affinity for GDP (Fig. S3*A*). A slight increase in affinity for GTP was observed for Rab1Q70L, phosphomimetic Rab1T75E, and WT-Rab1 preincubated with TAK1 and ATP (Fig. S3 *B* and *C*). The catalysis of nucleotide exchange by EDTA suggests that phosphorylation does not prevent activation of Rab1, and may in fact enhance GTP affinity.

Multiple studies have shown that phosphocholination and AMPylation of Rab1 switch II manipulate Rab1 function by blocking the ability of Rab1 to interact with GEFs and GAPs (16, 28, 30), because switch II serves as the primary interface for these binding events. To investigate whether phosphorylation of Rab1T75 may have a similar effect, we assayed the ability of the *Legionella* Rab1 GEF DrrA (GEF domain only, residues 340–533) to catalyze the displacement of mant-GDP from WT and mutant Rab1 in vitro (Fig. 4 *A* and *B* and Fig. S3*D*). DrrA$_{340–533}$ was selected for these assays as a result of numerous publications (30–32) describing similar experiments with this enzyme. We found no significant difference in the k_{cat}/K_m of DrrA$_{340–533}$ toward WT Rab1 and Rab1T75E, and only a slight difference with Rab1T75A; thus, we infer that it is unlikely phosphorylation of T75 prevents Rab1 interaction with GEFs or interferes with activation. Phosphomimetic mutant Rab1T75E yields similarly insignificant effects on the ability of a *Legionella* GAP, LepB (33), to stimulate Rab1 hydrolysis of GTP, as measured with the Promega GTPase-Glo system (34). Briefly, increasing concentrations of LepB with excess GTP held at a constant concentration are added to wells containing a constant concentration of Rab1 and allowed to react for 1 h. The amount of remaining, unhydrolyzed GTP in each condition is detected by luminescence-coupled assay, plotted against GAP concentration, and a LepB EC$_{50}$ is determined. We found no difference in LepB activity toward WT, T75E, or T75A (Fig. S4 *A* and *B*), which suggests phosphorylation does not interfere with inactivation of Rab1 by GAPs. Considering the GTP/GDP affinity, GEF assay, and GAP assay together, we conclude phosphorylation of Rab1 does not impact the ability of Rab1 to cycle between GDP- and GTP-bound states or interact with GAP and GEFs.

The subcellular location of Rab1 is also generally dictated by its nucleotide association, as recruitment of Rab1 to the membrane of the endoplasmic reticulum (ER) from the cytoplasm is concurrent with displacement of GDP and activation of Rab1 upon binding of GTP (35). Until this displacement occurs, inactive GDP-Rab1 is sequestered in the cytoplasm in complex with a GDI. Similar to their effect on GAPs and GEFs, *Legionella*-derived posttranslational modifications of Rab1 disrupt association with the GDI (31). We immunoprecipitated Flag-Rab1 from HEK-293 cells stably expressing Flag-Rab1 transfected with either vector or the combination of TAK1 and TAB1. The relative amount of GDI1 coprecipitating with Rab1 (GDI:Flag-Rab1) decreased in cells with highly phosphorylated Rab1 resulting from TAK1 and TAB1 overexpression (Fig. 4*C*). More strikingly, little to no GDI1 coimmunoprecipitates with Flag-Rab1T75E transiently overexpressed in HEK 293Ts, whereas Flag-Rab1T75A increases association with GDI1 (Fig. 4*D* and Fig. S4*C*). Disruption of the GDI:Rab1 complex by phosphorylation suggests pT75-Rab1 is available for activation and recruitment to the ER membrane. Thus, we examined the

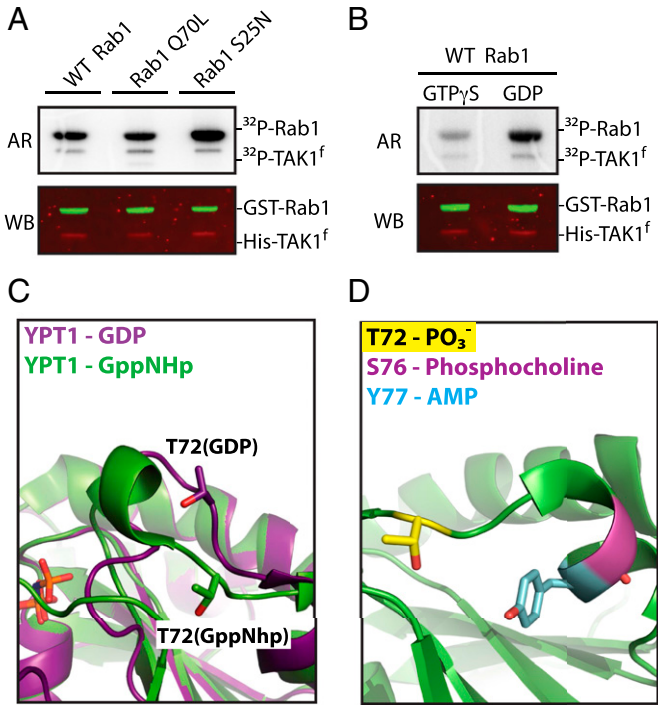


Fig. 3. TAK1 preferentially phosphorylates GDP-bound Rab1. (*A*) In vitro kinase assay of purified GST-Rab1 mutants (10 μM) and WT-TAK1^{f} (100 nM) imaged by autoradiography (AR) and Western blot (WB) as loading control. (*B*) In vitro kinase assay of WT-TAK1^{f} (100 nM) and purified GST-WT Rab1 (10 μM) loaded with the indicated nucleotide. (*C*) Alignment of Rab1-homolog Ypt1 structures bound to GDP (PDB ID code 2BCG) or GTP analog GppNHp (PDB ID code 1YZN). Ypt1 T72 aligns to Rab1 T75. (*D*) Mapping of posttranslationally modified Rab1 residues within switch II where S76 and Y77 (Rab1 S79 and Y80 as aligned to YPT1 structure PDB ID code1YZN) are sites of *Legionella*-derived modifications.

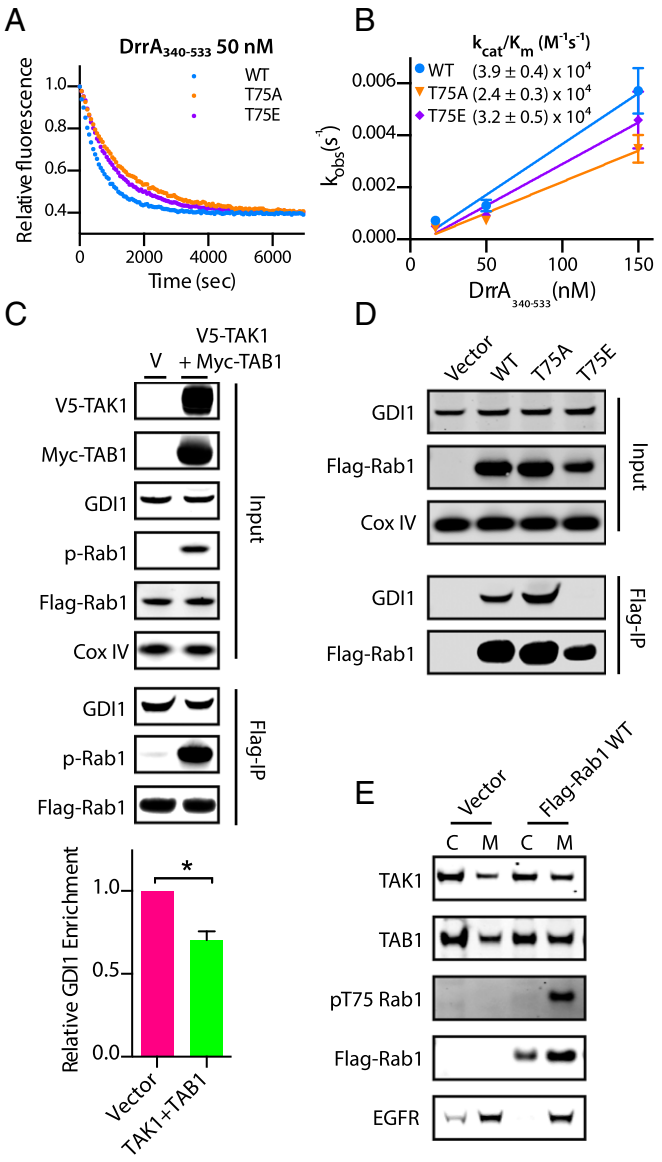


Fig. 4. Phosphorylation of Rab1 disrupts interaction with GDI but not GEFs. (*A*) Measurement of mant-GDP dissociation from GST-Rab1 mutants by $DrrA_{340–533}$ from a single representative experiment where each data point represents the mean of technical replicates ($n = 3$). (*B*) Observed rate constants (k_{obs}) for DrrA catalyzed mant-GDP dissociation with error bars for mean ± SD ($n = 2$) and extrapolated catalytic efficiencies (k_{cat}/K_m). (*C*) HEK-293s stably expressing Flag-Rab1 were transiently transfected with either empty vector (V) or full-length V5-TAK1 and Myc-TAB1. Lysates were subject to immunoprecipitation of Flag-Rab1 using α-Flag antibody-coupled magnetic beads and analyzed by Western blot for coimmunoprecipitation of GDI1. The bar graph represents the ratio of precipitated GDI:Flag-Rab1 ($n = 2$). *$P < 0.05$, unpaired *t* test. (*D*) HEK-293Ts were transiently transfected with vector, Flag-Rab1 WT, T75A, or T75E and subject to immunoprecipitation of Flag-Rab1. Quantitation is in Fig. S4*B*. (*E*) Cell fractionation of HEK-293Ts transiently transfected with empty vector or Flag-Rab1 with cytoplasmic (C) or membrane (M) fractions.

localization of pT75 Rab1 by cellular fractionation of HEK 293Ts transiently expressing Flag-Rab1 (Fig. 4*E*). Although total Flag-Rab1 is distributed between cytoplasmic and membrane fractions, pT75 Rab1 is exclusively detected in the membrane fraction, where Rab1 activation occurs. Taken together, these results suggest that phosphorylation of Rab1 by TAK1 may be an important precursor to GTP binding and activation by driving dissociation from the GDI.

Rab1 Phosphorylation Is Required to Maintain Golgi Structure. Rab1 is responsible for transporting vesicles from the ER to the Golgi. Disruption of Rab1 activity, by knockdown or overexpression of the dominant-negative Rab1S25N, results in fragmentation of the Golgi apparatus (36, 37). We tested the effect of overexpression of nonphosphorylatable T75A and phosphomimetic T75E Rab1 on Golgi structure to determine if the T75 site was critical for Rab1 function. Immunofluorescence was performed in HeLa cells transiently overexpressing GFP-Rab1 mutants (Fig. 5 *A* and *B*) and the Golgi was stained with a *cis*-Golgi marker, GM130. As previously shown, overexpression of Rab1S25N acts in a dominant-negative fashion, disrupting Golgi structure, whereas Rab1Q70L, the active-state mimic, has no effect on Golgi structure. Similarly, Rab1T75E has no effect on the Golgi. However, Rab1T75A acts in a dominant-negative fashion similar to Rab1S25N to cause extensive Golgi fragmentation.

To complement these results, we assayed the effect of inhibiting TAK1 with 5z-7-oxozeaenol (25) on Golgi structure by immunofluorescence. Inhibition of TAK1 for 6 h leads to a marked disruption of normal Golgi structure versus DMSO (Fig. 5 *C* and *D*). This effect is largely rescued by overexpression of GFP-Rab1T75E, but not WT or T75A, in the presence of inhibitor. We conclude from these data that the phosphorylation of Rab1, or ability of Rab1 to be phosphorylated, plays an important role in ER to Golgi vesicle transport and in maintaining proper Golgi structure.

Innate Immunity Kinase TAK1 and Pathogen *Legionella* Compete to Posttranslationally Modify Rab1. There is a well-established precedent for regulation of Rab1 function by posttranslational modification during microbial infection. The intracellular bacterial pathogen *L. pneumophila* uses posttranslational modifications of Rab1 in order to establish the *Legionella*-containing vacuole. *Legionella* effectors DrrA and AnkX are secreted into the host cell cytoplasm during infection and catalyze the AMPylation at Y80 and phosphocholination at S79 of Rab1, respectively (Fig. 3*D*) (15, 16, 30, 38). As discussed earlier, these modifications serve as locks on the Rab1 nucleotide state and block interactions with the host enzymes normally responsible for regulating Rab1. Two additional *Legionella* enzymes, SidD and Lem3, have cognate Rab1-demodifying activities (28, 39). *Legionella* maintains exquisite and tightly regulated control of Rab1 to mature its replication vacuole by carefully timing the sequential secretion of these effectors and subsequent recruitment of Rab1 to the *Legionella*-containing vacuole.

Because TAK1 is a kinase activated by pathogens, such as *Legionella*, and *Legionella* extensively modifies the Rab1 switch II region, we examined the interplay between TAK1-mediated phosphorylation of Rab1 and *Legionella* infection. HEK-293 cells stably expressing FCγIII receptor (to allow for opsonization and endocytosis of *Legionella* in HEK-293 cells) and Flag-Rab1 were infected with *Legionella* (WT), an isogenic strain lacking the Dot/Icm type IV secretion system (Δ*dotA*), an isogenic strain lacking the two known Rab1 posttranslational modifying enzymes, DrrA and AnkX (Δ*ankX,drrA*), or left mock-infected (Fig. 6*A*). Some basal phosphorylation of Rab1 was detected in the mock-infected (0 h) sample. Phosphorylation of Rab1 increased slightly at 1 h in the WT condition, then tapered to below basal levels at 4 and 6 h. Infection with both Δ*dotA* and Δ*ankX,drrA* strains lead to increased levels of pT75-Rab1 at 1 and 4 h, with levels remaining high at 6 h in Δ*dotA*. Deletion of AnkX and DrrA provided a moderate restoration of pT75-Rab1 levels versus WT infection, suggesting these enzymes may be responsible for outcompeting TAK1 for control of Rab1 during WT infection. We next considered the contribution of the AMPylation versus GEF activity of DrrA toward reducing pT75-Rab1 levels during WT infection. GST-Rab1 was incubated for 15 min with ATP and either WT-TAK1^{f} or full-length DrrA in the presence of excess GTP or GDP, then incubated for an

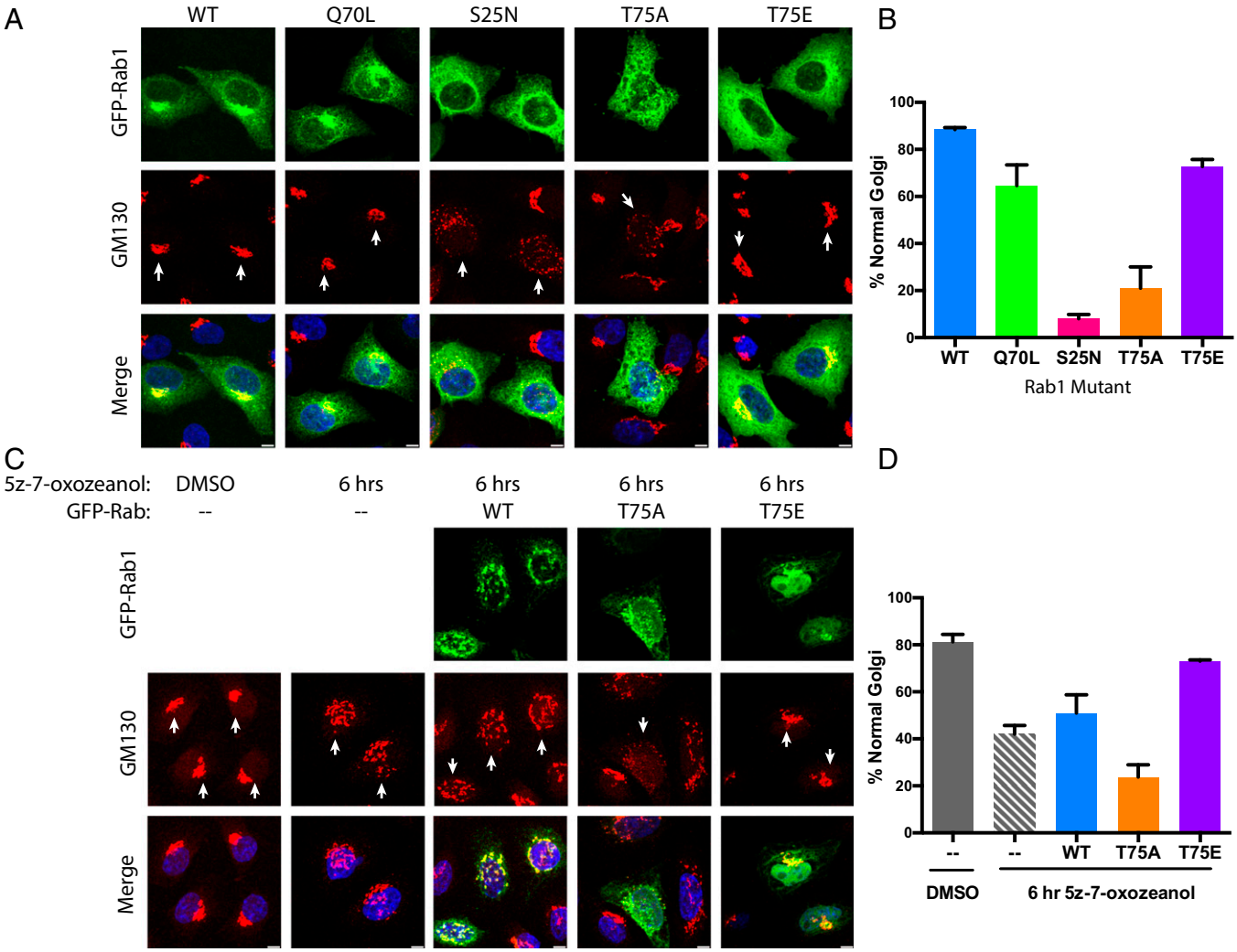


Fig. 5. Fragmentation of the Golgi is a result of overexpression of Rab1T75A or inhibition of TAK1. (*A*) Representative images of HeLa cells transfected with GFP-Rab1 in green and stained for GM130, a *cis*-Golgi marker in red, and DAPI in blue. (Scale bar, 5 μm.) (*B*) Quantitation of immunofluorescence experiment ($n = 3$ replicates, 33 cells per replicate). (*C*) Representative images of HeLa cells stained for GM130 after dosing with 2.5-μM TAK1 inhibitor 5z-7-oxozeaenol for 6 h and preceding transfection with GFP-Rab1 where indicated. (Scale bar, 5 μm.) (*D*) Quantitation of immunofluorescence experiment ($n = 3$ replicates, 33 cells per replicate).

additional 15 min after addition of the remaining enzyme. Aliquots were removed and quenched at 0, 15, and 30 min and analyzed by Western blot (Fig. 6*B*). AMPylation of Rab1 is not affected by preexisting phosphorylation in the presence of either nucleotide. However, phosphorylation of Rab1 by TAK1 is significantly hindered only when excess GTP is present, suggesting that it is the DrrA-catalyzed Rab1-GTP binding, which reduces phosphorylation levels, not the presence of AMPylation, disrupting the TAK1 phosphorylation site. Although the contribution of phosphocholination and other factors to outcompeting Rab1 phosphorylation remain to be determined, these results suggest that TAK1 may be outcompeted in part by *Legionella* for modification and control of Rab1 during infection by the GEF activity of DrrA.

Discussion

Here, we present an effort to identify a broad set of substrates of TAK1, an S/T kinase with crucial function in the innate immune system. We generated an ATP AS mutant of TAK1, termed AS-TAK1^{f}, to selectively label, isolate, and identify novel substrates. We identified over 200 substrate proteins of TAK1, with a subset validated by in vitro kinase assays. TAK1 demonstrated a striking preference for threonine in the identified phosphosites. A recent study demonstrated that kinases with a β-branched residue in the conserved DFG loop drives specificity for threonine over serine (40). Fittingly, TAK1 contains a threonine at this position, T178. Although only one substrate, Rab1, was studied in depth in this study, a number of these substrates fit with known characteristics of TAK1 signaling. Filamin A functions as a scaffold for MKK4 and JNK association and activation of JNK (41, 42). MKK4 is a direct substrate of TAK1, thus it is possible TAK1 also associates with Filamin A to stimulate MKK4/JNK signaling. Additionally, TAK1 is known to be a client of HSP90 (43). TAK1-mediated phosphorylation of a close relative, endoplasmin (HSP90B1), suggests TAK1 is capable of interacting with additional heat-shock proteins.

We focused in particular on a novel TAK1 substrate, the GTPase Rab1. We demonstrate TAK1 phosphorylates Rab1 at a single site within the dynamic switch II region in vivo. Given the preference of TAK1 for GDP-bound Rab1 in vitro, we believe phosphorylation of Rab1 occurs in the inactive state. However, the GTP affinity of phosphorylated Rab1 is unchanged, GEF DrrA catalyzes nucleotide exchange of Rab1T75E efficiently, Rab1T75E can perform GAP-catalyzed GTP hydrolysis normally, and

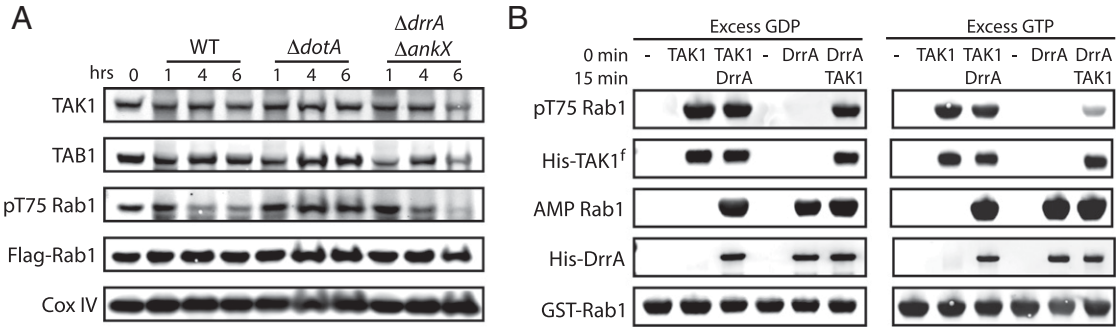


Fig. 6. pT75 Rab1 levels decrease during *Legionella* infection because of the GEF activity of DrrA. (*A*) HEK-293 cells stably expressing FCγIII receptor and Flag-Rab1 were mock-infected (0 h), infected with WT, secretion deficient (Δ*dotA*), or AnkX and DrrA-deficient (Δ*ankX,drrA*) *Legionella* for 1, 4, or 6 h and analyzed by Western blot. (*B*) Sequential modification of Rab1 by TAK1 and DrrA. Enzymes were added at 0 and 15 min, with aliquots of sample removed at 0 (just before enzyme addition), 15, and 30 min for analysis in the presence of excess GDP or GTP (30 μM).

phosphorylation is present only on membrane-associated Rab1, suggesting phosphorylated Rab1 has a normal catalytic cycle and associates with membranes. We find phosphorylation of Rab1 disrupts interaction with GDI1, an interaction that is stabilized by the residues of switch II (44). Combined, these results suggest phosphorylation of Rab1 may serve to disrupt association with the GDI, and perhaps push Rab1 toward membrane association and activation rather than sequestration. A strong Golgi fragmentation phenotype was observed by immunofluorescence upon overexpression of nonphosphorylatable but not phosphomimetic Rab1, as well as inhibition of TAK1, suggesting that the ability to be phosphorylated is essential for Rab1 function, specifically in maintaining Golgi structure, and perhaps more widely in ER to Golgi vesicle transport. Thus, we propose that TAK1 phosphorylation of Rab1 is a priming step for Rab1 activation and integral component of the Rab1 activity cycle.

We believe these findings are evidence of regulation of Rab1 function by endogenous posttranslational modification within the catalytic domain of the protein. Seen in the context of recent studies and existing data, modification—especially phosphorylation—of switch II may be a widespread endogenous mechanism of Rab family regulation. The Phosphosite.org database contains phosphoproteomic evidence for switch II phosphorylation of at least 15 additional Rab GTPases, and several other small GTPases. Recent work from Mann and colleagues (45) identified phosphorylation by LRRK2 of the switch II regions of Rab3a, Rab8a, Rab10, and Rab12 as a driver of membrane localization. In addition, a few GTPases outside the Rab family, including Cdc42, Rac1, and Ran, are thought to be regulated by modification of switch II (46–48). Switch II has long been recognized for its importance in dictating the Rab activation state; it has now becoming clear that posttranslational modification of this region allows further, external control of Rab function.

Our interest in Rab1 stemmed from the extensive literature describing the ability of *L. pneumophila* to manipulate Rab1 function by posttranslational modifications to ensure maturation of the *Legionella* replication vacuole within the host cell (35, 38). Here, we suggest that a similar and endogenous mechanism, TAK1-mediated phosphorylation of Rab1, serves to regulate Rab1 function in normal conditions. We also show that phosphorylation of Rab1 is stimulated by secretion-deficient *Legionella* (Δ*dotA*) infection, yet is reduced during WT infection. We believe that TAK1 phosphorylation is outcompeted by secreted *Legionella* factors, as evidenced by the observed reduction of TAK1 driven phosphorylation of Rab1 exposed to GEF DrrA and the rescue of pT75 Rab1 levels in Δ*ankX,drrA*-infected cells. Interestingly, *Legionella* also stimulate NF-κB, p38, and JNK signaling through TLR-independent mechanisms during infection (49, 50). TAK1 normally serves to respond to TLR signaling and activate these same pathways during infection. In addition, *Yersinia pestis* has been shown to inhibit innate immune signaling through acetylation and inactivation of TAK1 (51). Thus, we hypothesize that *Legionella* has evolved mechanisms to mimic, or perhaps directly manipulate, TAK1 function during infection to control the innate immune response, as evidenced by activation of NF-κB, p38, and JNK, and now by modification of Rab1. The unbiased identification of TAK1 substrates has revealed phosphorylation of Rab1 switch II, a hotspot for posttranslational modification, as a novel regulatory mechanism and potential unique component of innate immunity.

Methods

TAK1 was expressed in SF9 cells and purified as previously described (17). Covalent capture of TAK1 substrates was performed on 2 mg of lysate per sample labeled with 1% (wt/wt) of purified TAK1 and 250 μM N^6-furfuryl-ATPγS. Covalent capture of substrates was performed using Sulfolink resin with oxone elution. Samples were analyzed in technical duplicate using HCD or ETD fragmentation on a Thermo Fisher Scientific LTQ-Velos. Purification of Rab1 and DrrA from *Escherichia coli* was performed as described previously (15, 16). Infection of HEK-293 FCγIII cells stably expressing Rab1 with *Legionella* was performed as described previously (15), with slight modification as *Legionella* strains were grown in AYE broth overnight before infection. All experimental procedures are described in detail in *SI Methods*.

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